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Metabolomics analysis of TiO_2 nanoparticles induced toxicological effects on rice (*Oryza sativa* L.)☆

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The wide occurrence and high environmental concentration of titanium dioxide nanoparticles (nano-TiO_2) have raised concerns about their potential toxic effects on crops. In this study, we employed a GC-MS-based metabolomic approach to investigate the potential toxicity of nano-TiO_2 on hydroponically-cultured rice (*Oryza sativa* L.) after exposed to 0, 100, 250 or 500 mg/L of nano-TiO_2 for fourteen days. Results showed that the biomass of rice was significantly decreased and the antioxidant defense system was significantly disturbed after exposure to nano-TiO_2. One hundred and five identified metabolites showed significant difference compared to the control, among which the concentrations of glucose-6-phosphate, glucose-1-phosphate, succinic and isocitric acid were increased most, while the concentrations of sucrose, isomaltulose, and glyoxylic acid were decreased most. Basic energy-generating ways including tricarboxylic acid cycle and the pentose phosphate pathway, were elevated significantly while the carbohydrate synthesis metabolism including starch and sucrose metabolism, and glyoxylate and dicarboxylate metabolism were inhibited. However, the biosynthetic formation of most of the identified fatty acids, amino acids and secondary metabolites which correlated to crop quality, were increased. The results suggest that the metabolism of rice plants is distinctly disturbed after exposure to nano-TiO_2, and nano-TiO_2 would have a mixed effect on the yield and quality of rice.

1. Introduction

Titanium dioxide nanoparticle (nano-TiO_2) is one of the most abundant engineering nanomaterials produced in the world and is widely used in cosmetics, drugs, dyes, and many other consumer products (Gottschalk et al., 2009; Sun et al., 2014). Annual global production of nano-TiO_2 is more than 10,000 metric tons (U.S. EPA, 2009). Sun et al. (2014) have reported that the annual increase of nano-TiO_2 in the environment is 21 ng/L in surface waters and 89 μg/kg in sludge-treated soil; the annual input concentration in the sediment can reach 1.9 mg/L in the U.S., Europe and Switzerland. Nano-TiO_2 concentration in the environmental media, including surface water, sediment, natural and urban soils, was estimated to be in the range of μg/kg to low mg/kg, which is far higher than that of other nanoparticles (Gottschalk et al., 2009; Sun et al., 2014). The earliest report on the potential toxicity of nano-TiO_2 was in 1985, where tumors were found in the lung of rats after a lifetime exposure to nano-TiO_2 at 250 mg/L (Lee et al., 1985). In recent years, nano-TiO_2 has drawn great public concerns because of its potential toxic effects on organisms and ecosystems (Godwin et al., 2009; Hext et al., 2005; Klaine et al., 2008).

For many metal nanoparticles, the dissolved metal ions (Lin and Xing, 2008; Thuesombat et al., 2014) and oxidative stress induced by reactive oxygen species (ROS) (Burello and Worth, 2011; Xia et al., 2006) are usually considered to be critical factors in causing toxic effects. However, because of the low solubility of nano-TiO_2, the specific photoelectric properties of nano-TiO_2 that induced the production of ROS were seen as a major factor resulting in cell damage (Lee et al., 1985; Shukla et al., 2011; Szymańska et al., 2016; Xia et al., 2015). Li et al. (2012) found that nano-TiO_2 could induce three types of ROS: superoxide radicals ($O_2{\bullet}^-$), hydroxyl radicals (•OH), and singlet oxygen species($^1O_2$) under UV irradiation. It was observed that nano-TiO_2 generated the most •OH and $^1O_2$ among seven experimental nanoparticles including nano-TiO_2, nano-ZnO,

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nano-Al_2O_3, nano-SiO_2, nano-Fe_2O_3, nano-CeO_2, and nano-CuO. Moreover, in all seven nanoparticles, the total concentration of all three types of ROS generated by nano-TiO_2 was the highest.

Studies on the phytotoxicity of nano-TiO_2 found that nano-TiO_2 could be transported and accumulated in plant tissue (Jacob et al., 2013; Servin et al., 2012, 2013), which affected the physiological metabolism in plants (Gardea-Torresdey et al., 2014; Ghosh et al., 2010; Miralles et al., 2012; Song et al., 2013a, 2013b; Xia et al., 2015; Zheng et al., 2007). Servin et al. (2012, 2013) observed that nano-TiO_2 was absorbed and transported from root to leaf trichomes in cucumbers with no biotransformation, indicating that nano-TiO_2 was transportable in plants. Additionally, the ROS induced by nano-TiO_2 can break the cell membrane of plant, decrease the biomass, affect the enzymatic activities of the antioxidant defense system, decrease photosynthesis, and damage DNA (Gardea-Torresdey et al., 2014; Ghosh et al., 2010; Miralles et al., 2012; Song et al., 2013a, 2013b; Xia et al., 2015; Zheng et al., 2007). It was found that nano-TiO_2 induced DNA damage, inhibited the growth and increased lipid peroxidation in *Allium cepa* root at the concentration of 319 mg/L and induced DNA damage in *Nicotiana tabacum* leaf at 157 mg/L (Ghosh et al., 2010). However, studies also explored the advantages or non-toxic of nano-TiO_2 treatment on plants (Song et al., 2013b; Su et al., 2009; Yang et al., 2007; Zheng et al., 2007). Nanoanatase TiO_2 was shown to promote photosynthesis, improve plant growth, promote the vigor of aged seeds, and induce chlorophyll biosynthesis in spinach (Yang et al., 2007). Song et al. found that nano-TiO_2 was not toxic to the three plants species (oilseed rape, lettuce and kidney bean) in their experiment (Song et al., 2013b). Nevertheless, few studies focused on the mechanism of phytotoxicity of nano-TiO_2 at the metabolite level.

Metabolomics provide valuable information regarding the overall changes of small metabolites and biochemical pathways that might be altered in response to toxicants or environmental stressors (Zhang et al., 2013). It has also been shown as an effective tool to help understand the changes in chemical composition of crops with subsequent multivariate analysis (Hirai et al., 2004; Jonsson et al., 2005; Schauer and Fernie, 2006). GC-MS has been a long-standing approach used for metabolite profiling, offering a good balance between sensitivity and reliability to separate and identify the small molecular intracellular metabolites, and their intermediates in plant tissue (Lisec et al., 2006). Recently, metabolic responses to nano-TiO_2 were studied in mouse fibroblast cells (Bo et al., 2014; Liu et al., 2012), HpeG2 cells (Kitchin et al., 2014), human gingivitis cells (Garcia-Contreras et al., 2015), *C.* elegans (Ratnasekhar et al., 2015), and earthworms (Whitfield Åslund et al., 2012). There are researches that used metabolomics to study the phytotoxicity of C_{60}, nano-Cu, nano-$Cu(OH)_2$, and nano-CdO (Du et al., 2016; Kim et al., 2016; Večeřová et al., 2016; Zhao et al., 2016a, 2016b). In these researches, the metabolic changes on the root exudate, roots and leaves were indicated, which revealed important detoxification mechanisms defense the nanoparticles stress. However, there is still scant research that uses metabolomics to estimate the effects of nano-TiO_2 on the crops. Serving as the initial step of the food chain, crops provide a major route of pollutant exposure to higher species, including humans (Gardea-Torresdey et al., 2014; Miralles et al., 2012; Rico et al., 2011). Furthermore, these harmful effects can affect the yield and quality of crops. Previous researches on wheat, tomato and cucumber reported that nano-TiO_2 could be detected in crop tissues and fruits, which reduced the biomass, and modified the nutritive element content and macromolecular composition (Du et al., 2011; Servin et al., 2013; Song et al., 2013a). Kim et al. (2016) reported that nano-TiO_2 showed greater movement in the sediment than in the water in a paddy microcosm. Considering that rice (*Oryza sativa* L.) is a staple food consumed by more than half of the world's population (Rico et al., 2013a), it is essential to understand the interaction of rice plant with nano-TiO_2 to provide information on the metabolic influence of engineered nanoparticles (ENPs) on it.

Therefore, the primary aim of this work is to investigate the metabolic response of rice to nano-TiO_2 exposure. We measured the biomass and activities of the antioxidant defense system of roots and leaves of rice plants. Using GC-MS-based metabolomics, we evaluated the up- or down-regulation of small molecule metabolites and analyzed the connection among the metabolites using principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA). Significantly affected metabolic pathways were also identified. This research contributes to elucidate the potential influence of nanoparticles on the yield and quality of crops.

2. Materials and methods

2.1. Preparation of TiO_2 suspensions

The hydrophilic nanosized TiO_2 material (99.8% metals basis, anatase) used in this study was purchased from the Aladdin® Chemical Reagent Co., Ltd., China. The nano-TiO_2 has a mean transmission electron microscopy (TEM) imaged size of 20 nm and particle size of 293 $\pm$ 17 nm in deionized water. A nano-TiO_2 250 mg/L stock solution was characterized for monomer size and morphology by TEM (HRTEM, JEM, 2010; JEOL Ltd., Japan) and for hydrodynamic diameter by dynamic light scattering (Nano-ZS, Malvern Instruments, U.K.). The TEM images of nano-TiO_2 are showed in Supporting Information (SI) Fig. S1. The surface area of nano-TiO_2 was 79.5 m^2/g, determined by the multipoint Brunauer–Emmett–Teller (BET) method (Yang et al., 2006). Nano-TiO_2 was suspended in Hoagland nutrient solution (Lin and Xing, 2008). The experimental concentrations used in the study are 0, 100, 250, and 500 mg/L, which are higher than those observed in the soil, thus the obtained results should be considered as mechanism research rather than a simulation of processes in natural environment (Song et al., 2013b; Szymańska et al., 2016; Xia et al., 2015). All suspensions were stirred for 5 min, and then sonicated (100 w) for 30 min in a water-bath with continuous stirring.

2.2. Seed germination and plant growth

Seeds of rice (*Oryza sativa* L.) were obtained from Zhejiang Academy of Agricultural Sciences (Hangzhou, China). Rice seeds were sterilized for 12 h in a 3% H_2O_2 solution, then rinsed 3 times with Millipore water, and finally placed on sterilized perlite growth substrate in Millipore water to grow. After 7 days, similar plants were selected and transferred to 2 L glass pots containing 1.8 L nano-TiO_2 suspensions. The seedlings were fixed on the perforated sheet with a sponge, and their roots were submerged into the suspensions. Every pot had four bundles of seedlings, and each bundle contained six seedlings. The seedlings were grown in a greenhouse under natural light and temperature for 14 days. The absorption and transportation of nano-TiO_2 in roots, stems and leaves of the exposed rice plants were observed by TEM.

2.3. Titanium (Ti) analysis

At harvest, the roots and leaves were washed with deionized water and lyophilized. Dried tissues were ground by a high-throughput tissue grinder (TP-48P, ONEBIO, Shanghai, China), and then digested with a mixture of 5 mL of hydrofluoric acid (HF) and 5 mL of HNO_3 (v/v 1:1) using a graphite digestion system (ZEROM, ProD 48, Changsha, China) at 120 °C for 3 h (Schimpf et al., 2002).