kavaka

being
Transactions of the
Mycological Society of India

Golden Jubilee Year

Volume 59, Issue 4
December 2023

Golden Jubilee Issue

VOLUME 59, ISSUE 4 (DECEMBER) 2023
GOLDEN JUBILEE ISSUE

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ISSN 0379-5179
ISSN-L 0379-5179

http://www.fungiindia.co.in
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KAVAKA 59(4): 56-61 (2023) DOI: 10.36460/Kavaka/59/4/2023/56-61

Fungus Mediated Copper Oxide Nanoparticles against Fungi Isolated from Soft-rot Infected Ginger

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(Submitted on October 30, 2023; Accepted on November 1, 2023)

ABSTRACT

Ginger is one of the cash crops grown worldwide, and consumed daily as a spice food, and utilized as Ayurvedic medicine. Soft-rot or rhizome-rot, is a major rhizome-deteriorating fungal disease caused by various fungi like *Fusarium* spp. and *Pythium* spp. in ginger, leading to huge yield losses and economic losses. This study reported *in vitro* antifungal activity of *Phoma herbarum*, cell-free extract-mediated copper oxide nanoparticles (CuONPs) against *Pythium* and *Fusarium* isolates from soft-rot infected ginger, identified at the genus level microscopically. CuONPs were detected by a visible color change from blue to dark brick red precipitate and characterized by Ultra Violet (UV)-visible spectrophotometry (absorbance maxima at 630 nm) and Nanoparticle Tracking Analysis (average size 83 nm). Stability was confirmed by Zeta potential measurement (-23.5 mV), and Face Centered Cubic crystalline structure was elucidated by X-ray diffractometry, and roughly spherical crystals were visualized by Field Emission Scanning Electron Microscopy (FESEM). Fourier Transform Infra-red (FTIR) spectroscopy showed the presence of various functional groups that stabilized CuONPs. The i*n vitro* study showed significant antifungal activity of mycogenic CuONPs against test fungi, which was substantially comparable with a chemical fungicide, i.e., mancozeb. Accordingly, the findings supported the application of mycogenic CuONPs as a cutting-edge antifungal agent in the direction of sustainable agriculture.

Keywords: Mycogenic, Nanoparticles, *Phoma* sp., *In vitro*, Characterization, Agriculture

INTRODUCTION

Ginger (*Zingiber officinale* Rosc.) is a Southeast Asian perennial herb consumed daily as a spice crop and an essential additive of Indian traditional medicine. Major producers are India, China, Nigeria, Indonesia, Bangladesh, etc. In India, Karnataka, Mizoram, Orissa, Madhya Pradesh, and Kerala repeatedly account for the produce as dry (Bag 2018). At the same time, other states like Maharashtra, West Bengal, Meghalaya, and Arunachal Pradesh produce fresh ginger. Increased fungal attacks on the ginger crop have accounted for yield losses of up to 90% (Rai *et al.*, 2018). Thus, it is important to prevent post-harvest damage to the crop by fungi like *Fusarium* and *Pythium* (Plotto, 2004; Rai *et al.*, 2020, 2021a). Nanotechnology is gaining attraction as a new generation of advanced technology, enduring novel approaches to mitigate technological problems and their solutions. The particulate matter with one of its dimensions in the 1-100 nm range is considered for study under nanotechnology (Adil *et al.*, 2022). Nanomaterials studied are fabricated using various methodologies, such as chemical, physical, or biological methods (Rai *et al.,* 2021b). The biological method involves using plants, fungi, and bacteria as primary materials for nanoparticle synthesis (Rai *et al.,* 2021c). Fungi are a rich source of secondary metabolites like alkenes, amines, flavonoids, terpenes, proteins, lipids, etc. These secondary metabolites are explored for their ability to share electrons with ionic entities such as metal ions, e.g., copper ion ($Cu^{+/2+}$), zinc (Zn^{2+}), silver (Ag^{+}), gold (Au^{+}), etc. (Rai *et al.*, 2023). The sharing or release of electrons stabilizes the ions and nullifies their charge. Thus, the properties of precursor materials change drastically, and conversion to nano-form occurs. In the present study, the secondary metabolites from the fungus *P. herbarum*, cell free extracts, were explored to stabilize copper ions

($Cu^{+/2+}$) to convert them into CuO (copper oxide) nanoparticles (NPs).

As mentioned below, the CuONPs were further detected and characterized by various spectrophotometric methods and light scattering techniques. The CuONPs were then evaluated for their antifungal activity against the soft-rot-causing fungi, i.e., *Pythium aphanidermatum* and *Fusarium oxysporum* isolated from soft-rot infected ginger rhizomes.

MATERIALS AND METHODS

P. herbarum was isolated from soil and was microscopically examined and identified for the structure of the spore (**Figure 1a)**. Fungal pathogens *P. aphanidermatum* and *F. oxysporum* were isolated from the soft-rot infected ginger bought from the local market. The cultures were isolated in pure form and identified morphologically for their structure of sporangium and hyphae (**Figure 1c and d**). *P. herbarum* was cultured in potato dextrose broth for seven days. The mycelium was extracted and thoroughly washed with sterile distilled water (2-3 times). The washed fungal biomass was suspended in distilled water (sterile) for 24 hours. Extracellular secondary metabolites are secreted into the water and separated from fungal biomass by simple filtration through nitrocellulose filter paper. The extract collected was used to synthesize CuONPs from copper sulfate pentahydrate ($CuSO_4.5H_2O$, 100 mM) by boiling in a flask with Al^{3+} ions. The brown precipitate of CuONPs obtained was recovered by washing, drying at room temperature, and grinding in mortar and pestle into a fine powder. The procedure given by Shende *et al.* (2021) was modified and used to synthesize CuONPs. The CuONPs were further characterized using Ultra Violet (UV)-visible spectrophotometry (Nanodrop, Thermoscientific, Mumbai, India), Zeta potential analysis (Zetasizer NanoZS-90, Malvern, UK), Nanoparticle Tracking Analysis (NTA) (LM 20, Malvern, UK), Fourier Transform Infra-red (FTIR) (BrukerOptics, GmbH, Germany), and X-ray Diffraction (XRD) analysis. CuONPs were suspended in water to form uniform colloid (1 mg/mL), which were then used for assessment of their antifungal activity against fungal pathogens isolated from infected ginger, i.e., *P. aphanidermatum* and *F. oxysporum* on Potato dextrose agar (PDA) medium by Kirby-Bauer disc diffusion method (1966). The antifungal action was determined by the appearance of inhibition zones around the disc loaded with the nanoparticles compared with the commercially used fungicide i.e., mancozeb. The antifungal activity of CuONPs was statistically verified with one-way ANOVA parametric test for the significance of the results.

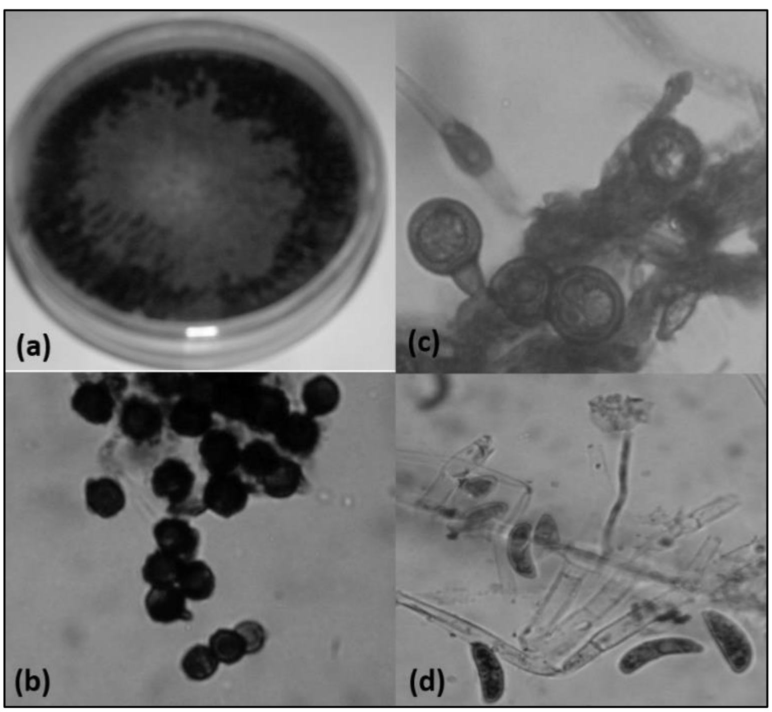

Figure 1: Fungal cultures, a, *P. herbarum*; b, Chlamydospores of *P. herbarum*; c, Oospores of *P. aphanidermatum;* d, Spores of *F. oxysporum*

RESULTS

The fungal isolates were stained with lactophenol cotton blue and were observed under the microscope (**Figure 1b and 1c**). CuONPs synthesized from $CuSO_4$ were confirmed by a change in color from blue to brown precipitate at the bottom of the flask **(Figure 2)** in the presence of *P. herbarum* extract.

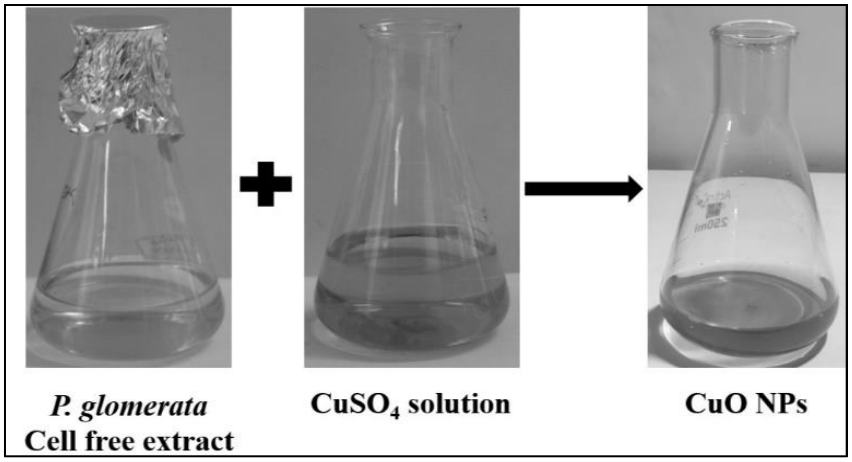

Figure 2: Synthesis of CuONPs using *P. herbarum* extract.

The spectrophotometric analysis showed the absorption maxima at 630 nm **(Figure 3a)**. Nanoparticle Tracking Analysis (NTA) showed the average size of CuONPs as 119 nm, with a mode value of 111 nm and standard deviation of 34 nm **(Figure 3b)**, and 2D distribution indicated the presence of major concentration below 100 nm with particle concentration of 3.8 x 10^8 per mL (**Figure 3c**). Zeta potential analysis of CuONPs showed an average value of -22.8 mV **(Figure 3d)** and a standard deviation of 7.34 mV. FTIR spectrum **(Figure 3e)** represents the peaks assigned to various biomolecules. The peaks were assigned to different functional groups of biomolecules present in the capping layer of *P. herbarum* stabilized CuONPs. The peaks at 3687 cm^{-1}, 3058 cm^{-1}, and 2793 cm^{-1} were assigned to the O-H stretch of poly hydroxyl compounds and OH-stretch of carboxylic acid, respectively. 2331 cm^{-1} and 1656 cm^{-1} belonged to multiple bonding of the nitrile group and C=O stretch of the keto group, respectively. Peaks at 1005 cm^{-1}, 878 cm^{-1}, and 632 cm^{-1} confirmed the phosphate group, aromatic P=O=C stretch, and halide group (Cl^- or Br^-). XRD pattern **(Figure 3f)** revealed the face centered cubic monoclinic structure of CuONPs (JCPDS 80-1268). Field emission scanning electron microscopy (FESEM) **(Figure 4)** revealed the formation of crystalline CuONPs with an average size below 100 nm (Kamel *et al.*, 2022).

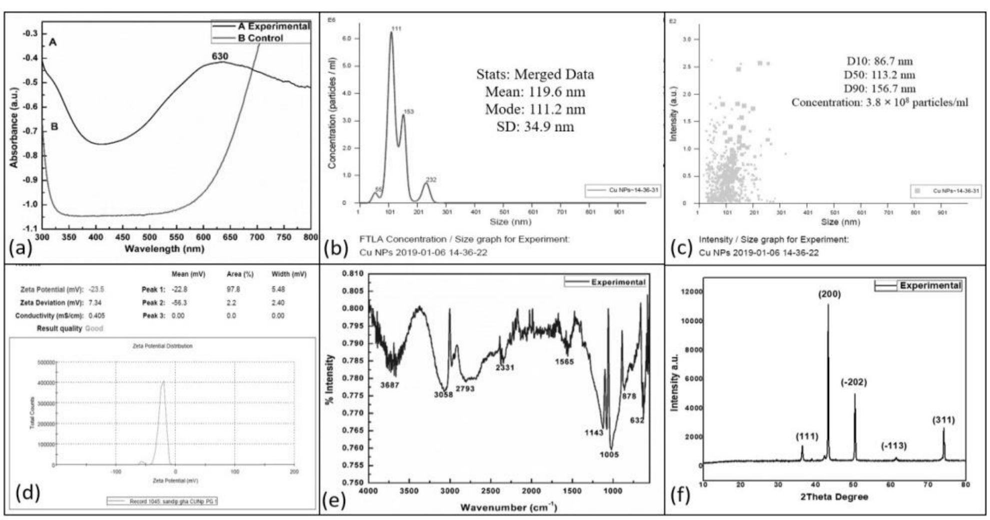

Figure 3: Characterization of CuONPs, a, UV-visible spectrum with absorption maxima of 630 nm; b, NTA analysis of CuONPs; c, 2D distribution of CuONPs; d, Zeta potential analysis; e, FTIR spectrum; f, X-ray diffraction of CuONPs.

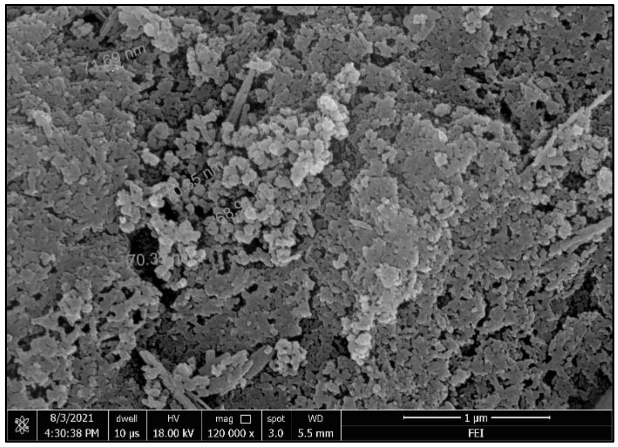

Figure 4: FESEM images of *P. herbarum* mediated CuONPs

Antifungal activity of CuONPs was demonstrated against both *P. aphanidermatum* and *F. oxysporum* by the Kirby-Bauer disc diffusion method, using 1 mg/mL of CuONPs suspension, which was loaded onto a separate disc, along with the positive control as a fungicide, mancozeb **(Figure 5a and b)**. **Figure 5c** depicts the comparative antifungal activity of CuONPs in the form of inhibition zones by the Kirby-Bauer disc diffusion method.

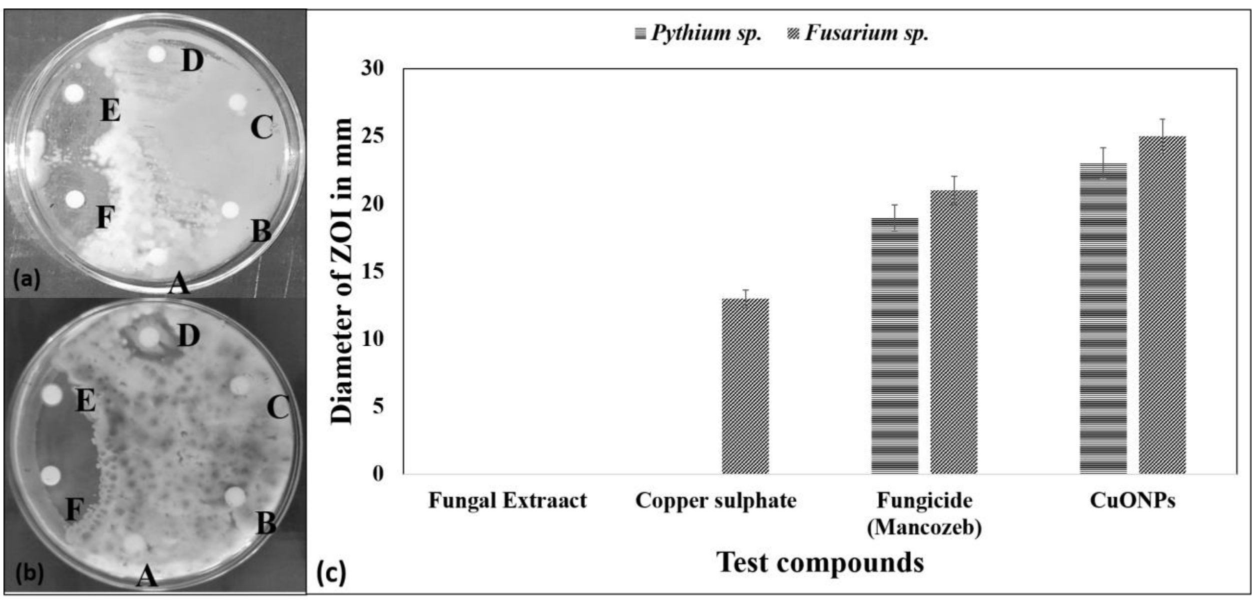

Figure 5: Antifungal activity of *P. herbarum* mediated CuONPs against. a, *Pythium aphanidermatum*; b, *Fusarium oxysporum*; [Where A, Water; B, *Phoma* sp. extract; C, Ketoconazole (20 g); D, Copper sulphate solution; E, Fungicide (Mancozeb); F, Copper oxide NPs (20g)]; c, Graphical representation of comparative zones of inhibitions.

DISCUSSION

Ginger is an important spice food consumed daily all over the world for their characteristics of enhancing flavor and medicinal properties. The common diseases of ginger arose from soil and water. The fungal pathogens are the major threat throughout their cultivation, production, and post-harvest till consumption stage. Different fungi, such as members of *Fusarium* and *Pythium*, are the major pathogens of ginger, causing rhizome- or root-rot that initiates from the collar region of ginger (Rai *et al.*, 2018). Post-manifestation, ginger leaves start yellowing due to reduced nutrient assimilation and fungal deterioration. This causes 60-90% of the yield losses of ginger. The present study focused on assessing *P. herbarium*, a soil-borne fungus-mediated CuONPs synthesis using the cell-free extract. The change in color from blue to brown indicated the formation of CuONPs in the reaction mixture in the presence of aluminum ions. The results correlate with the previous reports indicating the synthesis of stable CuONPs using *Aspergillus* strains (Nassar *et al.*, 2023).The absorption maxima indicated the highest surface plasmon resonance (SPR) shown by CuONPs. The value near 630 nm reflects the synthesis and confirmation of CuONPs (Shende *et al.*, 2021). The nanoparticles were analyzed with NTA, which showed that the particle average size was near 100 nm with a standard deviation of 34.9 nm. The results were in unison with the previous results (Kamble *et al.*, 2015; Ingle *et al.*, 2022; Luque-Jacobo *et al.*, 2023). Zeta potential was observed in the stability range, indicating the synthesis of stable CuONPs that forms stable colloid at room temperature (Gaikwad *et al.*, 2013; Gade *et al.*, 2014). FTIR analysis elucidated the presence of different functional groups in the capping layer of the CuONPs. The functional groups arose due to the encapsulation of the ionic core with biomolecules from the Phoma extract (Zhao *et al.*, 2022). XRD interpretation predicted the presence of FCC monoclinic crystal formation using Phoma extract. Also, FESEM images confirmed the average visible size of CuONPs under an electron beam was below 100 nm (Kamel *et al.*, 2022). CuONPs have previously been reported to exhibit antifungal activity against root-rot causing fungi in cucumber, where they help in maintaining thicker cell walls, mesophyll tissue, and root cortex after treatment (Kamel *et al.*, 2022; Hashem *et al.*, 2023). Nanotechnology is continuously paving the way to solve the losses due to fungi in agriculture (Athawale *et al.*, 2018; Rai *et al.*, 2018; Yadav *et al.*, 2023). The statistical analysis was performed using one-way ANOVA in MS Excel. The observed P-value was < 0.05, indicating the significance of the observed data.

CONCLUSION

The present study concluded that P. herbarum cell-free extract could be used to synthesize stable copper oxide nanoparticles by simply boiling in the presence of copper ion precursor. Nanoparticles exhibited significant antifungal activity against root-rot-causing fungi in ginger. Thus, emphasizing their use as a potential antifungal agent to replace the commercially available chemical fungicides. The application of biogenic nanoparticles is eco-friendly, cost-effective, and easy to handle, making them easy to use in agriculture by farmers.

ACKNOWLEDGMENTS

PG and AG would like to acknowledge this research is part of project No. UMO-2022/45/P/NZ9/01571 co-funded by the National Science Centre and the European Union Framework Programme for Research and Innovation Horizon 2020 under the Marie Skłodowska-Curie grant agreement No.945339.

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