RESEARCH PAPER

Antifungal Efficacy of green-synthesized copper nanoparticles from *Xanthium strumarium* against *Fusarium moniliforme* and their characterization

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Abstract

The increasing demand for sustainable, low-toxicity alternatives to conventional fungicides has driven interest in green-synthesized metallic nanoparticles for crop protection. The methanolic leaf extract of *Xanthium strumarium* was used in this work to prepare copper nanoparticles (CuNPs), with the phytochemicals of the plant serving as both capping and reducing agents simultaneously. A noticeable color shift and a Surface Plasmon Resonance peak at around 570 nm in UV-Visible spectroscopy demonstrated successful bio-reduction of Cu^{2+} to metallic copper. The nanoparticles (NPs) were further characterized by particle size analysis (Z-average 89.6 nm), zeta potential (-21 mV, indicating colloidal stability), XRD (face-centered cubic metallic copper; average crystallite size ≈ 22.3 nm), and electron microscopy (SEM/TEM), which revealed predominantly quasi-spherical particles below 30 nm with mild aggregation. The antifungal efficacy of the CuNPs against *Fusarium moniliforme*, the causal agent of rice bakanae disease, was evaluated in vitro and in vivo. Broth microdilution assays gave a minimum inhibitory concentration (MIC) of 125 µg/mL, while the poisoned-food technique demonstrated dose-dependent mycelial inhibition, reaching 67.1% at 250 µg/mL comparable to the standard antifungal drug - Amphotericin B (74.4%). In pot trials with *F. moniliforme*-infested Pusa Basmati (1509) rice seedlings, CuNPs treatment alleviated bakanae symptoms in a dose-dependent manner, improved development of root and shoot and preserving total content of chlorophyll (51.4% of healthy control at 250 µg/mL versus 21.6% in infected, untreated plants). Acute toxicity testing in zebrafish (*Danio rerio*) at 125 and 250 µg/mL produced no mortality over 168 hours, with only transient, non-lethal behavioral changes, indicating a favorable short-term biosafety profile. These results indicate that the CuNPs isolated from *X. strumarium* are an environmentally friendly nanomaterial to manage rice bakanae disease.

Keywords: Green synthesis, Copper nanoparticles, *Xanthium strumarium*, Nanoparticle characterization, Antifungal activity, *Fusarium moniliforme*, Rice seedlings, Zebrafish toxicity

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1. Introduction

The production of plants-based nanotechnology protection methods has gained popularity recently due to the ability of metallic nanoparticles (NPs) to be effective in their antimicrobial activity while utilizing less chemicals in agriculture (Pariona et al., 2019; El-Abeid et al., 2024). The NPs of copper (Cu) and copper oxide (CuO) among other nanomaterials can be considered highly significant owing to their ability to oxidize/reduce, broad biological effects, as well as to serve as green fungicides (Lungu et al., 2026; Liu et al., 2024). Plant-based synthesis of such NPs can be regarded as a green approach to the issue, since traditional chemical synthesis methods usually involve usage of toxic substances and harsh processing conditions (Lungu et al., 2026; Shon et al., 2025; Shaikh et al., 2021). In the case of green chemistry, plant-based phytochemicals may serve as stabilizing, capping and reducing agents simultaneously, thus simplifying the synthesis process and making it potentially more biocompatible (Lungu et al., 2026; Gaddigal et al., 2024; Kumar et al., 2016).There has been successful production of platinum (Pt), gold (Au), and silver (Ag) NPs through extracts of *X. strumarium*, which suggests that *X. strumarium* can be explored as an efficient botanic material for NPs biosynthesis. Biosynthesized NPs using *X. strumarium* leaf extract have been previously characterized by various techniques and exhibited antimicrobial or antifungal activity (Kumar et al., 2016; Kumar et al., 2018; Kumar et al., 2019; Shaikh et al., 2021). Physicochemical characterization methods such as UV-Visible spectroscopy (UV-Vis.), particle size analysis (PSA), scanning electron microscopy (SEM), zeta-potential analyzer (ZPA) and X-ray diffraction (XRD), transmission electron microscopy (TEM) are often employed to verify the plant-based CuNPs (Lungu et al., 2026; Mali et al., 2020; Manasa et al., 2021). In Cu biosynthesis by plants,

UV-Visible absorption serves as a reliable method for the early detection of NPs, SEM characterize morphology and core size, while zeta potential measurements may be employed to determine colloidal stability (Mali et al., 2020; Manasa et al., 2021). The green synthesized CuNPs generally have nanoscale size and spherical or quasi-spherical shape. The hydrodynamic diameters of the particles obtained by particle-size analysis can be larger than the microscopy sizes since the capping agents also contribute to the size of NPs (Lungu et al., 2026; Shon et al., 2025). The agronomical importance of CuNPs is due to the significant yield losses associated with the infection of crops by phytopathogenic fungi and a demand for cheaper alternatives to traditional fungicides (Pariona et al., 2019; El-Abeid et al., 2024). Bakanae has been described as a significant seed and soil-borne rice disease that is primarily caused by *Fusarium moniliforme* and whose symptoms include elongation, etiolation, chlorosis, poor root development, and poor seedling vigor (Shakeel et al., 2023; An et al., 2023; Jain et al., 2021). These symptoms are due to the production of gibberellins and other secondary metabolites by fungi. Several studies have specifically pointed out that the overproduction of gibberellins is responsible for the classical symptom known as the "foolish seedling" syndrome (Shakeel et al., 2023; Jing & Suga, 2021; Haritha & Choudhury, 2021). The antifungal properties of CuNPs prepared using greener methods have been demonstrated in many species of the Fusarium genus. For instance, plant-based CuNPs inhibited *Fusarium oxysporum* with maximum inhibition of mycelium (76.29 ± 1.52%) (Mali et al., 2020). In another study, chemically prepared CuNPs inhibited 93.98% of Fusarium growth at 450 ppm, which shows that CuNPs may be efficient against Fusarium regardless of different synthesis processes (Viet et al., 2016). Additionally, green CuNPs caused morphological damage to fungal mycelium, damaged cell membrane and tends to reactive oxygen species (ROS) production inside the cells, which demonstrates that antifungal effect of CuNPs does not depend on inhibiting growth only but includes other factors as well (Pariona et al., 2019). Although direct evidence on *F. moniliforme* remains limited, information from other Fusarium literature sources encourages evaluating CuNPs activity against *F. moniliforme* based on antifungal properties exhibited by CuNPs on other phytopathogenic Fusarium species in vitro and in plants (Pariona et al., 2019; El-Abeid et al., 2024). In greenhouse or plant-pathogen setups, green CuO NPs inhibited *Fusarium solani* infection in tomatoes by 46.30-91.17% at 100 to 250 mg/mL NPs concentration (El-Abeid et al., 2024). In the context of rice-pathogen interactions, biogenic CuNPs showed reduced infection symptoms and promoted plant growth while inducing antioxidants and defense reactions in the diseased plants, supporting the importance of in-vivo testing of the material in fungus-infected rice (Islam et al., 2025). These results together justify the evaluation of CuNPs from *X. strumarium* not only in-vitro against *F. moniliforme* but also in-vivo in the fungus-infected rice plants, in which direct antifungal properties may be involved in disease suppression (Tran et al., 2025). The proposed agricultural nanomaterial also requires toxicological testing since CuNPs could enter aquatic environments and have negative impact on non-target organisms (Liu et al., 2024; Sneka et al., 2025). Zebrafish are a well-recognized model for NPs safety testing and are commonly used for mortality assessment and developmental toxicity studies (Kaur et al., 2024; Liu et al., 2024). The research study is focused on synthesizing environmentally friendly *X. strumarium*-assisted CuNPs, their characterization using UV-Vis., XRD, PSA, ZPA, SEM and TEM, evaluating the antifungal efficacy against *F. moniliforme*, testing its application on fungus-infected seedlings of rice plant, and assessing its safety using the mortality rate of zebrafish.

2. Materials and Methodology

2.1 Materials

Cupric nitrate trihydrate, Potato Dextrose Broth (PDB), methanol, acetone, sodium hydroxide (NaOH), Potato dextrose agar (PDA), Amphotericin - B, resazurin sodium salt solution was procured from SRL. *Fusarium moniliforme* MTCC No.156 was procured from IMTECH Chandigarh. Pusa Basmati rice (1509) variety was procured from IARI, New Delhi.

2.2 Green Synthesis of CuNPs

A 10 mM aqueous solution of cupric nitrate trihydrate (50 mL) was made and pH was adjusted using NaOH in order to create the CuNPs. After adding the *X. strumarium* methanolic leaf extract dropwise, the mixture was vigorously magnetically stirred at 60°C for two hours. After synthesis, the reaction mixture was centrifuged at 10,000 × g for 10 minutes to pellet the NPs. To purify the pellet and eliminate residual biomolecules from the extract, it was resuspended in distilled water and subjected to two additional rounds of centrifugation under identical conditions (10,000 × g, 10 minutes each). The final purified CuNPs were then spread onto a petri plate and dried in an oven for 24 hours (Hasheminya et al., 2020).

2.3 Characterization of Synthesized CuNPs

NPs formed were characterized by using UV-Visible spectrophotometry by scanning the NPs suspension within a suitable range of wavelengths and determining the characteristic absorption peak of the CuNPs. UV-Visible spectrometry is usually performed for characterization purposes when carrying out green CuNPs synthesis. Determination of particle size distribution was done through

particle size analyzer, whereas a zeta potential analyzer was used to examine colloidal stability and surface charge. Hydrodynamic size is usually bigger than microscopic core size since hydrodynamic size measures the core and capping layer diameter. Size and surface morphology of particles were analyzed using SEM and TEM. Green-synthesized CuNPs' nanoscale size and form are often examined using electron microscopy (Shon et al., 2025; Trzcińska-Wencel et al., 2023; Kora, 2022; Lungu et al., 2026). Cu-Kα radiation (λ = 1.5406 Å) was used to analyze the crystalline nature and phase composition of the synthesized CuNPs using XRD over a 2θ range of 10°-90°. The average crystallite size was calculated by the major reflections using the Debye-Scherrer equation, $D = K\lambda/(\beta \cos\theta)$, where K = 0.9 (Rajesh et al., 2018).

2.4 In Vitro Antifungal Assays

2.4.1 Broth Microdilution Assay

Minimum inhibitory concentration (MIC) of CuNPs obtained from *X. strumarium* towards *F. moniliforme* was determined using the broth microdilution method. Two-fold serial dilution series of CuNPs were done in sterile PDB in sterile 96-well microplates for achieving the concentrations between 1000 to 7.8 µg/mL. Broth microdilution technique is widely used to assess the MIC values of metallic NPs, and the resazurin-based detection system is proposed since it excludes the possibility of optical interference caused by suspension of NPs. Standardized spore suspension of *F. moniliforme* was made and inoculated into each well to obtain the inoculum density of 1×10^5 spores/mL. The experiment included a growth control (fungal spores + PDB) and a sterility control (uninoculated PDB). The fungus-inoculated plates were then incubated at 28°C for 48 hours. Each well was then filled with a sterile 0.01% resazurin sodium salt solution (10 µl), and the mixture was incubated for four to six hours at 28°C. Lowest concentration of CuNP's (MIC) at which the blue color of resazurin remained unchanged was evaluated; pink or white color indicated the growth of the fungus (Trzcińska-Wencel et al., 2023; Chung et al., 2023; Kora. 2022).

2.4.2 Poisoned Food Technique

The impact of the CuNPs on the hyphae growth of *F. moniliforme* was conducted via the Poisoned Food method. PDA was prepared under sterile conditions and mixed with CuNPs in concentrations of 0, 62.5, 125, 250 µg/mL and positive control - Amphotericin B (2 µg/mL). The solutions were then poured in Petri plates and left to solidify. This test is frequently used for studying antifungal properties of NPs towards Fusarium species. Each plate was centrally inoculated with a mycelia disc, which was obtained from *F. moniliforme* (5-day old culture) with actively growing edge. Three replicates for each group were prepared and left to grow for 7 days. Poisoned food tests usually involve inoculation of fungi in a disc and subsequent evaluation of colony growth after growing fungus on nanoparticle-treated solidified nutrient media. Colony diameter was measured in millimetres at the conclusion of the experiment, and the following formula was used to determine the percentage of growth inhibition:

Inhibition (%) = $(C - T)/C \times 100$, where T is the colony diameter (treated plate with CuNPs) and C is the colony diameter in the control (no treatment) (Sánchez et al., 2023; Kalia et al., 2020; Kaur et al., 2016; Elamawi et al., 2020).

2.5 Effect of CuNPs on Bakanae disease management in controlled condition

2.5.1 Treatment of *Fusarium moniliforme*-Infested rice seeds with green-synthesized CuNPs

Conidia from 2 weeks old sub-cultures of *F. moniliforme* from PDA plates were suspended in sterile test tubes, and 25 mL of sterile distilled water (dH_2O) was introduced. The test tubes were then vortexed, and conidial suspension (1mL) was further diluted and plated onto PDA plates, and incubated for three days at 28 ± 2°C. The conidial suspension was standardized to 1×10^6 conidia/mL by adding sterile dH_2O. Seeds of rice (Pusa basmati - 1509) at a density of 100 seeds/100 mL dH_2O were kept under water for 10 minutes, and were vortexed for 3 minutes. For 1 hour, seeds were air dried and were then immersed in the conidial suspension for 24 hours at 25 ± 2°C. The fungus infected seeds were air dried for 1 hour. The seeds were grouped in to five experimental groups with 20 seeds for each group. Group 1 - These were non-infested, untreated seeds which were placed in sterile dH_2O, Group 2 - (CuNPs treatment I) Infested seeds were placed in dH_2O containing CuNPs in a concentration of 125 µg/mL, Group 3 - (CuNP treatment II) Infested seeds were placed in dH_2O containing CuNPs in a concentration of 250 µg/mL, Group 4 - (Antifungal control) Infested seeds were placed in sterile dH_2O containing Amphotericin-B antifungal drug in a concentration of 2 µg/mL and Group 5 - (Untreated) Infested seeds were placed in sterile dH_2O without any CuNPs treatment and without any antifungal drug treatment (Jahan et al., 2024).

2.5.2 Management of Bakanae disease

The trial experiment to treat bakanae disease was done in pots. The seedlings were grown in soil collected from riverside, Rohtak. The soil was autoclaved before and after the experiment. In total 15 pots were divided in 5 experimental groups and each experimental group consisted of three replicates- group 1: Non-infested seeds were incubated in sterile dH_2O, dried and sown in soil without using any CuNPs and antifungal drug treatment, group 2: Infested with fungi and treated with CuNPs (concentration: 125 µg/mL), group 3:

Infested with fungi and treated with CuNPs (concentration: 250 µg/mL), group 4: Infested with fungi and treated with antifungal drug (Amphotericin-B) at 2 µg/mL, group 5: Infested with fungi without using any CuNPs and antifungal drug. In each pot 15 seeds were planted at 5-inch interval. Seeds were infested with fungi (*F. moniliforme*) according to above mentioned method and disease symptoms were noted (Jahan et al., 2024).

2.6 Assessment of antifungal activity by measurement of total chlorophyll content

Spectrophotometric technique was performed to quantify the total chlorophyll content of the leaves (Komal et al., 2022). 0.1 g fresh leaf extracts of rice seedlings were extracted in 80% cold acetone solution, it was then centrifuged for 10 minutes at 4°C at 5000 rpm. Afterwards, 80% acetone was added to dilute the solution to 10 mL. Using a UV-visible spectrophotometer, the absorbance of the supernatant was measured at wavelengths of 663 nm (A663) and 645 nm (A645). The amounts of total chlorophyll, chlorophyll a, and chlorophyll b (mg/g fresh weight) were determined as follows:

Chl a (mg/g FW) = [12.7(A663) − 2.69(A645)] × V / (1000 × W)

Chl b (mg/g FW) = [22.9(A645) − 4.68(A663)] × V / (1000 × W)

Total Chl (mg/g FW) = Chl a + Chl b

where V = Final volume of extract (mL), and W = Weight of tissue sample (g).

2.7 Zebrafish Toxicity Study

The toxicity of the synthesized CuNPs was analyzed through the use of zebrafish (*Danio rerio*) as the test organism because zebrafish are often utilized in environmental and nanotoxicology studies. Exposure of organisms to CuNPs is relevant to environmental health as metallic nanomaterials can enter water bodies and cause toxicity in organisms. The zebrafish were exposed to CuNPs at two concentrations (125 and 250 µg/mL) and one negative control group. The survival was recorded after specific time periods ranging from 48 to 168 hours. The survival data was used to record the mortality and thus serve as the endpoint for the acute toxicity analysis. Assessment of zebrafish mortality is the commonly used technique for assessing the acute toxicity of NPs. The percentage survival and behavioral changes were calculated for each time point relative to the control group. Zebrafish models have been extensively applied in assessing the environmental impacts of NPs in order to adopt safer design principles (Griffitt et al., 2007).

2.8 Statistical Analysis

All quantitative results are expressed as the mean ± standard deviation (SD) of three biological replicates (n = 3) for each treatment group. Tukey's honestly significant difference (HSD) post-hoc test was employed for pairwise multiple comparisons, and one-way analysis of variance (ANOVA) was utilized to assess differences between treatment groups. A p-value of less than 0.05 was considered statistically significant. The statistical analysis was performed using GraphPad Prism.

3. Results and discussion

3.1 Green synthesis of CuNPs

Methanolic leaf extract of *X. strumarium* was employed as the reducing and capping agent to initiate nanoparticle nucleation in the reaction solution. Successful synthesis of CuNPs was first inferred from the visible color shift observed in the reaction mixture, and this was subsequently verified through UV-visible spectroscopic analysis. A distinct color progression was observed from an initial blue, transitioning to green, and ultimately settling into a reddish brown confirmed the reduction of Cu^{2+} ions into their nanoparticle form. This characteristic color change arises due to surface plasmon resonance (SPR) excitation associated with the newly formed CuNPs, offering an early visual confirmation of formation of NPs. After drying, the nanoparticle powder was brown in color. These results were consistent with other findings (Krishnaswamy et al., 2025; Vakili et al., 2025).

3.2 Characterization of green synthesized CuNPs

3.2.1 UV-Visible spectroscopy Analysis

UV-visible spectroscopy was used in the 200-800 nm wavelength range to examine the production of CuNPs synthesized using *X. strumarium* methanolic leaf extract. The spectra of three samples: the plant extract alone, cupric nitrate trihydate precursor solution, and the biosynthesized CuNPs were recorded and compared, as shown in Figure 1.

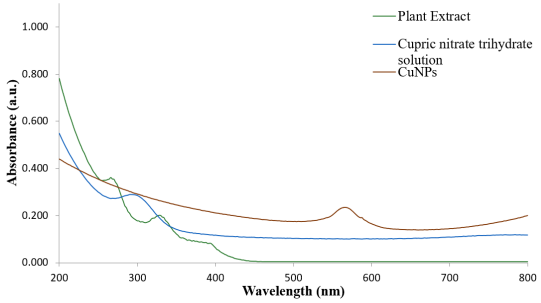


Figure 1. UV-Visible spectrum of *X. strumarium* leaf extract, cupric nitrate trihydrate solution and CuNPs

The absorbance in the UV-Visible spectrum of *X. strumarium* leaf extract show two prominent peaks at about 270 nm and about 330 nm, which corresponded to transition of the π-π* aromatic rings in the flavonoids and phenolic substances, respectively. The bioactive compounds (polyphenolic and flavonoid compounds) produce CuNPs by bio-reducing copper ions (Cu^{2+}) to metallic copper (Cu^0). UV-Visible spectrum of cupric nitrate trihydrate solution displayed a minor

peak at about 290-295 nm. The clear flat nature of the spectrum beyond 400 nm shows that the precursor existed in an ionized state of Cu^{2+} before interacting with the plant extract. The spectrum of the biosynthesized CuNPs is quite distinct from both the plant extract and cupric nitrate trihydrate solution. This gives clear optical proof of the CuNPs formation. An absorption peak appeared at about 570 nm of CuNPs. The Surface Plasmon Resonance (SPR) effect on metallic CuNPs is responsible for this absorption band. The SPR peak demonstrates the particles' relatively limited size distribution. This means that nucleation has been controlled effectively by the capping phyto-molecules from *X. strumarium*. This agrees with other works done using various green methods on CuNPs production where visible region surface plasmon resonance peaks have been observed to be in the 555-576 nm range or within 500-600 nm in case of biosynthesis of CuNPs (Mohamed, 2020; Tsilo et al., 2023). Other green synthesis of CuNPs has recorded different SPR positions such as 384 nm, 403 nm, 426 nm, and 481 nm indicating that the UV-Vis position does not correspond to a specific copper peak (Thiruvengadam et al., 2019; Murthy et al., 2020; Alshammari et al., 2023). The presence of peaks in the 270-330 nm range is also expected due to the presence of phytochemicals in the reducing medium. Phenolics, flavonoids, tannins, alkaloids, are the primary substances that cause reduction (Lungu et al., 2026; Ghosh et al., 2020; Villagrán et al., 2024).

3.2.2 Particle size analyzer (PSA) analysis

PSA was done to measure the size of CuNPs that were prepared in colloid form. The Z-average particle size was determined to be 89.6 nm. It clearly shows that the NPs synthesized lie in the nanometer range (<100 nm). Therefore, it confirmed that the NPs were successfully synthesized on nanoscale level using plant-based method of preparation. PDI (polydispersity index) was 0.262 showing that there is moderate variability in distribution of size of NPs. The size distribution graph obtained shows that there is one distinct size peak at 69.66 nm with 100% intensity as reported in Figure 2. The PSA result indicates that CuNPs produced were within the reported range of sizes of the CuNPs prepared using green synthesis methods having sizes ranges from 20-150 nm (Lungu et al., 2026; Mohamed, 2020; Shon et al., 2025).

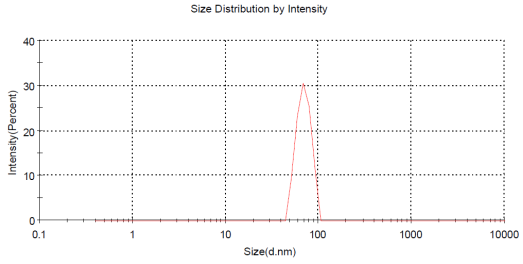


Figure 2. Particle size analyzer graph of green synthesized CuNPs

3.2.3 Zeta potential analyzer

The measurements of the zeta potential were conducted to calculate the charge of produced NPs. The result of zeta potential analysis was -21 mV as shown in Figure 3. This indicates that the NPs are negatively charged. Phytochemicals present in plant extract that coats the particles give them their negative charges. Zeta Potential Distribution graph shows only one distinct peak at -21 mV having 100% peak area, hence the particles are uniformly distributed, and there is a single species in the sample with a charge. The NPs have stabilized due to the presence of phytochemicals. Other similar plant-based CuNPs reveal that their zeta potential values range from -13.65 mV to -28.0 mV (Shon et al., 2025; Saha et al., 2023; Mali et al., 2020).

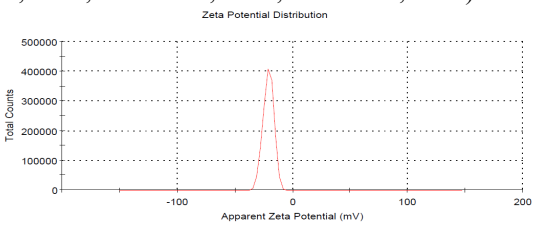


Figure 3. Zeta potential graph of green synthesized CuNPs

3.2.4 X-ray Diffraction

The bio-synthesized CuNPs' XRD pattern (Figure 4) showed distinct diffraction peaks at 2θ = 32.08°, 43.28°, 48.76°, 50.62°, 61.48°, and 66.14°. The two most intense and sharpest reflections, at 2θ = 43.28° and 50.62°, were closely aligned with the standard JCPDS card No. 04-0836 and indexed to the (111) and (200) planes of face-centered cubic metallic copper, respectively (Rajesh et al., 2018; Kausar et al., 2022). Using the Debye-Scherrer equation, the average crystallite size calculated from these two reflections was found to be 22.6 nm and 22.0 nm, respectively (mean ≈ 22.3 nm), which is consistent with the sub-30 nm particle dimensions observed in the TEM analysis. In addition to the dominant Cu^0 peaks, minor reflections were observed at 2θ = 32.08°, 48.76°, 61.48°, and 66.14°, which could be attributed to Cu_2O and CuO phases that result from the metallic Cu core's partial surface oxidation when exposed to ambient air; this behavior is frequently documented for green-synthesized Cu/CuO NPs (Amin et al., 2021). A weak, broad peak around 2θ ≈ 20–25° was also noted, which may correspond to the amorphous phytochemical layer derived from the *X. strumarium* leaf extract capping the NPs surface.

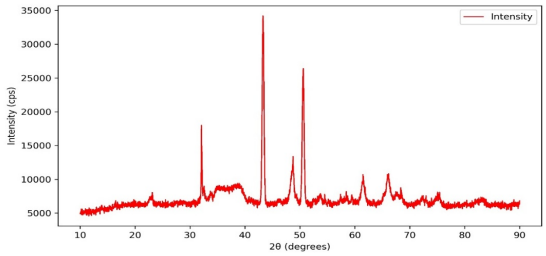


Figure 4. X-Ray Diffraction graph of green synthesized CuNPs

3.2.5 Scanning electron microscopy

The results obtained from SEM technique indicate the presence of quasi-spherical and spherical-shaped CuNPs as shown in Figure 5, indicating an inclination towards agglomeration due to their high surface energy. Quasi-spherical shaped particles and agglomeration shown by SEM images are common for copper NPs. Previous studies on green Cu/CuO NPs state the presence of spherical or near spherical-shaped particles along with aggregation or clustering because of the relatively high surface energy despite the phytochemical capping agents' presence (Amjad et al., 2021; Manasa et al., 2021). Several research have mentioned the difference between the particle size measured using DLS and SEM/TEM (Saha et al., 2023; Gaber et al., 2023; Shon et al., 2025). The effects of size and surface characteristics are known to influence the biological properties. Some studies have shown that the efficiency of CuNPs' having antimicrobial and antifungal qualities were strongly impacted by their size and surface features. Smaller particles have greater inhibition against fungi (Alshammari et al., 2023; Truong et al., 2023).

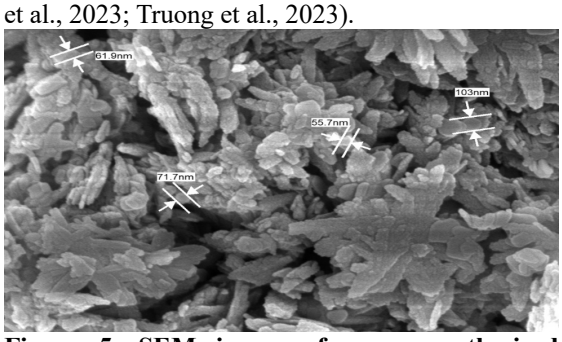
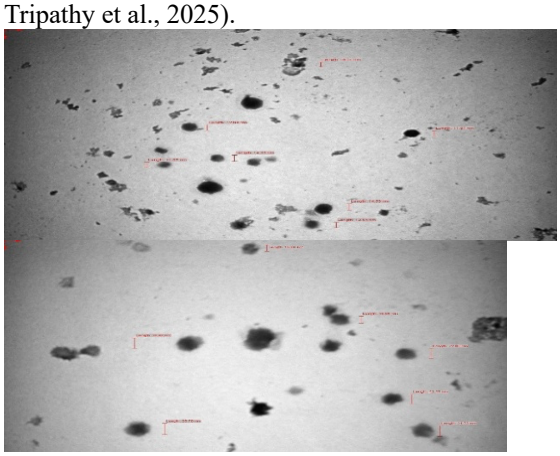


Figure 5. SEM image of green synthesized CuNPs

3.2.6 Transmission electron microscopy

Determining the size, shape, and dispersity of CuNPs produced by *X. strumarium* leaf extract through TEM study is crucial for comprehending the stability and effectiveness of these NPs in a variety of applications. The TEM micrographs as shown in figure 6 (a)(b), recorded at 200,000× magnification, show that the CuNPs are predominantly spherical with nanoscale dimensions of less than 30 nm, indicating that Cu^{2+} ions are bio-reduced to metallic Cu by the extract's phytochemicals (Saikia et al., 2023; Tripathy et al., 2025). This spherical shape aligns with previous research on green-synthesized CuNPs, which often report similar shapes and sizes due to the influence of plant-derived biomolecules serving as capping and reducing agents (Pullapukuri and Gopa, 2023; Dengale et al., 2026). The mild polydispersity and occasional aggregation observed in the TEM images are typical of green synthesis routes, where factors affect nucleation uniformity (Lee et al., 2011; Tripathy et al., 2025). The presence of faint, irregular patches surrounding the NPs is attributed to residual phytoconstituents like proteins, flavonoids, and phenolics from the *X. strumarium* extract, which adsorb onto the NPs surface, providing stabilization and preventing uncontrolled agglomeration (Dhir et al., 2023). This stabilization is crucial as it enhances the NPs stability and functionality, suitable for use in agriculture (Nkosi et al., 2025; Ghuge et al., 2026). Compared to chemically synthesized NPs, green-synthesized CuNPs offer benefits such as less toxicity and low energy consumption (Saranyaadevi et al., 2014; Tripathy et al., 2025).



(a)

(b)

Figure 6. (a), (b) TEM image of green synthesized CuNPs

3.3 Antifungal activity of CuNPs against *Fusarium moniliforme*

3.3.1 Determination of MIC by broth microdilution assay

The MIC of CuNPs synthesized by *X. strumarium* against *F. moniliforme*, measured as the lowest concentration at which there is no blue to pink color change in resazurin method, was 125 µg/mL. The antifungal efficacy of CuNPs has been demonstrated in various studies, with different synthesis methods and fungal targets. For instance, CuNPs synthesized by chemical reduction have shown substantial antifungal activity with inhibitory rates against Fusarium species as high as 93.98% at 450 ppm concentration (Viet et al., 2016). Similarly, other studies have reported MIC values for CuNPs against different fungi, highlighting the variability in fungal sensitivity to these NPs (Ghasemian et al., 2012). The research on CuNPs produced by *X. strumarium* with a MIC of 125 µg/mL for the Fusarium species offers a

fascinating combination of efficiency, matching the higher end of antifungal efficiency of NPs. Conventional synthetic fungicides typically exhibit MICs between 10-100 µg/mL, while plant-derived compounds often show higher MICs, ranging from 200-500 µg/mL, as evidenced by various studies on plant extracts and natural compounds against Fusarium species (Dhole NA, 2025; Bakar et al., 2018). Similarly, the methanolic extracts from several medicinal plants demonstrated inhibitory effects on Fusarium species, with concentrations ranging from 100-500 µg/mL (Bakar et al., 2018). The efficacy of CuNPs synthesized using natural methods, such as those from *Cinnamomum camphora* extract of fruit, has demonstrated efficacy against *F. oxysporum*, with an IC50 value of 154.39 µg/mL (Huang et al., 2019). This suggests that the *X. strumarium*-mediated CuNPs are competitive with other natural antifungal agents, offering a promising alternative to synthetic fungicides. The variability in antifungal activity of CuNPs is due to different synthetic processes, particle size distribution, and target organism that have a significant impact on how they behave (Huang et al., 2019).

3.3.2 Antifungal activity by poisoned food technique

The CuNPs have an inhibiting effect on the growth of *F. moniliforme* depending on their concentration. With a minimum concentration of CuNPs (62.5 µg/mL), 25% inhibition (31.00 ± 2.6 mm diameter of zone of inhibition) was observed. At higher concentrations, such as 125 µg/mL, level of inhibition increased up to 55% (22.33 ± 2.5 mm zone of inhibition) as mentioned in Figure 7(A),(B). This indicates an improvement in the antifungal activities of the NPs with increasing concentration. The highest concentration of 250 µg/mL showed 67.1% of inhibition (16.33 ± 1.5 mm diameter of zone of inhibition), which is comparable to the 74.4% of inhibition (12.67 ± 1.5 mm diameter of zone of inhibition) caused by the antifungal drug (Amphotericin B). CuNPs concentration had a significant impact on fungal colony width, according to a one-way ANOVA, and therefore on percentage growth inhibition ($F(4,10) = 137.17$, $p < 0.0001$, $\eta^2 = 0.982$). Tukey's HSD post-hoc test showed that all CuNP concentrations (62.5, 125, and 250 µg/mL) significantly decreased colony diameter in comparison to the untreated control ($p < 0.0001$), and each stepwise increase in CuNP concentration produced a further significant reduction (62.5 vs. 125 µg/mL, $p = 0.0046$; 125 vs. 250 µg/mL, $p = 0.044$), confirming a clear dose-dependent antifungal effect. Interestingly, there was no discernible difference in the colony diameter at 250 µg/mL CuNPs and amphotericin B ($p = 0.31$), indicating that the highest tested CuNPs concentration achieved antifungal efficacy statistically comparable to the standard antifungal drug. From the above results, it is evident that increasing concentration of CuNPs leads to increasing their inhibitory effects. It was shown that synthesized CuNPs had potent antifungal action against *F. moniliforme*. The efficiency was found to be similar to that of an antifungal drug at higher concentrations. There is an obvious dose response correlation between CuNPs concentration and the inhibitory rate as the concentration increased from 25% at 62.5 µg/mL, 55% at 125 µg/mL, and 67.1% at 250 µg/mL. This is correlated with other research on CuNPs against Fusarium species (Abbas et al., 2025; Mali et al., 2020; Khatami et al., 2019). Other studies have also noted potent inhibition of *F. oxysporum* or other Fusarium pathogens, where there was inhibition ranging from $59.25 \pm 0.57\%$ to $76.29 \pm 1.52\%$ at 0.12 to 0.24% CuNPs concentration. According to an investigation, at the maximum dosage of CuNPs (100 ppm), the inhibition rates were 80.12% for *F. oxysporum* and 94.89% for *F. solani*. The growth of *F. oxysporum* was almost totally inhibited since the E value recorded for the CuNPs sample was 100% at 80 ppm and 100 ppm concentration levels (Mali et al., 2020; Abbas et al., 2025; Truong et al., 2023). There are formulations that outperform traditional fungicides in certain conditions. One example is chitosan-loaded CuO nanocomposites which inhibited *F. oxysporum* isolate by 96.22 ± 1.35% at 1 mg/L and decreased tomato wilt infection by 91.5%, emphasizing the importance of formulating NPs (Mosa & El-Abeid, 2023). It has been found out that nanoparticle containing Cu can damage the surface of hyphae, disrupts the plasma membrane, cell wall, nuclei, and cytoplasm of Fusarium (Ibarra-Laclette et al., 2022; Gaber et al., 2023; Parada et al., 2024; Gandhi et al., 2024).

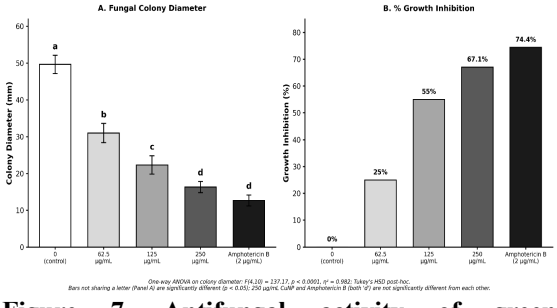


Figure 7. Antifungal activity of green synthesized CuNPs against *Fusarium moniliforme*

3.4 In-vivo antifungal activity

3.4.1 Effect of green CuNPs on bakanae management in controlled condition

The seedlings that survived in the five treatment groups exhibited a clear variation in terms of their shoot growth, leaf color, and root system that depended on their status concerning the infection

with the fungus and the treatments with CuNPs and the antifungals (Figure 8).

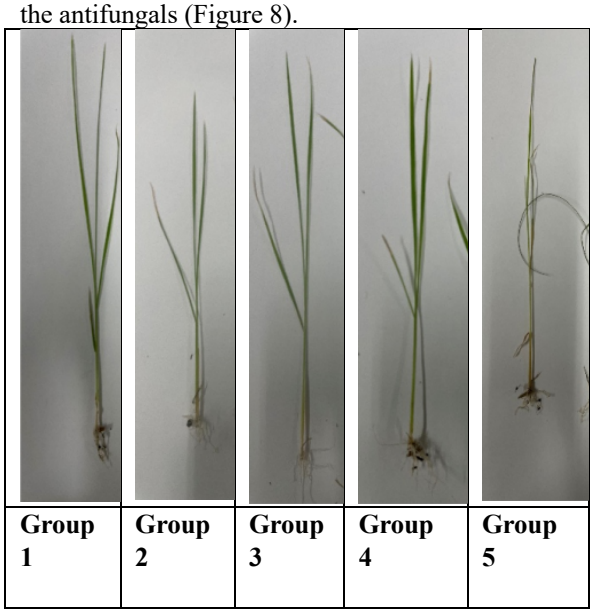


Figure 8. Rice seedlings with different treatments

The Group 1 (Non-infested, untreated) exhibited healthy traits with upright, dark green shoots of medium size and a healthy branched root system and was considered the healthy control against which the infected and treated groups were compared. On the other hand, Group 5 (Infested, untreated) was the most severely affected, exhibiting the classical bakanae disease phenotypes. It shows elongated, thin, greenish-yellowish color and etiolated shoots and leaves and weak root system with very few fibrous roots. *F. moniliforme*, the causative organism of bakanae, is very notorious for producing gibberellins and other secondary metabolites responsible for interfering with the natural growth and development of seedlings through abnormally elongated plants or even more severely infected ones resulting in stunted growth, poor root development and seedling mortality. The group 5 proved that the pathogen was successfully grown. In comparison to the aforementioned results, both CuNPs treated groups performed relatively well showing a dose-dependent trend. The Group 3 (CuNPs treatment: 250 µg/mL) was the most effective of the NPs treated samples with well-developed shoots with green colors, along with better developed roots close to the vigor displayed by the antifungal drug group. The Group 2 (CuNPs Treatment: 125µg/mL) had better shoot growth and well pigmented leaves together with developing roots relative to the fungus. The Group 4 (Antifungal drug: Amphotericin B - 2 µg/mL) generated the best grown seedlings compared to the other treated fungi-infected samples. This is due to its well-developed shoots, darkest green coloration, and largest developed root system. In general, it can be inferred that the effectiveness ranking, namely Group 1>Group 4>Group 3>Group 2>Group 5 confirmed the presence of an antifungal effect on *F. moniliforme* in rice seedlings through the use of CuNPs. It supports the interpretation that CuNPs exert a genuine, dose-responsive antifungal effect against *F. moniliforme* in rice seedlings. CuNPs can be a potential nanomaterials alternative for the conventional antifungal treatment of seeds against bakanae disease, pending further optimization and field testing. The higher dose of 250 µg/ml of NPs was more effective in inducing recovery compared to 125 µg/ml of NPs. Other research using NPs in greenhouse/net house conditions has also reported the dose-dependent reduction in Fusarium diseases and improved vigor or plant growth parameters (El-Abeid et al., 2024; Gandhi et al., 2024; Jahan et al., 2024). Copper salicylate NPs boosted the seedling performance and decreased blight of infected rice seeds and reduced seed rot (Gandhi et al., 2024).

3.4.2 Total Chlorophyll Content of Rice Seedlings in different treatments

Fungal infection by *F. moniliforme* caused substantial decrease in the total amount of chlorophyll in rice seedlings in contrast to the healthy control (Figure 9). The maximum quantity of total chlorophyll was found in the healthy seedlings (0.9291± 0.048 mg/g FW) whereas in infected seedlings the amount decreased substantially to 0.2017 ± 0.013 mg/g FW (21.6% of the healthy control). Treatment with CuNPs led to a dose-dependent recovery of the chlorophyll amount in infected seedlings. Treatment of seedlings with CuNPs at 125 µg/mL recovered 0.4009 ± 0.011 mg/g FW (43.1% of the healthy control) whereas in case of 250 µg/ml CuNPs treatment, the amount recovered to 0.4777 ± 0.044 mg/g FW (51.4% of the healthy control). The most recoverable seedlings were those exposed to amphotericin B (2 µg/ml) having the highest chlorophyll value (0.5751± 0.034 mg/g FW; 61.8% of the healthy control). A highly significant impact of the treatment on total chlorophyll concentration was verified by one-way ANOVA($F(4,10) = 189.70$, $p < 0.0001$). Tukey's HSD post-hoc test showed that chlorophyll content in all treated groups (CuNP 125 µg/ml, CuNP 250 µg/ml, and amphotericin B) was significantly higher than in the untreated infected control ($p < 0.001$ for all comparisons), confirmed that each treatment significantly mitigated infection-induced chlorophyll loss. However, chlorophyll content in all infected groups (treated and untreated) remain far lower compared to the non-infested, healthy control ($p < 0.0001$), indicating partial rather than complete physiological recovery. In general, the trend of chlorophyll content among different treatments (Healthy>Treated with amphotericin B>Treated with CuNPs (250 µg/mL)>Treated with CuNPs (125 µg/mL)>Infected-untreated) was very close to the antifungal efficacy order, which was observed

in the in-vitro test. The CuNPs inhibited growth of *F. moniliforme* leads to preservation of chlorophyll content and vigor in infected rice seedlings. Similar studies of Cu-based NPs in Fusarium-infected plants show decreased severity of the disease along with increased vigor and chlorophyll content. Sharp decrease in total chlorophyll in infested, untreated seedlings corresponds to the known biological characteristics of the disease because Fusarium infection in rice often results in chlorosis, elongation of seedlings, and decrease in vigor (Husna et al., 2024; Bashyal & Aggarwal, 2013). Low to moderate levels of CuNPs increase chlorophyll content under pathogenic pressure, though the phenomenon is dose-dependent. Fusarium root rot disease was significantly reduced by CuO NPs made from *Zizyphus spina* leaf extract (72.0 to 88.6%) in tomato plants. Chlorophyll pigments are increased by CuNPs or CuO NPs while reducing Fusarium disease, especially in formulations enhancing nutrients or defense activity (El-Abeid et al., 2024; Ashraf et al., 2021). Suppression of pathogens reduces losses in pigments though does not completely restore the physiological state of uninfected seedling after the damage caused by pathogens (López-Lima et al., 2021). One such important caution to mention is that while the positive effect on the plant and the biosafety may not always coincide with the maximum dose. Some investigations have proved to induce chlorophyll formation or growth promotion at a low concentration, at high concentration, some physiological damage has also been observed (Tiwari et al., 2024; El-Abeid et al., 2024).

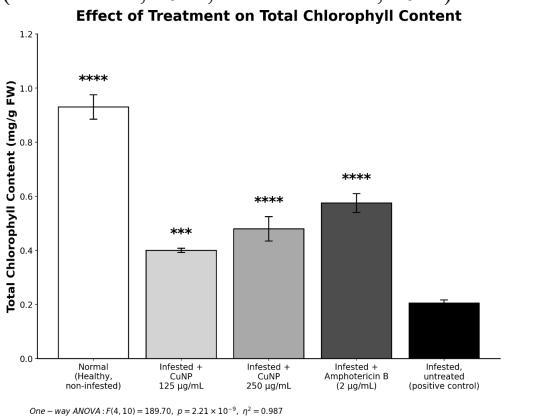


Figure 9. Total chlorophyll content of rice seedlings under different treatments

3.5 Acute Toxicity Assessment of CuNPs in Zebrafish

To determine the acute toxicity level of *X. strumarium*-produced CuNPs, Zebra fish (*Danio rerio*) was used in the study. Two concentrations of CuNPs (125 µg/mL and 250 µg/mL) were tested in addition to a control group. Separate aquaria were used to keep the fish exposed to each treatment concentration. There were no mortalities in either of the test groups in the whole observation period. It means that there were no lethal impacts on the fish due to the tested concentrations of CuNPs. Some fish from both test groups demonstrated some behavioral changes. Reduced swimming activities and floating near the water surface were observed in some fishes exposed to CuNPs. Also, some zebrafish demonstrated short loss of equilibrium and decreased feeding response in the first hours after exposure to CuNPs. These changes in the behavior were more pronounced in the 250 µg/mL CuNPs group relative to the 125 µg/mL one. All mentioned symptoms are not lethal. The lack of mortalities in both test groups shows that *X. strumarium*-produced CuNPs are quite safe at the tested concentrations. Reduced swimming activity, surface dwelling, loss of equilibrium for a few moments, and reduction in feeding behaviors are consistent with the toxicity profile of zebrafish, where Cu exposure is known to affect swimming behavior and social behavior (Santos et al., 2020; Santos et al., 2022). However, the increase in the level of mild stress symptoms among the 250 µg/mL group due to dose effect is also logical since Cu intoxication in the zebrafish can develop according to the principle of dose dependence and can be affected by elements including formulation, Cu ion dissolution, and particle size (Santos et al., 2020; Santos-Rasera et al., 2022; Batîr-Marin et al., 2025).

4. Conclusion

The present study shows that *X. strumarium* leaf extract may be used to green-synthesize CuNPs. Successful bio-reduction is verified by a distinctive surface plasmon resonance peak at around 570 nm and a nanoscale Z-average size of 89.6 nm, a stabilizing negative zeta potential of -21 mV, an XRD-confirmed face-centered cubic metallic copper phase (average crystallite size ≈ 22.3 nm), and SEM/TEM evidence of predominantly quasi-spherical particles below 30 nm. These CuNPs exhibited a clear, dose-dependent antifungal effect against *Fusarium moniliforme* in vitro, with an MIC of 125 µg/mL and mycelial growth inhibition rising from 25% at 62.5 µg/mL to 67.1% at 250 µg/mL (one-way ANOVA, $F(4,10) = 137.17$, $p < 0.0001$), a level statistically indistinguishable from the 74.4% inhibition achieved by amphotericin B ($p = 0.31$). This fungal inhibition was evident in pot experiments carried out on Pusa Basmati (1509) rice seedlings infested with *F. moniliforme* where application of CuNPs led to noticeable reduction of bakanae disease symptoms and significant preservation of total chlorophyll content relative to untreated infected controls (250 µg/mL: 51.4% of the healthy control versus 21.6% in infected, untreated plants; $F(4,10) = 189.70$, $p < 0.0001$), although chlorophyll levels in all infected groups remained below those of the healthy control, indicating partial rather than complete

physiological recovery. Acute toxicity testing in zebrafish recorded zero mortality at both 125 and 250 µg/mL over 168 hours, with only transient, non-lethal, dose-related behavioral changes such as reduced swimming activity and brief loss of equilibrium, supporting a favorable short-term biosafety profile. Taken together, these findings position *X. strumarium*-derived CuNPs as a promising, environmentally friendly nano-fungicide candidate for managing rice bakanae disease, combining antifungal efficacy approaching that of a conventional antifungal drug with a favorable short-term safety margin in a non-target aquatic organism. However, before practical deployment can be recommended, further work is needed to verify the long-term chemical phase stability of the particles, quantify in vivo efficacy with larger replicated trials, and perform longer-term ecotoxicity and field-validation studies.

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Disclosure statement: The authors confirm that there is no conflict of interest.

Data Availability: All datasets generated or analyzed during this study are included in the manuscript.

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