Green Synthesis, Characterization of Cu Nano Units and Evaluation of Antibacterial Properties towards the Bacterial Blight Control in *Anthurium andraeanum Lind* under *In-vitro* and *In-vivo* Conditions

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Abstract

Most types of metal nanoparticles exhibit antibacterial properties. Copper nanoparticles can generate reactive oxygen species (ROS) within bacterial cells, initiating a cascade of events that lead to bacterial cell death. In this study, copper nano units (Cu NUs) were synthesized through green synthesis method using anthurium leaf extract toward the control of bacterial blight disease in Anthurium andraeanum cv 'Rainbow' plants. Synthesized Cu NUs were characterized using scanning electron microscopy (SEM), transmission electron microscopy (TEM) and X-ray diffraction (XRD) techniques. Antibacterial properties of 30, 50 and 100 ppm of Cu NUs against Xanthomonas sp. which cause bacterial blight in anthurium plants were studied under in-vitro conditions using well diffusion test. Moreover, antibacterial properties of said NUs were studied at in-vivo levels by spraying Cu NUs at concentrations of 50, 100 and 200 ppm on bacterial blight infected anthurium cv 'Rainbow. According to SEM and TEM analysis, synthesized NUs remained within size of 25.36 ± 12.55 nm length and 16.18 ± 3.75 nm width exhibiting a polygonal shape. In well diffusion test, optimized 30 ppm concentration of Cu NUs formed an inhibition zone with a diameter of 20.6 ± 0.94 mm. In comparison, the antibiotic sulfamethoxazole (positive control) formed an inhibition zone with a diameter of 26.3 ± 0.47 mm. At in-vivo level, 200, 100 and 50 ppm all three concentrations were able to control disease in 90% of treated plants within 5, 6 and 7 weeks respectively. Overall, this research highlights the potential of Cu NUs as an effective alternative for controlling bacterial blight disease in anthurium cv 'Rainbow'.

Keywords: Copper nano units; Antibacterial properties; Bacterial blight disease control; Xanthomonas spp. Bacteria; Anthurium andraeanum

Introduction

The use of green ingredients to replace chemicals is increasingly observed across all industries today due to the eco-friendliness and to achieve sustainable economic development via green approaches. One of such techniques used widely in nanotechnology is utilizing green ingredients i.e. plant extracts and fruit juice to synthesize nanoparticles. (Siddiqi & Husen, 2020). Copper (Cu) nanoparticles have been recognized as one of the best materials which shows antibacterial properties. (Ntasiou et al., 2021). The use of different plant extracts to synthesise Cu NPs has been already tested. Those studies include use of Citrus fruit juice (Shende et al., 2015), *Hagenia abyssinica* leaf extract (Murthy et al., 2020) and *Eclipta prostrata* leaf extract (Chung et al., 2017) to synthesize Cu nanoparticles.

This study focuses on investigating the efficacy of copper nano units (Cu NUs) synthesized through

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green synthesis method using anthurium leaf extract toward control bacterial blight disease in *Anthurium andraeanum* plants. Most Interestingly, this study aims to develop a Cu NUs based curing method for bacterial blight disease in anthurium cv 'Rainbow' using its own leaves.

Anthurium andraeanum, a vibrant flowering plant from the Araceae family, is widely recognized as the flamingo lily. This plant, however, is susceptible to bacterial blight disease, a destructive condition caused by the plant pathogen bacteria, *Xanthomonas axonopodis pv. dieffenbachiae* (XAD) (Alvarez *et al.*, 2006). To combat this disease, anthurium growers traditionally resort to methods such as the physical removal of infected or dead parts and the application of chemical treatments. (Alvarez et al., 2006). Despite these efforts, these conventional methods often prove inadequate in commercial environments. Consequently, there is an urgent need for a more sustainable and effective strategy to control bacterial blight disease in *A. andraeanum* plants. Developing such a solution is crucial for enhancing global anthurium cultivation. However, the use of NPs is more effective and sustainable than the use of conventional methods and chemical treatments for controlling plant disease. Nanoparticles can manage diseases at much lower concentrations compared to chemicals. Most importantly, NPs can enhance plant tolerance by stimulating enzyme activity, regulating plant hormones, balancing ions, and boosting photosynthesis (Kumari et al. 2024).

The application of Cu NPs to treat bacterial blight disease caused by the plant pathogen *Xanthomonas spp.* in various plant species such as rice (Datta Majumdar et al., 2021) pomegranate (Mondal & Mani, 2012), and olive (Ntasiou et al., 2021) has already been studied. These studies demonstrated that Cu NPs effectively combat bacterial blight in those plants. The utilization of Cu NPs synthesized using chemical reduction method to control *in-vitro* growth of (XAD) which cause bacterial blight disease in *Anthurium andraeanum,* had been tested in a previous study. That study revealed that the *in-vitro* growth of (XAD) was controlled at a 50-ppm concentration of Cu NPs (Peiris et al., 2022). However, transitioning from *in-vitro* studies to *in-vivo* applications involve significant differences in parameters. In the context of controlling bacterial blight disease in anthurium plants using Cu NPs, the process involves the uptake of NPs into the plant through stomata or other leaf openings. Once getting inside the plant, NPs must traverse through plant tissues to reach bacterial cells and exert their antimicrobial action. Hence, sometimes, researchers have failed to control bacterial blights during the *in vivo* studies using low concentrations of NPs (Clift et al. 2011).

The main goal of this study was to develop efficient, cost effective and eco-friendly curing method for bacterial blight disease in *A. andraeanum* plants using Cu NUs. To achieve this goal, four objectives were pursed.

1. synthesize of Cu NUs, through a green synthesis method using anthurium leaf extract for controlling bacterial blight in anthurium plants.

2. Evaluation of the antibacterial properties of the synthesized NUs both *in vitro* and *in vivo* environments.

3. Application of synthesized Cu NUs to bacterial blight infected anthurium plants and determine the optimal concentration of Cu NUs that effectively controls bacterial blight without causing phytotoxicity.

4. Investigation of changes in disease progression, after applying copper Cu NUs, throughout the treatment period.

Materials and Methods

Chemicals and equipment

All chemicals and reagents used in this experiment were of analytical reagent (AR) grade, ensuring high purity. They were sourced from SISCO Research

Laboratory, Sigma-Aldrich, and HIMEDIA in India, and DAEJUNG Chemicals and Metals Co. Ltd. in Korea. Distilled water was used to dissolve the chemicals and reagents. Plant materials were collected from nurseries in the suburban areas of Colombo, Sri Lanka. The synthesized nano units (NUs) were characterized using advanced instrumentation facilities at the Sri Lanka Institute of Nano Technology (SLINTEC) in Pitipana, Homagama, Sri Lanka.

Synthesis of Cu NUs

Anthurium leaves were collected from anthurium plant nurseries in the suburban areas of Colombo, Sri Lanka. The leaves were cut using a sterilized knife, placed in a zip lock bag and sealed. Then, anthurium leaves were brought into the laboratory. In the lab, the anthurium leaves were washed with running tap water and liquid soap to eliminate dust and other impurities. The leaves were then sterilized by washing with 10% Clorox and liquid soap solution for 30 minutes, a process repeated three times, followed by rinsing with distilled water. The sterilized leaves were ground into a fine paste. Meantime a 100 ml of 5M $CuCl_{2(aq)}$ solution was prepared by dissolving 85.25 g of $CuCl_{2(s)}$ powder in 100 ml of distilled water. Anthurium leaf extract was obtained by extracting 200 g of finely ground, sterilized anthurium leaves into 100 ml of distilled water using the sonication method. Then leaf extract was filtered through a Whatman no 01-filter paper to separate solid particles. Then leaf extract was mixed with 5M $CuCl_{2(aq)}$ solution and mixture was stirred at 600 rpm for 3 hours at 70°C. The resulting precipitate was filtered through Whatman No. 1 filter paper and stored in a labelled sample container after being oven-dried for one day at 40°C.

Characterization of synthesized Cu NUs

The synthesized Cu NUs were analysed using SEM, TEM, and XRD techniques to determine their surface morphologies, crystalline structures, and chemical compositions. SEM images were captured using a Hitachi SU6600 FE-SEM (field emission scanning electron microscope) under magnifications of 1000x, 5000x, 15000x, and 25000x, with particle sizes analysed in the 1-500 nm range. TEM images were captured with the aid of an JEOL JEM-2100 high-resolution transmission electron microscope. XRD analysis was performed over a 2-theta range of 0-80° within 40 minutes using a Bruker D8 x-ray diffractometer.

***Xanthomonas* bacteria isolation and characterization**

Xanthomonas sp. bacteria were isolated from bacterial blight disease infected anthurium leaves extract and inoculated to *Luria-Bertani* (LB) Broth + Agar culture plates and incubated 3 days for 37 ^{0}C. Then a pure yellow colour *Xanthomonas* colony was isolated and sub-cultured on *LB Broth* + Agar culture plates and incubated 3 days for 37 °C. Isolated bacteria colonies were characterized by investigating their size, shape and gram negativity through light microscope observation after gram staining. pathogenicity of isolated bacteria colonies was tested.

Study of antibacterial properties of Cu NUs

Antibacterial properties of synthesized Cu NUs were tested against *Xanthomonas sp*. at *In-vitro* using the well diffusion method with 30, 50 and 100 -ppm concentrations and antibiotic sulfamethoxazole was used as the positive control. Distilled water was used as the negative control. All experiments were triplicated. Antibacterial activities of Cu NUs *in-vivo* were tested through application of Cu NUs in concentrations of 50, 100 and 200 ppm to 30 bacterial blights infected anthurium cv ‘Rainbow’. Antibiotic sulfamethoxazole was used as the positive control. A flow chart for Cu NUs treatment plan is depicted in Figure 1. *Xanthomonas sp.* bacteria were inoculated to 50 healthy anthurium plants, initially. After three weeks of inoculation, Cu NUs treatments were started, and continued up to 10th week applying Cu NUs to infected plants weekly using a foliar sprayer. While applying Cu NUs, diameters of disease lesions were measured weekly until 12th week from day of bacterial inoculation. The average lesion diameter for each group of plants treated with different concentrations of Cu NUs over time were graphed. A one-way ANOVA analysis was performed to analyse the significant variations of antibacterial activities statistically at different concentrations. All *in vivo* experiments were conducted under the optimum environmental condition suitable to anthurium plant growth.

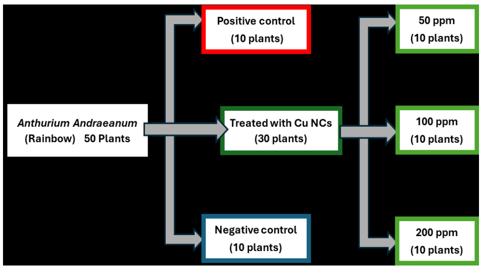

Figure 01. A flow chart for Cu Nus treatment plan

Results and Discussion

Synthesis and characterization of copper nano units

Pullman brown (#644117) colour Cu NUs were formed. Size, surface morphology, lattice structure and chemical structure of synthesized Cu NUs have been characterized using SEM and TEM techniques (Figure 2).

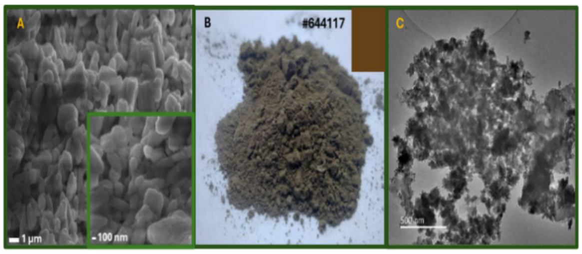

Figure 2. (A) SEM image of synthesized Cu NUs [(Mag ×15K)] **(B)** Cu NUs synthesized via green synthesis method **(C)** TEM image of synthesized Cu Nus Moreover, XRD diffractogram of the synthesized Cu NUs and polycrystalline patterns obtained through the selected area energy diffraction (SAED) patterns further proves the bulk and the nano structures of the synthesized Cu NUs as can be seen in Figure 3. XRD patterns of the synthesized Cu NUs showed distinct diffraction peaks at 35^{0} 38.20°and 43.00° and 51^{0} (Figure 3-A) of the spectrums correspond to the CuO (111), Cu_2O (200), Cu (111), and Cu (200), respectively, (JCPDS No. 00-001-1241) which represent crystal face-cantered cubic (fcc) of copper. (Powar et al., 2019). SAED evidence showed in Figure 3-B further supports for this claim. In addition to the peaks appeared, few sharp peaks can be observed between the range of 15-30° represents Cu $(OH)_2$ which consists as a residual component in the product (Lam et al. 2022). Table 1 summarizes all the parameters related to Cu Nus.

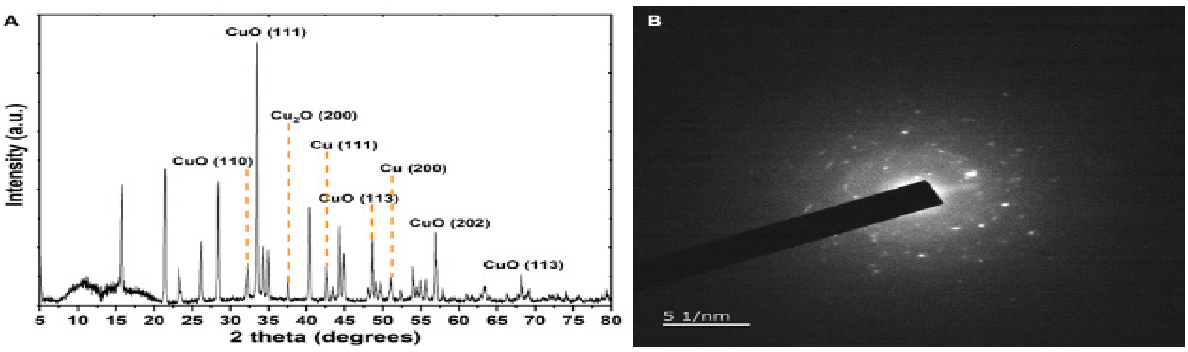

Figure 3. (A) Powder XRD patterns of synthesized Cu NUs **(B)** SAED patterns of synthesized Cu Nus.

Table 01. Characterization of Cu NUs

Surface morphology	Average particle length (nm)	Average particle width (nm)	Average Size of Agglomerates (d nm)	XRD Peaks	Composition	References
Polygonal shape	25.36 ± 12.55	16.18 ± 3.75	1003 ± 1098	35.00^{0} 38.20° 43.10° 51.67° 58.72^{0}	CuO (111) Cu_2O (200) Cu (111) CuO (112), CuO (202)	Lam et al. 2022 JCPDS No. 00-001-1241 JCPDS No. 00-001-1241 Lam et al. 2022

Isolation and characterization of *Xanthomonas sp.* Bacteria

Yellow colour bacteria colonies were observed on *LB* + agar culture plates after 24 hours of inoculation. Rod shape, gram negative bacterial cells were observed through light microscope after gram staining. In pathogenicity test, bacteria inoculated leaves were showed bacterial blight symptoms.

Antibacterial properties of Synthesized Cu NUs

In a well diffusion test, inhibition zones with diameters of 20.6 ± 0.94 mm, 22.3 ± 1.24 mm, and 23.3 ± 0.47 mm were observed at concentrations of 30 ppm, 50 ppm, and 100 ppm, respectively. In comparison, the antibiotic sulfamethoxazole (positive control) produced inhibition zones with diameters of 26.3 ± 0.47 mm, 27 ± 0.81 mm, and 27.6 ± 0.94 mm at the same respective concentrations. No inhibition zones were observed around the well filled with distilled water.

In plant applications, 200 ppm, 100 ppm, and 50 ppm of Cu NUs controlled the disease in 90% of treated plants within 5, 6, and 7 weeks, respectively. High concentrations (above 150 ppm) of antibiotic Sulfamethoxazole can be toxic to plants (Michelini et al. 2014). It may inhibit plant growth, affecting photosynthesis and disrupting nutrient uptake. Additionally, it may persist in the environment, potentially contaminating soil and water. (Straub, 2016) Because of those reasons, high concentrations above 200 ppm were not tested. Changes of average lesion growth with time after treating with 50, 100 and 200 ppm concentrations of Cu NUs can be seen in Figure 4.

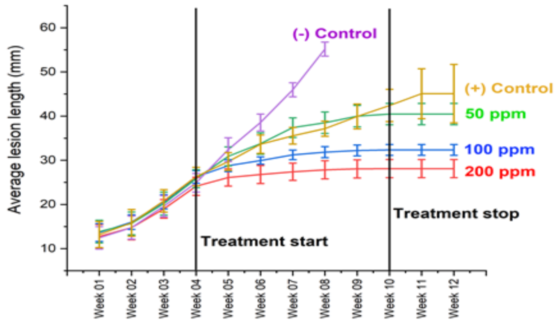

Figure 4. Changes of average lesion growth with time after treating with 50, 100 and 200 ppm concentrations of Cu NUs.

After applying the optimized 200 ppm concentration of Cu NUs, to the diseased leaves, the brownish and yellowish disease lesions remained unchanged in size and did not spread, even after four weeks. Additionally, it was observed that the newly formed leaves remained disease-free. In the negative control, plants treated with distilled water showed continuous disease spread throughout the leaves and stems, resulting in the death of infected leaves within 4-6 weeks. In the positive control, plants treated with the antibiotic sulfamethoxazole (100 ppm) showed moderate disease control in 30% of treated plants within 2 weeks of treatment initiation; however, the disease reappeared after 4-5 weeks.

Conclusion

In this study copper nano units were synthesized using anthurium leaves extract through a green synthesis against Xanthomonas sp. Cause bacterial blight disease in A. andraeanum. Synthesized Cu NUs were characterized using SEM, TEM and XRD techniques. Antibacterial properties of synthesized Cu NUs against Xanthomonas sp. were tested at In-vitro using well diffusion method, and stages of in-vivo through applying Cu NUs to bacterial blight infected plants. In a well diffusion test, inhibition zones with diameters of 20.6 ± 0.94 mm, 22.3 ± 1.24 mm, and 23.3 ± 0.47 mm were observed at concentrations of 30 ppm, 50 ppm, and 100 ppm, respectively. In comparison, the antibiotic sulfamethoxazole (positive control) produced inhibition zones with diameters of 26.3 ± 0.47 mm, 27 ± 0.81 mm, and 27.6 ± 0.94 mm at the same respective concentrations. In plant applications, 200 ppm, 100 ppm, and 50 ppm of Cu NUs controlled the disease in 90% of treated plants within 5, 6, and 7 weeks, respectively. In comparison, antibiotic sulfamethoxazole moderately controlled the disease in 30% of treated plants while showing some phytotoxic effects. According to all data 200 ppm concentration is the optimized concentration cure bacterial blight disease within short time. When considering the advantages, including cost-effectiveness, eco-friendliness, ease of use and production, the green synthesized Cu NUs would be a valuable approach to replace the use of chemicals and antibiotics to controlling bacterial blight disease in Anthurium andraeanum plants.

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