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Research Article

Induction of resistance in tomato against root-knot nematode *Meloidogyne javanica* with salicylic acid

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Abstract: The effect of salicylic acid (SA) on induction of resistance against root-knot nematode (*Meloidogyne javanica*) and the effect of *M. javanica* to induce biochemical defense responses in tomato (*Solanum lycopersicum* L.) roots at six-leaf stage were investigated. Meanwhile, the effect of different concentrations of SA on mortality of second stage juveniles of *M. javanica* was examined. Changes in the activity of cytoplasmic peroxidase (POX), catalase (CAT) and phenylalanine ammonia lyase (PAL) enzymes in the roots of tomato seedlings were measured during seven successive days after inoculation with *M. javanica* in greenhouse. SA was used as soil drench and leaf spray. The efficiency of treatments were evaluated by measuring diameter of galls, number of galls per plant, number of egg masses per plant, number of eggs per individual egg mass, root and foliage fresh weights. The results showed that use of SA as soil drench and leaf spray significantly reduce diameter of galls 28% and 32%, number of galls per plant 40% and 44%, number of egg masses per plant 45% and 49% and number of eggs per individual egg mass 53% and 55% compared to control (inoculated with nematode only). The activity of the enzymes (POX, CAT and PAL) increased in comparison with plants treated with distilled water. The maximum level of larva mortality was observed at 7 mM SA with no significant difference at concentration of 6 mM. SA caused 21.2% mortality of larvae at concentration of 5 mM.

Keywords: Salicylic acid, *Meloidogyne javanica,* Peroxidase, Catalase, Phenylalanine ammonia lyase

Introduction

Root-knot nematodes (*Meloidogyne* spp.) are phytopathogenic obligate endoparasites that infect many plant species and cause serious damage to agricultural crops per year (Abad *et al.*, 2008). Management of plant-parasitic nematodes has always been difficult, and the most successful strategy for many years has been the use of toxic fumigant nematicides, such as the most known methyl bromide (Oka *et al.*, 2000b). Also, effective nematicides such as ethylene dibromide (EDB) and dibromochloropropane (DBCP) have been withdrawn from the market due to their deleterious effects on humans and environment (Oka *et al.*, 2000b). Thus, new strategies for the control of plant-parasitic nematodes have actively been sought in the last few years, and investigation has focused on biological control, organic and inorganic amendments, naturally occurring nematicides and induced resistance (Oka *et al.*, 2000a). Plants have a number of

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defense mechanisms against pathogens. In addition to constitutive resistance, plants can activate protective mechanisms upon infection with widely different pathogens. The pathogen-induced resistance can be established in the tissue surrounding the site of initial infection (localized acquired resistance, LAR) and also in the distant, uninfected parts of the plant (systemic acquired resistance, SAR) (Hammerschmidt, 2009). Resistance to pathogens can be chemically induced by applying to plants SA and compounds which can mimic the action of SA, such as acibenzolar-S-methyl (ASM) and 2,6-dicholoroisonicotinic acid (INA) (Oostendorp *et al.*, 2001). SAR elicitors do not exhibit any direct antimicrobial activity and seem to be environmentally benign, unlike traditional pesticides. This form of resistance protects plants from a broad spectrum of pathogens and works systemically in many cases (Klessig and Malamy, 1994; Schneider *et al.*, 1996). The use of SA to induce resistance to nematodes is particularly interesting as it is a natural compound and non-phytotoxic at the appropriate dosage (Molinari, 2008). Activation of enzymes related to plant defenses against pathogens and accumulation of plant defense metabolites are the most important mechanisms of chemical inducers in plants. Many plant enzymes are involved in defense reactions against plant pathogens (Odjakova and Hadjiivanova, 2001). A plant exposed to pathogens also activates oxidative enzymes such as peroxidase (POX) (Ryan and Jagendorf, 1995). Antioxidant enzymes inactivate active oxygen forms induced by different stresses such as H_2O_2. The enzymatic actions such as catalase and peroxidase could lead to scavenging the accumulation of H_2O_2 in tissue (Tripathi, 2006). Catalase (CAT) plays an important role in the catabolism of hydrogen peroxide (H_2O_2). This enzyme catalyses the disruption of H_2O_2 into water and oxygen. Phenylalanine ammonia-lyase (PAL) is the entry point enzyme into phenylpropanoid metabolism, involved in the production of phenolics and phytoalexins that prevent establishment of the pathogens (Mariutto *et al.*, 2011).

The objectives of the present study were to monitor the activity of POX, CAT and PAL in the roots of tomato plants. Also the efficacy of SA against *M. javanica* was evaluated under greenhouse conditions. Meanwhile, the potential of different concentrations of SA (3, 4, 5, 6 and 7 mM) in killing J2 of *M. javanica* after 72 hour in *in vitro* were examined.

Materials and Methods

Nematode inoculum preparation

Infected tomato plants with root knot nematodes were collected from a tomato farm in Varamin (Tehran province, Iran) and single egg mass was used to establish a population on Early Urbana Y tomato variety for the experiments. Eggs were extracted according to Hussey and Barker (1973) and the species was identified as *M. javanica* according to Eisenback (1985).

Salicylic acid preparation

Salicylic acid (Merck, Germany) was purchased from the market. SA at concentrations of 3, 4, 5, 6 and 7 mM was prepared in sterile distilled water as instructed by the manufacturer.

Plant material

Tomato seeds (*Solanum lycopersicum* L.) cultivar Calj-N3 were surface sterilized with 1% sodium hypochlorite for 5 min, sown in pots (1 kg) containing sterile soil (Mixture of sand, field soil, humus 1:1:1 v/v/v) and grown in a greenhouse at 27 °C ± 5 and 16 h light/8 h dark cycle Six-leaf stage plants were used for inoculation with nematodes and SA.

Laboratory experiments

Effect of different concentrations of SA on mortality of *M. javanica* J2s in vitro

To determine the effect of SA on nematode mortality, concentrations of (3, 4, 5, 6 and 7 mM SA in sterile distilled water) were placed in sterile petri dishes containing 100 ± 5 second-stage juveniles (J2s) of *M. javanica*. Larvae were sterilized according to Mitchum

et al. (2004). Petri dishes containing distilled water were used as control. Treatments were replicated four times and kept in incubator at 25 ± 1 °C. After 72 hours the dead juveniles were counted and mortality percentages were calculated according to Abbott (1925). Nematodes were considered dead if they did not move when probed with a fine needle (Cayrol *et al.*, 1989).

Greenhouse experiments

Induction of resistance with SA in tomato against *M. javanica*

Seedlings with six leaves were treated with 5 mM solution of SA applied as leaf spray or soil drench (20 ml per plant). Three days after treatment with SA, seedlings were inoculated with 2000 second-stage juveniles (J2s) per individual tomato seedling (Sahebani and Hadavi, 2009). Treatments included: sterile distilled water; 2000 J2s of *M. javanica* (control); soil drench of SA alone and SA in combination with nematode, leaf spraying of SA alone and in combination with nematode. During spraying of the leaves with SA the soil surface underneath each plant was covered with plastic sheet to prevent run-off. Fifty days after inoculation of seedlings with pathogen, root and foliage fresh weights of plants, number of galls and egg masses per seedling and the number of eggs per individual egg mass were measured. Nematode eggs were extracted from the roots according to Hussey and Barker (1973). The experiment was performed with five replications.

Enzyme activity assays

Seedlings with six leaves were treated as mentioned above. Treatments included: sterile distilled water, 2000 J2s of *M. javanica*, leaf spraying of 5 mM SA alone and in combination with nematode. To evaluate the activity of some enzymes related to plant defense responses, root samplings were taken 1-7 days after inoculation with nematodes.

Protein extraction

Fresh tomato roots were washed and dried on filter paper. The homogenized tissue (1 g) was rinsed with the 1 mL of 10 mM sodium phosphate buffer (pH 6.0) at 4 ^{o}C into centrifuge tube. The tissue extracts were centrifuged at 12,000 g for 20 min at 4 ^{o}C. The supernatant was used for the enzymatic activity assays. The experiment was performed with four replications (Sahebani and Hadavi, 2008).

Peroxidase activity assay

Peroxidase activity was assayed according to Reuveni (1995) with some modification. The POX activity was assayed by measuring the increase in absorbance at λmax of 475 nm due to oxidation of guaiacol (Merck, Germany). Two ml reaction mixture consisted of 1780 µL of phosphate buffer (25 mM) with pH of 5.4, 20 µL of 0.05% guaiacol (w/v) and 200 µL of the root extract. Then 5 µL of 30% H_2O_2 (Merck, Darmstadt, Germany) was added to this mixture and absorption of light was measured. Enzyme activity was expressed as ΔA/min/ml root extract.

Catalase activity assay

The CAT activity was assayed by measuring the rate of disappearance of H_2O_2 at 240 nm using the method proposed by Kato and Shimizu (1987). Three mL of CAT assay reaction mixture containing 10 mM potassium phosphate buffer (pH 7), 100 µL root extract, and 0.035 mL H_2O_2 (3%) were used. The observed decline in the optical density at λmax of 240 nm was concluded to be an indication of the decrease in H_2O_2 content. The enzyme activity was calculated using the extinction coefficient (40 $mM^{-1}cm^{-1}$) for H_2O_2. The enzyme activity was expressed as ΔA/min/ml root extract.

Phenylalanine ammonia lyase activity assay

The activity of PAL was determined by following the accumulation of trans-cinnamic

acid at 290 nm, using L-phenylalanine (Merck, Germany) as PAL substrate according to (Chen *et al.*, 2000). The enzyme activity was expressed as total accumulation of trans-cinnamic acid / ml root extract.

Statistical analysis

All analyses were done using SAS version 9.0 (Statistical Analysis System Institute Inc., Cary, NC USA). Statistical design of experiments was completely randomized design. Data were analyzed statistically by using analysis of variance and comparisons of means were done at the 5% level of significance according to the Duncan's multiple range test.

Results

Evaluation of the effect of various concentrations of SA on mortality of *M. javanica* larva in vitro

The results showed that the maximum level of larva mortality occurred in 7 mM concentration of SA but had no significant difference with 6 mM concentration. SA caused 12.4, 16.5 and 21.2% mortality of larvae at concentrations of 3, 4 and 5 mM respectively (Fig. 1).

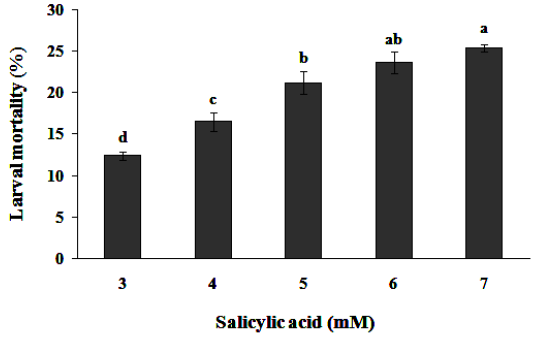

Figure 1 Effect of different concentrations of SA (3, 4, 5, 6 and 7 mM) on mortality of *Meloidogyne javanica* J2s. Values are means of four replicates. Vertical bars indicate ± standard error. Columns with different letter are significantly different according to Duncan's multiple range test ($P \leq 0.05$).

Greenhouse experiments

Study of the induction of resistance by SA in tomato against *M. javanica*

The results indicated that treatments were significantly ($p \leq 0.05$) different compared to control (plants inoculated with nematode only) in the diameter of galls, number of galls per plant, numbers of egg masses per plant and numbers of eggs per egg mass (Table 1). The maximum level of fresh root weight was observed in the control (inoculated with nematode only) and the minimum level was in treatment distilled water. The maximum level of fresh foliage weight was observed in the leaf spraying of SA treatment which had no significant difference with either soil drenching of SA or distilled water treatment. Plants inoculated with nematode and treated with SA (leaf spraying or soil drenching) compared to control (plants inoculated with nematodes) showed increase in foliage weight.

Peroxidase activity assay

The results showed that enzyme activity in plants treated with sterile distilled water did not significant change during the period of experiment (Fig. 2). Enzyme activity in control (nematode inoculated) increased steadily up to fourth day and then declined. There was, however, no significant difference between control and distilled water treatment on the seventh day. Greatest POX activity was observed in plants that were treated with SA + nematodes and SA on the first day after inoculation with nematodes. Thereafter POX activity dropped such that there was no significant difference between these treatments & control four days after inoculation.

Catalase activity assay

Plants treated with sterile distilled water did not show much change in catalase activity (Fig. 3). In the control (plants inoculated with nematodes), the maximum enzyme activity was observed in the third day. In this treatment, enzyme activity was not significantly different between the third and fourth days and gradually decreased after the

third day so that on the seventh day had no significant difference with distilled water treatment. The highest CAT activity was observed in plants treated with SA + nematode and SA on the first day.

Phenylalanine ammonia lyase activity assay

Results showed that enzyme activity in the control was maximum on fourth day after inoculation. There was no significant difference between SA and SA + nematodes treatments on the first day after inoculation with nematodes (Fig. 4).

Table 1 Control of root-knot nematode (*M. javanica*) in tomato with salicylic acid (SA) under greenhouse conditions.

Treatment	Diameter of gall (mm)	No. galls/plant	No. egg masses/plant	No. eggs/ individual egg mass	Fresh root weight (g)	Fresh foliage weight (g)
M + SA_{sd}	1.8 b	172.4 b	126.8 b	160.6 b	10.6 b	30.1 b
M + SA_{sp}	1.7 b	158.8 b	117.2 b	152.8 b	10.8 b	30.3 b
SA_{sd}	0.0 a	0.0 a	0.0 a	0.0 a	9.4 c	42.9 a
SA_{sp}	0.0 a	0.0 a	0.0 a	0.0 a	9.4 c	43.3 a
M (control)	2.5 c	286.4 c	229.2 c	344 c	11.6 a	26.1 c
DW	0.0 a	0.0 a	0.0 a	0.0 a	9.0 c	40.2 a

The data are means of five replications.
Means followed by different letters in each column show significant difference (Duncan's multiple range test, $P \leq 0.05$).
SA_{sd}: soil drenching of SA, SA_{sp}: leaf spraying of SA, M: *Meloidogyne javanica*, DW: distilled water.

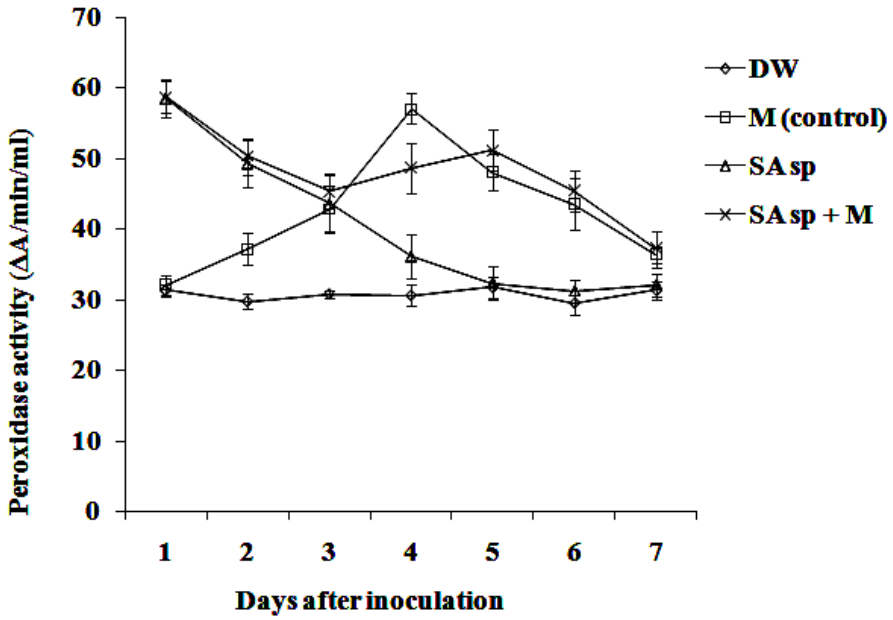

Figure 2 Effect of salicylic acid (SA) and *Meloidogyne javanica* on the activity of peroxidase (at 475 nm) in the roots of tomato, cultivar Calj-N3. Data are the average of four replicates. The values were significant according to Duncan's multiple range test ($P \leq 0.05$). Vertical bars indicate standard error of mean. DW: distilled water, M: *M. javanica*. SA_{sp}: Leaf spraying of SA.

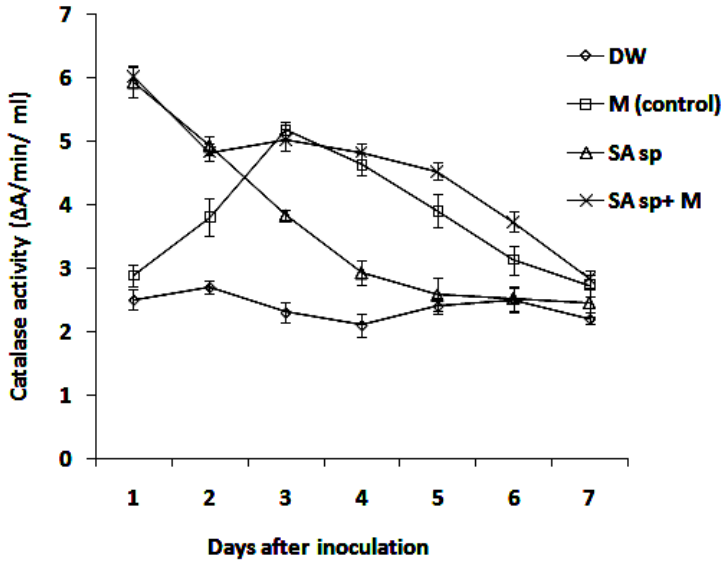

Figure 3 Effect of salicylic acid (SA) and *Meloidogyne javanica* on the activity of catalase (at 240 nm) in the roots of tomato, cultivar Calj-N3. Data are the average of four replicates. The values were significant according to Duncan's multiple range test ($P \leq 0.05$). Vertical bars indicate standard error of mean. DW: distilled water, M: *M. javanica*. SA_{sp}: Leaf spraying of SA.

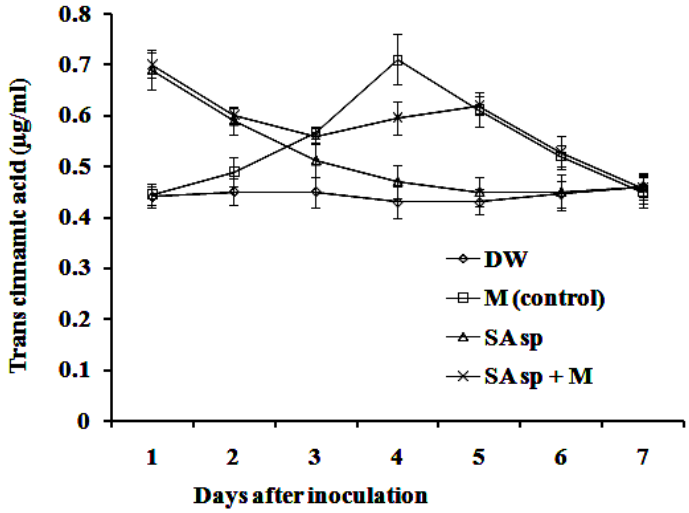

Figure 4 Effect of salicylic acid (SA) and *Meloidogyne javanica* on the activity of phenylalanine ammonia lyase (290 nm) in the roots of tomato, cultivar Calj-N3. Data are the average of four replicates. The values were significant according to Duncan's multiple range test ($P \leq 0.05$). Vertical bars indicate standard error of mean. DW: distilled water, M: *M. javanica*. SA_{sp}: Leaf spraying of SA.

Discussion

Induced Systemic Resistance (ISR) of plants against pathogens is a widespread phenomenon that has been intensively investigated with respect to the underlying signaling pathways as well as to its potential use in plant protection. Mukherjee *et al.* (2012) showed that shoot

weight of infected plants with nematodes and treated with SA were increased compared with infected plants without treatment, they also showed that numbers of root galls and eggs/g root decreased when plants were treated with SA. In this study, SA has been tested as an inducer of resistance against *M. javanica*. It was proved that various stages of nematode infestation process, i.e. penetration, establishment, development into gravid females and reproduction, may be act as SAR inhibition. The conclusions were that SA, correctly applied at the 5 mM dosage, can be used for root-knot nematode management.

Peroxidases represent a large group of oxidoreductases that catalyze the oxidation of substrate molecules using hydrogen peroxide as electron acceptor (Banci, 1997). These enzymes play a key role in important biological processes, such as biosynthesis of lignin, degradation pathways, and host-defense mechanisms (Passardi *et al.*, 2005; Davies *et al.*, 2008). Additionally, the chemical nature of peroxidase-catalyzed reactions, the oxidation of a wide variety of compounds with the help of hydrogen peroxide, has resulted in a wide range of peroxidase-based biotechnological applications. For example, peroxidases are utilized in biobleaching, wastewater treatment, and various analytical biosensors (Regalado *et al.*, 2004). Sahebani and Hadavi (2009) showed that guaiacol peroxidase activity in tomato (var. Early Urbana) treated with 10 mM SA occurred one day after treatment and progressively increased 2 days after treatment. The highest activity was observed 5 days after treatment. In this present study, treatment with SA had the highest activity of peroxidase 4 days after treatment. This difference could be due to differences in varieties of plants or the concentration of salicylic acid.

Catalase plays the role of a specific guaiacol peroxidase protecting cell from toxic effects of H_2O_2 (Ben Amor *et al.*, 2005). Korayem *et al.* (2012) showed that most sugar beet genotypes infected with *M.incognita* indicated significant increase in the activity of catalase and in this study increase in catalase activity was also observed in plants treated with *M. javanica* or SA. Kesba and El-Beltagi (2012) showed that CAT activity increased 46.97%, 57.97% and 68.25% in superior, superior/ freedom and freedom grape rootstocks when infected with *M. incognita* compared with healthy plants.

PAL is an important enzyme in the phenylpropanoid and flavonoid pathways, is involved in biosynthesis of trans-cinnamic acid, which is a precursor of a wide range of phenolic metabolites, e.g. phenylpropanoids and other compounds. Mukherjee *et al.* (2012) showed that PAL activity of tomato plants increased at 40 days after inoculation with *M. incognita* and in this study the highest activity of PAL was observed four days after inoculation with nematodes. Overall the results indicated that induction of resistance with SA can be of practical significance. Due to the problems caused by chemical control such as adverse effects on human health and environment, use of such alternative ways in management of root-knot nematodes and other pathogens is of remarkable importance.

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القای مقاومت علیه نماتد مولد گره ریشه (*Meloidogyne javanica*) در گیاه گوجه‌فرنگی با استفاده از سالیسیلیک اسید

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چکیده: اثر سالیسیلیک اسید در القاء مقاومت علیه نماتد مولد گره ریشه (*Meloidogyne javanica*) و نیز تأثیر *M. javanica* در القاء پاسخ‌های دفاع بیوشیمیایی در ریشه گیاه گوجه‌فرنگی در مرحله شش برگی مورد ارزیابی قرار گرفت. همچنین تأثیر غلظت‌های مختلف سالیسیلیک اسید بر لارو سن دو *M. javanica* نیز بررسی شد. تغییرات بیوشیمیایی در این تحقیق شامل تغییر در فعالیت آنزیم‌های پراکسیداز سیتوپلاسمی، کاتالاز و فنیل‌آلانین آمونیالیاز بود که طی هفت روز متوالی پس از مایه‌زنی با نماتد مورد بررسی قرار گرفت. آزمایش‌های گلخانه‌ای با استفاده از سالیسیلیک اسید پنج میلی‌مولار به‌صورت خیساندن خاک و محلول پاشی برگ انجام شد. اثر تیمارها با بررسی قطر گال، تعداد گال در هر گیاه، تعداد توده‌ی تخم در هر گیاه، تعداد تخم در هر توده و وزن تر اندام هوایی و ریشه گیاه مورد ارزیابی قرار گرفت. نتایج نشان داد که استفاده از سالیسیلیک اسید به صورت خیساندن خاک و محلول پاشی هوایی به ترتیب موجب کاهش ٪۲۸ و ٪۳۲ قطر گال‌ها، ٪۴۰ و ٪ ۴۴ تعداد گال در هر گیاه، ٪۴۵ و ٪۴۹ تعداد توده تخم در هر گیاه و نیز ٪۵۳ و ٪۵۵ تعداد تخم موجود در هر توده تخم در مقایسه با شاهد (گیاهان مایه زنی شده با نماتد) می‌گردد. فعالیت آنزیم‌های اشاره شده در تیمارهای مورد بررسی در مقایسه با گیاهان تیمار شده با آب مقطر استریل افزایش نشان داد. بیشترین درصد مرگ‌ومیر لارو در غلظت هفت میلی‌مولار سالیسیلیک اسید مشاهده شد که فاقد اختلاف معنی‌دار با غلظت شش میلی مولار و درصد مرگ‌ومیر لارو در غلظت پنج میلی‌مولار سالیسیلیک اسید ۲۱/۲ درصد بود.

واژگان کلیدی: سالیسیلیک اسید، *Meloidogyne javanica*، پراکسیداز، کاتالاز، فنیل آلانین آمونیالیاز