# Beneficial worm allies warn plants of parasite attack belowground and reduce aboveground herbivore preference and performance

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## Abstract

We investigated responses of tomato (*Solanum lycopersicum*) to two functional guilds of nematodes - plant parasite (*Meloidogyne javanica*) and entomopathogens (*Heterorhabditis bacteriophora*, *Steinernema feltiae* belowground, and *S. carpocapsae*) - as well as a leaf mining insect (*Tuta absoluta*) aboveground. Our results indicate that entomopathogenic nematodes (EPNs): 1) induced plant defense responses, 2) reduced root knot nematode (RKN) infestation belowground and 3) reduced herbivore (*T. absoluta*) host preference and performance aboveground. Concurrently, we investigated the plant signaling mechanisms underlying these interactions using biochemical and transcriptome analyses. We found that both entomopathogen and parasite triggered immune responses in plant roots with shared gene expression. Tomato plants responded similarly to presence of RKN or EPN in the rootzone, by rapidly activating polyphenol oxidase (PPO) and guaiacol peroxidase (GP) activity in roots, but simultaneously suppressed this activity in aboveground tissues. We quantified changes in gene expression in tomato that may play essential roles in defense response to RKN, which were also coincidentally triggered by EPN. For example, *PR-14* expression was greater in plants inoculated with EPN than in plants co-inoculated with both nematode functional guilds. Overall, EPN inoculation directly mediated enhanced plant defense and reduced subsequent RKN infection. Likewise, we show that EPNs modulate plant defense against RKN invasion, in part, by suppressing active expression of antioxidant enzymes. Inoculation of tomato roots with EPNs belowground reduced both host preference and performance of the aboveground herbivore, *T. absoluta*. Inoculations of roots with EPN also triggered an immune response in tomato via up-regulated phenylpropanoid metabolism and synthesis of protease inhibitors (PIs) in plant tissues, which could explain an observed decrease in egg laying and developmental performance exhibited by herbivores on EPN-inoculated plants. Our results support the hypothesis that subterranean EPNs activate a battery of plant defenses

associated with systemic acquired resistance (SAR) and/or induced systemic resistance (ISR) with concomitant antagonistic effects on temporally co-occurring subterranean plant pathogenic nematodes and terrestrial herbivores.

Keywords: multi-trophic interactions, biological control, molecular ecology, *Meloidogyne javanica*, entomopathogenic nematodes, *Tuta absoluta*, EPN-induced plant defense, phenylpropanoid biosynthetic pathway

## Introduction

Nematodes are a diverse group of ubiquitous roundworms (Cobb, 1914). Parasitic behaviour has evolved in nematodes multiple times (Blaxter, 2003). Nematodes can be parasites of plants, vertebrates, and arthropods and thus play several critical ecological roles or become serious pests affecting the lives of billions of people globally (Brown et al., 1995; Lamberti, 2012) and root knot nematodes (RKNs) can cause direct crop damage (Singh et al., 2013). 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 programs (Kaya and Gaugler, 1993; Gaugler, 2018).

While essential evolutionary differences exist between plant parasitic nematodes (PPNs) and EPNs, their overall lifecycles are remarkably similar. Ultimately, both plant and insect parasites must find and infect their host, which requires passage through a physical barrier (plant skin vs. insect cuticle). After this initial barrier is overcome, these vermiform parasites must then circumvent the host's immune response. Various strategies have evolved to accomplish this, including toxin release (Wang et al., 1994), or suppression of the innate immune/defense response (McSorley and Maizels, 2012). After infection, and following reproduction within the host, both plant and insect parasites leave the host as resources diminish beyond the point of sustaining further reproduction (Kaplan et al., 2012). In many species of nematode parasites, this stage of the livecycle is free-living and it is during this time that a complex environment must be navigated in search of a new host to repeat the lifecycle. During this free-living stage, nematodes can be highly responsive to specific cues derived from plants, insects or interactions among these trophic levels (Lewis et al., 1993).

Multi-trophic 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 aboveground ecosystems (Dicke and Sabelis, 1987; Turlings et al., 1990; Dicke et al., 1999). Recently, however, the development of new (bio) chemical and molecular techniques has enabled exploration of belowground communities and their interactions (Van Tol et al., 2001; Aratchige et al., 2004; Rasmann et al., 2005; Ali et al., 2012). These belowground communities provide a rich context for understanding multi-trophic interactions; in addition to having all the

components of above-ground systems (primary, secondary, tertiary predators, herbivores, volatile communication, etc.), they can be manipulated in laboratory and field settings.

Plants can directly affect the phytobiome with induced changes in their volatile profile in both the terrestrial and subterranean environments (Dicke, 2016). Furthermore, parasites can instigate unique cascades of effects through top-down regulation of herbivore populations above- and belowground that, in turn, function to regulate levels of herbivory (Kessler and Baldwin, 2002; Preisser et al., 2006; Van Dam et al., 2010; Dicke and Baldwin, 2010). Injury caused by herbivore feeding influences multitrophic interactions by indirectly attracting tertiary parasites of the herbivores, which is called indirect defense (Mumm and Dicke, 2010). Broadly, indirect defense is mediated by qualitative or quantitative changes in the volatile organic compounds (VOCs) released by plants in response to herbivory (Meiners and Hilker, 1997; De Moraes et al., 2001). 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 (HIPVs) on predators and expanding to address how subterranean predators (EPNs) may broadly modulate induced plant defense response (Jagdale et al., 2009; An et al., 2016) and ultimately impact plant-herbivore interactions (Helms et al., 2019; Li et al., 2020). In tomato, EPNs induce defense 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 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 odors from EPN-infected cadavers induce plant defense 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 and Bird, 1986). There are numerous examples showing that populations of PPNs decline upon exogenous applications of EPNs (Smitley et al., 1992; Grewal et al., 1997; Jagdale et al., 2002). 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 and Bird, 1986), stimulation of nematode predator population growth (Ishibashi and 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 defense 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 aboveground and displace or reduce PPN populations belowground (An et al., 2016; Helms et al., 2019; Kenney and 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 belowground 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 belowground, and an herbivore (aphids) aboveground, an emerging parsimonious hypothesis is that EPNs broadly modulate populations of aboveground herbivores and belowground nematode communities occupying different functional guilds indirectly via their broad-spectrum upregulation of plant defense.

Our overarching goal was to test the above hypothesis with a new example system that included the primary producer, tomato (*Solanum lycopersicum*) with two functional guilds of belowground nematodes - plant parasites and entomopathogens - as well as an aboveground mining insect, *Tuta absoluta*. Moreover, we described the consequences of inoculating the rootzone with EPN on the biochemical and post-transcriptional responses of plants and directly compared these with responses caused by RKN infection of roots or herbivore attack of leaves. Our specific hypotheses were that: 1) plants recognize and respond to entomopathogens and plant parasites similarly, i.e. '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 defense induced in response to perceived subterranean invaders (EPN or RKN) is a global effect concurrently reducing performance of root parasites belowground and foliavores aboveground 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, effectively priming defenses to reduce subsequent infection by the root parasite. Exposure of tomato roots to EPNs triggered a battery of immune responses in plant roots similar to that induced by the parasite itself, effectively priming tomato to withstand subsequent parasite attack belowground as compared with non-primed mock controls. Complementary experiments exploring the effects of belowground biota on aboveground 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 behavior were caused via indirect defense. 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 defense simultaneously explains reduced RKN performance belowground and herbivore performance aboveground.

## Materials and Methods

### 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. Then, fourteen-day-old seedlings of equivalent height were transplanted into pots filled and were grown for two weeks in a controlled-environment greenhouse. Plants used in experiments were approximately four-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. Since 2016, the rearing has been carried out in insect-proof screen cages in a growth chamber. 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 and 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).

### Experiment 1. Interactions between the root-knot nematode, *M. javanica* and entomopathogenic nematodes, *H. bacteriophora*, *S. feltiae*, and *S. carpocapsae*

The purpose of this experiment was to test the hypotheses that: 1) plants recognize and respond to entomopathogens (EPNs) as plant parasitic root knot nematodes (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 entomopathogenic nematode inoculation on post-transcriptional responses of host plants investigated subsequently.

### 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 (*SI Appendix*, Fig. S1). The experiment was arranged in a completely randomized design (CRD). Each treatment group was replicated eighteen 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 Jr 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 3 developmental groups: second-stage juvenile, third- to fourth-stage juvenile, or adult, according to Shukla et al. (2018). Therefore, tissue from three successive infected days 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 6 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.

**Estimation of defense-related enzyme activity**

Guaiacol peroxidase (GP) and polyphenol oxidase (PPO) are known essential defense-associated enzymes in plants and are widely used as measurements of plant defense against phytopathogens (Ye et al., 2013; Qin et al., 2015). Thus, these two enzymes were selected as markers for plant defense response to test this hypothesis. Four-week-old tomato seedlings were assigned to the following treatments: (i) mock-inoculated control (plants were treated with distilled water only), (ii) plant parasitic nematode alone (RKN—*M. javanica*), (iii) entomopathogenic nematode alone (EPN—*S. carpocapsae*), and (iv) 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.

**Experiment 2. Response of *T. absoluta* females to tomato plants inoculated with RKN or EPN**

We tested the hypothesis that plant defense in response to perceived subterranean invaders (EPN or RKN) reduces herbivore performance aboveground. We quantified (i) the oviposition preference of the tomato

leafminer, *T. absoluta* and (ii) larval development of leafminers in response to root invasion by the RKN, and/or inoculation with three species of EPNs (*H. bacteriophora*, *S. feltiae* or *S. carpocapsae*).

**Two-choice assays**

To evaluate whether *T. absoluta* egg-laying and development is affected by belowground 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 (*SI Appendix*, Fig. S2*A* and *B*). Choice tests were conducted separately with each nematode species: RKN, *M. javanica*, EPN *H. bacteriophora*, EPN *S. feltiae* or EPN *S. carpocapsae*. 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 (*SI Appendix*, Fig. S2*C*). 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 un-inoculated and root-inoculated plants were held in separate cages and permitted to mate (*SI Appendix*, Fig. S3*A* and *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 (*SI Appendix*, Fig. S3*C*). 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).

**No-choice assays**

Ten 4-week-old *S. lycopersicum* plants were allocated for each treatment group: plants without nematodes (Ctrl); roots inoculated with *M. javanica* (Mj), roots inoculated with *S. carpocapsae* (Sc), and roots inoculated with both *M. javanica* and *S. carpocapsae* (Mj+Sc) (*SI Appendix*, Fig. S4*A*). 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 (*SI Appendix*, Fig. S4*B*). 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 (*SI Appendix*, Fig. S4*C*).

## Experiment 3. Gene expression in tomato exposed to RKN, EPN, 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 defense 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 (Ctrl); (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 (*SI Appendix*, Fig. 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 third, seventh (prior to tomato leaf miner attack), fifteenth (7 d after moth egg release) and twenty-sixth day (17 d 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 d post-egg release, when the initial larvae began to pupate. The larvae were flash-frozen into 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, France) 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, BioTek, USA).

### 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 2 volumes of 100% ice-cold ethanol containing 0.1 mL of a 3M sodium acetate buffer solution, pH

5.5, in 1.5 mL microcentrifuge tubes and shipped on dry ice to Macrogen Inc. (Macrogen Inc., South Korea) for library construction and illumine sequencing. Upon receiving samples, the RNA was re-pelleted and quality control (QC) was checked with a 2100 Bioanalyzer (Agilent Technologies). RNA QC was confirmed as all samples had an RNA integrity number (RIN) ≥ 7.0 and lack of genomic DNA contamination prior to moving for library constructions and sequencing.

One μg of total RNA was applied as starting material and cDNA libraries were constructed using the Truseq stranded total RNA library preparation kit (Illumina, Inc., CA, USA). The libraries were quantified with quantitative real-time PCR (qRT-PCR) (Bustin et al., 2009) and paired-ends (2x100 bp) sequencing was conducted on a HiSeq2500 platform (Illumina, Inc., CA, USA).

**RNA-seq data analysis**

Qualities of the raw reads were checked through FastQC with default input parameters (*P*-values > 0.01, 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. The raw data were submitted to the NCBI Sequence Read Archive (SRA) database.

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 down regulated 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 ≤ 0.05).

**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 primers sequences for 21 tomato genes were designed through the Beacon Designer™ 8.21 software (Premier Biosoft International, Palo Alto, CA, USA). Detailed information of the primers is presented in Table 1. Two micrograms of DNA-free total RNA was used for cDNA synthesis using Revertaid first strand cDNA synthesis kit (Thermo Scientific, USA) following the 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 1). Relative expression levels of each target gene were determined according to the $2^{-\Delta\Delta Ct}$ method (Livak and 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 (Team, 2013; Santini et al., 2016).

To evaluate whether expression levels of candidate plant defense-related genes were affected by exposure to root knot-nematode, entomopathogenic nematode 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.

**Functional annotation and enrichment analysis**

Gene set enrichment analysis of DEGs was conducted with Blast2GO v.5.2 (Conesa et al., 2005) according to the BLASTx results (e-value cutoff of $1 \times 10^{-6}$) followed by mapping and annotation stages through the default parameters of Blast2GO. Subsequently, the following methods were applied on the set of differentially expressed genes (DEGs): AgriGO tool, which specifically focuses on agricultural species, was applied to conduct a singular enrichment analysis (SEA) (FDR correction and Fisher's exact test $\leq 0.1$) 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 (Warnes et al., 2009; Santini et al., 2016).

**Data Analyses**

Data collected from the nematode infection experiments were subjected to analysis of variance (ANOVA) SAS 9.4 software (Institute, 2015). In each trial, nematode counts and egg counts were compared among treatments and controls using Dunnett's test. Potential activities of both enzymes were analyzed with one-way ANOVA, and treatment means were compared by Tukey's post hoc test ($P$-values $\leq 0.05$) in SAS. Leafminer preference data collected in the choice and no-choice tests were analyzed with replicated G-tests (Sokal and Rohlf, 1995). This permitted us to analyze 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 and Rohlf, 1995).

In addition, expression of defense-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).

## Results

### Belowground presence of EPNs reduces RKN infection

Significantly fewer *M. javanica* juveniles were found inside tomato roots at 1-15 dpi in plants treated with 25 *S. carpocapsae* IJs/cm$^2$ than corresponding control plants inoculated only with *M. javanica* but without EPNs (Fig. 1). Also, at thirty dpi, fewer *M. javanica* galls, eggs masses, and eggs were recovered from plants growing in soil containing *S. carpocapsae* than from the control plants (Fig. 2*A-C*). Fewer *M. javanica* juveniles, galls, eggs masses, and eggs were extracted from plants roots inoculated with 25 *S. feltiae* IJs/cm$^2$ than from control plants at all sampling points, except for 5- 7 dpi (Fig. 1 and Fig. 2*A-C*). There were significantly fewer *M. javanica* juveniles within roots of plants treated with 25 *H. bacteriophora* IJs/cm$^2$ at 5-7 and 13-15 dpi than in roots of control plants (Fig. 1). Likewise, fewer *M. javanica* galls and eggs were extracted from plants roots inoculated with *H. bacteriophora* thirty dpi than from control plants (Fig. 2*A-C*). *M. javanica* eggs recovered from plants roots grown in soil inoculated with *S. carpocapsae* had a significantly lower hatching rate than eggs recovered from un-inoculated plants roots (Fig. 2*D*). However, neither the number of eggs produced per egg mass nor the number of egg masses produced per gall was affected by any of the treatments tested as compared with non-exposed control (*SI Appendix*, Fig. S6). These results indicated that presence of beneficial EPN species in the pots reduced RKN infection in tomato. We hypothesized that this could be due to a plant defense response triggered by exposure of plant roots to EPN in the soil. To evaluate this hypothesis, complementary biochemical analyses of plant responses upon root exposure to EPN or RKN were conducted. Only *S. carpocapsae* was included in

the subsequent experiments, because this EPN caused the greatest reduction of RKN infection (Fig. 1, Fig. 2*A-D*).

*GP activity* - Belowground presence of RKN *M. javanica*, and/or the beneficial EPN *S. carpocapsae*, reduced GP activity in aboveground plant tissue (Fig. 3*A*). However, a rapid induction of GP activity was observed in belowground tissues at 3-7 dpi with *M. javanica* and/or *S. carpocapsae*, and then activity returned to near pre-inoculation levels at 15-28 dpi (Fig. 3*B*). GP activity was lower at 15-28 d than at 3-7 dpi in both the mock control and inoculated treatments (Fig. 3*B*), indicating some natural fluctuation of enzymatic activity, which could have been due to plant ageing.

*PPO activity* - Similar to GP activity, PPO activity in shoots was also significantly reduced as a result of belowground presence of *M. javanica* and/or *S. carpocapsae* (Fig. 4*A*). In contrast, there was a large and statistically significant increase in PPO activity in the roots at 3-7 dpi with *M. javanica* and/or *S. carpocapsae* as compared with the mock control, which subsequently decreased significantly below mock control levels at 15-20 dpi and returned to basal levels at 28 dpi (Fig. 4*B*). 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 this activity in aboveground tissues.

**Aboveground attack by *T. absoluta* females on tomato plants reduced by RKN or EPN belowground**

To test if presence of RKN or EPN in the tomato growth substrate would affect plant acceptance by an aboveground 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 non-inoculated controls at 3-5 dpi; however, there was no strong pattern of preference between treatment and control plants for these two nematode species (Fig. 5*A* and *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 (Fig. 5*C* and *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 (*SI Appendix*, Fig. 7*A-D*). However, the duration of pupal stage was significantly longer and pupal mortality was higher in *T. absoluta* developing on *M. javanica* and *S. carpocapsae*-inoculated plants than on non-inoculated, control plants (Fig. 6*A* and *B*).

Then, we executed 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) (Fig. 7).

## Gene expression patterns in response to nematode inoculation

To better understand the effect of multiple interacting soil organisms on the host plant, we conducted transcriptome sequencing analyses of local and systemic response in plant roots after single- and multi-species exposure treatments comprised of root feeding and/or entomopathogenic nematode inoculations.

Transcriptome analysis was performed at 7 dpi to investigate the pathways influenced in the host that may shape these inter-species interactions. This time point after root inoculation by nematodes was chosen because: 1) the highest negative interaction between root-knot and entomopathogenic nematodes occurred at 7 dpi (Fig. 1) and 2) the greatest difference in oviposition preference by adult female tomato leafminer between tomato plants grown in substrate containing either *M. javanica* (Mj) or *S. carpocapsae* (Sc) was observed at this sampling point (Fig. 5-7).

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.

Quality control and filtering of sequencing reads was conducted for an average Phred quality score of 20 and any contamination adaptor sequences or low-quality reads were removed. Approximately 207,844,542 (207/223, 92.8%) trimmed reads were mapped on the tomato reference genome, and 36,962 tomato genes were identified.

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 $\geq \pm 1.5$ and adjusted *P*-values $\leq 0.05$) (*SII Appendix*, Table S1 and *SII Appendix*, Table S2). We detected 444 and 461 DEGs in response to RKN and EPN inoculation, respectively (*SI Appendix*, Fig. S8*A* and *SII Appendix*, Tables S1, *SII Appendix*, Tables 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 (*SI Appendix*, Fig. S8*A*). Moreover, 92 genes were up-regulated in both treatments, while 135 and 131 genes were exclusively up-regulated in response to RKN and EPN inoculation, respectively. Similarly, 141 and 162 genes were down-regulated exclusively in response to RKN and EPN, respectively (*SI Appendix*, Fig. S8*B*). The commonly up-regulated genes included those encoding phenylalanine ammonia-lyase, thioredoxin H, S glycoprotein, and major latex-like protein, whereas the 76 jointly down-regulated genes contained genes encoding xenotropic and polytropic retrovirus receptor, pectinesterase, and alpha-1 4-glucan-protein synthase (*SII Appendix*, Table S3).

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 (Kyndt et al., 2012; Postnikova et al., 2015;

Santini et al., 2016; Petitot et al., 2017; Shukla et al., 2018; Lee et al., 2019; Bali et al., 2019; Kumar et al., 2019; 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 ≤ 0.0001) 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 (Fig. 8*A* and *B*). For example, for the TSW12 gene, the $\log_2$ fold-change values (*P*-values ≤ 0.05) for RNA-seq and qRT-PCR were 5.05 and 15.27, respectively.

Gene ontology (GO) 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 (*SI Appendix*, Fig. S9*A* and *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 process, response to stimulus, and localization (*SI Appendix*, Fig. S10*A* and *B*). For the molecular function classification, many DEGs were in the categories of binding, catalytic activity, and transporter activity.

DEGs were also analyzed with the 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), signaling, secondary metabolites, transcription factors, and heat shock protein categories were up-regulated in response to RKN inoculation. Moreover, a similar overall pattern of gene up-regulation was observed in tomato roots exposed to EPN (*SI Appendix*, Fig. 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 RKN; these were further increased by EPN-inoculation (*SI Appendix*, Fig. S11). Overall, the analysis provided empirical evidence that expression of defense-related pathways was enhanced in *S. lycopersicum* in response to EPN exposure in the rhizosphere.

DEGs in response to RKN infection were allocated to 22 BINs (Fig. 9*A* and *SIII Appendix*, Table S4). Four BINs included only genes that were up-regulated (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 defense mechanisms, 25% of the genes (111/444) were allocated to 9 BINs, and mainly hormone metabolism (16/111), transcription factors (13/111), cell wall (7/111), and oxidative stress (15/111) (*SI Appendix*, Fig. S11 and *SIV Appendix*, Table S6).

In relation to hormone metabolism, we detected upregulation of genes associated with ethylene (ET) (six up- and one down-regulated) biosynthesis. Six auxin-associated genes were differentially expressed, four of them were up- and two were down-regulated. Furthermore, the genes for jasmonic acid (JA) biosynthesis, lipoxygenases and an allene oxide synthase were up-regulated. A brassinosteroid-related gene, BES1/BZR1

458 homolog protein 2, was down-regulated. From the transcription factors, nine (3 MYB, 3 AP2/EREBP, 1
459 WRKY, 1 DOF and 1 bZIP) were down-regulated and four (2 MYB, 1 WRKY, and 1 DOF) were up-regulated.
460 In relation to cell wall, a fucosyltransferase 7, and a xyloglucan endotransglucosylase/hydrolase 2 were up-
461 regulated. Conversely, a BURP domain-containing protein, a rhamnogalacturonate lyase, a pectate lyase-like
462 protein, an alpha-1 4-glucan-protein synthase and a pectinesterase were down-regulated. In relation to oxidative
463 stress, we detected six genes encoding peroxidases, as well as a thioredoxin H and glutathione-S-transferase
464 that were up-regulated and a thioredoxin reductase, two glutaredoxin family proteins, three glutaredoxins and
465 a GST that were down-regulated. Furthermore, a gene encoding a TIR-NBS-LRR protein, analogous to the R
466 genes that mediate resistance to Tobacco mosaic virus (Whitham et al. 1994) was induced in RKN-inoculated
467 plants.

468 In tomato plants grown only in presence of *S. carpocapsae*, the DEGs were allocated to 26 BINs (Fig. 9*B* and
469 *SIII Appendix*, Table S5). Three BINs included only up-regulated genes (fermentation, major CHO metabolism
470 and photosynthesis) and five BINs included only down-regulated genes (biodegradation of xenobiotics, metal
471 handling, minor CHO metabolism, N-metabolism and TCA/org transformation). Four BINs (major CHO
472 metabolism, metal handling, minor CHO metabolism and TCA/org transformation) were exclusive to this
473 treatment. TCA/org transformation liberates energy stores by oxidation of acetyl-CoA originating from
474 carbohydrates, fats, and proteins (Schmitz et al., 2014) and hence this activation suggests activation of energy
475 transport in the plant. Focusing on plant defense, 20.17% (93/461) of the genes were allocated to the PPN-
476 listed BINs, except beta glucanase and GST (*SI Appendix*, Fig. S11 and *SIV Appendix*, Table S7). In relation to
477 hormone metabolism, up-regulation of genes involved in auxin biosynthesis (four up- and four down-regulated)
478 was detected. Expression levels decreased for three genes involved in ET biosynthesis and signaling, i.e., 1-
479 aminocyclopropane-1-carboxylate oxidase-like protein and ethylene responsive factors. Moreover, a 1-
480 aminocyclopropane-1-carboxylate oxidase 1, a 2-oxoglutarate-dependent dioxygenase and a gibberellin 2-
481 oxidase 5 were induced. Two abscisic acid-associated genes were differentially expressed; one of them was up-
482 and one was down-regulated. A brassinosteroid-associated gene was up-regulated and one SA-associated gene
483 was down-regulated. Expression levels of MYB transcription factors, one WRKY and one AP2/EREBP were
484 induced. Also, a WRKY transcription factor, an AP2/EREBP and a DOF were down-regulated. In relation to
485 the cell wall, a xyloglucan endotransglucosylase/hydrolase 2, a beta xylosidase, two fasciclin-like arabinogalactan
486 protein 7s, a COBRA-like protein, and an expansin-1 were up-regulated, while a polygalacturonase, an alpha-1
487 4-glucan-protein synthase and a pectinesterase were down-regulated. In regard to oxidative stress, we detected
488 four up-regulated genes: a thioredoxin H and three genes encoding peroxidases, but found reduced expression
489 of five redox state-related genes: a thioredoxin reductase, three glutaredoxins and a glutaredoxin family protein.
490 Overall, an increase in gene expression levels related to hormone metabolism, oxidative stress, biotic stress, and
491 secondary metabolism was observed in tomato plants grown in presence of EPN (*SI Appendix*, Fig. S11).

Within the transcriptome data, several DEGs are related to the biosynthesis of different secondary metabolites, such as phenylpropanoid, isoprenoid, phenol, lignin and lignan. Although fifteen transcripts related to secondary metabolite production were differentially expressed between Mj and Sc inoculated and uninoculated controls (*SI Appendix*, Fig. S11 and *SIII Appendix*, Table S4, *SIII Appendix*, Table S5), 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 up-regulated and O-methyltransferase was down-regulated in response to RKN (*SI Appendix*, Fig. S11 and *SIII Appendix*, Table S4). We also found up-regulation of isoprenoid-related genes, including those encoding alpha-humulene/(-)-(*E*)-beta-caryophyllene synthase and transposase (*SI Appendix*, Fig. S11 and *SIII Appendix*, Table S4). Similarly, in response to EPN, we detected up-regulation of genes involved in PAL and down-regulation of AMP-dependent synthetase and ligase (regulator of phenylpropanoid biosynthesis). The genes encoding laccase 1a and a laccase were also up-regulated in the phenol pathway. In addition, a gene encoding alpha-humulene/ (-)-(*E*)-beta-caryophyllene synthase was up-regulated in response to EPN, indicating activation of terpenoid-mediated defense responses (*SI Appendix*, Fig. S11 and *SIII Appendix*, Table S5). This result indicated an effect of *S. carpocapsae* on secondary metabolite synthesis pathways, which may help explain the above behavioral results suggesting a nematode-induced plant-herbivore interaction.

In relation to the group of 168 genes differentially expressed in both treatment groups (Table S3), thirty are related to plant defense (*SI Appendix*, Fig. S12 and *SII Appendix*, Table S3, *SIV Appendix*, Table S8), fourteen were up-regulated and sixteen were down-regulated. Up-regulated genes were generally related to hormone metabolism, cell wall modification, redox state, signaling, secondary metabolites and PR proteins, which might reflect host responses to nematode-associated molecular patterns (NAMPs) (*SI Appendix*, Fig. S12 and *SIV Appendix*, Table S8).

**EPN presence in the substrate modulates tomato plant immune response against RKN**

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 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 *SV Appendix*, Table S9 and Fig. 11. There was a significant positive correlation between *PRP 1* and *DrTI* (Fig. 10 and *SV Appendix*, Table S9, Fig. 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

525 *GA*. The principal components associated with *M. javanica* (Mj)-inoculated roots and those associated with *M.*
526 *javanica* and *S. carpocapsae* (Mj+Sc)-inoculated roots were different from the principal components associated
527 with *S. carpocapsae* (Sc)-inoculated roots (Fig. 10).

528 **Discussion**

529 Our results are congruent with the hypothesis that tomato plants 'misrecognize' EPNs as RKNs and mount a
530 broad-spectrum immune response with indirect consequences on both RKN performance belowground and
531 herbivore performance aboveground. Both functional guilds of nematodes (EPN vs. RKN) caused
532 upregulation of coincident immunity related receptor complexes and signaling pathways. For example, plants
533 initially recognized EPNs as invaders by activating SAR, as indicated by the over-expression of *PR-14* (Fig. 10).
534 Expression of the *PX* gene suggests that tomato plants also responded with induced production of antioxidant
535 enzymes as protection against $H_2O_2$, which is typically generated as an early response to biotic challenges.

536 **Effect of EPNs on root defense against RKN**

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

554 The EPN-induced modulation of plant defense was transient and diminished over time, as is typically observed
555 for pattern-triggered immunity (PTI). At thirty dpi, RKN had developed into gravid females even in EPN-
556 exposed plants. It is also possible that some RKN J2s may have initially entered roots, and although their
557 development may have been to some degree retarded, they could have eventually built feeding sites and

558 reproduced. However, our data indicate that EPN-induced modulation of defense reduced tomato root
559 infection by approximately 50%, in terms of diminished RKN egg fecundity and fertility.

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

569 The physiological responses to both RKN and EPN inoculation were examined by GO enrichment analysis.
570 Notably, the group of genes related to defense response was significantly enriched in the DEGs investigated
571 by pairwise comparisons, suggesting that *S. carpocapsae* and/or *M. javanica* affected plant defense similarly, despite
572 occupying different functional guilds. Furthermore, plants inoculated with *S. carpocapsae* and/or *M. javanica*
573 exhibited significant enrichment of DEGs associated with the phenylpropanoid and flavonoid pathways. The
574 role of phenylpropanoids in plant defense is well-established (Vaganan et al., 2014). Flavonoids, isoflavonoids,
575 hydroxycinnamic acids, monolignols, and stilbenes are all classes of phenylpropanoids that function as host
576 defense molecules, acting as potential barriers and signaling molecules to induce defense against pathogen attack
577 (Dixon et al., 2002). These results suggest that inoculation of tomato with the entomopathogen, *S. carpocapsae*,
578 caused activation of genes involved in phenylpropanoid biosynthesis.

579 We also discovered that the lignin biosynthetic pathway was significantly enriched in the DEGs of plants
580 inoculated with *S. carpocapsae* and/or *M. javanica*, and it is known that lignin is produced upon nematode infection
581 and acts as a physical barrier (Caño-Delgado et al., 2003; Ji et al., 2015). In fact, nematode resistance is correlated
582 with higher lignin content among several plant species (Holbein et al., 2016). Our results also indicate that genes
583 involved in both hypersensitive response (HR) and oxidation–reduction (redox) processes were affected by *S.*
584 *carpocapsae* inoculation. The HR is a form of programmed cell death that is related to disease resistance (Morel
585 and Dangl, 1997), which involves excessive production of reactive oxygen species (ROS) (Lozano-Torres et al.,
586 2014; Shah et al., 2017). Therefore, it is also possible that detection of *S. carpocapsae* by plant roots leads to the
587 host redox status, which induces defense responses.

588 Tomato plants exposed to *S. carpocapsae* exhibited differential expression of the transmembrane receptor
589 tyrosine kinase (RTKs) signaling pathway, among which were the *Arabidopsis* homologues *FLS2* and *NILR1*.
590 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 signaling (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 transcription factors (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 and Somssich, 2007). A complex functional interaction occurring among preferential homoeologous alleles (*AtWRKY18*, *AtWRKY40*, and *AtWRKY60*) has been described in plant defense responses to a diversity of pathogenic microbes such as *Botrytis cinerea* and *Golovinomyces orontii* (Xu et al., 2006; Shen et al., 2006). WRKY TFs are believed to play a pivotal role in the regulation of signaling 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 defense-related genes (Gutterson and Reuber, 2004).

Our principal component analysis 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 non-specific 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, e.g. 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 defense signaling 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

principal component analysis also revealed that the two nematode guild treatments caused some non-overlapping 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 (Fig. 10). Overall, EPN inoculation directly mediated enhanced plant defense and reduced subsequent RKN infection. Our transcriptomic investigation is congruent with the hypothesis that plants mount a broad-spectrum defense 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 defense mechanisms, in particular those related to *PR*-genes expression, to facilitate successful colonization of their hosts (Mantelin et al., 2015; Goverse and Smant, 2014; Vieira and Gleason, 2019). For example, they induce an enzymatic response from the antioxidant system to neutralize toxic ROS (Molinari and 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 up-regulated 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 defense 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 defense 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, 2001).

**Effect of EPNs on aboveground herbivory**

Exposure of tomato roots to EPN also reduced aboveground 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 behavioral observations suggest that nematode inoculations may affect release of volatile organic compounds (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 defense responses (*SI Appendix*, Fig. S11 and *SIII Appendix*, Table S5). 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 defenses. Our future objectives include investigating the qualitative and quantitative changes to VOC production aboveground that may be caused by EPN exposure of tomato roots and relate those to aboveground 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 aboveground 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 aboveground foliage of potato exposed to *H. bacteriophora* IJs belowground as compared with non-exposed controls. Selection should favor avoidance of cues associated with potential predators by herbivores (Kats and 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 aboveground 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 belowground. It may benefit EPNs for plants to defend against RKN invasion by conserving available food for the insect larvae that EPN require for development.

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

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

715 Our data indicate that inoculation of tomato roots with EPN and RKN causes dramatic and overlapping
716 changes to a broad gene network associated with innate immune response. Furthermore, invasion of tomato
717 roots by RKN stimulates a reprogramming of the transcriptome that influences both the SA and JA pathways
718 (Kyndt et al., 2012; Postnikova et al., 2015; Santini et al., 2016; Petitot et al., 2017; Shukla et al., 2018; Lee et al.,
719 2019; Bali et al., 2019; Kumar et al., 2019; Zhou et al., 2020) and is in large part mimicked by EPNs which cause
720 a similar outcome. Recently, higher constitutive levels of abscisic acid (ABA) and JA, and basal expression of
721 ABA- and JA-related transcripts were described in a soybean genotype tolerant to the soybean aphid (Chapman
722 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 defense 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 defense reducing aphid performance on black mustard (*Brassica nigra*), the root knot nematode, *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 defense 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). Recently, it has been shown that ascarosides from plant parasitic nematodes 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 defense 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 likely 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 entomopathogenic nematodes (*S. carpocapsae*) interacting with tomato caused analogous and coincident enhanced plant defense responses against root knot nematodes (*M. javanica*) in the rootzone, and also reduced the preference and performance of a folivore (*T. absoluta*) aboveground. 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.

**Data available in article supplementary material:** The data that support the findings of this study are openly available in [datadryad.org] at https://doi.org/10.5061/dryad.x0k6djhjs.

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1040

**Table 1**. Sequences of forward (F) and reverse (R) primers designed from tomato transcripts identified with RNA-seq and used to quantify differential gene expression with quantitative real-time PCR (qRT-PCR).

| Gene | Primer | Primer sequence F (5´-3´) | Primer sequence R (3´-5´) | Amplicon length (bp) | Blast x description |
|---|---|---|---|---|---|
| | PSY[a] | TTAGACATCAAGCCGTTCAAGG | TCCAGCCACACAGTAACAGTA | 111 | Phytoene synthase 3 |
| | GluB[a] | GCTAAAGAAGGTAGTCCGAGAAAG | TTGGCTGCTTGTTTGGTGAA | 127 | Beta-glucanase |
| | GST[a] | TTGGTTGGATGGCGTATCTTCT | GTCTCTGGGTGGCAAGTGTT | 146 | Glutathione-S-transferase |
| | WIN2[a] | GACCAACTCCAACTCCGACTC | CAGCCATACTTACTCCGCCAT | 160 | Chitinase |
| TPS31 | TPS31[a] | CTTGCTTACCACTACATCCTAC | AATCTGACCTCTACCCTTCTC | 160 | Alpha-humulene/(-)-(*E*)-beta-caryophyllene synthase |
| | TPS33[a] | GTGAAGTATCAAGGTGGTGGAA | ATGACACGAGCCTGAGAGTAT | 133 | Alpha-humulene/(-)-(*E*)-beta-caryophyllene synthase |
| | PRP 1[a] | GCTGTGAAGATGTGGGTTGATG | CGTTGTCCTCTCCAGTTACCT | 200 | Pathogenesis-related protein 1a |
| | UDP[a] | GCTGTCTTGTATCTGATATGTTCC | TGGTGAGGCAAATTCGGTATA | 188 | UDP-glucosyltransferase HvUGT5876 |
| | HMG2.2[a] | GGTTCGGTTCTTGCTGGTGAA | GGACGCCTTGGTGACATCTT | 111 | 3-hydroxy-3-methylglutaryl coenzyme A reductase |
| | Pin2[a] | ACACTCCTCAACCCAAACAA | GGCATATCCCAAACCCAAGA | 194 | Proteinase inhibitor II |
| | PX[a] | GCTTTGTTGAGGGTTGTGATG | CGAGTGGCTAAGGCAAGAAT | 196 | Peroxidase 5 |
| | DrTI[a] | TCTTGGGAATCCGACTGTTTGT | GCCTGCTTTACCGTTTGTTGA | 103 | Kunitz trypsin inhibitor |
| | GA[a] | CGCTTCGTGTACTGCTTCTT | TCTATTGGCTCCTCTGGTGAA | 149 | Gibberellin-regulated protein 2 |
| | Q`a[a] | CTCCTTTGCTTGTCAACCTTT | GTTCTTGTATCCTCTTCCGTTATC | 125 | Beta-1 3-glucanase |
| | P450 CP7[a] | GTGAAGTCTCTGCTGTTGTTG | TTGCCTCCAGTAATCTCCATAG | 164 | cytochrome P450 |
| | ANL2[a] | GCATAGGTGAAGGCAAGAGT | CGACAGATACATCCACAATAATCC | 150 | Homeobox-leucine zipper protein PROTODERMAL FACTOR 2 |
| ACO4 | ACO4[a] | CGCAGGAGGCATCATACTTC | CCGAGTCCCATCTGTTTGTG | 196 | 1-aminocyclopropane-1-carboxylate oxidase |
| | LOX[a] | AATGCTGGTGGAGTTCTTGAG | ATCTGCTGGAAGTGCTTGTT | 111 | Lipoxygenase |
| | PR1a2[a] | TTGGGATGCCGACTTGGAAT | CCGCTAACACATTCATTCGTATCG | 192 | Pathogenesis-related protein |
| AOS3 | AOS3[a] | CACTTTCCCTCTACCTTACATCCT | AACCGCCATACGAATTGAATCC | 170 | Allene oxide synthase |
| TSW12 | TSW12[a] | GAGGCTGTTGTGGTGGTGTTA | GAACCGTGGAGCAGTCAGTG | 198 | Non-specific lipid-transfer protein |

| | | | | |
|---|---|---|---|---|
| TAC[b] | CTCACGCATTGACCACAAGT | CAGCACCAACCTCCTCATAATC | 149 | Tubulin alpha-3 chain |
| UBI[b] | TCGTAAGGAGTGCCCTAATGCTGA | CAATCGCCTCCAGCCTTGTTGTAA | 120 | Ubiquitin |

[a] Differentially expressed genes detected by RNA-seq approach.

[b] Endogenous reference genes used to normalize relative gene expression quantification.

## Result

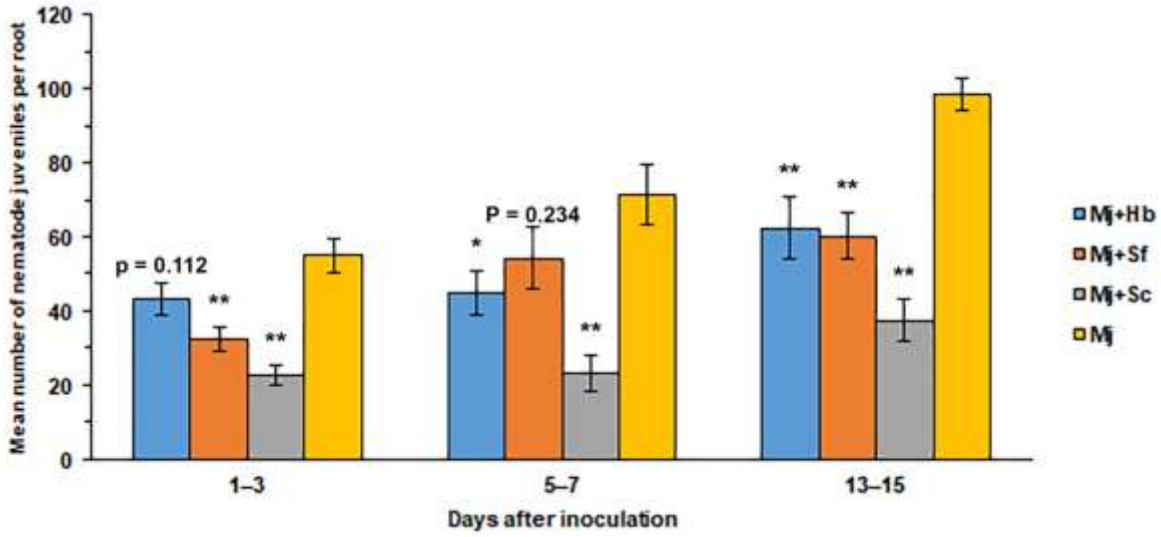


**Fig. 1**. *Meloidogyne javanica* (Mj) penetration into tomato seedling roots treated with 25 infective juveniles/cm² of *Heterorhabditis bacteriophora* (Hb), *Steinernema feltiae* (Sf), *S. carpocapsae* (Sc), or control roots receiving only *M. javanica*. Number of juveniles in roots 1, 2, 3, 5, 6, 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 ≤ 0.05) from the control according to Dunnett's test.

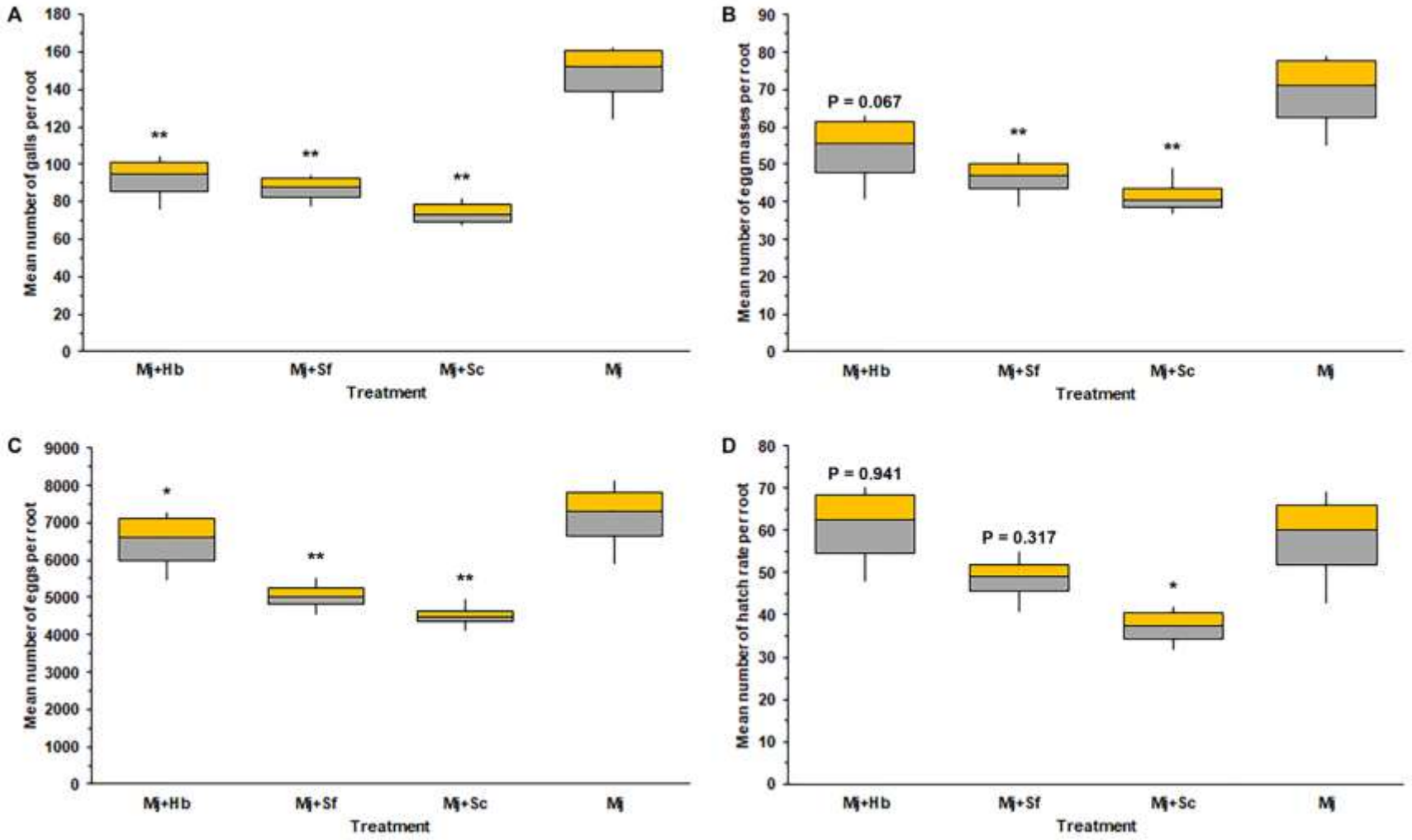


**Fig. 2**. Numbers of *Meloidogyne javanica* (Mj) galls, egg masses, eggs and egg hatch rate in tomato seedlings treated with 25 infective juveniles/cm² of *Heterorhabditis bacteriophora* (Hb), *Steinernema feltiae* (Sf), *S. carpocapsae* (Sc), and control. (*A*) Average number of galls per root; (*B*) 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 ≤ 0.05) from the control according to Dunnett's test.

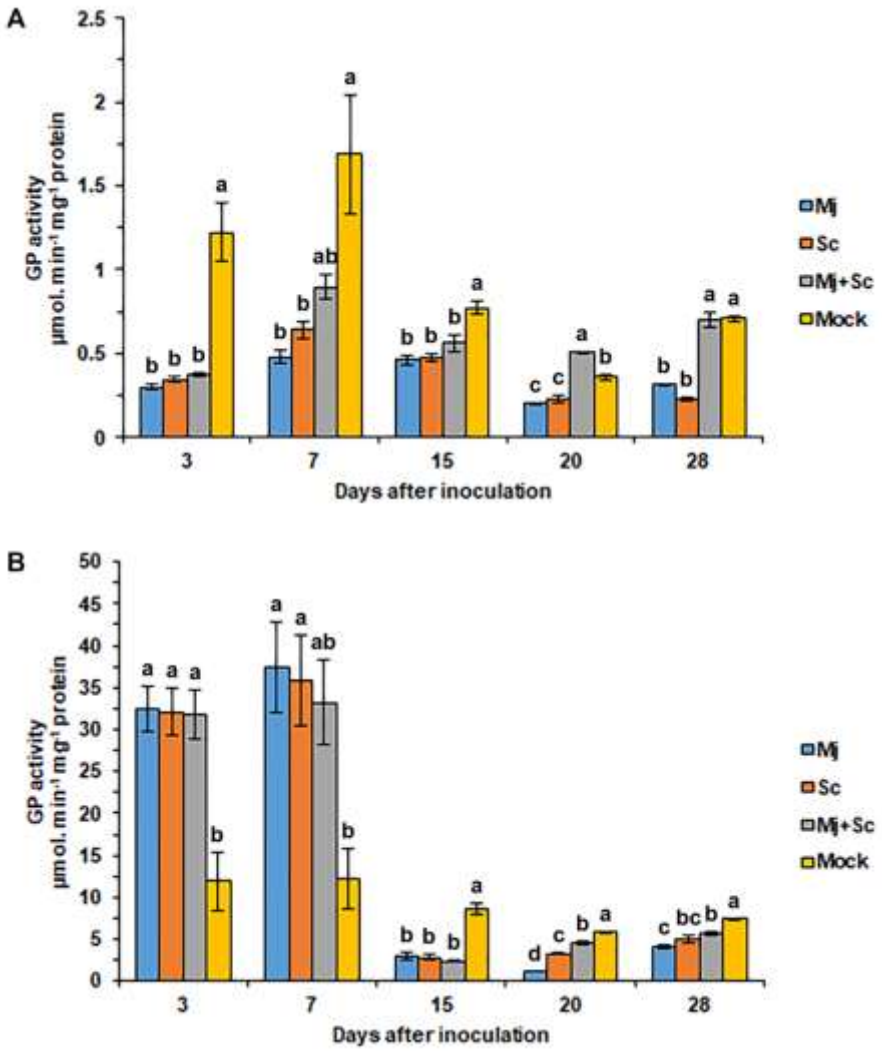


**Fig. 3**. Impact of *Steinernema carpocapsae* (Sc) inoculation on GP activity in (*A*) leaves and (*B*) roots of tomato. Means followed by the same letters are not significantly different according to Tukey's test a = 5% possibility level. Bars indicate standard error (n = 3).

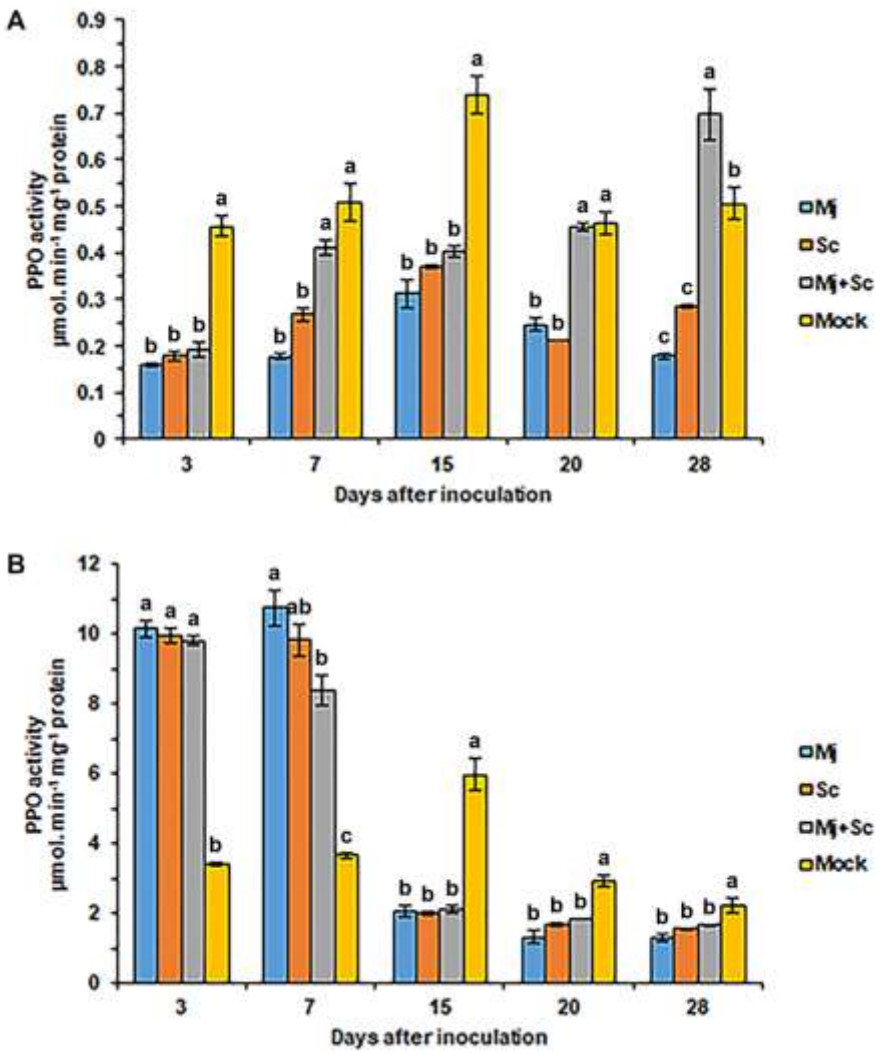


**Fig. 4**. Impact of *Steinernema carpocapsae* (Sc) inoculation on PPO activity in (*A*) leaves and (*B*) roots of tomato. Means followed by the same letters are not significantly different according to Tukey's test a = 5% possibility level. Bars indicate standard error (n = 3).

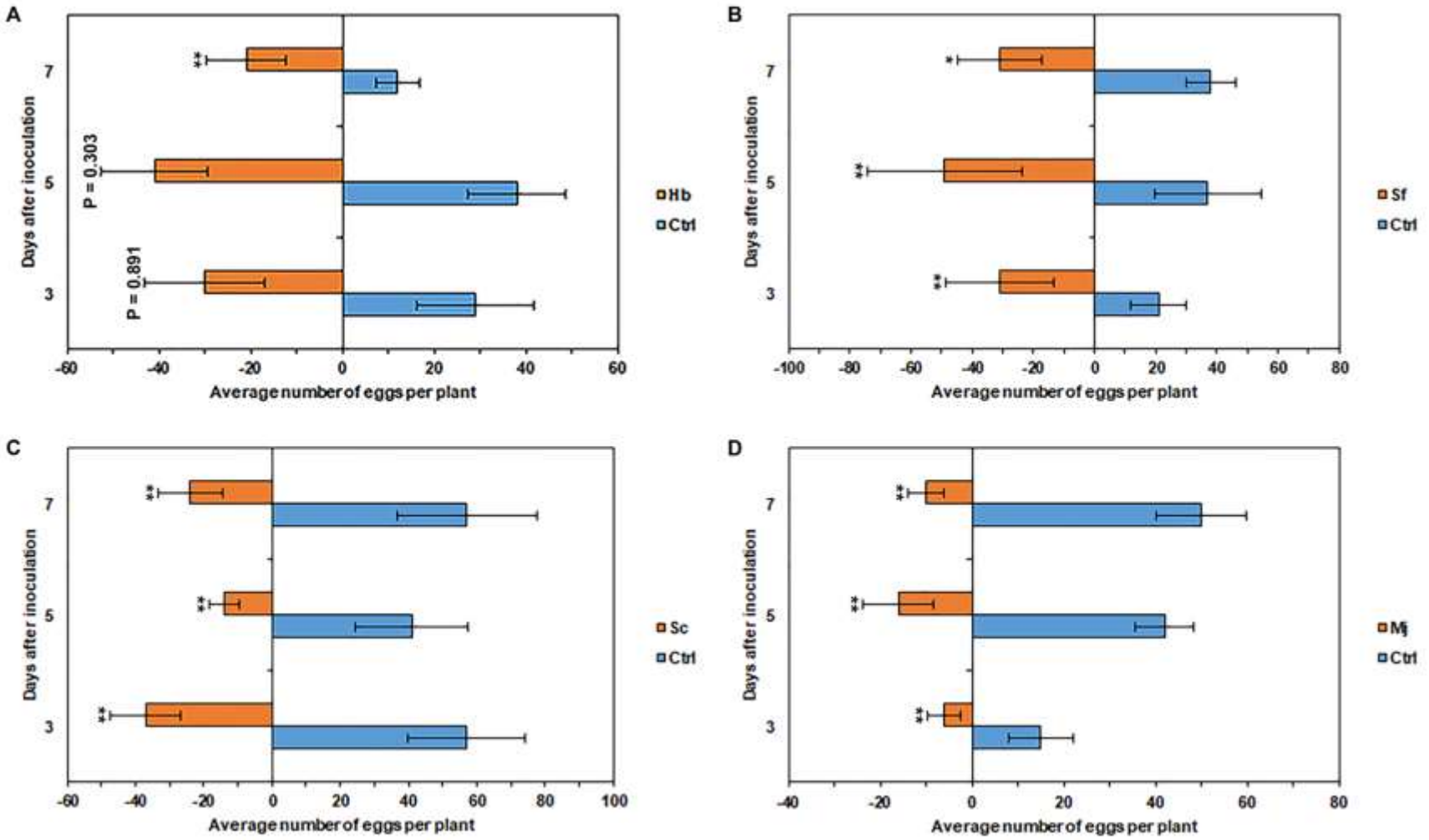


**Fig. 5**. Mean numbers (± SEM) of *Tuta absoluta* eggs detected per plant. Number of eggs at 3, 5 and 7 days post inoculation (dpi) in every insect-proof screen cage (n = 8). The females were permitted to choose between a control plant (blue bars) and a plant inoculated with (*A*) *Heterorhabditis bacteriophora* (Hb), (*B*) *Steinernema feltiae* (Sf), (*C*) *S. carpocapsae* (Sc) or (*D*) *Meloidogyne javanica* (Mj) nematodes surrounded by 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 ≤ 0.05, ** *P*-value ≤ 0.01.

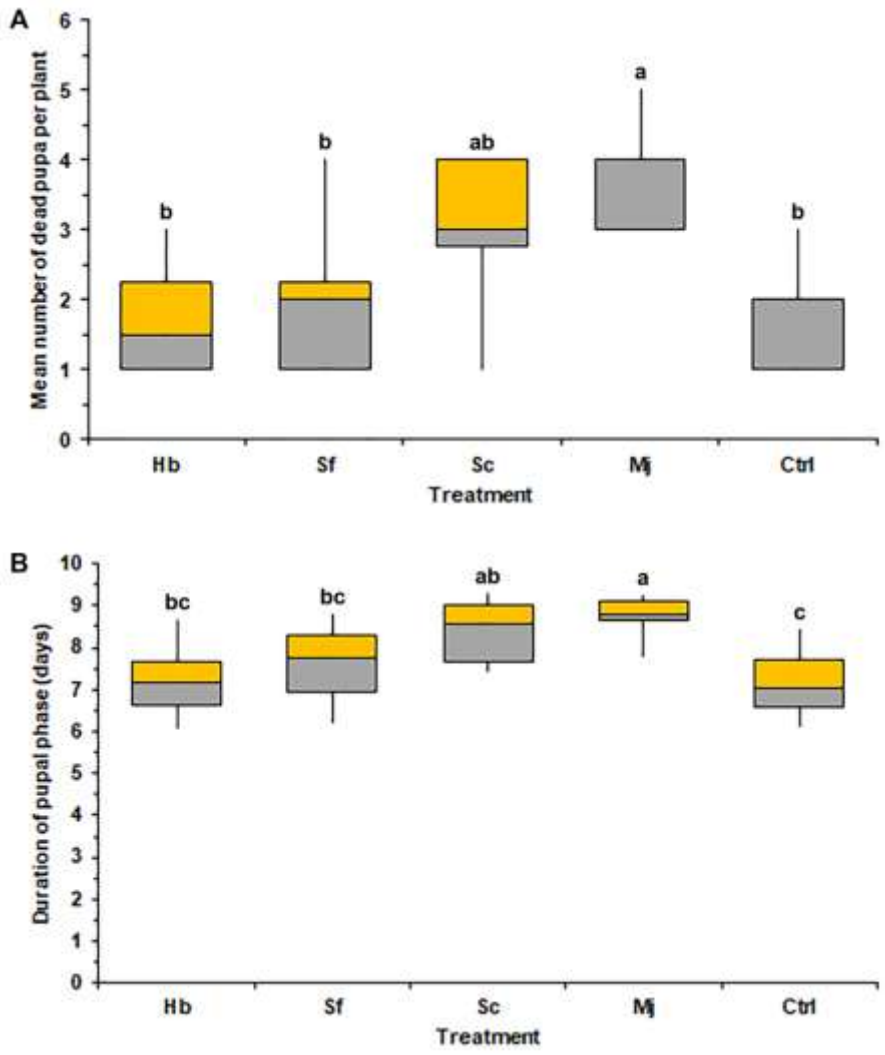


**Fig. 6**. Effect of *Meloidogyne javanica* (Mj) or *Steinernema carpocapsae* (Sc) on *Tuta absoluta* pupation. (*A*) Average (±SD) number of dead pupae. (*B*) Average (±SD) duration of pupal phase. Means followed by the same letters are not significantly different according to Tukey's test a = 5% possibility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

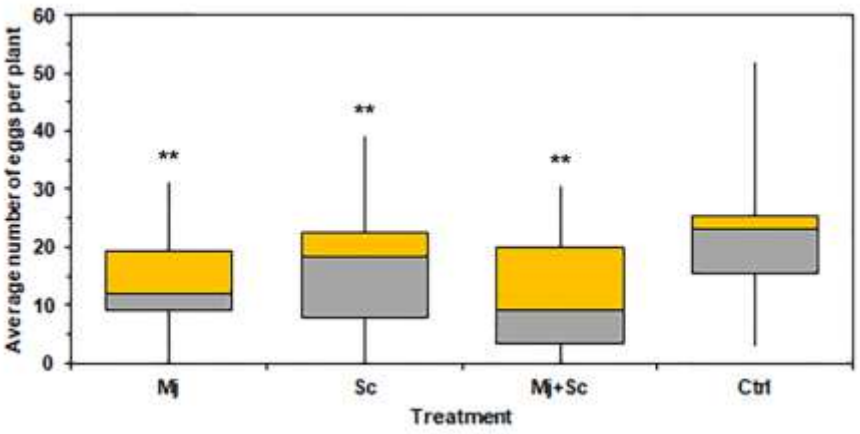


**Fig. 7**. Mean numbers (±SEM) of *T. absoluta* eggs detected per plant 12 h after three 2-day-old mated *T. absoluta* females were released on each plant (no-choice experiment; n = 6 per treatment group). Plants were either inoculated with *M. javanica* (Mj) or *S. carpocapsae* (Sc), simultaneously 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 ≤ 0.05, ** *P*-value ≤ 0.01.

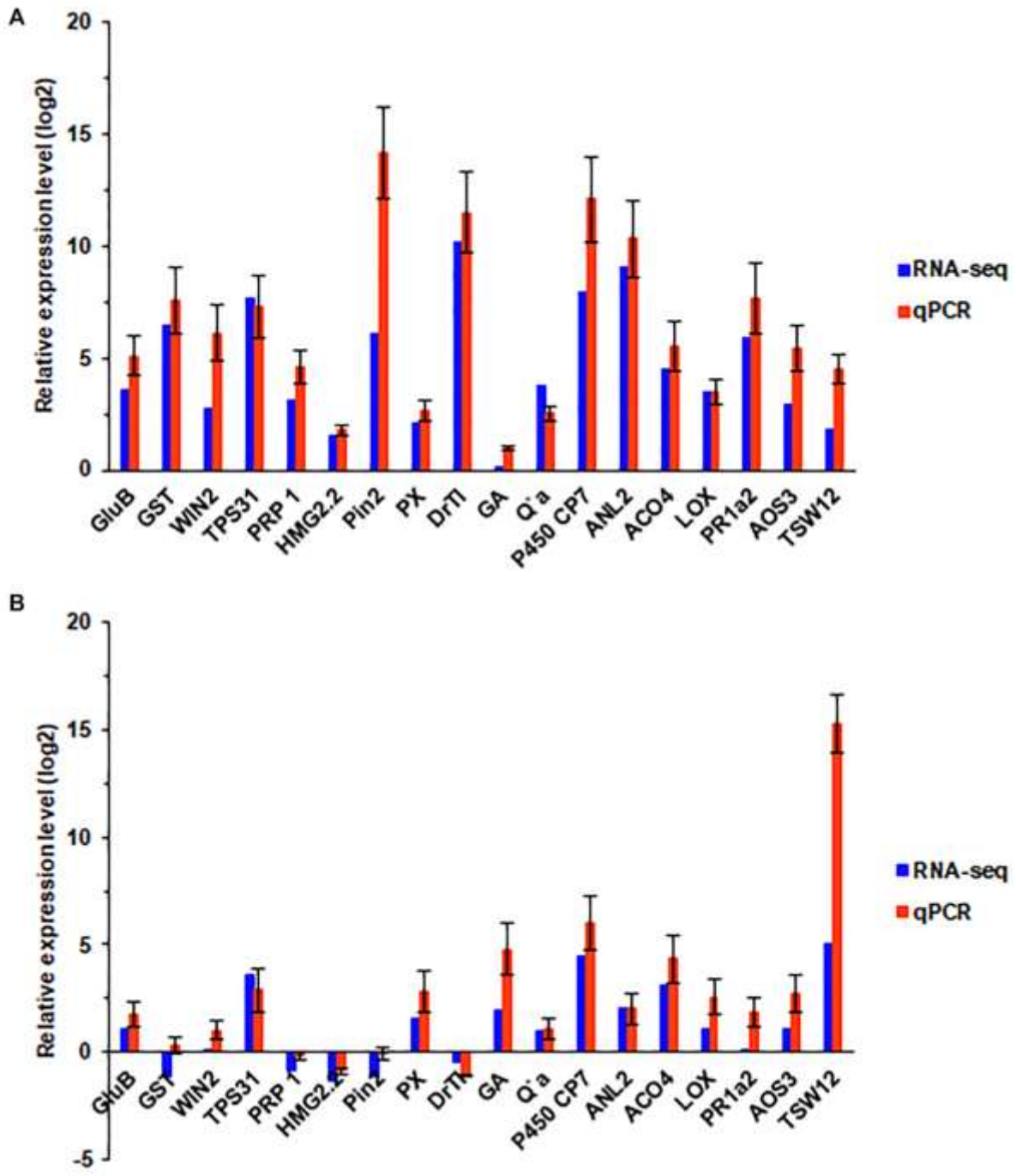


**Fig. 8**. Relative expression levels of 21 tomato genes in response to (*A*) *Meloidogyne javanica* or (*B*) *Steinernema carpocapsae* infection at 7 days post inoculation (dpi) provided from RNA-seq (blue bars) and quantitative real-time PCR (qRT-PCR) (orange bars) data. For every treatment group, 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*, Non-specific lipid-transfer protein. Note the consistency between RNA-seq and qRT-PCR data.

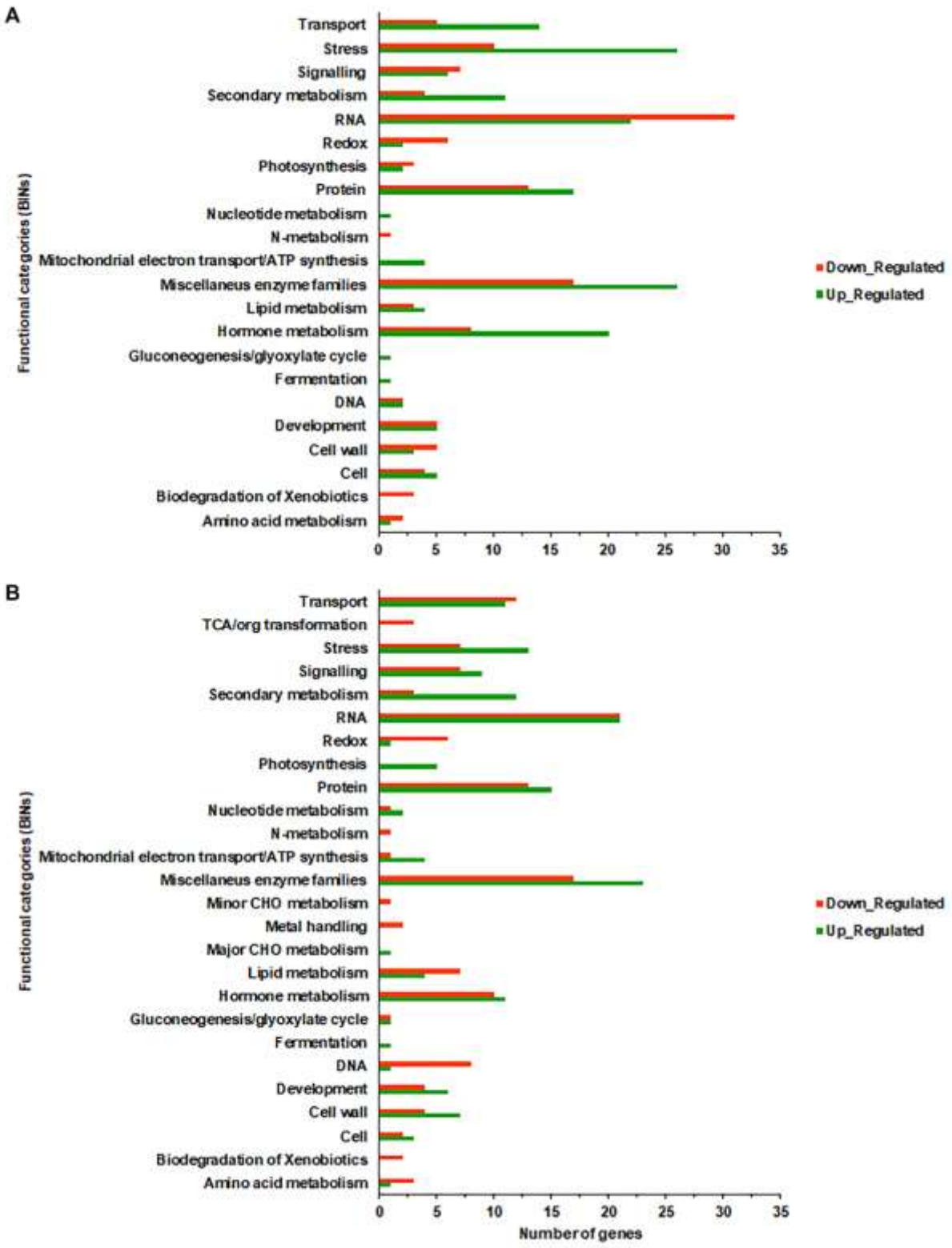


**Fig. 9**. A MapMan diagram of modulated genes from tomato ($log_2$ fold-change ≥ 1.5, false discovery rate (FDR) ≤ 0.05) according to their assignment to functional categories (BINs). The two diagrams indicate gene modulation in response to (A) *Meloidogyne javanica* or (B) *Steinernema carpocapsae*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

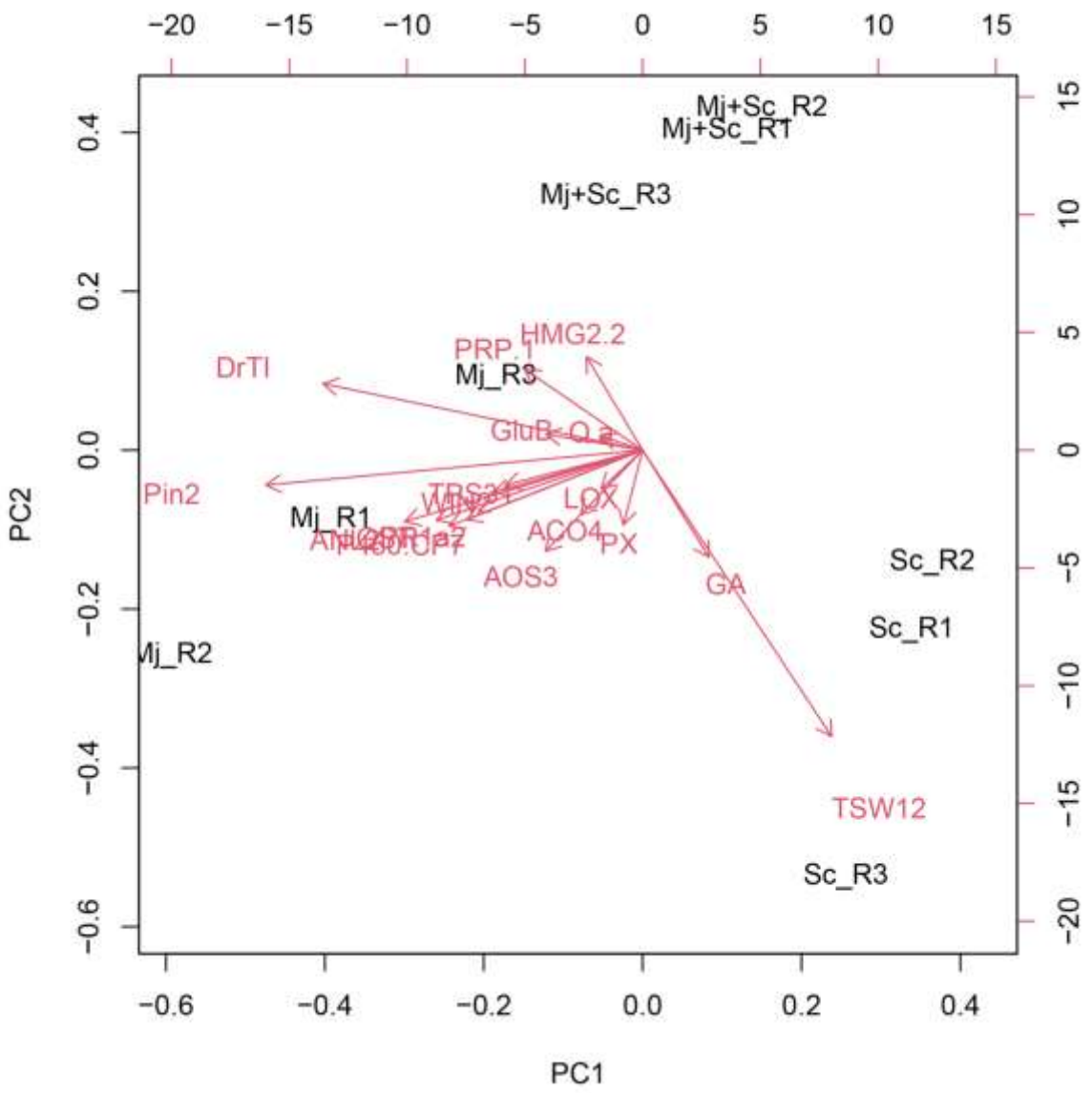


**Fig. 10**. Principal component analysis of defense-related gene ($log_2$ fold-change) expression in tomato in response to inoculation by *M. javanica* (Mj), *S. carpocapsae* (Sc), or *M. javanica* + *S. carpocapsae* (Mj+Sc) treatments for Beta-glucanase (*GluB*); Glutathione-S-transferase (*GST*); 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*); Non-specific 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.

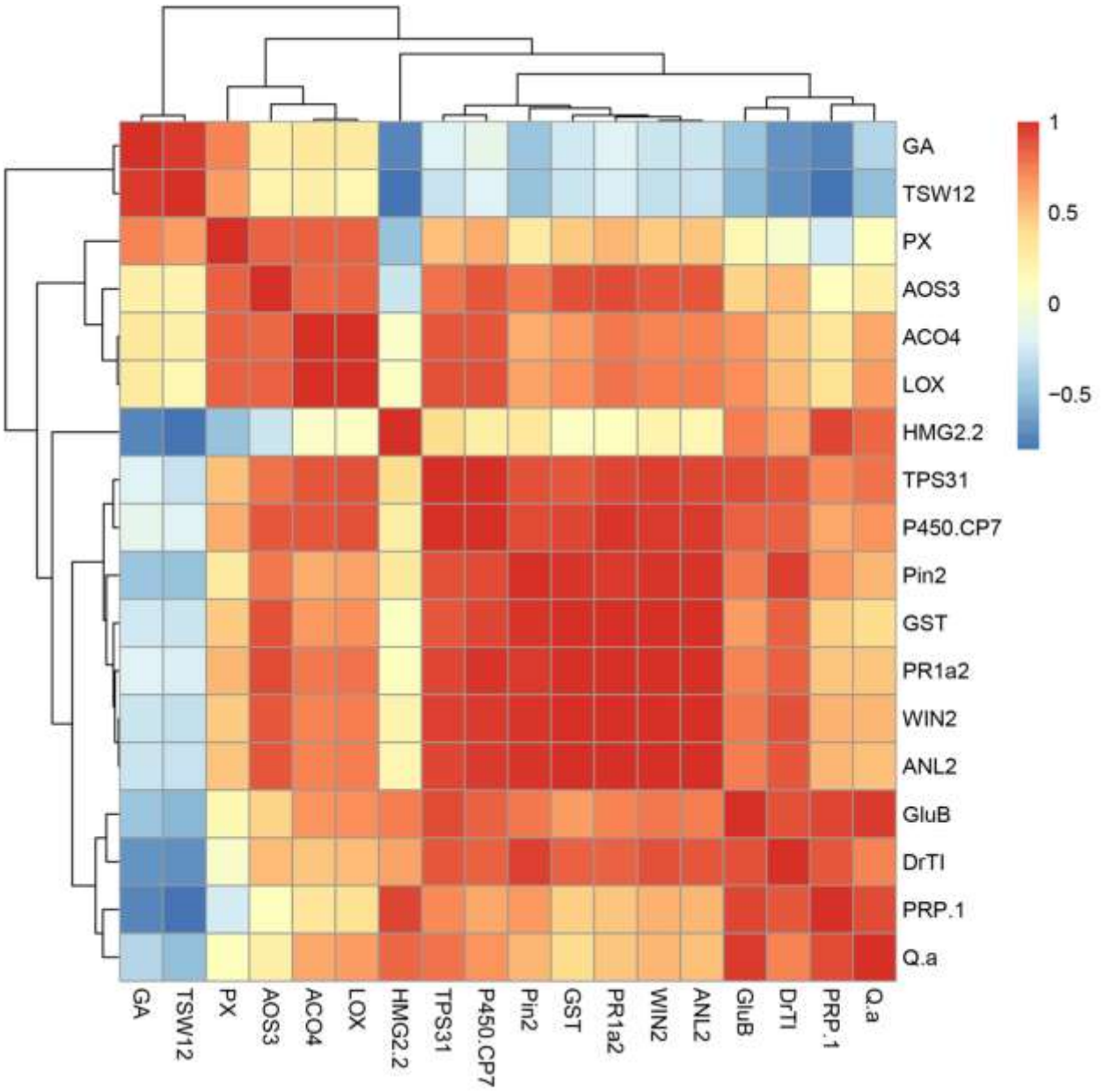


**Fig. 11**. Correlation matrix of genes involved in plant defense. The heatmap represents correlations between genes analyzed by the Pearson correlation test using R 4.0.3 software and visualized with R software. Squares indicate structural genes; Beta-glucanase (*GluB*); Glutathione-S-transferase (*GST*); 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*); Non-specific lipid-transfer protein (*TSW12*); The same color within the heatmap indicates the same level of gene expression.