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Copper Oxide Nanoparticles as Fungistat to Inhibit Mycotoxins and Hydrolytic Enzyme Production by *Fusarium incarnatum* Isolated from Garlic Biomass

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Garlic (*Allium sativum* L.) being infected by mycotoxigenic fungi is one of the primary factors limiting its nutritional and medical value. Therefore, there is an urgent need to repress mycotoxigenic fungi utilizing safer treatments, possibly involving nanoparticles. *Fusarium incarnatum* was isolated from garlic (*A. sativum* L.) that showed fungal contamination and their identification was confirmed using amplified and sequenced internal transcribed spacer nuclear ribosomal DNA regions, which confirmed the isolation of *F. incarnatum* from all cultivars. Copper oxide nanoparticles (CuONPs) at different concentrations showed inhibitory activity against *F. incarnatum* growth and mycotoxins, particularly at 400 ppm. The production of *F. incarnatum* mycotoxins, *i.e.*, beauvericins, fusarins, moniliformin, and enniatins, was inhibited to 62.8%, 45.4%, 58.1%, and 55.0%, respectively at 400 ppm of CuONPs compared to the control. Shrinkage of the *F. incarnatum* cell membrane and collapsing of the cell walls were recorded *via* transmission electron microscopy at 400 ppm, but negligible distortion appeared at 100 ppm of CuONPs. CuONPs at 100 ppm encouraged the activity of CMC-ase, zylanase, and amylase, while 200 and 400 ppm promoted less enzyme activity. The current findings suggest that CuONPs have a fungistatic effect on *F. incarnatum* and their mycotoxins.

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INTRODUCTION

Numerous fungi usually attack cereal grains, seeds, vegetables, fruits, as well as their foodstuff products either in the field or storage places. The ability of these fungi to produce mycotoxins has been documented under particular conditions, which minimizes the marketable and dietary value of these products, as well as results in undesirable health impacts on consumers including humans and animals (Abdelghany 2006). Many scientific papers have reported that *Fusarium* is considered one of the greatest economically

detrimental fungal genera for many reasons, *i.e.*, its phytopathogenic activity, potent producer for mycotoxins, and illness impacts on humans and livestock (Abdelghany 2014; Villani *et al.* 2019; Agriopoulou *et al.* 2020; Nofal *et al.* 2021a). Multiple documents have reported that *Fusarium* species are responsible for the production of several mycotoxins, *e.g.*, fumonisins, trichothecenes, zearalenone, enniatins, and fusaproliferin (Munkvold 2017; Jajić *et al.* 2019; Gautier *et al.* 2020). Recently, Choi *et al.* (2021) reported the presence of *Fusarium* spp. in millet samples with the highest frequency of mycotoxin being *F. asiaticum*, followed by *F. acuminatum, F. graminearum, F. incarnatum, F. equiseti*, and *F. tricinctum* besides other species of *Fusarium*. Occurrences of zearalenone, deoxynivalenol, fumnasine, and nivalenol in these millet samples were also recognized. The trichothecenes A and ZEN found in corn were *F. incarnatum* (Munkvold 2017). Yazid *et al.* (2021) showed that three isolates of *F. incarnatum* produced ZEN, while HT-2 and T-2 toxins were produced by one isolate.

Previously, different strains were isolated from Tunisian sorghum, and the presence of ZEA in sorghum samples varied according to the strain (Lahouar *et al.* 2017). Villani *et al.* (2019) mentioned that *F. incarnatum-equiseti* species complex (FIESC) covers 33 phylogenetically diverse species that have been isolated from diverse biological samples as well as agricultural soils. According to Villani *et al.* (2019), although miscellaneous mycotoxins were produced by FIESC, its potential to cause mycotoxin contamination in grains and other food products is unclear because the diversity of these species has only been recently documented.

Outside of the nutritional value, the therapeutic potential of garlic (*Allium sativum* L.) has been previously confirmed and is still the focus of studies, due to the presence of multiple bioactive molecules (Singh and Sahu 2020). Garlic is considered one of the oldest and most imported crops in all countries; therefore its worldwide production has been regularly increasing over the last two decades. Although numerous scientific reports mention the antifungal activity of garlic extract *in vitro* and *in vivo*, this activity is quite a paradox, since the garlic plant or its fruits can be colonized by numerous fungi, particularly *Fusarium* species comprising of *F. equiseti, F. proliferatum, F. oxysporum, F. acuminatum*, and *F. solani* (Stankovic *et al.* 2007; Ignjatov *et al.* 2018; Miriam *et al.* 2021; Paul *et al.* 2021). The deteriorative efficacy of *Fusarium* spp. as well as other fungi on plants are due to the secretion of cell wall-degrading enzymes (CWDEs), *e.g.*, lignocellulases, lipases, and proteases, which are responsible for the damage of the cell wall of plants (Nofal *et al.* 2021b; Al-Rajhi *et al.* 2022). Furthermore, the produced fungal toxins suppress the immune system of the plant, which helps the fungi successfully attack the plant (Lakshmipriya *et al.* 2020). Mallebrera *et al.* (2018) mentioned that *Fusarium* toxins enniatins and beauvericins encourage DNA fragmentation in addition to apoptosis through disorder in the mitochondrial pathways.

The search for effective approaches to eliminate pathogenic fungi and their toxins has become a global demand, so scientists turned to applying nanotechnology as a promising and potentially safe method for controlling fungal proliferation. Nanoparticles (NPs) have been applied as fungicide to restrict growth and mycotoxin production (Abdelghany *et al.* 2018a; Ganash *et al.* 2018; Jesmin and Chanda 2020). However, the activity of NPs on the production of mycotoxins has not been researched enough yet. More recent attention has been focused on the application of copper nanoparticles (CuNPs) and copper oxide nanoparticles (CuONPs) in these fields (Khamis *et al.* 2017; Abdelghany *et al.* 2020). It has been found that CuONPs suppresses *Fusarium culmorum*, *F. graminearum*, *F. oxysporum*, *F. solani*, *Penicillium chrysogenum*, *Aspergillus flavus*,

and *Alternaria alternate*, and *Aspergillus niger* growth (Ghasemian *et al.* 2012; Shende *et al.* 2015; Abdelghany *et al.* 2020). The effect of CuONPs exhibited genotoxicity in *F. solani* and *Penicillium digitatum* as a result of exposure to CuONPs (Khamis *et al.* 2017). The repression of mycotoxigenic fungi growth is an area of interest for numerous investigators; therefore, the current study aimed to evaluate the activity of CuONPs against the growth, ultrastructure, and mycotoxins, as well as hydrolytic enzyme production of *F. incarnatum* isolated from garlic (*Allium sativum* L.).

EXPERIMENTAL

Isolation and Identification of Mycotoxins Producing Fungus

Cloves of garlic (*Allium sativum* L.) that showed fungal infection (as shown in Fig. 1A) from a field in Menofia Governorate, Egypt were separated, peeled off, and the surface was disinfected using 5% NaOCl for 2 min. They were rinsed in sterile water using three successive baths of for 1 min each, then dried using sterile filter paper to remove the excess water. Small, infected tissue samples with symptoms of fungal appearance (1 cm in length) were plated onto potato dextrose agar medium (PDA) amended with streptomycin sulphate (3%) and incubated for 6 d at a temperature of 28 °C until fungal growth appeared. The hyphal tips of the appeared *Fusarium*-like colonies were purified by being transferred to PDA plates and were incubated at a temperature of 28 °C until the purified fungus appeared, which was kept for further study (Dhingra and Sinclair 1995). The purified fungus was morphologically identified by the presence of macroconidia, microconidia, chlamydospores, as well as by the observation of the growth rate and the color of the colony (Nelson *et al.* 1983; Leslie and Summerell 2006).

Molecular Identification of the Pathogen

The purified and morphologically identified fungus was subjected to molecular identification (Abdelghany *et al.* 2018b; Hami *et al.* 2021). For identification, fungal DNA was extracted using a Microprep Kit of quick-DNA fungi/bacteria (Zymo research; D6007) according to the procedure outlined by Sigma Scientific Services Company. Maxima Hot Start PCR Master Mix (Thermo; K1051) was utilized for the PCR. Amplification of the DNA was done using forward primer ITS1-F (5′- TCCGTAGGTGAACCTGCGG-3′) and reverse primer ITS4-R (5′- TCCTCCGCTTATTGATATGC-3′). Various thermal cycler conditions throughout the PCR protocol were used, starting with 1 cycle at a temperature of 94 °C for 6 min, which was required for the initial denaturation, followed by 35 cycles for 45 s at a temperature of 94 °C, which was required for another denaturation, then 35 cycles for 45 s at a temperature of 56 °C, which was required for annealing, and ending with 35 cycles for 60s at a temperature of 72 °C, which was required for extension. The fungus DNA was scanned *via* gel electrophoresis. Sequences homologous at NCBI (http://ncbi.nlm.nih. gov/BLAST) were analysis via BLAST. The neighbor joining manner was used for the reconstruction of the evolutionary tree.

Copper oxide Nanoparticles and Rizolex-T50

Copper oxide nanoparticles (CuONPs) were purchased from Nawah Scientific, Cairo, Egypt. Particles were less than 100 nm and came in powder form. The size was confirmed by scanning electron microscopy (FEI Company, Hillsboro, Oregon-USA) at EDRC, DRC, Cairo). Rizolex-T 50 (20% Tolclophos-methyl and 30% Thiram) was

applied as a chemical fungicide, which was purchased from Sumitomo Chemical, Tokyo, Japan.

Assessment of Copper Oxide Nanoparticles (CuONPs) Activity on Growth and Ultra-Structures of Pathogen

The inhibitory action of CuONPs at different concentrations against *F. incranatum* growth was achieved using Petri dishes containing Czapek agar media. Rizolex-T50 was applied as a positive control for fungal growth. The plates were centrally point-inoculated with a 0.5 mm disc from 5-day-old colony cultures of the pathogen, and then incubated for 7 d at a temperature of 28 °C. At the end of the incubation period, the radius of the colony was measured to calculate the inhibition %, according to Eq. 1,

$$\text{Inhibition \%} = \frac{(\text{CRC} - \text{CRT})}{\text{CRC}} \times 100 \qquad (1)$$

where CRC (mm) is the colony radius of the control and CRT (mm) is the colony radius of the treatment (Note: CuONPs nil medium was used as the control).

The tips of the growing fungus hypha at different concentration of CuONPs were cut and fixed using 5% gluteraldehyde for 1 d. Then, the samples were washed in phosphate buffer (pH 7.2) 3 times, followed by the elimination of the excess buffer. Next, the segments were rinsed for 2 h in osmium tetraoxide diluted up to 1%, followed by the removal of osmium tetraoxide. The prepared segments were dehydrated by a sequence of different levels of ethanol, ranging from 50% to 95%. The segments were dried with absolute ethanol, and then dipped in propylene oxide for 1 h. The segments of hyphae were placed in propylene oxide and Epon 812 resin at a 2 to 1 ratio, followed by being placed in pure resin for 12 h, before finally being placed in an oven for 48 h at a temperature of 60 °C . Blocks (50 nm) were segmented *via* ultra-microtome, then stained with uranyl acetate-lead citrate 500A and examined at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt *via* transmission electron microscopy (TEM) (C Joel Jem- 1200 EX II. Acc. Voltage 120 KV. MAG- medium) (Abdelghany *et al.* 2021).

Assessing Copper Oxide Nanoparticles (CuONPs) Activity on Mycotoxins production

Sterilized potato dextrose broth medium supplemented with different concentrations of CuONPs (50 mL in a 250 mL conical flask) was inoculated with *F. incranatum* and then incubated for 10 d at a temperature of 28 °C. At end of the incubation period, the fungal mycelia were decanted, and the filtrate medium was extracted with 50 mL of chloroform and methanol (at a 2 to 1 ratio), followed by desiccation to concentrate the extracted solvent (El-Taher *et al.* 2012). Then the extract was subjected to LC1620A liquid chromatography for mycotoxin analysis. The inject volume was 20 uL, the mobile phase water to acetonitrile to methanol was 5 to 4 to 1, the flow was 1.540 mL/min, and the current detector UV-Detector was at a wavelength of 254 nm. The mycotoxins were identified by comparing the retention time of the analyte with labeled internal mycotoxin standards.

Assessment of the Copper Oxide Nanoparticles (CuONPs) Activity on Hydrolytic Enzyme Production

Three substrates, including 0.5% carboxymethyl cellulose, 0.5% xylan, and 2% starch (Sigma-Aldrich, St. Louis, MO), were used for stimulating the production of CMC-

ase, xylanase, and amylase, respectively (El-Katatny 2010). The sterilized minimal synthetic medium (MSM) (NH$_4$NO$_3$ 1.0 g/L, K$_2$HPO$_4$ 0.9 g/L, KCl 0.2 g/L, MgSO$_4$·7H$_2$O 0.2 g/L, MnSO$_4$ 0.002 g/L, FeSO$_4$·7H$_2$O 0.002 g/L, ZnSO$_4$ 0.002 g/L, and agar 20 g/L) containing the appropriate substrate and different concentrations of CuONPs (at a pH of 5.5 adjusted by 50 mM acetate buffer) in a plate was inoculated with the isolated fungus in the center of plate and incubated for 5 d. At the end, the enzymes activities were visualized *via* staining the agar plates with 0.2% Congo red dye for 15 min, followed by washing with a 1 M NaCl solution for detecting the CMC-ase and xylanase (Herculano *et al.* 2011). The amylase was detected by flooding the plate with 1% iodine from 2% potassium iodide. The enzymatic index (EI) was determined by measuring the enzymatic halo (diameter of colony with the clear zone) according to Eq. 2,

$$EI = \frac{\text{Diameter of the degradation halo (mm)}}{\text{Average of the colony diameter (mm)}} \qquad (2)$$

Statistical Analysis

All the results were analyzed *via* SPSS software (version 14, IBM, Armonk, NY). Calculations were done for the mean ± SD (standard deviation) of three replicates.

RESULTS AND DISCUSSION

Fungal Identification of the Infected Cloves of Garlic Biomass

The collected samples of garlic were covered by a white mycelium (as shown in Fig.1A), with soft and spongy bulbs.
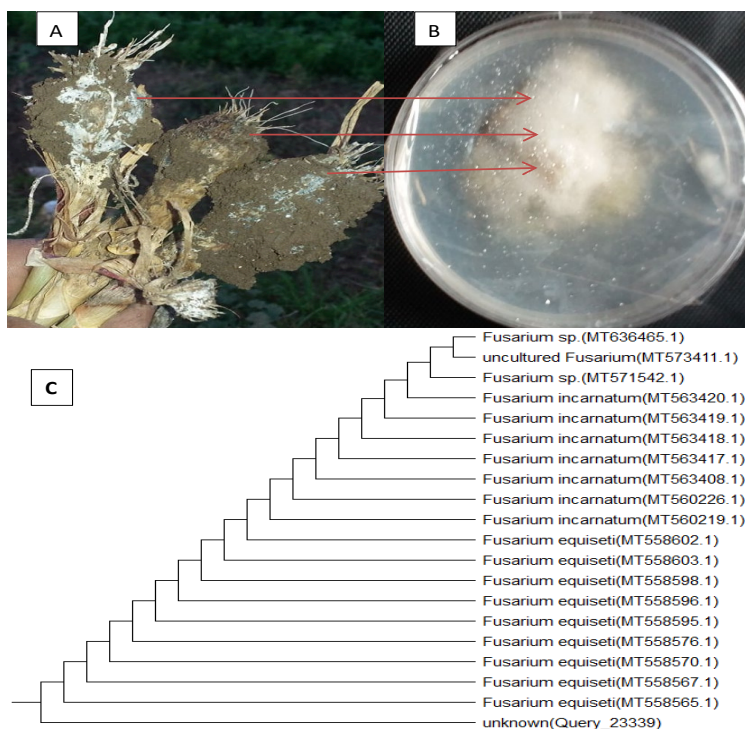

Fig. 1. Garlic samples infected with *F. incarnatum* (A); colony of *F. incarnatum* on a Petri dish containing PDA medium (B); and the phylogenetic tree of the *F. incarnatum* strain JL5-2 (C)

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The isolated fungus was morphologically identified as *F. incarnatum*, where at the initial stage the aerial mycelia were white (Fig. 1B) and turned to off-white, pale pink, and grey in the later phases, in addition to the microscopic examination. Molecular characterization of the isolate was conducted to recognize and confirm that the identity isolate was closely related to *F. incarnatum*. The phylogenetic tree demonstrated the similarity of the isolate to *F. incarnatum* (Fig. 1C). A BLAST search in the NCBI database *via* ITS sequences (Fig. 2) showed 99% to 100% similarities with *F. incarnatum*. *F.incarnatum* has been isolated from various plant material in different countries as well as other species, as mentioned in numerous reports. For example, it was detected on rapeseed in Iran (Nemati *et al.* 2015). Other *Fusarium* spp. were isolated from garlic, *e.g.*, *F. proliferatum* (Mondani *et al.* 2021). Recently, *F. proliferatum, F. solani, F. acuminatum, F. oxysporum, F. redolens*, and *F. subglutinans* have been detected in rot garlic (*A. sativum*) (Gálvez and Palmero 2021). Previously, *Penicillium*, *Aspergillus*, *Botrytis*, and *Fusarium* genera were detected in garlic bulbs in Egypt, but *Fusarium* spp. represented the most prevalent genera (Moharam *et al.* 2013).

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Fusarium sp. isolate LS479414 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and inter	798	798	100%	0.0	100.00%	MT636465.1
Uncultured Fusarium clone BFRIPFE1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, an	798	798	100%	0.0	100.00%	MT573411.1
Fusarium sp. strain A17 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete seq	798	798	100%	0.0	100.00%	MT571542.1
Fusarium incarnatum strain JL5-2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and inte	798	798	100%	0.0	100.00%	MT563420.1
Fusarium incarnatum strain JL3-4-1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and i	798	798	100%	0.0	100.00%	MT563419.1
Fusarium incarnatum strain CBB-2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and in	798	798	100%	0.0	100.00%	MT563418.1
Fusarium incarnatum strain JL3-3 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and int	798	798	100%	0.0	100.00%	MT563417.1
Fusarium incarnatum strain CBA-3 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and in	798	798	100%	0.0	100.00%	MT563408.1
Fusarium incarnatum strain CBB-1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and in	798	798	100%	0.0	100.00%	MT560226.1
Fusarium incarnatum strain CBA-2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and in	798	798	100%	0.0	100.00%	MT560219.1
Fusarium equiseti strain CB33-2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and inter	798	798	100%	0.0	100.00%	MT558602.1
Fusarium equiseti strain DH02-6-1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and int	798	798	100%	0.0	100.00%	MT558603.1
Fusarium equiseti strain TH01-3 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and inter	798	798	100%	0.0	100.00%	MT558598.1
Fusarium equiseti strain CB33-1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and inter	798	798	100%	0.0	100.00%	MT558596.1
Fusarium equiseti strain TH04-1-1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and int	798	798	100%	0.0	100.00%	MT558595.1
Fusarium equiseti strain TH01-3-1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and int	798	798	100%	0.0	100.00%	MT558576.1
Fusarium equiseti strain DH14-1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and inter	798	798	100%	0.0	100.00%	MT558570.1
Fusarium equiseti strain DH02-6 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and inter	798	798	100%	0.0	100.00%	MT558567.1
Fusarium equiseti strain CB20-5 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and inter	798	798	100%	0.0	100.00%	MT558565.1

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Sbjct  439  TCGTTACTGGTA   450
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Fig. 2. The ITS sequences of the closely related fungal strains retrieved from the NCBI GenBank database and the cluster analysis of *F. incarnatum*

Inhibitory Action of Copper Oxide Nanoparticles (CuONPs) Against Fungus Growth

The inhibitory action of CuONPs (size less than 100 nm shown in Fig. 3) in terms of *F. incarnatum* growth and sporogenesis was observed to be dependent on the concentration (as shown in Table 1). The obtained results indicated that the inhibition of growth reached up to 81.70% at 400 ppm when compared to the control zero %. According to the microscopic analysis and spore counting, sporulation (%) decreasing was recorded as the CuONPs concentration increased. Although the inhibitory action of the chemical fungicide (Rizolex-T50) and 400 ppm of CuONPs was approximately in a similar narrow spectrum (85.36 % and 81.70%, respectively), but the sporulation (%) was far apart from each other (10 and 29%, respectively).
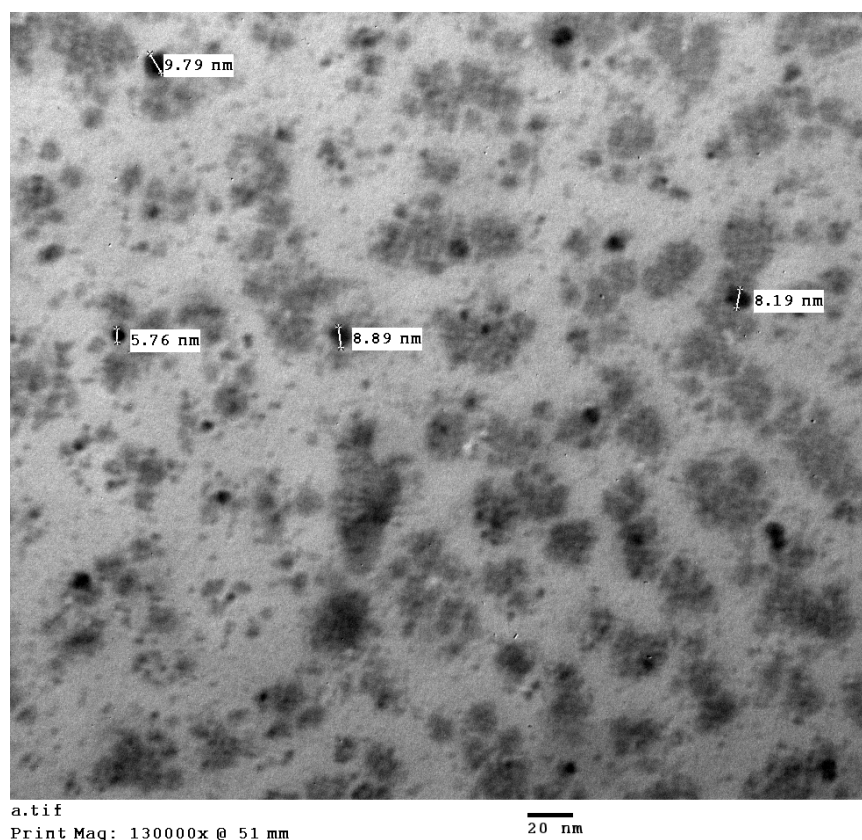

Fig. 3. Scanning electron micrograph of CuONPs. Magnification 12000X

Table 1. Colony Diameter, Inhibition and Sporulation of *F. incarnatum* at Various Copper Oxide Nanoparticles (CuONPs) Concentrations

CuONPs Concentration (ppm)	Colony Diameter (cm)	Inhibition (%)	Sporulation (%)
Control	8.2 ± 0.25	0.0	100
100	7.5 ± 0.15	8.53	97
200	3.5 ± 0.25	57.31	60
400	1.5 ± 0.11	81.70	29
Chemical fungicide	1.2 ± 0.33	85.36	10

Recent results showed the substantial suppression of *F. oxysporum* spore propagation after exposure to CuONPs (Ashraf *et al.* 2021). The inhibitory activity of CuONPs and chemical fungicide on fungal growth was confirmed and evaluated also in broth medium, which indicated that growth reduced with increasing CuONPs concentrations (Fig. 4).

The fungicidal activity of CuONPs was reported against multiple fungi. For instance, *Fusarium solani* and *Penicillium digitatum* had full inhibition at 60 µg/mL and 20 µg/mL, respectively (Khamis *et al.* 2017). In another study, the controlling effect of CuONPs was recorded for *Penicillium expansum, Alternaria alternata*, and *A. solani* (Nemati *et al.* 2015).
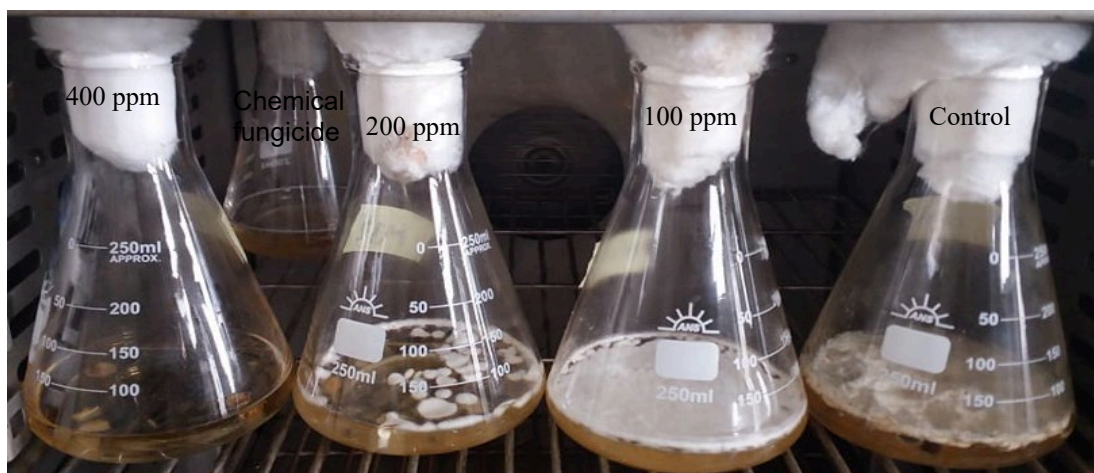

Fig. 4. Growth of *F. incarnatum* at different concentrations of CuONPs (100, 200, and 400 ppm) in broth medium

A mechanistic study of NPs against various fungi has been investigated. For instance, injury of the lipid bilayer in fungal cell membranes, as well as the formation of unusual swells on the surface of the hyphae, which led to hyphae deformation, were observed as a result of exposure to ZnO nanoparticles (He *et al.* 2011).

In the current research, the TEM showed that CuONPs caused alteration in the cell wall, as well as disruption of the cell membrane of *F. incarnatum*. Similarly, but on another fungus, CuNP treatments stimulated the distortion of *A. niger* hyphae at 200 and 300 ppm (Abdelghany *et al.* 2020).

In addition, sharp changes were observed in cell organelles of *Fusarium oxysporum* and *F. solani* hyphae treated with CuNPs (Pariona *et al.* 2019). Severe shrinkage of the cell membrane and the collapsing of the cell wall at high concentrations of CuONPs (400 ppm) are shown in Fig. 5.

Different levels of damage were observed in hypha or conidiospores but were dependent on the concentration; negligible deformation appeared at 100 ppm and was obvious at 200 and 400 ppm of CuONPs. It can be predicted that the oxidative stress stimulated by high levels of CuONPs, in addition to its toxicity mechanism, cause the disturbances of the metabolic pathways of the enzymes. Morphological disruptions in the *F. oxysporum* mycelia were observed recently by Ashraf *et al.* (2021), as well as reactive oxygen species (ROS) generation on the surface of the cell wall surface, which led to complete destruction.
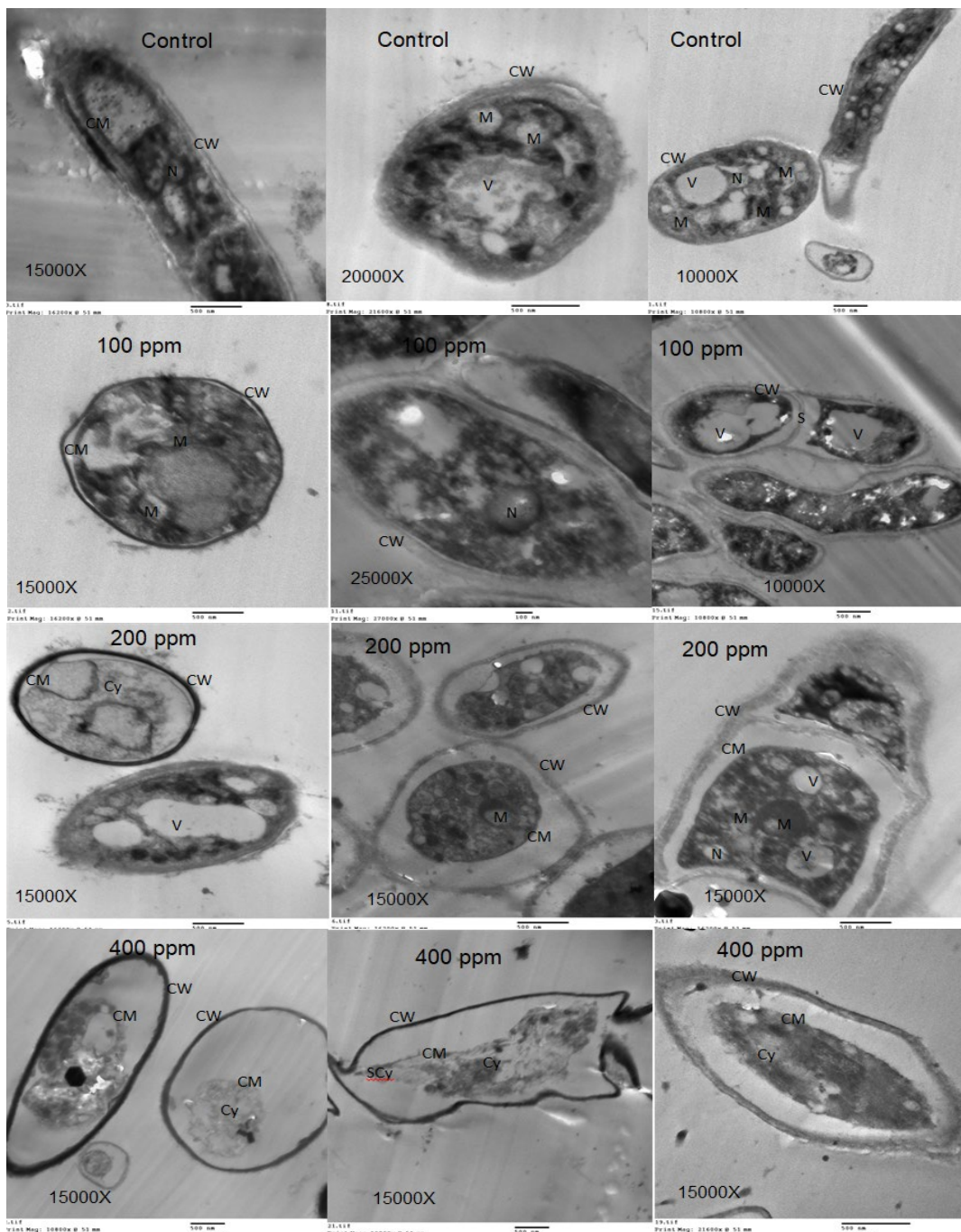
Fig. 5. The ultrastructures of *F. incarnatum* at different concentrations of CuONPs (100 ppm, 200 ppm, and 400 ppm) (Note: CW = Cell wall; S = septum; SCy = shrinkage cytoplasm; N = nucleus; V = vacuole; M = mitochondria; and CM = cell membrane)

Inhibition of Mycotoxin Production

NPs have been widely applied for inhibiting the growth of many microorganisms, but in terms of the inhibition of mycotoxins production is still in its early phases. Liquid chromatography detected the produced mycotoxins by *F. incarnatum* (as shown in Table 2 and Fig. 6) under the effects of CuONPs. The obtained findings indicated that at concentrations that did not fully inhibit growth, the CuONPs were able to limit and decrease the mycotoxin secretions of *F. incarnatum* (as shown in Table 2).
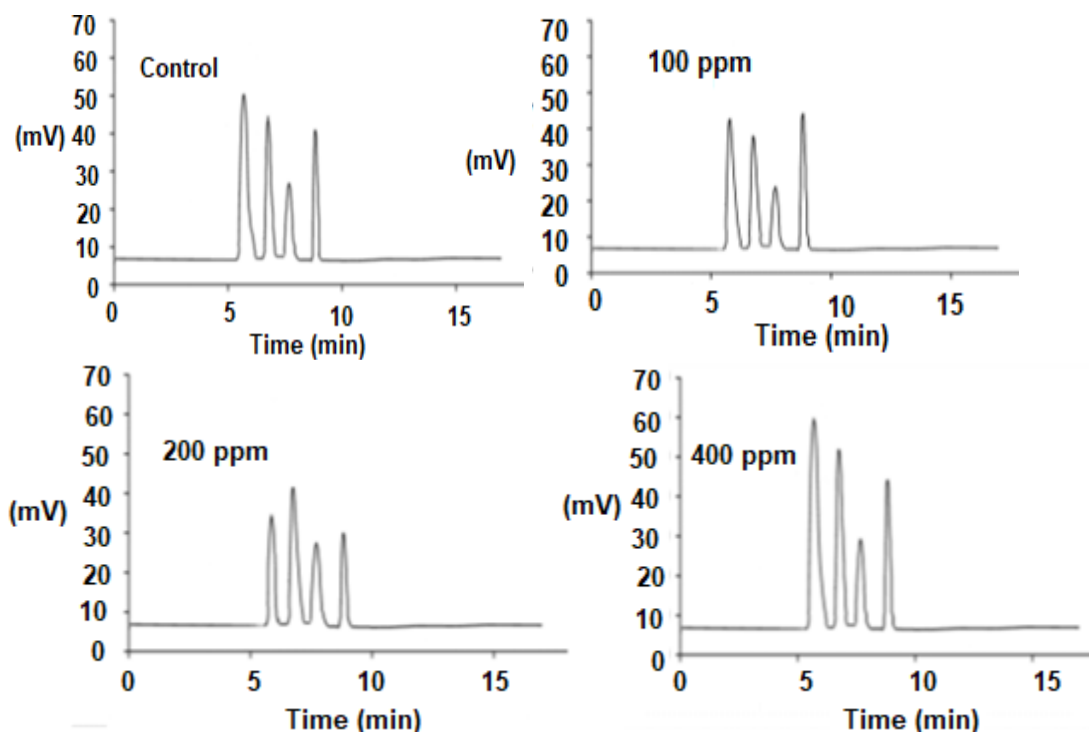
Fig. 6. The liquid chromatography chromatograms of the mycotoxins production of *F. incarnatum* at 100 ppm, 200 ppm, and 400 ppm of CuONPs

Table 2. Mycotoxin Production of *F. incarnatum* at Different Concentrations of Copper Oxide Nanoparticles (CuONPs)

CuONPs Concentration (ppm)	Mycotoxin Concentration (µg/mL)			
	Beauvericins	Fusarins	Moniliformin	Enniatins
Control	65.01 ± 0.01	58.71 ± 0.02	38.92 ± 0.01	55.63 ± 0.02
100	50.23 ± 0.02	44.84 ± 0.12	28.40 ± 0.35	40.24 ± 0.21
200	42.33 ± 0.02	39.98 ± 0.01	23.74 ± 0.01	33.43 ± 0.05
400	24.21 ± 0.12	32.03 ± 0.02	16.30 ± 0.01	25.01 ± 0.05
*I% Control/400	62.77 ± 0.02	45.44 ± 0.01	58.12 ± 0.01	55.04 ± 0.2
Retention time	11.5	8.8	10.24	13.00
Note: I is the inhibition % of the mycotoxins at 400 ppm compared to the control				

The changes in the productivity of mycotoxins may be due to the interference of CuONPs with the metabolic pathways of secondary metabolites. Four mycotoxins, *i.e.*, beauvericins, fusarins, moniliformin, and enniatins, were detected in the metabolized medium of *F. incarnatum*. Unfortunately, the complete inhibition of these mycotoxins did not occur until high concentrations of CuONPs were used. A recent idea focused on the application of engineered NPs at low levels to inhibit mycotoxins without killing the producer fungi, in order to avoid NPs toxicity in humans (Jesmin and Chanda 2020). A study conducted by Asghar *et al.* (2018) exhibited that the synthesis of aflatoxin B_1 was reduced under the effect of CuNPs. The inhibition % of beauvericins, fusarins, moniliformin, and enniatins synthesis at 400 ppm compared to the synthesis under the control treatment was 62.8%, 45.4%, 58.1%, and 55.0%, respectively (as shown in Table

2). The occurrence of these compounds, even in low concentrations, still can be unsafe, particularly in more consumed plant products. Seefelder *et al.* (2002) reported the presence of mycotoxins in garlic bulbs infected by fungi in Germany. Reduction of mycotoxins production under the effect of CuONPs may explained on the base of fungal growth retardation or the adsorption of mycotoxin on the nanoparticles.

Hydrolytic Enzymes

The obtained findings showed that *F. incarnatum* isolated from garlic was able to decompose the substrates *via* the hydrolytic enzymes CMC-ase, xylanase, and amylase with EI values greater than 1 (Table 3) at all the tested concentrations of CuONPs. The EI of each enzyme varied, which suggested different amounts of enzyme production depending on the CuONP concentration. An unexpected result was observed, where 100 ppm of CuONPs stimulated the production of enzymes with EIs of 1.31, 1.36, and 1.23 for CMC-ase, xylanase, and amylase, respectively, when compared with the EIs of the control or at 400 ppm of CuONPs. This finding was consistent with recent documentation by Abdelghany *et al.* (2020), which indicated that CuNPs at low level were efficient in terms of encouraging laccase production by *A. niger*, while it suppressed enzyme production at higher concentrations. The copper metal may interfere as a complementary for the composition of some enzymes. As mentioned previously, the synthesis of β-glucosidase, cellobiohydrolase, and β-xylosidase were repressed while Mn peroxidase was unaffected by the presence of CuNPs (Shah *et al.* 2010).

Table 3. Enzymatic Index of the CMC-ase, Xylanase, and Amylase Activities at Different Copper Oxide Nanoparticles (CuONPs) Concentrations

CuONPs Concentration (ppm)	CMC-ase	Xylanase	Amylase
Control	1.32 ± 0.01	1.36 ± 0.03	1.23 ± 0.03
100	1.42 ± 0.03	1.38 ± 0.06	1.41 ± 0.03
200	1.22 ± 0.01	1.29 ± 0.02	1.14 ± 0.06
400	1.17 ± 0.02	1.08 ± 0.01	1.05 ± 0.02

CONCLUSION

1. Inhibition efficiency against *F. incarnatum* increased as the CuONP concentration increased, up to 400 ppm. The risk of mycotoxins produced by *F. incarnatum* may be reduced by CuONPs, but further study is needed to reach the complete inhibition of its synthesis.

2. The application of CuONPs caused alteration in the ultrastructures of *F. incarnatum*, particularly at CuONP concentrations of 200 ppm and 400 ppm, as well as changes accompanied with decreasing in the activity of the hydrolytic enzymes, *i.e.*, CMC-ase, zylanase, and amylase.

Competing Interests

The authors declare that they have no conflict of interest.

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