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Synthesis of promising copper nanoparticles utilizing biocontrol agents, *Trichoderma virens* and *Chaetomium globosum*

LHAM DORJEE[1], DEEBA KAMIL[1], RAJESH KUMAR[1], M K KHOKHAR[2], RASHMI AGGARWAL[1] and ROBIN GOGOI[1*]

ICAR-Indian Agricultural Research Institute, New Delhi 110 012, India

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ABSTRACT

The pursuit of creating safe and efficient pesticides remains an ongoing aspiration. Recently, the potential efficacy of metal nanoparticles against diverse phytopathogens has come to light. Commonly used methods, viz. chemical and physical means of nanoparticle synthesis are associated with a range of drawbacks. Fortunately, the biological synthesis method ensures safe and non-toxic nanoparticles. The present study was carried out during 2022–23 at ICAR- Indian Agricultural Research Institute, New Delhi, which documented the successful synthesis of copper nanoparticles (CuNPs) utilizing the supernatant of potent fungal biocontrol agents, viz. *Trichoderma virens* and *Chaetomium globosum.* The reduction in the size of copper was determined by UV-Vis spectroscopy. The change in colour of the reaction mixture confirmed the reduction in the size of particles. Characterization using TEM measured spherical CuNPs of the size range 8 to 100 nm depending on the $CuSO_4$ concentration used. Furthermore, Fourier Transform Infrared Spectroscopy (FTIR) unveiled the presence of several functional groups that plays pivotal role in reducing and stabilizing particle size. The *in vitro* assay conclusively demonstrated the effectiveness of biosynthesized CuNPs, with significant inhibition at a concentration of 10 µg/ml in the case of *Xanthomonas euvesicatoria* and at 50 µg/ml in the case of *Enterobacter cloacae*. Our study marks the first instance of synthesizing CuNPs through the utilization of two aforesaid fungal species holding significant promise for antibacterial applications.

Keywords: Biosynthesis, *Chaetomium globosum*, CuNPs, Pathogens, *Trichoderma virens*

The global population is anticipated to reach 10 billion by 2050, consequently amplifying the demand for food by a staggering 50% (FAO 2017). Phytopathogens render an average crop loss of 16% (Ficke 2018) and repeated use of pesticides lead to the development of resistant strains. This necessitates novel innovative methods for crop protection. Recently, Nanoscience has earned a reputation for significant applications, including plant protection (Qasim *et al.* 2022) paving the way toward sustainable agriculture. Nanoparticles synthesized by chemical and physical methods are accompanied by numerous disadvantages, viz. high power consumption, high cost and toxic byproducts. The biogenic synthesis of nanoparticles encompassing fungi, bacteria and algae is an eco-friendly approach with benefits such as enhanced efficacy and reduced cost of production. Filamentous fungi shows promise due to their ability to release ample extracellular enzymes, tolerate metal toxicity, and facilitate easy biomass handling (Hammami *et al.* 2021).

[1]ICAR-Indian Agricultural Research Institute, New Delhi; [2]ICAR-National Research Centre for Integrated Pest Management, New Delhi. *Corresponding author email: r.gogoiiari@gmail.com

CuNPs have been synthesized using *Penicillium aurantiogriseum, P. citrinum* and *P. waksmanii* (Honary *et al.* 2013), *Aspergillus niger* (Noor *et al.* 2020), and *Trichoderma harzianum* (Consolo *et al.* 2020). Recently, Dorjee *et al.* (2023) reported the antifungal activity of CuNPs against important maize fungal pathogens. Significant antibacterial activity of CuNPs against several bacteria have been reported such as *Xanthomonas axonopodis* pv. *punicae* (Mondal *et al.* 2012), *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* (Ramyadevi *et al.* 2012), *P. syringae* (Banik and Luque 2017), etc.

Xanthomonas euvesicatoria and *Enterobacter cloacae* causing bacterial spots and lesions/brown necrosis on peppers, respectively are important emerging phytopathogenic bacteria (Kyeon *et al.* 2016, Garcia-Gonzalez *et al.* 2018). Considering the significance of these bacteria, there is a pressing need for an alternative method of management. Thus, the objective of this study was to synthesize toxic-free CuNPs using biocontrol fungi namely *Trichoderma virens* and *Chaetomium globosum,* and characterize them using UV-Vis spectroscopy, TEM, and FTIR to confirm the size reduction and functional group associated with synthesized CuNPs. Furthermore, *in vitro* antibacterial efficacy against two important bacterial phytopathogens, *X. euvesicatoria* and *E. cloacae* was conducted. To our knowledge, these two fungal species have not been exploited for CuNPs synthesis hitherto.

MATERIALS AND METHODS

Collection and maintenance of fungal, and bacterial culture: The fungal cultures, viz. *T. virens* and *C. globosum* with accession numbers 4177 and 6216, respectively were acquired from the Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi, previously proven as potential biocontrol agents (Srinivasa *et al.* 2014, Rashmi 2015) (Supplementary Fig. 1). The fungal cultures were maintained in Potato dextrose agar slants at 4–5°C. The two bacterial cultures *Xanthomonas euvesicatoria* and *Enterobacter cloacae* (Accession number: ITCCBU0007 and ITCCBY0001, respectively) were procured from Plant Bacteriology Lab, Division of Plant Pathology, ICAR-IARI, New Delhi. The 16S rRNA sequence GenBank accession numbers for the two bacteria are OR350453 (Lokesh Babu and Singh 2023) and OP897636 (Lokesh Babu and Singh 2022), respectively.

Biosynthesis of copper nanoparticles: The experiments were conducted during 2022–23 at the Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi. The fungal supernatant of two fungi under study namely *T. virens* and *C. globosum* was investigated for their ability to reduce copper particle size and stabilize the generated CuNPs. The study involved 4 different concentrations of $CuSO_4$ solution (1 mM, 3 mM, 5 mM, and 10 mM) prepared in distilled water (Type III water). The detailed standardized protocol is given in supplementary material (Supplementary Fig. 2).

Characterization of bio-synthesized copper nanoparticles: UV-Vis spectrophotometer (EPOCH L2 microplate reader, Agilent Technologies, USA) was used to ascertain the reduction in particle size in scan mode (300 to 800 nm). The reaction mixture (fungal Supernatant + $CuSO_4$) was assessed for absorbance with 3 replicates, measured at 24 h intervals over a span of 4–6 consecutive days. A control i.e. $CuSO_4$ without fungal supernatant was also included for comparison. Surface morphology was studied using TEM (Jeol 1011, Japan). The sample was sonicated, then deposited onto a 400-mesh carbon-coated copper grid, subsequently stained with 2% uranyl acetate solution, and allowed to air dry for an hour. Additionally, the average particle size was determined using 'ImageJ' and 'Origin Pro 2023' software. To ascertain the functional group associated, an FTIR (Nicolet 6700 FT-IR System, USA) analysis with a total of 32 scans was carried out by collecting the spectra of the sample at a resolution of 4/cm and wavenumber accuracy of 0.01/cm.

Efficacy against plant pathogenic bacteria: The efficacy of *T. virens* and *C. globosum*-mediated CuNPs (TV-CuNPs and CG-CuNPs) was evaluated *in vitro*. Nutrient agar media was used to cultivate the bacteria. Bacterial culture (OD = 1.0) was mixed in lukewarm media poured into petri plates and left undisturbed to solidify. A well was made at the centre of a plate containing solidified media. Different concentrations of CuNPs i.e. 10 to 50 µg/ml of volume 30 µl were dispensed in the well and the plates were incubated at 26±2°C for 48 h. The inhibition zone data were subsequently recorded using a scale.

Statistical analysis: A Complete Randomized Design (CRD) was adopted with 3 replications for *in vitro* experiments. Experiments were repeated twice for validation of the obtained results. Statistical analysis of the data obtained from these experiments was carried out using SAS 9.4 (SAS Institute, 2003, Cary, NC) following the prescribed procedure. To determine significant differences between treatment means, the Tukey HSD test ($P \leq 0.1$) was employed subsequent to the analysis of variance (ANOVA), as outlined by Gomez and Gomez (1984).

RESULTS AND DISCUSSION

Bio-synthesis of CuNPs: The present study demonstrated the ability of *T. virens* and *C. globosum* to synthesize CuNPs. There are few reports showing fungi as a remarkably potent candidate for nanoparticle synthesis paving the way to safe and non-toxic nanoparticles. The colour change of the reaction mixture confirms the formation of nanoparticles (Chaudhuri *et al.* 2016). The final colour appeared to be greenish-yellow in the case of *T. virens* which was initially light blue whereas *C. globosum* supernatant led to the transition of colour from pale bluish to light brown (Supplementary Fig. 3). Dorjee *et al.* (2022) also observed a change in reaction mixture colour into brown colour from the pale colour of $CuSO_4$ solution using *Macrophomina phaseolina* as a reducing agent whereas using mycelium-free extract of *Stereum hirsutum*, $CuCl_2$ solution turned to blue indicating the formation of nanoparticles (Cueves *et al.* 2015).

Characterization of biosynthesized CuNPs: UV-Vis spectroscopy exhibited a progressive observable trend in the data showing a distinctive absorption peak that intensified as the reaction time increased confirming the reduction of particle size. In the case of *C. globosum,* absorbance maxima for the reaction mixture was found at 310, 310, 330, and 330 nm for 1 mM, 3 mM, 5 mM, and 10 mM $CuSO_4$, respectively (Supplementary Fig. 4). *T. virens* mediated synthesis resulted in the absorbance maxima at 400 nm for 1 mM and 3 mM, at 410 nm for 5 mM, and 420 nm for 10 mM of $CuSO_4$ (Supplementary Fig. 5). CuNPs have been demonstrated to give characteristic peaks between 280–360 nm (Shankar *et al.* 2014) which supports the absorption peaks obtained in the present study using *C. globosum* supernatant. However, absorbance at a higher wavelength in the case of *T. virens* could be due to the colour of the supernatant used or may be due to collective excitations of electrons of other compounds present in the media. Dorjee *et al.* (2022) recorded the absorption of CuNPs at 360 nm using *Macrophomina phaseolina* which is in agreement with the result obtained using *T. virens.*

Similarly, Noor *et al.* (2020) reported absorption maxima at 480 nm wavelength of CuNPs synthesized using *Aspergillus niger.* In another study conducted by Natesan *et al.* (2021), distinct peaks of CuNPs were obtained at 520, 520 and 530 nm when *Streptomyces griseus, Trichoderma atroviride* and *Pseudomonas fluorescens*, respectively were used. The absorption peaks primarily arise from the excitation of surface plasmon resonance, a phenomenon contingent on both the particle size and the refractive index of the solvent (Creighton *et al.* 1991).

Transmission electron micrograph analysis of CuNPs synthesized revealed different shape forms and variable sizes depending upon the type of fungi (supernatant) used as reducing agents and the concentration of $CuSO_4$. TEM analysis for *T. virens* mediated CuNPs (TV-CuNPs) revealed monodisperse spherical particles in the range 7 to 50 nm (Av. particle size = 17.54 nm), 19 to 70 nm (Av. particle size = 30.52 nm), 43 to 82 nm (Av. particle size = 28.99 nm), and 45 to 90 nm (Av. particle size = 52.76 nm) when 1, 3, 5, 10 mM $CuSO_4$ solution were used (Fig. 1 Ai-Aiv). However, at 5 and 10 mM $CuSO_4$ agglomeration of particles was observed. *C. globosum* supernatant also led to the generation of CuNPs (CG-CuNPs) in the desired size range of 10–40 nm (Av. particle size = 15.80 nm), 24-70 nm (Av. particle size = 28.21 nm), 25–115 nm (Av. particle size = 42.92 nm), 40–120 nm (Av. particle size = 52.56 nm) with 1, 3, 5 and 10 mM of $CuSO_4$ solution as a precursor (Fig. 1 Bi-Biv). Earlier reports by Devi *et al.* (2013) support our findings where they exploited *T. virens* (isolate VN-11) for the synthesis of AgNPs in the size range of 8–60 nm. Consolo *et al.* (2020) also reported CuO NPs in the size range of 38 to 77 nm using *Trichoderma harzianum*. In line with our work, Ningaraju *et al.* (2021) exploited *C. globosum* for the synthesis of gold nanoparticles of spherical size with an average diameter of 23 nm.

FTIR spectrum of TV-CuNPs recorded broad bands at 3373.00/cm,1636/cm and 1079/cm which can be assigned to N-H stretching of aliphatic primary amine and secondary amine group whereas the peak at 1079/cm was due to C-N stretching of amine (Fig. 2A). The FTIR spectrum of CG-CuNPs exhibited two bands at 3326.61/cm and 1634.77/cm which correspond to N-H stretching of amide and C=O stretching of the amide group (Fig. 2B). In consensus with our work, peaks in a similar range were reported by Kamil *et al.* (2017). Some researchers have suggested that the presence of biomolecules, including aldehydes, carboxyl groups and primary and secondary amines, especially the bands I, II, and III of amides, could serve as potential means to reduce particle size and can act as effective capping agents in nanoparticle synthesis (Kamil *et al.* 2017). The presence

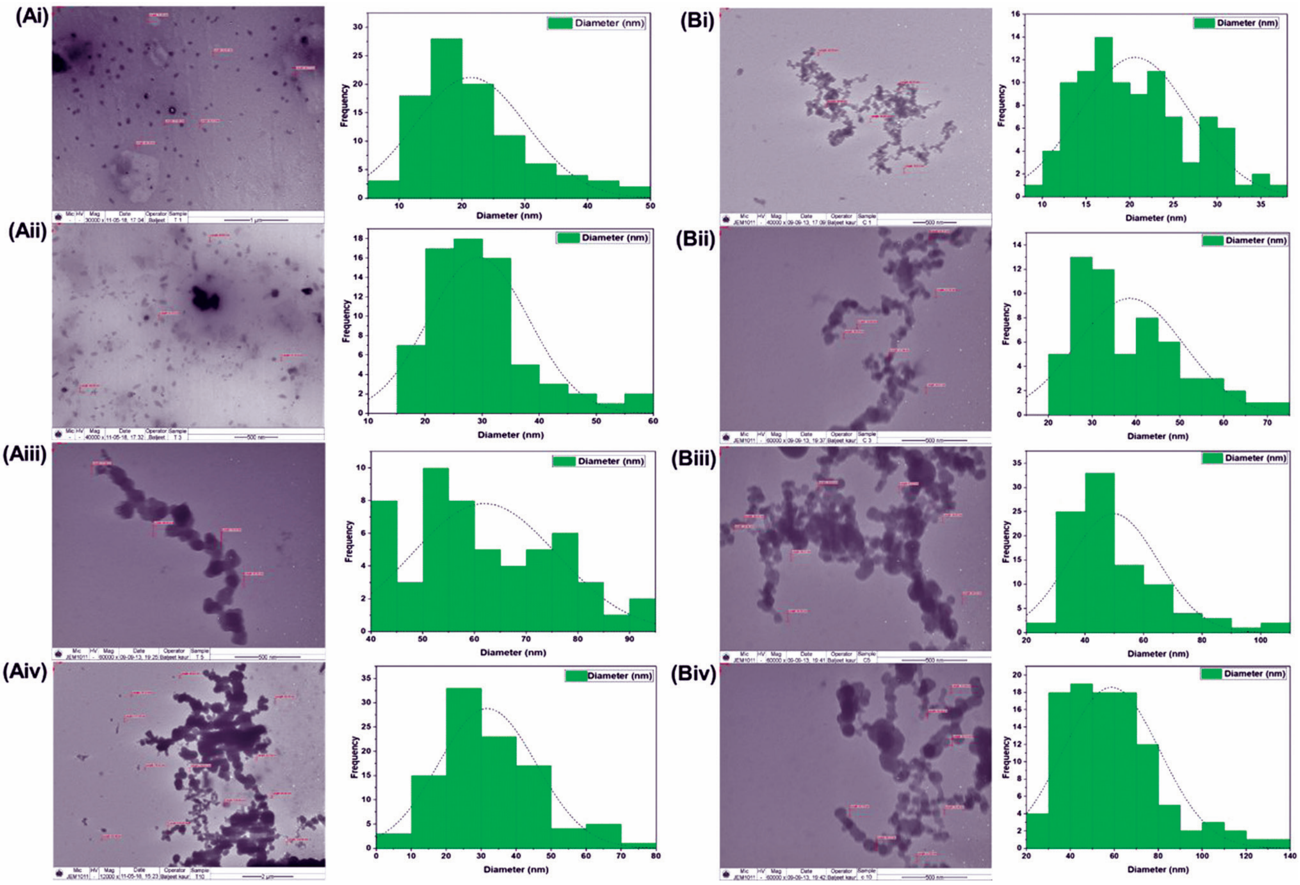

Fig. 1 TEM images of CuNPs synthesized using *Trichoderma virens* supernatant +1 mM (Ai), 3 mM (Aii), 5 mM (Aiii), and 10 mM (Aiv) $CuSO_4$ solution and *Chaetomium globosum* supernatant +1 mM (Bi), 3 mM (Bii), 5 mM (Biii), and 10 mM (Biv) $CuSO_4$ solution.

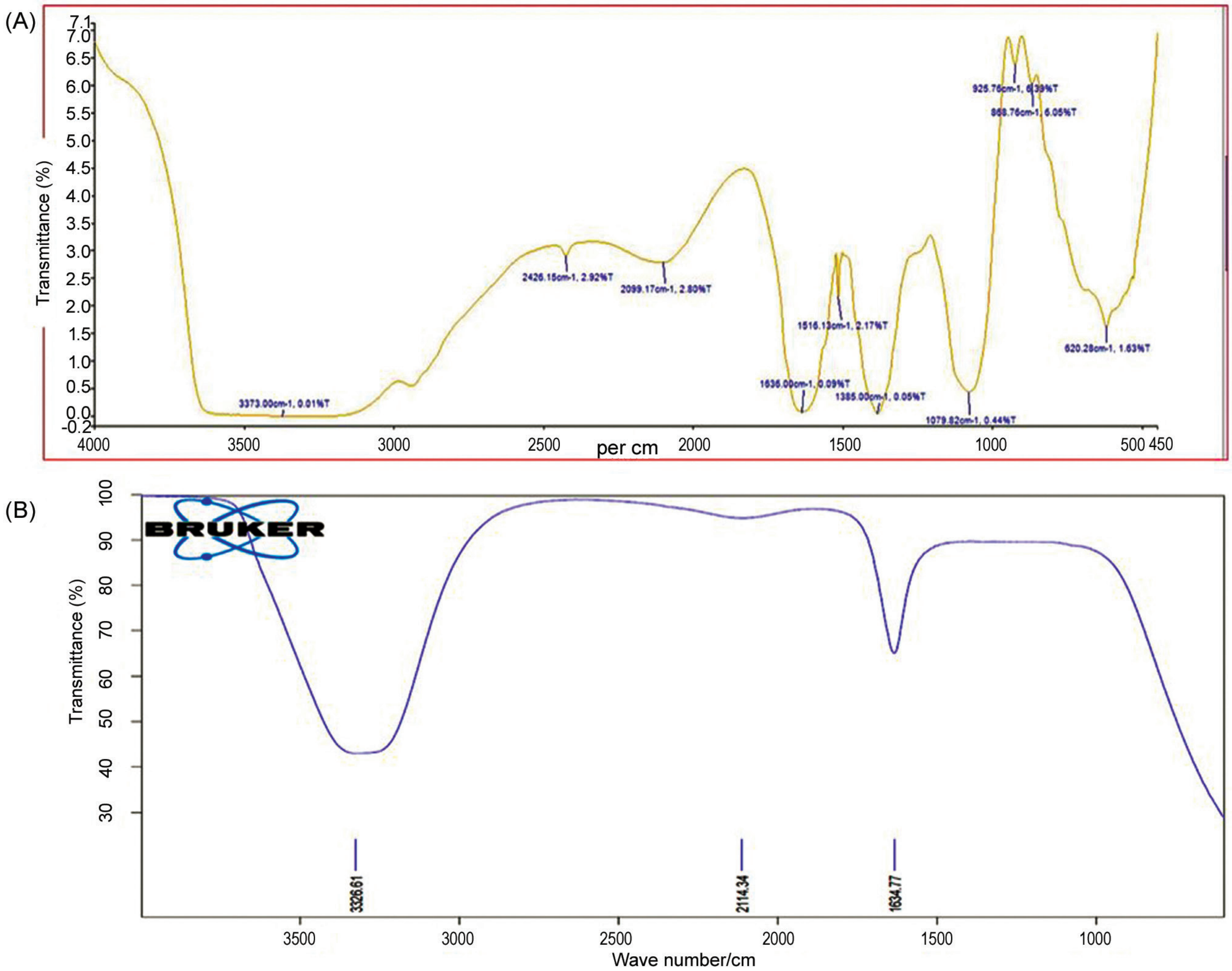

Fig. 2 FTIR Spectrum recorded from the reaction mixture containing *Trichoderma virens* + $CuSO_4$ (A) and *Chaetomium globosum* + $CuSO_4$ (B).

of primary amines, secondary amines, aliphatic amines, and amide groups plays a pivotal role in conferring stability to the synthesized CuNPs by acting as capping agents. To put it simply, they engage in a complex molecular dance that contributes to the fascinating dynamics of particle size reduction and stabilization.

Antibacterial activity of biosynthesized CuNPs in vitro: We observed a remarkable efficacy of TV-CuNPs and CG-CuNPs against the bacteria, viz. *X. euvesicatoria* and *E. cloacae*. For both TV-CuNPs and CG-CuNPs against *X. euvesicatoria,* significant inhibition was observed right from 10 µg/ml (1.30 cm and 1.37 cm, respectively) as compared to the control, and the highest zone of inhibition was observed at 50 µg/ml with values of 2.20 and 2.28 cm, respectively (Fig. 3A and B, Table 1). However, in the case of *E. cloacae*, only the highest concentration of TV-CuNPs used i.e. 50 µg/ml caused significant inhibition as compared to the control with an inhibition zone of 1.13 cm (Fig. 3C, Table 1). On the other hand, CG-CuNPs did not result in significant inhibition at different concentrations used against *E. cloacae* (Fig. 3D, Table 1). *E. cloacae* appeared to have some degree of tolerance to biosynthesized CuNPs which could be attributed to efflux pumps that actively pump out copper ions from the cell or reduce uptake by cell membrane modification reducing their intracellular concentration.

Another possible reason could be the formation of protective biofilms that shield them from the direct contact of copper nanoparticles and prevent cell damage. Copper nanoparticles act against bacteria by damaging cell membranes, generating reactive oxygen species, causing DNA damage, denaturing proteins, and releasing toxic copper ions which eventually lead to the disruption of bacterial function and cell death (Rosli *et al.* 2021). However, efficacy may vary depending on the size, shape, and surface chemistry of the copper nanoparticles, as well as the specific type of bacteria being targeted. In concurrence with our work, Nieto-Maldonado *et al.* (2022) reported the antibacterial activity of green synthesized CuNPs against *E. coli* and *Staphylococcus aureus* at 100 and 125 µg/ml with survival percentages of 0% and 10%, respectively. Similarly, Benassai *et al.* (2021) also documented the high antibacterial efficacy of CuNPs against *S. aureus, Bacillus*

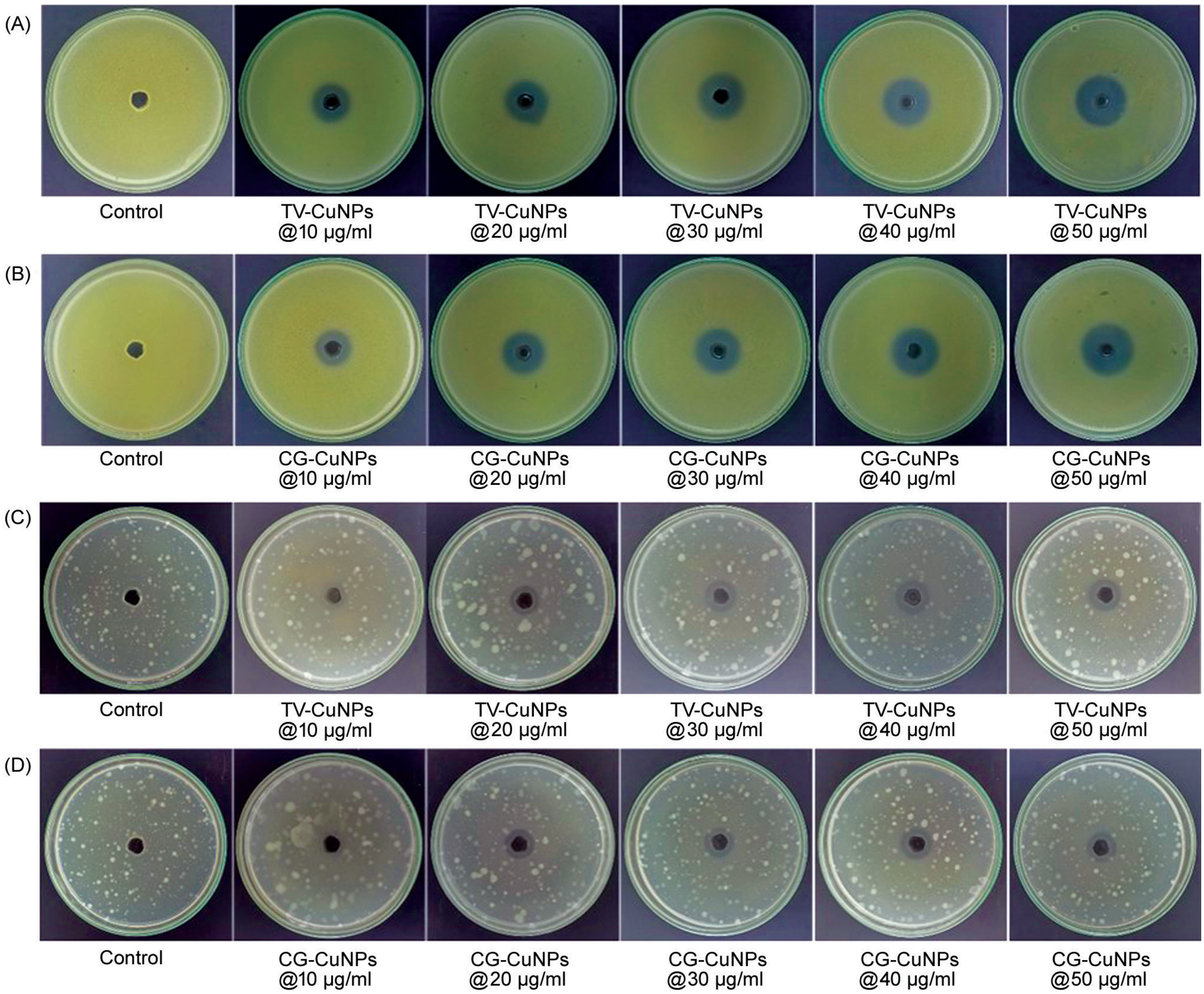

Fig. 3 The antibacterial efficacy of TV-CuNPs (A) and CG-CuNPs (B) against *Xanthomonas euvesicatoria*; TV-CuNPs (C) and CG-CuNPs (D) against *Enterobacter cloacae.*

subtilis, and *E. coli.* In support of our findings, Sharma *et al.* (2022) deduced an antibacterial efficacy of CuNPs due to higher permeability, generation of ROS and cytoplasmic component leakage against *Proteus vulgaris* and *E. coli.*

In conclusion, our study successfully introduces a straightforward, eco-friendly and highly reproducible approach for synthesizing stable CuNPs without any toxicity concerns using a supernatant of fungi that acted as a dual-purpose agent, functioning both as a reducing agent and a stabilizing agent. To the best of our knowledge, this

Table 1 The *in vitro* antibacterial efficacy of biosynthesized CuNPs recorded in terms of inhibition zone (cm)

Concentration	Inhibition zone (cm)			
	TV-CuNPs against *X. euvesicatoria*	CG-CuNPs against *X. euvesicatoria*	TV-CuNPs against *E. cloacae*	CG-CuNPs against *E. cloacae*
Control	0.00±0.00**d**	0.00±0.00**c**	0.00±0.00**b**	0.00±0.00**a**
10 µg/ml	1.30±0.06**c**	1.37±0.03**b**	0.66±0.03**ab**	0.53±0.03**a**
20 µg/ml	1.50±0.05**ab**	1.67±0.03**ab**	0.87±0.03**ab**	0.73±0.03**a**
30 µg/ml	1.73±0.03**abc**	1.83±0.03**ab**	0.96±0.03**ab**	0.83±0.41**a**
40 µg/ml	1.97±0.07**ab**	2.00±0.06**ab**	1.07±0.08**ab**	1.03±0.07**a**
50 µg/ml	2.20±0.06**a**	2.26±0.03**a**	1.13±0.06**a**	1.06±0.08**a**

Data are the mean of two experiments with three replications. Data (Mean ± Standard errors) with different letters are significant (Tukey, HSD, P≤0.01). TV-CuNPs, *Trichoderma virens* mediated CuNPs; CG-CuNPs, *Chaetomium globosum* mediated CuNPs study represents the first report of CuNPs synthesis using *T. virens* and *C. globosum* supernatants. The synthesis of CuNPs and their size in the nano range was verified through UV-Vis spectroscopy and TEM analysis, while FTIR analysis suggested the involvement of functional groups such as amine and amide groups, potentially contributing to particle size reduction and acting as effective capping agents. Furthermore, our findings demonstrated remarkable antibacterial activity of TV-CuNPs and CG-CuNPs exhibiting significant inhibition of two bacteria at a concentration of 10 µg/ml, indicating the high efficacy and promising potential of these biosynthesized CuNPs. The result demonstrates that CuNPs could be a potential means to manage bacterial pathogens. Moreover, environmental pollution exacerbated by the use of pesticides and the risk of development of antimicrobial resistance can be reduced using biosynthesized CuNPs, promoting sustainable agriculture and a healthier ecosystem. Nevertheless, it is imperative to focus efforts on standardizing a protocol that can facilitate the scaling up of the synthesis process for large-scale production with ensured consistency and reliability for disease management applications.

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