PHYSIOLOGICAL EFFECTS OF NANOPARTICLES ON DIFFERENT PLANTS IN RESPONSE TO PHOSPHORUS BIOAVAILABILITY

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By

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Environmental Science

Institute of Environmental Sciences and Engineering (IESE)

School of Civil and Environmental Engineering (SCEE)

National University of Sciences and Technology (NUST)

Islamabad, Pakistan

(2014)

Certificate

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Dedicated

To

My Loving Parents

For their endless love, support and encouragement

بسم الله الرحمن الرحيم

ACKNOWLEDGEMENTS

This piece of work would not have been accomplished without the blessings, wisdom and power given to me by Allah Almighty.

I would like to express my sincere gratitude to my esteemed and worthy supervisor ***Dr. Muhammad Arshad*** for his kind guidance during the study. His patience, support, and motivation helped me throughout this research work. I am thankful to ***Dr. Ishtiaq A. Qazi*** and ***Dr. Muhammad Fahim Khokhar*** for their kind help and very positive criticism throughout the project. My special thanks to external GEC member ***Dr. Nasar-um-Minullah,*** for providing the lab facilities at ASAB, NUST.

I am grateful to ***Dr. Muhammad Shahid*** for providing me help during analysis of H_2O_2 species. Many thanks also go to ***Dr. Andrew D. Richardson*** and ***Matthew A. Mickens*** for their insightful opinions and comments during chlorophyll content studies.

I would like to extend my heartfelt gratitude to my brother and friends especially Ms. Zahra, Hira Fatima, Farheen Mustafa, Shamsa Kanwal, Fatima Zohra and Bareera Maryam for their immense cooperation and encouragement throughout the research phase. Last, but definitely not the least, my deepest thank to all laboratory staff at IESE, SCME, ASAB and SMME, NUST for their help and cooperation.

Rafia Rafique

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LIST OF ABBREVIATIONS

ABBREVIATION	DESCRIPTION
µM	Micro mole
ATP	Adenosine triphosphate
CCI	Chlorophyll content index
CNTs	Carbon nanotubes
DNA	Deoxyribose nucleic acid
EDX	Energy dispersive x-ray spectroscopy
FAOSTAT	Food and agriculture organization statistics
H_2O_2	Hydrogen peroxide
ICP-AES	Inductive coupled plasma/atomic emission spectroscopy
JCPDS	Joint committee on powder diffraction standards
LHC II	Light-harvesting complex II
MN	Micronucleus
MT	Million tons
OS	Oxidative stress
QD	Quantum dot
ROS	Reactive oxidative species
Rpm	Revolution per minute
SD	Standard deviation
SEM	Scanning electron microscope
TCA	Trichloroacetic acid
Ti	Titanium
TNPs	Titanium dioxide nanoparticles
USDA	United states department of agriculture
XRD	X-ray diffraction
θ	Theta

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ABSTRACT

Advancement in nanotechnology and its extensive utilization has raised concerns about the effects of engineered nano-materials in agriculture and environment. The aim of present study was to assess the physiological responses of wheat and lettuce to exposure of 0, 20, 40, 60, 80, 100 mg TiO_2 nanoparticles (TNPs) kg^{-1} of soil. The effects of TNPs were investigated on the phytoavailability of phosphorus (P), root and shoot lengths, total fresh and dry biomass, chlorophyll content, H_2O_2 generation and DNA damage. The wheat and lettuce plants were exposed to TNPs for 60 and 75 days, respectively. It was found that uptake of P increased significantly ($p<0.05$) with decreasing pH as compared to control by lettuce, whereas wheat accumulated the maximum concentration of P at 60 mg kg^{-1} of TNPs. An increase in root and shoot lengths (35.3% and 39.2%), total fresh and dry biomass (46% and 52%), chlorophyll content (68%) and H_2O_2 generation (40%) was observed in lettuce at the highest level of TNPs applied as compared to the control. However, for wheat, the maximum increase in root and shoot lengths (42.2% and 35.7%), total fresh and dry biomass (60% and 72%) and chlorophyll content (29.7%) was observed at 60 mg kg^{-1} concentration level of TNPs in comparison with the control followed by a decrease at higher TNPs treatment levels. The results confirmed that wheat could not tolerate high concentrations (80 and 100 mg kg^{-1}) of TNPs due to overproduction of H_2O_2 content (80%) and DNA damages (40.5%). This study suggested the need to further investigate the possible consequences and effects of applying nanoparticles on agricultural crops.

Chapter 1

INTRODUCTION

1.1 BACKGROUND

Nanoparticles have average size of less than 100 nm and have unique properties depend on their phase, distribution, size and morphology (Christian *et al.,* 2008; Nel *et al.,* 2006). Therefore, these nanoparticles have gained much attention in number of consumer products, cosmetics, transportation, pharmaceutics, energy and agriculture. The manufacturing of engineered nanoparticles has reached to 58,000 metric tons per year worldwide in 2011-2020 (Maynard, 2006) which may result in their release to the environment. Furthermore, the behavior of these nanoparticles is different from their bulk forms to the environment and organisms (Taylor and Walton, 1993).

The present study looks on TiO_2 NPs (TNPs) which are extensively utilized in many products such as sunscreen, cosmetics, genomics, optics, toothpaste, pharmaceuticals and bio-analytical fields (Sun *et al.,* 2009). This production is expected to increase in the coming years because of its useful physico-chemical properties. This intensive use, production and disposal of nanoparticles in the environment may affect soil, soil nutrients and plants which are the most important part of ecosystem. These nanomaterials may alter seed germination, growth and biochemical processes of plants. That's why plants should be tested to examine their response to nanoparticles. Several recent studies published that TNPs could deposit on soil particles and alter soil properties (Fang *et al.,* 2009; Mattigod *et al.,* 2005). It is, therefore, important to study the effects of TNPs on soil nutrients such as phosphorus.

Phosphorus is the second major macronutrient after nitrogen and is considered as a key element for sustainable production of crops. It is important in many processes, including glycolysis, photosynthesis, nucleic acid synthesis, energy generation, respiration, membrane formation, redox reactions, nitrogen fixation, carbohydrate metabolism and enzyme inactivation/activation (Wu *et al.,* 2005). Although soils have phosphorus in large amount but only small fraction is available to the plants. The availability of phosphorus is not enough and about 30–40% crop yield of the world is limited due to lack of available phosphorus (Vance *et al.,* 2003).

Pakistan is an agricultural country and approximately 90% of Pakistan's soils are phosphorus deficient. Moreover, the added soluble phosphorus in soils also gets fixed which results in low crop yields (Ahmad *et al.*, 1992). Plants take up phosphorus in the form of orthophosphate (Pi) from the solution of soil, but its concentration is less in most of the soils. That's why fertilizers are used to satisfy the phosphorus requirements of plants (Bieleski, 1973). Furthermore, the deficiency of phosphorus affects root architecture, seed growth and therefore the crop yield (Borch *et al.*, 1999). According to previous assessments, the resources of phosphorus may be depleted in the world by the year 2050 (Vance *et al.*, 2003). Therefore, phosphorus accessibility to plants in sufficient amounts is a worldwide issue.

To overcome this issue, TNPs can be used to improve functionalities of crops. These nanoparticles could benefit the agriculture and save natural resources of phosphorus for good crop productivity. It has been reported in literature that macro- and micro-nutrients uptake by beans and other crops increased when Ti form applied to plants. This is also observed that reducing the phosphorus content from the fertilizer applied to the Ti- treated crops did not much affect the nutritional balance in the plants (Lopez-Moreno *et al.*, 1996). But effects of Ti varied with different nutritional requirements of plants (Carvajal and Alcaraz, 1998).

Any chemical and biological change in soil properties could affect plant productivity, growth and development (Arshad *et al.*, 2011). Different studies demonstrated that TNPs are taken up by plants and could affect the physiological functions of plants such as reactive oxidative species (ROS), micronuclei formation and chlorophyll content. ROS are highly reactive molecules and are produced due to the presence of one or more unpaired electrons. Under normal conditions, these products are generated because of metabolic processes and play a very important role in cell signaling. These include hydroxyl radical (·OH), hydrogen peroxide (H_2O_2), superoxide anion ($\cdot O2^-$) and singlet oxygen (electronically excited state of O_2). ROS levels increase dramatically due to the environmental stress conditions (heat, heavy metals or nanoparticle exposure). This can cause damage to the cell structure and DNA (Deoxyribose Nucleic Acid) which may result in a situation called oxidative stress (OS). Plants also have antioxidant defense system but occurrence of OS reduces the cell ability to respond through the defense system (Davies, 2005). Many reports show positive effects of TNPs on physiology of different plants. For instance, foliar spray of TNPs at 10 mg L^{-1} concentration on the leaves increased root and shoot length, root area, root nodule,

chlorophyll content of *Vigna radiata* and activity of acid phosphatase in rhizosphere (Raliya *et al.,* 2014). Other effects include the improvement of growth, nitrogen metabolism and photosynthesis of *Spinacia oleracea* even at low concentration i.e. 20 mg L^{-1} (Yang *et al.,* 2006; Hong *et al.,* 2005; Liu *et al.,* 2010). Application of TNPs can also improve the structure of chlorophyll, increase light absorbance, transfer of light energy to active electrons, and have effect on the process of photosynthesis (Morteza *et al.,* 2013). The uptake of TNPs was also studied in hydroponic conditions upon plants root exposure (Larue *et al.,* 2012b).

The effects of TNPs on physiology of plants are, however, contradictory. Some studies have reported that TNPs could also induce genotoxicity and have inhibitory effects on seed germination, root length and mitotic activity of *Vicia narbonesis* and *Zea mays* (Castiglione *et al.,* 2011). It is reported that TNPs have potential to produce ROS that can cause toxicity (Kang *et al.,* 2008; Barnard *et al.,* 2010). It is also observed that TNPs negatively affect the spinach plant species by inducing OS (Lei *et al.,* 2008). The ROS-mediated genotoxicity results in DNA damage and formation of micronuclei (Song *et al.,* 2012; Shahid *et al.,* 2014).

All above studies suggested that response of plants to TNPs varies with its nature, plant species and different growth stages along with their concentration levels. Therefore, there is a need to evaluate properties of these nanoparticles and their effects on plants.

Many studies have also described the effects of nanoparticles on different crops. In the present study, wheat (*Triticum aestivum*) and lettuce (*Lactuca sativa*) were selected as test plant species because of their importance as food crops in many countries including Pakistan. In Pakistan, production of wheat and lettuce was 25000 MT in 2014 (USDA, United States Department of Agriculture) and 350 tons in 2012 (FAOSTAT, Food and Agriculture Organization Statistics) respectively. Very few studies are available with respect to TNPs effects on crop plants. This research will help to understand the effects of TNPs on physiology of wheat and lettuce plants.

1.2 RESEARCH OBJECTIVES

The main aim of this study was to evaluate the effects of TNPs on plants as these nanoparticles are released in large quantities in the ecosystem. For this purpose, TNPs were

prepared in laboratory to assess their impacts on two plant species; Wheat (*Triticum aestivum*) and Lettuce (*Lactuca sativa*).

The effects of TNPs were explored with the following objectives:

- Studying the effects of TNPs on plants growth parameters i.e. root and shoot lengths, total fresh and dry biomass.
- Assessing the effects of TNPs on chlorophyll content, production of ROS and micronuclei formation in both plant species.

1.3 SIGNIFICANCE OF STUDY

The consumption of TNPs has been increasing in many commercial products which has raised concerns about their effects on environment that these nanoparticles may have. This study has the wide scope to demonstrate that how lettuce and wheat respond when grown in TNPs treated soil. Additionally, after TNPs application, physiological functions and nutritional qualities of crops can be improved or not such as phosphorus. In this way, this study gives us an insight of how and in which concentration of TNPs should be applied in a more safe and effective way for the betterment of agricultural crops.

Chapter 2

LITERATURE REVIEW

This chapter is structured to highlight the general background of nanoparticles and their interaction with plants in the environment. It also provides the review of physiological effects of TNPs on plant's growth, ROS, DNA damage, chlorophyll content and their uptake by the plants with a focus on the phosphorus bioavailability.

2.1 NANOTECHNOLOGY

Nanotechnology is a new field of modern research that focuses on the fabrication and manipulation of nanostructures, nanomaterials and nanoparticles of sizes ranging from 1-100 nm. Nanotechnology has gained much attention in numerous fields such as agriculture, environment, single electron transistors, catalysis, cosmetics, food, health care, chemical industries, biomedical sciences, space industries, drug-gene delivery, photo-electrochemical applications, optoelectronics and energy science (Colvin *et al.,* 1994). Synthesis and fabrication of nanomaterials along with modern study of their physico-chemical properties is opening the doors of fundamental frontiers in the current research age. Nanomaterials and nanostructures are considered as solution to many environmental and technological challenges in the field of agriculture, water treatment, solar energy and medicine, etc.

2.1.1. Nanotechnology in agriculture

Crop production is facing tremendous problems due to climate change and environmental stress conditions. Nanotechnology needs to be merged into new frontiers to overcome such type of environmental issues. Development of nanotechnology is leading to different nanomaterials for the degradation of pesticides and insecticides which are being employed in a variety of agriculture applications. Nair *et al.* (2010) reported that nano-biopesticides are safe for plants as compared to conventional chemical pesticides. Yu *et al.* (2007) used TNPs films for the photocatalytic degradation of pesticides (organochlorine). They demonstrated that production of hydroxyl radicle or electron transfer initiates the degradation process on the surface of TiO_2.

In 1997, a research group started their program to advance the bioavailability of a RPA 107382 "a novel insecticide" to the plants. Main goal of this program was to access

the capabilities of nanospheres (Boehm *et al.*, 2003) and to obtain stable, small sized nanoparticles having active ingredient encapsulation (by nanoprecipitation).

2.2 NANOPARTICLES

The term "nanoparticles" can be defined as a particle with one or more external dimensions in the size 1 nm to 100 nm (European, 2011). Nanoparticles possess exclusive biological, physical and chemical properties which are making them to behave differentially in both individual and bulk materials. Based on the core material, nanoparticles can be classified as organic and inorganic nanoparticles. Organic nanoparticles include 1), Fullerenes C70, C60 and their derivatives, 2), Carbon nanotubes (Single walled or multi-walled CNTs), while the inorganic nanoparticles comprise semiconductors (zinc oxide and titanium dioxide), noble metal nanoparticles (silver and gold), quantum dots (cadmium selenides) and magnetic. Among other nanomaterials, inorganic nanoparticles have superior properties that are increasing their demand in current research (Ju-Nam and Lead, 2008).

2.3 ROLE OF NAOPARTICLES IN AGRICULTURE

2.3.1. Nanoparticles in soil

Soil has always been fundamental to humans as it is main resource of food production and major source of trace elements (Mn, Cu, Zn, etc.). It has been reported that nanoparticles are found in air, water, soils, and organisms due to their increased production (Blaser *et al.,* 2008; Mueller and Nowack, 2008; Navarro *et al.,* 2008; Ma *et al.,* 2010). The use of nanoparticles in soil for agriculture is relatively a new domain and requires further exploration. According to a recent assessment, production of engineered nanoparticles was about 63–91% of over 260,000–309,000 in the year of 2010 that was ended up in landfills, with the balance released into soils (8–28 %), atmosphere (0.1–1.5 %), water bodies (0.4–7 %) (Keller *et al.,* 2013). Additionally, nanoparticles have been extensively used in environmental remediation e.g. soil, water (Zhang *et al.,* 2013; Ngomsik *et al.,* 2005; Uheida *et al.,* 2006). These are also found in soil when used in successive quantities and affect the plants growth by their deposition on soil particles. After deposition, they persist there for a long time or can be taken up by plants or biological organisms (Bystrzejewska-Piotrowska *et al.,* 2009). However, information on the fate of nanoparticles and their impacts is very limited.

2.3.2. Effects of nanoparticles on phosphorus

Phosphorous is the second most important limiting macronutrient which is essential for plant growth as it is a part of numerous macromolecules such as nucleic acids, phospholipids, and adenosine triphosphate (ATP). These macromolecules are necessary for growth and development of plants with consistent supply (Schachtman *et al.,* 1998). A study by Santner *et al.* (2012) demonstrated the increase in phosphorus uptake from nutrient solution in *Brassica napus* using Al_2O_3 nanoparticles. With the application of different concentrations of nanoparticles, plant P increased up to eight and forty fold.

2.3.3. Effects of nanoparticles on plants

The soil and plant's systems are closely inter-linked. Chemical and biological change in soil properties is bound to have effect on plant productivity, growth and development. The presence of engineered nanoparticles in soil could alter soil properties and affect the crop productivity. Plant cell wall acts as a barrier for external entries to enter within cell. Nanoparticles can cross this barrier and the sieving properties of the cell wall are based on its pore size (5-20 nm) (Fleischer *et al.,* 1999). It has been reported that plants may uptake and accumulate nanoparticles into their biomass (Ma *et al.,* 2010).

Inorganic nanoparticles among all engineered nanoparticles are widely used nanomaterials and found in many consumer products. These nanoparticles may enter to the environment and accumulate in plants due to their vast use. They may cause toxicity in plants. That's why their effects and possible mechanisms need to be studied. The effects of some of inorganic nanoparticles on different plants are discussed below:

2.3.3.1 Effects of Ag nanoparticles

Stampoulis *et al.* (2009) examined the effects of Ag nanoparticles suspensions on the seed germination and root growth of zucchini plants. The results shown no negative effects on germination of seed and growth whereas a decrease was observed in plant's biomass and transpiration when exposed to Ag nanoparticles for longer duration. It was also reported in literature that Ag nanoparticles caused genotoxicity and cytotoxicity in root tip of onion (Kumari *et al.,* 2009).

Lee *et al.* (2011) examined the toxic effects of Ag nanoparticle in agar as well as soil media on two plant species, *Phaseolus radiatus* and *Sorghum bicolor*. Agar plant tests

resulted in inhibition of growth with increasing concentration of nanoparticle for both crop plants. However, soil studies did not alter the growth rate of *P. radiatus*. The effects of Ag ions and Ag nanoparticles also varied in soil and agar. So, it has been attributed that toxicity of Ag nanoparticles is also different in soil environments.

2.3.3.2 Effects of Zn/ZnO nanoparticles

Zinc oxide (ZnO) nanoparticles are synthesized for many purposes. The increased production resulted in their release to the soil environment. Zhao *et al.* (2012) studied the route of Zn and ZnO nanoparticles in soil and uptake by *Zea mays*. The movement of ZnO nanoparticles in soil was low. The results of this study depicted that Zn was found in roots and shoots of corn plants when grown in ZnO nanoparticles amended soil. Moreover, ZnO nanoparticles also entered root epidermis and cortex. Images of confocal microscope also show that the aggregates of nanoparticles passed the endodermis via symplastic pathway. The suspensions of ZnO nanoparticles shown no negative effects on root growth and seed germination of zucchini seeds (Stampoulis *et al.,* 2009) whereas the ZnO nanoparticles with diameter 35 nm and 15-25 nm inhibited the seed germination of corn and rye grass, respectively (Lin *et al.,* 2007).

Two plants species; radish and rape were incubated in suspension of nano-Zn to determine the effects of ZnO nanoparticles on their root growth. Results show that growth of plant's roots was decreased significantly. It was observed that ZnO nanoparticles had no negative effects due to the selective permeability of seed coat. The same research group investigated phytotoxicity of ZnO on rhizosphere dissolution of nanoparticles (Lin *et al.,* 2008).

Mahajan *et al.* (2011) conducted the plant agar test to determine the effects of ZnO nanoparticles on mung and gram seedlings growth. The root and shoot length was also measured to check the effects of nanoparticles on plant growth. The images of scanning electron microscopy (SEM) confirmed the uptake of nanoparticles in roots. Good seedling growth was observed at 20 mg L^{-1} concentration level of nanoparticles.

2.3.3.3 Effects of magnetic nanoparticles

Magnetic nanoparticles permit a very specific localization of external agents which is very important in delivery of nanoparticulate to plants. Studies have shown the translocation and accumulation of magnetic nanoparticles with diameter < 50 nm in pumpkin plants

(Gonzalez *et al.*, 2008; Zhu *et al.*, 2008; Corredor *et al.*, 2009). The roots and leaves of plants images indicated successful uptake and translocation of magnetic nanoparticles. The plants also show no toxicity in response to the application of magnetic nanoparticles.

In topical studies, much attention has been given to genotoxicity of ferrofluids. These ferrofluids affected the chromosomes in young plant species (Racuciu and Creanga, 2009 and 2007; Pavel and Creanga, 2005; Pavel *et al.*, 1999). The tetramethylammonium hydroxide (TMA-OH) doped magnetic nanoparticles were used to determine their impacts on the growth of young maize plants (Racuciu and Creanga, 2006). The level of 'chlorophyll a' was decreased at high concentration of ferrofluids as compared to low concentration levels.

In another study, water based magnetic fluid was used during germination of maize seeds. At high concentration of magnetic fluids, the leaves had brown spots and OS due to excess iron treatment. It was also affected photosynthesis and metabolic process rate. The living plant tissues were also used to study the oxidative stress induced by the ferrofluid (Racuciu and Creanga, 2009). The magnetic effects were also produced by magnetic nanoparticles which influenced enzymatic activities and photosynthesis.

2.4 TITANIUM DIOXIDE

Among other inorganic nanoparticles, TNPs are produced by tons in the world and are included in number of commercial products. This large production results in their release to the environment. Titanium dioxide, one of the most suitable semiconductor material occurring naturally. It is the non-toxic material, inexpensive and having high surface-to-volume ratio. TiO_2 occur in nature in three forms anatase, rutile and brookite.

Table 2.1: Crystal systems of different phases of TiO_2

PHASE	CRYSTAL SYSTEM
Anatase	Tetragonal
Rutile	Tetragoanl
Brookite	Orthohombic

Photo-catalytic activity of TNPs makes them potential candidate for numerous technological applications that may result in the release of TNPs into the world ecosystem. The synthesis of TNPs reaches two million tons per year (Larue *et al.,* 2012a).

2.4.1. Why titanium dioxide (TiO_2)?

TNPs are used in this study to evaluate its impacts on soil and plants in detail. It is gaining attention in the field of research from history. TNPs are used for experimentation because;

- Titanium is the basic element on earth. All groups of plants (El Ghonemy *et al.,* 1977; Guha and Mitchell, 1965), aquatic organism (Dumon and Ernst, 1988), lichens (Takala and Olk konen, 1985) and fungi (Silverman and Muñoz, 1971) having mineral form of titanium. It is becoming higher in concentrations in the aquatic life (Dumon and Ernst, 1988).
- The fractions of atoms located at the outer surface of TNPs increases with the decreased size of TNPs. Fraction of atoms further enhances the surface area to volume ratio and catalytic activity (Xiaobo and Samuel, 2007). All these properties of TiO_2 are increasing their use in food, agriculture, cosmetics and medical fields, etc.
- TNPs having different ways of action which are making it important as both natural and synthetic element. Due to nanoscale size, it can be considered as a challenge to monitor their fate and mechanisms in environment. For this purpose, a lot of research is required to determine the possible impacts of TNPs.

2.4.2. Titanium dioxide in soils

Titanium is found in the earth crust as the 10^{th} most common element (McClendon, 1976). Two important minerals of titanium are rutile (titanium dioxide) and ilmenite (ferrous titanate) mainly found in sands (Dumon and Ernst, 1988). In particular, various soil, air and water pollutants can be reduced by TNPs because of their ability to degrade these photocatalytically. That's why their use is increasing (Higarashi and Jardim, 2002; Nagaveni *et al.,* 2004; Quan *et al.,* 2005; Aarthi and Madras, 2007). TNPs may cause potential health risks to the ecosystem and human body when used in excess quantities (Wiesner *et al.,* 2006). It is, therefore, necessary to study and understand the fate, life cycle, and transport mechanisms of TNPs with in the environment to assess the impacts of these on ecosystem and human health. Extensive use of TNPs might cause deposition on outer

surface of soil particles, where they interact differentially depending on their texture. It is found that TNPs are mobile in sandy soils and can pass 370 cm large soil column (Fang *et al.,* 2009) and they can immobilize anions with in soil particles (Mattigod *et al.,* 2005). Gottschalk *et al.* (2009) concluded from their study that TNPs have the greatest concern for the environment.

2.5 EFFECTS OF TITANIUM DIOXIDE NANOPARTICLES ON PLANTS

2.5.1. Effects of TiO_2 NPs on plant growth

The effects of TNPs were investigated on spinach seeds by measuring the growth and germination rate. Throughout the growth stage, the activity of Rubisco activase and light absorbance were improved by these nanoparticles with enhanced spinach growth (Hong *et al.,* 2005; Gao *et al.,* 2006 and 2008; Lei *et al.,* 2007; Linglan *et al.,* 2008; Xuming *et al.,* 2008; Mingyu *et al.,* 2008). TNPs (anatase) also improved nitrogen metabolism, as a result, absorption of nitrate in spinach got increased that might help to promote plant growth by converting inorganic nitrogen to organic one (Yang *et al.,* 2006).

A completely randomized block design was used to determine the effects of TNPs on physiological factors of barley. Five concentrations of TiO_2 were used; control, bulk TiO_2, 0.01, 0.02, and 0.03 percent. The nanoparticles were applied through spray during stem elongation and leaves formation stage. Different factors such as harvest index, grain yield, number of spikelets and weight were determined. The results had shown that spraying had significant effects on grain yield and number of spikelets. But harvest index and weight of spikelets had insignificant effects of TiO_2 (Moaveni *et al.,* 2011).

2.5.2. Accumulation of TiO_2 NPs

Studies have reported the effects and accumulation of TNPs in plants. Laure *et al.* (2012b) studied the response of wheat to TNPs grown in hydroponic conditions. The diameter of TNPs ranged from 12 to 140 nm. According to their estimates, TNPs with diameter less than 36 nm were translocated to the leaves but more reached in the roots i.e. 109 mg Ti/kg dry weight. Moreover, crystal structure of TNPs was not changed during transfer to tissues of wheat. TNPs with diameter 14 and 22 nm enhanced root length. At the same time, it did not affect seed germination, plant development and photosynthesis. Kurepa *et al.* (2010) also confirmed the uptake of the ultra-small anatase TNPs in *Arabidopsis*-

thaliana. Results demonstrated that TNPs were found in plant cells.

2.5.3. Effects of TiO_2 NPs on photosystem of plants

Another study compared the ability of anatase TNPs and TiO_2-quantum dot (QD) assembly for the solar energy conversion. Distribution and uptake of light in plant cells helped to improve the light harvesting contents. These QDs improved photosynthetic efficiency of plants by trapping solar energy (Kongkanand *et al.,* 2008). The QDs photoluminescence is also being used in imaging of cells.

Ze *et al.* (2011) studied the effects of TNPs on plant growth. They reported that TNPs increased the growth of plants by stimulating the process of photosynthesis. TNPs improved the light absorption capacity of chloroplast, enhanced light-harvesting complex II (LHC II), and also speeded up water photolysis, oxygen evolution and formation of electronic energy from light energy. The interaction of anatase TNPs with the content of LHC II on thylakoid membranes of spinach was also determined. The results depicted an increase in LHC II content (Lei *et al.,* 2007; Hong *et al.,* 2005).

2.5.4. Phytotoxic effects of TiO_2 NPs

Castiglione *et al.* (2011) investigated the phyto-toxicological effects of TNPs. The effects were studied on seed germination, seedling development, root mitosis, chromosomal aberrations and micronuclei release of *Vicia narbonensis* and *Zea mays*. The concentration levels of 0.2-4.0% of TNPs were used for both plants. Their findings revealed that TNPs inhibited germination in both plants during first 24 hours. The mitotic activity and root lengths were altered at higher concentration level of TNPs, indicating genotoxic effects of TNPs.

Boonyanitipong *et al.* (2011) studied the toxicity of ZnO nanoparticles and TNPs on germination of rice seedlings. It was concluded that ZnO nanoparticles had more negative effects on root growth in comparison with TNPs. Another study conducted by Du *et al.* (2011) shown that ZnO nanoparticles and TNPs reduced wheat biomass. TNPs remain in soil for long duration and might create problems for deep layer soils. But the solubility of TNPs is less as compared to ZnO nanoparticles which resulted in more uptake of toxic Zn by wheat. It has been found that anatase TNPs cause antioxidant stress in spinach when analyzed under UV-B radiation by increasing the production of oxygen. This stress results in reduction of hydrogen peroxide, superoxide radicals, and malonyldialdehyde. While the

activities of catalase, ascorbate peroxidase, superoxide dismutase and guaiacol peroxidase were enhanced, which negatively affected the actions of chloroplast (Lei *et al.*, 2008). Another study had shown that colloidal suspensions of inorganic nanoscale materials could affect the external water supply and quality of maize plantlets. These materials were accumulated in roots of plants through cell wall. The pore size of cell wall and water flow capacity through roots were affected by bentonite clay and TNPs. TNPs were adsorbed on plant roots that in turn led to inhibit transpiration and growth of leaf. The report also suggested that the effects of nanoparticles suspensions on shoot growth had less negative response if irrigated with them for long duration (Asli and Neumann, 2009).

Critically analyzing the literature cited, it is obvious that there are differential effects of nanoparticles in various plants species. The responses are also linked to the type of nanoparticles and dosage. Moreover, the exposure time and plant growth stage can modify the response to a particular type of nanoparticle. In short, the response of plants to nanoparticles varies with its nature, plant species and different growth stages along with their concentration level. In this context, there is need for comprehensive studies on different plant species coupled with already mentioned other factors. The current work is a humble contribution to understand the effects of TNPs on lettuce and wheat with reference to phosphorus availability in soil.

Chapter 3

MATERIALS AND METHODS

Experiments were carried out at Institute of Environmental Sciences and Engineering (IESE), National University of Sciences and Technology (NUST), Islamabad, Pakistan to assess the effects of nanoparticles on phosphorus availability in soil and test plants, chlorophyll content, ROS and DNA damage. The details of all kinds of experimentations are provided in this chapter.

3.1 CHEMICALS

Details of the chemicals used in the research work are given below.

Table 3.1: List of chemicals used in this study

CHEMICAL	SUPPLIER	PURPOSE
Titania (TiO_2) General Purpose Reagent (GPR)	Daejung Korea CAT No. 1053-4400	Nanoparticles preparation
Ethanol (C_2H_5OH)	BDH AnalaR, England Prod: 10107 7Y	Nanoparticles preparation & for micronuclei assay
Sodium bicarbonate ($NaHCO_3$)	Sigma-Aldrich, USA CAS: 144-55-8	P extraction
Sulfuric acid (H_2SO_4)	BDH AnalaR, England Prod: 10276 6H	P analysis
Ammonium molybdate tetrahydrate $(NH_4)_6MO_7O_{24}.4H_2O$	Sigma-Aldrich, USA CAS: 12027-67-7	Soil P analysis
Potassium antimony tartrate ($KsbO.C_4H_2O_6$)	Sigma-Aldrich, USA CAS: 28300-74-5	Same as above
Ascorbic acid ($C_6H_8O_6$)	Sigma Aldrich, UK CAS: 50-81-7	Same as above
Potassium dihydrogen phosphate (KH_2PO_4)	Honeywell, Frankfurt, Germany CAS: 7778-77-0	Source of P
Ammonium vanadate (NH_4VO_3)	PRS Panreac, E.U CODE No. 142352.1209	Plant P analysis

Nitric acid (HNO_3)	Merck KG AA, Germany CAT No. 1.00456.2500	Plant digestion
Per chloric acid ($HCLO_4$)	Fischer, Wan Chai, HK CAT No: P102511	Same as above
Hydrogen peroxide (H_2O_2)	Merck KG AA, Germany CAT No. 1.08600.1000	Source of hydrogen peroxide
Potassium iodide (KI)	Sigma-Aldrich CAS: 7681-11-01K	ROS determination
Trichloroacetic acid (TCA)	Uni-Chem CAS: 76-03-9	Same as above
Glacial acetic acid ($C_2H_4O_2$)	Sigma-Aldrich, USA CAS: 64-19-7	Roots fixation
Hydrochloric acid (HCl)	BDH AnalaR, England CAS: 7647-01-0	Micronuclei assay: roots hydrolysis
Potassium metabisulfite ($K_2S_2O_5$)	BDH AnalaR, England Prod: 10213 6G	Stain preparation
Basic fuchsin	Aldrich-Chemie, Germany CAT No. 42500	Same as above

3.2 PREPARATION AND SIZE CHARACTERIZATION OF TiO_2 NANOPARTICLES

3.2.1. Preparation of TiO_2 nanoparticles

For TNPs application in soil, first TNPs were synthesized using Liquid Impregnation (LI) method in IESE laboratory. Precisely, 6 g of titania (general purpose reagent, purity>99%) was added in 240 mL distilled water and 12 mL absolute ethanol was added to the solution and stirred vigorously to obtain homogenous solution at 325 rpm on magnetic stirrer (model: Stuart SB162). After 48 hours of stirring, the solution was sonicated at room temperature for 40 minutes. Sonication was done using JAC Ultrasonic 1505, JINWOO.

After complete precipitation, the slurry was allowed to settle overnight. The pH of TNPs was found to be 6.47. The final solution was dried at 105^{o} C for 12 hours in hot air oven. Finally, the dried material was powdered in mortar pestle and then calcined in muffle furnace (NEY M-525 series II) at 500 ^{o} C for 5 hours (Khan *et al.,* 2013).

3.2.2. Size and characterization of TiO_2 nanoparticles

The crystal structure of prepared sample of TNPs was characterized by X-ray diffraction (XRD) method and the average crystallite size was calculated through Scherrer's calculator using X'Pert Highscore. The XRD pattern of TiO_2 was obtained using STOE, Scintag Theta-Theta X-ray Diffractometer model with CuKα radiation in the 2Θ scan range of 20^o-80^o (λ= 0.154 nm) with a step of 0.5^o.

The surface morphology of TNPs was determined by SEM (JSM-6490A, JEOL) with an accelerating voltage of 20 kV. Before scanning, the powdered TNPs were diluted 100-fold in distilled water and then sonicated for 30 minutes. A drop of 10 µL diluted solution was placed on a carbon stub and air dried. The dry powder was sputter coated with gold in order to increase conductivity of surface. The coating of TNPs was done by using Atomic Ion Sputtering Device, JEOL, JFC-1500, Gold $250A^o$.

Energy dispersive X-ray spectroscopy (EDX) measurements were performed on a Compact Detector Unit (CDU) incorporated into SEM. The EDX spectrum was obtained at an acceleration voltage of 20 kV and collected for 50s. Pseudo-colors were used for mapping to represent the two-dimensional spatial distribution of energy emissions from the chemical elements present in the sample.

3.3 SOIL PREPARATION

For the application of TNPs in soil and to study their effects, first the soil was purchased from local nursery. A week before the start of the experiment, the representative soil was air dried for 3 days to remove moisture content. Air dried soil was pulverized using Ball Mill and then passed through sieve shaker to remove pebbles, roots and vegetation. With the help of sieve shaker, < 2 mm soil particle size was achieved. This clean and processed soil material was used in the experiments.

3.4 PLANT SPECIES AND PREPARATION

Wheat (*Triticum aestivum*) and lettuce (*Lactuca sativa*) were selected as test plant species to analyze the effects of TNPs. Wheat and lettuce belong to different angiosperm families; monocots and dicots respectively. The species were chosen as biological material considering their economic importance for agriculture and foods in world especially in Pakistan.

Seedlings of lettuce were obtained from local nursery. Seeds of wheat were obtained from the Ayub Agriculture Research Institute, Faisalabad, Pakistan. We used the hard variety of wheat 'Galaxy 70'. Healthy seeds were selected for experimentation. The seeds of wheat were soaked for six hours and their germination was conducted on wet soil (3 seeds per pot) at ambient temperature. Plastic pots were used for experimentation and purchased from local nursery.

The plants were grown in nanoparticles mixed soil with concentrations of 0, 20, 40, 60, 80, 100 mg kg^{-1}. Different studies have reported that the concentrations of TNPs below 100 mg kg^{-1} had significant effects on plants (Larue *et al*., 2012a; Feizi *et al.*, 2013; Salama, 2012; Arora, *et al.*, 2012; Azimi *et al.*, 2013).

3.4.1. Soil preparation for lettuce plant

Five concentrations i.e. 20, 40, 60, 80, and 100 mg kg^{-1} of TNPs were prepared for lettuce plants. There were four replicates for each concentration level. One and half kilogram soil was weighed for each treatment and then TNPs were mixed manually in 300g soil for each pot. The pots were filled with TNPs mixed soil one by one. Plastic pots were labeled accordingly. Freshly grown lettuce plants were purchased from local nursery. The age of seedlings was 15 days at the time of purchase. Roots and shoots of plants were washed carefully to ensure surface clarity. Plants were shifted to pots containing nanoparticles amended soil carefully and watered as per requirement.

3.4.2. Soil preparation for wheat plant

The concentrations of TNPs were 20, 40, 60, 80, 100 mg kg^{-1} for wheat plant. The five concentration levels were prepared by weighing calculated amounts of TNPs and mixed in the soil directly. Soil (1.5 kg) was weighed for four replicates of each level. Different concentrations of TNPs were mixed manually in 300 g soil for each replicate and then pots were filled with TNPs mixed soil one by one.

3.4.3. Control group

In every experiment, control group was also taken for comparison with the treated ones. For the control, nanoparticles were not added in the soil.

3.4.4. Experimental design

The experimental design was randomized complete block design. The plants were kept in locally made green house for growth.

3.5 CHARACTERISTICS OF EXPERIMENTAL SOIL

3.5.1. Soil pH

Soil pH measurement is useful because it can predict various chemical activities within the soil. Soil pH was calculated to check soil type (acidic, neutral or basic) and to determine suitability of soil for plant growth. To measure soil pH, soil: water (1:5) suspension was prepared by adding 10 g of dried soil (< 2 mm) in 100 mL glass beaker. 50 mL of distilled water was added using a graduated cylinder. It was stirred well using mechanical shaker at 180 rpm for 30 minutes. The pH of suspension was measured using combined electrode (model: HACH). The pH reading of each replicate was taken after 30 seconds (McLean, 1982). Initially, the average soil pH was around 7.6.

3.5.2. Moisture content

Ten grams of air dried soil (< 2 mm) was taken in a Petri dish. It was dried in hot air oven at 105°C overnight, with the lid unfitted. After this, Petri dish was removed from oven and then cooled in a desiccator for at least 30 minutes and re-weighed (Olsen *et al.,* 1954). Moisture content was calculated with the help of following formula:

$$\% \text{ moisture in soil} = \frac{\text{wet soil- dry soil}}{\text{dry soil}} \times 100$$

3.5.3. Soil texture

Saturation percentage (SP) method was used to investigate the quantitative measurement of soil texture and water holding capacity. To characterize the soil texture, 100 g air dried soil was taken into 100 mL container. Then distilled water was added gradually and mixed uniformly until a saturated paste was obtained. The SP equals the weight of water required to saturate the dry soil sample divided by the weight of the dried soil. The SP values ranged between 23 – 28% with an average of 24.7%. According to the following table, our soil type was classified as sandy loam soil (USDA textural soil classification).

Table 3.2: Soil texture classification (USDA)

SATURATION PERCENTAGE	SOIL TEXTURE
0 < 20	Sand or loamy sand
20 - 35	Sandy loam
35 - 50	Loam or silt loam
50 - 65	Clay loam
65 - 80	Clay
> 81	Organic soils

3.6 DETERMINATION OF EFFECTS OF TiO_2 NPs ON TEST PLANTS SPECIES

The effects of TNPs were determined after plants harvesting. The exposure time of TNPs for wheat and lettuce plants was of 60 and 75 days, respectively. Morphological parameters like root and shoot lengths, root and shoot weights of both plants were measured separately.

3.6.1. Plants length measurement

After harvesting of wheat and lettuce plants, roots and shoots were washed with distilled water, collected separately and their lengths were measured.

3.6.2. Plants biomass determination

Roots and shoots of lettuce and wheat plants were cut and their fresh biomass weighed one by one. Then they were kept in petri dishes for drying in oven at 80^{o}C with the lid unfitted. The dry biomass was taken after 48 hours. The dried samples were ground well and stored for phosphorus analysis.

3.7 PHOSPHORUS ANALYSIS

Phosphorus analysis was done in rhizosphere soil and test plant species, lettuce and wheat separately.

3.7.1. Phosphorus analysis in soil

The Phosphorus analysis in rhizosphere soil was measured using the ascorbic acid method (Olsen *et al*., 1954). The Olsen's method details of extractable soil phosphorus are as follow:

3.7.1.1 Preparation of reagents

A. 0.5M sodium bicarbonate solution ($NaHCO_3$): 42g of $NaHCO_3$ was dissolved in approximately 700 mL distilled water, shaken well and diluted to 1L. The pH was adjusted to 8.5 using 5N NaOH.

For the preparation of 5N NaOH, 50g NaOH was dissolved in distilled water and cooled and then volume was made to 250 mL.

B. Mixed reagent

(a) Ammonium molybdate tetrahydrate $(NH_4)_6MO_7O_{24}.4H_2O$: 12g of $(NH_4)_6MO_7O_{24}.4H_2O$ was dissolved in distilled water and diluted to 250 mL.

(b) Potassium antimony tartrate ($KsbO.C_4H_2O_6$): 0.291 g of $KsbO.C_4H_2O_6$ was dissolved in distilled water and then the solution was diluted to 100 mL.

(c) 5N H_2SO_4: 148 mL of concentrated sulfuric acid was diluted in distilled water and then cooled. After this, volume was made to 1L.

(d) Then both the dissolved reagents (a+b) was added in 1000 mL 5N H_2SO_4 and diluted to 2000 mL with distilled water. The solution was stored in dark and cool place in a pyrex bottle.

C. Color developing reagent: 0.528g ascorbic acid was weighed and added to 100 mL of mixed reagent. This reagent was prepared freshly for achieving accurate results.

D. Stock solution: For the preparation of stock solution, 2.5g potassium dihydrogen phosphate (KH_2PO_4) was dried in oven for one hour at 105 °C and then cooled in a desiccator. The dried chemical was stored in air tight bottle to avoid moisture. Exactly, 2.197g potassium dihydrogen phosphate (KH_2PO_4) was dissolved in distilled water and diluted to 500 mL with distilled water. The concentration of this solution was 1000 mg L^{-1}. Precisely, 10 mL stock solution was diluted to 100 mL final volume with distilled water. This solution contained 100 mg L^{-1} of phosphorus.

Standards: The standards were prepared from 100 mg L^{-1} stock solution as follows. Precisely, 0.125, 0.1875, 0.25, 0.3125, 0.375, 0.4375, 0.5, 0.5625, 0.625, 0.6875, 0.75,

0.875 and 1 mL was diluted to 25 mL with distilled water. These solutions contained 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.25, 2.5, 2.75, 3, 3.25, 3.5, 3.75 and 4 mg L^{-1} phosphorus respectively.

3.7.1.2 Procedure

2.5 g of air dried soil (< 2 mm) was weighed in a 250 mL conical flask. 50 mL of extracting solution (0.5M sodium bicarbonate solution) was added. Flask was stoppered and shaken for 30 minutes on a mechanical shaker at 180 rpm. The solution was filtered with Whatmann no. 42 filter paper, and 5 mL extract was taken for phosphorus analysis in a 25 mL volumetric flask. 5 mL of color developing reagent was added and flask was shaken to remove air bubbles and then diluted to 25 mL with distilled water. Bluish color was developed. The blue color was representing the concentration of phosphorus in the soil. The reading was taken after 15 minutes at 880 nm wavelength on spectrophotometer. A calibration curve was prepared for standards, by plotting absorbance at Y-axis and phosphorus concentration at X-axis. This calibration curve was used to calculate the concentration of phosphorus in the unknown samples.

3.7.2. Phosphorus analysis in plants

The effect of TNPs on available phosphorus in lettuce and wheat plants was analyzed. The ground plant samples were used for phosphorus analysis. The dried material was digested in concentrated nitric acid- perchloric acid (HNO_3- $HClO_4$) mixture (2:1) on hot plate. The analysis was done on roots and shoots of test plants separately. The phosphorus contents were measured at 430 nm by spectrophotometer with the help of vanadomolybdophosphoric acid colorimetric method (Ryan *et al.,* 2008).

3.7.2.1 Preparation of reagents

A. Nitrovanado-molybdic reagent was prepared by mixing following solutions in the same amounts.

(a) 5% ammonium molybdate solution (100 mL) ,

(b) 0.25% ammonium vanadate solution (100 mL)

(c) Diluted HNO_3 (HNO_3: H_2O :: 1:3) (100 mL)

B. 5% ammonium molybdate solution: 50g molybdate solution, $(NH_4)_6MO7O_{24}$. $4H_2O$ was dissolved in 700 mL warm distilled water (50^oC) and stirred well to dissolve. The -

solution was diluted to 1L after cooling.

C. 0.25% ammonium vanadate solution: 2.5g ammonium vanadate (NH_4VO_3) in boiling distilled water and then volume was made to 1L.

The solutions a, b, c were added in equal amounts.

Standard stock solution: 2.197g dried KH_2PO_4 was dissolved in 500 mL distilled water and it was 1000 mg L^{-1} phosphorus solution. Then, 10 mL of stock solution was diluted to 100 mL with distilled water. This solution contained 100 mg L^{-1} of phosphorus.

Standards: A series of standards were prepared from 100 mg L^{-1} stock solution as follows. Precisely, 0, 0.0625, 0.125, 0.1875, 0.25, 0.3125, 0.375, 0.4375, 0.5, 0.5625, 0.625, 0.6875, 0.75, 0.875 and 1 mL was taken and diluted to 25 mL with distilled water. These solutions contained 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.25, 2.5, 2.75, 3, 3.25, 3.5, 3.75 and 4 mg L^{-1} phosphorus, respectively.

3.7.2.2 Wet digestion method

Approximately 0.1g ground plant material was taken in 25 mL volumetric flask. 5 mL double acid mixture (HNO_3- $HClO_4$) was added to flask. Then it was shifted on hot plate in fume hood. Initially, temperature of hot plate was kept 150^oC. Brown fumes of nitrate came out when reaction was started. The temperature of hot plate was increased slowly. With the passage of time, formation of brown fumes was decreased and the color of the solution in the flask became light yellow. The heating was continued until clear transparent solution with white dense fumes appeared at the end. These white fumes indicated complete digestion process. Volumetric flasks were cooled. The clear solution was diluted and filtered with Whatmann no. 42 filter paper.

3.7.2.3 Procedure

After wet digestion of plant material, 2.5 mL of the aliquot taken into a 25 mL volumetric flask and 5 mL nitrovanado-molybdate reagent was added and volume was made up to the mark with distilled water. After one hour, the absorbance of the blank, standards, and for samples was measured using spectrophotometer at 430 nm wavelength as mg phosphorus kg^{-1}. A blank was also prepared with 5 mL nitrovanado-molybdate reagent for samples. A calibration curve was prepared to read phosphorus concentration in the samples.

3.8 TiO_2 NANOPARTICLES UPTAKE IN PLANTS

The presence of TNPs in roots and shoots of wheat and lettuce plants was recorded using SEM and EDX. To determine TNPs uptake in plants, all plants were washed thoroughly with distilled water and then oven dried at 80^{o}C for 48 hours. The images of thin sections of dried roots and shoots of both plants were acquired independently on SEM which is equipped with EDX.

3.9 ESTIMATION OF FOLIAR CHLOROPHYLL CONTENT

3.9.1. Plant material

Lettuce and Wheat plants used for this study were grown in a locally made green house and chlorophyll content was measured during month of March and April, 2014. Over the course of study, readings were taken after 30 and 45 days of TNPs exposure time for wheat and lettuce plants, respectively. Chlorophyll content measurements on plants were taken for 15 alternative days in the afternoons. There were four replicates for each treatment level. The measurements were made on each plant sample separately using thirty points averaging by hand-held chlorophyll absorbance meter. The readings were taken on the leaves of plants between the midrib and the leaf margin to avoid the placement of meter over major leaf veins.

3.9.1.1 Hand-held chlorophyll meter

Hand-held chlorophyll meter, CCM-200 plus was purchased from Opti-Sciences, England. The CCM-200 weighs 168g (battery not included), has a 0.71cm^2 measurement area, and calculates a Chlorophyll content index (CCI) based on the absorbance measurements. Peak chlorophyll absorbance is measured at 653 nm and non-chlorophyll absorbance (cell walls, veins, etc.) at 931 nm. Calibration was done every time the unit is powered up.

3.9.1.2 Data Analysis

For the analysis of data, arithmetic mean and calibration equation are used. The calibration equation converts CCI index values to chlorophyll content mg cm^{-2} (Richardson *et al.,* 2002).

$$y = -2.20e^{-03} + 3.09e^{-03}x - 5.63e^{-05}x^2$$

Where, y = Total chlorophyll content

X = Chlorophyll meter value

3.10 DETERMINATION OF H_2O_2 GENERATION

ROS have an unpaired electron in their outer shell that's why they are basically unstable molecules. The oxidization reactions occur when oxidative species interact with various cellular components of plants including DNA, lipids / fatty acids and proteins. The OS is generally the result of imbalance between the generation and the neutralization of ROS by antioxidant mechanisms. Among ROS, H_2O_2 is a very strong oxidant and capable of inactivating cell molecules even at a very low concentration, so it requires quick removal (Mishra *et al.*, 2006).

3.10.1. Procedure

The H_2O_2 was determined according to the protocol previously published by Islam *et al.* (2008). First, lettuce and wheat plants were harvested and roots were washed with distilled water to remove soil. Then these samples were frozen in liquid nitrogen and stored at -80^oC for analysis. 500 mg frozen root samples were homogenized with 3 mL of 0.1% (w/v) trichloroacetic acid (TCA) under liquid nitrogen, and was centrifuged at 12000 rpm for 30 minutes. The mixture assay contained 0.5 mL of the supernatant mixed with 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1M potassium iodide (KI). H_2O_2 content of the supernatant was measured at 390 nm and evaluation was done by comparing its absorbance with a standard calibration curve and expressed in µM g^{-1}.

Standards: A series of standards were prepared from 200 µM stock solution of H_2O_2 as follows. Precisely, 0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5 mL was taken from stock solution and diluted to 10 mL with distilled water. These solutions contained 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 µM H_2O_2 respectively.

3.11 MICRONUCLEI TEST

3.11.1. Principle

Micronuclei are produced as a result of chromosomal breakage during mitosis from lagging chromosomes. These are fragments of chromosomes that could not segregate during

the telophase. These fragments can be observed under the microscope in the form of small spherical cores separated from the principal core, called micronuclei.

3.11.2. Procedure

The roots of Wheat plants were used for micronuclei test after harvesting. The exposure time to the TNPs for the roots of wheat plant was 60 days while lettuce plants were exposed to NPs for 75 days. The roots of plants were washed with distilled water, and then 1 cm root ends were cut and placed in petri dishes containing Carnoy solution at 4^{o}C for 24 hours. After fixation, the roots were maintained in phosphate buffer saline (PBS) solution for 10 minutes and then they were hydrolyzed in 1N HCl for 15 minutes at 60^{o}C with the water bath. After that the roots were colored with Feulgen stain for 15-20 minutes at 60^{o}C with the water bath. Feulgen is coloring reagent that marks DNA. After staining, the root ends (2 mm) were cut and placed on glass slides. One drop of 45% acetic acid was added to avoid mixture drying and then roots were pressed with cover slip to prepare slides. The slides were examined under x40 magnification using a trinocular fluorescence microscope (Model No. OPTIKA B-353FL, Italy) available at Environmental Biotechnology Lab of IESE, NUST, Islamabad coupled with a digital camera DCM 130 with 1.3M pixels. The slides were observed under microscope to count the total number of cells with mitosis and cells with micronucleus (MN). The following relation was used to calculate micronucleus (Shahid *et al.*, 2011).

MN (%) = Number of micronuclei * 100 / total number of cells observed

3.11.3. Preparation of solutions

3.11.3.1 Carnoy solution

Carnoy solution was prepared by mixing glacial acetic acid and ethanol in the ratio of 1:3 respectively. Carnoy solution is a fixer which allows fixing the cells in metaphase.

3.11.3.2 Feulgen stain

For the preparation of feulgen stain, 150 mg basic fuchsin was weighed into 250 mL beaker. 30 mL boiling distilled water was poured over the basic fuchsin and then stirred well to dissolve. The solution was cooled to 50^{o}C and then filtered with Whatmann no. 1 filter paper. 4.5 mL 1N HCl was added and mixed. After that, potassium meta-bisulfite ($K_2S_2O_5$) was dissolved to decolorize the solution. This solution was then kept in dark for

24 hours. The prepared stain was stored in dark. The prepared solution was filtered through activated charcoal to further clarify if required.

3.12 STATISTICAL ANALYSIS

Differences between the values of control and treatment data sets were analyzed by using one-tailed t-test available in the analysis tool box in Excel. One-way ANOVA (Analysis of Variance) test was performed to identify statistically significant differences between treatments values and it was based on probabilities of $P<0.05$.

Chapter 4

RESULTS AND DISCUSSION

4.1 CHARACTERIZATION OF TiO_2 NANOPARTICLES

4.1.1. X-ray diffraction

The crystallite size and phase composition of the prepared TNPs were analyzed through XRD analysis as shown in Fig. 4.1. The peaks in the wide range of 2θ ($20^o < 2\theta < 80^o$) at 25.304º, 36.949º, 37.793º, 38.566º, 48.037º, 51.960º, 53.886º, 62.685º, 68.756º, 70.287º, and 75.046º can be attributed to the 101, 103, 004, 112, 200, 202, 105, 204, 116, 220 and 215 crystalline structure of anatase TNPs, respectively (Joint Committee on Powder Diffraction Standards, JCPDS Card no. 03-065-5714 of Anatase XRD). Strong diffraction peaks at 25.304º and 48.037º confirm that synthesized TNPs are in anatase phase (Ba-Abbad *et al.*, 2012).

4.1.1.1 Particle Size calculation

The average particle size has been estimated using Debye-Scherer calculator. Calculations show that prepared particles are less than 45.6 nm. Analysis of XRD peaks confirmed the small size, high purity, and crystalline structure of synthesized TNPs.

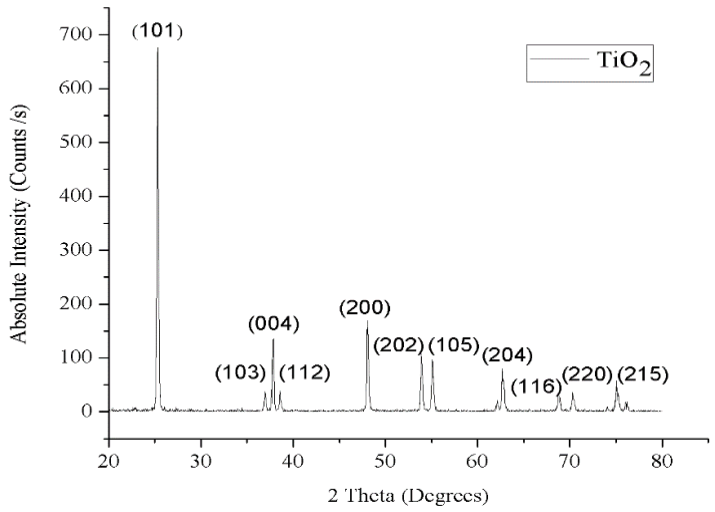

Figure 4.1: XRD pattern of synthesized TNPs

4.1.2. Scanning electron microscope (SEM) imaging

The surface morphology of TNPs was estimated by SEM. The image of the pure titania shows that particles are spherical in shape and distributed in the range of 11.93-18.67 nm (Fig. 4.2).

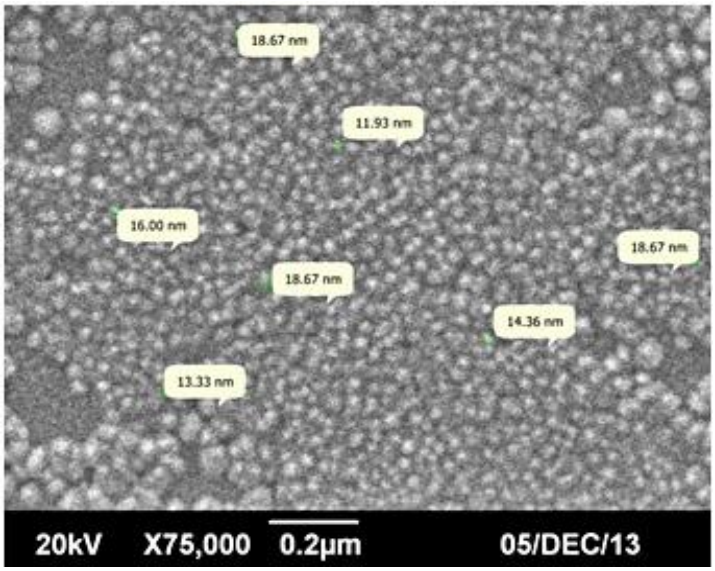

Figure 4.2: SEM image of TNPs

4.1.3. Energy-dispersive X-ray spectroscopy analysis

EDX is an analytical technique which is used for the chemical characterization or elemental analysis of a sample. Result indicated the presence of Ti and O elements in the representative sample as shown in Fig. 4.3.

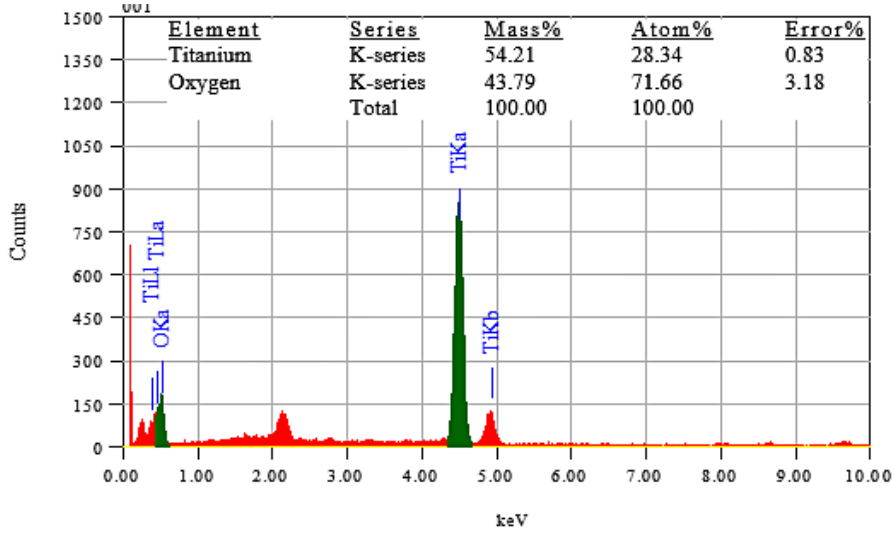

Figure 4.3: EDX analysis of TNPs

4.2 EFFECT OF TiO_2 NPs TREATMENT ON PLANT'S GROWTH

4.2.1. TiO_2 NPs effects on plants length

The effects of TNPs concentrations (0, 20, 40, 60, 80, 100 mg kg^{-1}) on wheat and lettuce plant's growth were investigated. Significant differences were observed in both plants as compared to the control.

4.2.1.1 Root and shoot length of wheat plant

After treatment with TNPs, the root and shoot lengths of wheat plants increased as compared to control. In wheat, root and shoot lengths were significantly ($p< 0.01$) increased at 60 mg kg^{-1} concentration level up to 42.2% and 35.7% respectively (Fig. 4.4). But at 100 mg kg^{-1} concentration level, the root and shoot lengths were decreased by 13.6% and 17.1% respectively as compared to 60 mg kg^{-1} concentration level. The bars show the SD of four replicates. The pictorial diagram of root and shoot lengths of wheat is given in Fig. 4.5, where Co represents the plant treated with no TNPs and C1, C2, C3, C4, C5 treated with 20, 40, 60, 80, 100 mg kg^{-1} of TNPs.

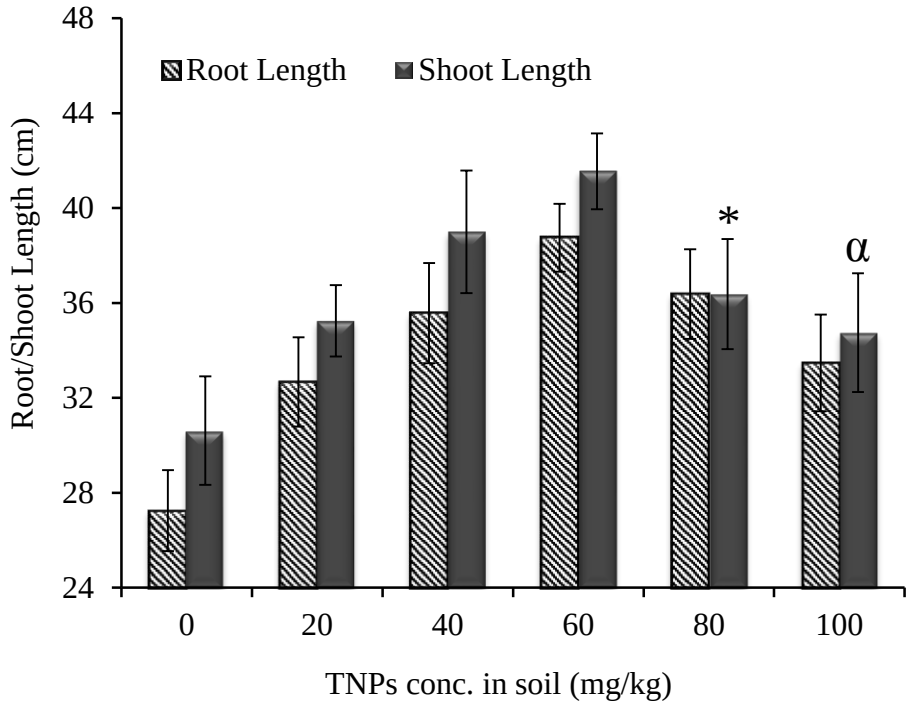

Figure 4.4: TNPs effects on root and shoot lengths of wheat. Error bars show SD and asterisk symbol (*) and α represent statistically significant difference at $p< 0.01$.

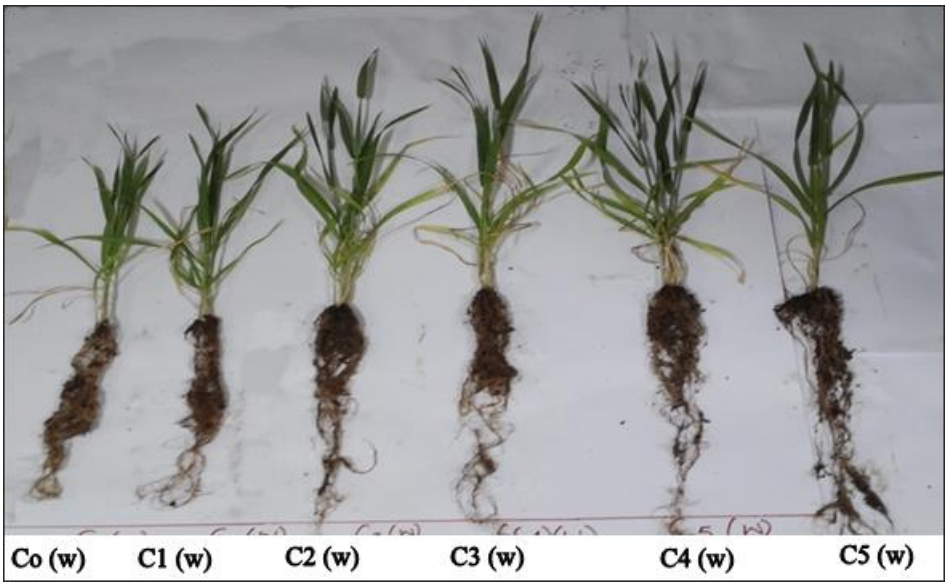

Figure 4.5: Effects of TNPs on root and shoot lengths of wheat plants

4.2.1.2 Root and shoot length of lettuce plant

Different concentrations of TNPs increased the root and shoot lengths of lettuce plants by 39.2% and 35.3% respectively in comparison with control as shown in Fig. 4.6. Moreover, the increase in shoot lengths was more significant than that of root lengths ($p < 0.05$). The pictorial representation of root and shoot lengths of lettuce is given in Fig. 4.7.

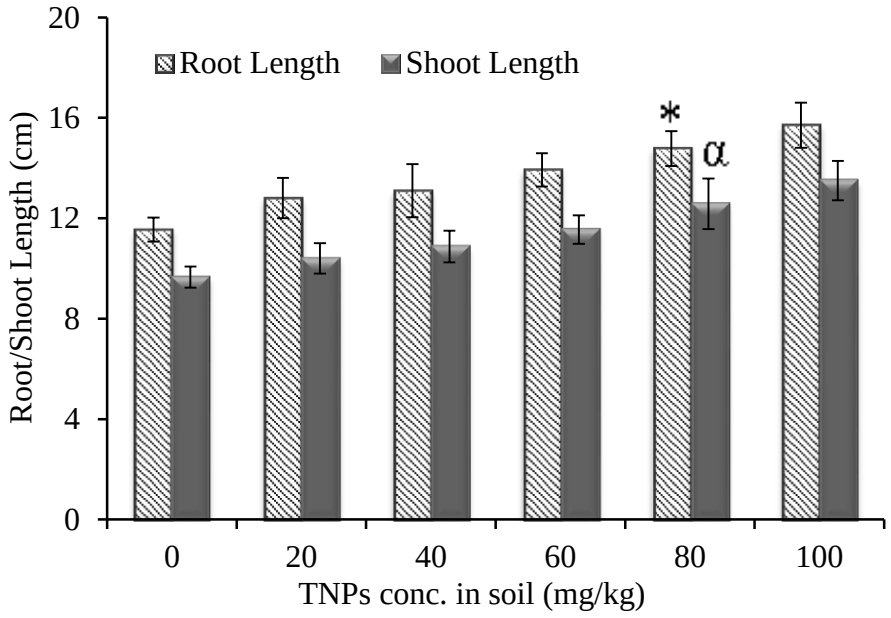

Figure 4.6: TNPs effects on root and shoot lengths of lettuce. Error bars show SD and asterisk symbol (*) and α represent statistically significant difference at $p < 0.05$.

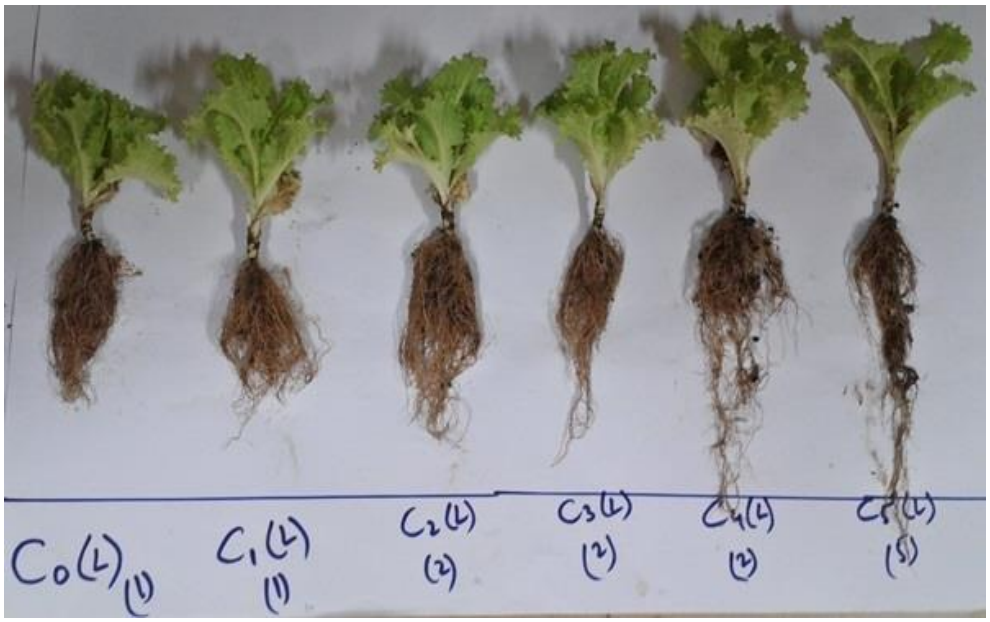

Figure 4.7: Effects of TNPs on root and shoot lengths of lettuce plants

4.2.2. TiO_2 NPs effects on plants biomass

4.2.2.1 Wheat plant biomass

The pictorial results of wheat biomass are given in Fig. 4.8. An increase in total fresh biomass by 60% and total dry biomass by 72% over control was observed ($p< 0.01$) at 60 mg kg^{-1} for wheat (Fig. 4.9). At the highest concentration 100 mg kg^{-1}, 9.6% decrease in fresh weight and 27.2% decrease in dry weight was observed as compared to 60 mg kg^{-1}.

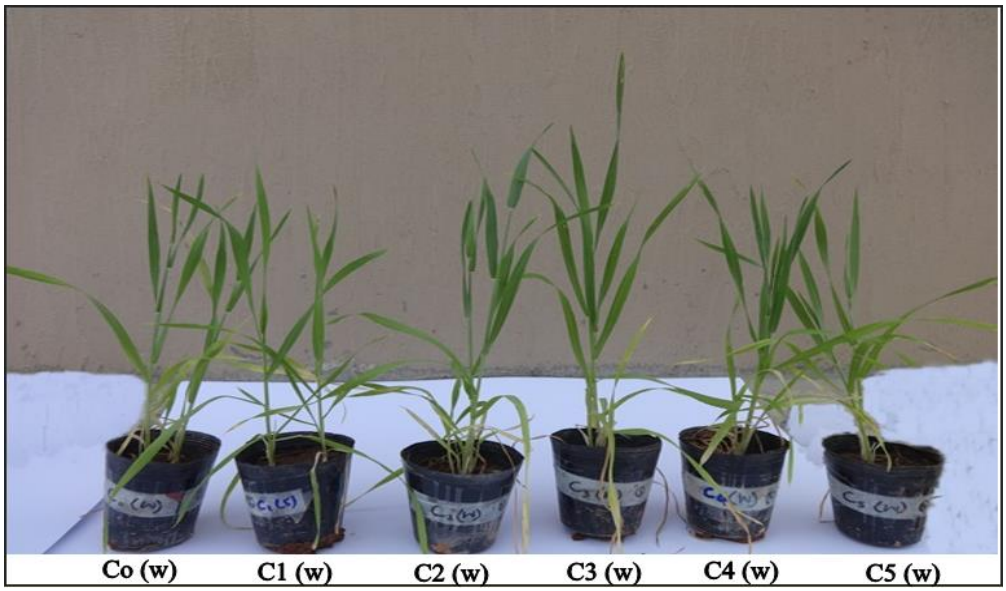

Figure 4.8: Effects of TNPs on wheat plants biomass

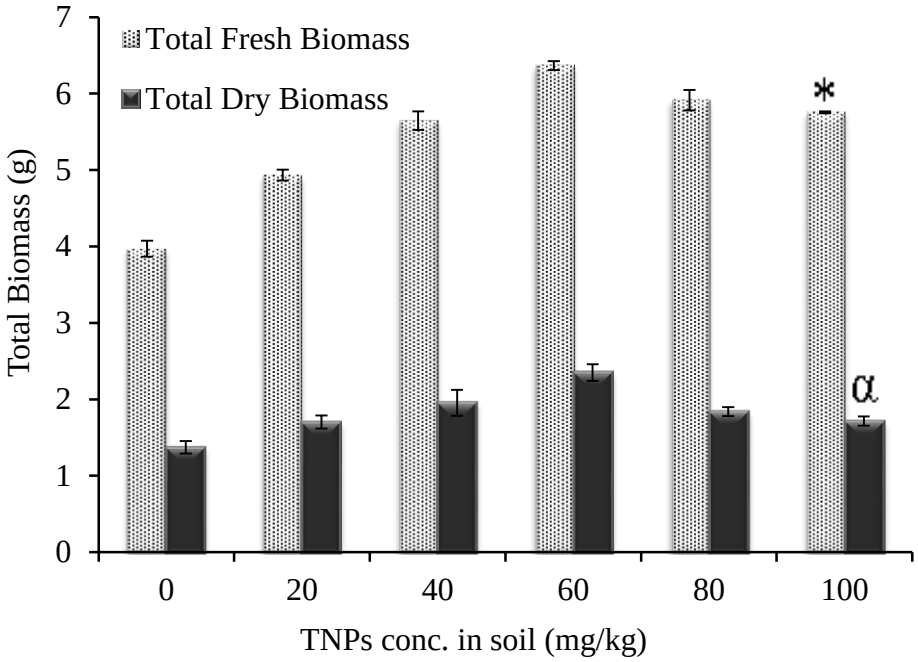

Figure 4.9: Effects of TNPs on wheat biomass. Error bars show SD and asterisk symbol (*) and α represent statistically significant difference at $p < 0.01$.

4.2.2.2 Lettuce plant biomass

The pictorial demonstration of lettuce biomass is given in Fig. 4.10. The total fresh and dry biomass was significantly ($p < 0.05$) higher as compared to the untreated plants by 46 and 52% respectively (Fig. 4.11).

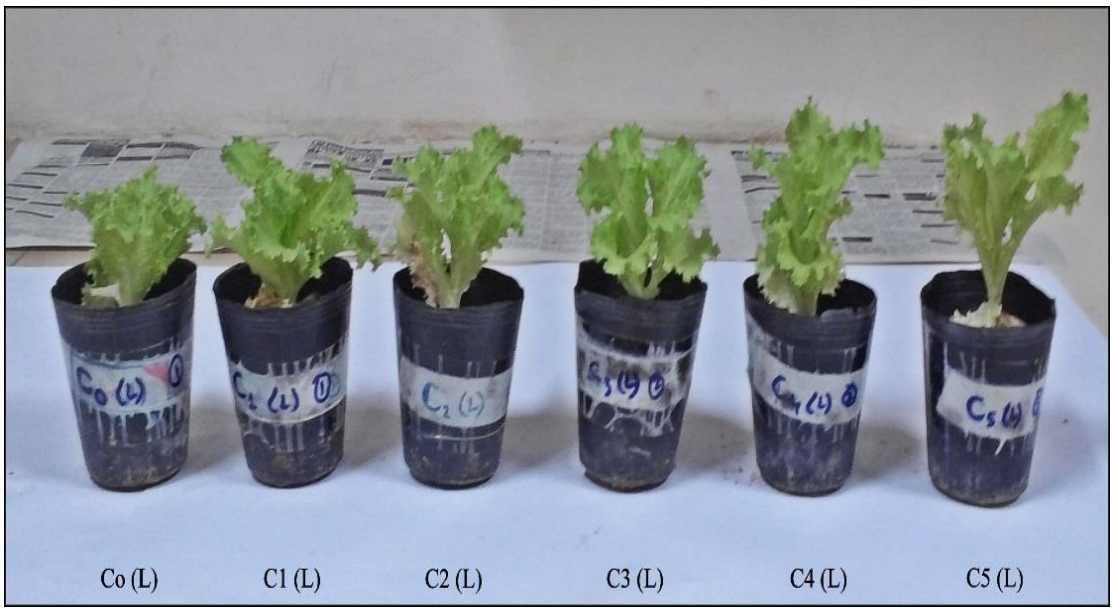

Figure 4.10: Effects of TNPs on lettuce plants biomass

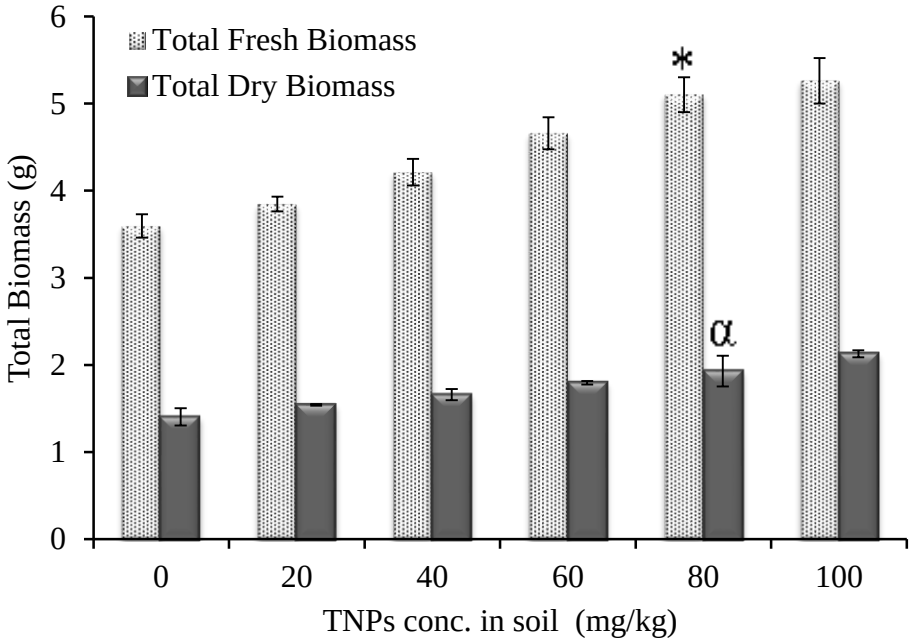

Figure 4.11: TNPs effects on lettuce biomass. The data is presented as mean SD and asterisk symbol (*) and α represent statistically significant difference at $p< 0.05$.

4.3 EFFECTS OF TiO_2 NANOPARTICLES ON PHOSPHORUS AVAILABILITY

4.3.1. Effects of TiO_2 NPs on soil pH and phosphorus availability

This study evaluated the effects of TNPs on rhizosphere pH and phosphorus availability in wheat and lettuce rhizosphere soil and plants.

4.3.1.1 Availability of phosphorus in wheat rhizosphere soil and plants

The effects of different treatments of TNPs on phosphorus concentration in plants and contribution of pH in phosphorus availability in wheat rhizosphere are presented in Fig. 4.12. The results shown that phytoavailability of phosphorus increased (54.80%) significantly as pH decreased ($p< 0.05$) from 7.24 to 6.44 at 60 mg kg^{-1} concentration level of TNPs. At the highest concentration 100 mg kg^{-1}, 19.7% decrease was observed in available phosphorus as compared to 60 mg kg^{-1} concentration level.

The concentration of phosphorus in wheat plants increased significantly ($P< 0.05$) by 45.7% at 60 mg kg^{-1} in comparison with control. Whereas, more addition of TNPs decreased the phosphorus concentration but the values are higher as compared to untreated plants. The concentration of P decreased at 100 mg kg^{-1} by 18.1% as compared to 60 mg kg^{-1} concentration level of TNPs.

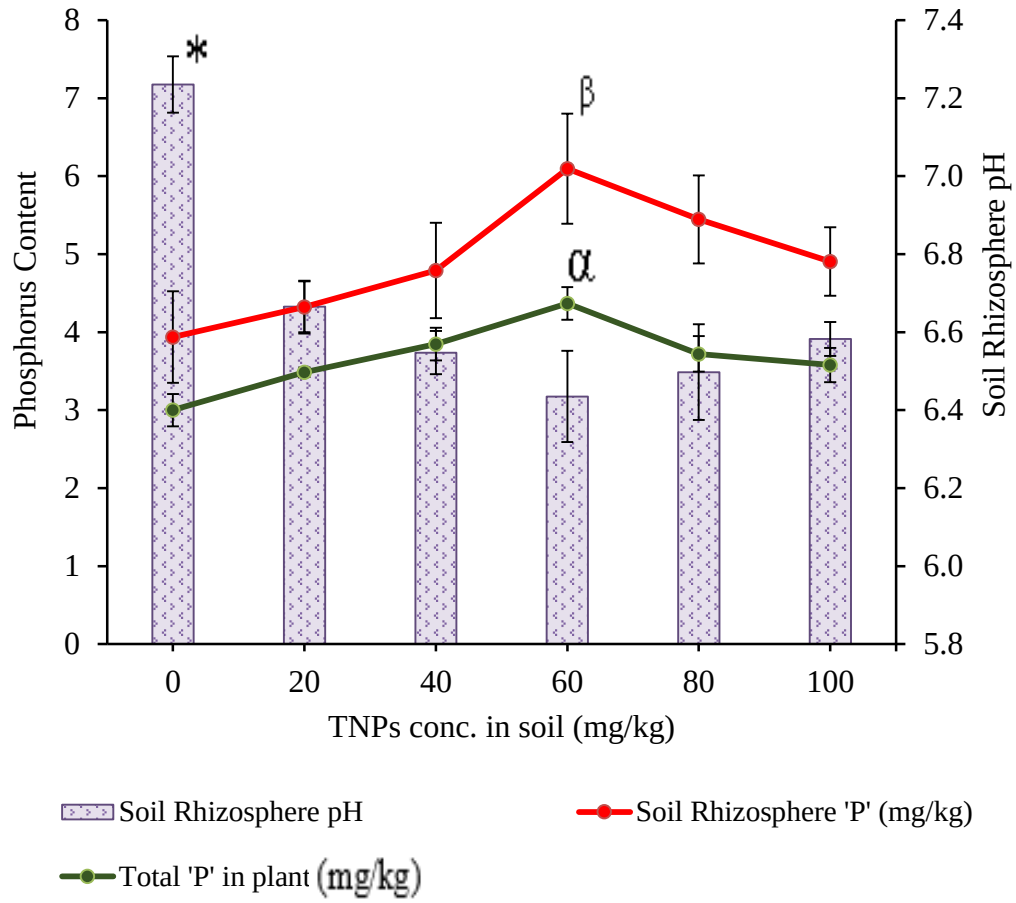

Figure 4.12: Effects of TNPs on wheat rhizosphere pH and P availability in soil and P contents in plants. Error bars show SD and asterisk symbol (*), α and β represent statistically significant difference at $p < 0.05$.

4.3.1.2 Availability of phosphorus in lettuce rhizosphere soil and plants

The effects of TNPs on lettuce rhizosphere pH and phosphorus availability in soil are shown in Fig. 4.13. With decrease in soil pH, the phosphorus availability increased with increasing concentration of TNPs. The concentration of phosphorus was significantly ($p < 0.05$) higher as compared to control at higher concentrations. The rate of P release ranged between 3.23 to 4.70 mg kg^{-1} (45.5%) and pH values 7.37 to 6.29 from the control to 100 mg kg^{-1}. Phosphorus concentration was increased significantly ($P < 0.05$) in lettuce plants by 62.2% as compared to control.

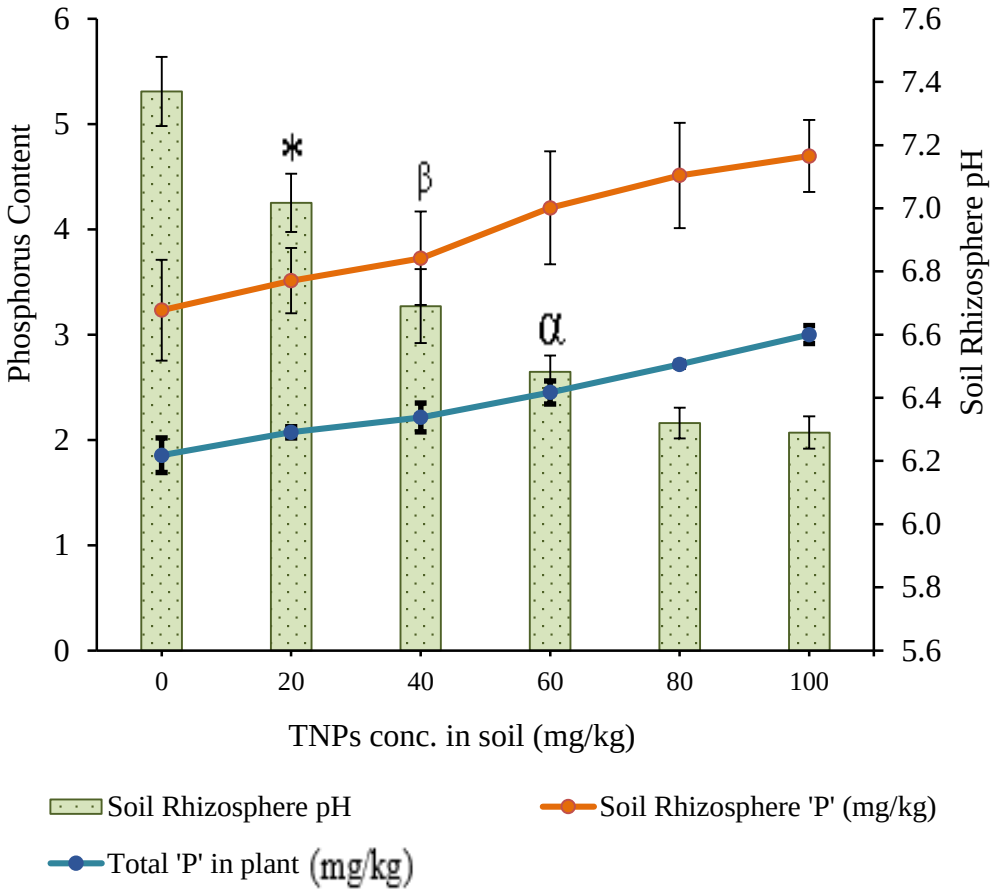

Figure 4.13: Effects of TNPs on lettuce rhizosphere pH and 'P' availability in soil and P contents in plants. Error bars represent SD and asterisk symbol (*), α and β indicate statistically significant difference at $p< 0.05$.

4.4 UPTAKE OF TiO_2 NPs

4.4.1. Observation of wheat plant using SEM-EDX

Figure 4.14 (A) shows SEM scans of leaves of control and Fig. 4.14 (C) represents the SEM image of wheat leaves when exposed to 100 mg kg^{-1} of TNPs. EDX results (Fig. 4.15B and D) of wheat roots indicated the presence of Ti in both control and treated plants.

4.4.2. Observation of lettuce plant using SEM-EDX

The results in Fig. 4.16A and B represent the control whereas Fig. 4.16C and D correspond to the results when exposed to 100 mg kg^{-1} of TNPs. It has been observed that Ti was observed only in leaves of treated plants in comparison with control plant's leaves. In roots of control plants (Fig. 4.17A and B) very low concentration of Ti was found as compared to the plants treated with high concentration of TNPs (Fig 4.17C and D).

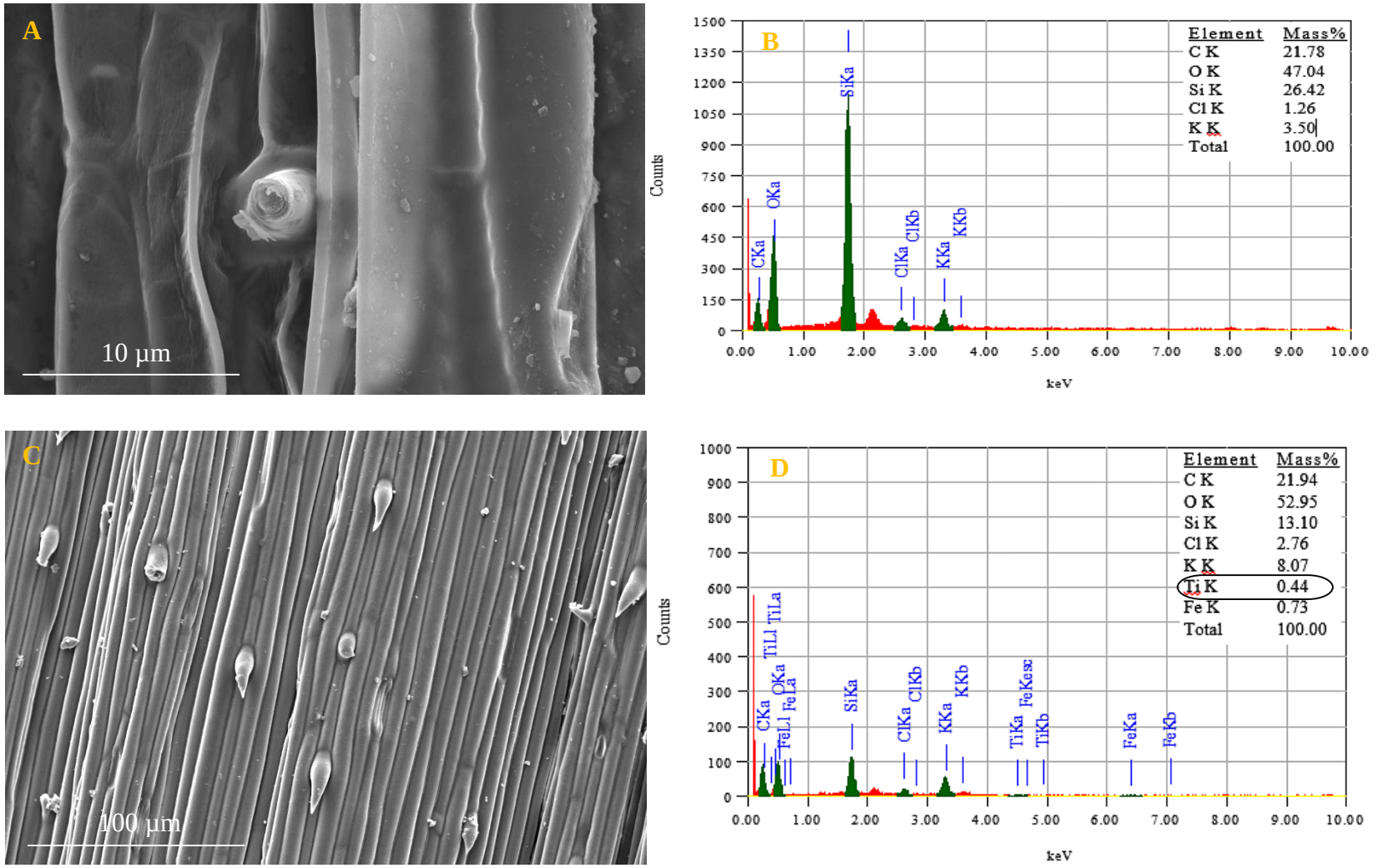

Figure 4.14: TNPs uptake in leaves of wheat, analyzed by SEM and EDX: A & B leaves (control group with no TNPs treatment), C & D leaves (100 mg kg^{-1} TNPs). The peaks show Ti mass% in the sample.

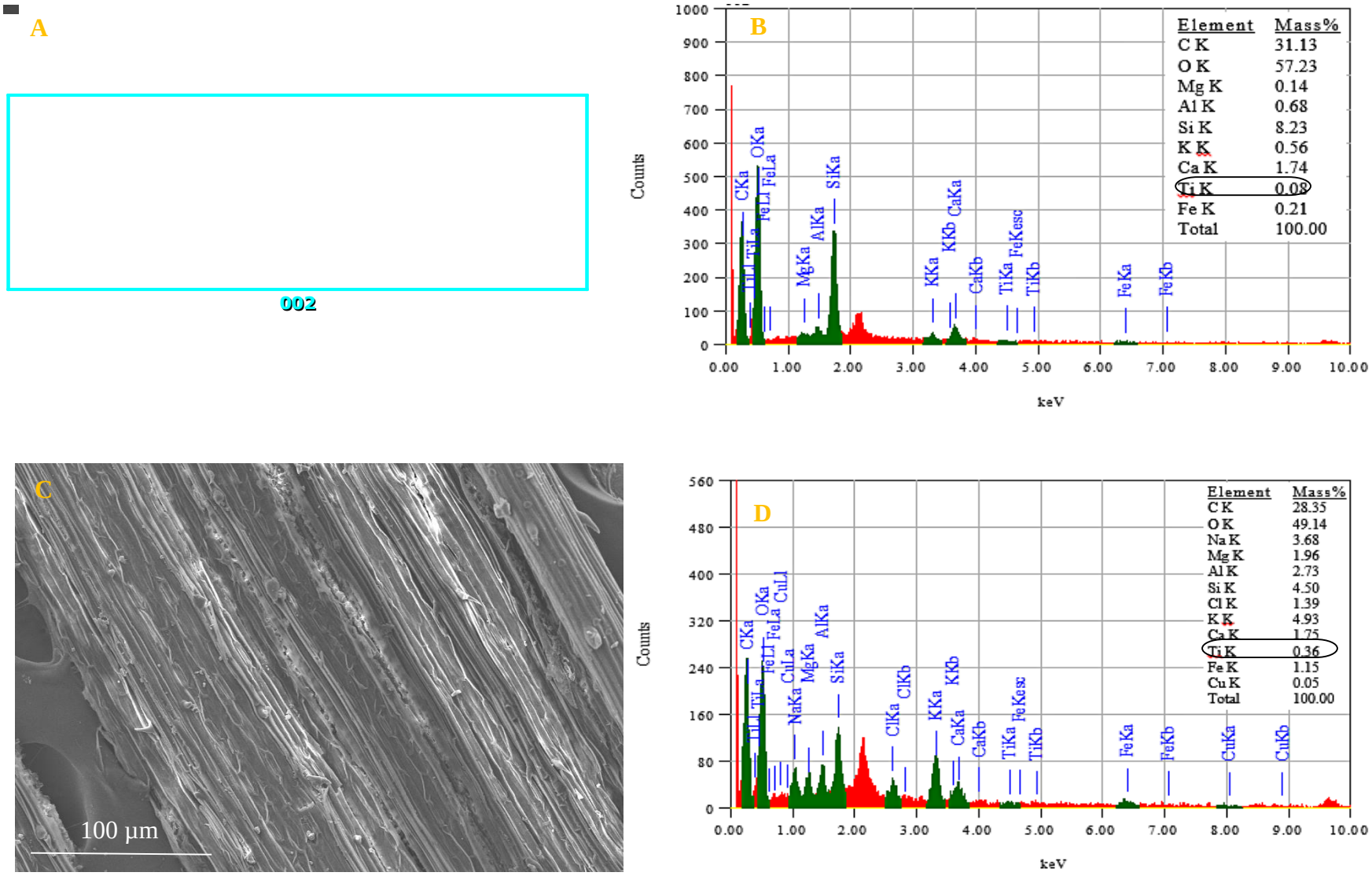

Figure 4.15: TNPs uptake in roots of wheat, analyzed by SEM and EDX: A & B roots (control group with no TNPs treatment), C & D roots (100 mg kg^{-1} TNPs). The peaks show Ti mass% in the sample.

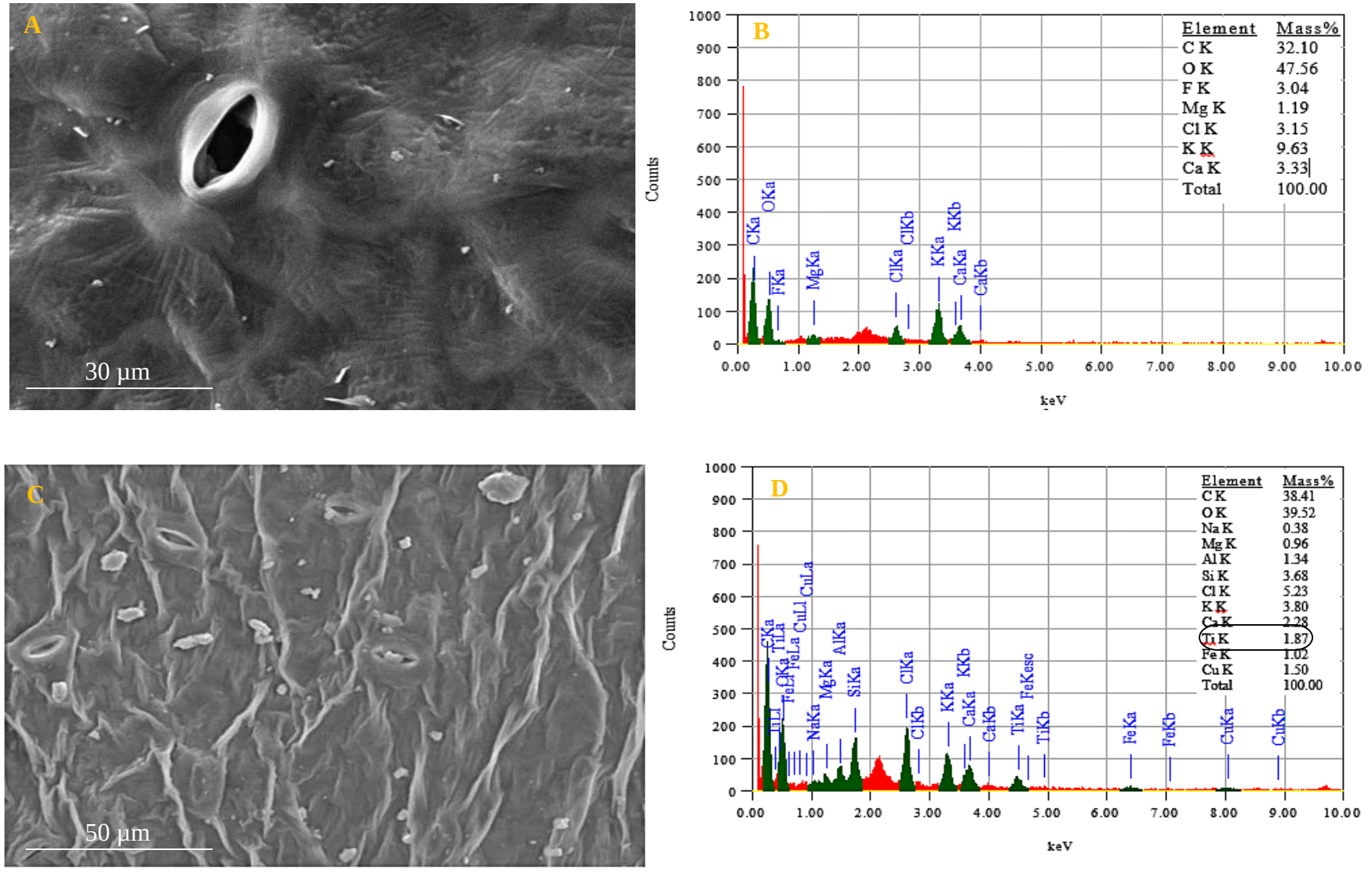

Figure 4.16: TNPs uptake in leaves of lettuce, analyzed by SEM and EDX: A & B leaves (control group with no TNPs treatment), C & D leaves (100 mg kg^{-1} TNPs). The peaks show Ti mass% in the sample.

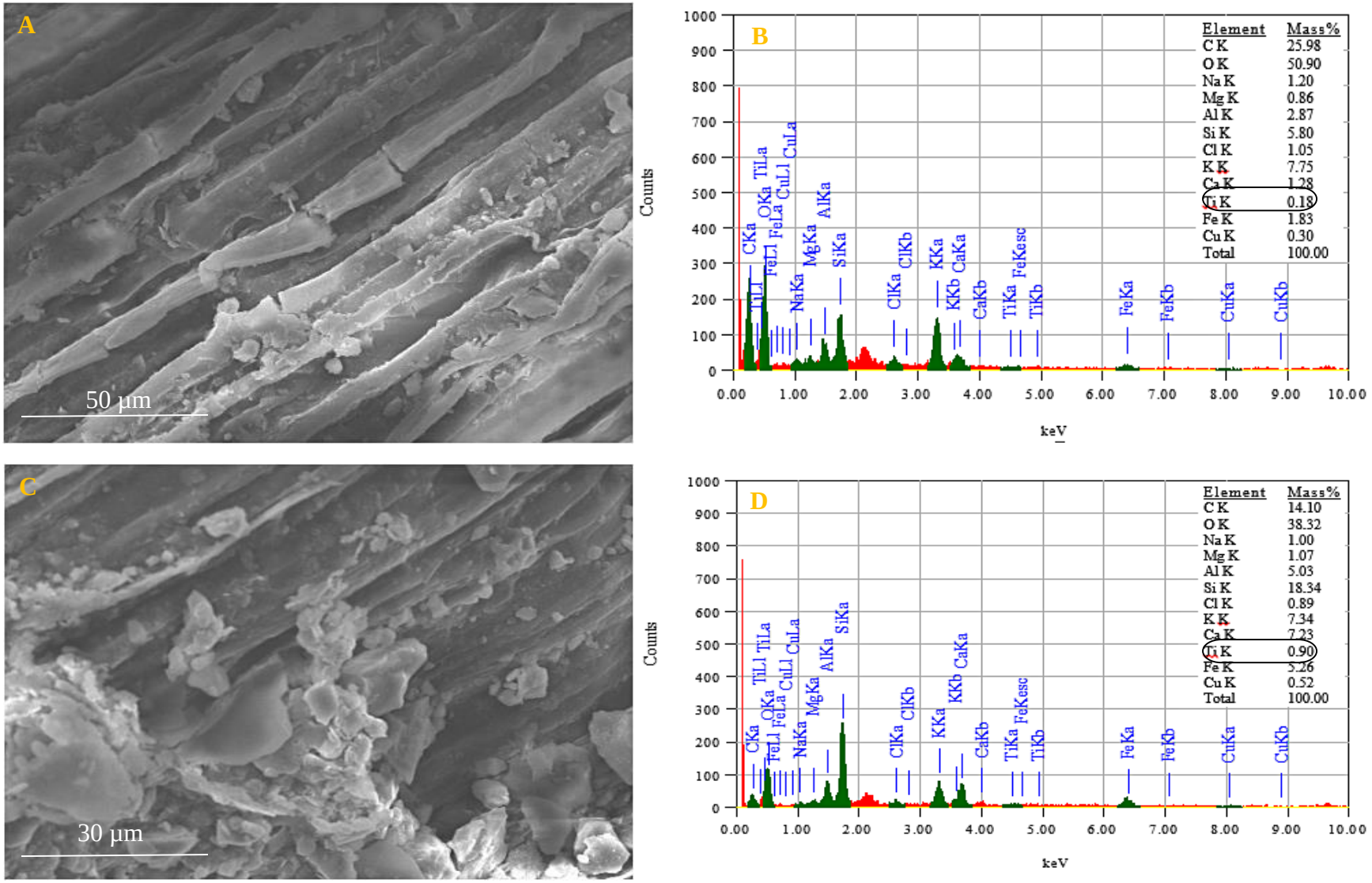

Figure 4.17: TNPs uptake in roots of lettuce, analyzed by SEM and EDX: A & B roots (control group with no TNPs treatment), C & D roots (100 mg kg^{-1} TNPs). The peaks show Ti mass% in the sample.

4.5 EFFECTS OF TiO_2 NPs ON PHYSIOLOGY OF PLANT SPECIES

4.5.1. Effects of TiO_2 NPs on chlorophyll content of plant species

4.5.1.1 Chlorophyll estimation of wheat plants

CCM measurements indicated that chlorophyll content of wheat was decreased consistently with an increase in TNPs concentrations (Figure 4.18). The chlorophyll contents were increased by 29.7% with increasing concentration of TNPs till 60 mg kg^{-1} and then decreased by 14.6% at 100 mg kg^{-1} as compared to 60 mg kg^{-1} concentration level. Similar results were found in wheat plants after 54 days, the leaves of plants shown a decrease in chlorophyll content. The sharp decrease was also found in all concentration levels during 48th day reading.

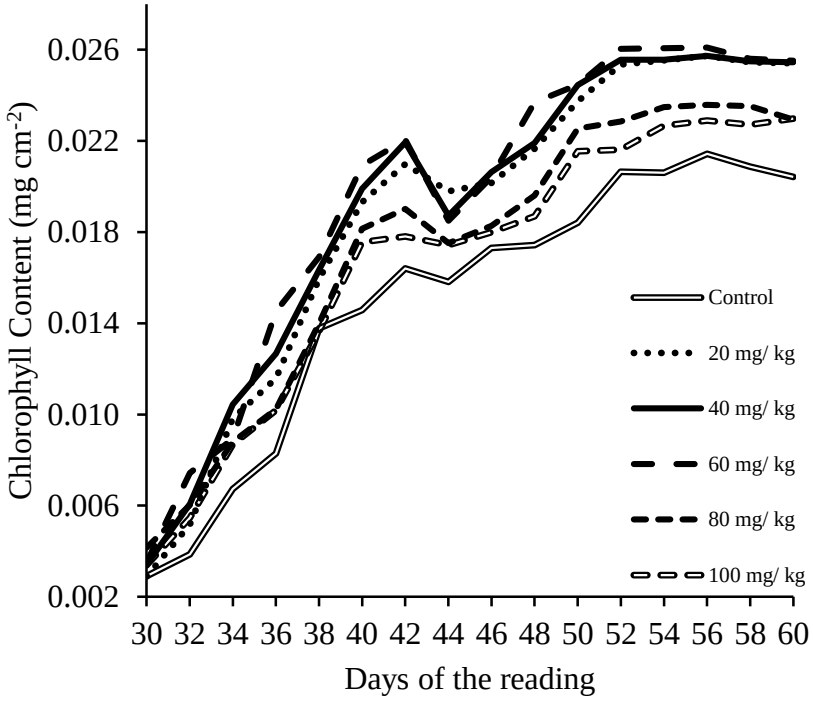

Figure 4.18: Chlorophyll contents in the leaves of wheat plants grown in soil, treated with 0 (control) - 100 mg kg^{-1} TNPs

4.5.1.2 Chlorophyll estimation of lettuce plants

An increase of 68% in total chlorophyll content was observed with increasing concentration of TNPs as compared to control (Figure 4.19). The value of total chlorophyll content was increased at 100 mg kg^{-1} as compared to the control.

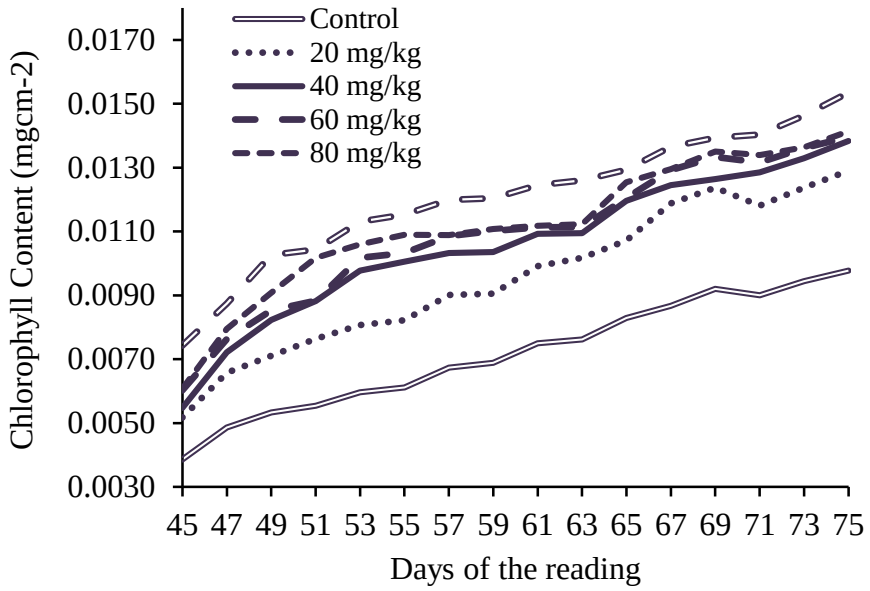

Figure 4.19: Chlorophyll contents in the leaves of lettuce plants grown in soil, treated with 0 (control) – 100 mg kg^{-1} TNPs

4.5.2. Effects of TiO_2 NPs on H_2O_2 content of plant species

The toxicity of TNPs was determined through the production of hydrogen peroxide (H_2O_2) species in wheat and lettuce roots. The results indicated that H_2O_2 generation is increased significantly ($P < 0.05$) in roots of both plants with increasing concentration of TNPs (Figure 4.20).

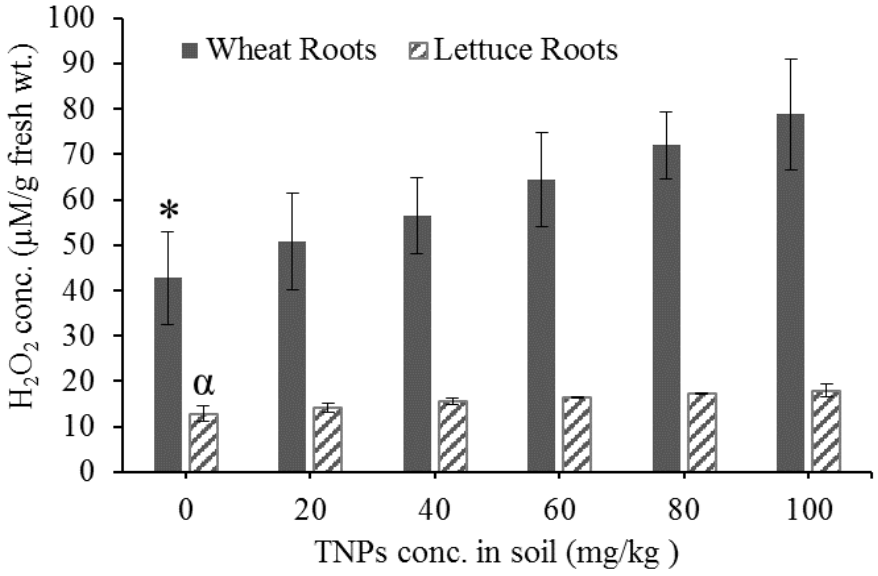

Figure 4.20: H_2O_2 conc. in root tissues of wheat and lettuce plants treated with TNPs. Asterisk symbol (*) and α represent statistically significant difference at $p < 0.05$.

Moreover, the increase in H_2O_2 content is not significant at low dose of nanoparticles (20 mg kg^{-1}) for lettuce. The results also confirmed that TNPs did not cause overproduction of H_2O_2 in lettuce roots (40% increased as compared to control). However, in wheat roots, 100 mg kg^{-1} treatment caused overproduction (80%) of H_2O_2 as compared to untreated plants which can be the reason of oxidative stress. The H_2O_2 production was increased in a dose-dependent (R^2= 0.99) manner for both plant species.

4.5.3. Effects of TiO_2 NPs on micronuclei generation

The microscopic images of wheat root meristems shown increase in number of micronuclei and root damage with increasing concentrations of TNPs. MN were found at 40, 60, 80, 100 mg kg^{-1} of TNPs concentration level (Fig. 4.21 and 4.22). The maximum numbers of cells with MN were found at 80 and 100 mg kg^{-1} (40.7% and 40.5%). The %MN and total number of MN increased in a concentration dependent fashion (R^2=0.9). However, cells with MN were not detected in root meristems of lettuce plants as shown in Fig. 4.23.

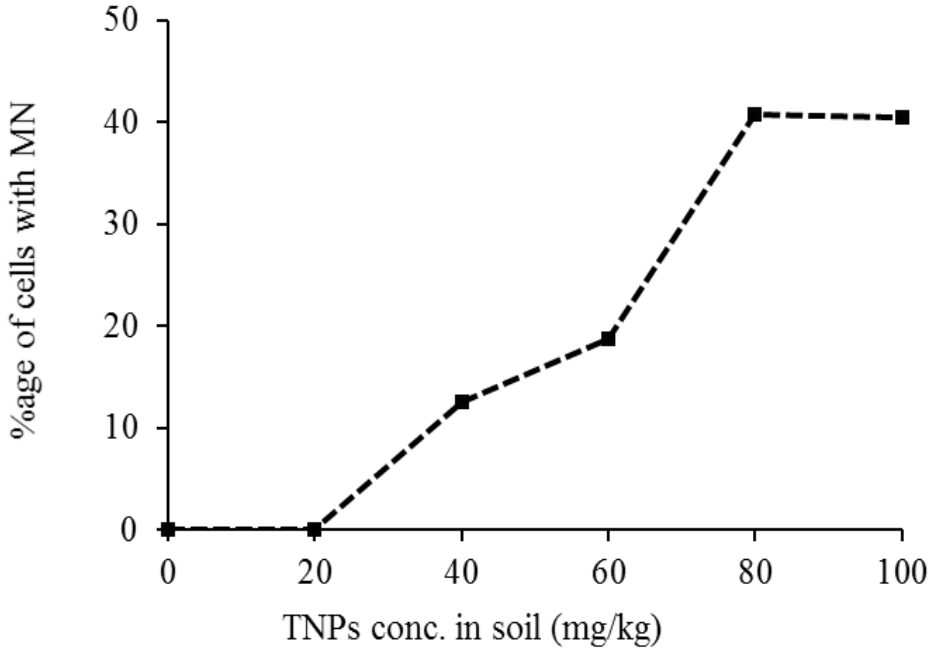

Figure 4.21: Effects of TNPs on the frequency of micronuclei induction in root-tip meristems of wheat plant

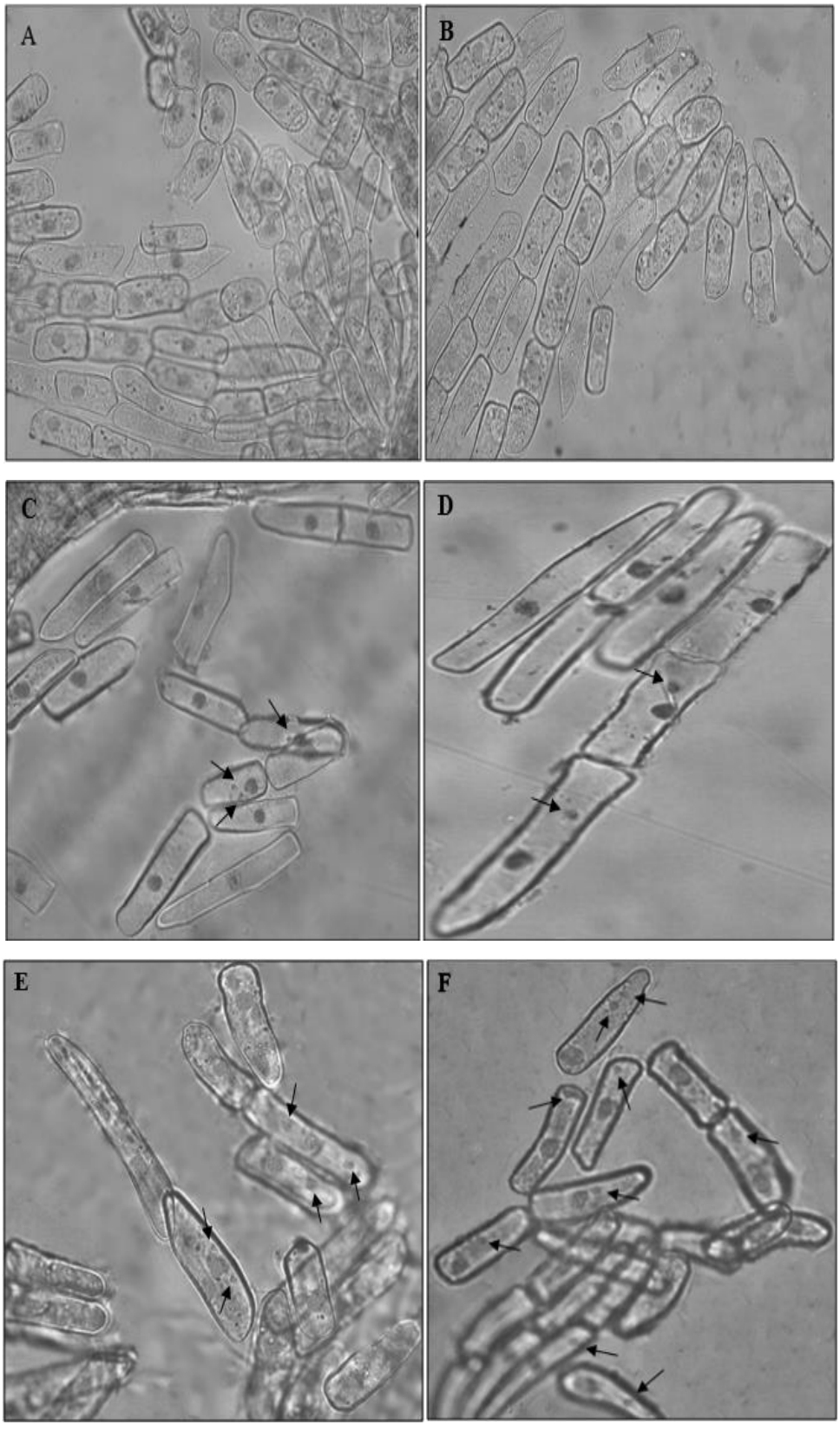

Figure 4.22: Microscopic images of wheat root-tip meristems. A) Cells with no TNPs treatment B) Treated with 20 mg kg^{-1} C) 40 mg kg^{-1} D) 60 mg kg^{-1} E) 80 mg kg^{-1} F) 100 mg kg^{-1} of TNPs. Small arrows point out the MN formed after exposure.

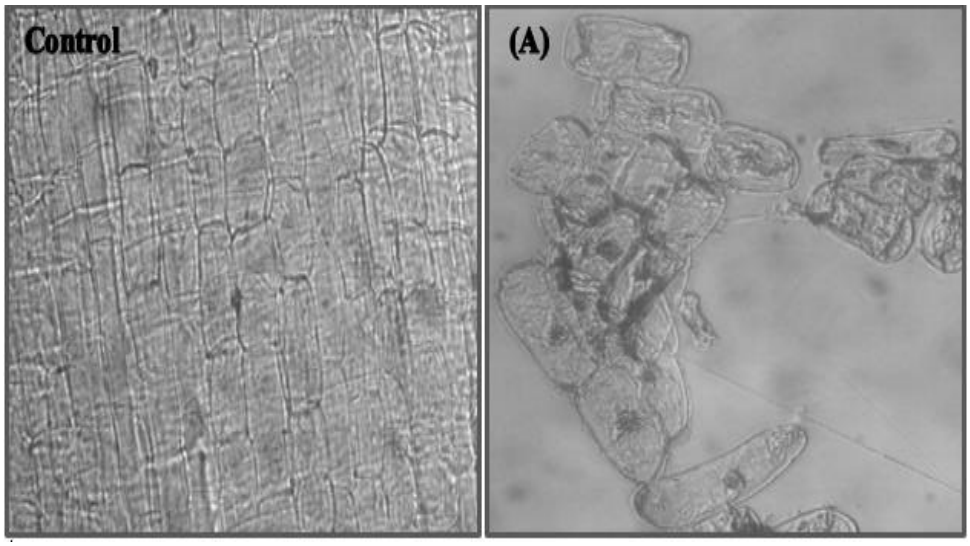

Figure 4.23: Microscopic images of lettuce root meristems, treated with 0 mg kg^{-1} (control) & (A) 100 mg kg^{-1} of TNPs

4.6 DISCUSSION

Release of TNPs in the environment may affect growth, oxidation-reduction reaction, photosynthesis, cell division and nutrients availability of plants. The effects of these nanoparticles mainly depend on type of plant species, soil chemistry and their physicochemical properties. Moreover, the way nanoparticles are applied to plants also plays an important role in the plant growth.

TNPs have three types and the anatase type exhibits a large specific surface area and the best photocatalytic activity with a band gap of 3.2 eV (Chen *et al.,* 2002). The present study demonstrated that anatase TNPs with range of 11.93 -18.67 nm and concentrations of 0, 20, 40, 60, 80, 100 mg kg^{-1} significantly affected growth of wheat and lettuce plants. At these concentration levels, both plants behaved differently.

4.6.1. TiO_2 NPs effects on plant's development

Lettuce plants' growth increased significantly with increasing concentrations of TNPs. This finding is in agreement with previous studies (Song *et al.,* 2013). However, best results of root and shoot length, fresh and dry biomass of wheat plants were found at 60 mg kg^{-1} concentration as compared to higher concentration levels. Reports indicated that high surface reactivity of TNPs might enlarge root pores and in turn, water absorption and nutrients availability to plants is improved (Larue *et al.,* 2012b). It is possible that wheat is more sensitive and permits the addition of TNPs in a limited range i.e. 20-60 mg kg^{-1} in our case. The low concentrations of TNPs have positive effects on root and shoot lengths, fresh and dry biomass whereas high concentrations decreased wheat plant growth.

Zheng *et al.* (2005) reported that best results of spinach growth were observed at low concentration levels of TNPs as compared to high concentration levels. In addition to concentration levels, exposure time and the way nanoparticles are applied to the plant is also important to understand the nanoparticles mobility in the soils and plants and their effects on plants growth. Mahmoodzadeh *et al.* (2013) carried out a pot culture experiment for 30 days on wheat plants by applying TNPs concentrations through leaf spray. They found highest value of fresh and dry weight of wheat roots at 100 mg L^{-1}. While we found maximum biomass, both the fresh and dry, at 60 mg kg^{-1} and TNPs were applied to wheat through soil.

4.6.2. TiO_2 NPs effects on phosphorus availability

The results of lettuce rhizosphere revealed that availability of phosphorus increased with increasing TNPs concentrations, also indicating that pH significantly influenced the phytoavailability of phosphorus especially at higher concentration levels of TNPs. But at high concentration, phosphorus availability in wheat rhizosphere and plant was decreased. The effects of TNPs on phosphorus availability and pH may involve combination of factors like large surface area, high reactivity of TNPs and root morphology. TNPs could attach to the root surfaces of wheat and lettuce. The attachment of TNPs to root pores considerably enhanced external surface area of roots due to high surface reactivity of TNPs. This increased surface area could increase phosphorus adsorption capacity for the plants. The phosphate adsorption is also increased with decreasing pH on TNPs surface. These nanoparticles made strong bonds with phosphate through surface complexes (Connor and McQuillan, 1999). It is postulated that bound phosphorus could be available to the plants in the presence of TNPs in soil. Recent research reported that application of TNPs enhanced activity of acid phosphatase which may lead to mobilization of phosphorus nutrient in the rhizosphere (Raliya *et al.,* 2014). Noticeably the increase of TNPs concentration doesn't continuously improve plant phosphorus absorption capacity in case of wheat. The value of phosphorus uptake increased with increasing TNPs concentrations, and then decreased with increasing concentration of TNPs. It has been observed that plant roots cell walls have different pore sizes (5-20 nm) and it also acts as a barrier for external entries (Fleischer *et al.,* 1999). This suggested that TNPs at high concentration could fill small pores of wheat roots and decreased the phosphorus uptake capacity of plants (Luo *et al.,* 2011). In this study, concentration of phosphorus might be decreased due to decrease in number of root pores at high TNPs level (80 and 100 mg kg^{-1}). This indicated that phosphorus absorption and uptake may also rely on the pore size of root cell wall.

4.6.3. Translocation of TiO_2 NPs in wheat and lettuce plants

The present study demonstrated that TNPs with diameter ranging from 11.93-18.67 nm were observed in wheat and lettuce roots and translocated to leaves upon root exposure. The translocation of Ti increased with increasing concentration of applied TNPs in both plant species. However, lettuce plants accumulated more Ti as compared to wheat plants at concentration level of 100 mg kg^{-1} of TNPs. The roots of control plants also contained traces of Ti, this might come from naturally occurring Ti in soils. But the leaves of both plant

species contained more Ti than roots when exposed to TNPs. The observations from SEM-EDX analysis indicated that translocation of Ti is concentration dependent. The other explanation can be the small size of TNPs. According to the previous study, TNPs of less than 36 nm are translocated to the leaves of wheat. Due to their small size, TNPs might be translocated to the leaves via water flow (Larue *et al.,* 2012a). Greater accumulation of Ti in lettuce plants could also be explained by its root morphology. The roots of lettuce have greater volume than wheat roots as shown in Fig. 4.5 and 4.7. The greater volume of roots increases the water absorption capacity. As a result of this, water flow could be more intense in lettuce than in wheat, leading to higher accumulation of Ti.

4.6.4. Effects of TiO_2 NPs on chlorophyll content of plants

Chlorophyll is very important pigment of plants which helps in conversion of light energy into electronic to chemical energy. An increase of the chlorophyll content value of plants has effect on photosynthesis. It is reported that photocatalytic ability of TNPs may lead to increase in chlorophyll content and photosynthetic reactions (Skupień *et al.,* 2007; Owolade *et al.,* 2008; Chen *et al.,* 2012). However, wheat shown decrease in chlorophyll content with increasing concentration. Moreover at 48^{th} day, the reading of total chlorophyll content value of wheat plants was decreased. The reduction in values can be attributed to the disturbance in availability of sunlight that is required to complete the photosynthetic reaction. After 56 days reading of wheat leaves, the values of chlorophyll decreased consistently due to less green color of leaves (Mukherjee *et al.,* 2014). At higher treatment levels of TNPs, concentration of phosphorus decreased in wheat as discussed earlier. It is also reported in literature that phosphorus deficiency resulted in declined photosynthetic rate (Mikulska *et al.,* 1998) as phosphorus (in the form of Adenosine Triphosphate, ATP) participates in plant photosynthesis, which supplies energy for the CO_2 fixation (Sivak *et al.,* 1986). ATP produced under phosphorus-deficient condition may be insufficient to support CO_2 fixation by the Calvin cycle (Kromer *et al.,* 1995). That may lead to decrease in photosynthetic rate at high concentration of TNPs.

4.6.5. Effects of TiO_2 NPs on reactive oxidative species (H_2O_2) of plants

Plants have different forms of ROS which are produced mostly under biotic or abiotic stress conditions (Shen *et al.,* 2010; Shahid *et al.,* 2011). It is observed that all plants also have attained biochemical defense against accumulation of intracellular ROS, which balanced the production of ROS even under unstressed conditions (Alscher *et al.,* 2002).

However, toxicity of nanomaterials may alter this balance of ROS by inducing an oxidative stress (Klaine *et al.,* 2008; Hu *et al.,* 2009). The H_2O_2 content is increased in roots of both plants with increasing concentration of TNPs. The application of TNPs did not show any toxic effects to lettuce. But enhanced production of H_2O_2 in wheat root tissues indicated that an increase in TNPs treatments might result in increased level of ROS species. A previous study reported that ZnO nanoparticles induced overproduction (61% higher as compared to control) of H_2O_2 species in stems of green peas (Mukherjee *et al.,* 2014). It is well known that TiO_2 is a semiconductor with wide-gap. Its electrons are promoted from its valence band to conduction band after absorbing photons with energy greater than 3.2 eV and leaves positively charged photogenerated holes (Arora *et al.,* 2010). These holes resulted in production of hydroxyl radicals when exposed to UV light and in dark also (Fenoglio *et al.,* 2009). These radicals may induce production of H_2O_2 species. It is also observed that production of H_2O_2 was increased in plant's roots as a result of phosphorus or other nutrients starvation (Shin *et al.,* 2005). It can be, therefore, concluded that biological effects of TNPs are dependent on plant species. However, there is need to clarify and understand its physiological mechanisms.

4.6.6. Effects of TiO_2 NPs on MN generation

It can be postulated that ROS were able to generate micronuclei upon treatment with TNPs. The production of oxidative species (e.g. H_2O_2) caused by nanoparticles exposure may result into many harmful effects to plant cells including DNA damages (Afaq *et al.,* 1998). Moreover, all results indicated that high TNPs treatments generate more H_2O_2 species and micronuclei in wheat as compared to lettuce. The negative effects of high concentration (319 mg L^{-1}) of TNPs resulted in DNA damage in *Nicotiana tabacum* and *Allium cepa* as well as in human lymphocytes due to oxidative stress (Ghosh *et al.*, 2010) . Phugare *et al.* (2011) reported that oxidative stress was the major reason of cytotoxicity and genotoxicity.

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

Based upon the experimental results and statistical analysis of the data the following conclusions were drawn from the present study.

- Phytoavailability of phosphorus and growth of lettuce increased with increasing concentration of TNPs. For wheat plants, the best results in terms of improved growth and phosphorus uptake were found at 60 mg kg^{-1} of TNPs as compared to the control.
- The SEM-EDX results depicted that uptake of TNPs increased with increasing concentration in both plants. But this translocation of TNPs reduced chlorophyll content and caused over production of H_2O_2 species in wheat at 80 and 100 mg kg^{-1} of TNPs. Moreover, maximum numbers of MN were also observed at 80 and 100 mg kg^{-1} concentration level.
- In case of lettuce, the chlorophyll content and production of H_2O_2 was increased with increasing concentration of TNPs in comparison with control. However, MN formation was not observed in root meristems after application of TNPs.
- Overall, the response to TNPs application was species dependent since TNPs improved plant growth and development in case of lettuce while had inhibitory effects in case of wheat at concentrations higher than 60 mg TNPs kg^{-1} of soil.

5.2 RECOMMENDATIONS

Based upon this research work, following recommendations can be made for future work.

- The results confirmed that TNPs can potentially help in phosphorus release. But further investigations are required to understand the speciation of phosphorus and soil characteristics in response to the application of TNPs in order to improve P availability.
- Further studies are required to test the possible effects of TNPs on other mineral elements i.e. nitrogen, potassium, etc.
- Soil type is an important factor in controlling P availability. Effects of nanoparticles in different textured soils need to be explored.
- In agriculture, fertilizer use for nutrient supply is an important factor. Application of the nanoparticles in combination with fertilizers and possible interactions can help to

improve crop productivity and ensure food security. So there is need to study this hypothesis as well.

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