A Novel Biological Approach to Copper Nanoparticles Synthesis: Characterization and its Application Against Phytopathogenic Fungi

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Research

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Abstract

Background: In nanotechnology, fungi are recognized as a good candidates for the creation of nanomolecules, so offering a cleaner alternative to synthesize novel resources with a varied array of potential requests in therapeutic and manufacturing fields.

Materials and methods: In this respect, a novel biological approach *Penicillium olsonii* have demonstrated excellent synthesis capacity to produce copper nanoparticles (CuNPs). Characteristics of CuNPs were detected by variable tools including UV-Vis spectrum, Scanning Electron Microscopy (SEM) and Fourier transform infrared spectroscopy (FT-IR)

Results: UV-Vis spectra with characteristic absorption peak was observed at 565nm. Biomolecules mediating the synthesis and stabilizing the nano-fungicide was studied with FTIR that showed different functional groups. SEM investigations confirmed that size of CuNPs were varied from 6-26 nm. The antifungal activity of CuNPs was evaluated by testing against three phytopathogenic fungi including *Fusarium oxysporum*, *Fusarium solani* and *Curvularia curvulatat* with growth inhibition 86.25, 32.92 and 68.42 %, respectively at 200ppm. *F. oxysporum* was more affected by CuNPs followed by *C. curvulata* and *F. solani*.

Conclusion: The current effort established that it's possible to achieve the production of CuNPs by *P. olsonii* with its fungicidal applicable potential.

Introduction

Nanoparticles have been synthesized through several physical and chemical processes; however, some chemical methods are costly and inefficient and generate hazardous wastes that are risky for the environment[1]. Therefore, there is an urgent need to develop environmentally friendly biological process for nanoparticle synthesis. Till date, the research in the field of biosynthesis has been mainly focused on Ag and Au nanoparticles, and there have been very few reports on the synthesis of Cu/CuO nanoparticles[2]. Microorganisms act as a biofactory and can also be used for the synthesis of metal nanoparticles. Fungi, due to their tolerance and bioaccumulation ability of metals, are taking the centre-stage of studieson biological metal nanoparticle generation. But a literature review, Varshney *et al*.[3] revealed only few studies on the biosynthesis of copper nanoparticles (CuNPs) using fungi. Majumber[4] used a fungal species (*Fusarium oxysporum*) to synthesize CuNPs (93–115 nm) at ambient temperature. Pavani *et al*.[5] used *Aspergillus* species of fungus for extracellular synthesis of CuNPs. *Penicillium vaksmanii, P. aurantiogriseum*, and *P. citrinum* have been used for the synthesis of CuNPs[6]. Also dead biomass of *Rhodotorula mucilaginosa* may considered an efficiently bioprocess, being fast and low-cost to production of CuNPs and also a probably nano-adsorbent of this metal ion in wastewater in bioremediation process[7]. CuNPs as well as other nanoparticles have been characterized by ultraviolet–visible absorption spectroscopy, X-ray diffraction[8], scanning electron microscopy[7, 9] transmission electron microscopy [10, 11] and atomic force microscopy [12, 13].

Several nanoparticles are being explored these days for their antimicrobial effects[14], which can be beneficial or harmful, depending on the context. Silver nanoparticles are the most widely studied and used as general antimicrobials [15, 16, 17, 18]. However, CuNP have also been reported as effective antimicrobials in several studies [14, 19]. Copper as a metal exhibit broad-spectrum biocidal activity, and several studies during the last two years found that copper demonstrates remarkable antibacterial activity at the nanoscale[20]. Copper is an essential element for living organisms and may be suitable for biomedical applications [21]. Yoon *et al*.[10] demonstrated that the antibacterial effects of silver and CuNPs using single representative strain of *E. coli* where the CuNPs showed superior antibacterial activity compared to the silver.

CuNPs showed significant antifungal activity against plant pathogenic fungi, *Fusarium oxysporum, Alternaria alternata, Curvularia lunata* and *Phoma destructive*[22]. At low concentration, CuNPs promoted the growth of the plant pathogenic fungi *Botrytis fabae, Fusarium oxysporum* f.sp. ciceris, *F.oxysporum f.sp. melonis, A. alternate* and sporulation of *Trichoderma harzianum* but at 800 mg/L completely inhibited mycelial growth of *A. Alternata*[23]. Non-biocidal effect of CuNPs against beneficial microbes was reported and indicates its potential use in the agri-ecosystem[23]. On the other hand if CuNPs used as fungicide, Ramesh *et al*. [24] reported that CuNPs enhance the germination and growth of some plants at lower concentrations, whereas high concentrations result in retarded growth and show good antimicrobial activity inhibiting the growth of pathogenic bacteria and pathogenic fungi. The purpose of this study was to synthesis CuNPs using a novel biological approach *Penicillium olsonii*.

Material And Methods

Screening for Copper Nanoparticles Synthesis

To explore CuNPs synthesizing agent, samples from soil of peri-urban agricultural areas next to some metal factories in Monoufeya Governorate, Egypt were collected and considered to be source for fungal isolation. Potato Dextrose Agar (PDA) medium was amended with different concentration of filtered and sterilized $CuSO4{\cdot}5H2O$ solution ranged from 200 to 1000 ppm and inoculated with particles of soil sample. Followed by incubation at 30 °C for 7 days. The appeared and recovered isolate at highest concentration was considered as copper-resistant fungi and subjected to a screening process for a biosynthesizes of CuNPs.

Identification of highest copper resistance

The copper resistant fungus was purified and identified using the keys of Pitt [25] and Domsch et al. [26] based on the macroscopic and microscopic characteristics including rate of colony growth, colony color and shape, reverse color, septation and diameter of mycelium, texture and size of conidia, shape and diameter of conidiophore. The identification was confirmed using molecular tool of PCR according to White *et al.* [27]. After incubation period 7 days, the growing fungus (0.2 g) on PD broth medium was removed and mixed with 300 ul sterile and distlled water in microcentrifuge tube with 95 ul solid tissue buffer (blue) and 10 ul proteinase K. Then mix thoroughly followed by the incubation at 55 °C for 2 hours. The reaction mixture was re-mix thoroughly, followed by centrifugation at 12,000 x g for 1 minute. The aqueous supernatant was transferred to a clean tube (300 ul) containing 600 ul Genomic Binding Buffer and mix thoroughly. The mixture was transfer to a Zymo-Spin™ IIC-XL Column in a Collection Tube and centrifuged at 12,000 x g for 1 minute. Discard the collection tube with the flow through. DNA Pre-Wash Buffer (400 µl) was added to the column in a new collection tube and centrifuged at 12.000 xg for 1 minute. Then700 µl g-DNA wash buffer was added to the reaction mixture and centrifuge at (12.000 xg) for 1 minute, followed by the empty of the collection tube. Then, 200 µl g-DNA wash buffer was added and centrifuged at 12.000 xg for 1 minute. Discard the collection tube. Discard the collection tube. Three µl elution buffer was added and incubated for 5 minutes, and then centrifuged at 12.000 xg for 1 minute. PCR reaction set-up: Twenty five µL MyTaq Red Mix, 8 µL DNA Template, 1 µl (20 Pico mol) Forward Primers, 1 µl (20 Pico mol) Reverse Primers, 15 µL Nuclease Free Water. Thermal Cycler Condition: Initial denaturation at 94 °C for 6 min (1 cycle), Denaturation at 94 °C for 45 s, Annealing at 56 °C for 45 s, Extension at 72 °C for 1 min (35 cycle), Final Extension at 72 °C for 5 min (1 cycle).

Biosynthesis of CuPNs

Penicillium olsonii inoculated in potato dextrose (PD) broth media for 7 days at 28 °C. The biomass was harvested after complete incubation by filtering through filter paper followed by repeated washing with distilled water to remove any medium component from the biomass for several times. Three grams of fungus biomass was brought in contact with 100 mL of sterilized double distilled water with concentration of copper sulfate (0.02M) to obtain a blue solution and incubated at 25 °C for 3 days. Control (Without copper) was also run along with the experimental flask.

UV-visible spectroscopic analysis

The production CuPNs was confirmed by qualitative testing of supernatant by UV-visible spectrophotometer. One ml of sample supernatant was withdrawn after 24 hr and absorbance was measured by between 300–800 nm at Regional Center For Mycology And Biotechnology, Cairo, Egypt. (RCMB).

Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM) analysis

The dried powder of CuNPs (2 mg) was mixed with 200 mg KBr (FTIR grade) and pressed into a pellet. The sample pellet was located into the sample holder and FTIR spectra were recorded in the range 450- 4000-500 cm^{-1} in FTIR spectroscopy at a resolution of 4 cm^{-1}. To estimate the size of CuNPs, it characterized by SEM (C Joel Jem-1200 EX II. Acc. Voltage 120 KV. MAG-medium) at RCMB.

Antifungal activity of CuPNs with using Poisoned Food Technique

Potato dextrose agar medium (PDA) with different concentration (50, 100, and 200 ppm) of CuPNs. About 25 ml of the growth medium was poured into each Petri-dish and allowed to solidify. Five mm disc of 5-day-old culture of the test fungi that provided by RCMB was placed at the center of the Petri-dish and incubated at 28 ± 2 °C for 7 days, the growth of fungal colony was measured in millimeter. PDA medium without the CuPNs served as control.

The fungitoxicity of CuPNs in terms of percentage inhibition of mycelia growth was calculated by using the formula:

$$\%\ \text{inhibition radial growth} = \left(\frac{RG1 - RG2}{RG1} X100\right) \quad (1)$$

Where, RG1 = the radial growth at control, RG2 = the radial growth at treatment[28].

Result And Discusion

Initial extensive screen for copper metal resistance fungi, it was discovered that *Penicillium olsonii* outperformed other fungi with regard to fungal growth with copper different metal concentration levels up to 800 ppm. The identification of *P. olsonii* funguswas confirmed using molecular characterization, which is based on ITS rDNA (Fig. 1) and applied for CuPNs synthesis. Identification of fungi to the species level by molecular manner is principally based on the variable nature of the DNA's ITS regions [29]. The 18S rRNA sequence of the *P. olsonii* isolate was searched on a database (Basic Local Alignment Search Tool (BLAST) using multiple sequence alignment (Fig. 1b) with theMEGA6 software. The results of alignment profile, concluded that the *P. olsonii* 18SrRNA amplicon closely matched other *P. olsonii* strains at more than 99%). Figure 1 illustrated the structured phylogenetic relatedness of the full sequence of *P. olsonii* 18S rRNA that was compared to the closely related strains from the database (blast.ncbi.nlm.nih.gov/Blast.cgi).

During the present study, the capacity of *P. olsonii* MT635310.1 to synthesize CuNPs was observed where the aqueous Cu^{2+} ions were reduced during exposure to the culture supernatant of *P. olsonii* MT635310.1. The color of this solution changes from blue to green color after 72 h of mixing fungus biomass with copper sulfate solution which indicated the formation of CuNPs extracellularly (Fig. 2). The present findings are in agreement with the recent findings [30]. The aforementioned studies reported that the appearance of green color color is due to reduction of copper sulfate and its bioconversion to CuNPs.

The finding appeared that the reaction solution has an absorption maximum around 565 nm attributed to the surface plasmon resonance band (SPR) of the CuNPs. In general, it has been reported that CuNPs display a surface plasmon peak at 580-590[31]. Although Sudhir *et al.*[32] demonstrated that the biogenic CuNPs were characterized by UV-Vis spectrophotometer showing a typical resonance (SPR) at about 631 nm which is specific for CuNPs. CuNPs were studied recently by [30] at 200–1000 nm wavelength range and it has absorbance at 550 to 650 nm.

The size distributions of CuNPs in the aqueous solution was evaluated by SEM images (Fig. 3). The synthesized CuNPs by *P. olsonii* were observed by SEM with almost of spherical shape and the particle sizes varied from 6–26 nm. According to Ponnusamy *et al.* [33] CuNPs size were varied from 5 to 50 nm in diameter. SEM measurements by Chalandar *et al.*[34] estimated that extracellular biosynthesis of CuNPs with the diameters 40 nm. It is also reported that *Penicillium citrinum*, *P. waksmanii* and *P. aurantiogriseum* produced spherical CuNPs of size 90–295 nm[6]. The size variation may be related to culture conditions and producing microorganism.

FTIR analyses of the nanoparticles biosynthesized using *P. olsonii* MT635310.1 was recorded (Fig. 4). The FTIR spectra of the CuNPs biosynthesized revealed the presence of different functional groups. The strong and broad band observed at 3,471 cm^{-1} indicates the presence of polyphenolic O-H group and primary amine O-H band. A narrow band at 1639.73 cm^{-1}, indicates the presence of amide I. While band at 1448.19 cm^{-1} corresponding to C-C stretching aromatic ring, C-O stretching carboxylic acid group assigned at 1,243 cm − 1, ring, C-O stretching carboxylic acid group assigned at 1242 cm^{-1}. Cuevas et al. [35] mentioned that bands at 1243 and 1244 cm^{-1} are designated for bending vibration movements in amides I and amides III. The weak bands at

657 cm − 1 may correspond to alkyl halides. These main bands indicate the presence of protein on the surface of the CuNPs and copper oxide nanoparticles. Ore results agreement with previous studies[36, 37].

It has been reported that CuNPs act as a fungicide against different species of phytopathogenic fungi such as *Fusarium solani, F. oxysporum, Neofusicoccum sp*.[38]. CuNPs showed various levels of inhibition on colony growth of *F. oxysporum*, *F. solani* and *C. curvulata*. CuNPs at concentrations of 50, 1000 and 200 ppm. Inhibition % of fungal growth increased with increasing CuNPs concentrations (Table 1 & Fig. 5). These results were agreement with recently result of Chalandar *et al*.[34] who show that antifungal properties of CuPNs increased by increasing the concentration of nanoparticles to 15%. The obtained results showed that the sensitivity of tested fungi to CuNPs depend on species, since *F. oxysporum* was more sensitive to different levels of CuNPs than *Fusarium solani*, for example the inhibition % was 86.25 and 32.92, respectively at 200 ppm. Similar results have been reported for the antifungal activity of CuNPs against different fungal species [38]. Furthermore, similar to present findings, Sudhir *et al*.[32] reported that *Fusarium culmorum* was found to be most sensitive to CuPNs followed by *F. oxysporum* and *F. Graminearum*. The best charitеria of copper was observed by Ramesh *et al*.[39] where, the application of CuPNs against plant pathogenic fungi exhibited improvement to plant growth. The previous author estimated the antifungal activity of CuPNs against the pathogenic fungi *F.culmorum*, *F. oxysporum*, *F. graminearum* and *Phytophthora infestans* beside CuPNs at concentrations below 100 ppm have been reported to enhance germination and growth of some plants. CuNPs not only repress the fungal growth but also inhibit the sporulation process. This may help in elucidating the antifungal mechanisms of CuNPs .The increment of CuNPs concentration was accompanied by reduction of sporulation rate that reached to 10.5, 20.2 and 26.5% at 200 ppm for *F. oxysporum*, *F. solani* and *C. curvulata*, respectively compared to the control (100%). Sporogenisis *of Aspergillus niger* has been recently described as sensitive to CuNPs [40]

Table 1
Antifungal activity of different concentrations of CuNPs

CuNPs concentration ppm	*F. oxysporum*			*F. solani*			*C. curvulata*		
	Colony radius (cm)	Growth Inhibition %	Sporulation %	Colony radius (cm)	Growth Inhibition %	Sporulation %	Colony radius (cm)	Growth Inhibition %	Sporulation %
Control	8.0 ± 0.1	0.0	100	8.2 ± 0.2	0.0	100	3.8 ± 0.2	0.0	100
50	3.4 ± 0.2	57.50	82.0	8.0 ± 0.1	2.44	95	3.6 ± 0.1	5.26	88.0
100	1.8 ± 0.2	77.50	39.4	7.2 ± 0.2	12.10	25.5	2.2 ± 0.1	42.10	50.6
200	1.1 ± 0.5	86.25	10.5	5.5 ± 0.4	32.92	20.2	1.2 ± 0.5	68.42	26.5

Morphological of the tested fungi hyphae after exposure to CuNPs was examined in an attempt to understand its inhibition mechanism. Therefore, the underlying mechanism by which CuNPs kill fungi is reflected by changes of fungal morphology (Fig. 6). From the observed micrographs, the hyphae had a bigger and swollen appearance particularly at 200 ppm of CuNPs treatment. Hyphae vaculation of *F. oxysporum* could be clearly observed at 200 ppm of CuNPs. On the other hand, spore size and shape of *F. solani* were dramtecaly changed at high concentration. Chlamydospores were observed in *C.curvulata*, beside disruption of hyphae. Abd El-Mongy and Abd El-Ghany [41] reported that chlamydospores were formed in fungi under stress conditions. Microscopic observation revealed that the CuNPs clearly damaged the hyphae of *Alternaria alternata* and *Botrytis cinerea* [42]. Effect of CuNPs Transmission electron microscopy revealed that CuSO4 and CuNPs treatments encouraged the deformed appearance of the *A. niger* at 200 ppm and 300 ppm, particularly CuNPs[40].

Conclusions

The present work demonstrated that it is possible to perform the biogenic synthesis of CuNPs using *P. olsonii* as appropriate fungus. It should be mentioned that CuNPS at concentrations of 50, 100 and 200 ppm inhibited growth as well as sporogenisis of phytopathogenic fungi.

Declarations

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For Faculty of Science, Jazan University

Author contributions

Mohamed A. AL Abboud designed the experiments, wrote the paper, analyzed and interpreted the data.

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Availability of data and materials

The data set (table and graphs) supporting this article's conclusion is available.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The author declare that there is no conflict of interests regarding the publication of this paper.

Research Involving Humans and Animals Statement

None

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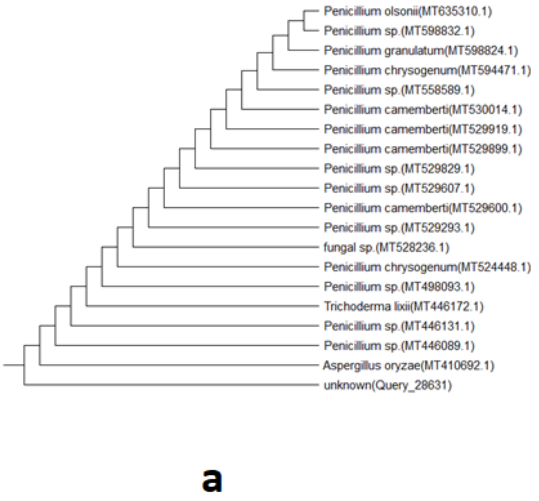
Figures



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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Penicillium olsonii internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and l	909	909	100%	0.0	100.00%	MT635310.1
Penicillium sp. isolate T36 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequen	909	909	100%	0.0	100.00%	MT598832.1
Penicillium granulatum isolate T27 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete	909	909	100%	0.0	100.00%	MT598824.1
Penicillium chrysogenum isolate FT3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, comple	909	909	100%	0.0	100.00%	MT594471.1
Penicillium sp. isolate F6001 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequ	909	909	100%	0.0	100.00%	MT558589.1
Penicillium camemberti clone SF_738 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and	909	909	100%	0.0	100.00%	MT530014.1
Penicillium camemberti clone SF_643 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and	909	909	100%	0.0	100.00%	MT529919.1
Penicillium camemberti clone SF_623 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and	909	909	100%	0.0	100.00%	MT529899.1
Penicillium sp. clone SF_553 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequ	909	909	100%	0.0	100.00%	MT529829.1
Penicillium sp. clone SF_331 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequ	909	909	100%	0.0	100.00%	MT529607.1
Penicillium camemberti clone SF_324 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and	909	909	100%	0.0	100.00%	MT529600.1
Penicillium sp. clone SF_17 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete seque	909	909	100%	0.0	100.00%	MT529293.1
Fungal sp. small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer	909	909	100%	0.0	100.00%	MT528236.1
Penicillium chrysogenum isolate air 7 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and	909	909	100%	0.0	100.00%	MT524448.1
Penicillium sp. isolate SCSIO06871 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complet	909	909	100%	0.0	100.00%	MT498093.1
Trichoderma lixii strain ZMXL3 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and interna	909	909	100%	0.0	100.00%	MT446172.1
Penicillium sp. strain ZMXR26 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and interna	909	909	100%	0.0	100.00%	MT446131.1
Penicillium sp. strain ZMQR8 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal	909	909	100%	0.0	100.00%	MT446089.1
Aspergillus oryzae isolate WC-3 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete se	909	909	100%	0.0	100.00%	MT410692.1

P. olsonii internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

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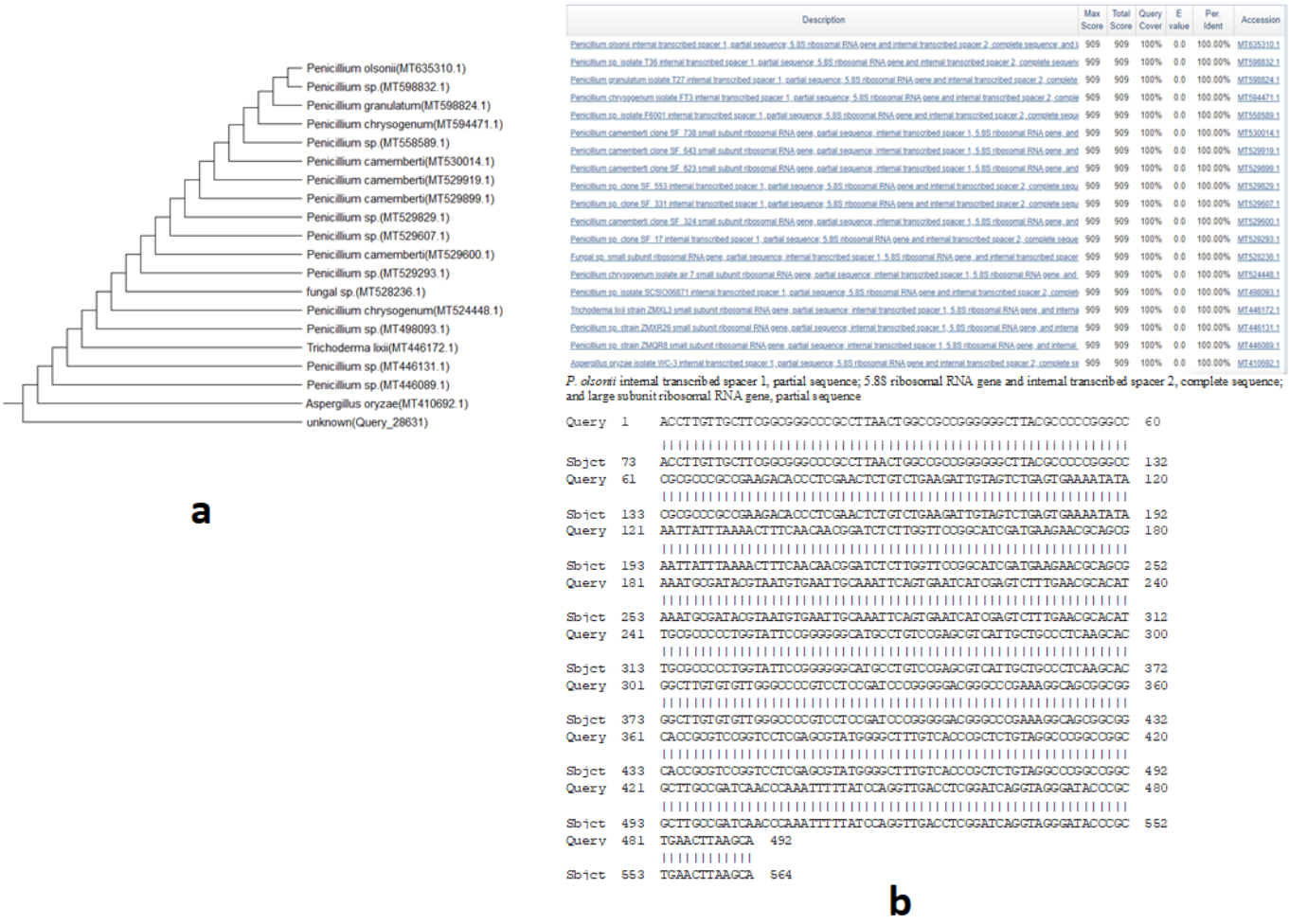
b

Figure 1

a. Phylogenetic relationships amoung the P. olsonii strain MT635310.1and the ITS sequences of closely related fungal strains retrieved from the NCBIGenBank database b. Nucleotide sequence alignment of Penicillium olsonii MT635310.1



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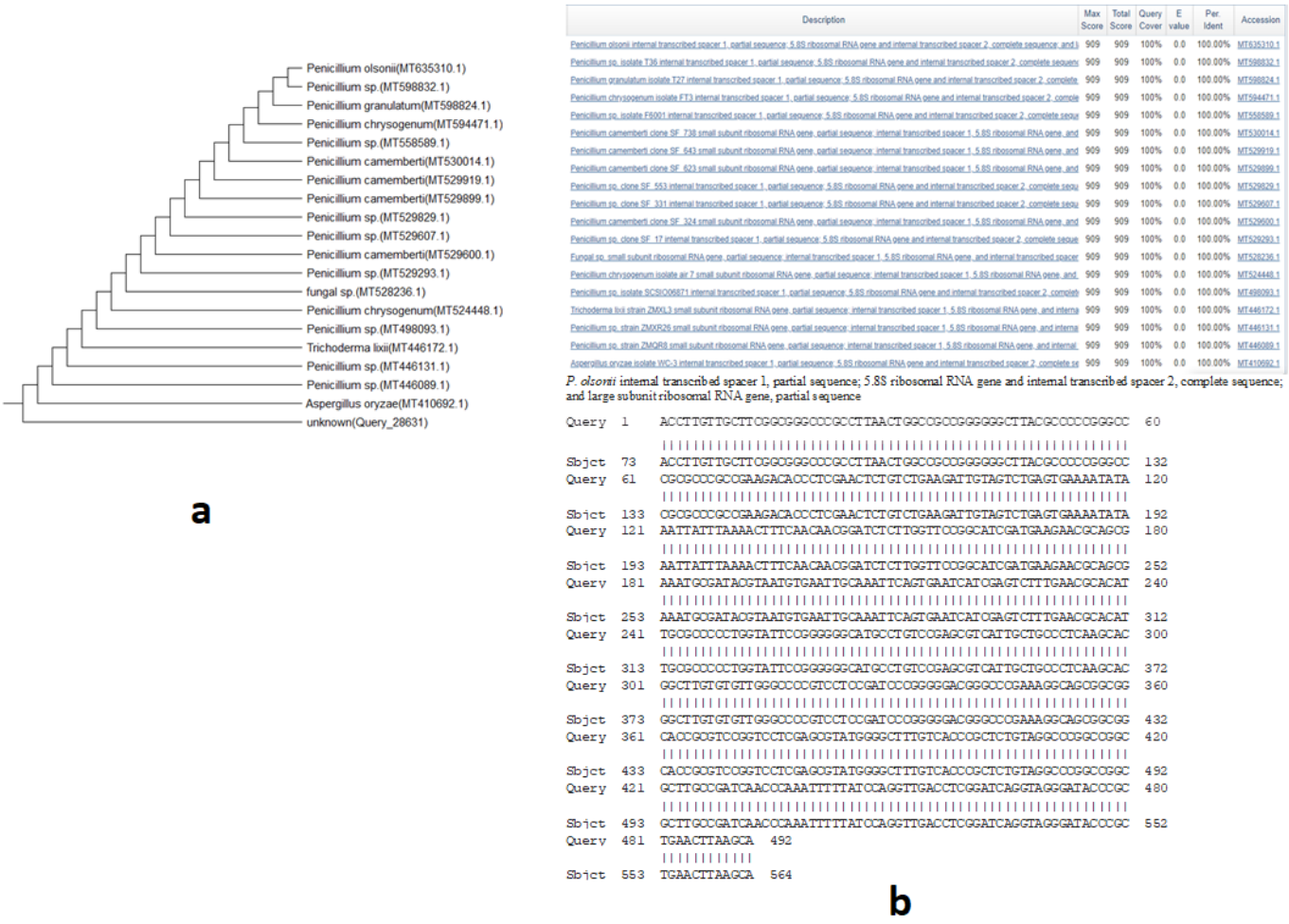

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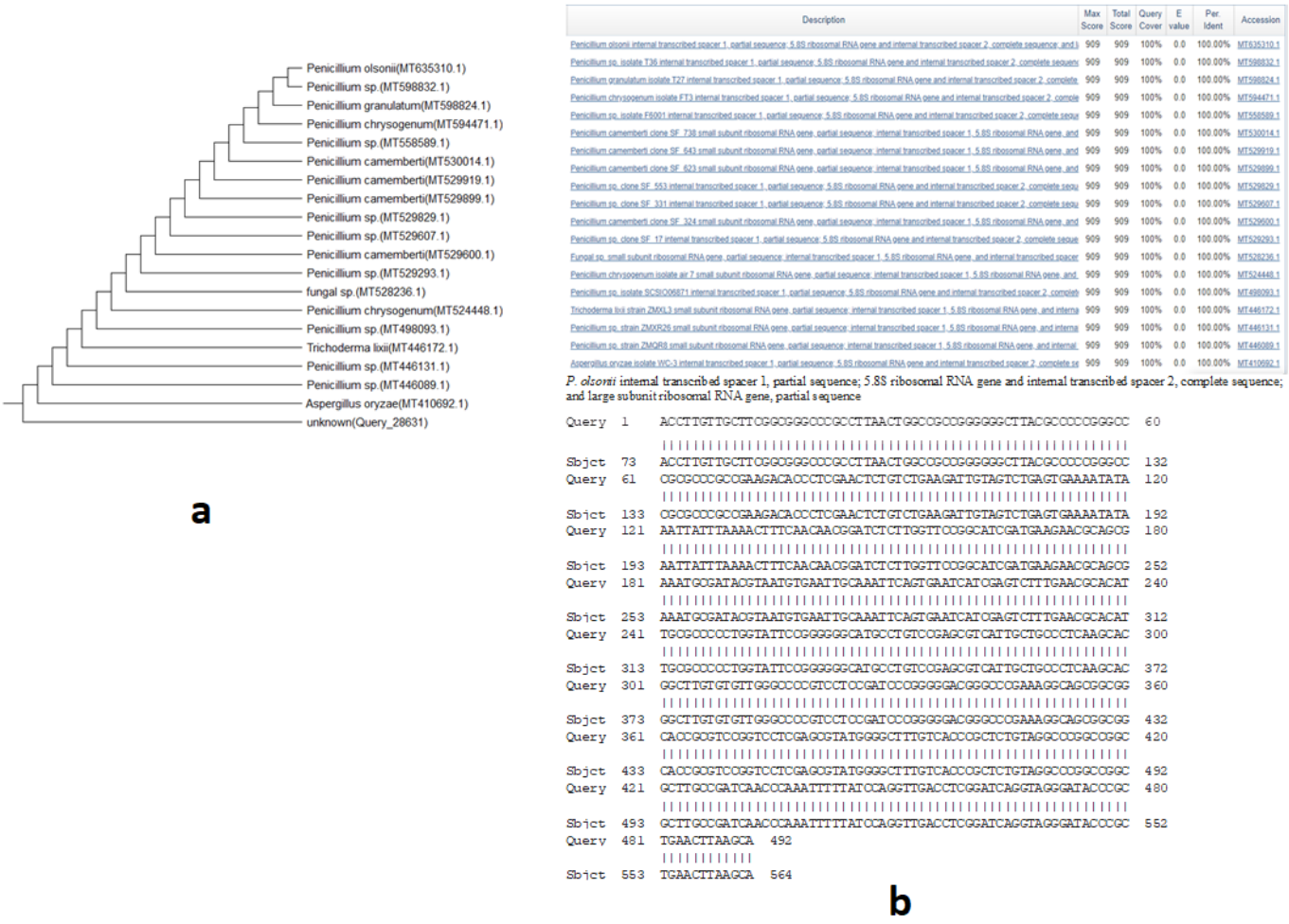

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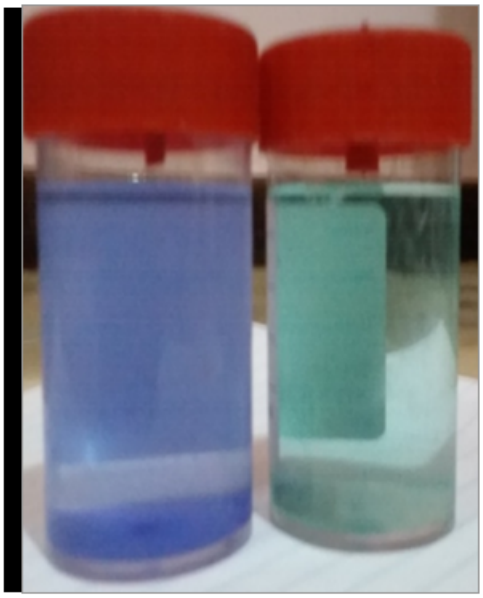
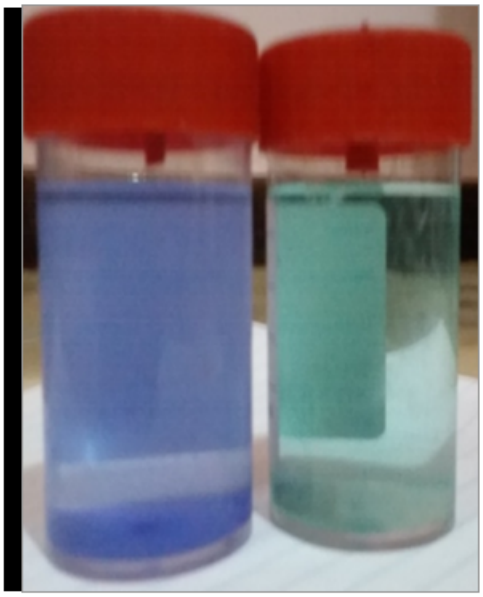


Figure 2

Conversion of copper sulfate to CuNPs by P. olsonii MT635310.1 (green filtrate after removing fungus biomass; Blue filtrate , copper sulfate without fungus biomass).



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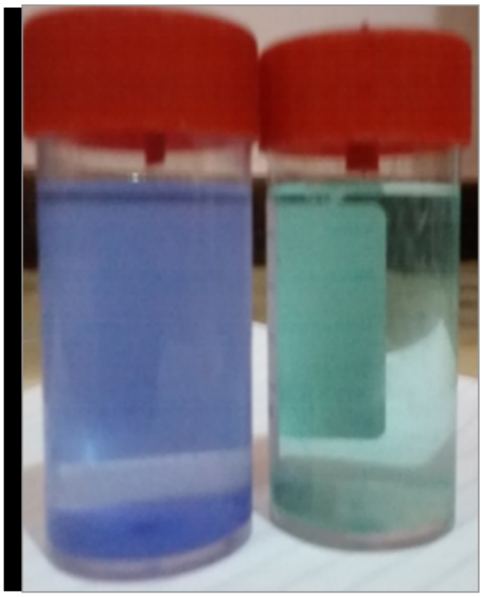

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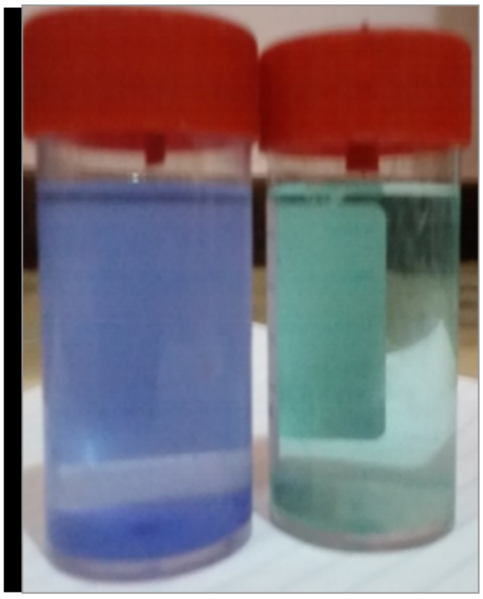

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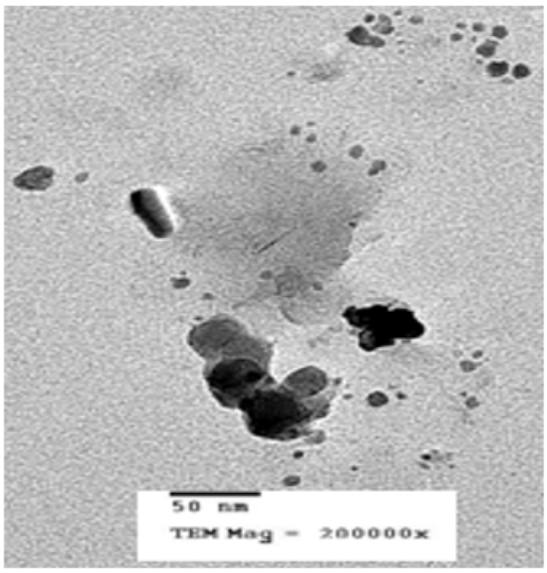
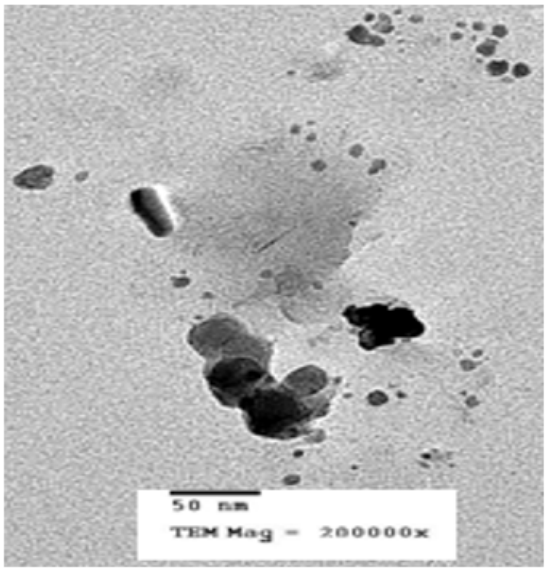


Figure 3

Scanning electron microscope of detected CuNPs produced by P. olsonii MT635310.1



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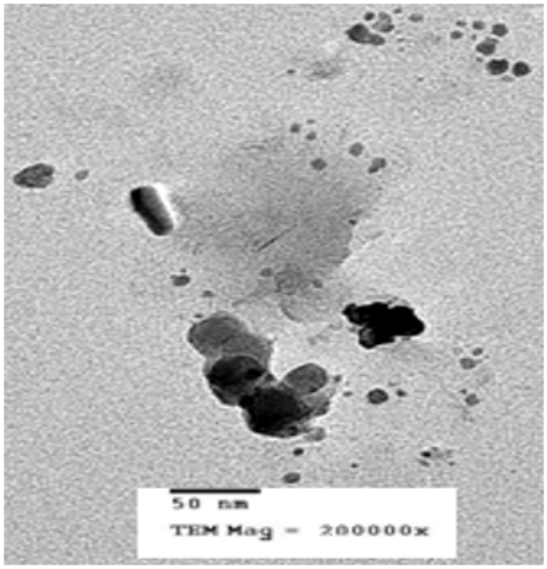

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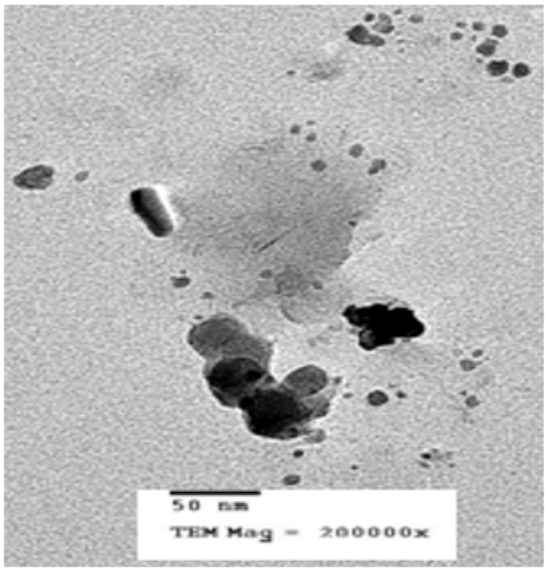

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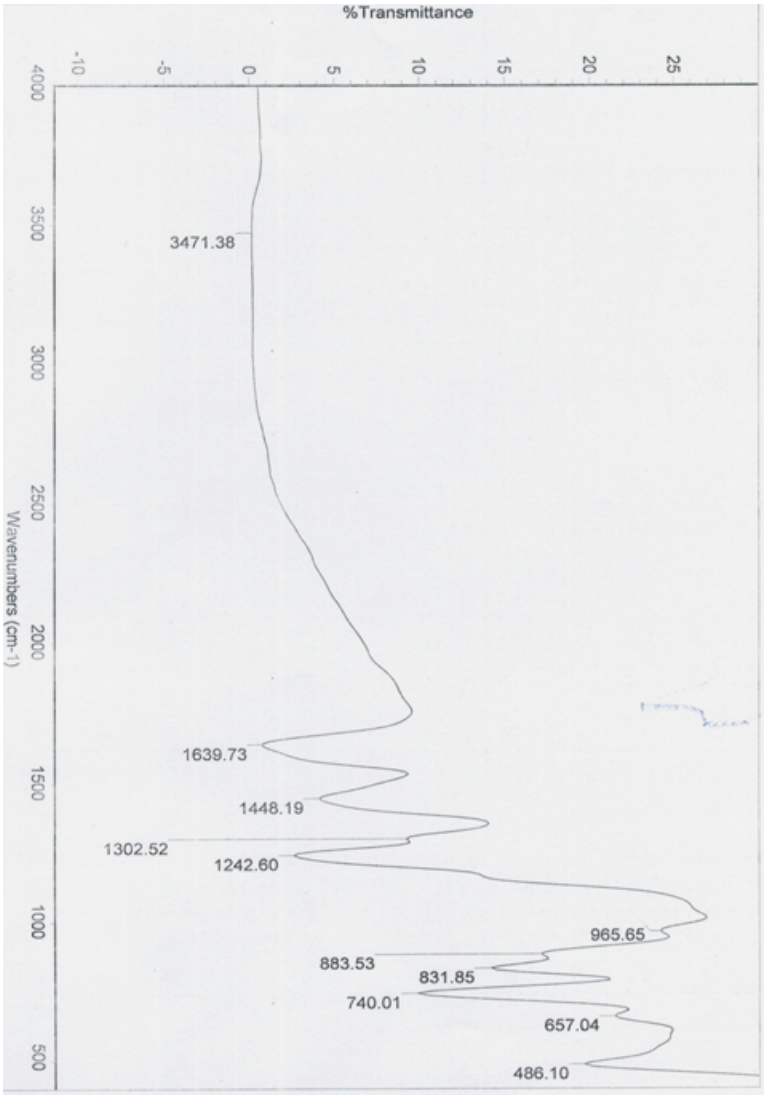
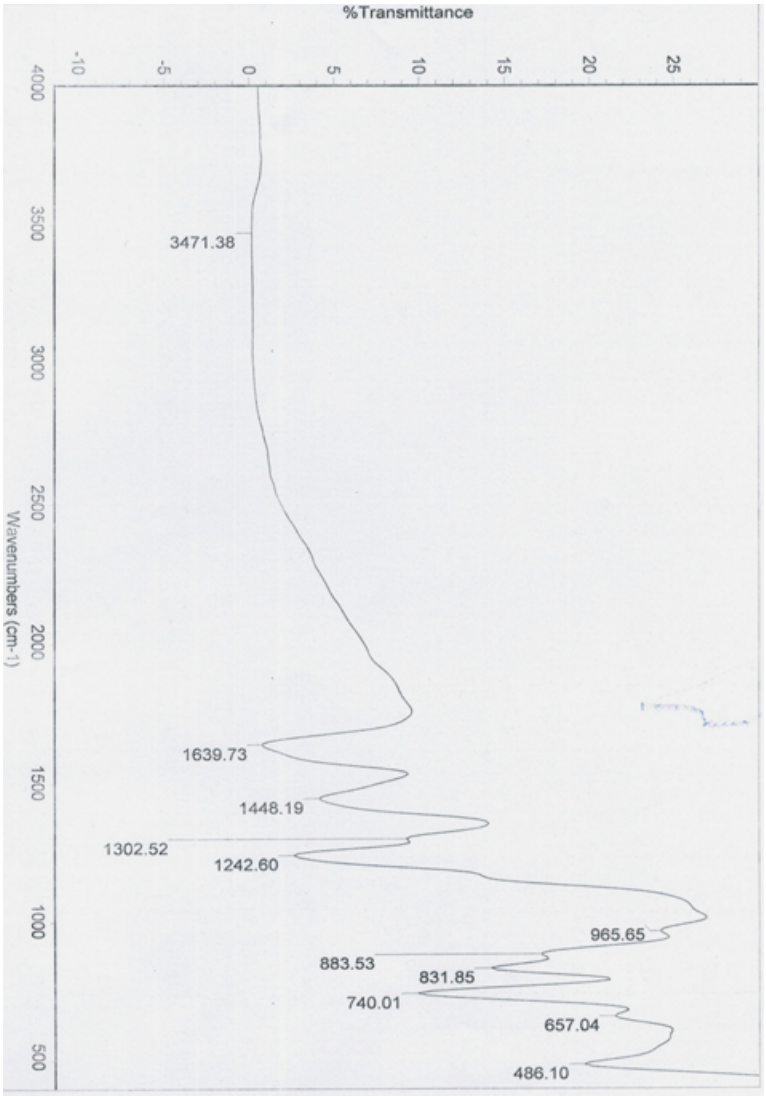


Figure 4

FTIR spectrum of CuNPs formed after 72 h of incubation of the biomass of P. olsonii MT635310.1



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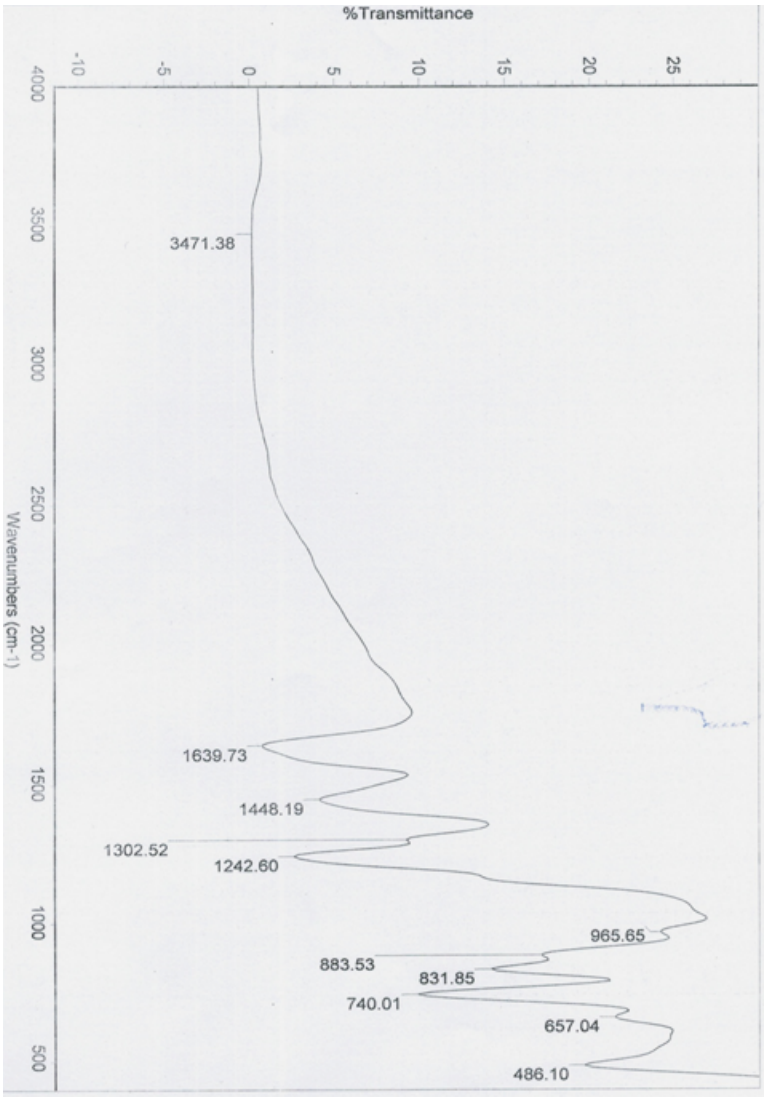

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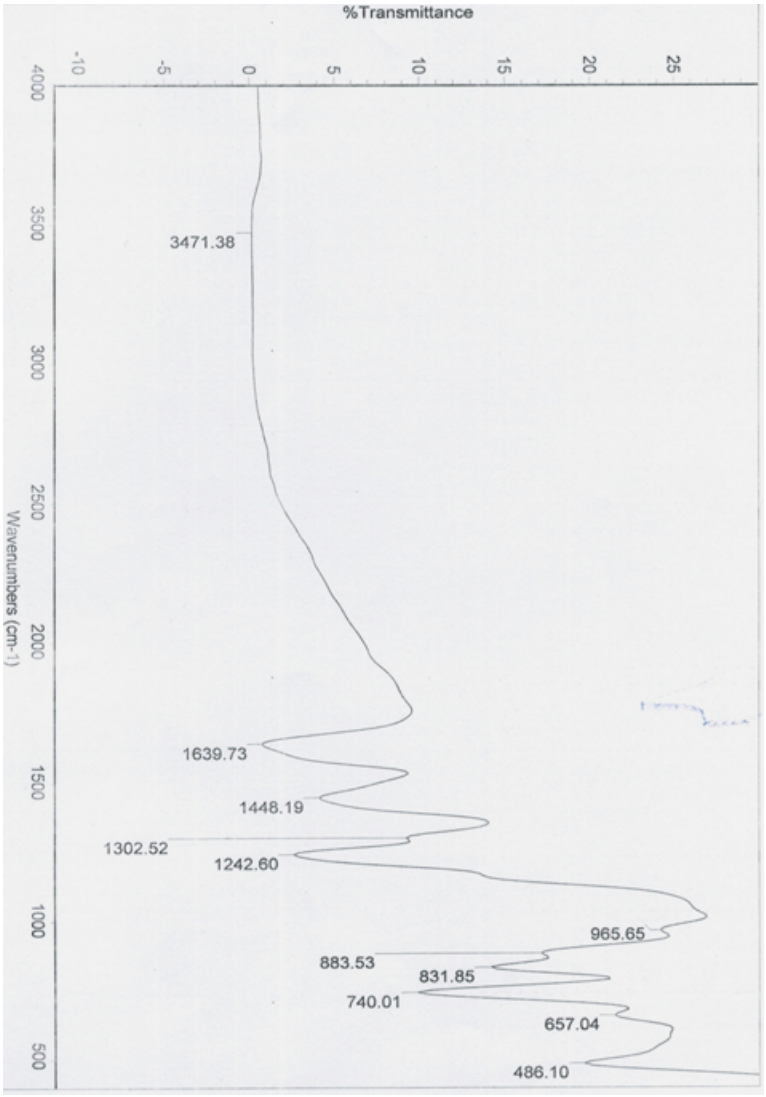

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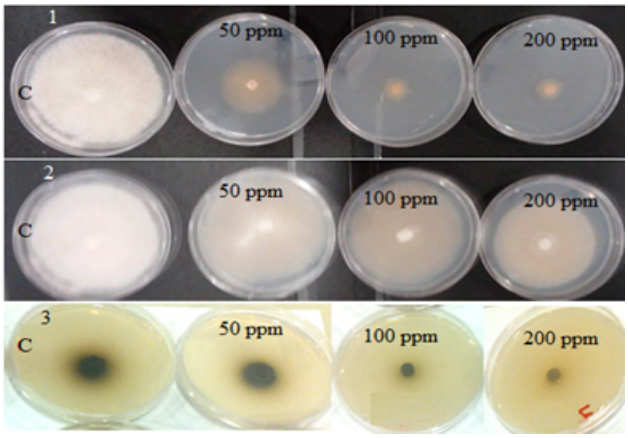
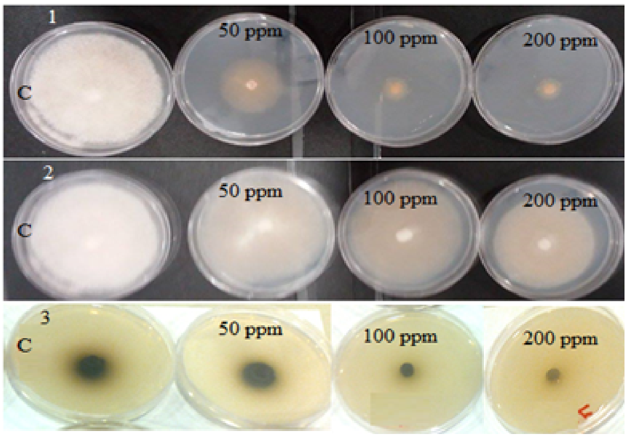


Figure 5

Antifungal activity of different concentrations of CuNPs against F. oxysporum (1), F. solani (2) and C. curvulata



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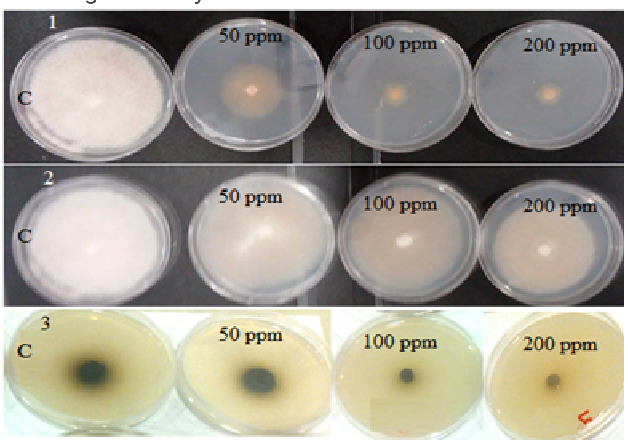

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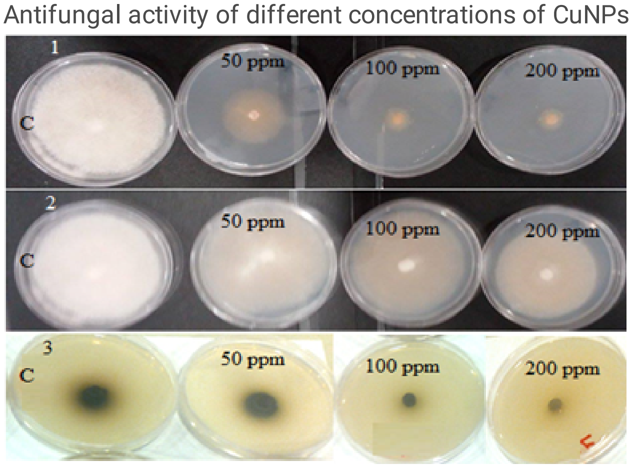

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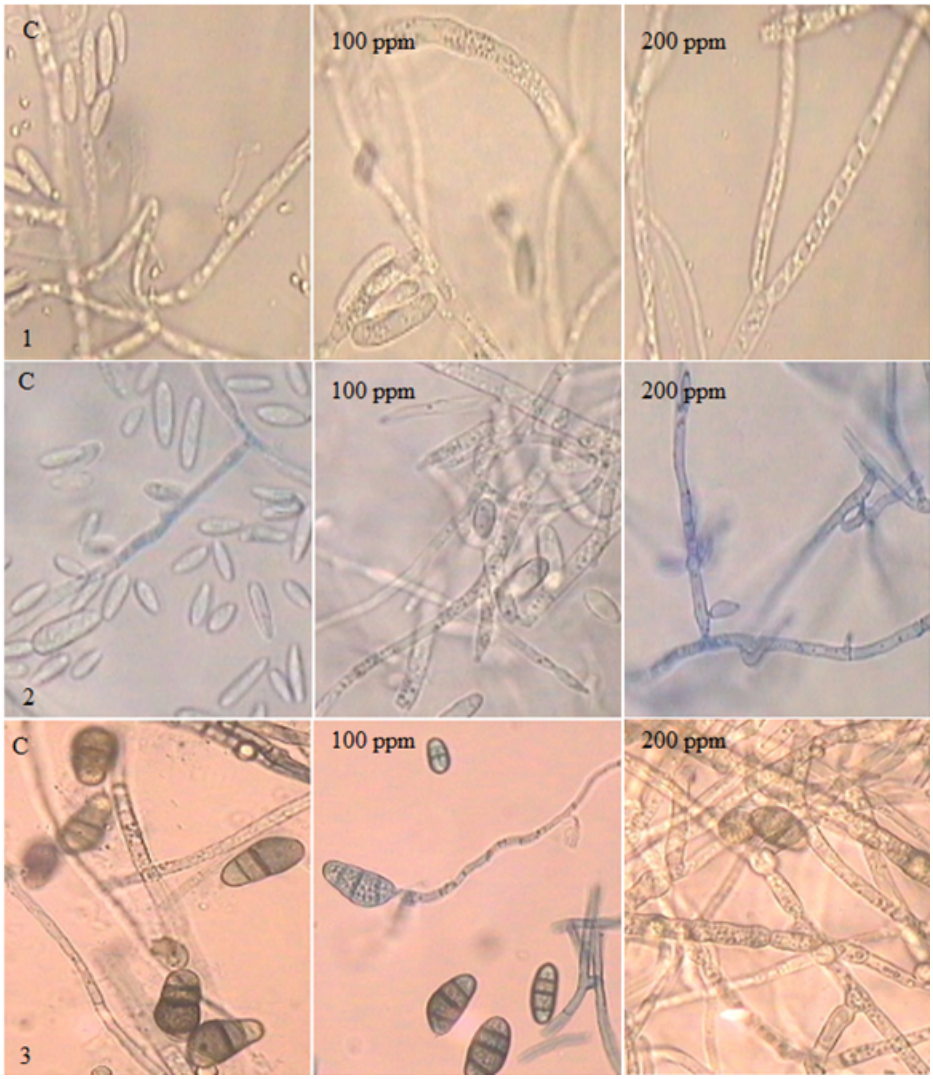
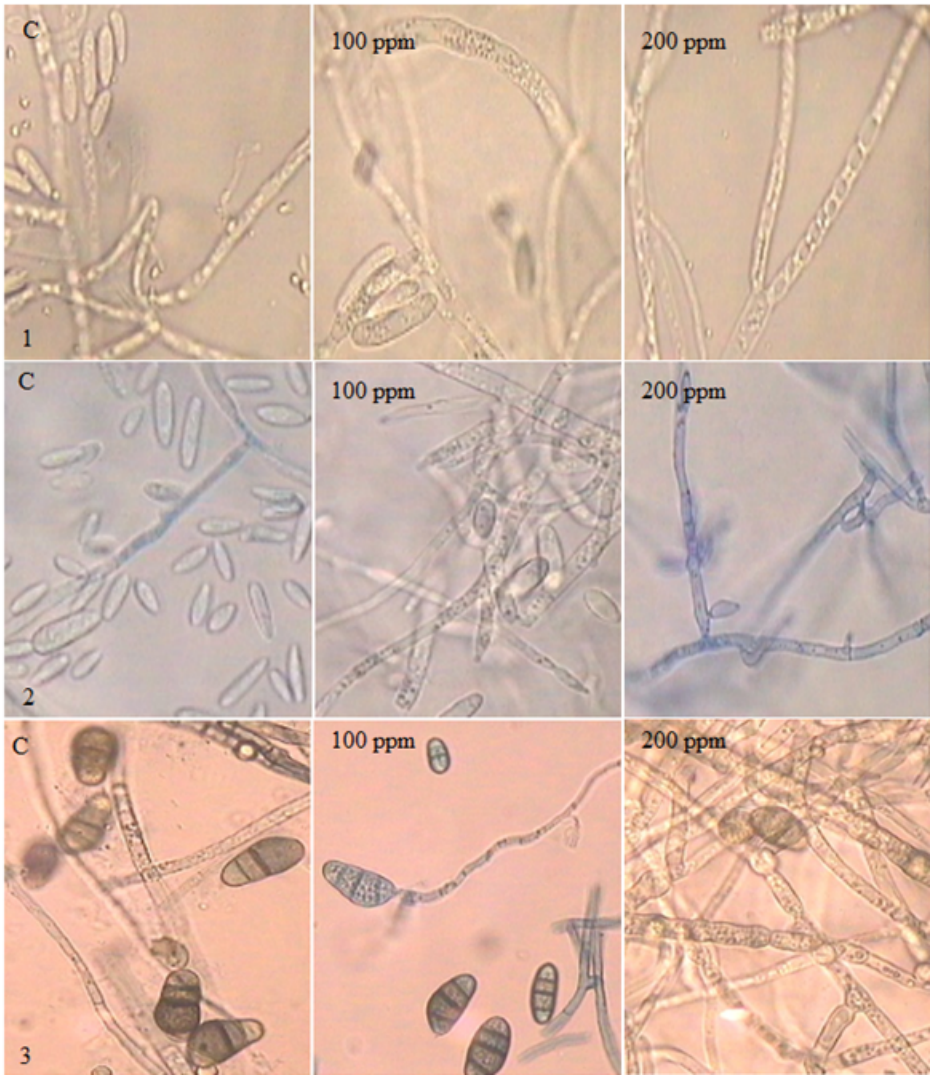


Figure 6

Morphological charaterization of at different concentrations of F. oxysporum (1), F. solani (2) and C. curvulata



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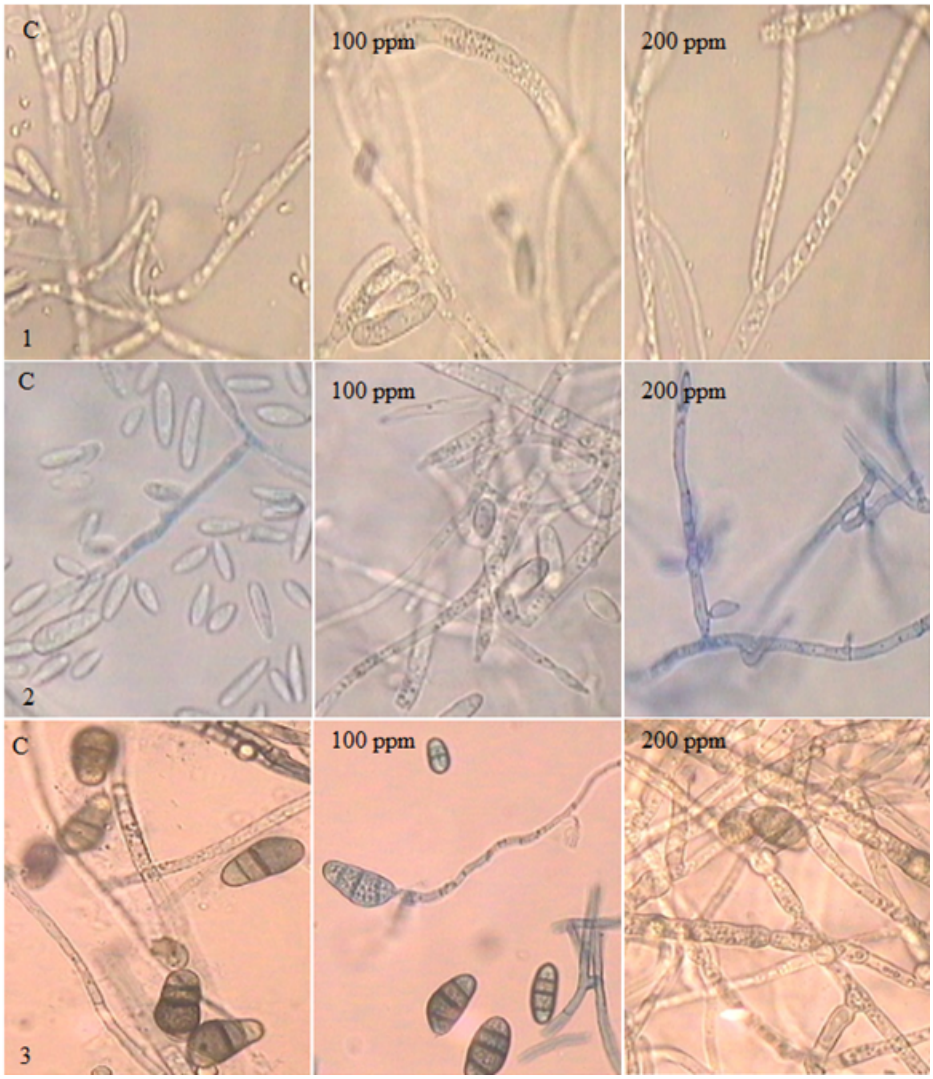

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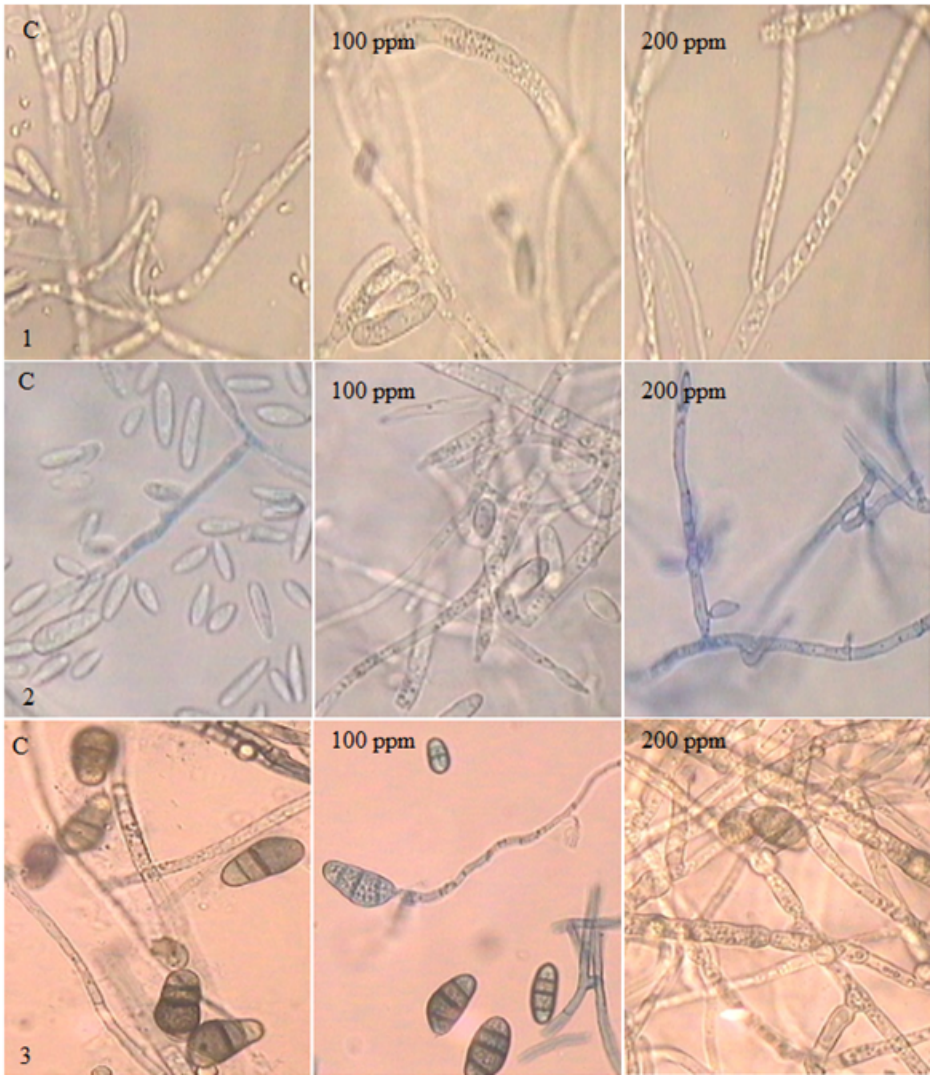

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