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Nematicidal Evaluation and Active Compounds Isolation of *Aspergillus japonicus* ZW1 against Root-Knot Nematodes *Meloidogyne incognita*

Qiong He [1], Dongya Wang [1], Bingxue Li [1], Ambreen Maqsood [1,2] and **Haiyan Wu [1,*]**

1   Guangxi Key Laboratory of Agric-Environment and Agric-Products Safety, Agricultural College of Guangxi University, Nanning 530004, China; heqiong3344@163.com (Q.H.); wdy15677131171@163.com (D.W.); 18437958381@163.com (B.L.); ambreenagrarian@gmail.com (A.M.)

2   Department of Plant Pathology, Faculty of Agriculture and Environmental Sciences, The Islamia University of Bahawalpur, Bahawalpur 63100, Pakistan

*   Correspondence: whyzxb@gmail.com

Abstract: The root-knot nematode is one of the most damaging plant-parasitic nematodes worldwide, and the ecofriendly alternative approach of biological control has been used to suppress nematode populations. Here the nematicidal activity of *Aspergillus japonicus* ZW1 fermentation filtrate against *Meloidogyne incognita* was evaluated in vitro and in greenhouse, and the effects of *A. japonicus* ZW1 fermentation filtrate on seed germination and the active compound of *A. japonicus* ZW1 fermentation filtrate were determined. The 2-week fermentation filtrate (2-WF) of *A. japonicus* ZW1 exhibited markedly inhibitory effects on egg hatching, and 5% 2-WF showed potential nematicidal activities on second-stage juveniles (J2s); the mortality of J2s was 100% after 24 h exposure. The internal contents of nematodes were degraded and remarkable protruded wrinkles were present on the body surface of J2s. The nematicidal activity of the fermentation was stable after boiling and was not affected by storage time. A germination assay revealed that 2-WF did not have a negative effect on the viability and germination of corn, wheat, rice, cowpeas, cucumbers, soybeans, or tomato seeds. The pot-grown study confirmed that a 20% fermentation broth solution significantly reduced root galls and egg numbers on tomatoes, and decreased galls and eggs by 47.3% and 51.8% respectively, over Czapek medium and water controls. The active compound from the *A. japonicus* ZW1 fermentation filtrate was isolated and identified as 1,5-Dimethyl Citrate hydrochloride ester on the basis of nuclear magnetic resonance (NMR) and LC-MS (liquid chromatograph-mass spectrometer) techniques. Thus, fermentation of *A. japonicus* ZW1 could be considered a potential new biological nematicide for the control of *M. incognita*.

Keywords: biocontrol *Aspergillus japonicus*; root-knot nematode; fermentation filtrate; biological control; seed germination

1. Introduction

Root-knot nematodes (*Meloidogyne* spp.) are economically important worldwide pathogens causing considerable damage to many crops, including cucumbers, tomatoes, rice [1–4], and even cotton [5,6]. *Meloidogyne incognita* is an important species of root-knot nematodes worldwide due to its direct impact on crop yields [7–9]. Specifically, it is capable of causing an estimated yield loss of 5–43% within vegetable crops cultivated in tropical and subtropical areas [10] and estimated $100 billion loss per year worldwide [11].

Due to their short life cycle and high reproduction rates, these root-knot nematodes have been particularly challenging to control. Previously, chemical nematicides are efficiently used to suppress

nematode populations, such as fenamiphos, sebufos, dazomet, and carbofuran [12]; however, these have been found to be harmful to both the eco-environment and human health due to their toxic effects. Thus, as a result of these negative impacts and the significant economic losses which can result from nematodes, new and alternative biological control options are urgently needed [13]. Therefore, the use of biological agents to suppress the population of plant-parasitic nematodes could provide an alternative strategy to sustainably manage plant-parasitic nematodes. Using biofumigation instead of harmful fumigants (like synthetic nematicide methyl bromide) to control nematodes is an increasingly feasible method of parasitic nematode management [14]. Plants such as *Melia azedarach* have been found to be potential sources of biofumigation plant material to control *Meloidogyne* spp. on tomato [15]. Moreover, microbial agents for the control of plant-parasitic nematodes is also a potential method; such as bacteria [16,17], fungi [18,19] and actinomycetes [20], which are nematophagous or antagonistic for root-knot nematodes. Specifically, *Arthrobotrys irregularis*, *Pochonia chlamydosporium*, *Paecilomyces lilacinus*, *Myrothecium verrucaria*, bacteria *Pasteuria usgae*, *Bacillus firmus*, *Burkholderia cepacia*, *Pseudomonas fluorescens*, and *Streptomyces avermitilis* [21,22] have been commercially used in many countries for the control of plant-parasitic nematodes. Some potential microbial sources were constantly obtained, volatiles from beneficial bacteria (*Bacillus* sp., *Paenibacillus* sp. and *Xanthomonas* sp.) can control *M. graminicola* second-stage juveniles (J2s) on rice and significantly reduced infection of susceptible rice [23]. Co-inoculation of *Streptomyces* spp. strains KPS-E004 and KPS-A032 showed success in suppressing root-knot nematode [24].

In our previous study, *A. japonicus* ZW1 culture filtrate was shown to have marked nematicidal activity against *M. incognita*. As a result, the main objective of this work was to evaluate the potential biological control of *A. japonicus* ZW1 against root-knot nematodes including: (1) the nematicidal activity of *A. japonicus* ZW1 fermentation filtrate on eggs and J2s within pot and in vitro experiments; (2) electron microscopic evaluation of J2 bodies after treatment with 2-week fermentation filtrate (2-WF); (3) effect of boiling and storage time on nematicidal activity stability of the fermentation filtrate; and (4) evaluation for the effect of *A. japonicus* ZW1 fermentation filtrate on the germination of various crop seeds.

2. Materials and Methods

2.1. Nematode Preparation

Tomato seeds (cv. Xin Bite 2 F1) were sourced from Yashu Garden Seeds Co., Ltd., (Guangzhou, China) and were used to generate seedlings for culturing the *M. incognita*. For the nematodes culture, one-month-old tomato seedlings were transplanted into pots ($7 \times 7 \times 8$ cm) with second stage juveniles of root-knot nematode-infected peat moss (Gui Yu Xin Nong Technology Co., Ltd., Nanning, China) and maintained at 25 °C with a 14 h light (22000 Lux) and 10 h dark photoperiod treatment within a GXZ-280C incubator (Jiangnan Instrument Factory, Ningbo, China). Tomato roots were collected 35 days after inoculation and were gently rinsed with tap water. Eggs were then extracted with 1% NaOCl [25] and hatched at 25 °C using the modified Baermann funnel method [26]. Eggs were put in 30 ìm pore sieves, nested in petri dishes (6 cm-diameter) containing 3 mL distilled water, and the fresh J2s in water were then collected on the day of experiment and used for subsequent experimentation.

2.2. Fermentation Filtrate Preparation

A. japonicus ZW1 from soil was deposited in the China Center for Type Culture Collection (accession number CCTCC No. M 2014641) and GenBank (accession number KR708636.1). One cm^2 potato dextrose agar (PDA) with a fresh culture of *A. japonicus* ZW1 (cultured 3-5 days at 25 °C) was inoculated in triangular flasks with 100 mL Czapek medium (NaNO$_3$ 0.2 g, KCl 0.05 g, FeSO$_4$ 0.001 g, K$_2$HPO$_4$ 0.1 g, MgSO$_4$ 0.05 g, Sucrose 3.0 g, H$_2$O 100 mL) and incubated in a MQD-S2R shaker (Minquan Instrument Co., Ltd., Shanghai, China) at 150 rpm and 25 °C [27] for 3 consecutive weeks, with 10 triangular flasks replicates per week. Czapek medium without inoculation was used as a

negative control. At the end of the 3-week period, fermentation broth from a total of 30 conical flasks was then filtered using 0.45 μm Millipore filters (Whatman, Clifton, NJ, USA) and 1-week fermentation filtrate (1-WF), 2-WF, and 3-week fermentation filtrate (3-WF) were prepared. The concentration of 2.5% (i.e., fermentation filtrate volume: sterilized water volume = 1:39), 5% (1:19), 10% (1:9), 20% (1:4) and 50% (1:1) of 1-week fermentation filtrate(1-WF), 2-week fermentation filtrate (2-WF), and 3-week fermentation filtrate (3-WF) were used and 20% Czapek medium and sterilized water were used as control.

2.3. Effect of Fermentation Filtrate on Meloidogyne Incognita Egg Hatching

Fresh eggs were treated with 2.5%, 5%, 10%, 20%, and 50% 1-WF, 2-WF, and 3-WF; and also 20% Czapek medium and sterilized water as controls. The specific experimental conditions were as follows: approximately 100 eggs and 200 μL of different concentrations of fermentation filtrate were dispensed into each well of 96-well plate, with 4 replicates for each treatment. Additionally, all experiments were performed in triplicate. The initial number of eggs was counted, and the hatched J2s were recorded using an inverted microscope (Ti-S, Nikon Instruments Inc., Tokyo, Japan) at 0, 3, 6, 9, 12, 15 d after exposure in the dark at 25 °C. The cumulative hatching rate was calculated using the following formula: cumulative hatching rate = (the number of hatched J2s)/(the initial number of eggs) × 100%.

2.4. Nematicidal Activity of Fermentation Filtrate on Meloidogyne Incognita J2s

Approximately 60 fresh J2s were contained in each well of a 96-well plate and treated with 200 μL of 2.5%, 5%, 10%, 20%, and 50% 1-WF, 2-WF, and 3-WF, 20% Czapek medium and sterilized water. The number of dead nematodes were counted using a Ti-S Nikon microscope (Nikon Instruments Inc., Tokyo, Japan) at 6, 12, 24, 48 h after treatment with the solutions and pictures were taken at each time point except for 48 h. It wax determined whether he bodies of dead J2s were straight and lacking movement even after mechanical prodding [28,29]. The test was conducted at 25 °C in the dark and the experiment was replicated 4 times. J2 mortality was calculated for each well as follows: mortality = (the number of dead J2s/total J2s) × 100%. This experiment was performed a total of three times.

2.5. Scanning Electron Microscopy Observations

J2s were treated with 10% 2-WF for 10 h and subsequently analyzed with scanning electron microscopy (SEM) using the approach as described below [30,31]. In preparation for the microscopic evaluations, J2 specimens were fixed in 2.5% glutaraldehyde with 0.1 M phosphate buffer (pH 7.2) at 4 °C overnight and subsequently washed 3 times in 0.1 M phosphate buffer. Afterwards, they were then fixed in 1% osmium tetroxide for 2 h, washed 3 times in 0.1 M phosphate buffer again, dehydrated in a graded series of ethanol, critical point dried with Quorum K850 critical dryers (Emitech, East Sussex, England, UK) and finally sputter coated with MSP-2S gold-palladium (IXRF, Austin, TX, USA). Prepared J2 specimens were observed using a SU8100 scanning electron microscope (Hitachi, Tokyo, Japan) operating at 3.0 kV accelerating voltage.

2.6. Transmission Electron Microscopy Observations

The technical approach was very similar to the aforementioned method described for 'scanning electron microscopy observations'; however, after J2s were dehydrated with ethanol, they were subsequently embedded in Araldite (Sigma-Aldrich, Sigma-Aldrich LLC., Darmstadt, Germany). To enable evaluation of the specimens, ultrathin sections (70 nm) were obtained using an EM UC7 ultramicrotome (Leica, Wetzlar, Germany) with a Diatome Ultra 45° diamond knife (Diatome Ltd., Helmstrasse Nidau, Switzerland). Sectioned samples were then stained with uranyl acetate and lead citrate using carbon film copper 500 mesh [30,32]. Sections of the J2 bodies were then observed using an HT7700 transmission electron microscope (Hitachi, Tokyo, Japan) operating at an 80.0 kV accelerating voltage.

2.7. Greenhouse Experiment

Thirty day old (3–4 leaf stage) healthy tomato seedlings (cv. Xin Bite 2 F1) were transplanted in a pot (785 cm^3) containing 250 g autoclaved and dried peat moss. A total of 2000 fresh J2s were inoculated in each pot at 3 days after transplanting. Subsequently, 130 mL of 20% and 50% 2-WF were used in this experiment and applied in pots. 20% of Czapek medium and tap water were utilized as controls. A randomized design with 6 replicates for each treatment group was used for the pot experiment and all materials were maintained after inoculation at 25 °C in a greenhouse with a 14 h light and 10 h dark photoperiod. Thirty-five days after transplantation, tomato roots were collected and gently washed with tap water to remove residual materials. Plant height, root fresh weight, and the total number of galls and eggs per plant root system were determined. The eggs were extracted separately from plants with a 1% NaOCl method as previously described [25] and were subsequently collected in beakers with water. Afterwards, 50 μL of a well-mixed egg suspension solution were transferred to a counting dish to enable egg count determination. Eggs were counted three times and the total number of eggs in the entire suspension was calculated. This experiment was repeated twice.

2.8. Effect of Boiling and Storage Time on Nematicidal Activity Stability of Fermentation Filtrate

Two-hundred mL of fresh 2-WF was dispensed into two 100 mL beakers respectively. One of the beakers was boiled in a microwave oven at 100 °C, whereas the second beaker was maintained at room temperature. The fermentation filtrate from two beakers were diluted to 10% and sterilized water was used as a control. Nematicidal activity was then conducted as described above and the experiment was triplicated.

For the analysis of storage time, the experiment was set up for 1-, 2-, and 3-week old 2-WF at 4 °C and 25 °C in dark, respectively; with 4 replicates for each treatment. After storage, the 2-WF solution was filtered through a sterile 0.45 μm polyethersulfone filter (Whatman, Clifton, NJ, USA) and subsequently diluted to a 10% solution in sterilized water. Sterilized water alone was used as a negative control. The nematicidal activity was measured as described above and this experiment was repeated 3 times.

2.9. Evaluation of the Strain Fermentation Filtrate on The Germination of Crop Seeds

In this study, the effect of 2-WF of *A. japonicus* ZW1 was evaluated on seed germination of various crops, e.g., from corn (Qingnong 13), wheat (Mianmai 41), cowpeas (Shanlv), cabbage (Green column), cucumbers (Liaoning 8), rice (Teyou 09103), tomatoes (Hongyingguo 808), and soybeans (Ludou 4). First, healthy seeds were surface sterilized with 2% NaOCl for 3 min and subsequently rinsed 5 times with sterilized water [33]. Seeds were treated with 10% and 20% 2-WF in triplicates across 3 independent experiments, with sterilized water used as a negative control. The sterilized crop seeds were then exposed to the fermentation filtrate in a moist chamber and incubated for several days in the dark at room temperature (25 °C). Sprouted seeds were counted every day until the seed germination rate no longer changed. The seed germination rate was calculated as: (number of germinated seed/total tested seeds) × 100%.

2.10. Isolation and Structural Determination of Aspergillus Japonicus ZW-1 Nematicidal Metabolites

Eight litre of *A. japonicus* ZW-1 2-week fermentation broth was filtered through 8 layers of muslin gauze, then concentrated to 500 mL using rotary evaporation (Hei-VAP Core ML G3, Instruments GmbH & Co. Heidolph, KG, Schwabach, Germany) at 55 °C. The crude extract (15.6 g) from *A. japonicus* ZW-1 fermentation broth was extracted with 1-butanol and evaporated at 40 °C until dry, dissolved in methanol (MeOH) and chromatographed on methylated sephadex LH20 (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) using MeOH as eluent to give two fractions, the two fractions were dissolved in distilled water to make 2.0 mg mL^{-1} aqueous solution for activity assay. One fraction showed activity against J2. This active fraction was dissolved in the chloroform, at which point white

crystals formed. The solution was filtered through cotton which was then washed 20 times using chloroform and dried at room temperature to get the purified active compound.

The chemical structures of the active compound were determined using nuclear magnetic resonance (NMR) analysis and high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) analysis. ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were acquired in MeOH with a Bruker AVANCE III HD600 spectrometer (Bruker Corporation, Faellanden, Switzerland) at 600 MHz for ^{1}H NMR spectra and 125 MHz for ^{13}C NMR spectra using tetramethylsilane as the internal standard. HR-ESI-MS analysis was performed using a Waters E2695 model ion trap mass spectrometer (Waters, Milford, MA, USA) [34]. The nematicidal activity of active compounds at different concentrations (1.25, 1.00, 0.75, 0.50, 0.25 mg mL^{-1}) was measured as described above and this experimental approach was repeated 3 times. Sterilized water was used as a control.

2.11. Statistical Analysis

Data were analyzed using SPSS 19.0. software (SPSS Inc. Chicago, IL, USA) and statistical significance was calculated using a one-way analysis of variance (ANOVA). The means of different parameters for each treatment group were compared among each other using a Fisher's protected least significant difference (LSD) test at $p < 0.05$. All figures for statistical analyses were made using Sigma Plot 10.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effect of Fermentation Filtrates on Hatching of Meloidogyne Incognita Eggs

The fermentation filtrate of *A. japonicus* ZW1 at various concentrations and different time points showed significant nematicidal activity against cumulative hatching rate of eggs. The cumulative hatching rate of eggs increased over time in the 1-WF, 2-WF, and 3-WF treatments (Figure 1). In relative comparison to 1-WF, *M. incognita* eggs exhibited higher sensitivity to 2-WF and 3-WF. Fifteen days after incubation, the cumulative hatching rates in 20% and 50% 1-WF were 71.1% and 30.1%, respectively, and were significantly lower in comparison to 2.5%, 5%, and 10% 1-WF and controls ($p < 0.05$). For the 2-WF treated samples, cumulative hatching rates in 5%, 10%, 20%, and 50% 2-WF were 42.5%, 36.0%, 24.3%, and 6.4%, respectively, 15 d after incubation. These values were significantly lower than that of the 2.5% 2-WF and control treatments ($p < 0.05$). Cumulative hatching rates in 5%, 10%, 20%, and 50% 3-WF treatments were 53.0%, 42.2%, 34.6%, and 21.2%, respectively, 15 d after incubation. These results were significantly lower than that of the 2.5% 2-WF and control treatments ($p < 0.05$).
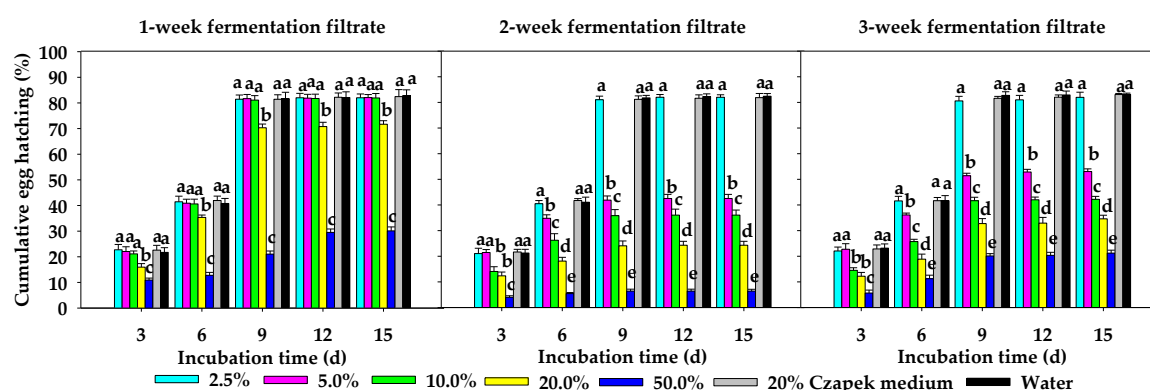

Figure 1. Cumulative *Meloidogyne incognita* eggs hatching rates in *Aspergillus japonicus* ZW1 fermentation filtrate. The bars represent the standard error. The same letter is not significantly different ($p < 0.05$) according to a Fisher's protected least significant difference (LSD) test.

3.2. Nematicidal Activity of Fermentation Filtrates on Meloidogyne Incognita J2s

The time of culturing influenced the nematicidal activity of the fermentation filtrate on J2s (Figure 2). In comparison to the 1-WF and control treatments, the mortality of J2s was higher in 2-WF and 3-WF treatments at different time points post incubation. In the 1-WF treatment, the mortality of J2s was less than 3.3% and no significant difference was observed after treatment for a 6 to 48 h period. Conversely, application of 2-WF and 3-WF resulted in a significantly higher mortality of J2s at different concentrations of the fermentation filtrates as compared to the controls ($p < 0.05$). When investigating 50% 2-WF and 3-WF, the mortality of J2s reached 100% after a 6 h incubation period. After the 48 h incubation period, the mortality of 2.5% 2-WF and 3-WF treatments reached 56.1% and 56.8%, respectively, and were all significantly higher than the controls ($p < 0.05$). From a morphological perspective, treatment with 2-WF resulted in differences in the J2 when compared to the controls (Figure 3). Specifically, microscopic observations revealed that the bodies of J2s in the 2-WF treatment were either straight or arched without movements at 6 h post-incubation (Figure 3, A2). However, bubbles (Figure 3, Bu) appeared in the body of J2s over time and protruded wrinkles (Figure 4, Wr) on the body surface and areas of intensive cytoplasmic vacuolization were observed (such as damaged areas; Figure 5, Da) at 10 h post-exposure to treatment with 2-WF.
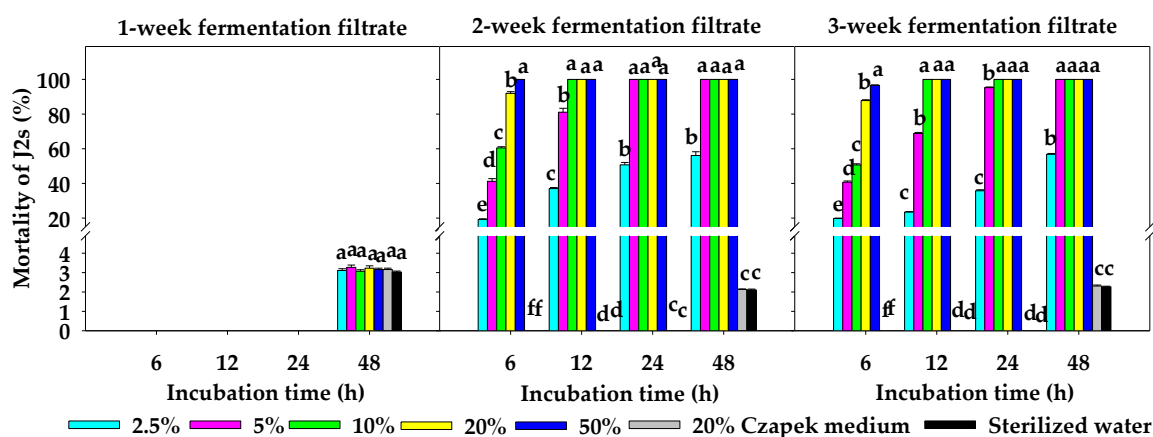
Figure 2. The mortality of *Meloidogyne incognita* J2s in *Aspergillus japonicus* ZW1 fermentation filtrate. Means with the same letter in each group designate no significant differences ($p < 0.05$) based on analysis with a Fisher's protected LSD test.

3.3. Greenhouse Experiment

Treatment with fermentation broth of *A. japonicus* ZW1 resulted in a significant reduction in the number of root galls and eggs per plant as compared to controls (Table 1). The number of root galls and eggs were 8.2 and 3488.9 per plant in the 50% fermentation broth treatment, respectively; whereas 16.8 and 6020 were observed per plant in the 20% fermentation broth treatment, respectively. In both treatments, the number of root galls and eggs was significantly lower than what was observed in controls ($p < 0.05$). The 50% fermentation broth decreased root galls by 78.6% and eggs by 69.4% per plant in comparison to treatment with the Czapek medium control (38.4 root galls and 11413.3 eggs) and 79.9% root galls and 72.0% eggs per plant compared with the tap water control (40.8 root galls and 12480.0 eggs, respectively), and root galls and eggs from the 20% fermentation broth treatment decreased by 56.3% and 47.3% per plant compared with the Czapek medium control (38.4 root galls and 11413.3 eggs, respectively), and 58.8% and 51.8% compared with the tap water control (40.8 root galls and 12480.0 eggs, respectively).
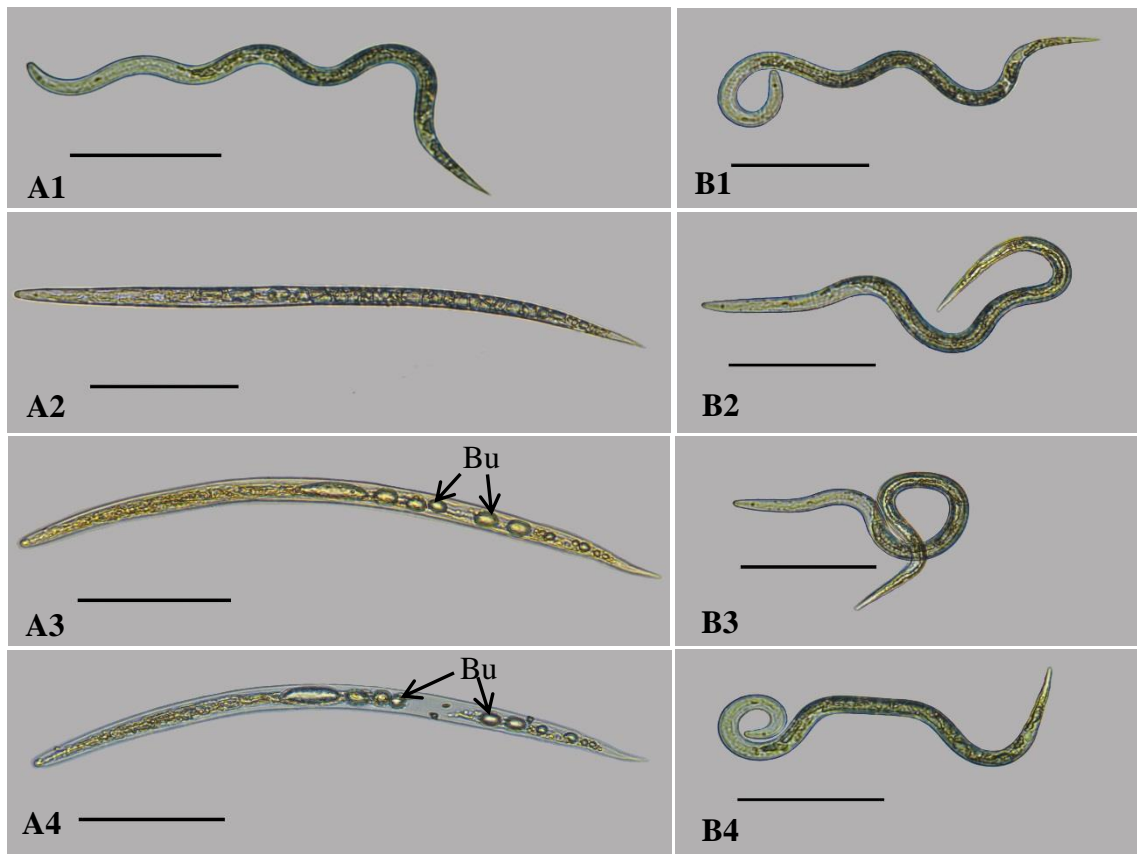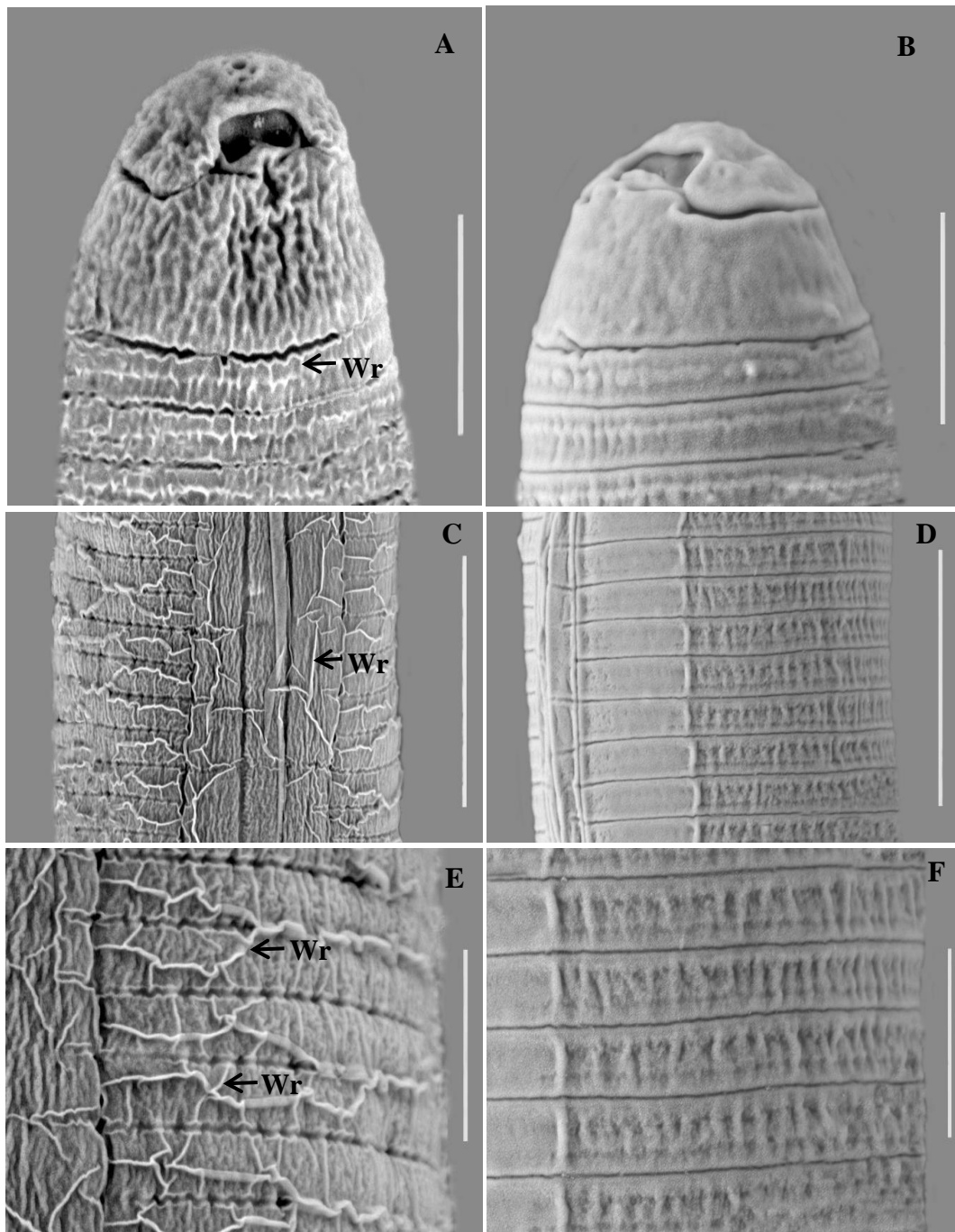
Figure 3. Morphology of second-stage juveniles of *Meloidogyne incognita* treated with 10% 2-week fermentation filtrate (2-WF) of *Aspergillus japonicus* ZW1. **A1**–**A4** were treated with 10% 2-WF; **B1**–**B4** were treated with sterilized water; **A1** and **B1** were treated at 0 h; **A2** and **B2** were treated at 6 h; **A3** and **B3** were treated at 12 h; and **A4** and **B4** were treated at 24 h. **Bu**: bubbles. Scale bars of **A1**–**A4** and **B1**–**B4** were 100 μm.

Table 1. Effect of *Aspergillus japonicus* ZW1 fermentation broth on the formation of galls and eggs on roots and the growth of tomato plants infected with *Meloidogyne incognita*.

Treatments	Plant Height (cm)	Fresh Root Weight (g)	Root Galls per Plant	Egg Number per Plant
50% Fermentation Broth	26.6 ± 0.6 a	0.6 ± 0.3 a	8.2 ± 1.7 c	3488.9 ± 155.6 d
20% Fermentation Broth	26.5 ± 0.6 a	0.9 ± 0.2 a	16.8 ± 1.4 b	6020.0 ± 214.9 c
Czapek Medium Control	26.9 ± 0.5 a	0.7 ± 0.1 a	38.4 ± 4.3 a	11413.3 ± 338.9 b
Tap Water Control	26.4 ± 0.6 a	0.8 ± 0.2 a	40.8 ± 3.8 a	12480.0 ± 200.4 a

Values represent means ± standard error of six replicate plants per treatment using the combination of two different experiments. Means with the same letter were not significantly different ($p < 0.05$) according to a Fisher's protected LSD test.

Figure 4. Visualization of the effect of 10% 2-WF of *Aspergillus japonicus* ZW1 on the morphology of *Meloidogyne incognita* J2s with scanning electron microscopy. (**A,C,E**) J2s treated with 10% 2-WF. (**B,D,F**) J2s treated with sterilized water. (**A,B**) Head region of J2. (**C–F**) The lateral field of J2. Scale bars of (**A,B,E,F**) and (**C,D**) were 2 and 5 μm, respectively. **Wr**: protruded wrinkles (black arrow).
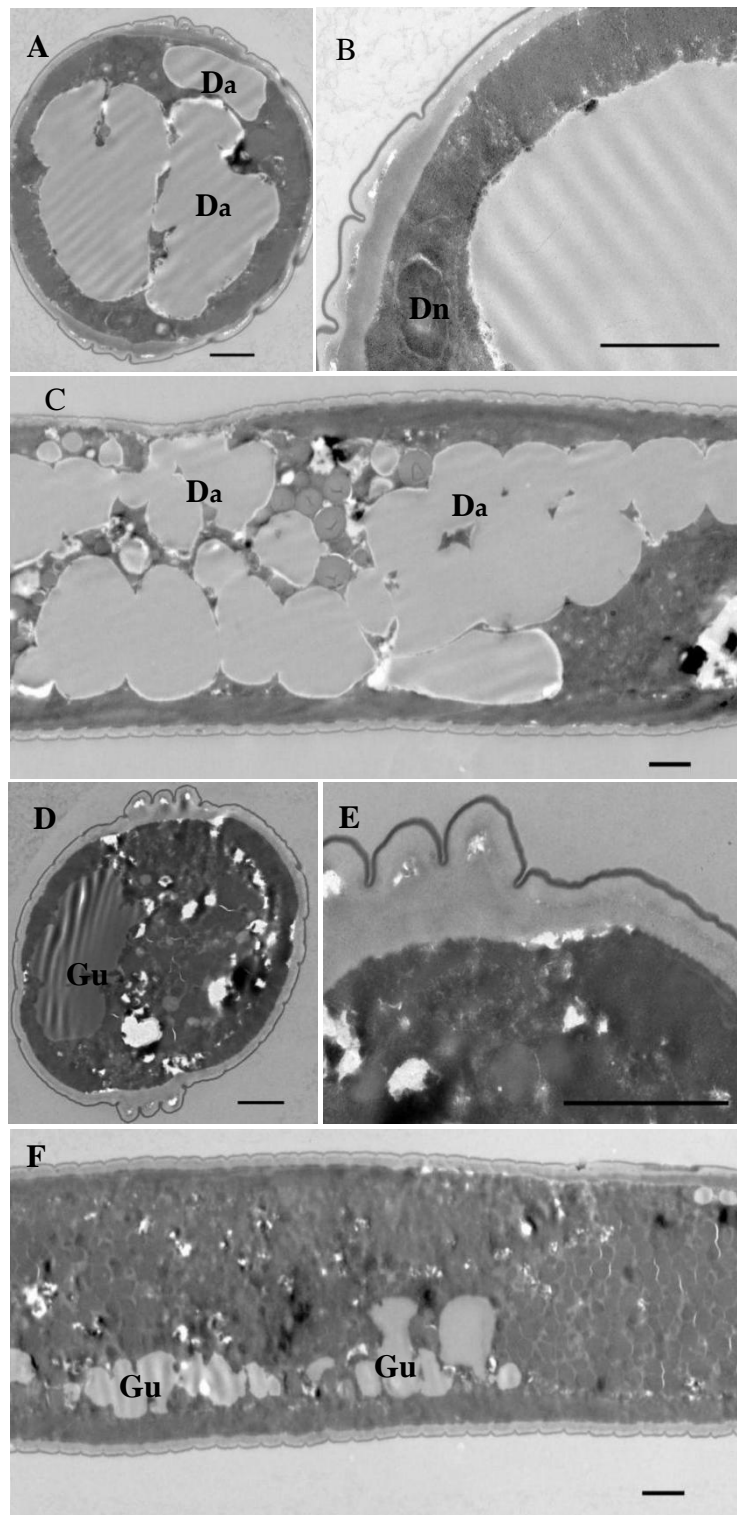
Figure 5. Cross-sections of *Meloidogyne incognita* J2 treated with 10% 2-WF of *Aspergillus japonicus* ZW1. (**A**–**C**) J2s treated with *A. japonicus* ZW1 fermentation filtrate. (**D**–**F**) J2s treated with sterilized water. Scale bars of **A**, **B**, **C**, **D**, **E**, and **F** were 2 μm. **Da**: damaged and area. **Gu**: gut. **Dn**: destructed nuclei.

3.4. Effect of Boiling and Storage Time on the Nematicidal Activity of Fermentation Filtrate

The mortality of J2s in fresh and boiled 10% 2-WF did not display any significant differences (Table 2). After a 48 h incubation period, the mortality of J2 reached 100.0% in both fermentation filtrates and was significantly higher than what was observed in the sterilized water treatment ($p < 0.05$).

Table 2. Nematicidal activity of the boiled fermentation filtrate of *Aspergillus japonicus* ZW1 on *Meloidogyne incognita* J2s.

Treatment with 10% 2-WF	Incubation Time (h)			
	6	12	24	48
Untreated	44.9 ± 5.6 a	91.2 ± 3.3 a	91.9 ± 3.4 a	99.2 ± 0.8 a
Boiled	40.5 ± 4.7 a	93.6 ± 2.4 a	96.6 ± 2.0 a	99.0 ± 1.0 a
Sterilized Water	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.1 ± 0.1 b

Values represent means ± standard deviation of three replicates. Means with the same letter are not significantly different ($p < 0.05$) according to a Fisher's protected LSD test.

No significant difference was observed in the mortality of J2s exposed to different storage conditions of 10% 2-WF (Table 3). Specifically, they all reached 100% mortality after a 48 h incubation period, which was higher than the sterilized water treatment ($p < 0.05$).

Table 3. Mortality of *Meloidogyne incognita* J2s in *Aspergillus japonicus* ZW1 fermentation filtrate under different storage conditions.

Treatments with 10% 2-WF	Storage Time	Incubation Time (h)			
		6	12	24	48
	1-week	58.9 ± 5.3 a	99.4 ± 0.6 a	100.0 ± 0.0 a	100.0 ± 0.0 a
4 °C	2-week	58.3 ± 2.2 a	98.8 ± 0.7 a	100.0 ± 0.0 a	100.0 ± 0.0 a
	3-week	60.8 ± 2.2 a	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a
	1-week	62.1 ± 1.8 a	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a
25 °C	2-week	55.6 ± 3.3 a	99.0 ± 0.6 a	100.0 ± 0.0 a	100.0 ± 0.0 a
	3-week	58.4 ± 4.4 a	98.7 ± 0.8 a	99.6 ± 0.4 a	100.0 ± 0.0 a
Sterilized Water	–	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	1.4 ± 0.8 b

Values represent the means ± standard error of four replicates; means with the same letter are not significantly different ($p < 0.05$) according to a Fisher's protected LSD test.

3.5. Effect of Fermentation Filtrate on Seed Germination

The 20% and 10% 2-WF did not influenced the germination of corn, rice, tomato, cowpea, and cucumber seeds (Table 4). Two days after incubation with 10% 2-WF, the wheat seed germination rate was 85.4% and was significantly higher than what was observed in the control ($p < 0.05$). After an extended period of time beyond the 48-h time period, this value did not increase any further. For soybean seeds treated with 10% 2-WF, germination was significantly lower than what was observed in sterilized water ($p < 0.05$) at day 1; however, there were no statistically significant differences 2–5 days post-incubation across 20% and 10% 2-WF and sterilized water treatments. For cabbage seeds, germination in 20% 2-WF was significantly lower than what was observed in 10% 2-WF and control treatments ($p < 0.05$).

Table 4. Seed germination (%) in different concentrations of 2-week *Aspergillus japonicus* ZW1 fermentation filtrate.

Seeds	Treatments	Incubation Time (d)					
		1	2	3	4	5	6
Wheat	20%	64.6 ± 4.5 a	78.1 ± 1.8 ab	78.1 ± 1.8 ab	78.1 ± 1.8 ab	78.1 ± 1.8 ab	–
	10%	63.5 ± 5.5 a	85.4 ± 3.8 a	85.4 ± 3.8 a	85.4 ± 3.8 a	85.4 ± 3.8 a	–
	Sterilized Water	60.4 ± 4.6 a	72.5 ± 1.3 b	73.5 ± 0.9 b	73.5 ± 0.9 b	73.5 ± 0.9 b	–
Corn	20%	10.4 ± 1.0 a	83.3 ± 1.0 a	88.5 ± 2.8 a	88.5 ± 2.8 a	88.5 ± 2.8 a	–
	10%	11.5 ± 4.5 a	81.3 ± 6.5 a	90.6 ± 4.8 a	90.6 ± 4.8 a	90.6 ± 4.8 a	–
	Sterilized Water	9.4 ± 4.8 a	78.1 ± 4.8 a	89.6 ± 2.1 a	89.6 ± 2.1 a	89.6 ± 2.1 a	–
Rice	20%	3.0 ± 1.8 a	94.0 ± 1.8 a	97.0 ± 1.8 a	97.0 ± 1.8 a	97.0 ± 1.8 a	–
	10%	4.0 ± 2.7 a	96.0 ± 4.0 a	97.0 ± 3.0 a	97.0 ± 3.0 a	97.0 ± 3.0 a	–
	Sterilized Water	1.0 ± 1.0 a	90.9 ± 3.0 a	93.9 ± 1.8 a	93.9 ± 1.7 a	93.9 ± 1.8 a	–
Tomato	20%	0.0	48.1 ± 2.9 a	73.1 ± 3.2 a	81.8 ± 3.3 a	87.5 ± 1.8 a	87.5 ± 1.8 a
	10%	0.0	51.6 ± 2.7 a	69.5 ± 3.6 a	79.5 ± 4.6 a	89.3 ± 3.3 a	91.3 ± 3.2 a
	Sterilized Water	0.0	46.6 ± 8.2 a	72.8 ± 4.2 a	85.0 ± 4.3 a	90.0 ± 2.5 a	91.0 ± 1.6 a
Soybean	20%	33.3 ± 2.8 ab	90.6 ± 4.8 a	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a	–
	10%	26.0 ± 5.2 b	86.5 ± 1.0 a	96.8 ± 1.8 a	99.0 ± 1.0 a	99.0 ± 1.0 a	–
	Sterilized Water	41.8 ± 3.8 a	90.6 ± 3.6 a	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a	–
Cowpea	20%	56.3 ± 3.6 a	99.0 ± 1.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a	–
	10%	64.3 ± 7.3 a	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a	–
	Sterilized Water	65.6 ± 3.6 a	99.0 ± 1.0 a	99.0 ± 1.0 a	99.0 ± 1.0 a	99.0 ± 1.0 a	–
Cucumber	20%	100.0	100.0	100.0	100.0	100.0	–
	10%	100.0	100.0	100.0	100.0	100.0	–
	Sterilized Water	100.0	100.0	100.0	100.0	100.0	–
Cabbage	20%	65.1 ± 4.6 b	72.1 ± 6.9 b	72.1 ± 6.9 b	72.1 ± 6.9 b	72.1 ± 6.9 b	–
	10%	89.0 ± 1.2 a	95.1 ± 1.8 a	96.1 ± 2.5 a	96.1 ± 2.5 a	96.1 ± 2.5 a	–
	Sterilized Water	77.9 ± 4.0 a	95.0 ± 3.7 a	95.0 ± 3.7 a	95.0 ± 3.7 a	95.0 ± 3.7 a	–

Values represent the means ± standard error of four replicates; means with the same letter were not significantly different ($p < 0.05$) according to a Fisher's protected LSD test.

3.6. Structural Confirmation of Nematicidal Substance from 2-WF

The active compound was a pale-yellow crystal, which can dissolve easily in water. The ^{1}H NMR spectrum in MeOH exhibited signals due to two methyl groups at δ 3.68 (each 3H, s, 7, 8-CH$_3$), 2.95, 2.85 (each 2H, AB system, d, J = 12.0 Hz, 2, 4-CH$_2$). The ^{13}C NMR and heteronuclear multiple-quantum correlation spectra revealed two carbonyl carbons at δ$_C$ 175.00 (s, C-6), 170.46 (s, C-1, C-5), two methoxy groups 72.84 (s, C-3), 50.78 (q, C-7, C-8), 42.63 (t, C-2, C-4). The electrospray ionization mass spectrometry (ESI-MS) data of active compound was identified the molecular formula of C$_8$H$_{12}$O$_7$ by the [M]$^-$ ion signal at *m/z* 219 [M]$^-$. The structure of the active compound was determined to be 1,5-Dimethyl Citrate hydrochloride ester (C$_8$H$_{12}$O$_7$ HCl, Figure 6) by the analysis of its spectroscopic data and comparison with the values in the literature [35].
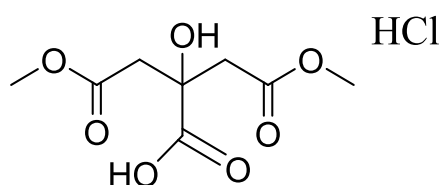
Figure 6. Chemical structures of active compound from *Aspergillus japonicus* ZW1 fermentation filtrate.

3.7. Effect of 1,5-Dimethyl Citrate Hydrochloride Ester on Meloidogyne Incognita J2s

1,5-Dimethyl Citrate hydrochloride ester had a strong toxic activity against J2s at low concentrations, and J2s mortality increased with the duration of exposure in different concentration of 1,5-Dimethyl Citrate hydrochloride ester (Table 5). There were significant differences in mortality between concentrations and control after exposure ($p < 0.05$). The mortality of J2s in concentrations of 1.25, 1.00, 0.75, 0.50, and 0.25 mg mL^{-1} of 1,5-Dimethyl Citrate hydrochloride ester were 91.7%, 57.7%,

36.9%, 20.8%, and 3.3% respectively at 48 h after exposure, which were significantly higher than that of sterilized water ($p < 0.05$).

Table 5. Mortality (%) of *Meloidogyne incognita* J2s in different concentrations of active compound from *Aspergillus japonicus* ZW-1 fermentation filtrate.

Concentration mg/mL	Incubation Time (h)			
	6	12	24	48
1.25	63.4 ± 0.9 a	72.9 ± 0.5 a	78.8 ± 0.6 a	91.7 ± 0.5 a
1.00	39.9 ± 0.7 b	44.4 ± 0.6 b	47.1 ± 0.4 b	57.7 ± 0.5 b
0.75	23.3 ± 0.8 c	31.4 ± 0.3 c	34.1 ± 0.7 c	36.9 ± 0.7 c
0.50	2.0 ± 0.3 d	4.8 ± 0.1 d	7.9 ± 0.2 d	20.8 ± 0.7 d
0.25	0.0 ± 0.0 e	0.0 ± 0.0 e	1.6 ± 0.1 e	3.3 ± 0.1 e
Sterilized Water	0.0 ± 0.0 e	0.0 ± 0.0 e	0.0 ± 0.0 f	0.0 ± 0.0 f

Values represent the means ± standard error of four replicates; means with the same letter each column were not significantly different ($p < 0.05$) according to a Fisher's protected LSD test.

Nematicidal activity of 1,5-Dimethyl Citrate hydrochloride ester was evaluated by comparing the median lethal concentrations (LC50) for different concentrations on *M. incognita* J2s under different exposure times. The concentrations at which 50% of the dead *M. incognita* J2s (LC50) were 1.0373, 0.9646, 0.9397, and 0.7614 mg mL^{-1} 1,5-Dimethyl Citrate hydrochloride ester for 6, 12, 24, and 48 h respectively. The LC50 values were decreasing with the enhanced of exposure time (Table 6).

Table 6. Toxicity of active compound to *Meloidogyne incognita* J2s at different treatment durations.

Exposure Time (h)	Slope (±SE)	Correlation Coefficient	LC50 (95%CI)	LC90 (95%CI)
6	4.8790(±0.2118)	0.9881	1.0373 (0.9112–1.1808)	1.5283 (1.2756–1.8312)
12	5.1225(±0.2843)	0.9800	0.9646 (0.8229–1.1308)	1.4059 (1.1282–1.7520)
24	5.1099(±0.1618)	0.9760	0.9397 (0.7922–1.1145)	1.9421 (1.4234–2.6498)
48	5.4928(±0.2180)	0.9596	0.7614 (0.6261–0.9260)	1.5469 (1.0971–2.1811)

LC-lethal concentration expressed in mg/mL active compound with 95% confidence intervals (CI). SE, standard error.

4. Discussion

In general, the management of parasitic nematodes is a challenging process and current control strategies are mostly dependent upon the application of nematicides [36]. However, many effective nematicides have been restricted for usage and have been banned from the market in recent years due to environmental concerns [37]. Biological options are gaining attention as promising new tools due to their environmentally-friendly and non-toxic characteristics. The potential for using microbes in controlling plant-parasitic nematodes has been documented [38] and effective microbes have been obtained from soil, plants, and the surface of nematodes [39–41]. *Aspergillus* spp. are very common in soil and are lethal to the nematode population; *A. niger* and *A. candidus* were the potential fungal agents to be used against plant-parasitic nematodes [35,42,43]. The results of this study indicated that fermentation of the *A. japonicus* ZW1 from soil was found to not only inhibit egg hatching but was also toxic to nematodes in vitro. The 2-WF was shown to be more toxic to J2s than 1-WF and 3-WF; this effect showed the presence of more active compounds in 2-WF, worth previous characterization. The similar behavior of several fungi and bacteria were also studied against plant parasitic nematodes. Among them a culture filtrate of the rhizosphere bacterium *Pseudoxanthomonas japonensis* isolated from soil exhibited strong nematicidal activity against the *M. incognita* [30]; a metabolite of *Xylaria grammica* KCTC 13121BP isolated from lichen showed strong J2 killing and egg-hatching inhibitory effects [44];

and a culture medium of *Stenotrophomonas maltophilia* and *Rhizobium nepotum* isolated from the surface of nematodes reduced the pathogenicity of wild pine wood nematodes [39].

Natural products have many limitations, such as natural laccases, which have poor stability of enzymatic activity [45]. As a result, it was important to determine and assess if the novel environmentally-friendly nematicides could be stable for practical and durable application opportunities. Consequently, in our present study, we were interested to determine the durability of the novel biological filtrates. Importantly, the toxic activity of the *A. japonicus* ZW1 fermentation filtrate was not effected by boiling, storage time (1-, 2-week, and 3-week) and warm/cold conditions (25 °C and 4 °C). Usually, the surface coating of nematodes was considered to play an important role in the external protection of nematode bodies, sensing, and communication [46,47]. The microbes and plant produced several acidic metabolites or proteinases that specifically degraded the outer membrane of host cells during primary infection [42,48,49]. In our study, wrinkles on the surface of the body of J2s in 2-WF were observed with scanning electron microscopy, and internal bubbles appeared in their body over time. Additionally, other prominent changes such as intensive cytoplasmic vacuolization areas were observed using transmission electron microscopy; suggesting that the activity of compounds produced by *A. japonicus* ZW1 targeted the skin of nematodes and changed its permeability [50]. Previous research showed acidoid (acetic acid) damage the nuclei of cells and led to intensive cytoplasmic vacuolization areas in the body of J2 *M. incognita* [28]. Nematicidal metabolites from the endophytic fungus *Chaetomium globosum* YSC5 significantly reduced the reproduction of *M. javanica* as well [51]. In our present study, nematicidal compound 1,5-Dimethyl Citrate hydrochloride ester from *A. japonicus* ZW1, first isolated and identified on the basis of NMR, LC-MS techniques, was different with the nematicidal compounds produced by *A. niger* (oxalic acid) and *A. candidus* (Citric acid and 3-hydroxy-5-methoxy-3-(methoxycarbonyl)-5-oxopentanoic acid). *M. incognita* J2 mortality reached 100% at 1 day, and egg hatching was suppressed by 95.6% at 7 days after treated with 2 mmol L^{-1} (180 μg mL^{-1}) oxalic acid [42]. 3-hydroxy-5-methoxy-3-(methoxycarbonyl)- 5-oxopentanoic acid was an isomer of 1,5-Dimethyl Citrate, which increased the mean percentage of immobile *Ditylenchus destructor* by 50% at a concentration of 50 mg mL^{-1} after exposure for 72 h [35]. In our study, *M. incognita* J2 treated with 1,5-Dimethyl Citrate hydrochloride ester, mortality reached 91.7% at 48 h after exposure to 1.25 mg mL^{-1} concentration, the LC50 was 0.7614 mg mL^{-1}, which exhibited the most potent toxic activity against the J2 of *M. incognita*. However, the interesting thing was that in in vitro bioassay, fermentation of the strain exhibited better nematicidal effects, and the mortality of J2s reached 100% after exposed to 5% concentration (approximately 100 μg mL^{-1} 1,5-Dimethyl Citrate hydrochloride ester) *A. japonicus* ZW1 fermentation filtrate at 24 h. Our speculation is that the nematicidal effect originated 1,5-Dimethyl Citrate hydrochloride ester combined with some other compounds produced by *A. japonicus* ZW1. Thus, we still need further study to find and proved other nematicidal activity compounds by metabonomics analysis.

No effect on the seed germination of corn, wheat, rice, cowpeas, cucumbers, soybeans, and tomatoes was observed for the 10% and 20% 2-WF treatments. In whole pot experiments, treatment with the fermentation broth of the strain suppressed root galls and egg populations for tomatoes. As a result, these results suggested that *A. japonicus* ZW1 produced and excreted metabolites that were toxic to root-knot nematodes but did not exert negative effects on seed germination. Thus, *A. japonicus* showed desirable, effective, and safe biocontrol properties against *M. incognita* for both in vitro and greenhouse conditions. Taken together, these observations suggest that the fermentation filtrate of *A. japonicus* ZW1 is safe for use as a biological control fungus against root-knot nematodes. However, further studies are warranted and necessary to evaluate the in vivo efficacy of the strain against root-knot nematodes or other plant-parasitic nematodes.

5. Conclusions

A. japonicus ZW1 fermentation filtrate exhibited a potential biocidal activity on *M. incognita* in vitro and in vivo. The *A. japonicus* ZW1 2-week fermentation filtrate exhibited markedly inhibitory

effects on egg hatching and nematicidal activities on J2s followed by 3-week fermentation filtrate. The *A. japonicus* ZW1 filtrate penetrated the body wall of *M. incognita* and caused intensive cytoplasmic vacuolization with remarkable protruded wrinkles appearing on the body surface of the J2s. Moreover, the nematicidal activity of the fermentation was stable after a boiling treatment and was not affected by storage time. *A. japonicus* ZW1 fermentation filtrate had no negative effect on the viability and germination of corn, wheat, rice, cowpeas, cucumbers, soybeans, and tomato seeds. The main active compound of 1,5-Dimethyl Citrate hydrochloride ester was first isolated and identified from the *A. japonicus* ZW1 fermentation filtrate. Finally, this work highlights the relevance of *A. japonicus* ZW1 fermentation filtrate as a potential new biological nematicide resource for the control of *M. incognita*.

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Conflicts of Interest: All authors declare they have no conflict of interest.

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