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Evaluation of Phytotoxicity of Biosynthesised Copper Nanoparticles on Seed Germination of Two *Vigna* Species

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Abstract

The production of metallic nanoparticles, especially Copper nanoparticles (CuNPs), has gained interest over the last few years. CuNPs with their unique physical and chemical properties can have both positive and negative effects, and therefore, the appropriate dosing concentration of CuNPs is important. The present research reports the production of CuNPs through a green synthesis method using *Burkholderia* sp. (NCBI Accn. No. OP646862). Biosynthesis parameters were optimized at 1 mM copper sulphate, with a 2:3 ratio of bacterial supernatant to copper solution, pH 8 and 48 h of incubation. The CuNPs showed a peak at 540 nm; Fourier transform infrared spectroscopy indicated proteins, amino acids, alkene and amine groups; Field-emission electron microscopy showed spherical particles; XRD indicated a crystalline size of 24.10 nm and the zeta size was 141.5 nm with moderate stability (−20.04 mV). The synthesized nanoparticles were checked for any phytotoxic effect on seed germination of *V. radiata* and *V. mungo*. However, at concentrations higher than 80 ppm, slight decline in radicle length was observed in both *Vigna* species. The optimum concentration that can be used for plant growth promotion is 40 ppm and 60 ppm in *V. mungo* and *V. radiata* respectively. While, higher concentration can be used to control plant pathogens as 100% seed germination in both the *Vigna* species.

Major Findings: CuNPs was successfully synthesized using *Burkholderia* species. CuNPs-primed seeds of both *Vigna* species showed 100% germination, hence there is no toxicity of the CuNPs, however, decrease in radicle length indicate phytotoxic effects above 80 ppm.

Keywords: Biosynthesis, Copper Nanoparticles, Legume Crops, Microbes, Seed Germination

1. Introduction

Plants experience retardation in growth with exposure to higher concentrations of copper, resulting in copper toxicity. Its deficiency in crop plants can lead to abnormal growth symptoms such as necrosis, distortion of young leaves, bending of the stem, and reduction in yield due to impaired growth at the vegetative phase[1].

The synthesis of nanoparticles with diameters ranging from 1 to 100 nm is one of the most promising fields of study in nanotechnology and because of their small size and high surface area, they eventually increase the state of activity compared to the bulk material[2].

There are several ways to synthesise metal nanoparticles, including chemical, physical and green approaches. Expensive chemicals are needed for the chemical synthesis approach, while the main disadvantage of the physical method is that it takes longer to synthesise a small quantity of NPs. Thus, scientists are concentrating on the biosynthesis of metal nanoparticles as a cost-effective and eco-friendly alternative to chemical and physical methods. CuNPs are synthesised from a variety of natural sources, including microorganisms and plants or plant parts[3-7]. Because of their high stability and ability to withstand or eliminate heavy metals, bacteria were chosen over all other microorganisms. Secondly, they are easy to culture and grow faster, which further adds to large-scale production of NPs in a sustainable way[8,9]. *Burkholderia* exhibits multiple plant growth-promoting (PGP) traits, including phosphate solubilisation,

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siderophore production, HCN production, as well as efficient in controlling plant pathogen[10,11] but less research has been done on it in the field of agriculture. The other species of *Burkholderia* have been reported to synthesise various types of nanoparticles due to the presence of enzymes and metabolites that help in the reduction of nanoparticles[12-14].

Therefore, considering its potential, the present study utilises the bacterium *Burkholderia* to synthesise CuNPs. Although fewer nanoparticles are leaking into the environment, their concentration, chemical composition, surface charge, and improper application can all have adverse impacts[15]. When these NPs are absorbed by plants, they enter the plant system by several internalisation processes that result in migration, accumulation, and other processes that impact plant growth in both favourable and negative ways[16,17]. The effects of these particles' bioaccumulation in the ecosystem must therefore be taken into account.

Seed priming with nanoparticles plays an important role in promoting plant growth and protecting them from soil-borne pathogens such as *Ralstonia solanacearum, Fusarium oxysporum, Macrophomina phaseolina, Alternaria alternata, Rhizoctonia solani, Colletotrichum* species, *etc*[18,19].

It ensures seed viability, promotes physiological seed development, and improves seed quality because it gives the seed-controlled hydration and permits seed germination without root emergence. It improves seed quality and assures quick, consistent germination[20].

Since seed germination and early plant growth are crucial phases of plant development, copper plays a significant role in crop development. Seed treatment/coating is one of the suggested and popular means of prevention of soil-borne fungal diseases at the germination and seedling stage. Despite promising results of CuNPs, its toxicity studies with potential antifungal effect w.r.t. seed germination in *Vigna* species are lacking. In light of this, the present study includes biosynthesis of CuNPs using *Burkholderia* and assessment of its dose on seed germination and radicle length of *Vigna* species.

2. Materials and Methods

2.1 Synthesis, Optimisation and Characterisation of Copper nanoparticles

Copper nanoparticles (CuNPs) were synthesised through biological processes[21] from the bacteria *Burkholderia* sp. (OP646862). The bacterial culture was kept at 28°C±2°C for 48 h in YEMB (Yeast Extract Mannitol Broth), which contained mannitol (10 g/L), dipotassium orthophosphate (0.5 g/L), magnesium sulphate (0.2 g/L), sodium chloride (0.1 g/L), tryptophan (2 mg/mL), and yeast extract (1 g/L). After centrifuging the bacterial cultures at 10,000 rpm for 15 mins at 4°C, the supernatant was filtered through an aseptic nylon membrane filter (25 mm, 0.2 µm) to produce a sterile filtrate (pH 6.5-7). For the synthesis of copper nanoparticles, the filtrate was combined with 1 mM copper sulphate solution in a 1:4 ratio and incubated in a rotatory shaker at 150 rpm and 35°C±2°C. The mixture was monitored until a dark green colour appeared.

2.1.1 Optimisation of CuNPs

In order to fasten up the synthesis of CuNPs, various reaction parameters such as pH, the volume ratio of bacterial supernatant and $CuSO_4$ solution, and incubation duration were adjusted[21,22].

pH: The sterile filtrate pH was adjusted from pH 4 to pH 9, added to a 1 mM $CuSo_4$ solution and incubated for 48 h at 35°C±2°C in a rotatory incubator shaker at 150 rpm. Bacterial supernatant to copper sulphate solution volume ratio: In order to determine the volume of bacterial supernatant needed for the effective synthesis of CuNPs, different volumes of bacterial supernatant and copper sulphate solution were utilised, holding the final amount up to 50 mL in the ratios of 1:4, 2:3, 3:2, 4:1 and 1:1. After that, the mixture was incubated for 48 h at 35°C±2°C and 120-150 rpm.

Incubation time: Nanoparticle synthesis was monitored at various time intervals, such as 3, 6, 12, 24, and 36 h, until a green colour showed consistently observed, in order to optimise the incubation period.

2.1.2 Characterisation of CuNPs

A range of analytical techniques, such as UV-vis spectroscopy (2202TS, Systronics, India), X-ray diffractometry (XRD, Bruker D8 Advance, Germany), Fourier Transform Infrared Spectroscopy (FTIR, Alpha, Bruker, Germany) and Field Emission Scanning Electron Microscopy (FESEM) with EDS (MIRA3, TESCAN, Brno, Czech Republic), were carried out at Banasthali Vidyapith, Rajasthan, India. In order to monitor the reduction of copper ions, a copper nanoparticle suspension was diluted ten times using deionised water. The absorbance was then

measured using a UV-vis spectrophotometer in the 300-800 nm range, with deionised water serving as a blank[23]. To determine the crystal structure, the biosynthesised CuNPs were sonicated (100 W, 40 kHz for 30 min) and characterised by X-ray diffractometry (XRD). The presence of a capped protein surrounding synthesised CuNPs was determined using Fourier Transform Infrared Spectroscopy (FTIR) in the 400-4000 cm^{-1} wavenumber range, and the size and shape of the nanoparticles were obtained with electron microscopy utilising scanning electron microscopy (SEM+EDS). The size and stability of AgNPs were determined by analysing Zeta-sizer (0.3 nm to 0.6 µm) and Zeta Potential (-100 mV to +100 mV) using the instrument, Zetasizer Nano ZSP (ZEN 5600) (Malvern Panalytical, UK) at Malaviya National Institute of Technology, Jaipur, India (Supplementary File 1).

2.1.3 Impact of CuNPs on Seed Germination of Vigna species

The collection of seeds was done from Krishi Vigyan Kendra (KVK), located in Banasthali Vidyapith, Rajasthan, India, for the PU 31 variety of *V. mungo* and the MH1142 variety of *V. radiata*.

Nanoparticle suspension: CuNPs powder was suspended in molecular water to make the nanoparticle suspension, which was then exposed to 30 mins of ultrasonic vibration at 100 W and 40 kHz. To prevent nanoparticle aggregation, small magnet bars were added to the suspensions for stirring.

2.1.4 Seed Germination Test of Vigna species by Standard Blotter Method (SBM)

In order to check the impact of copper nanoparticles on *V. mungo* (PU 31) and *V. radiata* (MH1142), mature seeds were thoroughly cleaned under running water for two to three times to get rid of any dust or contaminants. They were then surface sterilised with 95% ethanol for ten to twenty seconds, followed by a 2% sodium hypochlorite solution for 1-2 mins. Finally, they were washed five times with sterile dist. water and soaked in sterile dist. water for 24 h. To get rid of any remaining water, the seeds were then dried on a sterile blotting sheet. For 2 h, the soaked seeds were treated with varying concentrations of copper nanoparticle solution, ranging from 10 to 100 ppm.

Separate bedding was prepared for both *V. mungo* and *V. radiata* using glass Petri dishes with a diameter of 90 mm. The bottom of the Petri dish was first covered with a circular piece of Whatman filter paper, then another layer of cotton and Whatman filter paper. The bedding was moistened with 2 mL of sterile water, and each Petri dish was filled with ten seeds of PU 31 and MH1142 separately and kept in the dark. Radicle length, percent germination[24] and per cent growth change were assessed after 5-days.

Percent Germination = (Number of germinated seeds / Total number of seeds) *100

Percent growth change = (T-C/T) *100

where, 'T' is the mean radicle length of CuNPs-treated seeds and 'C' is the mean radicle length of control seeds.

2.2 Data Representation and Statistical Analysis

SigmaPlot 12.0 (Systat Software, Inc., Germany) was used to represent the data graphically, and Origin Pro (Origin 2021, OriginLab Corporation, USA) was used to prepare the XRD graph. X' Pert HighScore Plus 3.0 (PANalytical BV, Netherlands) was used for peak analysis. Statistical analysis was performed using Analysis of Variance, SPSS (IBM SPSS Statistics, version 20, USA). The reported data are the Mean± Standard Deviation (SD) of triplicates. Analysis of Variance (one-way ANOVA), Least Significant Test and DMRT (DUNCAN) were performed to analyse significant differences between means at $p \leq 0.05$.

3. Results and Discussion

3.1 Optimisation and Characterisation of CuNPs

3.1.1 Characterisation of CuNPs using UV-visible Spectroscopy

pH**:** After 48 h of synthesis, pH 8 was selected based on the appearance of green colour (Figure 1A) visually, which serves as a preliminary indication of nanoparticle synthesis. In the UV-Vis Spectrophotometric study of the synthesised copper nanoparticles under different pH, it was observed that at pH 8, the absorption was highest at 540 nm with a sharp peak (Figure 2A). Based on results obtained by varying pH, pH 8 was selected for further optimisation.

Bacterial supernatant and copper sulphate volume ratio**:** Based on colour appearance (Figure 1B) and spectrophotometric analysis (Figure 2B) of the produced nanoparticles under various ratios of copper sulphate

solution to bacterial supernatant, it was found that the ratio of 2:3 produced a sharp peak at 540 nm and produced better results than other volume ratios. This may be attributed to a higher concentration of biomolecules responsible for the reduction of copper ions into CuNPs. Thus, based on the results, the ratio 2:3 was further taken for optimisation of incubation time.

Incubation time: It was found that the colour intensity and UV–Vis spectra of the biosynthesised CuNPs increased consistently over time (Figure 2C) and became constant after 48 h of incubation.

The spectrophotometric indication of the surface plasmon resonance peak at 540 nm indicates successful synthesis of CuNPs[25]. According to reports, most bacterial enzymes produce silver nanoparticles with uniform sizes when the pH is alkaline[26]. Therefore, adjusting the pH can help regulate or modify both the shape and size of the nanoparticles[25,27]. The exact position of the SPR band may shift depending on individual particle properties, including size, shape and capping agents[28,29]. The volume ratio of the bacterial supernatant and copper sulphate solution plays a crucial role in balancing the available metal ions and the biomolecules (reductants/capping agents) present in the supernatant and determines the size and morphology of the resulting nanoparticles[30]. Studies have shown that a 1:2 ratio can sometimes maximise productivity due to the presence of an adequate amount of reductants/capping agents, leading to a balanced reaction as compared to 1:3 or 1:4[31]. When 5mM $CuSO_4$ was added to *Pseudomonas fluorescens* supernatant[32] and a flask with *Morganella sp*[33], the reaction mixture's colour changed from blue to dark green. *Shewanella oneidensis* reduced $CuSO_4$ solution to CuNPs in 96 h[34], *Salmonella typhimurium* reduced $CuSO_4$ solution to CuNPs in 20 mins[35], and *Pseudomonas fluorescens* reduced $CuSO_4$ solution to CuNPs in 90 mins[32].

3.1.2 Characterisation of CuNPs using XRD

The XRD spectrum (Figure 2D) shows the diffraction peaks of CuNPs at 28.5, 43.28, 53.3 and 74.06 which correspond to the 110, 111, 200, and 220 planes respectively confirming Face Centred Cubic structure (FCC) metallic copper (JCPDS no. 04-0784) and a diffraction peak around 28.5° indicates the (110) crystallographic plane of Cu_2O [36].

Additionally, the presence of extra peaks may indicate impurities or residual biomolecules from the culture medium or bacterial enzyme. The average crystalline size of the CuNPs is 24.10 nm as determined by Scherrer's formula[37,38].

$$Dp = (0.94\lambda) / (\beta Cos\theta)$$

Where, Dp = Average Crystallite size, β = Line broadening in radians, θ = Bragg angle, λ = X-Ray wavelength (0.15418 nm).

The study's peaks are in accordance with previous investigations that reported similar diffraction peaks for CuNPs[39].

3.1.3 Characterisation of CuNPs using SEM

The appearance and formation of copper nanoparticles are confirmed by the SEM image. The produced nanoparticles were almost spherical in shape with variable sizes ranging between 80-320 nm. The larger CuNPs could be due to slight agglomeration (Figure 3A, B). The EDX spectrum results confirmed the presence of copper with additional elements, which may be due to the culture medium and enzymes secreted by the *Burkholderia* (Figure 3C).

3.1.4 Characterisation of CuNPs using FTIR

Characteristic peaks observed at 3303.11, 1635.76, 1771.25, and 614 cm^{-1} correspond to N-H stretching, C=C stretching

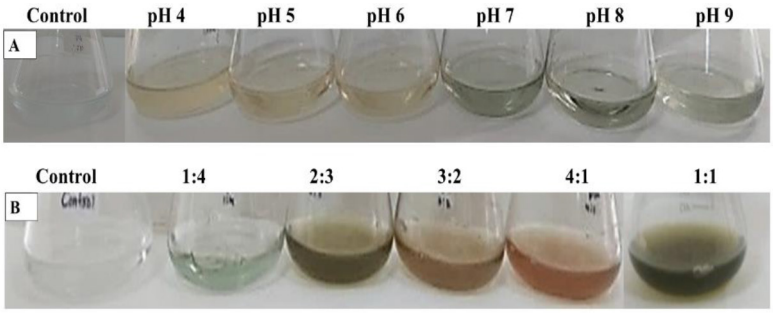


Figure 1. Visual appearance of optimised CuNPs at various parameters; A) pH and B) Bacterial supernatant and Copper sulphate volume ratio.

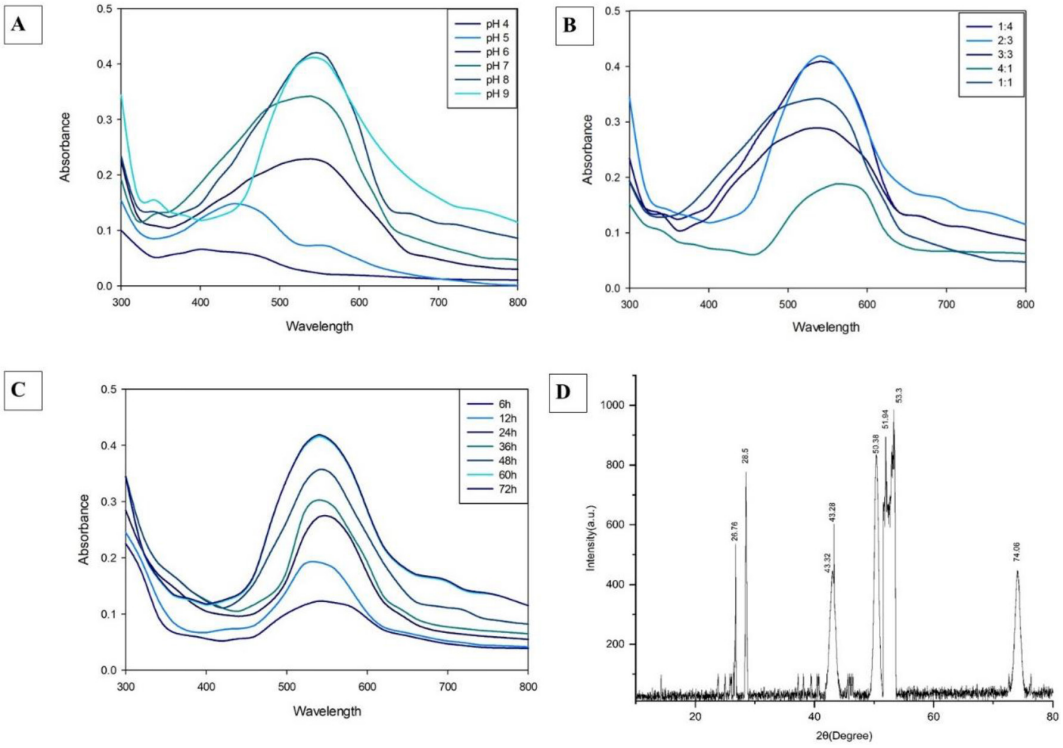


Figure 2. Characterisation of CuNPs. UV-Visible spectroscopy at various parameters: **A)** pH, **B)** Bacterial supernatant and Copper sulphate volume ratio, **C)** Incubation time; and **D)** XRD spectrum.

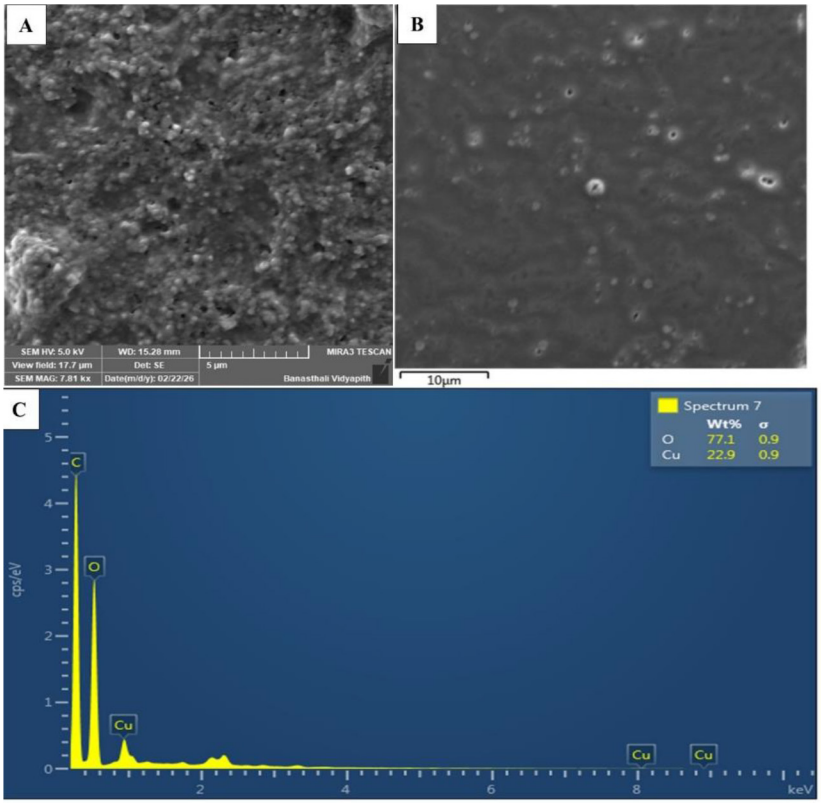


Figure 3. Characterisation of CuNPs. (A, B) Scanning electron microscopy and C) EDS spectrum.

and N-H bending, C-H bending and 614 cm^{-1} is related to Cu-NPs with oxygen from hydroxyl groups and formation of CuO[21]. Additionally, the spectra of CuNPs show the existence of amino acids, proteins, and enzymes as a source of oxidation of copper ions to copper nanoparticles (Figure 4). The CuNPs FTIR spectra investigation revealed distinct amide and amine group peaks, indicating that the CuNPs are protein-coated[21]. However, according to, proteins that coat CuNPs are known to decrease oxidation and improve long-term stability[40,41].

The *Vigna* seeds treated with CuNPs showed 100 percent germination at each concentration (10-100 ppm) including control but variation in radicle length was observed with changing concentration of CuNPs (Figure 5A, 5B). As compared to the control, CuNPs application improved radicle length at 40 ppm and 60 ppm in *V. mungo* and *V. radiata,* respectively. On increasing concentration, CuNPs showed a toxic effect on radicle length at concentrations higher than 80 ppm in both *Vigna* species. The statistical data reveal significant differences ($p < 0.05$) in radicle length from concentrations 20-80 ppm. Whereas at 100 ppm, the decline in radicle length was non-significant, with 2.2% and 13.02% in *V. mungo* and *V. radiata,* respectively (Figure 6).

CuNPs were known to exhibit dose-dependent effects where low concentrations (e.g., 25–50 mg/L or ppm) often promote or have little effect on seed germination percentage, while impacting radicle length by disturbing water uptake channels and enzymatic activity[42-44]. In this regard, numerous studies discovered that reduced size of soybean seeds at higher concentrations of CuONPs[45]. Studies employing the plant *Cicer arietinum* L. (chickpea) subjected to varying concentrations of copper (II) oxide nanoparticles (CuONPs) revealed that the NPs disrupted the plant's growth, delaying the development of roots and stems[46].

Thus, nano-primed seeds for short durations are effective in protecting the plant from pathogen infestation and that are present on the seed surface and not for internal infection[47]. Uptake of nanoparticles by the plant cells depends on shape, size, surface charge, polarity and interactions with the target cells[48]. Size of nanoparticles is one of the main concerns towards their entry to the plant tissue as plant barriers range from micrometre to nanometre in size[49]. NPs can enter through aquaporins (ubiquitous membrane proteins), involved in the transport of water and small solute particles[50,51].

There are two basic mechanisms through which nanoparticles can translocate (Figure 7):

Apoplastic pathway in which nanoparticles (50-100 nm) are transported through the extracellular gaps associated with adjacent cell walls and arteries[52].

Symplastic pathway in which nanoparticles are transported via the cytoplasm of adjacent cells[53].

According to previous studies, CuONPs at various concentrations can affect the growth and development of plants by increasing reactive oxygen species (ROS) generation and upsetting the equilibrium of necessary elements[54]. The toxicity phenomenon is very complicated and is dependent on lots of physicochemical parameters; hence, different metallic NPs with their special nature would have various toxicity mechanisms and indicate alterations in toxicity amount[55]. Copper nanoparticles (CuNPs) and copper oxide nanoparticles (CuONPs) act on seed germination through

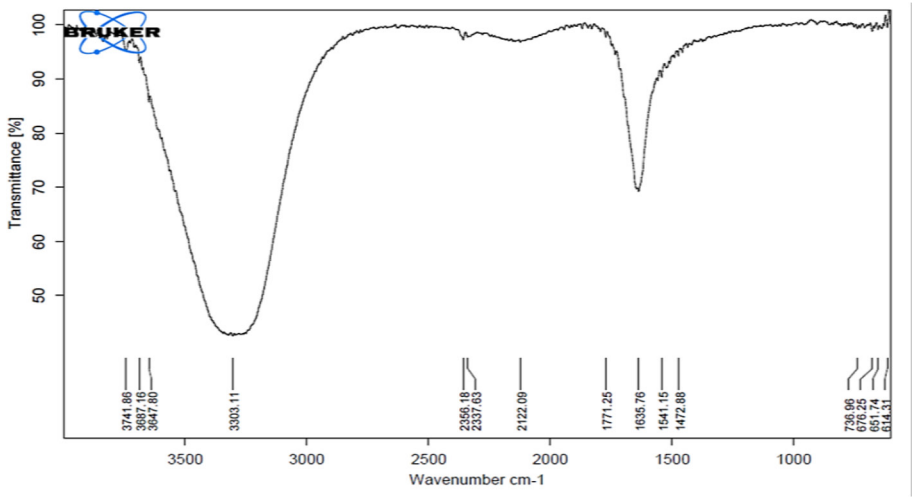


Figure 4. Characterisation of CuNPs by FTIR analysis Impact of CuNPs on Seed Germination of *Vigna* Species



Figure 5. Effect of CuNPs on radicle length. A) *V. mungo*; and B) *V. radiata.*

a biphasic, concentration-dependent mechanism, acting as growth stimulants at low doses and inhibitors at high doses. Their action is primarily driven by enhanced water uptake, modulation of Reactive Oxygen Species (ROS), and activation of metabolic enzymes[56,57].

Low concentrations of CuNPs produce a localised, beneficial level of reactive oxygen species (like hydroxyl radicals) that help loosen cell walls and trigger cell division. They boost the activity of antioxidative defence mechanisms, including Superoxide Dismutase (SOD), Catalase (CAT), and Peroxidase (POX), helping the seed withstand early-stage stress[58,59].

While at high concentration, NPs can clog the pores of the seed coat or accumulate on the root surface, hindering water and nutrient absorption, disturbing food reserve mobilisation and energy transfer required for growth[60]. In an experiment on *Coriandrum sativum,* higher CuNPs concentration leads to excessive Reactive Oxygen Species (ROS) production, causing lipid peroxidation and damage to cellular DNA, proteins, and membrane integrity[20].

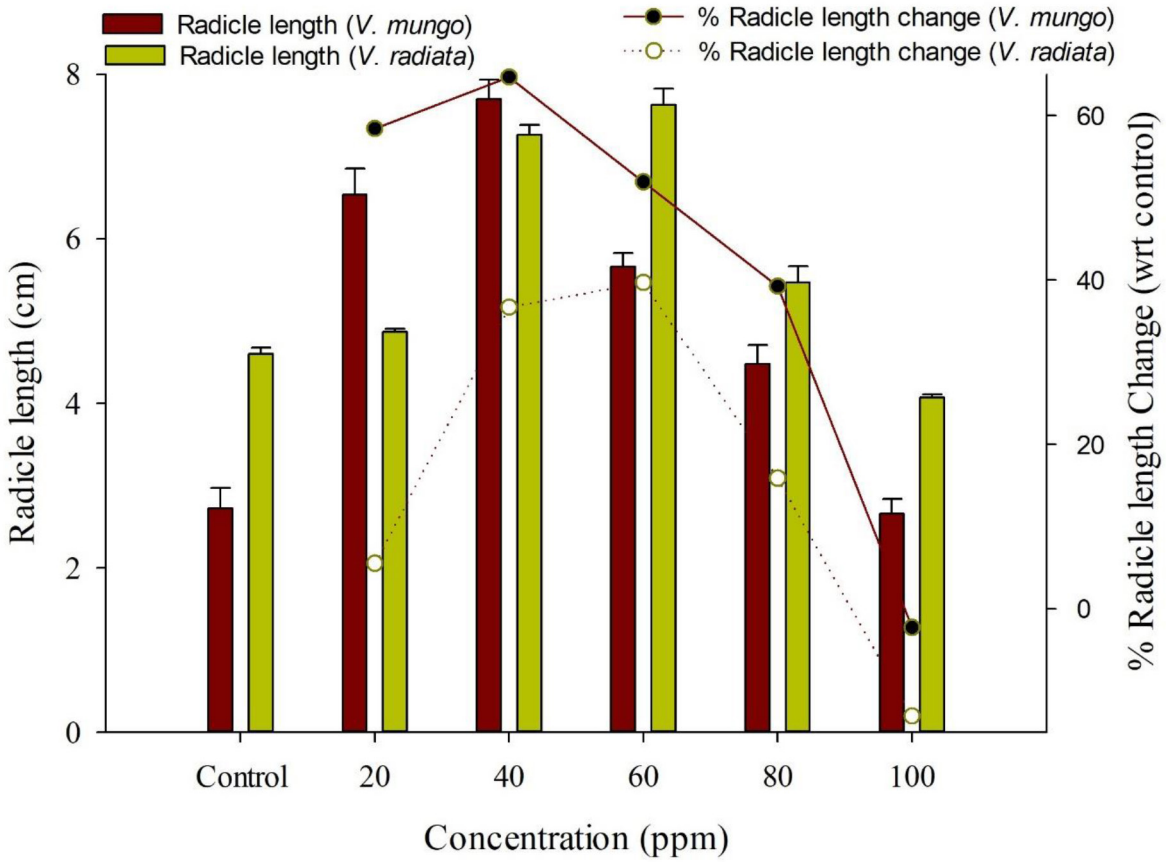


Figure 6. Graph showing effect of CuNPs on radicle length and % growth change of *V. mungo* and *V. radiata*. The bar displays standard error. (a-d) and (a'-e') indicate significant differences ($p < 0.05$), whereas NS and NS' indicate not significant ($p < 0.05$) for *V. radiata* and *V. mungo,* respectively.

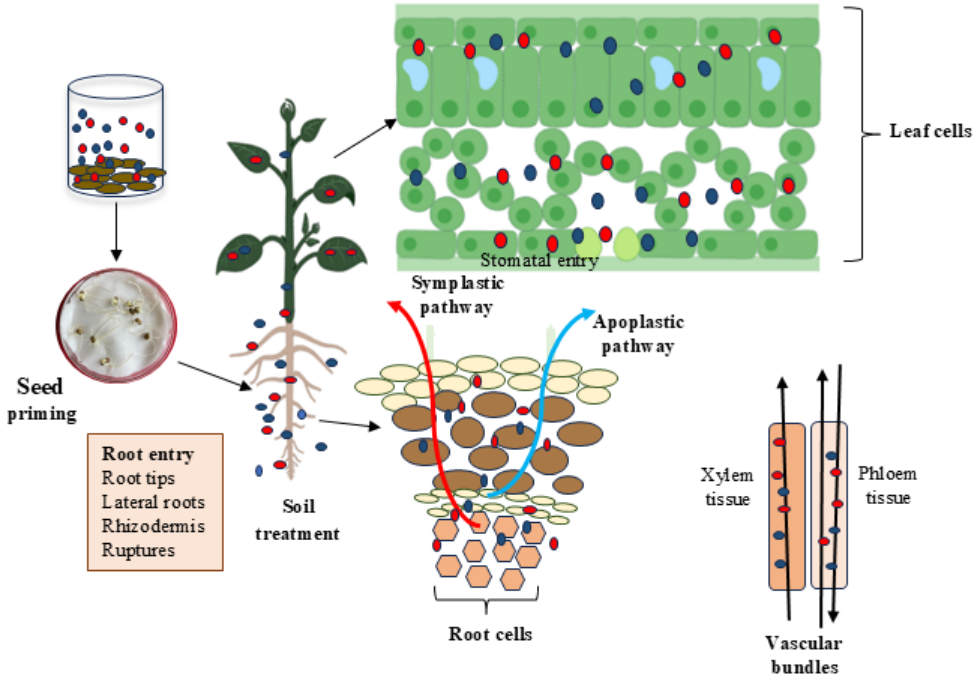


Figure 7. Possible mechanisms of uptake of CuNPs from primed seeds in crop plants. (Created using tools of biorender.com and Microsoft PowerPoint).

4. Conclusion

The current work presents a green route for the synthesis of copper nanoparticles using the bacterium *Burkholderia*. The FTIR spectrum revealed the presence of key functional groups that are consistent with those reported in the literature, validating the presence of enzymes, proteins, and amino acids that may be responsible for the synthesis and stability of CuNPs. Further characterisation revealed the size (80-320 nm), shape (nearly spherical) and moderate stability (-20.04) of CuNPs (Supplementary File 1). The highest growth-promoting concentration was obtained at 40 ppm and 60 ppm in *V. mungo* and *V. radiata,* respectively. While the phytotoxic effect of CuNPs was evident on the radicle length of germinated seeds of both the *Vigna* species decreased at concentrations higher than 80 ppm. This could be due to clogging of pores present on the seed coat, inhibiting water or nutrient uptake, generation of ROS and upsetting the equilibrium required for seed germination and growth.

As the CuNPs are widely used in a variety of applications, a comprehensive assessment of their advantages and disadvantages for plants needs to be conducted because these are small enough to enter cells and tissues. Although the present study highlights the growth-promoting and toxic concentration (>80 ppm) of CuNPs on seeds of *Vigna* species, further research is required before applying them to agricultural fields. In future, the efficacy of biosynthesised nanoparticles can be checked on seeds of other crops, controlling plant pathogens, and in agricultural fields.

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Author Contributions

Investigation, J.S. and A.B.; Original draft preparation, J.S.; Review and editing, A.B. and D.C.; Conceptualisation and supervision, D.C.

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