Mycogenic Copper Oxide Nanoparticles For Fungal Infection Management in Agricultural Crop Plants

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Abstract

Aspergillus infections are one of the significant threats to crop plants such as peanuts, cottonseed, maize, tree nuts, food, and feed. The fungal members of this genus can cause up to 100% losses in fruit plants under favorable conditions. It is thus a necessity to control these phytopathogenic fungi by a renewable, non-hazardous technology. *Phoma* spp. are well known to synthesize antifungal metabolites like 4,8-dihydroxy-6-methoxy-3-methyl-3,4-dihydro-1H-isochromen-1-one, Viridicatol, tenuazonic acid, alternariol, alternariol monomethyl ether, phomafungin, etc. In the present work, copper oxide nanoparticles (CuONPs) were synthesized using an aqueous extract of *Phoma glomerata* (MTCC-2210). They were assessed for in vitro antifungal activity against *Aspergillus flavus* and *Aspergillus niger*. CuONPs showed absorption maxima at 630 nm and an average size of 119.6 nm, which were stable at a zeta potential of -23.5 mV, forming the face-centered cubic (FCC) shaped crystalline structure as elucidated by XRD. FTIR confirmed the presence of various secondary metabolites from fungal extracts in the NPs capping. Kirby-Bauer disc diffusion assay indicated the antifungal activity with an average zone of inhibition of 19 $\pm$3 mm and 23 $\pm$4 mm diameter for *A. niger* and *A. flavus*, respectively. The serial dilution method

showed the MIC of 180 and 220 µg/ml against *A. niger* and *A. flavus*, respectively. MIC values were significantly compared to standard fungicide mancozeb. Green synthesized CuONPs using *Phoma* extract indicated significant fungicidal activity against test pathogens and paved their way to be applied in the agriculture fields for crop applications as a novel substitute to chemical fungicides.

Keywords: *Phoma glomerata*; *Aspergillus flavus*; *A. niger*; Copper oxide nanoparticles; Fungicide; Agriculture

1. Introduction

Crop plants under natural conditions in the agricultural field are prone to various biotic and abiotic stresses. Many previous studies suggest that biotic stress includes manifestations due to various pathogenic bacteria, viruses, and fungi [1-6]. Fungi are the most frequent crop pathogens as they can grow in any habitat with less moisture availability. Fruits, vegetables, and many significant crops like soybean, ginger, and other rhizomes [7, 8] are prone to fungal infections from water, air, and soil. *Aspergillus* is a plant pathogenic fungi causing infections in many crop plants. The different species causing infections are *A. parasiticus*, *A. flavus*, *A. carbonarius*, *A. niger*, and *A. ochraceus*. Out of these fungal pathogens, *A. flavus* and *A. parasiticus* are severely toxic as they produce a metabolite called aflatoxin, which is the toxin that contaminates crops in the field during harvest and storage [9].

On the other hand, *A. niger* and *A. ochraceus* produce 'ochratoxins.' Both these toxins, when consumed along with the infected foods, cause systemic health hazards in livestock animals and humans. Other members of the genus *Aspergillus*, such as *A. flavus* and *A. fumigatus*, are also human pathogens causing invasive aspergillosis and severe fungal asthma in immunocompromised individuals [10, 11]. Various antifungal agents have been used to treat human infections [12]. Crop

plants like maize are also very prone to *Aspergillus* infections [13, 14]. Thus, it is necessary to control these fungi at the farm level even before the uncontrolled manifestation and during the growth of plants.

Nanoparticles (NPs) are materials with at least one dimension in the 1-100nm [15]. Structurally, they are composed of an organic/inorganic core and capped with various bioactive metabolites from reducing material or functional compounds [16]. Green synthesis of Copper oxide nanoparticles (CuONPs) has been aimed to overcome the drawbacks of chemical methods that use toxic chemicals and cause damage to the environment. The method of green synthesis is meant for clean, easy, safe, cost-effective, and active sources for high output and purity of NPs [16-19]. Copper NPs are known for their antimicrobial anti-biofilm properties against many microbes. Thus, CuONPs can be used in the formulation of antimicrobials, replacing the antibiotics paving their way in medical and biotechnological applications [20]. The antifungal activity shown against plant fungal pathogens makes them an effective material to be used as nanofungicide in agriculture, replacing the hazardous chemical fungicides [21-23]. CuONPs have been reported to have antifungal activity against many crop-pathogenic fungi such as *Fusarium*, *Pythium*, *Alternaria*, etc. Copper salts have been used for centuries as an antifungal agent without the development of resistance in microbes. CuONPs have high dissolution ability due to their easy availability in situ [24]. Unique physiochemical properties make copper-based nanoparticles a suitable material to be used as a fungicide and for the development of various antifungal formulations. They have shown a broad range of activity against many major pathogenic fungi like *Fusarium*, *Saccharomyces*, *Alternaria* spp.

etc. [25]. Minimal environmental impact, excellent antimicrobial properties, and low cost make copper-based NPs the best antifungal materials with functional modification for their desired

activity [26]. Changes in the surface chemistry of CuONPs are responsible for their binding to living cells, resulting in toxicity effects. It is necessary to know the surface modifications and characterization of CuONPs. Nanoparticles enter the body *via* inhalation, ingestion, or direct contact. Exposure to higher concentrations harms living organisms, including humans, resulting in NPs toxicity. In vitro, toxicities are determined by ROS and oxidative stress, known as cytotoxicity. Damage to genetic material in the presence of nanoparticles is known as genotoxicity, accompanied by genetic alterations [27]. The toxicity effect of CuONPs results in the provoking defense mechanism of cells, ultimately killing or permanently damaging the cell [28]. The present study thus focuses on the fabrication of CuONPs using *Phoma glomerata* as a source of secondary metabolites for the reduction and stabilization of CuONPs and assessing their in vitro antifungal potential against a major phytopathogenic group of fungi, i.e. *Aspergillus* spp.. The main objective of the present study was the evaluation of the susceptibility of two crop pathogenic fungi i.e., *A. flavus* and *A. niger,* isolated from the farm soil. It was challenged against CuONPs synthesized by an aqueous extract of *P. glomerata* (MTCC-2210). The antifungal activity was assessed using the Kirby-Bauer disc diffusion method. The results evaluated the use of CuONPs as an antifungal agent for agricultural use.

2. Materials and methods

Materials used include potato dextrose agar, copper sulfate pentahydrate, conical flasks, stirrer, ultracentrifuge, oven, etc.

Culturing of *Phoma glomerata* and preparation of extract

P. glomerata (MTCC-2210) was grown in a potato dextrose broth with continuous stirring in a 500 ml conical flask to get an actively growing biomass. Biomass was collected aseptically after seven days of incubation at 27 ± 2 ^{o}C and suspended in sterile double distilled water for 1-2 hrs. Under the hypotonic conditions in the water medium, the metabolites from fungal biomass are

released into the water, which is followed by filtration to remove biomass and collecting the fungal filtrate for the synthesis of CuONPs from copper sulfate pentahydrate ($CuSO_4.5H_2O$) as a precursor.

Biosynthesis of CuONPs

CuONPs were synthesized by modifying the method suggested by Noor and colleagues [29]. The washed precipitate was collected in a petri plate and dried in the oven at 60 ^oC to remove moisture. The synthesized CuONPs were extracted and purified in a dry powder.

Characterization of CuONPs

Spectrophotometric and particle analysis

Further characterization of CuONPs was done by UV-visible spectrophotometry (Thermo Scientific NanoDrop 2000c, Ind.) [30], size determination by NTA (Nanoparticle Tracking Analysis, NTA LM 20, Malvern, UK), zeta potential measurement (Zetasizer Nano ZS-90, Malvern, UK).

Fourier Transform InfraRed (FTIR) spectrometry

Fourier Transform InfraRed (FTIR) spectrometry (Bruker optics, ATR, GmbH, Germany) and XRD (X-ray diffraction) analysis [30, 31].

FESEM and EDX analysis

The size and shape of CuONPs were confirmed by FESEM (Field Emission Scanning Electron Microscopy) analysis and elemental composition by energy dispersive X-ray spectrometry [32].

In vitro antifungal assessment and determination of MIC

Antifungal assessment was performed by Kirby-Bauer disc diffusion assay where cellulose paper discs of 10 mm diameter were placed on the PDA (potato dextrose agar) agar plates which were afterward loaded with test material i.e. CuONPs and other control materials individually [33] in

triplicate against the soil isolates of *A. flavus*, and *A. niger*. The plates were incubated at 30±2 ^{o}C in an incubator for 48-72 hrs to get visible fungal growth showing inhibition zones surrounding the cellulose disc. The zone measurement scale measured the zones. MIC (Minimal Inhibitory Concentration) was performed using the serial broth dilution method in a 96 well microtiter plate. Statistical analysis was done using two-way ANOVA (MS excel, Windows 2007; $P \leq 0.05$) to determination of the significance of the results.

3. Results

The CuONPs synthesized were detected visually by color change from light blue (Fig. 1a) to brick red (Fig. 1b) precipitate. The UV-visible spectrum indicated the absorption maxima at 630 nm, confirming the synthesis of CuONPs (Fig. 2). The broad spectrum indicated the formation of roughly spherical CuONPs. NTA analysis determines the size of NPs based on Brownian motion in the colloidal system. Particle-by-particle measurement under laser light indicated the average size of CuONPs under NTA as 119.3±34.9 nm (Fig. 3). The particle concentration was found to be 3.8 x 10^{8} /ml. Surface charge on the NPs is determined by measurement of zeta potential. Zeta potential of 23.5±7.34 mV (Fig. 4) indicated the formation of stable CuONPs using the *P. glomerata* extract.

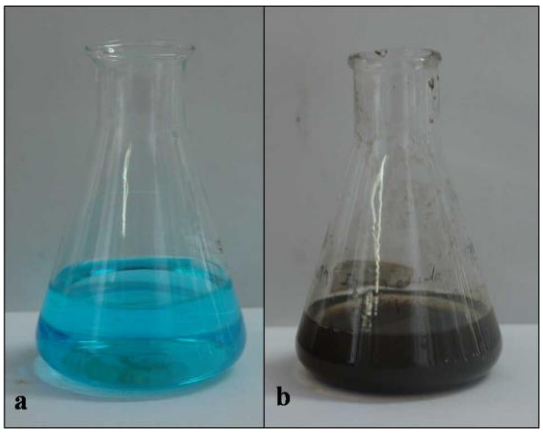

Fig. 1. Synthesis of nanoparticles using *P. glomerata* (a) Copper sulfate (100 mM); (b) brick red precipitate of copper oxide nanoparticles (CuONPs)

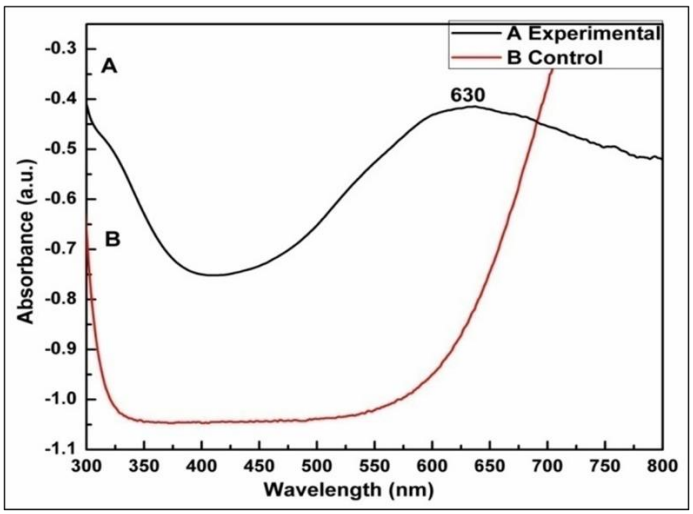

Fig. 2. UV-visible spectrum of *P. glomerata* mediated CuONPs showing absorption maxima at 630 nm.

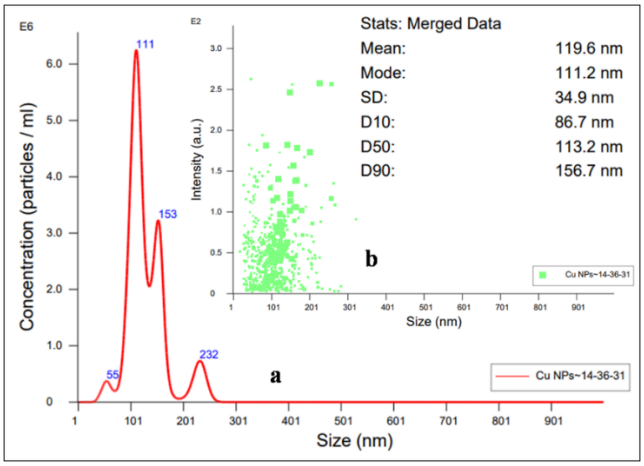

Fig. 3. Nanoparticle Tracking Analysis (NTA) of *P. glomerata* mediated CuONPs

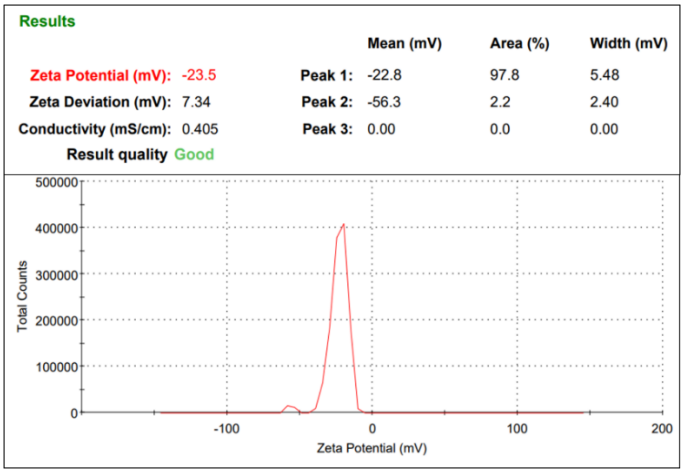

Fig. 4. Zeta potential analysis of *P. glomerata* mediated CuONPs with an average zeta potential of -23.5 mV.

FTIR spectrum (Fig. 5) indicated the presence of various functional groups from secondary metabolites from *P. glomerata* extract in the capping layer of the CuONPs as determined by the wavenumbers allotted to respective peaks. The peak at 3688 cm^{-1} belongs to OH- stretching vibrations. Other peaks at 2382, 1534, and 930 cm^{-1} represented the stretching vibrations of the aliphatic CH- bond, amide II, and C-O-C bonds, respectively. XRD analysis indicated the formation of face-centered cubic (FCC) crystalline (Fig. 6) structure of *P. glomerata* mediated CuONPs with monoclinic phase.

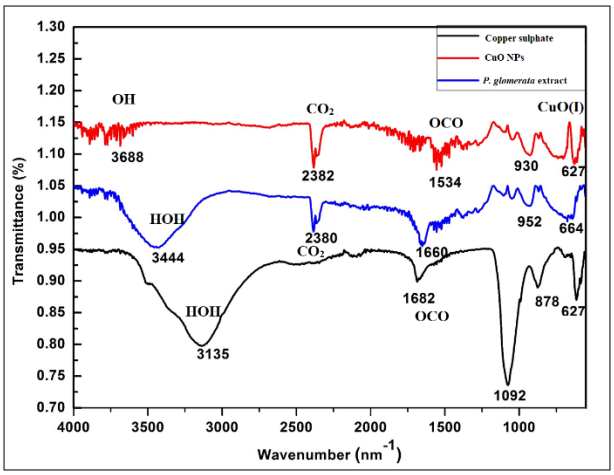

Fig. 5. Fourier Transform Infrared (FTIR) spectrum of *P. glomerata* mediated CuONPs.

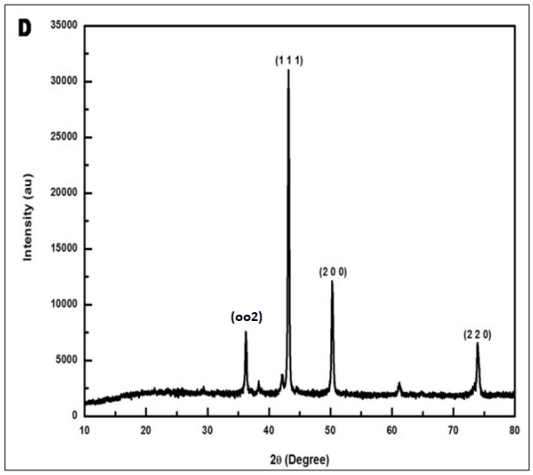

Fig. 6. X-ray diffraction pattern of *P. glomerata* mediated CuONPs indicating face-centered cubic (FCC) crystalline structure

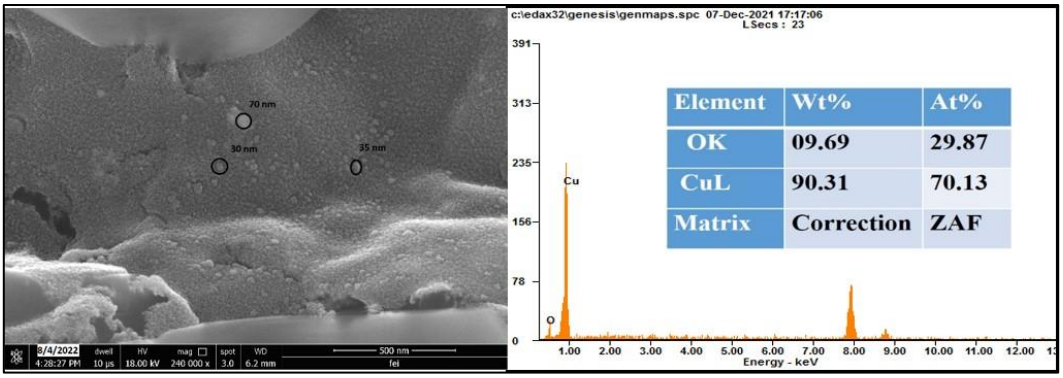

Fig. 7. (a) FESEM images of *Phoma*-mediated CuONPs and (b) EDX spectroscopy

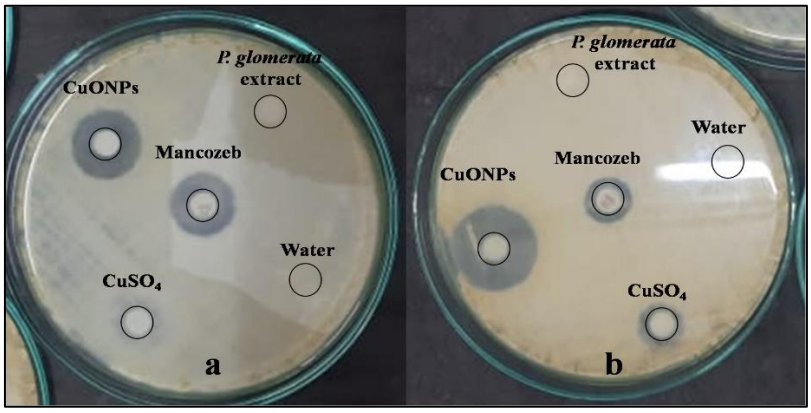

Fig. 8. *In vitro* assessment of antifungal activity of *P. glomerata* mediated CuONPs, fungicide mancozeb as a positive control, and *P. glomerata*, $CuSO_4$, water as negative control against (a) *A. niger* & (b) *A. flavus*

CuONPs were further characterized by FESEM (Field Emmission Scanning Electron Microscopy) to confirm their size and shape. FESEM images showed that CuONPs are roughly spherical in shape, and typical particle size is below 100 nm, as shown in figure 7(a). EDX analysis elucidated the elemental composition of CuONPs (Fig. 7(b)). It was confirmed that the CuONPs have 9.69 % by weight of oxygen and 90.39 % by weight of copper.

Further, *in vitro*, antifungal assessment of CuONPs by disc diffusion method represented the zones of inhibition (ZOI) of both the fungi as shown in figure 8(a) and 8(b). Mancozeb (ethylenebisdithiocarbamate, 85% w/w), a commercially used fungicide, was used as a positive control, which showed average ZOI of 18 mm and 14 mm against *A. niger* and *A. flavus*,

respectively. The average ZOI of CuONPs against *A. niger* and *A. flavus* was found to be 19 mm and 23 mm, respectively (Fig. 9). Copper sulfate (100 mM), aqueous extract of *P. glomerata*, and water were taken as negative controls. The lowest concentration (in µg/ml) of CuONPs showing inhibition of visible growth was considered as MIC value for CuONPs. The MIC value was observed as 180 and 220 µg/ml against *A. niger* and *A. flavus*, respectively. The results indicated the significant inhibition of fungal growth.

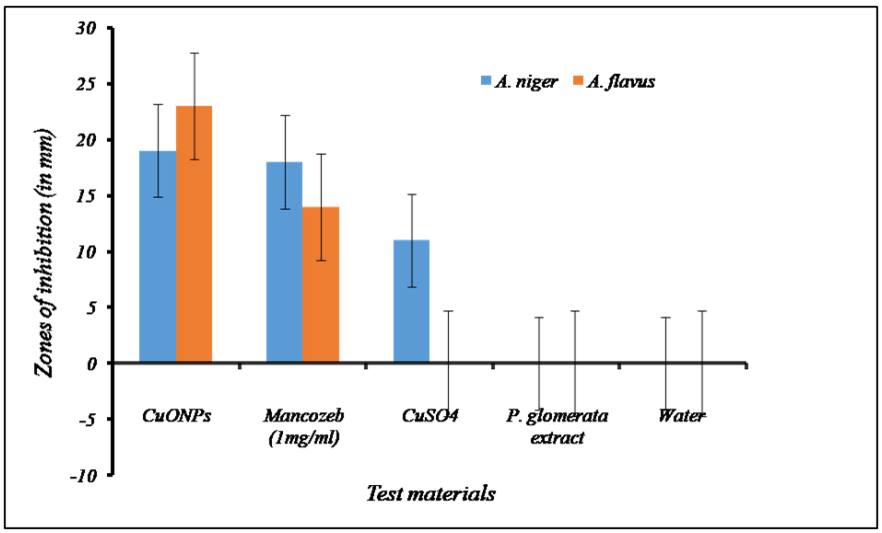

Fig. 9. Zones of inhibition of CuONPs against (a) *A. niger* & (b) *A. flavus*

Statistical analysis using two-way ANOVA resulted in the p-value significance of $P > 0.05$. Thus, the MIC results were shown to be significant as compared to the control, and this proved the significance of the results. This indicated that the antifungal activity of CuONPs is a function of two variables, i.e. size of NPs and their concentration.

4. Discussion

The genus *Aspergillus* is a well-known causative agent of a wide range of diseases in crop plants [13, 34]. They are mainly responsible for the rotting of plants and secondary foods from plants. *A. niger* causes leaf spot in ginger [35]. They can contaminate the food and food products at various stages, including pre-harvest, harvest, processing, and storage. Spoilage of food due to *Aspergillus*

species is presumably identified by the development of off-odor, pigmentation, discoloration, rotting, etc. As they are opportunistic plant pathogens, they can habitat the plant and plant based food products very quickly whenever favorable conditions are available [9]. In the case of *A. flavus, the* spermidine synthase gene is responsible for its expected growth and aflatoxin production during its infection in plants like maize [36]. This induces changes at the molecular level during transcription and also generates physical changes in the plants, such as improper kernel development [37]. *Aspergillus* species are known to produce various aflatoxins (B_1, B_2, G_1, G_2). Many of these are carcinogenic to humans [38]. The use of chemical fungicides in agricultural fields to control these fungal infections is a common practice worldwide. Fungicides like carbendazim, mancozeb, etc., are applied in bulk quantities despite their hazardous impact on the environment [39].

Nano-based fungicides or nanofungicides seem the best substitute to overcome the yield losses due to *Aspergillus* infection and minimize the environmental damages. Gold nanoparticles synthesized from broccoli (*Brassica oleracea*) and silver nanoparticles from various plant materials are routinely reported as efficient and effective antibacterial and antifungal agents against human pathogens [40, 41]. Other nanomaterials, like plant based zinc oxide NPs, have been shown to exhibit antimicrobial activity [42]. In the present study, *P. glomerata* aqueous extract was used to synthesize the copper oxide nanoparticles (CuONPs). The resulting red colored precipitate of CuONPs was purified and was further characterized [43, 44]. *P. glomerata* has previously also been explored for their ability to synthesize nanoparticles like silver NPs [45, 46]. Gold NPs have also been synthesized from *P. glomerata* [47]. In a similar manner, copper NPs have also been continuously reported to be synthesized by a green approach using fungal biomass and filtrates [48]. The CuONPs synthesized by *P. glomerata* showed an absorbance spectrum in a

similar range as reported by earlier researchers dealing with the mycosynthesis of NPs [49]. The average size of CuONPs was found to be 119.6 nm with a standard deviation of 34.9 nm by Nanoparticle Tracking analysis. The particle size is determined on the basis of the Brownian movement of the NPs in a colloidal state under mid infrared light [50]. A Zeta potential of -23.5 mV indicated the stable synthesis of CuONPs by *P. glomerata*. The results were in accordance with the previous studies on copper oxide NPs by Anandhavalli et al. [51]. The FTIR spectrometric analysis indicated the stabilization of CuONPs by various biomolecules from the *P. glomerata* extract, majorly those containing OH- group, followed by aliphatic CH- bond, amide II, and C-O-C bonds. The presence of hydroxyl and ethereal groups on the surface of the NPs indicated the electrostatic stability with the positively charged Cu ion core of CuONPs [52]. XRD analysis represented the Bragg's diffraction and Miller indices corresponding to face-centered cubic (FCC) structure of CuONPs. The '2θ' values correspond to peaks in X-ray diffraction pattern produced by nanoparticles about very well with monoclinic CuO (JCPDS N o. 801268). The results were in accordance with the earlier reports [51, 53].

Nanoparticles have multimodal action when interacting with the biological membranes. Primarily, they cause damage to the membrane, leaking the cytoplasmic content and death of cells. Secondly, NPs trigger the defense mechanism, thus producing various types of reactive oxygen species (ROS), which damage the genetic materials and kill the cell. Other mechanisms involve cellular protein damage, destabilization of the ribosomes, mitochondrial dysfunction, etc. Ultimately, all these indemnities lead to cell death [54]. *In vitro,* antifungal assessment of the CuONPs against test pathogens, i.e., *A. niger* and *A. flavus,* showed comparatively significant fungicidal activity at the test concentration of 1mg/ml. The MIC was calculated by serial broth dilution method and was shown as 180 and 220 µg/ml against *A. niger* and *A. flavus,* respectively.

The results were in comparison with the antifungal activity of CuONPs, as reported by Kanhed and colleagues [55] and El-Batal et al. [49]. Yadav and colleagues have comprehensively reviewed the various biogenic, especially mycogenic nanoparticles, and their applications as an antifungal agent against plant pathogenic bacteria and fungi [56]. They have quoted that the mycogenic nanoparticles have highly efficient, low-cost, safe, and resilient solutions in the prevention of plant diseases, which can revolutionize the agriculture sector.

5. Conclusion

In the present study, CuONPs were synthesized by an aqueous cell free extract of *P. glomerata*. The results indicated that the fungus *P. glomerata* has the potential for synthesis of stable CuONPs by green approach and were well versed with the previously reported studies. Antifungal potential against plant pathogenic fungi like *A. niger* and *A. flavus* indicated their application in the fabrication of fungicidal agents for agricultural as well as medicine purpose. Further, the thorough evaluation and assessment of CuONPs on farmers' field demonstration studies will assist researchers in designing a novel antifungal agent for crop plants. Thus, CuONPs are needed to be thoroughly explored for their characteristic physico-chemical properties and bio-catalytic activity. Future studies based on dose dependent activity and keen field testing will elaborate and support the public use and field application of CuONPs. It is thus recommended that fungal-mediated CuONPs are synthesized by the green method and will stand as a potential antifungal agent. This will ultimately be responsible for minimizing the hazardous effects of chemical fertilizers and losses to the environment.

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Declarations

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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Ethical approval

The present study involves no human participants. So, ethical approval is not required.

Availability of data and materials

The data and the information of materials will be made available on considerable request to the corresponding author.

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