EFFECT OF SILVER NANOPARTICLE (AgNPs) ON ‘KDML 105’ RICE *Oryza sativa* L. SEEDLINGS

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ภัควิรุฬห์ ถือสมบัติ : ผลของอนุภาคเงินระดับนาโนเมตรต่อการเจริญเติบโตของต้นกล้าข้าวพันธุ์ขาวดอกมะลิ 105 (EFFECT OF SILVER NANOPARTICLE (AgNPs) ON 'KDML 105' RICE *Oryza sativa* L. SEEDLINGS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.ศุภจิตรา ชัชวาลย์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร.สุพจน์ หารหนองบัว, รศ. ดร.สนอง เอกสิทธิ์, 121 หน้า.

งานวิจัยครั้งนี้มีจุดประสงค์เพื่อศึกษาผลของอนุภาคเงินระดับนาโนเมตรที่ขนาดแตกต่างกันคือ 20, 30-70, 70-120 และ 150 นาโนเมตร และความเข้มข้นแตกต่างกันคือ 0.1, 1, 10, 100 และ 1,000 มิลลิกรัมต่อลิตร ที่มีต่อการเจริญของต้นกล้าข้าวพันธุ์ขาวดอกมะลิ 105 ภายใต้สภาวะปกติและสภาวะเค็ม บนสมมุติฐานว่าอนุภาคเงินระดับนาโนเมตรจะส่งต่อการเจริญของต้นกล้าข้าวโดยอาจจะยับยั้งหรือส่งเสริมการเจริญเติบโตของต้นกล้าข้าวรวมถึงการเปลี่ยนแปลงทางสัณฐานวิทยาและสรีรวิทยาของต้นกล้าข้าว ผลการทดลองพบว่าภายใต้สภาวะปกติและสภาวะเค็มตัวชี้วัดการเจริญเติบโต ทั้งน้ำหนักสด, น้ำหนักแห้ง, ความสูงของต้นและความยาวรากจะลดลงซึ่งสอดคล้องกับการเพิ่มขึ้นของขนาดและความเข้มข้นของอนุภาคเงินระดับนาโนเมตรที่ใช้ในการแช่เมล็ดข้าวก่อนการปลูกเป็นเวลา 24 ชั่วโมง การส่งเสริมการเจริญของต้นกล้าข้าวพบภายใต้สภาวะเค็มพบ เมื่อแช่เมล็ดที่ความเข้มข้น 1 และ 10 มิลลิกรัมต่อลิตรที่ขนาดอนุภาค 20 นาโนเมตร โดยผลปรากฏว่าการแช่เมล็ดในสภาวะดังกล่าวก่อนการเพาะเมล็ดช่วยเพิ่มน้ำหนักสดและความยาวของรากเมื่อเปรียบเทียบกับชุดควบคุมและชุดทดลองอื่น ๆ จากการวิเคราะห์การสะสมอนุภาคเงินระดับนาโนเมตรในเนื้อเยื่อของต้นกล้าข้าว พบว่าการสะสมของอนุภาคเงินระดับนาโนเมตรอยู่ในระดับสูงเมื่อแช่ด้วยอนุภาคเงินระดับนาโนเมตรขนาดเล็ก อย่างไรก็ตาม การสะสมของอนุภาคเงินระดับนาโนเมตรขนาด 20 นาโนเมตรจะพบการสะสมในรากมากกว่าการนำส่งสู่ใบ ดังนั้นอนุภาคเงินระดับนาโนเมตรขนาดเล็กจะส่งผลเสียต่อการเจริญได้น้อยกว่าอนุภาคเงินระดับนาโนเมตรขนาดใหญ่ เมื่อแช่อนุภาคเงินขนาดใหญ่ 150 นาโนเมตรที่ความเข้มข้น 100 มิลลิกรัมต่อลิตรจะขัดขวางการทำงานของเซลล์ นอกจากนั้น ยังได้ศึกษาปริมาณไฮโดรเจนเปอร์ออกไซด์และกิจกรรมของเอนไซม์ต้านสารก่อออกซเดชันภายใต้สภาวะปกติและสภาวะเค็ม พบว่าเมื่อแช่เมล็ดด้วยอนุภาคเงินระดับนาโนเมตรจะทำให้ปริมาณไฮโดรเจนเปอร์ออกไซด์ทั้งภายใต้สภาวะปกติและสภาวะเค็มสูงขึ้นโดยปริมาณไฮโดรเจนเปอร์ออกไซด์จะสูงกว่าเมื่ออยู่ภายใต้สภาวะเค็ม และเมื่อแช่เมล็ดในอนุภาคเงินขนาด 150 นาโนเมตรที่ความเข้มข้น 100 มิลลิกรัมต่อลิตรจะทำให้ปริมาณไฮโดรเจนเปอร์ออกไซด์เพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับชุดควบคุม บ่งบอกถึงความเครียดที่มากขึ้นถูกชักนำโดยอนุภาคเงินระดับนาโนเมตรซึ่งสนับสนุนผลของอนุภาคเงินระดับนาโนเมตรยับยั้งการเจริญของต้นกล้าข้าว นอกจากนั้น การแช่เมล็ดในอนุภาคเงินขนาด 150 นาโนเมตรที่ความเข้มข้น 100 มิลลิกรัมต่อลิตรมีผลต่อการเพิ่มกิจกรรมของเอนไซม์ CAT, SOD, APX, GR และ GPX ผลการทดลองที่น่าสนใจคือภายใต้สภาวะเค็มเมื่อแช่เมล็ดที่ความเข้มข้น 10 มิลลิกรัมต่อลิตรที่ขนาด 20 นาโนเมตร ในรากที่แช่ด้วยอนุภาคนาโนเงินระดับนาโนเมตรพบว่ากิจกรรมของเอนไซม์ SOD และ APX จะสูงขึ้นเมื่อเปรียบเทียบกับรากที่ไม่ได้ผ่านการแช่ด้วยอนุภาคนาโนเงินระดับนาโนเมตร ผลการทดลองนี้บ่งบอกได้ว่าขนาดอนุภาคเงินระดับนาโนเมตรและความเข้มข้นที่เหมาะสมสามารถใช้เป็น seed priming agent สำหรับการปรับตัวภายใต้สภาวะเค็ม

สาขาวิชา วิทยาศาสตร์นาโนและเทคโนโลยี ลายมือชื่อนิสิต .....

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KEYWORDS: SILVERNANOPARTICLES / ORYZA SATIVA L. / SALT STRESS / ANTIOXIDANT ENZYMES

PAKVIRUN THUESOMBAT: EFFECT OF SILVER NANOPARTICLE (AgNPs) ON 'KDML 105' RICE *Oryza sativa* L. SEEDLINGS. ADVISOR: ASSOC. PROF. SUPACHITRA CHADCHAWAN, Ph.D., CO-ADVISOR: PROF. SUPOT HANNONGBUA, Dr.rer.nat., ASSOC. PROF. SANONG EKGASIT, Ph.D., 121 pp.

This research was aimed to study the effects of different sizes 20, 30-70, 70-120 and 150 nm and different concentration 0.1, 1, 10, 100 and 1,000 mg/L of AgNPs on rice *Oryza sativa* L. seedlings under normal and salt stress conditions based on the hypothesis that AgNPs had affected on rice seedlings growth by possibly inhibition or induction of rice growth, including morphological and physiological changes.The results revealed that under normal and salt stress conditions, all growth parameters, including fresh weight, dry weight, shoot height and root length were decreased relatively to the increasing size and concentration of AgNPs pretreatment by 24 hour-seed soaking prior to germination. The positive result was detected under salt stress condition, when the seeds were soaked with 1 or 10 mg/L of 20 nm AgNPs. The AgNP pretreatment significantly enhanced root fresh weight and root length in comparison with the control and other treatments. Based on the analysis of AgNPs accumulation in plant tissue, the higher uptake was found when the rice seeds were treated with smaller size of AgNPs. However, the high level of 20 nm AgNP uptake was trapped in the root rather than transported to the leaves. Therefore, the smaller size had the less negative effect on seedlings growth than the larger one, 150 nm diameter AgNPs. Cell disruption in the seedling leaves was detected, when the seeds were pretreated with 150 nm AgNPs at the higher concentration (100 mg/L). Moreover, hydrogen peroxide (H_2O_2) content and anti-oxidative enzyme activities were quantified under normal and salt stress conditions. When seeds pretreated with AgNPs, H_2O_2 was induced in both normal and salt stress conditions. The level of H_2O_2 contents in the salt treated plants was higher. The seed pretreatment with the 150 nm AgNPs at concentratin of 100 mg/L significantly increased hydrogen peroxide higher than control, suggesting the higher level of stress was induced by AgNP pretreatment. These supported the growth inhibition effects by AgNPs. Furthermore, 100 mg/L of 150 nm AgNP presoaking enhanced CAT, SOD, APX, GR and GPX activity. The interesting results were detected under salt stress condition when seeds were exposed to 10 mg/L of 20 nm AgNPs. SOD and APX activity in roots of seedlings germinated from the pretreated seeds were higher than the activities detected in no AgNP pretreatment control. This suggested that the appropriate size and concentration of AgNPs can be used as seed- priming agent for salt stress acclimation.

Field of Study: Nanoscience and Technology Student's Signature .....

Academic Year: 2014 Advisor's Signature .....

Co-Advisor's Signature .....

Co-Advisor's Signature .....

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CHAPTER I

INTRODUCTION

From the recent development of nanotechnology, there has been a rapid growth of research in the areas of nanomaterials and nanoscience because of their unique optical, mechanical, electrical and magnetic properties. These include their high surface to volume ratio, physiochemical properties, surface reactivity, structure and surface composition of nanoparticles (NPs) [1]. Metal and metal oxide NPs, such as those of Ag, Fe, ZnO, TiO_2 and MnO, have been exploited commercially for a variety applications. Moreover, they have already been used in numerous consumer products, including textiles, personal care products, food storage containers, laundry additives, home appliances, paints, and even food supplements [2], increasing their potential exposure levels to humans and the environment.

With the advancement in nanotechnology, silver NPs (AgNPs) have been applied in many industries, including daily products and medical products. To date, there has been the limited number of research on the toxicological impact of AgNPs when released into the environment. Worldwide, the present production of AgNPs is estimated to be around 500 tons per year and this is predicted to increase over the next few years [3]. AgNPs may be released into the environment by several routes, including during their synthesis, incorporation of the AgNPs into other goods, and recycling or disposal of these goods and AgNPs. Most safety investigations have focused on studying the effects of different nanomaterials on the morphology, behavior and function of cells, and on the selective ability to kill microbes, in order to understand how such structures could possibly affect animals and humans at various levels. However, evaluation of the toxicity of NPs to other components of the

environment is limited. NPs have been shown to have higher and unique toxicity than their corresponding bulk materials [4]. Thus, a better knowledge of nanomaterials, including their mode of interaction, uptake, accumulation and impact on the biosystems, and on their control measures to avoid nano-pollution in ecological systems, is of concern and increasing importance [5].

AgNPs have a dimension of 1–100 nm. The most important influences in determining the degree of bioaccumulation of AgNPs are likely to be similar to those that influence metal contaminants. For example, the processes that govern bioavailability and bioaccumulation of AgNPs in any circumstance are likely to be the result of the combined influences of the (i) concentration and (ii) nature of the NPs, (iii) the nature of the environment, (iv) the route of exposure, and (v) the biology and functional ecology of the organism involved [6].

From these parameters the level of bioaccumulation can be broadly predicted for the (i) species under study, (ii) the contaminant level of AgNPs, and (iii) the environmental conditions of the study. AgNPs released into an aquatic environment can be a source of dissolved/suspended silver ions or metals, respectively, and so potentially exert toxic effects on aquatic organisms [7, 8]. Although the toxicity of AgNPs has been examined to some extent, the mechanisms of the toxicity remain unclear. The impact of various types of NPs on higher plants has also been examined [9-13], whilst Ag^{+} ion toxicity *in vivo* has been researched in some detail in freshwater fish species, with derived LC_{10} values being as low as 0.8 μg/L for certain freshwater species [14]. At high concentrations (in the low μM range), NPs can impart important physiological consequences, such as blood acidosis that can ultimately lead to circulatory collapse and death, whilst multiwalled carbon nanotubes (MWCNT) can produce oxidative stress and cause lipid peroxidation in the brain of rainbow trout fish [15].

When growing, plants absorb relatively large amounts of essential and nonessential elements, which at certain concentrations may be toxic. Once stored within the plants, beneficial or toxic elements can be transferred along the food chain to consumers. Rice (*Oryza sativa* L.) is an important human food crop worldwide and is considered to be a model for monocot species for molecular biology research [16], and so it is a reasonable and suitable species for research on nanomaterial safety.

The objectives of this study are:

1. To determine the effects of different sizes and concentrations of silver nanoparticles on rice seedling growth.
2. To determine the accumulation of silver nanoparticles on rice plants
3. To determine the effects of silver nanoparticles on morphological and physiological responses.
4. To determine the effects of silver nanoparticles on physiological responses of plants under salt stress.

CHAPTER II

LITERATURE REVIEW

1. Rice (*Oryza sativa* L.)

Rice is an important crop of Thailand and the main food of Asia, Latin America and Africa[17] . Since rice has a genome significantly smaller than those of other cereals, it is a good model for genetic and molecular studies in plants[18]. In worldwide, there are rice areas more than 100 million ha and approximately 89% are in Asia. Forty-five percent of the rice grown area is rain-fed, 25% is upland. The large rain fed rice grown areas in Asia are in eastern India, Bangladesh, northeast of Thailand, Cambodia, and the island of Sumatra in Indonesia[19]. Two species of rice which consist of *Oryza sativa* and *Oryza glaberrima* are grown worldwide and in parts of West Africa.

1.1 Rice under salt stress

Soil salinity is a major constraint to food production because it limits crop yield and restricts use of land previously uncultivated. Salt stress caused by excess NaCl in the soil affects plant physiology in a variety of ways. Plants growth is restrained because water uptake is inhibited by the osmotic effect salinity. Moreover, Na^+ and Cl^- taken up by roots and transported to shoots via the transpiration stream may lead to toxic ion concentration in plant cells. The stresses created by a high salt concentration in the soil solution are 2-fold. First, many of the salt ions are toxic to plant cells when present at high concentrations externally or internally. Typically, sodium chloride constitutes the majority of the salts. Sodium ions are toxic to most plants, and some plants are also inhibited by high concentrations of chloride ions. Second, high salt represents a water deficit or osmotic stress because of decreased

osmotic potential in the soil solution. The deleterious effects of salinity on plant growth are associated with (1) low osmotic potential of soil solution (water stress), (2) nutritional imbalance, (3) specific ion effect (salt stress), or (4) a combination of these factors [20, 21]. All of these cause adverse physiological effects on plant growth and development at physiological and biochemical levels [22, 23] and at the molecular level [24, 25]. Plant salt tolerance has generally been studied in relation to regulatory mechanisms of ionic and osmotic homeostasis [26-28].

1.2 Effect of salt stress on plant response

General symptoms of damage by salt stress are growth inhibition, accelerated development and senescence, including death during prolonged exposure. Growth inhibition is the primary injury that leads to other symptoms although program cell death may also occur under severe salinity condition. An immediate effect of osmotic stress on plant growth is its inhibition of cell expansion due to the reduction of cell turgor pressure. The response of plants to adverse effects of salinity is regulated by different external and internal factors. Salt stress involves a combination of dehydration or osmotic-related stress effects, and damage due to excess sodium ions. Osmotic stress reduces the ability of plants to take up water and minerals. It not only reduces the growth rate in proportion to the salinity level, but also the tiller numbers in maize plants [29]. One of the biochemical changes occurring when plants are subjected to salt stress is the accumulation of reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet}$) [30]. ROS can seriously disrupt normal metabolism through oxidative damage to lipids, protein and nucleic acids and damage membrane functions [31]. Plant have involved to protect them from these potential phytotoxic effect such as the system that reacts with active forms of oxygen and keeps them at a low level (e.g. superoxide dismutase, catalase and peroxidases), and the system

that regenerates oxidized antioxidants (e.g. glutathione reductase and ascorbate peroxidase) [27]. Yu et al. (2014) studied effect of the enhanced salt to grapevine plant, salt stress resulted in the deterioration of plant growth and photosynthesis, and salt treatment to plantlets contributed to the enhanced ROS scavenging activity and a protection of photosynthetic apparatus from oxidative damage. Under salt stress, there was higher Na^{+} accumulation in roots than in stems and leaves, and Ca^{2+}, Mg^{2+} and P^{3+} content, as well as K^{+}/Na^{+} ratio were affected. It was demonstrated that activities of antioxidant defense enzymes changed in parallel with the increased H_2O_2. The salinity appeared to be associated with differential regulation of distinct SOD and POD isoenzymes. NaCl stress had little effect on the protein pattern in the roots. These reported provides some mechanism of woody plant responses to salt stress [27] . Moreover, Valifard et al. (2014) reported the effects of salt stress on volatile compounds, total phenolic content and antioxidant activities in *Salvia mirzayaniiis*. The results showed that both of total phenolic and some important volatile components was induced by moderate salinity and antioxidant enzymes activity were changed [32]. Qin et al. (2014) studied the effect of salt stress on growth and physiology in amaranth and lettuce, the results showed that the salt stress effect is insignificant on above-ground biomass output, leaf photosynthesis rate, F_v/F_m, photosynthesis pigment contents, activities of antioxidant enzymes, and inducing lipid peroxidation [33].

2. Antioxidant Systems

Plants subjected to various abiotic stresses resulting in producing reactive oxygen species (ROS) which were highly reactive and toxic substances. The ROS caused the damage to proteins, lipids, carbohydrates and DNA [34]. In order to plant protective

from various stresses, plants produced enzymatic and non-enzymatic mechanisms to eliminate or reduce their damaging effects [35]. The importance of antioxidant enzymes is their ability to scavenge ROS and thereby prevent oxidative damage. The antioxidant system comprises several enzymes such as superoxide dismutase (SOD), catalase (CAT), and guaiacol peroxidase (G-POD). Generated superoxide radicals were converted to H_2O_2 by the action of SOD, and the accumulation of H_2O_2 is prevented by the activities by APX, CAT, G-POD and GPX. Thus, the balance between ROS generation and detoxification determines the survival of the system. Besides, non-enzymatic anti-oxidative carotenoids (Car) such as carotene and xanthophylls can also eliminate ROS (e.g. $_1O^2$) and stabilize photosynthetic complexes [36].

2.1 Salt stress and antioxidant systems

Reactive oxygen species (ROS) caused the cellular damage during salt stress. Thai aromatic rice (cv. KDML105) is a well-known economically important Thai cultivar highly recognized in the international market (known as Thai Hom Mali Rice or Thai Jasmine Rice) as the world's best quality aromatic rice. However, 'KDML105' rice is sensitive to salt stress. It results in low yield and poor grain quality when it is grown under saline soils [37]. In plant species, the enzymatic components for ROS scavenging systems are superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POD), glutathione reductase (GR) and monodehydroascorbate reductase (MDAR). Non-enzymatic components included flavanones, anthocyanins, carotenoids and ascorbic acid (AA) [38]. Up to date, there are more reports in salt stress on plant including in rice (*Oryza sativa* L.), which were studied the inductive responses of the antioxidant enzymes by salt stress. The results showed that in rice leaves, salt stress preferentially enhanced the content of H_2O_2 as well as the activities of the SOD, APX, and G-POD, whereas catalase activity was decreased [39]. In wheat seedlings, the response of exogenous jasmonic acid (JA)

in salt-stress plants resulted in the effective protection from salt stress damage by enhancing activities of antioxidant enzymes and the concentration of anti-oxidative compounds to eliminated the excessive ROS caused by salt stress and presented a practical implication for wheat cultivation in salt-affected soils [40].

2.2 Enzymatic antioxidant system

Exposure of plants to unfavorable environmental conditions such as salt stress can increase the production of ROS e.g., $^1O_2$, O_2^-, H_2O_2 and OH^-. To protect themselves against these toxic oxygen intermediates, plant cells and its organelles like chloroplast, mitochondria and peroxisome employ antioxidant defense systems. The components of antioxidant defense system are enzymatic and non-enzymatic antioxidants (Figure 1) [41] . Enzymatic antioxidants include SOD, CAT, APX, MDHAR, DHAR and GR and non-enzymatic antioxidants are GSH, AA (both water soluble) carotenoids and tocopherols (lipid soluble).

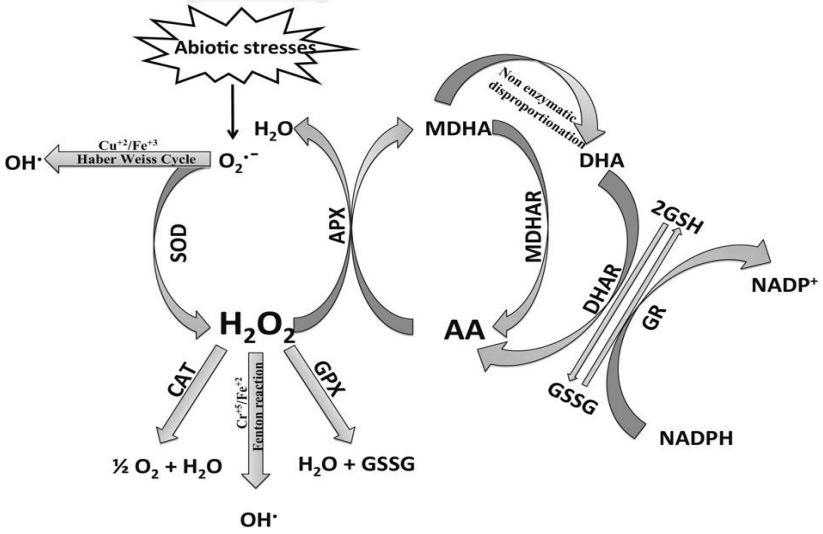

Figure 1. Reactive Oxygen Species (ROS) and antioxidants defense mechanism [41].

2.2.1 Superoxide dismutase (SOD) EC 1.15.1.1

Metalloenzyme SOD is the most effective intracellular enzymatic antioxidant which is ubiquitous in all aerobic organisms and in all subcellular compartments prone to ROS mediated oxidative stress. SOD has been proposed to be important in plant stress tolerance and provide the first line of defense against the toxic effects of elevated levels of ROS. The SODs remove O_2^- by catalyzing its dismutation, one O_2^- being reduced to H_2O_2 and another oxidized to O_2 [42].

2.2.2 Catalase (CAT) EC 1.11.1.6

CAT is a tetrameric heme containing enzymes with the potential to directly dismutase H_2O_2 into H_2O and O_2 and is indispensable for ROS detoxification during stressed conditions [43].

2.2.3 Ascorbate peroxidase (APX) EC 1.11.1.11

APX is thought to play the most essential role in scavenging ROS and protecting cells in higher plants, algae, *Euglena* and other organisms. APX is involved in scavenging of H_2O_2 in water-water and ASH-GSH cycles and utilizes ASH as the electron donor [35].

2.2.4 Glutathione reductase *(GR)*

GR is a flavo-protein oxidoreductase, found in both prokaryotes and eukaryotes which converts the oxidized dimer (GSSG) to the reduced monomer (GSH) [44]. It is a potential enzyme of the ASH-GSH cycle and plays an essential role in defense system against ROS by sustaining the reduced status of GSH.

2.2.5 Glutathione peroxidase (GPX) (EC 1.11.1.9)

GPX is a large family of diverse isozymes that use GSH to reduce H_2O_2 and organic and lipid hydro-peroxides, and therefore help plant cells from oxidative stress [42].

3. Nanoparticles

Nanoparticles (NPs) are widely used in extensive range of applications in different fields. NPs are nano-objects with all three external dimensions in the nanoscale, where nanoscale is defined as a size range from approximately 1 to 100 nm and show size-dependent properties that strikingly differ from those of the bulk material [45]. The NPs can be natural or synthetic. NPs naturally present in the environment was from natural events such as terrestrial dust storm, volcanic eruptions, erosion and forest fire. Moreover human activities introduce NPs in the environment, as by-products of simple combustion or generated by combustion engines, power plants and other thermo-degradation system. On the other hand, the advent of the nanotechnological industry is now exposing man to a new category of NPs, the engineered NPs, which encompass multiple chemical compositions, shapes and sizes. There has been a rapid growth of research in the areas of nanomaterials and nanoscience because of their unique optical, mechanical, electrical and magnetic properties. These include, high surface to volume ratio, physiochemical properties, surface reactivity, structure and surface composition [46]. The processes that govern

bioavailability and bioaccumulation of NPs in any circumstances are likely to be the result of the combined influences of 1) concentration of the NPs 2) the nature of the nanoparticle 3) the nature of the environment 4) the route of exposure and 5) the biology and functional ecology of the organism involved [47]. From these parameters bioaccumulation can be predicted for 1) the species under study, 2) the contaminant of silver nanoparticles and 3) the environmental conditions of the study. AgNPs may be released to the environment by several routes, including during synthesis, during

manufacturing and incorporation of the NPs in goods and recycling or disposal of goods and NPs.

3.1 Silver nanoparticles (AgNPs)

Ag NPs are among the most widely used NPs, basically due to their important antibacterial properties. Ag NPs have distinctive physicochemical properties, including high electrical and thermal conductivity, surface enhanced Raman scattering, chemical stability, catalytic activity, and non-linear optical behavior. These properties make them interesting to be used in inks, microelectronics, and medical imaging [48].With the advancement in nanotechnology, silver nanoparticles (AgNPs) have been applied in many industries, including daily products and medical products. To date, there has been the limited number of research on the toxicological impact of AgNPs, that are released to the environment. Worldwide, the present production of AgNPs is estimated at 500 tons per years and the increase in the volume manufacturing volume is predicted for the next few years [49].

Colloidal silver nanoparticles can be prepared by physical, biological and chemical methods. The chemical approach is mostly used and consists of the treatment of silver salts with a chemical reducing agent, such as hydrazine, ethylenediaminetetraacetic acid and above all sodium borohydride. However, most of these reducing agents are considered as non-environmentally friendly component. . Recently, efforts have been made for developing green methods to prepare silver nanoparticles, green synthesis of nanoparticles is the field of nanoparticle synthesis and assembly by utilization of biological systems such as yeast, fungi, bacteria and plant extracts. This technique is popular these days because of its vast reserves of plants that are easily accessible, widely distributed, safe to handle, availability of wide range of metabolites and minimize the wastes and energy cost [50].

3.2 Effect of nanoparticles on plant seedling responses

From the dispersion of AgNPs to the environment by several way that explain in 3.1, the reports showed positive or no effects of nanoparticles on plants**.** The effect of silver nanoparticles exposure was reported in rice (*Oryza sativa* L.) seedlings on physiological and molecular changes. The seedlings were exposed to different concentrations of AgNPs . Significant reduction in root elongation, shoot and root fresh weights, total chlorophyll and carotenoids contents were observed and AgNPs caused significant increase in hydrogen peroxide formation and lipid peroxidation in shoots and roots [51]. Seed germination percentage was significantly reduced under stress of nano-Cu. It was also resulted in the loss of root cells viability, severe oxidative burst, decline in carotenoids level and antioxidant enzyme activities [31]. Moreover, the researcher studied the effect of silver nanoparticles on rice plants and its rhizosphere bacteria, the results showed the effect on growth, photosynthetic pigment, production of the reactive oxygen species (ROS) and AgNPs may damage bacterium cell wall and transform them to protoplasts [52]. The studies on nano-SiO_2 were performed in tomatoes. It was reported that nano-SiO_2 enhanced seed germination percentage, seed germination index, seed vigor index, seedling fresh weight and dry weight [53]. However, negative effect of nickel oxide nanoparticles (NiO-NPs) was also reported on tomatoes. Due to the dissolution of Ni ions from NiO-NPs, it induced cell death and triggered the mitochondrial dependent intrinsic apoptotic pathway [54]. Other response especially to antioxidant enzyme were reported, in kidney bean, cerium oxide nanoparticles ($nCeO_2$) was found to induce antioxidant enzyme activity and soluble protein content [55]. ZnO nanoparticles effect on growth, metabolism and tissue specific accumulation in *Brassica juncea* were studied and the results showed the significant decrease in plant biomass, the

increase in proline content and lipid peroxidation. The estimation of the antioxidant enzymes, CAT, ascorbate peroxidase (APX), glutathione reductase (GR) and superoxide dismutase and SOD activities in different plant tissues was done [56]. ZnO nanoparticles was effected to soybean by delay plant development and reproduction [57].

CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. Plant materials

Jasmine rice (*Oryza sativa* L. cultivar Khao Dawk Mali 105 (KDML 105) was used in all experiments. The rice seeds were kindly provided by Pathum Thani Rice Research Center.

2. Silver nanoparticles

Silver nanoparticles with various size (20, 30-70, 70-120 and 150 nm) and concentration (0.1, 1.0, 10.0, 100.0 and 1,000 mg/L) were used in this study. They were provided by Sensor Research Unit, Department of chemistry, Faculty of science, Chulalongkorn university, which synthesized by green nanotechnology synthesis method (see in Appendix B).

3. Instruments

3.1 Equipment for plant growing

-150 ml glass bottles

-sand

-pots with 12.5 cm diameter

3.2 Equipment for plant growth determination

-aluminium foil

-1.5 ml Eppendorf tube

-forceps

-hot air oven

-ruler

-scissors

-balance: Sartorius CP423s (Scientific Promotin Co. USA)

3.3 Equipment for enzymatic and non-enzymatic antioxidant analysis

-motars and pestles

-spatula

-refrigerated centrifuge (Universal 32R, Hettich, Germany)

-spectrophotometer (Agilent Technology, USA)

-microplate reader (VERSA max, USA)

-centrifuge (Universalt6, Hettich, Germany)

-multi channel pipette

-plate

3.4 Equipment for anatomical study

-microtome MT-3

-slide glass

-cover slide

-stryrofoam

- Olympus DP70 photomicroscope.

-safranin-O dye

3.5 Equipment for AgNPs accumulation test

-Inductively coupled plasma optical emission spectrometry (ICP-OES; PerkinElmer Optima 4300 DV,German)

-centrifuge (Universalt6, Hettich, Germany)

-1,000 ml flask

-microwave

3.6 Equipment for silver nanoparticles characterizes

-transmission electron microscope (TEM) Hitachi H-7650

-scanning electron microscope (SEM) JSM-6510

- dynamic light scattering (DLS) ELS 8000 Otsuka Electronics Osaka Japan

- size analyzer (Microtrac Zetztrac model NPA 152-31a-0000-000-20M

4. Chemicals and reagents

4.1 Chemicals for silver nanoparticles treatment and salt stress

-silver nanoparticles

-WP No.2 nutrient solution (Vajrabhaya and Vajrabhaya, 1991) (see in Appendix A)

-10% polyethylene glycol 6000 (PEG6000)

-distilled water

-0.1% acetic acid

4.2 Chemicals for H_2O_2 determination

-phosphate buffer (pH 6.5) (see in Appendix A)

-hydroxylamine

-titanium sulphate

-sulfuric acid

4.3 Chemicals for enzymatic extraction and assay

4.3.1 Extraction buffer

-0.1 M potassium phosphate buffer (pH 7.0)

-2 mM ethylenediaminetetraacetic acid (EDTA)

-1 mg/ml dithiothreitol (DTT)

-1% (w/v) polyvinylpyrrolidone (PVPP)

-100μL/10ml phenylmethylsulfonyl fluoride (PMSF)

4.3.2 Catalase (CAT) activity assay

-50 mM potassium phosphate buffer (pH 7.0)

-100 mM H_2O_2

4.3.3 Superoxide dismutase (SOD) activity assay

-216 mM potassium phosphate buffer (pH 7.0)

-10 mM EDTA

-1.1 mM cytochrome C

-0.2 mM xanthine

-xanthine oxidase

4.3.4 Ascorbate peroxidase (APX) activity assay

-50 mM potassium phosphate buffer (pH 7.0)

-100 mM EDTA

-11.6 mM H2O2

-100 mM ascorbic acid

4.3.5 Glutaione reductase (GR) activity assay

-100 mM potassium phosphate buffer (pH 7.0)

-3.4 mM EDTA

-30 mM GSSG

-0.8 mM β-NADPH

4.3.6 Gluthathione peroxidase (GPX) activity assay

-100 mM potassium phosphate buffer (pH 7.0)

-0.5 mM EDTA

-1 mM NADPH

- 2 mM t-butyl hydroperoxide

-2 mM reduced glutathione

- glutathione reductase

4.4 Standard protein

-bovine serum albumin (BSA) (2 µg/ml) Sigma-Aldrich, USA

II. METHODS

1. Effects of the size and concentrations of AgNPs on 'KDML 105' rice *Oryza sativa* L. seedling growth.

1.1 Plant cultivation and growth measurement under normal and salt stress condition.

Rice seeds (150 seeds per treatment) were soaked in the aqueous AgNPs suspension of the specific AgNPs size, with a diameter of 20, 30–60, 70–120 or 150 nm (provided by Assoc. Prof. Dr. Sanong Ekasit and the preparation method was shown in appendix B) and concentration of 0.1, 1, 10, 100 or 1,000 mg/L for 24 h, and then sand germinated in a pot. After 7 days of germination, WP No.2 solution was added to the pot as the nutrient source for seedling growth and grown on sand for another 7 days before transferred to a fresh hydroponic WP No. 2 solution. When they were 14 days old, they were divided into two conditions;

1. In normal condition, rice plants were transformed to fresh hydroponic WP No. 2

2. In salt stress condition, rice plants were transformed to fresh hydroponic WP No.2 solution added with 0.5%NaCl.

They were grown in hydroponics system for 7 days in order to get the healthy root system and make them appropriate for root harvest and root growth determination. Seedlings were grown in a greenhouse under natural light and temperature.

1.2 Data collection

Plants were collected after 3 weeks of germination for shoot fresh weight, shoot dry weight, root fresh weight, root dry weight, shoot height and root length.

1.3 Silver nanoparticles accumulation

After germination for 21days, the treated rice seedlings were dried at 30 ^{0}C for 4 days and collected for the AgNPs accumulation analysis by inductively coupled plasma optical emission spectrometry (ICP-OES;Perkin Elmer Optima 4300DV, German). A representative sample of up to 0.5 g is digested in 9 mL of concentrated nitric acid and 3 mL of hydrofluoric acid for 15 minute using the microwave heating system. The temperature profile was specified to permit specific reactions and incorporates reaching 180±5 $^{\circ}$C in approximately less than 5.5 min and remaining at 180±5 $^{\circ}$C for 9.5 min for the completion to specific reaction (Dugo etal.,2012; Kailasa and Wu,2012;Ojeda and Rojas,2013). After cooling the vessel contents were filtered, centrifuged and then decanted. Then the sample volume was adjusted and analyzed by ICP-OES.

1.4 Effect of AgNPs on anatomy of rice leaves

Transverse sections were made of selected leaves from 21 day-old seedlings grown without exposure to AgNPs (control) or after exposure as seeds for 24 h to 20-nm and 150 nm-diameter AgNPs at 10 mg/L and 100 mg/L. Transverse cross-sections of 80 mm thick were made using a plant microtome MT-3, and specimens were examined and photographed under white light using an Olympus DP70 photomicroscope.

1.5 Experimental design and statistical analysis

A completely randomized design (CRD), with five replicates and four plants per replicate, was used to determine the effect of AgNPs on rice seedling growth. The growth parameters, in terms of the shoot and root fresh weights, shoot and root

dry weights, plant height and root length, were measured at 21days after germination. The results are expressed as the mean ± standard error (SE) of five replicates and data were analyzed using one-way analysis of variance (ANOVA) with Dunnett's post hoc test to determine the significance relative to the control. In all cases, $p < 0.05$ was considered significantly difference.

2. AgNPs effects on physiological changes during normal and salt stress

2.1 Growing condition and AgNPs and salt stress treatment

Rice seeds (150 seeds per treatment) were soaked in the aqueous AgNPs suspension of the specific AgNPs size, with a diameter of 20, 30–60, 70–120 or 150 nm (provided by Assoc. Prof. Dr. Sanong Ekasit and the preparation method was shown in appendix B) and concentration of 1, 10 and 100 mg/L for 24 hours. After 7 days of germination, WP No.2 solution was added to the pot as the nutrient source for seedling growth. After that (14 days old), were divided into two condition;

1. In normal condition, rice plants were transferred to fresh hydroponic WP No. 2.

2. In salt stress condition, rice plants were transferred to fresh hydroponic WP No.2 solution added with 0.5%NaCl.

Then, they were grown in hydroponics system and collected the data at 1, 3, 5 and 7 days after treatments under normal and salt stress condition

The hypothesis of this experiment is under salt stress; priming with AgNPs resulted in an increase in antioxidant enzyme activities and enhanced rice acclimation to salt stress.

2.2 Data collection

Plant root and leaves were collected from 14 days-old plants under normal and salt stress condition. For determination of hydrogen peroxide (H_2O_2), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPX) and ascorbate peroxidase (APX) were collected on day 1, 3, 5 and 7 days after treatments, normal and salt stress conditions.

2.3 Experimental design and statistical analysis

The experiment was performed with completely randomized design (CRD) with 5 replicates. For statistical analysis, the data were subjected to analysis of variance and the mean comparison was done using Duncan's multiple range test (DMRT) with $p<0.05$ using SPSS software (version 13.0). The data were shown as mean ± S.E. (standard error).

2.4 Measurement of some physiological changes

2.4.1 H_2O_2 content

One hundred milligrams of leaf tissues were extracted and were added with 1 ml phosphate buffer (pH 6.5) containing 1 mM hydroxylamine. After centrifuge at 13,000 rpm for 25 min, 0.5 ml of 0.2% titanium sulphate in 20% H_2SO_4 (v/v) (freshly preparation) was added to 1 ml of supernatant and then centrifuged at 10,000 rpm for 15 min. To determine H_2O_2 content, the supernatant was measured at 410 nm as described by Jana and Choudhuri (1982). The determination of H_2O_2 concentration was performed by using the standard curve (see in Figure C.1).

2.5 Determination of enzymatic antioxidants

2.5.1 Enzyme extraction

For enzyme extraction, 100 mg of leaf and root tissues were homogenized with 800 µl of extraction buffer (0.1 M sodium phosphate buffer (pH 7.0), 2 mM EDTA, 4 mM DTT, and 6 mg of polyvinylpyrrolidone) in the micro-centrifuge tube by using micro pestle. The whole extraction method was carried on ice. The homogenate was centrifuged at 12,000 rpm for 15 min. The supernatant was used as crude enzyme for the assays of enzyme activity.

2.5.2 Total protein determination

Protein content in the plant extract was measured by the method of Lowry (1951) (see in Appendix A) using bovine serum albumin (BSA) as a standard.

2.5.3 Measurement of superoxide dismutase (SOD) activity

Total SOD activity was measured according to the ability to inhibit the reduction of nitroblue tetrazolium chloride (NBT) by superoxide radicals generated photochemically at 25^0C. The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 8), 10.7 mM EDTA, 1.1 mM cytochrome C and 0.108 mM xanthine and 0.5 unit xanthine oxidase. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition in the reduction of NBT at 560 nm, expressed in units per milligram of protein.

2.5.4 Measurement of ascorbate peroxidase (APX) activity

Ascorbate peroxidase activity was determined according to the modified method of Nakano and Asada (1981). The substrate solution (50 ml) containing 48.6 ml of 50 mM sodium phosphate buffer (pH 7.0), 0.4 ml of 100 mM EDTA, 0.75 ml of 11.6 mM H_2O_2, 0.25 ml of 100 mM ascorbic acid was prepared. The oxidation of ascorbate was started by adding 200 µl of substrate solution into crude enzyme

extract in microtiterplate. The decrease in absorbance at 290 nm due to the oxidation of ascorbate was monitored at 25 ^{0}C. One unit of enzyme activity was defined as the oxidation of 1 µmol ascorbate per min.

2.5.5 Measurement of catalase (CAT) activity

Total CAT activity was monitored spectrophotometrically according to [58] with some modification in order to make it appropriate for microplate reader measurement. CAT activity was determined by the rate of H_2O_2 decrease at 240 nm at 37 ^{0}C. The 15 µl of crude enzyme was mixed with 160 µl of 50 mM potassium phosphate buffer pH 8.0 and 25 µl of 100 mM H_2O_2. The change in absorbance at 240 nm was measured for 1 minute; the rate of decomposition of H_2O_2 by CAT (mole H_2O_2 consumed min^{-1} mg $protein^{-1}$) was calculated.

2.5.6 Measurement of glutathione reductase (GR) activity

Glutathione reductase activity was assayed as described by Foyer and Halliwell (1976) at 25 ^{0}C. The oxidized glutathione (GSSG)-dependent oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) was followed at 340 nm in a 1 ml reaction mixture containing 100 mM potassium phosphate buffer (pH 7.0), 0.5 mM GSSG, 20 µl extract, and 0.1 mM NADPH. One unit of enzyme activity was defined as the rate of glutathione reduction at room temperature.

2.5.7 Measurement of glutathione peroxidase (GPX) activity

Glutathione peroxidase (GPX) activity was measured by a spectrophotometric method according to [59] with some modification for the determination using microplate reader. The reaction mixture (250 ml) contained 2 mM glutathione, 1 mM NADPH, 1 mM EDTA, 2 mM t-butyl hydroperoxide and 0.5 µl of glutathione reductase in 100 mM sodium phosphate buffer, pH 7.0 and 10 µl of crude enzyme. The

absorbance at 340 nm over a time period of 5 minutes was measure at 25 ^{0}C. The GPX activity was determined as the rate of NADPH oxidation.

CHAPTER IV

RESULTS

1. Effect of AgNPs concentrations and sizes on seedling growth

AgNPs with various sizes (20, 30-70, 70-120 and 150 nm) and concentrations (0.1, 1, 10, 100 and 1,000 mg/L) were used to study the effects on rice seedling growth. The similar patterns for dosage response were found in all AgNPs type tested. The larger size and higher concentration strongly inhibited growth than smaller size and lower concentration, especially, 1000 mg/L of 150 nm–AgNPs strongly inhibited rice growth (Table1-12). In normal condition, 0.1 and 1 mg/L of all AgNP sizes showed no significant effects on shoot fresh and dry weight (Table1 and Table 3). With higher concentrations of AgNPs (100 and 1000 mg/L), the shoot growth was more strongly inhibited than the root growth (Table 3 and Table 7). Shoot dry weight was reduced by 60–64 percent with 100–1000 mg/L AgNPs (Table 2), while the root dry weight was reduced by 32–36 percent (Table 3), compared to the control. Similar trends were also found in the shoot height (Table 9) and root length (Table 11). Moreover, in salt stress condition the similar responses to AgNPs were found. The shoot dry weight was reduced by 66-68 percent with 100-1000 mg/L (Table 4) while the reduction in root dry weight was ranging from 23–26 percent (Table 8). AgNPs treatment prior to salt stress caused the extremely decreased growth at high concentration and large size. However, when treated with 10 mg/L of 20 nm AgNPs prior to salt-stress stress, it enhanced root growth, resulting in the longer roots and higher dry weight than controls. The increase in root dry weight by 5.12% was detected (Table 8) and 17.42% increase in root length, when compared with control was found (Table 12).

Table 1. The effect of 24-h seed soaking AgNP at different concentrations (0.1, 1.0, 10.0, 100.0 and 1,000.0 mg/L) and sizes (20, 30-70, 70-120 and 150 nm) effect on shoot fresh weight 3-week-old seedlings in normal condition. Data are shown as the mean ± SE and are derived from five independent trials. Means with a different capital letter (concentrations) and lowercase letter (sizes) above them are significantly different ($P<0.05$).

Shoot Fresh Weight (mg)±standard error

Concentration (mg/L)	Sizes (nm)	20 nm	30-70 nm	70-120 nm	150 nm
	Control (no AgNPs)	988.4±0.01^{A}	988.4±0.01^{A}	988.4±0.01^{A}	988.4±0.01^{A}
0.1	990.00±0.02^{ns}	957.4±0.04^{AB}	930.4±0.08^{AB}	915.8±0.09^{A}	917.0±0.04^{A}
1	990.00±0.02^{ns}	932.4±0.02^{AB}	908.2±0.07^{AB}	897.4±0.02^{A}	884.8 ±0.03^{A}
10	990.00±0.02^{a}	943.2±0.03^{ABab}	890.8±0.03^{ABb}	904.2±0.02^{Ab}	876.6±0.02^{Ab}
100	990.00±0.02^{a}	854.2±0.06^{Bb}	802.4±0.03^{Bbc}	754.8±0.02^{Bbc}	705.8±0.03^{Bc}
1000	990.00±0.02^{a}	805.4±0.04^{Bb}	776.4±0.03^{Bb}	645.8±0.03^{Bc}	418.8±0.01^{Cd}

Table 2. The effect of 24-h seed soaking AgNPs at different concentrations (0.1, 1.0,10.0, 100.0 and 1,000.0 mg/L) and sizes (20, 30-70, 70-120 and 150 nm) effect on shoot fresh weight 3-week-old seedlings in salt stress condition. Data are shown as the mean ± SE and are derived from five independent trials. Means with a different capital letter (concentrations) and lowercase letter (sizes) above them are significantly different ($P<0.05$).

Shoot Fresh Weight (mg) ±standard error

Concentrations (mg/L)	Sizes (nm)	20 nm	30-70 nm	70-120 nm	150 nm
	Control (noAgNPs)	978.2±0.003^{A}	978.2±0.003^{A}	978.2±0.003^{A}	978.2±0.003^{A}
0.1	978.2±0.009^{a}	912.0±02^{ABab}	889.8±0.01^{Aab}	843.4±0.06^{Bb}	821.4±0.03^{Bb}
1	978.2±0.009^{ns}	901.4±0.02^{B}	916.6±0.01^{A}	907.2±0.03^{AB}	893.0±0.04^{AB}
10	978.2±0.009^{a}	914.2±0.01^{ABb}	897.4±0.03^{Ab}	912.0±0.01^{ABb}	891.3±0.02^{ABb}
100	978.2±0.009^{a}	866.8±0.01^{Bb}	690.2±0.03^{Bc}	694.3±0.02^{Bc}	633.8±0.05^{Cc}
1000	978.2±0.009^{a}	731.6±0.03^{Cb}	641.4±0.03^{Bbc}	598.8±0.03^{Cc}	568.0±0.03^{Cc}

Table 3. The effect of 24-h seed soaking AgNPs at different concentrations (0.1, 1.0, 10.0, 100.0 and 1,000.0 mg/L) and sizes (20, 30-70, 70-120 and 150 nm) effect on shoot dry weight 3-week-old seedlings in normal condition. Data are shown as the mean ± SE and are derived from five independent trials. Means with a different capital letter (concentrations) and lowercase letter (sizes) above them are significantly different ($P<0.05$).

Shoot Dry Weight (mg)±standard error

Concentrations (mg/L)	Sizes (nm)	20 nm	30-70 nm	70-120 nm	150 nm
	Control(noAgNPs)	106.6 ± 0.006^{A}	106.6 ± 0.006^{A}	106.6 ± 0.006^{A}	106.6 ± 0.006^{A}
0.1	106.6 ± 0.003^{ns}	104.40 ± 0.007^{A}	94.00 ± 0.008^{B}	91.80 ± 0.003^{B}	91.20 ± 0.004^{B}
1	106.6 ± 0.003^{ns}	89.60 ± 0.001^{B}	92.20 ± 0.006^{B}	91.20 ± 0.004^{B}	94.20 ± 0.003^{B}
10	106.6 ± 0.003^{a}	101.60 ± 0.001^{Aa}	91.86 ± 0.002^{Bb}	90.40 ± 0.003^{Bb}	89.80 ± 0.004^{Bb}
100	106.6 ± 0.003^{a}	84.00 ± 0.001^{Bb}	77.00 ± 0.003^{Cbc}	71.80 ± 0.004^{Bc}	67.20 ± 0.002^{Cc}
1000	106.6 ± 0.003^{a}	69.00 ± 0.002^{Cb}	61.00 ± 0.003^{Dbc}	54.40 ± 0.003^{Cc}	49.40 ± 0.002^{Dd}

Table 4. The effect of 24-h seed soaking AgNP at different concentrations (0.1, 1.0, 10.0, 100.0 and 1,000.0 mg/L) and sizes (20, 30-70, 70-120 and 150 nm) effect on shoot dry weight 3-week-old seedlings in salt stress condition. Data are shown as the mean ± SE and are derived from five independent trials. Means with a different capital letter (concentrations) and lowercase letter (sizes) above them are significantly different ($P<0.05$).

Shoot Dry Weight (mg)±standard error

Concentrations (mg/L)	Sizes (nm)	20 nm	30-70 nm	70-120 nm	150 nm
	Control (no AgNPs)	101.56±0.001^{A}	101.56±0.001^{A}	101.56±0.001^{A}	101.56±0.001^{A}
0.1	101.56±0.003^{a}	91.40±.0.002^{Bb}	88.80±0.001^{Bb}	87.40±0.002^{Bb}	87.80±0.002^{Bb}
1	101.56±0.003^{a}	90.40±0.003^{Bb}	88.20±0.002^{Bb}	87.40±0.001^{Bb}	85.40±0.002^{Bb}
10	101.56±0.003^{a}	91.80±0.002^{Bb}	89.00±0.001^{Bbc}	86.20±0.001^{Bbc}	84.8±0.002^{Bc}
100	101.56±0.003^{a}	80.40±0.00^{Cb}	77.60±0.001^{Cbc}	72.40±0.001^{Ccd}	68.20±0.001^{Cd}
1000	101.56±0.003^{a}	66.40±0.002^{Db}	60.20±0.002^{Dbc}	52.00±0.001^{Dcd}	43.80±0.003^{Cd}

Table 5. The effect of 24-h seed soaking AgNPs at different concentrations (0.1, 1.0, 10.0, 100.0 and 1,000.0 mg/L) and sizes (20, 30-70, 70-120 and 150 nm) effect on root fresh weight 3-week-old seedlings in normal condition. Data are shown as the mean ± SE and are derived from five independent trials. Means with a different capital letter (concentrations) and lowercase letter (sizes) above them are significantly different ($P<0.05$).

Root Fresh Weight (mg)±standard error

Concentrations (mg/L)	Sizes (nm)	20 nm	30-70 nm	70-120 nm	150 nm
	Control(noAgNPs)	402.60 ± 0.002^{A}	402.60 ± 0.002^{A}	402.60 ± 0.002^{A}	402.60 ± 0.002^{A}
0.1	402.60 ± 0.002^{a}	365.5 ± 0.009^{Bab}	350.20 ± 0.01^{Bab}	337.00 ± 0.02^{Bc}	309.60 ± 0.004^{Bc}
1	402.60 ± 0.002^{a}	357.00 ± 0.01^{ABb}	350.60 ± 0.007^{Bb}	339.00 ± 0.02^{Bb}	298.40 ± 0.003^{Bc}
10	402.60 ± 0.002^{a}	377.00 ± 0.01^{ABab}	363.20 ± 0.02^{Bab}	341.00 ± 0.02^{Bbc}	303.40 ± 0.01^{Bc}
100	402.60 ± 0.002^{a}	329.00 ± 0.01^{Ca}	306.20 ± 0.008^{Cb}	275.80 ± 0.01^{Cb}	262.00 ± 0.009^{Cc}
1000	402.60 ± 0.002^{a}	300.80 ± 0.01^{Db}	283.60 ± 0.01^{Cb}	234.40 ± 0.007^{Cc}	234.40 ± 0.004^{Cc}

Table 6. The effect of 24-h seed soaking AgNPs at different concentrations (0.1, 1.0, 10.0, 100.0 and 1,000.0 mg/L) and sizes (20, 30-70, 70-120 and 150 nm) effect on root fresh weight 3-week-old seedlings in salt stress condition. Data are shown as the mean ± SE and are derived from five independent trials. Means with a different capital letter (concentrations) and lowercase letter (sizes) above them are significantly different ($P<0.05$).

	Root Fresh Weight (mg)±standard error				
Concentrations (mg/L)	Sizes (nm)	20 nm	30-70 nm	70-120 nm	150 nm
	Control (noAgNPs)	395.48±0.001^{A}	395.48±0.001^{A}	395.48±0.001^{A}	395.48±0.001^{A}
0.1	395.48±0.004^{a}	366.20±.0.009^{Aab}	348.20±0.01^{Bab}	332.60±0.01^{Bbc}	315.40±0.006^{Bc}
1	395.48±0.004^{a}	379.40±0.01^{Aab}	379.60±0.003^{Aab}	348.80±0.009^{Bbc}	328.40±0.01^{Bc}
10	395.48±0.004^{a}	384.40±0.005^{Aa}	375.00±0.005^{ABab}	351.80±0.01^{Bbc}	327.0±0.007^{Bc}
100	395.48±0.004^{a}	327.00±0.005^{Bb}	310.60±0.005^{Cbc}	297.60±0.009^{Cbc}	301.80±0.006^{Bc}
1000	395.48±0.004^{a}	299.80±0.02^{Cb}	270.00±0.01^{Dc}	231.200±0.01^{Dd}	239.00±0.005^{Cd}

Table 7. The effect of 24-h seed soaking AgNPs at different concentrations (0.1, 1.0, 10.0, 100.0 and 1,000.0 mg/L) and sizes (20, 30-70, 70-120 and 150 nm) effect on root dry weight 3-week-old seedlings in normal condition. Data are shown as the mean ± SE and are derived from five independent trials. Means with a different capital letter (concentrations) and lowercase letter (sizes) above them are significantly different ($P<0.05$).

Root Dry Weight (mg)±standard error

Concentrations (mg/L)	Sizes (nm)	20 nm	30-70 nm	70-120 nm	150 nm
	Control (noAgNPs)	36.80 ± 0.001^{A}	36.80 ± 0.001^{A}	36.80 ± 0.001^{A}	36.80 ± 0.001^{A}
0.1	36.80 ± 0.001^{a}	32.60 ± 0.001^{Bab}	30.20 ± 0.001^{Bb}	29.60 ± 0.001^{Bb}	28.00 ± 0.001^{Bb}
1	36.80 ± 0.002^{a}	33.00 ± 0.007^{Bb}	31.20 ± 0.001^{Bbc}	28.00 ± 0.001^{Bbc}	27.80 ± 0.001^{Bc}
10	36.80 ± 0.002^{a}	33.60 ± 0.001^{ABab}	31.20 ± 0.008^{Bbc}	29.40 ± 0.002^{Bbc}	28.00 ± 0.002^{Bc}
100	36.80 ± 0.002^{a}	27.20 ± 0.02^{Cb}	24.40 ± 0.0005^{Cc}	23.40 ± 0.002^{Cc}	19.60 ± 0.0004^{Cd}
1000	36.80 ± 0.002^{a}	23.60 ± 0.001^{Db}	21.60 ± 0.0004^{Cb}	18.40 ± 0.002^{Dc}	14.60 ± 0.001^{Dd}

Table 8. The effect of 24-h seed soaking AgNPs at different concentrations (0.1, 1.0, 10.0, 100.0 and 1,000.0 mg/L) and sizes (20, 30-70, 70-120 and 150 nm) effect on root dry weight 3-week-old seedlings in salt stress condition. Data are shown as the mean ± SE and are derived from five independent trials. Means with a different capital letter (concentrations) and lowercase letter (sizes) above them are significantly different ($P<0.05$).

		Root Dry Weight (mg)±standard error			
Concentrations (mg/L)	Sizes (nm)	20 nm	30-70 nm	70-120 nm	150 nm
	Control (noAgNPs)	36.16 ± 0.004^{AB}	36.16 ± 0.004^{A}	36.16 ± 0.004^{A}	36.16 ± 0.004^{A}
0.1	36.16 ± 0.0005^{a}	$31.20\pm.0.005^{Bab}$	30.60 ± 0.001^{Bbc}	29.20 ± 0.001^{Bbc}	28.00 ± 0.001^{Bc}
1	36.16 ± 0.0005^{a}	34.80 ± 0.0006^{Aab}	32.20 ± 0.007^{Bb}	28.00 ± 0.008^{Bc}	26.60 ± 0.001^{Bc}
10	36.16 ± 0.0005^{ab}	38.40 ± 0.0007^{Aa}	32.20 ± 0.002^{Bb}	28.86 ± 0.002^{Bc}	27.00 ± 0.001^{Bc}
100	36.16 ± 0.0005^{a}	26.60 ± 0.001^{Cb}	24.00 ± 0.004^{Cc}	21.80 ± 0.007^{Cc}	19.20 ± 0.005^{Cd}
1000	36.16 ± 0.0005^{a}	23.60 ± 0.0006^{Db}	18.60 ± 0.006^{Db}	18.40 ± 0.001^{Dc}	14.60 ± 0.001^{Dd}

Table 9. The effect of 24-h seed soaking AgNPs at different concentrations (0.1, 1.0, 10.0, 100.0 and 1,000.0 mg/L) and sizes (20, 30-70, 70-120 and 150 nm) effect on shoot height 3-week-old seedlings in normal condition. Data are shown as the mean ± SE and are derived from five independent trials. Means with a different capital letter (concentrations) and lowercase letter (sizes) above them are significantly different ($P<0.05$).

Shoot Height (cm)±standard error

Concentrations (mg/L)	Sizes (nm)	20 nm	30-70 nm	70-120 nm	150 nm
	Control(noAgNPs)	29.98 ± 0.12^{A}	29.98 ± 0.12^{A}	29.98 ± 0.12^{A}	29.98 ± 0.12^{A}
0.1	29.98 ± 0.12^{ns}	29.25 ± 0.71^{A}	30.1 ± 0.61^{A}	28.35 ± 0.65^{AB}	28.35 ± 0.66^{B}
1	29.98 ± 0.12^{a}	29.20 ± 0.53^{Aab}	28.60 ± 0.61^{Aab}	27.70 ± 0.52^{BCb}	27.85 ± 0.55^{Bb}
10	29.98 ± 0.12^{a}	33.60 ± 0.001^{Aa}	31.20 ± 0.008^{Aa}	29.40 ± 0.002^{ABab}	28.00 ± 0.002^{Bb}
100	29.98 ± 0.12^{a}	29.75 ± 0.98^{Bb}	28.85 ± 1.02^{Bb}	28.40 ± 0.56^{Cb}	26.45 ± 0.36^{Bc}
1000	29.98 ± 0.12^{a}	23.45 ± 0.25^{Cb}	22.65 ± 0.76^{Cb}	19.90 ± 0.63^{Dc}	16.35 ± 0.49^{Cd}

Table 10. The effect of 24-h seed soaking AgNPs at different concentrations (0.1, 1.0, 10.0, 100.0 and 1,000.0 mg/L) and sizes (20, 30-70, 70-120 and 150 nm) effect on shoot height 3-week-old seedlings in salt stress condition. Data are shown as the mean ± SE and are derived from five independent trials. Means with a different capital letter (concentrations) and lowercase letter (sizes) above them are significantly different ($P<0.05$).

Shoot Height (cm)±standard error

Concentrations (mg/L)	Sizes (nm)	20 nm	30-70 nm	70-120 nm	150 nm
	Control (noAgNPs)	29.31 ± 0.51^{A}	29.31 ± 0.51^{A}	29.31 ± 0.51^{A}	29.31 ± 0.51^{A}
0.1	29.31 ± 0.51^{a}	$28.25\pm.0.22^{Ab}$	27.50 ± 0.29^{Abc}	26.85 ± 0.41^{Bcd}	26.30 ± 0.30^{Bd}
1	29.31 ± 0.51^{a}	28.15 ± 0.41^{Aab}	27.55 ± 0.71^{Abc}	26.9 ± 0.39^{Bbc}	26.15 ± 0.38^{Bc}
10	29.31 ± 0.51^{a}	28.10 ± 0.23^{Aab}	28.15 ± 0.78^{Aab}	27.3 ± 0.78^{ABbc}	26.2 ± 0.38^{Bc}
100	29.31 ± 0.51^{a}	25.90 ± 0.52^{Bb}	26.00 ± 0.63^{Ab}	25.15 ± 0.060^{Cb}	24.45 ± 0.24^{Bc}
1000	29.31 ± 0.51^{a}	23.10 ± 0.25^{Cb}	22.35 ± 0.76^{Bb}	19.90 ± 0.63^{Dc}	16.35 ± 0.49^{Cd}

Table 11. The effect of 24-h seed soaking AgNPs at different concentrations (0.1, 1.0, 10.0, 100.0 and 1,000.0 mg/L) and sizes (20, 30-70, 70-120 and 150 nm) effect on root length 3-week-old seedlings in normal condition. Data are shown as the mean ± SE and are derived from five independent trials. Means with a different capital letter (concentrations) and lowercase letter (sizes) above them are significantly different ($P<0.05$).

Root Length (cm)±standard error

Concentrations (mg/L)	Sizes (nm)	20 nm	30-70 nm	70-120 nm	150 nm
	Control (noAgNPs)	9.07 ± 0.05^{A}	9.07 ± 0.05^{A}	9.07 ± 0.05^{A}	9.07 ± 0.05^{A}
0.1	9.07 ± 0.05^{ns}	8.50 ± 0.45^{AB}	8.50 ± 0.48^{A}	7.85 ± 0.88^{AB}	7.70 ± 0.43^{BC}
1	9.07 ± 0.05^{a}	8.55 ± 0.28^{ABab}	8.35 ± 0.33^{Aab}	8.10 ± 0.18^{ABb}	7.90 ± 0.32^{Bb}
10	9.07 ± 0.05^{a}	8.80 ± 0.56^{ABa}	8.60 ± 0.24^{Ab}	8.05 ± 0.39^{ABc}	7.90 ± 0.32^{Bc}
100	9.07 ± 0.05^{a}	7.80 ± 0.22^{BCb}	7.45 ± 0.30^{Bbc}	7.15 ± 0.35^{BCbc}	6.90 ± 0.29^{Cc}
1000	9.07 ± 0.05^{a}	6.90 ± 0.20^{Cb}	6.85 ± 0.21^{Bb}	6.30 ± 0.21^{Cbc}	5.95 ± 0.27^{Dc}

Table 12. The effect of 24-h seed soaking AgNPs at different concentrations (0.1, 1.0, 10.0, 100.0 and 1,000.0 mg/L) and sizes (20, 30-70, 70-120 and 150 nm) effect on root length 3-week-old seedlings in salt stress condition. Data are shown as the mean ± SE and are derived from five independent trials. Means with a different capital letter (concentrations) and lowercase letter (sizes) above them are significantly different ($P<0.05$).

	Root Length (cm)±standard error				
Concentrations (mg/L)	Sizes (nm)	20 nm	30-70 nm	70-120 nm	150 nm
	Control (noAgNPs)	8.69 ± 0.06^{B}	8.69 ± 0.06^{AB}	8.69 ± 0.06^{A}	8.69 ± 0.06^{A}
0.1	8.69 ± 0.06^{ns}	$8.35\pm.0.21^{BC}$	8.40 ± 0.42^{B}	8.25 ± 0.13^{A}	8.30 ± 0.26^{A}
1	8.69 ± 0.06^{ns}	9.05 ± 0.56^{B}	8.90 ± 0.21^{A}	8.25 ± 0.19^{A}	8.00 ± 0.35^{A}
10	8.69 ± 0.06^{b}	10.50 ± 0.45^{Aa}	8.95 ± 0.28^{Ab}	8.35 ± 0.33^{Ac}	8.20 ± 0.39^{Ac}
100	8.69 ± 0.06^{a}	8.20 ± 0.39^{Cab}	7.80 ± 0.28^{CBab}	7.50 ± 0.32^{Bbc}	7.05 ± 0.45^{Bc}
1000	8.69 ± 0.06^{a}	6.90 ± 0.20^{Db}	6.85 ± 0.21^{Cb}	6.95 ± 0.21^{Bbc}	6.30 ± 0.12^{Cc}

2. Silver nanoparticles accumulation

After germination for 21 days, samples were collected for silver analysis by ICP-OES. The results are shown in Figure 2. The nutrient solution with seedlings germinated from treated seeds with 0.1 or 1.0 mg/L AgNPs at any sizes did not contain AgNPs at the detectable level. At higher concentration of AgNPs, the higher concentration of contaminated AgNPs in nutrient solution was found (Figure 2). When test in shoot and root tissue, various concentrations of AgNPs were found in shoot and root tissues depending on sizes and concentration of AgNP treatment (Figure 3). However, no detectable level of AgNPs was found in leaves. The AgNPs accumulated in leaves was found when seeds were treated with 100 or 1000 mg/L (Figure 3A). The increase in AgNP accumulation was found when the seeds were treated with higher concentration of AgNPs. When seeds were treated with the larger sizes of AgNPs at 1 mg/L, no detectable level of AgNPs was found in both roots and shoots. At higher concentration, 10 mg/L AgNPs with 70–150 nm in diameter, AgNP treated seeds developed with the accumulation of AgNPs in both shoot and root tissues (Figure 3C and 3D). However, seeds treated with 10 mg/L of 30–60 nm AgNPs resulted in AgNP accumulation in roots only (Figure 3B).

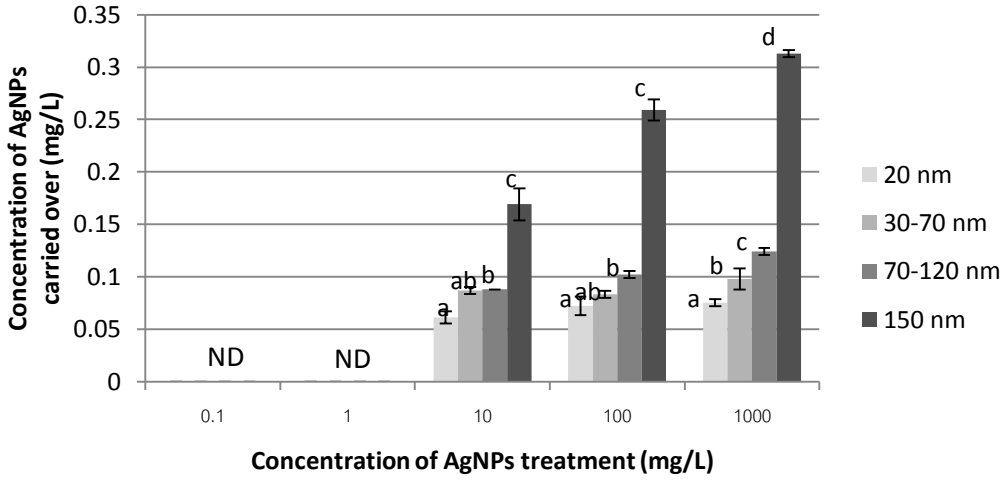

Figure 2. The concentration of AgNPs carried over in the nutrient solution. Data are shown as the mean± SE and are derived from three independent trials. Means with a different lower case letter above them are significantly different ($P<0.05$).

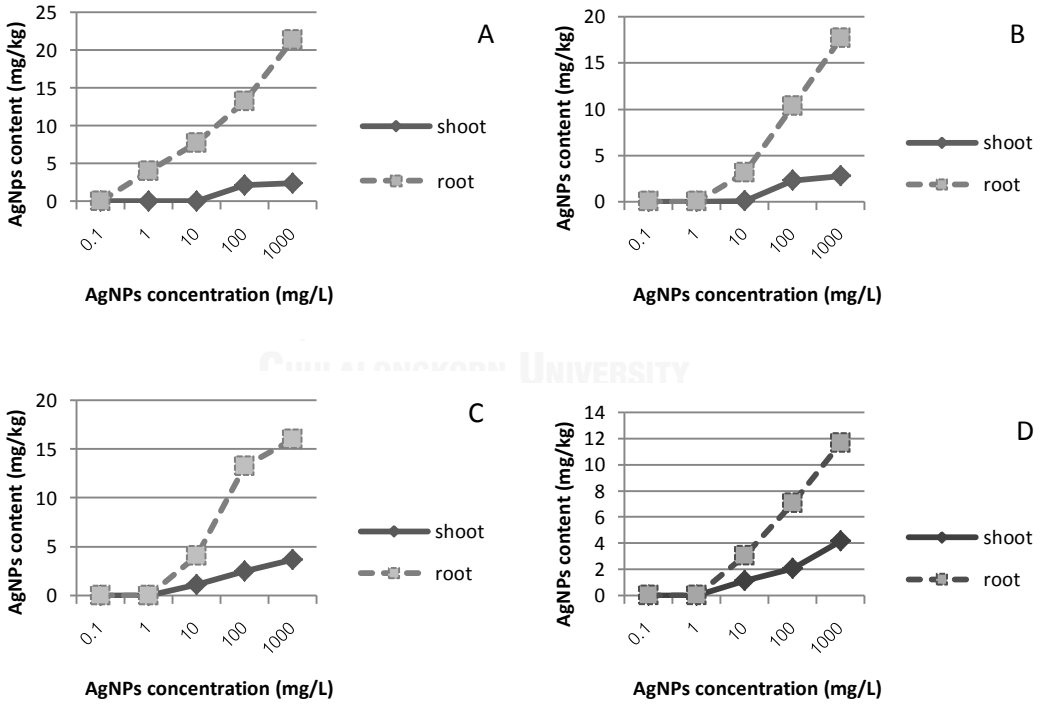

Figure 3. Bioaccumulation of AgNPs in root and shoot tissues when seeds were treated with 20 (A), 30–60 (B), 70–120 (C) and 150 (D) nm AgNPs at various concentrations. Data are shown as the mean±SE and are derived from three

independent trials. Means with a different lowercase letter above them are significantly different ($P<0.05$).

3. Effect of AgNPs on the rice seedling leaf anatomy

The anatomical changes in 21-day-old seedling leaves after exposure of the seeds to 20 or 150 nm-diameter AgNPs at 10 and 100 mg/L were evaluated. The 20-nm-diameter AgNPs at 10 mg/L showed only a slight effect on the rice leaf anatomy (Figure 4A), whereas at 100 mg/L (Figure 4B), and the larger 150-nm-diameter AgNPs at 10 mg/L. (Figure 4C) and 100 mg/L (Figure 4D) showed clear alterations in the seedling leaf anatomy in the mid-rib area, especially in the parenchyma cells connecting the upper and lower vascular bundles. In the control plants, about five to seven layers of parenchyma cells were found (Figure 4E), while more layers of smaller cells were found in the AgNP-treated seedlings (Figure 4A-D).

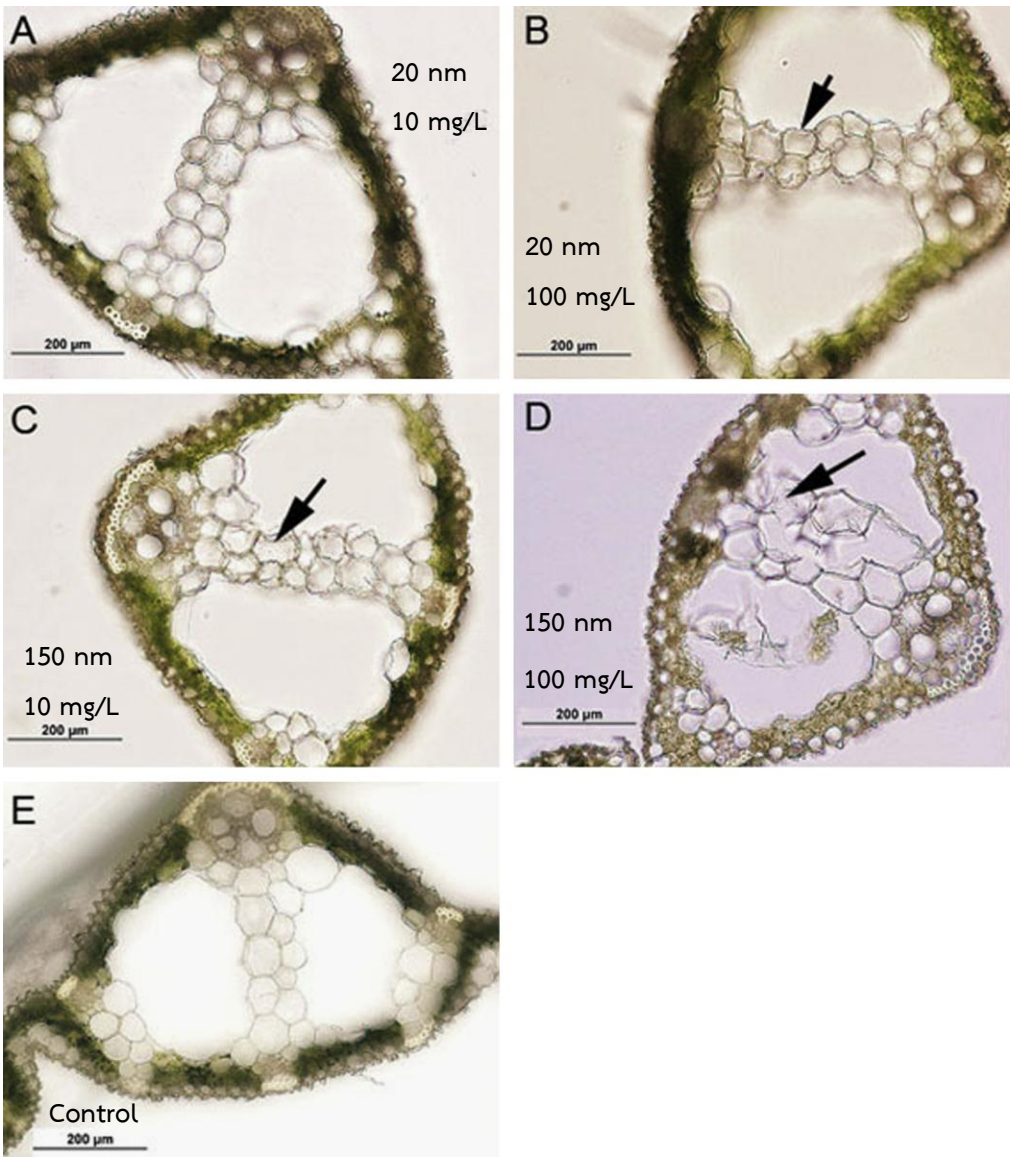

Figure 4. The effects of seed exposure to (A,B) 20-nm and (C,D) 150 nm-diameter AgNPs at a concentration of (A,C) 10 mg/L or (B,D) 100 mg/L on the leaf anatomy of 21d-old rice seedlings, compared with the (E) control plants. Arrows show abnormal of parenchyma cell, and scale bars represent 200 µm. Transverse section images shown (20X magnification) are representative of those seen from at least 50 such sections per sample and five independent samples.

4. Silver nanoparticles (AgNPs) effects on physiological changes during normal and salt stress.

4.1 H_2O_2 content

4.1.1 Normal condition

In normal condition, after the transfer of the plants to the fresh nutrient solution, H_2O_2 content was increased by time in both roots and shoots (Figure 5-8). The pre-treatment by seed soaking with AgNPs resulted in the increase of H_2O_2 content at some time points. The larger AgNP, 70-120 and 150 nm in diameters, tended to cause the higher level of H_2O_2 content than smaller size by 30% on Day1 and Day3 after treatment (Figure 5 and 7). When treated with the concentration of 100 mg/L 20 nm AgNP, plants had the significant increase in H_2O_2 content in both roots and shoots, which was 70-100% higher than controls (Figure 6 and 8).

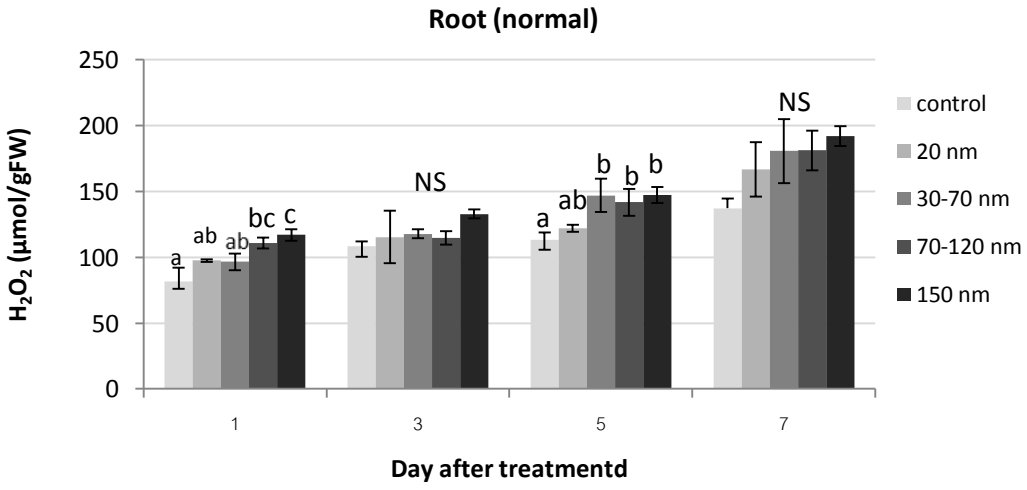

Figure 5. Hydrogen peroxide contents in 10 mg/L AgNPs pretreatment root after treatment for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly different of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly different of means at $p<0.05$.

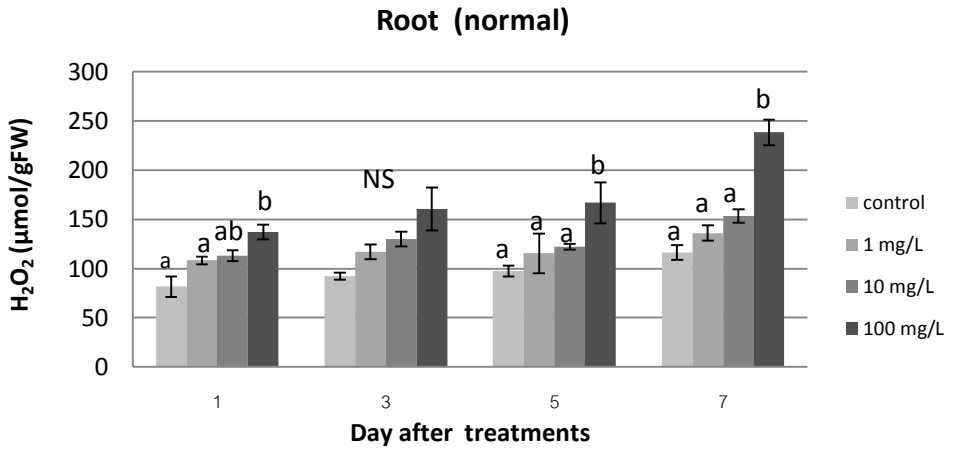

Figure 6. Hydrogen peroxide contents in 20 nm AgNPs pretreatment root after treatment for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly different of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly different of means at $p<0.05$.

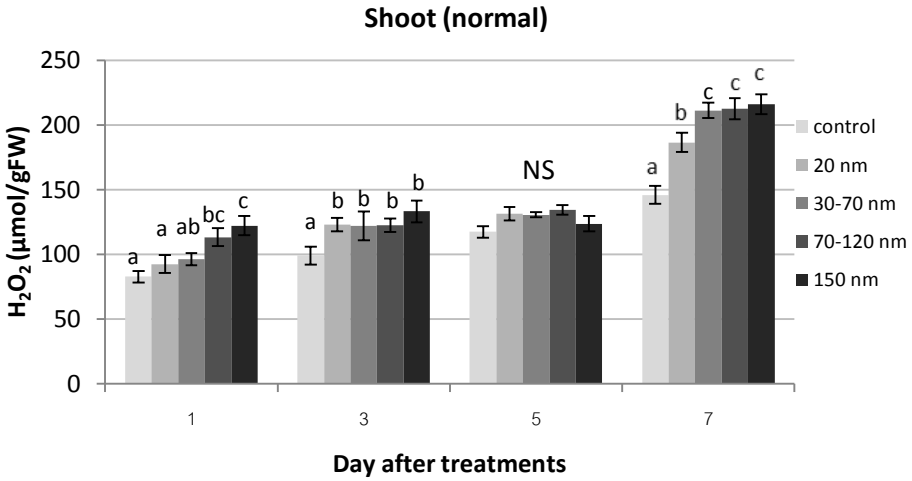

Figure 7. Hydrogen peroxide contents in 10 mg/L AgNPs pretreatment shoot after treatment for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly different of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly different of means at $p<0.05$.

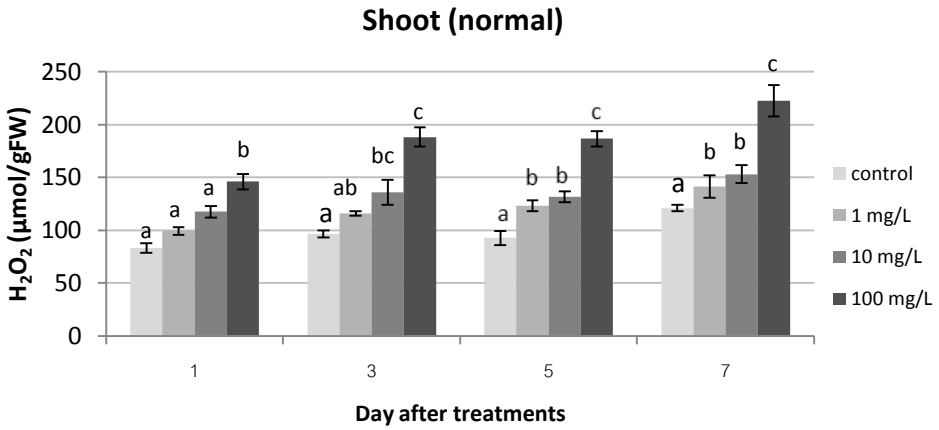

Figure 8. Hydrogen peroxide contents in 20 nm AgNPs pretreatment shoot after treatment for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

4.1.2 H_2O_2 content in salt stress condition

NaCl treatment induced an increase in H_2O_2 level in both roots and shoots. Moreover, the pre-seed soaking showed the tendency of the higher level of H_2O_2 content after salt stress (Figure 9-12). The larger AgNP pre-treatment showed higher H_2O_2 level than smaller ones (Figure 9). When compared to the plant grown in the normal condition (Figure 5-8), the level of H_2O_2 contents in the plants grown under salt stress treatment was higher. H_2O_2 content was about 80-290 µmol/gFW in roots and 80-240 µmol/gFW in shoots. The highest H_2O_2 level, 287µmol/gFW, was detected in salt stressed roots, when pretreated with 10 mg/L of 150 nm AgNP (Figure 9).

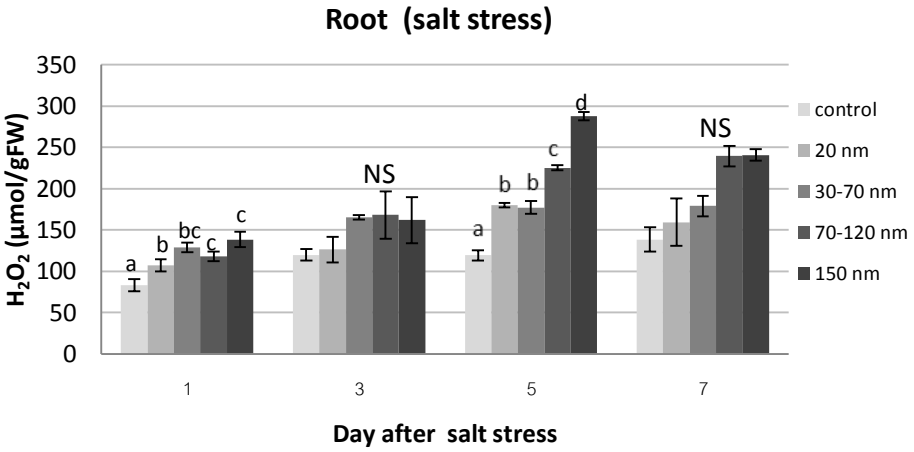

Figure 9. Hydrogen peroxide contents in 10 mg/L AgNP pretreatment root after exposure to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

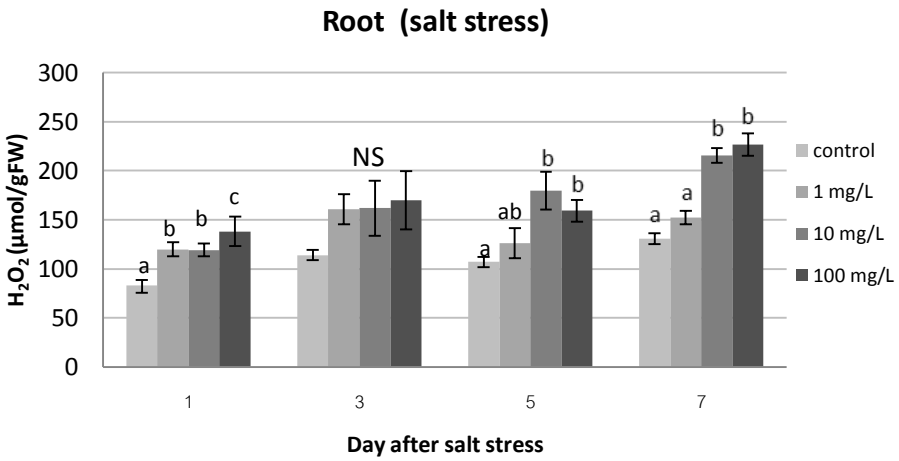

Figure 10. Hydrogen peroxide contents in 20 nm AgNPs pretreatment root after exposure to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

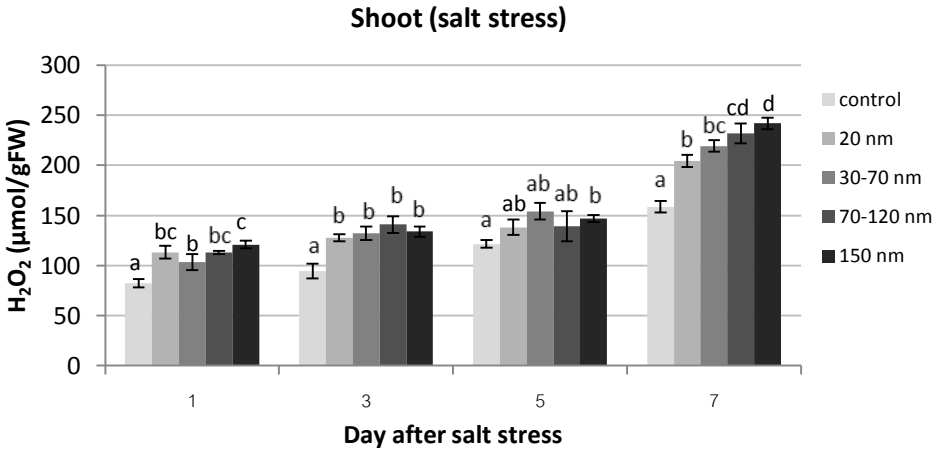

Figure 11. Hydrogen peroxide contents in 10 mg/L AgNP pretreatment shoot after exposure to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

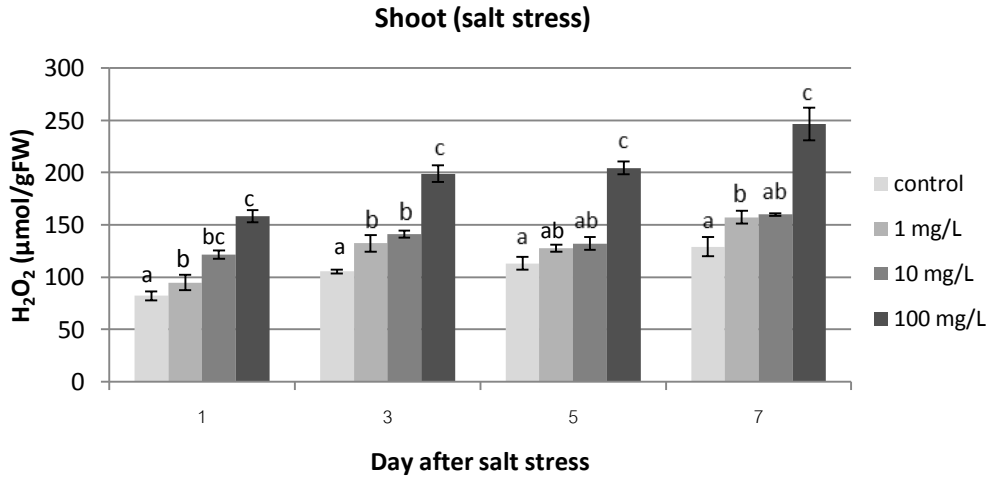

Figure 12. Hydrogen peroxide contents in 20 nm AgNPs pretreatment shoot after exposure to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

5. Effect of silver nanoparticles in normal and salt stress on anti-oxidative systems

5.1 Catalase activity (CAT)

5.1.1 Normal condition

The pretreatment by seed soaking in AgNPs solution with various sizes and concentration resulted in the significant increase in CAT activity when plants were transferred to the fresh nutrient solution. The strongest effects on CAT induction was detected, when seeds were soaked in 100 mg/L of 150 nm-AgNPs (Figure 13-16). The tendency of induction was found after a 1 day of treatment. However, it was increased in the lower level at 1 day after treatment after that was highly increased. Both in shoot and root CAT activity had similar tendency that a larger size had more CAT activity than smaller sizes (Figure 13 and 15). In root, normal condition; CAT activity induction was about 7.5 fold compared to control (Figure 13 and 14). In addition, in shoot; about 2.5 fold induction in CAT activity was detected.

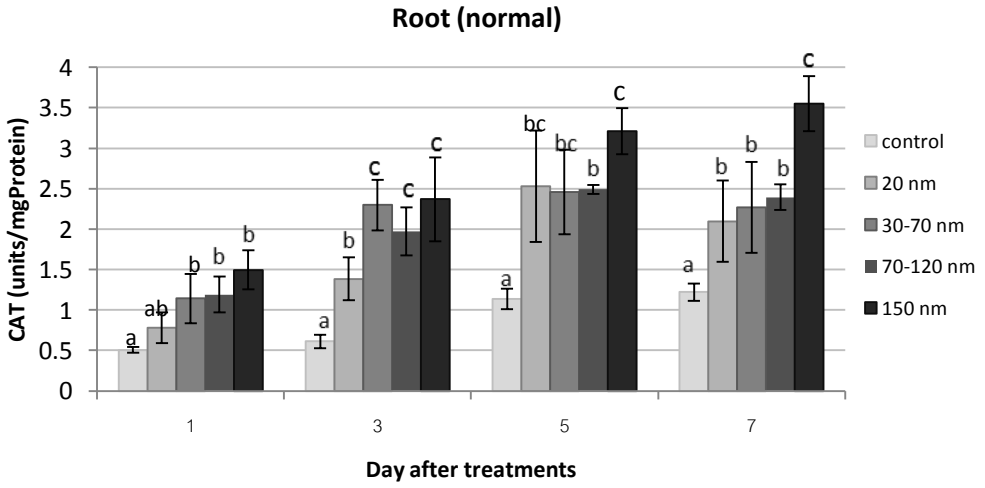

Figure 13. Catalase activity in 10 mg/L AgNPs pretreated root during the period of experiment on day 0, 1, 3, 5 and 7. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$

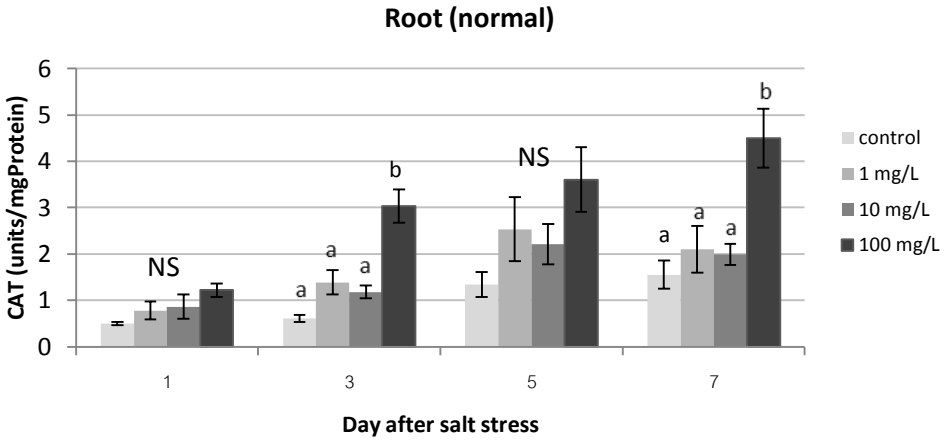

Figure 14. Catalase activity in 20 nm AgNP pretreated root during the period of experiment on day 0, 1, 3, 5 and 7. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

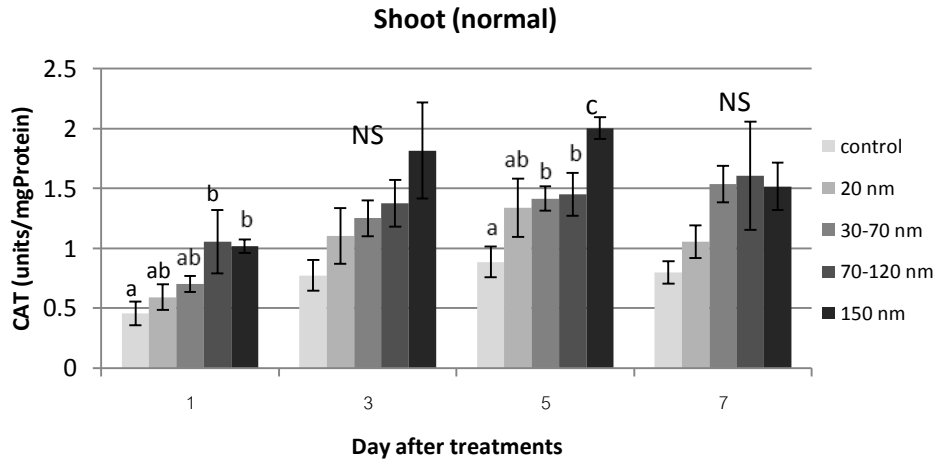

Figure 15. Catalase activity in 10 mg/L AgNP pretreated shoot during the period of experiment on day 0, 1, 3, 5 and 7. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

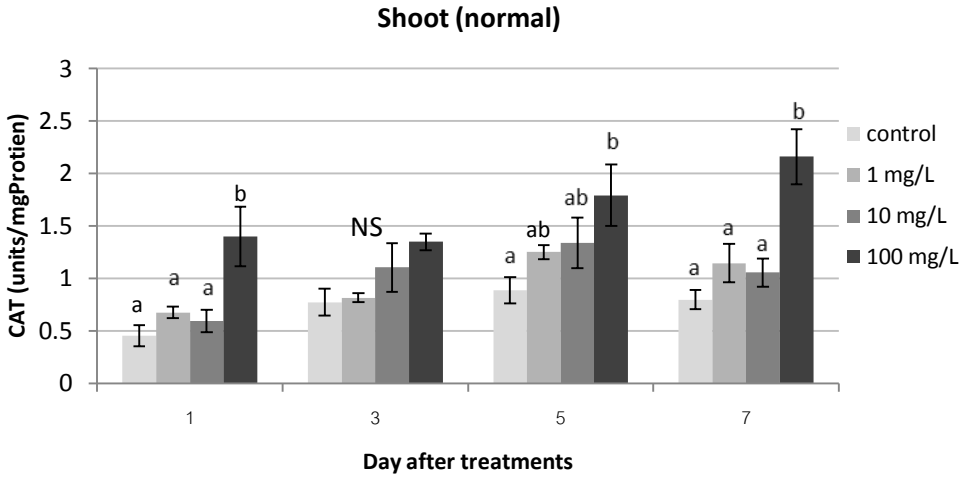

Figure 16. Catalase activity in 20 nm AgNP pretreated shoot during the period of experiment on day 0, 1, 3, 5 and 7. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

5.1.2 Salt Condition

Without AgNP pretreatment, salt stress caused slightly increase in CAT activity in roots. However, AgNP pretreatment at various sizes induced higher level of CAT activity under salt-stress condition (Figure 17). The larger AgNPs tended to show higher effect on CAT activity than smaller one. The high concentration (100 mg/L) induced the highest level of CAT activity, compared to the lower concentrations (Figure 17-18).

In shoot, pretreatment of 10 mg/L AgNP at various sizes did not significantly affect CAT activity. However, when treated with 20 nm AgNP at various concentrations, the higher concentration had more effects on CAT activity in shoots under salt stress condition (Figure 19-20).

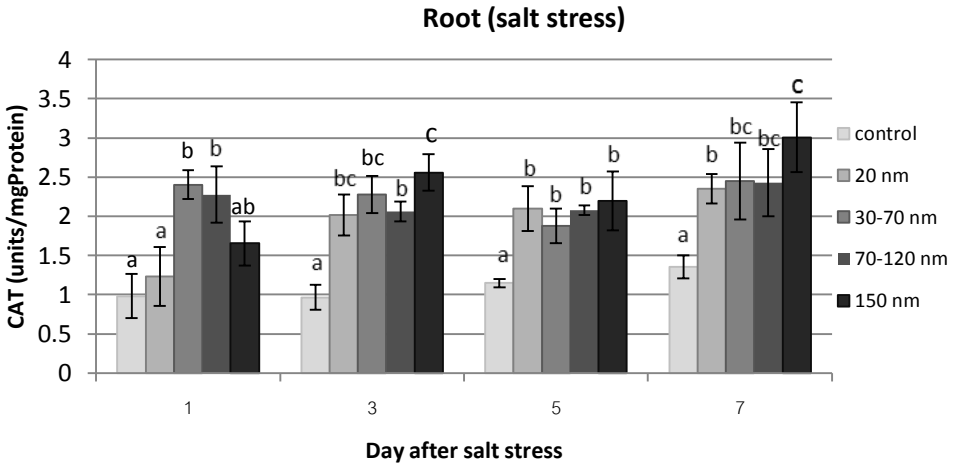

Figure 17. Catalase activity in 10 mg/L AgNPs pretreatment root after exposed to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

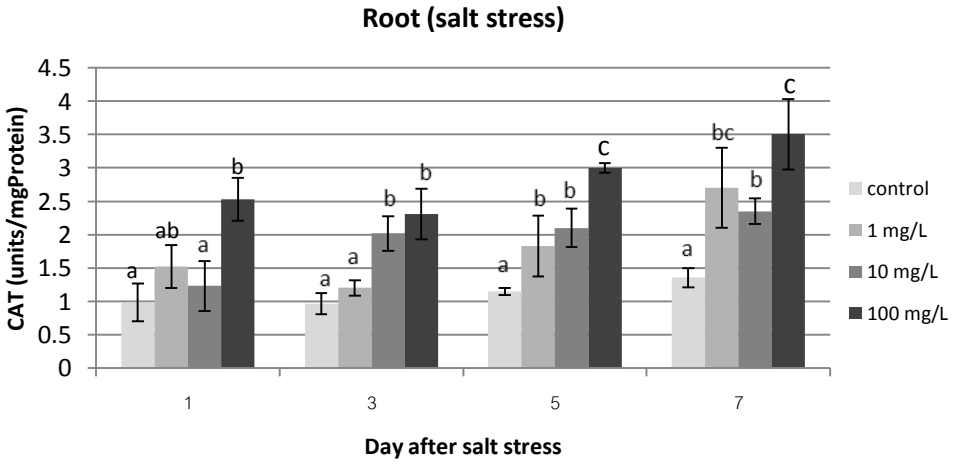

Figure 18. Catalase activity in 20 nm AgNPs pretreatment root after exposed to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

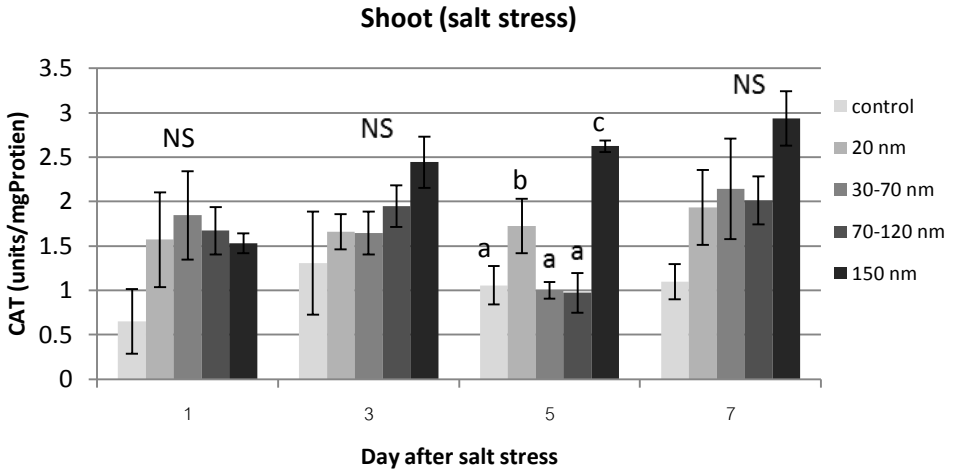

Figure 19. Catalase activity in 10 mg/LAgNPs pretreatment shoot after exposed to NaClfor 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at p<0.05 (Duncan's multiple range tests). NS is non-significantly difference of means at p<0.05.

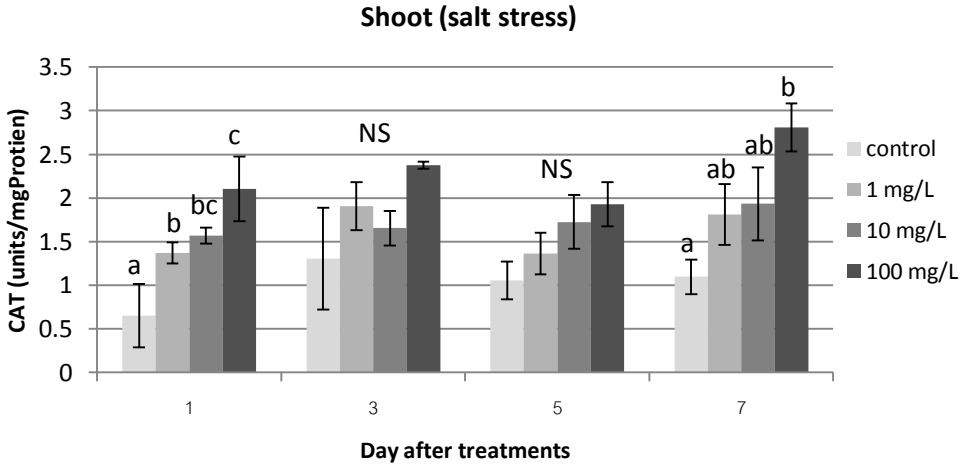

Figure 20. Catalase activity in 20 nm AgNPs pretreatment shoot after exposed to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at p<0.05 (Duncan's multiple range tests). NS is non-significantly difference of means at p<0.05.

5.2 Superoxide Dismutase (SOD)

5.2.1 Normal condition

When rice seeds were pretreated with various sizes of AgNPs at 10 mg/L, SOD activity was induced after 3 days of the transfer (Figure 21). The larger AgNPs showed more impact on SOD than the smaller ones.

When rice seeds were pretreated with various concentration of 20 nm AgNPs, the high concentration (100 mg/L) significantly induced SOD activity after 1 day of the transfer, while the lower concentration, 1 and 10 mg/L, induced SOD activity after 5-7 days of the transfer. The highest concentration showed the strongest induction of SOD activity in roots. It was induced up to 2 fold, when compared to the control (Figure 22). The ranges of SOD activity in root were 60-158 Unit/ mg protein.

In shoots, the similar response was found. The plant transfer cause slightly induction of SOD in shoots. The pretreatment of 10 mg/L AgNPs at various sizes tended to increase SOD activity. The larger AgNPs showed higher effects than the smaller ones (Figure 23).

When pretreated with 20 nm AgNPs at various sizes, the higher concentration, the higher SOD activity was detected. The significant induction of SOD was found after 5 days of the transfer. The range of SOD activity in shoot was 50-105 Unit/ mg protein (Figure 24).

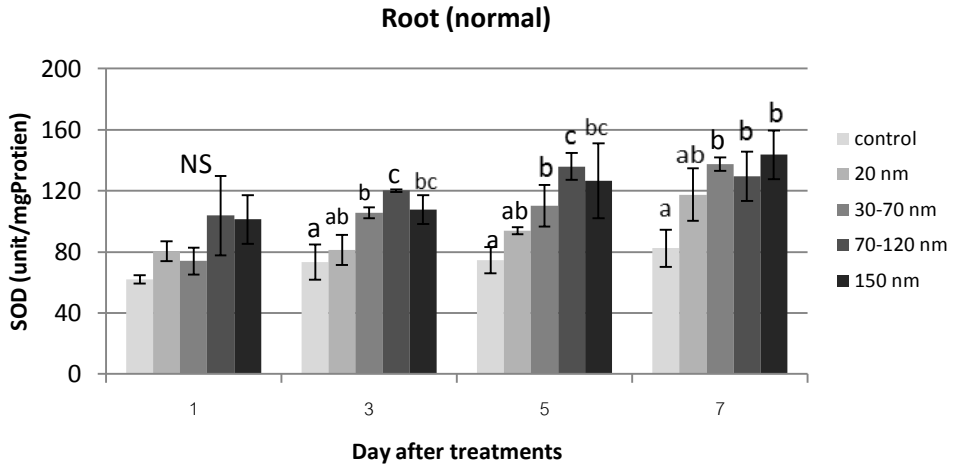

Figure 21. Superoxide dismutase activity in 10 mg/L AgNPs pretreatment root after transfer to fresh medium for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

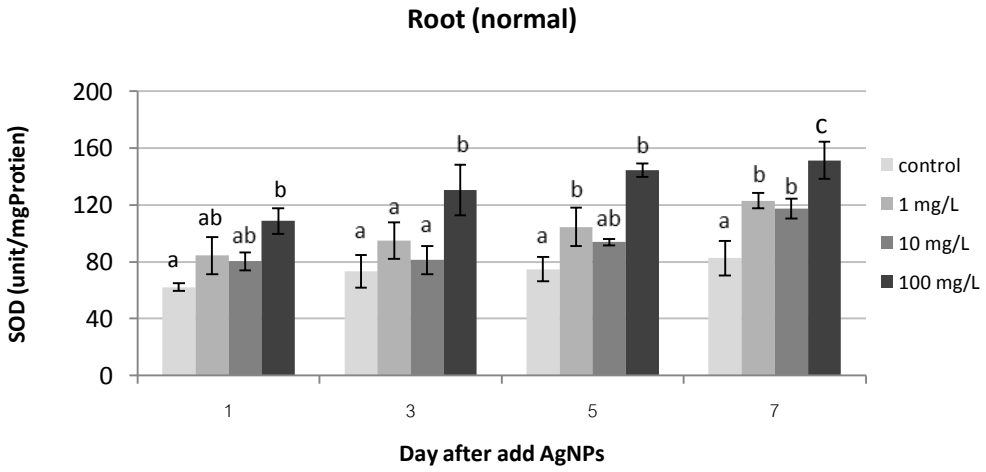

Figure 22. Superoxide dismutase activity in 20 nm AgNPs pretreatment root after transfer to fresh medium for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

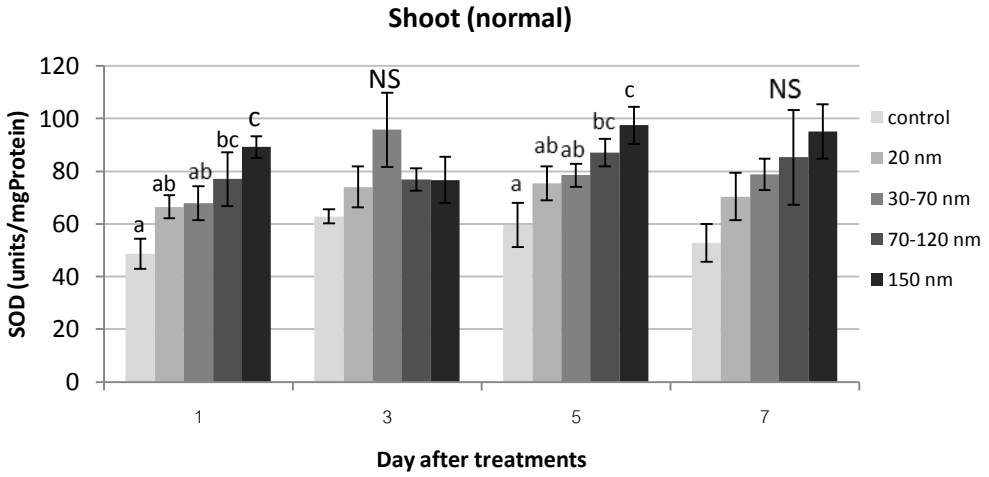

Figure 23. Superoxide dismutase activity in 10 mg/L AgNPs pretreatment shoot after transfer to fresh medium for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

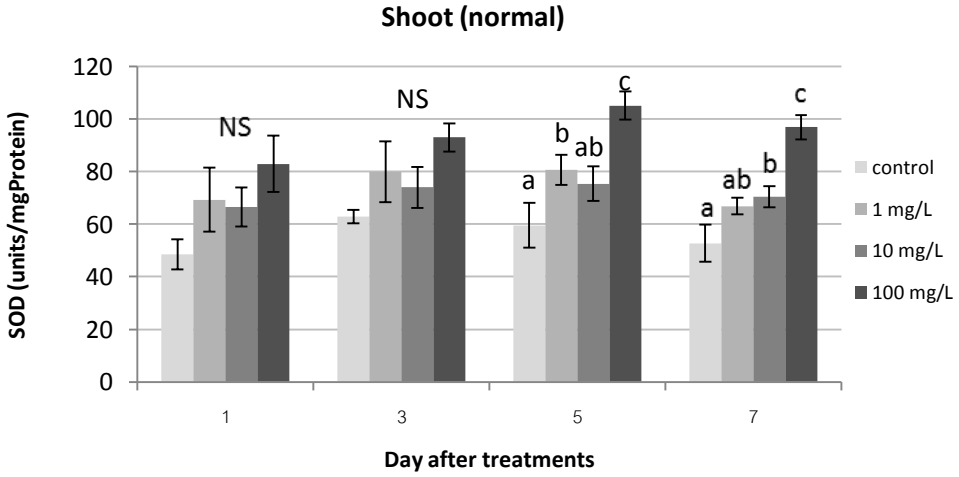

Figure 24. Superoxide dismutase activity in 20 nm AgNPs pretreatment shoot after transfer to fresh medium for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

5.2.2 Salt stress

Salt stress caused slightly induction of SOD in roots. However, with the pretreatment of 10 mg/L of AgNPs at various sizes during germination, SOD activity was significantly induced. AgNPs at the size of 30-70 nm to 150 nm significantly induced SOD activity after 3 days under salt treatment, while the pretreatment of 20 nm AgNPs significantly induced SOD activity in 5 days of salt treatment. SOD activity in the AgNPs treated roots was higher than controls up to 7 days of salt stress (Figure 25).

When pretreated the seeds with 20 nm AgNPs at various concentrations, the highest concentration (100 mg/L) resulted in the significantly higher SOD activity in roots after 1 day of salt stress, while the lower concentration pretreatment, 1 and 10 mg/L induced the higher level of SOD activity after 5 days of salt treatment (Figure 26). SOD activity was about 80-170 Units/mg protein in roots

The pretreatment of 10 mg/L AgNPs of all sizes caused the significant induction in shoots after 3 days of salt stress. Similar to the response in roots, the larger AgNPs showed the stronger induction than the smaller ones (Figure 27).

When pretreated the seeds with 20 nm AgNPs at various concentrations, 20 nm AgNPs at all concentration caused the induction of SOD activity in shoots after 3 days of salt stress. The higher concentration, the higher level of SOD activity was induced (Figure 28). SOD activity was about 50-130 Units/mg protein in shoots.

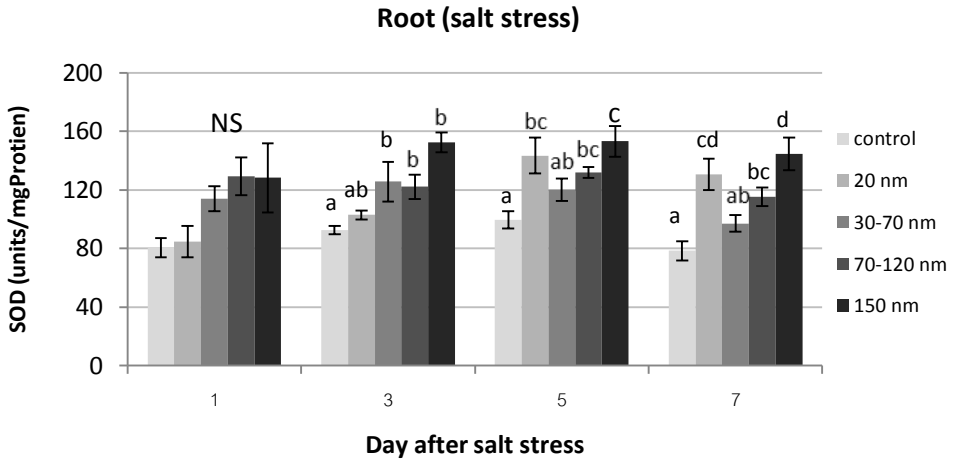

Figure 25. Superoxide dismutase activity in 10 mg/L AgNPs pretreatment root after exposed to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

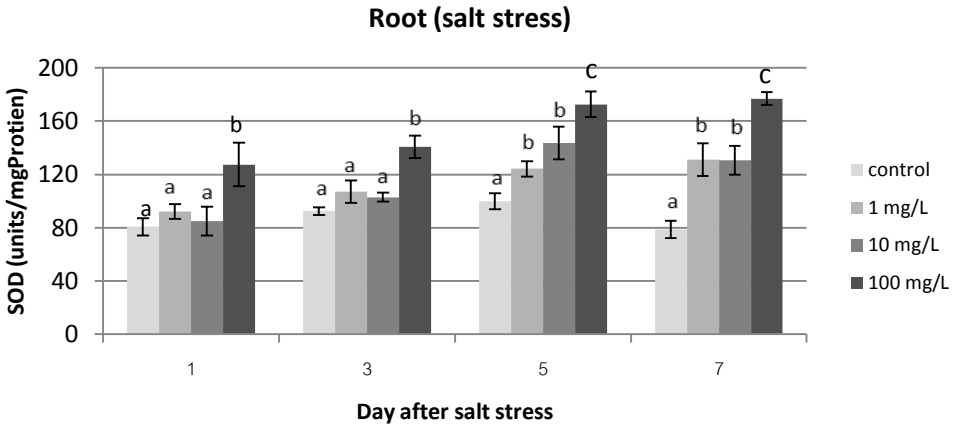

Figure 26. Superoxide dismutase activity in 20 nm AgNPs pretreatment root after exposed to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly differentce of means at $p<0.05$.

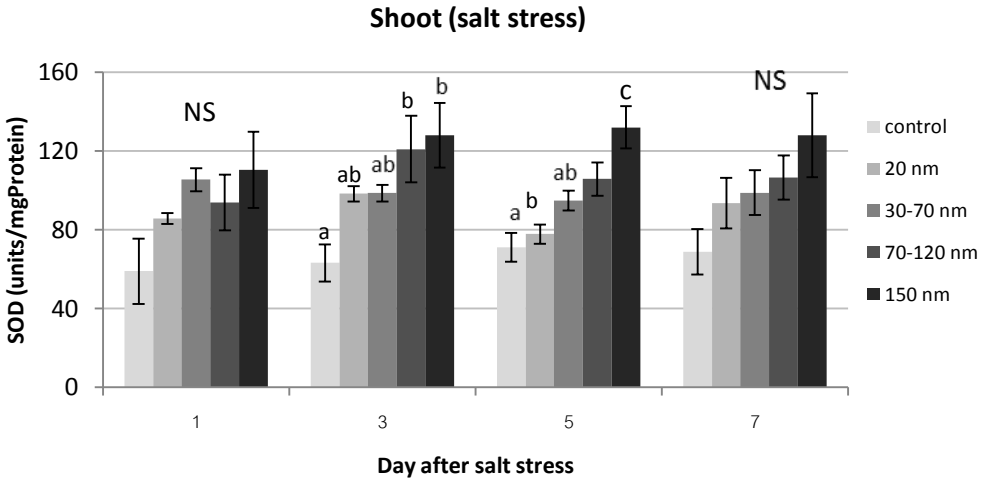

Figure 27. Superoxide dismutase activity in 10 mg/L AgNPs pretreatment shoot after exposed to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

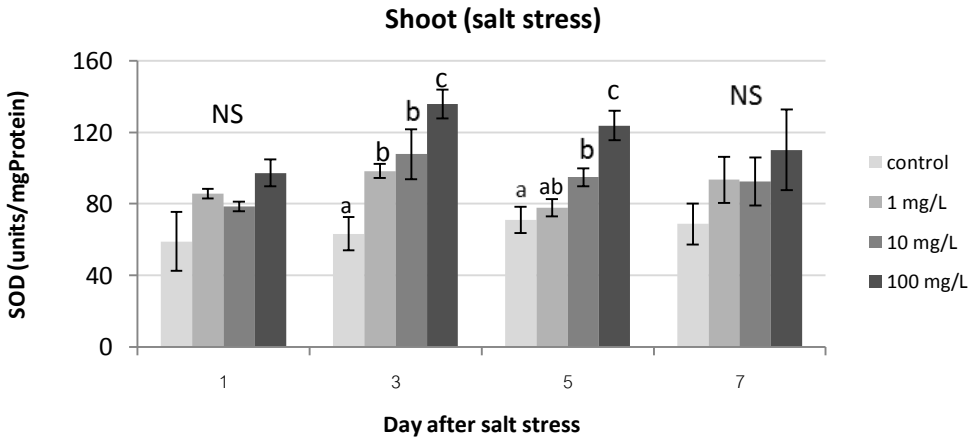

Figure 28. Superoxide dismutase activity in 20 nm AgNPs pretreatment shoot after exposed to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

5.3 Ascorbate Peroxidases (APX)

5.3.1 Normal condition

Pretreatment with AgNPs similarly affected on both roots and shoots. The larger 150 nm diameter AgNPs showed more impact on APX activity than smaller size (Figure 29). When rice seed pretreated with various concentrations of 20 nm AgNPs, the high concentration (100 mg/L) significantly increased APX acivity at 5 days after transfer. While lower concentration 1 and 10 mg/L slightly induced APX activity after 1 day after transfer. The highest concentration showed the highest induced APX activity on roots (Figure 30). It was induced up to 2 fold when compared to the control.

The plant transfer to new medium cused slightly increased APX activity on shoots. The pretreatments with larger size of 10 mg/L concentrations AgNPs significantly induced APX activity than smaller one (Figure 31). When rice seeds were pretreated with various concentration of 20 nm AgNP, the high concentration (100 mg/L) significantly induced APX activity after 5 day of the transfer, while the lower concentration, 1 and 10 mg/L no affected on APX activity when compared to the no AgNPs pretreatment (Figure 32). However, the range of APX activity in root were 10-42 Unit/mgProtien and shoot were 12-30 Units/mgProtein and 12-30 Unit, respectively.

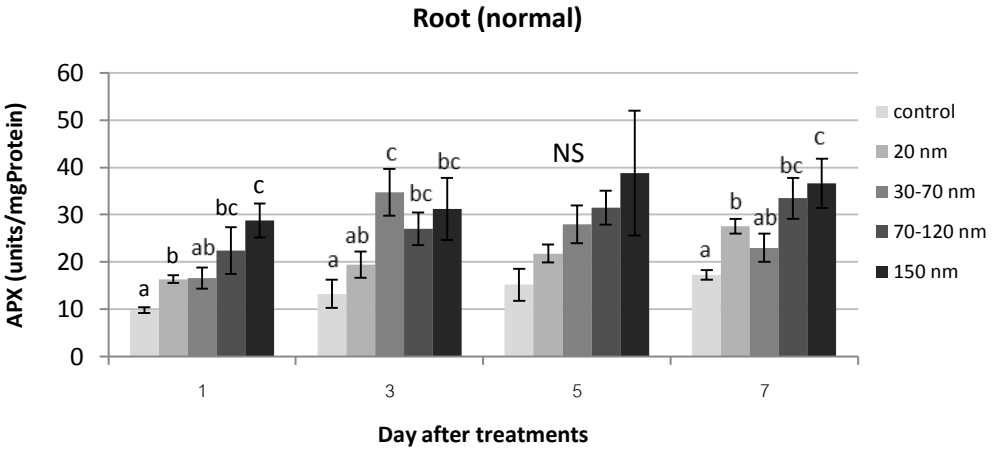

Figure 29. Ascorbate peroxidase activity in 10 mg/L AgNPs treatment root after transfer to medium solution for 0, 1, 3, 5 and 7. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

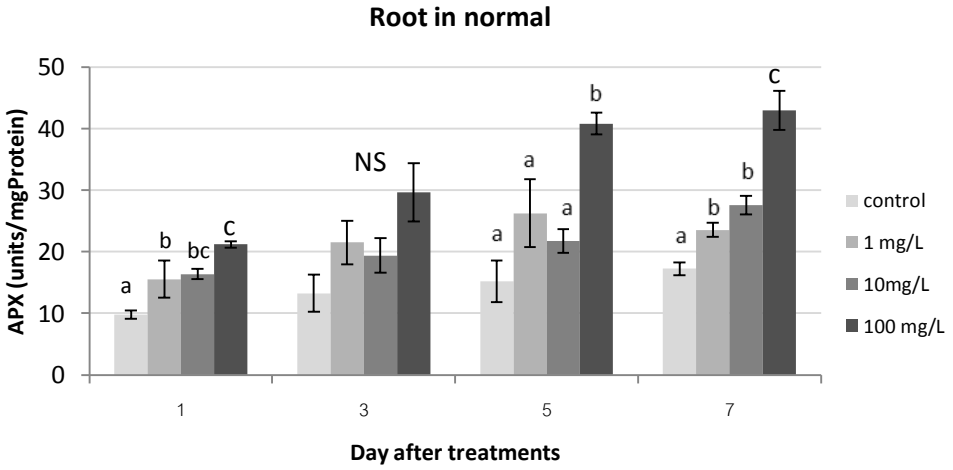

Figure 30. Ascorbate peroxidase activity in 20 nm AgNPs treatment root after transfer to medium solution for 0, 1, 3, 5 and 7. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

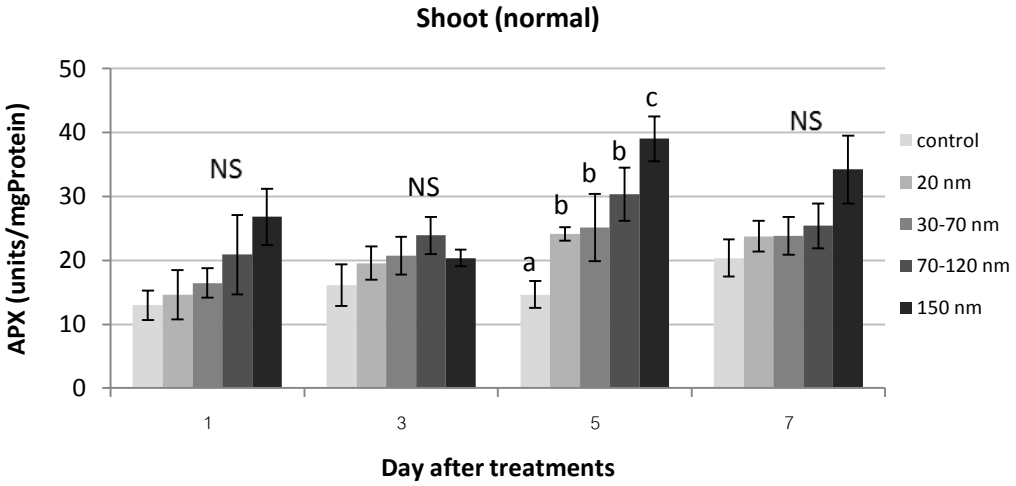

Figure 31. Ascorbate peroxidase activity in 10 mg/L AgNPs treatment shoot after transfer to medium solution for 0, 1, 3, 5 and 7. Error bars indicated S.E. Lowercase letter represent significantly difference of means at p<0.05 (Duncan's multiple range tests). NS is non-significantly difference of means at p<0.05.

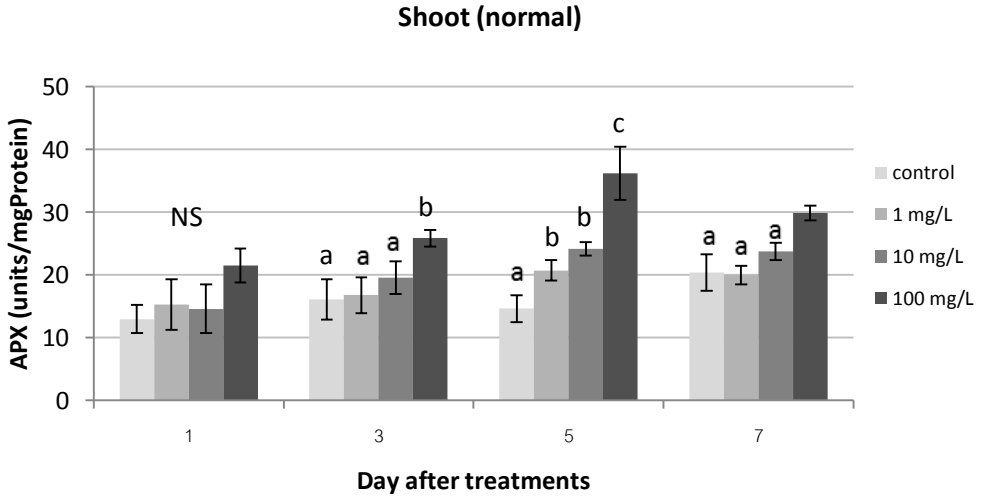

Figure 32. Ascorbate peroxidase activity in 20 nm AgNPs treatment shoot after transfer to medium solution for 0, 1, 3, 5 and 7. Error bars indicated S.E. Lowercase letter represent significantly different of means at p<0.05 (Duncan's multiple range tests). NS is non-significantly different of means at p<0.05.

5.3.2 Salt Stress

Salt stress had more affected on APX activity especially, the pretreatments of 10 mg/L at various concentrations nm diameter AgNPs during germination, significantly increased in 1 and 3 days of salt treatments (Figure 33). When pretreated the seeds with 20 nm AgNPs at various concentrations, the highest concentration (100 mg/L) resulted in the significantly higher APX activity in roots after 1 day of salt stress while after 5 days of salt stress at lower concentration 1 and 10 mg/L AgNPs induced APX activity similar with high concentration 100 mg/L AgNPs (Figure 34).

When pretreated the seeds with 10mg/L AgNPs at various sizes, 150 nm AgNPs caused the induction of APX activity in shoots after 3 days of salt stress (Figure 35). The highest concentration 100mg/L AgNPs, the higher level of APX activity was induced on 50 Unit/mgProtien (Figure 36). APX activity was about 15-40 Units/mg protein in shoots.

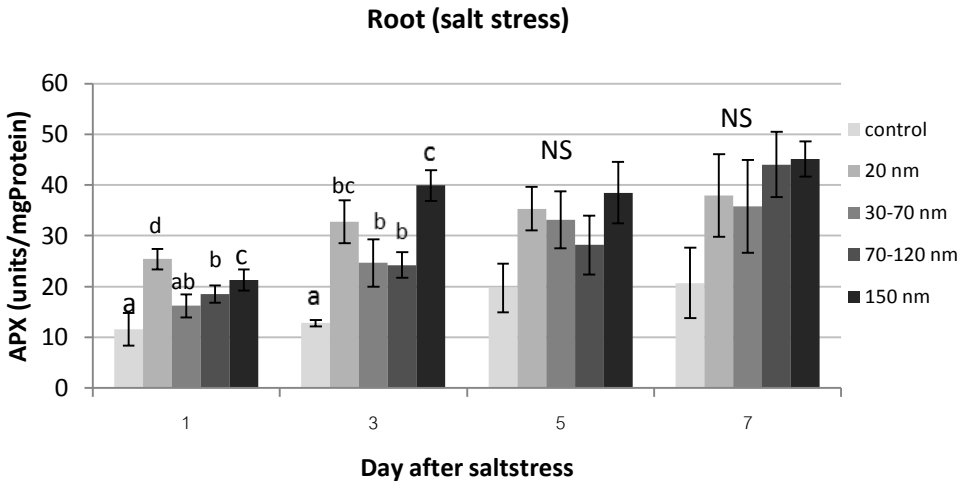

Figure 33. Ascorbate peroxidase activity in 10 mg/L AgNPs treatment root after exposed to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$

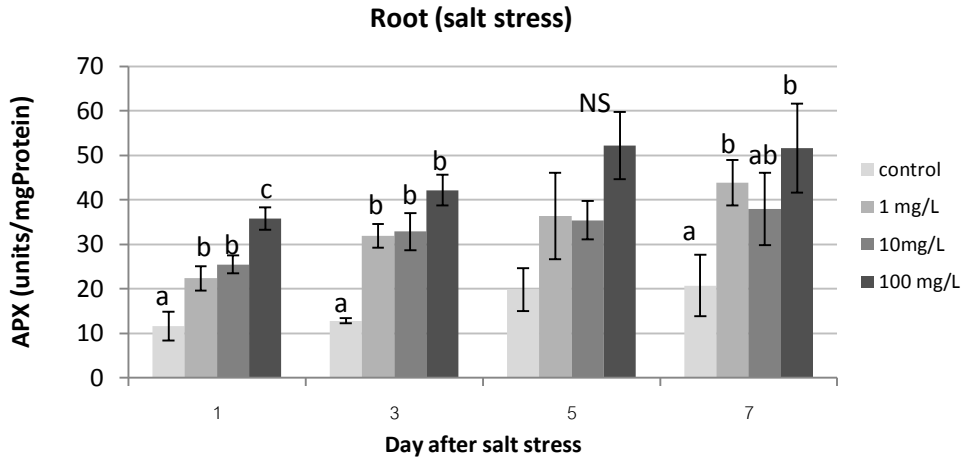

Figure 34. Ascorbate peroxidase activity in 20 nm AgNPs treatment root after exposed to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

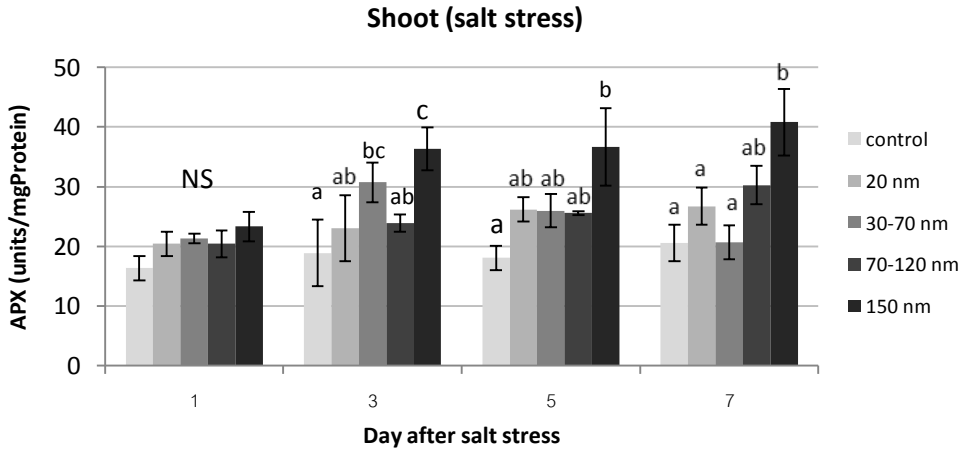

Figure 35. Ascorbate peroxidase activity in 10 mg/L AgNPs treatment shoot after exposed to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

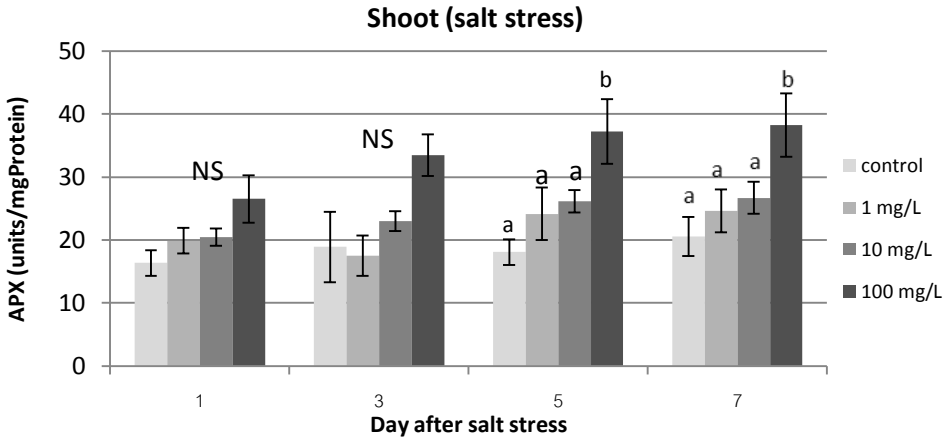

Figure 36. Ascorbate peroxidase activity in 20 nm AgNPs treatment shoot after exposed to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

5.4 Glutathione Reductase (GR)

5.4.1 Normal Condition

The pretreatment by seed soaking with AgNP of various sizes and concentration resulted in the significant increase in GR activity when transfer plants to the fresh nutrient solution. The larger size of AgNPs trended to show more effect on GR activity than smaller one about 33 % induced (Figure 37).When pretreatment with 20 nm diameter at various concentration of AgNPs, high concentration 100 mg/L induced the highest GR activity compared to lower concentration (Figure 38). In shoot, a large size AgNPs had more effected on GR activity (Figure 39) when treated with 20 nm AgNPs at various concentrations, the higher concentration had more effects on GR activity at 3 and 5 days after transfer (Figure 40). However, 100 mg/L inducedhighest GR activity about 56% enhancing, respectively (Figure 40).

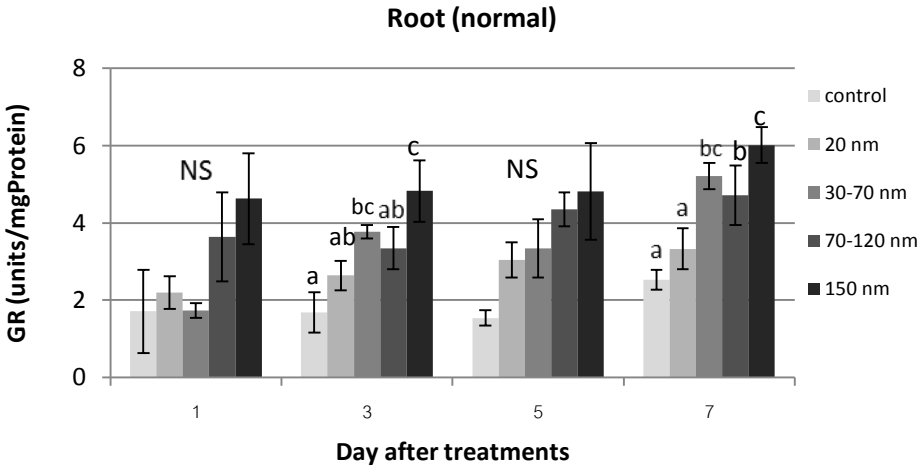

Figure 37. Glutathione reductase activity in 10 mg/L AgNPs pretreated root after transfer to fresh medium solution for 0, 1, 3, 5 and 7. Error bars indicated S.E. Lowercase letter represent significantly difference of means at p<0.05 (Duncan's multiple range tests). NS is non-significantly difference of means at p<0.05.

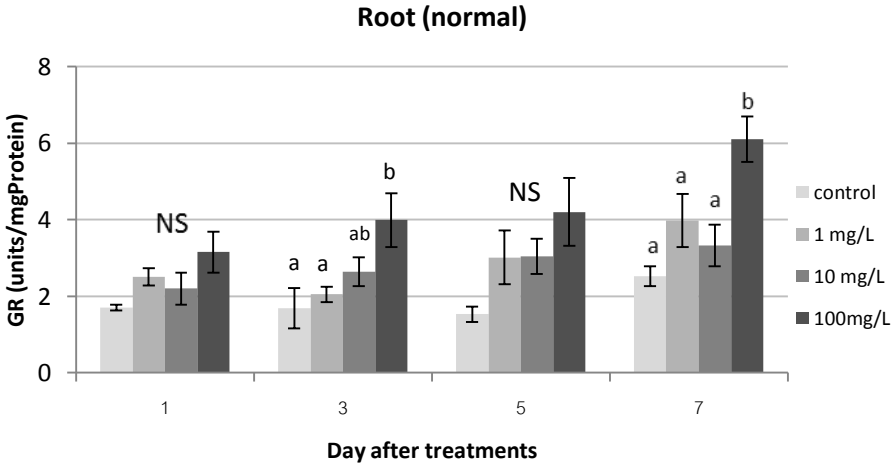

Figure 38. Glutathione reductase activity in 20 nm AgNPs pretreated root after transfer to fresh medium solution for 0, 1, 3, 5 and 7. Error bars indicated S.E. Lowercase letter represent significantly difference of means at p<0.05 (Duncan's multiple range tests). NS is non-significantly difference of means at p<0.05.

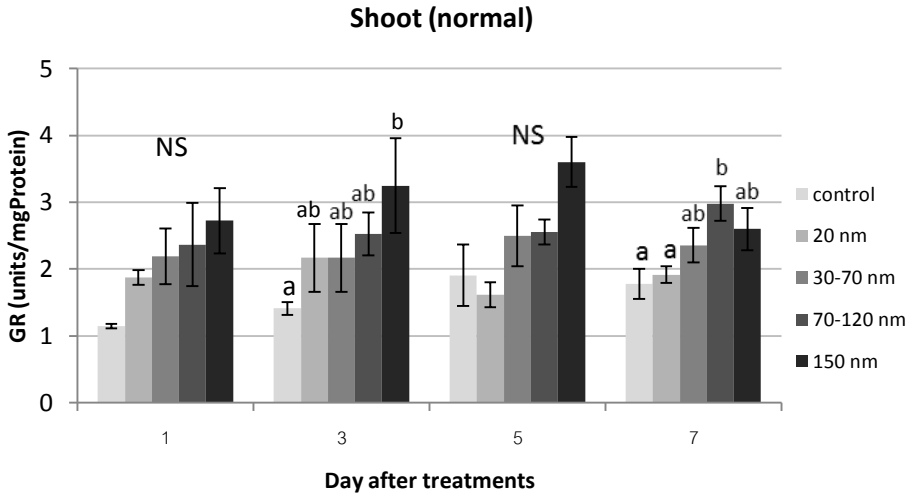

Figure 39. Glutathione reductase activity in 10 mg/L AgNPs pretreated shoot after transfer to fresh medium solution for 0, 1, 3, 5 and 7. Error bars indicated S.E. Lowercase letter represent significantly difference of means at p<0.05 (Duncan's multiple range tests). NS is non-significantly difference of means at p<0.05.

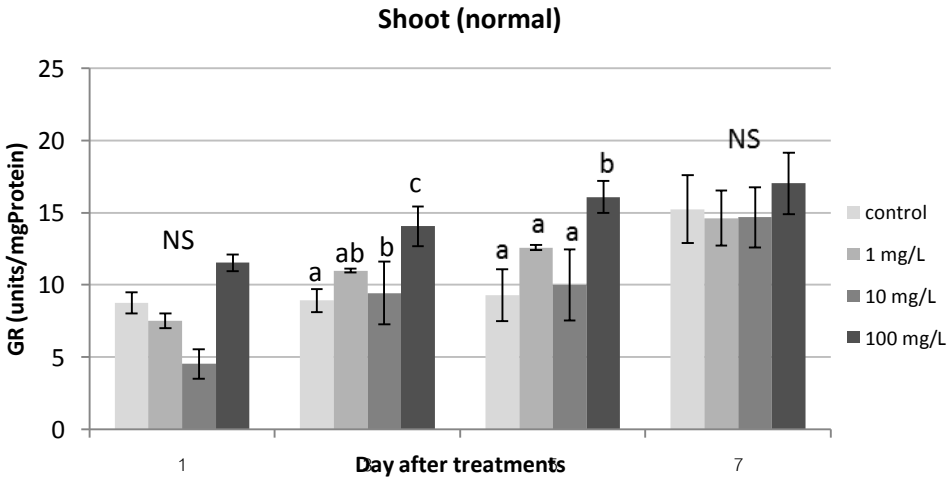

Figure 40. Glutathione reductase activity in 20 nm AgNPs pretreated shoot after transfer to fresh medium solution for 0, 1, 3, 5 and 7. Error bars indicated S.E. Lowercase letter represent significantly difference of means at p<0.05 (Duncan's multiple range tests). NS is non-significantly difference of means at p<0.05.

5.4.2 Salt stress

Salt stress induced an increase in GR activity both in roots and shoots. During germination soaked seed with 10 mg/L at various size, the large size 150 nm diameter showed more induced GR activity than smaller one about 33.33% induction on roots when compared to the no AgNPs pretreatments (Figure 41).The range of GR activity on roots were 1.5-6 Unit/mgProtien.

After 3 days of salt stress on shoot with pretreatment of 10 mg/L various size AgNPs 20 nm diameter was induced the highest GR activity on 25% induction when compared to the no treatments AgNPs control (Figure 43). At high concentration 100 mg/L) was dramatically enhanced GR activity about 25-29% enhancing respectively (Figure 44). However, the range of GR activity on shoots was 1.5-7 Unit/mgProtein.

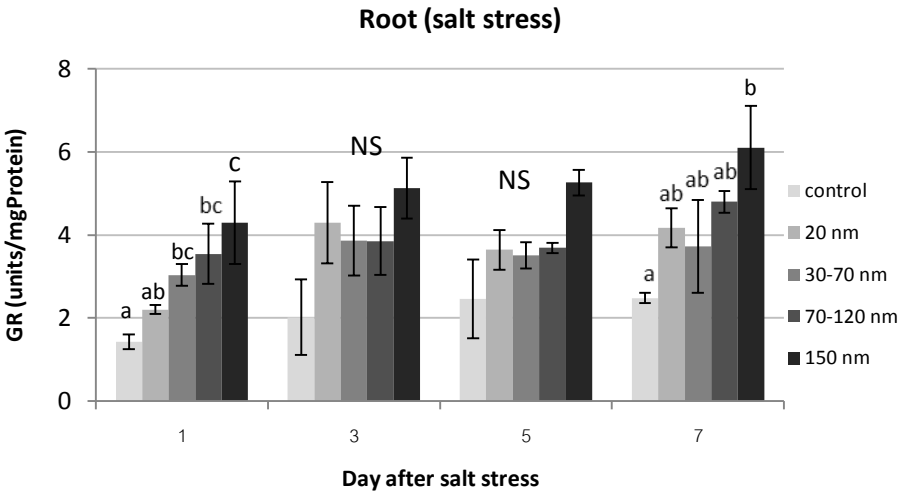

Figure 41. Glutathione reductase activity in 10 mg/L AgNPs pretreated root after exposed to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

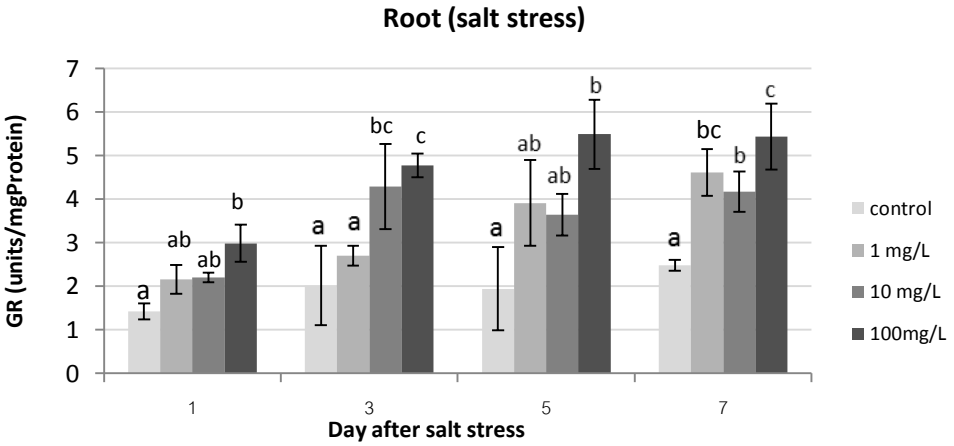

Figure 42. Glutathione reductase activity in 20 nm AgNPs pretreated root after exposed to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

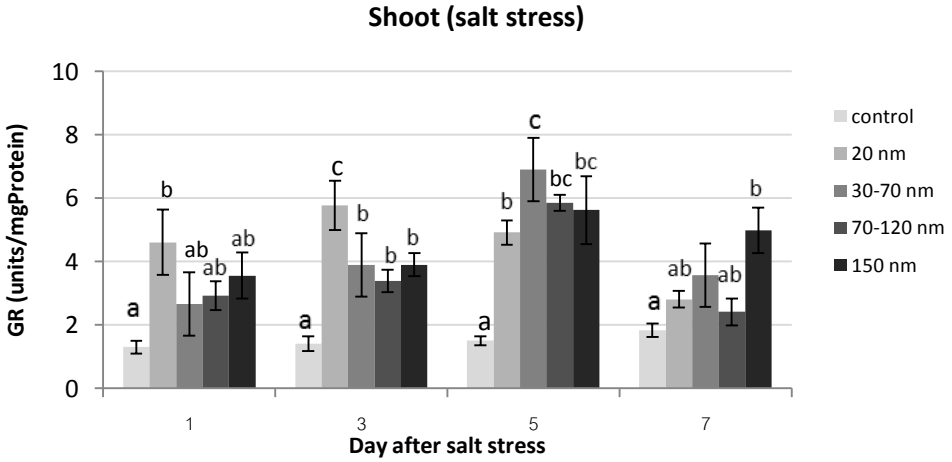

Figure 43. Glutathione reductase activity in 10 mg/L AgNPs pretreated shoot after exposed to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

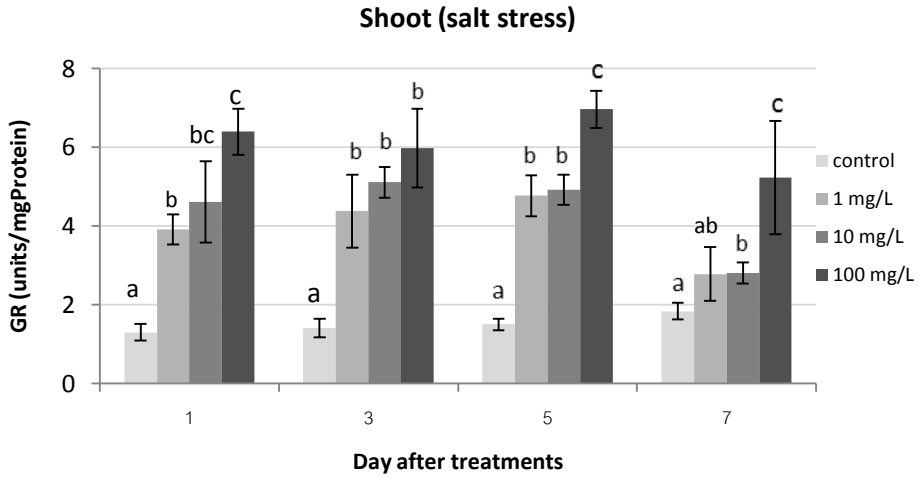

Figure 44. Glutathione reductase activity in 20 nm AgNPs pretreated shoot after exposed to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

5.5 Glutathione Peroxidase (GPX)

5.5.1 Normal condition

The strongest effects on GPX induction was detected after 7 days transfer to fresh medium, when seeds were soaked in the large diameter of AgNPs when compared to the no pretreated AgNPs and smaller one (Figure 45). In addition, GPX activity on roots that pretreatments with 20 nm diameter at various concentration of AgNPs showed the highest concentration 100 mg/L had more effected on GPX activity than lower concentration (Figure 46).

In shoot pretreatment with 10 mg/L at various sizes after 3 days transfer to new medium, 20 nm diameters showed highest GPX activity when compared to other sizes (Figure 47).And high concentration 100 mg/L AgNPs had more effected on GPX activity (Figure 48) However, the range of GPX activity were 1-5 Units/mgProtien on roots and 1-3 Unit/mgProtien on shoot, respectively.

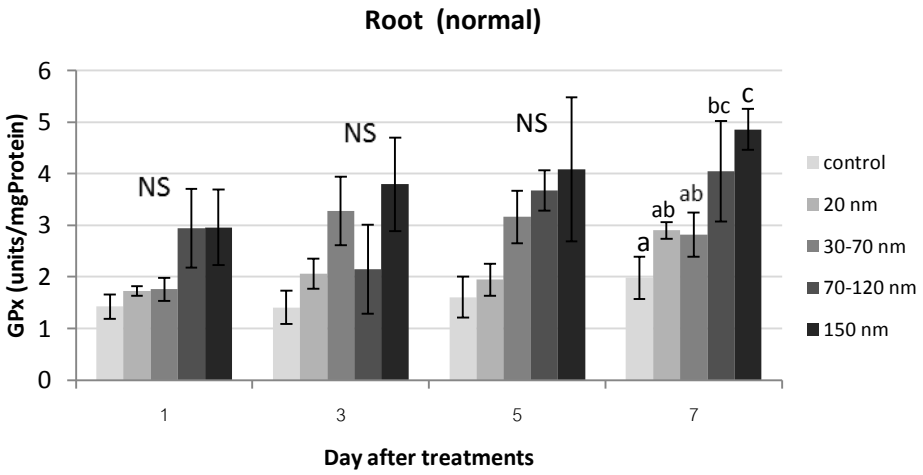

Figure 45. Glutathione peroxidase activity in 10 mg/L AgNPs pretreated root after transferred to fresh medium solution for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at p<0.05 (Duncan's multiple range tests). NS is non-significantly difference of means at p<0.05.

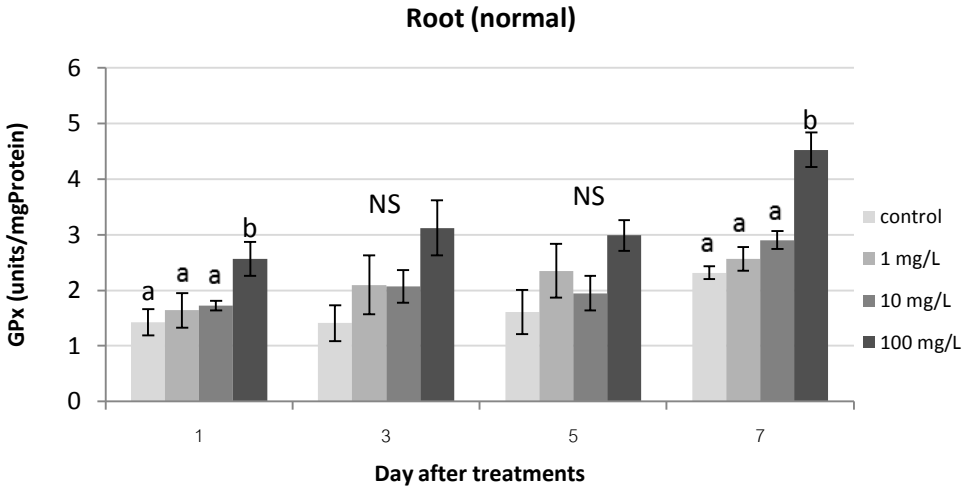

Figure 46. Glutathione Peroxidase activity in 20 nm AgNPs pretreated root after transferred to fresh medium solution for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at p<0.05 (Duncan's multiple range tests). NS is non-significantly difference of means at p<0.05.

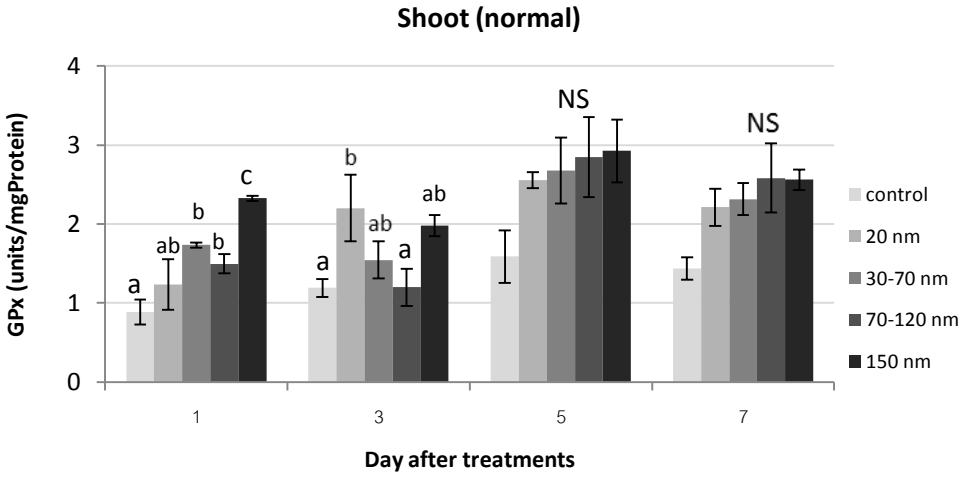

Figure 47. Glutathione peroxidase activity in 10 mg/L AgNPs pretreated shoot after transferred to fresh medium solution for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

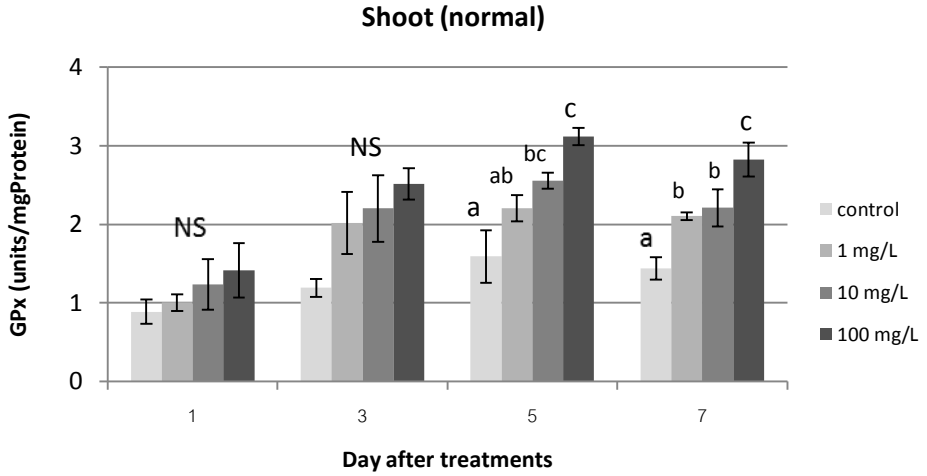

Figure 48. Glutathione peroxidase activity in 20 nm AgNPs pretreated shoot after transferred to fresh medium solution for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

5.5.2 Salt stress

Salt stress caused induction GPX activity both of roots and shoots. In root after 3 days salt stress, pretreatment with 10 mg/L at various sizes 20 nm diameter had more effected on GPX activity than other sizes while after 5 days of salt stress It's GPX activity decreased (Figure 49). When pretreatment with 20 nm diameter at various sizes, the high concentration 100 mg/L showed high GPX activity (Figure 50). The range of GPX activity was 1.5-5 Unit/mgProtien.

In shoot when pretreated the seeds with 20 nm AgNPs at various concentrations, the highest concentration (100 mg/L) resulted in the significantly higher GPX activity in roots after 1 day of salt stress, while after 5 days of salt treatment no effected on GPX activity (Figure 52). The pretreatment of 10 mg/L AgNPs of all sizes caused the significant induction in shoots after 1 day of salt stress GPX activity was significantly induced by AgNPs at the size of 20 nm diameter significantly induced GPX activity after 1 and 3 days under salt treatment. GPX activity was about 1-3.8 Units/mg protein in shoots.

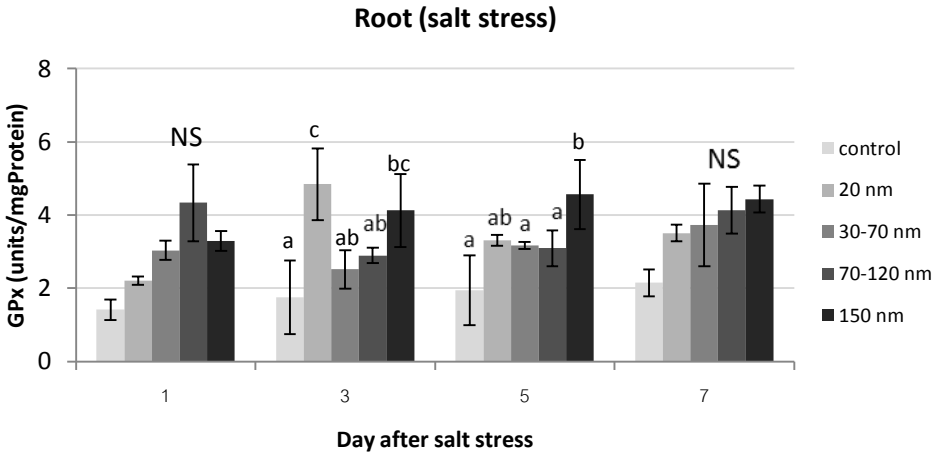

Figure 49. Glutathione Peroxidase activity in 10 mg/L AgNPs pretreated root after exposed to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

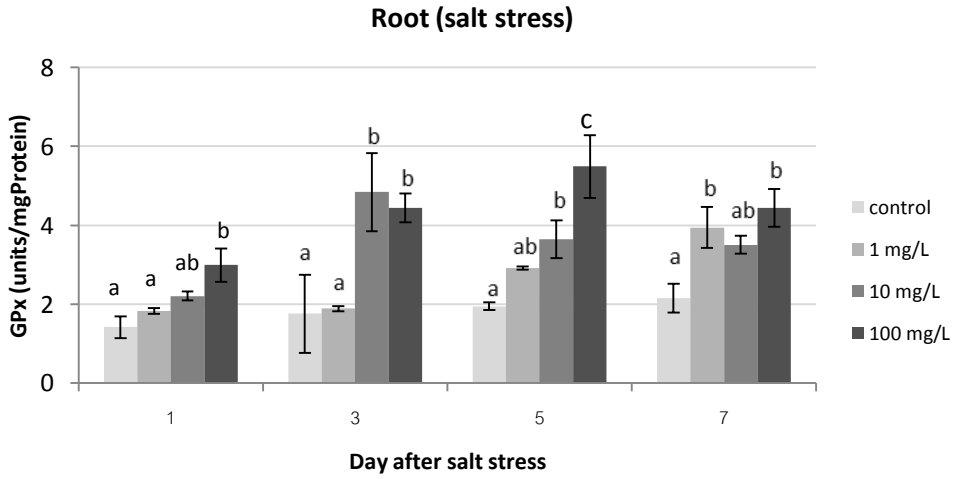

Figure 50. Glutathione Peroxidase activity in 20 nm AgNPs pretreated root after exposed to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

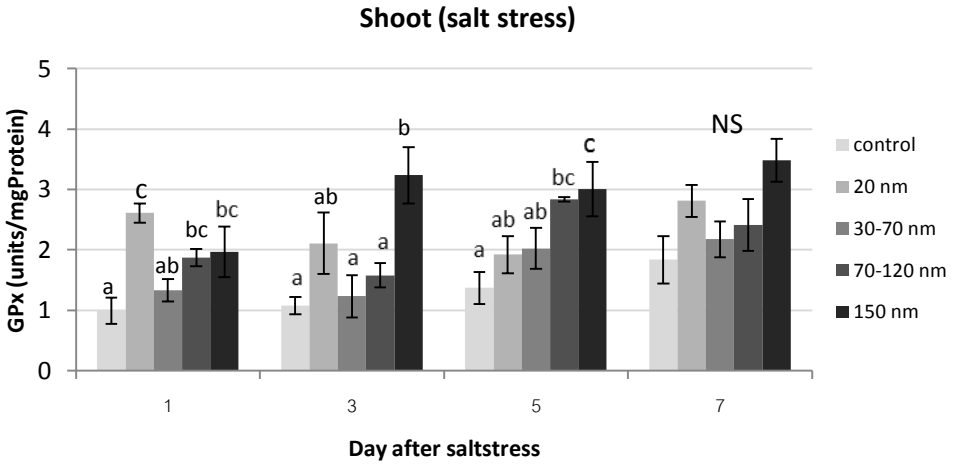

Figure 51. Glutathione Peroxidase activity in 10 mg/L AgNPs pretreated shoot after exposed to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

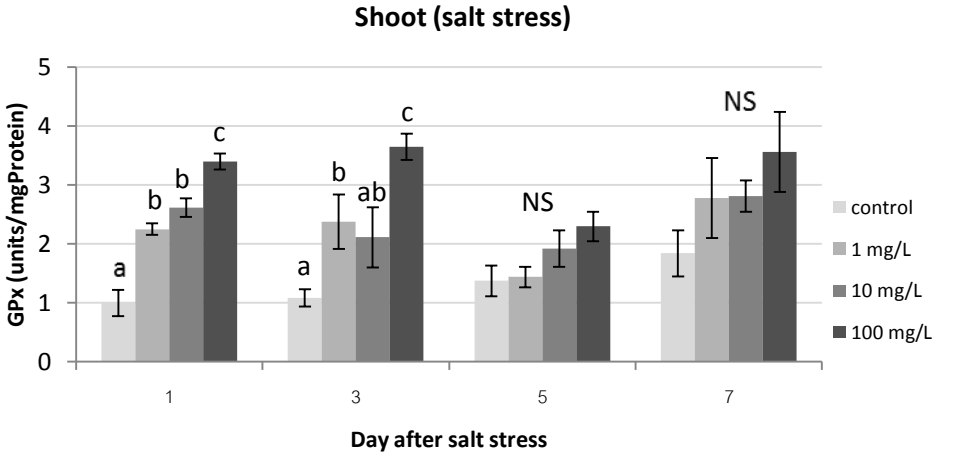

Figure 52. Glutathione Peroxidase activity in 20 nm AgNPs pretreated shoot after exposed to NaCl for 0, 1, 3, 5 and 7 day. Error bars indicated S.E. Lowercase letter represent significantly difference of means at $p<0.05$ (Duncan's multiple range tests). NS is non-significantly difference of means at $p<0.05$.

II Discussion

1. Effect of AgNPs concentrations and sizes on seedling growth

In normal condition, AgNPs had a negative effect on the growth of roots and Shoot in comparison with normal grown control. Exposure of the rice seeds to the 20-nm-diameter AgNPs showed a clear and dose-dependent inhibitory effect on their subsequent germination success and on the growth of those seedlings that did germinate. AgNPs at all concentrations affected the seedling growth, although this was not statistically significant at 10 mg/L for all parameters measured. The higher concentrations of AgNPs (100 and 1000 mg/L) strongly inhibited both the shoot and root growth (especially dry weight) (Table 2 and 4). In this study; believe that the AgNPs could block the water channels through adsorption and AgNPs possibly penetrate radically into roots and spoil the whole cellular metabolism and stages of cell division and affect the roots elongation, metabolism, genetic materials. It was recently reported that the NPs may induce the formation of new and large-size pores and routes for the internalization of large NPs through cell walls [5]. The obvious toxic effect of AgNPs could be noticed with the brownish and stiff roots, when exposed to AgNPs at any types and concentration. Phytotoxicity of metal oxide nanoparticles on seed germination and root development has recently been studied in *Allium cepa*; *A. cepa* with a well-developed root system in a hydroponic culture was identified before its exposure to different concentrations of the oxide NPs [60]. The molecular mechanism of the toxicity NPs in the plant roots is not clear and requires further investigation; however, it should be closely related to the chemical composition, chemical structure, particle size, and surface area of the NPs [61, 62]. When exposed with hydroxyapatite nanoparticles (HAP); The smaller sizes (<30 nm) showed more penetrated cross through the cell membrane while that 31.2 nm and

32.6 nm which were too big to enter into the cells [63]. Moreover, HAP nanoparticles encircled by a few of vacuoles were found in the cytoplasm. The cytoskeleton around the vacuoles disrupted. The nanostructure and Ca^{2+}concentration were considered as the main factors to cause cell apoptosis which was the reason of inhibition. [64]. Other NPs; the nZVI exhibited strong toxic effect on *Typha* at higher concentrations (>200 mg/L) but enhanced plant growth at lower concentrations. It's significantly reduced the transpiration and growth at high concentrations (1000 mg/L), respectively. Transmission electron microscope (TEM) and scanning transmission electron microscope (STEM) confirmed the internalization of nZVI in *Typha* root cells [65]. Lin and Xing (2007) studied effects of five types of nanoparticles (multi-walled carbon nanotube, aluminum, alumina, zinc, and zinc oxide) on seed germination and root growth of six higher plant species (radish, rape, ryegrass, lettuce, corn, and cucumber). Inhibition of root growth varied greatly among nanoparticles and plants [66]. On the contrary, nano-TiO_2 was reported to enhance photosynthesis and nitrogen metabolism and greatly improve spinach growth when administered to the seeds or sprayed onto the leaves [67]. The effect of surface characteristic NPs was found a slight reduction in root elongation in the presence of uncoated alumina NP but not with NP coated with phenanthrene. It was proposed that the surface characteristics of the alumina played an important role in phytotoxicity [68].

In salt-stress condition, AgNPs exhibited inhibition on growth of root and shoot especially at high concentration (100 and 1000 mg/L) and a large sizes of AgNPs (70-120 and 150 nm). But interestingly, concentration 1 and 10 mg/l and 20-nm sizes of AgNPs were enhanced in root growth as root dry weight and root length. Mark to positive effect in root length was significantly difference from control and other treatments (Table 5). The low concentration of NaCl could enhance plant growth, but at high concentration of NaCl, plant growth were inhibited. Camilla et al. (2012) studied the physiological change under salt stress in pea (*Pisum sativum*). It was

exposed with different NaCl concentration. The result showed no negative effect on growth of plant [69]. However, the positive effect on growth (fresh and dry weight) and another physiological change in soybean under different NaCl concentration was observed. This may be due to the acclimation phenomenon [70]. Moreover, positive effect on growth in salt stress condition was found in bean plant *Vicia faba* (L.). NaCl caused an increase in plant height and increased both fresh and dry weights of shoot [71]. These results for fresh and dry weights for the root system in this study, agree with the results presented by Rocio et al.(2005) in their study on lettuce (*Lactuca sativa* L.), where they reported that the treatment with salt increased the fresh weight by about 28%[72], and agree with the results of a study by Dantus et al. (2005) on cowpea (*Vigna unguiculata* L.), where they report that using 10 mM of NaCl increased fresh and dry weights of the shoot system of their seedlings[73]. The increase in fresh weight of plant may be due to the ability of the plant to increase the size of its sap vacuoles, which allows for the collection more water, and this in turn dissolves salt ions that have accumulated and leads to the subsequent increase in fresh weight [74].

2. Bioaccumulation of AgNPs in plant tissues

After seed soaking, the rice seeds were germinated on sand and then transferred to the nutrient solution. The carried over amount of AgNPs in the nutrient solution was determined as shown in Fig. 4. To study the bioaccumulation of AgNPs, Inductively coupled plasma optical emission spectrometry (ICP-OES) are still the dominant analytical techniques used for silver determination in environmental samples[75]. More research used ICP-OES technique to measure metal in sample such as using ICP-OES to measure metals in gum deposits of internal combustion engines[76], study the element determination in plant specimens[77] and study trace amounts of

Ag, Cd, Cu, and Zn in environmental samples[78]. The amount of silver in rice seeds was showed in Appendix D (Fig.D1). The nutrient solution with seedlings germinated from treated seeds with 0.1 or 1.0 mg/L AgNPs at any sizes did not contain AgNPs at the detectable level. At higher concentration of AgNPs, the higher concentration of contaminated AgNPs in nutrient solution was found (Fig. 4). However, the carried over concentration was less than 3000 fold dilution of AgNPs concentration for the seed-soaking. Various concentrations of AgNPs were found in leaf and root tissues depending on sizes and concentration of AgNPs treatment. As low as 1 mg/L of 20 nm diameter, AgNPs could be uptaken by rice seedlings as it could be detected in root tissues. However, no detectable level of AgNPs was found in shoot. This indicated that AgNPs were trapped in the roots and AgNPs transport may be inhibited by some mechanisms. When seeds were exposed to the higher concentration of 20 nm diameter AgNPs, the higher accumulation was found. The increase in AgNP accumulation was found when the seeds were treated with higher concentration of AgNPs. **.** Plant cells has a negative surface charge, which allows the transport of negatively charged compounds into the apoplast. Based on this knowledge about root physiology we could hypothesize that negatively charged NP could enter the apoplast of the root cortex and eventually also the xylem, but are not taken up by the cells. NPs with primary diameter smaller than 100 nm may thus cross root epidermis by passing through epidermis cell wall, possibly in root hairs. In root cortex radial transport of NPs can occur through the apoplast (cell walls and intercellular spaces) or the symplast (through cells) [79]**.** The accumulation of nanoparticles was reported by Larue et al., (2012) studied the impact of TiO_2 nanoparticles in wheat (*Triticum aestivum* spp.) the results showed TiO_2-NPs with smaller sizes can be accumulated in wheat roots parenchyma cell plant but do not reach the stele and shoots. The results provide evidence that the smallest TiO_2-NPs (14 nm) accumulate in roots and distribute through whole plant tissues without dissolution or crystal

phase modification. Moreover, wheat plants exposed to the smallest NPs during the first stages of development caused an increase of root elongation [80]. These data support the present study that only the small NPs (20 nm) may be more accumulated in rice root and that their impact is moderate. More evidence of nanoparticle penetration and accumulation in plant cells was reported by Zhang and et al. (2014). The uptake and accumulation of CuO nanoparticles and CdS/ZnS quantum dot by *Schoenoplectus tabernaemontaniin* was observed by Transmission electron microscope (TEM) [81]. The uptake of nanoparticles occurs *via* endocytosis or by phagocytosis in specialized cells. One hypothesis is that the coating of the NP by protein in the growth medium results in conformational changes of the protein structure, which triggers the uptake into the cell by specialized structures, limiting uptake to NP below about 120 nm [82].

3. Effect of AgNPs on the rice seedling leaf anatomy

The negative effect of 150 nm AgNPs at the concentration of 100 mg/L were detected. Cell disruption, especially in parenchyma cell was shown (Figure 4). The effect on root and leaf anatomy was reported but the mechanism of AgNPs penetration is not fully understood. The same result was also found on the other plants. The larger size of ZnONPs and TiO_2 caused more damage on plant cells than the smaller particles [83]. Other nanoparticles were also reported to cause morphological changes at high concentration treatment. It was found that grapheme showed phyto-toxicity on root morphology of cabbage, tomato, red spinach and lettuce seedlings, when exposed to high concentration (100mg/L) grapheme [84]. The AgNPs was shown to have more effects on maize (*Zea mays* L.) and cabbage (*Brassica oleracea* var. *capitata* L.) than zinc oxide nanoparticles. The structural changes were found in maize primary root cells while exposure to $AgNO_3$ leading to

cell erosion in root apical meristem. Moreover, a significant change was found in meta xylem [85].

4. Effect of silver nanoparticles on physiological responses under normal and salt stress

4.1 Hydrogen Peroxide Content

Exposure of plants to various abiotic stresses usually induces the generation of both H_2O_2 and reactive oxygen species (ROS). Such stresses include heavy metal, salinity, drought, atmospheric pollutants such as ozone, UV irradiation, temperature extremes, and mechanical wounding [86]. Hydrogen peroxide (H_2O_2) was a mainly toxic cellular metabolite. The generation of H_2O_2 is increased in response to various stresses. However, in this study, rice plant under normal and salt stress condition was induced H_2O_2 production. It's was detected in both root and shoot. Hydrogen peroxide (H_2O_2) contents showed greater increase under high concentration (100 mg/L) and large sizes (150 nm) of AgNPs (Figure 4-5). The level of H_2O_2 in pretreatment AgNPs followed by salt stress increased about 35-40% comparison with control (Figure 8-9). Furthermore, levels of H_2O_2 were highest under salt stress condition indicating that salinity was the main cause of stress inducing oxidative stress and induced antioxidant mechanism to detoxify ROS [87]. The same result was found in *Pancratium maritimum* L. Salinity affects plant growth and development; Hydrogen peroxide (H_2O_2) contents showed significantly increased under severe stresses [43]. However, during more severe and stress conditions including the pretreatment with AgNPs an uncontrolled accumulation of ROS may occur, an enhanced level of H_2O_2 indicate the presence of oxidative stress in plant cells[88]. The concentrations of H_2O_2 that are likely to be toxic will high rates of H_2O_2 production are normally balanced by very efficient antioxidant systems. Moreover,

H_2O_2 were induced by Cadmium in leaf of *Phaseolus aureus* and *Vicia sativa* [89] and NaCl induced an increased in H_2O_2 content in the tissue of *B. papirifera*. The content of H_2O_2 was changed in pararell with the increase of antioxidant enzyme [27].

4.2 Effects of AgNPs on anti-oxidative systems under normal and salt stress condition

ROS mainly include superoxide radicals ($O_2^{\cdot-}$), hydroxyl radicals ($^{\cdot}OH$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). To protect plants from the toxic action of ROS, cells possess a battery of antioxidants. The accelerated generation of reactive oxygen species (ROS) under salt and abiotic stress along with induction of ROS scavenging enzymes such as catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione peroxidase (GPX) and superoxide dismutase (SOD) [41]. The results showed that the enzymatic antioxidant activity was changed when 14 day-old rice seedlings were exposed to salt stress. The enzymatic activity significantly increased with same pattern; at high concentration (100 mg/L) and a large size (70-120 nm and 150 nm) of AgNPs was found to induce higher activity of the anti-oxidant enzymes than pretreatment with the smaller size AgNPs or the pretreatment at lower concentrations. These indicated that antioxidant enzymes played an important role in protecting the plant from salt-stress condition.

Catalase (CAT) has long been considered as one of the major antioxidant enzymes and mainly functions in the removal of excessive H_2O_2 by conversion to water and oxygen. In this result, the CAT activity exhibited more sensitivity and quickly response to the changes under high concentration (100 mg/L) and large size (150 nm) of AgNPs (Figure 12-15). The transfer process was also shown to induce CAT activity, especially in roots rather than shoots. These may be due to the fact that roots were the first tissue that sensed the changes of nutrient solution, and the

process slightly trigger the production of ROS, resulting the induction of antioxidant enzyme function to scavenge these molecules. Pretreatment with higher concentrations or the larger sizes of AgNPs showed stronger effect on CAT activity. On the other hand, in shoot, different size of AgNPs did not matter for the difference in CAT activity (Figure 18). The CAT activity under salt stress might be used to maintain growth and development under salt stress condition. This evidence support the root growth response under salt-stress that the priming with AgNPs by seed soaking enhanced root growth, but not shoot growth. These may be due to the higher induction of the anti-oxidant enzyme systems in roots, compared the level of the induction in shoots. The TiO_2 nanoparticles also affected the activity of CAT [90] while NiO-nanoparticles affected physiological response including CAT activity [54].

Superoxide Dismutase (SOD) is the most effective intracellular enzymatic antioxidant. SOD has been proposed to be important in plant stress tolerance and provide the first line of defense against the toxic effects by catalyzed dismutation, one O_2^- to H_2O_2 and another oxidized to O_2 [41]. Results are shown in Figure 20-27, the SOD activity was increased significantly when 1 mg/L of 20 nm AgNPs was used to prime at germination stage (Figure 20). The high concentration, 100 mg/L, of the same size of AgNPs induced the significantly higher SOD level in comparison of control and other concentration up to 2.3 fold (Figure 21). Under salt stress, the highest SOD activity level was detected in roots when pretreated with 10 mg/L of 20 nm AgNPs (Figure 24). SOD is a primary tool to scavenge free radicals. When oxidation damage happened, SOD induction can be seen. There are some reports in changes in antioxidant enzyme activity after abiotic stress. SOD was induced in wheat seedling under high temperature stress which suggested that SOD might play important roles under hot temperature stress [91]. Under salt-stress condition,

activities of antioxidant defense enzymes were changed in parallel with the increased H_2O_2 in *Broussonetia papyrifera* [27]

APX is primarily located in both the chloroplasts and cytosol. It is one of the most important enzymes playing a role in eliminating toxic H_2O_2 from plant cells. In this process, ascorbate was used as the specific electron donor [92]. In present study, the exposure with 100 mg/L of 150 nm AgNPs caused APX activity enhancement about 4 fold compared to controls. Interestingly, under salt stress in root tissues, when seedlings were primed with 10 mg/L of 20 nm AgNP, the APX activity was found significantly higher than control and other treatments (Figure 32). The increase of APX activity observed after salt stress in this study may be due to the increased H_2O_2 production (Figure 4-8), because APX play a role to eliminate excess H_2O_2.

In the case of GR activity, it was similar to other antioxidant enzyme responses. The pretreatment by the higher concentration or the larger AgNP size showed more impact on the enzyme activity. These phenomenon were similar in both normal and salt-stress condition (Figure 36-43). However, the remarkable induction of GR in shoots was found in salt-stress condition, when the seedlings were pretreated with AgNPs (Figure 42). The same result was reported in rice exposed to excess copper. NaCl stress can stimulate the expression of several antioxidant enzymes including GR activity [93].

GPX provides a mechanism for detoxification of peroxides in living cells by removing H_2O_2, neutralizing or scavenging free radicals. The result has shown in Figure 44-51, effect of AgNPs on GPX activity was found in same tendency; the higher concentration and larger sizes of AgNPs enhanced GPX activity both in root and shoots with no statistically significant. Under salt stress condition, plant response to stress by change GPX activity especially, at 20 nm 10 mg/L of AgNPs was found difference result when compared to under normal condition by increased GPX

activity (Figure 48-51). It is indicated that GPX play important role to detoxification and elimination excess free radical under stress environment to maintain growth and development.

In the present study, AgNPs induced oxidative stress in rice plant like other stresses. The pretreatment of AgNPs is expected to change the antioxidant enzyme activities as it can create the oxidative stress, which may acclimate the seedlings when they have to face the stress at later stages. SOD, CAT, APX, GR and GPX are the important anti-oxidative enzymes to prevent the excess ROS. Interestingly, under salt stress condition CAT, SOD, APX, GR and GPX activity was significantly different from normal condition. Acclimation to external environmental changes can occur in plants to internal adjustments within tissues and cells to maintain metabolism to proceed under altered conditions [69]. It was widely reported that many plant species increased the ability to tolerate salt stress after being exposed to low level of stress [71, 94-96]. The beneficial effects of acclimation included improved survival rate, higher growth rate, less biomass reduction and physiological (lower Na^+ accumulation in the shoot; better osmotic adjustment) characteristics [26]. However, the physiological mechanisms of acclimation under the resistance to salinity remain unclear. This study provides some evidences on how AgNPs priming could enhance salt-stress adaptation via the induction of antioxidant enzyme activities.

CHAPTER V

CONCLUSION

1. Effects of the sizes and concentrations of AgNPs on 'KDML 105'rice *Oryza sativa* L. seedlings.

Rice seeds were soaked at various sizes and concentrations of AgNPs. The larger sizes 150 nm diameter and high concentration 100 and 1,000 mg/L strongly inhibited rice growth. Under salt stress showed the similar responses as shown in normal condition. The larger sizes AgNPs, 150 nm diameter, and the higher concentration, 100 and 1,000 mg/L, strongly inhibited rice growth when compared to the smaller and the lower concentration ones.

On the other hand, positive effect was detected when treated with 10 mg/L of 20 nm-diameter AgNPs. It was shown that the pretreatment of 10 mg/L 20 nm AgNP could promote root growth as indicated by root dry weight and root length.

2. Study of AgNPs accumulation in rice tissue.

The higher uptake was found when the seeds were treated with the smaller 20 nm diameter of AgNPs. The AgNP was trapped in the roots rather than transported to the leaves. These resulted in the less negative effects on seedling growth, when compared to the seed soaking with the larger AgNPs with 150 nm diameter.

1. Effect of AgNPs on anatomy of rice leaves

The large size of 150 nm of diameter at 100 mg/L caused parenchyma cell deformation, while the smaller size AgNPs (20 nm) in diameter at 10 mg/L showed less effects.

2. AgNPs effects on H_2O_2 under normal and salt stress condition

H_2O_2 production in both shoots and roots was slightly induced by the transfer to the fresh nutrient solution with or without salt stress. The pretreatment of AgNPs was shown to enhance H_2O_2 production in both conditions in sizes and dose dependent manner, which was the larger and higher concentration led to the higher level of H_2O_2 production.

3. Effects of AgNPs on anti-oxidative systems under normal and salt stress condition

The enzymatic antioxidant activity was changed when 14 day-old rice seedlings were transferred to fresh WP No.2 solution and exposed to salt stress. The enzymatic activity significantly increased with same pattern; the pretreatment with the higher concentration (100 mg/L) of the large size (70-120 nm and 150 nm) of AgNPs was found to induce the higher activity of the anti-oxidant enzymes than pretreatment with the smaller size AgNPs or the pretreatment at lower concentrations. These suggested that antioxidant enzymes played an important role in protecting the plant from stress condition.

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APPENDIX A

REAGENT RECIPES

1. Modified WP nutrient solution (WP No.2) (Vajrabhaya and Vajrabhaya, 1991)

Table A.1 Modified WP nutrient solution

Chemicals	Content (mg/L)
Macroelements:	
Potassium nitrate (KNO_3)	580
Calcium sulfate($CaSO_4$)	500
Magnesium sulfate ($MgSO_4.7H_2O$)	450
Triple superphosphate	250
Ammonium sulfate ($(NH_4)_2SO_4$)	100
Microelements:	
Di-sodium ethylene diamine tetraacetate (Na_2EDTA) [a]	160
Ferrous sulfate ($FeSO_4.7H_2O$)[a]	120
Manganese sulfate ($MnSO_4.H_2O$)	15
Boric acid (H_3BO_3)	5
Zinc sulfate ($ZnSO_4.7H_2O$)	1.5
Potassium iodide (KI)	1
Sodium molybdate ($Na_2MoO_4.\ 2H_2O$)	0.1
Copper sulfate($CuSO_4.\ 5H_2O$)	0.05
Cobalt chloride ($CoCl_2.\ 6H_2O$)	0.05

a Preparation of FeSO4 stock concentration 30 g/l

1. Weight Na_2EDTA 40 g and $FeSO_4.7H_2O$ 30 g.
2. Dissolve Na_2EDTA 40 g in distilled water 500 ml and $FeSO_4.7H_2O$ 30 g in distilled water 500 ml at 70-90 °C, separately.
3. Add the Na_2EDTA solution into $FeSO_4.7H_2O$ solution, mix and then prepared this solution under the O_2 at the room temperature for 3-4 h until getting the clear solution.

2. Reagent Recipes

2.1 Lowry protein assay

2.1.1 Reagent A

(0.2% $CuSO_4$ + 0.4% Tartaric acid)	5 ml
20% Na2CO3	5 ml
0.8 N NaOH	10 ml
5% SDS	20 ml

2.1.2 Reagent B

Folin-Ciocalteu phenol	10 ml
Distilled water	50 ml

3. Potassium phosphate buffer, 0.1 M

Solution A: 27.2 g KH2PO4 per liter (0.2 M final) in water.

Solution B: 34.8 g K2HPO4 per liter (0.2 M final) in water.

Referring to Table A.1 for desired pH, mix the indicated volumes of solutions A and B, then dilute with water to 200 ml.

Table A.2 Preparation of 0.1 M potassium phosphate buffer

Desired pH	Solution A (ml)	Solution B (ml)	Desired pH	Solution A (ml)	Solution B (ml)
5.7	93.5	6.5	6.9	45.0	55.0
5.8	92.0	8.0	7.0	39.0	61.0
5.9	90.0	10.0	7.1	33.0	67.0
6.0	87.7	12.3	7.2	28.0	72.0
6.1	85.0	15.0	7.3	23.0	77.0
6.2	81.5	18.5	7.4	19.0	81.0
6.3	77.5	22.5	7.5	16.0	84.0
6.4	73.5	26.5	7.6	13.0	87.0
6.5	68.5	31.5	7.7	10.5	90.5
6.6	62.5	37.5	7.8	8.5	91.5
6.7	56.5	43.5	7.9	7.0	93.0
6.8	51.0	49.0	8.0	5.3	94.7

APPENDIX B

PROTOCOLS

1. Synthesis of Silver Nanoparticles

Ten milliliter of 0.1 M AgNO3 in nitric acid, derived as outlined in Section 2.1, was mixed with 5 mL of 50 mM sucrose in a 100-mL beaker and left for 45 min at room temperature for partial hydrolysis of the sucrose to glucose and fructose for the subsequent aldehyde-mediated reduction of Ag^{+} to Ag^{0}. Then, 5 mL of NaOH at a concentration of 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.1, 2.4, 2.7 or 3.0 M was added and mixed. The obtained silver powder is silver nanoparticles of various sizes. To ensure the complete conversion of silver ion into silver nanoparticle was performed according to Wongravee et al. (2013) by addition of reducing agent, $NaBH_4$ into Ag colloids. If Ag^{+} ion remains in the colloids, the intensity in extinction spectra (at 400 nm) will increase. The spectra obtained suggested no Ag^{+} remained in the system after the synthesis of AgNPs. With the complete conversion of Ag^{+} to Ag nanoparticle, the concentration of Ag nanoparticle stock solution was 5350 mg/L (approximately 5000 mg/L), and this stock solution was used to prepare the AgNP at the indicated concentration.

2.Characterization of the AgNPs

The size of the hydrated AgNPs obtained from each NaOH concentration (Section 2.2) was characterized by dynamic light scattering (DLS) using a particle size analyzer (Microtrac Zetztrac model NPA 152-31a-0000-000-20M). Each AgNP powder was washed with pure water five times by repeated centrifugation/ resuspension and then dispersed into pure water at 5 mg/500 mL by sonication for 1 min prior to

immediately being analyzed by DLS using DLS;ELS 8000 Otsuka Electronics Osaka Japan. In addition the anhydrous morphology and particle size of the AgNPs was evaluated with JEOL JSM-6510 a scanning electron microscope and Hitachi H-7650, transmission electron microscope (Figure 1A). The suspension of AgNP powder was washed and re-dispersed into pure water by sonication as outlined above, and then the suspension was dropped on the cleaned brass stub and air dried prior to examination.

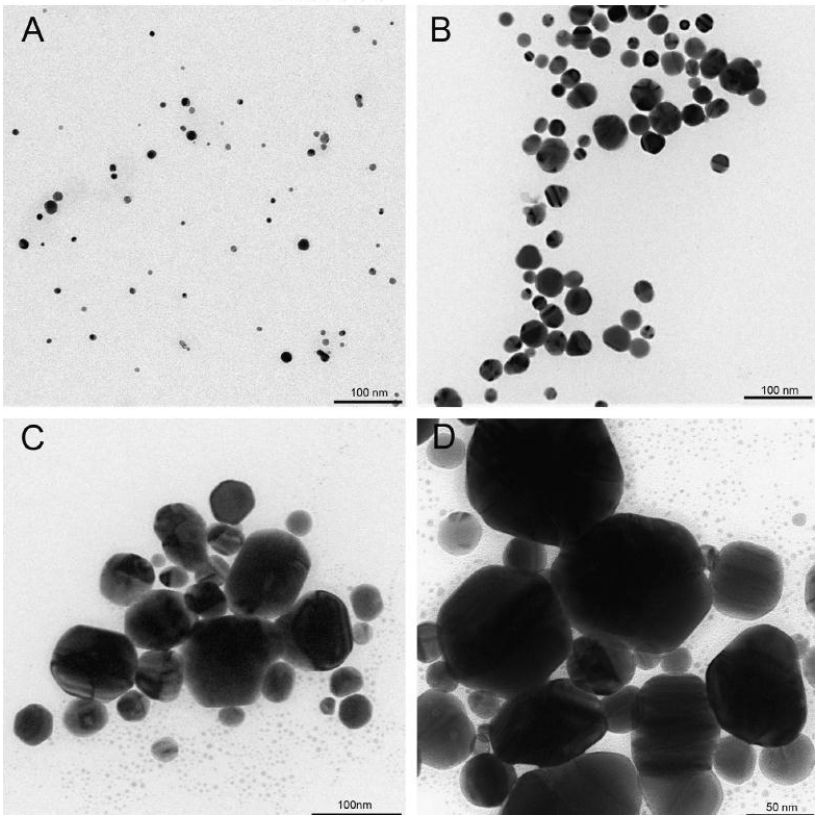


Figure B. 1 Transmission Electron Microscope (TEM) image of AgNPs (A) 20 nm, (B) 30-70 nm (C) 70-120 nm and (D) 150 nm

APPENDIX C

Standard Curve

Figure C. 1 H_2O_2 standard

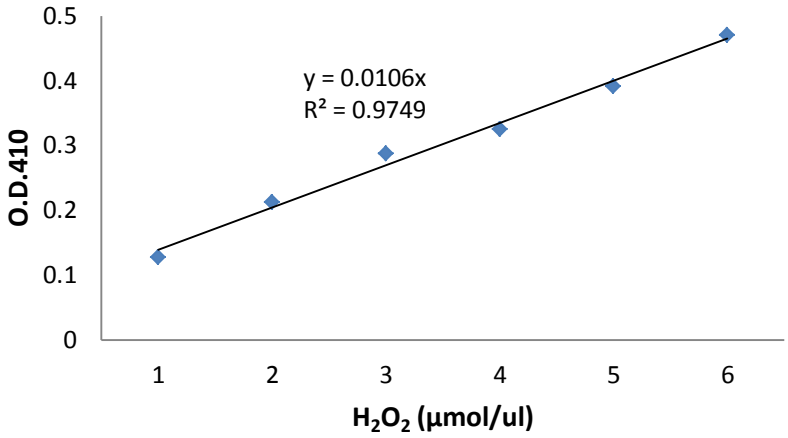
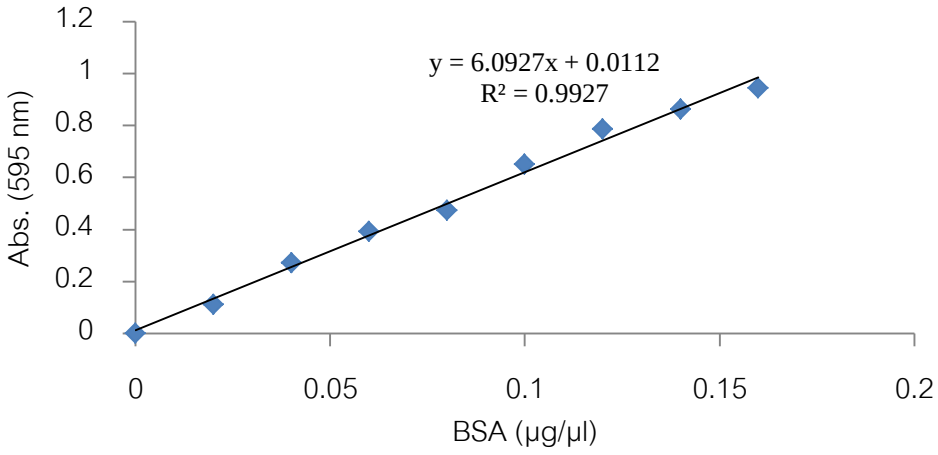


Figure C. 2 Standard protein



APPENDIX D

ICP-OES

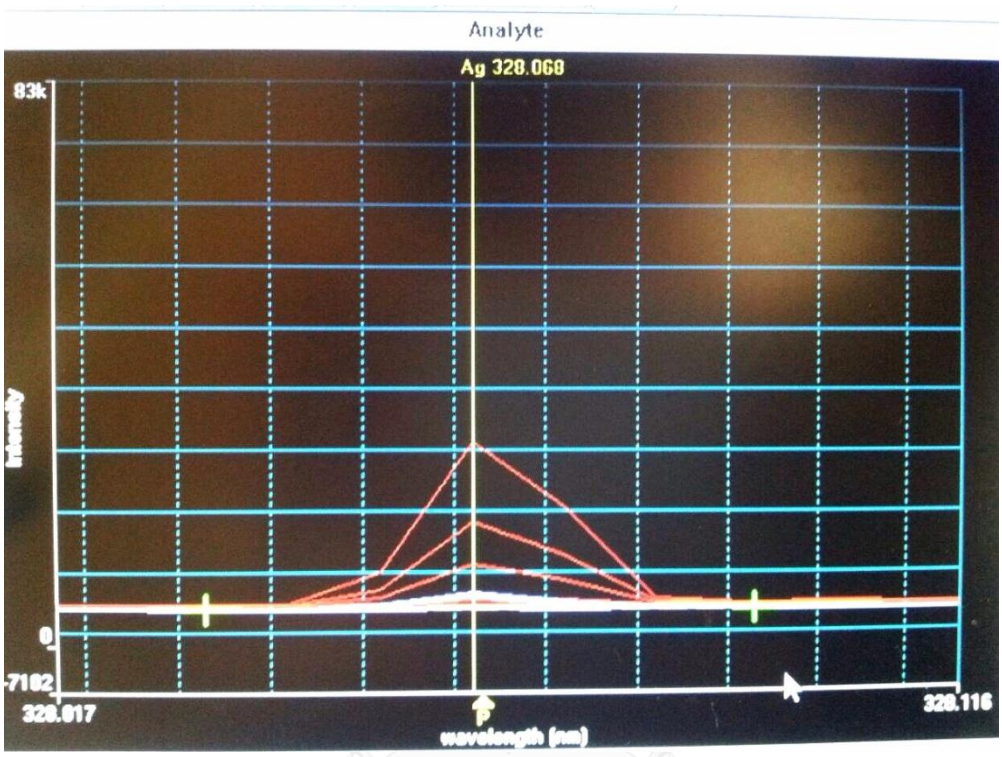


Figure D.1 Analytical of silver by ICP-OES ; Ag standard (red line), Sample (white line).

REFERENCES

VITA

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