TOXICITY OF TITANIA NANOPARTICLES IN RICE (*ORYZA SATIVA* L.) AND ITS RHIZOSPHERIC SOIL

Sayyada Phziya Tariq Wani
00000172558

Institute of Environmental Sciences and Engineering (IESE)

School of Civil and Environmental Engineering (SCEE)

National University of Sciences and Technology (NUST)

Islamabad, Pakistan

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By

S. Phziya Tariq Wani

(00000172558)

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CERTIFICATE

It is certified that the contents and form of the thesis entitled **"Toxicity of Titania Nanoparticles in Rice (*Oryza sativa* L.) and its Rhizospheric Soil"** submitted by Ms. Sayyada Phziya Tariq Wani has been found satisfactory for the partial fulfillment of the requirements of the degree of Master of Science in Environmental Science.

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

Member: _____
Dr. Imran Hashmi
Professor
IESE, SCEE, NUST

Member: _____
Dr. Deedar Nabi
Assistant Professor
IESE, SCEE, NUST

Member: _____
Dr. Khurram Yaqoob
Assistant Professor
SCME, NUST

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Signature with stamp: _____

Name of Supervisor: _____

Date: _____

Signature of HoD with stamp: _____

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Signature (Dean/Principal): _____

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DEDICATION

To my Mom for being my first language, my lighthouse; the rain that makes me blossom. To my Dad for being a real rock, my pillar, my hope, my eternal sunshine. This wouldn't have been possible without you two. You are my sun and moon and all my stars.

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Table of Contents

LIST OF ABBREVIATIONS

AWC	Available Water Capacity
CeO_2	Cerium Oxide
Cr_2O_3	Chromium(III) oxide
CuO	Copper Oxide
DHA	Dehydrogenase Activity
DNA	Deoxyribonucleic Acid
EC	Electrical Conductivity
ENPs	Engineered Nanoparticles
H_2O_2	Hydrogen Peroxide
HSD	Tukey's Honestly Significant Difference (HSD) Test
MDA	Malondialdehyde
Ni	Nickel
NOM	Natural Organic Matter
OM	Organic Matter
PCPs	Personal Care Products
pH	Potential of Hydrogen
RDW	Root Dry Weight
RL	Root Length
ROS	Reactive Oxygen Species
SDW	Shoot Dry Weight
SEM	Scanning Electron Microscope
SL	Shoot Length
OM	Organic Matter
TBARS	Thiobarbituric Acid Reactive Substances
TiO_2 NPs	Titanium dioxide Nanoparticles
TPF	Triphenylformazan
XRD	X-ray Diffraction
ZnO	Zinc Oxide

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The present study focused on the interactive effect of titanium dioxide nanoparticles (TiO_2 NPs) on rice (*Oryza sativa*) growth and changes in soil health for two contrasting soil textures (silt-loam and clay). For this purpose, the pot experiment was carried out, TiO_2 NPs (0, 500, 750 mg kg^{-1}) were applied through irrigation and plants were grown till the vegetative stage. After exposure period, plants were harvested; physiological parameters (root, shoot length; plant biomass), stress assay (H_2O_2 production, lipid peroxidation, leaf membrane injury index) and soil activities (microbial biomass C, dehydrogenase and soil respiration) were determined. The results showed an adverse effect of TiO_2 NPs on plant growth and soil microorganisms in both soil textures at 750 mg kg^{-1}. However, in clayey soil plants showed significant growth upon 500 mg kg^{-1} TiO_2 NPs application as compared to silt-loam. Root and shoot lengths were 2.1- and 0.47 –folds higher, root-shoot biomass was 4.2- and 2.2 –folds higher in clayey soil as compared to silt-loam at 500 mg kg^{-1} TiO_2 NPs treatment. H_2O_2 production, lipid peroxidation, and leaf membrane injury index were increased by 4.3-, 2.4-, and 1.9-folds in clay soil upon 750 mg kg^{-1} TiO_2 NPs application. Likewise, at the same level of TiO_2 NPs; microbial biomass, dehydrogenase, and respiration were decreased by 0.91-, 0.79-, and 0.78- folds respectively, in silt-loam soil. The results of the present study suggested that high concentrations of TiO_2 NPs could negatively affect plant growth and soil enzymatic activities. Therefore, detailed work is still required on the plant-soil-TiO_2 NPs interactions to assess their toxic influence before commercializing them as nano-fertilizers.

INTRODUCTION

1.1 Background

In recent years, nanotechnology has witnessed unprecedented growth, and nanoparticles, in general, have been highlighted due to their extraordinary and multifaceted capabilities. Their minute size (1-100 nm), provides them with "high surface area to volume ratio" which equips them with novel surface-properties (Ghosh *et al*., 2016). These very properties although beneficial, are also dangerous, increasing reactivity and enabling them to infiltrate cells leading to nanotoxicity in plants, soil microorganisms and eventually humans. Nanoparticles such as Titanium dioxide, Silver, Zinc Oxide, Copper Oxide, and Cerium Oxide are used in a myriad of applications from health and food to overall environmental areas including agriculture (Patil *et al*., 2016; Hossain *et al*., 2015).

TiO_2 NPs (Titanium Dioxide Nanoparticles) is one such nanoparticle that is overly synthesized and used in an array of applications: paints, sunscreens, in food additives, cosmetics, personal and medical care, solar cells, athletics, and treatment of wastewater (Cox *et al.,* 2017); Keller & Lazareva, 2013). Risk of environmental exposure has elevated in the past decade and has put agricultural regions and in general soil systems at a higher risk of exposure due to the unspecified release of nanoparticles (Keller and Lazareva, 2013; Ghosh *et al*., 2016). There is an urgent need to comprehensively assess the potential positive and negative effects of NPs before they are commercialized globally.

In most Asian countries, rice is one of the most common cereal crops to be produced and consumed. As rice is a good source of proteins, mineral elements, vitamins, carbohydrates and fiber, it is vital for growth and nourishment. In Asia alone, 140.4 million hectares is appointed for rice cultivation while globally rice is cultivated at 159.8 million hectares

(FAO, 2016). This leads to a total production of 740.9 million tonnes of rice and a total grain yield of 46 thousand hectograms per hectare worldwide (FAO, 2016).

1.2 Nanotechnology

In the manufacturing industry, TiO_2 NPs are among the 13 most used nano-materials (OECD, Paris, 2008). Worldwide annual production currently stands at 3000 tons and is still increasing exponentially (Piccinno *et al.,* 2012). TiO_2 NPs are found in three crystal phases: anatase, rutile, and brookite. The rutile phase being the most stable. Anatase and brookite are transition forms of rutile (Tan *et al*., 2018). To highlight a few select properties of TiO_2 NPs, it has a high refractive index which aids in creating whitening agents. It is exceedingly photocatalytic and increases chloroplast activity. Furthermore, it has great hydrophilic properties along with sterilization helping produce cleaning products. According to a study, a combination of anatase + rutile TiO_2 NPs resulted in a higher solar to electric energy conversion than pure phases of TiO_2 NPs (Han *et al.,* 2005). In plants, TiO_2 NPs stimulates carotene and chlorophyll a production, increases electron transfer and intensifies chloroplast activity (Hong *et al.,* 2005; Lie *et al.,* 2007)

Benefits aside TiO_2 NPs still aren't without fault. The International Agency for Research on Cancer has classified it has a possible carcinogen (group 2B) to humans (IARC, 2010). Size is a key factor in the toxicity, behavior, and reactivity of nanoparticles. With the amount of TiO_2 NPs used in global markets it is necessary to understand its effects on public health and the environment and taking into consideration the indirect sources of TiO_2 NPs, exposure is inevitable in the environment and on organisms, in particular, plants, as soil is a major recipient of nanoparticles (Simonin *et al.,* 2016).

1.3 Nanoparticles Application to Plants

Incorporation of Nanotechnology in agriculture is an emerging idea, effects of different nanoparticles have been observed on various plant species providing us with insight on their performance and effects on plant growth (Zahra *et al.*, 2015). To name a few, nanoparticles are used as growth regulators in plants, biosensors, in the production of fertilizers and pesticides on a nanoscale and to genetically improve plants (Rico *et al.*, 2013a). Because of its extensive use in almost everything, it is not surprising that TiO_2 NPs are also utilized in agrarian practices as well.

Plants as primary producers play an essential character in the working and upkeep of an ecosystem (McKee and Filser, 2016). Plant-TiO_2 NPs interactions and uptake serves as a possible pathway for NPs transportation (Rico *et al*., 2011) and is potentially the cause of phytotoxicity (morphological and cytotoxic effects) in plants at high concentrations (Tripathi *et al*., 2017; Shweta *et al*., 2016; Rico *et al*., 2015). Therefore, plant systems aid in the transference of numerous nanomaterials to diverse environmental biomes and among trophic levels. (Rico *et al*., 2011). Organisms in the ecosystem are likely to be victimized by TiO_2 NPs induced oxidative stress (Hong *et al*. 2014). Nevertheless, extensive research is still required on the plant-soil-NPs interaction to assess their toxic influence (Gardea-Torresdey *et al*., 2014).

1.4 Nanoparticles Interaction with Soil

Apart from agricultural practices, TiO_2 NPs can also enter soil through direct methods such as rain 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 a weak movement in soils, they'll eventually accumulate with time. Exposure modeling on the

concentrations of NPs also specifies that soils act as better sinks for NPs than water or air suggesting that the main source of NPs exposure to the environment is via soils (Gottschalk *et al*., 2009).

Risk assessment of their toxicity to soils is still in its infancy and there is a scarcity of knowledge on their influence on plant systems in field or soil setups, hence their fate in the environment and their behavior focusing on its toxic effects to plants and eventually humans require extensive studying (Rico *et al.,* 2013b).

1.5 Significance of the Study

Rice (*Oryza* sativa L.) is an important cereal crop and staple food of numerous Asian countries, inclusive of Pakistan. In Pakistan, rice cultivation covers 10 percent of the total agricultural area (Waseem, 2016). Its consumption is one of the possible routes of dietary exposure to toxins and nanoparticles (Zahra *et al*., 2017). Furthermore, the scope of nanotechnology in agriculture is still unclear and requires further exploration. Use of nanoparticles is known to have significant biological effects and positive impacts on physiological parameters of plants even at low doses.

But according to the perusal of literature, TiO_2 NPs depending on experimental elements, exhibit a dual nature of both advantageous and toxic effects. Hence, this study is designed to specifically explore the toxicity of nanoparticles on doses that are known to enhance nutrient availability, vegetative traits, and nutrient uptake simultaneously on rice and soil both. Furthermore, research in plant sciences can aid Pakistan in better combatting the emerging environmental challenges.

1.6 Objectives

Keeping in view the information from the literature, it was hypothesized that nanoparticles are likely to cause toxicity in plants and show a negative impact on soil health. Hence, the objectives of the present study were:

1. To assess the effect of soil texture on the growth of rice upon application of TiO_2 NPs.
2. To study the toxicity of TiO_2 NPs on soil enzymatic activity and rice plants.

1.7 The Scope of the Study

The study focused on the influence of TiO_2 NPs on rice growth in combination with soil textures. There are several studies that have observed the effects of TiO_2 NPs on rice but mostly are at seedling stages or with plants grown hydroponically excluding soil. There is a dearth of studies focusing on soil setups or field experiments. Therefore, this study has provided new reflections on how soil influences the behavior of TiO_2 NPs in soil and plant systems.

LITERATURE REVIEW

This chapter focuses on the related literature on the use of nanoparticles as nanoparticles, nano-fertilizers, their behavior in soil and how soil texture and its properties are likely to affect them, its possible routes in the environment besides agriculture and their toxic effect on both plant and soil systems.

2.1 Nano-Agriculture

Nano-Agriculture is an emerging field in science and has shown plenty of beneficial effects, such as improved agricultural output with low waste production and is also cost efficient (Kah *et al*., 2015), but it is still in its infancy and there are many things that require in-depth understanding before this can be commercialized on a global scale. Nano-agriculture doesn't just entail the use of nanoparticles; Multi and single-walled carbon nanotubes, coated nanoparticles, metal, and metal oxide nanoparticles all occupy a prominent position in studies on Nano-agriculture (Singh *et al*., 2015)

The term "nanoparticles" can be defined as "a particle with one or more external dimensions in the size 1 nm to 100 nm" (Auffan *et al*., 2009). Their minute size equips them with novel chemical, biological and physical properties that are different than their bulk material. These properties aid the plant in various ways such as an escalation in growth and development, increased crop yield, improved absorption of nutrients, and better resistance and control to diseases along with the enhanced ability to withstand environmental or external stress (Singh *et al*., 2015).

Even though new nanoparticles are manufactured continually, only a handful are used excessively in products, hence accumulation of these nanoparticles in the environment has been steadily growing over the years. Nanoparticles commonly used include: copper (Cu),

silver (Ag), silicon oxide (SiO_2), titanium dioxide (TiO_2), gold (Au), cerium oxides (CeO_x), zinc oxide (ZnO) whereas manganese (Mn), copper oxide (CuO) and iron (Fe) nanoparticles are advancing in reputation as well (Rico *et al*., 2015). Ample amount of literature on nano-toxicology is found on the above mentioned nanomaterials.

Agricultural regions are likely to face a higher risk of exposure, particularly because the NPs are likely to accumulate in the soil as time goes by (Keller and Lazareva, 2013). Exposure modeling has also indicated soils as the main sink of NPs compared to water and air (Gottschalk *et al*. 2009).

Due to increased exposure, global concerns for potential phytotoxic effects and their release into the environment from other sources apart from agricultural practices have raised the risks to the environment in general. This has resulted in the development of a daughter field, Nano-Toxicology, which solely focuses on studying the risks and dangers involved in their use in the environment.

2.2 TiO_2 NPs in the Environment

As our study focused on the effects of TiO_2 NPs on plant and soil, reviewed literature focused on TiO_2 NPs possible routes into the environment and its various interactions.

The figure below depicts the approximate amount of TiO_2 NPs in environmental spheres. According to literature, TiO_2 NPs emission makes up for "one-fourth of the estimated mass flow of engineered nanomaterials in a worldwide range". Among the various applications that TiO_2 NPs is used for, food, pigments, cosmetics, hair sprays, and shampoos and various PCPs (personal care products) are the chief contributors to the environment. Moreover, exposure model estimates show that TiO_2 NPs discharge is the highest in soil (1.38 folds higher) and groundwater sources (1.85 folds higher) followed by water and air

(Keller and Lazareva, 2013). This proposes that Titania nanoparticles have plenty of contact time and direct interaction with plants.

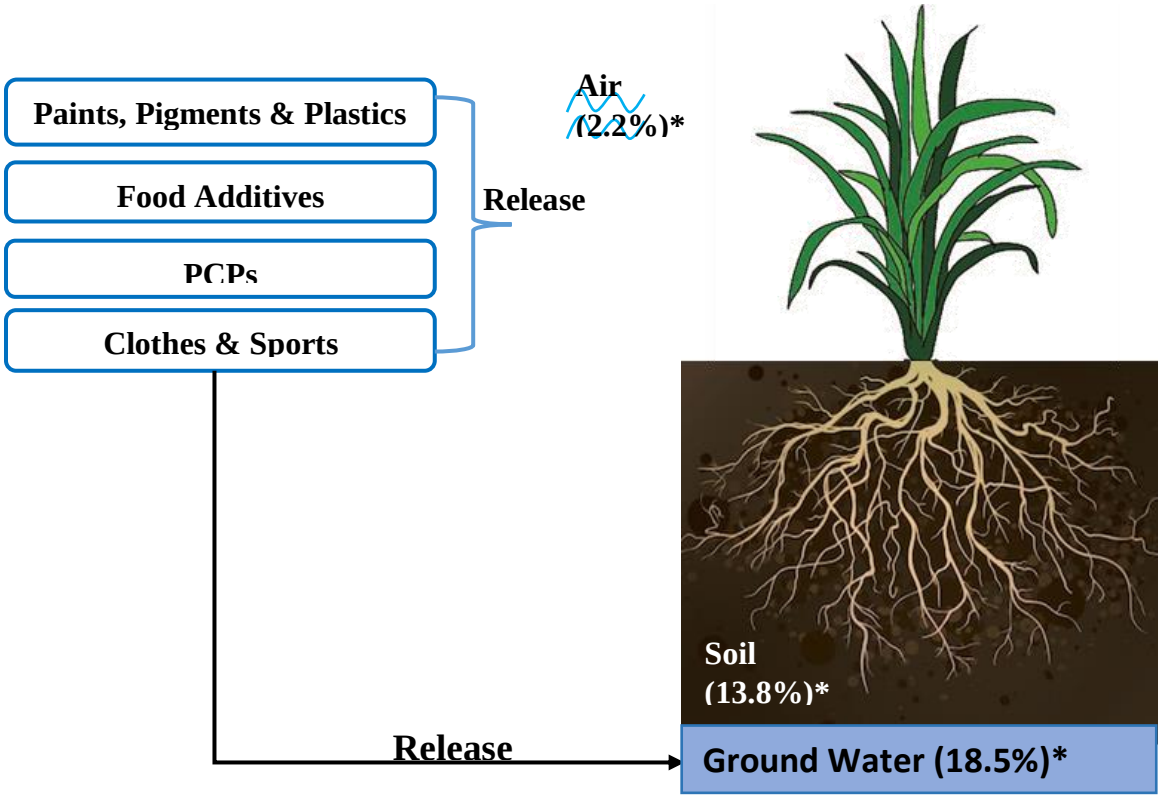

Figure 2.1: Graphic representation of uses of Titania nanoparticles and their dispersion in the environment *(Keller and Lazareva, 2013).

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 maintaining an ecosystem as they are primary producers. At the same time taking into account unspecified releases of TiO_2 NPs from indirect sources (landfills, wastewater sludge, and waste); the likelihood of nanoparticles interacting with plant systems before their uptake is high (McKee and Filser, 2016; Tripathi *et al.*, 2017). Moreover, as almost every other product contains TiO_2 NPs, it is expected that a large amount is discharged into the environment interacting with air, water, soil, and plants. This

brings physiological or genetic changes in plants depending on the size and how high the concentration of nanoparticles is. Their use as Nano-fertilizers, aids in them traveling up the food chain, eventually accumulating in higher trophic levels (Zhu *et al*. 2008).

Depending on the concentration, size, contact time, and experimental features, TiO_2 NPs have been known to display twofold attributes of both positive and negative effects. Several studies report a beneficial impact on overall plant growth development: Various studies conducted on spinach showed that use of TiO_2 NPs increased the rate of photosynthesis and "nitrogen metabolism" which aided in better plant growth (Yang *et al*. 2006). Likewise, a study conducted on mung bean with carbon dots (dosage: 0-1.0 mg mL^{-1}) showed a positive physiological response in its growth. The carbon dots boosted the uptake of nutrients and its utilization by the plant (Li *et al*., 2017). Another study was done by Parsad *et al* (2012) on peanuts with Nano-ZnO showed enhanced yield per pod at a concentration of 0.133 mg g^{-1}.

Alibadi *et al.* (2016), observed the effect of Titania and Aluminum nanoparticles in combination on wheat at four concentrations and found that 100 mg kg^{-1} showed an increase in root and shoot length. Also in 2016, Andersen *et al.* studied the effect of *n*CeO_2 and TiO_2 NPs on 10 different species of plants and found that they do not cause any damage or toxicity at germination and initial growth of the plant.

In contrast, various studies have also observed a negative impact as well: in 2016, Hung *et al.* noted growth inhibition in "*Bacillus thuringiensis*" when treated with Nano-SiO_2. Research conducted on rice seedlings showed decreased biomass and length of roots along with delayed germination rate and weight (Shaw and Hossain, 2013). In addition, a study focused on unique "earth oxides (Gd_2O_3, CeO_2, Yb_2O_3, and La_2O_3)" had injurious effects

to plant growth of tomatoes, rapeseeds, lettuce, cabbage, corn, wheat, radish and cucumber (Ma *et al*., 2010 and López-Moreno *et al*., 2010).

Plant-Nanoparticle interaction has been summarized below in figure 2.2. These interactions either result in an increase, decrease or alterations in plant systems which are either positive or negative. (Kumar *et al*., 2018).

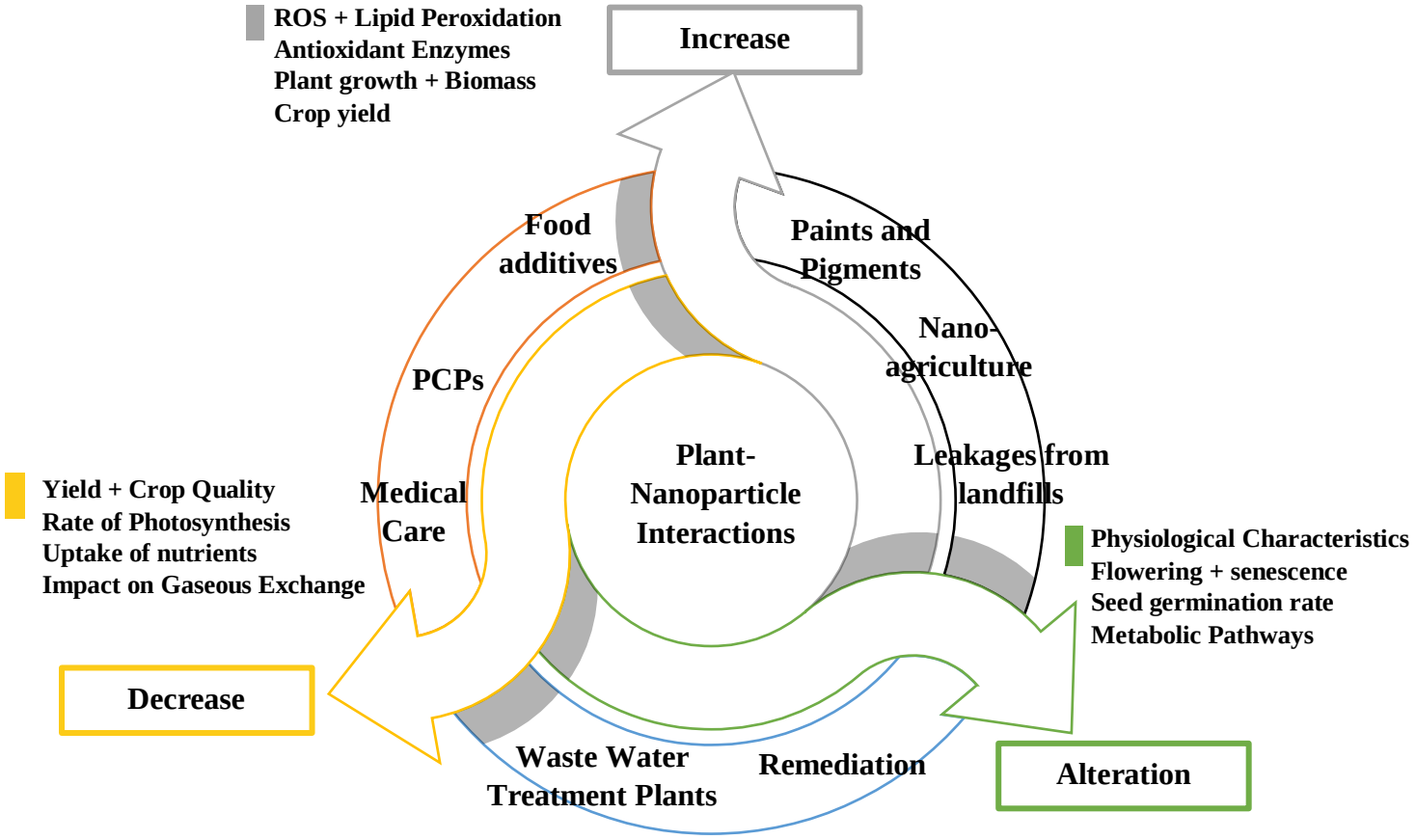

Figure 2.2: Plant-Nanoparticle interactions and its changes in plant systems.

2.3.2 Effect of TiO_2 NPs with Respect to Growth Stage

Thorough scrutiny of literature shows that numerous studies have been executed on seedlings, in Petri dishes, on full plant life cycles and at certain growth phases with plants grown hydroponically. These studies had a study period ranging from a week to about 2 to 3 months; before the plant started flowering or producing grain or fruit. Very few studies

focus on complete life cycles. Exposure to nanoparticles in most studies was through roots and leaves (foliar application)

Wu *et al*. (2017) tested TiO_2 NPs of 100, 200 and 500 mg L^{-1} dosage on rice over a period of two weeks. They observed a decrease in dry weight of roots and shoots along with a disruption in metabolic activities of the plant. They even found a dose-dependent increase of TiO_2 NPs in roots and shoots. Likewise, a study con ducted on lettuce with a concentration of 0.01, 0.1 and 1 g kg^{-1} showed that TiO_2 NPs caused hindrance in the uptake of phosphorus, calcium, and iron and also accumulated on root surface (Larue *et al*., 2016). Furthermore another study by Du *et al*. (2017) assessed TiO_2 NPs concentrations of 20 and 200 mg kg^{-1} on rice plants till maturity. Their results showed a decrease in crop yield and dry weight along with the increased accumulation of Titania, Magnesium, Calcium, and Phosphorous and Zinc in rice grains.

2.4 TiO_2 NPs and Phytotoxicity

As mentioned earlier, most NPs have a dual effect on plants, showing both beneficial and hazards sides. The chief reason for this is a combination of all three things: plant species (NPs Behave differently according to each species), growth media (Petri dishes, hydroponic or soil) and properties of NPs namely size and shape and coating. Also in the case of Metal and Metal oxide nanoparticles, the metals own inherent toxicity also plays a part.

2.4.1 TiO_2 NPs Induced ROS and Lipid Peroxidation

Reactive Oxygen Species (ROS) is a mutual term for reactive ions produced due to incomplete reduction of Oxygen (O_2). ROS present in plants includes Hydrogen peroxide (H_2O_2), superoxide radicals/anion (O_2^-), hydroxyl radicals ($^{\cdot}OH$), and singlet oxygen ($_1O^2$)

(Gechev et al. 2006). The reduction of oxygen from its ground state 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 revert back to an oxygen molecule or react with another proton to produce H_2O_2. Superoxide dismutase (SOD) enzyme catalyzes this reaction (Ślesak *et al.,* 2007).

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

These derivatives of O_2 have a powerful potential to oxidize which leads to damaging effects on plant systems such as injury to DNA, oxidation of lipids and proteins, and membrane damage and electrolyte leakage eventually resulting in cell fatalities (Meriga *et al.,* 2004; Sharma *et al.,* 2012). ROS is not only produced due to stress, but a normal functional metabolism can also produce ROS (Van Breusegem *et al.,* 2001). The imbalance of production and scavenging of ROS causes oxidative stress and literature suggests that metal centered Nanoparticles encourage oxidative stress in various plant species.

As H_2O_2 is likely to convert into a more reactive $^{\cdot}OH$ radical which even the plants own enzymatic system cannot detoxify. Because of its unpaired electron, it is prone to react with molecules and cause cell injuries such as peroxidation of lipid membranes, destruction of active sites and membranes proving fatal to the cell and eventually plants health (Ma *et al.,* 2015). Published literature supports the fact that exposure to metal nanoparticles shows a linear relationship between ROS generation and lipid peroxidation.

Several studies commemorate the fact that TiO_2 NPs possesses genotoxic capabilities: With a dosage range of 0.01, 0.1 and 1 mg L^{-1} TiO_2 NPs, a high amount of DNA damage was observed in onion only after 18 h of exposure (Demir *et al.,* 2014). A study in 2008 by Lin and Xing observed a particle size-dependent production of ROS and lipid peroxidation on the roots of rye grass's cell membrane surface. Furthermore, exposure of TiO_2 NPs, (0.0125, 0.025, 0.05 and 0.1 mg L^{-1}) on onion roots showed 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 that has small pores with a small very minute diameter's' (5 to 30 nm) which helps protect the cell from large contaminants, but TiO_2 NPs with a size lower than 30 nm can easily enter plant cell through these pores (Auffan *et al.,* 2009). Therefore, the size of a nanoparticle is a major characteristic that affects biological systems in plants. The literature stated that TiO_2 NPs were able to shrink root pore size, the flow of water and the roots ability to uptake water (Asli *et al.,* 2009). A study conducted on wheat found 140 nm TiO_2 NPs to be the limit past which no accumulation in plants was seen. In the same research they observed that smaller sized TiO_2 NPs (12, 22 and 25 nm) traveled from plant roots to leaves. While particle size 36 nm accumulated in the stem (Larue *et al.,* 2012a). In another study, it was found that particles above 30 nm were also found in root cells suggesting that TiO_2 NPs are capable of expanding cell pores or forming new ones (Larue *et al.,* 2012b). Besides, studies have already established the fact that bulk sized (in micrometer) TiO_2 particles do not show the same effects as TiO_2 NPs (Feizi *et al.,* 2012).

The limited amount of literature regarding the particle size of nanoparticles and its role in plant interaction requires an urgent need for more research in this area.

2.5.2 Crystal Phase

TiO_2 NPs have three crystal phases: anatase, rutile, and brookite. Each of them having a different interaction with plant systems. Several studies on this rectify the fact that crystal phase plays an important role in how NPs behave. Anatase is the most stable phase of TiO_2 and is likely to cause more damage than advantage to the plant systems. Studies report that anatase TiO_2 disrupted antioxidant system in duckweed and tomatoes (Song *et al.,* 2012; Song *et al.,* 2013) and hindered the rate of seed germination in rice (Jalill and Yousef, 2015).

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

In general, plants show a diverse response to TiO_2 NPs phases. This is due to the fact that the crystal phase brings changes in properties such as stability, cell volume, surface charge, and energy band gaps (Tan *et al.,* 2018). To summarize, the literature shows that anatase shows higher capabilities of toxicity rather than rutile, this is due to the fact that rutile

forms large masses that reduce its uptake and interactions with the plant (Clément *et al.*, 2013).

2.5.3 Doping of TiO_2 NPs

Nanoparticles are usually coated with either organic or inorganic complexes to improve optically and to accomplish less formation of aggregates. The type of surface coating impacts their solubility and toxicity. In addition, the coating of nanoparticles brings changes in their surface charge and area influencing their interactions with soil and plant systems. (Tan *et al.*, 2017). Various studies indicate that coated TiO_2 NPs show higher toxicity as they easily traverse through plant organs and have better access to plant cells for interactions (Foltête *et al.*, 2011; Wang *et al.*, 2011). But the results are not all negative, a study by Singh *et al.* (2016) saw an enhanced rate of seed germination in tomatoes and lentils. Studies stated that coating TiO_2 NPs with a small amount of metal increases their optical properties (Chen *et al.*, 2007). However, for a more rounded approach of coating NPs and its effect on the plant more research is required.

2.6 Effects of Soil Characteristics on TiO_2 NPs Toxicity

The soil is a chief and complex media. Adding soil to the nanoparticle-plant mix brings forth different interactions namely the effect of soil characteristics on NP behavior, how their change in behavior effects plant and soil systems and plant and soil microorganisms interaction. Soil properties that impact TiO_2 NPs performance include pH and soil's natural organic matter and particle size and soil texture. (Peralta-Videa *et al.*, 2011).

The figure below depicts the soil parameters that affect TiO_2 NPs, these factors usually have a cumulative effect rather than as individuals. They affect TiO_2 NPs surface charge, "zeta potential", and formation of agglomerates (Pachapur *et al.*, 2016).

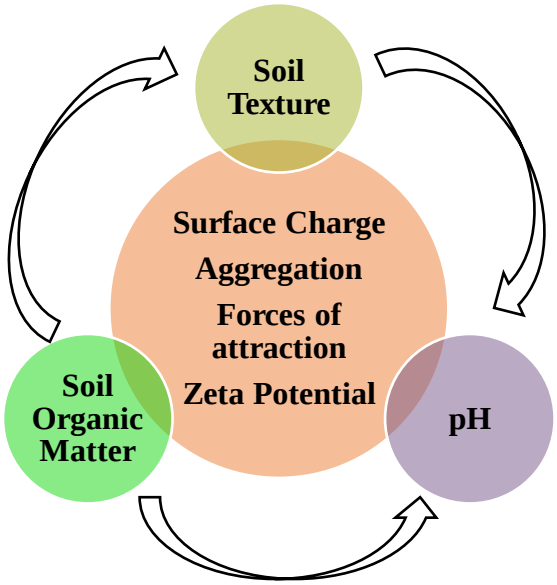

Figure 2.3: Soil Properties effecting TiO_2 NPs behavior (Parameters outside are the factors of soil affecting TiO_2 NPs properties)

2.6.1 Soil Texture

Clay content in soil plays an essential role in composition of the soil. There are two types of clays in soil: kaolinite and montmorillonite (bentonite is a lighter form of montmorillonite) (Bradly et al., 1999). A study observed the effect of clay on TiO_2 NPs behavior and found that higher the clay content, higher the rate of their movement within the soil. The high movement also equals to less formation of aggregates (Cai *et al.*, 2014). A study by Fang *et al.* (2009) observed movement and suspension of TiO_2 and found that they had a suspension rate of more than 10 days. These results were positively corroborated with the soils clay percentage and its organic matter but had a negative correlation with pH and surface charge of TiO_2 NPs. The study also stated that soils have a large particle size

and low EC which readily let TiO_2 NPs pass through while soils with high EC and Clay withheld them. The estimated distance of TiO_2 NPs in soil columns was within the range of 1.35 feet to 12.13 feet suggesting a threat to deeper layers of soil. (Fang *et al.,* 2009)

2.6.2 Soil pH and Organic Matter (OM)

As mentioned before, OM is one of the soil factors that affect the mobility of NPs by bringing changes in its surface properties. The soils own organic matter consists of humic and non-humic parts. Each part providing the soil with diverse solubility and reactivity according to its quantity. (Han *et al.,* 2014).

Interactions between OM and TiO_2 NPs include the removal of original coating (if NPs are coated), sorption on to non-coated NPs, formation or lack of agglomerates, and change in surface properties such as "hydrophobicity to hydrophilicity" (Wu *et al.,* 2017). Because of the negative charge on TiO_2 NPs surface (anatase and rutile both), positively charged OM particles are attaracted. They adsorbed on to TiO_2 NPs surface through hydrogen bonding and electrostatic forces. This gives the TiO_2 NPs increased movement and steadiness in soil along with reduced agglomeration. The reduction in agglomeration is in direct relation to the coating of OM. In simpler words, that higher the coating of OM on TiO_2 NPs, lower its chances of forming agglomerates (Lin *et al.,* 2010). A study on coated TiO_2 NPs with Citric acid and varying levels of pH found that coating of OM was reliant on two things: pH and surface area of TiO_2 NPs (Yang *et al.,* 2009). Likewise, an increase in pH from acidic to neutral showed more stability and less formation of clusters in TiO_2 NPs in a study by Mudunkotuwa *et al.* (2010). Results from another study concluded that TiO_2 NPs-OM interaction affected the nanoparticles bioavailability and this, in turn, was dependent on the soils pH, particle size and organic matter (Danielsson *et al.,* 2017).

2.7 Toxicity of TiO_2 NPs to Soil Organisms

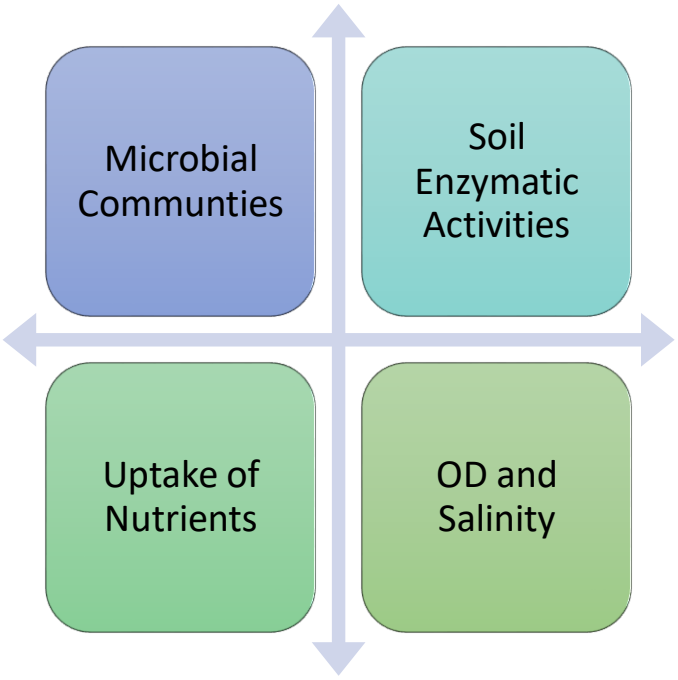

Figure 2.4: Effect of TiO_2 NPs on Soil Properties (Abbreviations: OD = oxygen demand)

Figure 2.4 displays soil parameters affected by TiO_2 NPs once they are mobile and available in the soil. Microbial communities are at a higher risk as they are essential to soil health. They are responsible for recycling nutrients and minerals and for the breakdown of organic matter (Bloomfield, 2016). As mentioned in the above figure, TiO_2 NPs can bring changes in the diversity of microbial communities by upsetting the balance of the following properties (either directly or indirectly); Oxygen Demand (OD), salinity or electrical conductivity (EC), soil enzymes and bacterial activities along with alterations in soil water and uptake of nutrients (Cai *et al.*, 2014; Ge *et al.*, 2013).

2.7.1 Effect of TiO_2 NPs on Soil Microbial Biomass

Numerous studies have reported that TiO_2 NPs bring a reduction and alteration in the diversity and population of microbial communities in the soil in an application-dependent manner (Ge *et al.*, 2013). A study by Xu *et al.* (2015) determined 3 different concentrations

(0.1, 0.5 and 1 g kg^{-1}) with control on rice paddy soils and discovered that TiO_2 NPs reduced the microbial community enough to pose a risk to the health of paddy soil systems. Similarly, research by Ge *et al.* (2011) applied TiO_2 NPs (dosage: 0, 500, 1000, 2000 mg kg^{-1}) on grassland soil and observed a highly negative impact on soil bacterial biomass. However, there are also studies that report no effects at all. A study by Simonin *et al.* (2015) tested the toxicity of TiO_2 NPs (concentration: 0.001 and 0.5 mg g^{-1}) on soil microbial biomass and found no significant effect on microbial communities despite different soil types. In a toxicity review of metal oxide nanoparticles by Suresh *et al.* (2013), TiO_2 NPs along with other significant NPs were reported as toxic. The above literature signifies that TiO_2 NPs have a negative effect on soil microbial communities and puts them at great risk.

2.7.2 Effect of TiO_2 NPs on Soil Enzymatic Activity

Because of TiO_2 NPs dual nature of performance depending on experimental setups, an array of diverse results are observed in regard to soil enzymes. A study reported a significant increase in urease activity at 0.091 g/L of TiO_2 NPs (Du *et al.,* 2011) while another study observed a significant decrease in urease activity at 1 g/L (Chai *et al.,* 2015; You *et al.,* 2017) in spite of soil texture and contact time. In another study applying metal oxide nanoparticles to tobacco, a reduction in dehydrogenase activity (DHA) was observed (Poborilova *et al.,* 2013). Nanoparticles are also known to inhibit soil enzymatic activity, several such instances are found in the literature. A study by Xu *et al.* (2015) found that Nano-CuO had a heavy inhibitory impact on enzymes in paddy soils. Another study in 2014 by Jośko *et al.* observed a noteworthy inhibition in DHA with ZnO nanoparticles.

There is a lack of studies observing the effect of TiO_2 NPs in soils along with plant systems. Further research is required to better understand whether they pose a risk or act as stressers that can be rectified or adjusted by the soil itself.

MATERIALS AND METHODS

This chapter describes the experimental framework adopted for this research. The pot experiment was carried out in the greenhouse at Institute of Engineering and Environmental Sciences (IESE), NUST, Islamabad, Pakistan. In the present study, rice was exposed to TiO_2 nanoparticles at two different dosages: 500 and 750 mg kg^{-1} with Control (0 mg kg^{-1}) via irrigation method. Rice was planted in two different soil textures (Silt-loam and Clay) to see its cumulative effect with Titania Nanoparticles.

The first phase and analysis focused on plants growth variations due to these treatments, the purpose of the second phase was to focus on the plant and soil toxicity. Keeping in view the main objectives of the study, the following methodology was adopted which is being discussed here in detail accordingly.

3.1 Preparation of TiO_2 Nanoparticles

3.1.1 Titania Nanoparticles Synthesis with Sol Gel method

Titanium dioxide nanoparticles were synthesized by using titanium isopropoxide, ethanol, distilled water, and hydrochloric acid at 1:15:60:0.2. Ethanol and HCl were added to distilled water and Titania isopropoxide was added drop by drop to the solution while it was being stirred on a hot plate at 600 rpm for 48h. The solution was oven dried at 105 ^oC for 48h; formed yellow crystals were ground with mortar-pestle and calcined at 450 ^oC for 6 hours (Gul *et al*., 2019).

3.2 Characterization of TiO_2 NPs

3.2.1 X-ray Diffraction (XRD)

This analytical procedure wass used to determine the crystalline structure and size. These were determined using Scherrer's calculator through X'Pert High score using a line width

of the (101) plane refraction peak for TiO_2. The XRD pattern of TiO_2 NPs was attained using X-Ray Diffractometer (Theta-Theta STOE, Germany) with Cu Kα radiation. Scan range of 20°-80° was used (2θ; λ = 0.154) was used with a step of 0.5° at 40 mA and 40 kV.

3.2.2 Scanning Electron Microscopy (SEM)

Titania nanoparticles' surface morphology was obtained by SEM (JSM-6490A, JEOL) with a 20 kV accelerating voltage. Before scanning, the powdered TiO_2 NPs were diluted 100-fold in distilled water and then sonicated for 60 minutes. A drop of 10 μL diluted solution was placed on a glass slide and air dried.

3.3 Soil Preliminary Analysis and Preparation for Pot Experiment

3.3.1 Soil Characterization

The major soil characteristics that were analyzed included soil pH, moisture content, soil texture, nitrate-nitrogen, total organic carbon, and extractable and total phosphorus.

3.3.2 Soil pH

pH of the soil was determined in order to check the chemical activities in soil. To measure soil pH, soil: water (1:5) suspension was prepared. 5 g of dried soil was added in 50 mL conical flask. 25 mL of distilled water was measured with a graduated cylinder. The resulting mixture was stirred correctly using an orbital shaker at 180 rpm; 30 minutes. The pH was measured using a combined electrode (HI 2211 pH Meter/ HANNA Instruments). The pH reading of each replicate was taken after 30 seconds (McLean, 1982 (ICARDA, 2013).

3.3.3 Soil Texture

Different texture of the soils was determined by saturation method (Malik *et al.,* 1984; ICARDA, 2013). The two different soil textures were classified as silt loam and clay.

3.3.4 Moisture Content

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

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

3.3.5 Total Organic Carbon

Total Organic Carbon was determined through Walkely and Black (1934) method (ICARDA, 2013). This was performed post harvesting as well. The soil was grounded and passed through a < 2 mm mesh sieve, measured and placed in a 500 mL Erlenmeyer flat bottom flask. 1 g of soil was measured and 20 mL of concentrated sulfuric acid (H_2SO_4) and 10 mL of 1N potassium dichromate ($K_2Cr_2O_7$) were added to the soil while mixing it to make sure that the soil was incorporated with reagents. After a rest of 30-min, addition of 10 mL of concentrated H_3PO_4 with 200 mL of distilled water and 10 drops of diphenylamine indicator prepared in sulfuric acid. This was titrated using ammonium ferrous sulfate hexahydrate as the titrant.

3.3.2 Soil Preparation for Pot Experiment

For this set up, two types of soils were used, silt-loam and clay. The soils were spread out and dried for a week. The dried soils were then grounded into fine form. Larger particles, gravel, roots, and shoots were removed further manually. Soils were sieved by the use of a sieve, size <2mm. For experiment, plastic pots of the diameter of 9 cm and height of 10 cm were used. This clean and processed soil material was used for the present experiment. The soil was weighed and 1 kg of soil was added to each pot.

3.4 Plant Cultivation

3.4.1 Sterilization of Seeds

Seeds of experimental plant species rice (*Oryza sativa* L.) were of *Super Basmati*, received from the Ayub Agricultural Research Institute, Faisalabad, Pakistan. Seeds were kept in a dry dark place under room temperature. Prior to use, sterilization of seeds was done by using 5% sodium hypochlorite solution and then washed with distilled water meticulously.

3.4.2 Growth of Seedlings

Seeds were sown in pots containing untreated soil (without fertilizer and TiO_2 NPs) and placed in a greenhouse at IESE, NUST. Six seedlings were planted per pot.

3.4.3 Preparation of Pots and Fertilizer Application

One kg soil was weighed for each plastic pot and was properly labeled. TiO_2 NPs suspension of three concentrations i.e. 0, 500 and 750 mg kg^{-1} was prepared. Control group without the addition of nanoparticles was maintained. Five replicates were there for each concentration of TiO_2 NPs. Recommended NPK was applied: N (70 mg kg^{-1}), P (40 mg kg^{-1}) and K (32.5 mg kg^{-1}) to all pots. Suspensions of urea 46% N, Potash containing 50% potassium and Di-ammonium Phosphate (DAP) containing 46% phosphorous and 18% Nitrogen were applied. Urea was added in two splits, first application was added 2 weeks after seedlings sprouted and the second was applied during the vegetative stage. Plants were irrigated after every 2 days to maintain paddy conditions.

3.4.4 Application of TiO_2 Nanoparticles

Rice plants were exposed to TiO_2 nanoparticles using irrigation method. TiO_2 NPs suspensions of 500 and 750 mg kg^{-1} were prepared and sonicated for 30 min prior to application by use of an ultra-sonicator (JAC-1505, Jinwoo, Korea). For soil application,

TiO_2 NPs suspensions of the desired dosage were added in the soil in previously labeled pots after seedlings sprouted. There were 5 replicates for each method. During the experimental phase, pots were kept in a greenhouse at IESE, NUST.

3.5 Morphological Parameter Measurement.

3.5.1 Plants Length Measurement

After harvesting of rice plants, roots and shoots were rinsed in distilled water, collected separately. Root and shoot lengths were measured.

3.5.2 Plants Biomass Determination

Roots and shoots of rice were cut and their fresh biomass weighed one by one. After weighing both were placed in an oven for 48 hours at a temperature of 70 °C. The plant material was reweighed for dry biomass.

3.6 Soil Toxicity Analysis.

3.6.1 Soil Microbial Biomass

A "rapid chloroform-fumigation-extraction method" by Witt et al., (2000) was used for the estimation of microbial carbon under different concentrations of Titania nanoparticles. For the experiment, moist soil samples were split into two portions at each test day for fumigation and non-fumigation in screw cap vials. Non-fumigated soil samples taken as control were extracted with 5 mL 0.50 M K_2SO_4 immediately, shaken for 60 min at 35 rpm, and filtered using Whatmann No. 42 filter papers. The extracts were frozen until further use.

For fumigation, 57 µL chloroform was added to each soil sample, followed by incubation for 24 h in dark at 25°C. After the incubation period, chloroform was allowed to evaporate from the samples by placing them in a fume hood for 30 min. The microbial carbon was

then extracted using potassium sulfate as done for non-fumigated samples. From the extracts, 1.60 mL was pipetted out in screw cap vials and 2.40 mL oxidant solution [0.128 g $K_2Cr_2O_7$ in 40 mL of DW and 200 mL of H_2SO_4] was added to it. The vials were then placed in the COD reactor at 150°C for 30 min to achieve biomass C oxidation. Spectrophotometric analysis of samples was then measured with UV-Spectrophotometer (Specord 200 plus Analytikjena Germany) at 350 nm (Cai et al., 2011). [Note if absorbance readings are higher than 1, dilute the sample with distilled water.]

Biomass C was determined using the formula as follows:

$$\text{Biomass} = {}^{EC}/_{K_{EC}}$$

Where EC is the difference of extractable C between the fumigated soil samples and the non-fumigated ones. The extractable part of microbial C (K_{EC}) for the proposed method was given as 2.64 which is specific for paddy soils.

3.6.1.1 Standard Preparation for Spectrophotometric Analysis

Microbial Biomass standard solution containing 137.5 mg L^{-1} glucose in a volumetric flask was diluted to prepare the final concentrations of 10-50 mg L^{-1}. These values were used as a standard to calculate soil microbial biomass. Distilled water was used as a blank and the absorbance was measured at 350nm.

3.6.2 Soil Dehydrogenase

The method followed for soil dehydrogenase was as described by Thalmann (1986). 2, 3, 5 Triphenyl tetrazolium chloride (TTC) was used for the estimation of dehydrogenase activity in soil. TTC is also known as Tetrazolium Red. Its initial solution is colorless but changes to red in the presence of dehydrogenase enzymes with the release of hydrogen ions. The colorless salt forms a red compound known as “formazan”.

The assay is as follows: A total of 6 grams of fresh moist soil was taken and split as sample and control for each sample. A total of 5 replicates was maintained for each treatment. To 3 grams of moist soil, 1.25 mL of 1% TTC (Triphenyltetrazolium chloride) was added. The soil was then mixed with 0.5 g of calcium carbonate ($CaCO_3$) and 0.5 g of anhydrous glucose. Control for each sample contained the same amounts of calcium carbonate and anhydrous glucose with 1% TTC being replaced with distilled water. These were all incubated for 24 hours at 30^{o}C in the dark. After incubation 10 mL of methanol was added. Bottles were shaken at 100 rpm in the dark at 30^{o}C for 2 hours. Resulting soil suspensions were filtered in the dark with Whatmann Filter paper no.1. Absorbance of the clear supernatant was measured with a UV-Spectrophotometer (Specord 200 plus Analytikjena Germany) at 546 nm.

For the preparation of calibration curve 0.3 grams of 1,3,5 Triphenylformazan in 500 mL of methanol to prepare a stock solution of 0.2 µmol/mL. Standards were prepared in the range of 0.004 - 0.1µmol/ml with methanol as blank. Results were calculated as follows:

$$\text{Dehydrogenase activity (TPF) (µg)/g dwt.} = \frac{TPF \times 45}{dwt \times 5}$$

Where,

TPF is the concentration of TTC – Control for each sample.

dwt. = 1 g of moist soils' dry weight.

5 = amount of moist soil (g)

45= amount of the solution added to the soil (ml)

[Values can be changed according to the amount of soil and solution used by you]

Dehydrogenase activity per gram dry soil was expressed in terms of microgram formazan per gram dry soil.

3.6.3 Soil Respiration

Respiration is an index of biological and metabolic activity of the soil microbial life. Therefore it is an indicator of the soil community's biological eminence. For this assay, Moebius-Clunes procedure from Cornell Soil Health Laboratory (2016) was adopted. Respiration was measured by capturing and quantifying the release of carbon dioxide from moist samples held in airtight jars. Greater the release of CO_2, the more active the soil microbial life. The method used is called "sealed chamber alkali trap respirometry." As the name implies, CO_2 is "trapped" in an absorbent and that lets us calculate the soils metabolic activity. Potassium hydroxide (KOH) is used as a trap. A change in its properties is directly proportional to the amount of CO_2 trapped.

3.6.3.1 Procedure

20 grams of soil was placed in an aluminum boat or aluminum foil perforated with 9 pinholes. The aluminum boat was placed on top of two stacked filter papers in the bottom of the glass jar. A trap assembly was set up as follows: a 10 ml glass beaker was attached to a plastic tripod filled with 9 ml of 0.5 M KOH this was placed on top of the aluminum boat. 7 ml of distilled water was pipetted on the side of the jar to wet the filter papers; this water was then picked up by the soil. The jar was capped and sealed shut and left to incubate for 4 days. As CO_3^{2-} concentration in the trap increases, the electrical conductivity of "trap" solution declines linearly with the absorption of CO_2. After incubation, the Electrical conductivity of the trap solution was measured. To determine the amount of CO_2 absorbed by the alkali trap, we measured the solution's electrical conductivity (EC). With the help of two constants: "EC_{Raw}" which is 0.5M KOH and "$EC_{saturated}$" 0.25M K_2CO_3 (if the alkali trap had absorbed its full capacity of CO_2) and our sample readings, we can

calculate the amount of CO_2 absorbed by the trap. Blank values were subtracted from the sample values to take into account CO_2 coming from the air. [For consistency, all the measurements should be taken at the same temperature.] Therefore:

$$\frac{(EC_{Raw} - EC_{sample})}{(EC_{Raw} - EC_{Sat})} = P$$

Where,

EC_{raw} is the electrical conductivity of pure 0.5 M KOH

EC_{sat} is the electrical conductivity of 0.25 M K_2CO_3

EC_{sample} is the electrical conductivity of the trap associated with a particular sample

P is the proportion of the trap capacity for CO_2 absorption that is actually used [P (trap capacity in mg) = amount of CO_2 in mg absorbed by the trap]

3.7 Plant Toxicity Assays.

3.7.1 Lipid Peroxidation and Membrane Integrity Index

Due to stress, production and accumulation of Reactive Oxygen Species (ROS) in plants is very common and often leads to membrane damage and lipid peroxidation. Plant roots are more sensitive to stress or contamination than any other part of the plant (Das *et al.*, 2017).

3.7.1.1 Lipid Peroxidation

Damage to membrane lipids caused by metal exposure was determined with TBARS (Thiobarbituric Acid Reactive Substances) and membrane integrity index following De Oliveria et al. (2017). Frozen *Oryza Sativa* L. plant material was homogenized with 1.5 ml of 5 % TCA in a freezing mortar pestle placed in an ice bath. After homogenization, homogenate was transferred to centrifuge tubes. Centrifuge was run for 10 min at 10,000g at 25^{o}C. To 1 ml of supernatant, 1 ml of 20% (w/v) TCA containing 0.5% (w/v) TBA was

added. This was heated at 95°C for 30 mins followed by cooling on ice. Measurement of absorbance was done at 532 nm and 600nm (at 600 nm to remove error due to unspecified turbidity) and values were subtracted. Calculation of TBARS (Thiobarbituric Acid Reactive Substances) was done using Lambert-Beer law with an extinction coefficient of 155 µ mol g^{-1} fw (fresh weight).

Effect of TiO_2 NPs on membrane firmness of plant roots was estimated by aid of uptake of Evans Blue dye test (Das et al., 2017). The stain is impenetrable through the membrane of living cells, hence why it serves as an index of the damage to the roots membrane stability. The electrolyte leakage evaluates the membrane integrity in answer to environmental stress. 0.25 g of Evans Blue dye was mixed in 100mL of 0.1M $CaCl_2$ at pH 5.6 for 10 mins or until dissolved. After roots were stained; washed with 100mM $CaCl_2$. Homogenization of roots was done using 1% (w/v) SDS (Sodium dodecyl sulfate), proceeded by centrifugation at 13,500g for 10 mins. Measurement of absorbance was at 600 nm. A graph of absorbance (OD 600) against treatment was plotted.

3.7.1.2 Membrane Integrity Index

Electrolyte leakage due to stress was also used as a measure to estimate membrane injury index by calculating electrical conductivity (De Oliveria et al., 2017). 0.1 g leaf samples of uniform size were placed in test tubes holding 10 mL volume of distilled water. Sets were maintained at 40°C for 30 mins and at 100°C for 15 mins in boiling water. Samples conductivity was measured at both temperatures C_{40} and C_{100} by a conductivity meter. The injury index was computed by the following formula:

$$\text{Injury Index} = \frac{C_{40}}{C_{100}} \times 100$$

3.7.2 Determination of H_2O_2 Generation

Reactive oxygen species are natural by-products of metabolic reactions in plants (photosynthesis, respiration). Due to an unpaired electron, ROS are considered free radicals which are unstable, anti-oxidants in plants are produced to irradiate these free radical species.

Oxidative stress occurs when there is an imbalance between ROS and Anti-oxidant species. Accumulation of ROS leads to rapid cell damage. H_2O_2 is one such very strong ROS. It is capable of causing DNA damage and inactivity in cells even at low concentrations.

3.7.2.1 Procedure

The H_2O_2 was determined following the protocol by Junglee et al. (2014). This is an optimized method combining colorimetric and extraction reaction. Hydrogen peroxide oxidizes iodide ions to iodine when added to a KI solution (initially colorless). The presence of iodide and iodine together leads to a reaction which produces triiodide eventually resulting in a yellowish solution whose absorbance can be read with a UV spectrometer.

0.1g of leaf tissue (fresh wt.) was weighed. For each sample, a control was also prepared. Frozen leaf tissue was grinded to a powder by liquid nitrogen; stored at -80°C till analysis. The frozen samples were homogenized with 1 mL of solution consisting of 0.25mL of 0.1% (wt./v) TCA (Trichloroacetic Acid), 0.5 mL of 1M KI and 0.25mL of Potassium phosphate buffer (10mM, pH 5.8). (This is the "one-step buffer: extraction and colorimetric reaction" combined).

Similarly for control, distilled water was added instead of KI and ground. Samples and solutions were all protected from light. Centrifugation of homogenate was done at 12,000

g at 4°C for 15 mins. Absorbance was measured at 350 nm. For each sample, control values were subtracted from sample values.

For quantification, a calibration curve was obtained by preparing an H_2O_2 standard solution in 0.1% TCA. 0, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2 and 0.5 Micromoles of hydrogen peroxide were used for devloping a standard curve.

3.8 Statistical Analysis

All the results presented here are as means with (± standard error). For each data set, statistical significance was determined by applying analysis of variance (ANOVA) with Honestly Significant Difference (HSD) Tukey test. Effects with $P < 0.05$ are referred to as significant.

RESULTS AND DISCUSSION

4.1 Characterization of TiO_2 NPs

4.1.1 X-ray Diffraction of Titania Nanoparticles

The crystalline size and phase composition of the prepared Titania nanoparticles were determined through XRD analysis as shown in Figure 4.1. The spectrum indicates that TiO_2 nanoparticles were crystalline and no amorphous phase was observed. Solid diffraction peaks at 25.30º (101), 37.8º and 48.03º confirm that the 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 compute the crystalline size of TiO_2 NPs and was found to be 54.6 nm.

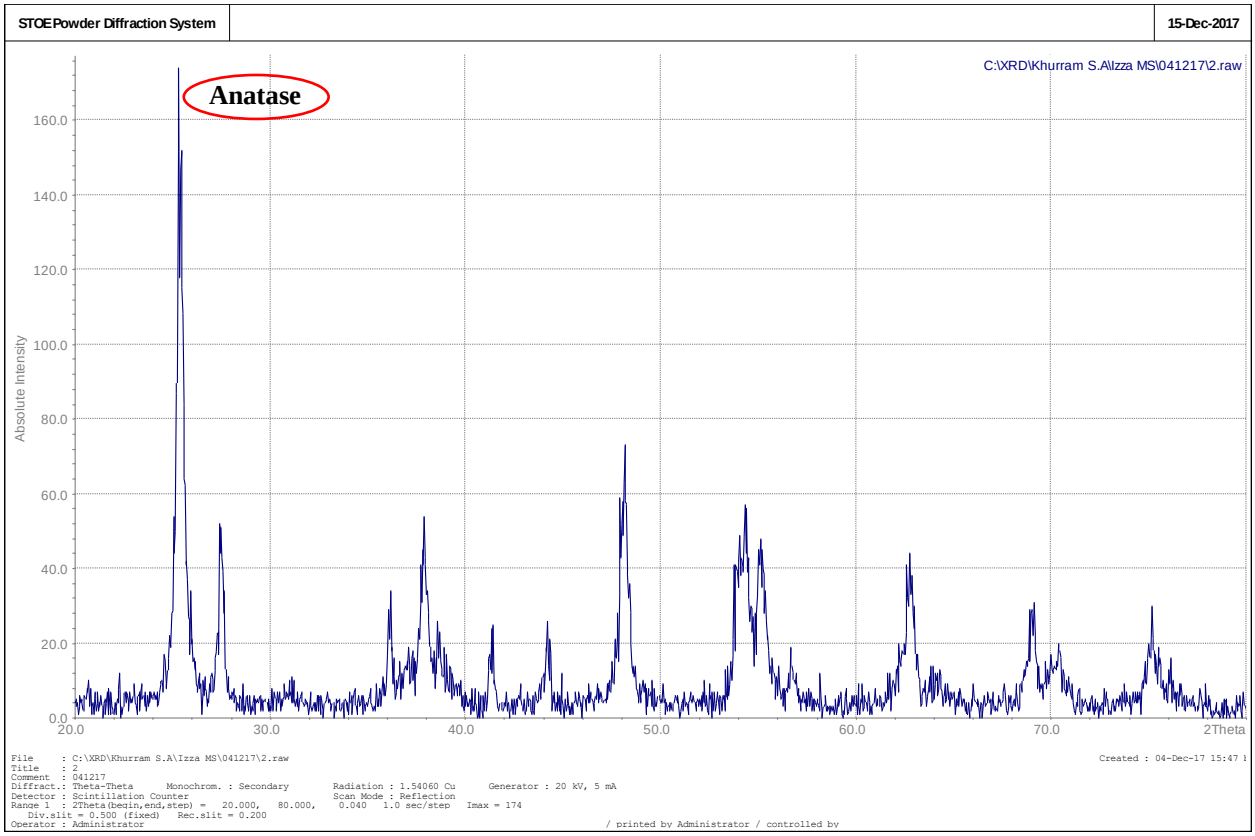

Figure 4.1: Phase identification of synthesized TiO_2 NPs through XRD

4.1.2 Scanning Electron Microscope (SEM) Imaging of TiO_2 NPs

Surface morphology of Titania nanoparticles was estimated by SEM. The image at a magnification of 12k X shows pure Titania particles, displaying that 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)

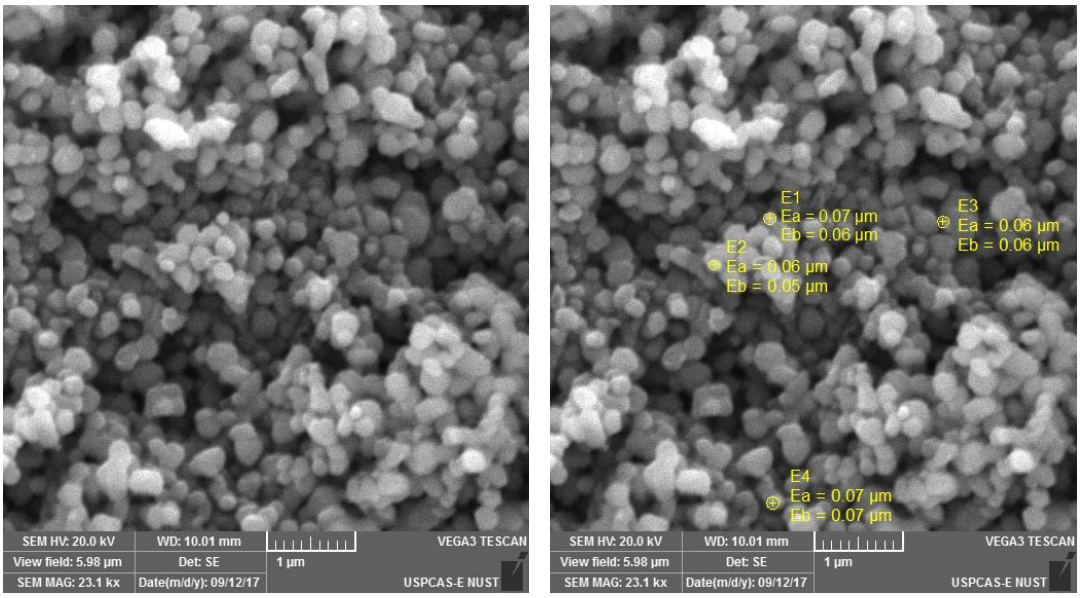

Figure 4.2: Morphological characterization of synthesized TiO_2 NPs through Scanning Electron Microscope (SEM)

4.2 Preliminary Characterization of Experimental Soil

Table 4.1 shows the results for certain physical and chemical characteristics of the experimental soil. Prior to pot experiment, different soil tests were performed for each soil including pH, electrical conductivity, total organic carbon, moisture content, water holding capacity and the concentrations of Nitrogen and Phosphorus. The texture of the soil used for this experiment was silt loam and clay, each with a pH of 7.25 and 8.70 respectively. Rice is best grown in soils with pH in the range of 5.5-6.5 however since most plants are highly adaptable they can flourish on pH values outside of this; pH 4-8 is also acceptable

for rice growth (Rice FAQs, Mian Abdul Majid, Pakistan Agricultural Research Council). Soil pH levels play a significant role in plant nutrient availability hence it should be taken into consideration to get a high plant yield.

Total organic carbon was 0.95% in silt-loam soil with nitrogen at 1.22 mg kg^{-1} and available phosphorus at 39 mg kg^{-1}. Clay soil had a higher level of total organic carbon: 1.8% with 28.8 mg kg^{-1} nitrogen and 64 mg kg^{-1} of available phosphorous. These concentrations were taken into consideration when calculating doses of nitrogen and phosphorus per pot.

Soil texture plays a pivotal role in rice production with respect to its water holding capacity or "available water capacity" (AWC). A soil's available water holding capacity is related to its organic matter. As clay soils are high in organic matter than sandy and silty soils, hence they have higher soil AWC's. (Dou *et al*., 2016). According to a study conducted on soils' organic matter (OM) and AWC by Hudson (1994), a rise in OM from 0.5%-3% doubled the soils AWC. Likewise, a loss in organic matter combined with compaction of soil affected the soils' AWC thereby affecting the crops' yield.

Soil texture also affects overall plant root growth. Larger roots generally have more potential to elongate therefore 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)	Clay (Swat)
pH	7.25	8.70
EC (µS/cm)	253	197
Moisture Content (%)	2.04	10.61
Water Holding Capacity (%)	40.6	60.0
Soil Organic Carbon (%)	0.95	1.80
Extractable Phosphorous (mg kg^{-1})	39.00	64.00
Nitrate Nitrogen (mg kg^{-1})	1.22	28.8

4.3 Effect on Growth Parameters

The first phase focused on changes in plant physiological growth parameters under applied treatments. Root and shoot length along with biomass was recorded. The results showed that growth at 750 mg kg^{-1} for both soils was very low.

4.3.1 Root and Shoot Length

Figures 4.3(a) and 4.3(b) depict the effect of TiO_2 NPs on plant root and shoot length for both soil textures. Maximum root and shoot length were both observed in clay soil at 500 mg kg^{-1} of TiO_2 NPs only. Root and shoot length increased by 42.8% and 13.2% respectively. Similarly, at the same dosage in silt-loam root and shoot length both decreased by 24.8% and 8.8%, correspondingly.

At 750 mg kg^{-1} in clay, root growth was comparatively better to control; an increase of 36.1% was observed while shoot growth decreased by 16% in comparison to control and

500 mg kg^{-1}. Comparing root and shoot length between the two treatments, a decrease of 6.7 and 29.2% were seen respectively.

Silt-loam showed overall poor growth at 750 mg kg^{-1}. Comparing 750 mg kg^{-1} and 500 mg kg^{-1}, in silt-loam, a decrease was seen in both root and shoot lengths by 8.8% and 4.4% correspondingly.

Increase in root-shoot length in clay at 500 mg kg^{-1} can be credited to the fact that 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).

Likewise increase in root length observed in clay for 750 mg kg^{-1} 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^{-1} of TiO_2 NPs and observed an increased in the growth of *Lactuca sativa* L. (Lettuce), root-shoot by 36.0% and 34.6%, individually. Another research conducted by Zahra *et al.* (2017) observed an increase of 13.8% in *Oryza sativa* L. (Rice) growth at 500 mg kg^{-1}. Likewise, a research conducted by Irum (2017) showed an increase in shoot length by about 84% when treated with 500 mg kg^{-1} TiO_2 NPs. 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^{-1} showed a decrease in root and shoot length (Aliabadi *et al*., 2016). Similarly, a study conducted in IESE by Nisar (2017) showed a decrease in both root-shoot lengths at a concentration of 750 mg kg^{-1} TiO_2 NPs.

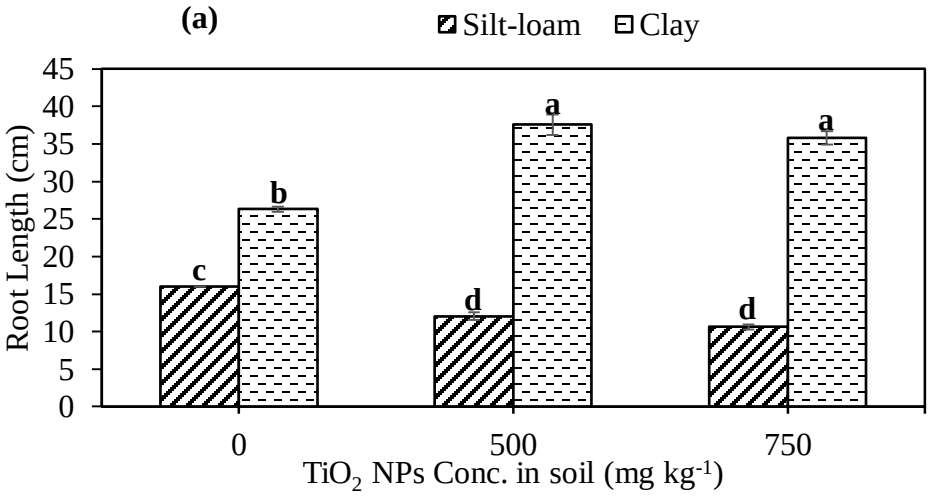

Figure 4.3 (a): Effect of TiO_2 NPs on root length (RL) in silt-loam and clay soil.

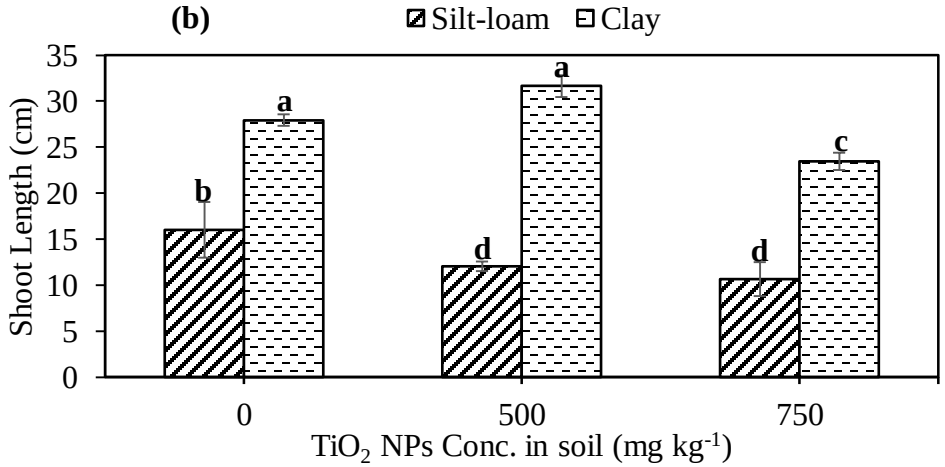

Figure 4.3 (b): Effect of TiO_2 NPs on shoot length (SL) in silt-loam and clay soil.

4.3.2 Root and Shoot Biomass

Figures 4.4 (a) and (b) show the impact of Titania nanoparticles on root and shoot dry weight in both soil textures. The results are in accordance with the trends seen in root and shoot length; root and shoot weight showed an increase at 500 mg kg^{-1} in clay while biomass for both (root-shoot) decreased as the concentration of TiO_2 NPs increased. Both

treatments showed an overall decrease in silt-loam with an increase in the concentration of TiO_2 NPs.

In silt-loam soil, a decrease was observed with increase in TiO_2 NPs concentration, root and shoot biomass both decreased by 43.4 and 21.2% in 500 mg kg^{-1} and 61.9 and 63.5% in 750 mg kg^{-1} compared to control. Between treatments, a decrease of 0.3 and 0.5% was seen in 750 mg kg^{-1}.

However, TiO_2 NPs performance was far better in clay soil at 500 mg kg^{-1}. 500 mg kg^{-1} showed the highest root and shoot biomass at 63.3 and 56.9% respectively compared to control. Conversely, 750 mg kg^{-1} showed a decrease of 18.5 and 34.3% in root and shoot biomass correspondingly compared to control.

Similar results were observed in a study by Zahra *et al.*, (2017); rice was treated at 500 mg kg^{-1} of TiO_2 NPs concentration, an increase of 45.9% was observed in total dry biomass compared to control. Likewise, an increase in plant biomass of *Lemna minor* has been reported at 200 mg L^{-1} of TiO_2 NPs concentration (Song *et al.*, 2012) Thus, our obtained results shown in table 4.2, coincide with the existing reports in the literature.

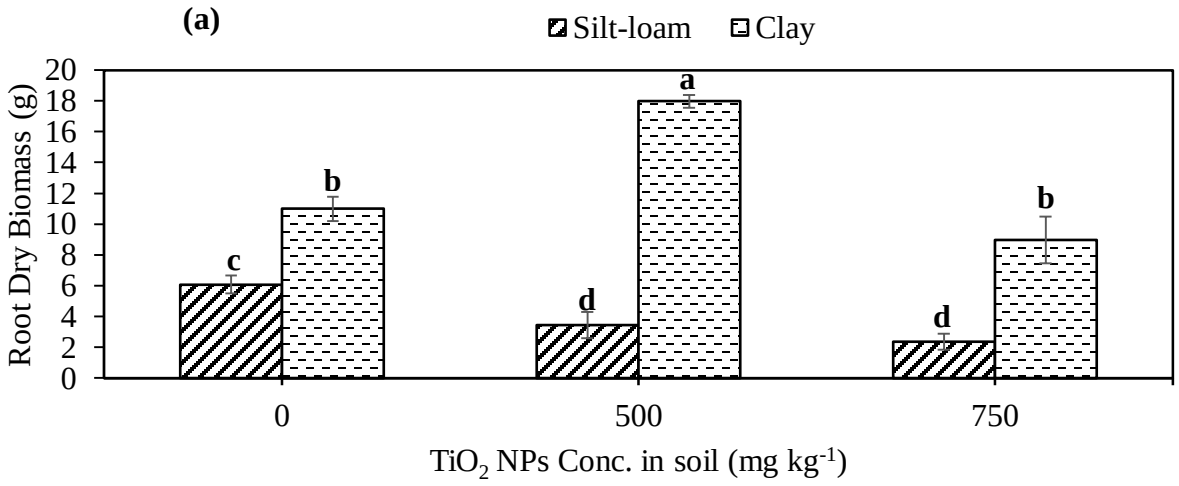

Figure 4.4 (a): Effect of TiO_2 NPs on root dry weight (RDW) in silt-loam and clay soil

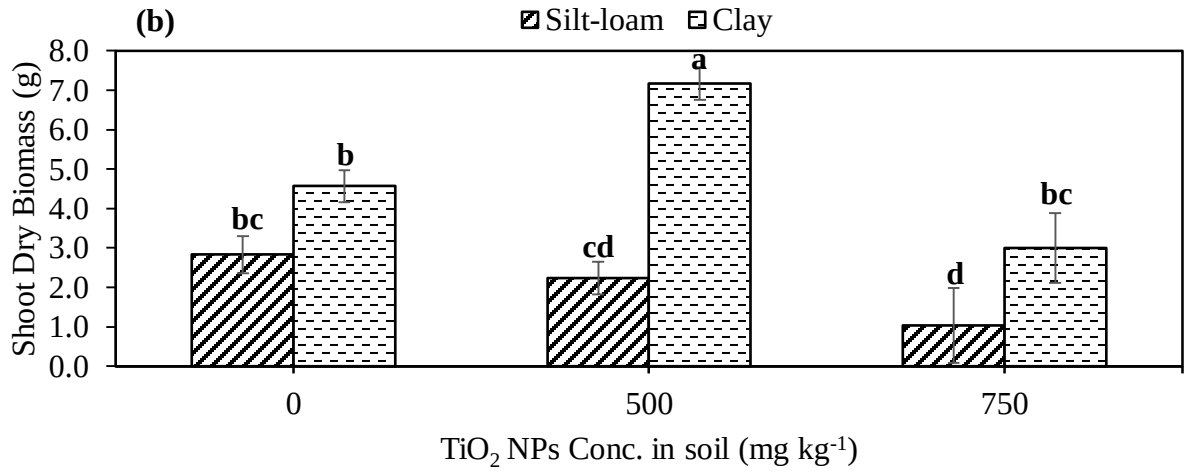

Figure 4.4 (b): Effect of TiO_2 NPs on shoot dry weight (SDW) in silt-loam and clay soil.

Table 4.2: Effect of TiO_2 NPs concentration on growth parameters of rice

TNPs (mg kg^{-1})	Treatments	RL (cm)	SL (cm)	RDW (g)	SDW (g)
Silt-Loam	**0**	16.00 ± 0.00^{c}	23.50 ± 3.04^{b}	$6.07 (\pm 0.59)^{d}$	$2.83 (\pm 0.47)^{c}$
	500	12.03 ± 0.50^{d}	21.43 ± 0.51^{b}	$3.43 (\pm 0.85)^{e}$	$2.23 (\pm 0.42)^{cd}$
	750	10.63 ± 0.32^{d}	20.40 ± 1.85^{b}	$2.37 (\pm 0.41)^{e}$	$1.03 (\pm 0.95)^{d}$
Clay	**0**	26.30 ± 0.30^{b}	27.93 ± 0.60^{a}	$11.00 (\pm 0.65)^{c}$	$4.57 (\pm 0.40)^{b}$
	500	37.57 ± 1.37^{a}	31.63 ± 1.18^{a}	$17.97 (\pm 0.34)^{a}$	$7.17 (\pm 0.42)^{a}$
	750	36.47 ± 0.89^{a}	29.5 ± 0.92^{a}	$13.97 (\pm 1.25)^{b}$	$5.67 (\pm 0.89)^{b}$

Where, mean values for the treatments within a column following different alphabet are significantly different at $p < 0.05$ by Tukey's Honestly Significant Difference (HSD) Test, RL=Root Length, SL=Shoot Length, RDW= Root Dry Weight, SDW =Shoot Dry Weight.

The lack of growth observed in silt-loam soil furthers the fact that soil texture plays an essential role in plant growth. Silt soils, in general, hold less water and hence fewer nutrients providing an easier passage through its aggregation. On the contrary, clay soil has finer particles that can retain nutrients and water better for hydrophilic rice to grow in than silty soils. According to a study done by Tsubo *et al.* (2007) in a rain-fed area of lowland Thailand, rice grown in soils with higher clay content had better growth and biomass than in soils with low clay content.

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 radishes 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^{-1} 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 Post-harvesting Soil pH and Organic Matter

Table 4.3 Changes in soil pH due to the planting of rice

Soil Texture	**Silt-loam**	**Clay**
Preliminary pH	7.25	8.70
Control	6.95	7.00
500 mg kg^{-1}	6.95	7.00
750 mg kg^{-1}	6.95	7.00

Table 4.3 shows the change in soil pH before and after rice growth. For plant growth, the soil is an indispensable and intricate medium. Plants interact with microbial communities, soil organic matter (SOM) and minerals present in the soil. Apart from soil texture, soil pH and its organic matter play a pivotal role in the fate of NPs. Therefore after NPs are introduced into a soil matrix, these factors affect the NPs performance, bioavailability, and kinesis. On the contrary, Titania Nanoparticles are very stable in soil (Schmidt & Vogelsberger, 2006). Moreover, the behavior of Titania nanoparticles is highly dependent on the clay content and composition of the soil (Tan *et al.,* 2018). But these soil factors do not influence the behavior of TiO_2 NPs on a separate level, rather they are linked with each other. Two such soil properties that work together are soil organic matter and pH. Taking into account our plant, *Oryza* sativa L., changes in these factors were also observed that further affected the behavior of TiO_2 NPs.

Usually, soil pH of paddy soils tends to increase or decrease in value due to submergence depending on its initial pH. The submergence of soil in water brings a series of chemical alterations out of which pH is the most important. Submergence causes the consumption of protons via reduction processes. The increase/decrease in pH is related to the soils organic matter as it is its' reduction to an oxidized form.

As soil pH affects many physical, chemical and biological properties of the soil, this, in turn, can affect the growth of rice plants indirectly or directly. However, the relationship between the reduction process and soil pH is not that linear. A reason for this is that upon submergence of soil, acid-forming radicals are produced due to microbiological activities. These radicals are responsible for lowering pH in alkaline soils (Yu, 1991).

In a study conducted by Yu (1987) on alkaline paddy soils, the pH decreased from 8.0 to 7.0 in the first two weeks of submergence and then remained steady, while the pH of the neutral soil more or less remained unchanged. These drops in pH are highly characteristic of paddy soils.

In a study focusing on the impact of engineered nanoparticles and soil characteristics, it was found that TiO_2 NPs does not affect soil pH or bring any changes in it rather pH affects its behavior as mentioned above (Simonin *et al.,* 2015). Hence the change in pH observed

here is formerly due to the growth of rice which further influenced the TiO_2 NPs performance in each soil type.

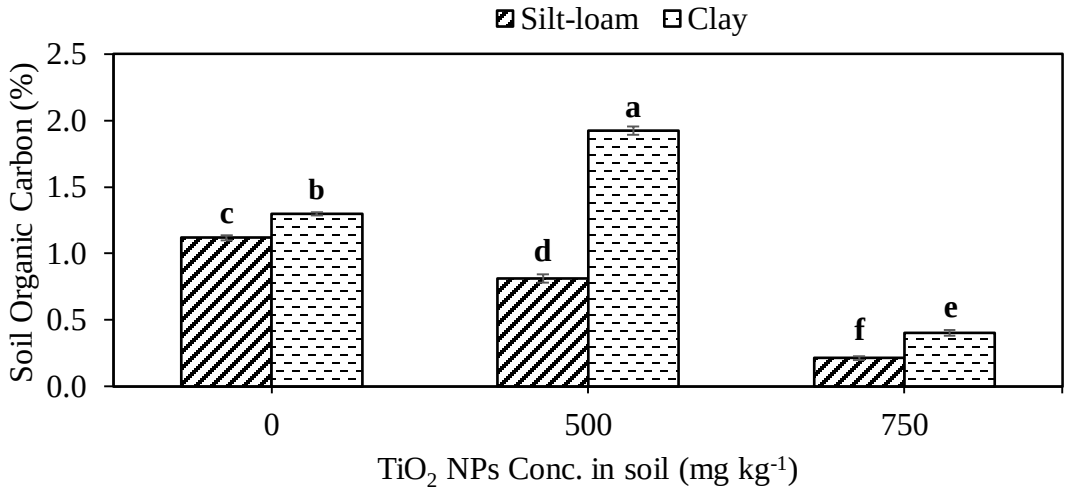

Figure 4.5: Changes in organic matter post harvesting and treatment.

Figure 4.5 shows changes in OM post plantation and treatment. As shown, there is a significant ($p > 0.05$) change in organic matter in both soil types. A decrease of 27.4% at 500 mg kg^{-1} and 80.8% at 750 mg kg^{-1} was seen in silt-loam at both treatments of nanoparticles. On the other hand, a 35.6% increase of OM was seen at 500 mg kg^{-1} of TiO_2 NPs. While, at 750 mg kg^{-1} a decrease of 67.6% in OM was noticed from control.

Just as rice paddy soil shows changes in pH, a similar phenomenon is observed when it comes to soil organic matter (OM). Cultivation of rice has shown to increase soil organic matter in soils and under long-term cultivation of rice over the years, it is known to increase the soils organic carbon storage and sequestration potential as well (Wang *et al.,* 2015).

The natural organic matter of a soil can influence the behavior of TiO_2 NPs by altering properties of the soil particle surface. According to several other studies, regardless of dosage, size or method of synthesis, anatase Titania nanoparticles with no surface coating have a negative charge. (Tan *et al.,* 2018, Simonin *et al.,* 2015) The organic matter present

in the soil would easily adsorb on to the negatively charged TiO_2 NPs bringing a change in the nanoparticles' surface properties and charge. This, in turn, can cause "steric hindrance" causing a change in how the NPs react, deposit or aggregate. Increased coating of soil organic matter on to Titania nanoparticles will decrease aggregation but increase the surface charge (Tan *et al.*, 2018). This is where pH comes into play. According to studies, the adsorption of organic matter onto Titania nanoparticles depends on pH. Mudunkotuwa & Grassian (2010) observed that at a pH of 6, Titania nanoparticles showed increased instability and formed huge aggregates but were stabilized in the presence of humic acids (citric acid). Likewise, studies on how OM plays a major role in stabilizing and dispersing Titania nanoparticles in soil show similar results (French *et al.*, 2009, Keller *et al.*, 2010). Thus, the bioavailability of Titania nanoparticles is highly dependent on pH and Organic matter, which affects the stability, mobility, and aggregation of Titania nanoparticles. This, in turn, can enhance or diminish the toxicity of TiO_2 NPs (Simonin *et al.*, 2015).

The coating of NPs by OM can be both beneficial and detrimental to the soil and plant. As the coating increases their bioavailability, it also leads to more interaction with the microorganisms in the soil. And depending on the dosage, exposure time and the toxicity of TiO_2 NPs it can either result in a "rich" soil or prove fatal to the community. Likewise, it is possible for NPs to aggregate and form large agglomerates which reduce their Nanotoxicity as it reduces direct interaction with the microorganisms in the soil (Wu *et al.*, 2010).

For our results in clay 500 mg kg^{-1}, as OM in clay soil was high it lead to less formation of aggregates and a better performance by the Titania nanoparticles which aided in an improved growth of rice. While at 750 mg kg^{-1}, just like in 500 mg kg^{-1}, a coating of OM

increased their bioavailability, it did so too at 750 mg kg^{-1} making them bioavailable, but as the concentration of TiO_2 NPs is too high, its effects can easily be seen in plants growth.

4.5 Soil Toxicity Assessments

4.5.1 Soil Microbial Biomass

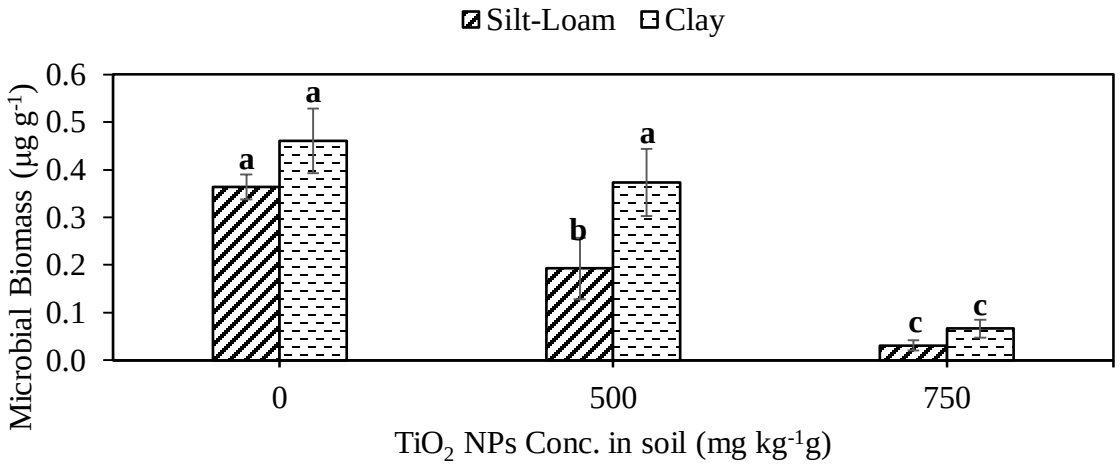

Figure 4.6: Changes in soil microbial biomass with an increase in TiO_2 NPs concentration.

The soil is the most essential and complicated matrix for plant growth. Plants interact with microbial communities, natural organic matter, and soil minerals. Besides biological indicators such as the soils indigenous organisms and its biotic characteristics play a vital role in the health of the soil as an ecosystems functionality is largely dependent on the workings of the soil microbial community.

The above figure shows the changes in the soil microbial biomass with an increase in the concentration of TiO_2 NPs. Both soils showed a noticeable decrease in the microbial community with an increase in TiO_2 NPs. Even though the best growth for rice was seen at 500 mg kg^{-1} in clay soil, there was still a negative effect of Titania on the microbial community. A decrease of 19% was observed in 500 mg kg^{-1} and a drastic decrease of 85% was seen at 750 mg kg^{-1} compared to control. Among treatments within the clay, a decrease of 66% was seen at 750 mg kg^{-1} compared to 500 mg kg^{-1}. Silt-loam showed a drastic

decrease of the microbial community at both treatments; 500 mg kg^{-1}: 47.0% and 750 mg kg^{-1}: 91.4% and comparison between treatments showed a decrease of 44%.

Nanoparticles can indirectly or directly affect the population, activity, and diversity of bacteria by bringing changes in the soils water content, total oxygen, total suspended solids, soil enzymes, electrical conductivity and nutritional elements (Tan *et al.*, 2017).

Exhaustive studies indicated that Titania nanoparticles brought noteworthy changes in the diversity of microbial biomass and reformed its composition with increasing dosage of TiO_2 NPs (Tan *et al.*, 2017). Many nanoparticles are reported to be toxic to the soils microbial community, hence directly affecting microorganisms. For instance, a study conducted in 2010 by Fang *et al.* showed that TiO_2 NPs damaged the cell membrane of *Nitrosomonas europaea*, which eventually lead to high permeability in cells and proved fatal to the cell resulting in its death. Furthermore, Fan *et al.* (2014) tested exposure of TiO_2 NPs on *Rhizobium*, a common nitrogen fixer, found in paddy soils, the results showed that it's damaged the bacteria's cell surface and changed its cell walls "polysaccharide composition". Moreover, it disrupted the symbiotic relationship between the bacteria and plant and caused a deferment in nitrogen fixing.

Contrastingly, Titania nanoparticles show a varying range of results, at best their influence on soil microorganisms is ambiguous as there are no standard methods to track their movements in the soil. According to different studies, it is either known to improve bacterial communities or bring a drastic decrease in them (Ge *et al.*, 2012, Burke *et al.*, 2014). Furthermore, soil texture aids in this diversity of results; in a study by Ge *et al.* (2011), TiO_2 NPs treated grassland soil reduced the microbial community severely while in comparison a study by Shah *et al.* (2014) observed an increase in soil richness. As

discussed before, this divergence in results is the cause of several factors working together such as the soil's properties (pH, OM, Texture, Clay content), the exposure time and dosage and the characteristic toxic properties of NPs used (Xu *et al.,* 2015).

In general, the specific properties of the NPs used, that is its "high surface area to volume ratio" and its Nano size enable them to travel and move through the soil medium, interacting with microorganisms and their compounds (Vittori-Antisari *et al.,* 2013). This results in the incapacitation of the microbial community. Hence, results suggest that TiO_2 NPs are injurious to the soils microbial communities. Also, toxicity on soil microbial activity is likely to have a grander impact than phytotoxicity (Kim *et al.,* 2011).

4.5.2 Dehydrogenase Activity

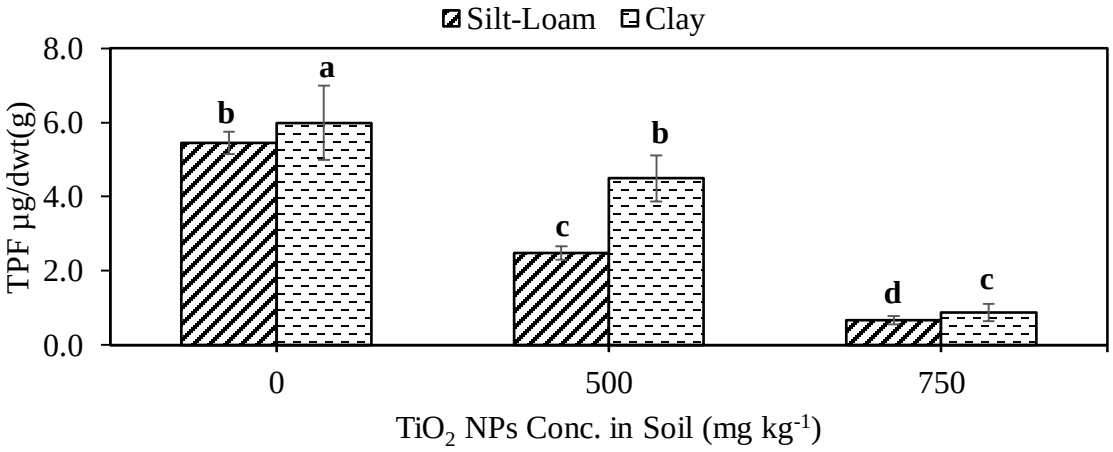

Figure 4.7: Changes in dehydrogenase activity with an increase in TiO_2 NPs

The figure above shows the changes in dehydrogenase activity with an increase in TiO_2 NPs for both soil textures. Overall there was a decrease in dehydrogenase activity at both soil textures at both dosages of TiO_2 NPs. Silt-loam showed a radical decrease in activity at both concentrations of TiO_2 NPs. At 500 mg kg^{-1} a decrease of 54.3% was observed while at 750 mg kg^{-1} a substantial decrease of 79.8% was seen compared to control. Within treatments, a decrease of 26.4% was noted. Equally, treatments on clay showed a decline

in enzymatic activity. 31.1% and 81.7% decline in activity was noted at 500 mg kg^{-1} and 750 mg kg^{-1} respectively in comparison to control. Between treatments, a drop of 50.6% was observed.

A soils biochemical and microbiological activity is often seen as an initial and delicate indicator of ecological stress in different ecosystems. Enzymes play a pivotal role in the cycling of soil nutrients, mineralization of organic carbon and transformation of plant nutrients. Furthermore, it reveals the soils "self-purifying" potential from impurities.

According to literature, even low concentrations of TiO_2 NPs are highly responsive to dehydrogenase activity (Nel *et al.,* 2006). Because of the small size and increased surface to volume ratio of nanoparticles, it increases the rivalry between substrates to adsorb to "enzyme binding sites". Furthermore, several studies show that enzyme activities in soils decreased in the presence of heavy metals and metal oxides with high inherent toxicity (Du *et al.,* 2011). Enzyme activities are inhibited when there is a reaction with active protein groups of enzymes or when complexes between substrates and binding sites are formed. This inhibits or makes the enzyme inactive (Dick *et al.,* 1997; Kizilkaya and Bayrakli, 2005). Besides, changes in the microbial community due to stress also affects soil enzymatic activities.

Several studies report a negative effect of nanoparticles on enzymatic activities of soil. ZnO, CuO, Cr_2O_3 and Ni NPs at concentrations of 10, 100 and 1000 $\mu g g^{-1}$ soil displayed inhibiting effects on the activities of acid and alkaline phosphatases, dehydrogenase, and urease (Jośko *et al.,* 2014). Similarly, ZnO NPs at a concentration of 2000 $\mu g g^{-1}$ soil) demonstrated inhibitory effects on the activities of acid phosphatase, dehydrogenase and ß-glucosidase (Kim *et al.,* 2011).

But as always, multiple factors contribute to the effects of nanoparticles. As stated by Jośko *et al.* (2014), soil contact time and the adaptation of microorganisms to stress factors play an essential role in a delaying or encouraging negative effects of NPs. And in this case, the growth of rice and the flooded conditions of soil may also aid in a variation of dehydrogenase activity. According to Zeng *et al.* (2007) who monitored dehydrogenase activity thorough out rice's life cycle; dehydrogenase showed an increase in the first two weeks after transplantation and from then on exhibited a linear decline with rice development. Furthermore, dehydrogenase activity is strongly influenced by soil moisture. Literature reports that flooded and anaerobic soil conditions have a higher DHA than non-flooded soils (Trevors, 1984; Subhani et al., 2001). However according to Wolińska and Stępniewska (2012), a decline in DHA with increase in soil moisture/ soil water content is based on the fact that flooded soils have an expressively increased "electron transport system" which in this case coupled with rice growth and TiO_2 NPs, shows a decline in DHA (Wolińska and Stępniewska., 2012).

4.5.3 Soil Respiration

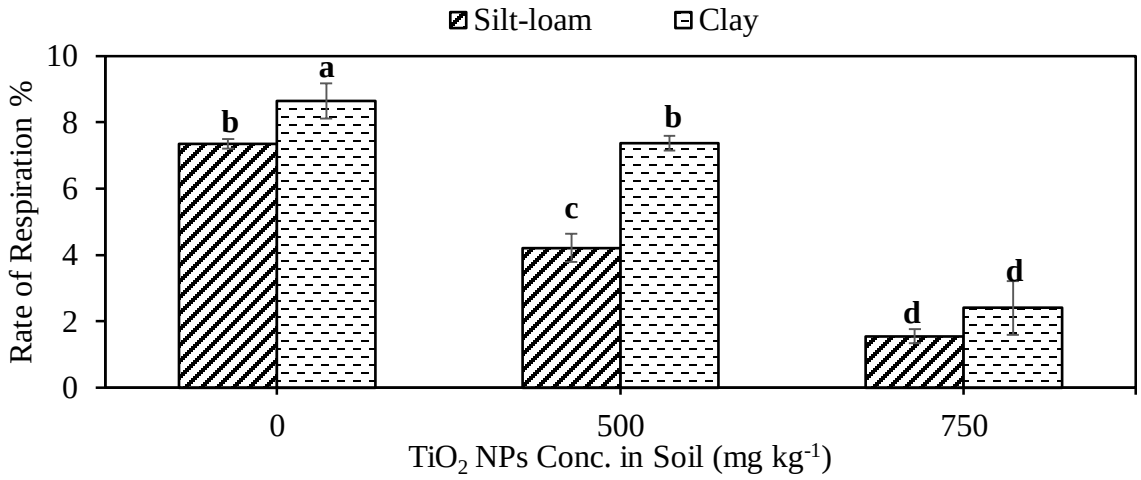

Figure 4.8: Rate of soil respiration with an increase in the concentration of TiO_2 NPs

As seen in figure 4.8, 750 mg kg^{-1} of Titania nanoparticles is causing toxicity in both soil textures. A decrease of 78.8% and 72.1% in respiration is seen in silt-loam and clay respectively in comparison to control. In the same way, a decrease in overall soil respiration is also observed at 500 mg kg^{-1}, with 42.6% and 14.8% in silt-loam and clay correspondingly in contrast to control. Soil respiration rate was closely associated with soil microbial biomass and enzymatic activity.

Soil respiration entails the richness and activeness of the soils microbial community and therefore is a direct measure of the soils biological processes. It specifies the health of the soil community thereby giving us a perception of decent soil structure: their ability to make nutrients available, mineralizing deposits, accepting changes and storing and safeguarding nutrient availability with time.

According to Zeng *et al.* (2007), Soil respiration in paddy soils has a zigzag trend with respect to rice growth. It tends to increase initially, then decrease and then increase linearly as rice reaches maturity. However, the decrease here is also due to the presence of Titania

nanoparticles. In a study by Ge *et al.* (2011) testing environmentally acceptable concentration of TiO_2 NPs, (0.5-2.0 mg kg^{-1}) after every 3-4 days, saw a decrease in overall soil respiration. Even at such a low dosage, TiO_2 NPs had a toxic effect on the soils bacterial community making it highly plausible to bring substantial changes in the soil processes beneficial to the environment. In the same way, CuO NPs at 10 mg kg^{-1} reduced bacteria's hydrolytic activity, redox potential, and basal respiration along with changes in the composition of the soil bacterial community (Frenk *et al.*, 2013).

The current literature generally shows both positive and negative effects of metal oxide nanoparticles on the soil microbial community and enzymatic activity. Additionally as mentioned before, soil properties namely pH, redox potential and organic matter play a huge role in how the nanoparticle is likely to affect the soil matrix. Hence why the same nanoparticles are likely to show distinct results depending on soil texture, dosage concentration and time of exposure. Therefore, it is imperative to explore the effects of a spectrum of Nano-metal oxides in different soil individualities. This will aid in setting up an organized assessment system to study their effects on soil.

4.6 Plant Toxicity Assays

Assays were performed on rice plant tissue to check the effect of Titania nanoparticles in two different soil textures. Toxicity was measured by quantifying electrolyte leakage with Evans blue dye binding assay and Conductivity assay. Along with measuring reactive oxygen species by lipid peroxidation through TBARS (Thiobarbituric Acid Reactive Substances) and by quantification of hydrogen peroxide production.

4.6.1 Reactive Oxygen Species and Membrane Damage

Reactive Oxygen Species are formed as a result of incomplete reduction of oxygen. The transfer of 1, 2 and 3 oxygen electrons result in O_2^- (superoxide radical), H_2O_2 (Hydrogen peroxide) and $HO^{\cdot}$ (Hydroxyl Radical) respectively. Out of this hydrogen peroxide has the longest half-life of 1 millisecond while the superoxide and hydroxyl radical have a relatively shorter half-life of 2-4 µs (Kao, 2014). ROS was originally documented as harmful by-products of aerobic metabolism removed by the plants' own antioxidant system. However, in the past decade, it has become clear that ROS has a dual role in the plant.

Rather than just being a destructive by-product it also a "signal transduction" molecule. The signaling helps in controlling plant growth and development along with reactions to changes in the environment and abiotic and biotic factors (Bailey-Serres *et al.*, 2016). ROS and lipid peroxidation work hand in hand, an increase in ROS levels eventually lead to cell membrane damage and generally their relationship is linear.

4.6.1.1 Effect of Titania NPs on Plant Hydrogen Peroxide content

In recent years, H_2O_2 has been scrutinized by scientists extensively, because of its small size and its ability to penetrate cell membranes and traverse into different cellular compartments, aiding it in its "signaling" functions. Acquired data proves that hydrogen peroxide monitors a myriad of a biological process such as firming of the cell wall, increasing overall resistance, photosynthesis, regulating growth and stress stimuli (Ismail *et al.,* 2014). Even with the benefits it possesses, it is still a harmful molecule and more so

at high concentration, hence a balance between removal and production of ROS is required.

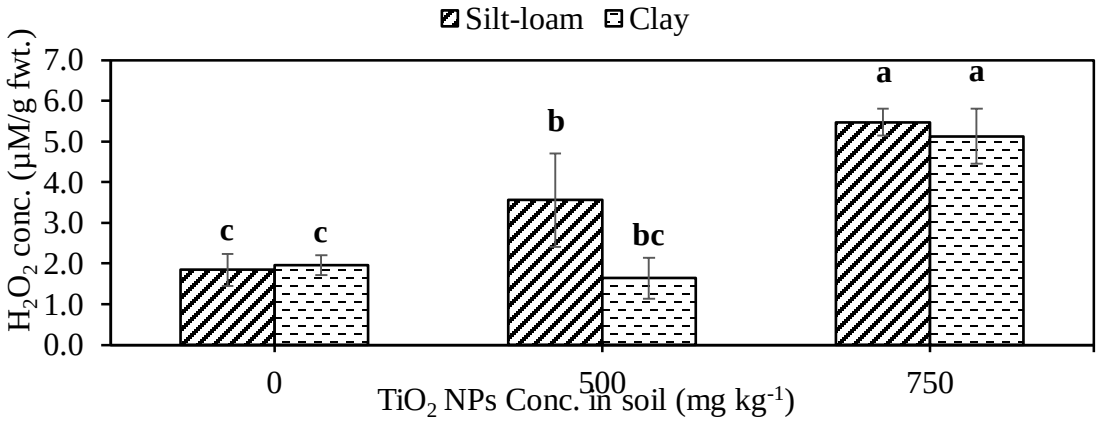

Figure 4.9: Effect of TiO_2 NPs on Plant Hydrogen peroxide production in both soil textures.

The graph shows an overall increase in H_2O_2 activity in both soil textures except for clay at 500 mg kg^{-1} of TiO_2 NPs. In silt-loam, an increase of 92.9% and 196.5% was seen at 500 mg kg^{-1} and 750 mg kg^{-1} compared to control. On the other hand, a decrease of 23% in hydrogen peroxide production was noted at 500 mg kg^{-1} in clay compared to control while a drastic increase of 431.8% was detected at 750 mg kg^{-1} in comparison to control.

Several studies observing the effect of nanoparticles and ROS formation have confirmed that NPs "cause" the increase in Reactive Oxygen Species in plants (Ma *et al.,* 2015; Rico *et al.,* 2015; Rafique *et al.,* 2018). As discussed above, copious amounts of ROS can eventually be fatal to plant systems causing damage to proteins, DNA and lipid membranes (Wani *et al.,* 2016; Kumar *et al.,* 2018). As a consequence of overproduction of ROS, the plant has a receptive antioxidant system that alleviates the damage. However, depending on the concentration of TiO_2 NPs, or other stresses, this is sometimes compromised as enzyme (antioxidant) activity is inhibited due to decreased synthesis because of changes in

active sites (Rao and Shekhawat, 2016). A study conducted by Du *et al.* (2011) showed that Titania and Zinc oxide NPs both prohibited enzyme (catalase, peroxidase, and protease) activity in wheat at concentrations of 100 and 50 mg kg^{-1}. In addition to this, the effect of hydrogen peroxide is mostly reliant on its concentration but factors like the plant's developmental stage, its production site in the plant, and exposure to various stresses also contribute to its biological effect (Petrov and Breusegem, 2012).

As conferred earlier, H_2O_2 is not just a toxic molecule, it also acts as a "signaling molecule" aiding in a myriad of physiological functions out of which one is an escalation in plant growth. Both of these functions can be observed in the different response of rice grown in clay soil at two concentrations of TiO_2 NPs. At 500 mg kg^{-1} production of H_2O_2 is lower than that of control, along with maximum growth (root and shoot length both). According to Gechev and Hille (2005), hydrogen peroxide acts as a "signaling molecule" at low concentrations. In addition, as an H_2O_2 molecule can easily traverse cell membranes; at low concentrations, it aids and encourages the transport of water and solutes between cells (Petrov and Breusegem, 2012). A research conducted by Benabdellah *et al.* (2009) reported that H_2O_2 exercises "concentration-dependent" effects on hydraulic uptake of roots in *Phaseolus vulgaris* (common bean). They found that H_2O_2 levels less than 1 µM increase conductivity of water whereas more than 1 µM decreases it. Furthermore, a study conducted on Arabidopsis roots showed that low concentrations promoted "plasmodesmal permeability" while higher concentrations repressed it (Rutschow *et al.,* 2011). This coincides with our results as highest root growth is observed at 500 mg kg^{-1} in rice grown in clay soil.

At 750 mg kg^{-1}, the concentration is 2 folds higher than in control in the same soil, this can be possible due to two reasons: either the antioxidant system has been compromised that is to say that enzymes that scavenge ROS have been inhibited due to the high concentration of NPs. Secondly, NPs higher than 40 nm, gather in the apoplastic space in plant roots, this can cause clogging of plant structures (stomata and plasmodesmata) that regulate the flow of water (García-Sánchez *et al.,* 2015).

In essence, low levels of the H_2O_2 act as "signaling molecules" as they produce a weak signal for stress which is easily mitigated by the plant by increasing the stream of nutrients and solutes. On the other hand, high levels of H_2O_2 send a stronger stress signal requiring the plant to remove damaged and stressed cells (Rutschow *et al.,* 2011).

4.6.1.2 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 causing the destruction of 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)

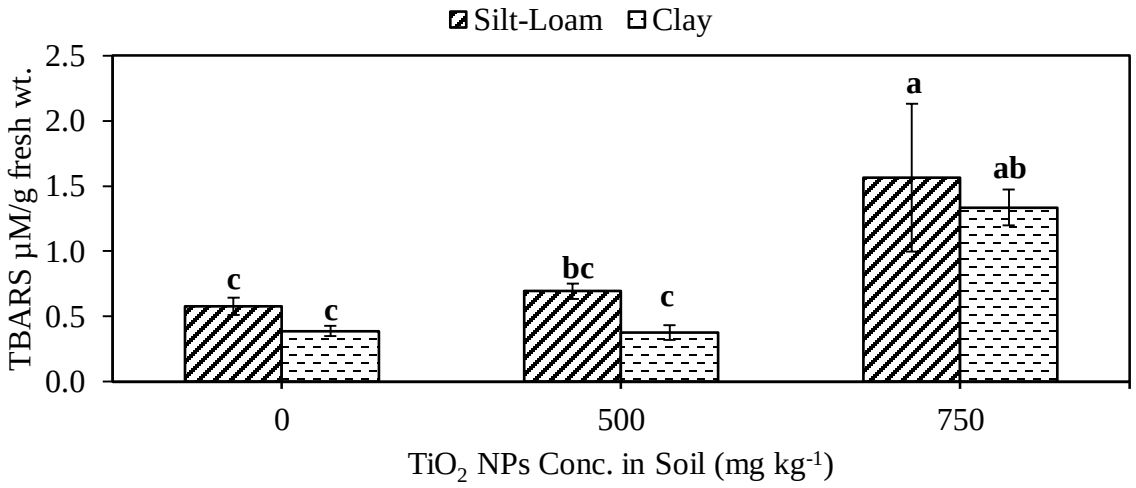

Figure 4.10: Lipid peroxidation in rice treated with TiO_2 NPs in contrasted soil.

The above figure shows lipid peroxidation in rice shoots treated with two concentrations of TiO_2 NPs. Highest production of TBARS is seen at 750 mg kg^{-1} in both soils. An increase of 171.3 and 245% was observed in silt-loam and clay respectively. At 500 mg kg^{-1} in clay soil, there was no difference in TBARS production while in silt-loam an increase of 20.1% was seen compared to control.

A definite amount 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^{-1} in silt-loam. Even though the 500 mg kg^{-1} 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^{-1} of nano-CeO_2 had an evident increase in H_2O_2 content but prompted low

membrane damage. 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)

But as observed in 750 mg kg^{-1}, the magnitude of lipid peroxidation increased with an increase in the concentration of TiO_2 NPs. Here a linear relationship was observed between H_2O_2 production and NPs. As supported by the literature, rice plants treated with Nano-Ag over a concentration of 30-60 mg L^{-1} also saw a negative impact on plants cell wall with an increase in the concentration of nanoparticles (Mirzajani *et al.,* 2013). 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 reactive oxygen species 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.

4.7 Phytotoxicity

Electrolyte leakage from plant tissue estimates membrane integrity in response to abiotic and biotic stress. The membrane acts as a "biomarker" to evaluate against environmental stresses and the cells ability to survive in the altered conditions. For evaluation of membrane integrity in both soils, Evans Blue Dye Binding Assay and Conductivity Assay were used. A membranes integrity can also be compromised due to high levels of ROS, so these results further cement the fact that increased levels of nanoparticles cause ROS which in turn creates mayhem in plant systems.

4.7.1 Evans Blue Dye Binding Assay

Evans Blue Dye binding assay works on the principle that the dye cannot enter the membranes of live cells rather dead or damaged cell only. Hence it acts as a good biomarker of cell death and therefore of the loss of root/shoot plasma membrane integrity (Das *et al.*, 2017). The graph below shows that membrane injury was highest in plants treated with 750 mg kg $^{-1}$ of TiO_2 NPs in both soils.

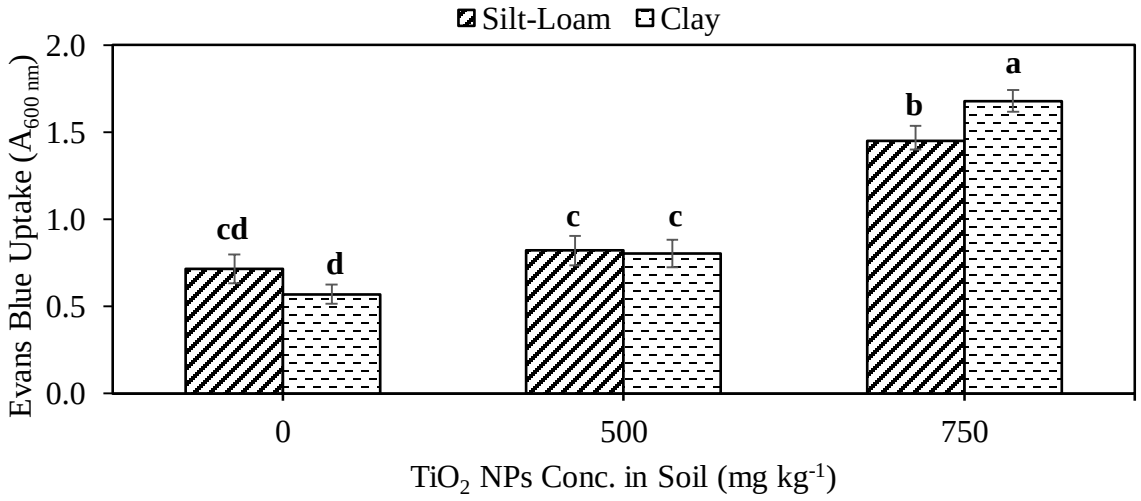

Figure 4.11: Membrane injury index using Evans Blue Dye Assay

The membrane injury index in the controls was 0.72 in silt-loam and 0.57 in clay, which increased by 15 and 40.9% in silt-loam and clay respectively at 500 mg kg^{-1}. With an increase in the concentration of TiO_2 NPs to 750 mg kg^{-1}, membrane injury increased to 102.6% and 194.3% in silt loam and clay respectively.

A study checking the effects of ZnO nanoparticles on hydroponically grown plants showed increased uptake of Evans Blue dye in root cells at all concentrations (200, 400 and 800 mg L^{-1}) of applied NPs (Ghosh *et al.*, 2016). In another study observing the impact of Copper oxide NPs on rice seedlings at concentrations of 40, 80 and 119 mg L^{-1} and the results were similar to the previous study, roots under nanoparticle stress had a higher uptake of Evans blue dye compared to control. Maximum cell death was observed at 119 mg L^{-1} as cell tissue was completely dark blue (Shaw and Zahed Hossain, 2013). Silva *et al.* (2016) explored the effects of pure anatase TiO_2 NPs and rutile + anatase TiO_2 NPs on wheat seedlings and found that pure anatase had less detrimental cytotoxic effects compared to rutile + anatase. At concentrations of 5, 100 and 150 mg L^{-1} Evans blue dye uptake was the highest for 100 and 150 mg L^{-1} compared to control. An increase of 85 and 112% was observed respectively.

A similar trend is experienced in the present study, with an increase in the concentration of TiO_2 NPs, Evans Blue uptake by plant roots increased as well. These results when read in correlation to ROS production resolve that higher levels of ROS cause stress which is a foreshadowing to increased lipid peroxidation and eventually cell death.

4.7.2 Conductivity Assay

Apart from membrane injury based on Evans Blue dye, electrolyte leakage from plant tissue was measured to determine membrane stability. Similar to results observed with Evans Blue dye binding assay, the data indicated low membrane integrity for both treatments in silt-loam soil.

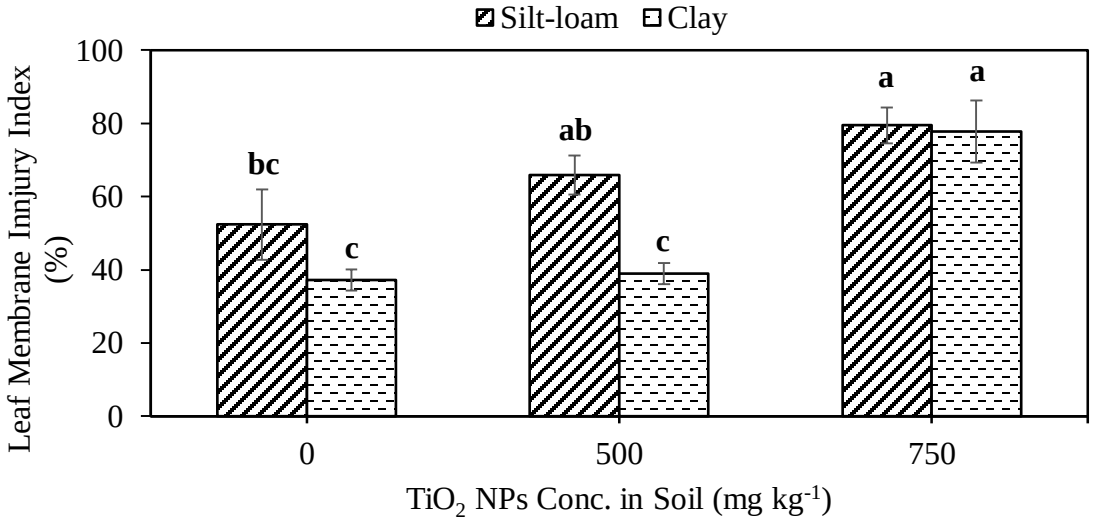

Figure 4.12: Shoot membrane integrity by conductivity assay

Plants grown at 750 mg kg^{-1} in both soils exhibited the highest membrane damage accounting for 52 to 109% in silt-loam and clay respectively compared to control. For silt-loam electrolyte leakage increased in a dose-dependent manner.

On the other hand, for clay, no significant change was observed between control and 500 mg kg^{-1} while 750 mg kg^{-1}, showed a marked increase in membrane damage (109%). In comparison to control, the results show that 750 mg kg^{-1} encouraged electrolyte leakage and that NPs concentration had a significant role to play here.

Similar results were observed by Rico *et al*. (2013c) on a study conducted on rice seedlings and nano-CeO_2 with concentrations of 62.5, 125, 250, and 500 mg L^{-1}, where the highest concentration of Nano-CeO_2 displayed the highest amount of membrane damage.

Furthermore, use of Copper nanoparticles of concentration 50, 100, and 200 mg L^{-1} on cucumbers grown hydroponically showed a significant increase in electrolyte leakage at 50 and 200 mg L^{-1} as well (Mosa *et al*., 2018).

It has been established by various studies that nanoparticles damage general growth and development of plants by disrupting their timing of flowering, fruiting, senescence, and dormancy. (Gardea-Torresdey *et al*., 2004; Vernay *et al*., 2008; Thul and Sarangi, 2015) along with numerous studies also confirming that NPs do in fact mediate oxidative stress in plants. This toxicological phenomenon brought about by NPs is measured by lipid peroxidation and electrolyte leakage; which affects the membranes permeability and fluidity and in the broader sense the procurement of nutrients to cells (Tripathi *et al*., 2016). Furthermore, there are very few studies observing the effects of metal oxide nanoparticles on full-growth cycle of plants or plants grown in soil. Most studies are conducted on seedlings or plants grown hydroponically. With soil, other factors such as the soils' own characteristic properties like pH and OM and particle size to name a few, may also play a role in how a nanoparticle interacts with a plant. For phytotoxic results, soils may aid in how available a nanoparticle is to the plant for uptake and this has already been extensively discussed in previous passages.

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.

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Considering the impact of TiO_2 NPs and the role of soil texture in how TiO_2 NPs impacted both growth and development of rice and the effect on soil microorganisms; the following conclusions can be summed up from present study:

- The overall growth of rice was better in clay soil than silt-loam.
- Noteworthy increase in root–shoot length (2.1- & 0.5-folds) and biomass (4.2- & 2.2-folds) was observed at 500 mg kg^{-1} of TiO_2 NPs.
- Poor growth was observed at all treatments of TiO_2 NPs in silt-loam compared to control.
- Soil characteristics such as pH, particle size and organic matter play a pivotal role in the bioavailability and movement of the nanoparticles through the soil matrix.
- At 750 mg kg^{-1} of TiO_2 NPs in Clay: Increase in H_2O_2 production, Lipid Peroxidation and Electrolyte Leakage by 4.3-, 2.4-, & 1.9-folds correspondingly.
- Phytotoxic results of TiO_2 NPs were markedly observed in plants grown in silt-loam soil in a dose-dependent manner.
- At 750 mg kg^{-1} in Silt-loam: Decrease in Microbial Biomass $_{Carbon}$, Dehydrogenase Activity, and Basal Respiration by 0.91-, 0.79- & 0.78-folds, respectively.

5.2 Recommendations

The present study has highlighted both positive and negative impacts of TiO_2 NPs application. Before using on an agricultural scale, extensive field trials need to be conducted possibly with lower TiO_2 NPs concentrations. Noteworthy effects were found on the *Oryza* sativa L. and soil health in response to the TiO_2 NPs application in combination with soil texture. Further

studies would help us better understand how these positive and negative effects come about and help us in mitigating and enhancing them. Following are the recommendations for the work to be done in the future:

- 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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