The impact of *Spodoptera exigua* herbivory on *Meloidogyne incognita* induced root responses depends on the nematodes’ life cycle stages

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Abstract

Induced responses to aboveground and belowground herbivores may interact via systemic signaling in plants. We investigated whether the impact of aboveground herbivory on root-knot nematode induced responses depends on the nematode’s life cycle stages. Tomato plants were infected with the nematode (*Meloidogyne incognita*) for 5, 15, or 30 days before receiving *Spodoptera exigua* caterpillars aboveground. We collected root materials after 24 h of caterpillar feeding. We investigated phytohormones and α-tomatine levels, and the expression of defense and glycoalkaloid metabolism (GAME) marker genes in tomato roots. Nematode infection alone increased the endogenous root levels of jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), α-tomatine, and the expression of the *GLYCOALKALOID METABOLISM 1 (GAME1)* gene mostly at 30 days post nematode inoculation. Caterpillar feeding alone upregulated *Lipoxygenase D* and downregulated *Basic-β-1-glucanase* and *GAME1* expression in roots. On nematode-infected plants, caterpillar feeding decreased JA levels, but it increased the expression of *Leucine aminopeptidase A*. The induction patterns of ABA and SA suggest that caterpillars cause cross-talk between the JA-signaling pathway and the SA and ABA pathways. Our results show that caterpillar feeding attenuated the induction of the JA pathway triggered by nematodes, mostly in the nematodes’ reproduction stage. These results generate a better understanding of the molecular and chemical mechanisms underlying frequent nematode-plant-caterpillar interactions in natural and agricultural ecosystems.

**Keywords:** aboveground-belowground interaction, systemic induced responses, root-knot nematodes, *Spodoptera exigua*, phytohormones, *Solanum lycopersicum*, steroidal glycoalkaloids
Introduction

Tomato is ranked the most consumed vegetable globally, with more than 170.8 million tons produced in 2017 alone (Omondi 2018; FAO 2019). This yield is approximately 30\% times more than a decade earlier (Oishimaya 2017). Like other crops, tomato plants experience high pest pressure by, e.g., nematodes, arthropods, bacterial, and fungal pathogens. This pest pressure reduces the growth and limits tomato yield (Kumar et al. 2016; van Dam et al. 2018; Garcia et al. 2018). Root-knot nematodes (RKN) are globally occurring, soil-borne pathogens that attack plants at their roots. The infective second-stage juveniles (J2) hatch in the soil, where they locate and infect the roots of a susceptible host. Upon penetrating the roots, the J2s migrate intercellularly until they reach the vascular tissues. There they establish their permanent feeding sites (Niebel et al. 1994; Williamson and Gleason 2003; Gheysen and Mitchum 2011). Their infection impairs the translocation of water and minerals from the roots to the shoots, which can limit the plant’s productivity and fitness (Abad et al. 2008; Jones et al. 2013). At the same time, aboveground (AG) herbivores, such as leaf chewing caterpillars, may be present on the plant. The leaf loss due to caterpillar feeding can adversely impact on primary plant processes, such as the rate of photosynthesis, which are directly related to the plant’s productivity (Meyer and Whitlow 1992; Mitchell et al. 2016). Together the damage caused by RKN and herbivorous insects can reduce crop production by approximately 20\% annually, making them agro-economically important crop pests (Karajeh 2008; van der Meijden 2015; Mitchell et al. 2016). Commonly, chemical pesticides are used to control crop pests, such as nematodes and insect herbivores. Although these pesticides might be effective, several of them are currently banned from use due to their detrimental effects on human health and the environment (Franco et al. 2015; Borel 2017). Efforts to identify natural plant resistance traits for AG and belowground (BG) herbivores may help to develop sustainable pest management strategies.
Plants rely on constitutive and inducible defense responses to protect themselves against attackers. Constitutive responses are described as the physical barriers, such as thorns and trichomes, and chemical traits, such as alkaloids and glucosinolates, usually expressed independently of herbivore or pathogen attack (Wittstock and Gershenzon 2002). Induced defenses are stimulated by herbivore feeding or pathogen attack, which results in the induction of specific plant phenotypic responses (Karban 2011; Boots and Best 2018). In addition, plants can tolerate herbivory via the re-allocation of resources to undamaged plant parts, followed by compensatory growth, or by increasing the rate of photosynthesis (Mauricio et al. 1997; Peterson et al. 1998; Retuerto et al. 2004; Boege et al. 2007; Núñez-Farfán et al. 2007; Fornoni 2011; Koch et al. 2016; Mitchell et al. 2016). These changes influence critical plant physiological processes and can adversely impact the performance of herbivores.

Plant hormonal signaling governs herbivore-induced defense responses. Among the many plant hormones described so far, jasmonic acid (JA), salicylic acid (SA), ethylene (ET), and abscisic acid (ABA) are the main signaling hormones that fine-tune plant defense responses upon attack (Pieterse et al. 2009, 2012). Interaction, or cross-talk, between phytohormonal pathways, results in specific defense responses, which tailor the defensive response to the particular attacker (Pieterse et al. 2009, 2012; Li et al. 2019). Induction of defense responses at the site of attack often results in systemic signaling to distal non-attacked plant parts, thereby protecting them against future attacks (Martínez-Medina et al. 2013; yan Dam et al. 2018). Moreover, systemic induced responses may influence the attraction, behavior, and performance of other organisms sharing the same host (Bruce 2014). As a consequence, induced responses play an essential role in indirect interactions between AG and BG herbivores feeding on the same plant (van Dam and Heil 2011).
Most studies investigating plant-mediated interactions between AG and BG herbivores focus on how AG herbivore-induced responses are affected by BG herbivory (Erb et al. 2009; Kumar et al. 2016; Hoysted et al. 2017; Arce et al. 2017; van Dam et al. 2018). Only a few studies analyzed how AG induced responses affect BG feeding herbivores or pathogens. These studies report that AG herbivory induces systemic responses in the roots of crops (e.g., potato, tomato) and grass species (Wang et al. 2017; Kafle et al. 2017; Hoysted et al. 2018). Both primary and secondary metabolites play a role in plant-mediated interactions between AG and BG insect herbivores. For example, AG feeding by aphids changes potato root exudates by reducing amounts of glucose and fructose, which diminish cyst hatching (Hoysted et al. 2018). Defoliation by clipping increases nitrogen concentration in roots, which in return increases the total abundance of two species of root-feeding nematodes (Wang et al. 2017). Similarly, AG feeding by Manduca sexta on Nicotiana attenuata induces jasmonate-dependent facilitation of plant-parasitic nematode (PPN) abundance in the field, and RKN (Meloidogyne incognita) reproduction in a greenhouse (Machado et al. 2018).

Collectively, these studies demonstrate that plant responses induced by AG herbivory can systemically affect BG defense responses.

The few studies available show that systemic induced responses triggered by AG herbivory cause different effects on root feeders (Huang et al. 2013; Wang et al. 2017; Kafle et al. 2017; Hoysted et al. 2018; Machado et al. 2018). Partly the differences in the observed interaction outcomes are due to variation in the timing and sequence of arrival of both AG and BG feeding organisms (Erb et al. 2011; Wang et al. 2017; Kafle et al. 2017). In nature, root herbivores commonly colonize the plant before shoot herbivores arrive. This natural sequence of pest arrival follows from the fact that roots develop first (Bezemer and van Dam 2005). For PPNs, such as RKNs, these factors are particularly relevant. As obligate root feeders, RKNs undergo different distinct life cycle stages. In the invasion stage, J2s enter the
root at the zone of elongation and move towards the vascular cylinder. Then they turn around and move several body lengths upwards before settling and initiating feeding (Robinson and Perry 2006). This movement occurs between the cells (intercellularly), which also reduces the elicitation of defense responses because only a few cells are damaged (Caillaud et al. 2008; Gheysen and Mitchum 2011). In the establishment stage, the juveniles become sedentary and inject various effectors to establish the so-called ‘giant cell’. This giant cell serves as their feeding site. The cells surrounding the giant cells undergo proliferation and enlargement, and, in due time, they become visible to the human eye as a gall or a “root-knot” (Rodiuc et al. 2014; Escobar et al. 2015). We refer to this stage, in which the nematode establishes a feeding site, as the galling stage. At their feeding site, the nematodes acquire resources and develop through three molts to mature and reach the reproduction stage. The female nematode’s body swells up and becomes pear-shaped. When the eggs are ripe, the females release their eggs into the rhizosphere, and another cycle begins (Caillaud et al. 2008; Gheysen and Mitchum 2011). In each infection stage, the nematodes’ growth and development depend on the injection of different effectors into the host cells (Quentin et al. 2013; Favery et al. 2016; Gheysen and Mitchum 2019). These effectors trigger different hormonal signaling pathways, including JA, SA, ET, and ABA (Caillaud et al. 2008; Kyndt et al. 2017; Gheysen and Mitchum 2019). Because hormones are generally involved in plant defense induction, systemic defense responses induced by AG herbivores might affect the nematodes and the local responses they induce in the roots. Moreover, the effect that AG herbivores may have on BG defense signaling triggered by root herbivores may depend on the life cycle stage in which the nematodes are at the time point of AG attack.

Here, we used tomato (Solanum lycopersicum ‘Moneymaker’) and two generalist crop pests, the RKN M. incognita and larvae of Spodoptera exigua (Hübner), as the study system to analyze the molecular mechanisms mediating interactions between AG herbivores and
nematodes. Previous studies showed that interactions between RKN and shoot herbivores can be governed by JA-dependent responses, evidenced by changes in jasmonates levels in *Nicotiana attenuata* (Machado et al. 2018) and the production of trypsin protease inhibitors in tomato (Arce et al. 2017). These interactions may also involve cross-talk between hormonal pathways, such as JA-SA (van Dam et al. 2018) and JA-ABA (Erb et al. 2009; Kyndt et al. 2017). Therefore, we measured phytohormone concentrations (JA, SA, ABA) and the expression of several marker genes for hormonal signaling; *Lipoxygenase-D* and *Leucine aminopeptidase-A* (JA markers), *Le4* (ABA marker) and *Basic-β-1,3-glucanase* (*GluB*) (ET marker) in roots (Table S1). Tomato is also known to produce steroidal glycoalkaloids, such as α-tomatine, as a defense to generalist herbivores (Friedman 2002; Cárdenas et al. 2015). Hence, we included measurements of α-tomatine and the expression of glycoalkaloid metabolism genes (GAME) *Jasmonate-responsive Ethylene Response Factor 4* (*JRE4*) and *GAME1*. We specifically analyzed how 24 h of AG feeding affected these defense-related traits in roots that were infected with *M. incognita* at 5, 15, and 30 days post nematode inoculation (dpi). These time points coincide with the invasion (5 dpi), galling (15 dpi), and reproduction (30 dpi) stages of this nematode. With this approach, we aimed to assess whether the nature of the interaction between shoot and root induced responses depends on the developmental stage of the RKN.
Material and Methods

Study plant, root and shoot organisms

In all our experiments, we used tomato (*S. lycopersicum* ‘Moneymaker’) as the model plant. The RKN *M. incognita* was used as root herbivore, and the larvae of the generalist herbivore *S. exigua* were used as shoot herbivores. We obtained *M. incognita* eggs from Rijk Zwaan (De Lier, The Netherlands) and maintained a glasshouse stock on tomato ‘Moneymaker’ for eight weeks. Similar to a previous study (Martínez-Medina et al. 2017), we initiated the colony from a single egg mass, and eight weeks later extracted eggs for use in the bioassay. We purchased *S. exigua* eggs from Entocare C.V. Biologische Gewasbescherming (Wageningen, The Netherlands) and maintained a laboratory colony on artificial diet, in a growth chamber set at 25 °C constant, 12 h photoperiod and 45 % relative humidity.

Plant growth condition and herbivores infection

The tomato seeds were obtained from Intratuin B.V (Woerden, the Netherlands). Before germination, the seeds were surface sterilized by immersion in 40 mL of 10 % sodium hypochlorite solution for four minutes. Afterward, the seeds were rinsed four times with water. Each round of rinsing was for 10 min. The sterilized seeds were placed on moistened glass beads and allowed to germinate at 27 °C in the dark for three days, followed by four days in a plant growth chamber (CLF PlantClimatic, CLF PlantClimatics GmbH, Wertingen, Germany). The growth conditions were 16 h:8 h day: night cycle, 55 % relative humidity (RH), and 60 % (65 μmol s⁻¹m⁻²) light intensity. One-week old seedlings were transplanted into sterilized 1:1 sand : soil mixture in 11x11x12 cm pots. They were grown in a glasshouse under at 26±3 °C: 23±3 °C day: night, with 16 h:8 h light: dark and RH was maintained at ~30 %. The plants were watered as required and supplemented weekly with 50 % strength
Hoagland solution. The plants were grown for three more weeks before using them in bioassays. We randomly selected healthy plants of similar size and appearance for our experimental treatments. We divided the plants into two groups; one group was inoculated with *M. incognita* eggs (3000 eggs mL\(^{-1}\)), and the other group was mock-inoculated with water. In the *M. incognita* inoculated plants, we set three-time points to coincide with the main nematode life cycle stages. These were 5 dpi (invasion stage), 15 dpi (galling stage), and 30 dpi (reproduction stage). At each of these time points, plants were subjected to four different treatments, each with ten biological replicates. The treatments were control (plants without herbivores or nematodes); belowground infection (plants challenged with *M. incognita*); aboveground herbivory (plants challenged with *S. exigua*); and both belowground infection and aboveground herbivory (plant challenged with both *M. incognita* in root followed by *S. exigua* feeding on leaves). We infested the plants assigned to leaf-feeding with one second-instar *S. exigua* caterpillar. The *S. exigua* caterpillars were confined to a 7 cm (diameter) round clip cage placed on one fully expanded leaf close to the tip (see Fig 4D in (Bandoly and Steppuhn 2016)). In plants without shoot herbivory, an empty clip cage was mounted on a leaf at a similar position to the one used in plants with shoot herbivory. The *S. exigua* larvae were allowed to feed for 24 h. Other studies showed that this time period suffices to affect defense metabolites and genes in roots. For example, 24 h of AG herbivory by *M. sexta* and *Spodoptera littoralis* on *N. attenuata* results in systemic induction of JA-related genes expression in roots (Fragoso et al. 2014). After this time, we harvested the roots by gently removing them from the pots. The soil was removed by soaking the whole root into a bucket filled with tap water. Then the roots were rinsed with running tap water and dried with filter paper. After quickly counting the number of galls (especially for roots collected at the galling and reproduction stages) (Figure S1), the roots were wrapped in clean labeled
aluminium foil, and immediately shock-frozen in liquid nitrogen. The root samples were stored at –80 °C, pending molecular and metabolite analyses.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from ~100 mg fresh weight per root sample according to the method described by (Oñate-Sánchez and Vicente-Carbajosa 2008). First-strand cDNA was synthesized from 1 µg DNase free mRNA using Revert Aid H-minus RT (Thermo Fisher Scientific Baltic UAB, Vilnius, Lithuania) following the manufacturer’s instructions. Real-time quantitative qPCR reactions and relative quantification of specific mRNA levels were performed according to (Martínez-Medina et al. 2017) by using a CFX 384 Real-Time PCR system (Bio-Rad Laboratories Inc. Singapore) and the gene-specific primers described in Table S1. These genes were selected from previously published articles where their involvement in tomato biotic interactions is reported (Uppalapati et al. 2005; Martínez-Medina et al. 2013; Yan et al. 2013; Abdelkareem et al. 2017). The data were normalized using the housekeeping gene (SIEF X14449), which encodes for the tomato elongation factor 1α, a commonly used and stable reference gene for data normalization in studies on induced responses in tomato (Miranda et al. 2013; Martínez-Medina et al. 2017). Data were analyzed by the 2^ΔΔct method (Livak and Schmittgen 2001).
Determination of phytohormone concentration

We extracted and quantified phytohormones following the protocol described by Machado et al. (2013). In brief, ~100 mg fresh weight per root sample was extracted with 1 mL ethyl acetate containing 40 ng of each of the following internal phytohormone standards: \(D_6\)JA and \(D_6\)SA, and \(D_6\)ABA. The extracts were vortexed for 10 min using a Thermomixer, then centrifuged at 15000 x g, 4 °C for 20 min, the supernatants were transferred to a new tube and evaporated to dryness at room temperature using a SpeedVac (Labconco Cooperation, Kansas, Missouri, USA). Remaining pellets were resuspended in 200 µL methanol: water (70:30) using an ultrasonic bath for 5 min and centrifuged at 15000 x g, 4 °C for 5 min. The supernatant was collected for phytohormone measurement using liquid chromatography (Bruker Advance UHPLC, Bremen, Germany) coupled to a mass spectrometer (Bruker Elite EvoQ Triple quadrupole, Bremen, Germany) (LC/MS EVOQ) (Schäfer et al. 2016). The separation was achieved on a Zorbax Eclipse XDB-C18 column (4.6x50 mm, 1.8 µm, 80 Å, Agilent technologies, Santa Clara, CA, USA). Mobile phase was composed of A; (0.05 % (v/v) aqueous formic acid) and B; (0.05 % (v/v) formic acid in 100% acetonitrile). The following gradient was used: 0 - 0.5 min, 5 % B; 0.5 - 0.6 min, 5 % - 50 % B; 0.6 - 2.5 min, 50 % - 100 % B; 2.5 - 3.5 min, 100 % B; 3.5 – 3.55 100 % - 5 % B; 3.55 - 4.5 min 5 % B at flow rate of 400 µL min\(^{-1}\). All solvents used were LC-MS grade. The column temperature was kept constant at 42 °C.

After separation, the compounds were nebulized by electron spray ionization in the negative mode using the following conditions: capillary voltage 4500 eV, cone gas 35 arbitrary units /350 °C, probe gas 60 arbitrary units /475 °C and nebulizing gas at 60 arbitrary units. The phytohormones were identified based on their retention time and the monitored mass to charge ratio \((m/z)\) transition. The \(m/z\) ratio of the phytohormones of interest were; \((m/z)\) 209.12 → 59.00 for JA; \((m/z)\) 263.13 → 153.00 for ABA and \((m/z)\) 137.02 → 93.00 for
SA. Samples were analyzed in a randomized sequence with acetonitrile samples in between as background controls. Data acquisition and processing were performed using the ‘MS data Review’ software (Bruker MS Workstation, version 8.2). Phytohormone levels were calculated based on the peak area of the corresponding internal standard and the amount of fresh mass of plant material (ng\(^{-1}\) mg\(^{-1}\) fresh weight).

**Determination of the root α-tomatine concentrations**

We extracted ~100 mg fresh weight of each root sample in a 2 mL Eppendorf tube with 1 mL solution containing 25% of acetate buffer (2.3 mL acetic acid, 3.41 mg ammonium acetate dissolved in 1 L of Milli pure water, pH 4.8) and 75% methanol. Tubes with extracts were inverted for 10 s and thoroughly mixed via shaking using a grinding ball mill (MM400, Retsch GmbH Leipzig, Germany) set at 30 Hz for 5 min. To remove the solid particles in the extracts, we centrifuged them at 15,000 x g for 15 min, and the supernatant transferred into a new 2 mL Eppendorf tube, and the pellet was re-extracted as above. We mixed the first and second supernatant and transferred 200 μL of the combined extracts into a 2 mL HPLC vial and added 800 μL of the extraction buffer to obtain a 1:5 dilution for each sample. The extracts were stored at -20°C, pending further analysis. Metabolites were characterized by injecting 1 μL of the extracts in a UPLC (Dionex 3000, Thermo Scientific). The chromatograph was equipped with a C18 column (Acclaim TM RSLC 120), 2.1 x150 mm external dimension, 2.2 μm particle size, and 120 Å pore size. The column was kept at 40°C. The mobile phases (LC-MS grade solvents) were composed of solvent A: 0.05% (v/v) aqueous formic acid and solvent B: 0.05% (v/v) formic acid in acetonitrile. The multi-step gradient for solvent B was; 0–1 min 5%, 1–4 min 28%, 4–10 min 36%, 10–12 min 95%, 12–14 min 95%, 14–16 min 5%, 16–18 min 5%. The flow was set to 400 μL min\(^{-1}\). We detected compounds using a maXis impact HD MS-qToF (Bruker Daltonics). Data were
acquired in positive mode. ESI ion source conditions were; endplate offset=500 V, capillary =4500 V, nebulizer =2.5 bar, dry gas=11 L min⁻¹, dry temperature =220 °C. Transfer line conditions were: funnels 1 and 2 = RF 300 Vpp, isCID energy = 0 eV, hexapole = 60 Vpp, quadrupole ion energy = 5 eV, low mass = 50 m/z, collision cell energy = 10 eV, collision RF 500 Vpp, transfer time = 60 µs, pre-pulse storage = 5 µs. The mass spectrometer operated with a mass range of 50 - 1500 m/z and a spectral acquisition rate of 3 Hz. Sodium formate clusters (10 mM) were used for calibrating the m/z values. These sodium formate clusters were a mix consisting of 250 mL isopropanol, 1 mL formic acid, 5 mL 1 M NaOH, and the final volume was adjusted to 500 mL. All analyses had a quality control sample, which was a pool of all the different experimental groups and time points. The quality control sample was analyzed at the beginning and the end of the batch and after every ten injections. The raw .d data files were processed using the program Compass DataAnalysis (Bruker Daltonics). The processing involved obtaining the extracted ion chromatogram (EIC) for a fragment of α-tomatine at the m/z value 578.4050 and m/z tolerance of ± 0.1. We selected the option compound list to automatically calculate the peak areas of each EIC per sample per study time point. All the peak areas for α-tomatine were tabulated and used for multivariate statistical analysis.

**Statistical analysis**

We created two datasets combining (1) phytohormone and α-tomatine levels, and, (2) defense markers and glycoalkaloid metabolism genes. In each combined dataset, we tested the effects of *M. incognita* (Mi; with vs. without), and *S. exigua* (Se; with vs. without), and their interactions on the defense variables (*i.e.*, the plant defense traits; phytohormone, α-tomatine and marker genes). Each dataset was analyzed using the permutational multivariate analysis of variance (PERMANOVA). PERMANOVA was chosen because our data lacked homogeneity of variance or normal distribution; PERMANOVA does not require this
because it uses a distribution-free permutation approach to partition the variance among treatments (Anderson 2017). The PERMANOVA analysis was run for each data set using the Adonis function, with the Gower dissimilarities method among samples, and 999 permutations in R v 3.6.1 software (R Core Development Team 2019). Where the PERMANOVA output showed significant effects for either factor or their interaction (Tables S2 and S4), we performed separate factorial linear model ANOVAs on each dependent variable, with *M. incognita* and *S. exigua* and their interaction as fixed factors. Once the main effect significantly affected any of the dependent variables, the differences among the four experimental treatments were tested using Tukey’s Honest significant difference test for multiple comparisons.
Results

Root infection by *Meloidogyne incognita* alone affects the expression of root-inducible defenses at different life cycle stages

We first considered how the nematode affected root inducible defenses at the invasion, galling, and reproduction stages. We found that *M. incognita* root infection enhanced the induction of JA, SA, ABA, and α-tomatine progressively during the infection process. In particular, the JA response in *M. incognita* infected plants became more pronounced with the progression of the nematode’s life cycle compared to controls (Figure 1A,E,I, blue boxplots). At both the invasion and galling stages, the levels of these signaling hormones were increased, but only at the reproduction stage, the increases became significant compared to control plants (Figure 1I,J,K,L, blue boxplots, Table S3). In contrast, root infection by *M. incognita* did not trigger changes in the expression of the defense marker genes. We found that the expression of *LoxD*, *LapA*, *Le4*, *GluB* (Figure 2, blue boxplots, Table S5), and *JRE4* (Figure 3, blue boxplots, Table S5) remained similar to those observed in control plants regardless of the nematodes’ root infection stage. However, we observed significant upregulation in the expression of the *GAME1* transcripts at the nematodes’ reproduction stage compared to control plants (Figure 3F, blue boxplot, Table S5). The increase in *GAME1* transcripts correlated with an increase in α-tomatine concentrations in nematode infected roots at the reproduction stage (Figure 1L, blue boxplot).
The impact of *Spodoptera exigua* feeding on root defense responses in tomato plants depends on plant age

Next, we analyzed the impact of *S. exigua* leaf herbivory on root defenses of tomato plants without nematode infection. Due to the experimental set-up, which was designed based on the life stages of the nematodes, the plants that received only caterpillars were 4.8 (coinciding with the invasion stage), 6.2 (coinciding with the galling stage), and 8 (coinciding with reproduction stage) weeks old. We found that *S. exigua* leaf herbivory did not affect the levels of JA, SA, ABA, and α-tomatine concentration in tomato roots compared to the control plants, regardless of plant age (Figure 1, green boxplots, Table S3). In contrast, *S. exigua* herbivory triggered differential effects on the expression of the hormonal signaling and GAME marker genes (Figures 2 and 3, green boxplots, Table S5). In the 4.8 (invasion stage), and 6.2 (galling stage) weeks old plants, the expression of the marker genes was not significantly different from controls (Figures 2A-H, and 3A-D, green boxplots, Table S5). Notably, when the plants were 8 weeks old, which coincided with the nematodes’ reproduction stage, the defense gene *LoxD* was upregulated compared to controls (Figure 2I, green boxplot, Table S5). The *LapA* and *Le4* expression levels were not significantly different compared to controls (Figure 2J,K, green boxplots, Table S5), while *GluB* was significantly downregulated compared to controls (Figure 2L, green boxplot, Table S5). The GAME gene *JRE4* was not affected while the *GAME1* was significantly downregulated compared to controls (Figure 3E,F, green boxplots, Table S5).
Effects of *Spodoptera exigua* on *Meloidogyne incognita* induced responses depend on the nematodes’ infection stage

Because our primary interest was to analyze the effect of *S. exigua* AG feeding on root responses induced by the *M. incognita* at different infection stages, we primarily focused on the comparison between *M. incognita*-infected plants (Mi treatment, blue box plots, Figures 1, 2, and 3) with the double infected plants (MiSe treatment, yellow boxplots, Figures 1, 2, and 3). We found that *S. exigua* herbivory on *M. incognita*-infected plants did not change JA levels at the invasion and galling stages compared to plants challenged with *M. incognita* alone (Figure 1A,E, yellow boxplots, Table S3). *Spodoptera exigua* herbivory on the *M. incognita* infected plant significantly decreased the JA levels at the nematodes’ reproduction stage compared to plants infected with *M. incognita* alone (Figure 1I, yellow boxplot, Table S3). *Spodoptera exigua* feeding on *M. incognita*-infected plants did not affect SA, ABA, and α-tomatine concentrations compared to plants challenged with *M. incognita* alone, regardless of the nematodes’ infection stage (Figure 1B-D,F-H,J-L, yellow boxplots, Table S3). Overall, we observed that the local nematode-induced responses dominated the nature of SA, ABA, and glycoalkaloid responses in roots (Figure 1, Table S3, main Mi effects). Similarly, *S. exigua* herbivory on *M. incognita*-infected plants triggered changes in the expression of marker genes depending on the nematodes’ root infection stages. At the invasion stage, the expression levels of both defense and GAME genes in double infected plants were similar to those with *M. incognita* infection alone (Figures 2A-D, and 3A,B, yellow boxplots, Table S5). At the galling stage, the JA biosynthesis marker *LoxD* overall increased in plants infected with *M. incognita* (Table S5, main Mi effect). Aboveground damage by *S. exigua* did not significantly alter this. A similar pattern was found for the expression levels of the other marker genes in plants with *M. incognita* and *S. exigua*; in the invasion and galling stage their expression levels were similar to plants with *M. incognita*
infection alone (Figures 2E-H, and 3C,D, yellow vs. blue boxplots, Table S5). During the reproduction stage, *S. exigua* herbivory on *M. incognita*-infected plants significantly upregulated *LapA*, (Figure 2J, yellow boxplot, Table S5), whereas it had no significant effect on the other marker genes compared to plants infected with *M. incognita* alone (Figures 2I,K,L, and 3E,F, yellow boxplots, Table S5). By comparing the double infected plants to control plants and those infected with *S. exigua* only, it became clear that the downregulation of *GluB* by *S. exigua* (Figure 2L, Table S5) is not affected by *M. incognita* infection. On the other hand, the significant main effects of *M. incognita* on the expression of *JRE4* and *GAME1* at the galling and reproduction stages were not changed by *S. exigua* feeding (Figure 3C,D, and E,F, blue and yellow boxplots, Table S5). Therefore, our results collectively suggest that *S. exigua* can affect nematode-induced root responses, in particular via the JA signaling pathway, depending on the nematodes’ infection stage.

**Discussion**

The goal of our study was to determine whether the impact of AG feeding on root defense responses induced by *M. incognita* depends on the nematodes’ life cycle. We tested this by exposing *S. exigua* caterpillars to tomato plants infected by *M. incognita* at different stages of the root infection cycle. We found that *S. exigua* affected *M. incognita* root-induced responses, mainly at the nematodes’ reproduction stage. In particular, the JA signaling pathway was affected, as evidenced by lowered levels of JA in double infected plants compared to plants infected with *M. incognita* alone. JA is known to regulate the GAME pathway in tomato via the *JRE4* transcription factor (Thagun *et al.* 2016). In this study, the attenuation of the JA pathway did neither lower α-tomatine concentrations nor the expression of the GAME genes (*JRE4* and *GAME1*) in double infected plants compared to plants challenged with *M. incognita* alone (Figure 4). This may be because the glycoalkaloid
biosynthesis transcriptional coordinator JRE4 can act downstream of JA signaling (Abdelkareem et al. 2017). Caterpillar feeding also enhanced LapA expression in double infected plants at the nematodes’ reproduction stage compared to plants challenged with M. incognita alone. LapA acts downstream of JA signaling as a modulator of late wound-induced responses (Fowler et al. 2009). LapA expression is induced by external application of ABA, MeJA, and ET (Chao et al. 1999). Here, the levels of ABA in double infected plants remained elevated, which could be related to the upregulation in LapA expression. Cross-talk between phytohormones is widely recognized as a mechanism to tailor herbivore-induced responses to specific – combinations of attackers (Pieterse et al. 2009; Zamioudis and Pieterse 2012). Cross-talk between the JA signaling pathway and both the SA and ABA pathways may also explain why glycoalkaloid levels remained increased in double infected plants at the nematodes’ reproduction stage compared to plants infected with M. incognita alone, despite lowered JA levels, GluB expression, and no effect on LoxD expression compared to M. incognita-infected roots (Figure 4). This cross-talk of SA and ABA with the JA pathway might also occur downstream of JA biosynthesis, e.g., at the level of transcription factors like MYC or ERF and in our case, JRE4 (Figure 4).

To date, the elicitation of root defenses by endoparasitic nematode infection at later time points in their life cycle is virtually undescribed; most papers focus on signaling events occurring at 1–7 days after infection (Kyndt, Denil, et al. 2012; Kyndt, Nahar, et al. 2012; Kammerhofer et al. 2015; Martínez-Medina et al. 2017). Here, we found that M. incognita infection at the invasion and galling stages did not elicit strong defense responses, either on the level of phytohormones, gene expression, or glycoalkaloid production. The lack of significant defense induction during the invasion stage can be partly attributed to how the RKNs migrate inside the roots. Once the J2s of RKN are inside roots, they avoid damaging plant cells by moving intercellularly through soft tissues of the host plant root tissues.
(Gheysen and Mitchum 2011; Gheysen and Jones 2013). Also, RKNs secrete effector proteins that play an essential role during both the penetration (invasion) and the establishment and galling phases. These effectors suppress host defense responses and help the nematode to establish a permanent feeding site (Abad and Williamson 2010; Mitchum et al. 2013). For instance, the rice pathogenic nematodes *M. graminicola* and *M. javanica* secrete the effectors, *Mg-MSP18* and *Mj-MSP18*, between 7-21 dpi to suppress the activation of their host’s immune responses, such as the hypersensitive response (Grossi-de-Sa et al. 2019). In our study, *M. incognita* did not induce significant root defenses at the galling stage. We correlate this lack of defense induction to the fact that *M. incognita* utilizes effector proteins to repress plant responses in roots during the galling stage. For example, in *A. thaliana*, *M. incognita* secretes the effector *Mi-CTR* at roots to suppress the expression of Pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), related defense genes, including *WRKY33,29, PDF1.2*, and *PR-1* (Jaouannet et al. 2013). The effect of *Mi-CTR* occurs after root invasion and initiation of the giant cells 21 dpi most likely to ensure successful establishment (Jaouannet et al. 2013).

Interestingly, when RKN reached the reproduction stage, we observed an induction of defense responses. We found that *M. incognita* infection increased all phytohormone levels measured, as well as the concentration of α-tomatine and the expression of its biosynthesis gene *GAME1*. Possibly, the swelling of the female bodies with the ripening eggs intensifies the cell damage at the feeding sites, leading to the observed hormonal and defense responses. It is remarkable, however, that the expression patterns of defense signaling marker genes are not affected the same way. Possibly the expression of defense marker genes might be regulated by effector proteins that are only secreted by female RKN during the reproduction stage. For instance, the *Misp12* effector is specific to *M. incognita* and secreted by mature females at least 28 dpi (Xie et al. 2016). Overexpression of *Misp12* suppresses the *Pathogen
related protein-1 (PR1) and phenylalanine ammonia-lyase-5 (PAL5) genes (SA pathway markers) in *N. benthamiana*. In *Misp12* silenced plants, an upregulation of the proteinase inhibitor 2 (Pin2) (JA pathway marker) is reported. The authors suggest that *Misp12* might be involved in the maintenance of giant cells during the reproduction stages (Xie et al. 2016).

The systemic effect of *S. exigua* feeding on root hormone levels and defense responses was much less pronounced than local nematode-induced responses. On the one hand, this may be because the caterpillars fed only for 24 h on the plant, while the nematodes were continuously feeding. In other studies, shoot feeding by herbivores, including *S. exigua* and *Pieris rapae*, was applied for 2–7 days before defense responses were observed in the roots (Danner et al. 2015; Kafle et al. 2017). Possibly, 24 h of AG feeding may have been too short to elicit strong systemic responses in tomato roots. Moreover, systemic responses are generally weaker than locally induced responses (van Dam et al. 2001; Babst et al. 2009; Ádám et al. 2018). For example, leaves feeding by diamondback moth caterpillars in *Brassica oleracea* revealed slight systemic JA responses in the roots compared to the local induction by *Delia radicum* (Karssemeijer et al. 2020). In another study, shoot feeding by *P. rapae* larvae on *Brassica rapa* plants elicits much lower root volatile emissions than local damage by *Delia radicum* larvae (Danner et al. 2015).

Interestingly we found that the age of the plant affects the systemic response as well. In our experimental set-up, we applied nematode eggs at one single time point. Consequently, the *S. exigua* caterpillars were placed on tomato plants that were at different ages and ontogenetic stages. The expression of some defense marker genes was significantly upregulated (Figure 2 I) or downregulated (Figure 3F) by *S. exigua* feeding only in the last batch of plants, which were 8 weeks old and flowering. It has been reported that herbivore-induced plant responses can significantly change as a function of plant ontogenetic stage (Quintero and Bowers 2011, 2012). For instance, the concentration of iridoid glycosides
(IGs) in *Plantago lanceolata* L. roots after AG herbivory was twice as high in mature plants compared to young plants (Quintero and Bowers 2011).

In nature, plants are likely to interact with AG herbivores and RKN at the same time. Here we found that *S. exigua* herbivory differentially affects the root-induced responses by *M. incognita* in tomato roots. These effects occurred in dependence on the life cycle of the nematode, whereby the impact was the strongest in the reproductive stages. Herbivore identity and sequence of arrival on the target host plant are some of the critical factors shaping interactions between AG-BG herbivores (Erb et al. 2011; Sarmento et al. 2011; Kafle et al. 2017). We conducted our experiment by first infecting the plants with RKN. This is likely the natural sequence of arrival because the roots develop before the shoots after seed germination. Moreover, nematodes are ubiquitous in natural systems. Roots are therefore likely to be invaded with nematodes before herbivores arrive on AG organs (Hoysted et al. 2018; van Dam et al. 2018). *Spodoptera exigua* feeding on *M. incognita*-infected plants reduced JA but not SA concentrations. In a similar study, *M. incognita* were allowed to colonize tomato plants that had experienced seven days of *S. exigua* feeding, followed by a lag phase of another seven days (Kafle et al. 2017). The authors found that after 14 days of *M. incognita* infection, the root JA levels decreased in tomato plants that were previously damaged by *S. exigua*. Combining our results with this study, we conclude that it may not matter whether the nematode or the AG herbivore infects first; AG feeding seems always to reduce RKN-induced JA levels in the roots.

Jasmonates are essential regulators of systemic signaling between AG and BG tissues (Wasternack 2007; Wasternack and Hause 2013). It has been established that JAs regulate the steroidal glycoalkaloid metabolism pathway via the *JRE4* transcription factor (De Geyter et al. 2012; Cárdenas et al. 2016; Thagun et al. 2016). Here the expression of *JRE4* was not altered by *S. exigua* feeding alone, nor did the caterpillar alter the *M. incognita*-induced
upregulation of this transcription factor. Notably, the expression of LapA (JA marker) was significantly upregulated in double infected plants compared to plants infected with M. incognita only, while LoxD expression was similar when S. exigua co-occurred with M. incognita. Our results suggest that the interaction between M. incognita and S. exigua might rely on the induction of late wounding responses regulated by LapA downstream of JA synthesis, e.g., on transcription factor level (Figure 4). Unfortunately, our experimental set-up did not allow us to precisely determine the role of LapA because the plants with RKN in different life cycle stages also differed in age. LapA might also be associated with plant development, especially in the flowering stage, as reported by Chao et al. (1999).

Finally, the induction of JA levels by M. incognita infection was accompanied by an increase in α-tomatine production. Increases in JA and α-tomatine concentrations upon nematode attack or exogenous application of elicitors, such as methyl-jasmonate (MeJA), have been reported in tomato and other plant species (Abdelkareem et al. 2017; Kafle et al. 2017). Glycoalkaloids are usually associated with increased generalist herbivore resistance (Ökmen et al. 2013; Abdelkareem et al. 2017). In our study, we did not measure the ecological consequences associated with these defense responses, e.g., for later arriving herbivores. Further studies to test the effects of α-tomatine on the performance of the RKNs may reveal their effectiveness as defenses against this generalist herbivore.
Conclusions

Our study examined the impact of AG chewing herbivores on root-induced responses by RKN at different life cycle stages. We found that both the AG chewing herbivore and the RKN affect root defenses. The effect of root infection by RKN alone, as well as the effect of AG herbivory on RKN-induced root defense responses, depends on the nematode’s life cycle stage. Studies testing the impact of long periods of AG herbivory on nematode induced root responses are needed to reveal how the interactions with BG responses might change over longer interaction times. Such studies will help to optimize tomato breeding efforts toward cultivars with high resistance to AG and BG insect pests and pathogens.
Data availability statement

The data underlying this study are published as open access at the iDiv Data Repository (http://idata.idiv.de/ddm/Data/ShowData/1816). The data can be cited as “Crispus Mbaluto., Ainhoa Martínez-Medina., and Nicole M. van Dam (2020) Induced defense response in tomato roots infected by root-knot nematode infection at different life cycle stages and with leaf damage by caterpillar, https://doi.org/10.25829/idiv.1816-16-2864

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Author contributions

CMM, AMM, and NMvD: conception of the idea and experimental design; CMM: execution, processing of samples, data analysis; EMA and MF: processing of samples, data analysis; CMM: writing of the initial manuscript; CMM: deposition of data in iDiv data repository; AMM and NMvD: critical revision of draft manuscripts and approval of the final manuscript for submission.

Conflict of interest statement

The authors declare that this research article was conceptualized, designed, and drafted in the absence of any commercial interest or financial obligations that could be construed as a potential conflict of interest.
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Figure legends

Figure 1 Phytohormones concentrations and relative peak area of α-tomatine. Mean concentrations (ng/mg fresh weight) of phytohormones and the relative peak area of α-tomatine in tomato plants infected with *Meloidogyne incognita* (Mi), infested with *Spodoptera exigua* (Se) or both (MiSe). Con = plant without herbivory. Boxplots are the mean (±SEM) of jasmonic acid (A,E,I); salicylic acid (B,F,J); abscisic acid (C,G,K); α-tomatine (D,H,L) per treatment (n=5) measured at the nematodes’ invasion (A,B,C,D), galling (E,F,G,H) and reproduction (I,J,K,L) stages. Different lower-case letters above the boxplots indicate significant differences in mean values between treatments, determined via multiple comparisons Tukeys HSD test after ANOVA at P ≤ 0.05.

Figure 2 Expression of defense marker genes. Relative expression of defense marker genes in tomato plants infected with *Meloidogyne incognita* (Mi), infested with *Spodoptera exigua* (Se) or both (MiSe). Con = plants without herbivory. Expression values are normalized over the expression of the housekeeping gene (*SIEF X14449*) encoding for tomato elongation factor 1α. Boxplots are mean (±SEM) expression values of *Lipoxygenase D* (LoxD); *Leucine aminopeptidase A* (LapA); abscisic acid-responsive *Le4* (Le4); *Basic-β-1-glucanase* (GluB) per treatment (n=5) measured at the nematodes’ invasion (A,B,C,D), galling (E,F,G,H) and reproduction (I,J,K,L) stages respectively. Different lower-case letters above the boxplots indicate significant differences in mean expression among treatments, determined via multiple comparisons Tukeys HSD test after ANOVA at P ≤ 0.05.
Figure 3 Expression of steroidal glycoalkaloid metabolism genes. Relative expression of steroidal glycoalkaloid metabolism genes in tomato plants infected with *Meloidogyne incognita* (Mi), infested with *Spodoptera exigua* (Se) or both (MiSe). Con = plants without herbivory. Expression values are normalized over the expression of the housekeeping gene (*SIEF XI4449*) encoding for tomato elongation factor 1α. Boxplots are mean (±SEM) expression values of jasmonate-responsive *ETHYLENE RESPONSE FACTOR 4* (*JRE4*, A,C,E); and glycoalkaloid metabolism 1 (*GAME1*; B,D,F) per treatment (n=5) measured at the nematodes’ invasion (A,B), galling (C,D) and reproduction (E,F) stages respectively. Different lower-case letters above the boxplots indicate significant differences in mean expression among treatments, determined via multiple comparisons Tukeys HSD test after ANOVA at P ≤ 0.05.

Figure 4 Interactions between root defense responses upon root and leaf herbivory.

Schematic illustration of induced defenses in tomato roots including the phytohormones jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), the glycoalkaloid α-tomatine, and defense genes (*Lipoxygenase D* (*LoxD*), *Leucine aminopeptidase A* (*LapA*), *Le4 abscisic acid-responsive*, *Basic-β-1-glucanase* (*GluB*), and glycoalkaloid metabolism (GAME) genes (*jasmonate-responsive ETHYLENE RESPONSE FACTOR 4* transcription factor (*JRE4*) and *GLYCOALKOID METABOLISM 1 GAME1*). The top panel represents phytohormones and gene expression induced in tomato roots by the caterpillar *Spodoptera exigua* on plants of different ages (4.8, 6.2, and 8 weeks). The bottom panel represents phytohormones and gene expression induced in tomato roots by the root-knot nematode (RKN) *Meloidogyne incognita* at different root infection cycle stages (invasion stage estimated at 5 days post nematode inoculation (dpi), galling stage estimated at 15 dpi, and reproduction stage estimated at 30 dpi). The middle panel shows the effect of *S. exigua* leaf feeding on root responses induced...
by *M. incognita* (MiSe) compared to those infected with *M. incognita* (Mi) alone at 30 dpi (hormonal cross-talk). White boxes: no response, yellow boxes: increase in trait levels or upregulation of gene expression, blue boxes: decrease in traits levels or downregulation of gene expression, and green box: hypothetical involvement. In the proposed hormonal cross-talk schedule in the middle, dotted red lines show negative cross-talk, the non-dotted black arrows show the steps in the JA pathway, and the dashed back arrowed line represents several unknown steps. In our cross-talk model, we propose that the increase in SA affects the JA pathway negatively at the level of the MYC transcription factor. At the same time, the increase in ABA levels blocks the ethylene (ET) pathway, which regulates the ETHYLENE RESPONSIVE FACTOR (ERF) branch of the JA pathway. We hypothesize that the absence of ET promotes the activity of the *JRE4* transcription factor, which enhances transcription of the GAME pathway. Based on the response of the defense marker gene *LapA* in MiSe plants at 30 dpi, we also hypothesize that this pathway leading to late JA responses is involved in the interaction.
Figure 1
Figure 2

(A) Invasion

(B) Invasion

(C) Invasion

(D) Invasion

(E) Galling

(F) Galling

(G) Galling

(H) Galling

(I) Reproduction

(J) Reproduction

(K) Reproduction

(L) Reproduction

Legend:

- Con: Control
- Mi: Treatment 1
- Se: Treatment 2
- MSe: Treatment 3

Significance:

- a: Significantly different from Con
- b: Significantly different from Mi
- ab: Significantly different from Se

Relative expression values are shown in the graphs.
Figure 3

(A) Invasion

(B) Invasion

(C) Galling

(D) Galling

(E) Reproduction

(F) Reproduction
Figure 4

**Spodoptera exigua**

Phytohormones and gene expression induced in roots by *S. exigua* at:

- 4.8 weeks
- 5.2 weeks
- 8 weeks

| 4.8 weeks | 5.2 weeks | 8 weeks |
|-----------|-----------|---------|
| JA, SA, ABA | JA, SA, ABA | JA, SA, ABA |
| α-tomatine | α-tomatine | α-tomatine |
| LoxD, LapA, Le4, GluB | LoxD, LapA, Le4, GluB | LoxD, LapA, Le4, GluB |
| JRE4, GAME1 | JRE4, GAME1 | JRE4, GAME1 |

**Effect of *S. exigua* on *M. incognita* induced response (MiSe vs Mi), at 30 dpi (hormonal cross-talk)**

- SA
- JA
- ET
- ABA
- MYC
- LoxD
- (GluB)
- LoxD
- LapA
- Le4
- GluB
- JRE4
- GAME1
- α-tomatine

**Phytohormones and gene expression induced in roots by *M. incognita* at:**

- (invasion, 5 dpi)
- (galling, 15 dpi)
- (reproduction, 30 dpi)

| Invasion (5 dpi) | Galling (15 dpi) | Reproduction (30 dpi) |
|-----------------|------------------|-----------------------|
| JA, SA, ABA | JA, SA, ABA | JA, SA, ABA |
| α-tomatine | α-tomatine | α-tomatine |
| LoxD, LapA, Le4, GluB | LoxD, LapA, Le4, GluB | LoxD, LapA, Le4, GluB |
| JRE4, GAME1 | JRE4, GAME1 | JRE4, GAME1 |

Colour key: no response, increase or upregulation, decrease or downregulation, hypothetical involvement

Source of root image: Carolina Escobar et al. (2015) and used with the author’s permission.