PHYTOAVAILABILITY OF NITROGEN IN RESPONSE TO APPLICATION OF TITANIA NANOPARTICLES IN SOIL

By

Komal Siddique

NUST201362290MSCEE65213F

Supervised by

Dr. Muhammad Arshad

Institute of Environmental Sciences and Engineering (IESE)
School of Civil and Environmental Engineering (SCEE)
National University of Sciences and Technology (NUST)
Islamabad, Pakistan

(2016)

PHYTOAVAILABILITY OF NITROGEN IN RESPONSE TO APPLICATION OF TITANIA NANOPARTICLES (TiO_2NPs) IN SOIL

By

KOMAL SIDDIQUE

NUST201362290MSCEE65213F

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

(2016)

Certificate

Certified that the contents and form of the thesis entitled **"Phytoavailability of Nitrogen in Response to Application of Titania Nanoparticles in Soil"** submitted by Ms. **Komal Siddique** has been found satisfactory for the requirement of the degree of Master of Science in Environmental Science.

Supervisor: ___________________
Dr. Muhammad Arshad
Associate Professor
IESE, SCEE, NUST, Islamabad

GEC member: ______________________

Professor Dr. Imran Hashmi
Associate Dean
IESE, SCEE, NUST, Islamabad

GEC Member: _____________________

Dr. Tariq Sultan
Principal Scientific Officer
Land Resources Research Institute
National Agriculture Research Centre (NARC) Islamabad

Dedication

I dedicate this thesis to my beloved Parents, who always supported and encouraged me to explore new horizons for myself, believed in my dreams and helped me become the person I am today.

To my maternal Grand Father (Late), who showed me the way and has always been a guiding light for me through the darkest of times and taught me to be humble and grateful for everything. I thank you for instilling confidence in me and telling me that it's ok to fall, what's important is what you do after you get back up.

Also, to life, for teaching me invaluable lessons about belief, the power of prayer, love and loss, seeing the silver lining, but most importantly about humility, compassion, understanding, and the strength within.

بسم الله الرحمن الرحيم

Acknowledgements

Thanks to ALLAH Almighty, the "Most Kind" and "Most Merciful" who blessed us with aptitude, skills and wisdom to accomplish this project. I express my sincere gratitude to my parents for their affection and moral support throughout my academic career.

My utmost gratitude to my supervisor *Dr. Muhammad Arshad* for his kind help during this research work. His patience, support, and guidance were a source of inspiration and patience during the study. I am grateful to *Dr. Imran Hashmi* in particular for his kind help and adding constructive comments throughout the project. My special thanks to external GEC member *Dr. Tariq Sultan and Dr. Tauseef Abbasi* for the lab facilities at NARC, Islamabad.

My special gratitude to the family for their prayers & support. My sincerest thanks to all friends and cousins (especially Ms. Shamsa Naz, Ms. Momna Izhar, Ms. Zahra Meer, Ms. Aneela Iqbal, Ms. Salma Alvi, Ms. Reenum Anwar and Ms. Tuba Azhar) for their continuous moral support and encouragement throughout the research. Last but not the least I would thank all the laboratory staff at IESE and SCME, NUST for their help and cooperation.

Komal Siddique

ABSTRACT

Although nitrogen (N) accounts for almost 78% of the air, it may only be absorbed by a small population of plants in its natural gaseous form (Leguminous plants and some other plants with root nodules) only through a process called biological nitrogen fixation. In present work, effort has been made to examine the effects of TiO_2 nanoparticles (TiO_2NPs) on growth of pea plants (*Pisum sativum* L). The seeds of pea were sown in soil containing different concentrations of TiO_2NPs i.e. 0, 50, 100, 250, 500, 750 mg kg^{-1}. Each treatment was further multiplied by four replicates. Liquid Impregnation Method and SEM-EDX analysis was performed for synthesis and characterization of nanoparticles. Plant's morphological parameters like root, shoot length, plant biomass, chlorophyll were taken into consideration in response to TiO_2NPs application. The results showed *Pisum sativum* L. growth is affected by Titania nanoparticles. The optimum concentration found was 250 mg kg^{-1} of nanoparticles. Nodulation number and nodule weight was also affected by TiO_2NPs, implying that Symbiotic Nitrogen Fixation (SNF) and nitrogen availability is affected. At optimum concentration, shoot length increased up to 61% as compared to control. Total dry biomass was increased up to 46%. In nutrient uptake mechanism, the nanoparticles affinities to adsorb nitrate and ammonium ions were the traits that could be optimized to improve the N efficiency for agricultural purposes and reduce N losses to the environment linked to their soil application.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AB-DTPA	Ammonium Bicarbonate (Diethylene Triamine Penta Acetic Acid)
CCI	Chlorophyll Content Index
CCM	Chlorophyll Content Meter
Cu	Copper
$CuSO_4$	Copper Sulfate
EDS	Energy Dispersive X-ray Spectroscopy
FAD	Flavin Adenine Dinucleotide
Fe_3O_4	Iron Oxide
FTIR	Fourier Transform Infrared Spectroscopy
GPR	General Purpose Reagent
H_3PO_4	Orthophosphoric Acid
K	Potassium
LI	Liquid Impregnation
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NaOH	Sodium hydroxide
NH_4HCO_3	Ammonium bicarbonate
NH_4OH	Ammonium hydroxide

NPs	Nanoparticles
NUE	Nitrogen Use efficiency
P	Phosphorus
SEM	Scanning Electron Microscopy
SNF	Symbiotic Nitrogen Fixation
TiO_2	Titanium dioxide
XRD	X-ray Diffraction Spectroscopy
ZnO	Zinc oxide

Chapter 1

INTRODUCTION

1.1 Background

Practically all macro and micro nutrients are vital but the most basic and imperative life-supporting element on Earth is Nitrogen (N). It is considered to be the major fundamental nutrient among other basic elements (P and K) which are deemed essential for plants growth thereby ensuring food security. Soil N is also a major decisive abiotic factor for the plant growing in nutrient-deficient soils (Chen *et al*., 2001).

Nitrogen amount presence in plants ranks as the fourth highest one, which is after C, O and H (Zhang and Xu, 2011). It is one of the major constituents of nucleic acids, proteins, lipids and some of the hormones in plants. It contributes 40%-50% of crop yield (Lu and Hu, 2006), meanwhile, it affects the plant life cycle by assembling, controlling or regulating the cytoplasm, nucleus and membrane system using these molecules. Nitrogen is also involved in the synthesis of some enzymes, co-enzymes and some prosthetic groups (NAD+, NADP+, and FAD+). Therefore, it is an understood fact that N level has an instantaneous effect on cell division and growth in plants (Tian *et al*., 2011).

Nitrogen in the biosphere exists mainly in three forms: gaseous N in the air, organic and inorganic N pools in soils. Although N_2 accounts for almost 78% of the air, it can only be absorbed by a small population of plants in its natural gaseous form (Leguminous plants and some other plants with root nodules) only through a process called biological Nitrogen fixation (Zhang and Xu, 2011). Some N-rich amino acids, amide, and urea, most of the organic N are hydrophobic and do not easily dissolve into soil solution to supply the plant growth. The organic N pool in soils consists of residue and compost of animal bodies, waste, plants, and microorganisms (Wang, 1999).

Inorganic N pool that accounts for about 1-2% of the total N in the soil mainly contains nitrate and ammonium. Some other transitional forms like nitrite and nitrous are also contained but at a very low and negligible level. The properties of inorganic N are high dissolvability, smaller molecule size, high mobility, and polarity. These characteristics and properties make it readily available for plants in soils, and therefore becomes the main N source for the plant growth (Zhang and Xu, 2011). Nitrate (NO_3^-) is the main source for plants in a field, even though many kinds of plants can absorb ammonium (NH_4^+). In order to ensure the optimal supply of nutrients to crops, fertilizers are regularly applied in order to maximize the crop yield as shown in Figure 1.1.

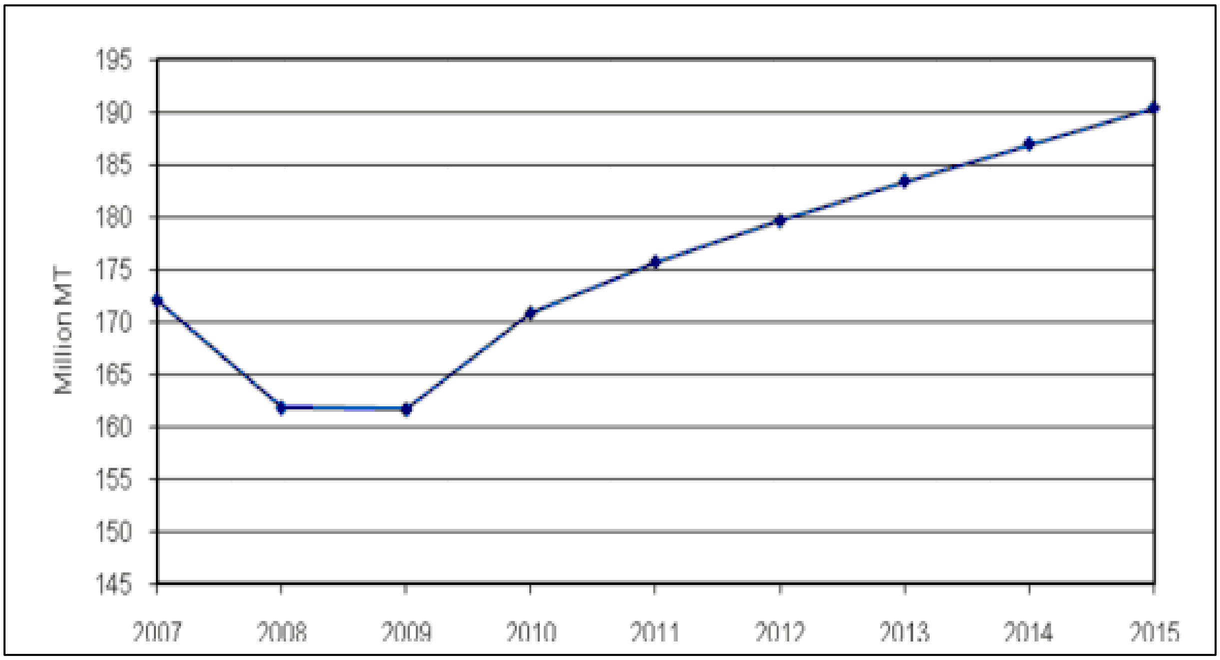

Figure 1.1 Global nutrients (N, P_2O_5, K_2O) consumption over past century (FAO, 2011)

Photosynthesis is affected by N level in plants. Nitrogen deficient soils reduce plant functions such as seed development, root structure building and eventually the typical crop yields. Nearly hundred percent of the cultivable soils in Pakistan are facing nitrogen deficiency, 80 to 90 percent are phosphorus deficient and about 30 percent are deficient in potassium. A wide range insufficiency of both macro and micronutrients has also started appearing in various parts of the world. Due to intensive cultivation and the increased output requirements, soil fertility is further depleting constantly and rapidly due to withdrawal of vital plant nutrients from the soils (GoP, 2013).

Very limited information regarding nano-particles influence on nutrients availability to crop plants is found. Therefore, in this study the design and aim was to identify the nanoparticles behavior in soil environment with focus on N partitioning into functional fractions that are available to plants (Dalen, 2012).

The constant provision of Nitrogen fertilizers is very critical and crucial in fulfilling the crop requirements. The ever increasing demand of fertilizers in agricultural production system makes it further high-priced to be used on cultivable lands by farmers. The trend has been especially noted in recent years in South Asia. It is noted that 58.5 percent of the world total fertilizer nutrient consumption is in Asia (mainly East Asia and South Asia as shown in Figure 1.2. The share of Asia in world consumption of three basic nutrients (NPK) is 62.1, 57.6 and 46.4 percent respectively (FAO, 2011). Large amounts of expensive nitrogenous fertilizers are required for productive agriculture. It is thus important to improve nitrogen use efficiency (NUE) of crop plants (Orsel *et al*., 2002).

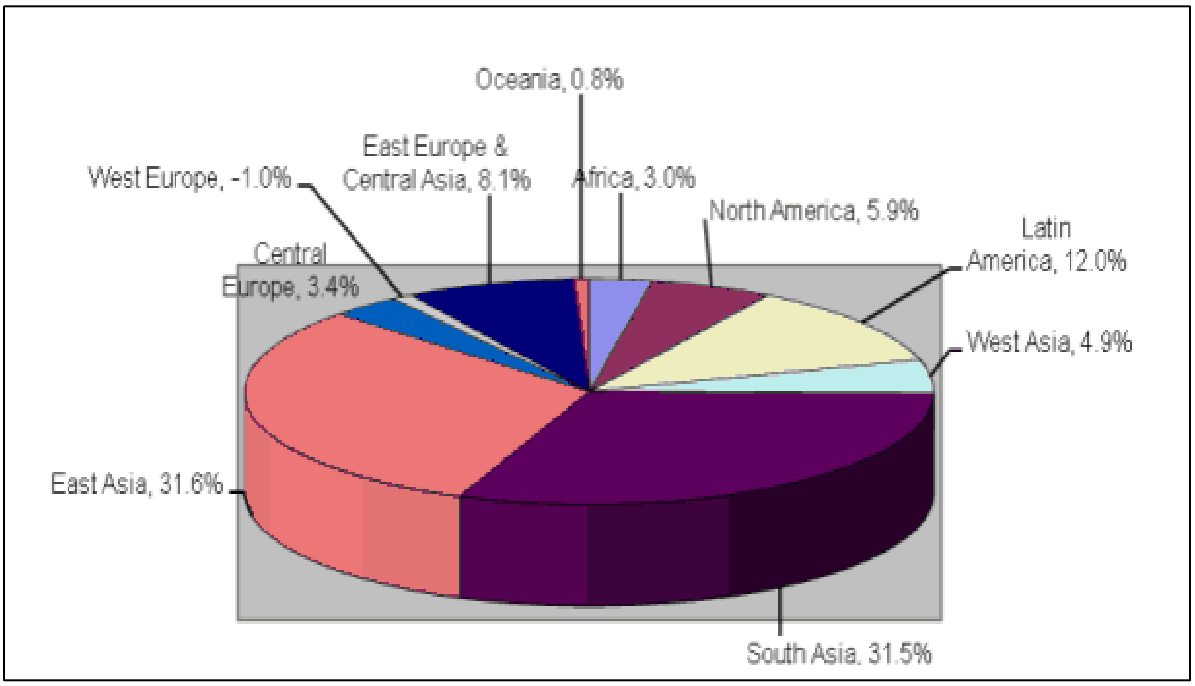

Figure 1.2 Regional and sub-regional share of world increase in nitrogen fertilizer consumption, 2011-2015 (FAO, 2011)

Currently, nanotechnology is formally aiming at fast development in the field of electronics, cosmetics, biotechnology and medical science etc. As this field is progressing rapidly, it is poised to soon become potentially a trillion-dollar industry. In the recent years, scientists have been working on different nano-particles effects on the plant growth and other plant mechanisms (Zheng *et al*., 2005).

Nanoparticles are commonly known as particles ranging between 1-100nm dimensions. (Ma *et al*., 2010). Among the synthetic nanoparticles, TiO_2 nanoparticles are used in a number of commercial products (Ghosh *et al*., 2010).

Due to the increased supply and demand of TiO_2 nanoparticles, these substances are expected and measured to have higher environmental exposure than other frequently used nano-materials i.e., nano-ZnO, nano-Ag, and carbon nanotubes (Gottschalk *et al*., 2009).

Likewise, Iron oxide (Fe_3O_4) nanoparticles have a wide-ranging applications in numerous fields of science and technology (Zhao *et al.*, 2006). Increased use of nanotechnology also calls for clarification of nanoparticles (NPs) effects on biological systems and nano-toxicity (Handy *et al*., 2008). Many studies on the NPs toxicity to animals and bacteria are available, but very limited work has been done on higher plants. Both fruitful and harmful effects of NPs during phyto-toxicity studies have been observed inevitably affecting the seed germination, root growth, and metabolic processes, such as photosynthesis of higher plants (Lin and Xing, 2007).

The effects of TiO_2NPs have been studied as photo-catalyst on the growth of spinach seeds. It was observed that while nano-particles helped improve light absorbing capacity on the one hand, they also promoted enzyme activity hence improving spinach growth in a positive manner. Moreover, nitrogen metabolism is enhanced which in turn promoted the absorption of nitrates in spinach and also increased the transformation of inorganic nitrogen in to organic nitrogen, consequently improving the fresh and dry weights of plant (Nair *et al*., 2010).

The rapid and swift advancement and possible release of engineered nanoparticles into the environment have raised many issues and concerns due to their exceptional properties. Different plants respond to or interact with nano-particles differently, usually depending upon the size, concentration, and physio-chemical properties of nano-particles and the plant species. Various plants respond differently at different growth phases either enhancing or hindering effects of different nano-particles on growth pattern. Presently a new area of research is now paying attention on short and medium term projects related to environmental and ecological impact of these nanoparticles (Ma *et al*., 2010).

Zinc deficiency is one of the main problems restraining agricultural productivity in alkaline calcareous soils. Application of nano-particles in such soils may enhance nutrient availability to plants. ZnO NPs are thus used to produce more soluble and diffusible sources of Zn fertilizers. (Milani *et al*., 2010). Similarly Fe_3O_4 nanoparticles can also be applied to reduce the Fe deficiency in soil system.

Nanotechnology applications in agriculture sector are one of the new dimension in which a lot of things need to be explored. As crop production faces many challenges, due to nutrient deficiencies thereby evolving needs for new plant-derived materials. This has resulted in increased interest in development of approaches to enhance availability of naturally reserved nitrogen in soils. So nanotechnology approach will be used to have a look for some solution and thus far holds a lot of potential.

1.2 Objectives

Keeping in view all the insights gained from the literature related to nanoparticles, soil and plants, this work is designed to assess and evaluate the phytoavailability of nitrogen in response to TiO_2 nano-particles and the growth response of Pea *(Pisum sativum).* So it may well be hypothesized that these nanoparticles could be successful in promoting the plant growth by escalating the phytoavailability of naturally bound nitrogen. In this study, the aim was to carry out the following objectives:

- To assess the impact of TiO_2 nanoparticles on nitrogen bioavailability.
- Growth response of pea *(Pisum sativum)* to nanoparticles application.

1.3 Scope of Study

In near future, manufacture and use of nano-materials is expected to rise. The use of these nano-materials for the edible plant growth is a recent trend. Few results however are available showing the influence of nanoparticles on nutrient absorption. The scope of the study is to provide a substantial evaluation of TiO_2 nanoparticles on nitrogen uptake and growth performance of pea plants. The current study has the potential to be adopted in field in near future. And this can work at both at the national and global level. This study will help to give new insight of how the adoption of modern crop technologies, in particular nanotechnology approaches, may be applied to broaden the nutrient availability to food crops.

To attain this purpose, sustainable management framework in agricultural systems must be developed based on meeting at least minimum N requirements for crops. Soil Nitrogen should be provided at the concentration level required for better crop yield. Similarly the study also helps to undertake the application in semi-arid or rain fed and other areas to use the resources sustainably by promoting less fertilizer use. Enriched dry land plant cultivars must have drought resistant ways that enable them to grow and persevere in areas with low moisture availability. Indeed, one of the most important nitrogen-fixing systems is *Rhizobium*-legume symbioses, which may have the possible ability to increase N input in dry lands (Ramos *et al*., 1999). The potentiality of legumes to form symbiotic mutualistic associations with certain bacterium and to harness their N_2 fixing ability from atmospheric N_2 into ammonia has a tremendous effect on natural and agricultural ecosystems.

All this would ultimately help to reduce the use of synthetic Nitrogen fertilizers in the long run, and can prove to be a stepping stone towards sustainable development. Besides these advantages it also helps to utilize the areas which are currently in environmental stress for plantation with increased productivity. Therefore, an increasing interest is emerging in the development of approaches to enhance availability of naturally reserved Nitrogen in soils.

Chapter 2

LITERATURE REVIEW

This chapter is structured to highlight the general background of nanoparticles and their interaction with plants in the environment.

2.1 State of the Art

In comparison to various other environmental issues, nano-particles have given new environmental challenges and introduced new dimensions for researchers and environmentalists all over the world. Nanotechnology has left no domain untouched including agriculture by its scientific novelties and growth prospects. Although, the use of nanotechnology in agriculture sector is still in the developmental stages, but it appears to have significant effects in different areas of this domain. Nanotechnology has great potential and can serve in various fields related to agriculture. Different and various types of nano-particles are being used in the fields of food production, processing, fertilizers & also the pesticides industry. In plants also nanoparticles can be used for a wide range of applications, including as a food supplement and as growth catalysts (Ali *et al*., 2014).

Nano-particles act as carrier agents along with agrochemical and other substances for the controlled release of nutrients. But while doing so we must not ignore the negative effects of nano-materials, which include phytotoxicity.

In this scenario, it is the need of hour to predict the effects of these nanoparticles on environment in the near future. (Zahra *et al*. 2015). The main area of focus of the present review was to cite the information related to the influence and effects of nano-particles function in pea plants, with both the positive and negatives effects in agro ecosystem.

Nanotechnology in real life scenarios has mystical powers to revolutionize every field touched by it (Chellaram *et al*., 2014). In the recent times, due to the advances in recognition of nanotechnology has led to increased application in various segments such as food systems (production, processing) including agriculture and fertilizers & pesticides industry. Agriculture is generally considered to be the main pillar of third world economies. Nanotechnology empowers agricultural researches by broad range of advancement in the treatment of plants using various NPs. Plants are one of the primary components of the environment and perform vital functions in maintaining equilibrium and balance across the entire ecosystem including the food chain and food web as nutrition supplement and a growth catalyst (Mishra *et al*., 2014). Our understanding of a wide range of crops and their biological parameters to potentially enhance not only their yield but also nutritional value while giving us control over plant diseases and pest incidences has been very much enhanced (Nair and Kumar, 2013).

Additionally, plants are also subjected to extensive human manipulations by these nano-particles. NPs reach plants through various ways, such as direct application, release via accidents, sediments or water, and atmospheric outcomes and even contaminations in the soil.

Nanoparticles (NPs) have average size of less than 100 nm and have unique properties that depend on their phase, distribution, size and morphology (Christian *et al*., 2008). Titanium dioxide nanoparticles (TiO_2NPs) are extensively utilized in composition of numerous commercial products such as paints, cosmetics, sunscreens, surface coatings and pigments (Kaida *et al*., 2003), and also for environmental remediation e.g. soil, water and air (Choi *et al*., 2006 and Esterkin *et al*., 2005). According to previous computer modeling assessments, this large production and utilization may lead to their release in the environment and about 136 mg TiO_2NPs would be present in per kg of sewage sludge (Gottschalk *et al*., 2009). This sludge if applied to agricultural lands, may affect the soil and the plants health in the long run. It has been reported that NPs are taken up by plant roots and from time to time translocated to their seeds, leaves and fruits (Nair *et al*., 2010). This accumulation, however, depends on the properties of NPs (shape, size, chemical composition, agglomeration) and plant species (Larue *et al*., 2012). Some studies have reported the positive effects (Raliya *et al*., 2014; Liu *et al*., 2005 and Yang *et al*., 2007) while others have found the negative effects (Castiglione *et al*., 2011 and Barnard, 2010) of TiO_2NPs on plants.

Although many studies have been performed and are still being performed to properly understand plant NP interactions, but so far no clear understanding has been developed signifying their impact either positive or negative. Different essential and non-essential elements taken by plants may pose toxic threats to plants under particular growing conditions (Ke *et al*., 2007). To study the impact of NPs over plants scientists internationally are divided into groups. Accumulated literature so far has indicated both beneficial as well as harmful effects of NPs.

2.2 Macro & Micro Nutrients of Soil

Both macro and micro nutrient deficiencies in soil affect the overall crop yields and quality all across the globe.

The application of nitrogen (N) fertilizers in agriculture has been increasing significantly since the 1970s. However, the over-fertilization could cause environmental problems, as well as low N use efficiency (NUE) (Chellaram *et al*., 2014). Nitrogen (an important macronutrient) in soil is an important marker of plant growth. (Wu and Zhao, 2010). Nitrogen quantity in plants ranks as the fourth largest one, which is after C, O and H. Soil N is also an influential abiotic factor for the plant growing in nutrient-deficient soils (Chen *et al*., 2001).

Huge amounts of N fertilizer are applied to achieve maximum agricultural production. On the other hand, the over-fertilization causes various environmental problems, such as high levels of nitrate contamination and eutrophication of water bodies.

2.3 Properties of Nanoparticles

Nano-particles (NPs) are classified as aggregates or components ranging anywhere between 1 and 100nm (Ball, 2002), which have specific physicochemical properties namely strength, optical and electrical features in comparison to their bulk counterparts (Nel *et al*., 2006). NPs with a higher surface/volume ratio and high reactivity are considered to be the building blocks of nanotechnology. Nano-particles possess the ability to cross cell walls and plasma membranes (Stern and McNeil, 2008; Farre *et al*., 2011) It is worth noting that these extraordinary properties differentiate them from bulk materials and bring about characteristic environmental fate and behaviors.

2.4 Fate of Nano-particles in Agriculture

Nanomaterial technology has reached new heights by playing a pivotal role in the fields of medicine, drug development, and even the information and communication sector. This could be credited to the characteristic features of NPs that include small size, shape, and larger surface area to mass ratio (Dowling *et al*., 2004; Biswas and Wu, 2005).

In the recent researches, nano-particles have been used in certain areas of agriculture but whether these nano-particles are unsafe or useful for plant growth is a sparingly studied subject. Various studies have been conducted to further explore and understand the effects of nano-particles in agriculture. At the moment, the possibility of plants exposure to nano-particles has increased to a greater extent with their enduring effects on soils. In general, on surfaces of soil particles adsorption reactions and diffusion processes occur that makes changes in the elemental composition and the matrices of soil compounds forming new soil complexes.

The extensive development in the field of nanomaterial technologies has caught the attention of public health groups about interventions towards the potent risk induced by NPs into the environment and human health. Thus, a stage is being set for a debate over the benefits and risks of engineered NPs (USEPA, 2007).

Ecotoxicity of nanomaterials, as well as the chemistry of nanoparticles is informed in recent studies (Handy *et al*., 2008; Nam and Lead, 2008).

2.5 **Application of Nanoparticles in Plant Studies**

Nano-particles are now being widely utilized in many fields and their interaction with the surrounding environments is one of the major issues and causes of concern. Nano-particles can enter the agro ecosystem by many and varid means including water, soil and plants. To date, different studies related to the application of nano-particles and their bioaccumulation in plants have been infrequently reported. Research in field of nanotechnology has been formerly aiming at fast development in different domains of electronics, biotechnology and biomedical science, etc. Likewise scientists have also been paying attention on the special effects and mechanisms of different nano-particles on various plants (Zheng *et al*., 2005).

NPs closely interact with their neighboring environment and plants forms an important base component of all such ecosystems. Consequently, NPs will inevitably interact with plants and such interactions such as uptake and the buildup of the plant biomass will greatly affect their chances and their transport in the surrounding environment. NPs could also adhere to the plant roots and exert toxicity either physical or chemical in plants. Publications on these types of issues have emerged quite recently concerning the interactions of NPs with plants (Lin and Xing, 2007; Lin *et al*., 2009). Most of these studies focus primarily on the potential toxicity of NPs to plants and thus far positive, negative or even negligible effects have been reported.

The positive impacts of TiO_2 have been reported in seed germination and growth. Various plants were observed to have shown improvement the area of light absorption and promoted the enhanced activity of Rubisco activase, thereby accelerating growth (Zheng *et al*., 2005; Gao *et al*., 2006, 2008; Xuming *et al*., 2008; Su *et al*., 2008). It was observed that Nano-TiO_2 also helped in improving nitrogen metabolism (Yang *et al*., 2006), which is responsible for promoting nitrate absorption in plants, preferably the formation of organic nitrogen from inorganic nitrogen, thereby resulting in increased and improved fresh and dry weight of the plants (Gao *et al.*, 2013).

2.6 **Entry Routes and Translocation of Different Nano-particles in Plants**

Plants offered a prospective route for the transfer of nano-particles to the environment and ultimately paved the way for them to be successfully bio accumulated. Different studies are now determining the response of nano-particles to plants growth and their possible mechanism. Plant cell walls do not allow the smooth entrance of any foreign agent including nano-particles into the plant cells. Diameter of pores in cell walls that generally ranges from 5 to 20 nm define the screening properties. Therefore nano-particles and their aggregates within that range could easily pass through the cell membrane & transferred to the above ground parts of the plants.

The morphology, especially the root structure, has also been one of the major concerns in uptake and utilization of Nitrogen by plants under water deficiency. Plant root systems play a pivotal role towards the uptake of water along with other nutrients, synthesis of plant hormones, and the storage of photosynthetic assimilates. In addition, nano-particles might induce different morphological changes in the root structures resulting in magnification of pores or stimulation of new cell wall pores which ultimately enhance uptake of nano-particles, their aggregates or complexes. Root length, depth, thickness, density, weight, root to shoot ratio, penetration, osmotic adjustment are all good indicators used for related researches (Luo and Zhang, 2001).

Formation of a cavity like structure around the nanoparticles by plasma membrane results in further internalization. By using embedded transportation carrier proteins or through ion channels, they are able to cross the membrane. Nano-particles may be attached to different cytoplasmic organelles and obstruct the metabolic processes. Conversely, accumulation of nanoparticles on photosynthetic facade induces foliar heating and can possibly alter the gaseous exchange due to stomatal obstruction. Studies on translocation and influence of different nano-particles within plants need to investigated further to underpin the whole mechanism of their behavior in plants (Nair *et al.*, 2010).

2.7 Pea (*Pisum sativum* L.) – A Nitrogen Fixing Crop

Pea (*Pisum sativum* L.) is an important pulse legume, nutritious and cool season vegetable crop. It is an important crop for nitrogen fixation. Nitrogen (N) is the one of the three key nutrients, which if available in limited supply can hinder and limit crop production under most situations. One of the major reasons for the failure in N supplies is its presence in soil in organic form which must be mineralized to be used by plants (Azam, 2001).Subsequently; plants can transform it into useable form of plant nitrogen viz., amino acids and proteins. The process of symbiotic N_2 fixation is carried out in the plant root nodules. Nodules that are fixing N_2 will be pink to red inside (effective) and those which do not do so are yellow to green (non-effective) in color (Anonymous, 2004).

In Pakistan, most of the cultivable soil has either nil or very low viable count of effective rhizobia and low N-supplying capacity because of low organic matter (0.3-1.0%) levels (Ladha *et al*., 1996) and approximately 1.0% of the total cultivated legume crops are inoculated (Aslam *et al*., 1997; 2000). Therefore it is strongly recommended that agri farmers should always inoculate their pea seeds with commercially available fresh inoculants of superior quality.

2.8 Effects of TiO_2 nano-particles on Plants

Although TiO_2 NPs are consumed in abundance in the form of daily use products, their uptake, translocation, and relevance has so far not been undertaken noticeably (Servin *et al*., 2012).

However, the effects of TiO_2 nano-particles on the germination as well as growth of pea plants seeds were studied in detail. These nano-particles acted as photocatalyst resulting in enhanced light absorbance.

TiO_2 nano-particles with anatase phase were observed to improve and help advance plant growth due to improved nitrogen metabolism that stimulated nitrate absorption in Pea and, amplified the conversion of nitrogen (inorganic to organic), consequently increasing the fresh and dry biomass of plants. Studies have also demonstrated the effects of nano-TiO_2 (rutile) and non nano-TiO_2 on the germination process and growth of naturally aged spinach seeds. During the growth stage, nano-TiO_2 has improved the chlorophyll content, proteins in pea as well as promoted antioxidant stress by lowering the buildup of superoxide radicals, hydrogen peroxide thus increasing the evolution of oxygen rate in pea chloroplast (Zheng *et al*., 2005).

The effects of TiO_2 nano-particles on plant growth were also studied in various other studies. Results with improved photosynthesis and growth in plants were reported due to the applied TiO_2 nano-particles but the main mechanism was vague. Generally, the absorption of light in chloroplast and light-harvesting complex II was supposed to stimulate by TiO_2 nanoparticles; thus increasing the transformation from light energy to electronic energy, water photolysis, and oxygen evolution (Ze *et al.*, 2011).

Studies showed that low concentrations of TiO_2 NPs were not found to be harmful to plants cell membrane and these findings have revealed their positive impact on the pea cells. The tested concentration levels did not induce any morphological effect, perhaps due to their short-term thermal exposure or low concentrations levels. TiO_2 NPs especially at 5 ppm concentration level reported to reduce cold-induced damages in sensitive and resistant pea genotypes. Such domino effect raises key questions about the possible mechanisms that direct these effects. It was thought to occur due to the activation of some defensive mechanisms in pea seedlings after absorption of TiO_2 NPs. These results are quite interesting in case of environmental stressed conditions. Furthermore, new findings could possibly pave the way for the use of NPs especially for increase of cold tolerance in major crops (Mohammadi *et al*., 2013).

The effects of TiO_2 nanoparticles on some of the physiological and chemical parameters in barley (*Hordem vulgare* L.) were studied during an experiment. The nano-particles were sprayed in their growth stages (stem elongation and 4 leaves stage) with five levels of TiO_2 which included: control, Bulk TiO_2, 0.01, 0.02, and 0.03 percentages (Moaveni *et al.*, 2011).

Larue *et al*. (2012) showed the accumulation of TiO_2 nano-particles with size lower than 140 nm in wheat (*Triticum aestivum*) roots. NPs can be easily transferred in to the leaves if their diameter is smaller than 36 nm. Accumulation reaches 109 mg Ti/kg dry weight in wheat roots, but their concentration was below the detection limit in wheat leaves. Enhanced wheat root elongation was observed when exposed to 14 and 22 nm TiO_2 NPs. On the contrary it neither affected wheat seed germination, nor vegetative development, photosynthesis or redox balance (Larue *et al*., 2012)

2.9 Effects of Silver nanoparticles on Plants

In some experiments silver NPs have also been used to study their effects on the seed germination as well as the root growth of zucchini plants (in hydroponics system). In this case decrease in the plant biomass and transpiration was observed along with their prolonged growth in presence of Ag nano-particles. Such type of research and results beg the need to further examine the ecotoxicity impacts caused by these nano-particles, their uptake, translocation and distribution in various plant tissues (Nair *et al*., 2010)

2.10 Related Research Done at IESE, NUST

Hanif *et al*. (2015) has done work on "Phyto-availability of phosphorus to *Lactuca sativa* in response to soil applied TiO_2 nanoparticles" and found increased phytoavailability of phosphorus due to TiO_2 nanoparticles and found enhanced root/shoot lengths (1.5 fold), total dry biomass (2 fold) & total P uptake by (4 fold).

Zahra *et al*. (2015) has done work on "Metallic nanoparticle (TiO_2 and Fe_3O_4) application modifies rhizosphere phosphorus availability and uptake by *Lactuca sativa*" and found significant effect on plant growth in terms of root and shoot length and biomass. Shoot length increased up to 36% by TiO_2 and 49% by Fe_3O_4 treated groups as compared to control. Total dry biomass was increased up to 1.4 by both nanoparticles.

Rafique *et al*. (2015) worked on "Growth response of wheat to titania nanoparticles application" and found phytoavailability of phosphorus and growth of lettuce increased with increasing concentration of TiO_2NPs. For wheat plants, the best results in terms of improved growth and phosphorus uptake were found at 60 mg kg^{-1} of TiO_2NPs as compared to the control.

Ullah and Arshad, (2014) worked on "Exposure-response of Triticum aestivum to titanium dioxide nanoparticles application: seedling vigor index and micronuclei formation" observed root elongation at 200 & at 400 mg/L it may decline. He also found low toxic effect on short term exposure as compared to control.

From the above cited detailed literature review, I hypothesized that by the application of nano-particles, nitrogen uptake by the plants may be enhanced. To test this hypothesis, methodology adopted is discussed in detail in Chapter 3.

Chapter 3

MATERIAL & METHODS

Mainly pot experiment was performed to assess the phytoavailability of nitrogen in response to TiO_2 nanoparticles application to soil. Pea (*Pisum sativum*) plant was selected for experiment. Plants were exposed to TiO_2 nano-particles over a period of three months. The whole experiment was conducted in a locally made greenhouse at IESE, SCEE, NUST. The first phase and analysis focused on plants growth due to the application of nanoparticles, the second phase purpose was to focus solely on the phytoavailability of nitrogen in response to these nanoparticles. Keeping in view the main objectives of the study, following methodology was adopted which is being discussed here in detail accordingly.

3.1 Preparation of Titania Nano-particles

3.1.1 Synthesis of TiO_2 nanoparticles by Liquid Impregnation method

For the present study, Titanium dioxide nanoparticles (TiO_2NPs) were synthesized by using Liquid Impregnation (LI) method in IESE laboratory. In 100 mL distilled water 5 g of Titanium dioxide powder, General Purpose Reagent (GPR) was added and placed on magnetic stirrer for 12 hrs. The mixture was then allowed to settle overnight and after that placed in oven at 105 °C for drying. The dried material was then grinded by using mortar pestle. The grind material is then placed in muffle furnace for 5 hrs at 400 °C (Zeb *et al.*, 2010).

3.2 Characterization of Titania Nano-particles

There are different techniques used to characterize nanoparticles for their phase identification, structure, shape, surface morphology and size of the particles. The techniques used for the present study were:

- X-ray Diffraction (XRD)
- Scanning Electron Microscopy (SEM)
- Energy Dispersive X-ray Spectroscopy (EDS/EDX)

3.2.1 X-Ray Diffraction XRD

The phase composition, crystalline size and crystal structure measurements for TiO_2NPs were characterized by X Ray Diffractometer (Theta-Theta STOE, Germany) with X-ray operating conditions at 40 kV and 40 mA. Absolute scan with step mode was used, the range for 2 theta angle was 20^o - 80^o. X'Pert High Score software package (PANalytical B.V. Almelo, Netherland) was used for the analysis of XRD results. The crystallite size of nanoparticles was estimated according to the line width of the (101), plane refraction peak for TiO_2 by using Scherer Formula.

3.2.2 Scanning Electron Microscopy SEM and EDS

TiO_2NPs' surface morphology was analyzed on Jeol, JSM 6490, SEM instrument (Japan) equipped with EDS (Jeol, JED 2300) and ion sputtering device (Jeol, JFC 1500). TiO_2 suspensions were prepared on quartz slides in ethanol and were directly observed under the microscope at different magnifications.

3.3 Soil Analysis & Preparation

3.3.1 Soil texture

Classification of soil was done on the basis of saturation percentage.

0-19% Sand

20-29% Sandy loam

30-45% Loam

46-60% Clay Loam

More than 60% Clayey

3.3.2 Soil pH

To ensure the appropriateness of soil for plant growth pH was measured. 10 g air-dried soil (< 2 mm) was added in 50 mL of distilled water by using a graduated cylinder. Mixture was prepared in a 100 mL beaker, stirred with a glass rod and allowed to stay for half hour. Suspension was stirred again after 1 hr, combined electrode (Cyberscan 500^{PH}) was put and reading was noted after 30 seconds (McLean, 1982).

3.3.3 Moisture content

10 g air-dried soil (< 2 mm) was taken in a Petri dish. It was then dried in oven at 105°C overnight. It was removed from oven; cooled in a desiccator for 30 minutes and then re-weighed. Moisture content was calculated by using the following relation:

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

3.3.4 Soil Preparation

When the soil was analyzed it was found to be suitable for experimentation therefore about 50 kg soil was obtained from National Agriculture Research Centre, Islamabad and spread out to dry for the subsequent two weeks with regular thorough mixing. By using sieve shaker roots, shoots and other material was removed and < 2mm of homogenized soil was obtained, weighed and subsequently mixed with nanoparticles in desired concentrations. Plastic pots (10 cm diameter and 9.5 cm height) were used for the experimentation.

3.4 Application of TiO_2 Nanoparticles in Soil

Nano-TiO_2 suspensions of proposed concentrations were prepared by weighing calculated amounts of nanoparticles separately and afterwards added in distilled water. Dispersion of these nanoparticles was prepared by using ultrasonicator (JAC Ultra Sonic 1505) for 40 minutes. For pea cultivation, a series of projected concentrations of nano-TiO_2 suspensions were added to the soil and vigorously mixed.

3.5 Plant Cultivation

Pea (*Pisum sativum*) seeds were sown in clayey loam soil at IESE. Soil was amended with different concentrations of TiO_2 NPs (0, 50, 100, 250, 500 and 750 mg kg^{-1}) at the time of sowing and watered for 90 exposure days. Freshly grown plants were then used for experimentation. There were 5 replicates for each concentration level. During experimental phase pots were kept at IESE, NUST.

3.6 Plant parameters (biomass, length, pH, moisture content %)

At the harvest, the whole plant was removed from the pot with at least minimal damage to the roots, by shaking the roots until the soil not tightly adhering to the roots was removed and then collecting the soil closely adhering to the root system by vigorously washing the roots in

100 mL distilled water. The pH of soil solutions was found and it was then filtered. The rhizosphere soil (approximately 5 g) collected near roots zone was analyzed for phytoavaliable nitrogen by Ammonium Bicarbonate DTPA Method.

Plants roots and shoots were collected separately, lengths were measured, and fresh biomass was weighed.

3.6.1 Plant Nodulation studies

Nodules of pea plants roots cores were collected, counted, washed, oven dried and weighed in order to collect the following data:

- Total number of nodules
- Fresh weight of nodules
- Dry weight of nodules

Shoots and roots dry biomass was weighed were then placed in oven at 70 °C for 48 h and. Moisture content percentage was calculated. Separately shoots and roots were ground with mortar pestle and the equivalent amounts were then kept in air tight sampling bags for N analysis.

3.7 Estimation of Foliar Chlorophyll Content

3.7.1 Plant material

Pea plant used for this study was grown in a locally made green house and chlorophyll content was measured during month of April, 2015. Over the course of study, readings were noted after 30 and 45 days of TiO_2NPs exposure time for pea plant. There were four replicates for each treatment level. The measurements were made on each plant sample separately using fifteen averaging by hand-held chlorophyll absorbance meter. The readings were recorded on the leaves of plants between the midrib and the leaf margin to avoid the placement of meter over major leaf veins.

3.7.2 Hand-held chlorophyll meter

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

3.7.3 Data Analysis

For the analysis of data, arithmetic mean and calibration equation were 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

3.8 Phytoavaliable Nitrogen in Soil

N is the most vital nutrient in agriculture due to its high requirements and low levels of available-N in many soils. Plant roots absorb nitrogen in forms of NH_4-N and NO_3-N ions. Breakdown of OM, manures or urea or ammonium-containing fertilizers produce ammonium ion. N breakdown/reactions (excluding gaseous forms of N) result in nitrate ions. Nitrate ions are highly soluble in water, and have a number of solutions so it is measured for estimating the need of N fertilizer for growing food crops.

The proposed technique of AB DTPA is a multi-element soil test reported by Soltanpour and Schwab for alkaline soils (1977). It was later revised by Soltanpour and Workman (1979) to overlook the use of carbon black. The extracting solution was 1M in the ammonium bicarbonate (NH_4HCO_3) and 0.005 M DTPA adjusted to pH 7.6. NO_3-N may be then determined in that extract.

Therefore, AB DTPA method has been adapted for the N analysis of soil in the Biotechnology Laboratory at IESE, NUST.

Apparatus

- Atomic absorption spectrophotometer
- Spectrophotometer suitable for measurement at 880 and 420-nm wavelength
- Accurate automatic dilutor
- Flame photometer
- Mechanical shaker

Reagents

A. Extracting Solution

To acquire 0.005 M DTPA, 1.97g *DTPA* was added to 800 mL DI water. Approximately 2 mL Ammonium hydroxide (NH_4OH) was then added to facilitate dissolution and to prevent spark when bicarbonate was added.

79.06 g of ammonium bicarbonate (NH_4HCO_3) was added and stirred gently until DTPA is completely dissolved. Ammonium hydroxide was added to adjust pH and the solution was prepared up to 1-L volume with water.

B. Hydrazine Sulfate Stock Solution ($H_2N_2H_2.H_2SO_4$)

- 27 g Hydrazine sulfate was dissolved in 750 mL DI water, diluted to 1-L volume, and mixed well.
- Hydrazine sulfate working solution was prepared diluting 22.5 mL stock solution to 1-L volume with DI water. This solution remains stable for 6 months.

C. Copper Sulfate Stock Solution ($CuSO_4.5H_2O$)

- 3.9 g copper sulfate pentahydrate was dissolved in 800 mL DI water, diluted to 10 L volume and mixed well.
- 6.25 mL of the stock solution was diluted to prepare 1L volume of copper sulfate working solution with DI water.

D. Sodium Hydroxide Stock Solution (NaOH), 1.5 *N*

- 60 g *sodium hydroxide* was added in 500 mL DI water, cooled and brought to 1-L volume with DI water.
- 0.3 N sodium hydroxide working solution was prepared by diluting 200 mL stock solution to 1-L volume with DI water.

E. Color Developing Solution for Nitrate-Nitrogen

5 g *sulfanilamide* and 0.25 g *N- (1-naphthyl)-ethylenediamine dihydrochloride* was added to 300 mL DI water. 50 mL 85% *orthophosphoric acid* (H_3PO_4) was then slowly added with continuous stirring, and 500 mL volume was prepared by adding DI water. This reagent should be prepared as required as it cannot be used after appearance of pink color.

F. Standard Stock Solutions

Nitrate-N:

0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 ppm NO_3-N containing working standards were prepared by given procedure:

Procedure

1. **Extraction Method**

10 g air-dried soil (2-mm) was weighed into a 125 mL conical flask. 20 mL extracting solution was then added, and shaken on a reciprocal shaker for 15 minutes at 180 cycles/minute with flasks kept open. The extracts were then filtered through Whatman filter paper No. 42.

2. **Nitrate-N**

1 mL of the soil extract was transferred to 25 mL test tube, 3 mL copper sulfate working solution, 2 mL hydrazine sulfate working solution, and 3 mL sodium hydroxide working solution was added. Solution was mixed and heated in a water bath at 38^{O}C for 20 minutes. Removed from water bath, 3 mL color-developing reagent was then added for NO_3-N. Solution was mixed and let stand at room temperature for 20 minutes. Absorbance was finally noted on spectrophotometer at 540-nm wavelength (Kamphake *et al.*, 1967). Standards calibration curve was obtained using absorbance values for standards.

Formula

For Nitrate – N in soil:

$$NO_3 - N\ (ppm) = NO_3 - N\ (ppm\ in\ extract) \times Dilution\ Factor$$

3.9 Nitrogen Analysis in Plant

Plants need a broad variety of proteins in order to grow, develop and mature in the right way. Amino acids are considered the building blocks of proteins. Nitrogen (N) is a major component of amino acids. Even plant chlorophyll contains nitrogen. Soil micro-organisms feed on soil N during the breaking down process of OM. Nitrogen helps in improving the quality of leafy vegetables and plants. It promotes rapid growth however, flowering and fruiting may be affected adversely if the supply is out of balance.

Dry plant matter normally contains anywhere between 1 to 5 % nitrogen. However sometimes it may be vary out of this range as well. Kjeldahl method is the most commonly used plant analysis method. Method is explained in detailed below:

Preparation of Reagents

a. **Reagent A:** 25 g ammonium heptamolybdate [$(NH_4)_6Mo_7O_{24}.4H_2O$] was dissolved in 500 mL warmed distilled water precisely (5% solution)

   (a) 1.25 g of ammonium metavanadate (NH_4VO_3) was dissolved in 500 mL boiled distilled water (0.25% solution)

   (b) When both these solutions were cooled down to room temperature then (b) was added to (a) and then 500 mL nitric acid ($HNO_3 : H_2O :: 1 : 3$) was added to the mixture in a volumetric flask. Solution was then allowed to cool further at room temperature.

b. **Reagent B:** 333.3 mL concentrated perchloric acid was added to 666.6 mL concentrated nitric acid in 1 L volumetric flask. This acid mixture was then allowed to cool down.

c. **Standard Stock Solution:** Exactly, 2.197 g oven dried potassium dihydrogen phosphate was dissolved in 500 mL distilled water (1000 ppm stock solution). 10 mL of the prepared solution was diluted with distilled water up to 100 mL (100 ppm sub stock solution).

A. Wet Digestion Method

Roughly, 0.1 g of ground plant material was added to 25 mL flask. 5 mL of the acid mixture was added to flask till the vigorous reaction stage was completed. Flask was then placed on hot plate in a fume hood and further heated. Temperature was increased slowly and gradually until all traces of nitric acid disappeared. Heating was continued on plate until dense fumes of perchloric acid just appeared and volume was made up to 25 mL. The plant digested material was filtered using the Whatmann filter paper No. 42, and extracts were stored at 4 °C for further analysis.

B. Measurement

1. Furthermore, 2.5 mL of the digested filtrate of ash plant material was taken into a 25 mL volumetric flask; 5 mL ammonium-vanadomolybdate reagent was added and volume was made with distilled water.

2. Precisely the sub-stock solution was pipetted out to 25 mL volumetric flask to select and set a series of standards. These solutions contain 0, 0.25, 0.5, 0.75, 1, 1.25, 1.50, 1.75, 2, 2.5, 2.75, 3, 3.5 and 4 mg kg^{-1} P, respectively. 5 mL mixed reagent was added and continued the same for the samples. A blank was also prepared with 5 mL ammonium-vanadomolybdate reagent as done for samples. Absorbance of the blank, standards, and for samples were noted after 1 hour at 430 nm wavelength. A calibration curve was prepared for standards, recording absorbance against respective N concentration. Nitrogen concentration for the unknown samples was estimated by using the calibration curve (Ryan *et al*., 2007).

3.10 **Statistical Data Analysis**

Plant parameters tests were carried out in triplicate. The statistical significance of results was checked by using Student's T-test (mean analysis) and standard deviation. Statistically significant differences were reported when the probability of the result assuming the null hypothesis ($p < 0.05$).

Chapter 4

RESULTS AND DISCUSSION

4.1 Characterization of Titania Nanoparticles

TiO_2NPs were prepared using Liquid Impregnation method in order to get the desired characteristic properties of the nanoparticles to be used for plant experimentation.

4.1.1 XRD Results of TiO_2 Nanoparticles

The phase composition, crystal structure and crystalline size measurements for the TiO_2 nanoparticles synthesized by Liquid Impregnation method were done using X Ray Diffractometer. XRD determines the crystalline phase of the nanoparticles by analyzing diffraction of X-rays. Average crystalline size of nanoparticles was determined by using Scherer formula (Younas, 2014).

$$\mathbf{L} = \frac{\mathbf{k\lambda}}{\boldsymbol{\beta} \cos\boldsymbol{\theta}}$$

Where,

L = Average particle size

k = 0.891, a shape factor of spherical particles

λ = 0.1542, wavelength of X-Rays

β = Full width of a diffraction line at half of maximum intensity (FWHM)

θ = Diffraction angle of crystal phase

TiO_2 nanoparticles used in this study ranged from 71.22-90.59 nm while average particle size of nanoparticles is 77 nm. Peaks of XRD results revealed that nanoparticles have crystalline structure (Figure 4.1). Strong diffraction peaks at 25.320° and 48.080° confirm that synthesized TiO_2NPs are in anatase phase (Ba-Abbad *et al*., 2012). Sample was compared with card no.

JCPDS 01-089-4921. The crystal structure was found to be tetragonal which was in agreement of that reported in literature.

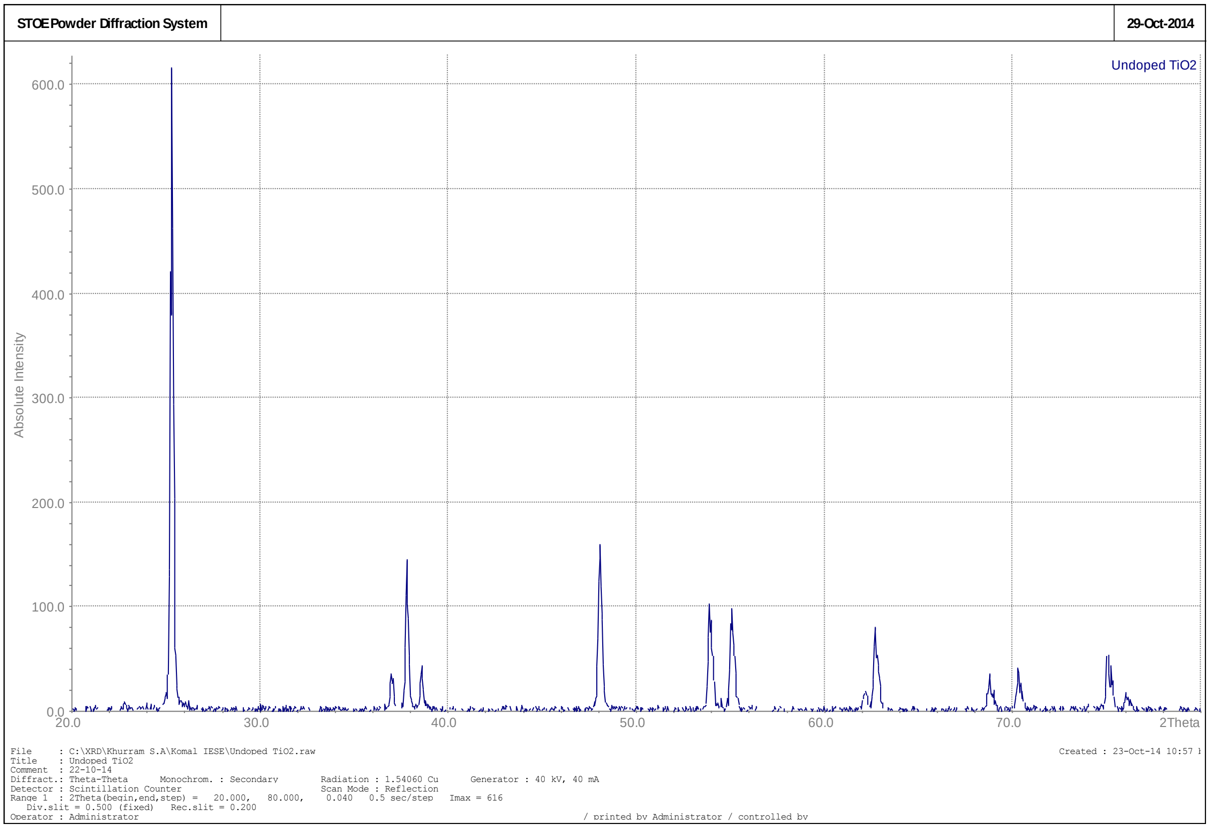

Figure 4.1 XRD Patterns of TiO_2 Nanoparticles

Peaks obtained at 25.320°, 37.760°, 37.800°, 37.840°, 48.080°, 53.880°, 55.080°, 62.680°, 68.760°, 70.320°, 75.040° and 76.000° were of (617), (113), (146), (104), (161), (104), (99), (81), (37), (37), (56) and (19) planes respectively.

4.1.2 Scanning electron microscopic (SEM) images

The surface morphology of TiO_2NPs was estimated by JEOL JSM-6460 SEM at 20,000 magnifications. The image of the pure titania shows that particles were spherical in shape and distributed in the range of 71.22-90.59 nm (Figure 4.2). Image of TiO_2 confirms the presence of a porous structure like a sponge with a large roughness and complexity.

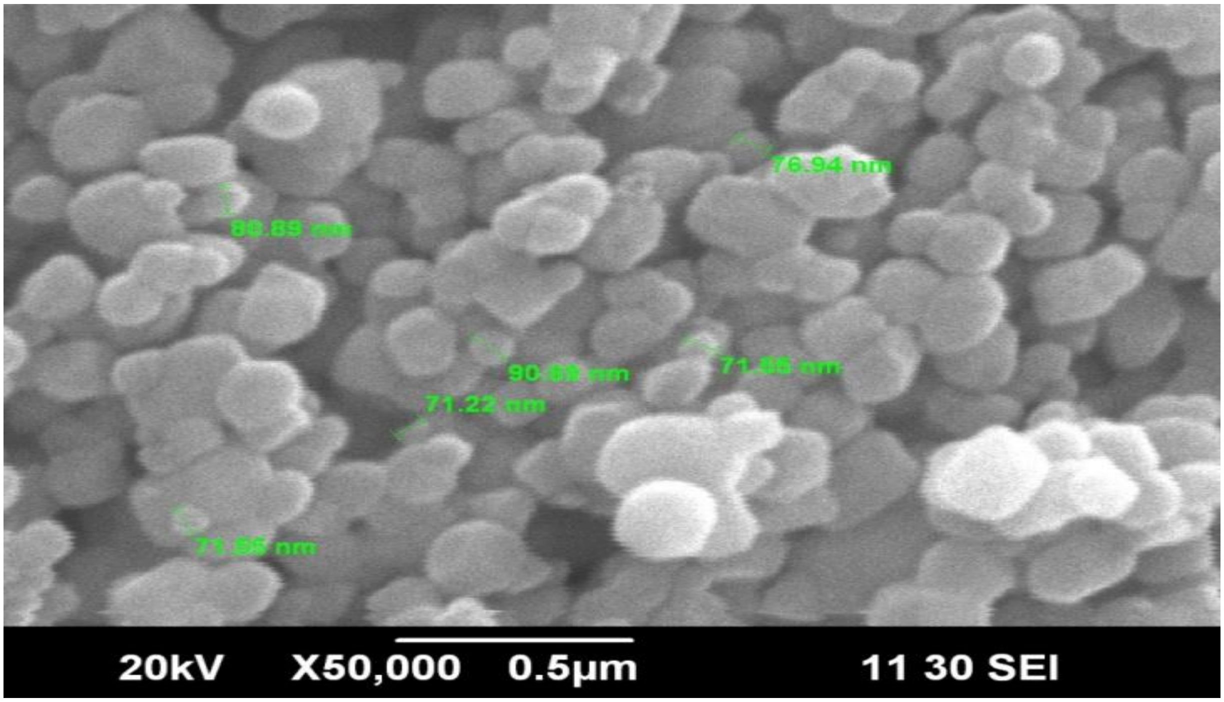

Figure 4.2 SEM images of TiO_2 NPs by Liquid Impregnation Method

4.1.3 Energy dispersive spectroscopy (EDS)

EDS examined the elemental composition of pure TiO_2 nanoparticles. Figure 4.3 and Table 4.2 shows the presence of Ti and O elements in the representative sample. It varies from point to point showing diverse composition of the prepared NPs that confirms the SEM results. It also confirms that the nanoparticles contain oxygen and titanium dioxide only while no foreign element or impurity is introduced into the synthesis process.

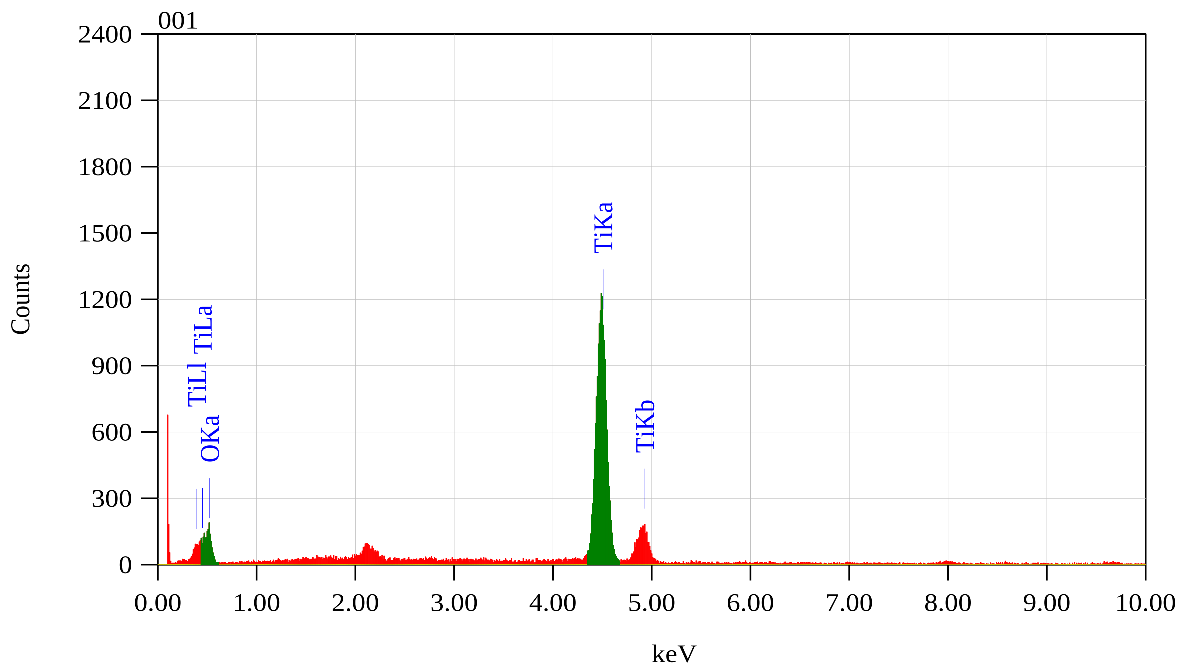

Figure 4.3 EDX analysis of TiO_2NPs

Material	Ti		O	
	Expected	Found	Expected	Found
TiO_2NP (Pure)	60	60.37	40	39.63

Table 4.1 EDS Results of TiO_2NP's

4.2 Growth Response of *Pisum sativum* L. to Nanoparticles

Figure 4.4 & 4.5 illustrated the influence of nanoparticles on physical growth parameters of *Pisum sativum.* The results demonstrated a significantly higher plant growth in those plants, which were treated by TiO_2NPs. With respect to control, plants exposed with TiO_2 NPs showed significant improvements in shoot and root lengths.

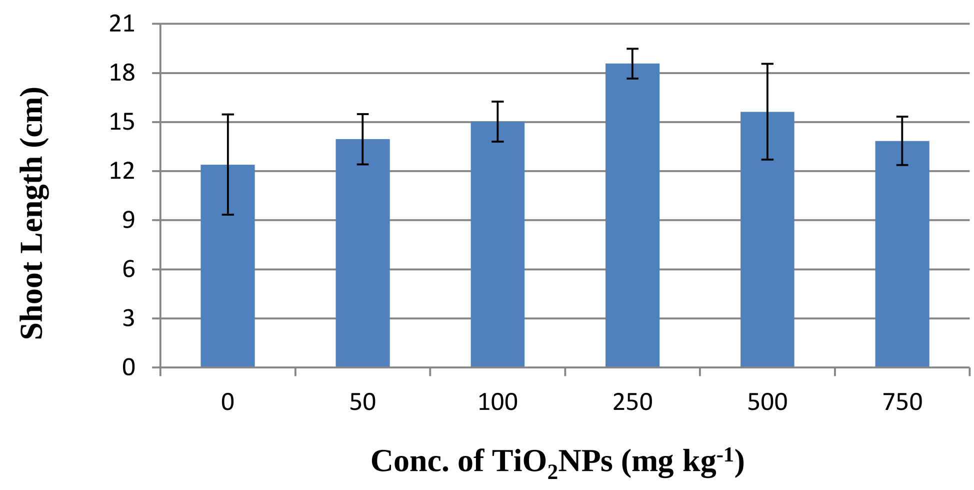

Figure 4.4 Shoot length of *Pisum sativum* in response to nanoparticles treatment

In Figure 4.4, the shoot length percentage showed the highest value (18.58 cm) in 250 mg kg^{-1} of TiO_2 nanoparticles whereas the lowest value (12.40 cm) was observed in control. Shoot growth increased up to 50% by TiO_2 nanoparticles over control and followed decreasing trend when the concentration of TiO_2 NPs was higher than 250 mg kg^{-1}.

It is believed that nanoparticles influence the growth of plants because of their antimicrobial properties. (Husen and Siddiqi, 2014). Jaberzadeh *et al*. (2013) reported that TiO_2NPs augmented wheat plant growth and provided components under conditions of water stress.

Similarly in another study, the effects of TiO_2 nanoparticles on the germination and growth of spinach seeds were studied and reported to improve plant growth. TiO_2 nanoparticles with anatase phase act as photocatalyst, enhanced light absorbance and promoted the activity of Rubisco activase resulting in increased spinach growth (Yang *et al*., 2007).

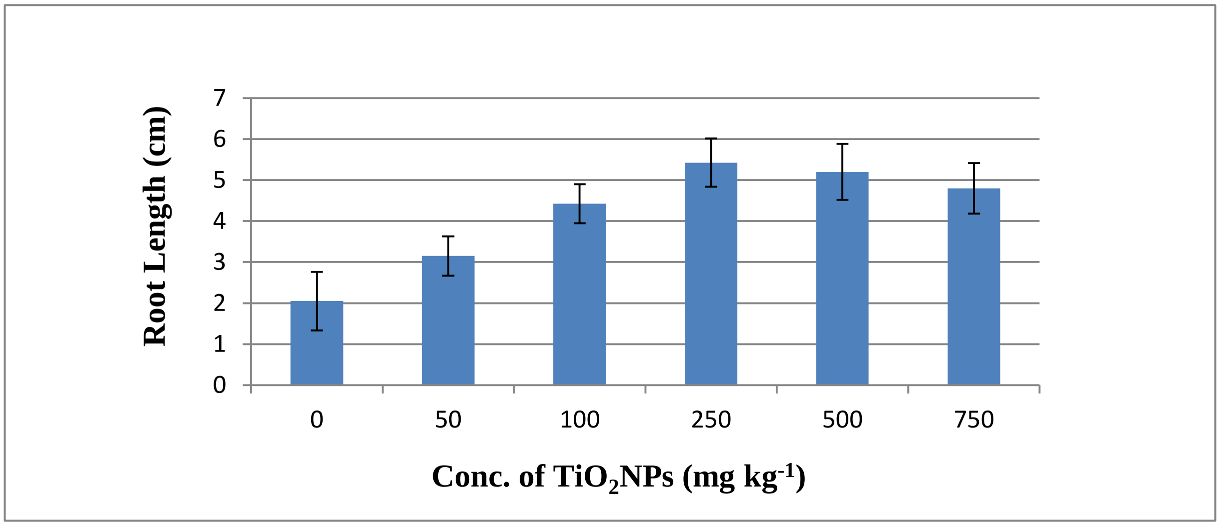

Figure 4.5 Root length of Pisum sativum in response to nanoparticles treatment

In the Figure 4.5, root length was observed to increase up to 5.425 cm (2.65 folds) in TiO_2 treated group as compared to 2.05 cm in control. Both shoot and root lengths in TiO_2 treatment was increased upto 250 mg kg^{-1} level of concentration further inhibiting lengths due to accumulation of TiO_2 in roots. As expected, it is observed that roots contained significantly higher levels of NP as compared to shoots.

In another study, TiO_2 nanoparticles were applied to wheat (*Triticum aestivum*). Enhanced wheat root elongation was observed when exposed to 14 nm and 22 nm TiO_2 NPs (Larue *et al*., 2012).

The main reason for this increased growth rate is due to the increased phytoavailability of nitrogen in soil. Nanoparticles retain the cell walls and simultaneously trap radicals molecules in the niche of bacterial cells indirectly regulating the activity of secondary metabolites, which are important in the process of nodulation and nitrogen fixation (Ghalamboran, 2011). Noticeably, nano-TiO_2 treatments showed a significant ($p < 0.05$) positive effect on the plant growth. Our results also coincides with the results of (Dhoke *et al*., 2013) who reported about 97.87 % increased shoot growth and up to 76.04% increased dry shoot biomass by using 20 mg L^{-1} of ZnO nanoparticles over control ($P < 0.05$) in mung seedlings while 6.38% and 26.61% increase in shoot growth and dry biomass respectively with dose of 1 mg L^{-1} ZnO nanoparticles in gram seedlings (Dhoke *et al*., 2013).

Alidoust & Isoda (2013) also examined the effect of NP Fe_2O_3, Fe_2O_3 NPs coated citrate, Fe_2O_3 citrate bulk and bulk Fe_2O_3 coating on soybean leaf physiology by exposure or soil. The authors reported that Fe_2O_3 NPs significantly increased root elongation and photosynthetic parameters compared to the corresponding bulk and citrate-coated Fe_2O_3 NPs via foliar application.

4.3 Fresh and Dry Biomass of *Pisum sativum* in Response to Nanoparticles

Use of TiO_2 enhanced *Pisum sativum* growth, plant fresh and dry biomass as compared to the control group. The total fresh and dry biomass of peas was increased with increase in the concentration of TiO_2NPs up to 250 mg kg^{-1} but decreased at higher treatment levels (Figure 4.6 and 4.7).

In almost all studies, the size of nanoparticles appears to be the decisive factor. Since the concentration of metal or metal oxide nanoparticles increases, the growth increases and reaches a maximum value after which either it becomes constant or delay in growth occurs. In such instances, the enzyme activity is either lost or the nanoparticles block the passage of other nutrients as a result of accumulation (Husen and Siddiqi, 2014).

In comparison with control, plants treated with TiO_2NPs showed improvements in total fresh (42% increase) and dry biomass (46% increase) significantly ($p < 0.05$) at 250 mg kg^{-1}. Zheng *et al*. (2005) reported that the significant results of spinach growth were observed at low concentration levels (< 2500 mg/L) of TiO_2NPs as compared to high concentration levels (> 2500 mg/L).

The total fresh and dry biomass showed the same trend as root and shoot length of *Pisum sativum* when exposed to TiO_2NPs. It may be explained by the fact that plant is composed of roots and shoots, and their growth changes will affect the biomass. The fresh and dry biomass of shoots in nanoparticles treated groups was enhanced by 2.1 folds respectively as compared with the untreated groups while the root fresh and dry weight increased to 3.5 and 4.1 folds at 250 mg /kg. It is postulated from the results that TiO_2NPs at 250 mg kg^{-1} proved to be more effective as compared to low and high treatment levels. It is also possible that *Pisum sativum* is more sensitive and permits the addition of TiO_2NPs in a limited range i.e. 50-250 mg kg^{-1} in our case.

Plant biomass (shoot and root) results were found in concurrence with the growth for nanoparticles concentrations and exhibited a strong correlation with applied concentrations. As the dry weight of a plant mostly defines its growth and strength. From the agricultural point of view these results are valuable to practice the improvement in productivity of crop plants with lesser amount of nanoparticles.

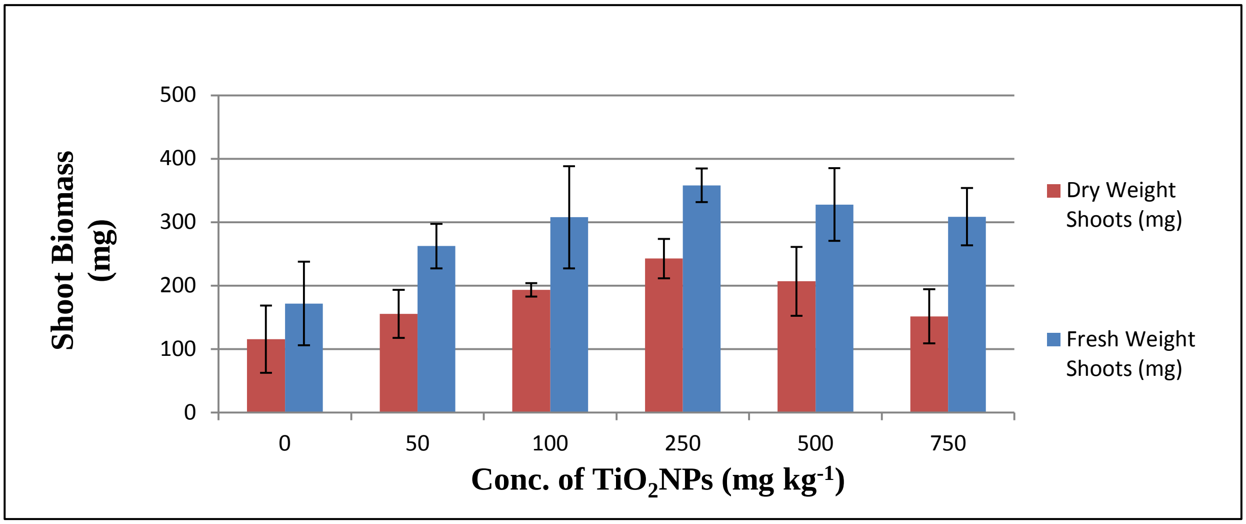

Figure 4.6 *Pisum sativum* shoot biomass in response to nanoparticles treatment

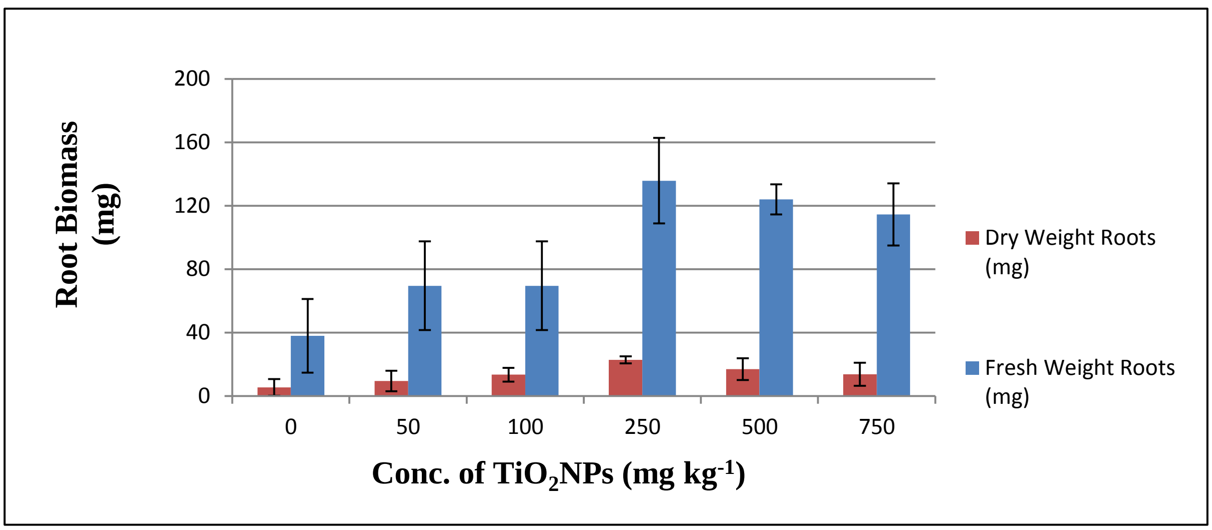

Figure 4.7 *Pisum sativum* root biomass in response to nanoparticles treatment

4.4 Effects of TiO_2 NPs on chlorophyll content of plant species

CCM measurements indicated that chlorophyll content of peas was increased consistently with an increase in TiO_2NPs concentrations (Figure 4.8). An increase of 54% in total chlorophyll content was observed with increasing concentration of TiO_2NPs as compared to control (Figure 4.8). The value of chlorophyll content was increased sharply at 50 mg kg^{-1} and 750 mg kg^{-1} concentration points.

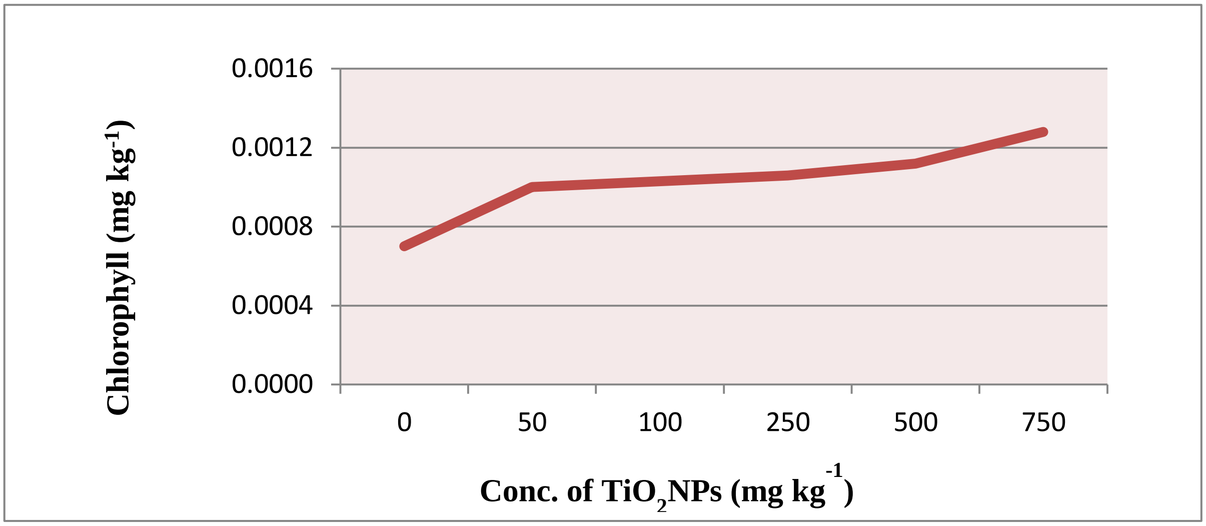

Figure 4.8 Chlorophyll contents in the leaves of *Pisum sativum* in response to TiO_2NPs

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 TiO_2NPs may lead to increase in chlorophyll content and photosynthetic reactions (Skupień and Oszmiański, 2007; Owolade *et al.*, 2008; Chen *et al.*, 2012).

4.5 Nitrogen Concentration in Shoots and Roots of *Pisum sativum*

Figure 4.9 presented the N concentration in shoots and roots of *Pisum sativum*. The concentration of N in shoots with TiO_2 treatment was 3.042 mg kg^{-1} treated group as compared to control i.e. 2.412 mg kg^{-1}. Overall, increased dosage of nanoparticles applied resulted in increased N concentration over respective control. The shoot N concentration showed 1.2 fold increase for TiO_2 treatment. Therefore, significant increase in this phase of experiment was found due to different concentrations of nanoparticles applied. The lowest value 1.29 mg kg^{-1} and highest value was 1.93 mg kg^{-1} (TiO_2) for root N concentration was observed with 1.5 fold increase.

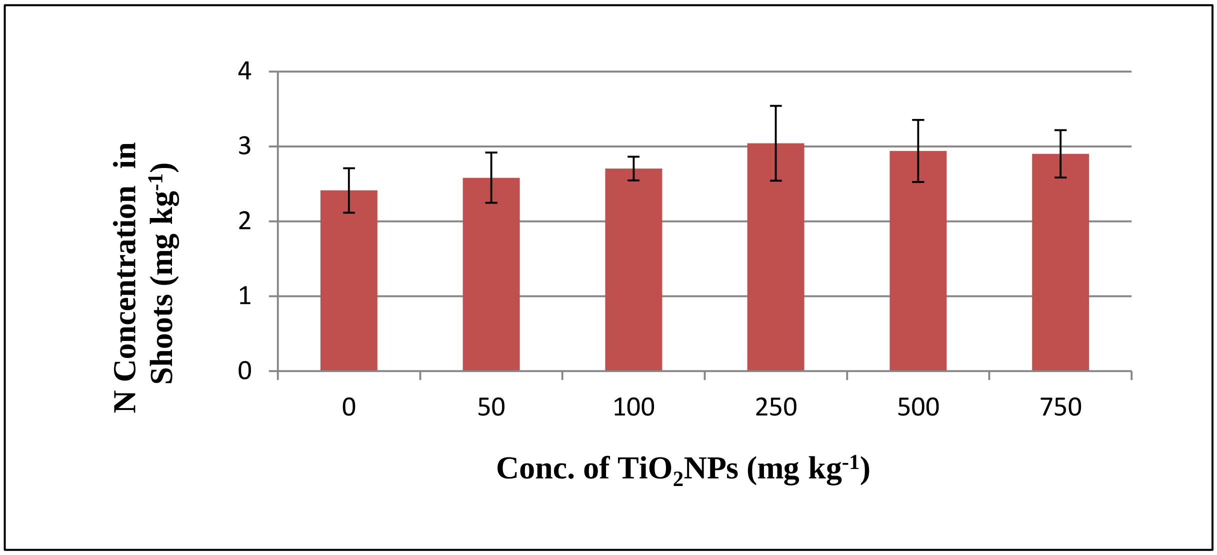

Figure 4.9 Nanoparticles effect on the Nitrogen concentration of plant shoots

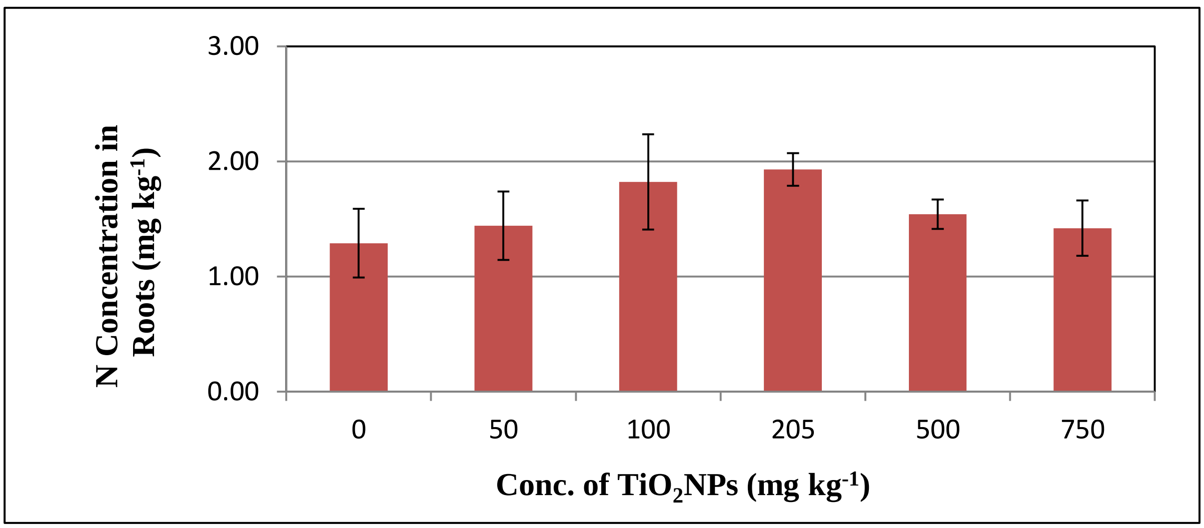

Figure 4.10 Nanoparticles effect on the Nitrogen concentration of plant roots

The N concentrations in shoot were found higher than the root N concentrations for TiO_2 treated group. The increase in both shoot and root N concentration from 0 to 250 mg kg^{-1} indicated its positive relationship with nano-TiO_2 application. This result is mainly due to the increased absorption of phytoavaliable N followed by increased conversion of atmospheric N to ammonium/nitrates ions.

Study revealed Nanosized TiO_2 particles may promote nitrogen metabolism in the plant leading to growth as a whole. The main reason for this increased growth rate is due to the increased phytoavailability of nitrogen in soil. Nanoparticles retain the cell walls and simultaneously trap radicals molecules in the niche of bacterial cells indirectly regulating the activity of secondary metabolites , which are important in the process of nodulation and nitrogen fixation (Ghalamboran, 2011).Similar trends have been shown in other studies, (Feizi *et al.,* 2012) and (Zheng *et al.*, 2005) that TiO_2 nanoparticles enhanced seedling germination, growth and promoted photosynthesis in wheat or spinach in comparison to control plants.

While in another study low or zero toxicity was observed when evaluated the effects of model nanoparticles (Au, Ag and Fe_3O_4) on plants and microorganisms (Barrena *et al*., 2009). In a phytotoxicity study conducted to estimate the effects of NPs on soil and no effects were observed in seed germination. In general NPs induced varied effects on inhibition of plant root elongation with respect to soil type (Josko and Oleszczuk, 2013). With reference to the effects of nanoparticles same species may show varied results depending not only on their size, shape and phase, but also on the concentration levels applied, experimental conditions and plant species as well as their mechanism of uptake (Castiglione *et al*., 2011). The literature review had revealed that the information on plants with respect to nanoparticles application was at the earlier stage. Nearly, there found no concluding studies on the subject matter; however, based on the limited information available, different observations had been cited in this regard.

4.6 Nodulation Number of *Pisum sativum* in response to TiO_2NPs

Figure 4.11 shows significant increase in nodule numbers per plant by nanoparticles application. Further, it is pertinent to mention here that increased number of nodules per plant has generated more nitrogen. The lowest average nodule numbers was obtained by control treatment and 50 mg kg^{-1} of the nanoparticles and the greatest average number of nodule was obtained by 750 mg kg^{-1} of the nanoparticles. Nodulation count increased by 7.5 folds.

It is reported that plant nodulation capacity is controlled mainly by genetic agents and environmental conditions (Cochran and Cox 1957). Increasing the number of bacterial cells in the bacteroid zone is an important factor beside increased number of nodules regardless of the effect of nutrients as assimilation occurs due to penetration of at least one bacterial cell into the meristem root hair cell

Ghalamboran (2011) reported in his study that magnetite nanoparticles and magnetite nano composites favorably effect number of nodules and nodule weight per plant. Symbiotic nitrogen fixation was enhanced by the effect of nanoparticles on secreted genistein from roots and nodf.

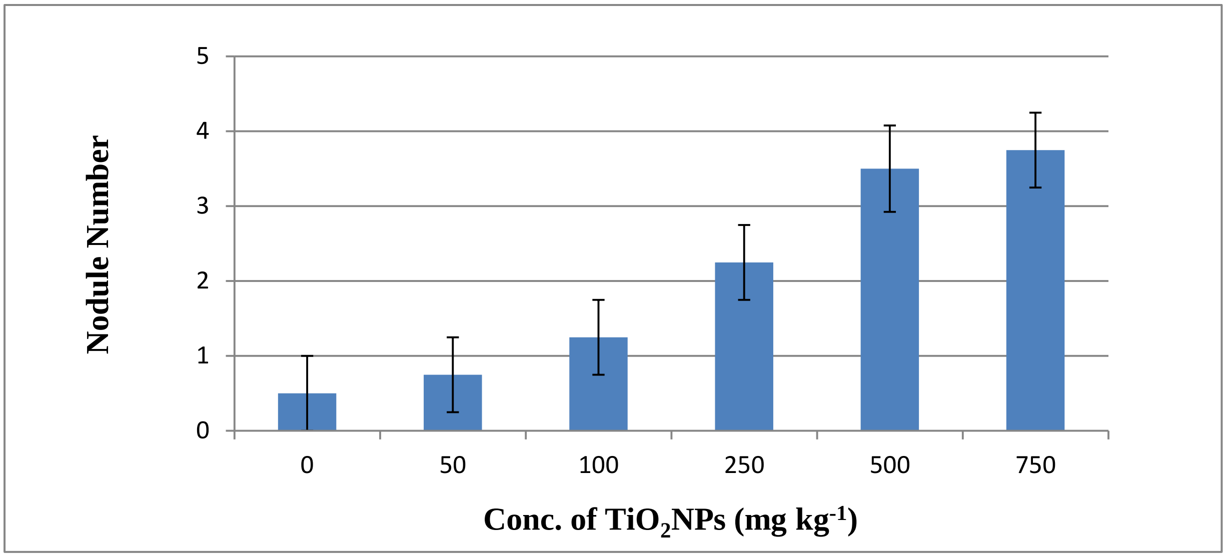

Figure 4.11 Nanoparticles effect on the number of nodules

4.7 Nodule biomass of *Pisum sativum* in response to TiO_2NPs

Figure 4.12 presented weight of nodules in reponse to TiO_2 nanoparticles. It was observed that dry weight of nodules at 750 mg kg^{-1} was 3.2 times greater than at 0 mg kg^{-1} (control group). Soil is a big pool of bacteria. In the present study there was no commercial inoculant used, but the presence of nodules in general and active ones in particular specified that soil was having their own native and effective strain of *Rhizobium leguminosaurum*.

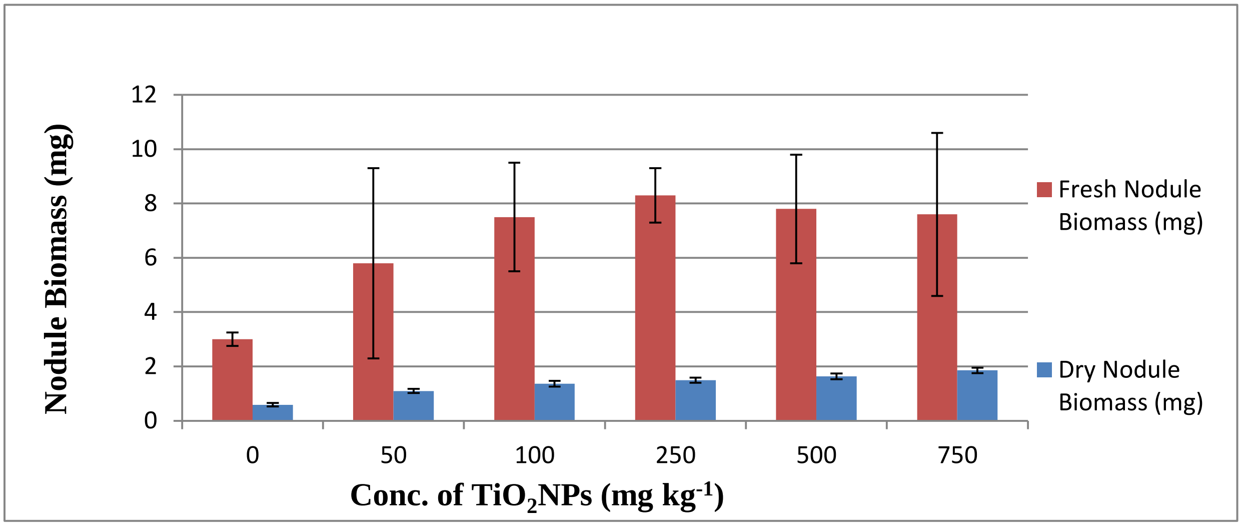

Figure 4.12 Nanoparticles effect on nodule biomass

4.8 pH of soil extract treated with TiO_2NPs

Figure 4.13 shows pH of soil extract treated with different nanoparticles concentrations.

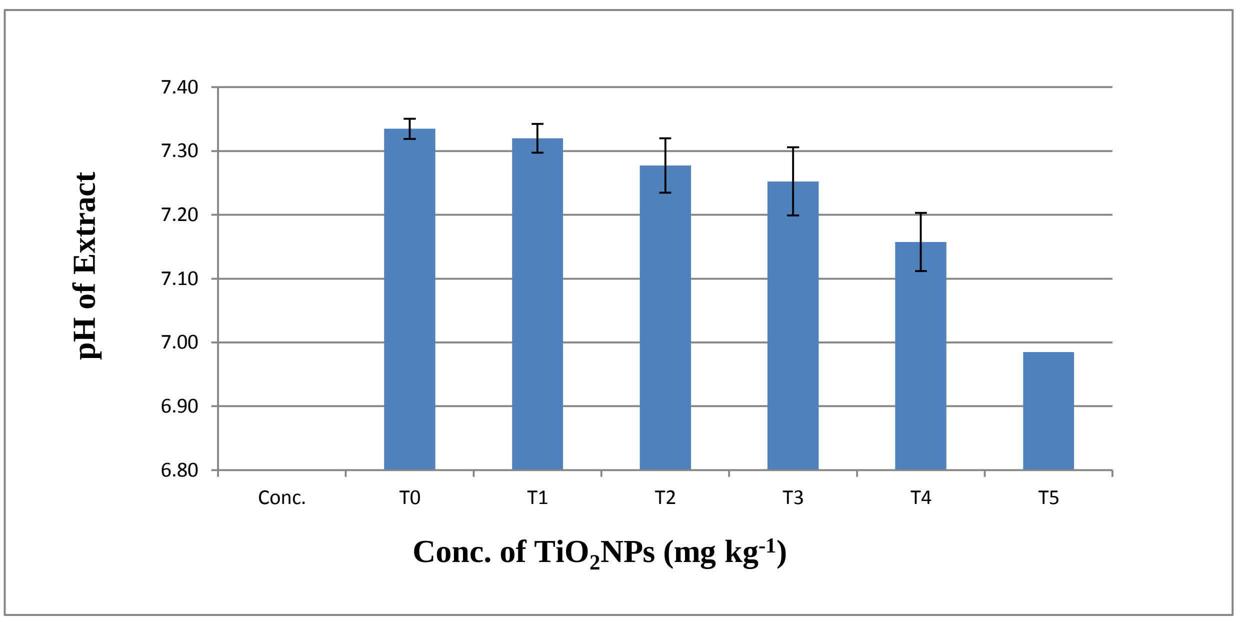

Figure 4.13 pH of soil extract treated with nanoparticles concentrations

Maximum yield of legume plants and fixed nitrogen by *rhizobia* was obtained at pH 7.25 and found significantly decreasing with decrease in pH below 7. Increase in TiO_2 NP concentration levels was also found to reduce pH.

Soil acidity is one of the important limiting factors for crop growth (Caires *et al*., 2008; Hawkins and Robbins, 2010). Peas like soybean are very sensitive to acidic soils, especially at low soil pH i.e., below 6 plants shows leaf scorching and yellowing. Decrease in shoot and root dry weights, nodules dry weight and number of nodules, shoot nitrogen concentration, and chlorophyll levels are also the results of acidic soils (Cline and Kaul, 1990; Caires *et al.*, 2008; Hawkins and Robbins, 2010). Conversely, plants may also have retarded growth under alkaline stress conditions (pH≥8). Studies like (Tang and Robson, 1993; Tang and Thomson, 1996; Tang *et al.*, 2006) confirmed that most legume species have decreased shoot growth, nodulation and nitrogen concentrations in shoots at high pH values.

In another study (Tang *et al.* 2006) reported some legume genotypes result in iron deficiency and poor nodulation under alkaline stress. They suggested impact of iron deficiency on plant biomass and nodulation was intensified with increasing pH (above 7.5). Dilworth *et al.* (2008) showed negative impact on symbiosis reaction activity and nutrients availability at pH range less than 4 and greater than 8.Taylor *et al.* (1991) advised that colonization of soils and soybean roots by *B. japonicum* may be negatively affected at pH< 4.6, further resulting in reduced cell numbers and nodulation.

Chapter 5

Conclusions and Recommendations

5.1 Conclusions

Application of different kinds of nanoparticles in the rhizosphere of *Pisum sativum* significantly affected plant growth in terms of root and shoot length, plant biomass, chlorophyll, available nitrogen and nodule formation.

1. *Pisum sativum* growth was affected by Titania nanoparticles. The optimum concentration point was 250 mg kg^{-1} of nanoparticles.
2. Nodulation number and nodule weight was affected by TiO_2NPs, implying that symbiotic nitrogen fixation (SNF) and nitrogen availability was affected. The optimum concentration was 250 mg kg^{-1} nanoparticles with 1.2 fold increase in shoots.
3. Shoot length increased up to 50% by TiO_2 treatment as compared to control. Total dry biomass was increased by 2.1 folds. In nutrient uptake mechanism, the nanoparticles affinities to adsorb nitrate and ammonium ions were the traits that could be optimized to improve the N efficiency for agricultural purposes.

5.2 Recommendations

TiO_2NPs help in N fixation & evasion of N deficiency in food crops. Following may be the future perspectives:

1. Varietal response of NPs should be checked in maximizing the nodule parameters (e.g. Fe_3O_4 NP consists of Fe that is an essential micronutrient for nitrogen-fixing bacteria).
2. Quantitative techniques may be used to determine nitrogenase activity and influence of TiO_2NPs on Nitrogen fixation.
3. There is need to do trials in combination with fertilizers.

References

Ali, M. A., Rehman, I., Iqbal, A., Din, S., Rao, A. Q., Latif, A. & Husnain, T. (2014). Nanotechnology, a new frontier in agriculture. *Advance Life Sciences*, *1*(3), 129-138.

Alidoust, D. & Isoda, A. (2013). Effect of gamma Fe_2O_3 nanoparticles on photosynthetic characteristic of soybean (*Glycine max* [L.] Merr.): Foliar spray versus soil amendment. *Acta Physiologiae Plantarum* , *35*, 3365-3375.

Anonymous. (2004). Inoculation of pulse crops (field peas, lentils, field beans, fababean soybeans). In: crops February 2004. *Manitoba Agriculture, Food and Rural Initiatives, Manitoba, Canada*.

Aslam, M., Mahmood, I. A., Sultan, T. & Ahmad, S. (2000). Inoculation approach to legume crops and their production assessment in Pakistan - A Review. *Pakistan Journal of Biological Sciences*, *3*(2), 193-195.

Aslam, M., Mahmood, I.A., Ahmad, S., Peoples M.B. & Herridge, D.F. (1997). Surveys of chickpea N_2 fixation in the Potohar and Thal areas of the Punjab, Pakistan. In: Extending nitrogen fixation research to farmer's fields. International workshop on managing legume nitrogen fixation in the cropping systems of Asia, 20-24 Aug. 1996, ICRISAT, Asia centre, India, 353-360.

Azam, F. (2001). Legume-bacterium (*Rhizobium*) association-symbiosis, a marriage of convenience, necessary evil or bacterium taken hostage by the legume. *Pakistan Journal of Biological Sciences*, *4*(6), 757-761.

Ba-Abbad, M. M., Kadhum, A. A. H., Mohamad, A.B., Takriff, M. S. & Sopian, K. (2012). Synthesis and catalytic activity of TiO_2 nanoparticles for photochemical oxidation of concentrated chlorophenols under direct solar radiation. *International Journal of Electrochemical Science*, 7, 4871-4888.

Ball, P. (2002). Natural strategies for the molecular engineer. *Nanotechnology, 13*, 15-28.

Barnard, A. S. (2010). One-to-one comparison of sunscreen efficacy, aesthetics and potential nanotoxicity. *Nature Nanotechnology*, *5*(4), 271-274.

Barrena, R., Casals, E., Colon, J., Font, X., Sanchez, A. & Puntes, V. (2009). Evaluation of the ecotoxicity of model nanoparticles. *Chemosphere*, 75, 850-857.

Biswas, P. & Wu, C. Y. (2005). Critical review: nanoparticles and the environment. *Journal of Air and Waste Management Association*, *55*, 708-746.

Caires, E. F., Garbuio, F. J., Churka, S., Barth, G., & Correa, J. C. L. (2008). Effects of soil acidity amelioration by surface liming on no-till corn, soybean, and wheat root growth and yield. *European Journal of Agronomy*, *28*(1), 57-64.

Castiglione, M. R., Giorgetti, L., Geri, C., & Cremonini, R. (2011). The effects of nano-TiO_2 on seed germination, development and mitosis of root tip cells of *Vicia narbonensis* L. and *Zea mays* L. *Journal of Nanoparticle Research*, *13*(6), 2443-2449.

Chellaram, C., Murugaboopathi, G., John, A. A., Sivakumar, R., Ganesan, S., Krithika, S., & Priya, G. (2014). Significance of nanotechnology in food industry. *APCBEE Procedia*, *8*, 109-113.

Chen, L., Liao, L., Wang, S., Huang, Z., & Gao, H. (2001). Effect of phenolics on~ (15) N nutrient absorption and distribution of *Cunninghamia lanceolata*. *Acta Phytoecological Sinica*, *26*(5), 525-532.

Chen, L., Zhou, L., Liu, Y., Deng, S., Wu, H. & Wang, G. (2012). Toxicological effects of nanometer titanium dioxide (nano-TiO_2) on *Chlamydomonas reinhardtii*. *Ecotoxicology and Environmental Safety* , *84*, 155-162.

Choi, H., Stathatos, E. & Dionysiou, D. D. (2006). Sol–gel preparation of mesoporous photocatalytic TiO_2 films and TiO_2/Al_2O_3 composite membranes for environmental applications. *Applied Catalysis B: Environmental*, *63*(1), 60-67.

Christian, P., Von der Kammer, F., Baalousha, M., & Hofmann, T. (2008). Nanoparticles: structure, properties, preparation and behavior in environmental media. *Ecotoxicology*, *17*(5), 326-343.

Cline, G. R., & Kaul, K. (1990). Inhibitory effects of acidified soil on the soybean/Bradyrhizobium symbiosis. *Plant and Soil*, *127*(2), 243-249.

Cochran, W.G. & Cox, G.M. (1957). Experimental designs (2^{nd} ed), *John Wiley and Sons, USA.*

Dalen, M. S. (2012). Understanding phosphorus dynamics of two alluvial soils grown with corn at different phosphorus rates (*Doctoral dissertation, Visayas State University, Philippines*).

Dhoke, S. K., Mahajan, P., Kamble, R., & Khanna, A. (2013). Effect of nanoparticles suspension on the growth of mung (*Vigna radiata*) seedlings by foliar spray method. *Nanotechnology Development*, *3*(1), 1-4.

Dilworth, M. J., James, E. K., Sprent, J. I., & Newton, W. E. (2008). Nitrogen-fixing leguminous symbioses. *Springer Science and Business Media*, *7*, 1-21.

Dowling, A., Clift, R., Grobert, N., Hutton, D., Oliver, R., O'neill, O. & Seaton, A. (2004). Nanoscience and nanotechnologies: opportunities and uncertainties. London: *The Royal Society & The Royal Academy of Engineering Report, 61*.

Esterkin, C. R., Negro, A. C., Alfano, O. M., & Cassano, A. E. (2005). Air pollution remediation in a fixed bed photocatalytic reactor coated with TiO_2. *AIChE Journal*, *51*(8), 2298-2310.

FAO–Fertilizer Organizations Working Group. (2011). Current world fertilizer trends and outlook to 2015.

Farre, M., Sanchis, J. & Barcelo, D. (2011). Analysis and assessment of the occurrence, the fate and the behavior of nanoparticles in the environment. *Trands Analyte Chemistry*, *30*, 517-527.

Feizi, H., Moghaddam, P.R., Shahtahmassebi, N. & Fotovat, A. (2012). Impact of bulk and nanosized titanium dioxide (TiO_2) on wheat seed germination and seedling growth. *Biological Trace Element Research 146*, 101-106.

Gao, F., Hong, F., Liu, C., Zheng, L., Su, M., Wu, X. & Yang, P. (2006). Mechanism of nano-anatase TiO_2 on promoting photosynthetic carbon reaction of spinach. *Biological Trace Element Research*, *111*(1-3), 239-253.

Gao, F., Liu, C., Qu, C., Zheng, L., Yang, F., Su, M. & Hong, F. (2008). Was improvement of spinach growth by nano-TiO_2 treatment related to the changes of Rubisco activase?. *Biometals*, *21*(2), 211-217.

Gao, J., Xu, G., Qian, H., Liu, P., Zhao, P. & Hu, Y. (2013). Effects of nano-TiO_2 on photosynthetic characteristics of *Ulmus elongata* seedlings. *Environmental Pollution*, *176*, 63-70.

Gewin, V. (2006). Nanobiotechnology: small talk. *Nature*, *444*(7118), 514-515.

Ghalamboran, M. R., Ramsden, J. J. & Ansari, F. (2011). Growth rate enhancement of Bradyrhizobium japanicum due to magnetite nanoparticles. *Journal of Bionanoscience*, *3*(1), 33-38.

Ghosh, M., Bandyopadhyay, M., & Mukherjee, A. (2010). Genotoxicity of titanium dioxide (TiO_2) nanoparticles at two trophic levels: plant and human lymphocytes. *Chemosphere*, *81*(10), 1253-1262.

GoP. (2013). The causes and impacts of power sector circular debt in Pakistan. *Islamabad: Planning Commission.*

Gottschalk, F., Sonderer, T., Scholz, R. W. & Nowack, B. (2009). Modeled environmental concentrations of engineered nanomaterials (TiO_2, ZnO, Ag, CNT, fullerenes) for different regions. *Environmental Science and Technology*, *43*(24), 9216-9222.

Handy, R. D., Owen, R. & Valsami-Jones, E. (2008). The ecotoxicology of nanoparticles and nanomaterials: current status, knowledge gaps, challenges, and future needs. *Ecotoxicology*, *17*(5), 315-325.

Hanif, H. U., Arshad, M., Ali, M. A., Ahmed, N. & Qazi, I. A. (2015). Phyto-availability of phosphorus to *Lactuca sativa* in response to soil applied TiO_2 nanoparticles. *Pakistan Journal of Agricultural Sciences*, *52*(1), 177-182.

Hawkins, B. J. & Robbins, S. (2010). pH affects ammonium, nitrate and proton fluxes in the apical region of conifer and soybean roots. *Physiologia Plantarum*, *138*(2), 238–247.

Husen, A. & Siddiqi. K.S. (2014). Carbon and fullerene nanomaterials in plant system. *Journal of Nanotechnology*, *12*, 1–10.

Jaberzadeh, A., Moaveni, P., Moghadam, H.R.T. & Zahedi, H. (2013). Influence of bulk and nanoparticles titanium foliar application on some agronomic traits, seed gluten and starch contents of wheat subjected to water deficit stress. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, *41*, 201–207.

Josko, I. & Oleszczuk, P. (2013). Influence of soil type and environmental conditions on ZnO, TiO_2 and Ni nanoparticles phytotoxicity. *Chemosphere*, 92, 91-99.

Kaida, T., Kobayashi, K., Adachi, M. & Suzuki, F. (2003). Optical characteristics of titanium oxide interference film and the film laminated with oxides and their applications for cosmetics. *Journal of Cosmetic Science*, *55*(2), 219-220.

Kamphake, L. J., Hnnah, S. A. & Cohen J. M. (1967). Automated analysis for nitrate by hydrazine reduction. *Water Research*, *1*, 205 – 216.

Ke, W., Xiong, Z. T., Chen, S. & Chen, J. (2007). Effects of copper and mineral nutrition on growth, copper accumulation and mineral element uptake in two *Rumex japonicus* populations from a copper mine and an uncontaminated field sites. *Environmental and Experimental Botany*, *59*(1), 59-67.

Ladha, J. K., Kundu, D. K., Coppenolle, A. V., Carangal, V. R., Peoples, M. B. & Dart, P. J. (1996). Legume productivity and soil nitrogen dynamics in lowland rice-based cropping systems. *Soil Science Society of America Journal*, *60*(1), 183-192.

Larue, C., Laurette, J., Herlin-Boime, N., Khodja, H., Fayard, B., Flank, A. M. & Carriere, M. (2012). Accumulation, translocation and impact of TiO_2 nanoparticles in wheat (*Triticum aestivum* spp.): influence of diameter and crystal phase. *Science of the Total Environment*, *431*, 197-208.

Lin, C., Fugetsu, B., Su, Y. & Watari, F. (2009). Studies on toxicity of multi-walled carbon nanotubes on *Arabidopsis* T87 suspension cells. *Journal of Hazrdous Materials, 170*, 578-583.

Lin, D. & Xing, B. (2007). Phytotoxicity of nanoparticles: inhibition of seed germination and root growth. *Environmental Pollution*, *150*(2), 243-250.

Lin, D. & Xing, B. (2008). Root uptake and phytotoxicity of ZnO nanoparticles. *Environmental Science Technology*, *42*, 5580-5585.

Liu, X. M., Zhang, F. D., Zhang, S. Q., He, X. S., Fang, R., Feng, Z. & Wang, Y. J. (2005). Effects of nano-ferric oxide on the growth and nutrients absorption of peanut. *Plant Nutrition and Fertilizer Science*, *11*, 14-18.

Lu, J.L. & Hu, A.T. (2006). Plant nutrition. *Higher Education Press*, Beijing: 23-25.

Luo, L. J. & Zhang, Q. F. (2001). The status and strategy on drought resistance of rice (Oryza sativa L.). *Chinese Journal of Rice Science*, *15* (3), 209-214.

Ma, X., Geiser-Lee, J., Deng, Y. & Kolmakov, A. (2010). Interactions between engineered nanoparticles (ENPs) and plants: phytotoxicity, uptake and accumulation. *Science of the Total Environment*, *408*(16), 3053-3061.

McLean, E. (1982). Soil pH and lime requirement. *Methods of soil analysis. Part 2. Chemical and microbiological properties*, 199-224.

Milani, N., McLaughlin, M. J., Hettiaratchchi, G. M., Beak, D. G., Kirby, J. K. & Stacey, S. (2010). Fate of nanoparticulate zinc oxide fertilisers in soil: solubility, diffusion and solid phase speciation. In *Soil solutions for a changing world: 19th world congress of soil science, Brisbane, QLD, Australia*, 1-6.

Mishra, V., Mishra, R. K., Dikshit, A. & Pandey, A. C. (2014). Interactions of nanoparticles with plants: an emerging prospective in the agriculture industry. *Emerging Technologies and Management of Crop Stress Tolerance, Biological Techniques, 1*, 159-180.

Moaveni, P., Talebi, A. H., Farahani, A. & Maroufi, K. (2011). Study of nano particles TiO_2 spraying on some yield components in barley (*Hordem vulgare* L.). In *International Conference on Environmental and Agriculture Engineering*, 119-115.

Mohammadi, R., Maali-Amiri, R. & Abbasi, A. (2013). Effect of TiO_2 nanoparticles on chickpea response to cold stress. *Biological Trace Element Research, 152*(3), 403-410.

Nair, R. & Kumar, D. S. (2013). Plant diseases—control and remedy through nanotechnology. in crop improvement under adverse conditions. *Springer New York*, 231-243.

Nair, R., Varghese, S. H., Nair, B. G., Maekawa, T., Yoshida, Y. & Kumar, D. S. (2010). Nanoparticulate material delivery to plants. *Plant Science*, *179*(3), 154-163.

Nel, A., Xia, T., Madler, L. & Li, N. (2006). Toxic potential of materials at the nano level. *Science*, *311*, 622-627.

Orsel, M., Krapp, A. & Daniel-Vedele, F. (2002). Analysis of the NRT_2 nitrate transporter family in Arabidopsis. Structure and gene expression. *Plant Physiology*, *129*(2), 886-896.

Owolade, O. F., Ogunleti, D. O. & Adenekan, M. O. (2008). Titanium dioxide affects disease development and yield of edible cowpea. *Electronic Journal of Environmental, Agricultural and Food Chemistry, 7*(50), 2942-2947.

Rafique, R., Arshad, M., Khokhar, M. F., Qazi, I. A., Hamza, A. & Virk, N. (2014). Growth response of wheat to titania nanoparticles application. *NUST Journal of Engineering Sciences (NJES)*, *7*(1), 42-46.

Raliya, R. Biswas, P. & Tarafdar, J. C. (2014). TiO_2 nanoparticles biosynthesis and its physiological effect on mung bean (*Vigna radiata* L.). *Biotechnology Report*, 50, 1-5.

Ramos, M. L. G., Gordon, A. J., Minchin, F. R., Sprent, J. I. & Parsons, R. (1999). Effect of water stress on nodule physiology and biochemistry of a drought tolerant cultivar of common bean (*Phaseolus vulgaris* L.). *Annals of Botany*, *83*(1), 57-63.

Richardson, A. D., Duigan, S. P. & Berlyn, G. P. (2002). An evaluation of noninvasive methods to estimate foliar chlorophyll content. *New Phytologist*, *153*, 185–194.

Ryan, J., Estefan, G. & Rashid, A. (2001). Soil and plant analysis laboratory manual, 2nd Ed. International Centre For Agricultural Research in the Dry Areas (ICARDA). Aleppo and National Agricultural Research Centre (NARC), Islamabad, Pakistan, 172.

Servin, A.D., Castillo-Michel, H., Hernandez-Viezcas, J.A., Diaz, B.C., Peralta-Videa, J.R. & Gardea-Torresdey, J.L. (2012). Synchroton micro-XRF and micro-XANES confirmation of the uptake and translocation of TiO_2 nanoparticles in cucumber (*Cucumis sativus*). *Plants Environmental Science Technology*, *46*, 7637-7643.

Skupień, K. & Oszmiański, J. (2007). Influence of titanium treatment on antioxidants content and antioxidant activity of strawberries. *Acta Scientiarum Polonorum Technologia Alimentaria*, *6*(4), 83-93.

Soltanpour, P. N., Workman, S. M., & Schwab, A. P. (1979). Use of inductively-coupled plasma spectrometry for the simultaneous determination of macro-and micronutrients in NH_4HCO_3-DTPA extracts of soils. *Soil Science Society of America Journal*, *43*(1), 75-78.

Stern, S.T. & McNeil, S.E. (2008). Nanotechnology safety concerns revisited. *Toxicology Science, 101*, 4-21.

Su, M., Liu, J., Yin, S., Ma, L. & Hong, F. (2008). Effects of nanoanatase on the photosynthetic improvement of chloroplast damaged by linolenic acid. *Biological Trace Element Research*, 124(2), 173-183.

Tang, C. & Robson, A.D. (1993). pH above 6.0 reduces nodulation in Lupinus species. *Plant and Soil*, *152*(2), 269-267.

Tang, C. & Thomson, B. D. (1996), Effects of solution pH and bicarbonate on the growth and nodulation of a range of grain legume species. *Plant and Soil, 186* (2),321-330.

Tang, C., Zheng, S. J., Qiao, Y. F., Wang, G. H. & Han, X. Z. (2006). Interactions between high pH and iron supply on nodulation and iron nutrition of Lupinus albus L. genotypes differing in sensitivity to iron deficiency. *Plant and Soil*, *279* (1-2), 153-162.

Taylor, R. W., Williams, M. L. & Sistani, K. R. (1991). N_2 fixation by soybean-Bradyrhizobium combinations under acidity, low P and high Al stresses. *Plant and Soil*, *131* (2), 293-300.

Tian, J., Guo, S. R., Sun, J., Wang, L. P., Yang, Y. J., & Li, B. (2011). Effects of exogenous spermidine on nitrogen metabolism of cucumber seedlings under high temperature stress. *Chinese Journal of Ecology*, *30* (10), 2197-2202.

Ullah, S. & Arshad, M. (2014). Exposure-response of *Triticum aestivum* to titanium dioxide nanoparticles application: seedling vigor index and micronuclei formation. *Science Vision, 2*, 57-61.

USEPA. (2007). Nanotechnology white paper. Document Number EPA 100-B-07001. Available at,www.epa.gov/osa.

Wang Z. (1999). Plant physiology. *China Agricultural Press, Beijing*: 80-110.

Wu, W. & Zhao, J. (2010). Advances on plants' nitrogen assimilation and utilization. *Chinese Agricultural Science Bulletin*, *26*(13), 75-78.

Xuming, W., Fengqing, G., Linglan, M., Jie, L., Sitao, Y., Ping, Y. & Fashui, H. (2008). Effects of nano-anatase on ribulose-1, 5-bisphosphate carboxylase/oxygenase mRNA expression in spinach. *Biological Trace Element Research*, *126*(1-3), 280-289.

Yang, F., Hong, F., You, W., Liu, C., Gao, F., Wu, C. & Yang, P. (2006). Influence of nano-anatase TiO_2 on the nitrogen metabolism of growing spinach. *Biological Trace Element Research, 110*(2), 179-190.

Yang, F., Liu, C., Gao, F., Su, M., Wu, X., Zheng, L. & Yang, P. (2007). The improvement of spinach growth by nano-anatase TiO_2 treatment is related to nitrogen photoreduction. *Biological Trace Element Research*, *119*(1), 77-88.

Younas, H., Qazi, I. A., Hashmi, I., Awan, M. A., Mahmood, A. & Qayyum, H. A. (2014). Visible light photocatalytic water disinfection and its kinetics using Ag-doped titania nanoparticles. *Environmental Science and Pollution Research*, *21*(1), 740-752.

Zahra, Z., Arshad, M., Rafique, R., Mahmood, A., Habib, A., Qazi, I. A., & Khan, S. A. (2015). Metallic nanoparticle (TiO_2 and Fe_3O_4) application modifies rhizosphere phosphorus availability and uptake by *Lactuca sativa*. *Journal of Agricultural and Food Chemistry*, *63*(31), 6876-6882.

Ze, Y., Liu, C., Wang, L., Hong, M. & Hong, F. (2011). The regulation of TiO_2 nanoparticles on the expression of light-harvesting complex II and photosynthesis of chloroplasts of *Arabidopsis thaliana*. *Biological Trace Element Research*, *143*(2), 1131-1141.

Zeb, A., Najmussaqib, Qazi, I.A., Shah, M.S., Awan, M.A. & Khan, F., (2010). Synthesis, characterization and arsenic adsorption studies of nascent and metal-doped titania nanoparticles. *International symposium on vacuum science and technology, Islamabad*, Pakistan.

Zhang, H. Z. & Xu, H. Y. (2011). Research progress on the enzymes during plant nitrogen assimilation. *Northern Horticulture*, *20*, 180-183.

Zhao, Y. M., Li, Y. H., Ma, R. Z., Roe, M. J., McCartney, D. G. & Zhu, Y. Q. (2006). Growth and characterization of iron oxide nanorods/nanobelts prepared by a simple iron–water reaction. *Small*, *2*(3), 422-427.

Zheng, L., Hong, F., Lu, S. & Liu, C. (2005). Effect of nano-TiO_2 on strength of naturally aged seeds and growth of spinach. *Biological Trace Element Research*, *104*(1), 83-91.