PHILIPP AGRIC SCIENTIST
Vol. 100 No. 1, 69–78
March 2017

ISSN 0031-7454

# Fungicidal Efficacy of Chemically-Produced Copper Nanoparticles against *Penicillium digitatum* and *Fusarium solani* on Citrus Fruit

**Youssef Khamis[1,2], Ayat F. Hashim[1,2], Rubina Margarita[3], Mousa A. Alghuthaymi[4] and Kamel A. Abd-Elsalam[1,2,*]**

[1]Agricultural Research Center – Plant Pathology Research Institute, 9 Gamaa St., 12619 Giza, Egypt
[2]Unit of Excellence in Nano-Molecular Plant Pathology Research Center – Plant Pathology Research Institute, 9 Gamaa St., 12619 Giza, Egypt
[3]A. N. Nesmeyanov Institute of Organoelement Compounds (INEOS), Russian Academy of Sciences, Moscow, Russia
[4]Biology Department, Science and Humanities College, Shaqra University, Alquwayiyah, Saudi Arabia
[*]Author for correspondence: Dr. Kamel A. Abd-Elsalam, Ph.D.; Plant Pathology Research Institute, Agricultural Research Center (ARC), Egypt; Tel.: +02-35724893; Fax: +02-35723146; e-mail: kamelabdelsalam@gmail.com

**The environmental problems caused by fungicides cannot be ignored. New eco-friendly and effective nano-agrochemicals as alternative chemical fungicides need to be explored. The fungicidal activity of copper nanoparticles (CuNPs) was tested against *Penicillium digitatum* (green mold) and *Fusarium solani* (Fusarium rot) *in vitro* and *in vivo*. Fungal growth inhibition was observed in the presence of different concentrations of CuNPs. *In vitro*, a complete growth inhibition was recorded at 20 and 60 µg mL$^{-1}$ for *P. digitatum* and *F. solani*, respectively. *In vivo*, CuNPs at 20 and 40 µg mL$^{-1}$ were tested as direct and indirect action against green mold and Fusarium rot, respectively. These two concentrations completely inhibited the decay caused by both pathogens when the pathogen and CuNPs were applied to the same wound (direct action). Fourier transform infrared (FTIR) spectroscopy identified the possible functional groups involved in the reduction and stabilization of CuNPs and the chemical composition of Cu. From the energy dispersive X-ray spectroscopy (EDX) spectrum, the formation of CuNPs was confirmed. The average particle size and distribution size were characterized by biophysical techniques such as Dynamic Light Scattering (DLS), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). TEM revealed the presence of spherical CuNPs with size ranging from 45 to 48 nm in diameter. Also, the genotoxicity exhibited by CuNPs was demonstrated by degradation of fungal DNA post-treatment even with concentrations at 20 and 40 µg mL$^{-1}$ of the nanoparticles against *P. digitatum* and *F. solani*, respectively. CuNPs are low-cost effective and less toxic to humans and animals. Thus, they may be a very promising alternative to synthetic fungicides offering a protection against green mold and Fusarium rot of citrus fruit.**

Key Words: copper nanoparticles, Fusarium rot, green mold, nano-agrochemicals

Abbreviations: ANOVA – analysis of variance, CTAB – cetyltrimethyl ammonium bromide, CuNPs – copper nanoparticles, DLS – dynamic light scattering, EDX – energy dispersive X-ray spectroscopy, FTIR – Fourier transform infrared, GRAS – generally regarded as safe, IPA – isopropyl alcohol, NPs – nanoparticles, PDA – potato dextrose agar, SEM – Scanning Electron Microscopy, TEM – transmission electron microscopy

## INTRODUCTION

Citrus fruits are susceptible to a number of postharvest diseases that cause significant losses during the postharvest phase. Among them, green mold and Fusarium rot caused by *Penicillium digitatum* Sacc. and *Fusarium solani* (Mart.), respectively, are common diseases that affect citrus fruits (Tripathi and Dubey 2003; Abd-Elsalam et al. 2015). Citrus postharvest diseases are commonly controlled worldwide by applying synthetic fungicides, especially imazalil, thiabendazole, sodium orthophenylphenate, fludioxonil, pyrimethanil or different mixtures of these compounds (Ismail and Zhang 2004; Smilanick et al. 2005; Palou et al. 2008). However, reducing fungal growth and reproduction, and toxin production by synthetic fungicides is a matter of public concern due to

environmental pollution and development of fungal resistance.

Low-toxicity chemicals, like food additives that are generally regarded as safe (GRAS) compounds (Youssef et al. 2012a, 2012b) and biocontrol agents (Canamas et al. 2008), have been successfully applied before or after harvest to reduce Penicillium molds. Bordeaux mixture, which combines copper sulfate with lime (calcium hydroxide), has been used successfully for more than 150 yr on fruits, vegetables and ornamentals (Johnson and Hofman 2009). Copper nano/microparticles are more effective than Bordeaux mixture in controlling plant pathogenic fungi, e.g., *Alternaria alternata, Alternaria solani* and *Fusarium expansum* (Nemati et al. 2015). Copper compounds have been shown to effectively kill a wide range of yeast and fungi, e.g., *Aspergillus carbonarius*, *Aspergillus fumigatus*, *Aspergillus niger*, *Colletotrichum gloeosporioides*, and *Phytophthora erythroseptica* (Borkow and Gabbay 2009; Oziengbe and Osazee 2012), thus proving their indispensability in agriculture.

The nanosize changes the chemical, physical and optical properties of metals, and hence, different nanoparticles are being used as potent antimicrobial agents (Rai et al. 2009). CuNPs, due to their interesting properties, low-cost preparation and many potential applications, have attracted a lot of interest in recent years. CuNPs play an important role as a novel antimicrobial agent (Kanhed et al. 2014). Recent studies have demonstrated the antimicrobial activities of various nanoparticle materials, including silver (Kim et al. 2008a, 2008b; Kumar et al. 2008), copper (Cioffi et al. 2005), titanium dioxide (Kwak et al. 2001), aluminum nanoparticles (Sadiq et al. 2011), magnesium oxide nanoparticles (Koper et al. 2002) and zinc oxide (Liu et al. 2009). Based on some studies which have reported the antifungal properties of CuNPs, researchers have recommended their use as disinfectant and antimicrobial agents (Ren et al. 2009). The prepared CuNPs are effective against *P. infestans* in lower copper doses compared with the commercial products (Giannousi et al. 2013).

Crops treated with safe nano-fungicides will gain added value because they are free from chemical residues, and effective in low dosage. They reduce food and feed spoilage and fungal pathogens, and help protect human health and sustain the universal demand for high product quality. Development of other eco-safe nano-biocide agents, like green nano-materials, is being investigated (Abd-Elsalam and Khokhlov 2015; Alghuthaymi et al. 2015). High charges and other disadvantages of physical and chemical controls of contaminated feeds have encouraged a search for alternative methods to control plant diseases (Jans et al. 2014).

Thus, the main aims of the current research were to (1) synthesize and characterize CuNPs, (2) investigate the *in vitro* antifungal activity of synthesized CuNPs against *P. digitatum* and *F. solani*, (3) evaluate their fungistatic and fungicidal activity against those pathogens, and (4) analyze the DNA of treated pathogenic fungi to determine the mode of action of CuNPs.

## MATERIALS AND METHODS

### Chemicals

Copper sulfate (Purity 98%, M.wt. 249.68) was purchased from the 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 ($NaBH_4$, Sigma-Aldrich) served 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.

### Synthesis of CuNPs

CuNPs were synthesized by reduction of copper sulfate ($CuSO_4.5H_2O$) with isopropyl alcohol (IPA) in the presence of the cationic surfactant cetyltrimethyl ammonium bromide (CTAB), following the Athawale et al. (2005) technique with slight modification. Copper nanoparticles were synthesized according to the method of Kanhed et al. (2014) with minor modification. First, 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 sulfate was added to the CTAB/(IPA) solution by using a dropper. 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.

**Characterization of Cu-based NPs**

*Fourier transform infrared (FTIR) spectroscopy*. CuNPs were analyzed by FTIR spectroscopy. For FTIR analysis, the nanoparticle solution was centrifuged at 25,000 × g for 25 min and the pellet was washed twice in deionized water to remove the unbound components. The purified sample was then dried and ground with KBr powder and pelleted for subsequent analysis on a Jasco FTIR 5300 spectrophotometer. The sample was directly placed in the zinc selenide crystal and the spectrum was recorded in the transmittance mode.

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

*Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDX)*. The morphology and chemical structure of the synthesized nanoparticles were examined by scanning electron microscopy (SEM, Quanta 250 Philips) equipped with an energy-dispersive X-ray spectrometer (EDX) (TEAM™).

*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 % phosphotungstic acid (PTA) and allowed to dry for 3 min. The coated grid was dried and examined under the TEM microscope (Philips, CM12). Then samples were observed by operating at 120 kV.

***In vitro* Effect of CuNPs against *P. digitatum* and *F. solani***

Agar plugs (5 mm in diam.) containing mycelia from the growing edge of 1-wk-old cultures of *P. digitatum* and *F. solani* were placed in the center of potato dextrose agar (PDA) Petri dishes. Five concentrations (5, 10, 20, 40 and 60 µg $mL^{-1}$) were used for *P. digitatum* while six concentrations (3, 5, 10, 20, 40 and 60 µg $mL^{-1}$) were used for *F. solani*. For each compound/concentration, five Petri dishes were utilized as replicates and the entire experiment was repeated twice. For each PDA plate, three holes (5 mm in diameter) at the corner of the plates were inoculated with 20 µL of CuNPs. The plates were incubated at 23 ± 1 °C for 1 wk. Colony diameter (mm) for each fungus was calculated as the average of the longest and the shortest diameter. Non-amended PDA plates served as control while copper sulfate was used as raw material for comparison.

***In vivo* Effect of CuNPs against Green Mold and Fusarium Rot**

*Pathogen suspension*. To produce fungal inocula for *P. digitatum* and *F. solani*, isolates were grown on PDA plates at 24 °C in the dark. Conidia were collected from 2-wk-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 $mL^{-1}$.

*Fruit samples*. Oranges [*Citrus sinensis* (L.) Osbeck] cv. Valencia late were obtained from a commercial market, selected for uniformity of size and absence of symptoms of any disorders, and immediately processed. Fruits were surface-sterilized with a 2% commercial bleach solution for 2 min, washed with tap water and air-dried at room temperature.

**Direct Antifungal Activity of CuNPs**

The fruits were wounded (5 mm deep × 3 mm wide) with a sterile nail-head at two equidistant points in the equatorial zone. For each treatment, 30 µL of CuNPs solution (20 and 40 µg $mL^{-1}$ for *P. digitatum* and *F. solani*, respectively) was applied into each wound. After 2 h, 10 µL of a $10^4$ conidia $mL^{-1}$ suspension of *P. digitatum* or *F. solani* were inoculated into the same wound. The control consisted of oranges treated with distilled water and then inoculated with the same concentration of the pathogen. Each treatment was replicated three times and each replicate consisted of four oranges with two wounds each. Replicates were individually wrapped in a plastic bag and incubated at 20 ± 1 °C and high relative humidity for 2 wk. The percentage of infected fruit (rot incidence, %) and decay lesions diameter (mm) were recorded for each fungal pathogen.

**Indirect Antifungal Activity of CuNPs**

Oranges were wounded once (5 mm deep × 3 mm wide) with a sterile nail-head along the equatorial axis. For each treatment, 30 µL of CuNPs solution (20 and 40 µg $mL^{-1}$ for *P. digitatum* and *F. solani*, respectively) were applied into each wound. Treated fruits were placed in a tray and individually wrapped in a plastic bag. After 48 h 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 *P. digitatum* or *F. solani* (Youssef et al. 2014). The control consisted of oranges 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 three times and each replicate consisted of four oranges. Replicates were individually wrapped in a plastic bag and conserved as described in the first experiment. Decay lesions diameter and rot incidence were recorded as described above.

**Fragmentation of Fungal Genomic DNA**

Fungal mycelium was produced in 20 mL of liquid medium (24 g $L^{-1}$ 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 to remove excess water. Mycelium was ground to a fine powder in liquid nitrogen using a mortar and DNA was extracted by using the method of Moslem et al. (2010). Five micrograms of fungal DNA for *F. solani* was treated with CuNPs (40 µg $mL^{-1}$), while *P. digitatum* DNA was treated with CuNPs (20 µg $mL^{-1}$) for a period of 2 h at 37 °C. 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 µg $mL^{-1}$ 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.

**Statistical Analysis**

The effect of copper treatments, concentrations and their interaction on *in vitro* colony diameter of pathogens was subjected to analysis of variance (ANOVA). Similarly, the effect of copper treatments, action type of inoculation and their interaction on lesion diameter *in vivo* was also subjected to ANOVA. All experiments were set up in a randomized complete block design with three replicates. The obtained data were subjected to statistical analysis using LSD test at $P < 0.05$ to evaluate the differences between various treatments. For disease incidence, mean values of treatments were compared by using Fisher's protected LSD test and judged at $P \leq 0.05$ level. Percentage data were arcsine transformed to normalize variance.

## RESULTS

**Synthesis of CuNPs**

Colloidal CuNPs were synthesized by the chemical reduction of copper sulfate with IPA. Droplet size was found to correlate well with the results obtained from droplet size analysis using dynamic light scattering. Mean diameter of CuNPs was recorded as 200–400 nm (Fig. 1a). Transmission electron microscopy of CuNPs gives the actual size and shape. TEM analysis revealed the formation of

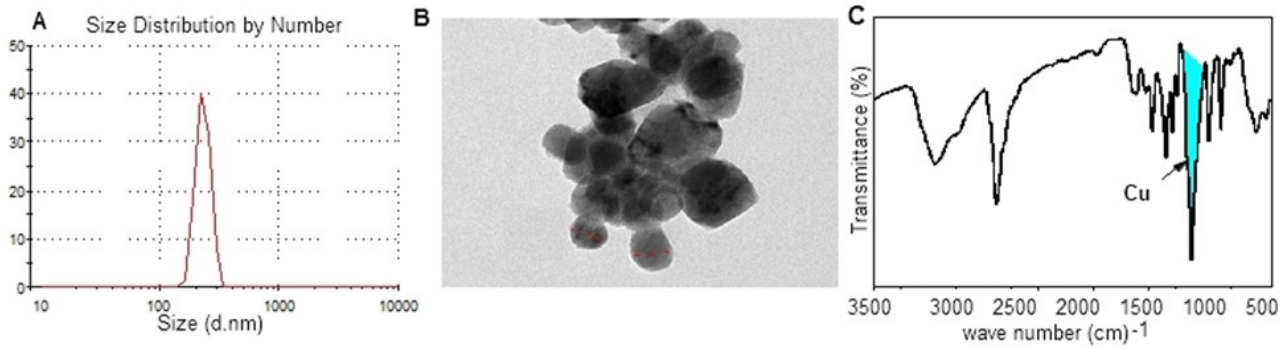


**Fig. 1. A:** Dynamic light scattering (DLS) of chemically-produced copper nanoparticles (CuNPs), **B**: Transmission electron micrographs (TEM), **C**: Fourier transform infrared (FTIR) spectroscopy of synthesized CuNPs.

spherical CuNPs measuring about 48 nm in diameter, uniformly distributed without any significant aggregation. TEM micrograph shows that the nanoparticle was crystalline with only limited surface oxidation (Fig. 1b). The FTIR spectra of CuNPs are shown in Fig. 1c. FTIR spectra identified the possible functional groups involved in the reduction and stabilization of CuNPs and chemical composition of copper. A coordination through the PEG ester bond to the copper is estimated to be due to electrostatic attraction. This ester bond is located at 1106 $cm^{-1}$ and is expected to shift to a lower wave number at 953 $cm^{-1}$. Absorption peak appears with CuNPs at 1630 $cm^{-1}$. The corresponding bond clearly appears to be involved in the interaction with CuNPs when coordinated to their surface. From the EDX spectrum, the formation of CuNPs was confirmed. The weight percentage of CuNPs is more than 65% and the rest consisted of impurities. SEM analysis indicated that CuNPs were created as aggregates and had variable morphology. The particle size observed in the SEM image (Fig. 2) is in the range of 10 µm (Shantkriti and Rani 2014; Chaudhari and Ingale 2016).

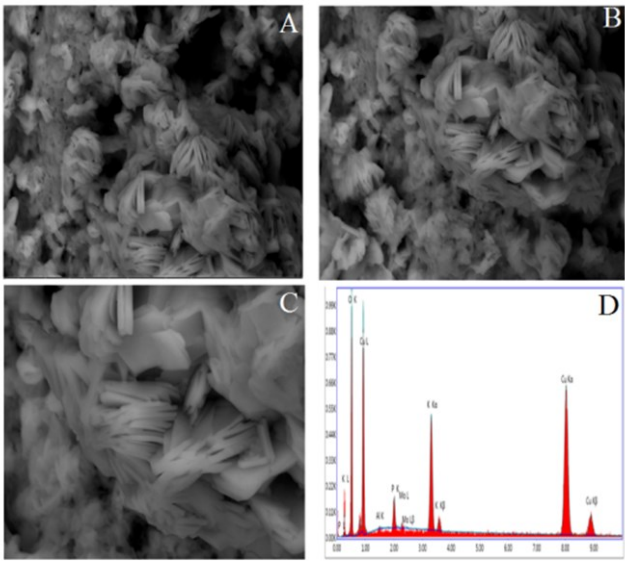


**Fig. 2.** Scanning electron microscopy (SEM) images **A**, **B**: mag 8000x, **C**: 16000x, **D**: energy dispersive X-ray spectroscopy (EDX) spectra of synthesized copper nanoparticles (CuNPs).

### *In vitro* Effect of CuNPs against *P. digitatum* and *F. solani*

Analysis of variance of the effects of copper treatments, concentration and their interaction on *in vitro* colony diameter of *P. digitatum* and *F. solani* are shown in Table 1 and Fig. 3, respectively. After 7 d of incubation, a complete inhibition of *P. digitatum* was achieved at 20 µg $mL^{-1}$ for both copper sulfate and CuNPs. The results of *in vitro* tests on the growth of *P. digitatum* and *F. solani* are shown in Table 2. The reduction (%) of *P. digitatum* colony diameter was 26%, 43%, and 100% at 5, 10, 20 µg $mL^{-1}$ for copper sulfate, and at 43%, 60%, 100% for CuNPs, respectively. For *F. solani*, a complete growth inhibition was evidenced at 60 µg $mL^{-1}$ for both copper sulfate and CuNPs. The reduction (%) in colony diameter was 46% and 82% for copper sulfate and CuNPs, respectively, at 40 µg $mL^{-1}$ (Table 2).

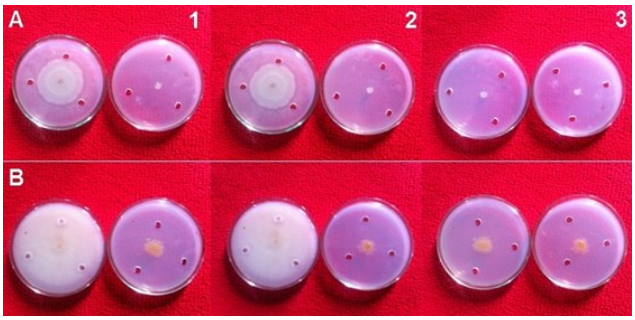


**Fig. 3.** Antifungal *in vitro* effect of copper nanoparticles (CuNPs) at three concentrations on *Penicillium digitatum* (A) and *Fusarium solani* (B). From right to left A1 and B1: control + copper, A2 and B2: control + CuNPs, A3 and B3: copper +

### *In vivo* Effect of CuNPs against Green Mold and Fusarium Rot

Analysis of variance of the effect of copper treatments, action type of inoculation and their

**Table 1.** Analysis of variance of the effects of copper treatments, concentrations and their interaction on *in vitro* colony diameter of *Penicillium digitatum* and *Fusarium solani*.

| Source of Variation[a] | *P. digitatum* | | | | | *F. solani* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | df | Mean Square | F Value | P>F | RC[b] | df | Mean Square | F Value | P>F | RC[b] |
| Replication | 2 | 107.23 | 0.975 | 0.390 | | 2 | 26.42 | 0.580 | 0.565 | 0.00016 |
| Concentration (C) | 4 | 2961.9 | 26.9 | 0.00 | 25.19 | 5 | 5429.4 | 119.22 | 0.00 | 87.02 |
| Treatment (T) | 2 | 15289.3 | 139.0 | 0.00 | 65.00 | 2 | 1092.69 | 23.99 | 0.00 | 7.00 |
| C × T | 8 | 549.60 | 4.997 | 0.00 | 9.35 | 10 | 180.90 | 3.97 | 0.001 | 5.80 |
| Error | 28 | 109.99 | | | | 34 | 45.53 | | | |

[a]Replication is random while each of concentration and treatment is fixed.
[b]Relative contribution (RC) to variation in colony diameter was calculated as percentage of sum of squares of the explained (model) variation.

**Table 2.** Effects of different concentrations of copper and copper nanoparticles (CuNPs) on colony diameter (mm) of *Penicillium digitatum* and *Fusarium solani* after 7 d of incubation at 24 ± 2 °C on potato dextrose agar (PDA).

| Treatment (T) | *P. digitatum* | | | | | *F. solani* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Concentration µg mL$^{-1}$ (C) | | | | | | | | | | |
| | 5 | 10 | 20 | 40 | 60 | 3 | 5 | 10 | 20 | 40 | 60 |
| Control | 77.50 | 77.50 | 77.50 | 77.50 | 77.50 | 72.50 | 72.50 | 72.50 | 72.50 | 72.50 | 72.50 |
| Copper sulfate | 57.30 | 44.10 | 0.00 | 0.00 | 0.00 | 71.00 | 64.00 | 59.30 | 55.60 | 39.00 | 0.00 |
| CuNPs | 44.30 | 31.30 | 0.00 | 0.00 | 0.00 | 72.00 | 63.00 | 48.50 | 45.60 | 13.00 | 0.00 |
| | LSD for T x C (P ≤ 0.05) = 16.95 | | | | | LSD for T x C (P ≤ 0.05) = 10.91 | | | | | |

**Table 3.** Analysis of variance of the effect of copper treatments, action type of inoculation and their interaction on lesion diameter *in vivo* caused by *Penicillium digitatum* and *Fusarium solani*.

| Source of Variation[a] | *Penicillium digitatum* | | | | | *Fusarium solani* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | df | Mean Square | F. Value | P>F | RC[b] | df | Mean Square | F. Value | P>F | RC[b] |
| Replication | 2 | 24.88 | 2.43 | 0.138 | | 2 | 0.222 | 0.14 | 0.869 | 0.0075 |
| Action type (M) | 1 | 16749.5 | 1638.90 | 0.00 | 98.23 | 1 | 8.000 | 5.13 | 0.047 | 0.14 |
| Treatment (T) | 2 | 138.88 | 13.589 | 0.004 | 0.41 | 2 | 2906.88 | 1868.38 | 0.00 | 98.35 |
| M x T | 2 | 206.88 | 20.243 | 0.00 | 1.213 | 2 | 44.66 | 28.7 | 0.00 | 1.51 |
| Error | 10 | 10.22 | | | | 10 | 1.56 | | | |

[a]Replication is random while each of concentration and treatment is fixed.
[b]Relative contribution (RC) to variation in lesion diameter was calculated as percentage of sum of squares of the explained (model) variation.

interaction on lesion diameter of *P. digitatum* and *F. solani* are shown in Table 3. Green mold and Fusarium rot diameter (mm) on oranges treated or untreated with copper sulfate or CuNPs are reported in Table 4. For green mold, in direct action (when the pathogen and compound is applied into the same wound), CuNPs gave the best treatment, completely inhibiting (100%) green mold, while the reduction (%) reached 73.9% for copper sulfate. In the case of indirect action (in which compound and pathogen were added into separate wounds), the reduction was 60% and 88.5% for copper sulfate and CuNPs, respectively. For Fusarium rot, in direct action, CuNPs completely inhibited (100%) the rot, while the reduction (%) reached 70% for copper sulfate. In the case of indirect action, the reduction (%) was 100% and 50.8% for CuNPs and copper sulfate, respectively (Table 4). Data in Table 5 shows the percentage of infected fruit by green and Fusarium rots. CuNPs completely inhibited the growth of both pathogens in the direct action. Also, when CuNPs were applied in separate wounds toward the pathogen, they completely stopped Fusarium growth and reduced the growth of green mold (Fig. 4).

**Genotoxic Potential of the Nano/Micro-sized Copper**

CuNPs were incubated with *P. digitatum* and *F. solani* DNA and studied for their DNA binding activity to explore the molecular basis of their antifungal activities. The genotoxicity exhibited by CuNPs was demonstrated by degradation of fungal DNA post-treatment even with 20 and 40 µg mL$^{-1}$ concentrations of the CuNPs for *P. digitatum* and *F. solani,* respectively. DNA strand scission induced by CuNPs

**Table 4.** Lesion diameter (mm) of *Penicillium digitatum* and *Fusarium solani* after 7 d of shelf-life at 22 ± 2 °C and high relative humidity (RH) on orange cv. Valencia late.

| Treatment (T) | Action type (M) | | | |
|---|---|---|---|---|
| | *Penicillium digitatum* | | *Fusarium solani* | |
| | Direct | Indirect | Direct | Indirect |
| Control | 112.67 | 104.67 | 45.33 | 42 |
| Copper sulfate | 29.33 | 42 | 13.33 | 20.66 |
| CuNPs | 0.0 | 12 | 0.0 | 0.0 |
| | LSD for T x M (P ≤ 0.05 = 5.17) | | LSD for T x M (P ≤ 0.05 = 2.01) | |

The concentration of copper and CuNPs was 40 µg mL$^{-1}$ for Fusarium and 20 µg mL$^{-1}$ for Penicillium. Fruits treated with water were utilized as control. Copper sulfate was included for comparison.

**Table 5.** Disease incidence (%) of *Penicillium digitatum* and *Fusarium solani* after 7 d of shelf-life at 22 ± 2 °C and high relative humidity (RH) on orange cv. Valencia late.

| Treatment (T) | Disease Incidence (%) | | | |
|---|---|---|---|---|
| | *Penicillium digitatum* | | *Fusarium solani* | |
| | Direct | Indirect | Direct | Indirect |
| Control | 100 (a) | 100 (a) | 100 (a) | 100 (a) |
| Copper sulfate | 41.67 (b) | 50 (b) | 33.33 (b) | 50 (b) |
| CuNPs | 0.0 (c) | 25 (c) | 0.0 (c) | 0.0 (c) |

Values followed by different letters are statistically different according to Fisher's protected LSD (P ≤ 0.05). Fruits treated with water were utilized as control. Copper sulfate was included for comparison.

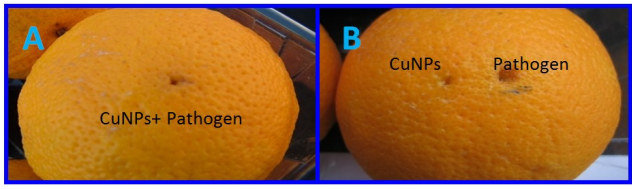


**Fig. 4.** Scheme of application of copper nanoparticles (CuNPs) and fungal suspension on citrus wounds. A – direct antifungal activity, B – indirect antifungal activity.

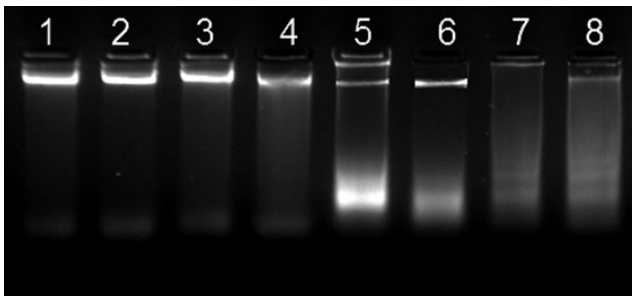


**Fig. 5**. Dose-dependent fungal DNA degradation action of copper and copper nanoparticles (CuNPs) in agarose gel electrophoresis. Lanes 1, 3: DNA for untreated *F. solani*, Lanes 2, 4: DNA for untreated *P. digitatum*. Lane 5: *F. solani* treated with copper, Lane 6: *P. digitatum* treated with copper, Lane 7: *F. solani* treated with CuNPs (40 µg $mL^{-1}$), Lane 8: *P. digitatum*, DNA treated with CuNPs (20 µg $mL^{-1}$) showing gradual degradation of the fragmented DNA bands.

leads to gradual degradation in the amount of linear DNA. Smear bands appeared lower in the gel which are the resultant fragmented DNA (Fig. 5).

## DISCUSSION

The main objective of the study was to produce stable CuNPs that demonstrated fungicidal activity against green mold caused by *P. digitatum*, and Fusarium rot caused by *F. solani* on citrus fruit. Significant losses can occur after harvest during storage, and citrus fruit would have no market value due to green mold and Fusarium rot diseases (Schiffmann-Nadel et al. 1987; Eckert and Eaks 1989). Nanotechnology is a new discipline which has developed innovative tools to revolutionize the agricultural and food industry through diagnosis and treatment of diseases, enhancing the ability of plants to absorb nutrients, to combat microbial and pest infections, to increase the efficiency of biocides, reduce pollution, and to clean-up existing pollutants (Misra et al. 2013).

In *in vitro* tests, a complete growth inhibition was observed at 20 and 60 µg $mL^{-1}$ for *P. digitatum* and *F. solani*, respectively. Cerioni et al. (2009) examined sodium hypochlorite, hydrogen peroxide and copper sulfate against *P. digitatum* and demonstrated that combination of sodium hypochlorite and hydrogen peroxide in the presence of copper sulfate produces a synergistic effect. The minimum inhibition concentration could not be determined because the concentrations as high as 500 mM did not affect the viability of conidia at different incubation times. Copper and copper compounds have been shown to effectively kill a wide range of yeast and fungi such as *Aspergillus carbonarius*, *Aspergillus fumigates*, *Aspergillus niger*, *Candida albicans*, *Cryptococcus neoformans*, *Trichoderma viride*, and others (Borkow and Gabbay 2009) proving their indispensability in agriculture worldwide. For green mold, in direct action, CuNPs provided the best treatment, completely inhibiting (100%) green mold incidence and lesion diameter. Copper sulfate showed high antifungal activity against citrus blue mold caused by *P. italicum* in both *in vitro* and *in vivo* tests and completely inhibited mycelial growth of the pathogen at 20 mM. *In vivo*, copper sulfate (200 mM) significantly reduced the incidence and severity of decay on mandarin (*Citrus reticulata* Blanco) cv. Clementine when compared with the control (Askarne et al. 2013).

Concerning Fusarium rot, in direct action, CuNPs completely inhibited (100%) the rot, while the percentage of reduction reached 70% for copper sulfate. In this context, Oziengbe and Osazee (2012) showed that copper sulfate could directly inhibit the growth of *Colletotrichum gloeosporioides in vitro* and potently induce defense reactions in mango fruit. Copper sulfate at 0.8 mg $L^{-1}$ gave significant reduction of *C. gloeosporioides* growth and conidia germination by 78.2% and 66.3%, respectively. In this study, CuNPs completely inhibited the growth of both pathogens in the direct action. Also, when CuNPs were applied in separated wounds toward the pathogen, they completely stopped Fusarium growth and reduced the growth of green mold. The properties of CuNPs depend largely on their synthesis procedures. Moreover, the production of CuNPs is even more challenging because CuNPs are quite sensitive to aqueous solutions and the air is stable at these conditions (Umer et al. 2012).

Based on the TEM of CuNPs, which gives the actual size and shape, the analysis revealed the formation of spherical CuNPs measuring about 48 nm in diameter, uniformly distributed without any significant aggregation. FTIR analysis provides a wide range of information about the strength of interatomic and intermolecular bonds within the NPs and can detect the presence of even thin oxide layers (Giannousi et al. 2013). In fact, independently of the mechanism, micro- or nano-copper ions are the active agent able to either kill (cidal effect) or inhibit the growth (static effect) of pathogenic fungi by several processes. Previous studies on the mechanism of action of CuNPs on fungi are not sufficient to determine the exact mechanism (Ingle et al. 2013). It seems reasonable to assume that the small-size range of CuNPs may add to its antifungal properties, since it can easily penetrate fungal cell membrane, inhibiting division of the cell, and finally lead to cell death. Elechiguerra et al. (2005) confirmed that particle accumulations in the cell membrane may lead to cell lysis.

In the current research, the genotoxicity exhibited by CuNPs was demonstrated by degradation of fungal DNA post-treatment even with concentrations at 20 and 40 µg $mL^{-1}$ of CuNPs for *P. digitatum* and *F. solani,* respectively. DNA strand scission induced by CuNPs leads to gradual degradation in the amount of linear DNA. The antifungal activity of the CuNPs may have resulted from their interaction with protein molecules, which results in the inactivation of protein molecules, and also from direct interaction with DNA molecules. This interaction may have caused a mutation in the DNA, and hence, the cessation of its replication ability (Nemati et al. 2015). Copper ions released may also interact with DNA molecules and intercalate with nucleic acid strands. CuNPs degrade DNA in a single oxygen-mediated fashion even in the absence of any external agents like hydrogen peroxide or ascorbate. The antifungal effect of copper sulfate on conidia and linear growth could be attributed to copper ions which can catalyze the production of highly hydroxyl radicals, with subsequent damage to lipids, proteins, DNA and other biomolecules. Extensive copper-induced disruption of membrane integrity inevitably leads to loss of cell viability (Kumbhar et al. 1991). CuNPs have a high antimicrobial activity against *B. subtilis*. This may be attributed to greater abundance of amines and carboxyl groups on cell surface of *B. subtilis* and greater affinity of copper towards these groups. Copper ions inside bacterial cells also disrupt biochemical processes (Ruparelia et al. 2008). In conclusion, the promising results that have been obtained may represent CuNPs as an effective antifungal agent for the control of green mold and Fusarium rot of orange fruit. However, the application of metal nano-materials is a relatively new approach in the field of postharvest pathology, and therefore needs further study.

## ACKNOWLEDGMENTS

Thanks are due to the Unit of Excellence in NanoMolecular Plant Pathology Research, Agricultural Research Center (ARC), Egypt. Current work was supported by the Science and Technology Development Fund (STDF), Egypt (STDF- RFBR program) [grant no. 13791]. This work was partially supported by STDF Basic and Applied Research Grants (no. 5555). Also, this work was partially funded by the Russian Foundation for Basic Research grant (RFBR-15-53-61030).

## REFERENCES CITED

ABD-ELSALAM KA, KHOKHLOV AR. 2015. Eugenol oil nanoemulsion: antifungal activity against *Fusarium oxysporum* f. sp. *vasinfectum* and phytotoxicity on cotton seeds. Appl Nanosci 5: 255–265.

ABD-ELSALAM KA, YOUSSEF K, ALMOAMMAR H. 2015. First morphogenetic identification of *Fusarium solani* isolated from orange fruit in Egypt. (Phyton) Int J Exp Bot 84: 128–131.

ALGHUTHAYMI MA, ALMOAMMAR H, RAI M, SAID-GALIEV E, ABD-ELSALAM KA. 2015. Myconanoparticles: synthesis and their role in phytopathogens management. Biotechnol Biotechnol Equip 29(2): 221–236.

ATHAWALE A, KATRE PP, KUMAR M, MAJUMDAR MB. 2005. Synthesis of CTAB–IPA reduced copper nanoparticles. Mat Chem Phys 91: 507–512.

ASKARNE L, BOUBAKER H, BOUDYACH EH, AIT BEN AOUMAR A. 2013. Use of food additives to control postharvest citrus blue mold disease. Atlas J Biol 2 (2): 147–153.

BORKOW G, GABBAY J. 2009. Copper, an ancient remedy returning to fight microbial, fungal and viral infections. Curr Chemical Biol 3: 272–278.

CANAMAS TP, VINAS I, USALL J, TORRES R, ANGUERA M, TEIXIDO N. 2008. Control of postharvest diseases on citrus fruit by preharvest applications of biocontrol agent *Pantoea agglomerans* CPA-2: Part II. Effectiveness of different cell formulations. Postharvest Biol Technol 49: 96–106.

CERIONI L, RAPISARDA VA, HILAL M, PRADO FE, RODRÍGUEZ-MONTELONGO L. 2009. Synergistic antifungal activity of sodium hypochlorite, hydrogen peroxide, and cupric sulfate against *Penicillium digitatum*. J Food Prot 72(8): 1660–1665.

CHAUDHARI AN, INGALE AG. 2016. *Syzygium aromaticum* extract mediated, rapid and facile biogenic synthesis of shape-controlled (3D) silver nanocubes. Bioprocess Biosyst Eng 39: 883–891.

CIOFFI N, TORSI L, DITARANTO N, TANTILLO G, GHIBELLI L, SABBATINI L. 2005. Copper nanoparticle/polymer composites with antifungal and bacteriostatic properties. Chem Mater 17(21): 5255–5262.

ECKERT JW, EAKS IL. 1989. Postharvest disorders and diseases of citrus fruits. In: Reuter W, Calavan EC, Carman GE, editors. The Citrus Industry. Vol. 5. Berkeley: University of California Press. p. 179–260.

ELECHIGUERRA JL, BURT J, MORONES JR, CAMACHO-BRAGADO A, GAO X, LARA HH, YACAMAN MJ. 2005. Interaction of silver nanoparticles with HIV-1. Nanobiotechnol 3(6): 1–10.

GIANNOUSI K, AVRAMIDIS I, DENDRINOU-SAMARA C. 2013. Synthesis, characterization and evaluation of copper based nanoparticles as agrochemicals against *Phytophthora infestans*. RSC Adv 3: 21743–21752.

INGLE AP, DURAN N, RAI M. 2013. Bioactivity, mechanism of action, and cytotoxicity of copper-based nanoparticles: A review. Appl Microbiol Biotechnol 98(3): 1001–1009.

ISMAIL M, ZHANG J. 2004. Postharvest citrus diseases and their control. Outlook Pest Manage 1(10): 29–35.

JANS D, PEDROSA K, SCHATZMAYR D, BERTIN G, GRENIER B. 2014. Mycotoxin Reduction in Animal Diets. In: Leslie JF, Logrieco AF, editors. Mycotoxin Reduction in Grain Chains. Chichester, UK: John Wiley & Sons, Ltd. doi: 10.1002/9781118832790.ch8

JOHNSON GI, HOFMAN PJ. 2009. Postharvest Technology and Quarantine Treatments. Wallingford, UK: CABI Publishing.

KANHED P, BIRLA S, GAIKWAD S, GADE A, SEABRA AB, RUBILAR O, DURAN N, RAI M. 2014. *In vitro* antifungal efficacy of copper nanoparticles against selected crop pathogenic fungi. Mater Lett 115: 13–17.

KIM KJ, SUNG WS, MOON SK, CHOI JS, KIM JG, LEE DG. 2008a. Antifungal effect of silver nanoparticles on dermatophytes. J Microbiol Biotechnol 18(8): 1482–1484.

KIM KJ, SUNG WS, SUH BK, MOON SK, CHOI JS, KIM JG. 2008b. Antifungal activity and mode of action of silver nano-particles on *Candida albicans*. Biometals 22 (2): 235–242.

KOPER OB, KLABUNDE JS, MARCHIN GL, KLABUNDE KJ, STOIMENOV PL. 2002. Nanoscale powders and formulations with biocidal activity toward spores and vegetative cells of bacillus species, viruses, and toxins. Curr Microbiol 44: 49–55.

KUMAR A, VEMULA PK, AJAYAN PM, JOHN G. 2008. Silver nanoparticle-embedded antimicrobial paints based on vegetable oil. Nat Mater 7(3): 236–241.

KUMBHAR AS, PADHYE SB, SARAF AP, MAHAJAN HB, CHOPADE BA, WEST DX. 1991. Novel broad-spectrum metal-based antifungal agents. Correlations amongst the structural and biological properties of copper 2-acetylpyridine N4-dialkylthiosemicarbazones. Biol Metals 4: 3141–3143.

KWAK SY, KIM SH, KIM SS. 2001. Hybrid organic/ inorganic reverse osmosis (RO) membrane for bactericidal antifouling. 1. Preparation and characterization of TiO2 nanoparticle self-assembled aromatic polyamide thin-film-composite (TFC) membrane. Environ Sci Technol 35(11): 2388–2394.

LIU Y, HE L, MUSTAPHA A, LI H, LIN M. 2009. Antibacterial activities of zinc oxide nanoparticles against *Escherichia coli* O157:H7. J Appl Microbiol 107 (4): 1193–1201.

MISRA AN, MISRA M, SINGH R. 2013. Nanotechnology in agriculture and food industry. Int J Pure Appl Sci Technol 16(2): 1–9.

MOSLEM MA, ABD-ELSALAM KA, BAHKALI AH, YASSIN MA. 2010. First morpho-molecular identification of *Penicillium griseofulvum* and *P. aurantiogriseum* toxicogenic isolates associated with blue mold on apple. Food-Borne Pathogen Dis 7: 857–861.

NEMATI A, SHADPOUR S, KHALAFBEYGI H, ASHRAF S, BARKHI M, SOUDI RM. 2015. Efficiency of hydrothermal synthesis of nano/micro sized copper and study on *in vitro* antifungal activity. Mater Manuf Process 30(1): 63–69.

OZIENGBE EO, OSAZEE JO. 2012. Antifungal activity of copper sulphate against *Colletotrichum gloeosporioides*. J Asian Sci Res 2(12): 835–839.

PALOU L, SMILANICK JL, DROBY S. 2008. Alternatives to conventional fungicides for the control of citrus postharvest green and blue molds. Stewart Postharvest Rev 2(2): 1–16.

RAI M, YADAV A, GADE A. 2009. Silver nanoparticles as a new generation of antimicrobials. Biotechnol Adv 27: 76–83.

REN G, HU D, CHENG EW, VARGAS-REUS MA, REIP P, ALLAKER RP. 2009. Characterisation of copper oxide nanoparticles for antimicrobial applications. Int J Antimicrob Agents 33(6): 587–90.

RUPARELIA JP, CHATTERJEE AK, DUTTAGUPTA SP, MUKHERJI S. 2008. Strain specificity in antimicrobial activity of silver and copper nanoparticles. Acta Biomater 4(3): 707–16.

SADIQ IM, PAKRASHI S, CHANDRASEKARAN N, MUKHERJEE A. 2011. Studies on toxicity of aluminum oxide ($Al_2O_3$) nanoparticles to microalgae species: *Scenedesmus* sp. and *Chlorella* sp. J Nanoparticle Res 13(8): 3287–3299.

SCHIFFMANN-NADEL M, CHALUTZ E, WAKS J, LOMANIEC E, YOFFE AZ. 1987. Increase of Fusarium rot in stored citrus fruit. J Phytopathol 120: 154–157.

SHANTKRITI S, RANI P. 2014. Biological synthesis of copper nanoparticles using *Pseudomonas fluorescens*. Int J Curr Microbiol App Sci 3(9): 374–383.

SMILANICK JL, MANSOUR MF, MARGOSAN DA, MLIKOTAGABLER F, GOOD-WINE WR. 2005. Influence of pH and $NaHCO_3$ on effectiveness of imazalil to inhibit germination of *Penicillium digitatum* and to control postharvest green mold on citrus fruit. Plant Dis 89: 640–648.

TRIPATHI P, DUBEY N. 2003. Exploitation of natural products as an alternative strategy to control postharvest fungal rotting of fruit and vegetables. Postharvest Biol Technol 32: 235–245.

UMER A, NAVEED S, RAMZAN N. 2012. Selection of a suitable method for the synthesis of copper nanoparticles. NANO: Brief Rep Rev 7(5):1230005 (18 pages).

YOUSSEF K, LIGORIO A, NIGRO F, IPPOLITO A. 2012a. Activity of salts incorporated in wax in controlling postharvest diseases of citrus fruit. Postharvest Biol Technol 65: 39–43.

YOUSSEF K, LIGORIO A, SANZANI SM, NIGRO F, IPPOLITO A. 2012b. Control of storage diseases of citrus by pre- and postharvest application of salts. Postharvest Biol Technol 72: 57–63.

YOUSSEF K, SANZANI SM, LIGORIO A, IPPOLITO A, TERRY LA. 2014. Sodium carbonate and bicarbonate treatments induce resistance to postharvest green mold on citrus fruit. Postharvest Biol Technol 87: 61–69.