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Growing rice (*Oryza sativa*) aerobically reduces phytotoxicity, uptake and transformation of CeO_2 nanoparticles

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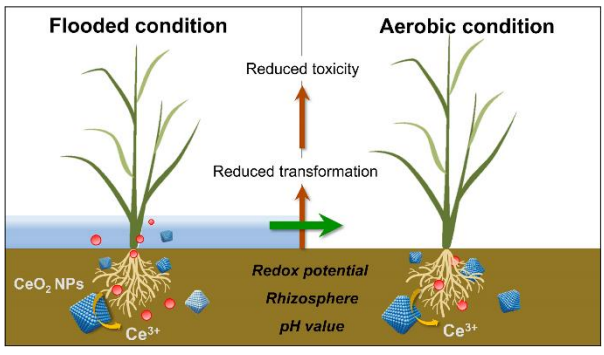
ABSTRACT

This study compared the impact and uptake of root administered CeO_2 NPs in rice growing under flooded and aerobic soil conditions, which are two water regimes commonly used for rice cultivation. CeO_2 NPs at 100 mg/kg improved photosynthesis and plant growth by reducing the oxidative damage and enhancing plant tolerance to stress, while higher concentration (500 mg/kg) of CeO_2 NPs negatively affected plant growth. More significant effects were observed under the flooded condition than the aerobic condition. CeO_2 NPs of 100 and 500 mg/kg resulted in 78% and 70% higher accumulation of Ce in shoots under the flooded condition compared to the aerobic condition. CeO_2 NPs partially transformed to Ce(III) species in soils and plants under both conditions. Higher extent of transformation under the flooded condition, which was partly attributed to the lower soil pH and redox potential under the flooded condition, leads to higher plant uptake of Ce. A higher extent of transformation in rhizosphere soil was observed. Higher plant transpiration rate under flooded conditions resulted in higher accumulation of CeO_2 species in shoots . This study for the first time reported that water regimes influenced the biotransformation of CeO_2 NPs and their uptake and impacts in rice plant.

Keywords: CeO_2 nanoparticles; plant; transformation; uptake; water regime; rhizosphere

Synposis: Growing rice aerobically reduces the transformation of CeO_2 nanoparticles and uptake of Ce species in plants, leading to less phytotoxicity.

Table of content graphic



INTRODUCTION

Terrestrial ecosystems represent a major sink of engineered nanomaterials (ENMs) after their entry into the environment, where plants represent the largest interface in the system. CeO_2 NPs may not only impair plant growth,[1] but also accumulate in plant organs[2] and fruit[3] and transfer to higher trophic levels of living organisms,[4] posing risks to human health and environmental safety[5]. Despite the adverse effects reported, the application of CeO_2 NPs for improving plant growth was also explored recently. For example, CeO_2 NPs showed potential to improve plant growth under stress conditions such as heat[6] and high salinity.[7] A more recent study suggested that CeO_2 NPs can capture reactive oxygen species (ROS) such as $OH^{\bullet -}$ in leaves and activate the K^+ efflux channels in mesophyll cells thereby improving the photosynthesis efficiency and carbon assimilation in *Arabidopsis thaliana*.[8] The beneficial effects of CeO_2 NPs are mainly derived from their well-known redox properties.[9] Cerium has two oxidation states, Ce^{3+} and Ce^{4+}, which alternate during redox reactions. This property enables the reversible stoichiometric storing and oxygen release as cerium changes oxidation states, enabling CeO_2 NPs to become a ROS scavenger. However, the biological effects of CeO_2 NPs on plant can be biphasic and need to be understood further to ensure safety as well as beneficial application.

It has been known that the effects of CeO_2 NPs on plant growth are highly related to the physicochemical properties of the NPs[10, 11] and plant species[12] as well as the growth environment[13] of the plant. Smaller-sized[12] or negatively charged[10] CeO_2 NPs may enter plant more easily thus causing higher toxicity. Different plant species (e.g., monocot and dicot) have different vascular structures and transpiration rates, which affects the uptake and translocation of CeO_2 NPs[14] and subsequent biological effects in plants. Hydroponic, soil or sand cultivated plants may show great variance in their responses to CeO_2 NPs exposure.[1] Studies have shown that biotransformation of

64 CeO_2 NPs is one of the critical mechanisms responsible for the different uptake and effects of CeO_2
65 NPs in plants.[15]

66 ENMs are highly dynamic and tend to transform in soil-plant systems, driven by various
67 physical, chemical or biological factors.[16] Despite being highly stable, CeO_2 NPs can transform after
68 interaction with plants, and the transformation mainly occur at the plant root interface,[17] where
69 rhizosphere composition such as organic acids and reducing substances play vital roles in the
70 transformation process.[15] Small-sized CeO_2 NPs are prone to transform more easily and release
71 more Ce^{3+} than larger ones, which can cause phytotoxicity depending on the sensitivity of the plant
72 species to the NPs and their transformation species.[12] Rod shaped CeO_2 NPs containing a higher
73 amount of reactive facets on the crystal surface showed higher reactivity and thus a higher extent
74 of transformation than octahedral or cubic CeO_2 NPs.[11] Changes in the composition of the culture
75 medium, e.g., presence of phosphate[18] and organic acids,[1] can also affect the transformation
76 process. Previous studies also suggests that plant species can affect the transformation of CeO_2 NPs
77 due to differences in the rhizosphere chemistry.[14] Despite these studies, there is still a lack of
78 systematic understanding of CeO_2 NPs transformation in complex soil environment as well as in
79 different agricultural practice scenarios (e.g., under various water regimes) and the impact of these
80 on the subsequent uptake and effect in plants.

81 Rice is a semi-aquatic plant species. Irrigated rice cultures, i.e. culturing rice under flooded
82 condition, account for about 75% of the global yield of rice grain.[19] Due to the high demand for
83 water resources of irrigated rice, aerobically cultured rice is an increasingly important strategy to
84 reduce water use and enhance water use efficiency.[20] The different water regimes used for the two
85 cultures may result in significantly different behaviours and impacts of contaminants in the rice
86 plants due to the change of the chemical and biological environments around the roots,[21] which
87 have been reported for contaminants such as heavy metals (e.g., Zn,[22] Cd,[23] Hg[24]) but have not yet

been studied for ENMs. The behaviour and impact of CeO_2 NPs in rice under different water regimes is currently unknown.

In this study, we hypothesized that changing water regime may affect the transformation, uptake, translocation and subsequent impacts of CeO_2 NPs in rice plants. Specifically, we investigated the impacts of increasing CeO_2 NPs concentrations on the growth of rice either in flooded or aerobic soils by measuring the phenotypic and physiological parameters including biomass, seedling elongation, photosynthesis, and organic and inorganic nutrients. Stress status and plant tolerance to the stress were evaluated by measuring components of the antioxidant system, DNA damage and proline levels in the plants. Uptake and transformation of CeO_2 NPs in soil and plants were measured and correlated with the observed biological effects.

MATERIALS AND METHODS

Chemicals

CeO_2 NPs (< 25 nm) were purchased from Sigma Aldrich. The primary size of the CeO_2 NPs characterized by transmission electron microscopy (TEM, JEOL, Japan) were 28 ± 13 nm (**Figure S1**). The crystal structure was cubic fluorite as verified by X-ray diffraction (XRD, D8 Advance, Bruker, USA) (**Figure S2**). The CeO_2 NPs have a hydrodynamic size (intensity) of 151 ± 23 nm and zeta potential of 18 ± 6 mV in deionized water, as analysed using a Zetasizer Nano ZS90 (Malvern, UK). The zeta potential was converted from electrophoric mobility using Smoluchowski equation. Other chemicals were purchased from Sigma Aldrich.

Soil preparation, plant cultivation and treatments

Sandy loam soil was collected from a local area in Beijing. The soil was air-dried and sieved through a 5 mm mesh. Soil characteristics were measured and are provided in Table S1. CeO_2 NPs powders

were mixed homogeneously into the soil to achieve final concentrations of 100 and 500 mg/kg CeO_2 NPs which are considered as low and high concentrations in this study. The concentrations were comparable with many existing plant studies in soil.[25, 26] In addition, rare earth elements are not rare, the average abundance of Ce in crust is 60 mg/kg and up to 900 mk/kg in soils nearby the rare earth element industry[27]. Indirect input of Ce into soils due to application of phosphate fertilizers which contain rare earth elements also leads to increasing soil Ce contents. The Ce contents reported in soils ranged from 15-350 mg/kg in United states.[28] A rhizobag pot system was used for plant cultivation following a previously described procedure.[29] In order to study the rhizosphere soil, a rhizobag made of 40 μm nylon mesh with a size of 10 x 10 cm^2 (D X H) was used. 0.3 kg soil amended with CeO_2 NPs was placed in each rhizobag and then transferred to a 2L light proof PVC pot. Another 0.7 kg amended soil was used to fill the gap between the rhizobag and the pot, with the total soil being 1 kg in each pot. Soil without CeO_2 NP amendment was used as control. Three replicates were set up for each treatment. The schematic show of the rhizobag experiment was provided in **Figure S3**.

Rice seeds (*Oryza sativa*) were purchased from the Chinese Academy of Agricultural Science. The seeds were sterilized in 5% H_2O_2 for 20 min and rinsed thoroughly with deionized water. The seeds were germinated in moist sand at 25 ^oC in the dark and grew for 10 days. Uniform seedlings were then selected and carefully transferred into the rhizobags with three seedlings per bag. In the flooded group, deionized water was supplemented to full saturation capacity of the soil and replenished every day to a standing water level of ~ 2 cm. In the aerobic group, deionized water was added to the soil to reach 70% of the water holding capacity (21%) of the soil. Soil Eh was measured at 6 cm below the surface on the day of exposure and 7, 14, 21 and 28 days after the exposure.

Impact of CeO_2 NPs on photosynthetic system and transpiration

The relative chlorophyll contents were measured on days 0, 7, 14, 21 and 28 using a portable SPAS-502 Plus. The first fully expanded leaf of each plant with five points on each leaf was measured. The optimum quantum yield (F_v/Fm) and photochemical efficiency of PSII (ΦPSII) was measured following a previously described method.[30] Briefly, the plants after 28 days of exposure were placed in the dark and the minimum fluorescence (F0) in the plant leaves was measured on a portable photosynthesis measurement system (Li-6400, LI-COR, USA). A pulse of white light was applied and the maximal fluorescence (Fm) was assessed. The plants were then exposed to saturating light (50 µmol m^{-2} s^{-1} for 3 min) to establish the maximal fluorescence (F_m') after light adaption. The minimal fluorescence (F_0') was then measured after turning off the actinic light. The Fv/Fm of PSII was calculated as (F_m-F_0)/F_m and the ΦPSII was calculated as (F_m' − F')/F_m'. The transpiration rate of plants were measured on the portable photosynthesis measurement system (Li-6400, LI-COR, USA).

Protein, sugar and starch contents in plant samples

The plants were harvested after 4 weeks of exposure. Roots and shoots were separated and rinsed in 0.01M HNO_3 and deionized water, and the fresh weight and root/shoot lengths were measured. Soluble protein contents in the samples were measured using a BCA assay kit (Thermo Scientific, USA) following the manufacturer's instructions.

Total soluble sugar content was measured following a previously described method.[1] Briefly, 0.5g samples were homogenized in 80% ethanol and centrifuged at 2000 rpm for 20 min. The supernatant was mixed with 5% phenol and 98% sulphuric acid followed by incubation in a water bath (30 °C) for 20 min. The absorbance of the solution was then measured at 490 nm using a UV-vis spectrometer. Glucose solutions with known concentrations were used as standards to calculate the sugar concentration in the samples.

Reducing sugar content was quantified according to a previous method described by Miller et al.[31] Briefly, 0.5g dry samples were homogenized in 80% ethanol and centrifuged at 2000 rpm for 20 min.

The supernatant was mixed with 3,5-dinitrosalicylic acid and boiled for 5 min. The absorbance was measured at 515 nm using a UV-vis spectrometer (LAMBDA 365, Perkin Elmer, USA). Glucose solutions with known concentrations were used as standards to calculate the sugar concentration in the samples.

The starch content was measured using a method described by McCready et al.[32] The residues after centrifugation for the extraction of total soluble sugar were re-suspended in deionized water. Perchloric acid was added and the mixture was centrifuged at 2000 rpm for 20 min. The supernatant was diluted by 10-fold with deionized water and processed following the same protocol as for total soluble sugar. Starch contents were quantified using glucose as standard with a factor of 0.9 applied for conversion of glucose to starch.

Content of Ce, macro- and micro- nutrient elements in plant samples

To measure the elemental content, fresh roots and shoots were separated and washed with 0.01M HNO_3 and deionized water (three times) to remove contaminants[33]. lyophilized. Dry samples were digested with a mixture of HNO_3/H_2O_2 (v/v ratio, 3:1) on a heating plate (80 ^oC for 1 h, 120 ^oC for 3 h, and 160 ^oC for another 0.5 h). The residues were diluted with deionized water for measurement. Ce contents were measured by inductively coupled plasma mass spectrometry (ICP-MS, Thermo X7, USA). Ce standard solution (0.1~10 mg/L) was used for external calibration. K, Ca, Mg, P, Fe, Cu, Zn and Mn were measured by inductively coupled plasma optical emission spectrometry (ICP-OES, Perkin Elmer, USA). Multi-element standard solutions (0.5~50 mg/L) containing the selected elements were used for external calibration. Blanks were analysed between every six samples. Spiking recovery experiments and analysis of certified reference material (GBW 07602 Bush Branches and Leaves) were performed for analytical method validation. The recoveries and limits of detection for all the elements are reported in Table S2.

Antioxidant enzyme activities, proline content and 8-OHdG content

The activities of the antioxidant enzymes including superoxide dismutase (SOD) and catalase (CAT), and the content of malondialdehyde (MDA) in plant tissues were measured using the relevant assay kits according to the manufacturer's instructions (Nanjing Jiancheng Biotechnology Co., Ltd).

Proline content was measured following the method described by Bates et al.[34] Briefly, fresh roots and leaves were homogenized with cooling by an ice bath and diluted with 3% sulfosalicylic acid to 10 mL followed by centrifugation at 1500 g for 10 min. The supernatant was collected and mixed with acidic ninhydrin and glacial acetic acid (100%) and allowed to react in boiling water for 1 h followed by addition of methylbenzene. The absorbance of the extractives was measured at 520 nm and the concentration was calculated based on calibration curve established with proline standard solutions.

The content of 8-hydroxy-2 deoxyguanosine (8-OHdG) was measured to determine the DNA oxidative damage. Fresh roots and leaves were ground in liquid nitrogen into powders followed by homogenization in a buffer solution containing 2% CTAB, 100 mM Tris-HCl, 2% Polyvinylpyrrolidone (PVP), 1.5 mM NaCl and 0.2 mM EDTA and 1% mercaptoethanol. DNA in the mixtures was then extracted using the DNA kit (Qiagen, France), and the 8-OHdG content was quantified using a DNA Damage ELISA kit (Stress Marq Biosciences, Inc, Thermo, USA).

Transmission electronic microscopy (TEM) observation of root sections

Fresh root apexes were cut and prefixed overnight in 2.5% glutaradehyde solution in phosphate buffered saline (PBS, pH 7.4). The samples were then washed with PBS three times followed by dehydration in gradient acetone and embedding in Spurr's resin. Ultrathin sections (90 nm) were obtained using an UC6i ultramicrotome (Lecia, Austria). To avoid artifacts, the sections were not stained by uranyl acetate. The sections were collected on copper TEM grids and observed on a JEM-1230 (JEOL, Japan) transmission electron microscope.

X-ray absorption spectroscopy (XAS)

Samples in treatments with 500 mg/kg CeO_2 NMs were used for XAS analysis. Rhizobag soils and pot soils were collected followed by lyophillization in a freeze-dryer. Fresh root and shoot samples were washed with deionized water and lyophilized. The samples were motor homogenized and pressed into thin pellets for XAS analysis. The XAS spectra were collected on beamline 1W1B at the Beijing Synchrotron Radiation Facility. The energy of the storage ring during the data collection was 2.5 GeV with current intensity of 50 mA. CeL_{III}-edge spectra of the soil and root samples were collected in transmission mode while the shoot samples were collected in fluorescence mode using a 19-element germanium array solid detector. The XAS spectra of $CePO_4$ and $Ce(CH_3COO)_3$ as well as CeO_2 NPs were collected as standards. ATHENA software was used to perform the data analysis including normalization, energy calibration and linear combination fitting (LCF) analysis.

Data analysis

Data were expressed as mean ± standard deviation (SD) (n=8). Statistical analysis was performed on IBM SPSS 19.0. One-way ANOVA and student t-test were used to evaluate the significance between data. $P < 0.05$ was considered significantly different.

RESULTS AND DISCUSSION

Different impacts of CeO_2 NPs on rice phenotype and photosynthesis under aerobic and flooded conditions

Under flooded conditions, CeO_2 NPs treatment did not significantly affect the fresh weight of rice seedlings (**Figure 1A**) compared to the untreated controls, while the dry weight of the roots increased by 22% at the concentration of 100 mg/kg and reduced the dry weight of roots and shoots at 500 mg/kg by 14% and 34%. CeO_2 NPs at 100 mg/kg enhanced the root and shoot elongation by 35% and 38%, respectively. While under aerobic conditions, CeO_2 NPs showed no significant impacts

on the fresh and dry biomass and the seedling length, with the exception of shoot length which was increased by 29% following 100 mg/kg CeO_2 NPs treatment.

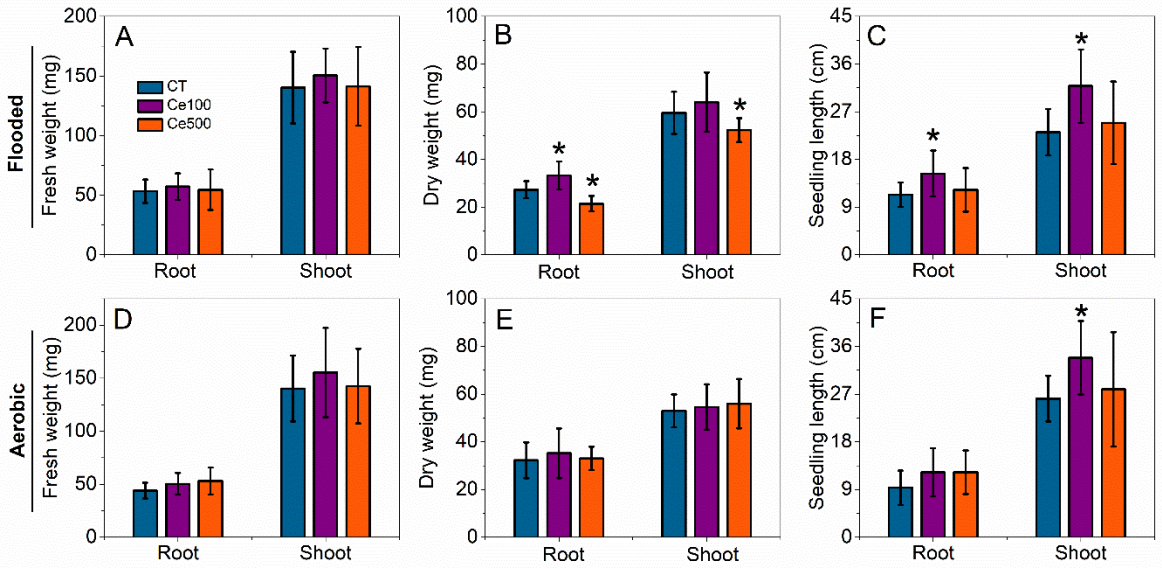


Figure 1. Fresh weight (A and D), dry weight (B and E) and seedling length (C and F) of rice under flooded (A-C) and aerobic (D-F) conditions. Asterisk (*) indicates significant difference compared to untreated control at $P < 0.05$.

The impacts of CeO_2 NPs on the photosynthesis of the rice plants were evaluated by measuring the relative chlorophyll content, PSII and ΦPSII. The effects of CeO_2 NPs on the relative chlorophyll content in leaves were different under the two conditions (**Figure 2A**). The relative chlorophyll content was increased by 40% following 100 mg/kg CeO_2 NPs treatment while decreased by 20% at 500 mg/kg under flooded conditions. However, CeO_2 NPs showed no effects on the relative chlorophyll content under aerobic conditions. The Fv/Fm is a commonly used indicator of stress in leaves since stress leads to damaged PSII with lowing of Fv/Fm.[35] The Fv/Fm was reduced by 17% following treatment with 500 mg/kg CeO_2 NPs **(Figure 2B)**, suggesting that the plant was in stress and the PSII was impaired. Although CeO_2 NPs of 100 mg/kg did not affect the Fv/Fm, they enhanced the ΦPSII by 11% and 19% under flooded and aerobic conditions, respectively (**Figure 2C**). CeO_2 NPs

at 500 mg/kg reduced the ΦPSII by 18% under flooded conditions while had no effects under aerobic conditions. These results suggest that CeO_2 NPs improved plant photosynthesis at 100 mg/kg while impairing the photosynthetic system if the dose was high (500 mg/kg) under flooded conditions. The results correlated with the results of biomass and seedling elongation, suggesting that the difference in the impact of CeO_2 NPs on the photosynthesis contributed to the different effects on plant growth under flooded and aerobic soil conditions.

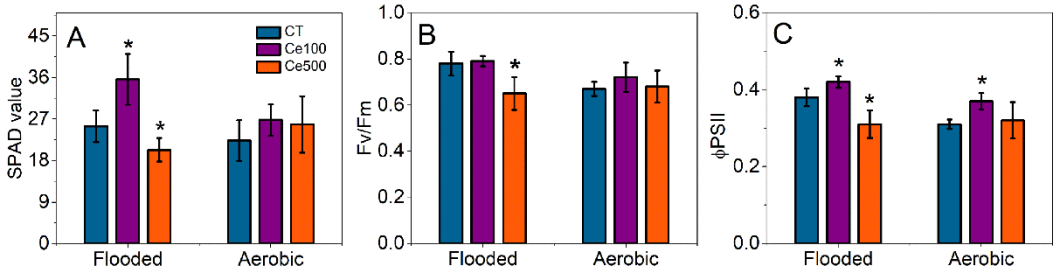


Figure 2. Effects of CeO_2 NPs on the relative chlorophyll content (A), Fv/Fm (B) and ΦPSII (C) in leaves under flooded and aerobic (B) conditions, Asterisk (*) indicates significant difference compared with untreated control at $P < 0.05$.

CeO_2 NPs caused more disturbance of the organic and mineral nutritional contents under flooded condition

To further explore the difference in the physiological impact of CeO_2 NPs on rice, we measured the effect on organic (**Table 1)** and inorganic (**Table 2)** nutrient contents in plants. Under flooded conditions, CeO_2 at 100 mg/kg significantly increased the organic nutrient contents. Although 100 mg/kg CeO_2 NPs did not affect the protein content in roots, it increased the protein content in shoots by 48%. Under flooded conditions, 100 mg/kg CeO_2 NPs also increased the contents of soluble sugar, reducing sugar and starch in both roots and shoots. At high concentration (500 mg/kg), CeO_2 NPs reduced the protein and soluble sugar content in root by 20% and 38%, respectively, but showed no effects on these organic nutrients in shoots (flooded conditions).

267 CeO_2 NPs showed similar patterns of effects on the organic nutrients under aerobic conditions
268 with less significant differences compared with that under flooded conditions. Under aerobic
269 conditions, 100 mg/kg CeO_2 NPs increased the starch content in roots by 81% but showed no effects
270 on other organic nutrients in roots compared with control. However, 100 mg/kg CeO_2 NPs increased
271 the contents of all the organic nutrients in shoots. CeO_2 at 500 mg/kg did not affect the organic
272 nutrients in either roots or shoots (aerobic conditions). These results suggest that CeO_2 NPs affected
273 the carbon metabolism of rice seedlings and the effects were different depending on the water
274 irrigation regime. Starch and sugar are two key components involved in carbon flux within most
275 plants. They are produced through fixation of atmospheric carbon via photosynthetic process.
276 Increase in sugar and starch contents provide sufficient energy source for the cellular respiration in
277 order to cope with ambient stress such as excess light, heat and high salinity. The increase of the
278 sugar and starch contents by CeO_2 NPs agree with the results observed for photosynthesis that more
279 profound impacts were observed under flooded conditions than under aerobic condition and that
280 only positive effects on plant growth were oserved under aerobic condition.

281

282 **Table 1.** Total soluble protein, total sugar, reducing sugar and starch content in plant roots and
283 shoots (unit: mg/g FW). * indicates significant difference at $p < 0.05$ compared with the
284 corresponding control. "F" indicates flooded. "A" indicates aerobic.

Treatments		Protein (mg/g FW)	Soluble sugar (mg/g FW)	Reducing sugar (mg/g FW)	Starch (mg/g FW)
Root	CT-F	14.2 ± 1.3	18.5 ± 2.9	7.9 ± 2.4	1.9 ± 0.3
	Ce100-F	15.7 ± 2.2	27.5 ± 4.6*	11.9 ± 1.6*	3.1 ± 0.4*
	Ce500-F	11.4 ± 2.5*	11.5 ± 3.3*	7.1 ± 3.0	2.3 ± 0.2
	CT-A	12.7 ± 2.5	19.7 ± 2.2	6.1 ± 1.4	1.6 ± 0.5
	Ce100-A	13.1 ± 3.0	23.3 ± 1.8	8.7 ± 2.9	2.9 ± 0.3*
	Ce500-A	11.7 ± 2.8	16.5 ± 3.6	8.3 ± 3.7	1.8 ± 0.4
Shoot	CT-F	8.3 ± 1.6	14.7 ± 2.4	8.3 ± 3.1	1.2 ± 0.2
	Ce100-F	12.3 ± 3.1*	18.5 ± 1.6*	13.9 ± 3.7*	2.1 ± 0.1*
	Ce500-F	7.3 ± 1.0	15.1 ± 3.0	11.0 ± 2.4	1.9 ± 0.3

CT-A	7.5 ± 1.6	12.3 ± 2.2	9.4 ± 1.8	1.4 ± 0.5
Ce100-A	9.4 ± 1.9*	15.7 ± 1.8*	12.9 ± 2.4*	2.1 ± 0.3*
Ce500-A	6.3 ± 2.5	10.7 ± 3.3	11.8 ± 3.2	1.7 ± 0.4

285

286 CeO_2 NPs did not affect the contents of macronutrients (K, Ca, Mg, P) in shoots, while 100 mg/kg
287 CeO_2 NPs increased the micronutrients including Fe, Zn and Mn in shoots under both flooded and
288 aerobic conditions. Specifically, the Fe, Zn and Mn contents were increased by 29%, 73% and 42%,
289 respectively, under flooded conditions, and were increased by 23%, 36% and 29%, respectively
290 under aerobic conditions. The positive effects of 100 mg/kg CeO_2 on plant growth might be related
291 to the increased Fe and Zn levels because both Fe and Zn are essential nutrients playing significant
292 roles in photosynthetic process. In contrast, 500 mg/kg CeO_2 NPs showed no effects on the content
293 of inorganic nutrients in shoots. Taken together, these results suggest that CeO_2 NPs showed more
294 significant positive impacts at 100 mg/kg or more severe negative impacts at 500 mg/kg on the
295 macro- and micro- nutrient elements uptake and accumulation in rice seedlings under flooded
296 conditions than under aerobic conditions.

297

298 **Table 2.** Contents of macro- and micro- element nutrients in plant shoots affected by CeO_2 NPs
299 treatment under flooded and aerobic conditions. *indicates significant difference at $p < 0.05$
300 compared with untreated control.

Treatments		Macronutrient (g/kg DW)				Micronutrient (mg/kg DW)			
		K	Ca	Mg	P	Fe	Cu	Zn	Mn
Flooded	CT	35.6 ± 1.3	1.6 ± 0.1	3.4 ± 0.2	4.4 ± 0.7	95 ± 10	5.2 ± 1.5	11 ± 3	43 ± 9
	Ce100	33.4 ± 2.0	1.7 ± 0.3	3.5 ± 0.3	4.0 ± 0.5	123 ± 14*	4.4 ± 0.9	19 ± 4 *	61 ± 8*
	Ce500	34.1 ± 1.8	1.6 ± 0.3	3.5 ± 0.4	4.2 ± 0.3	101 ± 21	4.1 ± 1.2	15 ± 4	48 ± 7
Aerobic	CT	38.1 ± 1.8	1.5 ± 0.2	3.7 ± 0.3	4.1 ± 0.5	87 ± 15	4.7 ± 1.3	14 ± 2	45 ± 5
	Ce100	36.7 ± 2.3	1.6 ± 0.2	3.4 ± 0.3	4.3 ± 0.7	107 ± 13*	4.0 ± 1.2	19 ± 3*	58 ± 7*
	Ce500	36.9 ± 3.1	1.6 ± 0.3	3.5 ± 0.2	4.5 ± 0.4	95 ± 19	4.3 ± 2.0	16 ± 3	47 ± 6

301

302 **Distinct stress responses in plants induced by CeO_2 NPs under aerobic and flooded conditions**

To further evaluate the stress induced by CeO_2 NPs exposure, we measured the antioxidant enzyme activity, proline content and the oxidative DNA damage marker (8-OHdG). The response patterns of the antioxidant enzymes and proline in plants to CeO_2 NPs were similar under flooded and aerobic conditions (**Figure 3**). CeO_2 NPs at 100 mg/kg enhanced the SOD and CAT activities in roots under both flooded and aerobic conditions, with a higher enhancement of CAT under flooded (145%) than aerobic (63%) conditions (**Figure 3A-3B** and **3E-3F**). The SOD and CAT activities in shoots were also enhanced by 100 mg/kg CeO_2 NPs treatment with the exception of SOD under aerobic conditions (**Figure 3E**). The MDA contents in roots were reduced by 100 mg/kg CeO_2 NPs under both flooded and aerobic conditions (**Figure 3C** and **3G**), while it was increased by 40% following 500 mg/kg CeO_2 NPs treatment. The MDA contents in shoots were not affected under aerobic condition. However, under flooded condition, CeO_2 NPs at 100 mg/kg reduced the MDA contents in shoots by 41%.

Plants have developed their own defense system against ambient stresses including enzymatic and non-enzymatic antioxidant system. There was no significant difference in the MDA contents between control groups under the two conditions, suggesting that water regime didn't cause stress to plants. The enhancement of SOD and CAT levels and reduced MDA levels in plant treated with 100 mg/kg CeO_2 NPs were observed under both conditions, suggesting that the CeO_2 NPs reduced the stress level in plants by improving the antioxidative capacity of plants. While plants failed to defense the stress in roots caused by high concentration (500 mg/kg) of CeO_2 NPs exposure under flooded condition but succeed under aerobic condition, as shown by the MDA content in plant.

We further examined the proline content in plants, which is a marker for plant stress (**Figure 3D** and **3H**). CeO_2 NPs at 100 mg/kg reduced the proline content in roots by 33% under both flooded and aerobic conditions. The proline content in shoots was also reduced in the 100 mg/kg CeO_2 NPs treatment under flooded conditions while no change was observed under aerobic conditions. High

concentration (500 mg/kg) of CeO_2 NPs caused enhanced accumulation of proline in roots under both flooded and aerobic conditions while it did not affect the proline content in shoots.

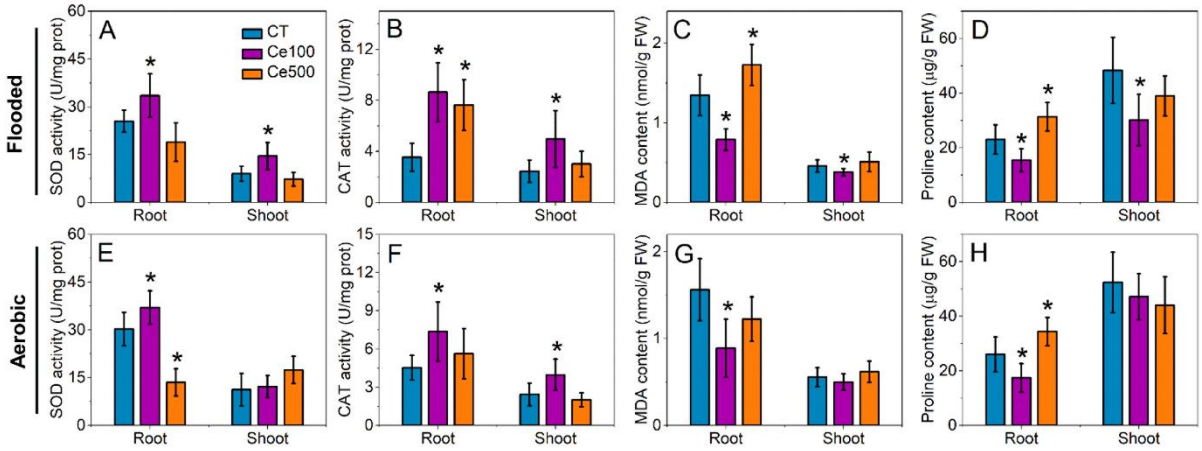


Figure 3. Stress response of rice plants to CeO_2 NPs exposure. (A-D) and (E-H) indicate the SOD activity, CAT activity, MDA content and proline content under flooded and aerobic conditions, respectively. * indicates significant difference at $p < 0.05$ compared with the corresponding control.

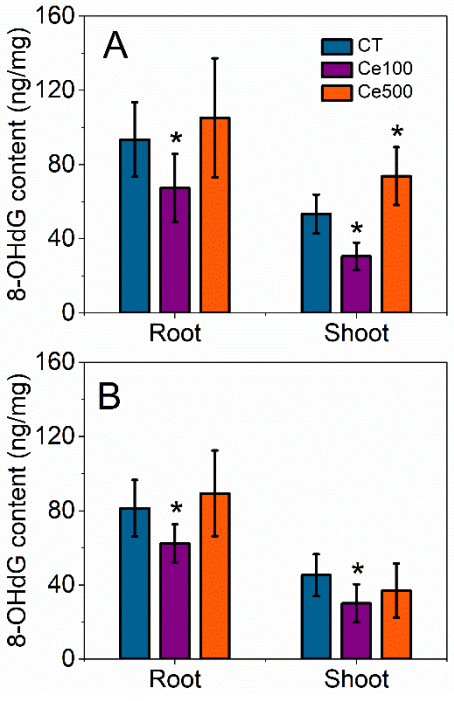


Figure 4. The content of 8-OHdG in plants affected by CeO_2 NPs under flooded (A) and aerobic (B) conditions. * indicates significant difference at $p < 0.05$ compared with the corresponding control.

The biomarker for oxidative DNA damage (8-OHdG) was measured to evaluate whether oxidative stress caused any genetic damage (**Figure 4**). The 8-OHdG contents in roots and shoots were reduced in the 100 mg/kg treatment under both flooded and aerobic conditions, while CeO_2 NPs of 500 mg/kg increased the 8-OHdG content in shoots under flooded conditions. Overall, our results suggest that 100 mg/kg CeO_2 NPs reduced the plant stress while 500 mg/kg CeO_2 NPs caused stress to plants, and either the positive effects at 100 mg/kg or the negative effects at 500 mg/kg seemed stronger under flooded conditions, compared to aerobic conditions.

Lower uptake of Ce in plants under aerobic than flooded conditions

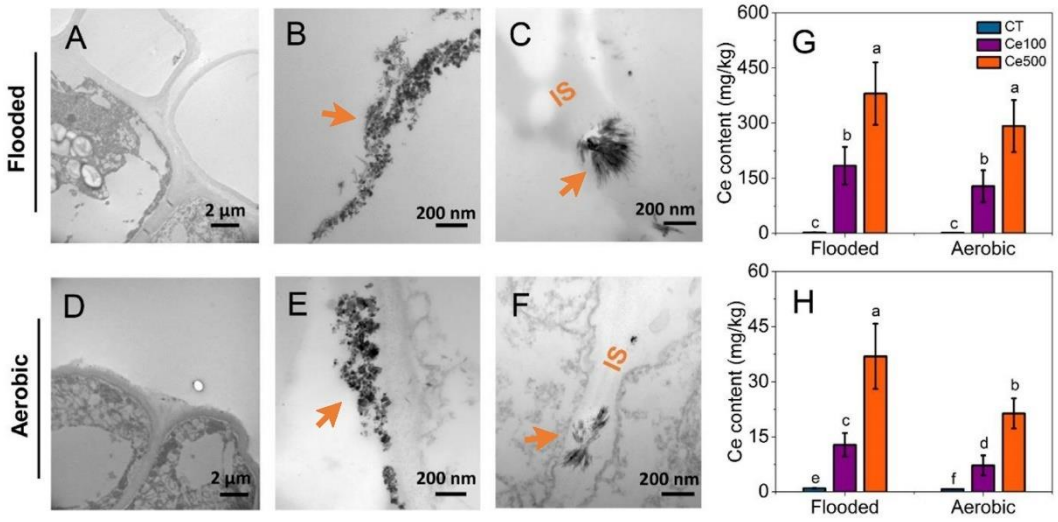


Figure 5. TEM images of root sections (A-F) of plants treated with 500 mg/kg CeO_2 NPs and Ce uptake in plants (G and H) treated with 100 and 500 mg/kg CeO_2 NPs. (A) and (D) are TEM images of roots in control group under flooded and aerobic conditions, respectively. (B) and (E) show the root surface. (C) and (E) show the intercellular space (IS). Arrows indicate the particles observed on root surface and in IS. (G) and (H) are the Ce contents in roots and shoots, respectively. Different lowercase letters indicate significant difference between treatments at $P < 0.05$.

Uptake of CeO_2 NPs in plants was first examined by TEM observation of root sections (**Figure 5A-5F**). There were no particles observed in control groups (**Figure 5A** and **5D**). CeO_2 NPs aggregates were observed along the root surface under both flooded and aerobic conditions (**Figure 5B** and **5E**). Needle-like clusters were observed in the intercellular space (**Figure 5C** and **5F**). Similar clusters were previously found in CeO_2 NPs treated cucumber, which were demonstrated to be $CePO_4$.[15] No difference in the CeO_2 NP uptake between flooded and aerobic conditions can be distinguished from the TEM images.

Ce uptake in plants was quantified by ICP-MS and dose-dependent Ce uptake in plants was observed (**Figure 5G and 5H**). The Ce contents in roots were not significantly different between the two conditions at the same exposure concentration (**Figure 5G**). However, the Ce contents in shoots treated with 100 mg/kg and 500 mg/kg CeO_2 NPs under flooded condition were 1.78 and 1.70 fold higher than those under aerobic conditions.

Less transformation of CeO_2 NPs in soil and plants under aerobic than flooded conditions

Transformation of CeO_2 NPs in soil and plants was analysed by synchrotron based XANES. As seen from **Figure 7**, the low-energy feature (a) and high-energy features (b and c) are respectively attributed to the Ce(III) and Ce(IV) compounds. This spectral difference is an important criterion for distinguishing Ce compounds of the two different oxidation states. The Ce L_{III}-edge XANES spectra in all the samples presented mainly the feature (b and c) of Ce(IV) while also showing a small peak (a) which is the feature of Ce(III), suggesting that transformation occurred. The fractions of CeO_2 NPs and its transformed products were obtained by LCF analysis using the XANES spectra of CeO_2 NPs, Ce acetate ($Ce(Ac)_3$) and $CePO_4$ as standard references (**Figure S4)**.

Figure 6A showed the results in rhizobag soils. A larger bump at the position of peak a in rhizobag soils under flooded condition than under aerobic condition can be clearly seen, suggesting

more transformation of CeO_2 NPs occurred under flooded condition. LCF analysis revealed 11% and 17% of Ce(III) species in rhizobag soils under aerobic and flooded conditions, respectively (**Figure 6A**). In contrast, the Ce presented mainly as CeO_2 in pot soils, with only 3% and 4% of Ce(III) species obtained by LCF (**Figure 6B**). A previous study by Ma et al. demonstrated that XANES and LCF analysis are very sensitive for distinguishing Ce species in plant matrix with error between 2% - 6%.[36] Therefore, the 3% or 4% reported here are within the error that can be negligible. The significantly higher Ce(III) species found in rhizobag soils than in pot soils suggest that rhizosphere chemistry played a significant role in the transformation of CeO_2 NPs. The rhizosphere, which contains large amounts of root excreted organic acids and reducing substances as well as abundant microorganisms, contributes significantly to the reduction and dissolution of CeO_2 NPs and their further transformation such as phosphorylation. The rhizobag soils are close to the root thus containing larger amount of root exudates than pot soils, therefore, more transformation of CeO_2 NPs can occur.

Higher fractions of Ce(III) (15% under aerobic and 28% under flooded conditions) were observed in root samples (**Figure 6C**) than in soil samples at the same condition, suggesting more transformation occurred in roots. The root surface has been reported to be the main site for transformation of CeO_2 NPs in plants.[17] A study by Ma et al. found that CeO_2 NPs that entered into plants remained as CeO_2 and did not transform further inside plant. Rico et al. found more transformation of CeO_2 NPs in hotspots on the root surface by synchrotron XRF/XANES than in soils. Our results are in accordance with the previous reports that roots played a critical role in CeO_2 NPs transformation.

The higher fraction of $CePO_4$ than that of $Ce(AC)_3$ in rhizobag soil can be attributed to the stronger affinity of Ce(III) for PO_4^{3-} ($Ksp=1 \times 10^{-23}$) than for the organic ligand. Reducing substance and organic acids in the root exudates are the dertemining factors that stimulate the reduction and release of Ce^{3+}. The Ce^{3+} can further bind with phosphates on the root surface or in the intercellular

space after entering the root[15]. Phosphates alone cannot lead to obvious transformation as comparable with that caused by root exudates, which has been demonstrated in a previous study, although they can bind with Ce(III) on the particle surface[37]. Therefore, more fractions of Ce(III) including $CePO_4$ and Ce carboxylates formed in rhizobag soils than in pot soils. Since $CePO_4$ accounted for the major part of the transformed species and the $CePO_4$ are needle-like clusters with low mobility, the upward translocation of Ce(III) was therefore restricted. As shown in **Figure 6D**, the majority of the Ce presented as CeO_2 in shoots under both aerobic and flooded conditions, with only 5% and 9% of $Ce(Ac)_3$ being detected, respectively, and no evidence of $CePO_4$.

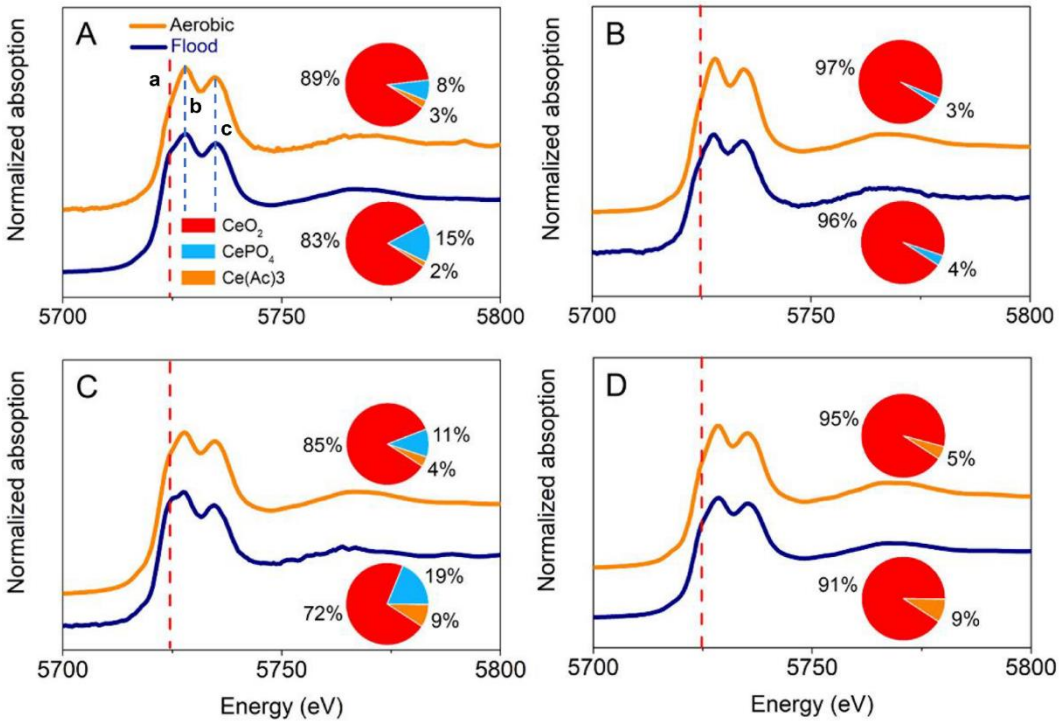


Figure 6. Ce L_{III}-edge XANES spectra and the fraction of Ce species (shown as pie charts) in rhizobag soil (A), pot soil (B), root (C) and shoot (D) samples collected from the 500 mg/kg CeO_2 NPs treatment groups. The dotted lines indicate the location of the characteristic peak of Ce(III) (a) and Ce(IV) (b and c) species.

The absolute contents of Ce(IV) and Ce(III) species were calculated by multiplying the total Ce content by the percentages of Ce(IV) and Ce(III). As shown in **Figure 7A**, the Ce(IV) content in roots under flooded (274 mg/kg) and aerobic (249 mg/kg) conditions were close, while the Ce(III) content in roots was higher under the flooded condition (106 mg/kg) than under the aerobic condition (43 mg/kg). The contents of Ce(IV) and Ce(III) in shoots were both higher under flooded than aerobic conditions (**Figure 7B**). The different transformation and translocation of CeO_2 NPs in soils and plants under different conditions can be explained by the following reasons. Firstly, the redox potential (Eh) was much lower in flooded soil (-188 mV) than in aerobic soil (-90 mV), and the Eh remained almost constant during the experiment (**Figure 7C**). Low Eh is favourable for the reduction of cerium from Ce(IV) to Ce(III). Moreover, flooding can cause variation of the soil pH, i.e. decrease of the pH of alkaline soil and increase of pH of acidic soil. As expected, the pH of flooded soil (5.1 ± 0.7) was lower than that of aerobic soil (7.7 ± 0.6). A lower pH is favourable for the dissolution of CeO_2 NPs and the release of Ce^{3+}. The lower Eh and pH caused higher transformation of CeO_2 NPs and subsequent translocation of Ce(III) species. The change of pH affects the surface charge of CeO_2 NPs, which can affect the uptake in plant. Previous studies suggest that the positive CeO_2 NPs adhere more to plant roots and translocate less into aboveground shoot than negative or neutral CeO_2 NPs.[38, 39] However, the surface charge of the CeO_2 NPs used here was nerutral at pH 7.8 (pH at aerobic condition) while was positive (21 mV) at pH 5 (pH at flooded condition) (Figure S5), which is not in accordance with the previous reports, suggesting that surface charge is not the main driver leading to the difference between flooded and aerobic condition. Secondly, inorganic ions and NPs share the same vascular system with the water and nutrients and translocate upwards from root to shoot following the water flow.[2] Therefore, the upward translocation of CeO_2 NPs or Ce^{3+} is highly dependent on the transpiration rate. Plants can adjust the opening or closure of the stomata on the leaves in response to different water irrigation regimes.[40] In flooded conditions, plants usually have a high transpiration rate. However, in aerobic conditions, plant reduce their transpiration rate by

restricting the opening of stomata.[40] As shown in **Figure 8**, the transpiration rate of rice was significantly higher in the flooded condition than in the aerobic condition for all groups. Under flooded condition, 100 mg/kg CeO2 NPs enhanced the transpiration rate (TR) by 38% at day 28, while 500 mg/kg CeO2 NPs reduced the TR by 30%. Under aerobic condition, 100 mg/kg CeO2 NPs enhanced the TR at day 14 and day 28 by 54% and 30%, respectively. The higher mobility of both particles or ions with water flow under flooded condition than aerobic condition led to high uptake and translocation of Ce in plants. It should be noted that in Fe^{2+} rich paddy soil, iron plaque can easily form on rice root surface. Previous studies suggest that iron plaque reduces the uptake of CeO_2 NPs in hydroponic conditions[41, 42], which is not in accordance with our results that Ce uptake was enhanced in flooded condition under which the iron plaque may form. The inconsistence may be related to that the difference in the actual amount of iron plaque on the root can cause contrasting effects. For example, it was reported that medium amount iron plaque increased Zn uptake while high amount of iron plaque reduced the Zn uptake in plant[43]. In these hydroponic studies, iron plaque was intentionally created by adding additional $FeSO_4$ as Fe source. The iron plaque formed in our study might be not significant enough thus its impacts on the Ce uptake are not comparable with the root exudates which contribute significantly to the release of Ce^{3+} and subsequent transformation. Moreover, while iron plaque can act as a physical barrier for root absorption of foreign substances, positive effects on the absorption and translocation of metals such as Zn, Pb, and Hg in plants have been reported[44]. More studies are required to gain further insights into the effects of iron plaque on the transformation and uptake of CeO_2 NPs in plants growing in realistic fields under different conditions (e.g., water regime, soil iron content, pH *etc.*).

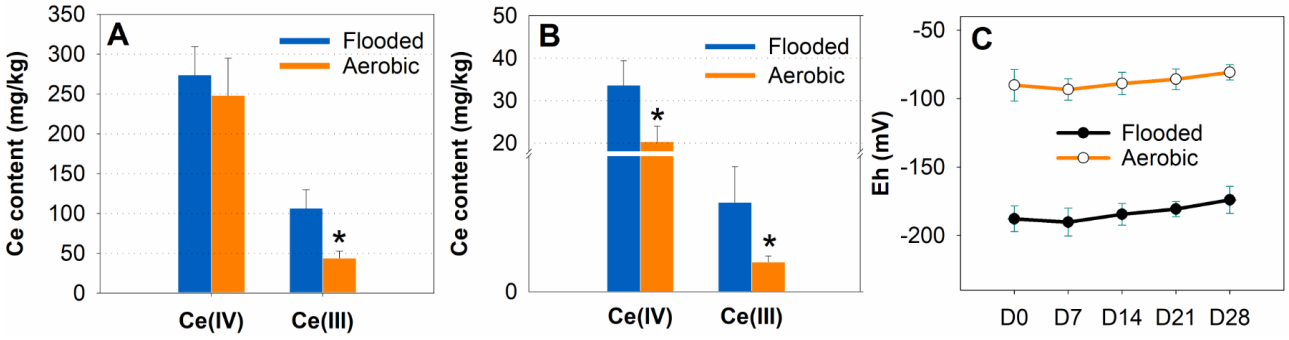


Figure 7. Ce(IV) and Ce(III) contents in roots (**A**) and shoots (**B**) calculated by multiplying the total Ce content by the percentages of Ce(IV) and Ce(III). (C) Eh of the soil in flooded and aerobic conditions at 0, 7, 14, 21 and 28 days. * indicates significant difference compared with untreated control at $P < 0.05$.

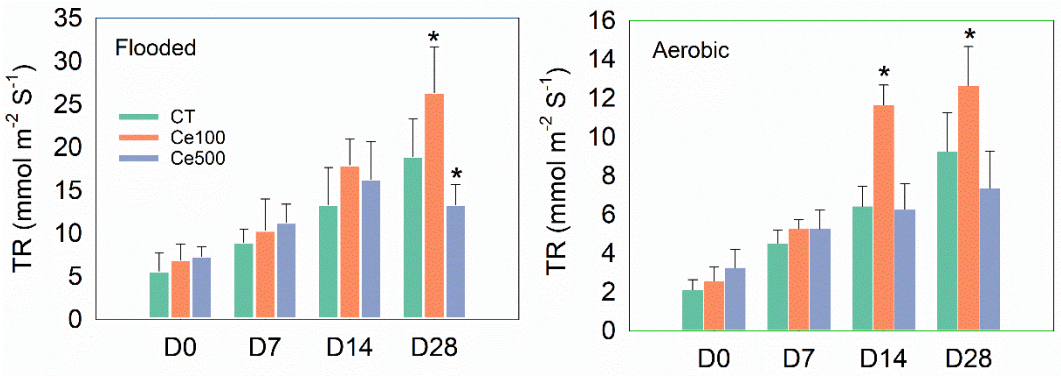


Figure 8. Transpiration rate of rice plant growing under flooded and aerobic ocnditions with CeO2 NPs treatments. * indicates significant difference compared with untreated control at $P < 0.05$.

Previous studies have suggested that the phytotoxicity of metal-based NPs such as CeO_2 NPs is highly linked with their transformation. For example, the phytotoxicity of CeO_2 NPs to *Lactuca* plants at germination stage was attributed mainly to the dissolution of CeO_2 NPs; a small amount of Ce^{3+} released at the root interface can significantly inhibit the seedling growth. Another study suggested particle-specific effects of CeO_2 NPs to *Arabidopsis thaliana*.[45] More recently, a life cycle study showed that CeO_2 NPs and Ce^{3+} showed different and even opposing effects on the soil grown

common bean (*Phaseolus vulgaris*); CeO_2 NPs impaired the photosynthesis and reduced the yield while Ce^{3+} enhanced the photosynthesis and improved the nutrition quality of pods, suggesting that the effects of CeO_2 NPs on plants cannot be solely explained by the ionic effect.[3] In agreement with this study, our results also suggest that both CeO_2 NPs and Ce^{3+} played distinct roles in the effects on rice plants. CeO_2 NPs caused more significant impacts on the growth of rice seedlings under flooded conditions than under aerobic conditions, which correlated well with the higher uptake and translocation of Ce in the plants. At 100 mg/kg, CeO_2 NPs showed stronger positive effects under flooded conditions than under aerobic conditions. As discussed previously, CeO_2 NPs are capable of capturing ROS thereby reducing oxidative stress in plants and improving photosynthesis and plant growth.[8] In addition to the CeO_2 NPs, the positive effects of low dose Ce^{3+} on plant growth have been long known, although the mechanisms are poorly understood. One possible mechanism that has been proposed is that Ce^{3+} can act as a substitute for Mg^{2+} to improve photosynthesis.[46] Indeed, the photosynthesis was enhanced by 100 mg/kg CeO_2 NPs treatment and higher improvement was observed under flooded conditions corresponding to the higher Ce(III) content in the shoots. Therefore, both CeO_2 and Ce^{3+} contributed to the positive effects at 100 mg/kg. In contrast, the high concentration of CeO_2 (500 mg/kg) caused negative impacts on rice growth, and the impacts correlated again with the total Ce and Ce(IV)/Ce(III) contents in the plants. More significant negative impacts were observed under flooded conditions than under aerobic conditions, which corresponded to the higher Ce(IV) and Ce(III) contents in the rice plants.

Environmental Implications and future perspectives

Taken together, these results suggest that the water irrigation regime influences the impact of CeO_2 NPs on photosynthesis, antioxidant system, plant stress, DNA damage and eventually the plant growth, by changing the local environmental in soil thus affecting the CeO_2 transformation and Ce

503 uptake and translocation in rice plants. It should be noted that CeO_2 NPs are a type of redox sensitive
504 NM, and thus whether changing water irrigation regimes can also influence the effects of other
505 ENMs on plant growth needs to be explored in the future. Even for ENMs that are not sensitive to
506 redox potential, the shift of redox potential can affect not only the soil chemistry, but also the
507 microbial activity and root rhizosphere chemistry that may significantly change the metal
508 bioavailability.

509 The positive effects on plant growth of CeO_2 NPs at 100 mg/kg imply the potential of CeO_2 NPs for
510 application in agriculture. Indeed, CeO_2 NPs have shown potential to improve plant growth under
511 stress caused by excess heat and light[6], high salinity[7], nitrogen deficiency or excess[47] maily owing to
512 its ROS scavenge property, while high dose of CeO_2 may also pose risk to plant growth.

513 Under realistic field condition, ENMs may co-exist with other conventional pollutants such as As, Hg
514 and Cd. It has been known that whereas aerobic condition may be beneficial for reducing
515 bioavailability of As[24], this may also lead to increase of the bioavailability of Cd[48], known to be
516 present in high quantities in many paddy soils with potential risks for food safety and human health.
517 The mutual effects of CeO_2 NPs and Cd or As has been reported[49, 50]. Although no effects on the Cd
518 or As accumulation was exerted by CeO_2 NPs, the uptake of Ce in plant was enhanced by the
519 presence of Cd or As. Note that these studies were performed in hydrponic and dry soils. More
520 studies are needed to gain insights into whether and how variation of redox potential due to the
521 change of water regime affects the mutual effects between these chemicals and ENMs.

522

523 **SUPPORTING INFORMATION**

524 TEM image of CeO_2 NPs (Figure S1); XRD patterns of CeO_2 NPs (Figure S2); Schematic show of the
525 rhizobag system (Figure S3); Ce $L\text{-}_{III}$ edge XANES spectra of standard reference samples including
526 CeO_2, $CePO_4$ and Ce carboxyaltes (Figure S4); Zeta potential of CeO_2 NPs as a function of pH

(Figure S5); Properties of the soil used in this study (Table S1); Limit of detection, precision and recovery data of ICP-OES for the selected elements (Table S2).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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