Copper nanoparticles hold promise in the effective management of maize diseases without impairing environmental health

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Research Article

Keywords: Copper nanoparticles, Plant pathogens, Maize, Management

Posted Date: August 2nd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1859037/v1

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Phytoparasitica on April 15th, 2023. See the published version at https://doi.org/10.1007/s12600-023-01060-3.

Abstract

A novel method of management of maize pathogens *in vitro* and *in vivo* using newly synthesized copper nanoparticles (CuNPs) has been documented in this study. CuNPs have been synthesized using $CuSO_4$ as a precursor, $NaBH_4$ and ascorbic acid as a reducing agent, and polyethylene glycol 8000 (PEG-8000) as a stabilizing agent. Characterization of CuNPs using a Transmission Electron Microscope (TEM) confirmed the nanoparticles' size range of 35–70 nm. Fourier transform infrared spectroscopy (FTIR) revealed the association of alcohol groups and allyl halides group with CuNPs. The synthesized CuNPs exhibited significant inhibition at 20 ppm of three pathogenic fungi namely *Macrophomina phaseolina, Bipolaris maydis*, and *Fusarium verticillioides*, and at 50 ppm against *Rhizoctonia solani*. Bactericidal property of CuNPs was evidenced against *Erwinia carotovora* and *Ralstonia solanacearum* at 30 ppm. Evaluation of CuNPs *in vivo* against two diseases *viz*., maydis leaf blight (MLB) and banded leaf and sheath blight (BLSB) culminated in a reduction in percent disease index (PDI). Seed treatment together with foliar spray @ 300 ppm of CuNPs resulted in a significant reduction of MLB. However, BLSB disease was reduced relatively less at the same aforesaid concentration nevertheless; it was evinced best in controlling BLSB disease. CuNPs were found inimical against beneficial fungi and bacteria. However, a positive effect was observed on soil enzyme activities namely dehydrogenase, urease, and alkaline phosphatase and maize seedling characters *viz*., shoot length, root length, number of roots per seedlings, fresh and dry weight.

Introduction

Maize (*Zea mays* L.), popularly known as the queen of cereals, is an important Kharif crop with astonishingly high genetic potential, cultivated in different agro-climatic conditions covering approximately 150 mha in about 160 countries (Parihar et al., 2011). The United States, China, and Brazil are the top three maize-producing countries approximately accounting for 563 of the 717 million metric tons/year (Ranum et al., 2014). Maize is regarded for its nutritive quality, containing approximately 72% starch, 10% protein, and 4% fat, providing an energy density of 365 Kcal/100 g. In addition, maize can be processed into various edible and industrial products such as starch, oil, alcohol, beverages, fuel, and ethanol (Ranum et al., 2014). Therefore, maize has a propitious industrial significance. However, plant pathogens pose a major constraint in its production, and a quest to overcome the problem has been endeavored for decades. With the constant tireless effort, many effective chemicals (fungicides, antibiotics, etc.) have been developed and introduced which can effectively manage various diseases rendered by phytopathogens. However, such conventional methods to manage phytopathogens have affected both environment and the farmer's economy due to its high toxicity and the liability of the applied fungicides getting wasted due to wind or surface runoff. It has been estimated that approx. 5.6 billion pounds of pesticides are used worldwide (Alavanja, 2009) and globally $38 billion is spent on pesticides each year (Pan-Germany, 2012). Its inimical effect on target organisms and the environment can be attributed to its high toxicity, non-biodegradable nature, and long residual activity (Aktar et al., 2009). Apparently, 80–90% of applied fungicides are wasted in the environment (Stephenson et al., 2003; Ghormade et al., 2011). Although the introduction of systemic fungicides took a major turn in the management of diseases, unfortunately, it has led to the development of resistance against fungicides because of their specific site of action. Therefore, the development of alternative antifungal agents like nano fungicides is of utmost need. Recently, nanoscience has emerged as an exciting yet unfathomed field having significant utility and application in agriculture as well. Nanotechnology can make disease management sustainable and eco-friendly by reducing toxicity and increasing the efficacy and shelf life of an antifungal agent. Concerning plant protection, the nanoparticles (Usually in the range of 10–100 nm) alone can be used directly as antifungal agents (Bramhanwade et al., 2016; Zain et al., 2016; Viet et al., 2016; Kanhed et al., 2014; Mondal et al., 2009) or as a nanocarrier of fungicides by entrapping or encapsulating which would result in controlled release of active ingredients increasing the efficacy (Mody et al., 2014). Metallic nanoparticles such as copper, silver, ferrous, titanium dioxide, zinc oxide, gold nanoparticles, etc. are widely being used as promising protectants. CuNPs can be exploited astoundingly to manage plant pathogens due to their remarkable antimicrobial properties. Moreover, nanoparticles are proven to show a synergistic effect when combined with biocontrol agents, generally recognized as safe substances (GRASS), biopolymers or essential oil, etc. (Beyki et al., 2014). Employing such a strategy would hold down pesticide usage, by its high efficacy and durability and also would delay the development of fungal resistance. Nanoparticles possess the potential to prevent the development of multidrug resistance in bacteria by hindering quorum sensing, preventing bacterial efflux pumps activity, biofilm formation, etc. (Baptista et al., 2018), and the development of fungicide resistance in fungi due to its multiple sites of action. However, there are certain limitations of nanoparticles generally associated with the synthesis which involves the use and generation of toxic chemicals (Casagrande et al., 2019). Moreover, nanoparticles bearing positive charges may result in cytotoxicity (Kutawa et al., 2021).

Copper was known to man since time immemorial as an essential element. Copper has drawn attention for its antimicrobial properties and various other advantages in several fields. Taking into account the myriad advantages of the copper compound, currently, they are of special interest to many for the synthesis of CuNPs. Several methods have been adopted for the synthesis of CuNPs such as chemical, physical, and biological methods. Chemical methods involve reducing agents, for instance, CuNPs synthesis by sodium hypophosphite as a reducing agent in ethylene glycol under microwave irradiation (Zhu et al., 2005), by ascorbic acid in the presence of chitosan using microwave heating (Zain et al., 2014), polyol method (Park et al., 2007), use of hydrazine and chitosan as reducing agent and stabilizer (Usman et al., 2013), etc.

In the present investigation, CuNPs synthesized by the chemical method were evaluated *in vitro and in vivo*. The CuNPs were evaluated for antimicrobial activities against five different fungi and two different bacteria. Under the net house conditions, the efficacy against two maize diseases namely MLB (Maydis leaf blight) and BLSB (Banded leaf sheath blight) was studied. The detrimental effect of synthesized CuNPs was ascertained against three different biocontrol fungi and two beneficial bacteria. An attempt was made to understand the influence of CuNPs on soil microbiome by

analyzing soil enzyme activities. Further, the CuNPs were also evaluated for their effect on maize seed germination, and other seedling characteristics to get an insight into its phytotoxic traits.

Experimental Details

Materials and synthesis

For the synthesis of CuNPs, chemicals *viz.*, copper sulphate ($CuSO_4$), sodium borohydride ($NaBH_4$), ascorbic acid ($C_6H_8O_6$), and polyethylene glycol 8000 (PEG-8000) of analytical grade were used. The CuNPs were synthesized by employing a modified standardized protocol (Kathad et al., 2014). A volume of 50 ml of $CuSO_4$ solution of 0.1M was poured in a 250 ml capacity flat bottom round flask. Ten ml of 0.01M PEG 8000 was added to it. The solution was stirred briskly using a magnetic stirrer for 30 min. After 30 min of constant stirring, 0.02 M ascorbic acids (20 ml) were added gradually to the mixture using a dropping funnel. The mixture was allowed to stir for 10 min followed by the addition of 0.01 M $NaBH_4$ (40 ml) by using a dropping funnel. The reaction mixture was then stirred for two more hours by heating at 50°C (Fig. 1).

Characterization Of Copper Nanoparticles

The synthesized CuNPs were subjected to transmission electron microscopy (TEM, Jeol 1011 100 kV, Japan) for morphological studies. The sample was prepared on a 400-mesh carbon-coated copper grid. Before placing on the carbon grid, the liquid sample was sonicated for 40 min at room temperature, and then one drop of the sample was placed on the grid using a micro-pipette. After 2–3 sec, the grid was stained with 2% uranyl acetate and the sample was allowed to dry for 1 h followed by the observation of the sample under the electron microscope. Fourier transform infrared (FTIR) spectroscopy (Nicolet 6700 FTIR System, USA) was carried out to determine the functional groups associated with the synthesized CuNPs. The sample was prepared by adding 100 mg of spectral-grade KBr which was further pressed under the pressure of 6,000 kg cm^{-2} for about 2 min which yielded a translucent KBr pellet. The pellet obtained was used for FTIR analysis. The sample's spectra were collected at a resolution and wave-number accuracy of 4 and 0.01 cm^{-1}, respectively, and in total 32 scans were made.

In vitro evaluation of the antifungal and antibacterial activity of synthesized CuNPs

Five different fungal pathogens were used in this study. Of these, four fungi *viz., Fusarium verticillioides, Rhizoctonia solani, Macrophomina phaseolina*, and *Bipolaris maydis* were procured from Maize Pathology Laboratory and one sclerotial fungus *Sclerotium rolfsii* (= *Athelia rolfsii*, Accession No. 8383) was obtained from Indian Type Culture Collection, ICAR-IARI, New Delhi. Potato dextrose agar (PDA) medium (200 g potato, 20 g dextrose, 20g agar, and 1 litre distilled water) was used to maintain the fungal cultures. A poisoned food technique (Nene and Thapaliyal, 1979) was adopted to evaluate the efficacy of synthesized CuNPs. Different concentrations of CuNPs (20 to 1000 ppm) were added to the media to ascertain their efficacy. Conventional fungicides (Carbendazim 50% WP, Hexaconazole 5% EC, Manocozeb 45% WP, Copper oxychloride) were also used as a negative control as per the recommendation. A cut-out disc (4–5 mm) of actively growing fungus mycelium (4–7 days old) was placed at the centre on the solidified PDA of previously labelled Petri plates. The plates were incubated at 28 ± 2°C in a BOD incubator. The radial growth of the test fungus was measured in all the treatments when complete growth was attained in the untreated control plates. For an antibacterial test of the synthesized CuNPs, cultures of *Erwinia carotovora* and *Ralstonia solanacearum* were obtained from the Bacteriology Laboratory of ICAR-IARI. The growth inhibitory activity of CuNPs against these bacteria was analyzed quantitatively in nutrient broth. The optical density (OD) value /CFU count (CFU ml^{-1}) of the nutrient broth amended with CuNPs with different concentrations (20 to 100 ppm) was recorded after incubation for 48 h using BioPhotometer (Mondal et al., 2010).

In vivo (Net house) evaluation of synthesized CuNPs against maize diseases causing pathogens

The CuNPs were evaluated two times from March to June and mid-July to mid-November, 2021 for their efficacy under the net house condition against two maize diseases namely maydis leaf blight (MLB) caused by *Bipolaris maydis* Shoemaker [*Cochliobolus heterostrophus* Drechs.] and banded leaf and sheath blight (BLSB) caused by *Rhizoctonia solani* f. sp. *sasakii* (Kuhn) Exner [*Thanatephorus cucumeris* Frank (Donk)] on maize varieties namely CM-500 and CM-501, susceptible for MLB and BLSB diseases, respectively obtained from Maize Pathology lab, IARI, New Delhi. Randomized block design (RBD) was followed for the experiment. The plot size for MLB disease was 9.90 m × 2 m and for BLSB, it was 11.60 m × 3 m. A total of seven treatments each with three replications were maintained. The spacing for CM-500 (MLB disease) was 60 × 15 cm (row × plant) and for CM-501 (BLSB disease), it was 60 × 20 cm (row × plant) (Fig. 1). In each row, 10 plants were accommodated. Inoculums of *B. maydis* were prepared using sorghum seeds. Half-filled conical flasks with cleaned and soaked seeds were sterilized for consecutive two days at 15 lbs for 30 min. Mycelia disc from actively growing culture plate was cut and dropped in sterilized sorghum flasks. The flask was incubated at 28°C for 8 days with intermittent shaking at two days intervals for uniform growth. Fully grown fungus on seeds was air-dried at room temperature for 5 days and then ground to powder. Fresh sorghum seed ground powder was mixed with fungus-laid seed powder in a 1:1 ratio.

Rhizoctonia solani f.sp. *sasakii* inoculums were prepared using barley seeds. Flask was quarter filled with overnight soaked seeds and autoclaved two times at 121°C (15 lbs) for 30 min for two consecutive days. Fresh mycelia discs were put mixed with sterilized barley seeds and incubated for 10 days with daily shaking of the flask (Ahuja and Payak, 1978).

MLB was inoculated in 30 days old plant (stage 5 of maize growth stages, FAO Manual, 1971) of CM-500 susceptible varieties by the whorl inoculation method of Payak and Sharma (1983). About 5 g inoculums powder was spread on the leaves of the central whorl in the evening hours. BLSB was inoculated with 15 days old barley grain culture of *R. solani* f.sp. *sasakii* on 40 days old plant of CM 501. Each plant was inoculated by inserting 3–4 barley grains in between the stalk and sheath of the second or third internodes from the soil surface. Sufficient humidity of the experimental plot was maintained using overhead water sprinklers two times a day from the next day of inoculation.

Evaluation of the efficacy of CuNPs on disease management was determined by a periodic recording of disease data. MLB disease was recorded twice at 20 (15 days after CuNPs spray) and 30 days (25 days after CuNPs spray) days after inoculation (DAI) using a 1–9 scale adopted in the All India Coordinated Maize Improvement Project (AICMIP), 2016. The scale had been modified from the rating scales of Balint- Kurti et al. (2006), and Mitiku (2014). The Percent disease index (PDI) i.e. severity was calculated by applying the formula described by McKinney (1923).

$$PDI = \frac{Sum of individual ratings}{Total No. of plant assessed \times Maximum disease rating} \times 100$$

In the case of BLSB, the length of the infected area in each inoculated plant was documented on 20 and 30 DAI. Disease severity was recorded by using a modified 1–5 rating scale based on the area affected (Payak and Sharma, 1983). The PDI was calculated by using the following formula.

$$PDI = \frac{Lesion length\,(in cm)}{Average length of internode\,(in cm)} \times 100$$

Effect Of Cunps On Beneficial Fungi And Bacteria

The inimical effect of CuNPs was ascertained against three beneficial and benign fungi namely, *Trichoderma virens*, *Paecilomyces lilacinus*, and *Chaetomium globosum*, and two bacteria namely, *Pseudomonas putida* and *Bacillus subtilis*. The poisoned food technique was adopted to evaluate its effect on fungi in the concentration range of 20 to 1000 ppm whereas the effect on beneficial bacteria was analyzed *in vitro* quantitatively in nutrient broth amended with CuNPs at 10 to 100 ppm concentrations (Mondal and Mani, 2012).

Determination Of The Effect Of Cunps On Soil Enzyme Activities

The experiment was carried out in earthen pots filled with one kg of soil collected from the maize field. Suspensions of chemically synthesized CuNPs and conventional copper oxychloride (Blitox) at 200 ppm and 400 ppm were poured into the potted field soil. Plain water was poured into the untreated control soil. The soil samples from the treated pots were collected periodically on the 0th, 15^{th,} and 30th day for the determination of activities. Each treatment was comprised of three replications. The collected soil samples were sieved and used for the analysis of the activities of three soil enzymes.

Assay of alkaline phosphatase activity

The estimation of alkaline phosphatase activity was performed by the method prescribed by Tabatabai and Bremner (1969). The chemicals acquired for the preparation of reagents were analytically pure. The reagents were prepared as per the prescription. Air-dried soil sample, 0.1 g was put in a test tube; to it, toluene (0.2 ml) was added, followed by the addition of 4 ml of modified universal buffer (pH 11) and 1 ml of 0.025 M p-nitrophenyl phosphate (PNP) solution. The test tubes were incubated at 37°C for an hour with constant shaking. After the incubation, 1 ml of $CaCl_2$ (0.5 M) and 4 ml of NaOH (0.5 M) were added to the mixture followed by filtration of soil suspension through Whatman filter paper No. 1. Simultaneously, a blank was also prepared in the same way except for the addition of the soil sample. The optical density (OD value) of the filtrate was measured by a spectrophotometer (Thermo fisher scientific, EVO 300 PC) at 440 nm wavelength. The phosphatase activity in each sample in terms of concentration of p-nitrophenyl was computed and expressed as a mole of p-nitrophenol released per gram dry soil per hour (µg PNPP/g soil/hour).

$$Concentration\,(x) = \frac{OD + 0.015}{0.005}$$

$$Activity = \frac{x \times V}{dw \times sw \times t}$$

Where, x = concentration of p-nitrophenol (µg ml^{-1} filtrate); V = total volume of soil solution;

dw = dry weight of 1g moist soil; sw = weight of soil sample; t = incubation time

Assay Of Dehydrogenase Activity

The 2-3-5-triphenyl tetrazolium chloride (TTC) reduction technique (Casida, 1977) was used to estimate the dehydrogenase activity under the influence of the CuNPs treatment. The reagents used in the experiment were analytically pure. Six grams of soil sample collected from the pots were taken in a glass screw tube of 30 ml capacity. A pinch of calcium carbonate ($CaCO_3$) was added to the soil sample. Then 1 ml of freshly prepared 3% TTC was added followed by vortexing of the soil sample. To ensure the submergence of the sample, 2 ml of distilled water was poured followed by incubation at 28–30°C for 24 h. Then 10 ml methanol was added totubes and was left undisturbed at room temperature for 30 min to allow it to change the color.

The appearance of red/orange color reveals the enzyme activity. Whatman filter paper no. 1 was used to filter the suspension. A spectrophotometer (Thermo Fisher Scientific, EVO 300 PC) was used at 485 nm using methanol as a blank to read the optical density of the filtrate. Dehydrogenase activity per gram of dry soil was expressed as milligram formazan per gram dry soil per hour (µg TPF released/g of soil/day).

$$Activity = \left(OD\frac{0.0655}{0.0106} \right) \times \frac{10}{6}$$

Where 10 = methanol added and 6 = soil taken in g

Assay of urease activity

The urease activity was determined by the method of McGarity and Myers (1967). Five grams of soil were taken in a tube and 2.5 ml of urea solution was added to it, subsequently incubating at 37°C for 2 h. Then 50 ml potassium chloride (KCl) solution was added to the sample by giving brisk stirring for 30 min. The filtrate was collected by centrifuging the samples at 10000 rpm for 10 minutes. One ml filtrate was taken in a vial and 9 ml distilled water was added to it. Then 5ml volume of sodium salicylate ($C_7H_5NaO_3$)/NaOH was added followed by the addition of 2 ml sodium dichloroisocyanurate ($C_3Cl_2N_3NaO_3$). The mixture was kept at room temperature for 30 min. Finally, the optical density value was taken at 690 nm wavelength using a spectrophotometer (Thermo Electron Corporation, 400 L/4). Concentration and activity were calculated by the following formula.

$$Concentration\,(x) = OD - \frac{0.004}{0.601}$$

$$Activity(\mu \mathrm{gNH4} - \mathrm{N/gFW/hr}) = \mathrm{ConcentrationinppmX52.5}$$

Effect of CuNPs on plant characters of maize seedlings

To determine the effect of CuNPs on seed viability, percent germination was considered as an indicator (Karimi et al., 2011). The experiment was conducted under lab conditions. A total of sixty healthy seeds of two different varieties *viz*., CM-500 and CM-501 were used for each treatment. A complete randomized design (CRD) was followed for three different treatments (*viz*., control, 100 ppm, and 300 ppm) with five replications in each. Seeds were soaked in 100 and 300 ppm solutions of CuNPs, and in distilled water (untreated control) for one night. The treated seeds were placed on the absorbent cotton pad by maintaining adequate space between them. The whole setup was incubated at 28°C for 6 days. On the 6th day, germination data were recorded and the percent germination was calculated. Further, the length of the shoot, as well as roots, were measured using a scale, and numbers of roots were counted and recorded treatment-wise. To determine the effect of CuNPs on the biomass of seedlings, fresh weight and dry weight were measured. Fresh weight (in mg) was recorded on the 6th day of whole seedlings by using a weighing microbalance (Sartorius). The fresh seedling samples were wrapped in aluminum foil, labeled treatment-wise, and oven-dried consecutively for 3 days at 50°C. After complete drying of the samples, dry weight (in mg) was recorded.

Statistical analysis

The lab experiments were conducted by adopting a complete randomized design (CRD) and the net house experiments were conducted in a randomized block design (RBD). The statistical analysis of the data (converted by angular transformation) generated in the experiments was performed by following the procedure of SAS 9.4 (SAS Institute, 2003, Cary, NC). The significant difference between the treatments' mean was determined after analysis of variance (ANOVA) (Gomez and Gomez, 1984) followed by the least significant difference test (LSD) ($P \leq 0.5$).

Results

Synthesis and characterization of copper nanoparticles

The color change indicated the formation of copper nanoparticles. The addition of ascorbic acid ($C_6H_8O_6$) led to the color change to light green confirming the size reduction. The addition of sodium borohydride ($NaBH_4$) led to a change in color from greenish-yellow to slightly yellow and transition to red which eventually turned into a brick red/brown color indicating further reduction (Fig. 2). The PEG-8000 conditioned a reaction medium and stabilized the synthesized CuNPs. The $C_6H_8O_6$ and $NaBH_4$ possibly could have reduced the Cu^{2+} to Cu^{+} and further to Cu^{0}. TEM analysis measured CuNPs of size 35–70 nm on a carbon grid that was spherical and appeared agglomerated (Fig. 3).

FTIR revealed prominent peaks at 3440, 2924, 1643, 1097, and 678.72 cm^{-1} (Fig. 4). The peaks at 3440 cm^{-1} correspond to the O-H stretching of the alcohol group. The peaks at 2924 and 1643 cm^{-1} overlap with C-H stretching, and N-H bending, respectively. The peaks at 1097 and 678.72 cm^{-1} can be assigned to the C-O stretching, and alcohol group; and the C-Cl stretching of allyl halides (Sharon et al., 2018).

In vitro fungicidal efficacy of synthesized CuNPs against maize pathogens

CuNPs rendered complete radial growth inhibition (100%) at 300 ppm against *R. solani* f.sp. *sasakii, B. maydis*, and *F. verticillioides* (Table 1, Fig. 5A, B, and C, Suppl. Figure 1A, B, and C). However, 1000 ppm of commercial fungicide; carbendazim for *R. solani* f.sp. *sasakii*, and *F. verticillioides* and

2000 ppm of Mancozeb for *B. maydis is* recommended. Concerning the concentration of CuNPs, significant inhibition was observed right from 50 ppm onwards in the case of *R. solani* f.sp. *sasakii* (34.20%), from 20 ppm in the case of *F. verticillioides* (13.34%) and *B. maydis* (39.34%). The CuNPs were also found highly effective against *M. phaseolina* in all the tested concentrations (Table 2, Fig. 5D, Suppl. Figure 1D). In the case of *S. roflsii*, significant inhibition (14.93%) was seen from 300 ppm and complete inhibition was achieved at 1000 ppm of CuNPs which was at par with commercial counterpart Hexaconazole at 1000 ppm (Table 2, Fig. 5E, Suppl. Figure 1E). It was also observed that with the increase in the concentration of CuNPs, the radial growth of all five fungal pathogens was consistently reduced.

Table 1
Efficacy of synthesized copper nanoparticles on the growth of pathogenic fungi

Rhizoctonia solani f.sp. *sasakii*			*Bipolaris maydis*			*Fusarium verticillioides*		
Treatment	**Radial growth (cm)***	**Inhibition (%)**	**Treatment**	**Radial growth (cm)***	**Inhibition (%)**	**Treatment**	**Radial growth (cm) ***	**Inhibition (%)**
Control	7.00 ± 0.0	0.00 (0.00 ± 0.0)[#] **f**	Control	7.00 ± 0.0	0.00 (0.00 ± 0.0)[#] **g**	Control	7.00 ± 0.0	0.00 (0.00 ± 0.0)[#] **g**
Car @1000 ppm	0.00 ± 0.0	100.00 (90.00 ± 0.0) **a**	Manc@1000ppm	0.00 ± 0.0	100.00 (90.00 ± 0.0) **a**	Carb @ 1000 ppm	0.00 ± 0.0	100.00 (90.00 ± 0.0) **a**
CN 20 ppm	6.93 ± 0.067	0.95 (3.24 ± ± 3.24) **f**	CN 20 ppm	4.18 ± 0.109	40.24 (39.34 ± 0.915) **f**	CN 20 ppm	6.62 ± 0.054	5.38 (13.34 ± 0.957) **f**
CN 50 ppm	5.70 ± 0.208	18.57 (25.36 ± 2.26) **e**	CN 50 ppm	3.63 ± 0.088	48.10 (43.89 ± 0.723) **e**	CN 50 ppm	6.36 ± 0.088	9.05 (17.42 ± 1.245) **e**
CN 80 ppm	4.78 ± 0.159	31.67 (34.20 ± 1.39) d	CN 80 ppm	2.66 ± 0.093	61.14 (51.87 ± 0.78) **d**	CN 80 ppm	6.16 ± 0.033	11.90 (20.17 ± 0.418) **d**
CN 100 ppm	3.18 ± 0.54	54.52 (47.61 ± 4.47) **c**	CN 100 ppm	2.30 ± 0.058	67.14 (55.01 ± 0.503) **c**	CN 100 ppm	5.33 ± 0.06	23.81 (29.18 ± 0.58) **c**
CN 200 ppm	0.86 ± 0.217	87.62 (69.68 ± 2.74) **b**	CN 200 ppm	1.60 ± 0.058	77.14 (61.42 ± 0.563) **b**	CN 200 ppm	2.15 ± 0.058	69.29 (56.32 ± 0.512) b
CN 300 ppm	0.00 ± 0.0	100.00 (90.00 ± 0.0) **a**	CN 300 ppm	0.00 ± 0.0	100.00 (90.00 ± 0.0) **a**	CN 300 ppm	0.00 ± 0.0	100.00 (90.00 ± 0.0) **a**
CN 400 ppm	0.00 ± 0.0	100.00 (90.00 ± 0.0) **a**	CN 400 ppm	0.00 ± 0.0	100.00 (90.00 ± 0.0) **a**	CN 400 ppm	0.00 ± 0.0	100.00 (90.00 ± 0.0) **a**
CN 500 ppm	0.00 ± 0.0	100.00 (90.00 ± 0.0) **a**	CN 500 ppm	0.00 ± 0.0	100.00 (90.00 ± 0.0) **a**	CN 500 ppm	0.00 ± 0.0	100.00 (90.00 ± 0.0) **a**

***Data are the mean of three replications. Data (Mean ± Standard errors) followed by different letters in each column indicate a significant difference (ANOVA, LSD, P ≤ 0.01). [#]Data within parentheses are Angular transformed value ± Standard errors. CN: Copper nanoparticles, Manc: Mancozeb 75%WP, Carb: Carbendazim 50% WP.**

Table 2
Efficacy of synthesized copper nanoparticles on the growth of pathogenic fungi

Macrophomina phaseolina			*Sclerotium rolfsii*		
Treatment	**Radial growth (cm)***	**Inhibition (%)**	**Treatment**	**Radial growth (cm)***	**Inhibition (%)**
Control	7.00 + 0.0	0.00 (0.00 ± 0.0)[#] **e**	Control	7.00 ± 0.0	0.00 (0.00 ± 0.0)[#] **i**
Carb @1000 ppm	0.00 ± 0.0	100.00 (90.00 ± 0.0) **a**	Hex @1000 ppm	0.00 ± 0.0	100.00 (90.00 ± 0.0) **a**
CN 20 ppm	6.50 ± 0.115	7.14 (15.29 ± 1.88) **d**	CN 20 ppm	7.00 ± 0.0	0.00 (0.00 ± 0) **i**
CN 50 ppm	3.053 ± 0.029	56.38(48.65 ± 0.24) **c**	CN 50 ppm	7.00 ± 0.0	0.00 (0.00 ± 0) **i**
CN 100 ppm	1.553 ± 0.023	77.81 (61.87 ± 0.23) **b**	CN 100 ppm	6.90 ± 0.058	1.43 (5.53 ± 2.886) **hi**
CN 200 ppm	0.00 ± 0.0	100.00 (90.00 ± 0.0) **a**	CN 200 ppm	6.76 ± 0.12	3.33 (8.58 ± 4.329) **h**
CN 300 ppm	0.00 ± 0.0	100.00 (90.00 ± 0.0) **a**	CN 300 ppm	6.53 ± 0.033	6.67 (14.93 ± 0.557) **g**
CN 400 ppm	0.00 ± 0.0	100.00 (90.00 ± 0.0) **a**	CN 400 ppm	6.47 ± 0.145	7.62 (15.73 ± 2.257) **g**
CN 500 ppm	0.00 ± 0.0	100.00 (90.00 ± 0.0) **a**	CN 500 ppm	6.23 ± 0.176	10.95 (19.08 ± 2.29) **f**
			CN 600 ppm	5.76 ± 0.033	17.62 (24.80 ± 0.356) **e**
			CN 700 ppm	5.46 ± 0.033	21.90 (27.89 ± 0.328) **d**
			CN 800 ppm	4.26 ± 0.067	39.05 (38.65 ± 0.56) **c**
			CN 900 ppm	3.83 ± 0.088	45.24 (42.24 ± 0.726) **b**
			CN 1000 ppm	0 ± 0.0	100.00 (90.00 ± 0.0) **a**

***Data are the mean of three replications. Data (Mean ± Standard errors) followed by different letters in each column indicate a significant difference (ANOVA, LSD, P ≤ 0.01). [#]Data within parentheses are Angular transformed value ± Standard errors. CN: Copper nanoparticles, Manc: Mancozeb 75%WP, Carb: Carbendazim 50% WP, Hex: Hexaconazole 5% SC.**

In vitro **bactericidal efficacy of synthesized CuNPs**

Two phytopathogenic bacteria *namely Ralstonia solanacearum and Erwinia carotovora* were taken into account under the present investigation. Among the CuNPs treatments, the highest OD (3.20 and 2.18) and CFU (3.20×10^9 and 2.18×10^9) values were found at 10 ppm while the least OD (1.16 and 0.54) and CFU (1.16×10^9 and 0.54×10^9) at 100 ppm for *E. carotovora* and *R. solanacearum*, respectively (Suppl. Table 1). In the case of *E. carotovora*, a significant reduction in bacterial growth was recorded from 30 ppm (OD 2.54) onwards as compared to the positive control (OD 3.05) (Fig. 6A and B). Similarly, the growth of *R. solanacearum* significantly reduced from the 30 ppm CuNPs (OD 1.98) onwards as compared to the control OD (2.34). The CuNPs treatments in the series, immediate to each other, did not reflect any significant difference (Fig. 6C and D). However, 100 ppm of CuNPs was perceived at par with the antibiotic streptomycin sulphate at 200 ppm in the case of *E. carotovora*. In the case of *R. solanacearum*, although 100 ppm of CuNPs was evidenced to be at par with 200 ppm of streptomycin sulphate in restricting bacterial growth, it was manifold better than the copper oxychloride (Blitox) at 1000 ppm.

Efficacy evaluation of CuNPs in vivo **for maize disease management**

The incidence of MLB disease was significantly less in treatments as compared to negative (inoculated) control in both seasons (Table 3). Among the treatments, the least incidence (20.37%) was recorded in T6 (Spray + ST@ 300 ppm), followed by T2 (24.07%), T5 (23.45%), and T4 (23.15%), however, no significant difference between the treatments *viz.*, T2, T4, T5, and T6 was observed. A maximum of 55.25% disease incidence was recorded in the negative control where no chemicals were applied, whereas the fungicide Mancozeb (2000 ppm) significantly restricted MLB incidence to 37.22%. Furthermore, significantly less disease incidence was evidenced when CuNPs were used as seed treatment and foliar spray at 300 ppm or seed treatment + spray at 100 and 300 ppm as compared to 2000 ppm of Mancozeb (Fig. 7A). The overall MLB incidence recorded on 1st scoring at 20 DAI showed a slight increase after 10 days (2nd scoring) in treatments in both seasons.

Table 3
In-vivo efficacy of synthesized copper nanoparticles (CuNPs) against maydis leaf blight of maize.

Treatment	MLB on maize variety CM 500						
	1st Season		2nd Season		Pool		
	Score* (1–9 scale)	PDI (%)	Score* (1–9 scale)	PDI* (%)	Score* (1–9 scale)	PDI* (%)	Inhibition %
T1: ST@100 ppm	2.83 ± 0.09	31.48 (34.11 ± 1.49) # **bc**	2.22 ± 0.06	24.69 (29.78 ± 0.41) **cd**	2.53	28.09 (31.95)	49.16
T2: ST @ 300 ppm	2.33 ± 0.34	25.92 (30.48 ± 2.49) **cd**	2.00 ± 0.17	22.22 (28.08 ± 1.26) **d**	2.17	24.07 (29.27)	56.43
T3: Spray @ 100 ppm	2.88 ± 0.48	32.09 (34.33 ± 3.36) **bc**	2.56 ± 0.24	28.39 (32.14 ± 1.71) **c**	2.72	30.24 (33.23)	45.27
T4: Spray @ 300 ppm	2.11 ± 0.20	23.48 (28.90 ± 0.65) **d**	2.06 ± 0.14	22.83 (28.51 ± 1.11) **d**	2.08	23.15 (28.70)	55.09
T5: ST + Spray@ 100 ppm	2.11 ± 0.11	23.45 (28.94 ± 0.83) **d**	2.11 ± 0.11	23.45 (28.94 ± 0.83) **d**	2.11	23.45 (28.94)	57.55
T6: Spray + ST@ 300 ppm	1.88 ± 0.11	20.98 (27.23 ± 0.88) **d**	1.78 ± 0.22	19.75 (26.29 ± 1.83) **d**	1.83	20.37 (26.76)	63.13
T7: Control	0.50 ± 0.33	5.56 (12.24 ± 4.43) **e**	0.44 ± 0.06	4.93 (12.78 ± 0.85) **e**	0.47	5.25 (12.51)	90.49
T8: Fungicide	3.33 ± 0.00	35.55 (36.37 ± 0.65) **b**	3.50 ± 0.19	38.89 (38.55 ± 1.26) **b**	3.42	37.22 (37.46)	32.63
T9: Negative Control	3.16 ± 0.00	55.56 (48.17 ± 0.62) **a**	4.94 ± 0.20	54.94 (47.82 ± 1.28) **a**	4.05	55.25 (47.99)	-

***Data are the mean of three replications. Data (Mean ± Standard errors) followed by different letters in each column indicate a significant difference (ANOVA, LSD, P ≤ 0.05). Disease data scored twice on 20 and 30 DAI, Seasons: 1st (March to June 2021) and 2nd (mid-July to mid-November 2021). T: Treatment, PDI: Percentage Disease index, Spray: Spray with CuNPs, ST: Seed treatment with CuNPs, Fungicide: Mancozeb 75%WP @2000ppm (for MLB) and Carbendazim 50% WP @1000 ppm (for BLSB). # Data within the parentheses are Angular transformed values.**

In the case of BLSB disease (Table 4), treatments except for T3 (spray @100 ppm) exhibited significantly less disease incidence as compared to the negative control (71.05%) (Fig. 7B). Among the different treatments of CuNPs under study, T6 (Spray + ST @ 300 ppm) performed best with the least PDI of 46.81% which was at par with Carbendazim spray (39.55%). Comparatively, seed treatments with CuNPs at the rate of 100 ppm (57.92%) and 300 ppm (55.33%) performed better than the spray with CuNPs at the rate of 100 ppm (67.05%) and 300 ppm (60.39%). Spraying with the lower dose of CuNPs (100 ppm) resulted in higher disease incidence, statistically at par with the negative control. In all the treatments, BLSB incidence was found increasing as evidenced by two times diseases scoring 20 and 30 DAI.

Table 4
In-vivo efficacy of synthesized copper nanoparticles (CuNPs) against banded leaf & sheath blight disease of maize *in vivo*

Treatment	BLSB on maize variety CM 501						
	1st Season		2nd Season		Pool		
	Lesion length (cm) *	PDI* (%)	Lesion length (cm) *	PDI* (%)	Lesion length (cm) *	PDI (%)	Inhibition %
T1: ST @100 ppm	11.99 ± 1.09	57.72 (49.44 ± 2.21) **bc**	11.62 ± 1.32	58.11 (49.70 ± 3.80) **c**	11.80	57.92 (49.57)	18.49
T2: ST @ 300 ppm	11.08 ± 0.52	55.39 (48.08 ± 1.49) **cd**	11.06 ± 0.19	55.28 (48.02 ± 0.55) **c**	11.07	55.33 (48.05)	22.12
T3: Spray @ 100 ppm	13.38 ± 0.84	66.89(54.92 ± 2.53) **ab**	13.44 ± 0.06	67.22 (55.06 ± 0.18) **ab**	13.41	67.05 (54.99)	5.63
T4: Spray @ 300 ppm	12.78 + 2.15	60.00 (50.89 ± 4.15) **bc**	12.16 ± 0.59	60.78 (51.22 ± 1.73) **bc**	12.47	60.39 (51.06)	15.01
T5: ST + Spray @ 100 ppm	10.77 ± 1.35	53.89 (47.29 ± 3.93) **cd**	10.83 ± 0.07	54.17 (47.37 ± 0.19) **cd**	10.8	54.03 (47.3)	23.96
T6: Spray + ST @ 300 ppm	9.41 ± 0.25	47.06 (43.29 ± 0.70) **de**	9.31 ± 0.54	46.55 (42.99 ± 1.56) **de**	9.36	46.81 (43.14)	34.13
T7: Control	0.00 ± 0.00	0.00 (0.00 ± 0.00) **f**	0.00 ± 0.00	0.00 (0.00) **f**	0.00	0.00 (0.00)	100
T8: Fungicide[@]	7.87 ± 0.14	39.39 (38.86 ± 0.40) **e**	7.94 ± 0.62	39.72(39.04 ± 1.80) **e**	7.91	39.55 (38.95)	44.33
T9: Negative Control	14.20 ± 0.89	71.00 (57.53 ± 2.857) **a**	14.22 ± 0.09	71.11 (57.46 ± 0.29) **a**	14.21	71.05 (57.49)	0

***Data are the mean of three replications. Data (Mean ± Standard errors) followed by different letters in each column indicate a significant difference (ANOVA, LSD, P ≤ 0.05). Disease data scored twice on 20 and 30 DAI, Seasons: 1st (March to June 2021) and 2nd (mid-July to mid-November 2021). PDI: Percentage Disease index, Spray: Spray with CuNPs, ST: Seed treatment with CuNPs. [@]Fungicide: Mancozeb 75%WP @2000ppm (for MLB) and Carbendazim 50% WP @1000 ppm (for BLSB). # Data within the parentheses are Angular transformed values.**

Determination of the inimical effect of CuNPs against beneficial fungi

The growth of *Trichoderma virens* was significantly inhibited by the CuNPs from the concentration of 80 ppm onwards as compared to the positive control, and complete inhibition (100%) was observed at 300 ppm which confirmed the inimical effect of CuNPs on *T. virens* (Fig. 8A and B, Supp. Table 2). In the case of *Chaetomium globosum*, significant inhibition in the growth was noticed right from 20 ppm (24.24%) (Fig. 8C and D, Supp. Table 2). With the increase in the concentration of CuNPs, radial growth of *C. globosum* was reduced and thus 100% inhibition was observed at 300 ppm. In the case of *Paecilomyces lilacinus*, a reduction in the radial growth was observed from 100 ppm of CuNPs (Fig. 8E and F, Suppl. Table 2). As compared to *T. virens* and *C. globosum*, *P. lilacinus* appeared to be slightly more tolerant since complete inhibition (100%) of growth was observed at higher concentrations (600 ppm) of CuNPs.

Determination of the inimical effect of CuNPs against beneficial bacteria

CuNPs against *Bacillus subtilis* exhibited a significant reduction in OD value from 20 ppm (0.52) onwards indicating a reduction in the growing number of the cell as compared to the control (0.98) (Table 5). The concentrations of 10 and 20 ppm turned out to be statistically significant but beyond 20 ppm, when two immediate treatments were compared, no significant reduction in OD or growth of bacteria was observed. The effect of CuNPs at 50 ppm and 90 ppm was at par with the Blitox (1000 ppm) and Streptomycin sulphate (200 ppm), respectively (Fig. 9A, Suppl. Figure 2A). As compared to *B. subtilis*, the OD value for *Pseudomonas putida* (Table 5, Fig. 9B, Suppl. Figure 2B) was higher and a significant reduction in growth was observed from 30 ppm onwards. The 100 ppm concentration of CuNPs was more harmful to both the beneficial bacteria than the Streptomycin sulphate @200 ppm and blitox @1000 ppm.

Table 5
Effect of synthesized CuNPs on the growth of beneficial bacteria

Treatment	*Bacillus subtilis*		*Pseudomonas putida*	
	Optical density* at 600 nm	CFU/ml (1OD = 1× 10^9)	Optical density at 600 nm	CFU/ml (1OD = 1× 10^9)
Control	0.98 ± 0.009 **a**	0.98 × 10^9	2.09 ± 0.016 **a**	2.089 × 10^9
Streptomycin @200 ppm	0.08 ± 0.001 **f**	0.08 × 10^9	0.33 ± 0.242 **g**	0.335 × 10^9
Blitox @ 1000 ppm	0.41 ± 0.180 **bc**	0.41 × 10^9	0.27 ± 0.006 **gh**	0.27 × 10^9
CN 10 ppm	0.87 ± 0.024 **a**	0.87× 10^9	1.03 ± 0.040 **cd**	1.03 × 10^9
CN 20 ppm	0.52 ± 0.002 **b**	0.51 × 10^9	1.92 ± 0.013 **a**	1.92 × 10^9
CN 30 ppm	0.50 ± 0.010 **b**	0.49 × 10^9	1.54 ± 0.010 **b**	1.54 × 10^9
CN 40 ppm	0.44 ± 0.010 **bc**	0.44 × 10^9	1.49 ± 0.021 **b**	1.49 × 10^9
CN 50 ppm	0.42 ± 0.015 **bc**	0.41 × 10^9	1.17 ± 0.044 **c**	1.71 × 10^9
CN 60 ppm	0.33 ± 0.025 **cd**	0.33 × 10^9	0.99 ± 0.029 cde	0.99× 10^9
CN 70 ppm	0.24 ± 0.022 **de**	0.24 × 10^9	0.80 ± 0.014 **ef**	0.80× 10^9
CN 80 ppm	0.09 ± 0.004 **ef**	0.09 × 10^9	0.86 ± 0.043 **def**	0.86 × 10^9
CN 90 ppm	0.08 ± 0.001 **f**	0.85 × 10^9	0.74 ± 0.011 **f**	0.74 × 10^9
CN 100 ppm	0.07 ± 0.005 **f**	0.07 × 10^9	0.08 ± 0.005 **h**	0.08× 10^9

***Data are the mean of three replications. Data (Means ± Standard errors) followed by different letters in each column indicate a significant difference (ANOVA, LSD, P ≤ 0.01). CN: Copper nanoparticles, OD: Optical Density. Blitox: Copper oxychloride, Streptomycin: Streptomycin sulphate**

Determination of the effect of CuNPs on soil enzyme activities

The soil dehydrogenase activity (µg triphenyl formazone released per gram soil per hour) of soil treated with CuNPs and copper oxychloride (COC) at two different concentrations *viz*., 200 and 400 ppm exhibited a significant difference on the first day when compared with the respective control (47.41 and 47.56 µg TPF/g soil/hr). The activity at 200 ppm for CuNPs and COC treated soil was 53.43 and 51.23 µg TPF/g soil/hr, respectively, and activities at 400 ppm were 61.77 and 54.27 µg TPF/g soil/hr, respectively (Fig. 10A and B, Suppl. Table 3). A significant difference in the effect was found when the concentrations of 400 ppm of two different compounds were compared. However, on the 15th day, an abrupt increase in dehydrogenase activity was observed in the case of soil treated with CuNPs at 200 ppm (70.89 µg TPF/g soil/hr) and 400 ppm (64.40 µg TPF/g soil/hr). The activity of soil treated with 200 ppm and 400 ppm of CuNPs on the 15th day was significantly higher than the activity on the 1st day. The COC-treated soil with 200 and 400 ppm remained at par with its mean activity value of 52.86 and 50.50 µg TPF/g soil/hr on the 15th day. Records of the 30th day revealed a fall of dehydrogenase activity in soil treated with CuNPs of 200 ppm (61.92 µg TPF/g soil/hr) and 400 ppm (61.61 µg TPF/g soil/hr). A decline in enzyme activity on the 30th day was apparent in the case of COC as well. The overall result demonstrates higher soil dehydrogenase activity in the chemically treated soil as compared to the untreated control, and CuNPs rendered the enhancement of dehydrogenase activity more than COC.

The alkaline phosphatase activity was found high in both COC and CuNPs treated soil as compared to control (Fig. 10C and D, Suppl. Table 4). Among the two chemicals, alkaline phosphatase activity in the soil treated with CuNPs was higher than the activity estimated for COC. In the case of COC at 200 ppm, the alkaline phosphatase activity was decreased from 466.80 to 445.19 µg pNPP/g soil/hr on the 15th day which was elevated on the 30th day (490.28 µg pNPP/g soil/hr). At a higher concentration of 400 ppm (COC), the enzyme activity increased from the base level of 497.79 (1st day) to 540.06 (15th day), and eventually decreased to 472.43 µg pNPP/g soil/hr (30th day). In the case of CuNPs at 200 and 400 ppm, a significant sharp increase of alkaline phosphatase activity was seen on the 15th day (784.25 and 832.15 µg pNPP/g soil/hr) respectively, however, the activity declined on the 30th day (772.04 and 713.81 µg pNPP/g soil/hr) respectively. As compared to COC, CuNPs treatment resulted in significantly higher activity.

The activity of urease in terms of µg NH_4-N/ g/soil/hr was found to be significantly high on the 15th day (3.931 µg NH_4-N/ g/soil/hr) in soil treated with CuNPs (400 ppm) which further reduced on the 30th day (3.669 µg NH_4-N/ g/soil/hr) (Fig. 10E and F, Suppl. Table 5). The difference among the treatments remained insignificant in the case of COC treatments as well as in CuNPs treatment except for CuNPs treatment at 400 ppm. A comparison of the CuNPs and COC treatments did not show any significant difference in the urease activity.

The changes in enzyme activities observed in this study demonstrated that the soil microbial communities and their activities are influenced by metallic nanoparticles or possibly certain genes responsible for specific enzymatic functions are influenced.

Effect of CuNPs on plant characters of maize seedlings

Two maize varieties *viz.*, CM-500 and CM-501 were evaluated after CuNPs treatment at 100 ppm and 300 ppm concentrations. Both varieties exhibited 100% germination. In the case of CM-500 (Fig. 11A) all the characters under consideration i.e. number of roots per seedlings, shoot length, root length, fresh weight, and dry weight exhibited a steady increase, which was statistically significant with the increase in the concentration of CuNPs upto 300ppm except for the dry weight of seedlings (Table 6). Similarly, an increase in all the seedling's characters was perceived in CM 501 (Fig. 11B).

Table 6
Effect of copper nanoparticles on different characters of maize seedling

Treatment	Variety CM-500*						Variety CM-501*					
	Seed germination (%)*	Root length (cm)	No. of roots	Shoot length (cm)	Fresh weight of seedling (mg)	Dry weight of seedlings (mg)	Seed germination (%)*	Root length (cm)	No. of roots	Shoot length (cm)	Fresh weight of seedling (mg)	Dry weight of seedlings (mg)
Control	100	2.87 ± 0.58 **c**	2.30 ± 0.20 c	2.59 ± 0.43 **c**	127.12 ± 22.08 **c**	16.56 ± 2.98 **a**	100	3.60 ± 0.72 **c**	2.93 ± 0.57 **b**	2.01 ± 0.46 **c**	144.72 ± 34.82 **c**	22.03 ± 4.99 **c**
CN 100 ppm	100	4.12 ± 0.72 **b**	2.95 ± 0.38 **b**	3.10 ± 0.62 **b**	155.57 ± 16.04 **b**	21.73 ± 2.69 **a**	100	4.89 ± 0.85 **b**	3.67 ± 0.42 **a**	2.57 ± 0.22 **b**	176.25 ± 26.58 **b**	26.10 ± 2.32 **b**
CN 300 ppm	100	6.11 ± 0.39 **a**	4.37 ± 0.24 **a**	5.52 ± 0.49 **a**	265.55 ± 31.39 **a**	30.13 ± 7.58 **a**	100	5.22 ± 0.41 **a**	3.52 ± 0.22 **a**	3.89 ± 0.41 **a**	229.92 ± 23.49 **a**	31.42 ± 1.29 **a**

*** Total of 60 seeds were taken for the seed germination test. *Data are the mean of five replications. (Data) Mean ± Standard errors) followed by different letters indicates a significant difference and with the same letter are insignificant from each other (ANOVA, LSD, P ≤ 0.01).**

Discussion

The chemical method of nanoparticle synthesis is considered a traditional method and is widely being used. Such method involves the use of certain chemical reagents as a reducing agent such as sodium borohydride ($NaBH_4$), potassium bitartrate ($KC_4H_5O_6$), sodium hypophosphite ($NaPO_2H_2$) (Zhu et al., 2004), hydrazine (N_2H_4) (Usman et al., 2013), glucose (C H O) (Suvarna et al., 2017), ascorbic acid ($C_6H_8O_6$) (Zain et al., 2014), diethylene glycol (Park et al., 2007). In addition, a stabilizing agent is used which prevents the agglomeration of synthesized nanoparticles by capping the nanoparticles. There are two important approaches to synthesis *viz.*, down strategy and bottom-up strategy. In the former strategy, the precursor is converted to particles in the nano-size range whereas, in the latter approach, nanoparticles are synthesized by joining atom by atom (Zielonka et al., 2017). In the present investigation $NaBH_4$ and the ascorbic acid act as a reducing agent, reducing the size of Cu precursor to CuNPs whereas PEG 8000 conditions a good reaction medium and stabilizes the formed CuNPs by capping those (Shameli et al., 2012). Possibly, Cu^{2+} under the influence of the reducing agent first gets converted to Cu^{+} and then to Cu^{0} resulting in the reduction of particle size. A similar result was obtained by Kaur et al. (2014) and Soomro et al. (2014) using $NaBH_4$ as a reducing agent which generated nanoparticles in size range from 40 to 80 nm and of 15× 14 nm size, respectively. The synthesis is influenced by factors such as concentrations of reactants, pH of the reaction medium, temperature, etc. Relation between the size of the nanoparticles and the concentration was shown by Zain et al. (2014) where they found that with the increase in the concentration of copper nitrate and silver nitrate, the size of the respective nanoparticles increased proportionately. The effect of temperature on the synthesis was reported by Rahimi et al. (2010). The temperature at 50°C with reducing agent to the precursor ratio (R/P) 2 and 8 and at 60°C with R/P 2 did not result in the formation. However, the temperature at 60°C with R/P 4 and 6 and temperature at 75°C with R/P as 4 led to the synthesis of copper nanoparticles without any precipitation. Although 85°C with R/P 4 could also generate nanoparticles but resulted in precipitation. Traiwatcharanon et al. (2015) studied the effect of pH on particle size. They observed the surface plasmon resonance peak to be at > 330 nm for acidic medium (pH 4 and 6 and) and at > 420 nm for basic medium (pH 8 and 10 nm) which implies that the AgNPs size was smaller in the acidic medium. It was shown that the concentration of acid to base ratio would influence the size of nanoparticles by influencing the formation of nuclei (Ahmed et al., 2012).

Change in color/optical properties is the first and foremost indication of nanoparticle synthesis. In our study, with the reaction time and addition of reactants color changed from light yellow, dark yellow to red and eventually to the dark brick brown-red color. A similar observation was reported by Umer et al. (2014), Rahimi et al. (2010), Khalid et al. (2015), and Jain et al. (2015) where they perceived the final colour of the reaction mixture to be dark brown in color.

Copper was known to possess antimicrobial properties since the 17th century and hence the water was being stored in utensils made of copper. Moreover, Cu is an important micronutrient to plants. Taking into account the importance, copper was exploited in the present investigation. In the present study, five important maize fungal pathogens were tested. As less as 200 ppm of CuNPs rendered 100% inhibition in *M. phaseolina* and 300 ppm exhibited complete control of *F. verticillioides, R. solani, f.* sp. *Sasakii*, and *B. maydis*. However, to attain complete inhibition of *S. rolfsii* a concentration of 1000 ppm was required, although significant inhibition was apparent from 80 ppm. Pertaining to the findings of the present investigation, similar effectiveness of CuNPs was reported by Viet et al. (2016) at 450 ppm against *Fusarium* sp., Bramhanwade et al. (2016) against *F. oxysporum* and *F. equiseti*, and Kanhed et al. (2014) against *Phoma destructiva*, *C. lunata*, *A. alternata*, and *F. oxysporum*, Banik et al. (2017) against *P. syrinagae, Phytophthora cinnamon, A. alternata* at 200, 100, 800 ppm, respectively. The aforesaid findings perceived the effectiveness of CuNPs better than the commercial fungicides. In agreement with previous reports, the present investigation confirms the better effectiveness of CuNPs over commercial fungicides (Mancozeb, Carbendazim, Copper oxychloride, and Hexaconazole) used, exhibiting significant inhibition of the fungal pathogens at the concentration of CuNPs as low as 20 ppm. The enhanced fungicidal activity of CuNPs is due to their reduced size or high surface area to volume ratio. Moreover, its ability to disrupt enzymes by binding to sulfydryl amino and carboxyl groups of amino acids and by virtue of their small size, CuNPs even disrupt the DNA helix of the microbes (Shobha et al., 2014). Furthermore, CuNPs are also found to be affecting membrane integrity and membrane lipids (Santo et al., 2008). In our study, we have demonstrated the effectiveness of CuNPs against two bacteria namely, *Ralstonia solanacearum* and *Erwinia carotovora* at a concentration of 30 and 20 ppm, respectively. The effectiveness against bacteria could be attributed to the ability of CuNPs to cross the bacterial cell wall, thereafter affecting the shape and functions of the cell membrane. It also affects bacterial DNA and enzymes, creates oxidative stress, and alters gene expression (Slavin et al., 2017). In the current study, CuNPs were evaluated against *Erwinia carotovora* and *Ralstonia solanacearum*, and they exhibited a significant reduction in growth, observed at 20 and 30 ppm, respectively. The bactericidal effect of CuNPs has been reported by Mondal and Mani (2009, 2012) and Mondal et al. (2010) against *X. axonopodis* pv. *phaseoli, X. oryzae* pv. *oryzae* and *X. axonopodis* pv. *punicae*, respectively at very low concentration (0.2 ppm) of CuNPs. Usman *et al.* (2013) also reported growth inhibition of several bacterial species (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella choleraesuis*, *Bacillus subtilis*, and *Candida albicans*) at 30 ppm which supports the findings of the present study. A more precise mechanism of CuNPs against *R. solanacearum* had been elucidated by You *et al.* (2018) where they found bacterial cytomembrane was highly damaged due to absorption of CuNPs; moreover, several genes related to pathogenesis were down-regulated. However, counterproductive results against the beneficial microbes namely *T. virens, C. globosum, B. subtilis*, and *P. putida* were obtained. A similar result was reported by Ruparelia et al. (2008) against *B. subtilis* strain MTCC 441 growth inhibition at 20 µg/ml (20 ppm). The findings of the present investigation support the earlier report of Banik et al. (2017), in which they observed an effective concentration of CuNPs at 200 mg/ml against *Pseudomonas syringae*.

To ascertain the reliability of *in-vitro* results, experiments in *in-vivo* (Net house) conditions are imperative. The efficacy evaluation *in-vivo* was carried out twice to confirm the reliability of synthesized CuNPs. The severity of two diseases under study *viz.*, MLB and BLSB with the treatment of CuNPs at 300 ppm (spray + seed treatment) were significantly reduced as compared to the treatment with respective commercial fungicides. Typical symptoms of both MLB and BLSB diseases started appearing at 3–4 DAI. The substantial decrease in PDI of both MLB and BLSB diseases could be due to the direct effect of CuNPs on the fungal pathogens as an antifungal agent, distressing the pathogen's physiology by various mechanisms. Another possible reason could be the activation of defense genes/mechanisms in maize plants after exposure to CuNPs. The results achieved in the present investigation are more or less in agreement with Chaudhary et al. (2017), who observed the maize plants treated with Cu-chitosan NPs suffered from less disease, due to induction in defense response through higher antioxidants such as peroxidase and superoxide dismutase and activation of defense genes polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) against *Curvularia lunata*, the incitant of Curvularia leaf spot (CLS) disease. A similar observation was recorded by Zhao et al. (2017), in which they observed an increase in phenolic compounds when maize plants were treated with $Cu(OH)_2$ nano-pesticide. The CuNPs in combination with chitosan-polyvinyl alcohol hydrogels (Cs-PVA) resulted in increased expression of defense genes in tomato plants under salt stress (Hernández et al., 2018) and reactive oxygen species (ROS) as well as peroxidase activity in finger millet against *Pyricularia grisea* (Sathiyabama and Manikandan, 2016).

Soil enzyme activities provide an idea about soil fertility and productivity (Tiwari et al., 1989). It is also a measure of microbial biomass and microbial activity (Klose and Tabatabai, 1999) and hence it is a direct indicator of soil quality (Pascual et al., 2000). Owing to their definite significance in organic matter transformation and phosphorous cycle, three different enzymes were targeted; dehydrogenase, alkaline phosphatase, and urease. Dehydrogenase has an important role in transferring hydrogen or electron from substrate to acceptor during the initial stages of oxidations; hence, considered an adequate tool to assess microbial oxidative activity (Ross, 1971). Phosphatase activity is vital for the release of phosphorous from organically bound phosphorous (Nannipieri et al., 2011). Hydrolytic conversion of urea into CO_2 and NH_4 is carried out by the urease enzyme, hence acting as a regulator of nitrogen economy in soil (Swensan and Bakken, 1998). The decrease in the activity of two enzymes on the 30th day is possibly due to the exhaustion of nutrients in the soil. A similar trend in change in activities was also reported by Gopal et al. (2012), where they noted the shoot-up in the activities of dehydrogenase, alkaline phosphatase, and acidic phosphatase on the 30th day when treated with nano hexaconazole, but a gradual fall in activity was observed which reached to a minimum on 60th day. You et al. (2018) reported adverse effects of four metal oxide nanoparticles, i.e., zinc oxide (ZnO NPs), titanium dioxide (TiO_2 NPs), cerium dioxide (CeO_2 NPs), and magnetite (Fe_3O_4 NPs) on the soil enzyme activities *viz*., invertase, urease, catalase, and phosphatase. McGee et al. (2017) also evaluated the effect of AgNPs, SiO_2NPs, and Al_2ONPs on soil enzyme activities of dehydrogenase and urease and observed a decrease in the activities. Contradicting earlier reports, the present study confirms no adverse effect of CuNPs on soil enzyme activities. However, better insight can be achieved about the effect of CuNPs on soil enzyme activities (microbial activities) by applying advanced approaches like meta-transcriptomics and metaproteomics.

The phytotoxicity of nanomaterial is under purview, therefore, to determine the effect of synthesized CuNPs on maize seed germination and seedling characters, a study under the lab conditions was carried out. Enhancing effect was observed in all the seedling characters taken into account after the CuNPs treatment which is possibly due to the role of copper as a micronutrient. It is well established that the concentration of copper in the range of 4 to 20 ppm (Landis et al., 2000) is required for the normal development and physiological function of the plant. The significance of copper as a micronutrient is enormous as it acts as the main factor that activates the enzymes responsible for catalyzing reactions in the plant. Another function of copper in a plant is protein regulation by producing 'vitamin A' which ensures protein synthesis, mitochondrial respiration, activation of several enzymes like polyphenol oxidase (PPO), superoxide dismutase (SOD), amino oxidase, etc. role in oxidative stress response (Landis and Steenis, 2000; Pich et al., 1996; Passam et al., 2007). An earlier report by Yasmeen et al. (2015) supports the present result, where they observed a significant increase in the percentage of seed germination of wheat seeds treated with copper (CuNPs), silver (AgNPs), and iron (FeNPs) nanoparticles. A similar result was obtained by Adhikari et al. (2012) when CuO nanoparticles were tested against the seeds of soybean and chickpea. Up to 200 ppm, no effect on germination was observed, but root development was inhibited at above 500 ppm of CuONPs. Also, the result of the present investigation is in impeccable agreement with the findings of Gautam et al. (2016) where they reported enhanced seed germination and seed vigor index (SVI) of soybean (*Glycine max* (L) Merr.) by 15% and 50.08%, respectively when treated with 200 ppm of CuO NPs However, the higher concentration drastically reduced the germination percentage as well as SVI. Consistent with earlier described results, 100–400 ppm of sulphur nanoparticles (SNPs) also reported enhancing the growth of *Cucurbita pepo* (summer squash) by increasing the number of leaves and branches stem girth and height of the plant. However, Salem et al. (2016) observed a slight reduction in growth at 400 ppm. Contradicting previous findings, Lin and Xing (2007) reported the inimical effect of five different types of nanoparticles *viz*., multi-walled carbon nano-tube, aluminium, alumina, zinc, and zinc oxide nanoparticles on root growth and seed germination of six plant species *viz*., cucumber, radish, lettuce, rape, ryegrass, and corn. They discern a harmful effect of 2000 ppm of Al_2O_3NPs on seed germination and root elongation of different plant species. On the other hand, ZnNPs and ZnONPs did inhibit seed germination at lower concentrations except for ryegrass and corn seeds, respectively. Hence the output of the present investigations presents the positive effect of CuNPs on seed germination and other plant characters by contributing as a micronutrient.

Conclusion

In conclusion, four important maize pathogenic fungi (*F. verticillioides, M. phaseolina, R. solani* f. sp. *sasakii*, and *B. maydis*) and two pathogenic bacteria (*E. carotovora* and *R. solanacearum*) were effectively managed by synthesized CuNPs. Fungal growth was inhibited completely at 200 or 300 ppm of CuNPs, however significant inhibition was observed at 20 ppm in the case of *M. phaseolina, B. maydis*, and *F. verticillioides* and 80 ppm in the case of *R. solani* f.sp. *sasakii.* Compared to other fungi, *S. rolfsii* appeared to be tolerant as its growth was checked at 1000 ppm. The growth of pathogenic bacteria was significantly reduced by CuNPs even at a concentration as low as 20 ppm. Economical and effective management of MLB and BLSB disease of maize was also possible using CuNPs which was manifold better than the commercial fungicides/bactericides without impairing the normal growth of the maize plant. Moreover, CuNPs proved to have a positive effect on maize seedlings' characters and exhibited no adverse effect on soil enzyme activities. The higher concentration (> 50 ppm) of CuNPs exhibited an inimical effect on beneficial organisms which indicated standardizing the concentration of CuNPs compatible with such widely used biocontrol agents. Our results demonstrate the high potential of nanoparticles as protectants in managing phytopathogens.

Declarations

Acknowledgments

The first author is thankful to Post Graduate School, ICAR-Indian Agricultural Research Institute, New Delhi for receiving the research support. The authors acknowledge the support of the National Bureau of Plant Genetic Resources (NBPGR), New Delhi, and the Institute of Advanced Studies for Science and Technology (IASST), Guwahati, Assam.

Conflicts of interest

We declare no conflict of interest.

Ethical approval

There are no requirements for formal consent.

Statement on the welfare of animals

This report does not involve any studies on animals.

Consent to participate

We hereby declare that we all participated in this study and in the development of this article.

Consent to publish

We, give consent to publish this article

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Figures

Figure 1

Experimental condition in the net house with maize varieties CM 500 and CM 501 susceptible for maydis leaf blight and banded leaf and sheath blight

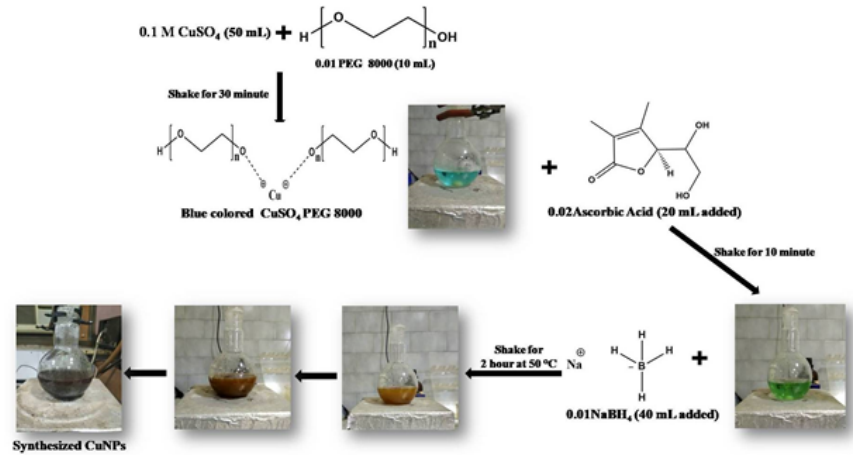

Figure 2

Standardized protocol for chemical synthesis of CuNPs using sodium borohydride ($NaBH_4$) and ascorbic acid ($C_6H_8O_6$)as a reducing agent and polyethyelene glycol (HO[CH2CH2O]n-H) as a capping agent

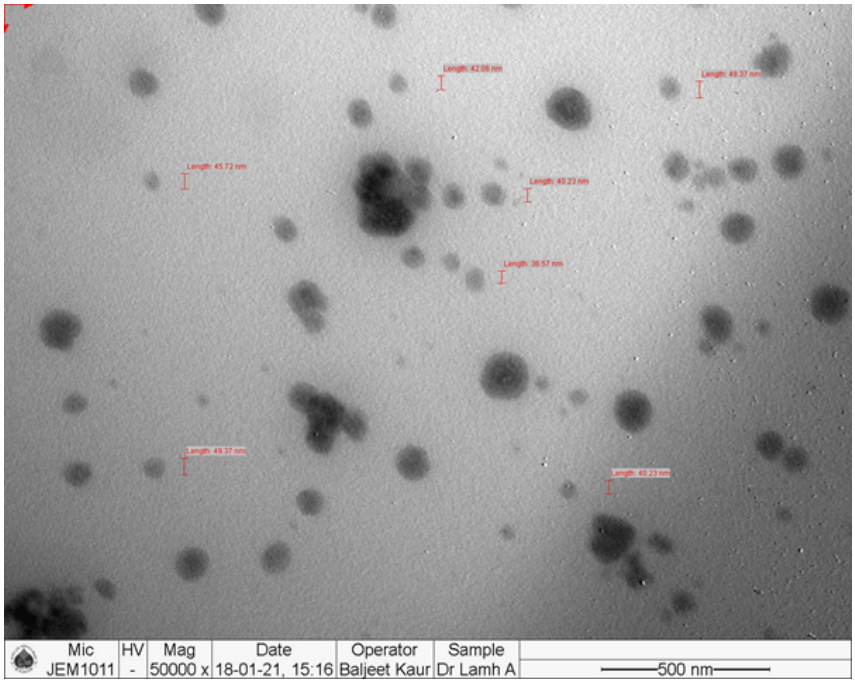

Figure 3

TEM image of copper nanoparticles synthesized by employing chemical method at 50000X magnification

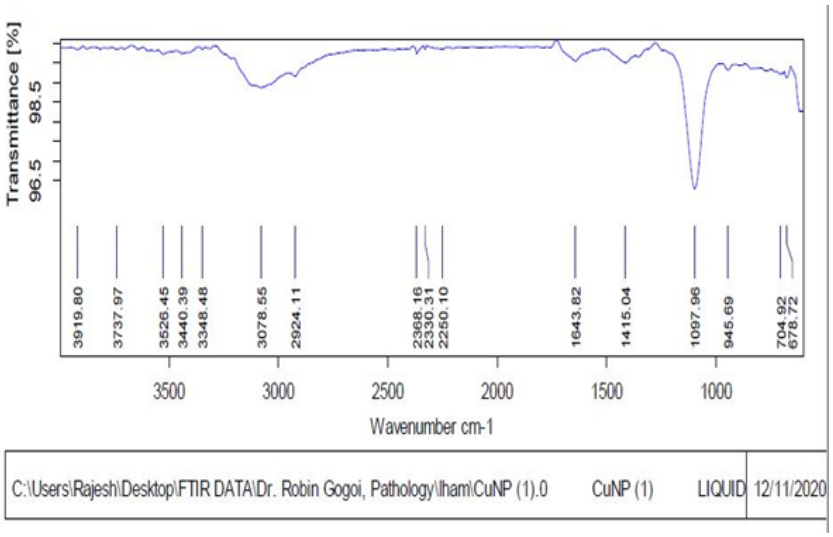

Figure 4

FTIR spectrum of synthesised CuNPs

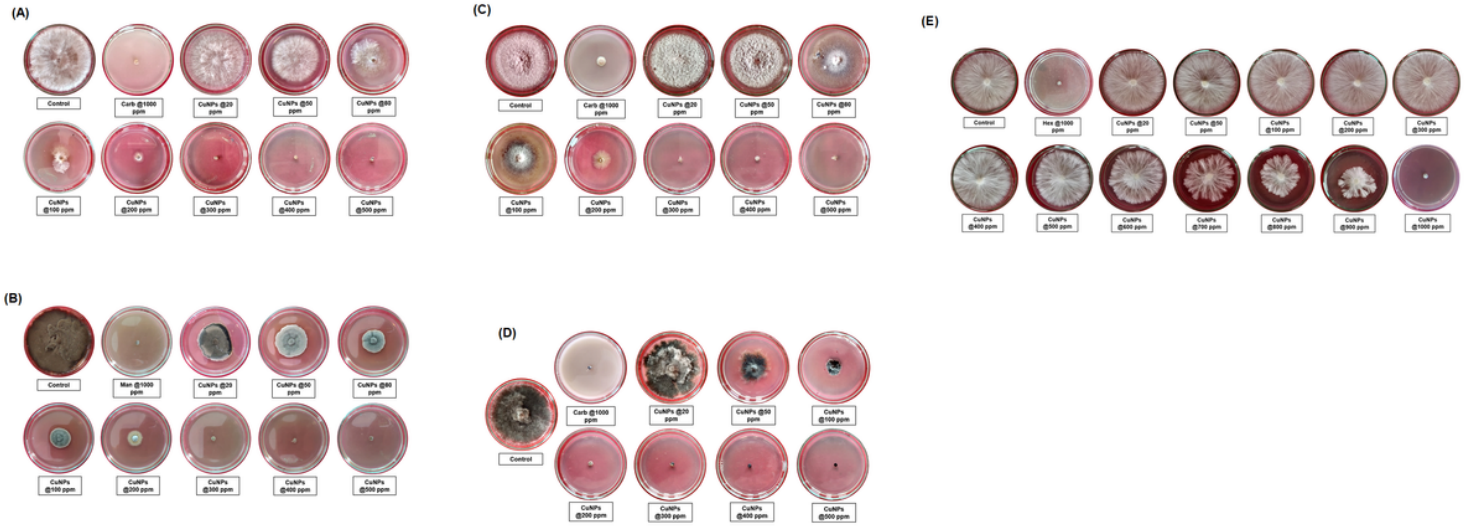

Figure 5

Concentration-dependent radial growth reduction of (A) *Rhizoctonia solani* f. sp. *Sasakii*, (B) *Bipolaris maydis*, (C) *Fusarium verticillioides*, (D) *Macrophomina*

phaseolina, and (E) *Sclerotium roflsii in vitro* exposed to various concentrations of chemically synthesized copper nanoparticles. Carb: Carbendazim 50% WP, Manc:Mancozeb 75 % WP, Hex: Hexaconazole 5% SC

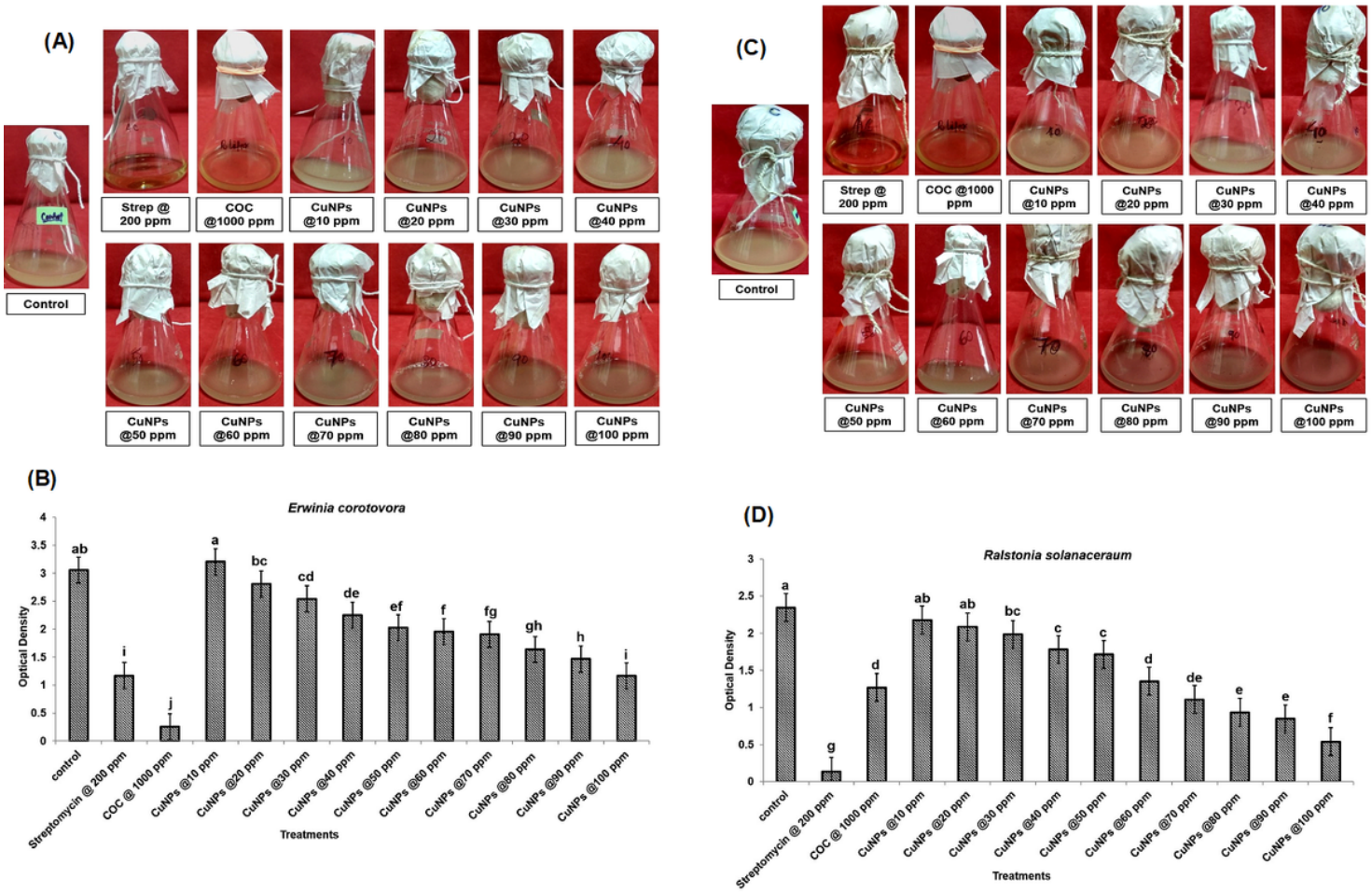

Figure 6

Concentration-dependent growth inhibition of *Erwinia carotovora* (A and B) and *Ralstonia solancearum* (C and D) *in vitro* exposed to chemically synthesized copper nanoparticles. Data (Mean ± Standard errors) with same letters are insignificant from each other and with different letters are significant in each graph (ANOVA,LSD,P≤0.01)

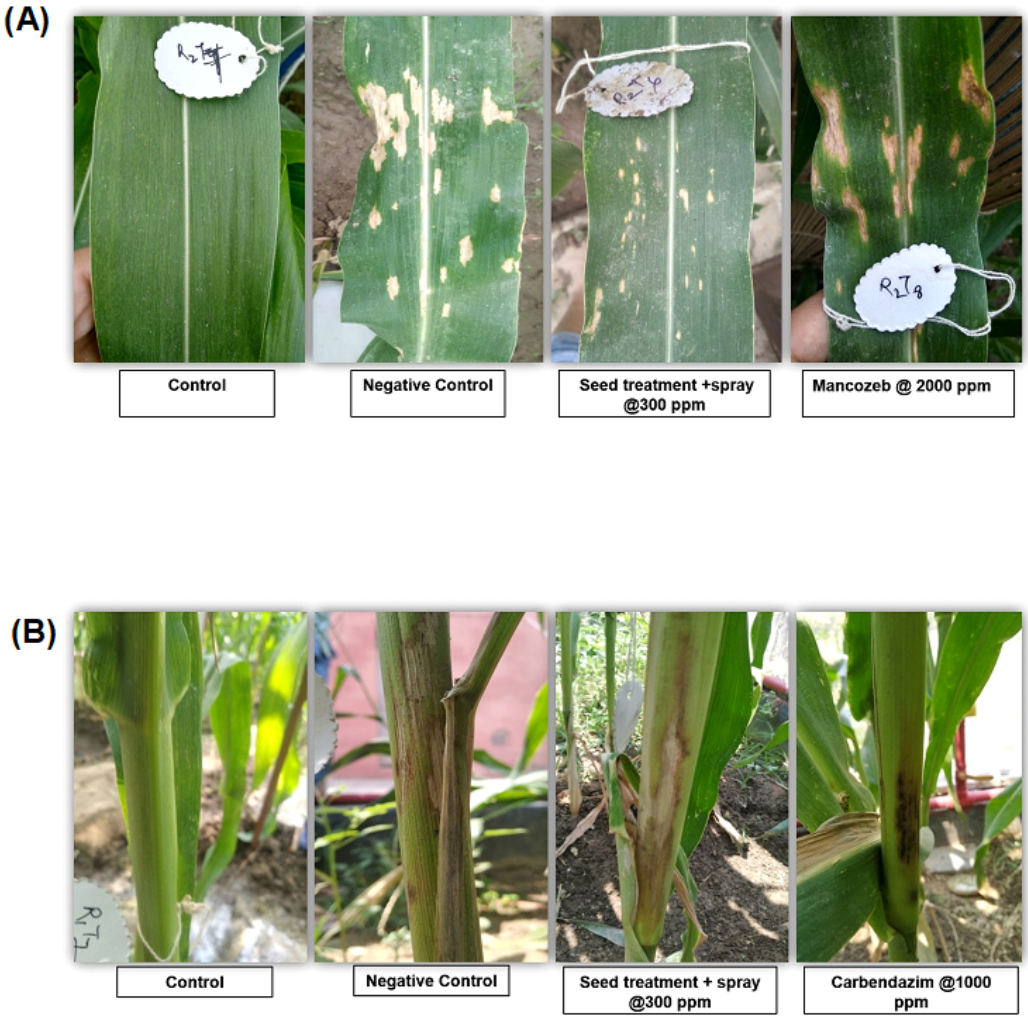

Figure 7

Disease symptoms on the artificially inoculated maize plants with various treatments: (A) Maydis leaf blight; (B) Banded leaf and sheath blight. *Control*: Without inoculation (healthy plants), *Negative control*: Inoculated with *pathogens* and without CuNPs/Fungicides spray

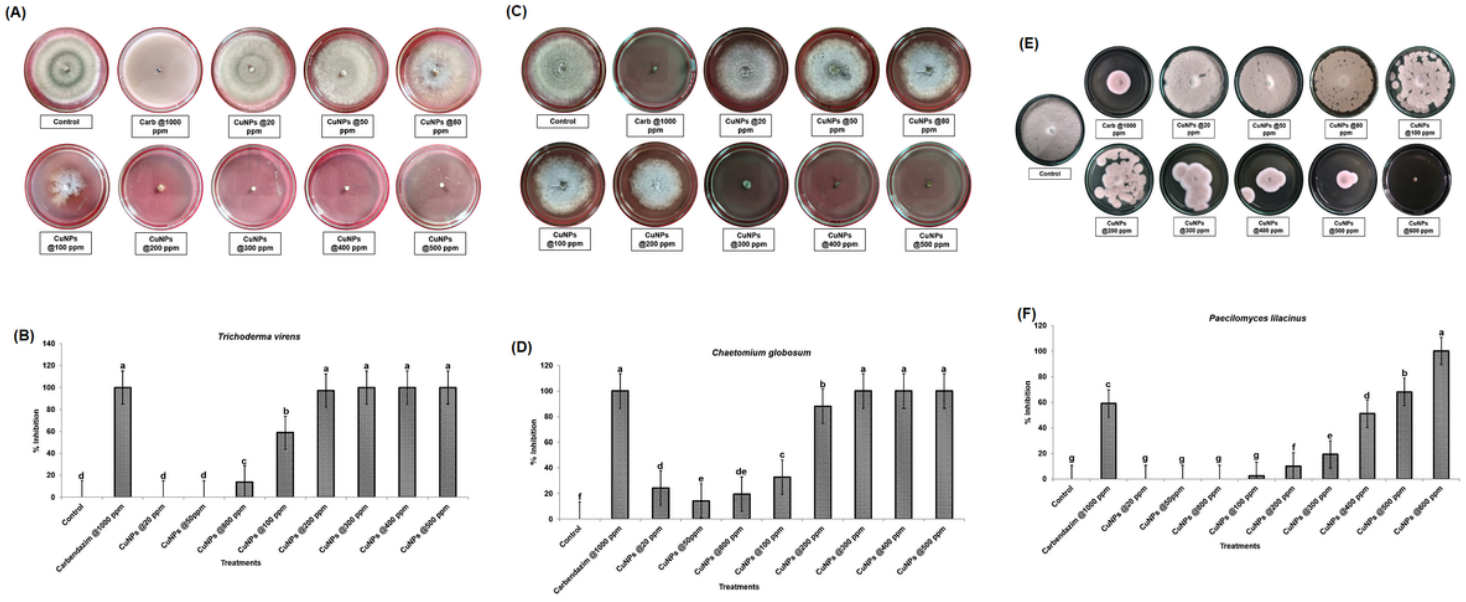

Figure 8

Concentration-dependent effect of synthesised CuNPs on the growth of *Trichoderma virens* (A and B), *Chaetomium globosum* (C and D), and *Paecilomyces lilacinus* (E and F). Data (Mean±Standard errors) with different letters are significant in each graph (ANOVA, LSD, P≤0.01). CuNPs: Copper nanoparticle, Carbendazim: Carbendazim 50% WP

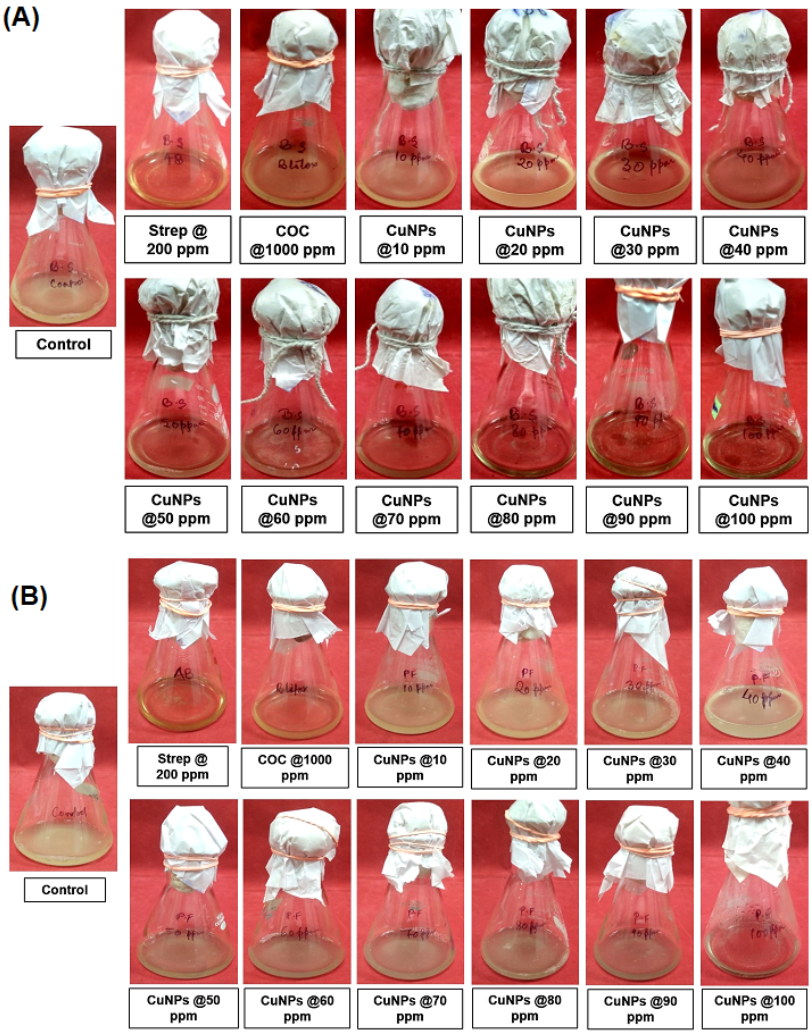

Figure 9

Concentration-dependent effect of synthesised CuNPs on the growth of (A) *Bacillus subtilis* and (B) *Pseudomonas putida*

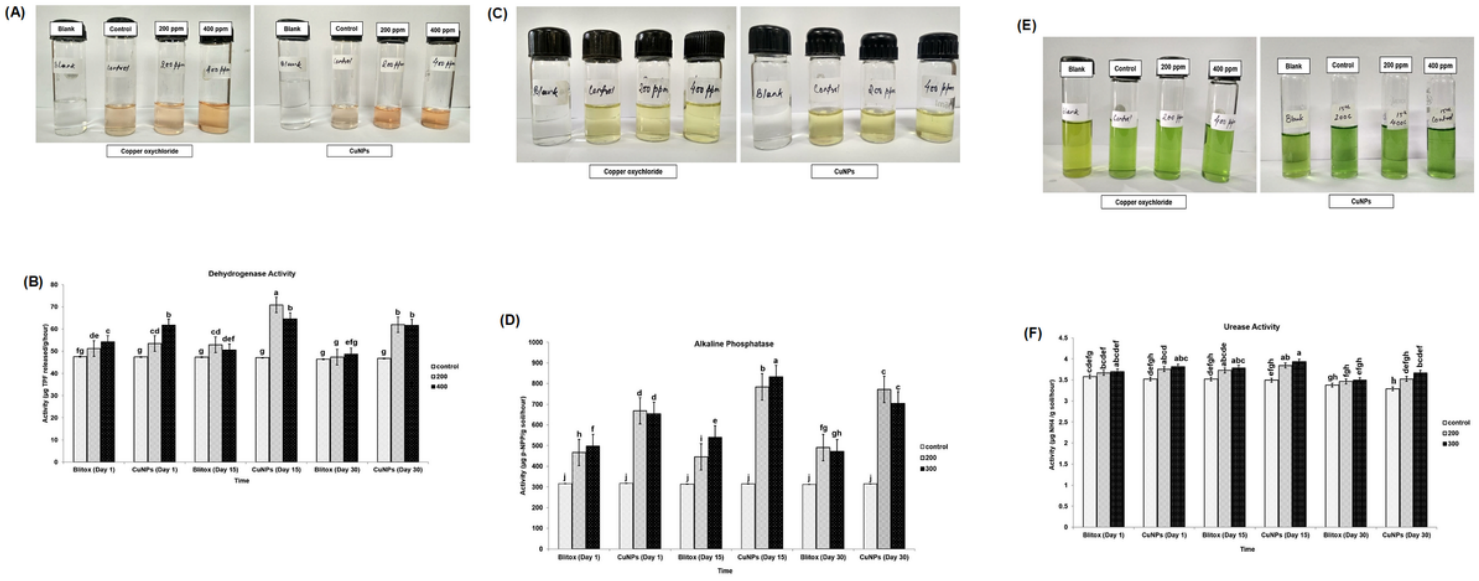

Figure 10

Effect of CuNPs on soil enzyme activities *viz.,* dehydrogenase (A and B), alkaline phosphatase (C and D), and urease (E and F). Data (Mean ± Standard errors) with different letters are significant from each other in each graph (ANOVA, LSD, P≤0.05)

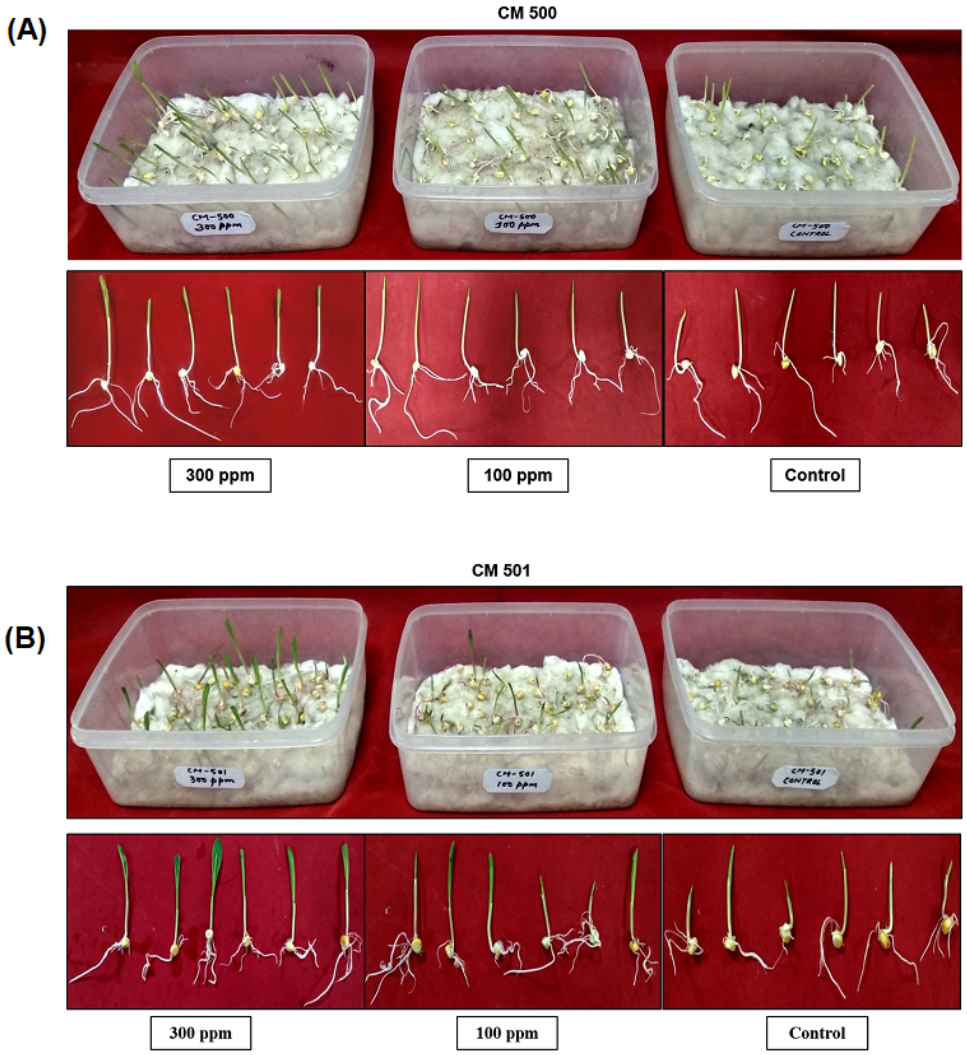

Figure 11

Effect of CuNPs on maize seedling’s characters after seed treatment with distilled water, 100 ppm and 300 ppm of CuNPs on maize varieties (A) CM-500 and (B) CM-501

Supplementary Files

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- Suplementaryfiles.docx