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Metabolomics Reveal the "Invisible" Responses of Spinach Plants Exposed to CeO_2 Nanoparticles

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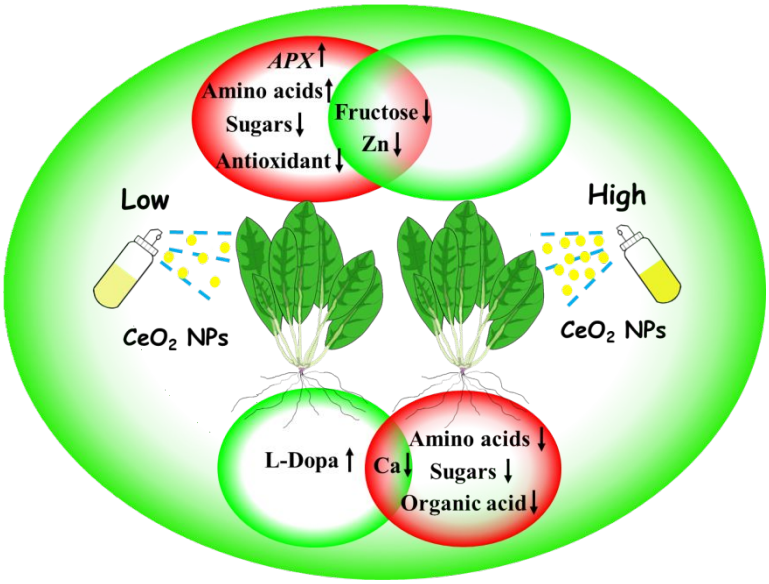
APX↑
Amino acids↑
Sugars↓
Antioxidant↓
Fructose↓
Zn↓
Low
High
CeO2 NPs
CeO2 NPs
L-Dopa↑
Ca↓
Amino acids↓
Sugars↓
Organic acid↓

TOC

ABSTRACT

Engineered nanoparticle (NP) with activities that mimic antioxidant enzymes have good prospects in agriculture because they can increase photosynthesis and improve stress tolerance. Here, the interaction between cerium oxide NPs with spinach plants (*Spinacia oleracea*) was investigated by integrating phenotypic and metabolomic analyses. Soil grown four-week-old spinach plants were foliar exposed for three weeks to CeO_2 NPs at 0, 0.3, and 3 mg per plant. Phenotypic parameters (chlorophyll fluorescence, photosynthetic pigment contents, plant biomass, lipid peroxidation and membrane permeability) were not affected. However, metabolomics analysis revealed that both doses of CeO_2 NPs induced metabolic reprogramming in leaves and roots in a non-dose-dependent manner. The low dose of CeO_2 NPs (0.3 mg per plant) induced stronger metabolic reprogramming in spinach leaves than high dose of CeO_2 NPs. However, the high dose of CeO_2 NPs triggered more metabolic changes in roots, compared to the low dose. Foliar spray of CeO_2 NPs at 3 mg/plant induced marked down-regulation of a number of amino acids (threonine, tryptophan, L-cysteine, methionine, cycloleucine, aspartic acid, asparagine, tyrosine, and glutamic acid). In addition, Zn decreased by 44% and 54% in leaves and Ca decreased by 38% and 32% in roots under exposure to CeO_2 NPs at 0.3 and 3 mg/plant, respectively. These results provide better understanding of the intrinsic phenotypic and metabolic changes imposed by CeO_2 NPs in spinach plants.

INTRODUCTION

Nanotechnology provides tools to improve plant production and protection, enabling a more sustainable agriculture.[1] Nano-enabled agrochemicals have been designed to encapsulate active ingredients within polymers enabling slow and target controlled release.[2] In addition, the unique physiochemical and photoelectric properties of engineered nanoparticles (NPs) can be used to enhance plant photosynthesis and growth.[3, 4] For example, single-walled carbon nanotubes have been reported to increase plant photosynthesis via enhanced energy capture.[3] CeO_2 NPs, a lanthanide metal oxide, exhibit excellent reactive oxygen species (ROS) scavenging activity.[5-7] The ROS catalytic scavenging properties of CeO_2 NPs have been utilized to protect plant photosynthesis from oxidative stress and improve plant growth performance, especially under stress condition.[8]

Recently, CeO_2 NPs (10 nm, -17 mV, Ce^{3+}/Ce^{4+} ratio of 35%, 50 mg/L) were found to enhance leaf mesophyll K^+ retention, therefore improving *Arabidopsis thaliana* salinity tolerance.[9] In addition to improve plant performance under stress conditions, both bare (10 nm, +45 mV, 8% Ce^{3+} ions) and polyvinylpyrrolidone-coated CeO_2 NPs (19 nm, -52 mV, 12% of Ce^{3+} ions), at 100 mg/kg, have been reported to increase photosynthesis in soybean plants.[10] Furthermore, CeO_2 NPs have been applied to combat pathogens. Adisa et al.[11] reported that CeO_2 NPs (8 nm) at 250 mg/L significantly suppressed fungal pathogen (*Fusarium* Wilt) in tomato (*Solanum lycopersicum*). In contrast, CeO_2 NPs (20 nm, +43 mV in DI water) have been reported to induce oxidative stress, lipid peroxidation and membrane damage in *Arabidopsis thaliana*[12] and *Lactuca sativa*.[13] Elevated antioxidative capacity also affects plant metabolism processes. Thus far, the

mechanism by which the CeO_2 NPs modulate plant metabolism is not understood. Rico et al.[14] found changes in the physiology and nutrient profile of second generation wheat plants, when the first generation plants were exposed to 500 mg CeO_2 NPs/kg. The reason for such changes remains unclear. The understanding of the mechanism of interaction between CeO_2 and physiological-biochemical functions of the plants is imperative for their sustainable application in agriculture.[15, 12, 16]

Much of the existing plant-NP interaction studies use relatively insensitive endpoints (germination, biomass and pigment content), which offer limited guidance to understand the interaction mechanisms between plants and NPs.[17] In contrast, "omic" based endpoints, such as transcriptomics, proteomics and metabolomics, can provide highly detailed, global and mechanistic information on plant responses to NP.[17] To date, only Reichman et al.[18] reported transcriptome profile changes of plants (Douglas-Fir) in response to CeO_2 NPs. Metabolomics allows the identification and quantification of thousands of small molecules in unexposed and NP exposed plants. Unlike other omics, low molecular weight metabolites are the closest link to phenotype. In addition, metabolites pool in organisms is much smaller than gene and protein pool. This technology can be used as a powerful tool to understand the molecular response of plants that grow under NP exposure. By using gas chromatography-mass spectrometry (GC-MS)-based metabolomics, we contributed to clarify the toxicity and detoxification mechanisms of CuNPs and AgNPs to *Cucumis sativus* plants.[19, 20] Therefore, using more sensitive endpoints can get deeper insights into the mechanism of plant responses to CeO_2 NPs, which is important for their sustainable application and safety design.

In this study, laboratory synthesized CeO_2 NPs (~4 nm, 20 mV in 10 mg/L DI water) were exposed for three weeks to four-week-old spinach plants at 0, 0.3, and 3 mg/plant. The physiological and biochemical parameters, including photosynthesis, biomass, and lipid peroxidation, were determined. GC-MS was applied to determine hundreds of low molecular metabolites in leaves and roots. The phenotypic analysis, combined with metabolic analysis, enabled a deep insight into the interaction between CeO_2 NPs and spinach plants. The obtained results are valuable for using CeO_2 nanomaterials in a safe and sustainable manner in agriculture.

MATERIALS AND METHODS

CeO_2 Nanoparticles Synthesis and Characterization. The CeO_2 NPs were synthesized as reported previously by Sreeremya et al.[21] More details regarding the synthesis method are provided in the Supporting Information. The synthesized CeO_2 NPs were characterized by transmission electron microscope (TEM) (JEM-200CX, JEOL, Japan) for for size, shape and morphology. The hydrodynamic diameter and zeta potential of CeO_2 NPs in exposure medium (10 and 100 mg/L in nanopure water) were measured using a Nano Zetasizer (Malvern, Nanosizer ZS90, USA). The CAT-mimic activity of CeO_2 NPs was determined according to Wang et al.[22] Specifically, 20 μL CeO_2 NPs stock solution (50 mg/L and 500 mg/L, final concentration is 10 mg/L and 100 mg/L) was mixed with 80 μL H_2O_2 solution (100 mg/L) and incubated for 3 h at 37 °C. 50 μL sulfuric acid (9 mol/L) was added subsequently to stop the reaction. Then 50 μL $KMnO_4$ (3.2 mmol/L) was added to react with remaining H_2O_2. The absorbance at 525 nm was determined using a microplate spectrophotometer (Varioskan LUX,Thermo scientific,

America). This experiment was repeated in triplicate. The superoxide dismutase (SOD) mimic activities of CeO_2 NPs were determined using SOD assay kit (Nanjing Jiancheng Bioengineer Ins., Nanjing, China), following the manufacturer's instructions. More details regarding the SOD assays are provided in Supporting Information.

Plant Growth and CeO_2 Nanoparticles Exposure. Spinach (*Spinacia oleracea*) seeds were purchased from Hezhiyuan Seed Inc. (Shandong, China). The spinach accession Octavia was provided by Syngenta and their F1 hybrid were used for this study. The organic potting soil Scotts Miracle-Gro (Scotts Co, Marysville, Ohio) as growth medium. The nutrient element content in potting soil was shown in **Table S1**. Plastic pot containing 100 g of soil were planted with one/pot. The plants were cultivated in a green house for 28 days at 25 °C during the day and 20 °C at night. The daily light integral was 180 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Since the potting soil is rich of nutrients, no additional nutrients were added during whole growth period. Four weeks old spinach plants were foliar applied with CeO_2 NPs (10 and 100 mg/L, 0.3 and 3 mg/plant as total amount applied) twice per week for three weeks. For each foliar application, CeO_2 NPs suspensions were freshly prepared by bath-solicitation (KH-140, Hechuang Utrasonic, Jiangsu, China) at 45 kHz for 30 min in cold water until a stable dispersion was achieved. A hand-held spray bottle was used for spraying. The final applied dosing are 0.3 and 3 mg CeO_2 NPs per plant, which are extremely low and environmental relevant dose. It should be pointed out that the calculated CeO_2 NPs dose may overestimate the amount received by the plants because only around 60-70% of the spray reaches the spinach leaves, the loss include spread into the air, but not to soil, because the soil were covered during spray.

Physiological and Biochemical Analysis. Chlorophyll fluorescence was measured using a MultispeQ fluorometer (Photosynq, Michigan, U.S.). The photosynthetic pigment and total phenolic were extracted according to Sesták et al.[23] The absorbance of chlorophyll *a, b* and carotenoids was measured at the wavelengths of 663 nm, 645 nm, and 470 nm. The total content of phenolic compounds was determined with the method of Rossi et al.[24] Membrane integrity was estimated by measuring ion leakage from leaf according to Liu et al,[25] with minor modifications (instead of weighing same amount of leaves, we cut leaves with four pieces of 1 cm × 1 cm segments). In addition, the content of malondialdehyde (MDA), a lipid peroxidation marker, was measured by the Thiobarbituric Acid Reactive Substances (TBARS) assay.[26] More details regarding these assays are provided in Supporting Information.

Ce and Other Mineral Analysis. Twenty mg of oven dried leaf, stem and root tissues (72 h at 70 °C) were digested with a mixture of 8 mL of H_2O_2 and 2 mL of plasma pure HNO_3 (v/v: 4:1) using a microwave oven system (Multiwave Eco, Anton Par) at 160 °C for 40 min. Ce, K, Na, Mg, Ca, Mn, Fe, Cu and Zn were analyzed using inductively coupled plasma mass spectrometry (ICP-MS) (NexION-300, PerkinElmer, USA) and inductively coupled plasma-optical emission spectrometer (ICP-OES) (Optima 8300, Perkin Elmer, USA). The standard reference materials NIST 1570A (spinach leaves) were also digested and analyzed as samples. The recoveries for all elements were between 90 and 99%.

Metabolite Analysis in Spinach Tissues. Spinach leaf and root metabolites were analyzed by gas chromatography-mass spectrometry (GC-MS). Details on sample preparation, GC-MS analysis, and multivariate analysis have been reported in previous

study.[19] Briefly, spinach tissues were ground into power in liquid nitrogen. Methanol was used to extract metabolites in spinach tissues by sonication, with of 2-chloro-l-phenylalanine (0.3 mg/mL) as internal standard. The derivatized samples were analyzed by using an Agilent 7890B gas chromatography system coupled to an Agilent 5977A mass selective detector (Agilent Technologies Inc., CA, USA). The column employed was a DB-5MS fused-silica capillary column (30 m × 0.25 mm × 0.25 μm; Agilent J & W Scientific, Folsom, CA, USA Agilent Technologies, Santa Clara, CA). Helium (> 99.999%) was used as the carrier gas at a constant flow rate of 1.0 mL/min through the column. The initial oven temperature was 60 °C, ramped to 125 °C at a rate of 8 °C/min, to 210 °C at a rate of 4 °C/min, to 270 °C at a rate of 5 °C/min, to 305 °C at a rate of 10 °C/min, and finally, held at 305 °C for 3 min. The injection volume was 1 μL with the injector temperature 260 °C in splitless mode. The temperature of MS quadrupole and ion source (electron impact) was set to 150 and 230 °C, respectively. The collision energy was 70 eV. Mass data was acquired in a full-scan mode (m/z 50-500), and the solvent delay time was set to 5 min. The QC samples were injected at regular intervals (every 10 samples) throughout the analytical run.

Multivariate Statistical Analysis. Un-supervised principal components analyses (PCA) and a supervised partial least-squares discriminant analysis (PLS-DA) clustering method were run based on GC-MS data via online resources (http://www.metaboanalyst.ca/).[27] Before PCA and PLS-DA analysis, the data normalization (normalization by sum) has been done for general-purpose adjustment for difference among samples, and data transformation (log transformation) was conducted to make individual features more comparable. PLS-DA uses a multiple linear regression technique to maximize the

194 separation between groups; this helps to understand which variables carry the class
195 separating information.[28] Variable Importance in Projection (VIP) is the weighted sum of
196 the squares of the PLS-DA analysis, which indicates the importance of a variable to the
197 entire model.[28] A variable with a VIP greater than 1 is regarded as responsible for
198 separation, defined as a discriminating metabolite in this study.[29] Biological pathway
199 analysis was performed based on GC-MS data using MetaboAnalyst 2.0.[30] The impact
200 value threshold calculated for pathway identification was set at 0.1.[29]

201 **Total RNA Extraction and Antioxidant Enzyme Gene Expression Analysis.** The
202 leaves were frozen in liquid nitrogen and ground using mortar and pestle. The resulting
203 powder was stored at -80 °C until further analysis. Total RNA was extracted from 0.1 g
204 of fresh leaf powder using a Spectrum Plant Total RNA kit (Sigma-Aldrich, St. Louis,
205 MO) according to the supplier's recommendation. Three independent biological
206 replicates per treatment were used. Quality and quantity of RNA was assessed using a
207 Nanodrop 2000 Spectrophotometer (Thermo scientific, USA). The integrity was
208 evaluated using agarose gel electrophoresis stained with ethidium bromide.
209 Quantification was performed with a two-step reaction process: reverse transcription (RT)
210 and PCR.

211 Each RT reaction has two steps. The first step is 0.5 μg RNA, 2 μL of 4×g DNA wiper
212 Mix, add Nuclease-free H_2O to 8 μL. Reactions were performed in a GeneAmp® PCR
213 System 9700 (Applied Biosystems, USA) for 2 min at 42 °C. The second step is add 2μL
214 of 5 × HiScript II Q RT SuperMix IIa . Reactions were performed in a GeneAmp® PCR
215 System 9700 (Applied Biosystems, USA) for 10 min at 25 °C; 30 min at 50 °C; 5 min at

85 °C. The 10 μL RT reaction mix was then diluted ×10 in nuclease-free water and held at -20 °C.

Real-time PCR was performed using LightCycler® 480 Ⅱ Real-time PCR Instrument (Roche, Swiss) with 10 μL PCR reaction mixture that included 1 μL of cDNA, 5 μL of 2 × QuantiFast® SYBR® Green PCR Master Mix (Qiagen, Germany), 0.2 μL of forward primer, 0.2 μL of reverse primer and 3.6 μL of nuclease-free water. Reactions were incubated in a 384-well optical plate (Roche, Swiss) at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s. Each sample was run in triplicate for analysis. At the end of the PCR cycles, melting curve analysis was performed to validate the specific generation of the expected PCR product. The design of the primers for *Spinacia oleracea* was based on the genome database of spinach (http://www.spinachbase.org/cgi-bin/spinach/index.cgi). RT-PCR was performed using primers listed in **Table S2**.

Data Analysis and Statistics. For the assay of biomass, total phenolics, MDA, ion leakage, photosynthetic pigments, significant differences between treatment means were evaluated by one-way analyses of Variance (ANOVA) followed by Tukey-Kramer Post-Hoc testing, performed by SPSS (PASW Statistics 18). PSII quantum yield, electron transport rate and antioxidant enzyme gene expression were statistically analyzed using an independent two sample *t*-test. Reference to a significant difference between treatment means is based on a probability of $p<0.05$, unless otherwise stated. Data are presented as mean±standard errors (n=5).

RESULTS AND DISCUSSION

CeO_2 NPs Synthesis and Characterization. TEM image reveals roughly spherical and well dispersed CeO_2 NPs with an average diameter of ~ 4 nm (**Figure S1**). DLS results showed the hydrodynamic size of 10 mg/L and 100 mg/L CeO_2 NPs are 194.8 ± 7.0 nm and 215.3 ± 4.8 nm, respectively, with a zeta potential of 20.2 ± 0.59 mV and 42.2 ± 1.3 mV, indicating strong positive charge. In addition, CeO_2 NPs exhibit strong catalytic activity to scavenge H_2O_2, indicating strong CAT mimicking activities (**Table S3**). Meanwhile, CeO_2 NPs also had shown strong $\bullet O_2^-$ scavenging capacities (approximately 50%), indicating strong SOD mimic activities (**Table S3**). This is consistent with previous report that less than 5 nm CeO_2 NPs exhibit ROS-scavenging activity by reversible binding of oxygen atoms and shuttling between Ce^{3+} and Ce^{4+} states on their surface.[31] However, Heckert et al.[32] believed that the ability of nanoceria to scavenge superoxide is directly related to Ce(III) concentrations at the surface of the particle.

Photosynthetic Pigments and Chlorophyll Fluorescence. After 3 weeks of exposure, none of the treatments showed toxicity symptoms (**Figure 1 A**). In addition, there were no significant difference in chlorophyll *a*, *b* and carotenoids levels between control and CeO_2 NPs exposed plants (**Figure 1 B).** Chlorophyll fluorescence parameters, such as photosystem II quantum yield (ΦII) and linear electron flow (LEF), were used as indicators of photosystem II efficiency.[33] During the three-week exposure period, chlorophyll fluorescence and electron transport rates were monitored after each foliar application. After the first foliar application, ΦII was significantly (*t*-test, $p < 0.05$) decreased by both doses of CeO_2 NPs exposure, compared with control (**Table S4**), indicating a stress response. After the 2nd foliar application, the low concentration (0.3 mg/plant) still significantly (*t*-test, $p<0.05$) decreased PSII quantum yield, relative to

controls. However, no quantum yield of photosystem II difference was evident between CeO_2 NPs treated and control plants after 3rd application until harvest, indicating an acclimation process. This indicates that plant adapted to or coped with CeO_2 NPs exposure by some unknown mechanism. Similarly, the electron transport rate was significantly ($p < 0.05$) decreased by the high dose of CeO_2 NPs at first foliar exposure, but the plants quickly recovered (**Table S4**). These data indicate that CeO_2 NPs, at the concentration tested, result in an early stage-response of the photosynthesis system; however, it does not markedly impact plant performance.

Cell Membrane Integrity and Antioxidants. The CeO_2 NPs used in this test were extremely tiny, with an average size of 4 nm (TEM). Thus, it was relatively easy for them to get into the intracellular space, via cuticle or stomata, inducing oxidative stress around cell membranes. However, there was no increase of ion leakage, indicating no damaging on leaf cell membrane (**Figure 2 A**). On the contrary, exposure to low dose of CeO_2 NPs significantly ($p \leq 0.05$) decreased electrolyte leakage, indicator of membrane integrity, which is beneficial for plant resistance to stress (**Figure 2 A**). These results are inconsistent with Salehi et al.[34] that foliar delivered CeO_2 NPs (10-30 nm) induced electrolyte leakage in leaves of *Phaseolus vulgaris L*. The reason for the different results are possibly due to the particle size and doses. In addition, the structure of bean and spinach leaves may contribute to the different performance.

Furthermore, there was no statistically significant effects on MDA content (**Figure 2 B**), indicating no lipid peroxidation occurred by CeO_2 NPs exposure. Moreover, there was no change in total phenolics content (**Figure 2 C**). After 3 weeks of exposure, the leaf fresh biomass was unchanged by CeO_2 NPs as well (**Figure 2 D**). Taken together,

the phenotypic, physiological and biochemical results pointed out that although exposure to CeO_2 NPs (0.3 and 3 mg/plant) result in an early stage-response in PSII quantum yield and the electron transport rate in spinach seedlings, it did not induce any stress response and did not impact plant biomass.

Ce and Other Mineral Content in tissues. The ICP-MS data showed that the Ce bioaccumulation in spinach leaves after 3 week exposure was 1.3 ± 1.1, 127 ± 41 and 916 ± 108 mg/kg dry weight, for the 0, 0.3, and 3 mg CeO_2 NPs per plant, respectively (**Figure S2 A**). Since the harvested leaves were washed thoroughly with nano pure water, the results reflect strong bound or absorbed Ce as either CeO_2 NPs or Ce^{3+} or Ce^{4+} ions on the spinach leaves. The previous study showed a typical diameter of a 4-week-old spinach stomata is between 10 to 15 μm,[35] which is large enough for nano-scale, even micro-scale, particles to reach mesophyll through the stomata. However, given the large size (~200 nm) revealed by DLS, we speculate that the foliar sprayed CeO_2 NPs remained outside the cell wall and they could have an influence on the ROS signaling events from cell to cell. The CeO_2 NPs may further enter into phloem, being transported to the root. However, there was no difference in Ce content in roots of control and CeO_2 treated plants (**Figure S2 C**), indicating no translocation from leaf to root. This is not consistent with Hong et al.[36] finding that CeO_2 NPs (8 nm) can be translocated from cucumber (*Cucumis sativus*) leaf to root as evidenced by ICP-OES and TEM. Wu et al.[4] reported that structural properties of nanoparticles such as size and charge might be the drivers governing their transport through plant cell walls. Further studies are needed to elucidate the different uptake mechanisms between different species and nanoparticles.

306 Results from the ICP analysis showed a significant decrease of Zn content in leaves of
307 plants exposed to 0.3 mg CeO_2/ plant (54%) and 3 mg/plant (44%), compared to control
308 (**Table 1**). In contrast, Zn content in roots remained unchanged under both doses of CeO_2
309 NPs exposure. This suggested that foliar spray of CeO_2 NPs inhibited Zn accumulation in
310 leaves, instead of uptake. Another interesting finding is that Ca content in root
311 significantly decreased 38 and 32%, respectively, in low and high dose of CeO_2 NPs
312 exposure (**Table 1**). The reason for CeO_2 NPs impact Zn and Ca accumulation is still
313 unknown and further studies are needed to elucidate the mechanism.

314 **Metabolic Changes in Spinach Leaf.** GC-MS-based metabolomics identified and
315 quantified 305 metabolites in spinach leaves. The significantly changed metabolites in
316 spinach leaves were first screened out by using one-way ANOVA. *P* value less than 0.05
317 were considered significant. We found that 6 out of 305 metabolites in spinach leaves
318 were significantly changed under CeO_2 NPs exposure, including picolinic acid, citraconic
319 acid, beta-hydroxypruvate, tryptophol, diglycerol and fructose (**Figure S3**). It is
320 noteworthy that most of those significantly changed metabolites were observed only in
321 plants exposed to the lower CeO_2 concentration.

322 To develop a visual plot for the non-biased evaluation of changes in the metabolic
323 profile between different groups, principle component analysis (PCA) was performed.
324 The PCA score plots showed that the group of the low concentration was separated with
325 control and the group of the high concentration (**Figure S4 A**). This is consistent with
326 the one-way ANOVA results, which showed that the low concentration of CeO_2 NPs
327 induced more metabolic changes in leaves, compared to high NP concentration. To get a
328 deeper insight into the metabolite changes induced by the low CeO_2 NP concentration,

329 the biased PLS-DA model was run based on the dataset of control and low dose data. The
330 PLS-DA score plot showed that the low CeO_2 concentration data were clearly separated
331 with control group along PC1, which explained 37.6% of the total changes (**Figure S4**
332 **B**). Variable importance in projection (VIP) value above 1 was considered responsible for
333 the separation (**Figure S4 C**). **Table S5** summarized the significantly changed
334 metabolites screened by VIP score of PLS-DA model and t-test *p* value. The metabolites
335 were discussed in the following part according to their category.

336 *Amino acid and Carbohydrate.* It is noteworthy that a number of amino acids,
337 including asparagine, aspartic acid, glutamine, L-cysteine, methionine, increased
338 28~958% under low dose of CeO_2 NPs exposure (**Figure 3 A**). Amino acids play an
339 important role in modulating plant physiological process, such as acting as osmolytes,
340 regulating ion transport, modulating stomatal opening, serving as precursors for the
341 synthesis of defense-related metabolites and signaling metabolites.[37] Cysteine and
342 methionine are sulphur-containing amino acids, which function in many basic and
343 essential process of plant life. Glutamic acid and aspartic acid are regulator for nitrogen
344 assimilation.[38] Thus, changes in the amino acid contents may indicate the perturbation of
345 primary N metabolism.

346 Different from amino acids which were up-regulated in response to CeO_2 NPs
347 exposure, the level of a number of carbohydrates, such as sucrose, fructose, glucose-1-
348 phosphate, levoglucosan, ribulose-5-phosphate, allose and xylose, were significantly ($p \leq$
349 0.05) decreased in leaves exposed to CeO_2 NPs (**Figure 3 B**). Carbohydrate provide
350 energy for plant normal growth and development and act as signaling molecues.
351 Additionally, they act as a bridge in the communications of protein, lipid and metabolism.

Sucrose decreased 38.8% and 33.3%, respectively, by low and high doses of CeO_2 NPs. Another soluble sugar, fructose, decreased 44.4% and 39.2%, respectively by low and high dose of CeO_2 NPs. The up-regulation of amino acids and down-regulation of sugars indicate that low dose of CeO_2 NPs affected nitrogen and carbon pool reallocation in spinach leaf.

Organic acids. It is noteworthy that some organic acids (mucic acid, 3-methylglutaric acid, 2,3-dimethylsuccinic acid, citraconic acid, picolinic acid, beta-hydroxypyruvate) shared same pattern in which their relative abundance was significantly decreased by the low concentration of CeO_2 NPs (**Figure 3 C**). Picolinic acid has been found to have a number of biological functions, and one of them is to induce rice defense-related genes and subsequent resistant enhancement against rice blast fungus *Magnaporthe grisea*. [39] Previous studies have indicated that organic acids and amino acids also play important roles in modulating metal ions transport from roots to leaves and their accumulation in leaves.[40, 41]

Antioxidants. Interestingly, a number of metabolites with ROS scavenging capacities, such as alpha-tocopherol, dehydroascorbic acid, 3-hydroxyflavone, hydrocinnamic acid, 3,4-dihydroxyphenylglycol, benzoic acid were decreased 27-79%, compared to control, in response to the low concentration of CeO_2 NPs (**Figure 3 D**). Alpha-tocopherol, the major liposoluble antioxidant found in leaves,[42] decreased under both doses of CeO_2 NPs. Dehydroascorbic acid (DHA), the oxidized form of ascorbate, significantly decreased 59% (p = 0.003) compared to control (**Figure 3 D**). In addition, 3-hydroxyflavone, hydrocinnamic acid and 3,4-dihydroxyphenylglycol (DHPG) are important phenolic antioxidants, which localized in chloroplast act a role as singlet oxygen scavengers and

stabilizers of the chloroplast envelope membrane.[43] Since CeO_2 NPs foliar applied exhibit $\bullet O_2^-$ scavenging capacities, we speculate that the SOD mimic activities of CeO_2 NPs conserved endogenous non-enzyme antioxidant system of spinach plants.

Antioxidant Enzyme Gene Expression in Spinach Leaves. In addition to low molecular weight antioxidant compounds, antioxidant enzyme also play an important role in quenching ROS; thus, they are an important component of a plant's defense system. Since non-enzyme antioxidants perturbation occurred upon exposure to 0.3 mg/plant of CeO_2 NPs, we then investigated whether same dose (0.3 mg) of CeO_2 NPs also affected the enzymic antioxidant system. We herein examined the expression of six antioxidant related genes, including copper/zinc superoxide dismutase (*Cu/Zn-SOD*), catalase (*CAT*), glutathione S-transferase (*GST*), glutathione peroxidase (*GPx*), ascorbate peroxidase (*APX*) and monodehydroascorbate reductase (*MDAR*) using RT-qPCR. It is known that *SOD* acts as the first line of defense against ROS by catalyzing the dismutation of $O_2^{\bullet -}$ to H_2O_2.[44] We especially interested to know how CeO_2 NPs impact *SOD* gene expression, given that CeO_2 NPs have *SOD* mimic activity. Interestingly, the gene expression levels of SOD were unchanged by exogenous antioxidants-CeO_2 NPs (**Figure S5**). *CAT, APX*, *GPX* and *MDAR* enzymes work to further convert H_2O_2 to nontoxic H_2O through different reactions.[45] We found that the expression of *APX* was significantly ($p < 0.05$) increased 6.5% in spinach leaves foliar sprayed with low dose of CeO_2 NPs compared to control (**Figure S5**). In addition, the gene expression level of *CAT* was slightly increased (p=0.07) by CeO_2 NPs. The up-regulation of *APX* and *CAT* was possibly due to the *SOD* mimic activities of CeO_2 NPs. The over-generated H_2O_2 catalyzed by *SOD* or CeO_2 NPs need to be timely reduced to H_2O by other enzymes, like *CAT* and *APX*. Hong et al.

reported that *APX* activities in cucumber leaves exposed to CeO_2 NPs decreased in a dose-dependent manner with CeO_2 NPs,[36] which is inconsistent with our results. The plant species, NPs size, and zeta potential may contribute to the differet performance. Other tested antioxidant enzymes (*GPX*, *MDAR* and *GSTU17*) were unchanged upon CeO_2 NPs exposure. Taken together, CeO_2 NPs have almost no effect on the expression of all the studied antioxidant enzymes.

Metabolic Changes in Spinach Root. In roots, a total of 208 compounds were identified and quantified by GC-MS. The PCA showed that control and CeO_2 NPs treated groups were well separated, even by unbiased PCA model, indicating notable metabolites difference between groups (**Figure S6 A**). This also indicates that more pronounced metabolic changes occurred in roots, instead of leaves. This is of great interest because root is not the organ which was directly exposed to CeO_2 NPs and are assumed to have less metabolic changes compared to leaf. In order to find out the responsible metabolites leading to the separation between groups, PLS-DA model was performed. The score plot (**Figure S6 B**) shows that the groups of both CeO_2 concentrations were clearly separated from the control group along first principal axis (PC1), which explained 43.7% of the total variability. Results from both PCA and PLS-DA indicate that foliar spray of CeO_2 NPs remarkably altered the metabolites profiles of spinach root, although roots were not directly exposed to CeO_2 NPs and Ce was not translocated from leaf to root. In addition, different from leaves where the low dose of CeO_2 NPs induced most the severe changes, the higher change in root metabolites occured at the high CeO_2 NPs concentration. However, the low group from the low NP concentration was separated from control and

high concentration group along PC2, which explained 15.5% of the total variability (**Figure S6 B**), indicating some metabolic changes are low dose CeO_2 NPs specific.

The metabolites responsible for the separation between CeO_2 NPs groups and control were screened by VIP score above 1 (**Figure S6 C**). Almost all metabolites with VIP score above 1, had a *p* value lower than 0.05 (*t*-test), which indicate remarkable changes of metabolic profile in spinach root. In addition, the majority of responsible metabolites decreased in a dose-dependent fashion upon exposure to CeO_2 NPs, which indicates a generally negative impact on root metabolism. It is noteworthy that a number of amino acids, including glutamic acid, aspartic acid, asparagine, tyrosine, tryptophan, L-cysteine, methionine, cycloleucine and threonine, were markedly decreased (t-test, $p<0.05$) at the high CeO_2 NP concentration (**Figure 4 and Figure S7**). Glutamic acid is involved in primary nitrogen metabolism that regulates ammonium assimilation in plants.[46] The decrease of glutamic acid might imply a lower capacity of nitrogen assimilation through the glutamine synthetase/glutamate synthase pathway. In addition, sulfuric acid decreased in a dose-depended way and was not detected in plants expose to the higher NP concentration. This may explain the decreases of two S-containing amino acids (methionine and L-cysteine). Methionine, after conversion to S-adenosyl-l-methionine (SAM), control the levels of several essential metabolites, including ethylene, polyamines, biotin, and phytosiderophores.[47] In addition, SAM regulate important processes such as the formation of chlorophyll, the formation of the cell wall, and the biosynthesis of a number of secondary metabolites.[48] Different from other amino acids, which decreased by high dose of CeO_2 NPs, phenylalanine was found significantly increased upon exposure to high dose of CeO_2 NP (**Figure 4**). Phenylalanine is the

443 precursor for a wide range of intermediary and secondary metabolites which play
444 important role in plant defense.[49] The up-regulation of phynylalanine may indicate the
445 stress response of spinach plant to NP.

446 In addition to amino acids, other N-containing compounds, such as 3-
447 hydroxynorvaline, S-carboxymethylcysteine, alpha-aminoadipic acid, nicotinoylglycine,
448 carbamoyl-aspartic acid, also decreased in spinach root exposure to high dose of CeO_2
449 NPs (**Figure S8**). The reason for the simultaneous down-regulation of amino acids upon
450 exposure to high dose of CeO_2 NPs is not known yet. One hypothesis is that down-
451 regulation of a number of amino acids may indicate the conversion of anabolism to
452 catabolism in plant. This implies potential plant growth retards. Another possibility is that
453 CeO_2 NPs impaired nitrogen assimilation and thus affected the synthesis of amino acids.
454 The decrease of amino acids may impair protein biosynthesis and impact plant growth,
455 because amino acids are building block of proteins. Long term exposure experiments are
456 needed in future.

457 In addition to amino acids, two saturated fatty acids including stearic acid and palmitic
458 acid, increased 5 and 3 fold ($p < 0.05$), respectively, upon exposure to high does of CeO_2
459 NPs (**Figure S9**). While un-saturated fatty acid, arachidonic acid decreased ($p < 0.05$) in
460 response to high dose of CeO_2 NPs. The changes of fatty acids profile may indicate that
461 the spinach root reprogramed membrane fluidity, which may be an active strategy for
462 plant to prevent the translocation of Ce or CeO_2 NPs from leaf to root.

463 Sugars including 3,6-anhydro-D-galactose, glucose-1-phosphate, erythrose, leucrose
464 were significantly decreased in roots by high dose of CeO_2 NPs exposure (**Figure S10**).
465 In addition, a number of organic acids, such as 2-Furoic acid, 2-methylfumarate,

466 citraconic acid, maleic acid, threonic acid, glycolic acid, were also significantly
467 decreased by high dose of CeO_2 NPs (**Figure S11**). Organic acids are intermediates of
468 major carbon metabolism (photosynthesis and respiration) in plant cells,[50] which
469 represent transitory or stored forms of fixed carbon.[51] They play an important role in the
470 maintenance of redox balance, production and consumption of ATP, support of protonic
471 and ionic gradients on membranes, and acidification of extracellular spaces.[51] Therefore,
472 the mechanism of organic acids profile changes under CeO_2 NPs exposure are a result of
473 reprogramming of multiple metabolic pathways.

474 We also observed that several lignins such as 1,2,4-benzenetriol, 3-hydroxybenzoic
475 acid, 4-hydroxybenzoic acid, nicotinic acid (**Figure S12**) decreased in a dose-dependent
476 manner with CeO_2 NPs. Lignin is an integral component of cell walls. The decreased
477 lignin may indicate the cell wall composition change. In addition, some metabolites
478 participate in nucleus process, such as thymine, uracil, ribitol and ribose (**Figure S13**)
479 were also decreased in presence of high dose of CeO_2 NPs. Furthermore, cytidine-
480 monophosphate (CMP), a nucleotide that is used as a monomer in RNA, was decreased in
481 a dose-dependent way with CeO_2 NPs.

482 Few metabolites were up-regulated in response to CeO_2 NPs. Among them, some
483 metabolites increase in a dose-dependent manner with CeO_2 NPs, such as gentiobiose,
484 maltotriose, ribulose-5-phosphate, allo-inositol, threitol, oxalic acid, and saccharic acid
485 (**Figure S14**). While indolelactate and phenylalanine only respond to high dose of CeO_2
486 NPs. Some of these metabolites might act as signaling and defense molecules such as
487 sugars, allo-inositol, phenylalanine and oxalic acid.[52]

Perturbed Biological Pathway in Spinach Leaf and Root. Results from biological pathway analysis show that low dose of CeO_2 NPs induced perturbation of four biological pathways, including glycine, serine and threonine metabolism, tyrosine metabolism, amino sugar and nucleotide sugar metabolism, and glycolysis or gluconeogenesis. In contrast, high dose of CeO_2 NPs only induced one biological pathway (Amino sugar and nucleotide sugar metabolism) disturbation (**Table S6**).

In root, low dose of CeO_2 NPs (0.3 mg/plant) perturbed two biological pathways, including isoquinoline alkaloid biosynthesis and tyrosine metabolism (**Table S7**). Isoquinoline alkaloid biosynthesis pathway generates alkaloids, which are indispensable compounds for plant defense against pathogenic organisms and herbivores. Notable, L-dopa changed in both pathways which increased 4.5-fold upon exposure to low dose of CeO_2 NPs. L-dopa (L-3,4-dihydroxyphenylalanine), as a non-protein amino acid, is a nitrogen-containing compound with strong allelopathic activity. Meanwhile, L-dopa is a precursor of many alkaloids, catecholamines, and melanin.[53] The activation of the two defense and signaling related pathways indicate low dose of CeO_2 NPs stimulate the spinach defense system. This may contribute to the conserved amino acids levels in spinach root exposed to low dose of CeO_2 NP.

Differently, high dose of CeO_2 NPs (3 mg/plant) induced eight biological pathways perturbations in spinach root, including alanine, aspartate and glutamate metabolism; glycine, serine and threonine metabolism, sulfur metabolism; arginine and proline metabolism; lysine biosynthesis; cysteine and methionine metabolism; tryptophan metabolism; valine, leucine and isoleucine biosynthesis (**Table S7**). Almost all of the perturbed pathways are related with nitrogen metabolism, except sulfur metabolism. This

finding is consistent with the previous discussion that low dose of CeO_2 NPs induced active defense response, but did not down-regulate biological pathway, while high dose of CeO_2 NPs perturbed nitrogen metabolism in spinach root.

Although the exposure is through foliar application, significant metabolic changes were observed in root tissues which are not directly exposed to NPs. The results also indicate that quantifying low molecular weight metabolites changes is a sensitive endpoint to investigate the response of plant to NPs, compared with phenotypic parameters, like biomass and chlorophyll content.

Environmetnal Implications. Nanotechnology is exhibiting promising perspectives in agriculture. In addition to act as nano-carrier to deliver genes and agrochemicals, nanoparticles with unique physiochemical properties can be directly used to enhance plant production and protection. Thus, elucidating the interaction mechanism between nanoparticles and plants is of great important for their safe and sustainable use in agriculture. Phenotypic-based endpoint cannot sensitively reflect the plant changes. In this study, metabolomics revealed invisible plant responses to CeO_2 NPs. Although no phenotypic parameters changed, low dose of CeO_2 NPs (0.3 mg/plant) induced pronounced metabolic reprogramming in spinach leaves. While high dose of CeO_2 NPs (3 mg/plant) induced nitrogen metabolism inhibition in root. In addition, foliar spray of both doses of CeO_2 NPs impacted the uptake of zinc and calcium. Results of this study remind that when benefit from NPs' unique properties, the hidden unexpected metabolic changes cannot be neglected. Meanwhile, more studies might be needed to determine the applicability of low dose of CeO_2 NPs in improving plant stress resistance.

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Additional Information

CeO_2 NPs synthesis method, assays for SOD and CAT mimic activities of CeO_2 NPs, membrane permeability, lipid peroxidation; soil element compositions (Table S1); the sequences of the primers (Table S2); ROS scavenging capacity of CeO_2 NPs (Table S3); effective quantum yield and electron transport rate (Table S4); significantly changed metabolites in leaf (Table S5); perturbed biological pathways in leaf (Table S6) and root (Table S7); TEM image and size distribution of CeO_2 NPs (Figure S1); cerium concentration in spinach tissues (Figure S2); ANOVA revealed significantly changed metabolites in leaf (Figure S3); multivariate analysis results of leaf (Figure S4) and root (Figure S6); gene expression of antioxidant enzyme (Figure S5); significantly changed amino acids (Figure S7), N-containing compounds (Figure S8), fatty acids (Figure S9), sugars (Figure S10), organic acids (Figure S11), lignins (Figure S12), nucleus related metabolites (Figure S13) and other metabolites (Figure S14); are available as Supporting Information.

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721 **Table 1.** Macro- and micro- nutrients content in spinach leaves (mg/kg)

	K	Na	Mg	Ca	Mn	Fe	Cu	Zn
				Leaf				
Control	6643 ± 1136 a	5181 ± 622 a	17864 ± 1183 a	12453 ± 1898 a	147 ± 7.6 a	114.9 ± 7.2 a	22.7 ± 2.4 a	**303.4 ± 44.1 a**
0.3 mg CeO_2 NP	6849 ± 2597 a	5371 ± 2150 a	16672 ± 1567 a	10548 ± 3237 a	151 ± 29.6 a	105.4 ± 20.8 a	26.9 ± 6.3 a	**139.6 ± 17.7 b**
3 mg CeO_2 NP	6202 ± 1177 a	4966 ± 1519 a	17408 ± 4480 a	11215 ± 1257 a	152 ± 53.1 a	167.2 ± 48.1 a	32.9 ± 6.2 a	**170.7 ± 30.9 b**
				Stem				
Control	12107 ± 1573 a	5295 ± 1368 a	4946 ± 471 a	3383 ± 427 a	53 ± 4.5 a	25.1 ± 5.5 a	15.3 ± 1.2 a	134.6 ± 42.4 a
0.3 mg CeO_2 NP	11382 ± 2177 a	4643 ± 1453 a	4349 ± 719 a	3269 ± 671 a	49.3 ± 13.1 a	21.1 ± 3.5 a	19.2 ± 4.5 a	185.3 ± 86.1 a
3 mg CeO_2 NP	13186 ± 1338 a	4972 ± 548 a	3985 ± 261 a	3206 ± 219 a	43.6 ± 6.9 a	22.9 ± 9.4 a	28.4 ± 13.3 a	174.3 ± 40.3 a
				Root				
Control	32588 ± 6112 a	11512 ± 2923 a	4282 ± 709 a	**10320 ± 1800 a**	253 ± 164 a	1451 ± 1144 a	45.3 ± 12.0 a	104.5 ± 8.3 a
0.3 mg CeO_2 NP	30561 ± 4833 a	8605 ± 500 a	4451 ± 576 a	**6447 ± 328 b**	329 ± 206 a	2020 ± 1447 a	56.8 ± 17.8 a	145.5 ± 19.8 a
3 mg CeO_2 NP	29841 ± 6833 a	10338 ± 2593 a	4547 ± 159 a	**7056 ± 1270 b**	373 ± 201 a	2489 ± 1337 a	58.5 ± 5.4 a	116.4 ± 21.5 a

722 The data are means of five replicates±standard deviation. Same letters within column indicate no significant difference and different
723 letters stand for significant differences at $p \leq 0.05$.

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Figure legend

Figure 1. Spinach plant images (**A**) and photosynthetic pigment content in spinach leaf (**B**). The 4-week-old spinach plants were foliar exposed to different doses of CeO_2 NPs (0, 0.3 and 3 mg/plant) for 3 weeks. Data are means of five replicates. Error bars represent standard deviation. Same letters stand for no statistical differences ($p \leq 0.05$).

Figure 2. Relative membrane permeability (**A**), lipid peroxidation (**B**), total antioxidants content (**C**) and biomass (**D**) of spinach leaves exposed to different doses of CeO_2 NPs (0, 0.3 and 3 mg/plant) for 3 weeks. Data are means of five replicates. Error bars represent standard deviation. Different letters stand for statistical differences at $p \leq 0.05$.

Figure 3. Heat map of significantly changed metabolites (A, amino acids; B, sugars; C, organic acids; D, phenolics) in spinach leaves exposure to 0.3 mg/plant CeO_2 NPs. The 4-week-old spinach plants were foliar exposed to different concentrations of CeO_2 NPs (0, 0.3 and 3 mg/plant) for 3 weeks.

Figure 4. Schematic diagram of TCA cycle and amino acid biosynthesis pathways of spinach root exposed to 0, 0.3 and 3 mg per plant CeO_2 NPs. Box-whisker plots shows the relative abundance of significantly changed amino acids in spinach plants exposed to 0.3 and 3 mg/plant CeO_2 NPs. A, B and C in box plot represent different doses of CeO_2 NPs (0, 0.3 and 3 mg/plant).

Figure 5. Veen diagram of perturbed biological pathways in leaf and root of spinach exposed to 0, 0.3 and 3 mg per plant CeO_2 NPs.

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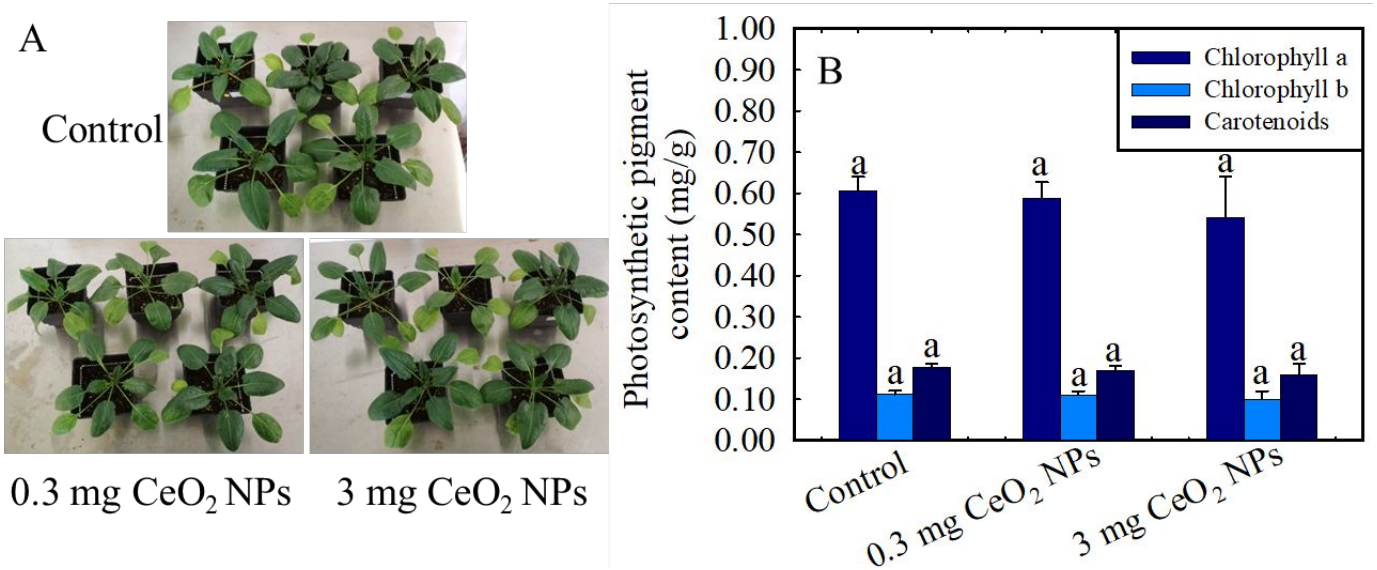


753

754

755 **Figure 1.**

756

757

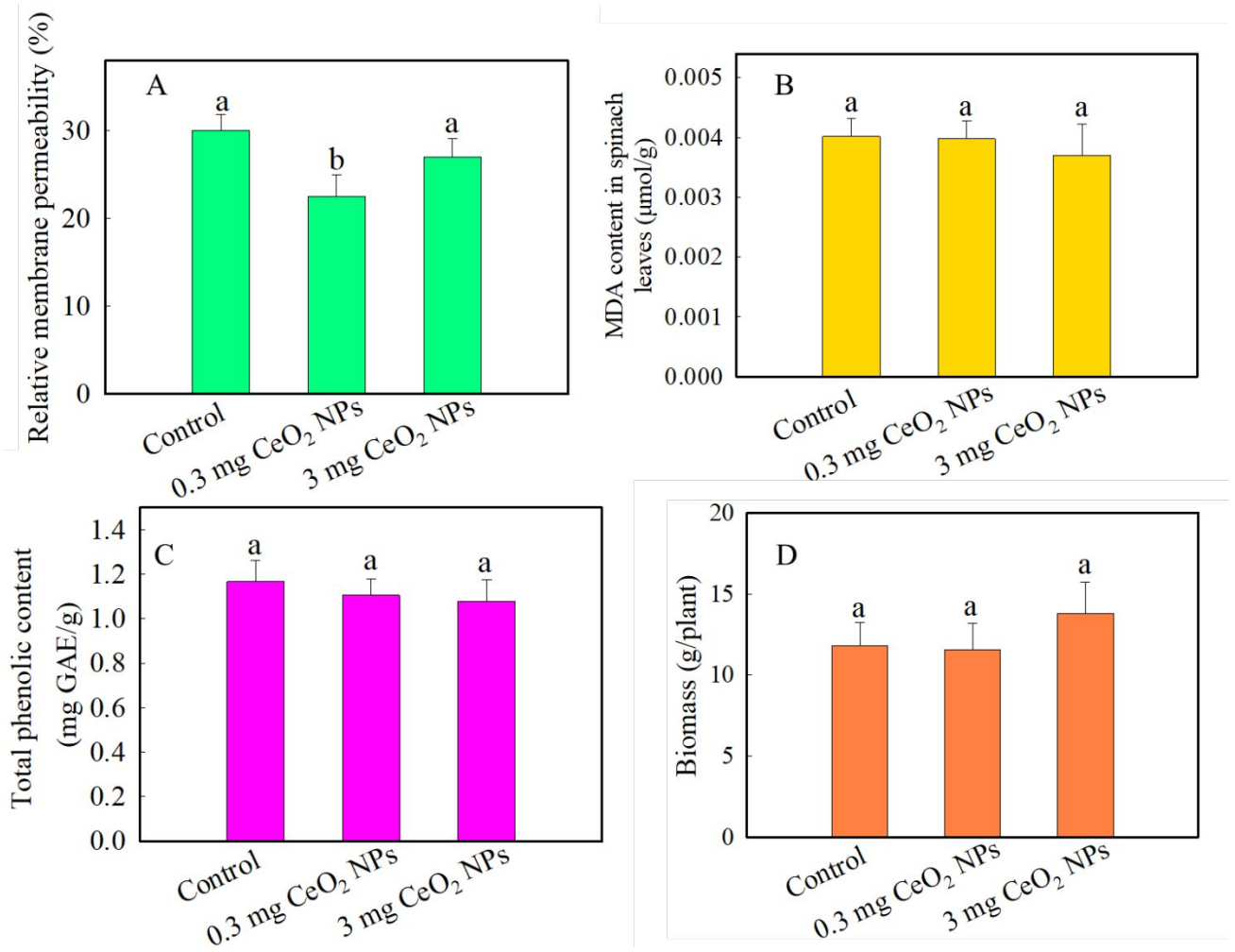
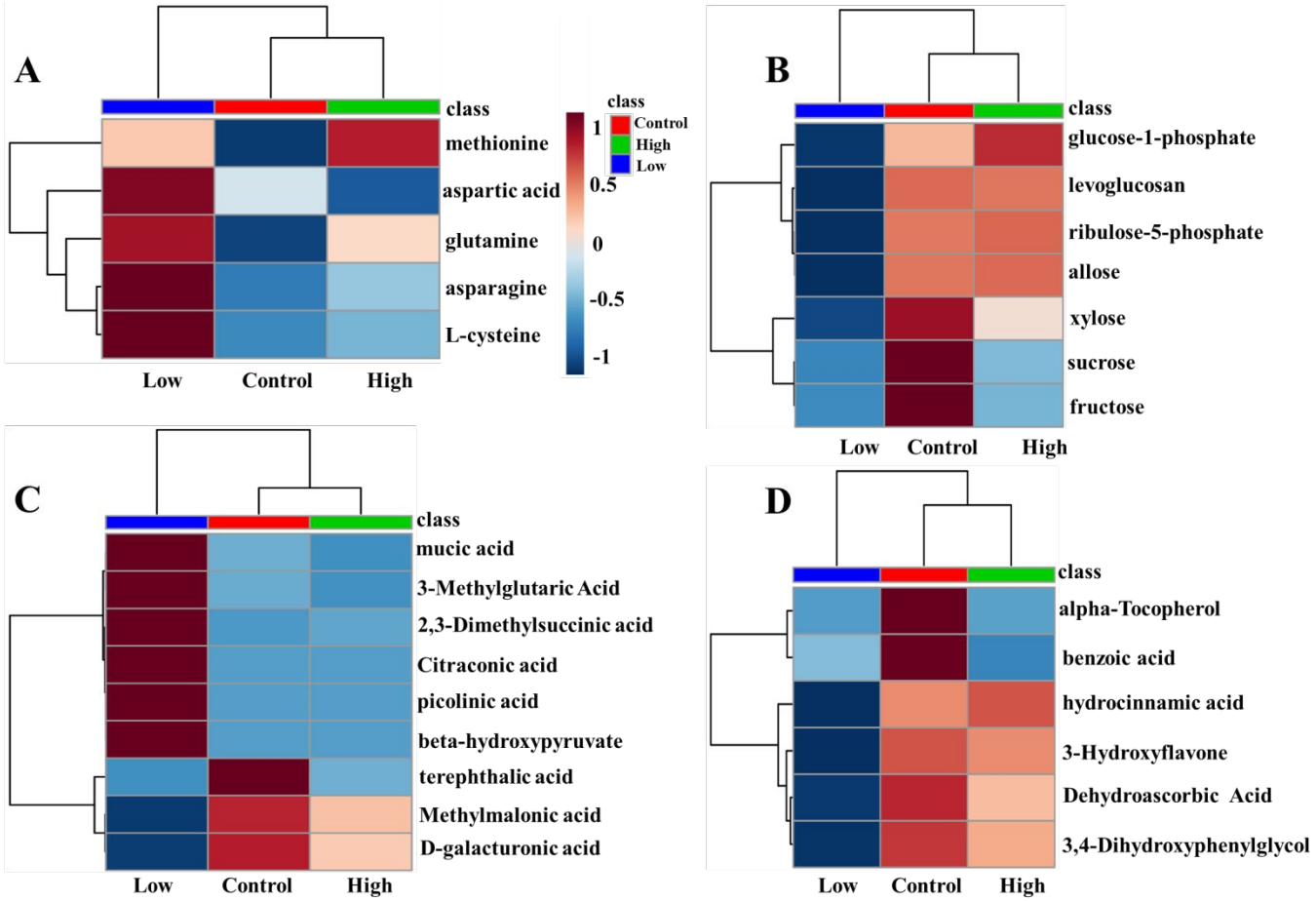

A
Relative membrane permeability (%)
0
10
20
30
a
b
a
Control
0.3 mg CeO2 NPs
3 mg CeO2 NPs
B
MDA content in spinach leaves (μmol/g)
0.000
0.001
0.002
0.003
0.004
0.005
a
a
a
Control
0.3 mg CeO2 NPs
3 mg CeO2 NPs
C
Total phenolic content (mg GAE/g)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
a
a
a
Control
0.3 mg CeO2 NPs
3 mg CeO2 NPs
D
Biomass (g/plant)
0
5
10
15
20
a
a
a
Control
0.3 mg CeO2 NPs
3 mg CeO2 NPs

Figure 2.

762



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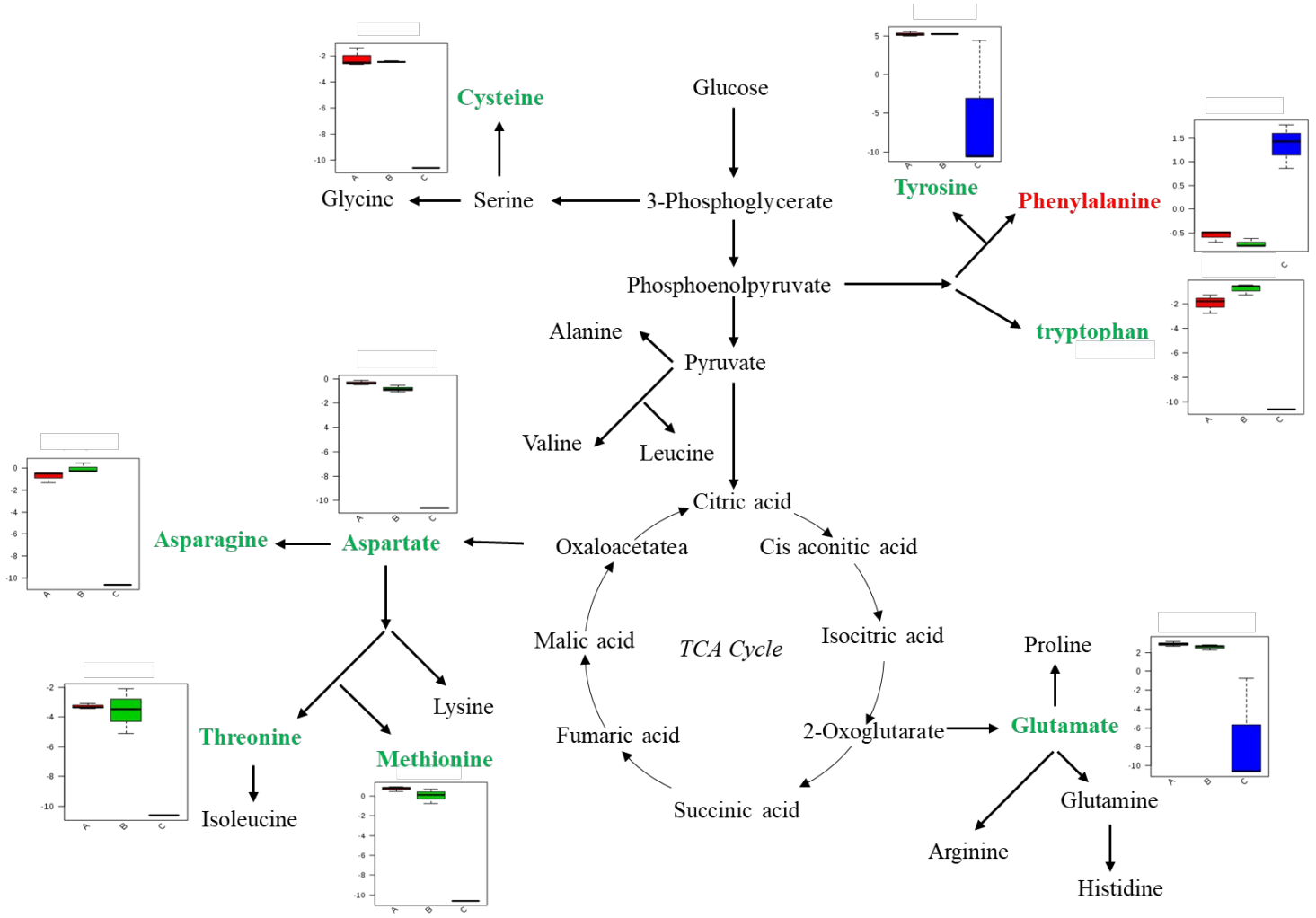
764

765 **Figure 3.**

766



767

768 **Figure 4.**