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# Toxicity of Molybdenum-Based Nanomaterials on the Soybean–Rhizobia Symbiotic System: Implications for Nutrition

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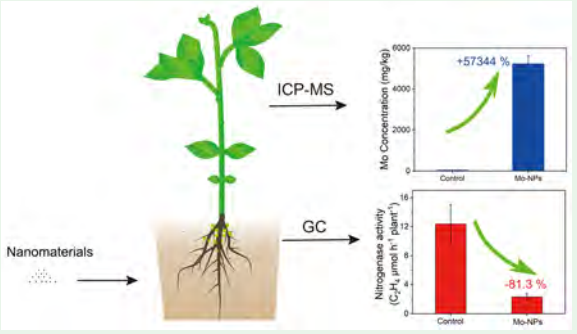
**ABSTRACT:** With the rapid development of nanotechnology, molybdenum (Mo)-based nanomaterials have been widely used in various fields, and the ensuing environmental behavior has also raised widespread concern. Here, we report the nanotoxic effects and mechanism of nano-Mo treatment on the soybean–rhizobia symbiotic system in the sterilized mixture of sand and vermiculite environment. Exposure to different concentrations and types of Mo-based nanomaterials was shown to have significant physiological and biochemical toxicity to soybean. The accumulation of Mo in plant tissues increased with the concentration of nano-Mo in the sand and vermiculite media. The Mo-based nanomaterials that enter plant tissues were found to affect not only the growth and development of plants, but also the microstructure of the roots and the activity of rhizobium in the symbiotic system, thereby weakening the nitrogen fixation capacity of the soybean–rhizobia symbiotic system. Meanwhile, the activity of superoxide dismutase (SOD) and peroxidase (POD) showed a significant increase, indicating that these enzymes were activated after exposure to nano-Mo, which helps to remove the reactive oxygen species. Furthermore, the treatment of high-concentration Mo nanoparticles was shown to have obvious epigenetic toxicity to plants. These results demonstrate that high concentrations of Mo nanoparticles can affect the agronomical and physiological parameters in soybean, which may impact human nutrition and health.




**KEYWORDS:** *nanotoxicity, molybdenum-based nanomaterials, soybean–rhizobia symbiotic system, root morphology and microstructure, nitrogenase activity*

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## 1. INTRODUCTION

Nowadays, the emergence of nanotechnology and the development of new nanomaterials have opened up a new application prospect for nanoagriculture and nanobiotechnology.[1] The widespread application of nanotechnology in agriculture has aroused great interest due to its potential to significantly increase agricultural productivity and efficiency with lower costs and less waste.[2] However, with the increasing application of nanotechnology in agriculture, new environmental behaviors have also attracted widespread attention.[3,4]

Molybdenum (Mo) and molybdenum (Mo)-based nanomaterials are broadly used in many fields, such as chemical engineering, photovoltaics, energy, environmental catalysis, biomedicine, and agriculture due to their unique physical and chemical properties.[5−8] The rapid increase in the use of Mo indicates the potential to release more toxic substances into the ecosystem.[9−12] Mo, a trace element in the soil, is an essential element for plant growth.[13] Like other metals, Mo is required for the growth of most plants, such as a cofactor for specific plant enzymes such as coenzymes MoCo and FeMo to participate in the reduction and oxidation of plants.[14] Additionally, Mo is known as an essential nutrient for plants, animals, and microorganisms.[15] However, the soluble molybdate anion is the only form of Mo available in plants and bacteria, and lack of Mo is lethal to organisms.[16] For instance, Mo deficient plants grow slowly with low chlorophyll content.[17] Previous studies have shown that Mo plays an important role in the normal nitrogen fixation of plants because it is an important component of nitrate reductases and nitrifying enzymes, which can control the reduction of inorganic nitrate and assist $N_2$ fixation.[18] Brkić et al. stated that Mo can increase the yield of legumes by stimulating nodulation and biological nitrogen fixation.[19] The legume plant–rhizobium symbiotic nitrogen fixation system is a special plant-microbial symbiosis type in the rhizosphere microecosystem, and has always been one of the focuses of biological nitrogen fixation research because of its high efficiency of nitrogen fixation.[20] In nature, biological nitrogen

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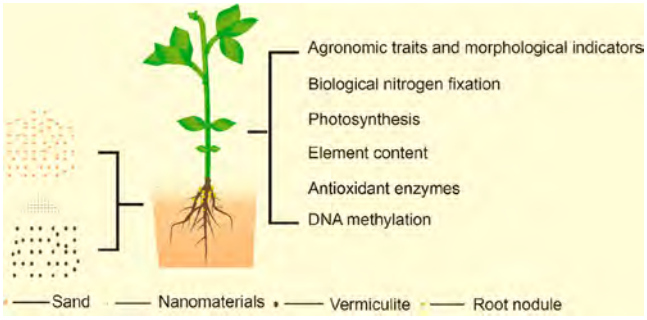
fixation is catalyzed by nitrogenase, an enzyme complex containing an iron−Mo cofactor (FeMo).[21]

Previous studies have investigated the effects of Mo-based nanomaterials on plant, animal, and microorganism, with different results being observed. For instance, Cui et al. reported that $MoS_2$ had no significant effect on the germination, malondialdehyde (MDA) content, and antioxidant enzyme activity of rice seeds, but significantly increased the length and biomass of rice roots and stems, chlorophyll content index (CCI), and aquaporin gene expression.[22] Nano $MoO_3$ has been shown to hinder the seed germination and growth of cowpea and disruption of bacterial cell walls in *Escherichia coli, Salmonella typhimurium, Enterococcus faecalis*, and *Bacillus subtilis*.[23,24] The acute aquatic toxicity evaluation of Mo (+VI) against *Daphnia magna* showed that Mo compounds vary significantly in their toxicity in solution, confirming that the toxicity of Mo in aquatic systems is closely related to the form of Mo salts used.[25]

Soybean, a very important crop that can fix nitrogen in the atmosphere through microbial symbiosis, is thought to contribute more than 50% of globally consumed edible oil and can also be used as protein materials for human consumption and livestock feed.[26] To our knowledge, the effects of exposure to Mo-based nanomaterials on the soybean−rhizobia symbiotic system in complex sand and vermiculite media and the related mechanism have not been reported so far. The purpose of this study was to clarify the effects of exposure to Mo-based nanoparticles on the complex soybean−rhizobia symbiotic sand and vermiculite environment and to explore the potential mechanisms. To this end, we grew soybeans in the sterilized mixture of sand and vermiculite for 30 days while adding commercially available Mo-based nanomaterials, such as nano-Mo powder (Mo), Mo dioxide ($MoO_2$), Mo trioxide ($MoO_3$), or nanosized Mo disulfide ($MoS_2$) (Scheme 1). The toxicity effects of nano-Mo exposure on the soybean−rhizobia symbiotic system was investigated by analyzing plant growth and development, biomass content, Mo nanoparticle distribution and content in tissues, nitrogenase activity, photosynthetic rate index, enzymatic activity related to reactive oxygen species (ROS), and the DNA methylation level.

**Scheme 1. Toxicity of Molybdenum-Based Nanomaterials on the Soybean−Rhizobia Symbiotic System**




## 2. EXPERIMENTAL SECTION

**2.1. Chemicals.** Mo nanomaterial was purchased from Aladdin (Shanghai, China); $MoO_2$, $MoO_3$, and $MoS_2$ were purchased from Nanuo Chemical (Guangzhou, China).

**2.2. Preparation of Soybean−Rhizobia Symbiotic System in Sand and Vermiculite Medium.** The planting substrate consisted of yellow sand and vermiculite (1:1). Briefly, nanomaterials were mixed with sand at a mass ratio (2, 20, 200, and 2000 kg/mg), followed by adding the same volume of vermiculite. After mixing with a blender (7.5 kg of yellow sand, the same volume of vermiculite, and 50 mL of double distilled (dd) water), the mixture was dispensed into eight bags and sterilized at 121 °C for 30 min, with one bag per pot for planting.

Soybean seeds (*Glycine max*) "Williams 82" were provided by Oil Crops Research Institute, Chinese Academy of Agricultural Sciences (Wuhan, China). Seeds were soaked in dd water in a dark environment at 28 °C for 24 h, planted in pots with the prepared planting substrate, eight pots per treatment, and then maintained in the greenhouse with a day/night temperature of 28 °C/23 °C and 14 h photoperiod. Meanwhile, Broughton and Dilworth (B&D) nitrogen-free nutrient solution was used once a week. At day 5 of the experiment, 1 mL of *Bradyrhizobium japonicum* USDA 110 (U.S. Department of Agriculture, Washington, DC) solution was added to each pot at 0.5 of OD600 nm for the optical density of the bacterial solution.

**2.3. Evaluation of Symbiotic Nitrogen Fixation Efficiency.** The nitrogenase activity of root nodules was measured by the acetylene reduction assay.[27,28] The root with nodules was collected, washed, and transferred to a sterile 40 mL headspace vial. Next, 4 mL of air was withdrawn from the head vial with a syringe, followed by injecting 4 mL of $C_2H_2$ into it, inverting the sample bottle in a 28 °C incubator for 2 h, and manually injecting 100 $\mu$L of gas from the headspace bottle into the equipped gas chromatograph. The analysis was performed under the following conditions: injection, manual injection; injector temperature, 180 °C; column, GS-Q 30 m × 0.320 mm; carrier gas, purity $N_2$; column flow, 5 mL/min; oven temperature, 100 °C; detector, FID; detector temperature, 250 °C; H2, 30 mL/min; air, 400 mL/min; makeup (N2), 25 mL/min.

**2.4. Evaluation of Photosynthesis Efficiency.** For chlorophyll analysis, the soybean leaves were cut into small pieces with scissors, weighed accurately, supplemented with 95% ethanol, and placed in the dark for 24 h. Chlorophyll a, chlorophyll b, and carotenoid contents were measured at 665, 649, and 470 nm, and calculated as described by Porra et al.[29]

Physiological measurement was performed as reported by Dudley et al.[30] Photosynthetic assimilation rate ($A$), transpiration rate ($E$), and intercellular $CO_2$ concentration ($C_i$) were measured by a portable photosynthesis analyzer LI-649 6800 (LI-COR Biosciences, Lincoln, NE) with a 6800-01A 650 (LI-COR Biosciences) fluorometer light source.

**2.5. Evaluation of Methylation Level.** Genomic DNA was extracted from soybean root, stem, and leaves as reported by Souza.[31] The methylation level was measured using the methylation sensitive amplification polymorphism (MSAP) technique as reported by Reyna-Lopez et al. with some modifications.[32]

**2.6. Statistical Analysis.** The values in the article are expressed as mean ± standard deviation (SD) from at least three experiments. The statistical significance of all data is determined by one-way analysis of variance (ANOVA) using Duncan's test at $p < 0.05$ level.

## 3. RESULTS AND DISCUSSION

**3.1. Characterization of Mo-Based Nanomaterials.** In this study, four kinds of Mo-based nanomaterials (Mo, $MoO_2$, $MoO_3$, and $MoS_2$) were used, and their characterizations are shown in Figure 1. The morphologies and particle sizes of Mo-based nanomaterials were determined by scanning electronic microscopy (SEM), and they were shown as particles with a spherical or lamellar structure (Figure 1A−D). The hydrodynamic diameters and zeta potentials of the Mo-based nanomaterials were determined by dynamic light scattering measurements (DLS, Nano S, Malvern), with the size of 246.33 ± 7.62 nm for Mo nanoparticles (Figure 1A), 309.07 ± 3.43 nm for $MoO_2$ nanoparticles (Figure 1B), 196.40 ± 9.82 nm for $MoO_3$ nanoparticles (Figure 1C), and 160.47 ± 1.65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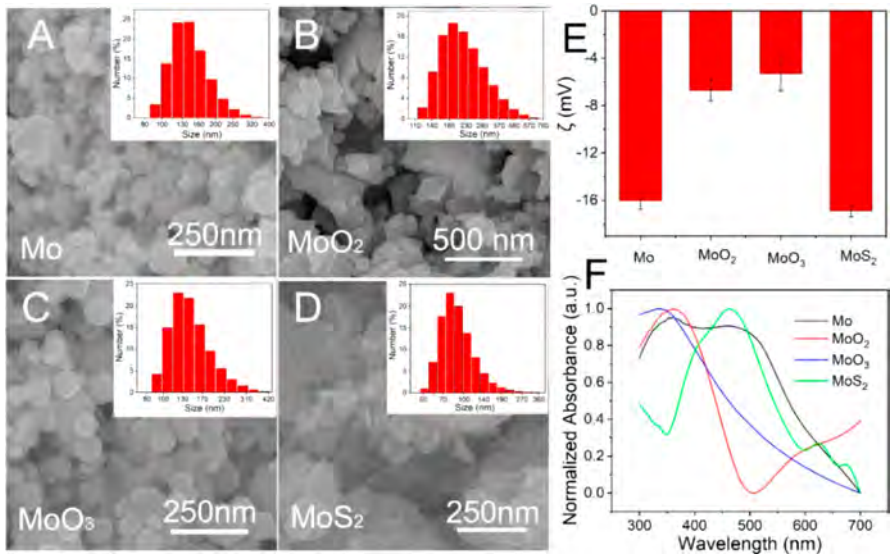


**Figure 1.** Characterization of nanomaterials. (A–D) SEM images of Mo (A), $MoO_2$ (B), $MoO_3$ (C), and $MoS_2$ (D), with the inset for the corresponding DLS data. ζ-Potential of nanomaterials (E). Normalized UV–vis absorption spectra of nanomaterials (F).

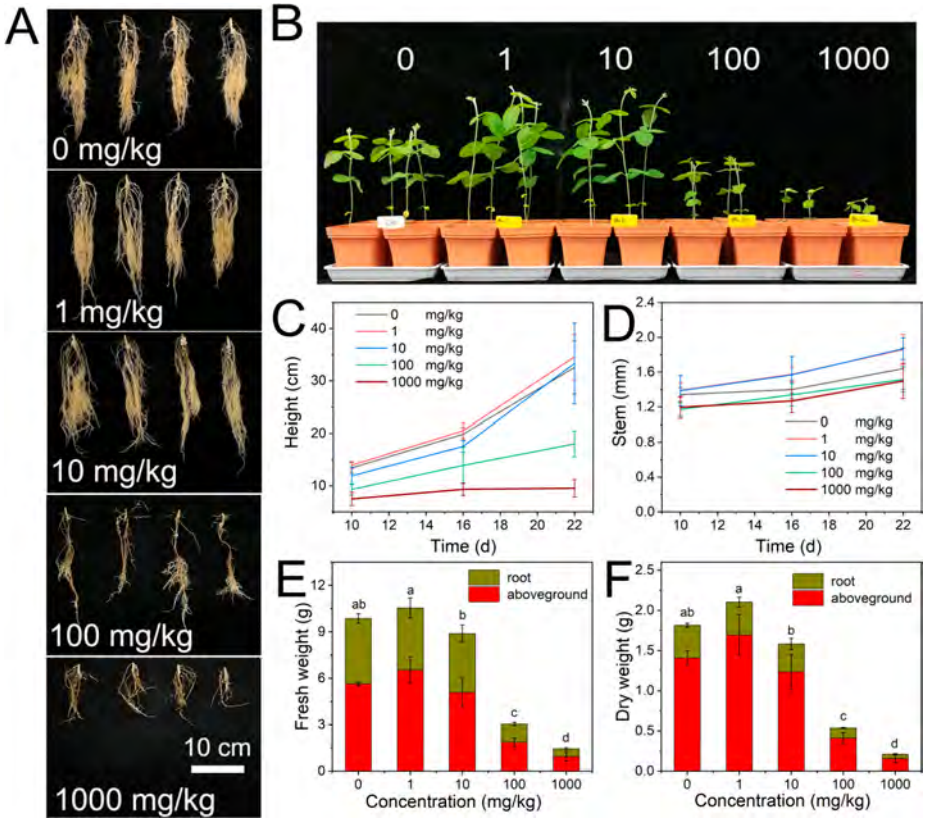


**Figure 2.** Growth of soybeans under the treatment of different concentrations of Mo nanomaterials. Photos of soybean roots grown for 30 days (A). Photos of soybean grown for 22 days (B). Soybean plant height and stem thickness grown for 10 days, 16 days, and 22 days (C, D). Fresh and dry weight of soybeans grown for 30 days (E, F). The error bars represent the standard error of the mean ($n$ = 4 plants for each treatment). The difference among data of each column followed by the same letter was not statistically significant ($p < 0.05$).

nm for $MoS_2$ nanoparticles (Figure 1D). Zeta potential data showed that all the four Mo-based nanomaterials were negatively charged (Figure 1E). The hydrodynamic diameter appears to be larger than the corresponding SEM diameter, which can be attributed to the surface coating and the accumulation of tiny particles in the water. In Figure 1F, the Mo-based nanomaterials showed a specific absorption in the UV–vis spectra. These experimental results indicate the successful synthesis of the Mo-based nanomaterials.

**3.2. Effect of Mo-Based Nanomaterials on Soybean–Rhizobia Symbiotic System.** The impact of Mo-based nanomaterials (Mo, $MoO_2$, $MoO_3$, $MoS_2$) on the symbiotic nitrogen fixation system was evaluated by exposing the soybean–rhizobia symbiotic system directly to different


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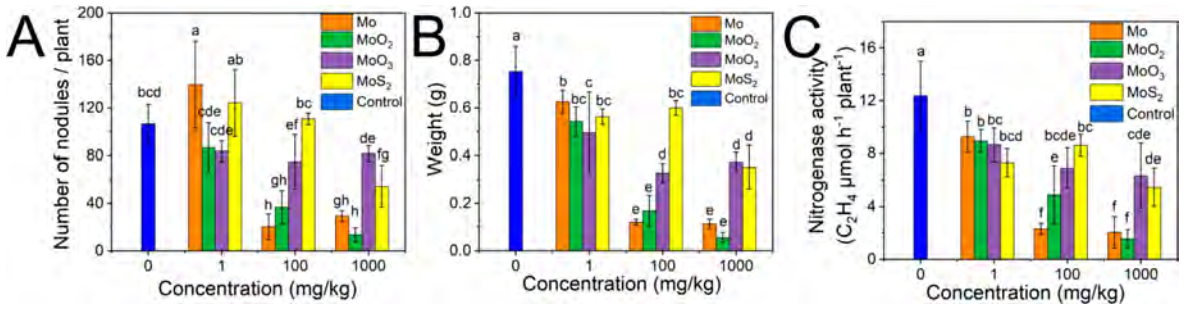


**Figure 3.** Nitrogen fixation capacity: nodule count per plant (A), nodule fresh weight per plant (B), and $N_2$ fixation potential per plant (C). The error bars represent the standard error of the mean ($n$ = 4 plants for each treatment). The difference among data of each column followed by the same letter was not statistically significant ($p < 0.05$).

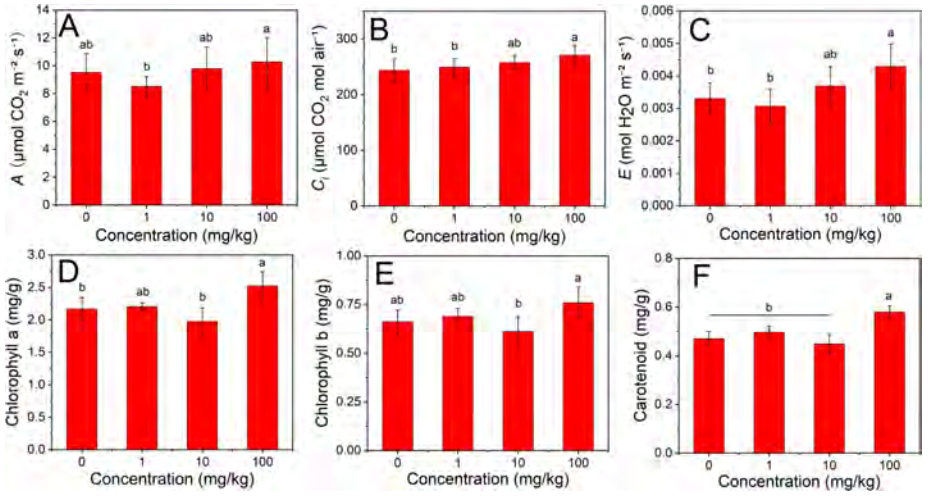


**Figure 4.** Photosynthesis of soybean under Mo treatment: The photosynthetic rate ($A$), intracellular $CO_2$ content ($C_i$), and transpiration rate ($E$) (A–C); chlorophyll a, chlorophyll b, and carotenoids (D–F). The error bars represent the standard error of the mean ($n$ = 4 plants for each treatment). The difference among data of each column followed by the same letter was not statistically significant ($p < 0.05$).

concentrations (1, 100, and 1000 mg/kg) of Mo-based nanomaterials mixed in yellow sand and vermiculite for 30 days and analyzing the morphology of soybean roots, growth, plant height, stem thickness, and fresh and dry weight (Figure 2 and Supporting Information, Figure S1). The different concentrations of Mo-based nanomaterials were shown to vary in their effects on the soybean. For Mo, $MoO_2$, and $MoO_3$, as their concentration increased, the toxicological effect on soybean roots became more obvious. Mo-based nanomaterials at 1 and 10 mg/kg promoted plant growth to some extent, when concentrations increased to 1000 mg/kg, the growth of the soybean root was almost completely suppressed (Figure 2A and Figure S1A); however, even at the high concentration of 1000 mg/kg, $MoS_2$ did not significantly affect root growth and seemed to promote root growth to a certain extent (Figure S1A). When exposed to higher concentrations of Mo, plants experienced more severe symptoms, such as stunted growth.[33] From soybean growth morphology (Figure 2B), we can see that the growth of soybeans is significantly inhibited with increasing Mo concentration, and soybeans almost stopped growing at 1000 mg/kg Mo treatment. These results can be confirmed by the plant height and stem thickness data at different experimental periods (10 days, 16 days, and 22 days) (Figure 2C,D, Figure S1B–D). Furthermore, we measured the fresh and dry weights of the soybeans (Figure 2E,F, Figure S1E,F), which also showed that the high-concentration Mo nanomaterial significantly inhibited the growth and development of soybeans. Previous studies have shown that, as Mo is a micronutrient required by plants, the effect of Mo-based nanomaterials on plant growth is concentration-dependent, which not only inhibits growth, but also affects root morphology.[34] Mo, as a nanofertilizer, can promote plant growth to some extent under low concentration treatment. At a soil Mo content exceeding 1000 mg/kg, the above-ground biomass of cultivated *Axonopus compressus* (Sw.) P. Beauv. decreases significantly.[35] Similar observations of phytotoxicity were also reported in various other crops when they were exposed to excess Mo.[36−38]

**3.3. Effect of Mo-Based Nanomaterials on Symbiotic Nitrogen Fixation (SNF).** To evaluate the effects of Mo-based nanomaterials (Mo, $MoO_2$, $MoO_3$, and $MoS_2$) on SNF, the nodule number, nodule weight, and nitrogen fixation ability were investigated after treating the SNF system with different concentrations (1, 100, and 1000 mg/kg) and different types of Mo nanomaterials for 30 days (Figure 3). In Figure 3A, the four types of Mo nanomaterials showed no significant effect on the number of nodules in the SNF system at a low concentration (1 mg/kg). However, as the concentration increased, the number of nodules was significantly decreased. Especially, when the concentration of Mo and $MoO_2$ nanomaterials increased to 1000 mg/kg, the number of nodules decreased sharply (Ck 106.5 ± 16.44, Mo 29.25 ± 4.5, $MoO_2$ 13.5 ± 5.80). Similar results were obtained for nodule weight (Figure 3B). The nitrogenase activity of root


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nodules were measured by the acetylene reduction method,[27] and the nitrogenase activity per plant is shown in Figure 3C. The Mo-based nanomaterials were seen to inhibit the whole-plant $N_2$ fixation potential even at a low concentration (1 mg/kg), and the inhibition effect became more obvious with the increase of the nanomaterial concentration.

Soybean production and its associated $N_2$ fixation may be susceptible to soil contaminants, including nanomaterials.[39] Previous experimental results indicated that cerium dioxide ($CeO_2$) nanoparticles could inhibit soybean growth and yield by changing leaf pressure, causing damage to the leaf surface, and reducing the potential for N2 fixation.[40,41] Holden et al. mentioned that treatment with multiwalled carbon nanotubes (MWCNTs), graphene nanoplatelets (GNPs), or carbon black (CB) can reduce the nitrogen-fixing capacity of the entire plant, with the highest reduction (over 91%) for low MWCNT as well as medium and low CB treatments.[39] The biological nitrogen fixation is known to be catalyzed by nitrogenase, an enzyme complex containing an iron−Mo cofactor, which means that the unavailability of Mo is lethal to the organism.

**3.4. Effect of Mo-Based Nanomaterials on Photosynthesis.** The ability of plants to produce carbohydrates is usually evaluated by photosynthesis, and their health status can be indicated by chlorophyll content.[42,43] The effects of Mo-based nanomaterials (Mo, $MoO_2$, $MoO_3$, and $MoS_2$) on photosynthesis were evaluated by measuring the photosynthetic rate ($A$), intracellular $CO_2$ content ($C_i$), transpiration rate ($E$), chlorophyll a, chlorophyll b, and carotenoids of the soybean plants. The net photosynthetic rate ($A$) showed no significant differences between the control and the treatment of Mo-based nanomaterials at different concentrations (1, 10, 100 mg/kg) (Figure 4A). Meanwhile, a comparison was made between the Mo treatment and the other three treatments ($MoO_2$, $MoO_3$, and $MoS_2$) and similar results were obtained (Figure S2). The intercellular $C_i$ results showed no significant differences between the control and the treatments at low concentrations (1, 10 mg/kg). When the Mo concentration increased to 100 mg/kg, the intercellular $C_i$ was significantly enhanced in the Mo nanomaterial treatment versus the control (Figure 4B). Similar results were also obtained in the transpiration rate assay (Figure 4C).

In addition, we investigated the effect of Mo nanomaterials on chlorophyll content. Chlorophyll a fluorescence from green plants can reflect photosynthesis in a complex way.[43] In soybean leaf tissues, the chlorophyll a and chlorophyll b content showed no significant difference between the control and the treatment of Mo nanomaterials at all the tested concentrations (Figure 4D,E). For the carotenoids (Figure 4F), no significant difference was observed between the control and the Mo treatment at low concentrations (1, 10 mg/kg), in contrast to a significant increase ($P < 0.01$) in the carotenoid content as the concentration was increased to 100 mg/kg. Previous studies have shown that chlorophyll content is not affected when soybeans are exposed to a nanomaterial concentration less than 400 mg/L nCuO.[44] Additionally, some metal oxide nanoparticles such as $TiO_2$, ZnO, and $CeO_2$ have been shown to reduce the chlorophyll content in peas, corn, rice, and tomatoes.[45] Studies have also shown that chlorophyll was not affected in mature bell pepper treated with copper nanoparticles (nCu) or Cu microparticles ($\mu$Cu), or in oregano treated with Cu NP.[46] Our results shown that chlorophyll content did not change significantly after Mo exposure. We speculate that the reason may be because the damage of the Mo nanomaterials to the plants was mainly reflected in the roots. After entering the plant tissues, whether the molybdenum nanomaterials affect the photosynthetic electron transfer and damage or hinder the function of the leaves needs further verification.

**3.5. Mo Content in Soybean Plant Tissues after Mo Nanomaterials Exposure.** When absorbed into plant tissues, the efficiency of engineering nanomaterials mainly depends on their types, concentrations, plant growth conditions, and tissue types.[47] The Mo concentrations of leaf, stem, and root after Mo exposure are shown in Figure 5. The Mo concentration

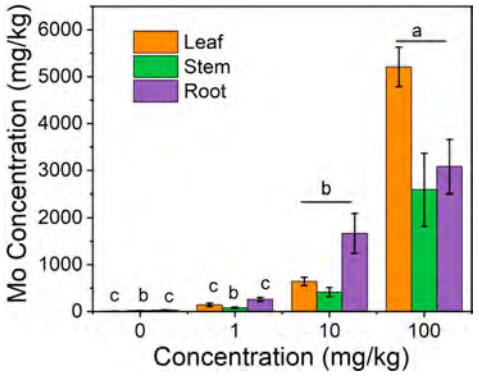


**Figure 5.** Mo concentrations in leaf, stem, and root after Mo exposure. The error bars represent the standard error of the mean ($n$ = 3 plants for each treatment). The difference among data of each column followed by the same letter was not statistically significant ($p < 0.05$).

was seen to increase to 15.661-fold in leaves, 4.110-fold in stems, and 8.226-fold in roots under 1 mg/kg Mo treatment. Under 10 mg/kg Mo treatment, the Mo concentration was increased to 70.758-fold in leaves, 20.996-fold in stems, and 53.039-fold in roots. Under 100 mg/kg Mo treatment, the Mo concentration was increased to 574.442-fold in leaves, 131.194-fold in stems, and 98.085-fold in roots (Figure 5). When exposed to 1 and 10 mg/kg Mo nanomaterials, the Mo concentration was the highest in roots, followed by leaves, and then stems. In plants exposed to 100 mg/kg Mo, the Mo concentration was significantly higher in leaves than in roots. The differences in the absorption and tissue distribution of different engineering nanomaterials by plants can be determined by the characteristics of the particles themselves, especially the differences in morphology and surface charge. Generally, the accumulation of Mo increases with the increase of Mo content in soil. Wang et al.[48] and Zhu et al.[49] found that under hydroponic conditions, particles with different surface charges vary in their distribution in plants. For example, nanoparticles with positive surface charges ($\zeta$-potential ≈ +20 mV) are more easily absorbed by plant roots and distributed in roots, while nanoparticles with negative surface charges ($\zeta$-potential ≈ −20 mV) tend to accumulate in plant leaves. These results confirm the importance of surface charge to plant absorption and distribution of engineering nanomaterials under environment-related conditions.[47]

**3.6. Effect of Mo Nanomaterials on the Structure of Soybean Tissues.** Biophysical structural damage caused by nanomaterials is one of the key reasons for nanotoxicity.[50] To evaluate the effects of Mo nanomaterials on the soybean structure, the microstructures of vein, root, and nodule slices were assessed and shown in Figure 6. In Figure 6A, the bast


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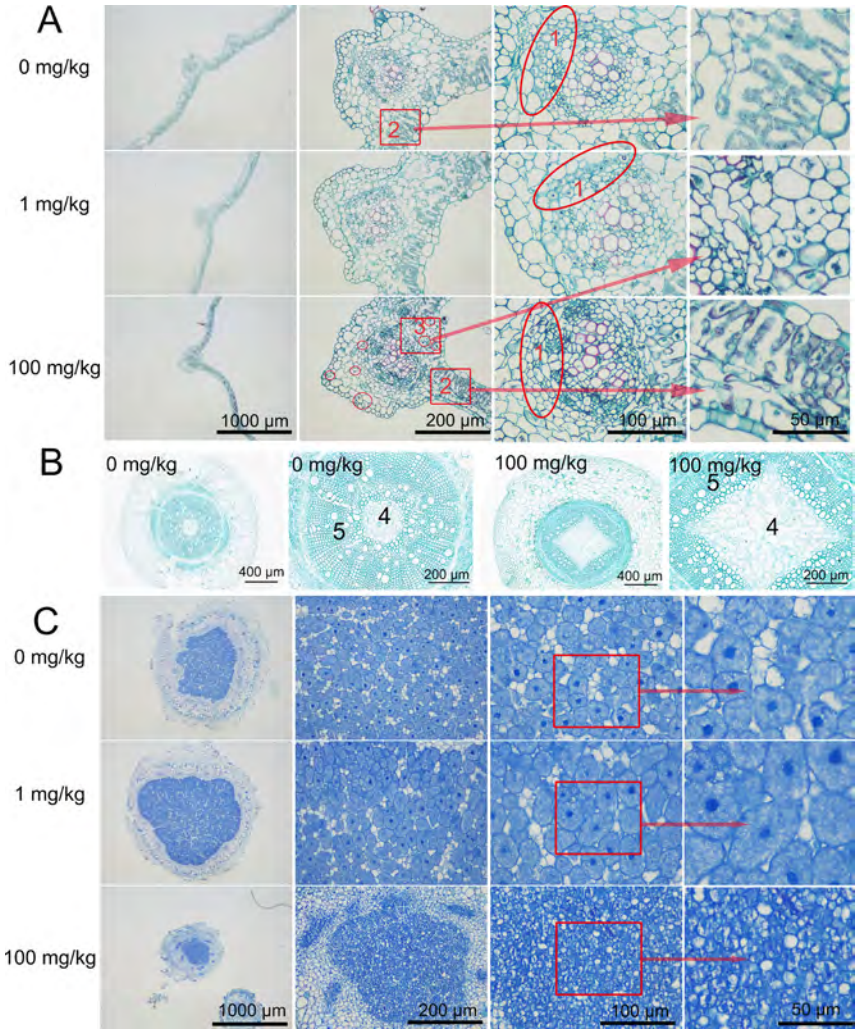


**Figure 6.** Photos for the microstructures of soybean taken with an optical microscope: leaf vein (A), bast fibers (1), palisade cell (2, 3). Root (B), pith (4), xylem (5). Nodule (C).

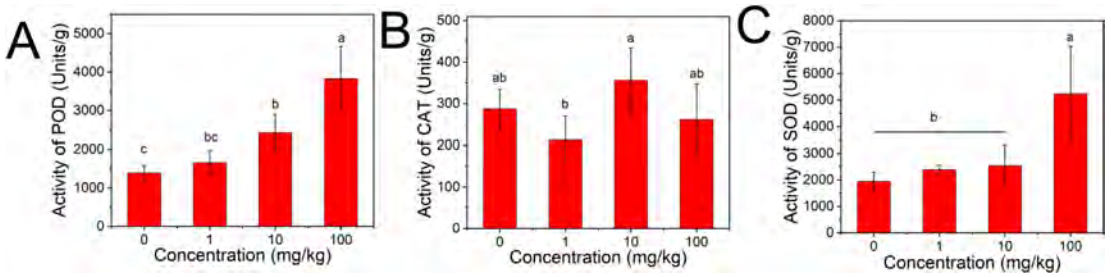


**Figure 7.** Effect of different concentrations of Mo nanoparticles on the activity of soybean root antioxidant enzymes: POD (A), CAT (B), SOD (C). The error bars represent the standard error of the mean ($n$ = 3 plants for each treatment). The difference among data of each column followed by the same letter was not statistically significant ($p < 0.05$).

fibers (red circle) in the veins are seen to be gone, with many floccules in the palisade cell under 100 mg/L Mo treatment, while in the control treatment, the bast fibers (red circle) can be clearly seen in the veins, with almost no floccule in the palisade. As the concentration of nanomaterials increases, the pith area of the root becomes larger (Figure 6B). Furthermore, we evaluated the effect of different concentrations (0, 1, 100 mg/kg) of Mo nanomaterials on the nodule morphology and rhizobium activity (Figure 6C). It was shown that the nodule has complete morphology and the rhizobium has a clear cellular structure under the control conditions, in contrast to a swollen rhizobium cellular structure under 1 mg/kg Mo treatment and no complete cell morphology under 100 mg/kg Mo treatment.

All the above results showed that the treatment of Mo nanomaterials can change the soybean structure, inhibit soybean growth, and affect soybean nitrogen fixation symbiosis system. Previous studies have shown that $CeO_2$ nanoparticles (NPs) can produce phytotoxicity by destroying cotton chloroplasts and vascular bundles and altering nutrient


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Table 1. DNA Methylation Patterns of Soybean Exposed to Different Concentrations of Mo Nanomaterials by MSAP[a]

| | 0 | | | 1 | | | 10 | | | 100 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MSAP band types | L | S | R | L | S | R | L | S | R | L | S | R |
| 14 d | | | | | | | | | | | | |
| I (unmethylated) | 428 | 514 | 385 | 411 | 462 | 354 | 445 | 520 | 352 | 333 | 224 | 241 |
| II (hemimethylated) | 334 | 310 | 319 | 373 | 395 | 452 | 365 | 333 | 218 | 360 | 257 | 180 |
| III (methylated) | 323 | 173 | 96 | 255 | 184 | 168 | 219 | 269 | 361 | 324 | 523 | 262 |
| IV (hypermethylated) | 721 | 809 | 1006 | 767 | 765 | 832 | 777 | 684 | 875 | 789 | 802 | 1123 |
| total amplified bands | 1806 | 1806 | 1806 | 1806 | 1806 | 1806 | 1806 | 1806 | 1806 | 1806 | 1806 | 1806 |
| hemimethylated bands (%) | 18.49 | 17.17 | 17.66 | 20.65 | 21.87 | 25.03 | 20.21 | 18.44 | 12.07 | 19.93 | 14.23 | 9.97 |
| full-methylated bands (%) | 57.81 | 54.37 | 61.02 | 56.59 | 52.55 | 55.37 | 55.15 | 52.77 | 68.44 | 61.63 | 73.37 | 76.69 |
| total methylated bands (%) | 76.30 | 71.54 | 78.68 | 77.24 | 74.42 | 80.40 | 75.36 | 71.21 | 80.51 | 81.56 | 87.60 | 86.66 |
| 28 d | | | | | | | | | | | | |
| I (unmethylated) | 386 | 400 | 388 | 337 | 447 | 493 | 569 | 652 | 567 | 640 | 697 | 425 |
| II (hemimethylated) | 325 | 311 | 203 | 254 | 292 | 157 | 202 | 210 | 175 | 211 | 227 | 162 |
| III (methylated) | 513 | 530 | 288 | 565 | 535 | 238 | 351 | 402 | 176 | 439 | 350 | 283 |
| IV (hypermethylated) | 582 | 565 | 927 | 650 | 532 | 918 | 684 | 542 | 888 | 516 | 532 | 936 |
| total amplified bands | 1806 | 1806 | 1806 | 1806 | 1806 | 1806 | 1806 | 1806 | 1806 | 1806 | 1806 | 1806 |
| hemimethylated bands (%) | 18.00 | 17.22 | 11.24 | 14.06 | 16.17 | 8.69 | 11.18 | 11.63 | 9.69 | 11.68 | 12.57 | 8.97 |
| full-methylated bands (%) | 60.63 | 60.63 | 67.28 | 67.28 | 59.08 | 64.01 | 57.31 | 52.27 | 58.91 | 52.88 | 48.84 | 67.50 |
| total methylated bands (%) | 78.63 | 77.85 | 78.52 | 81.34 | 75.25 | 72.70 | 68.49 | 63.90 | 68.60 | 64.56 | 61.41 | 76.47 |

[a]Notes: Type I is *Hpa*II/*Eco*RI and *Msp*I/*Eco*RI (1, 1); Type II is *Hpa*II/*Eco*RI and *Msp*I/*Eco*RI (1, 0); Type III is *Hpa*II/*Eco*RI and *Msp*I/*Eco*RI (0, 1); Type IV is *Hpa*II/*Eco*RI and *Msp*I/*Eco*RI (0, 0).

uptake.[51] In this study, the high concentration of Mo nanoparticles significantly altered the microstructure of the roots and the activity of rhizobium, thus affecting the soybean−rhizobia symbiotic nitrogen fixation system.

**3.7. Effects of Mo Nanomaterials on Antioxidant Enzymes in Soybean Root.** The engineered nanomaterials can induce oxidative stress by generating reactive oxygen species (ROS) and thus affect plants.[45] For plants, oxidative stress damage caused by ROS can be reduced by increasing the level of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), which play a key role in eliminating ROS from environmental stress.[52−54] Figure 7 shows the activity changes of antioxidant enzymes (SOD, CAT, and POD) in the SNF system after 30 days of exposure to different concentrations (1, 10, 100 mg/kg) of Mo nanoparticles. After exposure to Mo nanoparticles, the POD activity in the SNF system showed a dose-dependent increase, with the activity being increased to 1.193-fold, 1.745-fold and 2.761-fold in the roots under the 1 mg/kg, 10 mg/kg, and 100 mg/kg Mo treatment, respectively Figure 7A). Mo had no significant effect on the activity of CAT in the soybean roots at all the tested concentrations (1, 10, 100 mg/kg)(Figure 7B). The SOD activity was increased to 2.697-fold in the roots under the 100 mg/kg Mo treatment (Figure 7C).

Previous studies have shown that metal or metal oxide nanoparticles can cause oxidative stress damage to animal cells, bacteria, and soil organisms by producing ROS.[55] In this study, the activities of three essential antioxidant enzymes (SOD, POD, and CAT) were measured to evaluate the effect of Mo nanoparticle-induced oxidative stress on the soybean−rhizobia symbiotic system. Among them, SOD can convert superoxide radical ($O_2^-$) into hydrogen peroxide ($H_2O_2$) and oxygen ($O_2$), and $H_2O_2$ can be removed by CAT and POD.[56] In the present study, the SOD and POD activity showed a dose-dependent increase in the soybean−rhizobia symbiotic system after exposure to different concentrations of Mo nanoparticles, indicating that these enzymes were activated under nano-Mo exposure, which helps to remove active oxygen. These results are consistent with a previous study reporting that Mo can significantly improve the activities of SOD, POD, and CAT and thereby reduce the oxidative stress of ROS on Chinese cabbage.[57]

**3.8. Effects of Mo Nanomaterials on DNA Methylation Level.** Table 1 shows the methylation levels under the treatment of different concentrations (0, 1, 10, 100 mg/kg) of Mo nanoparticles versus the control in the root, stem, and leaf tissues as assessed by methylation sensitive amplification polymorphism (MSAP) (Table 1). In the bands amplified by MSAP, all fragments can be divided into four types: type I fragments, which are unmethylated sites and indicated by the presence of bands composed of two enzymes (1, 1); type II segments, which are hemimethylated sites and are only represented by *Eco*RI/*HpaII* bands (1, 0); type III segments, which are indicated by the presence of *Eco*RI/*MspI* (0, 1); type IV fragments, which are indicated by the absence of *Hpa*II/*Eco*RI and *Eco*RI/*MspI* in the bands (0, 0).[58] According to the above classification, types III and IV represent complete methylation in this study (Table 1). Using the 16 MSAP primer pairs (Table S1), a total of 1806 bands were revealed in root, stem, and leaf tissues, respectively. The full-methylated DNA bands accounted for 57.81%, 54.37%, and 61.02% of the total in the leaf, stem, and root tissues under the 14-day control condition versus 56.59%, 52.55%, and 55.37% under 1 mg/kg Mo treatment, 55.15%, 52.77%, and 68.44% under 10 mg/kg Mo treatment, 61.63%, 73.37%, and 76.69% under 100 mg/kg Mo treatment for 14 days in the leaf, stem, and root tissues,


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respectively. These results demonstrated that the full-methylated sites increased significantly in the leaf, stem, and root tissues with the concentrations of Mo nanomaterials increased, especially in stem and root, where the full-methylated sites increased by 19% (from 54.37 to 73.37%) and 15.67% (from 61.02 to 76.69%) under 100 mg/kg Mo treatment versus the control.

As the treatment time increased, we found that full-methylated DNA bands reached 60.63%, 60.63%, and 67.28% in the leaf, stem, and root tissues under the 28-day control condition versus 67.28%, 59.08%, and 64.01% under 1 mg/kg Mo treatment, 57.31%, 52.27%, and 58.91% under 10 mg/kg Mo treatment, and 52.88%, 48.84%, and 67.50% under 100 mg/kg Mo treatment for 28 days in the leaf, stem, and root tissues, respectively. The above experimental results indicate that in the symbiotic system of soybean and rhizobia, the DNA of different parts of soybean plants treated with different concentrations of molybdenum nanomaterials has a certain degree and regular DNA methylation modification, which may also be one of the important reasons for the differences in soybean morphology after treatment with different concentrations of nanomaterials.

Nanomaterials interact with lipids, proteins, and nucleic acids to affect their structure and function, leading to genotoxicity,[59] cytotoxicity,[60] and epigenetic toxicity,[61] but these toxic effects appear to be interdependent. Epigenetic toxicity is caused by DNA methylation and/or histone modification and/or changes in miRNA expression and function, and these epigenetic mechanisms can drive each other.[62] DNA methylation refers to the addition of a methyl group at position 5 of a cytosine residue, resulting in 5′ methylation of the residue. Methylation occurs on the cytosine residue of the CpG dinucleotide, with the cytosine base followed by the guanine base.[62] In 2008, Choi et al. first reported that engineering nanomaterials can cause significant epigenetic toxicity.[63] However, only a few studies have explored the epigenetic mechanisms (DNA methylation, histone modification, and miRNA expression changes) of plant- and microbial system-induced toxicity. Epigenetic toxicity of heavy metals has been well studied.[64,65] For example, previous studies have shown that heavy metals such as lead, arsenic, chromium, copper, nickel, and cadmium produce epigenetic toxicity by altering DNA methylation and histone modification. At the same time, different exposure time can lead to different epigenetic toxicity. For example, short-term exposure (1 week) of heavy metal chromium can reduce overall DNA methylation by inhibiting DNA methylase activity, while long-term exposure (10 weeks) results in increased DNA methylation.[66]

Variation of plant genome methylation level as a defense mechanism of plants against external environmental stress, through methylation level changes, regulates chromatin structure and related gene expression, thereby improving plant adaptability to adversity stress. In our study, we found that with the extension of the processing time of molybdenum nanomaterials (14 days, 28 days), the methylation level of high-concentration molybdenum nanomaterials (10 mg/kg, 100 mg/kg) showed a downward trend, especially the methylation level of the root (10 mg/kg treatment: 68.44% to 58.91%; 100 mg/kg treatment: 76.69% to 67.50%). During the processing of molybdenum nanomaterials, DNA methylation may be involved in the response to changes in the concentration of molybdenum nanomaterials, which may be one of the ways in which soybean plants regulate gene expression in response to the stress of molybdenum nanomaterials.

## 4. CONCLUSION

In this study, high concentrations of Mo-based nanomaterials have been demonstrated to induce significant nanotoxicity in the soybean−rhizobium symbiotic system. The generation mechanism of these nanotoxicities may include the following aspects: (1) The Mo accumulation in plant tissues increases with the increase of nano-Mo concentration in the sand and vermiculite media. (2) The Mo-based nanomaterials that enter the plant tissues affect the growth and development of plants, the microstructure of the roots, and the activity of rhizobium in the symbiotic system, thereby weakening the nitrogen fixation capacity of the system. (3) The activity of superoxide dismutase (SOD) and peroxidase (POD) is significantly increased after exposure to nano-Mo, indicating the activation of these enzymes, which helps to remove reactive oxygen species. (4) The treatment of Mo nanoparticles exhibits obvious dose-and-time dependent epigenetic toxicity to plants. These results indicate that the high concentration of Mo nanoparticles affect the agronomical and physiological parameters in the soybean−rhizobia symbiotic system, which may impact human nutrition and health.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsanm.0c00943.

> Soybean growth 30 days of root photos with $MoO_2$, $MoO_3$, and $MoS_2$ treatment; photosynthesis of soybean under $MoO_2$, $MoO_3$, and $MoS_2$ treatment; Mo concentrations in leaf, stem, and root after Mo exposure; sequences of adapters and primers used for MSAP analysis (PDF)

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### Author Contributions

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### Notes

The authors declare no competing financial interest.

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