Research Square

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4-Ethylbenzaldehyde from the volatilome of Annona muricata against Meloidogyne incognita

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Abstract

In the present study, we demonstrated the potent nematicidal activity of volatile organic compounds (VOCs) released by the *Annona muricata* leaf macerate. In addition, the toxicity of the compound 4-ethylbenzaldehyde was reported in the various phases of the *Meloidogyne incognita* life cycle. By biofumigation, even the lowest concentration of soursop leaf macerate tested (1.0%) caused a reduction in nematode hatching, infectivity and reproduction. Analysis of the soursop volatilome performed by gas chromatography coupled with mass spectrometry revealed 41 compounds, of which three, were selected for studies against the nematode. Only 4-ethylbenzaldehyde showed nematicidal activity *in vitro*, against *M. incognita* J_2 and hatching. As a soil fumigant, 4-ethylbenzaldehyde at dose of 1 mL per litre of substrate had an effect similar ($P < 0.05$) to that of the commercial fumigant Dazomet (250 µg mL^{-1}). In addition, this molecule, when applied directly to *M. incognita*-infested soil, significantly reduced nematode infectivity and reproduction compared to the negative control. Therefore, 4-ethylbenzaldehyde shows potential for the development of a new commercial nematicide with reduced toxicity to the environment and high efficacy against plant parasitic nematodes. However, field assays are necessary to confirm these results, which were obtained under controlled conditions.

Introduction

Plant-parasitic nematodes (PPNs) are major plant pathogens. Their ability to host different plant species and their high adaptability to different environments result in high economic losses [1]. The genus *Meloidogyne*, known as root-knot nematodes (RKNs), has a wide host range and is responsible for approximately 80% of the losses caused by PPNs [2]. Within the RKN group, *Meloidogyne incognita* parasitizes numerous plant species of economic importance and it is distributed worldwide [3].

PPN control is essentially carried out by the planting of resistant cultivars, by biological control or by applying synthetic nematicides, which for the most part is highly harmful to the environment [4]. For this reason, several chemical compounds are being withdrawn from the market, thus reducing control options [5]. Researchers have sought to identify compounds with low environmental toxicity along with effectiveness to control PPNs and supply the shortage of nematicide molecules.

All parts of the soursop plant *Annona muricata* have been studied in several areas of research with positive outcomes in medicine, agriculture and veterinary medicine. The leaves have phytotherapeutic properties against oxidative stress, diabetes, neuralgia, arthritis, malaria, inflammation, tumours and cancer. In addition, several studies have shown that soursop has an anthelmintic effect against parasites of small ruminant animals [6, 7, 8]. Some studies demonstrated the nematicidal and antifungal activities of acetogenins isolated from *Annonaceae*, making these compounds a promising source for new molecules for the development of potential botanical nematicides and fungicides [9]. However, studies on the effects of soursop emissions toxic to PPNs as well as identification of soursop comprising compounds and mode of action of its molecules against PPNs have not been carried out.

Volatile organic compounds (VOCs) are short-chain molecules (less than 20 carbons). VOCs released by plant extracts and root exudates act on interactions between microorganisms [10], contributing to the balance of the soil microbiome as antimicrobials or natural enemy attractants [11]. In the last decade, several studies have demonstrated the nematicidal activities of VOCs released by plants and microorganisms [12, 13, 14, 15]. In these studies, molecules that have shown control efficiency that are equivalent to the current commercial nematicides have been identified, e.g., γ-decalactone identified from castor bean cake emissions and benzylacetonitrile from papaya seed emissions [14, 15].

In this work, we analysed the effects of biofumigation with soursop leaf macerate on *M. incognita* infectivity and reproduction, evaluated the toxic effects of the VOCs emitted by different amounts of soursop leaf macerate on *M. incognita* second-stage juveniles (J2s) and identified soursop leaf volatilome molecules by gas chromatography coupled with mass spectrometry (SPME-CG-MS). Next, the effects of three isolated volatile molecules identified in the soursop leaf volatilome against the nematode were investigated, and the LC_{50} and LC_{95} values of 4-ethylbenzaldehyde were determined. The hatching of eggs exposed to 4-ethylbenzaldehyde was also evaluated, and the effects of 4-ethylbenzaldehyde fumigation on substrates infested with *M. incognita* eggs was analysed. Infectivity and reproduction were determined after the application of different concentrations of 4-ethylbenzaldehyde.

Results

Biofumigation with soursop leaf macerate

Biofumigation with soursop leaf macerate significantly ($P < 0.05$) reduced the number of *M. incognita* galls and eggs. The increase in the concentration of macerate reduced infectivity and reproduction in tomato roots in both experiments. At an 8% concentration, the number of galls and eggs per gram of root were reduced by 79% and 93% in the first experiment and 94% and 96% in the second experiment, respectively, compared to the control (Fig. 1).

Toxic effects of the volatiles released by the soursop leaf macerate on M. incognita J2s

Here for the first time, we demonstrated that VOCs released by soursop leaf macerate are toxic to *M. incognita* explaining the results obtained in the biofumigation test. There was a significant ($P < 0.05$) difference in J2 mortality between the amounts of macerate tested (Fig. 2). The increase in the amount of macerate was positively related with the mortality rate of J2s, reaching 100% at 2 g of soursop leaf macerate.

Characterization of the volatilome

In the emission of the soursop leaf macerate, 41 molecules were identified (Table 1). The chemical groups present in the chromatogram were alcohols, aldehydes, ketones, esters, terpenes and

sesquiterpenes. The compounds with medium and high intensities were hexanal, 2-hexenal, hexanol, coapene and E-caryophyllene. The remaining 35 compounds were identified with low intensity.

Table 1
Volatilomes identified in the soursop leaf macerate by SPME-GC-MS.

Compound	Chemical Group	RI Exp. [a]	RI Lit.[b]	Intensity[c]
(1) ethanol	Alcohol	-	-	v
(2) 3-methyl-butanol	Alcohol	731,797	734	v
(3) 3-hexen-1-ol	Alcohol	849,855	850	v
(4) hexanol	Alcohol	866,377	863	vvv
(5) 3-methyl-butanal	Aldehyde	650,943	654	v
(6) hexanal	Aldehyde	799,078	800	vv
(7) 2-hexenal	Aldehyde	848,696	847	vv
(8) hexanal	Aldehyde	866,377	863	vvv
(9) benzaldehyde	Aldehyde	957,143	952	v
(10) 2-ethylhexenal	Aldehyde	998,721	-	v
(11) 4-ethylbenzaldehyde	Aldehyde	1160,47	1164	v
(12) 2-nonanone	Ketone	1090,49	1091	v
(13) ethyl acetate	Ester	610,377	606	v
(14) ethyl hexanoate	Ester	992,537	997	v
(15) ethyl octanoate	Ester	1191,5	1195	v
(15) hexyl acetate	Ester	1007,08	1007	v
(17) α-pinene	Terpene	929,424	932	v
(18) sabinene	Terpene	966,738	969	v
(19) β-pinene	Terpene	972,281	974	v
(20) mircene	Terpene	982,942	988	v
(21) terpinene	Terpene	1009,07	1014	v
(22) limonene	Terpene	1026,11	1024	v

[a] Experimental retention indices calculated by injection of a homologous series of alkanes.

[b] Theoretical retention index according to the literature (RP Adams, Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, 4th Ed., Allured Publishing Corp., Carol Stream, 2007) (http://webbook.nist.gov/chemistry/). [c] The intensities of the compounds identified by GC–MS in the macerate were categorized as low (“v”), median (“vv”) and high (“vvv”) as estimated by the peak area of each compound in the chromatogram, which is proportional to the concentration of the compound.

Compound	Chemical Group	RI Exp. [a]	RI Lit.[b]	Intensity[c]
(23) trans-β-ocimene	Terpene	1042,92	1044	v
(24) -elemene	Sesquiterpene	1332,8	1335	v
(25) α-cubebene	Sesquiterpene	1347,84	1345	v
(26) longiciclene	Sesquiterpene	1370,84	1371	v
(27) copaene	Sesquiterpene	1376,31	1374	vv
(28) β-bourburene	Sesquiterpene	1385,42	1387	v
(29) β-elemene	Sesquiterpene	1391,12	1389	v
(30) E-caryophyllene	Sesquiterpene	1423,06	1417	vvv
(31) aromandrene	Sesquiterpene	1441,51	1439	v
(32) E-β-farnesene	Sesquiterpene	1454,37	1454	v
(33) α-humulene	Sesquiterpene	1462,62	1452	v
(34) germacrene D	Sesquiterpene	1489,08	1484	v
(35) β-selinene	Sesquiterpene	1492,72	1489	v
(36) viridiflorene	Sesquiterpene	1497,09	1496	v
(37) α-selinene	Sesquiterpene	1501,25	1498	v
(38) -cadineno	Sesquiterpene	1521,45	1516	v
(39) d-cadinene	Sesquiterpene	1527,68	1522	v
(40) α-calacrolene	Sesquiterpene	1544,64	1544	v
(41) caryophyllene Oxide	Sesquiterpene	1586,53	1582	v

[a] Experimental retention indices calculated by injection of a homologous series of alkanes.

[b] Theoretical retention index according to the literature (RP Adams, Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, 4th Ed., Allured Publishing Corp., Carol Stream, 2007) (http://webbook.nist.gov/chemistry/). [c] The intensities of the compounds identified by GC–MS in the macerate were categorized as low (“v”), median (“vv”) and high (“vvv”) as estimated by the peak area of each compound in the chromatogram, which is proportional to the concentration of the compound.

Nematicidal activity and LC_{50} and LC_{95} values of some compounds identified in the soursop leaf volatilome

against M. incognita J2s

Among the molecules tested (sabinene, caryophyllene oxide and 4-ethylbenzaldehyde), only 4-ethylbenzaldehyde showed nematicidal effects, killing 100% of *M. incognita* J2s at 250 µg mL^{-1} (Fig. 3). Sabinene and caryophyllene oxide had no effect on mortality and were excluded from subsequent tests (Table 2).

Table 2
Mortality tests of J2s exposed to the selected molecules for 48 hours.

Molecule	Concentration (µg mL^{-1})	Mortality (%)	
		Assay 1	Assay 2
Sabinene	250	0%	0%
Caryophyllene Oxide	250	0%	0%
4-Ethylbenzaldehyde	250	100%	100%

The LC_{50} and LC_{95} of 4-ethylbenzaldehyde and the commercial molecule fluensulfone were determined using probit analysis. 4-Ethylbenzaldehyde had an LC_{50} of 33 µg mL^{-1} and an LC_{95} of 88 µg mL^{-1}, which were lower than the values found for fluensulfone, which were an LC_{50} of 85 µg $mL^{-}1$ and an LC_{95} of 224 µg mL^{-1} (Table 3).

Table 3
Lethal concentrations (LC_{50} and LC_{95}) of the compound 4-ethylbenzaldehyde and the commercial molecule fluensulfone against *M. incognita* J2s expressed in micrograms per millilitre (µg mL^{-1}). The experiments were analysed together (P >0.05) considering 10 repetitions.

Molecule	LC_{50}	LC_{95}
4-Ethylbenzaldehyde	33	88
Fluensulfone	85	224

Hatching of M. incognita J2s from eggs exposed to 4-ethylbenzaldehyde

Both concentrations of 4-ethylbenzaldehyde (50 and 150 µg mL^{-1}) significantly reduced ($P < 0.05$) *M. incognita* hatching compared to the negative controls (Tween 80® and water). At the end of the 10-day evaluation period, the hatching of eggs exposed to 4-ethylbenzaldehyde (150 µg mL^{-1} for 48 hours) was reduced by 99% compared to the negative controls (water and Tween 80®). At a concentration of 50 µg mL^{-1}, the observed reduction was 90%. At a concentration of 150 µg mL^{-1}, the reduction was similar ($P < 0.05$) to that of commercial 200 µg mL^{-1} fluensulfone (Fig. 4).

4-Ethylbenzaldehyde fumigation of substrates containing M. incognita eggs

Fumigation with 4-ethylbenzaldehyde reduced ($P < 0.05$) the infectivity (galls g^{-1} root) and reproduction (eggs g^{-1} root) of *M. incognita* compared to the negative control (water), regardless of the concentration (Fig. 5). The doses of 4-ethylbenzaldehyde at 200 µL and 500 µL per litre of substrate reduced the infectivity by approximately 72–87%, respectively, compared to the negative control (water). At these doses, nematode reproduction was reduced by 87% and 97%, respectively. The highest dose of 1,000 µL caused a 100% reduction in infectivity and reproduction in both experiments. These values were similar ($P < 0.05$) to the effects observed from the commercial fumigant Dazomet. None of the doses used affected the growth and development of the root system of the plant.

Application of 4-ethylbenzaldehyde as a nonfumigant nematicide on substrates infested with M. incognita J2

Regardless of the dose, the application of 4-ethylbenzaldehyde in soil infested with *M. incognita* J2s caused a significant ($P < 0.05$) reduction in nematode reproduction compared to the negative controls water and Tween 80®. The infectivity was significantly reduced compared to Tween 80® at 150 µg mL^{-1} and at all doses compared to water (Fig. 6).

The application of 4-ethylbenzaldehyde at the four concentrations tested (85, 150, 300 and 600 µg mL^{-1}) reduced infectivity by 31%, 43%, 63% and 76%, respectively, compared to the negative control (water). The reduction in reproduction relative to the control (water) was greater than 90% at all concentrations tested, the largest (97%) being obtained at the highest concentration used (600 µg mL^{-1}). In addition, the reproduction at the 600 µg mL^{-1} concentration was statistically equal ($P > 0.05$) to that obtained for the positive control (fluensulfone).

Discussion

Here, for the first time was reported the toxicity of VOCs released by the soursop leaf macerate against *M. incognita.* In addition, we showed that 4-ethylbenzaldehyde, component of that volatile emission, has a strong nematicidal potential against different phases of the *M. incognita* life cycle.

Biofumigation with green manure from different sources has been effective in controlling PPNs [12, 13, 14, 15, 16]. In this study, the biofumigation with soursop leaf macerate reduced *M. incognita*'s infectivity and reproduction. The effectiveness of biofumigation with Annonaceae green manure probably involves volatile and non-volatile compounds. Non-volatile compounds like acetogenins are reported with a high concentration in Annonaceae species [17]. This compound has a pesticidal effect against various fly larva and mosquitoes, mites, aphides and nematodes [9, 18, 19]. In addition to the non-volatiles compounds, we demonstrated here that Annonaceae also released VOCs with strong nematicidal activity against *M. incognita* J2. The application of soursop leaf macerate at 8% concentration (w/w) in 1 ha at 10 cm depth spend 32 ton which is less than the recommended application with brassicas manure [20].

The emissions from plant manures are complex with different chemical groups compounds [13, 14, 15, 16]. In the macerate of the soursop leaf 41 molecules from different chemical groups were identified, such as terpenes, ketones, alcohols, phenols and esters, which can form interactions, resulting in complex mixtures, which exercise the function of protection, attraction, environmental adaptation and nematicidal action [21]. Some of the molecules comprising the soursop volatilome such as 3-methyl-1-butanol, 2-hexenal, benzaldehyde, mircene, limonene, 2-nonanone, have a toxic effect against nematodes [22, 23, 24, 25, 26]. Others molecules such as α-pinene, β-pinene, myrcene has not been shown to be toxic against nematodes [23, 24]. Lastly, molecules such as 4-ethylbenzaldehyde, aromandrene, α-humulene, α-calacrolene, have never been studied as a pesticide against nematotoides.

The 4-ethylbenzaldehyde is an organic compound, formed by a benzoic acid, derived from benzaldehyde. It has a sweet almond odor, naturally occurring in roasted chicken, grilled steak, cider, black tea and roasted peanuts [28]. Although there are no reports of the use of 4-ethylbenzaldehyde to control phytopathogens, the toxicity of benzaldehyde and oximes has been proven against nematodes, insects and other phytopathogens [28, 29, 30].

For the first time, the high toxicity to *M. incognita* from 4-ethylbenzaldehyde, has been demonstrated, with a LC_{50} 33 μg mL^{-1} lower than its analog benzaldehyde (171 μg mL^{-1}) [29]. This high toxicity demonstrated by 4-ethylbenzaldehyde maybe due to changes caused in the juvenile cuticle, which enabling the molecule penetrate into the J2 body, as was observed by [31] that placed *M. incognita* in contact with aldehydes. Those authors also found that aldehydes inhibit V-ATPase enzyme which regulate nutrition, osmoregulation, cuticle synthesis and nematode reproduction. Therefore, even without death, the deregulation of vital functions is operating by disturbing the ability of finding feeding plant as well as limiting penetration and feeding site establishment.

The nematode eggs are the primary means of survival of the RKNs in the soil, and its control is much importance. The 4-ethylbenzaldehyde causes also toxicity to *M. incognita* eggs by reducing hatching at the level of the commercial nematicide fluensulfone [32]. This finding differs from its analog benzaldehyde oxime which is unable to reduced egg hatching [30]. This result increases our expectancy that 4-ethylbenzaldehyde has great potential to become the active ingredient of a commercial nematicide in the future.

As happened by the soursop macerate emittion, 4-ethylbenzaldehyde, demonstrated fumigation action similar to the positive control Dazomet. Although some volatile compounds show phytotoxicity [33], no apparent phytotoxicity was observed by 4-ethilbenzaldehyde application in tomato at the dose used. Despite being a volatile molecule, 4-ethylbenzaldehyde, presented satisfactory results when applied as a non-volatile nematicide in substrate infested with *M. incognita* J2. This conformation indicates better stability compared to its analog, belzaldehyde oxime that failed on control *M. incognita* when placed directly to the substrate [30]. Such fact may be related to the instability of benzaldehyde in substrate, presenting high volatility and being easily converted into less toxic benzoic acid [30]. Therefore, 4-ethylbenzaldehydo is probably more stable than its analogues.

Accordingly, based on the widespread effect in different phases of *M. incognita* life cycle and its fumigant and non-fumigant action besides good substrate stability, 4-ethylbenzaldehyde is a promising molecule to develop new nematicide.

In conclusion the biofumigation with soursop leaf macerate reduces infectivity and reproduction of *M. incognita* in tomato roots*. In vitro* tests proved that part of the toxicity comes from the volatiles compounds released by the plant macerate of the soursop leaf. Among the molecules identified and selected for prospecting, 4-ethylbenzaldehyde had high toxic effect against different life-stages of *M. incognita*, a fact that was determined for the first time in this work. In addition, 4-ethylbenzaldehyde proved to be efficient as a fumigant and nonfumigante nematicide when applied in infested substrate.

Methods

Obtaining the plant material and nematode inoculum

Leaves of *A. muricata* (soursop) were always collected at 9 am between July and March of 2019 and 2020. The tree used for the experiments is located in the Engineering Department on the campus of the Federal University of Lavras - Lavras/MG-Brazil (21° 14' 45" S, 44° 59' 59" W). Only recently collected leaves were used to prepare the macerate. Maceration was performed using a manual grinder.

M. incognita second-stage juveniles (J2s) and eggs used in the experiments were obtained from pure populations multiplied in tomato plants, cv. Santa Clara, kept in the greenhouse. The purity of the inoculum used was confirmed by isoenzyme electrophoresis [34]. The *M. incognita* egg suspension was obtained according to the technique of [35]. The eggs suspension was incubated in a hatching chamber at 28 °C, and then J2s were obtained. Only the J2s that hatched after 48 hours were used in the tests.

Biofumigation with soursop leaf macerate

Soursop leaves were superficially sterilized using I) 70% alcohol for 1 min; II) 2% sodium hypochlorite (NaClO) for 2 min; and III) sterile distilled water for 2 minutes twice. The macerate was prepared using a manual shredder.

In plastic bags were placed 120 g of Tropstrato® HA substrate (Vida Verde Indústria e Comércio de Insumos Orgânicos Ltda., Mogi Mirim, São Paulo, Brazil) together with different macerate concentrations (0%, 1%, 2%, 4% or 8% (w/w)) and an aqueous suspension (5 mL) containing 5,000 *M. incognita* eggs. The contents of each plastic bag were homogenized by hand agitation. The mixture was poured into 300 mL plastic pots, adjusting the humidity to 60% of the field capacity. Then, the top of the pot was sealed with plastic film (PVC) and kept at 25 °C. After 5 days, the PVC was removed, and an approximately 20-day-old tomato seedling cv. Santa-Clara was transplanted into each pot. Forty-five days after transplantation, the number of galls and eggs per gram of root was evaluated.

Toxic effects of the volatiles released by soursop leaf macerate on *M. incognita* J2s

The methodology described by Barros et al. (2014) [12] was used to evaluate the nematicidal activity of VOCs emitted by soursop leaf macerate. Supelco® tubes (80 × 28 mm, Sigma-Aldrich, Bellefonte, PA, USA) were filled with 35 g of autoclaved sand (121 °C for 20 min, three times). Six quantities of macerate were used: 0, 0.5, 0.75, 1.0, 1.5 and 2.0 g. Tubes without the addition of plant material were considered as controls (0 g). The macerate was placed on the sand surface in the tube, and a 1.7 mL microtube was buried halfway in the sand. The lids of the tubes were tightly closed with a silicone septum and then sealed with PVC and incubated at 28 °C in the dark to form the gas chamber. After 72 hours, an aqueous suspension (1 mL) containing 200 *M. incognita* J2s was injected into the microtube using a syringe through the septum interposed between the silicone cap and the bottle, and the remaining hole was sealed with plastic adhesive. After 48 hours at 28°C, the tubes were opened, and 100 µL of the J2 suspension in the microtube was transferred to a polypropylene plate with 96 wells to estimate the number of mobile, immobile and dead J2s with the aid of an inverted objective microscope (Nikon TMS-F No. 211213, New York, USA). To estimate the number of dead nematodes, 20 µL of a 1.0 mol L^{-1} sodium hydroxide (NaOH) suspension was applied to each well of the plate [36]. J2s that remained immobile for approximately 3 minutes were classified as dead.

Characterization of the volatilome emitted from soursop leaf macerate

Two grams of leaf macerate was added to 20 mL solid phase microextraction (SPME) vials, and then the vials were hermetically closed. An empty vial was used as a control. The vials were prepared in triplicate and kept in an incubator at 28 °C. After 24 hours, the vials were subjected to VOC extraction and identification using SPME and gas chromatography-mass spectrometry (GC-MS), respectively. The parameters for SPME and GC-MS were identical to those described in previous work performed by our group research [14, 15, 16].

Nematicidal activity and determination of lethal concentrations (LC_{50} and LC_{95}) against *M. incognita* J2s of selected compounds identified in the soursop leaf volatilome

From the identification of VOCs by GC-SPME, three molecules were selected for toxicity studies. The three selected molecules were sabinene (75% purity), caryophyllene oxide (99% purity) and 4-ethylbenzaldehyde (98% purity). All molecules were purchased from Sigma-Aldrich (Bellefonte, PA, USA). Each molecule was initially tested at concentrations of 0 and 250 µg mL^{-1}. The molecules were dissolved in an aqueous solution containing 1% Tween 80® (Sigma-Aldrich). Solutions of the molecules (500 µL) with double the final desired concentration, along with an aqueous suspension (500 µL) containing 200 *M. incognita* J2, were poured into 1.7 mL microtubes, reaching the final concentrations of 0 and 250 µg mL^{-1}. The negative control was the solution used to dissolve the molecules (Tween 80®, 0.01 g mL^{-1}). Subsequently, the microtubes were sealed and incubated at 28 °C for 48 hours. After this period, the microtubes were homogenized and opened, and an aliquot of 100 µL of the suspension was transferred to a 96-well polypropylene plate well to evaluate the number of mobile and dead J2s as already described.

The lethal concentrations needed to kill 50% and 95% of *M. incognita* J2s (LC_{50} and LC_{95}) were determined for only the compound 4-ethylbenzaldehyde. Seven concentrations were evaluated: 0, 15, 30, 45, 60, 75 and 90 µg mL^{-1}. Concentration 0 was considered as a control, using only Tween 80®. For the positive control, the commercial nematicide Nimitz® was used, with 48% fluensulfone (active ingredient; 5-chloro-2-(3,4,4-trifluorobut-3-en-1-ylsulfonyl)-1,3-thiazole). To obtain the LC_{50} and LC_{95} of Nimitz®, concentrations of 0, 25, 50, 75, 100 and 150 µg mL^{-1} were used. Solution preparation, concentration curve construction and immobility and mortality assessment used the same methodologies already described.

Hatching of *M. incognita* J2s after egg exposure to 4-ethylbenzaldehyde

This experiment was conducted in 5.5 cm polypropylene Petri dishes. 4-Ethylbenzaldehyde was diluted with 0.01 g mL^{-1} Tween 80®, obtaining solutions with concentrations of 50 and 150 µg mL^{-1}. Fluensulfone at 200 µg mL^{-1} and Tween 80® at 0.01 g mL^{-1} and distilled water were used as positive controls and negative controls, respectively. Ten millilitres of the different concentrations of the molecule or the controls were pipetted into each plate. Next, a 0.025 mm (500 mesh) PVC sieve for egg retention was carefully placed inside each plate, and an aqueous suspension (50 µL) containing approximately 5,000 eggs was added inside of the sieve. Subsequently, the plates were closed and sealed with PVC and incubated at 28 °C for 48 hours. After this period, the sieve was removed, and the liquid was collected to

quantify the number of hatched J2s. To eliminate residual effects from the compounds, the remaining eggs in the sieve were subjected to washing with autoclaved distilled water. Then, 10 mL of distilled water was added to all plates, and the sieves with the eggs were returned to their respective wells. The plates were closed and incubated for 48 hours at 28 °C. Hatching evaluations took place every 48 hours, totalling five evaluations over a period of 10 days. In each evaluation, the procedure described above was followed.

4-Ethylbenzaldehyde as soil fumigant on substrates containing *M. incognita* eggs

The methodology used for this test was developed by Jardim et al. (2017) [29]. For this, 2 L polyethylene terephthalate (PET) bottles filled with Tropstrato® HA substrate (1.5 L) were used. Each bottle received a suspension (5 mL) containing approximately 15,000 *M. incognita* eggs. The concentrations of 4-ethylbenzaldehyde used were 200, 500 and 1,000 µL L^{-1} substrate. As a positive control, the nematicidal commercial fumigant Basamid® was used, which contains 98% of the active ingredient Dazomete (3,5-dimethyl-1,3,5-thiadiazinane-2-thione), at 0.25 mg L^{-1} substrate. Water was used as the negative control. The substrate humidity was adjusted to 60% of the field capacity. All bottles were closed and sealed with PVC. The mixture was homogenized by shaken hands and remained at 28 °C for three days. Each bottle was then opened, and the substrate was turned over and left to stand for five days, after which it was poured into plastic cups of 300 mL. One tomato seedling, approximately 20 days old, cultivar Santa-Clara, was transplanted into each pot. After 45 days, the number of galls and eggs per gram of root system were evaluated.

Application of 4-ethylbenzaldehyde as a nonfumigant nematicide on substrates infested with *M. incognita* J2

For this assay, concentrations of 85, 150, 300, 600 and 900 µg mL^{-1} 4-ethylbenzaldehyde were used. Tween 80® solution at 0.01 g mL^{-1} and water were employed as negative controls. Fluensulfone at a concentration of 200 µg mL^{-1} was used as a positive control. Solutions of 4-ethylbenzaldehyde and fluensulfone were prepared by dilution in 0.01 g mL^{-1} Tween 80® to obtain the desired concentrations. Tomato seedlings, approximately 20 days old, cultivar Santa-Clara®, were transplanted into 300 mL plastic pots containing Tropstrato® HA substrate. Four holes approximately 4 cm deep were made with a glass stick around the seedling stem. Subsequently, a suspension (4 mL) containing approximately 600 J2s was equally distributed into the holes. The 4-ethylbenzaldehyde solutions (4 mL) at different concentrations as well as the controls were immediately applied after infestation of the substrate with J2s. Then, the holes were filled with the same substrate, and the pots were kept in a greenhouse. The number of galls and eggs per gram of root system were evaluated 30 days after inoculation.

Experimental design and statistical analysis

For the statistical analysis and construction of the graphs the software SigmaPlot® and RStudio were used. The trials were conducted in a completely randomized design with five replicates per treatment. Each experiment was performed twice to confirm the results as a repetition on time. The assays without significant differences ($P > 0.05$) were analysed together, considering 10 repetitions per treatment. The data were previously submitted to normality (Shapitro-Wilk) and homogeneity (Bartlett) tests. Data that did not meet these assumptions were subjected to transformation. To obtain the lethal concentrations (LC_{50} and LC_{95}), the data were analysed using probit analysis. Once attending the assumptions described, the F test was applied to the analysis of variance (ANOVA). When the F test was significant ($P < 0.05$), the means of the different treatments were compared using Tukey's test ($P < 0.05$). For quantitative variables, regression analysis was performed using linear and non-linear regression models.

Declarations

Ethics statement

No specific permissions were required for the nematodes and plant used in this study. Nematode specie used in the experiments are plant pests and not protected by the government. All study/experimental protocols involving plant materials was conducted in accordance with institutional, national, and international guidelines and legislation.

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Figures

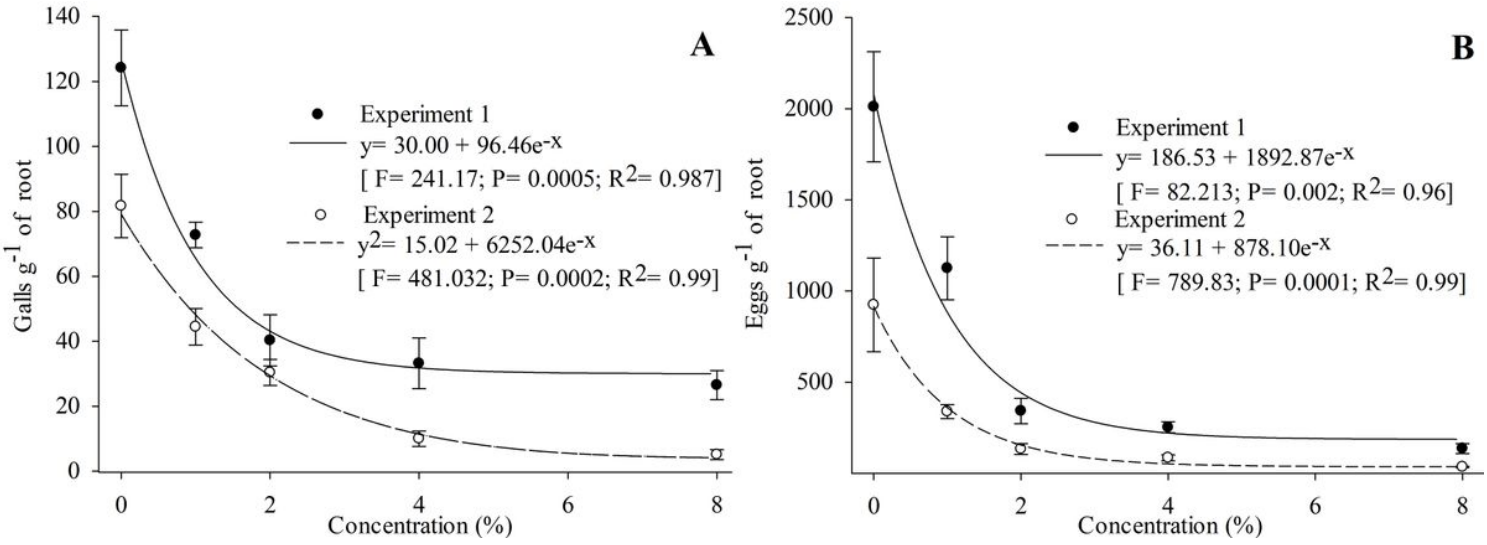

Figure 1

Number of galls (A) and eggs (B) in tomato roots after exposure of the M. incognita eggs to different soursop leaf macerate concentrations incorporated into the substrate and hermetically closed. The bars indicate the standard error of the mean. The data were transformed to log (x) and submitted to regression analysis.

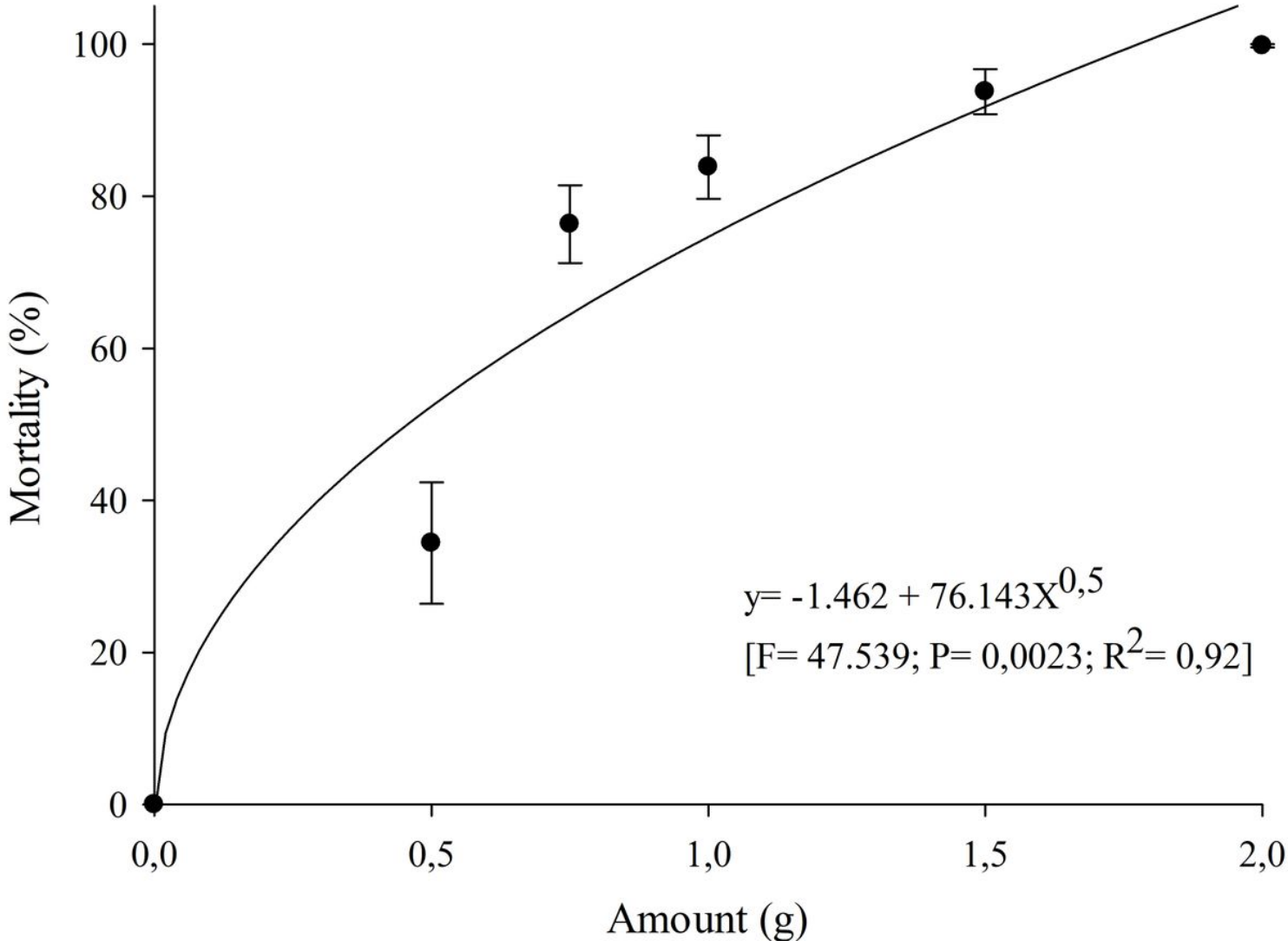

Figure 2

Meloidogyne incognita mortality after 48 hours of J2 exposure to VOCs emitted by different amounts of soursop leaf macerate. The experiments were analysed together ($P > 0.05$) considering the average of 10 repetitions. Bars represent the standard error of the mean.

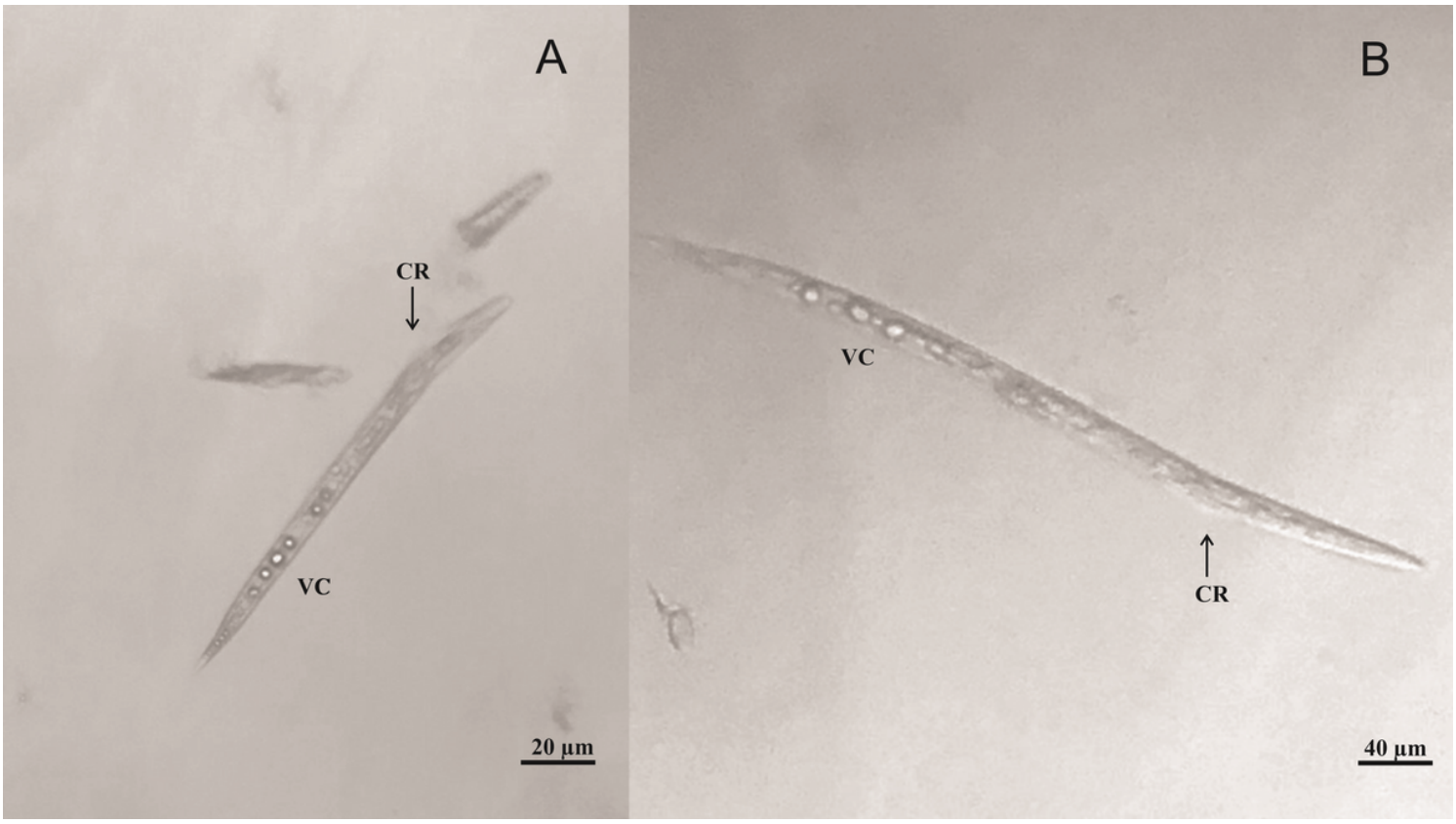

Figure 3

M. incognita body disruption after direct contact with different concentrations of 4-ethylbenzaldehyde. (A) Cuticular rupture (CR) and vacuolization (VC) after exposure to concentration of 250 µg mL^{-1}. (B) Cuticular rupture and vacuolization after exposure of the nematode to LC_{95} (88 µg mL^{-1}).

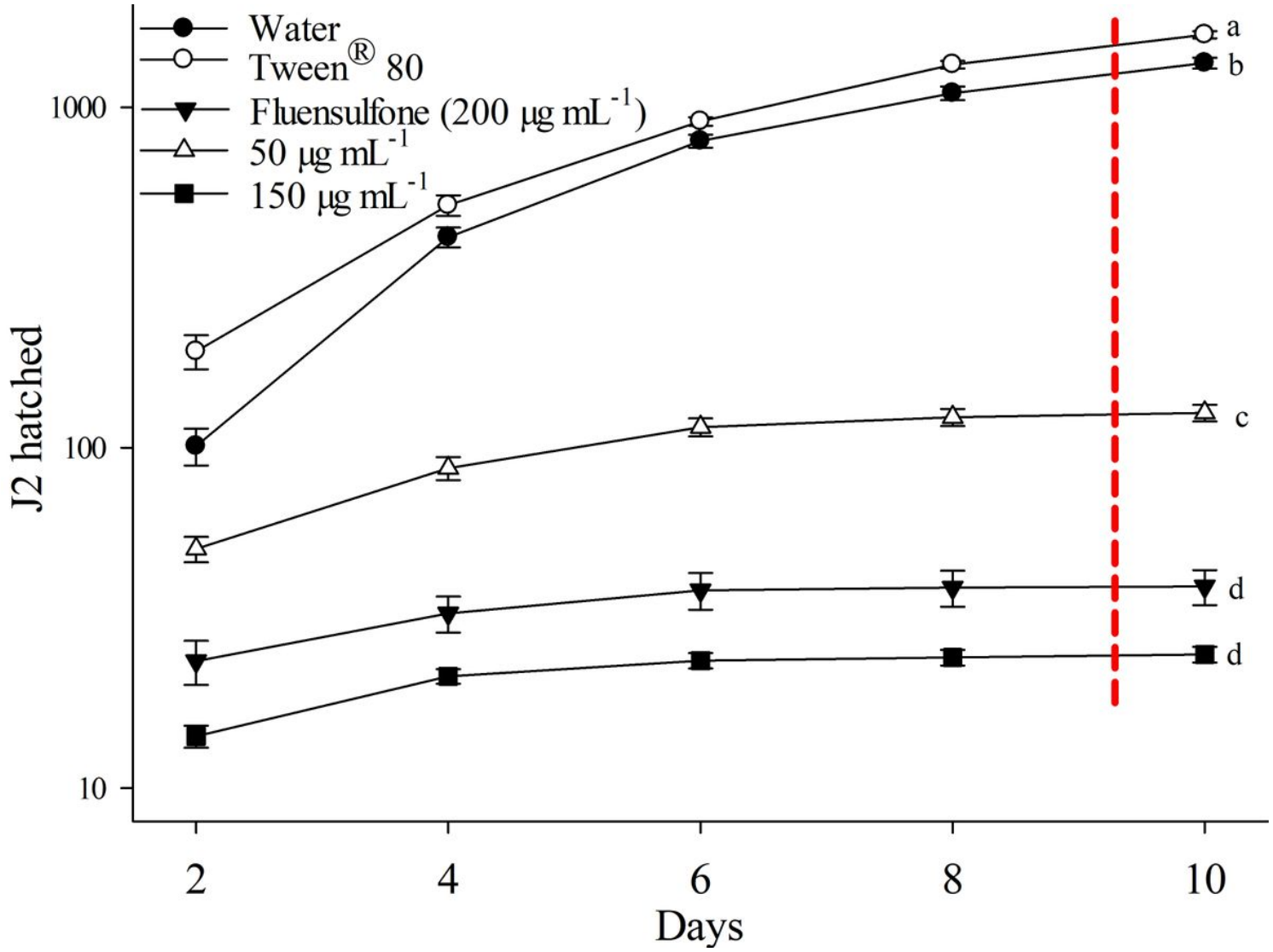

Figure 4

Number of M. incognita J2s hatched 10 days after exposure of the eggs to 4-ethylbenzaldehyde (50 and 150 µg mL^{-1}) for 48 hours. Negative controls: water and Tween 80®. Positive control: fluensulfone (200 µg mL^{-1}). Statistical analysis was performed with the total number of J2s hatched after the tenth day of evaluation. The experiments were analysed together ($P > 0.05$) considering 10 repetitions. The bars indicate the standard error of the mean. Means with the same letter do not differ statistically ($P > 0.05$) by Tukey's test.

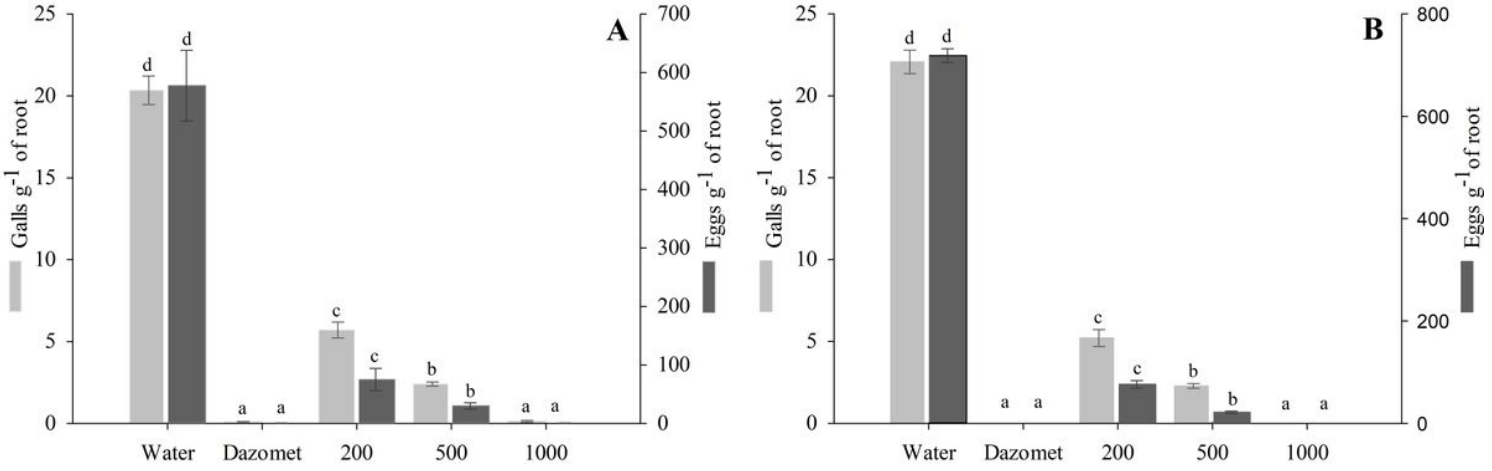

Figure 5

Experiment 1 (A) and Experiment 2 (B). Infectivity (galls g^{-1} of root) and reproduction (eggs g^{-1} of root) of M. incognita resulting from the fumigation of substrate with eggs after application of 4-ethylbenzaldehyde (200, 500, and 1,000 µL). Positive control: Dazomet (250 mg) and negative control: water. The bars indicate the standard error of the mean. Means with the same letter do not differ statistically ($P > 0.05$) using Tukey's test.

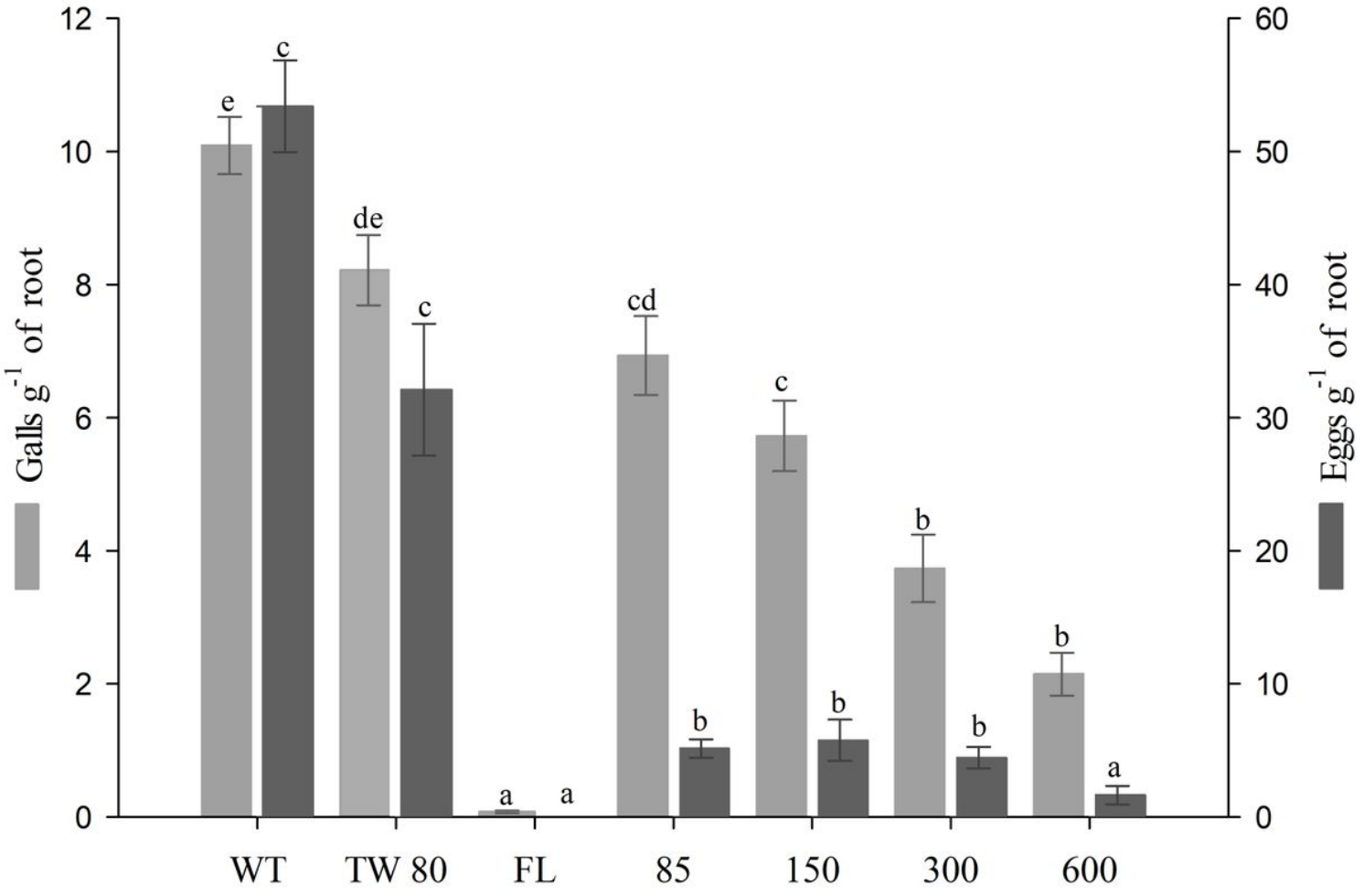

Figure 6

Infectivity (galls g^{-1} of root) and reproduction (eggs g^{-1} of root) of M. incognita resulting from the application of 4-ethylbenzaldehyde (85, 150, 300 and 600 µg mL^{-1}) in substrates infested with M. incognita J2s. Positive control: fluensulfone (FL) (200 µg mL^{-1}) and negative controls: water (WT) and Tween 80® (TW 80). The experiments were analysed together ($P > 0.05$) considering 10 repetitions. The bars indicate the standard error of the mean. Means with the same letter do not differ significantly ($P > 0.05$) by Tukey's test.