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Dose-Dependent Effects of Antioxidants on Root-Knot Nematode Infection in Vegetable Crops and Dosage Standardization for Preventive Measures

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Abstract: Different antioxidant compounds (ACs) were applied to vegetable plants as foliar spray or soil drench before inoculation with root-knot nematodes (RKNs). Different doses of salicylic acid (SA), methyl-salicylate (MetSA), methyl jasmonate (MetJA), and ascorbate (ASC) were tested; doses were chosen according to the size and weights of the plants to be treated. Generally, low doses of ACs increased nematode infection; conversely, when doses were raised, ACs acted as effective resistance inducers and reduced infection, measured as numbers of individuals developed in roots and reproduction rates. The activation of defense often occurred at the expense of plant fitness, although in infected plants, the benefits of treatments on plant growth were caused by relief from the symptoms monitored in untreated plants. Single pre-treatments of SA, MetSA, and ASC, in the proper amounts, almost halved infection variables; repeated applications of SA during nematode pathogenesis annulled the effectiveness of single pre-treatments. MetJA application was generally toxic to plants, and, also when provided in minimal amounts, this compound was always ineffective against nematodes. Other phenols and phenolic acids were tested at the same doses that were effective for SA with no reduction of infection except for duroquinone, which caused almost total suppression of infection, although associated with a decrease in root growth.

Keywords: antioxidants; ascorbate; salicylates; phenols; resistance induction; root-knot nematodes

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1. Introduction

Antioxidants are substances that, at low concentration compared with an oxidizable substrate, inhibit the oxidation of that substrate [1]. In aerobic organisms, most of the harmful oxidizing agents are reactive oxygen species (ROS), such as superoxide ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^\bullet$). Biotic stresses, such as attacks from soil-borne plant parasitic nematodes (PPNs), induce ROS generation at the sites of infection [2]. PPNs are small animal parasites of almost all crops worldwide. The most damaging and diffused nematodes are root-knot nematodes (RKNs), belonging to *Meloidogyne* spp. RKNs are sedentary endoparasites that, as vermiform invading juveniles (J2s), enter the root and migrate up to the vascular cylinder, where they establish their feeding site, become sedentary, develop into gravid females and reproduce [3]. Successful parasitism by these nematodes results in suppression of ROS generation exerted by different effectors secreted into root cells [4]. Conversely, if high levels of ROS are maintained in cells despite nematode action, building of feeding sites is contrasted by a hypersensitive reaction (HR), characterized by the death of the cells surrounding the head of invading juveniles, which can starve or leave the root [5]. Antioxidants, such as salicylic acid (SA), can act differently in the cells according to their concentration: they can have an anti-inflammatory effect and be ROS scavengers, or behave as pro-oxidants favoring inflammation and ROS generation [6].

SA, as such or in its methylated form (MetSA), is a plant hormone that has long been recognized to elicit a systemic acquired resistance (SAR) effective against foliar biotrophic

pathogens [7]. Exogenously added SA has been extensively reported to be effective in eliciting SAR and inhibiting infection in vegetable plants attacked by RKNs [8–10]. Therefore, SA can be an inducer of resistance against nematodes only if it is applied in such high amounts as to produce elevated concentrations in plants; high SA levels in cells support ROS generation and can trigger HR in response to the attempts of J2s to establish a feeding site in roots.

MetSA has already been reported to lessen RKN infection severity when plant roots are dipped in diluted solutions overnight [10]. Its eliciting effect was more beneficial than that of SA treatments in that it was more persistent and supported plant growth. On the other hand, another SA chemical analogue, acetyl-salicylic acid (Ac-SA), may induce resistance to pests by inhibiting ethylene generation that contributes to successful compatible plant–nematode interactions [11,12]. The effectiveness of treatments with the plant hormone jasmonic acid and its methylated form (Met-JA) in reducing infection by biotrophic parasites, such as RKNs that do not cause wounding during root penetration, is still a matter of debate [9]. Moreover, treatments with reduced ascorbate (ASC) were found to activate defense of rice against *M. graminicola* by means of its oxidation by ascorbate oxidase [13]. To obtain more insights into the actual ability of these chemicals in inducing resistance to RKNs, in this study, foliar sprays and soil drenches with different amounts of Met-SA/Met-JA and Ac-SA/ASC, respectively, were applied to vegetable plants. Polyphenol generation has long been considered as a response of plants to injury or invasion by pathogens, such as fungi, bacteria, viruses, and pests such as nematodes [14]. The resistant response to endoparasitic sedentary nematodes (ESNs) implies activation of the phenylpropanoid pathway that leads to synthesis of benzoic acid (BA) and SA, phytoalexins, chlorogenic acid and lignin [6,15]. BA together with a number of simple phenols, such as resorcinol (RESO), pyrogallol (PYRO) and guaiacol (GUA), were tested in this study as suppressors of RKN infection in vegetable plants, as they had been proved to have nematicidal activity and an inhibitory effect on egg hatching of *M. incognita* [16]. Lastly, treatments with duroquinone (DQ) were undertaken to investigate the impact it may have on nematode infection as a compound that diverts electrons from the alternative respiration to the mitochondrial cytochrome pathway, taking into account that nematodes use alternative respiration as ROS scavenger [5,17].

All of the phenolic compounds used as elicitors in this study are antioxidants, and antioxidants are generally recognized to play a major role in plant defense against biotic stresses [1]. Herein, phenols were applied separately, although an important trend of investigation uses phenolic mixtures extracted from different plant tissues as exogenously added antioxidants for plant protection from diseases and infections [18]. SA/MetSA have been revealed to be the most promising inhibitors of RKN infection; therefore, most of the experiments have focused on searching for the best amounts and application procedures to make their treatments a suitable practical method of control.

Finally, this investigation was undertaken to augment the environmentally friendly preventive practices available to farmers able to induce resistance in plants with an integrated nematode management alternative to pesticides [19].

2. Materials and Methods

2.1. Treatments of Vegetable Plants with Antioxidant Compounds

Roma VF (tomato, *Solanum lycopersicum* L.), Black Beauty (eggplant, *Solanum melongena* L.), and Theos (pepper, *Capsicum annuum* L.) were the cultivars used as vegetable plants susceptible to RKN infection. Rossol was used as the tomato cultivar resistant to RKNs. Seeds were germinated in a sterilized mixture of sand and soil (1:1 *w*/*w*) at 23–25 °C in a glasshouse. Seedlings at 4-leaf stage were singly transplanted into 100 cm^3 clay pots (100–150 g soil) filled with wild-collected loamy soil; soil in the pots was maintained at 23–25 °C by temperature-controlled benches located in a glasshouse (Figure 1).

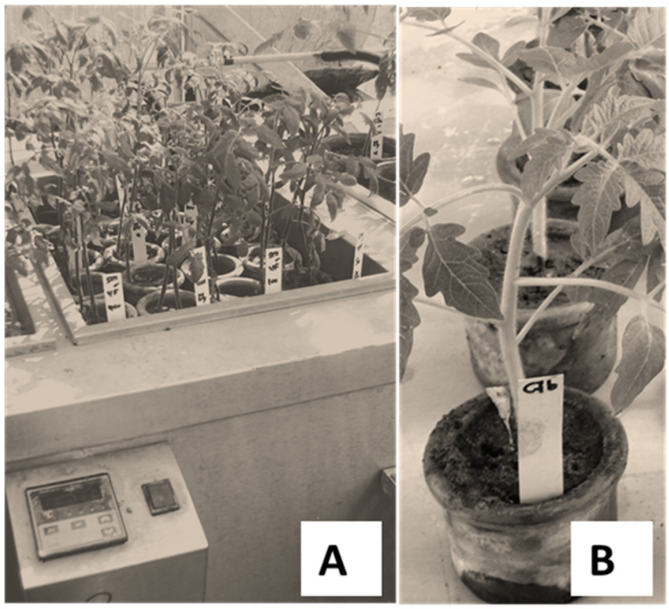

Figure 1. (**A**) Potted tomato plants growing in temperature-controlled benches; (**B**) single plants in 100 cm^3 clay pots.

Plants were provided with a 12 h light/dark regime and regularly watered with Hoagland's solution. Plants to be treated were grown to an average weight range of 3.0–5.0 g. The chemical structures of the antioxidant compounds used to treat the plants and test their effect on RKN infection are shown in Figure 2.

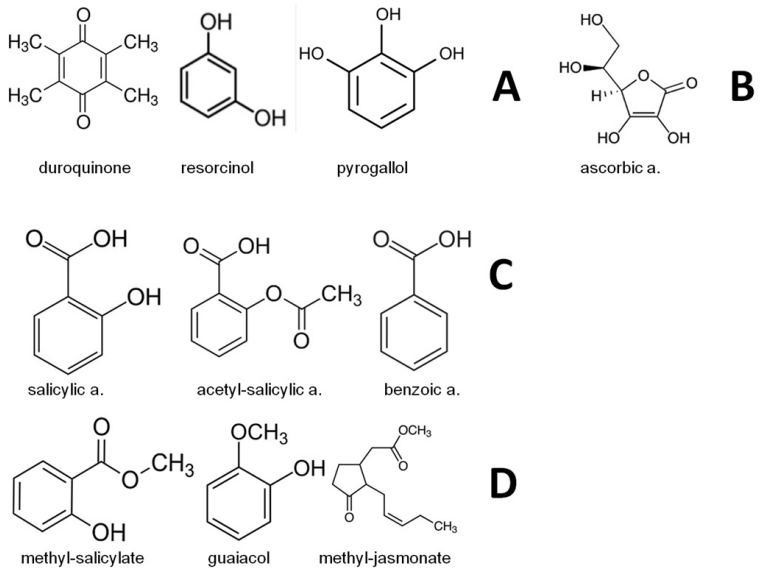

Figure 2. Chemical structures of the antioxidant compounds provided to plants as activators of defense against RKNs. (**A**) Hydro-benzenic species; (**B**) ascorbic acid; (**C**) phenolic acids; (**D**) methylated species.

Four different types of antioxidants were used: (Figure 2A) hydro-benzenic species (duroquinone "DQ", resorcinol "RESO", pyrogallol "PYRO"); (Figure 2B) ascorbic acid "ASC"; (Figure 2C) phenolic acids (salicylic "SA", acetyl-salicylic "AcSA", benzoic "BA"); (Figure 2D) methylated species (methyl-salicylate "MetSA", guaiacol "GUA", methyl-jasmonate "MetJA"). Applied dose ranges, type of application, treated plant species, and type of solvent for each tested antioxidant are shown in Table 1.

Table 1. Antioxidant compounds (ACs) used in the treatments, type of application, plant species to which treatments were applied, dose ranges, expressed as mg g^{-1} pfw (plant fresh weight at treatment), and type of dissolving medium for compounds soluble and insoluble in water. In bold, dose ranges able to inhibit nematode infection without costs or with acceptable costs to plant fitness.

AC	Type of Application	Plant Species	Applied Dose Ranges	Dissolving Medium
SA1-2-3	foliar spray	susceptible tomato	0.5–2.0-; **3.0–4.0**; 8.0–10.0	H_2O, pH 6.0 with KOH
SA1-2-3	soil drench	susceptible tomato	1.0–2.5; **4.0–10.0**; 13.0–30.0	H_2O, pH 6.0 with KOH
SA1	soil drench	resistant tomato	**2.5–6.0**	H_2O, pH 6.0 with KOH
SA1-2	soil drench	egg plant	0.8–1.7; **3.0–6.5**	H_2O, pH 6.0 with KOH
SA1	soil drench	pepper	**1.5–1.8**	H_2O, pH 6.0 with KOH
MetSA1-2-3	foliar spray	susceptible tomato	0.4–0.6; **2.0–5.0**; 10.0–20.0	Ethanol 95%
AcSA1	soil drench	susceptible tomato	5.0–7.0	H_2O, pH 6.0 with KOH
MetJA1-2	foliar spray	susceptible tomato	1.0–2.5; 6.5–8.3	Acetone
ASC1-2	soil drench	susceptible tomato	0.6–0.8; **1.5–5.0**	H_2O, pH 6.0 with KOH
BA1	soil drench	susceptible tomato	2.0	H_2O, pH 6.0 with KOH
GUA1	soil drench	susceptible tomato	**5.0**	Ethanol 95%
DQ1	soil drench	susceptible tomato	**2.5**	Ethanol 95%
PYRO1	soil drench	susceptible tomato	3.0	Ethanol 95%
RESO1	soil drench	susceptible tomato	5.0	H_2O

Solutions of SA, Met-SA, and MetJA were sprayed on the green parts of the plants; solutions of SA and other antioxidants were soil-drenched to plants. All water-soluble acidic compounds were dissolved by adding KOH to reach approx. pH 6. The tested antioxidant compounds (ACs) that were poorly soluble in water were dissolved first in minimal amounts of 95% ethylic alcohol or acetone; then, distilled water was added to achieve the fixed concentrations. Small volumes of solutions were sprayed on groups of 6 plants in pots, the surfaces of which were covered with aluminum foil to prevent the sprayed liquid from being absorbed into the soil. Plants were soil-drenched by pipetting a few ml of AC solution directly on the surface of the pot soil. Controls consisted of plants treated with solutions without ACs.

Each dose was applied 1 to 7 days before nematode inoculation. Tomato plants were soil-drenched with one range of SA doses (5.0–10.0) thrice: 1 day before and 7 and 14 days after nematode inoculation.

2.2. Procedures for Plant Inoculation with Nematodes

One population of the RKN *Meloidogyne incognita* (Kofoid *et* White) Chitw., long reared on susceptible tomato in a glasshouse, was used for plant inoculation. One lab-selected virulent isolate (*SM2V*) was used to break the resistance of the tomato cv. Rossol. Egg masses of heavily infested roots were manually excised, put on 10 cm diameter 500 mesh sieves, and incubated in tap water at 25 °C in the dark. Freshly hatched active second-stage juveniles (J2s) were collected until the third day of incubation and put in a refrigerator. J2s were then concentrated by filtering through 500 mesh sieves and counted in 1 mL suspension samples. Two holes were made in the soil at the base of each potted plant into which a few ml of a stirred J2 suspension were poured so as to inoculate each plant with 100–300 J2. Inoculations were performed 1–7 days after AC treatments. Inoculations of control and treated plants was performed, and groups of control and treated plants were also left uninoculated to test the effects of AC treatments on plant growth in the absence of nematodes.

2.3. Measurements of Plant Growth and Nematode Infection Variables

Plants were harvested 40 days after inoculation (DAI). Plant growth indices at the end of the experimental period were shoot (SW) and root (RW) weight, expressed in grams. These measurements were performed as soon as plants were uprooted and roots washed free of soil debris. A slight decrease in SW in AC-treated plants was considered as a result of the fitness costs associated with elicitation of highly effective defenses (priming)

against nematodes. Conversely, high decreases in SW (≥20% compared to untreated plants) were considered to have two possible meanings: (1) an excessive trade-off between major resistance to infection and impaired plant growth; (2) direct toxicity of the chemical dose to plants. In these cases, the relative doses were considered as not applicable, regardless of their suppressive effect on nematode infection. Furthermore, it should be noted that, normally, highly infected roots show a higher weight compared with less infected or healthy roots because of tissue hypertrophy caused by galls.

AC effects on the level of nematode infection were tested either on the reproduction rate of the nematode population or the level of damage caused to plants according to the degree of root galling. Numbers of galls are proportional to the amounts of sedentary developing individuals (sedentary forms, SFs: J3s, J4s and swollen females) in the roots. The numbers of SFs per root system were used to indicate the level of plant damage. Reproduction rate indicates the potential (reproduction potential, RP) of a population to multiply its initial J2 population density (P_i), which in the present experiment was equal to the numbers of inoculated J2s. Therefore, RP was calculated as:

$$RP = P_f/P_i$$

where P_f is the final population density; moreover, in small pots

$$P_f = EMs \times FF$$

P_f, then, may be calculated as the number of egg masses (EMs) multiplied by female fecundity (FF), that is, the average number of eggs contained in one EM [19]. Reproduction rate was also expressed by the numbers of EMs per root system (EMs) and FF. When plants were harvested at 40 DAI, only the inoculated J2s were able to develop in egg-laying gravid females and produce EMs under the used experimental conditions; conversely, J2s hatched from eggs laid in the pot soil were able to develop only into SFs, because the elapsed time was not sufficient for them to produce EMs. That is why total SF numbers may have exceeded the numbers of inoculated J2s.

From plants under each treatment, two root systems were chopped together to have one sample for detection of infection variables. Samples were divided into three subsamples that were weighed and used for extraction and counting of: (i) EMs; (ii) SFs; (iii) eggs. For EM detection, root tissue was immersed in a solution (0.1 g L^{-1}) of the colorant Eosin Yellow for at least 1 h and put in a refrigerator. EMs were red-colored and easily visible under a stereoscope (×6 magnification). Then, they were manually separated from the roots by forceps and counted. SF extraction was carried out by incubating the roots in a diluted mixture of pectinase and cellulase enzymes at 37 °C in an orbital shaker for 1–2 h. After a brief homogenization in physiological solution, sedentary forms were collected on a 90 µm sieve. Aliquots (2 mL) of stirring suspensions were pipetted into small Petri dishes, and SFs counted under a stereoscope (×12 magnification). Eggs were extracted by the sodium hypochloride method and counted (1 mL samples) under a stereoscope (×25 magnification) [20].

2.4. Experimental Design and Statistical Analysis

Groups of six plants per each dose of treatment were used in the experiments. Each functional range of AC amounts was constituted by at least three different tested doses; three dose ranges collected treatments with doses that were: (i) ineffective or significantly supportive of nematode infection; (ii) significantly suppressive of nematode infection; (iii) toxic to plants. Therefore, value means for indicators of plant growth and nematode infection came from at least nine replicates (3 replicates/experiment by 3 experiments). Values were always presented as means ± standard deviations ($n \geq 9$). For every dose or range of doses, means of control plants were separated from means of treated plants by a paired *t*-test (* $p < 0.05$; ** $p < 0.01$), using Excel software. In figures, data are shown by bars representing means ± standard deviations in percentage, where means of control plants

are set at 100%; in tables, means ± standard deviations are reported in absolute values along with percentages of difference when found significantly different by a paired *t*-test (* $p < 0.05$; ** $p < 0.01$).

3. Results

3.1. Effects of Different Doses of SA, Met-SA, and MetJA, Provided as Foliar Spray, on Plant Growth and Nematode Infection

Effects of sprayed SA on plant growth and nematode infection in tomato plants are shown in Figure 3. Three different dose ranges (SA1, SA2, SA3) had different impacts on plant growth and infection level.

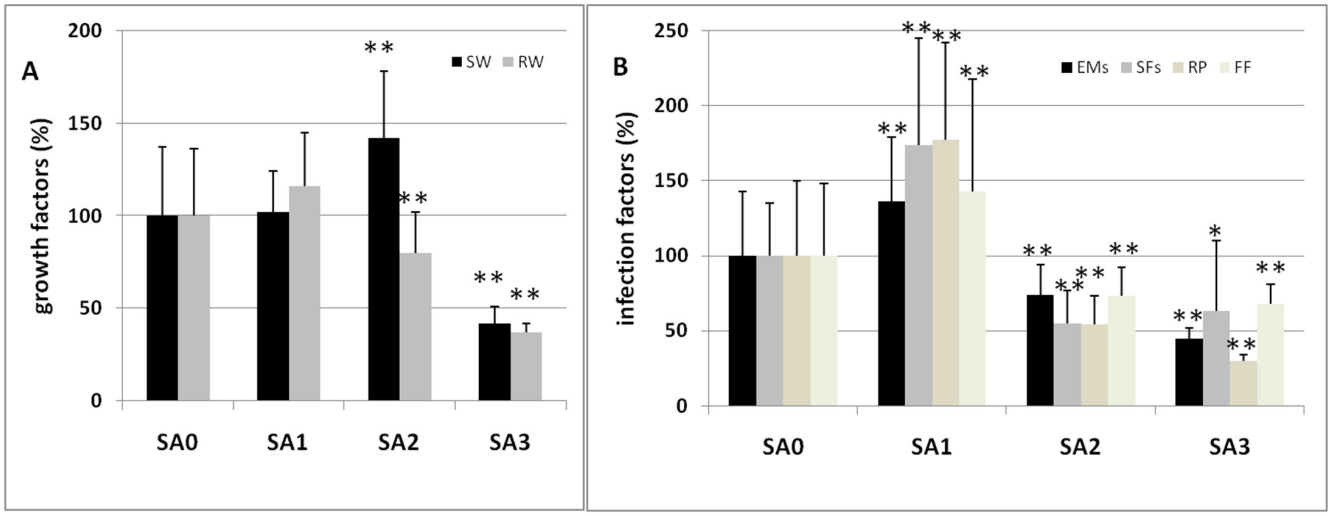

Figure 3. Effects of sprayed SA on plant growth (**A**) and nematode infection (**B**) in tomato plants 40 days after *M. incognita* inoculation. Growth factors were shoot (SW) and root (RW) weights. Infection was evaluated by the numbers of egg masses (EMs) per root system, the numbers of sedentary forms (SFs) per root system, reproduction potential (RP), and female fecundity (FF). Values for SA-treated plants are expressed in percentages with respect to untreated plants (set at 100%—SA0). Values ± standard deviations of controls were statistically differentiated from those of treatments by a paired *t*-test (* $p < 0.05$; ** $p < 0.01$). Dose ranges are expressed as mg SA g^{-1} plant fresh weight at treatment: SA1 0.5–2.0; SA2 3.0–4.0; SA3 8.0–10.0.

Only SA2 favored shoot growth (Figure 3A) and reduced about 50% of nematode infection (Figure 3B) in tomato plants. SA2 treatments markedly reduced both development and reproduction of nematodes with respect to untreated plants. Conversely, SA1 treatments increased about 50% nematode infection indicators. SA3 was toxic to plants and markedly reduced root growth; smaller roots were able to sustain only a contained nematode infection. Infection in untreated plants was heavy (SFs above 200) with high RPs (approx. 200); SA2 treatments were able to reduce control values of SFs to 44% and of RPs to 39% (Table S1).

Foliar sprays with three dose ranges of methylated salicylic acid had similar effects on both plant growth and nematode infection (Figure 4).

MetSA1 favored, whereas MetSA2 repressed, infection, and MetSA3 was toxic to plants. Surprisingly, MetSA2-mediated infection reduction did not result in higher plant growth, thus indicating that such MetSA amounts had a primary inhibiting effect on shoot growth. In these experiments, infection in untreated plants was characterized by a high number of small EMs, that is, egg masses with fewer eggs (low FF, Table S2). MetSA2 halved the severity of infection, while MetSA1 doubled it; MetSA1-mediated heavy infection of plants was evident in the highly galled root tissue (RW 55% higher than that of MetSA0). In uninoculated plants, SA2 caused an increase (20%) in SW and a decrease (−31%) in RW, while MetSA1 favored both shoot and root development (Table S3).

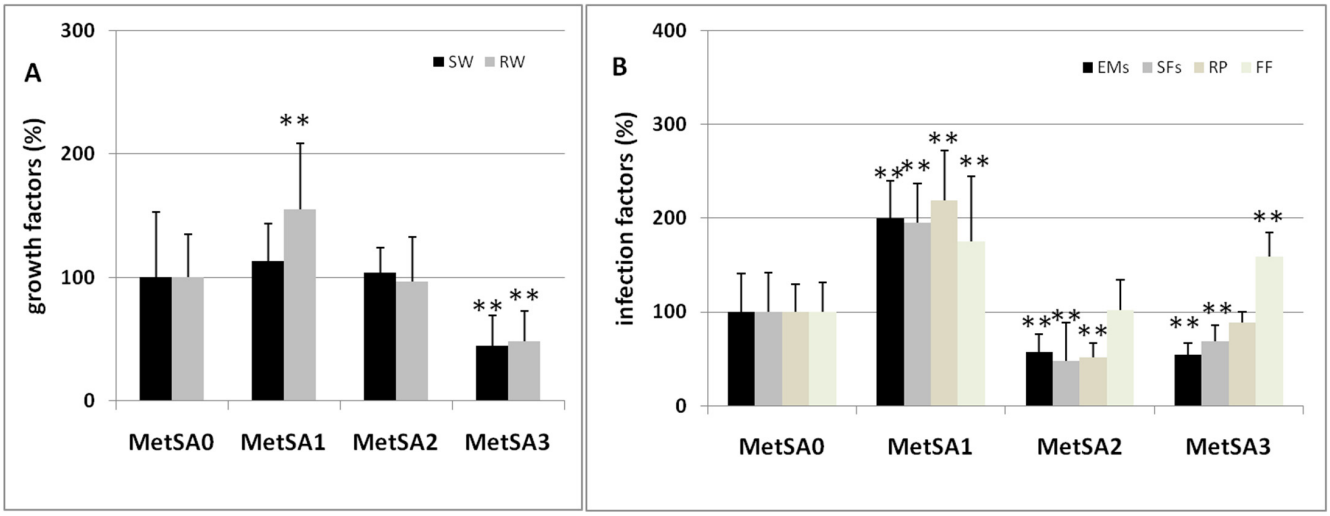

Figure 4. Effects of sprayed MetSA on plant growth (**A**) and nematode infection (**B**) in tomato plants 40 days after *M. incognita* inoculation. Growth factors were shoot (SW) and root (RW) weights. Infection was evaluated by the numbers of egg masses (EMs) per root system, the numbers of sedentary forms (SFs) per root system, reproduction potential (RP), and female fecundity (FF). Values for MetSA-treated plants are expressed in percentages with respect to untreated plants (set at 100%—SA0). Values ± standard deviations of controls were statistically differentiated from those of treatments by a paired *t*-test (** $p < 0.01$). Ranges of doses are expressed as mg MetSA g^{-1} plant fresh weight at treatment: MetSA1 0.4–0.6; MetSA2 2.0–5.0; MetSA3 10.0–20.0.

Treatments with two dose ranges of methyl-jasmonate gave poor results in terms of control of nematode infection (Table 2). Low doses (MetJA1) slightly reduced nematode reproduction (about 20%) and were ineffective against nematode development; higher doses (MetJA2) were toxic to plants.

Table 2. Plant growth and infection variables detected 40 days after inoculation with RKN J2s (200 J2/plant), in untreated (cntr) and MetJA-treated tomato. MetJA was applied to plants by foliar spray. Tomato plants were treated with two ranges of doses (expressed as mg MetJA g^{-1} plant fresh weight at treatment): MetJA1 1.0–2.5, MetJA2 6.5–8.3. Plant growth was assessed by shoot (SW) and root weights (RW) expressed in grams; nematode infection severity was indicated by egg mass per root system (EMs), sedentary forms per root system (SFs), female fecundity (FF), and reproduction potential (RP). Mean values ± standard deviations obtained from untreated and MetJA-treated tomato plants are differentiated by a paired *t*-test (* $p < 0.05$; ** $p < 0.01$). In parentheses, significant differences are expressed in percentages.

	Cntr	+MetJA1	Cntr	+MetJA2
SW	11.4 ± 4.3	9.7 ± 5.5 * (−14)	10.6 ± 3.8	6.8 ± 2.3 ** (−36)
RW	2.4 ± 1.5	2.0 ± 1.5 * (−15)	2.6 ± 1.2	1.3 ± 0.7 ** (−49)
EMs	70 ± 22	56 ± 25 ** (−21)	75 ± 47	62 ± 60
SFs	339 ± 102	310 ± 117	345 ± 92	154 ± 98 ** (−55)
FF	319 ± 182	283 ± 190	363 ± 154	366 ± 89
RP	60 ± 28	46 ± 23 ** (−23)	77 ± 28	70 ± 19

3.2. Effects of Different Doses of SA on Plant Growth and Nematode Infection Provided as Soil Drench

Tomato plants were soil-drenched with 3 dose ranges of SA (SA1 1.0–2.5; SA2 4.0–10.0; SA3 13.0–30.0, expressed as mg SA g^{-1} plant fresh weight at treatment). SA2 treatments were very effective in reducing nematode infection, and increasing plant growth (Figure 5). SA1 doses were too low to affect control parameters; SA3 elicited a plant response, although the resulting fitness costs were too high to consider such treatments as effective.

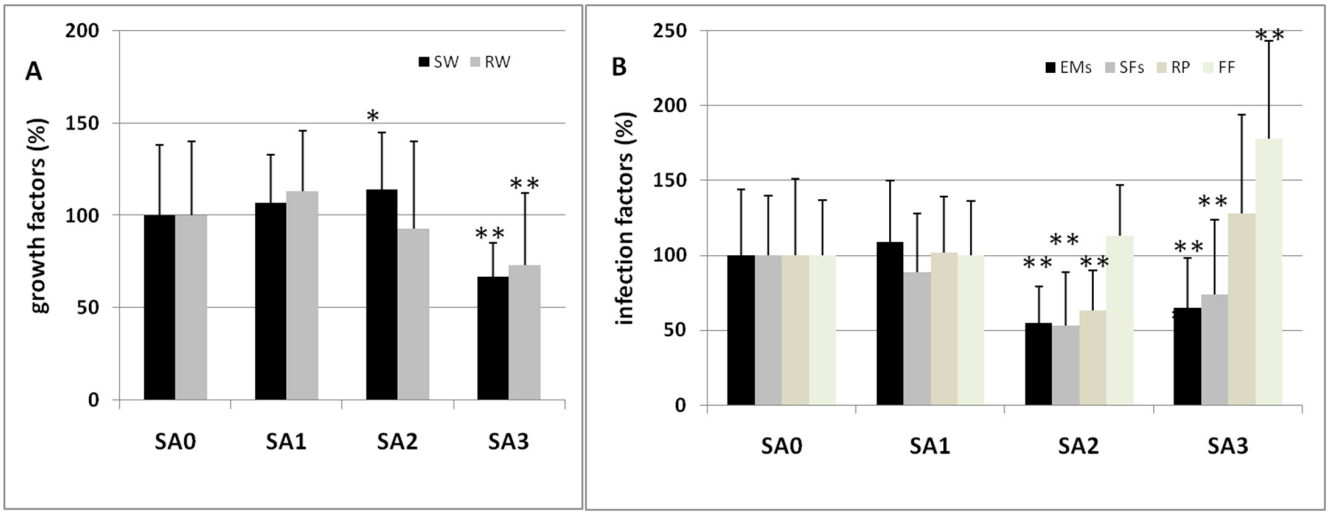

Figure 5. Effects of soil-drenched SA on plant growth (**A**) and nematode infection (**B**) in tomato plants 40 days after *M. incognita* inoculation. Growth factors were shoot (SW) and root (RW) weights. Infection was evaluated by the numbers of egg masses (EMs) per root system, the numbers of sedentary forms (SFs) per root system, reproduction potential (RP), and female fecundity (FF). Values for SA-treated plants are expressed in percentages with respect to untreated plants (set at 100%—SA0). Values ± standard deviations of controls were statistically differentiated from those of treatments by a paired *t*-test (* $p < 0.05$; ** $p < 0.01$). Ranges of doses were expressed as mg SA g^{-1} plant fresh weight at treatment: SA1 1.0–2.5; SA2 4.0–10.0; SA3 13.0–30.0.

In these experiments, nematode infection in control plants was very consistent with averages of about 150 EMs and about 450 SFs (Table S4); SA2 treatments, with only one pre-treatment of chemical, were able to halve the severity of infection. SA2 was found to restrain plant growth when nematode inoculation was not carried out (Table S5). However, the benefits to plant growth that these treatments gave because of the relief from infection damage outweighed the negative effect of the chemical on plant development.

Since the effectiveness of SA2 treatments was remarkable, a series of controls were carried out. The points to be addressed were: (i) what happens if application of SA2 is repeated during the initial nematode cycle in roots, for instance, 7 and 14 days after inoculation? Should the effectiveness increase?; (ii) are SA2 treatments effective against virulent populations developing in resistant tomato?; (iii) is acetyl-salicylic acid (AcSA) effective as SA?

Data from experiments to address these points are shown in Table 3. Increasing the number of SA2 applications apparently invalidated the effectiveness against nematodes observed with a single pre-application; SA2 was effective in reducing reproduction rates also of virulent populations in resistant tomato, but less effective in reducing the development rate; doses of AcSA similar to those used for SA were not effective at all.

Another very important question was to be answered: is the effectiveness of SA in reducing nematode infection maintained in other good hosts of RKNs, such as eggplant and pepper? The ranges of doses for eggplant were proved to be similar to those for tomato in their effects; surprisingly, pepper plants were found to be more sensitive to SA, in that the same low doses that did not work in tomato and eggplant were highly effective in pepper (Table 4). Low doses of SA (SA1) applied to eggplants strikingly increased infection factors by about three-fold those of untreated plants. If doses were raised to SA2, treatments caused 61% reduction in SFs in roots compared to infected control plants; reproduction rates were also reduced (about 25%), but by much less. Pre-applications of low doses of SA were sufficient to markedly reduce nematode infection in pepper; however, in this case, the very low numbers of EMs produced on roots of treated plants allowed females to lay many

more eggs (high FF) due to low competition for food, thus increasing the reproduction rate. This process reduced the differences in RP between the control and treated plants.

Table 3. Plant growth and infection factors detected 40 days after inoculation with RKN J2s (250 J2/plant), in untreated (cntr) and chemical-treated tomato. SA (5.0–10.0 mg g^{-1} plant fresh weight at treatment) was applied thrice to susceptible plants by soil drench: 1 day before inoculation and 7 and 14 days after inoculation. One pre-treatment of acetyl-salicylic acid (AcSA, 5.0–7.0 mg g^{-1} plant fresh weight at treatment) was also applied to susceptible plants. Resistant (res) plants were treated with 2.5–6.0 mg SA g^{-1} pfw and inoculated with a virulent population. Plant growth was assessed by shoot (SW) and root weights (RW) expressed in grams; nematode infection severity is indicated by numbers of egg masses (EMs) per root system, sedentary forms (SFs) per root system, female fecundity (FF), and reproduction potential (RP). Mean values ± standard deviations obtained from untreated and AC-treated tomato plants were differentiated by a paired *t*-test (* $p < 0.05$; ** $p < 0.01$). In parentheses, significant differences are expressed in percentages.

	Susc Cntr	**+3 × SA**	**Susc Cntr**	**+AcSA**	**Res Cntr**	**+SA**
SW	11.5 ± 4.6	13.0 ± 6.4 * (13)	11.7 ± 7.6	10.0 ± 6.2 * (−14)	11.6 ± 3.1	10.8 ± 2.3
RW	2.3 ± 0.7	2.8 ± 0.9 * (22)	1.5 ± 0.7	1.4 ± 1.2	3.5 ± 1.4	3.1 ± 0.8 * (−12)
EMs	92 ± 53	84 ± 53	95 ± 47	69 ± 35 * (−27)	98 ± 29	62 ± 16 ** (−37)
SFs	271 ± 74	201 ± 127 ** (−26)	247 ± 141	459 ± 322 * (86)	329 ± 111	246 ± 157 ** (−25)
FF	281 ± 97	406 ± 193 * (44)	nd	nd	328 ± 191	296 ± 170
RP	152 ± 59	191 ± 81 * (26)	nd	nd	182 ± 71	110 ± 30 ** (−40)

nd = not determined.

Table 4. Plant growth and infection factors detected 40 days after inoculation with RKN J2s (200 J2/plant), in untreated (cntr) and SA-treated eggplant and pepper. SA was applied to plants by soil drench in pots. Eggplants were treated with two ranges of doses (expressed as mg SA g^{-1} plant fresh weight at treatment: SA1 0.8–1.7, SA2 3.0–6.5; pepper with SA 1.5–1.8). Plant growth was assessed by shoot (SW) and root weights (RW) expressed in grams; nematode infection severity is indicated by egg mass (EMs) per root system, sedentary forms (SFs) per root system, female fecundity (FF), and reproduction potential (RP). Mean values ± standard deviations obtained from untreated and SA-treated tomato plants were differentiated by a paired *t*-test (* $p < 0.05$; ** $p < 0.01$). In parentheses, significant differences are expressed in percentages.

	Eggplant Cntr	**+SA1**	**Eggplant Cntr**	**+SA2**	**Pepper Cntr**	**+SA**
SW	7.7 ± 1.9	7.0 ± 1.9	6.7 ± 2.6	6.7 ± 2.3	11.3 ± 3.3	13.9 ± 8.1 * (22)
RW	1.7 ± 0.8	2.8 ± 1.3 ** (68)	1.7 ± 0.7	1.8 ± 0.9	3.7 ± 1.3	3.0 ± 1.6 * (−18)
EMs	43 ± 20	192 ± 106 ** (352)	40 ± 26	25 ± 24 ** (−35)	51 ± 14	22 ± 11 ** (−57)
SFs	130 ± 66	567 ± 355 ** (337)	247 ± 90	97 ± 39 ** (−61)	254 ± 95	119 ± 64 ** (−53)
FF	389 ± 126	262 ± 146 * (−33)	301 ± 124	312 ± 166	313 ± 108	525 ± 216 ** (68)
RP	44 ± 22	154 ± 81 ** (251)	57 ± 23	44 ± 16 * (−23)	78 ± 24	57 ± 28 ** (−28)

3.3. Effects of ASC and Other Antioxidants on Plant Growth and Nematode Infection Provided as Soil Drench to Tomato Plants

Low doses of ASC (ASC1) markedly increased the severity of infection mainly by favoring the penetration and development of J2s into sedentary forms (Table 5). Therefore, low doses of both salicylates and ASC promoted nematode parasitism in roots, causing infections heavier than those occurring in untreated plants. However, in the case of ASC1 treatment, plant weights were found to be higher than in untreated plants due to the high growth-promoting effect of ascorbate. When higher doses of ASC (ASC2, 1.5–5.0 mg g^{-1} pfw) were used, ascorbate did not show any growth-promoting effect, despite not having a repressive effect on nematode infection.

Table 5. Plant growth and infection variables detected 40 days after inoculation with RKN J2s (100 J2/plant), in untreated (cntr) and ascorbic acid (ASC)-treated tomato. Two ranges of doses of ASC (ASC1 0.6–0.8; ASC2 1.5–5.0 mg g^{-1} pfw) were applied to susceptible plants by soil drench 1 day before inoculation. Plant growth was assessed by shoot (SW) and root weights (RW) expressed in grams; nematode infection severity is indicated by egg masses (EMs) per root system, sedentary forms (SFs) per root system, female fecundity (FF), and reproduction potential (RP). Mean values ± standard deviations obtained from untreated and ASC-treated tomato plants were differentiated by a paired *t*-test (* $p < 0.05$; ** $p < 0.01$). In parentheses, significant differences are expressed in percentages.

	Cntr	+ASC1	Cntr	+ASC2
SW	6.7 ± 3.7	9.1 ± 4.5 * (35)	10.2 ± 5.1	10.7 ± 4.8
RW	1.8 ± 1.4	2.1 ± 1.0	1.4 ± 1.0	1.4 ± 0.8
EMs	31 ± 11	69 ± 28 * (127)	40 ± 24	22 ± 24 ** (−45)
SFs	106 ± 35	324 ± 174 ** (206)	151 ± 84	78 ± 69 ** (−48)
FF	433 ± 197	387 ± 193	311 ± 159	287 ± 201
RP	107 ± 23	129 ± 26 * (20)	86 ± 28	54 ± 31 ** (−37)

Single doses of the simple phenols PYRO (3.0 mg g^{-1} pfw), BA (2.0 mg g^{-1} pfw), GUA (5.0 mg g^{-1} pfw), DQ (2.5 mg g^{-1} pfw), and RESO (5 mg/g pfw) were soil-drenched to tomato plants and tested as elicitors of defense against RKNs (Table 6). PYRO favored nematode infection and impaired plant growth. Conversely, BA, although supporting nematode parasitism, had a positive effect on SWs. RESO did not affect the level of infection. GUA and DQ caused a moderate and an elevated repression of infection, respectively. DQ markedly reduced RWs; however, infection parameters were lowered also if expressed per g of roots.

Table 6. Plant growth and infection factors detected 40 days after inoculation with RKN J2s (100 J2/plant), in tomato plants untreated (cntr) and treated with benzoic acid (BA 2.0 mg/g pfw), guaiacol (GUA 5 mg/g pfw), duroquinone (DQ 2.5 mg/g pfw), pyrogallol (PYRO, 3 mg/g pfw), and resorcinol (RESO, 5 mg/g pfw) by soil drench 1 day before inoculation. Plant growth was assessed by shoot (SW) and root weights (RW) expressed in grams; nematode infection severity is indicated by egg masses (EMs) per root system and sedentary forms (SFs) per root system. Mean values ± standard deviations obtained from untreated and AC-treated tomato plants were differentiated by a paired *t*-test (* $p < 0.05$; ** $p < 0.01$). In parentheses, significant differences are expressed as percentages.

	Cntr	+BA	+GUA	+DQ	+PYRO	+RESO
SW	16.6 ± 7.6	20.8 ± 7.6 ** (25)	20.4 ± 9.4 ** (23)	15.2 ± 5.1	9.7 ± 3.6 ** (−42)	14.1 ± 4.2
RW	1.4 ± 0.7	2.3 ± 1.7 * (68)	1.7 ± 0.8 * (23)	0.8 ± 0.2 * (−45)	1.0 ± 0.5 * (−29)	1.1 ± 0.5 * (−23)
EMs	46 ± 12	100 ± 77 * (119)	37 ± 12 * (−19)	6 ± 2 * (−87)	94 ± 36 * (106)	44 ± 8
SFs	110 ± 31	428 ± 318 * (285)	81 ± 31 * (−26)	30 ± 3 * (−73)	247 ± 65 * (124)	120 ± 22

4. Discussion

Exogenously added SA has long been reported to suppress RKN parasitism in vegetable plants by inducing SAR [8]. High concentrations of external SA are needed to increase intracellular SA levels so as to prime plants against biotic challenges. This antioxidant molecule can act as a proinflammatory agent through the potentiation of ROS generation that, as oxidative burst, has been recognized to cause HR, cell death and lesion formation in tissues surrounding the invading juvenile [21,22]. In contrast, at low concentrations, SA acts as a powerful antioxidant with a protective role against oxidative stresses of biotic and abiotic origin [6]. The initial phases of nematode parasitism imply the generation of ROS, and H_2O_2 in particular, at the surface of cells and in the apoplasm [2]. Processes that favor this inflammatory response, such as high levels of apoplasmic SA, contribute to limit nematode invasion and settlement. Conversely, in unprimed plants, nematodes rearrange plant metabolic flux to enhance antioxidant enzyme activities and antioxidant generation,

thus suppressing such an inflammatory response [5]. Therefore, the choice of the antioxidant amounts that should be provided to plants in order to restrict nematode infection is extremely important. According to the provided amounts on a plant size basis, these compounds may act both as defense activators and suppressors. Of course, the provided amounts determine the quantities actually absorbed by plants; however, absorption by leaves of sprayed chemicals or chemicals bound to soil by roots may be quantitatively and qualitatively different.

In this study, foliar spray and soil drench of antioxidant aqueous solutions were both tested. The first observation was that the amounts of provided antioxidants must be referred to plant weights and expressed per gram of plant weight at treatment. The same amount can induce different effects on plant growth and defense responses to nematode attack, according to plant age and size [10]. Low doses of antioxidants had a generally promoting effect on nematode development and reproduction. At such low concentrations, these antioxidants contrast the defensive inflammatory response of plants against the invading parasites, thus allowing more juveniles to settle, develop, and reproduce in the roots. In healthy plants, foliar sprays with low amounts of salicylates did sustain plant growth; when plants were inoculated, this growth-promoting effect disappeared because of the severe symptoms from nematode-mediated disease.

Conversely, low doses of soil-drenched SA did not trigger any response in plants, probably because binding to pot soil impeded quantitative SA transfer into roots. Actually, the bioavailability of SA in soil is generally lower than in water solutions, as SA actively binds to soil humic acids [23]. It should be noted that the soil used in the experiments in this study was a loamy soil rich in humic substance. When the amount of soil-drenched SA was increased by many folds, SA did affect plant growth and response to nematodes, acted on healthy plants as a growth inhibitor, and reduced infection in inoculated plants; increasing doses (>10 mg g^{-1} pfw) did not improve the capability of SA to lessen infection parameters and caused an approx. 30% decrease in shoot and root weights. In these cases, treatments are not advisable because the trade-off between fitness costs and pest control is not convenient. Moreover, such excessive amounts of SA caused transient symptoms of leaf toxicity for many days after treatment. Therefore, it is evident that SA can be used as a defense activator against RKNs in vegetable plants only in a strict range of doses that must be arranged after a preventive screening carried out according to the specific plant–crop interaction, environmental conditions, soil texture, and preferred method of application.

The data presented herein are a clear demonstration that generalization of the efficacy/inefficacy of a certain resistance inducer is always misleading, because chemicals can act both as inducers or suppressors of resistance according to the provided amounts. In fact, repeated applications of soil-drenched SA impaired the successful suppressive effect of the single pre-treatment, instead of increasing it. Effective dosages can even not be the same for chemical analogues. Treatments with acetylated SA, at doses at which SA was effective, were conversely supportive of J2 development in roots. Comparably, it has already been reported that AcSA may suppress plant defense reactions in potato [24].

On the other hand, SA treatments were also effective at reducing nematode infection in eggplant and pepper as well as in a resistant tomato cultivar attacked by a virulent nematode population. However, when the numbers of egg masses per root system are strongly reduced, as occurred in SA-treated pepper (about 22 EMs), female fecundity usually increases because of the low competition for food. In these cases, the inhibitory effect of resistance inducers in terms of overall reproduction rates may be weaker.

Physiological costs of induced resistance have long been debated, thus questioning the convenience of such methods for disease control [25]. Therefore, in this study, the applied doses were considered as effective only if physiological costs of treatments were overcome or balanced by the physiological benefits of infection reduction. Otherwise, the application of activators becomes a disadvantageous practice in terms of crop development and yield. However, the sole strong reduction of nematode reproduction would decrease the initial population in the next crop to non-damaging limits [26].

Some chemicals can be mistaken for resistance elicitors because they inhibit root development and, consequently, apparently reduce parameters of nematode infection. Nematode infection is reduced because of feed scarcity and not because of augmented plant defense. MetJA has been found to be one of these chemicals, as already reported for JA [9]. Even minimal amounts of MetJA, sprayed onto leaves of tomato plants before nematode inoculation, already had a negative impact on root growth and no real effectiveness against nematode infection, which was lower, compared to controls, only because of the development of smaller roots. Higher amounts of MetJA were directly toxic to plants. It is known that a JA-dependent signaling pathway is activated upon wounding and wound-promoting attacks of herbivores and necrotrophic pathogens, as well as to be active in root growth inhibition [7,27]. In the specific tomato–RKN interaction, jasmonates do not counter the development of J2s; conversely, they probably support the establishment of functional feeding structures in the physiological reaction to nematode attack [9]. However, the average amounts used in studies reported in the literature probably cause concentrations higher than the physiological levels inside the plants, thus restraining root development and mimicking a positive response if plants are inoculated with nematodes.

Soil-drench treatments with ascorbate produced the same effects observed with SA treatments. High doses activated defense against RKNs, as has recently been reported for rice [13]. Duroquinone has been shown to function as a carrier accepting electrons from the NADH dehydrogenase portion of the respiratory chain and, as reduced durohydroquinone, donating electrons at a point between the natural quinone and cytochrome *b* or directly to cytochrome *b* [28]. Treatments with DQ almost completely reduced nematode infection in tomato, although, contextually, its inhibitory effect on root growth was evident. It could seem that DQ is another false defense activator, although if we measure infection factors per gram of root fresh weight, restriction of the infection is still observable. However, it is possible that such a high activation of defense against the pest induced by DQ required high metabolic efforts at the expense of root growth alone, since shoots were found not to be reduced in weight. It is generally known that the conversion of plant respiration towards the cyanide (CN)-resistant alternative respiration is promoted in roots of nematode-infected plants and functions as a scavenger of ROS [5,17]. Probably, duroquinone favors the rate of the electron transport chain through the mitochondrial cytochrome pathway, thus diverting electrons from CN-resistant respiration and countering nematode development. Benzoic acid is most probably converted into SA in challenged ROS-producing roots [6]. Probably, the dose tested as soil drench for BA was sufficient to reach root cells and be converted into very low amounts of SA that, at such low levels, has already been shown to act as an antioxidant suppressive of plant defense. Other antioxidant compounds had various effects on nematode infection. Hydro-benzenic species, such as resorcinol and pyrogallol, at the tested doses, did not affect infection and had a toxic effect on plants; conversely, one methylated species, guaiacol, had a slightly negative effect on infection and a positive effect on plant growth.

5. Conclusions

Antioxidants can be used as plant defense priming inducers against RKNs, as they have been found to halve nematode development and reproduction with a single pretreatment in proper amounts. Salicylates have been proved to be the most efficient along with ascorbate, while a possible role of duroquinone should be further investigated. However, doses should be set up in order to achieve elevated concentrations in root cells to induce a pro-oxidative and proinflammatory response that can counter the settlement and development of the invading nematode juveniles in roots but not provoke excessive costs in terms of plant fitness and growth in the long term. Wrong dosages, too low or too high, may worsen the infection factors or prove toxic to plants. The effective amounts of oxidants to be used for nematode management must be set according to the size and weight of plants at the time of treatment. Resistance inducers must be applied before possible infection by nematodes as a measure of prevention. Once nematodes are settled

in the roots and start to develop into females, induction of plant defense by treatments with activators does not impede the spread of the pest and severe plant damage; therefore, treatments with antioxidants such as SA, MetSA, and ASC should never be considered as a curative measure.

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