Manganese Nanoparticles: Impact on Non-nodulated Plant as a Potent Enhancer in Nitrogen Metabolism and Toxicity Study both in Vivo and in Vitro

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Supporting Information

ABSTRACT: Mung bean plants were grown under controlled conditions and supplemented with macro- and micronutrients. The objective of this study was to determine the response of manganese nanoparticles (MnNP) in nitrate uptake, assimilation, and metabolism compared with the commercially used manganese salt, manganese sulfate (MS). MnNP was modulated to affect the assimilatory process by enhancing the net flux of nitrogen assimilation through NR-NiR and GS-GOGAT pathways. This study was associated with toxicological investigation on in vitro and in vivo systems to promote MnNP as nanofertilizer and can be used as an alternative to MS. MnNP did not impart any toxicity to the mice brain mitochondria except in the partial inhibition of complex II–III activity in ETC. Therefore, mitochondrial dysfunction and neurotoxicity, which were noted by excess usage of elemental manganese, were prevented. This is the first attempt to highlight the nitrogen uptake, assimilation, and metabolism in a plant system using a nanoparticle to promote a biosafe nanomicronutrient-based crop management.

KEYWORDS: manganese nanoparticles, nitrogen metabolism, manganism, mitochondrial dysfunction

INTRODUCTION

Nitrogen assimilation is a vital process controlling plant growth and development. Plants biosynthesize both the suite of nitrogenous compounds that are present in all living organisms and many chemical species unique to plants. The most important and rate-limiting step is the assimilation of inorganic nitrogen into organic metabolites catalyzed by a series of enzymes through a high-flux process subjected to extensive regulation in all plants. Nitrogen moves along a complex branching and merging pathway, which interacts at numerous sites with the carbon flow, pH regulation, and ions, assimilating flow at the cell within whole plant level. Superimposed on these metabolic fluxes, nitrate and nitrogen metabolism influences plant development and architecture including changes in root physiology, the timing of senescence, and flowering. The largest requirement of nitrogen is the synthesis of amino acids, which function as the building blocks of proteins as well as precursors to many other metabolites. It is also prerequisite to nucleic acid, which is a cofactor to many physiological processes and a major component of chlorophyll. In addition, several plant hormones contain nitrogen and/or nitrogenous precursors. Plants can take up nitrogen from soil by roots in the form of nitrate ion (NO$_3^-$) and utilize it in nitrogen metabolism. Assimilation of NO$_3^-$ requires subsequent uptake, reduction, and conversion of NO$_3^-$ to ammonium ion (NH$_4^+$) and incorporation to organic compounds. However, the availability of nutrients including nitrogen within the plant system is the limiting factor for its productivity. Henceforth, greater attention is demanded for crop improvement within the realm of nitrogen metabolism in both nodulated and non-nodulated plants. Presently several nanoparticles have been designed to work in agricultural industry by changing their size, shape, and surface functionality. Most of the works demonstrated the improvement of the biocidal activity of a compound so that it can break the resistance of pests and fungi, whereas others showed detrimental roles in plant physiology and growth. Little attention has been paid in crop improvement to modulating the structural or functional properties of existing plant nutrients for the amelioration of plant nutritional deficiency. It has already been established that plant cells have the capacity to execute active transport of NH$_4^+$ during nitrogen assimilation. Some literature has shown that low availability of NO$_3^-$ concentration results from the decrease in the rate of nitrogen assimilation in acidic soil. Meanwhile, manganese salt (Mn), as a fertilizer for nitrogen metabolism, has been used in acidic soil for many years, which also influences the assimilatory process as a cofactor.

In this context, we introduce manganese nanoparticles (MnNP) within the framework that is already known to modulate the photochemical pathways in photosynthesis even better than its elemental/bulk counterpart. MnNP increase the...
activity of the electron transport chain by binding with the CP43 protein chain of photosystem II. They also enhance the oxygen evolution process, being a part of the water splitting complex in light reaction of photosynthesis; as a consequence, photophosphorylation capacity is improved, too. MnNP thus improve the photosynthetic ability of the plants, thereby producing more sugar, and can be useful in the agricultural sector for the improvement of plant productivity. As an afterthought, we have tried to investigate the route of nitrate uptake, assimilation, ammonium assimilation, and further downstream nitrogen metabolism in both source and sink tissues of legumes using MnNP to abate the problems of nitrogen metabolism. Thereafter, nitrogen reductase protein has been isolated from leaf tissue of treated plants followed by protein profiling and Western blot analysis to evaluate the mode of action of MnNP in accelerated nitrogen assimilation. Interestingly, MnNP are found to be more effective by both biochemical and molecular techniques in nitrogen assimilation and metabolism in contrast to their bulk counterpart, manganese sulfate (MS). To the best of our knowledge this is the first effort to use MnNP in the process of nitrogen metabolism for beneficial crop management study.

Mn is an essential trace element for human nutrition but also a toxicant at high concentration. Therefore, its biosafety impact has to be evaluated in light of in vivo experimentation for future research and development studies. As a consequence, MnNP have been injected intravenously to Swiss albino mice, and after 15 days of observation, the animals were sacrificed for biosafety studies following standard guidelines. Mn is also known to induce mitochondrial dysfunction and causes neurotoxicity, called manganism. Manganism is characterized by neuropsychiatric symptoms and extrapyramidal dysfunction such as hypokinesia, rigidity, and tremor that often resembles Parkinson’s disease. Therefore, detailed toxicity profiling of MnNP is required related to mitochondrial dysfunction and neurotoxicity; as a result, the effect of MnNP in energy metabolism and ETC activity in isolated brain mitochondria has been assessed. Biosafety studies with newly synthesized engineered nanoparticles are important because they may be found to be hazardous for biological systems; if it is related to the agricultural sector, then it is necessarily obligatory to profile the entire biosafety experiments, which ensure the complete screening of the particles in mammalian systems. In a recent work MnNP have been found to decimate the level of dopamine, imparting mitochondrial dysfunctioning and generation of more ROS in vivo as a result of metal toxicity in PC-12 cells such as free Mn2+ ions. Besides, there are several studies on metal toxicity performed in cell lines and mammalian systems to access the biological importance and environmental hazard measurements. In our previous paper we have reported that our custom-made MnNP are biosafe on the basis of acute oral toxicity parameter, although detailed research is explicitly demanded for its future endeavor in agricultural sectors. Herein, histopathological analysis of MnNP-treated mice is performed after intravenous injection with emphasis on brain mitochondrial toxicity studies. A series of detailed in vitro and in vivo studies including studies on human lymphocytes, hemolysis, and murine model system with an emphasis on brain mitochondria has been carried out. Our studies reveal that severe toxicity symptoms are avoided in the treated sets with minor alterations noted in complex II–III activity in ETC. Excess oxidative stress, severe mitochondrial dysfunction, and neurotoxicity are avoided, thereby paving the way toward the development of a biosafe crop management study based on nitrogen assimilation and metabolism with the aid of MnNP.

### MATERIALS AND METHODS

#### Characterization of MnNP

Custom-made manganese nanoparticles were purchased from MK Impex, Canada. The surface morphology was determined by field emission scanning electron microscope (FESEM; FEI Quantum-200 MX-2), and particle size was observed through a transmission electron microscope (TEM) (JEOL 2010). Surface topology of MnNP was obtained using atomic force microscopy (AFM). Hydrodynamic size distribution was obtained by dynamic light scattering (DLS) spectroscopy.

#### Experiments with Plant Model System

Seeds of mung bean (Vigna radiata) were purchased from Berhampur Pulse and Oil Research Centre, West Bengal. These seeds were selected from Chhattisgarh. Seeds were soaked in 5% sodium hypochloride solution for surface sterilization and imbibed with the treatment solutions (control; MnNP, 0.05, 0.1, 0.5, and 1 mg/L; MS, 0.05, 0.1, 0.5, and 1 mg/L) for a minimum of 4–6 h prior to germination for various analyses. After 24 h of germination, seeds were planted in pots filled with perlite supplemented with Hoagland’s solution with or without manganese solution (control plants were treated without manganese solution) for 15 days in a growth cabinet (GC-300, Lab companion) with 14 h daytime temperature of 25 °C and night temperature of 20 °C at a relative humidity of 40–60% and a light intensity of 440 μmol/m2/s. After 15 days of treatments, plants were uprooted and used to carry out further experiments.

#### Preparation of Leaf and Root Enzyme Extract for the Assays of Nitrogen Metabolism

Plant samples were ground with 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 DDT, and 1% (w/v) insoluble PVPP at cold. The homogenate was filtered and centrifuged at 3000g for 5 min. Supernatant was then centrifuged at 30000g for 20 min. The extract was then used for enzymatic assays.

- **Determination of NO3**−**.** Enzyme extract was added to 10% salicylic acid solution dissolved in 12 N sulfuric acid (w/v). NO3− content was measured as described by Cataldo et al.

- **Determination of Nitrate Reductase (NR) Activity.** The reaction mixture contained 100 mM phosphate buffer (pH 7.5), 100 mM KNO3, 10 mM cysteine, 2 mM NADH, and enzyme extract. After 30 min of incubation at 30 °C, reaction was stopped by 1 M zinc acetate. The nitrite formed was determined at 540 nm after azo coupling with sulfanilamide and naphthylethylene diamine dihydrochloride. The NR activity was expressed as micromoles of NO3− formed per gram fresh weight per hour.

- **Determination of Nitrite Reduction (NiR) Activity.** The reaction mixture contained 50 mM phosphate buffer (pH 7.5), 20 mM KNO3, 5 mM methyl viologen (MV), 300 mM NaHCO3, and enzyme extract. After 30 min of incubation at 30 °C, NR activity was determined by the disappearance of NO3− from the reaction medium and expressed as micromoles of NO3− per gram of fresh weight per hour.

- **Determination of Glutamine Synthetase (GS) Activity.** Enzyme extract was mixed with the reaction mixture containing 100 mM phosphate buffer (pH 7.5), 4 mM EDTA, 1 mM sodium glutamate, 450 mM MgSO4.7H2O, 300 mM hydroxylamine, and 100 mM ATP. Two controls were prepared, one without glutamine and another without hydroxylamine, and all of the treatments were incubated for 30 min at 28 °C. 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 aminoxyacetate, 1.5 mM NADH, 75 mM L-glutamate, and enzyme extract. Two controls, without ketoglutarate and without glutamine, were used to correct for endogenous NADH oxidation. The decrease in absorbance was recorded for 5 min at 28 °C. The activity was expressed as micromoles of NAD per gram fresh weight per hour.
SDS-PAGE and Western Blot of Isolated NR Protein. NR protein was purified from mung bean leaves following a standard procedure.26 NR protein was dissolved in a buffer containing 0.09% (w/v) n-decyl-β-D-maltopyranoside (β-DM), 20 mM NaCl, 70% (v/v) glycerol, and 20 mM HEPES (pH 7.5). After isolation of the protein, 12% SDS-PAGE was performed using a Bio-Rad gel apparatus, and subsequent Western blot analysis was also carried out by using NR antibody (Agrisera).

Experiments with Mouse Model System. Healthy young nulliparous, Swiss albino mice (average body weight = 20 g) of 8 weeks of age were kept in the animal house at 22 ± 2 °C, 60 ± 10% relative humidity, and 12 h light/dark cycle. The animals were kept in clean polyplyene cages and were provided with commercial rat pellet diet and deionized water. After 7 days of acclimatization, the mice were randomly assigned to control and treatment groups. There were five mice of each sex in each group. Water as control and MnNP as treatment were injected intravenously, and animals were weighed before and after the completion of the experiment. The animals were kept under close observation for 14 days. Skin and fur changes, eye secretion, and behavior patterns of the mice were observed. Special attention was paid to the clinical signs of toxicity including tremors, convulsions, salivation, nausea, vomiting, diarrhea, lethargy, etc. At the end of 14 days, they were sacrificed. Blood and serum from control and treated mice were analyzed for total count (TC), differential count (DC), platelet count (PLT), lactate dehydrogenase (LDH), creatinine, alkaline phosphatase (ALP), total protein (TP), cholesterol, triglyceride (TG), uric acid, and blood urea nitrogen (BUN), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), and phosphorus. Brain, heart, lungs, liver, stomach, kidney, spleen, uterus, and testis were carefully removed and fixed in 10% formalin solution containing neutral phosphate-buffered saline (PBS). Thereafter, the organs were embedded in paraffin, stained with eosin–hematoxylin, and examined under a light microscope.

Isolation of Mouse Brain Mitochondria. Mouse brain mitochondria (after 24 h of treatment) were isolated by a method based on differential centrifugation with digitonin treatment. The final mitochondrial pellet was suspended in isotonic buffer A (145 mM KCl, 50 mM sucrose, 1 mM EGTA, 1 mM magnesium chloride, 10 mM phosphate buffer, pH 7.4) and used immediately for the experiments related to measurement (after 24 h of treatment) were isolated by a method based on di

Measurement of Respiratory Chain Activity of Isolated Brain Mitochondria after MnNP Treatment. The activity of NADH–ferricyanide reductase (complex I) was assayed by using ferricyanide as the electron acceptor in a system containing 0.17 mM NADH, 0.6 mM ferricyanide, and Triton X-100 (0.1% v/v) in 50 mM phosphate buffer, pH 7.4, at 30 °C. The reaction was initiated by the addition of mitochondrial suspension (10–30 μg of protein) to the sample cuvette, and the rate of oxidation of NADH was measured by the decrease in absorbance at 340 nm.

The activity of succinate cytochrome c reductase (complex II–III) was assayed by following the succinate-supported reduction of ferricytochrome c to ferrocytochrome c at 550 nm in an assay mixture containing 100 mM phosphate buffer, 2 mM succinate, 1 mM KCN, 0.3 mM EDTA, and 1.2 mg/ml cytochrome c in a total volume of 1 mL. The reaction was initiated by adding mitochondrial suspension (10–30 μg) into the sample cuvette.

The activity of cytochrome c oxidase (complex IV) was assayed by measuring the rate of decrease in absorbance at 550 nm at room temperature following the oxidation of reduced cytochrome c (50 μM) in 10 mM phosphate buffer, pH 7.4. Ferricyanide (1 mM) was added to oxidize ferrocytochrome c in the blank cuvette and the reaction initiated in the sample cuvette by the addition of mitochondrial suspension (10–30 μg). The activity of the enzyme was calculated from the first-order rate constant.

(a) Measurement of Mitochondrial Respiratory Control Ratio (RCR). Polargraphic study of oxygen consumption using freshly isolated mitochondria allows measurement of respiratory control ratios (RCR) calculated as the ratio of state III to state IV. The chamber of the oxygraph was filled with fresh respiratory buffer along with ADP, glutamate, pyruvate, maleate, succinate, and DNP. A low (state IV) oxygen consumption rate was achieved. After that, 100–200 nmol with or without treated mitochondria was added for inducing state III respiration. After 2 min, the data were recorded, and RCR was calculated accordingly.

(b) Measurement of Reactive Oxygen Species (ROS). ROS generation in treated and untreated brain was determined using the nitrotetrazolium blue (NBT) reduction assay.28 The assay is based on the reduction of yellow water-soluble NBT powder to a blue insoluble substance upon reduction. The treated and control brains were incubated in NBT solution for 1 h. The reaction was stopped by the addition of acetic acid solution. After centrifugation (1 min, 12000g), intracellular reduced content was solubilized by adding 50% (v/v) acetic acid to the cell pellet followed by vortexing for 5 min. Cell debris was then pelleted, and the absorbance of the supernatant was determined at 595 nm using a UV–vis spectrophotometer.

(c) Protein Estimation. The protein was estimated after the membranes had been solubilized in 1% SDS according to Lowry’s method.

Experiments with Lymphocytes. Isolation of Lymphocyte from Blood. Human peripheral blood was obtained by venipuncture from healthy volunteers into heparinized vacutainers. Lymphocytes were isolated from fresh blood according to the method of Boyum et al.29 with some modifications using Histopaque.30 The cells were washed with PBS and resuspended in RPMI-1640 medium at a concentration of 10⁶ cells/ml for further use.

Treatment of Lymphocytes. Freshly isolated human lymphocytes were treated with different concentrations of MnNP and incubated for at least 3 h at 37 °C in RPMI-1640 medium. After incubation, cytotoxicity studies were carried out with treated lymphocytes.

(a) Cell Proliferation Assay (WST). The number of mononuclear cells remaining alive after reacting with the cellular mitochondrial dehydrogenase could be quantified by using a Biovision cell proliferation assay kit by measuring the absorbance of the dye solution at 440 nm. The formazan dye is produced by the breakdown of tetrazolium salt, and the amount of dye generated by the activity of dehydrogenase was directly proportional to the number of living cells.
Hemolysis. To perform in vitro hemolysis experiments, human blood was centrifuged twice at 3000 rpm for 15 min to remove residual plasma from the erythrocyte-enriched fraction of blood. RBCs were washed three times with PBS, pH 7.4, and resuspended in the same buffer. Six hundred and ninety microliters of the above RBC stock was suspended with 10 μL of MnNP treatments (1, 10, and 25 mg/L) along with control, and then the volume was adjusted to 1.5 mL with PBS for each of the treatments. The cells were then centrifuged again at 3000 rpm for 15 min at 4 °C and washed three times with PBS before measurement at 540 nm in a UV−vis spectrophotometer.32

Statistical Analysis. We tested for the effect of MnNP and MS for different biochemical and biosafety features. For each of the biosafety experiments, we considered three levels of concentration of the nanoparticles, 1, 10, and 25 mg/L, and compared the effects of the various concentrations with respect to the control and also relative to each other. For each concentration in the design, we had three replicates and so, the total numbers of experiments conducted for a specific nanoparticle were n = 12 (3 replicates for the 4 levels: control and 1, 10, and 25 mg/L). Although the data size was small, from a biological point of view, normality of the replicate data for each experiment could be assumed. Because there were three replicates for each concentration, two errors were attached to each concentration with a total error degrees of freedom of 8. The degrees of freedom for the prediction space was (number of levels − 1) 3. Therefore, in an ANOVA table, we performed the Fstatistic distribution and calculated the P value corresponding to this underlying distribution. In the case of biochemical assays of plants, four of each MnNP and MS concentrations were taken into account along with the control, so we had nine replicates altogether and for this case n = 27 (control, four concentrations of each MnNP and MS).

If the ANOVA F test was found to be significant, then the next step would be to check which level significantly affected the response, thereby influencing the F test to reject the null. This was achieved by using Tukey’s post hoc called HSD (honestly significant difference) test. We used alphabetic labels to check if one level was significantly different from the previous one as concentrations of the nanoparticles were considered as ordered factors. We also observed the box plot response for various levels to get a better indicative picture.

## RESULTS

Characterization of MnNP. The morphology of MnNP was investigated by using FESEM and TEM images. Cubic-shaped MnNP of size around 20.88 ± 0.44 nm were more or less homogeneously distributed as revealed from FESEM (Figure 1a) and TEM (Figure 1b) images, respectively. It was previously mentioned that the surface of MnNP contained hydroxyl groups, which contributed to their hydrophilic character. Hydrophilic character was one of the key features of MnNP to study their interaction with plant systems.14 Although due to the higher reactivity and oxophilicity of Mn,33 synthesis of MnNP was quite difficult; however, these custom-made MnNP were stable for a long time. Hydrodynamic radius measurement revealed a hydrodynamic radius of around 101.5 ± 13.395 nm, which changed insignificantly over time (Supporting Information (SI) Figure S1). Surface topology of MnNP was obtained from an AFM micrograph (Figure 1c), which justified the average 10 point height of around 6 nm. Chemical purity of the nanoparticles was determined by EDX analysis (SI Figure S2), where Mn was the major component in the sample powder.
Aqueous dispersion of MnNPs was fairly stable. No significant change in the hydrodynamic radius was noted up to 24 h under experimental condition. Even its XRD pattern was unchanged in aqueous dispersion after 24 h, which corroborated our previous report (SI Figure S3). To produce some response in nitrogen assimilation and metabolism, MnNP should be bioavailable to the root system. In our previous study we have already demonstrated the bioavailability of MnNP in roots through confocal microscopy; therefore, bioavailable MnNP on the root system could execute some response, which also triggered us to focus on a series of biochemical and molecular assays related to nitrogen assimilation and metabolism discussed below. Small-sized MnNP resulted in a better bioavailability with their nanosized effect, which was also consistent with the literature.

**Effect on Nitrogen Metabolism in Treated Plants.** The maximum NO$_3^-$ concentration was observed at the highest dose of MS in both root and leaf tissues, whereas a significant decrease in NO$_3^-$ concentration was noted in all of the MnNP-treated sets as shown in Figure S4 (SI). The amount of NO$_3^-$ present in the root was more than in the foliar tissue in all treatments. However, there were increases in both NR and NiR activities in root and leaf tissue of MnNP-treated plants in all treated sets, although the 0.05 mg/L MnNP dose was the most effective (Figure 2a,b). In non-nodulated plants activity of NR-NiR is the initial step for nitrogen metabolism, which is also a determining factor for the assimilation of inorganic nitrogen to organic metabolites in living tissues. Interestingly, NO$_3^-$ concentration in 0.05 mg/L MnNP treatment was lowest but NR and NiR activities were both maximum in the aforementioned dose. MnNP possibly influenced the process of enzymatic reduction during the course of assimilation; hence, NR and NiR activities were enhanced. The responses of GS and GOGAT, in the synthetic process of AA from the assimilated product of NR-NiR activities (Figure 2c,d), were similar. Maximum activity was found in 0.05 mg/L MnNP treatment in both root and leaf tissues, although functions of all of these enzymes were much greater in root tissue compared to foliar tissue. In all of the experiments, the highest doses of MS were found to be toxic to the plant tissues as expected. Elevated NR activity was further confirmed by protein profiling and Western blot techniques shown in Figure S5 (SI). NR, a homodimer or homotetramer of 110 kDa protein, is present in shoot mesophyll cells and is tightly regulated to reduce nitrate to nitrite. It plays a pivotal role in the nitrogen assimilatory pathway in plants. It was interesting to note that NR activity was increased biochemically; the result was further confirmed by SDS-PAGE and subsequent Western blot analysis of the protein. In both cases MnNP-treated plants showed enhanced activity with respect to control. This justified our hypothesis that MnNP modulated the activity of NR protein, the first assimilatory enzyme and also presumptive candidate responsible for nitrate inhibition step of nitrogen fixation. They bind to and inhibit nitrogenase and leghemoglobin,.
simultaneously increasing the activities of all interlinked proteins in the course of nitrogen metabolism.

**Toxicity Study of MnNP with Mouse Model System.** MnNP was injected intravenously at different doses, and the mice were sacrificed after 15 days of observation. No significant changes were noted in general appearance or weight gain between control and treated mice during the observational period (SI Table 1). Treated animals did not show histological abnormalities except in brain tissue at very high doses (SI Figure S6), and even pathological changes (SI Table 2) were negligible. Thus, MnNP were considered to be safe at the recommended dosage after 15 days of treatment, which justified its biocompatibility for future perspectives. Anomalies in brain tissues at higher MnNP concentration forced us to conduct detailed brain mitochondrial assays as it might trigger manganism to the mammalian system. In all toxicity studies we have only accounted for MnNP, as MS is a known toxicant to mammalian systems at experimental doses according to the literature.15,16

**Experiments with Mouse Brain Mitochondria.** As shown in Figure 3a, there was a dose-dependent loss of mitochondrial membrane potential after MnNP treatment, although significant inhibition was observed at the highest dose of 25 mg/L. A gradual increase in inorganic phosphate utilization was observed in dose-dependent manner with MnNP treatment shown in Figure 3b. Complex I activity of treated brain mitochondria (Figure 3c) was not significantly altered, but the activities of complex II–III and complex IV were significantly increased at the highest dose of MnNP treatment (Figure 3d,e). MnNP at much higher doses might impair the activity of complex II–III and/or complex IV, resulting in a reduction in energy conversion through electron transport chain (ETC). RCR was left unaltered even after MnNP treatment, showing no negative influence in the respiratory process as shown in Figure 3f. However, more ROS were found to be generated at 25 mg/L MnNP treatment (Figure 4), whereas the lowest dose (1 mg/L) showed no significant change in ROS level with respect to control.

**Experiments with Lymphocyte and Hemolysis.** Figure 5a represents the cytotoxicity of mononuclear cells by MnNP treatment; minor alteration was noted in treated sets with respect to control.

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**Figure 3.** Effect of MnNP on mice brain mitochondria. (a) Mitochondrial membrane potential. (b) Inorganic phosphate utilization. \( F = 76.18, P < 0.0001 \). (c) Complex I activity. \( F = 33.41, P < 0.0001 \). (d) Complex II–III activity. \( F = 746.35, P < 0.00000001 \). (e) Complex IV activity. \( F = 197.69, P < 0.0000001 \). (f) RCR. \( F = 43.07, P < 0.0001 \). Within each type of treatment, mean data (±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 4.** Effect of MnNP on ROS content of mice brain mitochondria: \( F = 27.79, P = 0.0001 \). Within each type of treatment, mean data (±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).
to control. Meanwhile, in the case of NO content (Figure 5b), LDH cell viability assay (Figure 5c), and ROS generation (Figure 5d), no significant alterations were noted between control and treated sets. Together MnNP did not impair dose-dependent cytotoxicity in any of the above-mentioned enzymatic assays expect in ROS content, although cytotoxicity was seen at higher doses in treated lymphocytes. Similar results were noted in hemolysis experiments. No significant hemolysis was noted in any of the treated sets (SI Figure S7); the highest dose of MnNP treatment (25 mg/mL) exhibited 5.31% hemolysis, which was comparable with the control one (4.96%).

**DISCUSSION**

This study revealed that MnNP might modulate the nitrogen metabolism process and thereby could be used as a suitable alternative for MS. MnNP, because of its size and properties, was absorbed readily by the root from soil and transported to the leaf, where it acted as a cofactor in the series of enzymatic reactions during the assimilation of nitrate salt into organic nitrogen compounds. That was probably the reason it did not participate in the process of NO$_3^-$ uptake and the level of NO$_3^-$ in the plant tissue was not influenced by uptake of MnNP. Nitrogen metabolism in plants is a linear pathway that involves the uptake and subsequent transport of nitrate within plant tissues, followed by nitrogen and ammonium assimilation and ultimately amino acid and protein synthesis. However, there had been complexities related to storage and remobilization of nitrate in different parts of the plants: de novo ammonium assimilation and its recycling in the course of photorespiration,$^{36}$ unpredictable interactions with carbon assimilatory pathways, which allowed malate to inhibit alkalinization$^{37}$ and also 2-oxoglutarate to act as the primary acceptor of ammonium in GOGAT pathways.$^{38}$ These complexities made it difficult to develop new methodologies and innovations to enrich the status of nitrate assimilation within this complex biological network.

The first step in nitrogen metabolism was the reduction of NO$_3^-$ to NO$_2^-$ in the cytosol by NR. MnNP at all doses directly or indirectly increased NR activity in foliar tissue with respect to control, whereas no significant alterations were noted in root tissue even at the highest dose. Therefore, MnNP could influence the entry of NO$_3^-$ to the cells, resulting in the hyperpolarization of the plasma membrane. The influence of MnNP over NR activity was supported by biochemical and molecular techniques. Enhanced expression of NR protein after MnNP treatment with respect to the control validated our speculation that MnNP could enhance protein activity. This in turn improved nitrogen status in the mung bean crop. It might stimulate NR activity, which was further validated by the enhanced activity of NiR, an enzyme required for the conversion of NO$_2^-$ to NH$_4^+$ in the cytosol. Both NR and NiR were induced by the same factors, explaining their similar responses to the micronutrient treatments.

Plants assimilate inorganic nitrogen into nitrogen-transport amino acids (AAs), glutamate and glutamine, which are destined to transfer nitrogen from source to sink tissues and to build up reserves during periods of nitrogen availability for subsequent use in growth, defense, and reproductive processes. It also

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**Figure 5.** Effect of MnNP on human lymphocyte cells. (a) Cytotoxicity assay by WST method. $F = 10.57, P < 0.01$. (b) NO content. $F = 197.69, P < 0.000001$. (c) Cell viability assay by LDH method. $F = 10.57, P < 0.01$. (d) ROS generation. $F = 21.26, P < 0.001$. Within each type of treatment, mean data ($±$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).
decimated the level of ammonium toxicity. The immediate step thereafter was to convert NH$_4^+$ to amino acid with the help of glutamine synthetase (GS) and glutamate synthase (2-oxoglutarate aminotransferase, or GOGAT). These two very important enzymes are involved in the primary assimilation of inorganic nitrogen from soil along with reassimilation of free ammonium into the plants. GS catalyzed the ATP-dependent assimilation of NH$_4^+$ into glutamine using glutamate as a substrate. Mn is a known cofactor in this reaction, and thereby the presence of MnNP triggered more GS activity and also the activity of GOGAT. Because GOGAT was known to be stimulated by elevated plastid levels of glutamine during ammonium assimilation, GOGAT was required to produce 2-oxoglutaramate, which ultimately produced dicarboxylic acids as carbon skeletons for glutamate, aspartate synthesis, and their corresponding amides. A couple of studies demonstrated the influence of high GS activity on vegetative growth and photosynthesis upliftment; even earlier flower and seed development were also observed under accelerated GS activity. MnNP triggered enhanced GS activity; thereby, it may function as an enzymatic cofactor in nitrogen assimilation in non-nodulated plants. It might work as a boon for agriculture as it was able to overcome the drawbacks of the commercially available MS salt at higher doses.

MnNP was already established as a micronutrient fertilizer over commercially available MS, but it was well recognized that excess use of Mn salt could induce manganism; therefore, the toxicity issues associated with MnNP could not be ruled out. As a consequence, we carried out a series of detailed toxicological evaluations on mononuclear cells and murine model system at relatively high dosages (1, 10, and 25 mg/mL) in contrast to the one used in nitrogen assimilation and metabolism. In murine model system MnNP was injected intravenously, and after 15 days of observation, mice were sacrificed for histopathological evaluation. From the results we confirmed that MnNP was biosafe for further implementation, although minor anomalies were noted in brain tissues. Therefore, we deliberately carried out a brain mitochondrial assay in the murine model system in light of manganism. The assays were carried out after 24 h of injection followed by isolation of brain tissues. In all of the experiments it was observed that 25 mg/L MnNP partially impaired the activity of complex II−III, but it had no effect in mitochondrial complex I, complex IV protein domains, and RCR activity. This justified that MnNP limited the efficiency of brain mitochondrial functions to some extent but did not hamper the entire physiological process. There was no loss of mitochondrial membrane potential at any of the dosages; increased phosphorylation capacity ensured no loss of phosphate utilization and hence ATP synthesis. The key function of the mitochondrial ETC was to supply reducing equivalents because the majority of electrons were provided by NADH. In our experiments, MnNP did not show any alteration in complex I activity of brain mitochondrial ETC like it did with its elemental counterpart. Therefore, MnNP could easily overcome the problem of inactivation of energy metabolism leading to mitochondrial dysfunction influenced by Mn salts. Partially inhibited complex II−III activity decreased electron transfer activity leading to ROS by leaking electrons from ETC complexes during respiration, particularly in complex III.

In addition, a detailed in vitro cytotoxicity experiment on human lymphocytes was carried out using MnNP of aforementioned dosages. No significant cytotoxic response was found in any of the treated sets with respect to control as noted from our detailed systematic evaluation. These results were consistent with the in vivo experiments mentioned earlier, which signified the biocompatibility of MnNP for biological perspectives. Interestingly, hemolysis experiments justified its biocompatibility as well, which also corroborated the results noted for mononuclear cells.

To the best of our knowledge, this is the first study in which MnNP has been used to enhance nitrogen metabolism in a plant model system. MnNP probably stimulated the activity of NR directly or indirectly as noted from biochemical and molecular techniques. They also enhanced the function of both GS and GOGAT enzyme activities, being an active cofactor in the biochemical reactions. They not only surpassed the difficulties raised due to low availability of inorganic ions during nitrogen assimilation but also alleviated enzymatic activities of the same physiological process. Hence, MnNP can be used as fertilizer for both carbon and nitrogen assimilatory processes. Biosafety experiments were carried out in both mouse and human mononuclear cells. Brain mitochondria of treated mice were isolated to check functional activities in respiration and energy production. Results demonstrated that MnNP were fairly biosafe, although minor toxic responses were noted at a very high dosage. We have good reason to believe that MnNP could be used as a fertilizer with great efficiency together with their biocompatibility relative to the commonly used salt counterpart, MS.

**ASSOCIATED CONTENT**

**Supporting Information**
Additional figures and tables as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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**REFERENCES**


