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Effects of Green Synthesized Copper-Derived Nano Particles extracted from *Piper betel* to Eliminate Contaminations in tissue culture of *Dracaena sanderiana* Sander ex Mast. cv 'Gold'

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Abstract

Micropropagation is often hindered by contamination from endophytic microbes. While antibiotics are used to eliminate these without harming plant tissues, they risk promoting antibiotic resistance. This study explores a green alternative by synthesizing copper nanoparticles (Cu-NPs) using antimicrobial-rich *Piper betel* leaf extract. The eco-friendly method uses plant biomolecules as natural reducing and stabilizing agents. The aim was to (i) produce Cu-NPs via eco-friendly plant-based synthesis, (ii) evaluate their antibacterial activity against *Escherichia coli* and *S. aureus*, and (iii) assess their effectiveness in reducing microbial contamination in plant tissue culture. Cu-NPs were produced by reacting leaf extract with cupric sulphate under controlled heating and stirring. Their antibacterial activity was tested against *E. coli* and *Staphylococcus aureus* via time-kill assays, and their efficacy in reducing contamination was assessed by applying them to *Dracaena sanderiana* explants in vitro. 200 ppm was identified as the optimal concentration for the antibacterial activity in *in vitro* establishment trials of *Dracaena sanderiana*, 50 ppm Cu-NPs proved to be the most effective concentration, resulting in 100% survival when used both for surface sterilization and as a supplement in the culture medium, and 80% survival when used only for surface sterilization. The findings suggest that green-synthesised Cu-NPs from *P. betel* show potential as an alternative to antibiotics in plant tissue culture, with promising effects on contamination control and reduced risk of antibiotic resistance.

Keywords: Copper nano particles, *dracaena sanderiana,* green synthesis, *in vitro* establishment, *piper betel*

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Introduction

Combining plant biotechnology with nanotechnology offers sustainable solutions for agriculture. A key challenge in tissue culture is contamination from endophytic microbes, which often survive surface sterilization. Conventional sterilants such as hypochlorites, alcohols, antibiotics, and fungicides are widelyused, yet contaminations from endophytes remains a concern. The repeated use of human antibiotics also raises ethical and scientific issues, including the risk of antibiotic resistance (Ahlawat et al., 2022).

Copper nanoparticles (Cu-NPs) are known for their antimicrobial properties and can be synthesized via ecofriendly plant-based methods. Green synthesis using plant extracts is a sustainable alternative to chemical or microbial methods, offering simplicity, cost-effectiveness, and reduced toxicity (Singh et al., 2023). Plant-derived biomolecules like flavonoids, terpenoids, and polyphenols act as natural reducing and stabilizing agents. (Yoonus et al., 2021).

Numerous studies have demonstrated the successful synthesis of Cu NPs using various plant sources recently (Shende et al., 2015; Tamil Elakkiya et al., 2022; Wanaguru et al., 2025). This study explores the green synthesis of Cu-NPs using *Piper betel* leaf extract, which contains alkaloids and phenolic compounds. The synthesized Cu-NPs were evaluated for their ability to reduce contamination and improve micropropagation of *Dracaena sanderiana* (lucky bamboo). As nodal explants are commonly used and prone to microbial contamination, they serve as an ideal model for evaluating sterilization strategies (Seneviratne et al., 2025). The study aims to identify optimal Cu-NP concentrations for improving in vitro establishment and contamination control in *D. sanderiana* (Ahlawat et al., 2022; Anju et al., 2021).

Materials and Methods

Green synthesis of Copper Nanoparticles using Piper betle L. cv. 'Maneru'

Piper betel L. cv. 'Maneru' leaves were collected from Malabe, Colombo, Sri Lanka, washed with tap water and soap, then cut into small pieces. A total of 200 g was blended with 100 mL distilled water and sonicated for 1 hour. The extract was filtered sequentially through cotton cloth, cotton wool, and Whatman No. 01 paper. A concentration of 1 M $CuSO_4{\cdot}5H_2O$ solution was prepared by stirring at 600 rpm and 70 °C. The P. betel extract was added dropwise and stirred under the same conditions for 18 hours. The resulting Cu-NP solution was kept in the dark for 5 hours, then filtered, oven-dried at 50 °C for 2 hours, and ground into fine powder. Previous studies confirmed the feasibility of this green synthesis method (Wanaguru et al., 2025).

Characterization of Cu-NPs

The surface morphologies, bulk & crystalline structure, nano structure and surface chemistry of the synthesized Cu-NPs were characterized through scanning electron microscopy (SEM), transmission electron microscopy (TEM), powder X-ray diffraction (PXRD) and Fourier transform infrared (FTIR) techniques. SEM imaging was conducted using Hitachi SU6600 FE-SEM (field emission scanning electron microscope) operated at an accelerating voltage of 10 kV. TEM imaging was performed using a JEOL – JEM – 2100 high resolution transmission electron microscope operated at an accelerating voltage of 200 kV. PXRD analysis was performed in 2 θ range 10- 80 degrees using a Bruker D8 X-ray diffractometer. The surface chemistry of the synthesized Cu-NPs was identified using an ABB-MB 3000 Fourier Transform

Infrared Spectrometer under attenuated total reflection mode (ATR-FTIR). The spectrum was recorded between 4000 – 400 cm^{-1} with a resolution of 8.0 cm^{-1} for 64 consecutive scans.

Time kill determination (Optical density method)

To investigate the antimicrobial activity of Cu NPs, different concentrations (50 ppm, 100 ppm, 200 ppm, 250 ppm) of green-synthesized Cu-NPs were tested on *E.coli* and *S. aureus* microorganisms in this experiment. Cu-NPs were dispersed in distilled water and sonicated for 1 hour. Each test involved adding 1 mL of bacterial culture (5×10^7 CFU/mL) to 10 mL of Cu-NP suspension. Samples were incubated at 37 °C with shaking at 200 rpm using a shaking incubator

NP-free bacterial suspensions in distilled water served as negative controls. The initial absorbance at 0 minutes and the final absorbance after 2 hours of incubation of all suspensions were measured using a VERNIER™ LABQUEST2 (INTL) associated with GO DIRECT SPECTROVIS PLUS (INTL) unit.

Application of Cu NPs to in vitro establishment of D. sanderiana

Preliminary surface sterilization

In this study, 200 single nodes (1.5 cm) of *D. sanderiana* were used to assess the impact of Cu-NPs on endophytes. Nodes were washed with soap and water, dried, and sprayed with 70% isopropyl alcohol (IPA), then air-dried for 10 minutes. After rinsing, they were placed in 500 mL distilled water with 2–3 drops of Dettol™ and agitated at 100 rpm for 10 minutes, followed by 180 rpm for another 10 minutes using a GEMMY orbital shaker (Model VRN-480). The soap solution was replaced with 500 mL of 10% Clorox™, agitated for 10 minutes, and repeated with fresh Clorox. Finally, nodes were rinsed three times with autoclaved distilled water under sterile conditions in a BIOBASE™ laminar airflow cabinet.

Surface sterilization with Cu-NPs

After preliminary cleaning, 200 single nodes were randomly divided into two experimental groups of 100 each. In Group 1, the nodes were assigned to five treatments (20 nodes per group). Three groups were immersed in Cu-NPs solutions at concentrations of 50 ppm, 100 ppm, and 150 ppm, respectively. Each treatment involved agitation for 30 minutes at 180 rpm using a GEMMY orbital shaker (Model VRN-480) to ensure uniform exposure. The fourth group received a combined antibiotic and fungicide treatment (Augmentin at 1 mL/100 mL and Daconil at 600 µL/1000 mL), while the fifth group was treated with a 10% Chlorox™ solution. After treatment, all nodes were cultured on full-strength Murashige and Skoog (MS) agar medium supplemented with 1 mg/L Benzyl aminopurine (BAP) and 0.5 mg/L Naphthaleneacetic acid (NAA), adjusted to pH 5.6–5.8. This medium did not contain Cu-NPs (Figure 1). Group 2 followed the same treatments, but Cu-NPs were also added to the culture medium at matching concentrations. The medium was sonicated to ensure uniform dispersion. A+F and Chlorox™ groups received their respective agents in both sterilization and medium. All cultures were maintained under controlled growth room conditions: 25 ± 1 °C temperature, 16-hour photoperiod with LED lighting at 40–60 µmol m^{-2} s^{-1}, and 70% relative humidity. Random placement of cultures ensured uniform environmental exposure across treatments (Figure 1). Random placement of cultures ensured uniform environmental exposure across treatments. The survival proportions were calculated for each treatment group, and the corresponding 95% confidence intervals (CIs) were obtained using the exact Clopper–Pearson method for binomial distributions.

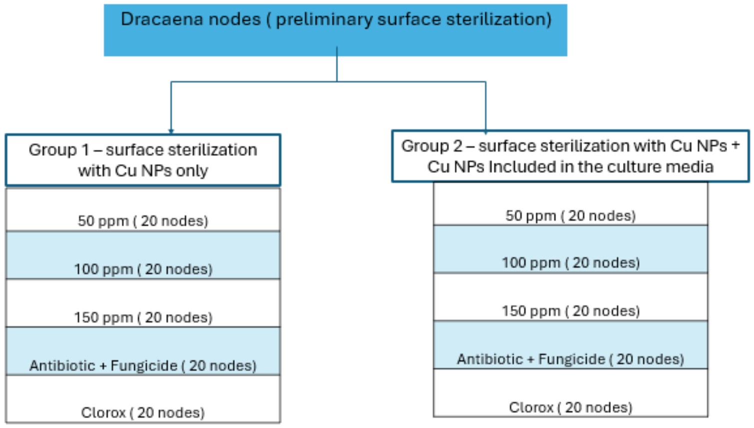

Figure 1: *Treatment plan for the sterilisation of D.sanderiana nodes using CuNPs*

Results

Copper nanoparticles synthesis and Characterization

Cu NPs synthesized via a green synthesis approach using *P. betle* leaf extract exhibited a distinct brown coloration, clearly visible to the naked eye, as shown in Figure 1-(a). This color change is a preliminary visual indicator of NP formation, commonly associated with surface plasmon resonance (SPR) phenomena in metallic NPs (Dadhwal et al., 2023). As a preliminary approach, spectrophotometric analysis was conducted to assess the optical properties of synthesized Cu-NPs. UV-Visible spectrophotometry revealed a strong absorbance peak at 373 nm (λ max) with an optical density of 0.665, which is consistent with reported SPR bands for Cu-NPs synthesized via plant-mediated methods (Akpanudo & Olabemiwo, 2024) as can be seen in Figure 1-(b). It is important to note that Cu NPs synthesized via plant-mediated approaches generally display SPR bands in the 330–400 nm range, consistent with their nanoscale characteristics (Aromal & Philip, 2012; Wanaguru et al., 2025). However, the broader peak that appeared suggests a wider size distribution of particle size or slight aggregation of the synthesized Cu NPs (Dhas et al., 1998).

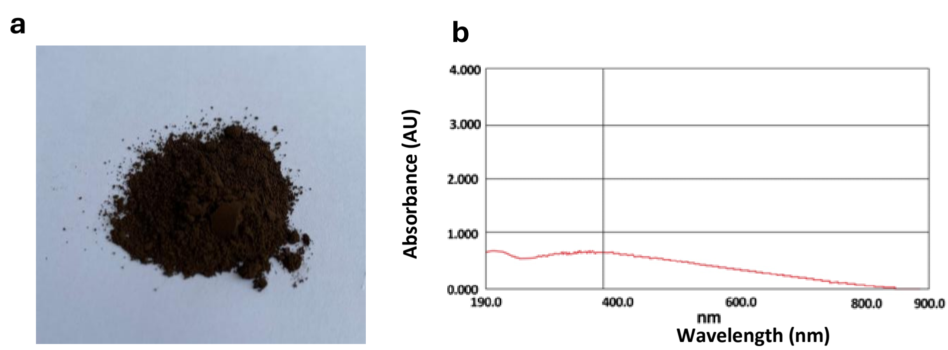

Figure 2: *(a) Green synthesized Cu NPs using Betel extract. (b) Absorbance spectrum of green synthesized Cu NPs*

XRD patterns of the synthesized Cu NUs showed distinct diffraction peaks at 26^{0}, 28.5^{0}, 33.5^{0}, 37^{0}, 40^{0}, 43^{0}, 48^{0}, 54^{0}, 56.8^{0}, ~70^{0} as visualized in Figure 2-(a), which corresponds to Cu_2O (1 1 1), CuO (1 1 0), CuO (1 1 0), CuO (1 1 1), Cu_2O (1 1 1), CuO (1 1 1), Cu (1 1 1), CuO (2 0 2), CuO (0 2 0), CuO (2 0 2), Cu (2 2 0) [CuO: JCPDS Card No. 01-089-5899, Cu_2O: JCPDS Card No. 01-078-2076, $Cu^{(o)}$: JCPDS Card No. 00-004-0836](Mobarak et al., 2022; Murthy et al., 2018) Bulk structural analysis revealed that the synthesized copper nanoparticles (Cu-NPs) predominantly consisted of copper oxide (CuO), with minor contributions from metallic copper (Cu) and cuprous oxide (Cu_2O). A broad hump observed in the XRD pattern (Figure 3a), between 6–25° is attributed to amorphous organic compounds—such as polyphenols, flavonoids, terpenoids, and alkaloids from Piper betel extract, which act as stabilizing capping agents. This interpretation is further supported by ATR-FTIR results (Figure 3b), where characteristic peaks were identified for Cu/Cu_2O (~615 cm^{-1}) and CuO (586, 555, and 505 cm^{-1}), confirming the presence of these phases and organic residues. Besides, the green mediated Cu-NPs acquired a rod-shaped morphology as can be seen in Figure 3-(c) with an average agglomerate size of 500 nm [Figure 3-(d)]. The particles were in a range of 35-95 nm, and the average particle size estimated was ~65 nm as can be seen in Figure 3-(e).

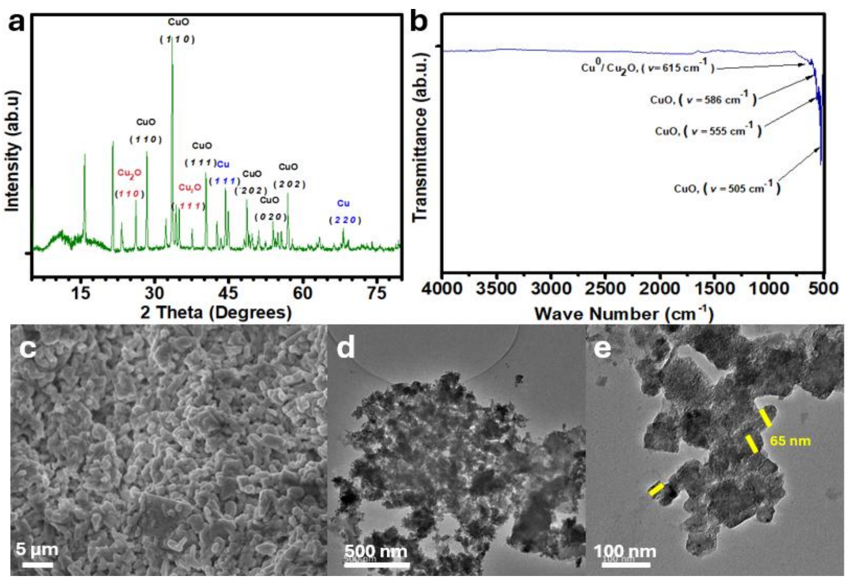

Figure 3 : *(a) PXRD profile of synthesized Cu-NPs, (b) FTIR spectrum of green synthesized Cu-NPs, (c) SEM visualization of Cu-NPS, TEM images of Cu-NPs under (d) 500 nm and (e) 100 nm.*

Time kill determination (Optical Density method)

Figure 4 illustrates the absorbance differences for S. aureus and E. coli treated with varying concentrations of Cu-NPs (50, 100, 200, 250 ppm), alongside positive (antibiotic) and negative (distilled water) controls. Cu-NPs showed a concentration-dependent antibacterial effect, with 200 ppm producing the greatest

absorbance drop and strongest inhibition in both species. This treatment outperformed all others, including the antibiotic control for S. aureus. At 250 ppm,
antibacterial activity declined, likely due to nanoparticle aggregation. Lower concentrations (100 ppm and 50 ppm) showed reduced efficacy but remained more effective than the negative
control. E. coli followed a similar trend, with peak inhibition at 200 ppm and reduced activity at 250 ppm.

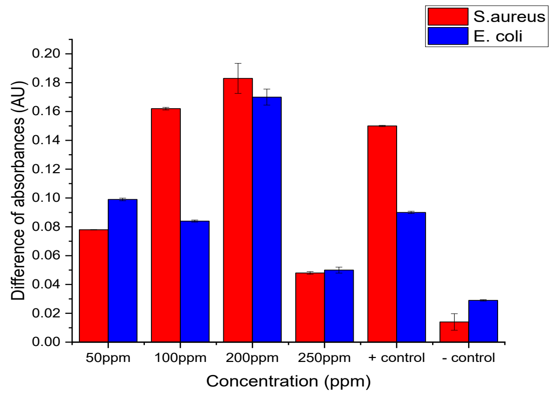

Figure 4: *The antibacterial properties of green synthesized Cu NPs against S. aureus and E. coli*

Application of Cu NPs to in vitro establishment of D. sanderiana

The study assessed survival rates of *D. sanderiana* nodes under various sterilization treatments across two experimental groups. In **Group 1**, where Cu-NPs were used only for surface sterilization, 50 ppm yielded the highest survival (80%), followed by 100 ppm (75%) and 150 ppm (70%). The A+F treatment also resulted in 70% survival, while Clorox™ showed the lowest (60%).
In **Group 2**, where Cu-NPs were applied both for sterilization and as a medium supplement, survival rates improved. The highest rate (100%) was observed at 50 ppm, with both 100 ppm and 150 ppm achieving 85%. A+F maintained a consistent 70% survival across both groups, while Clorox™ remained the least effective. Overall, 50 ppm Cu-NPs demonstrated the greatest efficacy, indicating its potential as an optimal concentration for enhancing explant survival and contamination control(Figure 5).

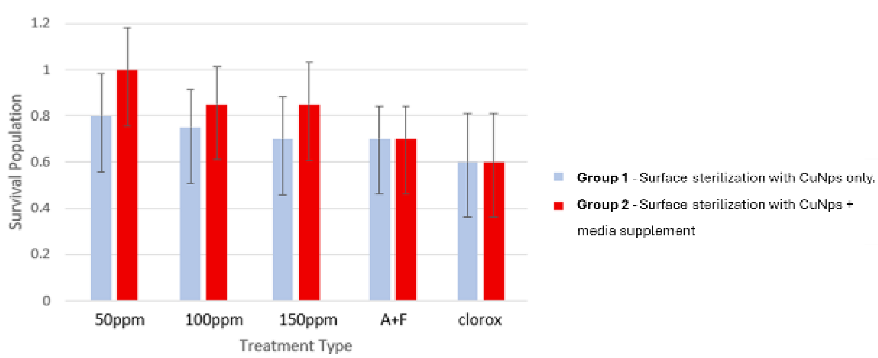

Figure 5: *Survival Rates of D. sanderiana of group 1 and group 2*

3.4. Statistical Analysis

In Group 1, survival proportions ranged from 0.60 in the Clorox™ control to 0.80 in the 50 ppm Cu-NP treatment. Confidence intervals (95% Clopper–Pearson) reflected variability due to the small sample size (n = 20). Specifically, 50 ppm showed 0.80 (CI: 0.56–0.94), 100 ppm 0.75 (CI: 0.51–0.91), and 150 ppm 0.70 (CI: 0.46–0.88). A+F and Clorox™ treatments yielded 0.70 and 0.60, respectively. In Group 2, higher survival was observed in copper nanoparticle treatments compared to Group 1. 50ppm achieved full survival (1.00; 95% CI: 0.83–1.00), while both 100ppm and 150ppm showed improved proportions of 0.85 (95% CI: 0.62–0.97). In contrast, A+F (0.70; 95% CI: 0.46–0.88) and the clorox (0.60; 95% CI: 0.36–0.81) produced identical results to those observed in Group 1.

Discussion

The successful green synthesis of Cu-NPs using *Piper betel* leaf extract highlights the dual role of plant-derived bioactive such as polyphenols, flavonoids, alkaloids, and terpenoids—as both reducing and stabilizing agents (Singh et al., 2023). These compounds facilitated the conversion of Cu^{2+} ions into nanoparticles while preventing aggregation through surface capping. The extract's concentration significantly influenced nanoparticle yield, with 200 g per 100 mL producing optimal results, underscoring the importance of extract volume in synthesis efficiency.

Quantitative information about the level of bacterial inhibition and the effectiveness of Cu-NPs treatments at different concentrations was obtained from these OD-based analyses from spectrophotometric analysis. Absorbance was recorded at 600 nm (OD600), a widely accepted wavelength for evaluating bacterial growth through turbidity in liquid cultures (Mira et al., 2022). This wavelength is chosen because it minimizes interference with bacterial physiology and reliably reflects cell density. The study demonstrates that copper nanoparticles (Cu-NPs) exhibit concentration-dependent antibacterial activity against E. coli and S. aureus, with 200 ppm showing the highest inhibition for both strains.

Interestingly, 250 ppm was less effective, likely due to nanoparticle aggregation, which reduces surface interaction with bacterial cells—emphasizing the importance of optimizing concentration. At 100 ppm, Cu-NPs displayed stronger antibacterial effects against E. coli than the antibiotic control, suggesting their potential as alternative antimicrobial agents, particularly where conventional antibiotics fall short. Additionally, E. coli showed greater resistance at lower concentrations compared to S. aureus, indicating strain-specific susceptibility.

The reduced effectiveness of Cu-NPs at higher concentrations, such as 150 ppm, may be attributed to nanoparticle aggregation over time, which limits their interaction with target organisms. Although the A+F treatment showed moderate antibacterial activity, it was less effective than Cu-NPs and offered no clear advantage at increased concentrations. Notably, Group 2—where Cu-NPs were used for both surface sterilization and media supplementation—demonstrated superior results, indicating that this combined approach enhances the elimination of both epiphytes and endophytes more effectively. The Clorox™ control's continuously poor performance supports Cu-NPs' potential as a more potent and environmentally responsible sterilizing option.

The broad-spectrum antimicrobial activity and biocompatibility of copper nanoparticles (Cu-NPs) indicate promising potential for use in the micropropagation of plant species *beyond D. sanderiana.* To fully harness this potential, future studies should investigate how different species respond to Cu-NP treatments, focusing on optimal concentrations, toxicity limits, and long-term effects on plant regeneration and growth.

Conclusion

This study confirms the green synthesis of Cu-NPs using *Piper betel* extract, with 200 g in 100 mL water yielding a stable brown precipitate. Antibacterial assays identified 200 ppm as the most effective concentration against *E. coli* and *S. aureus*. In *D. sanderiana* tissue culture, 50 ppm Cu-NPs achieved 100% survival when used for both sterilization and media supplementation, outperforming conventional methods. These findings highlight Cu-NPs as a sustainable, low-cost tool for improving agricultural practices. By utilizing local resources, this approach promotes eco-friendly innovation and supports broader adoption of nanotechnology in plant biotechnology.

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