PHYTOAVAILABILITY OF NUTRIENTS IN RESPONSE TO DIFFERENT NANOPARTICLES APPLICATION IN SOIL

Zahra

NUST2012-61044MSCEE65212F

Institute of Environmental Sciences and Engineering (IESE)

School of Civil and Environmental Engineering (SCEE)

National University of Sciences and Technology (NUST)

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Certified that the contents and form of the thesis entitled "**Phytoavailability of Nutrients in response to different Nanoparticles Application in Soil** " submitted by Ms. Zahra have been found satisfactory for the requirement of the degree of Master of Science in Environmental Science.

Supervisor: ....

Dr. Muhammad Arshad
Assistant Professor
IESE, NUST

GEC Member: ....

Dr. Ishtiaq A. Qazi
Professor /Associate Dean
IESE, NUST

GEC Member: ....

Dr. Saud Ahmed Khan
Assistant Professor
IESE, NUST

External Member: ....

Dr. Amir Habib
Assistant Professor
SCME, NUST

I dedicate this thesis to my beloved mother who would always be a source of inspiration for me and my respected, loveable and humble maternal grandmother and maternal grandfather (Mir Muhammad Azeem) who stood beside me at every moment in my life

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Contents

List of Abbreviations

Ag	Silver
ATP	Adenosine triphosphate
CDC	Critical Deficiency Concentrations
CeO_2	Cerium Oxide
CNTs	Carbon Nanotubes
Cu	Copper
DNA	Deoxyribonucleic acid
EDS	Energy Dispersive X-ray Spectroscopy
Fe_3O_4	Iron Oxide
FTIR	Fourier Transform Infrared Spectroscopy
H_3PO_4	Orthophosphoric acid
MWCNTs	Multi Walled Carbon Nanotubes
NMs	Nanomaterials
NP	Nanoparticles
P	Phosphorus
Pi	Inorganic Phosphorus
PO	Phosphates
SEM	Scanning Electron Microscopy
TiO_2	Titanium Dioxide
UV	Ultra Violet
XRD	X-ray Diffraction Spectroscopy
ZnO	Zinc Oxide

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ABSTRACT

One of the most recent aspects in the domain of environment and nanotechnology is the potential assessment of nanoparticles in soil and plants. We used soil medium for plant cultures to investigate the effects of TiO_2 and Fe_3O_4 nanoparticles on the phytoavailability of phosphorus in soil. For this purpose, TiO_2 and Fe_3O_4 nanoparticles were synthesized using Sol-gel and Co-precipitation methods, respectively. Characterization was done using X-Ray Diffraction (XRD), Raman spectroscopy, Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray Spectroscopy (EDS). In the present study, *Lactuca sativa* was exposed to TiO_2 and Fe_3O_4 nanoparticles (particle size 12 - 20 nm) with the concentration levels 0, 50, 100, 150, 200 and 250 mg/kg over a period of 90 days. The behavior of both these nanoparticles in the soil medium was monitored considering the plant biomass, root and shoot length, pH of rhizosphere soil, phytoavaliable phosphorus in soil and plant's phosphorus uptake. The growth of *Lactuca sativa* was promoted and enhanced phosphorus uptake per plant up to 2.9-fold by TiO_2 and 2.8-fold by Fe_3O_4 nanoparticles as compared to the control. Plants with TiO_2 nanoparticles treatment found to accumulate more phosphorus in their roots (TiO_2> Fe_3O_4> Control) while the phosphorus in shoots comply the following order (Fe_3O_4> TiO_2> Control). The total dry biomass of *Lactuca sativa* increased up to 1.4-fold at the highest concentration of nanoparticles applied (250 mg/kg). The FTIR results verified the change in peaks of functional groups of plant shoots in nanoparticles treated groups as compared to control while the Raman spectroscopy analysis of rhizosphere soil extract was performed to determine primary metabolites. Additionally, the translocation of nanoparticles into roots and shoots of *Lactuca sativa* was verified via SEM and EDS. The significant effects of nano-TiO_2 and Fe_3O_4 were attributed to their small size and high polarizing power, which allowed their passage into roots during the experimental phase, hence performing as catalysts for plant growth. In nutrient uptake mechanism, the nanoparticles affinity to adsorb phosphorus ions was the traits that could be optimized to improve the phosphorus efficiency for agricultural purposes.

Chapter 1

INTRODUCTION

1.1 Background

All macro- and micro-nutrients have their own key importance. Phosphorus is one of the most important life-supporting elements in all living organisms. It is one of the three fundamental nutrients for crop plants (N, P and K) which are essential for plants growth. It is taken up by the plant roots in the form of dihydrogen phosphate ion ($H_2PO_4^-$), hydrogen phosphate (HPO_4^{2-}) and orthophosphate (PO_4^{3-}). It is an essential constituent of adenosine triphosphate (ATP), deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). It also facilitates phospholipids in forming cell membranes (Dickinger, 2013).

Phosphorus deficiency can affect different plant functions, seed development, root structure and ultimately the standard crop yields. An adequate amount of phosphorus is initially required by the plants for optimal crop production. Therefore phosphorus is considered to be the yield limiting factor in numerous soils. Besides this, different varieties of crop plants response differently to phosphorus fertilizers along with the growing conditions, and the availability of other nutrients as well is equally important (Onasanya et al., 2009). In order to ensure the optimal supply of phosphorus to crops, phosphorus fertilizers are regularly applied but in growing season, the recovery of applied phosphorus by the crop plants becomes very low (10-30%), due to the immobilization of more than 80% of the phosphorus in soil (Holford, 1997).

1.2 Status of Soil Nutrients in Pakistan

According to the Agriculture Statistics of Pakistan, 100% soils in Pakistan are nitrogen deficient, 80 to 90% are phosphorus deficient and 30% potassium deficient. Due to intensive cultivation, soil fertility is further depleting constantly due to withdrawal of vital plant nutrients from the soils (GoP, 2013). Phosphorus availability is also related to variations in soil pH (Vance et al., 2003). In alkaline and acidic soils, phosphate (PO_4^{-3}) ions can certainly be adsorbed onto positively charged minerals like Ca, Fe and Al oxides, respectively (Hinsinger, 2001). Similarly the soil type, its physical and chemical properties also greatly influence the phosphorus nutritional contents (Karaman et al., 2001).

As a result of these limitations, there is growing interest in developing the ways to improve the accessibility of naturally bound phosphorus in soils which could save natural phosphorus resources for sustainable production of crops. It is likely to be attained by lowering the soil pH through organic acid production (Arshad, 2006) or any other process.

1.3 Nanotechnology Applications

Currently nanotechnology is aiming at fast development in the fields of electronics, cosmetics, biotechnology and medical science, etc. As this field is progressing rapidly and has a great potential, it can also serve in various fields related to agriculture. Nanoparticles are generally known as particles with exclusive properties including their size in nano range with large surface area and high surface energy (Ma et al., 2010).

1.4 Why TiO_2 and Fe_3O_4 Nanoparticles?

For the present study, TiO_2 and Fe_3O_4 nanoparticles were selected for the application on *Lactuca sativa* on the basis of information gained from the literature review and work done at IESE, NUST. We revealed that among the synthetic nanoparticles, TiO_2 nanoparticles are widely used in a number of applications. While from agricultural point of view, we found Fe_3O_4 nanoparticles more compatible for application in soil environment and plant physiology.

1.5 Significance of Study

In the recent years, scientists have worked on different nanoparticles' effects on the plant growth and other plant mechanisms (Zheng et al., 2005). There is little information available related to the influence of nanoparticles on nutrients availability to crop plants. Therefore, this study was designed to identify the nanoparticles behavior in soil environment with focus on phytoavaliable phosphorus which is important for better crop yield.

1.6 Objectives

Keeping in view all the insights gained from the literature related to nanoparticles, soil and plants, it was hypothesized that with the application of nanoparticles the phosphorus uptake by the plants can be enhanced. In this context, objectives of the present study were:

- Synthesis and characterization of TiO_2 and Fe_3O_4 nanoparticles (< 20 nm).
- To assess the impact of TiO_2 and Fe_3O_4 nanoparticles on phosphorus bioavailability.
- The growth response of lettuce *(Lactuca sativa)* due to nanoparticles application.

1.7 Scope of Study

The scope of the study is to provide an assessment of TiO_2 and Fe_3O_4 nanoparticles on phosphorus uptake and growth performance of *Lactuca sativa* plants. This study will help to give new insights of how modern crop technologies, in particular nanotechnology approaches, may be applied to improve the nutrient availability to food crops especially making insoluble phosphorus available to the plants.

Chapter 2

LITERATURE REVIEW

Key focus of this chapter is to provide the detailed information related to the application of nanoparticles on crop plants, their possible mechanisms and both positive and negatives effects in agroecosystem.

2.1 State-of-the-Art

Nanotechnology had left no domain untouched including agriculture by its scientific novelties. Although, the use of nanotechnology in agriculture is at the early stage, but it appeared to have significant effects in different areas. Nanotechnology has a great potential and can serve in various fields related to agriculture and environment. Different kinds of nanoparticles are being used in food production and food processing industry. They can also be used as fertilizers as well as pesticides. In plants, the same principles can be applied for a broad range of applications, particularly as nutrition supplement and as growth catalysts. Nanoparticles can be labeled to agrochemicals or other substances as carrier agent to plant system for the controlled release of nutrients. Doing so, the negative effects of nanomaterials must not be ignored, such as phytotoxicity. In this scenario, there is a need to predict the environmental effect of these nanoparticles in the near future.

2.2 Macro- and Micro-Nutrients of Soil

Both macro and micro nutrient deficiencies in soil affect the crop yields all over the world. Among soil nutrients, phosphorus is one of the major macronutrients that is essential for the plant's growth. After nitrogen, phosphorus is the second most important limiting factor for crop yield (Schachtman et al., 1998). In most of the soils, phosphorus is bound with Ca and Mg ions which is not readily available to plants. Thus creating the need for phosphate fertilizers to be applied which is no more beneficial as the most of the applied phosphorus also gets fixed due to immobilization.

2.2.1 Mineralization and Immobilization of Phosphorus

Phosphorus is present in two forms; organic and inorganic. The process of mineralization involves the release of inorganic phosphorus from disintegration of organic phosphorus compounds while immobilization occurs due to the assimilation of inorganic phosphorus from soil into organic phosphorus compounds. Both these phenomena are important sources and sinks for plant available phosphorus. In most of the soils, high concentration of organic and inorganic phosphates are present of which about 88 to 99 percent of the total inorganic phosphorus is bound by calcium and thus unavailable to plants. Numerous soils suffer phosphorus deficiency due to this reason all over the world (Gyaneshwar et al., 2002). Only inorganic form of phosphorus is available to plants which is mostly present in very low concentration.

2.2.2 Nutrient Pathways for the Phosphorus Uptake by Plants

There are two main nutrient supply pathways, for the plant roots to interact directly in soil medium; firstly mass flux and secondly diffusion. Mass flux refers to the pathway that allows the movement of solutes along with the flow of their solvent (i.e. water) towards the root surface, where they are consumed. This process is influenced by the transpiration in aerial parts of plant's following the movement of water towards plant roots and up to the shoots. Among different nutrients, for the uptake of N, Mn and Ca, roots followed this pathway. On the other hand, some nutrients like phosphorus are present in very low concentrations in soil solutions (even as low as 10^{-6} M) and hence their accumulated amount in plants cannot be clarified by simple mass flow, for this reason diffusional transport system seemed to be more important. In this case, as compared to the mass flow, not only the movement of the solvent is important, but also the movement of ions in the solvent from high concentration zones to low concentration zones. This phenomenon occurs until concentration equilibrium is achieved. Plants nutrient uptake mechanisms create such concentration gradients in the rhizosphere zone by actively lowering the concentration, which initiates a diffusional re-supply of ions. Continuous nutrient uptake leads to the development of a sink, where more ions diffuse. Besides phosphorus, this supply pathway was also followed by the Mn and Cu (Schilling, 2000).

2.3 Fate of Nanoparticles in Agriculture

In the recent researches, nanoparticles have been used in certain areas of agriculture but whether these nanoparticles are unsafe or useful for plant growth is a sparsely studied subject. Different studies have been conducted to understand the effects of nanoparticles on agriculture (Feizi et al., 2013; Santner et al., 2012). Most of the studies were conducted in hydroponics and of short period. Application of nanoparticles in different culture mediums respond differently.

2.3.1 Adsorption-Desorption of Nanoparticles in Soil

In soil medium, there are variety of ways of interaction with the soil solid phase (adsorption–desorption or precipitation–dissolution reactions) and speciation of phosphorus that governed the concentration of phosphorus ions in soil. These phenomena's are mainly dependent on (a) pH, (b) the concentrations of metallic cations like Ca, Fe and Al (c) the release of competing inorganic (bicarbonate, sulphate) and organic ligands (carboxylic anions) and gaseous (O_2/CO_2) exchanges. So the bioavailability of soil inorganic phosphorus can be enhanced by modifying these factors (Hinsinger, 2001).

In neutral and alkaline soils (pH $\geq$ 7), the dominant cations would be Ca^{+2} and Mg^{+2} (Hinsinger, 2001). The use of nanoparticles in soil could provide more adsorption sites to the phosphate ions due to their increased polarizing power. When considering organic ligand, oxalates and citrates exhibit strongest adsorption affinity and large concentration of these ligand required to desorb phosphorus ions up to a significant level (Jones and Brassington, 1998). Phosphates are mainly released by citric acid via organic ligand exchange and ligand-enhanced dissolution of mineral deposits. The process of ligand exchange occurs when the organic ligand interact for inorganic phosphate at a mineral surface site, as a result phosphate is released into the soil solution (Johnson and Loeppert, 2006). Ligand-enhanced dissolution at the surface is the process by which slow dissolution of the mineral surface occurs and adsorbed phosphate and Fe complexes release. Similarly, the exudation of piscidic acid (Ae et al., 1990) and phytosiderophores were reported to enhance phosphorus solubilization through the chelation of Fe due to their high affinity for divalent and trivalent metals (Murakami et al., 1989).

The ligand exchange process involved the replacement of phosphate ions for one or more surface hydroxyl groups, which releases OH^- ions into the soil solution.

Estimation of variation in the concentration of phosphate ions adsorbed and OH^- ions released stoichiometry gave key evidences related to the adsorption mechanism (Wang et al., 2013). Al and Fe phosphates are presumed to be the dominant phosphorus minerals in low pH soil with lower solubility (Dixon and Weed, 1989). Some studies also showed that the solubility of soil phosphorus can be further increased by decreasing the soil pH (Murrmann and Peech, 1969). For example, in another research work, lesser phosphorus adsorption occurred onto goethite due to decreased pH, with increased number of phosphorus ions in soil (Geelhoed et al., 2008). Citrate could increase the uptake of both phosphorus and Fe from the rhizosphere and projected a way related to the formation of a Fe–P citrate complex (Gardner et al., 1983).

2.3.2 Altering the Phosphorus Use Efficiency (PUE) by Nanoparticles

Improving the phytoavailability of phosphorus in soil must be at the forefront of plant nutrition research in the recent era especially by using the right phosphorus source at the right time, right rate, and right place ("The 4 R's") is essential for its effectiveness (IPNI, 2012). To begin with, PUE by nanoparticles application can be influenced by adsorption-desorption mechanism in the soil. Diffusional transportation and diffusion coefficients lie within low ranges (10-12 to 10-15 cm^2 s^{-1}) in soils that primarily ensure phosphorus supply (Marschner, 2012). Phosphorus-loaded Al_2O_3 nanoparticles were used to improve phosphorus uptake by *Brassica napus* in hydroponics. Plant phosphorus uptake was reported to increase about 8-fold at constant low free phosphate concentration, and about 40-fold because of passive, diffusion-based samplers (Santner et al., 2012).

Along with other parameters, soil pH is considered to be a critical factor for crop plant as it has a staged effect on the phytoavailability of phosphorus in soils. Yet, there are some studies that used nanoparticles for the slow release of nutrients by fertilizers (Corradini, 2010). An example would be the coating of fertilizer granules with synthetic nanoparticles to reduce the release of phosphorus. This could be extended over a longer period of time for the fast growing annual crops with a high demand of readily available phosphorus during their main growth period. Improving the solubility of phosphorus reserves and applied phosphorus in soils and preventing it from being adsorbed or precipitated by various soil compounds or even mobilizing is an important matter of investigation, both at the commercial as well as scientific research level.

Presently, only few of latest technologies went through extensive scientific research about this topic and reports about efficiency are varied with both positive and negative findings. Research attempts for reducing phosphorus retention by precipitation include the addition of polymers or polymeric organic acids or silicon-based compounds to complex possible precipitation partners in the soil to anticipate consequent reactions with Al, Ca, Fe and Mg. Desorbing phosphorus out of bound phosphorus pools with the addition of nanoparticles is very little addressed, thus we have discussed this in detail in chapter 4 with the results. All these strategies need to be further investigated for improving overall nutrients efficiency of crop plants.

2.3.3 Critical Concentrations of Nutrients and Nanoparticles

For the assessment of nutritional status in plant tissues, elemental analyses have to be performed and in additional estimation of phytoavailability of nutrients in soils requires appropriate knowledge about reactions of plant species to applied nanoparticles and their behavior under deficient conditions. For giving appropriate recommendations about the range of nanoparticles applied and nutrient concentrations in plant tissue, these guidelines need intensive investigations of plant growth and yield response curves related to the measured concentrations of nutrients in plant tissue and soil with respect to nanoparticles application. The specific and unspecific interactions of particular nanoparticles and nutritional elements have to be considered not only in the plant itself but also in the rhizosphere. Critical concentrations limits of nanoparticles in plants summarize this gained knowledge in terms of what concentration of nanoparticles could be helpful to achieve optimum crop yield in plants and at which level they cause toxicity effect. These critical range values are in the premature stage of optimization as they are dependent on wide range of factors like type and properties of nanoparticles, plant species and several other factors that are not discussed here in detail as they go beyond the scope of this work.

Interaction between different nutritional elements in plants and soil are in close conformity with each other. For example, nitrogen and phosphorus represent a non-specific interaction, which is of most importance when the concentration of both nutrients are near or at the critical deficiency concentrations (CDC). An increase or decrease of one element alone does not influence the CDC of the other but might be influenced in one or other way. But when both nutrients present in deficient concentrations, they definitely cause adverse cumulative effect (Jarrell and Beverly, 1981). Both potassium (K) and magnesium (Mg) ions compete for uptake by roots. It is an example for a specific interaction which also affects CDC of the related nutrients as high concentrations of K in the rhizosphere may induce Mg deficiency in plants (Parry and Hawkesford, 2012).

2.4 Application of Nanoparticles in Plant Studies

Nanoparticles are being widely used in various fields and their interaction with the surrounding environments is one of the major issues. Nanoparticles can enter the agroecosystem by different possible means including water, soil and plants. To date, different studies related to the application of nanoparticles and their bioaccumulation in plants have been reported. Scientists have also focused on the effects and mechanisms of different nanoparticles on plants (Zheng et al., 2005).

2.5 Entry Routes and Translocation of Different Nanoparticles in Plants

Plants offered a prospective route for the transfer of nanoparticles to the environment and ultimately paved way for their bioaccumulation into the food chain. Different studies have determined the response of nanoparticles to plants growth and their possible mechanism. Plant cell wall do not allow the smooth entrance of any external agent as well as nanoparticles into the plant cells. The screening property of cell wall depends on the diameter of pores present in the cell wall that mostly ranges from 5-20 nm (Rondeau-Mouro et al., 2008). Therefore nanoparticles and their aggregates within that range could simply cross the cell membrane and transferred to the aerial parts of the plants.

Nanoparticles might induce different morphological changes in the root structures resulting in magnification of pores or stimulation of new pores in the cell wall which ultimately enhance uptake of nanoparticles, their aggregates or complexes. During endocytosis, plasma membrane forms a cavity like structure around the nanoparticles resulting in further internalization. By using embedded transport carrier proteins or via ion channels, they are able to cross the membrane. In the cytoplasm, the nanoparticles may attached to different cytoplasmic organelles and disturbed the metabolic processes at that point (Jia et al., 2005). Conversely, nanoparticles accumulated on photosynthetic surface induced foliar heating that can alter the gaseous exchange due to stomatal disturbance. Consequently altering the different molecular and physiological functions of plants (Silva et al., 2006). Therefore, the translocation and influence of different nanoparticles within plants need to be investigated further to underpin the whole mechanism of their behavior in plants (Nair et al., 2010).

2.6 Influence of Different Nanoparticles on Plants

Scientists have focused on the effects of different nanoparticles on plants. These nanoparticles have definite effects on various plant parameters that are studied. Therefore it’s really important to understand the effects of different nanoparticles on crop plants which is discussed here.

2.6.1 Effects of Magnetic Nanoparticles

Magnetic nanoparticles are considered to be the most important due to their delivery at targeted sites in plants and other organisms. Uptake and translocation of these nanoparticles (< 50 nm) in pumpkin plants have been reported (Corredor et al., 2009). The well-defined translocation of magnetic nanoparticles can be monitored in roots and leaves of the experimental plants through magnetization signals in whole the plant. Toxicity impacts have not been observed on plant growth therefore suggested the safe usage of application in plants. These nanoparticles act as carrier agents for the release of various chemicals to the targeted sites by using external magnets. Such techniques are quite useful for particular application in crop plants kept in greenhouse.

In the recent years, the influence of ferro-fluids on the genetics was especially focused; that cause chromosomal aberrations in immature plants. The iron oxide nanoparticles coated with tetramethylammonium hydroxide (TMA-OH) was used as stabilizing agent in maize plants. The ‘Chlorophyll a’ level was reported to increase in the early growth stages at low concentration levels whereas at higher concentration level it was found restricted. Maize seeds were exposed to electromagnetic field in the presence of magnetic fluid, assimilatory pigments were observed to decrease as the concentration of magnetic fluid solution increased. Internalized magnetic nanoparticles in plant tissues absorbed the electromagnetic field energy that influenced the redox reactions. Redox reactions triggered the photosynthesis process resulting in increased level of in nucleic acid. The magnetic nanoparticles might also induce some magnetic effects on the enzymatic activities of plants that took part in photosynthetic and developmental processes. That's why it is necessary to optimize a suitable concentration ranges of ferro-fluids for further application on plant cultures to get better yield of crops with improved photosynthetic pigment levels (Racuciu and Creanga, 2006). In another experiment, three types of treatments including; Fe nanoparticles, Fe nanoparticles coordinated with organic fertilizer and with humic acid were applied to transfer iron and photosynthates to the leaves. Results revealed that iron oxide nanoparticles act as catalysts for iron transfer to the leaves of peanut. They found increased iron content in leaves about 218, 207 and 206 mg/kg respectively (Liu et al., 2010). Fe_3O_4 nanoparticles were introduced to pumpkin seedlings and results showed uptake of nanoparticles by the plant roots, stems and leaves (Zhu et al., 2008).

In another study, iron oxide nanoparticles were applied to soybeans**.** Iron oxide nanoparticles at the concentration level of 0.75 g L^{-1} were found to increase leaf and pod dry biomass. The maximum grain yield was gained by using 0.5 g L^{-1} iron oxide nanoparticles with 48% increase over the control (Sheykhbaglou et al., 2010).

2.6.2 Effects of Titanium Dioxide Nanoparticles

The effects of TiO_2 nanoparticles on the germination and growth of spinach seeds were studied. These nanoparticles act as photocatalyst, enhanced light absorbance and promoted the activity of *Rubisco activase* resulting in increased spinach growth. TiO_2 nanoparticles with anatase phase reported to improve plants growth due to improved nitrogen metabolism, more inorganic nitrogen was converted into organic nitrogen, consequently increased the fresh and dry biomass of plants by 91% and 99% as compared to control. Total nitrogen increased up to 23.35% along with improved chlorophyll and proteins of spinach (Yang et al., 2007). Studies also demonstrated the effects of nano-TiO_2 (rutile) and non-nano-TiO_2 on the germination and growth of naturally aged spinach seeds. During the growth stage, nano-TiO_2 has improved the chlorophyll content, proteins in spinach as well as increased the antioxidant stress due to lower accumulation of superoxide radicals that ultimately helped the spinach chloroplasts to release more oxygen in the presence of UV-B radiation (Zheng et al., 2005).

Studies also showed that low concentrations of TiO_2 nanoparticles were not found to be detrimental to plant cell membrane; these findings have highlighted their positive effects on chickpea cells especially when they were exposed to cold stress. The tested concentration levels did not induce any morphological effect, perhaps due to their short-term thermal exposure or low concentrations levels. TiO_2 nanoparticles especially at 5 mg/kg concentration level reported to reduce cold-induced damages in sensitive and resistant chickpea genotypes. Such domino effect raise key questions about the possible mechanisms that direct these effects. It was supposed to occur due to the activation of some defensive mechanisms in chickpea seedlings after absorption of TiO_2 nanoparticles, consequently supporting plants to cold stress. These results are quite interesting to further practice in case of environmental stressed conditions. The new findings possibly would pave the way for the use of nanoparticles especially for increase of cold tolerance in major crops (Mohammadi, 2013).

The effects of TiO_2 nanoparticles on plant growth have also been studied. Results with improved photosynthesis and growth in plants were reported due to the applied TiO_2 nanoparticles but the main mechanism was yet imprecise. Generally, the absorption of light in chloroplast and light-harvesting complex II was supposed to be stimulated by TiO_2 nanoparticles; thus enhancing the transformation of light energy to electronic energy, evolution of oxygen and water photolysis (Ze et al., 2011).

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

Another study demonstrated the uptake of sucrose coating of 43% TiO_2 nanoparticles with size less than 5 nm size in the model plant *Arabidopsis thaliana*. Results clearly demonstrated that small nano structures entered into plant cells, and got accumulated in distinct subcellular locations (Kurepa et al., 2010). Similarly, application of 40 mg/kg TiO_2 nanoparticles was found to improve the average germination time by 31.8% as compared to control (Feizi et al., 2013).

2.6.3 Effects of Carbon Nanotubes and Multi Walled Carbon Nanotubes

With the recent advancements in nanotechnology research, carbon nanotubes (CNTs) are used to deliver various biomolecules and drugs into the cells. Nanoparticles have capabilities to penetrate and migrate living plant tissues (Corredor et al., 2009). The effects of CNTs on tomato seeds were studied and effects on the germination and growth rates were observed. The seeds containing CNTs (10-40 mg L^{-1}) showed higher germination rate as compared to the untreated control. Further studies specified that carbon nanotubes are capable of penetrating the thick seed coat and improve water uptake in seeds, which in turn affects seed germination and growth of tomato seedlings (Khodakovskaya et al., 2009).

Both functionalized and non-functionalized CNTs were applied to six crop species (cabbage, carrot, cucumber, lettuce, onion and tomato) to study effects on root elongation. These plant species were normally used in phytotoxicity tests. The results showed that the non-functionalized carbon nanotubes had more effects on root lengths than functionalized nanotubes. Non-functionalized nanotubes reported to enhance root elongation in onion, cucumber but inhibited it in tomato. Functionalized nanotubes found to retard root elongation in lettuce plant. None of the nanotubes affected the cabbage and carrots crop species. Microscopic images showed the existence of nanotubes on root surfaces while uptake of nanotubes was not observed (Canas et al., 2008).

2.6.4 Effects of Aluminum Nanoparticles

Aluminum nanoparticles have been extensively used in different applications so there is more chance for their release in ecosystem and their interaction with higher plants. Different studies have been conducted using pure alumina nanoparticles (13nm) on various plant species including (corn (*Zea mays*), cabbage (*Brassica oleracea*), cucumber (*Cucumis sativus*), soybean (*Glycine max*) and carrot (*Daucus carota*)). Root elongation was found to reduce in studied plant species, consequently inhibiting plant growth (Yang and Watts, 2005).

Nanoparticles with proper surface modifications were reported to reduce the phytotoxicity effect. The aluminum oxide and aluminum oxide particles coated with carboxylate ligand (100 nm in size) showed no adverse effect on the red bean (*Phaseolus vulgaris*) and rye grass (*Lolium perenne)* growth. Concentration of aluminum (Al) was observed to increase by 2.5-fold over control in rye grass but no uptake of Al was observed in red beans. The study conformed the difference lied in uptake mechanism and distribution efficiency within the same type of nanoparticles by various plants (Doshi et al., 2008).

In a recent study, P-loaded Al_2O_3 nanoparticles were used as a source to release bound phosphorus in hydroponics. Phosphorus uptake by *Brassica napus* was reported to increase. Plant phosphorus uptake was reported to increase by 8-fold at constant, low free phosphate concentration, whereas increased by 40-fold in case of passive, diffusion-based samplers (Santner et al., 2012).

2.6.5 Effects of Zinc Oxide Nanoparticles

Zinc Oxide (ZnO) nanoparticles applied on zucchini seeds in hydroponic solution showed no negative effects on the seed germination and root growth whereas the seed germination of rye grass and corn was inhibited by nano scale zinc of 35 and 15–25 nm size, respectively (Lin and Xing, 2007a). Since ZnO nanoparticles were used to produce more soluble and diffusible sources of Zn fertilizers to overcome the Zinc deficiency which is one of the main problems restraining agricultural productivity in alkaline calcareous soils (Milani et al., 2010).

In another work, ryegrass plants treated in nutrient solution with ZnO nanoparticles showed toxic effects at higher dosages. Zn^{2+} ions were observed to be more toxic than ZnO nanoparticles. SEM images verified the uptake of ZnO nanoparticles and proved to damage the epidermal and cortical cells of plant. Nanoparticles aggregates formed could block the pores and channels, so there is need to do further research on this topic to lessen the risk of phytotoxicity assessment. Studies ought to emphasize on the production of innovative nanomaterials which on translocation causes the enlargement of plants' pore size and cell wall when interacted with cell proteins and polysaccharides, enhancing the nutrient uptake thus increasing the crop production.

The application of nanoparticles in soil and their influence in soil medium is of more importance as compared to hydroponics because this kind of studies paved way for the application in field and will definitely help to clarify the toxicity of nanoparticles in a better way and this needs extensive research. In another study, the passage of Zn and ZnO nanoparticles in soil and uptake by *Zea mays* was determined. The results of this study revealed that at various levels of concentration, ZnO nanoparticles showed slow movement in soil. The uptake of Zn by corn varied from

69 to 409 mg/kg in roots and 100 to 350 mg/kg in shoots, respectively, when grown in soils treated with ZnO nanoparticles from 100 to 800 mg nanoparticles kg^{-1} soil in one month exposure time. Confocal microscope images verified the entrance of ZnO nanoparticles in the root epidermis and cortex via apoplastic path. Nanoparticle in xylem vessels showed that the aggregates of nanoparticles passed the endodermis via symplastic path (Zhao et al., 2012).

The effects of nano-ZnO on the growth of seedlings of mung (*Vigna radiate*) and gram (*Cicer arietinum*) were also investigated in a study. Plant agar method was used to conduct this experiment to avoid settling of nanoparticles in test pots. Different concentrations of ZnO nanoparticles were added into agar medium, and their effects were studied by means of root and shoot growth parameters in seedlings. The presence and adsorption of nano-ZnO on the roots was verified through SEM (Mahajan et al., 2011).

2.6.6 Effects of Copper Nanoparticles

A study was conducted to demonstrate the effects of copper (Cu) nanoparticles on the mung bean and wheat by using plant agar culture media that allowed the nanoparticles remained in dispersed form. Growth inhibition of seedlings was found in mung bean. Mung bean was found to be more sensitive to copper nanoparticles as compared to wheat plants. The presence of Cu nanoparticles translocated across the cell membrane was confirmed via Transmission Electron microscopy (TEM) images. Increased bioaccumulation of these nanoparticles was observed with increased concentration in growth media. In another study, reduced length of emerging roots was observed in zucchini plants when exposed to Cu (Stampoulis et al., 2009). While in another work, presence of Cu nanoparticles caused to increase the shoot to root ratio in the germination of lettuce seeds as compared to control plants (Shah and Belozerova, 2009). Different types of flora and fauna respond in a different way to the nanomaterials, so before experimentation it is crucial to estimate their useful concentration that is considered to be safe and reduce the risks of ecotoxicity to its maximum.

2.6.7 Effects of Silver Nanoparticles

In an experiment silver (Ag) nanoparticles have been used to study their effects on the seed germination and root growth of zucchini plants (in hydroponics). Plant biomass and transpiration was observed to decrease in exposure to Ag nanoparticles. Similarly the plant growth was found to be prolonged. Such kind of studies urge the need to further investigate the ecotoxicity impacts caused by these nanoparticles (Nair et al., 2010).

2.7 Phytotoxicity of Nanoparticles in Edible Plants

The increased use of nanomaterials in various fields has raised a worldwide concern about their release and influence on surroundings and environment. Diverse results have been found in this regard. For this reason, toxicological effects of nanoparticles on soil and plants also got into attention in the recent decade. Therefore insight of phytotoxicity of nanoparticles in edible crop plants is a significant topic to be discussed here.

In some studies nanoparticles seem to be beneficial for plants, whereas decline in growth of plants was also reported in various studies for example in case of silver and copper nanoparticles. On treatment with silver nanoparticles, negative effects on the growth of phytoplankton and *Cucurbita pepo* were observed (Miao et al., 2007). To-date, bioaccumulation, bio-magnification and biotransformation of nanoparticles in food crops are not well defined. Very few nanoparticles and plant species have been studied in this perspective.

An evaluation of phytotoxicity of five different nanoparticles reported that only Zn and ZnO nanoparticles induced significant inhibition on seed germination and root growth of plant species. Inhibition was dominant in the seed incubation process as compared to the seed soaking process (Lin and Xing, 2007b). In a similar study, toxicity effects of ZnO and TiO_2 nanoparticles were studied in rice seed germination. Reduction in the percent seed germination from both nanoparticles was not significantly observed, whereas ZnO nanoparticles showed detrimental effects on rice roots at early seedling stage caused to stunt roots length and reduce number of roots. While TiO_2 nanoparticles found to have no effect on root length (Boonyanitipong et al., 2011).

In line to the above studies, ZnO and TiO_2 nanoparticles have also been reported to reduce the wheat's biomass, and thus harmful to the plants. The TiO_2 nanoparticles, considered to have low solubility, thus remained in the soil for long periods which might create potential environmental risks for deeper soil layers. Small-sized TiO_2 nanoparticles (20nm) were able to penetrate the plant cell wall. The ZnO nanoparticles, possess higher solubility than TiO_2, dissolved in the soil and reported to increase the wheat’s uptake of toxic zinc (Du et al., 2011). In another study, effects of different concentrations of nanosized TiO_2 and Fe_3O_4 on seedling growth of tomato were analyzed in hydroponics. In particular, morphological alterations caused by nanoparticles as well as tissue internalization and possible upward translocation of Fe_3O_4 and TiO_2 nanoparticles were focused. Root uptake and nanoparticles deposition over roots were observed through SEM equipped with EDS for chemical recognition (Giordani et al., 2012).

Among the phytotoxicity studies of different nanoparticles both positive and negative or insignificant effects have been stated. Among these, in one of the study both positive and negative effects were observed when seeds of alfalfa (*Medicago sativa*), corn (*Zea mays*), tomato (*Lycopersicon esculentum*) and cucumber (*Cucumis sativus*) were exposed to nanoceria at concentration levels 0–4000 mg L^{-1}. Uptake of nanoceria were significantly correlated with reduced corn germination by 30%, while the germination of tomato and cucumber was decreased by 30% and 20%, respectively at the concentration level of 2000 mg L^{-1}. On the contrary, root growth enhanced significantly in cucumber and corn with the exception of alfalfa and tomato where it was reduced. At all concentration levels, nanoceria enhanced the shoot elongation in the tested plant species (Lopez-Moreno et al., 2010).

As the human food chain instigated with plants, so at this stage, it's critical to understand how plants response differently to these nanomaterials which are frequently concentrated in our ecosystem. The cited review of reported literature indicated that the knowledge on defined behavior of nanoparticles is at the early stage. No conclusive studies on the nanotoxicity have been discussed in detail elsewhere; so with the limited information available we tried to present an overview of the effects of nanoparticles on the plants.

2.8 Related Research Work at IESE, NUST

An experiment was conducted to assess phytoavailability of phosphorus affected by TiO_2 nanoparticles. Soil was amended with TiO_2 nanoparticles with concentration levels: 0, 25, 50, 75 and 100 mg/kg. Concentration of phytoavaliable phosphorus in soil without plant culture and with lettuce culture was analyzed in experimental levels. In soil without plant culture, phosphorus was reported to increase up to 56% with the addition of TiO_2 nanoparticles at 100 mg/kg while soil with lettuce culture over 15 exposure days showed 83% increase in phosphorus with treatment of TiO_2 nanoparticles. The results also indicated increased root/shoot lengths by 1.5-fold, total dry biomass by 2-fold and total phosphorus uptake by 4-fold (Hanif, 2012).

Another study on the risk response of TiO_2 nanoparticles on wheat was conducted in hydroponics with concentration levels 0, 25, 50, 100, 200, 400 and 600 mg/kg. Physical growth parameters and cytotoxicity effects were studied. The results showed root elongation at 200 mg/L and at 400 mg/L it may decline. Low toxic effect on short term exposure as compared to control was also observed in this study (Sana-ullah, 2013).

From the above cited detailed literature review, we hypothesized that with the application of nanoparticles uptake of phosphorus by the plants can be enhanced. To test this hypothesis, the methodology adopted is discussed in detail in Chapter 3.

Chapter 3

MATERIALS AND METHODS

This chapter describes the experimental framework of the present study. The work was divided into two main phases. The first phase included synthesis and characterization of TiO_2 and Fe_3O_4 nanoparticles having size less than 20 nm. As the plant pore size range between 5-20 nm, so we expected that by using nanoparticles within this size range would not prevent diffusion of nanoparticle phosphate complexes towards the plasma membrane. Second phase starts with application of synthesized nanoparticles by conducting pot experiment. The whole experiment was conducted in locally made greenhouse at IESE, NUST. The first investigation focused on the plants growth and the second purpose was to focus solely on the phytoavailability of phosphorus in response to nanoparticles application in soil. Following methodology was adopted which is discussed here in detail accordingly.

3.1 Preparation of TiO_2 and Fe_3O_4 Nanoparticles

In order to achieve the desired properties of nanoparticles, following synthesis methods have been used.

3.1.1 Synthesis of TiO_2 Nanoparticles by Liquid Impregnation Method

In order to achieve the required size of nanoparticles, the first method used for the synthesis of TiO_2 nanoparticles was Liquid Impregnation (LI) method. In this method, 5 g of Titanium dioxide powder, General Purpose Reagent (GPR) was added into 100 mL distilled water and placed on magnetic stirrer for 12 h. The mixture was then allowed to settle overnight, after that placed it in oven at 105 °C for drying. The dried material was ground by using mortar-pestle. Then it was placed in muffle furnace for calcination at 500 °C for 6 h (Zeb et al., 2010).

3.1.2 Synthesis of TiO_2 Nanoparticles by Sol-Gel Method

TiO_2 nanoparticles were also synthesized using a slightly modified sol–gel process that was developed earlier for synthesis of molecularly imprinted titania (Lieberzeit et al., 2007). Briefly, Titania precursor was added in 0.5 M acidic solution.

Due to the reaction of $TiCl_4$ with water, HCl gas produced lowering the pH of the stock solution. As the $TiCl_4$ was added, color of the solution turned yellowish. After sometime, the solution became transparent. The solution was then neutralized with 0.5M NH_4OH till pH became 7, followed by stirring until the gel like network formed. It was centrifuged to form sol-gel. The supernatant was discarded and rest of the sol was dried in oven at 105 ^{o}C. Dry gel was ground and calcined at 500 ^{o}C for 6 h.

3.1.3 Synthesis of TiO_2 Nanoparticles by Sol-Hydrothermal Method

The sol was prepared through sol-gel process, neutralized with 0.5 M NH_4OH and hydrothermal treatment was done at 150 ^{o}C for 5h. Gel was dried in vacuum oven at 105 ^{o}C and dried material was then ground and calcined at 150 ^{o}C for 5 h.

3.1.4 Synthesis of Fe_3O_4 Nanoparticles by Solvothermal Method

In this experimental procedure for preparing superparamagnetic Fe_3O_4 nanoparticles, ferric chloride hexahydrate (6 g), urea (10 g) and citric acid (1.6 g) were added to 60 mL absolute Ethylene Glycol. The blend was placed into a stainless steel autoclave having Teflon-lined cup, at 200 ℃ for 18 hrs. The black slurry was then filtered off, washed with deionized water and absolute ethanol several times till the pH became 7. It was placed in vacuum oven for drying at 50 ℃ for 12 h. The dried clusters were ground into powered form (Maosheng et al., 2013).

3.1.4 Synthesis of Fe_3O_4 Nanoparticles by Co-precipitation Method

In this method, $FeCl_2$ (0.1 M) was added in $FeCl_3$ (0.2 M). NaOH was added into the salts mixture. The solution turned black. It was heated till the slurry rest behind. The slurry was washed with distilled water until the pH became 7 and then placed in oven at 80 ^{o}C. The magnetite (Fe_3O_4) clusters were ground into nanoparticle form (Kim et al., 2007).

3.2 Characterization of TiO_2 and Fe_3O_4 Nanoparticles

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 are:

- X-ray Diffraction
- Raman Spectroscopy

- Scanning Electron Microscopy
- Energy Dispersive X-ray

3.2.1 X-Ray Diffraction and Raman Spectroscopy Analysis

The phase composition, crystal structure and crystalline size measurements for the TiO_2 and Fe_3O_4 nanoparticles were performed using 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. Analysis of XRD results were done with X'Pert High Score software package (PANalytical B.V. Almelo, Netherland). The crystallite size of nanoparticles was estimated according to the line width of the (101) plane refraction peak for TiO_2 and (311) for Fe_3O_4 by using Scherer Formula.

Raman spectra for both synthesized nanoparticles was also obtained using the Micro-Ramboss, DPSS laser source, (Dongwoo Optron Co., Ltd. Korea). The spectrum was taken within the range of 200 cm^{-1} to 800 cm^{-1} using DM 320 monochromator and ANDOR DV 401A-BV CCD software to verify the results.

3.2.2 Scanning Electron Microscopy and Energy Dispersive X-ray Spectroscopy

The surface morphology of TiO_2 nanoparticles as well as Fe_3O_4 were analyzed on Jeol, JSM 6490, SEM instrument (Japan) equipped with EDS (Jeol, JED 2300) and ion sputtering device (Jeol, JFC 1500). Suspensions of TiO_2 and Fe_3O_4 nanoparticles in ethanol were made up on quartz slides and were directly observed under the microscope at different magnifications.

3.3 Preliminary Soil Screening and Preparation for the Final Experiment

3.3.1 Soil Classification

Classification of Soil was done on the basis of saturation percentage (Malik et al., 1984).

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 suitability of soil for plant growth, pH was measured. For this purpose, 10 g of air dried soil (< 2 mm) was taken in 100 m beaker. 50 mL of distilled water was added using a graduated cylinder. The mixture was stirred well for 30 min and left to stand. After 1 h, reading was taken by pH meter (McLean, 1982).

3.3.3 Moisture Content

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

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

3.3.4 Soil Preparation

After selecting the suitable soil for final experiment, 50 kg soil was purchased from local Modern Nursery, H-9 sector, Islamabad and spread out to dry for a week with regular mixing. Prior to any further usage, the dried soil was ground into fine form using ball mill at Particulate Technology Laboratory, SCME, NUST. Through mechanical sieve shaker of size < 2mm; roots, shoots and other materials were removed and fine homogenized soil was obtained. Desired amount of soil was weighed and subsequently added to pots. Plastic pots (10 cm diameter and 9.5 cm height) were used for the experimentation.

3.4 Application of TiO_2 and Fe_3O_4 Nanoparticles in Soil

Nano-TiO_2 and Fe_3O_4 suspensions of proposed concentrations were prepared by weighing calculated amounts of nanoparticles separately and added in distilled water. Dispersion of these nanoparticles were prepared by using ultrasonicator (JAC Ultra Sonic 1505) for 40 minutes. For *Lactuca sativa* cultivation, a series of desired concentrations of nano-TiO_2 and Fe_3O_4 suspensions were added to the soil and mixed vigorously. The concentrations of nano-TiO_2 and Fe_3O_4 were 0, 50, 100, 150, 200 and 250 mg/kg. For each concentration level, there were five replicates.

3.5 Plant Cultivation

Lactuca sativa seeds were sown in sandy loam soil in the greenhouse. Freshly grown plants of *Lactuca sativa* with 20 days age were then used for experimentation. Plant roots were washed carefully with distilled water to make sure surface clarity. Plants were then exposed to different concentrations of TiO_2 and Fe_3O_4 Nanoparticles by shifting to pots (one plant per pot) containing soil amended with nanoparticles. The plants were monitored daily and watered thrice a week for 90 exposure days. For each treatment level, there were five replicates. During experimental phase pots were kept in greenhouse at IESE, NUST.

3.6 Plant Parameters (Biomass, Length, pH, Moisture Content)

At harvest, whole the plant was removed from the pot and the roots were washed 100 mL distilled water. The pH of soil solutions was immediately determined and the solutions were filtered. The filtrate was placed at 4 ºC for Raman spectroscopy analysis of plant's primary metabolites. The rhizosphere soil (approximately 5 g) collected was analyzed for phytoavaliable phosphorus (Olsen et al., 1954).

Shoots and roots of the plants were collected separately, lengths were measured, and fresh biomass was recorded. Shoots and roots were placed in an oven at 70 °C for 48 h. The plant material was weighed for dry biomass. Moisture content percentage was calculated for the shoots. Both the shoots and roots were ground with mortar pestle separately and stored in air tight sampling bags for phosphorus analysis.

3.7 Analysis of Phytoavaliable Phosphorus in Soil

As phosphorus is a major nutrient, and is mainly found unavailable in alkaline and calcareous soils, so it is measured in most of the soil laboratories for estimating the need of phosphorus fertilizer for growing food crops. The modified method of (Olsen et al., 1954) is a simple, quick and inexpensive soil test which is generally acknowledged as an appropriate guide of phosphorus availability for alkaline soils, where the Ca^{++} precipitated as $CaCO_3$ thus increasing the solubility of calcium phosphate. Therefore, Olsen's test has been adopted for the phosphorus analysis of soil in the Biotechnology Laboratory at IESE, NUST.

Reagents

A. Extracting Solution

a) Sodium Bicarbonate Solution ($NaHCO_3$), 0.5 M

42 g of sodium bicarbonate were dissolved in about 700 mL distilled water and pH was adjusted to 8.5 with 5N NaOH. The volume was made up to 1-L with distilled water.

b) B. Sodium Hydroxide Solution (NaOH), 5 N

50 g of sodium hydroxide were dissolved in 200 mL distilled water and made the volume up to 250 mL with distilled water.

B. Mixed Reagent

a) 6 g of ammonium heptamolybdate $(NH_4)_6Mo_7O_{24}.4H_2O$ were dissolved in 125 mL distilled water.

b) 0.1455 g of antimony potassium tartrate ($KSbO.C_4H_4O_6$) were dissolved in 50 mL distilled water.

The dissolved reagents (a) and (b) both were added to a 1 L volumetric flask, then 500 mL of 5 N H_2SO_4 (74 mL concentrated H_2SO_4 in 500 mL DI) were added to the mixture. After mixing thoroughly, the volume was made up to 1 L with distilled water and stored in a Pyrex glass bottle in a dark and cool place.

C. Color Developing Reagent

For this purpose, 2.64 g of Ascorbic acid ($C_6H_8O_6$) were dissolved in 500 mL Mixed Reagent. Color developing reagent must be prepared freshly as needed because it could not be kept for more than 24 hours.

D. Standard Stock Solution

Accurately, 2.5 g potassium dihydrogen phosphate (KH_2PO_4) was oven dried for 1hat 105 °C, cooled in a desiccator then stored in air tight bottle. Exactly, 2.197 g potassium dihydrogen phosphate (KH_2PO_4) was dissolved in 500 mL distilled water. This solution contained 1000 mg L^{-1} stock solution. Precisely, 10 mL stock solution was diluted to 100 mL final volume with distilled water. This solution contained 100 mg L^{-1} phosphorus. A series of standards were prepared from the stock solution. These solutions contained 0, 0.25, 0.5, 0.75, 1, 1.25, 1.50, 1.75, 2, 2.25, 2.50, 2.75, 3, 3.5 and 4 mg/kg phosphorus respectively.

Procedure

Weigh 2.5 g air-dried soil (< 2 mm) into a 250-mL Erlenmeyer flask; add 50 mL sodium bicarbonate extracting solution ($NaHCO_3$). Placed on mechanical shaker for 30 minutes at 180 rpm. Blank was also prepared in one flask having all chemicals except soil. Filtered the solution using Whatmann filter paper No. 42. Then 5 mL of the filtered extract was pipetted out into 25 mL volumetric flask, 5 mL color developing reagent was added into it and made the volume up to mark with distilled water. It was shaken to remove the gas bubbles. Subsequently bluish color developed. The concentration of phosphorus in soil is directly proportional to the intensity of blue color developed. After 15 minutes, the samples were analyzed on the Spectrophotometer. The absorbance of blank, standards, and samples were recorded accordingly at 880 nm wavelength. The calibration curve for standards was prepared, plotting the absorbance of the samples and phosphorus concentrations on the y-axis and x-axis respectively. From the calibration curve, phosphorus concentrations were measured for the unknown samples by following formula.

$$\text{Phosphorus (mg/kg)} = \text{mg/kg P (from calibration curve)} \times \text{A} / \text{Wt} \times 25/\text{V} \ldots \quad \text{(Eq. 1)}$$

Whereas; A = Total vol. of the extract (mL)

Wt. = Wt. of air-dried soil (g)

V = Vol. of extract used for measurement (mL)

3.8 Analysis of Phytoavaliable Phosphorus in Plants

100 mg of both the ground shoots and roots saved in sampling bags for phosphorus analysis were then digested in 5 mL acid mixture of concentrated Nitric Acid and Perchloric Acid (HNO_3-$HClO_4$), 2:1 on hot plate at 180 °C for 1 h. The aliquots were filtered off by using Whatmann filter paper No. 42.

The phosphorus in plant extracts was determined using vanado-molybdo-phosphoric acid colorimetric method (Ryan, 2008). The details of this method are as follows:

Preparation of Reagents

a. **Reagent A:** Precisely 25 g ammonium heptamolybdate [$(NH_4)_6Mo_7O_{24}.4H_2O$] was dissolved in 500 mL warmed distilled water (5% solution) (a). 1.25 g of ammonium metavanadate (NH_4VO_3) was dissolved in 500 mL in boiling distilled water (0.25% solution) (b). When both solution were cooled 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 volumetric flask. Solution was allowed to cool 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. Acid mixture was then allowed to cool.

c. **Standard Stock Solution:** Precisely, 2.197 g oven dried potassium dihydrogen phosphate was dissolved in 500 mL distilled water (1000 mg/kg stock solution). 10 mL of this solution was diluted with distilled water up to 100 mL (100 mg/kg sub stock solution).

A. Wet Digestion Method

Precisely, 0.1 g of ground plant material was added to 25 mL flask. 5 mL of acid mixture was added to flask till the vigorous reaction stage was finished. Flask was placed on hot plate at 180 ^{o}C for 1h in a fume hood and heated. Temperature was increased slowly until all traces of nitric acid disappeared. Heating was continued until dense white fumes of perchloric acid just appeared leaving clear aliquots behind and volume was made up to 25 mL. The plant digested material was filtered using Whatmann filter paper No. 42, and extracts were stored at 4 °C for further analysis.

B. Measurements

1. Precisely, 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 up to the mark with distilled water.
2. The sub-stock solution was pipetted out to 25 mL volumetric flask to prepare 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 phosphorus respectively. 5 mL mixed reagent was added and continued as for the samples. A blank was also prepared containing all

the chemicals except plant material. Absorbance of the blank, standards, and for samples was read after 1h at 430 nm wavelength on Spectrophotometer (Model, Manufacturer??). A calibration curve was prepared for standards, plotting absorbance against respective phosphorus concentration. The phosphorus concentration for the unknown samples was estimated by using the calibration curve (Ryan, 2008). The total phosphorus uptake per plant was calculated from the following relation:

$$P\ uptake = [(shoot\ dry\ weight \times shoot\ P\ conc.) + (root\ dry\ weight \times root\ P\ conc.)]$$

3.9 Microscopic and Spectroscopic Analysis of Soil and Plant material (SEM, EDS, Raman and FTIR)

To determine the presence of nanoparticles in *Lactuca sativa* plants, shoots and roots were cut, and samples were washed with distilled water to remove electrolytes and soil from their surface, then oven-dried at constant 70 °C for 48 h. The samples were allowed to cool at room temperature. A fine piece of root and shoot was then observed under the Scanning Electron Microscopy (SEM). Energy Dispersive X-ray Spectroscopy (EDS) is a chemical microanalysis technique used in conjunction with SEM to illustrate the elemental composition of the sample. The EDS spectra was generated for the same plant's root and shoot observed under SEM.

Raman spectra of rhizosphere soil extract were obtained from Micro-Ramboss using diode laser (532 nm λ) as an excitation source to determine the presence of plant's primary metabolites in response to nanoparticles treatment.

FTIR spectra were recorded with a Nicolet - 6700 (Thermo-corporation). The powdered plant samples of *Lactuca sativa* shoots were scanned at room temperature (25±2 ºC) within spectral range of 800–3600 cm^{-1}. In the present study, we tried to relate the bands shifting and intensities of the peaks to the concentration of the corresponding functional groups especially phosphates interactions and OH groups in response to nanoparticle treatments.

3.10 Statistical Data Analysis

The statistical significance of results was checked by using Student's t-test (mean analysis) and standard deviation (Annexure II, Table 4.1). 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 TiO_2 and Fe_3O_4 Nanoparticles

As different synthesis methods have been used to get the desired properties of TiO_2 and Fe_3O_4 nanoparticles, so following results have been cited here for comparative analysis of different synthesis methods used.

Table 4.1: Comparative Analysis of Nanoparticles Synthesis Methods

Experiment	**Type of Nanoparticles and Methods**	**XRD (nm)**		**SEM (nm)**	
		1	**2**	**1**	**2**
1	TiO_2 by Sol-gel Method	15	14.9	16	-
2	TiO_2 by Sol-hydrothermal Method	114	-	-	33
3	TiO_2 by Liquid Impregnation Method	105	44	88	-
4	Fe_3O_4 by Co-precipitation Method	14.9	-	15	-
5	Fe_3O_4 by Solvothermal Method	-	-	10	-

Table 1 show the average particle sizes obtained from XRD and SEM measurements. In experiment 1, particle size was slightly larger with SEM than the observed crystallite size obtained from the XRD. In the comparative analysis, the results of XRD were coincident with the results obtained from the SEM analysis especially for the sol-gel and co-precipitation method which indicates the presence of single crystals. So the nanoparticles synthesized by these methods were used for further experimentation.

4.1.1 XRD Results of TiO_2 and Fe_3O_4 Nanoparticles

The phase composition, crystal structure and crystallite size of TiO_2 nanoparticles synthesized by sol-gel method and Fe_3O_4 by co-precipitation method were determined through XRD.

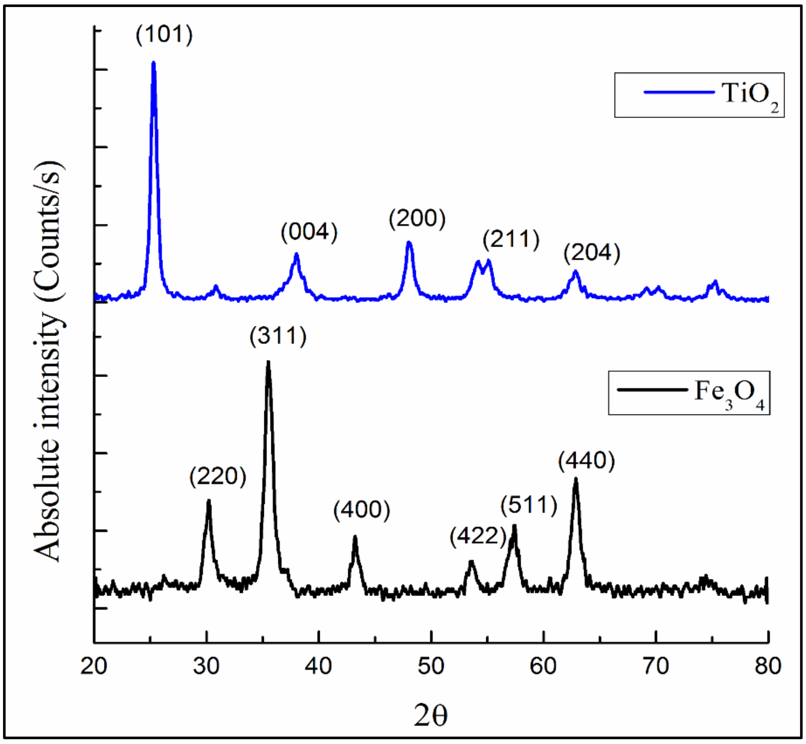


Figure : XRD Spectra of TiO_2 and Fe_3O_4 Nanoparticles

The spectrum in Figure 1 indicates that the TiO_2 nanoparticles were crystalline and no amorphous phase was observed. The peaks indicate the presence of anatase phase with (101), planes respectively. XRD card verifying result is 01-084-1286. The average crystallite size calculated by Scherer formula is 15 nm with tetragonal crystal system. The graph also showed the characteristic peaks corresponding to pure magnetite. The crystallite size of Fe_3O_4 nanoparticles was found to be 15 nm having cubic crystal structure. XRD card verifying result is 01-075-0033.

4.1.2 Raman Spectra of TiO_2 and Fe_3O_4 Nanoparticles

Raman spectra for both synthesized nanoparticles was also obtained using the Micro-Ramboss (DPSS, laser source) to verify the results.

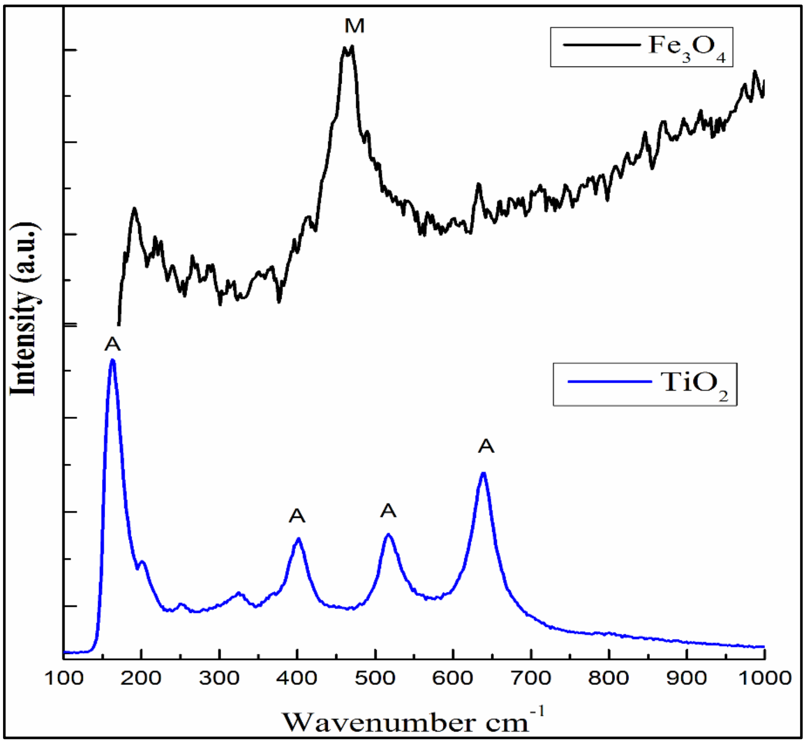


Figure : Raman Spectra of TiO_2 and Fe_3O_4 Nanoparticles

Figure 2 shows the Raman spectra verifying the composition of TiO_2 and Fe_3O_4 nanoparticles. Raman results obtained were consistent with XRD results. Raman spectra of TiO_2 showed peaks at active modes verify the anatase phase (Zheng et al., 2008). The Fe_3O_4 spectra showed major peak at 460 cm^{-1} which conformed the obtainment of magnetite nanocrystals with the surface partially oxidized to maghemite (Russo et al., 2012)

4.1.3 SEM Results of TiO_2 and Fe_3O_4 Nanoparticles

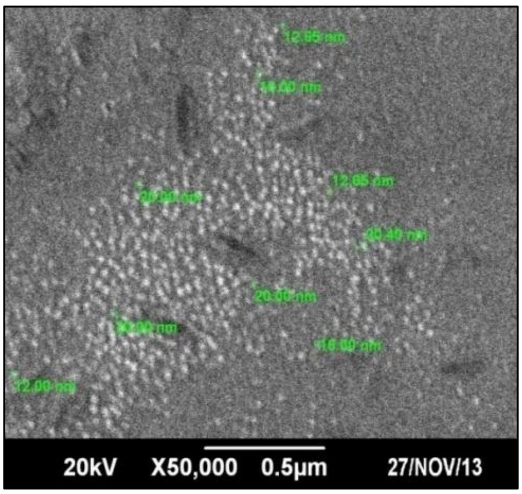


Figure SEM Image of TiO_2 Nanoparticles by Sol-gel Method

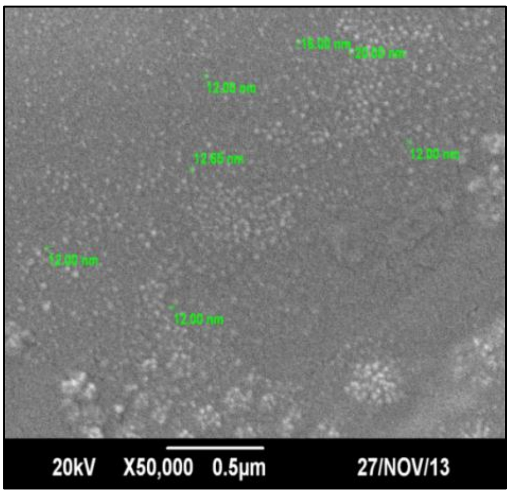


Figure SEM Image of Fe_3O_4 Nanoparticles by Co-precipitation Method

Figure 3 and 4 show the images of TiO_2 and Fe_3O_4 nanoparticles by sol-gel and co-precipitation method respectively at 50,000 magnifications (SEM, JEOL JSM-6490 A, Japan). Images of TiO_2 and Fe_3O_4 nanoparticles confirmed the size of these particles in nano range. i.e., within 12-20 nm range.

4.1.4 EDS Results of TiO_2 and Fe_3O_4 Nanoparticles

Energy Dispersive X-ray Spectroscopy is used to study the elemental composition of samples. Figure 5 and 6 showed the EDS spectra of TiO_2 and Fe_3O_4 nanoparticles synthesized by sol-gel and co-precipitation method, respectively. EDS spectra indicated the presence of pure of TiO_2 and Fe_3O_4.

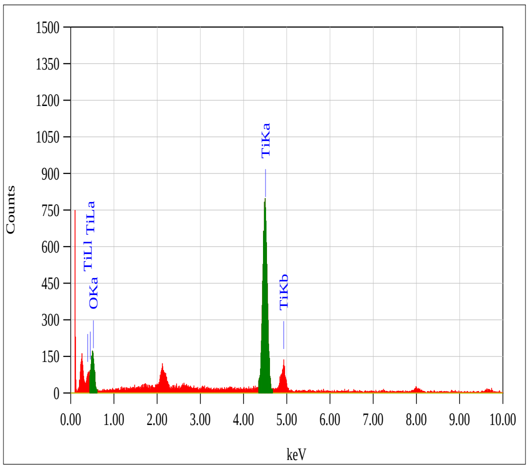


Figure : EDS Spectra of TiO_2 Nanoparticles

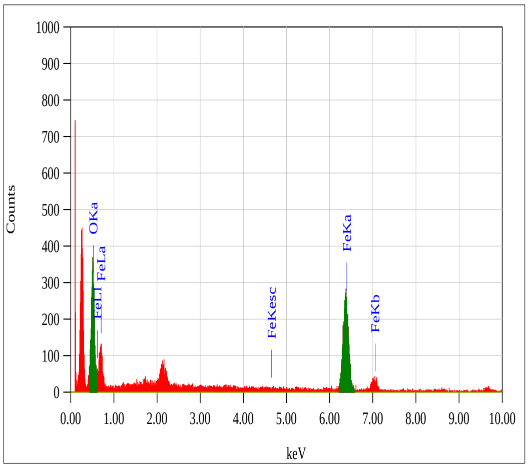


Figure : EDS Spectra of Fe_3O_4 Nanoparticles

4.2 Growth Response of *Lactuca sativa* to Nanoparticles

Figure 7 and 8 illustrated the influence of nanoparticles on physical growth parameters of *Lactuca sativa*.

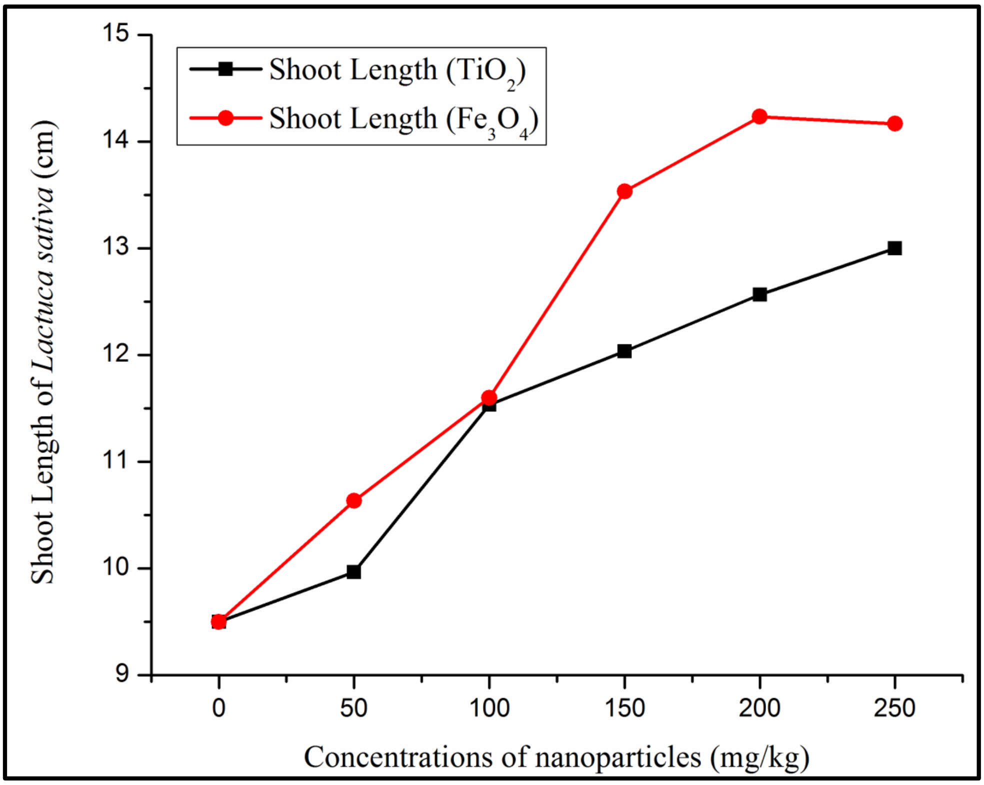


Figure : Shoot Length of *Lactuca sativa* in Response to Nanoparticles Treatment

In Figure 7, the shoot length percentage showed the maximum value (14.2 cm) in 200 mg/kg of Fe_3O_4 and (13 cm) in 250 mg/kg of TiO_2 nanoparticles whereas the lowest value (9.5 cm) was in control. Shoot growth increased up to 49% by Fe_3O_4 nanoparticles and 36% by TiO_2 nanoparticles over control.

The positive effects on the germination and seedling growth of spinach plants exposed to TiO_2 nanoparticle in solution were also reported (Zheng et al., 2005). 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).

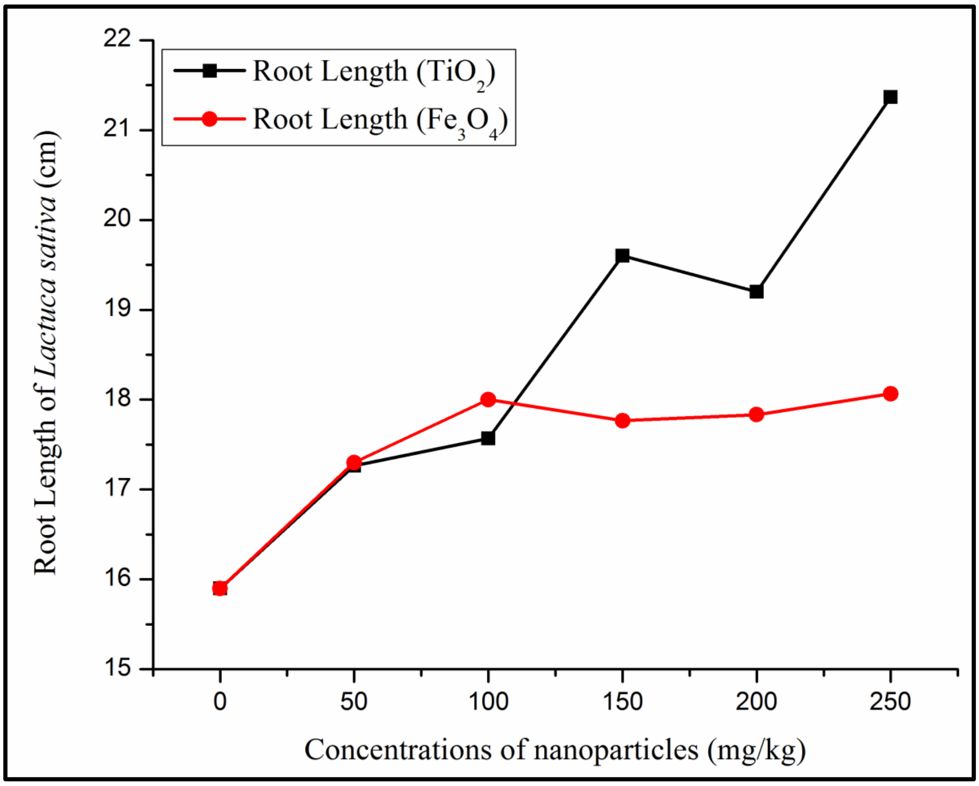


Figure : Root length of *Lactuca sativa* in Response to Nanoparticles Treatment

In Figure 8, root length was observed to increase up to 21.4 cm in TiO_2 treated group as compared to 18.1 cm in Fe_3O_4 treated group and 15.9 cm in control. The root length in TiO_2 treatment was increased more as compared to shoots as it might be accumulated in roots thus inhibiting the further shoot length. While opposite pattern was observed in Fe_3O_4 treatment in which shoot growth increased more as compared control. The values of shoot and root lengths were given as Mean ± SD (standard deviation) for five replicates in Annexure II (Table 4.1). In another study, TiO_2 nanoparticles were applied to wheat (*Triticum aestivum*). Enhanced wheat root elongation was observed when exposed to 14 and 22 nm TiO_2 nanoparticles (Larue et al., 2012).

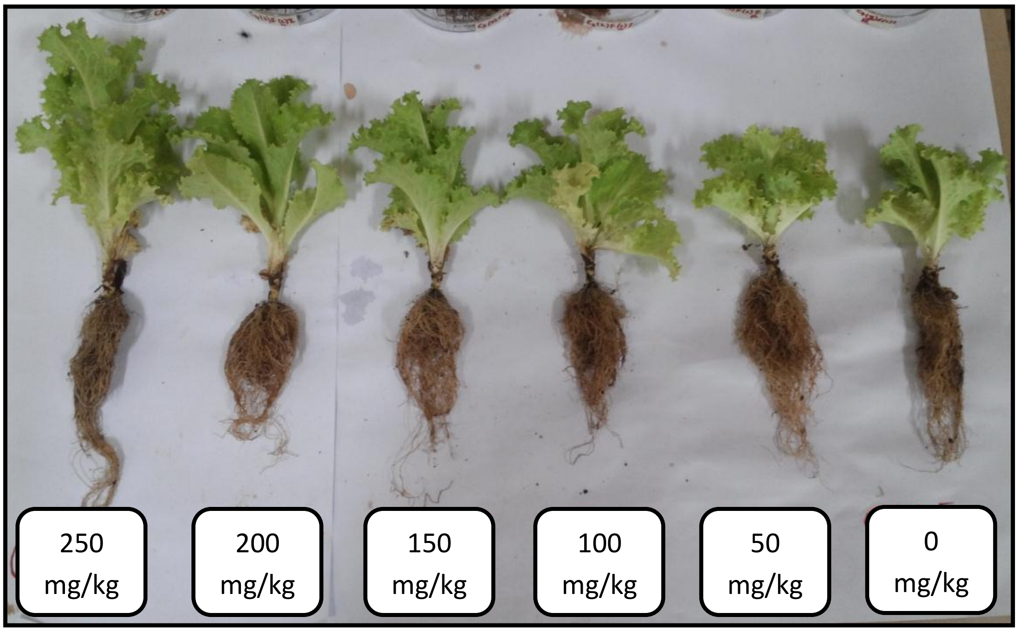


Figure : Pictorial Representation of *Lactuca sativa* Growth Response to Fe_3O_4 Nanoparticles

The main reason for this increased growth rate is due to the increased phytoavailability of phosphorus in soil that was bound and now readily available to the plant. Noticeably, nano-TiO_2 and Fe_3O_4 treatments showed a significant ($p < 0.05$) positive effect on the plant growth. Our results also coincides with the results of Mahajan et al. 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 (Mahajan et al., 2011).

4.3 Fresh and Dry Biomass of *Lactuca sativa* in Response to Nanoparticles

Use of TiO_2 and Fe_3O_4 nanoparticles enhanced *Lactuca sativa* growth, plant fresh and dry biomass as compared to the control group while percentage increase was less in exposure to higher concentrations of Fe_3O_4 as compared to TiO_2 nanoparticles. Figure 10 illustrated that both shoot and root fresh biomass increased due to both nanoparticles treatment.

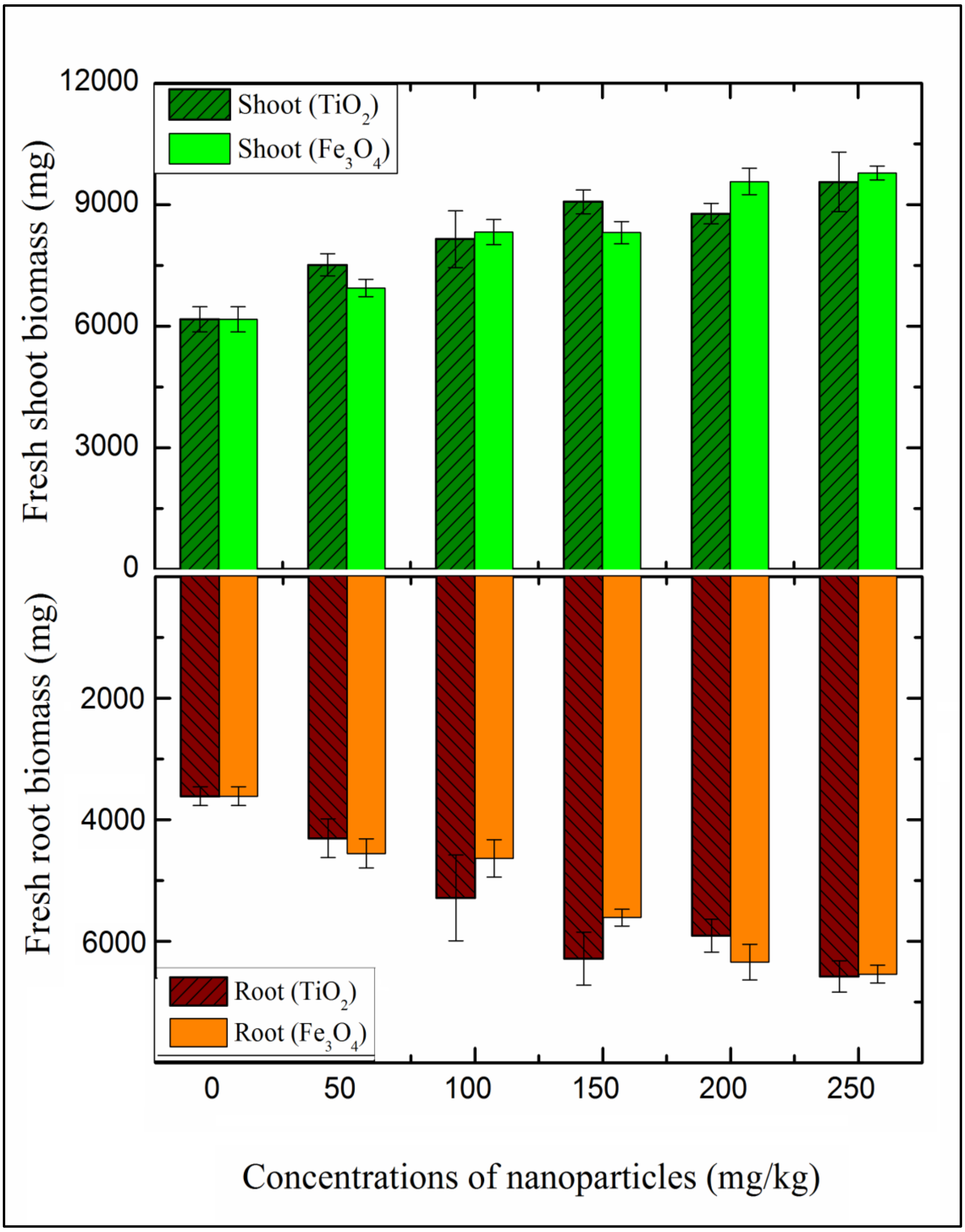


Figure : *Lactuca sativa* Fresh Biomass in Response to Nanoparticles Treatment

The fresh and dry biomass of shoots in nanoparticles treated groups was enhanced by 1.5-fold and by 1.2-fold, respectively as compared with the untreated group while the root dry weight increased 1.6-fold by both at 250 mg/kg. In another study, Fe_3O_4 nanoparticles have also been reported to increase soybean pod and leaf dry biomass (Sheykhbaglou et al., 2010).

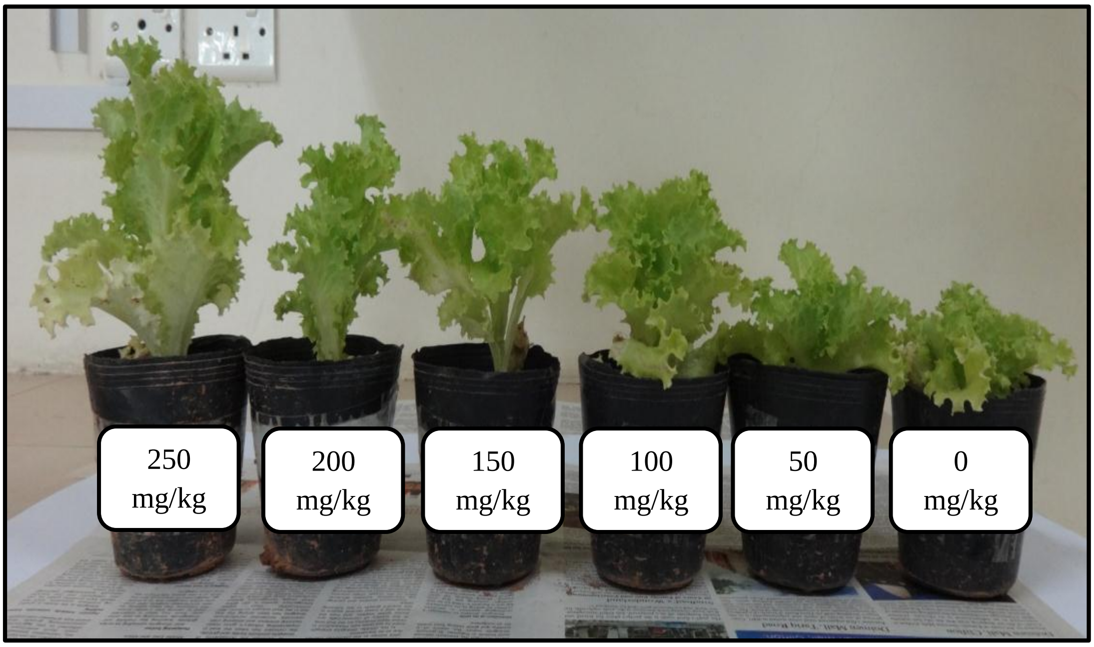


Figure : Pictorial Representation of *Lactuca sativa* Fresh Biomass in Response to TiO_2 Nanoparticles

Plant biomass (shoot and root) results were found in concurrence with the growth for corresponding nanoparticles concentrations and exhibited a strong correlation with applied concentrations.

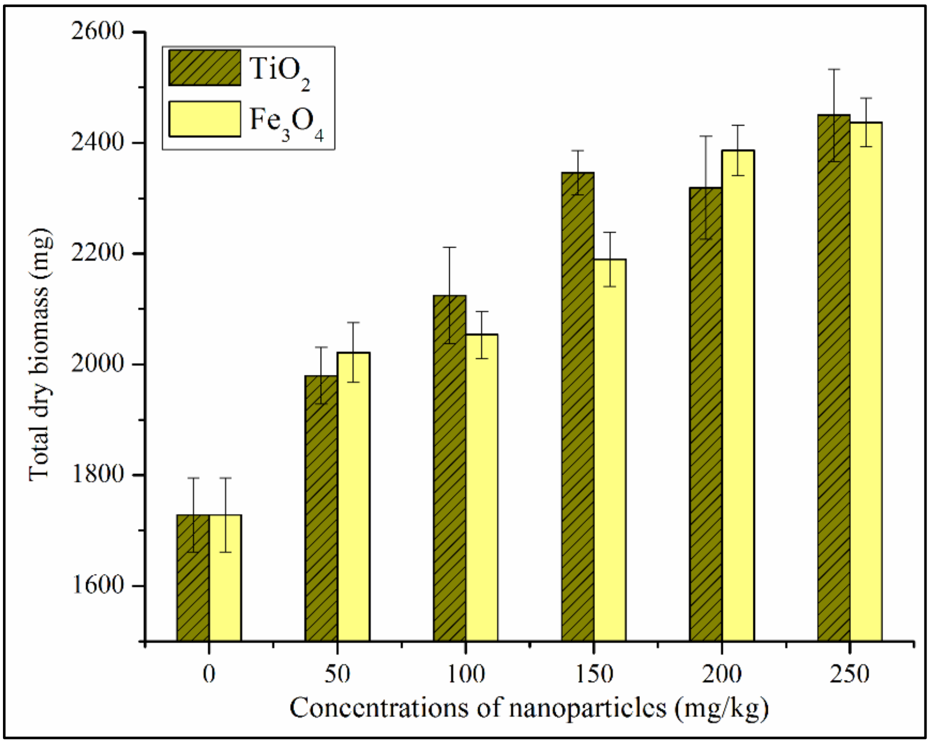


Figure : Total Dry Biomass of *Lactuca sativa* in Response to Applied Nanoparticles

Figure 12 shows that the total biomass of *Lactuca sativa* increased up to 1.4-fold at the highest concentration of nanoparticles applied. From the agricultural point of view, these results are valuable to practice the improvement in productivity of crop plants with lesser amount of nanoparticles. As phosphorus is known to be the limiting factor for plant growth, the increase in phytoavailability of phosphorus will ultimately increase the growth rate and biomass.

4.4 Moisture Content Percentage in *Lactuca sativa* Shoots

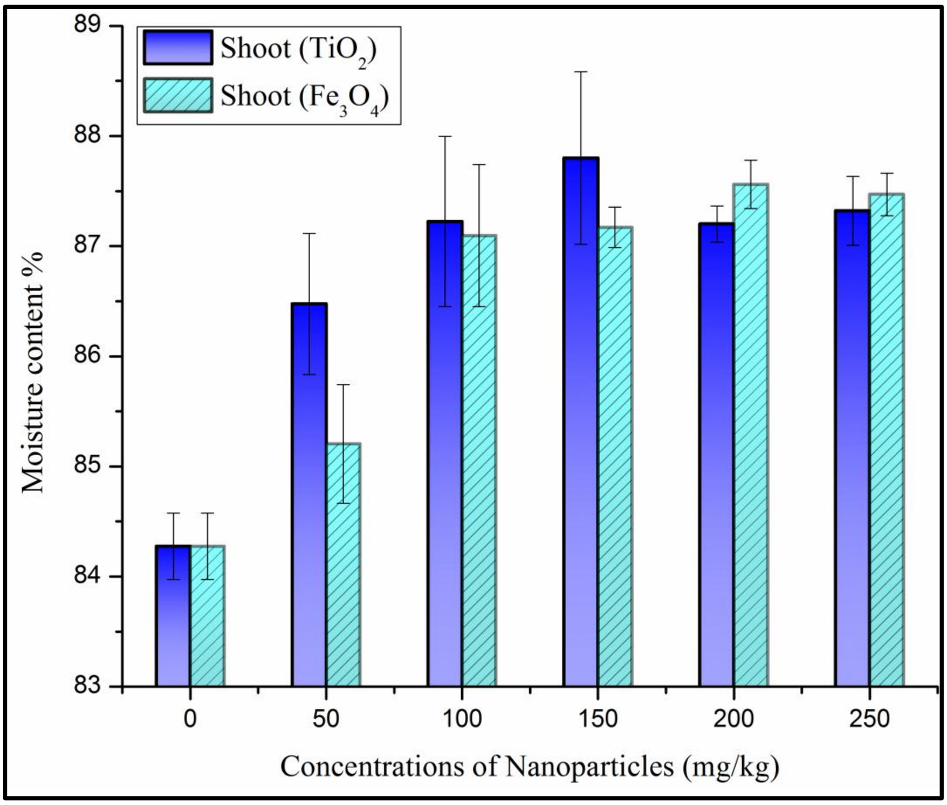


Figure : Moisture Content Percentage in *Lactuca sativa* Shoots in Response to Nanoparticles

Figure 13 presents an increased trend by shoot moisture content %age with respect to nanoparticles application. The percentage of moisture content increased with increased concentration of nanoparticles as compared to control. Due to their small size, these nanoparticles were absorbed by the roots, increasing the water uptake from soil and translocated to the aerial parts of the plant. This increased water movement also favored the increased nutrient uptake. In case of TiO_2 nanoparticles, once these were translocated to the shoots, they act as photocatalyst resulting in enhanced photosynthetic activity, improved chlorophyll formation and increased root exudation (plant metabolites). Similarly the iron oxide nanoparticles also acted as photocatalyst. Consequently both nanoparticles increased the moisture content of *Lactuca sativa* shoots. This was also verified by the FTIR spectra (Fig 20) of plant shoots treated with nanoparticles and control.

4.5 Phosphorus Concentration in Shoots and Roots of *Lactuca sativa*

Figure 14 presents the phosphorus concentration in shoots and roots of *Lactuca sativa*. The concentration of phosphorus in shoots with TiO_2 treatment was 2272 mg/kg and 2648 mg/kg for Fe_3O_4 treated group as compared to control i.e. 1371 mg/kg. Overall, increased dosage of nanoparticles resulted in increased phosphorus concentration over the respective control. The shoot phosphorus concentration showed increase up to 1.6-fold by TiO_2 and up to 1.9-fold by Fe_3O_4, respectively. A significant increase was found at different concentration levels of nanoparticles applied. The lowest value 1162 mg/kg and highest value was 2904 mg/kg (TiO_2) and 2439 mg/kg (Fe_3O_4) for root phosphorus concentration was observed as compared to control.

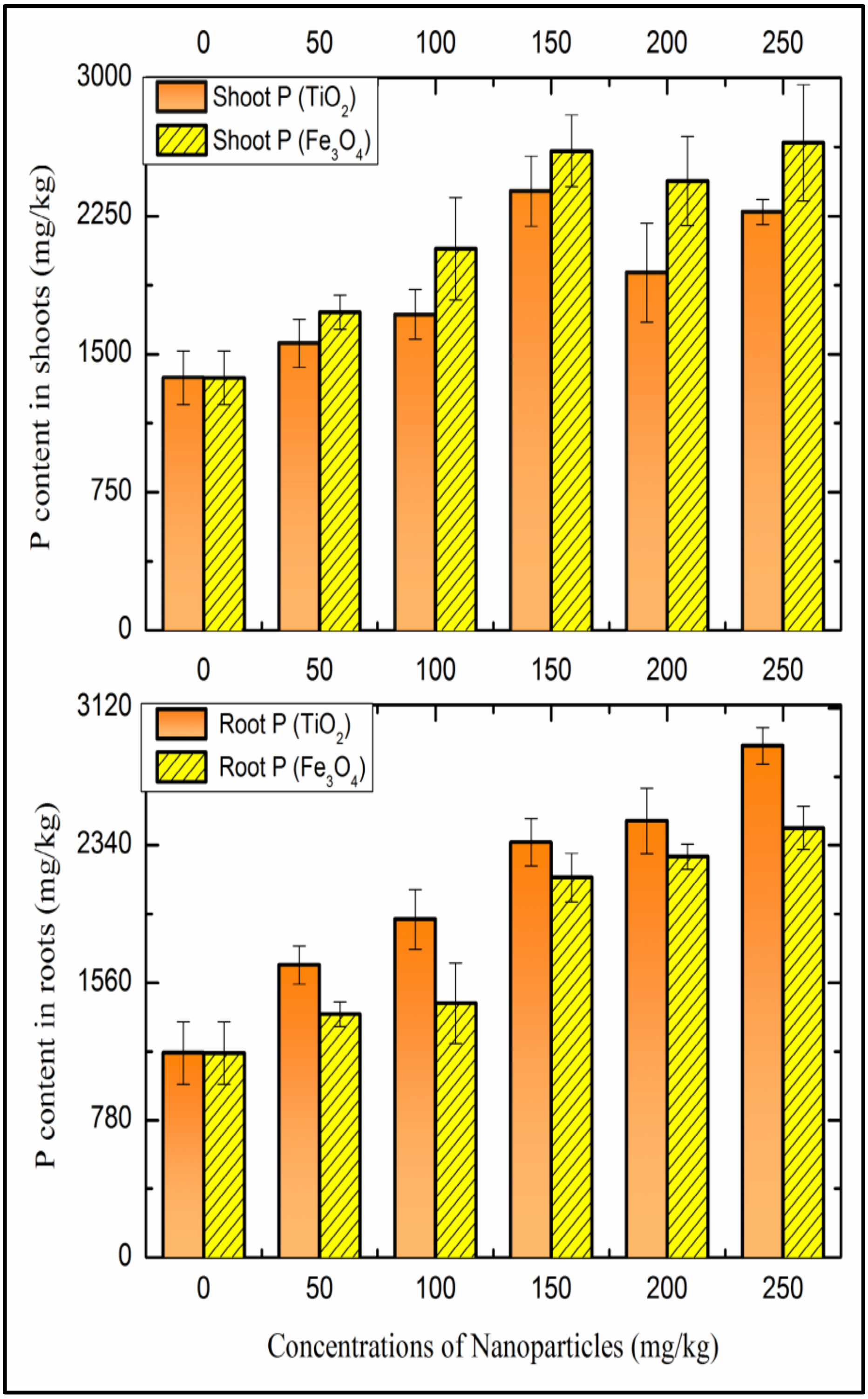


Figure : Nanoparticles Effect on the Phosphorus Concentration of Shoot and Root

The phosphorus concentrations in root were found higher than the shoot phosphorus concentrations for TiO_2 and vice versa in case of Fe_3O_4 treated group. The increase in both shoot and root phosphorus concentration from 0 to 250 mg/kg indicated its positive relationship with nano-TiO_2 and Fe_3O_4 application. This effect was certainly due to the increased absorption of phytoavaliable phosphorus (converted from bound to orthophosphate Pi form) due to applied nanoparticles and especially their small size that favored the diffusion into roots structure, and induced its positive functions during the phase of plant development.

Similar trends have been shown in other studies that TiO_2 nanoparticles enhanced seedling germination, growth and promoted photosynthesis in wheat and spinach in comparison to control plant (Feizi et al., 2012; Zheng et al., 2005).

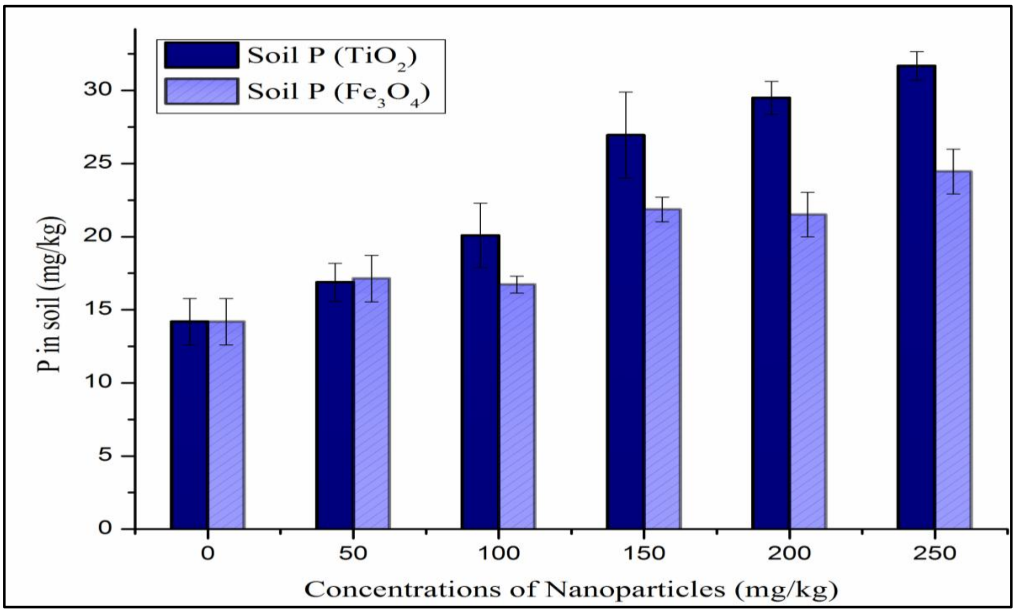


Figure : Phytoavaliable Phosphorus in Soil in Response to Nanoparticles Treatment

Figure 15 shows the relationship between phytoavaliable soil inorganic phosphorus (Pi) and applied nanoparticles. The range of phytoavaliable phosphorus in soil was between 16.9 to 31.7 mg/kg for TiO_2 and 17.1 to 24.5 mg/kg for Fe_3O_4 respectively. The minimum value (14.2 mg/kg) was obtained for soil without nanoparticles and maximum values were measured when *Lactuca sativa* grown on soil with 250 mg/kg nanoparticles. Plant phosphorus uptake from soil generally depended upon pH. The increase in soil Pi caused to increase the growth and biomass parameters of *Lactuca sativa* as well. An increase in concentrations of soluble phosphorus in the rhizosphere, instead of being depleted was also reported during the period of plant growth for rape and rice crops (Grinsted et al., 1982; Kirk et al., 1999).

Both Fe_3O_4 and TiO_2 nanoparticles dispersed in the soil were able to cover root epidermis and to form nanostructured agglomerates. Even if these agglomerates form rapidly, it is possible that small aggregates or individual nanoparticles can stay bioavailable. In fact, both Fe_3O_4 and TiO_2 nanoparticles were absorbed by the roots and translocated to the shoots as confirmed by the SEM and EDS. There were no apparent visual differences except growth parameters in plants treated with or without Fe_3O_4 or TiO_2 nanoparticles, representing that the nanoparticles did not pose any apparent toxicological effect to the plants at the tested concentration levels. Similar trend has been reported in other studies. In another study, the accumulation of Fe_3O_4 nanoparticles (20 nm) in roots (45.5%) and leaves (0.6%) of pumpkin plants in hydroponics while uptake was not observed when grown in soil medium (Zhu et al., 2008).

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 nanoparticles on soil and no effects were observed on seed germination. In general, nanoparticles induced varied effects on inhibition of plant root elongation with respect to soil type (Josko and Oleszczuk, 2013). Diverse results could be seen using the same species with reference to the effects of nanoparticles. Keeping in view that nanoparticles could behave differently depending 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).

4.6 Phytoavailability of Pi as Affected by pH Changes in the Rhizosphere

Figure 16 illustrated the uptake of phosphorus per plant vs. pH of rhizosphere soil in response to different concentrations of nanoparticles applied. In particular we focused our attention to the possible release of bound phosphorus in soil that was unavailable to plants due to Ca and Mg ions. Applying two types of nanoparticles TiO_2 and Fe_3O_4, we obtained a wide range of pH values in the rhizosphere soil of pot grown *Lactuca sativa*. The lower pH values were observed for Fe_3O_4 (range between 7.91 and 7.31) than TiO_2 (range between 7.87 and 7.57). Uptake of phosphorus per plant increased up to 2.9-fold by TiO_2 and 2.8-fold by Fe_3O_4 nanoparticles as compared to control. Similar trend had been observed in a study, in which Al_2O_3nanoparticles bound phosphorus increased plant phosphorus uptake at very low concentration of 0.01 g L^{-1} applied in nutrient solution for *Brassica napus* (Santner et al., 2012). The pH variation was probably due to the improved ability of root exudates to acidify (Arshad, 2006). In the present study, root-induced acidification of the rhizosphere treated with higher levels of nanoparticles resulted in an enhanced phytoavailability of Pi as compared to control.

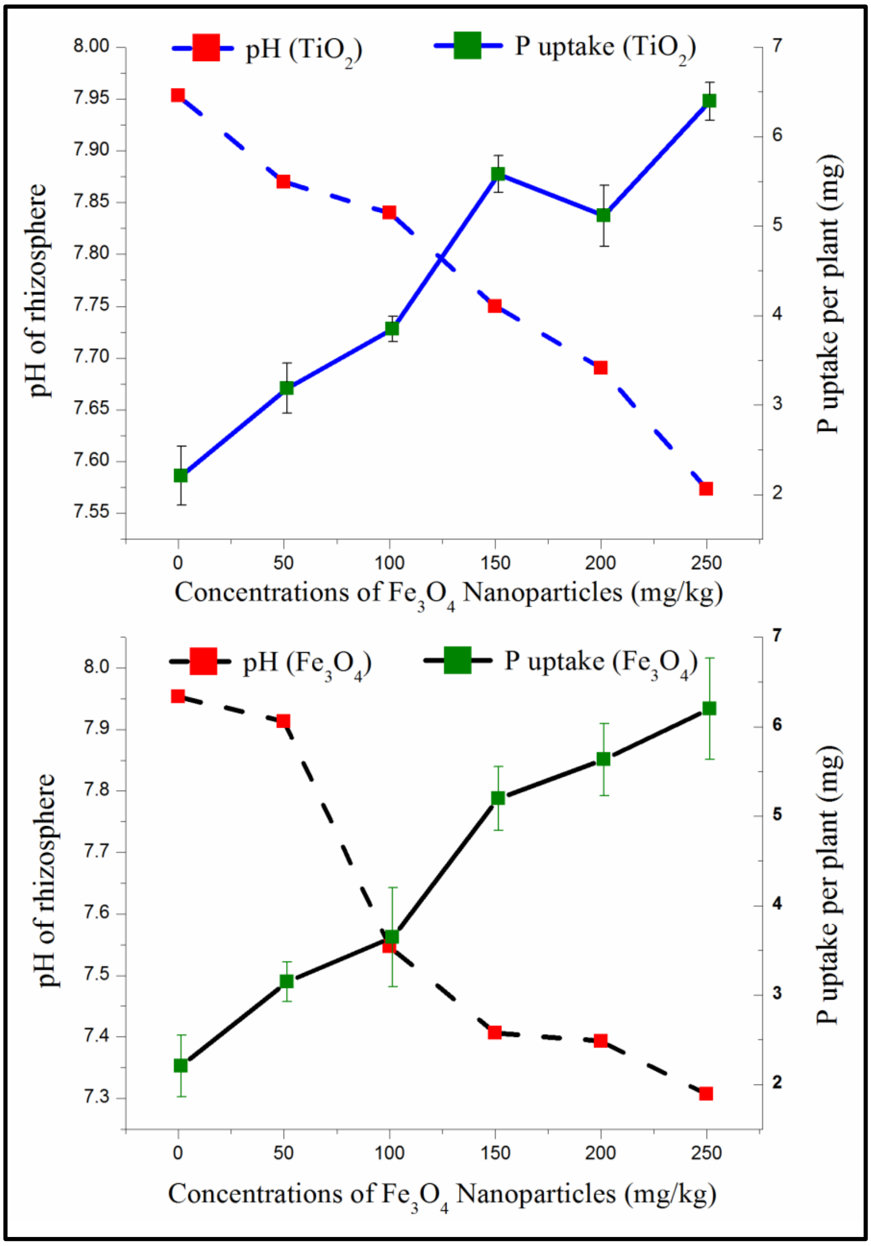


Figure : Influence of Rhizosphere pH on Phosphorus Uptake per Plant

As shown in Figure 16, the uptake of phosphorus per plant was increased as the pH of rhizosphere soil decreased. There were involved various possible aspects and mechanisms by which the plant roots could alter the phytoavailability of soil inorganic phosphorus (Pi) present in the rhizosphere. According to the past researches (Hinsinger, 2001), our observations are in conformity with the fact that there are variety of ways of interaction with the soil solid phase (adsorption–desorption or precipitation–dissolution reactions) and speciation of phosphorus that governed the concentration of phosphate ions in soil. These phenomena's mainly dependent on (a) pH, (b) the concentrations of metallic cations like Ca, Fe and Al (c) the release of competing inorganic (bicarbonate, sulphate) and organic ligands (carboxylic anions) and gaseous (O_2/CO_2) exchanges. So the bioavailability of inorganic soil could be enhanced by modifying these factors.

The pH of control group of rhizosphere soil was 7.95, the dominant cations would be Ca and Mg ions. The introduction of TiO_2 and Fe_3O_4 nanoparticles in soil provided more adsorption sites to the phosphorus ions due to the increased polarizing power of Fe^{+3} and Ti^{+2} as compared to Ca^{+2}, they made covalent bond with the phosphate group (PO_4^{3-}). So, when considering the sole mechanism of adsorption of phosphate ions onto Fe and Ti oxides, decrease in pH might be considered to cause stronger retention resulting in decreased mobility of Pi. While the desorption of sorbed P mostly occurred through a ligand exchange reaction as a result of plant root exudation that could alter the adsorption-desorption equilibrium towards improved desorption either due to the decrease in the concentration of phosphate ions in the soil or an increase in the concentration of competing anions produced. The plant roots induced depletion of phosphate ions (causes diffusion) and exudation of organic acids occurred that decreased the pH of rhizosphere (acidification). When considering organic ligand, oxalates and citrates exhibit strongest adsorption affinity and large concentration of these ligand required to desorb phosphate ions up to a significant level (Jones and Brassington, 1998). Similarly, the exudation of piscidic acid (Ae et al., 1990) and phytosiderophores were reported to enhance phosphorus solubilization through the chelation of Fe due to their high affinity for divalent and trivalent metals (Murakami et al., 1989). Within the diffusion layer, labile complexes dissociated where the free ions depleted, consequently improving the uptake, even if the intact complex was not readily taken up. The similar results were reported for uptake of Zn and Cu in spinach and tomato (Degryse et al., 2006). In the same way, we suggested that the exudation by *Lactuca sativa* roots could also result in solubilization of Fe and Ti bound phosphates in rhizosphere and resulting in increased uptake of phosphorus per plant. Conversely, the increased concentration of phosphate ions in the rhizosphere could be another possible reason for plant roots to enhance the phytoavailability of Pi. In a study, an unexpected lesser phosphorus adsorption was found to occur onto goethite due to decreased pH, thus increased number of phosphate ions in soil. Similar results were observed in another study with increased bioavailability of Pi for maize grown at acidic relative to neutral pH soil (Geelhoed et al., 2008). Our results for Fe_3O_4 nanoparticles coincided with these studies as the decrease in pH might cause lesser adsorption of Pi ions thus making them available to plants. Since the Al and Fe phosphates presumed to be the dominant phosphorus minerals in low pH soil and lower solubility (Dixon and Weed, 1989). Some studies also showed that the solubility of soil phosphorus can be further increased by decreasing the soil pH (Murrmann and Peech, 1969).

The root exudation pattern vary among the plant species (Neumann and Römheld, 1999) and can also be changed due to the environmental stresses as reported in case of Al toxicity and phosphorus deficiency or Fe deficiency (Ohwaki and Sugahara, 1997). In our experiment, the TiO_2 treated group increased the photosynthetic activity and amplified the uptake of P as verified in EDS spectra of shoots treated with TiO_2, Figure 5 (B). In a past study citrate was observed to increase the uptake of both phosphorus and Fe from the rhizosphere and projected a way related to the formation of a Fe–P citrate complex (Gardner et al., 1983). In the same way these nanoparticles could induce morphological alterations as well as tissue internalization in roots and possible upward translocation of PO_4^{3-} as the nanoparticles complexes. The uptake level depends upon the type and size of nanoparticles as well as on the plant species and soil properties (Smith et al., 2000).

Keeping in view a large number of mechanisms and reactions involved in soils and plants that represented a wide range of Pi forms with contrary geochemical behaviors, it was therefore difficult to infer that how the bioavailability of soil phosphorus will responded to a change in rhizosphere pH, up to what extent and in which way (positive or negative). It's apparent that pH was a critical factor to be considered as it might had a staged effect on the phytoavailability of Pi (Geelhoed et al., 2008).

According to a recent study, adsorption of phosphorus onto soil elements control phosphorus bioavailability from both agronomic and environmental viewpoints. Aluminum, iron, calcium and manganese are all involved in the surface adsorption of phosphorus. Other soil parameters that play significant role in controlling surface adsorption reactions include pH, organic matter content (OM), moisture, temperature and contact time between phosphorus and soil elements (Shafqat and Pierzynski, 2014).

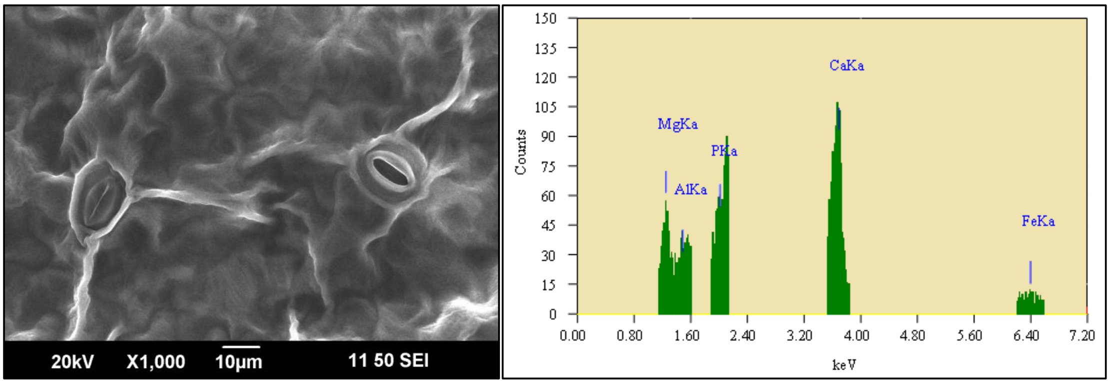
Similar to the studies of phosphate adsorption, in another study Se was added to the soil along with phosphate fertilizer that could readily be adsorbed to Al or Fe oxyhydroxides as a ligand-exchangeable species. Due to the possible mechanism of the ligand-exchangeable Se desorption from the soil occurred and translocated to plants (Altansuvd et al., 2014).

4.7 Microscopic and Spectroscopic Analysis of Soil and *Lactuca sativa*

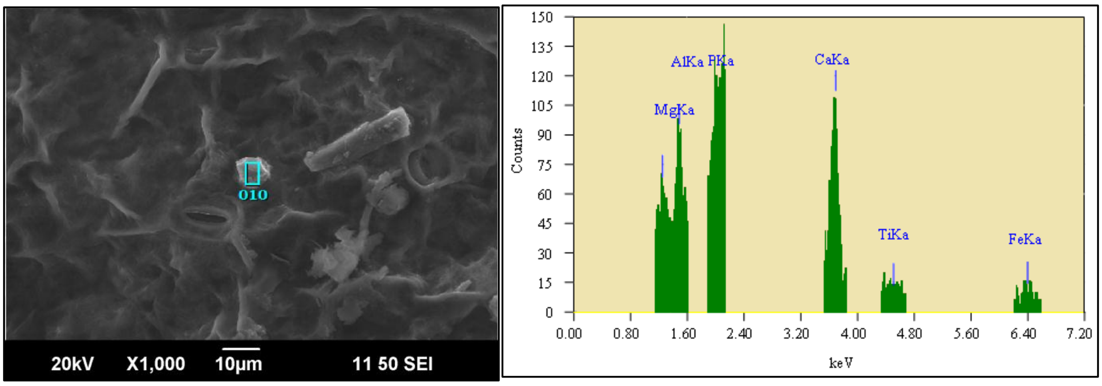
By using different spectroscopic techniques, nanoparticles influence have been determined.

4.7.1 SEM Images and EDS Spectra of *Lactuca sativa*

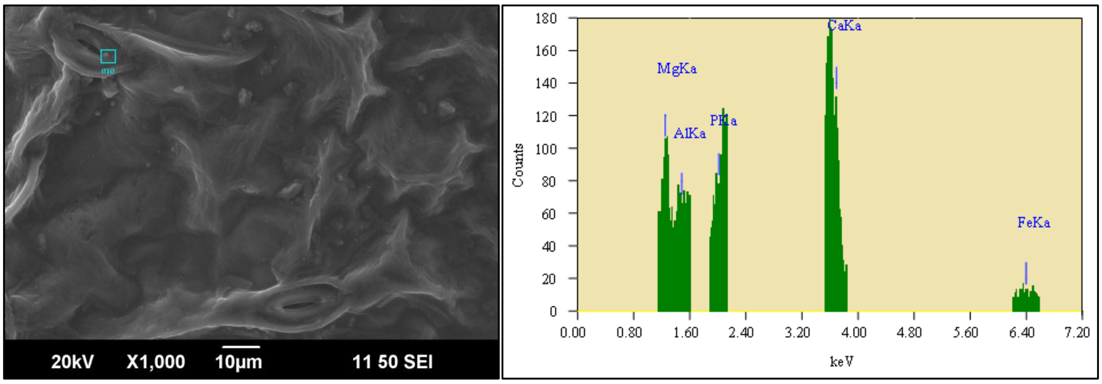
Figure 17 show SEM images of *Lactuca sativa* shoots taken at 1,000X. The control group (Fig 17A) illustrated clear surface as compared to other treated groups. Aggregates of TiO_2 and Fe_3O_4 nanoparticles were visible in Fig 17B and C. This means that nanoparticles were taken up by the plants via root and distributed in the aerial parts. They were transported by capillary action to distinct sites where the passage was wider than their size. When they reached a point where the passage was narrow nanoparticles got accumulated in the form of aggregates. The elemental presence of the applied nanoparticles were identified in the EDS spectra which confirmed the translocation of nanoparticles in shoots of *Lactuca sativa,* similarly it also showed that the TiO_2 treated group had increased phosphorus as compared to control and Fe_3O_4 group. There might be reason that the photosynthetic activity in plants exposed to TiO_2 nanoparticles have been enhanced more as compared to Fe_3O_4 nanoparticles.



(A) SEM image & EDS spectra of *Lactuca sativa* shoot (control)



(B) SEM image & EDS spectra of *Lactuca sativa* shoot (TiO_2)

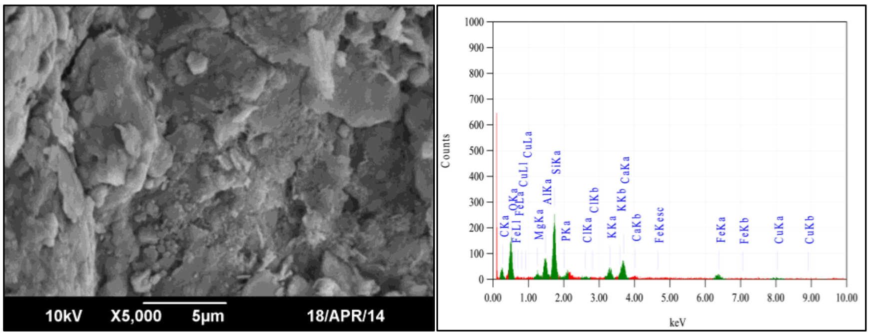


(C) SEM image & EDS spectra of *Lactuca sativa* shoot (Fe_3O_4)

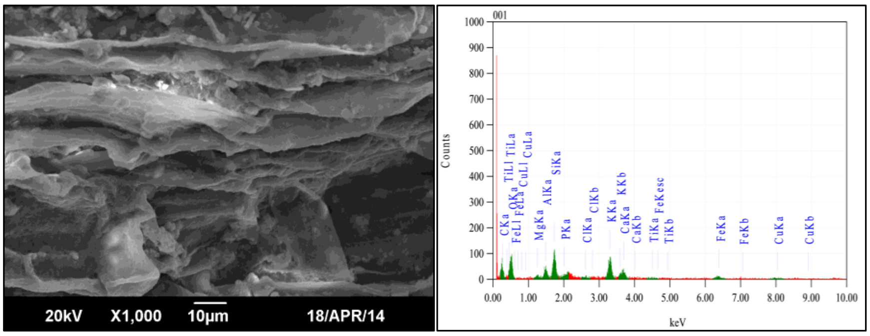
Figure : SEM and EDS of *Lactuca sativa* Shoots

The translocation of CeO_2 nanoparticles into pumpkin shoots was also reported in another study. Also, their confirmation was done via TEM and SEM that CeO_2 nanoparticles adhered to the root surfaces of plant. None of the plants showed reduced growth or any toxic effect during the experiment in hydroponics (Schwabe et al., 2013).

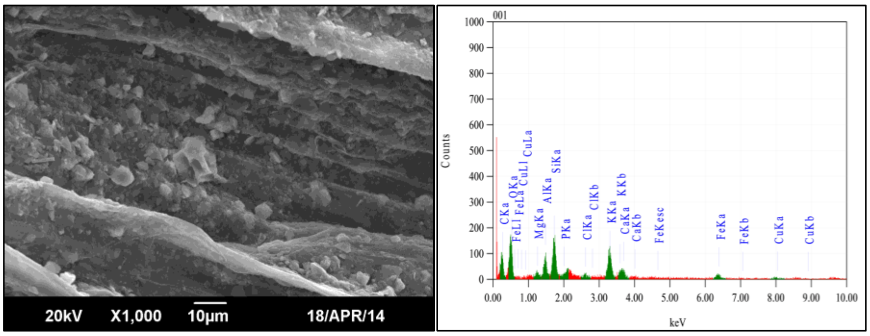
Figure 18 show SEM images and EDS spectra of root apex section of *Lactuca sativa* treated with TiO_2 and Fe_3O_4 nanoparticles and control. Both nanoparticles dispersed in the soil were able to cover root epidermis and to form nanostructured agglomerates. Even if these agglomerates form rapidly, it is possible that small aggregates or individual nanoparticles can stay bioavailable. In fact both nanoparticles were adsorbed by the roots and translocated to the shoots as confirmed by the SEM and EDS. Similar trend has been reported in another study as well (Giordani et al., 2012).



(A) SEM image & EDS spectra of Lactuca sativa root (control)



(B) SEM image & EDS spectra of *Lactuca sativa* root (TiO_2)



(B) SEM image & EDS spectra of *Lactuca sativa* root (Fe_3O_4)

Figure : SEM and EDS of *Lactuca sativa* Roots

At molecular level, the diameter of these nanoparticles could be the limiting factors for their penetration into the cell wall of the plants. If the size of the nanoparticles was too small it might diffused but if it was too large it might remained out of the cell and immobilized although it had already been penetrated in the plant cell wall. According to a recent study, the long MWCNTs (larger than 200 nm) got accumulated in subcellular organelles while the smaller ones (30-100 nm) were found into vacuoles, nucleus and plastids (Serag et al., 2013). Another study also reported the uptake of the very small sized nanoanatase TiO_2 in *Arabidopsis thaliana*. Results clearly verified that nanoparticles entered into plant cells, and got accumulated in distinct subcellular sites (Kurepa et al., 2010).

4.7.2 Raman Spectroscopy Analysis of Rhizosphere Soil Extract

Figure 19 show the Raman spectra of rhizosphere soil grown *Lactuca sativa* extract which was used to determine the plant's primary metabolites. These primary metabolites considered to be essential for the plants life as they directly influenced the normal growth, development and reproduction. Proteins and amino acids were among the main representatives of this group. Several Raman vibrational modes could be helpful for the interpretation of various amino acids and proteins occurring in plant tissues.

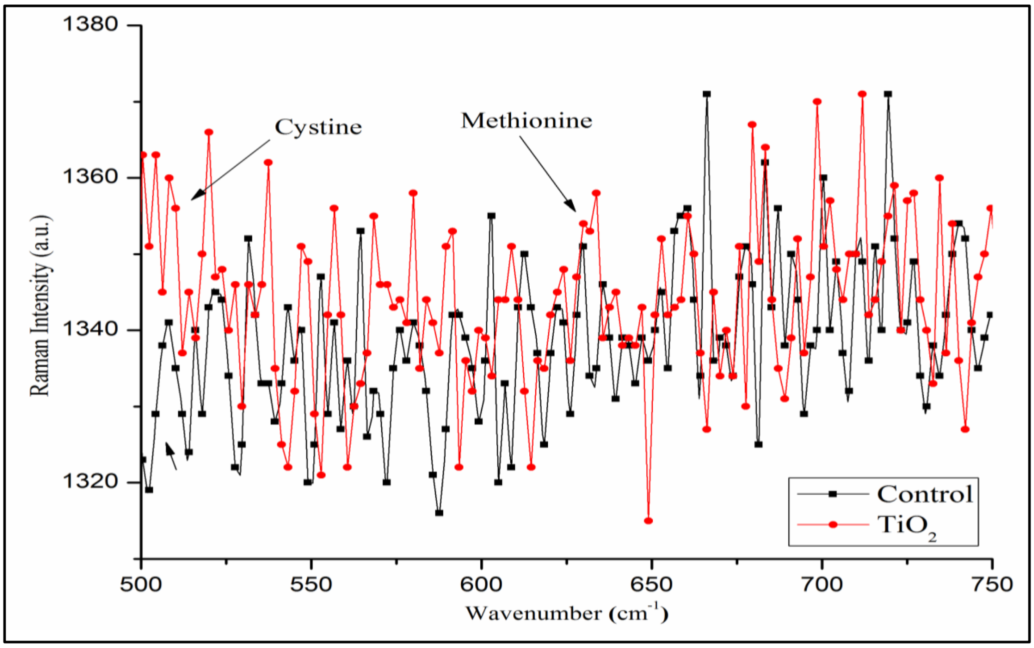


Figure : Raman Spectra of Rhizosphere Soil Extract

Applying Raman spectroscopy analysis the individual content of plants primary metabolites like cystine and methionine were found to increase in the extract by the appearance of a Raman peak at 510 cm^{-1} and 630 cm^{-1} wavenumber in the nanoparticle treated groups as compared to the values in control. Cystine and methionine proteins possessed particular amino acid structures like S–S and S–H, act as growth regulators and ultimately improved nutritional composition of crop plants.

4.7.3 FTIR Analysis of *Lactuca sativa* Shoots

The results of FTIR spectral data presented in Figure 20 verified the above discussed results showing that only the spectra is differently expressed in control and nanoparticle treated groups. Among these, the targeted groups in our study were phosphates (PO) groups. Interestingly, several of these bonds showed high similarity with both the nanoparticles treated groups as compared to control, while difference found to be lied at x-axis with a slight shift in wavenumber indicating the strength of these bonds in TiO_2 and Fe_3O_4 treated groups.

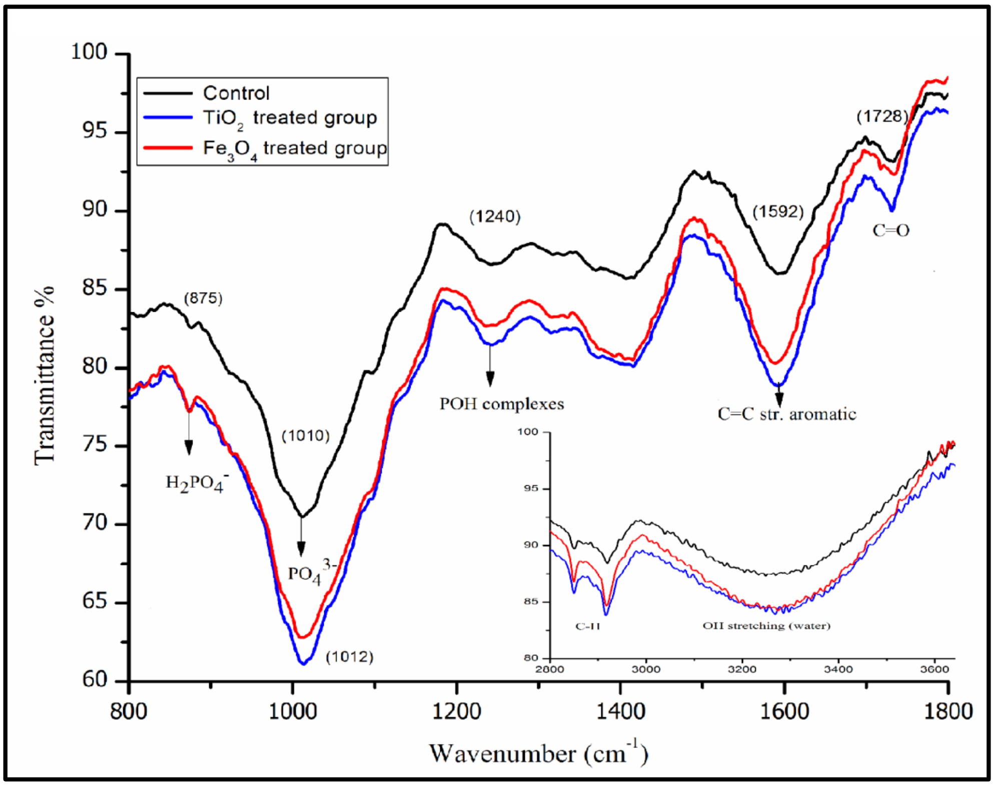


Figure : FTIR Spectra of *Lactuca sativa* Shoots

From the Figure 20, it could be seen that the stretching vibration of PO group was shifted from 1010 cm^{-1} to 1012 cm^{-1} with higher intensity in both nanoparticle treated groups as compared to control. These results revealed that chemical interactions between the nanoparticles and the functional groups occurred more strongly as compared to control. This result is consistent with the above mentioned results of soil phosphorus and phosphorus uptake by plants verifying that phosphate sorption to Fe and Ti-oxide mineral phases increased certainly as the pH of rhizosphere decreased. Therefore, resulting in increased uptake of phosphorus by the plants treated with nanoparticles as compared to the control. Similar results of increased phosphate complexes have also been reported to increase with the decreasing pH by (Elzinga and Sparks, 2007). In addition to intensity changes, significant changes in spectrum shape were observed. Similarly in nanoparticles treated group the OH bonds (water) were stronger as compared to control, related to the results of moisture content percentage in shoots. Thus *Lactuca sativa* shoot biomass with TiO_2 and Fe_3O_4 contained more phosphate, hydroxyl, and carbonyl groups on surface due to higher wavenumbers, and additional frequencies.

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Application of different kinds of nanoparticles in the rhizosphere of *Lactuca sativa* significantly affected 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-fold by both nanoparticles. Phosphorus availability was increased in the rhizosphere due to acidification as a result of root exudation up to 2.2-fold by TiO_2 and 1.7-fold by Fe_3O_4 nanoparticles. Nanoparticles taken up by the roots were translocated to the aerial parts and the presence was confirmed by microscopic and spectroscopic techniques. In nutrient uptake mechanism, the nanoparticles affinities to adsorb phosphate ions are the traits that could be optimized to improve the phosphorus efficiency for agricultural purposes. The usage of iron based nanoparticles can help in Fe fortifications in food crops and can help to evade Fe deficiency in individuals from poor and developing countries.

5.2 Future Perspectives

From the present study, we found significant effects on the *Lactuca sativa* in response to both types of nanoparticles (TiO_2 and Fe_3O_4). As agriculture sector is the backbone of economy in developing countries like Pakistan, this kind of studies could help to overcome the burden of nutrient deficit in soils providing better crop yield. Although interesting results have been found but there lies some limitations. Different factors such as experimental, environmental and climatic conditions especially temperature, humidity, sunlight, etc. affect the data sets. This work was done at small scale level in the laboratory at IESE, NUST. Pot experiment was performed in a local made greenhouse. Keeping in mind the different experimental conditions, these results may vary if experimentation tried in other parts of the country or regions across the globe with different environmental conditions. Similarly this work can also be tried in greenhouse with controlled conditions or growth chamber to better analyze the plant parameters. This kind of methodology can be adopted for different plant species, with different exposure time, at various growth stages and culture medium. Mode of application of nanoparticles can also be changed within the same and different plant species. Different results observed in case of different plant species, even if the same methodology was adopted. Besides these general aspects, we especially have to focus on the Fe_3O_4 nanoparticles as they can be used as a source of iron fortification in food crops. In Pakistan, iron deficiency in people is a common problem that needs to be solved. Fe_3O_4 nanoparticles found to increase the phosphorus concentration in plants along with iron. So it is more important to focus on the nutritional and grain compositional analysis of major food crops by using this methodology. Even in the 21st century, we have to solve the issues like unsustainable use of natural resources, low nutritional value of grains, depleting nutrients in soil and environmental issues like runoff and accumulation of fertilizers and pesticides. For this reason, we have to adopt a technology that could make the agriculture sector more productive in a cost-effective manner. This kind of studies in combination with fertilizers could be an effective option to search out the way for better application of these agrochemicals in a sustainable way. We further need to explore the potential of nanotechnology by up scaling of the present study through investigating the effects of nanoparticles at different stages in the life cycle of plant species. There is also need to do trails in combination with fertilizers and calculate the marginal rate of return.

Apart from the potential benefits of this kind of studies there are also some limitations that we could not ignore. At this stage we could not claim with surety that this kind of technology is fully safe for human health and environment or it is harmful. Risks are associated with chronic exposure of humans to these nanoparticles, interaction with flora and fauna and their possible bioaccumulation effects have not been fully considered yet. Therefore, these concerns should be considered seriously before applying this study from laboratories to the field. The other limitations include the safe range of nanoparticles concentration, scalability of research and development for prototype, industrial production and public's concern about health and safety issues. In this scenario, extensive research is necessarily required to resolve these concerns and provide conclusive studies.

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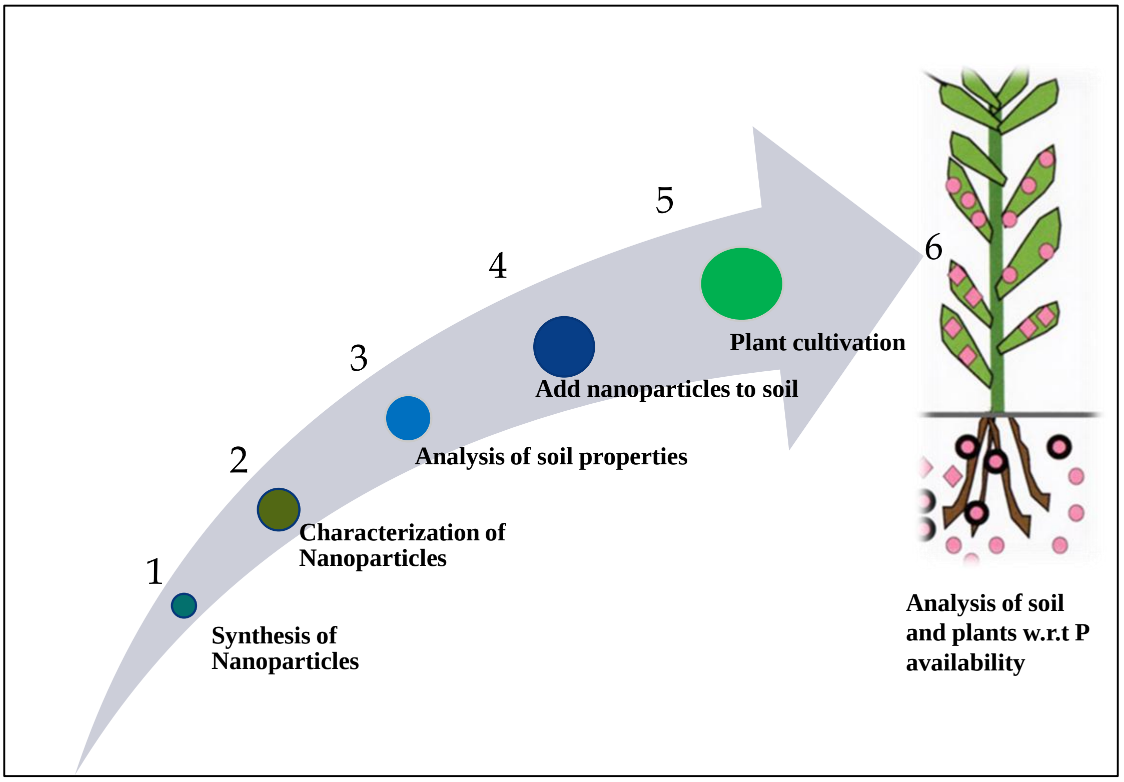
ANNEXURE I

Experimental Setup



ANNEXURE II

Table 4.1: Plant Growth and Biomass of *Lactuca sativa*

Influence of TiO_2 and Fe_3O_4 nanoparticles concentrations on plant growth and biomass of *Lactuca sativa*					
Concentration (mg/kg)	**Shoot length (cm)**	**Root length (cm)**	**Shoot dry weight (mg)**	**Root dry weight (mg)**	**Total Biomass (mg)**
Control	9.5	15.9	969.67	758.3	1728.0
TiO_2					
50	10.0*	17.3	1015.3*	964.3	1979.7
100	11.5	17.6	1037.7	1087.0	2124.7
150	12.0	19.6	1123.3	1223.0	2329.0
200	12.6	19.2	1106.0	1213.0	2319.0
250	13.0	21.4	1210.7	1239.6	2450.3
Fe_3O_4					
50	10.6*	17.3	1026.7*	994.6	2021.3
100	10.9*	18.0	1073.3	980.3	2053.7
150	13.5	17.8	1065.7	1124.0	2189.7
200	14.2	17.8	1190.3	1196.3	2386.7
250	14.2	18.1	1225.7	1211.3	2437.0
* Means, in each column, followed by similar values are not significantly different at the 0.05 % probability level (T-Test)					