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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-Fe_2O_3 NPs) and ferric ions (Fe^{3+}) on plant growth and molecular responses associated with the transformation and transport of Fe^{2+} 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-Fe_2O_3 NPs and Fe^{3+}. We found that γ-Fe_2O_3 NPs could enter plant roots but no translocation from roots to shoots was observed. 20 mg/L γ-Fe_2O_3 NPs had no impact on plant growth. 50 mg/L γ-Fe_2O_3 NPs significantly enhanced chlorophyll content by 23.2% and root activity by 23.8% as compared with control. However, 100 mg/L γ-Fe_2O_3 NPs notably increased MDA formation, decreased chlorophyll content and root activity. Although Fe^{3+} ions could be used by plants and promoted the synthesis of chlorophyll, they appeared to be more toxic than γ-Fe_2O_3 NPs, especially for 100 mg/L Fe^{3+}. The impacts caused by γ-Fe_2O_3 NPs and Fe^{3+} were concentration-dependent. Physiological results showed that γ-Fe_2O_3 NPs at proper concentrations had the potential to be an effective iron nanofertilizer for plant growth. RT-PCR analysis showed that γ-Fe_2O_3 NPs had no impact on AHA gene expression. 50 mg/L γ-Fe_2O_3 NPs and Fe^{3+} 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-Fe_2O_3 NPs and Fe^{3+} were significantly lower than control, indicating that all γ-Fe_2O_3 NPs and Fe^{3+} treatments could supply iron to *C. maxima* seedlings. Overall, plants can modify the speciation and transport of γ-Fe_2O_3 NPs or Fe^{3+} for self-protection and development by activating many physiological and molecular processes.

1. Introduction

Iron oxide nanoparticles (γ-Fe_2O_3 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-Fe_2O_3 NPs on plants. For example, Alidoust and Isoda (2013) showed that γ-Fe_2O_3 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-Fe_2O_3 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-Fe_2O_3 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-Fe_2O_3 NPs interact with plants. We propose that γ-Fe_2O_3 NPs may play three roles in plant growth. The first one is that γ-Fe_2O_3 NPs lead to phytotoxicity through

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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 γ-Fe_2O_3 NPs can be transformed by plants and taken up as iron micronutrient. Which role is dominant may depend on the concentration range of γ-Fe_2O_3 NPs (Liu et al., 2016). As a result, we conducted a series of concentration-dependent assays of γ-Fe_2O_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 γ-Fe_2O_3 NPs.

In addition, we measured the molecular changes of plants as triggered by different concentrations of γ-Fe_2O_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 γ-Fe_2O_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-Fe_2O_3 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 Ag NPs 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-Fe_2O_3 NPs and Fe^{3+} on plant growth. In this study, we investigated the plant responses to γ-Fe_2O_3 NPs or Fe^{3+} 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-Fe_2O_3 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/cm^3. 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-Fe_2O_3 NPs are present in supplementary materials. γ-Fe_2O_3 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-Fe_2O_3 NPs was 164.5 ± 11.3 nm and −11.7 ± 0.1 mV, respectively (Figs. S1B and C). In addition, the average hydrodynamic diameter and the zeta potential of γ-Fe_2O_3 NPs in 1/2 Hoagland's nutrient solution without iron was 267.8 ± 18.7 nm and −10.9 ± 0.2 mV, respectively (Figs. 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-Fe_2O_3 NPs, $FeCl_3$, or 50 μM 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 Fe^{3+} from γ-Fe_2O_3 NPs

In order to investigate the dynamic dissolution of Fe^{3+} from γ-Fe_2O_3 NPs under the same condition as the *Citrus maxima* seedlings exposures, we analyzed the concentration of soluble Fe released from the γ-Fe_2O_3 NPs. 100 mg/L of γ-Fe_2O_3 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-Fe_2O_3 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 dying method with Coomasie Brilliant Fluka G-250 (Wan et al., 2014). Coomassie Brilliant Blue G-250 solution was prepared by weighing 100 mg