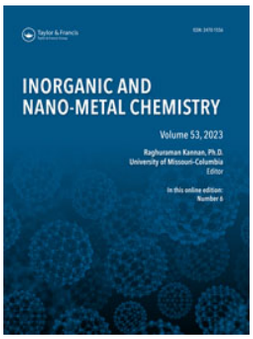

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Synthesis and characterization of copper-chitosan based nanofungicide and its induced defense responses in Fusarium wilt of banana

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ABSTRACT

In the present study, Copper-chitosan nanoparticles were synthesized and evaluated for their antifungal potential both *in vitro and in vivo* studies against Fusarium wilt of banana caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*). Cu-ChNPs were synthesized and characterized by Ultraviolet–Visible spectrometric (UV–Vis) studies, X-ray diffraction (XRD), Scanning electron microscopy (SEM), Fourier Transform Infrared (FTIR) analysis, as well as Transmission electron microscopy (TEM). The presence of pure Cu-ChNPs was confirmed using XRD analysis. The TEM images revealed the spherical shaped nanoparticles with 50 nm. The Cu-ChNPs have shown significant antifungal potential against *Foc* which showed 13 ± 2.5, 14 ± 2.1, and 14 ± 2.1 mm mycelial growth inhibition at 0.10, 0.20, and 0.30 mg/mL concentration, respectively. In pot experiments, 0.20 mg/mL concentration of Cu-ChNPs showed more effective in percentage efficacy of disease control (PEDC) in banana plants with the results of 73% against *Foc*. Further, the effectiveness of Cu-ChNPs in defense enzymes study indicates higher activities against *Foc* in banana plants and proved the effectiveness of Cu-ChNPs under greenhouse conditions. The overall results confirm the antifungal capabilities of Cu-ChNPs against Fusarium wilt in both *in vitro* and *in vivo* studies. Further, synthesized Cu-ChNPs may act as a nanofungicide for the delivery of agrochemicals.

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KEYWORDS
Chitosan; banana; *Fusarium* wilt; nanoparticles; plant defense enzymes

Introduction

Banana, the world's most staple fruit in production and volume, belongs to the family Musaceae, comprising 65 genera, includes wild and cultivated varieties. In India, the banana has been mentioned in ancient treatises, including the Ramayan (2000 BC) and Chilappthikaram (500 AD), describing its importance and its uses.[1] At present, India is the largest producer of bananas, and FAO sources estimated the yield up to 29 million tonnes/year, followed by China (11 million tonnes/year), the Philippines (7.5 million tonnes/year), and Brazil, on average.[2] Consequently, an infection with pests and disease decreases the production of bananas is one of the major limiting factors worldwide.[3] These banana cultivars are seriously threatened by one of the most important and destructive diseases known as Fusarium wilt, also known as Panama wilt, caused by the fungus *Fusarium oxysporum* f. sp. *cubense*.[4]

Fungicides are hazardous to the environment and enter the environment through water, air, and soil. It was estimated that 70–75% of applied fungicides are lost from the soil during run-off.[5] To overcome this problem, nanoparticles are gaining importance in controlling plant diseases.

In recent years, metal nanoparticles have expanded in various fields, including physics, chemistry, material science, agriculture, medicine, and biology, due to their unique physicochemical, magnetic, and optical properties. As a result, the production and applications of nanomaterials are rapidly increasing. Agricultural nanotechnology has been emerging at a tremendous pace.[6]

Chitosan is considered one of the most widely used biopolymers for nanoparticles preparation due to its unique structural features. Moreover, it enhances cross-linkage with glutaraldehyde, sodium tripolyphosphate (TPP), genipin, copper, etc.[7,8] Chitosan is also documented to possess an antimicrobial,[9] immunomodulatory,[10] and plant growth promotion agent.[11] Hence, chitosan-based nanoparticles exhibited various applications in agriculture, including plant growth.[12] Recently, chitosan-based nanoparticles have been evaluated as a potent inducer of antioxidant and defense enzymes.[13] Du et al.[14] synthesized chitosan nanoparticles loaded with copper ions, further assessed for antibacterial activity. The good fungicidal activity of copper at the nanoscale is higher than the copper salt. Ing et al.[15] reported chitosan nanoparticles coated with copper also exhibited antifungal activity against *Candida albicans*, *Fusarium solani*, and *Aspergillus niger*. For a few years, chitosan nanoparticles were evaluated against plant diseases to suppress pathogens, such as *Pyricularia grisea*, *Nigrospora sphaerica*, *N. oryzae*, *Botryosphaeria dothidea*, and *Alternaria tenuissima* in paddy crop.[16,17] Chitosan alone and synthesized Cu-chitosan nanoparticles proved their antifungal activity against

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Alternaria alternata, *Macrophomina phaseolina*, and *Rhizoctonia solani* in *in-vitro* studies.[18] Chitosan-based NPs treated plants showed a high level of defense responses due to the expression of defense related genes.[19]

Therefore, in the present study, Cu-chitosan nanoparticles were synthesized, and their physicochemical characterization was carried out by Ultraviolet–visible (UV–vis) spectroscopy, dynamic light scattering (DLS), Fourier transform infrared (FT-IR) spectroscopy, Transmission electron microscopy (TEM), X-ray diffraction (XRD), and Energy-dispersive X-ray microanalysis (EDX) spectroscopy. Further, synthesized nanoparticles were examined to evaluate the inhibition of mycelial growth and spore germination of *F. oxysporum* f. sp. *cubense* in *in vitro* and *in vivo* studies. Banana plants were sprayed with different concentrations of nanoparticles at regular intervals and observed for their defense through chitinase, peroxidase, phenyl ammonia-lyase, and polyphenol oxidase activities.

Materials and methods

Preparation of chitosan stock (Crab shell chitosan, Sigma Chem. Co., USA)

In the present study, one gram of Crab shell chitosan (Sigma Chem. Co., USA) was suspended in 40 mL deionized water and 9 mL of 1 M acetic acid. Later, it was dissolved by stirring slowly for 1–2 h at room temperature. The pH was adjusted to 6.0 using 1 M sodium acetate and made up to 100 mL with 0.05 M acetate buffer (pH 6.0). From this stock solution, 0.1% (w/v) solution was prepared by adding sterile distilled water and used for further treatment.[20]

Preparation of chitosan nanoparticles

Chitosan was dissolved at 0.5% (w/v) with 1% (v/v) acetic acid and adjusted to pH (5.0). Tripolyphosphate (TPP) was dissolved in distilled water at a concentration of 0.25% (w/v). The formation of chitosan nanoparticles was aided by adding TPP to chitosan solution (1:3) using a magnetic stirrer. The colloidal suspension was centrifuged at 10,000 rpm for 15 min. The precipitate was collected and washed several times with distilled water to remove the unreacted substances. The pure form of chitosan nanoparticles was stored at 4 °C.[20]

Synthesis of copper chitosan nanoparticles (Cu-ChNPs)

Cu–chitosan NPs were prepared based on the ionic gelation of chitosan with tripolyphosphate anions. The $CuSO_4$ (HiMedia, Mumbai, India) solution (0.01%) was added to the formulation forms precipitation. The formed precipitate was resuspended in acetone (90%, v/v), and the centrifugation was repeated three times to remove unreacted reagents. Finally, the precipitate was dissolved in water, dried overnight under a vacuum, and stored as described earlier.[20]

Physico-chemical characterization of NPs

UV-spectral analysis

Cu-ChNPs were analyzed spectroscopically using transmission mode in a wavelength ranging from 200 to 800 nm using a Shimadzu (UV-1800) spectrophotometer. Fourier Transformed Infrared Spectroscopy (FTIR). Infrared spectra were recorded on a PerkinElmer Spectrum FTIR spectrophotometer. FTIR spectrum of the synthesized nanoparticles sample was analyzed by PerkinElmer Spectrum Version 10.03.09 at a resolution of 600–4000 cm^{-1}. Using DLS, zeta potential wavelength was measured at 780 nm using a laser that ranges between 0.1 and 1000 µL at 25 °C and a scattering angle at 90 °C to analyze the data. The XRD technique was used for the determination of the structure of nanoparticles. XRD patterns were obtained on a desktop X-ray diffractometer operating at a voltage of 30 Kv, and a current of 15 mA with Cu radiation, and the diffraction intensities of nanoparticles were recorded from 10° to 90° 2θ angle using Rigaku II X-ray diffractometer according to Debye–Scherrer's formula.[21] Energy-dispersive X-ray diffraction (EDX) studies were used to determine the elemental composition done by drop coating sample on a glass substrate in a wide range of Bragg angles θ for 2 min^{-1} at a voltage of 40 kV and current 30 mA with Cu-K α radiation (1.5405 Å). Scanning electronic microscopy (SEM) (using Hitachi S-4500) was used to know the elemental analysis and morphology of the Cu-ChNPs, was determined using TEM analysis as described previously.[21]

Effect of pH and effect of reaction time of Cu-ChNPs

The synthesized nanoparticles were subjected to study the effect of pH at 4, 5, 6, 7, 8, and 9.[22] The reaction time of synthesized Cu-ChNPs was studied based on different time intervals, viz., 1st, 30th, 60th, and 90th day, which was further studied using UV–visible spectroscopy. Later, the synthesized nanoparticles were stored in a glass container under dark conditions accordingly.[22]

Isolation of the fungus

Pure culture of *Fusarium oxysporum* f. sp. *cubense* (*Foc*) was isolated from the susceptible cultivar Nanjangud rasabale banana plant and was used for further studies.

In-vitro antifungal assay

The antifungal activity of the synthesized Cu-ChNPs was examined using the agar well diffusion assay method. The antifungal activities of Cu-chitosan NPs were tested against the fungi *Foc*, on potato dextrose agar as the medium, and the nanoparticle solution was prepared at different concentrations, such as 0.10, 0.20, and 0.30 mg/mL NPs concentration. Five wells (8 mm each) diameter were formed by punching the Potato Dextrose Agar (PDA) with a sterile cork borer. In three wells, nanoparticles samples were added, whereas SAAF fungicide at 0.20 mg/mL was used as a

positive control, and distilled water was used as a negative control. Later, plates were seeded with *Foc* and incubated at 25 ± 2 °C under 12/12 h dark and light conditions. The radial growth of the fungus was measured after two weeks. Growth inhibition was expressed as the percent inhibition of radial growth relative to the control.[23]

Effect of Cu-ChNPs on Fusarium wilt disease in pot experiment

Two-month old Nanjanagudu rasabale, a susceptible banana cultivar, was grown in pots and sprayed with different concentrations of Cu-ChNPs like 0.01, 0.05, 0.10, 0.15, and 0.20 mg/mL, followed by bulk chitosan (BCH 0.2%), copper sulfate ($CuSO_4$) 0.2%, fungicide SAAF (0.2%) as a positive control and distilled water as another control sets were subjected to foliar spray under greenhouse conditions by following standard agronomic conditions. After ten days of foliar treatments, fungal spores (5×10^3 conidia mL^{-1}) of the test fungi were inoculated on banana plants, later covered with polythene bags to maintain high humidity (70%) and temperature at 25 ± 2 °C for 7 days to facilitate easy development of the disease in banana plants.[24] In Fusarium wilt, yellowing, and wilted leaves were selected to calculate the disease severity. The progress of diseases was observed for 7 days. Disease severities were recorded on a standard rating of 0–5 (grades 0 = 0% of leaf area infected/wilted and yellowing leaf; 1 = 1–5% of leaf area infected/wilted and yellowing leaf; 2 = 6–10% of leaf area infected/wilted and yellowing leaf; 3 = 11–20% of leaf area infected/wilted and yellowing leaf; 4 = 21–30% of leaf area infected/wilted and yellowing leaf; 5 = more than 31% leaf area infected/wilted and yellowing leaf) as proposed by Campbell and Madden.[25] Further, the percentage efficacy of disease control (PEDC) was calculated using the formula.[26]

$$\text{PEDC} = \frac{\text{Disease severity in control} - \text{disease severity in treatment} \times 100}{\text{Disease severity in control}}$$

The activity of defense enzymes and pathogenesis-related (PR) proteins

Induction of defense enzymes and pathogenesis-related (PR) proteins was studied in the Cu-ChNPs treated banana plants to examine the expression of defense-related proteins against *Foc* pathogen. Peroxidase (PO), Chitinase, Polyphenol oxidase (PPO), and Phenylalanine ammonia-lyase (PAL) activities were estimated, accordingly.

Treatment combinations were as follows:

1. Bulk Chitosan (BCH) (0.2%).
2. Copper sulfate ($CuSO_4$) (0.2%).
3. Fungicide (SAAF) (0.2%).
4. Copper-chitosan nanoparticles (0.01, 0.05, 0.1, 0.15, and 0.20 mg/mL).

Protein extraction

Treated banana leaf samples were collected between 24 h and 72 h after pathogen inoculation. Positive control was used as a fungicide (SAAF), and negative control was maintained by spraying distilled water (DW). Samples were ground in a pre-chilled mortar and pestle (1 g/2 mL) with 0.02 M Potassium phosphate buffer of pH 7.0. It was then centrifuged at 4 °C for 10 min at 10,000 rpm in a refrigerated centrifuge. The supernatant was collected and used as a source for protein/enzymes.

Chitinase (EC 3.2.1.14) assay

Five grams of chitin powder (Himedia Laboratories Pvt. Ltd. India) was dissolved in 60 mL of hydrochloric acid (HCl) with constant stirring for 30 min. Precipitated was kept at 4 °C overnight. The mixture was filtered through glass wool, and the filtrate was activated by adding excess water and ethanol (1:1). It was then centrifuged at 10,000 rpm for 10 min at 4 °C. The precipitate was washed with sterile distilled water through filter paper placed in a funnel fitted with glass wool till the pH of the colloidal chitin became neutral (pH 7.0) and stored at 4 °C for further use.[27]

Peroxidase (EC. 1.11.1.7) assay

The activity of peroxidase in the banana leaf samples was assessed by the method of Chance and Maehly.[28] The reaction mixture consisted of 3 mL of pyrogallol solution and 0.5 mL of enzyme extract. The spectrophotometer was adjusted to zero at 430 nm, followed by the addition of 0.5 mL of 1% (v/v) H_2O_2 and mixed thoroughly. The enzyme activity was expressed as a change in absorbance per minute per gram protein.

Polyphenol oxidase (EC. 1.10.3.2) assay

Polyphenol oxidase (PPO) activity was determined as per the procedure given by Taneja and Sachar.[29] The reaction mixture consisted of 1.5 mL of 0.1 M sodium phosphate buffer (pH 6.5) and 100 μL of the enzyme extract. To start the reaction, 0.01 M catechol was added. The activity was expressed as a change in absorbance at 495 nm per minute per gram of protein.

Phenylalanine ammonia-lyase (PAL) (EC 4.3.1.5) assay

Phenylalanine ammonia-lyase was determined per the procedure given by Moerschbacher et al.[30] The deamination of L-phenylalanine to trans-Cinnamic acid and ammonia was measured at 290 nm. The activities of all the enzymes were expressed in μmol/min/g tissue.

Results and discussion

The synthesis of chitosan nanoparticles was confirmed by the formation of an absorbance peak at 295 nm compared to

bulk chitosan, which showed a peak at 190 nm under UV–visible spectroscopy, as shown in Figure 1(A). The formation of Cu-ChNPs of absorbance peak due to surface plasmon resonance (SPR) vibration in chitosan-based NPs at 590 nm confirms the formation of copper-chitosan nanoparticles as shown in Figure 1(B). Reports by Mallick et al.[31] also revealed that the surface plasmon band for Cu-ChNPs was in the range of 570–600 nm.

The histogram obtained by dynamic light scattering (DLS) revealed the diameter of Cu-ChNPs in the range of 45–70 nm by intensity. The average diameter of the synthesized NPs was 50 nm. It was revealed in Figure 2(A). Choudhary et al.[32] reported the DLS of chitosan copper nanoparticles showed the size of NPs at 163.8 ± 13.3 nm. The zeta potential studies confirmed the stability of nanoparticles, which showed that the synthesized NPs have a positive surface charge of 9.6 mV and were found to be stable, as shown in Figure 2(A). Higher zeta-potential showed Cu-ChNPs as highly biologically active nanomaterials.[33,34] The positivity of the nanoparticles proved to be stable, and this is due to the higher surface charge, which correlates directly to the higher electrostatic repulsion between the particles.[35] Elemental analysis of Cu-ChNPs indicated three major peaks profile at 3 KeV, confirmed copper, chitosan, and oxygen elements (Figure 2(B)).

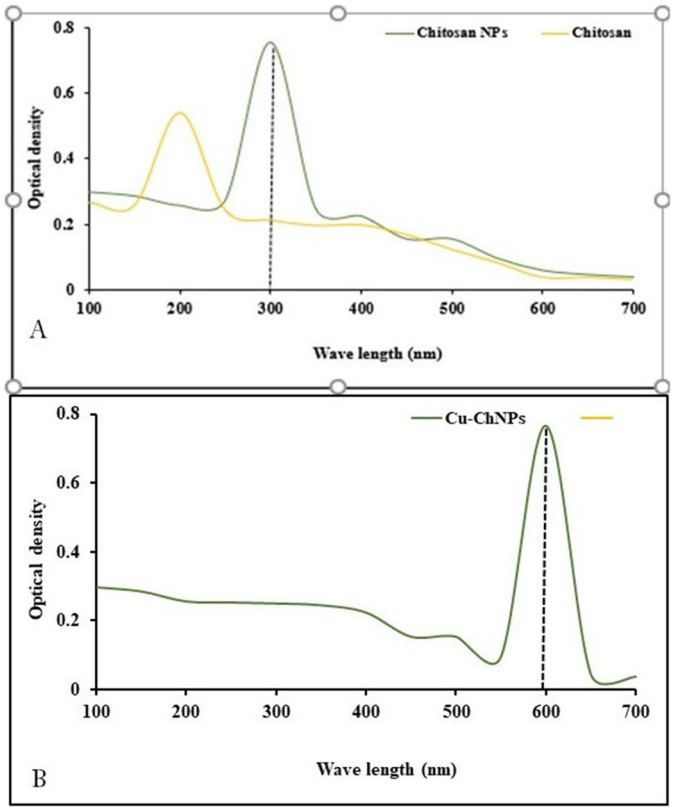

Figure 1. Synthesis of Cu-ChNPs *in vitro*.
(A) UV–visible spectra indicate the chitosan NPs alone and bulk chitosan showing at 295 nm. (B) Synthesized Cu-ChNPs at 590 nm. Bulk chitosan showing peak at 190 nm.

FTIR analysis

FTIR analysis of Cu-ChNPs was elucidated and presented in Figure 2(C). Dominant peaks were observed at 3286.57 cm^{-1}, 2920.27 cm^{-1}, 2112.56 cm^{-1} and followed by smaller peaks in bulk Cu-ChNPs indicated at 1622.28 cm^{-1}, 1318.99 cm^{-1}, 1031.87 cm^{-1}, and 635.71 cm^{-1} were due to the C–H (medium)- Alkynes HC–H stretch C–H (medium)-Alkanes CH_2, C = C (medium to weak)-Alkynes C = C stretch, respectively. The present findings are similar to the pattern spectral images reported by Saharan and Pal.[36]

The XRD study indicated the crystalline nature and phase of nanoparticles information, which gives the particle size as reported by the Debye Scherrer equation. The XRD results confirm the crystallinity of Cu-ChNPs. The diffraction peaks were recorded at 2θ values corresponding to the planes of (111), (200), (220), and (311), respectively, which can be indexed according to the facts of the face-centred cubic crystal structure of Cu-ChNPs as shown in Figure 3(A). The XRD corresponding planes obtained were matched with the JCPDS file number (JCPDS NO: 05-0061).[37] The SEM images of Cu-ChNPs showed spherical shape, fairly agglomerated, and exhibited highly crystalline because of the copper ions embedded in the chitosan backbone, as shown in Figure 3(B). From TEM analysis, the size and morphology of Cu-ChNPs were evaluated and demonstrated the spherical shape images, which are represented in 50 nm as shown in Figure 3(C). The Cu-ChNPs are well dispersed and were noted to be agglomerated. The TEM images showed the clear presence of copper ions embedded in the chitosan.[38]

The pH value influences the morphological characteristics of the biologically synthesized Cu-ChNPs. Usually, pH alters the charges of biological components present in the extract and improves their capacity to bind with metal ions and reduce them.[39] In the present study, the formation of Cu-ChNPs was studied over pH at 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0, as shown in Figure 4. Among them, pH 4.0 and 5.0 showed the least peak intensities due to the absence of nanoparticles. pH 7.0 showed higher peak formation of Cu-ChNPs, respectively, which at pH 6.0, 8.0, and 9.0 showed moderate peak. Raut et al.[40] have reported that large particles may be responsible for the change in peak intensity, which is confirmatory with the present findings. Cu-ChNPs revealed a time-dependent stability process up to the 90th day, as shown in Figure 5. A similar observation was reported by Choudhary et al.[19] to form copper chitosan nanoparticles and their storage.

Antifungal activity of Cu-ChNPs against Fusarium oxysporum f. sp. cubense

In the present study, using the Food Poisoning Technique (FPT) efficacy of Cu-ChNPs was tested against *Fusarium oxysporum* f. sp. *cubense* and exhibited mycelial growth inhibition on solid media (PDA), respectively. Cu-ChNPs 13 ± 2.5, 14 ± 2.1, and 14 ± 2.1 mm mycelial growth inhibition against *Foc* at 0.10, 0.20, and 0.30 mg/mL NPs concentration, as shown in Table 1. The Cu-ChNPs showed similar activity with respect to a positive control (SAAF fungicide at 0.20 mg/mL). It should be noted that the commercial

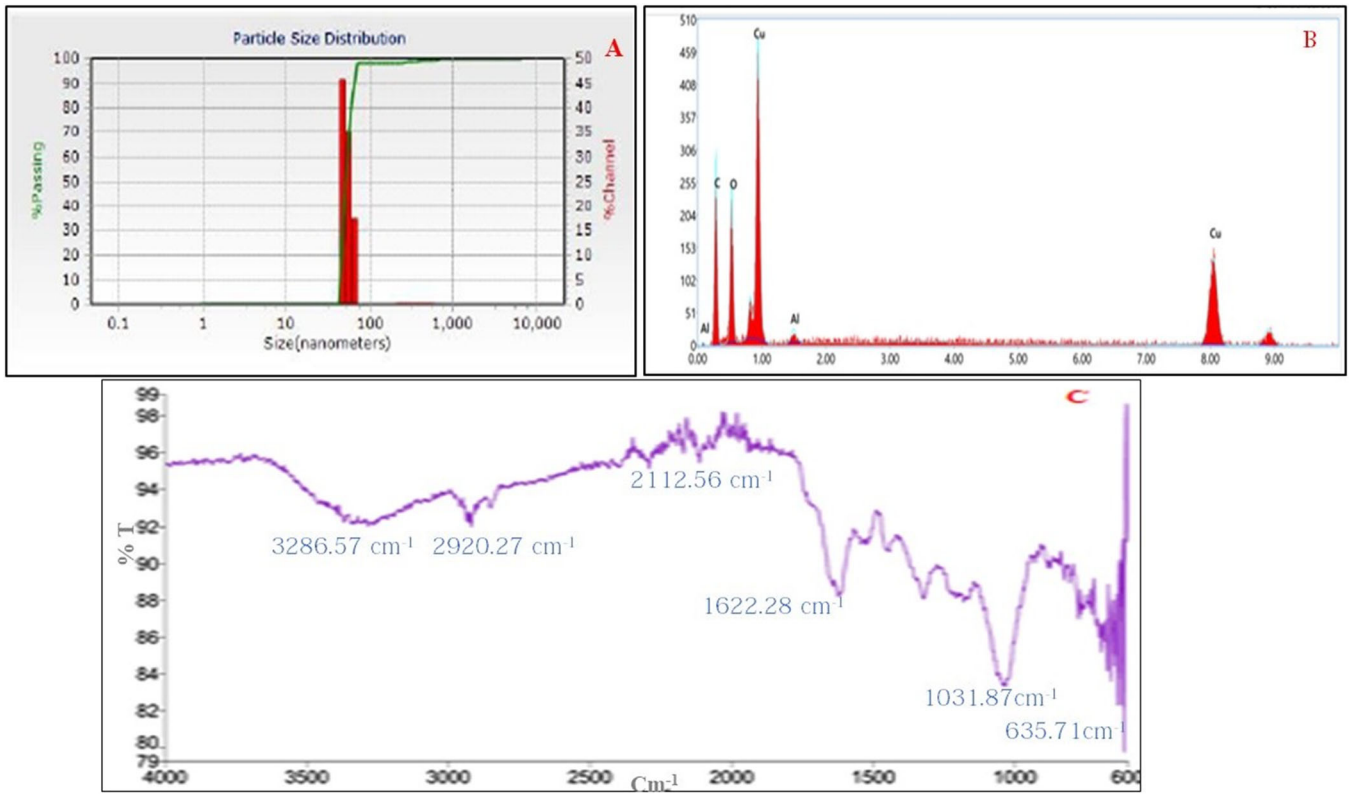

Figure 2. Characterization of synthesized Cu-ChNPs.
(A) DLS of Cu-ChNPs, showing nanoparticles size at 45–70 nm (B) EDAX spectrum of synthesized Cu-ChNPs, (C) FTIR spectra of Cu-ChNPs.

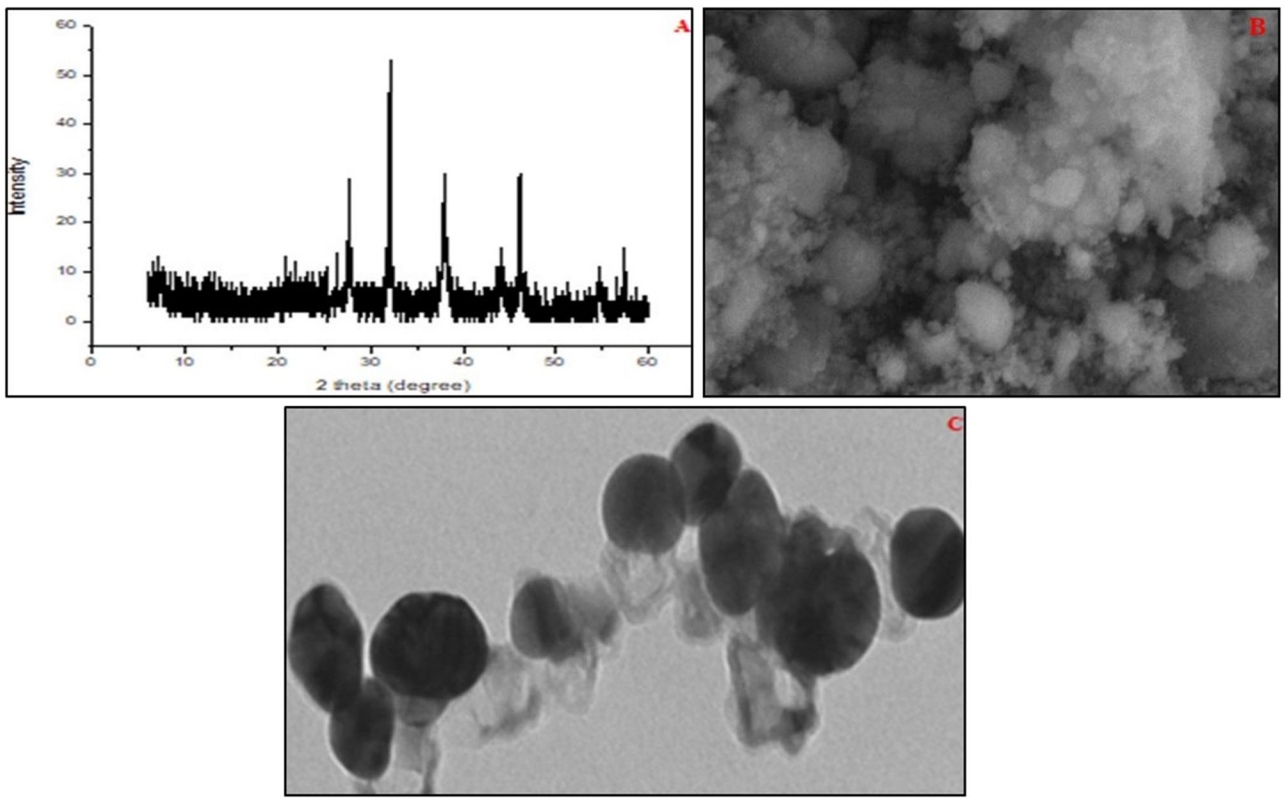

Figure 3. Characterization of synthesized Cu-ChNPs.
(A). XRD pattern of Cu-ChNPs, (B) SEM image of Cu-ChNPs, (C) TEM images showing the spherical shape of Cu-ChNPs surrounded by chitosan layer at 50 nm

fungicide used to evaluate the antifungal property was almost similar to the concentration of the Cu-ChNPs used. These results confirmed the potential inhibition activity against *Foc* using lower concentrations of NPs. Sathiyabama and Parthasarathy[12] reported 89% growth inhibition of *Alternaria alternata*, *Macrophomina phaseolina*, and

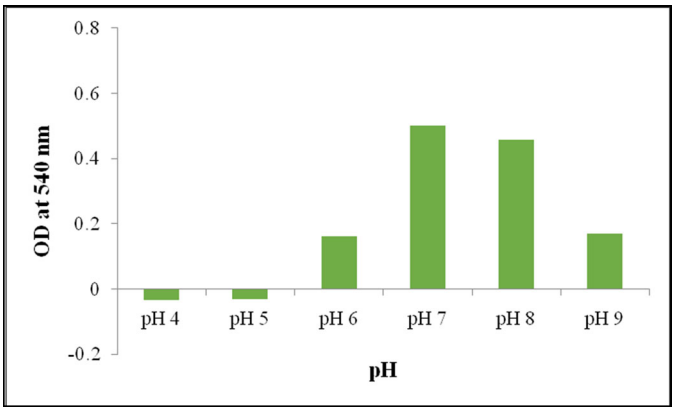

Figure 4. UV–visible absorption spectra of Cu-ChNPs at a various pH level.

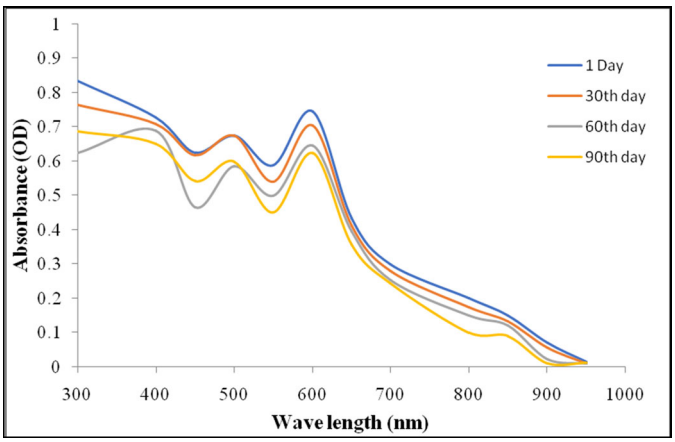

Figure 5. Stability of Cu-ChNPs biosynthesized at various time intervals up to 90th day of storage.

Table 1. Antifungal activity of Cu-ChNPs against *Fusarium oxysporum* f. sp. *cubense*.

Treatments	Colony diameter of *Fusarium oxysporum* f. sp. *cubense* (mm)*
Control	1.2 ± 0.7
Positive control (fungicide SAAF) 0.2 mg/mL	15 ± 0.4
Cu-ChNPs (0.1 mg/mL)	13 ± 2.5
Cu-ChNPs (0.2 mg/mL)	14 ± 2.1
Cu-ChNPs (0.3 mg/mL)	14 ± 2.1

*Each value is mean of triplicates. Each treatment is not significantly different at $p = 0.05$

Rhizoctonia. solani using 0.1% chitosan-copper NPs. In another study, 0.1% chitosan-copper NPs showed mycelial inhibition against *Sclerotium rolfsii* and *Rhizoctonia solani*.[41] The mechanism of nanoparticles on plant pathogens was not clearly known yet. However, the antifungal activity of nanoparticles may be due to the slow release of copper ions from the chitosan at the time of fungal infection that causes cellular leakage. Saharan et al.[20] reported Cu–chitosan NPs showed higher antifungal activity against *Fusarium oxysporum* at 0.10 and 0.12% under *in vitro* mycelial growth and spore germination. Bulk chitosan and 0.1% $CuSO_4$ were found less effective in inhibiting mycelia growth and spore germination as compared to synthesized NPs. Al-Hetar[42] showed bulk chitosan inhibited mycelial growth of *Foc* at different concentrations but showed

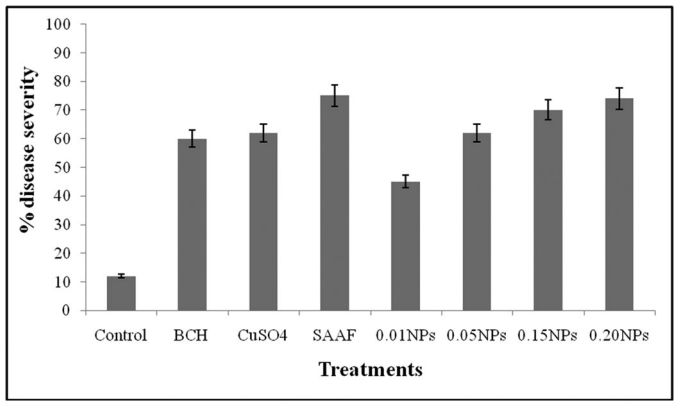

Figure 6. Effect of Cu-ChNPs on Fusarium wilt disease in pot experiments. Disease data were recorded after 15 days of inoculation using 1 to 9 standard disease rating scale. Each value is mean of triplicates and each replicate consisted of 3 plants samples. Each treatment is not significantly different at $p = 0.05$

maximum growth inhibition of 76.36% recorded at 8 mg/mL and the lowest inhibition at 0.1 mg/mL concentration.

Effect of Cu-ChNPs on Fusarium wilt disease in pot experiment

Under greenhouse conditions, pot experiments were conducted to assess the symptoms of Fusarium wilt, which were initiated after seven days of *Foc* inoculation. The early symptoms were appeared with wilt symptoms gradually extended to the whole plant, provoking intense wilting. A maximum percent efficacy disease control (PEDC) was recorded in banana plants sprayed with commercially available fungicide SAAF showed 75% followed by a PEDC of 73% in 0.20 mg/mL of Cu-ChNPs. The least PEDC was recorded 45% in 0.01 mg/mL when compared to control 12% as shown in Figure 6, respectively. It was noted that Cu-ChNPs were better than chitosan and fungicides when used alone, and also, the concentrations used for chitosan and fungicides were higher than Cu-ChNPs. This is because of the chitosan biopolymer, which is a strong elicitor of plant defense mechanisms as well as a higher antifungal activity when capped with copper nanoparticles.[20] A review of the literature divulges that among the chitosan-based nanomaterials, Cu–chitosan NPs results confirm the significant growth promotion as well as antifungal capabilities of Cu–chitosan nanoparticles.[43,44]

Effect of Cu-ChNPs on the activities of plant defense enzymes in pot experiments

To examine the activities of plant defense enzymes, leaf samples were collected after 24 h of foliar spray of the fungal pathogen. Cu-ChNPs at different concentrations substantially induced the enzyme activities in the leaf sample. Chitinase activity was significantly higher in all the treatments of Cu-ChNPs at 48 h. Similarly, control (DW), $CuSO_4$, and bulk chitosan treatments did not show much activity. In contrast, positive control (fungicide) treatment showed very similar to Cu-ChNPs treatments, as shown in

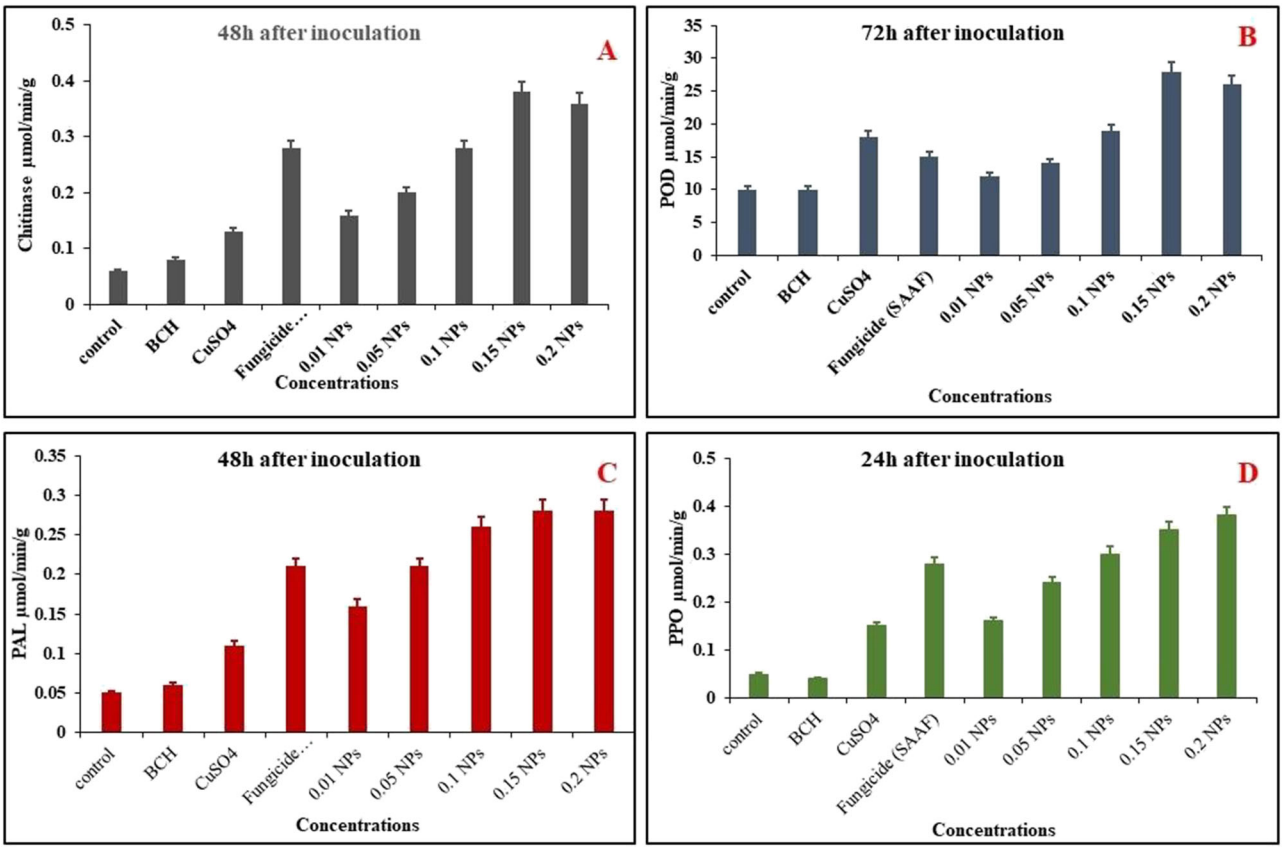

Figure 7. Effect of Cu-ChNPs on defense enzymes in banana plants challenged with the pathogen. (**A**) Chitinase (**B**) PO (**C**) PAL (**D**) PPO enzymes activity in banana plant leaves after 24 h of foliar spray. Each value is the mean of triplicates and each replicate consisted of 3 plants samples. Each treatment is not significantly different at $p = 0.05$ Control with distilled water. BCH (bulk chitosan, 0.01%) dissolved in 0.1% acetic acid. $CuSO_4$ (0.01%) and fungicide (0.01% of Bavistin).

Figure 7(A). Chitinase induction was elicited in banana plants in response to Cu-ChNPs. Kouzai et al.[45] reported that chitinase might provide crop protection against several fungal diseases. Chitinase induction was elicited in finger millet plants in response to Cu-chitosan NP. Protease inhibitors (PI) are involved in the defense response of the host plant against phytopathogens.[46] Satheesh and Murugan[47] also revealed the remarkable microbicidal potential of protease inhibitors from the leaves of *Coccinia grandis* against *Klebsiella pneumonia* and *Aspergillus flavus*.

Similarly, two-fold higher peroxidase activity was recorded at 72 h after *Foc* pathogen inoculation, treated with different concentrations of Cu-ChNPs compared to fungicide (SAAF), and bulk chitosan treated plants, as shown in Figure 7(B). Peroxidase is one of the important enzymes involved in plant defense enzymes under biotic and abiotic stress.[48] Furthermore, Cu-ChNPs (0.05 to 0.20%) plants showed 1.5–2 folds increased PAL activity at 48 h after inoculation of *Foc* pathogen compared to control and chitosan alone, as shown in Figure 7(C). The PPO activity was enhanced by two folds at 24 h after inoculation of *Foc* by Cu-ChNPs (0.15 and 0.20%) compared with control and bulk chitosan. Fungicide treatment showed lesser activity than Cu-ChNPs (0.15 and 0.2 µg/mL), as shown in Figure 7(D). The increased activity of chitinase, POD, PAL, and PPO might be associated with the production of chitin and lignin for the cell wall strengthening, which further acts as a mechanical barrier to invading plant pathogens.[49,50] High-level expression of PPO and peroxidase activity was reported in cucumber plants associated with resistance against *Pythium aphanidermatum*.[51]

Conclusion

Banana is a major commercial crop in India and has exponentially increased its production in recent years. Nanjanagudu rasabale cultivar has become very susceptible to Fusarium wilt. To manage the disease, commercially available fungicides are being used indiscriminately in agriculture, leading to environmental pollution. The present study shows that Cu-ChNPs can be used as nano-fungicide against the Fusarium wilt of Banana. The prepared Cu-ChNPs were spherically shaped with an average size of 50 nm as determined by TEM and DLS. Besides their antifungal activity, the Cu–chitosan NPs at 0.20 mg/mL showed maximum percent efficacy disease control similar to commercially available fungicide and increased the disease defense enzymes study, indicating higher activities against the Foc pathogen and proving the effectiveness of Cu-ChNPs under greenhouse conditions. Therefore, our study claims that Cu-ChNPs could be a new development for the generation of chitosan-based nanofungicides. The synthesized NPs have immense potential to be commercially explored for their use in sustainable agriculture.

Disclosure statement

No potential conflict of interest was reported by the authors.

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