THE IMPACT OF THE RHIZOSPHERE MICROBIAL COMMUNITY ON THE INTERACTIONS OF ENGINEERED NANOPARTICLES WITH PLANTS

A Thesis

by

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ABSTRACT

The use of engineered nanoparticles (ENPs) has drastically increased, resulting in the release of these particles into the environment; raising concerns over the impacts this could have on human food safety. Two ENPs of interest are cerium oxide nanoparticles (CeO_2 NPs) and graphene oxide nanoparticles (GO NPs). Both of these ENPs have shown that they can impact agricultural crops, but the role soil microorganisms in the rhizosphere can have in ENPs impacting plants is understudied. The rhizosphere contains microbes that are highly influenced by exudates produced from the roots. Interaction between ENPs and the rhizosphere community is important for understanding the potential environmental consequences.

The goals of this study were to: (1) assess the effects of the initial microbial community on the interactions of ENPs with plants, and (2) understand the physical and chemical transformation of ENPs in the rhizosphere and within plant tissues, and its impact on plant health. This was done by administering GO and CeO_2 NPs to soybeans at concentration levels of 0, 100 mg/kg, and 500 mg/kg. Two soil conditions were used, initially sterilized and unsterilized. Sterilizing the soil was used to eliminate the current microbial community. The GO study did not show any interactions between the nanoparticles and indigenous microbial community affecting the soybeans, but some physiological parameters were independently impacted by soil conditions and concentration levels. The CeO_2 NP samples showed significant interactions in parameters associated with photosynthetic process, as well as in biomass and total cerium accumulation. The presence of cerium significantly impacted the production of

nodules on the soybean roots, leading to the conclusion that the CeO_2 NPs were influencing nitrogen uptake by the soybeans. The results showed that CeO_2 NPs significantly interact with the microbial community in the rhizosphere in their interactions with plants and GO NPs probably did not.

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NOMENCLATURE

ENP	Engineered Nanoparticle
NP	Nanoparticles
WWTP	Wastewater Treatment Plant
Ce	Cerium
CeO_2	Cerium Oxide
GO	Graphene Oxide

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CHAPTER I

INTRODUCTION

The use of engineered nanoparticles (ENPs) has grown rapidly due to the distinctive properties that make them beneficial in a wide range of technologies. These unique properties, such as having more atoms at the grain surfaces and a high surface to volume ratio, allow ENPs to be more reactive than their bulk counterparts (Bandyopadhyay et al., 2012). This reactivity can also be a reason for concern, the rise in production has also led to the increase release of these particles into the natural environment. Of particular interest is the potential for these released ENPs to make their way into the human food supply.

A variety of studies have conducted research into how various ENPs affect agricultural crops (Yoon et al., 2013, El-Temsah et al., 2010, Rico et al., 2011). These studies showed that ENPs at varying concentration levels can have both positive and negative implications for certain crops. Although impacts can be seen, the interactions between ENPs and plants are still unclear. A zone of interaction that could be influencing many of the impacts seen in the previously mentioned studies is the rhizosphere. This region is one of the most important components in a terrestrial plant system, and there is a paucity of information on the ENP interactions with the rhizosphere microbial community and the implications of such interactions to ENP plant effects.

The rhizosphere is a thin region of soil that is highly influenced by the roots of plants, possessing a unique redox environment due to the release of plant exudates (Bais

et al., 2006). Roots produce exudates which can consist of sugars, amino acids, organic acids, polysaccharides and proteins; providing a nutrient rich environment to stimulate microbial growth in that region (Kuiper et al., 2003). In this nutrient rich region, complex chemical, physical, and biological interactions take place between the roots and its surrounding environment (Bais et al., 2006). The microenvironments created between plants and the microorganisms in the rhizosphere can affect ENPs uptake and mobility into plants (Schwab et al.,2015). The role of exudates and the microbial communities they create is imperative for plant health. It is necessary to understand whether ENPs interact with the microbial communities due to the pivotal role of these communities have on plant health.

The thesis will discuss how insights into the interactions of the rhizosphere microbial community and ENPs potentially affect ENP plant interactions. These potential interactions can impact the chemical and physical processes in rhizosphere, which together govern the fate and transport of ENPs in plant systems. This will shed light on the key roles the microbial community plays in ENP transformation and plant health; while significantly improving the understanding of ENPs on plants in terrestrial environments. This will be done by assessing the impacts that two ENPs (cerium oxide and graphene oxide) have on soybean plants (*Glycine max* (L.) Merr.) under two different soil conditions. These soil conditions (initially sterilized and unsterilized) will be used to assess how the soil microbial community impacts the transformation and accumulation of the ENPs, and their corresponding affects to the soybeans. Soybeans were used due to their significant production and use around the world.

CHAPTER II

LITERATURE REVIEW

Engineered Nanoparticles

Overview

During the 1980s a radical new device called an atomic force microscope was developed. This gave scientists the ability to observe and physically manipulate atomic units, which essentially marked the beginning of the nanotechnology field (Brar et al., 2010). The resulting ENPs produced are smaller than 100 nm in at least two dimensions for metal based particles and in at least one direction for other ENPs (Rico et al., 2011). They are used in fields for developing new materials, medicines, energy, electronics, and even environmental protection efforts (Cheng et al., 2016). The high surface-to-volume ratio of nanomaterials makes them highly reactive and catalytic, which is useful in a variety of industry sectors (Hossain et al., 2015). This high specific surface area, in addition to having an abundance of surface reactive sites, makes them quite useful, but these same properties can be a concern for potential health hazards (Navarro et al., 2008).

Rapid technological advancements have resulted in the rampant manufacturing of ENPs (Reddy et al., 2016), with production levels expected to reach over half a million tons by the year 2020 (Maurer-Jones et al., 2013). Currently it is estimated that over 15% of all products produced around the world involve nanotechnology in their production process (Shah et al., 2014). This billion dollar industry shows no signs of slowing down and if anything is actually in the exploration stage, meaning this field will

only continue to grow further (Reddy et al., 2016). Such remarkable growth in the nanomaterial industry has led to the increase of exposure levels to the natural environment. One study conducted a lifecycle analysis indicating that approximately 80% of carbon nanotubes could potentially end up in landfills (Lanphere et al., 2014). Organisms that interact significantly with their immediate environment (algae, fungi, plants) are at the greatest exposure risk to ENPs (Navarro et al., 2008), making it potentially detrimental to agricultural crops and the human food supply.

ENP Fate and Transport

While nanoparticles are a natural component of the environment and have always co-existed with organisms in the atmosphere, ocean, soil, and freshwater systems (Schwab et al., 2015), due to the development of the human society and its production of engineered nanoparticles (ENPs), these man-made nanoparticles are now entering natural systems. The transport and accumulation of ENPs in plants is dependent on size, type, chemical makeup, and reactivity (Rico et al., 2011). Once transported into the environment, ENPs can undergo aggregation, dissolution, sedimentation, and transformation by interacting with its surroundings (Reddy et al., 2016). In water, particles are found to aggregate and precipitate under certain conditions, but can re-suspend due to turbulence or exposure to certain organic matter (Schwab et al., 2015). NP movement through soil is found to be quite low, making it more likely for these particles to accumulate in the first few meters or centimeters of the soil, increasing the chances of accumulation into plant tissues (Schwab et al., 2015).

Aquatic and terrestrial plants can be exposed to ENPs from sewage sludge, ENP-incorporated pesticides or fertilizers, wastewater effluent, and atmospheric sources (Schwab et al., 2015, Shah et al., 2014). The most likely route of exposure from ENPs to agricultural crops would be through bio-solids collected at a wastewater treatment plant (WWTP) that are then applied onto agricultural lands (Dahle et al., 2014, Shah et al., 2014). In the United States, approximately 60% of the total bio-solids produced at WWTPs in a year are repurposed for use on agricultural lands (Shah et al., 2014). While the impacts from organic compounds, metals, and microorganisms in those bio-solids are not considered harmful to humans under correct management procedures, there is limited research available on the impacts due to ENPs (Shah et al., 2014). Once in the environment, the potential for uptake into crops is a concern. A review concerning the in-depth routes of nanoparticles to plant cells and tissues was done by Schwab et al. (2015). In a majority of studies reviewed the amount of accumulation into tissues was linear with ENP exposure rates. Out of the current known research, many indicate that the uptake, or translocation of ENPs occur in the apoplast of plant cells. A variety of factors can affect this accumulation, such as symbiotic microorganisms or mucilage compounds.

ENP Impacts on Plants

When ENPs accumulate in plant tissues or interact with their surrounding environment, plant health can be affected in a variety of ways. Sometimes positive effects are seen on growth and yield, which is often the reason for the success ENPs

have had when incorporated into fertilizers. One such example is when radish was exposed to aluminum nanoparticles (Al NPs) (1-100 nm) at the concentration of 2000 mg/L, improved root growth rates were seen (Lin et al., 2007). Other studies have shown negative impacts due to ENP contact. Zinc oxide nanoparticles (ZnO NPs) (< 50 nm) have been shown to negatively impact the development of soybean growth when exposed to a concentration level of 500 mg/kg in the soil, as well as negatively impacting soybeans in the developmental and reproductive stages (Yoon et al., 2013). El-Temsah et al. (2010) showed that barley grown in an aqueous solution had significantly lower germination rates when exposed to 10 mg/L of silver nanoparticles (Ag NPs) (5 and 20 nm).

Two particles of particular concern are graphene oxide nanoparticles (GO NPs), a stable carbon based material, and cerium oxide nanoparticles (CeO_2 NPs), a metal based ENP that could dissolute more readily. These particles are widely used and show potential for accumulation into the environment.

Cerium Oxide Nanoparticles

Overview

Of the rare earth metals, cerium is the most abundant, making up 0.0046% of the crust by weight (Collin et al., 2014). The toxicity, fate, and transport of ENPs can depend on its transformation from its original synthesized state; this transformation can be due to processes such as redox reactions, dissolution, agglomeration/aggregation, and reaction to bio-macromolecules (Maurer-Jones et al., 2013). Cerium in the form of CeO_2

nanoparticles, allows the Ce atom to exist in the trivalent (Ce^{3+}) and tetravalent (Ce^{4+}) state on the surface, allowing the NPs to store and release oxygen (Dunnick et al., 2015). This specific property has made CeO_2 NPs popular as a catalyst in the combustion of diesel fuels as a way to improve emission quality, as well as in a variety of other applications (Antisari et al., 2013, Dunnick et al., 2015). Along with the beneficial industry uses derived from this property, the redox capabilities of this particle can relate directly to the level of toxicity cerium may have on the environment (Collin et al., 2014). Higher ratios of Ce^{3+}/Ce^{4+} tend to be more toxic to plants in the environment, with one study showing the ability of excessive Ce^{3+} to produce hydrogen peroxide (toxic to plants) by consuming superoxide radicals (Pulido-Reyes et al., 2015). Other studies suggest that the reactive sites on CeO_2 NPs could act as scavengers of these free radicals (Hirst et al., 2009), exerting antioxidant effects (Celardo et al., 2011).

CeO_2 NPs Impact on Plants

Many studies have been conducted to gain insight on the potential effects CeO_2 NPs could have on agricultural plants. Results vary; some studies indicate potential toxicity while others indicate benefits to CeO_2 NP exposure. Lopez-Moreno et al. (2010) showed that when soybeans, tomatoes, and cucumbers were grown in an aqueous solution and exposed to 2000 mg/L concentrations of CeO_2 NPs (7 nm), germination rates were reduced. In a previous investigation, CeO_2 NPs at concentrations of 500 mg/kg were shown to improve plant biomass, height, and grain yield in wheat (Rico et al., 2014). Cao et al. (2017) showed that when dosed by CeO_2 NPs at 100 mg/kg,

soybeans exhibited an increase in the net photosynthesis rates, while decreasing in rate when exposed to a higher concentration of 500 mg/kg. When tomatoes, corn, and cucumbers were exposed to 4000 mg/L of CeO_2 NPs, root elongation rates were inhibited (Ma et al., 2010). At concentrations of 500 - 4000 mg/L, CeO_2 NPs were able to significantly increase root and stem growth for corn, alfalfa, and soybeans (Lopez-Moreno et al., 2010). Chemical and physical transformation of ENPs could be the underlying cause of many of the impacts that these particles have on agricultural crops. Zhang et al. (2016) showed that CeO_2 NPs were converted into ionic cerium in the rhizosphere and taken up into the plant through the roots. This study indicated that the reduction of Ce^{4+} in CeO_2 NPs is shown to be easier than in its bulk counterpart, and that the dissolution of this ENP and its redox conditions is key to understanding its potential toxicity to the surrounding environment (Majumdar et al., 2014).

Graphene Oxide Nanoparticles

Overview

Carbon nanomaterials are known for being light weight, have superior strength, and high conductivity levels; these materials include fullerenes, carbon nanotubes, and graphene (Zhang et al., 2015). Graphene is a carbon based material that has gained attention due to its potential in electronic, energy, medical, and environmental applications (Chowdhury et al., 2015). Graphene is a thin plane of atomically single graphite, with graphene oxide being composed of a sheet of this graphene with carboxylic groups at its edges and a basal plane made of phenol hydroxyl and epoxide

groups (Liu et al., 2011). GO NPs have been shown to have potential utilization capabilities in heavy metal detecting sensors, electrodes, and biomedical applications (Lanphere et al., 2014).

GO NPs Impact on Plants

Carbon nanomaterials have been shown to impact agricultural crops in both negative and positive ways. With one study showing single-walled nanotubes (SWNTs) to decrease yield rates of rice when exposed at concentrations of 400 mg/L (Lin et al., 2009). When tomatoes were exposed to multi-walled carbon nanotubes (MWCNTs) at concentrations of 10-40 mg/L, significant increases in germination, fresh biomass, and stem length were seen (Khodakovskaya et al., 2009). GO NPs are gaining popularity, but there is not as much available research on the impact GO has on agricultural crops as compared to carbon nanotubes (CNTs). Recent studies though show negative impacts due to contact with this ENP. When exposed to GO NPs at concentrations between 500-2000 mg/L it was seen that the leaves of cabbage, tomato, and red spinach were reduced in both number and size, associated with an increase in reactive oxygen species (ROS) production and the accompanying cell death from this (Begum et al., 2011). Another study showed that GO NP exposure at concentration levels of 25-100 mg/L caused shorter seminal root length of *Brassica napus* L. and that when exposed to concentration levels of 50-100 mg/L, fresh root weights decreased (Cheng et al., 2016).

Rhizosphere Interactions

Rhizosphere Overview

The rhizosphere was first termed by Lorenz Hiltner in 1904 as the zone of soil where microbes are influenced by the root system of a plant. Plants rely on the soil microbes of the rhizosphere for collecting nutrients and cycling the availability of key nutrients such as nitrogen and phosphorus (Burke et al., 2015). Changes in the relationship between plants and the microbial community in the rhizosphere can impact plants themselves. The rhizosphere has three parts: endorhizosphere (containing endodermis and cortical layers of root tissue), rhizoplane (contains the root surface along with the epidermis and mucilage), and finally the ectorhizosphere (this is soil near the root) (Badri et al., 2009).

Carbon modifies the surrounding soil environment drastically compared to the bulk soil, leading to the proliferation of microbes in any of the three regions of the rhizosphere (Haichar et al., 2014). The biological status and quality of soil is often determined by the composition and health of these existing microorganisms (Josko et al., 2014). A total of 5-21% of carbon fixed through the photosynthesis process is transferred into the rhizosphere through root exudates (Haichar et al., 2014). Root exudate composition is dependent on plant species, age, and external factors such as biotic and abiotic stressors (Badri et al., 2009). These exudates can be divided into two categories: low molecular weight compounds (amino acids, organic acids, sugars, and other secondary metabolites) and high molecular weight compounds (mucilage and proteins) (Haichar et al., 2014).

A range of highly complex chemical, physical, and biological interactions occur between the roots and its surrounding soil environment; these interactions can include root-root, root-insect, and root-microbe (Bais et al., 2006). Root exudates are suggested to have a significant role in eliciting the outcomes of such interactions and therefore creating the plant-rhizosphere dynamic (Ge et al., 2014, Bais et al., 2006). Many microbes in this region are considered to have root growth-stimulation or growth-inhibiting properties (Kuiper et al., 2003). The composition of root exudates can influence soil properties, either negatively or positively, such as if roots exude compounds that act as metal chelators, this will increase the availability of metallic soil micronutrients like manganese and zinc (Bais et al., 2006). With it being shown that polysaccharides found in the mucilage could potentially enhance accumulation of heavy metals or adsorb and inactivate their toxicity (Rico et al., 2011).

Legume-Rhizobia Symbiotic Relationships

The rhizosphere can contain a variety of organisms such as nematodes, fungi, bacteria, and arthropod herbivores; all capable of communicating or interacting with the plant (van Dam et al., 2016). Root exudates are key for plants to communicate with these organisms in the surrounding environment (Haichar et al., 2014). Chemical signaling is the way plants produce many of these interactions, one such example being when soybeans release isoflavone to attract *Bradyrhizobium japonicum* and the pathogen *Phytopthora sojae*, for use in the symbiotic nitrogen fixing relationship (Bais et al., 2006). Plant growth and productivity is supported by the rhizobia-legume interaction,

providing nitrogen to the plant (Huang et al., 2014). Plants get the majority of their nitrogen through the form of nitrates (NO_3^-) and also as ammonium ions. NO_3^- is produced through conversion of NO_2^- by nitrifying bacteria. When there is a lack of such nitrogen in the soil, leguminous crops will release flavonoids to attract nitrogen-fixing bacteria to produce nodulation factors (Pauly et al., 2006). Rhizobia and leguminous plants create symbiotic relationships that can develop root nodules, which are specialized plant organs that fix nitrogen (Ramu et al., 2002). Nodules are created on the roots housing these symbiotic bacteria, where they can then directly fix nitrogen from the air for use by the plant.

Reactive Oxygen and Nitrogen Species

Reactive oxygen and nitrogen species (ROS and RNS) are shown to be key players in the responses plants have to stresses (Pauly et al., 2006). These reactive species are signaling compounds for plant biotic interactions with their environments (Scheler et al., 2013), being recently discovered as signaling molecules that help the plant to recognize and respond to stress factors (Pauly et al., 2006). There is evidence that ROS and RNS play a role in the symbiotic relationships between legumes and rhizobium bacteria (Scheler et al., 2013). When RNS and ROS production are impaired, nodule development was impacted in the early stage of infection (Scheler et al., 2013). NO was shown to control the expression of genes involved in nodulation, this function along with its activating role in the plant and pathogen interaction shows the multiple purposes this reactive species has (Scheler et al., 2013). Recent developments show that

these reactive species are not only utilized during stress responses but throughout the lifespan of the plant, for growth and development stages as well (Pauly et al., 2006).

ENP-Microbe Interactions

Nanoparticles could interact with microbial communities in the soil through direct and indirect means; direct toxicity to the cells or indirectly through influencing the physicochemical soil properties (Antisari et al., 2013). ENPs may cause the production or release of ROS which can damage cells and DNA, as well as potentially release heavy metals which are toxic to microbial cells (Burke et al., 2015).The production of ROS, disturbing ion cell membrane transport activity, oxidation damage, and lipid peroxidation are some examples of chemical interactions that could take place between ENPs and microbial communities (Hossain et al., 2015). These particles can react with sulfhydryl, carboxyl groups, and potentially alter protein activity (Hossain et al., 2015). Indirect toxicity to plants and the surrounding system can be due to ENPs ability to often readily adsorb organic molecules or inorganic ions (Hossain et al., 2015).

It has been shown that Ag NPs can produce toxic effects to soil bacteria necessary for denitrification and nitrogen fixation (Yang et al., 2013). Ag NPs at concentrations of 800µg/kg slowed the nodulation process in faba bean (*Vicia faba* L.), as well as the nitrogenase activity (Abd-Alla et al., 2015). ZnO NPs significantly affected the root lengths of peas (*Pisum sativum* L.) and Zn^{2+} release had phytotoxic effects on the development of the peas (Huang et al., 2014). Due to the ZnO NPs and the ions it released during the early interactions between rhizobia and plant, the impact to

nodule development resulted in delayed nitrogen fixation (Huang et al., 2014). The presence of these nanoparticles also produced early onset of senescence to the nodules (Huang et al., 2014). In another study, it was shown that ZnO can decrease diversity of microbial community in corn microcosm (Kim et al., 2009). ZnO NPs produce toxic effects to Gram-negative Escherichia coli (E.coli) and that it is mainly attributed to the released free zinc ions (Zn^{2+}) (Bandyopadhyay et al., 2012).

GO NP Impacts on Microbes

There have been mixed reports on the antimicrobial activity of GO NPs. At concentrations of 80 mg/L of GO there was 3.3 times the amount of ROS in *Aradidopsis thanliana* T87 cells, which then mediated cell death through mitochondrial damage (Begum et al., 2013). Generating ROS in cells can be a primary mechanism for toxic damage, with key organelles producing ROS that include mitochondria, chloroplasts, and peroxisomes (Begum et al., 2013). It is also suggested that the antibacterial properties of GO is due to the stress inflicted on the membranes of cells induced by the sharp edges of the nano-sheets, resulting in physical damage to cell membranes, causing the cells to leak RNA and loss of membrane integrity (Liu et al., 2011). In another study though, it showed that when mammalian cells were exposed to concentrations of 25µg/mL of GO NPs the bacteria had higher growth rates (Ruiz et al., 2011), essentially showing no indication of this NP having antimicrobial properties.

CeO_2 NP Impacts on Microbes

As with GO NPs, there have been mixed results concerning the ability of CeO_2 NPs to significantly impact bacterial communities. A study by Ge et al. (2014) showed that CeO_2 NPs were not able to influence the bacterial richness in soil planted with soybeans or in unplanted soils. Although the richness of bacteria did not change, it is possible that the cerium influenced the composition of the bacterial community due to the effects seen on plant growth. It was surmised that root exudates were reduced due to stunted growth when plants were exposed to 1000 mg/kg of CeO_2 NPs, due to the fact that exudates production is often correlated to root and shoot biomass levels. This change in exudates could have impacted the composition of the bacterial community. In another study, nano-ceria at 31-125 mg/l inhibited the growth of the primary nitrogen fixing bacteria of alfalfa (Collin et al., 2014). The negative impact was attributed to the adsorption of NPs on the extracellular surface and the alteration of certain protein structures. It was also shown that CeO_2 NPs could exhibit antibacterial effects on *E. coli* due to surface attachment (Pelletier et al., 2010) or possibly from membrane damage (Thill et al., 2006).

The presence of ENPs in the rhizosphere could potentially create feedback loops in the plant-rhizosphere dynamic. ENPs could create a rhizosphere microbial shift which in turn influences plant response. The plant response to such a stress factor could then influence exudate composition which changes the entire rhizosphere makeup. Then going even further, this new rhizosphere environment may interact differently with the ENPs than at the initial time of exposure. Understanding the relationship ENPs have

with the rhizosphere and the microorganisms found in it is key to assessing the potential environmental risks these particles could have on plants in the long-term.

CHAPTER III

INTERACTION OF CERIUM OXIDE NANOPARTICLES WITH MICROBIAL SOIL COMMUNITY, AND ITS IMPACT ON SOYBEAN (*Glycine max* (L.) Merr.)

Introduction

Engineered nanoparticles (ENPs) have been increasingly used in fields such as medical, energy production, and manufacturing due to their specific reactive properties (high surface-to-volume ration and abundance of reactive sites) (Bandyopadhyay et al., 2012, Hossain et al., 2015). With the wide usage of ENPs, the environmental fate of these particles and their potential impact to the human food supply is a growing concern. These ENPs can enter an agricultural system through treated wastewater bio-solids or through pesticides and fertilizers containing ENPs (Ge et al., 2014). A range of studies have been conducted to determine the impact of ENPs on various agricultural crops, with both positive and negative results (Yoon et al., 2013, Shah et al., 2009, Rico et al., 2011).

In particular, cerium oxide nanoparticles (CeO_2 NPs) are one of the most widely used ENPs, making it a key particle of concern. There is an estimated global production of 10,000 tons of CeO_2 NPs per year and can be found in a variety of industrial products such as diesel fuel additives, petroleum refining catalysts, electronics, and automobile catalytic convertors (Collin et al., 2014). Just as many other ENPs are distributed through the application of WWTP bio-solids to agricultural lands; CeO_2 NPs are among these that could interact with the human food supply. In a previous investigation, CeO_2 NPs at concentrations of 500 mg/kg were shown to improve plant biomass, height, and

grain yield in wheat (Rico et al., 2014). When dosed by 100 mg/kg CeO_2, soybeans exhibited an increase in photosynthesis rates, while decreasing in rate when exposed to a higher concentration of 500 mg/kg (Cao et al., 2017). Although these studies, in addition to many others, showed that CeO_2 NPs can have an impact on agricultural crops, the mechanisms of interaction between nanoparticle and plant are challenging to assess.

It is hypothesized that many processes occur concurrently between nanoparticles and the root rhizosphere. The rhizosphere can be defined as the region of soil around the roots that is influenced by root activity (Haichar et al., 2014). This area of soil surrounding plant roots contains a variety of nutrients, bacteria, and enzymes (Bais et al., 2006). This region differs from its bulk soil counterpart due to the fact that the content found in the rhizosphere is highly influenced by the root itself (Anderson et al., 1993). Exudates from the roots can include secreted ions, free oxygen and water, enzymes, mucilage, and various carbon-containing primary and secondary metabolites (Bais et al., 2006). The bacteria in this region of soil are important to plant health, and leguminous plants, such as soybeans, often create symbiotic relationships with nitrogen-fixing bacteria in the soil. Previous studies indicate that ENPs can directly impact bacteria in the soil, with it being shown that nitrogen-fixing bacteria are identified as particularly sensitive to ENP exposure (Ge et al., 2014). Another study specifically tested the impact of CeO_2 NPs on microbial biomass carbon and nitrogen extracted from the soil, resulting in a significant decrease under a short-term 7 day exposure to the nanoparticles (Antisari et al., 2011). If ENPs are able to impact rhizosphere bacteria, this could impact plants themselves. This in turn, could affect the plant's production of exudates, which then

influences the rhizosphere and overall soil environment. The degree to which ENPs and the rhizosphere interact, and ultimately affect plants can have a much more significant impact to overall soil quality than previously thought.

While assessing the interaction between nanoparticles and plants, it is imperative to discuss the potential transformation that nanoparticles, in particular CeO_2 NPs, can have in this highly active region. Chemical and physical transformation of ENPs could be the underlying cause of many of the impacts that these particles have on agricultural crops. A previous study showed that CeO_2 NPs were converted into ionic cerium in the rhizosphere and taken up into the plant through the roots due to root exudation (Zhang et al., 2017). The reduction of Ce(IV) in CeO_2 NPs is shown to be easier than in its bulk counterpart, the dissolution of this ENP and its redox conditions is key to understanding its potential toxicity to the surrounding environment (Majumdar et al., 2014). Such transformations directly determine their accumulation and bioavailability to humans via food consumption, making it necessary to investigate further.

In order to obtain a more comprehensive understanding of CeO_2 NP interactions in the environment, a study was designed and conducted in a manner that maximized the ability to gain insights into nanoparticle-plant interactions. For this investigation, two primary objectives existed: (1) assess the effects of the initial microbial community on the interactions of ENPs with plants, and (2) understand the physical and chemical transformation of ENPs in the rhizosphere and within plant tissues, and its impact on plant health.

Materials and Methods

Cerium Oxide Nanoparticle

Polyvinylpyrrolidone (PVP)-coated CeO_2 NPs were purchased from US Research Nanomaterials, Inc (Houston, TX, USA). Characterizations of the NPs were reported in a previous publication (Cao et al., 2017). These NPs were in the form of a nanopowder dispersed in water, ranging in size between 6 and 24 nm. Through the use of an X-ray photoelectron spectroscopy analysis, the amount of Ce^{3+} on the surface was found to be approximately 12%.

Soil Preparation

Scotts Topsoil was purchased from a commercial outlet. The soil was packaged in Marysville, OH and contained a combination of peat, composted forest products, and sphagnum peat moss. Small potting containers were used to hold 150 grams of soil, acting as total mass of soil (M_t). These 150 grams of soil were then dried at 70˚C for 48 hours to determine its dry mass (M_d). Saturation level by mass (M_s) was determined by adding water to the dry soil until it could not absorb the water. The difference between M_s-M_d is the water capacity.

Soil was prepared in two conditions: sterilized and unsterilized. Sterilized soil was obtained by placing it in an autoclave (Panasonic MLS – 3781L) for 25 minutes at 121˚C. The soil was sterilized to eradicate any initial microbial community. The soil was not kept in a state of sterilization during the experiment; it is assumed that bacteria from the air and water would reenter the soil and establish a new microbial community.

Soybean Preparation

Soybeans (*Glycine max* (L.) Merr.) were purchased from Johnny's Selected Seeds (Winslow, Maine). Seeds were sterilized using 1.25% sodium hypochlorite solution and then rinsed with deionized water three times (Zhang et al., 2014). The seeds were germinated by placement into separate containers with sterilized and unsterilized saturated soil of a 2 inch depth. After 4 days they were transplanted into potting containers containing the 150 grams of topsoil.

Before seeds were transplanted into the soil, a dispersion of deionized water and CeO_2 NPs were added to the soil at concentration levels of 0 mg/kg (control), 100 mg CeO_2/kg of dry soil, and 500 mg CeO_2/kg of dry soil. Amount of water needed was determined to be 120 mL per replicate in order to maintain the previously determined level of saturation.

In total, each group (sterile and unsterilized) had three treatments with 5 replicates per treatment; for a total of 30 soybean seedlings. They were placed under UV lighting with a controlled light/dark cycling for 16 hours on and 8 hours off. Deionized water was given daily to maintain saturation and plants were kept at constant room temperature of 25˚C.

Photosynthesis Rate & Stomatal Conductance Analysis

Photosynthesis rate and stomatal conductance were measured to assess key physiological impacts. Measurements took place at day 11, day 18, and day 23 after CeO_2 exposure. These two parameters were measured simultaneously with a Licor-

6400XT (Lincoln, NE) in conjunction with an infrared gas exchanger. This equipment utilizes a lamp that keeps a constant quantum flux of 1000 µmol/m^2/s and a constant CO_2 exchange rate of 400 µmol/mol between two reference sensors. In addition, a flow rate of 500 µmol/s is utilized to control humidity within the chamber. These are set to mimic standard conditions.

Chlorophyll Fluorescence

Chlorophyll fluorescence values (F_v/F_m) were measured with the use of a continuous excitation chlorophyll fluorescence analyzer (OS1p, Opti-Sciences, Hudson, NH). Measurements took place on the same days the net photosynthesis rate was measured. Leaf clips were placed on the leaves of the soybeans and remained there for 30 minutes (Maxwell et al., 2000). These clips allowed the leaves to adjust to darkness and then be excited for measurement purposes.

Plant Harvest

After 27 days of growth, the plants were pulled from the soil gently and rinsed with deionized water to remove soil particles. The roots were separated from the shoots and weighed separately to obtain fresh weight. Roots were inspected for nodules, if found they were counted and then cut to determine inside coloring. Some fresh leaves were then used for chlorophyll analysis.

Chlorophyll Content Analysis

Fresh leaf tissues in the amount of 50 mg were weighed from each replicate and placed in a centrifuge tube with 12 mL of dimethyl formamide (DMF). The samples were vortexed for a minute and then kept in dark conditions for 24 hours.

After 24 hours, a chlorophyll analysis was then conducted using a UV-Vis spectrophotometer (model Lambda 35; Perkin Elmer). The procedures used for this were based off the methods of Moran (1982). A control of 1 mL of DMF was used to compare with a 1mL solution sample from each centrifuge tube to determine changes in absorbance due to the presence of chlorophyll. Light absorbance was read at wavelengths of 664 nm and 647 nm. These measurements were then used to calculate chlorophyll *a* and *b* values.

Ce Analysis in Plant Tissues

The total element of cerium in plant tissues (including Ce attached to root surface) was determined by strong acid digestion. First the tissues were dehydrated in an oven at 70˚C for 7 days. After fully dried, 0.5 grams of tissue material were added into a 4 mL solution of nitric acid (70% by volume) and sat overnight at room temperature. Then they were further digested in a DigiPREP MS hot block digester (SCP science, Clark Graham, Canada) at 95˚C until any remaining residual tissue was fully dissolved. The digestate was then cooled to room temperature, at which point it was further mixed with a 2 mL solution of H_2O_2 (30% by volume) and heated at 95˚C for two hours. This

solution was then analyzed through inductively coupled plasma mass spectrometry (ICP-MS, Perkin Elmer mod. DRCII, Waltham, MA).

Ce Speciation

Fresh root tissues rinsed with DI water three times were further rinsed with 5 mM $CaCl_2$ washing solution five times. This washing solution was used to collect cerium remaining on the surface of roots. 4 mL of this washing solution was then centrifuged through 10kDa Amicon Ultra-4 Centrifugal Filter (EMD Millipore, Billerica, MA, USA) units to separate the dissolved cerium from particulate cerium. Ce in the filtrate was then quantified by ICP-MS, and this fraction of Ce was considered as the dissolved Ce on the root surface. Another 4 mL of the same washing solution was acid digested and quantified by ICP-MS to determine total cerium on root surface. The difference was calculated as Ce in particulate form on root surface.

After collecting the washing solution, enzymatic digestion was performed on fresh root and shoot tissue to distinguish the particulate and dissolved cerium present in soybean tissues by use of a newly developed method (Zhang et al., 2017). Fresh root and shoot tissues were cut into small sections with a blade. 0.5 grams of this tissue were mixed into a 9 mL solution of 20 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer (pH=5, adjusted by NaOH). This mixture was homogenized in a centrifuge tube using a handheld homogenizer (150 W, Fisher Scientific). After the mixture was thoroughly homogenized, one mL of 30 mg/mL Macerozyme R-10 enzyme (bioWORLD, Ohio, USA) (prepared in 20 mM MES) was added to the solution. Then

this solution, now at 10 mL, was mixed on a shaker for 24 hrs at 37^{o}C. The dissolved and total Ce in this solution was then analyzed similarly as described above with the washing solution.

Statistical Analysis

Minitab was used to perform t-tests, one-way analysis of variance (ANOVA), and two-way ANOVA on the data obtained. Two-way ANOVAs are used to determine whether two independent factors have an interaction effect on the dependent factor. This was used to test the interaction of the independent factors (concentration and soil condition) on the dependent factor (any parameter tested). A one-way ANOVA determines if there are statistically significant differences between the means of three independent groups. This was used to determine statistical differences exhibited by the concentration level (0, 100 mg/kg, and 500 mg/kg) on each parameter tested. A t-test is used to determine if two groups of data are significantly different from one another. This was used to determine significance the two soil conditions (unsterilized and sterilized) at a particular concentration level.

Results and Discussion

Fresh Biomass

Fresh biomass levels were measured to determine whether the factors tested (soil condition and nanoparticle concentration) enhanced or inhibited plant growth. Graphs depicting the biomass of fresh shoots and roots are shown in Figure 3-1.

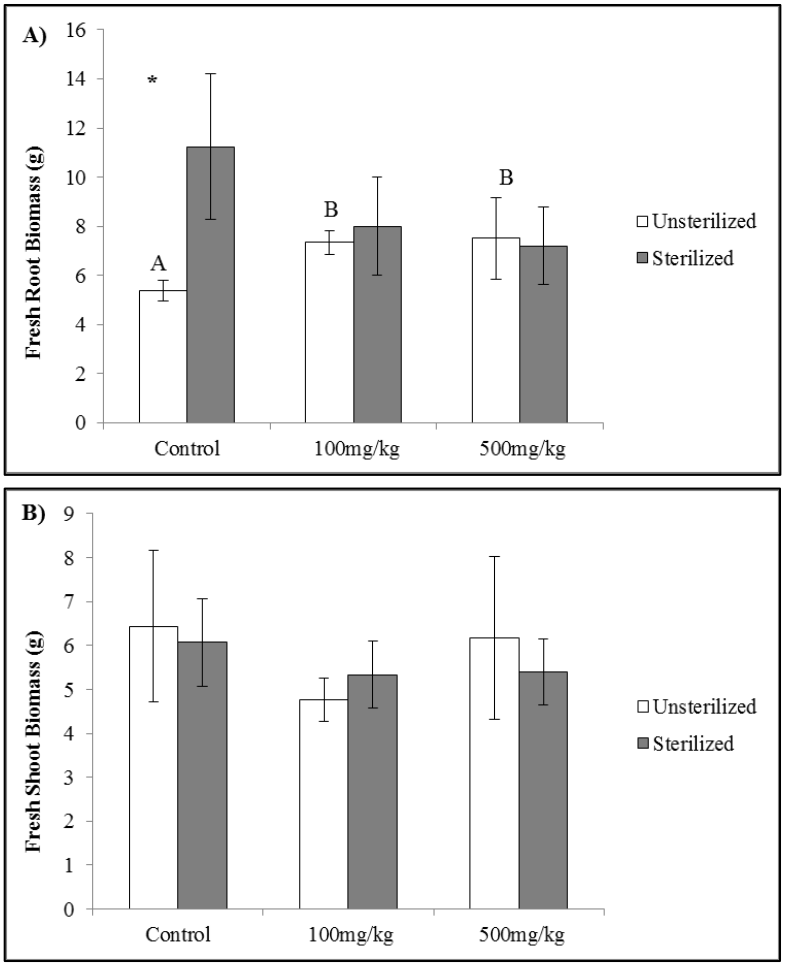

Fig.3-1 Fresh biomass levels after CeO_2 NP exposure. Root biomass (A) and shoot biomass (B) of soybean plants exposed to CeO_2 NPs. Soybeans were grown in two soil conditions (unsterilized and sterilized) and exposed to three different concentration levels (0, 100 mg kg^{-1}soil, or 500 mg kg^{-1}). Values represent mean ± SD (n=3), with the different letters indicating significant differences ($p \leq 0.05$) according to one-way ANOVA. Asterisks (*) indicate significant differences between soil conditions at a particular

Statistical analysis showed that no significant differences were found in the shoots across different concentration levels or soil conditions. A two-way ANOVA analysis showed no interaction effects between soil conditions and concentration levels.

For the fresh root biomass, average values in the sterilized group at 100 mg/kg and 500 mg/kg were lower compared to the control, but this decline was not considered significant. In the unsterilized group, root biomass significantly increased by 40% in the 500 mg/kg group compared to the control group. Only the control groups showed a significant difference in the root biomass between the two soil conditions, with the sterilized group being 89% higher than the unsterilized control group. Without the presence of nanoparticles, the soil condition appeared to play a significant role in the root biomass, but as higher concentrations of CeO_2 NPs were added, the two soil groups became comparable in biomass production. Two-way ANOVA analysis indicated that root biomass was directly impacted by the interaction between concentration level and soil condition.

Nodule Counts

When harvested, the soybean roots were visually assessed to count the number of nodules present. A graph depicting the nodule counts can be seen in Figure 3-2. The inside of all nodules were inspected, a reddish color prevailed indicating that the bacteria present were actively fixing nitrogen (Moll et al., 2016). In the control group for both soil types, where no CeO_2 NPs were administered, the nodule count was zero. As the dosing level increased, the number of nodules present significantly increased as well.

There was only a significant difference between soil conditions at the 100 mg/kg level, where in the sterilized group each sample consistently had greater than 10 nodules and the unsterilized group had only 1 or 2 present on a root sample. The two-way ANOVA indicated that the nodule count was independently affected by concentration and soil condition, but that these two factors did not interact.

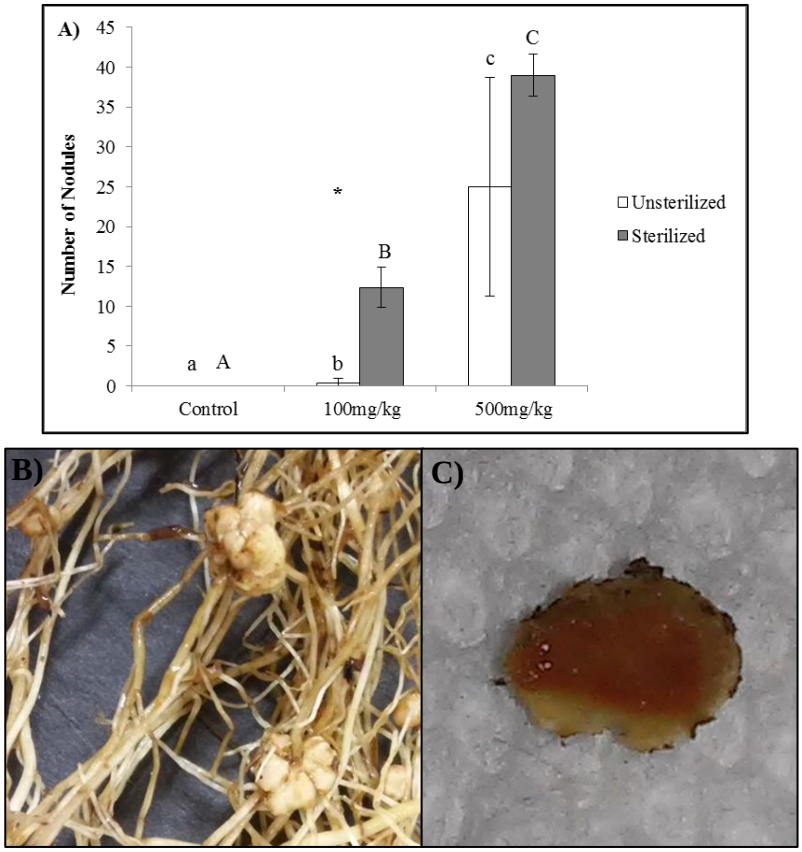

Fig.3-2 Nodulation results. Nodule counts (A) of soybean roots exposed to CeO_2 NPs. Soybeans were grown in two soil conditions (unsterilized and sterilized) and exposed to three different concentration levels (0, 100 mg kg^{-1}soil, or 500 mg kg^{-1}). Values represent mean ± SD (n=3), with the different letters indicating significant differences ($p \leq 0.05$) according to one-way ANOVA. Asterisks (*) indicate significant differences between soil conditions at a particular concentration level, according to t-test. Pictures of nodules found on roots (B). Nodule cut in half to display inside color (C).

Leguminous plants, such as soybeans, often create symbiotic relationships with nitrogen-fixing bacteria in the soil. Plants take up nitrogen in the form of ammonium ions or nitrate in the soil. When there is a lack of such nitrogen in the soil, leguminous crops will release flavonoids to attract nitrogen-fixing bacteria to produce nodulation factors (Pauly et al., 2006). Nodules are created on the roots housing these bacteria, where they directly fix nitrogen from the air for use by the plant. The presence of these nodules provides insight into how the plant is responding to its environment. The results indicate that the presence of cerium impacts how the soybean is responding to its environment and is somehow impacting the ability of the plants to uptake nitrogen.

Chlorophyll Content

Two types of chlorophyll were measured: chlorophyll *a* and chlorophyll *b*. Chlorophyll levels can be seen graphically in Figure 3-3. Significance between soil conditions was seen for chlorophyll *a* in the control group and at the 100 mg/kg group, with sterilized soil having significantly higher content levels. The same results were seen in chlorophyll *b* as well. Chlorophyll *a* was significantly impacted in the unsterilized soil group by concentration levels, with an 89% increase from the control group to the 500 mg/kg concentration group. While in the sterilized group, chlorophyll *a* content decreased by 19% (although not significantly) between the control group and the 500 mg/kg group. The sterilized soil group showed significant decreases in chlorophyll *b* content between the control and 500 mg/kg dosing group. Interaction effects between the two factors were seen for both chlorophyll *a* and *b*.

Chlorophyll is used during the photosynthetic process. It absorbs light and then transforms that light energy into chemical energy. Chlorophyll *a* is the primary pigment used to collect light waves, chlorophyll *b* acts as an accessory pigment, capturing energy and sending it to chlorophyll *a*. These results indicate that the interaction of CeO_2 NPs with the soil conditions impact one of the key components of the photosynthesis process.

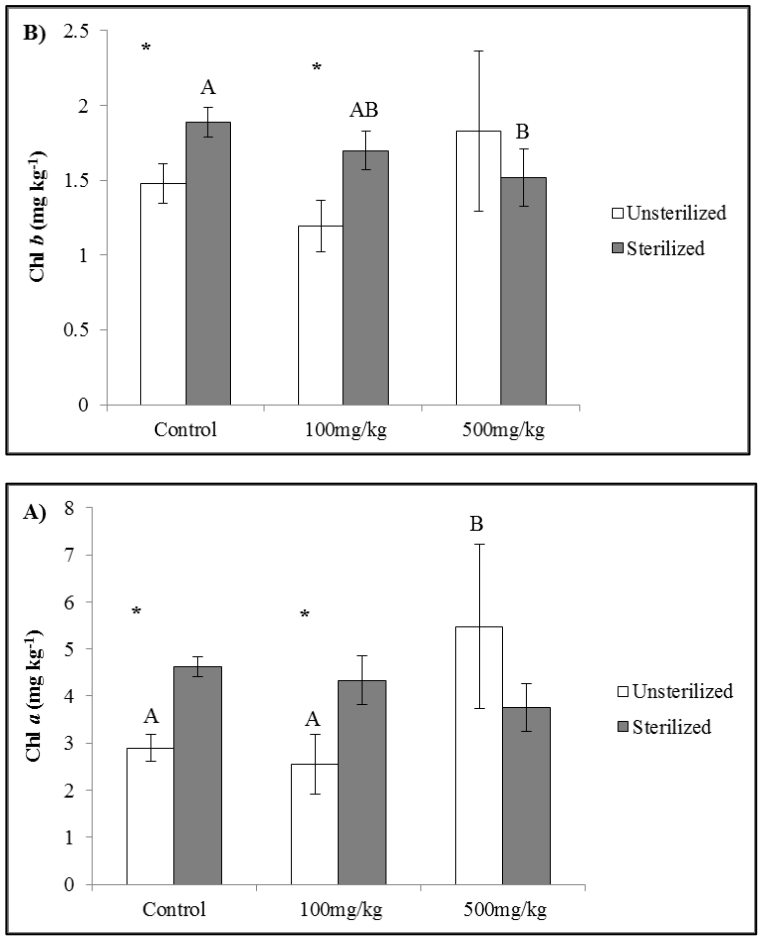

Fig.3-3 Chlorophyll content after CeO_2 NP exposure. Chlorophyll *a* (A) and chlorophyll *b* (B) content of soybean plants exposed to CeO_2 NPs. Soybeans were grown in two soil conditions (unsterilized and sterilized) and exposed to three different concentration levels (0,100 mg kg^{-1}soil, or 500 mg kg^{-1}). Values represent mean ± SD (n=3), with the different letters indicating significant differences ($p \leq 0.05$) according to one-way ANOVA. Asterisks (*) indicate significant differences between soil conditions at a particular concentration level, according to t-test.

Chlorophyll Fluorescence

Three measurements were taken while plants were growing (Figure 3-4). On day 11, the sterilized group showed the most significant differences between concentration groups, decreasing between the control and 100 mg/kg group, and then increasing again at the 500 mg/kg group. Similar effects could be seen in the unsterilized group on this day, but they were not significant. Day 18 measurements showed that at the 500 mg/kg concentration level, unsterilized soil and sterilized soil groups were significantly different from one another. It was not until day 25 that significant differences were seen for both soil conditions and concentration levels. Interaction effects between soil conditions and concentration levels were only significant at day 25.

When light is absorbed by a leaf, chlorophyll molecules become excited due to these light photons; de-excitation of the molecules takes place through three possible routes: photosynthesis, reemission of photons by chlorophyll *a* pigment (chlorophyll fluorescence), or heat dissipation (Cendrero-Mateo et al., 2015). By measuring chlorophyll fluorescence, insight can be gained into photosynthetic performance of plants and their ability to handle environmental stresses (Maxwell et al., 2000). Similar to the chlorophyll content results, this parameter for photosynthesis also appears to be influenced by the interaction between CeO_2 NPs are the existing microbial community of the rhizosphere.

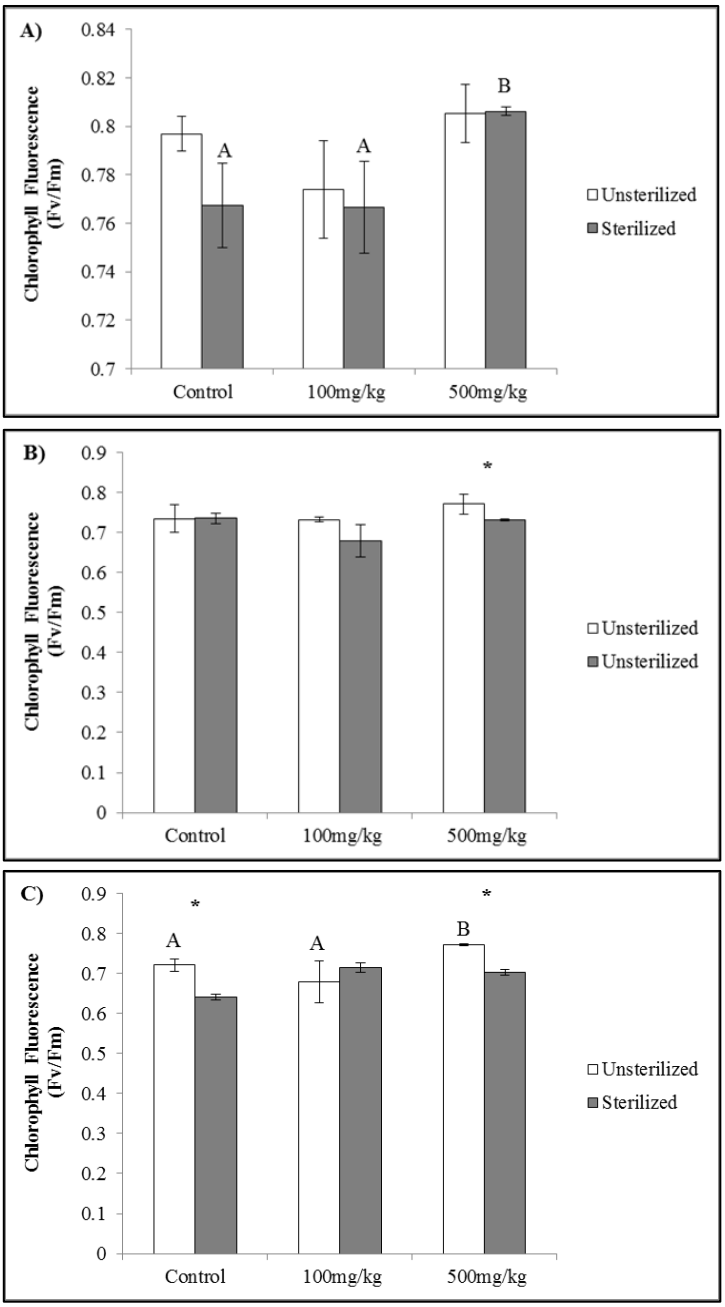

Fig.3-4 Chlorophyll fluorescence after CeO_2 NP exposure. Chlorophyll fluorescence (Fv/Fm) of soybean plants exposed to CeO_2 NPs at day 11 (A), day 18 (B) and day 25 (C) of plant growth. Soybeans were grown in two soil conditions (unsterilized and sterilized) and exposed to three different concentration levels (0, 100 mg $kg^{=1}$soil, or 500 mg $kg^{=1}$). Values represent mean ± SD (n=3), with the different letters indicating significant differences ($p \leq 0.05$) according to one-way ANOVA. Asterisks (*) indicate significant differences between soil conditions at a particular concentration level,

Stomatal Conductance

Three measurements were taken during the growing process (Figure 3-5). Concentration had a significant impact on stomatal conductance at both day 11 and day 25. Soil condition impacts were seen as significant at day 11 at the control and 100 mg/kg concentration levels. At day 18, soil condition was only significant in the 500 mg/kg concentration group. On day 25 soil condition significantly impacted values in the 100 mg/kg and 500 mg/kg groups. It appears that sterilizing the soil had a greater impact on the control initially, but as time progressed the control groups became less affected by

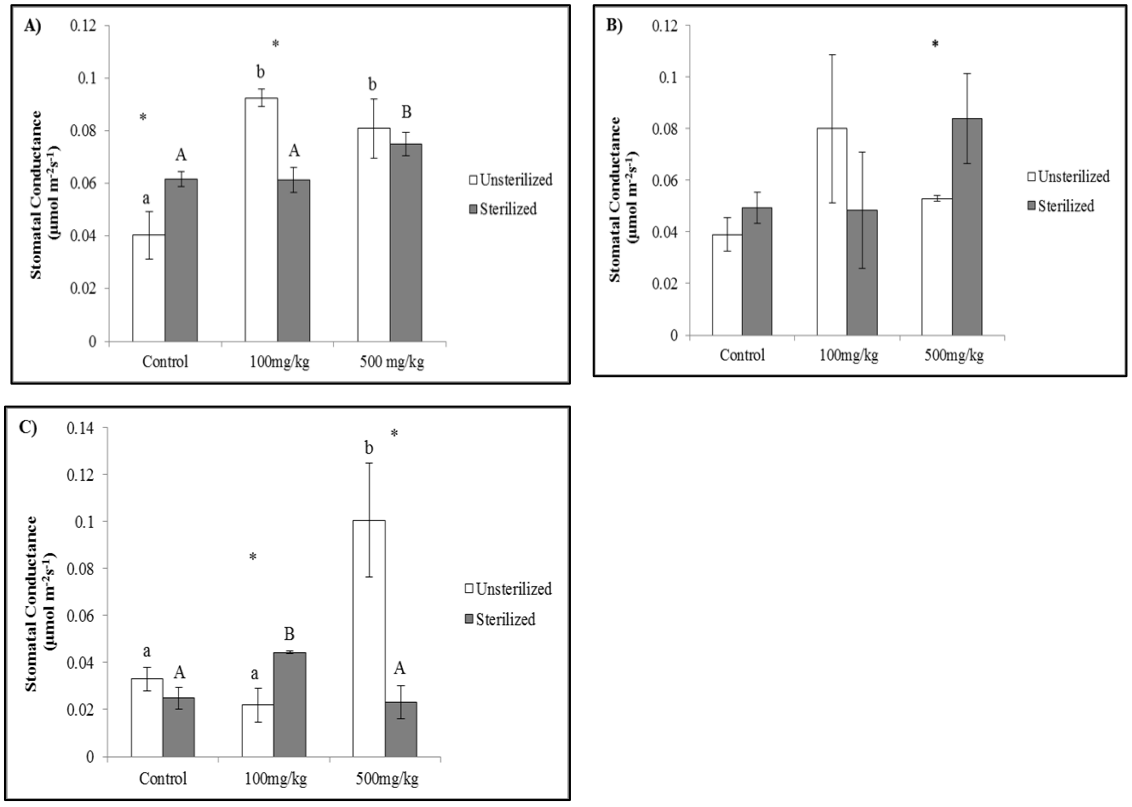

Fig.3-5 Stomatal conductance after CeO_2 NP exposure. Stomatal conductance of soybean plants exposed to CeO_2 NPs at day 11 (A), day 18 (B) and day 25 (C) of plant growth. Soybeans were grown in two soil conditions (unsterilized and sterilized) and exposed to three different concentration levels (0,100 mg $kg^{=1}$soil, or 500 mg $kg^{=1}$). Values represent mean ± SD (n=3), with the different letters indicating significant differences ($p \leq 0.05$) according to one-way ANOVA. Asterisks (*) indicate significant differences between soil conditions at a particular concentration level. according to t-test.

soil condition and the 500 mg/kg dosing group became more affected. Interaction effects were significant on day 11 and day 25.

Stomatal conductance is the rate at which CO_2 enters or water vapor exits stomata on the leaf surface. Stomata are pores on the leaf surface that facilitate gas exchange into the plant. This measurement relates directly to the effectiveness of the photosynthetic process and results show that these processes are impacted by both CeO_2 NPs and the soil conditions.

Photosynthesis Rate

This measurement was taken in conjunction with stomatal conductance; similar trends were observed (Figure 3-6). Day 18 showed no significance between soil conditions or dosing concentrations. On both day 11 and day 25, concentration level played a significant role in the photosynthesis rate. Soil condition affected plants during week one with the sterilized group being 38% higher than the unsterilized group for sample in the control group. As well as in the 100 mg/kg group, with the sterilized group being 15% lower than the unsterilized soil group. On day 25 the control group was no longer significantly affected by the soil condition, but the other two concentration groups were significantly affected by soil condition. With the unsterilized group being 59% lower in value than the sterilized group at concentration levels of 100 mg/kg and then the sterilized group being 67% lower than the unsterilized group at concentration levels of 500 mg/kg. When assessing the photosynthesis rates over time, it can be seen how the control group was impacted by soil condition initially, but then equalized by day 25, and

the 500 mg/kg group having the opposite results. Significant interaction effects were seen on day 11 and day 25, just as they were in the stomatal conductance results.

Photosynthesis is the process plants use to convert CO_2 and light energy into carbohydrates. The ability of a plant to effectively produce carbohydrates can give insight into the health of the system. From these results it is clear that NP and rhizosphere interaction took place, which impacted the photosynthesis parameters.

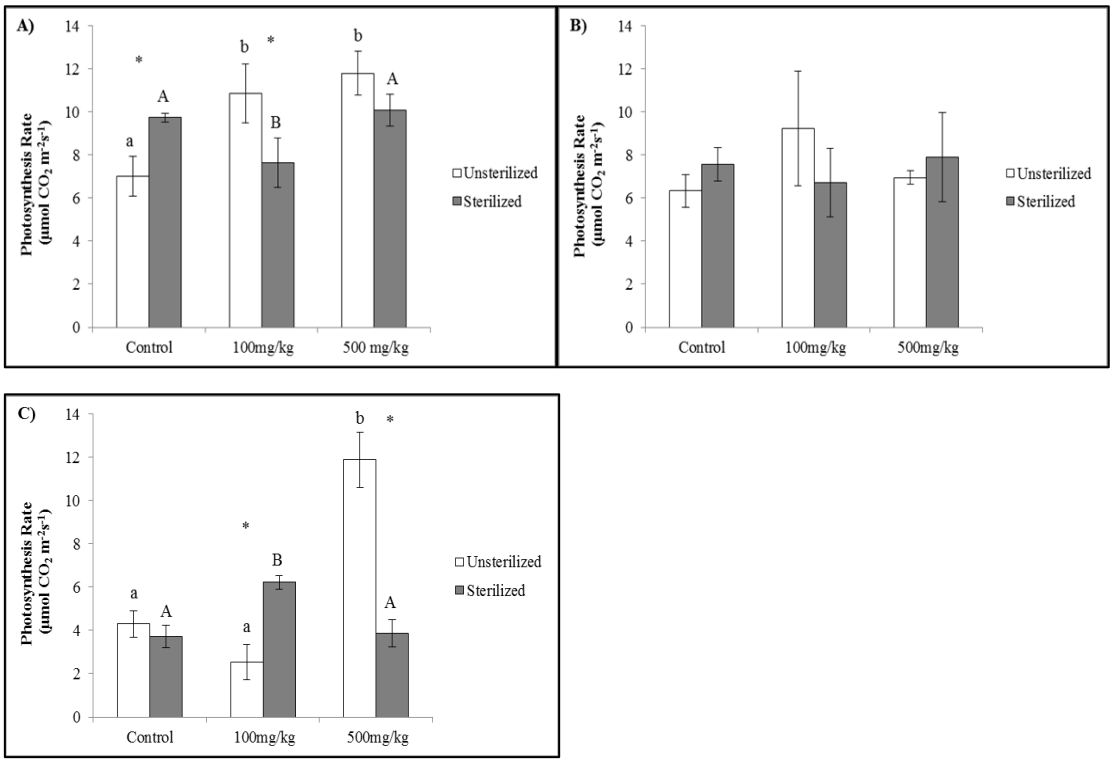

Fig.3-6 Photosynthesis rates after CeO_2 NP exposure. Net photosynthesis rate of soybean plants exposed to CeO_2 NPs at day 11 (A), day 18 (B) and day 25 (C) of plant growth. Soybeans were grown in two soil conditions (unsterilized and sterilized) and exposed to three different concentration levels (0,100 mg $kg^{=1}$soil, or 500 mg $kg^{=1}$). Values represent mean ± SD (n=3), with the different letters indicating significant differences ($p \leq 0.05$) according to one-way ANOVA. Asterisks (*) indicate significant differences between soil conditions at a particular concentration level, according to t-test.

Cerium Accumulation

Figure 3-7 shows total Ce levels in plant tissues obtained through the strong acid digestion of the dry soybean tissues. Total Ce associated with roots was impacted significantly by dosing concentrations, increasing in accumulation as the dosing concentration increased. There were no significant differences in the roots in Ce accumulation as a result of soil sterilization. For Ce accumulation in shoots, neither soil treatment nor concentration levels made a significant difference. Although the average

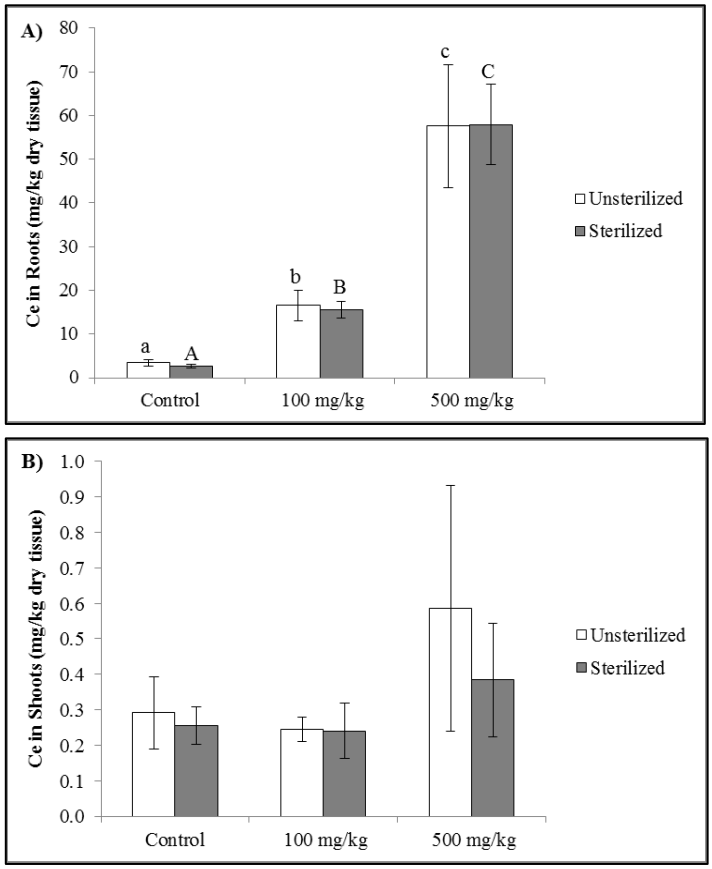

Fig.3-7 Total cerium accumulation. Accumulation levels within root tissue (A) and shoot tissue (B) of soybean plants. Soybeans were grown in two soil conditions (unsterilized and sterilized) and exposed to three different concentration levels (0,100 mg kg^{-1}soil, or 500 mg kg^{-1}). Values represent mean ± SD (n=3), with the different letters indicating significant differences ($p \leq 0.05$) according to one-way ANOVA. Asterisks (*) indicate significant differences between soil conditions at a particular concentration level, according to t-test.

value of accumulation for the shoots was higher at the 500 mg/kg level compared to the other two concentrations, this was not statistically significant. A two-way ANOVA indicated that there were no interactions between soil conditions and concentration levels.

Figure 3-8 and Figure 3-9 show the results obtained through the Ce speciation analysis using the enzyme digestion method. Total cerium was analyzed in soybean samples from the control and 500 mg/kg dosing groups. Figure 3-8 show the total Ce in shoots. No dissolved Ce was found in the shoots. Ce accumulation in the shoots was significantly higher in the 500 mg/kg compared to the control group for both soil conditions. Soil conditions were significantly different at the 500 mg/kg concentration level, with the unsterilized group being 54% lower than the sterilized soil group.

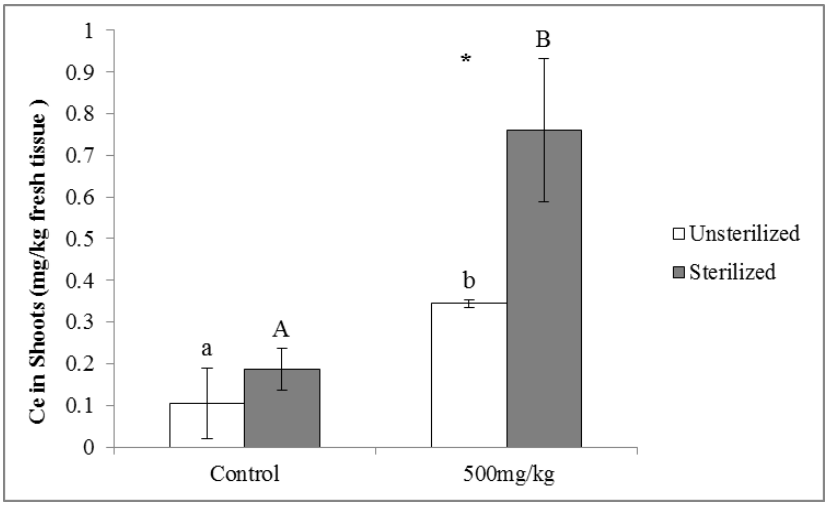

Fig. 3-8 Cerium accumulation in shoot tissue. Soybeans were grown in two soil conditions (unsterilized and sterilized) and analyzed at two different concentration levels (0 and 500 mg kg^{-1}). Values represent mean ± SD (n=3), with the different letters indicating significant differences ($p \leq 0.05$) according t-tests between concentration levels. Asterisks (*) indicate significant differences between soil conditions at a particular concentration level, according to t-test.

Figure 3-9 displays the total Ce in root tissues, dissolved Ce in root tissues, and total Ce found on root surface. No dissolved Ce was found on the root surface. The total Ce in the root significantly increased between the control and 500 mg/kg concentration level, for both soil conditions. There was a significant difference between the unsterilized and sterilized soils at the 500 mg/kg concentration level, with the sterilized group being 45% lower than the unsterilized group. On the root surface Ce was only seen at the 500 mg/kg concentration level. The difference in soil conditions was

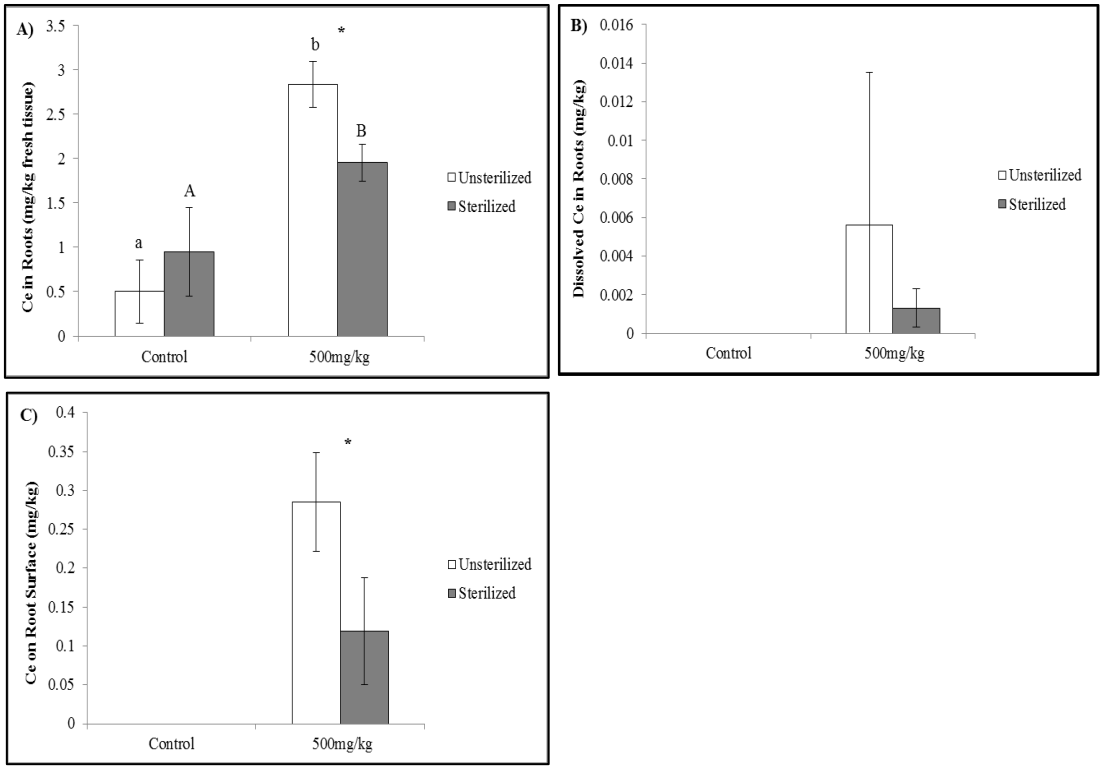

Fig. 3-9 Cerium speciation associated with root tissues. (A) Total cerium accumulation associated with root tissues ,(B) dissolved cerium content in root tissue, (C) cerium accumulation on root surface of soybean plants exposed to CeO_2 NPs. Soybeans were grown in two soil conditions (unsterilized and sterilized) and analyzed at two different concentration levels (0 and 500 mg kg^{-1}). Values represent mean ± SD (n=3), with the different letters indicating significant differences ($p \leq 0.05$) according t-tests between concentration levels. Asterisks (*) indicate significant differences between soil conditions at a particular concentration level, according to t-test.

significant, with there being 85% less Ce found on the root surface for plants in the sterilized soil group than in the unsterilized group. Dissolved cerium was found in the roots at the 500 mg/kg concentration level, but due to the high standard deviation of measurements, significance between soil conditions at this level could not be determined.

Summary of Results

A majority of the measurements indicated that the soil microbial community clearly impacted how the soybeans responded to CeO_2 NPs treatment. By compiling the data and results from the various tested parameters, four key conclusions were derived:

The first key observation was the impacts to the photosynthetic related parameters. By the third week of measurement, fluorescence, photosynthesis, and stomatal conductance were significantly impacted due to the independent effects of concentration and soil condition; as well as a significant impact due to the interaction of those two factors. Chlorophyll *a* and *b* were not measurably affected by the independent factors, but when those factors interacted, significant impacts were seen. At the highest concentration of CeO_2 NPs used (500 mg/kg), a clear trend could be seen, with each testing parameter being negatively impacted significantly more in the sterilized treatments than in the unsterilized treatments. All four of these factors are indicative of plant stress and related to the photosynthetic processes necessary for plant health. This indicates that the impact of CeO_2 NPs to a plants photosynthetic system processes are

directly linked to the existing soil microbial community, and if this community makeup changes so do the relative impacts to physiological parameters.

The second key conclusion was how often a measurement would show opposing effects across concentration levels depending on whether the soil was initially sterilized or not. Chlorophyll *a* levels dropped between control and the highest dosing in the sterilized group, while in the unsterilized group, levels increased between control and the 500 mg/kg concentration level. This was also seen in photosynthesis rate measurements, where on day 25 a peak was seen in the sterilized 100 mg/kg concentration level, but this was the lowest point for the unsterilized soil group. This showcases how the interaction effects between soil condition and concentration levels can impact the results of a particular measurement parameter. For example, when the soybeans were exposed to CeO_2 NPs, their root biomass decreased for sterilized conditions compared to control samples, but increased for samples in the unsterilized group. This was due to interaction effects, indicating how truly significant a role that a microbial community can have on the way CeO_2 NPs impact a plant system.

The third key impact seen in this experiment was the nodulation rates of the soybeans. The nodule count was an unexpected finding of this experiment. There was such a drastic increase in nodule production across concentration levels, signifying that the presence of CeO_2 NPs caused the soybeans to act in a manner as though there was a significant lack of nitrogen in the soil as compared to the control groups. This could indicate that the nanoparticles could be interfering with the nitrogen cycle taking place in the soil, which could have a variety of impacts to an agricultural system. Some research

has been conducted on the ability of reduced ceria to cause dissociation of nitrite (NO_2^-) (Nolan et al., 2006) and also it is commonly used as a catalyst due to its ability to reduce NO to N_2 (Sajith et al., 2009). Plants get the majority of their nitrogen through the form of nitrates (NO_3) and also as ammonium ions. NO_3 is produced through conversion of NO_2 by nitrifying bacteria, so it is possible that two things could be occurring. That the cerium could be affecting the nitrifying bacteria, therefore impacting their ability to produce nitrates in the soil, or directly reacting with the nitrite, limiting the amount available for the nitrification process. In a previous study using soybeans and CeO_2 NPs (concentrations at 0, 0.1, 0.5 and 1 g/kg), the soybean plants produced consistent nodulation (mean = 39 ± 3) per each plant (Priester et al., 2012). For that study the plants were grown continuously through the seed production stage, whereas in this experiment the soybeans were grown for only 27 days. It showed a decrease in nitrogen fixation at an 80% rate for the medium and high levels of cerium exposure, indicating that the nodules present were influenced by the NPs and could not fix nitrogen as effectively as the other groups. The control in this study showed no indication of nodulation and did not see drastic increases until the 500 mg/kg exposure level. A possible conclusion for this would be that the soil used for this experiment supplied enough nitrogen for the plant to exist for those 27 days, but when cerium came into play the plant was put under a nitrogen stress (through possible interference with the nitrogen cycle). But could the nodules produced be effective in nitrogen fixing? Although they indicated they were actively fixing nitrogen due to the reddish color, it is possible that they were not at maximum efficiency. This could relate to how in the sterilized group, where there were

increases in nodulation compared to the unsterilized group, the photosynthetic processes seemed to decrease in this sterilized group at higher concentration levels. Considering how nitrogen content is directly related to photosynthetic capacity (Evans, 1989), this decrease in nitrogen fixation may result in less efficient processes.

Lastly, the fourth key conclusion seen was in regards to the accumulation and transport of cerium within the soybean tissues. Similar to previous studies, higher concentration led to increased accumulation of cerium in soybean tissues (Cao et al., 2017). The Ce speciation analysis was able to show distinct impacts in total accumulation due to whether the soil was sterilized or not, leading to the conclusion that key processes and makeup of the rhizosphere directly interacts with the transport of cerium into soybean tissues. The higher accumulation of cerium into the shoots in the sterilized group, as shown in the fresh tissue analysis, could be connected to the decrease in the photosynthetic physiological parameters for those in the sterilized soil groups. This analysis only showed dissolved cerium within the soybean roots and none on the surface of the roots. Indicating that the transformation did not take place in the rhizosphere, but rather within the roots themselves. Unless the remaining dissolved ions on the roots surface were too low to gauge accurately. A previous study also showed dissolved cerium in the roots of radish, but the presence of dissolved ions on the root surface led to the conclusion that the dissolution was most likely occurring on the root surface (Zhang et al., 2017).

The results clearly show that CeO_2 NPs have an impact on various physiological parameters within soybeans. In addition to this, the impact created by these nanoparticles

is directly dependent on the composition of microbial community within the rhizosphere. These conclusions were able to assist in shedding light on the above- mentioned objectives. The initial microbial community influenced how the CeO_2 NPs were able to impact the photosynthetic related processes, nodulation, biomass, and cerium accumulation in soybeans. Two-way ANOVA results verify the interaction at a statistically significant level for many of the parameters. From previous studies it was shown that cerium can transform and it was assumed to do so in the rhizosphere. The results of this study only show transformation into a dissolved state occurring within the roots, since no dissolved cerium was found on the root surface. This study gives greater insight though into how accumulation and transport of the cerium relies on the composition of the rhizosphere, seeing significant differences in total cerium in the different soil conditions.

CHAPTER IV

INTERACTION OF GRAPHENE OXIDE NANOPARTICLES WITH MICROBIAL SOIL COMMUNITY, AND ITS IMPACT ON SOYBEAN (*Glycine max* (L.) Merr.)

Introduction

The production of engineered nanoparticles (ENPs) has been growing exponentially, creating a $3 trillion global market related to this technology (Xia et al., 2008). This growth in production rates has also led to increased release of ENPs to the environment. One particular ENP of interest is the carbon-based graphene oxide nanoparticle (GO NP). Increased attention has been focused on graphene-based nanomaterials due to their potential in electronic, energy, medical, and environmental applications (Chowdhury et al., 2015). Graphene oxide is a thin plane of atomically single graphite, with carboxylic groups at its edges and a basal plane made of phenol hydroxyl and epoxide groups (Liu et al., 2011).

Many studies show the impacts that ENPs have on agricultural plants, with one such showing that at exposure levels of 40µg/mL GO was able to penetrate vacuole and deposit in roots tips, causing a reduction in the biomass production (Zhang M. et al., 2015). It has also been reported that GO has shown signs of antibacterial properties, causing physical cell membrane damage due to the sharp edges of its structural makeup (Liu et al., 2011). Compared to other ENPs, there has been limited research conducted on the impacts of GO NPs to agricultural crops and especially limited insight in how these particles interact in the soil of the rhizosphere.

The rhizosphere contains exudates that attract microbial communities and influences the health and composition of the soil itself. Plant roots can secrete a wide variety of compounds into the rhizosphere and can dramatically change their environment through the exudates produced from their roots through the release of carbon compounds (Haichar et al., 2014). This carbon and nutrient rich region creates the ideal environment for the proliferation of microbial production. In addition to this, these exudates perform a variety of important roles, such as protecting against pathogens or releasing chemical signals to the surrounding soil environment (van Dam et al., 2016). If GO NPs are able to either impact plants or the exudates they produce, or influence the microbial community in the soil, it is important to understand the mechanisms behind such potential influences.

This experiment will assess how GO NPs can impact plant health at various concentrations. As well as determine whether these potential impacts can be influenced by the microbial community that is found in the rhizosphere. The importance of this study will be able to showcase how this carbon based NP could potentially interfere with the plant-microbe interactions that are key to soil health.

Materials and Methods

The methodology for this GO exposure assessment is identical to that of Chapter 3, except for two primary differences. The nanoparticles used differed and there was no ICP-MS accumulation measurements, since this nanoparticle was carbon based. Due to the similarity in methodology, the procedures will lack some of the detail found in the previous chapter.

Graphene Oxide Nanoparticles

Graphene oxide nanoparticles (GO NPs) were obtained as a powder through connection with Indranil Chowdhury at the National Exposure Research Laboratory. These particles were synthesized using a modified Hummers method. This involved treating natural graphite flakes (3061 grade material from Asbury Graphite Mills) with sulfuric acid and various oxidizing agents. Residual contaminants were then removed by filtering, washing, and centrifuging the treated graphite. This procedure can be found in greater detail in the Section 2.1 Supporting Information of Chowdhury et al. (2013).

Soil Preparation

Scotts Topsoil was placed in small potting containers with a total mass of 150 grams. After being dried, saturation levels and water capacity was determined. Soil was prepared in two conditions: sterilized and unsterilized. The soil was sterilized in an autoclave (Panasonic MLS – 3781L) to eradicate any initial microbial community. The soil was not kept in a state of sterilization throughout the experiment; it is assumed that

bacteria from the air and water would reenter the soil and establish a new microbial community.

Soybean Preparation

The soybeans (*Glycine max* (L.) Merr.) were sterilized using 1.25% sodium hypochlorite dispersion for 10 minutes (Zhang et al., 2014). The seeds were germinated by placement into separate containers with sterilized and unsterilized saturated soil of a 2 inch depth. After 4 days they were transplanted into potting containers containing the 150 grams of topsoil. Before seeds were transplanted into the soil, a dispersion of deionized water and GO NPs were added to the soil at concentration levels of 0 mg/kg (control), 100 mg GO/kg of dry soil, and 500 mg GO/kg of dry soil. Amount of water needed was determined to be 120 mL per replicate in order to maintain the previously determined level of saturation. There were 5 replicates per treatment, producing of total of 30 soybean seedlings. They were placed under UV lighting, kept at room temperature, and given deionized water to maintain saturation.

Photosynthesis Rate, Stomatal Conductance, & Chlorophyll Fluorescence Analysis

Photosynthesis rate, stomatal conductance, and chlorophyll fluorescence were measured to assess key physiological impacts. Photosynthesis rate and stomatal conductance were measured via use of a Licor-6400XT (Lincoln, NE). Chlorophyll fluorescence was tested through use of a continuous excitation chlorophyll fluorescence

analyzer (OS1p, Opti-Sciences, Hudson, NH). Measurements took place at day 10 and 23 of GO exposure.

Plant Harvest

After 25 days of growth, the plants were pulled from the soil gently and rinsed with deionized water to remove soil particles. The roots were separated from the shoots and weighed separately to obtain fresh weight. Roots were inspected for nodules and counted if found. Some fresh leaves were collected and then used for chlorophyll analysis.

Chlorophyll Content Analysis

Procedures used for chlorophyll content analysis were based off the methods of Moran (1982). Fresh leaf tissues in the amount of 50 mg were weighed from each replicate, placed in a centrifuge tube with 12 mL of dimethyl formamide (DMF), vortexed, and then kept in dark conditions for 24 hours. After 24 hours, a chlorophyll analysis was then conducted using a UV-Vis spectrophotometer (model Lambda 35; Perkin Elmer). These measurements were then used to calculate chlorophyll *a* and *b* values.

Statistical Analysis

Minitab was used to perform t-tests, one-way analysis of variance (ANOVA), and two-way ANOVA on the data obtained. Two-way ANOVAs were used to test the interaction of the independent factors (concentration and soil condition) on the

dependent factor (any parameter tested). A one-way ANOVA was used to determine statistical differences exhibited by the concentration level (0, 100 mg/kg, and 500 mg/kg) on each parameter tested. A t-test was used to determine significance the two soil conditions (unsterilized and sterilized) at a particular concentration level. Values were considered significant if $p \leq 0.05$.

Results and Discussion

Fresh Biomass

In Figure 4-1, roots showed that at each concentration level the unsterilized soybean group had a higher average biomass, this was not significant though. The shoot biomass levels were higher in sterilized plants per each concentration group, also not significant. The two-way ANOVA indicated that the two factors (soil condition and concentration levels) did not interact. Biomass was measured to determine whether the

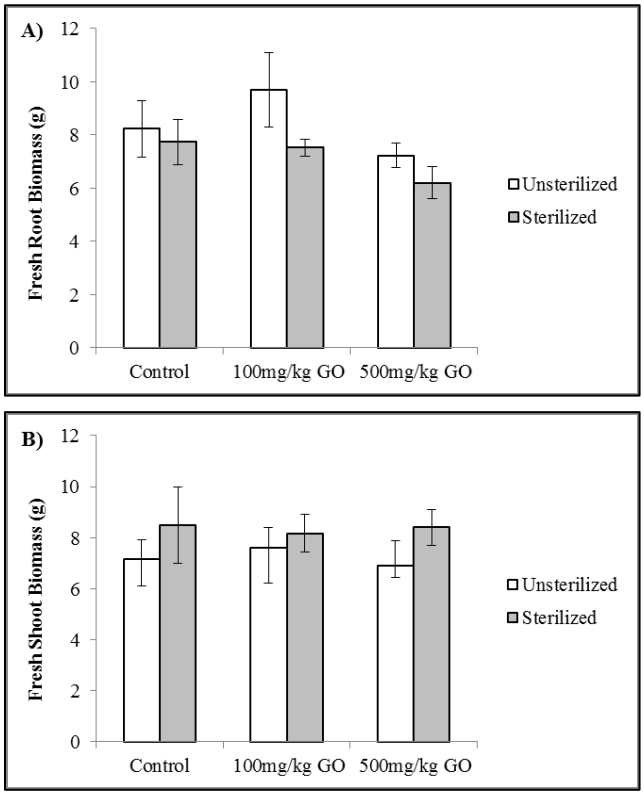

Fig.4-1 Fresh biomass levels after GO NP exposure. Root biomass (A) and shoot biomass (B) of soybean plants exposed to GO NPs. Soybeans were grown in two soil conditions (unsterilized and sterilized) and exposed to three different concentration levels (0,100 mg kg^{-1}soil, or 500 mg kg^{-1}). Values represent mean ± SD (n=3), with the different letters indicating significant differences ($p \leq 0.05$) according to one-way ANOVA. Asterisks (*) indicate significant differences between soil conditions at a particular concentration level, according to t-test.

factors tested (soil condition and nanoparticle concentration) enhanced or decreased plant tissue yield. No statistically significant differences were found between concentration levels or soil conditions, indicating that GO NPs do not have significant impacts to biomass production in soybeans.

Nodule Counts

No nodules were found in the samples at any concentration level.

Chlorophyll Content

Two types of chlorophyll were measured: chlorophyll *a* and chlorophyll *b*. These results can be seen in Figure 4-2. Both chlorophyll *a* and *b* levels were found to be significantly different across concentration levels in the unsterilized soil group, with the 500 mg/kg concentration being significantly higher than the other two concentration levels. For chlorophyll *a*, the 500 mg/kg group was 170% higher than the 100mg/kg group and 65% higher than the control group. For chlorophyll *b* content, the 500mg/kg group was 117% higher than the 100 mg/kg group and 55% higher than the control group. No significant differences were found amongst concentration levels for the sterilized soil groups.

The chlorophyll *b* content analysis also showed that, according to the t-tests, there were significant differences between the two soil conditions at both the control and 100 mg/kg concentration levels. In the control group, for chlorophyll *b*, the sterilized soil group had a 35% higher chlorophyll *b* content level than the unsterilized soil group. In the 100mg/kg group, the sterilized group was 42% higher than the unsterilized soil

group. According to the two-way ANOVA analysis, both the chlorophyll *a* and *b* content levels were impacted by soil condition and concentration levels. Although both these factors independently affected the content levels, the factors did not interact.

Chlorophyll is used during the photosynthetic process and can help provide insight into how this process is functioning under the imposed conditions. The results show that both factors (soil condition and concentration level) are in some way impacting chlorophyll content.

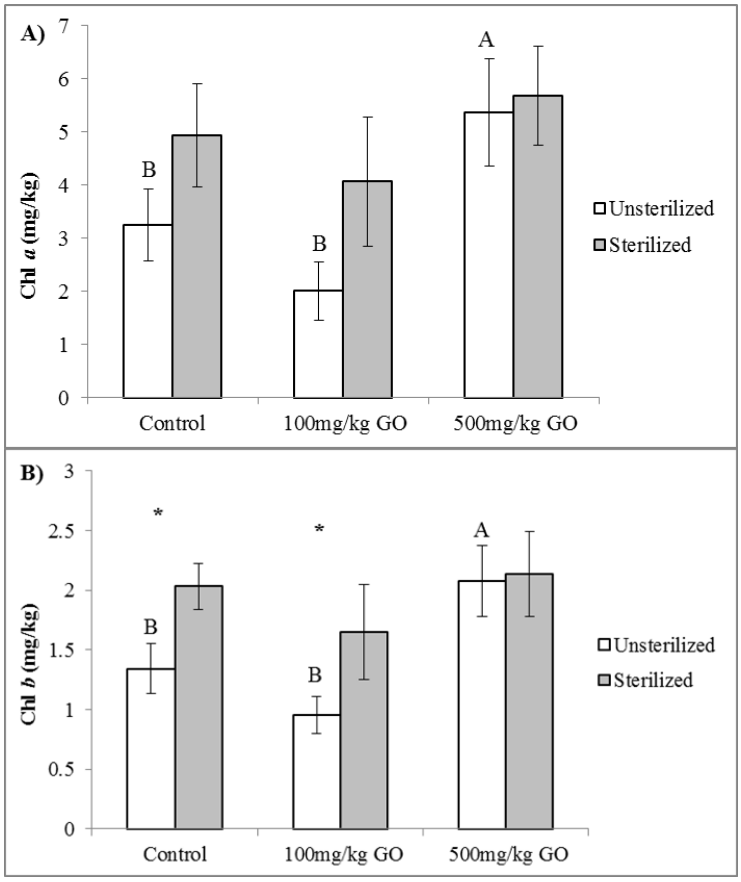

Fig.4-2 Chlorophyll content after GO NP exposure. Chlorophyll *a* (A) and chlorophyll *b* (B) content of soybean plants exposed to GO NPs. Soybeans were grown in two soil conditions (unsterilized and sterilized) and exposed to three different concentration levels (0,100 mg $kg^{=1}$soil, or 500 mg $kg^{=1}$). Values represent mean ± SD (n=3), with the different letters indicating significant differences ($p \leq 0.05$) according to one-way ANOVA. Asterisks (*) indicate significant differences between soil conditions at a particular concentration level, according to t-test.

Photosynthesis Rate

Two measurements were taken for this parameter on day 10 and day 23 (Figure 4-3). The only significant difference found was on the day 10 measurement. A t-test indicated that at the 100 mg/kg concentration level the two soil conditions had significantly different photosynthesis rates. The sterilized soil group showed a 30% higher rate than the unsterilized group. One-way ANOVAs indicated no significant differences across concentration levels on both days. Although not significant due to the

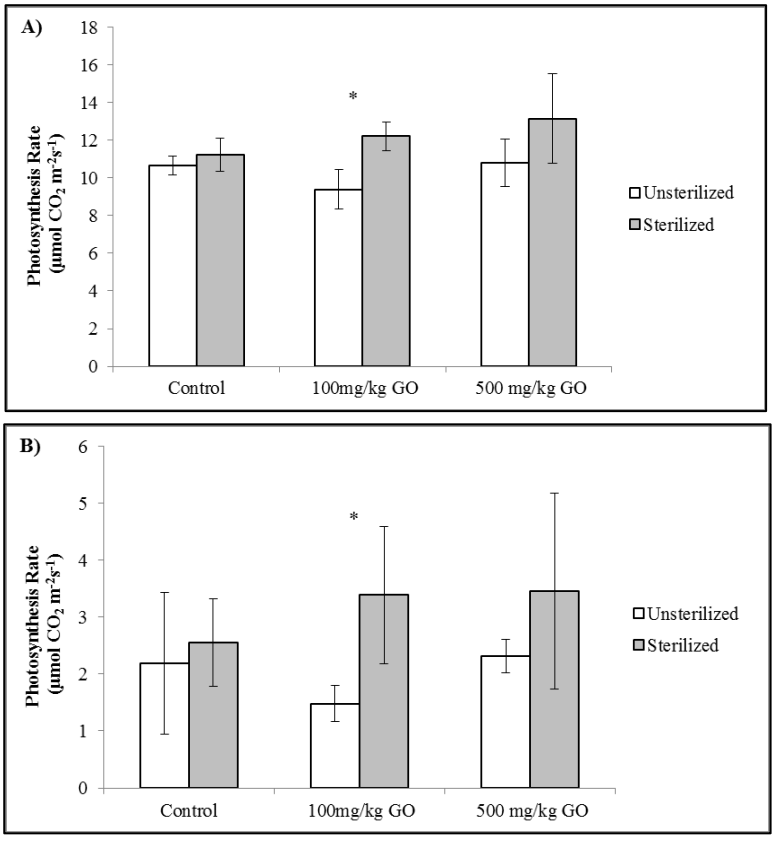

Fig.4-3 Photosynthesis rates after GO NP exposure. Net Photosynthesis rate of soybean plants exposed to GO NPs at day 10 (A) and on day 23 (B) of plant growth. Soybeans were grown in two soil conditions (unsterilized and sterilized) and exposed to three different concentration levels (0,100 mg $kg^{=1}$soil, or 500 mg $kg^{=1}$). Values represent mean ± SD (n=3), with the different letters indicating significant differences ($p \leq 0.05$) according to one-way ANOVA. Asterisks (*) indicate significant differences between soil conditions at a particular concentration level, according to t-test.

large standard deviations seen among the measurements, both days showed that average rate values were higher in sterilized plants at all concentration levels. In the two-way ANOVA analysis, photosynthesis rates were only affected by soil type and not concentration levels. Photosynthesis is the process plants use to convert CO_2 and light energy into carbohydrates. The ability of a plant to effectively produce carbohydrates can give insight into the health of the system, which is key to understanding the impacts the ENPs can have on plants. Results show that the soybeans do not appear to be stressed in a manner that is impacted their ability to photosynthesize.

Stomatal Conductance

Measurements were taken twice during the growing process, on day 10 and day 23 (Figure 4-4). In week one the unsterilized soil group at the 500 mg/kg concentration showed significantly higher values compared to the other unsterilized plants, with a 56% increase from control to the 500 mg/kg level. There were no significant measurements during week two, but averages were higher in all sterilized plants for this week. The two-way ANOVA indicated that there were no interaction effects between soil conditions and concentration levels.

Stomatal conductance is the rate at which CO_2 enters or water vapor exits stomata on the leaf surface. This measurement relates directly to the effectiveness of the photosynthetic process. Early during the growing process it appeared that concentration level impacted this particular photosynthesis related process, but as time passed no

significant impacts were seen. Indicating that GO NPs do not produce significant influences on stomatal conductance, similar to the results seen with photosynthesis rates.

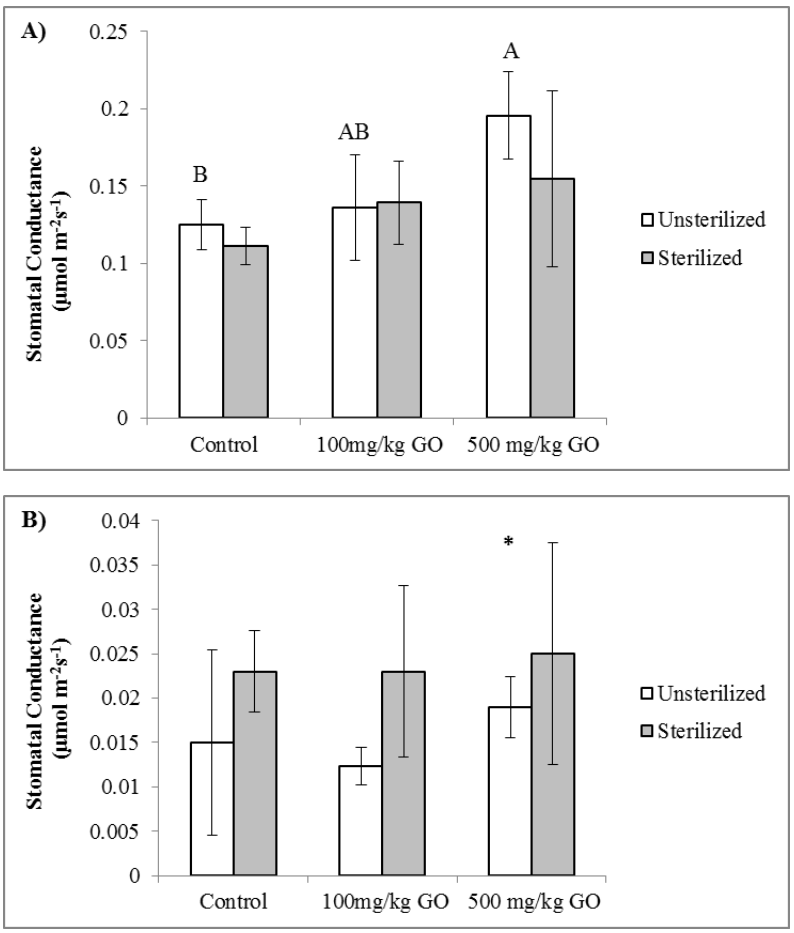

Fig.4-4 Stomatal conductance after GO NP exposure. Stomatal conductance of soybean plants exposed to GO NPs at day 10 (A) and day 23 (B) of plant growth. Soybeans were grown in two soil conditions (unsterilized and sterilized) and exposed to three different concentration levels (0,100 mg $kg^{=1}$soil, or 500 mg $kg^{=1}$). Values represent mean ± SD (n=3), with the different letters indicating significant differences ($p \leq 0.05$) according to one-way ANOVA. Asterisks (*) indicate significant differences between soil conditions at a particular concentration level, according to t-test.

Chlorophyll Fluorescence

As seen in Figure 4-5, two measurements were taken for chlorophyll fluorescence, on day 10 and day 23. No significant differences were found among concentration levels or soil conditions. Two-way ANOVA also showed no significant impacts. By measuring chlorophyll fluorescence, insight can be gained into photosynthetic performance of plants and their ability to handle environmental stresses (Maxwell et al., 2000). These results indicate that no stress was put on the soybeans due to soil condition or GO concentration exposure levels.

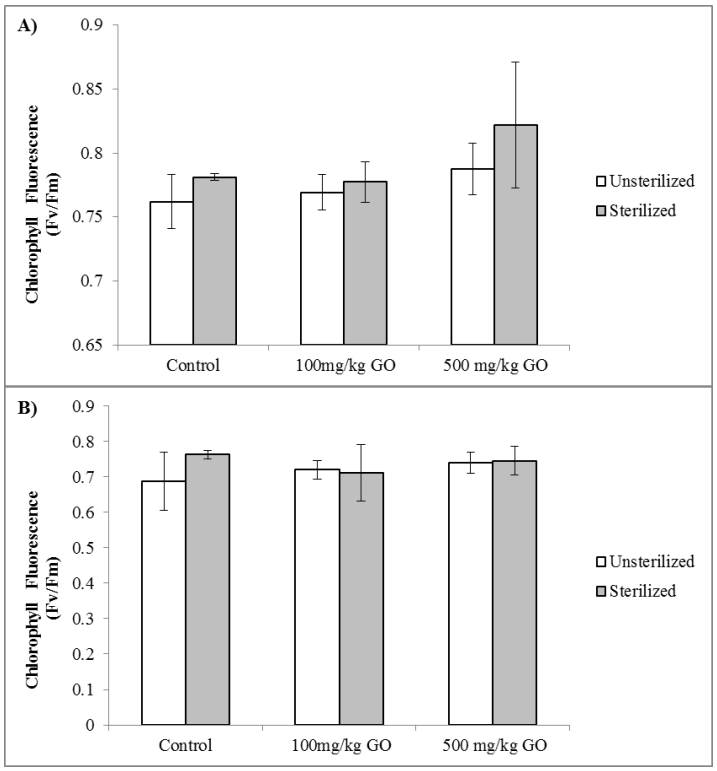

Fig.4-5 Chlorophyll fluorescence after GO exposure. Chlorophyll fluorescence (Fv/Fm) of soybean plants exposed to GO NPs at day 10 (A) and on day 23 (B) of plant growth. Soybeans were grown in two soil conditions (unsterilized and sterilized) and exposed to three different concentration levels (0,100 mg kg^{-1}soil, or 500 mg kg^{-1}). Values represent mean ± SD (n=3), with the different letters indicating significant differences ($p \leq 0.05$) according to one-way ANOVA. Asterisks (*) indicate significant differences between soil conditions at a particular concentration level, according to t-test.

Summary of Results

The two-way ANOVA determined that the sterilization of the soil did not interact with the GO NPs. Microbial community may have impacted the results independently, but they did not impact the ability of the GO NPs to have a more or less significant impact to the parameters. Photosynthetic processes were not drastically impacted, except for in the chlorophyll content analysis. Most values appeared to be slightly higher in the sterilized soil groups, but none of these were significant.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

Due to the important role that the rhizosphere microbial community has on plant and soil health, ENPs could pose a threat to this important relationship and was studied. Two experiments were conducted in an attempt to assess the interactions that ENPs may have with the rhizosphere microbial community. The first experiment used CeO_2 NPs, which are metal based and have been shown to dissolute more readily in the rhizosphere environment. The second experiment used GO NPs, which are carbon-based and typically more stable.

CeO_2 NPs Experiment Overview

Table 5-1 summarizes how, according to a two-way ANOVA analysis, each factor (soil condition and concentration level) impacted the measurement parameters in the experiment with CeO_2 NPs. Photosynthesis rates, stomatal conductance, chlorophyll fluorescence, chlorophyll content, and root biomass showed interaction effects between the two factors. This gives strong evidence that rhizosphere microbial communities can significantly impact how the CeO_2 NPs impact photosynthetic related processes. The measurement parameters were also independently influenced by the two factors. The nodulation impacts seen gave indications that CeO_2 NPs were somehow interacting with the nitrogen cycle in the soil or the ability of the soybeans to uptake nitrogen.

For this investigation, not only was the goal to assess interaction effects, but also to gain further insight into how this may affect the transformation and accumulation of

CeO_2 NPs in the soybean tissue. Results show that cerium was able to accumulate in the roots and shoots of the soybeans. Dissolved cerium was not found on the root surface, but in the root tissue indicating that ions were not being produced at a measurable amount on the root surface. Although interaction effects were not significant, both soil conditions and concentration levels affected accumulation levels.

This study provides evidence that CeO_2 NPs interact significantly with the rhizosphere environment and could therefore potentially change this environment through its relationship with the rhizosphere and plant.

Table 5-1. Two-way ANOVA results for CeO_2 NP experiment. The X indicates significance. Each parameter could be independently affected by soil conditions or concentration levels, and then also has significant interaction effects between the two independent factors. Significant differences at $p \leq 0.05$.

Measurement	Soil Condition	Concentration	Interaction
Photosynthesis (Day 11)	-	X	X
Photosynthesis (Day 18)	-	-	-
Photosynthesis (Day 25)	X	X	X
Conductance (Day 11)	-	X	X
Conductance (Day 18)	-	X	-
Conductance (Day 25)	X	X	X
Fluorescence (Day 11)	-	X	-
Fluorescence (Day 18)	X	X	-
Fluorescence (Day 25)	X	X	X
Chlorophyll *a*	-	-	X
Chlorophyll *b*	-	-	X
Shoot Biomass	-	-	-
Root Biomass	X	-	X
Nodule Count	X	X	-
Total Ce in Dry Shoots	-	-	-
Total Ce in Dry Roots	-	X	-

GO NPs Experiment Overview

Table 5-2 shows the two-way ANOVA results for the experiment with GO and soybeans. No key interaction effects were seen through the exposure of GO to soybeans. Although each factor, soil condition and concentration, independently had impacts, the results indicate that the rhizosphere community did not significantly influence the ability of GO to impact the parameters tested. Concentration levels were able to have the greatest impact in chlorophyll content and root biomass. This experiment does not provide strong evidence that GO NPs have significant interactions with the rhizosphere microbial community.

Table 5-2. Two-way ANOVA results for GO NP experiment. The X indicates significance. Each parameter could be independently affected by soil conditions or concentration levels, and then also has significant interaction effects between the two independent factors. Significant differences at $p \leq 0.05$.

Measurement	Soil Condition	Concentration	Interaction
Photosynthesis (Day 10)	X	-	-
Photosynthesis (Day 23)	X	-	-
Conductance (Day 10)	-	X	-
Conductance (Day 23)	-	-	-
Fluorescence (Day 10)	-	-	-
Fluorescence (Day 23)	-	-	-
Chlorophyll *a*	X	X	-
Chlorophyll *b*	X	X	-
Shoot Biomass	X	-	-
Root Biomass	X	X	-

Recommendations

Further work needs to be done to elicit the interactions that were seen, particularly for CeO_2 NPs. These particles are clearly interacting with the rhizosphere community. More research should be conducted that can assess what potential aspects this particle is interacting with. Is it reacting with specific bacteria or perhaps the exudates produced by the plants? How could this then change the microbial community? The effects of cerium exposure on nodulation pose an interesting find. This should be further investigated to assess whether these particles could have negative implications for nitrogen levels in the soil, this is imperative due to how important nitrogen cycles are for plant health. Specifically, further study questions address whether the cerium is interacting with the various nitrogen compounds, or if it is due to interaction with nitrifying bacteria. The GO experiment did not provide substantial evidence for interaction, but at different concentration levels and soil type, different results could potentially be obtained.

Each study should be further expanded with adjustments to soil type, rhizosphere composition, and concentration levels, due to how important these are in plant health and the impact ENPs have due to these factors. In addition, some molecular techniques may be applied to evaluate the microbial community shift in plant rhizosphere due to ENP exposure. These experiments showcase how the rhizosphere and potentially even the surrounding bulk soil play a key role in how ENPs influence plant crops. Some regions of the world may have soil conditions that are much more sensitive to certain ENP

contamination, and if applied to these particular soils, the impacts could be much more detrimental to overall soil health and an agricultural economy.

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