Copper Nanoparticle (CuNP) Nanochain Arrays with a Reduced Toxicity Response: A Biophysical and Biochemical Outlook on Vigna radiata

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ABSTRACT: Copper deficiency or toxicity in agricultural soil circumscribes a plant’s growth and physiology, hampering photochemical and biochemical networks within the system. So far, copper sulfate (CS) has been used widely despite its toxic effect. To get around this long-standing problem, copper nanoparticles (CuNPs) have been synthesized, characterized, and tested on mung bean plants along with commercially available salt CS, to observe morphological abnormalities enforced if any. CuNPs enhanced photosynthetic activity by modulating fluorescence emission, photophosphorylation, electron transport chain (ETC), and carbon assimilatory pathway under controlled laboratory conditions, as revealed from biochemical and biophysical studies on treated isolated mung bean chloroplast. CuNPs at the recommended dose worked better than CS in plants in terms of basic morphology, pigment contents, and antioxidative activities. CuNPs showed elevated nitrogen assimilation compared to CS. At higher doses CS was found to be toxic to the plant system, whereas CuNP did not impart any toxicity to the system including morphological and/or physiological alterations. This newly synthesized polymer-encapsulated CuNPs can be utilized as nutritional amendment to balance the nutritional disparity enforced by copper imbalance.

KEYWORDS: copper nanoparticles, encapsulated, photosynthesis, light reaction, dark reaction

INTRODUCTION

Copper (Cu) is an essential plant micronutrient in both algae and higher plants and plays an important role in numerous metabolic processes including photosynthesis, respiration, plant growth, and development, although at higher concentration, Cu may act as “food poison.” Excess Cu inhibits a large number of enzymes and interferes with several aspects of plant biochemistry, including photosynthesis, pigment synthesis, and membrane integrity. Perhaps its most important setback is to block photosynthetic electron transport chain (ETC), leading to the production of radicals that start peroxidative chain reactions involving membrane integrity. However, not a single effective strategy has been developed to combat this severe nutritional imbalance.

With recent developments in new technology, several smart nanoparticles have been introduced to improve the nutritional imbalance within plant systems. Manganese nanoparticles have been used to enhance the photosynthetic efficacy in mung bean system; nanotitanium dioxide (TiO2) enhanced the photosynthetic activity as well as nitrogen metabolism in spinach. Typical nanoparticles are used for crop management, but their phytotoxic effects cannot be ruled out. Zinc oxide nanoparticles and nanoscale zinc have been found to inhibit seed germination as well as root growth of six higher plant species, Raphanus sativus, Brassica napus, Lolium multiflorum, Lactuca sativa, Zea mays, and Cucumis sativus. Zinc oxide nanoparticles are readily internalized within the apoplast and tonoplast of the root endodermis and stele, resulting in shrinkage of the root tip of ryegrass. Use of nanoparticles may give rise to environmental toxic response; in this context, Doshi et al. have injected two types of nanoalumina into a sand column to evaluate ecotoxicological assessment. Several works have also been performed on various plant model systems such as wheat, rice, spinach, and onion to evaluate the potential efficacy of copper and copper oxide nanoparticles (CuO NPs). However, CuO NPs induce oxidative stress mediated damage within plant systems. Therefore, significant efforts are needed to design smart copper-based nanoparticles for crop management with a reduced toxic response to eliminate the aforementioned setbacks that copper oxide nanoparticles reinforced to the system.

In this study, we demonstrate an alternative strategy to combat the Cu-induced nutritional disorder, with enhanced plant growth, photochemical, and carbon and nitrogen assimilatory activities along with a reduced toxic response. To address the critical issues, copper nanoparticles (CuNPs) have been
CuNPs have been synthesized by a facile method with modifications. Synthesized CuNPs have been tested against mung bean plants and, as a comparison, a commercially available bulk counterpart, copper sulfate (CS) salt, is used under controlled laboratory conditions. At 0.05 mg/L concentration, CS promotes plant growth and development as per earlier literature. It also elevates the function of photosynthetic machineries; the rate of carbon and nitrogen assimilation as CS is known to be a cofactor in these metabolic processes. CS treatment at or above 0.5 mg/L resulted in severe tissue injuries, causing reduction in growth, even death, of the plants. Cu toxicity is a threat worldwide because of its tendency to produce harmful Cu chelates during interaction with soil colloids and nutrients, which are detrimental to the surrounding microfauna. In this study, we demonstrate a single-step designed encapsulation of CuNP within a biocompatible polymer to reduce its toxic response. Sustained release of nutrient interacts with biological sites, which controls the toxic response. Biochemical experiments have been carried out on isolated chloroplast (Chl) of mung bean plant treated with CuNPs, which demonstrates that CuNPs are more effective than CS to some extent. In a tandem process the effects of both nano and bulk counterpart of Cu in carbon and nitrogen assimilation have been checked. Interestingly, CuNP shows greater efficacy in contrast to CS. A series of biophysical techniques are used to confirm the bioavailability and uptake within the system. Oxidative stress markers, both antioxidative and non-antioxidative enzyme levels, show that CuNP does not inhibit any toxic response to the treated plant system. CuNP, both in physiological and biochemical aspects, worked significantly better than CS, which caused cellular toxicity because of dysfunctional metabolic pathways. The whole study is assisted by a probable mechanistic interpretation to outline its beneficial response. In light of these aforementioned analyses we envision CuNPs future prospect in crop management with reduced toxic response in contrast to its bulk counterpart. To the best of our knowledge this is the first effort to use biocompatible polymer-encapsulated CuNPs for augmentation of photosynthesis, elevated carbon and nitrogen assimilation, and improved morphological and physiological outputs within a plant system.

## MATERIALS AND METHODS

### CuNP Synthesis

Half a gram of copper sulfate was allowed to dissolve in 2 mL of water; to it was added 20 mL of PEG-200. The resultant mixture was sonicated in a sonication bath (100 Hz) for about 5 min to obtain a homogeneous dispersion. To the resultant dispersion was added dropwise an aqueous solution of sodium borohydride (0.1 M), and the reaction mixture was subjected to a microwave heating (450 W) until a characteristic reddish color was developed. The mixture was centrifuged and washed with ethanol three times to remove impurities and placed in a hot-air oven at 80 °C to obtain polymer-encapsulated CuNP.

### Plant Material and Growth Conditions

Mung bean seeds (Vigna radiata var. Sonali) were purchased from Berhampur Pulse and Oil Research Centre. Seeds (20 seeds/replicate; 3 replicates in total) were washed and deionized double-distilled water and surface sterilized with 5% sodium hypochloride solution for about 20 min. Then the seeds were washed thoroughly with deionized double-distilled water and imbibed with the treatment solutions (control; CuNP 0.05, 0.1, 0.5, and 1 mg/L; CS 0.05, 0.1, 0.5, and 1 mg/L) in the dark for 4-6 h prior to the germination. Detailed growth conditions of plants are described in the Supporting Information (SI).

### Morphological Parameters

Root and shoot lengths and fresh and dry weights of CuNP- and CS-treated plants were measured. Dry weight was recorded by drying the plants at 80 °C for 24 h.

### Estimation of Photosynthetic Pigment Content

Control and treated leaves were centrifuged twice with 80% alkaline acetone at 6000g for 20 min, and chlorophyll content was estimated with the supernatant spectrophotometrically in terms of micrograms of chlorophyll per gram of fresh tissue. Carotenoid was estimated according to the method of Davies with little modification, and the data were expressed in terms of optical density (OD) per gram of fresh weight.

### Isolation of Chloroplast

Mung bean leaves were homogenized in isolation buffer (330 mM mannitol, 30 mM HEPES, 2 mM EDTA, 3 mM MgCl₂, and 0.1% w/v BSA, pH 7.8). The homogenate was filtered and centrifuged. Supernatant was further centrifuged at 1000g for 5 min at 4 °C.

### Estimation of Fluorescence and Irradiance of Chloroplast by Photoluminescence (PL) Spectra

Chloroplast suspension contained 100 μM potassium phosphate buffer (pH 7.8), 2 μM NaCN, and 1.25 mM sucrose. The fluorescence spectra of the control and treated chloroplasts were recorded at 273 K under a 440 nm excitation wavelength. Photosynthesis versus irradiance (PI) of treated and untreated chloroplasts was calculated accordingly.

### Photoreduction Activities

The electron transport through the whole chain of photosynthesis was measured polarographically with an Oxygraph oxygen electrode (Hansatech Instruments, UK). Oxygen evolution was assayed in a medium containing chloroplasts equivalent to 378 μg/mL of chlorophyll. Hill activity was measured according to the method of Vishnani. Ferricyanide and nicotinamide adenine dinucleotide (NADP) phosphate (NADP) reduction were determined by spectrophotometric methods. Light-induced adenosine triphosphate (ATP) content of chloroplasts was measured by comparing the ATP level in the dark and after 1 min of illumination.

### Carbon Assimilation Assay

Chloroplast NADP-glyceraldehydes-3-phosphate dehydrogenase (GPDHase) activity was determined following the method of Taylor et al. The enzyme was activated by a solution containing 0.2 M tricine (pH 8.4) and 20 mM ATP added to 0.1 mL of the solution containing the extracted enzymes. After 5 min, reaction medium (62.5 mM tricine (pH 8.4), 16 mM MgCl₂, 7.8 mM ATP, and 0.2 mM NADPH) was added to it. Fifty millimolar glyceraldehyde 3-P was then added to initiate the reaction and the oxidation of NADPH monitored at 340 nm. Fructose-1,6-biphosphatase (FBPase) was activated by preincubation of enzyme extract with reaction medium (0.14 M tricine (pH 7.9), 14 mM MgCl₂, and 7 mM DTT). Sixty millimolar fructose-1,6-biphosphatase was added after 10 min and terminated after 15 min by the addition of 0.5 mL of 10% (w/v) TCA. This solution was cleared by centrifugation for 10 min at 12000g to determine the enzymatic activity. Ribulose-5-phosphate kinase (RBPase) activity was analyzed by 0.1 M tricine (pH 7.6) and 15 mM DTT for 1 h. After that, reaction medium (66 mM tricine (pH 7.6), 13 mM MgCl₂, 1.3 mM P-pyruvic acid, 1.3 mM ATP, 0.33 mM NADH, and 2 units each of phosphoribosyltransferase, pyruvate kinase, and lactic dehydrogenase) was added. The reaction wasinitiated by 30 mM ribose-5-P and the oxidation of NADH monitored at 340 nm.

### Nitrogen Metabolism

Preparation of Leaf and Root Enzyme Extract

Control and treated plants were homogenized at a ratio of 1:10 (w/v) in 50 mM phosphate buffer (pH 7.5) containing 2 mM EDTA, 1.5% (w/v) soluble casein, 2 mM DTT, and 1% (w/v) insoluble PVPP at cold temperature and centrifuged at 3000g for 5 min. Supernatant was again centrifuged at 30000g for 20 min. The extract was then used for enzymatic assays.

### Determination of NO₃⁻

Enzyme extract was added to a 10% salicylic acid solution dissolved in 12 N sulfuric acid (w/v). NO₃⁻ content was measured as described by Cataldo et al.[11] The results were expressed as micromoles per gram of fresh weight.

### Determination of Nitrate Reduction (NR) Activity

Enzyme extract was mixed in the reaction mixture containing 100 mM phosphate buffer (pH 7.5), 100 mM KNO₃, 10 mM cysteine, and 2 mM NADH. After 30 min of incubation at 30 °C, 1 M zinc acetate was added. The nitrite formed was determined at 540 nm, and NR activity was expressed as micromoles of NO₃⁻ formed per gram of fresh weight per hour.

### Determination of Nitrite Reduction (NIR) Activity

To the reaction mixture (50 mM phosphate buffer (pH 7.5), 20 mM KNO₃, 5 mM methyl viologen (MV), 300 mM NaHCO₃) was added enzyme extract at
After 30 min, NiR activity was determined by the disappearance of NO$_2^-$ from the reaction medium and expressed as micromoles of NO$_2^-$ formed per gram of fresh weight per hour.

**Determination of Glutamine Synthetase (GS) Activity.** Enzyme extract was added in the reaction mixture containing 100 mM phosphate buffer (pH 7.5), 4 mM EDTA, 1 M sodium glutamate, 450 mM MgSO$_4$·7H$_2$O, 300 mM hydroxylamine, and 100 mM ATP. The formation of glutamylhydroxamate was measured at 540 nm after complexing with acidified ferric chloride. GS activity was expressed as formed glutamylhydroxamate in grams of fresh weight per hour.

**Determination of NADH-Glutamate Synthase (GOGAT) Activity.** The reaction mixture contained 50 mM phosphate buffer (pH 7.5) with 0.1% (v/v) mercaptoethanol, 1 mM EDTA, 18.75 mM 2-oxoglutarate, 15 mM aminooxyacetate, 1.5 mM NADH, 75 mM L-glutamine, and enzyme extract. The decrease in absorbance was recorded for 5 min at 28°C. The activity was expressed as micromoles of NAD per gram of fresh weight per hour.

**Uptake of CuNP by Fluorescence Microscopy Study.** CuNPs were amine functionalized and conjugated with fluorescein-B-isothiocyanate (FITC) for uptake study. Ultrathin cross sections of treated plant samples were fixed in 2% glutaraldehyde followed by washing in graded ethanol and observed under confocal microscope.

**Electron Micrographic Study.** Transmission electron microscopy (TEM) and field emission scanning electron microscope (FESEM) of treated and untreated chloroplasts were performed for analyzing the interaction between chloroplast and CuNPs in photosynthesis. Chloroplasts were fixed with glutaraldehyde, stained with osmium tetroxide, and washed in graded ethanol for observation under an electron microscope.

**Cu Release Study by Inductively Coupled Plasma Optical Emission Spectroscopy (ICPOES).** Cu release from CuNPs was monitored by using ICPOES at pH 7, that is, the pH of the plant growth medium. One hundred milliliters of CuNP dispersion of the aforementioned concentrations in Milli-Q water was allowed to stir for 24 h. The resultant dispersion was centrifuged and subjected to digestion in the presence of ultrapure nitric acid followed by estimation against a standard.

**Enzyme Assays Related to Oxidative Stress.** Control and treated plants were ground in Tris-HCl buffer (pH 7.5) containing EDTA and polyvinylpyrrolidone followed by centrifugation at 0°C. The supernatant was used for enzymatic assays. Superoxide dismutase (SOD) activity was estimated following a standard procedure. Peroxidase (POD) activity was assayed according to the method of Chance et al. Peroxide content of plant tissue was estimated according to the method of Thurman et al. Glutathione reductase (GR) was assayed according to the procedure of Wang. Catalase (CAT) activity was assayed according to the method of Malík et al. The total peroxide content of plant tissue was estimated according to the method of Lowry et al.

**Estimation of Total Amino Acid (AA), Lipid, Protein, and Carbohydrate Contents.** AA content in plant samples was estimated according to the ninhydrin method. Protein contents were estimated according to the method of Lowry et al. Lipid content was estimated according to the Bligh method. Total carbohydrate content was estimated following Loewus.

**RESULTS AND DISCUSSION**

**Physicochemical Characterization of CuNP.** Herein we demonstrated a designed synthesis of CuNPs encapsulated within a polymeric matrix followed by evaluation of its photosynthetic efficacy, carbon and nitrogen assimilation, and
biosafety study in plant systems. In correlation bulk counterpart CS was also considered for a brief comparison. FESEM was used to evaluate the morphology of CuNPs, which justified spherical particles sheathed within a polymeric matrix (Figure 1a). TEM image showed that small spherical particles of almost 20 nm were formed during the synthesis (Figure 1b). In situ encapsulation of

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“Capital letters change across the row owing to significance of respective pairwise treatments, whereas the lower case letters change down the column.

Figure 2. (a) Morphology of control and treated plants after 15 days of treatment. (b) CuNP and CS on chlorophyll content of 15-day-treated mung bean plants. Chlorophyll a, F = 76.5579, P < 0.000001; chlorophyll b, F = 464.8072, P < 0.0000001. (c) CuNP and CS on carotenoid content of 15-day-treated mung bean plants. Data represent the mean ± SE (n, no. of samples = 3). Carotene, F = 37.9368, P < 0.00001; xanthophyll, F = 75.1419, P < 0.00001. Within each type of treatment, mean pigment content (±SE, n = 3) followed by the same upper case letter is not significantly different for a particular dose; within each dose mean pigment content followed by the same lower case letter is not significantly different (Tukey–Kramer HSD test).
CuNP within PEG-200 resulting in a chain-like unique morphology. This unique Cu nanochain array was composed of spherical CuNPs encapsulated within PEG-200 to yield a uniform dispersion, which was visible in the high-resolution image (Figure 1b, inset). It was well documented that the CuNP nanochain array was originally reddish; during synthesis a drastic blue to reddish coloration signified the formation of CuNP. A subsequent UV–vis absorbance at around 450–480 nm was noted, which also justified the formation of CuNPs (Figure 1c) and corroborated previous experimental findings. Polymeric encapsulation within PEG-200 resulted in suppression in crystallinity within CuNP in X-ray diffraction (XRD) pattern (SI, Figure S1). This amorphous nature of encapsulating agent was also consistent with the literature. Energy dispersive X-ray (EDX) analysis also justified that Cu is present as the main constituent. Additional peaks of impurities were avoided, which confirmed that CuNPs were obtained in their pure form (SI, Figure S2). The surface topology and height profile were obtained by atomic force microscopy (AFM). AFM image (SI, Figure S3) verified that the average height of the PEG-200 encapsulated CuNPs was 6–8 nm. Surface functionality of CuNPs was confirmed from Fourier transformed infrared (FTIR) spectra (Figure 1d). CuNPs were hydrophilic with the existence of surface hydroxyl and carbonyl/carboxyl groups. Spectral pattern confirmed C−H stretching, O−H bending, C≡O stretching, and C−O stretching, which arose due to surface PEG-200 during encapsulation of CuNPs. A sustained release of 0.0136 ppm from predesigned 0.05 mg/L CuNP nanochain arrays after 24 h was noted by ICP-OES under mimicking environment sealed inside a dialysis bag (SI, Figure S4). Sustained release of Cu from polymer-encapsulated nanochain array resulted in lower availability of Cu to the in vitro system in contrast to its bulk counterpart CS. As a result, toxicity symptoms were reduced to some extent in this smart designed nanochain array. Meanwhile, the hydrophilic character produced aqueous stability for the in vitro experiment. Altogether this paper claims well characterized, stable, polymer-encapsulated designed CuNPs to be useful for in vitro and in vivo plant experiments.

Increased Chlorophyll and Carotenoid Contents in CuNP-Treated Plants Even at a Dose of 1 mg/L. It is well established that in both Cu-deficient and -excess plants, a change in chlorophyll (Chl) content alters chloroplast structure and thylakoid membrane composition in leaves of spinach, rice, wheat, and bean in experimental growth conditions. Cu interferes with the biosynthesis of the photosynthetic machinery modifying the pigment and protein compositions of photosynthetic membranes. This is attributed to the reduction of Chl content and subsequent substitution of the central Mg ion of Chl by Cu in vivo, leading to the inhibition of photosynthesis.17 In our experiment in both Cu-deficient and CS-treated plants (at higher doses), similar results were observed. In the case of CuNP-treated plants, Chl-a content was increased specifically in all treatments, indicating CuNPs might have a momentous effect in photosynthesis, as Chl-a is key player in the light-dependent photosynthetic pathway (Figure 2b). Carotenoid, the other important factor, which protects the photosynthetic machinery from any abiotic stress, was simultaneously checked. In CuNP-treated plants, both xanthophyll and carotene were found to be increased significantly with respect to its CS and the control (Figure 2c). Therefore, photosynthetic machinery was prevented from unnecessary external stress during CuNP treatment.

CuNP at 0.05 mg/L Enhanced the Activity of Light Reaction in Photosynthesis. In natural photosynthesis, incident light electronically excites a membrane-bound protein–pigment complex, called photosystem (PS). The photo-generated electrons are rapidly delivered to reaction centers (both PSI and PSII) with the help of antenna pigments along the electron transport chain (ETC) through a series of oxidizing agents for regenerating NADPH cofactors. These cofactors drive redox enzymatic reactions to synthesize organic compounds in the Calvin cycle.

At the initial stage of light-dependent reaction, some associated pigments, called antenna pigments, along with some light-harvesting proteins capture solar energy and transfer it to the reaction center. When the energy stored in chlorophylls in the excited state is rapidly dissipated by excitation transfer, the excited state is said to be quenched.18 This quenching is believed to originate directly from the light-harvesting pigment bed and is influenced more by CuNP treatment than its bulk counterpart. Therefore, more intense energy transfer to the reaction center in the form of non-photosynthetic fluorescence was noted in CuNP treatment in contrast to CS treatment (SI, Figure S5). If the excited state of chlorophyll is not rapidly quenched by excitation transfer, it can react with molecular oxygen to form singlet oxygen. The extremely reactive singlet oxygen can damage many cellular components and hamper the photosynthetic efficiency of PSII.

Photosynthesis irradiance (PI) response was evaluated (SI, Figure S6) to understand the relationship between CuNP and pigment efficiency in terms of energy capture and subsequent transfer of exciton. Enhancement in response curve after CuNP treatment with respect to control supported that CuNP did not encourage photochemical damage on PSII; the subsequent quenching (SI, Figure S6) was due to non-photosynthetic change during energy transfer. This initial stimulation after CuNP treatment provided a strong basis to trace out next sets of photochemical assays to address photosynthetic efficiency.

From the reaction center, the excited PSII transfers an electron to pheophytin. On the oxidizing site of PSII, it is rereduced by water, generating molecular oxygen, which is a byproduct of photosynthesis. Pheophytin transfers electrons to a series of electron acceptors to cytochrome b_{5}f complex. A Cu–
protein component, called plastocyanin, then transfers electrons from the cytochrome complex to PSI. Thereafter, a series of electron acceptors transfers electrons to produce NADPH, which is an essential component to be used in a later stage, that is, dark reaction of photosynthesis. Therefore, photosynthetic efficacy was measured in light of a series of biochemical reactions in the presence of different electron acceptors.

Cu, being a metalloprotein component of plastocyanin, induces structural changes in photosynthetic membrane leading to the inhibition of electron flux. As a result, at any alteration of doses, it inhibits sites of photosynthesis starting from the Hill reaction, that is, dark reaction of photosynthesis, ET, and PSI activity. CuNP at all doses was found to enhance light reaction, reducing the harmful effect of Cu deficiency and/or toxicity. Polarographic study for efficiency of whole chain electron transport (Figure 3a) and subsequent oxygen evolution (Figure 3b) in light reaction corroborated the previous results. The effect was maximum at the dose of 0.05 mg/L compared to CS counterpart in the Hill reaction (Figure 3c), ferricyanide reduction assay (PSII activity) (Figure 3d), and ferricyanide–NADP reduction (PSI activity) (Figure 3e).

Photophosphorylation is the process of converting energy from a light-excited electron into the pyrophosphate bond of an adenosine diphosphate (ADP) molecule. During water splitting the liberated proton is bypassed to the ATPase complex by a proton gradient to form ATP from ADP, which is an important...
material for dark reactions. In CuNP-treated Chl, ATP content was found to be greater than its bulk counterpart CS. No apparent toxicity was observed even at the highest dose of CuNP as noted in CS-treated Chl (Figure 3f).

In brief, when light falls on the green cells CuNP encouraged the energy packets present in the pigment bed to transfer the electron readily to the reaction center without hampering the pigment machinery, that is, PSII or PSI. CuNP positively enhanced the efficiency of chloroplast in light-mediated entire electron transfer process (30.29% more than control Chl when measured polarographically). It stimulated the PSII activity (20.5% more activity was observed with respect to control), resulting in greater oxygen evolution. CuNP also promoted the PSI activity probably because of its interaction between Cu−protein components, plastocyanin. As a result, photophosphorylation activity was also improved after CuNP treatment. CuNP thus modulated the entire light reaction positively with effective influence on electron transport chain along with phosphorylation activity as seen in Scheme 1.

**CuNP Enhanced the Activity of Light-Regulating Enzymes in Dark Reaction of Photosynthesis.** In light-independent reaction, CO$_2$ and water are enzymatically combined with a 5-C molecule to generate two molecules of a 3-C intermediate, which is reduced to carbohydrate by enzymatic reactions driven by ATP and NADPH generated photochemically. CuNP was found to facilitate the light−dark modulation providing an on−off switch for three key enzymes in the Calvin cycle, namely, fructose-1,6-bisphosphate phosphatase (FBPase), ribulose-5-phosphate kinase (RBPase), and NADP-glyceraldehyde-3-phosphate dehydrogenase (GPDHase). When CuNP-treated Chl was subjected to evaluation of GPDHase (Figure 4a), RBPase (Figure 4b), and FBPase activity (Figure 4c), it was noted that CuNP exhibited greater efficacy in contrast to its bulk counterpart CS. Interestingly, 0.05 mg/L was found to be most effective among all of the dosages, although CuNP had a strong influence on FBPase activity among these three enzymes. This result indicated that CuNP might play a pivotal role in the dark reaction of photosynthesis.

**CuNP in Nitrogen Metabolism.** Plant growth and photosynthetic ability can be improved by enhancing the nitrogen supply within the system, and therefore it is essential to evaluate the interactive effect of CuNP on nitrogen metabolism in the plant. The first step of this process is the reduction of nitrate in cytosol, and the reaction is catalyzed by nitrate reductase (NR), in which nitrate ion is converted to nitrite ion. Because nitrite is a highly reactive, potentially toxic ion, plant cells immediately transport the nitrite generated by NR from cytosol into chloroplastic leaves and plastids in roots. Here, with the help of nitrite reductase (NiR), nitrite is transferred to ammonium ion. To avoid ammonium toxicity, plants rapidly convert...
ammonium ion into amino acids (AAs) with the help of glutamine synthase (GS) and glutamate synthase (GOGAT). GS combines ammonium with glutamate to form glutamine, whereas GOGAT assimilates glutamine translocated from roots to leaves. Once assimilated into glutamate and glutamine, nitrogen is incorporated to AA via transamination reactions. Therefore, the activities of all four enzymes are imperative in nitrogen assimilation and can be stimulated by essential heavy metals.36

In this paper, CuNP at modulated dose was found to be pertinent for use as an integral component in nitrogen fertilizer as it enhanced the activity of NR (Figure 5a), NiR (Figure 5b), GS (Figure 5c), and GOGAT (Figure 5d). Free nitrate ion concentration was much less in the system compared to the control (SI, Figure S7), ruling out the possibility of nitrate

Figure 5. CuNP and CS on nitrogen metabolism on 15-day-treated mung bean plants. (a) NR activity. CuNP leaves, $F = 33.57, P < 0.0001$; CuNP roots, $F = 3.39, P < 0.1$; CS leaves, $F = 25.56, P < 0.001$; CS roots, $F = 7.44, P < 0.1$. (b) NiR activity. CuNP leaves, $F = 19.87, P < 0.001$; CuNP roots, $F = 19.51, P < 0.01$; CS leaves, $F = 10.12, P < 0.01$; CS roots, $F = 12.8, P < 0.01$. (c) GS activity. CuNP leaves, $F = 39.21, P < 0.000001$; CuNP roots, $F = 23.66, P < 0.01$; CS leaves, $F = 31.68, P < 0.000001$; CS roots, $F = 23.44, P < 0.001$. (d) GOGAT activity. CuNP leaves, $F = 20.02, P < 0.0001$; CuNP roots, $F = 122.23, P < 0.000001$; CS leaves, $F = 26.03, P < 0.0001$; CS roots, $F = 223.94, P < 0.000001$. Within each type of treatment, mean data ($\pm$ SE, $n = 3$) followed by the same upper case letter are not significantly different for a particular dose; within each dose, means followed by the same lower case letter are not significantly different (Tukey–Kramer HSD test).

Figure 6. TEM images of (a) control and (b) CuNP-treated chloroplasts.
toxicity leading to cellular injuries within the system. CuNP treatment at all doses worked better than the bulk counterpart at their respective concentrations. Therefore, it justified that CuNP might have a significant and implicit role in both photosynthesis and nitrogen metabolism as it offsets all ominous effects of Cu toxicity caused in the agricultural sector.

Bioavailability and Uptake of CuNP. Due to strong binding of Cu by organic matter and other soil colloids, its mobility is severely restricted and the fraction of total Cu available for plant uptake is usually very low. Therefore, uptake and distribution of the CuNP within the plant system was important to execute the response. Compared to the bulk counterpart, availability of CuNP into the rhizosphere is expected to be very high because of its size, high surface by volume ratio, and unique physicochemical properties. Bioavailability of CuNP within the plant sample was verified by confocal microscopy. To demonstrate its bioavailability, CuNP was conjugated with FITC following our previous study. FITC-conjugated CuNP-treated mung bean plants were then observed under confocal microscopy. Leaf and root sections (SI, Figure S8a,b) demonstrated bright green color under confocal microscopy at 488 nm excitation wavelength, although the leaf section appeared to be brighter under confocal microscopy. A direct biomass distribution of CuNP-treated mung bean sample was observed by ICP-OES as shown in the SI (Table S1). Distribution of Cu was greater in leaf than in root, which corroborated the confocal results as well.

We assessed the morphology of treated and control Chl by electron microscopy experiments. Treated samples retained similar spherical morphology under scanning electron microscopy (SEM). No significant alterations in morphology were observed in the post-treated samples (SI, Figure S9a). EDX analysis associated with SEM verified the presence of CuNP within the Chl (SI, Figure S9b). Finally, the TEM image demonstrated the presence of small-sized CuNPs over the surface of Chl without altering its uniform spherical morphology (Figure 6).

Consequently, excess Cu causes reduction of Chl content, connected with partial destruction of grana and considerable modification of lipid–protein composition in thylakoid membranes depending upon the duration of Cu action. As a consequence, we checked the morphology of root and leaf sections under a light microscope; no abnormalities in tissue anatomy were found, which corroborated the previous results. In root tissues, epidermis pericycle and vascular bundles were also normal in position and shape (SI, Figure S10). In leaves, spongy and palisade parenchyma were normal in position and shape with respect to control; even no sunken stomata were observed (SI, Figure S11). Therefore, we conclude CuNP is readily taken up by the root from rhizosphere and is subsequently transported to leaves via shoot. CuNPs did not impart any structural changes in the root or leaf tissue, not even to the Chl, signifying its compatibility with the plant system.

Mechanistic Interpretation or Mode of Action of CuNP. Under physiological processes in plants, Cu exists as the Cu^{2+}/Cu^{+} system. Cu acts as a structural element in regulatory proteins and participates in photosynthetic ETC, mitochondrial respiration, oxidative stress response, cell wall metabolism, and hormone signaling. In its absence, plants develop specific deficiency symptoms, which affect mostly the early devel-
metalloenzymes catalyzing the disproportion of superoxide free radicals, generated by univalent reduction of molecular oxygen to hydrogen peroxide in different cellular compartments. No significant deviations of enzymatic activities with respect to control were observed at CuNP-treated plants at recommended doses as observed in Figure 7c. GR is a member of the flavoenzymes family, which catalyzes the NADPH-dependent reduction of glutathione disulfide to glutathione. In CuNP-treated sets GR levels showed minor alterations with respect to control, whereas CS showed drastic changes (Figure 7d). In both roots and leaves after CuNP treatment GR activities were increased with respect to control, but not in a dose-dependent manner because of the high affinity of Cu to bind with the -SH group of the protein moiety. Non-antioxidative protein components such as phenol (SI, Figure S13a), total peroxide (SI, Figure S13b), proline (SI, Figure S13c), and polyphenol oxidase (SI, Figure S13d) (POO) were also determined as plants have general strategies to induce the activities of these non-antioxidative proteins to overcome the oxidative stress due to imposition of abiotic stress. Except proline, no abnormal activities were observed in CuNP-treated root and leaf tissues. Proline is a known stress marker, which counterbalances the detrimental effect of stress to maintain the equilibrium of the cellular machineries. Therefore, the elevated activities of proline in CuNP-treated sets helped to surpass the possible harmful effect of plants as enforced by the nanoparticle action. This ensured the protective baseline for the plant from any abiotic stress that had been impaired at high concentration of CS to the system. Direct exposure or release of Cu²⁺ from CS could result from oxidative stress and damage within cellular network but deliberate embedding within a polymeric matrix resulted in controlled release of Cu from CuNP to yield the beneficial response as a micronutrient for crop management.

In a nutshell, biochemical and physiological implications as well as efficacy of newly synthesized polymer-encapsulated CuNP on plant systems had been established in comparison to its bulk counterpart CS. This not only promised to improve crop productivity by modulating photochemical pathway but also decimated the nutritional disorder by catalyzing the nitrogen assimilatory pathway and carbon assimilation. It reduced the toxicity issues developed by the commercially available salt CS, as it was biosafe at the physiological dosages used in these experiments. Ready uptake within the plant system verified its bioavailability; at the same time, strategic encapsulation within the biocompatible polymer matrix reduced the toxic response and ensured stability to some extent. At recommended dosages its efficacy in photosynthesis and biocompatibility were ensured in contrast to its bulk counterpart CS; cellular machineries were protected as well. Moreover, polymer-encapsulated CuNP might serve as a boon in the agricultural arena, being a suitable alternative of copper micronutrient, and hence it could be included in fertilizers amalgamated with other necessary macro- and micronutrients. We believe systematic modification in nanoparticle synthesis can lead to greater such beneficial response in the near future for crop management. Detailed toxicological profiling and thorough testing are also required prior to its commercialization, but it is an initial step that can provide a baseline for future research for sustainable development in agri-economics.
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mental effects on plants and soil communities.


