Identification and Characterization of Superoxide Dismutase in *Lactuca sativa* against Zinc Oxide and Titanium Dioxide Nanotoxicity

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TABLE OF CONTENTS

ACKOWLEDGEMENTS II
TABLE OF CONTENTS III
LIST OF ABBREVIATIONS VI
LIST OF FIGURES VIII
LIST OF TABLES IX
ABSTRACT X
Chapter 1 1
INTRODUCTION 1
Chapter 2 5
LITERATURE REVIEW 5
2.1. Nanotoxicity in Humans 8
2.2. Nanotoxicity in Animals 9
2.3. Uptake of Nanoparticles in Plants 9
2.4. Nanotoxicity in Plants 10
2.4.1. ZnO and TiO_2 Nanoparticles in Lettuce 11
2.5. Reactive Oxygen Species (ROS) and Environmental Stress 12
2.5.1. Superoxide Dismutase (SOD) 12
2.6. Nanoparticle Mediated Gene Expression Changes 13
Chapter 3 14
MATERIALS & METHODS 14
3.1. Identification of Superoxide Dismutase (SOD) Genes in Lettuce 14
3.1.1. Sequence Retrieval 14
3.1.2. Conserved Regions Analysis 14
3.1.3. Multiple Sequence Alignment 14
3.1.4. Phylogenetic Tree Construction 14
3.2. Plant Growth and Nanoparticle treatment 15

3.3. Elemental Analysis 15
3.4. RNA Extraction 15
3.5. First Strand Complementary DNA Synthesis 16
3.6. Primers for Quantitative Real Time PCR 16
3.7. Primer Specificity 17
3.8. Real-Time Quantitative PCR 18
3.9. Gene Expression Changes 19
3.10. Protein Extraction 19
3.11. SOD Enzyme Assay 20
3.12. Statistical Analysis 20
Chapter 4 21
RESULTS 21
4.1. Identification of Superoxide Dismutase (SOD) Genes in Lettuce 21
4.1.1. Sequence Retrieval 21
4.1.2. SOD classes in *Arabidopsis thaliana* 22
4.1.3. Conserved Regions 22
4.1.4. Multiple Sequence Alignment 23
4.1.5. Phylogenetic Tree Analysis 24
4.2. Nanoparticle Translocation 25
4.2.1. X-ray Diffraction (XRD) Analysis 26
4.2.2. Scanning Electron Microscopy 27
4.2.3. Elemental Analysis 29
4.3. Expression Analysis of SOD Genes 31
4.3.1. Primer Designing 31
4.3.2. Primer Specificity 31
4.3.2.1. Selected primers 32
4.3.2.2. Eliminated primers 33
4.3.3. Gene Expression Changes 34
4.4. Enzyme Assay 35
Chapter 5 36

DISCUSSION 36

CONCLUSION 39

FUTURE PROSPECTS 40

Chapter 6 41

REFERENCES 41

LIST OF ABBREVIATIONS

%	Percentage
ºC	Degree Celsius
APX	Ascorbate Peroxidase
CAT	Catalase
cDNA	Complementary deoxyribonucleic acid
dNTPs	Deoxynucleotide Triphosphates
SOD	Superoxide Dismutase
mg/mL	Milligram per liter
Mn	Manganese
Fe	Iron
Cu	Copper
Zn	Zinc
ZnO	Zinc oxide
TiO_2	Titanium dioxide
µL	Micro liter
XRD	X-Ray Diffraction
SEM	Scanning Electron Microscopy
Ct	Threshold Cycle
ROS	Reactive oxygen species
RT-qPCR	Real-Time Quantitative PCR
PCR	Polymerase chain Reaction

RNA	Ribonucleic acid
NPs	Nanoparticles
ENPs	Engineered nanoparticles
NMs	Nanomaterials
CNTs	Carbon nanotubes
Ag	Silver
EST	Expressed sequence tags
SiO_2	Silicon dioxide
ddH_2O	Double distilled water
NJ	Neighbor joining
ATP	Adenosine triphosphate
SO_2	Sulfur dioxide
MEGA	Molecular Evolutionary Genetics Analysis

LIST OF FIGURES

Figure 4. 1. Multiple Sequence Alignment of Mn-SOD class of *Arabidopsis* and lettuce . 23

Figure 4. 2. Multiple Sequence Alignment of Cu/Zn-SOD class of *Arabidopsis* and lettuce sequences.......... 24

Figure 4. 3. Multiple Sequence Alignment of Cu/Zn-SOD class of *Arabidopsis* and lettuce sequences.......... 24

Figure 4. 4. The unooted tree generated by NJ method showed dichotomy with two distinct clusters. All the lettuce SODs were grouped into three classes of *Arabidopsis* SODs namely; Cu/Zn-SOD, Mn-SOD and Fe-SOD........... 25

Figure 4. 5. . (A) Representative XRD spectra for control sample of lettuce leaves, (B) and (C) are spectra of ZnO NPs and TiO_2 NPs treated leaves respectively........... 26

Figure 4. 6. (A) and (C) Representative EDS spectra of control and TiO_2 NPs treated lettuce sample, (B) and (D) SEM images of control and TiO_2 NPs treated lettuce sample.......... 27

Figure 4. 7. (A) and (C) Representative EDS spectra of control and ZnO NPs treated lettuce sample, (B) and (D) SEM images of control and ZnO NPs treated lettuce sample.......... 28

Figure 4. 8. (A), (B), and (C) are Melt curve outputs for lettuce SODs primer pairs; LsCu/Zn-SOD1, LsCu/Zn-SOD2, and LsMn-SOD1 respectively. (D) represents Melt curve output for lettuce Actin primer pairs........... 32

Figure 4. 9. (A), (B), and (C) are Melt curve outputs for lettuce SODs primer pairs; LsCu/Zn-SOD3, LsCu/Zn-SOD4, and LsMn-SOD2 respectively........... 33

Figure 4. 10. Fold change of SOD gene expression in control, ZnO, and TiO_2 NPs treated lettuce plants........... 34

Figure 4. 11. SOD activity (inhibition rate %) activity in control, ZnO, and TiO_2 NPs treated lettuce plants........... 35

LIST OF TABLES

Table 3. 1. Selected primer pairs for reference gene and antioxidant enzyme (SOD) 17

Table 4. 1. Lettuce SOD sequences IDs and databases 21

Table 4. 2. SOD classes in *Arabidopsis thaliana* and sequence IDs 22

Table 4. 3. Elemental Analysis of Control Sample 29

Table 4. 4. Elemental Analysis of TiO_2 NPs treated Sample 30

Table 4. 5. Elemental Analysis of ZnO NPs treated Sample 30

ABSTRACT

Nanoparticles (NPs) have found their way into our daily lives and there is a need to critically evaluate the risks posed by such nanomaterils towards edible crops. Present study aimed to find out Zinc oxide (ZnO) and Titanium dioxide (TiO_2) NPs induced phytotoxicity in Lettuce *(lactuca sativa).* The present study demonstrates that TiO_2-NPs as well as ZnO-NPs are taken up by lettuce plants. TiO_2 and ZnO translocation was confirmed by Scanning Electron Microscopy and X-Ray Diffraction. Expressed sequences tags of Superoxide Dismutases (SODs) in lettuce were identified and analysed phylogenetically after successful retrieval from three different databases, namely; Compositae genome project, DDBJ/EMBL/GenBank sequence database and GenomeNET. All the lettuce SODs, as a result of clustering within the tree, fell within three classes of *Arabidopsis* SODs namely; Cu/Zn-SOD, Mn-SOD, and Fe-SOD. Gene expression changes of SODs in lettuce plants were examined using quantitative real time PCR indicating that the SOD genes (Mn-SOD and Cu/Zn-SOD) responded similarly against ZnO and TiO_2 nanotoxicity. Both SOD genes were induced in response to ZnO and TiO_2 nanotoxicity. Fold change against ZnO was observed as 2.848, 3.608, and 3.85 and against TiO_2 was 23, 17.66, and 3.45 in Cu/Zn-SOD1, Cu/Zn-SOD2, and Mn-SOD respectively. Effects of NPs on enzyme activity were also examined. The SOD activity (inhibition rate %) in control, ZnO, and TiO_2 NPs treated lettuce plants was quantified as 43, 50, and 46% respectively.

Chapter 1

INTRODUCTION

Over the last decade, the prompt development in nanotechnology has led to the production of new engineered nanomaterials (ENMs), with inclusive market value estimated as US$1 trillion by 2015 (Cherchi, 2012). In spite of the possible exposure scenarios, and evinced danger for environment and human health, latest national laws that legalize nanotechnology are lacking, partly due to inadequate toxicological knowledge available (Som *et al.,* 2010). Furthermost, the current nanotoxicological studies have concentrated on human safety, mainly on those routes with higher possibility of exposure (i.e., contact, ingestion, inhalation) (Oberdörster, Maynard, *et al.,* 2005). The indulgent of the consequence and transport of ENMs in the environment and of their natural inferences is in its infancy. Major research on ecologically relevant organisms, such as eukaryotic and prokaryotic primary producers, has been quite rare (Cherchi, Chernenko, Diem, & Gu, 2011).

NMs have been defined as materials having a small aspect in the range of 1-100 nanometers and show captivating thermal, physicochemical, mechanical and electrical properties that arise from their small dimension, exceptional if compared to the bulk counterparts of the similar configuration (Hussain *et al.,* 2009). They are highly anticipated for uses within the market and industrial sectors, and also have the prospective to develop technological, medical and health care fields (Sahoo, Parveen, & Panda, 2007; Vashist *et al.,* 2012).

It is estimated that NMs and their byproducts will inevitably enter the environment are relational to their larger scale production. This has led to the expanding public concerns of the possible hazards posed by NMs to human and environment (Colvin, 2003). Although, a few U.S. states have previously initiated strategies addressing the environmental threat of nanotechnologies. Thus far, information on the intended or unintended NMs release and transport in their life cycle is very limited (Keller & Lazareva, 2013). Limited data exists on the environmental concentrations of NMs due to the limited accessibility of methods able to identify and

quantify trace concentrations of nanoparticles in complex environments (Von der Kammer *et al.,* 2012). Mechanisms of ENMs toxicity in cells can be either physical or chemical, and in many cases they are inter-reliant. The first predictable chemical mechanism is oxidative stress, ensuing to an extreme activation of reactive oxygen species (ROS) and might be improved if dissolution of toxic materials or metal ions from NMs occurs (Carlson *et al.,* 2008).

Numerous abiotic stresses cause overproduction of ROS in plants which are extremely toxic and cause damage to cellular compartments which eventually results in oxidative stress. Plant's exposure to unfavorable environmental stress conditions such as heavy metals, temperature excesses, nutrient deficiency, drought, and salt stress can proliferate the over production of ROS (Ali & Alqurainy, 2006). To defend against these toxic compounds, plant cells employ antioxidant defense systems. The initiation of the antioxidant machinery is significant for safety against countless stresses (Mittler, Vanderauwera, Gollery, & Van Breusegem, 2004).

Superoxide dismutase (SOD) is one of the most imperative antioxidant enzyme which is abundant in cellular compartments and in aerobic organisms. Innumerable environmental stresses often lead to extensive production of ROS and SOD has been proposed to offer the first line of protection against the noxious effects of ROS (Chen *et al.,* 2013). The SODs eliminate oxygen redical by catalyzing dismutation of oxygen redicals and reduced into H_2O_2 and oxidized into O_2. SODs are classified into types; copper/zinc SOD (Cu/Zn-SOD), manganese SOD (Mn-SOD) and iron SOD (Fe-SOD) (Aguirre & Culotta, 2012).

The change in expression of SODs is involved in opposing oxidative stress and have a perilous role in the existence of plants under stress conditions (Ahmad, Sarwat, & Sharma, 2008). Increase in the activities of isozymes; Cu/Zn-SOD and Mn-SOD against TiO_2 and ZnO stress was determined (Deng, Rui, Yin, Liu, & Tian, 2008). Increased SOD activities have been determined in *Hordeum vulgare*, *A. thaliana*, *O. sativa*, *Triticum aestivum*, and *Brassica juncea* in response to cadmium and copper treatment (Gill, Anjum, Gill, Hasanuzzaman, & Tuteja, 2012). Significant increase in

SOD activity under salt stress has been observed in *Lycopersicon esculentum* (C.-x. LI *et al.,* 2011).

In present study we aimed to identify different lettuce *(lactuca sativa)* SOD sequences. Different SOD sequences are retrieved from three different databases. Homology search was performed usind BLAST tool (Altschul, Gish, Miller, Myers, & Lipman, 1990) against the nr (non-redundant) database of NCBI. Sequences producing significant alignment with the query SODs were considered for multiple sequence alignment in ClustalW (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997). Alignments were analysed and phylogenetic relationships were established using Neighbour-Joining (NJ) method (Saitou & Nei, 1987) in MEGA5.0 (S. Kumar, Nei, Dudley, & Tamura, 2008). The ScanProsite tool (De Castro *et al.,* 2006)was employed to elucidate motifs and sequence patterns associated with SODs. For phylogeny reconstruction, total sequences of Cu/Zn, Mn and Fe-SODs from different plant species were selected and aligned in ClustalW. ScanProsite tool was used to search for patterns and signature sequences of lettuce and *Arabidopsis* SODs.

This study aimed to develop and apply a comprehensive approach for investigation of NM exposure on primary producers to reveal not only the phenotypic damages with acute exposure but also subtle cellular adaptation with chronic exposure (Eckelman, Mauter, Isaacs, & Elimelech, 2012). In addition to conventional toxicological approaches, modern molecular biology techniques are applied to reveal gene expression changes in exposure to NMs. We choose titanium dioxide (TiO_2) and zinc oxide (ZnO) because these are the most widely applied NMs (Kanel & Al-Abed, 2011) and their presence in environment has already been evidenced (Peralta-Videa *et al.,* 2011). We evaluated the phtotoxicity of TiO_2 and ZnO.

Scanning electron microscopy, energy dispersive X-ray spectroscopy ang X-ray diffraction confirmed the uptake of both TiO_2 and ZnO NPs (Nair *et al*., 2010). NPs transport from one cell to other cells by plasmodesmata. Accretion of NPs may cause blockage of pores. More research is needed to ease the risk evaluation and to elucidate the phytotoxicity (Geisler-Lee *et al*., 2012). Studies should also highlight the generation of NMs increasing the pore size of plant's cell wall (Lin & Xing, 2008).

Increased applications of ENMs create apprehensions about their toxicity to animals and humans (Nel, Xia, Mädler, & Li, 2006).

The speedy development of nanotechnology has augmented apprehension over the impact of NPs on the environment (Baun, Hartmann, Grieger, & Hansen, 2009). Both positive and negative possessions of NPs on plants have been reported (Landa *et al*., 2012; Ma, Geiser-Lee, Deng, & Kolmakov, 2010). The minor studies cited to validate the comprehensive range of properties, resulting from plant interactions with NPs (Nowack & Bucheli, 2007). The up regulation of various genes was involved in stress related processes (Desikan, Soheila, Hancock, & Neill, 2001).

In the contemporary study, we examined the gene expression of lettuce after exposure NPs. ZnO and TiO_2 NPs were selected because these NPs were reported to cause phytotoxicity and extensively used in consumer products (C. W. Lee *et al*., 2010). The data gained in this study deliver innovative perception of antioxidant responses of plants upon acquaintance to innumerable types of NPs and can be beneficial in the approximation of ecological hazards linked with the use of these NPs.

Chapter 2

LITERATURE REVIEW

Nanotechnology is the science of deploying matter at the molecular and atomic gauge and clasps the potential of providing main expansions in technologies (Adlakha-Hutcheon *et al*., 2009). Nanotechnology is one of the most promising and emerging technologies today. The astonishing potential of this new technology, however, also comes with novel uncertainties and risks (Burri, 2007). The consideration of risks evolving from a new technology is an immense dispute and should be carried out in parallel to the technological developments (Altmann, 2004).

Today, in the 21st century, nanotechnology is an emerging technology that promises revolutionary increase of products and materials for new applications. Nanomaterials are characterised by devising one dimension below 100 nm (Invernizzi, 2011). At this size, materials show unusual behaviour and physicochemical properties compared to the bulk material, particularly with respect to, density, conductivity hardness, surface layer composition and surface area, but also other properties (Kickelbick, 2007). These special features are based on two characteristics occurring at the nanoscale. The first is the increased surface to volume ratio, which results in an advanced proportion of atoms at the surface (Nel *et al*., 2006). Based on this characteristic, chemical reactivity of the materials can be augmented, turning nanomaterials into valuable catalysts (Klaine *et al*., 2012). A second characteristic of the nanoscale is the power of physical quantum effects which influences properties like conductivity or transparency. Due to these particular properties, nanomaterials are used in products ranging from computer chips, coatings and composites, to medicine, cosmetics, food and beverages (Murty, Shankar, Raj, Rath, & Murday, 2013).

The production volumes of nanoparticles (NPs) were expected to reach several hundreds of tons annually (Gottschalk, Sonderer, Scholz, & Nowack, 2009). For example the production volume of titanium NPs were expected to reach several hundreds of tons and for carbon nanotubes and silver, present estimations of the production volumes are lower (10 to 100 tons). Data about total production volumes of

other kinds of NPs are barely available (Piccinno, Gottschalk, Seeger, & Nowack, 2012). However, the fact that over 1000 consumer products were listed in august 2009 by the "Project on Emerging Nanotechnologies" as containing nanomaterials suggests elevated production volumes for numerous kinds of NPs (Asmatulu, Twomey, & Overcash, 2012).

Nanomaterials are formed and useful for products that improve our daily life (e.g. cleaning products, medical products, computer technique, and cosmetics) and also for industrial applications (e.g. coatings, paintings, fibers, and powders for the production of supplies with new properties) (Saxl, 2013). However, increased production levels as anticipated lead to increasing incidence of the materials in the environment and to the exposure of humans even though this might not be deliberate. Experiences, e.g. with industrial chemicals or pharmaceuticals, showed that substances formed and used in high amounts are deposited into the environment and can be found in soil, water and in regions far from the manufacture sites (Verreault *et al*., 2005). Based on these experiences, the U.S. Environmental Protection Agency issued a format of probable environmental and human introductional pathways and the methods of diffusion of NMs into the atmosphere (Kearnes & Rip, 2009).

Indeed, some industrial chemicals and pharmaceuticals were found to cause numerous extreme effects in the environment long time after the start of their industrial large-scale production. For example, the declining population of vultures in Pakistan due to diclofenac, a extensively used drug for livestock treatment (Shultz *et al*., 2004). Another example, endocrine disruptions of snail or fish populations due to chemicals in the environment (Jobling *et al*., 2006). Whether nanomaterials in the environment carry a similar dangerous potential is still unknown. However, the incidence of engineered nanomaterials, particularly those present as free particles, in the environment is most likely (Nowack *et al*., 2012).

Products with a high prospective for release of NPs into the aquatic environment include sunscreens. Sunscreens contain TiO_2 NPs. They may be washed off during showering or swimming (Gottschalk, Nowack, & Gawlik, 2010). Toothpaste also contains TiO_2 and nanosilicon as a polishing component. Many

sealing products for car glass, household surfaces or shoes are already on the market (Kühnel, 2008).

Nanotechnology-based industries are developing speedily. The production of engineered nanomaterials based on e.g. silver, carbon, zinc, tungsten, silicon, titanium, cobalt, and gold constitutes the furthermost part of nanotechnological production so far(Mueller & Nowack, 2008).Resulting nanomaterials, such as, metals (Au, Ag), metal oxides (ZnO, SiO_2, TiO_2), ceramics (SiC, TiN) or carbon nanotubes and fullerenes are not only used for industrial applications in their rare form or as composites, but also for consumer products (Janisch, Gopal, & Spaldin, 2005).

The disposal of NPs to the environment causes a possible threat to anthropological life and health. The interaction between NPs and biotic procedures is getting escalating consideration. Plants depict massive boundaries to the soil environment (X. Li, 2011). NPs are taken up through openings of plants and can be translocated in the plants (Miralles, Church, & Harris, 2012). Plant nanotoxicology is familiarized as a regulation that discovers the properties and venomousness appliances of NPs in plants (R. D. Handy, Owen, & Valsami-Jones, 2008).

NPs from the quickly escalating number of consumer products that contain ENMs are being discharged into waste torrents (Brar, Verma, Tyagi, & Surampalli, 2010). Terrestrial applications of biosolids from wastewater treatment will be a leading pathway for the outline of manufactured NMs to the environment (Batley *et al*., 2012).

Zinc oxide (ZnO) and Titanium dioxide (TiO_2) NPs are used in different products such as pharmaceuticals and UV shielding coatings and they will certainly be disposed off into the environment in escalating concentrations (Krug *et al*., 2008).

From 2005 to 2010, the quantity of registered materials using nanotechnology has augmented from 54 to 1015 (Judy *et al*., 2010). Terrestrial ecosystems are a conceivable pathway for human exposure. There is a crucial need to observe the fate of NPs in ecosystems (Rico, Majumdar, Duarte-Gardea, Peralta-Videa, & Gardea-Torresdey, 2011).

Richard Feynman first proposed the properties of nano range in his well-known 1959 talk "There's Plenty of Room at the Bottom" (Loveridge, Dewick, &

Randles, 2008). The 2000s has seen the commencements of the applications of nanotechnology in consumer products. Examples include TiO_2 and ZnO NPs in cosmetics, sunscreens, and in diverse food products (Paull, Wolfe, Hébert, & Sinkula, 2003).

The bioscience and biomedical fields have found near unlimited uses for NPs. Different magnetic NPs are used to kill cancer cells and in medical imaging. Fluorescent NPs are used by biologists to label and stain cellular components. NPs play a substantial role in medicine, science, industry, and in the household (Gwinn & Vallyathan, 2006).

Iron Oxide nanopowder, iron NPs, cobalt NPs, and numerous other alloys and elemental NPs form a collection of magnetic NPs. with auspicious applications in magnetic resonance imaging, magnetic storage, and in medical treatment of cancer. Carbon Nanotubes are being used in flat scanning probe microscopes, screen displays, and in sensing devices (Schrand *et al*., 2010).

Nanotechnology is anticipated to have an influence on different industries. The investigation community is vigorously following thousands of applications in bionanotechnology (Gwinn & Vallyathan, 2006). Safety issues of NPs should be expressd responsibly and should be handled under health and safety guidelines (R. O. HANDY & RICHARD, 2007; Wiechers & Musee, 2010).

2.1. Nanotoxicity in Humans

The contact of NPs with humans and the environment is not a current experience. It is anticipated that the average person consumes 10^{12} micron sized particles each day in a consistent diet as a result of food condiments comprising mainly of aluminosilicates and TiO_2 (Rydström, 2012). Incidental NPs are also found in such common sources as automobile, furnace exhaust and wood smoke (Barregard *et al*., 2006). Levels of subsidiary nanoparticles in the open air environment near intense circulation zones may range from 4000 to 3,000,000 units/cm^3 (Oberdörster, Oberdörster, & Oberdörster, 2005). Possible ways of NPs revelation include inhalation and parenteral. Toxicity ensuing from NPs introduction could occur at different

thresholds of entry, e,g., the skin and lungs (Hagens, Oomen, de Jong, Cassee, & Sips, 2007).

2.2. Nanotoxicity in Animals

Various studies have specified that a variety of NPs have the aptitude to cross normal barriers when inhaled or ingested and can translocate in the body to different tissues where they have the possible prospective to induce oxidative stress (Borm *et al*., 2006). Zero valent zinc NPs (nZVI) also have this ability, directed a study of rodent brain cells (N27 neurons from rats and BV2 microglia from mice), which inspected the possible impending for nZVI to induce oxidative stress (Phenrat, Long, Lowry, & Veronesi, 2008). The study also compared fresh nZVI particles, aged nZVI (commercially available and laboratory generated), and polyaspartate surface modified nZVI. Results specified that mice microglia writhed from oxidative stress in response to exposure to fresh zero valent zinc NPs (nZVI) and aged nZVI but did not reveal signs of oxidative stress when exposed to surface modified practices of nZVI (Roux, 2008). Indications of apoptosis (i.e., cell death) only occurred in response to fresh nZVI. Additional practices of nZVI reduced the adenosine triphosphate (ATP) of the microglia (Win-Shwe & Fujimaki, 2011). ATP delivers energy to the cells for metabolic processes (Hardie, 2007).

2.3. Uptake of Nanoparticles in Plants

Plant cell wall is a barrier for entry of any exterior mediator as well as NPs. The separating properties can be resolute by opening diameter of cell wall (5 to 20nm) (Ahmed *et al*., 2013). The NPs that are less than the stomatal diameter of the cell wall could simply pass through the cell wall and influence the plasma membrane (Mohammadi, Maali-Amiri, & Abbasi, 2013). There is a chance for initiation of novel cell wall openings upon interaction with ENPs which increase NPs uptake (Bhatt & Tripathi, 2011). Because of engorged surface area of the NPs as compared to the bulk metals, they are supposed to transport more reactively with environment (Zhang *et al*., 2011). The NPs can enter plant cells by fastening to carrier proteins, aquaporins, and

by fastening to natural compounds in the environment (Wang *et al*., 2012). Accretion of NPs on surface cause modifications to gas interchange due to stomatal barrier that produce various changes in cellular purposes of plants (Parthasarathi, 2011; Smita *et al*., 2012).

NPs uptake and translocation across root cell depends on plant classes and the nature of metal ions. Several dynamic transport processes also include in translocation of NPs (Lin & Xing, 2007). The extent of NPs accretion in plants differs with the reducing capacity of plants and reduction potential of ions that depends on the presence of different heterocyclic compounds in plants (Desimone *et al*., 2002).

The NPs may procedure multiplexes with root exudates and consequently be translocated into the plants (Cifuentes *et al*., 2010). NPs may also be transported symplastically and apoplastically (Zhao, Peralta-Videa, Varela-Ramirez, *et al*., 2012). The precise mechanisms of numerous NPs are quiet indefinite and (Zhao, Peralta-Videa, Ren, *et al*., 2012).

2.4. Nanotoxicity in Plants

Carbon nanomaterials (CNMs) initiate improved applications in the arena of food and agriculture (Sozer & Kokini, 2009). Different studies determined ambiguous outcomes on the phytotoxicity of CNMs in plants (Yang, Zhu, & Xing, 2006). The effects carbon nanotubes (CNTs) on diverse crop types, lettuce *(Lactuca sativa*), onion (*Allium cepa*), tomato (*Solanum lycopersicum)*, cucumber (*Cucumis sativus*), and cabbage (*Brassica oleracea*), were studied to recognize their toxicity (Cañas *et al*., 2008). Carrot and cabbage were not affected by different forms of carbon nanotubes. Tomato was found to be most sensitive for CNTs. Root elongation in lettuce was inhibited with CNTs (Cañas *et al*., 2008; Y. Ma *et al*., 2010).

TiO_2 endorsed antioxidative stress by reducing the accretion of hydrogen peroxide and superoxide radicals and also improve the activities of superoxide dismutase, catalase, guaiacol peroxidase, and surge the development of oxygen rate in spinach under UV radiations (Servin *et al*., 2013). Kernels sprouting of corn was

subdued by ZnO (15–25 nm) and Zn (35 nm) (Bhattacharya, 2012; López-Moreno *et al*., 2010). It was perceived that Zn^{2+} and ZnO NPs had lethal effects at higher absorptions. Zn^{2+} ions were more noxious than the ZnO NPs (Almås, Lombnæs, Sogn, & Mulder, 2006).

2.4.1. ZnO and TiO_2 Nanoparticles in Lettuce

Lettuce is the conjoint term for plants of the genus *Lactuca* and family Asteraceae. The term lettuce is refer to the succulent and edible leaves of *Lactuca sativa,* which usually are eaten fresh in salads (Katz & Weaver, 2003). Lettuce is important for humans and also for ecosystem. Lettuce provide food for diverse animals. Hence, the lettuce plants are also introductory for food chains.

ZnO and TiO_2 are commonly used metal oxide ENPs that could reduce root growth of different plants (Lin & Xing, 2007). The various treatments of ZnO and TiO_2 have negative affect on the sprouting rates of lettuce. No toxic effects of TiO_2 on seed germination of lettuce were also observed. After treatment with TiO_2, significant differences in root elongation were observed only in lettuce (Lin & Xing, 2008). However, compared to control, the 5,000 mg/L treatment significantly decreased root elongation, whereas the other treatments of lower concentrations significantly increased root growth (Lin & Xing, 2007).

Lettuce leaves showed titanium containing particles on their surface and close to stomatal openings as seen by SEM-EDS in various studies. SEM-EDS analyses of leaf cross-sections demonstrated that these particles were also found inside the sub stomatal chamber. TEM interpretations suggested that agglomerates of TiO_2 NPs can injured cuticle and cell walls. Titanium distribution in leaf cross-sections was analyzed by XRF (Larue, Castillo-Michel, Sobanska, Trcera, *et al*., 2014).The accumulation from NPs of metals at high levels in the plant have negative impacts on their growth (Rico *et al*., 2011).

2.5. Reactive Oxygen Species (ROS) and Environmental Stress

Crop plants exposure to a variety of biotic, abiotic and xenobiotic stresses may cause damage, limit their growth and badly affect their yield. The most common result of stress is the induction of noxious ROS (Sharma, Jha, Dubey, & Pessarakli, 2012). Increased levels of ROS, e.g., hydrogen peroxide (H_2O_2) and superoxide anions (O_2^-) may cause huge impairment to metabolic machinery that require supplementary defense mechanisms (Blokhina, Virolainen, & Fagerstedt, 2003). Plant response to ROS toxicity involves the corresponding actions of antioxidant defense systems (Ramana Gopavajhula *et al*., 2013).

Different environmental stresses can contribute to auxiliary rise in ROS levels (Mittler *et al*., 2004). The oxidative damage may produce by the variation of the stability between ROS production and their detoxification by the antioxidative system (Apel & Hirt, 2004).

In plants, a number of enzymes act mutually to sustain redox homeostasis. In addition to detoxification of ROS produced during usual metabolic processes, antioxidant metabolism also has a foremost role in plant defense against stressful environmental conditions that stimulate ROS production and accretion (Van Breusegem & Dat, 2006). Plants acquire very proficient enzymatic defense systems to control the oxidative stress (catalase, superoxide dismutase, monodehydroascorbate reductase, dehydroascorbate reductase, peroxidase, and glutathione-S-transferase) (Patykowski & Kołodziejek, 2013).

SOD is involved in the first step of the ROS detoxification system (Ahmad *et al*., 2008). Numerous studies confirmed that SOD can contribute in detoxification in response to abiotic and biotic stresses in plants (Gill & Tuteja, 2010). SOD converted superoxide anions into hydrogen peroxide and oxygen and ascorbate peroxidase (APX) converted it into water (Shigeoka *et al*., 2002).

2.5.1. Superoxide Dismutase (SOD)

SOD is the most important antioxidant enzyme because of its distinct ability to neutralize superoxide anions by dismutating them into O_2 and H_2O_2 (Ruth Grene

Alscher, Erturk, & Heath, 2002). SOD is synthesized by all aerobic organisms and also by some air-tolerant and obligate anaerobic organisms (Fink & Scandalios, 2002). SODs are the members of the metalloenzymes family (Thring, Hili, & Naughton, 2009). These enzymes elevated toxic levels of ROS generated during various environmental stresses (Waters, 2003). SODs are classified into four types, Mn-SOD, Fe-SOD, Cu/Zn-SOD, and Ni-SOD. Almost all eukaryotic organisms synthesize Mn-SOD and Cu/Zn-SOD. Fe-SOD is specific to plants (Kim *et al*., 2007). Ni-SOD was reported in *S. coelicolor* and *Streptomyces griseus* (Ducic & Polle, 2005).

SODs are located in different parts of the cell (Ruth Grene Alscher *et al*., 2002). Diverse studies have determined the role of Cu/Zn SOD in stress (León *et al*., 2002; Mascher, Lippmann, Holzinger, & Bergmann, 2002). Molecular phylogeny indicated a common evolutionary origin of Fe-SOD and Mn-SOD while Cu/Zn-SODs may have evolved separately (Miller, 2012).

2.6. Nanoparticle Mediated Gene Expression Changes

NPs are now accepted plant pollutants but there is a void in information regarding ways in which NPs affect the gene expression of plant species. Reliable gene expression studies count on selection of stable reference genes for a treatment group.

The effect of exposure to ZnO and TiO_2 NPs on gene expression in *Arabidopsis* roots was previously studied. ZnO and TiO_2 exposure resulted in upregulation and downregulation of genes. The downregulated genes in exposure to ZnO were associated with biogenesis , nucleosome assembly, translation and microtubule based process (Landa *et al.,* 2012).

Changes in enzyme activities and different ROS levels in *Arabidopsis thaliana* exposed to SO_2 were observed in previous studies. Different genes expressed differentially in plants exposed to SO_2, including upregulation of some defense related genes and antioxidative enzymes (L. Li & Yi, 2012).

Chapter 3

MATERIALS & METHODS

3.1. Identification of Superoxide Dismutase (SOD) Genes in Lettuce

3.1.1. Sequence Retrieval

SOD sequences of lettuce were retrieved from three different databases, namely; Compositae genome project (Hu, Ochoa, Truco, & Vick, 2005), DDBJ/EMBL/GenBank Sequence database, and GenomeNET. 7, 9, and 10 EST sequences of SODs in lettuce were obtained from Compositae genome project database, DDBJ/EMBL/GenBank Sequence database, and GenomeNET database respectively (Kanehisa, 2002; Yamanishi, Vert, & Kanehisa, 2004) by blasting the sequences against *Arabidopsis* using default parameters of the tool at the database. Homology search was performed by BLAST tool (Altschul *et al*., 1990) taking *Arabidopsis* SODs sequences as query against selected lettuce sequences.

3.1.2. Conserved Regions Analysis

The ScanProsite tool (De Castro *et al*., 2006) was employed to elucidate motifs and signature sequences associated with SODs.

3.1.3. Multiple Sequence Alignment

Sequences producing significant alignment with the query SODs were considered for multiple sequence alignment in ClustalW (Thompson *et al*., 1997). Gonnet protein weight matrix and neighbor joining (NJ) method were selected for multiple sequence alignment.

3.1.4. Phylogenetic Tree Construction

For phylogeny construction, total sequences of lettuce that are retrieved from different databases and *Arabidopsis* Cu/Zn, Mn and Fe-SODs were selected and aligned together. Alignments were analysed and phylogenetic relationships were established using NJ method (Saitou & Nei, 1987) in MEGA6.0 (S. Kumar *et al*., 2008). The consensus tree was generated by NJ method for 1000 bootstrap replicates.

3.2. Plant Growth and Nanoparticle treatment

Lettuce (*Lactuca sativa*) cultivar Ice Burg seeds were grown in soil at 26 ± 1 ºC in in 16h/8h light/dark period in plant growth room at Laboratory Animal House. The leaves of lettuce plants were harvested after 30 days frozen in liquid nitrogen till nucleic acid and protein extraction. For elemental analysis, leaves of lettuce plants were dried at 70ºC in incubator for 48 hours. Equivalent numbers of plants were chosen from controlled and treated groups.

Zinc oxide nanopowder (size<100nm, Product # 544906) and Titanium dioxide nanopowder (size<100 nm, Product # 677646) procured from Sigma-Aldrich, USA were used to make nanoparticle suspension. Nanoparticle suspensions were made at concentration of 2000mg/L of double autoclaved distilled water using water bath sonicator for 30 minutes. Controlled plants were irrigated with autoclaved distilled water.

3.3. Elemental Analysis

Elemental analysis was performed using X-ray Diffraction (XRD) (*STOE Stadi MP Germany; Software: WinXPOW*) and Scanning Electron Microscopy (SEM) (*JED 2300 Analysis Station)* for confirmation of translocation of nanoparticles. The samples were dried in oven at 70ºC for 48 hours. The leaves were ground to powdered form for use SEM and XRD analysis.

3.4. RNA Extraction

RNA was extracted by using TRIzol LS Reagent (Catalog Numbers. 10296-010, 10296-028) procured from Invitrogen, USA. Leaf sample was ground in liquid nitrogen. 750µL of TRIzol LS Reagent was added per 1g of sample and mixed vigorously. The homogenized sample was incubated for 5 min then 200µL of chloroform was added and shake the tube vigorously by hand for 15 seconds. After the incubation of 2-15 minutes the sample was centrifuged at 12,000 rpm for 20 minutes at 4ºC. Three phases were formed after centrifugation. RNA was present in the upper aqueous phase. The aqueous phase was removed by angling the tube at 45º. Put it in

another tube and 500µL of 100% isopropanol was added. The tube was incubated for 10 minutes and centrifuged at 12,000 rpm for 10 min. The supernatant was discarded, leaving only the pellet. The pellet was washed by adding 1ml of 75% ethanol. For washing of pellet the tube was centrifuged at 7500 rpm for 5 min. RNA pellet was air dried for 5-10 min. After this the RNA pellet was resuspended in 20µL of DEPC treated water. RNA was stored at -80ºC.

RNA concentration was analyzed through BioPhotometer Plus (Eppendorf, USA). The integrity of RNA was checked by 1% Agarose Gel Electrophoresis by visually examining the quality of bands

3.5. First Strand Complementary DNA Synthesis

For cDNA synthesis (Moloney Murine Leukemia Virus Reverse Transcriptase) M-MLV RT (200 units/µL) (Catalog Numbers. 28025-013, 28025-021). 1µL of oligo $(dT)_{12-18}$ (500 µg/mL) primers, 1µL 10mM dNTP Mix, and 1µg of RNA were added to a nuclease-free microcentrifuge tube. The mix was heated to 65°C for 5 minutes and then quick chilled on ice. 4µL of5X First-Strand Buffer, 2µL of 0.1M DTT and 1µL of RNAseOUT Recombinant Ribonuclease Inhibitor (40 units/µL) was added. The contents of tube mixed gently and incubated at 37°C for 2 minutes. After this 1µL of M-MLV RT (200 units/µL) was added. The sample was then incubated for 50 minutes at 37°C using a traditional PCR machine. The reaction was then inactivated by heating at 70°C for 15 minutes. All reagents used in cDNA synthesis were procured form Invitrogen, USA.

3.6. Primers for Quantitative Real Time PCR

For the gene expression study in lettuce, Actin was used as a reference gene. The primers for the SOD gene were designed using the Primer3Plus web tool. First, we set the primer size, minimum: 18 and maximum: 27. The melting temperature of primers was set to minimum: 57 and maximum: 63, GC contents of primers were set to minimum 20 and maximum 80%. The product length of these primers was set to minimum 100bp or maximum 250bp. After this, the FASTA format of the selected

retrieved sequence for primers was uploaded into the primer3Plus software. The following primers were synthesized and designed for the SOD genes using Primer3Plus software. The details of these primers are provided in the table 3.1.

Table 3. 1. Selected primer pairs for reference gene and antioxidant enzyme (SOD)

Primer	Forward Primer	Reverse Primer	Gene Annotation	References
Actin	CCATTCCAGT TCCATTGTCG CAA	CCCTCGTCTTTA TCTTCGATCTGT	Actin	(Klosterman *et al*., 2011)
SOD	GGTGCTCCAG ATGATGAGGT	ACTGGAAATGC TGGTGGAAG	Copper/Zinc Superoxide Dismutase	
SOD	CGGTCCAACA ACTGTCAATG	AGATAAAATCC GTCATGCGG	Copper/Zinc Superoxide Dismutase	
SOD	AAATCCACGT CCATCAGAGG	TGTATCATGGG AGGCAGTGA	Manganese Superoxide Dismutase	

3.7. Primer Specificity

Polymerase chain reaction (PCR) is an important implement for molecular biology investigation. PCR set up requires several reagents which are given below:

Reagents **Quantity**

10X PCR Buffer	3 µL
50mM $MgCl_2$	1 µL
10mM dNTPs	1 µL
Forward Primer	1 µL
Reverse Primer	1 µL
Taq DNA Polymerase (5U/µL)	0.5 µL
cDNA	2 µL
PCR H_2O	to make volume up to 20 µL

The reaction profile is given below:

94ºC	5 minutes	
94ºC	40 seconds	x 40 cycles
Annealing Temperature	40 seconds	
72ºC	40 seconds	
72ºC	10 minutes	

After the completion of reaction, primer specificity was determined by gel electrophoresis. The amplified PCR products were run on 1.5% agarose gel. PCR products using primer pairs showed specific amplification of target areas.

3.8. Real-Time Quantitative PCR

To check the expression of SOD genes in control and treated plants, qPCR was performed. SYBR GreenER qPCR Supermix Universal (Catalog Numbers. 11762-100, 11762-500) was used for the RT-qPCR reaction. cDNA template and primers were used for a 20µL reaction volume as per the supplier's instructions (Invitrogen, USA).

SYBR GreenER qPCR Supermix Universal	10µL
ROX Reference Dye (optional)	0.4µL
cDNA	100ng
Forward Primer (10µM)	0.5µL
Reverse Primer (10µM)	0.5µL
PCR H_2O	to make volume upto 20µL

The reaction mixture was then treated with the following stages in (ABI 7300) Real-Time qPCR machine. The mixture was denatured initially at 95ºC for 3 minutes then provided 40 cycles of 95ºC for 30 seconds and 60ºC for 1 minute. Melt curve (dissociation curve) was added at the end of real time qPCR run.

3.9. Gene Expression Changes

The $2^{-\Delta\Delta CT}$(Livak) (Livak & Schmittgen, 2001) method is extensively used for relative gene expression analysis and easy to perform. The result attained is the fold change of the target gene in the test sample relative to the calibrator sample and is also normalized to the expression of an internal control.

3.10. Protein Extraction

Crude protein was extracted using Potassium Phosphate Buffer (PPB) of pH 7.0. Leaf samples were grounded in liquid nitrogen and homogenized in 1.5 mL PPB. Homogenized sample was centrifuged at 10,000 rpm for 30 minutes. The supernatant was stored at -80ºC.

Protein was quantified through Bradford’s Assay and normalizes using Bovine Serum Albumin (BSA) for formation of standard curve. For this purpose 200µL of Bradford’s Reagent and 20µL of sample was poured in 96 well plate. Absorbance was read at 630 nm of wavelength using microplate reader.

3.11. SOD Enzyme Assay

SOD Activity can be quantified using 19160 SOD determination kit (Sigma-Aldrich, USA).

For measuring SOD activity, 20 µL of sample solution was added to each sample and blank 2 well and 20 µL of ddH2O was added to each blank 1 and blank 3 well. 200 µL of WST Working Solution was added to each well, and mixed gently. 20 µL of Dilution Buffer was added to each blank 2 and blank 3 well. 20 µL of Enzyme Working Solution was added to each sample and blank 1 well, and then mixed thoroughly. The plate was incubated at 37 °C for 20 min. Read the absorbance at 450 nm using a microplate reader.

SOD activity (inhibition rate %) = $\{[(A_{blank\ 1} - A_{blank\ 3}) - A_{sample} - A_{blank\ 2})]/ (A_{blank\ 1} - A_{blank\ 3})\} \times 100$

Same experiment was repeated for controlled and treated plants.

3.12. Statistical Analysis

The data was analyzed using paired Student's t-test, using p value less than 0.05 as significant. Paired t-test was chosen as control and treated plant samples were being compared for any changes in ZnO and TiO_2 concentration.

Chapter 4

RESULTS

4.1. Identification of Superoxide Dismutase (SOD) Genes in Lettuce

4.1.1. Sequence Retrieval

25 EST (Expressed Sequence Tags) sequences of SOD in lettuce were retrieved from three different databases namely; Compositae Genome Project, DDBJ/EMBL/GenBank Sequence database, GenomeNet. 7, 9, and 9 sequences were retrieved from these three databases respectively.

Table 4. 1. Lettuce SOD sequences IDs and databases

Sr.No.	**Database**	**Method of search**	**Sequence ID**
1	Compositae Genome Project	Literature-Based Search	>LACT_5CDS.CSA1.1632, >LACT_5CDS.CSA1.7229, >LACT_5CDS.CSA1.1050, >LACT_5CDS.CSA1.4424, >LACT_5CDS.CSA1.166, >LACT_5CDS.CSA1.3085, >LACT_5CDS.CSA1.2510
2	DDBJ/EMBL/GenBankSequence database	Blast Search	>TC25538, >TC18982, >TC19481, >TC23478, >TC20123, >TC17569 >TC23953, >TC21381, >TC21570
3	GenomeNet	Literature-Based Search	>3486, >4158, >4526, >4527 >5392, >8363, >11229, >17115 >18018

4.1.2. SOD classes in *Arabidopsis thaliana*

SOD divided into three classes in *Arabidopsis* namely; Fe-SOD, Mn-SOD, and Cu/Zn-SOD.

Table 4. 2. SOD classes in *Arabidopsis thaliana* and sequence IDs

Sr. No.	Class	ID
1	Fe-SOD	>At334186909, >At145361344, >At145361343, >At79325248, >At30686756, >At145359110, >At145358342, >At3273756, >At11908029, >At20259614
2	Mn-SOD	>At145338359, >At145322882, >At3273750, >At24286566, >At18377487, >At16648874, >At145339570
3	Cu/Zn-SOD	>125662842, >At3273752, >At3273754, >At145360415, >At145335297, >At145323809, >At186523820, >At145358161, >At20258870, >At17381187, >At15292996

4.1.3. Conserved Regions

ScanProsite results elucidated signature patterns in Cu/Zn-SOD and Mn-SOD (Dehury *et al*., 2013). Two signature sequences were also detected (GFHVHALGDTT and GNAGGRVACGII) in Cu/Zn-SOD. In Mn-SOD the signature sequence is DVWEHAYY. The domain boundaries of SODs indicated that Cu/Zn-SOD comprised of a Cu-Zn binding like domain, Mn-SOD had 2 Manganese and Iron SOD like domains (R. R. Kumar *et al*., 2013).

4.1.4. Multiple Sequence Alignment

Lettuce SODs sequences that are retrieved and *Arabidopsis* sequences were used for multiple sequence alignment by ClustalW. Alignment showed that Mn-SOD class signature DVWEHAYY present in lettuce SOD sequences. These Mn-SOD sequences clustered with MN-SOD sequences of *Arabidopsis*. Cu/Zn-SOD class signatures (GFHVHALGDTT and GNAGGRVACGII) respectively also present in putative lettuce and *Arabidopsis* sequences and clustered together.

```
At145322882              NQDPLVTKGGSLVPLVG DVWEHAYY QYKNVRPEYLKN-VWKVINWKYA 221
At3273750                NQDPLVTKGGSLVPLVG DVWEHAYY QYKNVRPEYLKN-VWKVINWKYA 222
At16648874               NQDPLVTKGGSLVPLVG DVWEHAYY QYKNVRPEYLKN-VWKVINWKYA 222
At18377487               NQDPLVTKGGSLVPLVG DVWEHAYY QYKNVRPEYLKN-VWKVINWKYA 222
At145338359              NQDPLVTKGGSLVPLVG DVWEHAYY QYKNVRPEYLKN-VWKVINWKYA 222
TC18982                  NQDPLVTKGPSLVPLIG DVWEHAYY QYKNVRPDYLKN-IWKVINWKYA 219
11229                    NQDPLVTKGPSLVPLIG DVWEHAYY QYKNVRPDYLKN-IWKVINWKYA 219
LACT_5CDS.CSA1.1632_1    NQDPLVTKGPSLVPLIG DVWEHAYY QYKNVRPDYLKN-IWKVINWKYA 219
4527                     NQDPLVTKGATLVPLLG DVWEHAYY QYKNVRPDYLKN-IWKVINWKYA 209
18018                    NQDPLVTKGATLVPLLG DVWEHAYY QYKNVRPDYLKN-IWKVINWKYX 220
TC19481                  NQDPLVTKGATLVPLLG DVWEHAYY QYKNVRPDYLKN-IWKVINWKYA 220
LACT_5CDS.CSA1.7229_1    NQDPLVTKGATLVPLLG DVWEHAYY QYKNVRPDYLKN-IWKVINWKYA 224
At24286566               NQDPLVTKGSHLVPLIG DVWEHAYY QYKNARAEYLKN-IWTVINWKYA 227
TC25538                  AVNPLVWE---YHPLLA DVWEHAYY DFENRRPDYISVFLDKLVSWEAV 191
5392                     AVNSLVWE---YHPLLA DVWEHAYY DFENRRPDYISVFLDKLVSWEAV 244
17115                    AINPLVLD---YHPLLT DVWEHAYY DFQNRRPDYISVFLDKLVSWEAV 226
LACT_5CDS.CSA1.4424_1    AINPLVLD---YHPLLT DVWEHAYY DFQNRRPDYISVFLDKLVSWEAV 209
LACT_5CDS.CSA1.166_1     AINPLVLE---YHPLLT DVWEHAYY DFQNRRPDYVSVFLDNLVSWEAV 196
LACT_5CDS.CSA1.3085_1    AINPLVLD---YHPLLT DVWEHAYY DFQNRRPDYVSVFLDKLVSWEAV 196
```

Figure 4. 1. Multiple Sequence Alignment of Mn-SOD class of *Arabidopsis* and lettuce

```
At145358161               LGRAVVVHADPDDLGKGGHKLSKSGNAGSRVGCGIIGLQSSADAKL--- 164
At3273754                 LGRAVVVHADPDDLGKGGHKLSKSGNAGSRVGCGIIGLQSSADAKL--- 162
TC23953                   LGRAVVIHADPDDLGRGGHELSKTGNAGARVGCGVIGLQSSV------- 157
3486                      LGRAVVIHADPDDLGRGGHELSKTGNAGARVGCGVIGLQSSV------- 129
At3273752                 VGRAFVVHELKDDLGKGGHELSLTGNAGGRLACGVIGLTPL-------- 216
At17381187                VGRAFVVHELKDDLGKGGHELSLTGNAGGRLACGVIGLTPL-------- 216
TC17569                   VGRALVVHELADDLGKGGHELSLSGNAGGRLACGVVGLTPI-------- 222
4158                      VGRALVVHELADDLGKGGHELSLSGNAGGRLACGVVGLTPI-------- 210
LACT_5CDS.CSA1.1050_1     VGRALVVHELADDLGKGGHELSLSGNAGGRLACGVVGLTPI-------- 215
At20258870                VGRAVVVHADPDDLGKGGHELSLAGNAGGRVACGIIGLQG--------- 152
At15292996                VGRAVVVHADPDDLGKGGHELSLAGNAGGRVACGIIGLQG--------- 152
At145323809               VGRAVVVHADPDDLGKGGHELSLAGNAGGRVACGIIGLQG--------- 152
At145335297               VGRAVVVHADPDDLGKGGHELSLAGNAGGRVACGIIGLQG--------- 152
125662842                 VGRAVVVHADPDDLGKGGHELSLAGNAGGRVACGIFGLQG--------- 152
TC23478                   IGRAVVVHADADDLGKGGHELSKSGNAGGRVACGIIGLQA--------- 153
TC21570                   IGRAVVVHADADDLGKGGHELSKSGNAGGRVACGIIGLQA--------- 153
4526                      IGRAVVVHADADDLGKGGHELSKSGNAGGRVACGIIGLQ---------- 152
LACT_5CDS.CSA1.2510_1     IGRAVVVHADADDLGKGGHELSKSGNAGGRVACGIIGLQG--------- 153
```

Figure 4. 2. Multiple Sequence Alignment of Cu/Zn-SOD class of *Arabidopsis* and lettuce sequences

```
At186523820               LQF----VQDISGT-THVTGKISGLSPGF-GFHIHSFGDTTGCISTGP 67
At145358161               LQF----VQDISGT-THVTGKISGLSPGF-GFHIHSFGDTTGCISTGP 67
At3273754                 LQF----VQDISGT-THVTGKISGLSPGF-GFHIHSFGDTTGCISTGP 65
TC23953                   LQF----FQEANGV-THVSGKITGLKPGL-GFHIHALGDTTGCISTGP 64
3486                      -------------GV-THVSGKITGLKPGL-GFHIHALGDTTGCISTGP 36
At3273752                 VTL----TQDDSGP-TTVNVRITGLTPGP-GFHLHEFGDTTGCISTGP 124
At17381187                VTL----TQDDSGP-TTVNVRITGLTPGP-GFHLHEFGDTTGCISTGP 124
TC17569                   VTL----TQEDSGP-TTVNVKITGLTPGP-GFHLHEFGDTTGCISTGP 130
4158                      VTL----TQEDSGP-TTVNVKITGLTPGP-GFHLHEFGDTTGCISTGP 118
LACT_5CDS.CSA1.1050_1     VTL----TQEDSGP-TTVNVKITGLTPGP-GFHLHEFGDTTGCISTGP 123
At20258870                IFF----TQEGDGV-TTVSGTVSGLKPGL-GFHVHALGDTTGCMSTGP 61
At15292996                IFF----TQEGDGV-TTVSGTVSGLKPGL-GFHVHALGDTTGCMSTGP 61
At145323809               IFF----TQEGDGV-TTVSGTVSGLKPGL-GFHVHALGDTTGCMSTGP 61
At145335297               IFF----TQEGDGV-TTVSGTVSGLKPGL-GFHVHALGDTTGCMSTGP 61
125662842                 IFF----TQEGDGV-TTVSGTVSGLKPGL-GFHVHALGDTTGCMSTGP 61
TC23478                   ILF----EQETEGAPTTVTGNLSGLKPGP-GFHVHALGDTTGCMSTGP 62
TC21570                   ILF----EQETEGAPTTVTGNLSGLKPGP-GFHVHALGDTTGCMSTGP 62
4526                      ILF----EQETEGAPTTVTGNLSGLKPGP-GFHVHALGDTTGCMSTGP 62
LACT_5CDS.CSA1.2510_1     ILF----EQETEGAPTTVTGNLSGLKPGP-GFHVHALGDTTGCMSTGP 62
```

Figure 4. 3. Multiple Sequence Alignment of Cu/Zn-SOD class of *Arabidopsis* and lettuce sequences

4.1.5. Phylogenetic Tree Analysis

The consensus tree generated by NJ method, which showed dichotomy with two distinct clusters. All the lettuce SODs were grouped with three classes of *Arabidopsis* SODs namely; Cu/Zn-SOD, Fe-SOD, and Mn-SOD. Cu/Zn-SODs sequences fell in one cluster whereas Mn and Fe-SODs were grouped in second one. The phylogenetic analysis indicating evolution of the enzyme in different plants. Results of the phylogeny analysis indicate separate evolution of Cu/Zn-SOD from that of Fe and Mn-SOD which may have evolved from the same ancestral enzyme (Sheoran *et al*., 2013).

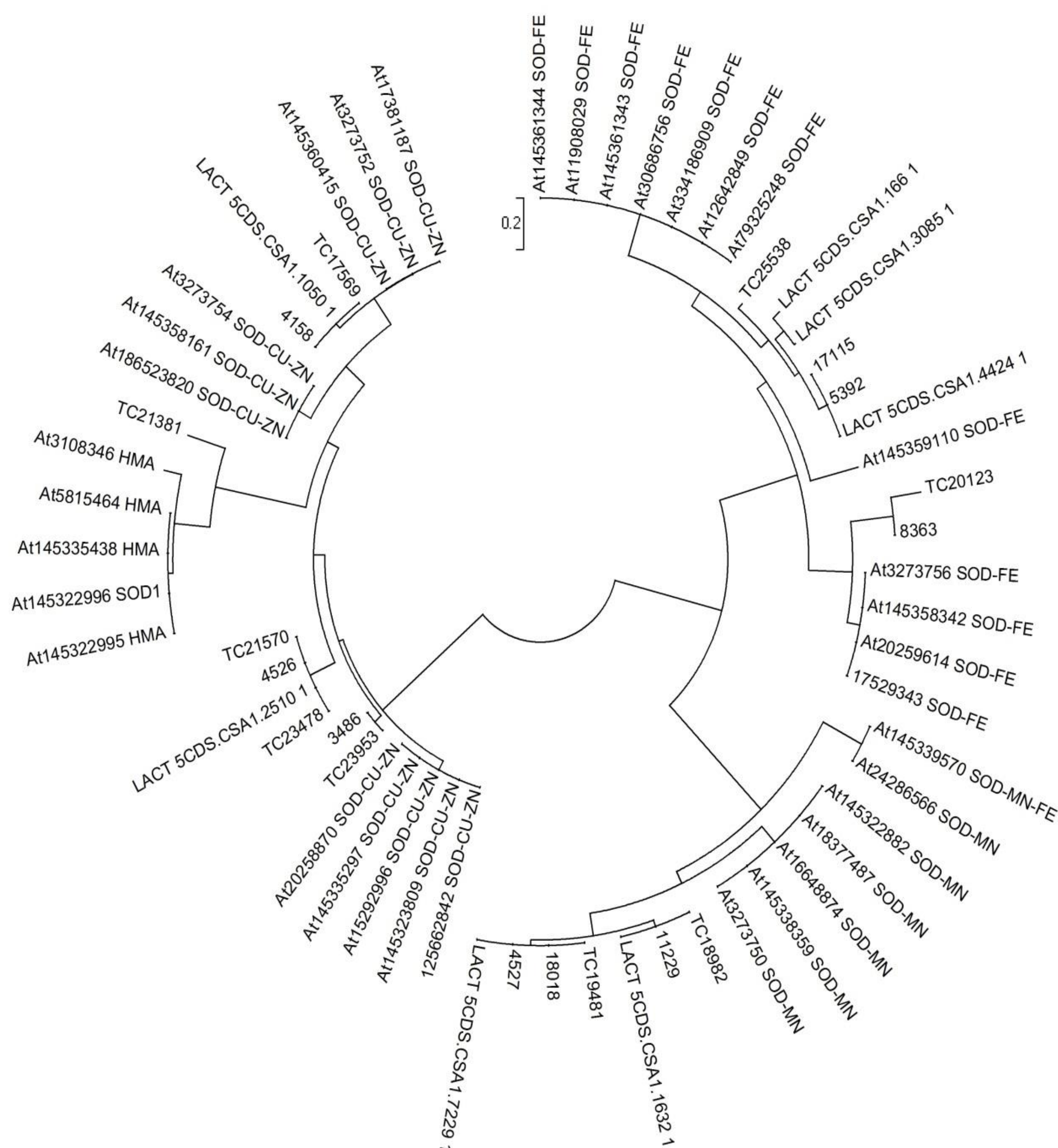

Figure 4. 4. The unooted tree generated by NJ method showed dichotomy with two distinct clusters. All the lettuce SODs were grouped into three classes of *Arabidopsis* SODs namely; Cu/Zn-SOD, Mn-SOD and Fe-SOD.

4.2. Nanoparticle Translocation

Translocation of ZnO and TiO_2 was confirmed in leaves of treated plants, compared to control plants, with X-ray diffraction (XRD) and Scanning Electron Microscopy (SEM) EDS.

4.2.1. X-ray Diffraction (XRD) Analysis

The XRD pattern obtained for the NPs with intense peaks in the whole spectrum of 2Θ values ranging from 20 to 80. The diffractions at 31.619° can be indexed to the (100) plane of the hexagonal ZnO NPs and The diffraction at the 27.527° can be indexed to the (001) plane of hexagonal TiO_2 NPs.

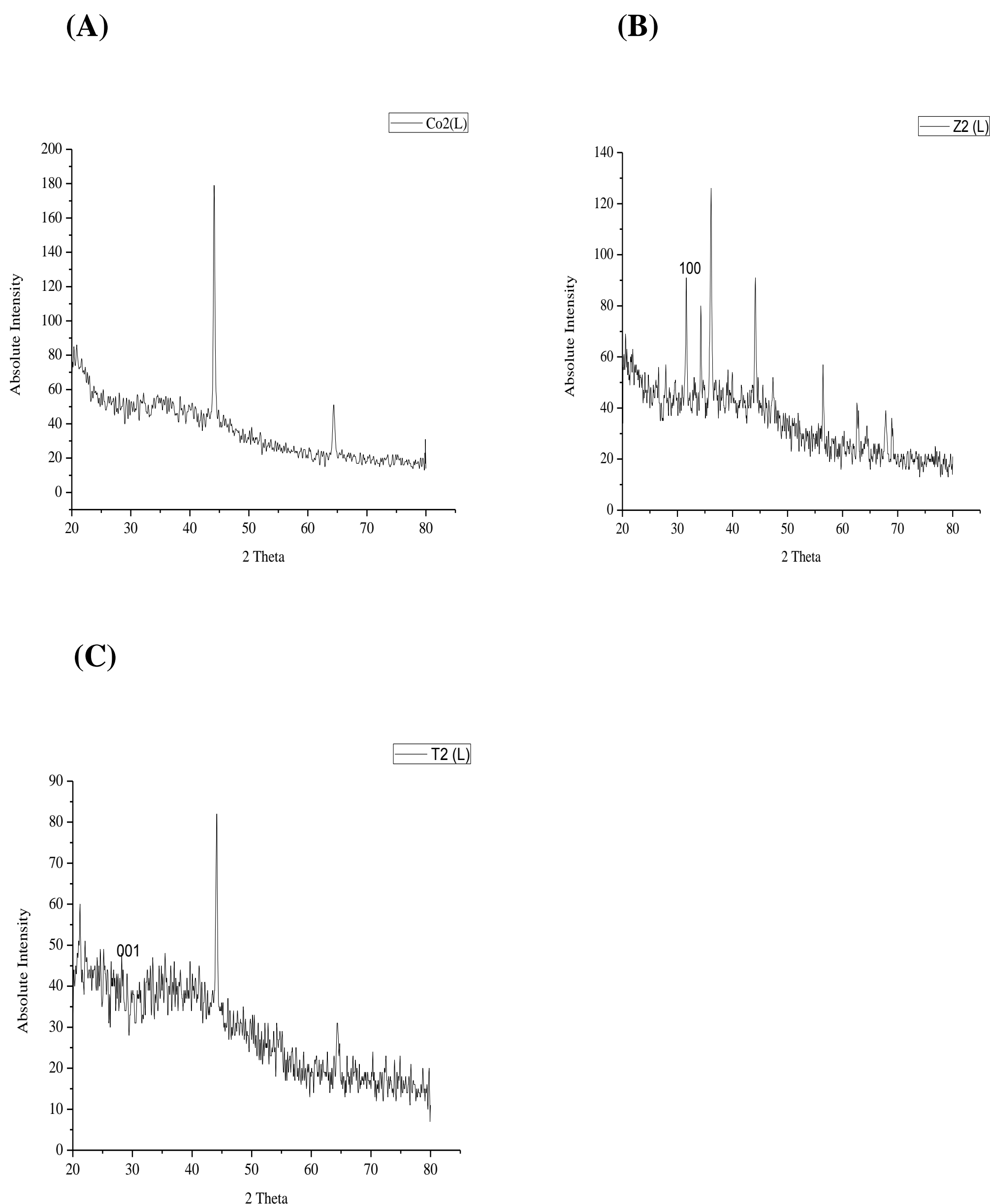

Figure 4. 5. . (A) Representative XRD spectra for control sample of lettuce leaves, (B) and (C) are spectra of ZnO NPs and TiO_2 NPs treated leaves respectively.

4.2.2. Scanning Electron Microscopy

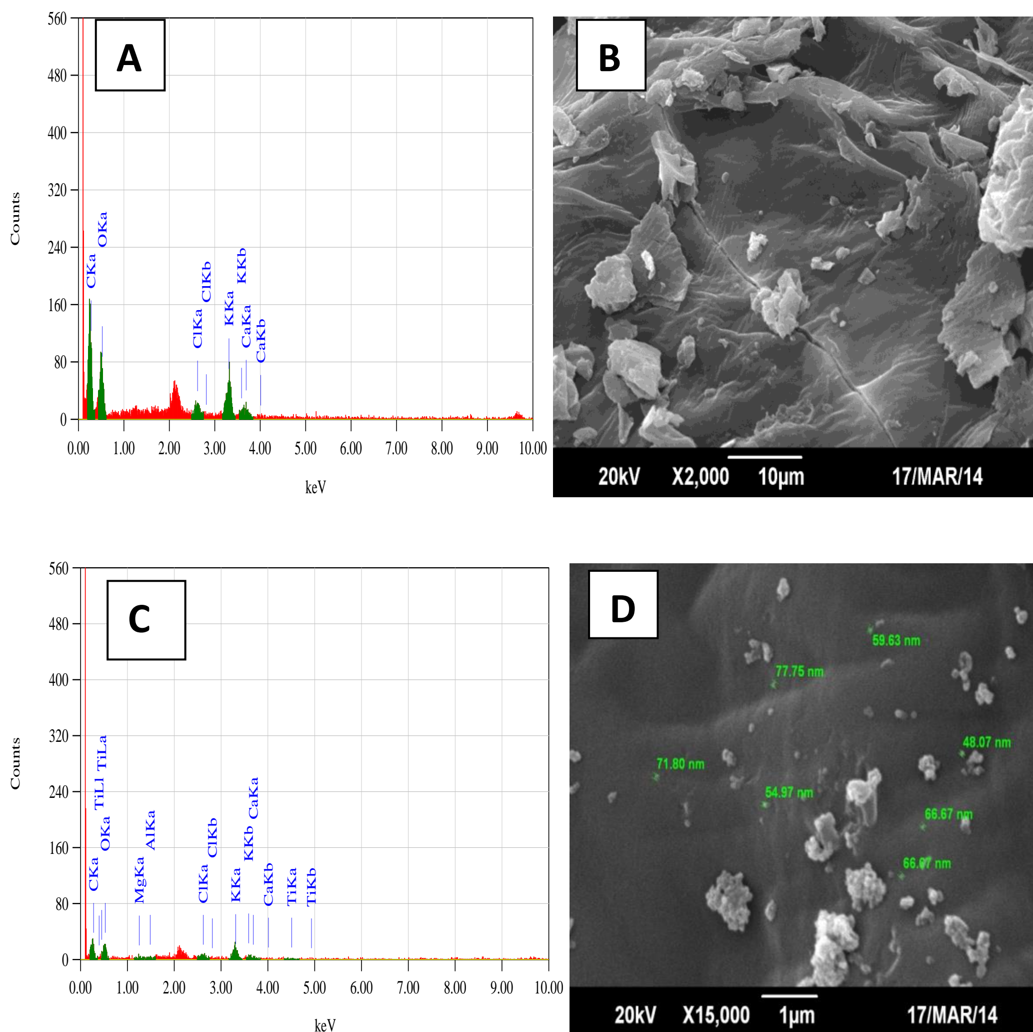

Figure 4. 6. (A) and (C) Representative EDS spectra of control and TiO_2 NPs treated lettuce sample, (B) and (D) SEM images of control and TiO_2 NPs treated lettuce sample

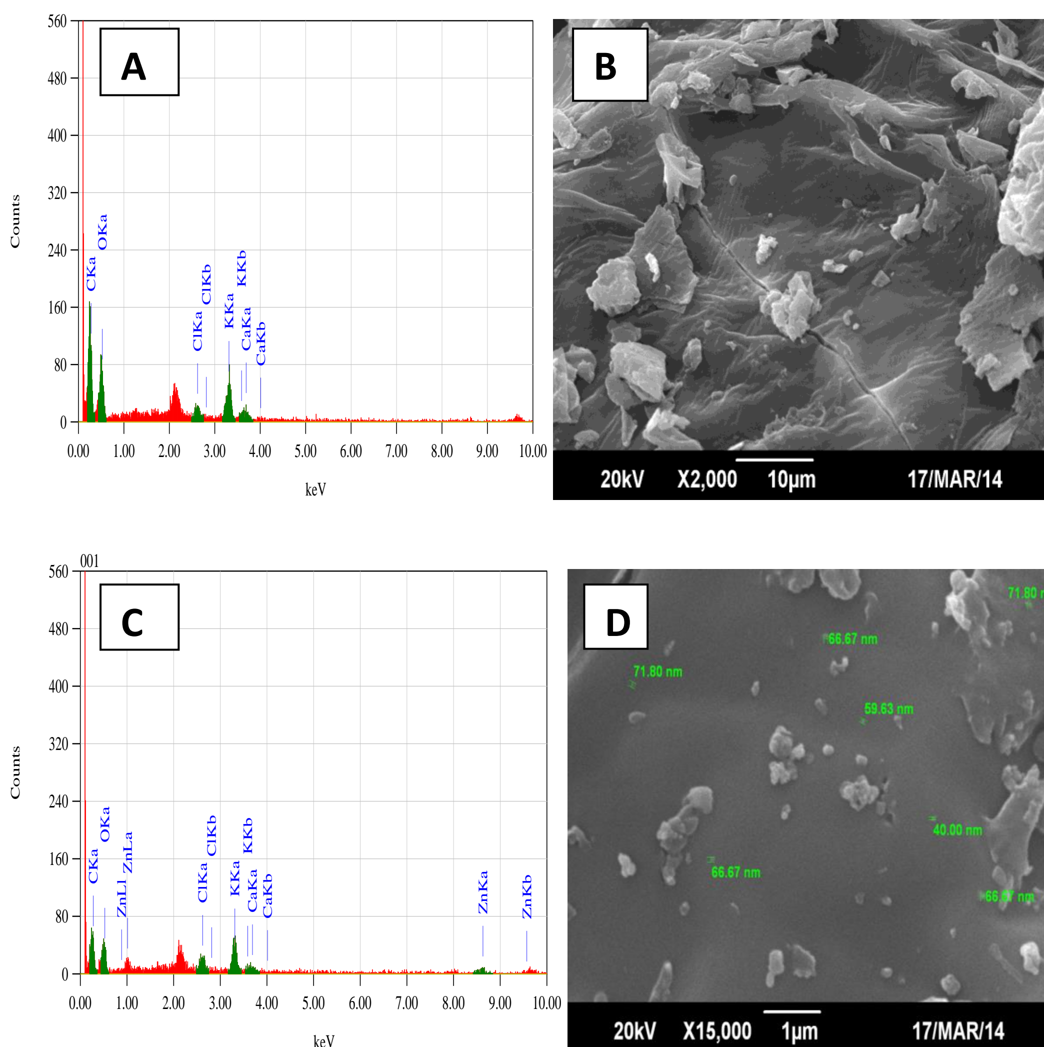

Figure 4. 7. (A) and (C) Representative EDS spectra of control and ZnO NPs treated lettuce sample, (B) and (D) SEM images of control and ZnO NPs treated lettuce sample

4.2.3. Elemental Analysis

Presence of NPs in treated plants were confirmed through elemental analysis. Tables 4.3, 4.4, 4.5 show the elemental analysis of control, TiO_2, and ZnO NPs treated samples respectively.

Table 4. 3. Elemental Analysis of Control Sample

Element	KeV	Mass%	Error %	Mol%	Compound	Mass %	Cation	K
C K	0.277	62.99	2.09	91.06	C	62.99	0.00	44.57776
O		6.31						
Cl K	2.621	4.27	2.87	2.09	Cl	4.27	0.00	8.2791
K K	3.312	21.78	4.98	4.84	K_2O	26.23	33.88	39.4888
Ca K	3.690	4.65	7.71	2.01	CaO	6.51	7.06	7.6545
Total		100.00		100.00		100.00	40.94	

Table 4. 4. Elemental Analysis of TiO_2 NPs treated Sample

Element	(KeV)	Mass%	Error%	Mol%	Compound	Mass%	Cation	K
C K	0.277	56.57	9.15	87.65	C	56.57	0.00	33.3604
O		9.98						
Mg K	1.253	3.11	17.79	2.38	MgO	5.16	4.92	4.0301
Al K	1.486	2.10	19.53	0.72	Al_2O_3	3.97	3.00	3.0547
Cl K	2.621	5.73	8.85	3.01	Cl	5.73	0.00	12.8958
K K	3.312	17.93	15.22	4.27	K_2O	21.59	17.64	38.1311
Ca K	3.690	2.47	22.80	1.15	CaO	3.45	2.37	4.9303
Ti K	**4.508**	**2.11**	**33.39**	**0.82**	**TiO_2**	**3.53**	**1.70**	**3.5975**
Total		100.00		100.00		100.00	29.62	

Table 4. 5. Elemental Analysis of ZnO NPs treated Sample

Element	(KeV)	Mass%	Error%	Mol%	Compound	Mass%	Cation	K
C K	0.277	53.17	3.50	87.34	C	53.17	0.00	25.9381
O		7.84						
Cl K	2.621	5.38	3.08	3.00	Cl	5.38	0.00	11.2404
K K	3.312	16.97	5.14	4.28	K_2O	20.44	21.26	34.4926
Ca K	3.690	1.90	7.58	0.94	CaO	2.66	2.33	3.6891
Zn K	**8.630**	**14.74**	**37.47**	**4.45**	**ZnO**	**18.34**	**11.04**	**24.6398**
Total		100.00		100.00		100.00	34.63	

4.3. Expression Analysis of SOD Genes

4.3.1. Primer Designing

Actin was used as reference gene for expression analysis. Six sequences are selected for primer designing. Selected sequences are given below:

1. >TC23478 for LsCu/Zn-SOD1
2. >TC17569 for LsCu/Zn-SOD2
3. >TC23953 for LsCu/Zn-SOD3
4. >TC21381 for LsCu/Zn-SOD4
5. >TC25538 for LsMn-SOD1
6. >TC19481 for LsMn-SOD2

4.3.2. Primer Specificity

Primer specificity was also checked for designed primers and actin primer pairs. Three primers were showed specificity namely; LsCu/Zn-SOD1, LsCu/Zn-SOD2, and LsMn-SOD1. LsCu/Zn-SOD3, LsCu/Zn-SOD4, and LsMn-SOD2 primers were eliminated because they were not specific.

4.3.2.1. Selected primers

Three primers were selected for expression analysis because they were showed specificity namely; LsCu/Zn-SOD1, LsCu/Zn-SOD2, and LsMn-SOD1. Actin was selected as a reference gene.

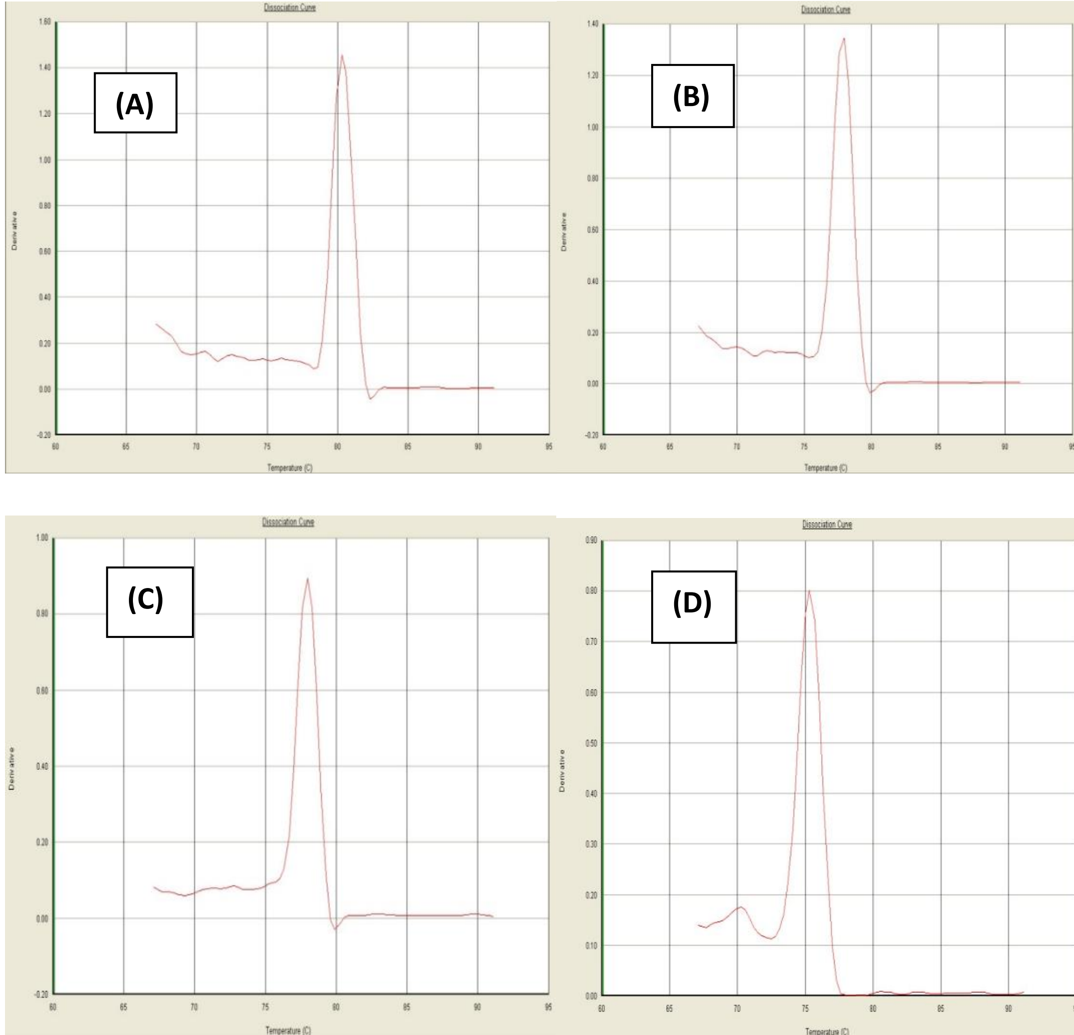

Figure 4. 8. (A), (B), and (C) are Melt curve outputs for lettuce SODs primer pairs; LsCu/Zn-SOD1, LsCu/Zn-SOD2, and LsMn-SOD1 respectively. (D) represents Melt curve output for lettuce Actin primer pairs.

4.3.2.2. Eliminated primers

LsCu/Zn-SOD3, LsCu/Zn-SOD4, and LsMn-SOD2 primers were eliminated because they were not specific.

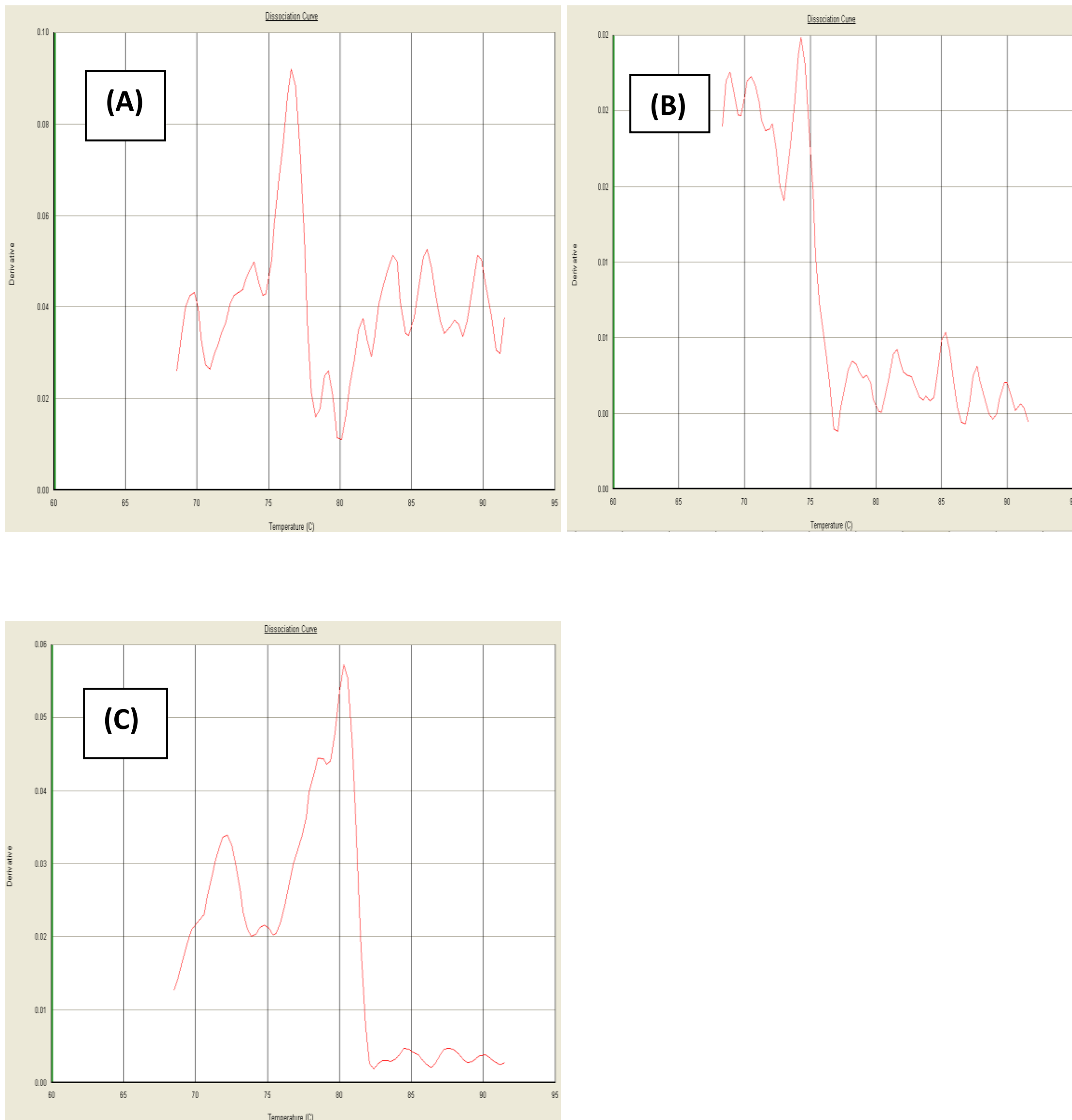

Figure 4. 9. (A), (B), and (C) are Melt curve outputs for lettuce SODs primer pairs; LsCu/Zn-SOD3, LsCu/Zn-SOD4, and LsMn-SOD2 respectively.

4.3.3. Gene Expression Changes

The effect of exposure to 2000 mg/L ZnO and TiO_2NPs on gene expression in lettuce was determined using real time quantitative PCR. After 30 days, ZnO andTiO_2 exposure resulted in up regulation of SOD genes, the expression difference was > 2-fold.

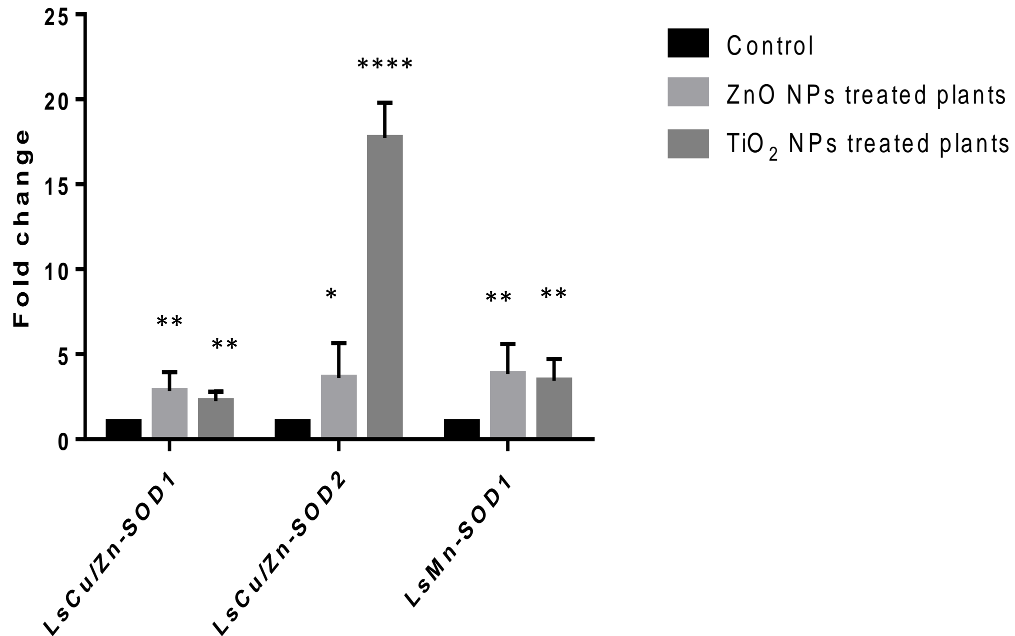

Figure 4. 10. Fold change of SOD gene expression in control, ZnO, and TiO_2 NPs treated lettuce plants.

4.4. Enzyme Assay

The SOD activity (inhibition rate %) was quantified by measuring the decrease in the color development at 450 nm. SOD activity in control, ZnO, and TiO_2 NPs treated lettuce plants was determined 43, 50, and 46% respectively.

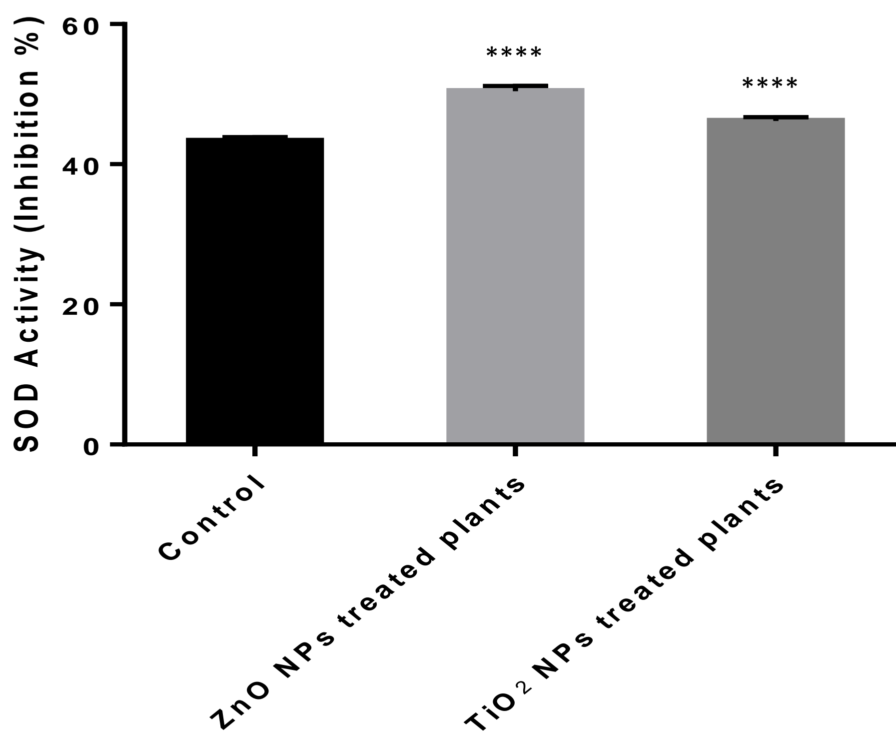

Figure 4. 11. SOD activity (inhibition rate %) activity in control, ZnO, and TiO_2 NPs treated lettuce plants.

Chapter 5

DISCUSSION

Nanoparticles in environment pose a risk for the plants and particularly for edible crops (Pokhrel & Dubey, 2013; Rico *et al*., 2011). There is a need for risk assessment of the increasing concentration of engineered nanomaterial in our environment (Warheit, Sayes, Reed, & Swain, 2008). ENPs are progressively used in different industries. NPs may cause severe toxicity and their overall effects remain largely unknown (Nowack & Bucheli, 2007).

SOD provides the first line of defense against ROS toxicity and oxidative stress. The molecular structural analysis of SOD is very important for understanding their role in response to different stresses (Ramana Gopavajhula *et al*., 2013). The information of the basic arrangement of amino acids is also very significant for understanding the molecular mechanisms by which proteins achieve their purposes (Fu, Subramanian, & Masters, 2000).

In this study, we established methods to test the phytotoxicity of two different NPs, TiO_2 and the other ZnO. Zinc is 24th most abundant element on earth and readily burns to form ZnO, whereas TiO_2 amongst the 10th most abundant compounds on earth (Csuros & Csuros, 2002).

The present study demonstrates that TiO_2 NPs as well as ZnO NPs are taken up by lettuce plant. TiO_2 and ZnO translocation was confirmed by XRD and SEM-EDS. Elemental analysis define the presence of NPs in TiO_2 and ZnO NPs treated lettuce plants. Mol% of TiO_2 and ZnO was 3.53 and 18.34 in TiO_2 and ZnO NPs treated lettuce plants respectively. Similar localization was observed previously in lettuces exposed to Ag NPs (Larue, Castillo-Michel, Sobanska, Cécillon, *et al*., 2014).

In present study, 25 EST sequences in lettuce were retrieved from different databases. Sequences producing significant alignment with the query SODs were considered for multiple sequence alignment in ClustalW (Thompson *et al*., 1997).

Alignments were analysed and phylogenetic relationships were established using NJ method (Saitou & Nei, 1987) in MEGA5.0 (S. Kumar *et al*., 2008). The ScanProsite tool (De Castro *et al*., 2006) was employed to elucidate motifs and signature sequences associated with SODs.

ScanProsite results elucidated two signature sequences (Signature 1: GFHIHAlGDtT and Signature 2: GNAGgRvACgiI) in Cu/Zn-SODs. In Mn-SOD the signature sequence is DVWEHAYY. The domain boundaries of SODs indicated that Cu/Zn-SOD comprised of a Cu-Zn binding like domain, Mn-SOD had 2 Manganese and Iron SOD like domains.

The consensus tree generated by NJ method showed dichotomy with two distinct clusters. All the lettuce SODs were grouped with three classes of *Arabidopsis* SODs namely; Fe-SOD, Mn-SOD, and Cu/Zn-SOD. Cu/Zn-SODs sequences fell in one cluster whereas Mn and Fe-SODs were grouped in second one. The phylogenetic analysis indicating evolution of the enzyme in different plants. Results of the phylogeny analysis indicate separate evolution of Cu/Zn-SOD from that of Mn and Fe-SOD which may have evolved from the same ancestral enzyme.

We have examined gene expression changes in lettuce plants. Our results indicate that the SOD genes (Cu/Zn-SOD and Mn-SOD) responded differently against ZnO and TiO_2 nanotoxicity. Both SOD genes were induced in response to ZnO and TiO_2 nanotoxicity. The effect of exposure to 2000 mg/L ZnO and TiO_2 NPs on gene expression in lettuce was determined using real time quantitative PCR. After 30 days, ZnO and TiO_2 exposure resulted in expression changes of SOD genes. SOD genes were induces in response to oxidative stress.

The effect of exposure to ZnO and TiO_2 NPs on gene expression in *Arabidopsis* was previously studied using microarrays. NPs exposure resulted in upregulation and downregulation of different genes. The downregulated genes were tangled with biogenesis , nucleosome assembly, translation and microtubule based process (Landa *et al.,* 2012).

ROS levels and antioxidant enzyme activities in *Arabidopsis thaliana* exposed to SO_2 were observed in previous studies. 494 genes differentially expressed in plants exposed to 30mg/m^3 SO_2 for 72 h, including upregulation of some defense related genes (L. Li & Yi, 2012).

We also examined effects of NPs on enzyme activity. Higher superoxide dismutase activity was observed at the concentration (2000mg/L) of TiO_2 and ZnO. The SOD activity was quantified by measuring the inhibition of the color development at 450 nm. SOD activity in control, ZnO, and TiO_2 NPs treated lettuce plants was determined 43, 50, and 46% respectively. SOD activity enhances in response to oxidative stress. Abiotic stresses have been associated with higher SOD activities. Increased antioxidant enzyme activity can prevent oxidative stress. High ROS levels and oxidative stress have been cited as common reasons for cellular damage induced by NPs, including ZnO and TiO_2 NPs.

CONCLUSION

These outcomes deliver significant information regarding plant detoxification mechanism for NPs at both transcriptomics and proteomics levels and also have inferences for defining the threat of NPs in consumer products. The interaction of plant cell with the NPs results in modification of plant gene expression and associated biological pathways which ultimately affect plant growth and development.

We conclude that improved antioxidant levels may play an imperative role in ROS detoxification, when plants are exposed to several stresses. Plants regulate to environmental stresses through activating their defence mechanisms. TiO_2 NPs as well as ZnO NPs are taken up by lettuce plant after being deposited in soil. SOD genes were induced in response to ZnO and TiO_2 nanotoxicity. ZnO and TiO_2 exposure resulted in expression changes of SOD genes. Higher SOD activity was also observed against TiO_2 and ZnO nanotoxicity. The facts noticeably specify that the mechanisms of phytotoxicity are extremely nanoparticle dependent even though a partial overlap in gene expression response.

FUTURE PROSPECTS

Development of the arena of nanotechnology means increase in risk posed by nanomaterials to the biotic modules of environment. Nano-pollution is no longer a conjectural scenario and hence need for wide research on the effects of engineered nanomaterials on environmental constituents, precisely edible plants, is unswervingly needed. Moreover, the effect of innumerable concentrations of nano-sized materials and their bulk counterparts needs to be evaluated to bring to light any differences between the interfaces of the two with plants.

The study needs to be inferred to more plants of edible value and must embrace genes from essential functional classes, e.g., cell development, energy pathway and electron transport chain proteins etc.

Chapter 6

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