**RESEARCH ARTICLE**

# Inhibitory effects of salicylic acid on *Meloidogyne javanica* reproduction in tomato plants

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## Abstract

Root-knot nematodes (*Meloidogyne* spp.), play a major role in loss of agricultural production**.** Natural substances**,** such as salicylic acid (SA) could possibly be involved in inducing host plant resistance against nematodes. The present study is concerned with exploring the effects of varying concentrations of SA as seed priming and soil drench on tomato growth parameters and the reproduction of the root-knot nematode *Meloidogyne javanica*. SA at 50 μM concentration caused only 2% of juvenile mortality under in vitro conditions. SA applied as 50 μM seed treatment caused 95% and, as a soil drench, 78% reduction in the number of egg masses that formed on tomato plants. The numbers of galls were reduced to a lesser extent. Final nematode density per gram of soil was reduced to less than 1 by the 50 μM SA seed treatment, and in other treatments decreased by between 70 and 88% compared with control plants. Our results indicate SA has potential to lower root knot nematode reproduction in tomato, and seed priming is a fairly easy method to work with.

**Additional key words**: induced resistance; seed priming; soil drench; root-knot nematode

## Introduction

Root-knot nematodes (*Meloidogyne* spp.), due to their widespread dissemination, broad host ranges, and interaction with other plant pathogens, are considered to be among the most damaging of plant parasitic nematodes, attacking a wide range of field crops, vegetables, fruit trees and ornamentals. To date, seven species and four races of *Meloidogyne* have been identified in Iran, with *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949 being the dominant species in tomato fields (Akhyani *et al.,* 1984; Mahdikhani *et al.,* 2003). Depending on the nematode species, different plant species exhibit various reactions, and economic damage thresholds range between 0.5 and 1 egg/g of soil (Sikora & Fernandez, 2005).

Resistant cultivars and nematicides have been widely used to control plant parasitic nematodes, but the use of many nematicides has recently been banned in many countries, owing to their adverse environmental effects (Oka & Cohen, 2001).

Plant cells are generally protected against pathogens by several layers of physical barriers (like the waxy cuticle on the leaf surface, the cell wall, and the plasma membrane, which deny access to most microbes) and a wide variety of chemicals that form a chemical barrier against microbes and pests. For example, saponins are glycosylated triterpenoids on the surfaces of many plant species. Their soap-like properties can disrupt the cell membranes of fungal pathogens (Bednarek & Osbourn, 2009). In addition to these nonspecific defense mechanisms, different classes of pathogens can be recognized by the cell surface-localized pattern-recognition receptors through highly conserved pathogen-associated molecular patterns (PAMPs). Both plants

and animals have independently evolved PAMP-triggered immunity as the first layer of active defense at the cellular level, highlighting the importance of this immune mechanism in preventing potential pathogen infection (Fu & Dong, 2013).

An avirulent pathogen causing local programmed cell death can induce systemic acquired resistance (SAR) and protect the rest of the plant from secondary infection for a period of weeks to months (Fu & Dong, 2013). To establish a successful infection, plant pathogens inject effectors into the host cells (Jones & Dang, 2006). Recognition of a pathogen effector by a host resistance protein can lead to effector-triggered immunity (ETI), characterized by rapid programmed cell death (PCD) known as the hypersensitive response; localized PCD can induce SAR through the production of the immune signal salicylic acid (SA), which provides resistance to a broad spectrum of pathogens (Fu *et al.*, 2012). In contrast to ETI, SAR is not associated with PCD, and instead promotes cell survival.

SAR is an induced immune mechanism in plants that may provide long-lasting protection against a broad range of microorganisms (Durrant & Dong, 2004; van Loon *et al.*, 2006). SAR can also be induced by exogenous application of the defense hormone SA or its synthetic analogs 2, 6-dichloroisonicotinic acid and benzothiadiazole S-methyl ester (Durrant & Dong, 2004).

Research suggests that SA is also involved in the *Mi-1*-mediated defense response to root-knot nematode in tomato (Branch *et al.,* 2004). Lin *et al*. (2013) reported that a salicylic acid methyltransferase (SAMT) gene from soybean plays a role in soybean defense against soybean cyst nematode (*Heterodera glycines* Ichinohe). SAMT modulates the level of SA by converting salicylic acid to methyl salicylate. In a pot experiment, soil drenching or leaf spraying with 5 mM SA increased the activity of the enzymes and phenolic compounds in tomato roots infected with *M. javanica* (Mostafanezhad *et al.,* 2014).

The objectives of this study were to evaluate the effects of exogenous applications of SA on second stage juvenile motility in vitro, also on tomato growth and *M. javanica* reproduction under in vitro conditions; furthermore to analyze and compare two methods of SA application (seed priming and soil drench) at two concentration levels of SA in a glasshouse study.

## Material and methods

### Nematode inoculum

Tomato (*Solanum lycopersicum*) seedlings cv. Early Urbana were transplanted into pots containing 500 g of soil infected with *M. javanica* and kept outdoors for three months. At harvest, plants were uprooted, roots were cut into 1 cm pieces, transferred to a 200 mL jar containing 100 mL of 0.5% commercial sodium hypochlorite, and shaken for 3 minutes to detach egg masses (Hartman & Sasser, 1985). The contents of the jar were immediately passed through 75 and 20 μm sieves and the material retained on the latter was rinsed 5 times and then transferred to a beaker.

To obtain second stage juveniles for the experiments, egg masses were placed onto a paper tissue supported in a basket sitting in shallow water; hatched juveniles passed through the tissue and were collected daily (Whitehead & Hemming, 1965).

### Effect of salicylic acid on juvenile mobility in vitro

Using a pipette, 0.2 mL of suspension containing ~100 juveniles was transferred to each well of tissue culture plates and 1 mL-aliquots of SA at concentrations of 5 μM or 50 μM were added to the wells; controls received 1 mL of water. The plates, with five replicates per treatment, were maintained in an incubator at 15 °C and, after 72 h, juveniles were probed with a needle. Motionless juveniles were counted under a stereomicroscope.

### Seed priming experiment

Tomato seeds of cv. Early Urbana were placed in a dish containing a few drops of washing-up liquid in water for 5 min and then rinsed several times with distilled water. Seeds were then disinfected with 1% commercial bleach solution for 15 min, rinsed with distilled water and dried on a filter paper.

Seeds were soaked in 5 μM and 50 μM concentrations of SA for 24 h and immediately sown in pots containing 165 g of sterilized soil; seeds soaked in water were used as controls. Three weeks later, each pot (with one seedling) was inoculated with 10 mL of a suspension containing ~ 2000 nematode eggs, which was added in three holes made in the soil around the plant roots. With 5 replicates/treatment, the pots were arranged randomly on a greenhouse bench for 2 months at 27 ± 2 °C and 16 h natural light supplemented with 150 lux artificial light when necessary. Pots were watered and fed with liquid fertilizer containing macro and micro elements (Kristalon ™, Yara Int. ASA, Norway) throughout the experiment.

After 81 days of seeding, the aerial parts of the plants were cut and the roots were removed from the soil, washed and blotted dry. All plant parts were weighed and the lengths of stems and roots were measured.

The numbers of galls and egg masses on the roots were counted under a stereomicroscope. The populations of eggs and juveniles on the roots were estimated by the method of Hartman & Sasser (1985). For this, the roots were chopped into 1 cm pieces, shaken in bleach solution and washed on a 20 μm sieve. The contents of the sieve were washed into a beaker standing on a magnetic stirrer. Using a pipette, 1 mL of the suspension containing eggs and juveniles was transferred to a counting slide and their numbers were counted under a stereomicroscope at 10× magnification.

The population of second stage juveniles in the soil of each pot was determined using the Whitehead & Hemming (1965)´s tray method. Finally, for each replicate, the overall population of nematodes was estimated from the sum of the eggs and juveniles on the roots and in the soil.

### Soil drench experiment

Disinfected tomato seeds were sown in pots containing 165 g of sterilized soil and, three weeks later, 10 mL of a suspension containing ~2000 nematode eggs was added in three holes made in the soil around the plant roots (one seedling per pot). Immediately, 30 mL of SA at either 5 μM or 50 μM (in water) were sprayed onto the surface of the soil in the pots, but control pots were sprayed only with water. There were four replicates of each treatment and the pots were arranged randomly on a greenhouse bench, where they were kept under the same watering and nutrient regime as the previous experiment. After two months plants were harvested following similar procedures and methods as pointed out in seed priming experiment.

### Statistical analyses

A completely randomized design was followed in all experiments. The generalized linear model with binomial distribution was performed on % dead second stage juveniles, and one way analysis of variance (ANOVA) on pot data to compare the results. Comparisons were drawn between the treatments using Duncan's multiple range tests in an SPSS 22 (IBM SPSS Statistics for Windows, vers. 22.0) spreadsheet.

# Results

## Effect of salicylic acid on juvenile mobility in vitro

As shown in Fig. 1, less than 2% of the juveniles were found immobile after 72 h of immersion in 5 or 50 μM SA solution ($p \leq 0.05$); but the number of dead juveniles increased with increasing concentration. In the GLM test, the F value of 13.899 was significant at the 0.001 level.

## Seed priming

The greatest number of galls was found on control plants; treated plants had fewer galls and plants treated with 50 μM SA had the fewest galls ($p \leq 0.05$) (Fig. 2a). The fewest egg masses were formed on the roots grown from seeds soaked in 50 μM of SA but, at a concentration of 5 μM, egg masses were still significantly fewer than on the control plants ($p \leq 0.05$) (Fig. 2b).

Seed priming with SA decreased the overall population of eggs and juveniles of *M. javanica* formed on the roots, resulting in a significantly smaller density/g of soil ($p \leq 0.05$) (Fig. 2c). Nematode densities were reduced to below 1/g of soil by the 50 μM SA treatment. Weights and stems and root lengths of tomato plants were not significantly different ($p \geq 0.05$) between control and treated groups (data not shown).

## Soil drench

Plant fresh weights and lengths of shoots and roots were similar ($p \geq 0.05$) in treated and untreated plants
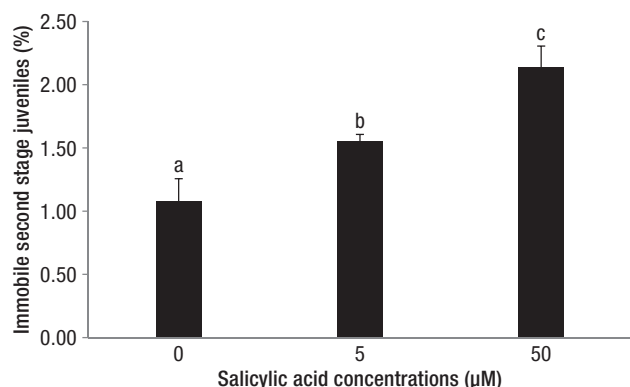


**Figure 1**. Direct effect of varying salicylic acid (SA) concentrations on second stage juvenile mobility of *M. javanica* in vitro. Columns with different letters are significantly different at the 5% level according to Duncan multiple range test. Bars represent the mean and standard error of the mean. Each value represents the mean of five replicates.
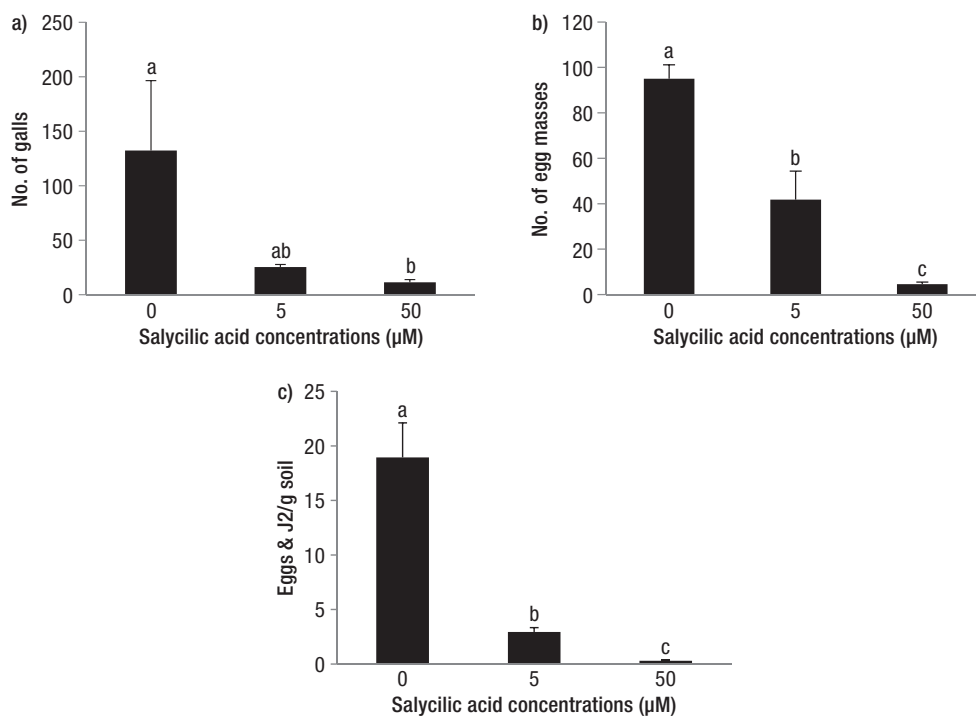
**Figure 2.** Effects of different concentrations of salicylic acid seed priming on the numbers of galls per plant (a), egg masses per plant (b) and final nematode density per gram of soil (c), of *Meloidogyne javanica* on tomato roots. Columns with different letters are significantly different at the 5% level according to Duncan's multiple range test. Bars represent the mean and standard error of the mean. Each value represents the mean of five replicates.

(data not shown). Soil drenches with SA reduced the numbers of galls, more in 50 µM than in 5 µM SA concentration ($p \leq 0.05$) (Fig. 3a).

Also, fewer egg masses were formed on the roots in pots to which SA soil drench was applied; the number of egg masses in 50 µM treated plants was reduced almost to one third of those in 5 µM treated plants ($p \leq 0.05$) (Fig. 3b).

The highest nematode population density of eggs and juveniles per gram of soil was observed in untreated soil (Fig. 3c). Addition of SA resulted in a drop in the overall population, with the density reduced to almost one third of that in the control in 5 µM treated soil and to less than one fifth in 50 µM treated soil ($p \leq 0.05$).

## Discussion

Treating plants with SA improved plant response in terms of reducing the overall population of the root-knot nematode *M. javanica* on tomato. Researchers have shown increasing interest in inducing resistance in plants via chemical inducers and plant extracts. Exogenous application of SA has been shown to activate SAR-associated genes in tobacco (Fraissinet-Tachet *et al.,* 1998), tomato (Ding *et al.,* 2002), and parsley (Thulke

& Conrath, 1998). Some of the genes activated during SAR induction by exogenous SA have been reported to encode a set of pathogenesis-related proteins (Ryals *et al.,* 1994). Due to their light molecular weight, these proteins accumulate significantly in infected plant tissues. The antimicrobial and enzymatic function of these genes has been suggested to play a potential role in induction and maintenance of SAR (Ohashi & Ohshima, 1992). The potential of several biotic resistance inducers, such as plant extracts, etheric oils and metabolic substances with resistance induction effects against pathogens, has also been tested (Zeller, 2006). Extracts from ivy (*Hedera helix*), mistletoe (*Viscum album*) and *Alchemilla vulgaris*, also essential oil of thyme, origanum, savory and cinnamon, have been described as natural antibacterial agents against the fire blight pathogen (*Erwinia amylovora*; Mende *et al.*, 1993).

In our study, SA applied as a 50 µM seed treatment caused 95%, and as a soil drench 78%, reduction in the number of egg masses (numbers of galls were affected to a lesser extent). These results are in agreement with results of Mostafanezhad *et al.* (2014), who also found that soil drench and spraying tomatoes with SA significantly reduced the diameter of *M. javanica* galls and numbers of galls and egg masses; treatments also increased the activity of enzymes and phenolic compounds. Mukherjee *et al.* (2012) showed that the num-
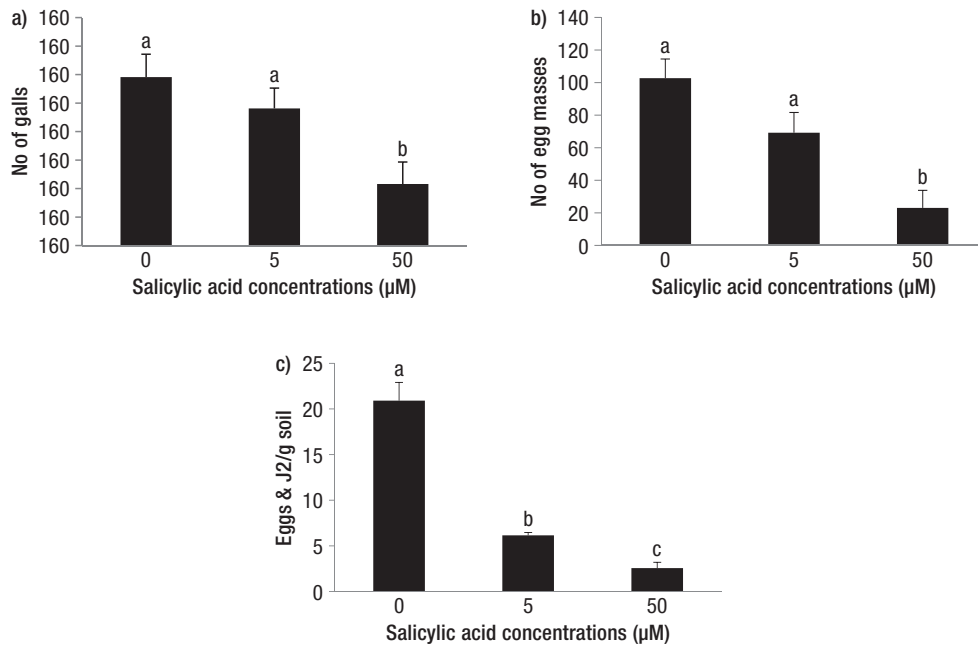
**Figure 3.** Effects of different concentrations of salicylic acid (SA) soil drench on the numbers of galls per plant (a), egg masses per plant (b) and final nematode density per gram of soil (c), of *Meloidogyne javanica* on tomato roots. Columns with similar letters are not significantly different at the 5% level according to Duncan's multiple range test. Bars represent the mean and standard error of the mean. Each value is the mean of four replicates.

bers of root galls and eggs per gram of root decreased when tomato plants infected with *M. incognita* were treated with SA. Our findings are similar to those obtained by Nandi *et al*. (2003), who when spraying SA onto leaves of cowpea and okra plants inoculated with eggs of *M. incognita* significantly reduced the numbers of galls and nematodes.

To ward off microbial pathogen infection, plants employ multiple layers of defenses (Nishimura & Dangl, 2010; Dempsey & Klessig, 2012). Development of necrotic lesions at the sites of pathogen infection/ inoculation, triggering of defenses in the uninoculated portions of the plant and a broad-spectrum, long-lasting resistance to pathogen infection known as SAR are amongst these mechanisms (Shah, 2009; Vlot *et al*., 2009). Induced resistance allows plants to resist attacks from pathogens and various parasites, including fungi, bacteria, viruses, nematodes and even herbivores (Hammerschmidt & Kuc, 1995; Sticher *et al*., 1997; Benhamou & Nicole, 1999; Kessler & Baldwin, 2002).

Activation of SAR in uninfected tissues requires transmission of signal from the infected tissue via the vascular system, generally the phloem. Early studies suggested that SA was the mobile signal (Shah, 2009; Dempsey & Klessig, 2012) but, in recent studies, several candidates for this long distance signal have been identified, including methyl salicylate (MeSA). Some of these putative signals work cooperatively to activate SAR and/or regulate MeSA metabolism.

Under the conditions of the experiments performed in this study, seed priming with SA proved to be more effective than soil drench in terms of reducing the number of galls and the final nematode population, the results being more pronounced at the 50 μM than at the 5 μM concentration. It is not clear why seed priming was more effective than soil drench. The effect could be attributed to the longer time available for SAR to build up in primed seed (three weeks between planting and nematode inoculation), while the soil drench with SA was applied at the same time as the nematodes were inoculated, perhaps allowing the nematodes to enter the plants and initiate their feeding sites before SAR was fully developed. Kempster *et al*. (2001) reported SA application as a root drench did not change the number of new females of *Heterodera trifolii* Goffart on white clover (*Trifolium repens*); however, it reduced fecundity and increased the proportions of distorted females and females with fewer eggs compared to controls. Molinari (2008) found 45 mM SA solution as soil drench or 0.5-1 mM root dip significantly reduced *Meloidogyne* reproduction (by 20 to 25%), while spraying the tomato seedlings was ineffective. Furthermore, foliar spraying or soil-drenching with 10 mM SA was either phytotoxic to tomato plants or did not induce resistance to *M. javanica* (Oka *et al.,* 1999).

The apparently varying results regarding the role of exogenous SA in inducing resistance to root-knot nematodes in tomato could be attributed to different

reasons, one of which being the use of different methodologies. It appears that the application of SA fails to inhibit nematode invasion significantly; however, it did exert an inhibitory effect on nematode reproduction (Kempster *et al.,* 2001; Molinari, 2008), which is in agreement with our findings. In some reports (Pankaj *et al*., 2005), application of SA was effective at concentrations higher than 200 mg/L. In another study (Nandi *et al.*, 2002), no mortality of nematodes occurred in vitro at 10 mM SA. SA is more likely to be involved in activating plant defense systems such as SAR. The decreases in the numbers of galls and eggs are probably due to the fact that the new juveniles formed by the nematodes are not healthy enough to create a new population. SA could possibly cause the formation of defective eggs (Pankaj *et al.*, 2005).

The effects of SA and other resistance inducers in protecting plants against nematodes need to be substantiated further. Induced resistance is a plant response that is influenced by various factors, essentially unknown as yet. For instance, how is the plant's response to elicitation affected by its developmental stage, what is the influence of both host and pathogen genotypes, of abiotic stress or of nutrition factors are still unanswered questions which need specific research (Adrian *et al.,* 2012). This should help the introduction of resistance inducers into future integrated nematode control management systems and reduce the use of nematicides. In summary, our results showed that exogenous application of SA reduced root knot nematode reproduction and final population on tomato; seed treatment was an effective and fairly easy method to work with. The use of SA and the methods of application under different conditions need further investigation.

## Acknowledgments

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