THE IMPACT OF CERIUM OXIDE NANOPARTICLES ON PHOTOSYNTHESIS AND WATER USE EFFICIENCY OF SOYBEAN (GLYCINE MAX (L.) MERR.)

A Thesis

by

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ABSTRACT

The widespread industrial uses of cerium oxide nanoparticles (CeO_2 NPs) and their unregulated disposal have raised increasing concerns about the consequences of these nanoparticles on the environmental health and safety. Previous studies on the interactions between CeO_2 NPs and plants reported inconsistent conclusions of CeO_2 NPs toxicity on various species. While many previous research have demonstrated the impacts of CeO_2 NPs on the physiological, biochemical and genetic processes, detailed understanding on the effects of CeO_2 NPs on plant photosynthesis in still elusive. There has also no study which investigated the impact of CeO_2 NPs on plant water use efficiency (WUE), a key parameter for crop yield. Therefore, this research aimed to provide new insights into the impact of CeO_2 NPs with different surface properties (uncoated and polyvinylpyrrolidone (PVP)-coated) on plant photosynthesis and WUE at various soil moisture contents. The concentration of CeO_2 NPs ranged from 0-500 mg/kg dry soil and the soil moisture content ranged from 55-100% θ_{fc}. WUE was estimated by measuring $\delta^{13}C$ of soybean tissues and photosynthesis was thoroughly studied by characterizing photosynthetic response curve with respect to varying photon intensities and CO_2 concentrations.

CeO_2 NPs exhibited hermetic effect on soybean that positive impact was observed on the soybean at 100 mg/kg while significant inhibition was shown at the highest concentration of (500 mg/kg) at water sufficient condition (100% θ_{fc}). The results also indicated that both types of CeO_2 NPs at the concentration of 100 mg/kg soil stimulated the growth and consistently enhanced the photosynthesis and WUE of the soybean during the 3-week treatment. Our results also indicated that the enhancive effect of CeO_2 NPs on plant photosynthesis and WUE was dependent upon the soil moisture content. While both types of CeO_2 NPs exhibited consistently positive impact on the photosynthetic performance of the soybeans at the moisture content of 70%, 85% and 100% θ_{fc}, CeO_2 NPs did not enhance the photosynthesis efficiency of the soybeans at 55% θ_{fc}, which suggested that the positive effect of CeO_2 NPs was limited by the soil moisture deficiency. Further examination of the results suggested that V_{cmax} (maximum carboxylation rate) was affected by CeO_2 NPs, indicating that CeO_2 NPs affected the Rubisco activity which governs carbon assimilation in the dark reactions of photosynthesis. In conclusion, CeO_2 NPs demonstrated significant impacts on the photosynthesis and WUE of soybeans and such impacts were affected by the surface properties of CeO_2 NPs, the concentrations and the environmental conditions.

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CHAPTER I

INTRODUCTION

Water use efficiency (WUE) is defined as the ratio of the net photosynthesis to water transpiration and it is one of the key ecosystem factors affecting plant water uptake, carbon cycle and crop yield. As a result, $\delta^{13}C$ has been commonly used as a substitute index of plant WUE (Lajtha, K., 2007). C_3 Plants such as soybeans tend to discriminate against the heavier isotope ^{13}C in favor of the major, lighter isotope ^{12}C due to several enzymatic and physical processes. A particular advantage of the $\delta^{13}C$ measurement is the long integration time than other instantaneous measurements. The method is well established, allowing rapid screening on the cerium oxide nanoparticles (CeO_2 NPs) effects on plant WUE at different environmental conditions.

Plant photosynthesis is the most important physiological process in plants and is subject to the influence of various environmental factors including environmental chemicals. The measurement of the net photosynthesis rate as well as the responses of plant photosynthesis to various light intensity and carbon dioxide concentration can provide significant information on the health of plants. Based on the performance of photosynthesis, various parameters such as the maximum rate of Rubisco carboxylase activity (Vc_{max}) and the maximum rate of photosynthetic electron transport (J_{max}) can be derived which reveal the plant carbon assimilation efficiency. (Sharkey et al 2007). Examination of all parameters associated with photosynthesis can be used to determine whether the plants are stressed and which part of the photosynthesis process is affected by certain environmental factors (Farquhar et al., 1980).

Even though there have been some reports on the impact of CeO_2 NPs on plant photosynthesis, the underlying mechanisms of the impact of CeO_2 NPs on photosynthesis and WUE are still poorly understood. With the rapid expansion of applications of CeO_2 NPs, a closer look at the impact of CeO_2 NPs on the photosynthesis and plant WUE would provide further insights on the impact of CeO_2 NPs in the environment.

This thesis would concentrate on four major questions: (1) How would CeO_2 NPs affect the WUE of plants? (2) What are the impacts of CeO_2 NPs on the photosynthesis of plants? (3) Would nanoparticles with various surface properties make any difference of the impacts of plants? (4) What role does the moisture content of soil play in the interactions between nanoparticles and plants?

Soybean (*Glycine max* (L.) Merr.) is a popular food crop around the world and is used as a model plant of the C_3 plants. This research was aiming to investigate mechanisms of how CeO_2 NPs would affect the photosynthesis process by assessing both photosynthesis-response parameters and WUE. The overarching goal of the project is to understand how CeO_2 NPs, with different physicochemical properties, may affect plant WUE and photosynthesis process differently. The overall hypothesis is that CeO_2 NPs will exert significant impact on some physiological and biochemical processes associated with plant water uptake and transport and the impact will vary according to the unique properties of nanoparticles and the environmental conditions.

CHAPTER II

LITERATURE REVIEW

Nanotechnology

The concept of nanotechnology was first brought up by Dr. Richard Feynman, a physicist at the California Institute of Technology, in 1959. After twenty-years of development, Dr. Norio Taniguchi further defined nanotechnology as techniques manipulating particles at molecular level of which sizes are smaller than 100 nm in at least two dimensions (Kang, 2010). Manufactured nanoparticles exhibit unique physiochemical properties compared with their bulk counterparts because of their extremely small size and consequently very large specific surface area. Due to their appealing characteristics resulted from the size and surface property change, nanoparticles related industry has grown into a trillion dollar industry, employing 2 million employees and having $3 trillion global market (Xia et al., 2008). Nanotechnology has been incorporated into various industries such as electronics, catalytic converters, cosmetics, pharmaceuticals, optical processes, imaging system (Koo et al., 2005), photodynamic therapy (Allison et al., 2008), and implantable biosensors (Vaddiraju et al., 2010).

Among the various types of nanoparticles, a few types of nanoparticles are commonly used such as carbon nanomaterials, polymeric and metallic nanomaterials. Carbon based nanomaterials including nanotubes, nanofibers and nanoparticles are widely used in adsorption, membrane manufacturing, ion exchange, reverse osmosis, and environmental sensing (Magrez et al., 2006). Specifically, synthesized multi-walled carbon nanotubes are considered as a potential component in wastewater filtration and pollutant remediation. Comparatively, engineered single-walled carbon nanotubes are more commonly used in microelectronic industry due to its unique semiconducting and semi-metallic properties (Cao & Rogers, 2009).

Polymer nanomaterials have undergone great development in the past decade. Synthesized polymer nanomaterials filled with organic or inorganic functionalized groups have the combined properties of nanomaterial and conventional polymer composites (Balazs et al., 2006). Given their gas impermeability, stability, and flame retardance, polymer nanomaterials are commonly used as aerospace materials, in renewable cells and automotive manufacturing. (Kang, 2010, Hussain et al., 2006). Properties of polymer nanomaterials can be specifically designed by artificial functionalization with the rapidly improving software engineering techniques as polymer nanomaterial usually constitutes only five percent filler substance of the total filling volume (Vaia & Wagner, 2004).

In addition to the carbon based and polymer nanomaterials, metallic nanoparticles also play a key role in nanotechnology industry. Metallic nanoparticles are broadly utilized in mechanical, chemical, fuel additive, pharmaceutical and consumer products due to their physiochemical flexibility (Cassee et al., 2011). With the rapid advancement on coating techniques and computer simulation, nanoparticles with unique surface properties can be synthesized, which are widely used in biomedical technology, targeted drug delivery and diagnostic imaging techniques (Mody et al., 2009). A recent research reported the potential methodology of drug delivery with coated CeO_2 NPs to break through the blood-brain barrier, which has hindered effective drug delivery for decades. Conventional drugs or tumor cures are blocked by the blood-brain barrier which is a part of the immune system keeping brain from any exoteric damage but also shutting out the drugs. Coated CeO_2 NPs can penetrate through the barrier which may drastically advance the brain tumor research. (Collin et al., 2014). Besides, metallic nanoparticles can also facilitate the development of imaging techniques such as computed tomography (CT), positron emission tomography (PET), magnetic resonance imaging (MRI), ultrasound (US), and surface-enhance Raman imaging (SERS) (Sharma et al., 2006). Moreover, energy cell industry is in great demand of metallic nanoparticles. For instance, mesoporous nanocrystalline titanium dioxide is widely applied on dye-sensitized solar cells due to its relatively low cost and diffusion efficiency. By changing the porosity, titanium dioxide exhibits the potential to increase photoelectric conversion efficiency (Benkstein et al., 2003). Additionally, silver nanoparticles are commonly used in consumer products such as washing machine, vacuum flask and membrane filter coating because of their outstanding thermal conductance and antibacterial characteristics (Wijnhoven et al., 2009).

Cerium Oxide Nanoparticles

Among all those metallic nanoparticles, cerium oxide nanoparticle is one of the most important and commonly encountered nanoparticles. Cerium is one of the most abundant rare earth elements on earth, representing roughly 0.0046% of the earth's crust by weight (Collin et al., 2014). Cerium naturally exists in ecological systems, ranging from 2 to 150 mg/kg dry soil, in the form of cerite, cerianite, and zircon (Collin et al., 2014). Cerium oxide and some other rare earth elements have been utilized in agricultural and gardening fertilizers to promote crop productivity since 1986.

To further enhance the reactivity, cerium oxide is increasingly manufactured at nanometer scale and these nanoparticles are widely used in catalytic converters, fuel additives, cosmetic products and chemical catalysts industry (Cassee et al., 2011). Automobile manufacturing and petroleum refining industry serve as the largest cerium consumers as it is commonly used in the automotive catalytic converters and fluid catalytic cracking process. Estimated by the U.S. Geological Survey (USGS), roughly 10,000 tons of CeO_2 NPs have been consumed per year since 2011, of which over eighty percent is produced in China (Collin et al., 2014). CeO_2 NPs market is further expanded because of their outstanding ultraviolet absorption capacity which can be applied on sunscreen coating. Cerium nanoparticles released into the environment have not been accurately estimated, but the release from different industrial pathways is expected to lead to considerable accumulation in soil, water and even atmosphere. Considering the great amount of annual production and release, potential accumulation, transport and enrichment in the ecosystem are becoming threats to environmental sustainability and human health. Therefore, researchers are particularly interested in its environmental effect and fate due to the accumulating evidences of some negative impact of engineered nanoparticles (ENPs) once they are released into the environment.

Mechanism of the catalytic property of CeO_2NPs mainly results from the oxidation states shift between Ce(III) and Ce(IV). CeO_2 NPs will enhance the mobility of lattice oxygen on the particle surface, which may result in higher reactivity and unique hazards to ecological receptors, due to their relatively larger surface area compared with the bulk composites. On the other hand, CeO_2 NPs can also exhibit anti-oxidative property as a result of their size (Asati et al., 2010). Asati et al (2010) reported that CeO_2 NPs show antioxidant property at pH 7.4 which can be potentially applied on pharmaceutical industry mediating radiation damage. Some studies also revealed that CeO_2 NPs can minimize oxidation stress and cure inflamed spinal cord neurons (Chen et al., 2006).

Toxicity of CeO_2 NPs is mainly defined by the surface content of Ce^{3+}. Generally, CeO_2 NPs containing higher ratio of Ce^{3+}/Ce^{4+} is more likely to exhibit greater toxicity to plants. Further experiment reveals that excessive Ce^{3+} is able to quickly consume the superoxide radicals producing hydrogen peroxide, which is harmful to plants (Pulido-Reyes et al., 2015). Besides, toxicity of CeO_2 NPs is also correlated with particle size and surface area that plant growth is more likely to be inhibited when it is treated by nanoparticles with decreasing size to surface area ratio. In contrast, the degree of agglomeration exhibits less significant impact on apparent toxicity (Van Hoecke et al., 2009). It was also reported that dispersed CeO_2 NPs are more toxic than agglomerated CeO_2 NPs that they inhibited growth of several selected marine and freshwater microalgae.

Ecosystem Transport and Fate

Due to the considerably great amount of cerium oxide nanoparticle usage, it is likely that CeO_2 NPs will continue to build up in the environment. Nanoparticles entering natural environment tend to aggregate forming larger particles depending on the surface charge, particle size and surrounding environment. On the other hand, size increment will change the transport, behavior, reactivity, uptake pathways by organisms, and toxicity of nanoparticles (Collin et al., 2014) Because of the complication of realistic agglomeration, transport and fate of released CeO_2 NPs, and property characterization are usually estimated by software modeling and simulation. (Hussain et al., 2006))

With the increase utilization and manufacturing of CeO_2 NPs, concerns about the fate of CeO_2 NPs releasing into soil and aquatic systems are growing. Although the transport pathways are not well understood, increasing evidences reveal that released nanoparticles will be enriched in plant tissues. Potentially harmful metallic nanoparticles will be further enriched in the food chain through their accumulation in edible tissues of plants, which leads to unpredictable consequences. (Miralles et al., 2012).

The impact of nanoparticles on plants varies with the plant species. Once the nanoparticles are transported into plant tissues, they will interact with plant cells, resulting in a series of modifications on plants. Mounting evidences suggest that CeO_2 NPs tend to be enriched in the roots of soil cultivated plants but will hardly translocate to above ground tissues. A recent study reported that cerium ions preferentially accumulate in the cell wall of the roots rather than shoots of rice seedlings (Liu, et al., 2012). Similarly, significant cerium accumulation was detected in the roots but not in the shoots of the wheat, suggesting that there are limited transport pathways for cerium uptake. (Rico, et al., 2014) Besides, Zhao et al. (2013) reported that engineered nanoparticles caused increasing accumulation of cerium in corn roots, which further confirm the cerium uptake preference by plant roots. However, CeO_2 NPs can be detected in shoots and fruits in hydroponics plants. Wang et al (2012) reported that Ce was detected in the edible tissues of hydroponically grown tomatoes exposed to 10 mg/L of CeO_2 NPs. Similarly, Miralles et al (2012) reported that CeO_2 NPs were detected in plant roots as well as the edible tissues, which indicated the probability of CeO_2 NPs getting into ecosystem and food chain.

Cerium Oxide Nanoparticles Impact on Plants

Previous studies have reported contradictory results on the impact of CeO_2 NPs on plants. Some reported the oxidative toxicity while the others demonstrated the antioxidant capability of free radical scavenging to protect organisms from oxidative damage (Pulido-Reyes, et. al., 2015). Increasing evidences suggest that CeO_2 NPs are able to alter the nutritional value, productivity and metabolism of some species under various concentrations. Priester et al. (2012) reported that soybeans cultivated in soil treated with CeO_2NPs and zinc oxide (ZnO) nanoparticles at different concentration were affected by both CeO_2 NPs and ZnO NPs. Moreover, seeds germination rate and roots elongation of tomato, corn cucumber were inhibited when they are exposed to 4000 mg/L CeO_2 NPs suspension while no significant impact was found on radish, wheat, cucumber and cabbage treated with 1000 mg/L CeO_2 NPs (Ma et al., 2010). No consensus has been established whether it can enhance or inhibit the metabolism and photosynthesis pathways of plants.

Interestingly, although CeO_2 NPs presents potential toxicity, it has been reported that CeO_2 NPs improved the yield of some crop species. Numerous previous studies have demonstrated that CeO_2 NPs could modify plant physiological and biochemical processes and affect plant growth and yield. For example, Yuan et al. (2001) reported that CeO_2 NPs-enriched fertilizer stimulated root growth in rice seedlings. CeO_2 NPs of up to 500 mg/kg soil concentration can enhance plant growth, shoot biomass and grain yield. (Rico, et. al., 2014) Typically, CeO_2 NPs at low concentrations are more likely to exhibit positive impact on soybeans. Shyam and Aery (2012) reported that cerium at low concentrations had positive impact on chlorophyll content, biomass productivity, and nitrate reeducates activity of cowpea plants (*Vigna unguiculata*). Likewise, CeO_2 NPs enhanced both the shoot and root length of corn at 0.1 mM concentration while they significantly damaged the shoot and root at higher concentrations (Diatloff et al., 2008). Similar results had been observed that 0.1mM CeO_2 NPs significantly increased the biomass of rice seedlings but both fresh weight and dry weight of rice dramatically declined when they were exposed to higher concentrations of cerium. (Liu, et al., 2012) Positive impact of CeO_2 NPs had also been found by Wang et al., (2012). The research involved with tomato exposed to CeO_2 NPs of varying concentrations (0.1 to 10 mg/L). Results showed slight positive impact on productivity and plant growth parameters. Overall, CeO_2 NPs at relatively lower range of concentration promotes plant growth and crop productivity.

Comparatively, some evidences suggest that plants exposed to CeO_2 NPs will suffer growth inhibition, enzyme malfunction, DNA damage and oxidative damage resulting from increasing reactive oxygen species. (Lopez-Moreno et al., 2010). According to Diatloff et al. (2008), CeO_2 NPs with the size less than 5 μm reduced biomass of corn shoots by up to 30%. Priester (2012) found that CeO_2 NPs undermined soybean growth and yield, and ceased the nitrogen fixation function at the concentration of 10 g/kg soil. Another study showed that exposure to TiO_2 NPs hydroponically quickly reduced the hydraulic conductivity of the primary root of corn seedlings and inhibited the corn leaf growth (Asli & Neumann, 2009). Except for the crops, experiments involved with some other species such as green algae indicated the negative impact of CeO_2 NPs on photosynthesis. Rohder et al (2014) cultivated green algae *Chlamydomonas renhardttl* with CeO_2 NPs enriched solution. The results showed that CeO_2 NPs at high concentration (100 μM) inhibited photosynthesis process of the *C. renhardttl*, with agglomerated CeO_2 NPs exhibited higher toxicity than dispersed CeO_2 . Moreover, CeO_2 NPs at high concentrations only inhibited seed germination and plant growth, but also led to DNA damage to some species. (Lopez-Moreno et al., 2010).

Additionally, nutritional values and mineral element contents of soybeans were changed by CeO_2 NPs. (Peralta-Videa, et al., 2014) L. Zhao et al., (2013) found that CeO_2 NPs at 400mg/kg could increase the starch content and globulin but reduce glutelin of cucumber while had no significant effects on macronutrients. Contents of mineral nutrition elements including Mg, Ca, K, Na, Fe, Mn, Zn and Mo were also altered within the rice seedlings treated by different concentration of cerium. (Liu, et al., 2012)

Inconsistency of the results might result from variances in exposure methods, culture media, properties of CeO_2 NPs, concentrations and species (Gui et al. 2015). A combination of environmental factors contributes to the uptake and accumulation of elements in plant tissues. In general, plant root uptake of minerals from the soil mainly depends on the availability of element species. Mineral accumulation preference in tissues, transport pathways and competition among element species also affect the uptake of nutrients. (Peralta-Videa, et al., 2014) Besides, nutrient adsorption by roots is influenced by plant species, soil type, pH and temperature. For instance, soybeans prefer to uptake more cationic element species in acidic soil (Wang et al., 2000). Organic matter in soil interacting with nanoparticles has significant impact on their properties, which may also change their mobility and bioavailability (Zhao et al., 2013, Zhang et al., 2012). Thus, nutrient uptake altered by CeO_2 NPs provides further insights on their impact on plants and the surrounding environment.

Some recent studies indicate that the plant water use efficiency (WUE) can be influenced by manufactured nanoparticles. For instance, titanium oxide nanoparticles dramatically reduced hydraulic conductivity of roots in corn seedlings (Asli and Neumann, 2009). Conversely, multi-walled carbon nanotubes were shown to be able to promote water transpiration and uptake as a result of declining friction in water transport pathways brought by their functionalized alignment. (Tripathi et al., 2011). A Separate study also showed that multi-walled carbon nanotubes improved water uptake by enhancing aquaporins activity, which is a key water channel protein regulating permeability of cell membrane, in Tabaco root cell walls (Khodakovskaya et al., 2012).

Plant Stresses

Plants are subject to the impact of a series of environmental factors including moisture content, soil type, temperature, light intensity, salinity, pH, and surrounding organic matter. Treated with nanoparticles, plants may go through germination inhibition, productivity reduction, and oxidative stress. Specifically, oxidative stress induced by nanoparticles generally leads to accumulating cellular reactive oxygen species (ROS) such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), and singlet oxygen (O_2^*), and reactive nitrogen species (NO^-), nitric oxide and peroxynitrite ($ONOO^-$) (Blokhina et al., 2003). Reactive oxygen and nitrogen species exhibit strong tendency to accept electrons because of their unbalanced electron, which may be harmful to cellular structures (Dat et al., 2000). Damage of reactive oxygen species origins from the unpaired electrons while damage of reactive nitrogen species mainly generated from the antimicrobial responses (Iovine et al., 2008). Although reactive oxygen species are naturally produced in quite a few biological processes in plant tissues, they will cause damage when they reach excessive amount.

Interestingly, plants have developed a series of protection mechanisms when faced with oxidative stress. Inherent antioxidant systems of plants maintain oxidative balance through removing free oxygen radicals with enzymatic and non-enzymatic scavengers.

Typically, enzymatic antioxidant includes superoxide dismutase (SOD) which is able to catalyze the conversion of superoxide radicals to H_2O_2, and catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) which can further scavenge the H_2O_2 (Beyer et al., 1987). Three types of SOD isoforms, including MnSOD, FeSOD, and

Cu/ZnSOD, have been shown to remove reactive oxygen species. Cu/ZnSOD functions more efficiently compared with the other two isoforms but its activity will be inhibited by increasing H_2O_2 concentration (Tepperman & Dunsmuir, 1990). Comparatively, MnSOD and FeSOD are able to protect DNA and redox-sensitive proteins from damaging by eliminating stress (Hopkin et al., 1992). In addition, GPX and APX are able to accelerate the conversion of H_2O_2 into oxygen and water (Gechev et al., 2006). At high level of H_2O_2, CAT serves as a more efficient scavenger which is 10,000-fold faster than natural degradation. However, it will mainly function at high level of H_2O_2 due to its low affinity with H_2O_2.

In addition to the enzymatic antioxidants, some small molecules can assist plants with nanoparticle induced stress mediation (Soren et al., 2015). These small molecules include ascorbic acid (vitamin C), reduced glutathione (GSH), peroxiredoxins (Prx), thioredoxins (Trx), glutaredoxins (Grx) and so forth (Dat et al., 2000). Specifically, for instance, ascorbic acid can serve as an electron donor neutralizing the electron depleted species. Comparatively, GSH can serve as the substrate of superoxide radical removal.

Although plants have sophisticated strategies to mediate oxidative stress, most of them are subject to excessive stress caused by manufactured nanoparticles. Based on previous studies, TiO_2 NPs could lead significantly higher reactive oxygen species in cucumber tissues over 150 days of exposure (Servin et al., 2013). Arising oxidative stress induced by NiO-NPs, ZnO-NPs, Ag-NPs, Fe_2O_3-NPs has also been reported in different plant species. CeO_2 NPs could induce higher level of hydrogen peroxide after corns (*Zea mays)* were treated 10 days. In the meanwhile, increasing concentration of catalase and guaiacol peroxidase were also detected (Zhao et al., 2013). Likewise, it was also found that CeO_2 NPs could stimulate catalase production in Cilantro. Treated with CeO_2 NPs at concentration from 1000 to 2000 mg/L, *Arabidopsis thaliana* L. tissues displayed dramatically higher level of anthocyanin, which is a small molecular antioxidant against oxygen radical damage (Wang R, et al., 2013). Similarly, Rico et al (2013) reported that rice seedlings could increase their free thiols and ascorbate production when they were exposed to CeO_2 NPs.

Photosynthesis

Photosynthesis in plants incorporates a complex of biological reactions converting solar energy into chemical energy at cellular level (Sharkey et al., 2007). As a primary driving force of global carbon cycle, photosynthesis accelerates the exchange of carbon between the atmosphere and terrestrial biosphere (Walker et al., 2014). In photosynthetic process, Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) serves as the major enzyme assimilating atmospheric CO_2, which catalyzes the CO_2 into ribulose-1,5-bisphosphate (RuBP) (Lin et al., 2014; Galmes et al., 2014). Accumulating studies have provided physiological insights of Rubisco activity such as the driving forces of Rubisco evolutionary adaption and the correlations among the Rubisco related kinetic parameters (Galmes et al., 2014).

Photosynthesis rate of plants responding to varying light intensity and CO_2 concentration provide significant information of leaf physiology including Rubisco activity. The light intensity response curve illustrates carbon assimilation function with respect to rising photon flux density along with physiological insights of maximum net photosynthesis rate and maximum quantum yield (Labo et al., 2013). Generally, CO_2 assimilation rate linearly increased with arising light intensity at light-deficient conditions, where the photosynthesis processes were limited by electron transport (Lambers et al., 2008). The slope at the light deficient conditions represented quantum yield indicating the efficiency of light adsorption and conversion into fixed carbon (Lambers et al., 2008). As the photon density arose, photosynthesis would be limited by carboxylation rate.

Complementary with the light intensity curve, the CO_2 concentration response curve demonstrates the plant responses with a series of photosynthetic parameters including Rubisco carboxylase activity (Vc_{max}), the maximum rate of photosynthetic electron transport (J_{max}), triose phosphate use (TPU), day respiration (R_d), and mesophyll conductance (g_m) (Sharkey et al., 2007).

Plants are able to adapt to varying surrounding conditions by changing their electron transfer and Rubisco activity. Carbon assimilation is a complex solar energy conversion reaction consisting of three major processes: Rubisco limited process, RuBP regeneration limited process, triose phosphate use (TPU) limited process (Sharkey at el., 2007). In general, the rate of photosynthesis can be predicted by the properties of ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco) assuming a saturating supply of substrate, RuBP. This state is called Rubisco-limited photosynthesis and normally occurs when the concentration of CO_2 [CO_2] is low. The limitation by Rubisco is associated with the low [CO_2] rather than *V*max of the enzyme (Sharkey at el., 2007).

The second process is limited by RuBP, which takes place when concentration of CO_2 arises, as increasing level of CO_2 accelerates the RuBP carboxylation process. Comparatively, TPU limited process will not correlate with carbon dioxide concentration because TPU produced by chloroplast will not keep increasing when it reaches the maximum value. TPU limitation can be used to estimate the theoretical maximum carbon assimilation rate of plants.

WUE and Carbon Isotope

Defined by the ratio of carbon assimilation to water transpiration, WUE is necessary to be increased by improving net carbon assimilation at relatively fixed stomatal conductance (Parry et al., 2005). Except for agronomic methods such as breeding crops with specific genotypes to achieve higher WUE (Gregory, 2004; Condon et al., 2004), WUE can also be promoted at the physiological level (Boyer, 1996; Parry et al., 2005). The methodology at the physiological level can be achieved by increasing concentration of catalytically active Rubisco sites to improve the carboxylation rate or bypassing photorespiration, or stimulating CO_2 diffusion in the mesophyll or improving mesophyll diffusion conductance to CO_2 (Galmes et al., 2005; Parry et al., 2005; Flexas et al., 2013).

Isotope ratios of elements could become locally enriched or depleted through a variety of kinetic and thermodynamic factors. In general, plants tend to discriminate against the heavier isotope ^{13}C in favor of the major, lighter isotope ^{12}C due to a series of enzymatic and physical processes. Thus, most plants incorporate less ^{13}C than the atmospheric CO_2 on which they rely for photosynthesis. Such discrimination varies among plants with different photosynthetic process. Measurement of the carbon isotope ratios can be used to differentiate between samples which otherwise share identical chemical compositions.

Specifically, the discrimination of ^{13}C generates from a few processes including the rubisco fractionation, stomatal conductance, mesophyll conductance, respiration and photorespiration. The C_3 pathway begins with the diffusion of CO_2 from the atmosphere into the air-filled spaces within the leaf. This diffusion occurs through the still air occupying stomatal pores. Such diffusion has an apparent fractionation of 4.4‰ due to the slower motion of the heavier ^{13}C-containing CO_2 molecules (Lajtha, & Michener, 2007). Within the leaf, the carboxylating enzyme ribulose bisphophate carboxylase/oxygenase (rubisco) discriminates further against the ^{13}C. These two dominant fractionation factors contribute 4.4‰ to 29‰ to the intercellular $\delta^{13}C$. Thus, the general $\delta^{13}C$ value varies from -12.4‰ to -37‰, with the median of -27‰ (Lajtha & Michener, 2007).

Carbon assimilation mechanisms and isotopic composition of C_4 plants considerably differ from those of C_3 plants. C_4 plant photosynthesis pathways are mainly controlled by a different type of enzyme name phosphoenolpyruvate carboxylase, which contributes roughly -6‰ to the carbon isotope fractionation. Total carbon isotope discrimination of C_4 plants usually lies at -14‰. Noticeably, CAM plants depending on the same kind of carboxylating enzymes as C_4 species can hardly be effectively distinguished by carbon isotope characterization due to their overlapping isotope composition range (Lajtha & Michener, 2007).

Climate Change and Food Supply Crisis

Thanks to the technologies of genotype screening, pesticides, fertilizers and irrigation, crop yield has increased by 100% than it used to be in 1960's (Tilman et al., 2002). However, food supply is still challenged by declining arable fields, unpredictable global climate change, water crisis and increasing population. To fulfill the climbing food demand led by growing population, it is necessary to increase crop productivity by 30% by 2050 (Tilman et al., 2002). Unfortunately, to achieve the 30% increment in crop yield, water use for irrigation has to be increased by 100%, which is unlikely given the limited fresh water resources. Furthermore, global climate change and degrading water quality would exacerbate the fresh water shortage and may further increase aridity in some regions (Dai, 2011; Sheffield et al., 2012). Due to the emission of greenhouse gas since the 20th century, global mean temperature has increased continuously (Flexas et al., 2013). According to the recent report released by Inter-Governmental Panel on Climate Change (IPCC), the global temperature is 0.74±0.18°C higher than it used to be in 1900 (Flexas et al., 2013). Consequently, the global warming leads to increasing surface water evaporation, decreasing the moisture content in soil. Besides, higher water vapor content in the atmosphere will bring increasing frequency and intensity of extreme precipitation, which has already been observed in previous studies. Faced with the declining availability of irrigation water and decreasing moisture content in soil, WUE is becoming a major concern to achieve more productive crop yield.

Among all those plant species, soybean serves as a major food crop that is currently the fifth largest crop in the global agricultural production according to the latest report of the Food and Agriculture Organization of the United Nations. Moreover, it is the second largest crop in the U.S. and produces roughly forty percent of the total annual production in the world, which build a $30 billion agricultural industry (Priester, et al., 2012). As CeO_2 NPs are widely used in fuel additives, crops are readily exposed to the deposit manufactured nanoparticles. Besides, different from other species, soybean depends on the symbiotic relationship with *Rhizobia* within nodules of their root systems. Nanoparticles may indirectly affect soybeans by interfering with the soil microbial community, which brings more complication to the investigation of legume species. Priester et al (2012) reported that CeO_2 NPs accumulation in roots significantly decreased the colonies of nitrogen fixation microbial community. Therefore, it is essential to get better insight of the integrated impact of CeO_2 NPs on soybean growth.

Overall, there is great disparity on the impact of CeO_2 NPs on soybeans. Most of the previous studies focused on nutritional value, mineral elements, biomass and some other plant growth related parameters. Therefore, for better understanding of the CeO_2 NPs, it is essential to investigate the nanoparticle impact from new perspectives including WUE and photosynthesis mechanism.

CHAPTER III

IMPACT OF CERIUM OXIDE NANOPARTICLES WITH DIFFERENT SURFACE PROPERTIES ON SOYBEAN PHOTOSYNTHESIS AND WATER USE EFFICIENCY

Introduction

Cerium oxide nanoparticles (CeO_2 NPs) have been incorporated into various industrial products such as fuel additives, automobile catalytic converters, electronic and optical devices, coatings, and paints (Collin et al., 2014). With the extent of industrial uses, the release and accumulation of CeO_2 NPs in the environment is inevitable. Priester et al. (2013) estimated that at most 1,255 tons of CeO_2 NPs would be used in diesel fuel additives annually, of which at least 6% could be released into the environment, by 2020.

The prospect of significant accumulation of CeO_2 NPs in the environment has raised increasing concerns about its potential implications on the environmental health and safety. It is well recognized that the toxicity of CeO_2 NPs depends on many factors including the particle size and shape, surface charge and reactivity, aggregation and so forth (Baalousha et al., 2012; Merrifield et al., 2013). Among them, surface charge plays a key role in colloidal stability and toxicity (Baalousha et al., 2012). For CeO_2 NPs, the reactivity and environmental impact of the NPs is also heavily affected by the relative ratios of Ce(III) and Ce(IV) on the NP surface. In general, larger CeO_2 NPs contain higher percentage of Ce (IV) oxidation state while smaller ones possess a larger fraction of Ce (III) (Merrifield et al., 2013).

Previous studies had revealed inconsistent evidences of CeO_2 NPs' impact on plants. For instance, wheat (*Triticum aestivum* L.) treated with CeO_2 NPs up to 500 mg/kg through the life cycle displayed faster plant growth, higher shoot biomass, and higher grain yield (Rico et al., 2014). Likewise, Gui et al. (2015) demonstrated that lettuce (*Lactuca sativa* L.) cultivated in soil with 100 mg/kg CeO_2 NPs grew faster than the controls. Conversely, some adverse effects have also been observed in the studies involved with CeO_2 NPs at relatively higher concentrations. Ma et al. (2010) reported that CeO_2 NPs inhibited seed germination of corn, tomato, and cucumber at the concentration of 4000 mg/L in suspension. Similarly, CeO_2 NPs at 4000 mg/L exhibited the potential to reduce root length of tomato (Lopez-Moreno et al., 2010). In addition, soybeans exposed to CeO_2 NPs up to 2000 mg/L could result in DNA damage and oxidative damage from increasing reactive oxygen species. (Lopez-Moreno et al., 2010).

The impact of CeO_2 NPs on plants in other perspectives including photosynthesis and water use efficiency (WUE) required further investigation for better understanding of the interactions between CeO_2 NPs and plants. Du et al. (2015) reported that the total chlorophyll content of wheat declined significantly after the plants were exposed to 400 mg/kg CeO_2 NPs. Comparatively, Zhao et al. (2013) reported that CeO_2 NPs at 800 mg/kg reduced the cucumber yield by 31.6% yet they had no measureable impact on either photosynthesis performance or chlorophyll content. Likewise, CeO_2 NPs up to 800 mg/kg exhibited inconsequential impact on the net photosynthesis, transpiration rate, stomata conductance and chlorophyll content in corn (Zhao et al., 2015). Conversely, Marchiol et al. (2016) found that CeO_2 NPs at 500 mg/kg enhanced the photosynthetic parameters of barley including the net photosynthesis rate and the transpiration rate. However, at 1000 mg/kg, CeO_2 NPs inhibited the photosynthesis of barley by reducing photosynthesis rate, stomatal conductance and transpiration rate (Marchiol et al., 2016).

WUE is another important index evaluating the crop yield driven by carbon assimilation and water transpiration of plants under stress. Seghatoleslami et al., (2015) reported that silver nanoparticles failed to exhibit appreciable effect on WUE of *Carum copticum*. Unfortunately, impact of nanoparticles on plants WUE was still poorly understood. Therefore, the study incorporated physiological measurements of biomass, stomatal conductance and carbon isotope composition to correlate $\delta^{13}C$ with WUE. Carbon isotope discrimination by plants had been shown to be an accurate substitute for direct WUE measurement for C_3 plants. The method was well established, allowing rapid screening on the CeO_2 NPs effects on plant WUE at different environmental conditions (Lajtha, & Michener., 2007). The fast output of the isotope analysis could provide an opportunity for high throughput screening on the CeO_2 NPs effects on plant WUE at different environmental conditions. To the author's knowledge, this study is the first attempt to incorporate natural carbon isotope abundance analysis in the assessment of CeO_2 NPs effects on plants.

Soybean (*Glycine max*) was cultivated in this study due to its considerable significance of global agriculture, which contributes roughly 40% of annual crop yield in the world (Priester et al., 2012). Furthermore, soybean involves a variety of industries worth $30 billion due to its enrichment of oil and protein. As soybean is usually cultivated with fuel-powered instruments, it is highly vulnerable to be polluted by CeO_2 NPs released from combustion engine exhaust.

Overall, this study aimed to provide comprehensive evaluation on the impact of CeO_2 NPs on soybeans. Specifically, the objectives of this study were (1): to determine the impact of CeO_2 NPs on soybean WUE represented by carbon isotope composition $\delta^{13}C$; (2) to understand the impact of CeO_2 NPs on plant physiological processes associated with plant photosynthesis and (3) to determine the impact of the surface properties of CeO_2 NPs on its interactions with plants. The combined information on the physiological, carbon isotopic, and photosynthetic perspectives presented valuable insights of the interactions between CeO_2 NPs and soybeans.

Materials and Methods

Cerium oxide nanoparticles: Uncoated CeO_2 NPs (10% by weight) was purchased from Sigma Aldrich (St. Louis, MO). Polyvinylpyrrolidone (PVP) coated CeO_2 NPs dispersion (20% by weight) was purchased from US Research Nanomaterials, Inc (Houston, TX). Transmission electron microscopic images of both nanoparticles obtained with a Tecnai G2 F20 transmission electron microscope (TEM) (FEI, Hillsboro, Oregon) are shown in TEM images below (Fig 3-1). Uncoated CeO_2 NPs displayed quadrilateral or polygonal shapes while coated CeO_2 NPs were mostly spherical or polygonal. Size of uncoated CeO_2 NPs ranged from 10 to 30 nm with an average of 19 nm. Comparatively, size of coated CeO_2 NPs varied from 6 to 24 nm with an average of

10 nm. In addition, measured by X-ray photoelectron spectroscopy, Ce^{3+} ratio of the two types of CeO_2 NPs were 8% and 12% respectively.

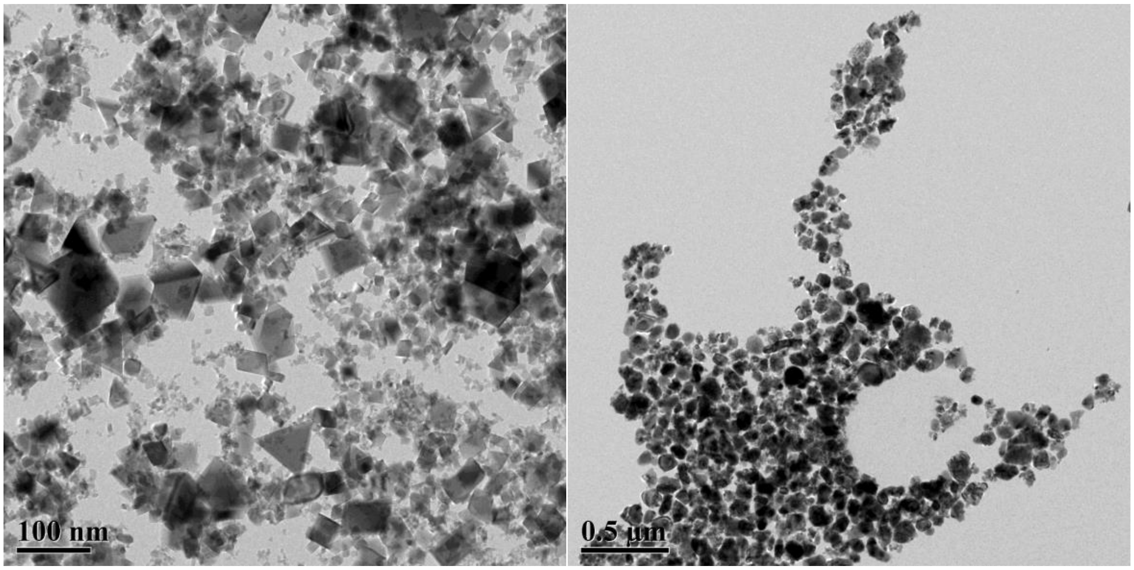

Fig 3-1. TEM images of uncoated CeO_2 NPs and PVP-coated CeO_2 NPs

Soil moisture capacity quantification: Potting soil (Micro-Gro) purchased from a commercial vendor was used in this study. 50 g potting soil was weighed into each pot and recorded as total weight (m_t). The soil was dried in an oven at 70 °C for 48 hours and then weighed as dehydrated soil (m_d). Water was then added to the dry soil to the saturation point where no more water could be adsorbed by the soil. Water capacity was derived from ($m_s – m_d$) where m_s was the weight of moisture saturated soil.

Plant growth and cultivation: The soybean seeds were purchased from Johnny Seeds (Fairfield, MN) and the potting soil as described above was used for plant growth. To ensure the homogenous mixing of soil and CeO_2 NPs, the mixture was placed on a shaker table for at least 24 hours, with the facilitation of frequent manual mixing. The targeted concentrations for CeO_2 NPs treated soil ranged from 0 mg – 500 mg/kg dry soil (0, 10, 100 and 500 mg/kg dry soil).

Each pot was filled with 50 g potting soil and then dried at 70 °C for 48 hrs. 52.3 g water (moisture saturation point derived from water capacity quantification measurement described above) was added to each pot containing the dried soil. Soybean seeds were germinated in water-sufficient soil 5 days prior to the transplant. Among the germinated soybean seedlings, 63 well grown seedlings with roughly the same height were transferred to the soil containing concentrations of CeO_2 NPs (uncoated and pvp-coated CeO_2 NPs). Including the control group without CeO_2 NPs, there were altogether seven treatments, with each having nine replicates. The soybeans were irrigated with sufficient deionized water every day for three weeks with controlled lighting (16/8 light/dark cycle) at room temperature.

Plant harvest and biomass weighing: The soybeans were pulled out from the soil gently and washed by deionized water thoroughly. Roots and shoots were separated and weighed to obtain their fresh weight. 6 plants from each treatment were then dried in an oven at 70 °C for 48 hours and weighed to obtain dry biomass of roots and shoots.

Photosynthesis: Stomatal conductance and photosynthesis rate of the soybeans were measured by Licor-6400XT at day 12, day 16 and day 20 after seedling transplant. In addition, net photosynthesis rate responding to varying light intensity and CO_2 concentration was determined with a portable IR gas analyzer (LI-6400; LI-COR, Lincoln, NE) equipped with a red/blue light source (6400–02B, LI-COR) at day 20 and

21. When the leaves were measured in the chamber, temperature was constant 25 ºC and CO_2 concentration was constant 400 mg $L^{-1.}$

For the light curve measurement, nine light levels (0, 50, 200, 400, 600, 800, 1000, 1200, and 1500 mmol m^{-2} s^{-1}) were applied with the red/blue light source connected to the Infra-Red (IR) gas analyzer. Top leaves of each soybean were selected for the measurement, which were allowed to adapt to each light level for at least 2 min until the physiological parameters stopped fluctuating. A_{max} (maximum photosynthesis capacity), R_d (respiration), and ϕ (quantum yield) were calculated by fitting data to the nonlinear regression model described by Hanson et al. (1987), with photosynthetic photon flux (PPF) levels as the independent variable.

For the CO2 curve measurement, nine concentration levels (100, 200, 300, 400, 600, 800, 1000, 1200 and 1600 mg/L) were applied with the CO_2 chamber connected to the Infra-Red (IR) gas analyzer. Top leaves of each soybean were selected for the measurement, which were allowed to adapt to each light level for more than 5 min until the physiological parameters little varied. Five parameters can be derived from non-linear curve fitting with the well-established model proposed by Sharkey et al. (2007), including V_{cmax} (maximum carboxylation rate), J (photosynthesis electron transport rate), TPU (triose phosphate use), R_{day} (day respiration) and g_m (mesophyll conductance).

Carbon isotope composition measurement: Two most fresh leaves of each soybean were dried at 70 °C to remove moisture and grounded into fine powders. Carbon isotope content was than quantified by gas chromatography isotope ratio mass spectrometer (GC-IRMS) (Finnigan DELTAplusXP, Waltham, MA) at the stable isotope geoscience facility at Texas A&M, which can serve as a reliable estimate of the overall WUE.

Chlorophyll content quantification: After the soybean was harvested, 20 ~ 30 mg fresh leaf tissue were weighed from each replicate. The fresh tissue was added into a centrifuge tube with 4 mL dimethyl formamide (DMF) and kept dark overnight. Chlorophyll extraction was then completed using the method described by Moran. 1 mL residual solution was extracted from each centrifuge tube. UV-Vis tests were performed by a UV-Vis spectrophotometer (model Lamba 35; PerkinElmer, Waltham, MA). Calibration was tested at zero absorbance using a blank of pure DMF and the absorbance of this blank was subtracted from the absorbance readings of each sample before calculation. The amount of absorbance was read at 664 and 647 nm. Absorbance readings were used to calculate leaf chlorophyll concentrations.

Chlorophyll fluorescence measurement: Leaf chlorophyll fluorescence (F_v/F_m) was measured using a continuous excitation chlorophyll fluorescence analyzer (OS1p, Opti-Sciences, Hudson, NH). The leaves were acclimated in darkness using lightweight leaf clips for at least 30 min before measurements (Maxwell and Johnson, 2000). Variable fluorescence (F_v) was derived from baseline (F_0) and maximum (F_m) fluorescence detected by the chlorophyll fluorescence analyzer. At the end, the ratio of variable fluorescence to maximum fluorescence (F_v/F_m) ratio was recorded.

Cerium content characterization: After the harvest, the soybean tissues were fully dehydrated by drying at an oven at 70 °C for 7 days before the dry weight determination. 0.5 g of the dry tissues were ground and added into 4 mL of 70% (v/v) nitric acid overnight. Afterwards, the residual was digested in a DigiPREP MS hot block digester (SCP science, Clark Graham, Canada) at 95 °C until the dry tissue was fully dissolved. After the digest cooled down to room temperature, the suspension was mixed with 2 mL of 30% (v/v) H_2O_2 and heated in the hot block at 95 °C for additional 2 hours. Detailed digestion procedures have been reported in a previous study (Dan et al., 2015). Cerium content was quantified by a Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Perkin Elmer mod. DRCII, Waltham, MA).

Results and Discussion

Biomass: Coated CeO_2 NPs consistently increased the fresh weight of soybeans at all concentrations (Fig 3-2 a). Conversely, uncoated CeO_2 NPs did not affect the fresh weight until it went up to 500mg/kg soil. A closer look at the fresh weight revealed some differential impacts of CeO_2NPs on roots and shoots (Fig 3-2 b and c). At the highest concentration (500mg/kg soil), both types of CeO_2 NPs tended to increase the fresh weight of shoots while they showed no significant impact on roots.

Besides, the dry weight of plant tissue was also examined (Fig 3-3). While coated CeO_2 NPs significantly increased the total dry weight consistently, uncoated CeO_2 NPs only exhibited measureable impact on the dry weight at 100mg/kg. When the dry weights of roots and shoots were examined, it indicated that the dry biomass increase caused by uncoated CeO_2 NPs at 100mg/kg soil was mainly attributed to the increase of shoot biomass.

Several studies reported that CeO_2 NPs could stimulate crop yield. For instance, Rico et al. (2014) addressed that grain yield of wheat increased by 36.6% exposed to 500 mg/kg CeO_2 NPs. Likewise, Gui et al. (2015) demonstrated that CeO_2 NPs at 100 mg/kg significantly enhanced the biomass of lettuce compared with the control. Similarly, CeO_2 NPs increased the fresh and dry weight of soybeans in our study, while the PVP-coated CeO_2 NPs exhibited greater impact on the biomass of soybean.

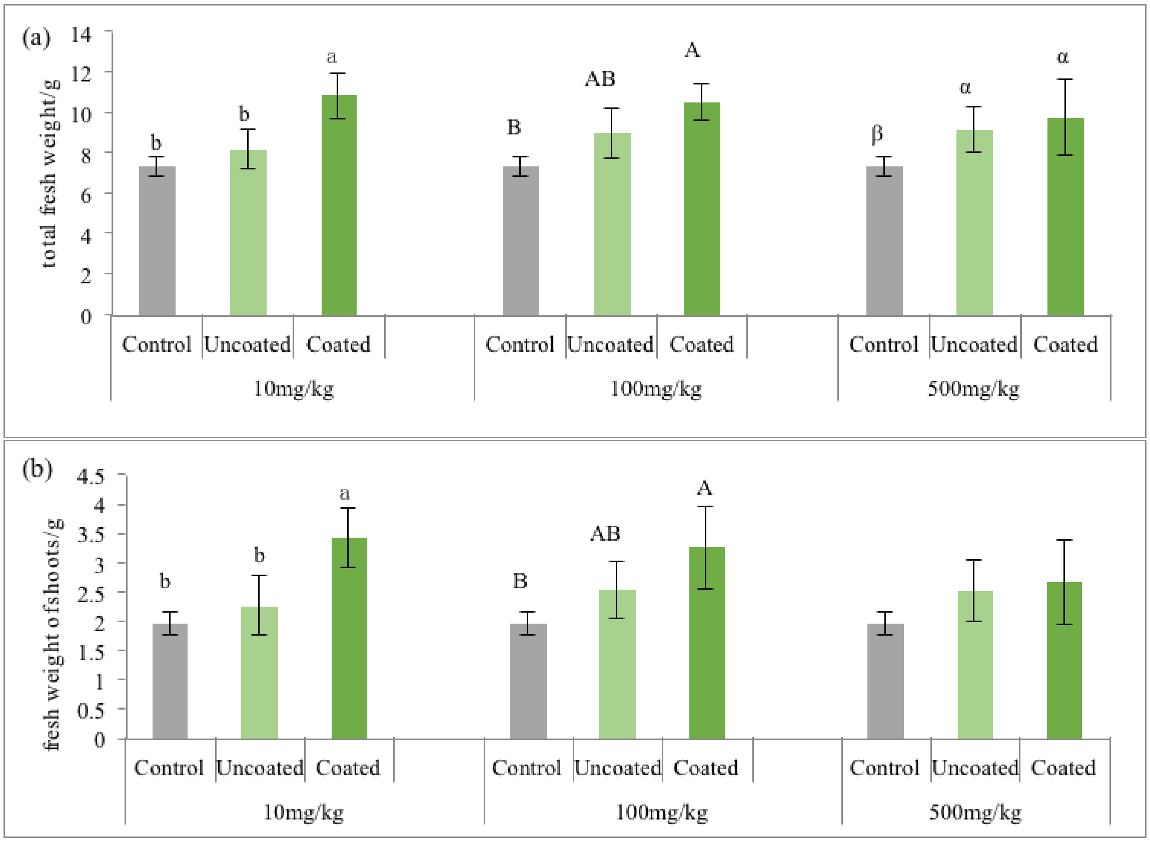

Fig 3-2. (a) Total fresh weight, (b) fresh weight of roots, (c) fresh weight of shoots. Values represent mean±SD (n=6). Different letters indicate significant statistical differences (at $p \leq 0.05$) according to the Tukey's test

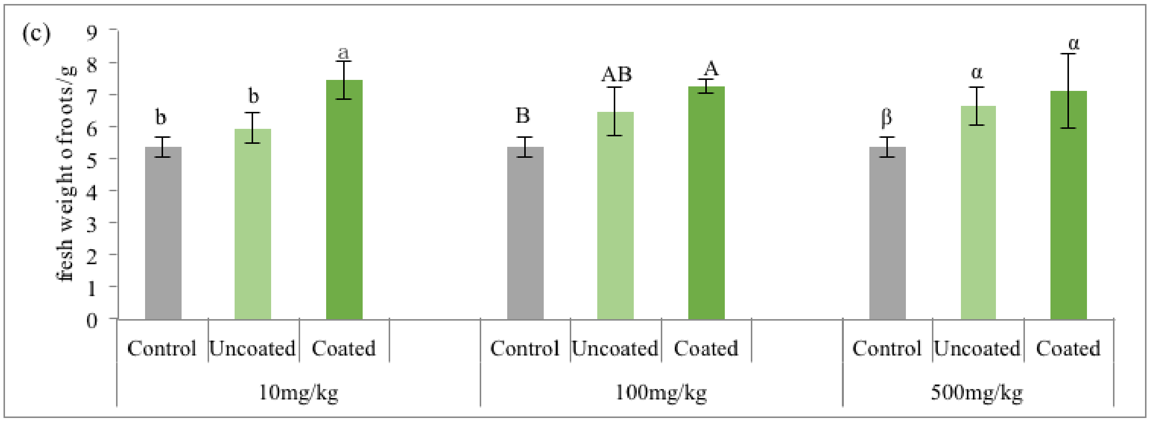

Fig 3-2. Continued

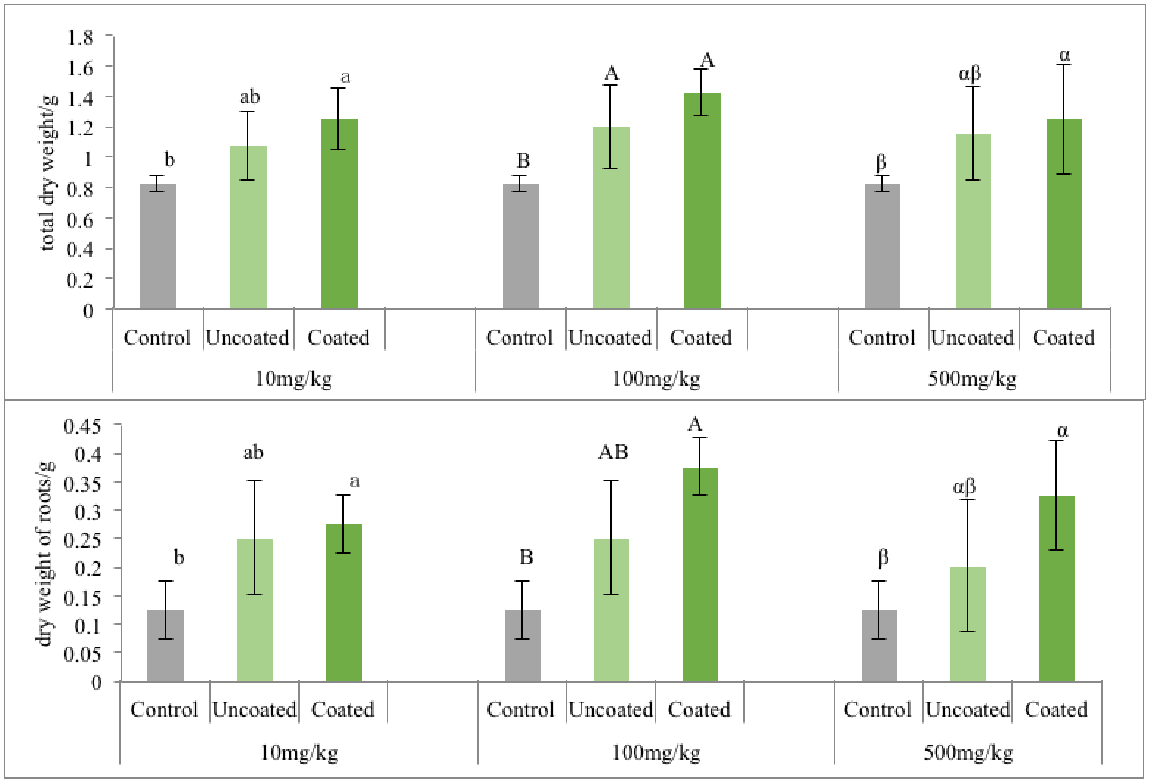

Fig 3-3. (a) Total dry weight, (b) dry weight of roots, (c) dry weight of shoots. Values represent mean±SD (n=6). Different letters indicate significant statistical differences (at $p \leq 0.05$) according to the Tukey's test

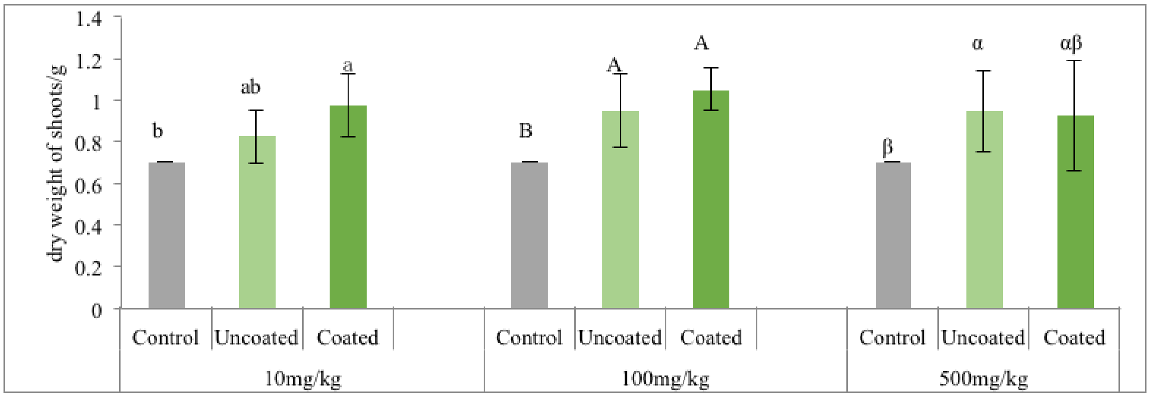

Fig 3-3. Continued

Stomatal conductance: Stomata play a key role in photosynthesis by controlling the CO_2 diffusion and water transpiration (Keenan et al., 2010). Water transpiration of soybean leaves varies over time due to the fluctuating microenvironment. It also changes over the lifespan of soybeans as the mature leaves are shaded by younger ones on the top canopy (Locke and Ort, 2014). More importantly, plants can adapt to varying environmental conditions including soil moisture and nutrient availability by changing their stomatal conductance and hydraulic conductance of leaves (Ocheltree et al., 2014). Thus, stomatal conductance can provide information on water transpiration and will further indicate stress induced by CeO_2 NPs.

Fig 3-4 illustrates the stomatal conductance of the soybean exposed to CeO_2 NPs at day 12, 16 and 20. At lower concentrations (10 and100mg/kg soil) soybeans treated with CeO_2 NPs exhibited higher stomatal conductance compared with the control, which suggested that the soybeans were not stressed by the CeO_2 NPs. Comparatively, CeO_2 NPs appeared to impose negative impact on stomatal conductance at the highest concentration (500 mg/kg). Interestingly, soybeans treated with CeO_2 NPs at 500 mg/kg soil slightly increased their stomatal conductance at day 16 and then significantly reduced the stomach conductance at day 20. While both types of CeO_2 NPs demonstrated similar effects on stomata conductance, significant differences were noticed between treated plants at lower concentrations. In both cases, the coated CeO_2 NPs seemed to have a stronger effect on stomata conductance than uncoated CeO_2 NPs.

Enhanced stomatal conductance indicated positive impact on gas exchange caused by CeO_2 NPs at lower exposure concentrations. A recent study published by Marchiol et al. (2016) demonstrated similar results that 1000 mg/kg TiO_2 NPs significantly enhanced stomatal conductance of barley by 89% compared with the control. However, gas exchange parameters of cucumber including stomatal conductance were not affected by CeO_2 NPs up to 800 mg/kg (Zhao et al., 2013). Likewise, more recent study indicated that no measurable effect was observed in stomatal conductance of corn treated with CeO_2 NPs at 400 and 800 mg/kg (Zhao et al., 2015).

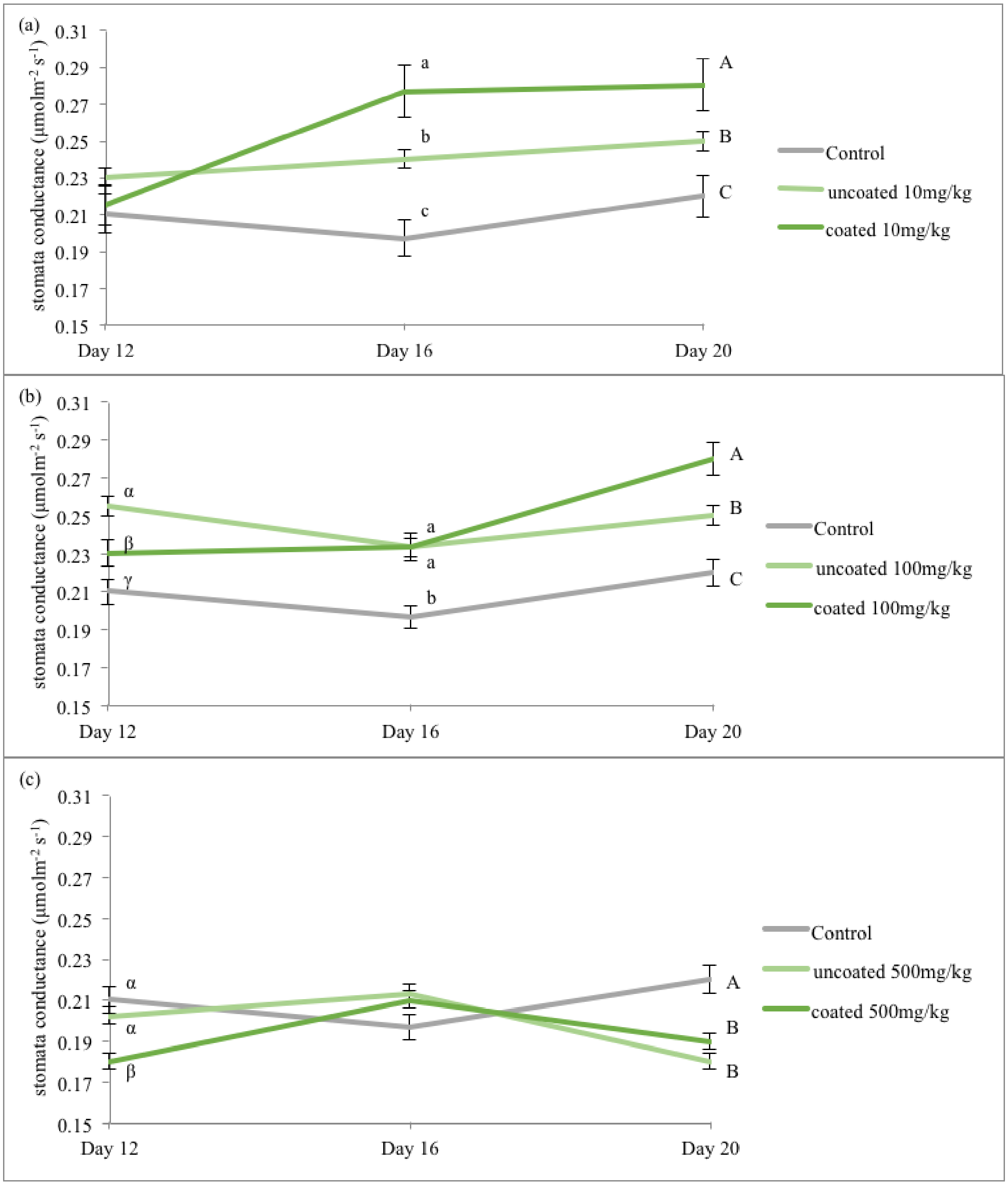

Fig 3-4. (a) Stomatal conductance over the three-week growth at 10mg/kg soil. (b) stomatal conductance over the three-week growth at 100mg/kg soil. (c) stomatal conductance over the three-week growth at 500mg/kg soil. Values represent mean±SD (n=4). Different letters indicates significant statistical differences (at $p \leq 0.05$) according to the Tukey's test

Water use efficiency: Defined by the ratio of carbon assimilation to water transpiration, WUE can be estimated by carbon isotope composition due to the ^{13}C discrimination during a series of photosynthesis processes including carbon dioxide diffusion and Rubisco activity. Specifically, as photosynthesis processes preferentially uptake lighter carbon isotope ^{12}C rather than heavier ^{13}C, more carbon isotope fractionation resulting in ^{12}C accumulation against ^{13}C that amounts to lower $\delta^{13}C$ in plant tissues. Given that variance of water transpiration and evaporation among all the treatments could be assumed to be ignorable at strictly controlled conditions, $\delta^{13}C$ is linearly correlated with WUE of the soybeans (Lajtha, & Michener, 2007).

Fig 3-5 displays the carbon isotope composition of soybean leaves treated with CeO_2 NPs at different concentrations. At 10 mg/kg, coated CeO_2 NPs significantly reduced $\delta^{13}C$ of soybean leaves, while uncoated CeO_2 NPs showed no significant impact although $\delta^{13}C$ slightly decreased. It have been noticed that CeO_2 NPs exhibited positive effect on soybean WUE at 100 mg/kg but inhibitive effect on WUE at the highest concentration.

Variance of ^{13}C might generate from CO_2 diffusion from the atmosphere into the air-filled spaces within the leaf with an apparent fractionation of 4.4‰ due to the slower motion of the heavier ^{13}C-containing CO_2 molecules (Lajtha, & Michener, 2007). Within the leaf, the carboxylation enzyme ribulose bisphosphate carboxylase/oxygenase (Rubisco) also discriminates against the ^{13}C. Rubisco activity serves as the dominant factor contributing to the intercellular $\delta^{13}C$ fractionation with a median of 27‰ (Lajtha, & Michener, 2007, Cao et al., 2011). Given that the $\delta^{13}C$ of soybean exposed to100 mg/kg uncoated and coated CeO_2 NPs reduced by 2.74% and 2.25% respectively compared with the control, CeO_2 NPs might interfere with CO_2 diffusion pathways or Rubisco activity of the soybean tested. It required further research to determine which process was affected by CeO_2 NPs leading to variance in WUE.

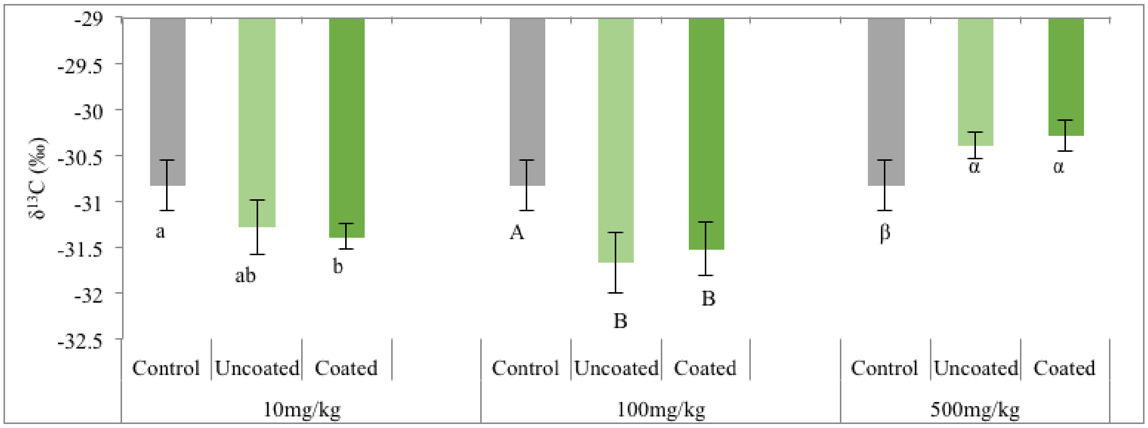

Fig 3-5. Carbon isotope discrimination of soybeans at different treatments. Values represent mean±SD (n=4). Different letters indicate significant statistical differences (at $p \leq 0.05$) according to the Tukey's test

Chlorophyll: Chlorophyll content of soybean was tested (Fig 3-6) as leaf pigments are considered as indicative of plant stress (Zhao et al., 2014). No significant differences were found in total chlorophyll content among the treatments except for the soybeans treated with coated CeO_2 NPs at 10mg/kg soil, which showed higher content of total chlorophyll than other treatments. However, significant variances could be seen when chlorophyll a and b were quantified separately. The results exhibited that at higher concentrations (100 and 500mg/kg soil) CeO_2 NPs treated soybeans consistently possessed more chlorophyll a but less chlorophyll b. Noticeably, results at the lowest concentration were slightly different. Uncoated CeO_2 NPs showed no significant impact on either chlorophyll a or b while coated ones increased chlorophyll a, and consequently the total chlorophyll content.

Changes in chlorophyll content induced by nanoparticles have been studied by several recent studies. CeO_2 NPs up to 800 mg/kg had no measurable impact on either chlorophyll a or b in corn and cucumber (Zhao et al., 2013; Zhao et al., 2014). Comparatively, Du et al. (2015) found that 400 mg/kg CeO_2 NPs significantly reduced chlorophyll content in wheat. Likewise, a more recent study found that chlorophyll content significantly declined in corn plants treated with 800 mg/kg ZnO NPs (Zhao et al., 2015). Decreasing chlorophyll might result from Zn released from ZnO NPs that interfered with the chlorophyll production by replacing the magnesium (Kupper et al., 1996). Nevertheless, mechanism of CeO_2 NPs impact on plant chlorophyll is yet to be elucidated. Inhibited chlorophyll a production generally indicates stress correlated with decreasing nitrogen concentration (Zhao et al., 2014). Subsequently, plants usually produce more chlorophyll b when they are stressed due to the wider absorption spectrum of chlorophyll b. However, the fact that CeO_2 NPs at concentration of 100 and 500 mg/kg did not affect chlorophyll content, with increasing chlorophyll a and decreasing chlorophyll b, suggested that no damage was exerted on the chloroplast of the soybeans.

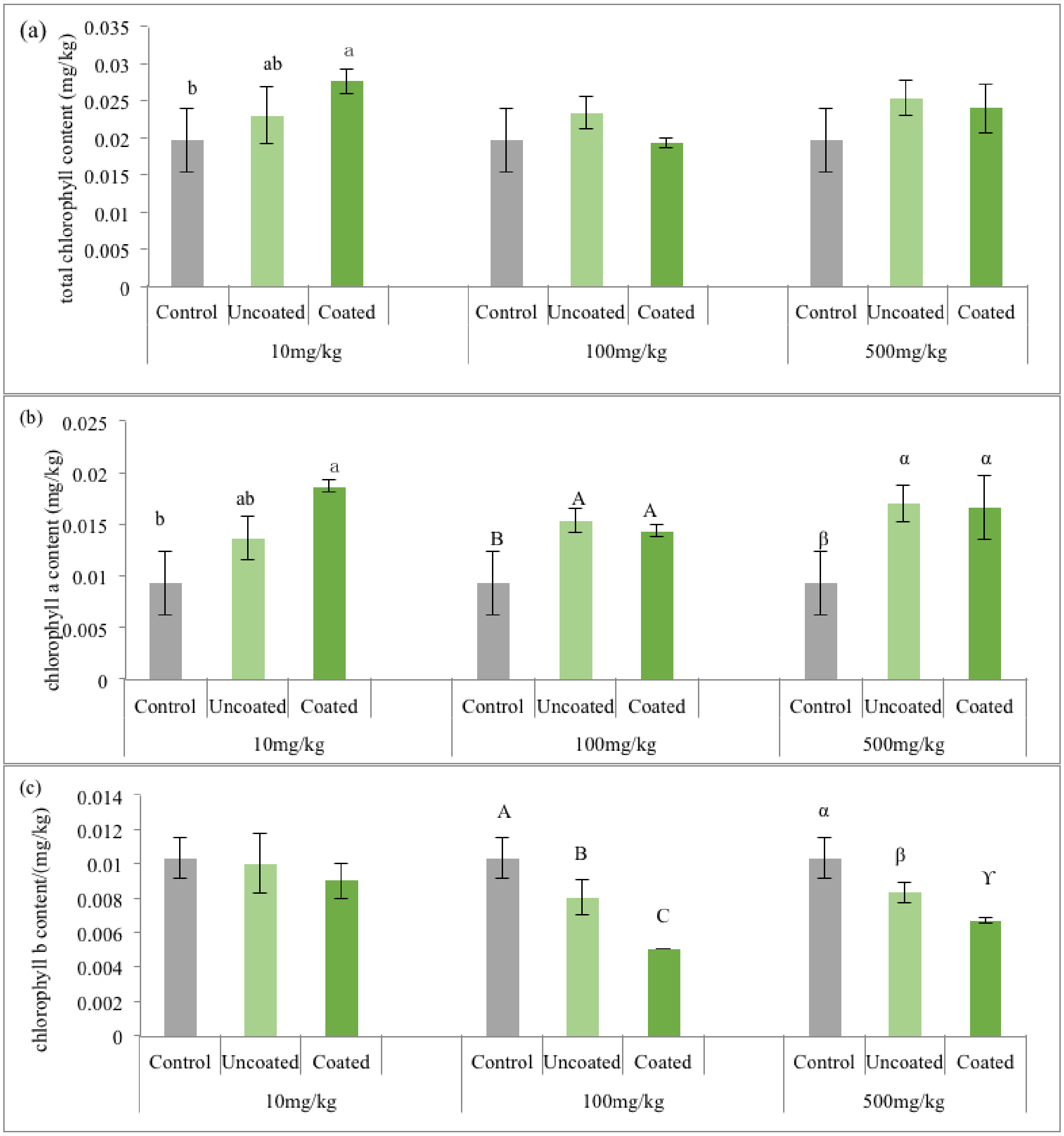

Fig 3-6. (a) Total chlorophyll content, (b) chlorophyll a content (c) chlorophyll b content. Values represent mean±SD (n=4). Different letters indicates significantly statistical differences (at p≤0.05) according to the Tukey's test

Photosynthesis rate: photosynthesis rate is measured at fixed light intensity and carbon dioxide concentration, which indicates the performance of the photosynthetic system in plants. Fig 3-7 shows that CeO_2 NPs at lower concentration (10 and 100mg/kg soil) enhanced photosynthesis rate in the three-week growth period. Conversely, photosynthesis rate of soybean exposed CeO_2 NPs at the highest concentration barely varied over time. When the treatments at different concentration were compared, soybean treated with CeO_2 NPs at 100 mg/kg showed the highest photosynthesis rate at day 20 compared with the treatments at other concentrations. Conversely, photosynthesis rate of soybean exposed to 500 mg/kg CeO_2 NPs became lower than the control at day 20, suggesting that an optimal concentration for positive impact might be exceeded. Similarly, Marchiol et al. (2016) reported 500 mg/kg CeO_2 NPs enhanced photosynthesis rate in Barley by 26% while 1000 mg/kg CeO_2 NPs treatment did not stimulate photosynthesis. Wang et al. (2016) observed that 200 and 300 mg/L ZnO NPs inhibited expression levels of chlorophyll synthesis genes along with the leaf photosynthesis in *Arabidopsis*. Previous research demonstrated that corn plants could adopt protection mechanism by producing antioxidant enzyme and heat shock proteins to alleviate stress induced by CeO_2 NPs (Zhao et al., 2013). Accordingly, soybean might overcome the stress caused by CeO_2 NPs at relatively low exposure level but could not tolerate extremely high concentration as the photosynthesis-related genes might be damaged. Lopez-Moreno et al. (2010) showed that CeO_2 NPs up to 3000 mg/L significantly affected the genetic integrity of soybean.

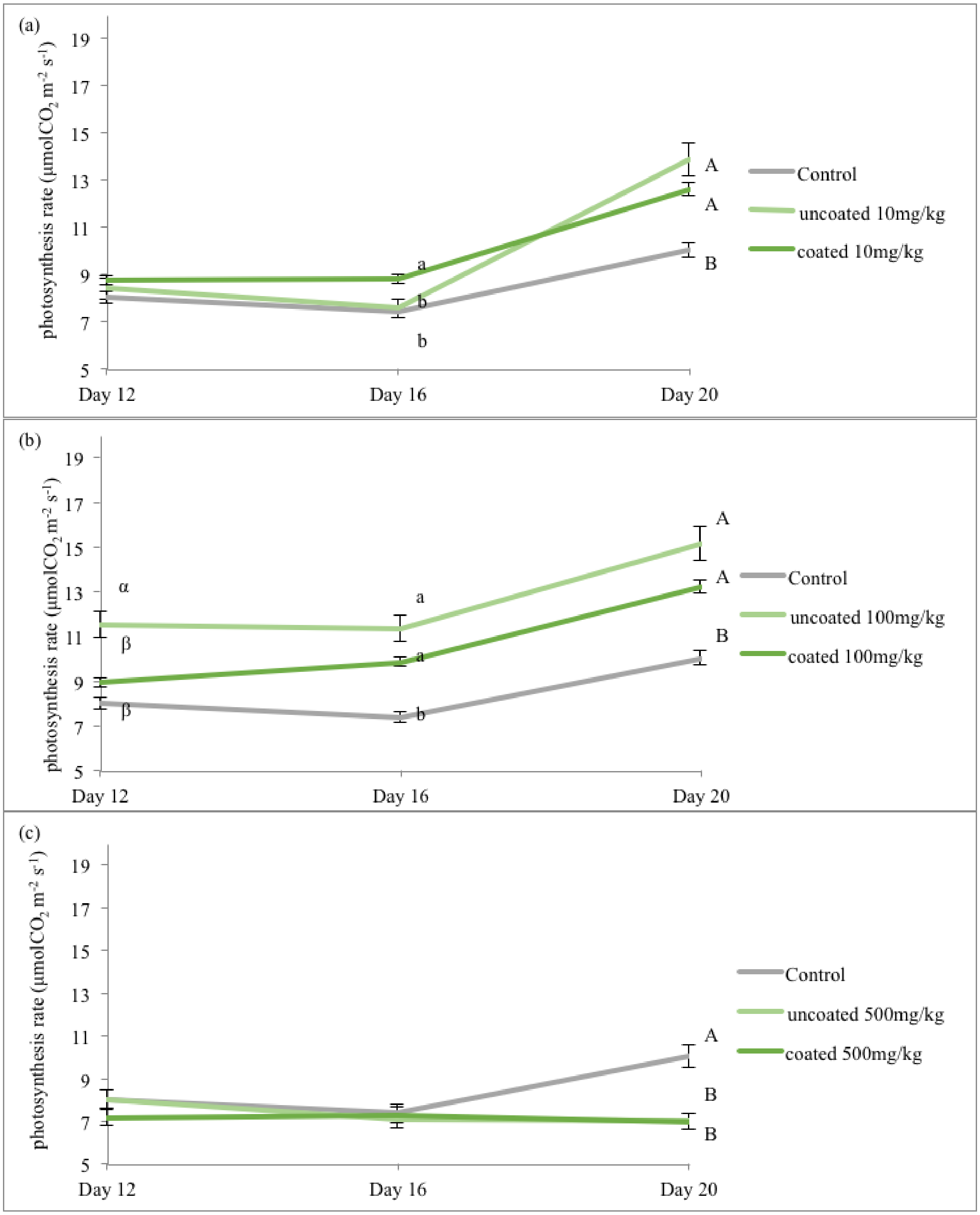

Fig 3-7. Photosynthesis rate over the three-week growth period at (a) 10mg/kg soil; (b) 100mg/kg soil; and (c) 500mg/kg soil. Values represent mean±SD (n=4). Different letters indicate significant statistical differences (at $p \leq 0.05$) according to the Tukey's test

Photosynthetic light response curve: Under the constant carbon dioxide concentration, carbon assimilation rate rises asymptotically with respect to the increasing photon density. The light photosynthetic response curve reveals several phases of photosynthetic light responses and provides insights on net photosynthesis rate (P_{Nmax}), respiration rate (R_d) and quantum yield (ϕ) (Lombardini et al., 2009, Greer & Weedon, 2012).

The net photosynthesis light-response curve (P_N/I curve) illustrates the change of the net photosynthesis rate of the soybean leaves as a function of increasing photon flux density (I) from dark condition to 2,000 μmol (photon) m^{-2} s^{-1} (Labo et al., 2013). Fig 3-8 displays the parameters including P_{Nmax}, R_d, and ϕ derived from the curve fitting. The results suggested that the soybeans exhibited significantly higher P_{Nmax} when they were treated with CeO_2 NPs at the concentration of 100 mg/kg soil. While P_{Nmax} of the control was 11μmol m^{-2} s^{-1}, it increased to 16.8 μmol m^{-2} s^{-1} in the soybeans treated by uncoated CeO_2 NPs. Similarly, coated CeO_2 NPs also enhanced the photosynthesis rate of the soybeans by 37.3% compared with the control group. No difference was observed among soybeans treated with CeO_2 NPs at the concentration of 500mg/kg soil and the control group. The results could be interpreted as that uncoated CeO_2 NPs at 100 mg/kg and coated CeO_2 NPs at 10, 100mg/kg soil were able to promote the photosynthesis capacity of the soybeans at light saturation conditions.

R_d is another key parameter characterizing the gas exchange affected by light intensity change of the soybeans. The fitted values indicated the CO_2 loss due to dark respiration processes. In most species, R_d fell in the range of 0.6 to 1.4μmol m^{-2} s^{-1}

(Lombardini et al., 2009). It exhibited that R_d of the soybeans treated with both types of CeO_2 NPs at the concentration of 100mg/kg soil were slightly higher than the control group, but no significant difference had been observed in the rest of treatments.

ϕ derived from the initial slope of the light response curves represents the energy converting efficiency from the energy absorbed from light into the chemical energy in fixed organic carbon, which typically amounted to 0.06 under favorable conditions and normal atmospheric CO_2 concentration (Lambers et al., 2008). In general, ϕ varies with the fluctuation in light absorptance due to the variance of chlorophyll content per unit leaf area. However, at constant chlorophyll content, ϕ is relatively constant unless the plants are stressed or inhibited by unfavorable conditions (Lombardini et al., 2009). Based on the following results, uncoated CeO_2 NPs treated soybeans exhibited lower ϕ value compared with the control group. The values varied among the other treatments but no significant difference was observed.

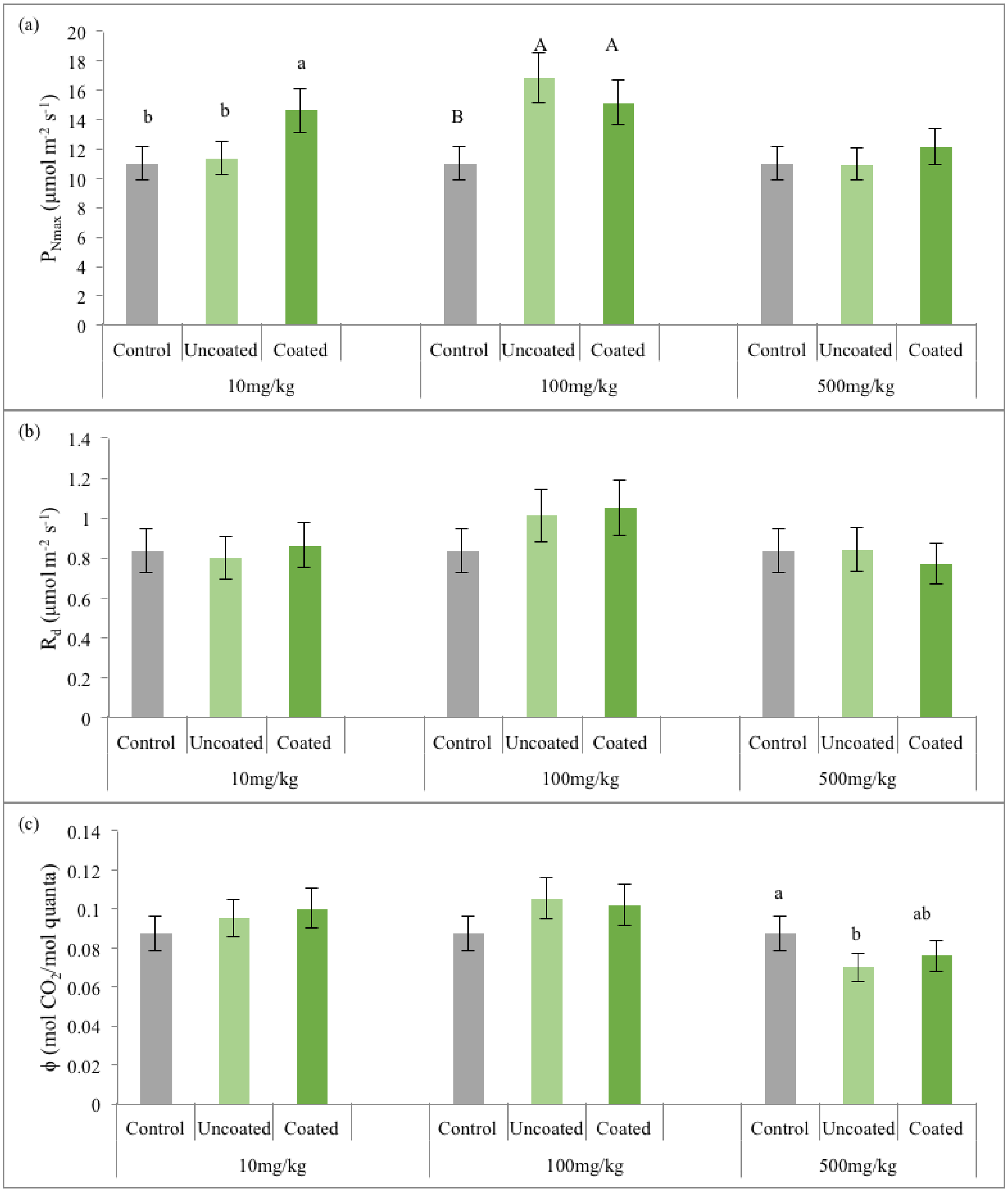

Fig 3-8. (a) Maximum photosynthesis rate (P_{Nmax}), (b) dark respiration rate (R_d), (c) quantum yield (ϕ) for the soybeans treated with CeO_2 NPs. Values represent mean±SD (n=3). Different letters indicate significant statistical differences (at $p \leq 0.05$) according to the Tukey's test

Photosynthetic CO_2 response curves: Complementary with the light curve, photosynthetic response to carbon dioxide concentration provides insights of biochemical parameters describing the gas exchange behavior of plants including maximum carboxylation rate (V_{cmax}) allowed by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), maximum rate of photosynthetic electron transport (J_{max}) relevant to Nicotinamide adenine dinucleotide phosphate (NADPH) regeneration, triose phosphate use (TPU), day respiration (R_d) and mesophyll conductance (g_m) (Sharkey et al., 2007, Miao et al., 2009). Table 3-1 presented the detailed results.

V_{cmax} was defined as the theoretical carboxylation rate of Rubisco at ribulose bisphosphate (RuBP) saturated conditions. As an indicative of Rubisco activity, V_{cmax} was estimated from the CO_2 response curves with the method described by Sharkey et al. (2007). In this case, significant enhancement was observed in V_{cmax} of the soybeans treated with CeO_2 NPs at the concentration of 100 mg/kg compared with the control group. However, the soybeans treated with coated CeO_2 NPs at 500 mg/kg exhibited lower V_{cmax} than the control group, suggesting that the Rubisco activity capacity was inhibited at this concentration.

Photosynthetic electron transport occurs in the membrane through the electron and proton transport chains, which could be influenced by water content and hydraulic conductance in the leaves because the electron transport chains were supported by water (Fan et al., 2012). Estimated J_{max} can provide information on the ability of plants to regenerate RuBP (Yang et al., 2015). At 100 mg/kg, uncoated CeO_2 NPs promoted the J_{max} of the soybeans tested while coated CeO_2 NPs showed intermediate effect.

Conversely, lower J_{max} was observed in the soybeans exposed to 500 mg/kg CeO_2 NPs compared with the control. According to pervious research, decreased photosynthetic electron transport rate could further inhibit photosynthetic phosphorylation, the synthesis of NADPH, and the regeneration of RuBP (Sharkey et al., 2007).

When photosynthesis rate was TPU-limited, it can be simplified as:

$$A = 3TPU - R_d$$

In addition to the consumption rate of triose phosphate, TPU also represents the carbon generated from other processes such as photorespiratory conversion of glycine or serine in the Calvin cycle (Sharkey et al., 2007). It represents the ability to convert triose phosphate into sugars such as starch and sucrose (Yang et al., 2015). Table 3-1suggests that no significant variance was observed among the treatments and controls.

Day respiration (R_{day}) is the rate of dark respiration during photosynthesis, which differs from dark respiration at night. R_{day} representing the dark respiration at light-sufficient conditions is generally lower than the dark respiration R_d in darkness (Yin et al., 2011). Coupled with the dark respiration results derived from the light response curves, the soybeans treated with CeO_2 NPs did not show significant effect on R_{day} compared with the control group.

Mesophyll conductance (g_m, μmol m^{-2} s^{-1} Pa^{-1}) controls the CO_2 transport from the substomatal cavity to the chloroplast, determining the partial pressure of CO_2 in the chloroplast (Lambers et al., 2008). Difference had been observed in the soybeans treated with CeO_2 NPs at the concentration of 500mg/kg. The results suggested that both types of CeO_2 NPs lowered g_m at the highest concentration, suggesting that the CO_2 diffusion pathways were partially blocked when the soybeans were stressed by CeO_2 NPs.

Table 3-1. V_{cmax} (maximum carboxylation rate), J_{max} (maximum photosynthesis electron transport rate), R_{day} (day respiration) and g_m (mesophyll conductance). Values represent mean (n=3). Different letters indicates significantly statistical differences (at $p \leq 0.05$) according to the Tukey's test

		V_{cmax}	J_{max}	TPU	R_{day}	g_m
		$\mu mol\ m^{-2}\ s^{-1}$	$\mu mol\ m^{-2}\ s^{-1}$	$\mu mol\ m^{-2}\ s^{-1}$	$\mu mol\ m^{-2}\ s^{-1}$	$\mu molm^{-2}s^{-1}Pa^{-1}$
10mg/kg	Control	48	89	6.71	0.67	8.64
	Uncoated	49.1	86	6.27	0.7	8.37
	Coated	49.35	90.5	6.58	0.68	8.59
100mg/kg	Control	48^b	89^b	6.83	0.67	8.64
	Uncoated	63.4^a	110.5^a	6.65	0.72	9.53
	Coated	61.7^a	102.5^{ab}	7.07	0.69	9.89
500mg/kg	Control	48^A	89^A	6.5	0.67	8.64^A
	Uncoated	41^{AB}	70.5^B	6.82	0.59	6.93^B
	Coated	37.5^B	68^B	6.67	0.63	6.81^B

Cerium uptake and accumulation: Fig 3-9 reveals that soybeans treated with uncoated CeO_2 NPs contained consistently higher cerium compared with both the control plants and plants treated with coated CeO_2 NPs. The significant difference in accumulation of both types of CeO_2 NPs might attribute to their opposite surface charge. While the positively charged uncoated CeO_2 NPs tended to adsorbed on the negatively charged root surface of soybeans, electrostatic repulsion inhibited the attachment of coated CeO_2 NPs to root membranes. Noticeable accumulation of coated CeO_2 NPs was only found at concentration of 500 mg/kg for coated CeO_2 NPs. Measurable cerium accumulation has also been detected in cucumber, barley and tomato plants exposed to

CeO_2 NPs in previous studies (Zhao et al., 2013; Marchiol et al., 2016; Wang et al., 2012). Moreover, the concentration of CeO_2 NPs was significantly higher in the roots than the shoots, which indicates limited upward transport rate in the first three weeks of the soybean lifespan. This agrees with a recent report that Ce content was significantly higher in roots of corn plants but no significant accumulation in above ground tissues was detected (Zhao et al., 2015). Likewise, CeO_2 NPs was not transported to aboveground tissues of soybean and wheat (Priester et al., 2012; Schwabe et al., 2013).

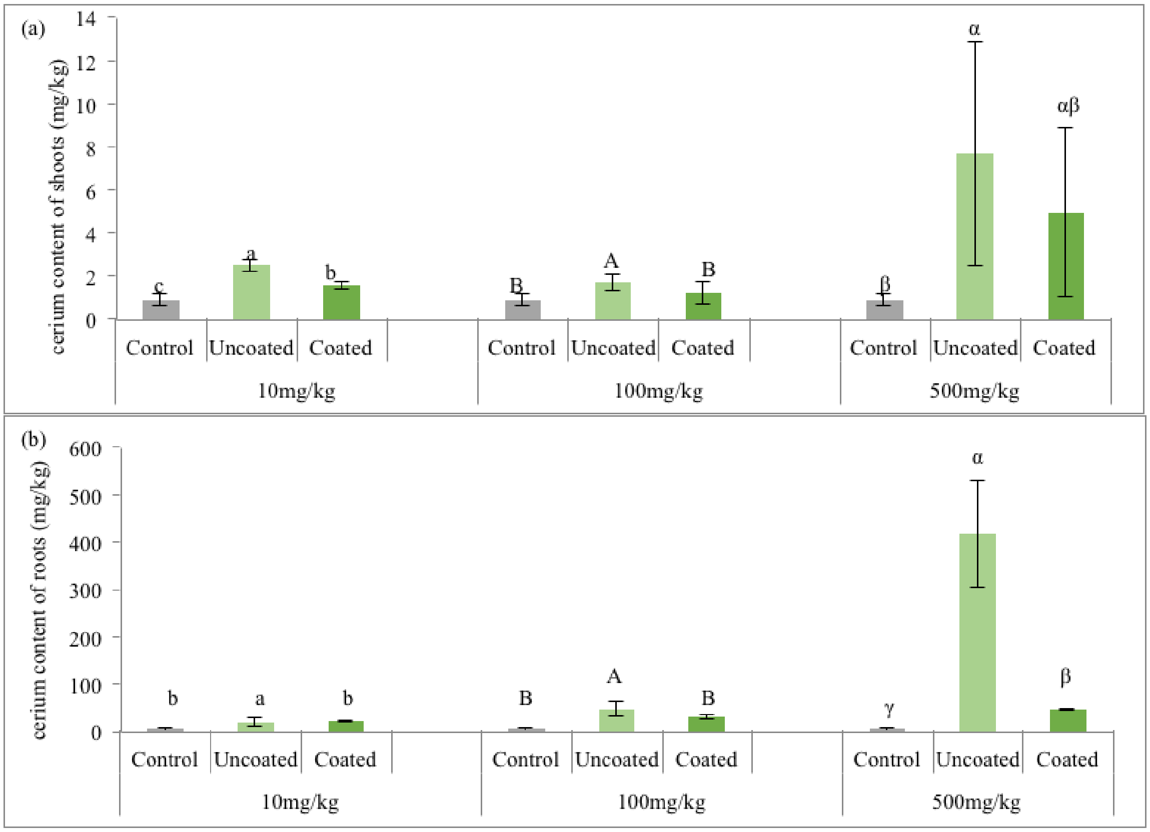

Fig 3-9. (a) Cerium content in shoots, (b) cerium content in roots. Values represent mean±SD (n=4). Different letters indicates significantly statistical differences (at $p \leq 0.05$) according to the Tukey's test

To the author's knowledge, it is the first research concerning WUE of plants affected by nanoparticles. Both the uncoated and the coated CeO_2 NPs at 100 mg/kg enhanced WUE of soybean while they reduced WUE at 500 mg/kg. Moreover, net photosynthesis rate was promoted by 100 mg/kg CeO_2 NPs but was inhibited by 500 mg/kg CeO_2 NPs. The positive impact of CeO_2 NPs at relatively lower concentration suggests a hermetic response of soybean, which was observed on some other studies (Marchiol et al., 2016; Wang et al., 2016). Comparatively, soybean could not tolerate CeO_2 NPs at 500 mg/kg indicating the optimal concentration of CeO_2 NPs on photosynthesis might be exceeded. Specifically, 100 mg/kg CeO_2 NPs could stimulate P_{Nmax}, V_{cmax} and J_{max} to promote photosynthesis of soybean. Conversely, negative impact of 500 mg/kg CeO_2 NPs on photosynthesis mainly results from the inhibited V_{cmax} and J_{max}.

Besides, the two types of CeO_2 NPs showed significant variance on plant uptake but basically no difference on their effects on photosynthesis. Toxicity of CeO_2 NPs depends on a variety of factors including particle size, surface area, morphology and oxidation states. Accordingly, coated CeO_2 NPs with average diameter of 10 nm is supposed to be more toxic to the uncoated CeO_2 NPs with average diameter of 19 nm, which exhibited impact at the same level regardless of variance in accumulated concentration. Besides, coated CeO_2 NPs with relatively higher ratio of Ce^{3+} can exhibit greater impact on plants that offset the effect of lower concentration in soybean tissues (Pulido-Reyes et al., 2015). However, further research is necessary for better understanding of the interaction between chloroplast and the CeO_2 NPs.

CHAPTER IV

IMPACT OF CERIUM OXIDE NANOPARTICLES ON SOYBEANS UNDER DIFFERENT MOISTURE CONTENTS

Introduction

Cerium oxide nanoparticles (CeO_2 NPs) are commonly used in oxygen gas sensors, solar cells and fuel additives. The widespread applications of CeO_2 NPs have raised concerns about their disposal into the environment and their potential impacts on the ecosystem. Mounting evidences have suggested that CeO_2 NPs could affect the crop yield and growth (Rico et al., 2014; Wang et al., 2012) and were able to alter the nutritional composition of plants (Rico et al., 2013; Peralta-Videa et al., 2014). Gui et al. (2015) reported that lettuce treated with 100 mg/kg CeO_2 NPs grew much faster than the controls. However, the researchers noticed that the growth of lettuce exposed to 1000 mg/kg CeO_2 NPs was inhibited. Wang et al. (2012) reported that CeO_2 NPs showed either inconsequential or slightly positive impact on tomato (*Solanum lycopersicum* L.). Separately, it was reported that wheat (*Triticum aestivum* L.) growth and yield was enhanced by CeO_2 NPs at concentrations from 125 to 500 mg/kg soil (Rico et al., 2014). While these studies are encouraging that low levels of CeO_2 NPs might be helpful for plant growth, detailed mechanisms were not provided by these studies. Knowledge was especially lacking on the mechanistic understanding of CeO_2 NPs on essential physiological and biochemical processes in plants. In addition, all these experiments were conducted in well controlled environments, without any limitations on available water to plants.

However, the increasing frequency and intensity of extreme weather events (e.g. extended droughts) due to global climate change have had a detrimental effect on crop yield (Routschek et al., 2014). There have been reports that the ongoing global climate change has caused extended low moisture content in some agricultural soils (Lock et al., 2014). While water deficient tolerance varies among plant species, severe drought could reduce hydraulic conductance of plant leaves (Locke et al., 2014). In a changing environment, plants are able to adapt to varying moisture availability by adjusting their stomatal conductance and other hydraulic properties (Zhou et al., 2014). In some species, they could partially close their stomata while hydraulic conductance exhibited no significant change until the threshold leaf water potential was reached (Wells et al., 2014). Photosynthesis rate may also decline along with the decreasing hydraulic conductance (Zhou et al., 2014). Alibas and Koksal (2015) stated that decreasing soil moisture content significantly decreased germination ratio and germination duration of soybeans, while the length, thickness, geometric mean diameter, and grain mass were also affected. These physiological changes affect plant water use efficiency (WUE), a key indicator of plant yield. Interestingly, some previous research has shown that the WUE of some plants, including *Hibiscus rosa-sinensis* and rice (*Oryza sativa L.*), increased under moisture deficit conditions (Flexas et al., 2013). The primary goal of this study was to investigate the impact of CeO_2 NPs on soybeans at different moisture contents. Specifically, the objectives are twofold: (1) to investigate the effects of CeO_2

NPs on the photosynthesis of soybeans; (2) to determine the effect of CeO_2 NPs on plant WUE estimated by the $\delta^{13}C$ in plant tissues.

Soybean (*Glycine max*) was chosen in this study due to its significance in global agriculture. Considering the increasing regional water scarcity due to the global climate change, investigation on the impact of CeO_2 NPs on plants at various moisture contents could provide insights to the interactions between plants and CeO_2 NPs in a more realistic environment. Soybean was treated with CeO_2 NPs at the 100 mg/kg soil at different field capacity (θ_{fc}, kg/kg), which was the soil moisture divided by the saturated water content in soil (Paredez et al, 2015; Wei et al., 2015). 100 mg/kg was chosen because our previous study demonstrated that CeO_2 NPs at this concentration significantly enhanced plant growth, including plant photosynthesis and WUE. The range of soil moisture levels was controlled between 55-100% of the field capacity based on the literature (Liu & Zhou 2015). For example, Paredez et al. (2015) showed that soybeans were cultivated in soil at the moisture thresholds of 60% and 75% of θ_{fc} to study the impact of moisture conditions on crop yield. Likewise, Wei et al. (2015) had also used 60% and 75% of θ_{fc} as the thresholds of the transpiration and yield modeling of soybeans in the field study. Lower water content was usually not adopted to avoid drought stress of the soybeans, which suggested that 60% of θ_{fc} should be the lower limit of water sufficient conditions for soybeans. We used slightly lower moisture content (55%) to investigate how CeO_2 NPs could affect plants undergoing drought stress.

Materials and Methods

Cerium oxide nanoparticles: Uncoated cerium oxide nanoparticle dispersion (Concentration wt. 10%) and PVP coated Cerium Oxide nanoparticle dispersion (Concentration wt. 20%) were used in the experiment, which were purchased from Sigma Aldrich and US Research Nanomaterials, Inc., respectively. Both nanoparticles were extensively characterized and the detailed information were reported in Chapter 3.

Soil moisture capacity quantification: 50 g potting soil was weighed into each pot and was dried at 70°C for 48 hours. The dry soil was weighed first and then was gradually hydrated to the saturation point, where no more water could be adsorbed. The field capacity was obtained by subtract weight of moisture saturated soil with the weight of dry soil.

Plant cultivation: The soybean seeds were purchased from Johnny Seeds (Fairfield, MN) and potting soil purchased from commercial vendors was used for plant growth. To ensure homogenous mixing, the mixed soil will be shaken on a shaker table for at least 24 hours, with the facilitation of frequent manual mixing. The targeted concentrations for CeO_2 NPs treated soil would be 100mg/kg soil.

Each pot was filled with 50g potting soil and then dried at 70°C for 48 hrs. 28.77, 36.62, 44.46 and 52.31g water was added to every 18 pots to achieve four θ_{fc} of 55%, 70%, 85%, and 100% respectively. Among the germinated soybean seedlings, 72 well grown seedlings with roughly the same length were picked out and were transferred to the soil with different treatments (uncoated and pvp-coated CeO_2 NPs) within which included 4 levels of moisture content (55%, 70%, 85% and 100% θ_{fc}). Considering the control group without CeO_2 NPs at different moisture content, there would be 12 treatments of which each included 6 replicas. The soybeans were cultivated at moisture-controlled conditions for three weeks with controlled lighting (16/8 light/dark cycle) at room temperature.

Photosynthetic response curve: Stomatal conductance and net photosynthesis rate of the soybeans were measured with a Licor-6400XT (Lincoln, NE) at the end of each week during the three-week growth period. Besides, photosynthetic response curves with respect to varying irradiance or carbon dioxide concentration were measured with Licor-6400XT (Lincoln, NE) before the soybeans were harvested. Licor-6400 is a portable IR gas analyzer equipped with a standard LI-6400, 2-3 cm leaf chamber and a red/blue light source.

Temperature in the chamber was kept constant at 25 ºC. Chamber CO_2 concentration was kept constant at 400 mg L^{-1} and irradiance generated from the red/blue light source was connected to the Infra-Red (IR) gas analyzer. Specifically, the photosynthetic photon flux (PPF) were programmed to be the nine levels including 0, 50, 200, 400, 600, 800, 1000, 1200, and 1500 mmol m^{-2} s^{-1}. The top leaves were measured at each light level for at least 2 min to achieve the steady state. A_{max} (maximum photosynthesis capacity), R_d (dark respiration), and ϕ (quantum yield) were calculated by fitting data to the nonlinear regression model described by Hanson et al. (1987), with PPF levels as the independent variable. From the photosynthesis response curve to varying carbon dioxide concentrations, five parameters can be derived from the non-linear curve fitting with the well-established model proposed by Sharkey et al. (2007), including V_{cmax} (maximum carboxylation rate), J (photosynthesis electron transport rate), TPU (triose phosphate use), R_{day} (day respiration) and g_m (mesophyll conductance).

Temperature in the chamber was kept constant at 25 ºC. Chamber CO_2 concentration was kept constant at 400 mg L^{-1} and irradiance generated from the red/blue light source connected to the Infra-Red (IR) gas analyzer. Specifically, the PPF were programmed to be the nine levels including 0, 50, 200, 400, 600, 800, 1000, 1200, and 1500 mmol m^{-2} s^{-1}. The top leaves were measured at each light level for at least 2 min to achieve the steady state. A_{max} (maximum photosynthesis capacity), R_d (dark respiration), and ϕ (quantum yield) were calculated by fitting data to the nonlinear regression model described by Hanson et al. (1987), with PPF levels as the independent variable. Besides, five parameters can be derived from non-linear curve fitting with the well established model proposed by Sharkey et al. (2007), including V_{cmax} (maximum carboxylation rate), J (photosynthesis electron transport rate), TPU (triose phosphate use), R_{day} (day respiration) and g_m (mesophyll conductance).

Carbon isotope composition measurement: Two top leaves of each soybean were sent to the Stable Isotope Geoscience Facility for carbon isotope composition analysis by gas chromatographs isotope ratio mass spectrometer (GC-IRMS, Finnigan DELTAplusXP) on an instantaneous basis, which can serve as a predictable estimate of the overall WUE over time.

Chlorophyll content quantification: At the end of the experiment, roughly 20 g fresh tissues of leaves were weighed from each soybean tested. The leaves were added into centrifuge tubes filled with 4 mL dimethyl formamide (DMF) and then kept in dark at 4 °C for 12 hrs. Chlorophylls extraction was completed following the method of Moran.

UV/vis test was performed on 1 mL residual extracted from each sample. Zero absorbance was calibrated by pure DMF before each measurement. The amount of absorbance was read at 664 and 647 nm using a UV-Vis spectrophotometer (model Lamba 35; PerkinElmer, Waltham, MA) and absorbance readings were used to calculate leaf chlorophyll concentrations.

Cerium content characterization: After all the measurements were done, the rest of the shoots and roots were fully dried at 70 °C for 7 days. Half gram of dry tissues of each replicats was carefully ground and mixed with 4 mL of 70% (v/v) nitric acid. The mixture was kept at room temperature overnight for pre-digestion, then was further digested in the DigiPREP MS hot block (SCP science, Clark Graham, Canada) at 95 °C for 3 hours until all dry tissues was dissolved. After the digested residual cooled down to room temperature, 2 mL of 30% (v/v) H_2O_2 was added into each residual. The solution was heated in the hot block at 95 °C for 2 more hours following the method of Dan et al. (2015). Then the cerium content was quantified by an inductively coupled plasma mass spectrometry (ICP-MS, Perkin Elmer mod. DRCII, Waltham, MA).

Results and Discussion

Biomass: At the lowest moisture content (55% of θ_{fc}), CeO_2 NPs did not affect the soybean biomass as indicated by the insignificant changes in total fresh weight (Fig 4-1). However, fresh weight of the CeO_2 NPs treated soybeans increased in all other moisture conditions. When the fresh weight of the roots and shoots were compared separately, both the fresh weight of roots and shoots displayed similar patterns of change as the total fresh weight. A comparison of the total fresh weight of soybeans with the same treatment at different moisture content revealed that their fresh weight at 70% and 85% moisture content were higher than those at 55% and 100% moisture content. The soybeans at the lowest moisture content probably experienced some drought stress, which resulted in less yield compared with those with sufficient water supply. Noticeably, the highest moisture content, which amounts to full saturation, led to slightly declining fresh weight.

When the dry weight of biomass was examined (Fig 4-2), a slightly different trend from the fresh weight was observed. The dry weight of soybeans at 55% θ_{fc} increased when plants were treated with both types of CeO_2 NPs while no measurable change was observed in fresh weight. It is clear that soybean exposed to both types of CeO_2 NPs at 100 mg/kg showed consistently higher biomass compared with the control. A closer look at the dry weight of root and shoot separately revealed similar trend as the total fresh weight.

Overall, 100 mg/kg CeO_2 NPs exhibited potential to enhance the growth of soybeans although the impact might be less significant at lowest moisture content. However, water deficit appeared to be mediated by CeO_2 NPs since the nanoparticles promoted the dry biomass of soybeans cultivated at 55% moisture content.

The ratio of fresh weight to dry weight (FW/DW) provides another indication of the growth status of plants. As an index of cell water content, FW/DW positively correlates with the level of sugar concentration in plant tissues (Park & Kim, 1993). In other words, theoretical cell density can be derived from the estimated water content in cells based on the FW/DW values. Based on the ratio of fresh weight to dry weight results (Fig 4-3), CeO_2 NPs treated plants at the lowest moisture content exhibited significantly lower FW/DW compared with the control group. The soybeans treated with coated CeO_2 NPs at 70% moisture content also showed lower FW/DW while no significant difference was detected between uncoated CeO_2 NPs treated ones and the controls. Consistent with previous experiments, soybeans with sufficient water supply (typically more than 85% moisture content) did not exhibit any significant variance in FW/DW values.

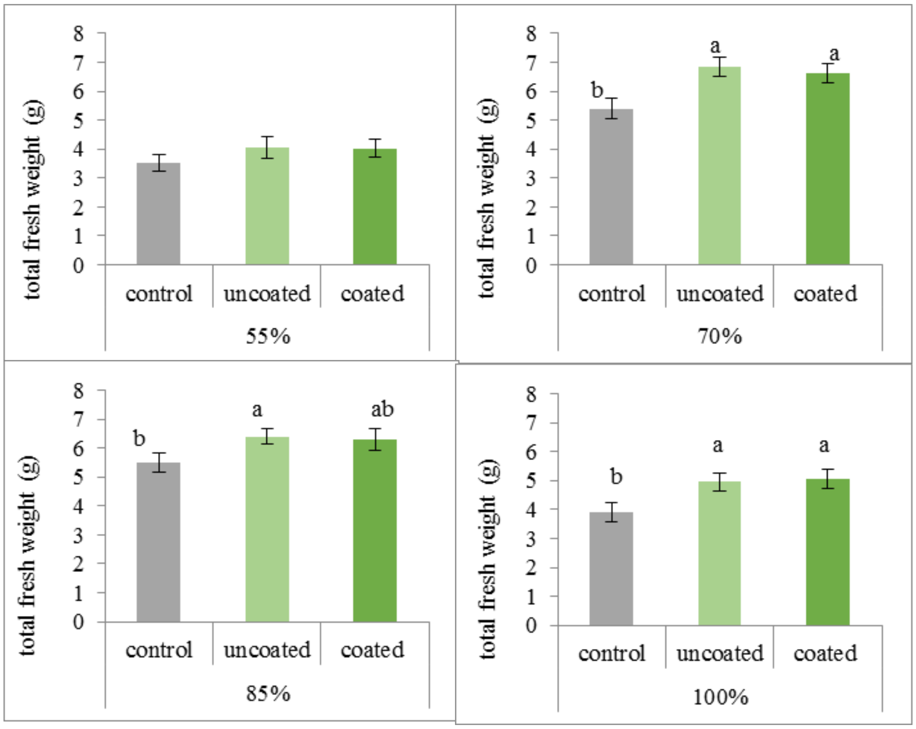

Fig 4-1. Total fresh weight of soybean exposed to 100 mg/kg CeO_2 NPs. Values represent mean±SD (n=6). Different letters indicates significantly statistical differences (at $p \leq 0.05$) according to the Tukey's test

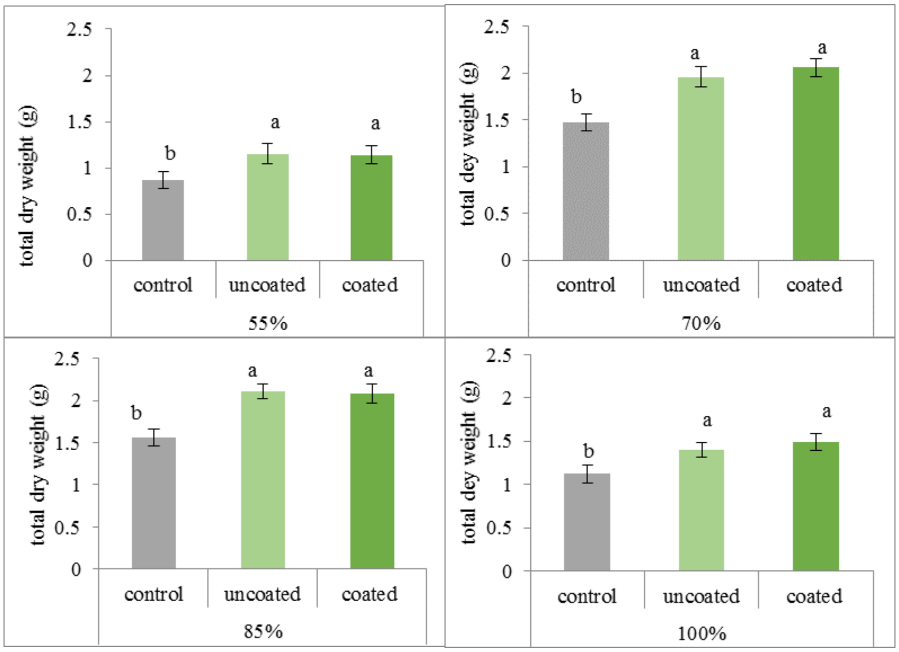

Fig 4-2. Total dry weight of soybean exposed to 100 mg/kg CeO2 NPs. Values represent mean±SD (n=6). Different letters indicate significant statistical differences (at p≤0.05) according to the Tukey's test

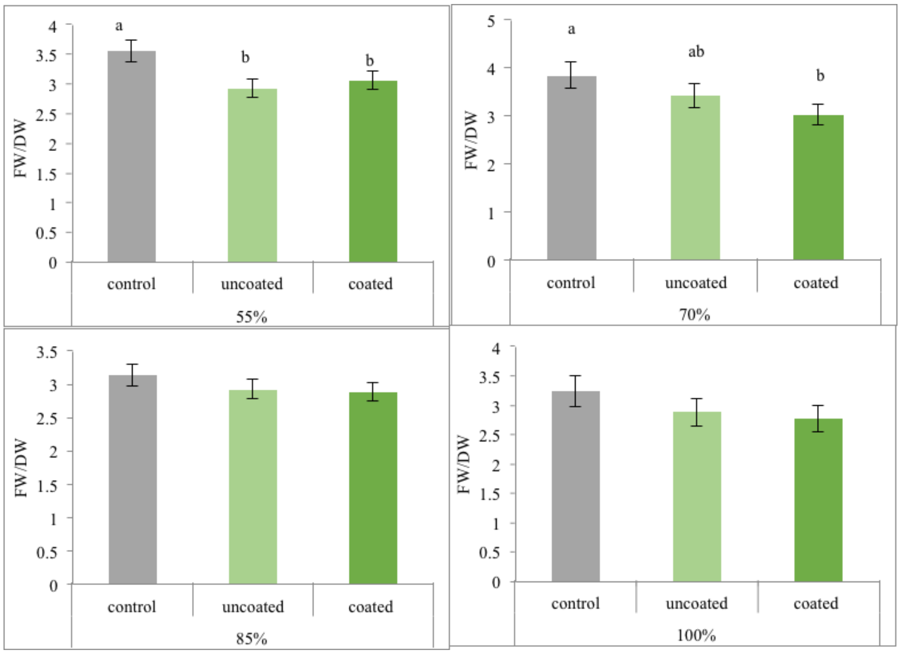

Fig 4-3. Ratio of fresh weight over dry weight. Values represent mean±SD (n=6). Different letters indicate significant statistical differences (at p≤0.05) according to the Tukey's test

Stomatal conductance: Fig 4-4 describes that at 55% moisture content, stomatal conductance of soybeans with all three treatments declined over time, which suggested that the plants were stressed by moisture deficit, as 60% of θ_{fc} was deemed to be the lower limit of water content for soybean cultivation in soil without suffering drought stress (Paredez et al., 2015; Wei et al., 2015). The stomatal conductance at 70% moisture content showed little variation and no significant difference was observed among the three treatments. Comparatively, stomatal conductance of the soybeans at 85% moisture content exhibited consistently increasing trends for all three treatments. Similarly, when the soil was moisture saturated, stomatal conductance of the soybeans treated with both types of CeO_2 NPs gradually arose while the control group maintained nearly constant. The result agrees with a recent study that 1000 mg/kg TiO_2 NPs enhanced the stomatal conductance of barley by 89% at water sufficient condition (Marchiol et al., 2016). Previous studies provided controversial results on stomatal conductance of plants exposed to nanoparticles. Zhao et al. (2013) reported that CeO_2 NPs up to 800 mg/kg exhibited no significant effect on the stomatal conductance of cucumber. Conversely, a more recent report of Wang et al. (2016) addressed that 300 mg/L ZnO NPs reduced stomatal conductance by 70% in *Arabidopsis*. Moreover, nanoparticles could be adsorbed by root surface of plants and then further interfere with water transpiration (Zhao et al., 2013; Zhang et al., 2012; Schwabe et al., 2013). In our study, 100 mg/kg CeO_2 NPs had positive impact on stomatal conductance, which is largely limited by moisture content. Decreasing water availability offset the positive effect of CeO_2 NPs and eventually inhibited the stomatal conductance of soybean when it was lower than 70% θ_{fc}.

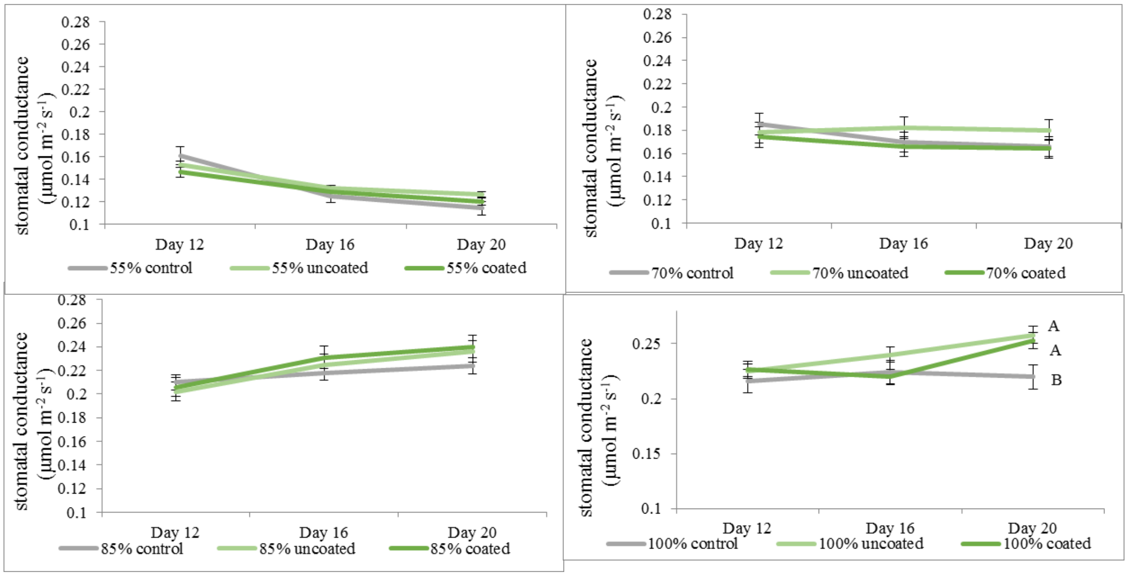

Fig 4-4. Stomatal conductance over the three-week growth at different moisture content. Values represent mean±SD (n=4).

Water use efficiency: Given that $\delta^{13}C$ of atmospheric CO_2 is -8‰, carbon isotopic values of plants generally vary from -25 to -35‰ (H. Craig, et, al., 1953). Carbon isotopic fractionation in plants stems from the discrimination against the heavier isotope ^{13}C due to enzymatic and physical processes. Accordingly, since WUE is defined as the photosynthesis rate to water transpiration, ^{13}C can serve as a time-integrated indicator of WUE based on the understanding of ^{13}C fractionation in photosynthesis (Lajtha, & Michener, 2007).

Fig 4-5 shows the carbon isotope composition of soybeans treated with CeO_2 NPs at different moisture contents. Both types of CeO_2 NPs at 100 mg/kg led to lower $\delta^{13}C$, which represented higher WUE at water abundant conditions. Although slightly lower $\delta^{13}C$ can be observed in plants treated with CeO_2 NPs at water-limited condition (55% θ_{fc}), no significant difference was found when compared with the control. It suggests that limited water availability may offset the positive impact of CeO_2 NPs on WUE of plants. Comparatively, $\delta^{13}C$ of the soybean exposed to CeO_2 NPs at higher moisture content (70%, 85% and 100% θ_{fc}) was significantly lower than the control. It should be noted that $\delta^{13}C$ at 85% and 100% θ_{fc} was consistently lower than it was at 70% θ_{fc} implying that moisture sufficiency had positive effect on WUE.

Physiologically, enhanced WUE indicates increased net carbon assimilation relative to stomatal conductance (Flexas et al., 2013, Klein et al., 2013). According to previous results, increased WUE may be attributed to the reduced stomatal conductance at 55% θ_{fc}. Conversely, since the stomatal conductance gradually increased at higher moisture content, increased WUE is supposed to result from promoted photosynthesis rate. Promoted photosynthesis could attribute to stimulated carboxylation rate by improving enzymatic kinetic characteristics or activating catalytically active Rubisco sites (Flexas et al., 2013; Galmes et al., 2005). Additionally, improved mesophyll conductance and reduced photorespiration could also enhance photosynthesis and WUE (Flexas et al., 2013; Parry et al., 2005). Therefore, further investigation in photosynthesis was necessary for better understanding of increased WUE of soybean.

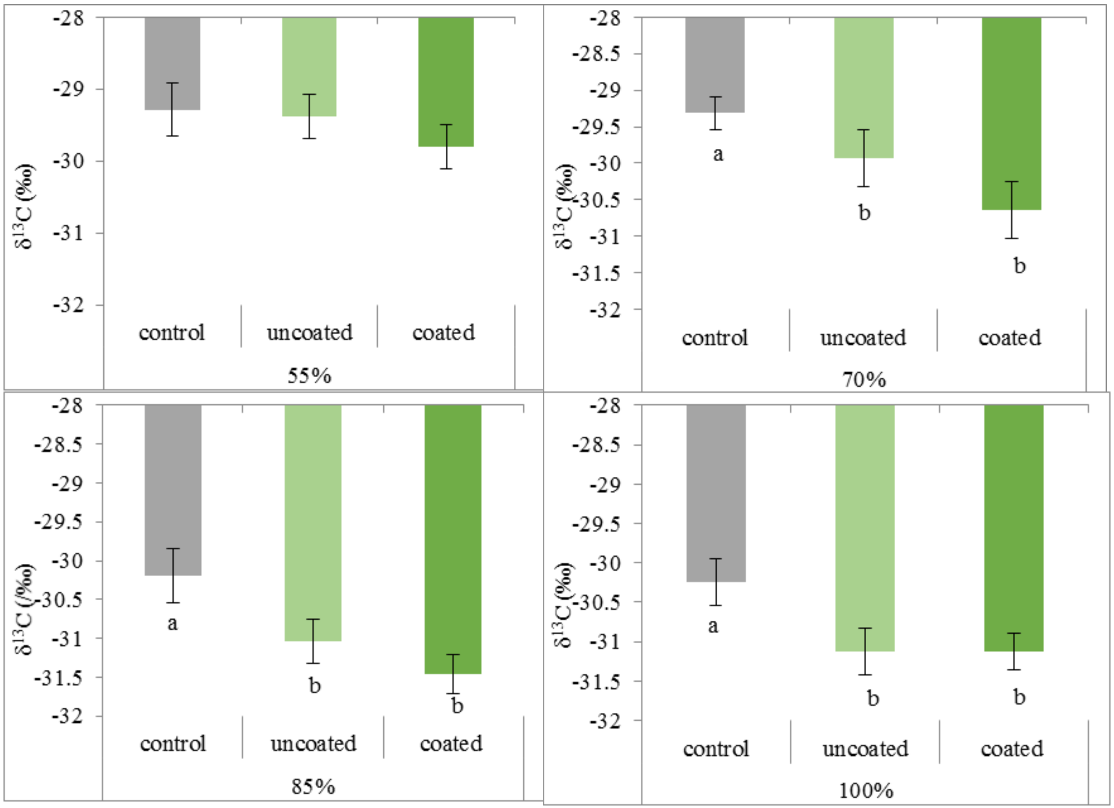

Fig 4-5. Carbon isotope composition $\delta^{13}C$ of soybeans at different moisture content. Values represent mean±SD (n=4). Different letters indicates significantly statistical differences (at $p \leq 0.05$) according to the Tukey's test

Chlorophyll content: Since chlorophyll is correlated with plant stress response, chlorophyll content in soybean exposed to CeO_2 NPs at different moisture content was quantified (Fig 4-6). Fig 4-6 (c) suggests that no measurable difference was detected in total chlorophyll content among the treatments at different moisture content, meaning that CeO_2 NPs did not affect the total chlorophyll concentration in soybean. However, at water abundant condition, chlorophyll a was increased by 20.2% in soybean exposed to coated CeO_2 NPs (Fig 4-6 a). In the meanwhile, uncoated CeO_2 NPs showed no significant effect on the plants. As the major photosynthetic pigment in plants, chlorophyll a concentration indicates photosynthetic stress in plants (Du et al., 2015). Comparatively, soybean treated with CeO_2 NPs exhibited relatively lower concentration of chlorophyll b compared with the control, but significant differences were only observed at 55% and 100% θ_{fc}. Specifically, chlorophyll b was reduced by uncoated CeO_2 NPs at 55% and 100% θ_{fc} while it was decreased by coated CeO_2 NPs only at 55% θ_{fc}.

In addition, the results suggested that chlorophyll could also be affected by moisture content. Total chlorophyll content at water sufficient condition (85% and 100% θ_{fc}) was consistently higher than it was at moisture limited condition (55% and 70% θ_{fc}). Similar trends could be observed in the results of chlorophyll a concentration at different moisture levels. Conversely, chlorophyll b concentration of soybean cultivated at higher moisture content was relatively lower than it was at moisture limited condition (55% θ_{fc}). It agrees with Sarker et al. (1999) that chlorophyll a/b ratio in wheat was significantly affected by soil moisture. Likewise, a few previous studies also reported that moisture stress could result in decreasing chlorophyll formation.

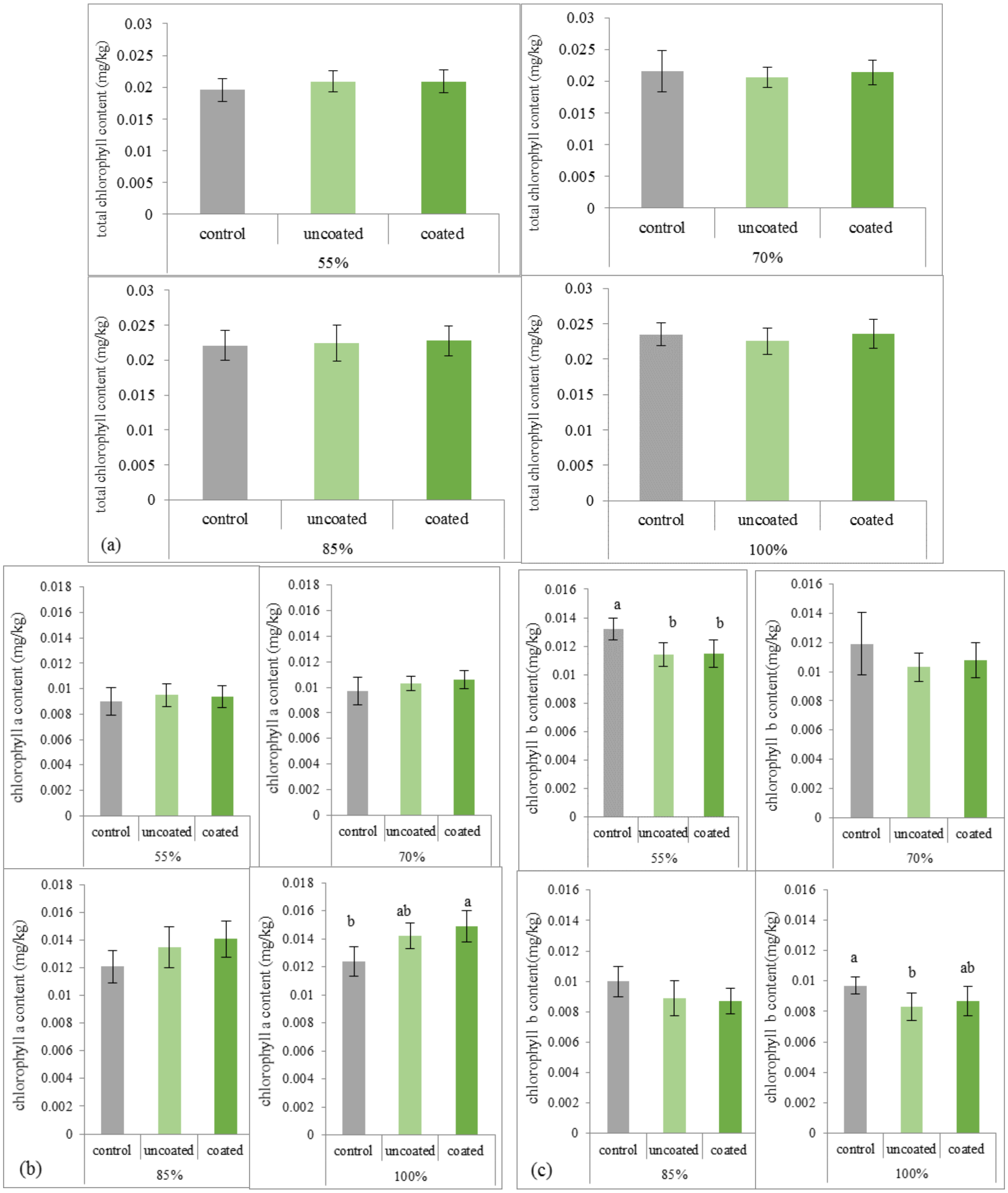

Fig 4-6. (a) Total chlorophyll content, (b) chlorophyll a content (c) chlorophyll b content at different moisture content. Values represent mean±SD (n=4). Different letters indicates significant statistical differences (at p≤0.05) according to the Tukey’s test

Photosynthesis rate: Photosynthesis rate of the soybeans at 55% θ_{fc} maintained a relatively low level ranging from 6.2 to 7.5 μmol $m^{-2}s^{-1}$, which was lower than observed for any other plants at higher moisture content. At 70% and 85% θ_{fc} the soybeans treated with CeO_2 NPs exhibited significantly higher photosynthesis rate compared with their respective controls at day 20, while the control group remained nearly constant over time. At water abundant condition, soybeans treated with CeO_2 NPs had outperformed the control group on photosynthesis rate since day 16. Consistent with our results, Marchiol et al (2016) reported that net photosynthesis rate of barley exposed to 500 mg/kg CeO_2 NPs was promoted by 26%. Likewise, corn plants exposed to 800 mg/kg CeO_2 NPs exhibited higher net photosynthesis rate than the controls at day 30 (Zhao et al., 2015).

Fig 4-7 displays that photosynthesis rate increased with respect to rising moisture content, which suggested the significance of water availability in the carbon assimilation processes of plants. While photosynthesis rate fell in the range 6.5-7.5 at 55% θ_{fc}, it gradually increased by 10-15 at 100% θ_{fc}. It agrees with previous studies that water stress could result in inhibited net photosynthesis rate due to decreased carbon assimilation per unit leaf area (Wang et al., 2009).

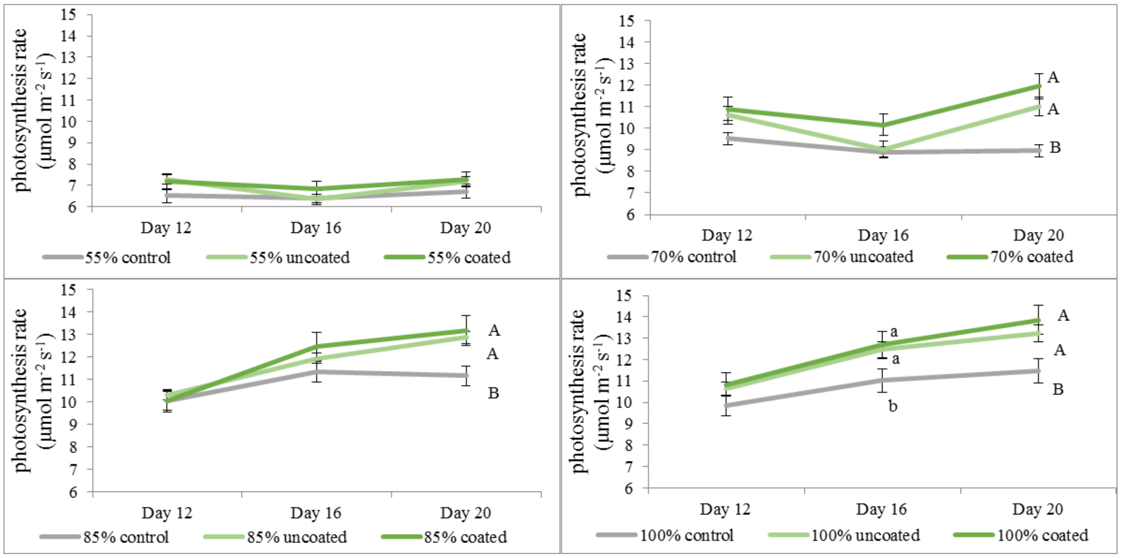

Fig 4-7. Photosynthesis rate at different moisture content. Values represent mean±SD (n=4). Different letters indicates significant statistical differences (at $p \leq 0.05$) according to the Tukey's test

Photosynthetic light response curves: Photosynthetic rate responding to varying irradiances could provide information on net photosynthesis rate (P_{Nmax}), respiration rate (R_d) and quantum yield (ϕ) (Lombardini et al., 2009, Salvucci et al., 1986). Specifically, P_{Nmax} represented the net CO_2 assimilation rate at infinitely high irradiance, which indicated the photosynthesis capacity associated with chlorophyll content, Calvin-cycle enzymes density, and volume of stroma (Lambers et al., 2008). R_d quantified the CO2 generated from dark respiration during photosynthesis while ϕ demonstrates the energy converting efficiency from the energy absorbed from light into the chemical energy in fixed organic carbon (Lambers et al., 2008).

Derived from the light response curves, the three parameters of the treatments under different moisture content are presented in Table 4-1. It was observed that soybeans treated with CeO_2 NPs under the moisture content of 70%, 85% and 100% θ_{fc} exhibited higher maximum photosynthesis rate compared with the control. However, at water limited conditions (55% θ_{fc}), P_{Nmax} was lower than it was at higher moisture content. It has been reported that water stress had significantly negative impact on plant photosynthesis rate (Escalona et al, 2012). Additional, P_{Nmax} at 85% θ_{fc} (11.5 to 15.8 mol/m^{2}s^1) was consistently higher than it was at 100% θ_{fc} (11.2 to 13.8 mol/m^{2}s^1), which agreed with Sanhueza et al. (2014) that N. *solandri* at slightly lower moisture content outperformed in P_{Nmax} compared with those at water saturated conditions.

No significant difference had been observed in R_d of the soybeans among all those treatments. However, the soybeans under the moisture content of 55% θ_{fc} exhibited relatively lower R_d compared with the treatments under higher moisture content. It should be noted that R_d at 85% θ_{fc} was close to or even slightly higher than its was at 100% θ_{fc}. Similar result was reported that respiration in *N. solandri* increased under unsaturated moisture conditions.

ϕ exhibited significant variances only at 85% $\theta_{fc,,}$ within which ϕ of soybean exposed to CeO_2 NPs was higher compared with the control . Moreover, soybeans showed relatively lower ϕ under 55% θ_{fc} compared with those cultivated under higher moisture contents, suggesting that the photosynthetic processes of the soybean leaves in water deficient conditions might be partially inhibited.

Table 4-1. Maximum photosynthesis rate (P_{Nmax}), dark respiration rate (R_d), quantum yield (ϕ) of the soybeans treated with CeO_2 NPs. Values represent mean (n=3). Different letters indicates significantly statistical differences (at $p \leq 0.05$) according to the Tukey's test

			Control	Uncoated	Coated
55% θ_{fc}	P_{Nmax}	μmol/m^{2}s^1	6.9	7.2	7.1
	R_d	μmol/m^{2}s^1	0.63	0.62	0.67
	ϕ		0.07	0.067	0.072
70% θ_{fc}	P_{Nmax}	μmol/m^{2}s^1	9.1^b	11.6^a	12.4^a
	R_d	μmol/m^{2}s^1	0.89	0.93	1.01
	ϕ		0.085	0.093	0.089
85% θ_{fc}	P_{Nmax}	μmol/m^{2}s^1	11.5 ^{b}	15.8 ^{a}	14.3 ^{a}
	R_d	μmol/m^{2}s^1	0.97	1.11	1.17
	ϕ		0.095^b	1.121^a	1.113^a
100% θ_{fc}	P_{Nmax}	μmol/m^{2}s^1	11.2^b	13.8^a	13.1^a
	R_d	μmol/m^{2}s^1	0.96	1.13	1.09
	ϕ		0.087	0.105	0.102

Photosynthetic CO_2 response curves: Photosynthetic performance responding to varying carbon dioxide concentrations revealed the maximum carboxylation rate (V_{cmax}) allowed by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), maximum rate of photosynthetic electron transport (J_{max}) relevant with NADPH regeneration, triose phosphate use (TPU), day respiration (R_{day}) and mesophyll conductance (g_m) (Sharkey et al., 2007). Table 4-2 presents the four parameters derived from the CO_2 response curves. V_{cmax} were significantly enhanced by CeO_2 NPs with different surface properties at water sufficient conditions (85% and 100% θ_{fc}) while it exhibited no variances among the soybeans at lower moisture contents. Inconsistency occurred at 70% θ_{fc} that only uncoated CeO_2 NPs increased the V_{cmax} of the treated soybeans while coated CeO_2 NPs had no significant impact on it. At moisture deficient conditions (55% θ_{fc}) V_{cma} of the soybeans were relatively lower than the rest of the subjects, which indicated the carboxylation rate was limited by water availability. Zhou et al. (2014) also reported that V_{cmax} in *Quercus* and *Eucalyptus* species was reduced with decreasing moisture content.

For J_{max}, it showed that CeO_2 NPs were able to stimulate photosynthetic electron transport rate of soybeans at 85% and 100% θ_{fc} while they failed to increase the J_{max} in plants grown in water deficient conditions. In addition, inconsistency between V_{cmax} and J_{max} was observed in the soybeans at 70% θ_{fc}. Coated CeO_2 NPs promoted J_{max} of the soybeans while they exhibited no significant impact on V_{cmax}. The water deficient treatments exhibited no difference in J_{max} among the soybeans treated with CeO_2 NPs and the control group, which indicated that moisture content should be the dominant factor in water deficient cultivation.

As shown in Table 4-2, CeO_2 NPs did not exhibit significant impact on R_{day}. Although R_{day} barely varied at the same moisture level, declining trend was observed with respect to decreasing moisture content. Coupled with the inhibited V_{cmax}, it indicated higher photorespiration occurred in soybean at water-limited condition (Yang et al., 2015). The photorespiration could be a conservative strategy the soybeans adopted to protect the tissues from photoinhibition, promote intercellular CO_2 to maintain Rubisco activity level (Yang et al., 2015), or scavenge excessive oxidants by catalases.

CeO_2 NPs exhibited positive impact in g_m for the soybeans at relatively higher moisture content (85% and 100% θ_{fc}), suggesting that CeO_2 NPs could stimulate the CO_2 diffusion and further promote the cellular CO_2 level. Comparatively, g_m was less sensitive at lower moisture content. At water deficient condition, soybeans were stressed by limited water availability, which was not alleviated by the CeO_2 NPs. Additionally, it should be noted that g_m was consistently lower at water limited condition than at water abundant condition, which agreed with Zhou et al. (2014) that g_m declined due to drought was observed in *Quercus* species. Previous studies addressed that inhibited g_m was the main reason leading to reduced photosynthesis under drought stress (Galmes et al., 2014; Zhou et al., 2014).

Table 4-2. Maximum carboxylation rate (V_{cmax}), maximum electron transport rate (J_{max}), day respiration rate (R_{day}), mesophyll conductance (g_m) of the soybeans treated with CeO_2 NPs at different moisture content of θ_{fc}. Values represent the average of three replicates (n=3). Different letters indicate significant statistical differences (at $p \leq 0.05$) according to the Tukey's test

θ_{fc}	Treatments	V_{cmax}	J_{max}	TPU	R_{day}	g_m
		$\mu molm^{-2}s^{-1}$	$\mu molm^{-2}s^{-1}$	$\mu molm^{-2}s^{-1}$	$\mu molm^{-2}s^{-1}$	$\mu molm^{-2}s^{-1}Pa^{-1}$
55%	Control	38.9	74.1	6.71	0.48	6.53
	Uncoated	37.6	75.1	6.95	0.43	6.46
	Coated	38	72.8	6.88	0.47	6.38
70%	Control	45^{b}	83.6^{b}	6.62	0.59	7.89
	Uncoated	49.3^{a}	85.8^{b}	6.8	0.62	8.02
	Coated	47.6^{ab}	86.6^{a}	6.54	0.59	7.94
85%	Control	51.2^{B}	90.5^{B}	7.05	0.56	8.97^{B}
	Uncoated	71.7^{A}	112.6^{A}	6.87	0.61	9.83^{A}
	Coated	70.5^{A}	109.3^{A}	6.95	0.6	9.91^{A}
100%	Control	46.7^{β}	86.9^{β}	6.79	0.6	8.78^{β}
	Uncoated	67.1^{α}	108.5^{α}	6.84	0.59	9.73^{α}
	Coated	62.4^{α}	104^{α}	7.08	0.63	9.91^{α}

Cerium accumulation: Due to the opposite surface charge of the two CeO_2 NPs, their accumulation in the soybeans differed significantly in both shoots and roots. Fig 4-8 illustrates the cerium content accumulated in soybean tissues. It showed that at the concentration of 100 mg/kg soil, considerable accumulation of uncoated CeO_2 NPs in roots and shoots was detected. Comparatively, although slightly higher concentrations of cerium were detected in soybeans treated with coated CeO_2 NPs than control plants, the difference was insignificant. Interestingly, a positive correlation between the moisture content and cerium accumulation in the soybeans has been noticed. In addition, much higher cerium concentration was detected in the roots of soybean compared with the shoots. Previous studies also reported that CeO_2 NPs are mostly adsorbed by the root surface of cucumber, pumpkin and wheat (Zhao et al., 2013; Zhang et al., 2012; Schwabe et al., 2015).

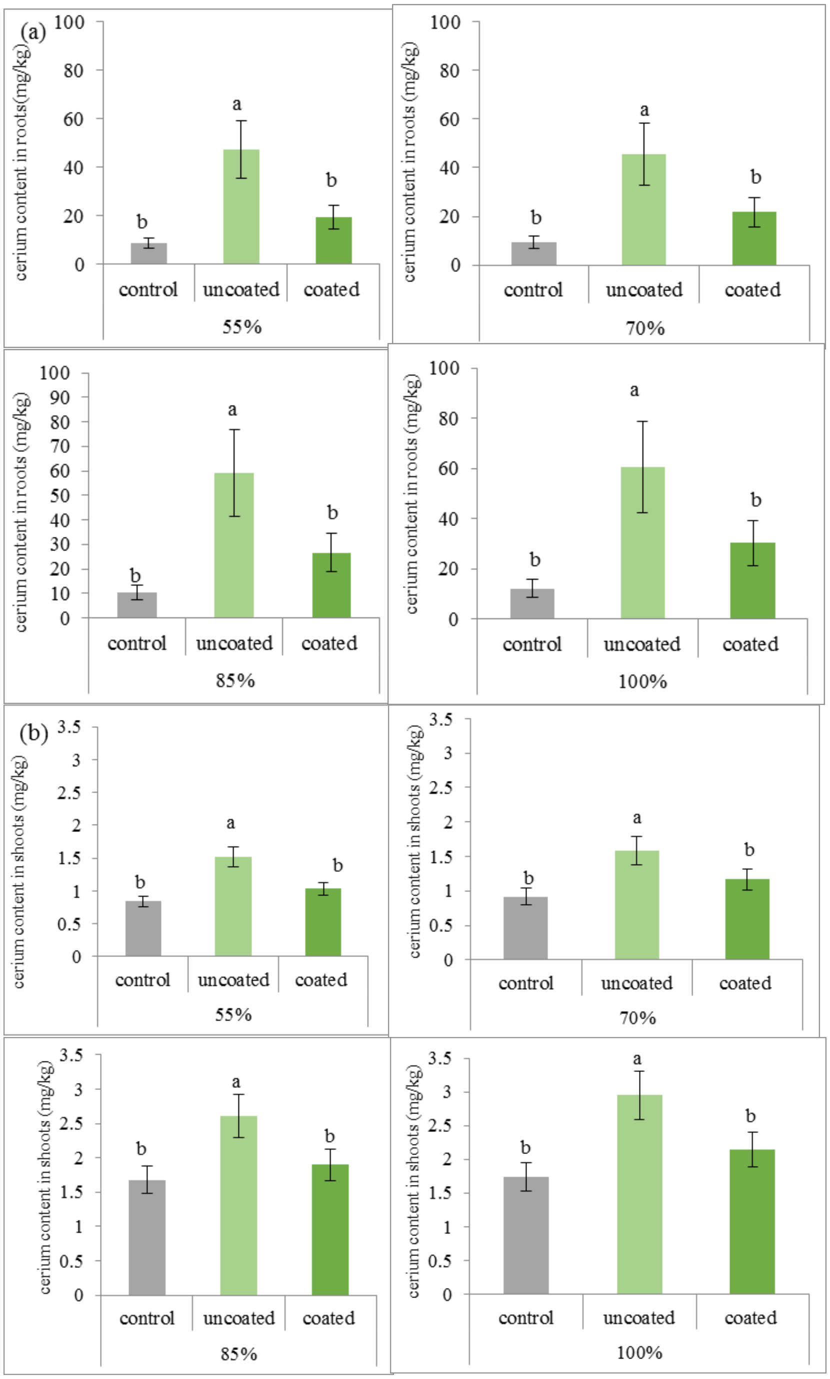

Fig 4-8. (a) Cerium content in roots (b) cerium content in shoots at different moisture content. Values represent mean±SD (n=4). Different letters indicates significant statistical differences (at $p \leq 0.05$) according to the Tukey's test

Consistent with the previous chapter, the results further confirmed the positive impact of 100 mg/kg CeO_2 NPs on soybean photosynthesis and WUE at water sufficient conditions. However, the positive effect was gradually limited by decreasing moisture content. At 55% θ_{fc}, soybean exposed to CeO_2 NPs did not exhibit any promoted photosynthetic parameters, while V_{cmax}, J_{max} and g_m were all stimulated when moisture content was larger than 85% θ_{fc}. Furthermore, photosynthetic parameters were consistently lower than those at water sufficient conditions, suggesting that drought stress exhibited negative effect on plant photosynthesis. Noticeably, V_{cmax} and J_{max} were slightly lower at 85% θ_{fc} compared with 100% θ_{fc} suggesting that the optimal moisture content might be exceeded at water saturated condition.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

In this research, soybeans were treated with two types of CeO_2 NPs at different concentrations and cultivated under varying moisture contents. At the concentration of 100 mg/kg, CeO_2 NPs exhibited consistently positive impact on photosynthesis and WUE.

Table 5-1. Summary of the impact of CeO_2 NPs at different concentration (↑ indicates positive impact, ↓ means negative impact, - suggests no significant impact)

		P	P_{Nmax}	R_d	ϕ	V_{cmax}	J_{max}	TPU	R_{day}	g_m
10mg/kg	Uncoated	↑	-	-	-	-	-	-	-	-
	Coated	↑	↑	-	-	-	-	-	-	-
100mg/kg	Uncoated	↑	↑	-	-	↑	↑	-	-	-
	Coated	↑	↑	-	-	↑	-	-	-	-
500mg/kg	Uncoated	↓	-	-	↓	-	↓	-	-	↓
	Coated	↓	-	-	-	↓	↓	-	-	↓

Above all, the soybeans treated with the two types of CeO_2 NPs at 100 mg/kg exhibited increased biomass and WUE, and outperformed the control group on the photosynthetic tests responding to varying light intensity and CO_2 concentration. Table 5-1 summarizes the photosynthetic parameters affected by CeO_2 NPs. Accordingly, 100 mg/kg CeO_2 NPs enhanced the P, P_{Nmax} and V_{cmax} while uncoated CeO_2 NPs additionally stimulated J_{max}. Conversely, 500 mg/kg CeO_2 NPs inhibited P, J_{max} and g_m while ϕ and V_{cmax} were decreased by uncoated and coated CeO_2 NPs respectively. Comparatively, 10 mg/kg CeO_2 NPs exhibited less significant impact on soybean that only P and P_{Nmax} were affected. Coupled with the increased total chlorophyll content of soybean treated with coated CeO_2 NPs, stimulated P_{Nmax} might result from higher chlorophyll concentration while the photosynthesis process was not significantly affected. Furthermore, although the two types of CeO_2 NPs did not differ from their effect on photosynthesis, they exhibited significant variance on accumulated concentration as a result of the electrostatic repulsion between coated CeO_2 NPs and root surface.

Table 5-2. Summary of the impact of CeO_2 NPs at different moisture content (↑ indicates positive impact, ↓ means negative impact, - suggests no significant impact)

		P	P_{Nmax}	R_d	ϕ	V_{cmax}	J_{max}	TPU	R_{day}	g_m
55%θ_{fc}	Uncoated	-	-	-	-	-	-	-	-	-
	Coated	-	-	-	-	-	-	-	-	-
70%θ_{fc}	Uncoated	↑	↑	-		↑		-	-	-
	Coated	↑	↑	-			↑	-	-	-
85%θ_{fc}	Uncoated	↑	↑	-	↑	↑	↑	-	-	↑
	Coated	↑	↑	-	↑	↑	↑	-	-	↑
100%θ_{fc}	Uncoated	↑	↑	-		↑	↑	-	-	↑
	Coated	↑	↑	-		↑	↑	-	-	↑

According to table 5-2, the experiment further confirmed the positive impact of CeO_2 NPs at 100 mg/kg at moisture sufficient conditions. Noticeably, soybean at 85% θ_{fc} outperformed in ϕ compared with the water saturated conditions. In addition, slight enhancement in P_{Nmax}, V_{cmax} and J_{max} at at 85% θ_{fc} were also observed, which suggests that the optimal moisture content for soybean photosynthesis might have been exceeded at water-saturated soil. Consistent with previous studies, plants would not benefit from increasing moisture content after it reached specific threshold and in some cases excessive water content might even cause detrimental effects on them (G. Striker, 2012). Although parameters at 70% θ_{fc} were lower than those at higher moisture content, positive impact of CeO_2 NPs on P and P_{Nmax} was observed. Besides, V_{cmax} and J_{max} were stimulated by uncoated and coated CeO_2 NPs respectively. However, when the moisture content further declined to 55% θ_{fc}, all the physiological and photosynthetic parameters further decreased and none of them was promoted by CeO_2 NPs. This indicated that water availability had considerable effect on plant photosynthesis and could offset the impact caused by CeO_2 NPs.

Fig 5-1 illustrates that V_{cmax} was linearly correlated with $\delta^{13}C$ based on the CO_2 response curve and carbon isotope composition data collected from the previous experiment. Negatively linear correlation between V_{cmax} and $\delta^{13}C$ was expected since Rubisco in the soybeans would preferentially uptake lighter ^{12}C against ^{13}C in the carbon assimilation processes. Subsequently, the linear regression provided a decreasing trend of $\delta^{13}C$ with respect to V_{cmax} with $R^2=0.82$, which suggested that roughly 82% of the variance could be explained by the linear equation. It further verifies the validity of the V_{cmax} derived from the CO_2 response curves and the carbon isotope discrimination in Rubisco activity.

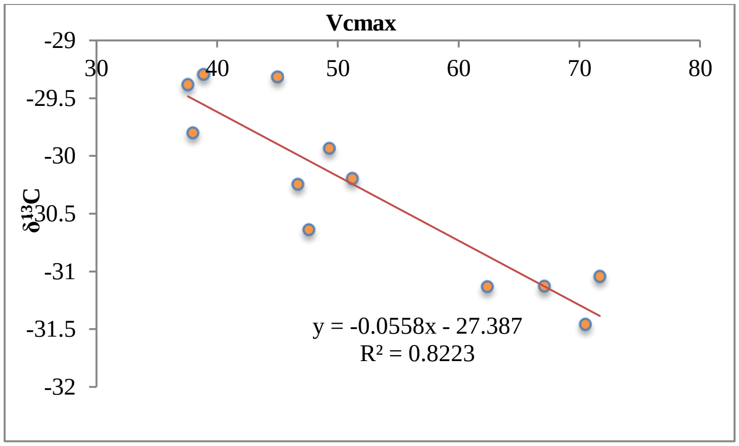

Fig 5-1. Linear regression of correlation between V_{cmax} and $\delta^{13}C$ Values represent mean (n=3)

The research left some questions that needed further study due to the limitation and restrains in the experiment. First of all, although positive impacts could be observed in the previous experiment, how did the CeO_2 NPs interact with the plant cells or chloroplast? Furthermore, while 100 mg/kg CeO_2 NPs exhibited consistently positive impact, is it the optimal concentration for soybean photosynthesis? Besides, although CeO_2 NPs promoted some of the photosynthetic parameters at relatively low moisture content (70%70% θ_{fc}), it requires further studies to verify whether the CeO_2 NPs were able to alleviate the drought stress. Lastly, while soybean at 85% outperformed the treatments at water saturated conditions, what is the optimal moisture content for photosynthesis and WUE of soybean?

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