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Piper nigrum Leaf Extract Mediated Synthesis of Copper Oxide Nanoparticles and their Antimicrobial Activity Against Soil Phytopathogens

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The bio-inspired synthesis of copper oxide nanoparticles using *Piper nigrum* leaf extract (BP-CuONPs) is reported in this study. The rapid reduction of copper (Cu^{2+}) ions was preliminarily confirmed using a UV–Vis spectrophotometer with peak formation at 270 nm. Further-ray diffraction

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(XRD) patterns confirmed the crystalline phase of copper oxide with a monoclinic crystal structure. Fourier Transformed Infrared (FTIR) spectroscopy of the nanoparticles revealed the presence of various functional groups, including alcohol, phenols, carboxylic acids, and amide containing alkaloids such as piperine, which serve as reducing and capping agents for the metal nanoparticles and indicate the presence of metal oxide nanoparticles. Energy dispersive X-ray (EDX) spectroscopy revealed that the weight percentage of copper was approximately 68.29%. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) revealed the formation of spherical BP-CuONP NPs with an average particle size of 5 nm- 24.5 nm. BP-CuONPs exhibited complete inhibition against four soil-borne fungal phytopathogens at 1500ppm.

Keywords: Bio- inspired synthesis; CuO NPs; Piper nigrum; electron microscopy; phytopathogens.

1. INTRODUCTION

"Soil is filled with countless microorganisms, many of which benefit both the soil and plants. However, some microorganisms can harm crops by damaging their roots and crowns, thereby causing significant economic loss. These persistent pathogens in the soil or on its surface are known as soil-borne plant pathogens, and can be caused by fungi, bacteria, nematodes, oomycetes, protozoa, and viruses. Soil-borne diseases can greatly affect crop yields, and when these pathogens form synergistic associations, they can cause significant economic losses that are difficult to manage. Soil-borne plant pathogens, such as Fusarium spp., Rhizoctonia spp., Pythium spp., Sclerotinia spp., Verticillium spp., and Phytophthora spp., can result in yield losses of up to 50-75% for important crops, such as wheat, maize, cotton, vegetables, and fruits" [1]. "*Fusarium oxysporum* strains can infect over 150 agricultural crop species, including bananas, tomatoes, melons, and cotton, and cause severe vascular wilt disease" [2]. "In cucurbitaceous crops, the pathogen is responsible for yield losses of approximately 30-80%" [3]. "Fusarium wilt of bananas, caused by *F. oxysporum* f. sp. *cubense*, is a major threat to banana cultivation worldwide, with Tropical Race 4 causing significant losses in Southeast Asian countries and affecting the lives of small producers" [4]. *"F. solani* mainly causes collar and root rot in economically important crops, such as beans and peas. *F. graminearum* and *F. verticillioides* cause cob rot in maize, both species are known to produce mycotoxins" [5]. "In *Brassica napus* and oil seed rape, the prevalent population of *Rhizoctonia solani* AG2-1 isolate is responsible for severe seedling diseases, establishment losses of up–80-100%, and final yield losses of up to 30%" [6,7,8]. "Rhizoctonia presents with a variety of symptoms including stem lesions, damping-off, crown rot, root rot, stem rot, and aerial web blight. Infections ultimately lead to wilting, stunting, and plant death. Phytophthora and Pythium, two species, cause damping-off and root rot disease under cool and wet conditions, affecting 5-80% of seedlings and resulting in significant economic losses for farmers" [9]. "Late blight, caused by Phytophthora spp., is one of the most destructive soil-borne diseases affecting potatoes and tomatoes, worldwide" [10]. "Globally, it causes an estimated $5 billion in annual losses" [11]. "Conventional methods of disease control include the use of chemical pesticides, which provide quick and reliable disease control, as well as significant drawbacks such as environmental pollution, health risks to humans and animals, and the development of increased resistance to pathogens. These issues necessitate exploration of safer and more sustainable alternatives. Nanotechnology, particularly phyto-nanotechnology, has emerged as a promising alternative. Nanotechnology leverages the unique properties of nanoparticles, including their small size, large surface-area-to-volume ratio, and high reactivity, thereby making them highly effective in various applications. Phyto-nanotechnology, which involves the use of plant-derived phytochemicals such as polyphenols and flavonoids to synthesize nanoparticles, offers several advantages over traditional chemical pesticides and can be engineered to have high specificity towards target pathogens, thereby minimizing the impact on non-target organisms and reducing environmental contamination. Nanoparticles alone can be directly utilized as antimicrobial agents and have been found to be effective against numerous soil-borne pathogens. They can be applied to the soil, seeds, roots, and foliage to provide protection against a variety of pests and pathogens, including fungi, bacteria, and viruses. Nanoparticles can penetrate plant systems and either act directly against pathogens or function as elicitor molecules, inducing local and systemic defence responses in plants. Metallic nanoparticles, including gold,

silver, titanium oxide, zinc oxide, and copper oxide, have garnered significant attention owing to their potential antimicrobial properties. Studies have demonstrated that these particles possess potent antifungal, antibacterial, and antiviral properties" [12,13,14]. "The increased surface area to volume ratio of nanoparticles facilitates enhanced interaction with microbial cells, resulting in improved efficacy at lower concentrations compared to conventional chemical pesticides. Among these, Copper oxide NPs possess unique crystal structures and high surface areas, making them highly valuable antimicrobial agents. These NPs are robust, stable, and have a longer shelf life than other organic antimicrobial agents" [15]. Many researchers have already reported using plant extracts such as Punica granatum [16], Ficus sycomorus [17], Eugenia caryophyllata [18], *Stachys lavandulifolia* and *citrus medica* [19] and *Ocimum basilicum* [20] for the synthesis of oxide NPs. Cu has been utilized to inhibit the growth of microorganisms for over two centuries, with reports indicating that it can reduce microbial concentrations by 99.9% [21,22]. Numerous studies have demonstrated that copper nanoparticles exhibit broad-spectrum antimicrobial activity against a wide range of fungi, including *Pythium aphanidermatum*, *Fusarium solani*, *Rhizoctonia solani*, *Phytophthora infestans*, *A. alternata and Botrytis cinerea* [23,24,25,26,27]. This study found that Piper nigrum leaves can effectively synthesize bioactive copper nanoparticles without negatively affecting the environment or budget. "Piper nigrum, or black pepper, is a perennial woody climbing liana belonging to the family Piperaceae. It is native to India, Indonesia, Malaysia, South America, and the West Indies, but is also widely cultivated in tropical regions. It is considered to be the 'King of Spices.' The presence of the pungent alkaloid piperine ($C_{17}H_{19}NO_3$) attributes a spicy taste to the seeds, leaves, and other parts of Piper [28]. It also contains small amounts of safrole ($C_{10}H_{10}O_2$), pinene ($C_{10}H_{16}$), sabinene ($C_{10}H_{16}$), limonene ($C_{10}H_{16}$), caryophyllene ($C_{15}H_{24}$), and linalool ($C_{10}H_{18}O$)" [29]. Shanmugapriya et al. [30] extracted alkaloids, phenolic compounds, saponins and flavonoids from the leaves of black pepper. Alkaloids have demonstrated antioxidant potential, functioning as reducing agents, free-radical scavengers, or complexing with pro-oxidant metals.

The present study was designed with a novel, rapid, and cost-effective route for the biosynthesis of copper oxide nanoparticles (CuONPs) using *Piper nigrum* Leaf Extract. The synthesized copper oxide nanoparticles obtained by the green method are under investigation for their effect on soil phytopathogens *viz*., *Rhizoctonia solani*, *Fusarium oxysporum*, *Pythium aphanidermatum*, *and Phytophthora capsici.*

2. EXPERIMENTAL

2.1 Materials

Copper sulphate pentahydrate [$CuSO_4 \cdot 5H_2O$] is analytical grade purchased from Merck, Darmstadt, Germany and used without further purification. Deionized distilled water was used in all experimental work. The pathogens like *Rhizoctonia solani*, *Fusarium oxysporum*, *Pythium aphanidermatum*, *Phytophthora capsici*, and *Sclerotium rolfsii* procured from repository of Department of Plant Pathology, CoA. Kerala Agriculture University, Vellanikkara. Agar and dextrose were purchased from Hi-Media Private Limited, Nagpur (India). All the chemicals were used in the experiments without further purification.

2.2 Preparation of *Piper nigrum* Leaf Extract

Fresh leaves of black pepper (*P. nigrum*) were collected from Thrissur district, Kerala, in December 2022, and the specimens were authenticated by an expert from the Department of Medicinal and Aromatic Plants of the College of Agriculture, Vellanikara, KAU. The leaves of the plants were thoroughly washed in tap water and air-dried for seven days. The dried leaves were ground into a fine powder using a mixer grinder. The powder thus obtained was stored in airtight glass jars for further use. Ten grams of dried powder was mixed in 100 ml deionized water and kept in a water bath at 60 °C for 20 min [31]. After cooling to room temperature, the extracts were filtered through a muslin cloth to remove any debris. The filtrate was centrifuged at 5000 rpm for 15 min (Eppendorf SE Centrifuge 5430 R Barkhausenweg 1 22339 Hamburg, Germany) and filtered through Whatman No.1 filter paper [32]. The final filtrate was then stored at 4 °C for further experiments.

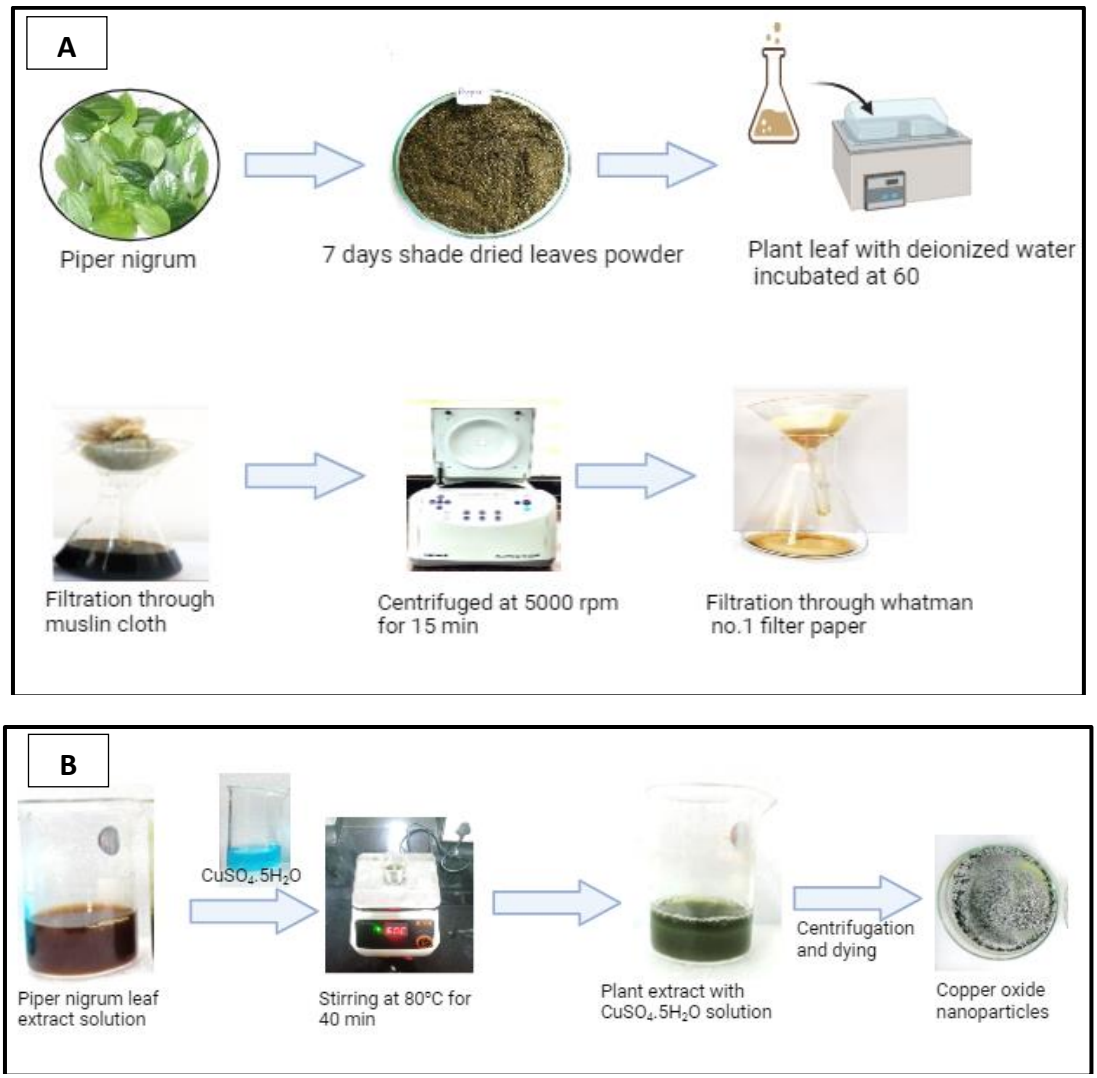


Fig. 1. Schematic representation of bio-inspired synthesis of copper oxide nanoparticles using *Piper nigrum* leaf extract; (A) Preparation of *Piper nigrum* leaf extract; (B) Biosynthesis of CuONPs from *Piper nigrum*

2.3 Biosynthesis of Copper Oxide Nanoparticles

The prepared plant extracts of black pepper were mixed with 5 ml of 0.3M copper sulphate solution. The pH of the reaction mixture was adjusted to 8 by adding 1M NaOH and stirring using a magnetic stirrer (LABQUEST by BOROSIL) at 80 °C for 40 min. The reaction mixture was centrifuged at 7830 rpm for 10 min to obtain black pepper mediated copper oxide nanoparticles (BP-CuONPs), which were washed in deionised water for twice. Further, CuO nanoparticles were dried in a hot air oven at 70 °C, transferred to a ceramic crucible, and heated in a muffle furnace, which was maintained at 500 °C for 2 h. The BP-CuONPs thus obtained were dissolved in di methyl sulfoxide (DMSO) and was analysed in UV vis spectrophotometer.

2.4 Characterization Techniques

The external and internal morphologies of the synthesized BP-CuONPs were examined using Field Emission Scanning Electron Microscopy (FESEM) coupled with Energy Dispersive X-ray (EDX)spectroscopy with a TESCAN Brono s.r.o Czech machine (Model: MAIA3 XMH) at the Sophisticated Analytical Instrument Facility (SAIF) at Mahatma Gandhi University, Kottayam. Transmission Electron Microscopy (TEM) analysis was conducted using a JEOL JEM 2100 with a LaB6 electron source operating at 200 kV from the International and Inter-University Centre

for Nanoscience and Nanotechnology (IIUCNN), MG University, Kottayam. The crystalline nature of the BP-CuONPs was assessed through Powder X-ray diffraction using an Aeris research bench top pan analytical rayon X-ray diffractometer at the Physics Department of St. Thomas College, Thrissur, Shimadzu, with CuK radiation at 1.5405 Å over a Bragg angle range of 20◦ ≤ 2 ≤ 80◦. Surface functional groups were confirmed by Fourier-transformed infrared (FTIR) spectra. The purified powder of plant-mediated metal nanoparticles was mixed with potassium bromide (KBr) to form a pellet and analyzed by FTIR (Thermo Nicolet iS50, 4000 cm^{-1} to 100 cm^{-1}) at the Sophisticated Test and Instrumentation Centre (STIC), CUSAT, Kochi. The UV-vis spectrum of copper oxide nanoparticles was recorded using a Schimadzu UV-1780 spectrophotometer in the wavelength range of 200–800 nm at a resolution of 1 nm. For this, 3 mg of the sample was diluted with 3 ml of dimethyl sulfoxide, and measurements were taken as a function of reaction time at the Department of Nano Science and Technology.

2.5 Antimicrobial Activity

The biosynthesized BP-CuONPs were evaluated against soil borne pathogens *viz*., *Rhizoctonia solani, Fusarium oxysporum, Pythium aphanidernatum and Phytophthora capsici* separately under *in vitro* condition by poisoned food technique using Potato Dextrose Agar (PDA) as basal medium. A 3000-ppm stock solution of the synthesized metal nanoparticles was prepared by dispersing 600 mg of powder form in 200 ml of deionised water. Different concentrations were prepared by mixing 33.33 ml, 50 ml and 66.6 ml of metal nanoparticles from stock with 66.7 ml, 50 ml and 33.4 ml of double strength PDA (Potatoes-400 g, Dextrose-40g, Agar 40g, dist. Water-1000 ml) medium to obtain 1000 ppm ,1500 ppm, 2000 ppm respectively. The PDA medium with the required concentration was poured into Petri plate. A control was maintained without metal nanoparticles and a 7 mm mycelial disc of five-day old fungal pathogen culture was inoculated at the centre of the plate and incubated at 23 ±10 ºC, until full growth was observed in control plates. Three replications were maintained for each treatment. Observations on colony diameter of the test isolates were recorded at an interval until the continued till untreated control plates showed complete growth of the test pathogen. The per cent mycelial growth inhibition of the test pathogens over untreated control was calculated by following formula.

$$Per\ cent\ inhibition\ (I)\ = \frac{C - T\ \times\ 100}{T}$$

where, I = Per cent inhibition, C= Radial growth (mm) of test pathogen in control and T= Radial growth (mm) of test pathogen in treatments.

3. RESULTS AND DISCUSSION

3.1 Confirmation of BP-CuONPs by X-ray Diffraction and Fourier-Transmission Infrared Spectroscopy

X-ray diffraction (XRD) analysis serves as a valuable tool for identifying phases and characterizing the crystal structure of nanoparticles. The X-ray diffraction pattern of synthesized BP- CuONPs, displayed in Fig. 2, revealed several Bragg reflection peaks at 2ɵ values of 32.45º, 35.57º, 38.69º, 48.77º, 53.57º, 58.75º, 61.72º, and 75.16º, assigned to the corresponding (110), (002), (111), (112), (020), (202), (113), (310), (220), and (004) planes, respectively. These XRD patterns indicated the highly crystalline nature of the synthesized nanoparticles with the monoclinic structure of CuO, as confirmed by JCPDS (Card No: 89-5895). These results are consistent with the findings by Nordin and Shamsuddin [33] on copper oxide nanoparticles synthesized using curry leaf extract, where the XRD analysis similarly indicated the presence of (110), (002), (111), (202), (020), (202), (113), (310), and (220) planes, confirming the monoclinic structure of copper oxide nanoparticles.

Fourier-transmission infrared spectroscopy (FTIR) analyses were conducted to ascertain the presence of diverse functional groups in biomolecules accountable for the reduction and stabilization of metal nanoparticles. The intense bands in the observed spectra were cross-referenced with standard infrared chart values to validate the functional groups. Fig. 3 shows the FTIR spectra of BP-CuONPs. The absorption peaks at 3419.2 cm^{-1} of BP-CuONps corresponds to hydrogen bonded O-H groups of alcohols and phenols [34]. The band at 1612 cm^{-1}can be allocated to the stretching vibration of C- OH bond from proteins (amide I). It may be due to the binding of one or more amide-containing alkaloids which is present in Piper like piperines [29]. The presence of peak at 1384.91

cm^{-1} and 1100.70 $cm^{-1}$of BP in CuONps may be attributed to the presence of carboxylic acids and amino groups. These biomolecules present in plant extract may act as reducing agent as well as possible reason for stability of copper oxide nanoparticles. Strong peak around 793.11 and 617.89 cm^{-1} of BP-CuONPs corresponds to the Cu-O stretching vibrations copper oxide nanoparticles in monoclinic state [35].

3.2 UV–vis Spectroscopy Analysis

The addition of *P.nigrum* leaf extract to a copper sulphate pentahydrate [$CuSO_4 \cdot 5H_2O$] solution resulted in colour change of the solution from blue to green. The colour changes arise from the excitation of surface plasmon vibrations by copper oxide nanoparticles [36]. The surface plasmon resonance (SPR) of CuO nanoparticles produced a peak centered near 270 nm as shown in Fig 4, indicating the reduction of $CuSO_4 \cdot 5\ H_2O$ into CuONPs. Similar reults were found in the study conducted by Atri et al. 2023 [37] on green synthesis of copper oxide nanoparticles using *Ephedra alata* plant extract, it was found that absorption peak at about 272 nm, confirmed good formation and pure phase of CuONps.

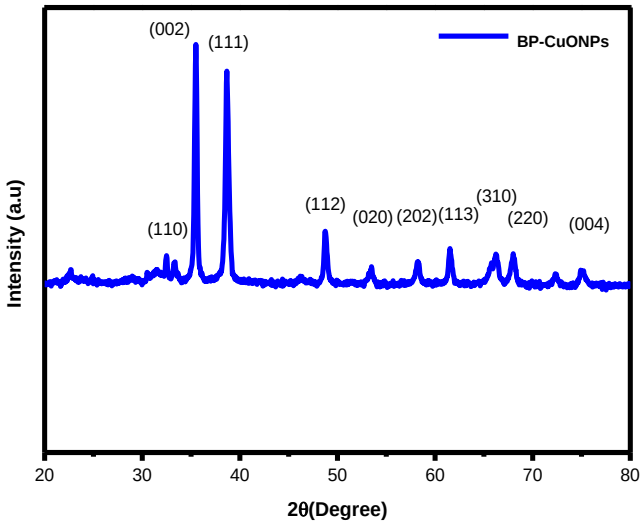


Fig. 2. XRD pattern of BP-CuONPs

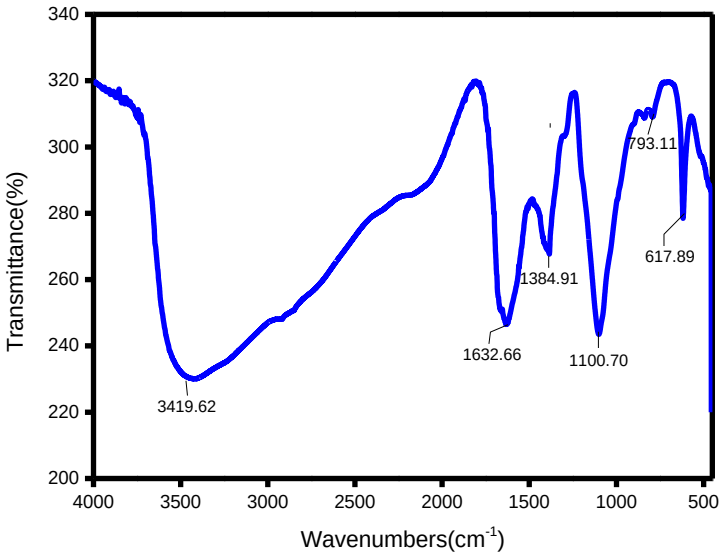


Fig. 3. FTIR spectrum of BP-CuONPs

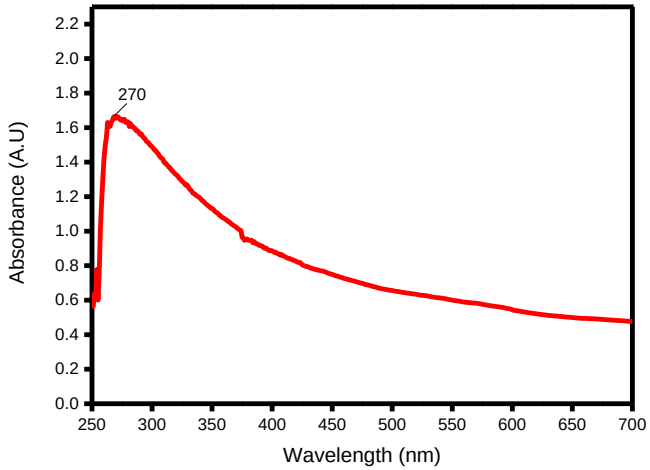


Fig. 4. UV Vis spectrum of BP-CuONPs

3.3 Scanning Electron Microscopy and Elemental Analysis of metal Nanoparticles

The particle size distribution and microstructure of the green synthesized metal and metal oxide nanoparticles were studied by the field emission scanning electron microscopy (FESEM) coupled with Energy Dispersive X-ray (EDX)spectroscopy. In the examination of BP-CuONPs, the FESEM analysis revealed the formation of spherical copper oxide nanoparticles with average diameters of 28.75 nm, respectively (Fig. 5). In the EDX analysis, copper peaks were detected at 1 keV, 8 keV and 9 keV while the oxygen peak was centred at 0.5 keV (Fig. 6). The weight percentages of copper and oxygen were found to be approximately 68.29% and 19.21% for BP-CuONPs (Table 1). Additionally, peaks corresponding to elemental constituents such as carbon, silicon, potassium, sulphur, and sodium were also detected. which may come along with plant extract which sometimes acted as a capping agent to nanoparticles. The table clearly shows that the major element is copper, which comprises more than 50% of the total constituent, along with oxygen, which clearly confirms the formation of pure copper oxide nanoparticles. This indicates the nanoparticles synthesis can be made easily by biological synthesis method.

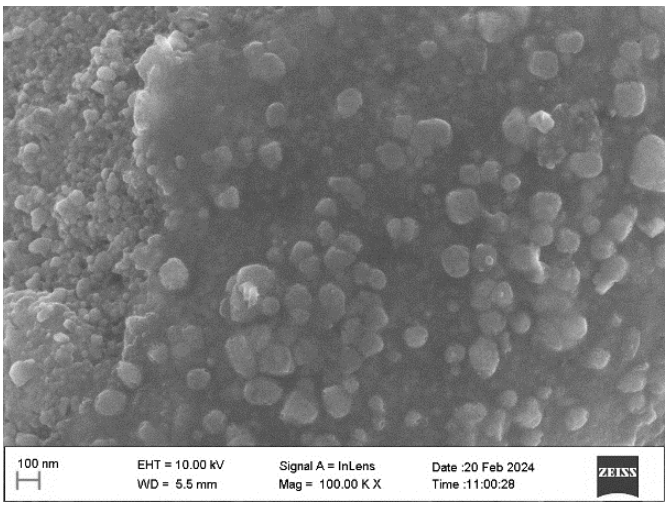


Fig. 5. SEM image of BP-CuONPs

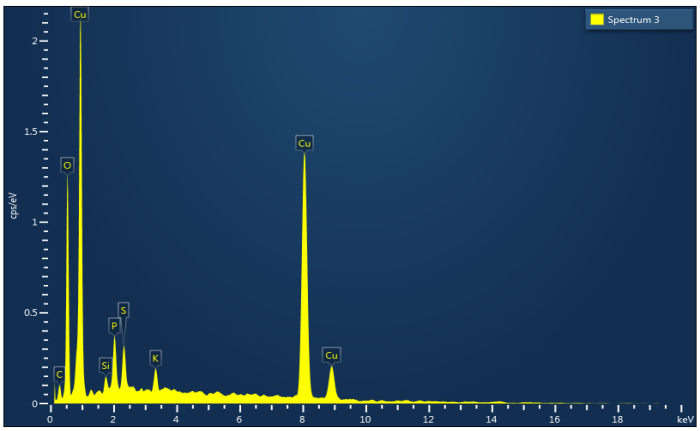
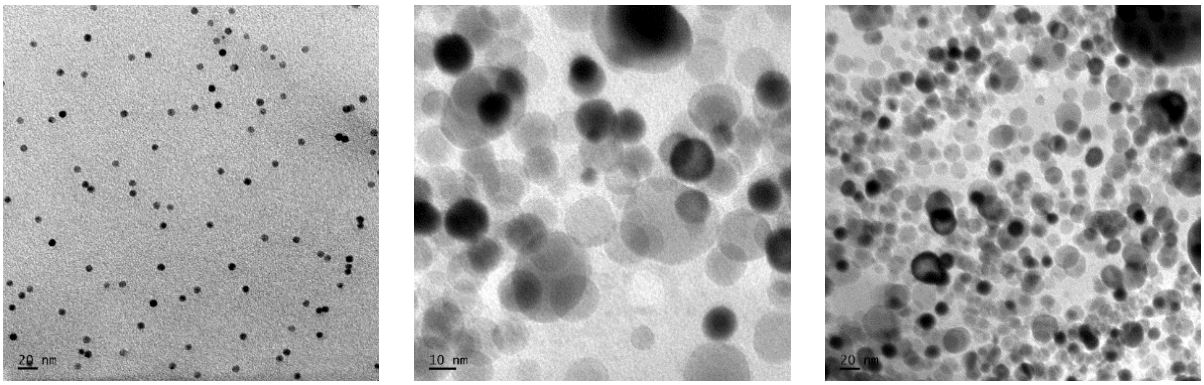


Fig. 6. EDAX spectrum of BP-CuONPs

Table 1. Elemental composition of BP-CuONPs

Element	Weight (%)	Atomic (%)
C K	5.5	15.52
O K	19.21	40.7
Si K	0.9	1.08
P K	2.62	2.87
S K	2.08	2.2
K K	1.4	1.21
Cu K	68.29	36.42
Total	**100**	**100**



(a) **(b)** **(c)**

Fig. 7(a,b,c). TEM images of BP-CuONPs

3.4 High Resolution Transmission Electron Microscopy (HRTEM)

HRTEM images of these BP-CuO NPs are depicted in Fig 7a, 7b and 7c. The analysis confirmed that the BP-CuO NPs were spherical or roughly spherical in shape with mean diameter 20 nm varied from 5 nm – 24.95 nm.

3.5 Antimicrobial Activity

The poisoned food technique was used to evaluate the antimicrobial potential of the BP-CuONPs. The results of the antimicrobial activity of BP- CuONPs tested at concentrations of 1000 ppm and 1500 ppm against *Rhizoctonia solani*, *Fusarium oxysporum*, *Pythium aphanidermatum*, *Phytophthora capsici*, and *Sclerotium rolfsii* were shown in Fig. 8. At both concentrations, *Pythium aphanidermatum* and *Phytophthora capsici* were completely inhibited, as shown in Figs. 9.a and 9.b and Figs. 10.a and 10.b. At 1000 ppm, *Rhizoctonia solani* showed partial inhibition at 22.22%, as seen in Fig 11.a, while *Fusarium oxysporum* did not exhibit any inhibitory response, as shown in Fig. 12.a. At 1500 ppm, *Fusarium oxysporum* was completely inhibited,

as seen in Fig 12.b, and *Rhizoctonia solani* demonstrated significant inhibition at 100 per cent, as shown in Fig 11.b. This study indicates that BP-CuONPs have promising efficacy against the tested organisms. These results align with the findings of Shende et al. [38], where copper oxide nanoparticles were effective against eleven fungal pathogens, including *Alternaria carthami*, *Aspergillus niger*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum f.sp carthami*, *Fusarium oxysporum f.sp. ciceri*, *Macrophomina phaseolina*, *Rhizoctonia bataticola*, *Colletotrichum lindemuthianum*, *Drechslera sorghicola*, *Rhizopus stolonifera*, and *Fusarium oxysporum f.sp. udum*. Similarly, Vanathi et al. [39] reported that *Eichhornia*-mediated copper oxide nanoparticles inhibited *Fusarium culmorum* and *Aspergillus niger* at 100 µg/ml.

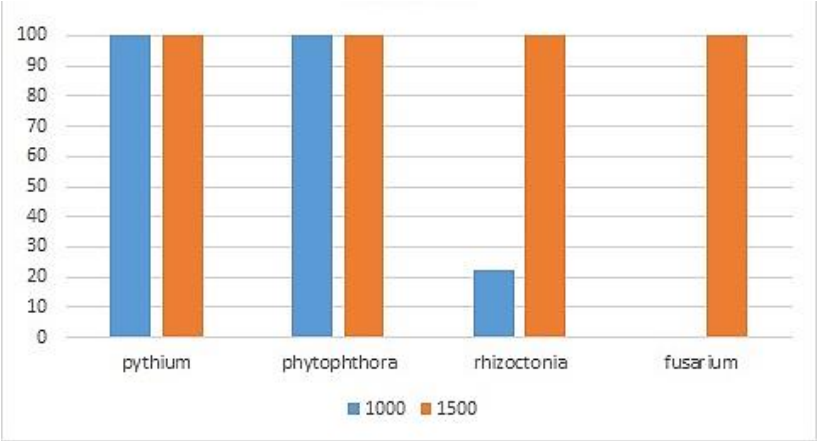


Fig. 8. Percent incubation at different concentration of synthesized BP-CuONPs

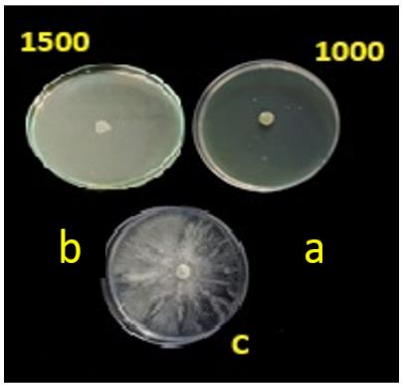


Fig. 9. Anti-microbial activity of BP-CuONPs against *Phytophthora capsica* at a) 1000 ppm b) 1500 ppm c) control

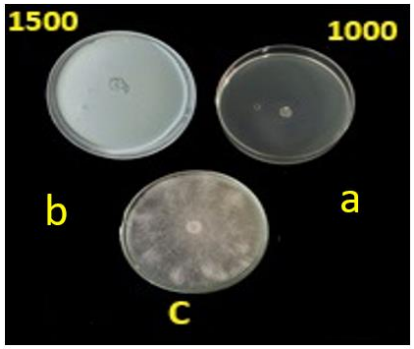


Fig. 10. Anti-microbial activity of BP-CuONPs against *Pythium aphanidermatum* at a) 1000 ppm. b) 1500 ppm. c) control

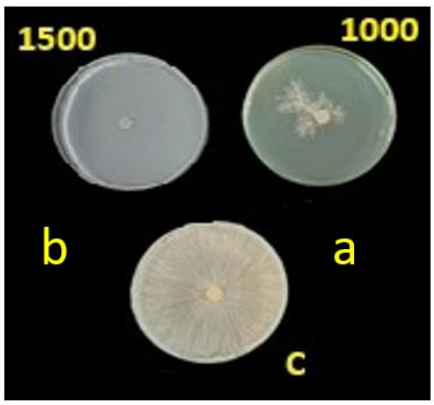


Fig. 11. Anti-microbial activity of BP-CuONPs against *Rhizoctonia solani* at a) 1000 ppm b) 1500 ppm. c) control

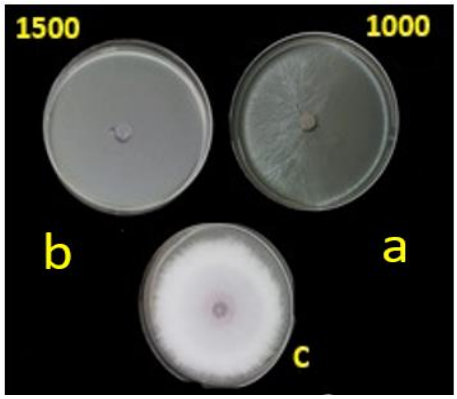


Fig. 12. Anti-microbial activity of BP-CuONPs against *Fusarium oxysporum* at a) 1000 ppm. b) 1500 ppm c) control

4. CONCLUSION

The synthesis of copper oxide nanoparticles (CuONPs) through green methods is a more environmentally friendly and safer option compared to chemical and physical methods. Our study developed a quick, eco-friendly, and practical technique for producing CuO nanoparticles using copper sulphate pentahydrate and *P. nigrum* leaf extract. The colour change resulting from surface plasmon resonance during the reaction with the extract ingredients confirms the formation of CuO nanoparticles, as demonstrated by XRD, FT-IR, UV–vis spectroscopy, SEM, and TEM. FTIR spectroscopy of the nanoparticles revealed the presence of various functional groups, including alcohols, phenols, carboxylic acids, and alkaloids, which served as reducing and capping agents for the metal nanoparticles and indicated the presence of metal oxide nanoparticles. In vitro studies have shown that these nanoparticles are effective against most soil-borne plant pathogens. Challenges such as the seasonal and regional availability of raw materials and non-uniform particle sizes need to be overcome for practical production. The unavailability of reliable toxicity data and the possibility of harm to human health need to be addressed in future studies. Future research should focus on scaling up this green technique to an industrial level while also considering its environmental and health impacts.

Highlights:

- Copper oxide nanoparticles were bio synthesized by precipitation method.
- Spherical shaped morphology was observed for the synthesised nanoparticles.
- Antimicrobial studies of copper oxide nanoparticles was conducted for *Rhizoctonia solani*, *Fusarium oxysporum*, *Pythium aphanidermatum* and *Phytophthora capsica* at different ppm.

- A complete inhibition of all the tested phytopathogens were observed at 1500ppm.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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