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SERVICES FOR SCIENCE AND EDUCATION

Nematicidal Assessment of Plant Mediated Green Synthesized Silver Nanoparticles Under Laboratory Conditions

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ABSTRACT

Plant mediated biosynthesis of silver nanoparticles (AgNPs) and the bioactivity under laboratory condition on plant parasitic nematode, Meloidogyne incognita, is discussed. In the present study, aqueous extracts of the aerial parts of a medicinal weed, Euphorbia geniculata was utilized to construct AgNPs. The synthesized AgNPs was characterized by Ultraviolet-visible (UV-Vis.), Fourier Transform-infrared (FT-IR), X-ray Diffraction (XRD) spectrometer and Scanning Electron Microscopy (SEM) analysis. The species identification of M. incognita was accomplished via the DNA-PCR technique. The green biosynthesized AgNPs demonstrated comparable nematicidal activity with the reference chlorpyrifos against Meloidogyne incognita identified through DNA assay in PCR.

INTRODUCTION

Nematodes are multi-cellular organisms and they are considered to be the most numerous organisms on earth.[1] These mostly microscopic worm-like creatures, range in sizes from 0.3 mm to over 8 m such as *Placentonema gigantissimum* that inhabits the placenta of a sperm whale.[2] They are so numerous that a handful of soil may contain thousands of them. Their habitat ranges from the ocean floor to the mountain tops.[2] Many of the nematodes are parasitic and feed on plants, bacteria, fungi, protozoa, other nematodes, animals and humans such as *Wuchereria bancrofti* that causes elephantiasis throughout the tropical region. Others are involved in the decomposition of organic matters and are referred to as free-living.[2, 3]

The knowledge of plant parasitic nematodes of *Meloidogyne* species has been well documented.[3] Knowing and managing plant parasitic nematodes is important in agriculture as these organisms are annually responsible for 15% crop loss that is about 78 billion US$ in monitory value globally.[3, 4] *Meloidogyne* are microscopic soil-borne nematode species and are known world-wide as the most plant parasitic pest that limits agricultural crop productivity.[5, 6] Among the root-knot *Meloidogyne* species, there are four major ones that affect agricultural

crops. In the order of their dominance and severity of damage to crops, they include *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla*.[7] *Meloidogyne hapla* mainly inhabits the cooler higher altitude soil environment while the other three species predominantly inhibit the warmer tropical lowlands.[6] Together they account for 95% of the root-knot nematodes in arable lands.[8] *Meloidogyne incognita* is the dominant of the *Moloidogyne* species worldwide and is found frequently in tropical and subtropical climates.[9] It feeds on a wide range of agricultural crops such as tomato, sweet potato and carrot to name a few, as well as other forest plants in general.[7] According to Lamberti and Taylor (1979), it accounts for about 64% of nematode population in tropical regions and about 70 - 80% in subtropical climates.[10]

From a number of methods used to identify nematode species, two commonly used at present are firstly, the identification based on perennial pattern morphology and secondly, the method based on PCR technique. The latter is the recent advancement that has enabled exact identification of the four major species of the root-knot nematodes and is based on their genetic marker.[7, 11]

Several methods are employed in the control and management of nematode populations and some of these include chemical, cultural, biological, sanitation, solarisation and botanical control.[7, 9] Among these methods, the chemical control method is the most common approach but it has its serious health and environmental issues. The tactic of botanical control is less practiced but safe, environment friendly, and the materials utilized are cheap and are readily available naturally. These biological materials are a rich source of biomolecules which can be utilized to construct metal nanoparticles such as silver nanoparticles that can be used as an alternative nematicide which is the basis of this research.

Technology of nano science is a rapidly emerging area of research that has application in all areas of sciences and engineering disciplines including agriculture.[12] Nanotechnology is based on the manipulation of atoms and molecules at nanoscale level (1-100 nm) because of the unique physical, chemical and biological properties demonstrated by certain nanomaterials.[13] Silver nanoparticle (AgNPs) is one such nanomaterial that has drawn attention for its application in agricultural research primarily due to its outstanding chemical and biological properties.[14] Among metal nanoparticles, silver is more biocompatible and has excellent antimicrobial properties due to its high surface-to-volume ratio, hence, its application as an antimicrobial and insecticidal agent.[14, 15]

In recent past, there have been numerous literature reports of promising nematicidal activities demonstrated by green synthesized AgNPs.[16, 17, 18] In the present investigation, aqueous extracts of a medicinal herb, *Euphorbia geniculata* (Euphorbiaceae), (Figure 1) was utilized to fabricate silver nanoparticles and assayed against *Meloidogyne incognita* to examine the nematicidal property of these nanoparticles under laboratory conditionals.

Euphorbia geniculata is an annual weed that grows up to 60 cm high in moist tropical and subtropical regions to an altitude of up to over 1000 m on a wide variety of soils and it is considered a pest that affects varieties of agricultural crops.[19] There has been no documented report of the ethno-botanical use of this plant. However, the medicinal use of a related species,

Euphorbia hirta is well documented.[19] No specific chemical information of *E. geniculata* is also found, but, triterpenoids, flavanoids, alkaloids, glycosides, tannins, steroids saponins, lignins, phytosterols, polyphenols and benzoic acid were found to occur in other related species including *E. Hirta*.[20] This study is a continuation of the biological assessment of AgNPs fabricated via aqueous extract of *E. geniculata*.[21]

MATERIALS AND METHODS

Preparation of Silver Nitrate Solution

Using an analytical balance, 9.5 g of silver nitrate was weighed and transferred into a 500 ml volumetric flask, dissolved and made up to volume with distilled water giving a concentration of 0.1177 moles L^{-1}. From this, 2.5 ml was transferred to 100 ml volumetric flask with a 5 ml pipette and diluted to mark with distilled water, giving a final concentration of 0.003 moles L^{-1}.

Sampling and Preparation of the Plant Sample

The aerial parts of fresh disease-free plant (Figure 1) were collected and 10 g of these were weighed and washed thoroughly with tap water and then rinsed with distilled water.



Figure 1: *Euphorbia geniculate*

Aqueous Extract of the Plant Sample

A 10 g of the plant material were chopped to smaller pieces with a kitchen knife and placed into 250 ml volumetric flask containing 200 ml of distilled water and heated on a bunsen burner to the first sign of boiling, then removed and cooled to room temperature. The solution was then filtered through a Whatman No. 1 filter paper and the filtrates were used to synthesize silver nanoparticles.

Synthesis of Silver Nanoparticles

Preparation of AgNPs:

A measuring cylinder was used to obtain 50 ml of aqueous plant extracts and transferred into 250 ml volumetric flasks and to these, 100 ml of 3.0 mM of silver nitrate were added, shaken and placed directly under the sun. A colour change from pale yellow to dark brown indicated

the formation of silver nanoparticles, followed by the agglomeration of the particles. After 2 h, the mixture was removed and placed in a room for agglomerated colloidal AgNPs to settle overnight before centrifugation at 2500 rpm. The supernatants were discarded and the AgNPs were washed twice with about 50 ml of distilled water by centrifugation per wash. The supernatants were discarded and the remaining AgNPs were dried and stored at 4^{o}C in a small sample bottle. About 250 mg of these nanomaterials were sent to India for spectral analysis.

Characterization:

Characterization of the synthesized AgNPs included UV-Visible spectroscopy, Fourier Transform Infra-red spectroscopy (FT-IR), X-ray Diffraction spectroscopy (XRD) and Scanning Electron Microscopy (SEM). The UV-vis. analysis of the phytosynthesized AgNPs was carried out using a Varian Cary 50 Bio UV-visible spectrophotometer. The absorption maxima (λ_{max}) of the coloured solution of AgNPs in a cuvette (1 cm path length) was taken from a wavelength range set at 200 – 700 nm and slit width at 1 nm. FT-IR analysis was carried out in the range of 450 to 4,000 cm^{-1}. XRD pattern was recorded using Cu Kα radiation (λ = 1.54060 Å) with nickel monochromator in the range of 2θ from 10° to 70°. The morphology and size range of the synthesized AgNPs were examined by SEM.

Molecular Identification of *Meloidogyne incognita*

Experimental Location:

Molecular identification of *Meloidogyne incognita* and laboratory tests were done at the Unitech Biotechnology Centre (UBC) in the Department of Agriculture of PNG University of Technology, (Unitech) Taraka Campus Lae, Morobe Province.

Bio-trapping of *Meloidogyne incognita:*

Beefsteak, a highly susceptible tomato variety was used to bio-trap *M. incognita*. Seeds were initially germinated in tray boxes using unsterilized soil for three weeks. The seedlings were then transplanted into 15 cm diameter polystyrene pots containing soil naturally infested with *Meloidogyne* spp. These were then raised in a glasshouse for about two months before they were harvested and brought to the laboratory for extraction of embedded females and isolation of egg masses.

Identification of *Meloidogyne incognita*:

The nematode species of *M. incognita* was identified using DNA-based PCR technique. Bi-directional oligo DNA primers (Table 1) were used to amplify the sequence characterized amplified region (SCAR) according to procedure described by Zijlstra *et al.* (2000).[11] DNA from nematodes were isolated according to procedure described by Powers and Harris (1993).[22] Female *Meloidogyne* spp. entrapped inside the infected root system of beefsteak tomato were milled using mortar & pestle and DNA was extracted according to Powers and Harris (1993).[22] PCR amplification was carried out in a total reaction volume of 25 µL containing 5 µL of DNA template (lysate), 2.5 µL 10x Kapa *Taq* buffer with dye and Mg^{2+} (Geneworks), 0.5 µL 10 mM dNTPs, 0.6 µL 10 µM Fine/Rinc primers, 0.5 µL 5 U/µL of *Taq* DNA polymerase (Geneworks), and nuclease-free water was added to final reaction volume. The PCR thermocycler was programmed with an initial denaturation at 94^{o}C for 2 min followed by 35 cycles at 94^{o}C for 30 s and 72^{o}C for 1 min. The reaction was terminated with 1 cycle at 72^{o}C for 5 min.

Pre-stained PCR products (DNA) were loaded into the well of 1% agarose gel with a molecular marker (Biorad). Loaded products were submerged in 1x TBE buffer containing 0.5 µg ml^{-1} ethidium bromide in mini gel tank and fractionated at 100v for 50 min. DNA bands specific to *M. incognita* were finally identified under a UV spectrophotometer using a UV box (Vilber Lourmat) and were recorded.

Table 1: Nucleotide sequences of *Meloidogyne* species-specific primers and expected sizes of their typical amplified DNA bands.[131]

	Primer Sequence	Size of DNA	
Primer Name	**(5'-3')**	**bps**	**Specificity**
Finc(1)	ctctgcccAATGAGCTGTCC	1200	*M. incognita*
Rinc	ctctgcccTCACATTAAG		
Mi-F(2)	TAGGCAGTAGGTTGTCGGG	1350	*M. incognita*
Mi-R	CAGATATCTCTGCATTGGTGC		

Laboratory Assay of Biosynthesized AgNPs against *M. incognita*

Rearing *M. incognita* on Beefsteak Tomato:

Seeds of beefsteak tomato were germinated and raised on heat-sterilized soil tray before being transplanted after two weeks into 15 cm diameter polystyrene pots containing sterilized soil. These seedlings were allowed to grow for one week before eggs and juveniles (J2s) from identified *M. incognita* were used to inoculate the seedling and were allowed to regenerate on roots of the host plant for subsequent studies.

Isolation of Eggs for Laboratory Susceptibility Test:

Eggs and J2s were harvested from roots of the infected beefsteak reared in the glasshouse. Infected tomato pots were fully submerged into a bucket of water and the soil was carefully washed off from the roots. To obtained mature eggs, the egg masses on infected roots were dipped into 0.05% sodium hypochlorite solution and gently swirled for 5 min. This freed the eggs from the roots and were separated by filtration using a 38 µm Endacott sieve and then washed into a sample vial to be used for testing and also to incubate to produce J2s. Those not for immediate use were stored in a refrigerator at 15°C.

Experimental Design and Treatments:

Experiments in the laboratory were arranged in small glass petri dishes on benches in replicates of three. There were nine sets of experiments which included blank (distilled water as positive control), commercial nematicide (chlorpyrifos, 1% w/v) as positive control, undiluted AgNPs, and 10x, 100x, 1000x, 2500x, 5000x and 10,000x dilution of AgNPs. For simplicity, each set of treatments were tested separately and simultaneously for both eggs and J2s.

Bioassay of AgNPs Against Eggs and J2s:

Eggs – Ten eggs extracted from roots of beefsteak tomato were transferred into nine different petri-discs (small) using sterile pipette tips and to these different treatments (distilled water, chlorpyrifos, and different dilutions of AgNPs) were added. The mortality of eggs was observed and recorded at 24 h intervals.

Juveniles (J2s) – Some egg suspensions extracted from roots of beefsteak tomato were transferred to a small petri-dish and incubated at room temperature overnight to allow them to hatch into J2s. From these, ten J2s were transferred into nine different small petri-dish and different treatments (distilled water, chlorpyrifos, and the different dilutions of AgNPs were added. The mortality of the J2s were then monitored and recorded at a 24 h interval.

RESULTS

Colour Change

The formation of silver nanoparticles was evident from the change of colour of the reaction mixture from a yellow to a dark brown solution within 20 min (Figure 2).



Figure 2: Formation of AgNPs indicated by the change of colour from yellow to dark brown solution.

Characterization

The Uv-vis spectrum (Figure 3) of the AgNPs generated from the aqueous extract of *Euphorbia geniculata* exhibits an absorption peak at 450 nm that is characteristic of the Uv-vis signal of the surface plasmon resonance of silver nanoparticles. FT-IR frequencies of the bio-molecules involved in the reduction of silver ion (Figure 4) shows peaks at 3410 (N-H), 2925 (aliphatic C-H), 2851(aliphatic C-H), 1449 (C-H bending) and 1031 cm^{-1}.[23] The XRD pattern of the AgNPs (Figure 5) shows three distinct diffraction patterns 2θ peak values at around 38°, 44° and 64° that are characteristic of the planes (111), (200) and (220) for the face-centered cubic silver.[24] Image from the scanning electron microscopy (SEM) (Figure 6) shows the morphology of the AgNPs to be mainly spherical and their size distribution ranges from 13 to 45 nm.

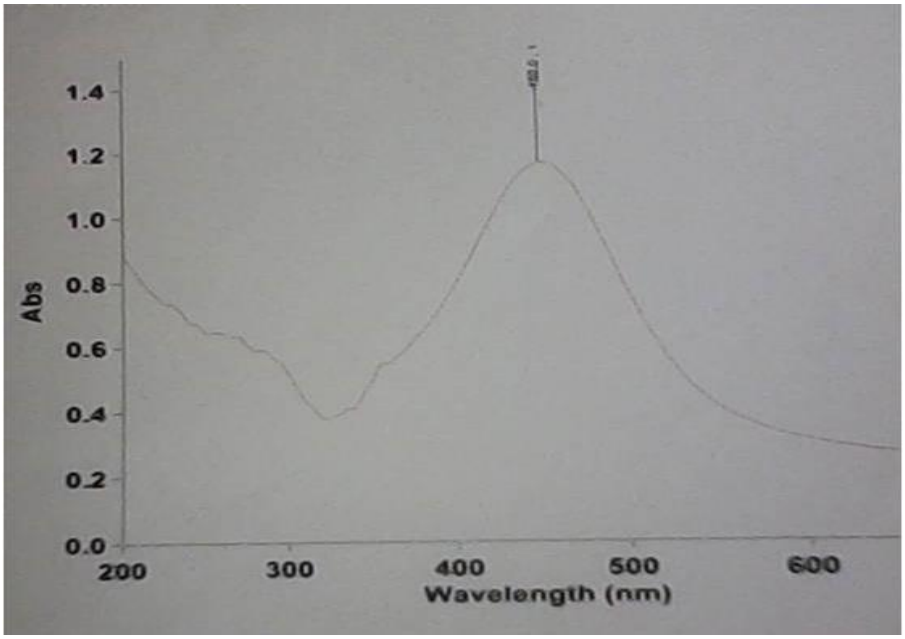


Figure 3: Uv-vis. Spectrum of AgNPs synthesized From Extract of *E. geniculata*

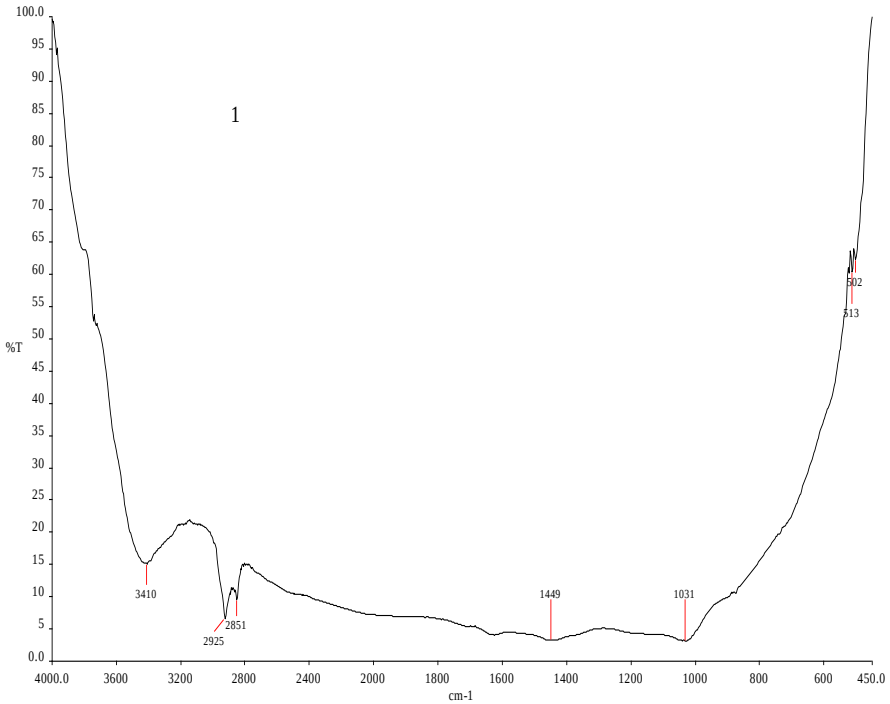


Figure 4: FT-IR Spectrum of AgNPs Synthesized From *E. geniculata*

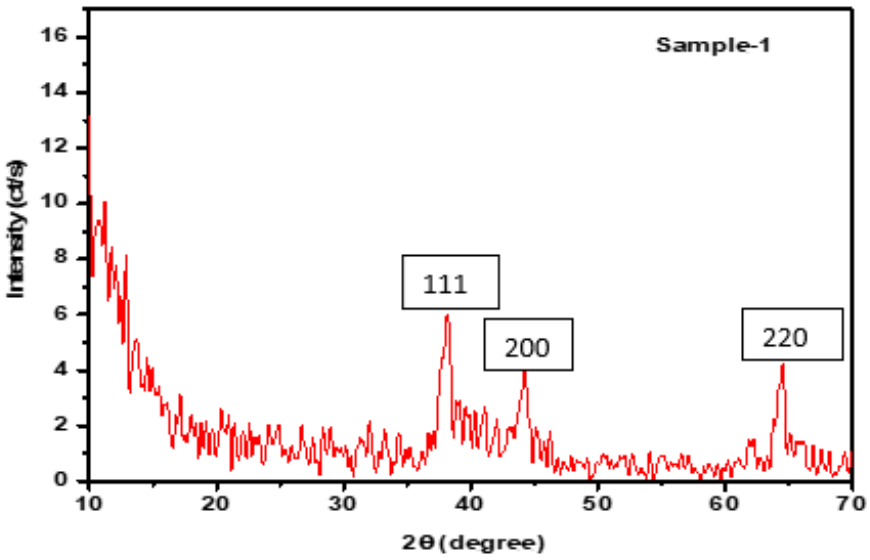


Figure 5: XRD Pattern of AgNPs Synthesized from Extract of *E. Geniculata*

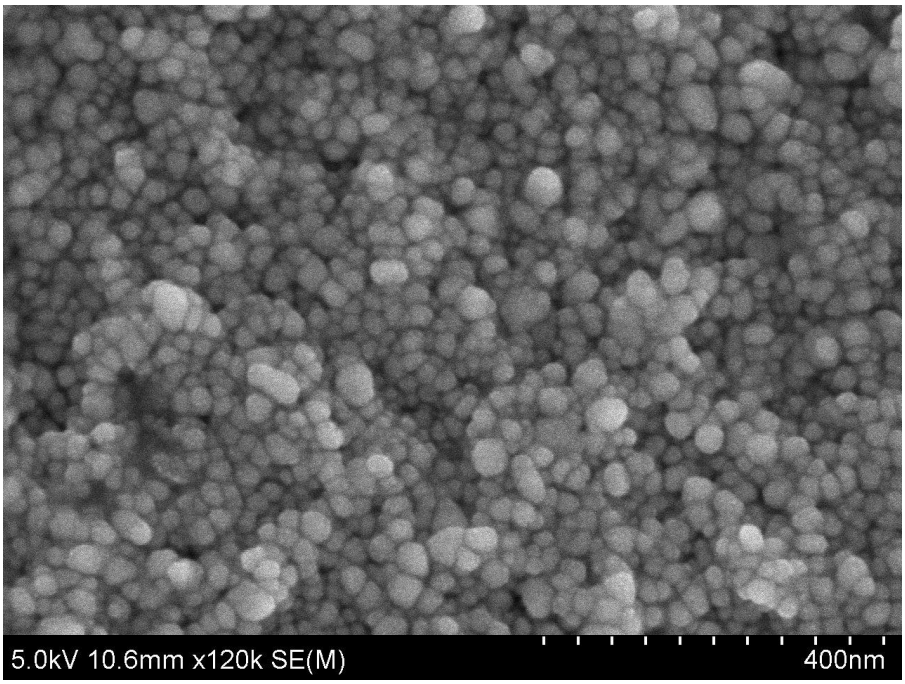


Figure 6: SEM Image of AgNPs Synthesized from Extract of *E. geniculata*

Identification of Nematode Species

Molecular Identification of *M. incognita:*

The nematode species *M. incognita* was identified using DNA-based technique. Oligo DNA primers used in PCR amplification process yielded expected DNA products for *M. incognita* (Figure 7). Lanes 3 to 6 on 1% agarose gel showed the expected DNA bands of 1200 bps using SCAR primers characteristic of *M. incognita*.

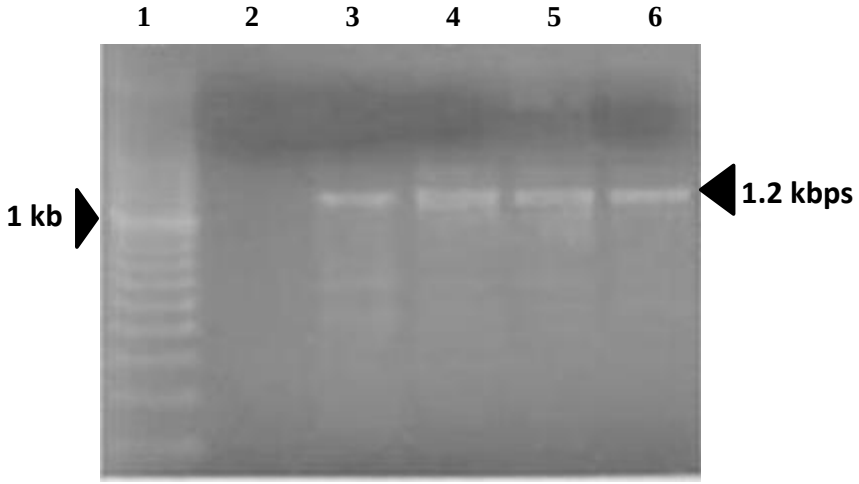


Figure 7: PCR products. Lane 1 is Biorad DNA marker; Lane 2 is negative control; Lanes 3 – 6 are positive amplifications (1200 bps) of *M. incognita* (Zijlstra *et al*., 2000)[11]

Laboratory Test on Mortality of J2s

The AgNPs tested against infective J2 showed promising toxic activity and the results are presented in Table 2. Tests revealed that undiluted AgNPs as well as dilutions at 10, 100, 1000 and 2500 caused 100% mortality in 24 h, showing the same outcome as the commercial nematicide, chlorpyrifos. No mortality was recorded from J2s treated with water alone. At that time interval, 50% mortality was recorded at higher dilution of 5000, while no deaths were observed at 10,000 dilutions of AgNPs (Table 2). The trend of mortality at the highest dilutions was similar to that observed from treatments with water alone. The undiluted AgNPs were kept as aqueous suspensions in ~30 ml of distilled water.

Table 2: Mortality of J2 *M. incognita* exposed to AgNPs synthesized from *E. geniculata*

Treatments	Average mortality of J2 from population of 10 juveniles as % of control				
Dilutions (1/x)	n	24	48	72	96
Control (H_2O)	10	0	0	0	50
Chlorpyrifos (1%)	10	100	100	100	100
Undiluted	10	100	100	100	100
10	10	100	100	100	100
100	10	100	100	100	100
1000	10	100	100	100	100
2500	10	100	100	100	100
5000	10	50	60	70	90
10,000	10	0	0	0	60

The undiluted AgNPs were kept as aqueous suspensions in ~30 ml of distilled water.

Laboratory Tests on Hatchability of Eggs

The effect of silver nanoparticles on the hatchability of the eggs of *M. incognita* was also examined and the results are presented in Table 3. The tests showed that AgNPs effectively suppressed the eggs from hatching during the 96-h observation period similar to the positive control, chlorpyrifos (1%). For the eggs treated with negative control (water), there was normal

life process that led to the hatchability of eggs from 48 h onward. Exposure of the eggs at 5000x and 10000x dilution of the AgNPs did not seem to affect the hatchability of eggs as the J2 *M. incognita* were observed to leave the eggs from 48 h onwards (Table 3)

Table 3: Egg hatchability of *M. incognita* exposed to AgNPs synthesized from *E. geniculata*

Treatments	Egg hatchability as % of control				
Dilution (1/x)	n	24	48	72	96
Control (H_2O)	10	0	40	70	100
Chlorpyrifos (1%)	10	0	0	0	0
Undiluted	10	0	0	0	0
10	10	0	0	0	0
100	10	0	0	0	0
1000	10	0	0	0	0
2500	10	0	0	0	0
5000	10	0	20	70	70
10,000	10	0	30	70	90

DISCUSSION & CONCLUSION

In biological assays, correct identification of target organism is an important aspect. This process facilitates tailored experimentation, data collection, analysis and interpretation. This study showed that *M. incognita* can be identified among other *Meloidogyne* species using species-specific DNA primers in PCR detection technique. Species-specific DNA product of approximately 1.2 kbps was amplified in PCR reactions and detected in agarose gel under UV trans-illumination. PCR technique in the use of single J2 nematodes proved that the technique is reliable in diagnosis and differentiation of the four major *Meloidogyne* spp. that affect crops. Population of *M. incognita* identified through this process were used in subsequent laboratory experiments.

Meloidogyne incognita is a significant harmful pest for most agricultural crops, reducing growth and yield of susceptible plants. The results show that infective juveniles of *M. incognita* exposed to AgNPs experienced high lethal effect and further, AgNPs showed strong suppressive effects on the hatchability of the eggs. The effect on both the J2 and the eggs was comparable to the commercial nematicide chlorpyrifos even at 2500x dilution of the nanomaterial. At 5000x dilution, the effect of the nanoparticles was negligible. From the egg hatchability tests, it was interesting to observe that chlorpyrifos at 1% concentration was rupturing the eggs unlike the nanoparticles, which only suppressed the eggs from hatching without rupturing them.

This research finding is seen to be consistent with previous research studies on laboratory assessment of silver nanoparticles when applied against *Meloidogyne*s spp.[25, 26] It further demonstrated the excellent biocidal properties of AgNPs phytosynthesized from species of Euphorbiaceae.[21, 27] Additionally, it further suggests that AgNPs of plant mediated biosynthesis can be utilized for biological applications in the control of agricultural pests.[21, 26, 27] The overall result under the laboratory conditions was encouraging and this led to further greenhouse and field assessment of the AgNPs against *M. incognita*.

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