

Research Article

Essential Metal-Based Nanoparticles (Copper/Iron NPs) as Potent Nematicidal Agents against *Meloidogyne* spp.

Gkanatsiou Ch¹, Ntalli N², Menkissoglu-Spiroudi U³, Dendrinou-Samara C^{1*}

¹Inorganic Chemistry Lab, Chemistry Department, Aristotle University of Thessaloniki, Thessaloniki, Greece

²Benaki Phytopathological Institute, Department of Pesticides' Control and Phytopharmacy, Athens, Greece

³Pesticide Science Laboratory, School of Agriculture, Faculty of Agriculture, Forestry and Environment, Aristotle University of Thessaloniki, Thessaloniki, Greece

***Corresponding Author:** Dendrinou-Samara C, Inorganic Chemistry Lab, Chemistry Department, Aristotle University of Thessaloniki, Greece, 54124 Thessaloniki, Greece, E-mail: samkat@chem.auth.gr

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Abstract

Root-knot nematodes (*Meloidogyne* spp.) cause great economic impact in the sector of agriculture while the number of existing commercial nematicides has been drastically reduced. The metal-based nanoparticles offer new possibilities as agrochemicals. In the current study, we shed light on the role of copper/iron based nanoparticles as bioactive agents against the root knot nematode *Meloidogyne* spp; along with the plant enhancement properties on the nematode infested host. Same sized PEGylated Cu, CuFe and CuFeO₂ NPs, were solvothermally synthesized characterized and evaluated against *M. incognita* and *M. javanica* nematodes, in terms of paralysis and biological cycle arrest in tomato plants. Comparisons were made against the commercial nematicide fosthiazate. All the tested compositions revealed nematicidal activity, but CuFe NPs were found best effective exhibiting the lowest EC₅₀ value calculated at 0.03 µg ai g/soil. Additionally, a fresh shoot and root weight increase was evident in tomato plants treated with CuFe NPs.

Keywords: *Meloidogyne incognita*; *Meloidogyne javanica*; Micronutrients; Nanoparticles; Nanoagrochemicals; Plant protection; Fertilizers

1. Introduction

Root-knot nematodes (*Meloidogyne* spp.) are the most notorious plant parasitic nematodes, amongst agricultural pests suppressing crops while causing annually significant crop losses to the production of fruits and vegetables [1, 2]. Till now, nematicidal control has been mainly based on synthetic organic chemical nematicides [3]. However, due to environmental problems, many of them such as the synthetic carbamate, organophosphate and

organophthalide pesticides have been recently banned (Council Directive 91/414/EEC, Regulation 2009/1107/EC OL and Directive 2009/128/EC). Therefore, there is an emergent need for the development of alternative nematode control measures and green nematicidal products that will impose less toxicity on non-target organisms, environment and humans [4].

Nanoagriculture involves the employment of nanomaterials, such as nanopesticides and nanofertilizers, in crop protection, providing sustainable options to improve plant's health [5-8]. In recent years, among a variety of nanostructures, metal-based nanoparticles (NPs) are gaining interest due to their unique physicochemical properties and biological activities. The impact of metal-based NPs on plant growth and their accumulation in food source according to the applied dose are evident. The effectiveness of metal-based NPs are species dependent and is driven by a series of factors, including the characteristics of the nanomaterial and the environmental conditions. The physicochemical properties of the NPs, known as the so-called 4s' parameters (size, shape, surface chemistry and structure), can highly affect their biological properties; while the environmental behaviour of the NPs (mobility, reactivity, toxicity) needs to be completely understood. Thus, the effects of metal-based NPs formulations in different soils and on diverse crop plants have to be explored to broaden the horizon of sustainable agricultural production of higher and safer yields [6].

Copper (Cu) and iron (Fe) are essential micronutrients in crop plants and play a very important role in biochemical functions such as the photosynthetic electron transport, the respiration of the mitochondria, the oxidative stress, the metabolism of the cell wall and the synthesis of the DNA [5, 6]. At the same time, copper formulations can be used as antagonists of plant antagonistic bacteria and fungi, with huge potential for use in pesticide formulations [2, 4]. Interestingly, Fe-based NPs exhibit great potential in the agricultural section as they are considered to be biocompatible and of low-toxicity [9]. Additionally, iron deficiency is a widespread agronomic issue caused by poor iron solubility in the vast majority of soils and consequential insufficient iron availability to plants [10]. Fe-based NPs are reported to have insignificant antimicrobial activity against many bacteria species, such as *Bacillus subtilis* and *Escherichia coli* [11] and their use is primarily as fertilizers. In fact, naked iron oxide (Fe_3O_4) NPs were found to increase the shoot and root length of tomato plants [12] while the application of Fe_2O_3 NPs to peanut seeds, resulted in stem and root growth and seed germination increase, indicating that Fe_2O_3 NPs can replace traditional Fe fertilizers in the cultivation [13].

On the other hand, Cu-based NPs reveal their effectiveness and specificity towards a broad spectrum of microorganisms [14, 15]. It is a matter of concern, though, which form of copper (Cu, Cu_2O , CuO) is more suitable, since it is linked to the flexible Cu(I)/Cu(II) redox activity and may undergo chemical transformations such as reduction or oxidation when applied to the soil [16]. However, the application of nanomaterials hardly reaches field experiments [17, 18] and the impact/ interaction of metal and metal oxide NPs on plants and soil are often controversial. The toxicity of the NPs in plants and the environment is correlated to the size, concentration and composition. In general, copper oxide NPs (CuO) are reported to be more toxic on plants, than metallic copper NPs, due to oxidative properties [19]. For instance, naked CuO NPs, of size <50 nm, prevented the growth of wheat in the

sand when tested at concentrations >10 mg CuO/kg [20]; while in the case of naked Cu NPs of 20 nm a dose dependent decrease occurred in mung bean and wheat growth at concentrations >200 mg/L and >800 mg/L respectively [21]. On the other hand, CuO NP were found to have a positive impact on waterweed aquatic plants (*Elodea densa*), by stimulating photosynthesis even at low concentrations <0.25 mg/L [19]. Meanwhile, studies concerning the interaction of Cu₂O NPs with soil and plants have not been reported. Specifically, applications of metal- based NPs, as nematicidal agents are very few. When ‘naked’ silver NPs were tested at 200 mg/mL against *Meloidogyne incognita* J2 100% immobility and mortality was revealed, while treatment at 0.0007% (w/w) of nano-silver completely controlled *M. incognita* in pot experiments; on the contrary, titanium oxide NPs tested at 800 and 400 mg/mL provoked 4.3 and 2% J2 mortality, respectively [22]. Interestingly, in other studies, when *M. incognita* J2 were immersed into 30-150 μ g/mL of silver NPs in water, $>99\%$ nematodes became inactive in 6 hours [23].

Previously we have reported on the enhanced antimicrobial activity of CuFeO₂ against *Bacillus subtilis*, *Escherichia coli* and *Saccharomyces cerevisiae* [24]. The composition of CuFeO₂ as a bimetallic oxide with an inert iron (III) and a reactive copper (I) metals in the presence of the biodegradable polyethylene glycol (PEG8000), helping avoid oxidation of the metal core and to protect plant cells from possible increased NPs toxicity by performing stealth properties [25], sounds promising. In continuing our studies in the field of nanoagrochemicals [16, 24, 26], we report on different species of copper/ iron-based NPs activity as nematicidals for the first time. In particular, in this study we report results on (1) the synthesis and characterization of the same crystallite sized Pegylated Cu, CuFe and CuFeO₂ NPs prepared solvothermally in the presence of the biodegradable polyethylene glycol (PEG8000) and (2) evaluation of the nematicidal activity of all tested compositions against *M. incognita* and *M. javanica* both in terms of J2s paralysis and biological cycle arrest of the parasite in host plant tissues. Last we report on the secondary effects on tomato plants’ growth, by counting fresh shoot and root weight.

2. Materials and Methods

2.1 Reagents

The reagents that were necessary for the preparation of nanoparticles are the following: Copper(II) nitrate trihydrate Cu(NO₃)₂·3H₂O (Merck, $\geq 99.5\%$, M=241.60 g/mol), Iron(III) nitrate nonahydrate Fe(NO₃)₃·9H₂O (Merck, $\geq 99.5\%$, M=404 g/mol), polyethylene glycol (PEG) 8000 (Alfa aesar), hydrazine hydrate N₂H₄·H₂O (Merck, about 100%, M=50.06 g/mol).

2.2 Preparation of Cu-based NPs

Cu NPs: 1 g (4 mmol) of Cu(NO₃)₂·3H₂O was dissolved in 6 mL of deionized water to obtain a blue solution. Then 0.1g (20.5 mmol) of N₂H₄·H₂O was added dropwise to the blue solution and the color changed to yellow, indicating the reduction of Cu (II). Successively, 1.5 g (1.9 mmol) of PEG 8000 was added. The resulting solution was stirred thoroughly and then transferred into a 23 mL Teflon-lined stainless-steel autoclave. The crystallization was carried out for 2 h, under autogenous pressure, at the temperature of 160°C. Then the autoclave was cooled naturally to room temperature. The supernatant liquid was discarded after centrifugation at 5000 rpm. A black-brown precipitate

was obtained and washed at least three times with ethanol, to remove the excess of ligands and the unreacted precursors.

CuFe and CuFeO₂ NPs: For the preparation of these compositions a modified synthetic protocol of our previous studies was followed according [27]. In brief for CuFe NPs preparation: Fe(NO₃)₃·9H₂O (0.5 mmol, 0.2 g) and Cu(NO₃)₂·3H₂O (0.5 mmol, 0.1 g) were dissolved in 8 mL of TEG, while for CuFeO₂ NPs: 0.5 mmol (0.2 g) Fe(NO₃)₃·9H₂O and 0.1 g (0.5 mmol) of Cu(NO₃)₂·3H₂O were dissolved in 8 mL deionized water. Then 0.1 g (39.5 mmol) of N₂H₄·H₂O was added dropwise to this solution and the color changed from yellow to brown. Successively a solution of 1.9 mmol (1.5 g) PEG 8000 was added. In both cases the resulting solution was stirred thoroughly and then transferred into a 23 mL Teflon-lined stainless-steel autoclave. The crystallization was carried out for 8 h, under autogenous pressure, at the temperature of 160°C. Then the autoclave was cooled naturally to room temperature. The supernatant liquid was discarded after centrifugation at 5000 rpm. A black-brown precipitate was obtained and washed at least three times with ethanol, to remove the excess of ligands and the unreacted precursors.

Composition	Surfactant (%)	Size by XRD (nm)	Size by DLS (nm)
Cu	40% PEG8000	40	200
CuFe	39% PEG8000	40	146
CuFeO ₂	20% PEG8000	36	257

Table 1: Experimental results and main characteristics of Cu, CuFe and CuFeO₂ NPs.

2.3 Ionic release measurements

The release of copper and iron ions from the Cu, CuFe and CuFeO₂ NPs into distilled (DI) water was studied by preparing a solution of 10 mg of NPs in 100 mL DI water. The solution was sonicated for 10 min. The suspension was kept in a rotary shaker under the same experimental conditions as in paralysis bioassays for 24 h and then the residual Cu²⁺ and Fe²⁺ concentration in the aqueous phase was determined using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES).

2.4 Dynamic light scattering measurements

Hydrodynamic diameters were determined with VASCO Flex™ Particle Size Analyzer NanoQ V2.5.4.0 dynamic light scattering (DLS), by electrophoretic measurements, at 25°C. For the measurements of all the samples, the dilution medium was filtered through a 0.2-micron membrane, at the concentration of 100 µg/mL because a high purity of the suspension was required in order to remove unwanted agglomerates and/or dust contamination.

2.5 NPs characterization

The characterization of the NPs was performed by X-ray powder diffraction (XRD) on a Philips PW 1820 diffractometer at a scanning rate of 0.050/3 s, in the 2θ range from 10 to 90°, with monochromatized Cu Kα

radiation ($\lambda=1.5406$ nm). TG analysis (SETA-RAM SetSys-1200) was carried out in the range from room temperature to 900°C at a heating rate of 10°C min⁻¹ under N₂ atmosphere, by Infrared spectra (280-4000 cm⁻¹) were recorded on a Nicolet and with FTIR 6700 spectrometer with samples prepared as KBr pellets.

2.6 Nematode bioassays

2.6.1 Nematode cultures: Two separate nematode species were raised by a single egg mass of *M. incognita* and another of *M. javanica* as characterized and kindly provided from the collection of E. A. Tzortzakakis of Hellenic Agricultural Organization-Demeter, Heraklion, Crete (ELGO). The obtained populations were reared on six-leaf stage tomato plants cv. Belladonna. Forty days after inoculation the roots were washed free of soil, cut into 2 cm pieces and eggs were extracted from a tomato plant roots, according to the sodium hypochlorite procedure [28] as described by Ntalli et al. [29]. Second-stage juveniles (J2) were allowed to hatch in modified Baermann funnels at 27°C. The first 3-day (J2) hatching was discarded. All freshly hatched (24 h) second-stage juveniles (J2) collected thereafter were used in the experiments.

2.6.2 Effect of NPs on J2 motility: Effects of Cu and CuFeO₂ or CuFe NPs on J2 motility, were tested at the dose rate of 25-200 µg/mL or 12.5-200 µg/mL, respectively, in CellstarR 96-well cell culture plates (Greiner bio-one). In all cases, NPs were dispersed in water to form test solutions, which were mixed in 96-well culture plates at a ratio of 1:1 v/v with suspensions of 20 J2 per well. Distilled water was used as control. All the plates were covered with plastic lids and kept in the dark at 27°C. The exact number of transferred J2 nematodes into the wells, was verified under an inverted microscope (Euromex, The Netherlands) at 40X. After 24, 48 and 72 h the mobile and immobile Juveniles (J2) were observed with the aid of the inverted microscope. The five treatment replications were completely randomized and each experiment was performed twice.

2.6.3 The NPs effect on the nematode's life cycle: The soil that was used for the pot experiments was an RKN-free clay loam soil containing 1.3% organic matter-pH 7.8 and was collected from a non-cultivated field of the University Farm. A 3-mm sieve was used in order to separate soil from debris. The sieved soil was then partially air-dried overnight. Measurements of soil moisture and maximum water holding capacity (MWHC) were performed according to Pantelelis et al. [30]. After the confirmation of the absence of nematodes using the modified Baermann funnel method, the soil was mixed with sand at a ratio of 2:1 to obtain the mixture hereafter referred to as soil. Plastic bags of 1.0-kg subsamples were prepared representing the experimental treatments. 12.500 J2 were applied to each plastic bag and the soil was mixed thoroughly by shaking, then sieved and equilibrated in the dark, for 24 h, at room temperature. The fortification of the soil with NPs took place at doses of 0.025 to 0.4 µg ai/gr soil. For this, appropriate amounts of CuFe, CuFeO₂ and Cu NPs were weighed, dispersed in distilled water and the produced solutions (10 mL each) were mixed with the soil so as to achieve the test concentrations.

A treatment with water and a treatment with Nemathorin150EC at the registered dose, 2 µg ai/gr soil (a.i. fosthiazate 15%, Hellafarm Co.) were used as controls. The soil in the plastic bags was mixed and sieved again, through a 3-mm sieve, to ensure uniform distribution and then returned into the plastic bags, mixed thoroughly by shaking and

left to equilibrate in the dark, for another 24 h, at room temperature. The moisture content of the soil never exceeded 24% of MWHC. After sieving again, the treated soil was divided into 200 g/ plastic pots (1kg × 5 treatments) and tomato plants cv. Belladonna were transplanted. Plants used for the experiment were 7-week old and at the six-leaf stage. The plants were watered every two days with 20 mL of water and were kept at 27°C, 60% RH at 16 h photoperiod. After forty days, the plants were uprooted and their roots were washed very gently in running tap water to remove all soil particles. In order to count nematode females in plant tissues, they were stained with an acid fuchsin solution [31]. The number of female nematodes per gram of root were counted at 10 × magnification under uniform illumination by transparent light within tissue sample. Measurements of fresh root and fresh shoot weight parameters were performed as well. The experiment was replicated once, and the treatments were always arranged in a completely randomized design with five replicates.

2.7 Statistical analysis

Nematode bioassays were performed twice with five replicates per treatment. Because ANOVA indicated no significant treatment by time interaction (between runs of experiment), means were averaged over the experiments and the data were analyzed (ANOVA) combined over time. The percentages of immotile J2 in the microwell assays were expressed as a percentage increase in the number of immotile J2 in the water control according to the Schneider Orelli's formula: corrected % = {[mortality % in treatment – mortality % in control]/[100 – mortality % in control]} × 100 [32]. Data was subjected to probit linear regression analysis and the concentration of nanoparticles (EC₅₀ values) required for 50% death/paralysis of nematodes was calculated. The data from the pot experiments were expressed as a percentage decrease in the number of females per gram of root corrected according to the control, using the Abbott's formula: corrected% = 100 × {1-[females number in treated plot/females number in control plot]}. A probit linear regression analysis was performed for EC₅₀ calculation. In this regression equation, the test compounds was the independent variable (x) and the number of female nematodes percentage decrease over water control, was the dependent variable (y). Treatments means for plant growth parameters were compared using Tukey's test at P=0.05 [33]. Statistical analysis was performed using IBM SPSS Statistics program while the dose-response curves were created using the program Origin Pro 8 (Graphing and Analysis).

3. Results

3.1 Characterization of Cu-based NPs

In the XRD patterns of the samples studied (Figure 1) the Bragg reflections correspond to the crystal structures of Cu, CuFe and CuFeO₂ NPs with Cards pdf.04-0836, pdf.03-065-7002 and pdf.75-2146 respectively. The reflections at almost 18,5° and 19,1° are associated with the presence of the organic coating (PEG8000) in Cu and CuFe NPs revealing the high crystallinity of the NPs. MDI's Jade software was used and lattice parameters were calculated. They were found to be a = 3.59 Å, 3.6170 Å, 3.05 for Cu and CuFe, respectively. For the rhombohedral structure of CuFeO₂ the lattice parameters were found to be a = 3.04Å and c = 17.12Å. The average crystalline sizes of pure Cu, CuFe and CuFeO₂ NPs were determined by using the Scherrer equation and taking the full width at half maximum (fwhm) of the most intense peak and were found to be 40 nm, 40 nm and 36 nm respectively.

FTIR spectra of Cu, CuFe and CuFeO₂ NPs with the corresponding pattern of PEG8000 is given in Figure 2. The surfactant PEG8000 was confirmed by the comparison of the two IR patterns in each sample. Peaks at 2880 cm⁻¹ are assigned to the stretching vibrations of methylene groups (-CH₂), in the surfactant molecules. There is a broad peak around 1635 cm⁻¹ ascribed to the hydrogen bonds which is formed from the hydroxide groups. The C-O-C ether stretch band at 1110 cm⁻¹ is indicative of the presence of PEG8000 on the samples. The vibrations around 1340 cm⁻¹ and 1470 cm⁻¹ are associated with (C-H) and (C-H₂) bending respectively. Finally, in the case of CuFeO₂ NPs we can observe a sharp peak at 424 cm⁻¹ indicating the metal oxide nature of the NPs. The amount of the surfactants on the samples was evaluated by thermogravimetric analysis (TGA). Under nitrogen atmosphere the temperature range was high as 800°C. The percentage of the cumulative organic content was estimated at 40, 39 and 20 wt% for the PEG8000- covered Cu, CuFe and CuFeO₂ NPs respectively (Figure 3).

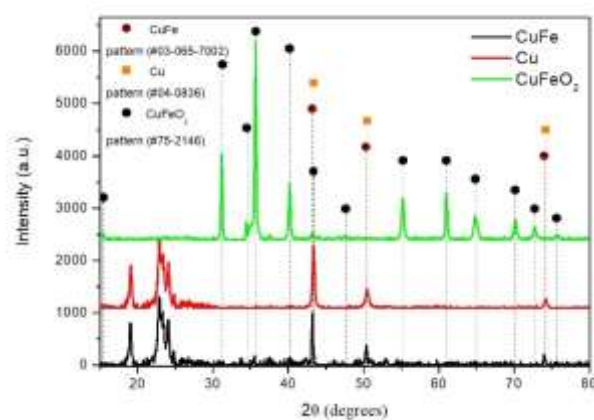


Figure 1: XRD patterns of Cu, CuFe, CuFeO₂ NPs.

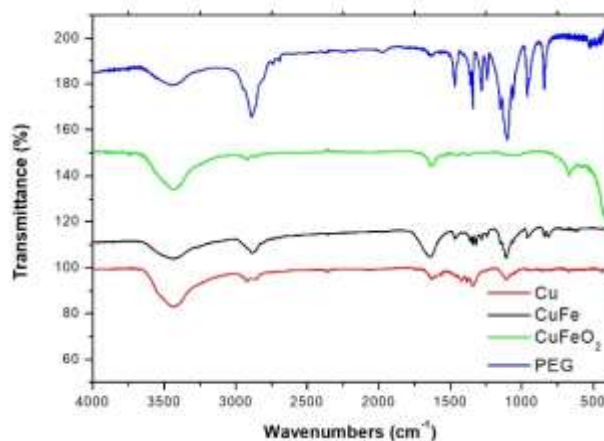


Figure 2: FTIR spectra of the samples Cu, CuFe, CuFeO₂ NPs.

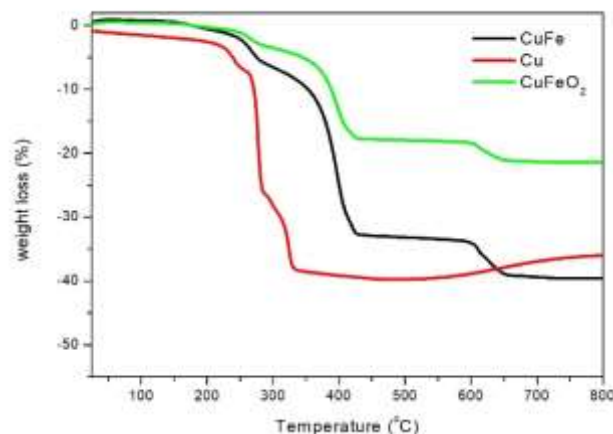


Figure 3: Thermogravimetric curves of Cu, CuFe and CuFeO₂ NPs.

3.2 Ion release measurements

The stability of the NPs was investigated in aqueous suspensions and the ions released from Cu, CuFe and CuFeO₂ NPs into the water were estimated after 24 h. The concentration of copper ions released in water after 24 h was found at 19.8, 13.3 and 1.6 µg/mL for CuFe, CuFeO₂, Cu, respectively (Table 2). On the contrary, release of Fe ions was found low at 0.5 and 0.2 µg/mL for CuFe and CuFeO₂ respectively.

Sample	Cu	Fe
Cu	1.6	-
CuFe	19.8	0.5
CuFeO ₂	13.3	0.2

Table 2: Concentrations (µg/mL) of Cu and Fe ions in water after 24 h dispersion of Cu-based NPs (100 µg/mL) in water.

3.3 Effect of NPs on J2 motility

The effect of Cu, CuFe, CuFeO₂ NPs on the motility of root-knot nematodes was evaluated. When the NPs were tested against *M. incognita* and *M. javanica*, a clear dose response relationship was established and after 24 h a significant paralysis of J2 was evident (Figure 4). The dose response curves of Cu, CuFe, CuFeO₂ NPs after 24 h of J2 immersion in NPs solutions are presented in Figure 4. The calculated EC₅₀ values for all tested NPs against both nematode species after J2 immersion for 24, 48 and 72 h, are shown in Table 3. Among the three tested compositions, CuFe NPs exhibited the highest nematicidal activity, causing 100% paralysis to both *Meloidogyne* species, after 72 h of J2 exposure at the lowest dose. The EC₅₀ values of Cu, CuFe and CuFeO₂ NPs, after 24 h immersion of *M. incognita* J2 in test solutions, was calculated at 75, 18 and 73 µg/mL respectively. A similar trend was shown 48 h after the experiments' establishment (Table 3), confirming once more the high nematicidal activity of CuFe NPs. Finally, increasing the exposure period to 48 or 72 h resulted in same EC₅₀ values (70 and 72 µg/mL 72 h after *M. incognita* J2 immersion to Cu and CuFeO₂ NPs, respectively, Table 3). This fact demonstrates that all

J2 found immotile after 24 h of immersion to test solutions had been dead and did not regain their activity later. The paralysis activity of the tested NPs against *M. javanica* was found similar (Table 3) and the EC₅₀ values of Cu, CuFe and CuFeO₂ NPs, after 24 h immersion of *M. javanica* J2 in test solutions, were calculated at 65, 15 and 75 µg/mL, respectively. Again, the activity of all tested NPs was retained after 72 h of exposure. Natural death in control for *M. incognita* and *M. javanica* did not exceed 5% for all assessment dates. In all cases, J2 found immotile after 72 h of exposure were recorded as dead because they did not regain their motility after moving to water. For comparison, copper sulfate and fosthiazate were used as chemical control, with estimated EC_{50/24h} of 32.9 and 0.79 µg/mL, or 24.5 and 0.89 µg/mL for *M. incognita* or *M. javanica* J2, respectively.

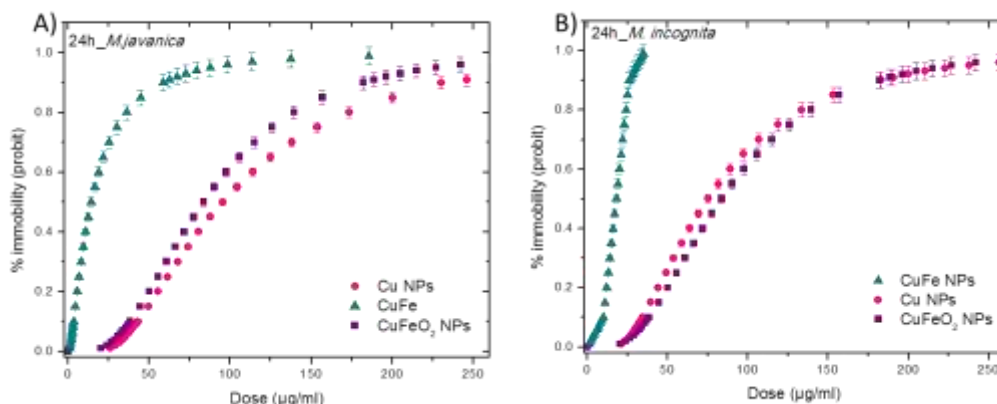


Figure 4: Dose response curves of (A) *M. javanica* and (B) *M. incognita* nematodes treated with Cu, CuFe and CuFeO₂ NPs solutions for 24 h. Each point represents the average percent number of paralyzed nematodes of five replicates per treatment (× two experiments replications) after elimination of natural death counts in the control.

Sample	EC ₅₀ (µg/mL), 24 h		EC ₅₀ (µg/mL), 48 h		EC ₅₀ (µg/mL), 72 h	
	<i>M. incognita</i>	<i>M. javanica</i>	<i>M. incognita</i>	<i>M. javanica</i>	<i>M. incognita</i>	<i>M. javanica</i>
Cu	75 ± 5	65 ± 4	72 ± 2	63 ± 5	70 ± 3	61 ± 2
CuFe	18 ± 3	15 ± 2	9 ± 2	13 ± 2	-*	-*
CuFeO ₂	73 ± 4	75 ± 3	73 ± 2	74 ± 4	72 ± 4	72 ± 3

*100% paralyzed at the lowest concentration dose.

Table 3: Estimated EC₅₀ values for Cu, CuFe and CuFeO₂ NPs calculated after 24, 48 and 72 h of *M. incognita* and *M. javanica* J2 immersion in test solutions and expressed as EC₅₀ ± SD (µg/mL).

3.4 Effect of NPs on the nematode’s life cycle

Cu, CuFe and CuFeO₂ NPs were evaluated *in vivo* in pot experiments (Table 4) against *M. incognita* and clear dose response relationships were established based on the numbers of females and galls/g of root tissues. The EC₅₀ values corresponding to the reduction of females/ g of root tissues for CuFe, Cu and CuFeO₂ NPs, were calculated at 0.03, 0.05 and 0.2 µg/g soil respectively (Table 4). Similar EC₅₀ values were found considering the reduction of galls/g of root tissues (data not shown). Fosthiazate tested at the registered dose of 2 µg ai g/soil resulted 100% efficacy.

Interestingly, a significant increase was observed in the fresh shoot and root weight of tomato plants treated at the three higher doses of CuFe NPs (Table 5) (Figure 5). In all cases, no phytotoxicity was observed in the tomato plants after treatment with metal composites. The infestation in control plants was 80 females/g of root tissues.

Sample	Females/g root reduction	
	EC ₅₀ (µg/g soil)	SD
Cu	0.05	0.01
CuFe	0.03	0.01
CuFeO ₂	0.20	0.02

Table 4: EC₅₀ values as calculated in pot experiments following *M. incognita* exposure to soils amended with Cu, CuFe and CuFeO₂ NPs, expressed as EC₅₀ ± SD (µg/g soil).

Treatment µg/g soil	Fresh shoot weight* (g)		Fresh root weight* (g)	
	Cu NPs	CuFe NPs	Cu NPs	CuFe NPs
0 (Control)	5.050 ^b	5.050 ^b	1.532 ^b	1.532 ^a
0.2	5.286 ^b	6.172 ^d	1.540 ^b	1.647 ^b
0.1	5.474 ^c	5.746 ^c	1.498 ^b	1.612 ^b
0.05	5.188 ^{bc}	5.344 ^{bc}	1.526 ^b	1.530 ^a
0.025	4.538 ^a	4.710 ^a	1.366 ^a	1.516 ^a

*Data are presented as means of five replicates with standard deviations. Means followed by the same letter in the same column are not significantly different according to Tukey’s test (P ≤ 0.05).

Table 5: Growth parameters (fresh shoot and root weight) of tomato plants (*Solanum lycopersicum*) in soil infested with *M. incognita* nematodes and containing the different rates of Cu and CuFe NPs.



Figure 5: Tomato plants and their roots treated with Cu, CuFe and CuFeO₂ NPs at the dose of 0.2 µg g/soil in comparison to the untreated-control plants.

4. Discussion

The root-knot nematodes, *Meloidogyne* spp. are difficult to control and the agricultural sector needs green and effective nematicides towards that direction. Nanotechnology could offer novel approaches for the solution of this problem since the performances of conventional pesticide formulations are often ameliorated through the construction of nanotechnology [34, 35]. A plethora of chemical, physical and biological techniques continues to evolve leading to the production of noble metal nanoparticles, some of whom are of use against nematodes [36]. Recent research on silver nanoparticles (Ag-NP), “naked” or of botanical products, showed significant nematicidal effect against *M. incognita* [37, 38]. In that vein, different compositions of Cu/Fe NPs were synthesized in the presence of the biocompatible surfactant PEG8000 and evaluated as nematicidal for the first time. Two nematode species were investigated, namely *M. incognita* and *M. javanica*, and similar EC₅₀ values were established. CuFe NPs was the best effective product in paralyzing *M. incognita*, with an EC_{50/24h} value of 18 µg/mL.

The nematicidal activity of the NPs was determined in the descending order of CuFe (Cu⁰) > Cu (Cu⁰) = CuFeO₂ (Cu¹⁺), with the last two compositions revealing similar efficacy. Copper salts have already been reported of nematicidal activity when used in combination with maleimide derivatives [39], while metal complexes have been found effective both against the root-knot nematodes and the soil born fungi *Fusarium oxysporum* [40]. In specific copper sulphate, principally used as a fungicide but also for treating copper-deficient soils, has been reported to exhibit an EC₅₀ value of 280 µg/mL against *M. javanica* [41] while Eloh et al. [39] reported an EC₅₀ value of 48.6 µg/mL against *M. incognita*. Also, application of a mixture of maleic acid and copper sulfate reduced RKN disease on tomato by 51.72% in a pot experiment, suppressed gall formation on melon and decreased nematode population density in the soil [42]. Regarding copper based NPs, in previous studies, against plant pathogenic bacteria, among NPs of the same sizes with varyin compositions such as Cu (Cu⁰), Cu₂O (Cu¹⁺) and CuO (Cu²⁺) NPs, metallic copper has been found the most potent [14].

In this study the CuFe NPs exhibited the highest activity, but we can't overlook the fact that in the case of CuFe NPs a higher ionic release occurs, after 24 h dispersion in water, than in the case of Cu NPs (19.8 µg/ml and 1.6 µg/mL, respectively). Considering that the nematicidal activity of copper sulfate has been determined at EC_{50/24h} = 32.9 µg/mL, the potent activity of CuFe NPs could partially be attributed to copper ions released 24 hours after dispersion of CuFe NPs in water. On the contrary iron has not significant antimicrobial properties [43], while the nematicidal activity of iron sulfate against *M. incognita* has been reported at 126 ± 48 µg/mL [39], much higher than the concentration of free iron (0.5 µg/mL) released after 24 h of dispersion and therefore the effect of copper/iron based NPs on *Meloidogyne* spp. has further to be studied.

Furthermore, Cu, CuFe and CuFeO₂ NPs arrest the biological cycle of *M. incognita* in tomato roots when used in pot experiments. Among the tested compositions, CuFe NPs revealed once again the highest efficacy and the lowest EC₅₀=0.03 µg/g soil. Finally, the nematicidal activity followed the same descending order of CuFe(Cu⁰) > Cu(Cu⁰) > CuFeO₂(Cu¹⁺). The fact that no phytotoxicity was observed in tomato plants treated with the tested compositions,

while the bimetallic CuFe NPs resulted in significant increase of fresh shoot and root weight in the three higher doses, indicate an additional beneficial role (Figure 5) (Table 5) and should be considered together with the efficacy parameters in case it would be addressed as a pest management tool.

On the other hand, both copper and iron are essential trace elements for plants' growth [44] participating in basic photosynthesis processes and bio functions of plants at the cellular level; while their lack may lead to structural damages [43]. Although recent published results report on root exposure of plants to NPs, the plant root uptake of NPs is still controversial [47]. Recent studies on Fe NPs revealed the fate of iron in soil being associated with its additive form. Coated NPs, as in the present study, were proved to alter the surface chemistry and interaction with the environment [45]. Specifically pegylation has been suggested to insert stealth characteristics to NPs and promote the biofunctions in comparison to the uncoated NPs [46]. Hydrodynamic sizes of the NPs were found under 260 nm (Figure 1S-Supplementary file) indicating aggregation effects. The particle size of NPs is one of the most important properties impacting their plant root uptake, although published results are contradictory [47]. NPs with sizes up to 3 μ have been reported to transport in plant tissues from root to shoot through the apoplastic pathway [47].

However, when NPs are applied to soil, usually biotic and abiotic transformations such as redox reactions, aggregation and dissolution of NPs occur, resulting in changes in their bioavailability, toxicity and fate of the NPs. Thus, NPs exposed to plants in the soil may retain different properties from their original ones. Therefore, it is possible their transformation into bioavailable ionic forms such as $\text{Cu}^{2+}/\text{Cu}^{1+}$ and $\text{Fe}^{2+}/\text{Fe}^{3+}$ and further transportation in the plant tissues. The fact that only CuFe NPs were found to enhance growth parameters might be attributed to a combined effect of the two elements. Plants evaluate iron in the ionic forms of Fe^{2+} and Fe^{3+} , but only Fe^{2+} stimulates growth parameters [43]. In fact, in case of CuFeO_2 where iron participates as Fe^{3+} there is no evidence of growth enhancement. Considering CuFe NPs, we assume that the metallic form of iron transforms to the ionic bioavailable Fe^{2+} ; although the combination with soil micronutrients cannot be excluded. This is in agreement with previous studies, reporting treatments of metallic Cu combined with metallic Fe NPs on wheat seeds resulting in an increase of sugar content [48]. Conclusively, copper/iron based NPs were found promising to control nematodes while CuFe NPs can be further suggested as plant growth enhancers.

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