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GREEN BIOSYNTHESIZED SILVER NANOPARTICLES USING MACROALGAE AND THEIR USAGE AS A BIOAGENT ON *MELOIDOGYNE INCOGNITA*

Rehab Y. Ghareeb[1]*, Mohamed A.M. EL-Saedy[2], Mahmoud M. Fathy[2], Elsayed E. Hafez[1] and Ayman B.A. Basyony[2]

[1*]Plant Protection and Biomolecular Diagnosis Dept., Arid Lands Cultivation Research Institute, City of Scientific Research and Technological Applications, New Borg El Arab, Alexandria, Egypt.
[2]Plant Pathology Dep., Faculty of Agriculture, Alexandria, University, Alexandria, Egypt.

Abstract

This study was conducted on two green algae to evaluate their water extracts and the green-synthesized nanosilver as nematicide on *Meloidogyne incognita*. The freshwater alga *Cladophora glomerata* and the marine alga *Ulva fasciata* were collected and identified. Both of the nematode and the collected algae were identified using the microscopic examination. The biosynthesized nanosilver was subjected to X-Ray Diffraction Analysis and the results shows the crystal diameter of nanoparticles prepared from *C. glomerata* and *U. fasciata* were 13.09 and 10.51 nm, respectively. Moreover, Transmission Electron Microscope indicated that diameter of nanoparticles prepared ranged from (9.79-13.64) and (9.48-13.01) nm in respective manner. The bioassay experiments revealed that nanoparticles showed high nematicidal activity on egg hatching and J2 mortality (up to 100%) compared with the chemical nematicide (Nemaphose 40%). The greenhouse experiment using the algal extracts, nanoparticles and Nemaphose 40%, indicated that nanoparticles had the highest effects on nematode egg hatching and the 2nd stage juveniles (J2). To examine the plant response toward the biosynthesized nanosilver, gene expression of the plant defense gene (chitinase gene) was tested using Real Time PCR. The results revealed that chitinase gene amplified from the infected tomato plants treated with *C. glomerata* extract shows high expression level compared to both control and plants treated with Nemaphose 40%. In addition, a high expression level of chitinase gene was noticed after 6 h of infected plants treated with *U. fasciata* extract. The same observation was noticed in plants infected with *M. incognita* and treated with nanosilver particles prepared from *C. glomerata* extract and lasts for 24 h post-treatment. Also, the biosynthesized nanosilver prepared from *U. fasciata* extract showed high expression of the chitinase after 6 h in infected plants treated with nanosilver and Nemaphose 40%. It can be concluded that the biosynthesized nanoparticles using *U. fasciata* and *C. glomerata* extract could be used as a potent nematicide against *M. incognita*.

Key words: Root-knot nematode, Nanosilver, Nematicides, Real Time PCR and Chitinase gene.

Introduction

Soil-borne pathogens are made many disasters in modern agriculture globally. Plant-parasitic nematodes are some of the most disaster pathogen in agriculture ecosystems by direct feeding, Virus transmission and infections of both bacteria and fungi were also facilitated by nematodes. Accordingly, nematode causes severe losses in the crop yield that it infected which exceeded more than $157 billion annually (Abad *et al.,* 2008). In Egypt and on many cultivated crops about 54 genera and 160 species of plant nematodes were recorded, weeds and grasses (Munif *et al.,* 2013). Moreover, nematodes; *Meloidogyne* sp., belonging to the order *Tylenchida*, are considered a limiting factor to their host's production (Korayem and Mohamed, 2010). Due that different chemical nematicides have been heavily used all over the world to control nematodes and due to the limitation of using the chemical nematicides because the majority of their affect both on human and environmental health. Accordingly, significant scientific interest was directed to the development of safe and effective control materials

***Author for correspondence :** E-mail : reyassin_ghareeb@yahoo.com

as a substitution for the chemically synthesized nematicides.

The phyto-synthesis of nanoparticles is one of the most common subjects in green- nanotechnology (Roy and Barik, 2010). Accordingly, different nanoparticles were synthesized biologically by using; plant extract, marine algae, fungi and bacteria (Seshadri *et al.,* 2012; Sinha and Paul, 2014; Muhsin and Hachim, 2014; Kannan *et al.,* 2013) and the resultant nanoparticles were used in different purpose. Khan and Fatima (2014) stated that bio-nanotechnology can be used to controlling different plant pathogens in addition for diagnosis their disease symptoms.

Nowadays biological synthesis of metallic nanoparticles gained an importance as it is reliable and eco-friendly. Previous literature revealed that the nanoparticles synthesis using algae as the source has been unexplored and underexploited. Recently there are a few reports that algae are being used as a bio-factory for the synthesis of metallic nanoparticles (Kwakye- Awuah *et al.,* 2008). The nematicidal effect of AgNPs was previously examined as anti-nematodes especially *Caenorhabditis elegans* (Lim *et al.,* 2012). In previous reports, it was noticed that a specific dose of AgNPs can inhibit the nematode reproduction (0.05 - 0.5 µg/ml) after 72 h post treatment and the dose of (5-50 µg/ml) is capable to inhibit the nematode growth for 1 to 3 days. The nematicidal activity of the biosynthesized AgNPs against nematodes because its playing an important role in disrupting numerous biological mechanisms inside the treated organism such as; permeability of cell membrane, synthesis of ATP and the defense system response either in eukaryotic (Ahamed *et al.,* 2010) or prokaryotic cells (Choi and Hu, 2008). Generally, AgNPs have a wide range as biocontrol materials against different plant pathogens (Jo *et al.,* 2009). The aim of this investigation is the biosynthesis of a nano-nematicide by using the algal extract for controlling *M. incognita* (root-knot nematode) infecting tomato plants. At the same time, examine the plant response under treatment with the synthesized nanoparticles compared with the chemically synthesized pesticide and control.

Materials and Methods

Macro algal sample collection

Two macro algae isolates were collected from Alexandria governorate, Egypt. The freshwater alga, *Cladophora* sp. was collected from El-Amria, Alexandria, Egypt. The marine alga, *Ulva* sp. was collected from the coastal area EL-Shatby coast, Alexandria, Egypt. Macroalgae were collected individually manually, packed in plastic bags and transferred directly to the laboratory for algal identification. Algal species were washed carefully with tap water three times and finally with distilled water. The clean marine algae were freeze-dried at -20°C and then, ground into powder and stored in a vacuum desiccator at room temperature for further studies (Azizi *et al.,* 2013).

Root-knot nematode collection

Root-knot nematode isolates were collected from infected tomato roots grown in Bader Center fields, El-Behera Governorate, Egypt during the season (2015-2017).

Tomato plants

The seed of tomato cultivar Super Strain B used in the greenhouse experiment and were obtained from the Faculty of Agriculture, Alexandria University, Egypt.

Macroscopic examination and identification of both nematodes and algal isolates

Both nematode and algal samples were examined under the light microscope (Olympus BX40 microscope, Japan). Taxonomic identifications were performed according to (John *et al.,* 2003 and Alves *et al.,* 2012).

Preparation of *Cladophora glomerata* and *Ulva* extracts

One gram of the algal fresh weight either from *C. glomerata* or *U. fasciata* were dispersed in 100 ml distilled water using a magnetic stirrer at 45°C for 40 min separately (Azizi *et al.,* 2013). The extracts were filtered through a Millipore filter (0.2 µm) and stored at -20°C for further studies.

Biosynthesized of silver nanoparticles Ag-NPs using the two algal aqueous extracts

Biosynthesized of Ag-NPs was according to the method of Azizi *et al.,* (2013; Ghareeb *et al.,* 2020). The Erlenmeyer flask containing 100 ml of aqueous solution (1 mM) of $AgNO_3$ were mixed with 100 ml of the aqueous extract of either *C. glomerata* or *U. fasciata* for 30 min under continuous stirring at 35°C then allowed standing at room temperature. The initial pH of the solution was 7.5 then decreased to 5.6 at the end of the reaction (2 h). The dark brown material was collected by centrifugation at 11000 g for 15 min and carefully washed three times with dH_2O and then was left in the oven overnight at 40°C. The dried material was ground into fine powder for analysis.

Characterization of Biosynthesized silver nanoparticles

UV–Vis Spectrophotometer

The sample solutions were analyzed at room temperature for UV–visible absorption using the Spectrophotometer (model T60-PG instruments). The absorbance of silver nanoparticles was measured at O.D.450 nm.

X-Ray powder Diffraction (XRD)

XRD measurements of the resulted silver nanoparticles were determined using X- Ray Diffraction model (XRD-7000, Shimadzu, Japan) with CuKα radiation (λ = 1.54060 Å). For determining the crystalline structure, the XRD measurements have been obtained by applying a step scanning method (2θ range from 5–80°) with a scan speed of 4θ/min. The average crystal size (D) was recorded according to (Elnouby, 2008).

Transmission Electron Microscope (TEM)

Samples of biosynthesized silver nanoparticles were prepared for TEM examination by dispersing small quantities of the dried sample into distilled water and depositing a few drops of the resulted 1DX suspension on the copper grid. Field Emission Transmission Electron Microscope (JEOL- JEM-2100F).

Fourier-Transform Infra-Red spectra (FTIR)

The chemical structures of silver nanoparticles were studied using Fourier-Transform Infra-Red Spectrometer (FTIR-8400S, Shimadzu, Japan). The silver bio-nanoparticles were documented in a range from 300 to 4500 cm^{-1} using KBr powder for obtaining the composition of these silver bio nanoparticles.

Root-knot nematode preparation

The root-knot nematode *(M. incognita*) eggs were isolated from infected tomato roots according to Hussey and Barker (1973). Second-stage juveniles (J2) of *Meloidogyne incognita* were acquired by using the Baermann plate technique (Ayoub, 1980).

The efficiency of two macroalgal extracts and silver bio-nanoparticles on egg hatching and juvenile mortality of *M. incognita (invitro)*

A laboratory study was achieved to examine the efficiency of two macroalgae extracts and their silver nanoparticles on egg hatching and J2 mortality%. Egg-masses were left into distilled water for 72h to the juveniles hatching and were collected. Nematodes suspension was adjusted to contain 30 eggs/0.2 ml for each egg hatch experiment and 5 J2/0.2 ml for J2 mortality %. All the plants treated either with algal extracts and/or AgNPs were inoculated with nematode eggs or J2. One treatment was left untreated to serve as a negative control. As well as, treatment was only treated with Nemaphose 40% (6 ml dH_2O +0.2 ml nematodes suspension + 15 µl Nemaphose 40%: Positive control). The other treatments (10) were divided into two groups 5 of each. The 1st group was treated with *C. glomerata* extract (6 ml Cl. Ex +0.2 ml suspension of nematode) and (S, S/2, S/4 and S/10) of silver nanoparticles [S= 6 ml N.S.Cl+0.2 ml nematode suspension; S/2= 3ml N.S.Cl +3 ml dH_2O +0.2 ml nematodes suspension; S/4=1.5 ml N.S.Cl+4.5 ml d.H2O +0.2 ml suspension of nematodes; and S/10=0.6 ml N.S.Cl +5.4 ml dH_2O +0.2 ml nematodes suspension]. Whereas, the 2nd group was treated with *Ulva* extract (6 ml U.Ex +0.2 ml nematodes suspension) and silver bio-nanoparticles formed from *U. fasciata* extract with the same concentration as previously mention. Each treatment was replicated five times. The numbers of hatched eggs and numbers of a live J2 were counted and recorded after 24, 48 h, one and two weeks from exposure at room temperatures. The mortality percentage and reduction in egg hatching were calculated according to the formula by Abbott, (1925).

The effect of the two algal extracts and their silver nanoparticles on root-knot nematode (*M. incognita*) under greenhouse conditions (*invivo*)

Eight treatments of forty pots were used in this experiment. Pots were divided into 5 groups. The pots were inoculated with J2 and eggs of *M. incognita* (15 egg masses/pot of 250 egg/one egg mass). The 1st treatment (5 pots) was left untreated with nematicide or algal extracts to serve as a control (inoculated only with MI). The 2nd treatment (5 pots) was treated with the nematicide Nemaphose 40% (1 ml)/pot (MI+ Nemaphose). The other 30 pots were split into 2 groups of 15 pots each. The 1st group was treated with *C. glomerata* extract (30 ml/pot) and silver nanoparticles (S and S/2). The 2nd group was treated with *U. fasciata* extract and silver nanoparticles (S and S/2) (30 ml/pot). Pots were watered as needed and fertilized weekly with 10 ml of commercial fertilizer (NPK fertilizer, with micronutrients, 2.5 g liter). Five replicates were used for each treatment.

Pot experiment

Tomato seedlings, 45 days old, cultivars Super stain B was used in this experiment. Tomato was grown in 25 cm in diameter plastic pots, each containing 2 kg soil and sterilized in autoclaving at 121°C for 1 h. Plants were maintained in the glasshouse of the Department of Plant Pathology, Faculty of Agriculture, Alexandria. Forty pots of eight treatments were used in this experiment. All pots were inoculated with J2 and eggs (15 egg masses/pot) of *M. incognita* (MI). Pots were divided into 4 groups. The 1st group of 5 pots was left untreated with nematicide or

algal extracts to serve as a negative control (MI). The 2nd group was treated with Nemaphose 40% (1 ml)/pot as a positive control (MI+ Nematicide). The other 30 pots were distributed into 15 pots with two groups. The 1st group was treated with *C. glomerata* extract, a complete concentration (S) and S/2 of their silver nanoparticles (30 ml/pot). While, the 2nd group was treated with *U. fasciata* extract, a complete concentration (S) and S/2 of their silver nanoparticles (30 ml/pot). Pots were arranged on a glasshouse bench in a randomized block design. Plants were watered as needed and after nematode inoculation, fertilized weekly. Plant was harvested 53 days after inoculation, carefully washed and the fresh and dry weights of root and shoot systems, numbers of nematode root galls, egg masses, eggs/egg mass and J2/200 g soil were determined. Also, numbers of J2/kg soil were determined (Liu *et al.,* 2013). Egg masses were stained using phloxin B stain (0.15 g/l tap water) for 15 minutes then washed with tap water (Hussey and Barker, 1973). Tomato leaves were collected from each treatment and the collected tissues were stored at -80°C for further study. Plant response toward the nematode infection was studied according to the type of treatment using Real time qPCR.

Plant response using Real Time qPCR

RNA isolation from tomato leaves (from MI inoculated and algal extract or their nanoparticle treated plants) was carried out according to the protocol of the TRIZOL RNA Isolation Protocol. The extracted RNA concentration was evaluated by measuring absorbance at $O.D._{260}$, Samples were stored at -80°C until further use. For the first strand cDNA synthesized using Reverse Transcriptase (Fermentas, USA) and buffer (5X) [50 mM Tris-HCl (pH 8.3 at 25°C), 250 mM KCL and 20 mM $MgCl_2$ and 50 mM DTT] in presence of random hexamer primer (Promega, USA) (Sambrook and Russell, 2001) and 5µl of RNA was added to (10 µl (5x) RT Buffer, 5µl (25mM) dNTPs, 5µl of primer, 0.5 µl (20 u/µl) of RT–enzyme, 24.5 µl H_2O). The mixture was incubated at 37°C for 60 minutes, then at 70°C for 10 minutes (for enzyme inactivation), followed by storage at 4°C until used. Tomato leaf samples were taken before and after inoculation with *Meloidogyne incognita* and treated either with algal extracts and/or algal nanosilver. The specific primers for chitinase gene F: GGCAGGCCATTGAAAAGTTCC and R: CTAATCGTCAATGATCCAAGCGG according to Hafez *et al.,* (2013) were used in this experiment. A 150 ng of cDNA as template (1µl), 1 µl of 25 pM/µl of each primer, 12.5 µL of 2x Quantitech SYBR® Green RT Mix (Fermentase Comp.), 9.5 µl of ddH_2O, for a total volume of 25 µl. Samples were spun before loading in the rotor's wells. The Real Time qPCR program was performed as follows: initial denaturation at 95°C for 10 min; 45 cycles of 94°C for 25 sec; annealing at 55°C for 1 min and extension at 71°C for 30 sec. Data acquisition performed during the extension step. This reaction was performed using Rotor-Gene 6000 system (Qiagen, USA). The ITS primers: ITS-1F: TCC GTA GGT GAA CCT GCG G; ITS-4R: TCC TCC GCT TAT TGA TAT GC, according to Hafez *et al.,* (2013) was used as a reference for total RNA.

Data analysis of the Real Time qPCR

The qPCR data were analyzed using the comparative Ct ($2^{-\Delta\Delta ct}$) method according to Livak and Schmittgen (2001).

Statistical analysis

Data were analyzed statistically using analysis of variance (ANOVA) and differences among the means were determined for significance at $p \leq 0.05$ using revised LSD test using the statically analysis system SAS (SAS Institute, 1997).

Results

Isolation and identification of macro algae

The preliminary identification of pure collected macroalgae based on light microscopic revealed the presence of two macro algae genera, *Cladophora* and *Ulva*. The complete examination revealed that; the fresh water alga was identified as *C. glomerata* and the marine alga was identified as *Ulva fasciata*.

Silver nanoparticles (AgNPs) synthesis by the algal aqueous extracts

The reaction mixture of algal crude extract and silver nitrate solution turned from yellow to dark brown color Fig. 1A and B.

The formation rate of silver nanoparticles, resulted from using of the mixture of algal extract and $AgNO_3$

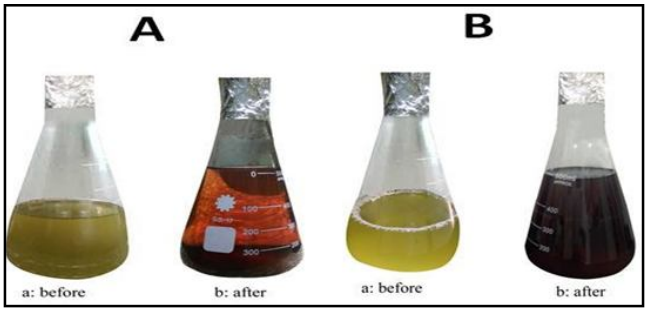

Fig. 1: The biosynthesis of the nanosilver using the two aqueous extracts of the two algal isolates (A) *Cladophora* sp., (B) *Ulva fasciata*.

solution of *C glomerata* and *U. fasciata* was increased with the time increase up to120 and 70 h, respectively Fig. 1. The highest OD reading (2.8 and 2.5) was recorded with *Cladophora* sp. and *Ulva fasciata* in a respective manner. After that, the intensity of nanoparticles formation was decreased Fig. 2.

The XRD pattern of the particles synthesized by *U. fasciata* was illustrated in Fig. 3a. The main peaks in the XRD patterns peak (32.4433) are indexed to the Monoclinic Ag_3O_4 phase with space group of p21/c (14) and lattice parameters a = 3.5787(23), b = 9.279(5), c = 5.6771(3) (ICDD Card No. 00-040-1054). Whereas, the peak (28.0450) is indexed to the Orthorhombic $AgNO_3$ phase with space group of pbca (61) and lattice parameters a = 6.995, b = (7.328), c = (10.118) (ICDD Card No. (00-006-0363). Peak 46.4650 is indexed to the Hexagonal Ag_2O phase with space group of P and lattice parameters a =4.727(3), c = (7.856(7) (ICDD Card No. 00-042-0874). Whenever, the XRD pattern of the particle biosynthesized using *Cladophora* sp. was illustrated in Fig. 3b. Main peaks of the XRD pattern were 38.466 and 45, are cubic silver phase with space group of Fm-3 m (225) and lattice parameters a = 4.074 (ICDD Card No. 00-003-0921). Peak 29.8066 is indexed to the Orthorhombic $AgNO_3$ phase with space group of p212121 (19) and lattice parameters a = 6.97, b = (7.34), c = (10.14) (ICDD Card No. 00-001-0856). Peak 32.56 is documented to the Ag_2O phase with space group of pn-3 (201) and lattice parameters a =4.76 (ICDD Card No. 010-076). Collectively, nanoparticles prepared using *Cladophora glomerata* was 13 nm.

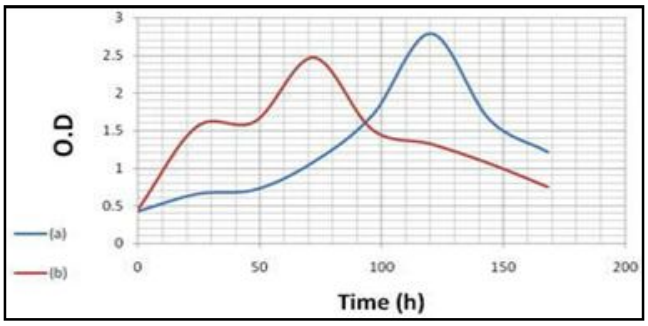

Fig. 2: The gradual formation of the biosynthesized nanoparticles in the aqueous extracts of the two selected algae measured in one week (as O.D.450nm) by the two algal. A: *Cladophora glomerata*. B: *Ulva fasciata*.

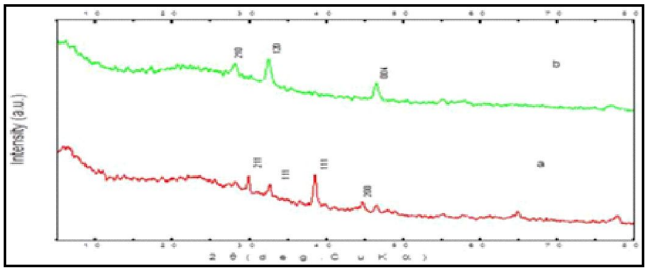

Fig. 3: XRD patterns of nanoparticles prepared using (a) *Ulva fasciata* and (b) *Cladophora glomerata*.

The Transmission Electron Microscope (TEM) observation of nanoparticles prepared using *Cladophora glomerata* was illustrated in Fig. 4. It is noticeable that the nanoparticles diameters were measured 9.8, 12.8, 13.3 and 13.6 nm Fig. 4A. The TEM observation of nanoparticles prepared using *U. fasciata* was illustrated in Fig. 4. The obtained nanoparticles were in spherical shape with diameter of 9.48, 12.83 and 13.01 nm Fig. 4B.

The Fourier-Transform Infra-Red (FTIR) spectra provided information about the local molecular environment of the organic molecules on the surface of nanoparticles. The results of FTIR analysis of the examined particles show different stretches of bonds located at different peaks; peaks (3425.69 and 3404.47); O–H stretch, H–bonded, alcohols and phenols. Peaks (2935.76 and 2933.83); C–H stretch, alkanes. The peak of (2546.12); O–H stretchcarboxylic acids and peak

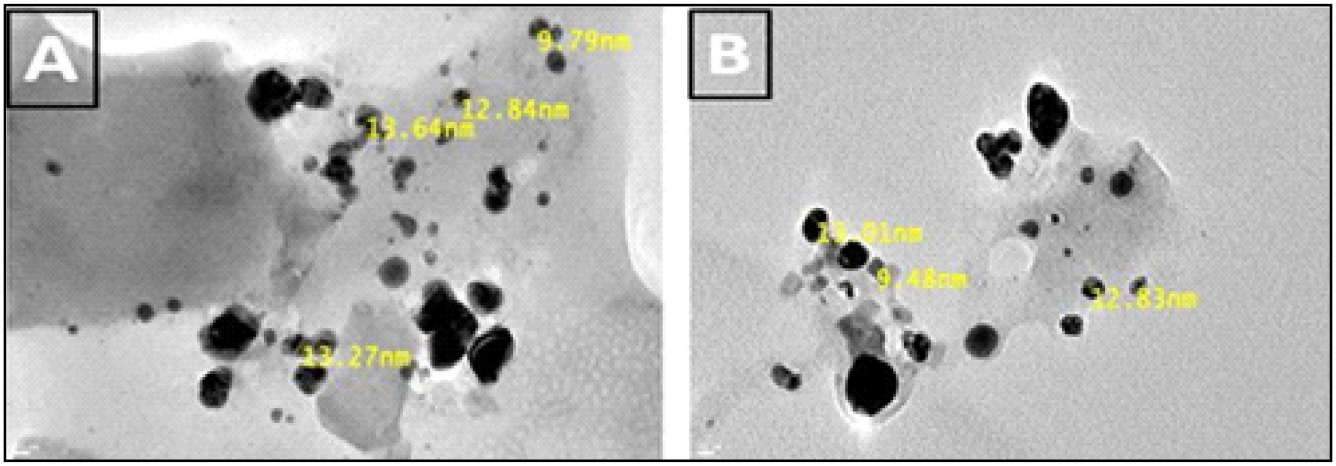

Fig. 4: TEM micrograph of biosynthesized nanoparticles; A: using *Cladophora glomerata* B: using *Ulva fasciata*.

(2150.7); –C≡C– stretch, alkynes. Peaks (1641.48 and 1647.26); – C=C– stretch, alkenes. Peaks (1413.87 and 1448.59); C–C stretch (in–ring), aromatics Fig. 5a. Peaks of (1263.42); C–N stretch, aromatic amines and peak (590.24); C–Br stretch, alkyl halides. Peaks (1055.10 and 1076.32); C–N stretch, aliphatic amines in Fig. 5b.

Bioassay evaluation of the two algal extracts and their prepared silver nanoparticles on *Meloidogyne incognita "invitro"*

The effects of *C. glomerata* and *U. fasciata* extracts and their prepared nanoparticles on egg hatch were recorded in table 1. Data showed that all treatments decreased *M. incognita* egg hatch by 83.3-100% compared to the negative control (MI alone), after 24 h and 48 h of exposure. However, 100% reduction was recorded after one and 2 weeks of exposure. Similar results were obtained with different nanoparticles concentrations (S, S/2, S/4 and S/10) or the nematicide (Nemaphose 40%) treatments. The effects of *C. glomerata* and *U. fasciata* algal extracts and the synthesized nanoparticles on J2 mortality % (M) of *M. incognita* (MI) after 24, 48 and a week was illustrated in table 2. Data indicated that all treatments increased mortality percentage % J2 of *M. incognita* by 80.9–100 % compared to the negative control, after 24 h and a week post treatment.

The efficacy of macroalgae species extracts and their biosynthesized silver nanoparticles against *M. incognita* (pot experiment-*invivo*)

Results tabulated in table 3 indicated the nematicidal effects of extracts both of *Cladophora* and *Ulva* algal, silver nanoparticles and Nemaphose 40% on the galls

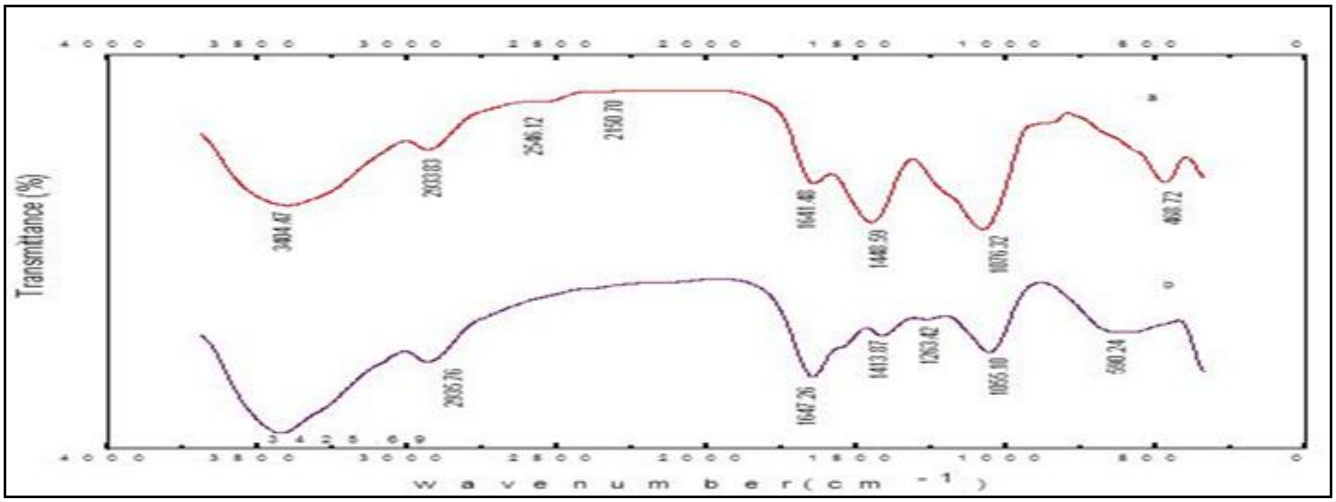

Fig. 5: FTIR spectra of nanoparticles prepared using (a) *Cladophora glomerata* and (b) *Ulva fasciata*.

Table 1: The effects of *Cladophora* (CEx) and *Ulva* (UEx) algal extracts and silver bionanoparticles (N) on egg hatch of *Meloidogyne incognita* (MI) after 24, 48, 1 and 2 weeks of exposure.

Treatments	Conc.	Exposure time, number of alive J2 (H) and reduction % (R)							
		24h		48h		1 week		2 weeks	
		H	R(%)	H	R(%)	H	R(%)	H	R(%)
(MI) (control)	-	1.2 a	-	6.4 a	-	15.8 a	-	30.2 a	-
MI+ Nemaphose 40%	-	0.2 b	83.3	0 b	100	0 b	100	0 b	100
MI+CEx	S	0 b	100	0.2 b	96.9	0 b	100	0b	100
MI+UEx	S	0 b	100	0.2 b	96.9	0 b	100	0 b	100
MI+NCEx	S	0 b	100	0 b	100	0 b	100	0 b	100
	S/2	0 b	100	0 b	100	0 b	100	0 b	100
	S/4	0 b	100	0 b	100	0 b	100	0 b	100
	S/10	0.2 b	83.3	0 b	100	0 b	100	0 b	100
MI+NUEx	S	0 b	100	0 b	100	0 b	100	0 b	100
	S/2	0 b	100	0 b	100	0 b	100	0 b	100
	S/4	0.2 b	83.3	0 b	100	0 b	100	0 b	100
	S/10	0 b	100	0.2 b	96.9	0 b	100	0 b	100

Data are means of 5 replicates, S = Full concentration, Values within a column followed by the same letter (s) are not significantly different at ($p \leq 0.05$).

numbers (G), the number of juveniles (J2)/200g soil, egg-masses (EM) in the infected tomato with *M. incognita* (MI) after 53 days post infection. It was observed that, algal extracts, nanoparticles and the nematicide treatments made a significant reduction in galls numbers and EM of *M. incognita* by 70.5–97.4 and 76.8-97 % in comparing with control plants, in respective manner. But, the number of eggs/ one egg mass was reduced only with S and S/2 treatments of NCEx by 63.5 and 46.6 % reduction, respectively and by 46.2 % reduction with S concentration of NUEx. However, data indicated that the numbers of J2 were reduced with all treatments by percentage ranged in between 56.4-87.2 except the treatment of *Ulva* extract (UEx) alone. The effects of *Cladophora* and *Ulva* algal extracts (S and S/2), the effect of Nemaphose 40% and silver nanoparticles on plants growth parameters of infected tomato by *Meloidogyne incognita* (MI) after 53 days post treatment were tabulated in table 4. Results indicated that silver nanoparticles S/2 of UEx treatment increased shoot fresh weight by 77.6%. While *C. glomerata* extract (CEx) showed that a high

Table 2: The effects of *Cladophora* and *Ulva* algal extracts and silver bionanoparticles on J2 mortality % (M) of *Meloidogyne incognita* (MI) after 24, 48 and a week of exposure.

Treatments	Conc.	Exposure time, number of alive J2 (L) and Mortality % (M)					
		24		48		week	
		L	*M*	L	*M*	L	*M*
(MI) (control)	-	4.2 a	-	4.2 a	-	4.2 a	-
MI+ Nemaphose 40%	-	0.6 b	85.7	0.2 bc	95.2	0.0 b	100
MI+CEx	S	0.6 b	85.7	0.6 b	85.7	0.4 b	90.5
MI+UEx	S	0.6 b	85.7	0.2 bc	95.2	0b	100
MI+NCEx	S	0.4 b	90.5	0c	100	0b	100
	S/2	0.6 b	85.7	0c	100	0b	100
	S/4	0.6 b	85.7	0.2 bc	95.2	0b	100
	S/10	0.8 b	80.9	0c	100	0b	100
MI+NUEx	S	0.19 b	95.2	0c	100	0b	100
	S/2	0.4 b	90.5	0c	100	0b	100
	S/4	0.8 b	80.9	0.2 bc	95.2	0.2 b	95.2
	S/10	0.8 b	80.9	0.4 bc	90.5	0.2 b	95.2

Data are averages of 5 replicates. Values within a column followed by the same letter(s) are not significantly different at ($p \leq 0.05$).

Table 3: The effect of *Cladophora*, *Ulva* algal extracts, silver nanoparticles and Nemaphose 40% on numbers of galls (G) and egg masses (EM)/tomato plant infected with *Meloidogyne incognita* (MI) and the number of juveniles (J2)/200 g of soil after 53 days.

Treatment	Con	Nematode parameters and reduction % (R)							
		G	R	EM	R	Eggs/EM	R	J_2/200	R
MI (control)	-	76.6 a	-	40.6 a	-	225.8 a	-	78.0 a	-
MI+ Nemaphos 40%	-	12.8 b	83.3	2.6 b	93.6	167.4 abc	-	34.0 bc	56.4
MI+CEx	S	9.2 b	88.0	2.2 b	94.6	203.6 ab	-	41.0 bc	47.4
MI+UEx	S	22.6 b	70.5	9.4 b	76.8	212.9 ab	-	47.0 ab	-
MI+NCEx	S	0.4 b	99.5	0.4 b	99	82.4 cd	63.5	10.0 cd	87.2
	S/2	2.0 b	97.4	0.6 b	98.5	120.6 bc	46.6	11.6 cd	85.1
MI+NUEx	S	0.6 b	99.2	0.6 b	98.5	121.2 bc	46.3	11.0 cd	86
	S/2	3.6 b	95.3	1.2 b	97	122.8 abc	-	16.0 bcd	79.5

Data are averages of 5 replicates. Values within a column followed by the same letter(s) are not significantly different at ($p \leq 0.05$).

Table 4: The effect of *Cladophora* sp., *Ulva fasciata* algal extracts, Nemaphose 40% and silver nanoparticles on some growth parameters of tomato plants infected with *Meloidogyne incognita* (MI) after 53 days.

Treatment	Con	Fresh weight(g)				Dry weight(g)			
		Shoot	I	Root	I	Shoot	I	Root	I
MI (Ve^+ control)	-	41.3 cd	-	14.5 c	-	8.7 c	-	2.5 a	-
MI + Nemaphose 40%	-	56.9 abcd	-	22.6 abc	-	12.9bc	-	4.2 a	-
MI + CEx.	S	57.5 abcd	-	20.7 abc	-	9.8 c	-	2.5 a	-
MI+UEx	S	43.4 c	-	24.4 abc	-	8.5 c	-	2.9 a	-
MI + NCEx.	S	73.4 a	77.6	27.1 ab	87	19.6 a	125.3	4.7 a	-
	S/2	61.8 abc	-	24.5 abc	-	15.8 ab	81.6	2.9 a	-
MI + NUEx.	S	64.4 ab	55.9	29.7 a	104.8	12.3 bc	-	5.5 a	-
	S/2	55.26 abcd	-	28.6 a	97.2	10.6 bc	-	5.2 a	-

Data are averages of 5 replicates. Values within a column followed by the same letter(s) are not significantly different at ($p \leq 0.05$).

decrease by (4.9%). Whereas results of the root fresh weights of tomato plants inoculated with *M. incognita* indicated that a high increase (104.8 %) with S concentration of nanosilver prepared from *Cladophora*. While the highest decrease of 42.8% was recorded with *Ulva* extract (UEx) in comparison to the positive control. Data using shoot dry weight clarified that nanosilver synthesized by *Ulva* (S/2) showed the highest yield (125.3%) whereas *Cladophora* extract show a decrease by (1.2%) in contrast with the positive control. On the other hand, data of using root dry weight indicated that the highest activity of the synthesized nanosilver by *Cladophora* (S and S/2) by 108-120 % while, *Ulva* and *Cladophora* extracts decrease by 0-16 %as compared with the positive control.

Real -Time PCR (qPCR)

The qPCR relative method was used in quantification statistical analysis which transformed the cycle of the threshold of PCR with the comparative level of gene expression. Magnitude patterns of chitinase gene at different intervals were examined using qPCR analysis. The highest expression pattern of the chitinase gene was obtained with the infected plants and treated with *C. glomerata* extract after 24 h from *M. incognita* inoculation compared with the control (MI alone) and Nemaphose 40% treatments, respectively Fig. 5. Similarly, the chitinase expression level was high in the infected tomato plants treated with *U. fasciata* extract after 6 h compared with plants treated with Nemaphose 40%.

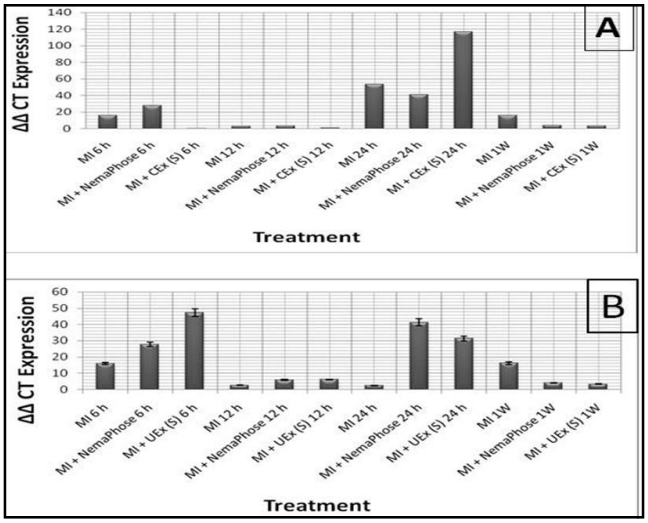

Fig. 6: ΔΔCt expression of chitinase gene in tomato plants infected with *M. incognita* and treated with *Cladophora* sp. extract (CEx) after 6, 12, 24 and 168 h (one week). B: ΔΔCt expression of chitinase gene in tomato plants infected with M. incognita and treated with Ulva fasciata extract (UEx) after 6, 12, 24 and 168 h (one week).

Further, the highest expression level of chitinase gene was noticed after 24 h in plants treated with *U fasciata* extract and Nemaphose 40% treatments, respectively compared with MI treatment. Whenever, chitinase gene was expressed in the low level of (< 6) in treated tomato with *U. fasciata* extract or Nemaphose 40% after 12 h after root-knot nematode inoculation Fig. 6.

Discussion

Biosynthesized nanoparticles have good characters such as; stability and biodegradability. It can be used in delivering pesticides, fertilizers and other agrochemicals, because they are smaller in size and have a charged large surface area (Singh *et al.,* 2012, Kannan *et al.,* 2013). Plants and microbes can be used in biosynthesized nanoparticles and the resulted in effective products with low cost (Chowdappa and Gowda, 2013).

The preliminary identification of pure collected algae based on light microscopic images and identification was performed on species level and it proved that the freshwater alga was *Cladophora* sp. and the marine alga was *U. fasciata*. The bio-synthesis of AgNps was confirmed by measuring the color changes reflected in the raised $O.D._{450}$ reading along with the experiment. Our observation was similar to that shown by Sharma *et al.,* (2015) who reported that the synthesis of AgNps was confirmed by visualizing the color change which shows surface plasmon resonance (SPR) peak at 425 nm. TEM observation of nanoparticles prepared using *Cladophora* sp. showed that the nanoparticles had diameters of 9.79, 12.84, 13.27 and 13.64 nm. The TEM observations of nanoparticles prepared using *U. fasciata* were in spherical shape with diameter of 9.48, 12.83 and 13.01 nm. Similarly, biosynthesis zinc oxide nanoparticles via the leaf extracts of the two plants *Pithecellobium dulce* and *Lagenaria siceraria* showed anti-bacterial effect according to (Prakash and Kalyanasundharam, 2015, Chung *et al.,* 2016). Sharma *et al.,* (2015) investigated the antimicrobial and nematode cytotoxic potential of silver nanoparticles synthesized by greener route using *Rheumemodi* root extract. The use of marine algae as control agents against plant-parasitic nematodes has been studied by many workers (Elsherbieny, 1995, Mansoor *et al.,* 2007). It well known that seaweed extracts are capable to increase plant resistance against a wide range of pathogens especially soil pathogens (Chung *et al.,* 2016). The physical and chemical properties of soil have the potential effect in controlling plant pathogen via different mechanisms such as organic matter which produce antimicrobial compounds during decomposition (Tenuta and Lazarovits, 2002) and

biological (Mazzola, 2004). The effect of *Cladophora* and *Ulva* algal extracts and silver nanoparticles on J2 mortality % of *M. incognita* indicated that all treatments increased J2 mortalityby 80.9–100 % compared to the negative control (MI alone). Studying the effect of *C. glomerata* and *U. fasciata* algal extracts, silver nanoparticles prepared from *C. glomerata, U. fasciata* extracts and Nemaphose 40% on the enhancement] the growth of tomato plants infected with MI and indicated that silver nanoparticles prepared from only "S" concentration of *Cladophora* and *Ulva* increased shoot fresh weight by 55.9-77.6 %. Also, root fresh weights of tomato plants were increased in plants inoculated with MI and treated with S concentration of nanosilver prepared from *C. glomerata* by 87%. While, the highest activity (104.8 and 97.2%) was achieved by S and S/2 concentrations of *Ulva* extracts. as compared to the positive control. Similarly, shoot dry weights were increased with only nano silver treatments of *Cladophora* by 81.6-125.3% compared to the positive control, these results are in agreement with that postulate by (Ehteshamul-Haque *et al.,* 2013; El-Ansary and Hamouda., 2014). Seaweeds contain many different ingredients that could be used as potential agent against the plant-parasitic nematodes (Sultana *et al.,* 2008, 2009). The use of brown algae, *Sargassum* spp., showed significant nematicide ($p<.05$) (Ara *et al.,* 1996) and *Meloidogyne* infected okra plant (Ara *et al.,* 1997; Tariq *et al.,* 2011).

Chitinase gene was highly expressed in the infected plants treated with *Cladophora* sp. extract after 24 h from *M. incognita* inoculation compared with the control (MI alone) and Nemaphose 40% treatments. Similarly, the expression altitude of chitinase gene was high in the infected and treated plants with *U. fasciata* extract after 6 h from nematode inoculation compared with the expression in plants treated with Nemaphose 40% or control treatments. Moreover, high expression of chitinase gene was noticed after 24 h in plants treated with *U. fasciata* extract or Nemaphose 40% treatments compared with MI alone treatment. The highest expression levels of chitinase gene were observed in the *M. incognita* infected plants and treated with S concentration (100%) of the biosynthesized nanosilver particles prepared from *Cladophora* extract after 6 and 24 h compared with either Nemaphose 40% or MI treatments. Then, the expression level increased to about 1.35- 6 folds after one week from nematode inoculation in plants treated with S concentration of the nanosilver particles prepared from *Cladophora* extract as compared with Nemaphose 40% or control treatments. Similarly, but with lower quantity than that recorded with nanoparticles prepared from *Cladophora* extract, high expression level of chitinase was obtained only after 6 h from nematode inoculation in the infected plants treated with the biosynthesized nanosilver prepared from S concentration of *Ulva* extract compared to that recorded in plants inoculated with nematode alone (control treatment). Chitinase have been known to be expressed in plant by pathogen infection or other biotic and abiotic factors (Mauch *et al.,* 1988). Chitinase enzyme is able to degrade chitin in plant attackers especially fungi because their cell walls contain high of chitin also the nematode eggs and egg masses (Neuhaus *et al.,* 1996, Ghareeb *et al.,* 2019). Jongedijk *et al.,* (1995) reported that the genes of chitinase, have been transferred to many plants species alone or together with β-1,3-glucanase genes and expressed into plants, its effect was studied against different plant pathogens. Various studies have shown that chitinase expression against phyto-pathogen systems is higher and induction is stronger in the resistant varieties in comparison to susceptible varieties in the sugar beet (Nielsen *et al.,* 1993), wheat (Anguelova *et al.,* 2001) and tomato varieties (Lawrence *et al.,* 2000, McFadden *et al.,* 2001).

Conclusion

This study conveys that the use of *Ulva fasciata* and *Cladophora glomerata* algal extracts and their synthesized nanoparticles may be used as nematicide bioagent against *Meloidogyne incognita*.

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