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RESEARCH ARTICLE

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Chemical cues linked to risk: Cues from below-ground natural enemies enhance plant defences and influence herbivore behaviour and performance

Anjel M. Helms[1,2] | Swayamjit Ray[2] | Nina L. Matulis[2] | Margaret C. Kuzemchak[2] | William Grisales[2] | John F. Tooker[2] | Jared G. Ali[2]

[1]Department of Entomology, Texas A&M University, College Station, Texas

[2]Department of Entomology, Center for Chemical Ecology, The Pennsylvania State University, University Park, Pennsylvania

Correspondence
Anjel M. Helms
Email: amhelms@tamu.edu

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Abstract

1. Chemical cues are essential for many ecological interactions. Previous studies of chemically mediated multitrophic interactions have typically focused on responses to cues from plants or herbivores above-ground. It is increasingly clear, however, that below-ground cues and those produced by organisms at higher trophic levels also have ecological importance. Prey animals often avoid predator odours to improve survival, and previous research has documented enhanced plant resistance following contact with below-ground natural enemies, though the ecological basis was unknown.
2. Here, we investigated plant and insect responses to chemical cues from below-ground natural enemies and explored the ecological significance of these cues for multitrophic interactions. More specifically, we examined the influence of odours emitted by entomopathogenic nematodes (EPNs), a natural enemy of insect herbivores, on the performance and behaviour of their insect prey and the defence responses of nearby plants.
3. Our findings revealed that EPN-infected insect cadavers emit a characteristic blend of volatile compounds with bioactivity in plants and insects. EPN chemical cues influenced both performance and preference of a specialist herbivore, Colorado potato beetle (CPB, *Leptinotarsa decemlineata*), feeding on its host plant, potato (*Solanum tuberosum*). CPB larvae consumed less leaf tissue and gained less mass feeding on plants exposed to EPN cues compared to control plants. Female CPBs laid fewer eggs on plants with EPN cues than on controls, indicating deterrence by EPN cues or EPN-altered plant defences.
4. Plant defences were enhanced by exposure to live EPNs or EPN chemical cues. Potato plants exposed to EPN infective juveniles induced higher amounts of the defence hormone salicylic acid (SA) and had higher expression of the pathogenesis-related gene *PR-1(PR4)* in foliar tissue. Exposing plants to EPN cues primed induction of SA and jasmonic acid in response to feeding damage by CPB larvae.
5. These findings suggest that herbivores avoid cues from their EPN natural enemies and plants respond to the beneficial nematodes by enhancing systemic defences

that reduce herbivore performance. This work has important implications for the chemical ecology of tritrophic interactions as we report that the third trophic level can play direct and indirect roles in plant defence.

KEYWORDS
below-ground chemical ecology, entomopathogenic nematodes, plant defence, tritrophic interactions

1 | INTRODUCTION

Semiochemicals or compounds that mediate ecological interactions can provide information related to an organism's survival, reproduction or physiological state (Raguso et al., 2015). Previous studies of tritrophic interactions have documented both plant and invertebrate responses to a variety of semiochemicals, with a diversity of cascading consequences for such responses (Ali, Campos-herrera, Alborn, Duncan, & Stelinski, 2013). Invertebrate herbivores and their natural enemies, for example, are often attracted to chemical cues associated with their host plants or prey (Badenes-perez, Gershenzon, & Heckel, 2014; Mccormick et al., 2014; Wiskerke, Dicke, & Vet, 1993). Relatively recent work has also revealed plant detection of herbivore-associated cues, including volatiles from herbivore-damaged plants and compounds from herbivores themselves, with plants responding by inducing or priming their defences (Helms et al., 2017; Hu & Erb, 2018; Manosalva et al., 2015; Orrock et al., 2018). The roles of semiochemicals from plants and herbivores, particularly in above-ground systems, have been fairly well characterized, whereas below-ground chemical cues and cues from natural enemies have received less attention. The ecological importance of these cues, however, is increasingly recognized, as evidenced in recent empirical work (Hermann & Thaler, 2018; Rasmann, Hiltpold, & Ali, 2012; Seo, Rivera, Stelinski, & Martini, 2018; Willett, Alborn, Duncan, & Stelinski, 2015). Chemical cues from natural enemies can warn prey organisms about their risk of attack, and we predict they could also provide information to plants about the presence of beneficial organisms that aid in plant defence. In this study, we investigated plant and insect herbivore responses to below-ground chemical cues from an herbivore natural enemy. We examined whether cues emitted by entomopathogenic nematodes (EPNs), an important natural enemy of soil-dwelling insects, influence herbivore performance and behaviour or alter plant defence responses.

Entomopathogenic nematodes (*Steinernema* and *Heterorhabditis*) are associated with symbiotic bacteria that aid nematode infective juveniles (IJs) in infecting and killing their insect hosts, creating a unique complex of host–vector–symbiont community interactions (Ciche, Darby, Ehlers, Forst, & Goodrich-Blair, 2006; Lewis, Campbell, Griffin, Kaya, & Peters, 2006). This tripartite complex, comprising EPNs, their bacterial symbionts and the infected host, produces a variety of metabolites with different roles in EPN ecology, development and reproduction (Hu, Li, & Webster, 1999; Hu & Webster, 2000; Kaplan et al., 2012; Lu et al., 2017). Based on studies from above-ground systems, it is clear that many prey species avoid chemical cues from potential predators (Hermann & Landis, 2017; Kats & Dill, 1998). Female insects also frequently use chemical cues to select suitable oviposition sites and to avoid plant defences, competition or elevated predation risk for their offspring (Kariyat et al., 2013; De Moraes, Mescher, & Tumlinson, 2001). To our knowledge, however, this has not been explored with EPNs or their associated cues.

Entomopathogenic nematode IJs often locate potential hosts while insects are feeding, using chemical cues emitted by herbivore-damaged plant roots (Ali, Alborn, & Stelinski, 2010; Rasmann et al., 2005). This suggests that EPNs and EPN-infected insect cadavers will frequently co-occur with herbivores in proximity to plant roots, such that roots would encounter EPNs and associated chemical cues. The majority of empirical work on EPNs has focused on their role in biological control of insect pests; however, a few recent studies have documented evidence that EPNs can interact directly with plants. These findings suggest EPNs trigger systemic resistance in plants against various pests; however, mechanisms underlying this resistance or plant responses to EPNs have not been identified, and the ecological significance of this plant response to an herbivore natural enemy remains unknown (An, Orellana, Phelan, Cañas, & Grewal, 2016; Jagdale, Kamoun, & Grewal, 2009).

Here, we ask (a) whether herbivores respond to cues from EPN-infected insect cadavers and (b) if plants respond to EPNs or associated chemical cues by altering their defensive status. To answer these questions, we examined responses of Colorado potato beetles (CPBs; *Leptinotarsa decemlineata*) and their potato host plant (*Solanum tuberosum*) to EPNs. EPNs primarily attack insects below-ground, and this includes foliar-feeding insects, like CPBs, at various life stages (e.g., pre-pupae) in or near the soil (Ebrahimi, Niknam, & Lewis, 2011; Stewart, Boiteau, & Kimpinski, 1998). We predicted that a relevant, EPN-susceptible, above-ground herbivore, such as CPB, would avoid chemical cues from EPNs and plants associated with these natural enemies. We also predicted that some plants, including potato, respond to EPNs or their chemical cues by activating or enhancing their defences. This prediction was based on the previous finding that EPNs increased plant resistance, and from our knowledge that plants respond to a wide variety of cues associated with risk of attack (An et al., 2016; Jagdale et al., 2009). An alternative prediction would be a reduction or relaxation of plant defences following exposure to EPNs, allowing plants to allocate resources to growth or reproduction instead of defence, when natural enemies

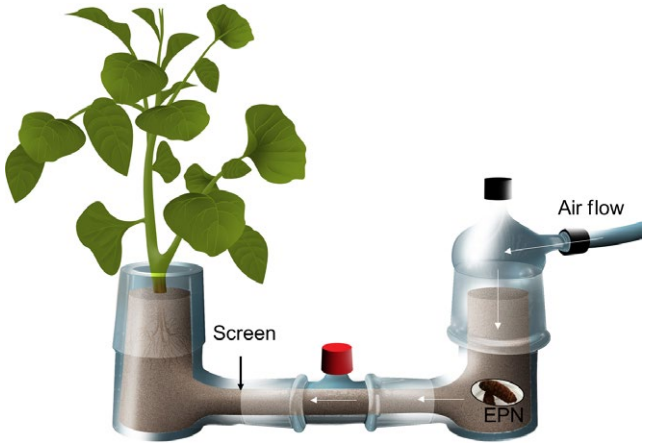

FIGURE 1 Potato plants were exposed to entomopathogenic nematode (EPN) chemical cues by pushing a gentle stream of clean air through the headspace of EPN-infected insect cadavers and onto roots

are available to reduce herbivore pressure. Although numerous studies have examined plant signalling to natural enemies and their role in indirect defence, no previous work has evaluated responses of plants to below-ground cues from organisms in the third trophic level or investigated how such responses might cascade out to affect the behaviour and performance of herbivores. By linking plant detection of chemical cues from organisms that play a beneficial role in their ecology, with herbivore detection of these natural enemies, we can gain additional insight into the complexity of adaptations in tritrophic interactions.

2 | MATERIALS AND METHODS

2.1 | Plants, insects and nematodes

Potato (*S. tuberosum* cv Yukon Gold) stock plants were grown from seed-potato cuttings. Experimental plants were vegetatively propagated from stock plants and used after 3–4 weeks. Plants were grown in individual pots in peat-based potting soil (Pro-Mix BX; Premier Horticulture Inc., USA) with 2 g Osmocote fertilizer (8–45–14 N–P–K; Scotts, USA) added to each pot. Plants were kept in an insect-free, climate-controlled greenhouse (16 hr light: 8 hr dark; 25°C: 22°C; 65% RH).

Colorado potato beetle (*L. decemlineata*) larvae and adults used in experiments were obtained from a colony maintained at the Pennsylvania State University (University Park, USA) that originated at the Vegetable Entomology Laboratory at Michigan State University (East Lansing, USA) (Hufnagel, Schilmiller, Ali, & Szendrei, 2017). Beetles were reared on *S. tuberosum* cv. Yukon Gold.

The EPN species *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* were used in this study because they are commercially available generalists found in natural environments and used for biological control. EPNs were cultured in last-instar wax moth larvae (*Galleria mellonella*) at 25°C. Infective juveniles that emerged from insect cadavers were collected in White traps (White, 1927) and stored in culture flasks at 4°C for up to 3 weeks.

2.2 | Collection and analysis of EPN volatiles

Volatile compounds emitted by EPN-infected cadavers were collected using dynamic headspace sampling and analysed by gas chromatography coupled to mass spectrometry (GC/MS) (n = 5). Seven EPN-infected cadavers (*G. mellonella* with *H. bacteriophora*) were placed in clean glass chambers with autoclaved, moistened sand (Quikrete®, USA). Freeze-killed, thawed *G. mellonella* (control cadavers) and blank sand were used as controls. Clean, filtered air was pushed into chambers at 100 ml/min and removed at 100 ml/min for 2 hr. Volatiles were trapped on adsorbent filters containing 45 mg of HayeSep® Q (Hutchison Hayes Separation Inc., USA) and eluted using 150 µl dichloromethane. A standard solution containing nonyl acetate (80 ng/µl) and *n*-octane (40 ng/µl) was added to each sample (5 µl).

Compounds were identified using an Agilent 6890 gas chromatograph and 5973 mass spectrometer with a splitless injector held at 250°C. After sample injection, the column (Rxi®-1 ms, 30 m, 0.25 mm id, 0.25 µm film thickness; Restek, USA) was maintained at 40°C for 2 min, then ramped at 10°C/min to 280°C. Tentative identification of target compounds was made by comparison of mass spectra and retention times with published data (NIST14 and Gothenburg Department of Chemical Ecology mass spectral library), and structure assignments were confirmed where possible by comparison of mass spectra and GC retention times with those of authentic standards.

2.3 | Plant exposure to EPNs or EPN chemical cues

In separate experiments, roots of potato plants were exposed to live EPNs, EPN cues or appropriate controls. To evaluate direct plant responses to EPNs, we exposed plants to EPN IJs by adding two 90 ml aliquots of water, each containing approximately 35,000 live IJs (*S. carpocapsae*), directly to the soil of each potted plant. Control plants received two 90 ml aliquots of water. One aliquot was added per day, for two consecutive days, to allow for adequate retention of water in the soil. To evaluate plant responses to EPN chemical cues, plant roots were exposed to EPN compounds without physical contact to EPNs. Clean filtered air was forced through the headspace of EPN-infected cadavers (*G. mellonella* with *H. bacteriophora*) and onto plant roots (Figure 1). Two weeks prior to experiments, plants were transplanted into clean glass chambers with peat-based potting mix (Pro-Mix BX; Premier Horticulture Inc.). Four EPN-infected cadavers were added to each of the EPN exposure treatment chambers in autoclaved, moistened sand (Quikrete®), and these were connected to plant chambers by a glass arm filled with sand. Control plants were exposed to headspace cues from either four freeze-killed, thawed *G. mellonella* (control cadavers) in sand, or sand only. Plant roots in all treatments were separated from the exposure-source chambers by 400 mesh screen (MSC Industrial Supply, USA) and a distance of

28 cm. The airflow rate into the chambers was 50 ml/min, with air exiting the system at the soil surface. Plants were exposed to the various treatments for 48 hr prior to taking any measurements and continuously through the duration of the sampling period.

2.4 | Larval performance assay

A no-choice feeding bioassay was conducted to determine the influence of plant exposure to EPN cues on herbivore performance. Larval mass gain and foliar consumption were compared among plants exposed to cues from EPN cadavers, control cadavers or empty sand controls (n = 10). Following the initial exposure, five *L. decemlineata* (CPB) neonates were caged on intact foliage. Larvae were monitored daily and moved to fresh leaves if more than half of the leaf tissue in the cage had been consumed. After 5 days, larvae were removed and weighed. Leaves of each plant were scanned and the total leaf area consumed by larvae was measured using Adobe Photoshop software.

2.5 | Insect herbivore oviposition preference assay

To determine the influence of chemical cues from EPNs on herbivore oviposition, a three-way choice test was conducted with adult female CPBs (n = 14). A single gravid female was placed in a cage containing three individually potted potato plants. One pot contained three EPN-infected cadavers (*G. mellonella* with *H. bacteriophora*), one contained three control cadavers (*G. mellonella*), and one was a soil control. Females were placed in the centre of the arena and allowed to lay eggs for 3 days. Then, the total numbers of eggs and clutches laid on each treatment were counted.

2.6 | Quantification of plant defence hormones and gene expression

Insect herbivore feeding assays were conducted to determine the influence of plant exposure to EPNs or their chemical cues on plant defence responses. Levels of jasmonic acid (JA) and salicylic acid (SA) were quantified as representative defences. After exposure to EPN IJs, EPN cues or the appropriate controls, one undamaged leaf from each plant (~100 mg tissue, n = 9, n = 7) was sampled. Fully expanded leaves of similar size from the upper-middle section of the plant were collected. In the EPN IJ-exposure experiment, three 3rd instar CPB larvae were added to each plant. In the EPN chemical cue-exposure experiment, two adult, female CPBs were caged on each plant and two 3rd instar CPB larvae were simultaneously added to a separate cage on these plants. After beetles fed for 5 or 20 hr, a recently damaged leaf was collected from each plant. Leaf tissue was flash frozen in liquid nitrogen and stored at −80°C until analysed. For quantification of JA and SA, endogenous phytohormones were extracted and derivatized to methyl esters, then isolated using vapour-phase extraction. The compounds were analysed by coupled GC/CI-MS using isobutane and selected ion monitoring (Schmelz, Engelberth, Tumlinson, Block, & Alborn, 2004). Relative amounts of JA and SA were quantified by comparing to 100 ng each dihydro-JA and labelled 2-hydroxy-benzoic acid, added as internal standards. Retention times and spectra were confirmed with standards of pure compounds.

Plant defence gene expression was measured for plants exposed to live EPN IJs (*S. carpocapsae*) with and without insect feeding damage (n = 9), in a similar design to the IJ-exposed phytohormone analysis. Following exposure to EPNs, a leaf of similar size and location was collected from each plant at 0, 5 or 20 hr of damage by three 3rd instar CPB larvae. Leaves were flash frozen in liquid nitrogen and stored at −80°C until analysed. Leaf tissue was homogenized in liquid nitrogen and RNA was extracted with TRIzol reagent (Life Technologies, USA) following the manufacture's protocol (~100 mg tissue in 1 ml TRIzol). RNA was quantified using a Nanodrop microvolume spectrophotometer (Thermo-Fisher Scientific, USA), and cDNA was made from 1 μg total RNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) with Oligo-dT. Quantitative real-time PCR (qRT-PCR) was performed with Fast Start SYBR Green Master Mix (Roche Applied Science, USA) in an Applied Biosystems 7900HT instrument with *ubiquitin* as a reference gene. Expression of *lipoxygenaseD* (*LoxD*), involved in JA biosynthesis, and *PR-1(P4)*, an SA-induced defence gene, was measured using compatible gene-specific primers (Chung et al., 2013). Relative abundance of gene transcripts was measured using the delta-delta CT method and calibrated using an undamaged plant (Livak & Schmittgen, 2001). Three qRT-PCR technical replicates were run for each sample.

2.7 | Statistical analyses

Statistical analyses were performed using the software program R (R Development Core Team, 2017). Larval performance data were analysed using nested ANOVA, after confirming the data met necessary assumptions. Gene expression and phytohormone data were analysed separately for each time point using one-way ANOVA comparisons with Tukey's Honest Significant Differences Test for post hoc multiple comparisons. These data were log-transformed to meet assumptions of normality and equal variance; however, data in figures are not transformed. Oviposition preference data were analysed using a chi-squared goodness-of-fit test (Kariyat et al., 2013).

3 | RESULTS

3.1 | EPN-infected insect cadavers emitted a characteristic volatile blend

The blend of volatiles emitted by EPN-infected cadavers was distinct from that of freeze-killed, thawed control cadavers (Figure 2). Compounds identified in the EPN odour blend included benzaldehyde, benzyl alcohol, acetophenone, nonanal and indole, and none of these were emitted by control cadavers (Table 1).

3.2 | Plant exposure to EPN chemical cues reduced performance of CPB larvae

Colorado potato beetle larvae feeding on potato plants exposed to chemical cues from EPNs gained less mass compared to larvae on

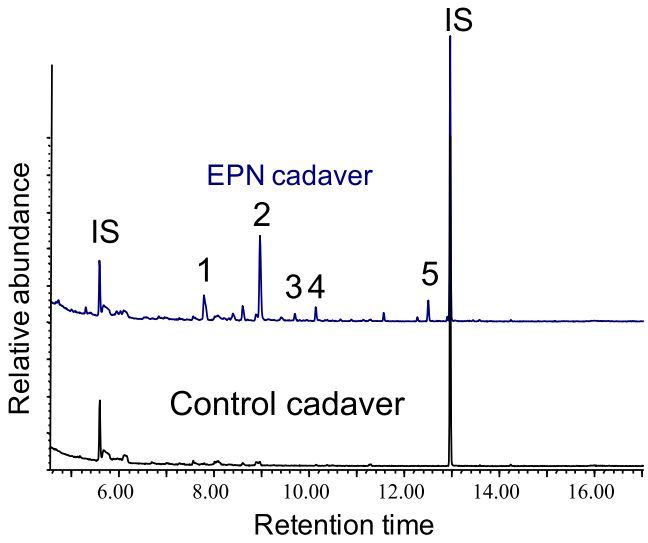

FIGURE 2 Gas chromatograms comparing volatiles emitted by entomopathogenic nematode (EPN)-infected insect cadavers and freeze-killed insects (Control cadavers). List of compounds identified in the EPN volatile blend: (1) benzaldehyde, (2) benzyl alcohol, (3) acetophenone, (4) nonanal, (5) indole

TABLE 1 Relative abundances of volatile compounds emitted by EPN-infected insect cadavers or freeze-killed insects (Control cadavers)

	EPN cadavers		Control cadavers	
Compounds emitted	**ng/g**	***SE***	**ng/g**	***SE***
Benzaldehyde	36.92	8.47	ND	—
Benzyl alcohol	104.83	24.25	ND	—
Acetophenone	13.53	3.62	ND	—
Nonanal	11.13	3.10	ND	—
Indole	21.07	2.31	ND	—

Note. EPN: entomopathogenic nematode; ND: not detected.

control plants (Figure 3a; Nested ANOVA, $F_{2,27} = 109.01$, $p < 0.001$; Plant × Odour $F_{2,27} = 9.38$, $p < 0.001$). Larvae also consumed less leaf tissue while feeding on plants exposed to EPN cues compared to larvae on controls (Figure 3b; ANOVA $F_{2,27} = 11.37$; $p < 0.001$).

3.3 | Presence of EPN chemical cues reduced oviposition by CPB females

Because CPBs are susceptible to attack by EPNs, and larval performance was reduced on EPN-exposed plants, we predicted that female CPBs would preferentially lay eggs on plants without cues from EPNs, to increase the performance and survival of their offspring. We found that CPB females laid approximately 30% fewer eggs on plants in the presence of EPN-infected insect cadavers compared to plants with control cadavers or soil controls (Figure 4; chi-squared goodness-of-fit test, $\chi^2_2 = 68.5$, $p < 0.001$). The observed difference in oviposition preference was related to clutch size as there was no difference in number of egg clutches on each treatment (Chi-squared goodness-of-fit test, $\chi^2_2 = 3.267$, $p = 0.195$).

3.4 | Plant EPN exposure induced systemic defence responses

We found that undamaged potato plants exposed to live EPN IJs induced higher levels of the defence hormone SA compared to unexposed control plants (Figure 5; ANOVA $F_{1,16} = 17.83$, $p < 0.001$). Levels of the defence hormone JA in undamaged, exposed plants were not different from controls ($F_{1,16} = 0.39$, $p = 0.54$). After 5 hr wounding by CPB larvae, SA levels were higher in EPN-exposed plants than controls (Figure 5; ANOVA $F_{1,16} = 4.38$, $p = 0.05$) and this trend persisted at 20 hr of continuous feeding damage (Figure 5; ANOVA $F_{1,16} = 4.12$, $p = 0.06$). JA levels were not different between EPN-exposed or control plants after 5 hr feeding damage by CPB larvae (ANOVA $F_{1,16} = 0.37$, $p = 0.55$) or 20 hr post-damage (ANOVA $F_{1,16} = 0.068$, $p = 0.80$).

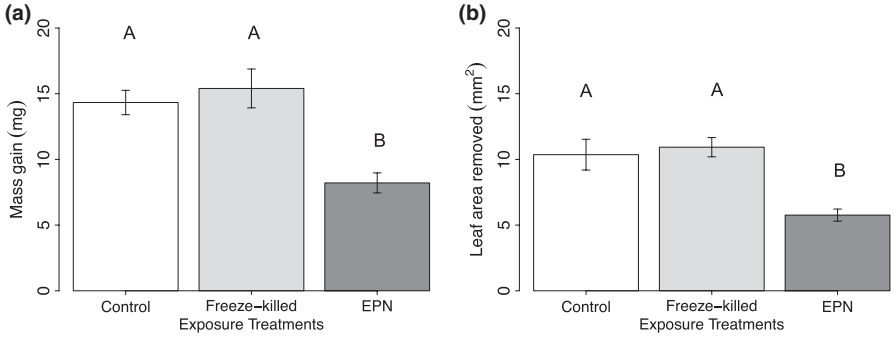

FIGURE 3 Plant exposure to entomopathogenic nematode (EPN) chemical cues reduced performance of Colorado potato beetle (CPB) larvae. (a) CPB larvae gained less mass feeding on plants exposed to EPN chemical cues (EPN) compared to larvae on plants exposed to control cadavers (freeze-killed) and unexposed control plants. (b) Larvae feeding on EPN-cue exposed plants consumed less leaf tissue than larvae on control plants. Bars marked with a different letter indicate significant difference (Tukey post hoc test, $p \leq 0.05$). Error bars correspond to standard errors

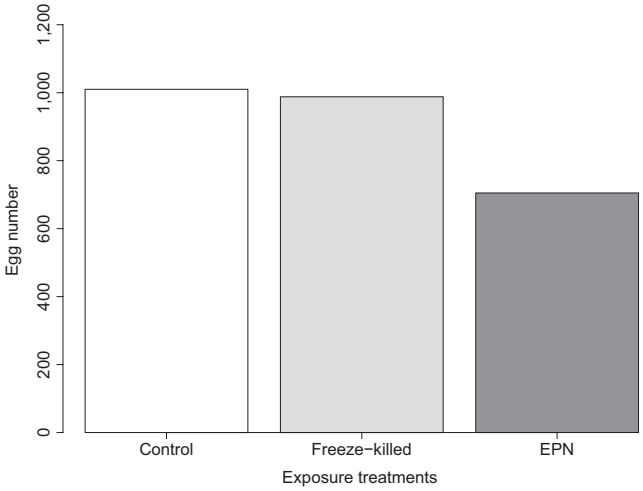

FIGURE 4 Presence of entomopathogenic nematode (EPN) chemical cues reduced oviposition by female Colorado potato beetles (CPBs). In a choice experiment, CPB females laid fewer eggs on plants in the presence of EPN-infected insect cadavers (EPN) compared to plants with control cadavers (freeze-killed) or soil controls

By comparing expression patterns of defence-related genes for EPN IJ-exposed and control plants, we found that undamaged, EPN-exposed plants had higher expression of the pathogenesis-related gene *PR-1(PR4)* (Figure 6; ANOVA $F_{1,16} = 12.87$, $p = 0.002$). There was no difference observed in expression of the wounding response-related gene *LoxD* between undamaged EPN-exposed and control plants (ANOVA $F_{1,16} = 0.53$, $p = 0.48$). Following CPB feeding damage, *PR-1(PR4)* expression was not different from control plants at 5 hr (Figure 6; ANOVA $F_{1,16} = 2.06$, $p = 0.17$) or 20 hr after wounding (Figure 6; ANOVA $F_{1,16} = 0.30$, $p = 0.59$). Expression of *LoxD* was not different between exposure treatments at 5 hr (ANOVA $F_{1,16} = 0.33$, $p = 0.57$) or 20 hr after CPB feeding damage (ANOVA $F_{1,16} = 2.28$, $p = 0.15$).

3.5 | Exposure to EPN chemical cues primed plant defences

Levels of SA and JA were not significantly different among undamaged plants exposed to EPN cues or unexposed control plants (Figure 7; ANOVA $F_{2,18} = 2.64$, $p = 0.10$; Figure 8; ANOVA $F_{2,18} = 1.31$, $p = 0.30$). However, following feeding damage by CPB larvae, plants exposed to EPN cues induced higher levels of both SA and JA compared to unexposed control plants (Figure 7; ANOVA $F_{2,14} = 3.88$, $p = 0.045$; Figure 8; ANOVA $F_{2,18} = 3.46$, $p = 0.05$). These findings indicate both SA- and JA-mediated defences were primed by plant exposure to EPN chemical cues, resulting in elevated defence responses to herbivore feeding damage. Plants exposed to EPN cues showed a trend of slightly higher levels of JA induced by CPB adult feeding compared to control plants, although differences were not statistically significant (Figure 8; ANOVA $F_{2,13} = 3.39$; $p = 0.07$). Levels of SA following feeding by adult CPB were not different among treatments (Figure 7; ANOVA $F_{2,14} = 0.23$, $p = 0.79$).

4 | DISCUSSION

Our findings indicate that plants and insect herbivores respond to below-ground chemical cues from organisms at the third trophic level. Potato plants induced or primed their defences following exposure to EPNs or EPN cues, respectively (Figures 5–8), which reduced the performance of CPB larvae (Figure 3). Female CPBs avoided EPN cues, laying fewer eggs on plants with EPNs (Figure 4). Previous studies have identified an adaptive value for

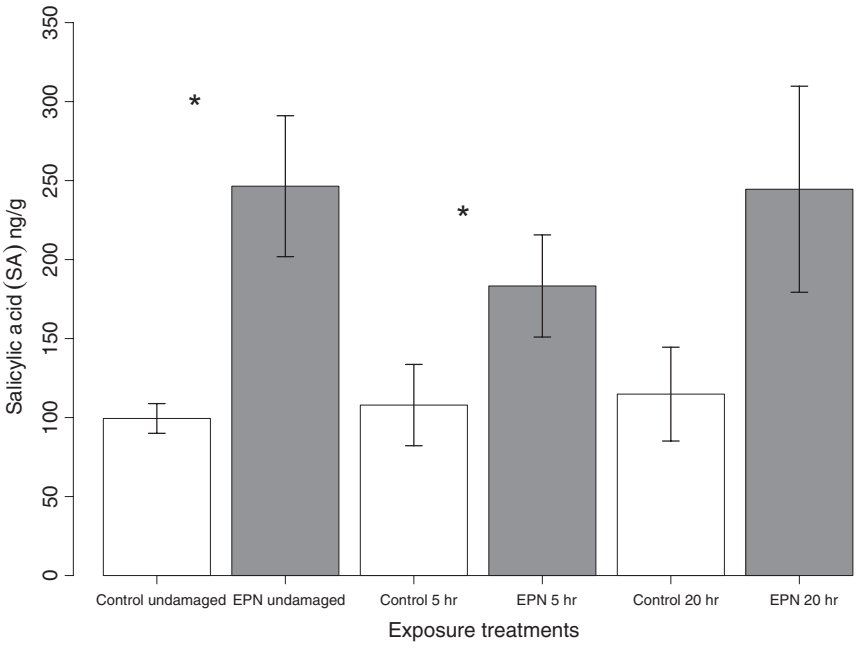

FIGURE 5 Plant exposure to live entomopathogenic nematodes (EPNs) induced higher levels of salicylic acid (SA) compared to unexposed control plants. After 5 hr wounding by Colorado potato beetle (CPB) larvae, SA levels were higher in EPN-exposed plants than unexposed controls. This trend persisted at 20 hr of continuous feeding damage. Pairs of bars marked with an asterisk indicate significant differences ($p < 0.05$). Error bars correspond to standard errors

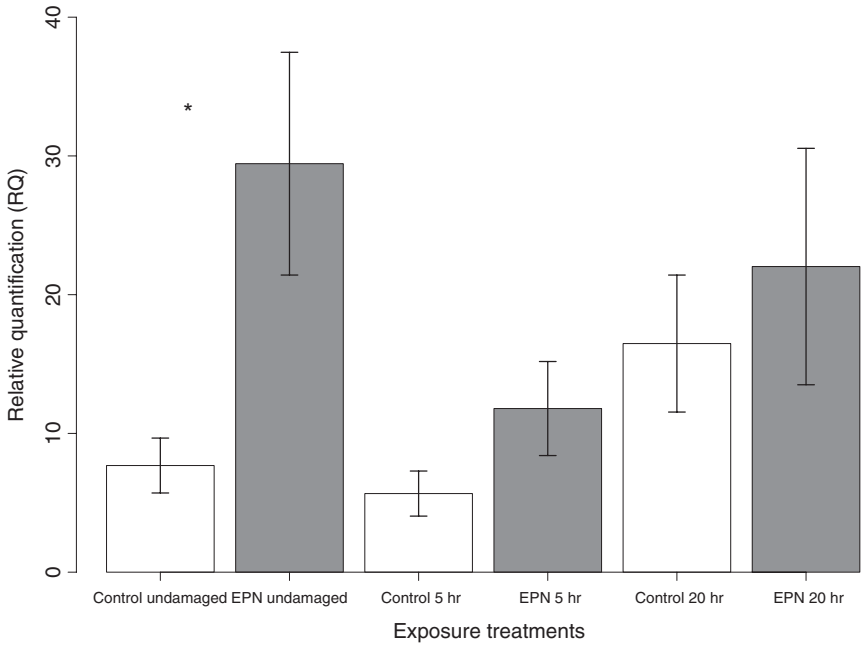

FIGURE 6 Plant exposure to live entomopathogenic nematodes (EPNs) increased expression of the defence gene *PR-1(PR4)*. Undamaged EPN-exposed potato plants had higher expression of *PR-1(PR4)*, but expression was not different from control plants after 5 hr feeding damage by Colorado potato beetle (CPB) larvae or 20 hr of damage. Pairs of bars marked with an asterisk indicate significant differences ($p < 0.05$). Error bars correspond to standard errors

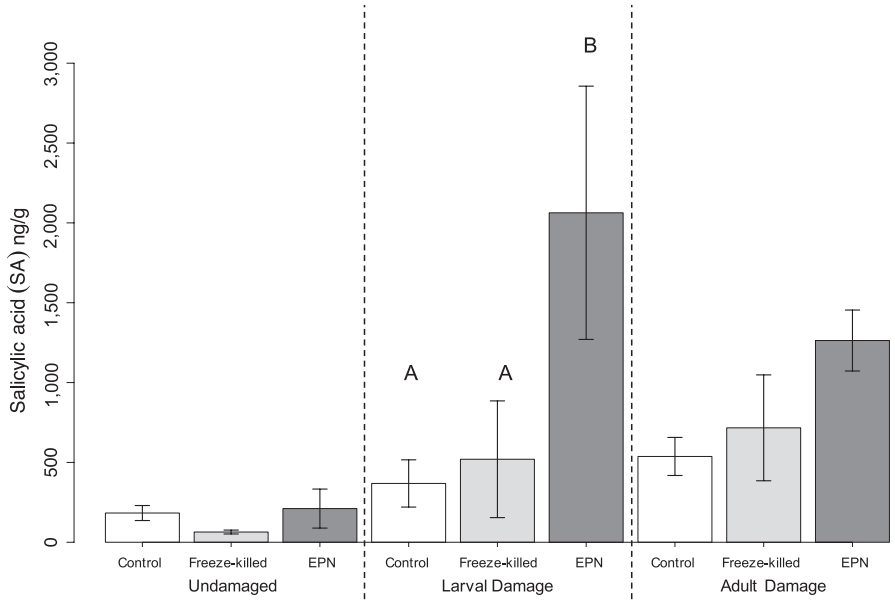

FIGURE 7 Plant exposure to entomopathogenic nematode (EPN) chemical cues primed induction of salicylic acid (SA). Levels of SA were not different among exposure treatments in undamaged plants. After feeding damage by Colorado potato beetle (CPB) larvae, SA levels were significantly higher in plants exposed to EPN chemical cues (EPN) compared to control-cadaver exposed (freeze-killed) and unexposed control plants. Levels of SA were not different among treatments following feeding damage by CPB adults. Groups of bars marked with different letters indicate significant differences ($p < 0.05$). Error bars correspond to standard errors

herbivores to detect and avoid chemical cues from natural enemies, as this can directly increase their performance or survival (Kats & Dill, 1998). Plants also appear to benefit from responding to chemical cues from an herbivore natural enemy, in this context, as enhanced plant defences led to reduced herbivore performance and damage (Figure 3). Recent theoretical work has suggested natural selection should favour a bias towards allocation of plant resources that make the least-costly error, which for plants is likely to be a failure to invest in defence when it is needed (Orrock et al., 2015). In general, we predict the benefit from plant response to a natural enemy should depend on the cost of allocation to defences and the reliability of an indirect indication of risk of attack.

There are a several potential ecological explanations for plants responding to an herbivore natural enemy. One possibility is that this response originated as a case of mistaken identity and overlapping cues, where plants detected EPN cues as a direct threat from pathogens or herbivores. EPNs rely on symbiotic bacteria to infect, kill and prevent putrefaction in their hosts (Ciche et al., 2006; Lewis et al., 2006). Following exposure to EPNs or their chemical cues, plants in this study had elevated defences typically associated with pathogens, plant-parasitic nematodes or phloem-feeding herbivores (Conrath et al., 2006; Erb, Meldau, & Howe, 2012; Manosalva et al., 2015). Based on previous characterizations of microbial volatiles, it is likely that compounds we identified from EPN-infected cadavers are produced by EPN symbionts and plants might associate these

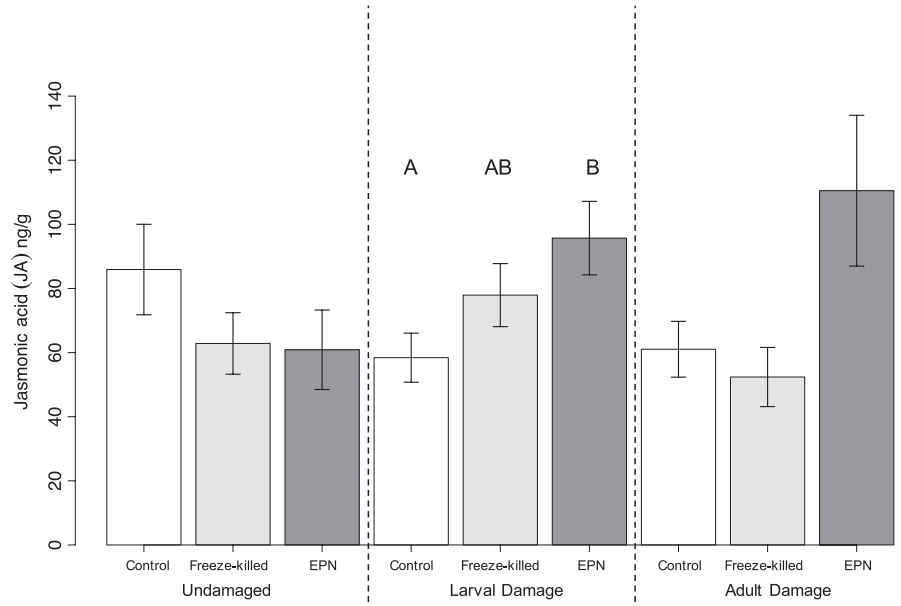

FIGURE 8 Plant exposure to entomopathogenic nematode (EPN) chemical cues primed induction of jasmonic acid (JA). Levels of JA were not different among exposure treatments in undamaged plants. Following damage by Colorado potato beetle (CPB) larvae, JA levels were higher in plants exposed to EPN chemical cues (EPN) compared to unexposed control plants. There was a trend towards higher levels of JA in EPN-exposed plants following feeding damage by CPB adults. Groups of bars marked with different letters indicate significant differences ($p < 0.05$). Error bars correspond to standard errors

cues with a microbial threat (Table 1) (Piechulla, Lemfack, & Kai, 2017; Tomberlin et al., 2017; Ullah et al., 2015). Notably, one of the compounds emitted by EPN cadavers, indole, is also produced by some plant species after herbivore damage and has been identified as a key defence priming signal in maize (Erb et al., 2015). Here, we documented priming of potato plant defences in response to EPN cues (Figures 7 and 8), suggesting plants might detect this compound as a cue associated with herbivore damage. Previous work has also documented increased plant resistance following exposure to plant-parasitic nematode ascarosides, a type of molecule also used in EPN chemical communication (Kaplan et al., 2012; Manosalva et al., 2015). This suggests plants might recognize broad taxonomically conserved cues EPNs share with their plant-parasitic relatives, and the observed plant response is an artefact of an over-generalized response to nematodes.

A second possibility is that plants respond to cues from these herbivore natural enemies because they perceive the presence of EPNs as an indication that herbivores are also present and pose a threat. Herbivores face strong selection pressure to avoid plant detection, suggesting plants may instead use indirect information about an herbivore threat. This could be a "better safe than sorry" approach, where plants are highly sensitive to environmental cues they associate with risk and respond by priming their defences (Orrock et al., 2015). As an alternative to inducing defences, priming arguably incurs little to no fitness cost for plants even in the absence of actual herbivore attack (Martinez-Medina et al., 2016; Yip, De Moraes, Mescher, & Tooker, 2017). An intriguing finding in this study was the subtle difference in plant responses to EPNs or their associated chemical cues. When plants were directly exposed to live EPN IJs, we observed systemic induction of pathogen-associated defences (Figures 5 and 6). When plants were exposed to chemical cues from EPNs, they instead primed their defences, inducing stronger defences following herbivore feeding damage (Figures 7 and 8). Previous studies have identified induction of plant defence following physical contact with herbivore-associated cues, including deposition of insect eggs or insect presence on leaves (Hilker & Fatouros, 2015; Peiffer, Tooker, Luthe, & Felton, 2009). In contrast, plant exposure to chemical cues associated with herbivores frequently results in defence priming (Helms, De Moraes, Tooker, & Mescher, 2013; Hu & Erb, 2018). These findings indicate that plants can modify their responses in a context-dependent manner, responding differently to physical or chemical cues. It is possible plants detect the physical presence of live EPNs as an indication of immediate danger and respond with direct induction of defence, possibly due to mistaken identity or correctly identifying EPN and preparing for future herbivore damage. EPN chemical cues, on the other hand, could represent a potential, though less urgent threat, leading to defence priming.

Enhancing plant signalling to higher trophic levels is another, non-mutually exclusive ecological explanation for plant response to EPNs. EPNs locate potential insect hosts using chemical cues emitted by herbivore-damaged plant roots and can provide an effective indirect defence against herbivores (Ali et al., 2010; Rasmann et al., 2005). We found that plants exposed to EPN cues primed induction of SA and JA in foliar tissue. While we have not determined the changes that occur in plant roots following exposure to EPN cues, we might expect a similar response in roots compared to foliar tissue (Bezemer & Van Dam, 2005). Production of many herbivore-induced plant volatiles (HIPVs), which attract natural enemies, is regulated

through induction of the jasmonate pathway, and priming of JA is often associated with increased HIPV production (Erb et al., 2015; Helms, De Moraes, Mescher, & Tooker, 2014). Some salicylate-mediated changes in plant volatiles also recruit herbivore natural enemies, including EPNs (Filgueiras, Willett, Junior, & Pareja, 2016). Recruiting and retaining higher numbers of EPN natural enemies could boost plant indirect defences and offer plants a competitive advantage, especially if they compete for natural enemies. Such changes in plant metabolites could also signal enemy-dense space to foraging herbivores that they should avoid, offering plants another strategy to avoid herbivore attack.

Herbivores can increase their chance of survival by avoiding cues reliably associated with their natural enemies (Kats & Dill, 1998). EPNs infect various life stages of CPBs, including larvae, pupae and adults, and we observed reduced performance of larvae on EPN-exposed plants (Figure 3) (Ebrahimi et al., 2011; Stewart et al., 1998). Female CPBs laid fewer eggs on plants in the presence of EPN cues (Figure 4). We interpreted this finding as evidence that they perceive EPN cues as a warning of a threat to the performance or survival of their offspring. Female insects often use chemical information to select suitable oviposition sites, often to avoid plant defence, competition or elevated predation risk (Hermann & Thaler, 2018; Kariyat et al., 2013; De Moraes et al., 2001). During the CPB oviposition experiment, one plant in each arena was exposed to EPN-infected cadavers for 3 days. Based on our finding of plant response to EPN cues, this exposure likely enhanced plant chemical defences, providing a feeding deterrent and additional cue of host plant suitability.

Despite the apparent negative consequence for EPNs of alerting and repelling potential prey, we suggest that the EPN-produced cues identified in this study are important for EPN ecology or linked to their metabolism in a way that limits selection against their production. The EPN-symbiont–host complex produces many compounds that are important for EPNs, including pheromones, insecticidal compounds, antimicrobial compounds and scavenging deterrents (Gulcu, Hazir, & Kaya, 2012; Hu et al., 1999; Hu & Webster, 2000; Kaplan et al., 2012; Lu et al., 2017). These metabolites provide a wealth of chemical information for other members of the ecological community, including the nematodes' insect prey and nearby plants. This apparentness to prey is a problem faced by predators from many taxonomic groups and it is unlikely that EPNs can modify their production of such compounds to overcome this challenge (Kats & Dill, 1998). An intriguing parallel with our system has been reported in recent studies documenting differences in plant defence responses to caterpillars infected with parasitoid wasps and their symbiotic polydnaviruses compared to uninfected caterpillars. While one study identified suppression of plant defences that benefitted the parasitoid, another study reported changes in HIPVs that betrayed parasitoids to their hyperparasitoids (Tan et al., 2018; Zhu et al., 2018). These findings provide further evidence that organisms at the third trophic level and their associated symbionts can influence plant responses, which sometimes benefit and are sometimes detrimental for the natural enemies.

Overall, this study demonstrates consequences of top-down chemical information exchange in a multitrophic interaction, where an herbivore and its host plant respond to cues from an herbivore natural enemy. These findings reveal an additional layer of complexity in the chemical ecology of multitrophic interactions with important implications for plant defence and predator–prey dynamics. Plant responses to EPNs have the potential to influence plant interactions with a variety of other organisms, including beneficial and pathogenic microbes, herbivores, and herbivore natural enemies. This sets the stage for future work examining the broader ecological consequences of such interactions, including in other multitrophic systems, and for studies identifying specific EPN cues involved in plant and herbivore responses.

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AUTHORS' CONTRIBUTIONS

A.M.H. and J.G.A. designed research; A.M.H., S.R., M.C.K., N.L.M. and W.G. performed research; A.M.H., S.R. and J.G.A. analysed data; A.M.H., S.R., J.F.T. and J.G.A. wrote the paper.

DATA ACCESSIBILITY

Data are available from the Dryad Digital Repository https://doi.org/10.5061/dryad.5tk7357 (Helms et al., 2019).

ORCID


Anjel M. Helms https://orcid.org/0000-0002-7430-5195

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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