Comparative Analysis of Tomato and Lettuce upon Application of Titania Nanoparticles and Titania GPR as Soil Amendment

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By

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It is certified that the contents and form of the thesis entitled **"Comparative Analysis of Tomato and Lettuce upon Application of Titania Nanoparticles and Titania GPR as Soil Amendment."** submitted by Ms. Sana Rizwan has been found satisfactory for the partial fulfillment of the requirements of the degree of Master of Science in Environmental Science.

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DEDICATION

To my Late Mom for being my inspiration throughout my whole life who makes me able to achieve my goals. To my Husband for always being supportive in every decision I made and for being humble throughout my research phase. And specifically, my friends who always stood by me and helped me in my thesis completion.

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LIST OF ABBREVIATIONS

AWC	Available Water Capacity
CeO_2	Cerium Oxide
CuO	Copper Oxide
DHA	Dehydrogenase Activity
DNA	Deoxyribonucleic Acid
EC	Electrical Conductivity
ENPs	Engineered Nanoparticles
H_2O_2	Hydrogen Peroxide
MDA	Malondialdehyde
Ni	Nickel
OM	Organic Matter
PCPs	Personal Care Products
pH	Potential of Hydrogen
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscope
OM	Organic Matter
TBARS	Thiobarbituric Acid Reactive Substances
TiO_2 NPs	Titanium dioxide Nanoparticles
TiO_2 GPR	Titania General Purpose Reagent
XRD	X-ray Diffraction
ZnO	Zinc Oxide

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ABSTRACT

Nanotechnology is the fastest growing industry in the field of agriculture due to its potential beneficial impacts on plants. Titania nanoparticles are used widely in agricultural practices due to their unique properties. Titania nanoparticles and Titania GPR (General Purpose Reagent) were applied to two different plant species to assess their comparative effects. For this purpose, *Lactuca sativa* L. (lettuce) and *Solanum lycopersicum* L.(tomato) were selected and cultivated in soil amended with previously reported concentrations of Titania nanoparticles i.e., 100 mg/kg for both the species. Different techniques such as Scanning Electron Microscopy (SEM), X-ray Diffraction (XRD) and SEM coupled with EDS (Energy Dispersive X-ray Spectroscopy) were used to monitor the presence of Titania nanoparticles and their effects on plants. Shoot and root lengths were increased up to 11.81% and 44.04%, respectively at 100 mg/kg of soil-applied Titania nanoparticles in lettuce relative to the control treatment. In the case of TiO_2 GPR applied soil, shoot and root lengths were increased by 7.38% and 22.28%, respectively. Decreased plant shoot length and increased root length were observed in tomato plants at 100 mg/kg of TiO_2 NPs. Whereas, decreased root length and decreased plant height were observed in the case of TiO_2 GPR amended soil. Fresh biomass of lettuce was increased up to 12.96% for TiO_2 NPs amended soil and 27.99% for TiO_2 GPR amended soil. Dry biomass of lettuce decreased in both treatments. In case of tomato, fresh biomass was decreased up to 22.02% and 37.48% upon application of TiO_2 NPs and TiO_2 GPR, respectively. Dry biomass was also decreased by 49.37% and 62.46% in case of TiO_2 NPs and TiO_2 GPR treatment groups. Toxicity in tomato was increased by 2.4% and 14.94% in case of TiO_2 NPs and TiO_2 GPR amended soil, respectively. Whereas toxicity in lettuce was increased by 12.28% and 59.65% in case of TiO_2 NP and TiO_2 GPR treatment groups, respectively. Better growth was observed in lettuce at 100 mg/kg of soil amended with TiO_2 NP whereas negative growth was

observed in case of tomato. The results of this study suggest that further experimentation is required to establish the possible consequences and impacts of nanoparticles with respect to food chain contamination and safety.

INTRODUCTION

1.1 Background

Nanotechnology has seen exponential growth in recent years, and nanoparticles, in general, have been highlighted because of their exceptional and multifaceted capabilities. Their high surface area to volume ratio, due to their small size (1-100 nm) provides them with novel surface properties (Ghosh *et al*., 2016). Their beneficial properties have been reported extensively, these very properties are also harmful, raising reactivity and allowing them to invade cells leading to nanotoxicity in plants, soil microorganisms and ultimately human beings. Nanoparticles such as Titanium dioxide, Silver, Zinc oxide, Copper oxide, and Cerium oxide are used in a variety of health and food applications, including in agriculture (Patil *et al*., 2016; Hossain *et al*., 2015).

TiO_2 NPs (Titanium Dioxide Nanoparticles) are one of the most widely available nanoparticles being utilized in wide range of applications such as paints, sunscreens, food additives, cosmetics, personal and medical care, solar panels, athletics and wastewater treatment (Cox *et al.*, 2017; Keller & Lazareva, 2013). The risk of environmental exposure has evolved over the last decade, placing agricultural regions and soil systems in general at a higher risk of exposure due to release of unspecified nanoparticles (Keller and Lazareva, 2013; Ghosh *et al*., 2016). Thus, thorough evaluation of the possible positive and negative consequences of NPs is essentially required before they are globally commercialized.

1.2 Nanotechnology

TiO_2 NPs are among the 13 most commonly used nano-materials in the industrial sector (OECD, Paris, 2008). Worldwide annual production of TiO_2 exceeds four million tons per year, and continues to rise exponentially (Ziental *et al*., 2020). TiO_2 NPs are in three crystal phases: anatase, rutile and brookite. The rutile phase is the most stable one while anatase and brookite are transition forms of rutile (Tan *et al*., 2018). To highlight a few of the selected properties of TiO_2 NPs, it has a high refractive index that helps to create whitening effect. It is highly photocatalytic and increases the activity of chloroplasts. In addition, it has strong hydrophilic properties along with sterilisation, favoring the production of cleaning products. According to a study, a combination of anatase and rutile TiO_2 NPs resulted in a higher solar to electric energy conversion than the pure phases of TiO_2 NPs such as anatase, rutile and brookite (Han *et al*., 2005). In plants, TiO_2 NPs stimulate the production of carotene and chlorophyll a, increases electron transfer and boosts the activity of chloroplast (Hong *et al.*, 2005; Lei *et al.*, 2007)

Apart from the advantages, TiO_2 NPs are still not without disadvantages. The International Agency for Research on Cancer has listed it as a potential carcinogen (Group 2B) for humans (IARC, 2010). Size is a crucial factor contributing to its toxicity, behavior, and reactivity of nanoparticles. With the amount of TiO_2 NPs used in global markets, it is important to consider its impact on public health and the environment. Taking into account the indirect sources of TiO_2 NPs, exposure is unavoidable in the environment and particularly in plant species as the soil is a major recipient of nanoparticles (Simonin *et al.*, 2016).

1.3 Nanoparticles Application to Plants

The integration of nanotechnology into agriculture is an evolving concept, the effects of different nanoparticles on different plant species have been observed, giving us an insight about nutrient management, food security and possible effects on plant growth (Zahra *et al.*, 2015). Due to its widespread usage in almost every field, it is not surprising that TiO_2 NPs are also used in agricultural practices. Nanoparticles, to name a few, are used as growth regulators in plants, biosensors, in the development of nanoscale fertilizers and pesticides and in the genetic improvement of plants (Rico *et al.*, 2013a).

Plants as primary producers play an important role in the functioning and preservation of the environment through nutrient cycling (McKee and Filser, 2016). Plant-TiO_2 NPs interactions serves as a potential transport route for NPs (Rico *et al*., 2011) and is potentially the main cause of phytotoxicity including morphological and cytotoxic effects in plants at higher concentrations (Tripathi *et al*., 2017; Shweta *et al*., 2016; Rico *et al*., 2015). Plant systems, therefore, lead to the transition of various nanomaterials to diverse environmental biomes and among different trophic levels (Rico *et al*., 2011). Organisms in the ecosystem are likely to be affected by oxidative stress caused by TiO_2 NPs (Hong *et al*., 2014).

1.4 Nanoparticles Interaction with Soil

Apart from agricultural activities, TiO_2 NPs can also penetrate into soil by direct methods such as rainwater erosion, atmospheric deposition and surface runoff or, indirectly, from landfills or waste materials. (Gottschalk *et al*., 2009; Tripathi *et al*., 2017). As most NPs have weak and slow soil movements, they will gradually accumulate over time (Gottschalk *et al*., 2009). Exposure modelling on NPs concentrations also states that soils serve as better sinks for NPs than water or air, indicating that the main source of NPs exposure to the environment is through soils (Gottschalk *et al*., 2009).

Risk assessment of their soil toxicity is still in its infancy, and there is a lack of information about their impact on plant systems in field or soil setups. Therefore, environmental fate and their toxic effects on plants and ultimately public health require thorough investigations (Rico *et al.*, 2013b).

1.5 Significance of the Study

Tomato and lettuce, two vegetable crops were selected for this study due to their high demand and high nutritional value. Tomato (*Solanum lycopersicum* L.) is one of the most widely cultivated vegetables worldwide. As it is a relatively short duration crop (3-4 months) and gives a high yield, it is economically attractive and the area under cultivation in Pakistan has increased from 123.56 (000 Ha) in 2010 to 150.00 (000 Ha) in 2015 (Qasim *et al*., 2018). Tomato belongs to the *Solanaceae* family. They are rich in minerals, vitamins, essential amino acids, sugars and dietary fibers. Tomato contains vitamin B and C, iron and phosphorus. Tomato fruits are consumed fresh in salads or cooked in sauces, soup and meat or fish recipies regardless of the culture and region. They can be processed into various products including purées, juices and ketchup. Canned and dried tomatoes are other economically important processed products. Yellow tomatoes have higher vitamin A content than red tomatoes, but red tomatoes contain lycopene, an antioxidant that may contribute to protection against carcinogenic substances (Khokar., 2013). On a global scale, the annual production of fresh tomatoes accounts for approximately 159 million tons. However, more than a quarter of this around 159 million tons are grown for the processing industry, making it world’s leading vegetable for processing industry. Tomato production in Pakistan was reported to 530 thousand tons in 2011 (Khokar.,2013) and 566 thousand tons in 2015 (Qasim *et a*l.,2018)

Lettuce, *Lactuca sativa* L. (Asteraceae) is one of the most widely utilized vegetables for food preparation purposes. It supplies water, polyphenols, carotenoids, fiber, Ca, Fe, K and antioxidants such as vitamins A, C and E (Serafini *et a*l., 2002; Nicolle *et al*., 2004; Guerrero and Rojano 2010). Among different types of lettuce, the most common ones are Romaine, Iceberg and Loose-leaf. Yet, they are all sensitive to damage caused during cultivation, harvest or transportation, and to attacks by microorganisms that affect their quality during distribution, processing or storage. In most cases, both damage and microbial attacks result from the mechanical and physiological fragility of the product (Pereyra *et al*., 2005; Martínez *et al*., 2008; Serrato *et al*., 2011).

Given the background, tomato and lettuce present an excellent model system to study the efficacy of NPs. Since the scope of nanotechnology in agriculture is still uncertain and requires further exploration, the use of nanoparticles is known to have important biological effects and beneficial effects on plant physiological parameters at low doses. Therefore, the current designed project would help to understand the use of NPs in the agriculture industry and enlisting their potential advantages and disadvantages. According to the literature, TiO_2 NPs, depending on experimental components, show a dual existence with both beneficial and toxic effects. The aim of this study was therefore to specifically explore the toxicity of nanoparticles at doses that are known to enhance the availability of nutrients, vegetative traits and nutrient uptake on lettuce and tomato plants simultaneously.

1.6 Objectives

Keeping in view the information from the literature, it was hypothesized that nanoparticles are likely to cause toxicity in plants. So TiO_2 NP and TiO_2 GPR treatments were used to assess the comparative effects of both these treatments on tomato and lettuce plants. Hence, the specific objectives of the present study were:

1. Assessing the effects of TiO_2 NP and TiO_2 GPR on lettuce and tomato growth parameters.
2. Analysis of uptake of TiO_2 NP and TiO_2 GPR in lettuce and tomato.
3. Toxicity assessment at given doses of treatments in lettuce and tomato plants.

1.7 Scope of the Study

The consumption of TiO_2 NPs has been increasing in many commercial products which has raised concerns about their effects on environment. This study has a wide scope to demonstrate that how lettuce and tomato respond when grown in TiO_2 NPs treated soil and TiO_2 GPR treated soil. In this way, this study gives us an insight of how TiO_2 NPs and TiO_2 GPR should be applied more safely and effectively for the betterment of agricultural crops.

LITERATURE REVIEW

This chapter is structured to highlight the general background of nanoparticles and their interactions with plants in the environment. It also provides a review of the physiological effects of TiO_2 NPs on plant's growth, reactive oxygen species (ROS), DNA damage, chlorophyll content and their uptake by the plants.

2.1 Nano-Agriculture

Nano-Agriculture is an evolving field of science and has shown a range of beneficial effects, such as increased agricultural production and is also cost-effective, but still in its infancy, and many aspects need in-depth understanding before it can be commercialized at a global scale (Kah *et al*., 2015). Nano agriculture does not only employ the use of nanoparticles but multi-and single-walled carbon nanotubes, coated nanoparticles, metal and metal oxide nanoparticles also hold a prominent role in nano-agricultural studies (Singh *et al*., 2015).

The word "nanoparticles" can be described as "particles with one or more external dimensions from 1 nm to 100 nm" (Auffan *et al.*, 2009). Their small size gives them novel chemical, biological and physical properties that are distinct from their bulk content. These properties help the plant in a number of ways, such as increased growth and development, increased crop yields, improved nutrient absorption, and improved disease tolerance and control, along with enhanced ability to withstand environmental or external stress (Singh *et al*., 2015).

While new nanoparticles are synthesized continuously, only a handful of nanoparticles are used excessively in commercial products, so the accumulation of these nanoparticles in the environment has been gradually building over the years. Nanoparticles widely used include copper (Cu), silver (Ag), silicone oxide (SiO_2), titanium dioxide (TiO_2), gold (Au), cerium oxides (CeOx), zinc oxides (ZnO), while manganese (Mn), copper oxide (CuO) and iron (Fe) nanoparticles are also well-known (Rico *et al.*, 2015). A sufficient body of literature on nanotoxicology can be found on the nanomaterials described above.

Agricultural production regions are likely to face a higher risk of exposure, especially as NPs are likely to accumulate in the soil as time passes (Keller and Lazareva, 2013). Exposure modeling also suggested soils as the key sink of NPs as compared to water and air (Gottschalk *et al.*, 2009).

As a result of increased exposure, global concerns over possible toxic effects and their release into the environment from various sources other than agricultural applications have raised risks to the environment in general. This has resulted in the emergence of a daughter field, Nano-Toxicology, which focuses solely on the study of the risks and hazards associated with NPs application and their accumulation in the environment.

2.2 TiO2 NPs in the Environment

As our study focused on the effects of TiO_2 NPs in plants, the reviewed literature highlighted the potential routes and interactions of TiO_2 NPs into the environment. The figure 2.1 shows the estimated amount of TiO_2 NPs in the environmental sphere. According to the literature, the emission of TiO_2 NPs represents "one-fourth of the estimated mass flow of engineered nanomaterials worldwide. Among various applications of TiO_2 NPs, food, pigments, cosmetics, hair sprays and shampoos and various PCPs (personal care products) are among the key contributors of NPs into the environment (Keller and Lazareva, 2013).

In addition, exposure model estimates indicate that the TiO_2 NPs discharge is the highest in soil (1.38 times higher) and groundwater (1.85 times higher) followed by air. (Keller and Lazareva, 2013). This indicates that Titania nanoparticles have sufficient exposure time to directly interact with plants in ecosystem.

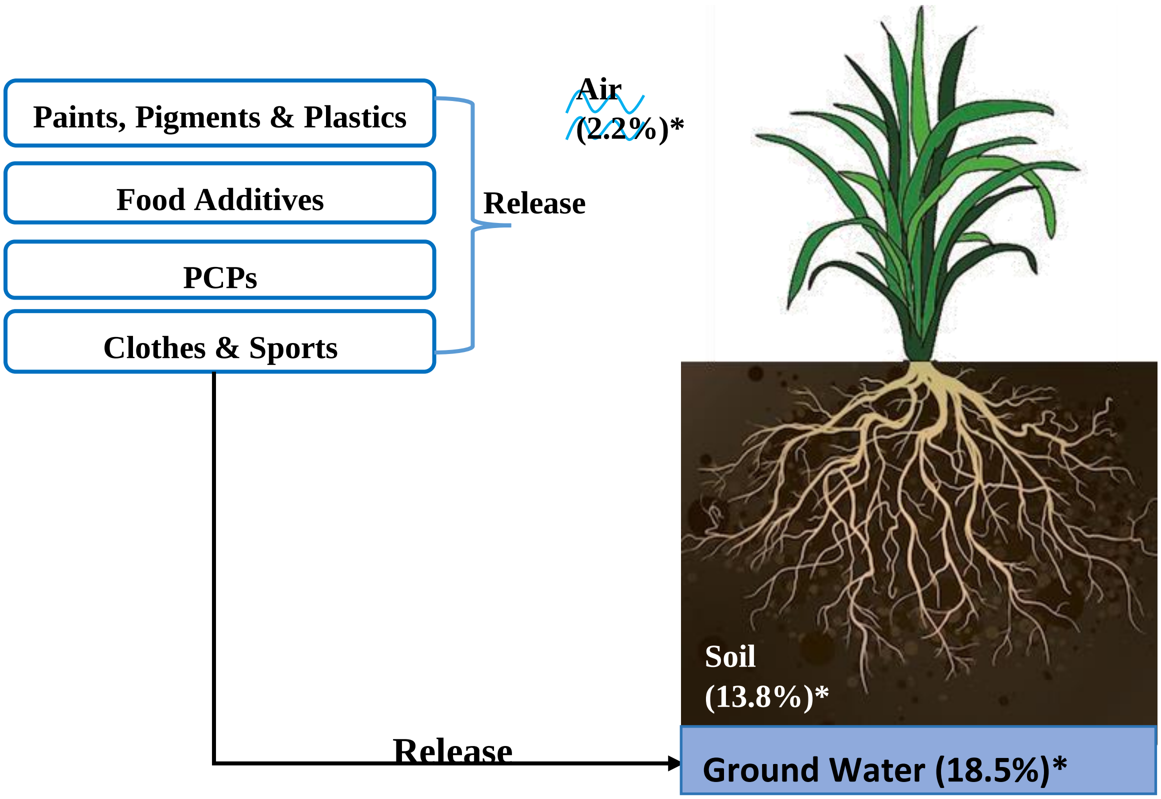


Figure 2.1: Graphic representation of uses of Titania nanoparticles and their dispersion in the environment *(Keller and Lazareva, 2013).

2.3 Effects of TiO_2 Nanoparticles on Plant Systems

2.3.1 Effect of TiO_2 Nanoparticles on Physiological Characteristics of Plants

Plants are valuable players in the maintenance of the ecosystem as they are primary producers. At the same time, taking into account unspecific releases of TiO_2 NPs from indirect sources such as landfills, wastewater sludge, and waste; the likelihood of nanoparticles interacting with plant systems prior to their uptake is high (McKee and Filser, 2016; Tripathi *et al*., 2017). Moreover, as almost every other product contains TiO_2 NPs, it is predicted that a significant amount is discharged into the environment interacting with air, water, soil and plants (Zhu *et al*., 2008). This results in physiological or genetic changes in plants depending on the size and concentration of nanoparticles. Their use as nano-fertilizers allows them to travel up the food chain, ultimately accumulating at higher trophic levels (Zhu *et al*., 2008). TiO_2 NPs have been known to show dual characteristics with both positive and negative effects, depending on the concentration, size, duration, contact time, and experimental characteristics. Several studies confirm a beneficial effect on the overall growth of plants: multiple studies on *Spinacia oleracea* L. (spinach) have shown that the use of TiO_2 NPs has increased the rate of photosynthesis and nitrogen metabolism, which has helped to enhance plant growth (Yang *et al*., 2006).

Similarly, a study of *Vigna radiata* L. (mung beans) with carbon dots (dosage:0-1.0 mg/mL) revealed a positive physiological response to their development. The carbon dots increased the absorption and use of nutrients by the plant (Li *et al*., 2017). Another research conducted by Parsad *et al* in 2012 on peanuts with Nano-ZnO showed an increased yield per pod at a concentration of 0.133 mg/g.

The effect of titania and aluminum nanoparticles in combination on wheat was observed by Alibadi *et al*. (2016) at four concentrations and found that 100 mg/kg showed an increase in root and shoot length. Andersen *et al*. have researched the impact of $nCeO_2$ and TiO_2 NPs on 10 different plant species in 2016 and found that they do not cause any harm or toxicity at the time of germination and initial plant development.

In comparison, some studies have found negative effects: in 2016, Hung *et al*. observed growth inhibition in *"Bacillus thuringiensis"* when treated with Nano-SiO_2. Research conducted on rice seedlings showed a decrease in biomass and root length, along with delayed germination and weight (Shaw and Hossain, 2013). In addition, a study was conducted to analyse the special "earth oxides (Gd_2O_3, CeO_2, Yb_2O_3, and La_2O_3)" and showed that they had injurious effects on the growth of tomatoes, rapeseed, lettuce, cabbage, corn, wheat, radish and cucumber (Ma *et al*., 2010 and López-Moreno *et al*., 2010).

Plant-Nanoparticle interactions are summarized below in Figure 2.2. These interactions either result in an increase, decrease or alteration in plant systems that are either positively or negatively (Kumar *et al*., 2018).

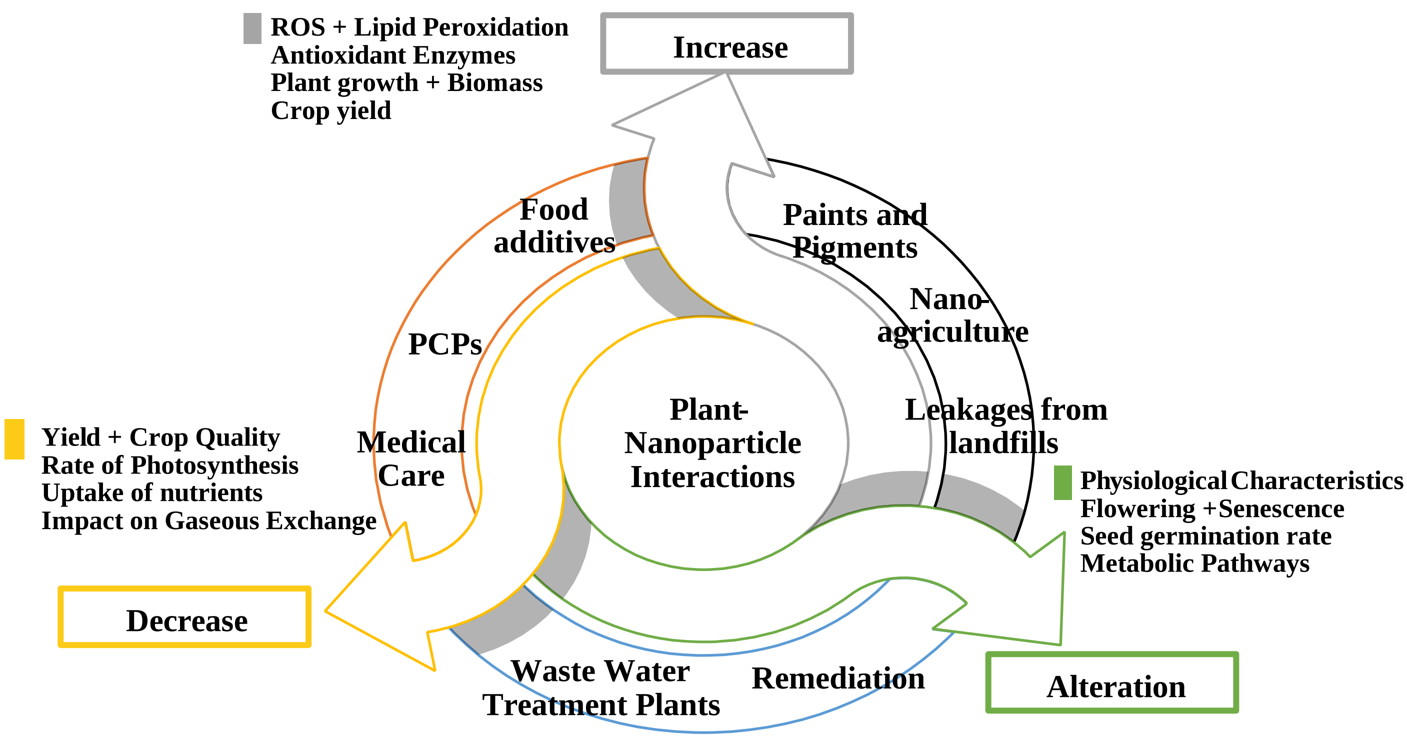


Figure 2.2: Plant-Nanoparticle interactions and its changes in plant systems.

2.3.2 Effect of TiO_2 NPs with Respect to Growth Stage

A detailed review of the literature shows that various experiments have been carried out on seedlings, in *Petri* dishes, on complete plant life cycles and at certain growth phases with hydroponically grown plants. These experiments had a period of research ranging from a week to approximately 2 to 3 months; before the plant began to flower or to develop grain or fruit. However, there are limited studies spanning across the whole generation time.

In most of the research, exposure to nanoparticles was through roots and leaves (foliar application), Wu *et al.* in 2017 observed TiO_2 NPs at 100, 200 and 500 mg/L in rice over a span of two weeks. They noticed a decline in the dry weight of the roots and shoots, as well as a disturbance of the plant's metabolic activities. They also noticed a dose-dependent rise in TiO_2 NP in roots and shoots.

Similarly, a study performed on lettuce with concentrations of 0.01, 0.1 and 1 g/kg found that TiO_2 NPs induced a loss of phosphorus, calcium and iron intake and also accumulated on the root surface (Larue *et al*., 2016).

2.4 TiO2 NPs and Phytotoxicity

As described earlier, most NPs have a dual effect on plants, both beneficial and hazardous. The key explanation for this is a combination of all three factors: plant species (NPs behave differently depending on each species), growth media (*Petri* dishes, hydroponic or soil) and NP properties (size and shape and coating). Also, in the case of metal and metal oxide nanoparticles, the inherent toxicity of metals also plays a key role in its activity.

2.4.1 TiO2 NPs Induced ROS and Lipid Peroxidation

Reactive Oxygen Species (ROS) is a common term for reactive ions formed due to incomplete reduction of Oxygen (O_2). ROS found in plants contains "Hydrogen peroxide" (H_2O_2), superoxide radicals / anion (O_2^-), hydroxyl radicals (-OH) and singlet oxygen ($1O_2$) (Gechev *et al*., 2006). The reduction of oxygen from the ground to the superoxide radical requires energy and the generation of "univalent intermediates" (Ślesak *et al*., 2007).

1. $O_2 + 1e \longrightarrow O_2^{-\cdot}$

As this extra electron is in its unpaired state, it makes the superoxide (a free radical which is highly unstable). It can either return to an oxygen molecule or react with another proton to produce H_2O_2. The enzyme Superoxide Dismutase (SOD) catalyzes this reaction (Ślesak *et al.*, 2007).

2. $2O_2^{-\cdot} + 2H^+ \longrightarrow H_2O_2 + O_2$

These O_2 derivatives have a powerful oxidizing potential which leads to damaging effects on plant systems such as DNA damage, lipid and protein oxidation, and membrane damage and electrolyte leakage resulting in cell death (Meriga *et al.*, 2004; Sharma *et al.*, 2012). ROS is not only produced because of stress, but natural functional metabolism may also produce ROS (Van Breusegem *et al.*, 2001). The imbalance in ROS development and scavenging induces oxidative stress and literature indicates that metal-centered nanoparticles promote oxidative stress in various plant species. The radical OH, which even the plants own as H_2O_2, is likely to transform to a more reactive enzymatic system, cannot be detoxified.

Because of its unpaired electron, it is likely to react with molecules and causes cell injuries such as lipid membrane peroxidation, degradation of active sites and membranes proving lethal to the cell, and ultimately affects plant's health (Ma *et al.*, 2015). Documented literature supports the fact that exposure to metal nanoparticles demonstrates a linear association between ROS generation and lipid peroxidation (Ma *et al.*, 2015).

Several studies acknowledge the fact that TiO_2 NPs possess genotoxic properties: with a dosage range of 0.01, 0.1 and 1 mg/L TiO_2 NPs, a high amount of DNA damage was found in onions only after 18 h of exposure (Demir *et al.,* 2014). A 2008 study by Lin and Xing showed a particle size-dependent production of ROS and lipid peroxidation on the roots of the rye grass cell membrane surface. In addition, exposure of TiO_2 NPs (0.0125, 0.025, 0.05 and 0.1 mg/L) to onion roots demonstrated oxidative stress and cell degeneration even at the lowest concentration (Pakrashi *et al.,* 2014).

2.5 TiO_2 NPs Characteristics Affecting their Behavior with Plants

2.5.1 Particle Size

Plant cells have a cell wall having small pores with a very minute diameter (5 to 30 nm) that helps to protect the cells from large pollutants, but TiO_2 NPs with a size of less than 30 nm can easily reach the plant cells via these pores (Auffan *et al.,* 2009). The size of a nanoparticle is therefore a key feature that affects the biological systems of plants. The literature claimed that the TiO_2 NPs were able to reduce the size of the root pore, the flow of water and the capacity of the roots to absorb water (Asli *et al.,* 2009). A study on wheat found that 140 nm TiO_2 NPs were the threshold above that no accumulation in plants was seen. They found in the same study that smaller TiO_2 NPs of size ranging from 12, 22 to 25 nm were transported from plant roots to leaves and particle size 36 nm accumulated in the stem (Larue *et al.,* 2012a). In another study, particles above 30 nm have also been identified in root cells, indicating that TiO_2 NPs are capable of expanding or developing new cell pores (Larue *et al.,* 2012b). In addition, studies have already confirmed that TiO_2 particles of bulk size do not show the same effects as TiO_2 NPs (Feizi *et al.,* 2012). Apart from a few studies cited above, there exists a knowledge gap to further understand the effect of NPs size and its effect as a functions of plant interaction.

2.5.2 Crystal Phase

TiO_2 NPs have three crystal phases i.e., anatase, rutile, and brookite. Each of them interacts differently with plant systems. Several studies rectify the fact that the crystal phase plays an important role in the actions and behaviour of NPs. Anatase is TiO_2's most stable phase and is likely to cause more damage over the advantages to plant systems. Studies report that anatase TiO_2 has damaged the antioxidant system in duckweed and tomatoes (Song *et al.*, 2012; Song *et al.*, 2013).

Likewise, rutile phase impacted the photosynthetic processes including the exchange of gases from chloroplasts along with the production of chlorophyll in spinach (Hong *et al.*, 2005a). Studies also showed a difference in uptake and movement in plant systems: a study by Cai *et al.* in 2017 showed that anatase transferred readily in rice roots but rutile did not. Another study on cucumber plants reported that the plant 's preferred uptake was a mixture of rutile and anatase rather than just rutile (Servin *et al.*, 2012). Studies on the brookite phase of TiO_2 NPs have not been undertaken due to its narrow range of applications.

Plants generally have a diverse response to the TiO_2 NP phases. This is due to the fact that the crystal phase causes changes in properties such as stability, cell volume, surface charge, and energy band gaps (Tan *et al.*, 2018). To sum up, the literature shows that anatase is more toxic than rutile, because rutile forms large agglomerates that reduce its uptake and plant interactions (Clément *et al.*,2013).

2.5.3 Doping of TiO2 NPs

Nanoparticles are typically coated with either organic or inorganic complexes for improvement of optical properties and less aggregate formation. The form of surface coating influences their solubility and toxicity. In addition, the coating of nanoparticles induces changes in their surface charge and the area affecting their interactions with soil and plant systems (Tan *et al.,* 2017). Various studies have shown that coated TiO_2 NPs exhibit higher toxicity as they move easily through plant organs and have better access to plant cells for interactions (Foltête *et al.,* 2011; Wang *et al.,* 2011). In contrary, a study by Singh *et al.* in 2016 showed an increased rate of seed germination in tomatoes and lentils. Studies have shown that the TiO_2 NP coating with a small amount of metal improves their optical properties (Chen *et al.,* 2007). However, further research is required to provide the conclusive evidence of coating NPs and its effects on plant system.

2.6 Effects of Soil Characteristics on TiO2 NPs Toxicity

Soil is the complex matrix and adding soil to the nanoparticle-plant mix creates different interactions due to the impact of soil characteristics on NP activity, how their behavioral changes affect plant and soil systems and plant and soil microorganism's interaction. The soil properties that impact the performance of TiO_2 NP include pH, soil organic matter, particle size and soil texture (Peralta-Videa *et al.,* 2011).

The figure 2.3 shows the soil parameters that affect the TiO_2 NPs, which typically have a cumulative effect rather than as individuals. They influence the surface charge of TiO_2 NPs, also known as "zeta potential" and the formation of agglomerates (Pachapur *et al.,* 2016).

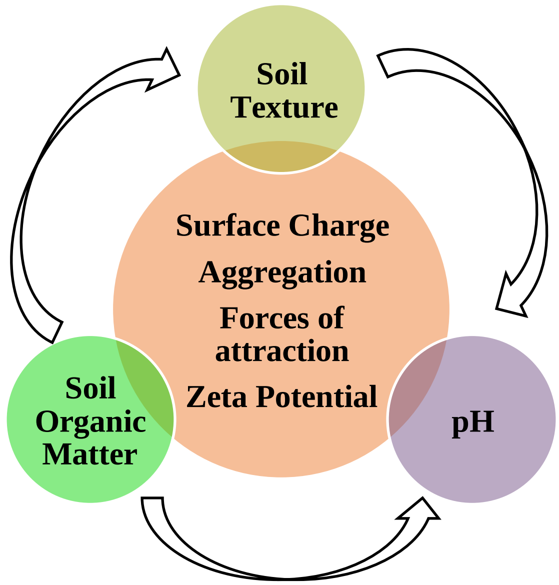


Figure 2.3: Soil properties affecting TiO_2 NPs behavior (Parameters outside are the factors of soil affecting TiO_2 NPs properties)

2.7 Related Research Work at IESE, NUST

An experiment was conducted to assess the 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. The concentration of phytoavailable phosphorus in soil without plant culture and with lettuce culture was analyzed in experimental levels. In soil without plant culture, phytoavailability of 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 phytoavailability of phosphorus to *Lactuca sativa* in response to soil applied TiO_2 nanoparticles showed that shoot and root lengths of lettuce was increased up to 49% and 62% respectively at 100mg/kg of soil applied TiO_2 nanoparticles as compared to the control group (Hanif *et al*.,2015).

Furthermore, a study on physiological effects of nanoparticles on different plants in response to phosphorus bioavailability showed that root and shoot lengths of lettuce was increased by 35.3% and 39.2% respectively, total fresh and dry biomass was increased by 46% and 52% respectively, chlorophyll content was increased by 68% and 40% was H_2O_2 generation at 100mg/kg TiO_2 NPs in soil (Rafia Rafique, 2014).

In addition to this, another study was performed on metallic nanoparticles (TiO_2 and Fe_3O_4) to modify rhizosphere phosphorus availability and uptake by *Lactuca sativa* which depicted that shoot length showed maximum values when treated with TiO_2 nanoparticles (200 mg/kg). This resulted into 49% increase in shoot growth by TiO_2 nanoparticles. In case of root length, maximum values were observed at 250mg/kg TiO_2 NPs. Similarly, the shoot and root dry weight increased by1.2-fold at 250 mg/kg in TiO_2 NPs treated groups as compared to the untreated one. The total biomass of *Lactuca sativa* was significantly ($p \leq 0.05$) increased up to 1.4-fold of the control at the highest concentration (250 mg/kg) of both NPs applied (Zahra *et al*.,2015).

MATERIALS AND METHODS

This chapter describes the experimental design adopted for the present study. In the present study, lettuce and tomato were exposed to TiO_2 nanoparticles at 100mg/kg and TiO_2 bulk at the same dosage i.e., 100mg/kg as soil amendment. Experiments were carried out at Institute of Environmental Sciences and Engineering, National University of Sciences and Technology, Islamabad, Pakistan to assess the effects of Titania nanoparticles and Titania anatase (bulk) in soil and plants. Furthermore, toxicity assessment tests were also performed to determine the harmful effect of given concentrations in both plant species.

So, the first phase of this study is focused on the effects of applied treatments on plant growth parameters i.e., physicochemical parameters of plants and comparison of tomato and lettuce plants. The second phase is focused on plant toxicity at the recommended dosages of both treatments. According to the objectives of the present study, the following methodology was adopted and discussed here in detail accordingly.

3.1 Preparation of Titania Nanoparticles

3.1.1 Synthesis of Titania Nanoparticles by Liquid Impregnation Method

For application in soil, Titania nanoparticles were synthesized using liquid impregnation (LI) method. For this purpose, 50g of Titania powder (General Purpose Reagent, Purity >99%) was added in 300mL of distilled water. The solution was allowed to stir for 24hr at 325rpm on a magnetic stirrer (STAURT SB 162) to obtain a homogenous solution. The solution was then allowed to settle for another 24hr. The slurry obtained after settling, was placed in hot air oven at 105^{o}C for 24hr till drying. Dried slurry was crushed by using mortar and pestle. The fine powder was then placed in muffle furnace (NEY-525 SERIES

II) for calcination at 550^{o}C for 6hr. And finally, the resultant fine powder was allowed to cool down at room temperature showing clear crystalline form of Titania nanoparticles (Fan *et al*.,2011).

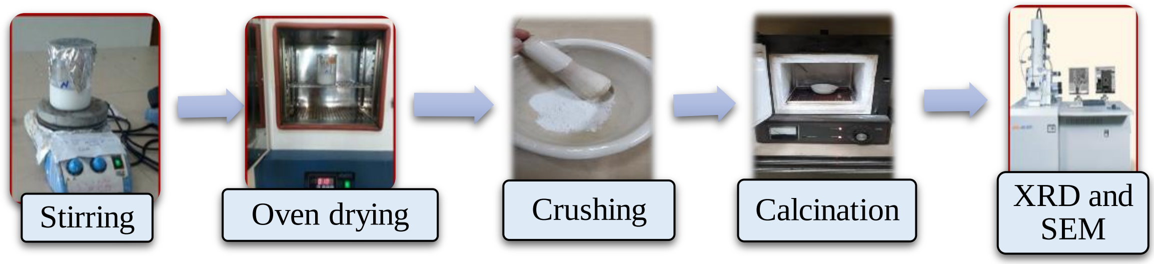


Figure 3.1: Synthesis of Titania NP by Liquid Impregnation Method.

3.2 Characterization of Titania Nanoparticles

3.2.1 Scanning Electron Microscopy (SEM)

The surface morphology and particle size of Titania nanoparticles were analysed using SEM (JSM-6490A, JEOL) with a 20KV accelerating voltage. This technique was used for the direct observation of morphology, topography and particle size of powdered samples at different magnitudes and resolution.

3.2.2 X-ray Diffraction:

The crystalline structure and size were determined using X-ray Diffraction (XRD, JEOL JDX-II). The average crystalline size of TiO_2 nanoparticles and TiO_2 bulk was determined using Scherer formula i.e., L 1/4 K1=b cos q (Danish *et al*., 2013). Here, L ¼ is average particle size, K ¼ is 0.891;1 ¼ is 0.1542, b ¼ is full width of a diffraction line at half of maximum intensity (FWHM) radian and q is ¼ of the diffraction angle of crystal phase. The XRD pattern of TiO_2 bulk and TiO_2 nanoparticles was attained using X-ray Diffractometer (Theta-Theta STOE, Germany) with Cu Kα radiation. Scan range was 20^{o}-

80^{o} (2θ; λ=0.154) with a step of 0.5^{o} at 40mA and 40kV. Analysis of XRD results was done using Origin software (Origin 2019b Graphing and Analysis).

3.3 Preliminary Soil Analysis for Pot Experiment:

The soil parameters that were analyzed prior to pot experiment include soil texture, soil pH, soil moisture content, nitrate-nitrogen, extractable and total phosphorus and total organic carbon.

3.3.1 Soil Texture:

Soil texture was determined using saturation percentage method (Malik *et al*.,1984). The USDA textural triangle was used to assign a textural class to the soil. For this purpose, 100g of air-dried soil was taken into 100mL glass beaker. Distilled water was then added gradually and mixed until saturated paste was obtained.

$$\text{Saturation Percentage (\%)} = \frac{\text{Weight of water to saturate dry soil sample}}{\text{Weight of dried soil}} \times 100$$

Table 3.1: Soil texture on the basis of saturation percentage:

Saturation Percentage (%)	Soil Texture
0-20	Sand or loamy sand
20-35	Sandy loam
35-50	Loam or silt loam
50-65	Clay loam
65-80	Clay
>81	Organic soil

3.3.2 Soil pH:

Soil pH was determined to assess the suitability of the soil for plant growth. The soil: water (1:5) suspension was prepared. For this purpose, 10g of air-dried soil was added in 100ml glass beaker and 50 ml of distilled water was added with the help of a graduated cylinder. Then the resulting mixture was stirred well by using a mechanical shaker at 180rpm for 30min. The pH of the suspension was then measured by using a combined electrode (HI2211 pH Meter/HANNA Instruments). The pH reading of each replicate was taken after 30 sec (McLean,1982).

3.3.3 Water Holding Capacity:

For determination of water holding capacity, 100mg of air-dried soil was taken. Funnels were taken and attached to ring stand. Placed the filter paper in funnel and filled the funnel with 100mL of sample. Then, 100mL of water was taken in graduated cylinder and added gradually to soil sample until its covered. Recorded the water added until the sample became saturated. After this, released the clamp and collected the excess water in graduated cylinder. Finally calculated the water retained in 100ml of soil sample and calculated the water holding capacity by using following formula:

$$\frac{Water\ retained\ (mL)}{100mL\ soil\ sample} = Water\ added(mL) - Water\ drained(mL)$$

$$Water\ Holding\ Capacity\ (\frac{mL}{L}) = \frac{Water\ retained\ (mL)}{100mL\ soil\ sample} \times 10$$

3.3.4 Soil Moisture Content:

For determination of soil moisture content, 10g of air-dried soil was taken in a *Petri* dish. It was dried in hot air oven at 105^{o}C for 24hr with the lid unfitted. After this, *Petri* dish was removed from hot air oven and cooled in a desiccator for 30min and re weighed. Following relation was used to calculate the moisture content of soil.

$$\% \text{ Moisture in soil} = \frac{\text{Wet soil - Dry soil}}{\text{Dry soil}} \times 100$$

3.3.5 Soil Organic Matter:

Soil organic matter/total organic carbon was determined using Walkley-Black method (Methods of soil, plants and water analysis, ICARDA) This was calculated before and after the application of treatments. For this purpose, 1g of soil was taken in 500ml glass beaker. then 10ml of 1N potassium dichromate ($K_2Cr_2O_7$) and 20ml of concentrated sulfuric acid (H_2SO_4) was added to the soil. The beaker was swirled to completely incorporate the soil with reagents and allowed the mixture to rest for 30min. After 30min, 200ml of distilled water was added along with 10ml of concentrated H_3PO_4. Furthermore, 10 drops of diphenylamine indicator were also added, and the beaker was placed on a magnetic stirrer. Finally, the mixture was titrated with 0.5M ferrous ammonium sulfate solution until the color changed from violet-blue to green. Two blanks were also prepared, containing all the reagents but no soil. The following relations were used to calculate organic matter in the soil.

$$M = 10/V_{blank}$$

$$\text{Oxidizable Organic Carbon (\%)} = \frac{[V_{blank} - V_{sample}] \text{ X } 0.3 \text{ X } M}{\text{Weight of air-dry soil(g)}}$$

$$\text{Organic Matter (\%)} = 1.724 \text{ X Total Organic Carbon \%}$$

3.3.6 Preparation of Soil for Pot Experiment:

The soil was prepared prior to pot experimentation. For this purpose, the soil was spread out on newspapers and air-dried for two weeks. The air-dried soil was grounded manually into fine particles using pestle and mortar. The soil was then sieved manually using <2mm sieve to remove larger particles, gravel, shoots and roots. Plastic pots of diameter 9cm and height 10cm were used for experiment. The processed and fine soil was used for the experiment so that our treatments can easily merge with all the soil particles. For every pot, 500g of soil was weighed by using weighing balance and weighed soil was added to each pot.

3.3.7 Preparation and Application of Fertilizer in the Soil:

The fertilizers were prepared by making suspensions of NPK (nitrogen, phosphorus and potassium) in distilled water and then 10ml was taken from stock solution and added to each pot. The recommended dosages of fertilizers were selected for both the plants. For lettuce, the recommended dosage of NPK was 100kg/ha, 50kg/ha and 90kg/ha respectively. For tomato, the recommended dosage of NPK was 100kg/ha, 90kg/ha and 60kg/ha respectively. The calculations were done accordingly to find out the exact amount of fertilizers for 500g soil. Urea was used as a source of nitrogen. For phosphorus, DAP (Di Ammonium Phosphate) was used and for potassium, KCl was used. After application of desired amount of fertilizer, the soil was mixed so that the fertilizer can merge and come to contact with every particle of soil.

Figure 3.2: Preparation of fertilizers for tomato and lettuce

3.3.8 Application of TiO_2 NP and TiO_2(GPR) in the Soil:

For soil application, recommended doses of TiO_2 NP (Hanif *et al.*,2015 and Haghighi *et al.*,2014) were selected and for comparison, the same doses of TiO_2 GPR were selected for both the plants. So, for lettuce, 100mg/kg TiO_2 was selected and 100mg/kg TiO_2 GPR was selected for comparison. For tomato, 100mg/kg for selected and 100mg/kg of TiO_2 GPR was used for comparison. Calculations were done accordingly to get the exact amount for 500g soil. For preparation, the recommended quantities of TiO_2 NP and TiO_2 GPR were added to distilled water after weighing using weighing balance. Suspensions of these two treatments were prepared using an ultra-sonicator (JAC Ultrasonic 1505) for 40min. The recommended doses of treatments were then applied to soil in each pot and mixed vigorously.

3.3.9 Plant Cultivation:

For the present experiment, seedlings of lettuce and tomato were bought from a local nursery in H-9, Islamabad. The age of plants was 20 days at the time of buying. Prior to pot experiment, the roots of both the plants were washed carefully with distilled water to make sure surface clarity. The seedlings were then shifted to the pots (one plant per pot) containing treatments and fertilizer as soil amendments. Five replicates were prepared for each treatment. The plants were monitored on daily basis and watered twice a week for 70 days of exposure. The pots were kept in greenhouse at IESE, NUST.

3.4 Morphological Parameters of Plants:

After harvesting, roots and shoots were washed with distilled water to ensure surface clarity and collected separately in zip lock bags. The pH of soil was also measured immediately after harvesting.

3.4.1 Shoot length Measurement:

Shoot length of lettuce and tomato were measured on weekly basis for a period of 70 days. Shoot height was measured using measuring tape. And the final measurement was taken after harvesting of both the plants.

3.4.2 Root length Measurement:

After harvesting, root length of lettuce and tomato was measured. The root lengths were measured by using measuring tape.

3.4.3 Plant's Fresh Biomass Determination:

After harvesting, roots and shoots of both plants were cut and their fresh biomass was weighed one by one by using weighing balance. The fresh biomass was recorded, and some samples were kept in zip lock bags in an ultra-freezer for further analysis.

3.4.4 Plant's Dry Biomass Determination:

After recording the fresh biomass, roots and shoots of both the plants were kept in oven at 70^{o}C for 48hr. After 48hr the dry biomass was recorded by weighing the dried plant material on weighing balance.

3.5 SEM Analysis of Plant Material for Uptake of TiO_2 NP and TiO_2 GPR:

Scanning electron microscopy (SEM) was used to determine the presence/uptake of TiO_2 NP and TiO_2 GPR in tomato and lettuce plants. For this purpose, samples were prepared prior to scanning. Shoots of both the plants were cut and washed separately with distilled water to remove soil and electrolytes from plant surface. The shoots were kept in petri dishes and placed in oven at 70^{o}C for 48hr to remove the moisture. The oven-dried plant material was then grounded into fine pieces and stored in Eppendorf tubes. Sputtering technique was used to coat fine pieces of plant material to avoid charge effects during SEM analysis at IST (Institute of Space Technology). The SEM coupled with EDS was used to analyse the elemental composition of plant samples and EDS spectra was generated to clearly show the elements in given samples.

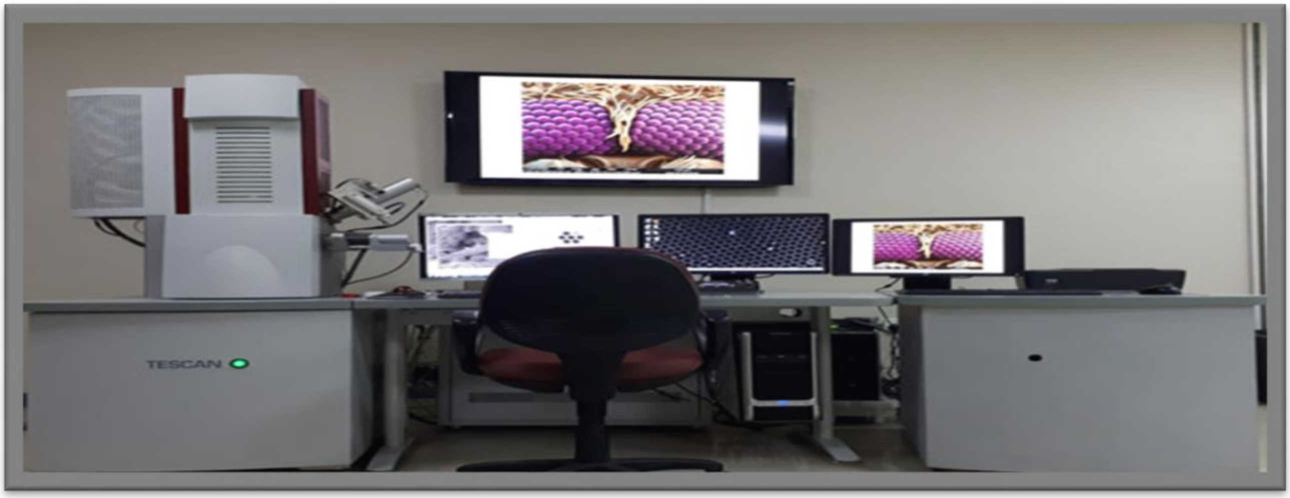


Figure 3.3: SEM coupled with EDS to show uptake of TiO_2 GPR and TiO_2 NP in tomato and lettuce shoots

3.6 Plant Toxicity Assays:

3.6.1 Lipid Peroxidation and Membrane Integrity Index:

Plants, under stress conditions, leads to the production and accumulation of ROS. ROS further leads to lipid peroxidation and membrane damage. Stress or any kind of contamination has more effect on plant roots as they are more sensitive than any other plant part (Das *et al*.,2017).

Procedure:

TBARS (Thiobarbituric Acid Reactive Substances) was used to determine the damage caused by metal exposure to the membrane lipids of plant sample by following the method used by de Oliveria *et al*. (2017). So, for this purpose, frozen plant material (tomato and lettuce) was cut into small pieces and homogenized by using 1.5ml of 5%TCA in freezing pestle and mortar placed in an ice bath. After homogenization, the homogenate was transferred to centrifuge tubes. The centrifuge was run at 10,000g for 10min at 25^{o}C. 1ml of supernatant was taken and 1ml of 20%(w/v) TCA containing 0.5% (w/v) TBA was added in supernatant. The mixture was heated for 30min at 95°C in water bath and then cooled down in the ice. The absorbance was then measured at 532nm and 600nm and values were subtracted. TBARS was calculated by using Beer-Lambert law with an extinction coefficient of 155µmol/g FW (fresh weight).

3.7 Statistical Analysis:

The statistical significance of results was calculated by Student's t-test (mean analysis) with ±standard deviation. For each data set, statistical significance was confirmed by applying one-way Analysis of Variance (ANOVA) with the Honestly Significant Difference (HSD) Tukey test. Effects with probability of less than 0.05 are referred to as significant.

RESULTS AND DISCUSSION

4.1 Characterization of TiO_2 GPR and TiO_2 NPs

4.1.1 X-ray Diffraction of Titania GPR and Titania Nanoparticles

The crystalline size and phase composition of the TiO_2 GPR and prepared Titania nanoparticles were determined using XRD analysis as shown in Figure 4.1. The spectrum shows that the TiO_2 GPR and TiO_2 nanoparticles were crystalline and that no amorphous phase was detected. Solid diffraction peaks at 2θ (101), confirm that the TiO_2 GPR and synthesized Titania nanoparticles were in the anatase phase. The favored crystal orientation was in the 101 plane (Vijayalakshmi *et al.,* 2012). Debye-Scherrer 's formula was used to measure the crystallite size of TiO_2 GPR and TiO_2 NPs which was found to be 33.6nm and 33.4nm respectively.

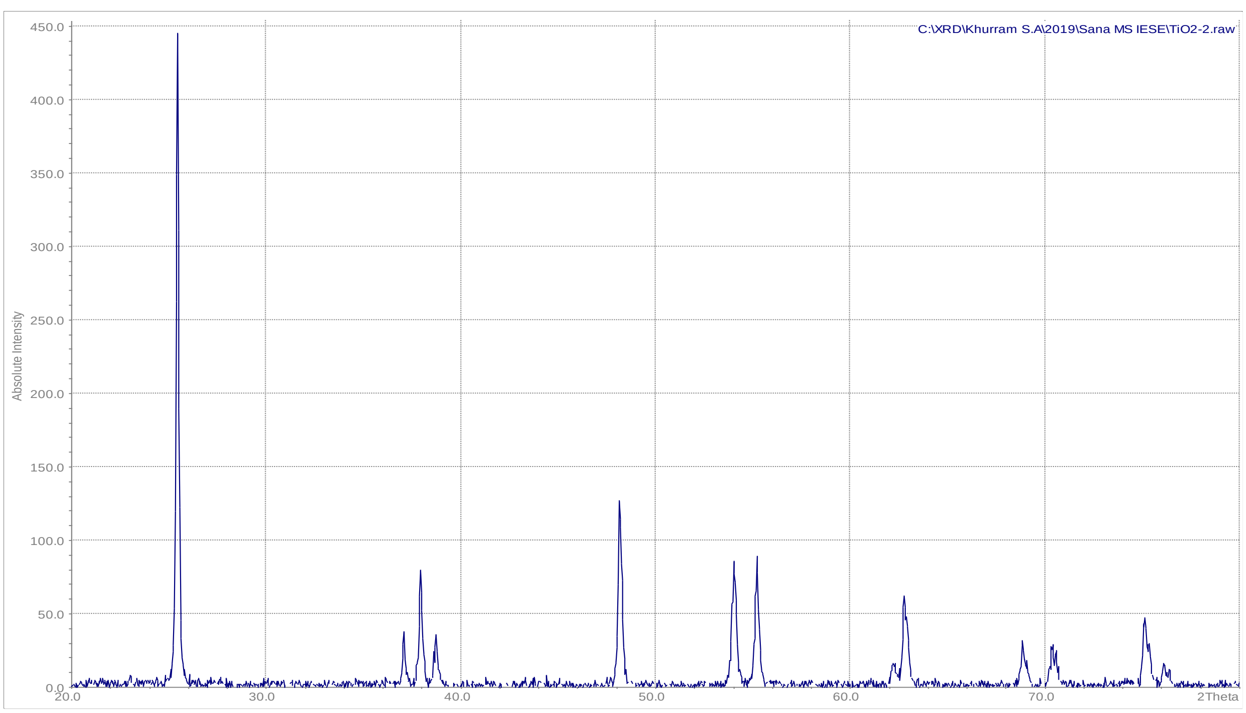


Figure 4.1: Phase identification of synthesized TiO_2 GPR through XRD

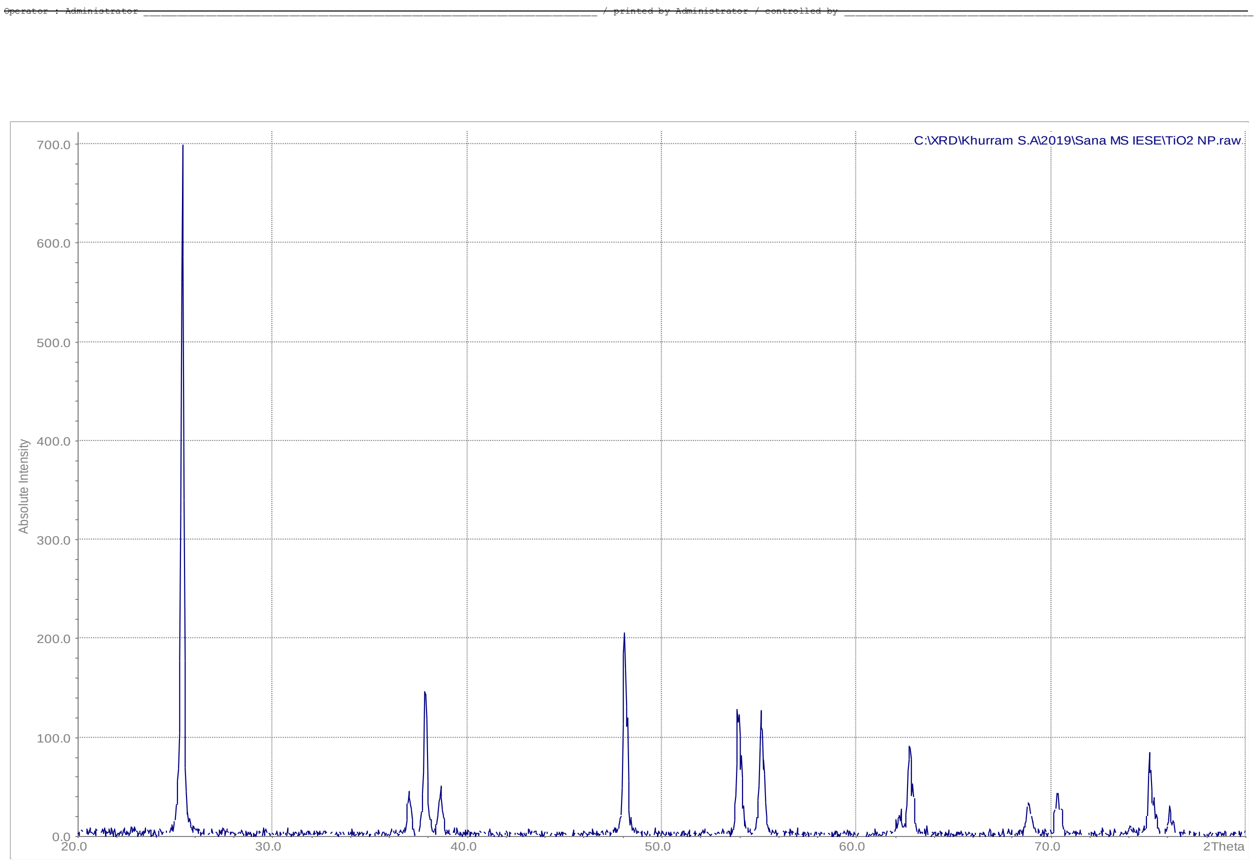
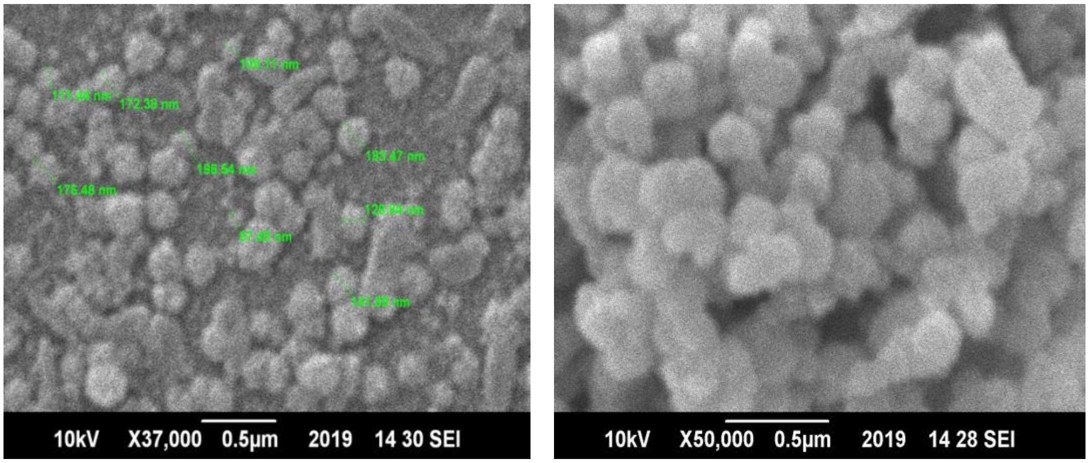


Figure 4.2: Phase identification of synthesized TiO_2 NPs through XRD

4.1.2 SEM Imaging of TiO_2 GPR and TiO_2 NPs

The surface morphology of Titania nanoparticles was examined by using SEM. The 10k X magnification picture shows pure Titania particles, indicating that the particles are spherically shaped (Figure 4.2). As the nanoparticles have zero dimensionality, it aids in increasing its specific surface area thereby increasing adsorption sites of ions (Bhatia, 2016).



a **b**

Figure 4.3: Morphological characterization of TiO_2 GPR (a) and synthesized TiO_2 NPs (b) respectively through Scanning Electron Microscope (SEM)

4.2 Preliminary Characterization of Experimental Soil

Table 4.1 shows the results for some physical and chemical characteristics of the experimental soil. Prior to the pot experiment, various soil tests were conducted, including pH, electrical conductivity, total organic carbon, moisture content, water holding capacity. The soil texture used for this experiment was a silt loam with a pH of 7.25. According to various literatures, sandy and silty loam soils are best for the growth of tomato and lettuce plants (Dou *et al*., 2016). Soil pH levels play a major role in the supply of plant nutrients, thus it should be taken into consideration to get a high plant yield. Total organic carbon was 0.95% in silt-loam soil.

The soil texture plays a crucial role in the development of tomatoes and lettuce in terms of its water holding capacity or "available water capacity" (AWC). The available water holding capacity of the soil is related to its organic matter. Sandy and silty are low in organic matter, hence their available water capacity is low (Dou *et al*., 2016).

The soil texture also influences the overall root growth of the plant. Larger roots usually have more ability to elongate, so they can provide better nutrients and water to the plant (Dou *et al*., 2016).

Table 4.1: Physiochemical properties of soil

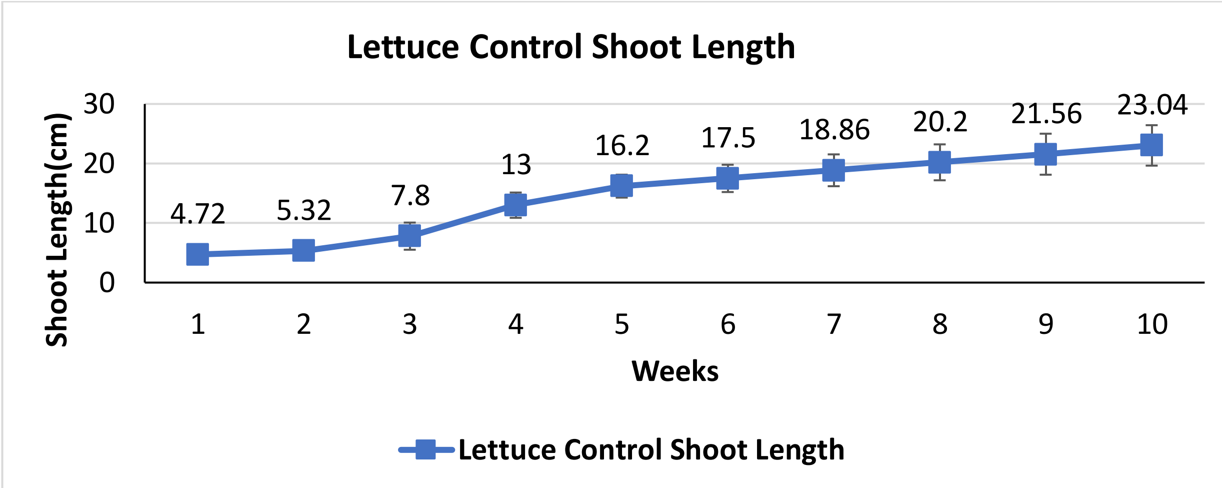
Soil Texture	Silt-loam (Islamabad)
pH	7.25
EC (µS/cm)	253
Moisture Content (%)	2.04
Water Holding Capacity (%)	40.6
Soil Organic Carbon (%)	0.95

4.3 Effect on Growth Parameters

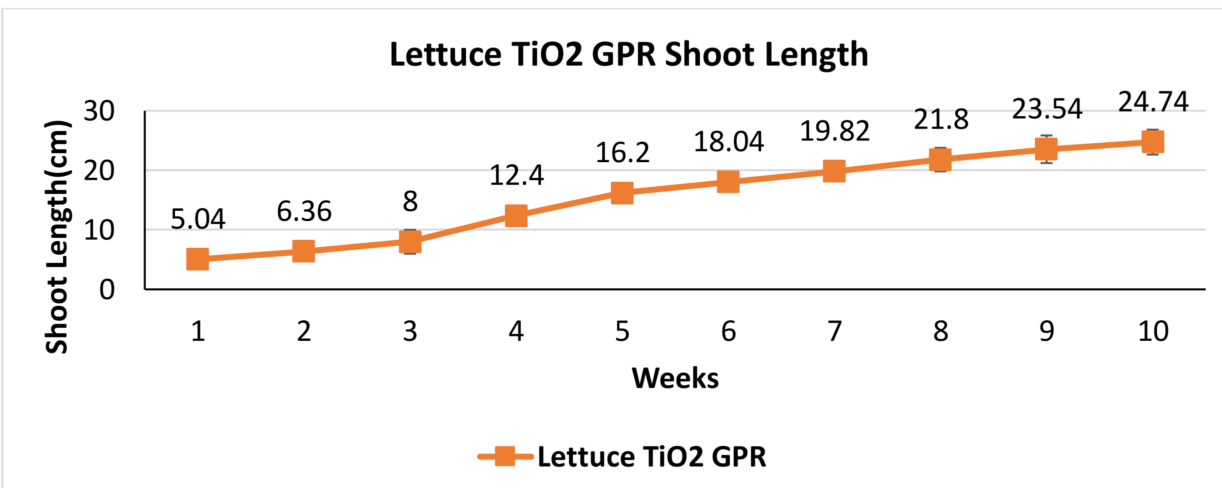
The first phase focused on changes in plant physiological growth parameters under the treatments applied. Root and shoot lengths were recorded along with biomass. The given concentrations of TiO_2 GPR and TiO_2 NPs was 100mg/kg in both the plants.

4.3.1 Root and Shoot Length in lettuce and tomato plant

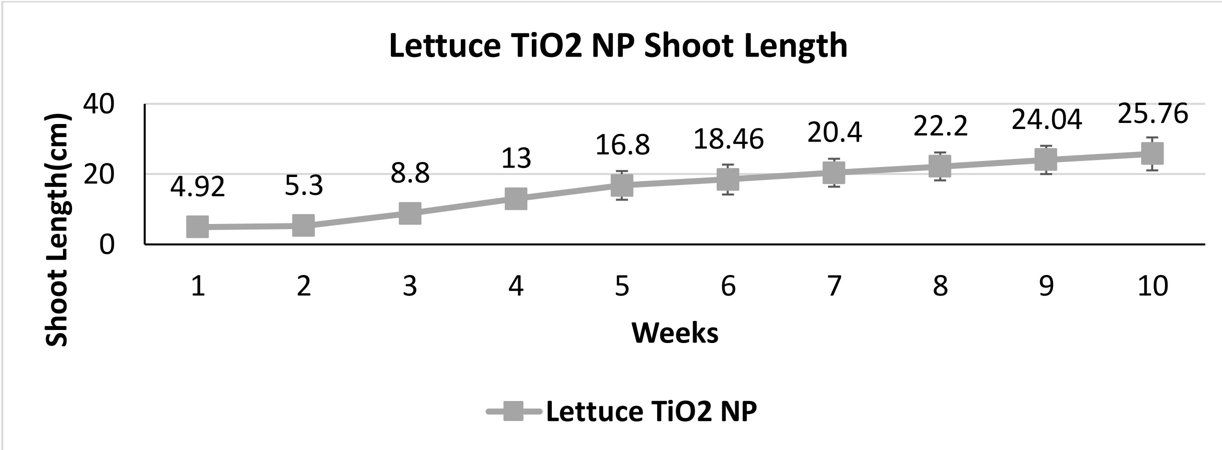
Figures 4.4(a), 4.4(b) and 4.4(c) depict the effect of TiO_2 NPs on plant shoot length for both the treatments in lettuce. Maximum shoot length was observed in soil applied TiO_2 NPs as compared to TiO_2 GPR and control group. The shoot length was increased up to 11.81% in case of TiO_2 NPs and 7.38% in case of TiO_2 GPR applied group.


Lettuce Control Shoot Length
Shoot Length(cm)
30
20
10
0
4.72
5.32
7.8
13
16.2
17.5
18.86
20.2
21.56
23.04
1 2 3 4 5 6 7 8 9 10
Weeks
Lettuce Control Shoot Length

(a)


Lettuce TiO2 GPR Shoot Length
Shoot Length(cm)
30
20
10
0
5.04
6.36
8
12.4
16.2
18.04
19.82
21.8
23.54
24.74
1 2 3 4 5 6 7 8 9 10
Weeks
Lettuce TiO2 GPR

(b)



(c)

Figure 4.4: Shoot lengths of lettuce plant in both the treatment groups along with their values.

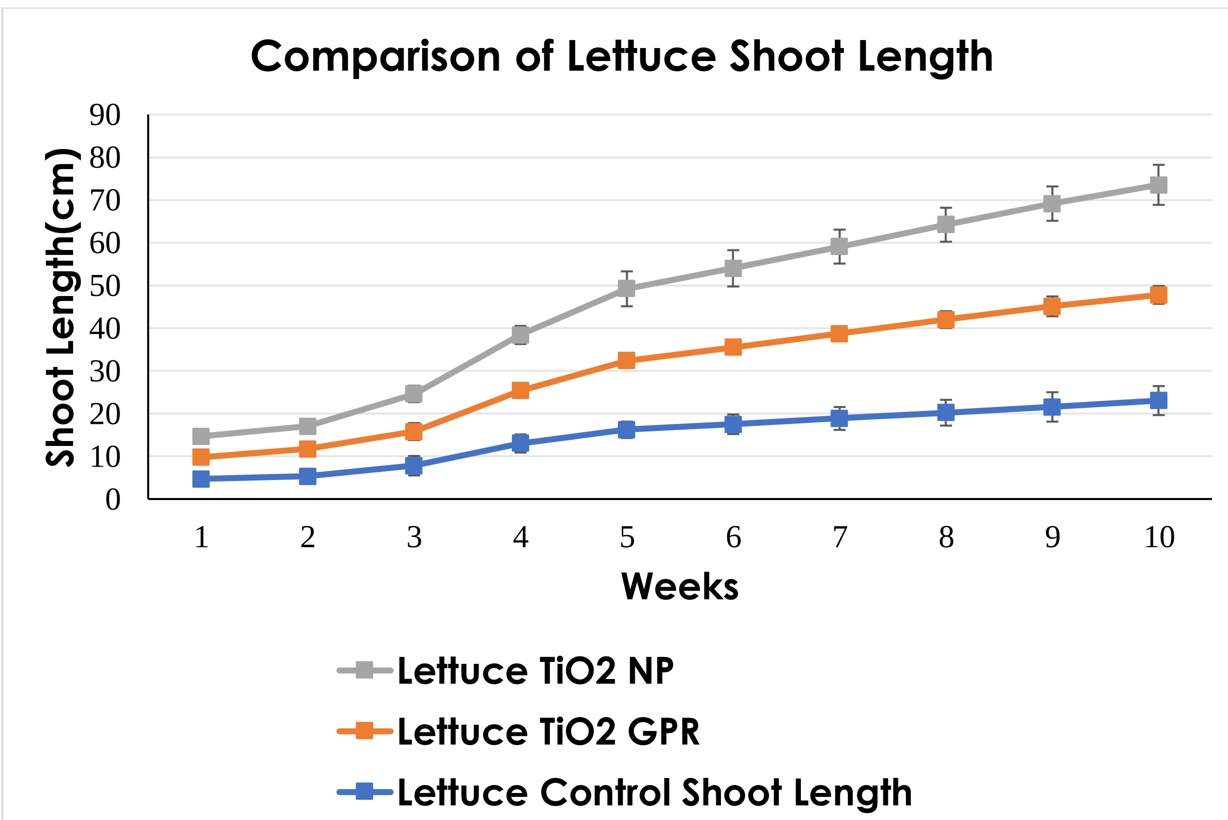


Figure 4.5: Comparison of lettuce shoot length in both the treatments as compared to control.

In lettuce, shoot lengths were increased up to 11.81% and 7.38% on application of TiO_2 NPs and TiO_2 GPR treated groups respectively. Increase in shoot length in both the treatment groups could be attributed to the fact that TiO_2 GPR and TiO_2 NPs promote plant growth by increasing plant light absorption capacity and photo energy transmission (Moaveni and Kheiri, 2011). Reports also indicate that high surface reactivity of TiO_2 NPs might enlarge root pores and in turn, water absorption and nutrients available to plants is improved (Larue *et al*., 2012a).

Figure 4.6 depicts the comparison of lettuce root length in both the treatment plants as compared to the control group.

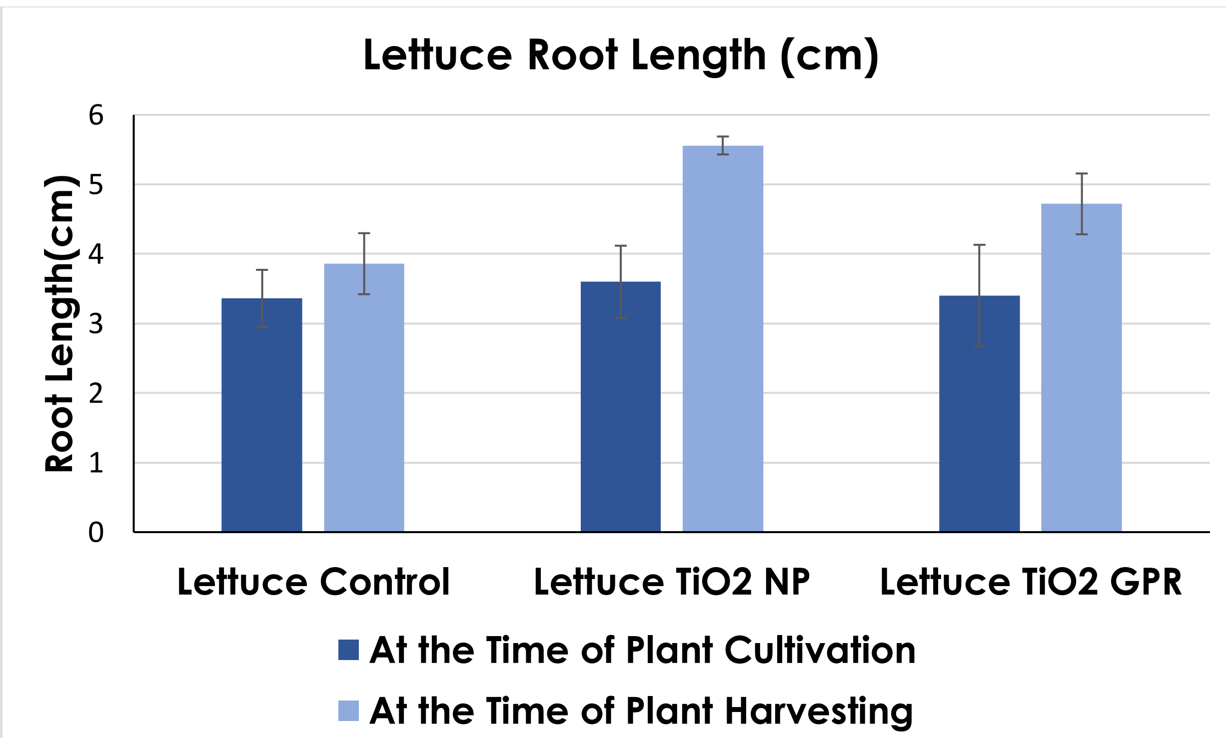


Figure 4.6: Comparison of lettuce root lengths in both the treatment groups as compared to control.

Figure 4.6 clearly shows that root length was increased by 44.4% in soil applied TiO_2 NP treatment group and 22.28% in case of TiO_2 GPR treatment group as compared to the control.

Increase in root length can be attributed to the fact that roots tend to elongate when there is a nutrient deficiency or unavailability in soils (Wissuwa, 2006). Zahra *et al.* (2015), used a concentration of 250 mg/kg of TiO_2 NPs and observed an increased in the growth of *Lactuca sativa* L., root-shoot by 36.0% and 34.6%, individually. However, some studies have also reported negative effects of TiO_2 NPs. A study conducted on wheat with concentrations of 1000 and 2000 mg/L showed a decrease in root and shoot length (Aliabadi *et al*., 2016).

Figure 4.7 shows the effect of both the treatment groups on tomato plant as compared to the control.

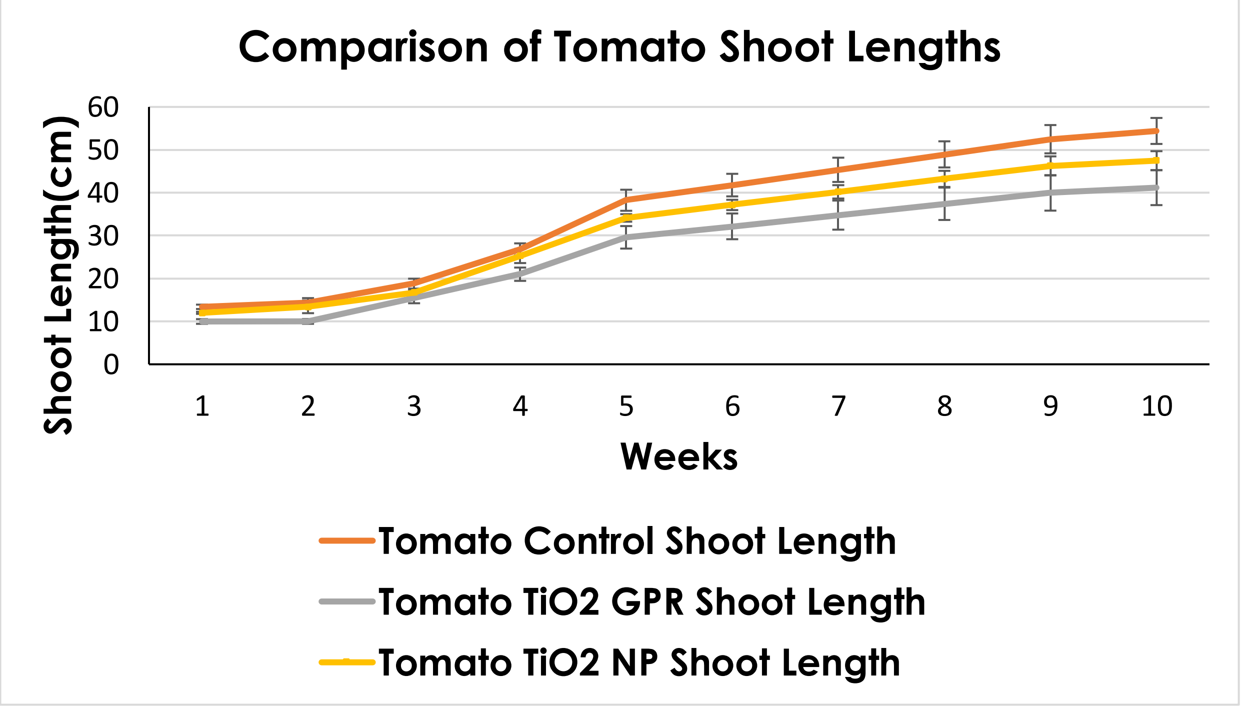


Figure 4.7: Comparison of tomato shoot lengths in both treatment groups as compared to control group.

Figure 4.7 clearly shows that both the treatments affected tomato shoot length in a negative manner. Decreased plant height was observed in the case of tomato plant.

Figure 4.8 shows the comparison of tomato root length after the application of both treatment groups.

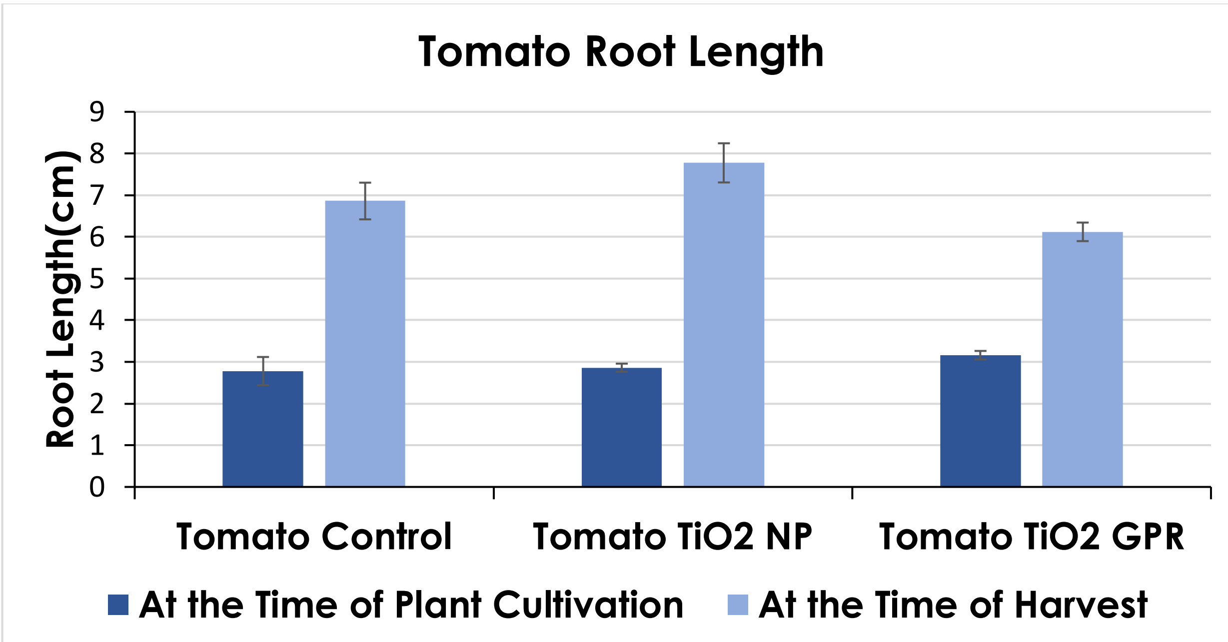


Figure 4.8: Comparison of tomato root length in both treatment groups as compared to control.

The figure clearly shows that there is an increase in root length in case of TiO_2 NP applied treatment group and in case of TiO_2 GPR group there is a clear decrease from the control group.

4.3.2 Fresh and dry Biomass of lettuce and tomato plants

Figures 4.9(a) and (b) show the impact of Titania nanoparticles and Titania GPR on fresh and dry biomass of lettuce and tomato plants, respectively. The results are not in accordance with the trends seen in root and shoot length; root and shoot length showed an increase in case of TiO_2 NP treatment group in lettuce. While fresh biomass was increased up to 12.96% in titania NP amended soil and 27.99% increase was observed in titania GPR amended soil. Whereas dry biomass of lettuce decreased in both the treatments.

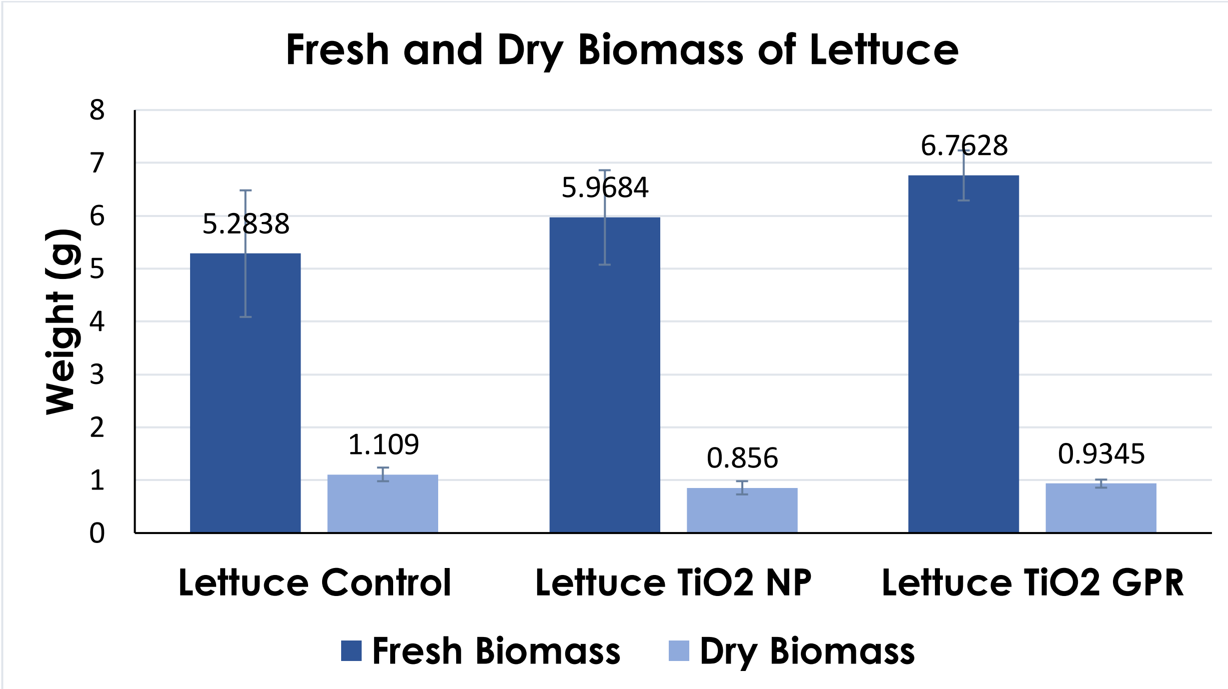


Figure 4.9(a): Comparison of fresh and dry biomass in both the treatment groups applied to lettuce.

Figure 4.9(b) shows the impact of both the treatment groups to depict the fresh and dry biomass of tomato plant.

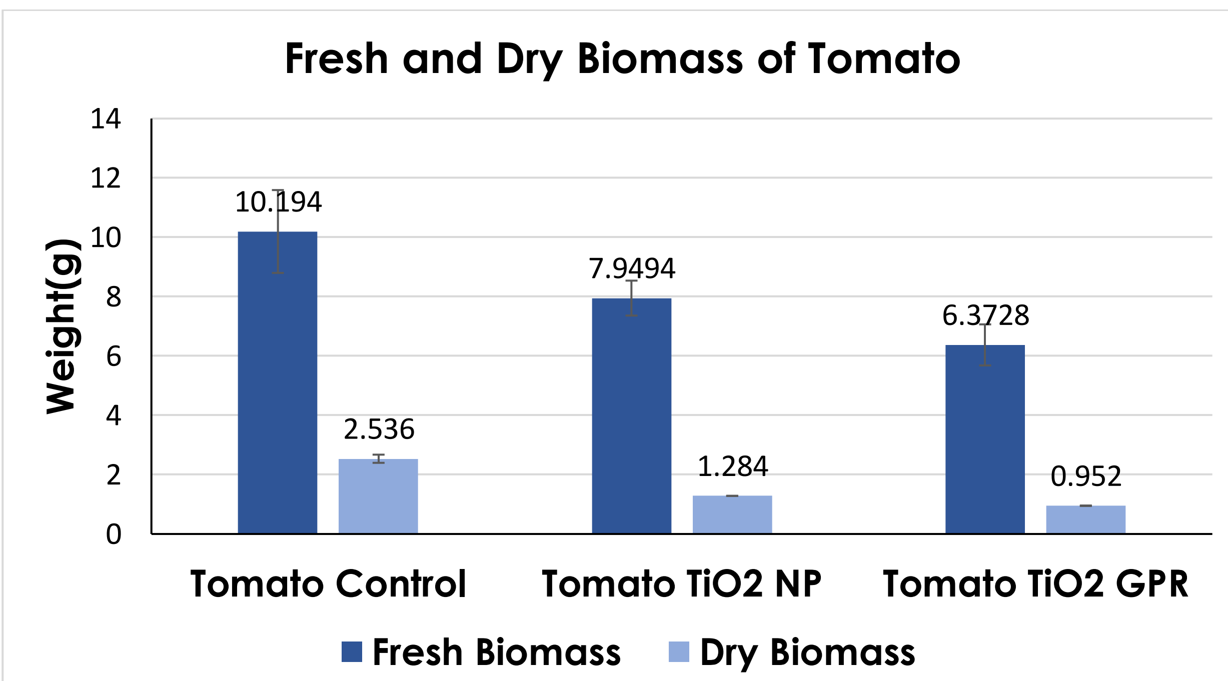


Figure 4.9(b): Fresh and dry biomass of tomato

In case of tomato plant, fresh biomass was decreased up to 22.02% and 37.48% upon application of TiO_2 NP and TiO_2 GPR respectively. Whereas, dry biomass was decreased by 49.37% and 62.46% in case of TiO_2 NP and TiO_2 GPR treatment groups, respectively. For calculation, mean values with the standard error were taken to see the significant difference and to plot graphs. In general, a performance of any nanoparticle is dependent on its environmental conditions or medium. (Song *et al.,* 2013) (Yang *et al.,* 2017). In a study by Zhang *et al.* (2015) the biomass of radish was compared in silty loam (2.21 % SOM) and loamy sand (11.87% SOM), the former had significantly higher root biomass even in the presence of 1000 mg/kg CeO_2 NPs. The results showed that root growth was higher in the loamy sand than silt loam. Similarly, different levels of phytotoxicity caused by CeO_2 NPs were found in lettuce seedlings incubated in potting mix soil (Gui *et al.*, 2015) and sand (Zhang *et al.,* 2017).

4.4 Plant Toxicity Assay

Plant toxicity assay was performed on tomato and lettuce plant tissue to check the effect of Titania nanoparticles and Titania GPR. Toxicity was measured by calculating reactive oxygen species by lipid peroxidation through TBARS (Thiobarbituric Acid Reactive Substances).

4.4.1 Lipid Peroxidation

An obvious indicator of stress in plant systems is the peroxidation of lipids as it is a chief cellular component targeted by reactive oxygen species. High ROS conditions bring the onset of free radicals that react with electrons in the lipid membranes eventually destroying the cell. This starts a chain reaction as unstable "lipid radicals" are formed which react with oxygen. Prolonged cycles can be fatal to cells and overall plant health. A byproduct of lipid peroxidation is Malondialdehyde (MDA) which apart from membrane damage brings an array of damaging effects on cells such as a disruption in ion transport, changes in membrane permeability, and loss of enzymatic activity hence resulting in cell death (Sharma *et al.*, 2012).

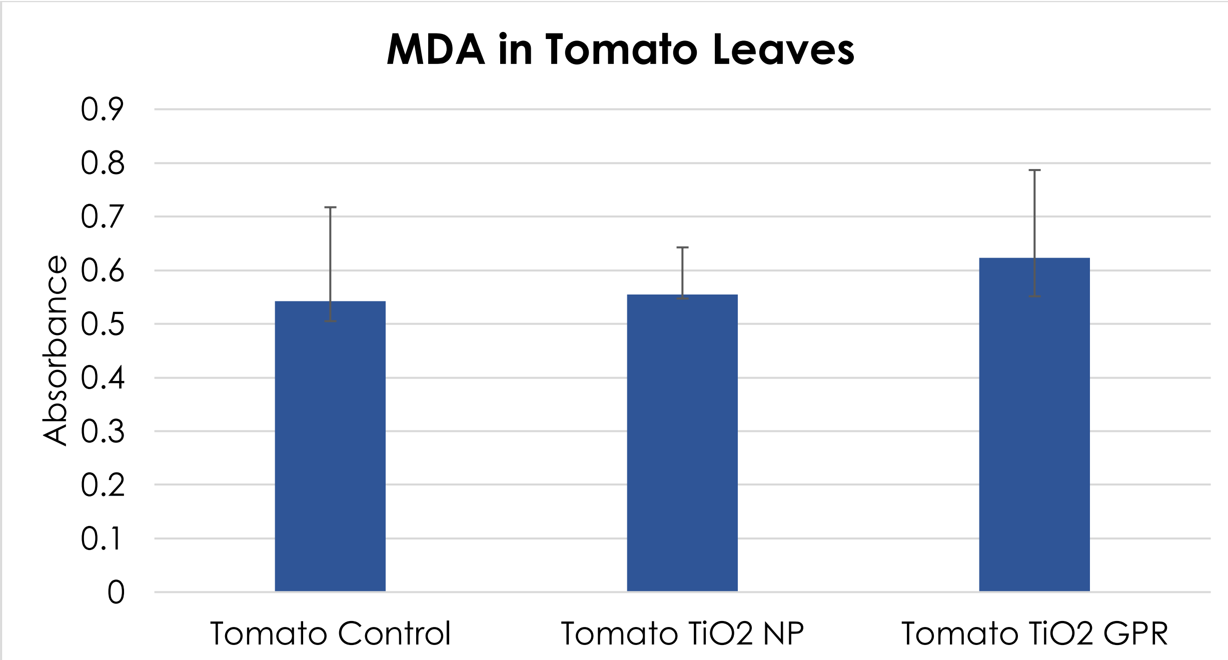


Figure 4.10(a): Lipid peroxidation in tomato treated with TiO_2 NPs and TiO_2 GPR.

The above figure shows lipid peroxidation in tomato shoots treated with TiO_2 NPs and TiO_2 GPR. The highest production of TBARS is seen in TiO_2 GPR treated group. Toxicity showed an increase of 2.4% and 14.94% in case of TiO_2 NP and TiO_2 GPR treatments, respectively as compared to the control group.

A number of studies on lipid peroxidation in conduit with H_2O_2 production have reported a linear relationship between the two; an increase in ROS results in membrane damage (Rico *et al.*, 2013c). A study conducted on peas (*Pisum sativum*) treated with Nano-ZnO showed a drastic amount of lipid peroxidation in comparison to control along with an overabundance of H_2O_2 (Mukherjee *et al.*, 2014). This is not the case for plants treated with 500 mg/kg in silt-loam. Even though the 500 mg/kg TiO_2 NPs had high H_2O_2 content, it showed low membrane damage. This inverse dose relationship can be explained by "hormesis" which is characteristic of when a "dose-response" to an environmental agent is stimulated by a low dose and shows a high inhibitory or toxic effect or vice versa. Another such instance is also reported by Rico *et al.* (2015) where the use of 500 mg/L of nano-CeO_2 had an evident increase in H_2O_2 content but prompted low membrane damage.

Figure 4.10(b) shows the lipid peroxidation and production of MDA in lettuce plants upon application of both treatments as compared to the control group.

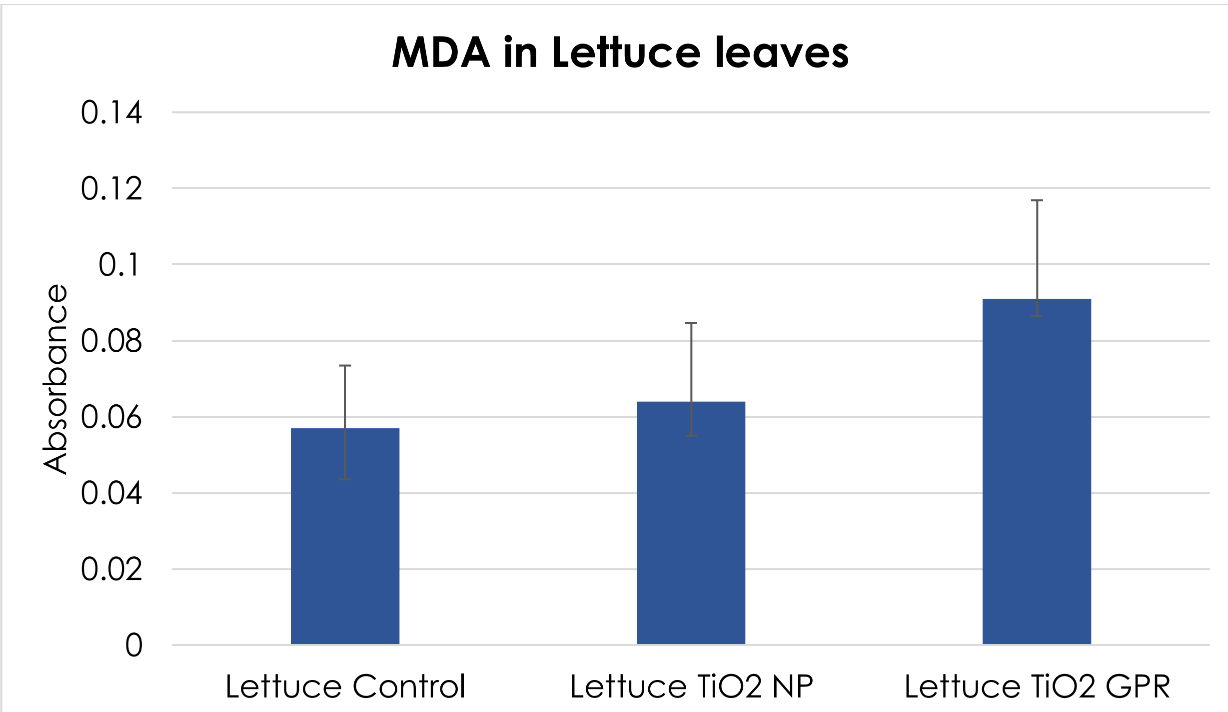


Figure 4.10(b): Lipid peroxidation in lettuce treated with TiO_2 NPs and TiO_2 GPR

Figure 4.10 (b) clearly depicts that toxicity was increased by 12.28% and 59.65% in case of TiO_2 NP and TiO_2 GPR treated groups, respectively. The highest TBARS production can be seen in TiO_2 GPR treated group which shows TiO_2 GPR is more toxic to plants.

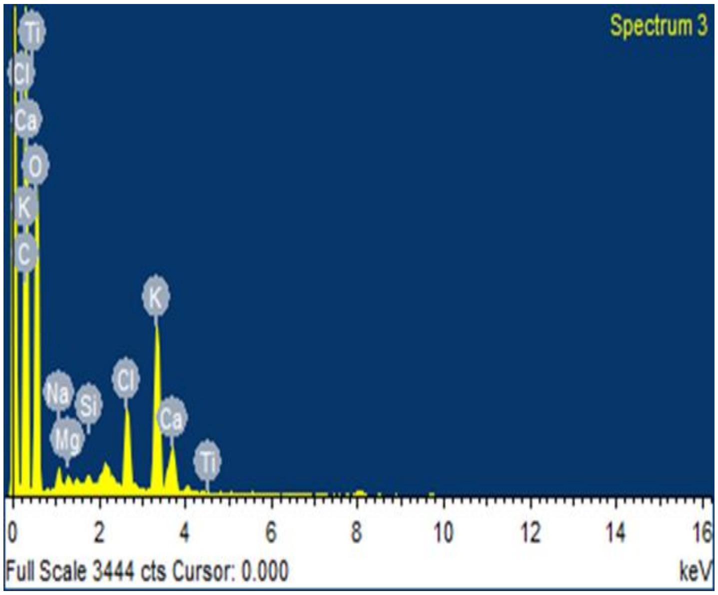
Another such instance was observed in a study conducted on pinto beans with 0.02% of Titania NPs which showed the highest amount of MDA production compared to control and other concentrations (0.03 and 0.05%) of TiO_2 NPs (Ebrahimi *et al.,* 2016). Even though this phenomenon is reported to be accurate for various environmental contaminants, there is very less discussion on whether it holds true for nanoparticles.

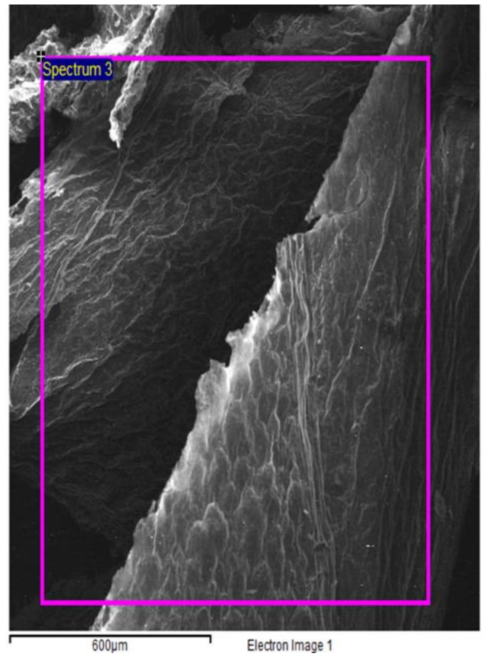
However, this is not consistent with the reported literature where oxidative stress and cell membrane damage has a linear relationship. Studies report a consistent linear relationship between the concentration of NPs and lipid peroxidation (Xu *et al.,* 2015) and also with oxidative stress (Rico *et al.,* 2013c). Another study on Nano-ZnO showed an increase in cell membrane damage with an increase in the concentration of NPs (Kumari *et al.,* 2011).

In essence, nanoparticles increase the production of ROS and therefore also peroxidation of lipids. A high level of ROS that the plant is unable to scavenge eventually leads to lipid peroxidation and this directly reflects the magnitude of cell damage in plants (Xu *et al.,* 2015). Chiefly, plant toxicity caused by metal nanoparticle exposure is a result of various factors including (but not limited to) the NPs size, shape, and mode of application. In addition, it is very difficult to observe NPs toxicity in soil because of the high likely hood of NPs agglomerating within the soil (Rico *et al.,* 2013)

4.5 TiO_2 NP and TiO_2 GPR uptake in tomato and lettuce:

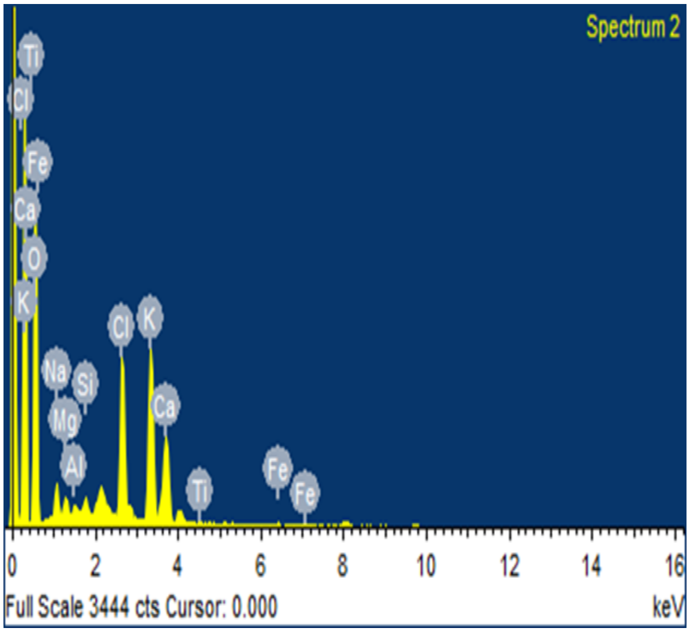
Figure 4.11 (a), (b) and (c) show SEM images of *Lactuca sativa* shoots. The control group (a) illustrated clear surface as compared to other treated groups. Aggregates of TiO_2 nanoparticles and Titania GPR were visible in Fig (b) and (c). This means that titania particles were taken up by the plants 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 titania particles were identified in the EDS spectra which confirmed the translocation of nanoparticles in shoots of *Lactuca sativa*.

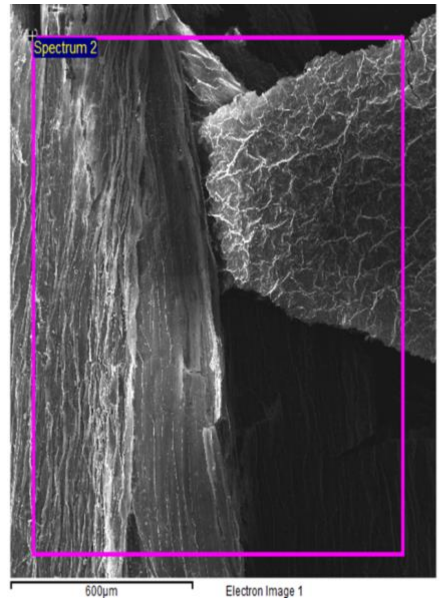




Element	Weight%	Atomic%
C K	50.10	59.68
O K	41.27	36.91
Na K	0.53	0.33
Mg K	0.21	0.13
Si K	0.13	0.07
Cl K	1.88	0.76
K K	4.62	1.69
Ca K	1.26	0.45
Ti K	0.00	0.00
Totals	100.00	

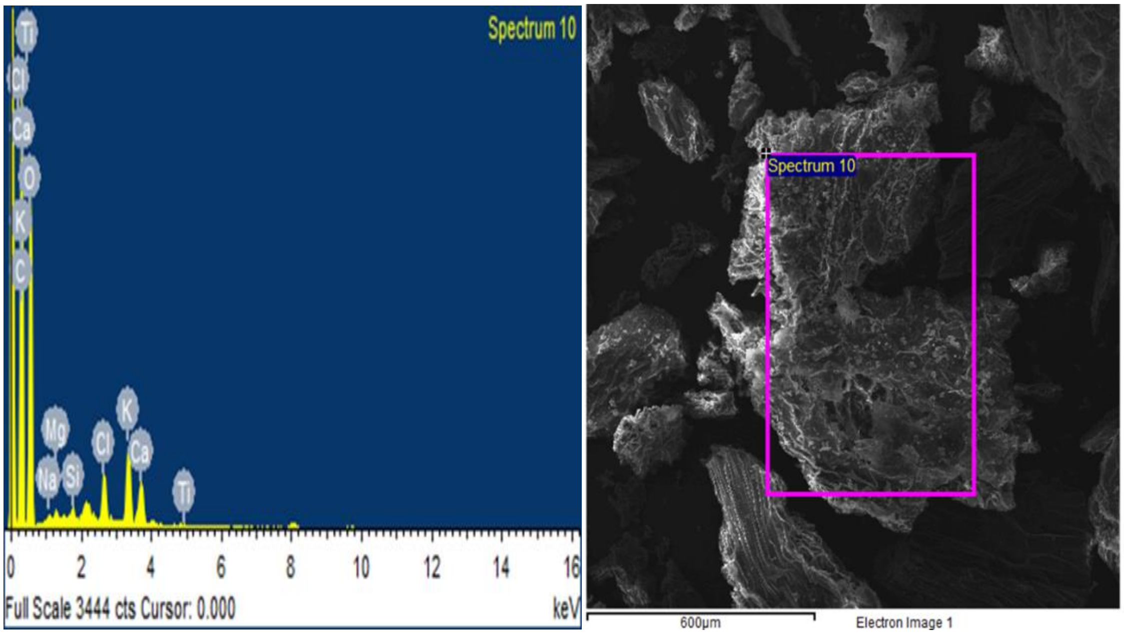
Figure 4.11(a): SEM image & EDS spectra of *Lactuca sativa* shoot (control)





Element	Weight%	Atomic%
O K	68.31	82.61
Na K	2.59	2.18
Mg K	1.10	0.88
Al K	0.39	0.28
Si K	0.55	0.38
Cl K	8.72	4.76
K K	11.17	5.53
Ca K	6.61	3.19
Ti K	0.03	0.01
Fe K	0.52	0.18
Totals	100.00	

Figure 4.11(b): SEM image & EDS spectra of *Lactuca sativa* shoot (TiO_2 NP)

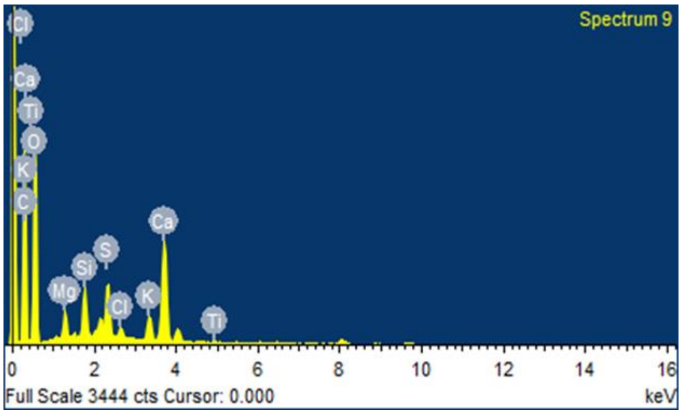


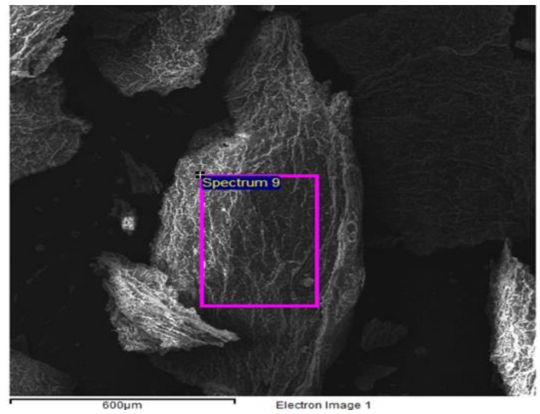
Element	**Weight%**	**Atomic%**
C K	47.87	56.66
O K	46.12	40.98
Na K	0.23	0.14
Mg K	0.22	0.13
Si K	0.22	0.11
Cl K	1.31	0.53
K K	2.55	0.93
Ca K	1.48	0.52
Ti K	0.01	0.00
Totals	100.00	

Figure 4.11(c): SEM image & EDS spectra of *Lactuca sativa* shoot (TiO_2 GPR)

Figure 4.12 (a), (b) and (c) show SEM images of tomato shoots. The control group (a) illustrated clear surface as compared to other treated groups. Aggregates of TiO_2 nanoparticles and Titania

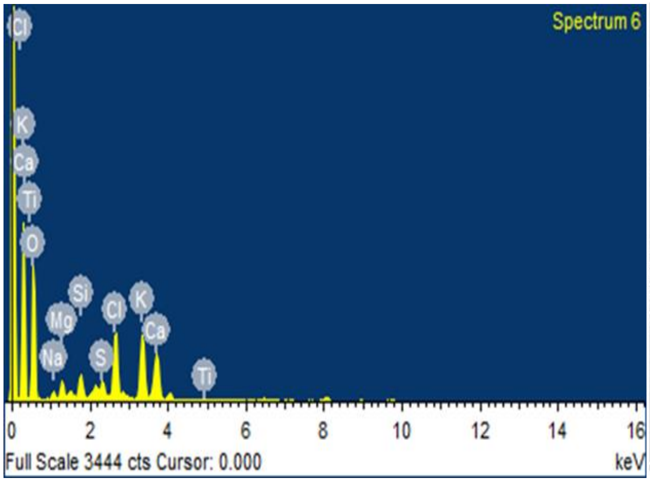
GPR were visible in Fig (b) and (c). The elemental presence of the applied titania particles were identified in the EDS spectra which confirmed the translocation of nanoparticles in shoots of tomato.

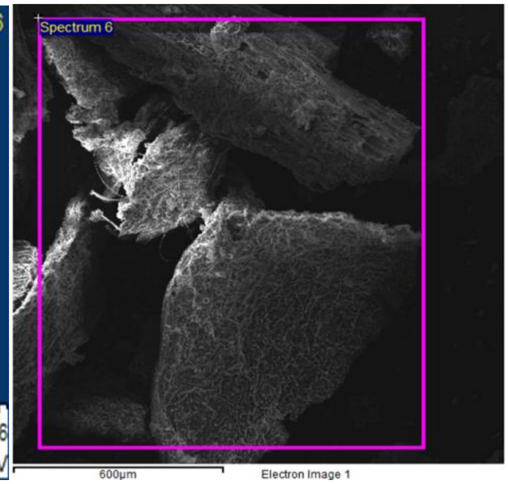




Element	Weight%	Atomic%
C K	41.97	52.02
O K	46.00	42.80
Mg K	1.08	0.66
Si K	1.60	0.85
S K	1.84	0.85
Cl K	0.51	0.21
K K	1.22	0.46
Ca K	5.73	2.13
Ti K	0.00	0.00
Totals	100.00	

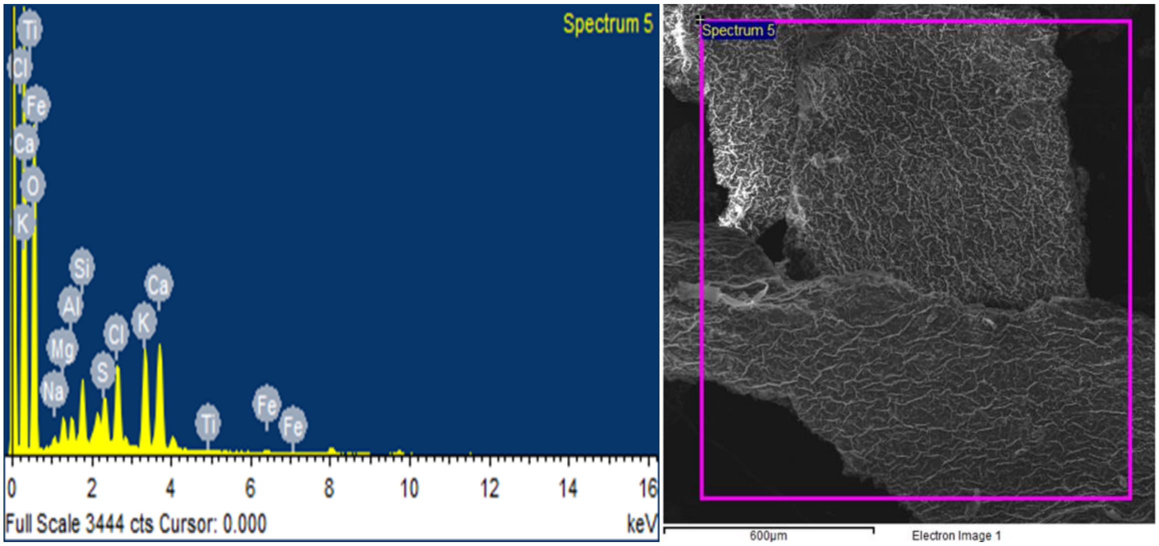
Figure 4.12(a): SEM image & EDS spectra of tomato shoot (control)





Element	Weight%	Atomic%
O K	**66.78**	**81.38**
Na K	**1.39**	**1.18**
Mg K	**2.18**	**1.75**
Si K	**2.27**	**1.58**
S K	**1.31**	**0.80**
Cl K	**8.43**	**4.64**
K K	**9.49**	**4.73**
Ca K	**8.02**	**3.90**
Ti K	**0.13**	**0.05**
Totals	**100.00**	

Figure 4.12(b): SEM image & EDS spectra of tomato shoot (TiO_2 NP)



Element	Weight%	Atomic%
O K	69.82	83.39
Na K	0.74	0.61
Mg K	1.63	1.28
Al K	1.29	0.91
Si K	2.85	1.94
S K	2.20	1.31
Cl K	5.17	2.79
K K	7.02	3.43
Ca K	8.61	4.10
Ti K	0.05	0.02
Fe K	0.63	0.22
Totals	100.00	

Figure 4.12(c): SEM image & EDS spectra of tomato shoot (TiO_2 GPR)

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 diffuse but large size would limit its diffusion and localized in the outer surface of cells even after penetration in the cell wall (Kurepa *et al*., 2010). According to a recent study, the long MWCNTs (larger than 200 nm) got accumulated in subcellular organelles while the smaller NPs (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*. A study reported that nanoparticles entered plant cells, and got accumulated in distinct subcellular sites (Kurepa *et al*., 2010).

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Considering the impact of TiO_2 NPs and TiO_2 GPR how they impacted both growth and development of tomato and lettuce, their uptake and the toxicity effects on both plants, the following conclusions can be summed up from the present study:

- Shoot length of lettuce increased up to 11.81 % in TiO_2 NP treatment group whereas tomato had more shoot length in the control group compared to treated group.
- Root length of lettuce increased up to 44.04% and increased plant height was observed in case of tomato in TiO_2 NP treatment group.
- Fresh and dry biomass of tomato was more in the control group whereas in lettuce it was higher in TiO_2 GPR treatment.
- Uptake of TiO_2 NPs was higher in tomato and lettuce causing drastic changes in elemental percentage as compared to control.
- TiO_2 GPR caused toxicity in both the plants with 14.94% in case of tomato and 59.65% in case of lettuce as compared to control and TiO_2 NP treatment group.

5.2 Future Recommendations

The present study has highlighted both positive and negative impacts of TiO_2 NPs application. Before using on an agricultural scale, extensive greenhouse and field trials are needed to be conducted possibly with lower TiO_2 NPs concentrations. Noteworthy effects were found on the lettuce and tomato plants in response to the TiO_2 NPs and TiO_2 GPR application. Moreover, multifactor parameter of plant production and yield indexes need to be included to reflect comprehensive evaluation of impact of NPs application. In depth study of toxicity effect to understand the underlying mechanism of physiological interruptions would also provide fundamental knowledge to design relatively safe NPs. It is also noticeable that study of whole value chain of food commodities is required to rule any possible public health and value chain hazard analysis rather than looking into a single or a fewer aspect of production of plant-based system. Thus, following future direction is suggested for research.

- Detailed mode of action studies are required to understand potential application of TiO_2 GPR and TiO_2 NPs in plants.
- Studies should be designed to assess plant protection advantages associated with the application of TiO_2 NP in various plant species.
- Intensive studies on better understanding of signaling pathways between ROS and NPs.
- Studies centering on soil rhizosphere chemistry, nanoparticles and root hair for better knowledge on how they influence each other.
- Field trials with lowered concentration of TiO_2 NPs to make it more economically feasible.

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