Comparative impacts of iron oxide nanoparticles and ferric ions on the growth of *Citrus maxima*

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Abstract: The impacts of iron oxide nanoparticles (γ -Fe₂O₃ NPs) and ferric ions (Fe³⁺) on plant growth and molecular responses associated with the transformation and transport of Fe²⁺ were poorly understood. This study comprehensively compared and evaluated the physiological and molecular changes of *Citrus maxima* plants as affected by different levels of γ -Fe₂O₃ NPs and Fe³⁺. We found that γ -Fe₂O₃ NPs could enter plant roots but no translocation from roots to shoots was observed. 20 mg/L γ -Fe₂O₃ NPs had no impact on plant growth. 50 mg/L γ -Fe₂O₃ NPs significantly enhanced chlorophyll content by 23.2% and root activity by 23.8% as compared with control. However, 100 mg/L γ -Fe₂O₃ NPs notably increased MDA formation, decreased chlorophyll content and root activity. Although Fe³⁺ ions could be used by plants and promoted the synthesis of chlorophyll, they appeared to be more toxic than γ -Fe₂O₃ NPs, especially for 100 mg/L Fe³⁺. The impacts caused by γ -Fe₂O₃ NPs and Fe³⁺ were concentration-dependent. Physiological results showed that γ -Fe₂O₃ NPs at proper concentrations had the potential to be an effective iron nanofertilizer for plant growth. RT-PCR analysis showed that γ -Fe₂O₃ NPs had no impact on AHA gene expression. 50 mg/L γ -Fe₂O₃ NPs and Fe³⁺ significantly increased expression levels of FRO2 gene and correspondingly had a higher ferric reductase activity compared to both control and Fe(II)-EDTA exposure, thus promoting the iron transformation and enhancing the tolerance of plants to iron deficiency. Relative levels of Nramp3 gene expression exposed to γ -Fe₂O₃ NPs and Fe³⁺ were significantly lower than control, indicating that all γ -Fe₂O₃ NPs and Fe³⁺ treatments could supply iron to *C. maxima* seedlings. Overall, plants can modify the speciation and transport of γ -Fe₂O₃ NPs or Fe³⁺ for self-protection and development by activating many physiological and molecular processes.

Keywords: γ -Fe₂O₃ NPs; Fe³⁺; *Citrus maxima*; micronutrient; impacts; gene expression

Capsule

The impacts caused by γ -Fe₂O₃ NPs and Fe³⁺ were concentration-dependent and plants could activate molecular responses to transform and utilize both γ -Fe₂O₃ NPs and Fe³⁺.

1. Introduction

Iron oxide nanoparticles (γ -Fe₂O₃ NPs) are one of the most widely explored and applied nanomaterials, which have a wide variety of applications in medical diagnostics, controlled drug release, separation technologies and environmental engineering due to their novel properties such as enhanced surface-to-volume ratio, superparamagnetism and inherent biocompatibility (He et al., 2011; Perez et al., 2002; Wang et al., 2016). Previous studies have reported both positive and negative effects of γ -Fe₂O₃ NPs on plants. For example, Alidoust and Isoda (2013) showed that γ -Fe₂O₃ NPs exhibited no adverse impacts on the growth of the soybeans (*Glycine max* (L.) Merr.) and produced a significant positive effect on root elongation after soil fertilization. Li et al. (2013) and Ren et al. (2011) observed that γ -Fe₂O₃ NPs could enhance seed germination, root growth, chlorophyll content in watermelon (*Citrullus lanatus*) planted in quartz sand and mung bean (*Vigna radiata* L.) grown in silica sediment. On the other hand, γ -Fe₂O₃ NPs were toxic and significantly decreased the sizes of two algal species cultures (*Nannochloropsis* sp. and *Isochrysis* sp.), due to the attachments of NP aggregates on the surface of algae and negative impact caused by these attached aggregates on photosynthetic or respiratory processes of algal species (Demir et al., 2015).

Regarding the previous reports, more efforts should be made to unravel the pathways that γ -Fe₂O₃ NPs interact with plants. We propose that γ -Fe₂O₃ NPs may play three roles in plant growth. The first one is that γ -Fe₂O₃ NPs lead to phytotoxicity through participation in the Fenton reaction and the production of hydroxyl radicals as reported previously (Halliwell and Gutteridge, 1992). Secondly, the aggregation and accumulation of NPs on plant root surface may inhibit the transmission of water and other nutritional components (Ren et al.,

2011). The last one is that $\gamma\text{-Fe}_2\text{O}_3$ NPs can be transformed by plants and taken up as iron micronutrient. Which role is dominant may depend on the concentration range of $\gamma\text{-Fe}_2\text{O}_3$ NPs (Liu et al., 2016). As a result, we conducted a series of concentration-dependent assays of $\gamma\text{-Fe}_2\text{O}_3$ NPs on *Citrus maxima* plants. Representative parameters such as biomass, root length, soluble protein content, lipid peroxidation, chlorophyll and elemental content, activity of antioxidant enzymes and root ferric reductase, root activity were measured to understand the plant responses to different levels of $\gamma\text{-Fe}_2\text{O}_3$ NPs.

In addition, we measured the molecular changes of plants as triggered by different concentrations of $\gamma\text{-Fe}_2\text{O}_3$ NPs, and analyzed gene expressions associated with the transformation and transport of Fe^{2+} . Generally speaking, plants evolve three ways to utilize Fe(III) oxides: protonation, chelation, and reduction (Guerinot and Yi, 1994). H^+ -ATPase (AHA), regulates plant roots to extrude protons, which lower the rhizosphere pH and solubilize iron (Guerinot and Yi, 1994). Ferric-chelate reductase (FRO2), reduces iron at the root surface and transports iron across the plasma membrane (Jeong and Connolly, 2009). The iron-regulated broad-range metal transporter (IRT1), a member of the ZIP family of metal transporters, is responsible for transport of reduced iron across the plasma membrane into root cells (Eide et al., 1996; Henriques et al., 2002). However, no homologous sequence of IRT1 was found in the citrus yet. The family of natural resistance-associated macrophage protein (Nramp) metal ion transporters is another gene associated with iron absorption. Nramp3 protein localizes in the vacuolar membrane, and can transport Fe^{2+} ; Nramp3 expression is upregulated by iron starvation. In the present study, in order to show the in-depth effects of $\gamma\text{-Fe}_2\text{O}_3$ NPs on *Citrus maxima*, the transcriptional modulation of genes involved in iron

uptake and transport viz. AHA, FRO2 and Nramp3, was analyzed at the molecular level using real-time polymerase chain reaction (RT-PCR) technique.

Furthermore, it is largely unknown whether the phyto-impacts are due to the exposure of γ -Fe₂O₃ NPs or the released ions. Many studies have attempted to figure out the effect sources upon NPs exposure, but the results are to some degree conflicting. Kim et al. (2009) reported that the Ag NPs solution contained a negligible concentration of free Ag⁺ ions, and therefore the cytotoxicity primarily arose from oxidative stress caused by AgNPs and is independent of Ag⁺. On the other hand, Kawata et al. (2009) reported that both Ag NPs and Ag⁺ contribute to the toxic effects. To our knowledge, no study has been reported to compare the impacts of γ -Fe₂O₃ NPs and Fe³⁺ on plant growth. In this study, we investigated the plant responses to γ -Fe₂O₃ NPs or Fe³⁺ at both physiological and molecular levels. The results were compared with iron-deficient treatment (control) and Fe(II)-EDTA treatment.

2. Materials and Methods

2.1 Experimental Design

γ -Fe₂O₃ NPs (99.5%, 20 nm) was purchased from Macklin Inc. (Shanghai, China). According to the data provided by the manufacturer, the NPs have a crystal phase of maghemite (Cubic), and true density of 5.15 g/cm³. The shape and size were determined by a Tecnai G2 20 TWIN transmission electron microscope (FEI, USA). The hydrodynamic diameter and zeta potential were determined by a Zetasizer Nano ZS90 dynamic light scattering spectrometer (Malvern Instruments Ltd., United Kingdom). The related results of characterization of γ -Fe₂O₃ NPs are present in supplementary materials. γ -Fe₂O₃ NPs had spherical morphology with an average diameter size of 20.2 ± 2.7 nm (Fig. S1A). The average

hydrodynamic diameter and the zeta potential of γ -Fe₂O₃ NPs was 164.5 ± 11.3 nm and -11.7 ± 0.1 mV, respectively (Fig. S1B and C). In addition, the average hydrodynamic diameter and the zeta potential of γ -Fe₂O₃ NPs in 1/2 Hoagland's nutrient solution without iron was 267.8 ± 18.7 nm and -10.9 ± 0.2 mV, respectively (Fig. S2A and B). *Citrus maxima* seeds were immersed in distilled water and germinated in moist perlite at 28 °C. Then uniform seedlings were transferred to a hydroponic system and exposed to 0 (control), 20, 50 and 100 mg/L of γ -Fe₂O₃ NPs, FeCl₃, or 50 μ M Fe(II)-EDTA amended 1/2 Hoagland's nutrient solution without iron. The plants were grown in an environmentally controlled growth chamber at 28/18 °C with a 16 h/8 h light/dark cycle; the light intensity was 2000 lx. Air was pump into the hydroponic system for 30 min every 3 h. 18 of seedlings were planted in each hydroponic container. The nutrient solution was replaced every 5 days. After 20 days of exposure, physiological parameters including fresh biomass, root length, soluble protein, chlorophyll content, lipid peroxidation, activity of antioxidant enzymes and ferric reductase, and root activity were measured.

2.2 Dynamic dissolution of Fe³⁺ from γ -Fe₂O₃ NPs

In order to investigate the dynamic dissolution of Fe³⁺ from γ -Fe₂O₃ NPs under the same condition as the *Citrus maxima* seedlings exposures, we analyzed the concentration of soluble Fe released from the γ -Fe₂O₃ NPs. 100 mg/L of γ -Fe₂O₃ NPs suspended in 1/2 Hoagland's nutrient solution without iron were placed in six of 10 mL of Eppendorf (EP) tubes, respectively. Ion-release experiments were conducted under the same condition as plant cultivation and the solution was shaken gently. After 12 h, one of the suspensions in EP tubes was centrifuged at 4000 rpm for 15 min. The dissolved iron concentrations in the supernatant

were measured by Avanta M atomic absorption spectrophotometer (GBC, Australia). The rest of five tubes of γ -Fe₂O₃ NPs suspension was measured subsequently every 24 h.

2.3 Root Length and Biomass Measurement

C. maxima plants were carefully removed from the hydroponic system after 20 days. The root lengths of the seedlings were measured with a ruler. The fresh biomass of *C. maxima* including roots and shoots was weighed by a FA1004C electronic analytical balance (Shanghai Yueping Scientific Instrument Co., Ltd, China).

2.4 Measurement of Lipid Peroxidation

Lipid peroxidation of roots and shoots of *C. maxima* plants was determined by using the method of Heath and Packer (1968). 0.3 g root or leaf tissues was ground in 2.0 mL of 10% trichloroacetic acid (TCA), and the mixture was centrifuged at 4000 rpm for 10 min. 1 mL supernatant was added into 2.0 mL of 0.6% thiobarbituric acid (TBA) and the mixture was boiled in water bath for 15 min and then cooled down at room temperature. After centrifugation at 4000 rpm for 10 min, the supernatant absorbance was measured at 450, 532 and 600 nm by a UV-752N spectrophotometer (Shanghai Precision Scientific instrument Co., Ltd, China).

2.5 Measurement of Soluble Protein Content

Soluble protein content was estimated using dyeing method with Coomassie Brilliant Fluka G-250 (Wan et al., 2014). Coomassie Brilliant Blue G-250 solution was prepared by weighing 100 mg coomassie brilliant blue G-250, adding 50 mL of 95% ethanol and 100 mL of 85% (w/v) H₃PO₄, and filling to the final volume (1000 mL) with distilled water at room temperature. 0.3 g of fresh leaves were grounded in 10 mL of 0.05 M phosphate buffer (pH

7.8), the mixture was centrifuged at 4000 rpm for 10 min. 0.1 mL of the supernatant and 0.9 mL of deionized water were added to 5 mL of 0.1% Coomassie Brilliant Blue G-250. After standing for 5 min, the supernatant absorbance was measured at 595 nm.

2.6 Measurement of Antioxidant Enzyme Activities

0.3 g of root or leaf tissue was separately homogenized in 10 mL of 0.05 M phosphate buffer (pH 7.8). The mixture was centrifuged at 4000 rpm for 10 min, and stored at 4 °C for further use. Superoxide dismutase (SOD) activity was determined by the ability to inhibit photochemical reduction of nitrobluetetrazolium (NBT) (Wang et al., 2004). The reaction mixture contained 0.5 mL supernatant, 0.5 mL of 130 mM methionine, 0.5 mL of 750 μ M NBT, 0.5 mL of 100 μ M EDTA-Na, 0.5 mL of 20 μ M lactochrome and 3.5 mL of 0.05 M phosphate buffer (pH 7.8). The thoroughly mixed sample was illuminated for 20 min, while the control sample prepared in distilled water was placed in the dark. The absorbance was measured at 560 nm. Catalase (CAT) activity was determined by adding 0.4 mL of supernatant into 3 mL of solution containing 0.1% H₂O₂ and 100 mM phosphate buffer (pH 7.0). The absorbance was measured at 240 nm every 30 s for 270 s. Peroxidase (POD) activity was estimated according to guaiacol colorimetric method (Zhang et al., 1995). The POD reaction mixture was prepared by adding 28 μ L guaiacol into 50 mL of 100 mM phosphate buffer (pH 7.0). After completely dissolving and cooling to room temperature, 19 μ L of 30% H₂O₂ was introduced. The assay mixture contained 0.1 mL of supernatant, 3 mL of POD reaction mixture and 0.9 mL of 100 mM phosphate buffer (pH 7.0). The absorbance was measured at 470 nm every 30 s for 270 s.

2.7 Measurement of Chlorophyll Content

Chlorophyll content was determined by a modified procedure according to Lichtenthaler (1987). 0.1 g fresh tissue was cut into pieces and added to 15 mL centrifuge tubes amended with 10 mL of 95% ethanol. The tested tubes were kept in the dark for 14 h, and the chlorophyll content was measured at 649 and 665 nm. Chlorophyll a, chlorophyll b, and total chlorophyll were determined by the following equations: $\text{Chla} = 13.95A_{665} - 6.8A_{649}$; $\text{Chlb} = 24.96A_{649} - 7.32A_{665}$; $\text{total chlorophyll} = \text{Chla} + \text{Chlb}$.

2.8 Metal Uptake Analysis

At harvest, root tissues were collected from *C. maxima* plants and cleaned by ultrasonic treatment (Ultrasonic Cleanser KQ2200B, 100W, 40 kHz, Kun Shan Ultrasonic Instruments Co., Ltd, China) in deionized H₂O for 5 s to remove the adsorbed $\gamma\text{-Fe}_2\text{O}_3$ NPs on root surface. Then shoot and root tissues were thoroughly washed with distilled water several times and dried in an oven at 60 °C for 48 h. 100 mg of oven-dried shoot and root tissues were separately digested in 3 mL of concentrated HNO₃ at 115 °C on a hot block for 1 h. After cooling to room temperature, 0.5 mL of 30% H₂O₂ was added to the digestions at 100 °C for 0.5 h. The iron content was analyzed by Avanta M atomic absorption spectrophotometer (GBC, Australia).

2.9 Measurement of Root Activity

Measurement of root activity was performed according to the triphenyltetrazolium chloride (TTC) method by following a modified procedure (Li, 2000). 0.3 g fresh root was mixed with 3 ml of 4% TTC and 3 ml of 1/15 M phosphate buffer (pH 6.89). The tubes were incubated at 37 °C for up to 2 h. The chemical reaction was stopped by adding 2 ml of 1 M sulfuric acid into the tubes. This step was followed by extraction of triphenylformazan (TPF) by replacing the original liquid with 6 mL of methyl alcohol and incubation at room

temperature for 24 h. The optical density (OD) values of TPF were recorded at 485 nm used to calculate the equivalent TPF concentrations, with which the root activity for each fresh root weight was calculated as follows:

$$\text{Root activity } (\mu\text{g TPF} \cdot \text{g}^{-1} \text{FW} \cdot \text{h}^{-1}) = \text{TPF reduction } (\mu\text{g}) / \text{fresh weight (g)} / \text{time (h)}.$$

2.10 Measurement of Ferric Reductase Activity

The activity of ferric reductase was determined by a 2,2-bipyridine colorimetric method (Li et al., 2013). 0.1 g of the excised roots was placed in an Erlenmeyer flask filled with 10 ml of an assay solution consisting of 0.1 mM Fe(III)-EDTA, 0.4 mM 2,2'-bipyridine. The flasks were placed in a dark room at 25 °C for 2 h, with periodic hand swirling at 15 min intervals. The absorbance of the assay solutions was recorded by a spectrophotometer at 520 nm.

2.11 Regulation of Gene Expression by RT-PCR

Total RNA was isolated from *C. maxima* roots using Trizol (Invitrogen, USA) according to the manufacturer's instructions. The quality and quantity of RNA preparation was verified by agarose gel electrophoresis and absorbance spectrophotometry ($A_{260}/A_{280} > 1.8$) (MAPADA, UV-1800 spectrophotometer). Primers for AHA (Forward-5'-TGACAAGTTCTGCCCTTTCTAC-3'; Reverse-5'-CATCCATACCAGCTCCTTTCTC-3'), FRO2 (Forward-5'-GTGTCTGTTGAAGGACCCTATG-3'; Reverse-5'-GCTCGCGGACTATGGAAATAA-3'), Nramp3 (Forward-5'-GCGTGTTGATTGCTACTGTTATT-3'; Reverse-5'-GATGAGCACGCCAACTAGAA-3') genes were designed based on the sequences available in NCBI genbank using the PrimerQuest (Integrated DNA Technologies,

Coralville, IA). The *C. maxima* Actin gene (Forward-5'-CAGCTGTGGAGAAGAGCTATG-3'; Reverse-5'-CGATCATGGATGGTTGGAAGA-3') was used as the internal control to normalize the gene expression levels. 1 µg of total RNA was used to synthesize cDNA using PrimeScript™ RT Master Mix (Perfect Real Time) (Takara, China) integrated with genomic DNA contamination removal step as per the manufacturer's instructions. The substrate specificity of the primers was verified by GeneAmp PCR System 9700 (ABI, USA) using a representative cDNA preparation and by visualizing on a 1.5 % agarose gel stained with ethidium bromide. RT-PCR analysis was carried out using the ABI 7500 PCR (ABI, USA) and accompanying software according to the manufacturer's instructions. The reaction mixture included 2 µL of cDNA, 1 µL of 0.2 µM corresponding forward and reverse primers, 25 µL of 2×UltraSYBR Mixture(with ROX) (CWBIO, China) in a final reaction volume of 50 µL. The reactions were run with an initial denaturing step for 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min and 72 °C for 45 s and a melting curve analysis was done. The gene expression analysis was done using plants collected from three biological replicates for each gene within each treatment and control. Relative quantity ($2^{-\Delta\Delta C_t}$ method) was then used to calculate relative gene expression level under different exposure conditions (Livak and Schmittgen, 2001).

2.12 Statistical Analysis

Each treatment was conducted with three replicates, and the results were presented as mean ± standard deviation (SD). The statistical analysis of experimental data was performed with the one-way ANOVA followed by Duncan's multiple comparison ($p < 0.05$) in the

statistical package IBM SPSS Version 22.

3. Results and Discussion

3.1 Dynamic dissolution of γ -Fe₂O₃ NPs

The dissolution curves of released Fe³⁺ from 100 mg/L γ -Fe₂O₃ NPs was showed in Fig. S3 in supplementary materials. The release of Fe³⁺ increased from 0-2 d and then reached equilibrium over a 5 d observation period. The final amount of Fe³⁺ release was approximately 14.2 ± 0.9 μ g/L. In this study, the magnitude of iron released was 10^{-6} of the total amount of Fe present in the γ -Fe₂O₃ NPs suspension, suggesting that negligible Fe³⁺ was dissolved from γ -Fe₂O₃ NPs. The higher ionic strength of 1/2 Hoagland's nutrient solution without iron may result in the aggregation of NPs and subsequently reduce the effective surface area, and finally limited the release of soluble Fe (He et al., 2013). It is noteworthy that this experiment was performed in γ -Fe₂O₃ NPs suspension without the growth of plant roots. In real scenario, the roots can affect the dissolution of Fe³⁺, likely through the enhanced expression of AHA gene.

3.2 Growth Analysis of Fe-Exposed *C. maxima* Plants

The impacts of γ -Fe₂O₃ NPs and their counterpart Fe³⁺ solutions at concentrations ranging from 20 to 100 mg/L on the growth of *Citrus maxima* are shown in Fig. 1. Visible signs of phytotoxicity are evident in *C. maxima* roots exposed to 100 mg/L Fe³⁺ (Fig. 1A). In addition, it is clear that many γ -Fe₂O₃ NPs aggregated on the root surface and the accumulation increased with increasing concentrations of NPs. In Fig. 1B, fresh biomass was not significantly altered except 100 mg/L Fe³⁺ treatment. Even though 100 mg/L γ -Fe₂O₃ NPs showed no significant difference from the control, the fresh biomass was 15.4% lower than

Fe(II)-EDTA treatment. As for root length, no apparent difference was observed at all exposures, except that of 100 mg/L Fe^{3+} treatment was reduced by 8.9% compared with control (Fig. 1C). Generally, the phytotoxicity of NPs was reported to increase with increasing suspension concentration (Alidoust and Isoda, 2013). However, in the current study, there was no obvious sign of *C. maxima* growth inhibition under $\gamma\text{-Fe}_2\text{O}_3$ NPs exposure at experimental concentrations at the seedling stages. The possible reason for the reduction of plant growth under 100 mg/L Fe^{3+} treatment might be due to the inappropriately high levels of free iron and the generation of cytotoxic hydroxyl radicals as a result of Fenton reaction (Halliwell and Gutteridge, 1992; Jeong and Connolly, 2009).

3.3 Lipid Peroxidation and Soluble Protein Content of *C. maxima* Plants

Lipid peroxidation, as an indicator of abiotic stress, can be indirectly measured by MDA formation (Ma et al., 2013). The formation of MDA in *C. maxima* as a function of $\gamma\text{-Fe}_2\text{O}_3$ NPs and Fe^{3+} exposure is shown in Fig. 2A. For *C. maxima* roots, $\gamma\text{-Fe}_2\text{O}_3$ NPs at 20 and 50 mg/L had no impact on MDA production, but at an exposure of 100 mg/L, the MDA levels were increased by 44.7% compared with the control plants. All Fe^{3+} treatments enhanced MDA formation. However, in contrast to Fe(II)-EDTA, no elevation of MDA production was observed under other treatments. As one of the most widely supplements for improving Fe availability to plants, Fe(II)-EDTA exhibited toxic effects to *C. maxima* roots for the evidently high level of MDA. It is reported that iron and ligand are absorbed separately and often in unequal proportions, and then iron is released from the ligand somewhere in or upon the root surface (Van Driel, 1964). Burström and Tullin (1957) confirmed chelate effects inside root cells. The high content of MDA induced by Fe-EDTA treatment might be attributed to the

toxic effects of free chelating agent. For *C. maxima* shoots, MDA content of 20 mg/L $\gamma\text{-Fe}_2\text{O}_3$ NPs had no significant difference from that of control or Fe(II)-EDTA. However, $\gamma\text{-Fe}_2\text{O}_3$ NPs at 50 and 100 mg/L led to an increase of MDA content by 38.1% and 43.1%, respectively, relative to the control plants. Moreover, the lipid peroxidation level was also significantly increased by 20-100 mg/L Fe^{3+} treatment. The elevation of MDA levels indicated that oxidative damage may occur in plants. Lipid peroxidation is a representative parameter of cell membrane integrity, and reaction oxygen species (ROS) generation is known to be a primary factor resulting in cell membrane damage through lipid peroxidation (Ma et al., 2015a). Excess ROS production was correlated to the significant increase in lipid peroxidation. Results revealed that $\gamma\text{-Fe}_2\text{O}_3$ NPs and Fe^{3+} treatment caused oxidative damage to either roots or shoots except 20 mg/L $\gamma\text{-Fe}_2\text{O}_3$ NPs. The lack of membrane damage upon 20 mg/L $\gamma\text{-Fe}_2\text{O}_3$ NPs exposure suggested that NPs at this concentration did not induce ROS formation or the plant's detoxification pathways were sufficient to remedy the induced stress (Ma et al., 2013).

Soluble proteins are an important part of plant active substances, and the accumulation of soluble proteins in plants favors the induction of antioxidant enzymes, detoxification enzymes, metabolic enzymes and some function factors for eliminating free radicals in plants under various stresses (Shang et al., 2013). For instance, Ge and Zhang (2015) noticed an increase of soluble protein content with the activation of SOD, CAT and POD under tetrabromobisphenol A stress. As depicted in Fig. 2B, 20-100 mg/L $\gamma\text{-Fe}_2\text{O}_3$ NPs and Fe^{3+} treatment had a notably higher soluble protein content by 36%-90% than control, while no significant difference was observed compared to Fe(II)-EDTA. The accumulation of soluble proteins can be considered as a self-protective response against oxidative stress. The increased

soluble protein content contributed to the production of antioxidant enzymes, which can remedy the induced stress.

3.4 Antioxidant Enzyme Activities of *C. maxima* Plants

SOD, CAT and POD are key enzymes of the antioxidant defense system in plants. Metalloenzyme SOD can accelerate the conversion of $O_2^{\bullet-}$ to H_2O_2 , while CAT and POD can catalyse H_2O_2 breakdown (Bowler et al., 1992). NPs can cause oxidative stress through ROS production, and thus induce an antioxidant defense in plants (Fig. 3A). For *C. maxima* roots, $\gamma\text{-Fe}_2\text{O}_3$ NPs had no significant impact on SOD activity compared to control plants (Fig. 3B). Similarly, no difference of SOD activity exposed to 20-100 mg/L $\gamma\text{-Fe}_2\text{O}_3$ NPs was statistically observed in corn roots (Li et al., 2016). Meantime, SOD activity of Fe^{3+} treatment was remarkably lower than that of control. The decreased activity of SOD may be explained by the fact that *C. maxima* plants lost the capability to regulate SOD activity when the ROS burst (Wang et al., 2011) and exhibited clear oxidative stress. On the other hand, for *C. maxima* shoots, 20 mg/L $\gamma\text{-Fe}_2\text{O}_3$ NPs had no impact on SOD activity, while SOD activities of other treatments were much lower relative to control, denoting that higher oxidative stress disrupted the regulation of SOD activity. Both SOD activity of Fe-EDTA treatment in *C. maxima* roots and shoots were at a low level. In Fig. 3C, no significant change in CAT activity of both *C. maxima* roots and shoots exposed to $\gamma\text{-Fe}_2\text{O}_3$ NPs was observed compared to control. CAT activity in *C. maxima* roots of 20 mg/L Fe^{3+} treatment was higher than control but no difference was found in that of 50 and 100 mg/L Fe^{3+} . Moreover, CAT activity of all treatments in *C. maxima* roots was lower than that of Fe(II)-EDTA. For *C. maxima* shoots, CAT activities of 20-100 mg/L Fe^{3+} treatment were increased by 1.6-2.9 times than

control. The increase of CAT activity contributed to the scavenging of H_2O_2 . In Fig. 3D, POD activity of 20 mg/L $\gamma\text{-Fe}_2\text{O}_3$ NPs remained unchanged in *C. maxima* roots, while that of 50 and 100 mg/L $\gamma\text{-Fe}_2\text{O}_3$ NPs exhibited significantly higher content than control. The elevated POD activity indicated that *C. maxima* plants strengthened their antioxidant system to deal with the ROS. For Fe^{3+} treatments, compared to the control, POD activity in *C. maxima* roots increased with the treatment of 20 and 50 mg/L Fe^{3+} , but decreased with the treatment of 100 mg/L Fe^{3+} . This reversal was due to the fact that *C. maxima* plants initially strengthened enzyme activities to remove excess ROS, but lost the ability to regulate POD activity when the ROS burst at high concentration of Fe^{3+} exposure. For *C. maxima* shoots, POD activities by 20-100 mg/L $\gamma\text{-Fe}_2\text{O}_3$ NPs and Fe^{3+} treatments were significantly higher than those of control plants. The enhanced POD activities lead to the elimination of the excess ROS. Furthermore, both CAT and POD activities of Fe(II)-EDTA in *C. maxima* roots and shoots were at high levels. The studies on SOD, CAT and POD activities suggested that both the $\gamma\text{-Fe}_2\text{O}_3$ NPs and Fe^{3+} treatments induced an antioxidant defense in *C. maxima* plants. Meanwhile, we observed that Fe(II)-EDTA can supply iron to plants and enhance chlorophyll content (Fig. 4B) but simultaneously induce oxidative stress on plants. It is known that Fe-chelate fertilizers such as Fe-EDTA have been recognized as the most widely supplements for improving Fe availability to plants (Abadía et al., 2011). However, synthetic Fe-chelate fertilizers have direct or indirect effects on the environment (Nowack, 2002) and may enhance mobilization of heavy and radioactive metals (Friedly et al., 2002; Means et al., 1978; Ylivainio, 2010). Therefore, new approaches should be developed to alleviate Fe deficiency and increase Fe fertilization efficacy in agroecosystems, such as developing nano Fe fertilizer

(Liu et al., 2016).

3.5 Chlorophyll Content and Iron Distribution in *C. maxima* Plants

The effects of γ -Fe₂O₃ NPs and Fe³⁺ exposure on *C. maxima* leaves are shown in Fig. 4A. 50 mg/L γ -Fe₂O₃ NPs could improve leaf chlorosis to some extent. While the color of *C. maxima* leaves exposed to 20-100 mg/L Fe³⁺ treatment was obviously greener than control, but visible signs of phytotoxicity, such as leaf wilting and malformation, were apparent at an exposure concentration of 100 mg/L. As expected, *C. maxima* leaves treated by Fe(II)-EDTA were healthier than other treatments. Chlorophyll content of *C. maxima* leaves under various treatments was shown in Fig. 4B. As the critical photosynthetic pigment, chlorophyll levels can be a significant indicator of toxicity to plants (Ma et al., 2015b). Basically, the results were in accordance with the images of *C. maxima* leaves. Compared with the control, 20 mg/L γ -Fe₂O₃ NPs had no obviously different chlorophyll content, while chlorophyll content of 50 mg/L γ -Fe₂O₃ NPs was significantly higher by 23.2%, and that of 100 mg/L γ -Fe₂O₃ NPs was decreased by 19.7%. It is known that the γ -Fe₂O₃ NPs have a high rate of agglomeration and low solubility, leading to precipitation due to gravitational forces (Alidoust and Isoda, 2013). The decrease of chlorophyll content upon 100 mg/L γ -Fe₂O₃ NPs exposure might be due to the aggregation and accumulation of γ -Fe₂O₃ NPs on the root surface. There is evidence that attached NPs on root surfaces hindered the water transpiration to the leaves (Asli and Neumann, 2009). On the other hand, oxidative damage could happen in chloroplast through interactions with metal-based NPs, ultimately disrupt the synthesis of chlorophyll or cause the degradation of chlorophyll in leaves (Ma et al., 2015a). However, 50 mg/L γ -Fe₂O₃ NPs induced stress but enhanced chlorophyll content as well, and Fe content in shoots of this

treatment was increased but not statistically significant (Fig. 4C), suggesting that the increase of chlorophyll content could be an alternative mechanism. Further experiment needs to be conducted at cell levels to figure out the correlative mechanism. On the other hand, it was observed that exposure to 20, 50, and 100 mg/L of Fe^{3+} treatments significantly enhanced the total chlorophyll content by 42.1%, 125.9% and 23.3%, respectively. The increased chlorophyll content of Fe^{3+} treatments indicated that despite toxic effects of Fe^{3+} , they can supply iron and contribute to the synthesis of chlorophyll. As expected, chlorophyll content of Fe(II)-EDTA was at a high level although lower than that of 50 mg/L Fe^{3+} treatment.

Iron distribution of *C. maxima* exposed to $\gamma\text{-Fe}_2\text{O}_3$ NPs and Fe^{3+} treatments was shown in Fig. 4C. Before oven-drying, the ultrasonic treatment of *C. maxima* roots could remove the adhered $\gamma\text{-Fe}_2\text{O}_3$ NPs on root surface. No significant difference was observed in Fe content of *C. maxima* roots between 20 mg/L $\gamma\text{-Fe}_2\text{O}_3$ NPs and control, whereas Fe levels of 50 and 100 mg/L $\gamma\text{-Fe}_2\text{O}_3$ NPs were significantly increased by more than 15-fold. Gui et al. (2015) reported that $\gamma\text{-Fe}_2\text{O}_3$ NPs, with particle sizes of less than 50 nm, penetrated the cell membrane and accumulated in the epidermis of the rice (*Oryza sativa* L. ssp. indica) root. The accumulation of $\gamma\text{-Fe}_2\text{O}_3$ NPs in *C. maxima* roots suggested that NPs could enter root cells of *C. maxima*. Interestingly, Fe level of 100 mg/L $\gamma\text{-Fe}_2\text{O}_3$ NPs was less than that of 50 mg/L $\gamma\text{-Fe}_2\text{O}_3$ NPs, which might be attributed to that $\gamma\text{-Fe}_2\text{O}_3$ NPs at high concentrations can easily form clusters and tends to block the porous plant cell wall (Ren et al., 2011), decreasing the uptake amount of NPs. For *C. maxima* shoots, no significant difference of Fe levels upon NPs exposure was observed, indicating that no measured translocation occurred in *C. maxima* plants. Several studies observed the uptake but no translocation of NPs by plants (Birbaum et

al., 2010; Lin and Xin, 2008; Wang et al., 2011). It is reported that NPs may share plant vascular system with water and nutrients and be transported via evapotranspiration after uptake into roots, but the NPs may also aggregate or even clog the vascular system, or leak into nearby tissues and cells, resulting in little or no translocation (Van Bavel et al., 1965; Wang et al., 2011). On the other hand, Fe concentrations were at high levels in *C. maxima* roots after Fe³⁺ treatments, especially the 100 mg/L Fe³⁺ treatment. As high levels of iron can be toxic due to the participation in the Fenton reaction, 100 mg/L Fe³⁺ treatment caused high phytotoxicity and inhibited the growth of *C. maxima* plants. In addition, Fe contents of three Fe³⁺ treatments in *C. maxima* shoots were higher than control by 1.64, 7.85 and 2.20 times, respectively. The extremely high Fe level of 50 mg/L Fe³⁺ treatment in *C. maxima* shoots promoted the synthesis of chlorophyll to a large extent. For Fe(II)-EDTA treatment, Fe level of *C. maxima* roots had no difference with control, but that of shoots was significantly increased by 62.2%. Undoubtedly, Fe(II)-EDTA could effectively provide iron nutrient for plants.

3.6 Root Activity and Ferric Reductase Activity of *C. maxima* Roots

Dehydrogenase activity, which is a comprehensive assessment index of root activity, reflects the metabolic activity level and the ability of roots to absorb nutrients and water (Taniguchi et al., 2008). Root activity of 50 mg/L γ -Fe₂O₃ NPs was 23.8% higher than control but similar to that with Fe(II)-EDTA (Fig. 5A). 20 mg/L γ -Fe₂O₃ NPs had no significant effect while 100 mg/L γ -Fe₂O₃ NPs decreased root activity of *C. maxima* plants as compared to control. Wang et al. (2012) reported that oxidized multiwalled carbon nanotubes (o-MWCNTs) can significantly enhance root dehydrogenase activity of wheat seedlings, which in turn

enhances the water uptake ability of the seedlings. In the present study, the increased root activity indicated that $\gamma\text{-Fe}_2\text{O}_3$ NPs at 50 mg/L could effectively enhance water uptake of plants and thus promote the metabolic activity levels of roots. However, the mechanism of the increased root activity by $\gamma\text{-Fe}_2\text{O}_3$ NPs is not clear yet, which could be a nano-specific behavior. The lower root activity might be due to the oxidative damage caused by 100 mg/L $\gamma\text{-Fe}_2\text{O}_3$ NPs, which could be supported by the much higher MDA content of *C. maxima* roots exposed to 100 mg/L $\gamma\text{-Fe}_2\text{O}_3$ NPs. Meanwhile, direct interactions between NPs and roots could be another reason. As mentioned before, NPs at high concentrations are easy to aggregate and form clusters. Particularly, magnetic interactions can enhance the aggregation of magnetic nanoparticles ($\gamma\text{-Fe}_2\text{O}_3$ NPs) (Hong et al., 2009). Martínez-Fernández et al. (2016) studied the correlation between the plant water relations and the adsorption of nanoparticles on the root surface by short-term exposure of *Helianthus annuus* roots to $\gamma\text{-Fe}_2\text{O}_3$ NPs in the hydroponic solution. They observed that NPs reduced root hydraulic conductivity, demonstrating that the root functionality were damaged. Results of Fe content showed that most of $\gamma\text{-Fe}_2\text{O}_3$ NPs at 100 mg/L were adsorbed on root surface (Fig. 4C). Based on this, it could be safe to draw a conclusion that 100 mg/L $\gamma\text{-Fe}_2\text{O}_3$ NPs affected root water transport, thus inducing adverse effect on root activity. Due to the toxicity caused by high content of Fe in roots, Fe^{3+} treatments had no positive impact on root activity. Since higher portion of iron translocated from root to shoots under 50 mg/L Fe^{3+} treatment, root activity of 50 mg/L Fe^{3+} treatment was higher than that of 20 and 100 mg/L Fe^{3+} treatments.

In *C. maxima* plants, Fe(III) is reduced by the ferric reductase, the resulting Fe(II) is transported across the plasma membrane and is available for plants (Schmidt, 2003).

Generally, if a plant suffers from iron shortage, the reductive system should be strongly activated (Bienfait et al., 1985). Ferric reductase activity of Fe(II)-EDTA was at a low level that was less than control (Fig. 5B), while ferric reductase activity of 50 mg/L γ -Fe₂O₃ NPs was 63.3% higher than control. The possible explanation is that the increased activity of ferric reductase could promote the reduction and utilization of iron. However, 100 mg/L γ -Fe₂O₃ NPs had a 59.2% lower ferric reductase activity. The lower activity could be due to the oxidative damage induced by 100 mg/L γ -Fe₂O₃ NPs, which disrupts the functions of *C. maxima* roots. Moreover, ferric reductase activities of 20 and 50 mg/L Fe³⁺ treatment were higher than control by 71.4% and 308.2%, respectively. The much higher ferric reductase activity indicated that plants can reduce and utilize Fe³⁺. However, ferric reductase activity of 100 mg/L Fe³⁺ treatment decreased by 72.4%. The toxicity of high level of iron injured plant roots and interfered with physiological functions of roots, leading to a lower ferric reductase activity.

3.7 Gene Expression Analysis of *C. maxima* Roots

Physiological responses of plant absorbing iron from Fe(III) oxides include increased acidification of the rhizosphere by H⁺-ATPase, and secretion of phenolics and organic acids to chelate iron, and reduction of Fe(III) to Fe(II) via a inducible, plasmalemma-bound Fe(III) reductase (Fig. 6D) (Guerinot and Yi, 1994). In Fig. 6A, as the relative levels of AHA gene expression upon all Fe-exposed treatments was less than 0.5 fold of the control, the presence of NPs had no impact on AHA expression. It is known that AHA pumps protons across the plasma membrane in response to iron deficiency, thus making the rhizosphere drive more iron into solution (Jeong and Connolly, 2009). However, in this study, no dramatic changes in AHA gene expression were observed under the exposure of γ -Fe₂O₃ NPs, indicating that plants treated with NPs were in iron-replete conditions. In Fig. 6B, 50 mg/L γ -Fe₂O₃ NPs and Fe³⁺ treatments significantly increased the relative levels of FRO2 gene expression by 66.3%

and 40.8%, respectively. The FRO2 gene encodes a ferric chelate reductase. Overexpression of FRO2 could result in higher root ferric reductase activity, which could further promote the iron utilization and enhance the tolerance of *C. maxima* seedlings to iron deficiency. Correspondingly, 50 mg/L γ -Fe₂O₃ NPs and Fe³⁺ treatments had a higher ferric reductase activity compared to both control or Fe(II)-EDTA. It is noteworthy that the FRO2 expression in 100 mg/L Fe³⁺ treated samples was suppressed, which was another proof of toxic effect of 100 mg/L Fe³⁺. Nramp is a member of iron-regulated metal transporters, which functions in diverse organisms from bacteria to human and plays a major role in metal ion homeostasis (Nevo and Nelson, 2006). The relative levels of Nramp3 gene expression under γ -Fe₂O₃ NPs and Fe³⁺ treatments were significantly lower than control (Fig. 6C). Nramp iron transporter system can allow the plant to rapidly respond to even small changes in iron requirements, e.g., Nramp transcription is up-regulated upon iron deficiency and down-regulated to a low level upon sufficient iron supply (Bereczky et al., 2003). The down regulation of Nramp3 gene expression relative to control implied that all NPs and Fe³⁺ treatments could supply iron to *C. maxima* seedlings. Besides, the relative levels of Nramp3 gene expression upon 100 mg/L of γ -Fe₂O₃ NPs and Fe³⁺ exposure were higher than Fe(II)-EDTA by 2.8-5.1 folds, which might be attributed to the aggregation of γ -Fe₂O₃ NPs and toxicity of Fe³⁺. It's reported that the overexpression of Nramp3 may lead to enhanced cellular Fe availability via vacuolar Fe release under Fe starvation (Thomine et al., 2003). The Fe starvation under 100 mg/L γ -Fe₂O₃ NPs or Fe³⁺ treatment may be caused by the aggregation of NPs on root surface (Ren et al., 2011), adverse effect on root water transport at higher concentrations (Martínez-Fernández et al., 2016), or damaged root function and activity as indicated in Fig. 5A. Therefore, the enhanced expression of Nramp3 gene exposed to 100 mg/L γ -Fe₂O₃ NPs and Fe³⁺ was a result of plant self-regulation. Overall, gene expression analysis manifested that both γ -Fe₂O₃ NPs and Fe³⁺ treatments could activate molecular responses in plant roots to transform and absorb external Fe. Correspondingly, the results of Fe content showed that Fe-exposed treatments led to significantly increased Fe content in roots, except 20 mg/L γ -Fe₂O₃ NPs and Fe(II)-EDTA treatments, which had higher Fe contents than control numerically but not statistically (Fig. 4C). The responses of *C. maxima* to γ -Fe₂O₃ NPs or Fe³⁺ at molecular levels could help us better understand the interactions between γ -Fe₂O₃ NPs or Fe³⁺ and plants. To our knowledge,

this is the first report evaluating the expressions of iron uptake and transport related genes under the exposure of $\gamma\text{-Fe}_2\text{O}_3$ NPs or Fe^{3+} .

In this manuscript, we showed different impacts of $\gamma\text{-Fe}_2\text{O}_3$ NPs and Fe^{3+} on the growth of *C. maxima* at physiological and molecular levels. It's clear that plants can utilize both forms of iron according to the results of physiological measurements and gene expressions. Uptake but no translocation of $\gamma\text{-Fe}_2\text{O}_3$ NPs occurred in *C. maxima* plants. Given the Fe^{3+} released from $\gamma\text{-Fe}_2\text{O}_3$ NPs and iron distribution in plants, both $\gamma\text{-Fe}_2\text{O}_3$ NPs and released Fe^{3+} could be responsible for the physiological and molecular responses of plants treated with $\gamma\text{-Fe}_2\text{O}_3$ NPs. Compared to control, 20 mg/L $\gamma\text{-Fe}_2\text{O}_3$ NPs had no significant impact on plant growth, while 50 mg/L $\gamma\text{-Fe}_2\text{O}_3$ NPs could significantly enhance chlorophyll content and root activity. Due to the higher aggregation and accumulation of $\gamma\text{-Fe}_2\text{O}_3$ NPs on the root surface, 100 mg/L $\gamma\text{-Fe}_2\text{O}_3$ NPs may inhibit the transmission of water and other nutritional components, causing a high level of lipid peroxidation, decreased chlorophyll content and root activity. It could be concluded that the impacts of $\gamma\text{-Fe}_2\text{O}_3$ NPs, growth promotion and/or oxidative stress, are dependent on the exposure concentration. On the other hand, all Fe^{3+} treatments induced high levels of MDA and caused damage to plant roots, although chlorophyll contents were increased compared to control due to the utilization of iron. In addition, 100 mg/L Fe^{3+} treatment produced a great toxicity to plants, e.g., reduced fresh biomass and root length. Collectively, $\gamma\text{-Fe}_2\text{O}_3$ NPs exposure resulted in less damage to *C. maxima* plants as compared to Fe^{3+} treatments. Furthermore, $\gamma\text{-Fe}_2\text{O}_3$ NPs at proper concentrations have the potential to be used as an iron nanofertilizer for plant growth. This research yields new insights into the mutual interactions between $\gamma\text{-Fe}_2\text{O}_3$ NPs or potentially released Fe^{3+} and plants, which will advance the agriculturally and environmentally friendly

applications of γ -Fe₂O₃ NPs.

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Figures and Captions

Fig. 1

Figure 1. *Citrus maxima* treated with different concentrations of $\gamma\text{-Fe}_2\text{O}_3$ NPs, Fe^{3+} and control groups. (A) Images of *C. maxima* roots exposed to different nominated concentrations of $\gamma\text{-Fe}_2\text{O}_3$ NPs, Fe^{3+} and control plants. (B) Fresh biomass of *C. maxima* including roots and shoots. (C) Root length of *C. Maxima*. Data are shown as mean $\pm$ SD of three replicates. Values of fresh biomass or roots length followed by different lowercase letters, respectively, are significantly different at $p \leq 0.05$.

Fig. 2

Figure 2. Lipid peroxidation (A) and soluble protein content (B) of *Citrus maxima* plants treated with different concentrations of γ -Fe₂O₃ NPs, Fe³⁺ and control plants. Data are shown as mean $\pm$ SD of three replicates. Values of MDA and soluble protein followed by different lowercase and uppercase letters, respectively, are significantly different at $p \leq 0.05$.

Fig.3

Figure 3. (A) Schematic diagram of the activation of antioxidant enzymes in plants to scavenge excessive ROS production induced by γ -Fe₂O₃ NPs. (B) Activity of superoxide dismutase (SOD), (C) activity of catalase (CAT) and (D) activity of peroxidase (POD) in root and leaf tissues of *Citrus maxima* plants treated with different concentrations of γ -Fe₂O₃ NPs, Fe³⁺ and control plants. Data are shown as mean $\pm$ SD of three replicates. Values of antioxidant enzyme followed by different lowercase and uppercase letters, respectively, are significantly different at $p \leq 0.05$.

Fig. 4

Figure 4. *Citrus maxima* treated with different concentrations of $\gamma\text{-Fe}_2\text{O}_3$ NPs, Fe^{3+} and control groups. (A) Images of *C. maxima* leaves exposed to different nominated concentrations of $\gamma\text{-Fe}_2\text{O}_3$ NPs, Fe^{3+} and control plants. (B) Chlorophyll content of *C. maxima* leaves. (C) Iron content of *C. maxima* including roots and shoots. Data are shown as mean $\pm$ SD of three replicates. Values of chlorophyll or Fe content followed by different lowercase and uppercase letters, respectively, are significantly different at $p \leq 0.05$.

Fig. 5

Figure 5. Root activity (A) and ferric reductase activity (B) of *C. maxima* roots treated with

different concentrations of $\gamma\text{-Fe}_2\text{O}_3$ NPs, Fe^{3+} and control plants. Data are shown as mean $\pm$ SD of three replicates. Values of root activity or ferric reductase activity followed by different lowercase letters, respectively, are significantly different at $p \leq 0.05$.

Fig. 6

Figure 6. Relative expression of iron uptake and transport related genes, including (A) H^+ -ATPase (AHA), (B) ferric-chelate reductase (FRO2), (C) natural resistance-associated macrophage protein (Nramp3) in response to different concentrations of $\gamma\text{-Fe}_2\text{O}_3$ NPs, Fe^{3+} and control plants in *C. maxima* roots. (D) Schematic diagram of the process of the absorption and translocation of iron in plant roots. Data are shown as mean $\pm$ SD of three replicates. Relative expression values of each gene followed by different lowercase letters, respectively, are significantly different at $p \leq 0.05$.

Graphical abstract