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Interaction mechanisms between α-Fe_2O_3, γ-Fe_2O_3 and Fe_3O_4 nanoparticles and *Citrus maxima* seedlings

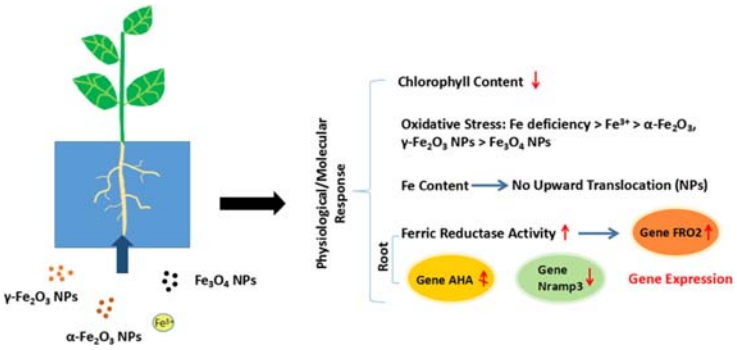
Junli Li [a,*], Jing Hu [a], Lian Xiao [a], Yunqiang Wang [b], Xilong Wang [c,*]

[a] School of Chemistry, Chemical Engineering and Life Sciences, Wuhan University of Technology, Wuhan 430070, China
[b] Institute of Economic Crops, Hubei Academy of Agricultural Science, Wuhan 430064, China
[c] Laboratory for Earth Surface Processes, College of Urban and Environmental Sciences, Peking University, Beijing 100871, China

HIGHLIGHTS

- Chloroplast in plant leaves is very sensitive to iron oxide NPs.
- Iron oxide NPs are not directly bioavailable to *Citrus maxima* seedlings.
- Both α- and γ-Fe_2O_3 NPs can enter plant root cells but through different pathways.
- The α- and γ-Fe_2O_3 NPs taken up by roots are not readily translocated to shoots.
- Plants exposed to 50 mg/L iron oxide NPs or Fe^{3+} are still in iron-deficiency state.

GRAPHICAL ABSTRACT



Citrus maxima seedlings exhibited dissimilar physiological and molecular responses to different types of iron oxide NPs.

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ABSTRACT

The interactions between α-Fe_2O_3, γ-Fe_2O_3, and Fe_3O_4 nanoparticles (NPs) and *Citrus maxima* seedlings were examined so as to better understand possible particle applications as an Fe source for crop plants. NPs toxicity to the exposed plant was investigated as well. The α- and γ-Fe_2O_3 NPs were accumulated by plant root cells through diapirism and endocytosis, respectively, but translocation to the shoots was negligible. Analysis of malondialdehyde (MDA), soluble protein content, and antioxidant enzyme activity revealed that Fe deficiency induced strong oxidative stress in *Citrus maxima* seedlings, which followed an order of Fe deficiency > Fe^{3+} > α-Fe_2O_3, γ-Fe_2O_3 NPs > Fe_3O_4 NPs. However, the chlorophyll leaf content of plants exposed to α-Fe_2O_3, γ-Fe_2O_3, Fe_3O_4 NPs and Fe^{3+} were significantly reduced by 31.1%, 14.8%, 18.8% and 22.0%, respectively, relative to the control. Furthermore, RT-PCR analysis revealed no up-regulation of AHA and Nramp3 genes in *Citrus maxima* roots; however, the relative FRO2 gene expression upon exposure to iron oxide NPs was 1.4–2.8-fold higher than the control. Ferric reductase activity was consistently enhanced upon iron oxide NPs exposure. These findings advance understanding of the interaction mechanisms between metal oxide NPs and plants, and provide important knowledge need for the possible application of these materials in agriculture.

1. Introduction

Iron (Fe) is an essential micronutrient for plants that is involved in respiration, photosynthesis, nitrate synthesis, nitrogen fixation, DNA

* Corresponding authors.
E-mail addresses: lijunli0424@sina.com (J. Li), xilong@pku.edu.cn (X. Wang).

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synthesis and hormone production (Hell and Stephan, 2003). However, Fe can also be toxic when it accumulates to an inappropriately high level; free Fe, including both Fe(II) and Fe(III), can participate in the Fenton reaction and generate cytotoxic hydroxyl radicals (Halliwell and Gutteridge, 1992; Jeong and Connolly, 2009). Although Fe is abundant in the earth's crust, it is present mainly in the insoluble Fe(III) in soils (Briat and Lobreaux, 1997), causing limited bioavailability in many soil types. Iron deficiency is the most widespread micronutrient deficiency to plants, especially for calcareous and alkaline soils, where Fe is nearly insoluble (Klatte et al., 2009). Furthermore, soluble inorganic Fe fertilization provides limited relief due to rapid conversion of Fe to the plant-unavailable Fe(III) form (Rengel et al., 1999). Therefore, new approaches need to be developed to alleviate Fe-deficiency in plants. The application of metal oxide nanoparticles (NPs) in a number of areas has been attracting increasing attention due to the unique physicochemical properties of these materials. As one of the most widely explored and applied nanomaterials, iron oxide NPs such as magnetite (Fe_3O_4), hematite (α-Fe_2O_3), and maghemite (γ-Fe_2O_3) have found many biotechnological applications (Ali et al., 2016; Assa et al., 2016; Espinosa et al., 2016). Importantly, Fe released from iron oxide NPs could be a potential nutrition source for plants. Prior to the possible development of nano Fe fertilizers, the interactions between iron oxide NPs and plants must be understood. Although Fe has ferromagnetic characteristics, different iron oxide forms exhibit dissimilar magnetic properties. Both Fe_3O_4 and γ-Fe_2O_3 possess ferromagnetic behavior; the difference is that Fe_3O_4 has Fe^{2+} and Fe^{3+} cations, whereas γ-Fe_2O_3 has only Fe^{3+} cation and vacancies in their sub-lattices (Belin et al., 2002; Kojima and Hanada, 1980; Morales et al., 1997). α-Fe_2O_3 has canted antiferromagnetism (Randrianantoandro et al., 2001), but does not possess periodic vacancies as compared to γ-Fe_2O_3 (Can et al., 2012). Additionally, the crystalline structures of these iron oxides are different; α-Fe_2O_3 has a rhombohedral crystalline structure whereas Fe_3O_4 and γ-Fe_2O_3 have cubic cells (Randrianantoandro et al., 2001). Recently, a number of investigations have reported the uptake, translocation and physiological effects of iron oxide NPs on plants. For example, Marusenko et al. (2013) evaluated the bioavailability of α-Fe_2O_3 NPs to *Arabidopsis thaliana* and showed that NPs with mean diameter of 40.9 nm (range in 22.3–67.0 nm) could not be taken up by the plant. Rui et al. (2016) observed that γ-Fe_2O_3 NPs increased root length, plant height, biomass, and chlorophyll levels of peanut (*Arachis hypogaea*) plants, suggesting that this material could be used to replace the traditional Fe fertilizers for peanut cultivation. Ghafariyan et al. (2013) observed that Fe_3O_4 NPs entered the roots of soybean (*Glycine max* L. Oxley) and were subsequently translocated to the shoots. NPs uptake and translocation increased chlorophyll levels in soybean leaves, but no overt signs of toxicity were noted under the hydroponic growth conditions.

Citrus maxima was selected as a model plant due to its susceptibility to iron deficiency. Iron oxide NPs previously were not only shown to be available to this species but also to enhance plant growth (Ghafariyan et al., 2013; Rui et al., 2016). Importantly, there is limited knowledge regarding the physiological and molecular response of plants to iron oxide NPs; as such, *Citrus maxima* seedlings were exposed to α-Fe_2O_3, γ-Fe_2O_3, Fe_3O_4 NPs, or Fe^{3+} so as to address this important knowledge gap. Representative physiological parameters such as soluble protein, chlorophyll content, lipid peroxidation, antioxidant enzyme activity, and ferric reductase/Fe content were determined so as to gain understanding of the plant's response to iron oxide NPs. Uptake of iron oxide NPs by plant roots was observed using transmission electron microscopy (TEM). Additionally, the transcriptional modulation of H^+-ATPase (AHA), ferric-chelate reductase (FRO2) and natural resistance-associated macrophage protein (Nramp3) and Fe superoxide dismutase (Fe-SOD) were analyzed at the molecular level by real-time polymerase chain reaction (RT-PCR) technique. A systematic analysis of these physiological and biochemical indicators will substantially advance understanding of the interactions between various iron oxide NPs and important crop species.

2. Materials and methods

2.1. Experimental design

The iron oxide NPs tested include α-Fe_2O_3 (99.5%, 30 nm), γ-Fe_2O_3 (99.5%, 20 nm), and Fe_3O_4 (99.5%, 20 nm) NPs. They were purchased from Macklin Inc. (Shanghai, China). The plant seeds were immersed in distilled water for germination in moist perlite at 28 °C. Since iron release from Fe-containing NPs could be a potential nutrition source for plants, the *Citrus maxima* (*C. maxima*) seedlings were then transferred to the hydroponic system which contained Hoagland's nutrient solution but was free of iron so as to eliminate confounding interactions related to the NPs effects on plant growth. A blank was included in Hoagland's nutrient solution with no Fe; comparison of the actual samples with the blank may help better understand the interactions between the plant and various iron oxide NPs. After acclimation for one week, *C. maxima* seedlings were grown in Hoagland's nutrient solution containing 50 mg/L of α-Fe_2O_3, γ-Fe_2O_3, or Fe_3O_4 NPs. The concentration was selected based on that used in previous studies (Li et al., 2016; Li et al., 2013; Hu et al., 2017). To evaluate role of dissolved Fe in plant growth, an ion equivalent (from $FeCl_3 \cdot 6H_2O$) of 50 mg/L Fe_2O_3 was also tested. The results were then used to compare the toxic effects of iron oxide NPs at different physiological and molecular levels. Others have similarly evaluated soluble metal salts at the same level as compared to metal oxide NPs (Bandyopadhyay et al., 2015; Zhang et al., 2014). In addition, the plant growth condition was identical to that described elsewhere (Li et al., 2016). After exposure for 20 days, physiological parameters including soluble protein, chlorophyll content, lipid peroxidation, antioxidant enzyme activity and ferric reductase levels were measured.

2.2. Plant response and TEM imaging of plant roots

The malondialdehyde (MDA) content was measured using the thiobarbituric acid method according to Heath and Packer (1968). The soluble protein content was determined using a dying method with Coomasie Brilliant Fluka G-250 as described elsewhere (Ren et al., 2011). The activities of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) were measured as described in our recent study (Li et al., 2016). Briefly, crude enzyme was extracted by grinding plant roots (0.3 g) or leaves (0.3 g) in 10 mL of 0.05 M pre-cooling phosphate buffer (pH 7.8). The mixture was then centrifuged at 4000 rpm for 20 min and stored at 4 °C for analysis. SOD activity was measured by the enzyme's ability to inhibit the photochemical reduction of nitrobluetetrazolium (NBT) (Wang et al., 2004); CAT activity was measured by its ability to catalyze the decomposition of H_2O_2; POD activity was estimated following the guaiacol colorimetric method (Zhang et al., 1995). Chlorophyll content was determined with a UV-752 N spectrophotometer (Shanghai Precision Scientific Instrument Co., Ltd., China) using 95% ethanol extracts as described in Lichtenthaler (1987), and the activity of ferric reductase was measured using a 2,2-bipyridine colorimetric method (Li et al., 2013). Roots from plants exposed to 50 mg/L α-Fe_2O_3, γ-Fe_2O_3, or Fe_3O_4 NPs were observed using TEM. Samples for TEM imaging were prepared following a standard procedure described elsewhere (Li et al., 2016).

2.3. Metal uptake

At harvest, shoot and root tissues of *C. maxima* seedlings were collected, and the roots were briefly immersed in 1 mM HNO_3 to remove surface adsorbed γ-Fe_2O_3 NPs (Servin et al., 2013). The roots and shoots were then thoroughly rinsed several times with distilled water and were dried for 48 h at 60 °C in a drying oven. One hundred milligrams of the oven-dried shoot or root tissues were separately digested in 3 mL of concentrated HNO_3 for 1 h at 100 °C on a hot block. After cooling down to ambient temperature, 0.5 mL of 30% H_2O_2 was added to the