Chemically-Produced Copper, Zinc Nanoparticles and Chitosan–Bimetallic Nanocomposites and Their Antifungal Activity against Three Phytopathogenic Fungi

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Abstract In the present study, metallic nanoparticles and bimetallic nanocompsites were synthesized and characterized using different physico-chemical methods. The average particles size and the distribution size were characterized by dynamic light scattering (DLS), and the morphology and structure of nanoparticles were investigated using transmission electron microscopy (TEM). An average hydrodynamic size for chemically-produced nanoparticles is 1.7-23.7 nm**,** a hydrodynamic size distribution value is near to calculated from TEM value**.** Antifungal activities of copper nanoparticles (Cu NPs), zinc oxide nanoparticles (ZnNPs) and chitosan, zinc oxide and copper nanocompsites (CS–Zn-Cu NCs) against three plant pathogenic fungi including *A. alternata, R. solani* and *B. cinerea*, were investigated in this study. The *In-vitro* application showed that CS–Zn-CuNCs at 90 µg ml^{-1}has the highest antifungal activity since it inhibited the growth of *B. cinerea* with inhibition zone 1.7cm compared to the other two concentrations and also the other nanoparticles. Zn NPs at 90 µg ml^{-1} significantly inhibited the growth of *R. solani* and the inhibition zone was 1.6 cm compared to concentrations of 30 and 60 µg ml^{-1}. CuNPs at 90 µg ml^{-1} inhibited the growth of *A. alternate* to 1.4 cm. The *in-vivo* application showed that Zn NPs at concentrations of 60 and 90 µg ml^{-1} against *R. solani* increased the percentages of survived seedlings to 85% and 86%, respectively, compared to 43.5% persisted seedlings in the concentration of 30 µg ml^{-1}. While Cu NPs at concentrations of 60 and 90 µg ml^{-1} significantly inhibited the growth of *A. alternate* to 5.20 mm and 4.95 mm, respectively, compared to 12.3mm of fungal growth when 30 µg ml^{-1} applied. CS–ZnO-Cu NCsat concentrations of 90 and 60µg ml^{-1}decreased the percentages of rotten strawberry fruits to 15±6.1 and 17±4.8, respectively, compared to19.0±5.6 when 30 µg ml^{-1}applied.

Keywords: Chitosan nanoparticles, Zn nanoparticles, Cu nanoparticles, nancomposites, Antifungal activities

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Introduction

Fungal pathogens are the main cause of significant economic loss during the growth of the crop and postharvest handling of fruits. *R. solani*, *A. alternata* and *Botrytis cinerea* can cause severe damage for number of economic crops either in the field or postharvest fruit diseases even when the most advanced preharvest and postharvest technologies are applied (Spadaro *et al*., 2004). Many recent research studies have already demonstrated antimicrobial activities of various nanoparticles such as silver (Kim *et al*., 2008; Kumar e*t al*., 2008), copper (Cioffi *et al*., 2005, Youssef *et al.,* 2017), chitosan (Xing *et al*., 2011) and zinc oxide (Liu *et al*., 2009). Copper nanoparticles had a high activity against Gram-positive bacteria, standard and clinical strains, including methicillin-resistant *Staphylococcus aureus*, comparable to silver nanoparticles and some antibiotics (Kruk *et al*., 2015). They also exhibited antifungal activity against *Candida* species. Also, the foliar applications of CuO NPs limit growth of the oomycete *Phytopthora* (Giannousi *et al*., 2013). Zinc oxide (ZnO) and magnesium oxide (MgO) nanoparticles at different concentrations brought about significant inhibition in the germination of spores of *A. alternata, F. oxysporum, R. stolonifer* and *Mucor plumbeus* (Wani and Shah, 2012). Zinc oxide (ZnO) and magnesium oxide (MgO) nanoparticles have been proposed as an anti-microbial preservative for wood or food products (Aruoja *et al*., 2009; Huang *et al*., 2006; Sharma *et al*., 2009). ZnO NPs toward plant pathogenic fungi including *Penicillium expansum* and *B. cinerea* was demonstrated in recent papers (He *et al.*, 2011). Also, it demonstrated against Aspergillus isolate (Jayaseelan *et al*. 2012). Dimkpa *et al*., 2013 reported that ZnO NPs are toxic to the wheat pathogen, *F. graminearum* both in medium and in a solid sand matrix. The combined copper-chitosan colloids are used as a new generation of copper-based bio-pesticides (Brunel *et al.,* 2013). Chitosan-based copper nanomaterials have been used as antifungal, antibacterial as well as plant growth promoting agents (Hardy *et al.,* 2004; Jaiswal *et al.,* 2012, Saharan *et al.,* 2013 and 2015). The aim of this work was to synthesize and characterize three types of nano-agrochemicals, also to determine theantifungal their activities against three plant pathogenic fungi *A. alternata, R. solani* and *B. cinerea.*

Material and methods

Chemicals

Copper sulfate (Purity 98%, M. wt. 249.68) was purchased from El-Nasr Pharmaceutical Co. for Chemicals (Egypt). Polyethylene glycol 6000

$(H(OCH_2CH_2)_nOH$- Sigma-Aldrich) was used as the capping agent. Sodium borohydride (NaBH4, Sigma-Aldrich) was used as the main reducing agent, while ascorbic acid ($C_6H_8O_6$, Sigma-Aldrich) was used as the antioxidant of colloidal copper. Sodium hydroxide (NaOH, Sigma-Aldrich) was used to adjust the pH.

Preparation of Chitosan nanoparticles

Chitosan (0.25g) was dissolved in 2% acetic acid, while stirring followed by sonication for 15 min. This was then filtered to obtain a clear solution. 20% Na_2SO_4 was then added drop wise to the chitosan solution, which was simultaneously stirred and sonicated. The solution was left undisturbed, centrifuged at 8,000 rpm to collect the CS nanoparticles.

Synthesis of Cu-based NPs

CuNPs were synthesized by reduction of copper sulphate with isopropyl alcohol (IPA) in the presence of the cationic surfactant CTAB following Athawale *et al.,* (2005) technique with slight modification. Copper (II) sulfate nanoparticles were synthesis according to the method of Kanhed *et al*. (2014) with minor modification. Firstly, 0.0030 M copper (II) sulfate and 0.09 M of CTAB were prepared in IPA. Reaction was carried out in clean dry 250 mL Erlenmeyer flasks open to air. Copper (II) sulfate was added drop-wise to CTAB/ (IPA) solution. The reaction mixture was stirred vigorously on a magnetic stir plate. IPA was used as a reducing agent in the synthesis of CuNPs. CTAB molecules catalyze the reduction of Cu^{2+} ions to Cu^0 with IPA. CTAB acts as a capping agent by surrounding the surface of CuNPs.

Synthesis of ZnO Nanoparticle

ZnO nanoparticle was prepared by conventional precipitation method with NaOH as precipitation agent. 0.4mg NaOH was added drop wise continually to aqueous 5.75mg Zinc sulphate solution in the molar ratio 1:2 with continuous stirring. The resulting slurry was continuously stirred for 24 h and precipitate obtained was filtered, washed thoroughly with distilled H_2O. The washing procedure was repeated several times and then collected residue was dried in oven at 100ºC for 12h grinned to fine powder.

Synthesis of Chitosan–bimetallic nanocomposites

For the synthesis of CS–Zn-Cu nanocomposite, 0.5g of chitosan was dissolved in 2% acetic acid. 20% Na2SO4 solution was added drop wise to the above solution under simultaneous stirring and sonication. 0.1g of synthesized ZnO and CuO nanoparticles were added to the solution which was stirred under sonication. The suspension was centrifuged and washed several times. Finally, the nanocomposite was collected and dried at 60 °C.

Characterization of NPs

Dynamic light scattering (DLS)

Measurement of Cu NPs distribution and size was performed by a dynamic light scattering method using Zetasizer Nano ZS (Malvern Instruments, UK) at room temperature. Prior to measurement, 30µl of the nanoparticle was diluted with 3ml of water at 25°C. Particle size data were expressed as the mean of the Z-average of 3 independent batches of the nanoparticles.

Transmission electron microscopy (TEM)

Twenty microliters of diluted samples was placed on a film-coated 200-mesh copper specimen grid for 10 min and the fluid excess was eliminated using filter paper. The grid was then stained with one drop of 3% phosphor tungstic acid (PTA) and allowed to dry for 3 min. The coated grid was dried and examined under the TEM microscope (Philips, CM 12). Then the samples were observed by operating at 120kV.

The effect of three types of NPs in vitro against R. solani, A. alternata and B. cinerea

The effect of three types of NPs on the mycelial growth *R. solani, A. alternata* and *B. cinerea* was evaluated. Agar plugs (5mm in diam.) containing mycelia from the growing edge of 1-week-old cultures of *R. solani, A. alternata* and *B. cinerea* were placed in the center of Petri dishes containing PDA. Three different concentrations (30, 60 and 90µg mL^{-1}) were used, three Petri dishes were utilized as replicates and the entire experiment was repeated twice. The plates were incubated at 23±1 °C for one week. Colony diameter (mm) for each fungus was calculated as the average of the longest and the shortest diameter. Non-amended PDA served as a control while copper sulfate was used as raw material for comparison.

Fungal pathogen suspension

To produce fungal inoculums for *A. alternata* or *B. cinerea*, isolates were grown on PDA plates at 24°C in the dark. Conidia were collected from two week-old plates by scraping them with a sterile spatula, and then suspended in sterile distilled water containing 0.05% Tween 80 (v/v). The resulting suspension was filtered through two layers of sterile gauze. The spore counts were made by a Thoma counting chamber (HGB Henneberg-Sander GmbH, Lutzellinden, Germany) and the suspension was diluted with sterile distilled water to obtain a final concentration of 10^4 conidia ml^{-1}.

Indirect antifungal activity of Cu NPs and Cu-Zn-Chitosan NPs

Tomato or strawberry fruits were wounded once (5 mm depth × 3 mm wide) with a sterile nail-head along the equatorial axis. For each treatment, 30 μl of copper nanoparticles solution were applied into each wound. Treated fruit were placed in a tray and individually wrapped into a plastic bag. After 48 hour of incubation at 20±1°C and high relative humidity, another wound was made approximately 5 mm apart from the previous one. This wound was air-dried and inoculated with 10 μl of a 10^4 conidia ml^{-1} suspension of *A. alternata* or *B. cinerea* (Youssef *et al.,* 2014). The control consisted of fruits treated in the first wound with sterile distilled water and then inoculated with the pathogen conidial suspension in the other one. Each treatment was replicated 3 times and each replicate consisted of 5 fruits. Replicates were individually wrapped in a plastic bag and incubated at 20 ±1°C and high relative humidity for two weeks. The percentage of infected fruit (rot incidence, %) and decay lesions diameter (mm) was recorded for each fungal pathogen.

Effect of seed dressing treatment with ZnNPs on R. solani in greenhouse

Fifty seeds were surface sterilized by gently shaking in 1% sodium hypchlorite solution for 3 min and rinsed six times for 5 min in sterile deionized water. Seeds were soaked in 30, 60, and 90μg for about 30 min. Infested soil was dispensed in 15 cm diameter clay pots and each pot was planted with 10 seeds. Pots were distributed on greenhouse benches in a randomized complete block design. The greenhouse was equipped with a heating system assuring that the minimum temperature in the greenhouse was maintained at 28°C. The test was repeated once with almost the same results. Percentage of infected seedlings was recorded 45 days from sowing.

Fragmentation of fungal genomic DNA

Fungal mycelium was produced in 20ml of liquid medium (24gl^{-1} of potato dextrose broth [PDB, Difco Laboratories]. Mycelium was harvested by filtration through mesh sieves (40 μm), washed with sterile water, and deposited onto Whatman paper no. 1 to remove excess water. Mycelium was ground to a fine powder in liquid nitrogen using a mortar and DNA was extracted by the method of Moslem *et al.,* (2010). Five micrograms of fungal DNA for *A. alternate*, *R. solani* and *B. cinerea* was treated with NPs and NCs (60μg ml^{-1}). Seven microliters of the isolated DNA and 3 μl of 10X loading dye were loaded in a lane of 1.5% (w/v) agarose gel containing 0.05 μgml^{-1} ethidium bromide, to check the quality of the DNA. For quantitative measurements, a charge-coupled device camera imaging system and UVIsoft analysis (Gel Documentation and Analysis Systems, Uvitec, Cambridge, UK) were used to capture the image.

Results

Nanomaterials Characterization

Dynamic light scattering (DLS)

Dynamic light scattering technique was performed to understand the size distribution and the stability of prepared CuNPs, ZnNPs and CS NPs have a size distribution range mainly within 1.6–23 nm, 1.5- 20 nm, and 1.9-28nm, respectively as shown in Fig. 1. An average hydrodynamic size for chemically-produced nanoparticles is 1.7-23.7nm**;**a hydrodynamic size distribution value is near to calculated from TEM value**.**

Transmission electron microscopy (TEM)

TEM characterization of three nanoparticles used in this study ranging from 6-100nm. The results showed that the biggest size was Cu NPs which was in the range of 50-100nm, the smallest size was ZnNPs which was in the range of 6-21 nm, while the size of CS NPs was in the range of 16.6-24.3nm (Fig. 2).
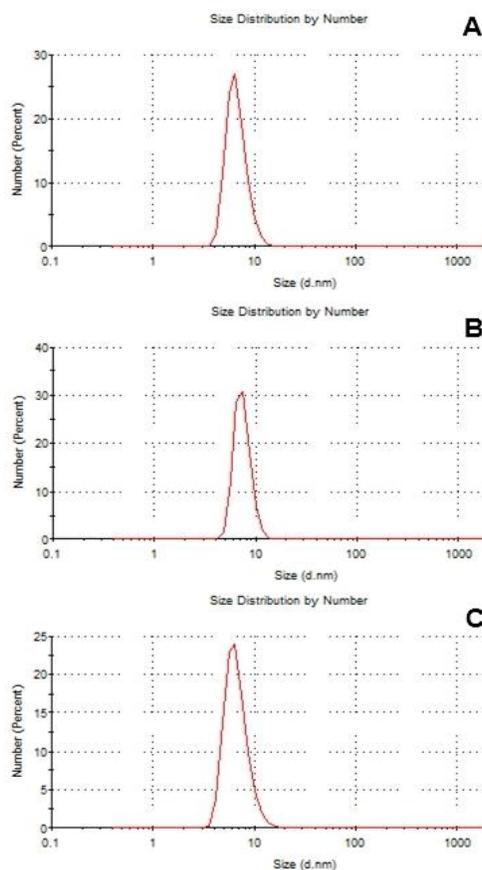
Figure 1. Dynamic light scattering spectroscopy for size distribution of (**A**) CuNPs, (**B**) ZnNPs and (**C**) CS NPs.
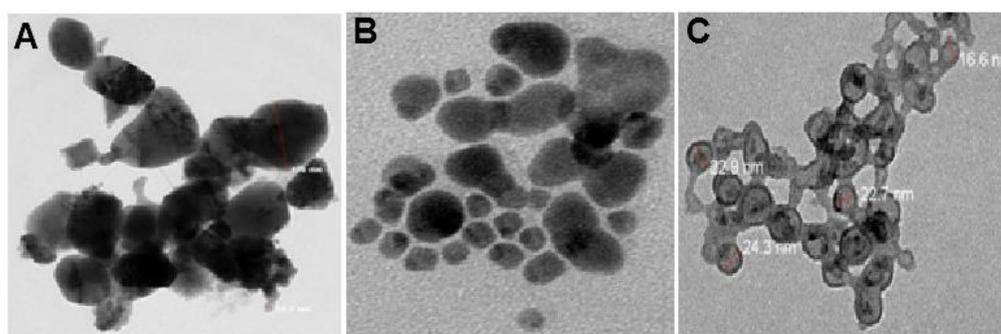
Figure 2. (**A**) Transmission electron microscopic image of CuNPs ranging from 50-100 nm, (**B**) Zn NPs, ranging from 6-21 nm, (**C**) CSNPs, ranging from 16.6-24.3 nm.

Antifungal activity of nanomaterials in-vitro

$A.$ $alternate$ showed lower resistance in response to 90µg ml^{-1} of Cu NPs which appeared as 1.4cm of inhibition zone compared to 0.8cm, 0.3cm and 0.0 cm of inhibition zone in response to 60 µg ml^{-1}, 30 µg ml^{-1}and control, respectively (Fig 3**A**). ZnNPs with concentration 90 µg ml^{-1} significantly inhibited the growth of $R.$ $solani$ and the inhibition zone was 1.6 cm, while it was 0.9 cm, 0.5 cm and 0.0 cm when the concentrations of 60 µg ml^{-1}, 30 µg ml^{-1} and 0 µg ml^{-1} were used, respectively (Fig.3**B**).The inhibition zone at 90 µg ml^{-1}against $B.$ $cinerea$was1.7 cm, while it was 0.8 cm, 0.3cm and 0.0 cm when the concentrations of 60µg ml^{-1}, 30µg ml^{-1} and 0µg ml^{-1} were used, respectively (Fig.3**C**). CS–Zn-CuNPs has the highest antifungal activity compared to the other nanomaterials (Fig. 4).
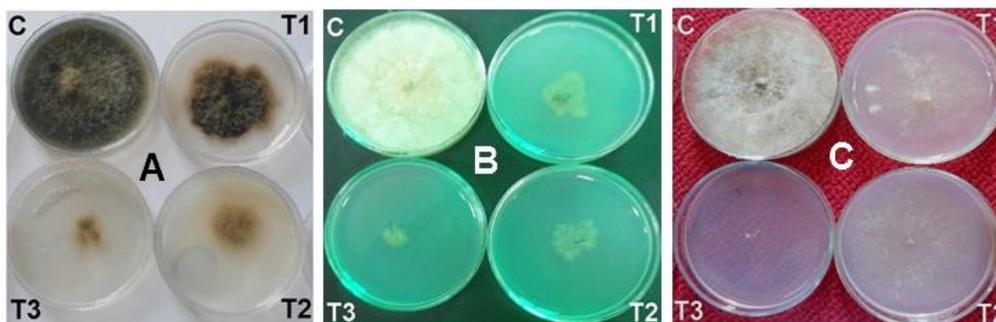
Figure 3. Fungicidal activity of (**A**) Cu NPs against $A.$ $alternate$ (**B**) ZnNPs against $R.$ $solani$ and (**C**) CS–Zn-Cu NCs against $B.$ $cinerea$, using three concentrations T1=30, T2=60, T3=90 µg ml^{-1}, C=control.
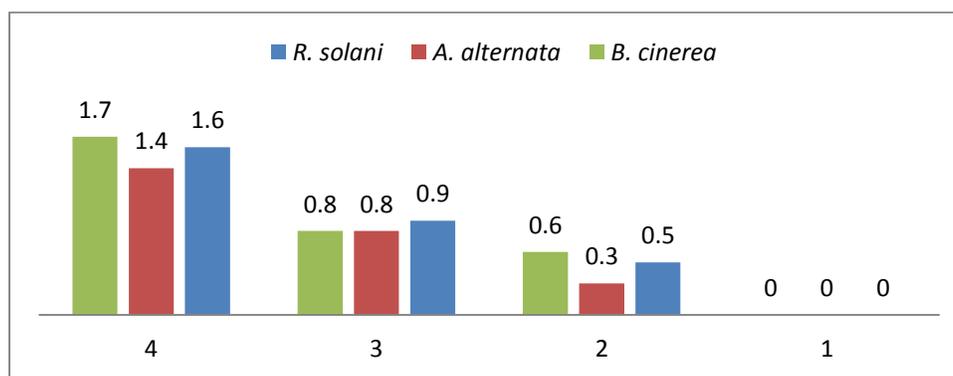
Figure 4. Fungicidal activity of ZnNPs against $R.$ $solani$, Cu NPs against $A.$ $alternata$, and CS–Zn-Cu NPs against $B.$ $cinerea$ using three concentrations 30, 60, 90 µg ml^{-1}.

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Indirect antifungal activity of CuNPs on tomato fruit rot caused by A. alternate

Antifungal activity of CuNPs (50-100nm) (Fig. 5) on tomato fruits rot caused by *A. alternate* increased gradually three weeks after treatment. The lesion diameter was 5 mm, 8.88 mm and 12.30 mm, one week, two weeks and three weeks respectively after treatment with 30µg ml^{-1}CuNPs compared to control. Tomato fruits, which were treated with *A. alternate* followed by injecting 60 µg ml^{-1}CuNPs showed lower diameter of lesions 2.6 mm, 2.84 mm and 5.2 mm, after one week, two weeks and three weeks respectively compared to control and 30 µg ml^{-1}CuNPs (Table 2). Antifungal activity of 90 µg ml-1 CuNPs increased to maximum level and the lesion diameter of tomato rot was 2mm, 2.34 mm and 4.95 mm (Fig. 5), one week, two weeks and three weeks respectively after treatment compared to control and other two concentrations of CuNPs (Table 2). Injecteing tomato fruits with 90µg ml^{-1}CuNPs before infecting the fruits with the suspension of *A. alternate*showed very little growth of the fungus after incubation for one week followed by very small symptoms. While Injected tomato fruits with *A. alternata* suspension in a standard incubation conditions showed a very typical after one week from the infection.

Table 2. Effect of CuNPs on tomato fruit rot incidence caused by *A. alternata* treated with three concentrations of CuNPs.

Treatments	Lesion diameter after (mm)		
	One weak	Two weaks	Three weaks
NPs -T1	5.00	8.88	12.30
NPs-T2	2.60	2.84	5.20
NPs-T3	2.00	2.34	4.95
Control	7.00	11.0	16.00

*Control = fruits treated with sterlized water, T1=30, T2=60, T3=90 µg ml^{-1}

Figure 5. Scheme of application of CuNPs and *A. alternata* suspension on tomato wounds using indirect antifungal activity (C=control treated with sterlized water, T= treated with 90 µg ml^{-1}Cu NPs) and incubated at 20°C for one week.

Effects of seed dressing treatment with ZnNPs on R. solani in greenhouse

ZnNPs (6-21 nm) (Fig. 4) antifungal activity against *R. solani* funguswere measured in the greenhouse in the coated cotton seeds (Cultivar Giza 90) with different concentration of ZnNPs. Antifungal activity of 30µg ml^{-1} against *R. solani* showed that the percentage of survived cotton seedling was 43.5% compared to 5.0% and 93.7% in the control-1 (infested soil) and control-2(autoclaved soil), repectively. Also the percentage of pre-emegence damping-off was 30.5% compared to 42.8% and 6.3% in control-1 and control-2, respectivly. The percentage of post-emergence damping-off was 26.0% compared to 52.2% and 0.0% in the control-1 and control-2 respectively. Percentage of survived cotton seddlings increased to 85.0% in the coated seeds with 60µg ml^{-1} of compared to 5.0% and 93.7% in control-1 and control-2 respectively. Also, the percentage of pre-emergence damping of decreased to 3.8% compared to 42.8% in control-1 and 6.3% in control-2.While the percentage of post-emergence damping-off in the coated seeds with 60µg ml^{-1} of was 11.2% compared to 52.2% in control-1 and 0.0% in control-2. The antifungal activity of 90 µg ml^{-1} of ZnNPs against *R. solani* increased to the maximum and reached to 86.0% of survived seedlings compared to 5.0% in control-1 and 93.7% control-2. Additionally, the percentage of post-emergence damping-off decreased to 6.4% in the coated cotton seeds with 90µg ml^{-1} of ZnNPs compared to 52.2% and 0.0% in control-1 and control-2, respectively. Interestingly, the percentage of pre-emergence damping-off in the coated cotton seeds with 90 µg ml^{-1} of ZnNPs was 7.6% compared to 3.8% in the concentration 60 µg ml^{-1} of ZnNPs (Table 3).

Table 3. Effect of ZnNPs on pre, post-emergence and survival of cotton seedlings (Cultivar Giza 90) caused by *R. solani* under greenhouse conditions

Treatments	Pre-emergence damping-off	Post-emergence damping-off	Survival
NPs -T1	30.5%	26.0%	43.5%
NPs-T2	3.8%	11.2%	85.0%
NPs-T3	7.6%	6.4%	86.0%
Control-1	42.8%	52.2%	5.0%
Control-2	6.3%	0.0%	93.7%

*Control-1 is infested soil, and Control-2 is autoclaved soil, T1=30, T2=60, T3=90 µg ml^{-1}

Indirect antifungal activity of CS–Zn-CuNPs on gray mold caused by B. cinerea

Strawberry fruits was treated with *B. cinerea* followed by injecting wounded parts with 30µl of 30µg ml^{-1} of CS–Zn-CuNPs (16.6-24.3 nm) (Fig.

4) showed gradual increase in the percentage of rotten fruits, 8.0±3.4%, 15.0±6.2 % and 19.0±5.6 % after one week, two weeks and three weeks respectively compared to control. While, antifungal activity of 60μg ml-1 of CS–Zn-Cu NPs against *B. cinerea* decreased the percentage of rotten strawberry fruits to 6±3.6%, 13±6.0% and 17±4.8% one week, two weeks and three weeks after treatment respectively in comparison to control and 30 μg ml-1 NPs (Table 1). Percentage of rotten strawberry fruits have been decreased to 5±4.0%, 13±6.0% and 17±4.8% one week, two weeks and three weeks respectively, after treatment with 90 μg ml-1 of CS–Zn-Cu NPs compared to control and other two concentrations (Table 4).

Table 4. Effects of combined CS–Zn-CuNCs on gray mold caused by *B. cinerea* on strawberry after treatment with three concentrations of NPs.

Treatments	% rotten strawberry fruits		
	One weak	Two weaks	Three weaks
NPs -T1	8.0±3.4	15.0±6.2	19.0±5.6
NPs-T2	6±3.6	13±6.0	17±4.8
NPs-T3	5±4.0	11±4.2	15±6.1
Control	25±6.0	23±5.6	21±7.6

*Control = fruits treated with sterlized water, T1=30, T2=60, T3=90 μg ml^{-1}

Genotoxic potential of the nano-sized copper

The synthesized nanoparticles and nancomposites were incubated with *A. alternate*, *R. solani* and *B. cinerea* DNA and studied for their DNA binding activity to explore the molecular basis of their antifungal activities. The genotoxicity exhibited by metallic nanoparticles was demonstrated by degradation of fungal DNA post-treatment even with concentration 60 μg ml^{-1} of the CuNPs, Zn NPs and Cu-Zn-chit-NCs, respectively. DNA strand scission induced by CuNPs leads to gradual degradation in the amount of linear DNA. Smear bands were appeared of lower in the gel which are the resultant fragmented DNA (Fig. 6).
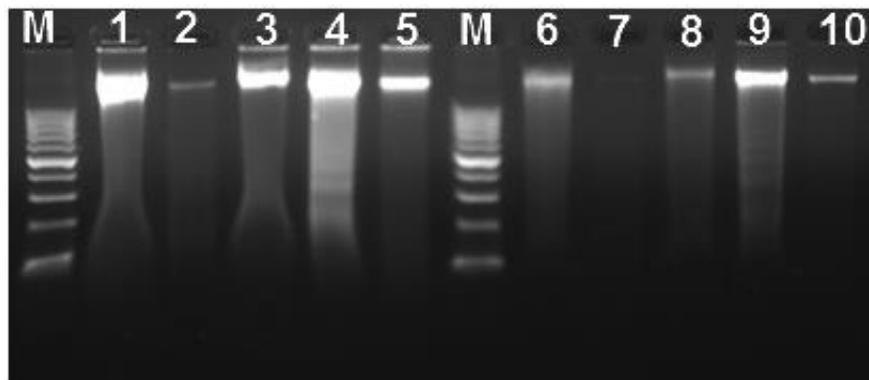
Figure 6. Dose-dependent fungal DNA degradation action of NPs and NCs in agarose gel electrophoresis. Lanes 1, 3: DNA for untreated *A. alternate*, *R. solani* and *B. cinerea* respectively, Lanes 4, 5: DNA for untreated fungal control. Lane 6: *A. alternate* treated with Cu NPs, Lane 7: *R.solani* treated with Zn NPs, Lane 8: *B. cinerea* treated with Cu-Zn- chit-NCs (60µg ml^{-1}), Lane 9-10: fungal DNA control treated with treated with Cu-Zn-chit-NCs (90µg ml^{-1}) showing gradual degradation of the fragmented DNA bands.

Discussion

Nanobiotechnology is playing an important role in the management of plant diseases. New characteristics of the combined nanostructures could improve the antifungal activity against different plant pathogens. Nanostructures in the new shape have incomparable physicochemical, biological and optical characteristics, and most of them have antimicrobial activity. Also development new nanocmposites could enhance the antifungal activities and induce the resistance in the host against different plant pathogenic organisms (Xu *et al.,* 2007). It has been testified that nanoparticles of Zn, Cu and chitosan exhibit a wide spectrum of antimicrobial activity against different bacterial (Chatterjee *et al*., 2012) and fungal species (Ouda, 2014). In this proof-of-concept manuscript, we developed and characterized a copper, zinc nanoparticles and chitosan–bimetallic nanocomposites. Also, we demonstrate their antifungal activity against *A. alternate*, *R. solani* and *B. cinerea* on tomato, cotton and strawberry respectively. An average hydrodynamic size for chemically-produced nanoparticles is 1.7-23.7 nm**,** a hydrodynamic size distribution value is near to calculated from TEM value**.** Transmission electron microscopic image of CuNPs ranging from 50-100nm, ZnNPs, ranging from 6-21nm, CS NPs, ranging from 16.6-24.3nm.The TEM of ZnNPs gives the actual size and shape and the analysis revealed the formation of spherical ZnNPs

measuring about 21 nm in diameter, uniformly distributed without any significant aggregation. Particle size and zeta potential are the important properties which may influence the antifungal activity of nanoparticles. Nanoparticles with different particle size or zeta potential may have different mechanisms of inhibition against fungi.

A. alternate showed lower resistance in response to 90 µg ml^{-1} of Cu NPs which appeared as 1.4 cm of inhibition zone compared to 0.8 cm, 0.3 cm and 0.0 cm of inhibition zone in response to 60 µg ml^{-1}, 30 µg ml^{-1}, 0.0 µg ml^{-1} of Cu NPs respectively. CuNPs have been demonstrated previously to suppress vegetative growth of some fungal species such as *A. flavus, A. niger, A. alternata, F. solani, P. chrysogenum* and *Candida albicans.*

In vitro studies showed that the antifungal activities of Zn NPs in the concentration of 90 µg ml^{-1}reached the highest activities against *R. solani* compared to 30 and 60 µg ml^{-1}of Zn NPs and the control. Zn NPs showed great enhancement in the antimicrobial activity due to their unique properties such as large surface area. However, Kasemets *et al*. (2009) found nano and bulk ZnO were of comparable toxicity against *S. cerevisiae*. Number of studies proposed that ZnNPs may cause structural modifications of microbial cell membrane, causing cytoplasm outflow and ultimately the death of bacterial cells (Brayner *et al*., 2006). *In vitro* studies on Cu-chitosan NCs proved their uniform size and stability, which may contribute to their higher antifungal activity against number of pathogenic fungi like, *A. alternata*, *M. phaseolina* and *R. solani.* Also, Cu-chitosan NPs showed maximum inhibition rate of spore germination of *A. alternate* (Saharan *et al*., 2013).

Also, current research study showed that application of 90 µg ml^{-1}CS–Zn-Cu nanocomposites (ranging from 16.6-24.3 nm) on strawberry fruits decreased the percentage of rotten strawberry fruits to 5±4.0%, 13±6.0% and 17±4.8% one week, two weeks and three weeks respectively. Application 60µg ml^{-1}CS–Zn-CuNPs NCs on strawberry fruits showed antifungal activities against *B. cinerea* but not as significant as the highest concentration of CS–Zn-Cu nanomaterials. Chitosan, zinc oxide and copper nanomaterials, each one of them has its unique and distinctive characters and mechanism as antimicrobial agent against wide range of microorganisms.

This new combinations of CS–Zn-CuNPs nanocomposites increased the antifungal activities against *B. cinerea* and increased the shelve life for strawberry fruits. This new combination was effective reducing strawberry fruits decay as a result of chitosan fungistatic effect plus the fungicide activities of both Cu and ZnO. Current study demonstrates the potential of chitosan nanomaterials as fruits preservatives against *B. cinerea* plus the antifungal activity of the new combination CS–Zn-Cu nanomaterials which may increase

the positive effect of nanomaterials against different post-harvest fungi. On the other hand new combinations of nanomaterials might be toxic to plant, pathogens, animal and human.

The biocidal activity of CuNPs could be attributed to the effect of the CuNPs and/or the copper ions discharged from CuNPs. Because of the great surface area of the nanoparticles, it could be strongly adsorbed onto the surface of the microbial cells resulting in; i) disruption of cell permeability and release of integral components (Raffi *et al*., 2010), ii) denaturing of number of functional biomolecules (Wei *et al.,* 2010), iii) stimulation of oxidative damage to the microbial cells. However, some studies have reported that Cu^{2+} is the stimulating force behind the antimicrobial properties of polymers containing Cu-nanocomposites (Delgado *et al.*, 2011). At the same time, the discharged copper ions might be moved inside the microbial cells or attached to their outer surfaces resulting in cell apoptosis via protein denaturation and disruption of cell membrane (Palza *et al.,* 2015). Briefly, Copper has strong biocidal activity with non-specific mode of action against microbial cells that make it ideal antimicrobial agent. Cu-chitosan nanoparticles enhanced enzymes activities involved in plant defense by chitosan participate in reactive oxygen species (ROS) scavenging system (Saharan *et al.,* 2013; Saharan *et al.,* 2015).CuNPs and Zn-chitosan nanocomposites nearly have the same mode of action for Cu-chitosan nanocomposites such as the production of reactive oxygen species and membrane disruption (Xie *et al.,* 2011; Ingle *et al.,* 2013). Therefore, ZnNPs may exhibit different antifungal activities against *B. cinerea* and other fungi. ZnNPs could be used as an effective fungicide in agricultural and food safety applications.

In the current work, the genotoxicity exhibited by the three types of nano-agrochemicals was demonstrated by degradation of fungal DNA post-treatment even with *A. alternate* treated with CuNPs, *R. solani* treated with Zn NPs, *B. cinerea* treated with Cu-Zn- chit-NCs (60 µg ml^{-1})*,* respectively. DNA strand scission induced by CuNPs leads to gradual degradation in the amount of linear DNA. Cu-chitosan NCs could penetrate into cell and interacts with cellular DNA or mRNA directly regulates the metabolisms and reproduction which could ultimately lead to death of fungal pathogens

In conclusion, antifungal activities of Cu NPs, ZnONPs and CS–Zn-CuNCs against three plant pathogenic fungi *A. alternata, R. solani* and *B. cinerea*, were investigated in this study. The *In-vitro* application showed that CS–Zn-CuNCs at 90 µg ml^{-1}has the highest antifungal activity since it inhibited the growth of *B. cinerea*. The *in-vivo* application showed that Zn NPs at concentrations of 60 and 90 µg ml^{-1} against *R. solani* increased the percentages of survived seedlings to 85% and 86%, respectively. Every type of nanoparticle

used in the current work has its own unique properties which make it destructive to specific organisms with a specific mode of action. Combination between more than one nanoparticle may enhance the antifungal activities of the new compound or decrease the activities of the nanomaterials because of interference between two different modes of action. Further studies are needed to investigate the toxicology of each size of nanoparticles and also discover the unique activities of new combinations of nanomaterials.

Acknowledgment

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