The Impact of Cerium Oxide Nanoparticles on the Salt Stress Responses of *Brassica napus*

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Table of Contents

Abstract

Dwindling high quality water resources and growing population are forcing growers to irrigate crops with water of high salinity. It is well recognized that salinity negatively affects plant physiology and biochemistry, and represents one of the most serious threats to crop production and food security. Meanwhile, engineered nanoparticles (ENPs) are increasingly detected in irrigation water and agricultural soils due to the rapid advancement of nanotechnology. Previous research has demonstrated that ENPs such as cerium oxide nanoparticles (CeO₂-NPs) exert significant impact on plant growth and production. However, almost all previous studies were conducted in well controlled environment. Knowledge on how ENPs affect plant development in a stressed condition is almost empty. The goal of the present study was to understand the physiological and biochemical changes in *Brassica napus* L. (canola) cv. 'Dwarf Essex' under synergistic salt stress and CeO₂-NPs effects. Two salinity levels: 0 (control) and 100 mM NaCl, and three CeO₂-NPs concentrations: 0 (control), 200 and 1000 mg kg⁻¹ dry sand and clay mixture, were employed. As expected, 100 mM of NaCl significantly hindered plant growth and negatively affected the physiological processes of canola. Plants treated with CeO₂-NPs had higher plant biomass, exhibited higher efficiency of the photosynthetic apparatus and less stress in both fresh water and saline water irrigation conditions. Overall, our results demonstrated that CeO₂-NPs led to changes in canola growth and physiology which improved the plant salt stress response but did not completely alleviate the salt stress of canola..

Keywords: cerium oxide nanoparticles, *Brassica napus*, salt stress, canola

Capsule abstract: Cerium oxide nanoparticles improved plant physiological responses to salt stress but did not completely alleviate the salt stress in canola.

Introduction

Global food demands are expected to grow by 70–110% by 2050 (Tilman et al., 2011) while arable lands are decreasing due to land degradation, urbanization and seawater intrusion (Munns et al., 2012). Global climate change further aggravates the problem, especially in arid and semi-arid regions, by forcing farmers to use salty water for irrigation (Qadir et al., 2007; Rengasamy, 2010; Sanoubar et al., 2016). It is estimated that more than 6% of the world's total land and approximately 20% of irrigated land are affected by salinity (Munns and Tester, 2008), which is a serious concern in agriculture. Meanwhile, nanotechnology is rapidly advancing and its growth is forecasted to reach \$3 trillion in final goods by 2020 (Roco, 2011). The rapid development of nanotechnology has resulted in significant increases in the applications and release of engineered nanoparticles (ENPs) into the environment (Ma et al., 2010; Ma et al., 2015; Schwabe et al., 2015). Most ENPs eventually find their way into agricultural soils through water irrigation or biosolids land applications. Because of their known impacts on plant health and development, the introduction of ENPs into agricultural systems may result in stronger effects on plants which are already suffering from various environmental restraints than those grown in well controlled environment (*e.g.*, green house). However, the synergistic effects of ENPs with different environmental stresses (*e.g.*, salt stress) on plant health have not been explored.

Cerium oxide nanoparticles (CeO₂-NPs) are widely used in the production of catalysts, sunscreen creams, microelectronics and polishing agents due to their unique catalytic and optic properties (EPA, 2009). They are also used as a diesel fuel additive to increase fuel combustion efficiency and decrease diesel soot emissions (Cassee et al., 2011). This last application entails great environmental occurrence and impact of these nanoparticles. Previous studies have demonstrated that CeO₂-NPs displayed strong effects on plant health both positively and

negatively, depending upon the plant species, exposure concentration, exposure duration and plant growth conditions (Ma et al., 2010; Ma et al., 2016; Ma et al., 2015; Wang et al., 2012; Zhao et al., 2014). For example, the root elongation of lettuce was significantly inhibited after exposure to 2000 mg L⁻¹ CeO₂-NPs suspension for five days, while the root elongation of tomato, radish, rape, wheat, cucumber, and cabbage were largely unaffected at the same exposure condition (Ma et al., 2010). Similar species-dependent responses of plants to CeO₂-NPs were also reported by other researchers (López-Moreno et al., 2010b). Recently, Pagano et al. (2016) investigated the molecular mechanisms for plant responses to CeO₂-NPs and reported that chloroplast in tomato and zucchini play important roles in plant responses to CeO₂-NPs. Overall, the study indicated low CeO₂-NPs toxicity both at the physiological and molecular level. A separate study showed that the toxicity of CeO₂-NPs is concentration dependent, with high concentrations of CeO₂-NPs (2000 and 4000 mg L⁻¹) causing DNA damage to the soybean seedlings, but such effect was absent at lower concentrations (López-Moreno et al., 2010a). In fact, CeO₂-NPs were shown to enhance plant growth under certain exposure conditions. Wang et al. (2012) found that irrigation with 10 mg L⁻¹ of CeO₂-NPs slightly improved tomato growth and yield. While the underlying mechanisms for the different impacts of CeO₂-NPs at different concentrations are not fully understood, this may be partially attributed to the dual valance states of Ce (Ce³⁺ and Ce⁴⁺) on the nanoparticle surface, which makes CeO₂-NPs behave as an antioxidant at certain conditions (Wang et al., 2012) and an oxidative stress inducer at other conditions (Ma et al., 2016; Ma et al., 2015).

Brassica napus L. (canola) is the third most important source of vegetable oil in the world, after soybean and palm oil. During the past 20 years, it has surpassed peanut, cottonseed, and most recently, sunflower, in worldwide production (Ash, 2012). This is mostly attributed to the pioneering plant breeding work initiated in the 1950s and 1960s in Canada which greatly reduced

the levels of two anti-nutritional compounds, erucic acid in the oil and glucosinolates in the meal, creating a new, high-value oil and protein crop known as canola in Canada and the United States (Sovero, 1993). In addition, canola has shown good salt and heat resistance (Maas and Grattan, 1999). However, the levels of these stresses in arid and semi-arid environments frequently exceed the thresholds for optimal canola production.

Given the chances of ENPs accumulation in saline agricultural soils, some studies examining the synergistic effects of these two conditions on plants have emerged. For instance, Soliman et al. (2015) reported that *Moringa* plants sprayed with Hoagland's solutions containing ZnO-NPs and Fe₃O₄-NPs enhanced growth parameters under both normal and saline conditions compared with controls in each condition. The researchers also found that spraying plants with nanoparticles-containing Hoagland solutions resulted in a significant decrease of Na⁺ and Cl⁻ and a significant increase of N, P, K, Mg, Mn, Fe, Zn, total chlorophyll, carotenoids, proline and carbohydrates in plant tissues. Considering the extent of CeO₂-NPs effects on plants and its unique redox chemistry on the surface, it is intriguing to investigate how these nanoparticles will affect plant responses to external abiotic stresses (*e.g.*, salt stress).

The main objectives of this study were (1) to investigate whether CeO₂-NPs at different concentrations (0, 200 and 1000 mg kg⁻¹ dry sand and clay mixture) could affect the physiological and biochemical processes in canola and (2) to determine if the synergistic presence of CeO₂-NPs and NaCl (100 mM) could alleviate the plant oxidative stress induced by NaCl.

Materials and Methods

CeO₂-NPs

The dispersion of CeO₂-NPs coated with Polyvinylpyrrolidone (PVP) was purchased from the US Research Nanomaterials, Inc. (Houston, TX). The Transmission Electron Microscopy (TEM) image of CeO₂-NPs used in this study is shown in Fig. 1. The CeO₂-NPs are roughly spherical, but polygonal nanoparticles with clear edges are also found. According to the TEM image, most CeO₂-NPs fell in the size range of 20-110 nm, with an average size of 55.6 nm. The size and size distribution of CeO₂-NPs shown in Fig. 1 was obtained by measuring 270 individual NPs with an image processing software ImageJ (ver. 1.49, National Institutes of Health, Bethesda, MD). The zeta potential of 200 mg L⁻¹ CeO₂-NPs dispersed in water at pH=7 was -51.8 mV, as measured by a dynamic light scattering instrument (Zetasizer Nano ZS90, Malvern Instruments Ltd., Worcestershire, UK). CeO₂-NPs concentrations (200 and 1000 CeO₂ mg kg⁻¹ dry mixture) were chosen because the majority of the previous studies on the toxicity of CeO₂-NPs to terrestrial plants fell in the range of 1–1000 mg/kg⁻¹ (Holden et al., 2014). Even though the chosen concentrations are substantially higher than most predicted concentrations of CeO₂-NPs in the environment, it should be cautioned that those predictive studies were often based on the assumption that the released CeO₂-NPs would be uniformly distributed in the studied media (*e.g.* water, or soil) (Gottschalk et al., 2013), while in reality, this assumption may not be true. For example, a study indicated that about 95% of CeO₂-NPs entering into a wastewater treatment plant was retained in the activated sludge (Limbach et al., 2008) and when these activated sludge were applied to agricultural soils as biosolids, the concentrations of CeO₂-NPs in agricultural soils could be significantly higher than the predicted concentrations of CeO₂-NPs in soil.

Plant species and growth conditions

Brassica napus (canola) cv. ‘Dwarf Essex’ seeds were purchased from Johnny’s Selected Seeds (Winslow, ME). This cultivar was selected because of its germination characteristics and

salt stress tolerance. The seeds were sterilized in 2.7% Clorox bleach for 10 minutes, and washed three times with DI water. They were then placed in 100 mm diameter $\times$ 15 mm depth polystyrene petri dishes with a thin layer of DI water above the supporting filter paper. Following germinations, young seedlings were individually transplanted into 9.5 cm diameter $\times$ 12 cm height plastic pot with closed bottom and 500 g of the mixture of sand (Quikrete, Atlanta, GA) and leca (Clay pebbles, Hydrofarm, Petaluma, CA) (1:1 v/v) on the third day after seed imbibition. Before plant transfer, known amount of CeO₂-NPs solution and 25% Hoagland solution (Phyto Technology Lab, Shawnee Mission, KS) were introduced to each pot to achieve the concentration of 200 or 1000 mg kg⁻¹ dry mixture and full saturation (100% water holding capacity) in each pot. Ten plants for each treatment (0, 200 and 1000 mg/kg) were grown at room temperature under fluorescent bulbs providing 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (*I*) (16 h light – 8 h dark photoperiod). Control plants were irrigated with 20 mL of DI water on the same day of CeO₂ feeding. After 10 days of plant transfer, five plants from each treatment were randomly selected to be treated with 100 mM NaCl once a week. All plants were fert-irrigated with full strength Hoagland (Hoagland and Arnon, 1950) for 40 days. The NaCl concentration was chosen because 60-120 mM (Yousuf et al., 2016; Zaghdoud et al., 2016) was deemed as the average salinity in saline soil and brackish water in most previous studies. Starting from day 12 (one week after NaCl treatment), the chlorophyll fluorescence, net photosynthesis rate and gas exchange rate were measured weekly until the end of experiment. At the end of the experiment all plants were carefully rinsed with deionized water, tapped dry with a paper towel and divided into root and leaves, and their fresh weights (FW) separately weighted. Part of the fresh plant leaves were used for proline extraction and chlorophyll determination. The root tissues and the rest of the leaf tissues from each replicates was dried in oven at 70 °C for more than 48 h for the final determination of dry biomass

(DW), concentrations of different ions and cerium in plant tissues. Detailed descriptions on the analytical procedures are provided below.

Chlorophyll analysis

At the end of the experiment, 20 to 30 mg of leaf lamina was collected using a paper puncher, avoiding major veins. Leaf portions were placed in dimethyl formamide (DMF), and chlorophylls extraction was completed using the method described by Moran (1982). The amount of absorbance was read at 664 and 647 nm using a UV-Vis spectrophotometer (Lamba 35; PerkinElmer, Waltham, MA). Absorbance readings were used to calculate leaf chlorophyll concentrations.

Chlorophyll fluorescence

Leaf chlorophyll fluorescence measurements were carried out using a continuous excitation chlorophyll fluorescence analyzer (OS1p, Opti-Sciences, Hudson, NH). Leaves were acclimated to the dark using lightweight leaf clips for at least 30 min before measurements were taken (Maxwell and Johnson, 2000). Baseline (F_0) and maximum (F_m) fluorescence were measured and variable ($F_v = F_m - F_0$) fluorescence and the ratio of variable fluorescence to maximum fluorescence (F_v/F_m) ratio were calculated from these data.

Photosynthetic light response curves, Net photosynthesis and gas exchange rates

Light response curves, net photosynthesis and gas exchange rates were measured using a portable Infra-Red (IR) gas analyzer (LI-6400; LI-COR, Lincoln, NE) equipped with a standard LI-6400, 2 × 3 cm leaf chamber and a red/blue light source (6400-02B, LI-COR). At the time of measurement, leaf temperature ranged between 25 and 30 °C and chamber temperature was kept constant at 25 °C. Chamber CO₂ concentration was kept constant at 400 mg L⁻¹ and light levels

were obtained using the red/blue light source connected to the gas analyzer, which was programmed to provide ten photosynthetic photon flux (I) levels (0, 20, 50, 100, 200, 500, 800, 1000, 1500, and 1800 $\text{mmol m}^{-2} \text{s}^{-1}$). Measurements were performed on top leaves. Leaves were allowed to adapt to each light level for 2 min before each point was recorded. Maximum gross photosynthesis rate ($P_{g_{\max}}$) [$\text{mmol (CO}_2\text{) m}^{-2} \text{s}^{-1}$], quantum yield at $I = 0$ (ϕ_{I0}) [$\text{mmol (CO}_2\text{) mmol}^{-1}$ (photons)], dark respiration rate (R_d) [$\text{mmol (CO}_2\text{) m}^{-2} \text{s}^{-1}$], and light compensation point (I_{comp}) [$\text{mmol (photons) m}^{-2} \text{s}^{-1}$] were calculated by fitting net photosynthetic light-response curves with the Microsoft Excel spreadsheet elaborated by Lobo et al. (2013) using the model proposed by Baly (1935). The measurements were initiated one week after the treatment with NaCl started.

Proline determination

Approximately 0.5 g of fresh leaves were placed in a mortar and was homogenized at room temperature in 10 mL of 3% aqueous sulfosalicylic acid using a pestle. The homogenate was centrifuged at 11,000 rpm for 5 min. Two mL of the supernatant was reacted with 2 mL acid-ninhydrin and 2 mL of glacial acetic acid in a test tube for 1 h at 100 °C, and the chemical reaction was terminated in an ice bath. The reaction mixture was extracted with 4 mL toluene by mixing vigorously with a stirrer for 15-20 s. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance was read at 520 nm using toluene for a blank. The proline concentration was determined from a standard curve and calculated on a fresh weight basis following Bates et al. (1973).

Mineral contents analyses

Approximately 0.5 g of plant dry material were grounded and submitted to the Texas A&M AgriLife Extension Service Soil, Water and Forage Testing Laboratory for the elemental analysis

of several nutrient ions. All samples were oven-dried at 65 ± 2 °C in a forced air oven for 16 h and then ground. Plant total N was determined by high temperature combustion process. Nitrate in plant tissues was first extracted with 1 M of KCl solution and then reduced to nitrite through a Cadmium column (Hach, Loveland, CO) before it was quantified by a UV-Vis spectrometer with a Flow Injection Analyzers (FIAlyzer-1000, FIALab, Seattle, WA). Plant minerals (Ca, K, Mg, Mn, Na, P) were determined by an inductively coupled plasma-optical emission spectrometry (ICP-OES, Spectroblue-OEP, Spectro Analytical Instruments GmbH, Kleve, Germany) after 70% (v/v) nitric acid digestion (Havlin and Soltanpour, 1980).

CeO₂ contents analyses

Approximately 0.5 g of plant dry tissues were digested using a DigiPREP MS hot block digester (SCP science, Clark Graham, Canada), following the EPA method 3050b (USEPA, 1996). Dry shoots and roots of three replicates were ground and mixed with 4 mL of 70% (v/v) nitric acid (Certified ACS Plus). The mixture was maintained at room temperature overnight for predigestion, and then was digested in the hot block at 95 °C for 4 hours. After cooling down to room temperature, 2 mL of 30% (w/v) H₂O₂ (Certified ACS) was added to the mixture and heated in the hot block at 95 °C for another 2 hours to further digest the undigested residues suspended in the mixture. Finally, the Ce in the digestate was quantified by an inductively coupled plasma mass spectrometry (ICP-MS, Perkin Elmer mod. DRCII, Waltham, MA).

Statistical analysis.

Data were subjected to analysis of variance by a completely randomized design. One-way ANOVA was performed and means separation between treatments was obtained using Tukey's

test. Data were analyzed using the Minitab 17 Statistical Software (Minitab Inc., State College, PA).

Results

Growth analysis

Significant differences in plant biomass between different treatments for both the FW and DW (Fig. 2) were observed. A clear trend can be seen (Fig. 2A, B) that CeO₂-NPs enhanced both the total FW and DW of plants even though the impact of 200 mg kg⁻¹ CeO₂-NPs was insignificant. However, 1000 mg kg⁻¹ of CeO₂-NPs significantly increased both the FW and DW of canola by 12% and 50% respectively in salt-free conditions using distilled water for irrigation, compared with the controls. The enhanced growth of canola was primarily due to the growth of roots. The FW and DW of plant roots increased by 20% and 100% respectively after treatment with 1000 mg kg⁻¹ of CeO₂-NPs and fresh water irrigation (Fig. 2E, F). No significant differences were found the biomass of leaves. Interestingly, with salt stress, 1000 mg kg⁻¹ of CeO₂-NPs also significantly increased the total FW of canola, but the increased growth of the total FW was primarily due to the improved growth of plant leaves. Under the synergistic effects of salt, 1000 mg kg⁻¹ CeO₂-NPs increased the FW and DW of plant leaves by 48% and 52% compared with the controls under salt stress. The root FW and DW were not affected by CeO₂-NPs under salt stress.

Chlorophyll Analysis

To understand whether CeO₂-NPs affect plant leaf physiology, chlorophyll content was determined (Fig. 3). The results show that chlorophyll contents increased significantly in plants treated with CeO₂-NPs in both salt free and salt stress conditions (Fig. 3). A closer look of the chlorophyll *a* and *b* indicated that most of the changes of the total chlorophyll content were due to

the stimulation of Chl *a* (Fig. 3A), which increased by 10% in salt-stressed plants exposed to 1000 mg kg⁻¹ CeO₂-NPs and by 27% exposed to 200 mg kg⁻¹ CeO₂-NPs compared with plants treated with NaCl only. The content of Chl *b* was largely unaffected except for the treatment at 200 mg kg⁻¹ CeO₂-NPs in salt-free conditions.

Photosynthetic responses analyses

To further quantify the environmental effects on overall plant photosynthetic apparatus, F_v/F_m, stomatal conductance, net photosynthesis and photosynthetic light response curves were determined. F_v/F_m revealed significant differences between salt and CeO₂-NPs treatment (Fig. 4A). Plant treated with CeO₂-NPs generally showed higher values of F_v/F_m, especially in salt stress condition. Net photosynthesis rate was stable over time at salt-free conditions. 1000 mg kg⁻¹ of CeO₂-NPs did not affect the net photosynthesis rate compared with the control plants in fresh water condition, but 200 mg kg⁻¹ CeO₂-NPs significantly reduced the net photosynthesis rate after three weeks. Irrigation with 100 mM NaCl drastically reduced the net photosynthesis rate of canola, especially in the first two weeks (Fig. 4B). Exposure to CeO₂-NPs substantially alleviated the impact of salt stress on plant net photosynthesis rate, especially in the first two weeks. The stomatal conductance showed clear differences between salt stressed plants and plants without exposure to NaCl. A decrease by 90% on average after the first week of NaCl treatment was noticed (Fig. 4C). Plants treated with 1000 mg kg⁻¹ CeO₂-NPs showed higher stomatal conductance (mmol H₂O m⁻² s⁻¹) compared with the control ones. Overall, the stomatal conductance was decreased by 45% when plants were treated with NaCl; no alleviation of salt stress mediated by CeO₂-NPs were reported for stomatal conductance. Photosynthetic light response curves are quite consistent with the observations for stomatal conductance and net photosynthesis data (Fig. 4D). The highest concentration of CeO₂-NPs increased P_{gmax} by 45% among the plants only exposed to CeO₂-NPs

and by 6% among the plant exposed to both CeO₂-NPs and NaCl (Tab. 1). ϕ_{I0} and R_d were not affected by CeO₂-NPs but they increased under NaCl stress condition. Significant differences were recorded for I_{comp} .

Proline

Proline protein was measured to understand the overall stress responses in Brassica plants. Salt stress significantly increased the proline concentration in plants. CeO₂-NPs did not alter the proline activity in salt free conditions. However, CeO₂-NPs slightly lowered the proline activity in salt-stressed plants, especially at higher concentrations (1000 mg kg⁻¹).

Plant uptake of sodium and other mineral nutrients

NaCl treatment significantly reduced plant nutrient uptake. In contrast, CeO₂-NPs demonstrated little effects on plant nutrient uptake. A general trend in N (%), P, K, Mg and Ca (ppm) showed a consistent and significant decrease in leaf concentration of these elements following NaCl treatment (Fig. 6). In particular, N, K and Ca decreased by almost 35% and P by 25% (Fig 6A, B, C, and D). Understandably, sodium showed a significant increase in leaves and roots in NaCl treated plants. The sodium concentrations in leaves without and with salt exposure were 979 ± 208 and $139,062 \pm 1,044$ ppm respectively. Interestingly, the exposure to CeO₂-NPs seemed to lower the plant uptake of sodium (Fig. 6E). The sodium concentration in plant leaves decreased to 125,194 ppm and 120,867 ppm for plants treated with 200 and 1000 mg kg⁻¹ CeO₂-NPs. Similar results could be found for sodium content in roots (Fig. 6E). Magnesium, an important mineral for chlorophyll generation, increased by 15% (200 mg kg⁻¹ CeO₂-NPs) and 8% (under 1000 mg kg⁻¹ CeO₂-NPs) in salt-free plant leaves, suggesting a correlation between Mg and chlorophyll concentrations in leaves with CeO₂-NPs treatment under salt stress free condition (Fig.

6F).. However, the impact of CeO₂-NPs on magnesium content in plant leaves was minimal in salt-stressed plants.

Plant uptake of cerium

Cerium content in plants increased with the increasing cerium concentration in the mixture (Fig. 7A, B). Noticeably, salt stressed plants treated with the higher CeO₂-NPs concentration demonstrated markedly higher concentrations of cerium in both leaves and roots compared with plants not experiencing salt stress. The cerium concentration in plant leaves was 150% higher in salt stressed plants than unstressed plants at both concentrations of CeO₂-NPs.

Discussion

This study investigated the synergistic impact of CeO₂-NPs and salt stress at concentrations of 200 and 1000 mg kg⁻¹ CeO₂-NPs and 100 mM NaCl on the plant physiology of *Brassica napus* L. (canola) cv. Dwarf Essex. Canola is one of the most widely cultivated oil crops in the world because of the healthy fatty acid composition of its oil and high protein content of its meal. Similar to many other crops, canola performance is negatively affected by salt stress, especially in arid and semi-arid areas (Long et al., 2015). Its salt tolerance mechanism at the molecular level remains unclear, despite of a few recent reports (Chen et al., 2010; Saadia et al., 2012; Xue et al., 2009). The expanding use of CeO₂-NPs as diesel fuel additive (Casseo et al., 2011) leads to an increase of their content in cultivated soils. This study represents the first comprehensive investigation in the effects of CeO₂-NPs on canola responses to salt stress.

The results indicated that canola plants exposed to high salinity stress (NaCl 100 mM) are impaired in their growth, consistent with the observations reported in earlier studies. In fact, it has

been reported that canola plants were unable to grow, flower, and produce seeds in the presence of 120 mM NaCl (Long et al., 2015; Zhang et al., 2001). Symptoms of salinity stress such as leaf discoloration were also observed in our study. Very importantly, CeO₂-NPs conferred some protection towards the salt stress, promoting physiological mechanisms which resulted in higher total biomass, even though the beneficial effects did not fully compensate for the negative effects of salt stress. One previous study demonstrated that low concentrations of CeO₂-NPs (up to 130 mg kg⁻¹ soil) did not induce any harmful effect on growth of tomato plants throughout their entire life cycle (Wang et al., 2012). To our best knowledge, this is the first study to report the effects of CeO₂-NPs on plant salt stress responses. In particular, we noticed that several aspects of the photosynthetic machinery were improved when canola plants were exposed to CeO₂-NPs. Firstly, a significantly higher chlorophyll content in CeO₂-NPs exposed plants was observed when comparing plants treated with 200 and 1000 mg/kg Ce₂O-NPs with their relative controls (e.g. plants treated with fresh water or saline water only) in both salt stress free and salt stressed conditions. The increase in chlorophyll content provides plants more capability to absorb light. A higher total chlorophyll content could be the result of an increase of Mg²⁺ in salt-free canola plants as confirmed by our mineral contents data. Similar results have been reported by Zhao et al. (2014). In their study on the exposure of cucumber plants to CeO₂-NPs at 400 mg kg⁻¹ dry soil they found that CeO₂-NPs significantly increased the uptake of Mg²⁺ ion, which is an essential element of the chlorophyll molecule. Secondly, we found that CeO₂-NPs enhanced the F_v/F_m ratio since higher values were found in plant treated with CeO₂-NPs. F_v/F_m ratio reflects the light reemission by chlorophyll molecules when light returns from excited state to ground state and is used as an indicator of the photosynthetic energy conversion in higher plants (Maxwell and Johnson, 2000). The elevated values of F_v/F_m suggested that CeO₂-NPs enhanced the plant light energy use

efficiency of the photosystem II. Thirdly, the photosynthetic measurements performed at varying light intensities revealed a beneficial role of CeO₂-NPs at 1000 mg kg⁻¹ on salt-unstressed plants as confirmed by the higher values of P_{gmax}. Further examination on other photosynthesis related parameters such as the stomatal conductance and P_N indicated that they were unaffected by CeO₂-NPs at the test conditions.

To obtain further insights on the impact of CeO₂-NPs on salt stressed plants, we analyzed the proline accumulation in leaves. It is well known that a high rate of salt uptake usually leads to sodium compartmentation through vacuoles (Saadia et al., 2012). Proline is considered the most common osmolyte in water- and salt-stressed plants (Jimenez-Bremont et al., 2006; Tripathi et al., 2007). In addition to reducing cytosolic osmotic potential, proline plays a vital role in protein protection against denaturation (Hong et al., 2000) as well as in scavenging reactive oxygen species (ROS). Overall, plants treated with salt showed a higher proline content. The lower content of proline found in plants exposed to higher concentration of CeO₂-NPs (*e.g.*, 1000 mg kg⁻¹ dry soil) suggested that CeO₂-NPs may play a role in stress protection mechanisms. However, in the absence of measures for other parameters related to the plant stress level it is premature to assign Ce₂O-NPs a role in osmoprotection. There have been contradictory reports about CeO₂-NPs regarding their roles as either oxidative stress inducer or antioxidant, capable of scavenging free radicals and protecting cells from oxidative damage (Pulido-Reyes et al., 2015). Even though the antioxidant properties of CeO₂-NPs are not fully understood, previous studies demonstrated that CeO₂-NPs could be both antioxidant and oxidative stress inducer depending upon the size, surface properties (*e.g.* surface charge), exposure duration and plant age (Ma et al., 2016; Ma et al., 2015). The antioxidant abilities of the CeO₂-NPs are due to the presence of both Ce³⁺ and Ce⁴⁺ (Dunnick et al., 2015). The mechanisms of toxicity rely primarily on the formation of abiotic ROS and on

attachment of the nanoparticles to the cell wall which affect cell viability and ultrastructure (Dunnick et al., 2015; Pulido-Reyes et al., 2015). In our current study, CeO₂-NPs were found to improve some photosynthetic activities in salt stressed plants (i.e. chlorophyll content). In our previous studies, we found that CeO₂-NPs displayed superoxide dismutase mimetic activities and the impact of CeO₂-NPs on several oxidative stress biomarkers varied at different growth stages of plants (Ma et al., 2015). We also noticed that the impact of CeO₂-NPs displayed generational patterns that plants exposed to CeO₂-NPs for only one generation responded to CeO₂-NPs differently from plants which have been exposed to CeO₂-NPs more than one generations (Ma et al., 2016; Wang et al., 2012). Consequently, it remains unclear whether CeO₂-NPs can provide long term protection for plants suffering from salt stress, but the results reported in this study are promising and worth further exploration even though the protection provided by CeO₂-NPs was not enough to fully eliminate the salt stress in this study. One caveat of the result is that very high concentrations of saline water was used in this study to mimic a severe salt stress condition. With the decrease of fresh water resources, many states resort to brackish water for agricultural irrigation which would exert certain levels of salt stress but not as severe as used in this study, it remains unknown whether CeO₂-NPs would provide full protection for plants from relatively low salt stress conditions.

It should be mentioned that while CeO₂-NPs conveyed benefits to the growth and physiology of plants at salt stressed condition, higher cerium concentration in salt treated plants was noticed than in plants without salt stress. The underlying mechanisms that why salt stressed plants showed significantly higher cerium in their tissues are unknown, possibly because the salt stress might have compromised the membrane properties of plant root cells. Mechanistic investigation is currently underway for the enhanced uptake of Ce by plants in salt stress

conditions. The implications of significantly higher cerium content in salt stressed plant tissues are not clear and need further examination.

Overall, this is the first study to report the synergistic salt stress and CeO₂-NPs effects on the physiological regulations in *Brassica napus*. The findings suggest a positive role of CeO₂-NPs in cultivated crops, likely through the enhanced photosynthetic machinery performance and proline synthesis regulation even though more investigations are needed to fully understand the role of CeO₂-NPs at relatively low salt stress conditions. The study provided insights on the potential applications of CeO₂-NPs for the reduction of salt stress impact in agriculture. The application of CeO₂-NPs at the tested concentrations did not alter the nutritional values of canola. However, the elevated uptake of Ce by salt stressed plants and its implications for food safety need further investigation. More investigations on the mechanisms at cellular levels with different crops and CeO₂-NPs of different properties will further enhance the understanding of the potential applications of engineered nanoparticles in agriculture.

Acknowledgement

The authors acknowledge the financial support of USDA-NIFA research grant #2011-67006-30181.

Table. 1 - Maximum gross photosynthesis rate (Pg_{max}), quantum yield at $I = 0$ (ϕ_{I0}), dark respiration rate (R_d), and light compensation point (I_{comp}) of *Brassica napus* plants exposed to CeO_2 -NPs at 200 and 1000 $mg\ kg^{-1}$ dry mixture and 100 mM NaCl. Data are means of 3 replicates $\pm$ standard deviation. Statistical differences calculated by one-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$) are indicated by letters.

Treatment	Pg_{max} [mmol (CO_2) $m^{-2}\ s^{-1}$]	ϕ_{I0} [mmol (CO_2) $mmol^{-1}$ (photons)]	R_d [mmol (CO_2) $m^{-2}\ s^{-1}$]	I_{comp} [mmol (photons) $m^{-2}\ s^{-1}$]
Control	8.72 $\pm$ 0.66 b	0.07 $\pm$ 0.03 bc	1.36 $\pm$ 0.27 b	23.95 $\pm$ 4.28 b
CeO_2 200 $mg\ kg^{-1}$	9.27 $\pm$ 0.74 b	0.03 $\pm$ 0.01 c	1.30 $\pm$ 0.64 b	34.91 $\pm$ 3.16 a
CeO_2 1000 $mg\ kg^{-1}$	11.09 $\pm$ 0.81 a	0.08 $\pm$ 0.01 abc	1.41 $\pm$ 0.18 b	20.08 $\pm$ 4.32 b
NaCl 100 mM	5.00 $\pm$ 0.02 c	0.20 $\pm$ 0.01 ab	3.17 $\pm$ 0.06 a	43.85 $\pm$ 2.03 a
NaCl 100 mM + CeO_2 200 $mg\ kg^{-1}$	5.00 $\pm$ 0.01 c	0.18 $\pm$ 0.04 a	3.00 $\pm$ 0.58 ab	34.94 $\pm$ 2.34 a
NaCl 100 mM + CeO_2 1000 $mg\ kg^{-1}$	5.26 $\pm$ 0.45 c	0.14 $\pm$ 0.05 ab	2.39 $\pm$ 0.46 ab	33.74 $\pm$ 0.48 a

Figure 1. Transmission Electron Microscopy (TEM) imaging (A) and particle size distribution (B) of CeO₂-NPs.

Figure 2. Fresh (A, C, E) and dry (B, D, F) weight of *Brassica napus* growing in the presence of 200 and 1000 mg kg⁻¹ dry mixture of CeO₂-NPs and 100 mM NaCl. A and B: Total biomass, C and D: Leaf biomass, E and F: Root biomass. Means labeled by different letters are significantly different by Tukey's post-hoc test ($p < 0.05$). Error bars represent the standard deviation ($n=5$).

Figure 3. Relative chlorophyll *a* and *b* content (A) and total chlorophyll content (B) in *Brassica napus* plants exposed to CeO₂-NPs at 200 and 1000 mg kg⁻¹ dry mixture and 100 mM NaCl. Means followed by different letters are significantly different by Tukey's post-hoc test ($p < 0.05$). Error bars represent the standard deviation ($n=5$).

Figure 4. Fv/Fm ratio (A), net photosynthetic rate (B), stomatal conductance (C) measured at different days after sowing (e.g., D12 means twelve days after sowing). Average response curves of net assimilation rate to increasing irradiance (D). All the measurements are referred to *Brassica napus* plants exposed to CeO₂-NPs at 200 and 1000 mg kg⁻¹ dry mixture and 100 mM NaCl. Means followed by different letters are significantly different by Tukey's post-hoc test ($p < 0.05$). Error bars represent the standard deviation ($n=5$).

Figure 5. Proline content in *Brassica napus* plants exposed to CeO₂-NPs at 200 and 1000 mg kg⁻¹ dry mixture and 100 mM NaCl. Means followed by different letters are significantly different by Tukey's post-hoc test ($p < 0.05$). Error bars represent the standard deviation ($n=5$).

Figure 6. Mineral contents in *Brassica napus* plants exposed to CeO₂-NPs at 200 and 1000 mg kg⁻¹ dry mixture and 100 mM NaCl. A: Nitrogen (%). B and C: Potassium and Calcium (ppm). E: Sodium (log₁₀). F: Magnesium (ppm). Means followed by different letters are significantly different by Tukey's post-hoc test ($p < 0.05$). Error bars represent the standard deviation ($n=5$).

Figure 7. Cerium content in leaves (A) and root (B) of *Brassica napus* plants exposed to CeO₂-NPs at 200 and 1000 mg kg⁻¹ dry mixture and 100 mM NaCl. Means followed by different letters are significantly different by Tukey's post-hoc test ($p < 0.05$). Error bars represent the standard deviation ($n=5$).

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