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Effects Of Cerium Oxide Nanoparticles In Cereals: Insights Into The Toxicity Mechanisms And Macromolecular Modifications

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Dedication

This work is dedicated to my parents whose love molded me to be the person that I am.
And whose guidance and training inculcated in me the love of learning.
May the cap on my head be a crown of honor on yours.
I honor you both.

I also dedicate this work to my wife who gave me strength to soar to greater heights.
None of this would have been possible without your love and support.
You're still the best thing that ever happened to me.
I love you.

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by

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DISSERTATION

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Abstract

Despite the inundation of studies on the interaction of engineered nanomaterials (ENMs) with plants, investigations involving complete life cycle (i.e from seedling establishment to full maturity) are still lacking. Assessments on the nutritional value of plants cultivated to full maturity in ENMs-treated soil are also missing. Cerium oxide nanoparticles (*n*CeO_2) have significant interactions with plants; however, there are no life cycle studies yet on their implications in cereals like rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), and barley (*Hordeum vulgare* L.). These cereals are globally important crops that support the economic activity, and nutritional and health needs of billions of people around the world. This research project was performed to determine the changes in the macromolecular and biochemical processes and their impacts on the physiological, yield, and nutritional properties in cereals. Experiments were completed in four parts: Parts I and II were conducted at germination stage and Parts III and IV included life cycle evaluations. Part I was devoted to rice seeds only. Rice seeds were germinated in *n*CeO_2 suspensions (0, 62.5, 125, 250, 500 mg L^{-1}) and biochemical assays, ICP-OES, GC-MS, and synchrotron micro-XRF analyses were performed on roots to measure the relationships between parameters like Ce uptake, oxidative stress, radical scavenging activities, and macromolecular contents. Results showed that Ce uptake increased as the external *n*CeO_2 increased without visible signs of toxicity. The *n*CeO_2 treatment (500 mg L^{-1}) decreased the fatty acid contents which resulted in reduced membrane damage, and reduced the lignin content despite the parallel increase in H_2O_2 content and peroxidase activities. Synchrotron micro-XRF also revealed the presence of Ce in the vascular tissues of the roots. In Part II rice, wheat, and barley seeds were exposed to the same *n*CeO_2 suspensions and the structural integrity of intact root xylem was analyzed using FTIR spectromicroscopy and principal component analysis (PCA). The PCA of FTIR spectra showed that *n*CeO_2 induced modifications in the biomolecular compositions of root xylem. Part III involved cultivation of low, medium, and high amylose rice varieties (LA, MA, and HA, respectively) in *n*CeO_2-treated soil (0 and 500 mg kg^{-1}) and the grains were analyzed for nutrient content, antioxidant property, and nutritional quality. The *n*CeO_2 treatment increased the accumulation of Ce in the grains of LA and MA by 997 and 1126%, respectively, compared to controls. The *n*CeO_2 treatment did not affect the sugar content but

decreased the starch content in HA and LA by 9 and 8%, respectively, compared to control, with concomitant reduction in the phenolic content and DPPH scavenging ability. In case of the protein fractions, glutelin was not detected in the treated grains. Relative to the untreated, globulin and prolamin decreased significantly in both treated MA and LA grains while albumin decreased in the treated LA grains only. On the other hand, the treated MA grains had dramatic reduction in lauric, valeric, palmitic, oleic, and total fatty acids contents. Similarly, the treated MA grains had improved K, Na, Fe, and Al contents compared to the untreated. In contrast, treated MA grains had reduced S content while treated LA grains had low Fe content. Part IV of this dissertation research was accomplished by growing wheat and barley to grain production in soil amended with *n*CeO_2 (0, 125, 250 and 500 mg kg^{-1}) and the agronomic, yield, and nutritional properties were examined. The *n*CeO_2 treatment promoted the growth and shoot biomass in both plants, but induced negative effects on yield parameters with wheat showing a more apparent delay in grain formation than barley. Despite the initial poor yield performance, grain yield in both *n*CeO_2-exposed plants was greatly improved at harvest. However, the highest *n*CeO_2 treatment completely halted the grain production in barley. Ce accumulation in wheat grains was not recorded but was tremendously enhanced in barley; Ce content in *n*CeO_2-treated barley grains registered up to 294% increase relative to control. In case of mineral content, the *n*CeO_2 treatment modified only the storage S and Mn in wheat grains but enhanced the accumulations of all elements, except for Na and B, in barley grains. Similarly, *n*CeO_2 modified the amino acid and fatty acid contents in both wheat and barley grains. This study provided insights on several issues and knowledge gaps in the current literature. The result showed that *n*CeO_2 impacted the growth and productivity of cereals which could entail adaptations of new planting and cultivation practices. *n*CeO_2 could enhance Ce accumulation in grains and compromise their nutritional value which may have unknown implications on human health and nutrition. In general, the findings afforded a more comprehensive understanding on the impacts of *n*CeO_2 on globally important agricultural crops.

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Chapter 1: Introduction

Engineered nanomaterials (ENMs) are manmade materials with one dimension under 100 nm and possess unique mechanical, catalytic, and optical properties.[1,2] Based on production estimates compiled by Lazareva and Keller,[3] the global production (in metric tons year^{-1}) for metal-containing ENMs ranged from 22 to 1,500,000 and that for carbon-containing ones was somewhere between 500-9,600,000. While these market information may be overly conservative,[4,5] the global production of ENMs grows exponentially[6] as ENMs properties can be easily manipulated/exploited to meet the growing demands for endless number of possible applications in nanotechnology industries.[7,8] However, material life cycle studies[8] reveal that these ENMs will ultimately be deposited in the environment, raising serious concerns on their environmental implications.

Cerium oxide nanoparticles (*n*CeO_2) are among the most important metal-oxide nanoparticles with a conservative annual global production estimate of 10,000 metric tons.[3] The cerium atom in *n*CeO_2 can shift between +3 and +4 oxidation states depending on the availability of oxygen, giving *n*CeO_2 the ability to store oxygen and act as catalysts for oxidation-reduction reactions.[7] Due to this unique property, *n*CeO_2 have important industrial uses in the areas of chemical mechanical planarization and polishing, catalytic converters, fuel catalysis, UV-coatings, and others.[5] *n*CeO_2 are highly stable in a range of environmental media with very low solubility (ng L^{-1}) in different soil types.[9] Since *n*CeO_2 are utilized commercially as fuel additive, they can easily spread in the environment and interact with different biological receptors.[10] As such, the Organization for Economic Co-operation and Development listed *n*CeO_2 among the priority ENMs to be assessed for human health and environmental implications.[11]

Plants are peculiar biological receptors that can offer fascinating insights regarding the effects of ENMs. Specifically, edible plants are being studied because they serve as major conduits for ENMs possible impacts on food chain and human nutrition. The different exposure pathways of plants to ENMs have been discussed in several reviews[12-15] and are schematically depicted in Figure 1.1. As seen in the figure, plants can be exposed to ENMs through the application of biosolids to agricultural fields. For example, agricultural soils in the U.S. are mixed with more than 60% of biosolids each year.[16] Biosolids

are rich in ENMs; they can be contaminated by up to 10^4 ppm depending on the type of ENMs.[17,18] As such, the regular application of biosolids would likely result in the accumulation of ENMs in agricultural fields.

Plants can also get exposed to ENMs through the applications of nano-enabled agricultural products and soil remediation technologies. In fact, dozens of nano-enabled agricultural products including pesticides, fertilizers, plant protectives, soil additives, and growth regulators are ready for commercialization in developing countries.[19] It is predicted that the beneficial impacts of ENMs application in agriculture may exceed those of farm mechanization and the green revolution.[20] Likewise, ENMs are used for removal of organic and inorganic pollutants or heavy metal contaminants in soil.[21,22] Thus, it is possible that the direct and repeated application of nano-enabled inputs could result in ENMs accumulation in soil through time. Other known routes of plants exposure to ENMs include atmospheric deposition, spillage, discharge, surface runoff, and wastewater reuse for food production.

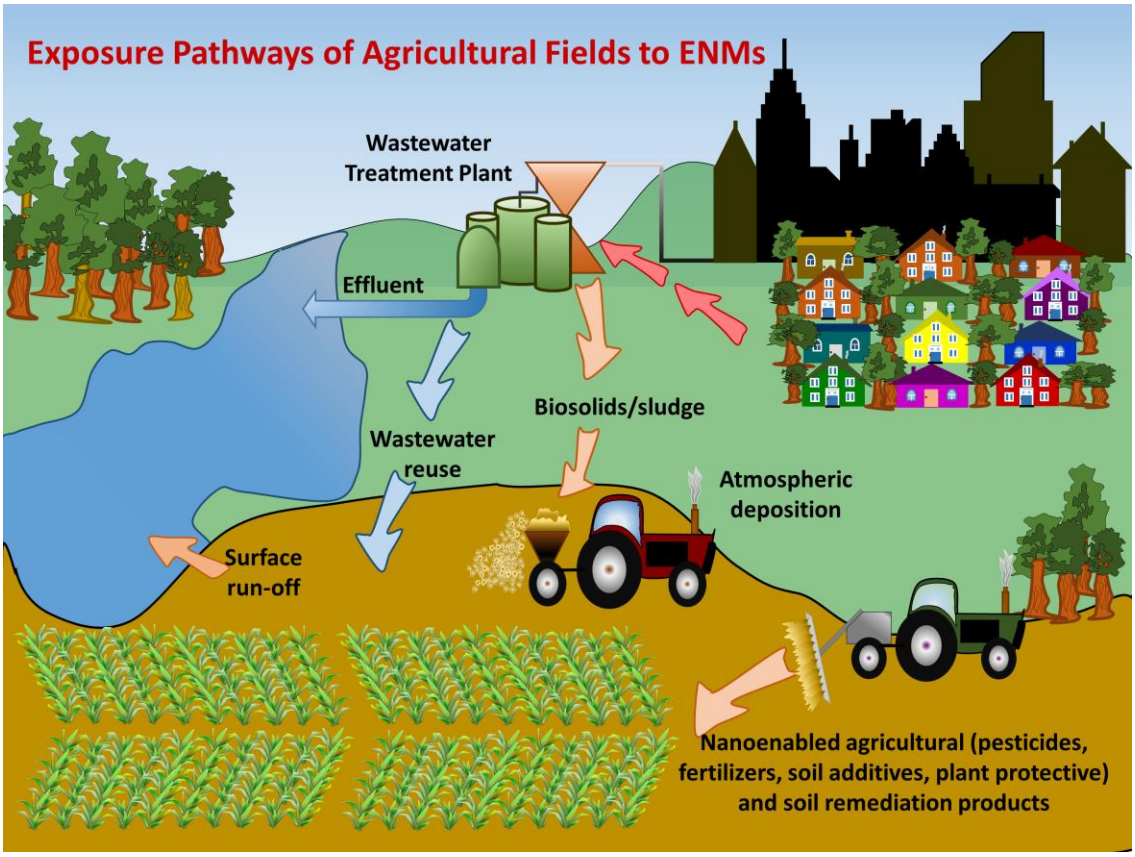

Figure 1.1: Exposure pathways of plants to engineered nanomaterials.

The interaction of ENMs with plants have been documented; however, majority of these studies were performed on short-term basis (i.e. germination phase or early seedling growth) with emphases on determining ENMs impacts on photosynthetic processes, oxidative stress, antioxidative enzyme activity, radical scavenging ability, and ENMs uptake, accumulation, and speciation in plant tissues.[23-25] Short-term assays provide a quick feedback and a unique opportunity for mechanistic studies of ENMs impacts in plants. Such studies highlighted the importance of species, tissues, and growth stage of plants, and/or the type, concentration, and mode of ENMs treatment on plant-ENMs interactions.[23] On the other hand, full life cycle studies from planting/transplanting to fruit harvest are critical to understanding plant's physiological and yield responses to ENMs exposure. However, there are limited number of reports on fully mature fruit/grain producing plants cultivated in realistic exposure scenario (i.e. soil) that focus on understanding plants responses in terms of physiological, yield, and nutritional properties of edible crops.[14] Thus, fundamental questions on the long-term risks and benefits of ENMs to plants and plant systems remain unanswered.

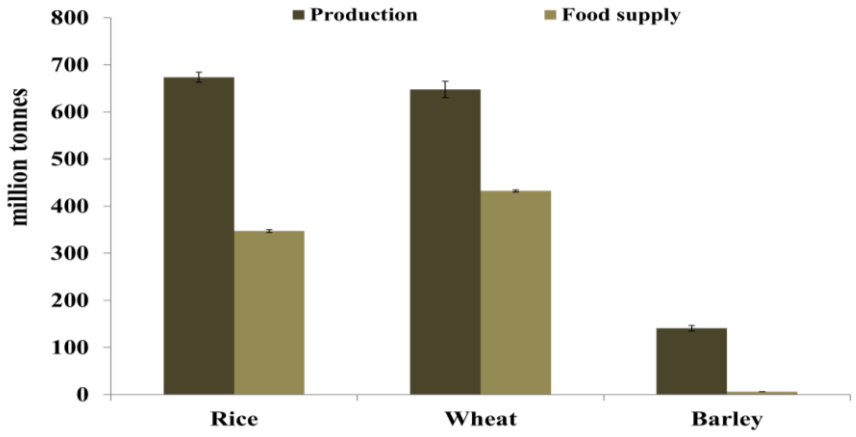

Figure 1.2: World's cereal production and food supply (2005-2009). Data taken from FAOSTAT 2012.[27]

Cereals are the world's most important staple food crops.[26] Major cereals include rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), and barley (*Hordeum vulgare* L.); rice and wheat are produced in almost similar magnitude but in a much larger scale compared to barley (Figure 1.2).[27] Owing to their similarities in botanical classification (i.e. they are grasses that belong to monocot family) and

farming/cultivation practices (i.e. annual plants with one planting yield and harvest),[26] examining their interactions with ENMs will provide profound insights on cereals responses to ENMs exposure or ENMs impacts to agricultural crops in general. Yet in the last four years (2010-2014), only a few studies on these cereals have been undertaken, with only one report performed using *n*CeO_2 and none involved a full life cycle study (Table 1.1). Given the widespread environmental dispersal of *n*CeO_2, it is paramount to understand its biochemical and molecular impacts on globally-important cereal crops. Furthermore, baseline information regarding *n*CeO_2 implications on the physiology, productivity, and quality of these cereals are yet to be reported.

Table 1.1: Engineered nanomaterials used on nanophytotoxicity studies in rice, wheat, and barley in the last four years (2010-2014).

cereal	engineered nanomaterials	references
rice	carbon nanomaterials, *n*Ag, *n*Au, *n*ZnO, *n*CuO, *n*TiO_2	28-34
wheat	*n*ZnO, *n*TiO_2, *n*CeO_2	35-42
barley	*n*CuO, *n*TiO_2	43-44

This research project was performed under the hypothesis that the plant response to *n*CeO_2 will be influenced by species and variety. The hypothesis was tested at short-term (i.e. germination phase) and long-term (i.e. from transplanting to grain production) exposures of rice, wheat, and barley. Short-term exposure involved a two-part study in a growth chamber (14-h photoperiod, 25/20°C day/night temperature and 65% relative humidity, light intensity of 340 µmole m^{-2} s^{-1}): Part I used rice seeds only and Part II utilized all three cereals. Long-term exposure was also accomplished in a two-part study in a greenhouse (14-h photoperiod, 25/20°C day/night temperature, 70% relative humidity): Part III involved an experiment on three rice varieties while Part IV employed wheat and barley. Specifically, biochemical and macromolecular changes were measured to determine the physiological changes in plants and nutritional quality of grains, agronomic and yield parameters were evaluated to assess the productivity of crops, and Ce accumulation in grain was quantified to elucidate the movement and

translocation of Ce in cereals. In the end, the study hoped to establish a more holistic understanding regarding the effects of species and variety on fate and toxicity of *n*CeO_2 in cereals.

The framework of the study is illustrated in Figure 1.3. The toxicity, physiological changes, and tissue/grain cerium accumulation in *n*CeO_2-exposed cereals at seedling to full maturity stage was investigated. The oxidative stress, cell membrane damage, and radical scavenging activity were assayed using confocal microscopy, biochemical assays, and colorimetric techniques. The modifications in macromolecular composition were analyzed by employing various chromatographic methods (GC-MS, GC-FID, HPLC) whereas structural changes were probed using FTIR spectromicroscopy. Ce accumulation and elemental uptake in tissues were quantified using plasma-based spectroscopy (ICP-OES, ICP-MS) whereas the localization of Ce in tissues was mapped using synchrotron x-ray absorption spectroscopy. The possible relationship between the biochemical and macromolecular changes was also examined. The experiments were laid out in randomized complete block design and all statistical analyses were performed using General Linear Model procedure in SAS statistical software.

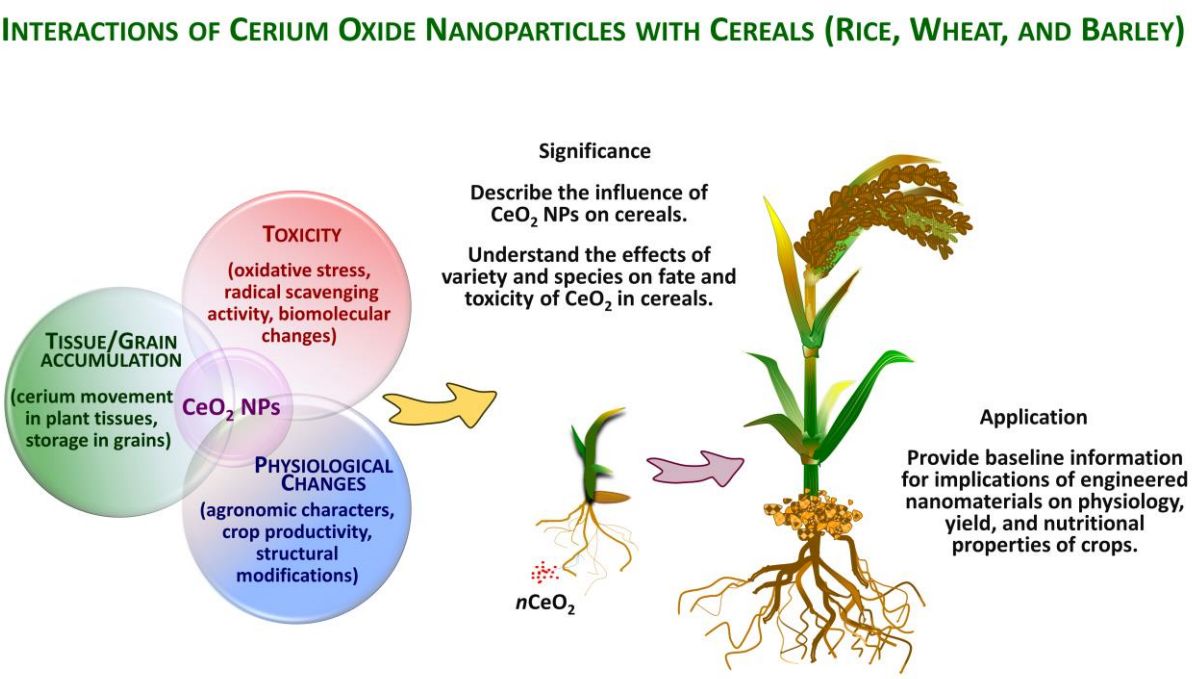

Figure 1.3: Schematic diagram for the study of interactions between cerium oxide nanoparticles and cereals (rice, wheat, and barley).

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Chapter 2: Cerium Oxide Nanoparticles Modify the Antioxidative Stress Enzyme Activities and Macromolecule Composition in Rice Seedlings[1]

Abstract

Cerium oxide nanoparticles (*n*CeO_2) have been shown to have significant interactions in plants; however, there are limited reports on their impacts in rice (*Oryza sativa*). Given the widespread environmental dispersal of *n*CeO_2, it is paramount to understand its biochemical and molecular impacts on a globally-important agricultural crop such as rice. This study was carried out to determine the impact of *n*CeO_2 on the oxidative stress, membrane damage, antioxidant enzymes' activities, and macromolecular changes in the roots of rice seedlings. Rice seeds (medium amylose) were grown for 10 days in *n*CeO_2 suspensions (0, 62.5, 125, 250 and 500 mg L^{-1}). Results showed that Ce in root seedlings increased as the external *n*CeO_2 increased without visible signs of toxicity. Relative to the control, the 62.5 mg *n*CeO_2 L^{-1} reduced the H_2O_2 generation in the roots by 75%. At 125 mg *n*CeO_2 L^{-1}, the roots showed enhanced lipid peroxidation and electrolyte leakage, while at 500 mg L^{-1}, the *n*CeO_2 increased the H_2O_2 generation in roots and reduced the fatty acid content. The lignin content decreased by 20% at 500 mg *n*CeO_2 L^{-1}, despite the parallel increase in H_2O_2 content and peroxidase activities. Synchrotron μ-XRF confirmed the presence of Ce in the vascular tissues of the roots.

Keywords: Antioxidative enzyme activities, Fatty acid, Lignin, *Oryza sativa*, Synchrotron μ-XRF

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2.1 INTRODUCTION

Engineered nanomaterials (ENMs) are extensively used in various industrial, technological, medical, agricultural and household products. Moreover, the number of nanotechnology patent applications grew by 34.5% from 2000 to 2008[1] while the number of nanoproducts increased worldwide by 521% from 2000 to 2011.[2] If the increase continues at this pace, the release of ENMs to the environment will inevitably have an impact in organisms, especially plants.[3] Although recent studies have shown diverse effects of ENMs in plants, the risk assessment of nanophytotoxicity is still at the initial stage and the knowledge on the influence of ENMs in plant systems is still greatly lacking.[4]

nCeO_2 are among the 10 most produced NPs in the world, with an estimated production of around 1000 tons/year.[5] They are heavily used in chemical mechanical planarization and fuel additives[5] that they can easily be dispersed in the environment. For example, a study in the United Kingdom showed a predicted discharge of 15.6-114.9 kg *n*CeO_2 $year^{-1}$ from vehicular use alone.[6] The current predicted environmental concentration (PEC) of *n*CeO_2 is much lower than the concentration that is known to have toxicological effects on organisms.[3,6] However, the PEC of *n*CeO_2 is expected to increase as their uses continue to expand.[3,6] *n*CeO_2 are highly stable and insoluble that they can persist in soil environment[7] and plants.[8-11] Recent reports have shown that *n*CeO_2 exists in nanoparticulate forms[8-11] and affect physiological processes in plants.[12,13]

The growing environment at seed germination and early seedling establishment can modulate the reproduction, productivity, and nutritional value of food crops.[14] Oxidative stress, membrane damage, and antioxidant enzyme activities are standard assays used for determining toxicity of ENMs on plants.[15-17] Modifications in these parameters could lead to the disturbance in fatty acids and lignin composition in plants.[14,18] For example, increased lipid peroxidation resulted in changes in fatty acid composition in the leaves of pepper (*Capsicum annum*)[19] and tomato seedlings (*Lycopersicon esculentum*).[20] Studies have also shown that inhibition of peroxidase (POX) activity or reduction in H_2O_2 generation led to reduced lignin polymerization in Norway spruce (*Picea abies*);[21] while transgenic tobacco (*Nicotiana tabacum*) overexpressing the POX gene, that enhanced the generation of H_2O_2, had increased lignification.[22] Majority of the nanophytotoxicity studies conducted at early

seedling establishment measured oxidative stress and membrane damage; however, studies describing their relationship with macromolecular content in plants are still missing.

Rice is the staple food of more than half of the world's population.[23] However, a survey of current literature revealed that rice-ENMs interactions have not been well explored yet. To date, there were only eight studies that reported the effects of Ag, Au, ZnO, TiO_2, CeO_2 and carbon-based ENMs on rice grown in different media (e.g. cultured/suspension cells and hydroponically grown to full maturity).[24-31] Moreover, the interactions of rice with *n*CeO_2 is not yet well understood.

If rice production fields are impacted with the very stable *n*CeO_2, this nanomaterial could affect rice production in an unknown manner. Thus, baseline information about the effects of the *n*CeO_2 at the early developmental stage of rice is pertinent. Hence, this study was conducted to determine the biochemical and macromolecular changes induced by *n*CeO_2 on germinating rice seedlings. Biochemical assays were performed to determine the oxidative stress, cell membrane damage and changes in antioxidative enzyme activities. GC-MS technique was employed to determine the modifications in macromolecular composition while synchrotron μXRF was performed to probe the translocation of Ce in the roots. The possible relationship between the biochemical and macromolecular changes was also examined.

2.2 MATERIALS AND METHODS

2.2.1 Preparation of *n*CeO_2 Suspensions

The *n*CeO_2 were obtained from the University of California Center for Environmental Implications of Nanotechnology (UC CEIN). The stability and aggregation of these NPs in aqueous media were previously published.[32] Keller et al.[32] reported that the *n*CeO_2 are rod with primary size of 8 ± 1 nm, particle size of 231 ± 16 nm in DI water, surface area of 93.8 m^2g^{-1} and 95.14% purity. The *n*CeO_2 suspensions were prepared at 0, 62.5, 125, 250, and 500 mg L^{-1} in Millipore water (MW) and sonicated in a water bath (Crest Ultrasonics, Trenton, NJ) at 25°C for 1 h. These concentrations were chosen because at <62.5 mg L^{-1}, the *n*CeO_2 did not induce visible signs of toxicity in the plants. The *n*CeO_2 suspensions were freshly prepared and characterized immediately after sonication following a

previously described method.[31] Characterization data of *n*CeO_2 suspensions is presented in the Supporting Information (SI, Appendix 1 Table 1.1).

2.2.2 Seed Germination

Rice seeds from a medium amylose rice variety (Neptune) were obtained from Louisiana State University Agricultural Center (Baton Rouge, LA). Rice seedlings were prepared following the procedure previously employed.[31] Disinfected seeds (30 seeds) were placed in Petri dishes (90mm diameter × 15mm depth) without membrane support and added with 5 mL *n*CeO_2 suspension. The seeds were germinated in the dark by wrapping the Petri dishes in aluminum foil, and incubated in growth chamber (Environmental Growth Chamber, Chagrin Falls, OH) with 14-h photoperiod, 25/20°C day/night temperature and 65% relative humidity. The germination rate was measured when 65% of control roots were 5 mm long, which was obtained at four days.[33]

For further analyses, ungerminated seeds were removed to prevent bacterial or fungal contamination of the media. The seedlings were allowed to grow for 6 days under light (340 μmol m^{-2} s^{-1}). Watering was done so that only the roots were submerged in the suspension. At harvest, the seedlings were washed with 1 mM HNO_3 and rinsed three times with ice-cold 2 mM $CaCl_2$[15] and MW.[31] The roots were thoroughly washed to avoid the possible problem from surface contamination that could compromise the accuracy of the succeeding analyses.

2.2.3 Analytical Methods

Plant roots were digested in a microwave accelerated reaction system (CEM Corp, Mathews, NC) using a mixture of plasma pure HNO_3 and H_2O_2 (1:4) and analyzed for Ce content by Inductively Coupled Plasma - Optical Emission Spectroscopy (ICP-OES) using the methods of analysis previously described.[34] Plasma pure HNO_3 (SCP Science, Champlain, NY) contains ≤1 μg L^{-1} trace metals and used for environmental applications in ICP-OES. Blank, spikes and reference standard material (NIST-SRF 1570a) were used to validate the digestion and analytical method. The H_2O_2 content was measured according to the method of Gay and Gibicki.[35] Membrane damage was evaluated by measuring the electrolyte leakage[36] and lipid peroxidation expressed as thiobarbituric reactive species (TBARS).[37] The

protein content was quantified by Bradford method.[38] Detailed description of the methods is provided in the SI (Appendix 1).

2.2.4 Antioxidative Enzyme Activity Assays

The crude enzyme extracts for catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), and dehydroascorbate reductase (DHAR) assays were prepared following the methods previously described[39] while extracts for ascorbate peroxidase (APOX) and guaiacol peroxidase (GPOX) were prepared following the method of Xu et al.[40] The CAT activity was assayed by monitoring the degradation of H_2O_2 (extinction coefficient 39.4 mM^{-1} cm^{-1}) at 240 nm.[41] One unit of CAT is defined as the amount of enzyme required to degrade 1 μmol of H_2O_2 per minute. GPOX activity was determined by monitoring the formation of guaiacol dehydrogenation product (6.39 mM^{-1} cm^{-1}) at 420 nm.[42] One unit of GPOX is defined as 1 μmol of guaiacol oxidized per minute. The SOD activity was determined by the inhibition of nitroblue tetrazolium reduction at 560 nm. One unit of SOD activity is the amount of enzyme that decreased the rate of nitroblue tetrazolium reduction by 50%. APOX activity was measured following the method of Nakano and Asada[44] by monitoring the decrease in ascorbate (2.8 mM^{-1} cm^{-1}) content at 290 nm. One unit of APOX is defined as 1 μmol of ascorbate oxidized per minute. The DHAR activity was estimated according to the method of Hossain and Asada[45] by following the increase in absorbance due to the formation of ascorbate (14 mM^{-1} cm^{-1}) at 265 nm. One unit of DHAR is 1 μmol of ascorbate formed per minute. The GR activity was determined according to the method of Foyer and Halliwell[46] by following the decrease in absorbance due NADPH oxidation (6.2 mM^{-1} cm^{-1}) at 340 nm. One unit of GR activity is defined as 1 μmol of NADPH oxidized per minute. All enzyme activity assays were done at 25°C using 1 mL volume of reaction mixture except for the SOD assay wherein 3 mL of final volume was used. The enzyme kinetics for the assays was recorded in a Perkin Elmer Lambda 14 UV/Vis Spectrometer (single-beam mode, Perkin-Elmer, Uberlinger, Germany).

2.2.5 GC-MS Analysis of Fatty Acid

The simultaneous extraction and methyl esterification of fatty acids (FA) was conducted following the method of Browse et al.[47] Briefly, fresh root tissues (50 mg) were cut in tiny pieces and

placed in screw capped glass tubes with 1 mL freshly prepared 5% H_2SO_4 in methanol; then, heated for 1.5 h at 80°C. After cooling to room temperature, 500 μL hexane and 1.5 mL 0.9% NaCl was added to extract the fatty acid methyl esters. The solution was vortexed, sonicated in a water bath at 10°C for 10 min, and centrifuged at 1500×*g* for 5 min at 4°C. The extraction was repeated three times. The pooled organic phase was kept in amber vials and stored at 4°C until use. The methods for GC-MS analyses were described in SI (Appendix 1). The double bond index (DBI) for polyunsaturated fatty acid (PUFA) was calculated according to Gajewska et al.[48]

2.2.6 Lignin Content Analysis

The Klason lignin content was analyzed by gravimetric technique described by Theander and Westerlund.[49] Briefly, fresh root tissues were ground using liquid nitrogen, washed 3 times with 80% ethanol at 70°C, and dried at 80°C. Dried tissue (100 mg) was acid hydrolyzed with 3 mL 72% H_2SO_4 at 30°C for 45 min and diluted to 4% before autoclaving at 120°C for 1 h. After cooling, the solution was filtered in a pre-weighed filter paper.

2.2.7 Synchrotron μ-XRF Data Acquisition

Root segments of 5-mm length from the region of elongation were sectioned at 30 μm thick slices and mounted onto Ultralene window film. μ-XRF mapping of Ce and other elements was done at 5.8 KeV excitation energy using a zone plate microfocused beam (0.8×0.3 μm^2) at beamline ID21 of the European Synchrotron Radiation Facility (ESRF, Grenoble, France) following the conditions described by Hernandez-Viezcas et al.[10] The X-ray fluorescence data were processed using PyMCA software.[50]

2.2.8 Statistical Analysis

The results are means ± S.E. (n=3). A one-way ANOVA using General Linear Model followed by Tukey's Honestly Significant Difference (HSD) test was performed using SAS statistical package Version 9.3 (SAS Institute, Cary, NC, USA). Statistical significance was based on a probability of $p \leq 0.05$.

2.3 RESULTS AND DISCUSSION

2.3.1 Cerium Accumulation from *n*CeO_2 Treatments

The Ce accumulation in rice seedling roots exposed to *n*CeO_2 concentrations varying from 0 to 500 mg L^{-1} are shown in Figure 2.1A. As shown in this figure, the amount of Ce in roots increased as the external *n*CeO_2 increased (310- 2884 mg kg^{-1} dry weight). However, there were no statistical differences among plants exposed to the 62.5 and 125 mg L^{-1} treatments. On the other hand, plants exposed to 250 mg L^{-1} had significantly more Ce compared to the 62.5 and 125 mg L^{-1} treated plants and significantly less Ce than plants exposed to 500 mg L^{-1} (Figure 2.1A). It is expected that significant amounts of Ce were absorbed by the roots and some of them reached the transport system;[8-11] though, the roots did not show any visible signs of toxicity like growth retardation or necrosis (Appendix 1 Table 1.2). The roots were thoroughly washed with 1 mM HNO_3, ice-cold 2 mM $CaCl_2$ and MW; however, it is highly probable that the amount of Ce measured was from both surface contamination and uptake.[31,51] Unfortunately, there is no proven method that could effectively remove all ENMs adsorbed on plants surface.

The uptake and translocation of *n*CeO_2 in plants is species-dependent. Studies have shown that Ce reaches the vascular or transport system of plants exposed to *n*CeO_2.[8-11] Furthermore, the uptake and translocation of Ce in the tissues and fruits/pods of tomato and soybean grown to fruit/pods production in *n*CeO_2-soil has been reported.[13,52] On the contrary, Birbaum et al.[53] found no evidence of translocation of *n*CeO_2 in corn exposed to aerosol of *n*CeO_2 while Schwabe et al.[54] observed that *n*CeO_2 were strongly adsorbed on root surface of wheat (*Triticum aestivum* L.) but translocated into the shoots of pumpkin (*Cucurbita maxima*). In this study, synchrotron μ-XRF analysis was performed to determine the uptake and distribution of Ce in the roots of rice seedlings.

2.3.2 Effects of *n*CeO_2 on H_2O_2 Generation, Lipid Peroxidation and Electrolyte Leakage

Figure 2.1 (B-D) shows the effect of *n*CeO_2 on the H_2O_2 content and membrane damage. Relative to the control, the 62.5 and 500 mg *n*CeO_2 L^{-1} decreased and increased the H_2O_2 content by 69 and 162%, respectively, whereas the 125 and 250 mg *n*CeO_2 L^{-1} did not change the H_2O_2 content in roots (Figure 2.1B). The lipid peroxidation at 125 mg *n*CeO_2 L^{-1}-treated roots was significantly higher

by 19 to 31% than those grown in the other treatments (Figure 2.1C). Similarly, the electrolyte leakage at 125 mg nCeO$_2$ L^{-1} was induced by 4 to 18% compared with the other nCeO$_2$-treated roots (Figure 2.1C). Since both lipid peroxidation and electrolyte leakage are indicators of membrane damage, the results suggest that the 125 mg nCeO$_2$ L^{-1} induced the most membrane damage in rice roots.

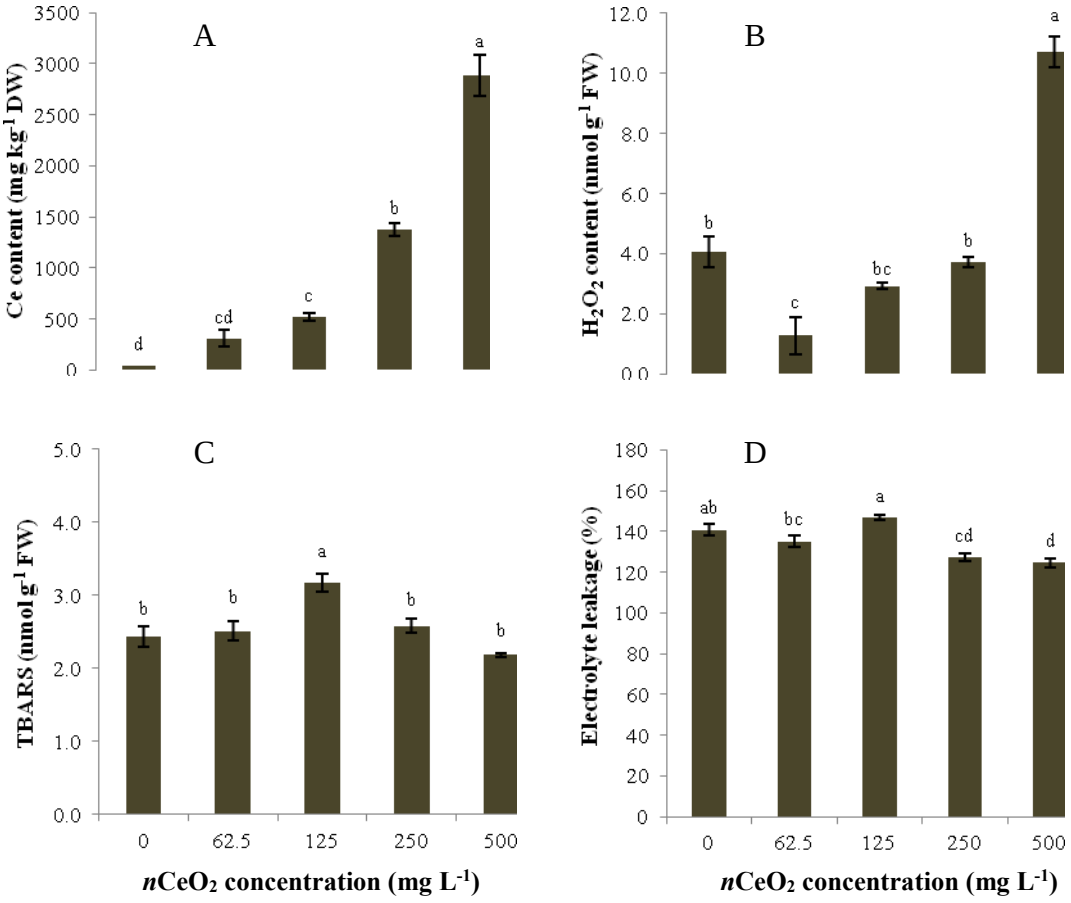


Figure 2.1: Ce concentration (A), H_2O_2 content (B), TBARS production (C), and electrolyte leakage (D) in the root of medium amylose rice variety germinated in nCeO$_2$ suspensions for 10 days. Values are means ± SE (n = 3). Same letters mean no statistical difference between treatments at Tukey's test ($p \leq 0.05$).

The results revealed that nCeO$_2$ induced changes at biochemical level without exhibiting visible symptoms of toxicity in the rice seedlings. The 62.5 mg nCeO$_2$ L^{-1} had significantly low H_2O_2 content probably due to the radical scavenging ability of nCeO$_2$ at low concentration.[55,56] It is also possible that the nCeO$_2$ SOD mimetic activity increased at increased nCeO$_2$ concentration, which promoted H_2O_2 generation.[57] The 125 mg nCeO$_2$ L^{-1} had low H_2O_2 content but caused the highest membrane damage,

while the 500 mg *n*CeO_2 L^{-1}, which had a markedly increased H_2O_2 content, induced low membrane damage. This inverse-dose effect relationship is in agreement with those observed in human lung epithelial cells wherein *n*FeO at lower concentration induced higher oxidative stress than the higher dose.[58,59] However, this is not in accordance with the widely reported trend between the oxidative stress and cell membrane damage observed in living and growing plants where the increase in oxidative stress would result in membrane damage.[60] The current findings indicate that the *n*CeO_2 is affecting other physiological processes in rice plants that resulted in the observed trend; thus, the antioxidant enzyme activities and fatty acid contents were further analyzed.

2.3.4 Effects of *n*CeO_2 on the Antioxidant Defense System in Roots

The plant cells have antioxidant defense system composed of enzymes such as CAT, GPOX, SOD, APOX, DHAR and GR. The SOD dismutates $O_2^{\bullet-}$ to H_2O_2 while CAT, GPOX and APOX catalyze the conversion of H_2O_2 to H_2O.[61] The DHAR and GR enzymes generate the ascorbate needed by APOX to catalyze the reduction of H_2O_2.[62] Together, the APOX, DHAR, and GR form a network of enzymes in the Halliwell-Asada pathway that effectively control oxidative stress. These enzymes were differentially affected by the concentrations of *n*CeO_2 used in this study (Figure 2.2). As seen in Figure 2.2(A-B), the response of the stress enzymes CAT and GPOX to the *n*CeO_2 at 62.5 mg L^{-1} (the lowest concentration tested) followed the same pattern; significantly increased, relative to control roots. However, the response was different at higher NP concentrations. While CAT in treated roots showed the same activity as in control roots, GPOX significantly increased at 250 and 500 mg *n*CeO_2 L^{-1} (Figure 2.2B). On the other hand, SOD exhibited significantly lower and higher activities in roots grown with 125 and 250 mg *n*CeO_2 L^{-1}, respectively (Figure 2.2C), compared with those grown in other treatments. In the case of APOX, only the roots of plants treated at 500 mg *n*CeO_2 L^{-1} showed increased activity compared to control roots (Figure 2.2D). Interestingly, the activities of GR (Figure 2.2E) and DHAR (Figure 2.2F) exhibited opposite response to the *n*CeO_2 treatment, except at 125 mg *n*CeO_2 L^{-1}. Relative to the control, the 125 mg *n*CeO_2 L^{-1} did not significantly change the GPOX, CAT and APOX activities, but resulted in lower SOD, DHAR and GR activities.

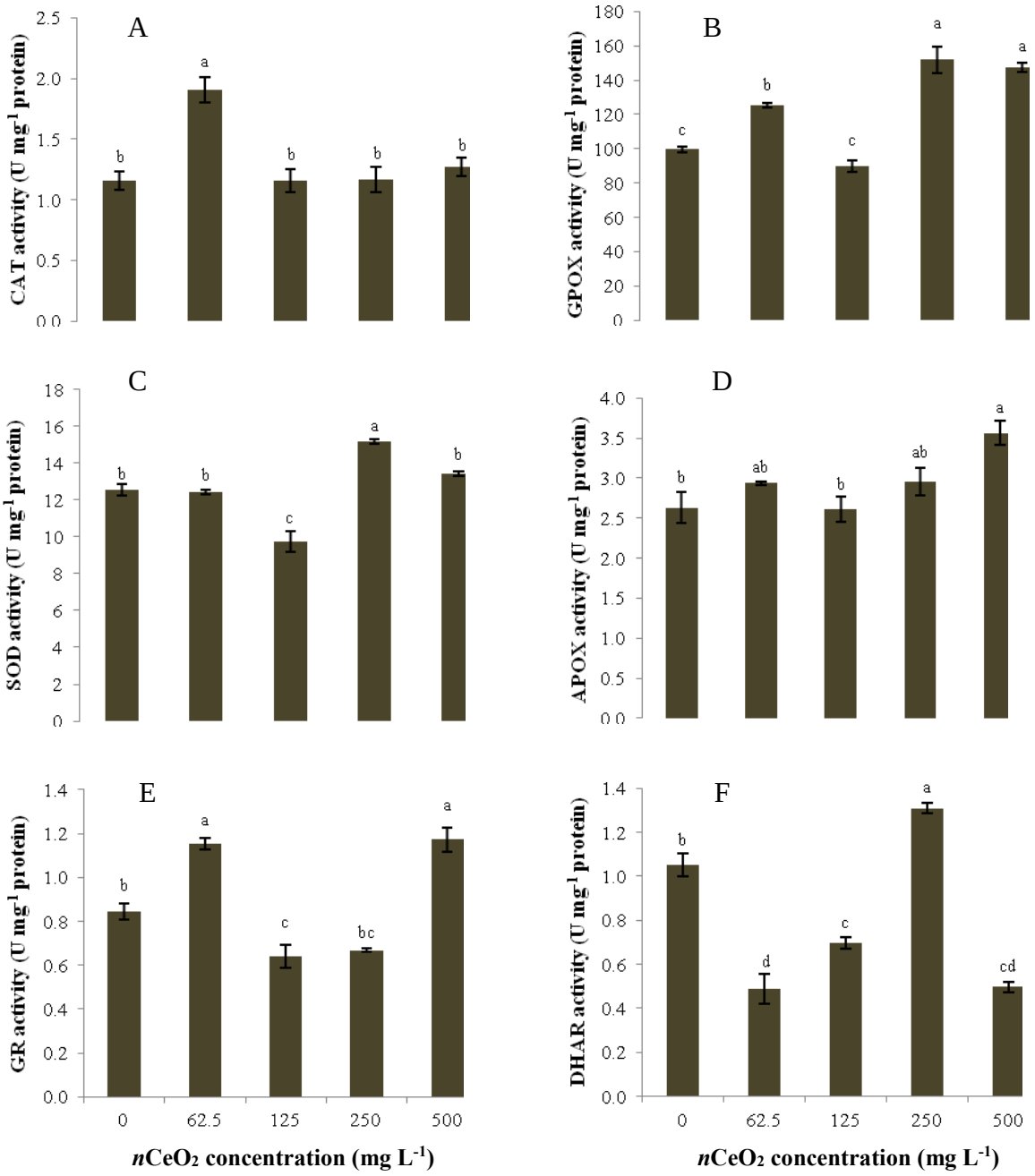


Figure 2.2: Enzyme activities of CAT, GPOX, SOD, APOX, DHAR and GR in the roots of medium amylose rice variety germinated in *n*CeO_2 suspensions for 10 days. Values are means ± SE (*n*=3). Same letters mean no statistical difference between treatments at Tukey's test ($p \leq 0.05$). Different scales were used due to the high variability in the unit activities of the enzymes.

Studies have shown that the antioxidant enzymes are activated by oxidative stress.[59,60] It seems that the increase in H_2O_2 observed at 500 mg *n*CeO_2 L^{-1} resulted in enhanced activities of GR, GPOX, and APOX. The high H_2O_2 generation in roots treated at 500 mg *n*CeO_2 L^{-1} (88-162% higher than the

rest of the treatments) was expected to aggravate the oxidative stress which would lead to increased lipid peroxidation.[62] However, this was not observed, suggesting that in rice roots, the enzymes GR, GPOX, and APOX, have a preponderant role in controlling the oxidative stress.[18,62] On the contrary, the high activities of CAT, GR, and GPOX relative to the control at 62.5 mg *n*CeO_2 L^{-1} did not correspond to the low H_2O_2 concentration observed in roots treated with this NP concentration (Figure 2.1B). It is possible that the high activity of these enzymes caused the significant reduction in the H_2O_2 content.

On the other hand, in roots treated at 125 mg *n*CeO_2 L^{-1}, the H_2O_2 content was low, but the lipid peroxidation and electrolyte leakage were greatly enhanced. The data suggest that at this concentration, the decreased activity of SOD, DHAR and GR resulted in an inefficient removal of H_2O_2; which led to enhanced membrane damage.[17,61] Previous reports indicate that reduction in GR activity is linked to lipid peroxidation. Schmidt and Kunert[63] reported an inverse relationship between GR activity and lipid peroxidation in bean (*Phaseolus vulgaris*) leaves treated with the herbicide acifluorfen-sodium. Although the GR activity at the 125 and 250 mg *n*CeO_2 L^{-1} treatments was similar, a lower lipid peroxidation in 250 mg *n*CeO_2 L^{-1} was found which may be due to parallel increase in SOD, GPOX and DHAR activities observed with this treatment. Previous studies have also shown that an enhanced DHAR activity results in reduced lipid peroxidation in plants.[64]

2.3.5. Effects of *n*CeO_2 on the Fatty Acid (FA) Contents in the Roots

The FA composition of the rice roots treated with *n*CeO_2 at concentrations varying from 0 to 500 mg L^{-1} is shown in Table 2.1. As seen in this table, the FA content was significantly increased or reduced by the *n*CeO_2 treatments, except palmitic acid (Table 2.1). The highest FA contents were observed in roots treated with 62.5 and 250 mg *n*CeO_2 L^{-1} (235.99 and 245.78 g FA ng^{-1} FW, respectively) while the lowest was found in roots treated with 500 mg *n*CeO_2 L^{-1} (187.52 g FA ng^{-1} fresh wt.). The 500 mg *n*CeO_2 L^{-1}-treated roots also showed the lowest content in unsaturated FA (C18:1, C18:2 and C18:3). The DBI and the proportions of each unsaturated FA were not significantly affected by the treatments (Appendix 1 Table 1.3). However, the total unsaturated FA was affected and followed the trend of the total FA wherein the 500 mg *n*CeO_2 L^{-1}-treated roots yielded the lowest total unsaturated FA (Appendix 1 Table 1.3, Table 2.1).

Table 2.1: Fatty acids content (ng g^{-1} FW) in the roots of medium amylose rice variety grown in *n*CeO_2 suspensions for 10 days. Values are means ± SE (n = 3). Same letters in each row mean no statistical difference between treatments at Duncan's test ($p \leq 0.05$).

Fatty acids	***n*CeO_2 concentration (mg L^{-1})**				
	0	**62.5**	**125**	**250**	**500**
Lauric acid (C12)	20.8 ± 0.1c	22.4 ± 0.2a	21.9 ± 0.4ab	21.0 ± 0.1bc	21.6 ± 0.4abc
Myristic acid (C14)	23.2 ± 0.6ab	24.2 ± 0.9a	24.3 ± 0.4a	25.0 ± 0.9a	21.9 ± 0.3b
Valeric acid (C15)	20.5 ± 0.2bc	21.8 ± 0.7ab	22.0 ± 0.1a	21.6 ± 0.6ab	21.2 ± 0.2c
Palmitic acid (C16)	51.6 ± 9.1a	58.7 ± 9.7a	56.5 ± 10.4a	64.0 ± 5.5a	37.9 ± 0.5a
Stearic acid (C18)	24.2 ± 2.9ab	26.7 ± 1.6ab	24.0 ± 1.7ab	29.1 ± 0.9a	22.6 ± 1.4b
Oleic acid (C18:1)	21.6 ± 0.6ab	23.5 ± 1.1a	22.9 ± 0.8ab	23.4 ± 0.6a	20.7 ± 0.3b
Linoleic acid (C18:2)	25.7 ± 2.9ab	29.8 ± 3.3ab	25.6 ± 3.5ab	31.4 ± 1.8a	21.2 ± 0.9b
Linolenic acid (C18:3)	25.0± 2.7ab	29.0 ± 3.3ab	24.6 ± 2.9ab	30.2 ± 1.7a	21.5 ± 0.9b
Total	212.5 ± 17.4ab	236.0 ± 20.2a	221.8 ± 13.2ab	245.9 ± 9.5a	187.5 ± 3.8b

The effect of NPs on FA composition in plants has not been reported yet. However, related studies on plants exposed to metals have shown that the increase in membrane damage result in the reduction of polyunsaturated fatty acid.[14] In the present study, the control, 62.5 and 250 mg *n*CeO_2 L^{-1} treatments resulted in low membrane damage and high content in unsaturated FA. Despite the high abundance of unsaturated FA as substrate for peroxidation, membrane damage was kept under control by the high activity of some of the antioxidant enzymes. Furthermore, the treatments did not affect the DBI, and the proportion of C18:3 and unsaturated FAs, indicating that the peroxidation did not affect the content of FA.

The results obtained with 125 mg *n*CeO_2 L^{-1} suggest that the high electrolyte leakage and lipid peroxidation could have resulted from high amount of unsaturated FA available and low antioxidant enzyme activities to inhibit the peroxidation. At 500 mg *n*CeO_2 L^{-1}, the treated roots exhibited both low membrane damage and unsaturated FA content (Figure 2.1, Table 2.1). The low electrolyte leakage and lipid peroxidation could be due to low unsaturated FA content, since FA is the substrate for peroxidation, and high antioxidant enzyme activities that inhibited the peroxidation. A study on wheat

(*Triticum aestivum*) exposed to 50 or 100 μM Ni also showed that the lower membrane damage in roots, compared with that of the shoots, was attributed to the lower abundance of unsaturated FA in the roots.[48] However, it is not clear whether the decrease in FA content at 500 mg *n*CeO_2 L^{-1} was due to the defense mechanism or NP-induced damage in the plant.

2.3.6 Effects of *n*CeO_2 on Lignin Content in the Roots

Table 2.2 shows the effect of *n*CeO_2 on lignin content in rice roots. Relative to the lignin content in control roots (74 mg g^{-1} DW), only 500 mg *n*CeO_2 L^{-1}-treated roots (59 mg g^{-1} DW) had significantly reduced lignin content while the other *n*CeO_2 treatments did not significantly change (63-72 mg g^{-1} DW) (Table 2.2). This result is contrary to the generally observed enhanced lignifications in plants exposed to stress.[65]

Table 2.2: Lignin content in the root of medium amylose rice variety germinated in *n*CeO_2 suspensions for 10 days. Values are means ± SE ($n = 3$). Same letters mean no statistical difference between treatments at Duncan's test ($p \leq 0.05$).

***n*CeO_2 concentration (mg L^{-1})**	**Lignin content (mg g^{-1} DW)**
0	73.63 ± 2.25a
62.5	71.77 ± 3.19ab
125	64.03 ± 1.33ab
250	62.98 ± 3.70ab
500	58.83 ± 2.89b

Peroxidases (POX) and H_2O_2 have been implicated in lignin synthesis.[18] POX catalyzes the oxidoreduction between H_2O_2 and monolignols (RH) resulting in monolignol radicals ($2RH + H_2O_2 \rightarrow 2R^{\bullet} + 2H_2O$), which can then form the lignin polymer.[18,66] The balance between POX activity and H_2O_2 content is a controlling factor in lignin synthesis.[18,21,22,66] The current study revealed that despite the enhanced POX activity (GPOX and APOX), accompanied by increased H_2O_2 content in the 500 mg *n*CeO_2 L^{-1}-treated roots (Figures 2.1 and 2.3), the lignin content significantly decreased. These results

indicate that *n*CeO_2 disturbs lignin biosynthesis differently than bulk metal particles would. Further studies are needed to understand the disturbance mechanism.

2.3.7 Synchrotron μ-XRF Analysis

The spatial distribution of Ce in the roots of rice seedlings was determined by using synchrotron μ-XRF. Figure 2.3A shows the map of the root vascular cylinder of rice seedling germinated with 500 mg *n*CeO_2 L^{-1} wherein red color stands for Ce, blue for K and green for Ca. Figure 2.3B presents the μ-XRF Ce intensity map. As seen in Figure 2.3A, Ce was taken up by roots and moved into the transport system of rice plant even for a short exposure time of 10 days. The low concentration of Ce in roots, due to the short exposure, did not allow their speciation. However, previous studies have confirmed that *n*CeO_2 undergo limited chemical transformation and most of the Ce found in *n*CeO_2-treated plants are in the form of NPs.[8-11] The uptake and translocation of Ce in the form of NPs in cucumber (*Cucumis sativus*), alfalfa (*Medicago sativa*), tomato, and corn (*Zea mays*) treated with *n*CeO_2 suspensions have been observed.[8,9,11,67] Recent reports have proven that Ce translocated from root to shoot, and accumulated in the pods, of soybean largely in the form of NPs.[10,13]

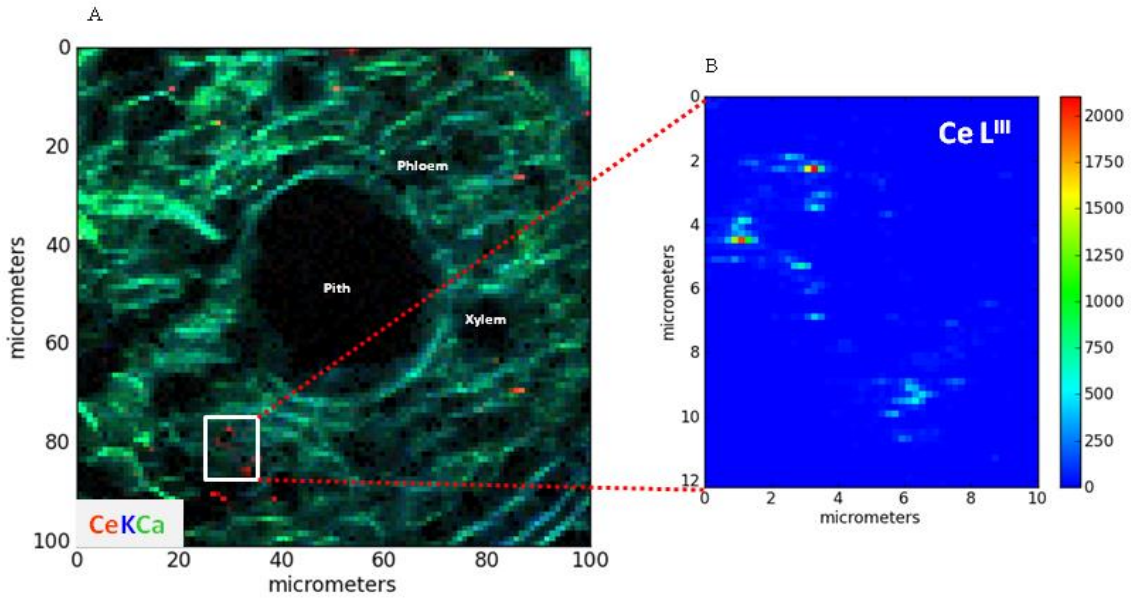
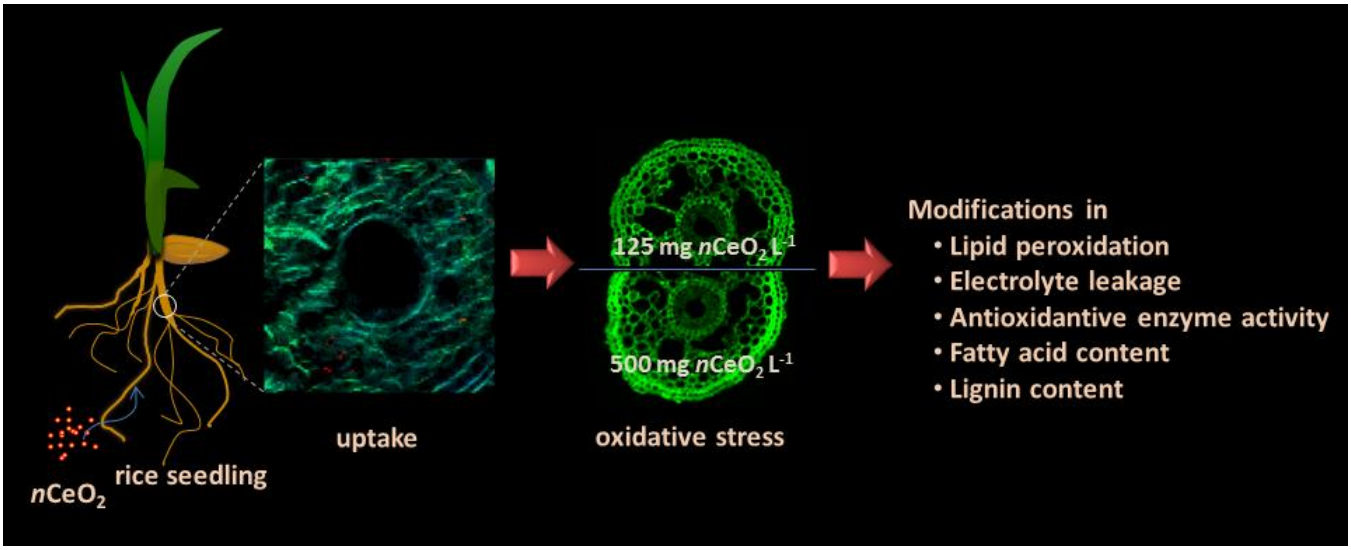


Figure 2.3: Images of the transversal section of roots from rice seedlings germinated in 500 mg *n*CeO_2 L^{-1} suspensions for 10 days. (A) Tricolor μXRF map (red = Ce, blue = K, green = Ca). (B) μ-XRF temperature map of same location in (A) showing intensity in counts per second.

2.4 CONCLUSION

In summary, the findings of the present study demonstrate that plants from medium amylose rice variety are affected by *n*CeO_2. The plants treated with 125 mg *n*CeO_2 L^{-1} showed reduced antioxidant enzyme activities and high FA content, resulting in increased membrane damage. In addition, plants treated with 500 mg *n*CeO_2 L^{-1} exhibited enhanced enzyme activities and low FA content that led to low membrane damage. The latter treatment also produced markedly reduced lignin content, despite high H_2O_2 content and POX activities. Furthermore, Ce translocated into the vascular tissues of rice seedling roots and altered the biomolecular components of the xylem; however, it is too early to determine if the changes will cause negative impact on rice plants that further studies are needed.



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Chapter 3: Differential Effects of Cerium Oxide Nanoparticles on Rice, Wheat, and Barley Roots: An Infrared (FTIR-IMS) Spectromicroscopy Study[2]

Abstract

Cerium oxide nanoparticles (*n*CeO_2) find extensive industrial applications that concerns regarding their environmental exposure have been raised. This study includes structural analysis of intact root xylem of rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), and barley (*Hordeum vulgare* L.) seedlings exposed to *n*CeO_2 suspensions (0, 62.5, 125, 250 and 500 mg L^{-1}). Infrared spectromicroscopy was applied to determine compositional alterations in the root xylem while principal component analysis (PCA) was carried out to examine spectral differences between *n*CeO_2 treatments. Results demonstrated that *n*CeO_2 at ≥125 mg L^{-1} changed the region of spectra around 1696-1760 cm^{-1} in rice root; 125 and 250 mg L^{-1} modified 1744-1792 cm^{-1} in wheat; and 62.5 and 125 mg L^{-1} altered 1727-1760 cm^{-1} in barley. PCA afforded the clustering of *n*CeO_2 treatments at 0 and 62.5 mg L^{-1} in rice and wheat and 0 and 500 mg L^{-1} in barley. Furthermore, major peaks at 1744 or 1760 cm^{-1} appeared in primary PC and 1728 cm^{-1} in secondary PC score loadings. These findings illustrated that *n*CeO_2 induced compositional modifications in the root xylem of cereals.

Keywords: FTIR-IMS, Infrared spectromicroscopy, PCA, Principal component analysis

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3.1 INTRODUCTION

Engineered nanomaterials (ENMs) are now present in thousands of personal care (cosmetics and sunscreen), agricultural (fertilizer and pesticides) and industrial products (paints and fuel cells).[1] As such, widespread concerns regarding the environmental threats of ENMs exposures have been equally expressed. Cerium oxide ENMs (nCeO$_2$) have been particularly studied because of their vehicular and fuel applications that can possibly lead to increased environmental contamination.[1] Since nCeO$_2$ is very stable in soil and different environmental media[2-4] and has been found to transfer within plants tissues unaltered,[5-9] it is very likely that they interact with plants in nanoparticulate forms. The impacts of ENMs on plants employing physiological, biological, and life cycle studies have been studied. However, structural analyses of intact plant tissues exposed to ENMs have not been reported yet.

Xylem is a major structural support in plants that transports water and nutrient ions into the shoots. Ions pass through the xylem cell wall, unloaded into the xylem and move into the transpiration stream.[10] The ion exchange capacity in the xylem cell wall affects the movement of micronutrient ions in the xylem vessel that alterations in the xylem composition could affect ion transport in the plants.[10,11] Although studies have proven that nCeO$_2$ accumulates and induces biochemical changes in plants, [5-9,12-14] structural analysis of root xylem exposed to nCeO$_2$ have not been performed yet.

Structural analysis of native plant tissues provides a major advantage over wet chemical analyses in providing rich information on the spatial distribution and chemical composition of plants microstructures which would have been otherwise lost when performed under conventional wet chemical analysis.[15,16] Moreover, wet chemical analyses are performed using composite samples making it difficult to examine the response of individual cell structure to external stimuli.[17] As an alternative, infrared spectromicroscopy (FTIR-IMS) allows high-throughput assessment and localization of changes in whole plant tissue.[15-18]

Infrared spectromicroscopy (FTIR-IMS) is a powerful tool for gathering chemical information from native cell structures of a growing and developing plant.[15,16,19] It has been extensively employed in determining structural changes in plants exposed to biotic and abiotic stressors. For example, FTIR-IMS was used to investigate the macromolecular modifications in the epidermis, cortex, and xylem of

sunflower (*Helianthus annuus*) and maize (*Zea mays*) roots treated with dinitrotoluenes,[20] and macromolecular changes in the root and stem tissues of Mexican Palo Verde (*Parkinsonia aculeata*) exposed to Cr.[21] Similarly, alterations in the carbohydrate chemistry of leaves due to chemical and enzymatic degradation,[22] and profiling of the chemical fingerprint of the inner and outer surfaces of distinct types of plant tissues have been assessed by employing FTIR-IMS technique.[17] Recently, FTIR-IMS has been utilized to analyze the chemical makeup of secondary xylem in *Populus tremula* and *Arabidopsis thaliana*.[16]

Principal component analysis (PCA) is a chemometric technique commonly employed in the analysis of FTIR spectroscopic data.[15] PCA reduces the dimensionality and complexity of FTIR data and highlights subtle spectral variations that discriminate between plant tissues.[15,18,23,24] PCA of spectral data has been applied in the rapid analysis of compositional changes in the xylem of transgenic aspen,[23] chemical and enzymatic alterations in leaf carbohydrates,[22] chemical differences between monocots and dicots,[18] and rapid screening of cell wall mutants.[24] Combined application of PCA and FTIR-IMS analyses of native plant tissues exposed to ENMs has yet to be reported.

This paper reports the use of FTIR-IMS in studying the impact of *n*CeO_2 on the compositional fingerprint of root xylem during the early development of rice, wheat and barley seedlings. PCA was employed to confirm that the chemical differences were induced by the treatments and not due to experimental variations only.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of *n*CeO_2 Suspensions

The *n*CeO_2 were obtained from the University of California Center for Environmental Implications of Nanotechnology (UC CEIN). The *n*CeO_2 are rod with primary size of 8 ± 1 nm, particle size of 231 ± 16 nm in DI water, surface area of 93.8 m^2 g^{-1} and 95.14% purity.[3] The stability and aggregation of these ENMs in aqueous media have been reported.[3] The *n*CeO_2 suspensions were prepared at 0, 62.5, 125, 250, and 500 mg L^{-1} in Millipore water (MW) and sonicated in a water bath (Crest Ultrasonics, Trenton, NJ) at 25°C for 1 h. The *n*CeO_2 suspensions were freshly prepared and

sonicated just before characterization. Characterization data is presented in the Supporting Information (SI) Appendix 2 Table 2.1.

3.2.2 Seedlings Preparation

Rice seeds from a medium amylose rice variety (Neptune) were obtained from Louisiana State University Agricultural Center (Baton Rouge, LA) while spring wheat (Jubilee) and spring barley (Millennium) seeds were procured from the University of Idaho Southwest Idaho Extension Cereals Program (Parma, ID). The seedlings were germinated and prepared following the previously described procedure.[13] "The seeds were soaked in 4% NaClO for 15 min and rinsed three times with Millipore water (MW). Clean seeds (30 seeds) were placed in Petri dishes (90 mm diameter × 15 mm depth) containing 5 mL *n*CeO_2 suspension. The Petri dishes wrapped in aluminum foil were incubated in the dark at 25°C. The germination rate was measured when 65% of control roots were 5 mm long,[25] which was obtained at four days for rice and three days for wheat and barley. For other analyses, the seedlings were left to grow for 6 days for rice and 4 days for barley and wheat in an environmental growth chamber (Chagrin Falls, OH) under the following conditions: 14-h photoperiod, 25/20°C day/night temperature, 65% relative humidity, and light intensity of 340 μmol m^{-2} s^{-1}. During the growing period, any ungerminated seeds were removed while water was added as needed to submerge the roots only. At harvest, the roots were rinsed successively with 1 mM HNO_3, ice-cold 2 mM $CaCl_2$[7] and MW for three times."

3.2.3 Analysis of Cerium Content

The roots were dried in an oven, ground to powder, and digested in a microwave accelerated reaction system (CEM Corp, Mathews, NC) using a 1:4 mixture of plasma pure HNO_3 and H_2O_2.[26] The cerium content was quantified using ICP-OES (Perkin Elmer) wherein blank, spikes and reference standard material (NIST-SRF 1570a) were used to validate the digestion and analytical method.

3.2.4 Infrared spectromicroscopy (FTIR/IMS) Sample Preparation and Data Analysis

FTIR/IMS analysis was conducted following the method of Zhao et al.[21] Briefly, seedling roots were frozen in liquid nitrogen. A 7-mm length of root cuts from the region of elongation were frozen

onto specimen blocks by Tissue Tek (Sakura Finetek, Torrance, CA), and cryosectioned using Minotome Plus (Triangle Biomedical Sciences, Durham, NC) at 10 μm thick slices. The frozen slices were thaw-mounted onto low-e microscope slides (Kevley Technologies, Indianapolis, IN). FTIR-IMS analysis was performed using a Perkin Elmer Spotlight 300 Microscope System (FTIR Microscope) coupled to the FTIR spectrometer 100. The sample's visible image was collected in reflection mode at a resolution of 4 cm^{-1} with 120 scans at a signal-to-noise ratio of 12,000:1. Then a box was made on the visible image to select the analysis area and a total absorbance image was collected at a resolution of 4 cm^{-1} with 64 scans. IR spectra around the root xylem were collected in a range of 1800 to 800 cm^{-1}. The spectra were baseline corrected, smoothed, area normalized, displayed in the absorbance mode, and subjected to Fourier self deconvolution using Perkin Elmer Spectrum software (Version 6.0.2.0025, Perkin Elmer, Shelton, CT). A total of 21 spectra per sample collected from 7 xylem structures were used.

3.2.5 Data Analysis

A one-way ANOVA using General Linear Model followed by Tukey's Honestly Significant Difference (HSD) test based on a probability of $p \leq 0.05$ was performed using SAS statistical package Version 9.3 (SAS Institute Inc., Cary, NC, USA). Principal component analysis (PCA) of deconvolved spectra was carried out using Matlab R2012b software (MathWorks, Inc., Natick, MA, USA).

3.3 RESULTS AND DISCUSSION

3.3.1 Cerium Uptake in Root and Physiological Response of Seedlings

The data on Ce concentration in the roots of wheat and barley is shown in Table 3.1. Results revealed an increased Ce content at increased *n*CeO_2 concentration in the suspension reaching up to thousand-fold increase in 500 mg *n*CeO_2 L^{-1} treatment compared to the control. A similar trend has also been reported for rice.[14] There are conflicting reports on Ce uptake and translocation in seedlings exposed to *n*CeO_2. For example, wheat grown in hydroponics spiked with *n*CeO_2 and corn under *n*CeO_2 aerosol exposure showed no evidence of Ce translocation in the plants tissues.[27,28] On the contrary, our previous work demonstrated the movement of Ce up to the vascular region of the rice root,[14] which was in accordance

with the observed Ce uptake in several crops.[6-9,27] No reports can be found on Ce uptake in barley. The current findings suggest that rice, wheat and barley could accumulate Ce in their root tissues.

The physiological response of seedlings to *n*CeO_2 treatment was assessed and displayed in Appendix 2 SI Table 2.2. It is evident from the data that the germination rate and root elongation of the seedlings were neither enhanced nor inhibited even with the presence of Ce in the roots. Similar to our earlier reports on rice,[13,14] the wheat and barley seedlings developed normally without displaying any phytotoxic symptoms like stunting, wilting and chlorosis. Previous studies also showed that *n*CeO_2 did not affect the germination rate and root elongation of wheat.[27,29] On the other hand, the growth response of barley exposed to *n*CeO_2 has never been reported yet.

Based on our previous findings in rice, *n*CeO_2 could induce significant modifications in plants at the biochemical and molecular levels despite the absence of apparent toxic response.[13,14] It was found that *n*CeO_2 had a remarkable influence on the oxidative stress, membrane damage, and macromolecular compositions of rice roots despite the notable absence of changes in germination rate and root growth. As a consequence, it is highly probable that *n*CeO_2 provoke modifications on an important structural support of plants such xylem. Thus, *in situ* analysis of root xylem was performed.

Table 3.1: Cerium content (mg kg^{-1} d wt) in the roots of wheat and barley germinated for 7 days in different *n*CeO_2 concentrations. Values are means ± SE, $n = 4$. Same letters mean no statistical difference between treatments at Tukey's test ($p \leq 0.05$).

***n*CeO_2 concentration (mg kg^{-1})**	**spring wheat (Jubilee)**	**spring barley (Millennium)**
0	8.7 ± 1.3d	9.4 ± 0.8c
62.5	324.6 ± 16.2c	349.9 ± 29.7bc
125	470.3 ± 19.7c	577.5 ± 74.7bc
250	854.2 ± 56.5b	807.7 ± 78.4b
500	2331.3 ± 127.6a	2579.6 ± 333.6a

3.3.2 IMS Analysis of the Roots Xylem

Figure 3.1 shows the visible images and total absorbance area maps of roots of rice, wheat, and barley treated with *n*CeO_2. As seen in the figure, the cortex section of the roots was still loosely formed while the pith and xylem had visibly formed that they could be clearly marked. Figures 3.2-3.4 present the deconvolved spectra recorded from the xylem of each root. Deconvolution technique was performed to uncover and enhance the resolution of small bands hidden in the overlapping bands of the original spectra.[30] The bands were grouped in different regions following the assignments used by D'Souza et al.[31] and the specific band assignments were presented in Table 3.2. The bands in the 1800-1500 cm^{-1} region are characteristic bands for proteins, wherein 1700-1600 cm^{-1} corresponds to amide I bands[32] while 1600-1500 cm^{-1} is attributed to the amide II bands.[33] Amide I is mainly attributed to C═O stretching vibrations of the peptide bond while amide II is due to N─H bending vibrations plus C─N stretch.[34,35] The region between 1500-1200 cm^{-1} displays the C─H bending vibrations of CH_3, CH_2, and CH functional groups,[36,37] whereas 1200-900 cm^{-1} is assigned for the carbohydrate fingerprint bands.[18,38] The carbohydrate fingerprint region yields complex absorption from various polysaccharides that is mainly due to C─O, C─C, C─O─C and C─O─P as well as CH_3 and CH_2 rocking modes.[36,37] Table 3.2 displays the assignment of IR bands to distinct functional groups in different class of macromolecules.

Rice. Figure 3.2 depicts the xylem spectra of rice root at 1800-800 cm^{-1} region. Inspection of the spectra reveals that the spectrum from the control had fewer numbers of peaks than the rest of the treatments. The peak at 1728 cm^{-1} appeared in the control, 62.5 and 125 mg *n*CeO_2 L^{-1} treatments, but disappeared in 250 mg *n*CeO_2 L^{-1} and shifted to 1760 cm^{-1} in the 500 mg *n*CeO_2 L^{-1} treatment. On the other hand, band at 1696 cm^{-1} appeared only at 125 and 250 mg *n*CeO_2 L^{-1} treatments. The bands at 1696, 1728, and 1760 cm^{-1} correspond to C═O stretching of the ester group.[31,35,39] Previous studies suggested that the modifications in these bands indicate the involvement of COOH in the chelation of heavy metals.[31,39] At the amide I region, two bands from 1632 and 1600 cm^{-1} appeared in all treatments with an apparent higher intensity at the *n*CeO_2 treatments. In case of the amide II region, only one band at 1520 cm^{-1} appeared in the control, which was also present in all *n*CeO_2 treatments. A peak at 1552 cm^{-1} also exists in all *n*CeO_2 treatments except in 250 mg *n*CeO_2 L^{-1} where it shifted to 1568 cm^{-1}. These

bands represent N─H bending vibrations plus C─N stretch in proteins suggesting that the *n*CeO_2 treatment promoted protein production in the roots.[31]

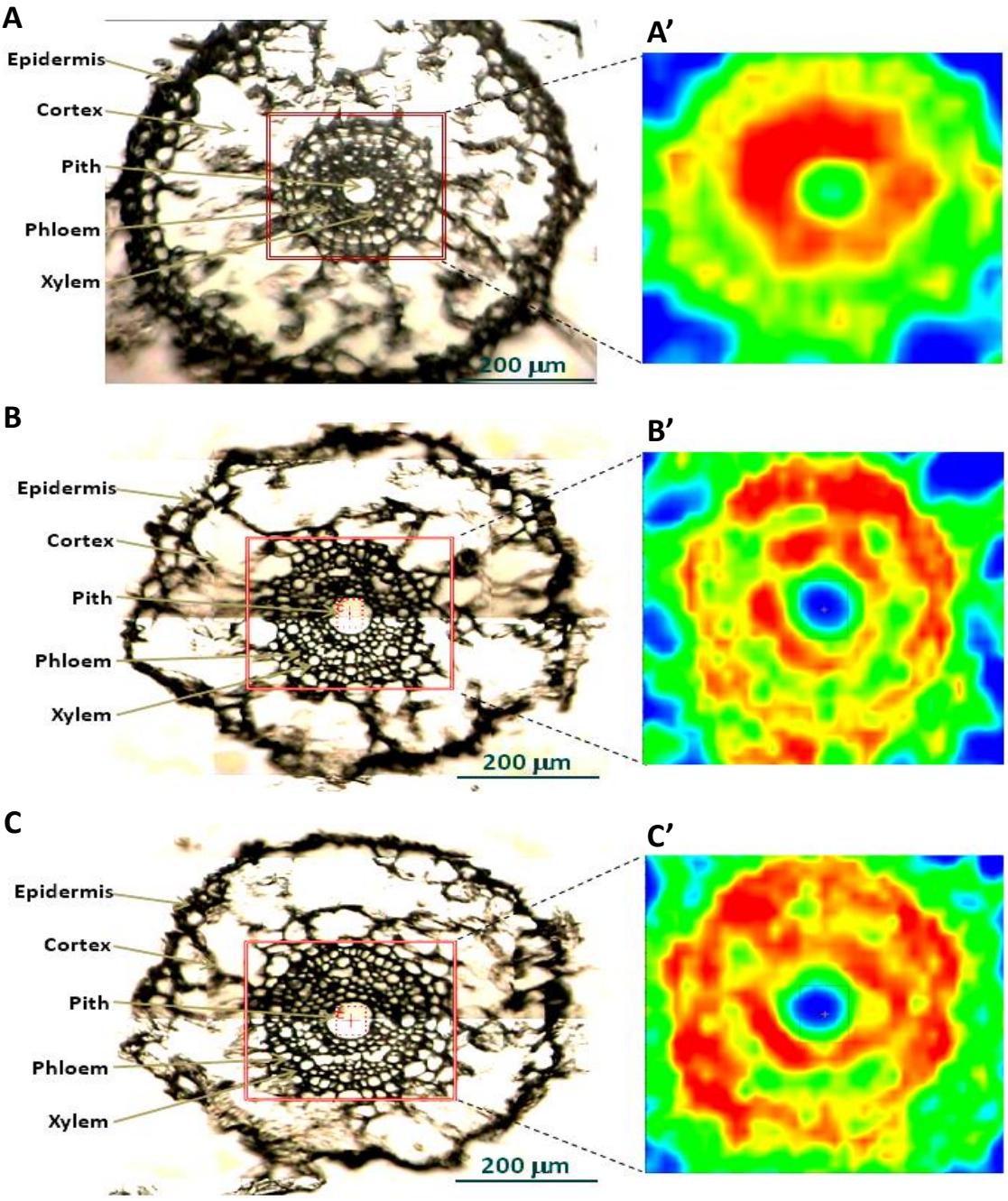

Figure 3.1: Sample and visible total absorbance images of the root of rice (A, A'), wheat (B, B'), and barley (C, C'). The rectangle on the visible image represents the studied area, the red color represents high intensity and the blue color little or no intensity in the total absorbance maps. The sample's visible image was collected in reflection mode at a resolution of 4 cm^{-1} with 120 scans while total absorbance image was collected at a resolution of 4 cm^{-1} with 64 scans.

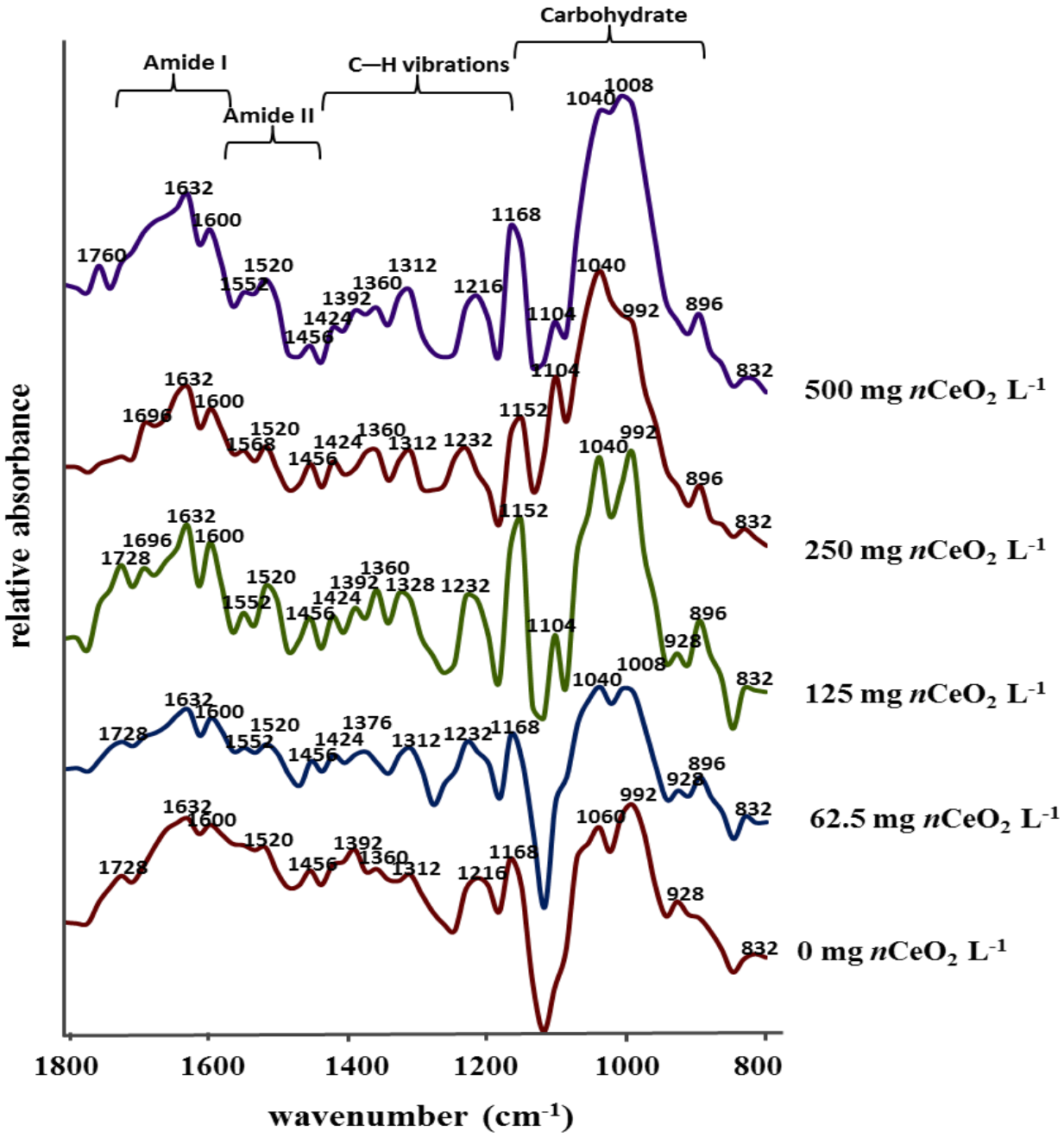

Figure 3.2: Infrared spectra of the root xylem of rice. The spectra were baseline corrected, smoothed, area normalized, displayed in the absorbance mode, and subjected to Fourier self deconvolution using Perkin Elmer Spectrum software (Version 6.0.2.0025, Perkin Elmer, Shelton, CT). Band assignments: Amide I (1700-1600 cm^{-1}),[32] Amide II (1600-1500 cm^{-1}),[33] C─H bending vibrations (1500-1200 cm^{-1}),[36,37] Carbohydrate (1200-900 cm^{-1}).[18,38]

Table 3.2: Assignment of IR bands in IR spectra of root xylem of rice, wheat and barley germinated in different *n*CeO_2 concentrations.

Frequency range (cm^{-1})	Type of vibration	Present in	ref
1744-1790	C═O stretching of carboxyl ester	lipids, esterified pectins	17, 30, 31, 35
1696-1728	C═O stretching of phenolic ester	phenolics	31, 41
1648-1664	C═O stretch plus C─N stretch	protein	31, 39
1632	aromatic C═C stretch	hydrocinnamic acid in lignin	31, 41
1600-1616	COO^- asymmetric stretch	acidic groups of pectic polysaccharides	38, 41
1536-1568	N─H deformation plus C─N stretch	protein	31
1504-1520	C═C phenolic stretch	guaiacyl ring of lignin	38, 41
1456-1472	C─H bend of OCH_3	cellulose and/or pectin	40
1408-1424	C─H bend from asymmetric CH_3	lipids, polysaccharides, cellulose	30, 41
1360-1392	C─H bend from symmetric CH_3	lipids, polysaccharides, cellulose	23, 41
1312-1344	C─O vibration	syringyl ring	23
1216-1248	C─O─H deformation asymmetric	cellulose, hemicelluloses	41
1152-1168	C─O─C antisymmetric stretching	cellulose, hemicelluloses	23, 36, 37
1104	O─H association band	cellulose, hemicelluloses	23
1040-1072	C─O stretching vibration	cellulose, hemicelluloses	23, 36, 37
992-1024	-	carbohydrate fingerprint region	36-38
912-928	-	carbohydrate fingerprint region	38
880-896	C─O stretching vibration	cellulose, hemicelluloses	23
816-848	aromatic C─H wag	aromatic ring associated with lignin	18

In the 1500-1200 cm^{-1} region, results showed that the control had five bands: 1456 cm^{-1} representing C─H bend of OCH_3 in cellulose and/or pectin,[40] 1392, 1360 and 1312 cm^{-1} indicating C─H bends from symmetric CH_3 in lipids, polysaccharides and cellulose, and 1216 cm^{-1} probably showing C─O ─H asymmetric deformation.[18] For the treated, the band at 1392 cm^{-1} appeared only in 125 and 500 mg *n*CeO_2 L^{-1} treatments while peaks at 1360 and 1312 cm^{-1} shifted to 1376 and 1328 cm^{-1} in the 62.5 and 125 mg *n*CeO_2 L^{-1} treatments, respectively. Likewise, 1216 cm^{-1} shifted to 1232 cm^{-1} in all

nCeO_2 treatments except 500 mg L^{-1} suggesting a stronger C─N stretching and C─N─H in-plane bending of protein.[41] However, the appearance of a new band at 1424 cm^{-1}, representing C─H bends from asymmetric CH_3 in lipids, polysaccharides and cellulose,[18] in the *n*CeO_2 treated-roots provided a peak for distinguishing between *n*CeO_2 treatments and control.

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The data showed three peculiar bands in the carbohydrate fingerprint region. The peak at 1104 cm^{-1} demonstrating O─H association band in cellulose[23] was evident only at ≥125 mg *n*CeO_2 L^{-1} treatments while peak at 928 cm^{-1} representing CH_2 and CH_3 rocking[41] was apparent only at ≤125 mg *n*CeO_2 L^{-1} treatments. A new band also appeared at 896 cm^{-1} representing C─H deformation vibration in cellulose in all *n*CeO_2 treatments.[23] On the other hand, the peak at 1060 cm^{-1} in the control downshifted to 1040 cm^{-1} in all *n*CeO_2 treatments. It is also apparent from the data that the peaks at 1104 and 896 cm^{-1} were more prominent in 125 mg *n*CeO_2 L^{-1} compared to those in 250 and 500 mg *n*CeO_2 L^{-1} treatments. Results indicated that the *n*CeO_2 treatments modified the carbohydrate composition of the root xylem. Similarly, ENMs induced alterations in the carbohydrate fingerprint region in rice and *Ulmus elongata*.[42,43]

The results showed several peaks that can be used to discriminate *n*CeO_2 treatments from the control. For example, the bands at 1552, 1424 and 896 cm^{-1} consistently appeared at *n*CeO_2 treatments signifying that the concentration or the chemical environment for protein and cellulose were modified.

Together with these bands, two more prominent peaks in 1696 and 1104 cm^{-1} appeared at 125 and 250 mg *n*CeO_2 L^{-1} treatments, and in 1760 and 1104 cm^{-1} manifested at 500 mg *n*CeO_2 L^{-1} treatment indicating that in addition to protein and cellulose, lipids were also modified at ≥125 mg *n*CeO_2 L^{-1} treatments. These findings suggest that low *n*CeO_2 treatment promoted the formation of chemical species containing C─H, C─N and C─N─H, probably in the form of protein and cellulose, whereas, higher *n*CeO_2 treatment enhanced the production of C═O and O─H containing species in the form of lipids. These observations were consistent with the previous report on rice showing increased fatty acid content at increased *n*CeO_2 concentration in the treatment.[14]

Wheat. The deconvolved spectra of the wheat root xylem under different treatments are displayed in Figure 3.3. Wheat spectra had less number of peaks compared to rice, but also revealed major differences between *n*CeO_2 treatments. The control and 62.5 and 500 mg *n*CeO_2 L^{-1} treatments showed peaks at 1744 and 1664 cm^{-1}. At 125 and 250 mg *n*CeO_2 L^{-1} treatments, the peak at 1744 cm^{-1} shifted to higher wavenumbers (1760 and 1792 cm^{-1}, respectively) while the band at 1664 cm^{-1} downshifted to 1648 cm^{-1}. However, the band at 1536 cm^{-1} remained visible in all treatments. These bands represent C═O stretch (1744, 1760 and 1792 cm^{-1}), C─O plus C─N stretch (1648 and 1664 cm^{-1}), and N─H bending vibrations plus C─N stretch (1536 cm^{-1}) suggesting that chemical environment of amide bond was affected.

The region between 1500 and 1200 cm^{-1} in the control had 4 prominent peaks at 1472, 1392, 1344, and 1264 cm^{-1} representing C─H bend of OCH_3, C─H bend from symmetric CH_3, C─O vibration in syringyl ring, and C─O─H deformation in cellulose/hemicellulose, respectively. These peaks eventually reduced to 3 peaks in the *n*CeO_2 treatments. The band at 1344 cm^{-1} shifted to 1328 cm^{-1} in 250 mg *n*CeO_2 L^{-1} whereas the peak at 1264 cm^{-1} shifted to lower wavenumbers (1232-1248) in ≥125 mg *n*CeO_2 L^{-1} treatments. The peaks at 1472 (C─H bend of OCH_3) and 1392 (C─H bend from symmetric CH_3) cm^{-1} became closer at 1456 and 1392 cm^{-1} in the 62.5 mg *n*CeO_2 L^{-1} treatment which ultimately fused to one band at 1424 cm^{-1} (C─H bend from asymmetric CH_3) in ≥125 mg *n*CeO_2 L^{-1} treatments. Only the formation of band at 1424 cm^{-1}, which represents the C─H bend from asymmetric CH_3 in lipids, polysaccharides or cellulose, resulted in significant change in band assignment.

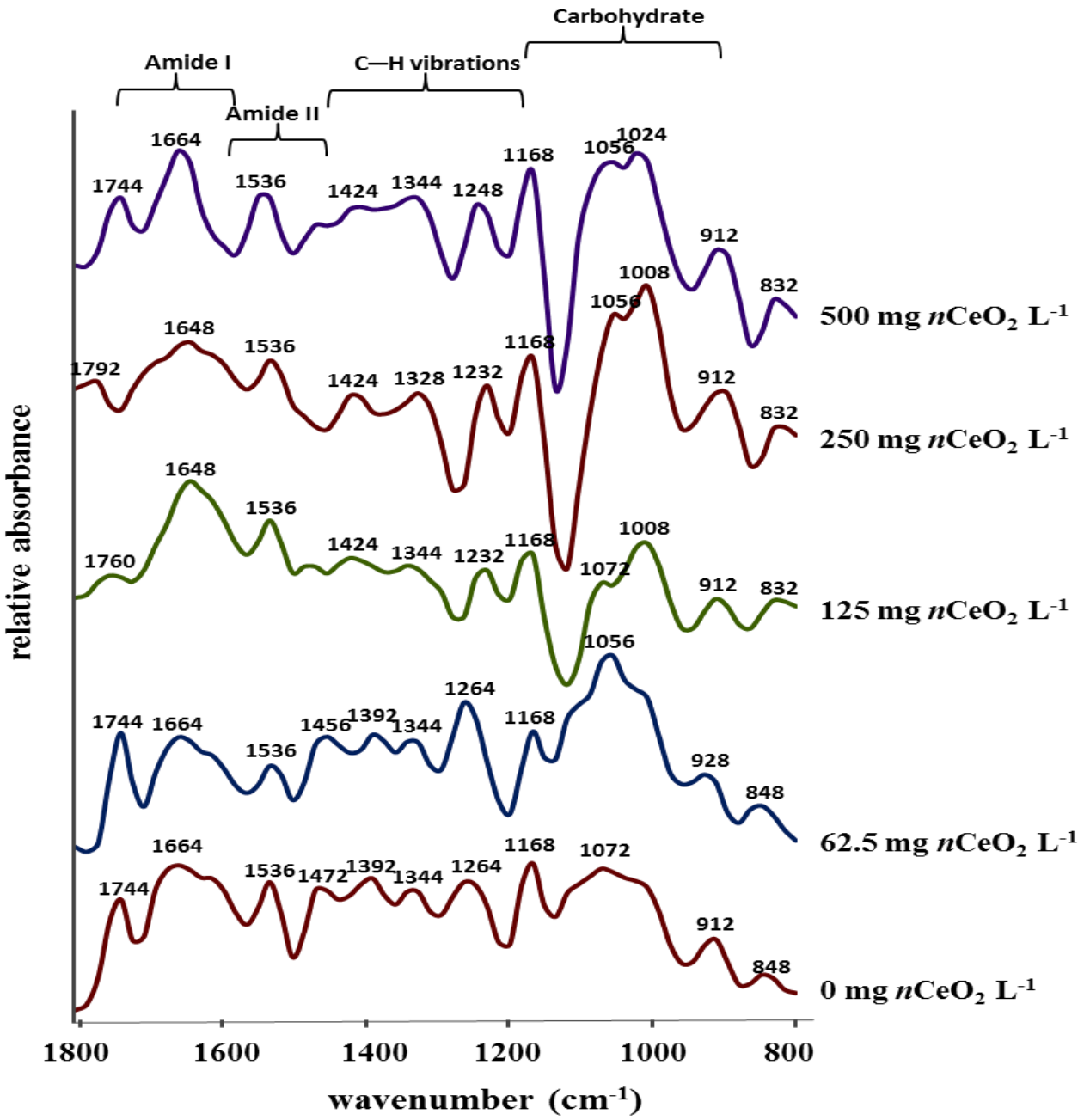


Figure 3.3: Infrared spectra of the root xylem of wheat. The spectra were baseline corrected, smoothed, area normalized, displayed in the absorbance mode, and subjected to Fourier self deconvolution using Perkin Elmer Spectrum software (Version 6.0.2.0025, Perkin Elmer, Shelton, CT). Band assignments: Amide I (1700-1600 cm^{-1}),[32] Amide II (1600-1500 cm^{-1}),[33] C─H bending vibrations (1500-1200 cm^{-1}),[36,37] Carbohydrate (1200-900 cm^{-1}).[18,38]

In the carbohydrate region, the lone peak at 1072 cm^{-1} from C─O stretching in cellulose/hemicellulose split up to two distinct peaks at ≥125 mg $nCeO_2$ L^{-1} treatments with a concomitant pushing of 848 cm^{-1} down to 832 cm^{-1}. It also interesting to note that the merging of two

distinct bands at 1472 and 1392 cm^{-1} into one (1424 cm^{-1}), the breaking up of one band (1072 cm^{-1}) into two separate bands, and the shifting of 1264 and 848 cm^{-1} bands to lower wavenumbers (1232-1148 and 832 cm^{-1}, respectively) occurred simultaneously at ≥125 mg *n*CeO_2 L^{-1} treatments.

However, the 125 and 250 mg *n*CeO_2 L^{-1} treatments yielded the most different spectra among all the treatments. The results revealed that the shifting of the band from 1744 cm^{-1} to 1760-1792 cm^{-1}, from 1664 cm^{-1} to 1648 cm^{-1}, and from 848 cm^{-1} to 832 cm^{-1} with the emergence of a new band at 1424 and 1008 cm^{-1} were concurrent with each other. The movement of the bands to 1760-1792 cm^{-1} suggests that the carboxyl group also played an important role in the response mechanism of wheat exposed to *n*CeO_2. The peaks at 1648, 1424, 1008 and 832 cm^{-1} could indicate the formation of pectic polysaccharides wherein the peak at 1008 cm^{-1} is indicative of starch, bands at 1424 and 832 cm^{-1} are diagnostic for pectic polysaccharides, and the band at 1648 cm^{-1} signals the presence of proteins.[18]

Barley. The barley root xylem also showed differences in the structural components. As seen in the Figure 3.4, control showed peak at 1728 cm^{-1} (C═O stretching of phenolic ester) which shifted to 1760 cm^{-1} (C═O stretching of carboxyl ester in lipids/esterified pectins) in 62.5 and 125 mg *n*CeO_2 L^{-1} treatments. The amide I region showed prominent peaks at 1648 and 1616 cm^{-1}. The band at 1648 cm^{-1} shifted down to 1632 cm^{-1} in 125 mg L^{-1} treatment while the peak at 1616 cm^{-1} disappeared in all *n*CeO_2 treatments. Two peaks (1552 and 1504 cm^{-1} from N─H deformation plus C─N stretch and C═C phenolic stretch, respectively) in the amide II region emerged in the control; however, these peaks disappeared in all *n*CeO_2 treatments and were replaced by a new peak at 1520 cm^{-1} (C═C phenolic stretch). The shifting of the band from 1728 cm^{-1} to 1760 cm^{-1} is indicative of the role of the carboxyl group similar to those observed in rice and wheat. However, the emergence of peak at 1520 cm^{-1} suggests the lignifications in the xylem.[18]

The barley spectra also showed several peaks in the region between 1500-1200 cm^{-1}. The peak at 1456 cm^{-1} (C─H bend of OCH_3) remained in ≥250 mg *n*CeO_2 L^{-1} treatments, but disappeared in 62.5 mg *n*CeO_2 L^{-1} treatment and shifted to lower frequency (1472 cm^{-1}) but similar vibration type in 125 mg *n*CeO_2 L^{-1} treatment. The band at 1424 cm^{-1} (C─H bend from asymmetric CH_3) also shifted to lower frequency (1408 cm^{-1}) in the same vibration type for all *n*CeO_2 treatments except for 125 mg *n*CeO_2 L^{-1}

treatment where it shifted further down to 1392 cm^{-1} (C─H bend from symmetric CH_3). The peaks at 1360 (C─H bend from asymmetric CH_3) and 1312 (C─O vibration) cm^{-1} showed opposite trend. The band at 1360 cm^{-1} disappeared in all *n*CeO_2 treatments except 500 mg *n*CeO_2 L^{-1} treatment while the band at 1312 cm^{-1} remained evident in all *n*CeO_2 treatments except 250 mg *n*CeO_2 L^{-1} treatment. A new peak emerged at 1344 cm^{-1} (C─O vibration) in 250 mg *n*CeO_2 L^{-1} treatment which could probably be due to the merging of the two peaks at 1360 and 1312 cm^{-1}. On the other hand, the peak at 1248 cm^{-1} shifted to 1216 and 1232 cm^{-1} in the *n*CeO_2-treated roots. The loss of band at 1248 cm^{-1} in *n*CeO_2 treatments with the simultaneous appearance of peaks at around 1216, 1408, 1392 cm^{-1} suggest the formation of lipids or polysaccharides rather than cellulose. Meanwhile, the emergence of bands at 1472-1456 cm^{-1} alongside with that 1520 and cm^{-1} denotes lignin formation.

The carbohydrate region showed interesting peaks at 1040-992 cm^{-1} representing C─O stretching vibration in cellulose/hemicellulose. Similar to that of the wheat, the control had a lone peak at 1040 cm^{-1} which split up into two peaks in *n*CeO_2 treatments. The peak at 1040 cm^{-1} shifted to 1056 cm^{-1} in ≥125 mg *n*CeO_2 L^{-1} treatment, while a peak at 992 cm^{-1} emerged in 62.5 and 125 mg *n*CeO_2 L^{-1} treatments which shifted to 1008 cm^{-1} in ≥250 mg *n*CeO_2 L^{-1} treatments. The peak at 896 cm^{-1} in the control remained at 250 mg L^{-1} treatments, but moved to a lower wavenumber (880 cm^{-1}) in 62.5 and 125 mg *n*CeO_2 L^{-1} treatments. Likewise, the band at 832 cm^{-1} indicative of aromatic C─H wag associated with lignin in the control shifted to 816 cm^{-1} in *n*CeO_2 treatments except 500 mg L^{-1}. The modifications in the bands in the carbohydrate fingerprint region was similar to those observed in rice and wheat suggesting that polysaccharides also played important role in the interaction between *n*CeO_2 and cereals.

In general, root xylem showed different sensitivity to *n*CeO_2 treatments. It is apparent from the data that the *n*CeO_2 treatment producing significant effects in the root xylem induced changes in the ester group as indicated by the shifting of the band from 1744 cm^{-1} to 1760-1792 cm^{-1}. It is also evident from the results that the significant treatment provokes alterations in the conformation or content of carbohydrate and in the C─H bending vibrations in the 1500-1200 cm^{-1} region. However, it is not

possible to distinguish with certainty which treatment induced significant chemical variations in the root xylem of rice, wheat and barley. Thus, principal component analysis (PCA) was performed.

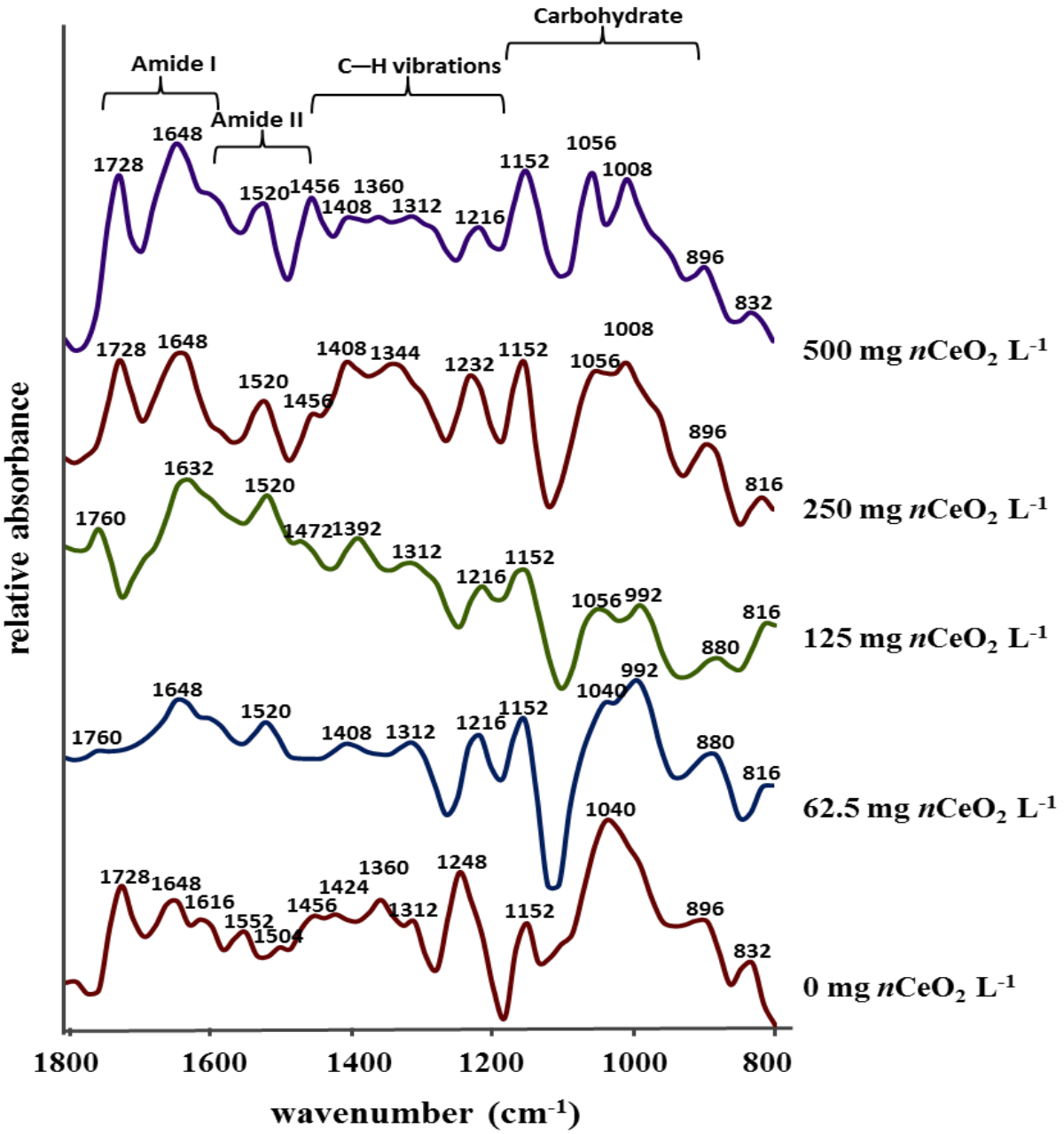


Figure 3.4: Infrared spectra of the root xylem of barley. The spectra were baseline corrected, smoothed, area normalized, displayed in the absorbance mode, and subjected to Fourier self deconvolution using Perkin Elmer Spectrum software (Version 6.0.2.0025, Perkin Elmer, Shelton, CT). Band assignments: Amide I (1700-1600 cm^{-1}),[32] Amide II (1600-1500 cm^{-1}),[33] C─H bending vibrations (1500-1200 cm^{-1}),[36,37] Carbohydrate (1200-900 cm^{-1}).[18,38]

3.3.3 PCA of Chemical Variations in the Root Xylem

PCA was employed to differentiate between the macromolecular compositions of root xylem of rice, wheat and barley. A total of 21 spectra from each treatment were used in the analysis.

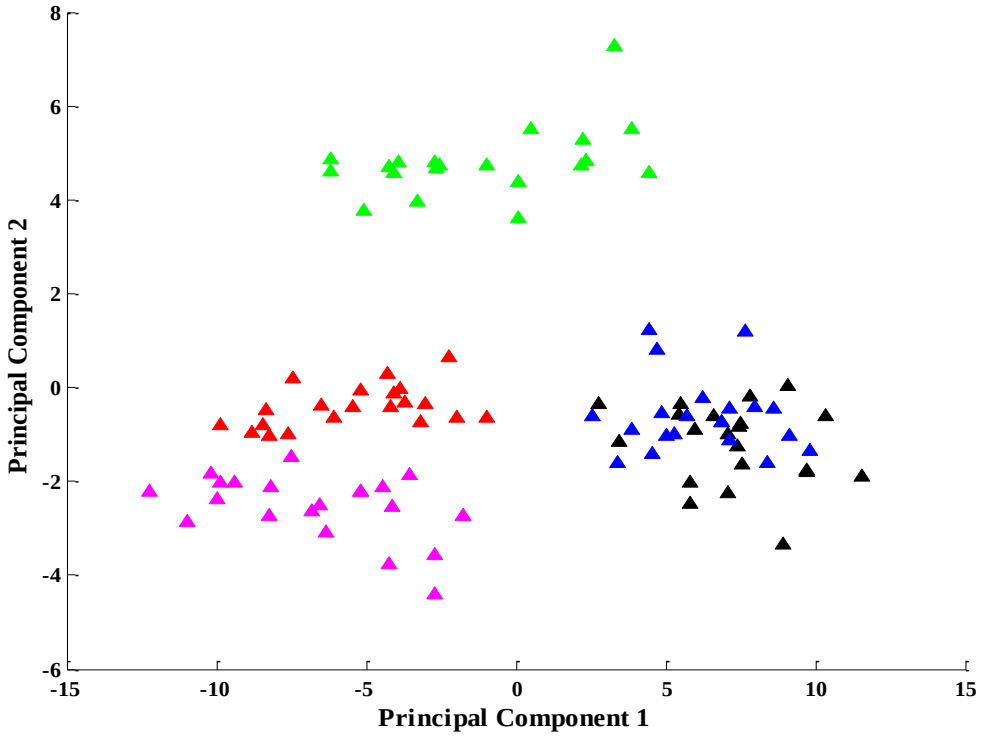


Figure 3.5: PCA score plots of rice. Black, blue, green, magenta, and red represents the control, 62.5, 125, 250, and 500 mg *n*CeO_2 L^{-1} treatments, respectively.

PCA of Root Xylem of Rice. Figure 3.5 depicts the score plot generated from the first two PCs and while Figure 3.5 displays the relative or PC loadings for rice. PC1 accounted for 64% while PC2 explained for 11% of the spectral variations. The PCA plot for rice revealed three distinct clusters namely: control and 62.5 mg *n*CeO_2 L^{-1}, 125 mg *n*CeO_2 L^{-1}, and 250 and 500 mg *n*CeO_2 L^{-1} treatments. PC1 separated 250 and 500 mg *n*CeO_2 L^{-1} treatments from control and 62.5 mg *n*CeO_2 L^{-1} treatments, whereas PC2 discriminated 125 mg *n*CeO_2 L^{-1} from the rest of the treatments. Both PCs discriminated control and 62.5 mg *n*CeO_2 L^{-1} from the ≥125 mg *n*CeO_2 L^{-1} treatments as well as 250 and 500 mg *n*CeO_2 L^{-1} treatments from the rest of the treatments. This finding suggests that 62.5 mg *n*CeO_2 L^{-1} did not induce changes on the root xylem that were different from the control whereas 250 and 500 mg

nCeO_2 L^{-1} treatments generated similar effects on the roots which were significantly different from the control and 62.5 mg *n*CeO_2 L^{-1}. On the other hand, 125 mg *n*CeO_2 L^{-1} caused alterations in the chemical makeup of the root xylem that were different from the rest of the treatments.

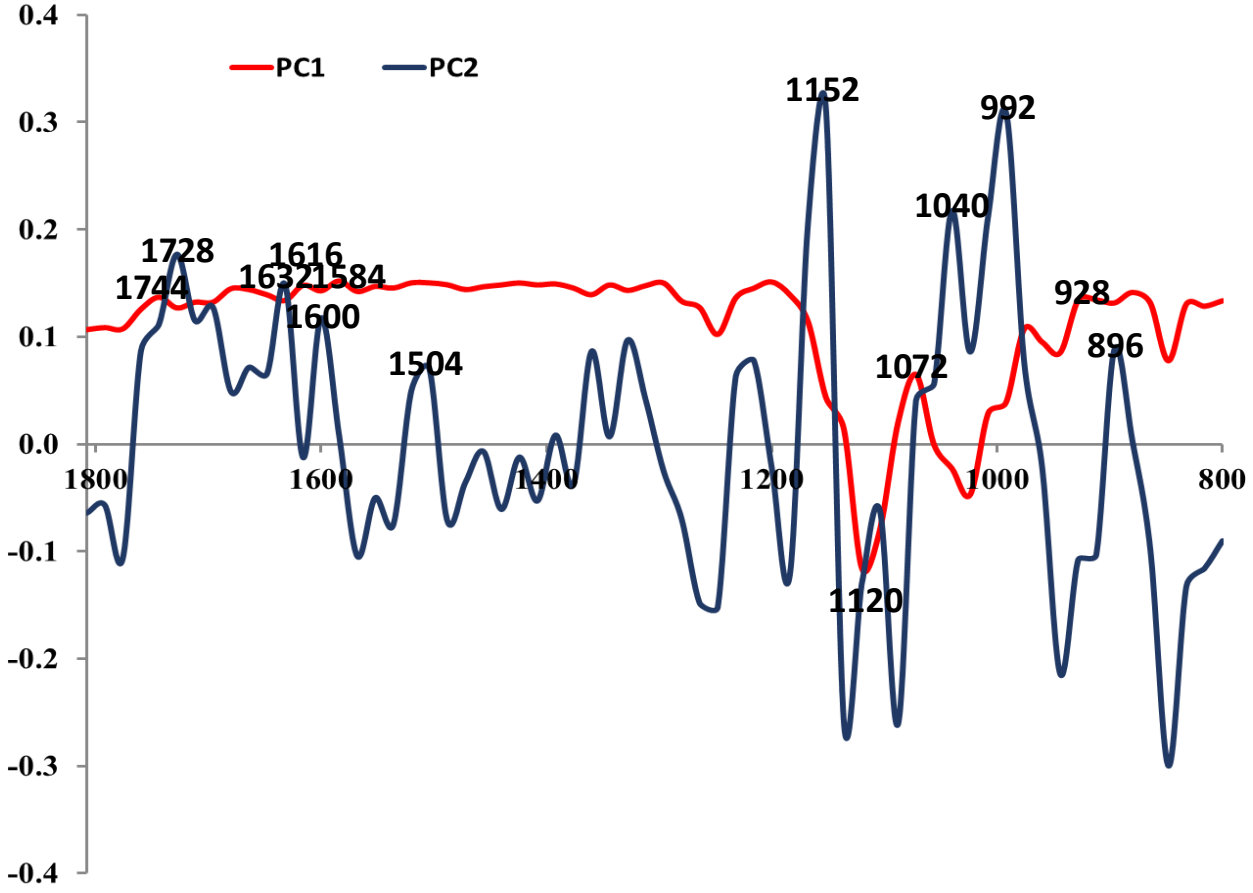


Figure 3.6: PCA score loadings for rice.

Inspection of the relative loading of PC1 in Fig. 3.6 revealed prominent negative peak at 1120 cm^{-1} which was a major dip in the spectra of control and 62.5 mg *n*CeO_2 L^{-1} treatments (Fig. 3.2). The positive peaks at 1744, 1616 and 1584 cm^{-1} could indicate presence of lipids and pectic polysaccharides or lignin. In the case of PC2, the score loading displayed notable bands at 1728, 1632, 1600, 1504, 1152, 1040, 992 and 896 cm^{-1} which were indicative of pectic polysaccharides. The bands at 1632 and 1504 cm^{-1} could possibly indicate presence of lignin; however, the absence of peak at 845 cm^{-1} signified negligible source of variability from lignin.

PCA of Root Xylem of Wheat. A more distinct separation between treatments in wheat was achieved using PC1 and PC3 as shown in Fig. 3.7. The score loadings of these PCs are also displayed in Figure 3B. As seen in Figure 3.7, PC1 scores contained over 33% of the variations which discriminated ≥125 mg *n*CeO_2 L^{-1} treatments from control and 62.5 mg *n*CeO_2 L^{-1} treatments. PC1 also afforded a more defined separation of 125 and 250 mg *n*CeO_2 L^{-1} from the rest of the treatments. On the contrary, PC2 explained 26% of the variability that allowed distinct separation between the 125 and 250 mg *n*CeO_2 L^{-1} treatments only.

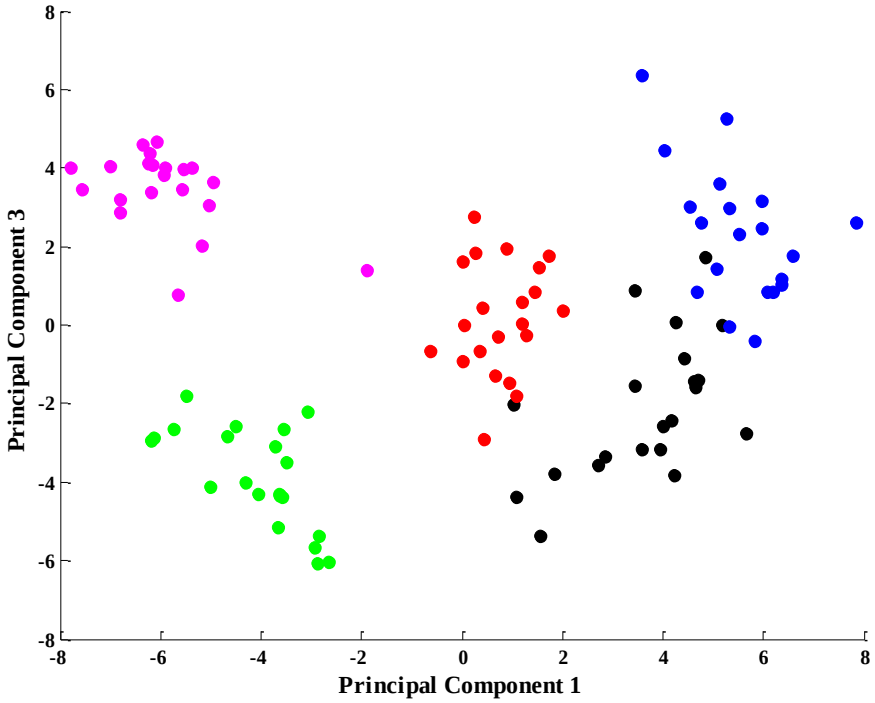


Figure 3.7: PCA score plots of wheat. Black, blue, green, magenta, and red represents the control, 62.5, 125, 250, and 500 mg *n*CeO_2 L^{-1} treatments, respectively.

PC1 score loadings revealed major positive peaks at 1744, 1456, 1392, 1244, 1264 and 1104 cm^{-1} whereas those of PC2 yielded prominent peaks at 1728, 1040, 940, 880 and 832 cm^{-1} (Fig 3.8) The peaks in PC1 indicate separation by pectic polysaccharide content while those in PC2 suggest discrimination by carbohydrate content. The control and 62.5 mg *n*CeO_2 L^{-1} treatments were separated from the rest of the treatments primarily due to the protein compositions. On the other hand, the major

peaks in PC2 signified that the 250 mg nCeO$_2$ L^{-1} treatment contained more cellulose or starch contents than the 125 mg nCeO$_2$ L^{-1} treatment.

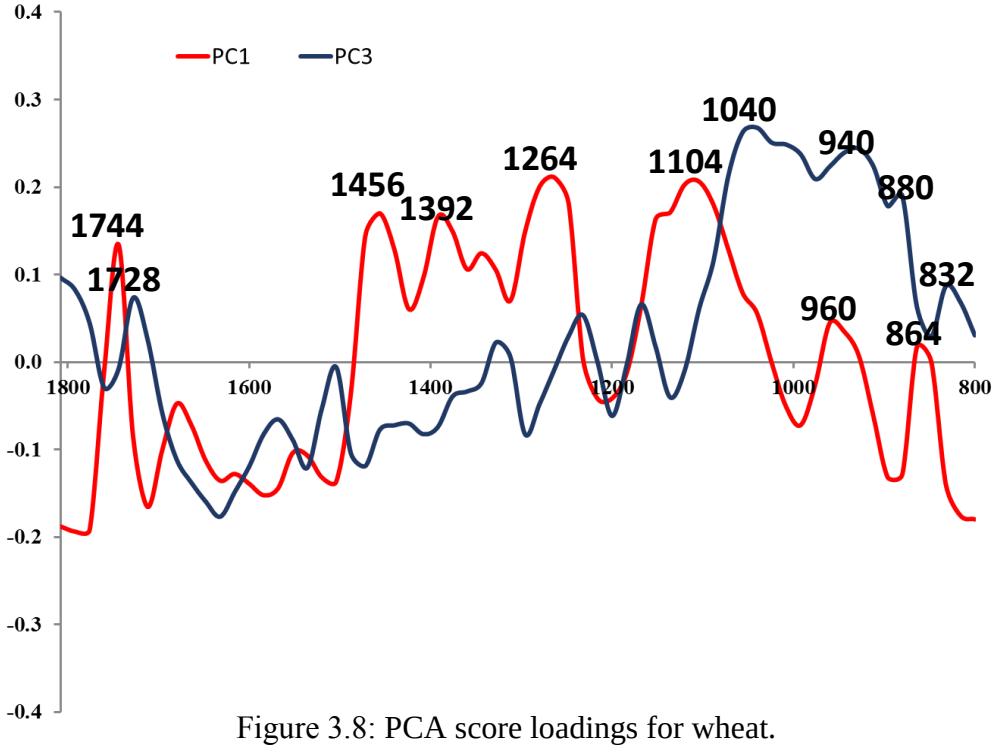


Figure 3.8: PCA score loadings for wheat.

PCA of Root Xylem of Barley. PCA also revealed variability in the spectra of barley root xylem. Figure 3.9 (upper part) shows the distinct separation between the 125 mg nCeO$_2$ L^{-1} and the rest of the treatments using the combination of PC1 and PC2 whereas Figure 3.9 (bottom part) displays the discrimination between the 62.5 mg nCeO$_2$ L^{-1} treatment and the rest of the treatment through PC1 and PC3. It is also apparent from Figure 3.9 that PC1 could cluster control and 500 mg nCeO$_2$ L^{-1} treatments separate from the 62.5, 125 and 250 mg nCeO$_2$ L^{-1} treatments. Figure 3.10 illustrates the score plots using PC1 and PC2 and PC1 and PC3. PC1, PC2 and PC3 represented 30, 21 and 14% of the chemical variations, respectively. The score loading in PC1 revealed sharper peaks in the region between 1600-1500 cm^{-1} suggesting that separation by PC1 was primarily due to the modifications in the amide I and II regions. In case of PC2, separation arose from the changes in bands corresponding to carbohydrate, while in PC3, discrimination could be attributed to the alterations in CH_3 bending vibrations and loss of peaks in the carbohydrate fingerprint region.

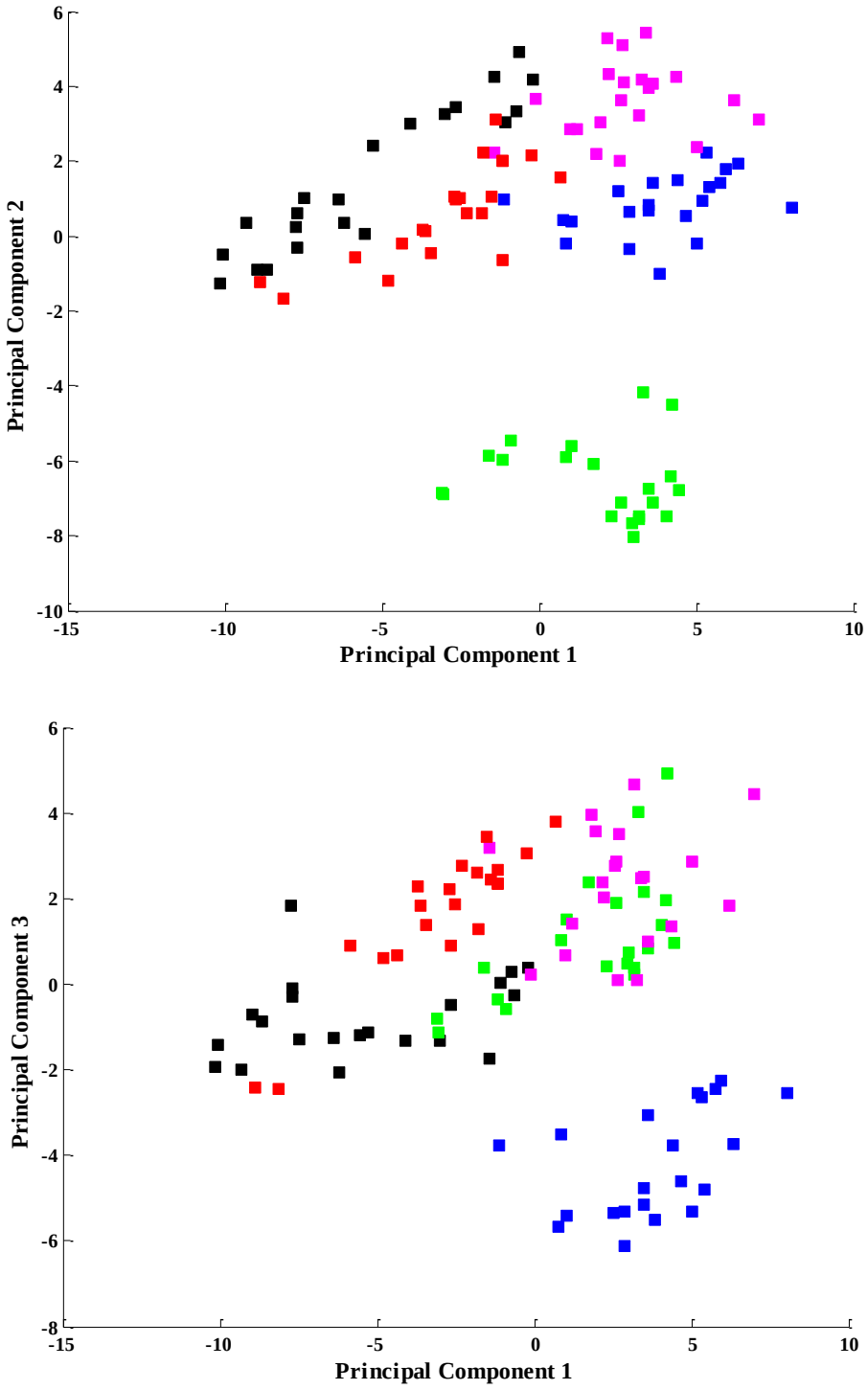


Figure 3.9: PCA score plots of barley. Black, blue, green, magenta, and red represents the control, 62.5, 125, 250, and 500 mg *n*CeO_2 L^{-1} treatments, respectively.

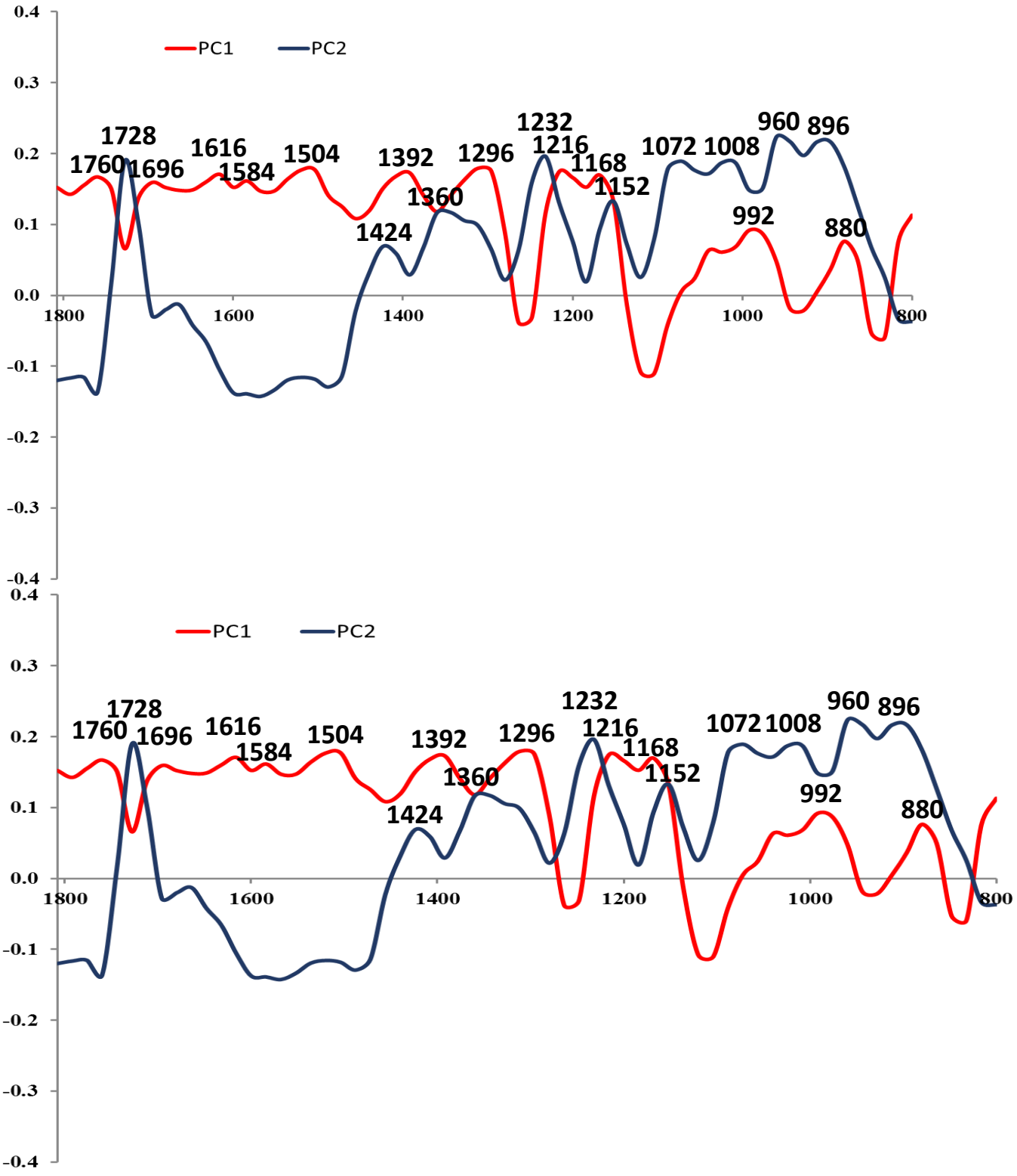
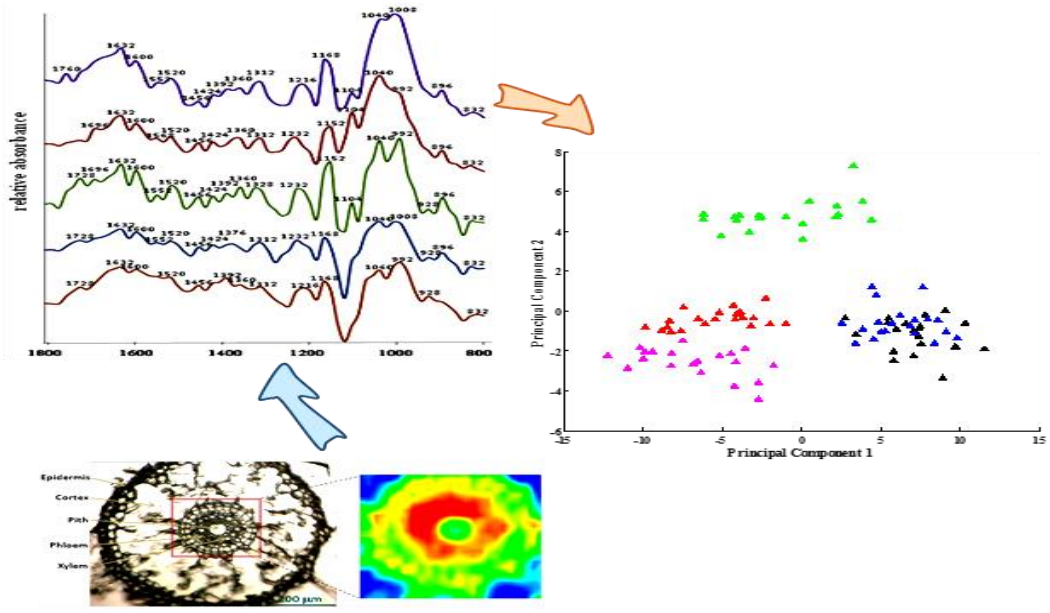


Figure 3.10: PCA score loadings for barley.

It is interesting to note that for all three seedlings, the PC score loadings that achieved spectra separation contained major peaks at 1744 or 1760 cm^{-1} representing C═O stretching of carboxyl ester in lipids or esterified pectins for primary PC and 1728 cm^{-1} indicating C═O stretching of phenolic ester for secondary PC. This finding is greatly related to the recorded band shifting from 1728 cm^{-1} to 1744 or 1760 cm^{-1} in spectra that was significantly affected by the *n*CeO_2 treatments (Figure 1), signifying that spectra separation was achieved primarily by the differences in the bands at 1744 or 1760 cm^{-1} and secondarily by the peaks at 1728 cm^{-1}.

3.4 CONCLUSION

In summary, FTIR-IMS and PCA revealed the subtle differences in the spectra of rice, wheat and barley root xylem which allowed for the distinct separation between *n*CeO_2-treated seedlings. Relative to the control of rice and wheat, 62.5 mg *n*CeO_2 L^{-1} treatment did not induce significant compositional changes while ≥125 mg *n*CeO_2 L^{-1} treatments induced significant spectral variability in the root xylem. In the case of barley, 500 mg *n*CeO_2 L^{-1} treatment had similar spectral features with the control whereas 62.5, 125 and 250 mg *n*CeO_2 L^{-1} treatments provoked compositional modifications in the root xylem. The findings revealed that *n*CeO_2 induce modifications in the root xylem of cereals.



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Chapter 4: Effect of Cerium Oxide Nanoparticles on the Quality of Rice (*Oryza sativa*) Grains[3]

Abstract

Despite the remarkable number of publications on the interaction of engineered nanomaterials (ENMs) with plants, our knowledge on the implications of ENMs in the nutritional value of food crops is still limited. This research was performed to study the quality of rice grains harvested from plants grown in soil treated with cerium oxide nanoparticles (*n*CeO_2). Three rice varieties (high, medium and low amylose) were cultivated to full maturity in soil amended with *n*CeO_2 at 0 and 500 mg kg^{-1} soil. Ce accumulation, nutrient content, antioxidant property, and nutritional quality of the rice grains were evaluated. Results showed that rice grains from *n*CeO_2 treated plants had less Fe, S, prolamin, glutelin, lauric and valeric acids, and starch. Moreover, the *n*CeO_2 reduced in grains all antioxidant values, except flavonoids. Medium and low amylose varieties accumulated more Ce in grains than high amylose variety, but the grain quality of medium amylose variety showed higher sensitivity to the *n*CeO_2 treatment. These results indicate that *n*CeO_2 could compromise the quality of rice. To the authors' knowledge, this is the first report on the effects *n*CeO_2 on rice grains quality.

Keywords: Antioxidant capacity, Grain quality, Nutritional value, Protein fractions, Starch

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4.1 INTRODUCTION

The production of engineered nanomaterials (ENMs) has rocketed to an extent that the environmental contamination and exposure to ENMs is a growing concern.[1] Cerium oxide nanoparticles (*n*CeO_2) are heavily used in applications such as chemical mechanical planarization, fuel catalysis, UV-coatings, and paints; with a conservative annual global production estimate of 1,000 tonnes.[2] The *n*CeO_2 are stable in a range of environmental media[3] and they have been found, mostly, in nanoparticulate form in different food crops.[4-6] Reports indicate that *n*CeO_2 induced physiological changes in soil-grown soybean (*Glycine max* (L.) Merr.) and corn (*Zea mays* L.).[5,7] However, fundamental questions remain on how *n*CeO_2 affects the quality of food crops.

Rice (*Oryza sativa* L.) is an important food crop feeding more than half of the world's population.[8] It is more valuable than corn (*Zea mays* L.) and wheat (*Triticum aestivum* L.) for human nutrition because it can provide superior energy per hectare and support more people per unit of land.[9] The effects of abiotic stresses on the nutrient content,[10-11] antioxidant property (phenolic contents and radical scavenging ability),[12] and nutritional quality (starch, sugar, protein, and fatty acid contents)[13-15] of rice have been studied. Studies also revealed that drought impacts the protein and starch syntheses and carbohydrate metabolism in developing rice grain.[16] As the environmental release of ENMs is a concern, it is imperative to investigate their effects on the quality of a major agricultural crop like rice.

Reports have shown that ENMs influence the elemental concentrations,[17-19] phenolic content and radical scavenging ability,[20-22] protein levels,[20,23-25] and carbohydrate contents in plants.[22,24,26,27] However, these studies have been performed at early growth stages and short exposure time; thus the effect of ENMs on the quality of fruits or grains is still unknown.

The impacts of ENMs on the quality of fruits and seeds harvested from plants cultivated to full maturity are increasingly being investigated. Reports showed that *n*Au altered the total and reducing sugars and oil contents in seeds harvested from mustard.[28] Others have shown that *n*Ag elevated the total soluble solids in fruits of cucumber (*Cucumis sativus* L.),[29] but did not change the polyphenol contents in borage (*Borago officinalis* L.).[30] Recently, fullerol [$C_{60}(OH)_{20}$] has been shown to improve the phytomedicine contents of fruits from soil-grown bitter melon (*Momordica charantia* Descourt.).[31]

However, studies on the nutritional value of the edible portions of plants grown until full maturity in ENMs-contaminated soil is still greatly lacking.

The varietal differences of rice play a role in the accumulation of toxicants in plant tissues. The heavy metals Cd, As, Cr, Cu, Hg, Ni, Pb and Zn accumulated differently in grains of different rice varieties.[32-34] Studies have also confirmed the variety-dependence in nutrient, phenolic, sugar, protein, and fatty acid contents in rice under different abiotic stresses.[10-15] The impacts of ENMs on plants through their life cycle have been investigated;[5,28-31] however, the effects of plant variety on ENMs-plant interactions have yet to be understood.

This study reports the interaction of *n*CeO_2 with three rice varieties (high, medium and low amylose contents) grown until grain production in *n*CeO_2-amended soil. IR and plasma-based spectroscopic techniques as well as biochemical assays were used to study the effects of *n*CeO_2 and rice varieties on Ce accumulation, nutrient content, antioxidant property, and nutritional quality of rice grains. This study hopes to shed light on the effect of *n*CeO_2 on the nutritional profile of rice grains.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of *n*CeO_2 Suspensions

The *n*CeO_2 (Meliorum Technologies, Rochester, NY) were procured from the University of California Center for Environmental Implications of Nanotechnology. The *n*CeO_2 were previously characterized by Keller et al.[35] The *n*CeO_2 are rod with primary size of 8 ± 1 nm, particle size of 231 ± 16 nm in DI water, surface area of 93.8 m^2g^{-1} and 95.14% purity.[35] The amount of *n*CeO_2 necessary to prepare 500 mg *n*CeO_2 kg^{-1} soil was suspended in 400 mL Millipore water by sonicating in a water bath (Crest Ultrasonics, Trenton, NJ) at 25°C for 30 min with occasional stirring. The 500 mg *n*CeO_2 kg^{-1} treatment was chosen because in preliminary studies, rice plants exposed to this concentration did not show phenotypical changes.

4.2.2 Pot Soil Preparation

Twelve plastic pots (24 cm diameter × 25 cm high) were filled with five kilograms of soil (Earthgro® potting soil) previously mixed with *n*CeO_2 suspension to have a final concentration of 500

mg kg^{-1}. The soil was equilibrated for 3 days before rice seedlings were transplanted. Twelve pots were also prepared with untreated soil (control). The pots were irrigated with distilled water to maintain saturation for the whole duration of the experiment.

4.2.3 Rice Cultivation

Rice seeds from high, medium, and low amylose varieties (Cheniere, Neptune, and 10AY004, respectively) were provided by Louisiana State University Agricultural Center (Baton Rouge, LA). Thirty-day old seedlings were transplanted into the pots and placed in a greenhouse (14-h photoperiod, 25/20°C day/night temperature, 70% relative humidity). Each pot was fertilized with 200 mL Yoshida nutrient solution[36] per week. The grains were harvested 135 days after transplanting and dried at 80°C. Brown rice, obtained by removing the rice hull, was powdered, sieved to pass mesh number 40 (W.S. Tyler, USA), and stored at -4°C until further use. The description of seedlings preparation is presented in the Supporting Information (SI) in Appendix 3.

4.2.4 Cerium, Macro, and Micronutrient Concentration in Rice Seeds

Rice grains (100 mg) were microwave digested (CEM $Mars_X$ Mathews, NC) using a mixture of plasma pure HNO_3 and H_2O_2 (1:4).[37] Elemental analysis was performed using Inductively Coupled Plasma - Optical Emission Spectroscopy while Ce quantification was achieved using ICP - Mass Spectroscopy following previously described method.[37] Blank, spikes and standard reference material (NIST-SRF 1570a) were used to validate the digestion and analytical method.

4.2.5 Analysis of Antioxidant Property

The extract for the analysis of antioxidant property was prepared based on Adom and Liu.[38] The total phenolic and flavonoid were estimated according to Dewanto et al.[39] and Jia et al.,[40] respectively. The DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS [2,2-azino-bis-(3-ethylenebenzothiozoline-6-sulfonic acid)] radical cation scavenging ability were determined based on Williams et al.[41] and Arts et al.,[41] respectively. The description of methods is shown in Appendix 3.

4.2.6 Sugars and Starch Analysis

Total and reducing sugars were extracted following previously described method.[43] Starch and total sugar were quantified according to Dubois et al.[44] while reducing sugar content was determined based on Nelson-Somogyi.[45] The non-reducing sugar content was obtained from the difference between total and reducing sugars. The methods are shown in Appendix 3.

4.2.7 Protein Analysis

The protein content of rice grains was fractionated according to Chen and Bushuk.[46] Protein from rice grain (500 mg) was extracted sequentially with 8 mL each of water, 0.5 M NaCl, 70% ethanol, and 0.05 M acetic acid, and labeled as albumin, globulin, prolamin and glutelin fractions, respectively. The protein content was quantified by Bradford method.[47]

4.2.8 Fatty Acid Analysis

Fatty acids in rice grains were esterified and extracted according to Browse et al.[48] and analyzed by GC-MS using GERSTEL Twister ™ Desorption Unit (Gerstel, Inc., Baltimore, MD, USA). A series of working calibration standards with concentrations of 0.5, 1, 10, 50, and 100 μg L^{-1} were applied for retention time identification and response curve generation. A total of 3 replicates were used. Appendix 3 presents the detailed description for GC-MS analysis.

4.2.9 FT-IR/ATR Analysis

The spectra of powdered rice were collected using FT-IR/ATR spectrometer 100 (Perkin Elmer, Shelton, CT) applying these settings: 2 cm^{-1} resolution, 4 number of scans, and air as background. The second derivative spectrum was calculated using Spectrum software (Version 6.0.2.0025, Perkin Elmer, Shelton, CT). A total of 3 replicates were used for the analysis.

4.2.10 Statistical Analysis

This study investigated two-factors and their interaction. *n*CeO_2 treatments (A) was the first factor which is composed of control and 500 mg *n*CeO_2 kg^{-1} referred to as untreated and treated, respectively. Rice varieties (V) is another factor which includes high, medium and low amylose varieties (HA, MA and LA, respectively). Data was analyzed using SAS statistical package Version 9.3 (SAS

Institute, Cary, NC, USA). A two-way ANOVA using General Linear Model was performed with the significance of the varietal means tested with Tukey's Honestly Significant Difference test based on a probability of $p \leq 0.05$ except, when otherwise stated. General treatment means were compared using Least Significant Differences. All values are in dry weight basis.

4.3 RESULTS AND DISCUSSION

4.3.1 Treatment Effects on Parameters Investigated

The ANOVA of parameters measured is presented in Appendix 3 SI Table 3.1-3.2. Results showed that *n*CeO_2 factor (A) significantly affected the Ce, K, Ca, S and Fe concentrations and all antioxidant properties, except flavonoid content. *n*CeO_2 also affected the albumin, prolamin and starch contents. ANOVA also revealed a large varietal (V) effect on most of the parameters, except for Cu concentration, DPPH, and total and non-reducing sugars. On the other hand, the interaction between A×V was significant for all protein fractions, lauric and valeric acids, phenolic content, Ce, K, Zn, Fe, Mn, and Al concentrations. These findings are in agreement with reports showing variety as an important factor in rice grains quality.[11,32,49]

4.3.2 Cerium Concentration in Rice Grains

The accumulation of ENMs like *n*CeO_2, *n*$C_{60}(OH)_{20}$, and *n*C_{70} in soybean pods,[6] bitter melon fruits,[31] and rice grains,[50] respectively, have been documented. Ce accumulation from the *n*CeO_2 treatment in rice grains is presented in Table 4.1. Data showed that Ce concentration in the HA variety did not change with *n*CeO_2 treatment. On the other hand, Ce concentrations in treated MA and LA varieties were elevated by 1126 and 996%, respectively, compared to control. Comparison between *n*CeO_2 treated plants showed that MA and LA accumulated more Ce than the HA variety. In the treatment means, the treated yielded Ce content that is remarkably higher by 805% than the untreated indicating that *n*CeO_2 treatment is a significant factor for Ce accumulation in rice grains. Similarly, *n*CeO_2 treatment greatly increased Ce concentration in soybean pods and tomato (*Solanum lycopersicum* L.) fruits.[5,51] Further studies should be performed to determine the speciation of Ce in the grains.

Table 4.1: Cerium concentration (μg kg^{-1} DW) in rice grains cultivated in soil treated or not with 500 mg $n$$CeO_2$ kg^{-1}. Values are means ± SE, n = 3. Between rice varieties, means with the same letter are not significantly different at Tukey's test ($p \leq 0.10$). Between $n$$CeO_2$ treatments, ns is not significant at $p \leq 0.05$; *, **, *** indicate significance at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

rice variety	untreated	500 mg $n$$CeO_2$ kg^{-1}
high amylose	115 ± 48a	224 ± 17b^{ns}
medium amylose	156 ± 10a	1912 ± 383a***
low amylose	169 ± 29a	1853 ± 460a**
mean	147 ± 21	1330 ± 341***

4.3.3 Nutrient Contents of Rice Grains

Dietary minerals such as micro (Fe, Zn, Se, and Cu) and macro-nutrients (Ca and Mg) are primarily obtained in diets that are reliant on grains.[11] These minerals are often lacking in human diets that biofortification is undertaken to boost their concentrations in food crops.[52] Table 4.2 shows the nutrient profile in grains harvested from $n$$CeO_2$-treated and untreated soils. As seen in the table, element concentrations in grains of the three rice varieties were different from each other except for Al, Cu, Mn, and Na in the untreated and Cu, Na, and Zn in the treated samples. Comparison between treatments showed that relative to the control, S concentration in treated HA was lower by 6.2% while Fe concentration in treated LA was significantly reduced (~69% lower). For the MA variety, K, Na, Fe, and Al concentrations in the treated plants were markedly higher than in untreated plants (20.7, 7.6, 425, and 174.2% higher, respectively), while S concentration in treated plants significantly dropped by 7.5% compared with untreated samples. In case of the treatment means, K and Ca concentrations in treated were higher than the control by 8.8 and 25.5%, respectively, whereas S and Fe concentrations in treated were lower than the untreated samples by 5.9 and 30.4%, respectively.

The increase in K and Ca in grains, although negatively impacts the eating quality, is beneficial for human nutrition.[53] On the other hand, the decrease in Fe could exacerbate the globally prevalent problem on Fe deficiency for those whose diets are primarily based on rice,[54] whereas reduction in S could affect protein and glutathione synthesis, and the antioxidant capacity of grains.[55] The effects of

nCeO_2 on K, Ca, and Fe were in agreement with those observed in rice shoots treated with Ce^{3+} (0.5 and 1.0 mM Ce^{3+}) wherein the uptake of K and Ca increased while the uptake of Fe decreased.[56] On the contrary, *n*Pd decreased the Ca concentration in kiwifruit (*Actinidia deliciosa*) pollen.[17] On the other hand, carbon nanotubes did not change the Ca accumulation in *Spartina alterniflora*,[18] and *n*TiO_2 did not affect the K concentration in *Ulmus elongata*.[19]

Table 4.2: Macronutrient concentrations (mg kg^{-1} DW) in rice grains cultivated in soil treated or not with 500 mg *n*CeO_2 kg^{-1}. Values are means ± SE, $n = 4$. HA, MA, LA = high, medium, and low amylase variety, respectively. Between rice varieties, means with the same letter are not significantly different at Tukey's test ($p \leq 0.05$). Between *n*CeO_2 treatments, ns is not significant at $p \leq 0.05$; *, **, *** indicate significance at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

Element	untreated				500 mg *n*CeO_2 kg^{-1}			
	HA	MA	LA	Mean	HA	MA	LA	Mean
P	66.1 ± 0.8a	55.7 ± 1.7b	69.0 ± 0.2b	63.6 ± 1.8	65.2 ± 0.2a^{ns}	57.7 ± 2.8b^{ns}	69.7 ± 0.8a^{ns}	64.2 ± 1.7^{ns}
S	161.8 ± 1.7b	183.4 ± 3.4a	164.6 ± 2.7b	169.9 ± 3.2	151.7 ± 3.6b*	169.6 ± 4.2a*	158.5 ± 1.7ab^{ns}	159.9 ± 2.8**
K	413.6 ± 5.5b	316.2 ± 7.7c	438.9 ± 1.7a	389.5 ± 16.2	424.9 ± 5.1ab^{ns}	381.7 ± 25.2b*	464.5 ± 11.6a^{ns}	423.7 ± 13.3**
Mg	163.5 ± 1.2b	160.1 ± 5.1b	176.8 ± 1.1a	166.8 ± 2.7	163.6 ± 1.8b^{ns}	153.4 ± 4.6b^{ns}	178.2 ± 2.8a^{ns}	165.1 ± 3.5^{ns}
Ca	6.9 ± 1.7b	18.0 ± 1.5a	21.2 ± 0.6a	15.3 ± 2.0	11.0 ± 12a^{ns}	22.2 ± 3.4a^{ns}	24.5 ± 1.4b^{ns}	19.2 ± 2.1*
Na	9.4 ± 0.1a	10.5 ± 0.2a	9.7 ± 0.4a	9.9 ± 0.2	10.2 ± 0.7a^{ns}	11.3 ± 0.2a*	10.2 ± 0.5a^{ns}	10.5 ± 0.3^{ns}
Zn	2.3 ± 0.3b	4.4 ± 0.2a	5.2 ± 0.3a	4.0 ± 0.4	3.2 ± 0.4a^{ns}	3.1 ± 0.7a^{ns}	4.8 ± 0.3a^{ns}	3.7 ± 0.4^{ns}
Fe	1.5 ± 0.1b	0.4 ± 0.1c	4.9 ± 0.2a	2.3 ± 0.6	1.1 ± 0.3b^{ns}	2.1 ± 0.1a*	1.5 ± 0.1ab*	1.6 ± 0.2**
Cu	2.6 ± 0.2a	2.8 ± 0.1a	2.7 ± 0.1a	2.8 ± 0.1	2.9 ± 0.2a^{ns}	2.5 ± 0.1a^{ns}	2.8 ± 0.1a^{ns}	2.7 ± 0.1^{ns}
Mn	0.78 ± 0.14a	0.90 ± 0.02a	0.79 ± 0.05a	0.83 ± 0.05	0.54 ± 0.03b*	0.93 ± 0.02a^{ns}	0.86 ± 0.02a^{ns}	0.77 ± 0.05^{ns}
Al	8.9 ± 3.1a	6.2 ± 0.8a	20.4 ± 5.6a	11.8 ± 2.7	8.8 ± 1.4b^{ns}	17.0 ± 1.2a*	12.3 ± 0.1b^{ns}	12.7 ± 1.2^{ns}

The Na/K, Na/Ca and Mg/K ratios are indicators of stress and quality in plants. These ratios were measured and displayed in Appendix 3 SI Table 3.3. In the treatment means, the Na/K was not affected by *n*CeO_2, while Na/Ca ratio notably decreased in the treated (0.586) relative to the untreated samples

(0.742), suggesting an increased competitive inhibition between the uptake of Na and Ca.[18] It has been reported that the accumulation of K and Ca and reduction in Na/Ca ratio by carbon nanotubes mitigated the harmful effects of Cd in *S. alterniflora*.[18] In the present study, the data also revealed that Mg/K ratio, an indicator of eating quality of rice,[53] was greatly reduced in the treated (0.391) compared to the untreated (0.435) samples. The decrease in Mg/K ratio with concomitant increase in K and Ca contents indicates poor eating quality,[53] signifying that *n*CeO_2 caused deterioration in the eating quality of rice.

4.3.4 Antioxidant Property of Rice Grains

Rice grains contain phenolic compounds and possess electron or radical scavenging ability that help reduce the risk of chronic diseases.[38,57] The influence of *n*CeO_2 on the phenolic content and radical scavenging ability of rice grains are presented in Table 4.3. In the untreated samples, phenolic and flavonoid concentrations were highest in HA and LA, respectively, while ABTS was lowest in HA. Similar trend was observed in treated grains. DPPH in both treated and untreated samples remained the same. Comparison between *n*CeO_2 treatments revealed that there were no changes in the antioxidant activities in MA, except for flavonoid concentration in treated samples, which increased by 12.5% relative to the control. On the other hand, the *n*CeO_2-treated HA and LA grains displayed marked reduction in phenolic contents (28.2 and 32.9%, respectively) and DPPH scavenging ability (42.8 and 34.3%, respectively) compared to the untreated ones. In case of treatment means, phenolic content, DPPH and ABTS were significantly reduced in treated samples by 24.1, 27.9, 12.8%, respectively, compared with untreated samples. Moreover, the data showed that HA and LA varieties were more sensitive to changes in antioxidant value compared to MA.

The results are in agreement with the inverse relationship found between magnetic nanoparticles concentration and antioxidant activity in tobacco BY-2 cells, but opposite to the direct relationship observed between *n*Ag concentration and antioxidant activity in castor seedlings reported in literature.[20,21] The decrease in phenolic content and radical scavenging ability indicate antioxidative activity of plants under metal stress since it plays a protective role in metal chelation and reactive oxygen scavenging.[58,59] *n*CeO_2 exhibits antioxidant-like property and has been found to influence the antioxidative enzymes in corn and rice,[7,60] but there is a dearth of information regarding its effects on the

phenolic compounds and radical scavenging ability in plants. The current findings indicate that *n*CeO_2 causes a negative effect on the antioxidant capacity of rice grains which could translate to reduced nutritional value of rice grain.

Table 4.3: The antioxidant property of rice grains cultivated in soil treated or not with 500 mg *n*CeO_2 kg^{-1}. Values are means ± SE, $n = 4$. HA, MA, LA = high, medium, and low variety, respectively. GAE = gallic acid equivalent. Between rice varieties, means with the same letter are not significantly different at Tukey's test ($p \leq 0.10$). Between *n*CeO_2 treatments, ns is not significant at $p \leq 0.05$; *, **, *** indicate significance at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

treatment	**rice variety**	phenolic content (μg GAE g^{-1})	flavonoid content (μg catechin g^{-1})	DPPH scavenging (%)	ABTS scavenging (%)
untreated	HA	959 ± 7a	99 ± 5b	16.6 ± 1.9a	3.1 ± 0.2b
	MA	464 ± 5b	88 ± 6b	10.6 ± 2.9a	4.3 ± 0.1a
	LA	420 ± 20b	116 ± 5a	16.9 ± 0.7a	4.4 ± 0.1a
	mean	628 ± 80	102 ± 4	14.7 ± 1.4	3.9 ± 0.2
500 mg *n*CeO_2 kg^{-1}	HA	689 ± 29a***	93 ± 2b^{ns}	9.5 ± 0.3a**	2.7 ± 0.5b^{ns}
	MA	453 ± 31b^{ns}	99 ± 4b*	11.1 ± 0.5a^{ns}	3.8 ± 0.1ab^{ns}
	LA	282 ± 6c***	115 ± 5a^{ns}	11.1 ± 1.0a*	3.8 ± 0.1a^{ns}
	mean	477 ± 57***	103 ± 4^{ns}	10.6 ± 0.4**	3.4 ± 0.2*

4.3.5 Protein Content in Protein Fractions of Rice Grains

The effect of *n*CeO_2 on the concentration of albumin, globulin, prolamin and glutelin fractions of rice grain proteins are presented in Table 4.4. In control treatment, MA grains had the highest concentration in albumin, globulin and prolamin while LA had the lowest. The concentration of glutelin did not vary between rice varieties. For the *n*CeO_2 treated plants, globulin did not change among the rice varieties while glutelin was not detected, suggesting these protein fractions are most sensitive to *n*CeO_2 toxicity. Comparison between treatments showed that the protein contents, except for glutelin, did not

change in HA. On the other hand, the protein contents in LA increased in treated (2.79-8.88 mg g^{-1}) compared with untreated (2.41-7.72 mg g^{-1}), and those of MA, except for albumin, decreased in the treated (4.32-8.51 mg g^{-1}) relative to the untreated (6.84-9.71 mg g^{-1}). Comparison between treatment means revealed that *n*CeO_2 affected the protein contents in all fractions except globulin. The *n*CeO_2 treatment increased the protein content in albumin by 7.3%, but greatly decreased that in prolamin by 17.4%, compared to the control. In general, the *n*CeO_2 decreased the protein content by 17% in MA and increased by 19% in LA relative to the untreated but did not change in HA indicating that *n*CeO_2 is detrimental to MA, beneficial to LA, and insignificant to HA.

Table 4.4: Protein content (mg g^{-1} d wt) of rice grains cultivated in soil treated or not with 500 mg *n*CeO_2 kg^{-1}. Values are means ± SE, n = 4. HA, MA, LA = high, medium, and low variety, respectively. GAE = gallic acid equivalent. Between rice varieties, means with the same letter are not significantly different at Tukey's test ($p \leq 0.10$). Between *n*CeO_2 treatments, ns is not significant at $p \leq 0.05$; *, **, *** indicate significance at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

treatment	**rice variety**	albumin	globulin	prolamin	glutelin
untreated	HA	5.45 ± 0.12ab	9.87 ± 0.18a	3.90 ± 0.09b	0.27 ± 0.03a
	MA	5.95 ± 0.05a	9.71 ± 0.42a	6.84 ± 0.11a	0.21 ± 0.04a
	LA	5.11 ± 0.21b	7.72 ± 0.20b	2.41 ± 0.10c	0.31 ± 0.03a
	mean	5.50 ± 0.13	9.10 ± 0.33	4.38 ± 0.56	0.27 ± 0.02
500 mg *n*CeO_2 kg^{-1}	HA	5.08 ± 0.11b^{ns}	9.09 ± 0.48a^{ns}	3.76 ± 0.02b^{ns}	nd
	MA	5.75 ± 0.25b^{ns}	8.51 ± 0.10a**	4.32 ± 0.03a**	nd
	LA	6.87 ± 0.17a***	8.87 ± 0.10a*	2.79 ± 0.04c***	nd
	mean	5.90 ± 0.24**	8.82 ± 0.17 ^{ns}	3.62 ± 0.19***	-

Studies have demonstrated that ENMs induce modifications on the protein levels in plants at early seedling stage.[18,20,22-25] However, its mechanism is not yet clear. Further studies are needed to elucidate the mechanism by which *n*CeO_2 alter the protein content in the grain. It is possible that *n*CeO_2

affect the gene expression for protein synthesis during grain development similar to those observed in rice under drought conditions.[16]

4.3.5 Starch and Sugar Content of Rice Grains

Rice is considered a valuable crop for human nutrition since it contains more energy to support more people per unit of land.[10] Table 4.5 presents the effects of *n*CeO_2 on the starch and sugar contents of rice grains. Results showed that the *n*CeO_2 treatment did not change the sugar contents but affected the starch concentration. HA and LA grains yielded starch contents in the treated sample that were lower than the untreated by 9.2 and 7.9%, respectively. Similarly, treatment means showed that *n*CeO_2 significantly decreased the starch concentration by 7.8% compared with the untreated. In general, the results demonstrated that *n*CeO_2 greatly reduced the starch concentration in HA and LA varieties.

Table 4.5: Starch and sugar content (mg g^{-1} d wt) of rice grains cultivated in soil treated or not with 500 mg *n*CeO_2 kg^{-1}. Values are means ± SE, $n = 4$. HA, MA, LA = high, medium, and low variety, respectively. GAE = gallic acid equivalent. Between rice varieties, means with the same letter are not significantly different at Tukey's test ($p \leq 0.10$). Between *n*CeO_2 treatments, ns is not significant at $p \leq 0.05$; *, **, *** indicate significance at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

treatment	**rice variety**	starch	total sugar	reducing sugar	non-reducing sugar
untreated	HA	731 ± 17a	8.00 ± 0.59a	3.10 ± 0.13a	4.90 ± 0.46a
	MA	663 ± 24a	8.86 ± 0.27a	2.58 ± 0.16ab	6.28 ± 0.22a
	LA	723 ± 7a	8.26 ± 0.80a	2.17 ± 0.17b	6.09 ± 0.82a
	mean	706 ± 13	8.37 ± 0.33	2.62 ± 0.14	5.76 ± 0.34
500 mg *n*CeO_2 kg^{-1}	HA	664 ± 10a*	9.25 ± 0.52a^{ns}	2.81 ± 0.17a^{ns}	6.44 ± 0.38a^{ns}
	MA	625 ± 24a^{ns}	9.00 ± 0.50a^{ns}	2.48 ± 0.10a^{ns}	6.52 ± 0.60a^{ns}
	LA	666 ± 5a*	7.49 ± 0.74a^{ns}	2.34 ± 0.08a^{ns}	5.14 ± 0.69a^{ns}
	mean	651 ± 10***	8.58 ± 0.39^{ns}	2.54 ± 0.09 ^{ns}	6.03 ± 0.35 ^{ns}

The modifications in starch and sugar contents of ENMs-treated plants as indicators of toxicity have been reported.[18,22,24,26,27] In case of studies in mature plants, foliar applications of *n*Au increased the amount of total and reducing sugars in the harvested seeds from *Brassica juncea* L.,[28] while spraying of *n*Ag resulted in high total soluble solids in fruits of cucumber.[29] The mechanism of starch modification by $nCeO_2$ in rice grains has yet to be elucidated. Related studies revealed that Ni disrupted the conversion of starch into sucrose resulting in reduced carbohydrate levels in rice roots[61] while Cd triggered dramatic perturbations in starch and sugar syntheses in rice roots and shoots.[43] Another study revealed that drought effected the carbohydrate metabolism involved in starch biosynthesis in developing rice grain.[16]

4.3.6 Fatty Acid Content in Rice Grains

Rice provides 3% of dietary fat in rice-consuming countries.[62] The effects of $nCeO_2$ on the FA profile of rice grains are presented in Table 4.6. In untreated grains, the MA variety displayed the highest amounts of lauric and valeric acids. In the $nCeO_2$-treated plants, the HA variety yielded the highest concentration of myristic acid while LA had the highest amount of valeric acid. Comparison between treatments revealed that concentrations of FA in HA and LA varieties did not change. However, compared to control, the $nCeO_2$-treated MA grains had a significant decrease in lauric, valeric, palmitic, and oleic acids (41.9, 41.2, 33.2, and 42.7%, respectively) as well as total FA (36.6%). Comparison between treatment means showed that lauric and valeric acids were most sensitive to $nCeO_2$ treatments (Table 4). However, the most abundant FAs (palmitic, oleic, and linoleic acids), which comprise 95% of the total FA in rice grains, were not affected.

Recent reports showed that *n*S and *n*ZnO altered the lipid content in bean and corn tissues[25] while *n*Au increased the oil content of seeds harvested from mustard.[28] A survey of the current literature reveals that ENMs promote membrane damage which most likely affects the FA profile in plants, similar to those observed under heavy metal stress.[63] The current understanding on the membrane damage in plants due to ENMs exposure should be explored to understand their effects on FA contents in plants.

Table 4.6: Fatty acid contents (μg kg^{-1} d wt) in rice grains cultivated in soil treated or not with 500 mg *n*CeO_2 kg^{-1}. Values are means ± SE, $n = 4$. HA, MA, LA = high, medium, and low amylase variety, respectively. Between rice varieties, means with the same letter are not significantly different at Tukey's test ($p \leq 0.05$). Between *n*CeO_2 treatments, ns is not significant at $p \leq 0.05$; *, **, *** indicate significance at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

treatment	rice varieties	lauric acid (C12)	myristic acid (C14)	valeric acid (C15)	palmitic acid (C16)	oleic acid (C18:1)	linoleic acid (C18:2)	linolenic acid (C18:3)	total
untreated	HA	275 ± 18b	317 ± 93a	333 ± 6b	3701 ± 756a	2127 ± 504a	4944 ± 1289a	1192 ± 456a	12888 ± 3071a
	MA	424 ± 21a	281 ± 40a	527 ± 15a	5054 ± 557a	3156 ± 463a	6574 ± 769a	1164 ± 89a	17181 ± 1882a
	LA	260 ± 10b	229 ± 41a	329 ± 2b	3368 ± 399a	1735 ± 292a	4237 ± 714a	686 ± 77a	10846 ± 1488a
	mean	320 ± 20	276 ± 35	397 ± 23	4041 ± 365	2340 ± 275	5252 ± 572	1014 ± 158	13638 ± 1378
500 mg *n*CeO_2 kg^{-1}	HA	283 ± 16a^{ns}	315 ± 49a^{ns}	324 ± 4b^{ns}	3811 ± 484a^{ns}	2081 ± 290a^{ns}	4801 ± 677a^{ns}	867 ± 93a^{ns}	12483 ± 1571a^{ns}
	MA	246 ± 6a***	174 ± 24b^{ns}	310 ± 5b***	3378 ± 508a*	1810 ± 347a*	4275 ± 962a^{ns}	705 ± 107a^{ns}	10898 ± 1911a*
	LA	282 ± 9a^{ns}	285 ± 18ab^{ns}	343 ± 2a^{ns}	4113 ± 434a^{ns}	2320 ± 318a^{ns}	5669 ± 730a^{ns}	857 ± 82a^{ns}	13868 ± 1552a^{ns}
	mean	270 ± 7***	258 ± 23^{ns}	326 ± 4***	3768 ± 268^{ns}	2070 ± 180^{ns}	4915 ± 455^{ns}	810 ± 54^{ns}	12417 ± 961^{ns}

4.3.7 FT-IR Analysis of Rice Grains

Recently, FTIR was employed in determining changes in the chemical makeup of mustard and rice tissues exposed to multi-walled carbon nanotubes and *n*Ag, respectively,[27,64] *Ulmus elongata* seedlings sprayed with *n*TiO_2,[19] and tomato (*Lycopersicum esculentum* L.) seeds germinated in *n*Ag.[65] However, FTIR analysis of ENMs-treated grains has not yet been reported. Figure 4.1 displays the FTIR spectra of rice grains harvested from the *n*CeO_2 treated plants. All rice varieties showed differences between the spectra of untreated and treated rice grains. Both the carbohydrate and amide regions in all varieties showed changes in the IR intensities; however, a more dramatic modification was observed in the lipid regions of both HA and MA. It is interesting to note that *n*CeO_2 caused changes in the IR intensity similar to those reported in *n*TiO_2-sprayed *U. elongata* leaves[19] and *n*Ag-treated tomato seeds,[65] but no shifting of bands similar to those observed in the roots of *n*Ag-treated rice[64] were found.

4.3.8 Pearson's Correlations

Some parameters showed significant Pearson's correlations with Ce as shown in Table 4.7. As seen in this table, Ce was positively correlated with Ca, Na and albumin, and negatively correlated to

phenolic and starch. The positive correlation between Ce and Ca concentrations is in agreement with reports showing increased absorption of Ca in rice and *Arabidopsis thaliana* treated with Ce^{3+}.[56,67] A high Na concentration could be toxic in rice grain; however, the simultaneous increase in Ca, which was statistically higher in treated compared with untreated grains (Table 4.2), indicates that Ca could have mitigated the detrimental effect of Na.[68] In addition, higher accumulation of Ce in albumin, compared to other protein fractions, in rice tissues treated with Ce has also been reported.[69] The inverse relationship between Ce and phenolic concentrations is in agreement with the generally observed role of phenolic compounds in sequestering heavy metals in plants.[58] It is possible that the reduced phenolic concentration obtained in the present study could be due to the increased Ce content in the grains (Table 4.3). The negative correlation between Ce and starch concentrations suggest that *n*CeO_2 will have negative impact on the nutritional value of rice, since, in dry weight, this contains 90% starch and provides 27% of dietary energy supply in more than 33 developing countries.[62]

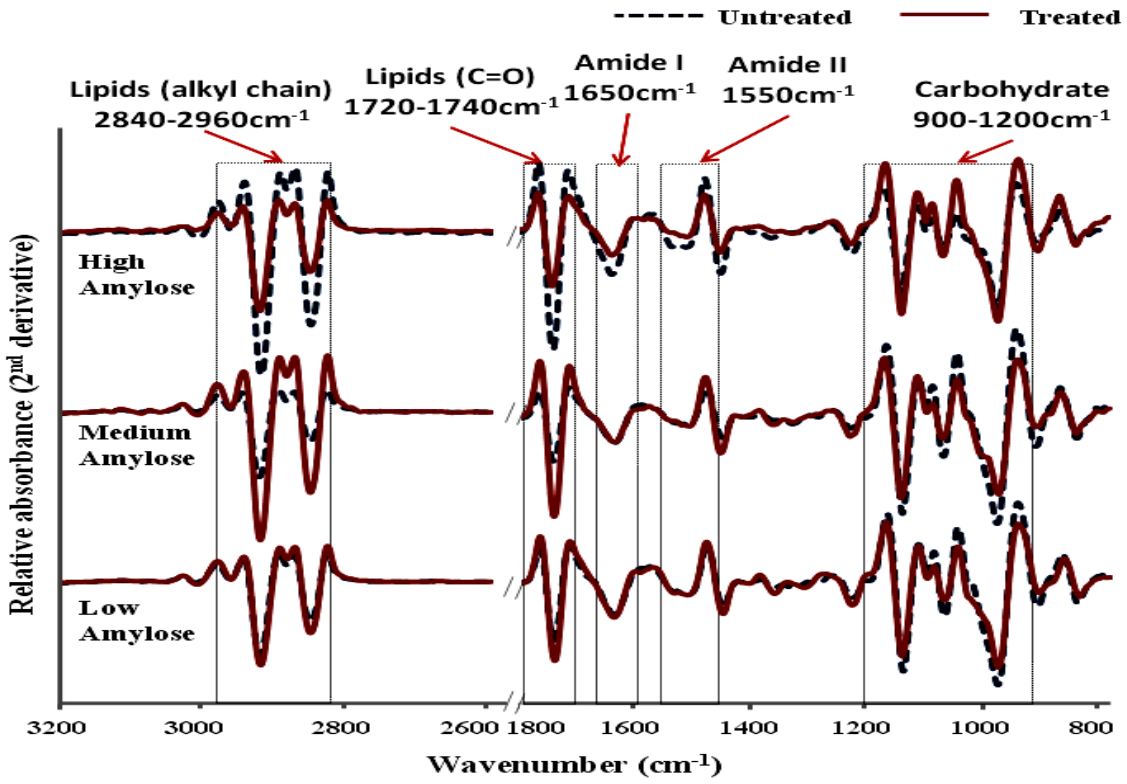


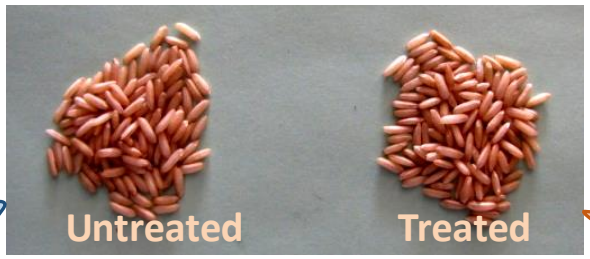
Figure 4.1: FTIR spectra of rice grain powder of different rice varieties harvested from plants cultivated in 500 mg *n*CeO_2 kg^{-1} soil. Assignment of spectra region was adapted from Dokken et al.[66]

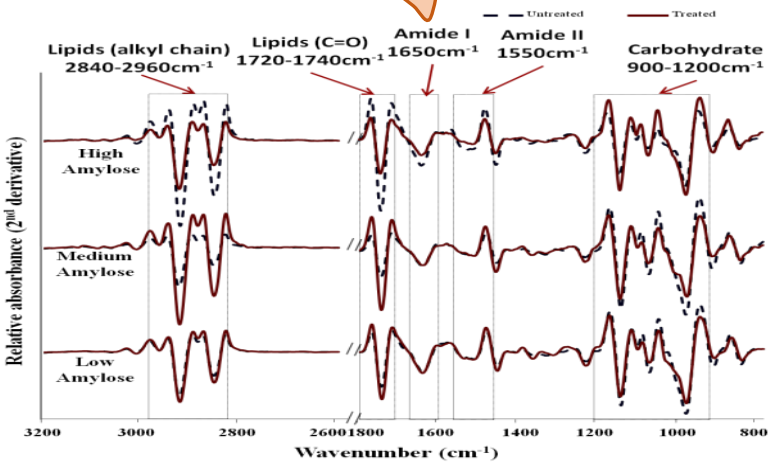
Table 4.7: Pearson's correlations parameters positively correlated with Ce concentration in grains of rice plants grown in soil treated or not with 500 mg nCeO$_2$ kg^{-1}. *, ** significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.

Ca	0.56025*
Na	0.48145*
phenolic content	-0.51249*
albumin	0.64734**
starch	-0.59486**

4.4 CONCLUSION

The findings demonstrate that nCeO$_2$ modified the nutritional value of rice, which may have a long term negative effect in food quality. This study provides the first proof that nCeO$_2$ can have significant impacts on the nutritive value of rice.





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Chapter 5: Cerium Oxide Nanoparticles Impact Yield and Modify Nutritional Parameters in Wheat (*Triticum aestivum* L.)[4]

Abstract

The implications of engineered nanomaterials on the productivity and quality of food crops are not yet well understood. The impacts of cerium oxide nanoparticles (*n*CeO_2) on growth and yield attributes and nutritional composition in wheat (*Triticum aestivum* L.) were examined. Wheat was cultivated to grain production in soil amended with 0, 125, 250, and 500 mg *n*CeO_2 kg^{-1} (control, *n*CeO_2-L, *n*CeO_2-M, and *n*CeO_2-H, respectively). At harvest, grains and tissues were analyzed for mineral, fatty acids, and amino acids contents. Results showed that, relative to the control, *n*CeO_2-H improved plant growth, shoot biomass, and grain yield by 9.0, 12.7, and 36.6%, respectively. Ce content in roots increased at increased *n*CeO_2 concentration but did not change across treatments in leaves and grains indicating a lack of Ce transport to the aboveground tissues. *n*CeO_2 elevated the K, P, S, Na, and Mn contents but decreased the Fe accumulation in roots, and modified the S and Mn storage in grains. *n*CeO_2-L modified the amino acids content, and increased the linolenic acid by up to 6.17% but decreased the linoleic acid by up to 1.63%, compared to the other treatments. This suggests nanoceria have the potential to modify crops quality with unknown consequences for living organisms.

Keywords: Amino acids, Engineered nanomaterials, Fatty acids, Spike formation/maturity

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5.1 INTRODUCTION

Engineered nanomaterials (ENMs) alter the agronomic traits like plant growth, biomass production, and chlorophyll content, and directly influence the yield, productivity, and quality of edible plants grown to full maturity.[1] Unfortunately, very few reports cover the life cycle of plants treated with ENMs to make a conclusive assessment of their long-term risks and benefits to crops. Thus, more studies in plants covering growth stages from planting/transplanting to harvesting are needed to assess the implications of ENMs on crop physiology and food quality.

Cerium oxide nanoparticles (*n*CeO_2) are widely used engineered nanoparticle that would probably end up in landfills and agricultural soils.[2] The stability of *n*CeO_2 affords them to remain in nanoparticulate form in a range of environmental media[3] and pose risks to plants. Although an increasing number of studies reveals that *n*CeO_2 interacts with plants,[1] their implications on the agronomic traits, yield, and nutritional value of food crops have not been well explored yet.

Wheat is a major cereal crop with nearly 70% of its 600 million tons annual production used in food supply.[4,5] The interaction of wheat to *n*CeO_2, *n*TiO_2, and *n*ZnO have been examined,[6,7] but the growth, yield and grain nutritional value of *n*CeO_2-cultivated wheat is yet to be reported. Since ENMs modify agronomic and yield potential of plants which are the key factors contributing to the success of wheat cultivation,[5,8] it is crucial to examine their effects on the productivity and quality of an important crop like wheat.

In this study, the responses of wheat grown to full maturity in *n*CeO_2-amended soil were investigated. In particular, the agronomic and yield components, accumulation of Ce, and macro- and micro-elements in different tissues, and grain nutritional quality were examined. This study hopes to provide insights regarding risks and benefits of *n*CeO_2 in wheat productivity and quality.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of *n*CeO_2-amended Soil

The *n*CeO_2 (Meliorum Technologies, Rochester, NY) were provided by the University of California Center for Environmental Implications of Nanotechnology. Characterization studies showed

that *n*CeO_2 are rod with primary size of 8 ± 1 nm, particle size of 231 ± 16 nm in DI water, surface area of 93.8 m^2g^{-1}, and 95.14% purity.[9] The amount of *n*CeO_2 necessary to prepare the concentrations of 125, 250, and 500 mg kg^{-1} in two kilograms of soil (Miraclegro® potting soil) was sonicated in 125 mL Millipore water (MW) at 25°C for 30 min in a water bath (Crest Ultrasonics, Trenton, NJ) before mixing thoroughly with the soil.[10] The soil was placed in plastic pots (24 cm diameter × 25 cm high) and allowed to equilibrate for 3 days before starting the experiment. Untreated soil for control was prepared using only MW.

5.2.2 Wheat Cultivation

Wheat (*Triticum aestivum* L. var. Jubilee) seeds were provided by the University of Idaho Southwest Idaho Extension Cereals Program (Parma, ID). Nine-day old seedlings were prepared,[10] and transplanted in five hills with four seedlings each totaling twenty seedlings per pot. The pots were placed in the greenhouse (14-h photoperiod, 25/20°C day/night temperature, 70% relative humidity) and irrigated three times a week with distilled water (200-300 mL) to maintain saturation. Yoshida nutrient solution[11] was used to fertilize the plant as described in the Appendix 4 SI Table 4.1. Wheat green bug (*Schizaphis graminum*) appeared 65 days after transplanting (DAT) which was immediately sprayed with 5 mg malathion L^{-1} (Ortho® MAX® Malathion). At harvest (94 DAT), spikes, flag leaves, and roots were collected, washed thoroughly with MW, and oven-dried at 80°C for 5 days. Roots were cleaned thoroughly for 30 min in running tap water and rinsed for 5 min in distilled water. The grains were de-hulled and stored at 4°C until use. Samples for analysis were powdered and passed through mesh number 40 (W.S. Tyler, USA).

5.2.3 Evaluation of Agronomic Traits and Yield Components

Agronomic traits (plant height, chlorophyll content, number of tillers, peduncle length, and number of internodes) were assessed using five measurements from randomly selected plants in each pot. Plant height and chlorophyll content were measured at heading stage (30 DAT). Plant height was determined from soil surface to the flag leaf while chlorophyll content was measured in the flag leaf using SPAD chlorophyll meter (Spectrum Technologies, Aurora, IL). The number of spikes was counted when the spikes were fully exposed while physiological maturity was recorded when the spike was

completely dry. Shoot biomass from each pot was determined at harvest. For the yield components, the following parameters were measured using five random samples: spike length, number of spikes, number of spikelets/spike, number of grains/spike, gain yield/spike, 100-grain weight, and grain yield/pot. The five measurements from each pot for agronomic and yield components, except grain yield/pot, were averaged and used as one replicate measurement for statistical analyses.

5.2.4 Cerium, Macro, and Micronutrient Concentration in Wheat

A microwave accelerated reaction system (CEM Mars$_X$ Mathews, NC) was used to digest the roots, leaves, and grains in a mixture of plasma pure HNO_3 and H_2O_2 (1:4) and analyzed for elemental composition using ICP-OES/MS (Perkin Elmer, Waltham, MA) according to the method previously employed by Rico et al.[10] Blank, spikes and standard reference material (NIST-SRF 1570a) were used to validate the digestion and analytical method.

5.2.2 Amino Acid Profiling

Amino acid analysis was performed following the method of Anjum et al.[12] Briefly, the powdered grains (50 mg) were mixed with 10 mL of 6 N HCL in a test tube which was subsequently evacuated by nitrogen, sealed, and placed in an oven (110°C) for 24 h. The hydrolyzate was filtered and dried under vacuum (60°C) before dissolving in 1 mL phosphate buffer (pH 2.2). An aliquot (100 µL) was diluted 10 times with buffer, filtered in a 45 µm syringe filter, and analyzed using Biochrom 20 amino acid analyzer (Biochrom, USA).

5.2.2 Fatty Acid Analysis

Fatty acid content was estimated according to Asekova et al.[13] The powdered grains (500 mg) were placed in a 5 mL solution of chloroform:hexane:methanol (8:5:2 v/v/v) overnight. Derivatization was performed by mixing 100-µL extract with 75 µL of methylating reagent (0.25 M methanolic sodiummethoxide: petroleum ether: ethyl ether [1:5:2, v/v/v]). After 10 h, the solution was diluted to 1 mL using hexane. Fatty acid content was analyzed using an Agilent (Palo Alto, CA) Series 7890 capillary gas chromatograph fitted with a flame ionization detector with an AT-Silar capillary column (Alltech Associates, Derfield, IL). The temperatures of the oven, injector, and detector were set at 210,

250, and 230°C, respectively. Standard fatty acid mixtures (Animal and Vegetable Oil Reference Mixture 6, AOACS) were used as calibration reference standards.

5.2.2 Data Analysis

The effect of *n*CeO_2 treatment on measured parameters was determined using the General Linear Model procedure in SAS statistical package Version 9.3 (SAS Institute, Cary, NC, USA). Statistical significance between means was tested with Tukey's honestly significant difference test at $p \leq 0.05$.

5.3 RESULTS AND DISCUSSION

5.3.1 Cerium Accumulation in Wheat Tissues

The accumulation of ENMs and their component metals in edible portions of the plant is becoming a concern. Table 5.1 presents the Ce content in the different wheat tissues. In the roots, control and *n*CeO_2-L yielded Ce content (1974 and 16828 μg kg^{-1}, respectively) significantly lower than those in *n*CeO_2-M and *n*CeO_2-H treatments (80933 and 111121 μg kg^{-1}, respectively). The Ce contents in the *n*CeO_2-treated leaves and grains were in the range 430-442 μg kg^{-1} and 155-263 μg kg^{-1}, respectively. However these values did not reach statistical differences compared with values recorded in their respective controls (600 and 248 μg kg^{-1}, respectively). Similarly, the Ce concentration in the hull did not change significantly across the treatments (412-450 μg kg^{-1}) strongly suggesting the lack of Ce translocation in wheat grains. A study revealed that *n*CeO_2 were stable in 16 different types of soil exhibiting 3.1% dissolution rate at pH 4.[3] Microscopy techniques have also shown that *n*CeO_2 accumulated largely in nanoparticulate form in food crops cultivated in soil and hydroponic suspension.[1] The current findings are in good agreement with previous reports in wheat showing that *n*CeO_2 adsorbed on the root surface,[7] but are in stark contrast with the remarkable increases in Ce content in rice grains, soybean pods, and tomato and cucumber fruits [10,14-16] signifying the differences in root storage and aboveground tissues transport of Ce in plants.

Table 5.1: Cerium concentrations (μg kg^{-1} d wt) in different tissues of wheat cultivated to grain production in *n*CeO_2-amended soil. Values are means ± SE, $n = 3$. Same letters mean no statistical difference between treatments at Tukey's test ($p \leq 0.05$).

***n*CeO_2 concentration (mg kg^{-1})**	**root**	**leaves**	**hull**	**grains**
0	1974 ± 549c	600 ± 85a	435 ± 20a	248 ± 96a
125	16828 ± 332c	442 ± 19a	412 ± 9a	155 ± 45a
250	80934 ± 200b	431 ± 50a	450 ± 12a	186 ± 32a
500	111121 ± 650a	430 ± 64a	428 ± 7a	263 ± 44a

5.3.2 Modifications in Growth Characters

The agronomic characters of *n*CeO_2-exposed wheat are presented in Table 5.2 and Figure 5.1. It is evident from Table 5.2 that *n*CeO_2 had no effect on the exposed peduncle length, number of tillers, number of internodes and chlorophyll content. *n*CeO_2-H improved plant height and shoot biomass by up to 5.32% and 12.71%, respectively, compared to the rest of the treatments. Previous studies found that *n*CeO_2 had no impact on the growth, gas exchange and chlorophyll content of cucumber,[17] but improved the dry stem and leaf biomass in soybean,[14] and slightly enhanced the plant growth but not the chlorophyll content in tomato.[15] On the contrary, *n*ZnO- or *n*TiO_2-exposed wheat had reduced biomass production.[6]

Table 5.2: Growth characters of wheat cultivated to grain production in *n*CeO_2-amended soil. Values are means ± SE, $n = 3$. Same letters mean no statistical difference between treatments at Tukey's test ($p \leq 0.05$).

***n*CeO_2 concentration (mg kg^{-1})**	**plant height (cm)**	**exposed peduncle length (cm)**	**number of tillers**	**number of internodes**	**chlorophyll content (SPAD unit)**	**shoot biomass (g)**
0	43.6 ± 0.7b	10.4 ± 1.0a	6.7 ± 0.2a	4.5 ± 0.1a	38.7 ± 0.8a	18.1 ± 0.2b
125	45.1 ± 0.5b	10.3 ± 0.1a	5.9 ± 0.2a	4.7 ± 0.1a	40.6 ± 0.8a	18.5 ± 0.6b
250	45.4 ± 0.5ab	10.5 ± 0.1a	5.8 ± 0.3a	4.3 ± 0.2a	39.2 ± 0.8a	19.2 ± 0.1ab
500	47.5 ± 0.2a	11.1 ± 0.9a	6.5 ± 0.1a	4.5 ± 0.1a	39.3 ± 0.4a	20.4 ± 0.3a

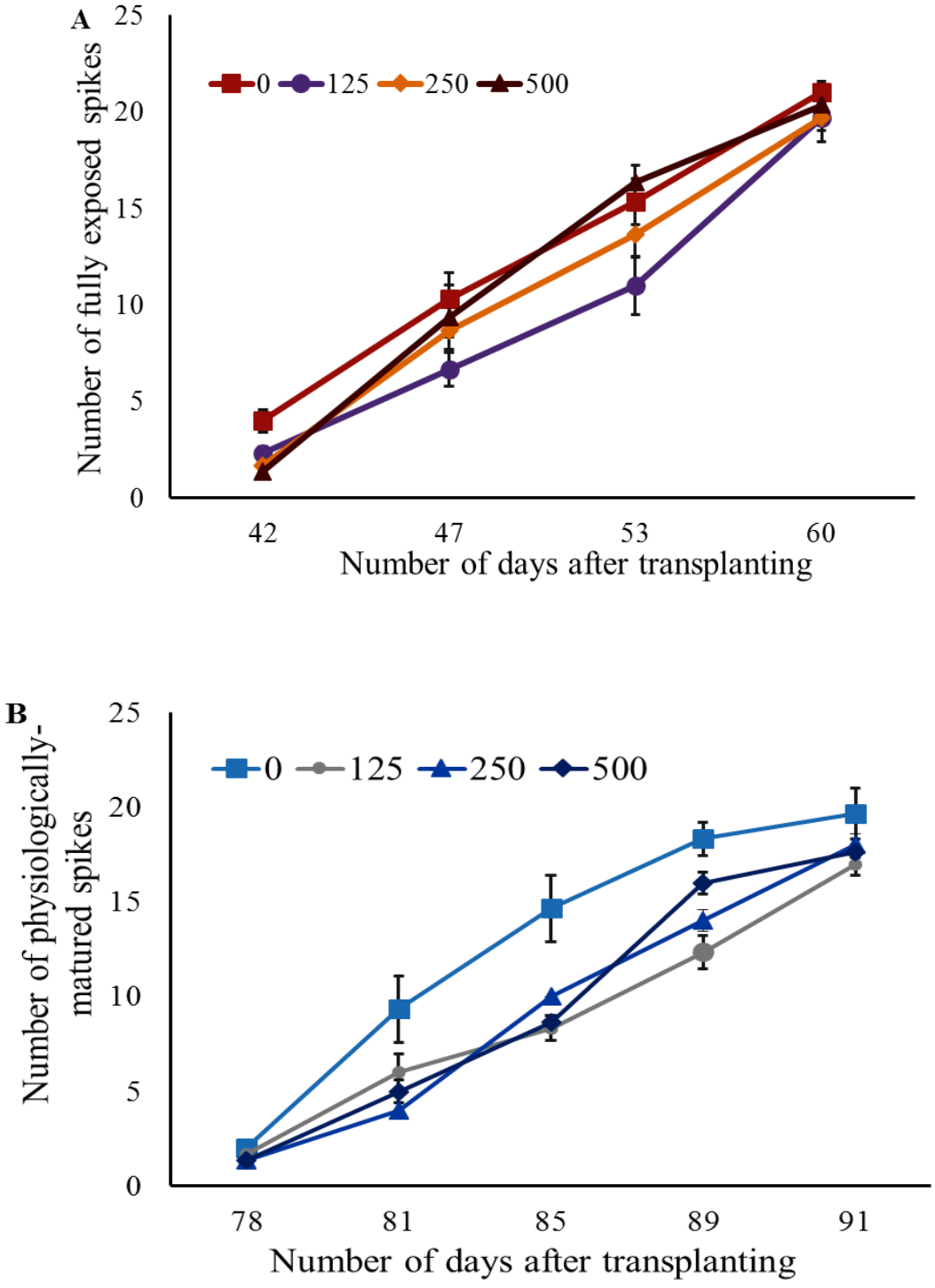


Figure 5.1: Spike formation (A) and spike physiological maturity (B) in wheat cultivated to grain production in *n*CeO_2-amended soil. 0, 125, 250, and 500 denote control, *n*CeO_2-L, *n*CeO_2-M, and *n*CeO_2-H, respectively. Each data point is mean ± SE (n = 3).

In addition, *n*CeO_2 treatment caused a 6-day delay in spike formation and physiological maturity in wheat compared to control (Figure 5.1A, B; Appendix 4 SI Tables 4.2 and 4.3). Wang et al.[15] found that *n*CeO_2 prolonged by 2 days the fruit ripening in tomato. Although the extended period of time for spike formation and physiological maturity could improve yield in wheat,[8] it may have significant impacts on seeding time since correct planting date is important for growth stages and phenological development to occur at appropriate environmental conditions.[18]

5.3.3 Changes in Yield Parameters

The impact of metal oxide ENMs on the crop yield is not yet well explored. Table 5.3 displays the influence of *n*CeO_2 on yield parameters of wheat. *n*CeO_2 altered all yield parameters, except for spike length and 100-grain weight. The number of spikelets per spike was highest (42.5) at *n*CeO_2-H and lowest (30.8) in control. However, the number of grains per spike in *n*CeO_2-L (25.3) and *n*CeO_2-H (26.0) were higher than that of the control (21.9) and *n*CeO_2-M (21.6). The grain yield per spike was significantly enhanced at *n*CeO_2-H (808.9 mg), but markedly reduced at *n*CeO_2-L (663.8 mg). In case of yield, only *n*CeO_2-H recorded a significantly higher yield per pot (12.7 g) compared to control (9.3 g). Current literature shows varying effects of *n*CeO_2 on the productivity of food crops; *n*CeO_2 had no impact on pod yield in soybean,[14] enhanced fruit production in tomato,[15] and reduced yield in cucumber.[17]

Table 5.3: Yield parameters of wheat cultivated to grain production in *n*CeO_2-amended soil. Values are means ± SE, $n = 3$. Same letters mean no statistical difference between treatments at Tukey's test ($p \leq 0.05$).

***n*CeO_2 concentration (mg kg^{-1})**	**spike length (cm)**	**number of spikelets per spike**	**number of grains per spike**	**grain yield per spike (mg)**	**100-grain weight (g)**	**yield per pot (g)**
0	7.9 ± 0.2a	30.8 ± 0.8c	21.9 ± 0.5b	761.8 ± 9.3ab	2.65 ± 0.03a	9.3 ± 0.4b
125	7.7 ± 0.1a	36.1 ± 0.4b	25.3 ± 0.7a	663.8 ± 20.8c	2.63 ± 0.09a	10.2 ± 0.3ab
250	7.8 ± 0.2a	34.9 ± 1.1bc	21.6 ± 0.3b	738.9 ± 8.4b	2.71 ± 0.09a	10.5 ± 0.2ab
500	8.3 ± 0.1a	42.5 ± 1.5a	26.0 ± 0.8a	808.9 ± 8.2a	2.83 ± 0.04a	12.7 ± 0.4a

Wang et al.[15] found that longer vegetative stage enhanced fruit yield in *n*CeO_2-exposed tomato. Similarly, the superior yield at *n*CeO_2-H was consistent with the delayed spike formation and maturity as well as improved plant height, shoot biomass, number of grains and spikelets per spike, and grain yield per spike. It is interesting to note that the reduced yield in the control could be due to low number of grains whereas, at *n*CeO_2-L, the superior number of grains per spike and low grain weight did not result in improved yield.

5.3.4 Mineral Accumulation

Table 5.4 presents the elemental contents in roots, leaves and grains of wheat. The *n*CeO_2 treatments did not modify the Ca, Zn and Cu contents (Appendix 4 SI Table 4.4) but altered the accumulations of K, P, S, Na, Fe and Mn in roots, P, Mg and Fe in leaves, and S and Mn in grains. In roots, K content markedly improved at *n*CeO_2-H (18173 mg kg^{-1}) compared to the rest of the treatments (8081-12658 mg kg^{-1}) while P accumulation significantly increased at *n*CeO_2-M (3686 mg kg^{-1}) relative to control and *n*CeO_2-L (2417 and 2903 mg kg^{-1}, respectively). S content was elevated at *n*CeO_2-M (2445 mg kg^{-1}) and *n*CeO_2-H (2772 mg kg^{-1}) in the roots but decreased at *n*CeO_2-L and *n*CeO_2-M (1621 mg kg^{-1}) in the grains compared to their respective controls (1667 and 1726 mg kg^{-1}, respectively). Mg accumulation in leaves was enhanced at *n*CeO_2-M (4559 mg kg^{-1}) relative to the control (3944 mg kg^{-1}). The Na content in roots increased in all *n*CeO_2 treatments (3526-4121 mg kg^{-1}) relative to the control (2447 mg kg^{-1}) whereas Fe content in all *n*CeO_2 treatments in roots (388-426 mg kg^{-1}) and *n*CeO_2-M and *n*CeO_2-H in leaves (97 and 101 mg kg^{-1}, respectively) decreased compared to their controls (455 and 117 mg kg^{-1}, respectively). In contrast, Mn content in roots and grains did not differ between *n*CeO_2 treatments (56-72 and 48-52 mg kg^{-1}, respectively); however, *n*CeO_2-H yielded superior Mn content (72 mg kg^{-1}) in roots compared to control (49 mg kg^{-1}) while *n*CeO_2-M had reduced Mn accumulation (48 mg kg^{-1}) in grains relative to the control (57 mg kg^{-1}).

These findings suggest that *n*CeO_2 modulated the elemental contents in roots and leaves without very detrimental impact on the nutritional content of grains. It is possible that the modulation in P storage was due to the association of Ce to PO_4^{3-} as observed in *n*CeO_2-exposed soybean and cucumber.[1] The increase in S concentration was opposite to that recorded in *n*CeO_2-treated soybean wherein S

accumulation did not change in all tissues analyzed.[19] Likewise, *n*CeO_2 altered Na and Fe concentrations in wheat roots and leaves which were not observed in previous studies in soybean.[19] Unfortunately, there is a lack of understanding in current literature regarding the aggregation, association, and mobility of elements in *n*CeO_2-amended soil that could help explain the changes in their accumulations in wheat. In grains, only S concentration at *n*CeO_2-L and *n*CeO_2-M and Mn concentration at *n*CeO_2-M had marked reductions compared to their respective controls. Previous reports also revealed that *n*CeO_2 altered the S and Mn accumulations in rice grains[10] and cucumber fruit,[16] respectively, but not in soybean pods.[19] At this point it is difficult to determine the cause of the reduced accumulations of S and Mn in grains, but their decreases may have negative implications on food quality.

Table 5.4: Elemental concentration (mg kg^{-1}) in different tissues of wheat cultivated to grain production in *n*CeO_2-amended soil. Values are means ± SE, $n = 3$. Same letters mean no statistical difference between treatments at Tukey's test ($p \leq 0.05$).

***n*CeO_2 concentration (mg kg^{-1})**	**K**	**P**	**Mg**	**S**	**Na**	**Fe**	**Mn**
			roots				
0	8081 ± 191c	2417 ± 111c	910 ± 86a	1667 ± 51b	2447 ± 72c	455 ± 86a	49 ± 5b
125	9600 ± 346c	2903 ± 250bc	1079 ± 122a	2224 ± 182ab	3526 ± 125b	408 ± 75b	56 ± 7ab
250	12658 ± 319b	3686 ± 162a	1184 ± 47a	2445 ± 76a	4103 ± 102a	388 ± 60b	57 ± 4ab
500	18173 ± 620a	3173 ± 28ab	1257 ± 45a	2772 ± 177a	4121 ± 164a	426 ± 27b	72 ± 2a
			leaves				
0	25181 ± 481a	2935 ± 162ab	3944 ± 93b	2706 ± 122a	280 ± 12a	117 ± 2a	162 ± 15a
125	24783 ± 878a	2626 ± 173b	4011 ± 81ab	2701 ± 115a	264 ± 29a	112 ± 5ab	136 ± 14a
250	23345 ± 665a	3076 ± 355ab	4559 ± 7a	2501 ± 31a	248 ± 19a	97 ± 1c	129 ± 2a
500	22973 ± 482a	3684 ± 106a	3956 ± 218b	2518 ± 123a	213 ± 9a	101 ± 2bc	159 ± 7a
			grains				
0	5079 ± 94a	5262 ± 93a	1906 ± 30a	1726 ± 55a	18 ± 1a	50 ± 1ab	57 ± 1a
125	4787 ± 132a	5064 ± 137a	1803 ± 15a	1621 ± 34b	20 ± 1a	46 ± 3b	50 ± 1ab
250	4765 ± 28a	5250 ± 85a	1886 ± 35a	1621 ± 10b	22 ± 2a	53 ± 1ab	48 ± 2b
500	5295 ± 268a	5584 ± 220a	1824 ± 83a	1648 ± 33ab	22 ± 1a	60 ± 3a	52 ± 1ab

5.3.5 Amino Acid Content in Grains

The effects of *n*CeO_2 on the amino acid (AA) contents of wheat grains was examined and the data is displayed in Table 5. Results showed that *n*CeO_2 did not modify the concentrations of alanine, cysteine, leucine, methionine, proline, and glutamic acid. *n*CeO_2-L had generally elevated AA content but statistical difference relative to the control was only achieved in aspartic acid, glycine, lysine, histidine, and arginine. The control, *n*CeO_2-M and *n*CeO_2-H had similar levels of AA except for superior amounts at *n*CeO_2-H treatment compared to control and *n*CeO_2-M for histidine and phenylalanine, respectively. Among the essential AA, *n*CeO_2-L increased the lysine and histidine contents (15.33 and 13.67 μg g^{-1}, respectively) compared to control (13.24 and 11.76 μg g^{-1}, respectively). The amide-NH_3 was highest at *n*CeO_2-L and *n*CeO_2-M (26.50 and 26.30-μg g^{-1}, respectively) and lowest in the control (24.05 μg g^{-1}), whereas total AA contents did not change between treatments although it had the tendency to increase at *n*CeO_2-L (540.73 μg g^{-1}) compared to the rest of the treatments (494.72-499.08 μg g^{-1}).

These findings revealed that *n*CeO_2 interfered with the AA synthesis in wheat with *n*CeO_2-L demonstrating the tendency to improve AA content in wheat grains. Currently, there is no other study regarding the effects of ENMs on AA or protein synthesis in cereal grains that makes the interpretation of the present results difficult. However, studies exhibited that AA metabolism or protein content was modified in ENMs-exposed wheat and rice[10,20] while various ENMs (*n*Ag, *n*S, *n*SiO_2, *n*TiO_2, and *n*ZnO) modified protein levels in different crops.[1]

5.3.6 Relative Fatty Acid Abundance in Grains

The influence of *n*CeO_2 on the relative fatty acid (FA) abundance in wheat grains was analyzed and presented in Table 6. The analysis revealed that the *n*CeO_2 treatments did not alter the relative FA abundance of palmitic, oleic, and stearic acids. In contrast, *n*CeO_2-L caused a remarkable reduction in the relative FA content of linoleic acid by up to 1.63% relative to the rest of the treatments but afforded a marked increase in the relative FA content of linolenic acid by up to 6.17% relative to the rest of the treatments. These data revealed that *n*CeO_2 also disturbed the FA synthesis and storage in wheat grains.

Table 5.5: Amino acid content (μg g^{-1}) in wheat grains harvested from wheat cultivated in *n*CeO_2-amended soil. Values are means ± SE, $n = 3$. Same letters in each row mean no statistical difference between treatments at Duncan's test ($p \leq 0.10$).

Amino acids	***n*CeO_2 concentration (mg kg^{-1})**			
	0	**125**	**250**	**500**
Alanine	17.10 ± 1.12a	18.31 ± 0.83a	15.16 ± 0.29a	15.54 ± 0.50a
Cysteine	7.56 ± 0.20a	7.88 ± 0.19a	7.29 ± 0.14a	7.41 ± 0.23a
Glutamic acid	148.97 ± 2.59a	158.53 ± 3.83a	139.28 ± 3.84a	154.45 ± 7.96a
Leucine	32.61 ± 0.95a	36.42 ± 1.28a	32.76 ± 1.69a	33.84 ± 1.88a
Methionine	5.51 ± 0.97a	6.12 ± 0.42a	5.65 ± 0.05a	5.92 ± 0.18a
Proline	54.49 ± 2.15a	55.96 ± 1.55a	55.51 ± 2.44a	52.51 ± 2.23a
Arginine	24.64 ± 0.42b	29.96 ± 1.39a	25.08 ± 0.68b	24.53 ± 1.02b
Aspartic acid	23.29 ± 0.43b	26.77 ± 0.99a	22.46 ± 0.44b	22.67 ± 0.66b
Glycine	19.13 ± 0.31b	21.83 ± 0.80a	18.39 ± 0.32b	18.68 ± 0.74b
Histidine	11.76 ± 0.30b	13.67 ± 0.29a	12.74 ± 0.39ab	13.34 ± 0.04a
Isoleucine	15.23 ± 0.39ab	17.02 ± 0.66a	14.24 ± 0.29b	14.64 ± 0.58b
Lysine	13.24 ± 0.41b	15.33 ± 0.54a	12.88 ± 0.30b	13.89 ± 0.46ab
Phenylalanine	22.87 ± 0.40ab	25.01 ± 0.71a	21.36 ± 0.49b	24.61 ± 0.52a
Serine	22.92 ± 0.31ab	24.70 ± 0.77a	21.59 ± 0.49b	21.82 ± 0.97ab
Threonine	14.66 ± 0.31ab	16.10 ± 0.51a	13.91 ± 0.27b	13.99 ± 0.45b
Tyrosine	14.77 ± 0.21ab	16.52 ± 0.53a	14.24 ± 0.36b	15.15 ± 0.41ab
Valine	21.95 ± 0.45ab	24.10 ± 0.83a	20.74 ± 0.32b	20.73 ± 0.84b
Amide-NH_3	24.05 ± 0.19b	26.50 ± 0.50a	26.30 ± 0.25a	25.36 ± 0.49ab
Total	494.72 ± 11.38a	540.73 ± 15.61a	479.60 ± 9.34a	499.08 ± 18.46a

Similar to that observed in the AA analysis, *n*CeO_2-L provoked significant changes on FA concentrations indicating the impact of this treatment on the nutritional value of wheat grains. Previous studies have shown that *n*CeO_2 modified the FA content in rice grains[10] while *n*Au stimulated oil production in mustard seeds (*Brassica juncea*).[21] The modifications in AA and FA contents signify that *n*CeO_2 could have serious implications on the nutritional value of wheat grains.

Table 5.6: Fatty acid content (% relative fatty acid) in wheat grains harvested from wheat cultivated in *n*CeO_2-amended soil. Values are means ± SE, $n = 3$. Same letters in each row mean no statistical difference between treatments at Duncan's test ($p \leq 0.10$).

***n*CeO_2 concentration (mg kg^{-1})**	**palmitic (C16)**	**stearic (C18)**	**oleic (C18:1)**	**linoleic (C18:2)**	**linolenic (C18:3)**
0	18.70 ± 0.10a	1.98 ± 0.10a	14.85 ± 0.18a	59.65 ± 0.13a	4.63 ± 0.06b
125	18.46 ± 0.12a	1.88 ± 0.08a	15.64 ± 0.33a	58.98 ± 0.16b	4.82 ± 0.01a
250	18.70 ± 0.07a	1.84 ± 0.02a	15.02 ± 0.07a	59.71 ± 0.10a	4.54 ± 0.03b
500	18.60 ± 0.07a	1.85 ± 0.03a	14.67 ± 0.23a	59.96 ± 0.08a	4.66 ± 0.03ab

5.3.7 Pearson's Correlation

Table 7 displays the correlations between measured parameters and Ce content in roots or grains. Data illustrates that in roots, Ce content was positively correlated with K, S and Na at $p \leq 0.01$ and with P, Mg, and Mn at $p \leq 0.05$. In grains, root Ce content was positively correlated with Fe at higher significance level (0.77308, $p \leq 0.01$) than Na (0.68055, $p \leq 0.05$). Likewise, root Ce content manifested stronger positive correlations ($p \leq 0.01$) with plant height, shoot biomass, number of spikelets, and yield per pot than with spike length ($p \leq 0.10$). Furthermore, the data displayed that root Ce content was negatively correlated with threonine, serine, alanine and valine at greater significance level ($p \leq 0.05$) than in aspartic acid, glycine and isoleucine ($p \leq 0.10$). Only linoleic acid displayed a positive correlation with root Ce content (0.63156, $p \leq 0.05$). The highly significant positive correlations between Ce and growth parameters are in agreement with the fertilizer effect of Ce. However, the improvement in growth and yield was accompanied with detrimental impacts on AA compositions of grains especially since four essential amino acids were negatively affected. On the other hand, the positive correlation of Ce with P, S, Mg, Mn and Na contents in roots could indicate important implications or associations of these elements with *n*CeO_2. Unfortunately, there is limited understanding on this aspect in current literature.

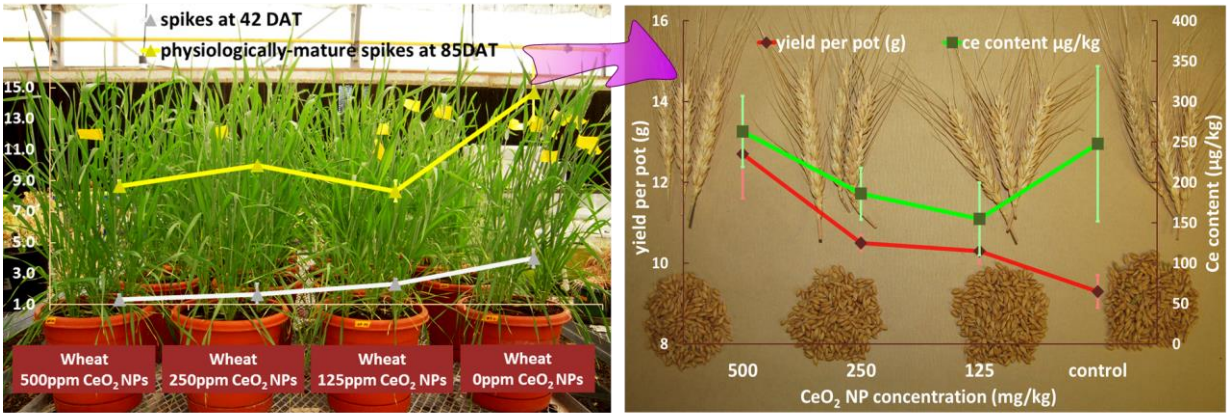
Table 5.7: Pearson's correlations between cerium content in roots or grains and measured parameters in wheat cultivated in *n*CeO_2-amended soil. *, **, *** indicate significance at $p \leq 0.10$, $p \leq 0.05$, and $p \leq 0.01$, respectively.

	r	*p* values	significance
Ce content in roots			
Elemental content in roots			
K	0.94436	<0.0001	***
P	0.68376	0.0142	**
Mg	0.70770	0.0100	**
Mn	0.66793	0.0176	**
S	0.86067	0.0003	***
Na	0.84243	0.0006	***
Growth/yield components			
Spike length	0.50375	0.0950	*
Plant height	0.81039	0.0014	***
Shoot biomass	0.86938	0.0002	***
Number of spikelets	0.77962	0.0028	***
Yield per pot	0.76626	0.0037	***
Elemental content in grains			
Na	0.68055	0.0149	**
Fe	0.77308	0.0032	***
Nutritional components in grains			
Aspartic acid	-0.52992	0.0764	*
Glycine	-0.50954	0.0906	*
Isoleucine	-0.54759	0.0653	*
Threonine	-0.60554	0.0369	**
Serine	-0.60142	0.0386	**
Alanine	-0.64724	0.0229	**
Valine	-0.62534	0.0297	**
Linoleic acid	0.63156	0.0276	**
Ce content in grains			
Elemental content in grains			
Ca	0.59941	0.0394	**
Growth/yield components			
SPAD	-0.56499	0.0556	*
Nutritional components in grains			
Threonine	-0.50398	0.0948	*
Isoleucine	-0.50237	0.0960	*
Lysine	-0.51175	0.0890	*

In grains, Ce content displayed positive correlation with Ca (0.59941, $p \leq 0.05$) similar to those observed in nCeO$_2$-exposed rice grains[10] and Ce^{3+}-treated rice seedlings.[22] On the other hand, the negative correlations between Ce and chlorophyll content, threonine, isoleucine and lysine suggest that Ce content could have significant impacts on essential amino acids in grains.

5.4 CONCLUSION

In conclusion, the study revealed that nCeO$_2$ improved the plant height, biomass, and grain yield, and modified the amino acids and fatty acids contents despite the apparent lack of Ce transport and accumulation in the aboveground tissues of wheat. The findings underscore the significance of examining the long-term impacts of ENMs on the productivity and quality of food crops.



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Chapter 6: Physiological and Biochemical Response of Soil-grown Barley (*Hordeum vulgare* L.) to Cerium Oxide Nanoparticles[5]

Abstract

The current knowledge on the long-term impacts of engineered nanomaterials in food crops is limited. A soil microcosm study was performed to examine the impacts of cerium oxide nanoparticles (*n*CeO_2) on the physiology, productivity, and macromolecular composition of barley (*Hordeum vulgare* L.). The plants were cultivated in soil treated with *n*CeO_2 at 0, 125, 250, and 500 mg kg^{-1} (control, *n*CeO_2-L, *n*CeO_2-M, and *n*CeO_2-H, respectively). Accumulation of Ce in leaves/grains and its effects on plant stress and nutrient loading were analyzed. The data revealed that *n*CeO_2-H promoted plant development resulting in 331% increase in shoot biomass compared to control. *n*CeO_2 treatment modified the stress levels in leaves without apparent signs of toxicity. However, plants exposed to *n*CeO_2-H treatment did not form grains. Compared to control, *n*CeO_2-M enhanced grain Ce accumulation by as much as 294% which was accompanied by remarkable increases in P, K, Ca, Mg, S, Fe, Zn, Cu, and Al. Likewise, *n*CeO_2-M enhanced the methionine, aspartic acid, threonine, tyrosine, arginine, and linolenic acid contents in the grains by up to 617, 31, 58, 141, 378, and 2.47% respectively, compared to the rest of the treatments. The findings illustrated the beneficial and harmful effects of nanoceria in barley.

Keywords: Amino acids, Engineered nanomaterials, Fatty acids, Oxidative stress

[5] Rico, C. M.; Barrios, A. C.; Tan, W. J.; Rubenecia, R.; Lee, S. C.; Varela-Ramirez, A.; Peralta-Videa, J. R.; Gardea-Torresdey, J. L. Physiological and biochemical response of soil-grown barley (*Hordeum vulgare* L.) to cerium oxide nanoparticles. *Environmental Science and Pollution Research* (submitted and under review)

6.1 INTRODUCTION

Management of environmental impacts of engineered nanomaterials (ENMs) requires understanding of their potential risks and benefits in plants. ENMs exert beneficial or harmful effects on food crops; however, the current understanding on ENMs-edible plants interaction provides little insight on the long-term impacts of ENMs on crop physiology and food quality. There is limited number of reports available in full grown plants to make a conclusive assessment of the long-term risks and benefits of ENPs in plants.[1] Only less than 30 studies from more than 200 reported in the last two years. Thus, more studies involving plants complete life cycle are needed to fully assess the sustainable use of ENMs in agricultural productivity.

Cerium oxide nanoparticles (*n*CeO_2) are widely utilized in catalytic applications, mechanical planarization, and fuel additives.[2] Modeling studies have predicted that *n*CeO_2 could contaminate agricultural lands through biosolid application.[3] Since *n*CeO_2 has very high stability in soil, i.e. <3.1% dissolution rate at pH 4 in 16 soil types,[4] there is a high chance that *n*CeO_2 would interact with agricultural plants and trigger unknown consequences on plant physiology, crop productivity, food quality, and even human nutrition. Therefore, efforts to understand these implications are crucial to evaluate safer use and disposal of these nanoparticles.

Barley is not a major cereal crop compared to rice and wheat;[5] still, it is widely cultivated with global grain production from 2005-2009 reaching to around 140 million tonnes.[6] Unfortunately, very limited number of studies on the effects of ENMs on barley at short exposure scenarios,[7,8] and none in long term studies, have been reported. Therefore, examining the ENMs-barley interactions could provide a broader appreciation on the influence of *n*CeO_2 on growth and reproduction of agricultural crops.

Herein, the responses of barley cultivated to grain production in *n*CeO_2-amended soil were reported. Special emphases were given on plant stress, alterations in growth and productivity, modifications in macromolecular components, and Ce accumulation in different tissues. This study hopes to shed light on the safety or toxicity of *n*CeO_2 in barley.

6.2 MATERIALS AND METHODS

6.2.1 Preparation of *n*CeO_2-amended soil

The *n*CeO_2 (Meliorum Technologies, Rochester, NY) were provided by the University of California Center for Environmental Implications of Nanotechnology. Characterization studies showed that *n*CeO_2 are rod with primary size of 8 ± 1 nm, particle size of 231 ± 16 nm in DI water, surface area of 93.8 m^2g^{-1}, and 95.14% purity.[9] The *n*CeO_2-amended soil was prepared as previously described; "The amount of *n*CeO_2 necessary to prepare the concentrations of 125, 250, and 500 mg/kg in two kilograms of soil (Miraclegro® potting soil) was sonicated in 125 mL Millipore water (MW) at 25°C for 30 min in a water bath (Crest Ultrasonics, Trenton, NJ) before mixing thoroughly with the soil." Untreated soil was mixed with MW only. Soil was placed in plastic pots (24 cm diameter × 25 cm high) and aged for 3 days before the experiment in the greenhouse (14-h photoperiod, 25/20°C day/night temperature, 70% relative humidity, light intensity of 340 μmole m^{-2} s^{-1}. Pots were prepared in triplicate.

6.2.2 Barley cultivation

Barley (*Hordeum vulgare* L. var. Millennium) seeds, supplied by the University of Idaho Southwest Idaho Extension Cereals Program (Parma, ID), were sterilized in 4% sodium hypochlorite solution for 10 min, and germinated for three days in Petri dishes wrapped in aluminum foil. After 9 days, the seedlings were transplanted in group of four, totaling twenty four seedlings per pot and cultivated to full maturity in the greenhouse. The pots were irrigated to saturation using 200-300 mL distilled water. Yoshida nutrient solution[11] was used as fertilizer; however, due to the vigorous growth of the treated plants, the nutrient solution was only added four times (Appendix 5 Supporting Information (SI) Table 5.1). Malathion (5 mg/L) (Ortho® MAX® Malathion) was sprayed at 90, 129, and 143 days after transplanting (DAT) to control the infestation of green bug (*Schizaphis graminum*). Spikes were harvested (169 DAT) and oven-dried at 80°C for 5 days. The grains were de-hulled and powdered to pass mesh number 40 (400 μm) (W.S. Tyler, USA) before storing at 4°C.

6.2.3 Evaluation of agronomic traits and yield components

The plant height, chlorophyll content, shoot biomass, and water content were determined at 48 DAT. Plant height and chlorophyll content were assessed using five measurements from randomly

selected plants in each pot. Plant height was measured from soil surface to the flag leaf while chlorophyll content was estimated by taking SPAD chlorophyll (Spectrum Technologies, Aurora, IL) measurements in the flag leaf. The fully exposed and completely dried spikes were counted just before harvest and recorded as the number of spikes formed. For shoot biomass and water content determination, six plants were harvested from each pot and the weight was recorded before and after oven drying. In the case of yield components, the following parameters were measured using five random samples from each pot: spike length, number of spikes, number of spikelets/spike, number of grains/spike, and gain yield/spike. Grain yield from each pot was measured and recorded as grain yield/pot. The average of five measurements from each pot for agronomic and yield components, except grain yield/pot, was obtained and inputted as one replicate measurement for statistical analyses.

6.2.4 Measurement of stress levels

The H_2O_2 content, K leakage, and UV absorbing substances (UVAS) were analyzed to measure oxidative stress in plants. Flag leaves were used for all analyses. Confocal laser scanning microscopy was employed to estimate H_2O_2 content in leaves. The tissues were incubated for 15 min in the dark with 2',7'-dichlorofluorescein diacetate. Digital fluorescence images were captured with a laser scanning microscope (LSM 700; Zeiss, New York, NY), equipped with a Plan-Apochromat 20x/0.8 objective and 488 nm laser. Image acquisition and analysis was achieved by using ZEN 2009 software (Zeiss). The natural fluorescence from leaves not incubated with dye was also measured. The H_2O_2 content was estimated from the difference in intensities between the dye-treated and untreated samples. The analysis was replicated six times. Membrane damage was determined by measuring the leakages of K and UV absorbing substances from the cells (K leakage and UVAS ratio, respectively) following the method of Navarri-Izzo et al.[12] and Lutts et al.[13], respectively. Detailed description of the method was presented in the SI (Appendix 5). For better understanding of plant stress, enzyme activities of catalase, ascorbate peroxidase, dehydroascorbate reductase, and glutathione reductase (CAT, APX, DHAR, and GR, respectively) were also measured. The assays were performed according to the methods described by Rico et al.[14]

6.2.5 Analytical methods

Quantitative analyses of cerium and nutrient elements in barley leaves and grain was performed using ICP-OES/MS (Perkin Elmer, Waltham, MA) following to the method previously employed by Rico et al.[10] The amino acid content of grains was analyzed using Biochrom 20 amino acid analyzer (Biochrom, USA) according to the method Anjum et al.[15] For the fatty acid analysis, the sample was prepared and analyzed based on the method described by Asekova et al.[16] The description of sample preparation and analysis was presented in the SI (Appendix 5).

6.2.6 Statistical analysis

The analysis of variance was carried out using the General Linear Model procedure in SAS statistical package Version 9.3 (SAS Institute, Cary, NC, USA). Statistical differences between means were tested with Tukey's honestly significant difference test ($p \leq 0.05$) or Duncan's multiple range test ($p \leq 0.10$).

6.3 RESULTS AND DISCUSSION

6.3.1 Cerium uptake in leaves and grains

Several reports have already confirmed that *n*CeO_2 stay largely in nanoparticulate form in soil and plants. The accumulation of Ce in barley tissues is shown in Table 6.1. As seen in the table, the Ce accumulations in the *n*CeO_2-treated leaves (524-701 μg kg^{-1}) did not achieved statistical difference between treatments whereas in grains, only control, *n*CeO_2-L, and *n*CeO_2-M produced grains which accumulated 200, 449, and 787 μg Ce kg^{-1}, respectively. Previous reports revealed differences in Ce uptake in grains. For example, medium and low amylose rice varieties stored more Ce (1912 and 1853 μg kg^{-1}) than high amylose variety (224 μg kg^{-1}) when exposed to *n*CeO_2-H treatment.[10] On the contrary, *n*CeO_2 treatment did not result in significant difference in Ce accumulations in wheat grains.[17] On the other hand, 100 and 500 mg kg^{-1} *n*CeO_2 treatments resulted in Ce accumulations in soybean pods by up to 101 and 133 μg kg^{-1}, respectively, while 400 and 800 mg *n*CeO_2 kg^{-1} resulted in about 90 and 1270 μg Ce kg^{-1}, in cucumber fruit, respectively.[18,19] Wang et al.[20] also reported low but detectable amount of Ce in tomato fruits collected from *n*CeO_2-exposed tomato plants. These studies strongly

suggest Ce accumulation in agricultural crops which may have unknown implications in food quality and human nutrition.

Table 6.1: Cerium concentrations (μg kg^{-1} d wt) in different tissues of barley cultivated to grain production in *n*CeO_2-amended soil. Values are means ± SE, $n = 3$. Same letters mean no statistical difference between treatments at Tukey's test ($p \leq 0.05$).

***n*CeO_2 concentration (mg kg^{-1})**	**leaves**	**grains**
0	571 ± 40a	200 ± 5c
125	595 ± 140a	449 ± 51b
250	524 ± 73a	787 ± 58a
500	701 ± 92a	-

6.3.2 Elemental uptake

ENMs could modulate mineral uptake in exposed plants. Table 6.2 displays the elemental concentrations in leaves and grains of barley. In leaves, *n*CeO_2 treatment did not change the accumulations of Ca, Mg, Mn, B, and Al, but increased the uptake of some elements. All *n*CeO_2 treatments elevated K concentration by 33-56% compared to control; *n*CeO_2-H had 33 and 46% more Zn and 36 and 53% more Cu compared to *n*CeO_2-L and control, respectively. Fe accumulation was significantly higher at *n*CeO_2-M and *n*CeO_2-L (38 and 54%, respectively) relative to control. Conversely, *n*CeO_2-H decreased P accumulation by 13 and 18% compared to *n*CeO_2-L and control, respectively, while *n*CeO_2-H reduced S uptake by 12% with respect to control, and *n*CeO_2-H decreased Na concentration by 37 and 46% compared to *n*CeO_2-M and control, respectively. . For the grains, the concentrations of Na and B did not change across treatments whereas the accumulations of all other elements increased remarkably with the increase of *n*CeO_2 in the treatment. The accumulations of K, Mg, Zn, Cu, and Al in *n*CeO_2-L and control were similar but were significantly lower than those of *n*CeO_2-M by up to 41, 38, 60, 41, and 222%, respectively. On the other hand, *n*CeO_2-M markedly enhanced the concentrations of P, Ca, S, Fe, and Mn compared to *n*CeO_2-L (higher by 20, 54, 11, 45, and 35%, respectively) and control (higher by 41, 127, 38, 82, and 102%, respectively).

Table 6.2: Elemental concentration (mg kg^{-1} d wt) in different tissues of barley cultivated to grain production in *n*CeO_2-amended soil. Values are means ± SE, $n = 3$. Same letters mean no statistical difference between treatments at Tukey's test ($p \leq 0.05$).

Element	leaves				grains		
	0	125	250	500	0	125	250
K	34308 ± 1378b	45755 ± 3139a	50286 ± 3030a	53483 ± 460a	6061 ± 113b	7143 ± 441b	8566 ± 310a
P	6111 ± 162a	5787 ± 184a	5467 ± 123ab	5028 ± 121b	5011 ± 70c	5917 ± 319b	7069 ± 127a
Ca	4936 ± 178a	4582 ± 126a	4651 ± 48a	4741 ± 252a	174 ± 6c	258 ± 27b	396 ± 10a
Mg	3188 ± 21a	2891 ± 73a	2930 ± 69a	3110 ± 199a	1200 ± 11b	1498 ± 47b	1657 ± 50a
S	3460 ± 73a	3127 ± 134ab	2787 ± 90b	3045 ± 25b	1362 ± 48b	1610 ± 134ab	1904 ± 46a
Na	5886 ± 198a	4365 ± 113bc	5087 ± 495ab	3187 ± 198c	154 ± 8a	150 ± 8a	187 ± 25a
Fe	52.8 ± 2.7b	81.1 ± 3.3a	72.8 ± 6.4a	65.6 ± 1.0ab	30.8 ± 1.0c	38.8 ± 1.5b	56.2 ± 1.4a
Mn	9.2 ± 0.4a	14.8 ± 3.0a	10.5 ± 0.7a	15.3 ± 0.4a	11.5 ± 0.8c	17.1 ± 0.7b	23.2 ± 0.6a
Zn	15.0 ± 1.2b	16.5 ± 1.1b	18.8 ± 1.3ab	21.9 ± 0.1a	25.8 ± 2.1b	26.8 ± 0.4b	41.2 ± 0.7a
Cu	8.4 ± 0.3c	9.4 ± 0.6bc	11.7 ± 0.8ab	12.9 ± 0.1a	15.2 ± 0.2b	15.1 ± 0.2b	21.3 ± 0.5a
B	10.3 ± 0.3a	10.2 ± 1.1a	10.8 ± 1.0a	7.9 ± 0.6a	10.2 ± 0.4a	9.6 ± 0.2a	8.8 ± 0.4a
Al	21.7 ± 1.9a	21.4 ± 1.5a	24.3 ± 4.4a	14.3 ± 0.6a	10.5 ± 1.0b	24.4 ± 2.0b	33.9 ± 1.4a

The *n*CeO_2 treatment modified the elemental uptake in barley leaves and grains that resulted in enriched elemental content in grains. The data suggests that *n*CeO_2 modulated the complex processes controlling the metal loading in grains, i.e chelation, membrane transport, and deposition as phytate.[21] Unfortunately, there is a lack of understanding in the current literature on how ENMs modify elemental uptake in plants. Since metal loading is the major hurdle in increasing elemental content in plant,[22] further studies on how *n*CeO_2 boosted this process in barley grains should be performed. On the other hand, it is highly possible that the notable increase in nutrient uptake negatively affected the processes involved in spike and grain formations in *n*CeO_2-H-treated plants. For example, failure to produce seeds in soybean treated with *n*ZnO (500 mg kg^{-1}) was attributed to the significant accumulation of Zn (Yoon et al. 2013).[23]

6.3.3 Changes in growth and productivity

Knowledge on the impacts of ENMs on crop physiology and productivity of plants is still lacking. The growth and yield responses of barley grown to full maturity in *n*CeO_2-amended soil are displayed in Table 6.3 and Table 6.4, respectively. All growth parameters were affected by the treatment; chlorophyll content increased in all *n*CeO_2 treatments by 32-47% compared to the control whereas, relative to *n*CeO_2-L and control treatments, the *n*CeO_2-H increased plant height, dry shoot biomass, and water content by 23 and 34%, 115 and 330%, and 145 and 395%, respectively.

Table 6.3: Agronomic characters of barley cultivated to grain production in *n*CeO_2-amended soil. Values are means ± SE, $n = 3$. Same letters mean no statistical difference between treatments at Tukey's test ($p \leq 0.05$).

***n*CeO_2 concentration (mg kg^{-1})**	**plant height (cm)**	**chlorophyll content (SPAD unit)**	**dry shoot biomass (mg)**	**water content (g)**
0	50.1 ± 2.4b	22.5 ± 0.4b	250 ± 16b	1.8 ± 0.1c
125	54.9 ± 0.7b	31.6 ± 1.6a	503 ± 113b	3.6 ± 1.0bc
250	65.0 ± 1.4a	29.6 ± 0.3a	737 ± 103ab	6.3 ± 1.0ab
500	67.2 ± 0.5a	33.1 ± 0.6a	1077 ± 183a	8.7 ± 1.2a

For the yield components, the *n*CeO_2-H did not produce fully matured spikes. Compared to the control, *n*CeO_2-M reduced the number of spikes by 52% but improved the spike length and number of spikelets per spike by 32 and 89%, respectively. On the other hand, *n*CeO_2-L recorded similar number of spikes and spike length but lower number of spikelets per spike (52% reduction) compared to control. In the case of the number of grains per spike, grain yield per spike, and yield per pot, values had an increasing trend from control to *n*CeO_2-M (6.3-7.6, 153.0-212.6 mg, and 0.99-1.56 g, respectively) but did not reach statistical differences between treatments. It is highly probable that despite the very low number of spikes registered in *n*CeO_2-M, the higher number of grains per spike and grain yield per spike caused the increasing trend in yield per pot. These results suggest that at significant concentration, *n*CeO_2 may have serious implications in barley production.

Table 6.4: Yield parameters of barley cultivated to grain production in nCeO$_2$-amended soil. Values are means ± SE, n = 3. Same letters mean no statistical difference between treatments at Tukey's test ($p \leq 0.05$).

nCeO$_2$ concentration (mg kg^{-1})	number of spikes	spike length (cm)	number of spikelets per spike	number of grains per spike	grain yield per spike (mg)	yield per pot (g)
0	10.3 ± 0.3a	2.3 ± 0.1b	8.1 ± 0.6c	6.3 ± 0.9a	153.0 ± 18.6a	0.99 ± 0.1a
125	9.3 ± 1.3a	2.4 ± 0.1b	11.4 ± 0.4b	6.4 ± 0.9a	179.4 ± 19.9a	1.18 ± 0.3a
250	5.0 ± 0.5b	3.1 ± 0.1a	15.4 ± 0.8a	7.6 ± 0.9a	212.6 ± 30.4a	1.56 ± 0.4a

Studies have indicated that ENMs provoke physiological changes in plants. For example, nCeO$_2$ promoted plant growth in soybean, wheat, and tomato,[10,18,20] but did not affect the growth in cucumber nor chlorophyll content in cucumber, wheat, and tomato.[10,19,20] nCeO$_2$ has been found to prolong the grain/fruit production in wheat and tomato that resulted in improved yield.[10,20] The current result also showed a significant delay in spike maturity and harvest (169 DAT) compared with the usual harvest time (~90 DAT), which may have considerable consequences on seeding time/planting date and phenology.[24] The delay probably caused the increasing trend in grain yield that despite the low number of spike in nCeO$_2$-M, a numerically higher grain yield than the control was obtained. Short exposure studies revealed that nCuO and nTiO$_2$ interfered with the physiology and growth of barley;[7,8] unfortunately, long term studies are lacking to make meaningful analysis of the current results.

6.3.4 Oxidative stress in plants

The oxidative stress and antioxidative activities were measured to help understand the responses of barley to nCeO$_2$ exposure. The results, as displayed in Figures 6.1 and 6.2, revealed that nCeO$_2$ modulated the oxidative stress in barley leaves. The H_2O_2 estimated by confocal fluorescence intensity in nCeO$_2$-H was 166% higher than in control; however, the intensity in nCeO$_2$-L was 26% lower than control. The K leakage in nCeO$_2$-H was higher by 177-187% with respect to rest of the treatments whereas UVAS ratio in control was superior by 131% relative to those in nCeO$_2$-L and nCeO$_2$-M. Likewise, Figure 6.3 showed that the antioxidative enzyme activities were modulated: CAT activity did

not differ between treatments, APOX activity was not detected in control but 3.5-fold higher at *n*CeO_2-L and *n*CeO_2-M compared to *n*CeO_2-H, DHAR activity was lower by 17-21% in *n*CeO_2-H compared to the other treatments, and GR activity decreased by 18% in *n*CeO_2-H relative to control. CAT and APOX reduce H_2O_2 to H_2O while GR and DHAR generate low molecular weight antioxidants (glutathione and ascorbate, respectively). APOX also utilizes ascorbate to carry out its functions.

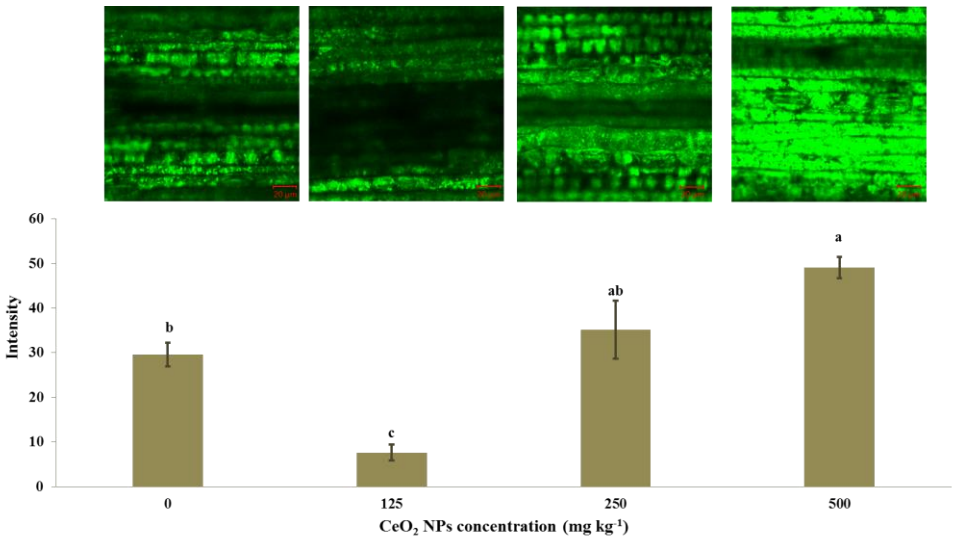


Figure 6.1: H_2O_2 generation in barley leaves at 48 DAT in *n*CeO_2-amended soil. 0, 125, 250, and 500 denote control, *n*CeO_2-L, *n*CeO_2-M, and *n*CeO_2-H, respectively. Each data point is mean ± SE (n = 6). Means with the same letter are not significantly different at Tukey's test ($p \leq 0.05$).

The enhanced H_2O_2 production at *n*CeO_2-H could be related to its high biomass and water content and may not necessarily due to higher oxidative stress since *n*CeO_2-H had lower antioxidative enzyme activities that indicated low oxidative stress. In contrast, the lower water content and increased APOX activity could have caused the low H_2O_2 content found in *n*CeO_2-L. The K leakage and UVAS ratio had opposite trend; K leakage was greatly enhanced at *n*CeO_2-H while UVAS ratio was extremely improved in control. It is possible that the high K content in *n*CeO_2-H contributed to the substantial increase in K leakage suggesting that the increase in K leakage was not solely due to membrane damage. On the other hand, the high UVAS ratio (indicator of protein or amino acids leakage) in control was probably due to increased membrane damage because control, despite having very low soluble protein (3.87 mg g^{-1}) compared to *n*CeO_2-H (26.81 mg g^{-1}) (Appendix 5 SI Table 5.2), still showed enhanced

UVAS ratio. GR activity was higher in control and lower in the *n*CeO_2-treated leaves; this trend was consistent with the S content being elevated in control and reduced in the *n*CeO_2 treatments. Moreover, in the control, the improved GR and DHAR activities probably covered for the suppressed APOX activity.

The current literature is replete with short-term studies regarding measurements of oxidative stress and radical scavenging activity in plants. In fact, in reports involving cereals alone, the list is already lengthy (references are provided in the SI, Appendix 5). Yet these studies may have serious shortcomings in providing a more comprehensive assessment of the relationship between stress, development and reproduction in plants. Here, the results indicated an increased stress levels at *n*CeO_2-H, but it is highly possible that the enhanced stress level was due to the growth, biomass, and water content of the plants in this treatment.

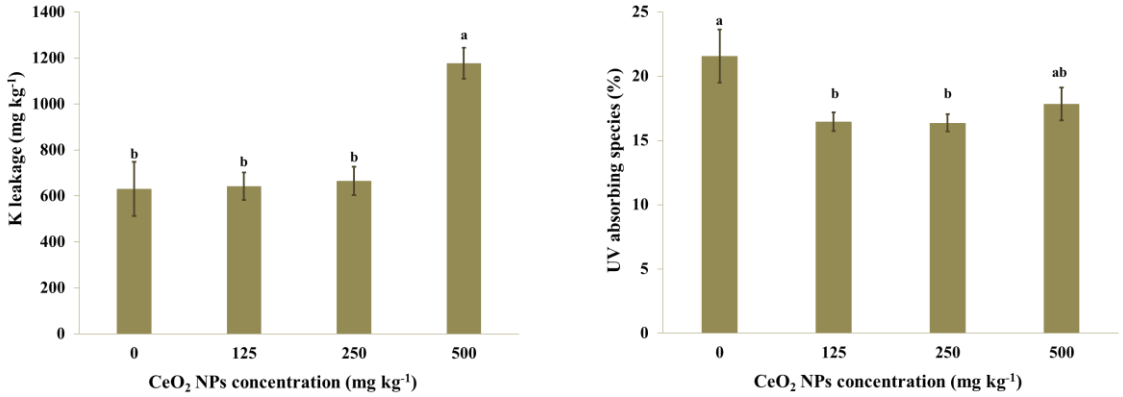


Figure 6.2: K leakage and UV absorbing species in barley leaves at 48 DAT in *n*CeO_2-amended soil. 0, 125, 250, and 500 denote control, *n*CeO_2-L, *n*CeO_2-M, and *n*CeO_2-H, respectively. Each data point is mean ± SE (n = 3). Means with the same letter are not significantly different at Tukey's test ($p \leq 0.05$).

6.3.5 Macromolecular Contents in Grains

ENMs modify the macromolecular content in edible plants. In this study, changes on the amino acid (AA) and fatty acid (FA) concentrations were analyzed and presented in Table 6.5 and Table 6.6, respectively. As seen in the data, *n*CeO_2 modified only the contents of methionine, arginine, threonine, tyrosine, and aspartic acid. *n*CeO_2-M increased the methionine, aspartic acid, threonine and tyrosine contents by up to 617, 31, 58 and 141% respectively, compared to the rest of the treatments. On the

other hand, the *n*CeO_2-M and *n*CeO_2-L treatments improved arginine content by 378 and 68%, respectively, compared to control. The rest of the AA did not change statistically across the treatments; however, *n*CeO_2-M registered higher numerical values for several AA (i.e. alanine, leucine, aspartic acid, lysine, phenylalanine, serine, threonine, tyrosine and valine) and total AA suggesting the possibility of enhanced production of these AA at increased *n*CeO_2 in the treatment. In the case of fatty acid content, *n*CeO_2 treatments did not elicit changes in the synthesis of oleic and linolenic acids but modified the production of the rest of palmitic, stearic, and linoleic acids. In comparison to control, *n*CeO_2-L decreased the palmitic acid content by 1.93% but enhanced the stearic acid concentration by 19.05%. In opposite, *n*CeO_2-M elevated the linolenic acid content by up to 2.47% compared to the rest of the treatments.

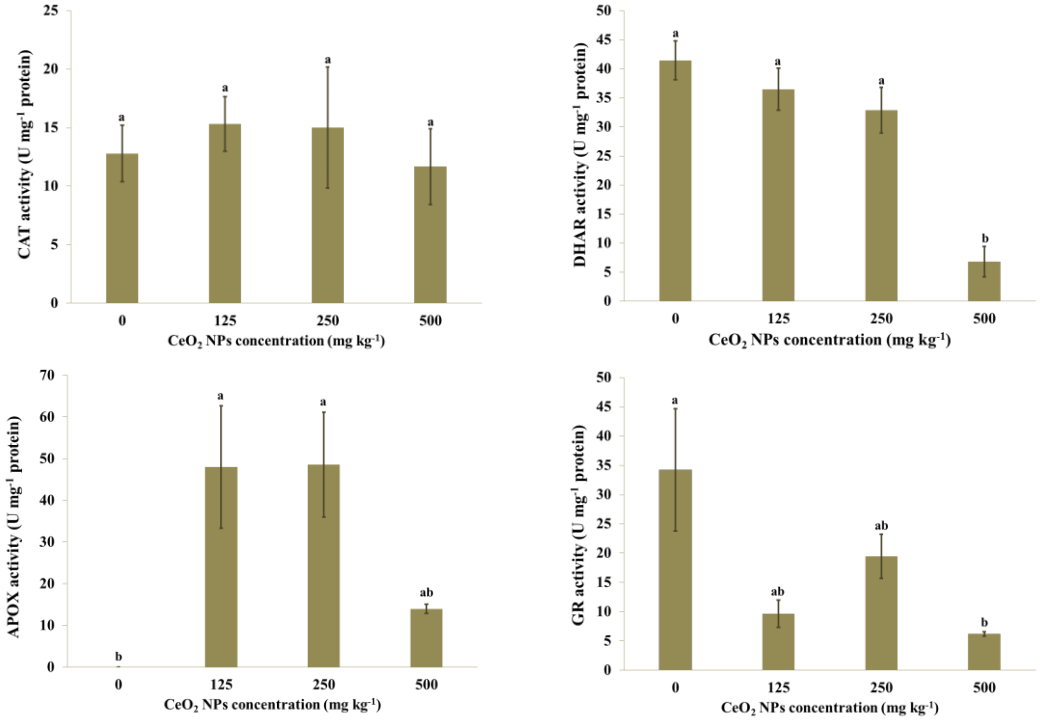


Figure 6.3: Antioxidative enzyme activities in barley leaves at 48 DAT in *n*CeO_2-amended soil. 0, 125, 250, and 500 denote control, *n*CeO_2-L, *n*CeO_2-M, and *n*CeO_2-H, respectively. Each data point is mean ± SE (n = 3). Means with the same letter are not significantly different at Tukey's test ($p \leq 0.05$). Abbreviations are catalase (CAT), ascorbate peroxidase (APOX), dehydroascorbate reductase (DHAR), and glutathione reductase (GR). One unit of CAT is the amount of enzyme needed to degrade 1 µmol of H_2O_2 per minute. One unit of GPOX is defined as 1 µmol of guaiacol oxidized per minute. One unit of APOX or DHAR activity is defined as 1 µmol of ascorbate oxidized or formed, respectively, per minute. One unit of GR activity is defined as 1 µmol of NADPH oxidized per minute. Different scales were used due to the high variability in the unit activities of the enzymes.

Table 6.5: Amino acid content (μg g^{-1}) of grains harvested from barley cultivated to grain production in *n*CeO_2-amended soil. Values are means ± SE, $n = 3$. Same letters across the row mean no statistical difference between treatments at Duncan's test ($p \leq 0.10$).

amino acids	*n*CeO_2 concentration (mg kg^{-1})		
	0	125	250
Methionine	4.36 ± 0.42b	5.80 ± 1.98b	31.24 ± 0.58a
Arginine	13.08 ± 2.32c	37.19 ± 3.25b	62.53 ± 2.10a
Aspartic acid	126.56 ± 8.37b	123.05 ± 2.92b	160.84 ± 18.95a
Threonine	65.86 ± 5.58b	74.82 ± 2.65ab	103.79 ± 18.44a
Tyrosine	36.64 ± 4.31b	60.48 ± 5.15ab	88.35 ± 25.95a
Alanine	61.10 ± 5.39a	67.62 ± 1.24a	88.72 ± 25.02a
Cysteine	6.57 ± 1.56a	8.32 ± 0.70a	6.25 ± 0.77a
Glutamic acid	500.49 ± 52.65a	473.87 ± 14.55a	573.74 ± 189.40a
Leucine	67.48 ± 8.50a	76.86 ± 7.37a	130.75 ± 56.94a
Proline	373.96 ± 31.15a	345.97 ± 10.78a	395.94 ± 25.49a
Glycine	75.02 ± 5.91a	77.02 ± 0.51a	82.79 ± 32.99a
Isoleucine	41.30 ± 5.48a	50.30 ± 2.50a	47.51 ± 24.69a
Lysine	42.16 ± 3.19a	44.30 ± 1.78a	71.24 ± 22.36a
Phenylalanine	35.56 ± 2.78a	38.29 ± 3.61a	61.42 ± 20.57a
Serine	26.70 ± 2.39a	31.97 ± 2.41a	45.86 ± 16.55a
Valine	82.62 ± 8.99a	101.92 ± 1.26a	125.94 ± 36.55a
Amide-NH_3	78.37 ± 5.12a	84.36 ± 2.56a	99.52 ± 24.45a
Total	1637.84 ± 121.38a	1702.14 ± 36.82a	1816.96 ± 448.64a

These results are in agreement with previous reports. Studies have found that long-term exposure with *n*CeO_2 provoked negative effects on the AA and FA synthesis in rice and wheat grains[10,17] whereas short-term treatments with various ENMs (*n*Ag, *n*S, *n*SiO_2, *n*TiO_2, and *n*ZnO) modulated protein production in different crops.[1] In comparison with the wheat grains, *n*CeO_2 triggered changes in fewer AA but more FA in barley grains. These findings illustrate the ability of ENMs to modulate the AA and FA contents in crops. Nevertheless, there is so little known on the mechanism how ENMs interfere with the AA synthesis in food crops and if these changes could have significant effects on human nutrition.

Table 6.6: Fatty acid content (relative % abundance) of grains harvested from barley cultivated to grain production in *n*CeO_2-amended soil. Values are means ± SE, $n = 3$. Same letters across the row mean no statistical difference between treatments at Duncan's test ($p \leq 0.10$).

fatty acids	*n*CeO_2 concentration (mg kg^{-1})		
	0	125	250
Palmitic acid	21.72 ± 0.12a	21.30 ± 0.13b	21.54 ± 0.12ab
Stearic acid	0.84 ± 0.03b	1.00 ± 0.05a	0.89 ± 0.07ab
Oleic acid	15.11 ± 0.31a	14.99 ± 0.50a	14.86 ± 0.20a
Linoleic acid	55.17 ± 0.12b	54.76 ± 0.49b	56.11 ± 0.28a
Linolenic acid	6.62 ± 0.11a	6.81 ± 0.11a	7.10 ± 0.29a

6.3.6 Pearson's correlations

The Pearson's correlations of Ce content in the grains with yield components and amino acid content was analyzed and displayed in Table 6.7. Results demonstrated that only Na accumulation was not correlated with Ce content. In contrast, the uptake of all elements were positively and strongly correlated (r = 0.83885-0.96487; $p \leq 0.01$), with the exception of B which was negatively correlated (r = -0.69503; $p \leq 0.05$), with the Ce content. In addition, the Ce content was significantly and positively correlated with aspartic acid, tyrosine, methionine, and arginine. In the case of yield components, the Ce uptake in the grains was negatively and strongly correlated (r = -0.69503; $p \leq 0.01$) with the number of spikes but positively correlated to a lesser degree ($p \leq 0.05$) with the number of spikelets per spike (r = 0.72663) and yield per pot (r = 0.58781).

The results showed that the accumulation of Ce in the barley grains was accompanied by enhanced storage of mineral elements (P, K, Ca, Mg, S, Fe, Zn, and Cu) and amino acids (aspartic acid, tyrosine, methionine, and arginine) as well as improved number of spikelets per spike and yield per pot. These data are opposite than those recorded in wheat. For example, Ce accumulation in wheat grains was positively correlated with Ca only and negatively correlated with the chlorophyll content and amino acids like threonine, isoleucine, and lysine.[10] Further studies should be performed to understand how *n*CeO_2 caused a tremendous increase in the accumulation of these elements in barley grains. This finding could be significant because enhancing the mineral contents of food crops is a common approach to

mitigate human malnutrition.[25] The decreased number of spikes at increased Ce uptake in grains indicates that high concentration of Ce was harmful to spike formation; however, the reduction in number of spikes probably promoted spikelet/grain formation and yield. A similar has also been observed in wheat.

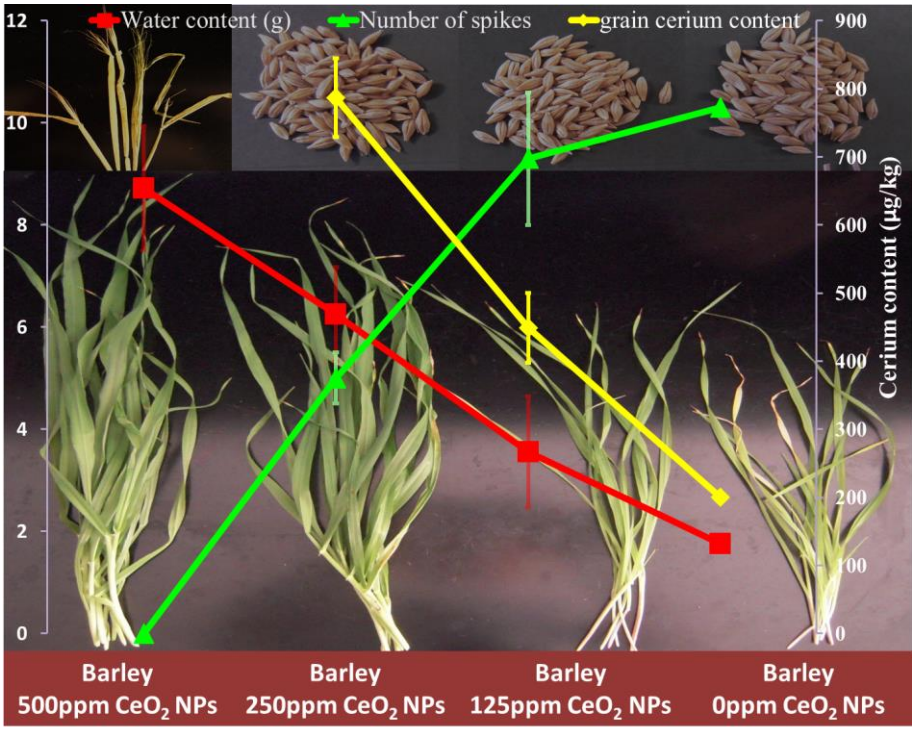
Table 6.7: Pearson's correlations between cerium content in grains and measured parameters in barley cultivated in *n*CeO_2-amended soil. *, **, *** indicate significance at $p \leq 0.10$, $p \leq 0.05$, and $p \leq 0.01$, respectively.

	r	***p* values**	**significance**
Elemental content in grains			
P	0.89052	0.0013	***
K	0.87238	0.0022	***
Ca	0.94225	0.0001	***
Mg	0.88226	0.0016	***
S	0.84020	0.0046	***
Fe	0.96487	<0.0001	***
Mn	0.92941	0.0003	***
Zn	0.88236	0.0016	***
Cu	0.83885	0.0047	***
Al	0.89215	0.0012	***
B	-0.69503	0.0377	**
Yield components			
Number of spikes	-0.88328	0.0016	***
Spikelets per spike	0.72663	0.0266	**
Yield per pot	0.58781	0.0960	*
Macromolecular contents in grains			
Aspartic acid	0.65003	0.0581	*
Tyrosine	0.67224	0.0473	**
Methionine	0.86113	0.0029	***
Arginine	0.98310	<0.0001	***

6.4 CONCLUSION

In summary, *n*CeO_2 greatly improved the plant height, biomass, and chlorophyll content but reduced the spike production in barley. At increased amount of *n*CeO_2 in the treatment, Ce and mineral uptake in leaves and grains also increased. The *n*CeO_2 treatment did not necessarily provoke oxidative

stress in leaves; however, productivity was compromised at the highest *n*CeO_2 treatment (500 mg kg^{-1}). The result corroborates the findings of previous studies demonstrating that ENMs could provoke significant changes on the productivity and quality of food crops.



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Chapter 7: Summary and Conclusions

The effects of *n*CeO_2 on terrestrial plants are not well understood yet. This research aimed at uncovering the physiological, biochemical, and agronomical responses of three major cereals to environmentally relevant *n*CeO_2 concentrations. The germination study showed that *n*CeO_2 caused dramatic changes on the physiology and biochemical composition in cereal roots. Ce traversed the different barriers and deposited in the vascular tissues of roots. Results also revealed that toxicity responses to *n*CeO_2 exposure was dependent on the relationship between stress levels (oxidative stress, membrane damage, and radical scavenging activity) and macromolecular changes (fatty acid and lignin contents). Furthermore, FTIR studies strongly suggest the involvement of COOH in the chelation of Ce.

The full life cycle study exhibited different responses in terms of physiological, productivity, Ce uptake, and macromolecular changes of cereals to *n*CeO_2 cultivation. The significant results are summarized in Table 7.1. As seen in the table, the *n*CeO_2 treatment did not affect the growth of rice but enhanced the growth and shoot biomass production of both wheat and barley with up to 331% increase in dry biomass in treated barley than control. Similarly, *n*CeO_2-exposed wheat and barley plants manifested a delay in spike formation and maturity; nevertheless, total yield increased at harvest probably due to enhanced number of spikes, spike length, number of spikelets per spike, number of grains per spike, and grain yield per spike. However, the grain production in barley was completely halted in the highest *n*CeO_2 treatment (500 mg kg^{-1}). In case of Ce accumulation in grains, the low and medium amylose rice varieties stored up to 1126% more Ce compared to the control while high amylose variety did not exhibit Ce accumulation in grains. On the other hand, Ce accumulated heavily in the grains of barley (up to 294% increase compared to control) but no apparent translocation in wheat was observed. For elemental uptake in grains, *n*CeO_2 enhanced the concentration of the majority of the nutrient elements (K, P, Ca, Mg, S, Fe, Mn, Zn, Cu) in barley, but only altered the uptake of Ca and Fe in rice and S and Mn in wheat. In addition, the macromolecular contents (starch, sugar, fatty acids, and protein) of grains were affected. The starch content in low and medium amylose rice varieties decreased by up to 9% compared to control while that of the high amylose variety was not affected. On the contrary, *n*CeO_2 disturbed the synthesis of only a few amino acids (methionine, arginine, threonine,

tyrosine, and aspartic acid) in barley grains but disrupted the production of several amino acids (aspartic acid, glycine, lysine, histidine, arginine, isoleucine, phenylalanine, serine, threonine, tyrosine, and valine) in wheat grains. The full life cycle study showed that barley is most sensitive to *n*CeO_2 in terms of physiological changes while rice is the least sensitive. Wheat was the least Ce accumulator while barley and low and medium amylose rice varieties were high Ce accumulators. In general, the study demonstrated the influence of species and cultivars on the toxicity of *n*CeO_2 in cereals.

Table 7.1: Responses of cereals from long-term exposure to *n*CeO_2.

parameters	**rice**	**wheat**	**barley**
plant development	no apparent changes on plant growth and development	enhanced plant growth and shoot biomass at increased *n*CeO_2 in the treatment; delayed spike formation and maturity	enhanced plant growth and shoot biomass at increased *n*CeO_2 in the treatment; delayed harvest
productivity	no apparent changes on yield performance	improved yield at higher *n*CeO_2 treatment	reduced number of spikes and improved grain yield per spike, but no overall change in total yield
Ce accumulation in grains	variety dependent; medium and low amylose varieties accumulated Ce but not the high amylose variety	no apparent Ce accumulation in the leaves, hull, and grains	heavy accumulator of Ce in grains, but no accumulation in leaves
elemental uptake	the uptake of Ca and Fe was affected	the uptake of S and Mn was affected	the accumulation of K, P, Ca, Mg, S, Fe, Mn, Zn, Cu increased significantly at increased *n*CeO_2 in the treatment
macromolecular modification in grains	starch and glutelin protein fraction, and antioxidant capacity were affected	most amino acids were affected	only few amino acids were affected

The findings and the possible health/environmental implications and future studies on *n*CeO_2 exposure to food crops are summarized in Figure 7.1. The study indicated negative impacts of *n*CeO_2 on the agronomic traits, yield, and productivity of cereals which would imply that cereals exposure to *n*CeO_2 may require changes in planting and cultivation practices. The accumulation of Ce in the grains also implies that Ce could be transferred to humans through dietary uptake with unknown effects to human health. In addition, the *n*CeO_2 modifications of the nutritional composition of the grains suggest

that nCeO$_2$ could affect billions of people who rely on grains for nutritional needs. Although the study, provided insights and understanding on the impacts of nCeO$_2$ on cereals, several issues and important questions are needed to be resolved in further studies. For example, the mechanistic differences in entry and sequestration of nCeO$_2$ in the roots of different species and varieties of cereals should be explored. The mechanisms may include facilitation or prevention of root entry by ion transporters and root exudates or immobilization in roots by binding to cell wall or extracellular exudates. Understanding the mechanisms involved in root-shoot movement of nCeO$_2$ is also needed. Unravelling the mechanisms involved in the amino acid and fatty acid syntheses probably using omics techniques should also be performed to provide understanding on how nCeO$_2$ modify the grain quality. Furthermore, anatomical studies on seed development can be explored to investigate how nCeO$_2$ affect seed initiation, improve seed formation, or abort seed pollination.

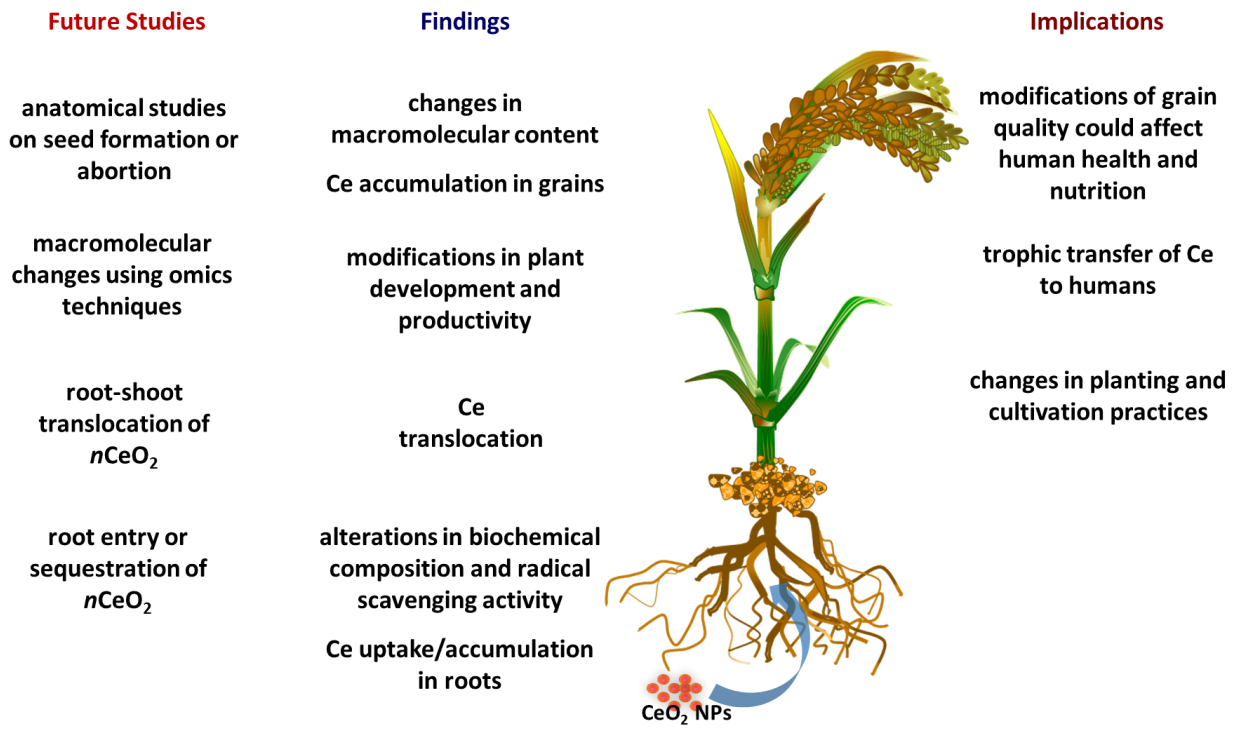

Figure 7.1: Schematic diagram summarizing the findings, implications and future studies on the interaction of nCeO$_2$ with cereals.

Appendix

1 SUPPORTING INFORMATION FOR CHAPTER 2: CERIUM OXIDE NANOPARTICLES MODIFY THE ANTIOXIDATIVE STRESS ENZYME ACTIVITIES AND MACROMOLECULE COMPOSITION IN RICE SEEDLINGS

1.1 Electrolyte leakage

The electrolyte leakage was conducted according to Lutts et al.[36] Fresh root samples were cut into pieces and placed in 10 mL Millipore water. After 2 hours incubation, the initial conductivity (EL_i) leakage was recorded. The samples were autoclaved at 121°C for 20 min, cooled down to RT, and the final conductivity (EL_f) was measured. Electrolyte leakage was calculated as $EL = (EL_i/EL_f) \times 100$.

1.2 GC-MS Analysis of Fatty Acids

1.2.1 Chemical Standards

The fatty acid methyl ester standards namely: tetradecanoic acid methyl ester; pentadecanoic acid methyl ester; hexadecanoic acid methyl ester; octadecanoic acid methyl ester; 9Z-octadecanoic acid methyl ester; 9Z,12Z-octadecadienoic acid methyl ester; 9Z,12Z,15Z-octadecatrienoic acid methyl ester were supplied by Accustandard (New Haven, Connecticut, USA).

1.2.2 Thermal Desorption GC-MS Analysis

The empty thermal desorption tubes were spiked with 2 μL samples and subjected to thermal desorption via a GERSTEL Twister ™ Desorption Unit (TDU) with a CIS 4 cryo-injector (Gerstel, Inc., Baltimore, MD, USA) in splitless mode. With a constant transfer temperature of 275°C, initial temperature of 50°C increased by 60°C min^{-1} until final temperature of 270°C was reached and held for 3 min. Desorbed sample was cryo-focused in the CIS 4 at -40°C before injection. Temperature was programmed to increase 12°C s^{-1} until final temperature of 275°C and held for 3 min.

A 6890N gas chromatograph and 5973 mass selective detector (Agilent Technology, Santa Clara, CA, USA) were subsequently used for separation and detection, respectively, with a Helium flow of 1.2 mL min^{-1}. Separation was performed using a HP-5MS (30m × 0.25mm × 0.25 μm) from Agilent Technologies. Initial oven temperature of held at 50°C for 1 min before two ramps (8 and 10 °C min^{-1}) allowed oven to reach temperatures of 260°C and 300°C, respectively. Final temperature was held for

10 min. Method was run under constant flow of 0.2 mL min^{-1}. The MS detector operated in scan mode had electron impact energy of 69.9 eV.

1.3 Results

SI Table 1.1. Size, zeta potential, pH and Ce ion concentration of *n*CeO_2 suspension. Values are means ± SE ($n = 3$). Means with the same letter are not significantly different at Tukey's test ($p \leq 0.05$) except for Diameter which was at Tukey's test ($p \leq 0.10$).

***n*CeO_2 concentration (mg L^{-1})**	**pH**	**Zeta potential (mV)**	**Diameter (nm)**	**Ce ion (mg L^{-1})**
62.5	5.44 ± 1.36a	29.0 ± 1.4b	857.3 ± 7.1a	0.32 ± 0.02b
125	5.48 ± 0.21a	35.3 ± 0.7a	527.4 ± 66.5c	1.08 ± 0.37ab
250	5.24 ± 0.84a	30.8 ± 1.0b	685.6 ± 35.9b	1.69 ± 0.35ab
500	5.09 ± 0.21a	20.4 ± 0.2c	616.2 ± 8.5bc	2.11 ± 0.32a

SI Table 1.2. Germination rate and seedling growth of medium amylose rice variety germinated and grown in *n*CeO_2 suspension for 10 days. Values are means ± SE ($n = 3$). Means with the same letter are not significantly different at Tukey's test ($p \leq 0.05$).

***n*CeO_2 concentration (mg L^{-1})**	**Germination rate (%)**	**Root length (mm)**	**Shoot length (mm)**
0	80.0 ± 2.2a	18.5 ± 2.0a	9.2 ± 0.3a
62.5	84.4 ± 4.8a	18.2 ± 1.7a	8.7 ± 0.4a
125	86.7 ± 0.1a	17.8 ± 2.5a	9.2 ± 1.0a
250	86.7 ± 0.1a	18.7 ± 0.2a	9.7 ± 0.4a
500	80.7 ± 3.3a	21.3 ± 2.5a	10.3 ± 0.5a

SI Table 1.3. Unsaturated fatty acid contents of roots of medium amylose rice variety germinated and grown in *n*CeO_2 suspension for 10 days. Values are means ± SE (n = 3). Means with the same letter are not significantly different at Duncan's test ($p \leq 0.05$).

***n*CeO_2 concentration (mg l^{-1})**	**Total unsaturated fat (ng g^{-1} FW)**	**Unsaturated fat (% of total fat)**	**C18:3 (% of total fat)**	**C18:3/C18:1 ratio**	**C18:3/C18:2 ratio**	**Double bond index**
0	72.37 ± 6.25ab	34.07 ± 1.13a	11.75 ± 0.50a	1.15 ± 0.09a	0.97 ± 0.01a	2.04 ± 0.02a
62.5	82.27 ± 7.67a	34.82 ± 0.41a	12.21 ± 0.41a	1.23 ± 0.09a	0.97 ± 0.01a	2.06 ± 0.02a
125	73.06 ± 6.07ab	32.93 ± 1.62a	11.05 ± 0.90a	1.08 ± 0.15a	0.97 ± 0.02a	2.02 ± 0.04a
250	85.04 ± 3.99a	34.60 ± 0.97a	12.27 ± 0.47a	1.29 ± 0.05a	0.96 ± 0.01a	2.08 ± 0.01a
500	63.36 ± 1.89b	33.78 ± 0.37a	11.45 ± 0.26a	1.04 ± 0.04a	1.02 ± 0.03a	2.01 ± 0.01a

2 SUPPORTING INFORMATION FOR CHAPTER 3: DIFFERENTIAL EFFECTS OF CERIUM OXIDE NANOPARTICLES ON RICE, WHEAT, AND BARLEY ROOTS: AN INFRARED MICROSPECTROSCOPY (FTIR-IMS) STUDY

SI Table 2.1. Size, zeta potential, pH and Ce ion concentration of *n*CeO_2 suspension. Values are means ± SE (n = 3). Means with the same letter are not significantly different at Tukey's test ($p \leq 0.05$) except for Diameter which was at Tukey's test ($p \leq 0.10$).

***n*CeO_2 concentration (mg L^{-1})**	**pH**	**Zeta potential (mV)**	**Diameter (nm)**	**Ce ion (mg L^{-1})**
62.5	5.44 ± 1.36a	29.0 ± 1.4b	857.3 ± 7.1a	0.32 ± 0.02b
125	5.48 ± 0.21a	35.3 ± 0.7a	527.4 ± 66.5c	1.08 ± 0.37ab
250	5.24 ± 0.84a	30.8 ± 1.0b	685.6 ± 35.9b	1.69 ± 0.35ab
500	5.09 ± 0.21a	20.4 ± 0.2c	616.2 ± 8.5bc	2.11 ± 0.32a

SI Table 2.2. Germination rate and seedling growth of medium amylose rice variety germinated and grown in *n*CeO_2 suspension for 7 days. Values are means ± SE (n = 4). Means with the same letter are not significantly different at Tukey's test ($p \leq 0.05$).

***n*CeO_2 concentration (mg L^{-1})**	**spring wheat (Jubillee)**			**spring barley (Millenium)**		
	Germination rate (%)	**Root length (mm)**	**Shoot length (mm)**	**Germination rate (%)**	**Root length (mm)**	**Shoot length (mm)**
0	60.0 ± 4.5a	7.0 ± 0.2a	4.8 ± 0.2a	59.2 ± 3.2a	6.0 ± 0.7a	5.1 ± 0.3a
62.5	68.3 ± 3.2a	5.4 ± 0.6a	4.4 ± 0.3a	50.0 ± 4.1a	5.2 ± 0.4a	4.3 ± 0.5a
125	55.8 ± 3.7a	6.2 ± 0.4a	4.8 ± 0.2a	67.5 ± 2.5a	6.6 ± 0.5a	5.4 ± 0.2a
250	51.7 ± 4.4a	7.0 ± 0.3a	4.6 ± 0.4a	64.2 ± 0.8a	6.8 ± 0.2a	5.5 ± 0.2a
500	60.8 ± 3.4a	6.6 ± 0.7a	4.5 ± 0.3a	62.5 ± 7.1a	6.2 ± 0.4a	5.3 ± 0.2a

3 SUPPORTING INFORMATION FOR CHAPTER 4: EFFECT OF CERIUM OXIDE NANOPARTICLES ON THE QUALITY OF RICE (*ORYZA SATIVA* L.) GRAINS

3.1 Preparation of Seedlings

The seeds were soaked in 4% NaClO for 15 min, washed three times with Millipore water (MPW), and placed in Petri dishes with 5 mL MPW. The Petri dishes were covered with aluminum foil and kept at 25°C in dark. After 4 days, the germinated seedlings were exposed in a growth chamber (Environmental Growth Chamber, Chagrin Falls, OH) with 14-h photoperiod, 25/20°C day/night temperature, 65% relative humidity, and 340 μmol m^{-2} s^{-1} light intensity.

3.2 Sample Preparation for Antioxidant Property Analysis

The grain powder (100 mg) was added with 1 mL 80% methanol and agitated overnight (100 rpm) at room temperature. The mixture was centrifuged (22,000×*g*, 15 min) and the supernatant was recovered before repeating the extraction using 1 mL methanol for 1 h. The pooled supernatants were allowed to evaporate at 45°C to ~500 μL and added with MW to a final volume of 2 mL. The extracts were kept at -20°C until use.

3.3 Determination of Total Phenolic and Flavonoid Contents

The total phenolic content was estimated following the method described by Dewanto et al.[39] The extract (100 μL) was mixed with Folin-ciocalteu phenol reagent (200 μL) and left to stand for 1 min at RT. Then, 3 mL 5% Na_2CO_3 was added and left to stand for 1 h in the dark at RT. The absorbance at 760 nm was measured using a spectrophotometer (Eppendorf 5417R, Hamburg, Germany). Gallic acid was used as a standard, and total phenolic content was expressed as gallic acid equivalents (GAE).

Total flavonoid content was measured according to the method of Jia et al.[40] The extract (500 μL) was mixed with 75 μL 5% $NaNO_2$, and left to stand for 5 min at room temperature. The mixture was sequentially added with 150 μL 10% $AlCl_3$, 500 μL 1 M NaOH, and 275 μL MPW. The absorbance at 510 nm was recorded. Catechin was used as a standard, and total flavonoid content was expressed as catechin equivalent.

3.4 Scavenging Ability by the DPPH Radical Assay

The DPPH scavenging ability of rice grain was determined following the method of Williams et al.[41] The extract (200 μL) was mixed with 2.8 60 μM DPPH, and left to stand in the dark for 30 min at RT. The absorbance at 515 nm was recorded, and BHT was used for comparison. The scavenging ability was calculated using the formula: scavenging ability (%) = $[(Abs_{511nm\ of\ control} - Abs_{511nm\ of\ sample})/Abs_{511nm\ of\ control}] \times 100$.

3.5 Scavenging Ability by ABTS Radical Cation Assay

ABTS radical cation scavenging ability of rice grain was analyzed following the method of Arts et al.[42] The extract (30 μL) was mixed with 3 mL ABTS radical cation solution, and left to stand in the dark for 7 min at RT. The absorbance at 734 nm was recorded, and BHT was used for comparison. ABTS radical cation solution was prepared according to previously described method.[39] The scavenging ability was calculated using the formula: scavenging ability (%) = $[(Abs_{7341nm\ of\ control} - Abs_{734nm\ of\ sample})/Abs_{734nm\ of\ control}] \times 100$.

3.6 Sample preparation for analysis of total, reducing, and non-reducing sugars

The rice grain (100 mg) were homogenized in 10 mL 80% ethanol, boiled in water bath (80 °C) for 30 min and centrifuged at 22,000×*g* for 20 min. The extraction was repeated 3 times and the extracts were pooled together. The volume of the extract was reduced to 5 mL by evaporation and the final volume was made up to 25 mL with MW.

3.7 Sample preparation for starch analysis

Starch was extracted from the dried residue from sugar extraction. The dried residue was added with 2 mL water and boiled in water bath for 15 min. The solution was cooled to room temperature and added with 2 mL concentrated H_2SO_4, stirred for 15 min and diluted to 10 mL with water. The mixture was centrifuged at 3,000×*g* for 20 min and the supernatant was obtained. The extraction was repeated using 50% H_2SO_4, and the supernatants were pooled and diluted to 40 mL with water.

3.8. Total sugar analysis

The analysis of total sugar was done according to Dubois et al.[44] Briefly, 100 μL of the extract was diluted to 1 mL with water and added with 1 mL 5% phenol and 5 mL 96% H_2SO_4. The solution was vortex and allowed to cool down for 10 min before placing in a water bath (30 °C) for 20 min. The same procedure was carried out in a series of standards and water as blank. The absorbance at 490 nm was recorded and the amount of total sugar was determined from the standard calibration curve. Glucose was used as standard.

3.9 Reducing sugar analysis

The reducing sugar content was determined following the method of Nelson-Somogyi.[45] A 100 μL sample was diluted to 2 mL with water and added with 1 mL alkaline copper tartrate. The solution was placed in a boiling water bath for 10 min, cooled down and added with 1 mL arsenomolybdolic acid reagent. The volume was adjusted to 10 mL with water, and stood for 10 min before reading the absorbance at 620 nm. The same procedure was done for a series of standards and water as blank. The amount of reducing sugars was calculated using the standard curve. Glucose was used as standard.

3.10 GC-MS Analysis of Fatty Acids

3.10.1 Chemical Standards

The fatty acid methyl ester standards namely: tetradecanoic acid methyl ester; pentadecanoic acid methyl ester; hexadecanoic acid methyl ester; octadecanoic acid methyl ester; 9Z-octadecanoic acid methyl ester; 9Z,12Z-octadecadienoic acid methyl ester; 9Z,12Z,15Z-octadecatrienoic acid methyl ester were acquired from Accustandard (New Haven, Connecticut, USA). The mirex internal standard was also purchased from Accustandard (New Haven, Connecticut, USA). These solutions were used to prepare a stock solution containing 1000 mg L^{-1} of each FAMEs and mirex. Using the stock solution, a series of working calibration standards with concentrations of 0.5, 1, 10, 50, and 100 μg L^{-1} were prepared. The calibration standards were applied to identify the retention time and generate the response curve. A total of 3 replicates with at least 2 runs per replicate were used.

3.10.2 Thermal Desorption GC-MS Analysis

The empty thermal desorption tubes were spiked with 2 μL samples and subjected to thermal desorption via a GERSTEL Twister ™ Desorption Unit (TDU) with a CIS 4 cryo-injector (Gerstel, Inc., Baltimore, MD, USA) in splitless mode. With a constant transfer temperature of 275°C, initial temperature of 50°C increased by 60°C min^{-1} until final temperature of 270°C was reached and held for 3 min. Desorbed sample was cryo-focused in the CIS 4 at -40°C before injection. Temperature was programmed to increase 12 °C s^{-1} until final temperature of 275°C that was held for 3 min.

A 6890N gas chromatograph and 5973 mass selective detector (Agilent Technology, Santa Clara, CA, USA) were subsequently used for separation and detection, respectively, with a Helium flow of 1.2 mL min^{-1}. Separation was performed using a HP-5MS (30m × 0.25mm × 0.25 μm) from Agilent Technologies. Initial oven temperature was held at 50 °C for 1 min before two ramps (8 and 10 °C min^{-1}) allowed oven to reach temperatures of 260°C and 300°C, respectively. Final temperature was held for 10 min. Method was run under constant flow of 0.2 mL min^{-1}. The MS detector operated in scan mode had electron impact energy of 69.9 eV. The MS detector operated in scan mode had electron impact energy of 69.9 eV.

3.2 Results

SI Table 3.1. ANOVA for nutrient content and antioxidant properties in rice grains harvested from plants cultivated in soil treated or not with 500 mg nCeO$_2$ kg^{-1}. ns, not significant at $p \leq 0.05$; *, **, *** significant at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

Parameters	nCeO$_2$ concentration (A)	Rice variety (V)	A×V	CV
Nutrient content				
Ce	26.10***	6.03*	5.39*	66.55
P	0.25ns	41.38***	0.52ns	4.48
K	11.81**	37.26***	2.66*	5.99
Ca	6.77*	32.16***	0.03ns	21.22
Mg	0.43ns	22.12***	0.92ns	3.84
S	16.44**	23.32***	0.84ns	3.66
Na	4.03ns	3.99*	0.08ns	8.06
Zn	0.85ns	16.11***	3.88*	21.09
Fe	24.56***	82.61***	110.09***	18.27
Cu	0.20ns	1.28ns	2.17ns	8.92
Mn	1.06ns	8.71**	3.65*	15.39
Al	0.15ns	3.87*	5.95*	44.91
Na/K	0.81ns	32.53***	1.49ns	9.75
Na/Ca	4.88*	23.02***	1.93ns	22.60
Mg/K	52.45***	49.68***	23.00***	3.57
Antioxidant value				
phenolic content	97.36***	409.32***	27.92***	6.37
flavonoid content	0.19ns	21.09***	2.83ns	7.37
DPPH scavenging ability	11.30**	2.29ns	3.60ns	20.64
ABTS scavenging ability	7.72*	19.76***	0.14ns	11.81

SI Table 3.2. ANOVA for nutritional value in rice grains harvested from plants cultivated in soil treated or not with 500 mg nCeO$_2$ kg^{-1}. ns, not significant at $p \leq 0.05$; *, **, *** significant at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

Parameters	***n*CeO$_2$ concentration (A)**	**Rice variety (V)**	**A×V**	**CV**
Nutritional content				
Fatty acids				
lauric acid (C12)	17.70***	11.84***	30.16***	11.90
myristic acid (C14)	0.19ns	1.62ns	1.35ns	45.99
valeric acid (C15)	145.14***	96.80***	156.39***	4.88
palmitic acid (C16)	0.39ns	0.51ns	2.75ns	33.62
oleic acid (C18:1)	0.76ns	0.83ns	3.38ns	42.07
linoleic acid (C18:2)	0.22ns	0.23ns	2.25ns	42.55
linoleic acid (C18:3)	1.51ns	0.83ns	1.33ns	54.63
Total	0.57ns	0.40ns	2.80ns	37.38
Protein fractions				
albumin	8.68**	11.12***	26.02***	5.75
globulin	1.40ns	8.74**	9.48**	6.45
prolamin	160.30***	824.48***	219.69***	3.68
Starch and sugars				
starch	16.28***	6.64**	0.37ns	4.86
total sugar	0.18ns	1.68ns	1.46ns	14.04
reducing sugar	0.40ns	12.74**	1.39ns	10.88
non-reducing sugar	0.37ns	1.22ns	2.45ns	19.12

SI Table 3.3. Some important elemental ratios in rice grains harvested from plants cultivated in soil treated or not with 500 mg *n*CeO_2 kg^{-1}. Values are means ± SE (n = 4). HA, MA, LA = high, medium, and low amylose variety, respectively. Between rice varieties, means with the same letter are not significantly different at Tukey's test ($p \leq 0.05$). Between *n*CeO_2 treatments, ns is not significant at $p \leq 0.05$; *, **, *** indicate significance at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

		Na/K	**Na/Ca**	**Mg/K**
untreated	HA	0.023 ± 0.001b	1.162 ± 0.146a	0.396 ± 0.003b
	MA	0.033 ± 0.000a	0.625 ± 0.041b	0.506 ± 0.004a
	LA	0.023 ± 0.001b	0.438 ±0.023b	0.403 ± 0.003b
	mean	0.026 ± 0.002	0.742 ± 0.117	0.435 ± 0.015
500 mg *n*CeO_2 kg^{-1}	HA	0.024 ± 0.001ab^{ns}	0.829 ± 0.134a*	0.385 ± 0.008a^{ns}
	MA	0.030 ± 0.002a^{ns}	0.484 ± 0.071ab^{ns}	0.405 ± 0.015a***
	LA	0.022 ± 0.002b^{ns}	0.444 ± 0.028b^{ns}	0.384 ± 0.004a^{ns}
	mean	0.025 ± 0.001^{ns}	0.586 ± 0.075*	0.391 ± 0.006***

4 SUPPORTING INFORMATION FOR CHAPTER 5: CERIUM OXIDE NANOPARTICLES IMPACT YIELD AND MODIFY NUTRITIONAL PARAMETERS IN WHEAT (*TRITICUM AESTIVUM* L.)

4.1 Results

SI Table 4.1. Fertilization record of wheat cultivated to grain production in *n*CeO_2-amended soil.

Number of days after transplanting	Amount of Yoshida nutrient solution (mL)
0	200
1	100
2	100
5	100
6	100
8	100
21	200
24	200
29	200
34	200
42	100
57	200
61	200
67	100
71	100

SI Table 4.2. Number of spikes in wheat cultivated in *n*CeO_2-amended soil. Values are means ± SE (n = 3). Means with the same letter are not significantly different at Tukey's test ($p \leq 0.05$).

***n*CeO_2 concentration (mg kg^{-1})**	**Number of days after transplanting**			
	42	**47**	**53**	**60**
0	4.00 ± 0.58a	10.33 ± 1.33a	15.33 ± 1.20a	21.00 ± 0.58a
125	2.33 ± 0.33ab	6.67 ± 0.88a	11.00 ± 1.53a	19.67 ± 1.20a
250	1.67 ± 0.67b	8.67 ± 0.33a	13.67 ± 1.20a	19.67 ± 0.67a
500	1.33 ± 0.33b	9.33 ± 1.67a	16.33 ± 0.88a	20.33 ± 0.33a

SI Table 4.3. Physiological maturity of wheat cultivated in *n*CeO_2-amended soil. Values are means ± SE (n = 3). Means with the same letter are not significantly different at Tukey's test ($p \leq 0.05$).

***n*CeO_2 concentration (mg kg^{-1})**	**Number of days after transplanting**				
	78	**81**	**85**	**89**	**91**
0	2.00 ± 0.01a	9.33 ± 1.76a	14.67 ± 1.76a	18.33 ± 0.88a	19.67 ± 1.33a
125	1.67 ± 0.33a	6.00 ± 1.00a	8.33 ± 0.67b	12.33 ± 0.88c	17.00 ± 0.58a
250	1.33 ± 0.33a	4.00 ± 0.01a	10.00 ± 0.01b	14.00 ± 0.58bc	18.00 ± 0.58a
500	1.33 ± 0.33a	5.00 ± 0.33a	8.67 ± 0.33b	16.00 ± 0.58ab	17.67 ± 0.33a

SI Table 4.3. Elemental concentration (mg kg^{-1}) in different tissues of wheat cultivated to grain production in *n*CeO_2-amended soil. Values are means ± SE, $n = 3$. Same letters mean no statistical difference between treatments at Tukey's test ($p \leq 0.05$).

***n*CeO_2 concentration (mg kg^{-1})**	**Ca**	**Zn**	**Cu**
		roots	
0	4642 ± 805a	45 ± 4a	50.8 ± 4.3a
125	3761 ± 672a	56 ± 5a	78.6 ± 19.6a
250	4315 ± 296a	49 ± 3a	67.1 ± 3.1a
500	4828 ± 305a	53 ± 2a	60.3 ± 5.0a
		leaves	
0	6219 ± 205a	24 ± 3a	3.1 ± 0.4a
125	6301 ± 346a	32 ± 3a	2.8 ± 0.2a
250	7360 ± 192a	24 ± 2a	3.1 ± 0.2a
500	6416 ± 332a	24 ± 2a	2.9 ± 0.2a
		grains	
0	387 ± 1a	75 ± 2a	7.3 ± 0.2a
125	380 ± 6a	75 ± 3a	7.3 ± 0.2a
250	378 ± 8a	71 ± 1a	6.9 ± 0.2a
500	386 ± 7a	73 ± 1a	7.3 ± 0.2a

5 SUPPORTING INFORMATION FOR CHAPTER 6: PHYSIOLOGICAL AND BIOCHEMICAL RESPONSE OF SOIL-GROWN BARLEY (*HORDEUM VULGARE* L.) TO CERIUM OXIDE NANOPARTICLES

5.1 Materials and Methods

5.1.1 Measurement of membrane damage

Membrane damage was determined by measuring the leakages of K and UV absorbing substances from the cells (K leakage and UVAS ratio, respectively). The measurement of K leakage was performed according to Navarri-Izzo et al.[12] The leaves were cut into small pieces, washed three times, placed in 15 mL MW, and incubated with shaking at RT for 4 h. The supernatant was collected and analyzed for K content by ICP-OES. UVAS ratio was estimated following the method of Lutts et al.[13] The samples were incubated in 10 mL MW for 24 h and an aliquot was used to read the absorbance at 280 nm. The aliquot was added back to the original solution before freezing the solution for 6 h. The absorbance was measured again and the UVAS was calculated by taking the ratio between the initial and final absorbance reading.

5.1.2 Cerium, Macro, and Micronutrient Concentrations in Wheat

Quantitative analyses of elemental concentrations in barley tissues were performed according to the method previously employed by Rico et al.[10] The samples were digested in a mixture of plasma pure HNO_3 and H_2O_2 (1:4) using a microwave accelerated reaction system (CEM Mars$_X$ Mathews, NC). ICP-OES/MS (Perkin Elmer, Waltham, MA) were used for the analyses. Blank, spikes and standard reference material (NIST-SRF 1570a) were used to validate the digestion and analytical method.

5.1.3 Amino acid profiling

The method described by Anjum et al.[12] was employed to analysis of amino acid contents in barley grains. Briefly, a 5 mL 6 N HCl was used to homogenized the powdered grains (25 mg) in a test tube which was subsequently evacuated by nitrogen, sealed, and further incubated in an oven (110°C) for 24 h. The solution was filtered and dried under vacuum (60°C) before dissolving in 1 mL phosphate buffer (pH 2.2). An aliquot (100 µL) was diluted 10 times with buffer, filtered in a 45 µm syringe filter, and analyzed using Biochrom 20 amino acid analyzer (Biochrom, USA).

5.1.4 Fatty acid analysis

Fatty acid content in barley grains was estimated according to Asekova et al.[13] The powdered grains (500 mg) were digested in a 5 mL solution of chloroform:hexane:methanol (8:5:2 v/v/v) overnight. A 100-µL extract was obtained and mixed with 75 µL of methylating reagent (0.25 M methanolic sodiummethoxide: petroleum ether: ethyl ether [1:5:2, v/v/v]) for 10 h before diluting the solution to 1 mL using hexane. Fatty acid content was analyzed using an Agilent (Palo Alto, CA) Series 7890 capillary gas chromatograph fitted with a flame ionization detector with an AT-Silar capillary column (Alltech Associates, Derfield, IL). The temperatures of the oven, injector, and detector were set at 210, 250, and 230°C, respectively. Standard fatty acid mixtures (Animal and Vegetable Oil Reference Mixture 6, AOACS) were used as calibration reference standards.

5.2 Results

SI Table 5.1. Fertilization record of barley cultivated to grain production in *n*CeO_2-amended soil.

Number of days after transplanting	Amount of Yoshida nutrient solution (mL)
0	200
1	200
26	200
122	300

SI Table 5.2. Soluble protein content in leaves of wheat cultivated in *n*CeO_2-amended soil at 48 days after transplanting. Values are means ± SE (n = 3). Same letters in each column mean no statistical difference between treatments at Tukey's test ($p \leq 0.05$).

***n*CeO_2 concentration (mg kg^{-1})**	**soluble protein (mg g^{-1})**
0	3.87 ± 0.37a
125	4.86 ± 0.37ab
250	5.74 ± 0.63b
500	26.81 ± 0.66b

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Vita

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Mr. Rico is an excellent writer with strong publication record. His collaborative work with other researchers resulted in 16 articles fully published in high impact factor journals and several other manuscripts under review or in preparation. He is the senior author of eight publications. His first paper, entitled "*Interaction of Nanoparticles with Edible Plants and Their Possible Implications in the Food Chain*" was published in the Journal of Agricultural and Food Chemistry. This paper was chosen as best research paper selected from more than 40 major peer-reviewed American Chemical Society (ACS) journals and Chemical & Engineering News. It was featured in the ACS PressPac and over a hundred newspapers, magazines and websites, and has been cited in more than 160 articles published in scientific journals. It was also featured in the ACS Global Challenges/Chemistry Solutions – an ACS project that identifies the world's most pressing sustainability challenges and explores the solutions emerging from chemistry. He is also a co-author in other papers published in high-impact factor journals. Most notable are the papers "Applications of synchrotron μ-xrf to study the distribution of biologically important elements in different environmental matrices: a review" that was selected as the journal cover, and "*In situ* synchrotron x-ray fluorescence mapping and speciation of CeO_2 and ZnO nanoparticles in soil cultivated soybean (*Glycine max*)" which was also featured in the ACS PressPac, Stanford Synchrotron Radiation Lightsource, and European Synchrotron Radiation Facility.

His outstanding performance as researcher allowed him to be invited as peer reviewer in high ranking journals like the Environmental Science & Technology, Journal of Hazardous Materials, Journal

of Agricultural and Food Chemistry, Science of the Total Environment, Environmental Science and Pollution Research, and Industrial Biotechnology. He is a consistent recipient of honors and awards including the Dodson Research Grant, the Frank B. Cotton Trust Scholarship Award, and several travel grants from UTEP and professional organizations. Recently, he was offered the Research Associateship Award by the National Research Council, The National Academies. He will continue his research at the National Health and Environmental Effects Research Laboratory, Western Ecology Division, Office of Research and Development, US Environmental Protection Agency in Corvallis, Oregon. His career goal is to become a reputable researcher in the field of analytical and environmental chemistry and join the academe or national laboratory in the future.

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18. **Rico, C.M.**; Majumdar, S.; Duarte-Gardea, M.; Peralta-Videa, J. R.; Gardea-Torresdey, J. L. Interaction of nanoparticles with edible plants and their possible implications in the food chain. *Journal of Agricultural and Food Chemistry* 2011, *59*, 3485-3498.

Selected platform/poster presentations:

1. **Rico, C. M.**, Barrios, A. C., Tan, W. J.; Peralta-Videa, J. R., Gardea-Torresdey, J. L. Cerium oxide nanoparticles modify crop physiology and grain quality in cereals. *UTEP Graduate Research Expo*, November 14, 2014, El Paso, Texas.
2. **Rico, C. M.**, Barrios, A. C., Hong, J., Morales, M. I., McCreary, R., Lee, W. Y., Peralta-Videa, J. R., Gardea-Torresdey, J. L. The interaction of CeO_2 nanoparticles with rice: Impacts on productivity and nutritional value. *Society for Risk Analysis 2013 Annual Meeting*, December 8-11, 2013, Baltimore, Maryland.
3. **Rico, C. M.**, Hong, J., Barrios, A. C., Morales, M. I., McCreary, R., Lee, W. Y., Peralta-Videa, J. R., Gardea-Torresdey, J. L. CeO_2 nanoparticles induce biochemical but not phenotypical modifications in rice. *SETAC North America 34th Annual Meeting*, November 17-21, 2013, Nashville, Tennessee.
4. **Rico, C. M.**, Barrios, A. C., Hong, J., Peralta-Videa, J. R., Gardea-Torresdey, J. L. Cerium oxide nanoparticles compromise the quality of rice (*Oryza sativa* L.) grains. *2nd Annual Conference of Sustainable Nanotechnology Organization*, November 3-5, 2013, Santa Barbara, California.
5. **Rico, C. M.**, Morales, M. I., Barrios, A. C., Hong, J., McCreary, R., Lee, W. Y., Varela-Ramirez, A., Peralta-Videa, J. R., Gardea-Torresdey, J. L. The impact of CeO_2 nanoparticles in rice. *UTEP Chemistry Research Day*, May 3-4, 2013, El Paso, Texas.
6. **Rico, C. M.**, Morales, M. I., Barrios, A. C., McCreary, R., Hong, J., Peralta-Videa, J. R., Gardea-Torresdey, J. L. Physiological impacts of cerium oxide nanoparticles in rice plants. *UC-CEIN Nano EH&S Forum*, May 8-9, 2013, Los Angeles, California.
7. **Rico, C. M.**, Hong, J., Morales, M. I., Barrios, A. C., Peralta-Videa, J. R., Gardea-Torresdey, J. L. The impact of CeO_2 NPs on rice roots: the relationship between enzyme activity, membrane damage, and lipid content. *1st Sustainable Nanotechnology Organization Conference*, November 4-6, 2012, Arlington, Virginia.

8. **Rico, C. M.**, Morales, M. I., Hong, J., Barrios, A. C., Peralta-Videa, J. R., Gardea-Torresdey, J. L. CeO_2 nanoparticles enhance lipid peroxidation in the shoot of germinating rice seeds. *SETAC North America 33rd Annual Meeting*, November 11-15, 2012, Long Beach, California.
9. **Rico, C. M.**, Hong, J., Morales, M. I., Barrios, A. C., McCreary, R., Lee, W. Y., Varela-Ramirez, A., Peralta-Videa, J. R., Gardea-Torresdey, J. L. The impact of CeO_2 NPs on rice roots: the relationship between enzyme activity, membrane damage, and macromolecule contents. *UTEP Graduate Research Expo*, November 9, 2012, El Paso, Texas.

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