

Article

Evaluation of Multiple Impacts of Furfural Acetone on Nematodes In Vitro and Control Efficiency against Root-Knot Nematodes in Pots and Fields

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Abstract: Root-knot nematodes (RKNs) seriously endanger agricultural development and cause great economic losses worldwide. Natural product furfural acetone (FAC) is a promising nematicide with strong attractant and nematicidal activities, but baseline information about the impact of FAC on the reproduction, egg hatching, feeding, and growth of nematodes and its pest control efficiency in field are lacking. Here, the inhibition effects of FAC on nematodes in vitro and its RKN control efficiency in pot and field were investigated. FAC inhibited the egg hatching of *Meloidogyne incognita* by 91.7% at 200 mg/L after 2 days and suppressed the reproduction, feeding, and growth of *Caenorhabditis elegans* in vitro. In pot experiments, FAC in various dosages reduced the disease index of plant root significantly. In field experiments, FAC exhibited control effect on RKNs equivalent to commercial nematicides avermectin and metam sodium, with a reduction in disease index by 36.9% at a dose of 50 mg/plant. FAC also reduced the population density of RKNs in soil, with a reduction rate of 75.3% at the dose of 750 mg/m². No adverse effect was detected on plant growth after FAC application. These results provide compelling evidence for development of FAC as an appropriate alternative for current nematicides.

Keywords: furfural acetone; nematode; *Meloidogyne incognita*; biological control; reproduction; egg hatching; feeding; field experiment

1. Introduction

Plant parasitic nematodes (PPNs) are among the most serious plant parasites that cause plant diseases, and affect the growth of crops, resulting in economic losses [1,2]. Diseases caused by PPNS lead to more than 150 billion U.S. dollars in economic losses worldwide each year [3], more than half of which is caused by root-knot nematodes (RKNs) [4]. RKNs (*Meloidogyne* spp.) have been considered to be one of the most damaging nematode group in the world because they cause great global economic losses to the majority of cultivated plant species in subtropical and tropical regions [5]. Therefore, the presence of RKNs in cultivated crops must be controlled.

Multiple management methods—including crop rotations [6], crop interplants [7], development of nematode-resistant varieties [8], and application of chemical nematicides [9]—have been applied to control RKNs. Chemical nematicides are still the primary means to control RKNs because of their high efficacy, easy application, and rapid onset [10]. However, frequent and excessive use of chemical nematicides increases risks to ecological systems and even human health [11], which lead to restricted

use or even total ban of the most effective chemical nematicides. Therefore, environmentally friendly and sustainable alternatives are urgently needed for RKN control.

Biological control is an environmentally acceptable means to reduce pest losses. A great number of microorganisms [12–14], plants [15,16], and their metabolites [17] have been reported to possess good nematicidal activity. Volatile organic compounds (VOCs) produced by microorganisms or plants have especially good prospects for agricultural applications because of their multiple effects and integrated control strategies against RKNs [18–20]. Furfural, a nematicidal VOC produced by several plants [18] with inhibition effects on the respiration and motility of *M. incognita* [21], reduces root galls in greenhouse and microplot experiments [22], and has been developed into the commercial nematicide CropGuard® [21]. The product development of furfural and the study of its anti-nematode activity are good examples for the study of other VOCs.

Furfural acetone (FAC) is, also known as 4-(2-furyl)-3-buten-2-one. The molecular formula of FAC is $C_8H_8O_2$, and its molecular weight 136.15 g/mol. FAC has been recognized as a flavoring agent and food additive by the Joint FAO/WHO Expert Committee on Food Additives [23]. FAC is a VOC produced by several microbes or plants [24,25], and has been considered as a promising alternative to currently available nematicides because of its nematicidal, fumigant, and attractant activities against *M. incognita* [24]. However, the effects of FAC on the reproduction, egg hatching, feeding, and growth of nematodes and control efficiency on RKNs in the field are still unknown.

The reproduction, feeding, and growth of *M. incognita* all almost occur in the root of plants and are difficult to directly observe in vitro under current experimental conditions. *Caenorhabditis elegans* is a model nematode to research the biological activity of natural compounds on PPNs and it is easy to breed and reproduce in the laboratory [26–28]. Therefore, *C. elegans* is a good model to test the inhibit effect of nematicides on PPNs.

This study mainly aimed to determine (i) the effects of FAC on the reproduction, egg hatching, feeding, and growth of nematodes; and (ii) the control efficiency of FAC on RKNs in pots and field. The results indicated that FAC inhibited the egg hatching of *M. incognita* and suppressed the reproduction, feeding, and growth of *C. elegans* in vitro, and exhibits control effect on RKNs similar to commercial chemical nematicides in field experiments.

2. Results

2.1. Effect of FAC on the Egg Hatching of *M. incognita*

The results indicated that FAC in various concentrations had adverse effect on the egg hatching of *M. incognita*, especially when the concentration was above 100 mg/L (Table 1). The egg hatching was remarkably inhibited at the concentration of 500 mg/L. the average numbers of worms hatched from a single egg mass after 2 and 4 days of 500 mg/L FAC treatment were only 2.44 and 2.67, respectively, whereas those from an egg mass treated with distilled water (control) were 47.58 and 74.31, respectively (Table 1). Hence, FAC has a strong inhibition effect on the egg hatching of *M. incognita*.

Table 1. Effect of FAC on the egg hatching of *M. incognita*.

Concentration (mg/L)	2 Days		4 Days	
	Hatched Worms/Egg Mass	Inhibition Rate (%)	Hatched Worms/Egg Mass	Inhibition Rate (%)
0	47.6 ± 5.8 a ¹	(0.0)	74.3 ± 9.3 a	(0.0)
50	40.1 ± 4.9 a	15.8	67.8 ± 10.1 a	8.8
100	16.9 ± 3.3 b	64.6	19.7 ± 5.3 b	73.5
200	4.0 ± 1.5 c	91.7	4.6 ± 1.5 b	93.8
500	2.4 ± 0.6 c	94.9	2.7 ± 0.7 b	96.4

¹ Data are shown as mean ± standard error ($n = 3$), and different lowercase letters indicate significant difference among treatments by LSD test ($p < 0.05$).

2.2. Effect of FAc on the Reproduction, Feeding, and Growth of *C. elegans*

L4 worms were incubated with different concentrations of FAc at 20 °C for 48 h to assess the effect of FAc on the reproduction of *C. elegans*. As shown in Figure 1, FAc significantly affected the reproduction of *C. elegans*. FAc caused a consistent concentration-dependent decline in the brood size of a single worm, with an approximately 75% decrease in brood size after exposure to 200 mg/L FAc for 48 h.

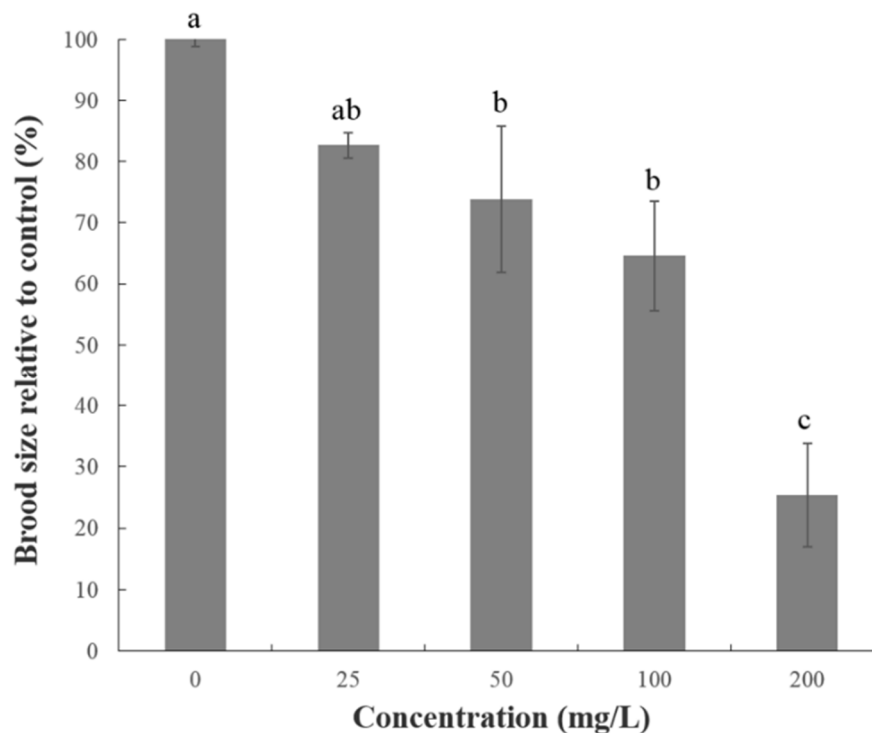


Figure 1. Reproduction assay of *C. elegans* immersed in FAc solution in various concentrations for 48 h. Data are shown as mean \pm standard error ($n = 3$), and different lowercase letters indicate significant difference among treatments by LSD test ($p < 0.05$).

In the presence of *Escherichia coli* OP50, *C. elegans* ingested food by rhythmic and sustained pharyngeal pumping. The pharyngeal pumping frequency of *C. elegans* decreased with increasing concentration of FAc. The pharyngeal pumping was reduced significantly when the concentration of FAc was above 25 mg/L after 48 h exposure, with an inhibition rate of 37.72% at the dose of 200 mg/L (Table 2).

Table 2. Effect of FAc on the food pharyngeal pumping of *C. elegans*.

Concentrations (mg/L)	Pumping ¹ (Hz)	
	24 h	48 h
0	183.7 \pm 2.7 a ²	175.1 \pm 3.9 a
25	163.7 \pm 8.8 ab	155.4 \pm 2.7 b
50	164.3 \pm 7.8 ab	147.9 \pm 4.5 b
100	148.1 \pm 7.8 b	135.5 \pm 4.5 c
200	117.8 \pm 2.9 c	109.1 \pm 3.5 d

¹ Pumping was counted for a period of 1 min and expressed as pumps/min (Hz). ² Data are shown as mean \pm standard error ($n = 3$), and different lowercase letters indicate significant difference among treatments by LSD test ($p < 0.05$).

FAC at various concentrations caused visible suppression of nematode growth with an obvious dose effect (Figure 2A), leading to 55% decrease in size even at a low concentration of 25 mg/L (Figure 2B). Hence, FAC has a significant inhibition effect on the reproduction, feeding, and growth of *C. elegans*.

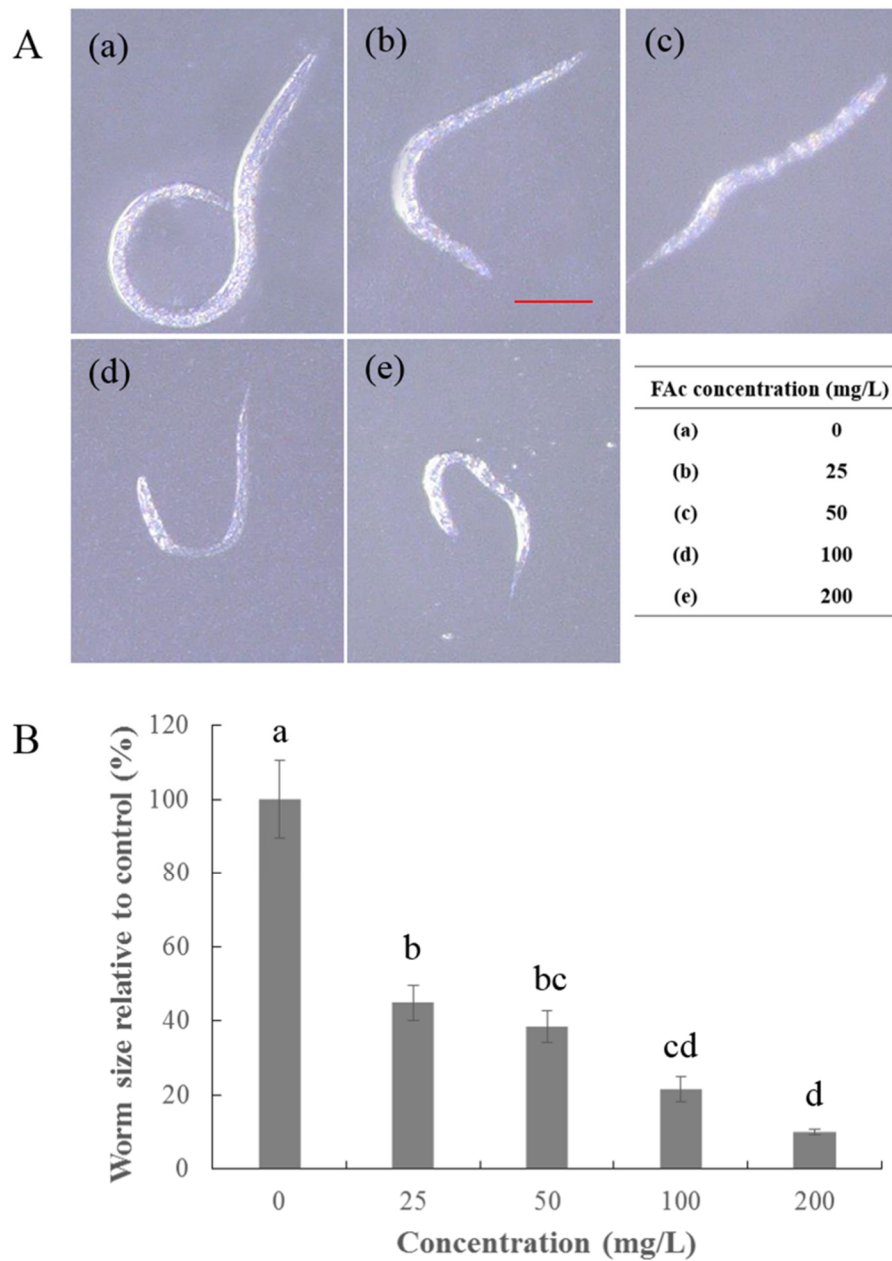


Figure 2. Growth assay of *C. elegans* treated with FAC. **(A)** (a–e) Wild-type L2 worms were cultured in gradient doses of FAC in a concentration range of 0–200 mg/L, incubated at 20 °C for 48 h, and photographed using a 3D digital microscope (Keyence VHX-6000, Keyence, Osaka, Japan). The scan bar is 100 μ m. **(B)** The size of worms cultured in FAC as a percentage of the size of worms cultured in distilled water (control). Data are shown as mean \pm standard error ($n = 3$), and different lowercase letters indicate significant difference among treatments by LSD test ($p < 0.05$).

2.3. Efficiency of FAc against RKNs in Pots

The disease index of the tomato root irrigated with FAc solution decreased significantly compared with that in the control group. The control effect of FAc at the dosage of 100 mg/pot was 58.82% compared with that of the control and showed no significant difference from that (64.71%) of avermectin at the dose of 10 mg/pot (Figure 3A). FAc also exerted good control effect on RKNs when applied as a fumigant, with control effects of 43.45%, 52.38%, and 73.81% at doses of 10, 50, and 250 mg/pot, respectively, which are lower than that (100%) of metam sodium (Figure 3B). In summary, FAc irrigated into the rhizosphere of the tomato plant or applied as a fumigant at different dosages have good control efficacy against RKNs in pots.

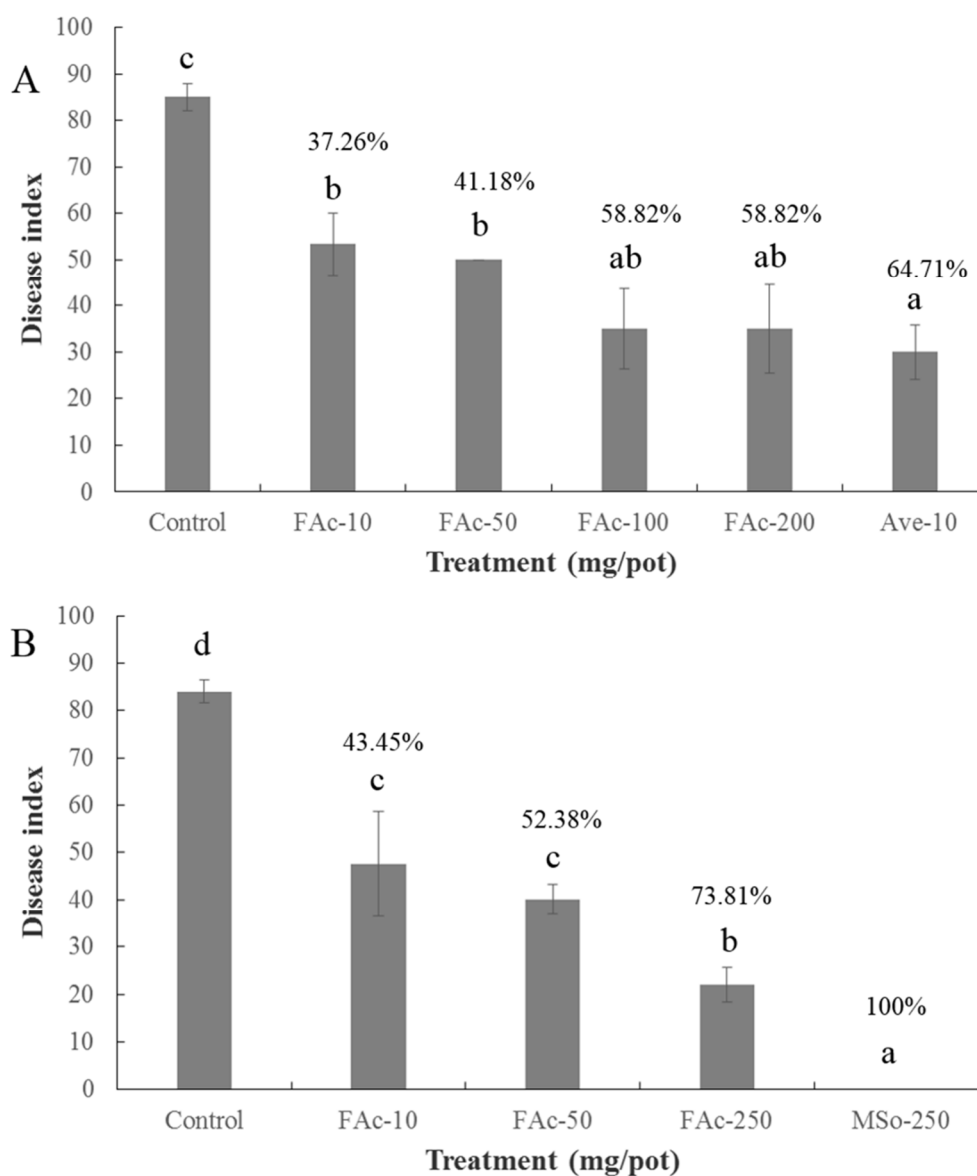


Figure 3. Control efficiency of FAc, avermectin (Ave), and metam sodium (MSo) on RKNs in pots. (A) Chemicals were irrigated into the rhizosphere of the plant. (B) chemicals were applied as fumigants. Data are shown as mean \pm standard error ($n = 5$), and different lowercase letters indicate significant difference among treatments by LSD test ($p < 0.05$). The numbers above the lowercase letters indicate the control effect of each group compared with the control.

2.4. Efficiency of FAc against RKNs in the Field

FAc irrigated into the rhizosphere of the tomato plant or applied as a fumigant on RKNs in the pot experiments exhibited strong control efficacy, though FAc was less efficient than either avermectin or metam sodium. The RKN control effect was evaluated further in field experiments by irrigation of FAc into the rhizosphere of the tomato plant or application of it as a fumigant. RKNs infected and formed numerous large root galls in the control group, while fewer and smaller galls were observed on the roots after treatment with different dosages of FAc or commercial chemical nematicides through the two different application methods (Figure 4).



Figure 4. Root of tomato plants after treatment with (A,E) water (control), (B) 10 mg/plant FAc, (C) 50 mg/plant FAc, (D) 10 mg/plant avermectin, (F) 375 mg/m² FAc, (G) 750 mg/m² FAc, and (H) 750 mg/m² metam sodium for 60 days in the field. (A–D): Chemicals were irrigated into the rhizosphere of the plant. (E–H): Chemicals were applied as fumigants.

After irrigation of FAc into the rhizosphere of the tomato plants for 60 days in the field, the disease index in the FAc-treated groups decreased significantly compared with that in the control group, with a control effect of 30.2% at the dose of 10 mg/plant, which is equivalent to the control effect (29.5%) of 10 mg/plant avermectin (Table 3). When applied as a fumigant, FAc showed satisfactory nematode control activity, with control effects of 17.1% and 19.9% at doses of 375 and 750 mg/m², respectively, close to the control effect (11.2%) of 750 mg/m² metam sodium (Table 3). In addition, FAc applied at various dosages via two different methods reduced the population density of RKNs in the soil, with great reduction rates of 43.1%, 47.9%, 53.1%, and 75.3% at doses of 10 mg/plant, 50 mg/plant, 375 mg/m², and 750 mg/m², respectively (Table 3).

Table 3. Suppression of RKNs by FAc on tomato plants in the field experiments.

Treatment	Disease Index	Control Effect (%)	Number of RKNs/30 g Soil	Reduction Rate of RKNs (%)
Irrigation ¹				
Control ²	82.4 ± 2.2 b ⁷	(0.0)	432 ± 93 a	(0.0)
FAc ³ 10 mg/plant	57.5 ± 2.8 a	30.2	246 ± 62 a	43.1
FAc 50 mg/plant	52.3 ± 2.9 a	36.9	225 ± 97 a	47.9
Ave ⁴ 10 mg/plant	58.1 ± 3.0 a	29.5	401 ± 31 a	7.2
Fumigant ⁵				
Control	73.6 ± 2.6 b	(0.0)	1617 ± 1323 a	(0.0)
FAc 375 mg/m ²	61.1 ± 3.8 a	17.1	759 ± 259 a	53.1
FAc 750 mg/m ²	59.0 ± 2.6 a	19.9	400 ± 51 a	75.3
MSo ⁶ 750 mg/m ²	65.4 ± 3.2 a	11.2	726 ± 314 a	55.1

¹ Irrigation means the chemicals were irrigated into the rhizosphere of the plant. ² Control means the same volume of water was applied to the field. ³ FAc means furfural acetone. ⁴ Ave means avermectin. ⁵ Fumigant means the chemicals were applied as fumigants. ⁶ MSo means metam sodium. ⁷ Data are shown as mean ± standard error ($n = 3$), and different lowercase letters indicate significant difference among treatments by LSD test ($p < 0.05$).

The growth of tomato plants was improved when FAc was applied as a fumigant, except that the height of plants treated with 375 mg/m² FAc was slightly decreased compared with that of control. FAc irrigated into the rhizosphere of the tomato plant could improve the growth of plants at the dose of 10 mg/plant, and affected the growth of plants slightly at the dose of 50 mg/plant. However, no consistent and significant differences in the fresh weight on the ground, plant height, and stem thickness of tomato plant were found among the treatments (Table 4). It indicated that FAc has no adverse effect on plant growth in the field.

Table 4. Growth effect of FAc on tomato plants in the field experiments

Treatment	Fresh Weight on the Ground (g)	Plant Height (cm)	Stem Thickness (cm)
Irrigation			
Control	618 ± 78 a ¹	89.5 ± 3.1 a	3.64 ± 0.13 a
FAc 10 mg/plant	737 ± 109 a	96.5 ± 3.7 a	3.76 ± 0.11 a
FAc 50 mg/plant	593 ± 72 a	85.3 ± 4.4 a	3.59 ± 0.14 a
Ave 10 mg/plant	655 ± 16 a	94.7 ± 2.8 a	3.72 ± 0.10 a
Fumigant			
Control	667 ± 52 a	101.0 ± 3.0 a	3.46 ± 0.09 a
FAc 375 mg/m ²	724 ± 77 a	98.7 ± 3.4 a	3.70 ± 0.15 a
FAc 750 mg/m ²	749 ± 44 a	105.4 ± 2.9 a	3.71 ± 0.11 a
MSo 750 mg/m ²	821 ± 42 a	106.1 ± 2.7 a	3.63 ± 0.08 a

The means of Control, Irrigation, Fumigant, and abbreviations are the same as that in Table 3. ¹ Data are shown as mean ± standard error ($n = 3$), and different lowercase letters indicate significant difference among treatments by LSD test ($p < 0.05$).

3. Discussion

Nematicides, such as avermectin [29], methyl bromide [30], and metam sodium [31], exhibit nematicidal or fumigant activity against RKNs, and most of them are toxic to animals and unfriendly to the environment [32–34]. Many RKNs have developed resistance to these nematicides that are continuously applied in the agricultural field for a long period worldwide [35]. Therefore, environmentally friendly and sustainable alternatives are urgently needed for RKN control. FAc has been reported to have attractant and nematicidal activities on RKNs [24]; in the present study, FAc inhibited the egg hatching of *M. incognita*, suppressed the reproduction, feeding, and growth of *C. elegans* (Figures 1 and 2, Tables 1 and 2). *C. elegans* is a model nematode to research the biological

activity of natural compounds on PPNs [26–28]. Therefore, FAc may also suppress the reproduction, feeding, and growth of RKNs. As a VOC, FAc can spread quickly in the soil and then exert its inhibition effects on RKNs of various age stages via its multiple nematocidal activities. FAc is a natural product of several microorganisms and plants with nematocidal activity [24,25] and is relatively safe to humans because it can be used as a food additive in alcohol free drinks, ice, candies, gelatins, and other products of current life [23,36]. In addition, FAc can be chemically synthesized by using low-cost sugar cane residues [36], and can be degraded by soil microorganisms [37]. In the field experiment, FAc irrigated into the rhizosphere of the tomato plant or applied as a fumigant in the field could alleviate plant root-knot disease, exhibited similar control effect with commercial nematocides avermectin and metam sodium (Table 3), decreased the population density of RKNs in the soil (Table 3), and did not adversely affect the growth of tomato plants (Table 4). In conclusion, FAc possesses many advantages over current nematocides and has considerable potential to be developed as an appropriate alternative for current nematocides.

Many VOCs exhibit nematocidal, fumigant, or chemotactic activities against RKNs [19,20,24]. However, few works have investigated the effects of VOCs on the egg hatching, reproduction, feeding, and growth of nematodes. A previous study reported that FAc had strong nematocidal and attractant activities on RKNs [24], and the present work showed that FAc inhibited the egg hatching of *M. incognita*, and suppressed the reproduction, feeding, and growth of *C. elegans*. The multiple nematocidal activities of FAc made it possible to inhibit RKNs of different instars. The synergistic effect of multiple inhibition effects and attractant activities of FAc on nematodes may enhance its control efficiency on RKNs in agricultural production and achieve a strong RKN control effect in the field experiments (Table 3).

In the field experiments, FAc exerted similar nematode control effect to commercial nematocides when applied in the same dose via two different methods. However, the increasing of control effect on RKNs is not obvious with the rising of FAc dosage in the field experiments (Table 3). Low-dose FAc can achieve satisfactory nematode control effect in the field experiments and reduce the costs in agricultural applications. In addition, FAc irrigated into the rhizosphere of the tomato plant could reduce the disease index of plant root by 30.2% and 36.9% at doses of 10 and 50 mg/plant, respectively. However, when applied as a fumigant, FAc exhibited control effects of 17.1% and 19.9% at doses of 375 and 750 mg/m² (150 and 300 mg/plant in average), respectively. Considering the cost and control efficiency against RKNs, FAc irrigated into the rhizosphere of the tomato plant could be a better application method compared with application as a fumigant. This study is the first to evaluate the control effect of FAc on RKNs in the field via two different application methods. The results lay a foundation for establishing methods of applications of subsequent VOCs in the field experiment. In contrast to commercial nematocides that can only be used as non-fumigants or fumigants, FAc can be used in the field via irrigation or fumigation because of its multiple impacts on RKNs. Therefore, a more reasonable application method of FAc can be selected in agricultural application. In conclusion, multiple application methods of FAc can enable farmers to select the more reasonable application method according to farmland and environment conditions.

VOCs can control RKNs or pathogens directly by killing, repelling, or inhibiting egg hatching [19,38,39]. Recent studies have also found that VOCs can promote plant growth and suppress pathogens by affecting the structure and abundance of microorganisms in the plant rhizosphere [40]. The control effect of FAc is lower than that of avermectin and metam sodium in pot experiments (Figure 3), but is similar to the same dose of avermectin and metam sodium when used in the field (Table 3). Hence, compared with experiments conducted in pot, FAc applied in the field may not only control RKNs by killing it or inhibiting its egg hatching directly, but also may have some other indirect mechanisms, such as affecting the microbial structure of the plant rhizosphere to enhance its inhibition efficiency. However, the specific mechanism of action is still unclear and deserves further research.

In vitro, FAc inhibited the egg hatching of *M. incognita*, and suppressed the reproduction, feeding, and growth of *C. elegans*. FAc irrigated into the rhizosphere of the tomato plant or applied as a fumigant in the field showed that it could alleviate plant root-knot disease, exhibited similar control

effect to commercial nematicides avermectin and metam sodium, decreased the population density of RKNs in the soil, and did not adversely affect the growth of tomato plants. Taken together, FAc has a considerable potential to be developed as an appropriate alternative to current nematicides.

4. Materials and Methods

4.1. Chemicals

Furfural acetone (purity $\geq 98\%$) was purchased from TCI (Tokyo Chemical Industry, Tokyo, Japan) and dissolved in distilled water or S medium at various concentrations to determine its effects on nematodes in vitro. Meanwhile, FAc was dissolved in dimethylsulfoxide to a concentration of 200 g/L and diluted to various concentrations with distilled water for pot and field experiments.

4.2. Nematodes

Meloidogyne incognita individuals were maintained on the roots of tomato (*Solanum lycopersicum*) grown in the greenhouse at 25 ± 1 °C. The egg masses of *M. incognita* were removed from the infected roots with needles, and washed three times in distilled water. The egg masses were placed in 24-well culture plates with distilled water at 20 °C for 3 days. Freshly hatched J2 juveniles were collected in a sterile tube. The fresh egg masses and J2 juveniles were used immediately.

Wild-type *C. elegans* (N2 Bristol strain) was provided by the Caenorhabditis Genetics Center and maintained at 20 °C on nematode growth media (NGM) seeded with *E. coli* OP50 [28].

4.3. Effect of FAc on the Egg Hatching of *M. incognita*

The influence of FAc on the egg hatching of *M. incognita* was determined by immersing the egg masses of *M. incognita* in FAc solutions in 96-well plates. Three egg masses and 200 μ L of FAc solution dissolved in water were added to each well, and distilled water was used as the control. The number of J2s that hatched from the egg masses was counted under an inverted microscope (Olympus IX73, Olympus, Tokyo, Japan) after 2 and 4 days of exposure at 20 °C. The experiment was repeated three times, with three replicates at each concentration of FAc. The inhibition rate of *M. incognita* egg hatching was calculated by using the following equation.

Inhibition rate (%) = [(hatched worms per egg mass in control – hatched worms per egg mass in treatment)/hatched worms per egg mass in control] \times 100.

4.4. Effect of FAc on the Reproduction of *C. elegans*

The effect of FAc on the reproduction of nematodes was performed in 96-well plates according to a described method [41]. Each well was added with 5 μ L of *E. coli* OP50 cultured in S medium ($OD_{600} = 2.0$), a single L4 wild-type *C. elegans*, and 115 μ L of FAc solution dissolved in S medium. The S medium instead of the FAc solution was used as control, and five wells were assayed for each concentration. The plates were placed at 20 °C for 48 h, and the number of eggs in each well was counted under an inverted microscope (Olympus IX73). The experiment was repeated three times, and the number of eggs produced by a single worm in each group was calculated and compared with the control.

4.5. Effect of FAc on Pharyngeal Pumping of *C. elegans*

The effect of FAc on the pharyngeal pumping of *C. elegans* was examined as a described method [42] to determine its influence on the feeding of nematodes. A total volume of 100 μ L of FAc solution dissolved in water was added to 900 μ L of NGM to final concentrations of 0, 25, 50, 100, and 200 mg/L. About 1 mL of the FAc-NGM mixture was pipetted into each well of a 24-well plate and left to dry overnight. Each well of the 24-well plate was added with 50 μ L of the OP50 culture ($OD_{600} = 0.6$). The plates were left overnight to allow the OP50 lawn to grow at 20 °C. Six synchronized L4 wild-type *C. elegans* were placed in each well of the 24-well plate filled with *E. coli* seeded FAc-infused NGM

at 20 °C. Pharyngeal pumping was counted at 24 and 48 h post-exposure. The worms were viewed under a 3D digital microscope (Keyence VHX-6000, Keyence, Osaka, Japan) and pumping rates were determined by visual observations. One pharyngeal pump was defined as a complete forward and backward movement of the grinder in the pharynx. The rate was counted for a period of 1 min and expressed in Hz. The experiment was repeated three times, with five replicates at each concentration of FAc.

4.6. Effect of FAc on the Growth of *C. elegans*

The growth assays for *C. elegans* were conducted according to a previously described method [41] with some modifications. Briefly, 10 µL of *E. coli* OP50 culture (OD₆₀₀ = 2.0), 135 µL of FAc solution dissolved in S medium, and 5 µL of 20 L2 wild-type *C. elegans* were added to each well of 96-well plates. The S medium without FAc instead of the FAc solution was used as control. The plates were incubated at 20 °C on a rocking platform at a speed of 150 r/min for 48 h. After incubation, the worms of three wells treated with the same concentration of FAc were gently mixed and washed by distilled water three times. About 10 µL of the worms were pipetted onto a glass slide containing 15 mM sodium azide as an anesthetic. At least 10 nematodes in each concentration were photographed under a 3D digital microscope (Keyence VHX-6000). The size of these worms was calculated by using Image J1.33 software (NIH, Bethesda, MD, USA). The experiment was repeated three times, and the average size of the worms was plotted against that of the control group for comparison.

4.7. Control Efficacy of FAc against RKNs in Pots

The soil used in the pot experiment was collected from the RKN-infested field at Institute of Vegetables, Wuhan Academy of Agricultural Sciences (30.710038° N, 114.476825° E, Wuhan, Hubei, China), and mixed with organic matter at the ratio of 1:1. The seeds of tomato ZhongShu No.4 were surface disinfested in 2% sodium hypochlorite for 5 min, rinsed three times in distilled water, and incubated in organic matter for 2 weeks. Two-week-old plants of the same size were selected for pot experiments. Round plastic pots (18 × 18 × 12.5 cm) were filled with about 1 kg of the soil mixture. One two-week-old tomato seedling was transplanted into each pot and incubated in the greenhouse at 22–25 °C.

The two different application methods of FAc in the pot experiment were irrigation of FAc solution into the rhizosphere of the tomato plant and fumigation. When applied by irrigation, FAc was introduced into the rhizosphere soil of the plant by adding 50 mL of FAc solution 2 days after transplant. When used as a fumigant, 50 mL of the FAc solution was mixed with 1 kg of soil and covered with a plastic film for 10 days in the greenhouse at 25 °C. The plastic film was opened and deflated for 7 days. Each 1 kg of soil after fumigation was placed into round plastic pots and a single tomato seedling was transplanted into each pot.

When FAc was irrigated into the rhizosphere of plants, the experiment contained six treatments: FAc dissolved in water (10, 50, 100, and 200 mg/pot), 1.8% avermectin dissolved in water (10 mg/pot), or water alone (control). When FAc was used as a fumigant, the experiment comprised five treatments: FAc dissolved in water (10, 50, and 250 mg/pot), 45% metam sodium dissolved in water (250 mg/pot), or water alone (control). Each treatment had five replicates, and the disease severity of the plant root was assessed 60 days after transplant.

The disease severity of plant root was determined by rating the roots on each plant on a 0 to 10 scale based mainly on the types of roots galled by RKNs after 60 days. The scale 0 to 10 represented as follows: 0, no knots on roots; 1, few small knots but difficult to find; 2, small knots only and main root is clean; 3, some large knots visible and main roots is clean; 4, larger knots predominate and main roots is clean; 5, 50% of roots infected and knotting on parts of main roots; 6, knotting on main roots; 7, majority of main roots knotted; 8, all main roots knotted with few clean roots visible; 9, all roots severely knotted and plant usually dying; 10, all roots severely knotted and plant usually dead [43].

Disease index was calculated by using the following equation [44]. Nematode control effect was calculated by using the following equation [45].

Disease index = $[\sum (\text{the number of diseased plants with severity rating } i \times \text{rating } i) / (\text{total number of plants} \times 10)] \times 100$.

Control effect (%) = $[(\text{disease index in control} - \text{disease index in treated}) / \text{disease index in control}] \times 100$.

4.8. Control Efficacy of FAc against RKNs in Field

The field experiment was conducted in RKN-infested loamy soil at the Institute of Vegetables, Wuhan Academy of Agricultural Sciences, at a site used to cultivate tomato plants before this batch of field experiments. The plots with tomato root-knot index of 7 were used to conduct field experiments. The tomato seedlings (ZhongShu No.4) of the same size grown in a seedling room for 15 days were used to transplant in the field experiments. FAc was irrigated into the rhizosphere of the plants or used as fumigant in the field. When applied by irrigation, 50 mL of FAc solution was irrigated into the rhizosphere of the plant 2 days after transplant; this experiment consisted of four treatments: FAc dissolved in water (10 and 50 mg/plant), 1.8% avermectin dissolved in water (10 mg/plant), or water alone (control). When FAc was used as a fumigant, two trenches with depth of 15 cm and length of 3.5 m were dug on the left and right sides of each plot (3.5 m in length and 1.6 m in width). A total of 5 L of FAc solution dissolved in water was spread evenly in the trench, and the soil was covered immediately. The soil moisture was adjusted to 70%. Each plot was covered by the plastic film and fumigated for 10 days. Tomato seedlings of the same size were transplanted to the plots 7 days after peeling off the film. This experiment involved four treatments: FAc dissolved in water (375 and 750 mg/m²), 45% metam sodium dissolved in water (750 mg/m²), and the same volume of distilled water (control). Each treatment consisted of three plots arranged randomly, and each plot included 14 plants. Compound fertilizer (China-Arab Chemical Fertilizer Co., Ltd., Qinhuangdao, China) was applied at the suggested dosage of 25 kg/mu in each plot before transplant to ensure normal plant growth. Disease severity, fresh weight on the ground, plant height, and stem thickness were assessed 60 days after transplant. The disease severity of plant roots and the control effect of chemicals were counted as above.

The tomato root soil of each plot was collected by picking a tomato from the front, middle, and back of each plot. The tomato root was pulled out, the soil attached to the root was collected, and three soil samples in the same plot were mixed. Afterward, 30 g of the soil sample in each plot was then selected to detect the population density of RKNs in the soil. Nematodes in the soil were collected into 90 mm Petri dishes by sugar centrifugation flotation [46]. Identification of RKN by its size and morphology [47] from nematodes isolated from soil, and the number of RKNs was counted under an inverted microscope (Olympus, IX73). Reduction rate of RKNs (%) = $[(\text{number of RKNs in control} - \text{number of RKNs in treatment}) / \text{number of RKNs in control}] \times 100$.

4.9. Statistical Analyses

All data were analyzed by SPSS (Statistical Package for the Social Sciences), version 22.0 software (SPSS, Chicago, IL, USA) and shown as mean \pm standard error (SE) ($n \geq 3$). Least significant difference (LSD) test was employed to test for significant differences among treatments at $p = 0.05$. Different lowercase letters indicate significant difference among treatments.

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References

- Wei, L.H.; Shao, Y.; Wan, J.W.; Feng, H.; Zhu, H.; Huang, H.W.; Zhou, Y.J. Isolation and characterization of a rhizobacterial antagonist of root-knot nematodes. *PLoS ONE* **2014**, *9*, e85988. [[CrossRef](#)]
- Zhang, S.W.; Gan, Y.T.; Ji, W.H.; Xu, B.L.; Hou, B.H.; Liu, J. Mechanisms and characterization of *Trichoderma longibrachiatum* T6 in suppressing nematodes (*Heterodera avenae*) in wheat. *Front. Plant Sci.* **2017**, *8*, 1491. [[CrossRef](#)]
- Li, J.; Zou, C.G.; Xu, J.P.; Ji, X.L.; Niu, X.M.; Yang, J.K.; Huang, X.W.; Zhang, K.Q. Molecular mechanisms of nematode-nematophagous microbe interactions: Basis for biological control of plant-parasitic nematodes. *Annu. Rev. Phytopathol.* **2015**, *53*, 67–95. [[CrossRef](#)] [[PubMed](#)]
- Kim, T.Y.; Jang, J.Y.; Jeon, S.J.; Lee, H.W.; Bae, C.H.; Yeo, J.H.; Lee, H.B.; Kim, I.S.; Park, H.W.; Kim, J.C. Nematicidal activity of kojic acid produced by *Aspergillus oryzae* against *Meloidogyne incognita*. *J. Microbiol. Biotechnol.* **2016**, *26*, 1383–1391. [[CrossRef](#)] [[PubMed](#)]
- Abdelnabby, H.; Wang, Y.H.; Xiao, X.Q.; Wang, G.F.; Yang, F.; Xiao, Y.N. Impact of direct and indirect application of rising furfural concentrations on viability, infectivity and reproduction of the root knot nematode, *Meloidogyne incognita* in *Pisum sativum*. *Microb. Pathog.* **2016**, *96*, 26–34. [[CrossRef](#)] [[PubMed](#)]
- Win, P.P.; Kyi, P.P.; Maung, Z.T.Z.; Myint, Y.Y.; Cabasan, M.T.N.; De Waele, D. Crop rotation sequencing to minimize yield losses of summer-irrigated lowland rice in Myanmar caused by the rice root-knot nematode *Meloidogyne graminicola*. *Int. J. Pest Manag.* **2020**, *66*, 319–331. [[CrossRef](#)]
- Xie, G.H.; Cui, H.D.; Dong, Y.; Wang, X.Q.; Li, X.F.; Deng, R.K.; Wang, Y.; Xie, Y. Crop rotation and intercropping with marigold are effective for root-knot nematode (*Meloidogyne* sp.) control in angelica (*Angelica sinensis*) cultivation. *Can. J. Plant Sci.* **2017**, *97*, 26–31. [[CrossRef](#)]
- Jordan, S. Yield to the resistance: The impact of nematode resistant varieties on alfalfa yield. *Nat. Resour. Model.* **2018**, *31*, e12150. [[CrossRef](#)]
- Giannakou, I.O.; Anastasiadis, I. Evaluation of chemical strategies as alternatives to methyl bromide for the control of root-knot nematodes in greenhouse cultivated crops. *Crop Prot.* **2005**, *24*, 499–506. [[CrossRef](#)]
- Desaeger, J.A.; Watson, T.T. Evaluation of new chemical and biological nematicides for managing *Meloidogyne javanica* in tomato production and associated double-crops in Florida. *Pest Manag. Sci.* **2019**, *75*, 3363–3370. [[CrossRef](#)]
- Coyne, D.L.; Cortada, L.; Dalzell, J.J.; Claudius-cole, A.O.; Haukeland, S.; Luambano, N.; Talwana, H. Plant-parasitic nematodes and food security in Sub-Saharan Africa. *Annu. Rev. Phytopathol.* **2018**, *56*, 381–403. [[CrossRef](#)] [[PubMed](#)]
- Hu, H.J.; Gao, Y.; Li, X.; Chen, S.L.; Yan, S.Z.; Tian, X.J. Identification and nematicidal characterization of proteases secreted by endophytic bacteria *Bacillus cereus* BCM2. *Phytopathology* **2020**, *110*, 336–344. [[CrossRef](#)]
- Li, L.L.; Tan, J.J.; Chen, F.M.; Hao, D.J. Colonization of *Bacillus cereus* NJSZ-13, a species with nematicidal activity in Masson pine (*Pinus massoniana* Lamb.). *J. For. Res.* **2020**, *3*, 1025–1033. [[CrossRef](#)]
- Park, E.J.; Jang, H.J.; Park, C.S.; Lee, S.J.; Lee, S.; Kim, K.H.; Yun, B.S.; Lee, S.W.; Rho, M.C. Evaluation of nematicidal activity of *Streptomyces yatusensis* KRA-28 against *Meloidogyne incognita*. *J. Microbiol. Biotechnol.* **2020**, *5*, 700–707. [[CrossRef](#)]
- Jayasinghe, U.L.B.; Kumarihamy, B.M.M.; Bandara, A.G.D.; Vasquez, E.A.; Kraus, W. Nematicidal activity of some Sri Lankan plants. *Nat. Prod. Res.* **2003**, *4*, 259–262. [[CrossRef](#)] [[PubMed](#)]
- Andres, M.F.; Gonzalez-Coloma, A.; Munoz, R.; De la Pena, F.; Julio, L.F.; Burillo, J. Nematicidal potential of hydrolates from the semi industrial vapor-pressure extraction of Spanish aromatic plants. *Environ. Sci. Pollut. Res.* **2018**, *25*, 29834–29840. [[CrossRef](#)]
- Zhai, Y.L.; Shao, Z.Z.; Cai, M.M.; Zheng, L.Y.; Li, G.Y.; Yu, Z.N.; Zhang, J.B. Cyclo (l-Pro-l-Leu) of *Pseudomonas putida* MCCC 1A00316 isolated from Antarctic soil: Identification and characterization of activity against *Meloidogyne incognita*. *Molecules* **2019**, *24*, 768. [[CrossRef](#)]
- Ntalli, N.G.; Vargiu, S.; Menkissoglu-Spiroudi, U.; Caboni, P. Nematicidal carboxylic acids and aldehydes from *Melia azedarach* fruits. *J. Agric. Food Chem.* **2010**, *58*, 11390–11394. [[CrossRef](#)]
- Zhai, Y.L.; Shao, Z.Z.; Cai, M.M.; Zheng, L.Y.; Li, G.Y.; Huang, D.; Cheng, W.L.; Thomashow, L.S.; Weller, D.M.; Yu, Z.N.; et al. Multiple modes of nematode control by volatiles of *Pseudomonas putida* 1A00316 from Antarctic soil against *Meloidogyne incognita*. *Front. Microbiol.* **2018**, *9*, 253. [[CrossRef](#)]

20. Huang, D.; Yu, C.; Shao, Z.Z.; Cai, M.M.; Li, G.Y.; Zheng, L.Y.; Yu, Z.N.; Zhang, J.B. Identification and characterization of nematocidal volatile organic compounds from deep-sea *Virgibacillus dokdonensis* MCCC 1A00493. *Molecules* **2020**, *25*, 744. [[CrossRef](#)]
21. Fourie, H.; Van Aardt, W.J.; Venter, C.; Tiedt, L.R. The effects of CropGuard (R) on the motility, ultrastructure, and respiration of two *Meloidogyne* species. *Nematropica* **2014**, *44*, 93–100.
22. Rodriguez-Kabana, R.; Kloepper, J.W.; Weaver, C.F.; Robertson, D.G. Control of Plant-parasitic nematodes with furfural-A naturally occurring fumigant. *Nematropica* **1993**, *23*, 63–73.
23. Joint FAO/WHO Expert Committee on Food Additives. Evaluation of certain food additives: Eighty-sixth report of the joint FAO/WHO expert committee on food additives. *WHO Tech. Rep. Ser.* **2019**, *1014*, 1–170.
24. Cheng, W.L.; Yang, J.Y.; Nie, Q.Y.; Huang, D.; Yu, C.; Zheng, L.Y.; Cai, M.M.; Thomashow, L.S.; Weller, D.M.; Yu, Z.N.; et al. Volatile organic compounds from *Paenibacillus polymyxa* KM2501-1 control *Meloidogyne incognita* by multiple strategies. *Sci. Rep.* **2017**, *7*, 16213. [[CrossRef](#)] [[PubMed](#)]
25. Sanchez-Recillas, A.; Arroyo-Herrera, A.L.; Araujo-Leon, J.A.; Nunez, E.; Andrade, R.O. Spasmolytic and antibacterial activity of two *Citrus sinensis* Osbeck varieties cultivated in Mexico. *Evid. Based Complement. Altern. Med.* **2017**, *2017*, 3960837. [[CrossRef](#)] [[PubMed](#)]
26. Kurz, C.L.; Ewbank, J.J. *Caenorhabditis elegans* for the study of host-pathogen interactions. *Trends Microbiol.* **2020**, *8*, 142–144. [[CrossRef](#)]
27. Costa, J.C.; Lilley, C.J.; Urwin, P.E. *Caenorhabditis elegans* as a model for plant-parasitic nematodes. *Nematology* **2007**, *9*, 3–16.
28. Ayuda-Durán, B.; González-Manzano, S.; González-Paramás, A.M.; Santos-Buelga, C. *Caenorhabditis elegans* as a model organism to evaluate the antioxidant effects of phytochemicals. *Molecules* **2020**, *25*, 3194.
29. Huang, W.K.; Sun, J.H.; Cui, J.K.; Wang, G.F.; Kong, L.A.; Peng, H.; Chen, S.L.; Peng, D.L. Efficacy evaluation of fungus *Syncephalastrum racemosum* and nematocide avermectin against the root-knot nematode *meloidogyne incognita* on cucumber. *PLoS ONE* **2014**, *2*, e89717. [[CrossRef](#)]
30. Hutchinson, C.M.; McGiffen, M.E.; Ohr, H.D.; Sims, J.J.; Becker, J.O. Evaluation of methyl iodide as a soil fumigant for root-knot nematode control in carrot production. *Plant Dis.* **1999**, *1*, 33–36. [[CrossRef](#)]
31. Mao, L.G.; Jiang, H.Y.; Zhang, L.; Zhang, Y.N.; Sial, M.U.; Yu, H.T.; Cao, A.C. Replacing methyl bromide with a combination of 1,3-dichloropropene and metam sodium for cucumber production in China. *PLoS ONE* **2017**, *11*, e0188137. [[CrossRef](#)] [[PubMed](#)]
32. Nishimura, M.; Umeda, M.; Ishizu, S.; Sato, M. Cytotoxicity of methyl bromide: Effect of methyl bromide on cultured mammalian cells. *J. Toxicol. Sci.* **1980**, *4*, 321–330. [[CrossRef](#)] [[PubMed](#)]
33. Zhu, W.J.; Zhang, Z.W.; Wang, X.S.; Xu, S.W.; Li, M.; Li, S. Effects of avermectin on microsomal cytochrome P450 enzymes in the liver and kidneys of pigeons. *Environ. Toxicol. Pharmacol.* **2014**, *2*, 562–569. [[CrossRef](#)] [[PubMed](#)]
34. Bernal-Gonzalez, M.; Solis-Fuentes, J.A.; Duran-Dominguez-de-Bazua, M.D. Metam Sodium (MS) in water and cane juice at different processing conditions according to the industrial case. part 1. effects of matrix, pH, temperature, processing time, and photolysis. *Water Air Soil Pollut.* **2017**, *12*, 456. [[CrossRef](#)]
35. Zhu, J.W.; Dhammi, A.; van Kretschmar, J.B.; Vargo, E.L.; Apperson, C.S.; Roe, R.M. Novel use of aliphatic n-methyl ketones as a fumigant and alternative to methyl bromide for insect control. *Pest Manag. Sci.* **2018**, *3*, 648–657. [[CrossRef](#)]
36. Fakhfakh, N.; Cognet, P.; Cabassud, M.; Lucchese, Y.; Rios, M.D.D.L. Stoichio-kinetic modeling and optimization of chemical synthesis: Application to the aldolic condensation of furfural on acetone. *Chem. Eng. Process.* **2008**, *47*, 349–362. [[CrossRef](#)]
37. Wierckx, N.; Koopman, F.; Ruijssenaars, H.J.; de Winde, J.H. Microbial degradation of furanic compounds: Biochemistry, genetics, and impact. *Appl. Microbiol. Biot.* **2011**, *6*, 1095–1105. [[CrossRef](#)]
38. Pedroso, L.A.; Campos, V.P.; Pedroso, M.P.; Barros, A.F.; Freire, E.S.; Resende, F.M.P. Volatile organic compounds produced by castor bean cake incorporated into the soil exhibit toxic activity against *Meloidogyne incognita*. *Pest Manag. Sci.* **2019**, *2*, 476–483. [[CrossRef](#)]
39. Bui, H.X.; Hadi, B.A.R.; Oliva, R.; Schroeder, N.E. Beneficial bacterial volatile compounds for the control of root-knot nematode and bacterial leaf blight on rice. *Crop Prot.* **2020**, *135*, 104792. [[CrossRef](#)]
40. Raza, W.; Wang, J.N.; Jousset, A.; Friman, V.P.; Mei, X.L.; Wang, S.M.; Wei, Z.; Shen, Q.R. Bacterial community richness shifts the balance between volatile organic compound-mediated microbe–pathogen and microbe–plant interactions. *Proc. R. Soc. B Biol. Sci.* **2020**, *1925*, 20200403. [[CrossRef](#)]

41. Manan, A.; Bazai, Z.A.; Fan, J.; Yu, H.F.; Li, L. The Nif3-family protein YqfO03 from *Pseudomonas syringae* MB03 has multiple nematicidal activities against *Caenorhabditis elegans* and *Meloidogyne incognita*. *Int. J. Mol. Sci.* **2018**, *19*, 3915. [[CrossRef](#)] [[PubMed](#)]
42. Kudelska, M.M.; Lewis, A.; Ng, C.T.; Doyle, D.A.; Holden-Dye, L.; O'Connor, V.M.; Walker, R.J. Investigation of feeding behaviour in *C. elegans* reveals distinct pharmacological and antibacterial effects of nicotine. *Invertebr. Neurosci.* **2018**, *18*, 14. [[CrossRef](#)] [[PubMed](#)]
43. Bridge, J.; Page, S.L.J. Estimation of root-knot nematode infestation levels on roots using a rating chart. *Int. J. Pest Manag.* **1980**, *26*, 296–298. [[CrossRef](#)]
44. Zhou, L.H.; Yuen, G.; Wang, Y.; Wei, L.F.; Ji, G.H. Evaluation of bacterial biological control agents for control of root-knot nematode disease on tomato. *Crop Prot.* **2016**, *84*, 8–13. [[CrossRef](#)]
45. Cheng, X.K.; Liu, X.M.; Wang, H.Y.; Ji, X.X.; Wang, K.Y.; Wei, M.; Qiao, K. Effect of emamectin benzoate on root-knot nematodes and tomato yield. *PLoS ONE* **2015**, *10*, e0141235. [[CrossRef](#)]
46. Freckman, D.H.; Virginia, R.A. Extraction of nematodes from dry valley antarctic soils. *Polar Biol.* **1993**, *13*, 483–487. [[CrossRef](#)]
47. Seesao, Y.; Gay, M.; Merlin, S.; Viscogliosi, E.; Aliouat-Denis, C.M.; Audebert, C. A review of methods for nematode identification. *J. Microbiol. Methods* **2017**, *138*, 37–49. [[CrossRef](#)]



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