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The Role of Jasmonic Acid in Plant – Nematode Interaction

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Acknowledgment

To my Lord and Savior, Jesus, my source of everything! His living Word sustains me…

Be strong and courageous. Do not be terrified; do not be discouraged, for the Lord your God will be with you wherever you go – Joshua 1:9

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To my IMaHS colleagues and friends… for the ☺

To my Family, for the love…

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List of Abbreviations

JA= Jasmonic acid

MeJA = Methyl Jasmonate

SHAM = Salicylhroxamic acid

J2/J2's = Second stage juvenile/s

PDF1.2A = plant defensin 1.2A

COI1 = Coronatine insensitive 1

JAR1 = Jasmonate resistant 1

Dai = Days after inoculation

Abstract

Heterodera schachtii Schmidt, has been considered for more than a century the most important nematode pest of sugar beet. In plant–nematode interactions, plant defense responses to parasitic nematodes have the potential to become part of management strategies to increase agricultural productivity. The volatile MeJA was known to activate systemic response of plants attacked by harmful pathogens. At certain external stimuli (eg. wounding), the jasmonate biosynthesis is activated leading to gene expression changes and hormone accumulation.

The aim of the current study was to elucidate the role of JA for the *Arabidopsis thaliana – Heterodera schachtii* interaction.

The results revealed that MeJA or SHAM shoot application had no effect on nematode attraction. However, MeJA treatment significantly lowered the infection rate at 24h and 48h after J2 inoculation, as well as, significantly reduced female development, while SHAM shoot application was found to have no significant effects. The quantitative expression analysis on JA-related genes showed a significant down-regulation of *PDF1.2A* in the roots but was up-regulated in the shoots after 24h from J2 inoculation without hormone application. Hormone application showed different gene expression in roots, *COI1 and JAR1* were down-regulated, whereas, *PDF1.2A* was up-regulated. The results of this work clearly show that JA is involved in the *A. thaliana – H. schachtii* interaction and imply that elevated JA levels causes unfavorable condition nematode root infection and development.

Keywords: *Heterodera schachtii, Arabidopsis thaliana,* MeJA, *COI1, JAR1, PDF1.2A*

1 Introduction

1.1. Plant Parasitic nematodes

Plant parasitic nematodes are principally aquatic animals requiring free moisture for activity; they inhabit the moisture films surrounding soil particles and the moist environment of plant tissues. Nearly all plant parasites spend a portion of their life cycle in the soil. Most nematodes are adapted to the specific climates and living conditions of the northerly and southerly latitudes or to high elevations. Spread of nematodes and thereby the damage they cause is typically associated with coarsely textured soil with relative large pore spaces. However, substantial nematode damage has been observed in nearly all soil types. (Bridge and Starr, 2007).

Agricultural importance of plant parasitic nematodes is ranked immediately after insects, fungi, bacteria, and viruses. Plant parasitic nematodes comprise about 15% of the total known nematode species (Wyss 1997). These cause in total approximately 125 billon $ of yield losses worldwide (Chitwood, 2003). They attack all kinds of plant and can infect roots, stems, leaves, crowns, inflorescence, flowers and developing seeds. Nematodes cause about 10-12% yield loss when various crops are considered. The degree of damage to a particular crop is influenced by the plant species or cultivar, the nematode species, level of soil infestation and the prevailing environment around the host and nematode (Khan, 2008). A severe infection may result in as much as 80-90% yield loss in an individual field and sometimes plants fail to give yield reaching the economic threshold (Khan 2008). Plant parasitic nematodes may damage plants directly, causing

disruption or necrosis of infected plant tissues and indirectly creating entry points for other soil born plant pathogens. Further, there are several virus- transmitting nematode species known. Moreover, several nematode species survive on or within planting material, with which they can spread over long distances; therefore, they are included in lists of quarantine pests by many countries (Lamberti et al., 2007).

Plant parasitic nematodes can be conveniently classified based on their mode of parasitism (Bridge and Starr, 2007): (1) ectoparasites - generally the nematodes remain on the surface of the plant tissues, feeding by inserting the stylet into cells that are within reach (e.g. *Anguina spp., Ditylenchus spp.*); (2) migratory endoparasites – all stages of the nematodes can completely penetrate the plant tissues, remaining mobile and vermiform and feeding as they move through tissues; they often migrate between soil and roots(e.g. *Pratylenchus spp., Radopholus spp., Rotylenchus*[some] *spp.*); (3) semi-endoparasites –juvenile nematodes only partially penetrate the roots, leaving the posterior half to two-thirds of the body projecting into the soil(e.g. *Rotylenchus spp., Tylenchus spp.*), and (4) sedentary endoparasites - juvenile nematodes completely enter the plant tissues, develop a permanent feeding site, become immobile and develop adult; females often swell into obese bodies(e.g. *Globodera, Meloidogyne, Heterodera spp.*).

1.1.1 Sedentary endoparasitic sugarbeet cyst nematode: distribution and economic importance

Within the genus *Heterodera* more than sixty-five species have been described (Lamberti et al., 2007). Among them, attention has been given to the most damaging

species, some of which have been known for a long time as economically important agricultural pests, whereas others have only been described and have more restricted distribution (Lamberti et al., 2007). The beet cyst nematode, *Heterodera schachtii* Schmidt, has been considered for more than a century the most important nematode pest of sugar beet (Cooke, 1993). It was observed by Schacht in 1859, while Schmidt described it in 1871 as *Heterodera schachtii* (Evans and Row 1998). This species occurs in all the major sugar beet growing areas, especially in eastern and western Europe and the western USA. It is also present in limited areas of New York and Ontario. The species has also been found on table beet in west Africa (Bridge and Starr, 2007). Furthermore, this species is not only destructive for sugar beets but also attacks a range of *Chenopodiaceae* and *Cruciferae* (Lilley et al., 2005), including *A. thaliana*(Sjimons, 1991).

The economic threshold for *H.schachtii* is 1-2 eggs/g soil, in sandy loam soils. Yield losses can be over 60% but more likely to be 20-30% in mineral and organic soils. Continuous cropping on the same land is the cause of severe nematode population build up and yield loss (Bridge and Starr, 2007). The optimum temperature for egg hatching and development is 25 ^{o}C, and an average of three generations for the sugar beet cycle have been shown on spring-sown crops and up to five on fall-sown crops in southern California (Lamberti et al., 2007).

Plants severely infested with *H. schachtii* suffer from reduced growth and wilting under dry conditions (Figure 1A) leading to plant death, often appearing in patches (Figure 1B) in the field. Invasion and feeding on roots by the infective juveniles causes new, secondary lateral roots resulting in the production of a mass of extra small root, a so

called root-beard (Figure 1C) emerging from the swollen main root. Under these conditions the size of the sugar beet is severely reduced resulting in yield losses (Bridge and Starr, 2007).

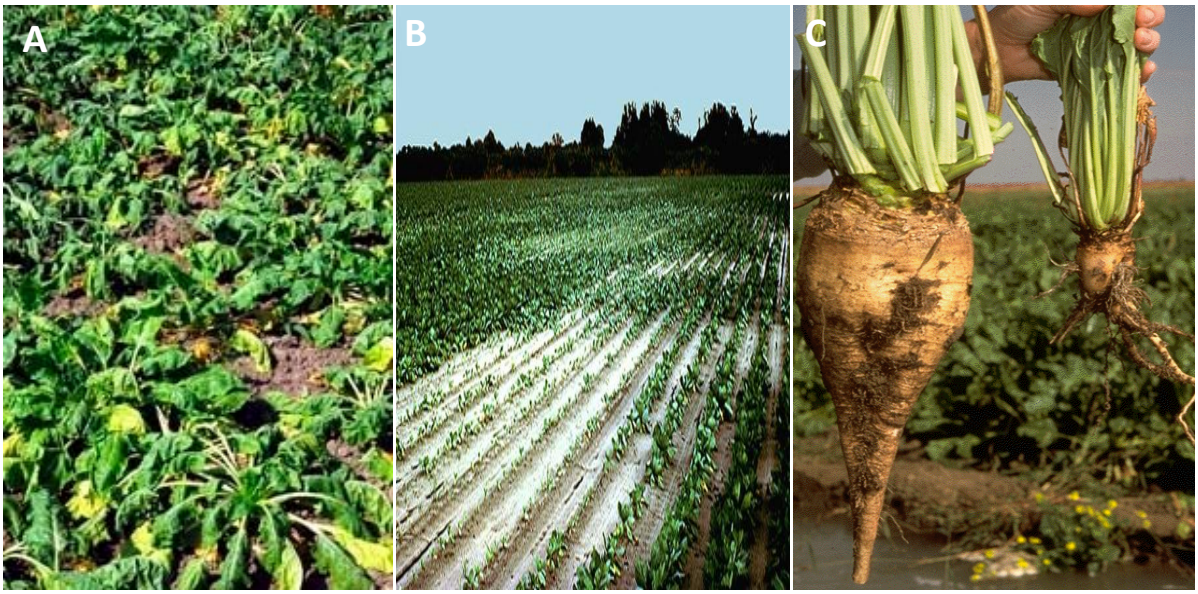


Figure **1A** - Beet plants infested with *H.schachtii* wilting in a field (courtesy of schaeden.rheinmedia.de); **1B** - Severe field damage to beet by *H.schachtii* occurring in patches (courtesy of plantwise.org); **1C** - Root-beard appearance of beet root (right) infected with *H.schachtii* (courtesy of nematology.ucdavis.edu)

1.1.2. Life cycle of a cyst nematode

The cyst nematode has a simple life cycle, generally taking 3-6 weeks (Abad and Williamson, 2010) (Figure 2). The *H. schachtii* shows marked sexual dimorphism, having wormlike adult males and globose or lemon-shaped adult females. The cyst which is the final life stage contains up to 500 eggs and can survive in the soil and on infected plant parts for several years (up to twenty years, depending on the species) (Lamberti et al., 2007). Generally, eggs within cysts are stimulated to hatch only by root exudates of host plants during suitable temperature and soil moisture conditions (Lamberti et al., 2007).

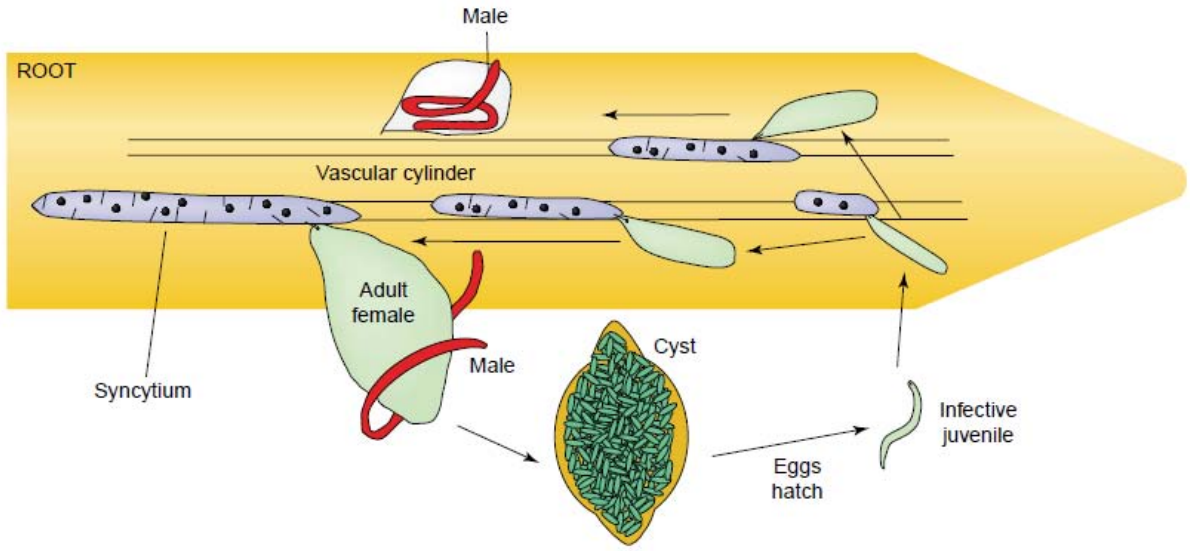


Figure 2. Life cycle of *H.schachtii* cyst nematode (Williamson and Gleason, 2003)

The second-stage juvenile (J2) emerging from the egg is the only infective stage and penetrates young roots near to their tips (Lamberti et al., 2007) (Figure 3A, 3B). Around actively growing roots there exist several gradients of volatile compounds, including amino acids, ions, pH, temperature and CO_2. It is evident that the nematodes use their chemosensory sensilla, the amphids, to orientate towards the roots using at least some of these gradients (Perry and Moens, 2011). Perry (2005) separated gradients into 3 types: 'long distance attractants' that enable nematode to move to the root area, 'short distance attractants' that enable the nematode to orientate to individual roots, and 'local attractants' that are used by the endoparasitic nematodes to locate the preferred invasion site. The orientation of cyst nematodes to the preferred invasion site, the root tip, is well established but the active factors that constitute the 'local attractants' are unknown. The nematode may orient to an electrical potential gradient at the elongation zone of the root tip but the relative importance of electrical and chemical attractants for

the root tip location has not been evaluated; in addition the elevated temperature at the zone of root elongation may influence nematode perception (Perry and Moens, 2011).

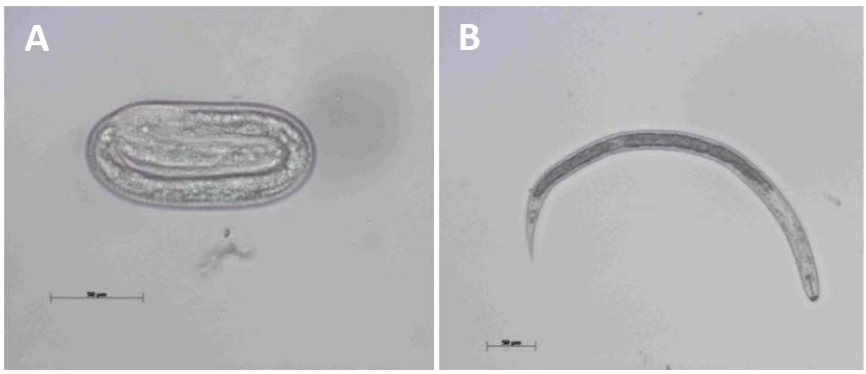


Figure **3A** - Egg of *Heterodera schachtii ;* **3B** - freshly hatched J2 (Courtesy of Kammerhofer, unpublished data, 2011)

After locating a suitable host the J2 uses a combination of physical and chemical means to penetrate the root. Thrusts of the stylet are used along with a set of endogenous cell wall degrading and modifying enzymes to weaken cell walls as the nematode enters the host cells to migrate intracellularly (Sobczak and Golinowski, 2011). During migration in the outer root tissue the juvenile behaves very destructively. Stylet thrusts are quick and numerous and the head movements are very rapid and vigorous. This behavior changes and becomes more delicate and exploratory when the J2 approaches the vascular cylinder. The J2 probes to each cell until a suitable cell that does not respond to J2 probing is found. Once migration is complete, the nematode chooses a cell that will become the initial syncytial cell (ISC) (Sobczak et al., 1999).

After selection of an ISC, the J2s enters a preparation phase which lasts for approximately 7 h. During this time no secretion emanates from the stylet orifice and the metacorpal bulb is motionless. The activity of oesophageal glands also changes: the

number of granules in the ampullae and extensions of both subventral glands decreases whereas that in the dorsal gland increases (Wyss, 1992). When the preparation phase is complete the stylet is withdrawn and reinserted. This time secretions from the nematode oesophageal gland cells are injected into the cytoplasm of the ISC and the metacarpal bulb begins to pump. After this injection of secretions the juvenile begins to withdraw food from the selected cell. It withdraws food in cycles lasting for few hours and consisting of three distinctive stages. During the first stage, lasting for 1 – 2 h, the stylet is inserted into the syncytium and the nematode withdraws food; in the second stage the stylet is withdrawn and reinserted; and in the third stage nematode gland secretions are injected into the syncytium where they form a feeding tube (Wyss and Zunke, 1986).

Once feeding commences the nematode continue development. The J2's undergo three moults to the third, fourth, and adult stages (Golinowski et al., 1996). The infective J2 is sexually undifferentiated (Sobczak and Golinowski, 2011), which means the sex chromosomes are absent (reviewed by Perry and Moens, 2011). Sex is determined by environmental conditions, with frequency of males in unfavorable conditions (Triantaphyllou, 1985). The first difference in the organization of the genital primordium indicating the sex of the juvenile appear at the end of the J2 stage shortly before the moult to the J3 (Wyss, 1992). The J4 male (Figure 4A) and adult male revert to the vermiform body shape, at this stages they do not take up food from the syncytium but use energy reserves stored as lipid droplets in their bodies. Then, it leaves the root and seeks for females to fertilize. The female remains sedentary, continues to withdraw food until the shape of its body becomes spherical (Figure 4B, 4C). After mating the female

starts to produce fertilized eggs. The syncytium remains functional for as long as the attached nematode continues feeding (Golinowski et al., 1996). Müller et al. (1982) cited by Perry and Moens (2011) calculated that during development the total food consumption of a female was 29 times greater than that of a male. When a female cyst nematode dies, its body wall forms a protective enclosure for the eggs. The first-stage juvenile (J1) moults within the egg to produce the J2, which hatches under favorable conditions.

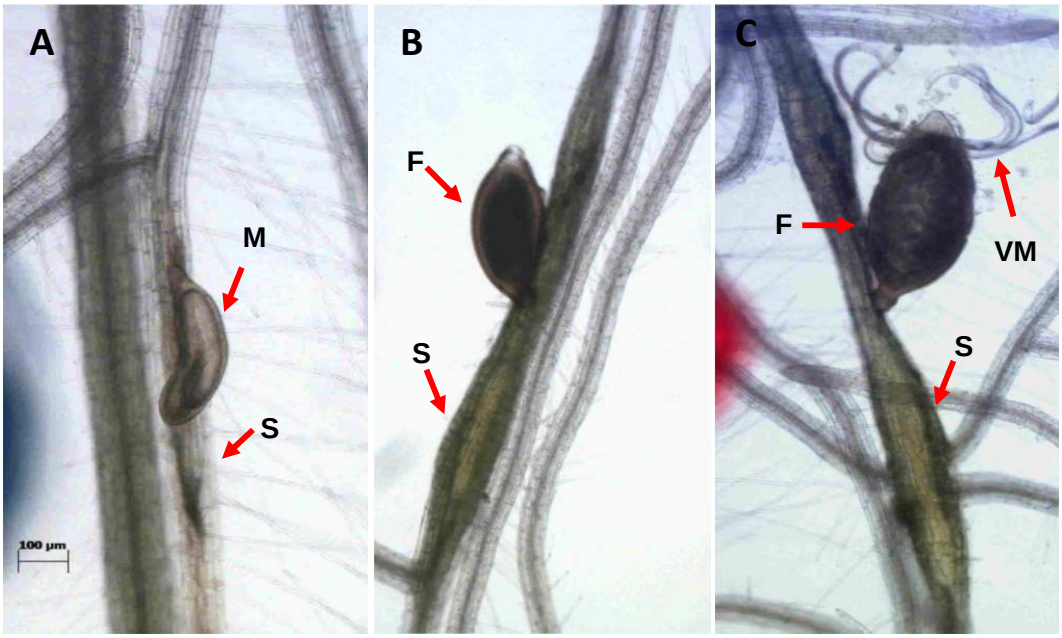


Figure 4A – male (M); 4B – female (F); 4C - vermiform males (VM) surrounding the lemon-shaped females (F), syncytium (S) (Courtesy of Kammerhofer, unpublished data, 2011)

1.1.3. Management practices

Heterodera species have notorious history of global dispersal that is enhanced by their ability to withstand desiccation in the protective cyst stage (Szalanski et al., 1997) and are thus difficult to control.

Over the years a number of soil fumigants have been tested and used to suppress cyst nematodes. Soil fumigation with 1,3-D or dazomet gives very goods yield increases of sugarbeet in infested soil, also non-fumigant chemicals such as aldicarb or oxamyl (Lamberti et al., 2007). However, the use of synthetic nematicides are becoming less and less acceptable in many countries (Bridge and Starr, 2007).

With the restriction on the use of synthetic nematicides, interest in the development of safe, sustainable and economically viable nematode management strategies has increased. One such strategy includes the use of 'biofumigants', referred to as the release of volatile breakdown products, like isothiocyanates from *Brassica* roots suppressing soil-borne pest and pathogens (Ploeg et al., 2007). These crops are used in crop rotation or intercropping schemes.

Nematicidal *Brassicaceae* can accumulate the majority of glucosinolates either in the root system (catch effect) or in the stems and leaves (biofumigant effect). The first process is the most suitable to control cyst nematodes. Brassicaceae catch crops attract the juvenile stages of endoparasitic nematodes working as a trap, since these, after root penetration, are poisoned by hydrolysis products and are not successful in completing their developmental cycle in 10–12weeks, that is the intercropping time. The nematicidal effect of a catch crop is produced during the whole cultivation time, while its incorporation as green manure shows an overall amendment effect, increasing the organic matter amount and improving soil fertility, being the biofumigant effect during incorporation only secondary (Curto, 2007).

The definition of either resistant or tolerant sugar beet variety was recently described. A resistant variety is able to limit the nematode reproduction, while a tolerant variety is able to decrease the productive losses, if compared with a susceptible one. The selection of sugar beet genotypes tolerant to *H. schachtii*, achieved only recently interesting productive performances. The new genotypes derive from crosses between cultivated selections of sugar beet (*Beta vulgaris L. ssp. saccharifera*) and spontaneous species, such as *Beta maritima* and *Beta procumbens*, both carriers of resistance genes to the cyst nematode (Curto, 2007).

Varieties of fodder radish (*Raphanus sativus var. oleiformis* Pres.) and yellow mustard (*Sinapis alba L*.), have been selected for its resistance to *H. schachtii* and their ability to reduce *H. schachtii* soil populations (Krall et al., 2000) are used as green crop in sugar beet rotations. A resistant trait has been successfully transferred to rapeseed (Budahn et al., 2009).

In plant–nematode interactions, plant defense responses to parasitic nematodes have the potential to become part of management strategies that increase agricultural productivity. Both constitutive and induced defense mechanisms contributing to disease resistance are observed in plants and a range of secondary metabolites is induced in plants after nematode invasion. Induction of phytoalexin synthesis in the roots in response to nematode parasitism has been recognized (Soriano et al., 2004).

1.2. Plant defense mechanisms

Plants protect themselves against harmful organisms applying basal and inducible defense mechanisms. Such mechanisms include among others the formation of cell wall polymers, secondary metabolites and proteins with antimicrobial activities. Local defense responses are triggered by recognition of elicitors derived from the invaders. Subsequently, signaling compounds are initiated, which spread systemically throughout the plant tissues from the infection site, eventually, an increased resistance to secondary infections in distal tissues is also established. This spread of resistance is collectively termed ‘systemic acquired resistance’ (SAR) (Hamamouch, 2011).

Towards the early 19th century, Wiesner (1892) as cited by (Koo and Howe, 2009) worked on the idea that plants actively respond to tissue injury dates. During that time, a so-called wound hormone “traumatin” was purified from the bioassay. The discovered hormone exhibits mitogenic properties related to wound healing. Traumatin is a reactive fatty acid derivative (12-oxo-*trans*-10- dodecenoic acid) produced by the hydroperoxy lyase branch of the lipoxygenase pathway. Apparently, the activity of traumatin was limited only to specific bean cultivars, so the concept of a wound hormone was put aside. However, this early work helped to regain interest in biochemical research on lipoxygenase-based pathways for oxidative metabolism of polyunsaturated fatty acids. Today, the concept that biotic agents activate local and systemic host defense responses is a cornerstone of current theories of plant immunity. Oxylipins play a central role in many of these defense signaling pathways. In both plants and animals, oxylipins derived from membrane lipids are sentinels of wound stress. These signaling

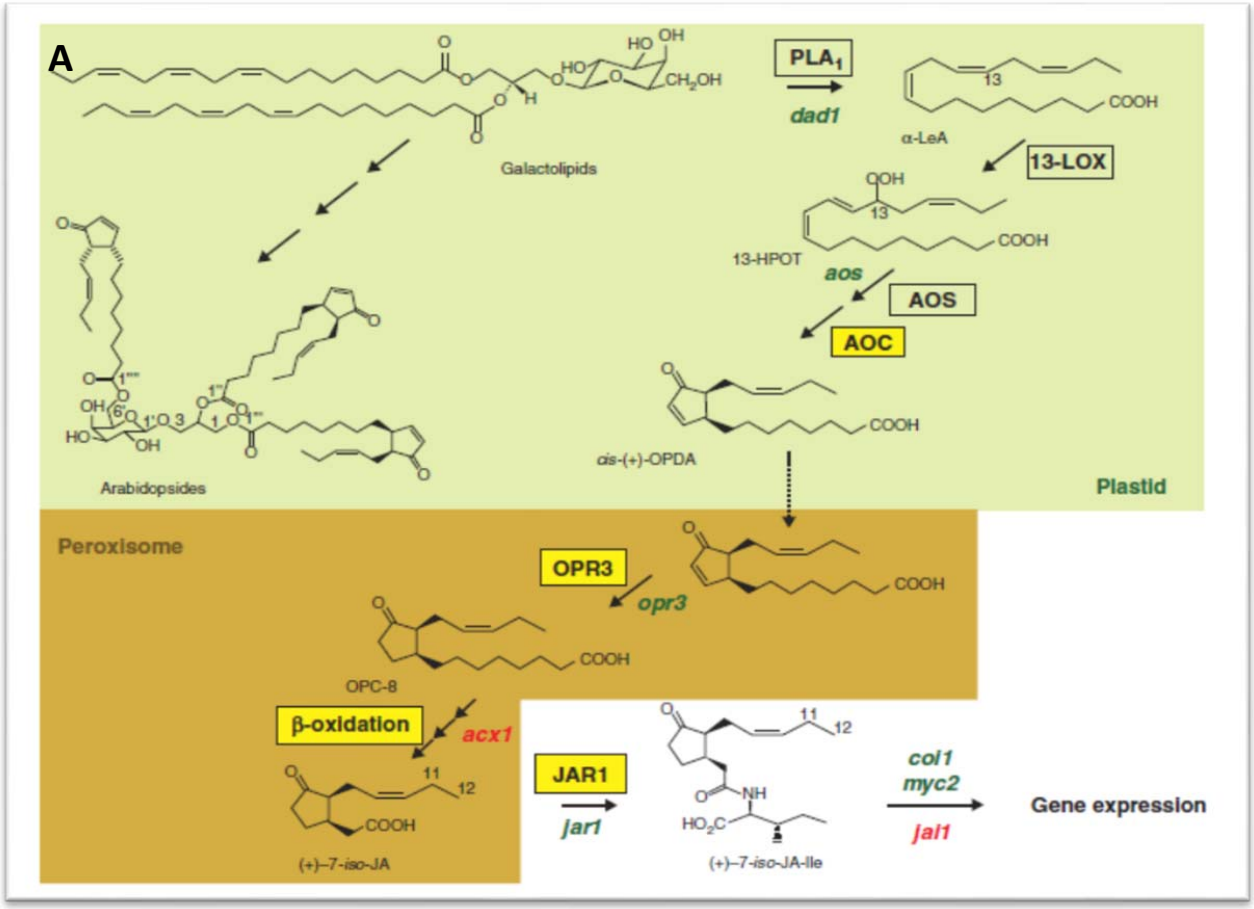
compounds are typically synthesized *de novo* in specific cell types upon activation of lipases that release fatty acids from membrane lipids (Koo and Howe, 2009).

1.3. Jasmonic acid biosynthesis and signaling

Jasmonic acid (JA), and its methyl ester (methyl jasmonate, MeJA) are members of the oxylipin family. These are linolenic acid (LA)-derived cyclopentanone-based compounds that are widely distribution in the plant kingdom (Creelman and Mullet, 1997). Figure 5A shows the reaction steps, names of enzymes and substrates involved in JA biosynthesis (Wasternack and Hause, 2013). The fatty acid substrate of JA biosynthesis is α-linolenicacid (18:3) (α-LeA) released from galactolipids of chloroplast membranes. It is generally accepted that a phospholipase1(PLA1) releasing α-LeA from the *sn1* position of galactolipids is responsible for the generation of JA. Oxygenation of α-LeA is the initial step in JA biosynthesis. The oxygen has to be inserted in the C-13 position by a lipoxygenase (LOX). Among the six *LOX* genes of *Arabidopsis*, four of them are 13-*LOX*s (*LOX2*, *LOX3*, *LOX4*, *LOX6*), their functions are still only partly understood. *LOX2* was suggested to be involved in the wound response for a long time and subsequent studies revealed that *LOX2* was responsible for the bulk of JA formation in the first hours upon wounding (Glauser et al., 2009)). Enzymes in JA biosynthesis such as LOX, allene oxide synthase (AOS) and allene oxide cyclase (AOC) are partially associated with chloroplast membranes. For AOS the level of the protein within the envelope is affected by rhomboids, a family of intra-membrane serine proteases of inner envelope membrane (Farmaki et al., 2007). A positive feedback loop of JA biosynthesis is currently suggested by the SCF^{COI1}–JAZ regulatory module that is known to be active in the expression of *LOX, AOS, AOC, OPR3* and *ACX* (Knopf et al., 2012). The cloning

of JAR1 as a member of the *GH3* gene family (Staswick and Tiryaki, 2004), which belongs to the large group of enzymes formingacyl-adenylate/thioester intermediates, was a breakthrough in the JA field. This enzyme catalyses the final step in the formation of the bioactive JA compound.

JA is further catabolized by JA carboxyl methyltransferase (JMT) to form its volatile counterpart MeJA (Figure 5B) (Cheong and Choi, 2003).



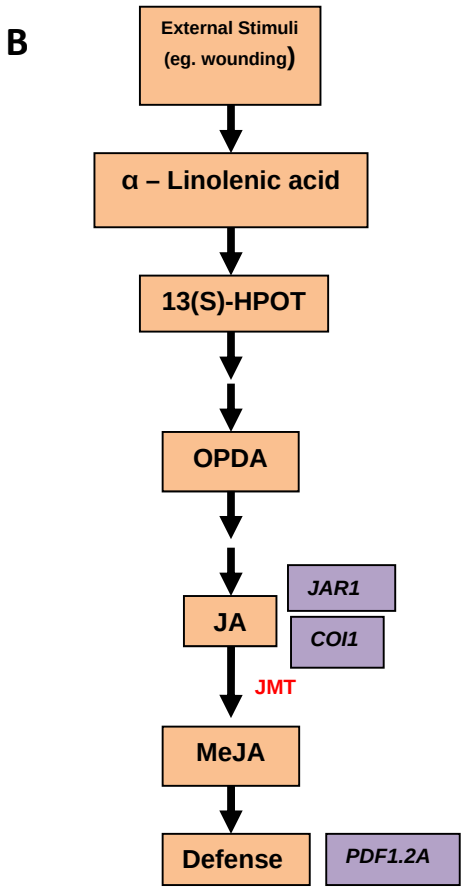


Figure **5A** - JA Biosynthesis of jasmonic acid (JA)/JA-Ile from a-linolenic acid generated from galactolipids. Enzymes have been crystallized are given in yellow boxes. Steps impaired in mutants of Arabidopsis (green) or tomato (red) are ed. acx1, acyl-CoA-oxidase1; AOC, allene oxide cyclase; AOS, allene oxide synthase; coi1, coronatine insensitive1; delayed anther dehiscence1; 13-HPOT, (13S)-hydroperoxyoctadecatrienoic acid; jai1, jasmonic acid insensitive1; JAR1, no acid synthetase; a-LeA, a-linolenic acid; 13-LOX, 13-lipoxygenase; myc2, bHLHzip transcription factor MYC2; OPR3, reductase3; OPC-8, 3-oxo-2-(2-pentenyl)-cyclopentane-1-octanoic acid; cis-(+)-OPDA; cis-(+)-12-oxophytodienoic acid; phospholipase A1 (Wasternack and Hause, 2013); **5B** – MeJA biosynthesis and the genes expressed in the pathway.

The role of JA for plant defense was first described by Vijayan et al. (1998) showing that the *Arabidopsis* JA-deficient triple mutant *fad3/fad7/fad8* was killed by *Pythium jasmonium* (previously *Pythium mastophorum)* whereas neighboring wild-type plants remained healthy. Some remarkable features of plant responses, such as production of repellent volatiles against herbivorous insects, or the massive transcriptional reprogramming in response to wounding, are under the control of the JA pathway (Stinzi

et al., 2001). This transcriptional reprogramming included a considerable numbers of front-line defense-related genes encoding wound-related and pathogenesis-related proteins (Liechti and Farmer, 2002). Accordingly, MeJA application to roots of oat (*Avena sativa*) and spinach (*Spinacia oleracea*) and to shoots of tomato (*L. esculentum*) enhanced resistance to root-knot nematodes (RKNs). Similar to these studies MeJA was shown to be an effective elicitor of systemically induced defense mechanisms against migratory root-rot nematodes RRNs in rice roots (Nahar et al., 2012). Further, application of exogenous JA significantly protected mutant plants and reduced the incidence of disease to a level close to that of wild-type controls (Browse, 2009). Some studies showed that exogenous application of JA or MeJA can further promote senescence and act as a growth regulator. In addition, other research described the role of JA in vegetative development, fruit development, and pollen viability (Creelman and Mullet, 1997).

The accumulation of JA in response to wounding and pathogen elicitors has been proposed to alter wound-induced expression of several genes (Bell et al., 1995) (Table 1). Changes in gene expression underlying inducible responses to pathogens and herbivores are known to be multifarious, and were suggested as pattern of multiple, independent, but networked defense response pathways (Moran and Thompson, 2011).

Next to JA biosynthesis the effect of JA is dependent on downstream signaling pathways. In these, several genes are involved such as the CORONATE-INSENSITIVE 1(*COI1*), which is the F-box subunit of anE3 ubiquitin ligase of the type SKP1-CUL1-F-box (SCF).When hormone concentrations are low or undetectable, JAZ proteins bind transcription factors such as MYC2 and thus impair the transcription of jasmonate-

responsive genes. In case of induced JA biosynthesis increased hormone levels are perceived by *COI1* what favors binding of COI1 to JAZ proteins via their Jas motif. This promotes ubiquitination of JAZ proteins and their subsequent degradation by the 26S proteasome. The transcription factors are relieved from JAZ-mediated repression and free to recruit the RNA polymerase transcriptional machinery to the promoter of jasmonate-responsive genes. Thus, COI1 is essential for transcription of JA-responsive genes (Yan et al., 2009).

The JASMONATE RESISTANT 1 (JAR1) locus encodes an enzyme that conjugates JA to isoleucine, which was recently shown to function directly in COI1-mediated signal transduction. JAR1 is essential for pathogen defense, but was shown to play a minor role in transcriptional modulation of genes induced by mechanical wounding (Suza and Staswick, 2008).

The *PDF1.2* gene of Arabidopsis encodes a JA-responsive pathogen defense genes. Its expression was described to be induced by pathogen challenge both locally at the site of inoculation by incompatible fungal pathogen and systemically in remote non-inoculated regions of the plant (Penninckx et al., 1996). The activation of this gene occurs via a jasmonate/ethylene-mediated signaling pathway, rather than via the SA-dependent pathway of defense gene activation. This gene is commonly used as a marker for characterization of jasmonate-dependent defense responses (Brown et al., 2003).

Table 1. *A. thaliana* genes used for the current gene expression analysis.

Gene*	Biological process, Involved in	Cellular component located in	Plant structure, Expressed in
COI1 (Coronatine insensitive 1)	defense response, jasmonic acid and ethylene-dependent systemic resistance, jasmonic acid mediated signaling pathway, response to jasmonic acid stimulus, response to wounding,signal transduction, ubiquitin-dependent protein catabolic process	Nucleus	leaf apex, leaf lamina base, petiole, root, shoot apex, shoot system, stem,vascular leaf
JAR 1 (JASMONATE RESISTANT 1)	induced systemic resistance, jasmonic acid mediated signaling pathway, jasmonic acid and ethylene-dependent systemic resistance, jasmonic acid metabolic process, negative regulation of defense response, response to jasmonic acid stimulus, response to wounding, systemic acquired resistance	Cytoplasm, vacuole nucleus	leaf apex, leaf lamina base, petiole, root, shoot apex, shoot system, stem, vascular leaf
PDF 1.2A (PLANT DEFENSIN 1.2A)	defense response, jasmonic acid and ethylene-dependent systemic resistance,response to ethylene stimulus, response to insect, response to jasmonic acid stimulus	Cell wall, extra cellular region	leaf apex, leaf lamina base, petiole, root, shoot apex, shoot system, stem, vascular leaf

Source: * The Arabidopsis Information Resources (TAIR, http://www.arabidopsis.org)

Aim of this study

Plant–nematode interactions research paved a way to study hormone-based plant defense signaling especially in root tissues (Nahar et al., 2012). The distinct host range of the sugarbeet cyst nematode *H. schachtii* has been exploited to use the interaction with *Arabidopsis thaliana* roots as a model system. Its translucent roots growing on artificial media have made it possible to study the behavior of the nematodes inside the root. Further, the *Arabidopsis–H. schachtii* model system has been used extensively for gene expression analyses and for functional studies of diverse Arabidopsis mutants (Mazarei et al, 2004).

Thus, the aim of the study was to elucidate the role of JA for the *Arabidopsis – H. schachtii* interaction. Therefore, two major questions were studied:

1. Does MeJA application affect nematode attraction, infection and development?
2. Does nematode infection trigger expression of genes involved in JA signaling and biosynthesis?
3. Does MeJA shoot application modulate these potential gene expression changes?

a. Structure of the Study

Plant Material	*A. thaliana* (Col 0)
Attraction tests	Percentage of J2s attracted to root exudates of control or hormone treated plants
Infection tests	Infection rate of J2s 24, 48, 72h after inoculation in control and hormone treated plants
Development tests	Percentage females and males developed 15 days after hormone application on control and hormone treated plants
Gene expression analysis	Gene expression analysis (Quantitative RT-PCR) of the target genes *COI1, JAR1, PDF1.2A* in control, nematode-infected and hormone-treated plants

2 Materials and Methods

2.1. Cultivation of *A. thaliana*

In the following experiments *A. thaliana* wild-type (Col 0) was used as the test plants.

Seed sterilization

Sterilization of *A. thaliana* (Col 0) seeds was performed under sterile conditions in a laminar flow hood. The seeds of about 1/16 the volume of a1,5mL-Eppendorf tube was sterilized for 8 minutes using a sterilization solution of 1:5:4 ratio NaClO (bleach):70% EtOH:sterile d.H_2O, respectively. Then the seeds were washed twice with 70% EtOH, and finally, washed thrice with sterile d.H_2O. The seeds were poured evenly into a sterile filter paper for drying.

Media preparation

For 1L of 0.2 Knop media, the following were prepared as described by (Sijmons et al., 1991):

- Daichin agar----------8.0g
- Sucrose----------20.0g
- B5 (Gamborg)----------1.0ml
- StocksolutionI----------2.0ml
  II----------2.0ml
  III----------2.0ml
  IV----------0.4ml
  V----------0.2ml

(composition of stock solution can be referred to appendix)

pH was adjusted to 6.4.

Planting

Ten sterilized seeds of *A. thaliana* (Col 0) were sown in two rows in Petridishes (Ø = 9 cm) containing 0.2% Knop media and cultivated under axenic conditions. The plates were placed in the growing chamber with controlled conditions (16h light/ 8h darkness at 21 ^{o}C) for 11 days. When roots started to grow, the Petridishes were arranged in a nearly vertical position (Figure 6A) to stimulate a downward root growth (Figure 6B).

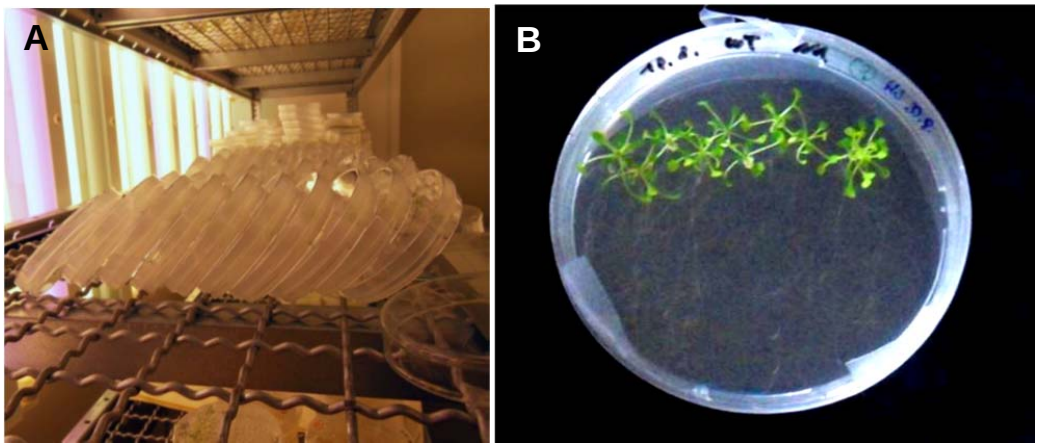


Figure **6A** - Petridishes in the growth chamber; **6B** - *A.thaliana* plants in a Petridish.

2.2. *Heterodera schachtii* second-stage juveniles (J2s) Cultivation and Hatching

Approximately 300 cyst of *H. schachtii* were collected from the nematode stock culture maintained on mustard plant *cv. "Albatros."* The cysts were put into a sterile 100 µm sieve inside a beaker containing 3mM $ZnCl_2$ solution (Figure 7). This was done under aseptic condition in the sterile bench. The beaker with the cyst was covered with aluminum foil, sealed with parafilm and was placed in a dark chamber. Second-stage juvenile (J2) were released from the cyst 2-3 days later.

Prior to inoculation, the J2s were sterilized with 0.05% $HgCl_2$ solution for 2-3 minutes on a 20µm sieve inside a beaker, and subsequently washed 3-4 times with sterile d.H_2O (Figure 7). Using a sterile pipette, the sterilized J2's were transferred to a staining block and a 0.7% gelrite was added.

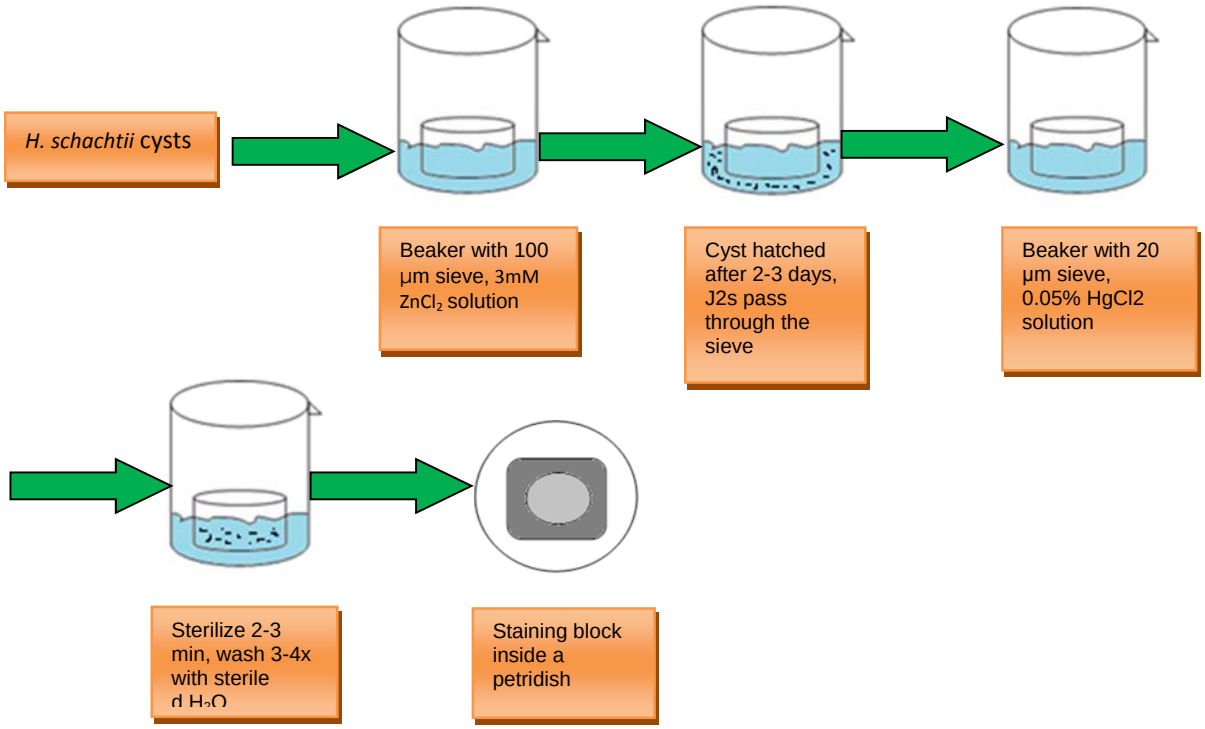


Figure 7. *H. schachtii* (J2's) hatching and sterilization

2.3. *Heterodera schachtii* Attraction Test

2.3.1. Preparation of attraction test arenas

Water agar (0.2%) was poured into Petridish (Ø = 6 cm). Before it solidified, a solid cylinder (2.4 cm long) was placed in the middle of the plate to form a mold for the tunnel. After the water-agar solidified an improvised well was created displacing an agar-disc with a 1ml pipette tip at both ends of the tunnel. The wells and the tunnel were

filled with water before placing the exudates and J2's in order to facilitate the J2's mobility.

2.3.2. Preparation of exudates

Eleven day-old *A. thaliana* plants were treated with hormone (MeJA), hormone inhibitor (SHAM) or water (control) (for details see section 3.4). 24h later root exudates were sampled by collecting agar discs with a 20µl pipette tip from areas where most of the roots were located and carefully placed inside the wells of the attraction test arenas (Figure 8). In the wells the following treatments were compared: MeJA vs. control; SHAM vs. control; MeJA vs. SHAM. The J2's were placed directly in the middle of the tunnel. The arenas were kept in darkness by covering with an opaque material. After 4h, the number of J2's in both wells was counted under the microscope (Olympus SZ51). All tests were replicated 3 times.

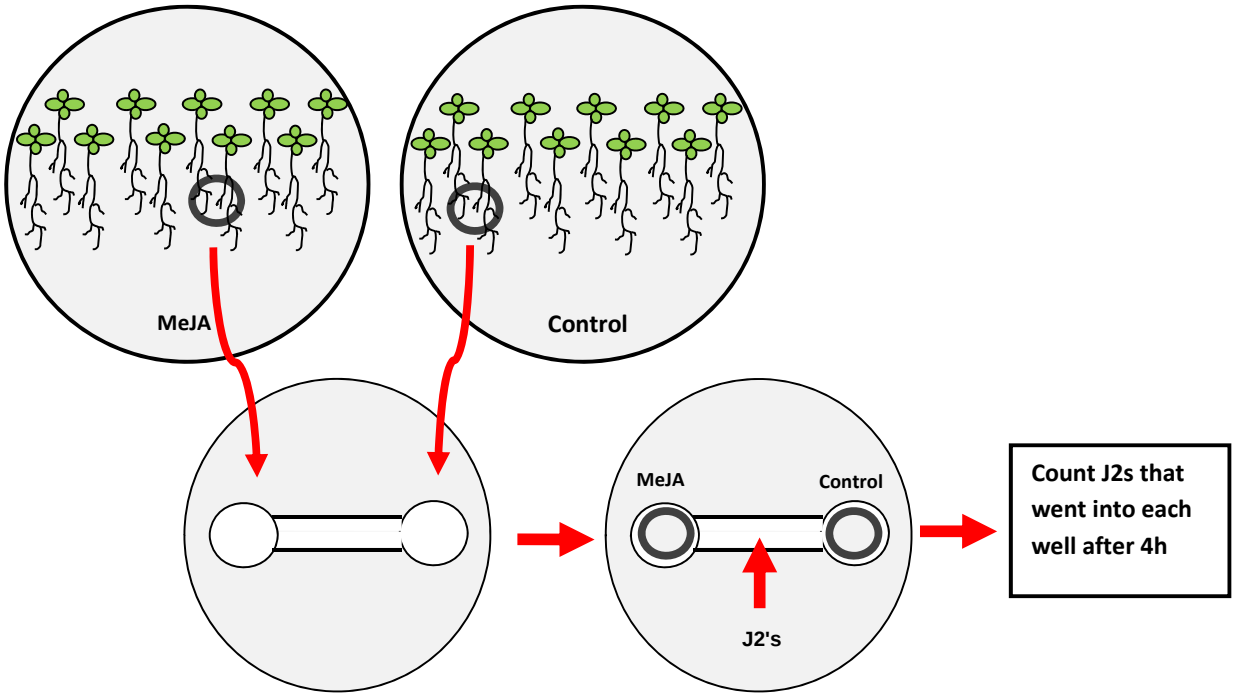


Figure 8. Attraction test

2.4. *Heterodera schachtii* Infection Assay

Eleven-day old *A. thaliana* seedlings were treated with hormone, inhibitor or control by carefully dropping two times 3µl/leaf (6µl/plant) solution on top of the leaf avoiding it from dripping to the medium to ensure that it was absorbed by the plant (Figure 9):

The following concentrations were applied:

- MeJA – 60 *µ*M
- Control (for MeJA) - H2O + 0.1% EtOH + 0.02% Tween 20
- SHAM – 150 *µ*M
- Control (for SHAM) – H2O + 0.02% Tween 20

After hormone application, the roots were classified using a rating scheme (see Appendix). The seedlings were placed back to the growing chamber, laid on a horizontal position and were covered with white paper preventing direct exposure to light.

2.4.1. J2 infection rate

24 hours after hormone application, the roots were inoculated with approximately 50 freshly hatched J2s per plant. The plates were put in the dark chamber for 24 h and were then put back into the growing chamber and covered with white paper. At 24, 48 and 72h after inoculation, the infection sites on the roots were counted under the microscope (Olympus SZ51).

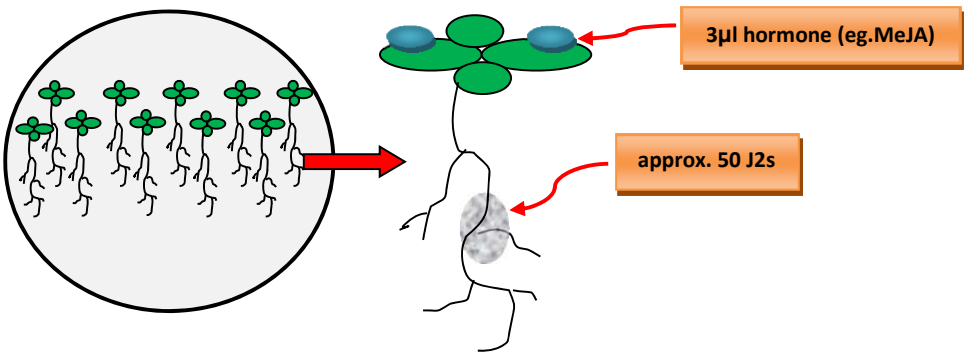


Figure 9. Hormone application and J2 inoculation

2.4.2. Female and male nematode development rate

Fifteen days after J2 infection, the numbers of males and females (Figure 4A, 4B) that developed were counted under the microscope (Olympus SZ51). All tests were replicated 3 times.

2. 5. Gene Expression Analysis

2.5.1. RNA extraction and cDNA synthesis

Shoot and root samples of treated and non-treated as well as inoculated and non-inoculated plants were sampled and immediately shock frozen in liquid nitrogen. Frozen plant material of approximately 100 mg was used for RNA extraction using a RNeasy® Plant Mini Kit (Qiagen, http://www.qiagen.com/) according to the manufacturer's protocol, including DNase I (Qiagen) digestion. The gained RNA concentration was measured using the Nanodrop 2000/2000c Spectrophotometer (Thermo Fisher Scientific). For cDNA synthesis, Superscript III reverse transcriptase (Invitrogen™,

www.invitrogen.com) and random primers (oligo(dN)6) according to the manufacturer's instructions were used, and amplified using a Peltier Thermal Cycler (MJ Research).

2.5.2. Primer design and establishment

A primer design software was used (Blast® Assembled RefSeq Genomes, http://blast.ncbi.nlm.nih.gov/Blast.cgi) to obtain a suitable forward primer sequence (Table 2). The primers were supplied by Invitrogen™. Reverse and forward primers (*COI1, JAR1, PDF1.2A*) were diluted 10μM. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to test primer efficiencies and specificities.

Table 2. Target genes used in this study and their primer pair used for qRT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
COI1	CGAGACAAGGAATGTAATCGG	TGAGCCAAAGCGATTAATCC
JAR1	GCTACATTTGCTGTGATTCCG	GGTATCGATACAACCCTGCG
PDF1.2A	TTGTTCTCTTTGCTGCTTTCG	TTTCCGCAAACCCCTGAC

2.5.3. Quantitative-PCR

Quantitative PCR was performed using an ABIPrism 7300 sequence detector (Applied BioSystems, http://www.appliedbiosystems.com/). Each quantitative PCR sample contained 12.5 μl Platinum SYBR Green qPCR (Invitrogen™),2μl cDNA, 0.5 μl forward and reverse primers (10 μM), and distilled water was added to obtain a 25 μl total reaction volume. All samples were tested as 3 biologically independent replicates and were tested in technical triplicates. Distilled water with no cDNA was used as a control

to rule out false-positives. In addition, dissociation runs were performed to control the possible formation of primer dimers. *The UBP22* was used as an internal reference. Samples were diluted 1:2 and 1:50 for *UBP22*. The QPCR conditions were 95°C for 10mins, 95°C for 15sec, 60°C for 1min (40 cycles). Results were analyzed using the Applied BioSystems7300 Real Time PCR systems. Relative expression was calculated using the ΔΔCt method.

2.5.4. Experimental set-up

Gene expression of *COI1, JAR1* and *PDF1.2A* were tested in a 13-day old *A. thaliana* roots and shoots according to Table 3.

Table 3. Gene expression analysis on *A. thaliana* (Col 0) genes involved in plant signaling and defense

Gene expression analysis	**Plant tissue analyzed**	**Without hormone applied (-)**		**With hormone applied (+)**	
		ni (-)	**Inf (+)**	**ni (-)**	**Inf (+)**
1. Effect of *H.schachtii* infection on the expression of *COI1, JAR1, PDF1.2A* genes	Roots				
2. Effect of *H.schachtii* infection on the expression of *COI1, JAR1, PDF1.2A* genes	Shoots				
3. Effects of MeJA applied on the leaves on the expression of *COI1, JAR1, PDF1.2A* genes	Roots				
4. Effect of MeJA applied on the leaves on the expression of *COI1, JAR1, PDF1.2A* genes in non-infected roots	Roots				
5. Effect of MeJA applied on the leaves on the expression of *COI1, JAR1, PDF1.2*A genes in infected roots	Roots				
6. Effect of MeJA applied on the leaves on the expression of *COI1, JAR1, PDF1.2*A genes in infected roots	Roots				

The set-ups for the qRT-PCR were performed as shown in Table 4 and Table 5.

Table 4. Lay-out of a qPCR plate on root and shoot samples of infected and non-infected *A. thaliana* (Col 0) plants (n=3).

COI 1	***JAR 1***	***PDF1.2A***	***UBP***
ni	ni	ni	ni
ni	ni	ni	ni
ni	ni	ni	ni
inf	inf	inf	inf
inf	inf	inf	inf
inf	inf	inf	inf
Ntc	Ntc	Ntc	Ntc

ni – non-infected; inf – infected; Ntc - blank

Table 5. Lay-out of a qPCR plate on root samples of infected and non-infected and treated and non-treated *A. thaliana* (Col 0) plants. (n=2).

COI 1	***JAR 1***	***PDF1.2A***	***UBP***	***COI 1***	***JAR 1***	***PDF1.2A***	***UBP***
MeJA ni	MeJA ni	MeJA ni	MeJA ni	Control ni	Control ni	Control ni	Control ni
MeJA ni	MeJA ni	MeJA ni	MeJA ni	Control ni	Control ni	Control ni	Control ni
MeJA ni	MeJA ni	MeJA ni	MeJA ni	Control ni	Control ni	Control ni	Control ni
MeJA inf	MeJA inf	MeJA inf	MeJA inf	Control inf	Control inf	Control inf	Control inf
MeJA inf	MeJA inf	MeJA inf	MeJA inf	Control inf	Control inf	Control inf	Control inf
MeJA inf	MeJA inf	MeJA inf	MeJA inf	Control inf	Control inf	Control inf	Control inf
Ntc	Ntc	Ntc	Ntc	Ntc	Ntc	Ntc	Ntc

ni – non-infected; inf – infected; Ntc– blank

3 Results

The role of MeJA on *H. schachtii* infecting the *A. thaliana* (Col 0) plant was determined through descriptive methods (Attraction test, infection rate, and female and male development) and molecular analysis (expression of *COI1, JAR1* and *PDF1.2A*).

3.1. Attraction tests

Nematodes use their chemosensory sensilla to orientate towards the roots following existing gradients (eg. volatiles, soluble metabolites, REDOX) surrounding the actively growing roots (Perry and Moen, 2011). In order to study the effect of MeJA or SHAM shoot application on the attractiveness of root exudates for J2s nematode attraction assays were performed. Four hours after exposure the J2s showed no significant preference among treatment contrasts (Figure 10) but tended towards the exudates from the treated plants. Interestingly, comparing SHAM, the inhibitor of lipoxygenase in jasmonate biosynthesis in plant cells (Zhu et al., 2006), and MeJA, movement of the J2s was low, and they only slightly favored the roots of SHAM - treated *A. thaliana* plants.

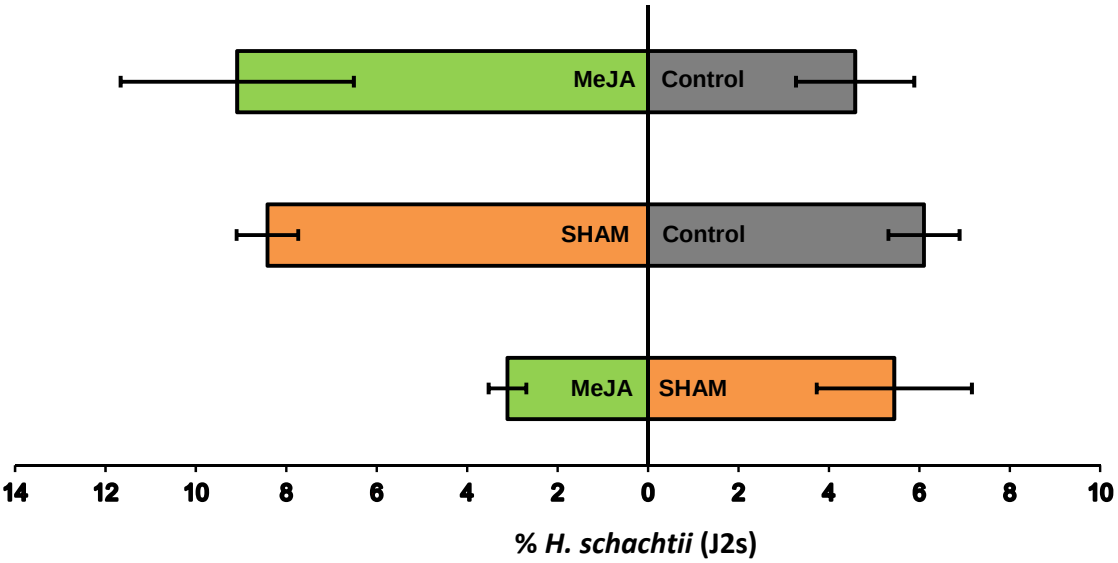


Figure 10.Nematode attraction assay towards root exudates obtained MeJA-treated, SHAM-treated or respective control treated *A. thaliana* plants. Values are means ±SE, n=3, *no significant differences were observed (T-test, $p<0.05$).

3.2. Infection tests

3.2.1. Infection rate 24h after inoculation of J2's

Next to attraction the actual infection activity of the J2's was studied in treated and non-treated plants. In this experiment, 6µl MeJA, SHAM or controls (see Material & Methods) were applied on the leaves11day-old *A. thaliana* plants. 24h later they were inoculated with J2s of *H. schachtii* and again 24h later the infection sites were marked. The results revealed that J2 infection was significantly reduced in MeJA treated plants of more than 4% compared to the control treatment (Figure 11). However, there was no significant difference on the infection rate after 24h on SHAM – treated plants compared to its respective control.

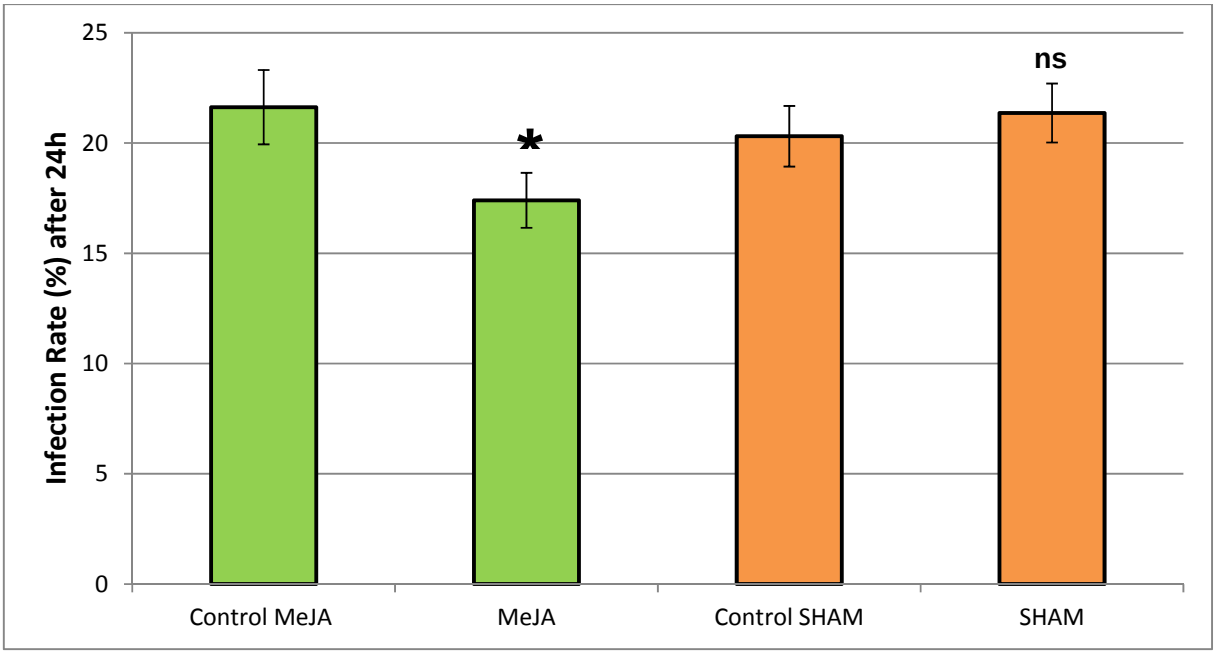


Figure11. Infection rate of *H.schachtii* J2's on *A. thaliana* (Col 0) roots one day after MeJA or SHAM shoot application. Values are means ± SE, n=3,*- significant; ns- no significant difference (P<0.05, T-test).

3.2.2. Infection rate 3 days after J2 inoculation

In the currently applied culture condition the majority of the J2s infect plant roots 3 dai. Thus, the total infection rate on the roots of treated and non-treated *A. thaliana* (Col 0) plants was not only counted at 24h after J2 inoculation but also on the succeeding 2 days at 48h and 72h. The trend line (Figure 12A) shows that J2 infection was significantly inhibited in the MeJA- treated *A. thaliana* plants at 24h and 48h, but balanced at 3dai showing no significant difference between MeJA-treated and the control plants at that stage. Again, there was no difference observed on infection rate on SHAM-treated plants (Figure 12B) at all tested time-points (24, 48 and 72h).

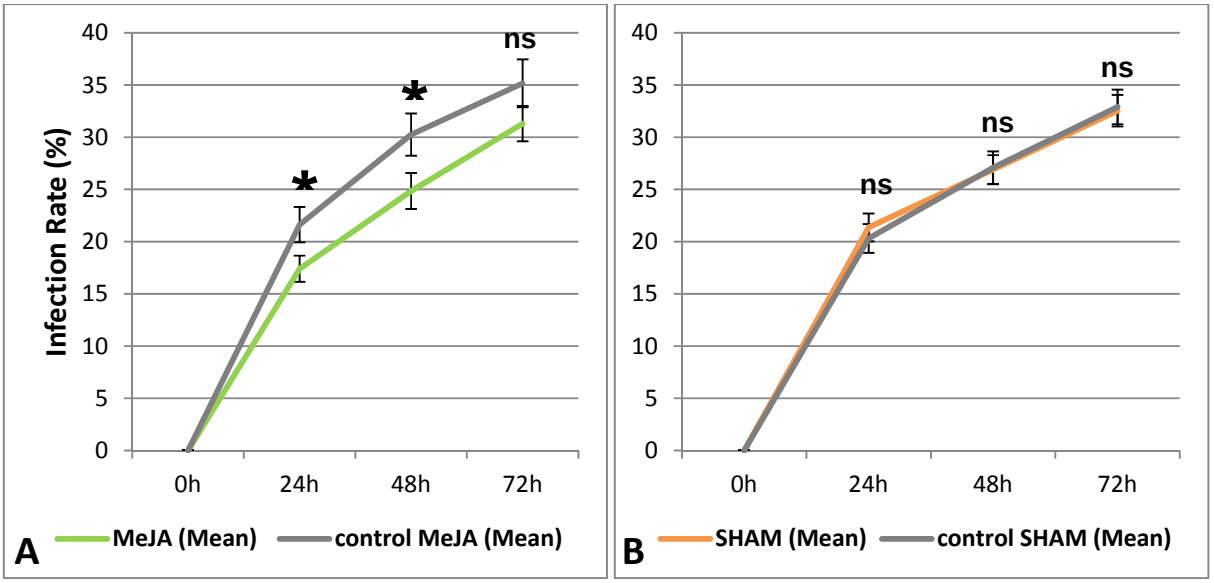


Figure**12A** Infection rate of *H. schachtii* J2s on *A. thaliana* (Col 0)roots 24h, 48h and 72h after MeJA and **12B** SHAM shoot application. Values are means ± SE , n=3, *- significant; ns- no significant difference (P<0.05, T-test).

3.3. Nematode development test

3.3.1. Development rate of both females and males

Two weeks after inoculation *H. schachtii* developed into adult female and male nematodes. Thus, the total development rate of both was counted (Figure 13). In all treatments less than 25% of the inoculated J2s totally developed to both females and males. The results of the infection test revealed no significant differences in nematode development among treatments.

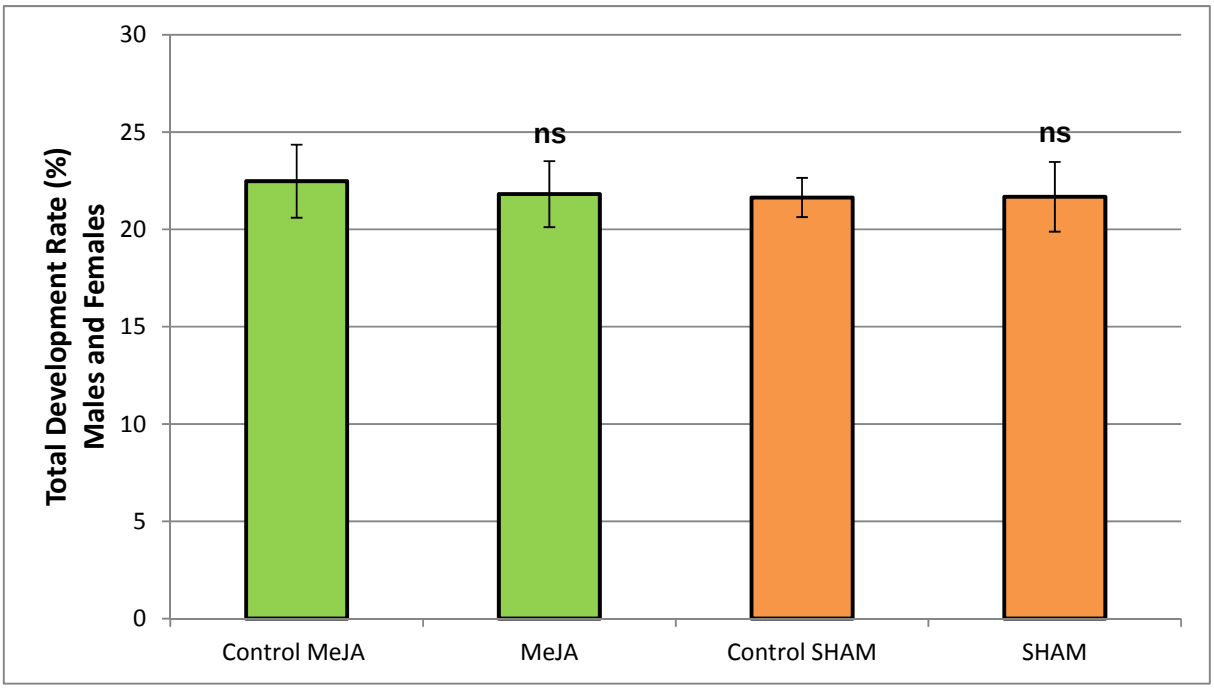


Figure 13. Total development rate of *H.schachtii*on *A. thaliana* (Col 0) roots 15 days after MeJA or SHAM shoot application. Values are means ± SE , n=3, ns- no significant difference (P<0.05, T-test).

3.3.2. Female Development rate (%)

The sexual differentiation of the non-determined J2's was suggested to depend on external factors (Triantaphyllou, 1985). In order to study if MeJA or SHAM shoot application affect living conditions the development rates of females and males were separately illustrated. As shown in Figure 14, there was a significantly reduced number of females that developed in MeJA-treated plants total nematode development was surmounted by a considerably high number of males. The average males that developed in MeJA-treated plants were above 11% while the rest of the treatments range from 8-10%. No significant difference in sexual differentiation was observed between SHAM-treated *A. thaliana* plants and its control.

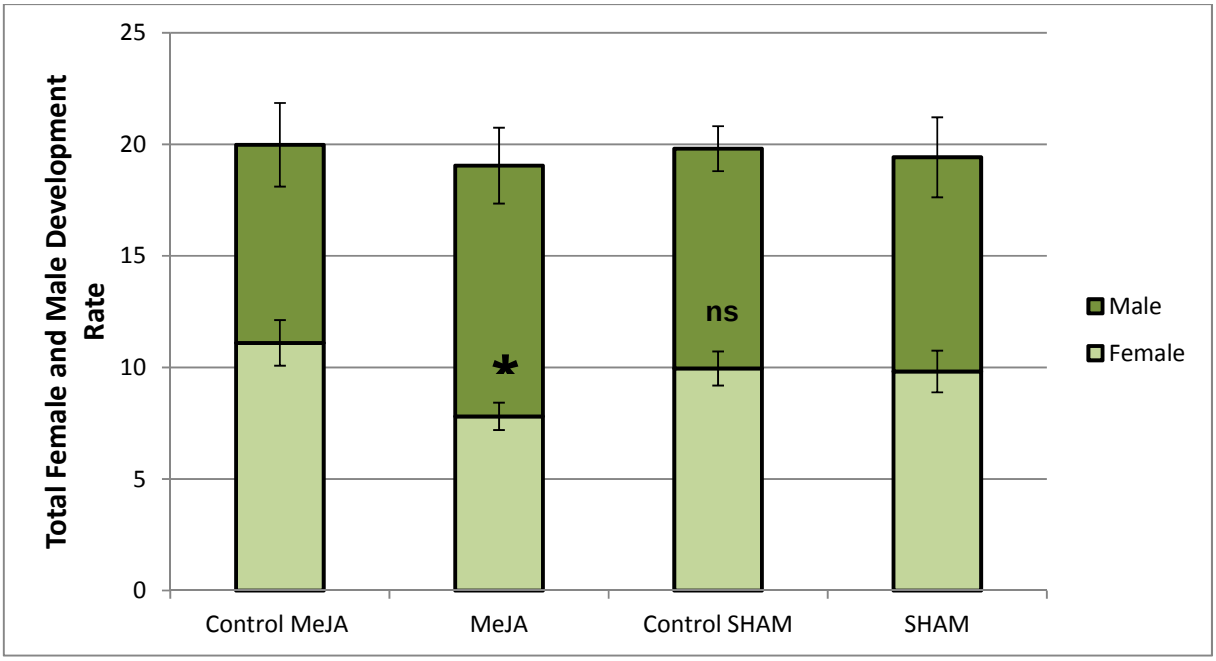


Figure14.Total female and male development rate of *H.schachtii* on *A. thaliana* (Col 0) roots 15 days after MeJA or SHAM shoot application. Values are means ± SE or SD?, n=?, ns- no significant difference (P<0.05, T-test).

3.3.3. Female/male ratio

In order to underline this shift in sexual development the female/male ratio was calculated. Figure 15 clearly shows a highly significant difference between MeJA-treated plants and the control with a p-value (two-tailed T-test) of 0.000135 which means less than 0.01% significance level. The female/male ratio in non-treated plants (control MeJA) was almost 50% higher as compared to MeJA-treated plants. While plants treated with SHAM and its control revealed no significant difference. Numerically, SHAM-treated plants exemplified higher female/male ratio than MeJA-treated plants.

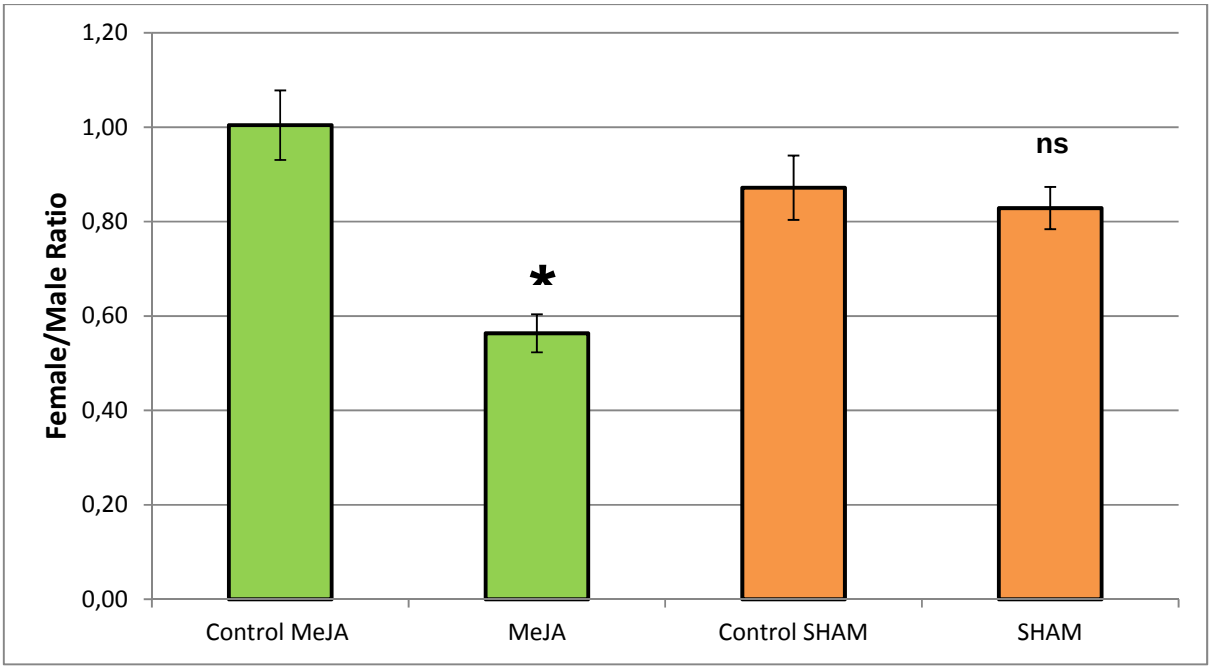


Figure 15. Female/male ratio of *H.schactii* on *A. thaliana* (Col 0) roots 15 days after MeJA or SHAM shoot application. Values are means ± SE n=3, ns- no significant difference (P<0.05, T-test).

3.4. Gene expression analysis

The second aim of the work was to test if *H schachtii* infection and/or MeJA shoot application affect the expression of the JA-related genes *COI1*, *JAR1* and *PDF1.2A*. Therefore, quantitative gene expression analyses were performed on roots and shoots of infected and treated plants that were compared to the expression levels in the respective controls. Since the shoot application of SHAM had no significant effect on nematode attraction, infection and development these experiments were focused on MeJA-treated plants.

3.4.1. Effect of *H.schachtii* infection on the expression of *COI1*, *JAR1* and *PDF1.2A* genes in the roots

First, the effect of *H. schachtii* J2's 24h after infection on the expression of *COI1*, *JAR1* and *PDF1.2A* genes in *A. thaliana* roots was quantified using the quantitative RRT-PCR (qRT-PCR). The values were expressed in log_2-scale.Result revealed that *PDF1.2A* was significantly down-regulated in roots of infected plants (Figure 16), whereas, J2 infection has no significant effect on *COI1 and JAR1*.

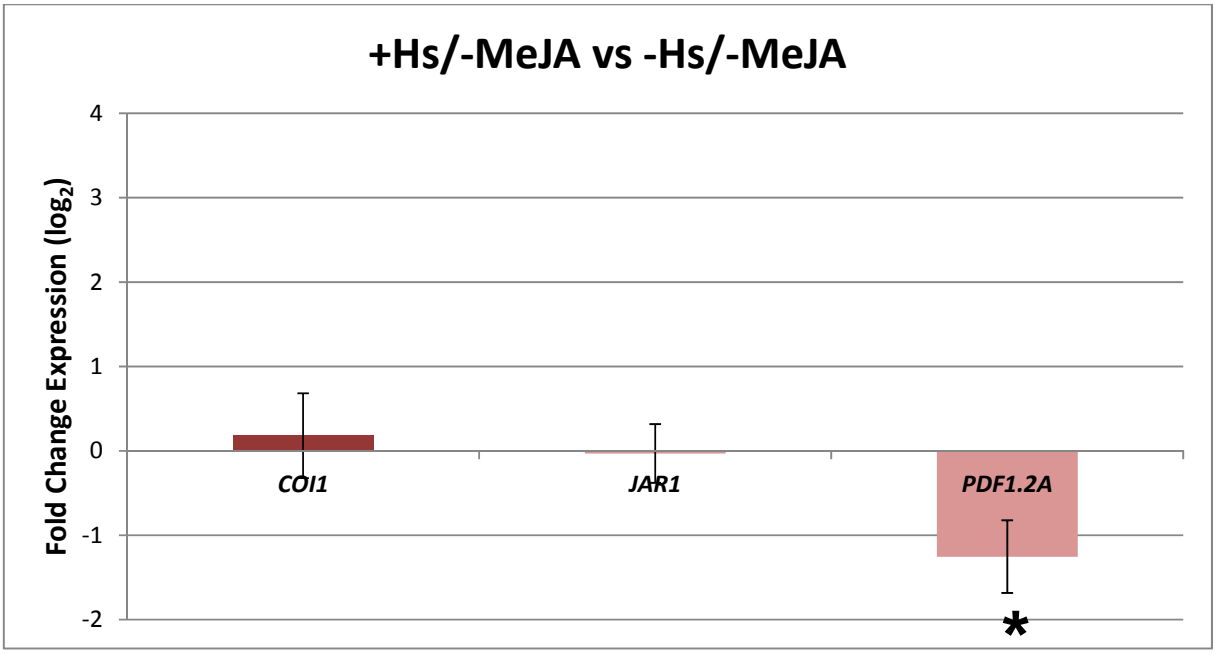


Figure16.Quantitative RT- PCR comparing gene expression in *H.schachtii*-infected to non-infected *A. thaliana* roots (without JA-treatment). Values are means ± SE o, n=3, *- significant gene expression changes (P<0.05, T-test).

3.4.2. Effect of *H. schachtii* infection on the expression of *COI1*, *JAR1* and *PDF1.2A* genes in the shoots

Next, the potential systemic effect of *H. schachtii* root infection on the tested JA-related genes was studied. The fold change expression of *COI1*, *JAR1* and *PDF1.2A* in the

shoots 24h after infection (Figure 17) showed a reverse response as compared to the genes expressed in the roots (Figure 16). The *PDF1.2A* gene was significantly up-regulated while *COI1* and *JAR1* were slightly down- and up-regulated, respectively.

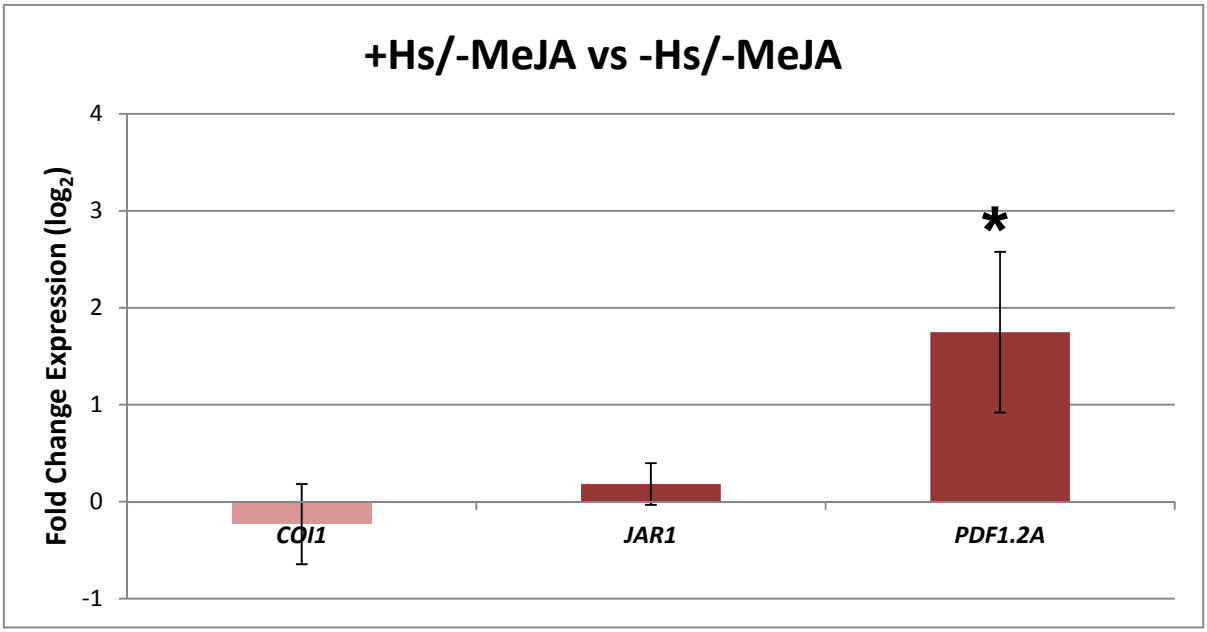


Figure 17. Quantitave RT- PCR comparing gene expression in *H.schachtii*-infected to non-infected *A. thaliana*shoots (without JA-treatment). Values are means ± SE , n=3, *- significant gene expression changes (P<0.05, T-test).

3.4.3. Effect of MeJA shoot application on the expression of *COI1*, *JAR1* and *PDF1.2A* genes in non-infected roots

In order to elucidate if MeJA shoot application has systemic effects on the roots the expression of JA-related genes was tested in non-infected roots. All the studied genes were slightly up-regulated in roots upon MeJA shoot application, *COI1* was significantly down-regulated (Figure 18).

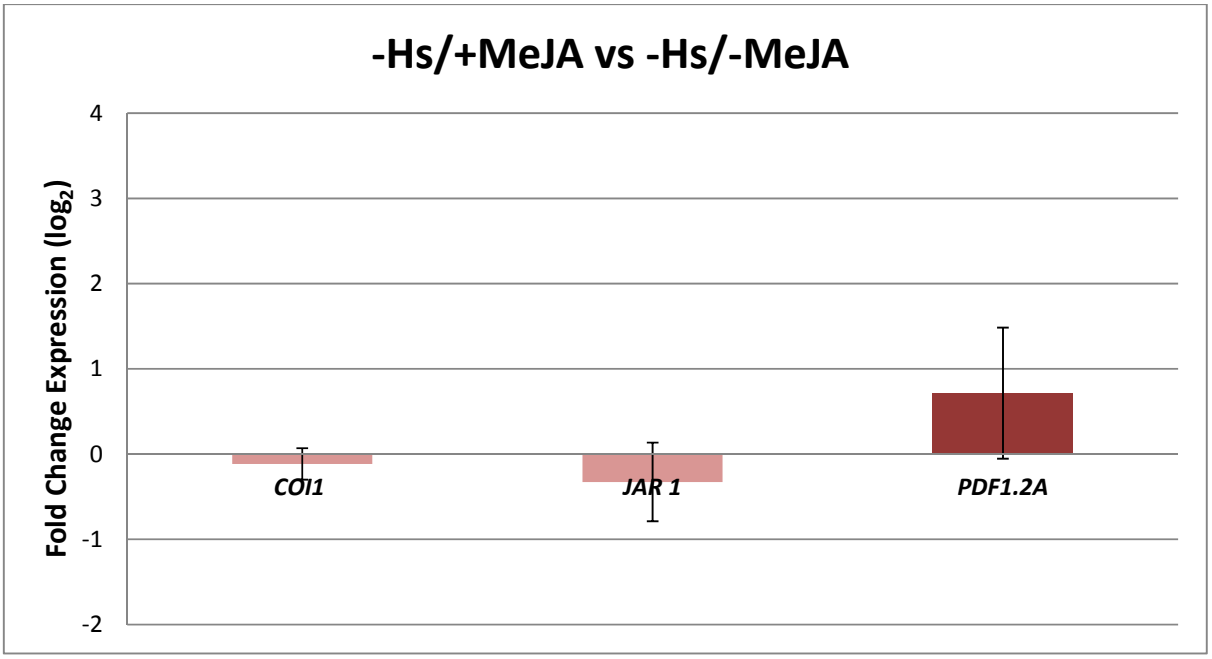


Figure 18. Quantitave RT- PCR comparing gene expression in JA-treatedto control treated *A. thaliana* roots (without *H. schachtii* inoculation). Values are means ± SE n=3.

3.4.4. Effects of MeJA shoot applcation on the expression of *COI1*, *JAR1* and *PDF1.2A* genes in *H. schachtii*-infected and non-infected roots

In the present study it was shown that MeJA shoot application on the 11 day-old *A.thaliana* plants reduced nematode infection rate 24h (Figure 11) and female development 15 days after *H. schachtii* J2 inoculation (Figure 14). The effect of MeJA on the expression of the tested genes was again studied by qRT-PCR. The roots infected after 24h as well as the non-infected ones of MeJA treated plants were analyzed in this experiment. The application of MeJA on the leaves trigged a significant up-regulation of *PDF1.2A,* a significant down-regulation of *JAR1* and down-regulation of *COI1*in the infected roots compared to the non-infected roots (Figure 19).

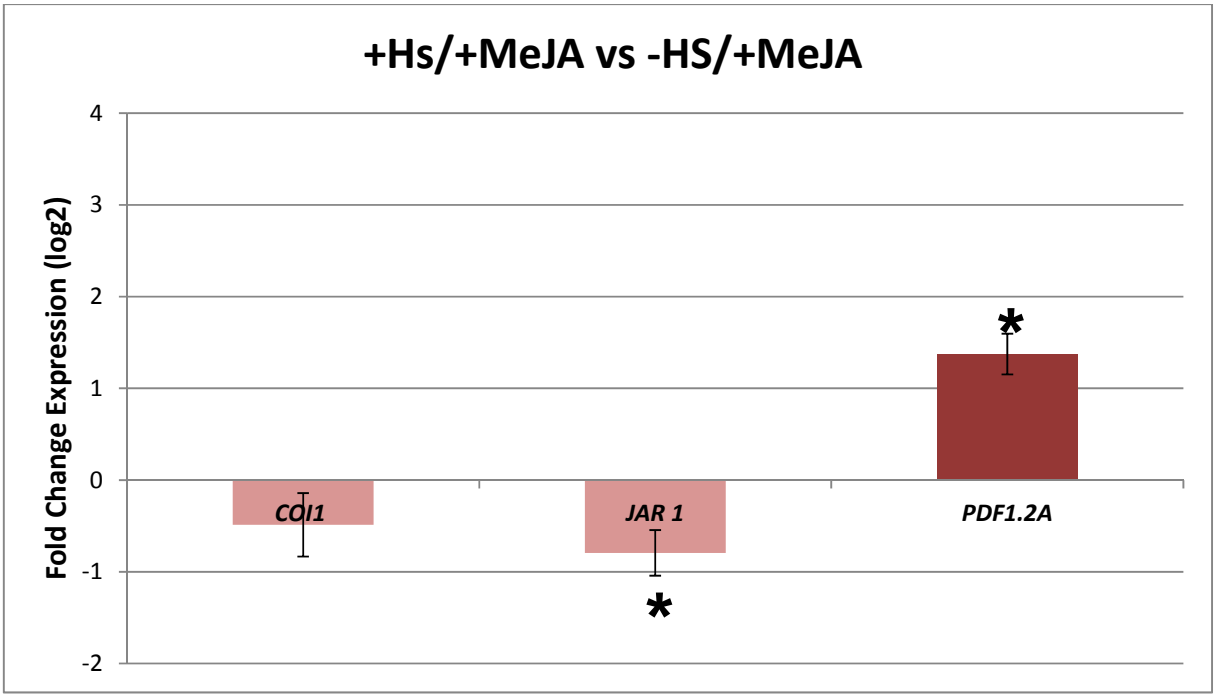


Figure 19.Quantitave RT- PCR comparing gene expression in JA-treated *H.schachtii*-infected to JA-treated non-infected *A. thaliana* roots. Values are means ± SE, n=2, *- significant gene expression changes (P<0.05, T-test).

3.4.5. Effect of MeJA shoot-application on the expression of *COI1*, *JAR1* and *PDF1.2A* genes in *H. schachtii*-infected roots

The effect of MeJA-treated plant infected with *H.schachtii* versus non-treated and infected plants can be reflected in Figure 20. The *PDF1.2A* gene was clearly up-regulated, however, *COI1* and *JAR1* were both down-regulated significantly.

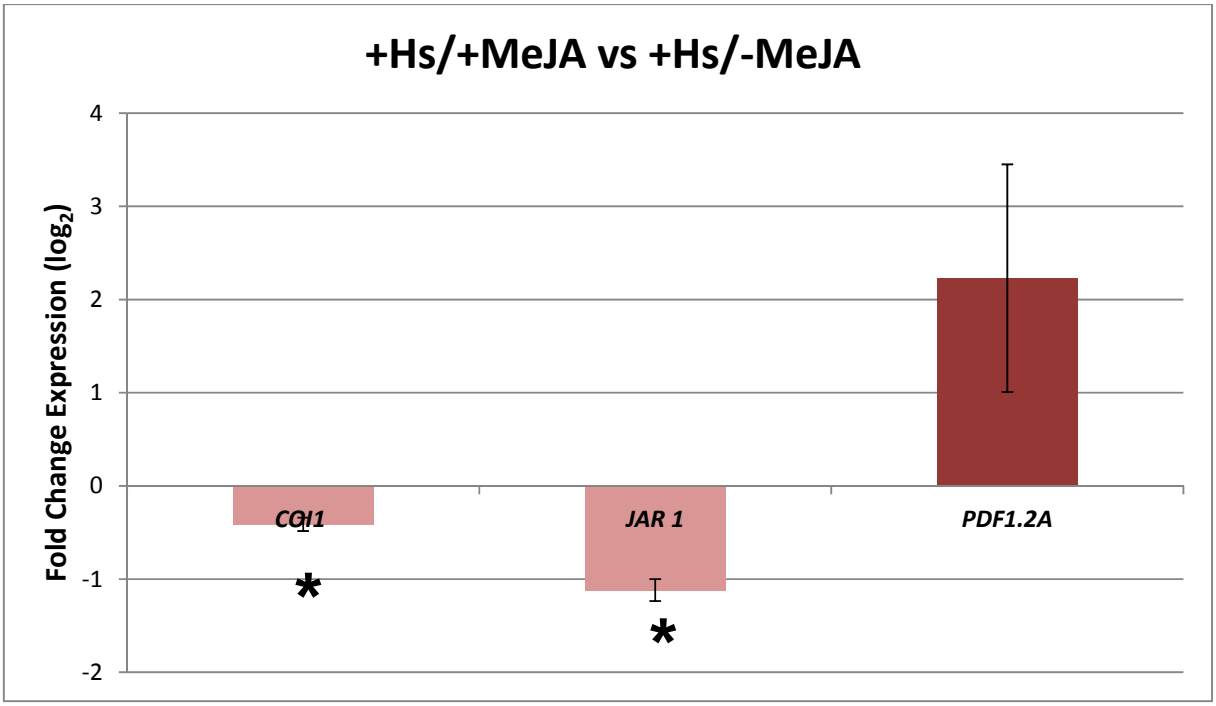


Figure 20.Quantitave RT- PCR comparing gene expression in JA-treated *H.schachtii*-infected to control *H. schachtii*-infected *A. thaliana* roots. Values are means ± SE , n=2, *- significant gene expression changes (P<0.05, T-test).

3.4.6. Effect of MeJA applied on the leaves on the expression of COI1, JAR1, PDF1.2 genes in infected roots

As shown in Figure 21, MeJA-treated plants infected with *H. schachtii* nematodes up-regulated the *PDF1.2A* gene, while down-regulating *COI1* and *JAR1*. Only *JAR1* was found to be significant.

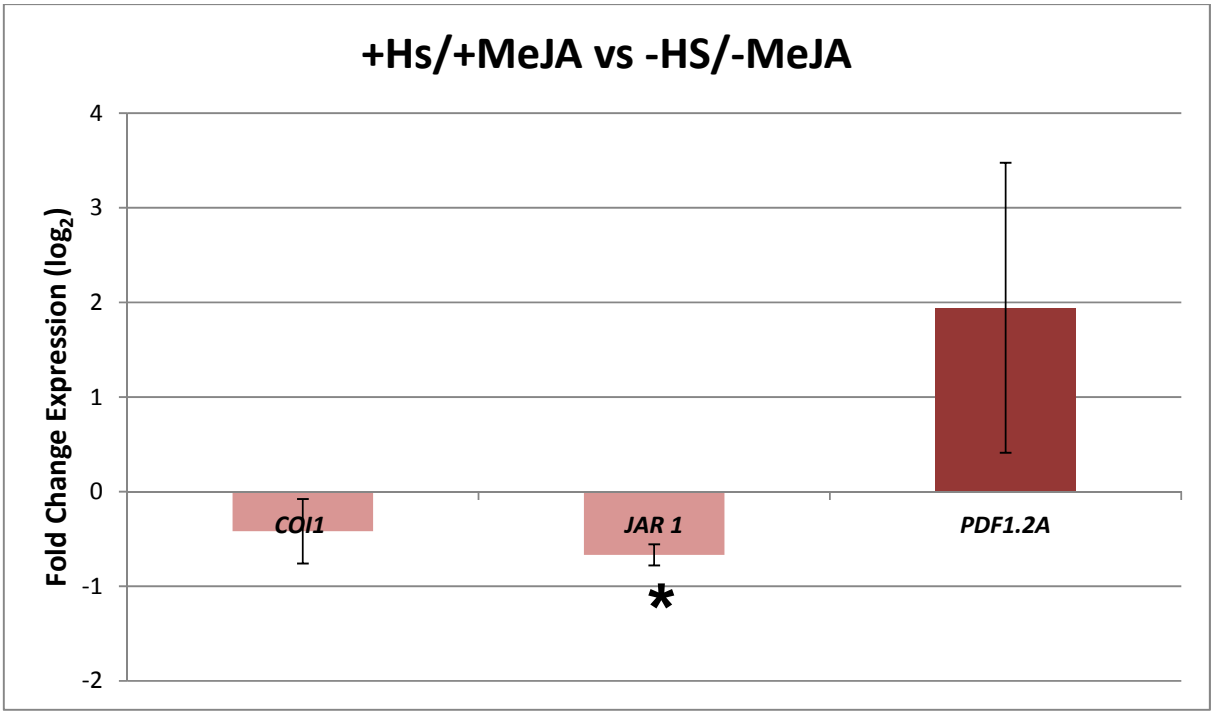


Figure 21.Quantitave RT- PCR comparing gene expression in JA-treated *H.schachtii*-infected to control *H. schachtii*-non-infected *A. thaliana* roots. Values are means ± SE , n=2, *- significant gene expression changes (P<0.05, T-test).

4 Discussion

The *A. thaliana – H. schachtii* interaction is an efficient model to study the role of changing hormone levels in compatible plant-nematode interactions. *H. schachtii* attacks *Arabidopsis* and can complete its whole life cycle within 6 weeks (Sjimons, 1991). The translucent roots of *A. thaliana* growing on artificial media have facilitated behavior studies on different nematode species inside the root (Wyss and Grundler, 1992). Further, *A. thaliana* is an excellent model plant to perform gene expression studies and it is used to investigate the important subgroup of oxylipins: the jasmonates (Acosta and Farmer, 2010).

4.1 MeJA shoot application affects nematode infection and development

Jasmonic acid (JA) is a prohormone that is conjugated to hydrophobic amino acids to produce regulatory ligands (Acosta and Farmer, 2010).It is known to be transported from foliage to roots (Baldwin et al., 1994; Zhang and Baldwin, 1997), where it can have a wide range of effects on development and metabolism. JA influences root growth and nutrient partitioning and plays a major role in plant defense responses. Potential JA-triggered changes in plant metabolism, partitioning and defense response may serve as solid basis to develop alternative control strategies against plant parasitic nematodes and may thus help to reduce reliance on hazardous nematicides (Cooper and Googin, 2005)

Roots of different species differ dramatically in their feature to attract individual species of plant parasitic nematodes (Abad and Williamson, 2010). The sugar beet cyst nematode recognizes the occurrence of host root specific gradients of e.g. volatile

compounds around actively growing roots (Perry and Moens, 2011). However, currently there is little information what compounds may attract or repel the plant feeders. Accordingly, a study by Fujimoto et al.(2011) showed that root-knot nematodes(RKN) maybe repelled by substances exuded from the roots of tomato plants. In the present study it was investigated if potential changes in root exudates triggered by JA and SHAM shoot application may affect nematode attraction. The results showed no significant difference on the attractiveness of the root exudates of *A. thaliana* to *H. schachtii* J2's in between treatment contrasts (MeJA vs. control, SHAM vs. control and MeJA vs. SHAM), nevertheless, the J2's tended to migrate towards exudates of hormone-treated plants.

Further, the rate of *H. schachtii* J2 infecting *A. thaliana* (Col 0) roots was determined in this study. It was found out that the rate of infection at 24h and 48h was significantly reduced in *A. thaliana* leaves treated with 60µM MeJA. It was reported that foliar application of MeJA on tomato at 0.5mM or at higher concentrations repressed RKN infection (Fujimoto et al., 2010) and a concentration of 100µM sprayed on rice seedling effectively reduce migratory root-rot nematode (RRN) infection (Nahar et al., 2012). Also application of MeJA directly to the roots of oats lowers root infection of *H. avena, P. neglectus* (Soriano et al., 2004) and RKN on spinach (Cooper and Goggin, 2005). However, in the current work the application of MeJA showed no longer significant effects against *H.schachtii* at 72h after J2 inoculation. In this study, it may be possible that the nematodes are attracted to the roots of hormone-treated plants but later found out it is not suitable for them. A study by Choi et al. (1999) and Kouassi et al. (2005) observed emigration of J2 of *M. incognita* and *M. javanica* out of the roots of peanut and

tomato resistant genotype. Nevertheless, the correlation between attraction and host suitability is not perfect, and little is known about the plant signals that modulate nematode behavior. Fujimoto et al. (2010) reported a suppressive effect of MeJA lasted for 7 days in tomato infected with RKN and the effect still remained but weakened when two weeks had passed from the application of MeJA. These results indicate that the role of MeJA is specific in different plant-nematode interactions. The exogenous application of MeJA to roots or shoots was shown to enhance resistance to parasitic nematodes (Fujimoto et al., 2011), possibly by elevating the level of compounds toxic to nematodes like phytoectosteroids, flavonoids and proteinase inhibitors. Further, in plants JA is closely associated with the production of secondary metabolites such as flavonoids (Gundlach et al., 1992).

The production of such chemicals may not only affect nematode infection and syncytium establishment, but also nematode development and syncytium maintenance. After infection the cyst nematodes select a single cell, often in the vascular parenchyma, to induce a feeding cell, generally known as the initial syncytium cell (reviewed by Abad and Williamson, 2010). In Arabidopsis, the initial syncytial cells are preferably procambium or pericycle cells within the central cylinder (Golinowski et al., 1996). In this study, the development rate *H. schachtii* cyst nematode was evaluated fifteen days after J2 inoculation and the occurrence of males and females was investigated. It was observed that total nematode development was not affected by MeJA shoot application reflecting the infection rate observed at 72h after inoculation. Nevertheless, there was a reduction of female development and a highly significant difference on female/male ratio on MeJA treated plants compared to other treatments. This result implies that MeJA–

treated *A. thaliana* causes unfavorable condition for *H. schachtii* cyst nematode development. Cyst nematodes possess sexual differentiation that is determined by environmental conditions, with the frequency of males increased under unfavourable circumstances (Abad and Williamson, 2010). Shoot application with the JA-inhibitor SHAM had no effect on nematode attraction, infection and development. Thus, reduced JA-signaling trigged by SHAM may not induce improved conditions for *H. schachtii*. Further, the chemical may not be translocated from shoots to roots of *A. thaliana* plants or may not affect signaling compounds delivering systemic messages explaining the failing effects.

Hence, in the current work nematode resistance was not achieved, however, the data give a solid base for further research achieving reduced nematode populations. Resistant against nematodes, as defined by Cook and Evans (1987) and by Roberts (2002), are plants that support low or no nematode development and reproduction. With some cyst nematode resistance genes, including tomato gene *Hero A* and *H1*, nematode development occurs on the host but is characterized by reduced reproduction and a high ratio of male to female nematodes (Rice et al., 1985; Sobczak et al., 2005). As for several of the RKN resistance genes, a feeding site is initiated, but develops poorly and atrophies; the nematodes that do develop are mostly males (Abad and Williamson, 2010). Reduced reproduction of RKN species was found in *A. thaliana* and of *H. glycines* on transgenic soybean with other constructs designed for post-transcriptional gene silencing of specific nematode target genes (Huang et al., 2006).

4.2. The expression of JA-related marker genes is affected by MeJA shoot application and nematode infection

The study of plant–nematode interactions has progressed considerably in recent years, switching from descriptive steps to detailed approaches such as gene expression and silencing. Accordingly, in the current study genes involved in JA-signaling were selected to study potentially altered expression patterns during nematode infection and MeJA-treatment. The hormone is perceived by the receptor protein COI1, eventually liberating transcription factors allowing gene expression (Acosta and Farmer, 2010). JAR1 catalyzes the final step in the formation of a bioactive JA compound (Wasternack and Hause, 2013). And the *PDF1.2* gene encodes a plant defensin, a commonly used marker for the characterization of JA-dependent plant defense responses (Brown et al., 2003). For these gene expression analyses, 24h after nematode inoculation was selected as representative sampling time point covering nematode root-penetration, intracellular migration and syncytium induction.

It was found out in this study that *H. schachtii* infecting on the roots of non-treated *A. thaliana* (Col 0) plant significantly down-regulated *PDF1.2A* but had no effect on *JAR1* and *COI1* gene expression. This indicates that nematodes trigger the suppression of JA-related defense. Accordingly, Hermsmeier et al. (2000) reported that several defense-related genes are down-regulated in roots after nematode infection, suggesting that the nematodes actively suppress the host defense mechanisms. This phenomenon was described in *A. thaliana* infected with the sugar beet cyst nematode and after infection of susceptible soybean with soybean cyst nematode (Bekal et al., 2003) . Effectors synthesized in the oesophageal glands that are potentially responsible for

regulating plant defense responses have been identified such as the oesophageal gland-specific chorismate mutase (Huang et al., 2005). During intracellular migration the cyst nematodes cause severe cell damage what may lead to plant defense as well as JA-triggered signaling. Impairment of these mechanisms may support successful nematode infection.

While *PDF1.2A* was down-regulated in the roots, the reverse occurred in the leaf samples 24h after J2 inoculation. This indicates that the shoot defense system was activated during nematode root infection. Hofmann et al. (2010) identified clear systemic effects of nematode parasitism of *H. schachtii* infecting *Arabidopsis* roots. Hamamouch (2011) observed that JA-dependent 'Systemic Acquired Resistance' (SAR) appeared to be induced in leaves of *H. schachtii* infected *A. thaliana*, as indicated by an increase in *PR-3* transcript level.

MeJA shoot application triggered minor but no significant up-regulation of *PDF1.2A* and down-regulation of *COI1* and *JAR1*. This indicates that MeJA is either translocated towards the roots or that it induces JA-related systemic signaling. When MeJA was applied to the shoots of *H. schachtii* infected *A. thaliana* roots, there was a significant up-regulation of the marker gene *PDF1.2A* and a significant down-regulation of *JAR1* when compared to treated but non-infected roots. The same trend was observed when comparing MeJA treated and non-treated but infected roots or comparing treated plus infected with non-treated plus non-infected roots. This indicates that MeJA shoot application affects systemically the expression of JA-related genes that are suppressed by nematode-infection only. These data clearly show the opposed effects of JA treatment and nematode infection. It was shown before that tomato plants have an

induced resistance response to root-knot nematode when JA is applied to the plant (Cooper et al., 2005). Nahar et al. (2012) reported that exogenous MeJA is a potent inducer of systemic root defense against RRN attack in rice, while MeJA application on the shoots is actively inducing systemic defense against this migratory nematode in rice roots activating the expression of the pathogenesis-related (PR) genes *OsPR1a* and *OsPR1b* in rice roots.

The expression of *COI1* and *JAR1* was only regulated when plants were both MeJA treated and nematode infected. Yan et al., 2009 simulated a molecular model on the possibility of *COI1* as binding sites for JA and found unfavorable binding with MeJA but it was efficiently bounded to JA-Ile. Suza and Staswick (2008) reported that *JAR1* gene is strongly dependent on *COI1*, JAR1 enzyme catalyzes the formation of JA-Ile.

The data obtained in this study suggest that nematodes aim to reduce JA-based plant responses and that increased JA levels lead to unfavourable conditions for nematode infection and development.

5 Summary

The aim of the current study was to elucidate the role of JA for the *Arabidopsis thaliana* – *Heterodera schachtii* interaction. Therefore, three major questions were studied: (1) does MeJA or SHAM (inhibiting JA-signaling) shoot application affect nematode attraction, infection and development?,(2) does nematode infection trigger expression of genes involved in JA signaling and biosynthesis? and (3) does MeJA shoot application modulate these potential gene expression changes?

In this study, the attraction test shows no significant difference on the attractiveness of the roots of treated *A. thaliana* to *H. schachtii* J2's in between treatment contrasts (MeJA vs. control, SHAM vs. control and MeJA vs. SHAM) , nevertheless, the J2's tended to migrate towards exudates of hormone-treated plants. It was found that the rate of infection at 24 and 48h was significantly reduced in *Arabidopsis* leaves treated with 60µM MeJA. It was observed that there was a reduction of female development and a highly significant difference on female/male ratio on MeJA treated plants compared to other treatments. This result implies that MeJA –treated *A. thaliana* causes unfavorable condition for *H.schachtii* cyst nematode development.

There was no significant effect found for the application of SHAM (MeJA inhibitor) on the attraction, infection and female and male development test.

It was found out in this study that *H. schachtii* infecting on the roots of non-treated *A. thaliana* (Col 0) plants, 24h after inoculation the infection there was a significant down-regulation of *PDF1.2A* in the root samples. However, the reverse effect was found in

leaf samples, *PDF1.2*A was up-regulated in the shoots. This indicates that the whole shoot defense system was activated during nematode infection in the roots.

The expression of *COI1* and *JAR1* was found to have little or no effect on the infected roots.

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Attachment 1. Bonitурschema *A. thaliana*

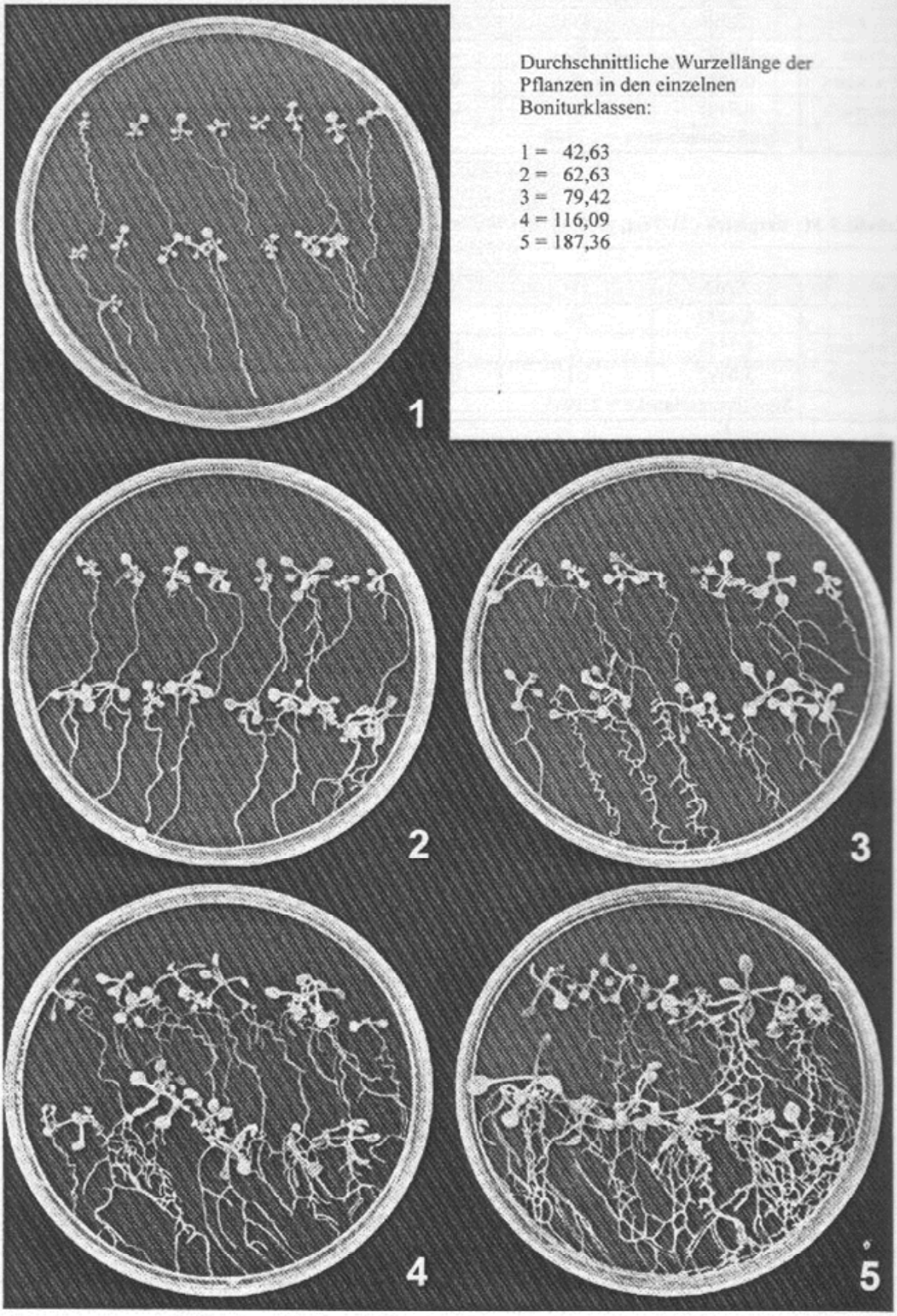

Abbildung 9.2: Bonitурschema für Resistenztests an *A. thaliana*. Die Wurzelentwicklung wird in 5 Boniturklassen eingeteilt. Die durchschnittlichen Wurzellängen in jeder Boniturklasse