A HYDROPONICS-BASED ASSESSMENT OF NUTRIENTS UPTAKE AND RELATIVE GROWTH OF *ORYZA SATIVA* IN THE PRESENCE OF TITANIA NANOPARTICLES

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CERTIFICATE

It is certified that the contents and form of the thesis entitled "A Hydroponics-based Assessment of Nutrients Uptake and Relative Growth of *Oryza sativa* in the Presence of Titania Nanoparticles", submitted by Ms. Zainab Zahid, has been found satisfactory for partial fulfillment of requirements for the degree of Master of Science in Environmental Science.

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DEDICATION

I dedicate this research work to my beloved parents and grandparents (late), who have always remained a source of inspiration for me. It is only due to their endless and unconditional love, support, and prayers that I am able to achieve anything.

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All praise to The Allah Almighty who has the most knowledge of what lies in this whole universe and who teaches us in the most righteous way. With my deepest gratitude, O Allah! I ask you for knowledge that is of benefit.

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Zainab Zahid

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LIST OF ABBREVIATIONS

0D, 1D, 2D, 3D	Zero, one, two, three dimensional
B	Boron
Cd	Cadmium
CNTs	Carbon nanotubes
DMSO	dimethyl sulphoxide
DW	Dry weigh
EC	Electrical conductivity
ENMs	Engineered nanomaterials
FW	Fresh weight
FWHM	Full width of a diffraction line at one half of maximum intensity
H_2O_2	Hydrogen peroxide
IRRI	International Rice Research Institute
K	Potassium
KI	Potassium iodide
Mm	Millimeter
mS cm^{-1}	Millisiemens per centimeter
nAg	Silver nanoparticles
$nCeO_2$	Cerium oxide nanoparticles
NH_3-N	Ammonia-nitrogen
NO_2^-N	Nitrite-nitrogen
NO_3^-N	Nitrate-nitrogen
nPVP-CeO_2	Polyvinyl pyrrolidone-coated nanoparticles
nSi	Silicon nanoparticles
$nTiO_2$	Titania nanoparticles
nZnO	Zinc oxide nanoparticles
P	Phosphorus
PVC	Polyvinyl chloride
Rpm	Revolution per minute

RWC	Relative water content
SEL	Size exclusion limit
SEM	Scanning Electron Microscopy
Si	Silicon
SI	International system of units
SPIONs	Superparamagnetic iron oxide nanoparticles
SVI	Seedling vigor index
TBARS	Thiobarbituric Acid Reactive Substances
TFW	Turgid fresh weight
TiO_2	Titania
TKN	Total Kjeldahl Nitrogen
WUE	Water use efficiency
XRD	X-Ray Diffraction

ABSTRACT

Titanium dioxide nanoparticles ($nTiO_2$) are promising agents for improving plant morphological characteristics and physiological mechanisms but show a plant specific dose-dependent response. *Oryza sativa* (rice) was grown under hydroponics-based greenhouse pot experiment, with administration of increasing concentrations of anatase $nTiO_2$ (~28 nm). Different doses, including t1 (100 mg L^{-1}), t2 (200 mg L^{-1}), t3 (300 mg L^{-1}), t4 (400 mg L^{-1}), and t5 (500 mg L^{-1}) $nTiO_2$ NPs along t0 (control), were administered for six weeks in five replicates each. The change in Hoagland solution's pH, electrical conductivity (EC), and temperature were recorded with regular intervals, whereas nutrients content (NPK) was analyzed after harvesting. The highest plant height was observed in control followed by 300 mg L^{-1} treatment. In general, the data showed a continuous decrease in pH and EC of Hoagland solution, with a slight increase in pH during last week. The carbohydrates content was significantly ($p < 0.05$) higher from 100-300 mg L^{-1} $nTiO_2$ compared to the control but decreased below control at higher doses. Following a similar trend, chlorophyll content increased by 20% at 300 mg L^{-1} and decreased by 1.5 folds at 500 mg L^{-1}. Results suggested a decrease in rice tolerance to increased lipid peroxidation, at higher doses. These findings corroborated with a decrease in rice nutrients content, marking total Kjeldahl nitrogen (TKN) reduction by 2.1 and 2.2 folds, P reduction by 1.5 and 2.4 folds, and K reduction by 1.2 and 1.5 folds in 400 and 500 mg L^{-1} $nTiO_2$ application, respectively. The significant impact of $nTiO_2$ was obtained due to its unique properties owing to small size, which facilitated its role as a catalyst for enhancing plant growth, however the mediation of stress mechanisms in rice could further be optimized to improve the nutrients efficiency.

Keywords: titania nanoparticles, nutrients analysis, *Oryza sativa*, hydroponics

Chapter 1

Introduction

1.1. Background

The global population is increasing at an unprecedented rate. It is projected to reach 8.5 billion in 2030 with a further rise to 9.7 billion in span of just 20 years till 2050 (World Population Prospects, 2019). According to the *The World Population Prospects: 2015 Revision*, 2015, Asia alone is estimated to have the highest rate of population growth (2015-2050) with 17% of a projected rise in the population. With the increasing pressure on availability of the natural resources, this rapid increase in the population has presented a huge food security challenge (Tito et al., 2018). Intensification of agricultural practices (increase in crop yield production per unit of area) and unsustainable crop production practices have led to the degradation of natural soil. Anthropogenic activities have also dominantly aggravated the issue leading to restricted availability of suitable land and water for agricultural activities. The changing environmental conditions have further restricted the options needed for the sustainable production of required quantity and quality of food crops (Myers et al., 2017).

It has been estimated that out of 13.4 billion hectares of the globally available land, only 2.9 billion hectares could be brought under cultivation excluding its 20% of the legally protected forest cover (Pingali et al., 2008). Moreover, in order to meet the bioenergy needs, a rapid increase in the land demand for feed and fuel production have also resulted in a sharp decrease in land availability for agricultural activities (Kampman et al., 2008). Climate change and increasing global temperature have similarly challenged the agricultural systems around the world with varying intensity of the negative impacts obtained across the geographical regions. It has been estimated that climate change has induced approximately 5-15% decrease in the crop yield per degree centigrade of the rise in global temperature and has posed a huge socioeconomic threat for the agrarian economies (Premanandh, 2011). Furthermore, along the food production and sufficiency issue, the undernourishment is also prevalent in Asia, with 12% of the total population has been affected by it (FAO, 2015). It has been reported that Bangladesh, India, and Pakistan collectively account for 36% of the global undernourished people (Ahmed et al., 2013).

To help improve the agricultural production and meet nutrients needs of the people, the use of external inputs and amendments including chemical fertilizers have continually increased in the last century. Since the natural nutrients reserves are released slowly over time and are not readily available for plants uptake, the chemical fertilizers have proved to be of great benefit to mobilize the nutrients release rate and consequently the uptake by food crops (Chen, 2006). Aside the benefits, the over application of fertilizers have also been found to overwhelm the actual rate of absorption which ultimately results in the transformation of the nutrients to biologically unavailable form. Hence, it has been reported that plants can uptake only 30-50% of the applied fertilizer and the remaining amount is lost in the soil (Wang et al., 2018; Zulfiqar et al., 2019). Additionally, high rates of the chemical fertilizers put farmers at economic disadvantage with lower profit return. Thus, owing to intensive use of chemical fertilizers for improving the production of crops, nutrients management has appeared as a major challenge for maintaining a sustainable agricultural system. The over application of chemical fertilizers may cause damage to human or environmental health through leaching, water pollution/ eutrophication, and greenhouse gas emissions. It may also lead to an irreparable damage to the overall soil structure and system by altering its physical, chemical, or biological properties (Iqbal et al., 2021; Zulfiqar et al., 2019). This situation calls for an action to adapt to the current situation and meet the increasing global food demand.

In order to meet the increasing food need around the globe and to ensure food security, food production should be increased by 70% till 2050 (Noel, 2016). Moreover, according to the *OECD-FAO Agricultural Outlook*, (2017), required increase in the food production to ensure the global food security is expected to come from increasing the crop yield growth rather than expansion of the cultivated land area. With some regional exceptions, such as sub-Saharan Africa and Latin America, the data projections have shown that 93% rise in the rice production is expected to come through crop yield intensification, whereas land expansion for wheat cultivation is expected to increase by only 1.8%. Given such circumstances, it is necessary to devise strategies and interventions which could help inefficient management of the agricultural systems and provide distinct improvements beyond those of traditional methods.

The modern technologies such as nanotechnology have been regarded as a potentially promising option for significant improvement in the agricultural production and overall yield with added benefits to improve the crop nutrients management (Sekhon, 2014; Zahra et al., 2020). Nanotechnology refers to the materials that have a range or fall in nanometers scale, and are measured as one billionth of a meter or 10^{-9} meters in length. A substantial increase in the use of nanotechnology is being carried out in the fields of agriculture and food industry due to its range of benefits. Engineered nanomaterials (ENMs) are widely being used in form of nano fertilizers, nano pesticides, and nano biosensors for agricultural improvements (Mandal & Banerjee, 2020). Studies have shown significant improvement in plant growth in response to the ENMs application either via direct use in soil, seed coating, or foliar treatment (Gilbertson et al., 2020). They stimulate the plant growth by aiding release of growth-promoting substances from roots which ultimately increase the abundance of soil microarthropods. These microbes also aid the mineralization process and increase the soil fertility by improving the nutrients cycling through enhanced mineralization of the compound. Additionally, nanomaterials also improve the physical, chemical, and biological properties of soil, thus acting as a conditioner to improve the overall properties (Hasan et al., 2020).

ENMs have porous structure and a high surface area owing to which they can either act as a direct nutrient source, a nutrient carrier, or an intermediate to mobilize the mineralization process. Furthermore, the metal and metal oxide-based nanomaterials have a slow release mechanism which improves the plants' mineral uptake efficiency (Gilbertson et al., 2020). Therefore, instead of the conventional chemical fertilizers, the application of metal or metal-oxide based nanomaterials is beneficial for agricultural improvements coupled with low inputs, less nutrients runoff, and enhanced efficiency (Kalia & Sharma, 2019). Among the metal-oxide based nanomaterials, titanium dioxide nanoparticles/ titanium (IV) oxide nanoparticles/ titania nanoparticles ($nTiO_2$) are widely being used for improvement in the physiological, biochemical, and morphological characteristics of plants (Myhara et al., 2000). Titanium is inert in environment since its mineral forms i.e. anatase, rutile, and brookite are not generally soluble (Lyu et al., 2017) and therefore, unlike other metal oxide nanoparticles, $nTiO_2$ are unlikely to discharge phytotoxic ions upon dissolution (Hu et al., 2020). However, a few studies have reported

Ti to be mobile in rocks due to weathering (Lyu et al., 2017). The high photocatalytic potential, chemical stability, and nontoxicity of $nTiO_2$ make them highly suitable for the environment (Ajmal et al., 2019) and due to the extensive use, their global annual production volume has been estimated to be around >10,000 tons (Zhu et al., 2020). Multiple studies have reported $nTiO_2$ to increase the yield of crops by improving the photosynthesis rate (Waghmode et al., 2019), protein content (Chhipa, 2017), nutritional quality (Hu et al., 2020), plant biomass (Jiang et al., 2017), and by mediating the antioxidant stress (Saxena et al., 2016) along other abiotic stresses including salinity, drought, cold stress, and flooding (Almutairi, 2019). However, the impacts may vary based on the selected plant species, applied dose, properties of nanoparticles (size and shape), application medium (soil or hydroponics), and presence of other non-targeted compounds (Bellani et al., 2020; Zand et al., 2020).

Agriculture sector holds great importance in economic growth of the developing countries as the quality and yield of food crops play an important role to determine the economic stability. Moreover, cereal crops primarily contribute to overall dietary intake of important mineral nutrients all around the world (Sabagh et al., 2020) and Asia is the home for most important cereal crops including wheat, maize, barley, rice, jowar, and bajra and accounts for providing a major portion of the calorie intake for the region (Ahmad et al., 2017; Khan et al., 2020; Zhang et al., 2020). The intensification in population and agriculture along the low production of the crops is also crucial for developing economies like Pakistan. The cultivation of major crops such as rice, wheat, cotton, maize, and sugarcane directly account for 5.3% of the overall GDP of the country along 25.6% through value addition in agriculture (Ahmad et al., 2017). Furthermore, about 64% of the rural population and 43.5% of the labor force is directly employed to work in the agricultural sector and therefore it plays a major role in supporting their livelihood (Ahmad & Afzal, 2020). Rice is cultivated in 100 countries around the world, however Asia alone accounts for about 90% of the world's rice production and consumption (Rao et al., 2017). After wheat, rice is ranked as the second largest consumed staple food crop in Pakistan. Given its high consumption, about 11% of the total agricultural area in Pakistan is based on the cultivation of Basmati and International Rice Research Institute (IRRI) varieties with 90% of the produce coming from two

provinces i.e. Sindh and Punjab (Chandio et al., 2018, Chandio et al., 2020a). According to Mughal & Fontan Sers (2020), the South Asian countries including Pakistan, India, and Bangladesh are globally ranked among the largest rice producers, whereas the former two are top rice exporters. Therefore, given the extensive cultivation of rice as a food and cash crop, rice was selected as an experimental plant.

Studies have shown that due to the aggravating water management issues, the hydroponics provide a relatively promising solution to create a paradigm shift in the crop cultivation from soil-dependent to a soil-less system (Premanandh, 2011). The agriculture sector has been reported to utilize a large volume of water and is responsible for the global withdrawal of 70% of the total freshwater. Due to the changing diet preferences, low precipitation, high evapotranspiration, and increasing aridity in regions like South Asia, Southern Europe, East Australia, and Africa, the irrigation requirements may increase further in future; making the water requirement to increase by 7 times in the future (Akbar & Gheewala, 2020; Farooq & Gheewala, 2018; Farooq & Gheewala, 2019). Moreover, it is a well-established fact that rice (*Oryza sativa*) is highly water-intensive crop and the initial 15 days of the seedlings transplantation are followed by an intensive puddling and ponding of water (Naseer et al., 2020). At on average, 1 kg of the rice cultivation takes about 2500 L of the water with the upper and lower limit ranging from 800-5000 L (Basha et al., 2017). Thus, changing pattern of water availability has impacted the crop production with significant impacts obtained on the water intensive crops including rice which has huge implications in eventually disrupting the food markets and trade (Chandio et al., 2020b; Ozcan & Strauss, 2016).

1.2. Significance of the Study

The study holds a great significance to present an overview of the plant-nanoparticles interaction in a soil-less medium. Being based on the hydroponics technique, it provided an opportunity to investigate the rice growth pattern and quality of the post-harvest nutrients solution as a baseline study for further experimental optimization. Moreover, with restricted use of the conventional chemical fertilizers, it also presented an option to scale up this experimental study in arid regions of the

Pakistan and analyze the nanoparticles-driven enhanced nutrients mobilization for improving plant growth under the limited supply of water.

1.3. Objectives

Since nanoparticles have been reported to improve the plant growth, it was hypothesized that $nTiO_2$ could be applied in a dose-dependent manner to enhance the nutrients uptake and rate of photosynthesis along with mediation of the oxidative stress in *Oryza sativa*. Based on this hypothesis, the objectives of the study were formulated as under:

1. Quantitative assessment of nutrients' uptake by plants grown hydroponically.
2. Estimation of the lipid peroxidation in response to hydrogen peroxide (H_2O_2) production.

Chapter 2

Literature Review

The key focus of this chapter is to review the potential benefits of the nanoparticles application on food crops, their uptake mechanism, phytotoxicity, and likely ecotoxicity symptoms as a result of nanoparticles bioaccumulation in plants.

2.1. Nanotechnology

Nanotechnology has emerged as one of the most promising technology of the 21st century with its application across different fields. The word "nano" is derived from a Greek prefix meaning "dwarf" and is referred to the scale of one thousand millionth of a meter (10^{-9} m) in the International System of Units (SI). Here, it is important to establish a difference that nanoscience is the study of materials that are on scale of nanometers (1-1000 nm), whereas nanotechnology is referred to the application and use of nanosized materials (Bayda et al., 2019). The chemical, physical, mechanical, and thermal properties of nanomaterials drastically change compared to the bulk material and therefore, they show enhanced properties with reduction in size to nanoscale (Rafique et al., 2020).

Historically, the concept of nanotechnology was first introduced by an American physicist and a Nobel Prize laureate Richard Feynman. During the annual meeting of the American Physical Society at the California Institute of Technology in 1959, Feynman delivered a lecture "There's Plenty of Room at the Bottom", where he made a hypothesis "Why can't we write the entire 24 volumes of the Encyclopedia Britannica on the head of a pin?" (Sharon, 2019). After fifteen years, in 1974, Norio Taniguchi who was a Japanese scientist defined the term nanotechnology. He defined it as: "Nanotechnology mainly consists of the processing of separation, consolidation, and deformation of materials by one atom or one molecule" (Bayda et al., 2019).

2.2. Classification of Nanomaterials

Materials with any internal or external structures on the nanoscale dimension are called nanomaterials. According to the British Standards Institution, this scale may fall between 1-1000 nm (Gupta et al., 2021). Based on the dimensionality, nanomaterials can be divided into four different classes namely zero-dimensional (0D), one dimensional

(1D), two dimensional (2D), and three dimensional (3D). The 0D nanomaterials have all three dimensions (x,y,z) within the nano range, 1D nanomaterials have two dimensions (x,y) under nanoscale, 2D nanomaterials have only one dimension under nanoscale, and 3D nanomaterials don't have any dimension within the nanoscale. The examples of nanomaterials which fall under respective categories are given in Table 1 (Aversa et al., 2018; Prajitha et al., 2019; Saleh, 2020).

Table 1: Examples of nanomaterials based on dimensionality

Dimensions of Nanomaterials	Examples
0D, all dimensions < 1000 nm	nanoparticles, quantum dots, nanoshells, nanorings, fullerenes
1D, two dimensions < 1000 nm	nanotubes, nanowires, nanofibers, nanorods
2D, one dimension < 1000 nm	crystalline/ amorphous and single/ multilayered nanofilms, nanosheets, graphene, nanoplates, nanolayers, and nanocoatings
3D, all dimensions > 1000 nm	graphite, polycrystals, bulk powder, nano-diamond, nanoflowers

The nanomaterials are classified into different groups based on several criteria. On basis of the occurrence, the nanomaterials are generally divided into three categories (Figure 1). These categories are: (i) natural nanomaterials which are generated in the environment as result of the forest fires or volcanic eruptions etc., (ii) non-ENMs formed as a by-product of any anthropogenic activity e.g. through operation of any power plant or an incinerator, and (iii) ENMs are synthesized for specific use in the industry either as pure particles or in form of a composite (Saleh, 2020).

Similarly, the engineered nanoparticles are classified into four different types based on their composition (Figure 2). This classification includes (i) inorganic metal/ metal-oxide based nanoparticles which are prepared from metals such as silver, copper, iron, alumina, zinc, titanium, and silica and are sometimes coated into silica to enhance their biocompatibility, (ii) carbon based nanoparticles which are entirely composed of the carbonaceous material and are found in different shapes such as hollow spheres and tubes with major examples including fullerenes, carbon nanotubes (CNTs), and graphene, (iii) quantum dots which are inorganic semiconductor particles and possess unique optical and

electronic properties due to their quantum mechanics, and (iv) silica based nanoparticles made entirely of silica and are highly biocompatible to use from environmental safety perspective (Ha et al., 2012; Saleh, 2020).

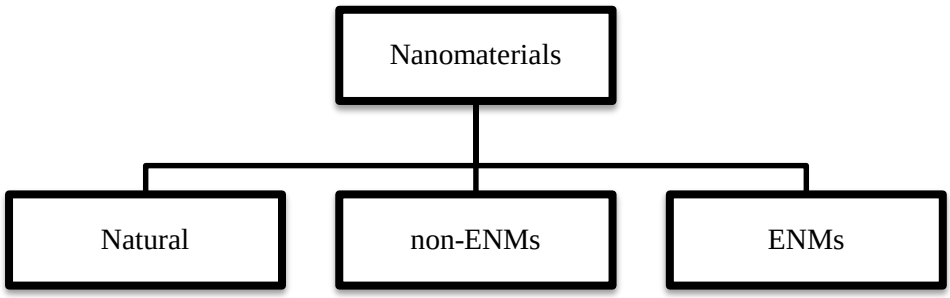

Figure 1: Classification of nanomaterials on basis of occurrence

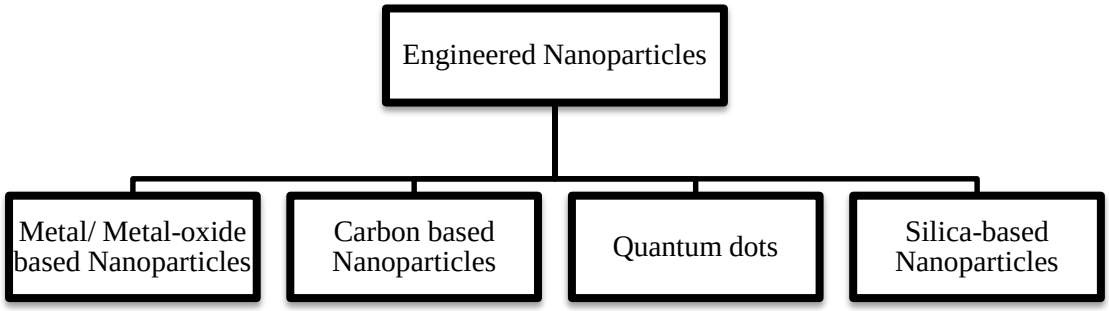

Figure 2: Classification of nanomaterials on basis of composition

2.3. Methods of Synthesis

Nanomaterials or nanoparticles can be synthesized through different methods which may impact their reactivity and functioning depending on the core properties. These factors include composition, size, shape, surface properties, purity, and stability of the nanoparticles. The scientist community has widely adopted two fundamental approaches of nanomaterials synthesis known as the top-down and bottom-up approaches, which differ on basis of the starting material for the preparation. The top-down approach uses bulk material as a starting material based on the size reduction through physical methods such as mechanical milling, thermal or laser ablation, sputtering or vapor deposition, and pulsed electrochemical etching. The bottom-up approach uses smaller molecules for production of the nanomaterials through chemical or biological/ green methods. Chemical methods include sol-gel process, co-precipitation,

microemulsion, atomic/ molecular condensation, laser pyrolysis, hydrothermal synthesis, sonochemical synthesis, or microemulsions, whereas the biological/ green synthesis is done through using plant extracts, biological enzymes, agricultural waste, bacteria, or fungus (Balasooriya et al., 2017; Jamkhande et al., 2019; Prajitha et al., 2019; Singh et al., 2018).

Based on the mentioned approaches, nanoparticles can be engineered through physical, chemical, and biological methods involving the electrochemical changes, and chemical, or photochemical reduction. Researchers have found that metal and metal oxide nanoparticles differ from the bulk counterparts owing to their surface, optical, thermal, and electrical properties. Since metal and metal oxide nanoparticles' synthesis is carried out by adding a reducing or oxidizing agent, this may be attributed to the factors such as type of synthesis technique, reaction kinetics of metal ions with reducing agent, and adsorption process of stabilizing agent with metal. These factors may also strongly influence the morphology (structure and size), stability, and physicochemical properties of nanoparticles (Jamkhande et al., 2019; Rastogi et al., 2017).

2.4. Mobilization of Nanoparticles in Plants

2.4.1. Application and Uptake of Nanoparticles

The application of nanoparticles in plants can be administered either through seeds priming (Acharya et al., 2020), foliar spray to target the vegetative organs particularly leaves (Sharifi et al., 2016), or exposure via roots (irrigation method) (Sanzari et al., 2019). In hydroponics, the nanoparticles suspension is added directly in the aerated setup, whereas soil spiking is done either by using nanoparticles in form of a dry powder or suspension (Deng et al., 2017; Waalewijn-Kool et al., 2012). These modes of delivery seem to impact the nanoparticles uptake efficiency as well as plant response to exposure. Moreover, the applied dose of nanoparticles may not get instantly activated right after exposure and it has to pass the chemical and physiological barriers for uptake and translocation to be available for plants (Elemike et al., 2019; Rastogi et al., 2017).

Nanoparticles can be taken up by plants through their primary interaction on the plant surface and can enter passively by foliar spraying through different plant organs

(Salama et al., 2021; Sanzari et al., 2019). Cuticle is plant's lipophilic barrier composed of different biopolymers and waxes. It covers the shoot surfaces leaving access only through natural openings, controls the unwanted exchange of solutes, and prevents the water loss. This layer is generally impenetrable to nanoparticles but research has shown that $nTiO_2$ could produce holes in it, making its way into the plant (Sanzari et al., 2019). It may allow the uptake of solutes via diffusion. The permeation of nonpolar solutes occurs through lipophilic pathway and polar aqueous pores allow the permeation of polar solutes through hydrophilic pathway. However, the effective size which could penetrate through cuticular pathway ranges from about 0.6-4.8 nm. This phenomenon needs to be studied further throughout the plant's lifecycle and in link with other factors which may impact the uptake of nanoparticles (Lv et al., 2019).

Trichomes, the extensions of above-ground epidermal cells, forming the fine outgrowths or appendages on plant organs may entrap the nanoparticles on plant surface from where they make way into the plant tissues (Wang et al., 2021). Due to the large size (approximately 25 μm length and 3-10 μm width), stomatal aperture may also allow the nanoparticles to enter into plants but due to less amount of data on the actual size exclusion limit (SEL), there is still an uncertainty regarding range of nanoparticles which could easily penetrate the plants (de la Rosa et al., 2021; Lv et al., 2019). Research has shown that other parts of the plants, including stigma, hydathode, and lenticels, as well as damages or wounds as a viable route for nanoparticles entry in plants tissues (Kumar et al., 2020). Roots come in direct contact with nanoparticles and mediate its uptake through thin permeable cuticle and root hairs. Size of root pores also has a strong influence on nanoparticles uptake (Salama et al., 2021). Symbiotic organisms, membrane transporters, mucilage, and root exudates additionally influence nanoparticles availability (Rico et al., 2011).

2.4.2. Nanoparticles Internalization and Translocation in Plants

The nanoparticles internalization is limited by different factors including the size of nanoparticles, cell wall pore size, thickness, and its biochemical composition i.e. cellulose, hemicellulose, and pectin. Upon interaction with plants, cell wall acts an initial barrier which hinders the entry of nanoparticles into cell. The SEL of the cell wall (5-

20 nm) mediates the uptake of the particles through passive diffusion or active transport (Ahmed et al., 2021; Hubbard et al., 2020; Ullah et al., 2020). However, nanoparticles may easily enter through quickly growing and dividing cells e.g. root tips, due to their high SEL. Since the newly formed cell walls are thinner than mature tissues, they also facilitate nanoparticles to enter in the cells (Hubbard et al., 2020). Upon internalization of nanoparticles in the plant tissues, they are either translocated through apoplastic pathway (Singh et al., 2021), get accumulated inside the cell wall or extracellular spaces (Avellan et al., 2017), or move across the cell membrane (Pérez-de-Luque, 2017). Only selected molecules could pass across the cell membrane due to its polar nature. Cell membrane mediated nanoparticles progression to protoplasm occurs via symplastic pathway. It may take place through passive diffusion, facilitated uptake (through carrier proteins), ion channel transport (after dissolution of nanoparticles), endocytosis, or formation of new pores. The nanoparticles may then accumulate in cell membrane or are translocated to different parts of the plants (Chichiriccò & Poma, 2015; Pérez-de-Luque, 2017; Singh et al., 2021).

The cellular translocation of nanoparticles to different plant organs is carried out by vascular tissues i.e. xylem and phloem, following the apoplastic and symplastic pathway. The apoplastic pathway acts as a non-selective route which transports the material through the extracellular spaces between cell walls of neighboring cells, from where it can enter the vascular tissues, whereas the symplastic transportation of cytoplasmic material is mediated by plasmodesmata. Plasmodesmata are the cytoplasmic channels connecting the adjacent cells for inter-cellular movement of molecules (Ahmed et al., 2021; de la Rosa et al., 2021; Miralles et al., 2012). Once nanoparticles enter cytoplasm, they are either further transported to xylem tissues or begin to accumulate in the cytoplasmic organelles causing interference with various metabolic functions occurring in the cell (Banerjee et al., 2019; Liu et al., 2020). The presence of casparian strips in root endodermis may block the apoplastic pathway causing the nanoparticles to traverse the protoplast of endodermal cells to ultimately reach the vascular tissues like xylem. Once in the xylem, nanoparticles begin to translocate towards the aerial parts of the plant along other different organs (Mittal et al., 2020).

2.5. Unique Properties of Titanium Dioxide (TiO_2) and $nTiO_2$

Titanium (Ti) is a transition metal with atomic number 22. It is classified as the 9th most abundant element making about 0.57% of the earth's crust (National Center for Biotechnology Information, 2021). It is found in the mineral forms as rutile (TiO_2), ilmenite ($FeTiO_3$), and sphene ($CaTiSiO_5$), or iron ores such as leucoxene ($Fe_2O_3TiO_2$) (Free, 2019). Titania (TiO_2), the transition metal oxide, exists in three crystalline forms namely anatase (tetragonal), rutile (tetragonal), and brookite (orthorhombic) (Lavacchi et al., 2021). Anatase is metastable, whereas the rutile is most stable among all due to its thermodynamic properties. Anatase and brookite can be transformed to rutile when subjected to high temperature, whereas brookite is at first transformed to anatase phase upon calcination to around 700 °C, and change to rutile when the temperature is further increased (Alsheheri, 2021).

The $nTiO_2$ belong to the category of most used nanoparticles. They possess unique characteristics due to low particle size, large surface to volume ratio, and improved optical properties (Elemike et al., 2019). $nTiO_2$ are used for sustainable agricultural practices due to their unique photocatalytic properties, chemical stability, hydrophilic nature, and biocompatibility (Dastan, 2017; Waghmode et al., 2019). TiO_2 and $nTiO_2$ hold a commercial value and are used as pigment in production of paints, paper, electronic materials, inks, rubbers, sunscreens, food colors, toothpaste, white chocolate, and other products due to their white color, high brightness, and refractive index (Birlik & Dagdelen, 2020; Rodríguez-González et al., 2019). Due to useful properties of TiO_2 such as high transmittance (in the visible range), wide bandgap (3.2 eV), and thermal and chemical stability, it is also categorized among the most regularly used semiconductor oxides and photocatalyst (Ajmal et al., 2019; Parangi & Mishra, 2019). Moreover, $nTiO_2$ can be synthesized through an easy and non-toxic sol-gel method which is not only independent of the time and specifications such as revolution per minute (rpm), but is also a low cost method which allows production of nanoparticles of desired size and shape (Dastan, 2017).

2.6. Application of Nanoparticles for Crops Improvement

Nanoparticles are known to play a substantial role in promoting the overall crop yield by increasing the seedlings growth, plant biomass, and grains production (Priyanka et al., 2019). Nanoparticles induced increase in plant root length has been observed in wheat (Ullah et al., 2020), chili (Afrayeem & Chaurasia, 2017), eggplant (Baskar et al., 2018), maize (Itroutwar et al., 2020), lettuce (Hayes et al., 2020), rice (Khan et al., 2020), and sorghum (Sharif et al., 2021). A study conducted on soybean crops (*Glycine max*) showed improvement in the plant fresh weight at 10, 100, and 500 mg kg^{-1} polyvinylpyrrolidone-coated nanoparticles (nPVP-CeO_2) treatment. At 500 mg kg^{-1}, an increase in fresh weight of plants treated with non-coated cerium oxide ($nCeO_2$) nanoparticles, along with a significant increase of 24.3% and 32.7% in the fresh shoot biomass of both $nCeO_2$ and nPVP-CeO_2 treated plants was observed (Cao et al., 2017). Nanoparticles could significantly enhance the uptake of essential nutrients, even under environmental stress, and act as a carrier to enhance the delivery of fertilizers in plants. It has been found that N accumulation in roots of sorghum increased as a result of zinc oxide nanoparticles (nZnO) application under drought (Ma et al., 2018).

Application of silicon nanoparticles (nSi) on wheat, under cadmium (Cd) stress, enhanced the light use efficiency of crops resulting in improved chlorophyll content. nSi also increased the rate of photosynthesis which enhanced the plant growth (Ali et al., 2019). Similarly, the superparamagnetic iron oxide nanoparticles (SPIONs) have been evaluated to enhance the chlorophyll content in soybean plants without causing any toxic symptoms (Ghafariyan et al., 2013). Additionally, the water use efficiency (WUE) of plants is one of the major parameters for crop growth which determines the ratio of carbon used during photosynthesis and water lost through transpiration (Cao et al., 2017). Nanoparticles may lead to increase in the WUE of plants, which ultimately causes high rate of nutrients uptake along with water flow. As reported by Mahmoud et al. (2020), the combined treatment of zinc (Zn), boron (B), silicon (Si), and zeolite nanoparticles on potato grown under the saline conditions increased the WUE of plants by increasing the availability of water and its absorption via roots.

Among all the nanoparticles, $nTiO_2$ have been extensively used to enhance fertilizers uptake and reduce the metallic stress (Gul et al., 2020; Hu et al., 2020). Various studies have been conducted on $nTiO_2$, which suggested that dose dependent application of $nTiO_2$ could promote a positive impact on plants (Gao et al., 2008; Vuong, 2019; Zheng et al., 2005). A study conducted on spinach confirmed the increased plant growth with foliar spraying, and also when their seeds were administered with $nTiO_2$. The seeds priming with $nTiO_2$ enhanced the activity of numerous enzymes and also promoted the rate of nitrates adsorption, which accelerated the inorganic nitrogen transformation into organic nitrogen (Zheng et al., 2005). Under Cd stress, $nTiO_2$ have been reported to reduce the metal toxicity and increase the production of biomass along with improvement in chlorophyll content of *Oryza sativa* (Zhang et al., 2020). It has been found that $nTiO_2$ promoted growth by increasing the gas exchange rates in leaves of *Oryza sativa*, and also enhanced the production of antioxidant enzymes (Rizwan et al., 2019).

2.7. Nanoparticles Induced Toxicity

Varying concentrations of nanoparticles have been found to cause toxicity of various levels in different categories of plant species, resulting in biochemical, physiological, morphological, and genetic effects (Tripathi et al., 2016). Upon interaction with plant roots, nanoparticles either form chelates with root exudates through binding, or are translocated to shoots, where they tend to accumulate in the aerial parts (Mustafa & Komatsu, 2016; Tripathi et al., 2017). Although the usage of nanoparticles in agricultural activities has proven to be of great benefit to plants and crops, but if not in suitable environmental conditions, concentrations, or form, nanoparticles may induce stress leading to unfavorable impacts indicated by low seed germination, reduced root and shoot length, low plant biomass, decreased transpiration, imbalanced regulation of genes dealing with stress, increased damage to DNA, and self-death of the plant cells (Tripathi et al., 2017). The inhibitory effects on wheat growth beyond optimum $nTiO_2$ concentration have been reported by Rafique et al. (2014). Dağhan (2018) estimated reduction in the dry biomass of *Zea mays* at high $nTiO_2$ concentrations, whereas silver (Ag) nanoparticles have been found to reduce the root and shoot biomass in *Oryza sativa* (Nair & Chung, 2014). A study carried out to find the impacts of multiple concentrations i.e. 20, 200, and 2000 mg kg^{-1} of silver nanoparticles (nAg) on wheat showed reduction

in the crop yield and quality with increase in the exposure dose. Relative to the control, at the highest concentration, root and shoot biomass decreased by 73% and 60%, respectively (Yang et al., 2018).

Investigations have showed $nTiO_2$ to significantly inhibit the plant growth promoting bacteria, including nitrogen fixers and phosphate solubilizers (Chavan et al., 2020). *Anabaena variabilis*, a cyanobacterium, has also been reported to have inhibited growth and nitrogen fixation activity in response to $nTiO_2$ exposure (Cherchi & Gu, 2010). Servin and colleagues (2013) have also reported a reduction of carbohydrates in cucumber, owing to application of high concentrations $nTiO_2$. They further suggested that nanoparticles may alter the chemical environment of the carbohydrates and macromolecules, affecting the nutritional quality of the plant and the fruit. Additional evidence was collected by Morales et al. (2013), who observed alteration in the chemical environment of carbohydrates in *Coriandrum sativum*, causing a change in its nutritional content.

Oxidative stress is also among the major phytotoxic effects on plants, induced by nanoparticles application (Huerta-García et al., 2014). Production of reactive oxygen species (ROS) leads to oxidative stress, lipid peroxidation, and damage to protein and DNA structure, thus disrupting their functionality (Arruda et al., 2015; Siddiqui et al., 2015). $nTiO_2$, nZnO, cerium oxide ($nCeO_2$), as well as nAg have been found to induce oxidative stress signaling pathways in plants (Mustafa & Komatsu, 2016). Servin et al. (2013) reported oxidative stress in cucumber, in response to $nTiO_2$ exposure, whereas increased H_2O_2 production as a result of increasing concentrations of $nTiO_2$ was also reported by Rafique et al. (2018). Photosynthesis is among the key factors of plant systems, whereby, light energy is converted to chemical energy, which forms the basis of the primary energy source of the food chain. Nanoparticles application tends to affect the photosynthetic processes. Though, the impacts are positive, such as enhanced light absorbance, thus transformation into chemical energy, and increased carbon dioxide assimilation, nanoparticles tend to have unfavorable impacts on the system too. Application of increasing concentrations of $nTiO_2$ caused decreased chlorophyll content in *Zea mays* (Dağhan, 2018), and rutile phase $nTiO_2$ have been observed to cause damage

to chloroplast and its internal organelles, leading to deformed shaped chloroplasts (Iswarya et al., 2015).

$nTiO_2$ are more biodegradable compared to the widely used carbon nanomaterials such as graphene and porous carbon, which are derived from natural sources, but induce toxic impacts on human health (Raliya et al., 2017; Rodríguez-González et al., 2019). In comparison to other metal/ metal oxide-based nanoparticles, since $nTiO_2$ are relatively inert in behavior and do not release phytotoxic ions upon dissolution. They can be used in a dose-dependent manner to promote plant growth. The International Agency for Research on Cancer (IARC) has categorized TiO_2 as a "possibly carcinogenic to humans", and therefore it falls under Group 2 carcinogen (Hu et al., 2020), whereas the allowed Ti concentrations in food and personal care products are under 10 µg mg^{-1} of food and 100 µg mg^{-1} of a product, respectively (Rodríguez-González et al., 2019). Based on the established scientific research, it is evident that nanoparticles may induce phytotoxicity, when applied in higher doses, and may also enter different environmental matrices (e.g., soils or sediments). Lack of detection methods and low reliability of techniques has further made the detection and estimation of nanoparticles a difficult task (Mahdi et al., 2017; Navratilova et al., 2015).

2.8. Significance of Hydroponics and Cultivation of Food Crops

Hydroponics brought a paradigm shift in agricultural production from a soil-dependent to soil-less medium in 1930s. The water recirculation in a system not only provides an effective method for crop production under sufficient nutrients supply, it significantly reduces the water consumption pattern in a closed system (Premanandh, 2011). With introduction of multiple benefits like minimum or no soil contamination, controlled nutrients composition, high productivity, and better quality have made it a better alternative to conventional agricultural system (Sapkota et al., 2019). A comparative study based on soil and soil-less system was conducted for growing vegetable plants including pea (*Pisum sativum*), okra (*Abelmoschus esculentus*), and moong (*Vigna radiata*). The hydroponic cultures of four weeks old pea and okra seedlings showed higher root-shoot ratio showing the more suitability of hydroponics-based system for growing leafier biomass. Additionally, high chlorophyll content was

found in okra and moong seedlings which could be attributed to the leaf expansion and larger area (Sankhalkar et al., 2019).

2.9. Related Research Work Done at IESE

In a study investigating the effect of exposure-response of $nTiO_2$ (0-1000 mg kg^{-1}) on 10 different wheat cultivars, it was found the only *Galaxy* cultivar sustained the whole exposure range during germination by improving the seedling vigor index (SVI) and height stress tolerance index (Zahra et al., 2019). In another experiment with 60 days $nTiO_2$ application in *Triticum aestivum* up to 100 mg kg^{-1}, the root and shoot length, and plant phosphorus content was found higher between 20 and 60 mg kg^{-1} nanoparticles treatment, and reduced with any further increase in the applied dose. The chlorophyll content also increased by 32.3%, indicating high photosynthetic activity, and improvement in the overall growth (Rafique et al., 2018). Earlier, Ahmad (2017) also studied the effects of soil pH on availability of phosphorus in *Triticum aestivum* upon $nTiO_2$ application. The results drawn from the study indicated an improvement in plant growth and nutrients availability. The maximum growth was recorded at pH 7.3 with the highest applied $nTiO_2$ dosage of 50 mg kg^{-1} which was attributed to a subsequent increase in reactivity, polarizing power, and the photocatalytic activity of nanoparticles at higher concentration.

In 2016, Naima Waseem reported positive impact of $nTiO_2$ application on the *Super Basmati* genotype of *Oryza sativa* as the crop showed an improvement in overall physiological parameters, nutrients availability, and chlorophyll content. About 15% increase in the shoot length and 38% increase in the phosphorus content was reported in plants. Moreover, this genotype was also found to be resistant to the negative impacts of nanoparticles even at the germination stage. Focusing the nutrients mobility in plants, Hanif and coworkers (2015) studied the phytoavailability of phosphorus to *Lactuca sativa* in response to soil applied $nTiO_2$ and found a positive correlation between these two. They reported 1.5 folds increase in the overall plant length along 2 folds increase in the total dry biomass, and 4 folds increase in the total P uptake. A study based on the application of metallic nanoparticles, $nTiO_2$ and magnetite (Fe_3O_4), on *Lactuca sativa* also concluded similar findings. The shoot length significantly increased up to 36% and

49% upon $nTiO_2$ and nFe_3O_4 application, compared to control, whereas total dry biomass increased by a similar percentage i.e. 1.4 folds under both treatments (Zahra, 2014).

Likewise, the growth and nutrients uptake response of wheat and lettuce to $nTiO_2$ showed positive results on both species. For lettuce, a dose dependent increase in the plant growth and phytoavailability of phosphorus was observed. Wheat plants had highest rate of growth and phosphorus uptake at 60 mg kg^{-1} $nTiO_2$ application in comparison to control, whereas with further increase in the dose, this positive impact was reversed as indicated through reduction in plant biomass along reduced root and shoot length (Rafique et al., 2014). Sana Ullah (2013) carried out a hydroponics-based study to observe the risk response of $nTiO_2$ on wheat with a maximum applied dosage of 600 mg kg^{-1}. Results showed that roots could only elongate at the applied dose of 200 mg L^{-1} and followed a downwards trend at 400 mg L^{-1}.

On basis of the detailed literature review and previous studies carried out at IESE, it could be hypothesized that $nTiO_2$ may improve the overall plant growth by enhancing the nutrients mobilization and uptake, thus consequently improving the other parameters such as amount of chlorophyll, rate of photosynthesis, and mediation of oxidative stress. To test this hypothesis, the detailed methodology adopted is discussed in Chapter 3.

Chapter 3

Materials and Methods

This chapter gives a detailed overview of the materials and methods adopted and applied for conduction of the experiment.

3.1. Synthesis of $nTiO_2$

The synthesis of nanoparticles was carried out via liquid impregnation method using titania powder as a precursor. A solution was prepared using 50 g titania powder and 300 mL distilled water followed by stirring for 24 hours at a moderate speed. It was then left to settle for the next 24 hours followed by 12 hours of oven drying at 105℃ (Figure 3). The dried slurry was properly crushed and put in the muffle furnace for at 550℃ for 6 hours (Husnain et al., 2016).

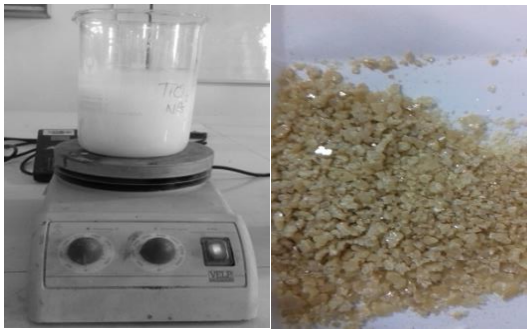

Figure 3: Synthesis of $nTiO_2$ through liquid impregnation method

3.2. Characterization of Nanoparticles

3.2.1. Scanning Electron Microscopy (SEM) Analysis

SEM analysis was carried out to get particle size and study surface morphology of $nTiO_2$ at various magnifications. The sample was prepared by taking a very small number of nanoparticles (approx. 100 mg) in 10 mL distilled water followed by sonication for 15 minutes. It was then spread on a glass slide with the help of a dropper and allowed to air dry. The slide was then carefully cut in a 1x1 dimension and analyzed for particle size under different magnifications. The accelerating voltage was set as 20 kV. In order to avoid any contamination and interferences, Atomic Ion Sputtering Device (JEOL, JFC-1500) was used for gold coating of nanoparticles.

3.2.2. X-Ray Diffraction (XRD) Analysis

X-Ray Diffractometer, Theta-Theta STOE, Germany was used to identify the crystalline phase and average crystallite size of nanoparticles. It was operated at 20 kV and 5 mA, and Cu Kα radiations (λ = 0.154 nm) were used. The diffraction peaks were collected with an acquisition time of 1.0 second per step and in the 2θ range of (20º-80º) with a 0.04º step size. Average crystal size was obtained using the Scherrer equation through Origin Pro (8.5.0).

$$D = \frac{K\lambda}{\beta\cos\theta}$$

Where, D = Crystallite size (nm)

K = 0.891 (Scherer constant)

λ = 0.1542 (Wavelength of the x-ray source)

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

θ = The diffraction angle of crystal phase (radian)

3.3. Preparation of Hoagland Nutrients Solution

Followed by the germination for 5 days, seedlings were transferred to the nutrients solution containing defined concentrations of $nTiO_2$ (0, 100, 200, 300, 400, and 500 mg L^{-1}). Composition of the Hoagland nutrients solution (Table 2) was adapted from (Hoagland & Arnon, 1950). All the chemicals for preparation of solution A were dissolved in distilled water and a solution of 100 mL was prepared. Solution B was made by pouring 0.5 mL concentrated H_2SO_4 in distilled water and total volume was made as 100 mL. All the chemicals for solution C were dissolved in 400 mL distilled water and put on heating at 70℃ until the color turned to yellow-brown and left for cooling. It was further diluted with distilled water making 500 mL total volume. All chemicals required for preparation of 10X Hoagland stock solution were dissolved in distilled water with further addition of 30 mL of solution A and 3 mL solution B. Final volume was adjusted to 3 L and stored at 4℃ until further use. Hoagland nutrients solution (1X) was prepared using 3 L prepared 10X stock solution, 150 mL of solution C, and bringing its final volume to 30 L with distilled water. It was prepared just before use.

Table 2: Composition of the Hoagland nutrients solution

Chemicals	**Weigh**
Solution A*	
Boric acid (H_3BO_3)	280 mg
Manganese sulfate ($MnSO_4.4H_2O$)	340 mg
Copper sulfate ($CuSO_4.5H_2O$)	10 mg
Zinc sulfate ($ZnSO_4.7H_2O$)	22 mg
Ammonium heptamolybdate ($(NH_4)_6MO_7O_{24}.4H_2O$)	10 mg
Solution B*	
Concentrated sulphuric acid (H_2SO_4)	0.5 mL
Solution C*	
Disodium ethylenediaminetetraacetate dihydrate (Na_2EDTA)	3.36 g
Iron sulfate ($FeSO_4$)	2.79 g
Hoagland Stock Solution (10X)*	
Calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$)	28.2 g
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	15.6 g
Potassium nitrate (KNO_3)	19.8 g
Diammonium phosphate $(NH_4)_2HPO_4$	20.64 g

**Stored at 4 ℃*

3.4. Seeds Germination

Healthy rice seeds (Super Basmati variety) obtained from Rice Research Institute, Kala Shah Kaku, Lahore were surface sterilized with 5% (w v^{-1}) calcium hypochlorite $(CaClO)_2$ for 10 minutes and washed thoroughly with distilled water. They were then germinated in an incubator at 33℃ for a period of 5 days. Germination setup was made to ensure that roots do not entangle each other. The germination solution containing 10 µM boric acid (H_3BO_3) and 600 µM concentration of calcium chloride ($CaCl_2$) was prepared up to a volume of 5 L and each pot was filled equally. PVC plastic pots were covered at the top by a nylon mesh and seeds were uniformly spread over it. The containers were then put in an incubator for 5 days to germinate (Figure 4).

Figure 4: Seeds germination for the pot experiment

3.5. Experimental Setup

Oryza sativa was grown in hydroponics. The experiment was randomly arranged in a greenhouse with a total of 5 replicates of each treatment along with control for 6 weeks of plant growth (Figure 5). Air diffusers were connected to the Polyvinyl chloride (PVC) flexible tubing and distributed into each pot to ensure continuous and uniform air flow to plant roots for 8 hours a day. Pots were covered with polystyrene sheets having 6 holes in each, stuffed with cotton for anchoring plants. The young rice saplings were transferred to the Hoagland nutrients solution and grown under ambient temperature and humidity in the greenhouse for whole growth experiment.

Figure 5: *Oryza sativa* greenhouse pot experiment setup

3.6. Physicochemical Analysis during Pot Experiment and Harvesting

The Hoagland's solution pH, electrical conductivity (EC), and temperature were monitored and noted with regular intervals throughout the experimental phase (Figure 6). Plants were monitored until they reached maturity followed by harvesting. Fresh weight of the harvested plant tissues including root and shoots were noted separately. Measurements for length were made followed by the washing of roots using 0.1 M hydrochloric acid (HCl) to help remove nanoparticles adhered to the roots' surface. Plant roots were rinsed thrice with distilled water and stored in ultra-freezer till further analysis. The post-harvest Hoagland nutrients solution was stored at 4℃ for the nutrients' analysis.

Figure 6: Measurement of the Hoagland nutrients solution's pH and EC

3.7. Post-Harvest Physicochemical Analysis of Hoagland Nutrients Solution

3.7.1. Nitrite-Nitrogen (NO_2^--N) Analysis

The NO_2^--N in Hoagland solution was analyzed via photometric/ colorimetric method (Baird et al., 2017). The pH of Hoagland nutrients solution was measured and adjusted between 5-9 using 1N HCl or ammonium hydroxide (NH_4OH). A 25 mL sample was measured, followed by addition of 1 mL color reagent. Upon addition of color reagent, the solution developed pink color (Figure 7). It was then swirled gently and allowed to rest for around 10 minutes. The absorption was analyzed at 543 nm via UV-Visible Spectrophotometer.

Figure 7: Hoagland solution's NO_2^--N content measurement

3.7.2. Nitrate-Nitrogen (NO_3^--N) Analysis

The NO_3^--N in Hoagland solution was analyzed via photometric/ colorimetric method (Baird et al., 2017). Precisely measured 0.5 mL (1 N) HCl was poured in 25 mL of the Hoagland solution sample. After preparation, all samples were run on UV-Visible Spectrophotometer at 220 nm and 275 nm for measuring the absorbance. To obtain absorbance due to NO_3^-, the standard curve was plotted by subtracting two times the absorbance reading at 275 nm from the reading at 220 nm, for both samples and standards. Using the sample absorbance, concentration was then directly calculated from the standard curve.

3.7.3. Total Kjeldahl Nitrogen (TKN) Analysis

The Kjeldahl nitrogen measured the presence of organic nitrogen and ammonia-nitrogen (NH_3-N) in given samples (Baird et al., 2017). A 20 mL of sample volume was measured and added in digestion tube along 3 g potassium sulphate (K_2SO_4), 0.1 g copper sulphate ($CuSO_4$), and 5 mL concentrated H_2SO_4 and set for digestion at 350℃ for around 20 minutes till the digestion was completed and white fumes got separated from translucent digested solution. After digestion, the distillation was carried out in the Automatic Kjeldahl Distillation Unit, therefore after cooling, the Kjeldahl tube containing digested sample was then set at Automatic Kjeldahl Distillation Unit with distillation time of 5 minutes, borate buffer injection volume of 20 mL, H_3BO_3 injection volume of 30 mL, and 70 mL injection volume of 30% sodium hydroxide (NaOH). The NH_3 was stripped by means of steam distillation, condensed during cooling, and trapped in H_3BO_3. Therefore, an empty 250 mL conical flask was placed at the outlet to receive H_3BO_3 with dissolved NH_3.

Followed by distillation, around 7-8 drops of mixed indicator were dropped in the flask containing distillate. After addition of indicator, the solution was then titrated against the 0.02 N H_2SO_4 to quantify TKN. The reading for the endpoint was noted when solution turned from mustard yellow to pink. A blank was also run using distilled water which showed pink endpoint indicating absence of nitrogen content in the sample. The final concentration was calculated using following formula:

$$\text{TKN (mg L}^{-1}) = \frac{\text{(A-B) x 280}}{\text{sample volume (mL)}}$$

Where, A = Volume of H_2SO_4 titrated for sample

B = Volume of H_2SO_4 titrated for blank

3.7.4. Phosphorus (P) Analysis

P content was quantified using vanadomolybdophosphoric acid colorimetric method (Estefan et al., 2013). Filtered Hoagland solution sample (10 mL) was poured in a conical flask (100 mL) using measuring cylinder and 2 mL vanadate molybdate reagent was added in it. The solution was swirled gently and left for 10 minutes for color to

develop (Figure 8). When the solution developed yellow color, it was analyzed for the absorbance at 470 nm using UV-Visible Spectrophotometer.

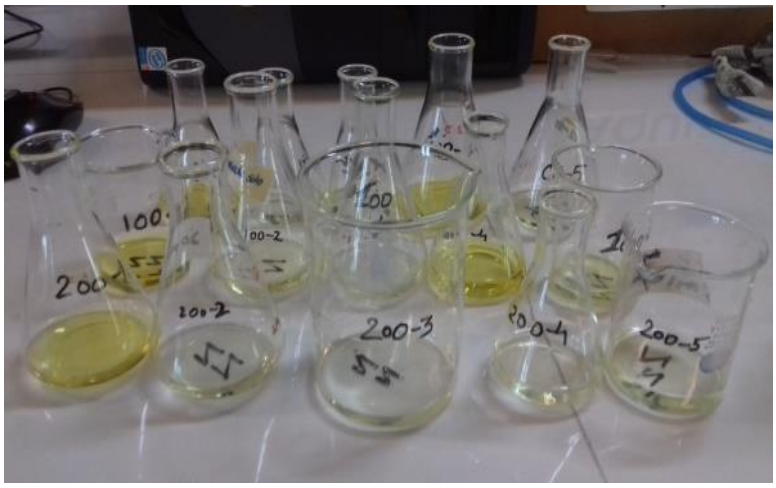

Figure 8: Measurement of the Hoagland solution's P content

3.7.5. Potassium (K) Analysis

The K content in water samples was quantified through flame photometer (Estefan et al., 2013). Hoagland nutrients solution samples were prepared by simple filtration and analyzed on flame photometer. The wavelength used to analyze K was 766 nm and distilled water was aspirated subsequently after each reading. A standard curve was obtained by running K standards and concentration was calculated based on the obtained values.

3.8. Plants Analysis

Plant growth parameter such as shoot length was measured on weekly basis throughout the experiment, whereas root length was only measured at the time of harvesting. Other physicochemical analyses were carried to analyze the overall quality of the harvested crop.

3.8.1. Measurement of the Relative Water Content (RWC)

The RWC was measured as per methodology described in the Handbook of Plant Ecophysiology Techniques (Roger, 2001). Around 5 sets of Eppendorf tubes (0.5 mL and 1.5 mL) were taken for 5 replicates and labeled with same number. All 1.5 mL Eppendorf tubes were filled with a pre-cooled deionized water. Fresh youngest developed leaf was taken from plant samples, cut in 4 small pieces of 0.5 cm^2 (apical and basal parts were

avoided for taking the leaf samples), and put in 0.5 mL Eppendorf. These tubes were sealed and placed on ice to stop the evaporation of water and growth (if any). These tubes were then weighed for measuring plant fresh weight (FW). These plants were then put back into 1.5 mL Eppendorf tubes filled with deionized water, placed in ice using icebox, and put in the refrigerator. To measure the fully turgid fresh weight (TFW), after 4 hours, the tissues were taken out carefully with help of a forceps and excess water was gently removed using tissue paper. These tissue samples were transferred to the previously used 0.5 mL tubes and reweighed. Followed by this, tissues were put for over drying 48 hours at 70 °C temperature. The dry weight (DW) of plant was noted and RWC was calculated using following formula:

$$\text{RWC} = \frac{\text{FW} - \text{DW}}{\text{TFW} - \text{DW}} \text{ x } 100$$

Where, FW = Fresh weight

DW = Dry weight

TFW = Turgid fresh weight

3.8.2. Chlorophyll Content Analysis

The chlorophyll content of fresh plant biomass was calculated using dimethyl sulphoxide (DMSO) for extraction (Hiscox & Israelstam, 1979). Fresh leaf biomass (100 mg) was cut into fractions and put in a glass vial containing 7 mL DMSO. The vials were collectively placed in an oven and incubated at 65℃ until the chlorophyll completely got extracted from leaves and they turned transparent-white. The extract was then diluted up till 10 mL with DMSO (Figure 9) and analyzed immediately for absorbance at 645 and 663 nm on UV-Visible Spectrophotometer. The samples were analyzed against a DMSO blank and final readings were obtained using Arnon equation mentioned below. If the value of absorption appeared higher than 0.7, the extract was diluted to 50% using 90% DMSO and adjusted accordingly.

$$\text{Chlorophyll a} = (12.7\ A_{663}) - (2.69\ A_{645})$$

$$\text{Chlorophyll b} = (22.9\ A_{645}) - (4.68\ A_{663})$$

$$\text{Total Chlorophyll} = (20.2\ A_{645}) + (8.02\ A_{663})$$

Where, A_{663} = Absorbance at a wavelength of 663 nm

A_{645} = Absorbance at a wavelength of 645 nm

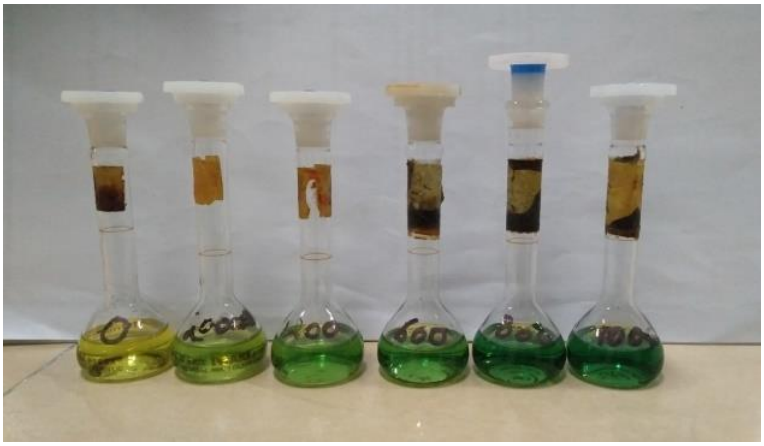

Figure 9: Estimation of *Oryza sativa's* chlorophyll content

3.8.3. Carbohydrates Content Analysis

The carbohydrates in plant samples were analyzed using Morris Anthrone Method (Ludwig & Goldberg, 1956). Fresh plant sample (100 mg) was taken in a glass tube and hydrolyzed in a hot water bath with 5 mL of 2.5 N HCl for 3 hours. The temperature of the extract was brought down to room temperature and neutralized with solid sodium carbonate ($Na_2\ CO_3$) until the effervescence ceased. The solution was centrifuged at 3000 rpm for 15 minute after dilution with 100 mL distilled water. From each sample, 1 mL of the supernatant was collected, placed in an ice, and 4 mL of ice-cold Anthrone ($C_{14}H_{10}O$) reagent was added in it. Samples along with the working standard solutions were heated in a boiling water bath for around 8 minutes and cooled at room temperature. Standards were prepared according to the methodology described by Serna-Saldivar (2012). Absorbance was read at 630 nm and the calibration curve was obtained.

3.8.4. Thiobarbituric Acid Reactive Substances (TBARS) Assay

This assay is based on potassium iodide (KI) oxidation by H_2O_2 in an acidic medium. Accurately measured 50 mg of fresh plant biomass was taken and homogenized with 1.5 mL of 5% TCA using liquid nitrogen. After homogenization, the homogenate was transferred to 1.5 mL Eppendorf tubes and centrifuged for 10 min at 10,000 g at room temperature (25℃). From this sample, 1 mL of the supernatant was transferred to second tube, and 1 mL of 20% (w v^{-1}) TCA containing 0.5% (w v^{-1}) TBA was added in it.

This solution was heated at 95℃ for 30 mins, cooled in ice, and absorbance was measured at 532 nm and corrected for unspecific turbidity by subtracting the value of absorbance at 600 nm. The TBARS content was calculated as nmol g^{-1} fresh weight, and extinction coefficient used for measurement was 155 mM^{-1} cm^{-1} (Das et al., 2017).

3.8.5. Total Kjeldahl Nitrogen (TKN) Analysis

TKN in plant samples was digested and distilled according to methodology described by Estefan et al. (2013). The plant samples were oven-dried at 60℃ overnight and cooled in the desiccator. Carefully weighed 0.2 g of the dried plant sample was taken in the digestion tube followed by addition of 3 g K_2SO_4, 0.1 g $CuSO_4$, 20 mL distilled water, and 7 mL concentrated H_2SO_4. The mixture was mixed well and set for digestion at 350℃ for around 20 minutes till the digestion was completed and white fumes got separated from translucent digested solution. After digestion, the distillation was carried out in the Automatic Kjeldahl Distillation Unit, therefore after cooling, the Kjeldahl tube containing digested sample was then set at Automatic Kjeldahl Distillation Unit (Figure 10) with distillation time of 5 minutes, borate buffer injection volume of 20 mL, H_3BO_3 injection volume of 30 mL, and 70 mL injection volume of 30% sodium hydroxide (NaOH). The NH_3 was stripped by means of steam distillation, condensed during cooling, and trapped in H_3BO_3. Therefore, an empty 250 mL conical flask was placed at the outlet to receive H_3BO_3 with dissolved NH_3.

Followed by distillation, around 7-8 drops of mixed indicator were dropped in the flask containing distillate. It was then titrated against 0.02 N H_2SO_4 to quantify TKN and endpoint was noted. A blank was also run using distilled water. The TKN content in plants was calculated using the formula for sediments analysis as described by Baird et al. (2017):

$$\text{TKN (mg kg}^{-1}) = \frac{\text{(A-B) x 280}}{\text{sample dry weigh (g)}}$$

Where, A = Volume of H_2SO_4 titrated for sample

B = Volume of H_2SO_4 titrated for blank

Figure 10: TKN distillation assembly unit

3.8.6. Total Phosphorus (P) Content Analysis

The P in plant extracts was determined through vanadomolybdo phosphoric acid method (Estefan et al., 2013). The plant samples were oven-dried overnight at 60℃ and cooled in a desiccator. Precisely weighed 100 mg dried sample was put in a 100 mL beaker and digested along 5 mL of mixture containing nitric acid (HNO_3) and perchloric acid ($HClO_4$) (2:1). It was digested on a hot plate set at 180℃ till the dense white fumes got separated from translucent aliquot. It was cooled and a solution with final volume of 25 mL was prepared by dilution with distilled water. The clear digested aliquot (2.5 mL) was added in a 25 mL volumetric flask along 5 mL ammonium-vanadomolybdate reagent. This solution was diluted up to 25 mL with distilled water. One blank with distilled water was kept along the samples. The absorbance of blank, standards and samples was read after 30 minutes at 410 nm on UV-Visible Spectrophotometer. A calibration curve was obtained for standards and total P concentration of the unknown plant samples was calculated.

3.8.7. Potassium (K) Content Analysis

The plant samples were oven-dried overnight at 60℃ and cooled in a desiccator. Determination of the K content was carried out through wet digestion method (Estefan et al., 2013). Precisely weighed 1 g grounded plant sample was added in 100 mL digestion flask and 5 mL of the HNO_3 and $HClO_4$ mixture was added to maintain the relative ratio

of 2:1. The digestion flask was put on hot plate and the temperature was slightly increased with time. Digestion was carried out till the liquid became colorless and dense white fumes got separated from aliquot. After cooling, distilled water was added to bring solution's total volume up to 25 mL. The samples were filtered using Whatman no. 42 filter paper and analyzed at 767 nm on Flame Photometer (Figure 11).

Figure 11: Flame photometer used for measurement of the K content

3.9. Statistical Data Analysis

The statistical analysis was applied using XLSTAT. The p-values were calculated separately for each parameter to test the validity of null hypothesis, using one-way ANOVA. Tukey's Honest Significant Difference (HSD) test was applied to carry out pair-wise comparisons between treatments and Dunnett's test was applied to make a comparison with control, with a confidence interval of 95%.

Chapter 4

Results and Discussion

This chapter includes the results obtained from the analyses carried out throughout the experimental phase. The discussion is based on the data evidence from previous studies, to test the validity of hypothesis. The nanoparticle treatments are mentioned as control (t0), 100 mg L^{-1} (t1), 200 mg L^{-1} (t2), 300 mg L^{-1} (t3), 400 mg L^{-1} (t4), and 500 mg L^{-1} (t5) $nTiO_2$, respectively.

4.1. Characterization of Nanoparticles

The SEM monograph in the Figure 12 showed surface morphology of $nTiO_2$ synthesized through liquid impregnation method. The particles were found to be spherical with distinct edges, but less monodispersity was observed due to the aggregate formation.

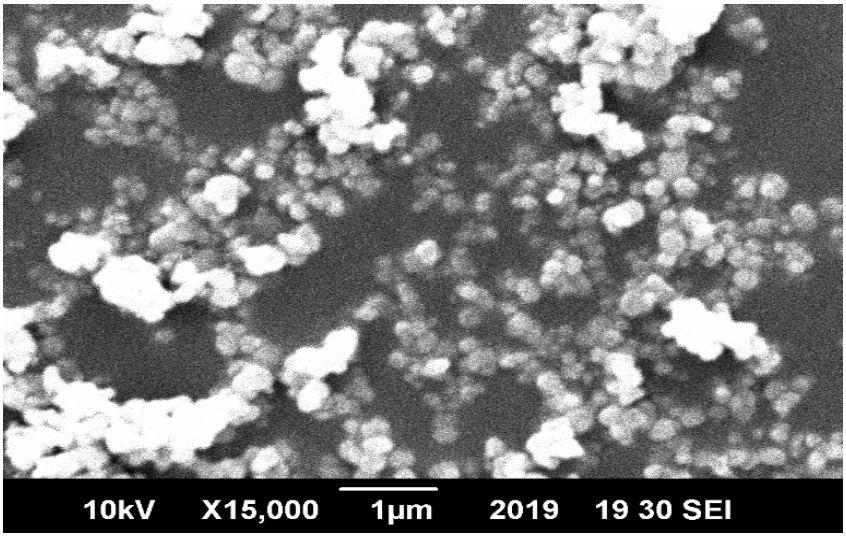

Figure 12: SEM monograph of $nTiO_2$

The x-ray diffraction pattern of the synthesized $nTiO_2$ is shown in Figure 13, and the peak details are given in Table 3. Sharp diffraction peaks indicated the crystallinity of the nanoparticles (Tanaka et al., 2016). The average particle size was calculated to be ~28 nm based on average crystallite size, using Scherrer equation, whereas the x-ray diffraction peaks at 25.4° and 48° confirmed the synthesis of anatase TiO_2 (Theivasanthi & Alagar, 2013).

Table 3: Diffraction peak details obtained via Origin Pro

Peak Position (2θ)	FWHM	Crystallite size D (nm)
25.414	0.262	30.969
37.088	0.240	34.805
37.925	0.299	28.080
38.690	0.258	32.513
44.328	0.362	23.667
48.159	0.313	27.743
54.009	0.344	25.917
55.182	0.340	26.359
62.787	0.347	26.812
68.880	0.378	25.465
70.398	0.409	23.748
75.163	0.405	24.738

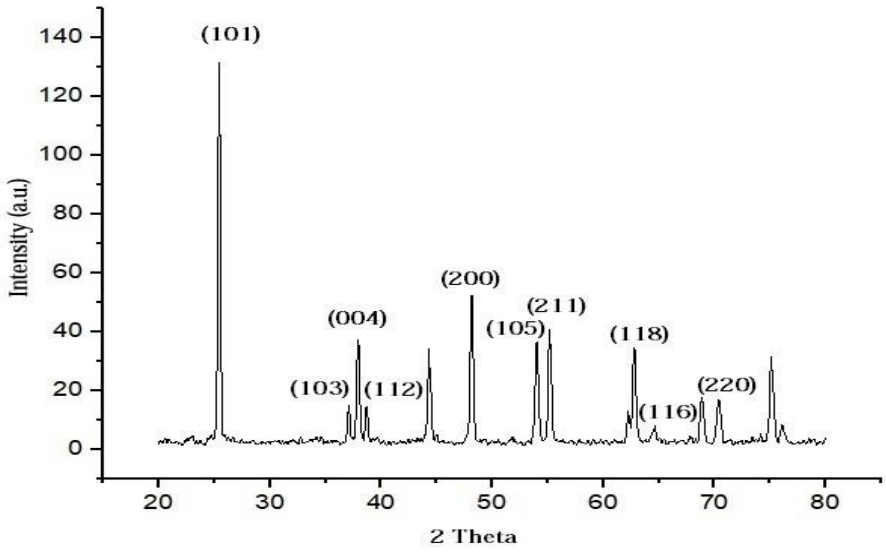

Figure 13: X-ray diffraction pattern of $nTiO_2$

4.2. Changes in the Hoagland Nutrients Solution's pH, EC, and Temperature

The pH of Hoagland nutrients solution was measured on weekly basis throughout the six weeks of plant growth experiment. Treatment-wise comparison and week-based data of the pH is given in Figure 14 and Table 4. Nanoparticles significantly impacted the pH of Hoagland solution with $p<0.05$. Compared to the control, there was 0.05 folds, 0.17 folds, 0.36 folds, 0.20 folds, and 0.23 folds increase in the pH values of treatments t1-t5, respectively. The treatments t3 and t5 were found to be significantly different than control, whereas maximum value was recorded for t3.

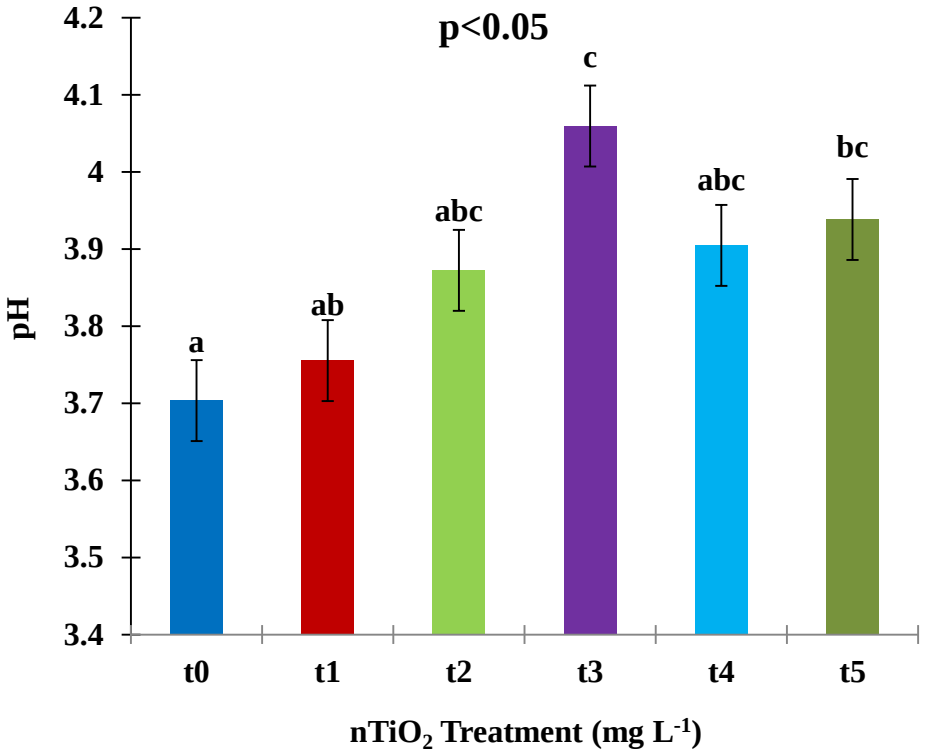

Figure 14: Treatment-wise comparison of the Hoagland solution's pH

Statistical analysis of the data obtained for Hoagland solution's pH showed that the values obtained for the week 1, 2, 3, and 5 were statistically significant ($p<0.05$). Overall, pH followed a decreasing trend throughout the first five weeks of growth experiment (Table 4). This reduction in Hoagland solution's pH could be linked to the formation of root exudates releasing low molecular weight organic acids such as lactic acid, citric acid, and oxalic acid (Ghoto et al., 2020). However, this rhizosphere acidification was not constant and in the sixth week a slight increase in pH was observed.

This increase was recorded as 1.98%, 1.10%, 2.56%, 1.86%, 6.37%, and 3.75% in t0-t5, respectively. This increase could be attributed to the mineralization of acids over time, causing pH to return to the original value (Macias-Benitez et al., 2020).

Table 4: Changes in the post-harvest Hoagland nutrients solution's pH

Treatment	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
t0	4.304[b]	3.882[b]	3.608[b]	3.478	3.440[ab]	3.508
t1	4.318[b]	4.020[b]	3.780[ab]	3.488	3.444[ab]	3.482
t2	4.342[b]	4.230[ab]	3.992[ab]	3.544	3.518[ab]	3.608
t3	4.798[a]	4.554[a]	4.252[a]	3.582	3.552[a]	3.618
t4	4.798[a]	4.468[a]	4.056[ab]	3.494	3.204[b]	3.408
t5	4.452[b]	4.294[ab]	4.010[ab]	3.594	3.572[a]	3.706

Figure 15 shows the trend of changing EC of Hoagland nutrients solution under different $nTiO_2$ treatments. EC is an indicator of the amount of nutrients present in solution, and are available for plant uptake (Kaur et al., 2016). The EC did not follow a uniform trend and was not found to be statistically insignificant ($p>0.05$). It decreased by 12%, 35%, 17%, 22%, and 42% in t1, t2, t3, t4, and t5, compared to the control. Lower EC indicated higher uptake and hence lower residual nutrients in the solution. The highest uptake was noted in the 200 and 500 mg L^{-1} $nTiO_2$ treatments. Moreover, literature suggests that high EC also causes salinity issues, but improved plant growth parameters indicated that application of $nTiO_2$ mediated the negative impact likely to be caused by salinity stress (Gohari et al., 2020).

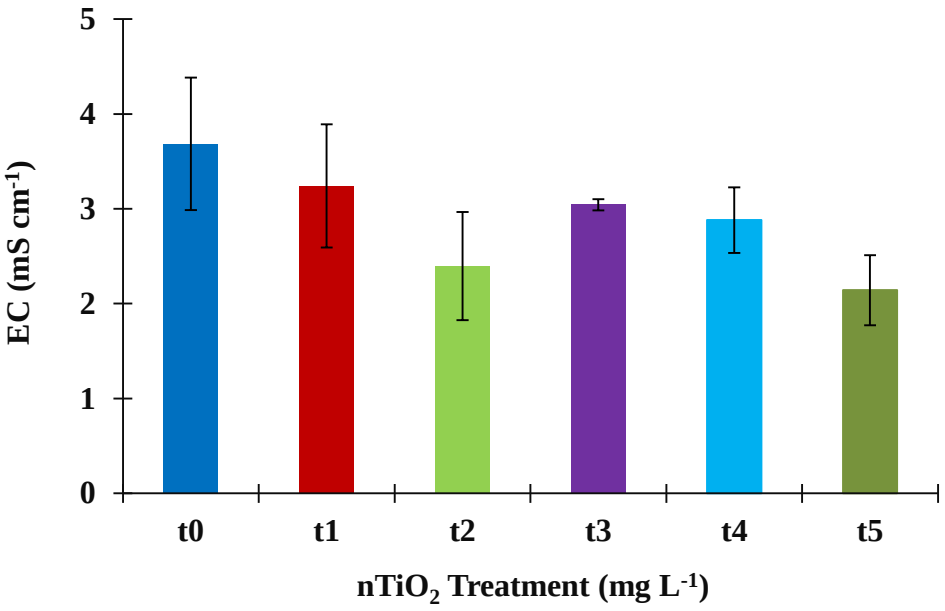

Figure 15: Average EC of the Hoagland nutrients solution

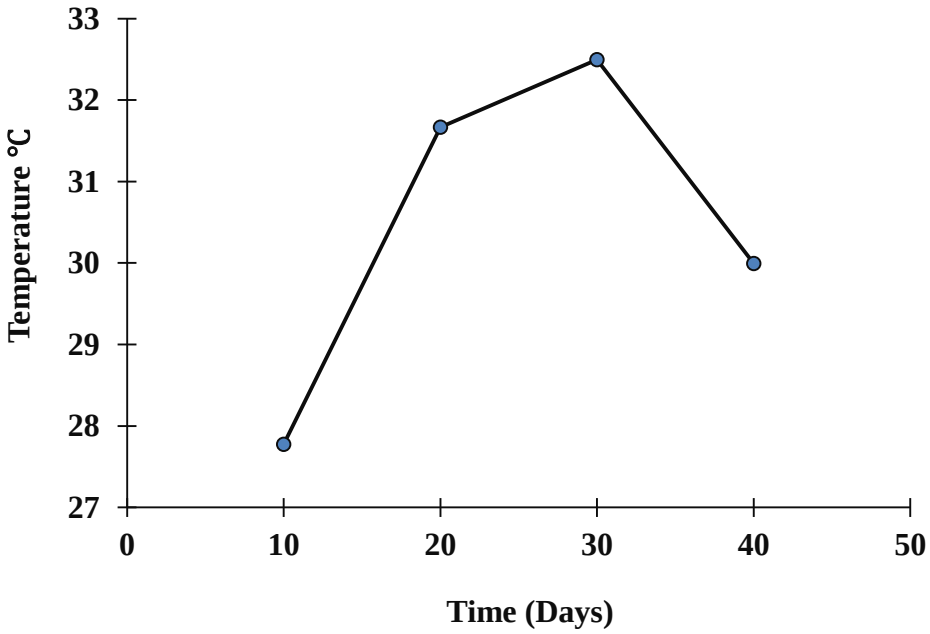

Figure 16: Hoagland nutrients solution's temperature

The temperature was recorded at regular intervals during 6 weeks of growth experiment (Figure 16). The average of four data points was calculated to be 30.5℃. Literature suggests that this temperature favors the cultivation of rice, with optimum range falling between 25℃ and 35℃ (Ghadirnezhad & Fallah, 2014). The water

temperature is an important parameter which impacts biochemical processes and rate of nutrients availability to plants in flooded conditions. Moreover, the high specific heat capacity of water also makes temperature a critical parameter for healthy plant growth under flooded conditions, as the warming of flooded fields gets slower (Krishnan et al., 2011).

4.3. Growth Response of Rice to $nTiO_2$

The effect of $nTiO_2$ on physical plant growth parameters including root and shoot length is illustrated in Table 5. After being continually grown in a hydroponic system for 6 weeks, the root length was highest in t4 followed by t5, t0, t3, t2, and t1. Statistical analysis showed that root length was statistically significant ($p<0.05$). Similarly, the highest shoot length was recorded in control (t0) followed by t3, t4, t5, t2, and t1; however, statistically it did not bring any significant information to explain variability in the data. Compared to the control, plant root length increased by 1.20 and 1.14 folds in 400 and 500 mg L^{-1} $nTiO_2$ treatment. The shoot height of t3 decreased by 1.03 folds compared to the control, besides being the highest among $nTiO_2$ treated plants. Based on the above findings it was concluded that control showed better root and shoot length due to complete absence of any nanoparticles induced stress.

Table 5: *Oryza sativa's* root and shoot length

$nTiO_2$ Treatment (mg L^{-1})	Root length (cm)	Shoot length (cm)
t0	$8.74^{ab} \pm 0.44$	15.51 ± 1.63
t1	$6.86^{b} \pm 0.41$	12.70 ± 1.08
t2	$7.09^{b} \pm 0.18$	12.92 ± 1.57
t3	$8.56^{ab} \pm 0.40$	15.09 ± 1.14
t4	$10.46^{a} \pm 0.32$	14.60 ± 1.40
t5	$9.96^{a} \pm 0.63$	14.07 ± 1.00

Supported by the findings of RWC and nutrients content (NPK), the increased plant height was due to readily available nutrients in solution. The nanoparticles additionally helped in NPK internalization in all treatments irrespective of the dose.

4.4. Relative Water Content in *Oryza sativa* Shoots

Leaf RWC refers to the absolute amount of water required by a plant to reach artificial full saturation (González & González-Vilar, 2001). The RWC of all treatments, except 100 mg L^{-1} $nTiO_2$ application, was statistically significant ($P<0.05$) compared to the control. Figure 17 presents the trend of RWC, where it increased till 300 mg L^{-1} $nTiO_2$ concentration and then followed a downwards trend. This decrease was noted to be 0.48% and 17.56% in t4 and t5, compared to t3. It has been reported that nanoparticles may increase the water uptake by penetration into root tissues, or enhance the activity of nitrate reductase enzyme, which further mobilizes the water uptake process (Mondal et al., 2011). The reduction in the RWC observed under higher concentrations of nanoparticles might have occurred due to deposition or adsorption of nanoparticles on the root hairs, blocking the route of water entry and thus low water intake by plants (Boykov et al., 2019)

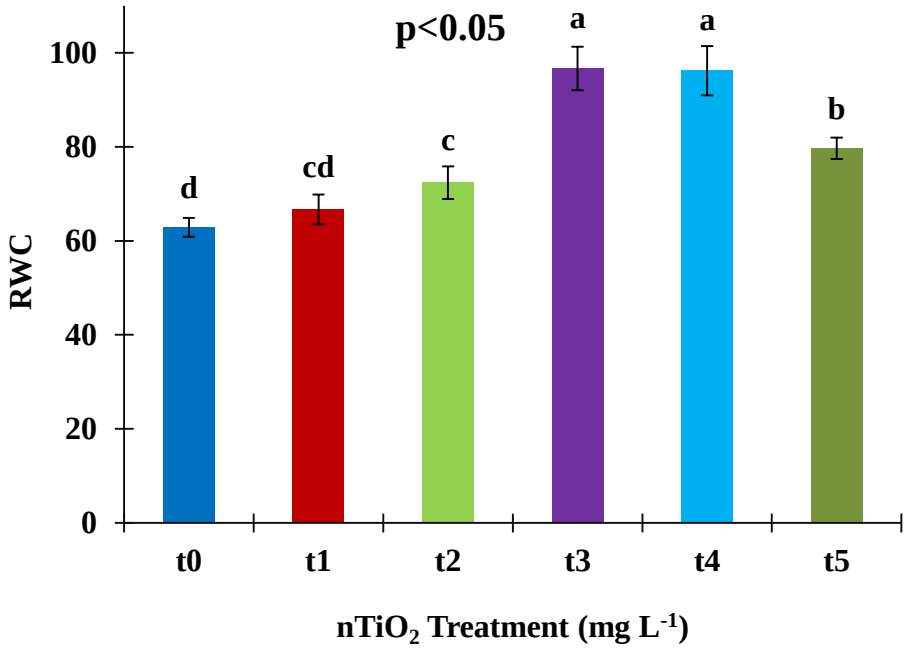

Figure 17: Relative water content in *Oryza sativa* shoots

4.5. Nitrogen (N) Content in Post-Harvest Hoagland Nutrients Solution and *Oryza sativa*

Figure 18, 19, 20, and 21 showed the impact of $nTiO_2$ on NO_2^--N, NO_3^--N, Hoagland solution's TKN, and rice TKN concentration. The former two did not show statistically significant values for Hoagland solution for any treatment. Lower nutrients concentration of the post-harvest Hoagland solution indicated higher uptake of nutrients by plants, thereby leaving small amount of the residual nutrients content behind. As represented in figure 16, the NO_2^--N content for all the treatments except t3 and t4 was higher than control. It was found to be the lowest in t4 (2.66 mg L^{-1}) followed by t3 (2.78 mg L^{-1}), and the highest in t5 (4.72 mg L^{-1}).

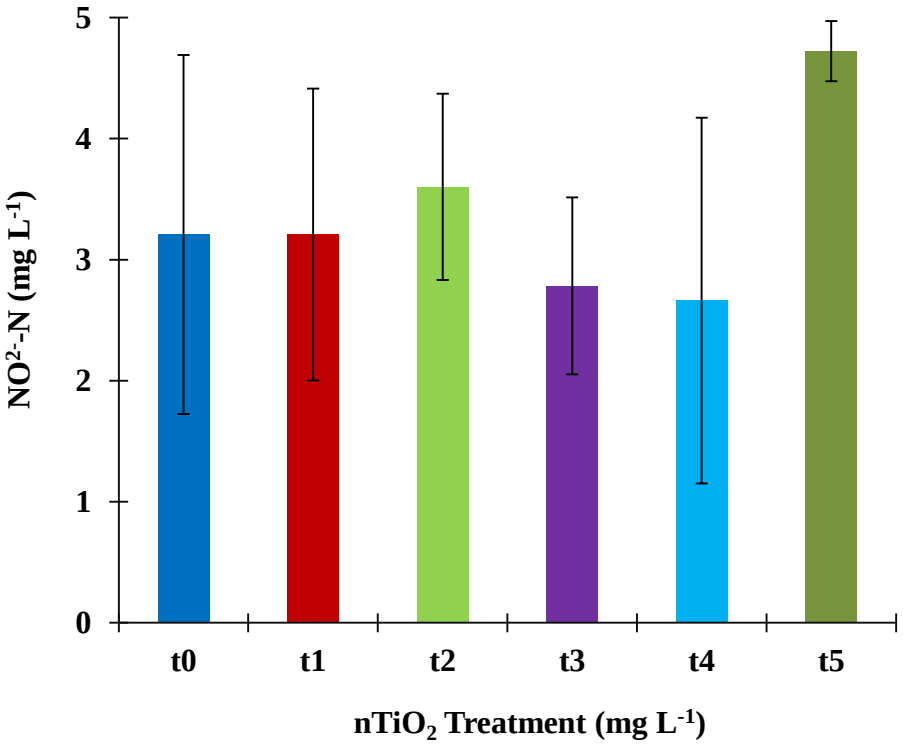

Figure 18: NO^{2-}-N content in Hoagland solution

The NO^{3-}-N concentration (Figure 17) was noted to be minimum at t4 (7.01 mg L^{-1}) followed by t3 (7.03 mg L^{-1}), and maximum at 200 mg L^{-1} (7.3% higher than control) followed by 500 mg L^{-1} (0.9% higher than control) $nTiO_2$ concentration. Only these two treatments were found to be higher than the control.

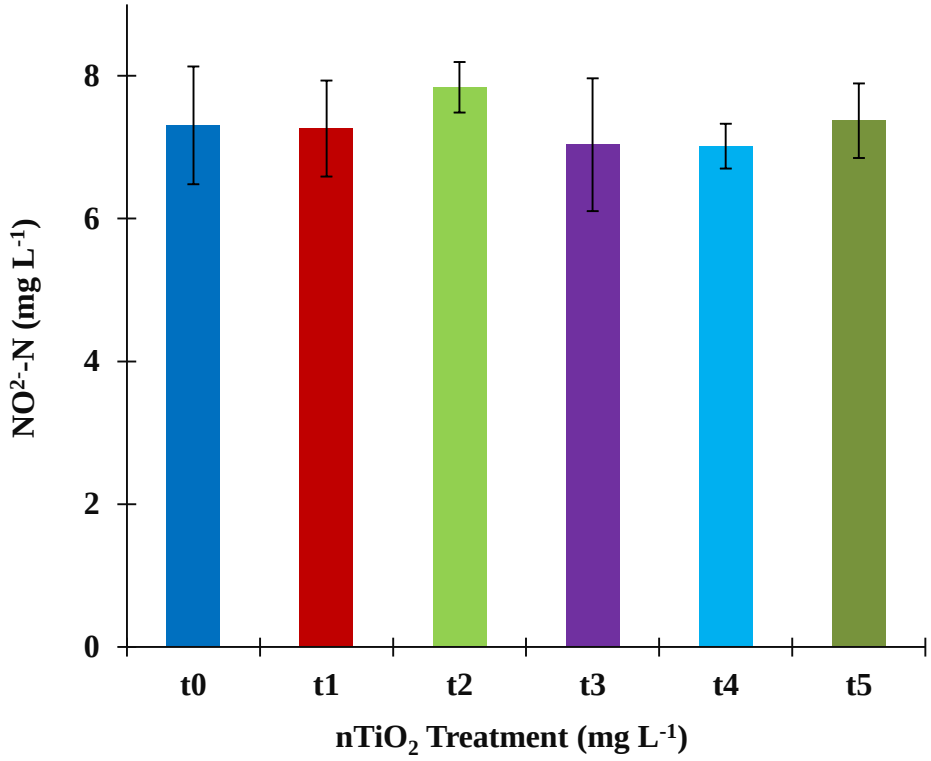

Figure 19: NO^{3-}-N content in Hoagland solution

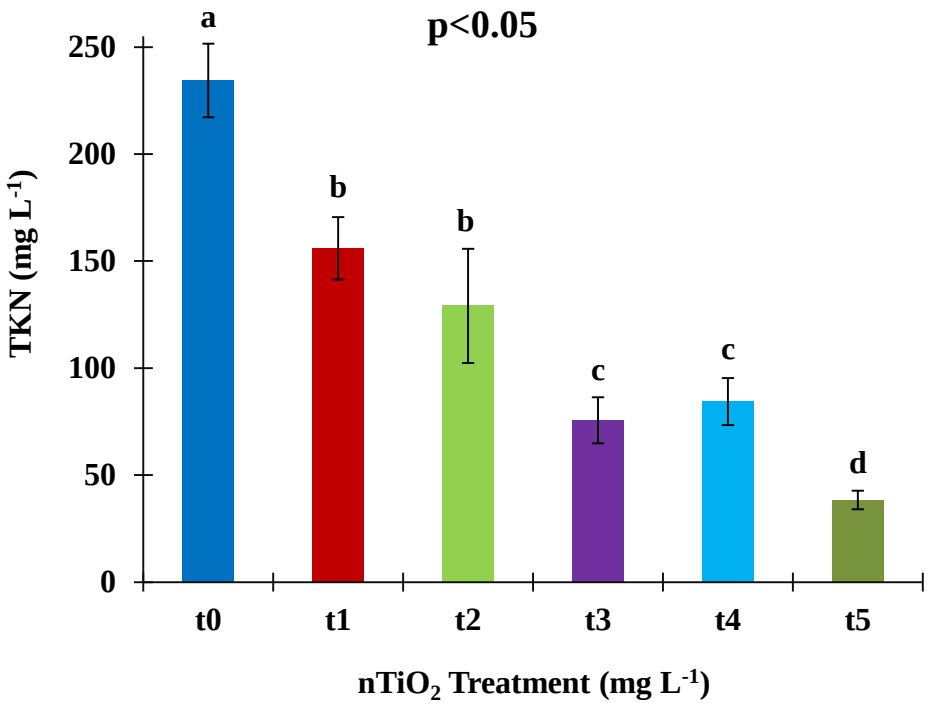

Figure 20: TKN content in Hoagland solution

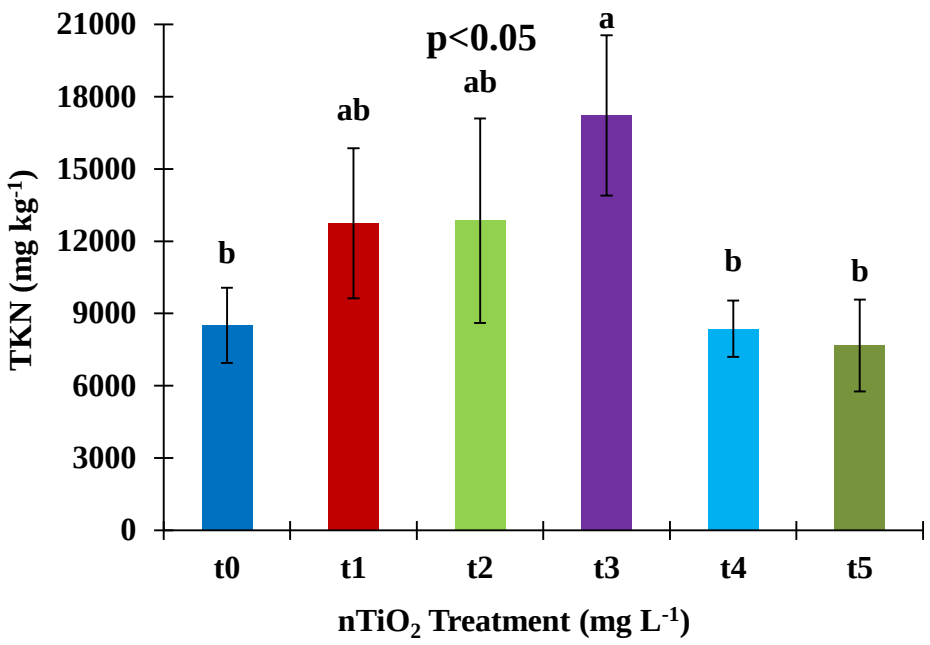

Figure 21: TKN in *Oryza sativa*

The TKN content in both, post-harvest Hoagland nutrients solution and rice was statistically significant ($p<0.05$). The TKN in Hoagland nutrients solution (Figure 18) was found to be 234, 156, 129, 76, 84, and 38 mg L^{-1} in t0-t5, respectively. Downwards trend in data indicated the decrease in residual TKN with increasing concentration of $nTiO_2$, hence more uptake with highest rate of nanoparticles application. All treatments were also found to be statistically significant for Hoagland solution compared to the control. The TKN content in plants (Figure 19) was measured to be 1.5 folds, 1.5 folds, and 2 folds higher in t1, t2, and t3, as compared to the control. Maximum amount of TKN was recorded in t3 which also showed statistically significant value among all 5 treatments. Additionally, lowest amount of TKN in *Oryza sativa* was present in t5 followed by t4. This indicated that though the amount of N in Hoagland solution was low (indicating uptake by *Oryza sativa*), the actual amount taken up by plants was not relatively high. This could be attributed to the nutrient's loss or adsorption of nutrients on the surface of nanoparticles. Total N content (Table 6) was calculated on the basis of NO_2^--N, NO_3^--N, and TKN (ammonia N and organic N), as presented in Table 6. Dose at which total N was the lowest in the Hoagland solution was t5 followed by t3, indicating maximum N uptake by plants grown in respective treatments. The findings for t3 also correlate with the higher TKN content in plants, however nutrients adsorption under

higher doses of nanoparticles might have resulted in lower N available for plants uptake in t5.

Table 6: Hoagland nutrients solution's total N content

Total N Content	t0	t1	t2	t3	t4	t5
NO_2^--N (mg L^{-1})	3.21	3.21	3.60	2.78	2.66	4.72
NO_3^--N (mg L^{-1})	7.30	7.26	7.84	7.03	7.01	7.37
TKN (mg L^{-1})	234	156	129	76	84	38.36
Total N (mg L^{-1})	81.62	55.48	46.84	28.47	31.32	16.82

$nTiO_2$ act as a catalyst to oxidize the ammonia N into nitrites and nitrates, using their photocatalytic properties (Altomare et al., 2015). At an optimum concentration, the $nTiO_2$ has been reported to regulate the enzymes required for nitrogen metabolism in plants, which enhance the nitrate uptake. These enzymes particularly include glutamate dehydrogenase, nitrate reductase, and glutamic-pyruvic transaminase glutamine (Rao & Shekhawat, 2014). Additionally, since these enzymes enhance the plants ability to uptake nitrogen, they also increase the plant physical growth parameters, chlorophyll content, and carbohydrates production (Samadi et al., 2014).

4.6. Phosphorus (P) Content in Hoagland Nutrients Solution and *Oryza sativa*

The P content in Hoagland solution and rice is shown in Figure 22 and 23. The P content in both, post-harvest Hoagland solution and plant, was found to be statistically significant ($p<0.05$), indicating that 36% and 59% of the variability in P content is explained by the $nTiO_2$ treatment. Compared to the control, only two treatments including t5 in Hoagland and t3 in *Oryza sativa* appeared to be statistically different with a confidence interval of 95%. However, $nTiO_2$ facilitated the uptake of the P till 300 mg L^{-1} concentration. $nTiO_2$ led P increase have also been reported by Zahra et al. (2017). Additionally, the low pH of Hoagland solution also followed a decreasing trend which is in coherence with the evidence that low pH increased the P availability to plants by adsorption of phosphate (PO_4^{3-}) ions (Zahra et al., 2015). The decreasing trend of the P in Hoagland nutrients solution could be attributed to the increase in its uptake by plants but

since the plant's P content did not correlate with the findings, it was suggested that P adsorption was restricted owing to high concentration of the $nTiO_2$ resulting in agglomerates.

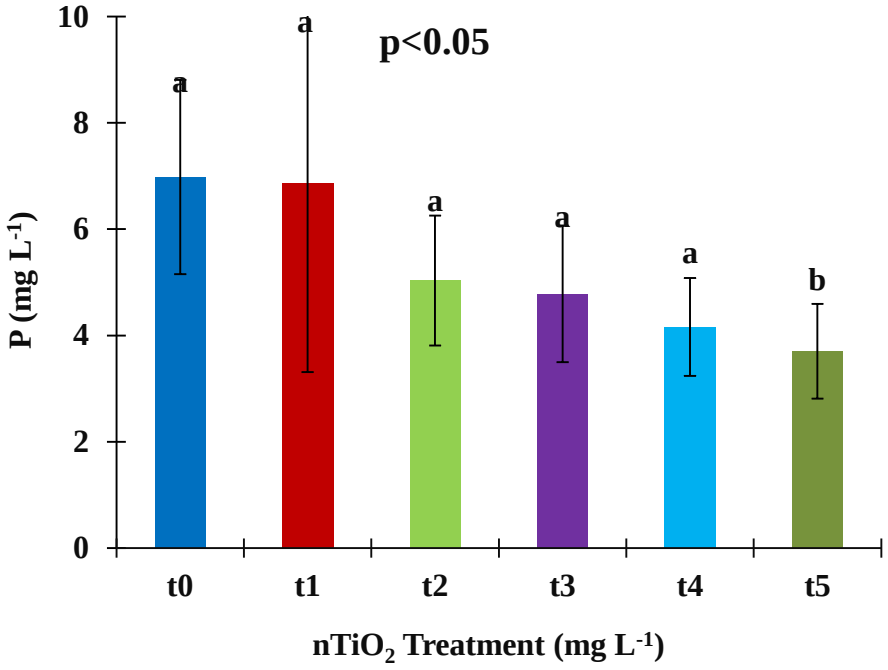

Figure 22: Hoagland solution's P content

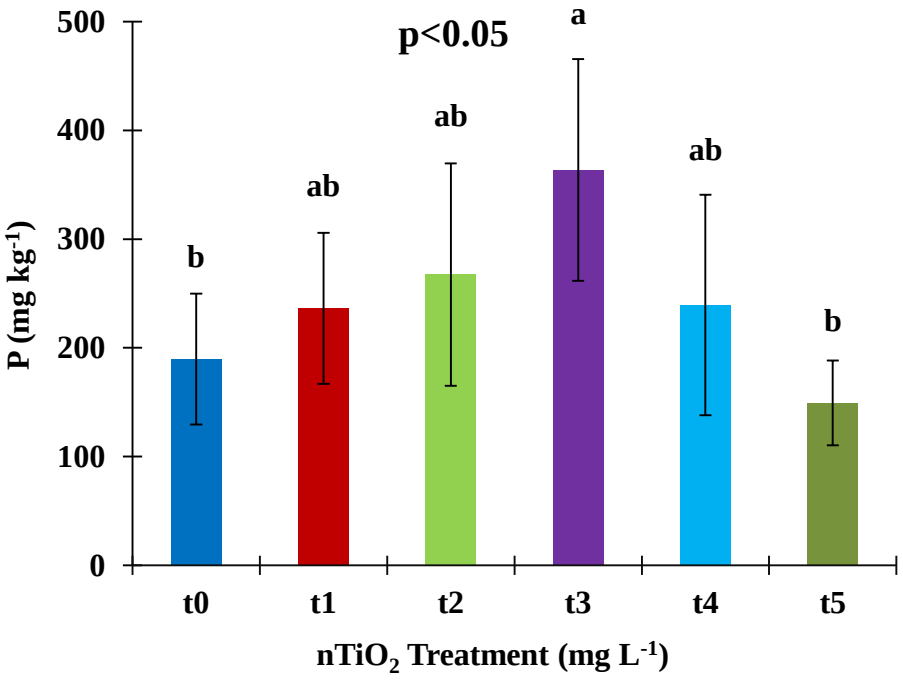

Figure 23: P content in *Oryza sativa*

4.7. Potassium (K) Content in Hoagland Nutrients Solution and *Oryza sativa*

The K content in Hoagland nutrients solution and rice is shown in Figure 24 and 25. Based on the data for K content in Hoagland nutrients solution and rice, it was found to be statistically significant ($P<0.05$) in both. However, in comparison to control, only t3 in plants brought the significant variance. The $nTiO_2$ treatment (t1, t2, t3, and t4) increased the K content in shoots by 13%, 2%, 26%, and 6%, and reduced the K concentration in t5 by 13.8%, as compared to control. The dose at which the nanoparticles performed better causing the highest K content was at t3, which was also found to be significantly different than control. This concentration indicated the better performance of $nTiO_2$ as a nutrients' enhancement agent.

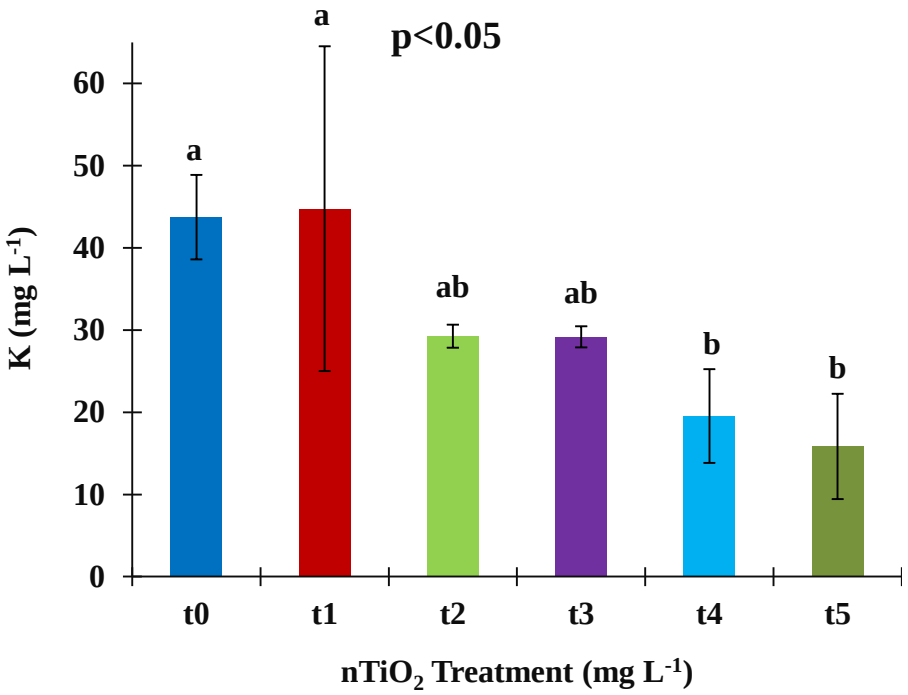

Figure 24: Hoagland solution's K content

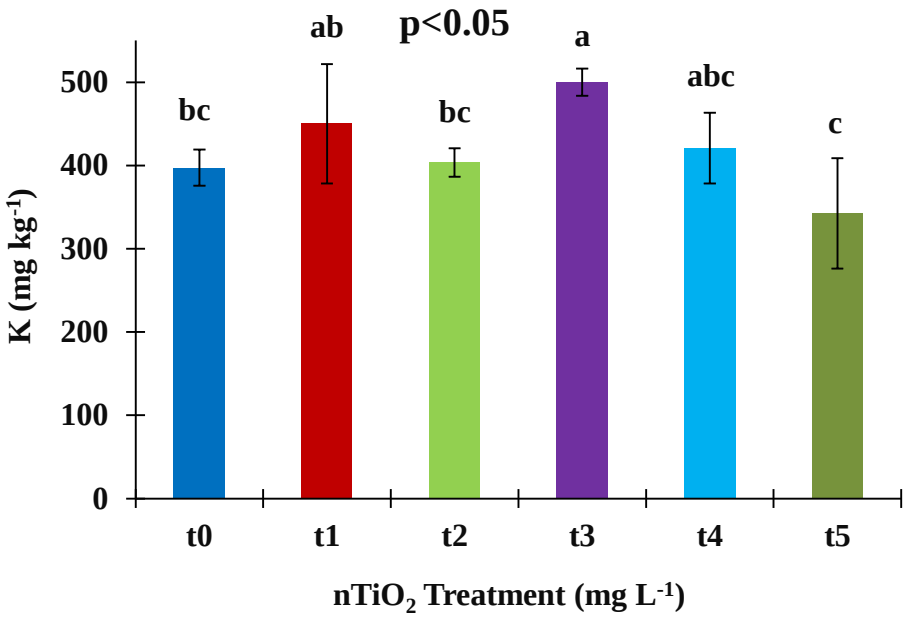

Figure 25: K content in *Oryza sativa*

4.8. Estimation of Chlorophyll Content, Carbohydrates, and Lipid Peroxidation in *Oryza sativa*

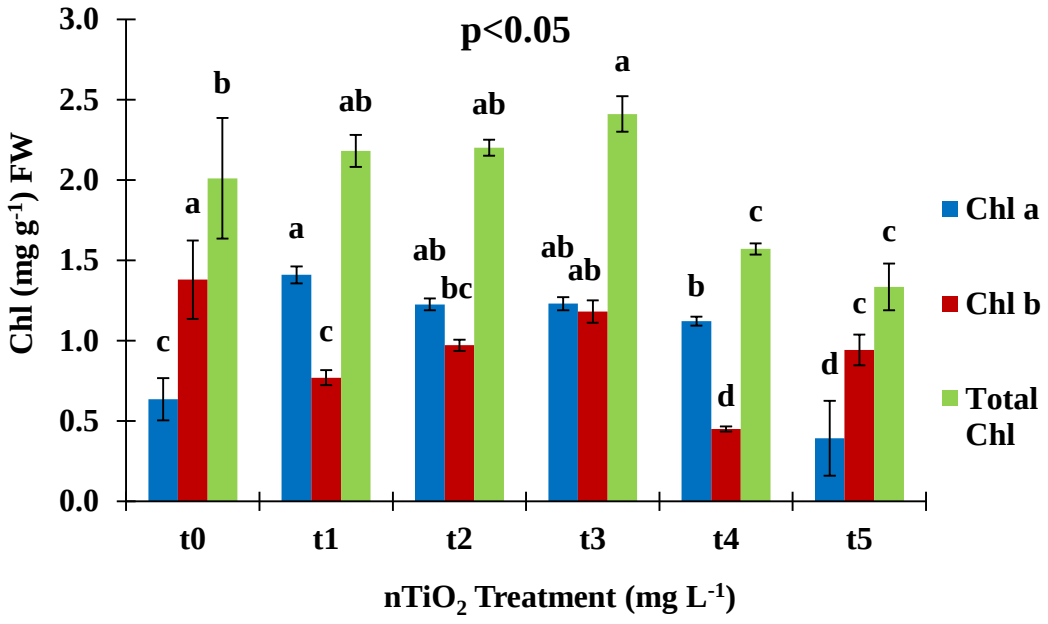

Figure 26: Chlorophyll a, chlorophyll b, and total chlorophyll content in *Oryza sativa*

Figure 26 shows the chlorophyll a, b, and total chlorophyll content in plants, measured after harvesting. Nanoparticles significantly impacted the chlorophyll content

($p<0.05$), while an overall decreasing trend was observed in the chlorophyll a with increasing concentration of nanoparticles. The maximum amount of chlorophyll a (1.41 mg g^{-1}) was found in t1, the treatment with 100 mg L^{-1} $nTiO_2$ concentration, and lowest (0.39 mg g^{-1}) was recorded in 500 mg L^{-1} $nTiO_2$ treatment. Similar trend in nanoparticles treated rice was reported by Samart et al. (2018). The chlorophyll b did not follow any specific trend and its maximum concentration was found in control, followed by 300 mg L^{-1} $nTiO_2$ treatment. Total chlorophyll content was also highest for t3 making 2.41 mg g^{-1} of plant fresh weight. Results indicated that, at higher concentrations, the nanoparticles limited the synthesis of photosynthetic pigment which could be attributed to production of ROS and oxidative damage of the chloroplast membranes, low efficiency of CO_2 assimilation, and interruption of energy transfer from PSII to the Calvin cycle (Ghoto et al., 2020).

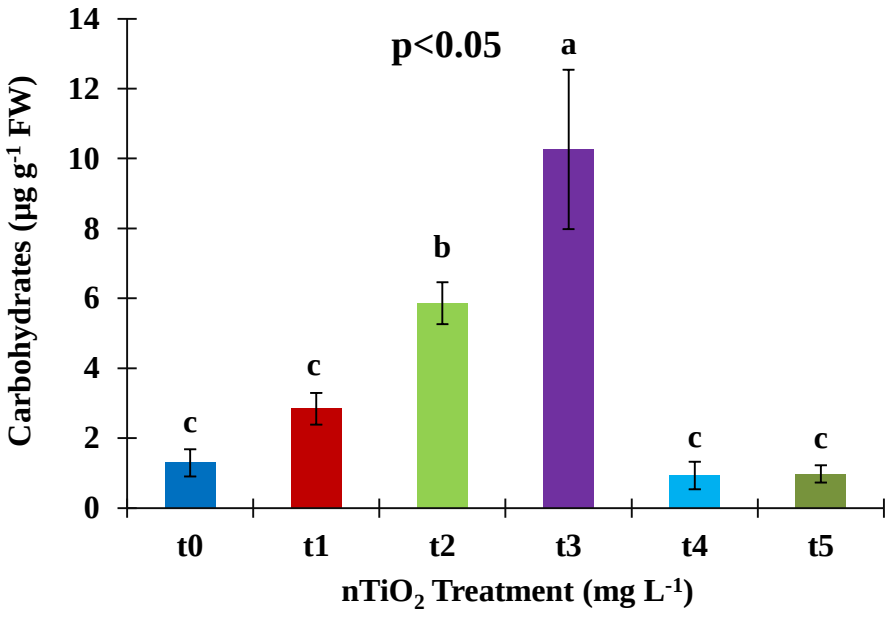

Figure 27: Carbohydrates content in *Oryza sativa*

The carbohydrates content under different nanoparticles doses is shown in Figure 27. Following the same trend, as that of total chlorophyll, the highest carbohydrates content was found to be 10.26 µg g^{-1} of the fresh weigh in t3, followed by 5.86 µg g^{-1} in t2. At lower concentrations, $nTiO_2$ stimulated the activity of metabolic pathways including carbohydrates, but significantly reduced its production at higher doses (Song et

al., 2020). Compared to the control, carbohydrates content increased in t1, t2, and t3 by 2, 5, and 8 folds, whereas it decreased in both t4 and t5 by 1.3 folds.

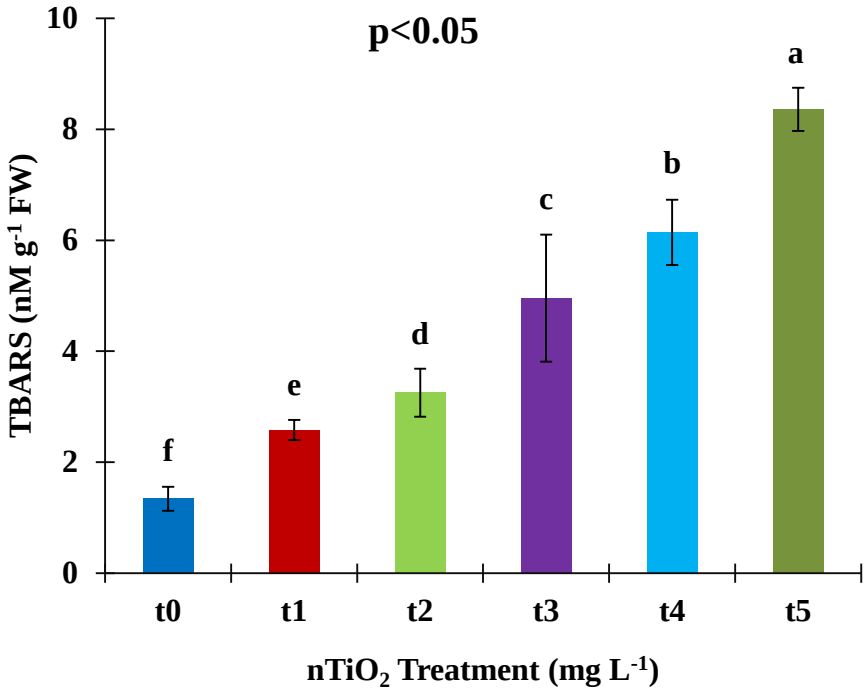

Figure 28: Estimation of TBARS production in *Oryza sativa*

The lipid peroxidation is expressed as TBARS content. Figure 28 shows a significant increase in the TBARS content with increase in the applied nanoparticles dose. When compared to control, all treatments showed a significant variation, marked with 1.9, 2.4, 3.6, 4.8, and 6.2 folds increase in the TBARS production in 100, 200, 300, 400, and 500 mg L^{-1} $nTiO_2$ treatments, respectively. This could be attributed to low activity of antioxidant enzymes, including catalase, glutathione reductase, superoxide dismutase, and ascorbate peroxidase, under nanoparticles stress due to higher dose (Jahan et al., 2018).

The above findings helped in establishing a correlation between lipid peroxidation, plant photosynthetic rate, and carbohydrates content. Plants benefitted from the nanoparticles stimulated metabolic processes but could not cope up with the stress once the concentration exceeded the optimum limits, causing clear toxicity symptoms.

Chapter 5

Conclusions and Recommendations

Conclusions

On basis of the results obtained from 6 weeks long hydroponics-based experiment, it was observed that comparatively better plant growth and improved physiological functioning of *Oryza sativa* was observed at 300 mg L^{-1} $nTiO_2$, considering it to be the optimum dose. At higher doses, i.e. 400 and 500 mg L^{-1}, $nTiO_2$ induced toxicity symptoms in rice as indicated by the decrease in chlorophyll content, carbohydrates production, and lipid peroxidation of cell organelles. Furthermore, at pH 4.0, the 300 mg L^{-1} $nTiO_2$ application facilitated the $nTiO_2$ mediated nutrients (NPK) uptake along a 1-fold increase in the photosynthetic pigment, and improved carbohydrates content by 10 folds in plants. The t3 treatment also showed a better tolerance to dose-dependent oxidative stress (8.25 nM g^{-1} FW), as measured through estimation of TBARS content. At lower doses, $nTiO_2$ did benefit some plant parameters, but the relative change was not significant. Since the study was conducted under optimum environmental conditions, it was concluded that specific properties of $nTiO_2$ such as size, photocatalytic activity, reactivity, and polarizing power have a significant impact on its behavior and interaction with the plant systems.

Recommendations

On basis of the experimental design, results, and conclusion, following recommendations are proposed to be considered for future studies:

1. A very limited number of studies are available which document the nanoparticles induced effect on plants gene expression, therefore the co-relation of nutrients uptake with gene expression could be explored for tracing the link of nutrients mobilization at the molecular level.
2. Little information is available on the usage of $nTiO_2$ as an amendment for crops growth experiment in a Hoagland medium. Future experiments should include the translocation studies for plant-specific dose optimization and upscaling of hydroponics for commercial viability.

3. Given experiment was conducted on *Oryza sativa* in a greenhouse. Plants may respond differently under changing environmental factors, nanoparticles dosage, and composition of Hoagland nutrients solution at different growth stages. Owing to such uncertainty, no conclusive statement could be made. Therefore, future studies should be planned to address these gaps for getting a complete picture of impacts of nanoparticles on a single plant species.

It is evident that at this stage any claim regarding viability of the application of nanotechnology for crops production could not be made. Multiple parameters and analysis throughout the lifecycle of *Oryza sativa* need to be further studied, which could not be explored due to the limited scope of this study. Moreover, the impact of environmentally compatible concentrations of nanoparticles on human health, flora, and fauna also needs to be studied in detail for ensuring save field trials and commercial viability of nanotechnology for agriculture.

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