ผลของอนุภาคขนาดนาโนของไททาเนียมไดออกไซด์และซิงค์ออกไซด์ต่อรากข้าว *Oryza sativa* L.

นางสาวประภัสสร บุญญานิติพงษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาพฤกษศาสตร์ ภาควิชาพฤกษศาสตร์
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ปีการศึกษา 2554

EFFECTS OF TITANIUM DIOXIDE AND ZINC OXIDE NANOPARTICLES ON ROOTS OF RICE *Oryza sativa* L.

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Botany

Department of Botany Faculty of Science

Chulalongkorn University

Academic Year 2011

Thesis Title	EFFECTS OF TITANIUM DIOXIDE AND ZINC OXIDE NANOPARTICLES ON ROOTS OF RICE *Oryza sativa* L.
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ประภัสสร บุญญานิติพงษ์ : ผลของอนุภาคขนาดนาโนของไททาเนียมไดออกไซด์และซิงค์ออกไซด์ต่อรากข้าว *Oryza sativa* L. (EFFECTS OF TITANIUM DIOXIDE AND ZINC OXIDE NANOPARTICLES ON ROOTS OF RICE *Oryza sativa* L.) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร.บุญธิดา โฆษิตทรัพย์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร.ประบัติ คุมา, 70 หน้า

งานวิจัยนี้มีจุดประสงค์ในการศึกษาผลของอนุภาคขนาดนาโนของไททาเนียมไดออกไซด์และซิงค์ออกไซด์ที่มีต่อการงอกของเมล็ดและการเจริญของรากข้าว (*Oryza sativa* L.) การศึกษาในครั้งนี้ทดลองใช้ข้าวพันธ์ปทุมธานี 1 (Pathum Thani 1) และวัดค่าพารามิเตอร์สี่ค่าคือ ก) การงอกของเมล็ด ข) ความยาวราก ค) จำนวนราก และ ง) ความยาวรากสัมพัทธ์ หลังให้เมล็ดและต้นกล้าได้รับอนุภาคขนาดนาโนที่มีความเข้มข้นต่างๆ จากการศึกษาพบว่า อนุภาคขนาดนาโนทั้งสองชนิดไม่มีผลยับยั้งการงอกของเมล็ดข้าว นอกจากนี้ ในขณะที่ อนุภาคขนาดนาโนของไททาเนียมไดออกไซด์ไม่มีผลต่อความยาวราก จำนวนราก และความยาวรากสัมพัทธ์ อนุภาคขนาดนาโนของ ซิงค์ออกไซด์มีผลให้ความยาวรากและจำนวนรากลดลงอย่างมีนัยสำคัญ สำหรับการวัด ความยาวรากสัมพัทธ์ พบว่าอนุภาคขนาดนาโนของซิงค์ออกไซด์ทำให้เกิดการลดลงของความยาวรากได้ตั้งแต่ความเข้มข้นที่ระดับ 10 mg/L เมื่อทำการศึกษาต่อไปโดยศึกษาผลของอนุภาคขนาดนาโนของซิงค์ออกไซด์ต่อกายวิภาคศาสตร์ของรากข้าว พบว่ามีออแกเนลล์ที่มีลักษณะเป็นถุงกลมขนาดเล็ก (globules) ภายในเซลล์บริเวณชั้นคอร์เทกซ์ของรากข้าวที่ได้รับอนุภาคขนาดนาโนของซิงค์ออกไซด์ ยังไม่มีการตรวจสอบของเหลวที่อยู่ใน globules แต่คาดว่าเป็นอนุภาคขนาดนาโนของซิงค์ออกไซด์ ซิงค์ไอออน หรือสารอื่น ๆ ที่พืชผลิตขึ้น ซึ่งในอนาคตควรมีการศึกษาถึงการเจริญของ globules รวมถึงองค์ประกอบที่อยู่ข้างในด้วย ในภาพรวมการศึกษาในครั้งนี้ทำให้ทราบว่าอนุภาคขนาดนาโนของสารประกอบบางชนิด เช่น อนุภาคนาโนของซิงค์ออกไซด์ สามารถทำให้เกิดความเป็นพิษได้อย่างชัดเจนต่อการเจริญของรากข้าวซึ่งเป็นพืชเศรษฐกิจที่สำคัญ ผลเสียที่เกิดจากอนุภาคขนาดนาโนต่อการผลิตพืชและสิ่งแวดล้อมควรได้รับการตระหนัก และควรเน้นถึงวิธีการจัดการรวมทั้งการกำจัดของเสียที่มีอนุภาคขนาดนาโนปนเปื้อนอยู่ เพื่อป้องกันการรั่วไหลออกสู่สิ่งแวดล้อม

สาขาวิชา.....พฤกษศาสตร์……...ลายมือชื่อนิสิต.....

ปีการศึกษา...2554.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5272404323 : MAJOR BOTANY

KEYWORDS: zinc oxide / titanium dioxide / nanoparticles / rice root / toxicity

NAME: MISS PRAPATSORN BOONYANITIPONG

EFFECTS OF TITANIUM DIOXIDE AND ZINC OXIDE NANOPARTICLES ON ROOTS OF RICE *Oryza sativa* L.

ADVISOR: ASSIST. PROF. BOONTHIDA KOSITSUP, Ph.D., CO-ADVISOR: PRABHAT KUMAR, Ph.D., 70 pp.

The present study investigated the effects of nanoparticles of titanium dioxide (nano-TiO_2) and zinc oxide (nano-ZnO) on rice (*Oryza sativa* L.) seed germination and root development. In this work, seeds of the rice cultivar Pathum Thani 1 were used and four different parameters: a) seed germination percentage, b) root length, c) root number and d) relative root growth were analyzed after treating rice seeds and seedling with different concentrations of nanoparticles. The results show that there was no reduction in the percentage of seed germination for both ZnO and TiO_2 nanoparticles. Also, whilst nano-TiO_2 had no effects on root length, root number and relative root growth of rice seedlings, nano-ZnO was observed to cause significant reduction of both root length and number. For the relative root growth measurements, the nano-ZnO exposure also caused decrease in root elongation even at low concentration as 10 mg/L nano-ZnO. Further study on the effects of the nanoparticles on anatomical structure of root treated with nano-ZnO was performed. Globules were observed in the cortical cell treated with nano-ZnO, leading to the conclusion that the globules were formed specifically to nano-ZnO. The fluids within the globules were not analyzed in this study, but it might either contain nano-ZnO, zinc ion, or possibly other substances produced by plant cells. Further studies on the development of the globules and their fluid compositions are recommended. Overall, this study shows that direct exposure to some types of nanoparticles i.e. nano-ZnO can cause significant toxicity on root development of economically important rice plants. This detrimental effect of nano-ZnO should be of great concern for both plant production and the ecosystems, and emphasize the need for responsible treatment and disposal of wastes containing nanoparticles so that they are not released into the unprotected environment.

Field of Study…Botany……. Student's Signature……..

Academic Year….2011…… ……. Advisor's Signature……..

Co-advisor's Signature……..…

ACKNOWLEDGEMENTS

Completion of this thesis is made possible by help and support from many people and institutions. I acknowledge the partial financial support and encouragement from Professor Joydeep Dutta, Vice President for Academic Affairs and the Director of the Center of Excellence in Nanotechnology (CoEN), who mentored me like one of CoEN's students with his valuable time, creativity, and constructive suggestions. It is also a pleasure to acknowledge Assistant Professor Dr. Boonthida Kositsup and Dr. Prabhat Kumar, who were my advisor and co-advisor and, by being very supportive and helpful, guided me to a better understanding and knowledge of my topic. I also thank the entire CoEN team and all of my friends in Nanocenter for discussions and general encouragements. Dr. Abha Mishra, Dr. Sunandan Baruah, Professor Gabor Hornyak, Assistant Professor Dr. Manit Kidyoo, Dr. Anchalee Chaidee and Assistant Professor Dr. Chumpol Khunwasi are also acknowledged for their suggestions and help.

Sincere thanks are also extended to all of my friends in the Department of Botany, Chulalongkorn University, Mr. Edward Woods and my family for their encouragement.

This research was supported by the Department of Botany, Faculty of Science, Chulalongkorn University and the Development and Promotion of Science and Technology talents project, the National Nanotechnology Center, belonging to the National Science & Technology Development Agency (NSTDA), Ministry of Science and Technology (MOST), Thailand, and the Centre of Excellence in Nanotechnology (CoEN), Asian Institute of Technology.

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LIST OF ABBREVIATIONS

CNTs	carbon nanotubes
DNA	deoxyribonucleic acid
ENPs	engineered nanoparticles
fCNTs	functionalize carbon nanotubes
mg/L	milligram per liter
nano-Al_2O_3	aluminium oxide nanoparticles
nano-CeO_2	cerium nanoparticles
nano-Fe_3O_4	magnetite nanoparticles
nano-SiO_2	silicon dioxide nanoparticles
nano-TiO_2	titanium dioxide nanoparticles
nano-ZnO	zinc oxide nanoparticles
nm	nanometer
ROS	reactive oxygen species
UV	ultraviolet
Zn^{2+}	zinc ion

CHAPTER I

INTRODUCTION

Nanotechnology comprises a range of technologies involving materials with particle sizes less than 100 nm (Guzman et al., 2006). The production and use of nanoparticles has increased rapidly over the past two decades with increasing applications in many industrial sectors, including paints, textiles, pharmaceutics, electronics, cosmetics and environmental remediation (Allianz Group and the OECD, 2005; Guzman et al., 2006). Investments in nanotechnology research, production and applications are growing internationally, with the annual value for nanotechnology-related-products estimated at US$1 trillion by 2011-2015 (Roco, 2005). It was previously estimated that after 2010, that the annual production of engineered nanoparticles (ENPs) is expected to be $10^4 – 10^5$ tons per year (The Royal Society, 2004).

Nanoparticles refer to particles, which have at least one dimension with a size less than 100 nm including ambient spherical and engineered particles (Oberdorster et al., 2005). A classification system for engineered nanoparticles, defines groups such as metal based materials, carbon based materials, dendrimers and nanoparticles-combine-composites (Lin and Xing, 2007). The nanoparticles have unique properties due to their small size, high specific surface area, catalytic efficiency, surface energy, abundant reactive sites and strong adsorption, and these properties have been utilized for many different industrial applications. However, the fact that the development of nanotechnology seems to be escalating with no regulation (Colvin, 2003) is a cause for concern. The unique properties of the artificial nanoparticles may result in them having significant effects on organisms if released into the environment (Maynard et al., 2006; Wiesner

et al., 2006; Kumar and Chen, 2008). For example, there are possible detrimental effects of ZnO and TiO_2 on human skin cells (skin fibroblasts) in a study by Dechsakulthorn et al. (2007). They reported that 50% inhibition concentration (IC_{50}) of nano-ZnO was about 49.56 mg/L, which is less than IC_{50} of nano-TiO_2 (about 2696 mg/L) indicating that nano-ZnO was more toxic than nano-TiO_2 on this category of skin cells. Nano-ZnO had been generally considered to be non-toxic (Kumar and Chen, 2008), but there were published findings that demonstrated significant toxicity under laboratory studies on rodent cells (Brunner et al., 2006).

In this regard, it is likely that with their increasing use in consumer products, ENPs are likely to find their way into the environment in the aquatic, terrestrial and atmospheric phases of the biosphere, both by application-related means and also by waste disposal (Colvin, 2003; Nowack and Bucheli, 2007; Service, 2008). This discharge into the environment has resulted in considerable concern about the potentially harmful effects of those ENPs due to the current lack of knowledge of how organisms react to their contamination.

As the metal oxide nanoparticles, titanium dioxide (nano-TiO_2) and zinc oxide (nano-ZnO) are commonly used in industrial applications (Kumar and Chen, 2008; Dietz and Herth, 2011), these were chosen for the present study. Zinc oxide is used in a range of applications such as sunscreens and other personal care products, electrodes and biosensors, photocatalysis and solar cells (Kumar and Chen, 2008). Uses for titanium dioxide nanoparticles also include personal care products, pigments, photocatalysis, sensors, solar cell and memory devices (Remillard et al., 2000; Li et al., 2002; Baruah and Dutta, 2009). Titanium dioxide (TiO_2) and zinc oxide (ZnO), are part of the metal oxide nanoparticle category and they are important in the field of heterogeneous

catalysis for catalytic support of a wide variety of metals (Biener et al., 2005). Due to their visible transparency and ultraviolet (UV) blocking ability in nanoparticulate form, metal oxide nanoparticles have found numerous applications relating to sunscreen products (Klaine et al., 2008). With their common industrial utilisation, they have been tested for toxicity with many organisms such as human cells, fish, freshwater microalgae and plants (Dechsakulthorn et al., 2007; Franklin et al., 2007; Lin and Xing, 2007; Reeves et al., 2008; Lee et al., 2010).

Currently, many researchers are continuing to examine the effects of nanoparticles on human and animal cells (Dechsakulthorn et al., 2007; Franklin et al., 2007). Relatively fewer studies have focused on the interaction of nanoparticles with plants. However, in conjunction with the evolving science of nanotechnology, there is a need for industrial standards to be defined for their safe use, particularly on their potential adverse effects on various plant species, including commercial food crops (USEPA, 2007).

Considering plants as an essential component of all ecosystems, it is of utmost importance to undertake systematic studies to understand the effects, both beneficial and also harmful, of the various nanoparticles. Their interaction with plants could cause effects on plant growth. Also, the uptake and accumulation of ENPs in plants may affect cell functions, whilst their absorption by plant roots could result in toxicity from either or both physical and chemical reactions (USEPA, 2007; Monica and Cremonini, 2009; Ma et al., 2010). Increasing numbers of publications have emerged recently concerning the interactions of ENPs with plants (Battke et al., 2009; Lin and Xing, 2007; Lin et al., 2009). Most of these studies are focused on the potential

toxicity of ENPs to plants and a range of beneficial, detrimental and inconsequential effects have been reported (Menard et al., 2011).

Among the positive effects observed on plants, nano-TiO_2 was reported to promote the growth of spinach through an increase in photosynthetic rate and nitrogen metabolism (Hong et al., 2005; Yang et al., 2006). It was also demonstrated that Carbon nanotubes (CNTs) could improve root growths of onion and cucumber plants, but conversely would decrease the root lengths of tomato plants. In that same study, nanotube sheets were reported to have formed on the root surfaces, but none of them were observed to have entered the root cells (Canas et al. 2008). A more recent study showed that CNTs could penetrate the seed coat of tomato plants and increase seed germination rate and seedling growth in that species (Khodakovskaya et al., 2009).

Overall, most of the reports in the literature are demonstrating phytotoxicity of ENPs. For instance, nano-aluminum oxide (Al_2O_3) was found in one study to have a negative effect on root elongation of corn, cucumber, soybean, cabbage and carrot (Yang and Watts, 2005) whilst nano-ZnO was shown in another study to be a highly toxic substance that could terminate root growth of tested plants (radish, rape, ryegrass, lettuce, corn and cucumber) (Lin and Xing, 2007). Similar research was studied on the toxicology of nano-Al_2O_3, nano-silica (SiO_2), nano-magnetite (Fe_3O_4) and nano-ZnO on *Arabidopsis thaliana*, the results showing that nano-ZnO could inhibit germination at a concentration of 400 mg/l (Lee et al., 2010). Recent studies on rare earth oxide nanoparticles reported inhibition of root growth of some plant species (radish, rape, tomato, lettuce, wheat, cabbage and cucumber) (Ma et al., 2010).

The penetration of nanoparticles into plant cells was also reported in many cases, but variably with or without showing adverse effects (Khodakovskaya et al., 2009; Lin et al., 2009; Birbaum et al., 2010; Cifuentes et al., 2010). Nano-cerium oxide (CeO_2) had no ability to translocate in maize (Birbaum et al., 2010), while fullerene (C_{70}) could be absorbed and passed through to the next generation of rice plant. However, no acute toxicity was exhibited in the latter case (Lin et al., 2009). Another study reported that magnetic carbon-coated nanoparticles could penetrate cell walls of roots (Cifuentes et al., 2010). Overall, the current phytotoxicity profile of ENPs is still being established, but due to their speculative and hypothetical status, the effects of their unique characteristics are poorly understood and more studies on toxicity are required, especially on commercially important food crops.

This study consists of two stages. The first stage was to examine the effects of nano-TiO_2 and nano-ZnO on rice seed germination, then root elongation (in first 7 days) and relative root growth in the developing rice seedlings. The second stage comprised observations of the root structure in the same seedlings that were treated with nano-ZnO. The observation of the effects from these two types of nanoparticles on rice seedling development is important for understanding their toxicity, especially on such a commercially important food crop. It is important to determine the specific types of nanoparticles that could cause significant toxicity in the subject species and emphasizes the need in the future for ecologically responsible treatment and disposal of wastes containing nanoparticles. The objectives of this study are:

1. To examine the effects of nano-TiO_2 and nano-ZnO on rice seed germination, and the root elongation and relative root growth of rice seedlings.
2. To study the root structure of the rice seedlings treated with nano-ZnO and comparing with the control group.

CHAPTER II

LITERATURE REVIEW

1. Present uses of nanoparticles

Nanotechnology is a range of technologies involving particles manufactured at nanometer scale, with widespread applications involving many industries (Allianz Group and the OECD, 2005; Guzman et al., 2006). The implementation of nanotechnology has been rapidly increasing since the 1990s (Nanonet website). Products based on nanotechnologies were estimated to number more than 800 and there is the expectation of many more nanotechnology-derived products in the market within the next few years (Maynard et al., 2006). It was estimated that by 2014 more than 15% of all products on the global market will have some kind of nanotechnology incorporated into their manufacturing process (Dawson, 2008). The growth in nanotechnology can be illustrated by the number of patents relating to nanomaterials and illustrated in Figure 2.1 below. The acceleration in patenting is remarkable with a doubling of patents every 2 years (RCEP, 2008).

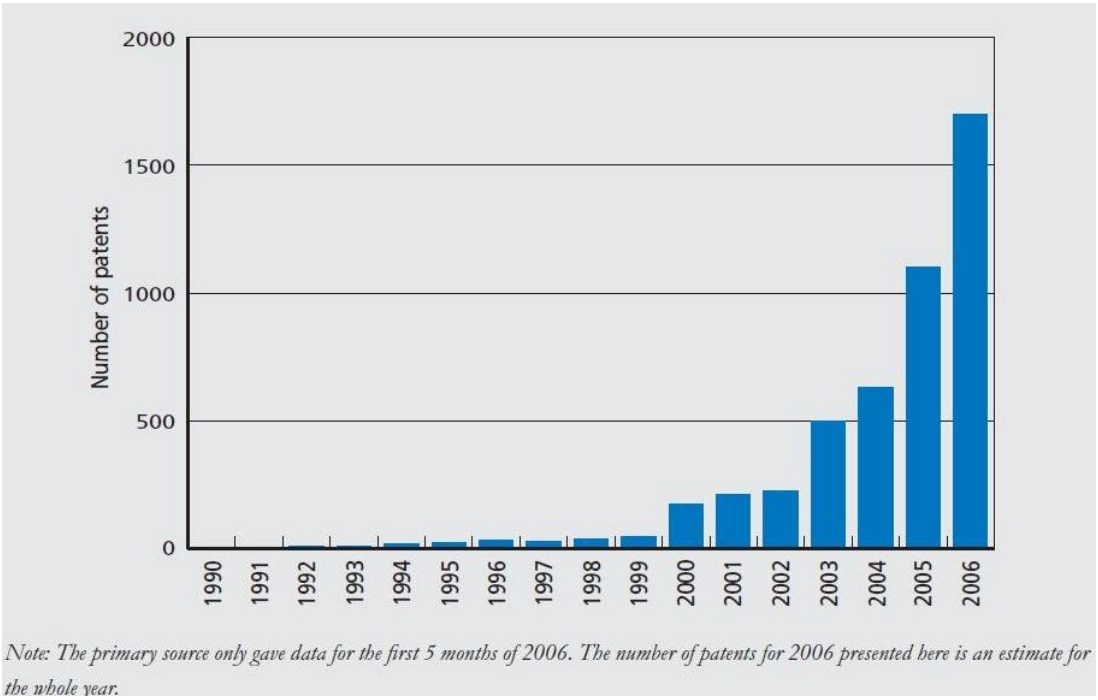

Figure 2.1 The number of patents registered globally from 1990-2006 (RCEP, 2008).

Current annual global production of ENPs is raising each year proportional to their rapidly increasing applications in various industries and products (The Royal Society, 2004). However, most aspects of nanotechnology are developing with no regulation (Colvin, 2003). Presently, nanotechnology-related products can be classified into two main classes, nanomaterials fixed on a substrate and free nanoparticles, which more easy to release into the environment (RCEP, 2008). Nanoparticles can also be released into atmosphere in the form of aerosols, and also may contaminate soil and surface water (Piotrowska et al., 2009). They can be released into the environment as bare nanoparticles, functionalized nanoparticles, aggregates or embedded in a matrix (Nowack and Bucheli, 2007). Nano-silver (Ag), various forms of carbon, nano-ZnO, nano-TiO_2 and nano-iron oxide constitute the majority of nanomaterials common usage.

In the case of metal oxides such as TiO_2 and ZnO, they already have applications in cosmetics, paints, textiles and in the longer term, could also be used for targeted drug treatments (Kumar and Chen, 2008; RCEP, 2008). Moreover the nano-metal oxides, nano-TiO_2 and nano-ZnO, are important substances in the region of heterogeneous catalysis (Biener et al., 2005) and with their ultraviolet (UV) blocking ability, they also have broad applications in the manufacture of sunscreens (Klaine et al., 2008). These kind of nano-products (free nanoparticles) have unconstrained physical limits and can easily be released into the environment, and becoming present in water, soil and also the air (Buzea et al., 2007). Once in the environment, they can persist for a long time and may contaminate biological ecosystems. They can also cause toxic effects through biodegradation or accumulation in the food chain (SCENIHR, 2006).

2. Toxicity from nanoparticles

Not all nanoparticles are toxic; and toxicity can depend on the unique properties of their small size such as chemical composition, surface area and surface energy (Buzea et al., 2007). Many types of nanoparticles were report to be non-toxic (Goodman et al., 2004); and others can be rendered non-toxic (Derfus et al., 2003), while others have beneficial effects (Schubert et al., 2006). Therefore, the different types and compositions of nanoparticles can have a wide range of toxicological properties. Therefore, their effects and risks should be evaluated on a case-by-case basis (SCENIHR, 2006). Nanoparticles have a range of sizes up to several dozens of nanometers: and their dimensions can be similar to complexes of protein molecules (Piotrowska et al., 2009). However, they differ from proteins in term of chemical composition, shape, size, density,

aggregation, type of surface and unique physiochemical properties; e.g. magnetic, optical and electrochemical properties (Aitken et al., 2004). In fact, the properties that can make nanomaterials useful could be the same properties that make them toxic to many organisms (USEPA, 2007).

The environmental side-effects of nanomaterials should also be evaluated from their life-cycle perspective (Figure 2.2) to properly ascertain their environmental impact not only in the present but also in the future (USEPA, 2007). The different stages of the production and consumer life-cycle have different associated environmental effects and risks.

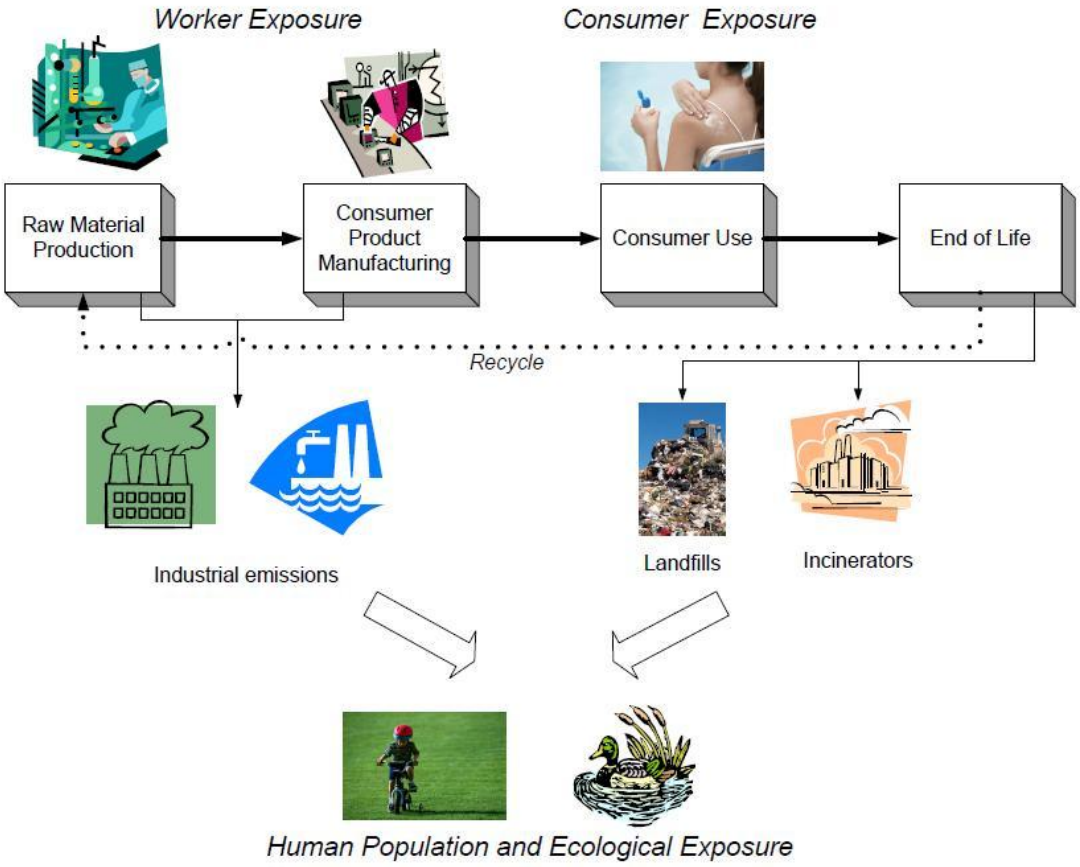

Figure 2.2 Life cycle perspective to risk assessment (USEPA, 2007).

The size of nanomaterials, lies in the range between molecules/atoms and bulk materials (Nel et al., 2006). The small size of the particles changes the physiochemical properties of the material and also can lead to their uptake and interaction with biological tissues (Oberdörster et, al., 2005), with their uptake being dependent on the actual dimensions of the nanoparticle types (Chithrani et al., 2006). Their small size of all the nanoparticles results in a larger relative surface area than other forms of the same substances, and this characteristic, as well as the ability of nanoparticles to generate reactive oxygen species, both play a major role in their toxicity (Nel et al., 2006).

3. Effects of nano-toxicity on plant life

Plants are crucial components of all ecosystems, and their interaction with ENPs could play a key role in the fate and transport of the nanoparticles when released into the environment. Their uptake and accumulation by plant life (Monica and Cremonini, 2009), and the adherence of the nanoparticles to plant roots could cause toxicity from both their physical and chemical properties and is a major concern as their ultimate effects could be profound (USEPA, 2007).

There is a relatively little research on the effects of nanoparticles on plants (Menard, 2011, and Lin and Xing, 2007) in relation to the numerous published nanotoxicology articles that have focused on animals and bacterial cells. In addition, modern agriculture has used nanotechnology in numerous applications ranging from reproductive technology to energy byproducts and also disease prevention (Nair et al., 2010).

In contrast with those of animals, the cells of most plants have cell walls that become a primary site for interaction and a cell barrier for the entrance of ENPs (Navarro et al., 2008). The

biological impacts of ENPs and their biokinetics are dependent on many factors as discussed (size, chemical composition, surface structure, solubility, shape and aggregation), (Nel et al., 2006) and vary on different plant species as well (Lee et al., 2010). Hence future studies should cover a broad range of permutations to adequately define nanotoxicity in plants (USEPA, 2007).

4. Titanium dioxide (TiO_2) nanoparticles

Nano-sized TiO_2 comprises the most frequently used nanoparticles and is therefore is expected to significantly contaminate the environment due to their production at an industrial level (Klancnik et al., 2011). In this regard, there has been a recent report of a specific occurrence of nano-TiO_2 in the environment (Kaegi et al., 2008) and calculations of its overall expected environmental concentrations have also been made (Mueller and Nowack, 2008). Commercial production of nano-TiO_2 has been estimated at more than ten thousand metric tons per year between 2011 and 2014 (UNEP, 2007). The key characteristics of nano-TiO2 are their photocatalytic activity and ultraviolet (UV) light absorbing properties, resulting in the use of nano-TiO_2 for a wide variety of applications (Mueller and Nowack, 2008). Nano-TiO_2 actually has the ability to generate reactive oxygen species (ROS) in the presence of UV light (Chan et al., 2011), as well as in its absence (Reeves et al., 2008). The toxicity of nano-TiO_2 in the absence of UV light was reported by Baun et al. (2008) and Lee et al. (2009). However, the precise mechanisms of toxicity of nano-TiO_2 and other metal nanoparticles are still largely unclear (Griffitt et al., 2008) as most studies remain focused on broadening the range of empirical observations. For instance, there has been research on the effects of nano-TiO_2 on a variety of animal cells, algae, freshwater invertebrates and fish (Oberdörster et al., 2005; Cattaneo et al.,

2009; Kahru and Dubourguier, 2010) in order to determine their toxic effects, but, as discussed before, there are relatively fewer studies of their effects on plants (Menard et al., 2011).

Results of some of that research studying the effects of nano-TiO_2 on plants, published by Seeger et al. (2008) indicated no significant toxic effects to willow trees. They experimented on willow trees with two types of nano-TiO_2 particles: a) 25 nm in diameter and b) < 10 nm, with short-term test endpoints. They concluded that woody species are not vulnerable to nano-TiO_2 in the condition used in the experiment (, 1 – 100 mg/L). On the other hand, the genotoxicity of nano-TiO_2 was evaluated for onion and tobacco plants, and with 100 nm nano-TiO_2 treatment, toxicity was observed (Ghosh et al., 2010). That toxicity was determined by observations of DNA damage, and inhibition of growth. In the same study, increased lipid peroxidation on onion roots was observed at concentrations of 319 mg/L, and there was induced DNA damage in leaf cells at a nano-TiO_2 concentration of 157 mg/L. In different experiments, it was observed that nano-TiO_2 (15 nm) had low toxic potential on growth but affected the mitotic index in root tips of onion plants (Klancnik et al., 2010).

In summary, more empirical studies on the toxicity of nanoparticles are needed as an ongoing requirement to provide sufficient data for a complete classification. Toxicity data must also be considered for their relevant characteristics, and then the critical mechanisms for their toxicity can be theorized (Menard et al., 2011).

5. Zinc oxide (ZnO) nanoparticles

Zinc oxide nanoparticles are commonly used in cosmetics products as well as in textiles (Nel et al., 2006). A key characteristic is their antibacterial property on a broad spectrum of

micro-organisms, with smaller particle sizes being more effective (OECD, 2009). Studies on the nano-ZnO have resulted in ecotoxicity data being available for the varied life forms of bacteria, algae, crustaceans and nematodes (Adams et al., 2006; Heinlaan et al., 2008; Aruoja et al., 2009; Wang et al., 2009).

In addition, the toxicity of nano-ZnO on some species of plant has been examined. In that study, nano-ZnO affected the seed germination rate of corn and actually terminated the elongation of roots of the radish, rape, ryegrass, lettuce and cucumber plants (Lin and Xing, 2007). Evidence of root uptake of nano-ZnO was also reported a year later by Lin and Xing (2008); and they found that Zn content in the roots of ryegrass in the presence of (1000 mg/L) nano-ZnO was higher than a similar treatment with a solution of (1000 mg/L) Zn^{2+}. It is likely that nano-ZnO treatment could result in the uptake of nano-ZnO by the roots, and few of them could transport to the shoot. Conversely, the roots from the Zn^{2+} treatment may have a lower Zn content because Zn^{2+} could be absorbed by the roots and then be transported to the shoots more easy. That hypothesis is supported by the observation that the Zn content of the shoots subjected to the Zn^{2+} treatment was higher than corresponding nano-ZnO treatment.

In other studies, seed germination observation and root elongation measurements were used to observe nano-ZnO toxicity compared to other type of nanoparticles (nano-Fe_3O_4, nano-SiO_2, and nano-Al_2O_3). Using that methodology, Lee et al. (2009) reported that concentrations of 400 mg/L nano-ZnO inhibited seed germination of *Arabidopsis thaliana*, which is the plant that has had its genome sequenced and therefore is more relevant for further study. The effect of Zinc ions from nano-ZnO solution was also investigated and tested to determine the

toxicity. No toxic effects were observed due to the solution of the zinc ions, as the concentrations in the nano-ZnO solution was lower than the threshold of zn^{2+} that have been shown to be toxic to plants.

6. Seed germination and root elongation test

The Seed germination rate, expressed as a percentage, is widely used to test the phytotoxicity of chemicals on plant species (USEPA, 1996). The seed germination rate and the root elongation measurements have been described as simple and sensitive parameters for detecting the toxicity from heavy metals and other pollutants (Wong and Bradshaw, 1982). In a different publication, many plant species have been recommended for ecotoxicity testing by using seed germination and root elongation methods (Wang, 1990). Lettuce, cabbage, oats, carrot, cucumber, tomato, wheat and rice have also been recommended plant species in several papers (OECD, 1984; FDA, 1987; USEPA, 1996). In another publication, a phytotoxicity test for effluent toxicity testing was reported and is a relatively new approach in comparison with the previous tests that used the fathead minnow fish. Instead, *Daphnia magna*, or green alga, can use for testing in higher plants (Peltier and Weber, 1985). Other studies (e.g. Wang, 1990) have also explored the potential of using higher plants for ecotoxicological studies. In that paper, a typical condition for this test was described, which should make the seed germination test become more precise. The seeds should have a seed germination rate exceeding 85% for the non treated control group under normal conditions which are defined in their paper.

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Plant materials

Rice seed (*Oryza sativa* L. "Pathum Thani 1")

1.2 Nanoparticles

Degussa P 25 nanoparticles (Degussa, Germany)

Zinc oxide nanopowder (Sigma-Aldrich, USA)

1.3 Instruments and chemical solution

1.3.1 Equipment for seed germination, root elongation and relative root growth test

Aluminum foil

Beaker 100 ml

Blades

Digital balance

Erlenmeyer flask 100 ml and 250 ml

Filter paper

Forceps

Graduated cylinder 10 ml and 50 ml

Graduated cylinder 100 ml

Incubator (GFL, England)

Micropipette

Plastic Petri dish 90 x 15 mm

Scissors

Sodium hypochlorite

Ultrasonic vibrator

Vernier caliper

1.3.2 Equipment for rice root cross section

Adhesive glue

Aluminum foil

Blades

Cover slip

Digital balance

Dissecting needles

Eyedropper

Eppendorf tubes 1.5 ml

Erlenmeyer flask 250 ml

Filter paper

Forceps

Glass bottles 5 ml

Glass slide

Graduated cylinder 50 ml

Light microscope (Olympus, Japan)

Micropipette

Microtome (NK system, Osaka, Japan)

Microwave

Pasteur pipette

Plastic Petri dish 90 x 15 mm

Polyethylene foam

Refrigerator

Ruler

Safranin O dye

Scissors

2. Methods

2.1 Nanoparticle solution and characterization

Dispersions of the two types of nanoparticles used in this study were prepared at the laboratory of the Center of Excellence in Nanotechnology, Asian Institute of Technology in Bangkok, Thailand.

2.1.1 Titanium dioxide nanoparticle

Titanium dioxide nanoparticles (nano-TiO_2) suspensions were prepared by dispersing Degussa P25 (Degussa, Germany) nanoparticles in Milli-Q water through ultrasonication (300 W, 40 kHz) for 30 minutes (Lin and Xing, 2007).

2.1.2 Zinc oxide nanoparticles

Zinc oxide nanoparticles (nano-ZnO) were prepared from commercial ZnO nanopowder (Sigma-Aldrich, USA) using the same method as in 2.1.1.

Particle size distribution of the nanoparticles was determined through measurements carried out on Transmission Electron Microscopy (TEM) (JEOL JEM 2010, Japan, operated at 120 kV) images using Scion Image processing software (SCION Corporation, USA).

2.2 Varying concentration using nano-TiO_2

Rice seeds were sterilized in a 2.5% sodium hypochlorite solution for 15 minutes (min) (Lin and Kao, 1996). After rinsing three times with Milli-Q water, they were soaked in nano-TiO_2 suspensions at various concentrations (10, 100, 500, 1000, 1500, 2000 and 5000 mg/L) for 24 hours (h) in an incubator at ambient laboratory conditions (30±1^{o}C, 63% RH) in the dark. Milli-Q water was used as a control. A piece of filter paper (Whatman No. 42, Maidstone, England) was placed into each Petri dish (90 mm × 15 mm), 4 ml of Milli-Q water was added and 20 seeds were then transferred into each dish. Three replications were used for each concentration. The Petri dishes were sealed with parafilm and placed in an incubator. After 7 days, the seed germination was recorded by counting the germinated seeds in each Petri dish that had coleoptiles (shoots) longer than 2 mm (Lin and Xing, 2007); and the remainder were considered as not germinated. Root lengths were measured using a ruler or a vernier caliper. The measurement starts from the point that the primary root (radical root) emerge from the seed to the root tip (Mishra and Salokhe, 2008). Three seedlings were randomly chosen from each Petri dish to measure root length.

2.3 Seed germination and root elongation test (nano-TiO_2 and nano-ZnO)

2.3.1 Seed germination and root elongation test using nano-TiO_2

A similar process of seed sterilization was followed as in 2.2. Then the seeds were soaked in nano-TiO_2 suspensions at various concentrations which were 100, 500 and 1000 mg/L (selected from experiment 2.2), and at various soaking times which were 24, 48 and 72 h, in an incubator at ambient laboratory conditions in the dark. Milli-Q water was used as a control. A piece of filter paper was put into each Petri dish, 4 ml of Milli-Q water was added, and 20 seeds were then transferred into each dish. Three replications were used for each concentration. The Petri dishes were sealed with parafilm and placed in the incubator. After 7 days, the seed germination was recorded by counting the germinated seeds that had coleoptiles longer than 2 mm (Lin and Xing, 2007); and the remainder were considered as not germinated. Root lengths were measured using a ruler or a vernier caliper. The measurement starts from the point that the primary root emerge from the seed to the root tip (Mishra and Salokhe, 2008). Three seedlings were randomly chosen from each Petri dish to measure root length. Additionally, the numbers of roots (primary root, seminal root and nodal root, which had a length longer than 5 mm) were counted.

2.3.2 Seed germination and root elongation test using nano-ZnO

A similar process was used as in experiment 2.3.1 except that the seeds were treated with nano-ZnO (10, 100, 500 and 1000 mg/L) at different soaking times (24, 48 and 72 h). Milli-Q water was used as a control.

2.4 Relative root growth test

2.4.1 Relative root growth test using nano-TiO_2

Rice seeds were sterilized using the same process as in experiment 2.2. Then seeds were soaked overnight in Milli-Q water in an incubator under similar condition as discussed in experiment 2.2. Following that, the seeds were transferred onto filter papers, placed in Petri dishes (90 mm × 15 mm) containing 4 ml of Milli-Q water with 4 seeds per dish, and were allowed to germinate in the dark for 2 days prior to nanoparticle exposure. After that, 4 ml of nano-TiO_2 suspension at different concentrations (10, 100, 500 and 1000 mg/L) was added into each Petri dish. Milli-Q water was used as a control. The bottoms of the dishes were marked underneath to ensure the exact site of each seedling. Then seedling were transferred onto filter paper and put on the marked sites at ambient laboratory conditions as described above. The length of the seminal root of each seedling was measured before (L_{before}) and after (L_{after}) exposure to nano-TiO_2. Root elongation (RE) during the exposure period was calculated using Eq. (1) below. Relative root growth (RRG) was calculated using Eq. (2) (Schildknecht, 2002, cited in Yang and Watts, 2005).

$$RE = L_{after} - L_{before} \quad (1)$$

$$RRG = \frac{RE_{sample}}{RE_{control}} \quad (2)$$

2.4.2 Relative root growth test using nano-ZnO

A similar process of exposure was followed as in experiment 2.4.1, except the seeds were exposed to nano-ZnO suspension (10, 100, 500 and 1000 mg/L).

2.5 Zinc ion test

To determine whether the role of the dissolved metal species was involved in nanoparticle toxicity or not, we measured concentrations of zinc ions in the supernatants of nanoparticle suspension at a concentration of 1000 mg/L, after centrifuging (Gemmy Industrial Corp., Taiwan) at 5000 rpm for 30 minutes, and determined by an inductively coupled plasma optical emission spectrometer (ICP-OES) (Perkin-Elmer, Optima 2100 DV, USA) at 206.2 nm wavelength. An analysis determined that the highest zinc ion concentration was 40 mg/L. The effects of zinc ions on root length were tested by using zinc ion solutions prepared by dissolving zinc acetate ($Zn(CH_3COO)_2 \cdot 2H_2O$) in Milli-Q water to prepare 4 concentrations of zinc ions (0, 10, 30 and 50 mg/L). The measurement of the primary root length was performed as in experiment 2.2.

2.6 Rice root structure (nano-ZnO)

In those cases that exhibited adverse effects on root growth from treatment with nano-ZnO in the preliminary work, the roots were further examined by cross section. Root samples from control and 1000 mg/L nano-ZnO were planned to be collected from day 1 - 7 after being transferred to Petri dishes, and then fixed in a fixative solution. Following that, each root was embedded in 6% agarose in eppendorf then cut by using a Plant Microtome MT-3 (NK system, Osaka, Japan) at 3 different parts which were basal part, middle part and near the root tip separated by using root ratio (Fig. 1). The root samples were stained using safranin O before being photographed by an Olympus BX51 microscope and a DP70 digital camera system (Olympus, Japan). Three roots from each treatment were used. Some samples were taken by a

scanning electron microscope (SEM) (JEOL JSM-6301F with Oxford ISIS 300 energy-dispersive spectroscopy, EDS).

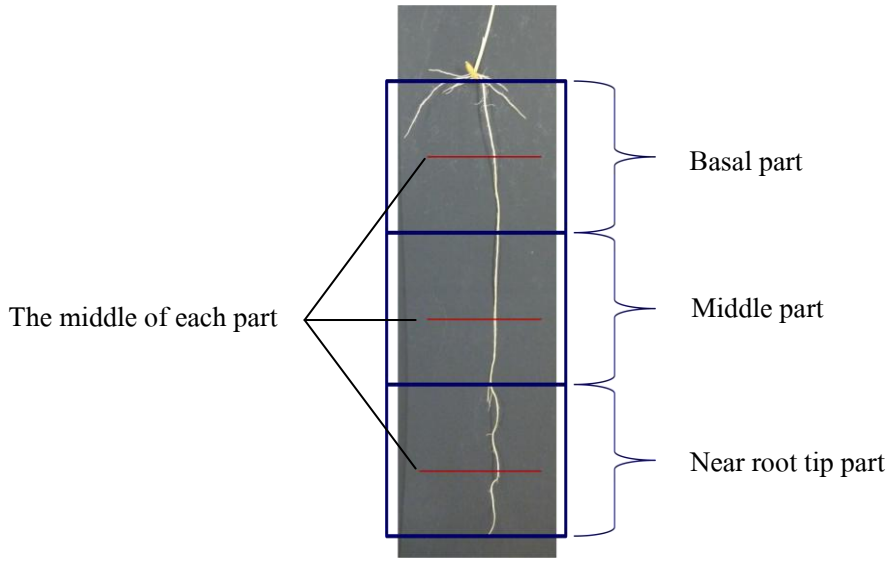

Figure 3.1 A diagram of a primary root of rice with a description of each part using root ratio. The middle of each part will be cut to compare root structure between the roots from control and nano-ZnO treatment.

2.7 Statistical analysis

Each treatment was conducted with three replicates, and the results are presented as mean±SE (standard error of the mean). The data was analyzed using the SPSS GLM procedure in SPSS to determine single or the interaction effects of factors. Whenever a significant interaction was detected, the level of one factor was compared to each level of the other factors by all pair-wise multiple comparison procedures (Fisher's LSD), unless mentioned otherwise. A significance level ($\alpha = 0.01$) was used in all analyses.

CHAPTER IV

RESULTS AND DISCUSSION

1. Nanoparticle solution and characterization

1.1 Titanium dioxide nanoparticles

Through the TEM micrograph, the particle size of nano-TiO_2 ranged between 10 – 50 nm. The size ranging between 21 – 30 nm was found in the majority, which is in accordance with the size reported by the company (average size 21 nm).

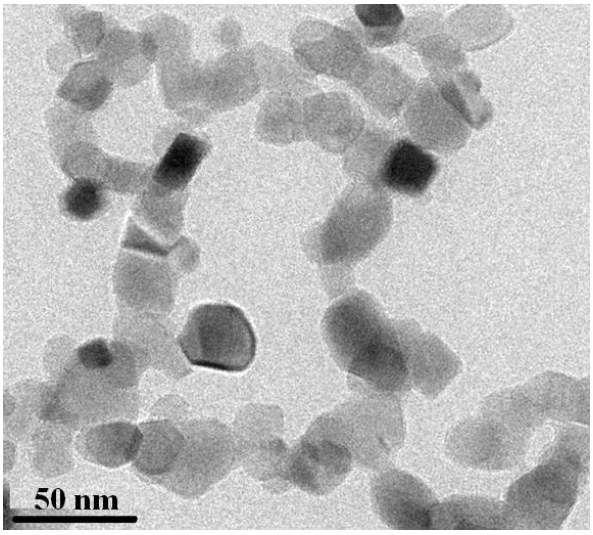

Figure 4.1 TEM micrograph of nano-TiO_2 particles after being dispersed in Milli-Q water.

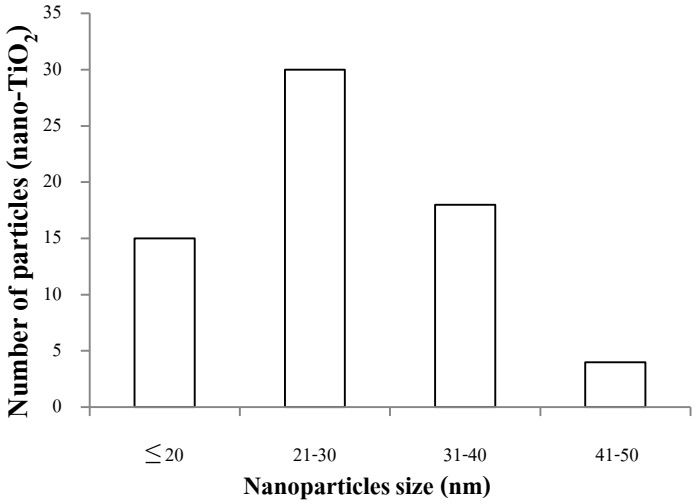

Figure 4.2 Particle size distribution of nano-TiO_2 (a total of 72 particles).

1.2 Zinc oxide nanoparticles

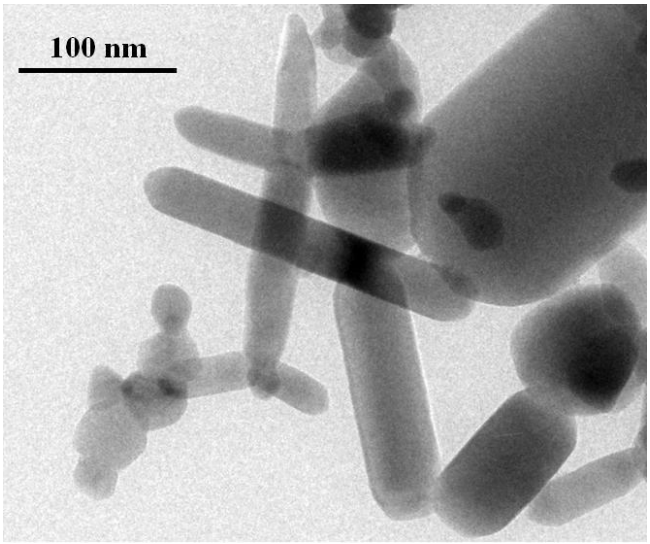

Figure 4.3 TEM micrograph of nano-ZnO particles after being dispersed in Milli-Q water.

Nano-ZnO had particle sizes between 30 – 200 nm. The majority ranged between 30 – 100 nm, which is in accordance with the range advertizing by the company (< 100 nm).

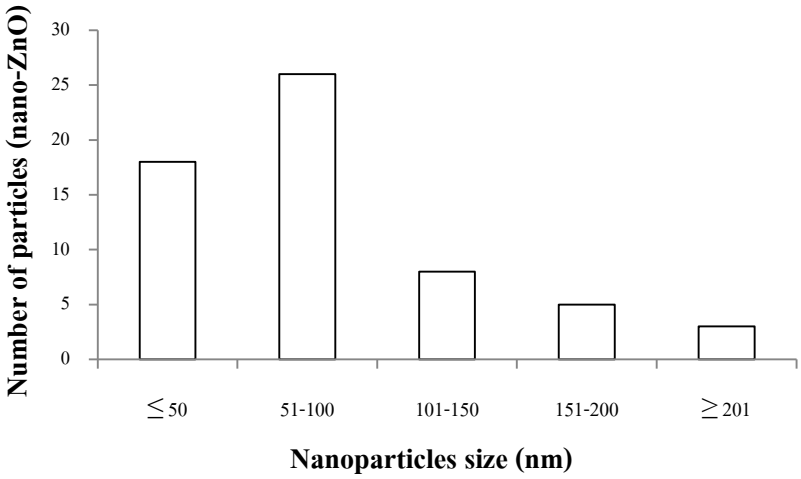

Figure 4.4 Particle size distribution of nano-ZnO (a total of 60 particles).

2. Varying concentration using nano-TiO_2

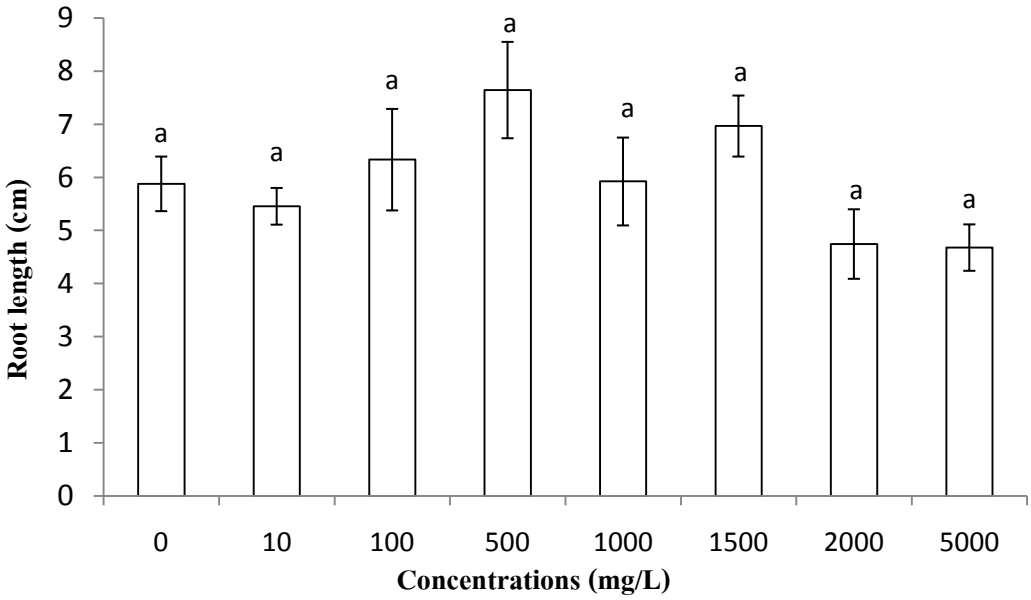

Figure 4.5 Effect of nano-TiO_2 at different concentrations on rice root length. The values are given as mean ± SE followed by the same case small lettersand are not significantly different (p = 0.01), Fisher's LSD.

All treatments led to 100 % germination of seeds, showing that nano-TiO_2 did not adversely affect rice seed germination. The results showed that there were no significant effects from nano-TiO_2 concentration between 0 – 5000 mg/L, which means nano-TiO_2 had no reducing effect on root length (Figure 4.5). However, the possibility that longer exposure time to nanoparticle solution (soaking time) could affect seed germination or root length still exists. In order to prove that hypothesis, concentrations of 100, 500 and 1000 mg/L were selected for experiments, regarding to their aggregation (the higher the concentration the faster the aggregation's rate) and also based on literature review.

3. Seed germination and root elongation test (nano-TiO_2 and nano-ZnO)

3.1 Seed germination and root elongation test using nano-TiO_2

Table 4.1 Effect of nano-TiO_2 at different concentrations and soaking times on rice root length.

Soaking time (day)	Root length (cm) Nano-TiO_2 concentrations			
	Milli-Q water	100 mg/L	500 mg/L	1000 mg/L
1	5.19±0.19aA	5.46±0.35aA	5.47±0.35aA	5.22±0.27aA
2	4.84±0.33aB	4.81±0.31aB	4.19±0.34aB	4.77±0.32aB
3	3.69±0.26aB	3.93±0.28aB	5.09±0.66aB	4.11±0.39aB

*values expressed as mean ± SE followed by the same case small letters within row and upper case letters within columns are not significantly different ($p = 0.01$), Fisher's LSD.

Table 4.2 Effect of nano-TiO_2 at different concentrations and soaking times on number of roots.

Soaking time (day)	Root length (cm) Nano-TiO_2 concentrations			
	Milli-Q water	100 mg/L	500 mg/L	1000 mg/L
1	5.44±0.29aA	5.00±0.24aA	5.33±0.24aA	4.89±0.42aA
2	5.11±0.39aA	5.44±0.24aA	5.89±0.31aA	5.11±0.45aA
3	5.89±0.42aA	5.78±0.36aA	5.11±0.45aA	4.67±0.50aA

*values expressed as mean ± SE followed by the same case small letters within row and upper case letters within columns are not significantly different ($p = 0.01$), Fisher's LSD.

All treatments led to 100 % germination of seeds even at longer soaking times. No interaction effect (concentration*soaking time) was found ($p = 0.13$). Table 4.1 shows that nano-TiO_2 concentrations had no effect on root length but soaking times did. A soaking time of 24 h was the optimum soaking time for rice seeds. However, with longer soaking times (48 and 72 h), root length decreased, which might be because of O_2-lack condition, resulting in a decreasing root length (Turner et al., 1980). Table 4.2 shows that nano-TiO_2 had no inhibiting effect on the number of roots.

These results demonstrate that nano-TiO_2 had no negative effect on either root length or the number of roots similar to those of Seeger et al. (2009) who found no significant differences in growth of willow trees by nano-TiO_2 in term of growth, transpiration and water use efficiency. Moreover, nano-TiO_2 has the possibility of being used as a promoting plant growth chemical considering the reports from some research which found that nano-TiO_2 could promote growth, increase photosynthesis and nitrogen metabolism in some plant species such as spinach (Hong et

al., 2005; Yang et al., 2006), despite the report from Ghosh et al. (2010) which found that nano-TiO_2 had a negative effect on onion and tobacco in term of damaging DNA and increasing lipid peroxidation, leading to genotoxicity. It is possible that nano-TiO_2 might only have a minor effect on plant growth; however this isconcerned with root length and number of roots.

3.2 Seed germination and root elongation test using nano-ZnO

Table 4.3 Effect of nano-ZnO at different concentrations and soaking times on rice root length.

Day	Root length (cm) Nano-ZnO concentrations				
	Milli-Q water	10 mg/L	100 mg/L	500 mg/L	1000 mg/L
1	6.74±0.48 abA	7.89±0.48 aA	5.73±0.36 bA	1.42±0.13 cA	1.19±0.05 cA
2	5.19±0.44 abB	6.09±0.22 aB	4.39±0.34 bB	0.82±0.10 cB	0.41±0.04 cB
3	4.67±0.25 aB	4.36±0.31 abC	3.56±0.19 bB	0.91±0.07 cB	0.61±0.06 cB

*values expressed as mean ± SE followed by the same case small letters within row and upper case letters within columns are not significantly different ($p = 0.01$), Fisher's LSD.

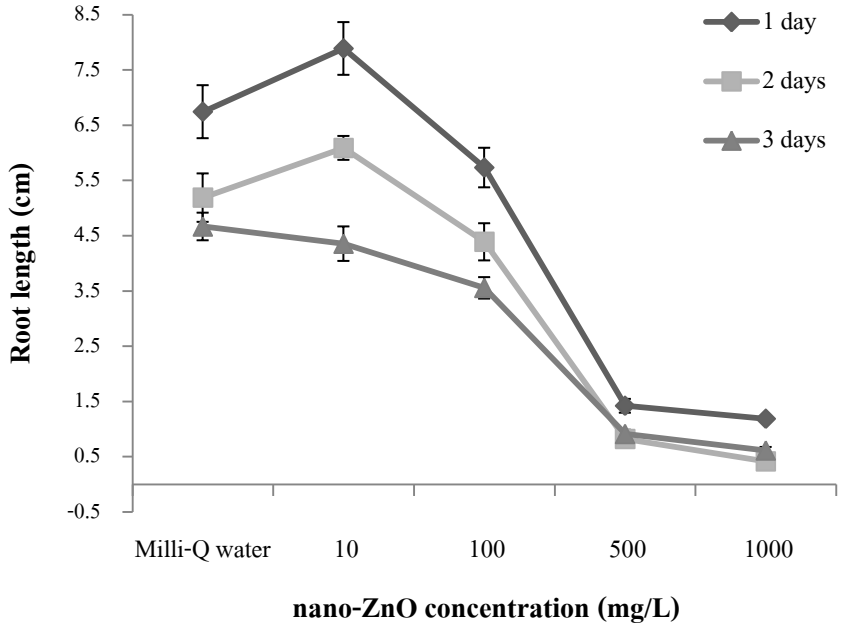

Figure 4.6 Effect of nano-ZnO at different concentrations and soaking times on rice root length. The value points are given as mean ± SE.

All treatments from nano-ZnO led to 100 % germination of seeds as did the results from nano-TiO_2 but opposite to the report from Lee et al (2009) which found that 400 mg/L of nano-ZnO could inhibit seed germination of *Arabidopsis thaliana* and 2000 mg/L of nano-ZnO could reduce seed germination of corn (Lin and Xing, 2007). These results show that plant species is one of the main characters, which could vary the results of nanoparticles.

The effects of nano-ZnO on rice roots was apparent as shown by root length (Table 4.3 and Figure 4.6); concentration was greatly involved with the reduction effect on root length, and soaking period also affected it (p = 0.002), higher concentrations showed a reducting effect starting from 100 mg/L and greatly inhibited it at concentrations of 500 and 1000 mg/L (Figure

4.8). Longer soaking time seems to be one more factor which induced inhibition of root growth, possibly due to the lack of O_2 similar to the result from nano-TiO_2.

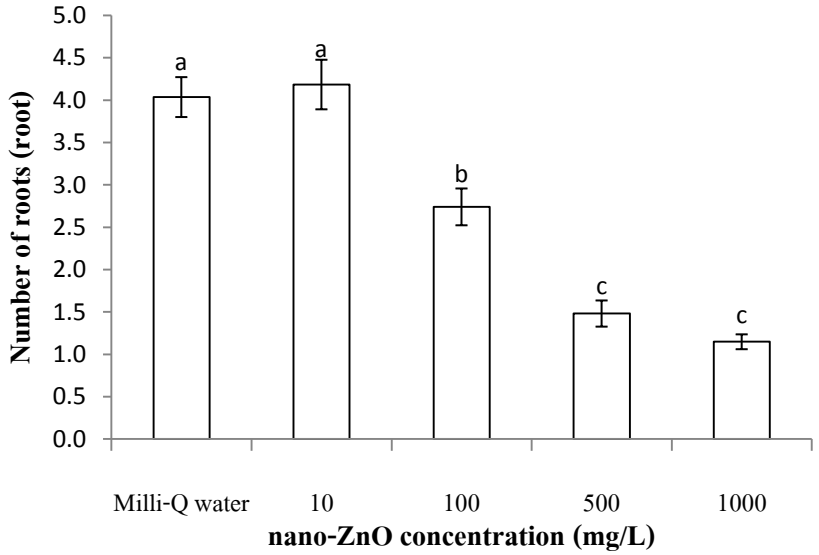

Figure 4.7 Effect of nano-ZnO at different concentrations on number of rice roots. The values are given as mean ± SE followed by the same case small letters are not significantly different (p = 0.01), Fisher's LSD.

For the number of roots (Figure 4.7), no interaction effect (concentration*soaking time) from nano-ZnO was found (p = 0.67), but it was affected by the nano-ZnO concentration (p = 0.00) starting from 100 mg/L. For further analysis, a relative root growth test was conducted in the following experiments.

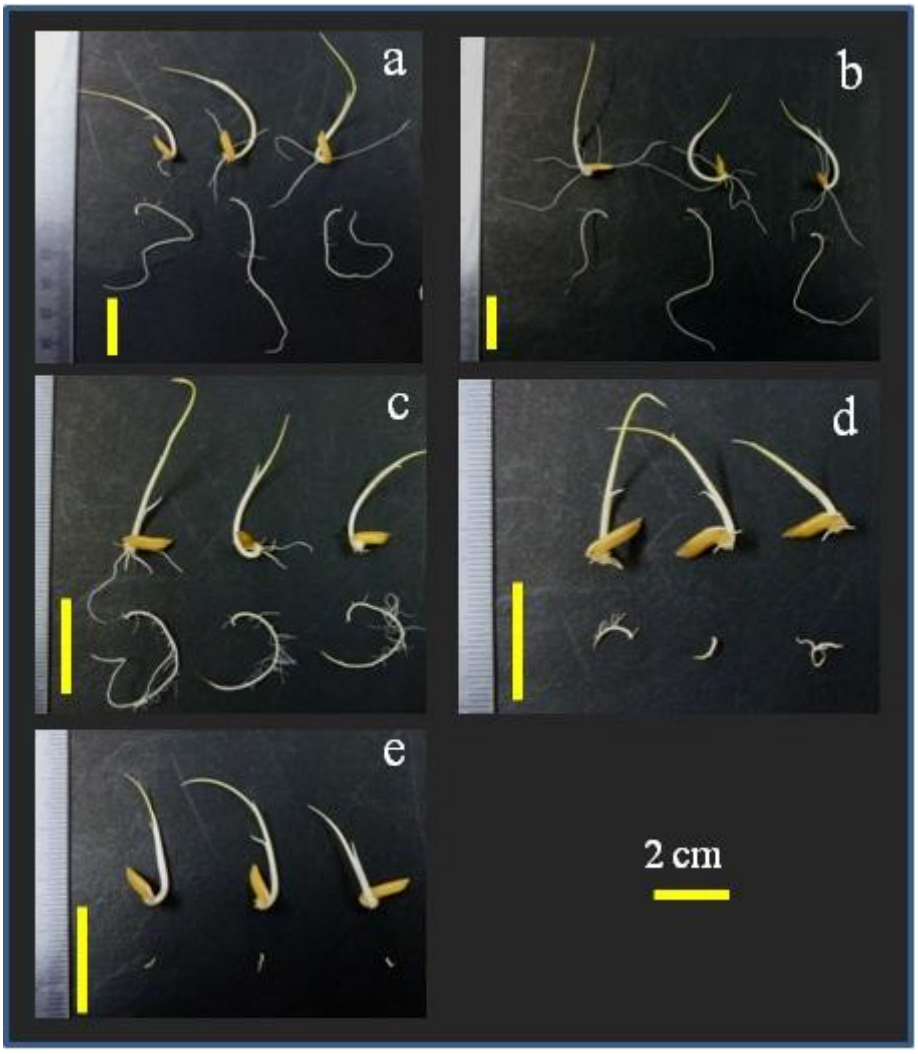

Figure 4.8 Effect of nano-ZnO at different concentrations on rice roots: a) Milli-Q water (control), b) 10 mg/L nano-ZnO, c) 100 mg/L nano-ZnO, d) 500 mg/L nano-ZnO and d) 1000 mg/L nano-ZnO, the bar = 2 cm.

4. Relative root growth test

4.1 Relative root growth test using nano-TiO_2

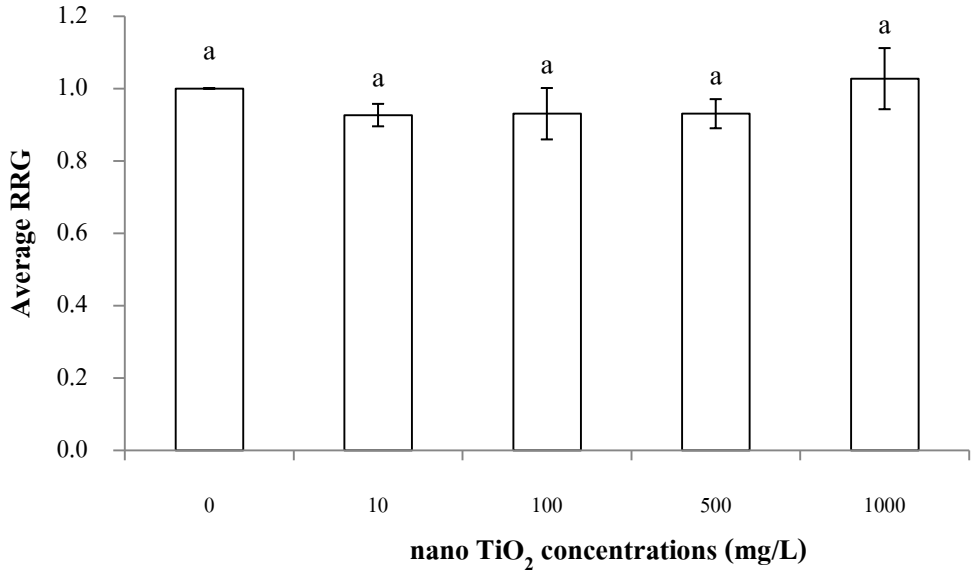

Figure 4.9 Effect of nano-TiO_2 at different concentrations on relative root growth (RRG) values. The values are given as mean ± SE followed by the same case small letters are not significantly different ($p = 0.01$), Fisher's LSD.

This experiment was performed to ensure the results from the previous studies on seed germination and root elongation. It is different from those experiments in that this case allows the seeds to germinate before expose to the nanoparticle solution. In this regard, the results confirmed that nano-TiO_2 had no reducing effect on root growth, which could be seen because the relative root growth values were not significantly different from the control (Figure 4.9). This result suggests that nano-TiO_2 is possibly a harmless substance to this plant species.

4.2 Relative root growth test using nano-ZnO

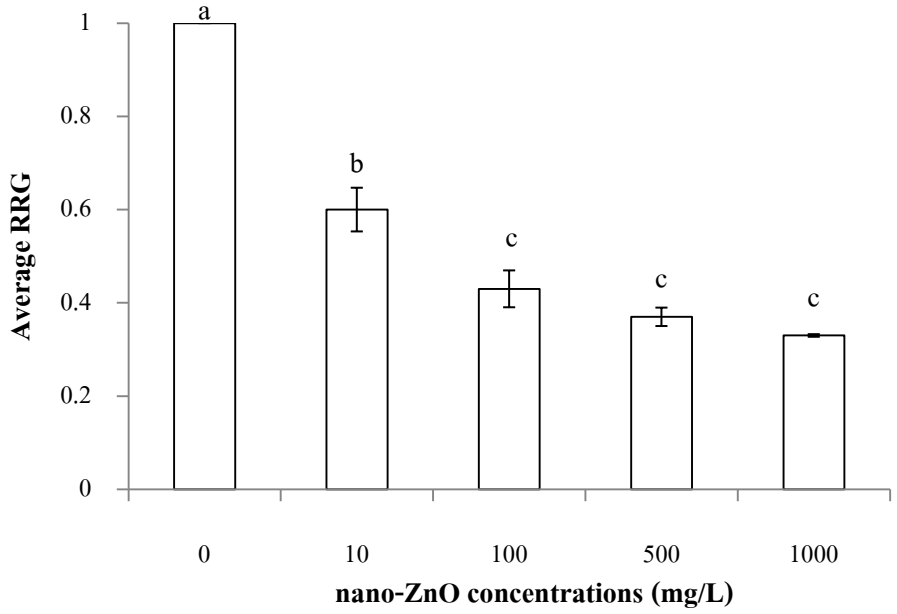

Figure 4.10 Effect of nano-ZnO at different concentrations on Relative root growth (RRG) values. The values are given as mean ± SE followed by the same case small letters are not significantly different (p = 0.01), Fisher's LSD.

Relative root growth values demonstrated that higher concentrations of nano-ZnO had a reducing effect on root elongation (p = 0.00) (Figure 4.10), and when the emergent roots were directly exposed to nanoparticle suspensions, the reduction effect started from the lowest concentration in this experiment (10 mg/L), in contrast to the soaking method where we found the toxicity started at 100 mg/L of nano-ZnO at 3 days soaking time. A similar report from Yang and Watts (2005) found that alumina nanoparticles (nano-Al_2O_3) at 2000 mg/L could inhibit root elongation in five plant species. However, in this case, nano-ZnO was found to be more severe than nano-Al_2O_3 in terms of reducing root elongation. Photographs comparing the roots before

and after expose to Milli-Q water, nano-TiO_2 and nano-ZnO are shown here in Figure 4.11 – 4.13.

The hypothesis that nano-TiO_2 could have the same effect on rice root growth as nano-ZnO, because it has some common properties with nano-ZnO, could be rejected. From the observations in this study, nano-TiO_2 showed no significant negative effect on root length, number of roots or root elongation, which is opposite from nano-ZnO, which showed a reducing effect in all parameters, except seed germination. Lin and Xing, (2007) also found that nano-ZnO had no inhibiting effect on seed germination of radish, rape, ryegrass, lettuce, and cucumber, but found the reduction effect on root length of all tested plants as mentioned above.

The main characteristics which make each nanoparticle result in different ways are chemical composition, surface area and surface energy (Brunner et al., 2006). However, the specific surface area reported by the company of nano-ZnO is 15-25 m^2/g, which is less than the specific surface area of nano-TiO_2, which is 35-45 m^2/g, suggesting that some others surface characteristics, such as chemical composition, could play a key role that makes these two nanoparticles provide different results. This hypothesis could be supported by the work of Yang and Watts (2005) who found that nano-Al_2O_3 with a surface-coat by phenanthrene had a lesser reduction effect on root elongation of tested plants (corn, cucumber, soybean, cabbage and carrot) than nano-Al_2O_3 without coating, meaning that the coating process regarding the surface characteristics could be an important factor on nanotoxicity.

Moreover, nano-ZnO has properties which exhibit very high surface reactivity when compared to nano-TiO_2, and due to the efficient generation of hydroxyl, leads to high reaction

and mineralization rates which could also support the possible factor of its toxicity (Carraway et al., 1994; Baruah et al., 2009). These results provide a guideline for the need for more studies to be undertaken on surface characteristic properties to clarify and provide information of nanotoxicity from surface characteristic of nanoparticles.

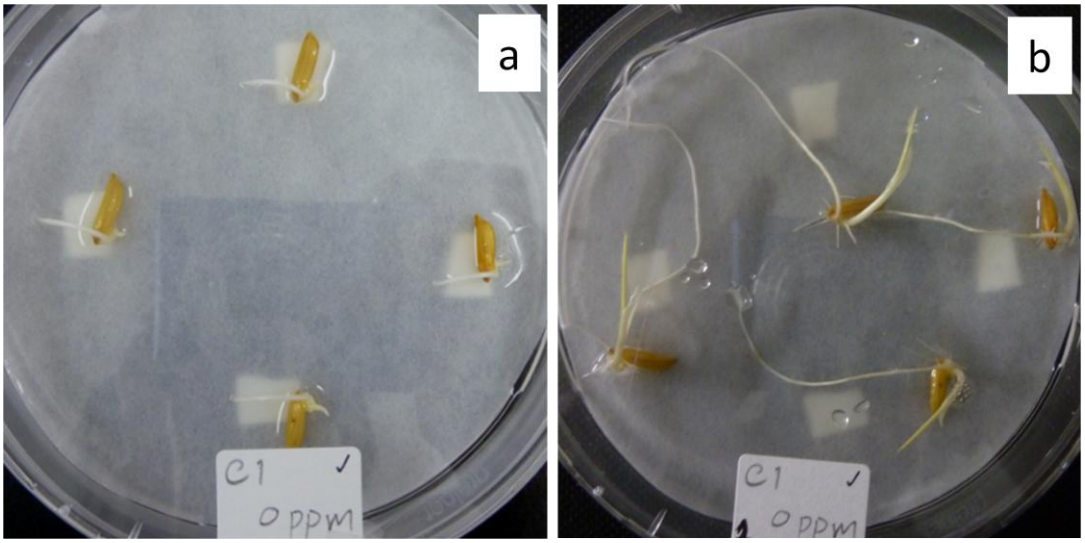

Figure 4.11 Relative root growth (RRG) test (control): a) rice roots before exposure to Milli-Q water; b) 2 days after the exposure.

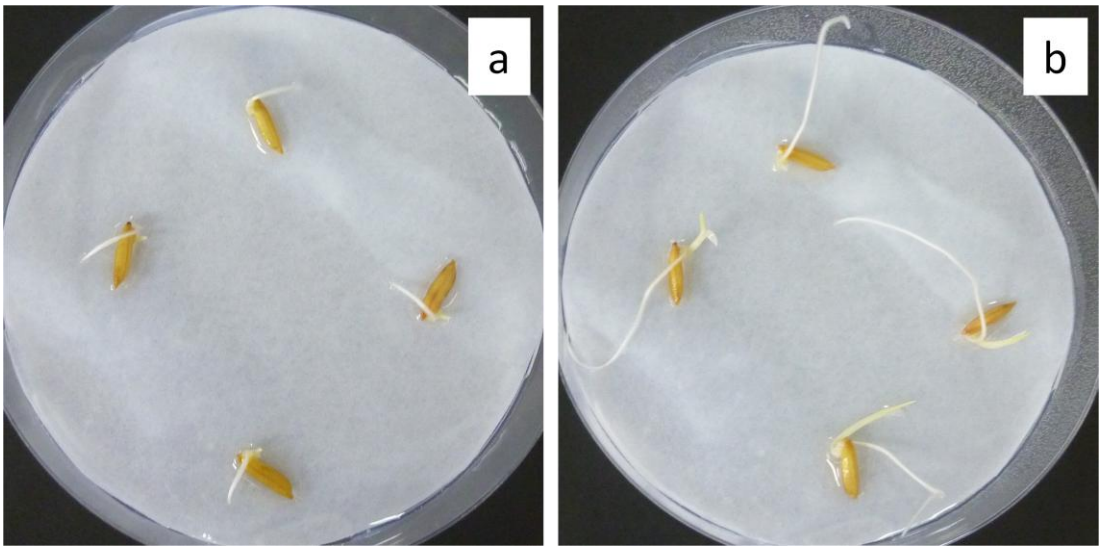

Figure 4.12 Relative root growth (RRG) test (nano-TiO_2): a) rice roots before exposure to nano-TiO_2 at 1000 mg/L water; b) 2 days after the exposure.

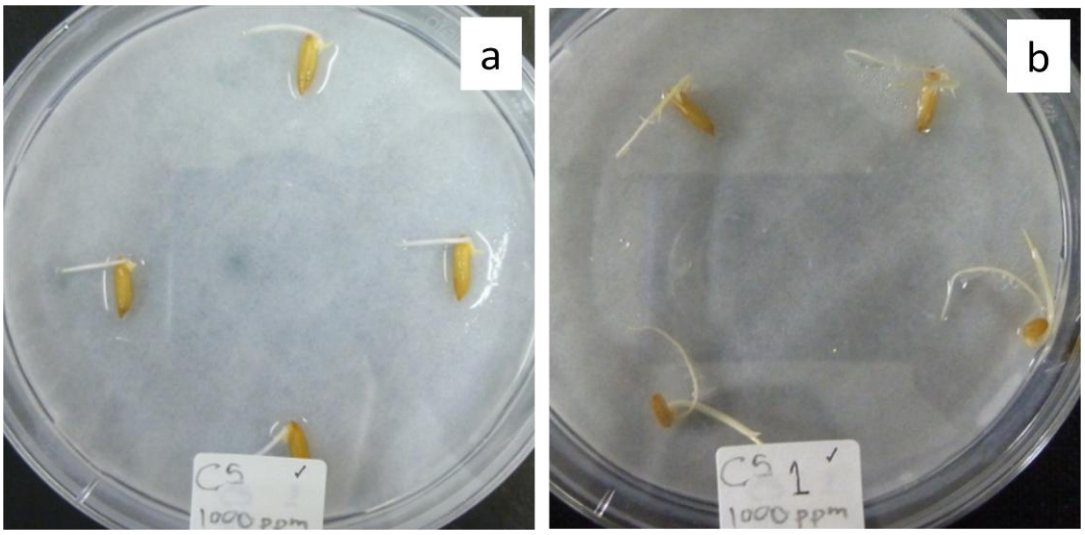

Figure 4.13 Relative root growth (RRG) test (nano-ZnO): a) rice roots before exposure to nano-ZnO at 1000 mg/L water; b) 2 days after the exposure.

5. Zinc ion test

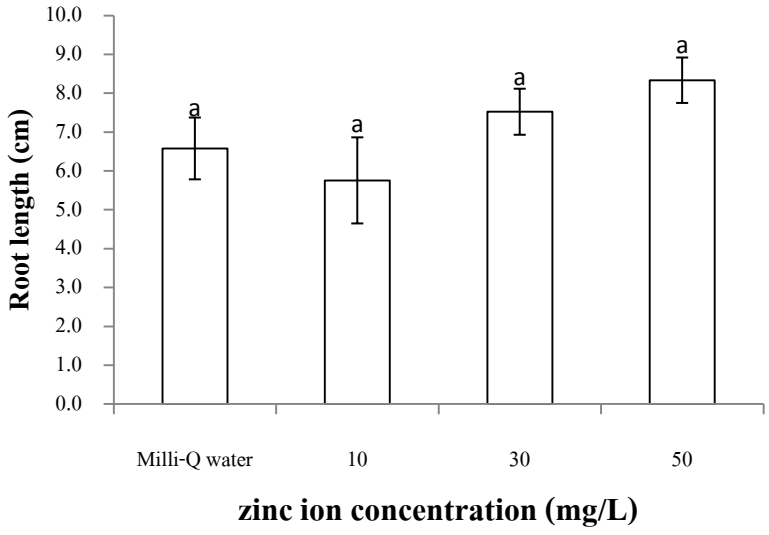

Figure 4.14 Effect of zinc ion at different concentrations on root length. The values are given as mean ± SE followed by the same case small letters are not significantly different (p = 0.01), Fisher's LSD.

A zinc ion test was performed in order to determine whether or not the role of the dissolved metal species (Zn^{2+}) was involved in nanoparticle toxicity. Figure 4.14 shows that zinc ion

concentration had no reducing effect on root length. The concentration of zinc ion found in nano-ZnO solution at 1000 mg/L was about 40 mg/L. The results from this experiment help to confirm that toxicity from nano-ZnO could not come from ion dissolution, in agreement with the report from Ling and Xing (2007) and Lee et al. (2009).

6. Rice root structure (nano-ZnO)

From the previous experiment, it was clear that nano-ZnO had adverse effects on root length, number of roots and root elongation. For further study, the observations of the rice root anatomy were carried out, in order to elucidate possible harmful effects in the roots treated with nano-ZnO. When observed via transverse thick section (80 μm in thickness), the rice root structure treated with nano-ZnO 1000 mg/L was found to be able to create amounts of globule in the parenchyma cells at the cortex area. The observations were noticed from rice roots collecting from day 4 until day 7, because roots from earlier days from nano-ZnO treatment were unable to be collected. Moreover, this observation also compared root structure between control and nano-ZnO treatment at 3 different parts; basal part, middle part and near root tip, in order to cover all root parts, not only one specific area in the root.

The results from this observation suggested that globules occurred in larger amounts at basal part and middle part of root, following by near root tip part which found lesser globules as compare to the parts mentioned previously. Here we hypothesized the possibility that this evidence could come from the period of the soaking process, during the imbibitions stage which is the beginning of seed germination (Wierzbicka and Obidzinska, 1998), the majority of water

enters the seed via the micropylar seed end (an open pore, which radical root always penetrated from) (Manz et al., 2005). With this process, nano-ZnO could either enter the seed via the micropylar seed end and accumulate in the seed, or penetrate into the embryo and endosperm. When radical roots sprout from the seed during germination, the zone of cell division (which is immediately behind the root tip) is likely to have a high level of sensitivity to the nanoparticles and may absorb some of them. This would explain the observed presence of globules in the basal part and middle part of the roots, which correspond to the zone of maturation (which in turn is related to the presence of root hairs and lateral roots which developed in this zone). Furthermore, the zone of cell division may continue producing new cells that are less sensitive to nano-ZnO treatment than the earlier generation of cells. This hypothesis is consistent with observations that fewer globules were present near the later-formed root tips. However, the division of cells could not continue that far due to the inhibiting effect from nano-ZnO, the root seems to be stunted in its growth or continues at a very low rate, the roots from this treatment became very short (between 0.4 - 1.2 cm) when compared to the control.

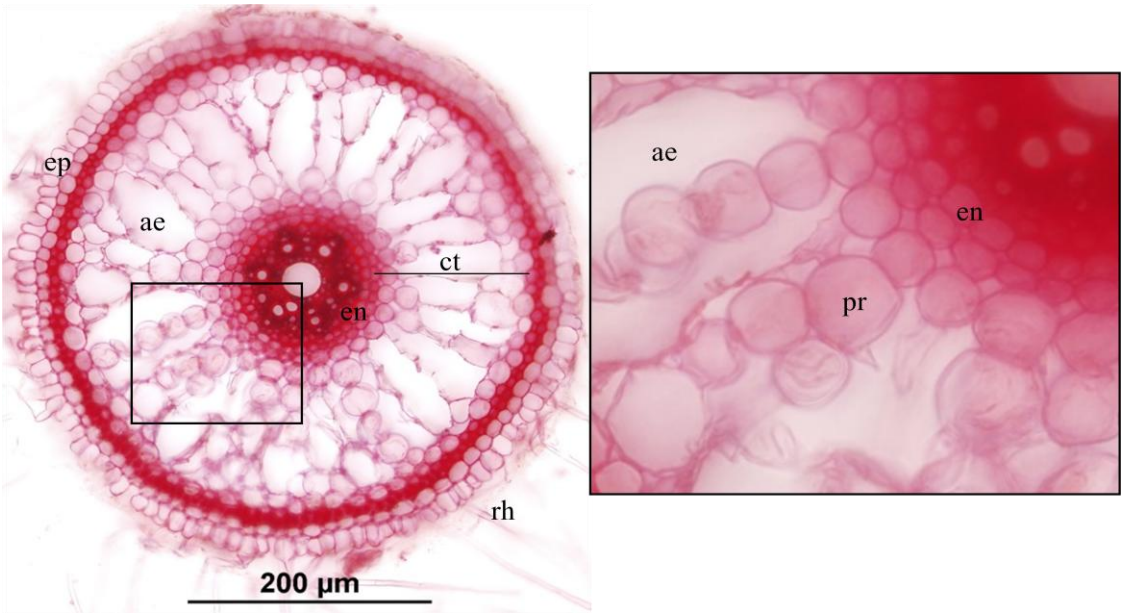

Figure 4.15 Rice root in control treatment, 4 day-old, basal part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma, rh = root hair.

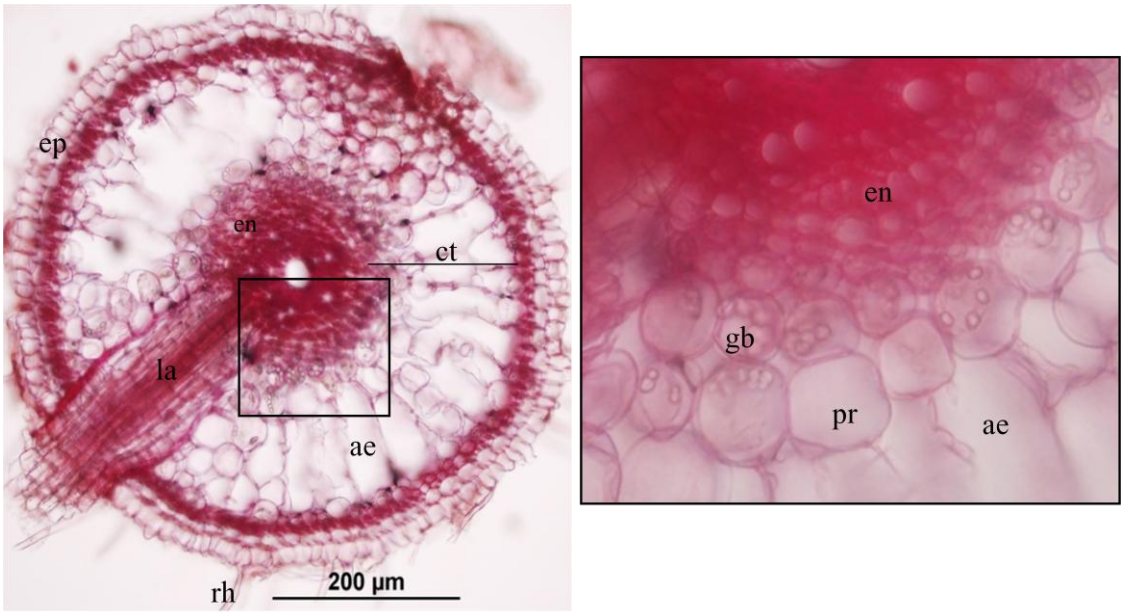

Figure 4.16 Rice root in 1000 mg/L nano-ZnO treatment, 4 day-old, basal part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma , rh = root hair, gb = globules.

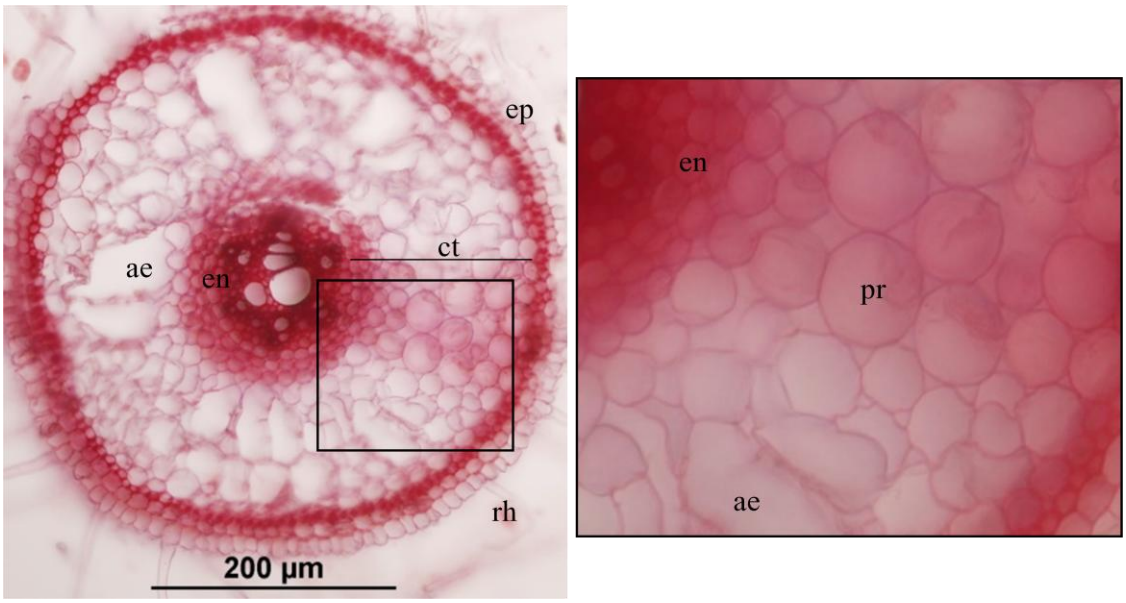

Figure 4.17 Rice root in control treatment, 5 day-old, basal part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma, rh = root hair.

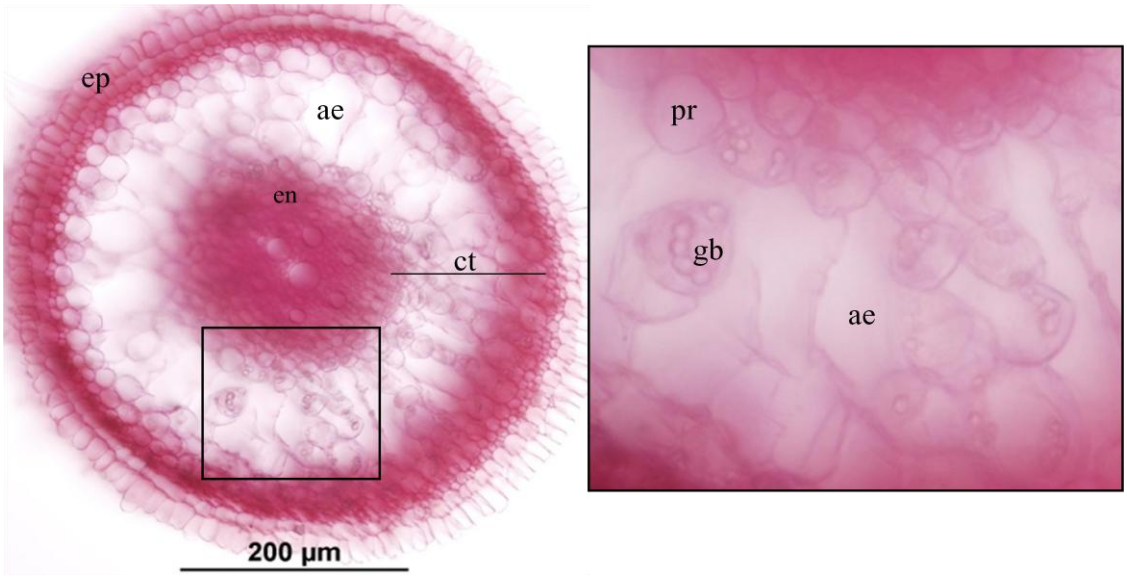

Figure 4.18 Rice root in 1000 mg/L nano-ZnO treatment, 5 day-old, basal part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma , gb = globules.

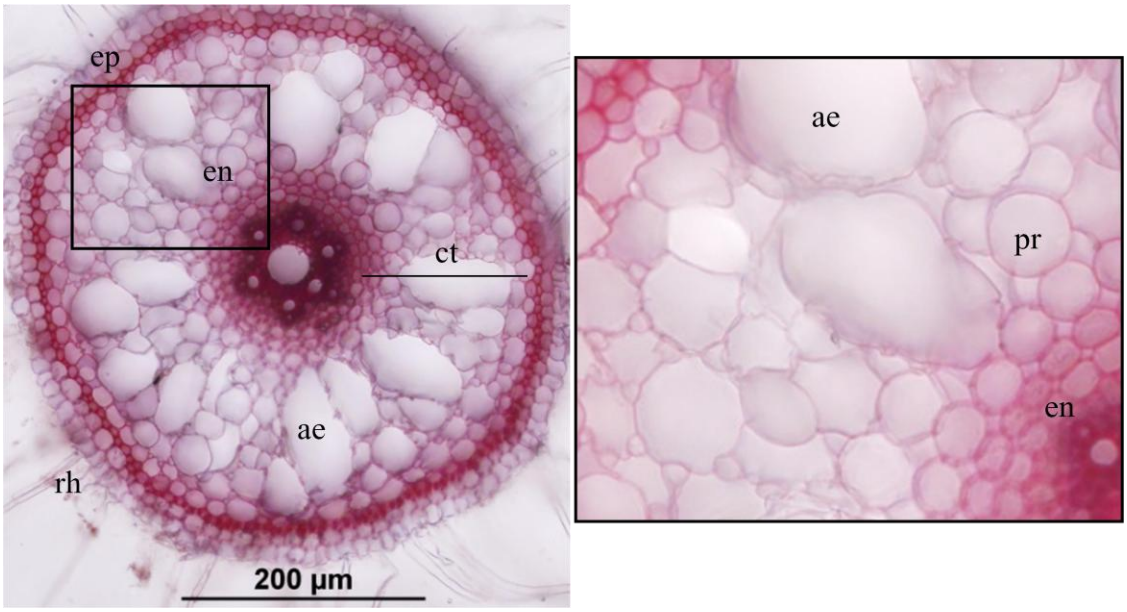

Figure 4.19 Rice root in control treatment, 6 day-old, basal part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma, rh = root hair.

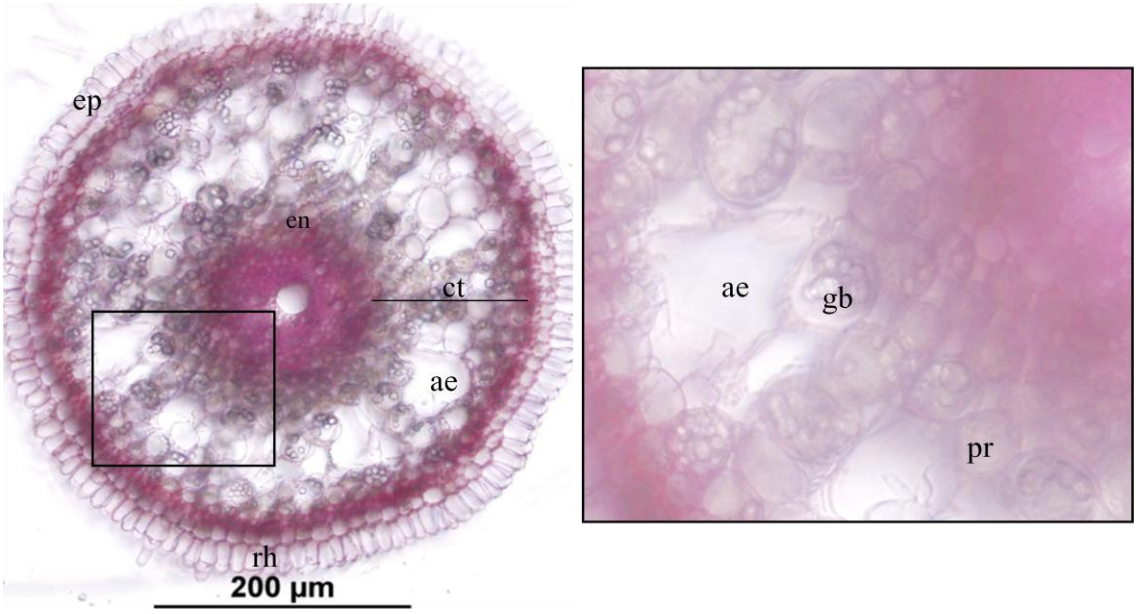

Figure 4.20 Rice root in 1000 mg/L nano-ZnO treatment, 6 day-old, basal part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma , rh = root hair, gb = globules.

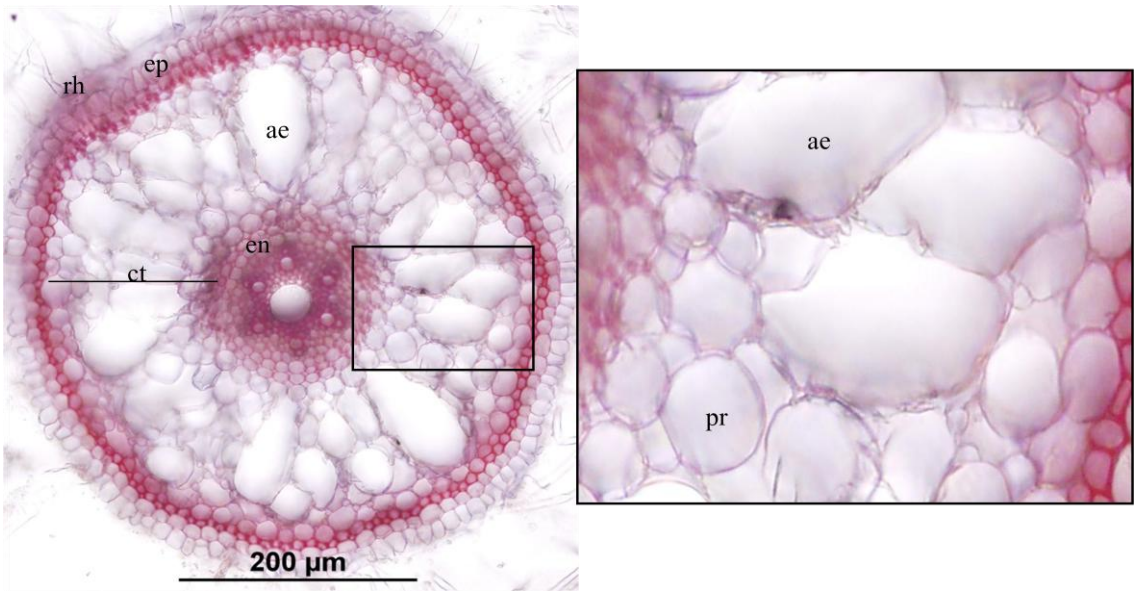

Figure 4.21 Rice root in control treatment, 7 day-old, basal part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma, rh = root hair.

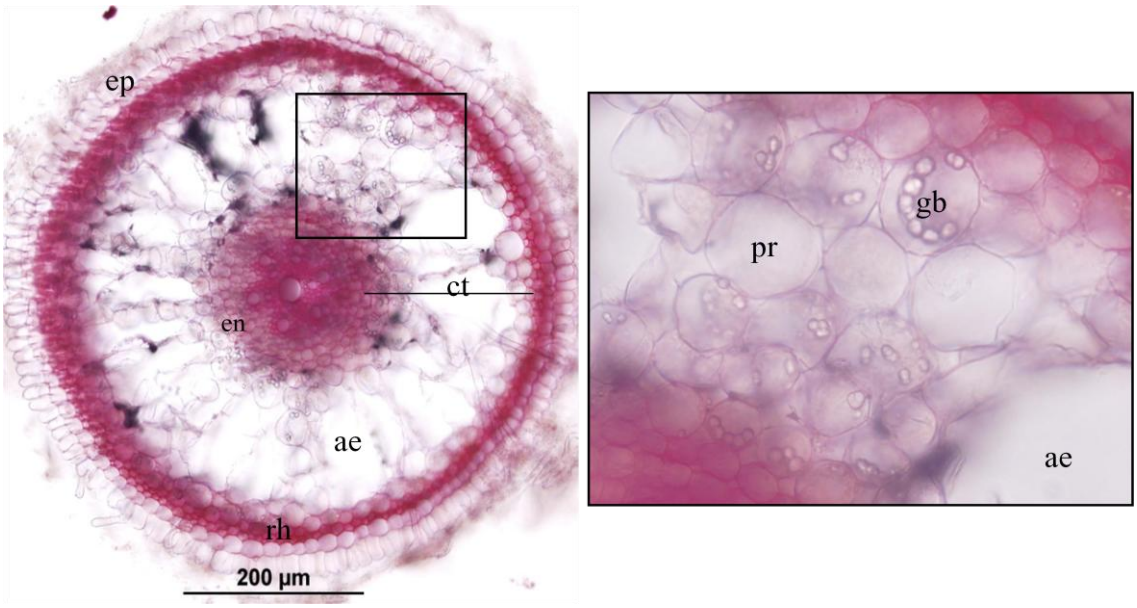

Figure 4.22 Rice root in 1000 mg/L nano-ZnO treatment, 7 day-old, basal part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma , rh = root hair, gb = globules.

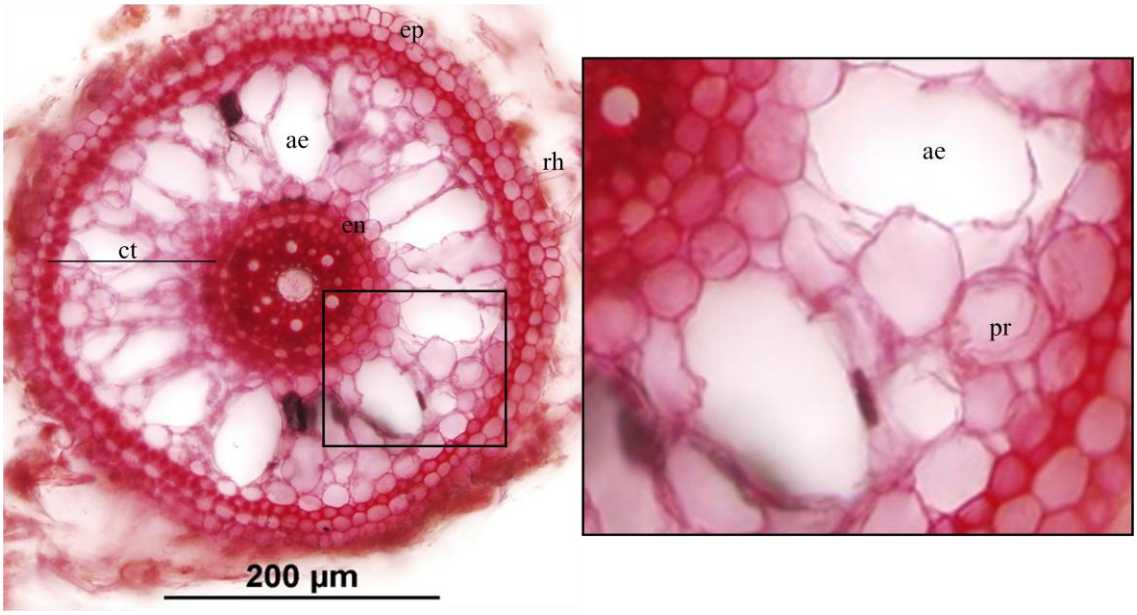

Figure 4.23 Rice root in control treatment, 4 day-old, middle part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma, rh = root hair.

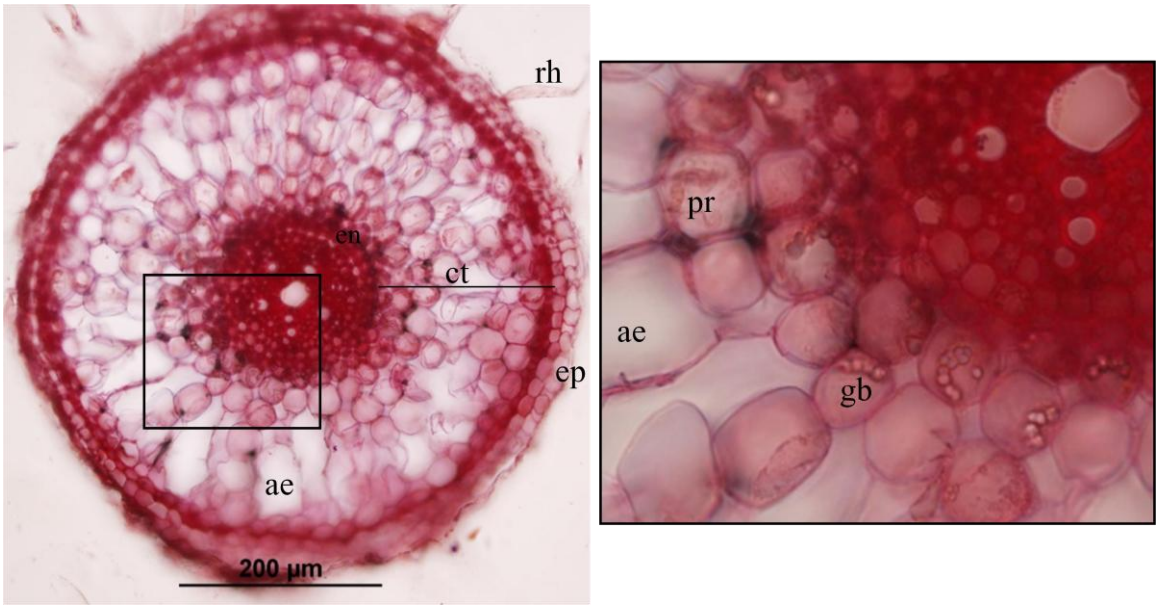

Figure 4.24 Rice root in 1000 mg/L nano-ZnO treatment, 4 day-old, middle part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma , rh = root hair, gb = globules.

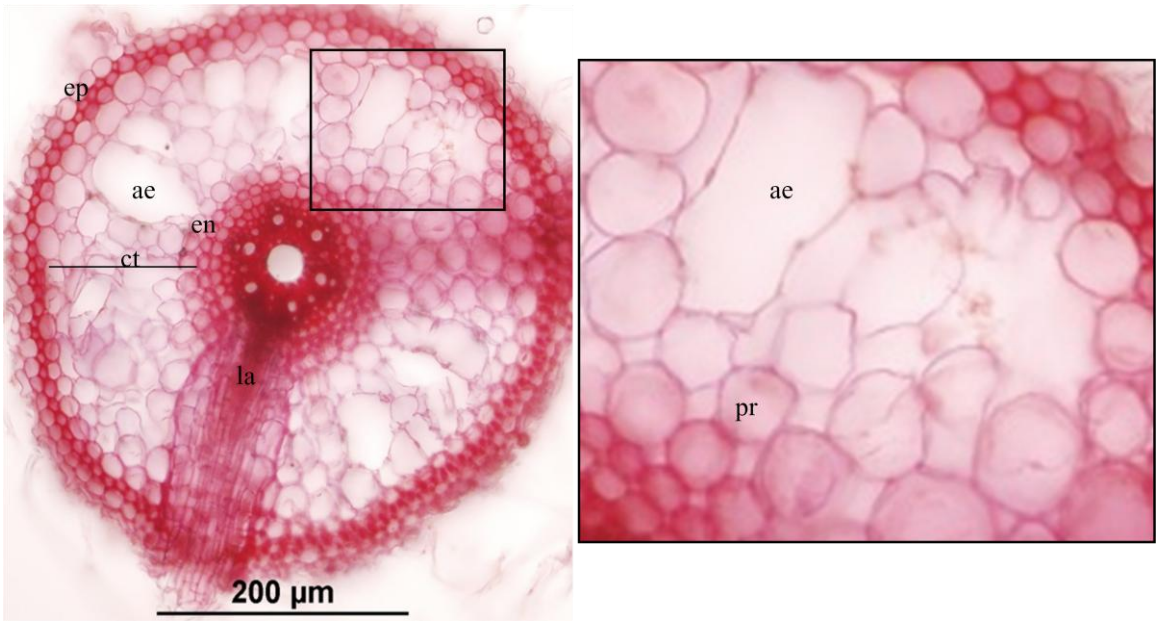

Figure 4.25 Rice root in control treatment, 5 day-old, middle part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma, rh = root hair, la = lateral root.

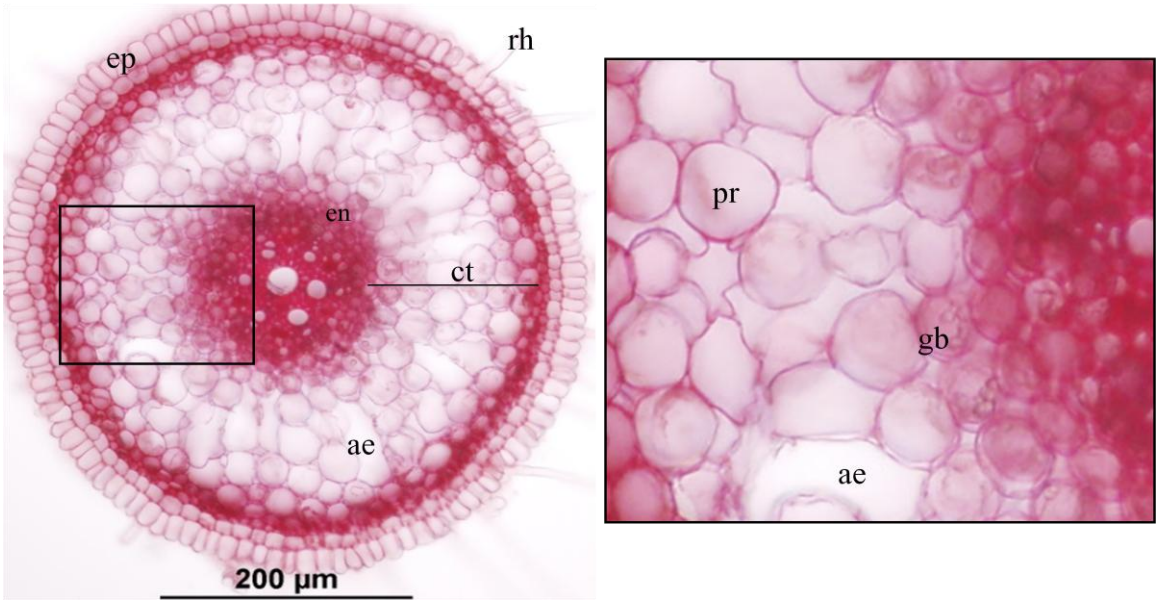

Figure 4.26 Rice root in 1000 mg/L nano-ZnO treatment, 5 day-old, middle part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma , rh = root hair, gb = globules.

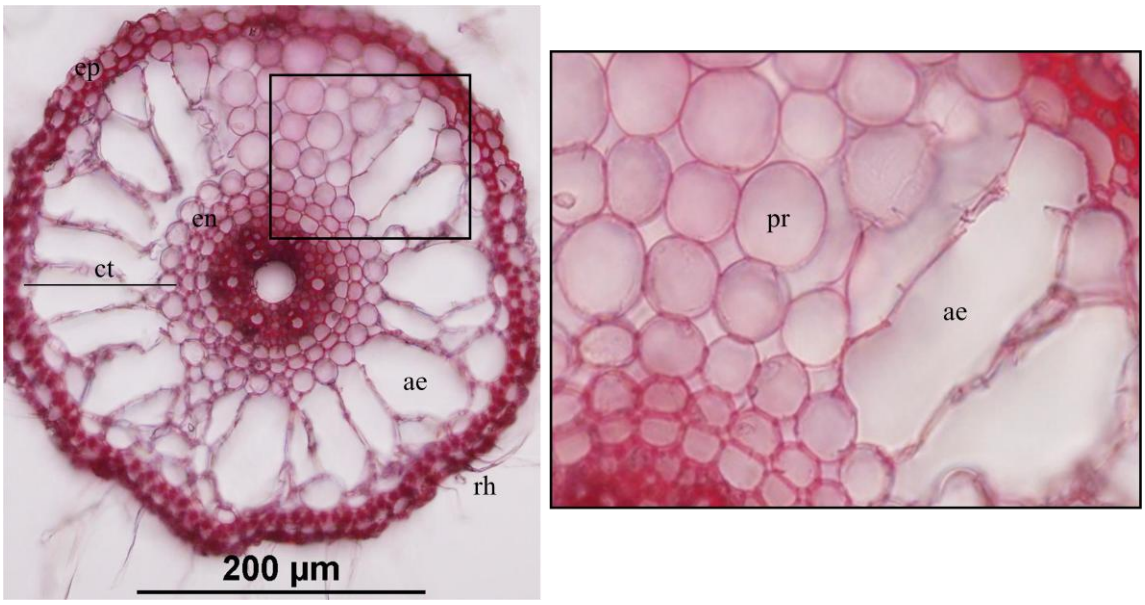

Figure 4.27 Rice root in control treatment, 6 day-old, middle part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma, rh = root hair.

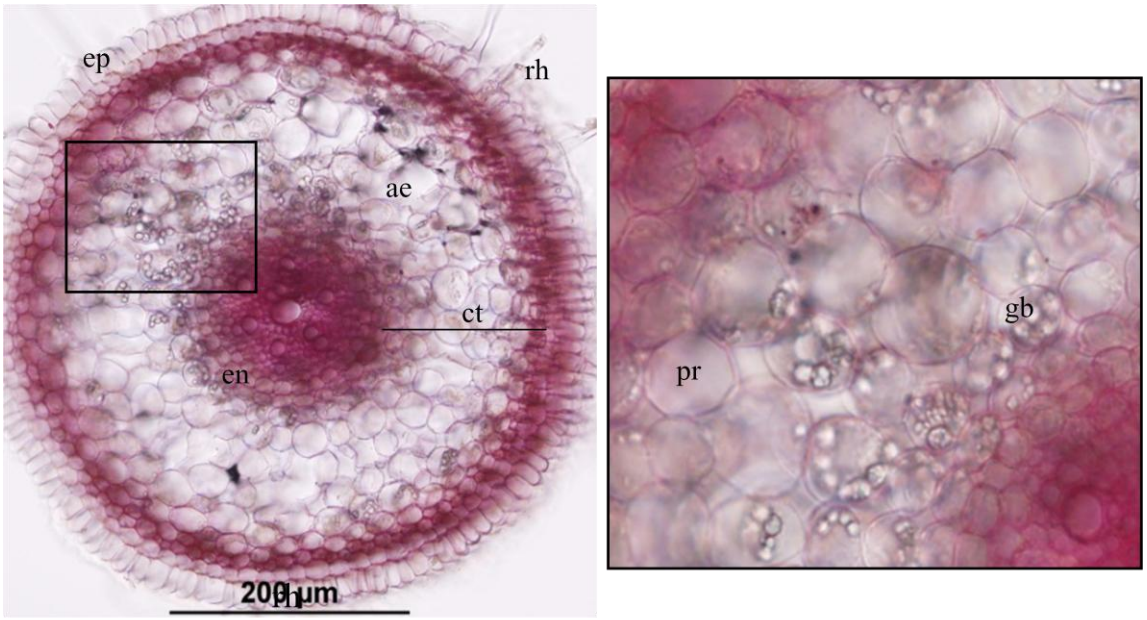

Figure 4.28 Rice root in 1000 mg/L nano-ZnO treatment, 6 day-old, middle part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma , rh = root hair, gb = globules.

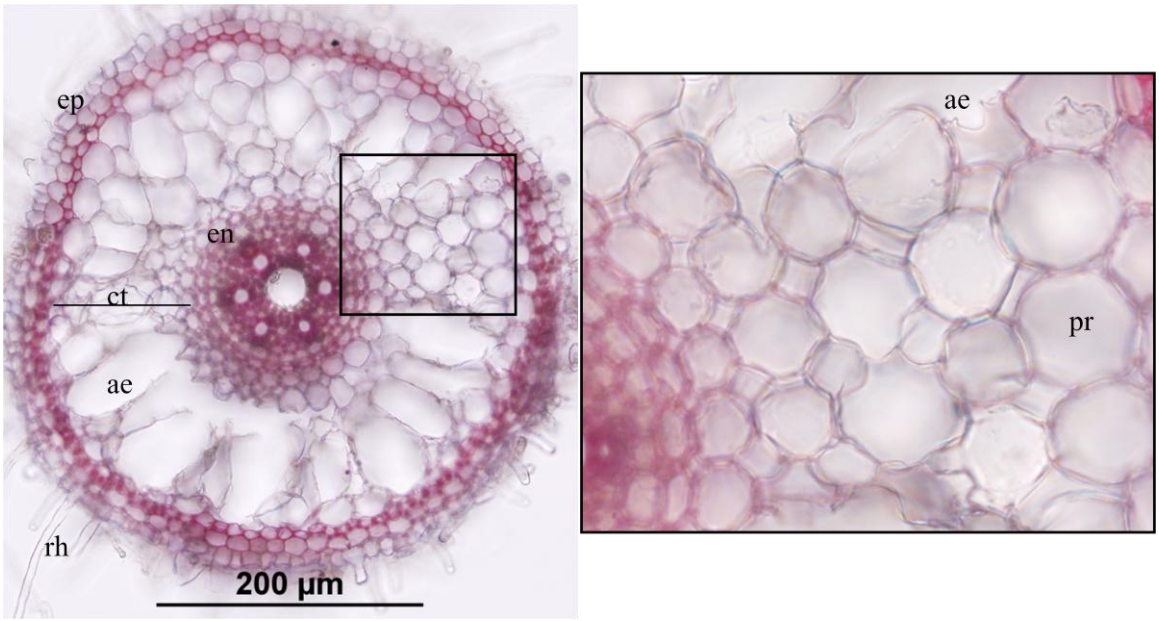

Figure 4.29 Rice root in control treatment, 7 day-old, middle part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma, rh = root hair.

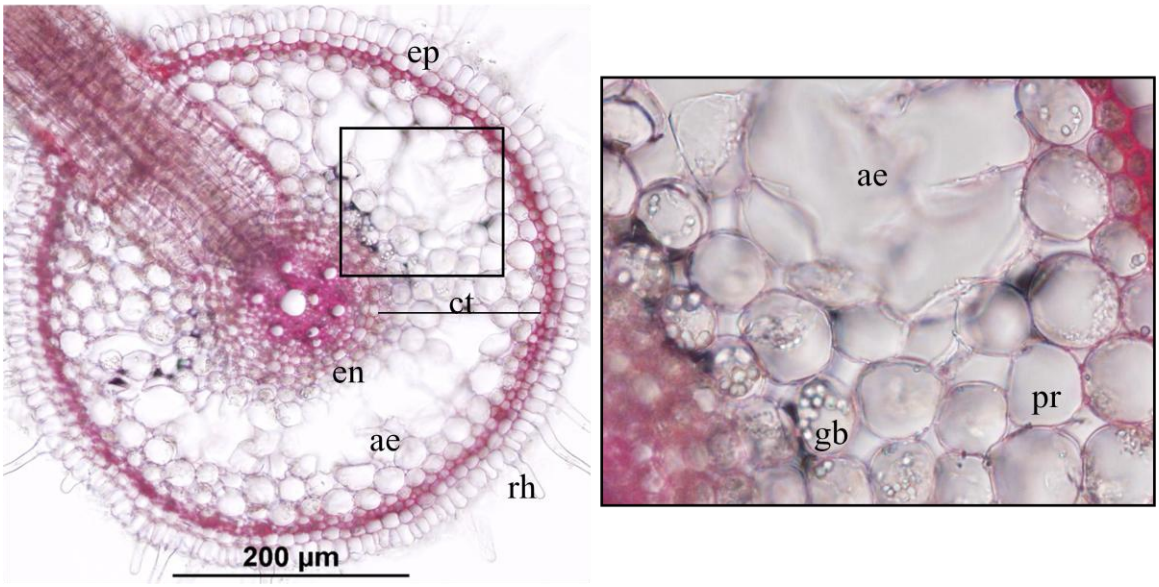

Figure 4.30 Rice root in 1000 mg/L nano-ZnO treatment, 7 day-old, middle part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma , rh = root hair, gb = globules.

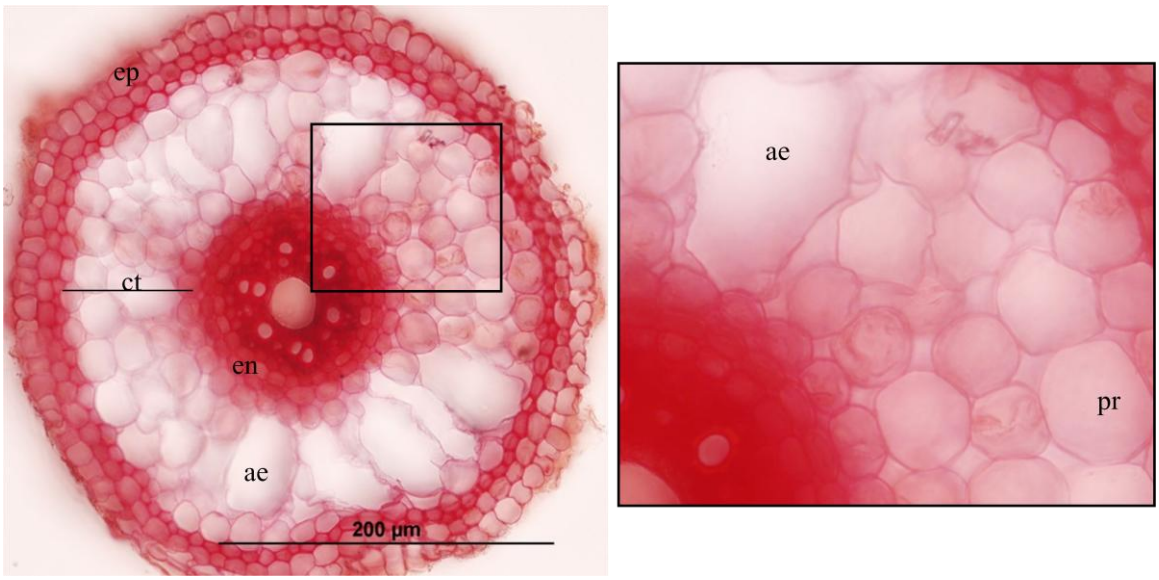

Figure 4.31 Rice root in control treatment, 4 day-old, near root tip part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma, rh = root hair.

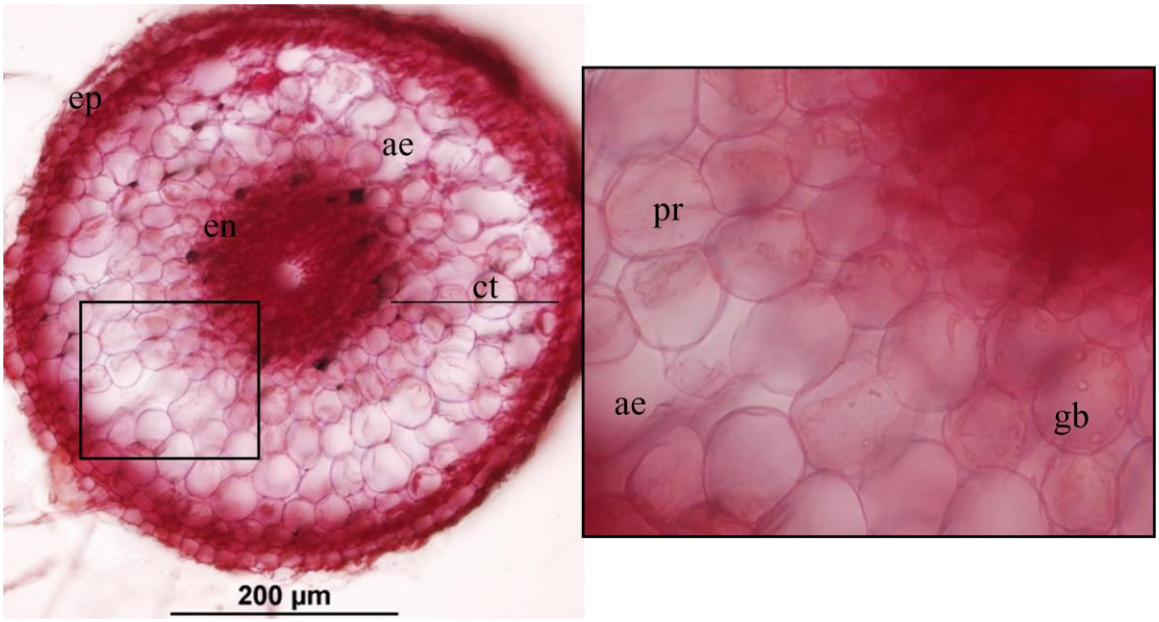

Figure 4.32 Rice root in 1000 mg/L nano-ZnO treatment, 4 day-old, near root tip part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma , rh = root hair, gb = globules.

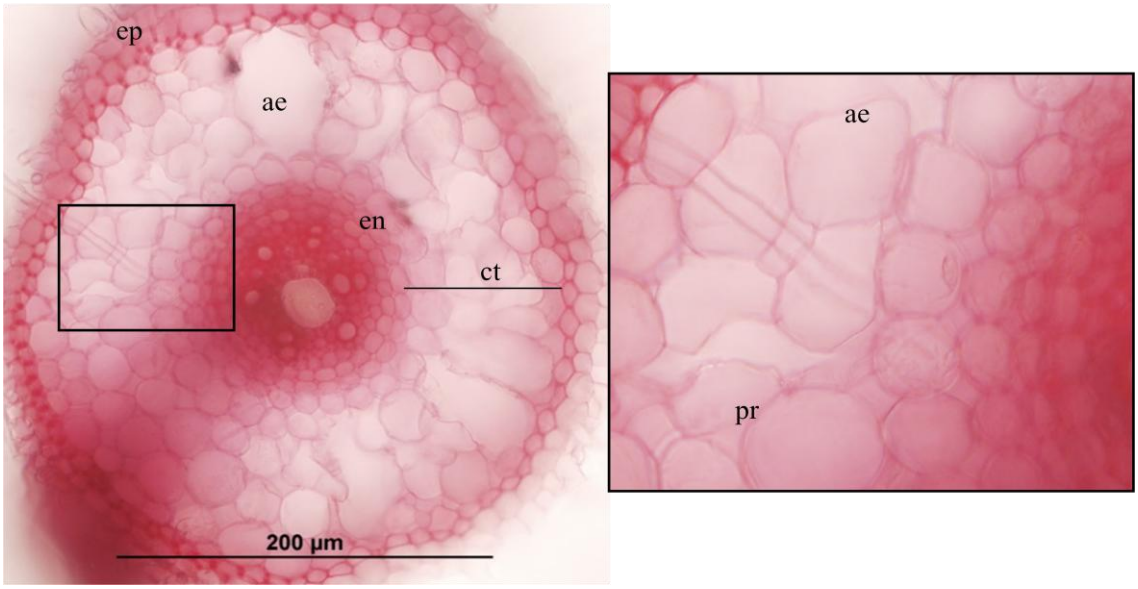

Figure 4.33 Rice root in control treatment, 5 day-old, near root tip part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma, rh = root hair.

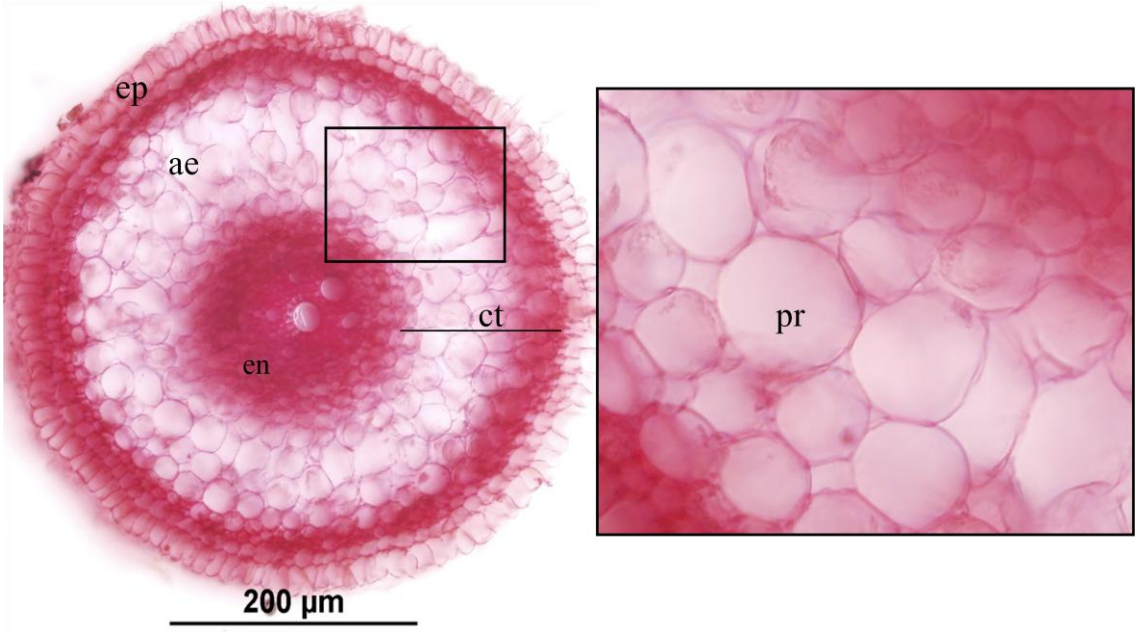

Figure 4.34 Rice root in 1000 mg/L nano-ZnO treatment, 5 day-old, near root tip part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma , rh = root hair, gb = globules.

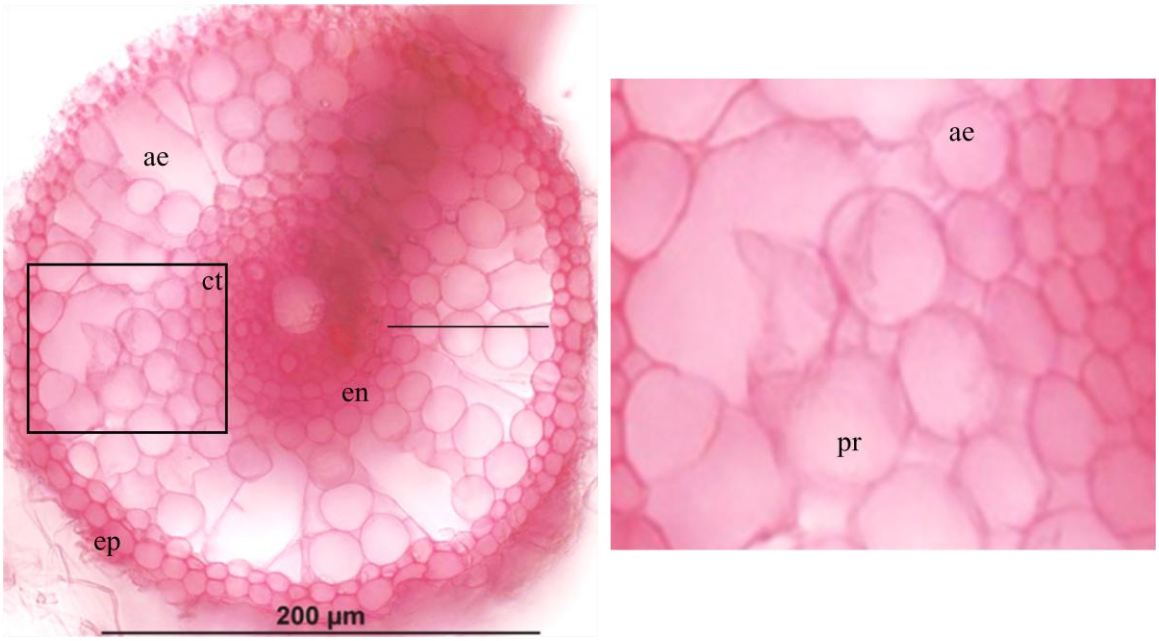

Figure 4.35 Rice root in control treatment, 6 day-old, near root tip part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma, rh = root hair.

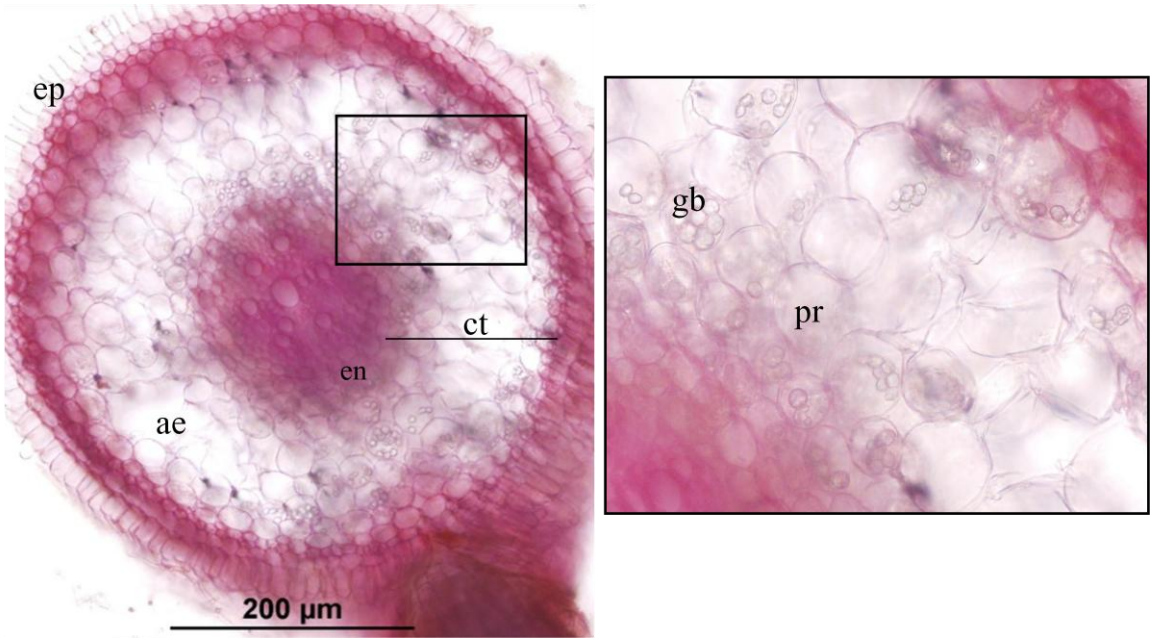

Figure 4.36 Rice root in 1000 mg/L nano-ZnO treatment, 6 day-old, near root tip part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma , rh = root hair, gb = globules.

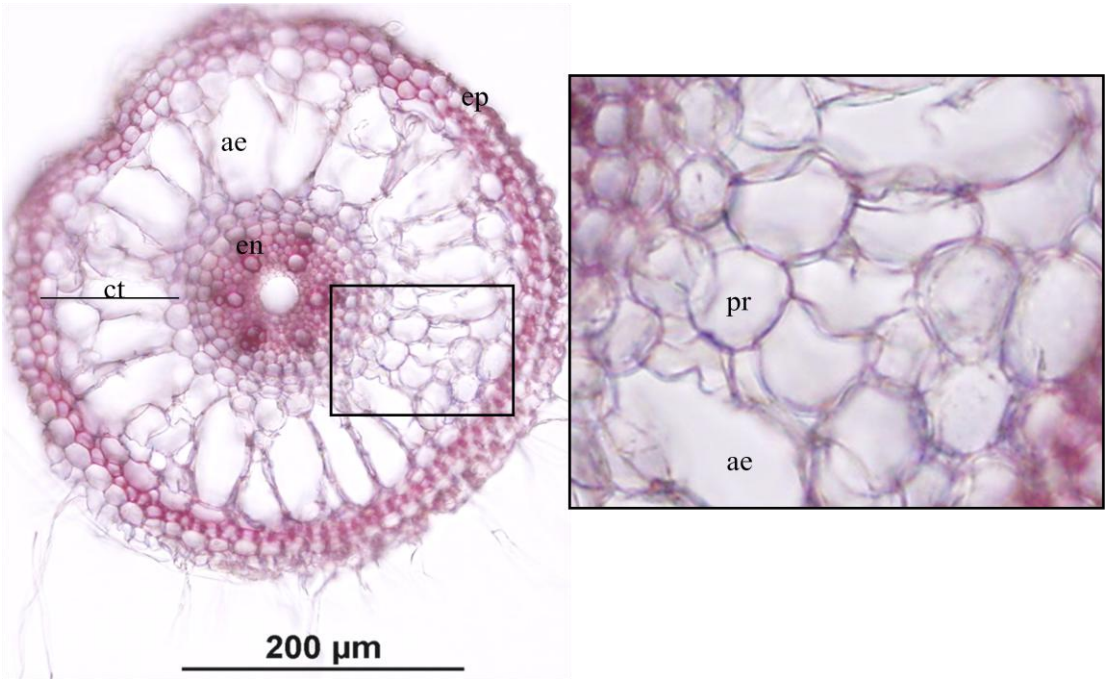

Figure 4.37 Rice root in control treatment, 7 day-old, near root tip part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma, rh = root hair.

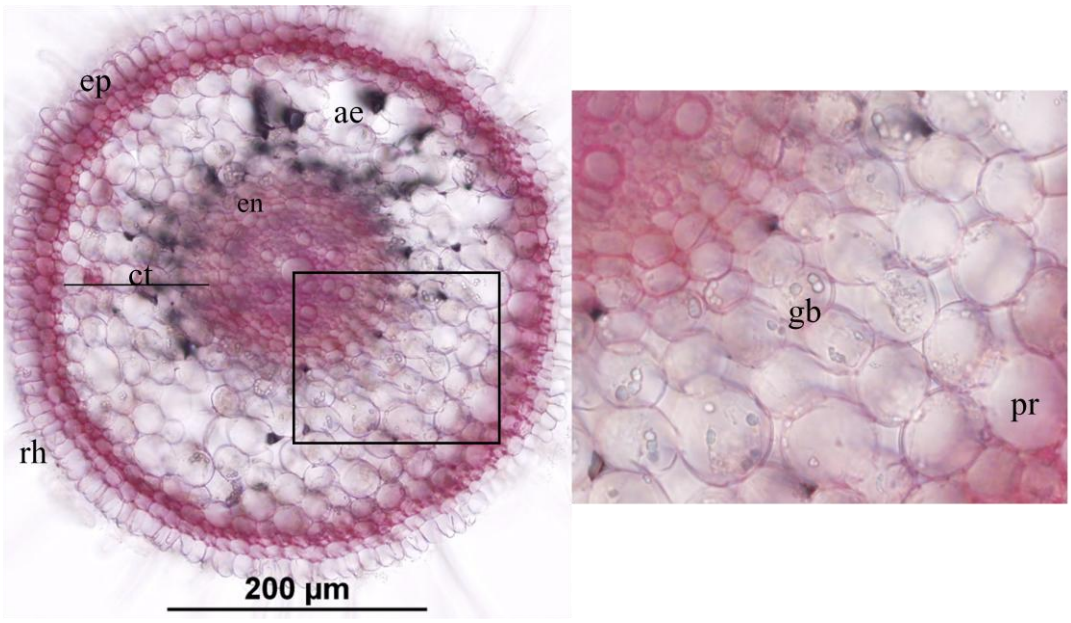

Figure 4.38 Rice root in 1000 mg/L nano-ZnO treatment, 7 day-old, near root tip part: ep = epidermis, en = endodermis, ct = cortex, ae = aerenchyma, pr = parenchyma , rh = root hair, gb = globules.

The possible hypothesis of the occurrence of those globules was given previously. Figure 4.15 – 4.22 represents the roots cut from basal part, nano-ZnO-treat roots showed amount of globules as described, following by Figure 4.24 – 4.30, which represents the roots cut from middle part. Globules were found in larger amount compared with the roots cut from near root tip part (Figure 4.31 – 4.38).

Both roots from control and nano-ZnO treatment were found to have 1 layer of epidermis, 1 layer of exodermis, 7-8 layers of cortical parenchyma (cortex layer) and 1 layer of endodermis with no disruption. Aerenchymas (air space) were found in the cortex layer in both treatments, and the vascular bundles were formed normally. However, the globules, which were found in nano-ZnO treatment, made the difference between the roots from the 2 treatments.

For further analysis, samples from both control and nano-ZnO treatment were analyzed under scanning electron microscope (SEM) which also found many globule like clusters in the parenchyma cell at the cortex layer (Figure 4.39-4.40). From the analysis by an energy dispersive spectroscopy (EDS), one of the functions in SEM that could identify particular elements, zn^{2+} which we expected to be found was not there (the globule). This could be because this analysis can be conducted on only the outer part of the globule.

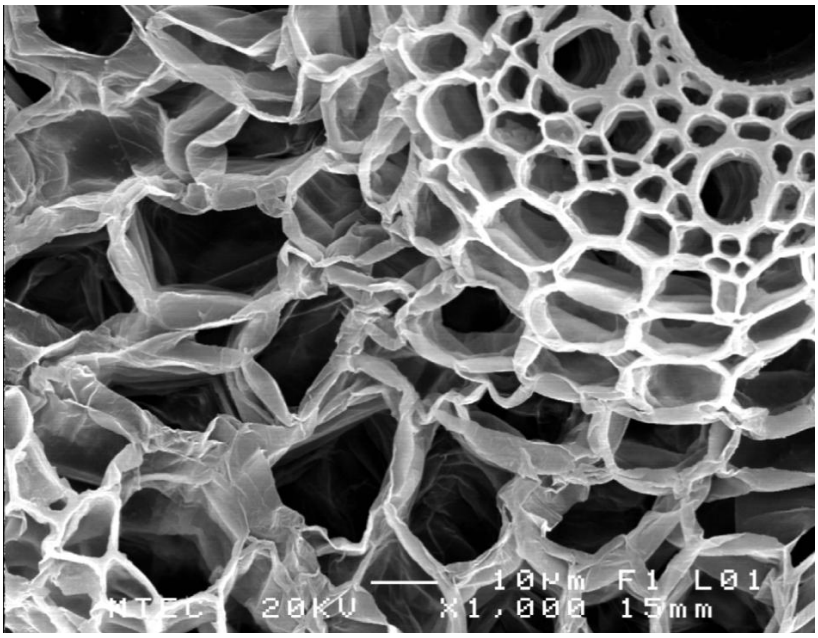

Figure 4.39 SEM micrograph of rice root in control treatment.

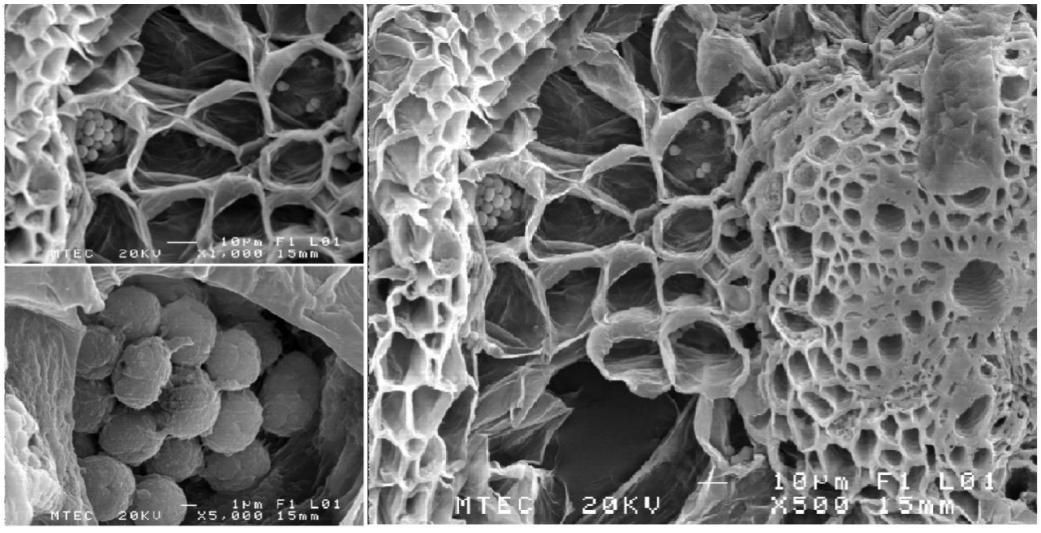

Figure 4.40 SEM micrograph of rice root in 1000 mg/L nano-ZnO treatment.

This study shows new results on the globules which rice root cells produced in the condition of excess of nano-ZnO from outside and the obvious fact that nano-ZnO is one of the factors that caused a reducing effect on rice root. Although, the conclusion of whether those globules contained nano-ZnO, other elements was not able to be determined, the previous study on other

plant species showed the possibility that nano-ZnO can pass though cell walls (Lin and Xing, 2008).

However, there were many hypotheses from literature review which described the possible reasons of the toxicity of nano-ZnO, which we can classify into two main groups here.

1). Nano-ZnO entered into the cell: Lin and Xing, (2008) mentioned this hypothesis in their studies, which found the dark spot (via transmission electron microscope, TEM) inside the endodermis, vascular and stele. The transportation mechanism also described that nano-ZnO might pass through the cell wall, accumulate in the cell or transport into other parts of root tissues from the epidermis to the cortex layer and stele, disturbing cell function resulting in cell death because of their chemical toxicity of materials.

2). Hydrogen peroxide (H_2O_2) generated from nano-ZnO: it was reported that nano-ZnO can generate hydrogen peroxide (H_2O_2), a strong oxidizing agent, (Osamu, 2001; Sawai, 2003; Ghule et al., 2006; Liu et al. 2009) which could be one of the main causes of toxicity to rice root. Exogenous H_2O_2 was reported to be able to inhibit root growth of rice seedlings (Lin and Kao, 2001). H_2O_2 is a necessary substance for a cell wall stiffening process which is considered to be one of the mechanisms resulting in inhibition of growth (Fry, 1986; Schopfer, 1996). H_2O_2 was also demonstrated to be able to inhibit auxin-mediated growth of maize coleoptiles (Schopfer, 1996). Therefore, H_2O_2 which is generated from nano-ZnO could be the main reason of the root inhibition effect, by inducing a stiffening process and/or inhibition of root growth mediated hormone.

Overall, this study demonstrated possible effects of these two metal oxide nanoparticles on rice, an important food crop for many countries, pointing out the need for responsible ecological disposal of wastes containing metal oxide nanoparticles. Therefore, the challenge for further studies is the uptake kinetics and interaction mechanisms within the cells. Surface characteristics could also be one of the main factors causing nanotoxicity, with a large surface area and surface energy; it could affect bioavailability, reactivity and catalytic properties of nanoparticles (Yang and Watts, 2005; Guzman, 2006). Moreover, bioavailability and toxicity of nanoparticles have not yet been described in many groups of organisms especially in food crops (USEPA, 2007; Navarro et al., 2008).

CHAPTER V

CONCLUSION

1. Nano-TiO_2 had no significant effect on rice seed germination, root length and relative root growth.
2. Nano-ZnO had no significant effect on rice seed germination but it had adverse effect on root length depended on concentration and seed soaking time.
3. Relative root growth values showed that nano-ZnO was highly toxic to root elongation. While nano-TiO_2 did not show reduction effect.
4. Nano-ZnO affected the production of globules within cortical cell.

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APPENDIX

APPENDIX

REAGENT RECIPES

1. Reagent for Fixation solution (FAA

FAA (Formalin-Acetic-Alcohol) (100 ml)

Ethyl alcohol	50 ml
Glacial acetic acid	5 ml
Formaldehyde (37 – 40 %)	10 ml
Distilled water	35 ml

BIOGRAPHY

Ms. Prapatsorn Boonyanitipong was born on July 21, 1986 in Ubon Ratchathani. After she finished school in 2004 from Phrapathom Witthayalai School in Nakhon Pathom, she has gotten the scholarship from Development and Promotion of Science and Technology Talents Project, and then she was enrolled in the Department of Biology, Faculty of Science at Silpakorn University and graduated with the degree of Bachelor of Science in 2008. She also has gotten the same scholarship to study for the degree of Master in Botany, Faculty of Science, Chulalongkorn University since 2008.