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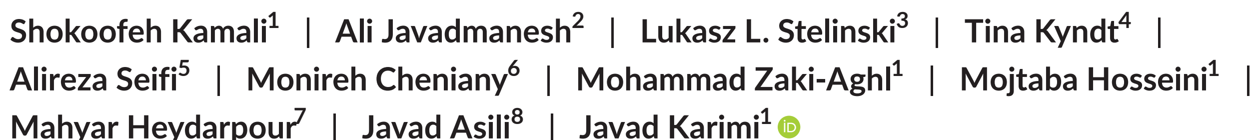
Beneficial worm allies warn plants of parasite attack below-ground and reduce above-ground herbivore preference and performance



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Abstract

Antagonistic interactions among different functional guilds of nematodes have been recognized for quite some time, but the underlying explanatory mechanisms are unclear. We investigated responses of tomato (*Solanum lycopersicum*) to two functional guilds of nematodes—plant parasite (*Meloidogyne javanica*) and entomopathogens (*Heterorhabditis bacteriophora*, *Steinernema feltiae* below-ground, and *S. carpocapsae*)—as well as a leaf mining insect (*Tuta absoluta*) above-ground. Our results indicate that entomopathogenic nematodes (EPNs): (1) reduced root knot nematode (RKN) infestation below-ground, (2) reduced herbivore (*T. absoluta*) host preference and performance above-ground, and (3) induced overlapping plant defence responses by rapidly activating polyphenol oxidase and guaiacol peroxidase activity in roots, but simultaneously suppressing this activity in above-ground tissues. Concurrently, we investigated potential plant signalling mechanisms underlying these interactions using transcriptome analyses. We found that both entomopathogens and plant parasites triggered immune responses in plant roots with shared gene expression. Secondary metabolite transcripts induced in response to the two nematode functional guilds were generally overlapping and showed an analogous profile of regulation. Likewise, we show that EPNs modulate plant defence against RKN invasion, in part, by suppressing active expression of antioxidant enzymes. Inoculations of roots with EPN triggered an immune response in tomato via upregulated phenylpropanoid metabolism and synthesis of protease inhibitors in plant tissues, which may explain decreased egg laying and developmental performance exhibited by herbivores on EPN-inoculated plants. Furthermore, changes induced in the volatile organic compound-related transcriptome indicated that *M. javanica* and/or *S. carpocapsae* inoculation of plants triggered both direct and indirect defences. Our results support the hypothesis that plants "mistake" subterranean EPNs for parasites, and these otherwise beneficial worms activate a battery of plant defences associated with systemic acquired resistance and/or induced systemic resistance with concomitant antagonistic effects on temporally co-occurring subterranean plant pathogenic nematodes and terrestrial herbivores.

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KEYWORDS
biological control, entomopathogenic nematodes, EPN-induced plant defence, *Meloidogyne javanica*, molecular ecology, multitrophic interactions, phenylpropanoid biosynthetic pathway, *Tuta absoluta*

1 | INTRODUCTION

Multitrophic interactions have often been investigated within the context of herbivory given that plants, as accessible autotrophs, are central players joining communities across trophic levels (Schmitz, 2008). Traditionally, such interactions have been examined in the context of above-ground ecosystems (Dicke et al., 1999; Dicke & Sabelis, 1987; Turlings et al., 1990). Recently, however, the development of new (bio) chemical and molecular techniques has enabled exploration of below-ground communities and their interactions (Ali et al., 2012; Aratchige et al., 2004; Rasmann et al., 2005; Van Tol et al., 2001). These below-ground communities provide a rich context for understanding multitrophic interactions because all of the components of above-ground systems (primary, secondary, tertiary predators, herbivores, volatile communication, etc.) are present and they can be manipulated in laboratory and field settings. Nematodes are a diverse group of ubiquitous roundworms serving many ecological roles in subteranen communities (Cobb, 1914). Parasitic behaviour has evolved multiple times among nematodes (Blaxter, 2003), which can act as parasites of plants, vertebrates, or arthropods (Brown et al., 1995; Lamberti, 2012). Plant parasitic nematodes (PPNs) cause indirect harm as virus vectors and root knot nematodes (RKNs) cause direct crop damage during feeding, with global agricultural loss up to 100 billion USD annually (Brown et al., 1995). However, the insect parasitic guild of nematodes, known as entomopathogenic nematodes (EPNs), are highly effective biocontrol agents of many well-known pests of cultivated crops and hold great promise as natural control agents within integrated pest management programmes (Gaugler, 2018; Kaya & Gaugler, 1993).

Plants can directly affect the phytobiome with induced changes in their volatile profile in both terrestrial and subterranean environments (Dicke, 2016). Furthermore, parasites can instigate unique cascades of effects through top-down regulation of herbivore populations above- and below-ground that, in turn, function to regulate levels of herbivory (Dicke & Baldwin, 2010; Kessler & Baldwin, 2002; Preisser et al., 2006; Van Dam et al., 2010). Injury caused by herbivore feeding influences multitrophic interactions by indirectly attracting tertiary parasites of the herbivores, which is called indirect defence (Mumm & Dicke, 2010). Broadly, indirect defence is mediated by qualitative or quantitative changes in the volatile organic compounds (VOCs) released by plants in response to herbivory (De Moraes et al., 2001; Meiners & Hilker, 1997). For example, citrus roots fed upon by larvae of the citrus root weevil, *Diaprepes abbreviatus*, release pregeijerene into the rhizosphere (Ali et al., 2011). This volatile is attractive to EPN which attack the weevil larvae (Ali et al., 2011).

Investigations of chemically mediated above-below ground interactions are moving beyond the effects of herbivore-induced plant volatiles on predators and expanding to address how subterranean predators (EPNs) may broadly modulate induced plant defence response (An et al., 2016; Jagdale et al., 2009) and ultimately impact plant-herbivore interactions (Helms et al., 2019; Li et al., 2020). In tomato, EPNs induce defence mechanisms that are remarkably similar to those induced by pathogenic organisms, including increased H_2O_2-scavenging enzymes, catalase, and peroxidase, as well as expression of the *PR1*-gene in leaves (Jagdale et al., 2009). Furthermore, enhanced systemic resistance induced by EPNs has broad spectrum effects on organisms using those plants as hosts, simultaneously reducing performance of both chewing and sap-sucking herbivores, as well as growth of pathogenic bacteria (An et al., 2016). More recent examples have confirmed that EPNs themselves (Helms et al., 2019; Li et al., 2020), as well as the odours from EPN-infected cadavers induce plant defence responses, as measured by induction of *PR-1* and, salicylic acid (SA)-accumulation, with concomitant negative effects on herbivore performance (Helms et al., 2019).

Antagonistic interactions among different functional guilds of nematodes have also been recognized for quite some time (Bird & Bird, 1986). There are numerous examples showing that populations of PPNs decline upon exogenous applications of EPNs (Grewal et al., 1997; Jagdale et al., 2002; Smitley et al., 1992). Several hypotheses have been proposed to explain the apparent antagonism of EPNs against PPNs including physical exclusion of PPNs by buildup of EPNs in the rootzone (Bird & Bird, 1986), stimulation of nematode predator population growth (Ishibashi & Choi, 1991), and allelopathy (Grewal et al., 1999). Given the more recent discovery that EPNs induce systemic plant resistance, it has been suggested that EPN-induced plant defence may explain the antagonistic effect of EPNs on PPN performance and population density (Jagdale et al., 2009).

A growing body of evidence indicates that EPNs indirectly reduce herbivore performance above-ground and displace or reduce PPN populations below-ground (An et al., 2016; Helms et al., 2019; Kenney & Eleftherianos, 2016); however, the potential costs and/or benefits of these effects remain debated. Indirect antagonistic effects caused by EPNs via systemic plant resistance has emerged as a likely hypothesis explaining these phenomena (Jagdale et al., 2009), yet it has not been established whether above- versus below-ground plant responses are an inextricably linked plant immune response to all invaders versus more targeted effects. Based on a recent investigation by Li et al. (2020), which included an interaction between EPNs and RKNs below-ground, and an herbivore (aphids) above-ground, an emerging parsimonious hypothesis is that EPNs broadly modulate populations of above-ground herbivores and below-ground nematode communities

occupying different functional guilds indirectly via broad-spectrum upregulation of plant defence.

The purpose of this study was to compare mechanisms of plant response to nematodes occupying two different functional guilds. Our specific hypotheses were that: (1) plants recognize and respond to entomopathogens and plant parasites similarly, that is, "confusing" entomopathogens as invaders; (2) the antagonistic effect of entomopathogens on plant parasite performance is mediated indirectly by activation of systemic acquired resistance (SAR) in plants; and (3) plant defence induced in response to perceived subterranean invaders (EPN or RKN) is a global effect concurrently reducing performance of root parasites below-ground and folivores above-ground via conserved mechanisms involving SAR and/or induced systemic resistance (ISR). Our results confirm that exposure of plant roots to various species of EPNs modulated immune response in tomato and reduced subsequent infection by the root parasite. Complementary experiments exploring the effects of below-ground biota on above-ground multitrophic interactions revealed that *T. absoluta* female moths avoided laying eggs on tomato plants with roots infested by RKNs or exposed to EPNs, compared with mock controls. Transcriptomic analysis suggested these effects on herbivore behaviour were caused via indirect defence. Also, development and survival of leafminer pupae were reduced on plants whose roots were exposed to RKN or EPN, corroborating our biochemical and transcriptomic observations and indicating that tomato immune response is similarly triggered by both nematode functional guilds. Collectively, our results support the hypothesis that EPN-induced modulation of plant defence simultaneously explains reduced RKN performance below-ground and herbivore performance above-ground.

2 | MATERIALS AND METHODS

2.1 | Plant, insect and nematodes

The RKN-susceptible tomato cultivar, *S. lycopersicum* cv. "Moneymaker" (MM) was used in all the experiments. "MM" seeds were kindly provided by Professor Gary B. Dunphy (McGill University, Montreal, Canada). Tomato seeds were germinated on trays in an environmental chamber and 14-day-old seedlings of equivalent height were transplanted into pots. These were grown for 2 weeks in a controlled-environment greenhouse. Plants used in experiments were approximately 4-weeks-old.

The immature stages of *T. absoluta* were gathered from infested tomato foliage (*S. lycopersicum* var. Newton) in a commercial greenhouse in Mashhad, Razavi Khorasan, Iran. This culture has been reared continuously in insect-proof screen cages in a growth chamber since 2006. Tomato plants (cv. MM, 3–5-weeks old) grown under the above described conditions were provided to larvae three times per week until pupation (Hickel, 1990).

The larvae of the greater wax moth, *Galleria mellonella*, were used as a host for rearing *Heterorhabditis bacteriophora*, *Steinernema feltiae*, and *S. carpocapsae* (Kaya & Stock, 1997). A nematode population of *Meloidogyne javanica* was originally isolated from infested tomato roots and soil from Mashhad (Razavi Khorasan, Iran) according to the method described by Coolen and d'Herde (1972).

2.2 | Experiment 1: EPNs reduce RKN infestation by inducing plant defence

The purpose of this experiment was to test the hypotheses that: (1) plants recognize and respond to entomopathogens (EPNs) as plant parasitic RKN, (2) plant response to EPNs reduces performance of RKN, and (3) the antagonistic effect of entomopathogens on plant parasite performance is mediated indirectly via activation of SAR in plants. The ancillary objectives of this initial experiment were to identify appropriate time points and determine the most effective species for investigating effects of root-knot and/or EPN inoculation on post-transcriptional responses of host plants investigated subsequently.

2.2.1 | Nematode infection experiments

For *M. javanica* penetration and development tests, 264 seeds were planted as previously described. Four-week-old tomato seedlings were inoculated with approximately 1–2 ml of aqueous suspension including 370 newly hatched RKN J2s per plant. Immediately thereafter, suspensions of *H. bacteriophora*, *S. feltiae*, or *S. carpocapsae* were applied to seedlings at a concentration of 25 infective juveniles' nematodes (IJs)/cm^2. Because replicates were performed with multiple generations of nematodes, at least four positive control treatment plants (inoculated just with *M. javanica* and distilled water) were established for every day/treatment combination throughout the experiment (Figure S1). The experiment was arranged in a completely randomized design (CRD). Each treatment group was replicated 18 times (12 + 6). At 1, 2, 3, 5, 6, 7, 13, 14 and 15 days post inoculation (dpi), 12 tomato seedlings from each treatment were slowly separated from the plastic pot to examine roots for *M. javanica* penetration and development through acid/fuchsin staining (Bybd et al., 1983; Thies et al., 2002). Nematodes inside the roots were visualized and counted under the stereomicroscope (Discovery v.20; Zeiss). Samples were categorized into three developmental groups: second-stage juvenile, third- to fourth-stage juvenile, or adults, according to Shukla et al. (2018). Therefore, tissue from three successive days of infection was pooled in order to enrich the tissue for the specific nematode stage: 1, 2 and 3 dpi was pooled to represent stage 1 (attack of J2s/beginning of feeding sites); 5, 6 and 7 dpi as stage 2 (parasitic J2s/ establishment of feeding sites); and, 13 and 14, 15 dpi as stage 3 (feeding J2s and J3s/ development of feeding sites). Thirty days after nematode application, the remaining six tomato seedlings in each treatment were evaluated for *M. javanica* infection. Each plant was removed from the growth tube by flushing it with water. Each root was cut into small pieces (approximately 1 cm in length) and mixed thoroughly, and

0.5 g of root tissue (wet weight) was obtained from each plant for analysis. The total numbers of galls and egg masses were counted for each plant under a dissecting microscope. To estimate egg hatch rate, the eggs were kept in the same petri dish for 1 week at 27°C, and the ratio of hatched eggs was calculated. This information was obtained for each plant, which allowed us to approximate: (1) the mean number of viable infective juvenile *M. javanica* per plant 1 week after egg extraction (hatch rate multiplied by total egg production), (2) the mean number of egg masses per gall, and (3) the mean number of eggs per egg mass.

2.2.2 | Estimation of defence-related enzyme activity

Guaiacol peroxidase (GP) and polyphenol oxidase (PPO) are known essential defence-associated enzymes in plants and are widely used as measurements of plant defence against phytopathogens (Qin et al., 2015; Ye et al., 2013). Thus, these two enzymes were selected as markers for plant defence response to test this hypothesis. Four-week-old tomato seedlings were assigned to the following treatments: (1) mock-inoculated control (plants were treated with distilled water only), (2) PPN alone (RKN—*M. javanica*), (3) EPN alone (EPN—*S. carpocapsae*), and (4) RKN + EPN. Three pots were established per treatment group, each with one plant and arranged in a CRD with three replicates. Three tomato leaflets and the entire root mass were sampled on 3, 7, 15, 20, and 28 dpi per replicate; all tissues samples were kept at –20°C before enzymatic measurement.

2.3 | Experiment 2: EPN or RKN inoculation reduces above-ground herbivore preference and performance

We tested the hypothesis that plant defence in response to perceived subterranean invaders (EPN or RKN) reduces herbivore performance above-ground. We quantified (1) oviposition preference of tomato leafminer, *T. absoluta*, and (2) larval development of leafminers in response to root invasion by RKN, and/or inoculation with three species of EPNs (*H. bacteriophora*, *S. feltiae* or *S. carpocapsae*).

2.3.1 | Two-choice assays

To evaluate whether *T. absoluta* egg-laying and development is affected by below-ground nematode inoculation of tomato roots, two-choice oviposition assays were conducted using mock-infested plants as controls. An oviposition preference test was performed by releasing three mated 2-day-old females into cages containing one control and one nematode-inoculated plant (Figure S2A,B). Choice tests were conducted separately with each nematode species: *M. javanica* (RKN), *H. bacteriophora* (EPN), *S. feltiae* (EPN) or *S. carpocapsae* (EPN). Ninety-six insect-proof screen cages were established simultaneously. After 12 h, the number of eggs laid on nematode-inoculated and control plants was determined as a rate of oviposition preference (Figure S2C). Eggs were counted every other day beginning 3 and 7 days following plant inoculation. Tests were performed during scotophase when *T. absoluta* oviposition occurs in nature (Proffit et al., 2011).

Afterward, we standardized the number of eggs from the oviposition preference test to 20–25 per plant by removing extraneous eggs with a paintbrush. Plants were checked three times daily to evaluate herbivore developmental stage (egg, larvae, and pupae) and to determine mortality. When larvae pupated, the pupae were harvested, weighed, paired, and their emergence rates were recorded. To evaluate the fecundity and longevity of offspring, emerging adults from either un-inoculated or root-inoculated plants were held in separate cages and permitted to mate (Figure S3A,B). Twenty-eight *T. absoluta* pairs were evaluated for each treatment group. Twenty-four hours after mating, females were released into single cages, and their fecundity was assessed by counting the number of eggs laid on tomato leaflets (Figure S3C). Adult females were fed with a 10% honey solution on a wet piece of cotton wool. Females were kept until they died or did not lay eggs for four successive days (Arce et al., 2017).

2.3.2 | No-choice assays

Ten 4-week-old *S. lycopersicum* plants were allocated for each treatment group: plants without nematodes (control); roots inoculated with *M. javanica* (Mj), roots inoculated with *S. carpocapsae* (Sc), and roots inoculated with both *M. javanica* and *S. carpocapsae* (Mj + Sc) (Figure S4A). All plants in the Mj and Mj + Sc treatment groups were inoculated with 1–2 ml of *M. javanica* suspension in distilled water containing 370 nematodes. For those treatments that also included the EPN, the nematode suspension included *S. carpocapsae* at 25 IJ/cm^2. Plants designated as the control treatment group were inoculated with the similar amount of distilled water without nematodes. On the seventh day after nematode treatment, all plants were relocated to single insect-proof screen cages (Figure S4B). Three mated, 2-day-old *T. absoluta* females were released and allowed to oviposit for 12 h. Afterwards, the number of eggs on each plant was determined (Figure S4C).

2.4 | Experiment 3: Transcriptomic analysis of tomato exposed to EPNs, RKNs, and/or leafminer herbivory

The purpose of this experiment was to test the hypothesis that plants respond to perceived subterranean invaders (EPN or RKN) via conserved defence mechanisms involving SAR and/or ISR. Four-week-old *S. lycopersicum* seedlings were allocated, at random, to the following eight treatment groups: (1) plants without nematode or tomato leaf miner (control); (2) roots inoculated with *M. javanica*

(Mj), (3) roots inoculated with *S. carpocapsae* (Sc), (4) shoots exposed to *T. absoluta* (Ta), (5) roots inoculated with *M. javanica* and *S. carpocapsae* (Mj + Sc), (6) roots inoculated with *M. javanica* and shoots exposed to *T. absoluta* (Mj + Ta), (7) roots inoculated with *S. carpocapsae* and shoots exposed to *T. absoluta* (Sc + Ta), (8) roots inoculated with *M. javanica* + *S. carpocapsae* and shoots exposed to *T. absoluta* (Mj + Sc + Ta). The experimental pots for each nematode treatment were inoculated with 370 freshly hatched RKN J2s and/or 25 EPN IJs/cm^2 of the nematode species described above. On the seventh day after inoculation with nematodes, four *T. absoluta* eggs were released onto *S. lycopersicum* plants in Ta, Mj + Ta, Sc + Ta and Mj + Sc + Ta treatment groups (Figure S5). This trial was performed in insect-proof screen cages and arranged in a CRD with six biological replicates per treatment (un-inoculated and inoculated), consisting of 150 plants in total.

Tomato tissues were collected for differential gene expression analysis on the 3rd, 7th (prior to tomato leaf miner attack), 15th (7 days after moth egg release) and 26th day (17 days after moth egg release) after inoculation with nematodes. For each time point, six tomato plants were collected from each treatment group. Leaves and entire intact root systems from control and inoculated tomato plants were carefully separated from the potting soil, washed with deionized water, dried with sterile paper towels and instantly frozen into liquid nitrogen to prevent RNA degradation (Van Dam et al., 2018). At the end, moth larval mass was determined at 17 days post-egg release, when the initial larvae began to pupate. The larvae were flash-frozen in liquid nitrogen and freeze-dried. The larvae that fed on tomato plants from the same treatment were pooled. Both plant tissue and larval samples were kept at −80°C until RNA extraction. The dried samples were ground with porcelain mortar and pestle. Total RNA was extracted with RNeasy Plant Mini Kit (Qiagen) with additional on-column DNase I digestion. In order to decrease biological variation, the number of samples from each treatment group was decreased from six to three by combining two samples. The RNA quality and lack of residual genomic DNA was investigated on a 1.2% denaturing agarose gel electrophoresis. The concentration of RNA and its purity was estimated through a microplate spectrophotometer (Epoch).

2.4.1 | RNA extraction, library construction, and sequencing

Based on the root penetration trials and oviposition assays results, the 7 dpi time point was chosen for transcriptomic analysis. Equal amounts of RNA from three individual plants from the Mj and Sc treatment groups or the controls at 7 dpi were used for library constructions. RNA samples from each treatment were prepared in two volumes of 100% ice-cold ethanol containing 0.1 ml of a 3 M sodium acetate buffer solution, pH 5.5, in 1.5 ml microcentrifuge tubes and shipped on dry ice to Macrogen Inc. (Macrogen Inc.) for library construction and illumina sequencing. Upon receiving samples, the RNA was repelleted and quality control (QC) was checked with a 2100 Bioanalyser (Agilent Technologies). RNA QC was confirmed as all samples had an RNA integrity number ≥7.0 and lack of genomic DNA contamination prior to moving for library constructions and sequencing.

One microgram of total RNA was applied as starting material and cDNA libraries were constructed using the Truseq stranded total RNA library preparation kit (Illumina, Inc.). The libraries were quantified with quantitative real-time PCR (qRT-PCR) (Bustin et al., 2009) and paired-ends (2 × 100 bp) sequencing was conducted on a HiSeq2500 platform (Illumina, Inc.).

2.4.2 | RNA-seq data analysis

The Illumina platform produced a total of 223,269,372 reads, 101 bp in length, including 70,210,844 reads (31.4%) from the Mj samples and 79,217,798 reads (35.4%) from the Sc samples. Raw sequence data are available at NCBI BioProject database (http://www.ncbi.nlm.nih.gov/bioproject) under accession number PRJNA732672. Approximately 207,844,542 (207/223, 92.8%) trimmed reads were mapped on the tomato reference genome, and 36,962 tomato genes were identified. Qualities of the raw reads were checked through FastQC with default input parameters (*p*-values >.01, Phred quality score <20, mean error rate <0.2%, sequence quality score >10, duplicate sequences<20%) (Andrews, 2010). The next-generation sequence data were processed through the modified Tuxedo pipeline (Trapnell et al., 2012). Low-quality bases and adapter sequences of paired reads were trimmed through the TRIMMOMATIC v.0.30 program (Bolger et al., 2014). Subsequently, trimmed reads were mapped independently to the tomato GENOME v. 2.50 (https://solgenomics.net) through TOPHAT v.2.0.4 on default parameters. Sequence alignment files were input into the software CUFFLINKS and CUFFMERGE v.2.2.1 to assemble potential transcripts.

Differential gene expression analyses were conducted through CUFFDIFF v.2.2.1. The expression levels of each gene were normalized with fragments per kilobase of exon per million mapped reads (FPKM) values. All computed *p*-values were adjusted for multiple testing with Benjamini and Hochberg's method through a false discovery rate (FDR) of 5% and genes were estimated to be differentially expressing with FDR ≤0.05. cDNA libraries from *M. javanica* and/or *S. carpocapsae* inoculated plants were compared to their respective control to determine up- and downregulated genes. Gene expression was considered significantly different between treatments when their relative expression levels indicated at least a 2-fold-change ($\log_2$ fold-change ≥1.5 or ≤ −1.5) difference between un-inoculated and inoculated samples (*p*-value ≤ .05).

2.4.3 | Gene expression validation using qRT-PCR

The RNA-seq results were validated using qRT-PCR with the similar set of root tissues as applied for transcriptome analysis. Specific primer sequences for 21 tomato genes were designed with

the BEACON DESIGNER 8.21 software (Premier Biosoft International). Detailed primer information is given in Table S1. Two micrograms of DNA-free total RNA was used for cDNA synthesis using the Revertaid first strand cDNA synthesis kit (Thermo Scientific) following instructions provided by the manufacturer. Subsequently, all nine root samples (three un-inoculated and three inoculated tomatoes for each of the treatments) were used for target gene amplification with qRT-PCR in three technical replicates. Amplifications were conducted on a LightCycler 96 Real-Time PCR System (Roche Life Science). The reaction mixtures included 1.5 µl of cDNA, 10 µl of 2x SYBR Green Real Time PCR Master Mix, 0.7 µl (10 µM) of each primer, and dnase/rnase-free distilled water was added to a final volume of 20 µl. The thermal cycling conditions were as follows: 95°C for 5 min; then 45 cycles of 95°C for 15 s and 58 to 62°C for 30 s. Reaction specificity was confirmed by obtaining the dissociation curve for each reaction. Two tomato genes, encoding Tubulin alpha-3 chain (TAC) (Shukla et al., 2018) and Ubiquitin (UBI) (based on our RNA sequencing results), were used as endogenous control reference genes to normalize gene expression levels (Table S1). Relative expression levels of each target gene were determined according to the$2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001), in comparison with untreated control samples. Pearson's correlation coefficient was used to compare the transcriptomic and qRT-PCR results in the statistical software R (R Core Team, 2013; Santini et al., 2016).

To evaluate whether expression levels of candidate plant defence-related genes were affected by exposure to root knot-nematode, EPN or simultaneously with both nematode species inoculation/infection, gene expression was traced with qPCR using target-specific primers with the root tissues harvested from the controlled trial.

2.4.4 | Functional annotation and enrichment analysis

Gene set enrichment analysis of differentially expressed genes (DEGs) was conducted with BLAST2GO v.5.2 (Conesa et al., 2005) according to the BLASTX results (*e*-value cutoff of 1×10^{-6}) followed by mapping and annotation stages through the default parameters of BLAST2GO. Subsequently, the following methods were applied on the set of DEGs: AgriGO tool, which specifically focuses on agricultural species, was applied to conduct a singular enrichment analysis (FDR correction and Fisher's exact test ≤ 0.1) through the *S. lycopersicum* v2.4 data set as the reference. Significant gene ontology (GO) terms in the biological process category were visualized using the TreeMap view obtained by REVIGO analysis (Supek et al., 2011). Furthermore, the protein sequences were aligned with BLASTX against all general plant databases supported in the Mercator web application (Lohse et al., 2014). Every transcript of a protein-coding gene was allocated to a functional bin and a BLAST cutoff of 80 was selected. The Mercator-generated mapping file was used to assign the protein sequences to Mapman functional pathways (Thimm et al., 2004; Usadel et al., 2005). Eventually, common genes that were expressed in both treatment groups were graphically presented with a hierarchically clustered heatmap (Santini et al., 2016; Warnes et al., 2009).

2.4.5 | Data analyses

Data collected from the nematode infection experiments were subjected to analysis of variance (ANOVA) SAS 9.4 software (SAS Institute, 2015), followed by the Dunnett's post-hoc test for comparison of means. Potential activities of both enzymes were analysed with one-way ANOVA, and treatment means were compared by Tukey's post hoc test (p-values ≤ .05) in SAS. Leafminer preference data collected in the choice and no-choice tests were analysed with replicated *G*-tests (Sokal & Rohlf, 1995). This permitted us to analyse the overall distribution of eggs found on pairs of un-inoculated and nematode-inoculated tomatoes (Gp, equal to Chi-square test) and deviation of the data from a 1:1 distribution (Gt). Gp or Chi-square values are based on total numbers; the multiples of rows and columns in the distribution table. The Gt value takes into consideration multiple replications of paired treatments and permits identification of heterogeneity between the replications (Sokal & Rohlf, 1995).

In addition, expression of defence-related genes among the three nematode inoculation treatments was examined using principal component analysis (PCA). To investigate correlations of each principal component with gene expression, the Pearson bivariate correlation was performed (Jackson, 1980).

3 | RESULTS

3.1 | Entomopathogenic nematodes reduce RKN infestation

Significantly fewer *M. javanica* juveniles were found inside tomato roots of plants treated with 25 *S. carpocapsae* IJs/cm^2 at 1–15 dpi than in corresponding control plants inoculated only with *M. javanica* but without EPNs (Figure 1). Also, fewer *M. javanica* galls, eggs masses, and eggs were recovered from plants growing in soil containing *S. carpocapsae* than from the control plants at thirty dpi (Figure 2a–c). Fewer *M. javanica* juveniles, galls, eggs masses, and eggs were extracted from plant roots inoculated with 25 *S. feltiae* IJs/cm^2 than from control plants at all sampling points, except for 5–7 dpi (Figure 1 and Figure 2a–c). There were significantly fewer *M. javanica* juveniles within roots of plants treated with 25 *H. bacteriophora* IJs/cm^2 than in roots of control plants at 5–7 and 13–15 dpi (Figure 1). Likewise, fewer *M. javanica* galls and eggs were extracted from plant roots inoculated with *H. bacteriophora* than from control plants at 30 dpi (Figure 2a–c). *M. javanica* eggs recovered from plant roots grown in soil inoculated with *S. carpocapsae* had a significantly lower hatch rate than eggs recovered from un-inoculated plant roots (Figure 2d). However, neither the number of eggs produced per egg

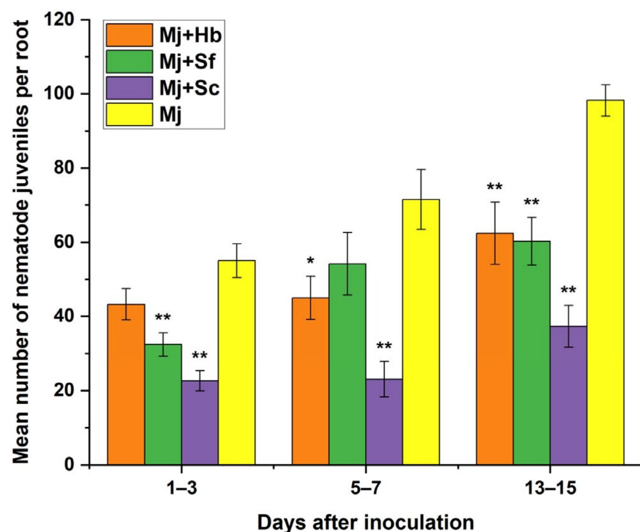

FIGURE 1 *Meloidogyne javanica* (Mj—Root knot nematode) penetration into tomato seedling roots treated with 25 infective juveniles/cm^2 of *Heterorhabditis bacteriophora* (Hb—Entomopathogenic nematode), *Steinernema feltiae* (Sf—Entomopathogenic nematode), *S. carpocapsae* (Sc—Entomopathogenic nematode), or control roots receiving only *M. javanica*. Number of juveniles in roots 1, 2, 3, 5, 6, 7, 13, 14 and 15 days after infestation ($n = 4$). *M. javanica* stained with acid fuchsin following infection of tomato roots at 1–15 days post inoculation (dpi). Significantly different (*p-value ≤ .05; **p-value ≤ .01) from the control according to Dunnett's test

mass nor the number of egg masses produced per gall was affected by any of the treatments tested as compared with nonexposed controls (Figure S6).

3.2 | Entomopathogenic nematodes induce plant defence

3.2.1 | GP activity

Below-ground presence of RKN *M. javanica*, and/or the beneficial EPN *S. carpocapsae*, reduced GP activity in above-ground plant tissue (Figure 3a). However, a rapid induction of GP activity was observed in below-ground tissues inoculated with *M. javanica* and/or *S. carpocapsae* at 3–7 dpi, and then activity returned to near preinoculation levels at 15–28 dpi (Figure 3b). GP activity was lower at 15–28 days than at 3–7 dpi in both the mock control and inoculated treatments (Figure 3b), indicating some natural fluctuation of enzymatic activity, which could have been due to plant ageing.

3.2.2 | PPO activity

Similar to GP activity, PPO activity in shoots was also significantly reduced as a result of below-ground presence of *M. javanica* and/or *S. carpocapsae* (Figure 4a). In contrast, there was a large and statistically significant increase in PPO activity in roots with *M. javanica* and/or *S. carpocapsae*, as compared with the mock control, at 3–7 dpi, which subsequently decreased significantly below mock control levels at 15–20 dpi and returned to basal levels at 28 dpi (Figure 4b). These data indicate that tomatoes respond similarly to presence of RKN or EPN in the rootzone, by rapidly activating PPO and GP activity in roots, but simultaneously suppressing these activities in above-ground tissues.

3.3 | Entomopathogenic nematode or RKN inoculation reduces above-ground herbivore preference and performance

To test if the presence of RKN or EPN in the tomato growth substrate would affect plant acceptance by an above-ground herbivore, we conducted choice assays to quantify oviposition preference of *T. absoluta*. Female *T. absoluta* laid more eggs on *H. bacteriophora*-inoculated plants than control plants at 7 dpi and on *S. feltiae*-inoculated than on paired noninoculated controls at 3–5 dpi; however, there was no strong pattern of preference between treatment and control plants for these two nematode species (Figure 5a,b). However, when given the choice, female *T. absoluta* preferentially and consistently laid significantly more eggs on nematode-free control plants than those inoculated with either *M. javanica* or *S. carpocapsae* at 3–7 dpi (Figure 5c,d).

Presence of *M. javanica* or various EPN species in the plant substrate had no significant impact on larval developmental period and mortality rate, nor did it affect the fertility or lifespan of newly emerged *T. absoluta* females (Figure S7a–d). However, the duration of the pupal stage was significantly longer and pupal mortality was higher in *T. absoluta* developing on *M. javanica* and *S. carpocapsae*-inoculated plants than on noninoculated, control plants (Figure 6a,b).

In no-choice assays, adult female *T. absoluta* laid significantly more eggs on control treatments (average: 30.5) than plants that were grown in substrate containing *M. javanica* (13.8), *S. carpocapsae* (17.1), or the combination of the two species (15.6) (Figure 7).

3.4 | Differentially expressed genes following inoculation by EPNs or RKNs

The comparison of roots of tomato plants grown in the presence of nematodes with their corresponding controls identified a total of 905 significant DEGs ($\log_2$ fold-change ≥ ±1.5 and adjusted p-values ≤ .05) (Tables S2 and S3). We detected 444 and 461 DEGs in response to RKN (*M. javanica*) and EPN (*S. carpocapsae*) inoculation, respectively (Figure S8A and Table S1 Table S2). Among them, 217 and 227 genes were down- and upregulated, respectively with RKN inoculation, whereas 238 and 223 genes were down- and upregulated, respectively, with EPN inoculation at 7 dpi (Figure S8A). Moreover, 92 genes were upregulated in both treatments, while 135 and 131 genes were exclusively upregulated in response to RKN and

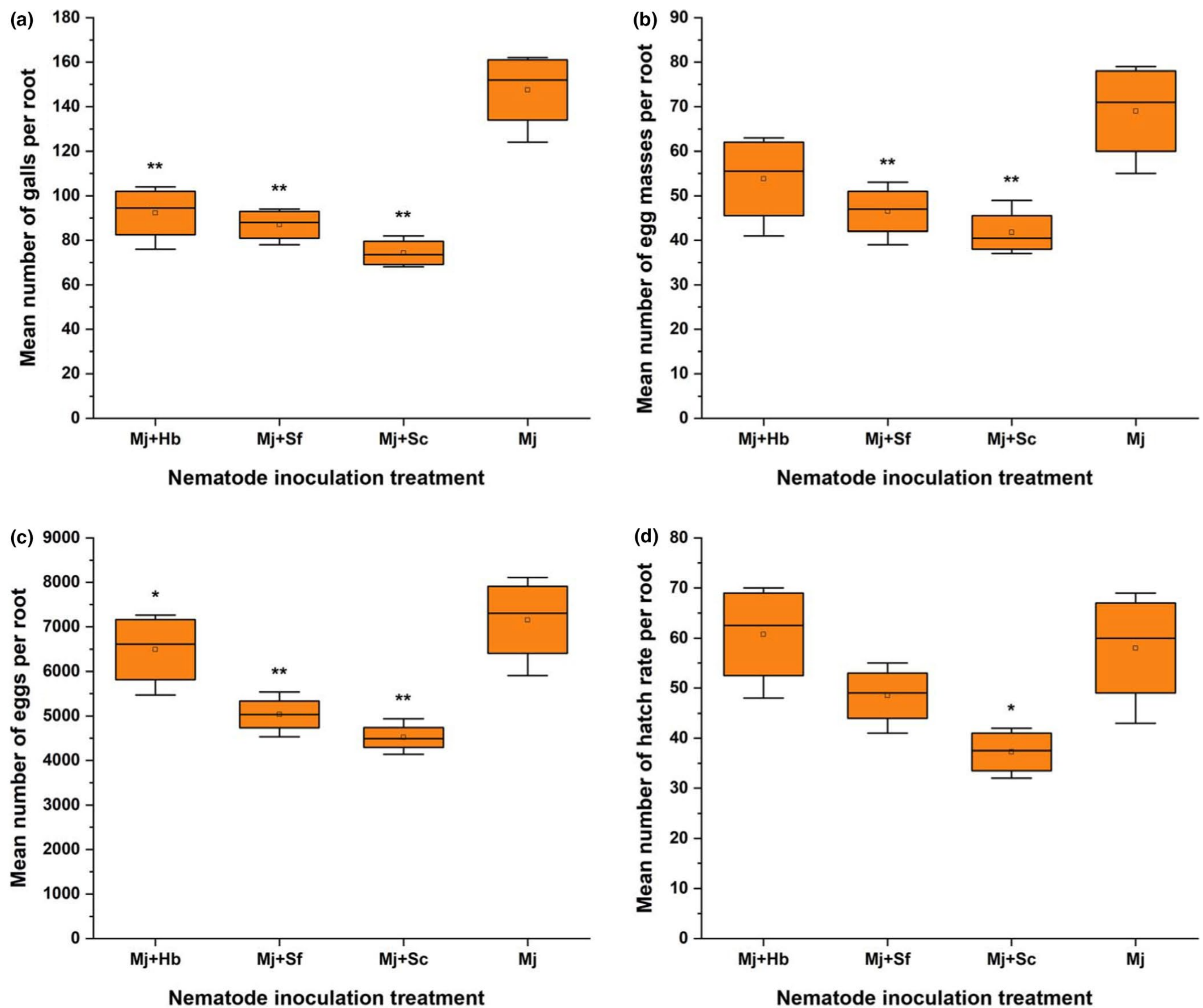

FIGURE 2 Numbers of *Meloidogyne javanica* (Mj—Root knot nematode) galls, egg masses, eggs and egg hatch rate in tomato seedlings treated with 25 infective juveniles/cm^2 of *Heterorhabditis bacteriophora* (Hb—Entomopathogenic nematode), *Steinernema feltiae* (Sf—Entomopathogenic nematode), *S. carpocapsae* (Sc—Entomopathogenic nematode), and control. (a) Average number of galls per root; (b) average number of egg masses per root; (c) average number of eggs extracted per root; (d) average hatch rate of eggs extracted per root ($n = 6$). Bars indicate standard deviation of the mean. Significantly different (*p-value ≤ .05; **p-value ≤ .01) from the control according to Dunnett's test

EPN inoculation, respectively. Similarly, 141 and 162 genes were downregulated exclusively in response to RKN and EPN, respectively (Figure S8B). The commonly upregulated genes included those encoding phenylalanine ammonia-lyase, thioredoxin H, S glycoprotein, and major latex-like protein, whereas the 76 jointly downregulated genes contained genes encoding xenotropic and polytropic retrovirus receptor, pectinesterase, and alpha-1 4-glucan-protein synthase (Table S4).

The relative changes in gene expression data through qRT-PCR were congruent with the RNA-seq results, as formerly presented for other host plant-nematode interactions (Bali et al., 2019; Kumar et al., 2019; Kyndt et al., 2012; Lee et al., 2019; Petitot et al., 2017; Postnikova et al., 2015; Santini et al., 2016; Shukla et al., 2018; Zhou et al., 2020). The Pearson's correlation coefficients of transcript levels between RNA-seq and qRT-PCR data were 0.83 and 0.82 (p-values ≤ .0001) for Mj and Sc root samples, respectively. It is noteworthy that the qRT-PCR was more sensitive compared to RNA-seq in tracing the expression of the target genes (Figure 8a,b). For example, for the TSW12 gene, the $\log_2$ fold-change values (p-values ≤ .05) for RNA-seq and qRT-PCR were 5.05 and 15.27, respectively.

Gene ontology enrichment analysis on DEGs was executed through the BLAST2GO software, allowing sequence annotation for 71.17% and 75.27% of the DEGs in the Mj and Sc samples, respectively (Figure S9A,B). For the biological process classification, a large number of DEGs were located in the categories of metabolic process, cellular process, biological regulation, regulation of biological

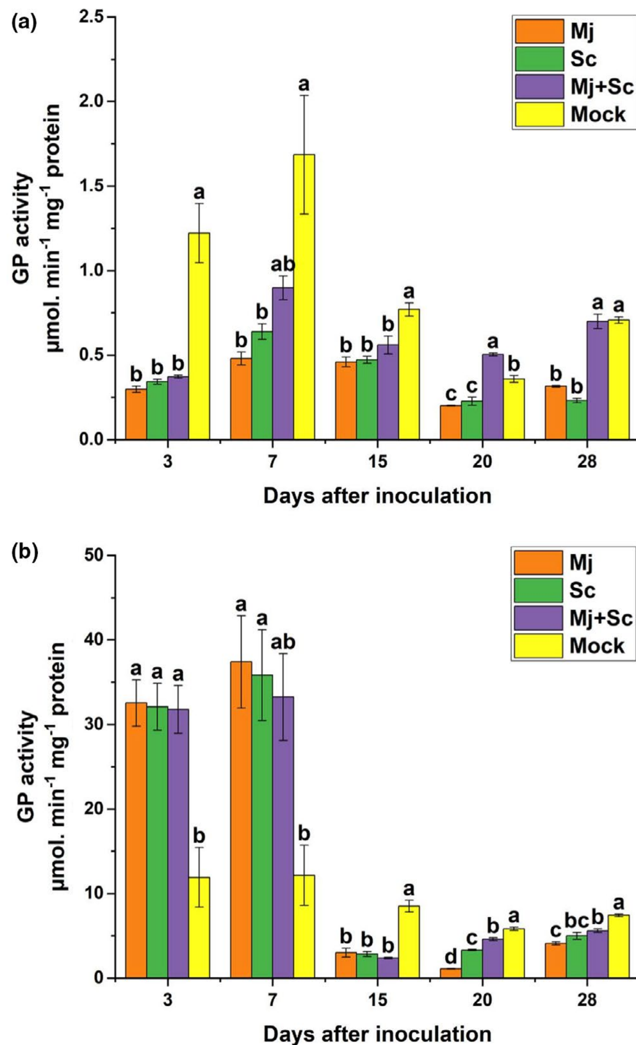

FIGURE 3 Effect of *Steinernema carpocapsae* (Sc—Entomopathogenic nematode) inoculation on guaiacol peroxidase (GP) activity in (a) leaves and (b) roots of tomato. Means followed by the same letters are not significantly different according to Tukey's test a = 5% possibility level. Bars indicate standard error ($n = 3$)

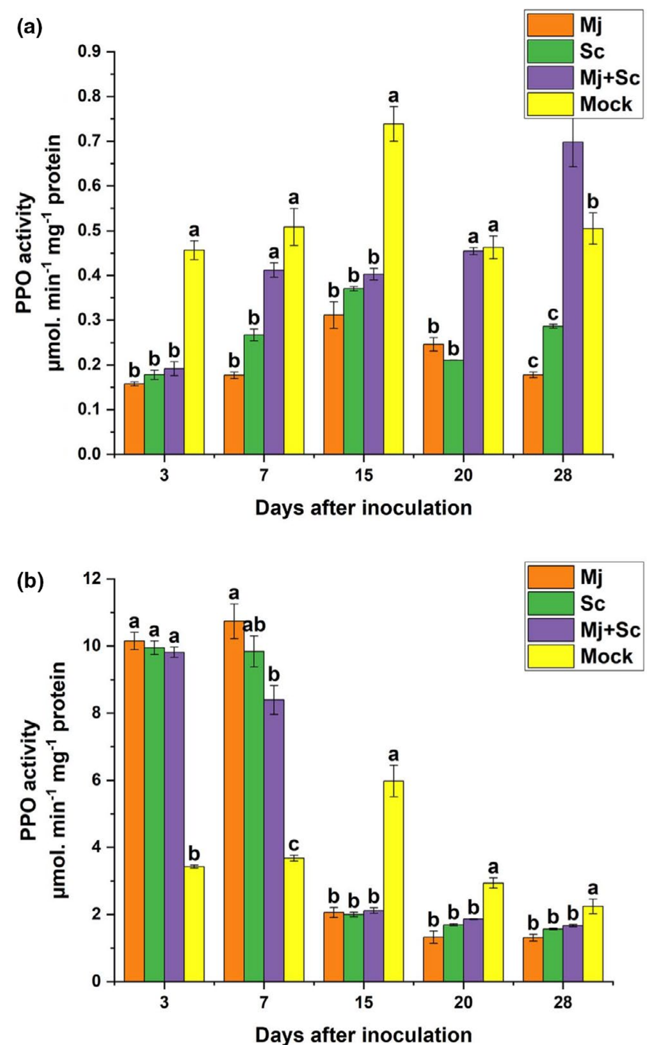

FIGURE 4 Effect of *Steinernema carpocapsae* (Sc—Entomopathogenic nematode) inoculation on polyphenol oxidase (PPO) activity in (a) leaves and (b) roots of tomato. Means followed by the same letters are not significantly different according to Tukey's test a = 5% possibility level. Bars indicate standard error ($n = 3$)

process, response to stimulus, and localization (Figure S10A,B). For the molecular function classification, many DEGs were in the categories of binding, catalytic activity, and transporter activity.

Differentially expressed genes were also analysed with MAPMAN software with focus on biotic stress and secondary metabolism pathways. Under biotic stress, the transcripts related to hormone metabolism, cell wall modification, beta glucanase, proteolysis, redox state, containing peroxidases and glutathione S-transferases (GST), signalling, secondary metabolites, transcription factors (TFs), and heat shock protein categories were upregulated in response to RKN inoculation. Moreover, a similar overall pattern of gene upregulation was observed in tomato roots exposed to EPN (Figure S11). Similarly, transcripts encoding enzymes involved in secondary metabolite production, such as phenylpropanoid, terpenoid, phenol, as well as lignin and lignan biosynthesis, were induced in response to *M. javanica* (RKN); these were further increased by EPN-inoculation (Figure S11). Overall, the analysis provided empirical evidence that expression of defence-related pathways was enhanced in *S. lycopersicum* in response to EPN exposure in the rhizosphere.

3.5 | Modulation of hormone and secondary metabolite biosynthesis in tomato by *M. javanica* (RKN)

Differentially expressed genes in response to *M. javanica* (RKN) infection were allocated to 22 BINs (Figure 9a and Table S5). Four BINs included only genes that were upregulated (fermentation, gluconeogenesis/glyoxylate cycle, mitochondrial electron transport/ATP synthesis, and nucleotide metabolism) and two BINs included only downregulated genes (biodegradation of xenobiotics and N-metabolism). Focusing only on plant defence mechanisms, 25%

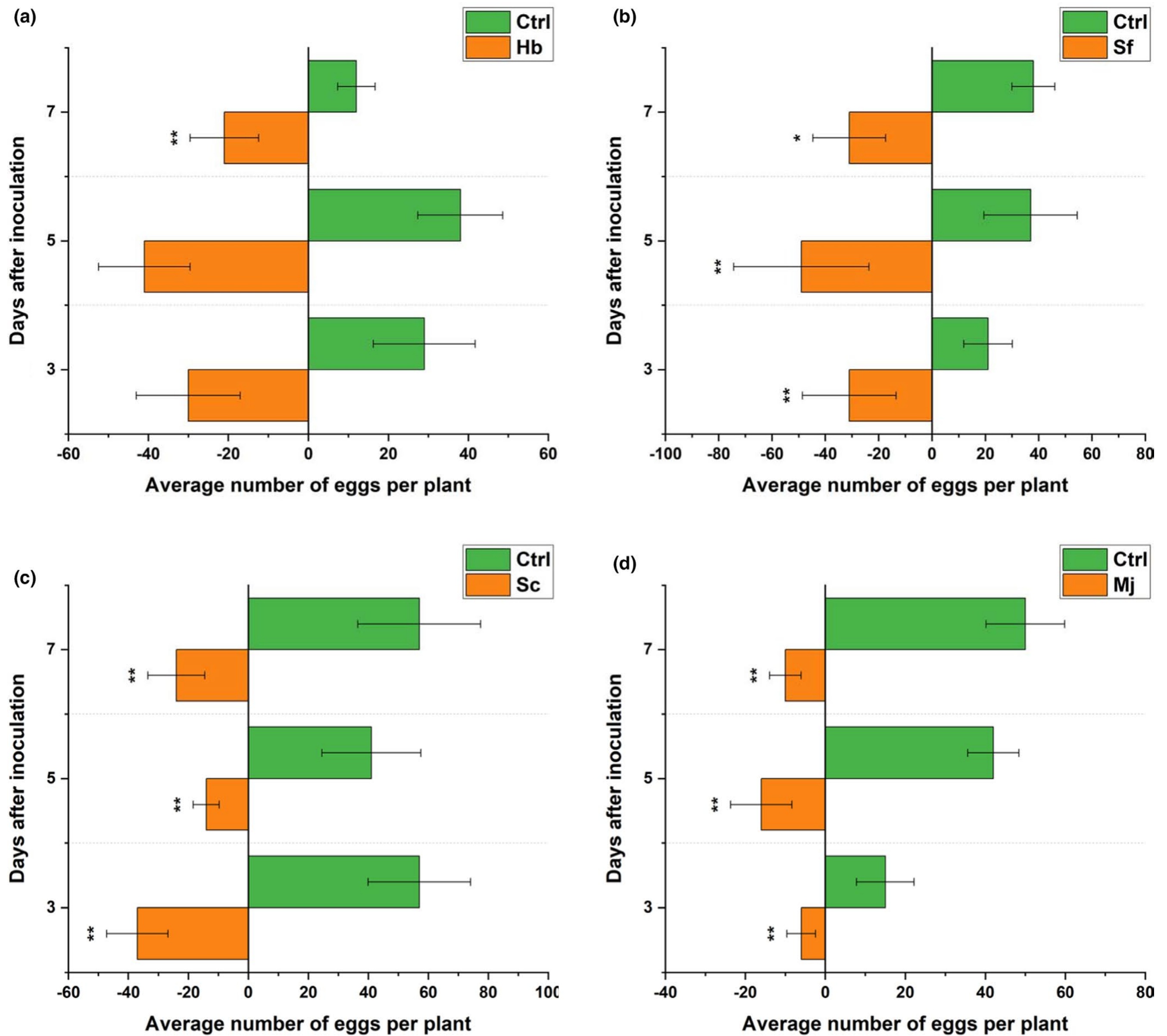

FIGURE 5 Mean numbers (±SEM) of *Tuta absoluta* eggs detected per plant. Number of eggs at 3, 5 and 7 days post inoculation (dpi) per insect-proof screen cage ($n = 8$). Females were permitted to choose between a control plant (blue bars) and a plant inoculated with (a) *Heterorhabditis bacteriophora* (Hb—Entomopathogenic nematode), (b) *Steinernema feltiae* (Sf—Entomopathogenic nematode), (c) *S. carpocapsae* (Sc—Entomopathogenic nematode) or (d) *Meloidogyne javanica* (Mj—Root knot nematode) nematodes within an individual cage. Asterisks indicate whether the overall distributions of the eggs deviated from a 1:1 distribution (replicated G-test per time point, Gpooled, d.f. = 1); *p-value ≤ .05, **p-value ≤ .01

of the genes (111/444) were allocated to nine BINs, and included mainly hormone metabolism (16/111), TFs (13/111), cell wall (7/111), and oxidative stress (15/111) (Figure S11 and Table S7).

In relation to hormone metabolism, we detected upregulation of genes associated with ethylene (ET) (six up- and one downregulated) biosynthesis. Six auxin-associated genes were differentially expressed; four were up- and two were downregulated. Furthermore, genes coding for jasmonic acid (JA) biosynthesis, lipoxygenases and an allene oxide synthase were upregulated. A brassinosteroid-related gene, BES1/BZR1 homologue protein 2, was downregulated. Among TFs, nine (3 MYB, 3 AP2/EREBP, 1 WRKY, 1 DOF and 1 bZIP) were downregulated and four (2 MYB, 1 WRKY, and 1 DOF) were upregulated. In relation to cell wall related proteins, a fucosyltransferase 7, and a xyloglucan endotransglucosylase/hydrolase 2 were upregulated. Conversely, a BURP domain-containing protein, a rhamnogalacturonate lyase, a pectate lyase-like protein, an alpha-1 4-glucan-protein synthase and a pectinesterase were downregulated. In relation to oxidative stress, we detected six genes encoding peroxidases, as well as a thioredoxin H and glutathione-S-transferase that were upregulated and a thiore/doxin reductase, two glutaredoxin family proteins, three glutaredoxins and a GST that were downregulated. Furthermore, a gene encoding a TIR-NBS-LRR

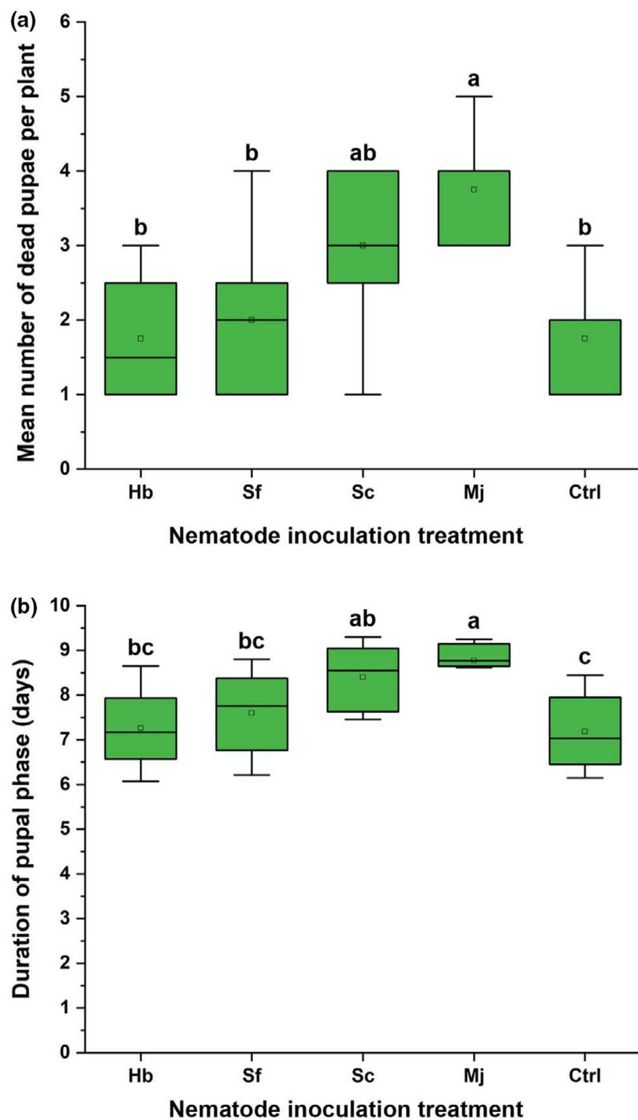

FIGURE 6 Effect of *Meloidogyne javanica* (Mj—Root knot nematode) or *Steinernema carpocapsae* (Sc—Entomopathogenic nematode) on *Tuta absoluta* pupation. (a) Average (±SD) number of dead pupae. (b) Average (±SD) duration of pupal phase. Means followed by the same letters are not significantly different according to Tukey's test a = 5% possibility level. Bars represent standard deviation ($n = 8$)

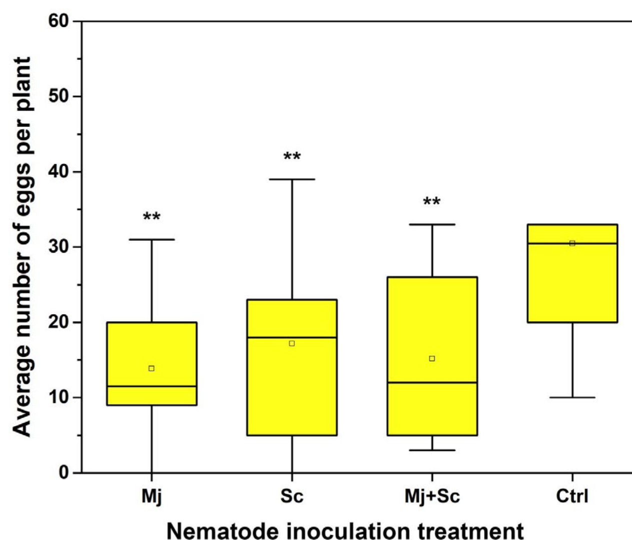

FIGURE 7 Mean numbers (±SEM) of *T. absoluta* eggs detected per plant 12 h after three 2-day-old mated *T. absoluta* females were released on each plant (no-choice experiment; $n = 6$ per treatment group). Plants were either inoculated with *M. javanica* (Mj—Root knot nematode) or *S. carpocapsae* (Sc—Entomopathogenic nematode), simultaneously with plant parasitic and entomopathogenic nematodes (Mj + Sc) or mock inoculated seven days before female moths were released. The asterisks indicate whether the overall distributions of the eggs deviated from a 1:1 distribution (replicated G-test, Gpooled, d.f. = 3); **p*-value ≤ .05, ***p*-value ≤ .01

protein, analogous to the R genes that mediate resistance to Tobacco mosaic virus (Whitham et al., 1994) was induced in RKN-inoculated plants.

3.6 | Modulation of hormone and secondary metabolite biosynthesis in tomato by *S. carpocapsae* (EPN)

In tomato plants grown only in presence of the *S. carpocapsae* (EPN), the DEGs were allocated to 26 BINs (Figure 9b and Table S6). Three BINs included only upregulated genes (fermentation, major CHO metabolism and photosynthesis) and five BINs included only downregulated genes (biodegradation of xenobiotics, metal handling, minor CHO metabolism, N-metabolism and TCA/org transformation). Four BINs (major CHO metabolism, metal handling, minor CHO metabolism and TCA/org transformation) were exclusive to this treatment. TCA/org transformation liberates energy stores by oxidation of acetyl-CoA originating from carbohydrates, fats, and proteins (Schmitz, 2008) and hence this activation suggests activation of energy transport in the plant. Focusing on plant defence, 20.17% (93/461) of the genes were allocated to the PPN-listed BINs, except beta glucanase and GST (Figure S11 and Table S8). In relation to hormone metabolism, upregulation of genes involved in auxin biosynthesis (four up- and four downregulated) was detected. Expression levels decreased for three genes involved in ET biosynthesis and signalling, that is, 1-aminocyclop ropane-1-carboxylate oxidase-like protein and ethylene responsive factors. Moreover, a 1-aminocyclopropane-1-carboxylate oxidase 1, a 2-oxoglutarate-dependent dioxygenase and a gibberellin 2-oxidase 5 were induced. Two abscisic acid-associated genes were differentially expressed; one of them was up- and one was downregulated. A brassinosteroid-associated gene was upregulated and one SA-associated gene was downregulated. Expression levels of MYB TFs, one WRKY and one AP2/EREBP were induced. Also, a WRKY TF, an AP2/EREBP and a DOF were downregulated. In

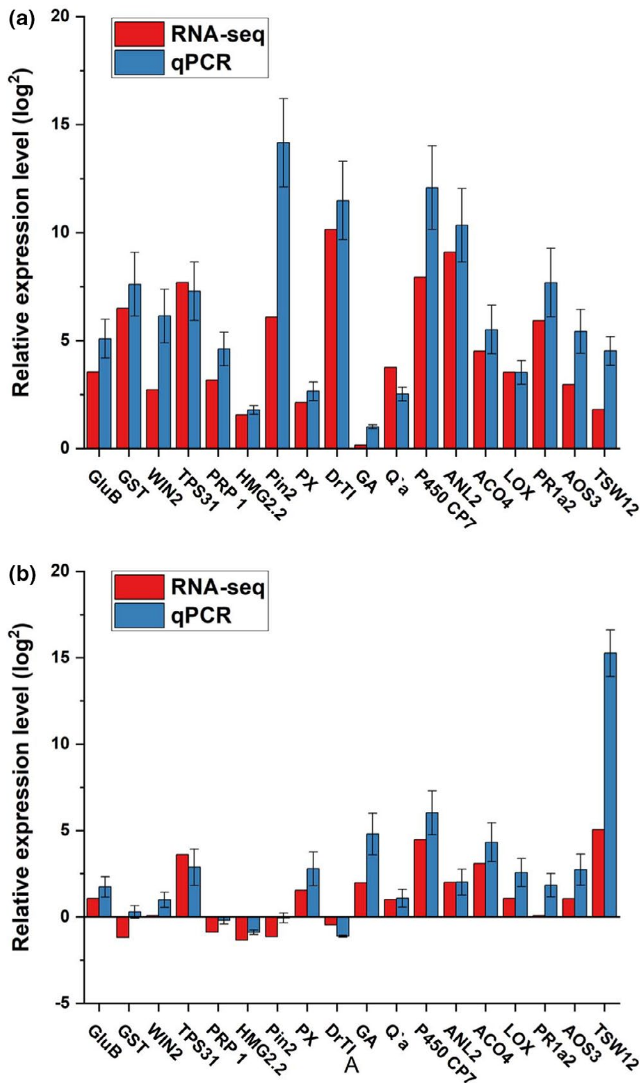

FIGURE 8 Relative expression levels of 21 tomato genes in response to (a) *Meloidogyne javanica* (root knot nematode) or (b) *Steinernema carpocapsae* (entomopathogenic nematode) infection at 7 days post inoculation (dpi) provided from RNA-seq (blue bars) and quantitative real-time PCR (qRT-PCR) (orange bars) data. For every treatment group, only significant changes in genes expression are shown. Genes encoding for tubulin alpha-3 chain and ubiquitin were applied to normalize the expression values for each candidate gene. *GluB*, Beta-glucanase; *GST*, glutathione-S-transferase; *WIN2*, chitinase; *TPS31*, alpha-humulene/(-)-(*E*)-beta-caryophyllene synthase; *PRP 1*, pathogenesis-related protein 1a; *HMG2.2*, 3-hydroxy-3-methylglutaryl coenzyme A reductase; *Pin2*, proteinase inhibitor II; *PX*, peroxidase 5; *DrTI*, Kunitz trypsin inhibitor; *GA*, gibberellin-regulated protein 2; *Q`a*, Beta-1 3-glucanase; *P450 CP7*, cytochrome P450; *ANL2*, homeobox-leucine zipper protein PROTODERMAL FACTOR 2; *ACO4*, 1-aminocyclopropane-1-carboxylate oxidase; *LOX*, lipoxygenase; *PR1a2*, pathogenesis-related protein; *AOS3*, allene oxide synthase; *TSW12*, nonspecific lipid-transfer protein. Note the consistency between RNA-seq and qRT-PCR data

relation to the cell wall, a xyloglucan endotransglucosylase/hydrolase 2, a beta xylosidase, two fasciclin-like arabinogalactan protein 7s, a COBRA-like protein, and an expansin-1 were upregulated, while a polygalacturonase, an alpha-1 4-glucan-protein synthase and a pectinesterase were downregulated. With regard to oxidative stress, we detected four upregulated genes: a thioredoxin H and three genes encoding peroxidases, but found reduced expression of five redox state-related genes: a thioredoxin reductase, three glutaredoxins and a glutaredoxin family protein. Overall, an increase in gene expression levels related to hormone metabolism, oxidative stress, biotic stress, and secondary metabolism was observed in tomato plants grown in presence of *S. carpocapsae* (EPN) (Figure S11).

3.7 | *M. javanica* (RKN) and *S. carpocapsae* (EPN) induce overlapping defence response in tomato

Within the transcriptome data, several DEGs are related to the biosynthesis of different secondary metabolites, such as phenylpropanoid, isoprenoid, phenol, lignin and lignan. Although 15 transcripts related to secondary metabolite production were differentially expressed between *M. javanica* (RKN)- and *S. carpocapsae* (EPN)-inoculated and uninoculated controls (Figure S11 and Tables S5 and S6), the secondary metabolite transcripts induced in response to the two nematode species were generally overlapping and showed an analogous profile of regulation. In the phenylpropanoid biosynthetic pathway, genes involved in phenylalanine ammonia lyase (PAL) and alcohol dehydrogenase were upregulated and O-methyltransferase was downregulated in response to *M. javanica* (RKN) (Figure S11 and Table S5). We also found upregulation of isoprenoid-related genes, including those encoding alpha-humulene/(-)-(*E*)-beta-caryophyllene synthase and transposase (Figure S11 and Table S5). Similarly, in response to *S. carpocapsae* (EPN), we detected upregulation of genes involved in PAL and downregulation of AMP-dependent synthetase and ligase (regulator of phenylpropanoid biosynthesis). The genes encoding laccase 1a and a laccase were also upregulated in the phenol pathway. In addition, a gene encoding alpha-humulene/(-)-(*E*)-beta-caryophyllene synthase was upregulated in response to *S. carpocapsae* (EPN), indicating activation of terpenoid-mediated defence responses (Figure S11 and Table S6). This result indicated an effect of *S. carpocapsae* on secondary metabolite synthesis pathways, which may help explain the above behavioural results suggesting a nematode-induced plant-herbivore interaction.

In relation to the group of 168 genes differentially expressed in both treatment groups (Table S4), 30 are related to plant defence (Figure S12 and Tables S4 and S9), 14 were upregulated and 16 were downregulated. Upregulated genes were generally related to hormone metabolism, cell wall modification, redox state, signalling, secondary metabolites and PR proteins, which might reflect host responses to nematode-associated molecular patterns (NAMPs) (Figure S12 and Table S9).

A principal components analysis was conducted to determine the main contributions to gene expression associated with various nematode inoculation treatments. The first (*x*-axis) and second (*y*-axis) principal components accounted for 95.62% of the variance in the total data (PC1 variance of 73.68% and PC2 variance of 21.94%). Figure 10

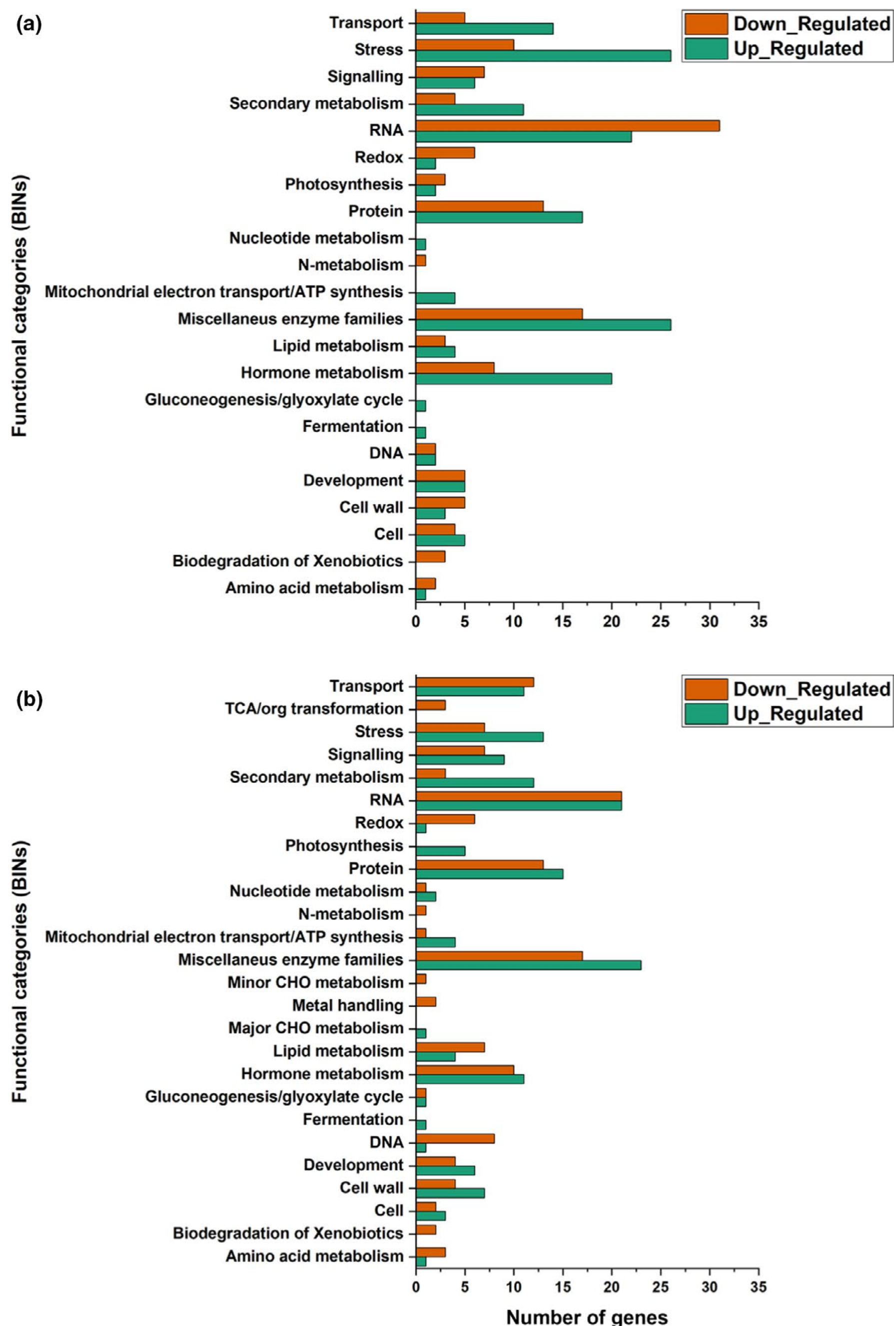

FIGURE 9 A MapMan diagram of modulated genes from tomato ($\log_2$ fold-change ≥1.5, false discovery rate (FDR) ≤ 0.05) according to their assignment to functional categories (BINs). The two diagrams indicate gene modulation in response to (a) *Meloidogyne javanica* (root knot nematode) or (b) *Steinernema carpocapsae* (entomopathogenic nematode) inoculation at 7 days post inoculation (dpi). BINs colored green are significantly upregulated, while those in red are significantly downregulated

represents a biplot analysis of data into PCs where the expression level of the *TSW12* gene and *GA* in plants corresponds to PC 1 and expression of *HMG2.2a*, *PRP 1*, *DrTI*, *GluB* and *Q`a* corresponds to PC 2. The correlation matrix among these parameters is shown in Table S10 and Figure 11. There was a significant positive correlation between *PRP 1* and *DrTI* (Figure 10 and Table S10, Figure 11). *HMG2.2a* and *PRP 1* were also significantly positively correlated. *HMG2.2* showed a significant negative correlation with *TSW12* and *GA*. However, *TSW12* exhibited a significant positive correlation with *GA*. The principal components associated with *M. javanica* (Mj)-inoculated roots and those associated with *M. javanica* and *S. carpocapsae* (Mj + Sc)-inoculated roots were different from the principal components associated with *S. carpocapsae* (Sc)-inoculated roots (Figure 10).

4 | DISCUSSION

Our results are congruent with the hypothesis that tomato plants "misrecognize" EPNs as RKNs and mount a broad-spectrum immune response with indirect consequences on both RKN performance below-ground and herbivore performance above-ground. Both functional guilds of nematodes (EPN vs. RKN) caused upregulation of coincident immunity related receptor complexes and signalling pathways. For example, plants initially recognized EPNs as invaders by activating SAR, as indicated by the overexpression of *PR-14* (Figure 10). Expression of the *PX* gene suggests that tomato plants also responded with induced production of antioxidant enzymes as protection against H_2O_2, which is typically generated as an early response to biotic challenges.

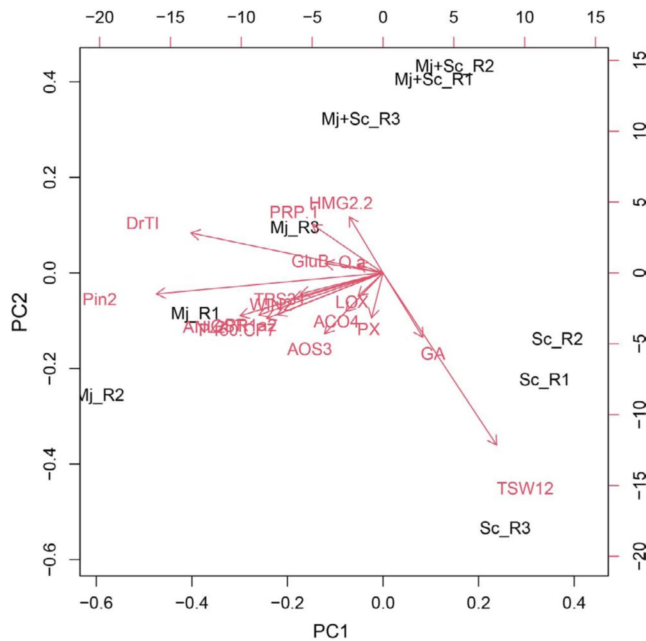

FIGURE 10 Principal component analysis of defence-related gene (log_2 fold-change) expression in tomato in response to inoculation by *M. javanica* (Mj—Root knot nematode), *S. carpocapsae* (Sc—Entomopathogenic nematode), or *M. javanica* + *S. carpocapsae* (Mj + Sc) treatments for Beta-glucanase (*GluB*); Glutathione-S-transferase (*GST*); Chitinase (*WIN2*); Alpha-humulene/(-)-(*E*)-beta-caryophyllene synthase (*TPS31*); pathogenesis-related protein 1a (*PRP 1*); 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMG2.2*); proteinase inhibitor II (*Pin2*); peroxidase 5 (*PX*); Kunitz trypsin inhibitor (*DrTI*); gibberellin-regulated protein 2 (*GA*); Beta-1 3-glucanase (*Q`a*); cytochrome P450 (*P450 CP7*); homeobox-leucine zipper protein PROTODERMAL FACTOR 2 (*ANL2*); 1-aminocyclopropane-1-carboxylate oxidase (*ACO4*); lipoxygenase (*LOX*); pathogenesis-related protein (*PR1a2*); allene oxide synthase (*AOS3*); nonspecific lipid-transfer protein (*TSW12*); (Mj) roots inoculated with *M. javanica*, (Sc) roots inoculated with *S. carpocapsae*, (Mj + Sc) roots inoculated with both *M. javanica* and *S. carpocapsae*. R1–R3 indicates expression patterns from three separate biological replicates

4.1 | Entomopathogenic nematodes modulate tomato plant immune response to reduce RKN infection

Entomopathogenic nematode-conferred immunity in plants can restrict RKN penetration into the root. More specifically, activation of this type of immune response in tomato roots exposed to EPNs subsequently limits the ability of invading juveniles to build feeding sites. However, our results indicate that the number of sedentary nematodes inside the roots was considerably reduced in plants exposed to EPN. We demonstrate that exposing plants to EPN elicited a complex machinery of plant defence responses within 3 days of inoculation, when only mobile invasive forms of RKN were detected. It appears that immunity was induced before feeding sites were built, when attacking juvenile RKN were still searching for cortical cells to penetrate in the apical elongation zone of roots. Our results suggest that plant immune response was triggered rapidly after EPNs contacted plant roots. Although plant defence is typically thought of as being triggered upon initial contact with invading pathogens (in this system: RKN J2s), comprehensive plant immunity probably includes other mechanisms including preemptive responses, such as the EPN-induced modulation that we describe here. In the current study system, such modulation of defence response acts to limit construction of feeding sites by RKN, thus decreasing subsequent populations of RKN sedentary forms. For those RKN which evade this defence response and successfully construct feeding sites, subsequent development and reproduction do not appear to be affected. Overall, our results support the hypothesis that previously observed antagonism between EPNs and PPNs in the rootzone (Kenney & Eleftherianos, 2016) is mediated indirectly via plant defence against PPNs induced by EPNs.

The EPN-induced modulation of plant defence was transient and diminished over time, as is typically observed for pattern-triggered immunity (PTI). At thirty dpi, RKN had developed into gravid females even in EPN-exposed plants. It is also possible that some RKN J2s may have initially entered roots, and although their development may have been to some degree retarded, they could have eventually built feeding sites and reproduced. However, our data indicate that EPN-induced modulation of defence reduced tomato root infection by approximately 50%, in terms of diminished RKN egg fecundity and fertility.

4.2 | Transcriptomic analysis reveals similar host plant response to different nematode guilds

We inoculated tomato with *S. carpocapsae* and/or *M. javanica* and performed transcriptome analysis to comprehensively understand how plants respond to either of these highly specialized nematode life history strategies individually, as well as to the simultaneous interaction of both nematode types. We detected candidate resistance genes in tomato that may play essential roles in defence response to the RKN, *M. javanica*, which were also coincidentally triggered when roots were exposed to the EPN, *S. carpocapsae*. Furthermore, we attempted to identify the mechanisms underlying plant response to the EPN specifically. Overall, 905 DEGs were identified, and inoculation of tomato with *S. carpocapsae* induced 461 DEGs compared with mock controls (water injection). Notably, *S. carpocapsae* inoculation caused upregulation of 223 genes compared with mock-treated plants, which suggests a robust transcriptional response in plants caused by the entomopathogen.

The physiological responses of plants to both RKN and EPN inoculation were examined by GO enrichment analysis. Notably, the group of genes related to defence response was significantly enriched in the DEGs investigated by pairwise comparisons, suggesting that *S. carpocapsae* and/or *M. javanica* affected plant defence similarly, despite occupying different functional guilds. Furthermore, plants inoculated with *S. carpocapsae* and/or *M. javanica* exhibited significant enrichment of DEGs associated with the phenylpropanoid and flavonoid pathways.

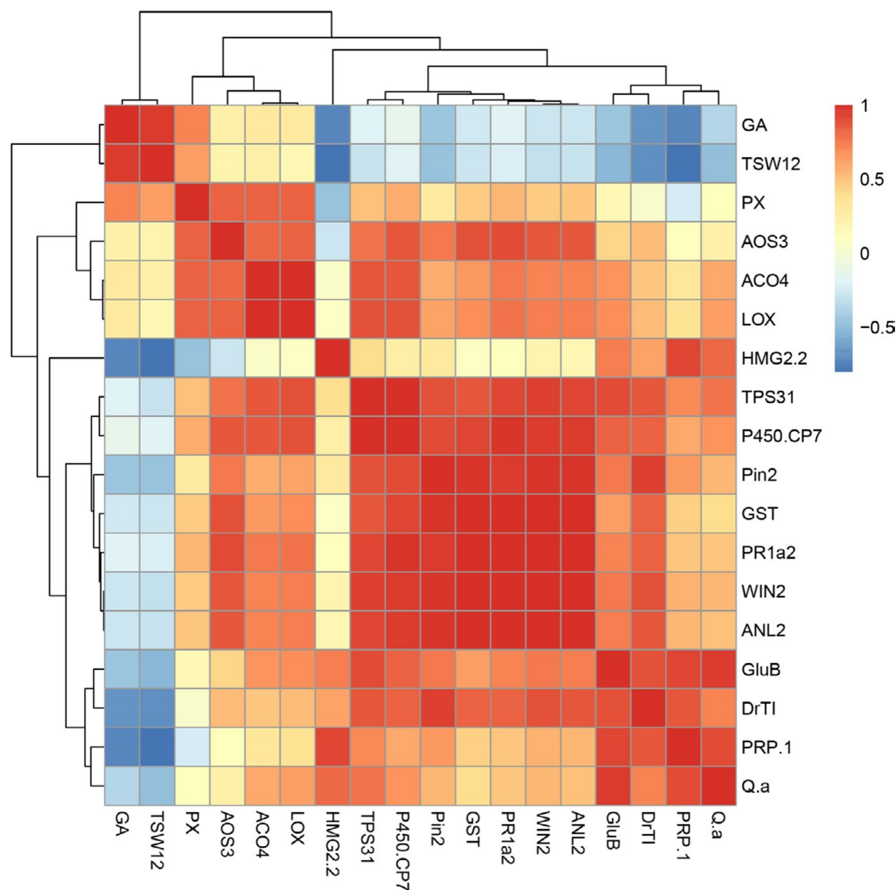

FIGURE 11 Correlation matrix of genes involved in plant defence. The heatmap represents correlations between genes analyzed by the Pearson correlation test using R 4.0.3 software and visualized with R software. Squares indicate structural genes; Beta-glucanase (*GluB*); glutathione-S-transferase (*GST*); Chitinase (*WIN2*); Alpha-humulene/(-)-(*E*)-beta-caryophyllene synthase (*TPS31*); Pathogenesis-related protein 1a (*PRP 1*); 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMG2.2*); proteinase inhibitor II (*Pin2*); peroxidase 5 (*PX*); Kunitz trypsin inhibitor (*DrTI*); gibberellin-regulated protein 2 (*GA*); Beta-1 3-glucanase (*Q`a*); cytochrome P450 (*P450 CP7*); homeobox-leucine zipper protein PROTODERMAL FACTOR 2 (*ANL2*); 1-aminocyclopropane-1-carboxylate oxidase (*ACO4*); lipoxygenase (*LOX*); pathogenesis-related protein (*PR1a2*); allene oxide synthase (*AOS3*); nonspecific lipid-transfer protein (*TSW12*). The same colour within the heatmap indicates the same level of gene expression

The role of phenylpropanoids in plant defence is well-established (Vaganan et al., 2014). Flavonoids, isoflavonoids, hydroxycinnamic acids, monolignols, and stilbenes are all classes of phenylpropanoids that function as host defence molecules, acting as potential barriers and signalling molecules to induce defence against pathogen attack (Dixon et al., 2002). These results suggest that inoculation of tomato with the entomopathogen, *S. carpocapsae*, caused activation of genes involved in phenylpropanoid biosynthesis.

We also discovered that the lignin biosynthetic pathway was significantly enriched in the DEGs of plants inoculated with *S. carpocapsae* and/or *M. javanica*, and it is known that lignin is produced upon nematode infection and acts as a physical barrier (Caño-Delgado et al., 2003; Ji et al., 2015). In fact, nematode resistance is correlated with higher lignin content among several plant species (Holbein et al., 2016). Our results also indicate that genes involved in both hypersensitive response (HR) and oxidation–reduction (redox) processes were affected by *S. carpocapsae* inoculation. The HR is a form of programmed cell death that is related to disease resistance (Morel & Dangl, 1997), which involves excessive production of reactive oxygen species (ROS) (Lozano-Torres et al., 2014; Shah et al., 2017). Therefore, it is also possible that detection of *S. carpocapsae* by plant roots leads to the host redox status, which induces defence responses.

Tomato plants exposed to *S. carpocapsae* exhibited differential expression of the transmembrane receptor tyrosine kinase (RTKs) signalling pathway, among which were the *Arabidopsis* homologues *FLS2* and *NILR1*. PTI is the basal plant immune pathway that is activated upon recognition of PAMPs with the help of surface- localized receptor-like kinases (RKs), such as *FLS2* and *NILR1*, which trigger downstream signalling (Nürnberger et al., 2004; Thomma et al., 2011). In *A. thaliana*, the leucine-rich repeat receptor-like kinase, termed *NILR1*, is required for induction of innate immunity to parasitic nematodes (Mendy et al., 2017). Therefore, an interaction between NAMPs and RTK receptors could lead to activation of a PTI–response upon detection of *S. carpocapsae* by tomato roots.

We also compared the transcript levels of TFs among plants inoculated with *S. carpocapsae* or with *M. javanica*. Among the DEGs, WRKY TF was primarily affected, followed by MYB, and ERF. WRKY

TFs are involved in several different plant developmental processes, most notably in innate immune system response and senescence (Eulgem & Somssich, 2007). A complex functional interaction occurring among preferential homoeologous alleles (*AtWRKY18*, *AtWRKY40*, and *AtWRKY60*) has been described in plant defence responses to a diversity of pathogenic microbes such as *Botrytis cinerea* and *Golovinomyces orontii* (Shen et al., 2006; Xu et al., 2006). WRKY TFs are believed to play a pivotal role in the regulation of signalling networks through the phytohormones SA and JA, mostly in complex cross-regulation (Xu et al., 2006). ERF TFs are regulators of *PR*-genes, as well as ET-, SA-, and JA - mediated defence-related genes (Gutterson & Reuber, 2004).

Our PCA revealed that expression of candidate resistance genes (*GluB*, *PRP 1*, *HMG2.2a*, *DrTI*, *GA*, *Q`a*, and *TSW12*) was correlated. Beneventi et al. (2013) also reported a significant positive correlation between expression of a nonspecific lipid transfer protein (nsLTP) (also referred to as "pathogenesis-related" protein p14) and penetration of *M. javanica* into soybean roots. nsLTPs play a key role in general plant stress response and thus increased nsLTP expression in *M. javanica* -infected roots is perhaps unsurprising. These results indicate that expression of both *TSW12* and *PRP 1* genes in roots inoculated simultaneously with *M. javanica* and *S. carpocapsae* (Mj+Sc), in concert with nsLTP response, could form a complex that competitively binds to fungal elicitin receptors with a lipid-derived molecule interaction, for example, JA or lysophosphatidylcholine (lysoPC). These elicitins are small cysteine-rich secreted proteins (SCRSPs) on plasma membranes, such as those secreted by the plant pathogen *Phytophthora*, with structural pattern similarities to nsLTPs (Liu et al., 2015). During invasion of roots by *M. javanica*, such elicitins may limit nematode entry or jeopardize development of *M. javanica* J2s in the syncytium, reducing the likelihood of *M. javanica* infestation.

Interestingly, overall gene expression was higher in EPN-inoculated than RKN-infested plants. nsLTPs are known to be involved in long-distance defence signalling related to SAR (Maldonado et al., 2002). Also, nsLTP proteins characterized from different plant species show strong in vitro antimicrobial properties (Gizatullina et al., 2013). With regard to plant growth and development, nsLTPs play an important role in embryogenesis, seed development and germination, and during nodule organogenesis (Liu et al., 2015). This was reflected in the correlation analysis, where a significant positive correlation was observed between *TSW12*, and *GA*. The PCA also revealed that the two nematode guild treatments caused some nonoverlapping responses in plants, which also differed from when they were presented in combination. For example, *TSW12* expression was greater in plants inoculated with EPN than in the plants co-inoculated with EPN+RKN (Figure 10). Overall, EPN inoculation directly mediated enhanced plant defence and reduced subsequent RKN infection. Our transcriptomic investigation is congruent with the hypothesis that plants mount a broad-spectrum defence response when encountered by EPNs that is remarkably similar to that induced by RKN infection. This would seem to confirm that hypothesis that plants misrecognize EPNs as invaders.

Plant parasitic nematodes can overcome plant defence mechanisms, in particular those related to *PR*-genes expression, to facilitate successful colonization of their hosts (Goverse & Smant, 2014; Mantelin et al., 2015; Vieira & Gleason, 2019). For example, they induce an enzymatic response from the antioxidant system to neutralize toxic ROS (Molinari & Leonetti, 2019). Expression levels of the *PX* gene quantified here were approximately 2-fold higher in roots of plants treated with *M. javanica* or *S. carpocapsae* compared with those inoculated simultaneously with both *M. javanica* and *S. carpocapsae*. Therefore, we putatively demonstrated for the first that the interaction between *M. javanica* and *S. carpocapsae* in the rhizosphere is associated with inhibition of a nematode-primed *PX* gene that is normally upregulated by RKNs alone as early as 7 dpi. A similar suppression of nematode-induced GP enzyme activity was detected in roots of plants inoculated with *M. javanica* + *S. carpocapsae* at the same stage after inoculation. This plant defence modulation caused by *S. carpocapsae* appears to be absent by 15–28 dpi. Antioxidant enzyme activity causes degradation of H_2O_2 that favors nematode development; thus, suppression of these enzymes mediated by EPNs may augment plant suppression of parasite (RKN) invasion. Accumulation of SA in primed plants following exposure to EPNs is congruent with the hypothesis that modulation of *PRP 1* and *PR-14* genes by each nematode functional guild (EPN or RKN) is conserved. In addition, accumulation of endogenous SA leads to increased H_2O_2 activity (Molinari, 2007). EPNs may modulate plant defence against RKN invasion, in part, by suppressing active expression of antioxidant enzymes. Genes conferring resistance to RKNs in tomato, which prevents development of RKN juveniles in roots, are associated with a marked reduction in root catalase (CAT) and ascorbate peroxidase (APX) activity following inoculation (Molinari, 1990).

4.3 | Entomopathogenic nematodes reduce above-ground herbivory by inducing plant defence

Exposure of tomato roots to EPN also reduced above-ground herbivore host preference and performance congruent with the hypothesis that EPNs triggered a broad-spectrum SAR and/or ISR. Tomato plants treated with *M. javanica* and/or *S. carpocapsae* exhibited decreased attractiveness to adult female *T. absoluta* compared to controls. These behavioural observations suggest that nematode inoculations may affect release of VOCs by tomato plants, which was consistent with observed changes in the VOC-related transcriptome. The significant differences observed between the VOC-transcriptomes of plants treated with *M. javanica* and/or *S. carpocapsae* versus untreated plants are further corroborated by genes involved in both the octadecanoid and SA pathways. In the presence of the EPN, *S. carpocapsae*, tomato plants showed stronger expression of a gene encoding alpha-humulene/(-)-(E)-beta-caryophyllene synthase, indicating activation of terpenoid-mediated defence responses (Figure S11 and Table S6). Large-scale transcriptome reprogramming reducing the performance of aphids on tomato has also

been shown following root infection with the fungus, *Trichoderma harzianum* (strain T22) (Coppola et al., 2019). Metabolic changes induced following infestation of this fungus included substantial accumulation of isoterpenoids (Coppola et al., 2019), similar to the EPN-induced responses observed here. These results suggest that EPNs triggered secondary metabolite synthesis pathways, reducing herbivore performance via both direct and indirect defences. Our future objectives include investigating the qualitative and quantitative changes to VOC production above-ground that may be caused by EPN exposure of tomato roots and relate those to above-ground plant-herbivore interactions.

In addition to changes in VOC production, several key enzymes associated with both early and late stages of phenylpropanoid metabolism were differentially expressed in plants exposed to *M. javanica* and/or *S. carpocapsae*. Phenylpropanoid metabolism produces a rich source of secondary metabolites, including molecules with antimicrobial properties that exhibit direct repellency to herbivores (Didry et al., 1999; Naoumkina et al., 2010; Vogt, 2010). Therefore, our transcriptomic analyses suggest that EPN inoculation may have caused indirect antibiotic or antixenotic effects against above-ground herbivory. Furthermore, we observed evidence for reinforcement of physical barriers, such as cell wall formation and lignification, in response to EPN presence (Naoumkina et al., 2010).

Congruent with our results, Helms et al. (2019) recently reported that Colorado potato beetles, *Leptinotarsa decemlineata*, laid fewer eggs on above-ground foliage of potato exposed to *H. bacteriophora* IJs belowground as compared with nonexposed controls. Selection should favour avoidance of cues associated with potential predators by herbivores (Kats & Dill, 1998), which is congruent with our results, if EPN-treated tomato plants are characterized by specific chemical and/or visual cues that affected host preference of *T. absoluta*. Although reducing herbivore performance above-ground may be considered an apparent negative consequence for EPNs in cases where potential future prey are repelled, we speculate that selection may favor an EPN-mediated cue that "warns" plants to defend against RKN invasion since these vermiform root parasites compete for the same resource (roots) that is used by the arthropod hosts of EPNs below-ground. It may benefit EPNs for plants to defend against RKN invasion by conserving available food for the insect larvae that EPN require for development.

Leaf consumption by *T. absoluta* caterpillars on tomato did not appear affected by *M. javanica* and/or *S. carpocapsae* inoculation; however, pupal duration increased while survival decreased on nematode inoculated as compared with control plants. Developmental delays observed in herbivores are usually related to the activity of digestive enzyme inhibitors and simultaneous compensative hyperproduction of counteracting enzymes (Brioschi et al., 2007; Brito et al., 2001; Chen et al., 2005). Inoculation of plants with *M. javanica* and/or *S. carpocapsae* enhanced production of protease inhibitors (PIs) in plant tissues, presumably as a result of JA pathway activation. This was further confirmed via observed upregulation of genes encoding representative classes of PI molecules, such as threonine deaminase (TD), PPO, and leucine aminopeptidases (LAP), which are known to cause developmental delays and inhibit pupation. Likewise, inoculation of tomato plants with related *Steinernema* species decreases leaf herbivory by *Spodoptera exigua* (An et al., 2016) via upregulation of the octadecanoid pathway, which results in accumulation of JA in shoots. Furthermore, developmental and defence mechanisms in plants are further fine-tuned via the arginine catabolic pathway (Winter et al., 2015), and the observed upregulation of key genes involved in this pathway following EPN inoculation here would suggest that EPN-induced defence in tomato is a well-regulated response.

An exceptional aspect of the tomato–*S. carpocapsae* interaction described here is the rapid impact of EPN inoculation on response of TFs involved in regulation of defence-related genes. Our results indicate that protein-coding genes for several TF families related to defence such as ERF, WRKY, and MYB were upregulated, similar to the relationship described between tomato roots and *M. incognita* (Lee et al., 2019). These TFs are known to be associated with innate immunity in plants. For instance, AP2/ERF proteins are associated with expression of JA-responsive genes in *Arabidopsis*; these are known as octadecanoid-responsive components that induce the expression of several JA- and ET-related defence genes (Pré et al., 2008). More specifically, *OsERF3* is a positive regulator of resistance against chewing herbivores in rice, affecting induction of MAPK gene cascades and hormone synthesis (Lu et al., 2011). In addition, the MYB family of TFs activates JA signalling pathways and is associated with plant resistance against aphids and lepidoptera. Likewise, *AtMYB44* regulates resistance against *Myzus persicae* (Sulzer) and *Plutella xylostella* (Linnaeus) by activating *EIN2*-affected defences in *Arabidopsis* (Coppola et al., 2019).

Our data indicate that inoculation of tomato roots with RKN stimulates a reprogramming of the transcriptome that influences both the SA and JA pathways (Bali et al., 2019; Kumar et al., 2019; Kyndt et al., 2012; Lee et al., 2019; Petitot et al., 2017; Postnikova et al., 2015; Santini et al., 2016; Shukla et al., 2018; Zhou et al., 2020) and is in large part mimicked by EPNs which cause a similar outcome. Recently, higher constitutive levels of abscisic acid (ABA) and JA, and basal expression of ABA- and JA-related transcripts were described in a soybean genotype tolerant to the soybean aphid (Chapman et al., 2018). Similarly, we observed induction of transcripts related to ABA and the aforementioned JA in response to *S. carpocapsae* inoculation in the current investigation. Another recent investigation demonstrated that herbivory by subterranean nematodes induces plant defence responses with vastly different effects on herbivore performance, depending on nematode feeding strategy (Van Dam et al., 2018). While the cyst nematode, *Heterodera schachtii*, induced SA-pathway associated defence reducing aphid performance on black mustard (*Brassica nigra*), the RKN, *M. hapla*, induced responses associated with the JA pathway enhancing aphid performance and infestation (Van Dam et al., 2018).

The specific functional mechanism(s) by which EPNs trigger host defence responses in plants remains an open question, but there are likely candidates. Dideoxysugar derivatives, termed ascarosides, are a highly conserved group of multifunctional

pheromones produced by nematodes (Kaplan et al., 2011, 2012). Recently, it has been shown that ascarosides from PPNs are NAMPS that induce PTI in exposed plants, increasing broad-spectrum resistance (Manosalva et al., 2015). Given their ubiquitous and promiscuous functions among nematode species, it seems likely that ascarosides associated with the EPNs investigated here may cause a similar effect. Alternatively, it is possible that other molecules or effectors associated with EPNs may mediate the observed induced defence response in plants. It has been suggested that plants may mistake EPNs for a microbial threat given the similarity between volatiles identified from EPN-infected cadavers and probably associated with their symbionts and those from pathogenic microbes (Helms et al., 2019).

Collectively, our results describe a comprehensive picture of the multitrophic interactions and underlying transcriptional and biochemical changes that occur in tomato inoculated with *S. carpocapsae* or *M. javanica*. We demonstrate that EPNs (*S. carpocapsae*) interacting with tomato caused analogous and coincident enhanced plant defence responses against RKNs (*M. javanica*) in the rootzone, and also reduced the preference and performance of a folivore (*T. absoluta*) above-ground. Furthermore, inoculation of tomato with EPN or RKN caused enhanced activity of GP and PPO in roots, but not shoots, as well as induced expression of genes associated with antioxidant enzymes. The conferred immunity appears to occur systemically to decrease the process of feeding site construction by parasitic nematodes as well as egg laying and development by folivores. Continued investigation is needed to explore the consequences of using EPNs as part of a growing strategy in integrated pest management, given the peculiar and unpredictable interactions these beneficial microorganisms might have with the existing soil microbiome of various plant species.

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AUTHOR CONTRIBUTIONS

SK and JK conceived the ideas. SK, AS, AJ, TK and JK designed methodology; SK collected the data; SK, AS, M Haydarpour, MZ and M Hosseini analyzed the data; SK, AJ, LS, TK, AS, MC, JA, M Hosseini and JK validated the results. SK, LS, TK, MC, M Hosseini, and JK interpreted results. SK provided visualization. SK and JK prepared the initial draft. SK, LS, TK and JK edited the final manuscript. All authors approved the final manuscript. JK provided funding and supervision.

DATA AVAILABILITY STATEMENT

All data are available in public form and corresponding accession numbers and doi are presented. The raw data of DNA sequences raw sequence data are available at NCBI BioProject database (http://www.ncbi.nlm.nih.gov/bioproject) under accession number PRJNA732672. Supplementary data of the project was submitted during manuscript submission as "online publication". All data are both available either as "supplementary files" for online publication and also on DRYAD as: Shokoofeh Kamali, Ali Javadmanesh, Lukasz Stelinski, et al. Beneficial worm allies warn plants of parasite attack below-ground and reduce aboveground herbivore preference and performance. *Authorea*. 13 July 2021. The assigned doi is: 10.22541/au.162620679.92139255/v1. The doi for DRYAD is https://doi.org/10.5061/dryad.x0k6djhjs

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