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Modulation of above-belowground plant-herbivore interactions by entomopathogenic nematodes

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ABSTRACT

Plants indirectly mediate above-belowground interactions between root- and shoot- herbivores via changes in primary and secondary metabolism. Such effects can cascade up to affect higher trophic level organisms such as predators, however, to what extent predators can in turn influence plant-mediated above-belowground interactions needs to be further elucidated. In this study, we examined the effect of entomopathogenic nematodes (EPNs) (*Heterorhabditis bacteriophora* and *Steinernema carpocapsae*) on the interactions between a belowground herbivore, the root-knot nematode (RKN) *Meloidogyne arenaria,* and an aboveground herbivore, the green-peach aphid *Myzus persicae* on tobacco (*Nicotiana tabacum)* plants. We found that the effect of RKNs on aphid population growth and reproduction was negative only when EPNs were inoculated near the rhizosphere. We also observed that the addition of EPNs in the rhizosphere annihilated the positive effect of the aphids on the RKNs feeding on tobacco roots. While aphids and RKNs feeding, respectively, reduced the root and shoot biomass independently of EPNs presence in the rhizosphere, the concentration of glucose and nicotine in tobacco leaves was modified by the presence of EPNs in a species-dependent manner. Our study reveals that tobacco plants detect the presence of EPNs in the rhizosphere and in turn they respond by modifying their interactions with the above- and belowground herbivores. Therefore, soil-dwelling organisms that are not directly associated with the plants can also have community-wide effects that are mediated by changes in plant primary and secondary chemistry.

1. Introduction

Soil organisms impact the functioning of plants, as well as higher trophic level organisms such as herbivores and predators above ground (Bardgett and van der Putten, 2014; Yang et al., 2018). Plant-mediated effects of soil organisms, including herbivores and decomposers, on aboveground communities are attributed to both primary and secondary metabolite changes in the foliage of the plants (Bezemer and van Dam, 2005; Heinen et al., 2018; Wurst, 2013). Similarly, systemic plant-induced changes of nutrient and chemistry may also occur on plants exposed to foliar herbivores, which subsequently affect the performance of soil organisms, such as root herbivores (Bardgett et al., 1998; Soler et al., 2012).

Concerning changes in primary metabolites, it has been generally postulated that root feeders' attack on belowground tissues leads to physiological changes mimicking drought stress (Erb and Lu, 2013; Khan et al., 2010). Accordingly, after root herbivory, water content in leaves decreases and in turn, nutrients such as nitrogen, amino acids and carbohydrates increase in shoots, ultimately improving the performance of foliar-feeding herbivores, in particular the phloem-feeders (Masters et al., 1993). This mechanism has been verified in several systems (Kergunteuil et al., 2018a). For instance, root feeders *Otiorhynchus sulcatus* beetle larvae favored the growth of aphid populations in the field, an effect that was mediated by changes in carbon, nitrogen

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and phosphorous (Johnson et al., 2013). However, such effects are highly context dependent. For instance, root induction of plants by herbivory or the damage-related phytohormone jasmonic acid resulted in either decrease (van Dam and Oomen, 2008), or no change of total sugar levels in leaves (Ryalls et al., 2016). From the other side, aboveground herbivory can also alter the concentration of amino acids, nitrogen and sulfur in roots, which has been shown to affect the performance of belowground herbivores (Steinbrenner et al., 2011; Wang et al., 2017).

A second key mechanism underlying above-belowground interactions relies on plant-wide systemic changes in secondary metabolites concentrations in plant tissues following herbivore attack (Eisenring et al., 2018; Huang et al., 2017; Rasmann and Agrawal, 2008). A wide array of secondary metabolites, such as glucosinolates in cabbage (van Geem et al., 2016), terpenoids and flavonoids in tomato (Su et al., 2018) or alkaloids in tobacco (Kaplan et al., 2008) have been shown to either increase or decrease across all organs of the plants in response to feeding of either above- or belowground herbivores, subsequently influencing the performance of the herbivores feeding on the other parts of the plants (Bezemer and van Dam, 2005). Moreover, induced changes in plant nutritional and defensive strategies by aboveground and belowground herbivores affect the growth and physiological status of one another, which further influences higher trophic level organisms such as predators and parasitoids (Kergunteuil et al., 2018b). However, whether these higher trophic-level organisms (i.e. predators) in turn impact the performance of the herbivores, and the plant-mediated above-belowground interactions is still largely unknown.

Aboveground, current evidence is showing that non-consumptive effects of predators can modify herbivore behavior, and in turn plant nutritional and defense qualities (Kafle et al., 2017; Machado et al., 2018). Belowground, these effects are scantly studied, or only investigated in relation to root-feeding nematodes (e.g. De Deyn et al., 2007). Apart from root-feeding nematodes, free-living nematodes are also naturally present in the soil, and are involved in above-belowground interactions through soil nutrient cycling or trophic interactions (Heinen et al., 2018; Johnson et al., 2016). In addition, soils of all continents contain entomopathogenic nematodes (EPNs) that are obligate predators of arthropods, and are usually used as biocontrol agents against soil-dwelling insects (van den Hoogen et al., 2019). EPNs, once inside an arthropod host, they release their symbiotic bacteria in the hemocoel, where they proliferate; causing septicemia that kills the host in 2–5 days (Dillman et al., 2012). EPNs interact with plants, particularly through volatile and non-volatile secondary metabolites released from plants after root-feeding insect attack (Rasmann and Turlings, 2016; Rasmann et al., 2005). Interestingly, besides repressing root-feeding insects, EPNs were also found to inhibit the population growth of plant-parasitic nematodes (Caccia et al., 2013). This effect was likely mediated by the EPNs-associated bacteria releasing nematicidal substances, such as stilbene derivatives and ammonia, in the rhizosphere (Kenney and Eleftherianos, 2016; Kepenekci et al., 2018). In addition, it was previously shown that EPNs could also induce systemic plant resistance against foliar-feeding herbivores, including foliar nematodes (Jagdale et al., 2009), or chewing and sucking insects (An et al., 2016). For instance, the EPN species *Heterohabditis bacteriophora* and *Steinernema carpocapsae* reduced the performance and preference of Colorado potato beetles (*Leptinotarsa decemlineata*) on potato plants by interfering with the salicylic acid-dependent pathway in foliar tissue (Helms et al., 2019).

In the present study, we analyzed the effects of EPNs on plant-mediated above-belowground herbivore interactions. To this end, we exposed tobacco plants (*Nicotiana tabacum*) to foliar-feeding aphids *Myzus persicae* and belowground root-knot nematodes *Meloidogyne incognita*, and examined how the two herbivores as well as their interactions are affected by the rhizosphere inoculation of two EPN species *Heterorhabditis bacteriophora* and *Steinernema carpocapsae*. We hypothesized that (1) aboveground aphids and belowground RKNs will negatively affect each other due to the induced changes in both primary and secondary compounds, but (2) the outcome of interactions between aphids and RKNs depends on the presence of EPNs, and (3) the effects of EPNs on above-belowground interactions depend on the species of EPNs..

2. Materials and methods

2.1. Plants, nematodes and aphids

Seeds of *Nicotiana tabacum* var. MS k326 were purchased from Yuxi Zhongyan Tobacco Seed Company (Yunnan, China). The seeds were sown in germination trays filled with high phosphorus organic nursery substrate (provided by China tobacco Yunnan industrial Co., Yunnan, China). The major components of the substrate were peat, powdered rock phosphate, and vermiculite. The trays were floated in sterilized water and placed in a tunnel-shaped greenhouse that was fenced with one 1-m-high nylon net (250 μm in diameter) at each side and fully covered by a plastic film. The tunnel was exposed to natural light, and the temperature in the tunnel was manually adjusted by lifting or replacing the plastic film when needed. This experiment was done in Yuxi, Yunnan, China (102.53°N, 24.38°E) in September 2016.

The root knot nematode (RKN) *Meloidogyne arenaria* (Meloidogynidae) is an obligate endoparasite of several plant species (Arens et al., 1981). Typically, *M. arenaria* infects plant roots at the 2nd juvenile stage, and reproduces in the roots for multiple generations, ultimately causing significant reduction in plant productivity (Nicol et al., 2011; Sun et al., 2006). For our experiments, egg masses of *M. arenaria* were collected from severely infected roots of tobacco plants sampled from a cultivated tobacco field in Yuxi county, Yunnan province, China (102.58°N, 24.27°E). Egg masses were sterilized using 1% NaClO for 1 min, rinsed thoroughly with tap water and then transferred in sterilized water. The egg masses were incubated in the dark in a growth chamber at 25 °C for 48 h. The newly hatched *M. arenaria* juveniles were collected and counted under an inversed-light microscope (Olympus CKX41, Japan) to determine the density of nematodes in the suspension. Eventually, the density of the RKNs was adjusted to 375 individuals per mL.

The aphid species *Myzus persicae* is among the most abundant herbivore pest of several crop species, including tobacco plants in the region of Yuxi county (Yang et al., 2009). Adults of *M. persicae* were collected from the same field where RKNs were collected, and reared on tobacco plants *Nicotiana tabacum* L. in mesh cages (3 × 5 × 1.6 m) in the same greenhouse as described above until use in the experiment.

Two EPN species *Heterorhabditis bacteriophora* (Hb) and *Steinernema carpocapsae* (Sc) were used in our study. The two EPN species were provided by USDA-ARS, Southeastern Fruit and Tree Nut Research Lab, Byron, GA, and reared on late-instar waxworms (*Galleria mellonella*) larvae. Third instar dauer juveniles recovered from the White traps (White, 1927) were maintained in distilled water at a density of 250 IJs /mL until the bioassays.

2.2. Experimental design

To measure the effect of EPNs on the performance of above- and belowground herbivores as well as on plant-mediated above-belowground interactions, we ran a full-factorial design experiment (experiment 1) that included three treatments: RKN (two levels: presence / absence) × aphids (two levels: presence / absence) × EPNs (three levels: Hb, Sc, and no EPNs). A total of 180 pots (14 × 22 × 22 cm) were prepared for the experiment (n = 15 pots per treatment). Each pot was filled with 2 kg sterilized growing substrate (moisture = 14% w/w), consisting of sterilized sand and peat (v:v = 1:2) with N, P and K (KLASMANN, Germany). The substrate was sterilized by autoclave at 121 °C for 20 min. Twelve weeks after the seed germination, one individual seedling was transplanted into the middle of each pot. All

plants were watered every 3 days ad libitum. One week after the transplanting, 4 mL of RKN suspension consisting of 1500 *M. arenaria* individuals was inoculated into the sterilized soil near the roots within each corresponding pot. At the same time, the pots were also inoculated with 1000 individuals of one-week-old either *H. bacteriophora* or *S. carpocapsae* in 4 mL suspension (depending on the assigned treatment). Nematode-free treatments (no RKNs or EPNs) received 4 mL distilled water instead.

After nematode inoculation, all plants were caged individually using an insect mesh (80 × 80 × 120 cm) to avoid potential unsolicited herbivory. Successively, all the pots were randomly placed in the aforementioned greenhouse and rotated once per week to avoid position effect. Three weeks after the nematode inoculation, ten 4th instar *M. persicae* were added on a fully expanded leaf on half of the plants using a fine brush. The number of aphids on each individual plant was counted at 3, 6, 9 and 12 days after the introduction. Fifteen days after the commencement of the aphid treatment, all plants were harvested. Plant shoots were cut off at the level of soil surface and the aphids were gently washed off using water. The youngest fully expanded leaf of each plant was sampled using a blade, and immediately flash–frozen in liquid nitrogen, and later stored at −80 °C for metabolic analysis. The remaining shoot tissues were oven dried at 80 °C for 48 h. Plant roots were carefully washed off the soil media and kept at −20 °C for the nematode extraction. Frozen roots were thawed and blended in 1% NaClO solution for 30 s. The obtained suspension was decanted through a 74-μm and a 23-μm mesh sieves. All the material from the 23-μm sieve was collected in 50 mL Eppendorf tubes for nematode counting (Ruan et al., 2012) and the rest root material was oven-dried to determine plant root biomass.

A parallel experiment (experiment 2) was also established to examine the isolated and combined effects of RKNs and EPNs on the reproduction potential of aphids. We prepared 60 plants with 10 replicates per treatment, consisting of 1) plants with aphids, and RKNs only, 2) plants with aphids, RKNs, and Hb, and 3) plants with aphids, RKNs, and Sc. Two weeks after nematodes' inoculation as described above, one neonate aphid was added into a clip-cage attached to a fully expanded leaf of each plant. The aphids in the clip-cages were checked daily, and all the newly-born aphid nymphs were removed and counted over a period of 16 days.

2.3. Metabolic measurement

From experiment 1, at the end of the experiment, we collected leaf samples, which were freeze-dried for 24 h (Christ ALPHA1-4D, Germany) and ground into powder and sieved through a 380-μm mesh sieve. Twenty mg of leaf powder were transferred into a 2-mL Eppendorf tube and suspended in 1.5 mL extraction solvent containing isopropanol/acetonitrile/water (3:3:2, v:v:v). After a 200 W ultrasonic bath (Scientz SB-5200D, China) for 40 min, the samples were centrifuged at 10000*g* for 10 min. A subsample of 400 μL of the supernatant was transferred into a 2.5-mL Eppendorf tube and dried by N-EVAP concentrator (Hengao HGC-24A, China), and 100 μL of methoxyamine hydrochloride in pyridine (20 mg/mL) was added to each tube for derivatization. The obtained suspension was vortexed for 1 min and incubated at 37 °C for 90 min. Subsequently, the suspension was added with 80 μL of MSTFA and incubated at 37 °C for another 30 min. The supernatant (70 μL) was transferred to a conical insert of a 2 mL glass vial for GC/MS analysis. Thirty-five μL of nonadecanoic acid (0.8 μg/mL) was finally added to the glass vial as internal standard (Zhang et al., 2013; Zhao et al., 2016).

For chromatographic analyses, 1 μL of each sample was injected into an Agilent GC–MS system (Agilent 7890-5975C, Agilent, Santa Clara, California, USA) with a split ratio of 10:1 (inlet temperature 290 °C), and separated on a fused-silica capillary column (Agilent HP-5ms, USA; 30 m × 0.25 mm i.d., 0.25 μm thin layer). Helium gas was applied as carrier gas with a flow rate of 1 mL/min. The initial oven temperature was set at 70 °C for 4 min, ramped to 290 °C at 5 °C/min, and held for 10 min (Zhang et al., 2013; Zhao et al., 2016). Detection was done on the same GC–MS system for sample injection. Quantification of metabolites was done by calculating the ratio of individual compounds' peak area relative to the total peak area following corrections using the area of the internal standard.

2.4. Statistical analyses

2.4.1. EPNs-mediated RKN effect on aphid population growth

All statistical analyses were performed using the R statistical package, version 3.5.1 (R Core Team, 2018). For experiment 1, the interactive effect of RKNs and EPNs on the number of aphids at 3, 6, 9 and 12 days after first inoculation on leaves (i.e. aphids' population growth rate) were analyzed using a repeated-measures linear mixed effect model analysis, with RKN (+/−), EPN treatment (+/−), and their interaction as fixed factors, and days post-inoculation as a random factor (function *lmer* in the package *lme4* in R (Bates et al., 2015)). Pairwise treatment comparisons were done with Tukey HSD post-hoc tests (function *emmeans* in the library *emmeans* (Searle et al., 1980)). Aphid counts were log-transformed prior analysis to meet homoscedasticity assumptions.

2.4.2. EPNs-mediated RKN effect on aphid reproduction

For experiment 2, the effect of the two EPNs (Hb and Sc) on below-to-aboveground interaction (i.e. effect of RKNs on aphid reproduction after two weeks of plant growth) was analyzed by computing the Cohen's d effect size (Cohen, 1988) between plants with aphids only and plants with both aphids and RKNs. For this we used the function *cohen.d* with hedges correction in the package *effesize* in R (Torchiano, 2018). Specifically, we measured the effect size in the absence of EPNs, and the, sequentially, with Sc and Hb. Significant differences in effect sizes between EPN treatments (no EPNs, Sc and Hb) were estimated with a Welch modified two-sample *t*-test (function *tsum.test* from the package *PASWR* (Arnholt, 2012)) after Benjamini and Hochberg p-value adjustment. With these analyses, we were thus able to visualize plant mediated above-belowground interactions between RKNs and aphids in the presence or absence of EPNs.

2.4.3. EPNs-mediated effect of aphids on RKNs

For experiment 1, the effect of the two EPNs (HB and Sc) on above-to-belowground interaction (i.e. the effect of aphids on RKNs numbers after 15 days post-inoculation was analyzed by computing the Cohen's d effect size as described above. In this case, the response variable was the numbers of RKNs.

2.4.4. Plant traits

The effect of RKNs, aphids and EPNs on plant shoot and root biomass as well as the concentrations of glucose and nicotine in the plant leaves were analyzed using three-way ANOVAs with main factors including; RKNs (+/−), aphids (+/−) and EPNs (no EPNs, Sc, Hb). Metabolites data were log-transformed to meet the homoscedasticity assumptions.

3. Results

3.1. Aphid population growth and reproduction

We found that the presence of EPN species decreased aphid population growth, to the same extent for both species (Fig. 1; EPN effect, $F_{2,351} = 9.26$, $p = 0.0001$; and pairwise analysis results: Hb vs. Sc: $t = 0.311$, $p = 0.94$; Hb vs. Control: $t = -3.56$, $p = 0.001$; Sc vs. Control: $t = -3.87$, $p = 0.0004$). On the other hand, we found no effect of RKN treatment on aphid growth (Fig. 1; RKN effect, $F_{1,351} = 0.003$, $p = 0.95$, and RKN by EPN interaction effect; $F_{2,351} = 1.02$, $p = 0.36$).

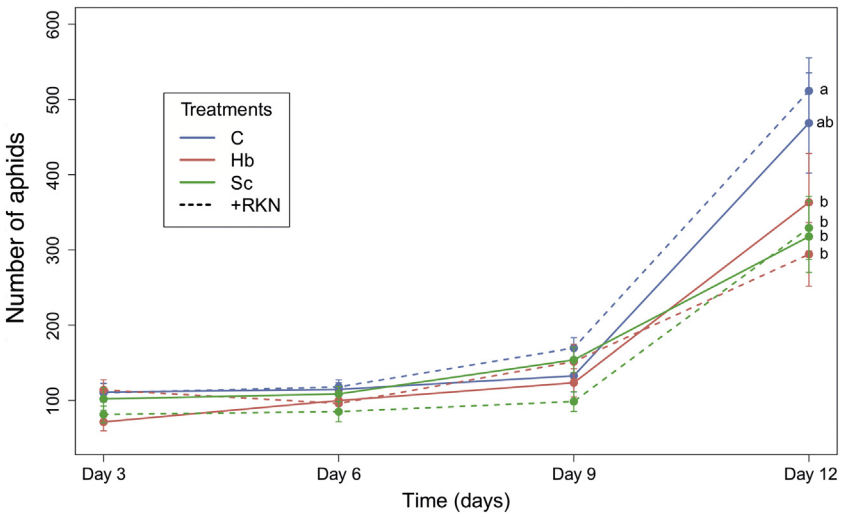

Fig. 1. Effect of root herbivory and entomopathogenic nematodes (EPNs) on aboveground aphid population growth. Shown are mean (± SE) number of aphid *Myzus persicae* at 3, 6, 9, 12 days post-inoculation on leaves of tobacco *Nicotiana tabacum* plants grown in soil that were previously inoculated with either root-knot nematode (RKN, dotted lines,) *Meloidogyne arenaria*, or not (solid lines). Moreover, the rhizosphere of plants were also inoculated with entomopathogenic nematodes *Heterorhabditis bacteriophora* (Hb, red lines), *Steinernema carpocapsae* (Sc, green lines), or neither (C, blue lines). Different letters indicate differences among treatments (p = 0.05; TukeyHSD after repeated-measures ANOVA, n = 15). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

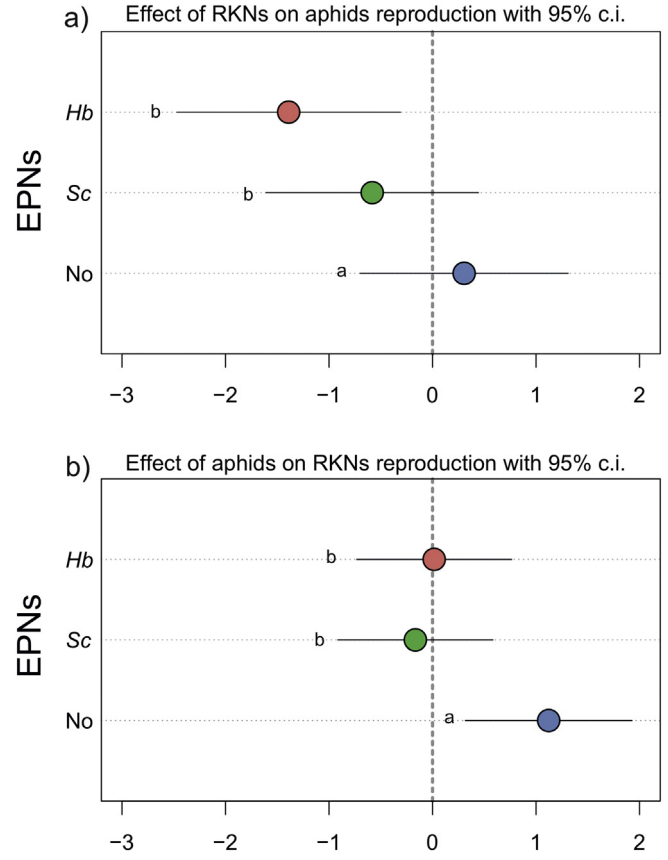

Fig. 2. Effect of entomopathogenic nematodes on above-belowground plant-herbivore interactions. Shown are the mean Cohen's d effect sizes of a) inoculation of root-knot nematodes (RKN) *Meloidogyne arenaria* to soil on the reproduction of foliar-feeding aphids *Myzus persicae* and b) the introduction of aphids *M. persicae* on reproduction of RKN *M. arenaria* on tobacco *Nicotiana tabacum* two weeks after the inoculation of either entomopathogenic nematodes species *Heterorhabditis bacteriophora* (Hb, red dots) and *Steinernema carpocapsae* (Sc, green dot) or neither (No, blue dots) to the rhizosphere. Horizontal bars indicate 95% confidential interval (n = 8–10 per treatment). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Cohen's d effect size analyses indicated that inoculation of RKNs in the soil did not significantly affect aphid reproduction when RKNs were alone in the rhizosphere. However, we found a negative effect of RKNs on aphid reproduction when EPNs, particularly Hb, were also added to the rhizosphere (Fig. 2a).

3.2. Number of root-knot nematodes at harvest

Addition of aphids to plants alone significantly increased the number of RKNs on tobacco roots (Fig. 2b). However, this aphid effect was dampened when EPNs were also inoculated in the soil, independently of the EPN species added (Fig. 2).

3.3. Plant traits

Shoot biomass was 13% lower for plants exposed to aphid feeding compared to plants without aphids (Fig. 3a, Table 1). On the contrary, root biomass was reduced by RKNs by 25% (Fig. 3b, Table 1), and by aphid feeding by 12% (Fig. 3b, Table 1). EPNs had no effect on plant biomass (Table 1).

The concentration of glucose in plant leaves was neither affected by aphids nor by RKNs, but by the presence of EPNs in the soil (Fig. 3c, Table 1). Overall, EPN presence increases glucose concentrations by 67% in the leaves of tobacco plants. This EPN effect was particularly noticeable with Hb treatment alone.

The concentration of nicotine in leaves was idiosyncratically shaped by the presence or absence of EPNs depending on the presence or absence of aphids and RKNs (Fig. 3d, see three-way interaction in Table 1). For instance, in the absence of herbivory, EPNs decreased the amount of nicotine, but in the presence of aphids, EPNs presence increased nicotine concentration, an effect slightly dampened by RKNs feeding.

4. Discussion

We found that soil-dwelling entomopathogenic nematodes (EPNs) in the rhizosphere of tobacco plants affected plant-mediated above-belowground interactions between aphids and root-knot nematodes (RKNs). Their presence induced a negative effect of RKNs on aphid performance, and eliminated the positive effect of aphids on RKNs. EPNs presence in the soil did not change plant biomass production, but, in conjunction with aphids and RKNs, they modified primary (glucose) and secondary (nicotine) metabolites. Below, we expand on each of these findings.

We found that soil inoculation of EPNs reduced the population of aphids feeding on tobacco plants and this effect only occurred after relatively long time subsequent aphid addition to the plants (i.e. 12 days post-inoculation). This result is in line with the findings of the

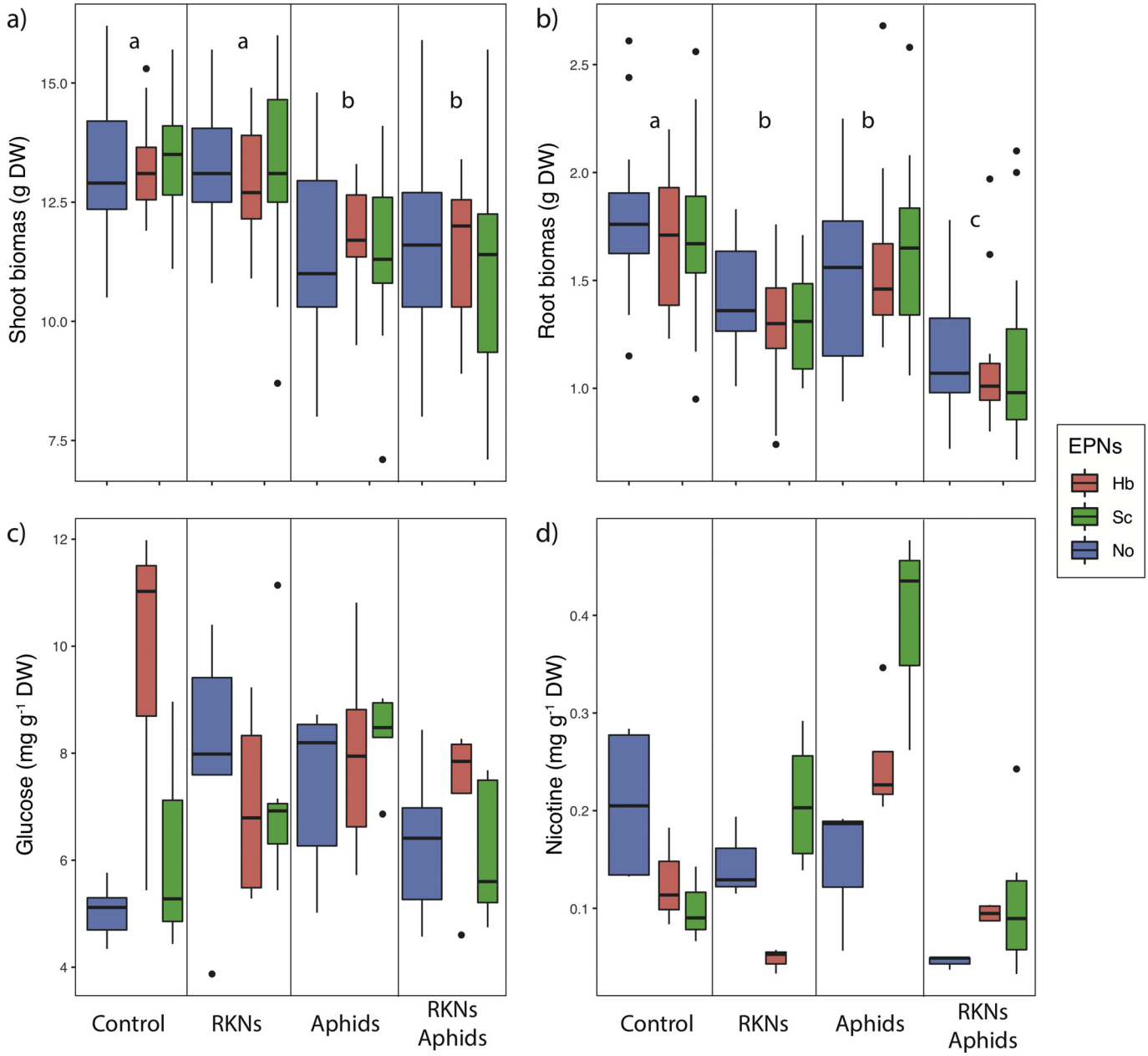

Fig. 3. Effect of above- and belowground herbivory, and entomopathogenic nematodes (EPNs) on plant primary and secondary metabolites. Shown are the mean (± SE) a) shoot biomass, b) root biomass, c) glucose and d) nicotine concentrations of leaves of tobacco *Nicotiana tabacum* after two weeks of introduction with either foliar-feeding aphids (Aphids) *Myzus persicae*, root-knot nematodes (RKNs) *Meloidogyne arenaria*, both aphids and root-knot nematodes (RKNs + Aphids) or neither (Control) to plants grown in soil inoculated with EPN species *Heterorhabditis bacteriophora* (Hb, red boxes), *Steinernema carpocapsae* (Sc, green boxes) or neither (No, blue boxes) (see Table 1 for main effects of treatments after three-way ANOVAs). Different letters above bars indicate differences among main factors (aphids, or RKN). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

aphid reproduction experiment, in which EPNs negatively affected the reproduction rate of the aphids on tobacco plants. In previous studies, it was also observed that EPNs can affect aboveground herbivores (Jagdale et al., 2009). For instance, a study by An et al. (2016) found that the addition of EPNs in the rhizosphere decreased the population of whitefly *Bemisia tabaci*, the growth of *Spodoptera littoralis* noctuid butterfly caterpillars, as well as the infection of the bacterial pathogen *Pseudomonas syringae* on tomato plants. These results thus confirm that soil application of EPNs can result in a broad-spectrum systemic induced resistance in plants, likely through changes in primary and secondary metabolism (An et al., 2016). Along these lines, it was previously shown that several defense-related genes, as well as production of defensive enzymes and hormones, were activated following application of EPN and their symbiotic bacteria in the rhizosphere of *Hosta* sp. and *Arabidopsis thaliana* (Jagdale et al., 2009). Here, we found that EPNs affected leaf nicotine content of tobacco plants in an aphid presence dependent manner. Particularly, the presence of the nematode *H. bacteriophora* in the rhizosphere decreased nicotine in leaves when aphids were absent, but increased nicotine when aphids were present (see significant EPNs by aphid interaction in Table 1). Together, previous findings and ours suggest that plants may respond to the presence of EPNs near their roots, likely through chemical signals (Ali et al., 2010; Rasmann et al., 2012), but the exact mechanisms mediating such interactions should be studied further.

We also found that primary metabolites in tobacco leaves were also affected by the presence of EPNs in the rhizosphere. Particularly, the

Table 1
Three-way ANOVA table for assessing the effects of aphids *Myzus persicae* (presence/absence), root-knot nematodes *Meloidogyne arenaria* (RKNs; presence/absence), and free-living entomopathogenic nematodes species *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* (EPNs; Sc/Hb/no) on the shoot biomass, root biomass, glucose and nicotine concentrations in the foliage of tobacco species *Nicotiana tabacum* at harvest. Degrees of freedom (*df*), F and p-values are also shown ($n = 15$ for shoot and root biomass; $n = 3$–8 for glucose, and n = 3–6 for nicotine). The up and downward arrows indicate increases and decreases in the amount of plant trails, respectively.

Source	*df*	Shoot biomass			Root biomass			Glucose			Nicotine		
		F	p-Value		F	p-Value		F	p-Value		F	p-Value	
Aphids (A)	1	**46.22**	**< 0.001**†	↓	**13.47**	**< 0.001**	↓	0.00	0.98		0.81	0.37	
RKN (R)	1	1.04	0.31		**62.52**	**< 0.001**	↓	2.60	0.11		**23.50**	**< 0.001**	↓
EPN (E)	2	0.02	0.98		0.30	0.74		**4.59**	**0.01**	↑	**4.28**	**0.02**	↓
A × R	1	0.03	0.86		0.18	0.67		3.85	0.05		**18.04**	**< 0.001**	↓
A × E	2	0.53	0.59		1.07	0.35		1.87	0.16		**7.18**	**< 0.001**	↑
R × E	2	0.24	0.79		0.41	0.67		2.36	0.10		0.13	0.88	
A × R × E	2	0.03	0.97		0.18	0.83		**4.68**	**0.01**	↑	**8.12**	**< 0.001**	↓
Residuals		168			168			58			31		

† Bold values indicate statistical significance at p = 0.05.

presence of *H. bacteriophora* increased glucose content in the leaves in an aphid and RKNs dependent manner, in which, the effect of EPNs on leaf glucose concentration was positive without both herbivores, but EPNs had no effect on glucose concentration when herbivores were also present. Therefore, we could not detect a direct link between sugar content in leaves and aphid growth, as was previously observed (Cao et al., 2017). However, we observed that the presence of EPNs in the rhizosphere stimulated the induction of nicotine in leaves in conjunction with aphid presence. Therefore, a direct effect of nicotine on aphid performance seems to have emerged (Devine et al., 1996). To confirm these findings, however, further tests should be performed by for example using glucose- or nicotine-deficient mutant of tobacco plants (von Dahl and Baldwin, 2007; Zhu et al., 2005).

Previous studies found that aboveground herbivores usually negatively affect belowground herbivore survival, but tend to increase population growth rates (Johnson et al., 2012). In line with these observations, we indeed found a positive effect of leaf aphids on the population growth of belowground herbivore RKN in plant roots. This may be because the feeding of aphids enhanced the nutritional quality of the roots, such as N content, which further promoted the reproduction of RKNs (Wang et al., 2017). Alternatively, aphid feeding has also been shown to reduce defense compounds (e.g. nicotine) levels in roots, which in turn potentially helped on promoting the growth of RKNs (Machado et al., 2018; McCarville et al., 2014). However, in our study, when EPNs were also inoculated to soil, the positive effect of aphids on RKNs was annulled, regardless of the EPN species added. It is possible that EPNs directly interfere with the population growth of RKN (Kenney and Eleftherianos, 2016). However, we can exclude this possibility in our study as we did not find a direct inhibition of EPNs on the RKN reproduction (Fig. S1: effect of EPNs on RKNs without aphids; $F_{2,42} = 1.83$, $p = 0.17$). The alternative explanation is that the addition of EPNs may directly repress the infective abilities of RKNs (but not reproduction), thus reducing the positive effect of aphids on RKNs in our study (Kepenekci et al., 2018). This is indicated by the fact that root biomass was reduced by RKN alone, but this effect disappeared when EPNs were also added to the rhizosphere. Also, the negative effect of EPNs on RKNs may be generated by the positive indirect effect of EPNs on plant defenses, ultimately resulting in the suppression of the aphid feeding behavior and RKN reproduction (Manosalva et al., 2015). This was supported by the result of increased nicotine content in plant leaves and the reduced aphid population growth at the presence of EPNs in the current study, but this needs to be confirmed mechanistically in future studies.

5. Conclusions

Our study showed that inoculation of EPNs in soil reduces the population growth of aphids on tobacco plants, an effect likely mediated by an induced change of plant secondary metabolites, such as nicotine, more than primary metabolites, such as glucose in this case. Soil inoculation of EPNs also suppressed the population development of RKNs by counteracting the positive effects of aphids. These findings collectively show that free-living soil-dwelling EPNs may strongly modulate above-belowground herbivore interactions via multiple direct and indirect pathways. This study highlights the complexity of above-belowground community interactions as well as the necessity of integrating non-herbivorous high-trophic level organisms to fully understand community-level dynamics in both natural as well as agricultural systems.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2019.103479.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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