Electronic Supplementary Material (ESI) for Environmental Science: Nano.

Defense Mechanisms and Nutrient Displacement in *Arabidopsis thaliana* upon Exposure to CeO_2 and In_2O_3 Nanoparticles

Chuanxin Ma[a], Hong Liu[ad], Huiyuan Guo[a], Craig Musante[b], Sanem Hosbas Coskun[c], Bryant C. Nelson[c], Jason C. White[b], Baoshan Xing[a*], and Om Parkash Dhankher[a*]

[a]Stockbridge School of Agriculture, University of Massachusetts Amherst, MA 01003, United States

[b]Department of Analytical Chemistry, The Connecticut Agricultural Experiment Station, New Haven, CT 06504, United States

[c]Material Measurement Laboratory, National Institute of Standards and Technology, 100 Bureau Drive, Gaithersburg, Maryland 20899, United States

[d]College of Resources and Environment, Fujian Agriculture and Forestry University, Fuzhou, 350002, China

Corresponding authors:

* Om Parkash Dhankher: parkash@umass.edu; Phone: 413-545-0062.

* Baoshan Xing: bx@umass.edu; Phone: 413-545-5212; Fax: 413-577-0242.

Numbers of Pages: 14

Numbers of Figures: 3

Numbers of Tables: 3

S1. Assay for total protein

Modified Bradford method was used to quantify total protein content in *A. thaliana* seedlings. Briefly, a 0.5 g sample of homogenized tissue was mixed with 5 mL of 10 mM Tris-HCl (pH 7.2) vigorously for 5 min using a vortex mixer.100 μL of supernatant and 1900 μL of Bradford reagent were mixed in Eppendorf tube thoroughly. The mixture was incubated at ambient temperature for 15 min. The absorbance of protein-dye complex was measured at 595 nm. Bovine serum albumin (BSA) was used to set up standard curve of protein.[1]

S2. Assays for activities of antioxidant enzymes

Catalase (CAT) was extracted in 25 mM KH_2PO_4 with pH 7.4. Decreased absorbance in the reaction that contained 100 μL of supernatant and 1900 μL of reaction buffer (10 mM H_2O_2) was recorded at 240 nm for 3 min. The H_2O_2 extinction coefficient was 23.148 mM^{-1} cm^{-1}.[2]

Ascorbate peroxidase (APX) was extracted in 100 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 0.1 mM ascorbate and 2% β-mercaptoethanol. The reaction buffer was made of 50 mM phosphate buffer (pH 7.0) and 0.6 mM ascorbic acid. The total 2 mL reaction system contained 100 μL of enzyme extract and 1900 μL of reaction buffer. Decreased absorbance was monitored at 290 nm for 3 min after initiating the reaction with 10% (v/v) H_2O_2.[3]

Superoxide dismutase (SOD) was extracted in 50 mM phosphate (pH 7.8) containing 0.1% (w/v) ascorbate, 0.1 % (w/v) bovine serum albumin (BSA), and 0.05% (w/v) β-mercaptoethanol. Nitroblue tetrazolium (NBT) was used to indirectly determine SOD activities. Briefly, 100 µL of enzyme extract and 1900 µL of 50 mM phosphate buffer (pH 7.8) containing 9.9 mM L-methionine, 57 µM NBT, 0.0044% (w/v) riboflavin and 0.025% (w/v) Triton X-100 were mixed in cuvette and placed under fluorescent tube light (light intensity: 250 µmol $m^{-2}s^{-1}$) for 20 min. Reduction in the absorbance of NBT was recorded at 560 nm.[3]

Peroxidase (POD) was extracted in 50 mM phosphate (pH 7.0) containing 1% (w/v) polyvinylpyrrolidone. Briefly, 50 µL of enzyme extract was mixed with reaction buffer containing 1.75 mL of 50 mM sodium phosphate buffer (pH 7.0) and 0.1 mL of 4% guaiacol in cuvette and 0.1 mL of 1% (v/v) H_2O_2 was used to initiate the reaction. Increased absorbance was recorded at 470 nm for 2 min.[4]

Polyphenol oxidase (PPO) was extracted in the same buffer as stated in POD extraction. The reaction mixture consisted of 200 µL of enzyme extract and 2.8 mL of 10 mM catechol. PPO activity was recorded by measuring its ability of oxidizing catechol at 410 nm.[4, 5]

Phenylalanin ammonialyase (PAL) was extracted in 0.1 M sodium borate buffer (pH 8.8). 100 µL of enzyme extract was used to react with 2.9 mL of reaction buffer containing

100 mM sodium borate buffer (pH 8.8) and 50 mM 1-phenylalanine at 37 °C for 1 hr. The absorbance change was monitored at 298 nm.[4, 5]

Glutathione S-transferase (GST) was extracted in 50 mM phosphate buffer (pH 7.5) containing 1 mM Ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT). 1-Chloro-2,4-dinitrobenzene (CDNB) was used to conjugate with thiol group of glutathione (GSH) and form GS-DNB conjugate. The increase of absorbance recorded at 340 nm for 5 min represents GST activity.[3]

Glutathione reductase (GR) was extracted in 100 mM phosphate buffer (pH 7.5) containing 0.5 mM EDTA. A sample of 100 μL of enzyme extract was added into a reaction buffer containing 500 μL of 2 mM oxidized GSH (GSSG), 50 μL of β-Nicotinamide adenine dinucleotide phosphate, reduced (NADPH) and 350 μL of assay buffer (100 mM potassium phosphate buffer with 1 mM EDTA). The decrease in absorbance was recorded at 340 nm for 2 min.[6]

S3. Histochemical staining for hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet-}$) production

For the H_2O_2 staining assay, *A. thaliana* seedlings were soaked in 1mg/mL DAB solution at pH 3.8. The plant tissues were vacuum-infiltrated at 100 to 150 mbar for 1 min, and this step was repeated 3 times. The plant tissues were then incubated for 5 h in a high humidity environment until dark colored precipitates were observed. Before observing the levels of H_2O_2 in the plant tissues under light microscopy, chlorophyll was removed

by 95% ethanol washes. Relative color intensity was calculated in histogram function in Adobe Photoshop CS version 8.0.

For the $O_2^{\bullet -}$ staining assay, the plant tissues were immersed into a staining mixture containing 0.1% (w/v) NBT, 10 mM sodium azide, and 50 mM potassium phosphate and then vacuum-infiltrated at 100 to150 mbar for 1 min, which was again repeated 3 times. The plant tissues were incubated in the mixture for 15 min, and then the infiltrated seedlings were exposed to cool fluorescent light for 20 min at room temperature. The samples were treated with 95% ethanol to stop the reaction and to remove the chlorophyll.[7] Blue staining was then observed in each *A. thaliana* seedling by light microscopy.

S4. Analysis of the total N in *A. thaliana* tissues

Dry shoot and root tissues were ground to a fine powder for determination of total Kjeldahl nitrogen.[8] Briefly, a 50 mg plant tissue and a mixture of 1.5 g potassium sulfate and 0.125g cupric sulfate were mixed in a Kjeldahl flask. A volume of 3.5 mL sulfuric acid was added into each sample for digestion at 160 °C until a clear solution was observed. The sample was further digested at 390°C for 40 min. All digests were cooled in a hood prior to the addition of 46.5 mL DI H_2O into each flask (50 mL final volume). The total levels of N were measured using the QC8500 analyzer (LACHAT Instruments, WI, USA).

S5. Nanoparticle characterization

Table S1 shows the hydrodynamic diameter and zeta potential of CeO_2 and In_2O_3 NPs dispersed in deionized water and 1/2X Hoagland's solution. Generally, the hydrodynamic diameters of CeO_2 and In_2O_3 NPs in the deionized water were significantly smaller than in 1/2X Hoagland's solution, suggesting that ion strength is one of the main factors that can determine the NP aggregation in solution. However, two different concentrations of both NPs had no impact on either hydrodynamic diameter or zeta potential regardless of solution types. Interestingly, as compared to the zeta potential values from the deionized water treatment, 1/2X Hoagland's solution altered the surface charges of both NPs from positive to negative.

S6. Distribution of cerium and indium in *A. thaliana* tissues

With CeO_2 NPs treatments, the Ce content in 1000 mg/L CeO_2 NP-treated root was increased by approximately 4.3 times relative to the Ce content in the 250 mg/L CeO_2 NP-treated root (Figure S2A). Similarly, there was a dose-response increase of Ce content in the shoot. However, regardless of exposure doses, no difference of In content in root was evident while there was a slight increase in shoot In levels. (Figure S2B). Dose-response fashion was shown in soybean seedlings exposed to 0-4000 mg/L CeO_2 NPs suspension.[9] Potting experiments conducted by Zhao *et al.* (2013) suggested that the concentrations of Ce in cucumber upon exposure to 800 mg/kg CeO_2 NPs decreased from root to fruits; approximately 200-fold higher Ce concentration in CeO_2 NPs treated fruits suggested that food safety could be of major concerns.[10] Another study demonstrated that Ce mainly accumulated in corn root and was barely transported to the aboveground part

(leaf and corn cob).[11] These results suggest that the uptake and tissue distribution of NPs depends on the type of NPs and plant species.

S7. Total protein concentration in *A. thaliana* seedling

Total protein concentration in *A. thaliana* seedlings was measured (Figure S3). The presence of CeO_2 and In_2O_3 NPs significantly altered the total protein concentration in *A. thaliana* as compared with the control. The CeO_2 NP exposure resulted in approximately 30% reduction in the total protein concentration and a 12.5% reduction in total protein concentration was noted upon In_2O_3 NP exposure. CeO_2 NP exposures had more impact on the total protein decreases relative to In_2O_3 NP exposures. However, no difference between two exposure doses was observed. A previous study demonstrated that Ag NPs significantly reduced the total protein content by approximately 50% in leaves of *Bacopa monnieri*.[12] Another study demonstrated that 100 mg/kg Ag NPs did not alter the protein content in lettuce leaves via foliar exposure.[13] These results suggest that metal-based NPs could interact with the protein associated within the photosynthesis system, carbohydrate transport, and defense mechanism, and subsequently result in oxidative stresses and decreases of photosynthesis rate/ chlorophyll content and plant biomass.[12] Our results showed that CeO_2 and In_2O_3 NPs can alter the total protein contents in plants and thus may have play a role in altering the nutritional quality of the food crops.

References:

1. N. J. Kruger, *The protein protocols handbook*, 2009, 17-24.

2. L. Zhao, B. Peng, J. A. Hernandez-Viezcas, C. Rico, Y. Sun, J. R. Peralta-Videa, X. Tang, G. Niu, L. Jin and A. Varela-Ramirez, *ACS nano*, 2012, **6**, 9615-9622.
3. V. Dixit, V. Pandey and R. Shyam, *Journal of Experimental Botany*, 2001, **52**, 1101-1109.
4. G. Jing, H. Huang, B. Yang, J. Li, X. Zheng and Y. Jiang, *Chemistry Central Journal*, 2013, **7**, 19.
5. M. Su, K. Chen, Z. Ye, B. Zhang, J. Guo, C. Xu, C. Sun, J. Zhang, X. Li and A. Wu, *Journal of the Science of Food and Agriculture*, 2012, **92**, 1448-1454.
6. M. Schaedle and J. A. Bassham, *Plant Physiology*, 1977, **59**, 1011-1012.
7. N. Jambunathan, *Plant stress tolerance: methods and protocols*, 2010, 291-297.
8. D. Diamond, in *Lachat Instruments Inc.*, Lachat Instruments Inc., 1992.
9. M. L. López-Moreno, G. de la Rosa, J. Á. Hernández-Viezcas, H. Castillo-Michel, C. E. Botez, J. R. Peralta-Videa and J. L. Gardea-Torresdey, *Environmental Science & Technology*, 2010, **44**, 7315-7320.
10. L. Zhao, Y. Sun, J. A. Hernandez-Viezcas, A. D. Servin, J. Hong, G. Niu, J. R. Peralta-Videa, M. Duarte-Gardea and J. L. Gardea-Torresdey, *Journal of agricultural and food chemistry*, 2013, **61**, 11945-11951.
11. L. Zhao, Y. Sun, J. A. Hernandez-Viezcas, J. Hong, S. Majumdar, G. Niu, M. Duarte-Gardea, J. R. Peralta-Videa and J. L. Gardea-Torresdey, *Environmental Science & Technology*, 2015, **49**, 2921-2928.
12. C. Krishnaraj, E. Jagan, R. Ramachandran, S. Abirami, N. Mohan and P. Kalaichelvan, *Process Biochemistry*, 2012, **47**, 651-658.
13. C. Larue, H. Castillo-Michel, S. Sobanska, L. Cécillon, S. Bureau, V. Barthès, L. Ouerdane, M. Carrière and G. Sarret, *Journal of hazardous materials*, 2014, **264**, 98-106.

Table S1. Characterization of CeO_2 and In_2O_3 NPs in DI water and 1/2X Hoagland's solution

Treatment	Solution	DLS (nm)	Zeta potential (mV)
250 mg/L	DI water	229±56.7	28.59±3.34
In_2O_3 NPs	1/2X Hoagland's solution	1795.9±57.5	-8.68±1.70
1000 mg/L	DI water	221.4±6.5	32.28±1.95
In_2O_3 NPs	1/2X Hoagland's solution	1779.5±73.4	-10.38±1.52
250 mg/L	DI water	249.4±2.5	43.09±2.11
CeO_2 NPs	1/2X Hoagland's solution	3352.8±691.5	-4.24±1.02
1000 mg/L	DI water	209.1±1.0	43.58±2.39
CeO_2 NPs	1/2X Hoagland's solution	3532.6±1075.9	-6.12±0.81

Table S2. A list of primer sequence used in qRT-PCR

Gene name	Primer sequence (5'-3')
Actin-F	CGTGACCTTACTGATTAC
Actin-R	TTCTCCTTGATGTCTCTT
FRO-F	GCTTCCGCCGATTTCTTAAGGC
FRO-R	AACGGAGTTATCCCGCTTCCTC
IRT-F	ACTTCAAACTGCGCCGGAAGAATG
IRT-R	AGCTTTGTTGACGCACGGTTC
FER-F	CAACGTTGCTATGAAGGGACTAGC
FER-R	ACTCTTCCTCCTCTTTGGTTCTGG

qRT-PCR amplification program:

For genes encoding iron transporters: 95 °C for 15 min; 95 °C for 15s, 59 °C for 30s, 72 °C for 10 s, repeating 40 cycles; 72 °C for 10 min; 95 °C for 15 s, 59 °C for 15 s, melting curve for 20 min; 95 °C for 15 s.

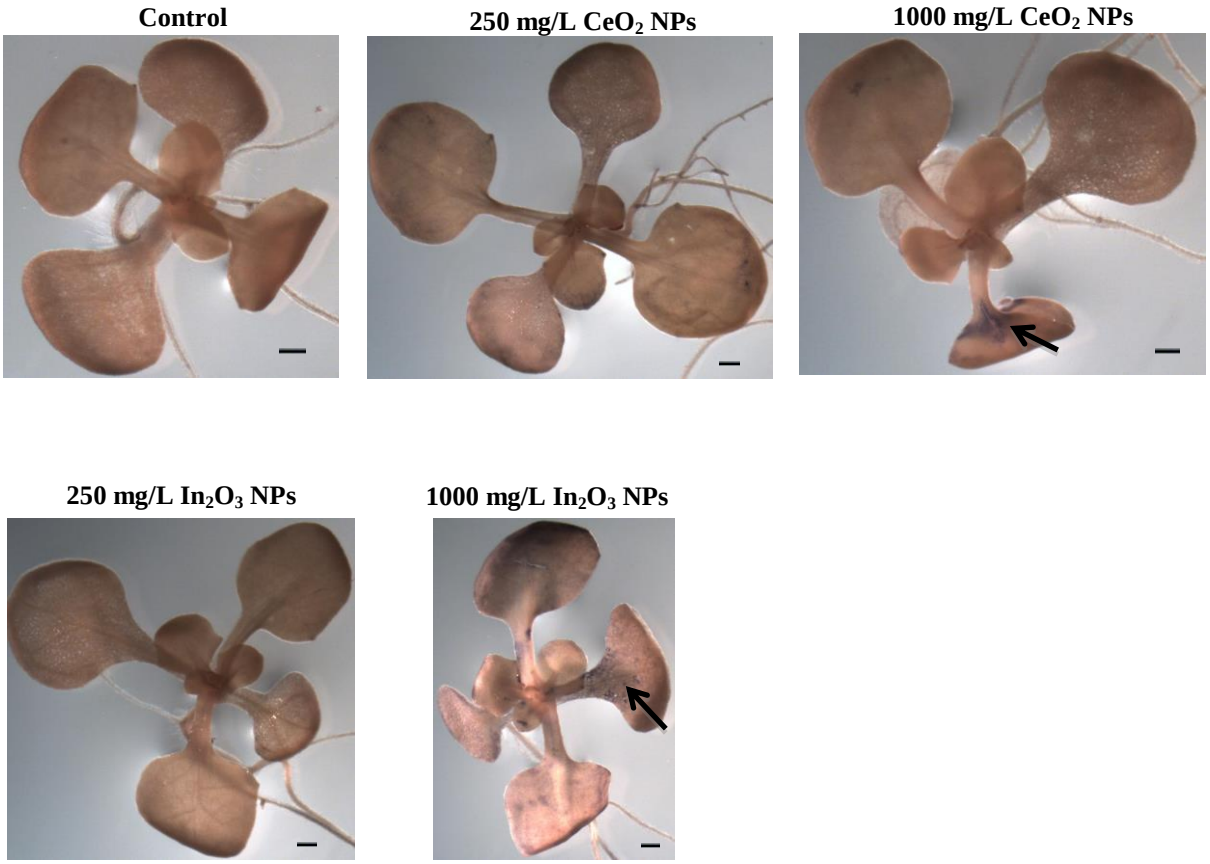

Figure S1. Images of histochemical staining of superoxide anions in CeO_2 and In_2O_3 NP treated *A. thaliana* for 7 d. The scale bar in each image represents 1 mm. The black arrow points out the blue stain in *A. thaliana* seedlings treated with 1000 mg/L CeO_2 and In_2O_3 NPs

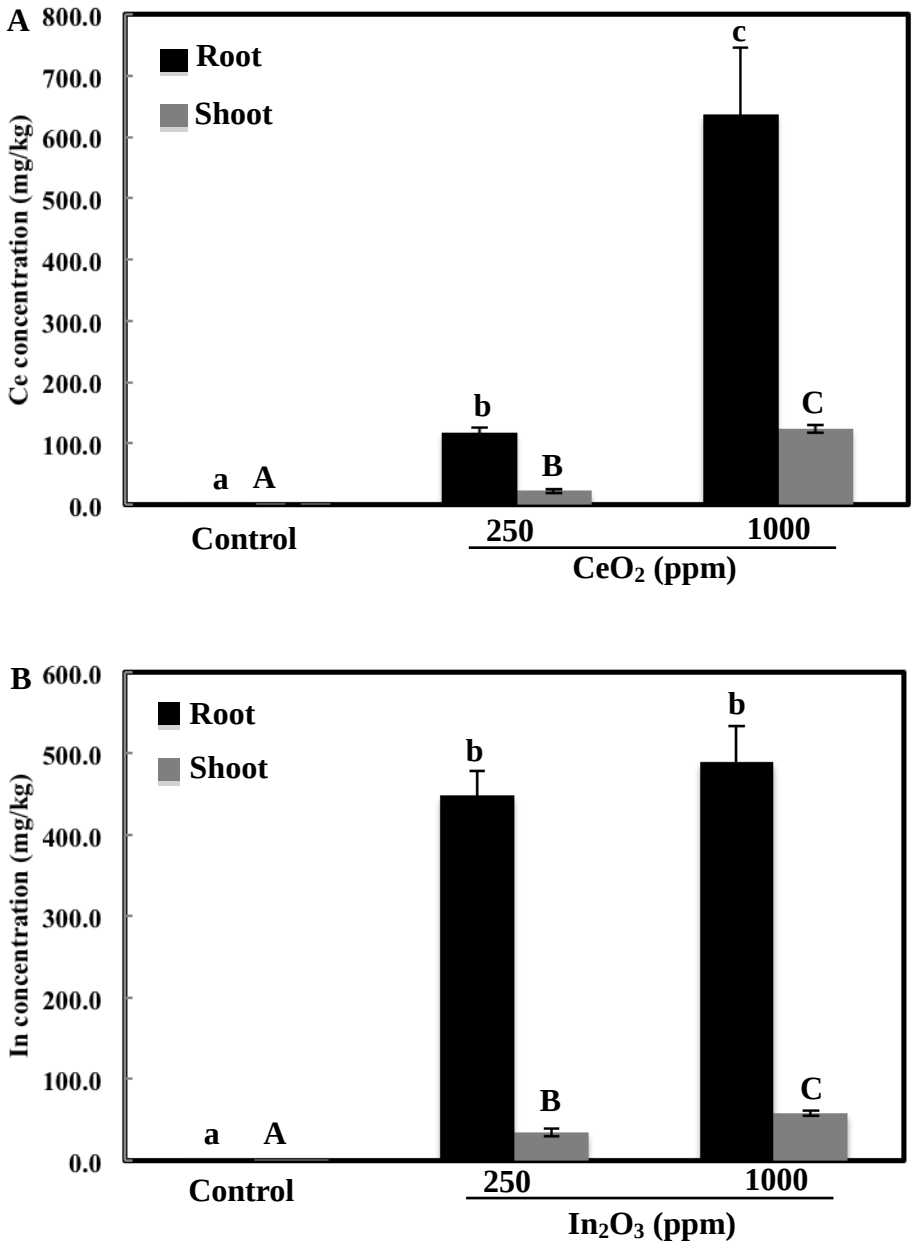

Figure S2. Ce and In content (ICP-MS) in shoots and roots of Arabidopsis. Arabidopsis was exposed to CeO_2 and In_2O_3 NPs in hydroponic system for 5 d. (**A**) Ce content in shoot and root of Arabidopsis treated with 250 and 1000 mg/L CeO_2 NPs; (**B**) In content in shoot and root of Arabidopsis treated with 250 and 1000 mg/L In_2O_3 NPs. Data are mean ± standard error of 4 or 5 replicates. Values of metal uptake followed by double asterisks indicate statistically significant differences at $p \leq 0.01$ compared to control group.

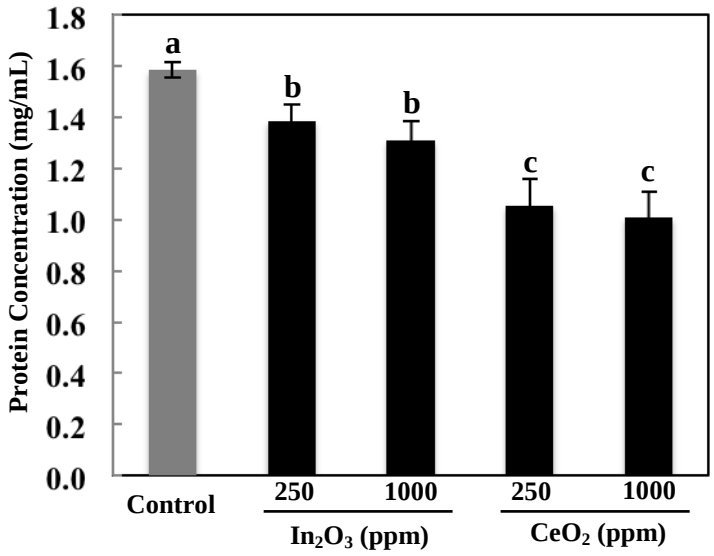

Figure S3. Total protein content in *A. thaliana* seedlings treated with different concentrations of In_2O_3 and CeO_2 NPs. Data are mean± standard error of 3 replicates. Values of total protein content followed by different letters indicate that the data points are significantly different at $p \leq 0.05$.

Table S3. The levels of other elements in shoot and root of *A. thaliana* exposed to CeO_2 and In_2O_3 NPs for 5 d

Plant tissue	Nutrient	Control	CeO_2 NPs (mg/kg)		In_2O_3 (mg/kg)	
			250	1000	250	1000
Shoot (mg/kg)	N	61260±2315.4 A	56818.0±6149.1 A	59034.5±1182.3 A	59810.4±3134.7 A	59884.1±3245.3 A
	S	7710.0±437.9 A	6966.6±74.1 A	6849.4±313.8 A	7509.1±585.2 A	7201.2±166.5 A
	Na	998.2±86.5 A	1089.2±48.6 A	1104.4±92.0 A	1011.0±41.6 A	1112.4±99.1 A
Root (mg/kg)	N	36815.8±876.8 ab	31321.7±5109.4 ab	35390.1±2739.2 ab	29914.1±2781.4 a	42847.2±4243.1 b
	S	5160.1±501.4 a	6087.3±104.0 a	6069.4±433.8 a	6240.6±367.2 a	6144.8±254.4 a
	Na	1015.9±80.8 a	1460.5±129.9 bc	1523.5±58.3 c	1426.5±132.0 bc	1168.1±52.3 ab

Note: Data are mean ± standard error of 4 or 5 replicates. Values of each element content followed by different letters indicate that the data points are significantly different at $p \leq 0.05$