IMPACTS OF SILVER NANOPARTICLES ON BACTERIAL SPECIES *B. SUBTILIS* AND *E. COLI* AND THE MAJOR CROP PLANT *Z. MAYS*

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Plant and Soil Sciences

Summer 2014

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ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my advisor, Dr. Yan Jin for her unlimited encouragement and mentoring. Without her guidance this thesis would not have been possible. I would also like to thank Dr. Harsh Bais, who provided me with an excellent foundation in rhizosphere ecology and plant-microbe interactions. Additionally, Dr. Chin-Pao Huang and Dr. Angelia Seyfferth have been wonderful committee members, offering their continued guidance and support. Special thanks go to William Bartz, Caroline Golt, Shannon Modla, Deborah Powell, Jean Ross, Jerry Pourier, and Teclemariam Weldekidan for their assistance.

I am also thankful to the many post-doctoral researchers and fellow graduate students with whom I worked, including Venkatachalam Lakshmanan, Olga Lazouskaya, Gang Li, Amanda Roberson, Rafael Saldana, Gopinath Selvaraj, Carla Spence, Taozhu Sun, Gang Wang, Jing Yan, and Wenjuan Zheng. I am extremely grateful to have shared the laboratory with such bright and talented scientists.

I would also like to thank my parents and brother for their unconditional support and encouragement.

Finally, I would like to thank my fiancée, Jacqueline O'Neill. She has been a constant source of compassion, encouragement, and reassurance throughout this process, and I am forever grateful for her love.

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ABSTRACT

This thesis examines the impacts of citrate-coated silver nanoparticles (c-AgNPs) on two species of bacteria (*Bacillus subtilis* and *Escherichia coli*), the major crop plant *Zea mays*, and the beneficial plant-microbe relationship between *Z. mays* and *B. subtilis*. AgNPs are an increasing component of antimicrobial consumer, industrial, and military products. This has led to widespread scientific concern for the ecological safety outside their intended use. An overview of their history, use, and toxicity was used to inform the design of experiments and resulting data. Growth inhibition and sub-lethal toxic effects were used to assess the effects of c-AgNP exposure to bacteria. Similar analytical methods were used to quantify the response of *Z. mays* to c-AgNP exposure. Results showed that exposure to c-AgNP significantly reduced the growth of bacterial populations and alters their growth kinetics. *Z. mays* experienced significant sub-lethal effects due to exposure, including reduced root length and biomass, and hyper-accumulated Ag in root tissues. Beneficial interactions between *B. subtilis* and *Z. mays* were reduced as both species suffered sub-lethal effects of exposure to c-AgNPs.

Chapter 1

INTRODUCTION

1.1 Introduction to Silver Nanoparticles

Silver nanoparticles (AgNPs), defined as engineered individual silver particles or small aggregates of silver particles measuring no more than 100 nm in any dimension (USEPA, 2007), are used in a variety of applications, including commercial antimicrobial products such as bandages and socks and other textiles, as well as various military and industrial products (Morones et al., 2005). In fact, AgNPs are one of the most commonly used nanomaterials in consumer products (Fabrega et al., 2011; El-Temsah and Joner, 2010; Klaine et al., 2008). The increasing use of AgNPs in such applications has greatly increased environmental risk of exposure (Gottschalk et al., 2009; Mueller and Nowack, 2008; Nowack and Bucheli, 2007; USEPA, 2012) and associated scientific concern.

Numerous studies on the toxicity of AgNPs to aquatic organisms (Wijnhoven et al., 2009; Lee et al., 2012b; Morones et al., 2005) and soil-dwelling nematodes (Kim et al., 2012; Yang et al., 2012; Meyer et al., 2010; Roh et al., 2009;) have been published, and the influence of AgNPs on bacteria (Suresh et al., 2010; El Badawy et al., 2011) and a select number of plant species is becoming increasingly known (Geisler-Lee et al., 2013; Qian et al., 2013; Yin et al., 2011; El-Temsah and Joner, 2010). However, studies on the influence of AgNPs on specific plant-microbe interactions are lacking. Because plant-microbe interactions are ubiquitous in both natural and agricultural soils (Berg, 2009; Berg et al., 2005), it is critical to not only understand how each organism is impacted by exposure to AgNPs, but if and how those critical interactions between the organisms are altered.

1.2 Literature Review

In the following research, I integrate traditional toxicology with microbiology, plant developmental biology, and colloid science to investigate the influence of AgNPs on the growth and survival of two species of bacteria and the major crop plant *Zea mays*, while also determining if exposure to AgNPs limits plant-microbe interactions. I present an overview of the significant literature pertaining to AgNPs in the review below. Studies of specific relation to this research and the larger picture of AgNP use and safety are further highlighted.

1.2.1 Silver Nanoparticles

Silver has been used for nearly a century for its antimicrobial and biocidal properties (Nowack et al., 2011). Over the past 20 years, the military and industry have begun to make use of silver's antimicrobial properties by applying silver in nano-form to their products, such as field dressings, socks, and even washing machines and dishwashers (Ma et al., 2010; Klaine et al., 2008). Recent studies have shown that silver is the most widely used metallic nanoparticle in consumer products (Maynard and Michelson, 2014; Benn and Westerhoff, 2008; Klaine et al., 2008). Some debate over the safety and regulation of AgNPs has arisen recently, resulting in increased research and policy inquiries by the government and interest groups (Nowack et al., 2012; Stone et al., 2010; Gottschalk et al., 2009; Blaser et al., 2008). This research focuses on the scientific concern for AgNPs' ecological safety outside their intended use.

1.2.2 Antibacterial Activity

Presently, there are two major explanations for how AgNPs are toxic to bacteria – as well as other species such as algae, nematodes, and fish (Kittler et al., 2010; Meyer et al., 2010; Choi et al., 2008; Navarro et al., 2008). Several authors attribute toxicity to some nano-scale property or combination of such properties differing from bulk ionic silver (Sharma et al., 2014; Levard et al., 2012; Navarro et al., 2008) while others identify the release of ionic silver from AgNPs as the primary mechanism of toxicity (Kittler et al., 2010; Choi and Hu, 2009; Choi et al., 2008). Nano-scale properties that may differentiate toxicity from bulk species include increased specific surface area and reactivity or photocatalytics that alter how organisms, especially microorganisms such as bacteria, interact with the particles (Meyer et al., 2010). These properties are ultimately altered through environmental interactions based on particle behavior and environmental conditions (Fig. 1).

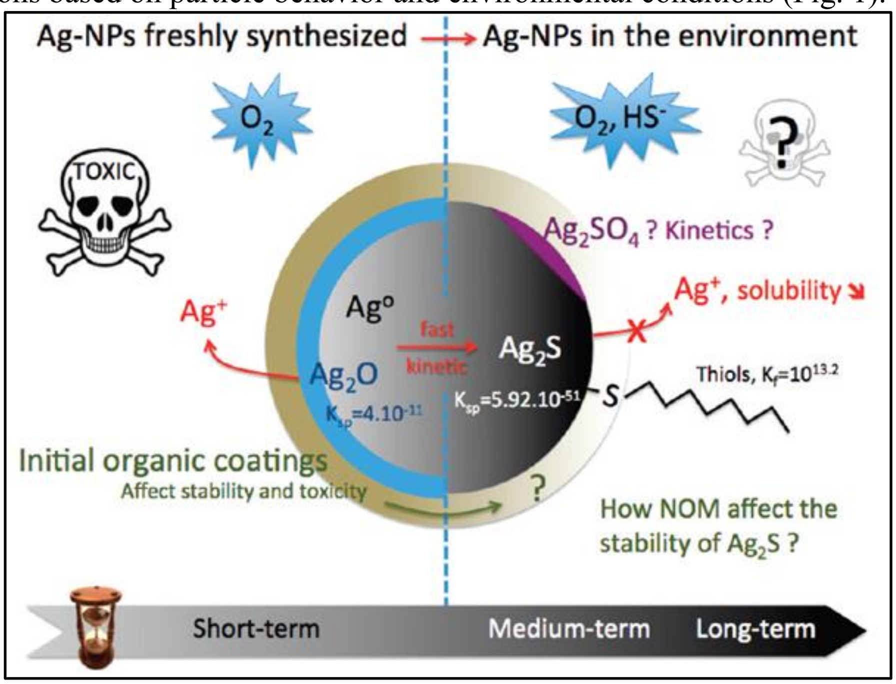

Figure 1: Current understandings and questions of environmental transformations of AgNPs (From Levard et al., 2012).

Particles in natural environments will quickly undergo transformation, including dissolution, transformation through redox reactions, and complexation with thiols and natural organic matter, and the rate at which transformations occur is strongly linked to the stability of the particle coating (Levard et al., 2012).

1.2.2.1 AgNP-Specific Toxicity

AgNP-related bacterial toxicity mechanisms are becoming increasingly understood, and possible mechanisms include attachment of particles or particle aggregates to cellular membranes. Such interaction may result in changes to membrane permeability and the cytosol redox cycle, accumulation of intracellular radicals, or disruption of ATP synthesis (Nel et al., 2009; Lok et al., 2006; Morones et al., 2005; Sondi and Salopek-Sondi, 2004). Several authors have demonstrated robust evidence for toxicity mechanisms directly related to AgNPs (El Badawy et al., 2011; Suresh et al., 2010; Choi and Hu, 2008). For example, Choi and Hu showed greater levels of inhibition by AgNPs than equal concentrations of ionic silver (2008). El Badawy et al. (2011) showed greater levels of toxicity due to treatment with surface-charged AgNPs, whereby cell-NP interactions resulted in the disruption of the organism's cellular membrane (Fig. 2) and ultimately cell death. Furthermore, Suresh et al. (2010) showed significant growth inhibition of *B. subtilis* and *E. coli* in AgNP solutions with less than 5% Ag^+ by mass. Such Ag^+ concentrations are more than 10x less than previously identified threshold concentrations for Ag^+ toxicity (Suresh et al., 2010; Li et al., 1997).

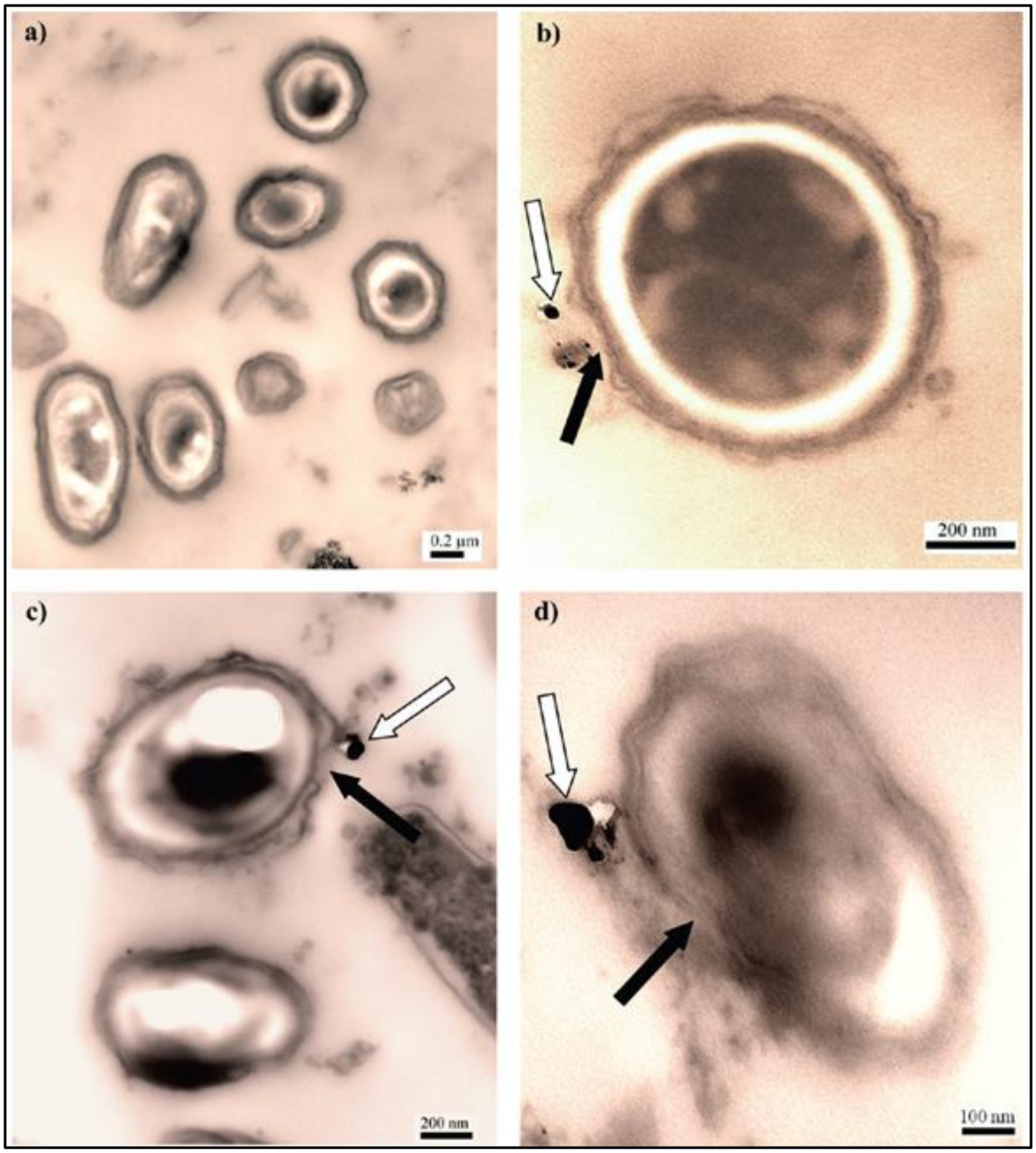

Figure 2: TEM micrographs showing impacts of AgNPs on cellular membranes of *B. subtilis*. A) Control cells. B-D) Cells exposed to AgNPs. White arrows refer to AgNPs and black arrows refer to the cellular membrane disruptions. (From El Badawy et al., 2011).

1.2.2.2 Ionic Ag-Related Toxicity

However, several studies have also shown toxicity related to the extended release of ionic silver. This popular viewpoint is partially justified by the historical use of Ag^+ antimicrobial agent (Maynard and Michelson, 2014) and even the recent incorporation of AgNPs into consumer products marketed as antimicrobial. The mechanism behind Ag^+ toxicity is well-studied; for bacteria, the positive charge of the ion promotes sorption onto the negatively charged cell wall, resulting in the deactivation of cellular enzymes, and the disruption of membrane permeability (Sambhy et al., 2006; Ratte, 1999). Additionally, uptake of Ag^+ has been shown to generate intracellular reaction oxygen species (ROS) leading to cell lysis and death (Ratte, 1999). Other mechanisms include deleterious interactions with nucleic acids and sulfur-containing metabolic enzymes (Ahamed et al., 2008; Morones et al., 2005). Rizzello and Pompa provide an excellent schematic of AgNP-related Ag+ toxicity to bacteria (Fig. 3) (2014). Choi et al. (2008) found that autotrophic and heterotrophic bacterial species were susceptible to exposure to ionic silver species, and that observed AgNP-toxicity could not be specifically linked to the particles themselves due to a lack of cell membrane disruption. These authors also observed a steady shift in the color of AgNP suspensions from yellow to dark brown during their experiments, indicating oxidative dissolution of AgNPs to Ag^+ species. Evidence of diffusion of ionic silver across cell membranes by sorbed AgNPs has also been observed (Choi and Hu, 2009). Kittler et al. (2010) found that Ag^+ present in aged suspensions of AgNPs resulted in greater toxicity to human mesenchymal stem cells than fresh AgNP suspensions with comparatively less Ag^+.

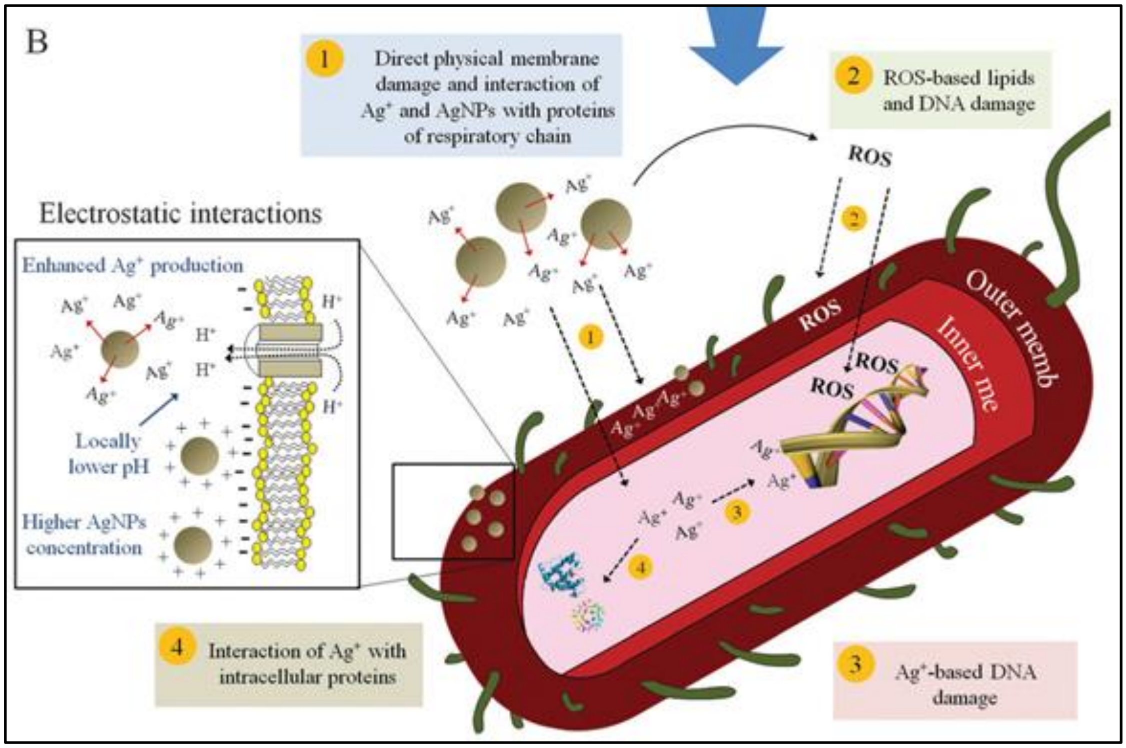

Figure 3: Proposed mechanisms of Ag^+ toxicity to bacterial cells, including blocking of respiratory chain, collapsing membrane potential and stopping ATP production, promoting the runaway production of ROS and damaging membrane lipids and DNA, and influencing metabolic activity by binding to intracellular proteins and chromosomes. (From Rizzello and Pompa, 2014).

1.2.2.3 Discrepancies in Toxicity Mechanisms

There currently exists a fundamental lack of concrete knowledge on the toxicity of AgNPs, though significant progress has been made for some classes of organisms. There is significant evidence for both AgNP and Ag^+ toxicity, yet most studies are inconclusive. The literature is currently incomplete and sometimes contradictory, sometimes providing evidence for both mechanisms of toxicity. For instance, several studies have shown significant growth inhibition by 1.0 mg/L AgNP with no observed disruption of cell membranes using LIVE/DEAD analysis (Choi et al., 2009; Choi and Hu, 2009). However, other studies showed almost complete cell membrane disruption by similar concentrations (El Badawy et al., 2011). Advanced imaging techniques such as transmission electron microscopy (TEM) and scanning electron microscopy (SEM) have been used extensively by most authors, and in each case significant sorption of AgNPs onto cell surfaces has been observed (El Badawy et al., 2011; Suresh et al., 2010; Choi et al., 2008, 2009). Several authors have also shown evidence of localized uptake of small AgNPs (Choi and Hu, 2009; Choi et al., 2008).

Such discrepancies have not gone unnoticed in the literature, as several review articles have recognized and commented on the variability of results. For example, Levard et al. (2012) state:

> Although the toxicity of Ag-NPs is partly explained by the release of Ag ions, it remains unclear if Ag-NPs are a direct cause of enhanced toxicity. For example, Navarro et al. (2008) presented evidence that toxicity is mainly the result of Ag ions and that Ag-NPs contribute to toxicity as a source of dissolved Ag ions. In contrast, Fabrega et al. (2011) showed a specific nanoparticle effect that could not be explained by dissolved Ag^+. Similarly, Yin et al. (2011) demonstrated that gum arabic-stabilized Ag-NPs more strongly affected the growth of *Lolium multiflorum*, a common grass, more than the equivalent dose of Ag ions added as $AgNO_3$. They concluded that growth inhibition and cell damage can be directly attributed either to the nanoparticles themselves or to the ability of Ag-NPs to deliver dissolved Ag^+ to critical biotic receptors. Recently Sotiriou et al. (2010) proposed that the antibacterial activity of Ag-NPs depends on their size. They provide some evidence that when Ag-NPs are small and release many Ag ions, the antibacterial activity is dominated by these ions. However, when relatively large (mean diameter >10 nm) Ag-NPs are employed, the concentration of released Ag^+ is lower, and the particles themselves also influence Ag-NP antibacterial activity.

The conflicting nature of the literature may point toward some combination of the two different mechanisms. One such combination of mechanisms may be that the dissolution of Ag^+ from AgNPs is a nano-specific property related to the incredibly large surface area to volume ratios exhibited by most particles. However, because the size and coating of AgNPs is varied by use and manufacturer, the amount of dissolution across studies is difficult to compare. Ma et al. have shown that these properties are strongly related to particle dissolution (2012), thus providing the first evidence and argument that any toxicity resulting from dissolved Ag is then fundamentally linked to the specific properties of the initial AgNP source. Other authors have consistently found that particle coatings play an integral role in behavior ranging from stability and dissolution to toxicity (Yang et al., 2012; El Badawy et al., 2011; Meyer et al., 2010)

1.2.3 Phytotoxicity

There is increasing evidence for sub-lethal toxic effects of exposure to metallic NPs for a variety of plant species, including the model system *Arabidopsis thaliana*, and more ecologically and agriculturally relevant species such as *Lolium multiflorum*, *Triticum aestivum*, and *Z. mays*. There are five main modes of biological interaction with NPs that could potentially lead to toxicity. These include: chemical effects, physical toxicity (association of NPs with cell structures or mechanical clogging), catalytic effects, surface effects, and changes to environment (Dietz and Herth, 2011). To date, most studies have focused on surface effects (especially phenotypic responses), while some have evaluated chemical effects, physical toxicity, and to some extent changes in local environments.

Particle-plant associations can occur via the root or the shoot (Dietz and Herth, 2011), and may lead to long distance translocation given the proper conditions and NP size (Fig. 4). Most studies of metal-NP toxicity to plant species have focused on the root system as the point of origin for particle-plant association. This is justified in most cases through the application of biosolids and the major influence of root system health on plant development and overall health.

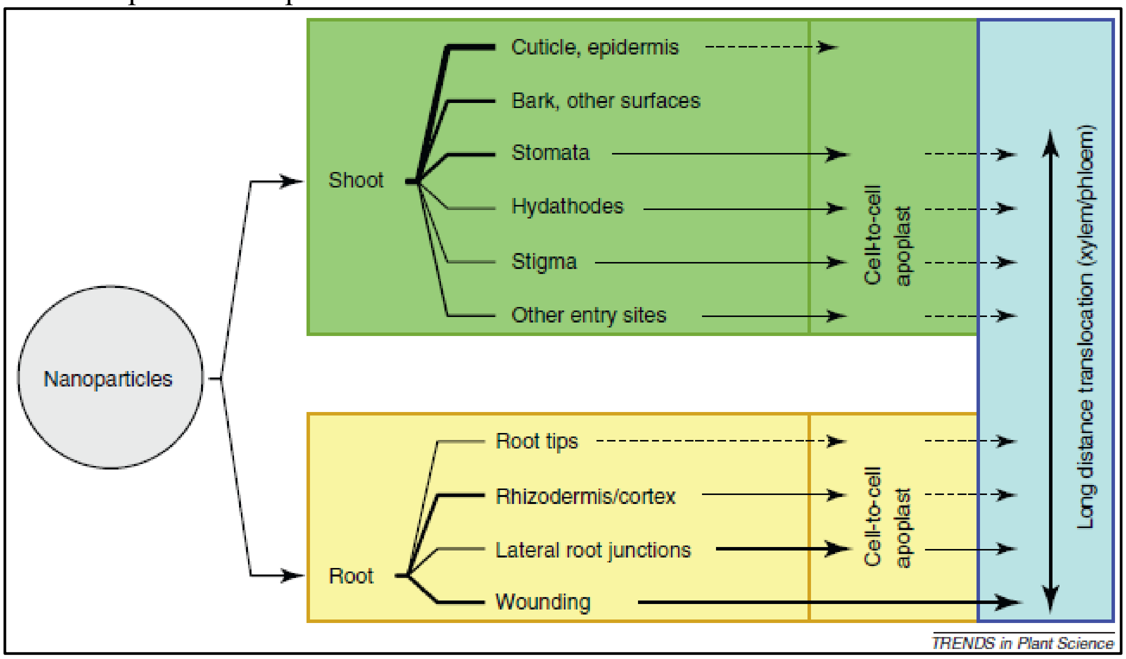

Figure 4: Current understanding of the potential pathways for particle-plant associations. (From Dietz and Herth, 2011).

Several studies on the impacts of AgNP exposure on the model plant system *A. thaliana* have shown significant sub-lethal toxicity. Localized uptake and storage of AgNPs in root caps and intracellular space resulted in browning of root tips and limited development of root hairs in developing *A. thaliana* seedlings (Fig. 5) (Geisler-Lee et al., 2013). *A. thaliana* was further shown to be susceptible to AgNP exposure by Qian et al. (2013). These authors demonstrated significant inhibition of root growth by AgNPs, as well as disruptions to the plants' thylakoid membrane structure and a decrease in chlorophyll content.

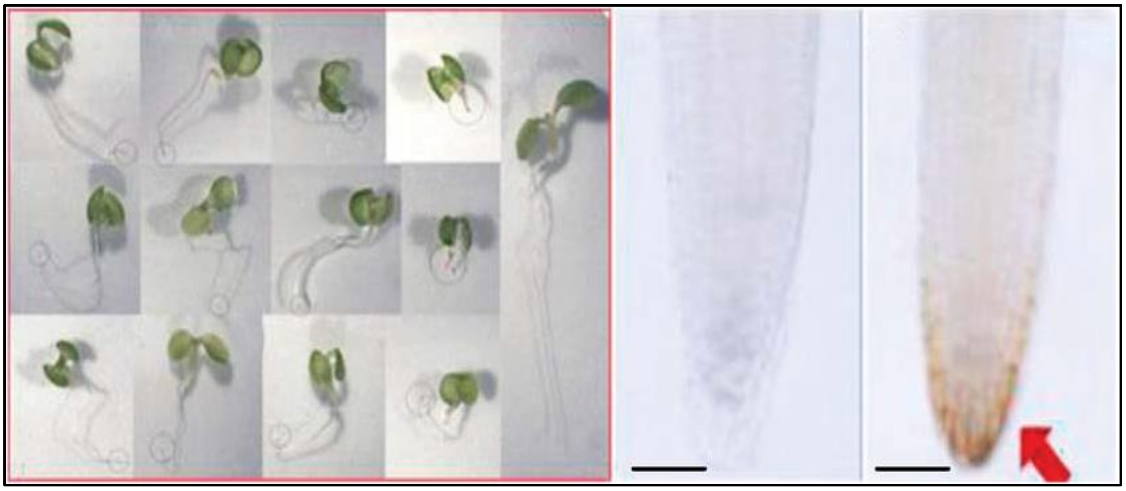

Figure 5: Phenotypic difference in *A. thaliana* seedlings exposed to AgNPs, including reductions in shoot length and browning of root tips. (From Geisler-Lee et al., 2013).

Studies on more ecologically and agriculturally significant plants have also been conducted. Yin et al. (2011) showed significant growth inhibition, cell damage and alterations to root morphology in *L. multiflorum* exposed to AgNPs. Roots grown in 5 mg/L AgNP nutrient solution accumulated ~ 100 mg/kg Ag with a bioconcentration factor close to 30. These results indicate that Ag can become hyperaccumulated in the root systems of plants grown in NP-spiked media. The authors showed similar uptake rates and bioconcentration factors for higher concentrations of Ag, indicated a dose-response effect. However, the high root [Ag] was not necessarily reflected in the shoots of exposed seedlings, as shoot [Ag] and bioconcentrarion factors were 2 orders of magnitude lower, a well understood phenomenon and typical finding (Yin et al., 2011). Pokhrel and Dubey (2013) showed substantial sub-lethal effects in *Z. mays* seedlings exposed to AgNPs, including alterations in root morphology due to structural changes in primary root cells. AgNPs have also been shown to disrupt the growth of *T. aestivum* (Dimpka et al., 2013). The authors showed decreased biomass due to induced branching of roots, as well as reductions in root and shoot length (Fig 6). Significant accumulation of Ag in the roots was also observed, indicating some degree of uptake and translocation.

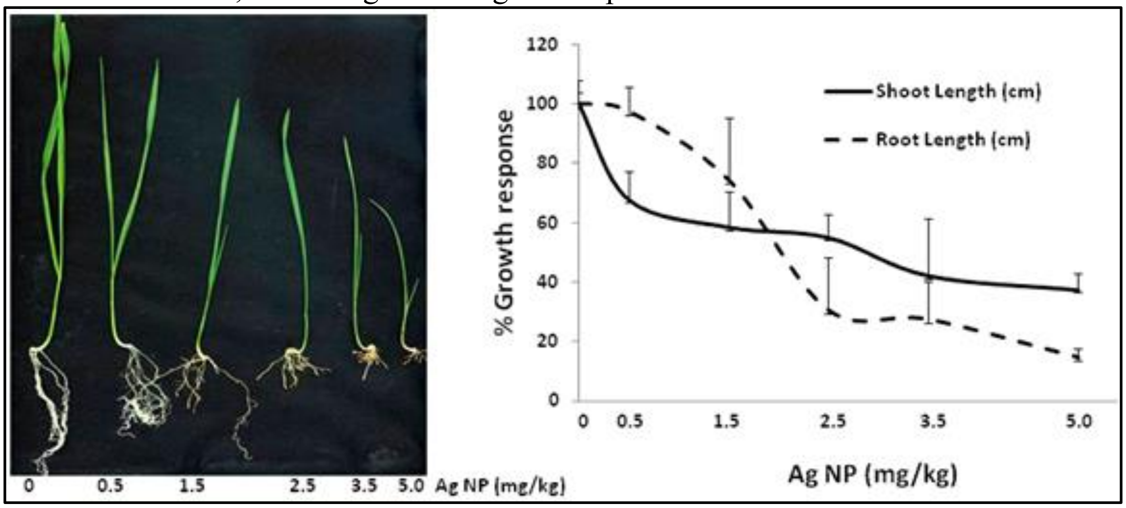

Figure 6: Dose-response effect on wheat seedlings due to exposure to AgNPs (From Dimpka et al., 2013).

As with the mechanism of toxicity to bacteria and other microorganisms, there is some debate over the mechanism of toxicity in higher plants. However, there seems to be more robust evidence for some nano-specific mechanism. Yin et al. (2011) argue that growth inhibition and altered root morphology in *L. multiflorum* were the result of AgNP-specific interactions. They specifically cite an increased surface area to volume ratio that promotes increased surface interactions for smaller particles. They also showed that the addition of Ag^+-binding ligands did not significantly reduce the toxic effects of AgNPs. Additionally, Geisler-Lee et al. (2013) showed that exposure to AgNPs resulted in increased Ag content in *A. thaliana* roots than exposure to equivalent concentrations of $AgNO_3$. Still, there is minimal evidence of translocation of particles larger than 5 nm, indicating that any plant-particle associations will be mainly limited to the roots (Yin et al., 2011; Shane et al., 2000). Additionally, Dimpka et al. (2013) showed that $AgNO_3$ applied in doses equivalent to the soluble fraction of their AgNPs did result in significant reduction in plant growth.

To date there is limited vigorous evidence linking toxicity to dissolved Ag species. Lee et al. (2012a) showed significant toxic effects of $AgNO_3$ released from AgNPs to *Phaseolus radiatus* and *Sorghum bicolor* when grown on agar media, but no discernable toxic effects when grown in soil media. Reductions in root and shoot length in *Hordeum vulgare* and *L. multiflorum* exposed to AgNPs was attributed to the presence of Ag^+ in solution (El-Temsah and Joner, 2010). However, as with other organisms, sorption of AgNPs to root surfaces may result in the diffusion of Ag^+ across the membrane where it can be accumulated and result in runaway production of ROS and eventually cell apoptosis (Kim et al., 2009; Choi et al., 2008). Additionally, Ag^+ has been shown to restrict ethylene activation in plants (Stampoulis et al., 2009) and inhibit mitochondrial function (Knee, 1992).

1.2.4 Disruption of Mutually Beneficial Relationships

Few studies have addressed the impacts of AgNPs on symbiotic relationships, which can be altered in different ways. Most relevant work to date has focused on individual constituents known to participate in such mutually beneficial relationships. Extensive work has been done on the effects of AgNP exposure to nitrogen-fixing bacteria as well as nitrifying and denitrifying bacteria (Bharadway, 2012).

In separate studies, both Priester et al. (2013) and Chen et al. (2003) found that the symbiotic relationship between soybean plants and N_2-fixing bacteria was disrupted by exposure to a variety of metal or metal NPs. Chen et al. (2003) showed significantly reduced numbers of thalli in rhizobium exposed to bulk Cd, along with drastic morphological changes (Fig. 7).

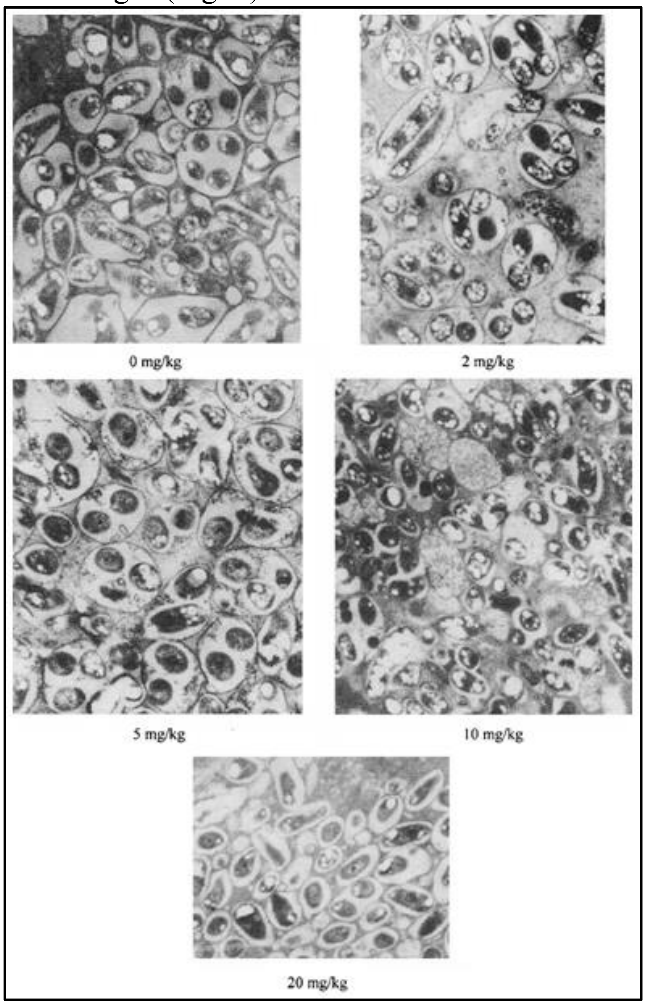

Figure 7: Impacts of Cd exposure to rhizobia include reduction in thalli numbers and alterations to thalli morphology (From Chen et al., 2003).

However, no studies of the impacts of AgNPs on such symbioses have been completed to date. Fundamental knowledge of the principles of these positive relationships can provide clues as to how participating organism will response to

AgNP exposure (Priester et al., 2013). Specifically, direct disruption via toxicity to one or more participants should reduce efficiency of relationship, while indirect disruptions may occur through alteration of soil conditions such as pH, or through larger disruptions of the food web (Priester et al., 2013). However, the lack of understanding of how AgNPs exert toxicity and impact soil processes has led to the inability to describe how whole soil ecosystem processes such as plant-microbe interactions are affected.

1.3 Research Goals and Objectives

The interpretation of toxicity across organisms and classes of organisms should be based on particle characteristics, not just concentration (Stone et al., 2010). Additionally, mechanisms are likely to be different for different types of organisms: bacteria may be affected by cell membrane disruption or direct uptake, while plants may be affected by physical blockage of pores by adsorption of NPs (Dietz and Herth, 2011; Navarro et al., 2008). Until these knowledge gaps are addressed, studies of how biomass production, organic matter breakdown, nutrient cycling, are affected by AgNPs will remain superficial and incomplete. In this research, I addressed knowledge gaps pertaining to bacterial and plant toxicity and the disruption of beneficial relationships between beneficial bacteria and their plant hosts upon exposure to AgNPs. Specifically, this research aimed to quantify and characterize AgNP toxicity to bacterial species *Bacillus subtilis* and *Escherichia coli*; determine the effects of AgNP exposure on the major crop plant *Z. mays*, and to quantify reductions of the beneficial plant-microbe relationship between *Z. mays* and *B. subtilis*.

Chapter 2

SILVER NANOPARTICLES LIMIT INTERACTIONS BETWEEN THE SOIL BACTERIUM *B. SUBTILIS* AND THE MAJOR CROP PLANT *Z. MAYS*

2.1 Introduction

Silver nanoparticles (AgNPs), defined as engineered individual silver particles or small aggregates of silver particles measuring no more than 100 nm in any dimension (USEPA, 2007), are used in a variety of applications, including commercial antimicrobial products such as bandages and socks and other textiles, as well as various military and industrial products (Morones et al., 2005). In fact, AgNPs are one of the most commonly used nanomaterials in consumer products (Fabrega et al., 2011; El-Temsah and Joner, 2010; Klaine et al., 2008). The increasing use of AgNPs in such applications has greatly increased environmental risk of exposure (Gottschalk et al., 2009; Mueller and Nowack, 2008; Nowack and Bucheli, 2007; USEPA, 2012) and associated scientific concern.

Numerous studies on the toxicity of AgNPs to aquatic organisms (Wijnhoven et al., 2009; Lee et al., 2012; Morones et al., 2005) and soil-dwelling nematodes (Kim et al., 2012; Yang et al., 2012; Meyer et al., 2010; Roh et al., 2009;) have been published, and the influence of AgNPs on bacteria (Suresh et al., 2010; El Badawy et al., 2011) and a select number of plant species is becoming increasingly known (Geisler-Lee et al., 2013; Qian et al., 2013; Yin et al., 2011; El-Temsah and Joner, 2010). However, studies on the influence of AgNPs on specific plant-microbe interactions are lacking. Because plant-microbe interactions are ubiquitous in both natural and agricultural soils (Berg, 2009; Berg et al., 2005), it is critical to not only understand how each organism is impacted by exposure to AgNPs, but if and how those critical interactions between the organisms are altered.

Here, we investigate the impacts of AgNPs on the growth behavior and kinetics of two bacterial species, *B. subtilis* and *E. coli*; their impacts on the growth of *Z. mays* seedlings; and their effect on the beneficial plant-microbe interaction between *B. subtilis* and *Z. mays*.

2.2 Materials and Methods

2.2.1 Silver and Silver Nanoparticles

Citrate-coated AgNPs (c-AgNPs) were purchased in 2 mM citrate suspension at pH 7.6 from Ted Pella Inc. (CA, USA) with the following manufacturer's specifications: 1.14 mg/mL Ag, 2.7 x 10^{12} particles/mL, 40.6 ± 3 nm average Transmission Electron Microscope (TEM) diameter, 53.7 nm hydrodynamic diameter (HDD), and -40.7 mV zeta potential. $AgNO_3$ and all other reagents used in this research were analytical grade purchased from Thermo Fisher Scientific (MA, USA).

2.2.2 Biological Materials

Cultures of *B. subtilis* strain FB17 and *E. coli* strain OP50 were provided courtesy of the Bais Lab at the University of Delaware. Cultures were prepared from reserved glycerol stocks and plated on solid Luria-Bertani (LB) plates prior to use. *B. subtilis* strain FB17 was chosen due to its proven participation in plant-beneficial interactions with the model system *A. thaliana* (Kumar et al., 2012; Bais et al., 2004). *E. coli* strain OP50 was chosen as a well-studied common laboratory strain of the bacterium. Additionally, as a Gram-negative bacterium, *E. coli* has a different cell membrane structure than the Gram-positive *B. subtilis* (Ruparelia et al., 2008; Yoon et al., 2007). Seeds of the *Z. mays* cultivar Missouri 17 (Mo-17) were collected from the University of Delaware Greenhouse seed stock (Source ID: 09.1.19716.00359).

2.2.3 Determination of c-AgNP Morphology, Stability, and Ion Release

Characteristics important to the fate, transport, and environmental interactions of c-AgNPs as identified by Stone and colleagues (2010) were quantified prior to testing their effects on bacteria and plants. These characteristics include morphology (size, size distribution, and shape), stability, and ion release.

2.2.3.1 Morphology

Manufacturer-provided particle size and morphology was verified using a Libra 120 TEM (Zeiss AG, DK). Copper grids were treated with poly-l-lysine for 15 min, washed 3x with NanoPure water and dried before being incubated over the AgNP solution for 1 h. After incubation, grids were washed 3x and subsequently dried for 1 h. Images were taken at an accelerating voltage of 120kV. Collected images were processed and analyzed for average TEM diameter and shape using ImageJ v.1.46 software (National Institutes of Health, MD, USA).

2.2.3.2 Stability

Particle stability was determined by measuring HDD and zeta potential of 5.0 mg/L c-AgNP solutions in LB-liquid, basal Plant Nutrient Solution (PNS), and a solution of maize excretes in PNS (PNS+RE). A complete composition of PNS is found in Appendix A (Seyfferth and Parker, 2007; Pedler et al., 2000). Root excretes were collected after growing maize roots in PNS for 7 d. The combined solution was harvested and filtered through sterile 0.22 μm PES membranes (Argos Technologies, IL, USA) and preserved at 4°C. HDD was determined by dynamic light scattering (DLS) and zeta potential by electrophoretic mobility using a Mobius Mobility Instrument (Wyatt Technology, CA, USA).

2.2.3.3 Ion Release

The dissolution of AgNPs in deionized water (DIW), LB-liquid, and PNS+RE was quantified by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) using modified methods from Lee et al. (2012a) and Ma et al. (2012). Batch experiments were conducted by preparing a 1.0 mg/L AgNP solution in each medium and incubating at room temperature for 48 h in DIW and LB-liquid, and for 7 d in PNS+RE. Two 1 mL aliquots of the sample were taken at each sampling; the first was filtered through a 0.025 µm membrane filter (Millipore, MA, USA) for aqueous phase Ag concentration (Ag_{aq}) determination. The filtrate was then acidified with 9 mL of 1% v/v HNO_3 and allowed to digest for at least 24 h before ICP-MS analysis. The second aliquot was taken for total Ag concentration determination, and was immediately acidified and digested for 24 h. Solutions were analyzed on an Agilent 7500cx ICP-MS (Agilent Technologies, CA, USA). Concentrations of Ag_{aq} obtained from ICP-MS were then compared to theoretical dissolved concentrations calculated using a modified form of the Ostwald-Freundlich equation (Eq. 1):

$$S_r = S_{bulk} \times e^{(2\gamma V_m / RT \times r)} \quad (1)$$

where S_r is the solubility (mg/L) of AgNPs with radius r (m), S_{bulk} is the accepted solubility (mg/L) of a silver particle with a flat surface, γ is the surface tension of the particle (J m^{-2}), V_m is the molar volume of the particle (m^3/mol), R is the gas constant (8.314 J/mol*K), and T is the temperature (K) (Ma et al., 2012).

2.2.4 Bacterial Susceptibility to c-AgNPs

B. subtilis and *E. coli* were exposed to varying concentrations of c-AgNPs and the best-estimated concentrations of $AgNO_3$ based on literature reports and dissolution results. c-AgNPs were applied at doses of 0.1, 1.0, and 5.0 mg/L (El Badaway et al, 2011; Stebounova et al, 2011, Suresh et al, 2010) and $AgNO_3$ was applied at concentrations of 0.1 and 1.0 mg/L based on literature values (Ma et al, 2012; Stebounova et al, 2011; Zhang et al., 2011).

2.2.4.1 Determinations of Minimum Inhibitory Concentrations and Half Maximal Effective Doses

To determine the minimum inhibitory concentration (MIC) of c-AgNPs batch experiments were carried out in Costar® 2 µL 96-well plates (Corning Inc., NY, USA). Fifty µL of freshly prepared bacterial culture was diluted in 150 µL of Ag-spiked LB-liquid and incubated at 30°C and 200 rpm for 8 h. Ag-free LB-liquid was used as a control media to assess the standard growth of the bacteria. The absorbance of the cultures at 630 nm (OD_{630}) was taken every 1 h through the log phase of growth and every 2 h to the stationary phase using a Dynex Opsys MR Microplate Reader (Dynex Technologies, VA, USA). Bacteria-free Ag-spiked LB-liquid at each concentration was used to eliminate any artifacts of the suspended particles on the OD_{630}. Each treatment was replicated 8 times, and experiments were run 3 separate times to ensure reproducibility of data. Dynamic growth curves were generated from the collected data and the MIC was taken as the lowest concentration of c-AgNPs that showed growth less than the control group.

The half maximal effective concentration (EC_{50}) was calculated by averaging growth of each treatment over the full 8-h exposure and determining the percent inhibition. Data was analyzed using Toxicity Relationship Analysis Program (TRAP) v.1.22 (USEPA, 2013) using the dose-response model as follows:

$$Y = \frac{Y_0}{1+e^{4S(X-X50)}} \qquad (2)$$

where Y is the response, Y_0 is the response of the control, S is the slope of the curve, X is the dose concentration, and X_{50} is the dose which has an effect on 50% of the population (Metzler et al., 2012; Metzler et al., 2011; Huang et al, 2010).

2.2.4.2 Growth Kinetics

Absorbance data was transformed to bacterial number by convention: 1.0 OD = 5.0×10^8 cells/mL (Bio-Rad Laboratories, Inc., 2002) and then plotted in *Mathematica v. 9* (Wolfram Research Company, USA) and using a modified form of the logistic function (eq. 3) in order to determine the effect of c-AgNP exposure on bacterial growth kinetics. This form of the logistic function is used to model bacterial growth, and is written as:

$$N_t = \frac{K}{1+\frac{K-N_0}{N_0}e^{-rt}} \qquad (3)$$

where N_t is the number of bacteria at time t, K is the carrying capacity, N_0 is the initial bacterial population, all expressed as cells/mL, and r is the growth rate of the population expressed as cells mL/h. Obtained values included K, N_0, and r. These parameters were than used to assess the impact of c-AgNPs on the bacterial population over the entire 8-h exposure.

Impacts of c-AgNPs on growth kinetics can also be assessed by comparing maximum specific growth rates of control and exposed cultures using the following equation (Schacht et al., 2013):

$$\mu = \frac{lnx_1 - lnx_2}{t_1 - t_2} \qquad (4)$$

where μ is the specific growth rate between two time points (cells/mL/h), and x is the cell concentration (cells/mL) at some time t (h) (Schacht et al., 2013).

2.2.4.3 Bacteria-Particle Associations

Bacteria-particle associations were investigated using a Hitachi S4700 field-emission Scanning Electron Microscope (SEM) (Hitachi, JP). Samples were prepared by addition of 1 mL of AgNP solution to freshly prepared cultures of bacteria with O.D.600nm ~ 2.00 and vortexing for 1 min to ensure adequate mixing of bacteria and NPs. Mixtures were then fixed in 2% gluteraldehyde for 1 h and then washed 1x PBS followed by a 1-h incubation in 1% Osmium Tetroxide. Samples were rinsed with distilled water and dehydrated in an ethanol dilution series before being dried and mounted on aluminum stubs and coated with Au/Pd on a Denton Vacuum Bench Top Turbo III sputter-coater (NJ, USA). All images were taken at 3.0kV to avoid charging of the c-AgNPs and damaging the sample.

2.2.5 *Z. mays* Susceptibility to c-AgNPs

Z. mays seeds were sowed in sterile Pro-Mix soil (Premier Tech Horticulture, PA, USA) and grown for 10-14 d in a greenhouse at 25°C and 65% relative humidity under natural day-night light cycles. Prior to use, seedlings were removed from soil and rinsed with tepid tap water to remove soil and soil aggregates. Root systems of the seedlings were then soaked in sterile distilled water for 30 min to further remove bound soil.

2.2.5.1 Phytotoxicity Assays

Z. mays seedlings were grown in PNS spiked with varying concentrations of c-AgNPs based on results from bacterial toxicity experiments. c-AgNPs were applied at

1.0 and 5.0 mg/L, and $AgNO_3$ was applied at a concentration of 0.1 mg/L. Ag-free PNS was used as a control. After rinsing and soaking, seedlings were transferred to sterile double Magenta boxes with 30 mL of sterile PNS. Boxes were then transferred to a shaker table at a rotating speed of 100 rpm and placed under a plant lighting system on a 12-h day/night light cycle, where they grew for 7 days. Each treatment was replicated 3 times and experiments were repeated 3 separate times to ensure reproducibility.

After 7 d seedlings were removed from the Magenta boxes and roots triple rinsed in sterile DIW. Morphology of roots and plant health were examined by digital photographs of the seedlings. Roots were then separated from the shoot, and the shoot discarded. Small sections of the primary root and tertiary/fine roots were taken and preserved for analysis by FE-SEM. The remaining roots were weighed for wet biomass. Roots were then dried for 48 h at 70°C (Yin et al, 2011). After removal, roots were cooled in a desiccator and dry root biomass was weighed.

Dried roots were preserved at 4°C prior to digestion in preparation of analysis for Ag content by ICP-MS. Roots were digested by adding 2 mL of concentrated trace metal grade HNO_3 and heating at 120°C for 30 min. Digestion tubes were removed from the heating block and the solution cooled to room temperature. Two mL of trace metal grade H_2O_2 (30% w/w) was then added and the tubes were reheated at 120°C for an additional 30 min (Geisler-Lee et al, 2013). The remaining solution was cooled and diluted gravimetrically with DIW. A blank and an $AgNO_3$ reference standard were digested following the same procedure. Digested and diluted samples were stored at 4°C prior to analysis by ICP-MS.

Remaining PNS+RE was collected and filtered as previously described. Collected PNS+RE solutions were acidified with 1% HNO_3 and allowed to digest for 24 h. Samples were then analyzed for total Ag by ICP-MS.

2.2.5.2 Detection of AgNPs on *Z. mays* Roots by Field Emission Scanning Electron Microscopy

Harvested *Z. mays* roots were thoroughly rinsed in sterile DIW and sliced into 1 mm sections of primary roots and root hairs for analysis under FE-SEM. Sections were fixed in a solution of 2% paraformaldehyde and 2% gluteraldehyde in 0.1M sodium cacodylate buffer at pH 7.4. Preserved sampled were stored at 4°C prior to preparation for imaging. The samples were then washed in 0.1M phosphate buffer, dehydrated in ethanol stepwise from 25% to 100% then dried in a Tousimis Autosamdri 815B critical point dryer (MD, USA). Samples were then mounted on SEM mounts and coated with carbon using a Denton Vacuum Bench Top Turbo III sputter-coater (NJ, USA). All images were taken at 3.0kV to avoid charging of any c-AgNPs and damaging the root tissue.

2.2.6 Susceptibility of Bacteria-Inoculated *Z. mays* to c-AgNPs

Z. mays exposures to c-AgNPs were repeated with modifications to assess the impact of exposure on plant-microbe interactions. After removal from soil and washing/soaking to remove soil particles, the root systems of *Z. mays* seedlings were inoculated with 1 mL of *B. subtilis* culture at a concentration of $1.0x10^5$ cells/mL. The plants were then placed in sterile plant nutrient solution and placed under a growth lamp on a shaker table as previously described. The same endpoints of root length, mass, morphology, and Ag content were used to assess impacts *Z. mays* seedlings.

After 7-d of growth, 1 mL of the remaining nutrient solution was plated on LB-solid media and incubated for 24 h at 30°C to quantify *B. subtilis* growth.

2.2.7 Statistical Analysis

Results were analyzed using IMB SPSS Statistics Software v.22 (NY, USA). One-way Analysis of Variance (ANOVA) was used to compare means between experimental treatments, and Tukey's post-hoc tests were used to identify which treatments were different. Independent t-Tests were used to compare differences across experiments.

2.3 Results and Discussion

2.3.1 Particle Characterization

2.3.1.1 Size and Morphology

More than 600 individual c-AgNPs were identified in all collected TEM micrographs (Fig. 8A). The mean TEM diameter of c-AgNPs in stock solution was determined to be 44.9 ± 7.2 nm (Fig. 8B). Of all analyzed particles, 52.1% were in the 40-49 nm size range, and no analyzed particles were less than 25 nm or greater than 72 nm. Particles were roughly spherical with some polygonal features, such as sharp corners or defined edges (Fig. 8A).

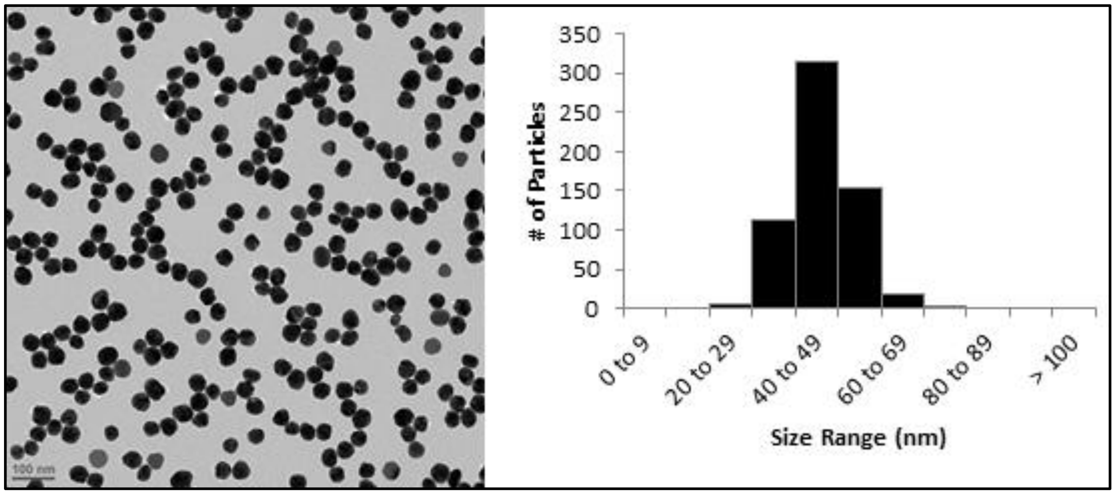

Figure 8: A) TEM micrograph of c-AgNPs in stock solution. Scale bar is 100 nm. B) Size Distribution of c-AgNPs in stock solution. Mean TEM diameter = 44.9 ± 7.2 nm, n = 607.

2.3.1.2 Decreased Particle Stability in Tested Media

c-AgNPs were less stable in tested media compared to stock solution, as indicated by larger HDDs and less negative zeta potentials (Table 1). Particles are smallest but least stable in LB-liquid, though still more than 3x the size of c-AgNPs in stock solution. c-AgNPs are the largest but most stable in sterile PNS. c-AgNPs are ~15% smaller in PNS+RE, indicating that low molecular weight carbon compounds – such as the organic acids excreted or exuded by *Z. mays* roots – may act as a stabilizer (Akaighe et al., 2011), though this cannot be confirmed given the observed increase in zeta potential.

Table 1: HDD and Zeta Potential of c-AgNPs in Various Media

Solution	**HDD / nm (Avg. ± SD)**	**ζ / mV (Avg. ± SD)**
Stock*	53.7	-40.7
LB-Liquid	180.2 ± 11.2	-12.8 ± 2.4
PNS	222.6 ± 18.2	-17.1 ± 1.8
PNS+RE	188.2 ± 11.4	-14.8 ± 3.2

*No SD was provided by the manufacturer for these values

Although c-AgNPs have larger HDDs and less stable zeta potentials, they appear stable over the duration of various experiments (solution color shifts and settling out of particles were not observed).

2.3.1.3 Ion Release in Tested Media is Negligible

Batch experiments conducted in DIW, LB-liquid, and PNS+RE revealed negligible dissolution of Ag^+ from c-AgNPs in the time period studied. Maximum dissolution occurred in DIW, while c-AgNPs suspended in LB-liquid and PNS+RE dissolved considerably less. Expected concentrations obtained using Eq. 1 were generally found to overestimate the dissolution of Ag^+ in the tested media (Ma et al., 2012). For particles with a radius of 45 nm, and assuming S_{bulk} = 0.009 mg/L, γ = 1 J/m^2, and V_m = 6.02 x 10^{-4} m^3/mol, maximum S_r was calculated as 0.18 mg/L. This value is similar to the final concentration of Ag^+ observed in DIW (0.10 ± 0.02 mg/L), but roughly 50x higher than the final concentration in LB-liquid (0.004 ± 0.002 mg/L). Ag^+ concentrations in PNS+RE after 7d were below instrument detection limit. These findings agree with several authors touting the increased stability of c-AgNPs over AgNPs stabilized with other coatings (Ma et al., 2012; Kittler et al., 2010; Zhang et al., 2011).

2.3.2 Suppression of Bacterial Growth

Exposure to c-AgNPs resulted in the suppression of growth of both *B. subtilis* and *E. coli*. Eight-hour growth experiments showed differences between non-exposed control populations and populations exposed to even low concentrations of c-AgNPs. Dynamic growth curves generated from absorbance data in Fig. 9 show qualitative MICs of 0.1 mg/L c-AgNPs for both *B. subtilis* and *E. coli*. B. subtilis was significantly inhibited by exposure to 5.0 mg/L c-AgNPs ($P < 0.05$), while inhibitions of E. coli by c-AgNPs were not statistically significant ($P > 0.05$). These observations are in general agreement with previously reported values (Suresh et al., 2010; Krishnaraj et al., 2010; Verma et al., 2010; Gade et al., 2008). *B. subtilis* was significantly more affected by exposure to 5.0 mg/L c-AgNPs than *E. coli* ($P < 0.05$). Additionally, dose-response effects of exposure to c-AgNPs were more pronounced in the *B. subtilis* culture than for *E. coli*, as further indicated by 8-h average inhibition and EC_{50} calculations.

Both species were relatively unaffected by exposure to 0.1 mg/L $AgNO_3$ (Fig. 10), a concentration equivalent to literature-reported solubility of c-AgNPs (Ma et al., 2012; Zhang et al., 2011). The dynamic growth curves show a slight lag in the growth behavior of these cultures, specifically in the time to reach the log and stationary phases. This phenomenon has previously been observed in cultures exposed to AgNPs (Suresh et al., 2010). However, cultures had statistically significant ($P < 0.05$) greater cell concentrations after 8-h than control. And while the cultures outperformed control, *E. coli* concentrations were significantly greater than *B. subtilis* (P < 0.05). Both species were completely growth-inhibited by exposure to 1.0 mg/L $AgNO_3$. Complete toxicity at this level of Ag^+ has been previously observed (Li et al., 1997) and is a testament to the incredible antimicrobial power of this form of silver. In fact, toxicity was so high that further kinetic analysis was made impossible, as growth did not fit the logistic model of growth.

c-AgNPs were significantly more toxic to both species of bacteria than equivalent concentrations of soluble Ag ($P < 0.05$). These findings are in agreement with previously reported data (El Badawy et al., 2011; Suresh et al., 2010). The difference in toxicity between equal concentrations of c-AgNPs and Ag^+ is clear, as the latter was 5x more toxic to *B. subtilis* and 12x more toxic to *E. coli*.

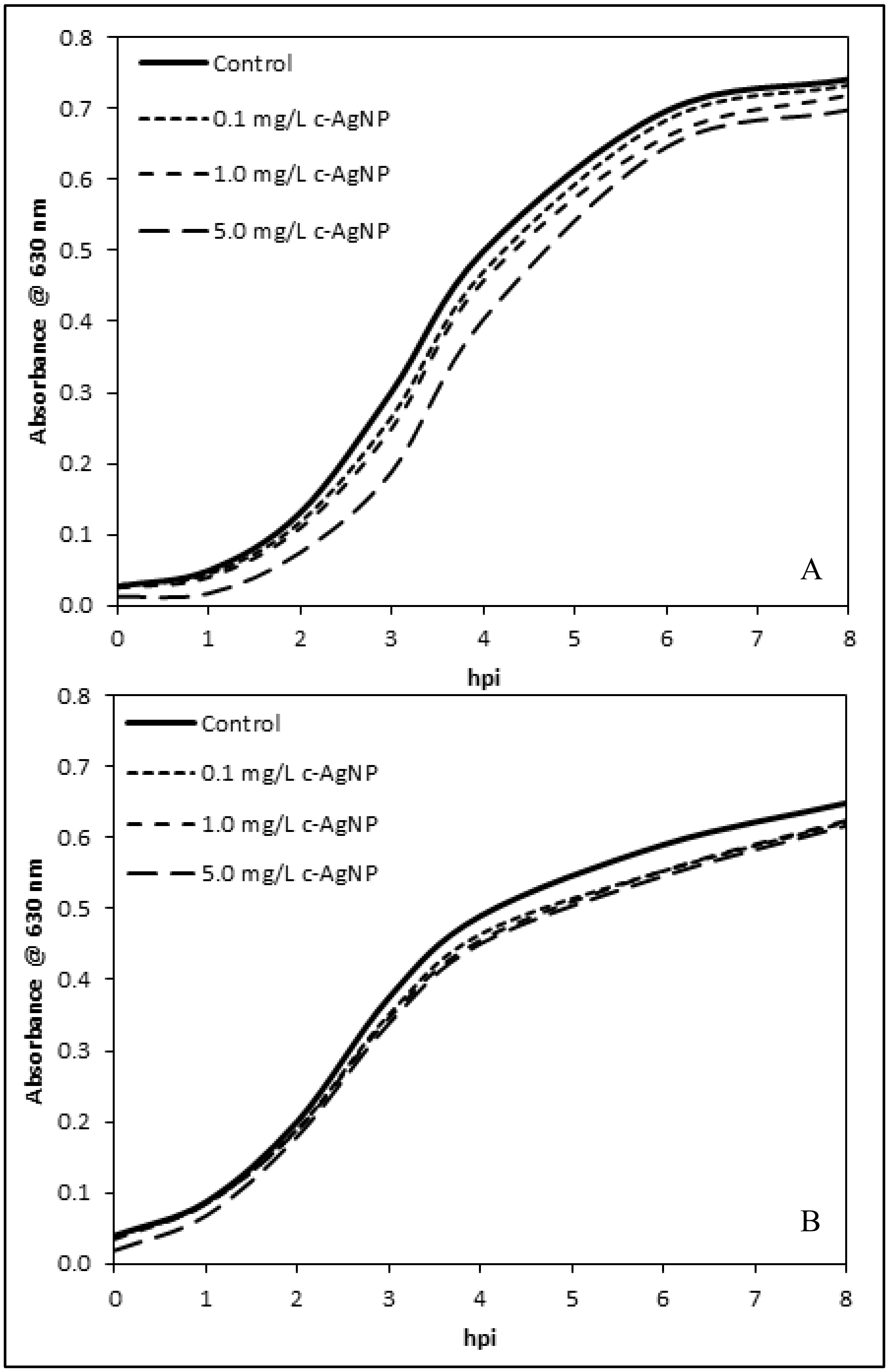

Figure 9: A) Dynamic growth curve for *B. subtilis* exposed to c-AgNPs; B) Dynamic growth curve for *E. coli* exposed to c-AgNPs.

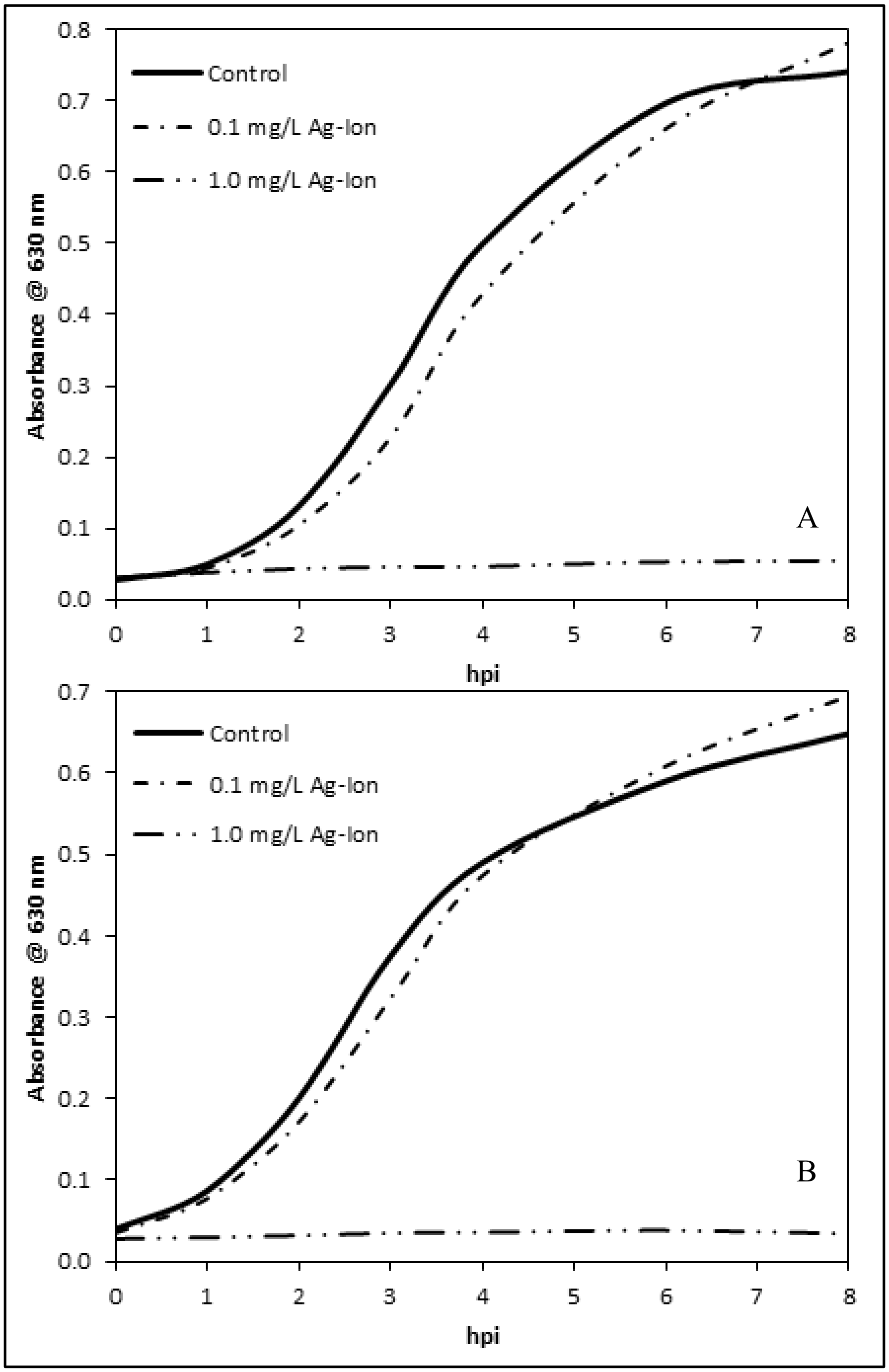

Figure 10: A) Dynamic growth curve for *B. subtilis* exposed to $AgNO_3$; B) Dynamic growth curve for *E. coli* exposed to $AgNO_3$.

Calculated EC_{50} values further demonstrate the increased susceptibility of *B. subtilis* to c-AgNPs compared to *E. coli*. The Toxicity Relationship Analysis Program (TRAP) v. 1.22 (USEPA, 2013) was used to determine the EC_{50} for c-AgNPs for both species of bacteria; *B. subtilis*: 6.5 ± 3.6 mg/L, *E. coli*: 10.9 ± 5.8 mg/L. In each instance, the calculated EC_{50} value is higher than any of the experimental concentrations used in this study, which reduced the predictive power of the software (as indicated in the large standard error). Thus it may prove more beneficial to examine the effects of c-AgNPs on the growth kinetic behavior of each species (Table 2). The carrying capacity (K) for both species of bacteria was significantly reduced by exposure to 5.0 mg/L c-AgNPs, while the 0.1 mg/L $AgNO_3$ had a significantly higher K than the untreated control. No significant differences were observed for the 0.1 and 1.0 mg/L c-AgNP treatments. However, there is still an observable dose-response effect for *B. subtilis* that mirrors the response observed in the MIC experiments. The weak dose-response observed in the MIC experiments for *E. coli* is also seen in K. Growth rates (r) generally followed the same trend as K. In logistic growth, r tends to decrease as populations increase; thus higher growth rates are associated with smaller populations typifying boom and bust growth behavior (Vandermeer, 2010). This explains the general decrease in r observed in cultures treated with increasing concentrations of c-AgNPs. However, this trend was not statistically significant due to relatively high degrees of variability inherited during the curve-fitting process. Using μ_{max} instead of r alleviates some of the fitting variability associated with the logistic function by focusing on growth between only two time points (Schacht et al., 2013). The trend in r is also apparent in μ_{max}, with a general decrease across increasing c-

AgNP concentrations; μ_{max} was significantly greater for the cultures exposed to 5.0 mg/L c-AgNP for both species of bacteria.

Table 2: Kinetics Parameters

Treatment		Parameter (Avg. ± SD)		
		K*	***r****	**μ_{max}#**
B. subtilis	Control	3.72 ± 0.21	1.181 ± 0.230	0.999 ± 0.285
	0.1 mg/L c-AgNP	3.75 ± 0.26	1.148 ± 0.270	0.931 ± 0.207
	1.0 mg/L c-AgNP	3.76 ± 0.33	1.193 ± 0.295	1.029 ± 0.231
	5.0 mg/L c-AgNP	3.67 ± 0.25†	1.321 ± 0.288	1.584 ± 0.674†
	0.1 mg/L $AgNO_3$	3.96 ± 0.27†	0.981 ± 0.176†	0.901 ± 0.374
E. coli	Control	3.18 ± 0.19	1.007 ± 0.120	0.850 ± 0.103
	0.1 mg/L c-AgNP	3.02 ± 0.23†	0.998 ± 0.153	0.840 ± 0.166
	1.0 mg/L c-AgNP	3.03 ± 0.20	0.966 ± 0.103	0.926 ± 0.174
	5.0 mg/L c-AgNP	2.97 ± 0.23†	1.057 ± 0.134	1.501 ± 0.427†
	0.1 mg/L $AgNO_3$	3.42 ± 0.18†	0.915 ± 0.042†	0.815 ± 0.139

*Logistic function parameters: *K:* $1.0x10^8$ cells/mL and *r*: cells/mL/h

#Specific growth rate parameters: μ_{max}: cells/mL/h

†Statistically different from control ($P < 0.05$)

The alteration of kinetics due to exposure to c-AgNPs is most significant for cultures treated with 5.0 mg/L c-AgNPs. Although dose-response was not as strong for *E. coli* as for *B. subtilis*, the general suppression of *K* and elevation of *r* and μ_{max}

indicate a suppressive effect due to exposure to c-AgNPs. Treatment with low levels of $AgNO_3$ resulted in better performing cultures, while higher concentrations led to complete inhibition of bacterial growth. The added nitrate may be responsible for the increased performance of the bacterial culture exposed to 0.1 mg/L $AgNO_3$, considering its extremely low background concentration in the untreated LB-liquid. Overall though, these findings support previous studies that found high levels of $AgNO_3$ to be extremely toxic to bacteria (Suresh et al., 2010; Li et al., 1997).

2.3.3 c-AgNPs Sorb onto Bacterial Cell Surfaces

c-AgNPs and small aggregates sorbed to both species of bacteria during exposures (Fig. 11). The differing surface charges of the bacteria did not appear to alter sorption behavior, and sorption of c-AgNPs did not induce any observable morphology changes on cellular surfaces. This lack of impact on cellular surfaces is contrary to previously reported data. El Badawy et al. (2011), Suresh et al. (2010) and Choi and Hu (2009) showed significant impacts on cell membranes of different bacteria types due to exposure to AgNPs. Impacts include loss of cell height and restricted morphology, as well as cell wall pitting and formation of lumps. Possible explanations for differing results include a lack of resolution due to charging of cellular surfaces, as well as shorter incubation times prior to sample analysis.

Interestingly, the citrate coating of the particles may have provided an additional carbon source the bacteria, as several colonies formed on top of masses of c-AgNP aggregates (Fig. 11B and D). This finding provides important insight into the role of particle coatings to overall behavior, especially toxicity. Further investigation is necessary to quantify the utilization of the citrate component of c-AgNPs by bacteria, and to determine the time-dependent effects of any utilization and resulting exposure of fresh Ag surfaces that may exert toxicity.

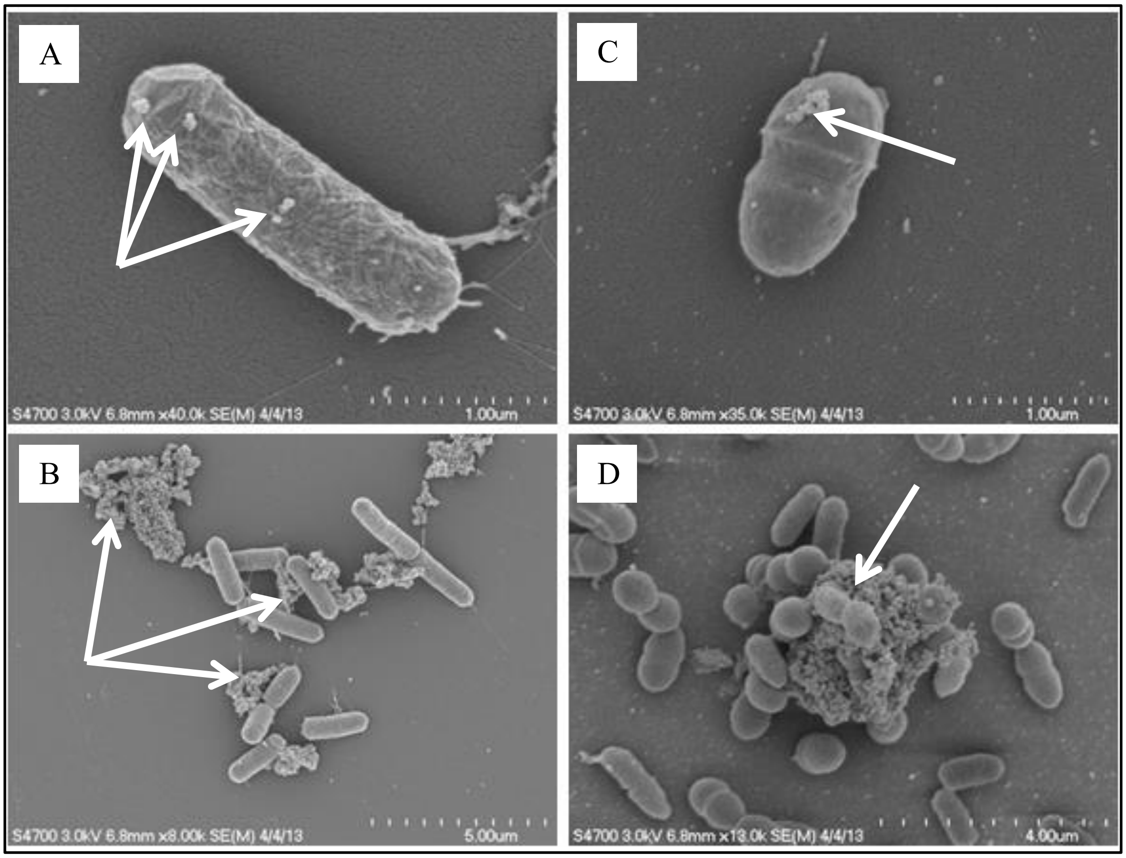

Figure 11: FE-SEM micrographs of *B. subtilis* (A) and (B) and *E. coli* (C) and (D). Arrows denote c-AgNPs or aggregates

2.3.4 Comparing Effects of Exposure between Bacterial Species

Experimental results revealed that the highest concentration of c-AgNPs had a more pronounced effect on *B. subtilis* than *E. coli*. Additionally, the EC_{50} value for *E. coli* was nearly double that for *B. subtilis*. This phenomenon is somewhat in contrast to previous findings that indicated the negative surface charge of c-AgNPs limits their toxic effects to Gram positive bacteria such as *B. subtilis* and increasing their effects on Gram negative bacteria such as *E. coli* (El Badawy et al., 2011). The increasingly touted mechanism for surface-charge dependent associations of AgNPs and bacterial surfaces does not completely explain the observed toxicity. Under this mechanism, the lipopolysaccharide-rich cell membrane of Gram negative bacterial species tends to have a neutral to slightly positive surface charge, thereby increasing cell-particle interactions and resulting toxicity (El Badawy et al., 2011). The carboxyl, phosphate, and amino groups present in the cell membranes of Gram positive bacterial species results in a strongly negative surface charge (van der Wal et al., 1997), supposedly increasing repulsion and limiting cell-particle interactions.

This phenomenon was not observed in the tested bacterial cultures; instead, these findings support previous data showing that the lipopolysaccharides of the outer membrane of Gram-negative bacteria provides innate resistance to NPs (Suresh et al., Ruparelia et al., 2008; Yoon et al., 2007; Brayner et al., 2006). Comparatively, the lack of a protective outer membrane and periplasmic space in Gram-postive bacteria such as *B. subtilis* may increase the likelihood of cell-particle interactions compared to Gram-negative species.

Another facet of the observed toxicity is related to the carbon-rich citrate coating. It is well known in the microbial ecology field that citrate is a ubiquitous compound that acts as a carbon source for both species tested here (Brocker et al., 2009; Yamamoto and Sekiguchi, 2000; Meyer et al., 2001, 1997; Bott et al., 1995), and thus it is not surprising to observe such behavior. This phenomenon may override surface charge dependent interactions and the impacts of cellular membrane differences, and toxicity may simply occur as bacteria exploit the citrate coating for its carbon, exposing fresh and highly toxic Ag surfaces (Fig. 11B and D) or releasing Ag^+ into solution.

2.3.5 Phytotoxicity of AgNPs on *Z. mays* roots

Exposure to c-AgNPs results in sub-lethal phytotoxicity to *Z. mays* seedlings, including reduced root length and biomass, (Figs. 12 and 13). Control plants had a root length of 17.0 ± 6.1 cm and a root biomass of 0.579 ± 0.307 g. Plants exposed to 1.0 mg/L AgNPs had respective lengths and biomass of 13.2 ± 6.8 cm and 0.454 ± 0.307 g. Increasing the c-AgNP concentration to 5.0 mg/L resulted in a root length of 12.9 ± 4.1cm and a root biomass of 0.480 ± 0.144 g. Treatment with 0.1 mg/L Ag^+ as $AgNO_3$ resulted in the shortest roots (11.1 ± 0.9 cm) and lowest root biomass (0.418 ± 0.222 g).

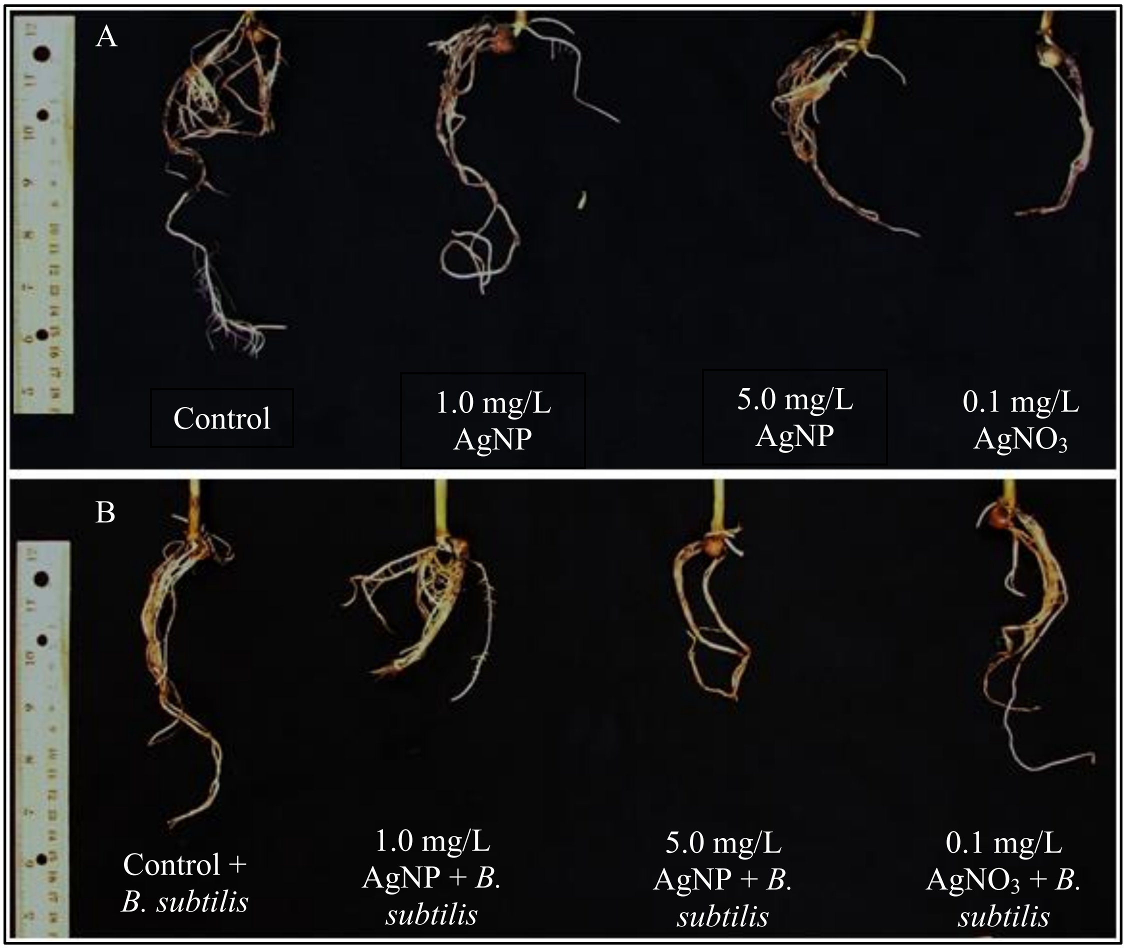

Figure 12: A) Digital photograph of *Z. mays* roots after 7-d exposure; B) Digital photograph of *B. subtilis*-inoculated *Z. mays* seedlings after 7-d exposure.

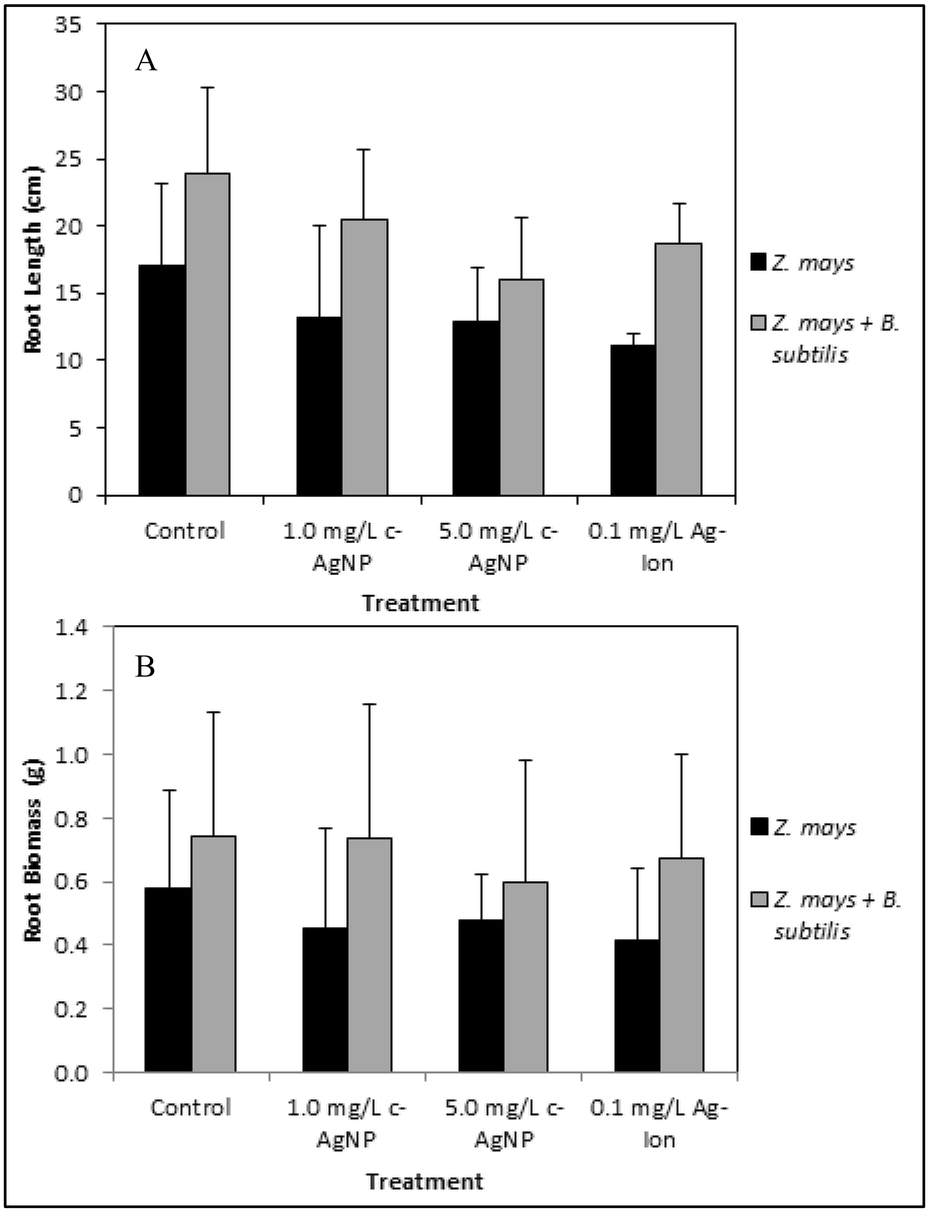

Figure 13: Root length (A) and Wet Root Biomass (B) of *Z. mays* seedlings after 7-d exposures. Error bars are ± 1 SD.

While a limited dose-response is evident, differences between control and Ag treatments were not statistically significant ($P > 0.05$), likely due to relatively high degrees of variability within treatments. However, these same general trends and phenomena have been observed in a variety of plant species exposed to AgNPs, including *L. multiflorum* (Yin et al, 2011) and the model system *A. thaliana* (Geisler-Lee et al, 2013). Yin et al. found no difference in root length and biomass between control plants and those exposed to 1.0 mg/L AgNP. However, they did show some significant effects when increasing exposure concentrations to 5.0 mg/L and above. El-Temsah and Joner found that exposure to AgNPs of various sizes resulted in significant decreases in shoot length of *L. perenne*, although root length was not measured (2010).

Ag content of *Z. mays* roots increased with c-AgNP treatment concentration (Fig. 14). Control plants had Ag contents lower than the instrument detection limit. Roots of seedlings exposed to 1.0 mg/L AgNP had an average Ag content of 2.44 ± 2.90 $x10^{-5}$ mg. Increasing the exposure concentration to 5.0 mg/L led to an average root Ag content of 1.15 ± 2.03 $x10^{-4}$ mg, while treatment with 0.1 mg/L $AgNO_3$ led to an average root Ag content of 1.92 ± 5.78 $x10^{-6}$ mg. Seedlings grown in 5.0 mg/L c-AgNP nutrient solution accumulated more Ag than all other treatments, but differences were not statistically significant ($P > 0.05$).

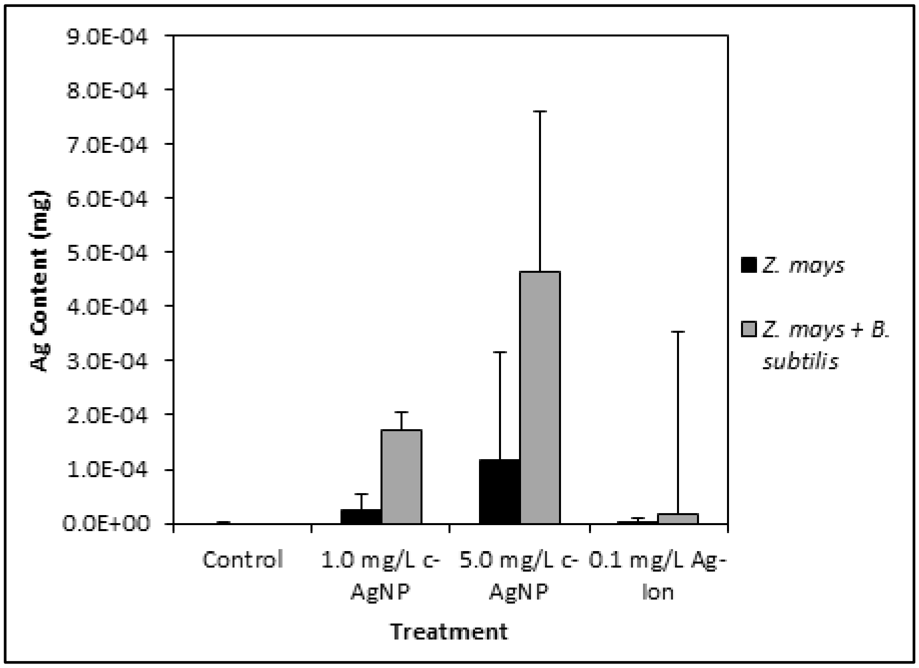

Figure 14: Ag content of *Z. mays* roots after 7-d exposure to c-AgNPs. Error bars are ± 1 SD.

Yin et al. (2011) found a similar trend in concentrations of Ag in the roots of exposed *L. multiflorum* plants. They also observed a weak-dose response in the bioconcentration factor for plants exposed to a range of [Ag] for gum-arabic coated 6 nm AgNPs. The authors found greater [Ag] in their root systems at similar concentrations than in the present study, likely due to the large size difference (6 nm compared to 45 nm). Macroscopic root features of *Z. mays* seedlings (Fig. 12) are consistent with previous findings, are likely representative of smaller-scale alterations in morphology, including shortened or missing root hairs, highly vacuolated and collapsed cortical cells, and broken epidermal and root cap cells (Geisler-Lee et al., 2013; Yin et al., 2011).

2.3.6 Stunting of Tertiary and Fine Roots

c-AgNP treated plants had fewer tertiary roots than control plants (Fig. 12). Biomass differences are not necessarily reflective of this due to the overwhelmingly larger mass of primary and secondary root structures. And while primary and secondary root structure and architecture were not impacted by exposure to c-AgNPs, fine roots were significantly impacted by exposure to AgNPs (Fig. 12). Stunting of these roots is a common response in plants exposed to AgNPs (Geisler-Lee et al., 2013; Yin et al., 2011). Impacts on the development of tertiary roots and root hair structures, though not as significant as damages to primary or secondary roots, reduces the plant's ability to regulate moisture and nutrient uptake (Eissenstat et al., 2000; Davies and Zhang, 1991), and their ability to communicate through excretions and exudations (Zobel, 2005).

These same authors have shown "browning" of root tips caused by exposure to AgNPs. It is unknown whether this phenomenon is the result of uptake of AgNPs or by complexation of Ag^+ with secondary plant compounds. However, significant localized uptake of AgNPs into the intracellular spaces within the root cap and associated border cells was observed, indicating some nano-specific impact (Geisler-Lee et al., 2013).

2.3.7 Decreased Beneficial Interactions in Bacteria-Inoculated *Z. mays* Exposed to c-AgNPs

Inoculation of *Z. mays* seedlings with *B. subtilis* resulted in increased root length and root biomass for all treatments (Figs. 12 and 13). Root length and biomass increased 35% and 15% respectively between control groups. Treatment of inoculated *Z. mays* seedlings with c-AgNPs reduced the root length and biomass compared to control. Root length and biomass were still greater than non-inoculated seedlings, indicating that treatment with bacteria can mitigate the effects of c-AgNPs through general growth promotion. However, increases in root length and biomass were less significant for Ag-treated seedlings than control (24% and 15% respectively for seedlings treated with 5.0 mg/L c-AgNP). Inoculation with *B. subtilis* dramatically increased the root length and biomass of *Z. mays* seedlings treated with 0.1 mg/L $AgNO_3$ (68% and 34%, respectively). While dose-response is clear, differences in root length and biomass were not statistically significant between treatments or between experiments for the control group and seedlings exposed to 1.0 mg/L c-AgNPs ($P >$ 0.05). Biomass differences were significantly different for seedlings exposed to 5.0 mg/Lc-AgNPs, while differences in root length were significantly different for those exposed to 0.1 mg/L $AgNO_3$.

Interestingly, roots of seedlings inoculated with *B. subtilis* had higher Ag contents than seedlings alone across all concentrations (Fig. 14). Control group seedlings again had Ag contents less than the instrument detection limit. Treatment with 1.0 and 5.0 mg/L c-AgNPs resulted in root Ag contents of $1.72 \pm 0.35 \times 10^{-4}$ mg and $4.63 \pm 2.99 \times 10^{-4}$ mg, respectively. The increase in root Ag content for seedlings exposed to c-AgNPs compare to control was not statistically significant. Seedlings exposed to 0.1 mg/L $AgNO_3$ had root Ag contents of $0.16 \pm 3.36 \times 10^{-5}$ mg. These contents represent marked increases of over non-inoculated seedlings. Increases in root Ag content were statistically significant for seedlings exposed to 5.0 mg/L c-AgNPs ($P < 0.05$). However, all other increases were not statistically significant.

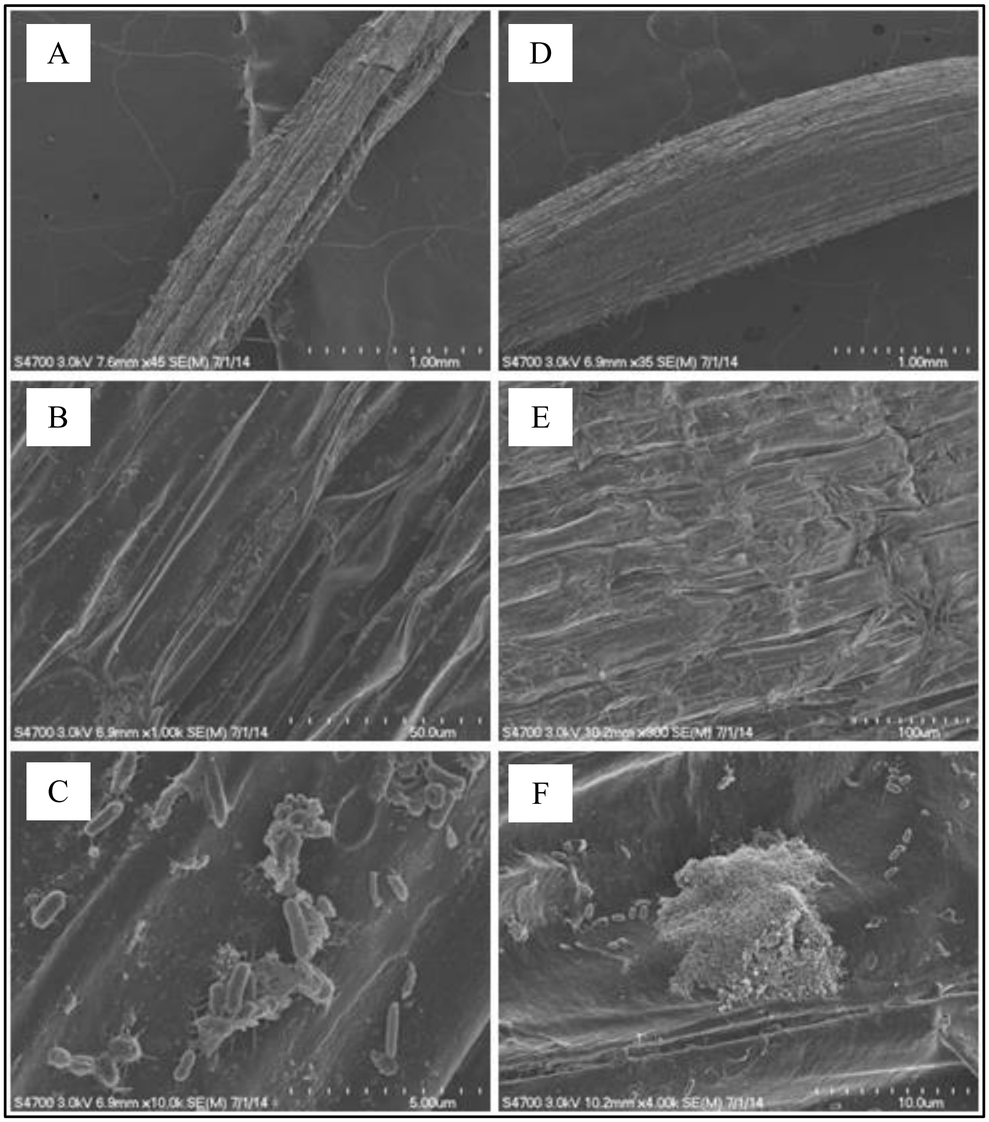

Figure 15: FE-SEM micrographs of *Z. mays* roots. A-C) Control; D-F) 5.0 mg/L c-AgNP.

Analysis of roots by FE-SEM (Fig. 15) showed large aggregates of c-AgNPs on root surfaces (Fig. 15F). Smaller particles were sorbed in smaller random clusters over surfaces. In each case, the presence of c-AgNPs or aggregates was also associated with bacterial populations (Fig. 15F). Images are markedly similar to those taken of bacteria after incubation with c-AgNPs (Fig. 11). Like c-AgNPs, bacteria were mostly randomly distributed across root surfaces (Fig. 15C), but were more concentrated in areas of greater local topography. There were no observable differences in c-AgNP sorption or bacterial distribution between primary roots and tertiary roots or root hairs (images not shown).

Bacterial concentrations in solution were also reduced by treatment with c-AgNPs (Fig 16). Control group bacteria numbered 9.39 $x10^7 \pm 3.57$ $x10^7$ cells/mL. Treatment with c-AgNPs reduced numbers to 5.83 $x10^7 \pm 0.80$ $x10^7$ and 3.92 $x10^7 \pm$ 0.60 $x10^7$ cells/mL for 1.0 mg/L and 5.0 mg/L, respectively. This reduction is significantly different from control ($P < 0.05$). Bacteria exposed to 0.1 mg/L c-$AgNO_3$ numbered 7.38 $x10^7 \pm 1.48$ $x10^7$ cells/mL. FE-SEM images show qualitative evidence of reduced bacterial numbers, though quantitative differences could not be determined (Fig 15). Inhibition levels of bacteria in these inoculation experiments were greater than in previous experiments (Fig. 9A), which showed inhibitions of 11.0 ± 6.0% and 33.3 ± 23.0 for *B. subtilis* exposed to 1.0 and 5.0 mg/L c-AgNPs, respectively. Bacteria inoculated on *Z. mays* seedlings showed inhibitions of 42.0 ± 11.4% at 1.0 mg/L c-AgNP, and 56 ± 11% at 5.0 mg/L c-AgNP (Fig. 16). Additionally, bacteria exposed to 0.1 mg/L $AgNO_3$ did not outperform control in inoculation experiments as in previous growth inhibition experiments (Figs. 9A and 16).

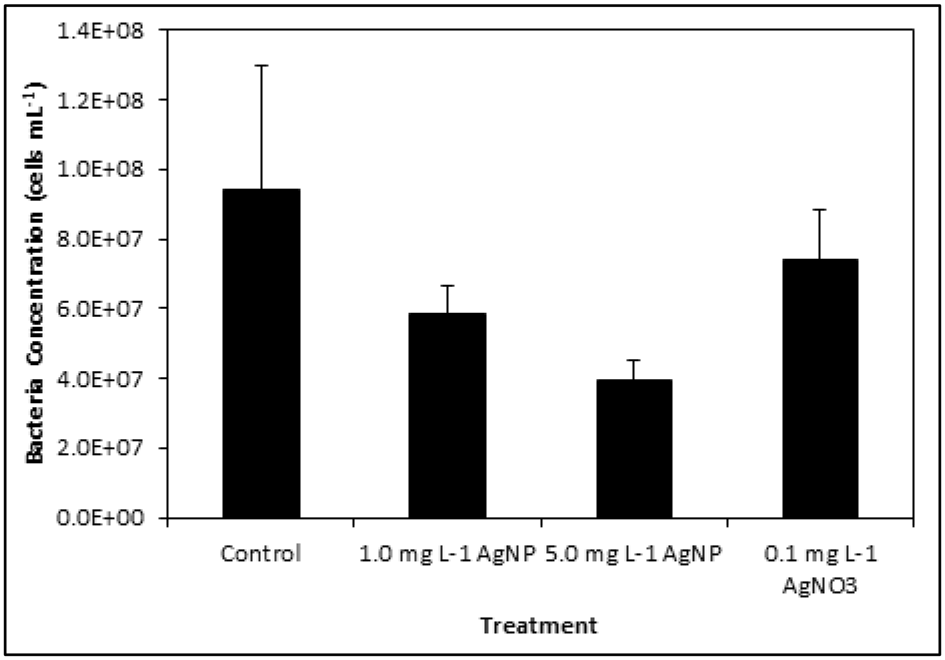

Figure 16: Concentrations of bacteria in PNS+RE after 7-d exposure. Error bars are ± 1 SD.

A possible simple mechanism for reduced bacterial populations is competition for binding/colony sites on the root surfaces. As the c-AgNPs sorb to the roots of *Z. mays* seedlings, the effective available area for bacterial colonization is reduced. It is well known that bacteria have preferred root sites for colonization and that their ability to form and maintain stable relationships with their plant hosts is strongly linked to their colonization ability (Bais et al., 2004; Lutenberg and Dekkers, 1999; Schippers et al., 1987). Thus available surface area reductions (by sorption of c-AgNPs, natural soil particles, or other microbes) can have a significant impact on the colonization effort (Schippers et al., 1987). Our data show limited evidence for this mechanism; however the magnitude of the root sites occupied by c-AgNPs is comparably smaller than those free from c-AgNPs. Still, these findings demonstrate the ecological significance of exposure to even low levels of c-AgNPs and are indicative of the delicacies required to maintain a healthy and mutually beneficial relationship.

2.4 Conclusion

Though c-AgNPs have been routinely found to be less toxic than AgNPs with other coatings (Sharma et al, 2014), they impart sub-lethal toxicity on both Gram positive and Gram negative bacteria and the major crop plant *Z. mays*. These sub-lethal effects of exposure to c-AgNPs ultimately resulted in dramatically reduced beneficial interactions between *Z. mays* and the bacterium *B. subtilis*. Reductions in such beneficial plant-microbe interactions are of great concern due to the indiscriminate nature of silver's antimicrobial activity. While the mechanism of toxicity to both bacteria and plants remains unknown, increasing evidence points towards a combination of effects imparted by AgNPs and ionic Ag alike. Further research is needed to elucidate the exact mechanism of reductions in beneficial interactions between soil microbes and their plant hosts.

Chapter 3

CONCLUSION AND SYNTHESIS

3.1 Introduction

Because the use of AgNPs in consumer and other types of products has risen dramatically, their release into the environment has become an increasing concern to scientists and policy makers (Gottschalk et al., 2009; Mueller and Nowack, 2008; Nowack and Bucheli, 2007; USEPA, 2012). And while their impacts on aquatic organisms and certain species of bacteria and plants are becoming increasingly well-known, the mechanism and degree of toxicity remain unclear. Additionally, the effects of AgNPs on inter-species relationships have yet to be studied. In order to accurately assess the impacts of c-AgNPs on such relationships and begin to gain an understanding of how they may impact whole soil ecosystem processes, their impacts on individual organisms must first be well-understood and quantified.

In this research, the impacts of exposure to c-AgNPs on bacterial species *B. subtilis*, *E. coli*, and the major crop plant *Z. mays* were quantified and characterized according to concentration as well as particle characteristics (Stone et al., 2010). Furthermore, the effects of c-AgNPs on the beneficial plant-microbe interaction between *Z. mays* and *B. subtilis* were similarly quantified and characterized.

3.2 Summary of Findings and Connection to Current Scientific Understanding

Experimental results demonstrate that c-AgNPs exert significant sub-lethal toxicity to bacteria as well as plants. The findings are in general agreement with the literature, and the demonstration of the disruption of beneficial plant-microbe interactions between *Z. mays* and *B. subtilis* is an important contribution to the growing body of knowledge concerning AgNP toxicity.

3.2.1 c-AgNP Stability and Dissolution

The nature of particle stability and dissolution are vastly important when considering the mechanism of toxicity to both bacterial species and plants. It is thus necessary to consider how measured values compare to the literature.

The decreased stability of c-AgNPs in tested solution compared to stock suspension is evidence that NPs undergo environmental transformations, as stated and diagramed (Fig. 1) by Levard et al., (2012). However, the lack of solution color change and settling out of particles indicated that c-AgNPs were relatively stable over the time periods tested, a finding in agreement with literature reports of stability on the order of weeks (Ma et al., 2012; Kittler et al, 2010).

The low dissolution of c-AgNPs to Ag^{+} in the tested media is also in general agreement with the literature (Ma et al., 2012; Kittler et al., 2010, Zhang et al., 2011). Use of the modified Ostwald-Freundlich equation (Eq. 1) accurately predicted dissolution in DIW, but overestimated dissolution in LB-liquid and PNS+RE, further supporting claims of the importance of environmental conditions to particle fate (Levard et al., 2012).

3.2.2 Bacterial Toxicity

c-AgNPs resulted in observable reductions in the growth of *B. subtilis* and *E. coli* during 8-h exposures at concentrations as low as 1.0 mg/L, and statistically significant reductions to *B. subtilis* at 5.0 mg/L c-AgNPs. Increasing concentrations to

5.0 mg/L resulted in even more significant reductions and alterations in growth kinetics of both species. These findings support literature reports of significant toxicity at such concentrations (El Badawy et al., 2011; Suresh et al., 2010).

FE-SEM images show significant sorption of c-AgNPs or aggregates onto bacterial surfaces, as previously observed (El Badawy et al., 2011; Suresh et al., 2010; Choi and Hu, 2009). However, cell membrane damage was not observed in the analyzed images, contrary to prior findings. Images do show that both species may initially colonize the c-AgNPs, making use of the easily available carbon before succumbing to the toxic effects of the underlying Ag (Brocker et al., 2009; Yamamoto and Sekiguchi, 2000; Meyer et al., 2001, 1997; Bott et al., 1995).

The more significant impacts of c-AgNP exposure on the Gram-positive *B. subtilis* may be more related to its cellular membrane structure than composition and surface charge (Suresh et al., 2010). Gram-positive species lack a protective outer membrane and periplasmic space present in the cellular membranes of Gram-negative species such as *E. coli*, and this triple layer membrane offers innate protection against effects of c-AgNPs or Ag^+.(Suresh et al., 2010; Ruparelia et al., 2008, Yoon et al., 2007; Brayner et al., 2006). The relatively thin and less rigid cell membrane leaves Gram-positive species more susceptible to sorption of NPs, subsequent morphological changes including cell wall pitting, as well as diffusion of Ag^+ across the membrane to inside the cytoplasm (El Badawy et al., 2011, Choi and Hu, 2009).

$AgNO_3$ applied at 0.1 mg/L (a concentration equal to the predicted maximum soluble fraction of c-AgNPs) resulted in statistically significant increases in populations for both bacterial species. Increasing $AgNO_3$ concentrations to 1.0 mg/L resulted in complete culture collapse, a well-observed and reported phenomenon

(Suresh et al., 2010; Li et al., 1997). Combined with the significant sorption of c-AgNPs onto surfaces, these data suggest that the primary mechanism of toxicity is related to nano-scale properties, not Ag^+. However, the potential breakdown of the stabilizing citrate coating by bacteria may introduce fresh Ag^+ into solution at rates greater than predicted or measured in dissolution experiments.

3.2.3 Sub-lethal Toxicity to *Z. mays*

Reductions in root length and biomass of *Z. mays* seedlings exposed to c-AgNPs are in agreement with studies of such impacts on other major plant species. Results mirror findings by Yin et al. (2011) that significant responses were not observed until Ag concentrations reached 5.0 mg/L. Accumulation of Ag in root tissues at concentrations greater than the treatment solution are indicative of some degree of bioconcentration, a phenomenon also observed by other authors (Geisler-Lee et al., 2013; Yin et al., 2011).

3.2.4 Impacts on Plant-Microbe Interactions

Inoculation of *Z. mays* seedlings with *B. subtilis* resulted in observable increases in biomass and root length across all experimental treatments, indicating at least general growth promotion (Beauregard et al., 2013; Kumar et al., 2012; Mohamed and Gomaa, 2012; Bais et al., 2004). However, inoculated seedlings exposed to AgNPs showed less dramatic increases in endpoints. Increases in root Ag content compared to non-inoculated plants may be related to the accelerated breakdown of the citrate coating by bacterial activity and subsequent increased availability. However, increased root Ag content did not increase sub-lethal effects compared to non-inoculated seedlings, except at 5.0 mg/L c-AgNPs. These findings are in general agreement with previously reported instances of disruptions of beneficial plant-microbe relationships (Priester et al., 2012; Chen et al., 2003), though few studies of this nature exist.

3.3 Limitations and Recommendations for Future Research

There are two categories of potential limitations to this research. The first concerns limited test subjects; bacterial toxicity was tested on only two species of bacteria, and phytotoxicity on only one species, albeit a major crop plant. While the bacteria tested represent the two major classes (Gram-positive and Gram-negative), a more diverse selection of species within these classes may have provided additional insight into specific toxicity mechanisms, as well as inter- and intra-species response to c-AgNP exposure. Testing the toxicity of c-AgNPs to other major crop plants such as rice (*Oryza* spp.), wheat (*Triticum* spp.) and soy (*Glycine* spp.) would increase the broader impacts of this research. Additionally, testing these species may show differences in responses between monocots and dicots, as well as grain and legume species.

The second stems from the narrow concentration range, comparatively large size of tested c-AgNPs, and the use of only one type of coated AgNP. The narrow concentration range was most constraining when calculating the EC_{50} values for *B. subtilis* and *E. coli*, where c-AgNP concentrations were not high enough to reduce bacterial populations by 50%. Higher concentrations also may have resulted in greater sub-lethal toxicity in exposed *Z. mays* seedlings. Other authors used concentrations have demonstrated significant effects of exposure at concentrations between 10 and 50 mg/L, depending on the size of the NPs used (Yin et al., 2011). However, using higher concentrations reduces the applicability to natural systems, thus our lower concentrations are still justified. The (relatively) large size may have been responsible for the lower degree of toxicity observed in experiments compared to the literature. Several authors have shown that smaller particles (ranging from 6 to 20 nm in diameter) have the most impact on bacteria and plants alike (Geisler-Lee et al., 2013; El Badawy et al., 20l1; Yin et al., 2011; Choi and Hu, 2009). As previously mentioned, citrate has been shown by several authors to create the most stable and least bio-interactive particles (Ma et al., 2012; El Badawy et al., 2011; Yang et al., 2012). Using other coatings, such as polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA), gum-arabic (GA), or branched polyethyleneimine (BPEI) may have produced different results owing to altered surface properties and dissolution behavior.

Additional sources of error and limitations include cross-contamination of 96-well plates in MIC experiments that reduced usable data, and fungal contamination of *Z. mays* seedlings during exposures that likewise reduced sample size.

3.4 Conclusion

This research contributes to the growing evidence for a new paradigm concerning NP toxicity in general in which it is not necessary to discriminate between NP-specific and ionic-specific toxicity effects. Because NPs have different dissolution characteristics than their bulk counterparts (Ma et al, 2010; Kittler et al, 2008), the release of ionic species from NPs is novel property in and of its self. Additionally, the interaction of these coatings with biological materials may further alter particle characteristics. What is critical, however, is that c-AgNPs did impart toxic effects on *B. subtilis*, *E. coli*, and *Z. mays*, as well as the beneficial plant-microbe interactions between *Z. mays* and *B. subtilis*.

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Appendix A

PLANT NUTRIENT SOLUTION COMPOSITION

Table 3: Plant Nutrient Solution

Salt	Molecular Weight (g/mol)	Stock Solution (mol/L)	Aliquot per 1 L (mL)	Final Concentration (µM)
$Ca(NO_3)_2*4H_2O$	236.15	0.9500	2.0	1900.0
NH_4NO_3	80.06	0.0500		100.0
KNO_3	101.11	0.5000	2.0	1000.0
$MgSO_4*7H_2O$	246.48	0.5000	1.0	500.0
KH_2PO_4	136.09	0.2400	0.333	80.0
H_3BO_3	61.83	0.0100	1	10.0
$Na_2MoO_4*2H_2O$	241.95	0.0001		0.1
$ZnCl_2$	136.28	0.0080	1	8.0
$MnCl_2*4H_2O$	197.91	0.0006		0.6
$CuCl_2*2H_2O$	170.48	0.0020		2.0
$NiCl_2*6H_2O$	237.71	0.0001		0.1
$FeCl_3*6H_2O$	270.30	0.0200		20.0
HEDTA	374.45	0.0577		57.7
HCl (1 M)				
MES	213.24	0.5000	2	1000.0
NaOH	40	02500		500.0

Appendix B

PARTICLE SIZE ANALYSIS

Table 4: Particle Size Analysis

Particle #	Line Length (pixels)	Diameter (nm)*	Particle #	Line Length (pixels)	Diameter (nm)*
1	53.67	58.23	31	40.20	43.62
2	53.37	57.91	32	62.29	67.58
3	50.99	55.32	33	43.86	47.59
4	59.46	64.51	34	50.36	54.64
5	55.46	60.17	35	43.17	46.84
6	58.14	63.08	36	52.35	56.80
7	55.17	59.86	37	54.59	59.23
8	50.99	55.32	38	56.60	61.41
9	55.61	60.34	39	52.84	57.33
10	58.14	63.08	40	46.17	50.09
11	44.41	48.18	41	50.16	54.42
12	51.42	55.79	42	50.60	54.90
13	41.18	44.68	43	50.99	55.32
14	48.17	52.26	44	49.48	53.69
15	52.04	56.46	45	46.04	49.95
16	60.00	65.10	46	44.72	48.52
17	51.26	55.62	47	52.61	57.08
18	56.57	61.38	48	46.39	50.33
19	52.00	56.42	49	45.69	49.57
20	56.82	61.65	50	44.94	48.76
21	52.80	57.29	51	47.20	51.21
22	58.58	63.56	52	48.33	52.44
23	50.00	54.25	53	43.68	47.39
24	40.79	44.26	54	48.66	52.80
25	66.48	72.13	55	51.46	55.83

Particle #	Line Length (pixels)	Diameter (nm)*	Particle #	Line Length (pixels)	Diameter (nm)*
26	57.27	62.14	56	38.47	41.74
27	54.92	59.59	57	44.05	47.79
28	54.00	58.59	58	43.68	47.39
59	41.23	44.73	93	36.88	40.01
60	46.17	50.09	94	41.04	44.53
61	40.79	44.26	95	47.41	51.44
62	48.04	52.12	96	37.36	40.54
63	52.84	57.33	97	39.85	43.24
64	46.18	50.11	98	46.52	50.47
65	39.45	42.80	99	46.82	50.80
66	38.47	41.74	100	44.18	47.94
67	33.29	36.12	101	41.18	44.68
68	44.00	47.74	102	37.95	41.18
69	46.86	50.84	103	35.61	38.64
70	44.72	48.52	104	34.99	37.96
71	43.27	46.95	105	36.06	39.13
72	30.07	32.63	106	40.20	43.62
73	46.69	50.66	107	41.76	45.31
74	44.05	47.79	108	43.27	46.95
75	45.61	49.49	109	35.61	38.64
76	46.04	49.95	110	58.14	63.08
77	34.06	36.96	111	34.05	36.94
78	42.05	45.62	112	32.56	35.33
79	39.29	42.63	113	33.29	36.12
80	38.47	41.74	114	38.83	42.13
81	34.53	37.47	115	26.83	29.11
82	46.04	49.95	116	46.86	50.84
83	37.74	40.95	117	41.23	44.73
84	45.34	49.19	118	37.95	41.18
85	48.37	52.48	119	29.73	32.26
86	40.00	43.40	120	32.98	35.78
87	44.41	48.18	117	41.23	44.73
88	39.40	42.75	118	37.95	41.18
89	36.72	39.84	119	29.73	32.26
90	34.93	37.90	120	32.98	35.78

Particle #	Line Length (pixels)	Diameter (nm)*	Particle #	Line Length (pixels)	Diameter (nm)*
91	41.23	44.73	121	37.36	40.54
92	40.20	43.62	122	42.76	46.39
123	35.78	38.82	158	43.86	47.59
124	39.70	43.07	159	30.59	33.19
125	34.93	37.90	160	32.80	35.59
126	54.59	59.23	161	43.08	46.74
127	44.00	47.74	162	42.52	46.13
128	36.06	39.13	163	37.95	41.18
129	45.69	49.57	164	34.06	36.96
130	35.38	38.39	165	32.56	35.33
131	42.05	45.62	166	32.31	35.06
132	35.44	38.45	167	30.27	32.84
133	36.72	39.84	168	40.25	43.67
134	34.00	36.89	169	36.88	40.01
135	46.04	49.95	170	42.05	45.62
136	41.23	44.73	171	38.47	41.74
137	44.72	48.52	172	37.76	40.97
138	36.88	40.01	173	32.00	34.72
139	43.17	46.84	174	34.06	36.96
140	41.76	45.31	175	49.40	53.60
141	34.00	36.89	176	38.42	41.69
142	37.20	40.36	177	38.47	41.74
143	47.41	51.44	178	36.77	39.90
144	46.04	49.95	179	37.95	41.18
145	51.42	55.79	180	30.46	33.05
146	40.50	43.94	181	41.62	45.16
147	37.74	40.95	182	36.88	40.01
148	35.44	38.45	183	34.99	37.96
149	36.72	39.84	184	42.52	46.13
150	38.83	42.13	185	38.63	41.91
151	42.43	46.04	186	38.42	41.69
152	39.60	42.97	187	41.23	44.73
153	38.47	41.74	188	38.05	41.28
154	46.17	50.09	189	43.27	46.95
155	34.93	37.90	190	44.41	48.18

Particle #	Line Length (pixels)	Diameter (nm)*	Particle #	Line Length (pixels)	Diameter (nm)*
156	27.20	29.51	191	39.45	42.80
157	36.88	40.01	192	50.00	54.25
193	41.23	44.73	229	37.58	40.77
194	42.19	45.78	230	33.11	35.92
195	42.38	45.98	231	33.29	36.12
196	36.88	40.01	232	35.44	38.45
197	34.53	37.47	233	40.79	44.26
198	38.05	41.28	234	35.44	38.45
199	49.48	53.69	235	36.77	39.90
200	44.27	48.03	236	39.45	42.80
201	37.58	40.77	237	41.04	44.53
202	37.74	40.95	238	42.94	46.59
203	30.53	33.13	239	36.77	39.90
204	33.29	36.12	240	46.00	49.91
205	43.86	47.59	241	45.25	49.10
206	39.60	42.97	242	37.20	40.36
207	33.29	36.12	243	35.38	38.39
208	39.85	43.24	244	36.72	39.84
209	36.06	39.13	245	31.30	33.96
210	36.22	39.30	246	44.41	48.18
211	26.68	28.95	247	34.00	36.89
212	45.69	49.57	248	36.77	39.90
213	30.59	33.19	249	42.76	46.39
214	36.50	39.60	250	38.83	42.13
215	39.40	42.75	251	47.71	51.77
216	34.23	37.14	252	28.28	30.68
217	40.79	44.26	253	34.93	37.90
218	50.16	54.42	254	29.73	32.26
219	41.23	44.73	255	39.45	42.80
220	34.99	37.96	256	43.27	46.95
221	42.52	46.13	257	36.22	39.30
222	33.53	36.38	258	37.74	40.95
223	39.60	42.97	259	44.72	48.52
224	43.86	47.59	260	46.39	50.33
225	29.12	31.60	261	40.35	43.78
226	41.76	45.31	262	46.00	49.91

Particle #	Line Length (pixels)	Diameter (nm)*	Particle #	Line Length (pixels)	Diameter (nm)*
227	22.80	24.74	263	47.54	51.58
228	31.24	33.90	264	38.00	41.23
265	45.61	49.49	301	35.38	38.39
266	43.27	46.95	302	43.17	46.84
267	37.20	40.36	303	46.17	50.09
268	38.42	41.69	304	38.63	41.91
269	29.12	31.60	305	33.11	35.92
270	37.74	40.95	306	42.19	45.78
271	39.40	42.75	307	42.05	45.62
272	38.05	41.28	308	46.00	49.91
273	39.70	43.07	309	41.04	44.53
274	42.43	46.04	310	49.40	53.60
275	46.00	49.91	311	37.58	40.77
276	50.60	54.90	312	41.18	44.68
277	36.22	39.30	313	40.25	43.67
278	36.06	39.13	314	40.50	43.94
279	32.56	35.33	315	36.50	39.60
280	41.23	44.73	316	44.18	47.94
281	45.34	49.19	317	40.45	43.89
282	48.70	52.84	318	38.21	41.46
283	40.50	43.94	319	40.20	43.62
284	41.62	45.16	320	40.05	43.45
285	39.53	42.89	321	44.72	48.52
286	26.08	28.30	322	36.06	39.13
287	40.79	44.26	323	29.73	32.26
288	36.77	39.90	324	31.24	33.90
289	35.38	38.39	325	41.23	44.73
290	39.29	42.63	326	38.83	42.13
291	34.06	36.96	327	43.27	46.95
292	31.30	33.96	328	38.83	42.13
293	37.36	40.54	329	43.86	47.59
294	48.08	52.17	330	43.17	46.84
295	31.24	33.90	331	35.44	38.45
296	51.92	56.33	332	49.03	53.20
297	37.95	41.18	333	37.95	41.18
298	44.94	48.76	334	36.88	40.01

Particle #	Line Length (pixels)	Diameter (nm)*	Particle #	Line Length (pixels)	Diameter (nm)*
299	25.61	27.79	335	35.61	38.64
300	39.29	42.63	336	38.42	41.69
337	32.06	34.79	373	48.66	52.80
338	50.12	54.38	374	34.23	37.14
339	53.85	58.43	375	38.05	41.28
340	47.07	51.07	376	50.36	54.64
341	36.77	39.90	377	36.88	40.01
342	41.04	44.53	378	36.06	39.13
343	34.23	37.14	379	48.66	52.80
344	39.45	42.80	380	43.08	46.74
345	39.45	42.80	381	49.68	53.90
346	50.60	54.90	382	47.41	51.44
347	40.20	43.62	383	52.15	56.58
348	38.63	41.91	384	42.38	45.98
349	41.23	44.73	385	44.05	47.79
350	52.13	56.56	386	35.61	38.64
351	41.76	45.31	387	36.05	39.11
352	41.18	44.68	388	43.08	46.74
353	50.04	54.29	389	43.27	46.95
354	32.31	35.06	390	42.94	46.59
355	44.05	47.79	391	39.40	42.75
356	38.42	41.69	392	54.59	59.23
357	41.23	44.73	393	48.83	52.98
358	53.25	57.78	394	53.37	57.91
359	33.53	36.38	395	38.83	42.13
360	50.99	55.32	396	38.47	41.74
361	36.77	39.90	397	40.79	44.26
362	46.17	50.09	398	37.95	41.18
363	53.85	58.43	399	53.85	58.43
364	53.25	57.78	400	44.05	47.79
365	40.50	43.94	401	42.38	45.98
366	36.77	39.90	402	50.64	54.94
367	34.00	36.89	403	41.04	44.53
368	47.41	51.44	404	43.36	47.05
369	48.33	52.44	405	50.00	54.25
370	49.40	53.60	406	37.36	40.54

Particle #	Line Length (pixels)	Diameter (nm)*	Particle #	Line Length (pixels)	Diameter (nm)*
371	52.35	56.80	407	45.69	49.57
372	50.99	55.32	408	48.33	52.44
409	54.04	58.63	445	48.08	52.17
410	41.23	44.73	446	46.69	50.66
411	46.82	50.80	447	42.52	46.13
412	37.58	40.77	448	48.00	52.08
413	42.43	46.04	449	38.83	42.13
414	46.69	50.66	450	53.81	58.38
415	49.52	53.73	451	36.77	39.90
416	36.88	40.01	452	42.19	45.78
417	48.00	52.08	453	38.05	41.28
418	36.72	39.84	454	44.72	48.52
419	48.08	52.17	455	48.66	52.80
420	41.04	44.53	456	40.45	43.89
421	40.50	43.94	457	56.60	61.41
422	38.00	41.23	458	40.50	43.94
423	42.05	45.62	459	43.86	47.59
424	41.23	44.73	460	40.25	43.67
425	44.05	47.79	461	44.94	48.76
426	41.62	45.16	462	34.41	37.33
427	32.80	35.59	463	38.83	42.13
428	47.54	51.58	464	44.18	47.94
429	40.00	43.40	465	40.79	44.26
430	53.25	57.78	466	38.00	41.23
431	52.35	56.80	467	32.80	35.59
432	45.65	49.53	468	42.19	45.78
433	51.61	56.00	469	36.06	39.13
434	42.19	45.78	470	37.58	40.77
435	42.76	46.39	471	32.25	34.99
436	48.04	52.12	472	56.04	60.80
437	38.42	41.69	473	34.06	36.96
438	50.99	55.32	474	37.76	40.97
439	38.42	41.69	475	37.20	40.36
440	39.29	42.63	476	44.27	48.03
441	43.91	47.64	477	54.00	58.59
442	36.50	39.60	478	43.68	47.39

Particle #	Line Length (pixels)	Diameter (nm)*	Particle #	Line Length (pixels)	Diameter (nm)*
443	36.72	39.84	479	41.04	44.53
444	37.58	40.77	480	47.71	51.77
481	40.25	43.67	517	42.05	45.62
482	36.06	39.13	518	38.47	41.74
483	34.93	37.90	519	34.53	37.47
484	46.69	50.66	520	40.00	43.40
485	50.12	54.38	521	48.17	52.26
486	39.45	42.80	522	34.53	37.47
487	51.25	55.61	523	48.37	52.48
488	39.45	42.80	524	44.72	48.52
489	36.72	39.84	525	44.05	47.79
490	40.79	44.26	526	41.23	44.73
491	49.19	53.37	527	45.65	49.53
492	43.91	47.64	528	43.27	46.95
493	41.04	44.53	529	36.50	39.60
494	44.41	48.18	530	45.61	49.49
495	40.25	43.67	531	42.00	45.57
496	39.85	43.24	532	47.54	51.58
497	39.45	42.80	533	46.86	50.84
498	40.45	43.89	534	46.39	50.33
499	32.06	34.79	535	35.38	38.39
500	41.62	45.16	536	46.69	50.66
501	43.91	47.64	537	48.08	52.17
502	43.08	46.74	538	44.05	47.79
503	39.40	42.75	539	40.45	43.89
504	38.63	41.91	540	50.16	54.42
505	45.65	49.53	541	36.00	39.06
506	42.43	46.04	542	45.25	49.10
507	42.83	46.47	543	42.19	45.78
508	48.41	52.52	544	46.04	49.95
509	41.23	44.73	545	40.00	43.40
510	37.36	40.54	546	54.00	58.59
511	39.70	43.07	547	45.61	49.49
512	40.45	43.89	548	36.88	40.01
513	42.05	45.62	549	48.17	52.26
514	52.50	56.96	550	43.27	46.95

Particle #	Line Length (pixels)	Diameter (nm)*	Particle #	Line Length (pixels)	Diameter (nm)*
515	43.17	46.84	551	34.99	37.96
516	34.99	37.96	552	45.34	49.19
553	40.79	44.26	583	49.40	53.60
554	46.04	49.95	584	46.04	49.95
555	38.00	41.23	585	27.86	30.23
556	36.06	39.13	586	39.60	42.97
557	40.00	43.40	587	32.98	35.78
558	30.00	32.55	588	32.25	34.99
559	41.23	44.73	589	36.06	39.13
560	48.37	52.48	590	46.69	50.66
561	42.05	45.62	591	40.20	43.62
562	43.17	46.84	592	34.00	36.89
563	28.68	31.12	593	37.36	40.54
564	34.00	36.89	594	44.94	48.76
565	31.62	34.31	595	31.62	34.31
566	36.06	39.13	596	52.04	56.46
567	36.72	39.84	597	44.05	47.79
568	38.21	41.46	598	45.69	49.57
569	51.22	55.57	599	36.00	39.06
570	52.80	57.29	600	43.68	47.39
571	42.93	46.58	601	49.52	53.73
572	35.78	38.82	602	50.99	55.32
573	44.94	48.76	603	44.05	47.79
574	35.61	38.64	604	42.05	45.62
575	30.53	33.13	605	45.69	49.57
576	30.00	32.55	606	46.17	50.09
577	43.71	47.43	607	47.54	51.58
578	36.88	40.01			
579	32.56	35.33			
580	39.45	42.80			
581	45.61	49.49			
582	49.68	53.90			

* Diameter = Pixel Length x 1.085

Appendix C

STATISTICAL TABLES

Table 5: ANOVA Results for Inhibition of *B. subtilis* and *E. coli* by c-AgNPs

ANOVA

Absorbance

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	16.221	11	1.475	872.771	.000
Within Groups	.488	289	.002		
Total	16.709	300			

Table 6: Tukey's Post-Hoc Results for Inhibition of *B. subtilis* and *E. coli* by c-AgNPs

Multiple Comparisons

Dependent Variable: Absorbance

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
1	2	.001849	.011922	1.000	-.03744	.04113
	3	.022471	.011522	.726	-.01549	.06044
	4	.043143*	.011346	.009	.00576	.08053
	5	-.041000*	.011346	.018	-.07839	-.00361
	6	.687571*	.011346	.000	.65019	.72496
	7	.092591*	.009022	.000	.06286	.12232
	8	.119571*	.011033	.000	.08322	.15593
	9	.117048*	.011346	.000	.07966	.15443
	10	.124311*	.011033	.000	.08795	.16067
	11	.046446*	.010894	.002	.01055	.08234
	12	.707702*	.011033	.000	.67135	.74406
2	1	-.001849	.011922	1.000	-.04113	.03744
	3	.020622	.013355	.927	-.02338	.06463
	4	.041294	.013203	.081	-.00221	.08480
	5	-.042849	.013203	.058	-.08636	.00066
	6	.685722*	.013203	.000	.64222	.72923
	7	.090742*	.011269	.000	.05361	.12788
	8	.117722*	.012935	.000	.07510	.16035
	9	.115198*	.013203	.000	.07169	.15870
	10	.122461*	.012935	.000	.07984	.16509
	11	.044597*	.012817	.028	.00236	.08683
	12	.705853*	.012935	.000	.66323	.74848
3	1	-.022471	.011522	.726	-.06044	.01549
	2	-.020622	.013355	.927	-.06463	.02338
	4	.020671	.012843	.904	-.02165	.06299
	5	-.063471*	.012843	.000	-.10579	-.02115
	6	.665100*	.012843	.000	.62278	.70742

	7	.070120*	.010845	.000	.03438	.10585
	8	.097100*	.012567	.000	.05569	.13851
	9	.094576*	.012843	.000	.05226	.13689
	10	.101839*	.012567	.000	.06043	.14325
	11	.023975	.012445	.742	-.01703	.06498
	12	.685230*	.012567	.000	.64382	.72664
4	1	-.043143*	.011346	.009	-.08053	-.00576
	2	-.041294	.013203	.081	-.08480	.00221
	3	-.020671	.012843	.904	-.06299	.02165
	5	-.084143*	.012685	.000	-.12594	-.04234
	6	.644429*	.012685	.000	.60263	.68623
	7	.049448*	.010658	.000	.01433	.08457
	8	.076429*	.012406	.000	.03555	.11731
	9	.073905*	.012685	.000	.03211	.11570
	10	.081168*	.012406	.000	.04029	.12205
	11	.003304	.012282	1.000	-.03717	.04378
	12	.664559*	.012406	.000	.62368	.70544
5	1	.041000*	.011346	.018	.00361	.07839
	2	.042849	.013203	.058	-.00066	.08636
	3	.063471*	.012843	.000	.02115	.10579
	4	.084143*	.012685	.000	.04234	.12594
	6	.728571*	.012685	.000	.68677	.77037
	7	.133591*	.010658	.000	.09847	.16871
	8	.160571*	.012406	.000	.11969	.20145
	9	.158048*	.012685	.000	.11625	.19985
	10	.165311*	.012406	.000	.12443	.20619
	11	.087446*	.012282	.000	.04697	.12792
	12	.748702*	.012406	.000	.70782	.78958
6	1	-.687571*	.011346	.000	-.72496	-.65019
	2	-.685722*	.013203	.000	-.72923	-.64222
	3	-.665100*	.012843	.000	-.70742	-.62278
	4	-.644429*	.012685	.000	-.68623	-.60263
	5	-.728571*	.012685	.000	-.77037	-.68677
	7	-.594980*	.010658	.000	-.63010	-.55986
	8	-.568000*	.012406	.000	-.60888	-.52712

	9	-.570524*	.012685	.000	-.61232	-.52872
	10	-.563261*	.012406	.000	-.60414	-.52238
	11	-.641125*	.012282	.000	-.68160	-.60065
	12	.020130	.012406	.900	-.02075	.06101
7	1	-.092591*	.009022	.000	-.12232	-.06286
	2	-.090742*	.011269	.000	-.12788	-.05361
	3	-.070120*	.010845	.000	-.10585	-.03438
	4	-.049448*	.010658	.000	-.08457	-.01433
	5	-.133591*	.010658	.000	-.16871	-.09847
	6	.594980*	.010658	.000	.55986	.63010
	8	.026980	.010324	.277	-.00704	.06100
	9	.024457	.010658	.483	-.01066	.05957
	10	.031720	.010324	.094	-.00230	.06574
	11	-.046145*	.010175	.001	-.07967	-.01262
	12	.615111*	.010324	.000	.58109	.64913
8	1	-.119571*	.011033	.000	-.15593	-.08322
	2	-.117722*	.012935	.000	-.16035	-.07510
	3	-.097100*	.012567	.000	-.13851	-.05569
	4	-.076429*	.012406	.000	-.11731	-.03555
	5	-.160571*	.012406	.000	-.20145	-.11969
	6	.568000*	.012406	.000	.52712	.60888
	7	-.026980	.010324	.277	-.06100	.00704
	9	-.002524	.012406	1.000	-.04340	.03836
	10	.004739	.012121	1.000	-.03520	.04468
	11	-.073125*	.011994	.000	-.11265	-.03360
	12	.588130*	.012121	.000	.54819	.62807
9	1	-.117048*	.011346	.000	-.15443	-.07966
	2	-.115198*	.013203	.000	-.15870	-.07169
	3	-.094576*	.012843	.000	-.13689	-.05226
	4	-.073905*	.012685	.000	-.11570	-.03211
	5	-.158048*	.012685	.000	-.19985	-.11625
	6	.570524*	.012685	.000	.52872	.61232
	7	-.024457	.010658	.483	-.05957	.01066
	8	.002524	.012406	1.000	-.03836	.04340
	10	.007263	.012406	1.000	-.03362	.04814

	11	-.070601*	.012282	.000	-.11107	-.03013
	12	.590654*	.012406	.000	.54977	.63153
10	1	-.124311*	.011033	.000	-.16067	-.08795
	2	-.122461*	.012935	.000	-.16509	-.07984
	3	-.101839*	.012567	.000	-.14325	-.06043
	4	-.081168*	.012406	.000	-.12205	-.04029
	5	-.165311*	.012406	.000	-.20619	-.12443
	6	.563261*	.012406	.000	.52238	.60414
	7	-.031720	.010324	.094	-.06574	.00230
	8	-.004739	.012121	1.000	-.04468	.03520
	9	-.007263	.012406	1.000	-.04814	.03362
	11	-.077864*	.011994	.000	-.11739	-.03834
	12	.583391*	.012121	.000	.54345	.62333
11	1	-.046446*	.010894	.002	-.08234	-.01055
	2	-.044597*	.012817	.028	-.08683	-.00236
	3	-.023975	.012445	.742	-.06498	.01703
	4	-.003304	.012282	1.000	-.04378	.03717
	5	-.087446*	.012282	.000	-.12792	-.04697
	6	.641125*	.012282	.000	.60065	.68160
	7	.046145*	.010175	.001	.01262	.07967
	8	.073125*	.011994	.000	.03360	.11265
	9	.070601*	.012282	.000	.03013	.11107
	10	.077864*	.011994	.000	.03834	.11739
	12	.661255*	.011994	.000	.62173	.70078
12	1	-.707702*	.011033	.000	-.74406	-.67135
	2	-.705853*	.012935	.000	-.74848	-.66323
	3	-.685230*	.012567	.000	-.72664	-.64382
	4	-.664559*	.012406	.000	-.70544	-.62368
	5	-.748702*	.012406	.000	-.78958	-.70782
	6	-.020130	.012406	.900	-.06101	.02075
	7	-.615111*	.010324	.000	-.64913	-.58109
	8	-.588130*	.012121	.000	-.62807	-.54819
	9	-.590654*	.012406	.000	-.63153	-.54977
	10	-.583391*	.012121	.000	-.62333	-.54345
	11	-.661255*	.011994	.000	-.70078	-.62173

*. The mean difference is significant at the 0.05 level.

1: *B. subtilis* Control; 2: *B. subtilis* 0.1 mg/L c-AgNP; 3: *B. subtilis* 1.0 mg/L c-AgNP; 4: *B. subtilis* 5.0 mg/L c-AgNP; 5: *B. subtilis* 0.1 mg/L Ag-Ion; 6: *B. subtilis* 1.0 mg/L Ag-Ion; 7: *E. coli* Control; 8: *E. coli* 0.1 mg/L c-AgNP; 9: *E. coli* 1.0 mg/L c-AgNP; 10: *E. coli* 5.0 mg/L c-AgNP; 11: *E. coli* 0.1 mg/L Ag-Ion; 12: *E. coli* 1.0 mg/L Ag-Ion

Table 7: ANOVA Results for Growth Kinetics of *B. subtilis* and *E. coli* exposed to c-AgNPs

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
K	Between Groups	1.126	9	.125	60.124	.000
	Within Groups	.529	254	.002		
	Total	1.655	263			
N0	Between Groups	.039	9	.004	46.977	.000
	Within Groups	.024	254	.000		
	Total	.063	263			
r	Between Groups	3.540	9	.393	10.716	.000
	Within Groups	9.324	254	.037		
	Total	12.864	263			

Table 8: Tukey's Post-Hoc Results for Growth Kinetics of *B. subtilis* and *E. coli* Exposed to c-AgNPs

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
K	1	2	-.004903	.01229	1.000	-.04415	.03434
		3	.011376	.01249	.996	-.02851	.05126
		4	.042542*	.01229	.022	.00330	.08179
		5	-.047240*	.01229	.006	-.08648	-.00800
		6	.110478*	.00967	.000	.07959	.14137
		7	.140975*	.01193	.000	.10287	.17908
		8	.138683*	.01229	.000	.09944	.17793
		9	.151573*	.01193	.000	.11347	.18968
		10	.061941*	.01177	.000	.02434	.09954
	2	1	.004903	.01229	1.000	-.03434	.04415
		3	.016279	.01425	.980	-.02922	.06178
		4	.047446*	.01407	.029	.00250	.09239
		5	-.042336	.01407	.084	-.08728	.00260
		6	.115381*	.01186	.000	.07751	.15325
		7	.145879*	.01376	.000	.10193	.18983
		8	.143587*	.01407	.000	.09865	.18853
		9	.156476*	.01376	.000	.11252	.20043
		10	.066845*	.01363	.000	.02333	.11036
	3	1	-.011376	.01249	.996	-.05126	.02851
		2	-.016279	.01425	.980	-.06178	.02922
		4	.031167	.01425	.469	-.01433	.07667
		5	-.058615*	.01425	.002	-.10411	-.01312
		6	.099102*	.01207	.000	.06057	.13763
		7	.129599*	.01394	.000	.08508	.17412

	8	.127308*	.01425	.000	.08181	.17281
	9	.140197*	.01394	.000	.09567	.18472
	10	.050566*	.01381	.011	.00648	.09466
4	1	-.042542*	.01229	.022	-.08179	-.00330
	2	-.047446*	.01407	.029	-.09239	-.00250
	3	-.031167	.01425	.469	-.07667	.01433
	5	-.089782*	.01407	.000	-.13472	-.04484
	6	.067935*	.01186	.000	.03007	.10580
	7	.098433*	.01376	.000	.05448	.14239
	8	.096141*	.01407	.000	.05120	.14108
	9	.109030*	.01376	.000	.06508	.15298
	10	.019399	.01363	.919	-.02411	.06291
5	1	.047240*	.01229	.006	.00800	.08648
	2	.042336	.01407	.084	-.00260	.08728
	3	.058615*	.01425	.002	.01312	.10411
	4	.089782*	.01407	.000	.04484	.13472
	6	.157717*	.01186	.000	.11985	.19559
	7	.188215*	.01376	.000	.14426	.23217
	8	.185923*	.01407	.000	.14098	.23086
	9	.198812*	.01376	.000	.15486	.24277
	10	.109181*	.01363	.000	.06567	.15269
6	1	-.110478*	.00967	.000	-.14137	-.07959
	2	-.115381*	.01186	.000	-.15325	-.07751
	3	-.099102*	.01207	.000	-.13763	-.06057
	4	-.067935*	.01186	.000	-.10580	-.03007
	5	-.157717*	.01186	.000	-.19559	-.11985
	7	.030497	.01149	.199	-.00619	.06719
	8	.028205	.01186	.344	-.00966	.06607
	9	.041095*	.01149	.015	.00440	.07778
	10	-.048537*	.01132	.001	-.08470	-.01237
7	1	-.140975*	.01193	.000	-.17908	-.10287
	2	-.145879*	.01376	.000	-.18983	-.10193
	3	-.129599*	.01394	.000	-.17412	-.08508
	4	-.098433*	.01376	.000	-.14239	-.05448
	5	-.188215*	.01376	.000	-.23217	-.14426

		6	-.030497	.01149	.199	-.06719	.00619
		8	-.002292	.01376	1.000	-.04624	.04166
		9	.010598	.01345	.999	-.03234	.05354
		10	-.079034*	.01331	.000	-.12153	-.03654
	8	1	-.138683*	.01229	.000	-.17793	-.09944
		2	-.143587*	.01407	.000	-.18853	-.09865
		3	-.127308*	.01425	.000	-.17281	-.08181
		4	-.096141*	.01407	.000	-.14108	-.05120
		5	-.185923*	.01407	.000	-.23086	-.14098
		6	-.028205	.01186	.344	-.06607	.00966
		7	.002292	.01376	1.000	-.04166	.04624
		9	.012889	.01376	.995	-.03106	.05684
		10	-.076742*	.01363	.000	-.12026	-.03323
	9	1	-.151573*	.01193	.000	-.18968	-.11347
		2	-.156476*	.01376	.000	-.20043	-.11252
		3	-.140197*	.01394	.000	-.18472	-.09567
		4	-.109030*	.01376	.000	-.15298	-.06508
		5	-.198812*	.01376	.000	-.24277	-.15486
		6	-.041095*	.01149	.015	-.07778	-.00440
		7	-.010598	.01345	.999	-.05354	.03234
		8	-.012889	.01376	.995	-.05684	.03106
		10	-.089631*	.01331	.000	-.13212	-.04714
	10	1	-.061941*	.01177	.000	-.09954	-.02434
		2	-.066845*	.01363	.000	-.11036	-.02333
		3	-.050566*	.01381	.011	-.09466	-.00648
		4	-.019399	.01363	.919	-.06291	.02411
		5	-.109181*	.01363	.000	-.15269	-.06567
		6	.048537*	.01132	.001	.01237	.08470
		7	.079034*	.01331	.000	.03654	.12153
		8	.076742*	.01363	.000	.03323	.12026
		9	.089631*	.01331	.000	.04714	.13212
N0	1	2	.000608	.00259	1.000	-.00767	.00889
		3	.002588	.00263	.993	-.00583	.01100
		4	.009033*	.00259	.020	.00075	.01731
		5	-.002424	.00259	.995	-.01070	.00586

	6	-.023467*	.00204	.000	-.02999	-.01695
	7	-.023229*	.00251	.000	-.03127	-.01519
	8	-.024073*	.00259	.000	-.03235	-.01579
	9	-.015693*	.00251	.000	-.02373	-.00765
	10	-.021299*	.00248	.000	-.02923	-.01336
2	1	-.000608	.00259	1.000	-.00889	.00767
	3	.001980	.00300	1.000	-.00762	.01158
	4	.008425	.00297	.130	-.00106	.01791
	5	-.003032	.00297	.991	-.01252	.00645
	6	-.024076*	.00250	.000	-.03207	-.01608
	7	-.023837*	.00290	.000	-.03311	-.01456
	8	-.024681*	.00297	.000	-.03416	-.01520
	9	-.016301*	.00290	.000	-.02558	-.00703
	10	-.021907*	.00287	.000	-.03109	-.01272
3	1	-.002588	.00263	.993	-.01100	.00583
	2	-.001980	.00300	1.000	-.01158	.00762
	4	.006445	.00300	.499	-.00316	.01605
	5	-.005011	.00300	.813	-.01461	.00459
	6	-.026055*	.00254	.000	-.03419	-.01792
	7	-.025816*	.00294	.000	-.03521	-.01642
	8	-.026660*	.00300	.000	-.03626	-.01706
	9	-.018281*	.00294	.000	-.02768	-.00889
	10	-.023887*	.00291	.000	-.03319	-.01458
4	1	-.009033*	.00259	.020	-.01731	-.00075
	2	-.008425	.00297	.130	-.01791	.00106
	3	-.006445	.00300	.499	-.01605	.00316
	5	-.011457*	.00297	.006	-.02094	-.00197
	6	-.032501*	.00250	.000	-.04049	-.02451
	7	-.032262*	.00290	.000	-.04154	-.02299
	8	-.033106*	.00297	.000	-.04259	-.02362
	9	-.024726*	.00290	.000	-.03400	-.01545
	10	-.030332*	.00287	.000	-.03951	-.02115
5	1	.002424	.00259	.995	-.00586	.01070
	2	.003032	.00297	.991	-.00645	.01252
	3	.005011	.00300	.813	-.00459	.01461

	4	.011457*	.00297	.006	.00197	.02094
	6	-.021044*	.00250	.000	-.02903	-.01305
	7	-.020805*	.00290	.000	-.03008	-.01153
	8	-.021649*	.00297	.000	-.03113	-.01217
	9	-.013269*	.00290	.000	-.02254	-.00399
	10	-.018875*	.00287	.000	-.02806	-.00969
6	1	.023467*	.00204	.000	.01695	.02999
	2	.024076*	.00250	.000	.01608	.03207
	3	.026055*	.00254	.000	.01792	.03419
	4	.032501*	.00250	.000	.02451	.04049
	5	.021044*	.00250	.000	.01305	.02903
	7	.000239	.00242	1.000	-.00750	.00798
	8	-.000605	.00250	1.000	-.00860	.00739
	9	.007774*	.00242	.048	.00003	.01552
	10	.002168	.00239	.996	-.00546	.00980
7	1	.023229*	.00251	.000	.01519	.03127
	2	.023837*	.00290	.000	.01456	.03311
	3	.025816*	.00294	.000	.01642	.03521
	4	.032262*	.00290	.000	.02299	.04154
	5	.020805*	.00290	.000	.01153	.03008
	6	-.000239	.00242	1.000	-.00798	.00750
	8	-.000844	.00290	1.000	-.01012	.00843
	9	.007536	.00283	.198	-.00153	.01660
	10	.001930	.00280	1.000	-.00704	.01090
8	1	.024073*	.00259	.000	.01579	.03235
	2	.024681*	.00297	.000	.01520	.03416
	3	.026660*	.00300	.000	.01706	.03626
	4	.033106*	.00297	.000	.02362	.04259
	5	.021649*	.00297	.000	.01217	.03113
	6	.000605	.00250	1.000	-.00739	.00860
	7	.000844	.00290	1.000	-.00843	.01012
	9	.008380	.00290	.116	-.00090	.01765
	10	.002774	.00287	.994	-.00641	.01196
9	1	.015693*	.00251	.000	.00765	.02373
	2	.016301*	.00290	.000	.00703	.02558

		3	.018281*	.00294	.000	.00889	.02768
		4	.024726*	.00290	.000	.01545	.03400
		5	.013269*	.00290	.000	.00399	.02254
		6	-.007774*	.00242	.048	-.01552	-.00003
		7	-.007536	.00283	.198	-.01660	.00153
		8	-.008380	.00290	.116	-.01765	.00090
		10	-.005606	.00280	.603	-.01457	.00336
	10	1	.021299*	.00248	.000	.01336	.02923
		2	.021907*	.00287	.000	.01272	.03109
		3	.023887*	.00291	.000	.01458	.03319
		4	.030332*	.00287	.000	.02115	.03951
		5	.018875*	.00287	.000	.00969	.02806
		6	-.002168	.00239	.996	-.00980	.00546
		7	-.001930	.00280	1.000	-.01090	.00704
		8	-.002774	.00287	.994	-.01196	.00641
		9	.005606	.00280	.603	-.00336	.01457
r	1	2	.032838	.05163	1.000	-.13197	.19765
		3	-.011554	.05247	1.000	-.17905	.15594
		4	-.140182	.05163	.173	-.30499	.02463
		5	.200504*	.05163	.005	.03569	.36532
		6	.174573*	.04064	.001	.04483	.30431
		7	.182917*	.05013	.012	.02287	.34296
		8	.214677*	.05163	.002	.04986	.37949
		9	.124442	.05013	.283	-.03560	.28449
		10	.266147*	.04947	.000	.10823	.42406
	2	1	-.032838	.05163	1.000	-.19765	.13197
		3	-.044392	.05986	.999	-.23548	.14669
		4	-.173020	.05912	.104	-.36176	.01572
		5	.167667	.05912	.130	-.02108	.35641
		6	.141735	.04982	.127	-.01730	.30077
		7	.150079	.05782	.225	-.03451	.33467
		8	.181839	.05912	.070	-.00690	.37058
		9	.091604	.05782	.855	-.09299	.27620
		10	.233309*	.05725	.002	.05056	.41606
	3	1	.011554	.05247	1.000	-.15594	.17905

	2	.044392	.05986	.999	-.14669	.23548
	4	-.128627	.05986	.495	-.31971	.06246
	5	.212059*	.05986	.017	.02097	.40315
	6	.186128*	.05069	.011	.02431	.34794
	7	.194471*	.05857	.034	.00748	.38146
	8	.226231*	.05986	.007	.03514	.41732
	9	.135996	.05857	.379	-.05099	.32299
	10	.277701*	.05800	.000	.09253	.46287
4	1	.140182	.05163	.173	-.02463	.30499
	2	.173020	.05912	.104	-.01572	.36176
	3	.128627	.05986	.495	-.06246	.31971
	5	.340686*	.05912	.000	.15194	.52943
	6	.314755*	.04982	.000	.15572	.47379
	7	.323098*	.05782	.000	.13850	.50769
	8	.354858*	.05912	.000	.16612	.54360
	9	.264623*	.05782	.000	.08003	.44922
	10	.406329*	.05725	.000	.22358	.58908
5	1	-.200504*	.05163	.005	-.36532	-.03569
	2	-.167667	.05912	.130	-.35641	.02108
	3	-.212059*	.05986	.017	-.40315	-.02097
	4	-.340686*	.05912	.000	-.52943	-.15194
	6	-.025931	.04982	1.000	-.18497	.13311
	7	-.017588	.05782	1.000	-.20218	.16701
	8	.014172	.05912	1.000	-.17457	.20291
	9	-.076063	.05782	.949	-.26066	.10853
	10	.065643	.05725	.979	-.11711	.24839
6	1	-.174573*	.04064	.001	-.30431	-.04483
	2	-.141735	.04982	.127	-.30077	.01730
	3	-.186128*	.05069	.011	-.34794	-.02431
	4	-.314755*	.04982	.000	-.47379	-.15572
	5	.025931	.04982	1.000	-.13311	.18497
	7	.008343	.04827	1.000	-.14575	.16243
	8	.040104	.04982	.998	-.11893	.19914
	9	-.050131	.04827	.990	-.20422	.10396
	10	.091574	.04757	.652	-.06030	.24345

7	1	-.182917*	.05013	.012	-.34296	-.02287
	2	-.150079	.05782	.225	-.33467	.03451
	3	-.194471*	.05857	.034	-.38146	-.00748
	4	-.323098*	.05782	.000	-.50769	-.13850
	5	.017588	.05782	1.000	-.16701	.20218
	6	-.008343	.04827	1.000	-.16243	.14575
	8	.031760	.05782	1.000	-.15283	.21635
	9	-.058475	.05649	.990	-.23882	.12187
	10	.083230	.05590	.896	-.09523	.26169
8	1	-.214677*	.05163	.002	-.37949	-.04986
	2	-.181839	.05912	.070	-.37058	.00690
	3	-.226231*	.05986	.007	-.41732	-.03514
	4	-.354858*	.05912	.000	-.54360	-.16612
	5	-.014172	.05912	1.000	-.20291	.17457
	6	-.040104	.04982	.998	-.19914	.11893
	7	-.031760	.05782	1.000	-.21635	.15283
	9	-.090235	.05782	.866	-.27483	.09436
	10	.051470	.05725	.996	-.13128	.23422
9	1	-.124442	.05013	.283	-.28449	.03560
	2	-.091604	.05782	.855	-.27620	.09299
	3	-.135996	.05857	.379	-.32299	.05099
	4	-.264623*	.05782	.000	-.44922	-.08003
	5	.076063	.05782	.949	-.10853	.26066
	6	.050131	.04827	.990	-.10396	.20422
	7	.058475	.05649	.990	-.12187	.23882
	8	.090235	.05782	.866	-.09436	.27483
	10	.141705	.05590	.255	-.03676	.32017
10	1	-.266147*	.04947	.000	-.42406	-.10823
	2	-.233309*	.05725	.002	-.41606	-.05056
	3	-.277701*	.05800	.000	-.46287	-.09253
	4	-.406329*	.05725	.000	-.58908	-.22358
	5	-.065643	.05725	.979	-.24839	.11711
	6	-.091574	.04757	.652	-.24345	.06030
	7	-.083230	.05590	.896	-.26169	.09523
	8	-.051470	.05725	.996	-.23422	.13128

	9	-.141705	.05590	.255	-.32017	.03676

*. The mean difference is significant at the 0.05 level.

1: *B. subtilis* Control; 2: *B. subtilis* 0.1 mg/L c-AgNP; 3: *B. subtilis* 1.0 mg/L c-AgNP; 4: *B. subtilis* 5.0 mg/L c-AgNP; 5: *B. subtilis* 0.1 mg/L Ag-Ion; 6: *E. coli* Control; 7: *E. coli* 0.1 mg/L c-AgNP; 8: *E. coli* 1.0 mg/L c-AgNP; 9: *E. coli* 5.0 mg/L c-AgNP; 10: *E. coli* 0.1 mg/L Ag-Ion;

Table 9: ANOVA Results for Length, Biomass, and Root Ag Content of *Z. mays* Seedlings

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Length	Between Groups	878.906	7	125.558	4.979	.000
	Within Groups	1210.488	48	25.219		
	Total	2089.394	55			
Biomass	Between Groups	.762	7	.109	1.114	.370
	Within Groups	4.693	48	.098		
	Total	5.455	55			
AgContent	Between Groups	.004	7	.001	10.641	.000
	Within Groups	.003	48	.000		
	Total	.006	55			

Table 10: Tukey's Post-Hoc Results for Length, Biomass and Root Ag Content of *Z. mays* Seedlings

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Length	1.00	2.00	3.8014	2.4402	.772	-3.930	11.533
		3.00	4.1389	2.4402	.690	-3.592	11.870
		4.00	5.9264	2.4402	.251	-1.805	13.658
		5.00	-6.8944	2.6467	.179	-15.280	1.491
		6.00	-3.4611	2.6467	.891	-11.847	4.924
		7.00	.9889	2.8010	1.000	-7.886	9.863
		8.00	-1.6944	2.6467	.998	-10.080	6.691
	2.00	1.00	-3.8014	2.4402	.772	-11.533	3.930
		3.00	.3375	2.5109	1.000	-7.618	8.293
		4.00	2.1250	2.5109	.989	-5.830	10.080
		5.00	-10.6958*	2.7121	.006	-19.288	-2.103
		6.00	-7.2625	2.7121	.155	-15.855	1.330
		7.00	-2.8125	2.8629	.975	-11.883	6.258
		8.00	-5.4958	2.7121	.476	-14.088	3.097
	3.00	1.00	-4.1389	2.4402	.690	-11.870	3.592
		2.00	-.3375	2.5109	1.000	-8.293	7.618
		4.00	1.7875	2.5109	.996	-6.168	9.743
		5.00	-11.0333*	2.7121	.004	-19.626	-2.441
		6.00	-7.6000	2.7121	.118	-16.193	.993
		7.00	-3.1500	2.8629	.954	-12.220	5.920
		8.00	-5.8333	2.7121	.399	-14.426	2.759
	4.00	1.00	-5.9264	2.4402	.251	-13.658	1.805
		2.00	-2.1250	2.5109	.989	-10.080	5.830
		3.00	-1.7875	2.5109	.996	-9.743	6.168
		5.00	-12.8208*	2.7121	.001	-21.413	-4.228
		6.00	-9.3875*	2.7121	.023	-17.980	-.795
		7.00	-4.9375	2.8629	.672	-14.008	4.133

		8.00	-7.6208	2.7121	.116	-16.213	.972
	5.00	1.00	6.8944	2.6467	.179	-1.491	15.280
		2.00	10.6958*	2.7121	.006	2.103	19.288
		3.00	11.0333*	2.7121	.004	2.441	19.626
		4.00	12.8208*	2.7121	.001	4.228	21.413
		6.00	3.4333	2.8993	.933	-5.753	12.619
		7.00	7.8833	3.0409	.184	-1.751	17.518
		8.00	5.2000	2.8993	.627	-3.986	14.386
	6.00	1.00	3.4611	2.6467	.891	-4.924	11.847
		2.00	7.2625	2.7121	.155	-1.330	15.855
		3.00	7.6000	2.7121	.118	-.993	16.193
		4.00	9.3875*	2.7121	.023	.795	17.980
		5.00	-3.4333	2.8993	.933	-12.619	5.753
		7.00	4.4500	3.0409	.822	-5.184	14.084
		8.00	1.7667	2.8993	.999	-7.419	10.953
	7.00	1.00	-.9889	2.8010	1.000	-9.863	7.886
		2.00	2.8125	2.8629	.975	-6.258	11.883
		3.00	3.1500	2.8629	.954	-5.920	12.220
		4.00	4.9375	2.8629	.672	-4.133	14.008
		5.00	-7.8833	3.0409	.184	-17.518	1.751
		6.00	-4.4500	3.0409	.822	-14.084	5.184
		8.00	-2.6833	3.0409	.986	-12.318	6.951
	8.00	1.00	1.6944	2.6467	.998	-6.691	10.080
		2.00	5.4958	2.7121	.476	-3.097	14.088
		3.00	5.8333	2.7121	.399	-2.759	14.426
		4.00	7.6208	2.7121	.116	-.972	16.213
		5.00	-5.2000	2.8993	.627	-14.386	3.986
		6.00	-1.7667	2.8993	.999	-10.953	7.419
		7.00	2.6833	3.0409	.986	-6.951	12.318
Biomass	1.00	2.00	.125125	.151931	.991	-.35624	.60649
		3.00	.099375	.151931	.998	-.38199	.58074
		4.00	.161000	.151931	.962	-.32036	.64236
		5.00	-.160500	.164793	.976	-.68261	.36161
		6.00	-.157000	.164793	.979	-.67911	.36511
		7.00	-.017000	.174400	1.000	-.56955	.53555

	8.00	-.093000	.164793	.999	-.61511	.42911
2.00	1.00	-.125125	.151931	.991	-.60649	.35624
	3.00	-.025750	.156336	1.000	-.52107	.46957
	4.00	.035875	.156336	1.000	-.45944	.53119
	5.00	-.285625	.168862	.693	-.82063	.24938
	6.00	-.282125	.168862	.705	-.81713	.25288
	7.00	-.142125	.178251	.992	-.70687	.42262
	8.00	-.218125	.168862	.897	-.75313	.31688
3.00	1.00	-.099375	.151931	.998	-.58074	.38199
	2.00	.025750	.156336	1.000	-.46957	.52107
	4.00	.061625	.156336	1.000	-.43369	.55694
	5.00	-.259875	.168862	.783	-.79488	.27513
	6.00	-.256375	.168862	.794	-.79138	.27863
	7.00	-.116375	.178251	.998	-.68112	.44837
	8.00	-.192375	.168862	.945	-.72738	.34263
4.00	1.00	-.161000	.151931	.962	-.64236	.32036
	2.00	-.035875	.156336	1.000	-.53119	.45944
	3.00	-.061625	.156336	1.000	-.55694	.43369
	5.00	-.321500	.168862	.555	-.85650	.21350
	6.00	-.318000	.168862	.569	-.85300	.21700
	7.00	-.178000	.178251	.972	-.74275	.38675
	8.00	-.254000	.168862	.801	-.78900	.28100
5.00	1.00	.160500	.164793	.976	-.36161	.68261
	2.00	.285625	.168862	.693	-.24938	.82063
	3.00	.259875	.168862	.783	-.27513	.79488
	4.00	.321500	.168862	.555	-.21350	.85650
	6.00	.003500	.180521	1.000	-.56844	.57544
	7.00	.143500	.189332	.994	-.45636	.74336
	8.00	.067500	.180521	1.000	-.50444	.63944
6.00	1.00	.157000	.164793	.979	-.36511	.67911
	2.00	.282125	.168862	.705	-.25288	.81713
	3.00	.256375	.168862	.794	-.27863	.79138
	4.00	.318000	.168862	.569	-.21700	.85300
	5.00	-.003500	.180521	1.000	-.57544	.56844
	7.00	.140000	.189332	.995	-.45986	.73986

		8.00	.064000	.180521	1.000	-.50794	.63594
	7.00	1.00	.017000	.174400	1.000	-.53555	.56955
		2.00	.142125	.178251	.992	-.42262	.70687
		3.00	.116375	.178251	.998	-.44837	.68112
		4.00	.178000	.178251	.972	-.38675	.74275
		5.00	-.143500	.189332	.994	-.74336	.45636
		6.00	-.140000	.189332	.995	-.73986	.45986
		8.00	-.076000	.189332	1.000	-.67586	.52386
	8.00	1.00	.093000	.164793	.999	-.42911	.61511
		2.00	.218125	.168862	.897	-.31688	.75313
		3.00	.192375	.168862	.945	-.34263	.72738
		4.00	.254000	.168862	.801	-.28100	.78900
		5.00	-.067500	.180521	1.000	-.63944	.50444
		6.00	-.064000	.180521	1.000	-.63594	.50794
		7.00	.076000	.189332	1.000	-.52386	.67586
AgContent	1.00	2.00	-.0000244	.00352505	1.000	-.0111928	.0111439
		3.00	-.0001154	.00352505	1.000	-.0112838	.0110528
		4.00	-.0000019	.00352505	1.000	-.0111703	.0111663
		5.00	.0269391*	.00382345	.000	.01482529	.0390529
		6.00	-.0001717	.00382345	1.000	-.0122855	.0119420
		7.00	-.0004630	.00404636	1.000	-.0132831	.0123569
		8.00	-.0001533	.00382345	1.000	-.0122671	.0119605
	2.00	1.00	.00002446	.00352505	1.000	-.0111439	.0111928
		3.00	-.0000910	.00362725	1.000	-.0115832	.0114011
		4.00	.00002246	.00362725	1.000	-.0114697	.0115146
		5.00	.0269635*	.00391787	.000	.01455060	.0393765
		6.00	-.0001472	.00391787	1.000	-.0125602	.0122656
		7.00	-.0004386	.00413570	1.000	-.0135416	.0126644
		8.00	-.0001288	.00391787	1.000	-.0125418	.0122841
	3.00	1.00	.00011549	.00352505	1.000	-.0110528	.0112838
		2.00	.00009103	.00362725	1.000	-.0114011	.0115832
		4.00	.00011349	.00362725	1.000	-.0113786	.0116056
		5.00	.0270546*	.00391787	.000	.01464164	.0394675
		6.00	-.0000562	.00391787	1.000	-.0124691	.0123567
		7.00	-.0003475	.00413570	1.000	-.0134506	.0127555

	8.00	-.0000378	.00391787	1.000	-.0124507	.0123751
4.00	1.00	.00000199	.00352505	1.000	-.0111663	.0111703
	2.00	-.0000224	.00362725	1.000	-.0115146	.0114697
	3.00	-.0001134	.00362725	1.000	-.0116056	.0113786
	5.00	.0269411*	.00391787	.000	.01452814	.0393540
	6.00	-.0001697	.00391787	1.000	-.0125826	.0122432
	7.00	-.0004610	.00413570	1.000	-.0135641	.0126420
	8.00	-.0001513	.00391787	1.000	-.0125642	.0122616
5.00	1.00	-.0269391*	.00382345	.000	-.0390529	-.0148252
	2.00	-.0269635*	.00391787	.000	-.0393765	-.0145506
	3.00	-.0270546*	.00391787	.000	-.0394675	-.0146416
	4.00	-.0269411*	.00391787	.000	-.0393540	-.0145281
	6.00	-.0271108*	.00418838	.000	-.0403808	-.0138408
	7.00	-.0274021*	.00439281	.000	-.0413198	-.0134844
	8.00	-.0270924*	.00418838	.000	-.0403624	-.0138223
6.00	1.00	.00017172	.00382345	1.000	-.0119420	.0122855
	2.00	.00014726	.00391787	1.000	-.0122656	.0125602
	3.00	.00005622	.00391787	1.000	-.0123567	.0124691
	4.00	.00016972	.00391787	1.000	-.0122432	.0125826
	5.00	.0271108*	.00418838	.000	.01384082	.0403808
	7.00	-.0002913	.00439281	1.000	-.0142090	.0136263
	8.00	.00001842	.00418838	1.000	-.0132515	.0132884
7.00	1.00	.00046306	.00404636	1.000	-.0123569	.0132831
	2.00	.00043860	.00413570	1.000	-.0126644	.0135416
	3.00	.00034756	.00413570	1.000	-.0127555	.0134506
	4.00	.00046106	.00413570	1.000	-.0126420	.0135641
	5.00	.0274021*	.00439281	.000	.01348446	.0413198
	6.00	.00029134	.00439281	1.000	-.0136263	.0142090
	8.00	.00030976	.00439281	1.000	-.0136079	.0142274
8.00	1.00	.00015330	.00382345	1.000	-.0119605	.0122671
	2.00	.00012884	.00391787	1.000	-.0122841	.0125418
	3.00	.00003780	.00391787	1.000	-.0123751	.0124507
	4.00	.00015130	.00391787	1.000	-.0122616	.0125642
	5.00	.0270924*	.00418838	.000	.01382239	.0403624
	6.00	-.0000184	.00418838	1.000	-.0132884	.0132515

	7.00	-.0003097	.00439281	1.000	-.0142274	.0136079

*. The mean difference is significant at the 0.05 level.

1.00: *Z. mays* Control; 2.00: *Z. mays* 1.0 mg/L c-AgNP; 3.00: *Z. mays* 5.0 mg/L c-AgNP; 4.00: *Z. mays* 0.1 mg/L Ag-Ion; 5.00: *Z. mays + B. subtilis* Control; 6.00: *Z. mays + B. subtilis* 1.0 mg/L c-AgNP; 7.00: *Z. mays + B. subtilis* 5.0 mg/L c-AgNP; 8.00: *Z. mays + B. subtilis* 0.1 mg/L Ag-Ion.