Supporting Information

CuNP nanochain arrays with a reduced toxicity response: a biophysical, biochemical outlook on *Vigna radiata*.

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Physico-chemical characterization of CuNP

The surface morphology of CuNP was determined by SUPRA 40 Field Emission Scanning Electron Microscope (FESEM) (Carl Zeiss) and atomic force microscopy (AFM) and particle size was observed through transmission electron microscope (TEM, JEOL 2010). Powder X-Ray Diffraction (XRD) measurements were performed using a Philips diffractometer of X'pert Company. The surface functional group was determined by FTIR spectroscopy (Perkin Elmer, Spectrum Two). The elemental compositions of the nanoparticles were confirmed by EDX analysis. Thermogravimetric analysis (TGA) was monitored by using Perkin-Elmer (diamond series) instrument. Confocal microscopic analysis was carried out by using high content confocal microscope (BD pathway 855) while normal light microscopic images were acquired by using Carl-Zeiss Axiolab microscope. Biochemical assays were performed using UV-Vis absorbance spectrometer of Perkin Elmer. Cu release and/Cu content was monitored by using inductively coupled plasma optical emission spectroscopy (ICP-OES) (Thermo Scientific ICAP-6300 DWO).

Plant material and growth conditions.

20 seeds / replicate were rinsed with deionized double distilled water and then soaked with sodium hypochloride solution for surface sterilization for 20 min. After that seeds were washed thoroughly with deionized double distilled water for several times and imbibed with treatment solutions for atleast 4-6 hr. After that they were kept in the petridishes for germination in dark. 3 replicates were taken for each of the treatment doses. After 24 h of germination, seeds were planted in pots (20 plantlets in each pot and 3 pots per treatments) filled with perlite supplemented with Hoagland's solution with MnNP and MS solution in case of treatments while only Hoagland's solution was used for control. They were grown for 15 days in a growth cabinet (GC-300, Lab companion) with 14 h day, 25 °C; night temperature of 20 °C and RH 40 to 60 %, light intensity 440 µmoles/m^2/s.

Estimation of Photosynthetic Pigment content.

Chlorophyll content.

Fresh leaves with 80 % alkaline acetone were centrifuged twice at 6000 g for 20 min and after centrifugation, combined supernatant were collected. Chlorophyll content was estimated spectrophotometrically at 645 nm and 663 nm and calculations were done according to Arnon's Formula (1949) [1].

Chl *a* = 12.7 x (OD at 663) - 2.69 x (OD at 645) x V/1000 x 1/W

Chl *b* = 22.7 x (OD at 645) - 4.68 x (OD at 663) x V/1000 x 1/W

Total Chlorophyll= 20.7 x (OD at 645)+8.02 x (OD at 663) x V/1000 x 1/W

Where, OD= Optical density, V= Final volume of 80 % acetone-chlorophyll extract and W= Fresh weight of the tissue taken. Chlorophyll content was estimated in terms of µg chlorophyll/ gm fresh tissue.

Carotenoid content.

Fresh leaves were extracted with 80 % alkaline acetone and pigment extract was prepared following the same method as for chlorophyll content. Carotenoid was estimated according to the method of Davies (1965) with little modification [2]. Equal volume of cyclohexane was mixed with the pigmented alkaline acetone solution in a separating funnel. The aqueous acetone solution was re-extracted with cyclohexane; the hexane fractions were combined and washed with an equal volume of water. Xanthophyll was removed from the upper hexane layer containing carotene by repeated extraction with 90 % methanol. Carotene and xanthophyll were measured by utilizing the values of absorbance at 425 nm and 450 nm respectively and the data were expressed in terms of OD/gm fresh wt.

Isolation of chloroplast.

Mature and fully expanded deveined mung bean leaves were homogenized in chloroplast isolation buffer (330 mM mannitol, 30 mM HEPES, 2 mM EDTA, 3 mM MgCl, and 0.1 % w/v BSA, pH 7.8) using blender for 15 sec. The homogenate was rapidly filtered through eight layers of cheesecloth and

centrifuged for 5 minutes at 250 g. Supernatant was further centrifuged at 1000 g for 5 min at 4 ºC. A pellet of chloroplast was then resuspended in isolation buffer and stored at - 80 ºC, till further use.

Photo reduction Activities.

Whole Chain Electron Transport.

The electron transport through the whole chain of photosynthesis, i.e. from water to methyl viologen (MV) (oxygen uptake) was measured polarographically with an Oxygraph oxygen electrode (Hansatech Instruments, UK) [3]. Assay medium (3 mL) consisted of 50 mM HEPES (pH 7.5), 10 mM NaCl, 1 mM NH_4Cl, 3 mM $MgCl_2$, 1.0 mM NaN_3, and 0.5 mM MV. Chloroplast was added to the above reaction mixture to a total concentration of 378 µg/mL. Entire reaction was monitored for 5 min at a stretch to determine activity of ETC.

Oxygen evolution measurement.

Oxygen evolution was assayed in a medium containing chloroplasts equivalent to 378 µg/mL of chlorophyll [4]. The 2.9 mL assay medium consisted of sorbitol, 0.33 M; NaEDTA, 2.0 mM; $MgCl_2$, 1.0 mM; $MnCl_2$, 1.0 mm; and HEPES, 50 mm, adjusted to pH 7.6 at 20° C with NaOH. Sodium 2,6-dichlorophenolindophenol, 0.88 mM, served as oxidant in all assays and was added to the medium immediately prior to injection of the chloroplasts. The isolated Chlorophyll solution was illuminated for 1 min with 500 µmol m^{-2} s^{-1} visible light (400–700 nm) illumination, and then the assays of photochemistry reaction were carried out.

Hill reaction in chloroplast.

Hill activity was assayed according to the method of Vishniac (1957) [5]. Fresh leaves were extracted in sucrose-phosphate buffer (0.4 M sucrose in 0.05 M phosphate buffer) at pH 6.2 and centrifuged at 1000 g for 5 min. The pellet was discarded and supernatant was collected. The supernatant was centrifuged at 5000 g for 15 min. The pellet containing chloroplasts was taken and sucrose-phosphate buffer was added to it to make the volume 5 mL. 1 mL chloroplast suspension, 4 mL sucrose-phosphate buffer and 0.5 mL

0.03 % 2, 6-dichlorophenol indophenol (DCPIP) were added in a test tube and initial absorbance was recorded at 610 nm. After 5 min of saturating radiance, optical density values were again measured at 610 nm. Hill activity was expressed as µmole DCPIP reduced per hour per mg chlorophyll.

Photophosphorylation.

Ferricyanide and NADP reduction were determined by the spectrophotometric methods of Trebst [6]. Ferricyanide reduction was measured using a mixture (1.45 mL) containing chloroplasts (378µg/mL Chl/mL); 86 mm sorbitol, 50 mM Tricine (pH 8.1), 50 mM NaCl, 5 mM $MgCl_2$, 2 mM K_2HPO_4, 2 mM ADP, and 1 mM ferricyanide. Immediately following 1 min of saturating irradiance, trichloroacetic acid was added to a final concentration of 2 %. Chloroplasts were pelleted by centrifugation and the absorbance of the supernatant was determined at 420 nm. Dark controls showed no ferricyanide reduction. The experimental conditions for measuring NADP reduction were identical except for the deletion of ferricyanide from the reaction medium and the addition of 3 µM purified spinach ferredoxin (Sigma) and 0.66 mM NADP. After 1 min of irradiation the reaction mixture was centrifuged and determined at 340 nm.

ATP content measurement.

Light-induced ATP synthesis of chloroplasts was measured by comparing the ATP level in the dark and 1 min after illumination [7]. 1 mL reaction mixture contained 0.4 M Sucrose, 50 mM Tris-HCl (pH 7.6), 10 mM NaCl, 5 mM $MgCl_2$, 2mM ADP, 10 mM Na_2HPO4, and intact chloroplasts with 378 µg/mL. After illumination for 1 min, 10 % TCA was immediately added to the illuminated and dark-controlled samples and neutralized with 3 M Na_2CO_3. ATP content was then analyzed by ATP Colorimetric Assay Kit (Biovision).

Enzyme assays related to oxidative stress: Estimation of superoxide dismutase (SOD) content, total peroxide content, glutathione reductase (GR) content, catalase (CAT) content, peroxidase content, polyphenol oxidase content, phenol content, proline content.

Superoxide dismutase (SOD) content was estimated following standard procedure [8]. Fresh tissue was homogenized in a cold chamber maintained 4 °C. In 50 mM Tris-HCl buffer (pH 7.5), containing 0.1 mM EDTA and 10 % polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 10,000 g for 20 mins at 0 °C. The supernatant was used for enzyme assay. The reaction mixture contained of following 80 mM Tris-HCl buffer (pH 8.9) containing 0.12 mM ethylenediaminetetraacetic acid (EDTA) and 10.8 mM N,N,N',N'-tetra methyl ethylene diamine, 3.3 X 10^{-3} % bovine serum albumin, 6 mM nitro blue tetrazolium, 0.6 mM riboflavin in 5 mM potassium hydroxide and enzyme extract. The mixture was illuminated in glass test tubes of uniform thickness. The test tube containing reaction mixture were exposed to light by immersing the test tubes in cylindrical glass container 3/4th of which was filled with water and maintained at 25 °C and placed between 2 fluorescent tube lights, 20 waltz each. The reaction was initiated and terminated by turning the light on and off respectively. Protein content of enzyme was estimated according to Lowry et al (1951) and the enzyme activity was expressed as % inhibition / min/ mg protein.

The enzymatic activity of peroxidase was assayed according to Chance and Maehly[9]. Plant material was extracted in 0.1 M phosphate buffer (pH 7). The homogenate was centrifuged in cold at 10,000 g for 20mins and supernatant was used for enzyme assay after making a definite volume in each set. The reaction mixture contained 0.1 M phosphate buffer (pH 7), 10 % H_2O_2, 0.5 % catechol and enzyme extract. The absorbency of the solution was recorded at 0, 30, 60 seconds after incubation at 420 nm. Soluble protein was estimated according to Lowry et al and enzyme activity was expressed in terms of change in OD at 420 nm/ min/ mg protein.

The total peroxide content of plant tissue was estimated according to the method of Thurman et al [10]. Plant tissue was homogenized with cold 5 % trichloroacetic acid (TCA) at 4 °C and the homogenate was centrifuged at 17000 g at 0 °C for 10 mins. The supernatant was immediately used for estimation of total peroxide by ferrithiocyanate method. Each reaction mixture contained enzyme extract, 50 % TCA, 10 mM ferrous ammonium sulphate, 2.5 M potassium thiocyanate and distilled water. The absorbance of

ferrithiocyanate complex formed was read at 480nm and was compared with a standard curve prepared with known concentration of hydrogen peroxide (H_2O_2). Total peroxide content was expressed in terms of mM peroxide present per gm fresh wt.

Glutathione Reductase was assayed according to Foyer and Halliwell [11]. The supernatant was used for GR assay. The assay mixture contained 50 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 0.15 mM NADPH, 10 mM GSSG and 0.1 mL of crude enzyme extract. The total reaction volume was 1.0 mL. The activity of GR was assayed by monitoring glutathione-dependent oxidation of NADPH at 340 nm.

The activity of CAT was assayed according to Beers and Sizer [12]. Fresh samples were homogenized in 50 mM Tris-NaOH buffer (pH 8) containing 0.5 mM EDTA, 2 % PVP and 0.5 % triton X 100. The homogenate was centrifuged at 22,000 g for 10 min at 4 °C and after dialysis; supernatant was used for enzyme assay. Assay mixture in a total volume of 1.5 mL contained 100 mM potassium phosphate buffer (pH 7), 200 mM H_2O_2 and enzyme extract. The decomposition of H_2O_2 was followed at 240 nm by decrease in absorbance. Enzyme specific activity was expressed as mM of H_2O_2 oxidized/ min / mg protein.

Activity of polyphenol oxidase was assayed according to the standard methods [13]. Plant tissue was extracted in 0.1 M phosphate buffer (pH 7) and centrifuged in cold at 10,000 g for 20 mins. Supernatant was used as enzyme source. Reaction mixture contained enzyme extract, crushing buffer and 0.1 % catechol. Absorbency of the solution was recorded at 0 and 30 min at 480 nm. Specific activity of enzyme was expressed in terms of change in OD at 480 nm/hr/mg protein.

Estimation of proline was done according to the method of Bates et al (1973)[14]. Plant material (both leaf and root) were homogenized with 0.1 ml sulphosalicyclic acid and centrifuged at 5000 g for 30 mins and supernatant was taken. To the supernatant, glacial acetic acid and acid ninhydrin were added for boiling in water bath for 1hr.Then the mixture was extracted with 10ml of toluene in a separating funnel and OD was recorded at 520 nm.The quantity of proline present in tissue was calculated from the standard curve

prepared by OD values of known concentration of proline solutions. Proline content was expressed in terms of µg proline / gm fresh wt.

Phenol content was estimated according to the method of Malik and Singh (1980)[15]. Plant tissue was extracted in 0.3 N hydrochloric acid in methanol and centrifuged twice at 10000 g for 20 mins. Supernatant was then evaporated to dryness. To 0.1 ml of the solution, Folin phenol reagent was added. After 3 mins, 35 % sodium carbonate was added and each set was then allowed to stand for 1 hr. Absorbance was recorded at 630 nm.A standard curve was prepared with known concentration of gallic acid.

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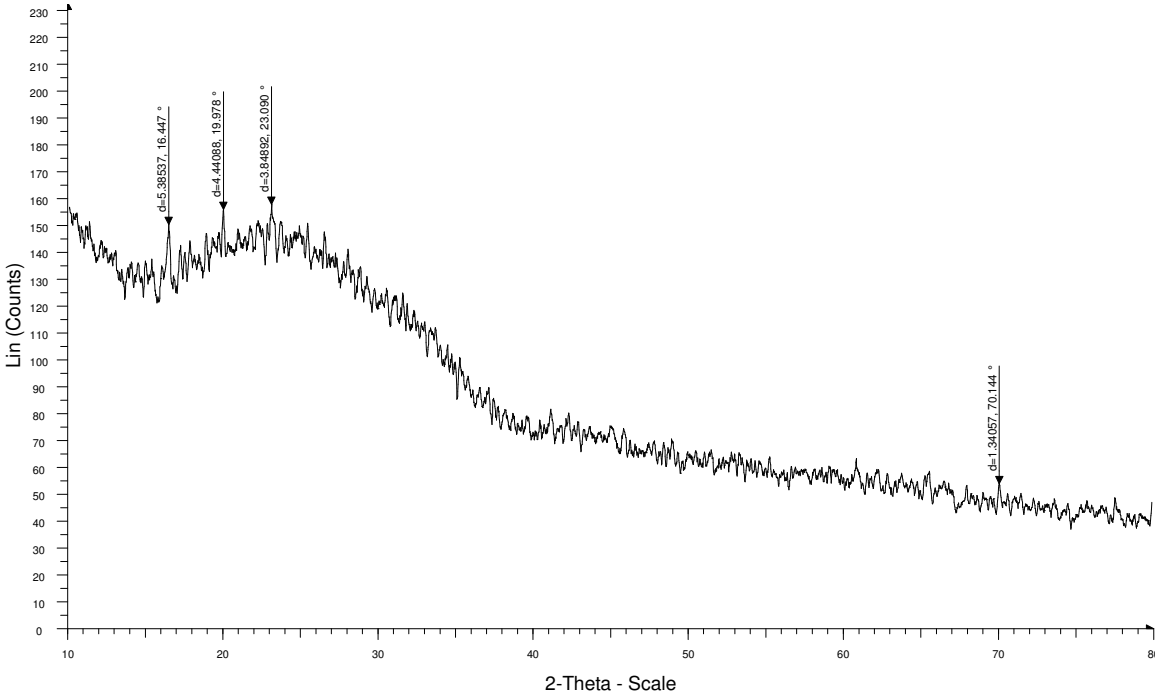
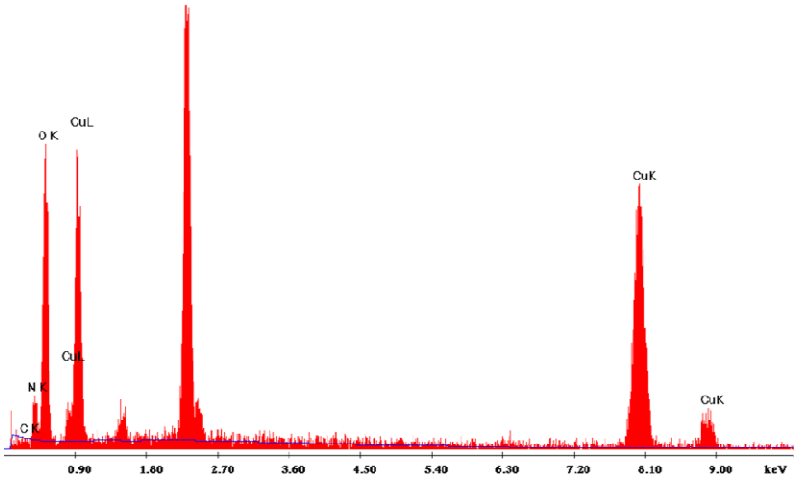
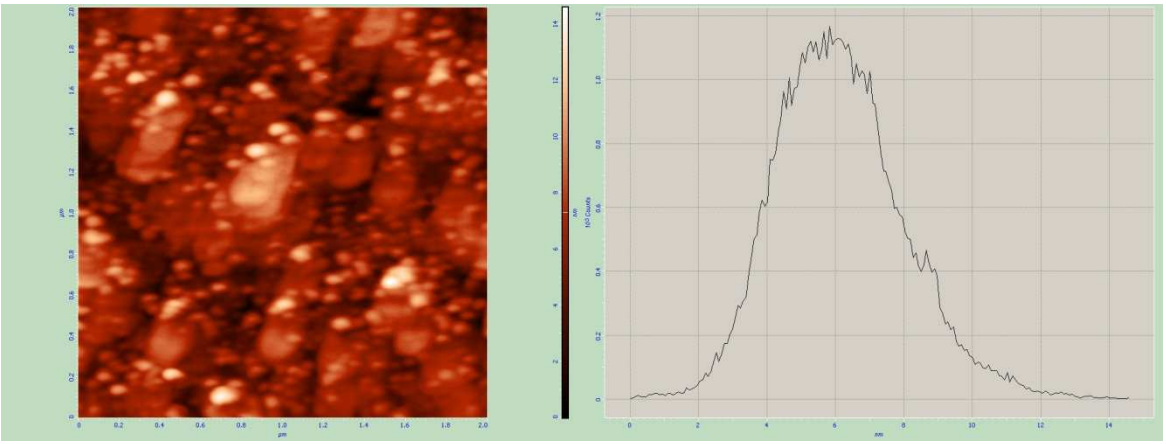


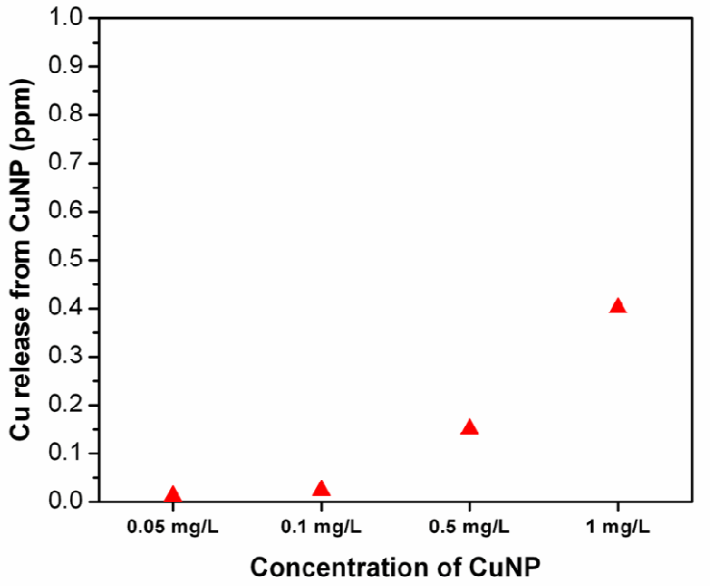
SI Figure S1. XRD pattern showing crystallinity of CuNP nanochain-array. Particles are amorphous in nature.



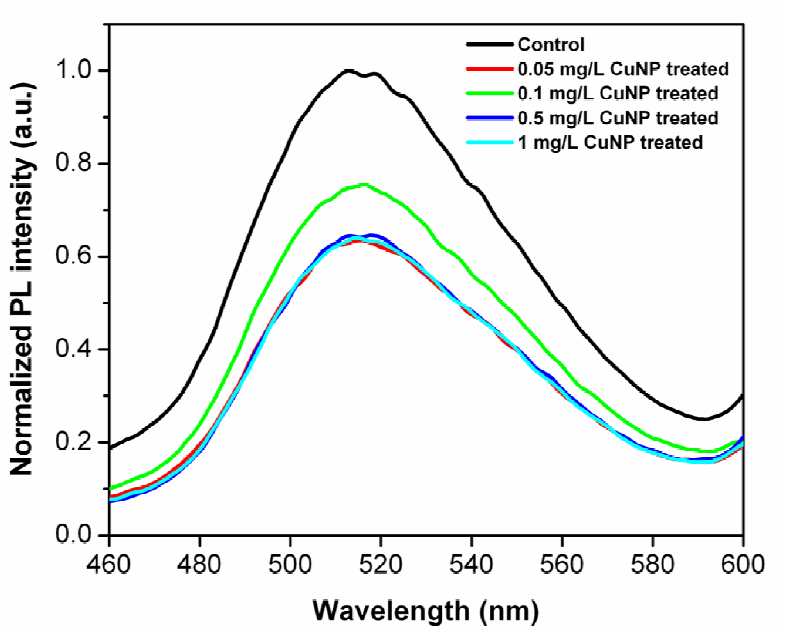
SI Figure S2. EDX analysis of CuNP nanochain-array showing presence of copper in EDX spectra



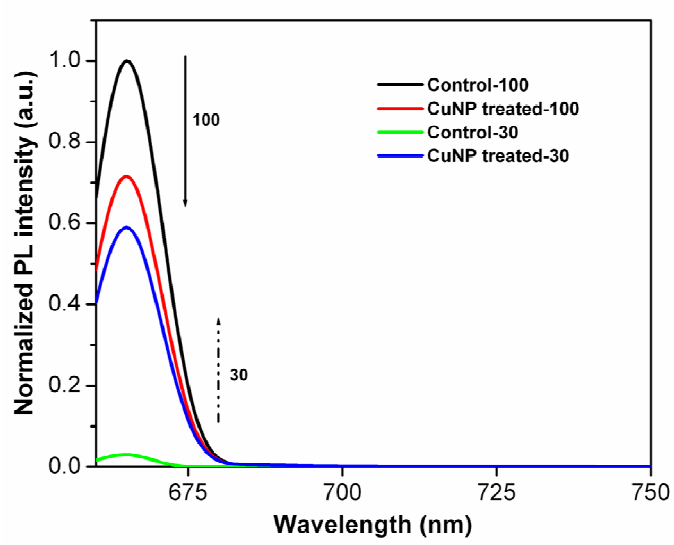
SI Figure S3. AFM image and particle height distribution of CuNP nanochain-array



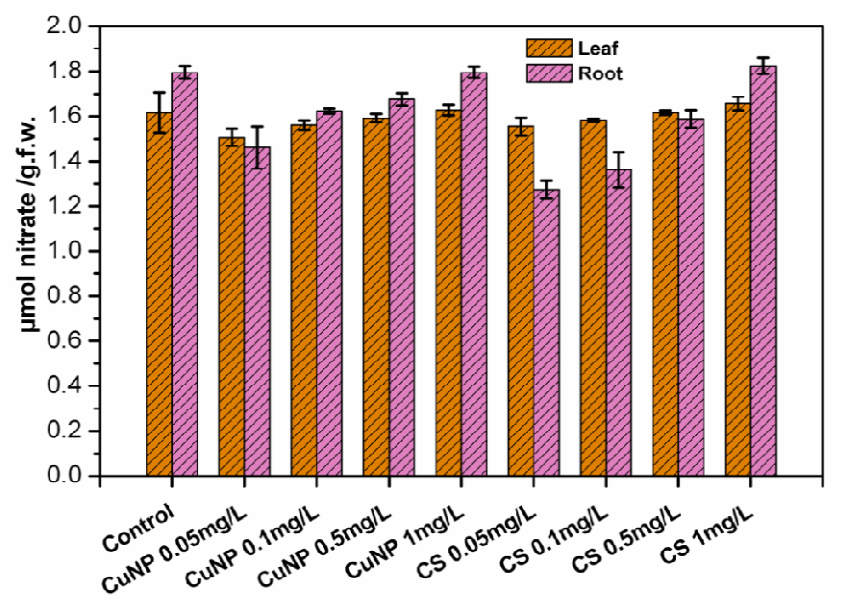
SI Figure S4. Cu release from CuNP by ICP-OES.



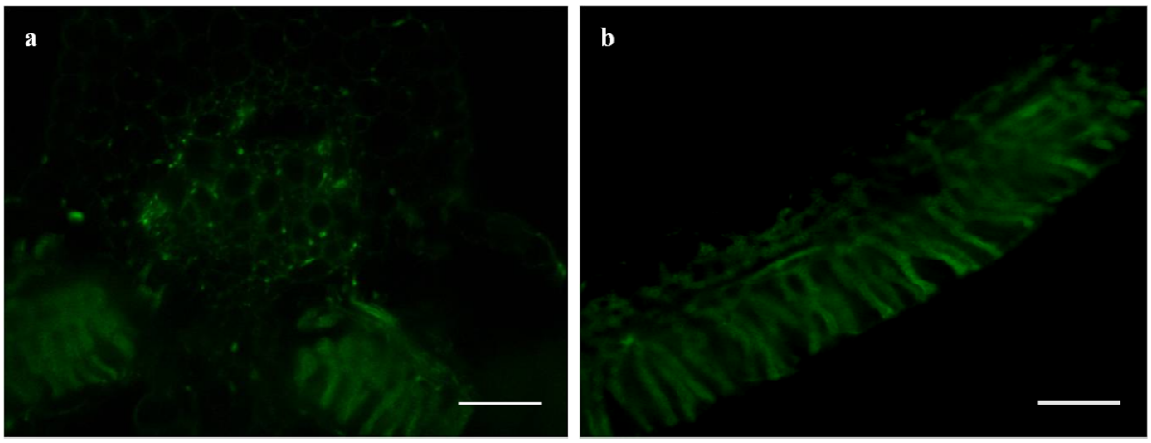
SI Figure S5. PL measurements of control and CuNP treated chloroplast



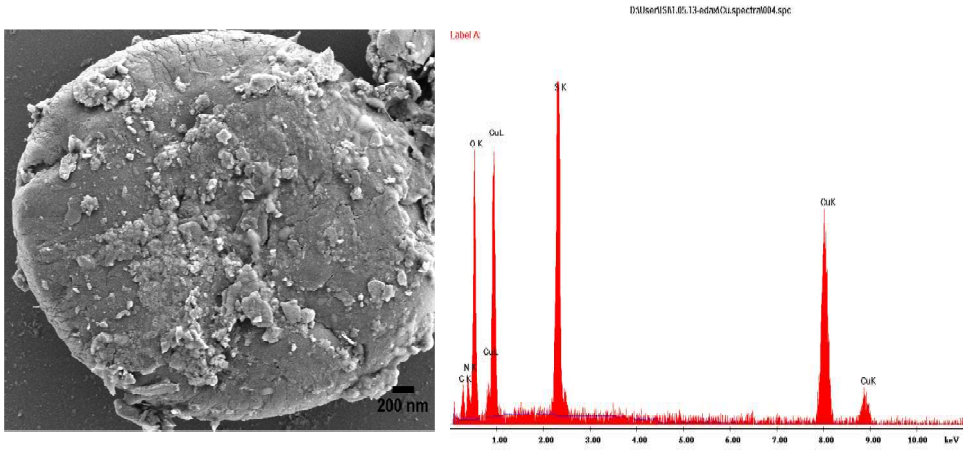
SI Figure S6. PI index measurements of control and treated chloroplasts



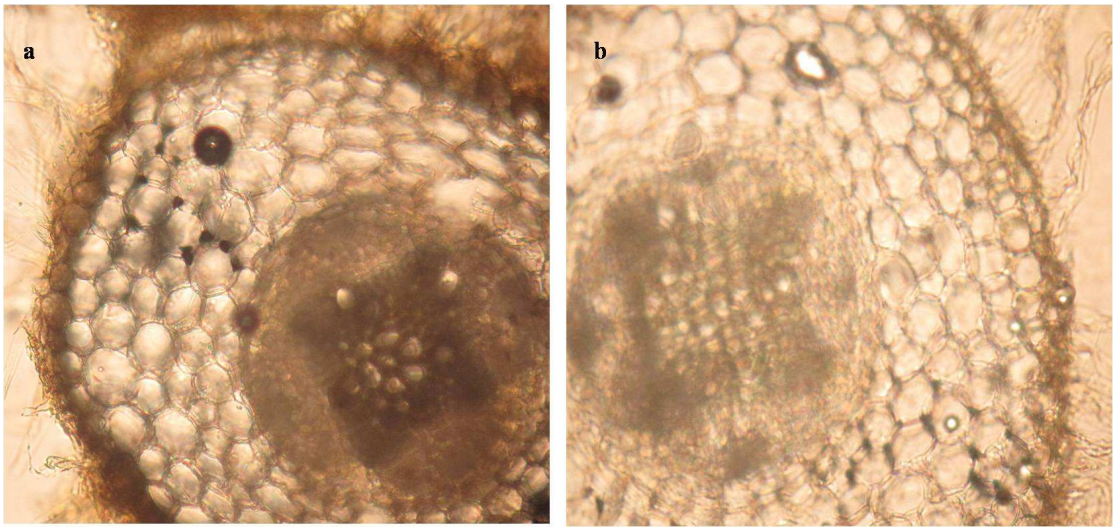
SI Figure S7. Measurement of nitrate content of 15 days treated CuNP and CS treated plants.



SI Figure S8. Bioavailability study of CuNP treated (a) Root (b) Leaf.



SI Figure S9. (a) FESEM image of Chloroplast-CuNP, (b) EDX analysis of the same demonstrating the Cu content.

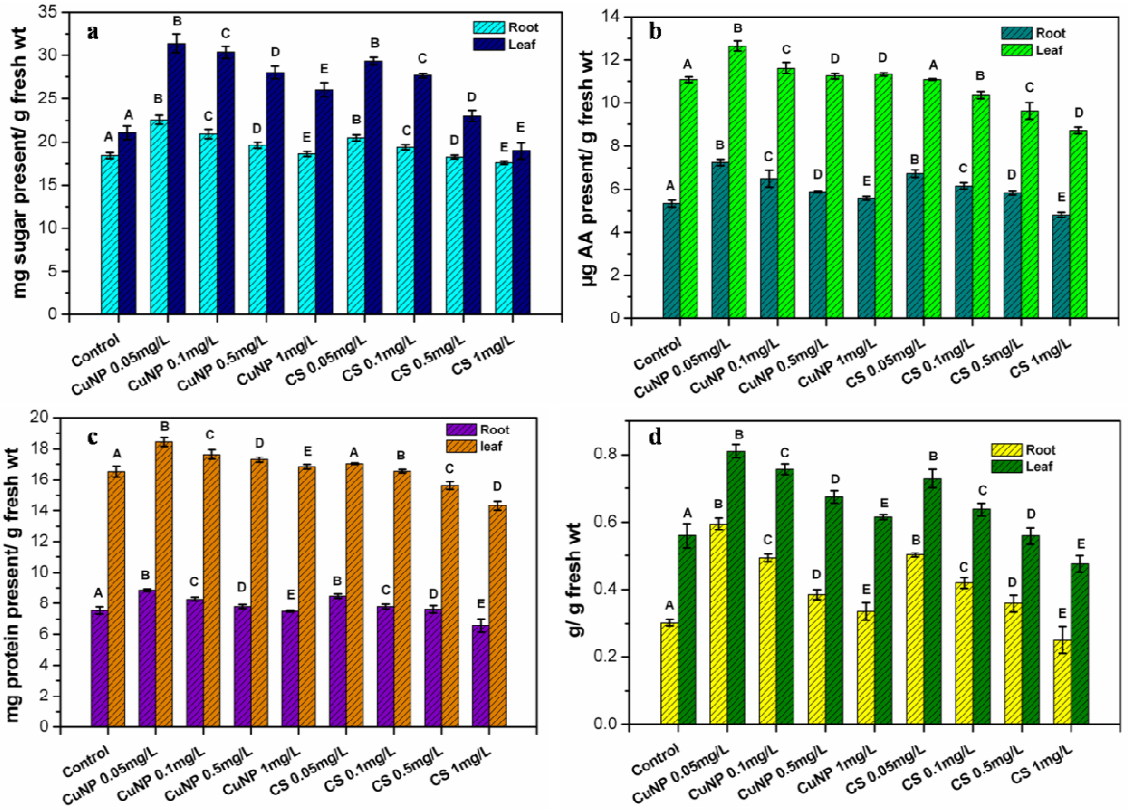


SI Figure S10. Cross sectional light microscopic images of (a) control root sample and (b)CuNP treated root sample.

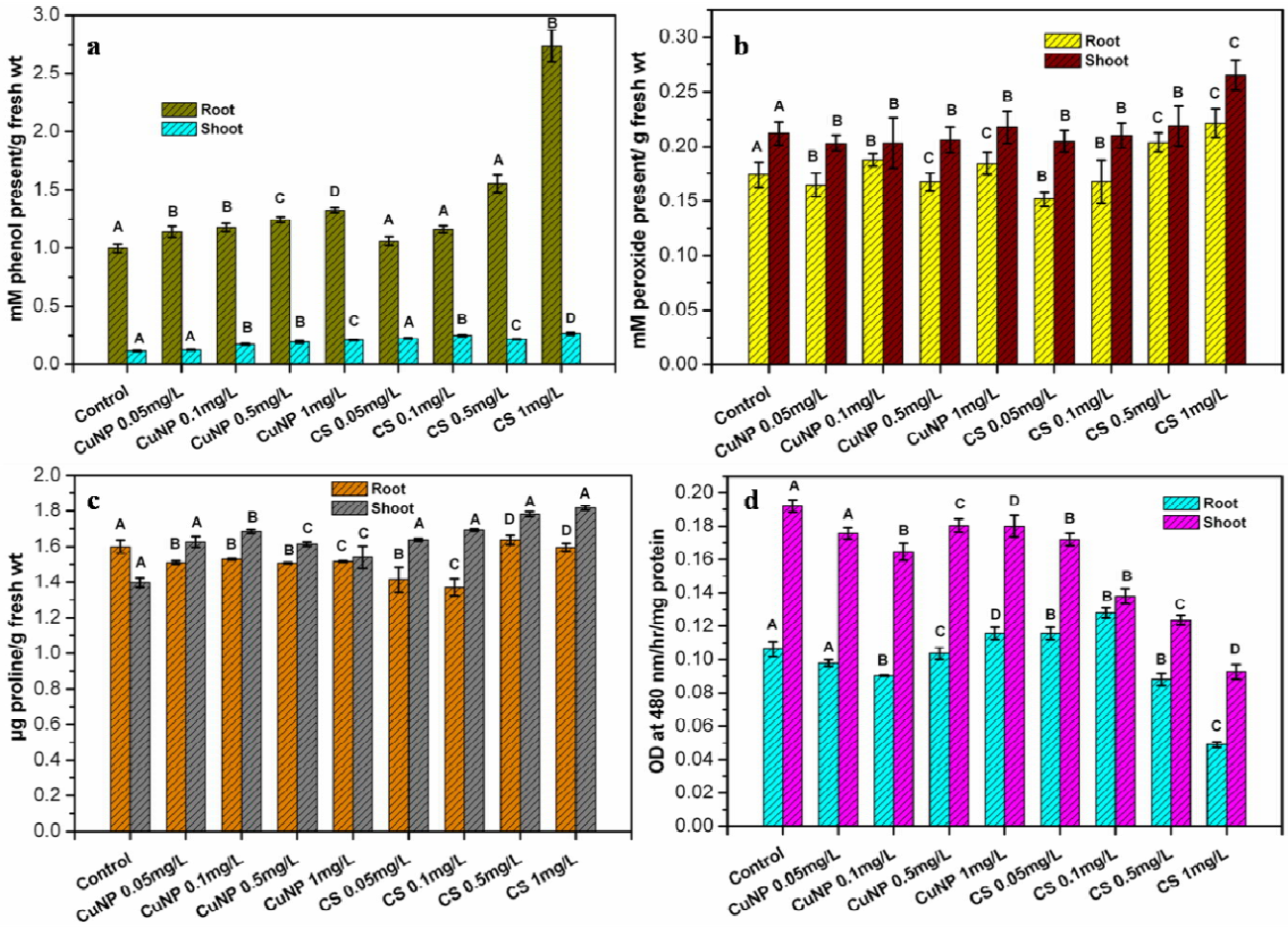
SI Figure S11. Cross sectional light microscopic images of (a) control leaf sample and (b) CuNP treated leaf sample.

SI Table S1: Cu content estimation by ICP-OES

Type of Sample	Cu content in Control (mg/Kg)	Cu content in treatment (mg/Kg)
Root	20.92 ±0.034	21.85 ±0.19
Leaf	23.76 ±0.01	26.49 ±0.45



SI Figure S12. (a) Sugar content, (b) AA content, (c) Protein content, (d) Lipid content of 15days treated plants. Data represent mean ± SE (n, no. of samples = 3). Sugar: root F= 71.3516, P< 0.00001; leaf F= 48.7339, P< 0.0001; AA: root F= 45.878, P< 0.00001; leaf F= 34.0207, P< 0.00001; Protein: root F= 51.6772, P< 0.0001; leaf F= 23.9719, P< 0.00001; Lipid: root F= 67.2403, P< 0.0001; leaf F= 159.5553, P< 0.00001.



SI Figure S13. (a) Phenol content, (b) Total peroxide, (c) Proline, (d) PPO content of 15days treated plants. Data represent mean ± SE (n, no. of samples = 3). Proline: root F= 21.182, P< 0.001; leaf F= 22.766, P< 0.001; total peroxide content: root F= 9.645, P< 0.01; leaf F= 5.795, P< 0.1; polyphenol oxidase: root F= 13.336, P< 0.001; leaf F= 2.140, P< 0.1; phenol: root F= 41.959, P< 0.0001; leaf F= 3.15, P< 0.1.