Novel approach to enhance *Bradyrhizobium diazoefficiens* nodulation through continuous induction of ROS by manganese ferrite nanomaterials in soybean

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**Abstract**

**Background:** The study of symbiotic nitrogen fixation between (SNF) legumes and rhizobia has always been a hot frontier in scientific research. Nanotechnology provides a new strategy for biological nitrogen fixation research. However, how to construct abiotic nano-structure-biological system, using the special properties of nanomaterials, to realize the self-enhancement of biological nitrogen fixation capacity is important.

**Results:** In order to construct a more efficient SNF system, in this study, we applied manganese ferrite nanoparticles (MF-NPs) with sustainable diatomic catalysis to produce reactive oxygen species (ROS), thus regulating the nodulation pathway and increasing the number of nodules in soybean (*Glycine max*), eventually enhancing symbiotic nitrogen fixation. Symbiosis cultivation of MF-NPs and soybean plants resulted in 50.85% and 61.4% increase in nodule weight and number, respectively, thus inducing a 151.36% nitrogen fixation efficiency increase, finally leading to a 25.70% biomass accumulation increase despite no substantial effect on the nitrogenase activity per unit. Transcriptome sequencing analysis showed that of 36 differentially expressed genes (DEGs), 31 DEGs related to soybean nodulation were upregulated in late rhizobium inoculation stage (12 d), indicating that the increase of nodules was derived from nodule-related genes (Nod-R) continuous inductions by MF-NPs.

**Conclusions:** Our results indicated that the nodule number could be effectively increased by extending the nodulation period without threatening the vegetative growth of plants or triggering the autoregulation of nodulation (AON) pathway. This study provides an effective strategy for induction of super-conventional nodulation.

**Keywords:** Reactive oxygen species, Nodulation, Autoregulation of nodulation, Manganese ferrite, Soybean
**Background**

Due to the long-term evolution of nitrogen-fixing organisms in the biosphere, the biological nitrogen fixation (BNF) is considered as one of the most environmentally friendly and sustainable methods to obtain nitrogen [1]. As the main source of human plant protein, soybean plays an indispensable role in human food supply [2]. By promoting soybean nodulation and enhancing total nitrogen fixation is considered as one of the most effective and convenient methods to increase yield. In recent years, the application of nanotechnology in agriculture has gradually attracted attention [3], and nanotechnology may be a potential breakthrough for sustainable agricultural development [4, 5]. Increasing the nodulation of legumes through functional nanoproducts is a breakthrough attempt.

Reactive oxygen species (ROS), as highly reactive oxygen derivatives, includes superoxide anion (O$_2^−$), hydroxyl radicals (-OH), hydroperoxyl radicals (-HO$_2$), and hydrogen peroxides (H$_2$O$_2$). These radicals are produced during aerobic metabolism under biotic and abiotic stresses [6, 7]. Despite its toxicity, ROS can serve as signals to trigger metabolic regulations in response to environmental stresses. ROS is produced during nodulation in legumes under the induction of nodulation factors (NFs) and participates in infection formation, contributing to cell wall reconstruction, matrix glycoprotein (MGP) cross-linking, and actin microfilament nucleation and branching [8]. ROS regulates nodulation in conjunction with Ca$^{2+}$ through spatial and temporal alterations [9]. Symbiotic production of ROS is primarily accomplished through cytoplasmic membrane NADPH oxidases respiratory burst oxidase homologue (Rbohs) [10, 11], and Rbohs overexpression stimulates rhizobium infection and nodule formation, resulting in an increase in the number of symbiotic microsomes and nitrogen fixation efficiency [12]. Conversely, the inhibition of Rbohs decreases ROS production and downregulates the expression of rhizobium-induced peroxidases (RIPs), NIN, and ENOD2 [13, 14]. Collectively, ROS in legumes is a group of signaling molecules regulating nodulation. In the process of nodulation, in order to control the stress caused by excessive nodules on the plant, a microRNA (miRNA), as a feedback regulation switch, can reduce the amount of GmNNC1 protein by shearing GmNNC1 mRNA, and alleviate the inhibitory effect of transcription repressor GmNNC1 on GmENOD40, thus allowing GmENOD40 to further activate the signal transduction pathway, finally initiating the development of nodules [15]. When there are too many root nodules, root-derived nodulation-specific CLAVATA/ESR-related (CLE) peptides GmRIC1 and GmRIC2 [16] induce the autoregulation of nodulation.
(AON) through shoot-derived inhibitor (SDI) such as cytokinin to inhibit the expression of miR172c, thereby avoiding excessive nodulation of soybeans [15, 17].

The reaction between H$_2$O$_2$ and Fe$^{2+}$ is called Fenton reaction (FTR). The factors affecting FTR include pH, temperature, reaction time, H$_2$O$_2$ concentration, and Fe$^{2+}$ concentration. The FTR reaction equation was as follows (Eq. 1):

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + (\text{OH})^- + \text{OH}^- \quad (1)$$

Further research has revealed that some materials such as, manganese-based, cobalt-based, and copper-based materials show high efficiency in FTR-like. Nanomaterials-mediated FTR is also widely used in fields such as biomedicine [18, 19] and environmental protection [20–24]. The capacity to produce -OH in FTR-like between Mn$^{2+}$ and H$_2$O$_2$ is lower than that in the Fenton reaction between H$_2$O$_2$ and Fe$^{2+}$, but the former (with Mn$^{2+}$ participation) can extend induction time of ROS, and adding a certain amount of Mn$^{2+}$ to the FTR system can significantly promote FTR efficiency. It has been reported that the reaction of Mn$^{2+}$ and H$_2$O$_2$ can produce O$_2^-$ [25]. Fe$^{3+}$ produced in FTR can be reduced to Fe$^{2+}$ to form a sustainable FTR under the mediation of O$_2^-$. The corresponding reaction equations were as follows [26, 27] (2–4):

$$\text{H}_2\text{O}_2 + \text{Mn}^{2+}/\text{Fe}^{2+} \rightarrow \text{Mn}^{3+}/\text{Fe}^{3+} + \text{OH}^- + \text{OH}^- \quad (2)$$

$$-\text{OH} + \text{H}_2\text{O}_2 \rightarrow \text{HOO}^- + \text{H}_2\text{O} \quad (3)$$

$$\text{HOO}^- \rightarrow \text{H}^+ + \text{O}_2^- \quad (4)$$

In the iron-manganese diatomic FTR system, the scavenging capacity of -OH and O$_2^-$ is inhibited since the Mn$^{3+}$ generated in the system is reduced by the Fe$^{2+}$ ion. Previous studies have revealed that the reduction of Mn$^{3+}$ by Fe$^{2+}$ is thermodynamically favorable [28, 29]. The corresponding reaction Eqs. (5–7) were as follows.

$$\text{Fe}^{3+} + \text{1e}^- \rightarrow \text{Fe}^{2+}, \varepsilon^o = 0.77 \text{ V} \quad (5)$$

$$\text{Mn}^{3+} + \text{1e}^- \rightarrow \text{Mn}^{2+}, \varepsilon^o = 1.51 \text{ V} \quad (6)$$

$$\text{Fe}^{2+} + \text{Mn}^{3+} \rightarrow \text{Fe}^{3+} + \text{Mn}^{2+}, \varepsilon^o = 0.73 \text{ V} \quad (7)$$

The higher reduction capacity of Mn$^{3+}$ than Fe$^{3+}$ promotes the reduction of Mn$^{3+}$ and the production of Mn$^{2+}$, thus extending the catalytic reaction time of H$_2$O$_2$ decomposition. Therefore, the FTR mediated by the MF-NP diatomic system is recyclable and sustainable.

All above evidence supports that nanoparticles can induce ROS production through FTR. Based on this, we assumed that legume nodulation could be induced by ROS generated from FTR with optimized conditions to extend nodulation time, thus achieving the same desirable effect as regulating Rbohs gene expression. The sustainability of nanoparticle-induced FTR generating ROS and extension of the nodulation period overcome the adverse impact of excessive nodulation on plant vegetative growth. Our findings provide a reference for developing strategies of excessive nodulation.

**Materials and methods**

**Substrate preparation**

MnFe$_2$O$_4$ (MF-NPs, Purity: 99.5%) and MnO$_2$ (M-NPs, Purity: 99.9%) were purchased from Shanghai Bike New Material Technology Co., Ltd.; Fe$_2$O$_3$ (F-NPs, Purity: 98.0%) from Aladdin Reagent Co., Ltd. (Shanghai, China); FeCl$_2$ and MnCl$_2$ (analytical purity) from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). This experiment was performed with the soybean variety “Williams 82” and rhizobium USDA110 strain. Before USDA110 inoculation, nanomaterials (MF-NPs, M-NPs, F-NPs, and F-NPs+M-NPs) were dissolved in 1.2 L ultrapure water under ultrasonication at the concentrations of 0.1, 1, 10, 50, and 100 mg L$^{-1}$ with the metal ion concentration in ionic materials (FeCl$_2$ at 1.1, 10.10, 54.98, 109.95 mg L$^{-1}$ and MnCl$_2$ at 0.55, 5.45, 27.27, 54.55 mg L$^{-1}$) consistent with that in MF-NPs. After mixing materials and 6L vermiculite and stirring thoroughly, the mixture was divided into groups according to concentrations, sterilized at 121 °C for 20 min, and transferred to an ultra violet (UV)-sterilized plug tray after complete cooling. The control group (CK) was not mixed with any material in ultrapure water.

**Planting conditions**

The soybean seeds were surface-sterilized in sodium hypochlorite under stirring for 4 min, rinsed 6 times with sterile water, transferred into a blank agar petri dish, and incubated at 25 °C in a dark incubator for germination. The germinated soybeans were planted into sterile plug tray containing matrixes with a planting depth of 3 cm. After planting, each plug tray with 32 plants in total was watered with 2 L of nitrogen-free nutrient solution every three days. Next, the seedlings were cultured at a greenhouse (14 h-light/10 h-dark cycle at a light intensity of 150 μmol m$^{-2}$ s$^{-2}$).
Rhizobium inoculation
After activation of rhizobia, a single colony was collected and inoculated into YMA liquid medium for 72 h at 28 °C in a shaker (150 rpm) for further use. Afterwards, rhizobia were washed with sterile water. The absorbance value (OD600) of the bacterial liquid was adjusted 0.5. After 1-week planting of the seedlings, the base matrix around each plant root was inoculated with 1 mL bacterial liquid.

Sampling time
On day 4 post inoculation, root samples were collected to observe the curling of root hairs and the staining of H2O2 and O2−. The roots were sampled on day 4, 8, and 12 post rhizobium inoculation for transcriptome sequencing analysis. On day 12, 16, and 20, the samples were collected to observe the nodule development status. On day 28, samples were collected to evaluate the growth indexes including nodule weight, nodule number, plant height, and root length. After the nodule was fully mature (on day 28), the root system with nodules was collected to determine the nitrogenase activity, and the mature nodule sections were observed to evaluate the development morphology of the symbiosis zone and the biological activity of rhizobia.

Nitrogenase activity assay
The nitrogenase activity was measured as our previously study described [30]. The difference is that the sample used for GC detection in this study was 200 μL. The standard curve data (Additional file 1: Fig. S24) were converted to C2H4 concentration, C2H4 yield (μmol min−1) was normalized to mass (μmol h−1 g−1), and nitrogenase activity was calculated by the following equation:

\[ \phi = \frac{M}{m \times t} \]

where \( \phi \) indicates nitrogenase activity (μmol h−1 g−1); \( M \), ethylene production (μmol); \( m \), nodule weight (g); \( t \), reaction time (h).

H2O2 decomposition assay
A total of 2.5 mM H2O2 was mixed separately with MF-NPs, F-NPs, M-NPs, F-NPs+M-NPs, MnCl2, or FeCl3 in 20 mL PBS at room temperature. Then, 1 mL reaction solution was centrifuged at 10,000 g for 3 min to collect the supernatant for test using the H2O2 content assay kit (Solarbio, Beijing, China). After the reaction was completed, the absorbance of the solution at 415 nm was measured to evaluate the H2O2 concentration. The above operation was repeated at 10 min, 30 min, 1 h, 3 h, 5 h, 12 h, 24 h, 3 d, 7 d, and 14 d during the reaction.

Hydroxyl radical generation
The nanomaterials (at 1.25 mg mL−1 concentration) and ionic materials (with the ion concentration in ionic materials consistent with that of MF-NPs) were separately prepared into mother liquid, and 1 M methylene blue (MB) solution was prepared. Subsequently, 100 μL material mother liquid (nanomaterials or ionic materials) and 200 μL of MB solution were mixed with 2 mL of 0.3% H2O2 solution under shaking. After reaction for 0, 6, 12, 18, 24, 30, 36, 42, and 48 h, spectral scanning was performed, and OD660 was measured for quantitative analysis.

Detection of H2O2 and O2−
H2O2 and O2− in plant roots in situ were visualized by DAB and NBT staining, as previously described [31]. Briefly, the root H2O2 content was analyzed by staining the roots with 0.1 mg mL−1 DAB in 50 mM Tris buffer (pH 5.0). The O2− content was measured after root incubation with 2 mM NBT in 20 mM K-phosphate containing 0.1 M NaCl (pH 6.1). Root images were taken under brightfield light.

Transcriptomic analyses of soybean
On day 4, 8, and 12 post Rhizobium inoculation into vermiculite media with or without 10 mg L−1 MF-NPs (as CK), the whole root tissues were collected from the plants, immediately put into an Eppendorf tube, and stored in liquid nitrogen. Total RNA was extracted using an plant RNA extraction kit (TransGen Biotech, Beijing, China). The obtained total RNA was sequenced on Illumina NovaSeq sequencing platform, and the mRNA sequencing libraries were constructed (MetWare Biological Science and Technology Co., Ltd. Wuhan, China). Subsequently, the sequencing reads were mapped to the Glycine max genome using hisat2 software. The differentially expressed genes (DEGs) were identified using DESeq software with P value <0.05, the false discovery rate (FDR)<0.05, and a |log2 Fold Change| of ≥ 1 as thresholds.

Bacterial live and dead stain
Frozen microtome (Leica CM1950) was used for microsection to observe the live and dead bacteria in nodules. Each slice was stained with the mixture of SYTO9 and propidium iodide (PI) stains and held in the dark for 30 min, followed by observation under a fluorescence microscope. The bacteria with intact cell membranes showed green fluorescence, in contrast to red fluorescence for bacteria with damaged membranes.
Statistical analysis
All the data were presented as the mean ± standard deviation (±SD). The experiments were performed in triplicates. Significant differences between groups were determined by independent sample t-test and statistical differences were analyzed by applying the one-way ANOVA. The significance levels were determined at *P < 0.05, **P < 0.01, and ***P < 0.001.

KEGG pathway analysis of DEGs
KEGG pathway analysis method is explained by Irudayaraj [32].

Results
Characterization of nanoparticles
In this study, nanomaterials (MnO₂ nanoparticles, M-NPs; Fe₂O₃ nanoparticles, F-NPs; and MnFe₂O₄

Fig. 1 Material characterization. a SEM. b Element line scan. c Zeta-potential (n = 3). d Size distribution. e XPS spectra of MF-NPs in Fe2p region (e-1) and Mn2p region (e-2)
nanoparticles, MF-NPs) and conventional ionic materials (FeCl₂ and MnCl₂) were derived from ordinary commercial production. Nanomaterials were characterized accordingly. Scanning electron microscopy (SEM) (Fig. 1a) and transmission electron microscopy (TEM) (Additional file 1: Fig. S1) results indicated that nanomaterials used in this study were irregular granules. Line scanning results indicated that the content of manganese and oxygen in M-NPs was 40.12% and 59.88%, respectively (Fig. 1b), that the content of iron and oxygen in F-NPs was 52.14% and 47.86%, respectively, and that the content of iron, manganese, and oxygen in MF-NPs was 29.64%, 10.84%, and 59.52% (Additional file 1: Table S1). The zeta potential value (Fig. 1c) of M-NPs, F-NPs, and MF-NPs in deionized water (pH 6.5) was \(-6.8 \pm 0.3\) mV, \(-9.2 \pm 0.3\) mV, and \(-1.5 \pm 0.2\) mV, respectively, and the average particle size distribution was 223.43 nm for MF-NPs, 59.34 nm for F-NPs, and 108.71 nm for M-NPs (Fig. 1d). Fourier transform infrared (FTIR) spectroscopy showed no specific surface functional groups in MF-NPs (Additional file 1: Fig. S2). The photoelectron spectrum of MF-NPs showed that Fe 2p3/2 peaks for FeO, Fe₃O₄, and Fe₂O₃ appeared at binding energies of 709.9 eV, 710.7 eV, and 711.8 eV, respectively [33, 34], coupled with satellite peaks at a binding energy of 713.4 eV and 718.8 eV in the Fe 2p region (Fig. 1e). The Mn 2p3/2 peaks for MnO, Mn₃O₄, and Mn₂O₃ appeared at binding energies of 641.2 eV, 642.2 eV, and 643.2 eV, respectively, coupled with satellite peaks at a binding energy of 644.4 eV [33, 35].

**Synergistic ROS generation of MF-NPs**

The mechanism by which MF-NPs catalyzed ROS production was studied by comparing the Mn-based nano-oxides, Fe-based nano-oxides, and ionic compounds (Fig. 2a). The determination results of the increase value in dissolved oxygen and the number of O₂ bubbles showed that under M-NPs, M-NPs+F-NPs, and FeCl₂ exposures, O₂ generation capability was increased within 30 min, which was faster than under MF-NP exposure (Fig. 2b and Additional file 1: Fig. S3). A time-dependent H₂O₂ assay at pH 6.5 indicated that nano-sized M-NPs and M-NPs+F-NPs decomposed almost all H₂O₂ within 1 h, and FeCl₂ also achieved similar effects within 5 h (Fig. 2c). Under MF-NP exposure, about 10% H₂O₂ residue was still detected 1 week later. Only a weak H₂O₂ decomposition was observed under F-NP and FeCl₂ exposures. After adding H₂O₂ to reaction system (pH 6.5), ·OH concentration in the presence of NPs was evaluated by detecting the fading degree of methylene blue (MB) induced by ·OH (Fig. 2d). The ·OH yield under MF-NP exposure was
Response to MF-NPs exposure and Fenton reaction

To evaluate the response of soybean growth to different nanomaterials, soybeans were planted in substrates containing different nanomaterials and their physiological and nitrogen fixation indexes were analyzed at different inoculation time points. The plant height analysis indicated a general decrease in the height of the soybean plants treated with a certain concentration of manganese-based materials (M-NPs+F-NPs, M-NPs, MF-NPs, MnCl₂), especially that treated with MF-NPs. However, almost no obvious change in plant height was observed for the treatment with F-NPs and FeCl₂ at all concentrations (Additional file 1: Figs. S5, S6). The stem diameter (Additional file 1: Fig. S7) analysis showed a completely opposite trend to that of plant height, with a larger diameter in the groups treated with M-NPs+F-NPs, M-NPs, MF-NPs, and MnCl₂ than in the groups treated with F-NPs and FeCl₂, and the most significant stem diameter difference was observed in the MF-NP treatment group at all concentrations, which might be due to dwarfing induced by ROS. Root length analysis indicated that root growth was obviously promoted by all the treatments except F-NP treatment at certain concentrations, which might be due to the advantages of iron and manganese as massive element in plant physiological growth (Additional file 1: Figs. S8, S9). Biomass analysis (Additional file 1: Fig. S10) showed only a significant increase in MF-NP and MnCl₂ treatment groups, which was consistent with the increasing trend of nodule number (Additional file 1: Fig. S11) and weight (Additional file 1: Fig. S12), suggesting that biomass increase might be related to the increase in nitrogen nutrition. Under the treatment with different concentrations of MF-NPs, the plant exhibited a height decrease of 15.04%, 13.99%, and 18.10% in response to 10 mg L⁻¹, 50 mg L⁻¹, and 100 mg L⁻¹ MF-NPs, respectively (Fig. 3a); a stem diameter increase of 10.4% at 1 mg L⁻¹, 11.88% at 10 mg L⁻¹, 8.05% at 50 mg L⁻¹, and 10.06% at 100 mg L⁻¹ (Fig. 3b); a biomass increase at 0.1 mg L⁻¹, 1 mg L⁻¹, and 10 mg L⁻¹, with a maximum increase of 25.70% (1.05 g) at 10 mg L⁻¹ (Fig. 3c); a maximum nodule weight increase of 0.28 g at 1 mg L⁻¹ (Fig. 3e) and a maximum nodule number increase of 71 at 10 mg L⁻¹ with various degrees of increase at 1 mg L⁻¹, 10 mg L⁻¹, and 50 mg L⁻¹ (Fig. 3d). The growth phenotype investigation showed that the root length exhibited a significant increase of 25.36%, 26.19%, and 20.92% at 1 mg L⁻¹, 10 mg L⁻¹, and 50 mg L⁻¹, respectively, and that plant growth promotion effect was weakened at a high concentration of 50 or 100 mg L⁻¹ (Fig. 3f, g).

The response of soybean growth and nitrogen fixation indicators to Fenton reaction was examined at the optimal promoting concentration (10 mg L⁻¹) under two different acidity matrixes (pH 6.5) and (pH 5.2) conditions. As shown in Fig. 3m, in the case of low pH-induced ROS redundancy, no significant difference was observed in plant height (Fig. 3h). Under 0.01% and 0.1% H₂O₂ exposure, the stem diameter at pH 6.5 matrix was respectively 6.62% and 8.71% higher than that at pH 5.2, and under 0.01% and 0.1% H₂O₂ exposure at pH 6.5, the stem diameter was 10.05% and 11.89% higher than that without H₂O₂ exposure at the same pH value (Fig. 3i). Biomass exhibited an increase of 15.06% under 0.01% H₂O₂ exposure at pH 6.5 matrix relative to pH 5.2 (Fig. 3j). Meanwhile, the nodule number (Fig. 3k) and weight (Fig. 3l) under 0.01%, 0.1%, and 0.3% H₂O₂ exposure at pH 6.5 were 1.35, 1.78, 1.43 (number) and 1.40, 1.25, and 1.47 (weight) times as much as those at pH 5.2, respectively. The weight under 0.01% H₂O₂ exposure at pH 6.5 was 15.32% higher than that without H₂O₂ exposure at pH 6.5. Root length (Fig. 3m, n) showed significant difference between pH 5.2 and pH 6.5 at low H₂O₂ concentrations (0.01% and 0.1%) and tended to be stable with the increase of H₂O₂ concentration, but excessively high H₂O₂ concentration (>0.1%) induced the suppression of the root length (independent of pH) and all growth indicators (regardless strong or weak acid).

Seedling roots response to MF-NP exposure

The effect of MF-NP exposure on the ROS level in soybean roots was examined by tissue staining and localization of soybean root tips and root maturation zone. The roots were stained with either 3, 3-diaminobenzidine (DAB) or nitroblue tetrazolium (NBT) to observe H₂O₂ and O₂⁻ production, respectively. Compared with the control, MF-NP-treated plants showed the accumulation of H₂O₂ and O₂⁻ in the root tips (Fig. 4b, c) and root maturation zone (Fig. 4d, e). The degree of coloring was calculated with ImageJ software [31]. The content of H₂O₂ and O₂⁻ in the MF-NPs treatment group was 6.52 and 2.08 times as high as that in the control group in the root tip, and 2.73 and 2.99 times in the root maturation...
zone. The detection results of total ROS content in root tips and root maturation zone were consistent with those described above (Additional file 1: Fig. S13).

Evaluation of nodulation and nitrogen fixation efficiency

Nodulation starts from the flavonoid-induced Rhizobium infection, leading to the expression of nodulation factor (Nod-F). In nodulation process, ROS first induces root hair curling, and then the development of infection lines and nodule primordia [36]. In this study, we counted the early curled root hairs. As shown in Fig. 4f and g, MF-NPs exposure increased the curling of root hairs in the maturation zone, which was 3.38 times of that in the control group, thus providing a prerequisite for the development and growth of mature nodules. With the further growth of nodules, early immature nodules visible to the
naked eye were formed, and their number at different stages was used to locate the nodule development period. The difference in the distribution of immature nodules was observed between the control group and MF-NP treatment group during the 20-day growth period (Fig. 5a). The number of immature nodules in the 20-day growth period was found to be 7.43 times of that in the control group, indicating that the exposure to 10 mg L\(^{-1}\) MF-NPs could prolong the early development of the nodules, leading to a continuous increase in the number of nodules (Fig. 5b).

Root phenotype and nodule development were used to evaluate the influence of MF-NP treatment on the growth and development of underground parts (Fig. 6a). Analysis of root morphology showed no obvious suppression effect of MF-NPs on root, and further analysis of the nodule section showed no obvious difference in the cell morphology and size between the infected area and the non-infected area, and the development of the transport tissue was normal. Fluorescence staining with SYTO\(_9\) and PI showed that the infected rhizobia in the cells of the nodule exhibited viability, and that the number of total

**Fig. 4** ROS distribution in roots and root hair curling. a ROS production mediated by MF-NPs under abiotic stress. b, c Root tip phenotype and relative ROS content in root tip on day 7 post inoculation. root tip (n = 3) and (n = 3) grown in vermiculite (10 mg L\(^{-1}\) MF-NPs exposure) stained with NBT (to detect O\(_2\)\(^{-}\)∙). d, e Root maturation zone phenotype and relative ROS content in root maturation zone with H\(_2\)O\(_2\) stained with DAB. f Morphological changes of root hair on day 4 post inoculation with rhizobia. g Number of curled root hair (treatment number, n = 9)
infected rhizobia (green) and the dead rhizobia (red) in MF-NPs treatment group was comparable to that in the control group (Fig. 6b). These observations indicated that MF-NPs exposure had no obvious effect on the growth and development of soybean roots and nodules, and it could even increase root length.

The acetylene reduction method (ARM) was used to evaluate the nitrogenase activity in the mature nodules. All the treatments resulted in no significant difference in the nitrogenase activity per unit mass nodule, implying that the exposure to these nanomaterials would neither promote nitrogenase activity nor impose stress on the nitrogen fixation function of nodules (Fig. 6c). Furthermore, the total nitrogen fixation within 2 h in a single soybean plant (Fig. 6d) was evaluated by analyzing the reduction activity of the whole plant nodules. The 2.51-, 2.37-, 1.99-time increase in total nitrogen fixation under MF-NPs treatment (10 mg L\(^{-1}\), 50 mg L\(^{-1}\), and 100 mg L\(^{-1}\)) and 1.79-time increase under MnCl\(_2\) (27.27 mg L\(^{-1}\)) treatment, relative to the control group.

Gene expression analysis
The mechanism by which MF-NPs exposure induced nodulation was investigated by transcriptome sequencing analysis of root samples from control and treatment groups at different stage (early stage, day 4; middle stage, day 8; and late stage, day 12 post inoculation). First, principal component analysis (PCA) was performed to determine the impacts of MF-NPs on soybean (Fig. 7a). A total of 595 DEGs were detected in the early stage with 100 up-regulated and 495 down-regulated, and 2676 DEGs in the late stage with 912 up-regulated and 1764 down-regulated. Hierarchical clustering analysis showed significant differences in different stages (Additional file 1: Figs. S14a, c and S15). However, only 38 DEGs (35 up-regulated and 3 down-regulated) were detected in the middle stage, indicating that MF-NPs exposure in the middle stage made little difference in the transcription level (Additional file 1: Fig. S15b). The Venn diagram showed the relatively few overlapping DEGs in three different stages (Fig. 7b).

The relationship between DEGs and nodulation metabolism was further explored by KEGG, NR, SwissProt, TrEMBL, KOG, GO and Pfam as a reference. The 5, 0, and 36 DEGs in the early, middle and late stages were found to be nodulation-related (Nod-R) with 2, 0, and 31 up-regulated, respectively (Fig. 7c). The late-stage DEGs were shown in Additional file 1: Table S2. The promotion effect of MF-NPs exposure on nodulation was mainly found in the early and late stages, especially

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**Fig. 5** Early nodulation response to MF-NPs exposure. a Distribution of immature nodules at the various stage (on day 12 (early), 16 (middle), and 20 (late) post rhizobium inoculation, respectively) with/without MF-NPs exposure. b Number of nodules (n = 9)
in the late stage. We further analyzed flavonoid-related (Fla-R) DEGs (Fig. 7d, Additional file 1: Table S3) and ROS-related (ROS-R) DEGs (Fig. 7e, Additional file 1: Table S4) at late stage. Of 61 ROS-R DEGs, 51 down-regulated and 10 up-regulated. In total, 47 differential Fla-R DEGs were detected, of which 41 down-regulated and 6 up-regulated. In order to study the expression patterns of DEGs under different processing conditions, the FPKM of the DEGs was normalized, followed by K-means cluster analysis (Fig. 7f). Based on the expression patterns, Nod-R DEGs were clustered in class 12, and no significant difference between treatment group and control group in the early and middle stages, but a significant increase in the expression level of Nod-R DEGs was observed under MF-NPs exposure in the late stage. After optimization and clustering, the ROS-R DEGs were clustered into class 4, class 5, class 7, class 10, and class 11, all of which showed an opposite trend to class 12, which might be due to the feedback regulation triggered by the MF-NP-induced ROS increase. The Fla-R DEGs were mainly clustered into Class 4, Class 5, and Class 11, which was overlapped with the clustering of ROS-R, suggesting a synergistic relationship between these two types of DEGs. Our results were consistent with previous reports that expression of flavonoids could induce ROS expression [37, 38]. In addition, our results indicated that the expression patterns of Fla-R DEGs were completely opposite to those of Nod-R DEGs. Class 2 and class 3 showed the trend similar to that of Class12, indicating these 3 classes of DEGs might have synergistic effect, which remains to be further investigated.

GO enrichment analysis classified DEGs into biological process, cellular component and molecular function according to gene functions. Different genes in organisms exert biological functions through interactions. The 50 most significantly enriched GO-Terms were selected and classified according to GO database annotations. In the early stage (Additional file 1: Fig. S16), DEGs were mainly enriched in the pathways related to photosynthetic system inhibition and ROS metabolism. In the middle stage, DEGs were mainly enriched in the pathways (Additional file 1: Fig. S17) involved in cation transport. In the late stage (Fig. 8a), DEGs were mainly enriched in the pathways related to the secondary metabolism. Flavonoids (yellow box) serve as the initial inducers of legume nodulation. Our results indicated
Fig. 7 Global expression profiles of soybean genes at different nodulation stages. a Principal component analysis (PCA) of RNA-Seq data. b Venn diagram of E-CK (early stage), M-CK (middle stage), and L-CK (late stage) without NPs exposure. E-MF, M-MF, and L-MF indicate early-stage, middle-stage, and late-stage MF-NPs (10 mg L⁻¹) exposure, respectively. c–e Relative expression heatmap of c Nod-R genes, d Fla-R genes, and e ROS-R genes. f K-means diagram of differential expressed genes (DEGs)
that the expression level of Fla-R genes was significantly reduced in the late stage, which might be due to the feedback regulation induced by the ROS increase in the early and middle stages. This was consistent with the expression of ROS-R genes in root samples (Fig. 8a). The microorganism-plant symbiosis system related to nodulation was significantly enhanced (as shown in Fig. 8a red box and Additional file 1: Fig. S18), which was specifically manifested as the enhancement in nodulation, nitrogen fixation, endocytic vesicle membrane, bacteroid-containing symbiosome, peribacteroid membrane, and other functions. The obtained GO terms were subjected to topGO directed acyclic graph (DAG) analysis. During symbiont process, the microorganism-plant interspecies interactions occurred nodulation upstream (red box in Fig. 8b and Additional file 1: Fig. S18). Changes in the metabolism of ROS were also reflected in multiple hierarchical pathways (Fig. 8b and Additional file 1: Fig. S19). The KEGG pathway analysis revealed the top 20 most significantly enriched pathways (Fig. 8c) with “biosynthesis of secondary metabolites” pathway exhibited the highest enrichment degree,
which might be due to peroxide stress induction in soybean roots (Fig. 8c, Additional file 1: Fig. S20). KEGG pathway analysis indicated that “Flavonoid synthesis” pathway was significantly enriched, which was consistent with the results of GO enrichment analysis (Fig. 8c, Additional file 1: Fig. S21). KEGG pathway analysis also showed that glutathione metabolism pathway was significantly enriched, which might be due to the continuous increase in ROS, and this pathway enrichment contributed to the metabolic balance between root tissues and relieve oxidative stress (Fig. 8c and Additional file 1: Fig. S22). In addition, the enrichment of nitrogen metabolism pathway was observed, which might be explained by the increase in nitrogen fixation in some mature nodules in the late stage (Fig. 8c and Additional file 1: Fig. S23).

MF-NPs-mediated AON response

According to previous studies, the relevant auto-regulation of nodulation (AON) pathway genes were identified [15, 17]. To evaluate the regulatory effect of MF-NPs exposure on the AON pathway, we analyzed the expression levels of the related genes, including NFR1/ NFR5 (nodulation factor), GmNINa (nodulation gene), ENOD40s (nodulation response gene), NNC1 (nodule number control), miR172c (fine-tuning rhizobia infection and nodule organogenesis), GmRIC1 and GmRIC2 (specific CLAVATA/ESR-related (CLE) peptides in soybean responsible for producing root-derived nodulation), and GmNARK (nodule autoregulation receptor kinase) (Fig. 9). In the nodulation pathway, our study showed that the expression levels of Fla-R and ROS-R genes in MF-NPs exposure groups were overall down-regulated, while Nod-R genes showed a up-regulation expression trend. The expression of nodulation factors (NFR1/S) was low and not significantly different between MF-NPs exposure group and CK, which might be cause of the down-regulation of the ROS-R genes. In addition, GmNINa, miR172c, and ENOD40s all showed an up-regulation trend with significantly increasing ROS levels during this process. Therefore, the up-regulated expression of nodulation genes (GmNINa, miR172c, and ENOD40s) and the low expression of nodulation factors (NFR1/S) might be due to the increase in exogenous ROS. In the AON pathway, NARK made no obvious response to significant up-regulation of GmRIC2 and GmRIC1 expressions, which might...
be attributed to the inhibition of CLE peptides responsible for long-distance transportation. Therefore, MF-NPs exposure could enhance the expression of nodulation genes, meanwhile inhibiting the AON pathway, thereby achieving an increase in the number of nodules.

**Discussion**

Divalent manganese ions have been reported to produce Fenton-like reactions, and they have a better hydroxyl radical induction effect in the FTR system [29, 39]. Although M-NPs, M-NPs+F-NPs, and FeCl₂ showed strong H₂O₂ removal efficiency, most of their catalytic products was oxygen rather than -OH. Our data showed that FeCl₂ had a high ∙OH generation efficiency, but ∙OH products was oxygen rather than ∙OH. Our data showed that FeCl₂ had a high ∙OH generation efficiency, but ∙OH generation persistency was not satisfactory. Therefore, MF-NPs might be the best option for long-term ROS induction. In previous studies, MF-NPs have been widely used as an inducer of -OH originated from the FTR between Fe²⁺ and H₂O₂ and the Fenton-like reaction among Mn²⁺, Fe²⁺, and H₂O₂ in diatomic compounds [39]. The redox reaction between iron and manganese can further increase the yield of -OH, thus enriching the ROS type and content.

During plant growth, Mn ions participate in processes such as photosynthesis, respiration, protein synthesis, and hormone response [40]). Previous studies have shown that maize (Zea mays) growth is inhibited only under 200 mg kg⁻¹ Mn exposure [41], whereas sunflower growth is not inhibited until Mn concentration reaches 5300 mg kg⁻¹ [42]. Theoretically, exposure to 0–100 mg L⁻¹ MF-NPs will not impose a serious stress on plant growth, thus the dwarfing of soybean plant is not caused by the stress of manganese ions. ROS stress has been reported to affect the plant root and height [43]. Therefore, in this study, the change of soybean plant height might result from ROS regulation. Our data showed that despite a significant plant height reduction, the total biomass of soybean plant was significantly increased, which can be attributed to the increase in the total nitrogen fixation efficiency of nodules in a single plant. However, this advantage disappeared under exposure to high concentrations of MF-NPs, suggesting an inhibitory effect of excessively high ROS stress on total nitrogen fixation. Our elemental composition analysis indicated that biomass was obviously higher in the system containing iron and manganese double ions than that in CK group, which was consistent with our assumption about the FTR between iron and manganese ions (Fig. 1b, Additional file 1: Table S1). Although the combination of M-NPs+F-NPs also contained iron and manganese ions, such a combination failed to exhibit a promoting effect, which might be due to the physical distance between the two ions. The MF-NPs exposure group exhibited a significant accumulation of nodule number and weight, which might be explained by the positive regulatory effect of ROS on the early nodulation system of soybean. Our results are consistent with one previous report [12].

Our data showed that the H₂O₂ and O₂⁻ in the root were mainly derived from the induction of MF-NPs (Fig. 4a). Under MF-NPs exposure, H₂O₂ or O₂⁻ can be converted into -OH through FTR to further enrich and enhance the ROS [44]. Previous studies have revealed that nanoparticles can significantly increase H₂O₂ and O₂⁻ in plant roots, and the application of nanomaterials enhances plant resistance to the stresses such as drought, insect, and disease [45–47]. Therefore, nanomaterials conducive to enhancing plants'stress resistance need to be further developed and utilized.

The ROS regulatory gene Rbohs is used as a target gene to promote ROS yield, the overexpression of Rbohs can increase ROS yield, whereas RNA interference (RNAi) often decrease ROS yield [48]. In this study, under the optimal concentration (10 mg L⁻¹) of MF-NPs exposure, Rbohs overexpression exhibited similar effect on the ROS generation to the pH-dependent FTR. Within an appropriate H₂O₂ concentration range (0.01–0.3%), the excessively acidic matrix exhibited significant inhibition on the soybean development, indicating that -OH indirectly inhibited the soybean growth through Fenton reaction, while high concentration of H₂O₂ (>0.3%) directly inhibited root growth. Based on all above findings, it could be concluded that only a proper ROS concentration could induce an increase in the number and weight of nodules.

Nitrogenase can directly supply nitrogen nutrient for soybean, thus nitrogenase activity is used to evaluate nitrogen fixation of soybean. In our previous study, ROS was found to irreversibly damage nitrogenase activity [30]. In the present study, we did not observe a significant inhibition of unit nitrogenase activity by ROS, which might be due to the leghaemoglobin-supplied strong reducing conditions and MF-NPs exposure-induced ROS degradation [49]. In the early root development and early nodulation, the increasing evidence support that nodulation efficiency is highly dependent on ROS accumulation [50]. FTR provides a relatively high ROS in early nodulation, but the reaction between Fe²⁺ and Mn²⁺ tends to be complete when ROS is not needed in large amount during the nodule maturity stage. Therefore, the ROS exposure will not have an inhibitory effect on nitrogenase activity, resulting in the significant increase in nitrogen fixation efficiency of a single plant.

In this study, transcriptomics analysis confirmed the early experimental phenomena from the genetic level and metabolic pathways. In previous studies, the induction of carbon nanomaterials has been reported to increase the expression of the Lotus japonicus early nodulation
genes [51], but the related action mechanism remains unclear. Our study provides evidence for the exploration of this mechanism. In the study of toxicity effect of engineered nanomaterials (ENM) (Ag, ZnO, and TiO2) on the legume Medicago sativa A17 [52], the early nodulation genes have been found to be significantly down-regulated, and the inhibition of nodulation under the ENM treatment might be primarily be due to abiotic stress induced by Zn ions. In non-legume rice, fluorescent silica (F-SiO2) ENM treatments have been revealed to increase biomass accumulation, and transcription analysis has unveiled that F-SiO2 ENM treatment could upregulate the expression of the genes related to glucose metabolism and carbon fixation [53]. These previous findings are in line with the expression patterns of the related genes in MF-NPs-exposed soybean in the middle stage post inoculation in our study. The combined treatment of titanium dioxide and carbon dioxide can enhance the antioxidative activity of rice, down-regulate the expression of the genes related to flavonoids and other secondary metabolites, thereby enhancing photosynthesis [54]. This is in accordance with our findings of the inhibitory effect of MF-NPs on the expression of flavonoid-related genes in this study.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12951-022-01372-2.

Additional file 1: Fig. S1. TEM images of (a) MF-NPs, (b) M-NPs, and (c) F-NPs. Fig. S2. Fourier transform infrared (FTIR) spectra of F-NPs, M-NPs and MF-NPs. Fig. S3. Comparison of the efficiency of oxygen production by different materials. Fig.S4. Ultraviolet scan curve of MB fading at different reaction time points in the presence of NPs and CMs. Fig. S5. Aboveground phenotypic images of soybean plants treated with different NPs. Fig. S6. Soybean plant height under exposure to different materials. *P < 0.05, **P < 0.01, and ***P < 0.001 respectively indicate three different significance levels. Fig. S7. Stem diameter of soybean plant under exposure to different materials. *P < 0.05, **P < 0.01, and ***P < 0.001 respectively indicate three different significance levels. Fig. S8. Phenotypic image of root system of soybean treated with different NPs. Fig. S9. Root length of soybean treated with different NPs. *P < 0.05, **P < 0.01, and ***P < 0.001 respectively indicate three different significance levels. Fig. S10. Total biomass of soybean under exposure to different NPs. *P < 0.05, **P < 0.01, and ***P < 0.001 respectively indicate three different significance levels. Fig. S11. Number of soybean nodules under exposure to different NPs. *P < 0.05, **P < 0.01, and ***P < 0.001 respectively indicate three different significance levels. Fig. S12. Weight of soybean nodules under exposure to different NPs. *P < 0.05, **P < 0.01, and ***P < 0.001 respectively indicate three different significance levels. Fig. S13. Quantitative analysis of total ROS in root. *P < 0.05, **P < 0.01, and ***P < 0.001 respectively indicate three different significance levels. Fig. S14. Number of differentially expressed genes (DEGs) detected in the three stages (early, middle, and late) post inoculation. Red indicates the significantly up-regulated DEGs. Green indicates the significantly down-regulated DEGs. Blue indicates DEGs with no significant expression change. Fig. S15. Heat map of cluster analysis of differentially expressed genes in the three different stages post inoculation. Fig. S16. GO enrichment histogram of differentially expressed genes (DEGs, early stage). Fig. S17. GO enrichment histogram of differentially expressed genes (DRGs, middle stage). Fig. S18. Directed acyclic graph (DAG) of enriched GO terms (late stage). Fig. S19. Directed acyclic graph (DAG) of GO enrichment analysis of ROS metabolism-related genes (late stage). Fig. S20. KEGG pathway of biosynthesis of various secondary metabolites—part 2 in response to MF-NPs treatment. Fig. S21. KEGG pathway of flavonoid biosynthesis in response to MF-NPs treatment. Fig. S22. KEGG pathway of glutathione metabolism in response to MF-NPs treatment. Fig. S23. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of nitrogen metabolism in response to MF-NPs treatment. Fig. S24. Standard curve of nitorgenase activity. Table S1. Elemental content of NPs. Table S2. Expression of nodulation related (Nod-R) genes in the roots in response to MF-NPs treatment. Table S3. Expression of flavonoid related (Fla-R) genes in the roots in response to MF-NPs treatment. Table S4. Expression of reactive oxygen species related (ROS-R) genes in roots in response to MF-NPs treatment.

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Authors’ contributions
JM, ZS, HH planned and designed the research. JMYZ performed performed most of the experiments, JL and JM contributed to data analysis and wrote the article, ZS and HH supervised the project. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets analysed in the current study are available from the corresponding author on reasonable request.

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Not applicable.

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Competing interests
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