NUTRIENT AVAILABILITY AND GENE EXPRESSION IN RESPONSE TO NANOPARTICLES IN RICE (*Oryza sativa*)

By

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CERTIFICATE

It is certified that the contents and forms of the thesis entitled **"Nutrient Availability and Gene Expression in Response to Nanoparticles in Rice (*Oryza sativa*)"** submitted by Ms. Naima Waseem has been found satisfactory for the requirements of the degree of Master of Science in Environmental Science.

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I dedicate this thesis to my beloved parents and siblings who would always be a source of inspiration for me and stood beside me at every moment in my life

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Contents

LIST OF ABBREVIATIONS

TNP	Titania Nanoparticles
P	Phosphorus
N	Nitrogen
Si	Silicon
PiTs	Phosphate Transporters
RRIK	Rice Research Institute Kala-Shahkaku
SEM	Scanning Electron Microscopy
EDX	Energy Dispersive X-ray Spectroscopy
XRD	X-Ray Diffraction Spectroscopy
UV	Ultra Violet
PHSTI	Plant Height Stress Tolerance Index
SVI	Seedling Vigor Index
SLSI	Shoot Length Stress Index
RLSI	Root Length Stress Index

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ABSTRACT

Nanotechnology is a fast growing industry now-a-days which is having considerable impacts on society, economy, and also on environment. Present study was designed to assess the effects of TNP on *Oryza sativa* for an exposure time of 70 days. The core objectives were; i) to identify the best respondent genotype of *Oryza sativa* in response to TNP, ii) to determine the growth parameters and phosphorus availability in soil medium and iii) identification of genes involved in phosphorus uptake in *Oryza sativa*. TNP were synthesized by Liquid Impregnation method and characterized via SEM, EDX and XRD. Six concentration levels (0, 100, 200, 300, 400, 500 mg L^{-1}) were used in seed germination test and applied to five *Oryza sativa* genotypes. Seeds of *Super Basmati* treated with TNP showed better response in germination percentage, shoot length stress index (SLSI), root length stress index (RLSI), seedling vigor index (SVI) and plant height stress tolerance index (PHSTI) as compared to other four genotypes. In soil medium, *Super Basmati* was treated with six concentration levels of TNP (0, 100, 200, 300, 400, 500 mg kg^{-1}). In comparison with control, plants treated with TNP showed 15 and 35% improvement in shoot length and shoot biomass, respectively at 400 mg kg^{-1}. Whereas 18% increase in root length and 73% improvement in root biomass was recorded at 400 mg kg^{-1}. Availability of phosphorus also improved to 0.38 folds in shoots while 1.3 folds in roots at 500 mg kg^{-1}. Morphological differences were found among treated and control plants that were confirmed by Scanning Electron Microscopy analysis. It was observed that there was significant increase in silica bodies in treated shoots, which enhances the plant strength against fungal attack and pests. *In silico* study was done by blasting the Phosphate Transporter gene (OsPT1) with other cereal crops and *Arabidopsis thaliana*. Sequences were retrieved from different databases and it was confirmed that there was great similarity between all studied cereal crops in terms of phosphate transporters (PiTs) genes with respect to rice. Results indicate towards the advantageous characteristics of TNP along with environmental benefits which encourage its application on cereal crops.

Chapter 1

INTRODUCTION

1.1 Background

Rice (*Oryza sativa)* is considered as one of the most important cereal crops for human consumption and it occupies about one-fifth of the land area covered by all cereals. Asia is the largest consumer and cultivator of rice crop in the whole world. *Oryza sativa* occupies almost 10% of the total cultivated area in Pakistan (Rabbani *et al.,* 2010). Worldwide about 40,000 genotypes of *Oryza sativa* are present which are further categorized as *japonica, indica, glutinous* and *aromatic.* But major subspecies are the *indica* (long grained) mostly cultivated in tropics and subtropics including China, India, Sri Lanka, Pakistan etc. while *japonica* (short grain) is cultivated usually in temperate, high elevations and upland areas in East Asia, Southeast Asia and South Asia (Yu *et al.,* 2002; Garris *et al.,* 2005; Lu *et al.,* 2009).

1.2 Nanotechnology

Nanotechnology is a fast growing industry now-a-days which is posing considerable impacts on society, economy, and also on environment. Nano-materials are increasingly being used for many commercial products and purposes (Lin *et al.,* 2007). Due to the quality of nano-materials such as zirconia and titania of trapping the heavy metals along with attraction of bio-organisms makes them exceptional candidates for the filters that can be used for liquid separations in industrial processes or for the waste stream purification (Vaithianathan, 2007). Why

application of nanoparticles is very vast? It is because forces of attraction are weak on larger scale while these are strong at nano-scale, and surface area to volume ratio is also very large in nanoparticles (Rico *et al.,* 2007).

1.3 Nanoparticles Application to Plants

Nanotechnology also has the emerging scope in the field of agriculture; impacts of different kinds of nanoparticles have already been studied on various plant species. Impact of silver nanoparticles (AgNPs) on corn, watermelon and zucchini were determined through the parameters of seed germination and plant growth (Almutairi *et al.,* 2015). Among all engineered nanoparticles, titanium dioxide nanoparticles are the most widely used nanoparticles in the world. It was reported that TNP have no effect on root length, whereas zinc oxide NPs have detrimental effects at early seedling stage in *Oryza sativa* (Boonyanitipong *et al.,* 2011).

During the exposure of 30 days, it was observed that there was an increase in biomass and photosynthesis in rice after the application of TNP (Da Costa *et al.,* 2015). Size is a key factor in the toxicity, behavior and reactivity of nanoparticles. In *Brassica napus* (canola), there was enhancement in the germination rate and root growth at higher concentrations of TNP (Mahmoodzadeh *et al.,* 2013). TNP application on *Triticum aestivum* showed increase in biomass and root, shoot length up to 60 mg kg^{-1}. It may lead to detrimental effects at higher concentrations (Rafique *et al.,* 2014). TNP exposure to *Lactuca sativa* also showed an increase in phosphorous availability, biomass and growth compared to the control (Zahra *et al.,* 2015).

Currently NPs have vast applications in various fields but in the field of agriculture, there is a need to explore the new dimensions.

1.4 Significance of Study

Nanoparticles application is an emerging technology which is now in its developmental stage and is being used in many fields. They have significant biological effects on plants, and could show beneficial effects on various physiological parameters even at low doses. But in agriculture, dimensions of nanotechnology are still not so clear. This study is designed to explore the possible impacts on vegetative traits, nutrient availability and genes involved in nutrient uptake.

1.5 Objectives

Keeping in view the above information, it was hypothesized that nutrient uptake by the plants can be enhanced using TiO_2 nanoparticles. The objectives of the present study are:

- Screening of rice genotypes in response to TiO_2 NPs through seed germination test
- Determination of growth response and P uptake
- *In silico* identification of genes involved in P uptake mechanism

1.6 Scope of the Study

Agricultural countries like Pakistan need advance research in plant sciences to cope up with the emerging environmental challenges. This study would help in assessment of effects of TNP on *Oryza sativa* and also would give new insights on positive impact of TNP on environment. If TNP help in P uptake, fertilizer use will be decreased and that can ultimately help to improve the environment.

Chapter 2

LITERATURE REVIEW

This chapter focuses on the related literature on the importance of nanotechnology and uses, agricultural aspects and role of nanoparticles in plants.

2.1 Nanotechnology

In recent years, rapid innovations in nano-sciences and nano-technologies have opened up new prospects for many consumer and industrial sectors. That's why now-a-days nanotechnology is being used almost in every field of life.

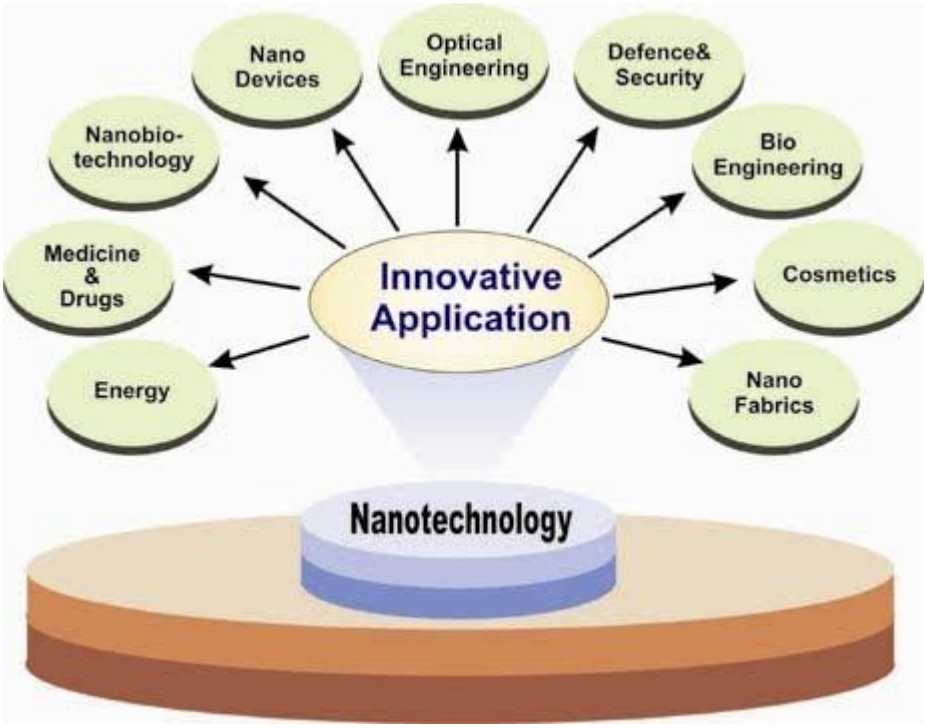

Figure 2.1: Applications of Nanotechnology (Khan F. H. 2013)

2.2 Nanotechnology and Agriculture

Currently, agricultural sector is facing numerous global challenges including urbanization, climate change, environmental issues (i.e. accumulation and run-off of fertilizers and pesticides) and sustainable utilization of resources. Such situations are more aggravated by the growing food demands because of rapid population growth. Developments in the field of technology and science offered potential solutions for better agriculture production systems.

Various technologies being developed not only have the potential to improve farm productivity but also helped in reducing the environmental issues associated with the agricultural production. Nanotechnology is one of such technologies which are helping in the field of sustainable agriculture (Chen and Yada, 2011). Advancements in bio-nanotechnology research are focusing on improving plant resilience against various environmental stresses including salinity, drought and diseases (Branton *et al.,* 2008).

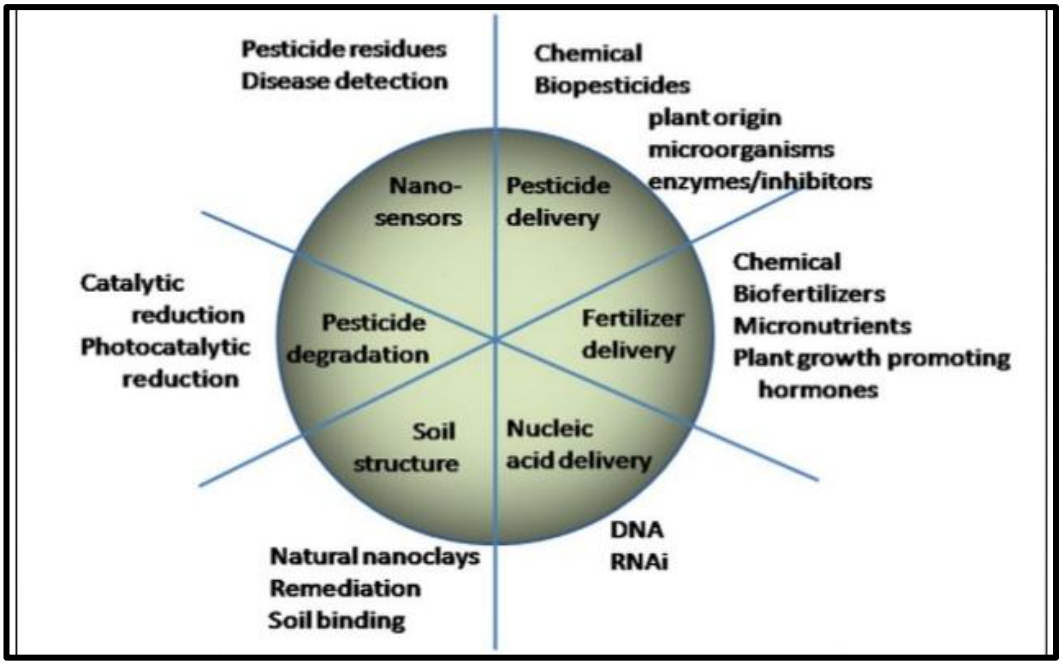

Figure 2.2: Applications of Nanotechnology in Crop Protection and Plant Nutrition

2.3 Nutrient Deficiency and Nanotechnology

Deficiency of both micro and macro nutrients in soil affects the crop yield. Essential soil macronutrients are nitrogen (N), phosphorus (P) and potassium (K). Among these macronutrients lack of P is second limiting factor in good crop yield (Schachtman *et al.,* 1998). P is present in organic and inorganic forms. Mostly in soils large amount of inorganic and organic phosphates are present; but 88 to 99 percent of these phosphates are bound by calcium and thus become unavailable to plants. This is the reason that many soils are phosphorus deficient (Gyaneshwar *et al.,* 2002).

In hydroponics, phosphorus-loaded Al_2O_3 nanoparticles were used to increase phosphorus uptake by *Brassica napus*. It was reported that there was about 8-fold increase at constant low free phosphate concentration and almost 40-fold because of passive, diffusion-based samplers (Santner *et al.,* 2012). Zinc oxide (ZnO) nanoparticles synthesized from soil fungi increased the mobilization and availability of phosphorus in mung bean rhizosphere (Raliya *et al.,* 2016). Titania and iron nanoparticles also have positive impact in terms of availability of phosphorus in *Lactuca sativa* (Zahra *et al.,* 2015). Application of biologically synthesized zinc nanoparticle significantly improved the plant biomass, root, shoot length, root area and chlorophyll content. ZnO nanoparticles also enhanced 48.7% alkaline phosphatase, 73.5% acid phosphatase and 72.4% phytase in clusterbean rhizosphere in comparison with control in six weeks old plants (Raliya and Tarafdar, 2013).

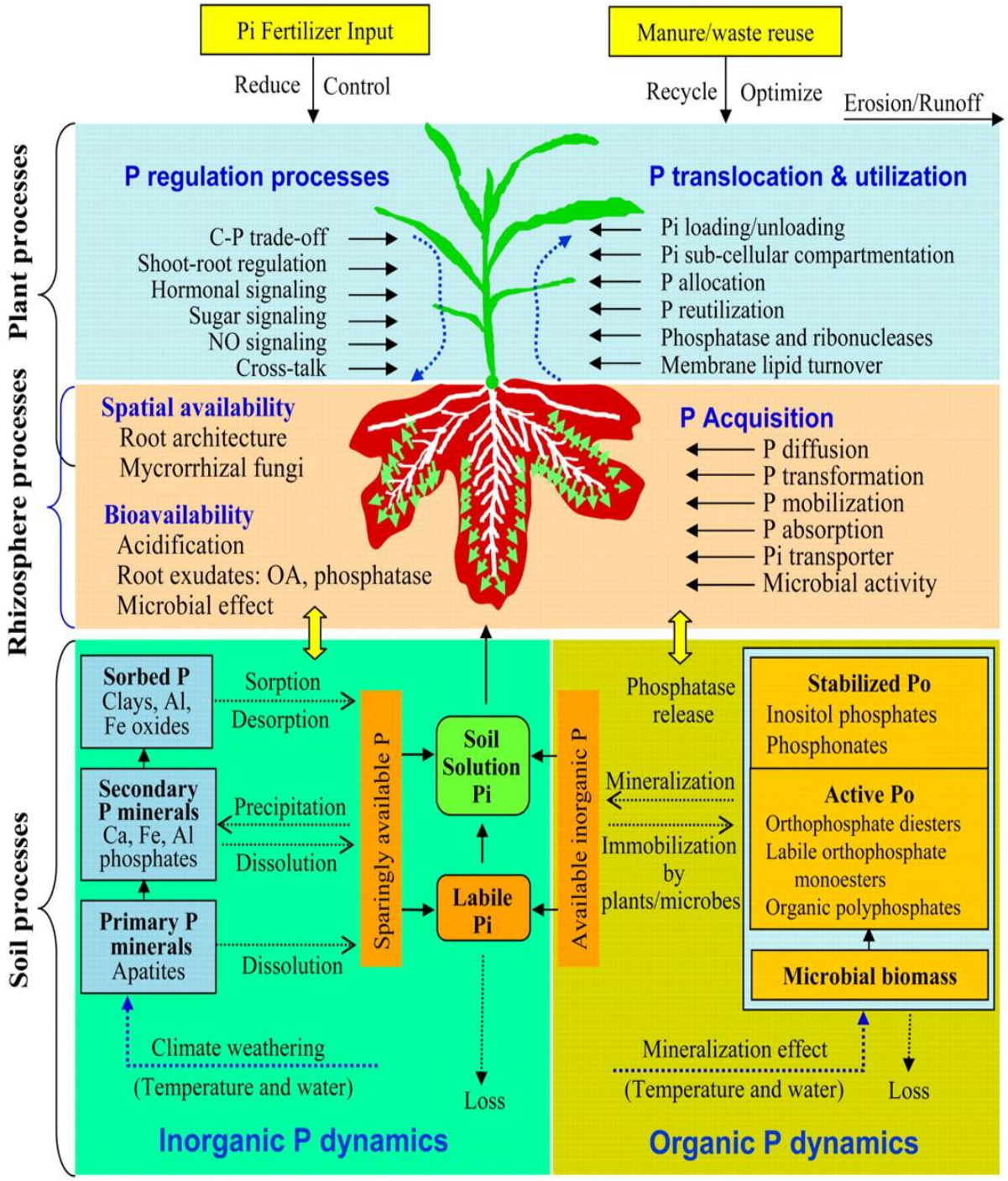

Figure 2.3: Phosphorus Mechanism in Soil and Plant (Shen *et al.*, 2011)

2.4 Impact of Different Nanoparticles on Plants

It is very important to know about the effects of nanoparticles on plants especially cereal crops. Some studies of application of nanoparticles on plants are discussed here.

Nanoparticles have the capabilities to penetrate in living plant tissues (Corredor *et al.,* 2009). The effects of carbon nanotubes on tomato seeds were studied and impacts on germination and growth rates were observed. The seeds containing carbon nanotubes (10-40 mg L^{-1}) showed higher germination rate as compared to the untreated control. Further studies specified that carbon nanotubes are capable of penetrating the thick seed coat and thus improved water uptake in seeds, which in turn affects seed germination and growth of tomato seedlings (Khodakovskaya *et al.,* 2009).

Lu et al. (2002) studied the combined effects of nano-SiO_2 and nano-TiO_2 and observed significant increase in nitrate reductase, catalase (CAT), superoxide dismutase (SOD) and peroxidase (POD) activity of germinating seeds of soybean. Wang et al. (2001) confirmed that nano-SiO_2 treatment could be the cause of increased strength and resistance to disease which results in increased yields of rice.

Effect of copper nanoparticles on the growth of plant seedlings of mung bean and wheat were studied and their bioaccumulation was investigated. All tests were conducted in plant agar media. Growth inhibition of a seedling exposed to different concentration levels of copper nanoparticles was examined. Copper nanoparticles were toxic to both plants and also were bioavailable (Lee *et al.,* 2008).

2.5 Influence of Titania Nanoparticles on Plant

Titania is a naturally occurring mineral that mostly exists in three crystalline forms including rutile, anatase and brookite, and in an amorphous form (Reyes-Coronado *et al.*, 2008). Rutile phase is the most common form of titania found in nature (EPA, 2009).

Titania nanoparticles had positive effect on the germination, chlorophyll formation and shoot growth of aged spinach seeds (Zheng *et al.*, 2005). Seeger et al. (2009) observed no hindering effects of nano-sized titania particles on the growth, water use efficiency and transpiration rate in willow trees. Studies showed that, presence of TNP also help in reducing oxidative stress caused by UV-B radiation (Lei *et al.*, 2008).

The effects of titania nanoparticles (rutile) were studied on the photochemical reaction of chloroplasts of spinach. The results showed that when spinach was treated with 0.25% TNP, the rate of oxygen evolution of chloroplasts was accelerated and chloroplast coupling was improved. It suggested that photosynthesis was promoted by rutile TNP exposure and might be related to activation of photochemical reaction of chloroplasts of spinach (Hong *et al.*, 2004).

Enhancement in spinach growth in relation with anatase TNP was studied and it was observed that all spinach leaves kept green by nanoanatase TNP treatment and all old leaves of control turned yellow white under culture with N-deficient solution. There was significant increase in fresh weight, dry weight, total nitrogen

contents, chlorophyll, and protein of spinach treated with anatase TNP as compared to the control (Yang *et al.*, 2007).

Hanif et al. (2015) conducted a study on soil with plant culturing and without plant. Phytoavaliable P in soil increased up to 56% after incubation of 72 h at room temperature in petri dishes on 100 mg kg^{-1} TNP concentration level. Similar trends were observed for phytoavaliable P in the soil after growing *Lactuca sativa* and given exposure of 14 days. Root and shoot lengths were increased up to 62% and 49% respectively at 100 mg kg^{-1} as compared to the control treatment. Phosphorus uptake per plant was increased five-folds with reference to the control (Hanif *et al.*, 2015).

Effects of titanium dioxide nanoparticles (TNPs) on early growth characteristics of *Triticum aestivum* (Wheat) were studied and growth and cell nuclei integrity were evaluated under hydroponic conditions. Maximum vigor index of 2,722 was obtained at concentration level 200 mg L^{-1} and highest germination rate (96.67%) was also found. But vigor index dropped at 600 mg L^{-1}. Micronuclei assay was done; large numbers of micronuclei were formed in root tips and exhibited the signs of toxicity. Results showed TNP concentrations up to 200 mg L^{-1} promoted growth and enhanced seed germination but may cause toxicity at higher concentration levels of TNP (Sana Ullah & Arshad, 2014).

2.6 Phosphate Transporters (PiTs)

Phosphorus (P) is one of the fundamental mineral elements vital for plant development and growth. P is an essential constituent of phospholipids and nucleic

acids. P plays significant roles in signal transduction, photosynthesis, respiration process and energy transfer. (Plaxton & Carswell, 1999). A phosphate (Pi) is taken up from the soil by the plant roots and translocate within the plant through phosphate transporters (PiTs) (Raghothama, 1999).

Large numbers of genes that help in encoding PiTs have been identified from various plant families, e.g. *Arabidopsis*, cereals, legumes and solanaceous species. At present, availability of fully sequenced genomes allows identification of the Pi transporters and determining the entire network of Pi-relocation activities. The genome of *Arabidopsis thaliana* contains nine putative high-affinity transporters (Paszkowski, 2006; Bucher, 2007; Chen *et al.,* 2007; Ai *et al.,* 2009; Jia *et al.,* 2011; Nagarajan *et al.,* 2011). Majority of transporters showed expression mostly in the roots while transcript levels were strongly induced by low Pi supply or by inoculation with arbuscular mycorrhiza (Mudge *et al.,* 2002; Paszkowski *et al.,* 2002; Javot *et al.,* 2007; Ai *et al.,* 2009).

A study explained that in *Oryza sativa* OsPT1 which is first member of Pht1 phosphate transporters family, expressed abundantly and constitutively in different cell types of both roots and shoots (Sun *et al.,* 2012). Whereas OsPT9 and OsPT10 redundantly function in Pi uptake and expressed in the root epidermis, root hairs and lateral roots with their expression being specifically induced by Pi starvation (Wang *et al.,* 2014).

2.7 Computational Models

In recent years scientists have extracted genetic sequences i.e. DNA, RNA, and protein sequences from numerous organisms. These sequences hold the information about the functioning and construction of these organisms but yet we

are mostly unable to understand them. It has long been known that these sequences contain many kinds of **'motifs'**. Motifs are the re-occurring patterns associated with specific biological functions such as splice junctions, transcription factor binding sites and protein to protein interaction sites. Thus, much research has been devoted to computer algorithms for automatic discovery of refined and recurring motifs in sequences. Many computer algorithms are used for discovering motifs and help us to determine the functions of many biological molecules. Some models are specialized for discovery of DNA motifs including A-GLAM, BioProspector, AlignACE, MDscan, Weeder, RSA Tools and YMF. Others, such as MEME and Gibbs can discover motifs in either DNA or protein sequences (Frith e*t al.,* 2008). RHYTHM, GLAM2 and MEME are the computational models used during research work for the identification of motifs present in phosphate transporter genes of both *Arabidopsis thaliana* and *Oryza sativa*.

2.7.1 RHYTHM

RHYTHM is a web server, which predicts buried vs. exposed residues of helical membrane proteins. Both secondary and tertiary structures information is calculated within only a few seconds. The forecast applies structural records from a growing data base of pre-calculated packing files and evolutionary information from the sequence patterns conserved in a representative dataset of membrane proteins ('Pfam-domains'). The Pfam database is a broad set of protein families and domains currently covering 72% of identified protein sequences (Sammut *et al.,* 2008). RHYTHM uses two types of position specific matrices; 'channels' (to account for different geometries of packing in channels and transporters) and 'membrane-coils' (membrane proteins). Output result provides information on the secondary structure and topology of the protein, and especially on the contact type of every residue and

its conservation. This data can be downloaded as a text file for analysis and statistics, a graphical file for illustration and a PyMOL file for modeling purposes (Rose *et al.,* 2009).

2.7.2 Gapped Local Alignment of Motifs (GLAM2)

Sequence motifs are the important tools in the field of molecular biology. Biological motifs fall into three somewhat distinct classes. The first comprises short motifs often found at functional sites of biopolymers, such as binding sites, cleavage sites and attachment sites. The second consist of longer protein motifs associated with globular structural domains. Finally, the recurring motifs can arise from evolutionarily recent duplications i.e. DNA transposons. GLAM2 is primarily aimed at short motifs for functional sites. The GLAM2 algorithm is used for discovering gapped motifs and a companion scanning algorithm GLAM2SCAN use identified motifs and scan against different protein databases (Frith *et al.,* 2008).

2.7.3 Multiple Em for Motif Elicitation (MEME)

MEME discovers one or more motifs present in protein or DNA sequences. Multiple motifs are retrieved by fitting a mixture model to the data by probabilistically erasing the occurrence of motifs thus found and repeating the process again and again to find the successive motifs. The program estimates that how many time a motif occurs in each sequence in dataset (Bailey and Elkan, 1994). To illustrate an intuitive and statistically valid method for combining independent sources of evidence that yields a p-value for the complete evidence, and to apply it to the problem of detecting simultaneous matches to multiple patterns in sequence homology searches. Sorting sequences by this p-value effectively combines the information present in multiple motifs, leading to highly accurate and sensitive sequence homology searches (Bailey and Gribskov, 1998).

From the above mentioned literature review, we hypothesized that with the application of nanoparticles positive impacts on plant growth was observed and TNP help in better uptake of nutrients by the plants. To test this hypothesis, the methodology adopted is discussed in detail in Chapter 3.

Chapter 3

MATERIALS AND METHODS

This chapter describes the experimental framework adopted during the conducted research work. The work is divided into three major levels. At the first level synthesis of titania nanoparticles and screening of rice varieties in response to TNP through seed germination test was done. At the second level phytoavailability of phosphorus was determined. And finally at the third level, the determination of gene expression involved in nutrient uptake mechanism was identified. All the methodologies followed throughout the study are described here in detail.

3.1 Titania Nanoparticles Synthesis

TNP were synthesized by using the Liquid Impregnation method. In this method, 25g of Titanium dioxide powder, General Purpose Reagent (GPR) was added into 500 mL distilled water and placed on magnetic stirrer at 350 rpm for 24 h. then suspension was allowed to settle overnight. After that it was placed in the oven at 105 ºC for drying. The powder left after oven drying was crushed with the help of pestle and mortar. Then after putting the grounded powder in the china dish it was placed in muffle furnace for calcination at 500 ºC for 6 h (Zeb *et al*., 2010).

3.1.1 Characterization of Nanoparticles by using XRD and SEM

Characterization of synthesized Titania nanoparticles was done by XRD and SEM. The crystallite size measurements, crystal structure and phase composition for the TNP was identified by X-ray diffraction (XRD, Theta/Theta STOE, Germany) at

40 keV and 40 mA. TNPs surface morphology was analyzed via Scanning Electron Microscopy (SEM, Jeol, JSM 6490 A, Japan) equipped with Energy Dispersive X-ray spectroscopy and an ion-sputtering device.

3.2 Screening of Different Rice Genotypes and their Response to TNP through Seed Germination Test

3.2.1 Selection of Rice Genotypes

Seeds of best known and cultivated five rice genotypes of Pakistan were chosen for seed germination test. Seeds of these genotypes were collected from the Rice Research Institute, Kala Shahkaku (RRIK) Pakistan. Genotypes including KSK Basmati, Super Basmati, P.S 2, Basmati 86 (basmati) and IRRI 9 (non-basmati) were used as experimental material. Six concentration levels of TNP including control as 0 mg L^{-1}, and TNP concentrations as 100, 200, 300, 400 and 500 mg L^{-1} respectively were exposed to the selected rice genotypes.

3.2.2 Seed Sterilization and Preparation of Suspensions

Seeds were sterilized by Calcium hypochlorite ($CaCl_2O_4$) solution and then soaked for about 20 minutes. After washing with distilled water, seeds were soaked for 24 hours to sprout.

Suspensions of TNP were made in 10 mL vials for each concentration level as mentioned earlier. For suspensions nanoparticles were weighed in required quantities, after that 10 mL distilled water was added in each vial. Then these suspensions were sonicated for dispersion of TNP by using ultrasonicator (model JAC Ultra Sonic 1505), for almost 30 minutes.

3.2.3 Preparation of Petri Dishes

Petri plates were washed, dried and then autoclaved to avoid any fungal and bacterial contamination. After autoclave, plates were again dried in oven to remove the moisture content. Filter papers were placed in every petri plate and plates were labeled according to the concentration levels. There were four replicates for each concentration level. Suspensions were poured in the respective labeled petri plates.

3.2.4 Seed Germination Test

After preparation of petri plates for the experiment, seeds which were soaked earlier and sprouted were taken. Ten seeds were placed in each labeled petri plate. All work was done in the laminar flow hood under the sterile conditions, to reduce the factor of contamination. Plates were sealed by using polypropylene sheets to avoid the moisture loss, and then placed in incubator for seven days at 27 ºC. Seeds germinated were checked at third day, and finally the readings of roots and shoots were taken at the seventh day of test.

Table 3.1: Parameters Used for Germination Test

Sr. No.	Germination Parameters
1	Germination Percentage (GP %) = (Gf/n) × 100
2	Seedling Vigor Index = GP % × Seedling Length (Root Length + Shoot Length)
3	Shoot Length Stress Tolerance Index (SLSI) = SLT/SLC ×100
4	Root Length Stress Tolerance Index (RLSI) = RLT/RLC × 100
5	Plant Height Stress Tolerance Index (PHSTI) = (Plant height of stressed plants/ Plant height of control plants) x 100

3.3 Soil Analysis and Preparation for Experiment

3.3.1 Classification of soil

Soil was classified on the basis of saturation percentage (Malik *et al*., 1984). Classification was as follows:

0-19% Sand

20-29% Sandy Loam

30-45% Loam

46-60% Clay Loam

More than 60% Clayey

3.3.2 Soil pH and Moisture Content

Soil pH was measured for ensuring its suitability for the growth of plant. 10 g air dried soil (<2 mm) was taken in 100 mL beaker and then 50 mL distilled water was added into it. It was stirred for 30 minutes and then mixture was left for some time. Reading was taken by using pH meter after an hour (McLean, 1982). For moisture content again 10 g air dried soil was taken and placed in oven overnight at 105 ºC. And then re-weighted after cooling. Moisture content was calculated by:

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

3.3.3 Soil Preparation

Soil from the nursery of National University of Science and Technology was finally selected for the final experimentation. Soil was firstly spread out and dried for a week. The dried soil was grounded into fine form by using ball mill at

Particulate Technology Lab, SCME, and NUST. Roots and shoots were removed further by using mechanical sieve shaker of size <2mm. For experiment plastic pots of diameter of 5 cm and height of 6 cm were used. Soil was weighed and 1250 g soil was added to each pot.

3.4 Application of Titania Nanoparticles in Soil

Suspensions of desired concentrations were prepared by weighing the calculated amounts and by the addition of distilled water. For the well dispersion of TNP, suspension were sonicated by using ultrasonicator (model JAC Ultra Sonic 1505), for almost 30 minutes. Suspensions were added in the soil and then mixed vigorously. The concentrations of TNP were 0, 100, 200, 300, 400 and 500 mg kg^{-1}. For each concentration there were eight replicates.

3.5 Plant Cultivation

Seeds of *Super Basmati* were sown and placed in green house at IESE, NUST. After 25 days the seedlings were shifted to the pots for exposure to nanoparticles. Plants were monitored on daily basis and watered twice a week over a period of 70 days.

3.6 Estimation of Chlorophyll Content

Chlorophyll content was measured by using hand-held chlorophyll meter (CCM-200 plus). Three readings of plants were taken during the period of 70 days. The chlorophyll measurements were taken from each plant samples of all treatment levels. There were eight replicates for each concentration level. To avoid the risk of placement of chlorophyll meter over the major leaf veins, readings were taken from

the leaf area between the midrib and the leaf margin. Taken measurements were the thirty points averaging of the hand-held chlorophyll absorbance meter.

3.7 Vegetative Parameters (pH, Length and Biomass)

Whole plant was taken from the pot at the time of harvest. Roots were washed with 300 mL distilled water. The pH of soil solutions was instantly measured. Root and shoot lengths were measured using scale (cm). Roots and shoots were separately placed in an oven at 80 ℃ for 48 h and biomass was recorded. Both the roots and shoots were ground by using mortar pestle then kept in sampling bags for further analysis.

3.8 Determination of Phytoavaliable Phosphorus in Soil

Phosphorus is one of the major nutrients and necessary for good plant growth. The improved blue method (Olsen *et al.,* 1954) is a quick, inexpensive and convenient for alkaline soil testing in terms of phosphorus availability. Thus, Olsen's test has been used for the analysis of soil phosphorus.

Reagents

A. Extracting Solution

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

84 g of sodium bicarbonate was dissolved in about 2 L distilled water. 5N NaOH solution was used to adjust pH to 8.5. The volume was made up to mark.

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

50 g of sodium hydroxide was dissolved in 250 mL distilled water.

B. Mixed Reagent

a) 3 g of ammonium heptamolybdate $(NH_4)_6Mo_7O_{24}.4H_2O$ dissolved in 62.5 mL distilled water.

b) 72.75 mg of antimony potassium tartrate ($KSbO.C_4H_4O_6$) dissolved in 25 mL distilled water.

The dissolved reagents (a) and (b) both were added to a 500 mL volumetric flask, then 250 mL of 5 N H_2SO_4 (37 mL concentrated H_2SO_4 in 250 mL distilled water) were added to the mixture. After mixing thoroughly, the volume was made up to 500 mL with distilled water and kept in a Pyrex glass bottle in a cool and dark place.

C. Color Developing Reagent (CDR)

2.64 g of Ascorbic acid ($C_6H_8O_6$) dissolved in 500 mL Mixed Reagent. CDR must be prepared freshly when needed because it cannot be stored more than 24 h.

D. Standard Stock Solution

Exactly, 0.7 g potassium dihydrogen phosphate (KH_2PO_4) was oven dried for 1h at 105 °C, cooled in a desiccator then stored in air tight bottle. Precisely, 439.4 mg potassium dihydrogen phosphate (KH_2PO_4) was dissolved in 100 mL distilled water. Prepared solution contained 1000 mg L^{-1} stock solution. Precisely, 10 mL stock solution was diluted to 100 mL final volume with distilled water. This solution contained 100 mg L^{-1} phosphorus. A series of standards were prepared from the stock solution. These solutions contained 0, 0.25, 0.5, 0.75, 1, 1.25, 1.50 and 2 mg/kg phosphorus respectively.

Procedure

2.5 g air dried soil was taken into 250 mL Erlenmeyer flask; 50 mL sodium bicarbonate extracting solution ($NaHCO_3$) was added into it and shakes for 30 minutes on mechanical shaker at 180 rpm. Three blank were also prepared having all chemicals in them except soil. Solution was filtered using Whatmann filter paper No. 42. About 5 mL of filtrate was pipetted out into volumetric flask of 25 mL, then

5 mL CDR was added and made volume up to the mark using distilled water. It was shaken well to remove the air bubbles. Gradually bluish color was developed. Concentration of phosphorus present in soil is directly proportional to the intensity of blue color developed. The more the blue color is; the more phosphorus is present. Samples were run on UV/Vis Spectrophotometer after 15 minutes. Absorbance of blanks, standards, and samples was noted at 880 nm wavelength. A calibration curve for the standards was prepared by plotting phosphorus concentrations on the x-axis while absorbance of the samples on the y-axis. Phosphorus concentrations for the unknown samples were measured by following formula.

Phosphorus (mg/kg) = mg/kg P (from calibration curve) × A / Wt × 25/V... (Eq.1)

Whereas;

A = Total vol. of the extract (mL)

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

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

3.9 Determination of Phytoavaliable Phosphorus in Plants

Exactly, 100 mg of both the roots and shoots were ground and saved in sampling bags were digested in 5 mL acid mixture comprising concentrated Nitric Acid and Perchloric Acid (HNO_3-$HClO_4$) in 2:1. Placed on hot plate for 1 h. at 180 °C. The extracts were filtered with the help of Whatmann filter paper No. 42. The concentration of phosphorus in plant filtrates was calculated using vanado-molybdo-phosphoric acid colorimetric method (Ryan, 2008; Zahra *et al.* 2015). Detailed method is given below:

Preparation of Reagents

a. Reagent A: Accurately 12.5 g ammonium heptamolybdate [$(NH_4)_6Mo_7O_{24}.4H_2O$] was dissolved in 250 mL warm distilled water (soln. a). 625 mg of ammonium metavanadate (NH_4VO_3) was dissolved in 250 mL in boiling distilled water (soln. b). Add soln. b was added to soln. a when cooled to room temperature and then 250 mL nitric acid (HNO_3 : H_2O :: 1 : 3) was added to the mixture in volumetric flask. Allowed the solution to be cooled at room temperature.

b. Reagent B: 141.6 mL concentrated perchloric acid was added to 283.4 mL concentrated nitric acid in 500 mL volumetric flask. Acid mixture was then allowed to cool.

c. Standard Stock Solution: Precisely, 0.7 g oven dried potassium dihydrogen phosphate was dissolved in 100 mL distilled water (1000 mg kg^{-1} stock solution). 10 mL of this solution was diluted with distilled water up to 100 mL (100 mg kg^{-1} sub stock solution).

A. Wet Digestion Method

Precisely, 100 mg of ground plant material was added to the volumetric flask of 25 mL. Then 5 mL of acid mixture was added to the flask. Flask was placed on hot plate at 180 ^oC for 1h. The temperature was slowly increased until all traces of nitric acid were disappeared and dense white fumes of perchloric acid appeared and left clear aliquot behind. Volume was made up to the mark with distilled water. The digested plant material was filtered by using Whatmann filter paper No. 42, and extracts were stored at 4 °C for further experimentation.

B. Measurements

1. Exactly, 2.5 mL of the digested filtrate was taken into 25 mL flask then added 5 mL ammonium-vanadomolybdate reagent and volume was made up to the mark with distilled water.

2. The sub-stock solution was pipetted out to 25 mL volumetric flask for the preparation of series of standards. These solutions contained 0, 0.25, 0.5, 0.75, 1, 1.25, 1.50 and 2 mg kg^{-1} phosphorus respectively. Five milliliter mixed reagent was added and continued as for the samples. Blanks were also prepared having all chemicals except plant material. The absorbance of the blanks, standards, and for samples was taken after 1h at 430 nm wavelength on UV/Vis Spectrophotometer. Calibration curve was prepared for standards by plotting absorbance against phosphorus concentrations. Concentration of phosphorus for unknown samples was assessed by using calibration curve (Ryan, 2008).

Total phosphorus uptake per plant was estimated from the following relation:

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

3.10 SEM and EDX of Soil and Plant samples

Roots and shoots after oven drying at 80 °C for 48 h were allowed to cool at room temperature. Sufficient pieces of shoot and root of both treated and control concentration levels were taken and observed under the Scanning Electron Microscopy (SEM). The Energy Dispersive X-ray Spectroscopy (EDS) was also done which was used to elucidate the elemental composition of sample. Soil samples of control and higher concentration levels were also examined under SEM and EDX

3.11 Databases Search and Phylogenetic Tree

3.11.1 Sequence Retrieval

Sequences of phosphate transpoters (PiTs) genes of rice (*Oryza sativa*) and *Arabidopsis thaliana* cited in literature were retrieved and confirmed from databases named as Rice Annotation Project DataBase (RAP-DB), Oryzabase, TAIR and NCBI (Paszkowski *et al.*, 2002; Mudge *et al.*, 2002).

3.11.2 Multiple Sequence Alignments

Sequences of PiTs genes of both *Oryza sativa* and *Arabidopsis thaliana* were used for further multiple sequence alignments in Clustal W and Clustal Omega (Thompson *et al.*, 1997) these alignments were also confirmed in MEGA 6.0 (Kumar & Chen, 2008).

3.11.3 Construction of Phylogenetic Tree

For phylogenetic tree construction, 13 sequences of *Oryza stiva* and 9 sequences of *Arabidopsis thaliana* were analyzed and phylogenetic relationships were established by using Maximum Likelihood Method in MEGA 6.0 (Kumar *et al.*, 2008). The consensus tree was generated by Maximum Likelihood Method for 1000 bootstrap replicates with amino acid substitution method and with Jones-Taylor-Thornton (JTT) model method.

3.11.4 Computational Models for Conserved Motif

Computational models were used for the determination of conserved similar motifs present in sequences of both *Oryza sativa* and *Arabidopsis thaliana*. Conserved motifs were determined by using three software tools named as

RHYTHM, Gapped Local Alignment of Motifs (GLAM2) and Multiple Em for Motif Elicitation (MEME).

3.12 Statistical Analysis of Data

Statistical significance of findings was checked by applying Tukey's test (Honest significant difference); single factor ANOVA, standard deviation and Student's t-test (mean analysis). When the probability of the results was less than 0.05 ($p < 0.05$), results were considered statistically significant.

Chapter 4

RESULTS AND DISCUSSION

4.1 Characteristics of Synthesized TNP

The surface morphology of TNP was analyzed through SEM and EDX. (a) SEM confirmed the size of these TNP in range of 14−22 nm while (b) EDX indicated the presence of elements, oxygen (O) as 40.05% by mass and titania (Ti) as 59.95% by mass in the representative sample (Figure 4.1). The XRD analysis of TNP indicated the peak and crystalline form with the anatase phase at the (101) plane.

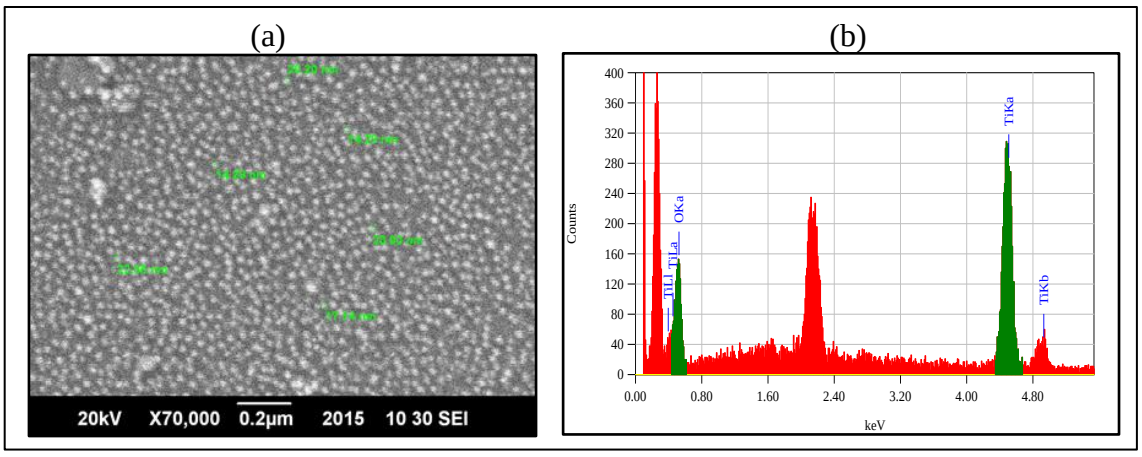

Figure 4.1: Scanning Electron Microscope and Energy Dispersive X-ray Spectroscopy Images of TNP

4.2 Effects on Seed Germination of Rice

4.2.1 Effect on Seed Growth Parameters (Shoot & Root Length)

Results regarding observed root and shoot length of rice genotypes grown in petri dishes upon treatment with the suspensions of TNP have been presented in Table 4.1.

Table 4.1: Root and Shoot Length of Germinated Seeds in Response to TNP Treatment Levels

	Treatment Levels (mg L^{-1})					
Genotypes	0	100	200	300	400	500
ARL	5±0.7	5.1±1.3	5.3±0.7	3.5±0.5	4.1±0.9	3.6±1.0
ASL	4.3±0.6	3.9±0.3	3.9±0.5	3.5±0.3	3.2±0.8	3.3±0.8
BRL	4.6±0.9	5±1.1	5±1.3	4.7±1.1	4.3±0.9	4.3±0.9
BSL	4.3±1.2	4.2±1.6	4.1±0.7	4±1.0	4.4±0.7	4.1±0.4
CRL	5.9±1.4	5.2±1.7	5.5±1.3	4.6±0.7	5±1.5	4.5±0.5
CSL	3.8±0.3	3.5±1.3	4.3±.5	3.5±0.3	4±1.5	3.9±0.6
DRL	7.4±0.8	6±0.8	6.6±1.1	5.8±1.2	5.9±1.8	5.9±2.3
DSL	5±0.7	4.3±0.5	4.6±0.3	3.5±0.6	4.1±0.5	3.6±1.0
ERL	6.6±0.4	6.7±1.2	4.9±0.7	5.1±1.0	6.5±1.5	5.4±0.8
ESL	4.9±0.5	5±0.7	4.7±0.4	4±0.5	5±0.5	4.7±0.3

A is genotype KSK Basmati, B is Super Basmati, C is P.S. 2, D is Basmati 86, E is IRRI 9, RL represents root length, SL represents shoot length

It describes that as compared to control, KSK Basmati showed the decreasing trend in root length as the concentration increases. On the contrary, both the root and shoot length of upper Basmati showed almost similar trend at all treatment levels. While the other genotypes P.S.2, Basmati 86, and IRRI 9 showed random increase and reduction in both root and shoot length. For example, in Basmati 86 and P.S. 2 increased lengths were only observed at 200 and 400 mg L^{-1} of TNPs concentration levels and decrease in length at other concentration levels. Control of Basmati 86 has the maximum mean root length which was 7.4 cm. The treated seeds of IRRI 9 showed the largest root length 6.7 cm at the treatment level 100 mg L^{-1}. The minimum mean root length was 3.5 cm at concentration level 300 mg L^{-1} of KSK Basmati.

In case of shoot, maximum mean length was found in control of Basmati 86, and treatment level 100 and 400 mg L^{-1} of RRI 9 that was 5 cm. For KSK Basmati, the minimum mean shoot length 3.2 cm at concentration level 400 mg L^{-1} was observed. Therefore, we found that Super Basmati showed better response upon application of TNP as compared to the other tested genotypes.

4.2.2 Seed Germination Percentage

Highest total seed germination percentage was 93% of IRRI 9 followed by 88% with Super Basmati while KSK Basmati had the lowest germination percentage that was 83% (Figure 4.2).

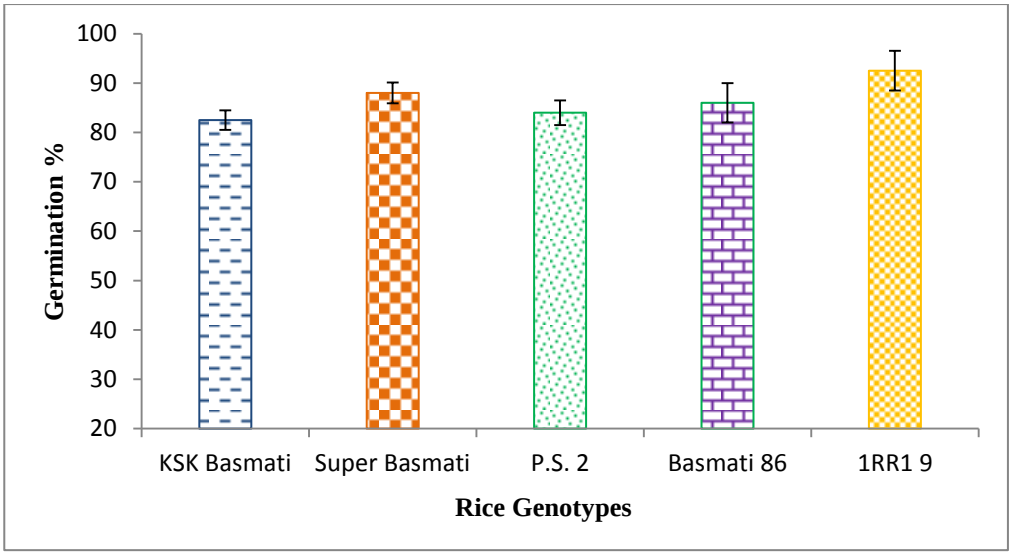

Figure 4.2: Total Germination Percentage of Rice Genotypes

4.2.3 Genotypes Seedling Vigor Index (SVI)

Seedling Vigor Index symbolizes the seeds growing capability, health and germinating tendency of seedlings. If vigor of seeds increases under the given stress, it means seeds can withstand in the extreme and harsh conditions. Decrease in SVI show decline in plant growth rate and leads to reduced crop yield (Zheng *et al.*, 2005; Deng *et al.*, 2014). Figure 4.3 depicts the decreasing trends in vigor indices of KSK Basmati and Basmati 86 as compared with control. While Supper Basmati showed the increasing trend over control. Both P.S.2 and IRRI 9 had increased SVI at only two treatment levels of different concentration. It was proved that TNP had positive impact on wheat seed vigor index up to 200 mg L^{-1} but had decreasing vigor index at concentration level 400 mg L^{-1} or higher (Sana Ullah & Arshad, 2014). Lu et al. (2015) demonstrated that there was significant increase in vigor index of tomato by adding nanosilica powder and higher vigor indexes were observed at 5 and 7 g L^{-1}. Higher vigor index 1127 was recorded at treatment level 400 mg L^{-1} in IRRI 9.

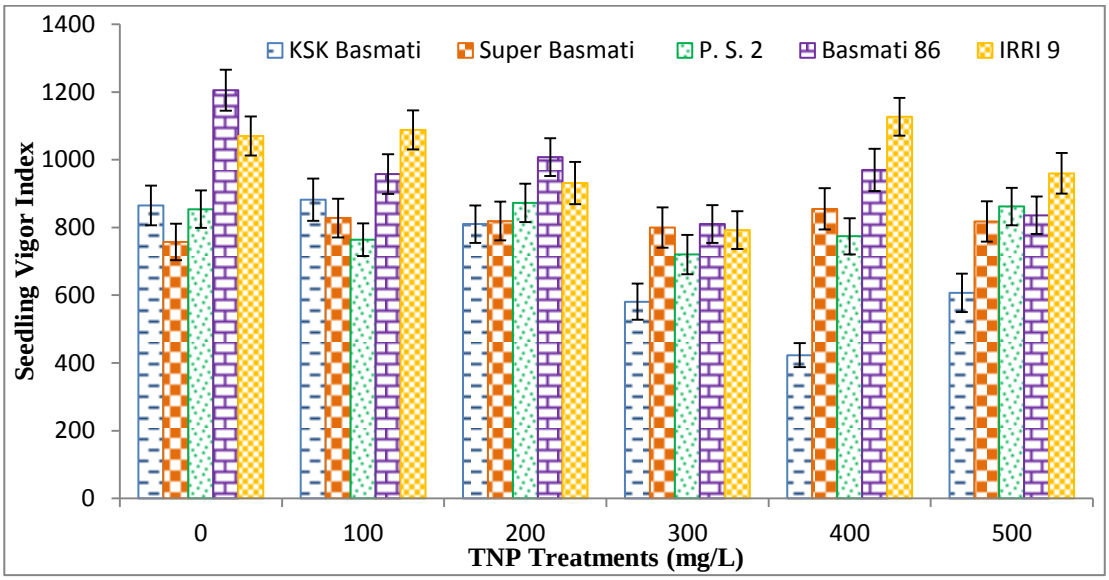

Figure 4.3: Effect of TNP (mg L^{-1}) on Seedling Vigor Index of Rice Genotypes

4.2.4 Plantlet Growth Stress Indices

Shoot and root stress tolerance indexes represents the tolerance capacity of seedlings in stress conditions. SLSI showed declining trend at all treatment levels in KSK Basmati and Basmati 86 whereas RLSI decreases in Basmati 86 and IRRI 9 in comparison with control. Genotypes Super Basmati, P.S.2 and IRRI 9 had improved SLSI at concentration level 400 mg L^{-1}. Highest RLSI recorded value was 109.18 at 200 mg L^{-1} of genotype Super Basmati. Super Basmati showed the more sustained and increasing trend in both SLSI and RLSI (Annexure I). RLSI demonstrated increase in values at lower concentration levels of TNP while decrease at TNP concentration level of 50 µg m L^{-1} in onion (Raskar *et al.*, 2013).

4.2.5 Plant Height Stress Tolerance Index (PHSTI)

To identify and confirm the best respondent variety among these five rice genotypes PHSTI was also calculated. Depicted graph (Figure 4.4) illustrates the

visual difference about plant height stress responses of genotypes. KSK Basmati and Basmati 86 again showed the negative decreasing response to TNPs while there was significant increase in PHSTI of Super Basmati in contrast to P.S. 2 and IRRI 9 when compared with control.

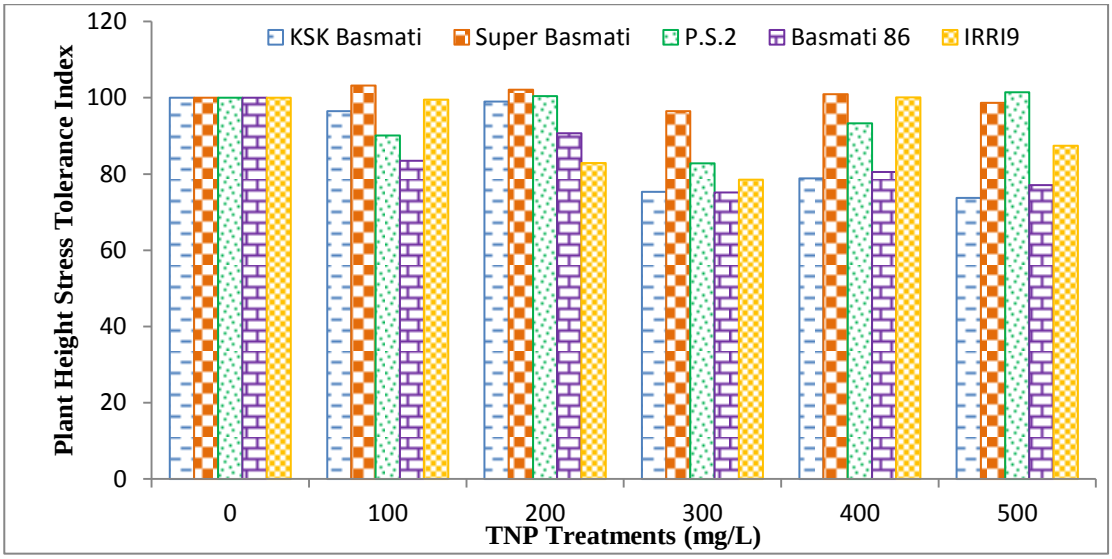

Figure 4.4: Plant Height Stress Tolerance Index of Rice Genotypes in Response to Titania Nanoparticles

4.2.6 Findings of Seed Germination Test

From this experiment it was determined that at all the tested treatment levels of TNPs improvement in growth parameters occurred. Seeds of Super Basmati, P.S.2 and IRRI 9 treated with TNPs showed increase in GP%, SLSI, RLSI, SVI and PHSTI as compared to KSK Basmati and Basmati 86. All analysis proved that Super Basmati is best and more resistant among all other genotypes in response to TNP application. So Super Basmati was used further for soil medium experiment.

4.3 Determination of Growth Parameters

4.3.1 Shoot and Root Length

Following figures describes findings regarding plant length both in terms of shoot and root length.

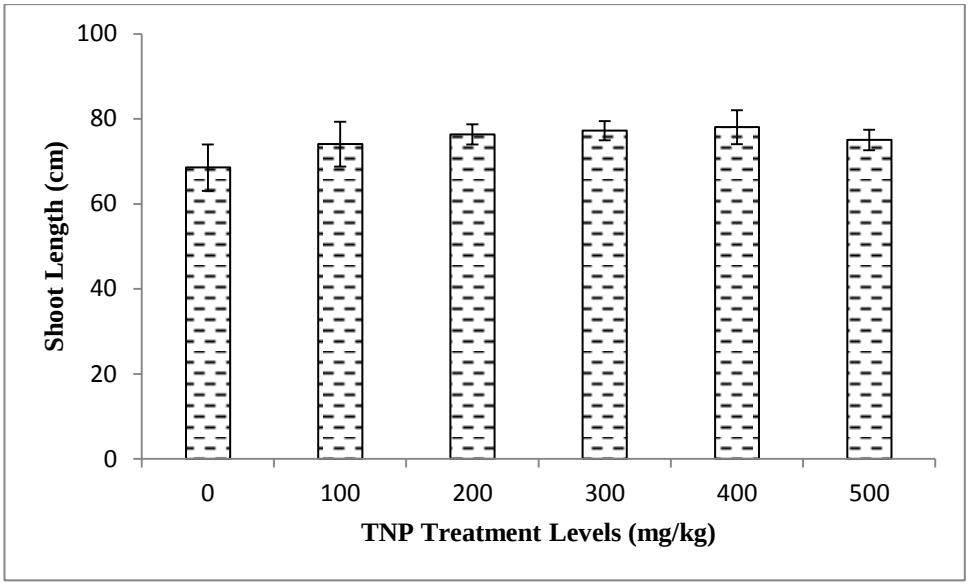

Figure 4.5: Shoot Length of Super Basmati in Response to TNP

Maximum mean shoot length 78.6 cm was observed when treated with 400 mg kg^{-1} of TNP, while shoot length of 68.5 cm was found in control. This showed that there was 15% increase in shoot growth treated with TNP relative to that of control. Values in graph represent the mean (±SD) of 5 replicates. In a previously reported study, 49 and 36% increase were found in the shoot growth of lettuce when treated with Fe_3O_4 and TiO_2 NPs in comparison with control (Zahra *et al.,* 2015).

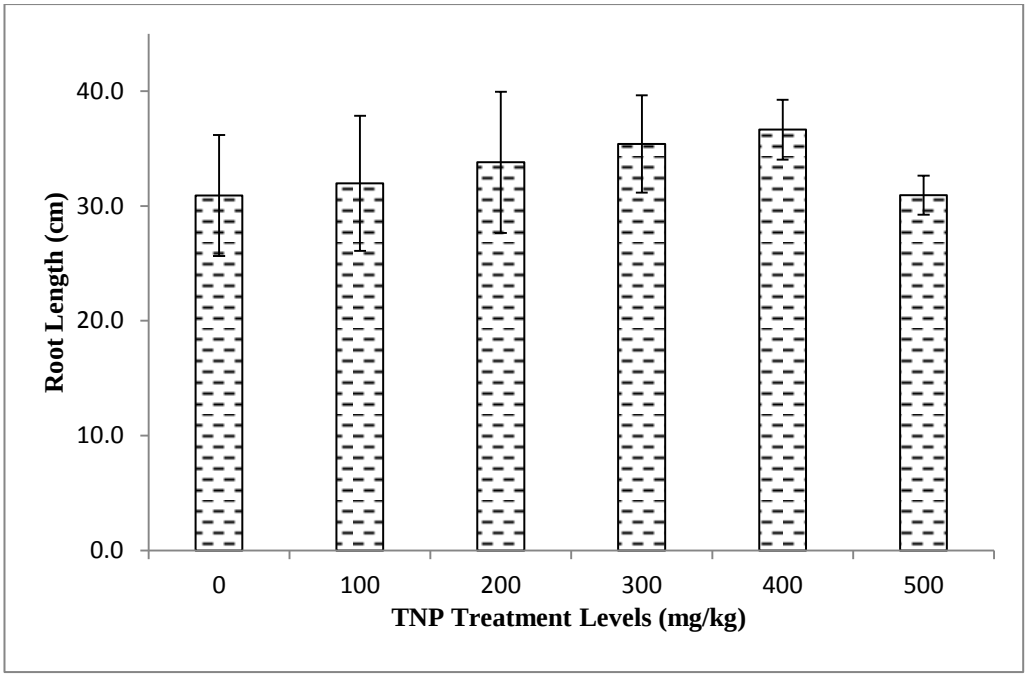

Figure 4.6: Root Length of Super Basmati in Response to TNP

Root length with the maximum mean value of 36.6 cm at 400 mg kg^{-1} was observed as compared to 30.9 cm value of control. It showed increase of 18% on treated concentration as compared with control (Figure 4.6).

4.3.2 Biomass

Similar to as increase in root and shoot length, biomass also improved in TNPs treated groups as compared to the control. Figure 4.7 depicts the shoot biomass; maximum observed value was 3.1 g at 400 mg kg^{-1} whereas for control value was 2.3 g. It showed 35% increase in shoot biomass of treated group as compared with control. Values in the graph represent the mean (±SD) of 5 replicates.

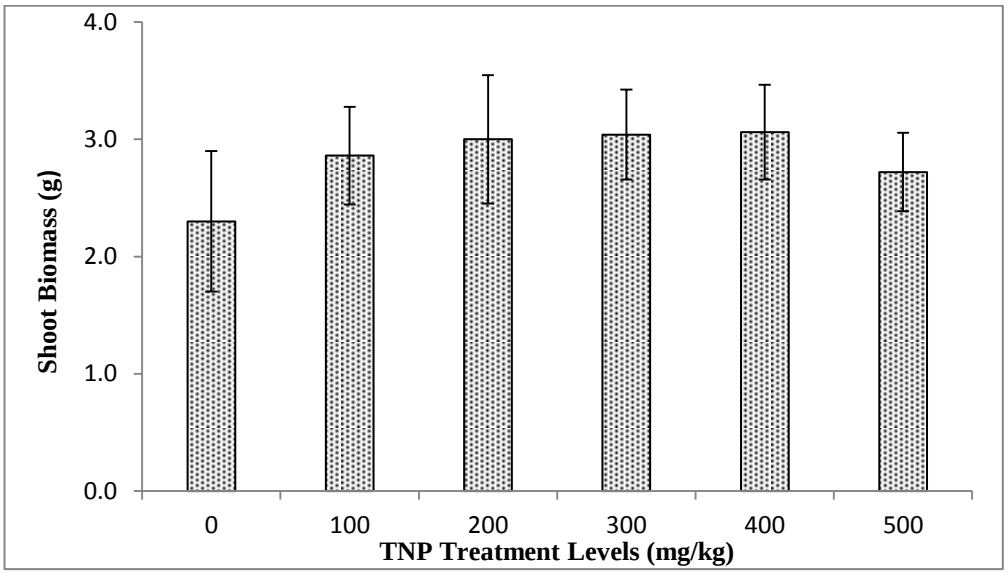

Figure 4.7: Shoot Biomass of Super Basmati in Response to TNP

Maximum root biomass was weighed as 1.9 g also at 400 mg kg^{-1} treatment level of TNP while root biomass of control was 1.1 g. There was 73% improvement in root biomass of treated group in comparison with control (Figure 4.8). Values in the graph represent the mean (±SD) of 5 replicates.

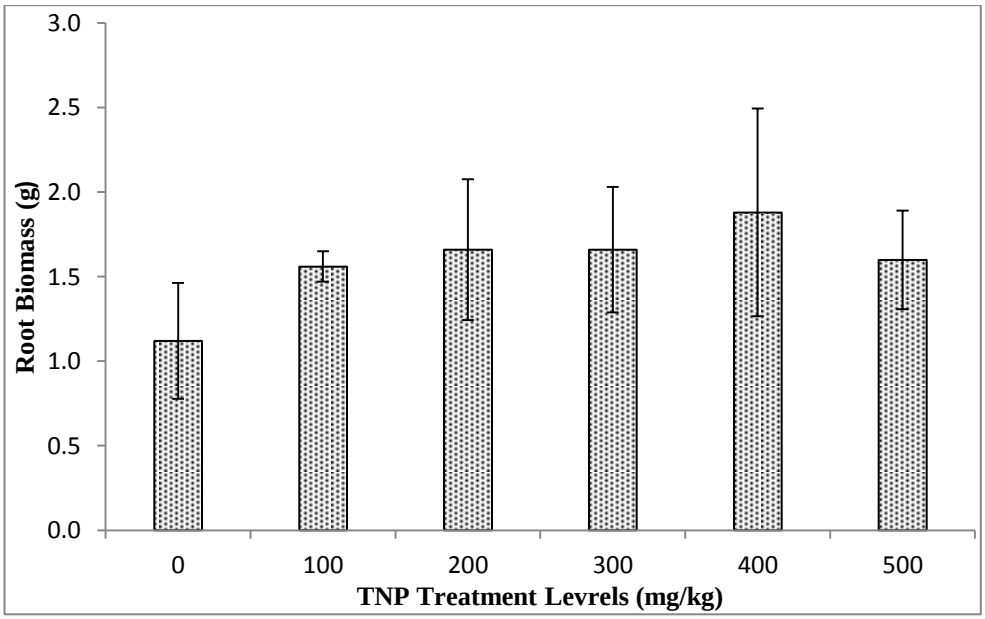

Figure 4.8: Root Biomass of Super Basmati in Response to TNP

Therefore, the total biomass of *Oryza sativa* was significantly increased upon treatment with TNPs as compared to the control. According to pervious literature, upon application of TNPs in wheat improvement in fresh and dry biomass was reported up to 60% and 72% respectively as compared to control (Rafique *et al.,* 2014). In another study, it was found that at concentration levels (< 2500 mg/L) of TNPs, best growth results of spinach were observed (Zheng *et al.,* 2005). Recently, Zahra et al. (2015) reported about 36% increase in shoot length and biomass was reported to improve up to 1.2 fold in comparison with control.

4.4 Chlorophyll

Chlorophyll is one of the essential pigments present in plants; it helps in the conversion of light energy into chemical energy which further enhances the photosynthetic activity in plants. In case of Super Basmati, at the given concentration levels, three chlorophyll readings were taken during the exposure time of 70 days, approximately with the gap of 15 days on fortnightly basis. Results regarding chlorophyll content in Super Basmati have been shown in Figure 4.9.

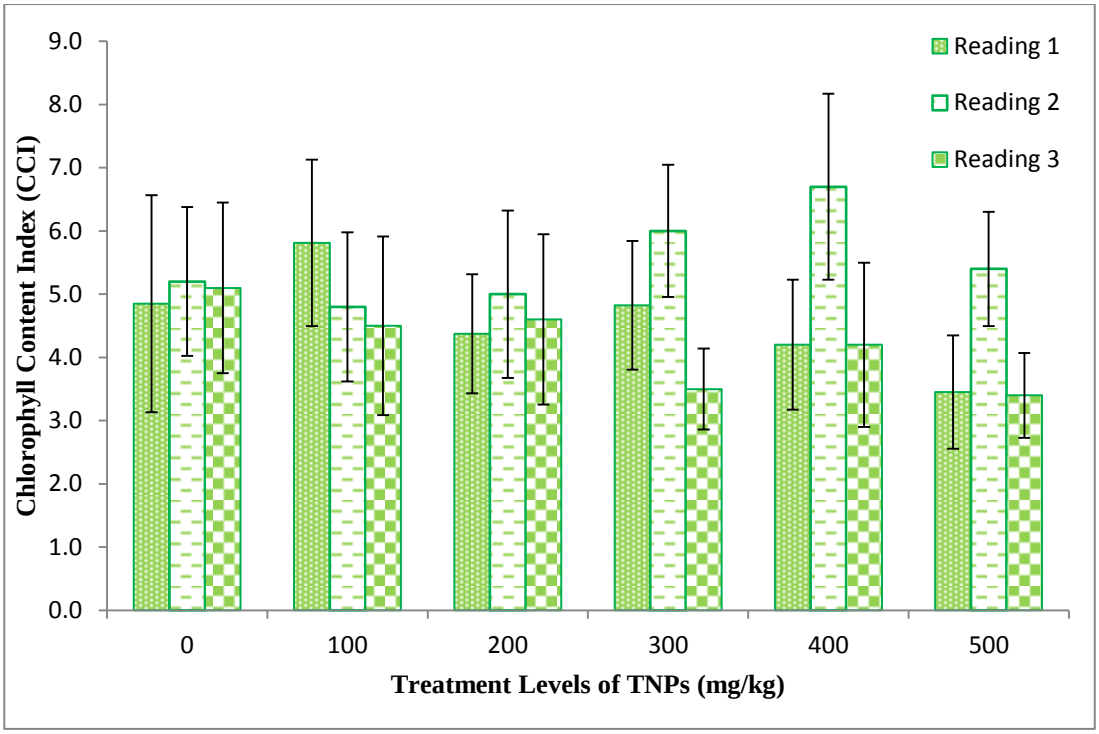

Figure 4.9: Chlorophyll Content Calculated in CCI at all Treatment Levels of TNP

It was observed that during first reading taken on 35th day of Super Basmati exposure to TNP, the highest mean reading of chlorophyll was 5.8 CCI at the treatment level 100 mg kg^{-1}. The second chlorophyll measurements showed maximum reading 6.7 CCI at concentration level 400 mg kg^{-1}. During third fortnightly reading taken on 65th day of exposure, there was decrease in chlorophyll value in treated plants as compared to control.

These variations in chlorophyll contents might occur due to several reasons including at first plants were in the growing phase and have somehow less chlorophyll. At the time of second reading, there was broad surface leaf area and the leaves were lush green in color that's why more chlorophyll values were observed. During third reading, the chlorophyll again decreases because the leaves started to turn brown at this stage. In literature, it was reported that TNPs photocatalytic activity increased the chlorophyll content (Skupień *et al.,* 2007; Owolade *et al.,*

2008; Chen *et al.,* 2012). Mukherjee et al. (2014) reported that chlorophyll content decreases consistently with the time due to the less green color of the leaves. TNPs application to spinach improved the 37.48% chlorophyll content, 23.35% total nitrogen concentration and 91 and 99% fresh and dry biomass respectively in comparison with the control (Yang *et al.,* 2007).

4.5 Soil pH

Soil pH is an important parameter for plant growth; pH measures the acidity and alkalinity of a soil. The pH range from 6.8-7.2 is termed nearby neutral. Rice can be cultivated under diverse climatic and soil environments. For normal growth of rice pH range of 5.0 to 8.0 is suitable but neutral soil pH is more favorable for better crop production. Figure 4.10 depicts the rhizosphere soil values starting from 7.8 at control without treatment of TNP was slightly alkaline but decreasing gradually as concentration increases and lowest at 400 mg kg^{-1} which was 7.0 which was neutral. Generally decrease in pH increases the uptake of phosphorus (Zahra *et al.,* 2015).

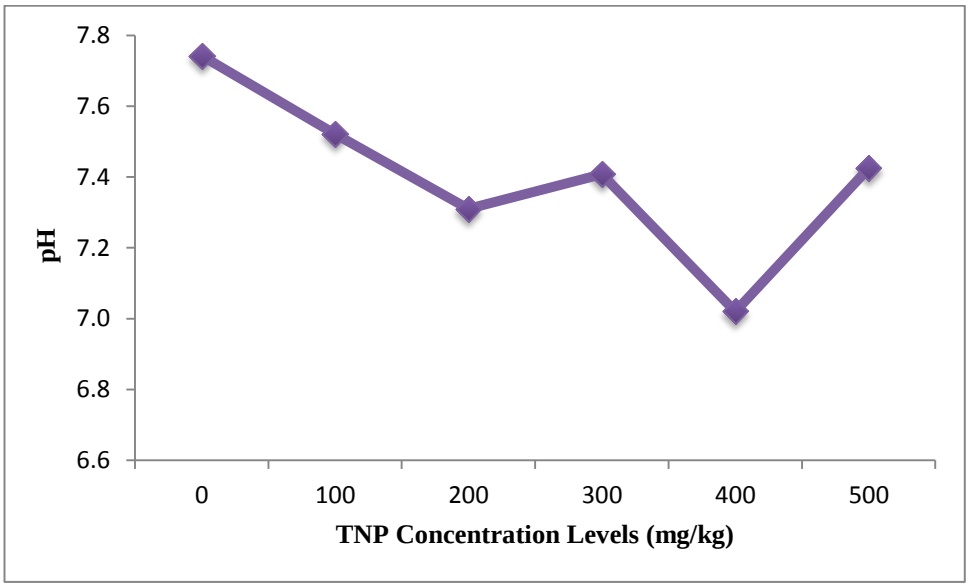

Figure 4.10: Rhizosphere Soil pH Measured at Treatment Levels of TNP

4.6 Available Phosphorus to Super Basmati

4.6.1 Phosphorus Availability in Shoots of Super Basmati

Figure 4.11 illustrates the phosphorus (P) uptake in shoots of Super Basmati at all concentration levels. Maximum P uptake was observed at 500 mg kg^{-1} and calculated as 901 mg kg^{-1} while it was 651 mg kg^{-1} in case of control. It showed increase of almost 0.38 folds as compared with control. Values shown in graph are mean (± SD) of five replicates.

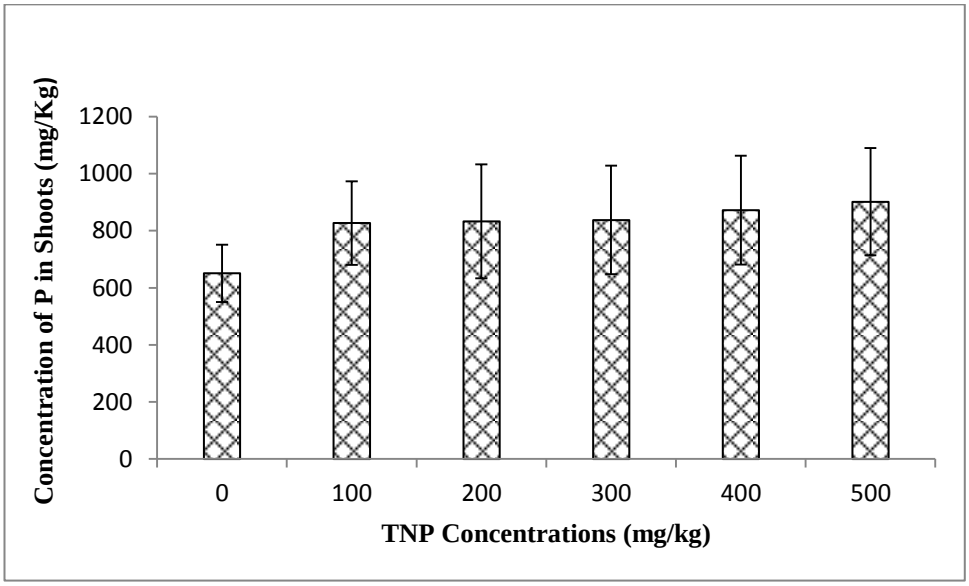

Figure 4.11: Phosphorus Uptake in Shoots at All Treatments Levels of TNP

4.6.2 Availability Phosphorus in Roots of Super Basmati

Available P in roots of Super Basmati was also higher at 500 mg kg^{-1} calculated as 1539 mg kg^{-1} whereas it was 668 mg kg^{-1} uptake in roots of control. There was 1.3 folds increase in treated group in contrast with control. All the values presented in the graph were mean (± SD) of five replicates.

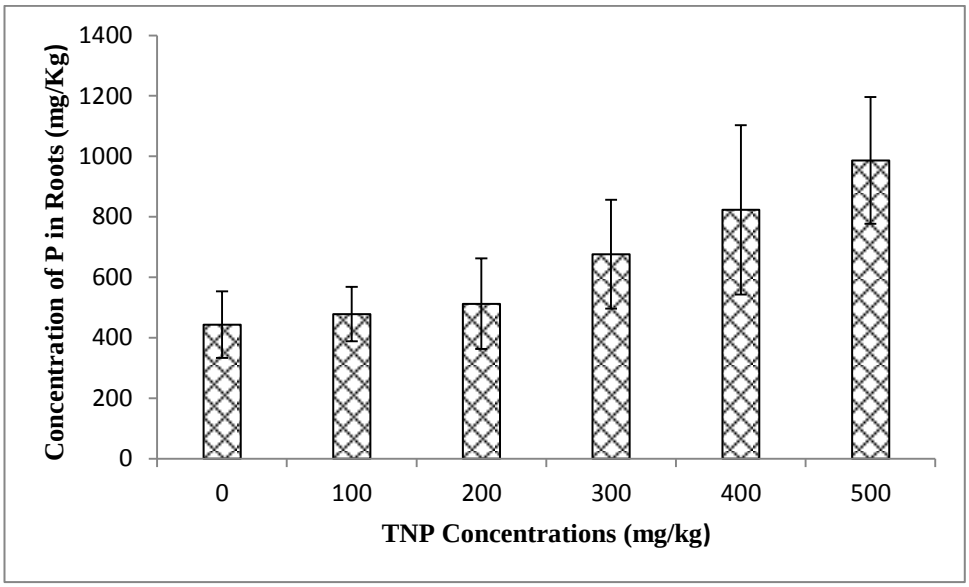

Figure 4.12: Phosphorus Uptake in Roots at All Treatments Levels of TNP

It was proven from the previous literature that nanoparticles application on crops enhance the phosphorus availability to the plants. Zinc oxide (ZnO) nanoparticles synthesized from soil fungi increased the mobilization and availability of phosphorus in mung bean rhizosphere (Raliya *et al.,* 2016). Titania and iron nanoparticles also have positive impact in terms of availability of phosphorus in *Lactuca sativa* (Zahra *et al.,* 2015). Same trend was observed when TNP were applied on Super Basmati, there was increase in available phosphorus in both roots and shoots as concentration level of TNP increased.

4.7 Microscopic Analysis of Super Basmati

Microscopic analysis by SEM determined the impact of nanoparticles on shoot, root and soil.

4.7.1 SEM Analysis of Shoots

SEM image of shoot of Super Basmati is shown in Figure 4.13. Trichomes were fine out growths and appendages on plant. In previous literature two types of silica bodies are reported in rice one are small scatter bodied encircled in yellow color whereas silica bodies were also sandwiched between two walls and make ladder like structure depicted in Figure 4.13 (Yamanaka *et al.*, 2009, Yang *et al.*, 2015).

Figure 4.13: SEM Image of Shoot of Super Basmati

Yellow circles in Figure 4.14, showed the wart-like protuberance (WP) containing more silica cells while stomas were stomata surrounded by safeguard cells. Silicon is predominantly deposited in wart-like protuberance silica cells of the epidermis.

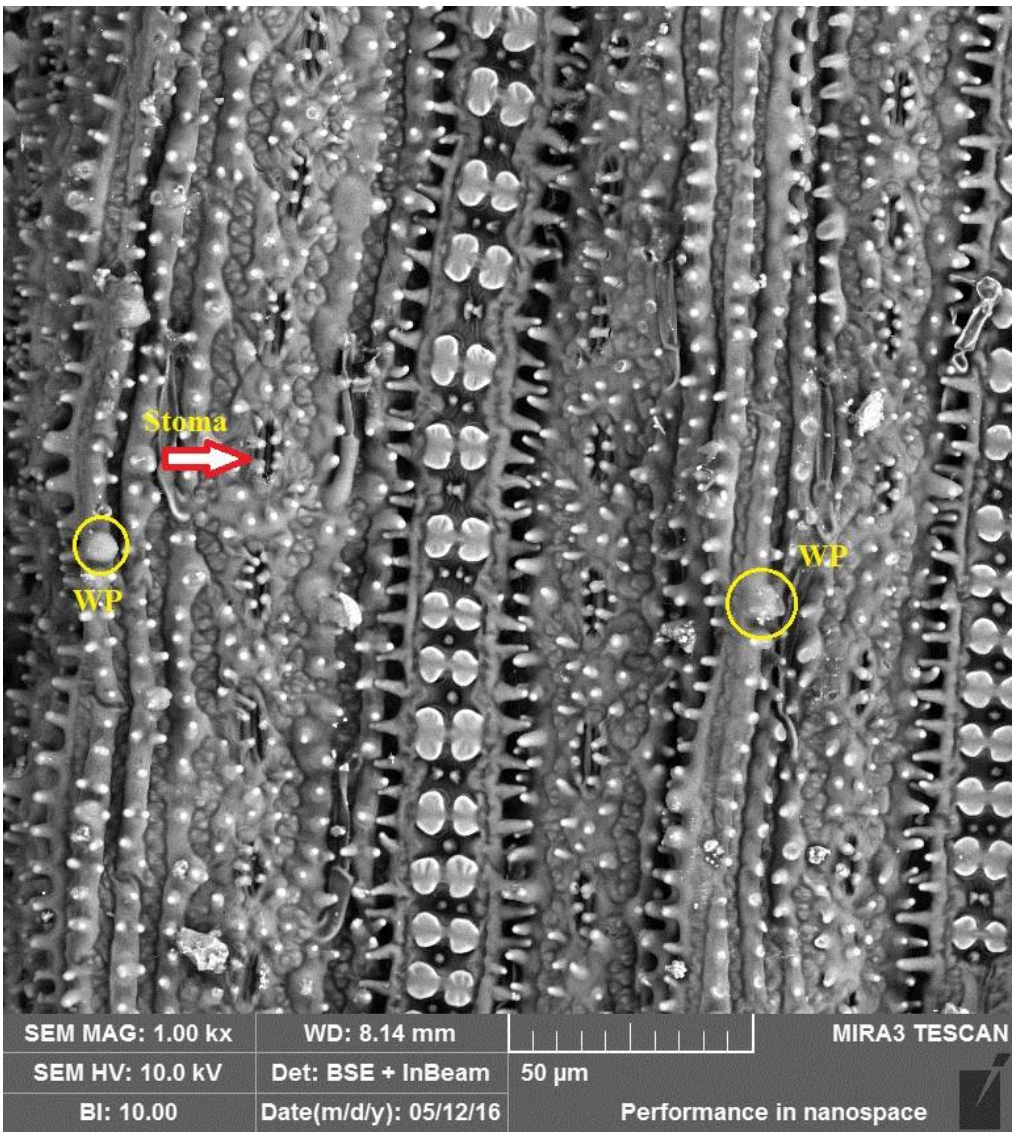

Figure 4.14: SEM Image of Shoot of Super Basmati

4.7.2 SEM Images of Control and Treated Shoots of Super Basmati

Image of control group shows the less number of WPs, have defined features and organized ladder like structure and cell layer are closely embedded in each other.

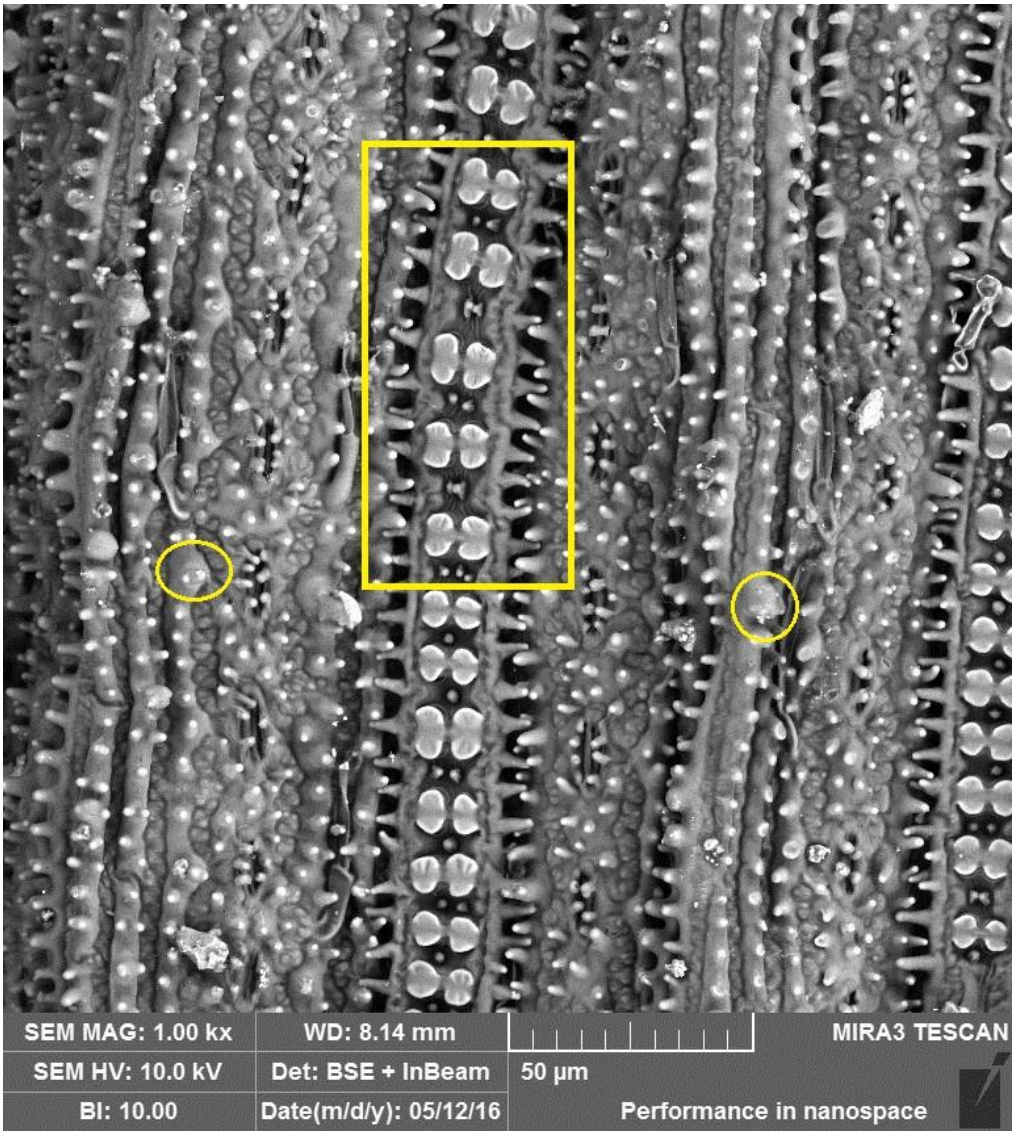

Figure 4.15: SEM Image of Shoot at 0 mg Kg^{-1}

While in this figure of 500 mg kg^{-1} situation is different. More number of WPs was found, undefined and merged features, deformed ladder like structure and cells layers are expanded.

Silicon application to rice in an experiment led to more pronounced cell silicification and more elaborate and larger papillae (WP) (Ning *et al.*, 2014). The elaborate papillae formed in silicon treated leaf epidermal surface might increase the resistance to fungal penetration (Zhang *et al.*, 2006; Cia *et al.*, 2008).

Our findings showed the same results at 500 mg kg^{-1} treatment level of TNP, more silicification means more and elaborated papillae (wart-like protuberances). It showed that TNP treatment, enhanced the WPs which results in more silica bodies and strengthen the plant defense mechanism against fungal and pest attacks.

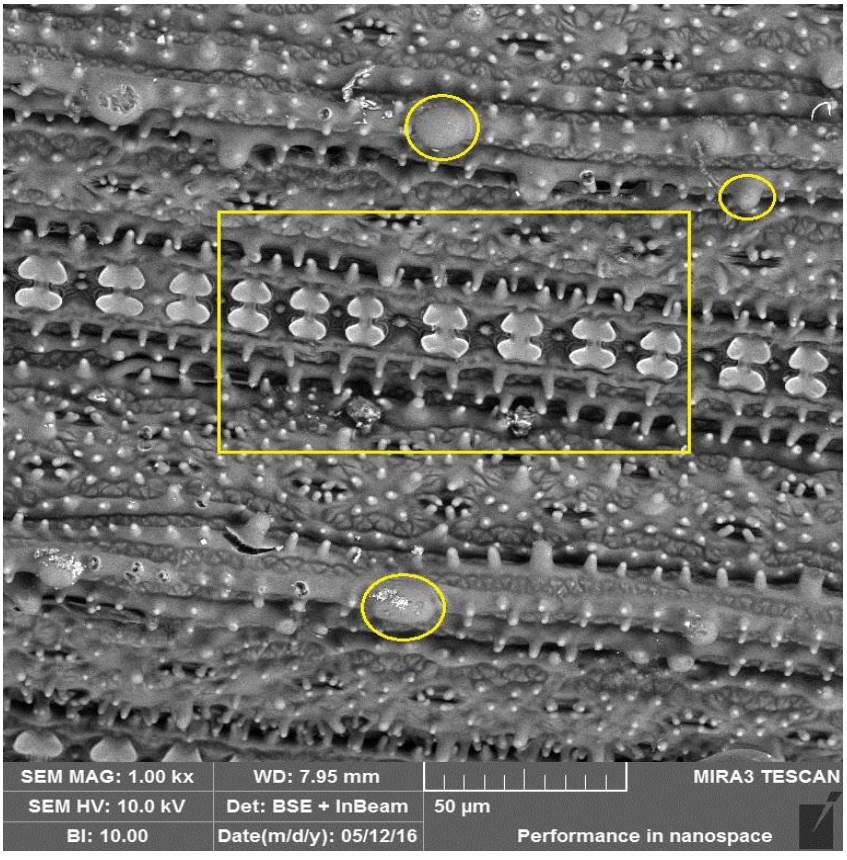

Figure 4.16: SEM Image of Shoot at 500 mg Kg^{-1}

Silicon (Si) is considered as the second most abundant element on the earth. It is also present in plants in equivalent amounts to those of macronutrients such as phosphorus, calcium and magnesium (Epstein, 1999). Silicon is major inorganic constituent of higher plants and deposited in the form of amorphous silica gel. Gramineae can either be stunted or very weak if silicon is absent, so silicon has been considered essential for the normal plant growth and development (Kim *et al.*, 2002).

Some authors suggested that physical barriers provided by silicon deposition in cell walls contribute to boosted resistance (Yoshida, 1965; Hayasaka *et al.,* 2008). While recent studies suggest that silicon plays a biochemical role in mediating plant resistance to pathogens (Rodrigue *et al.,* 2003; Liang *et al.,* 2005; Sun *et al.,* 2010).

4.7.3 SEM Analysis of Roots

Figure 4.17 and 4.18 depicts the SEM analysis of roots of Super Basmati at 0 and 500 mg kg^{-1} respectively. There was smooth surface of control root in comparison with treated, roughness means more surface area and absorption of nutrients.

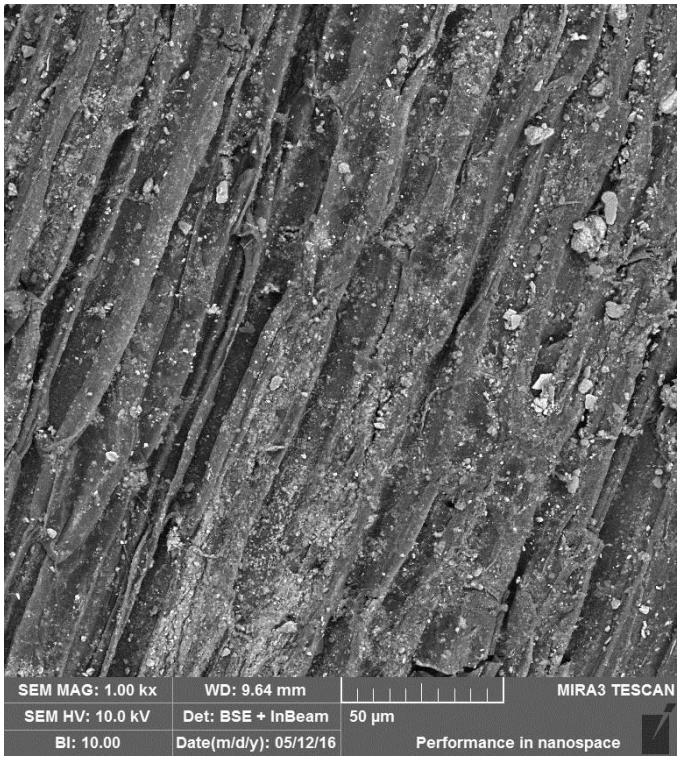

Figure 4.17: SEM Analysis of Root at 0 mg Kg^{-1}

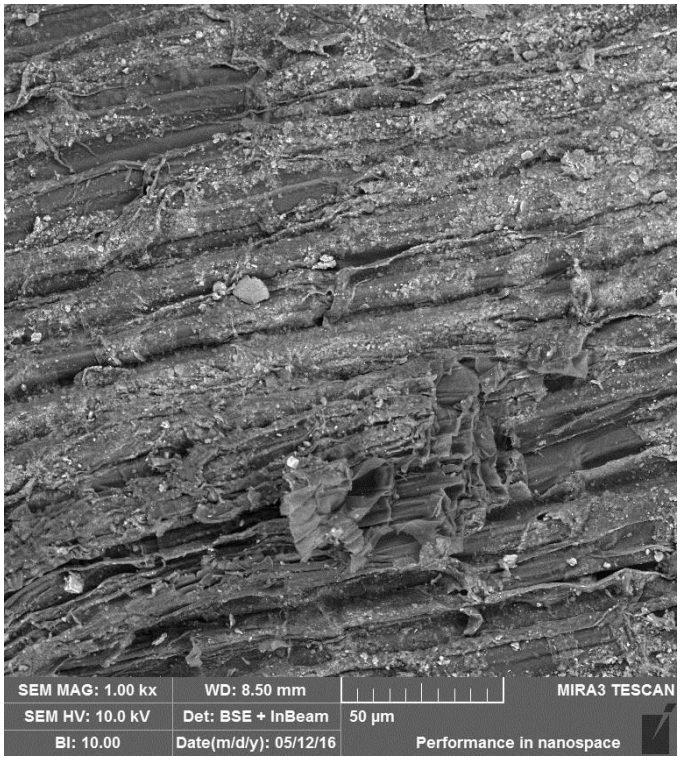

Figure 4.18: SEM Analysis of Root at 500 mg Kg^{-1}

4.8 Phosphate Transporter Genes

4.8.1 Phosphate Transporters (PiTs) Genes Retrieval

Sequences of phosphate transporters (PiTs) genes of rice (*Oryza sativa*) and *Arabidopsis thaliana* cited in literature were retrieved from databases named as Rice Annotation Project Data Base (RAP-DB), Oryzabase, TAIR and NCBI.

4.8.2 Phylogenetic Tree Analysis

Phylogenetic relationships were established by using Maximum Likelihood Method in MEGA 6.0 (Kumar *et al.,* 2008). The consensus tree was generated by Maximum Likelihood Method for 1000 bootstrap replicates (Figure 4.19). All the phosphate transporter genes of both species falls in same clad .While phosphate genes (AtPT8, AtPT9, OsPT9 and OsPT10) showed maximum similarity as cited in literature (Wang *et al.,* 2014). Genomic distance of the tree was 0.1 which shows the maximum similarity among the genes of both species. Another tree was constructed containing NAC16 gene which makes the separate clad as an out-group and used as a baseline to show the clustering (Figure 4.20). Phylogenetic tree of different cereal crops including wheat, barley, sorghum and maize with *Arabidopsis* and *Oryza sativa* is given in Annexure II.

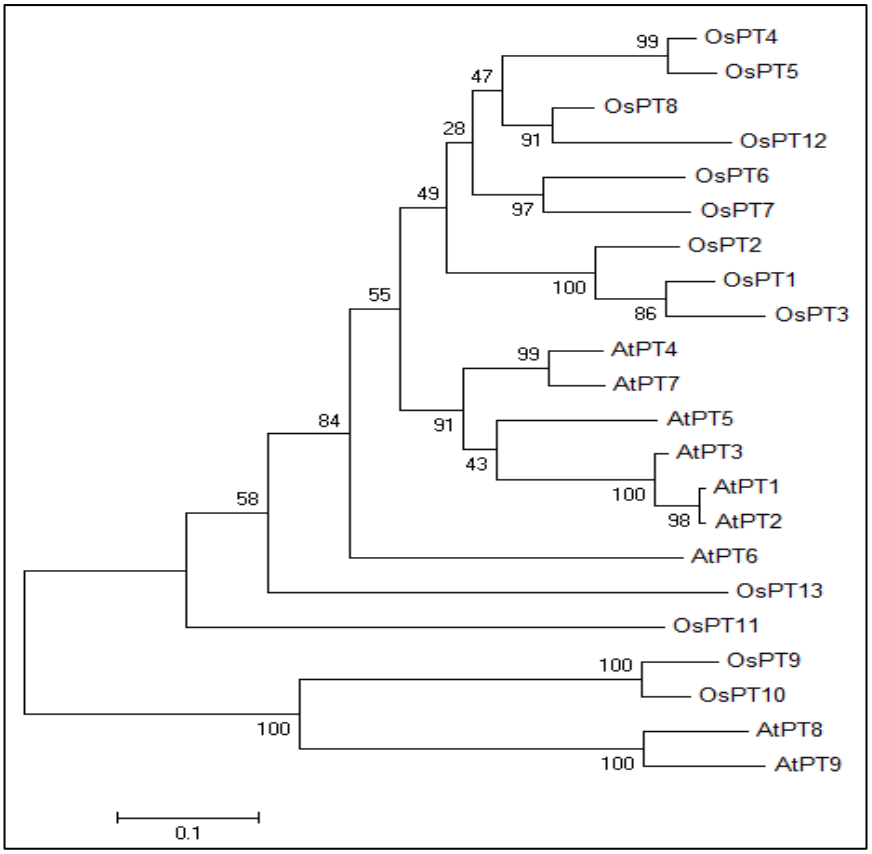

Figure 4.19: Maximum Likelihood Phylogenetic Tree at 1000 Bootstrap Replicates

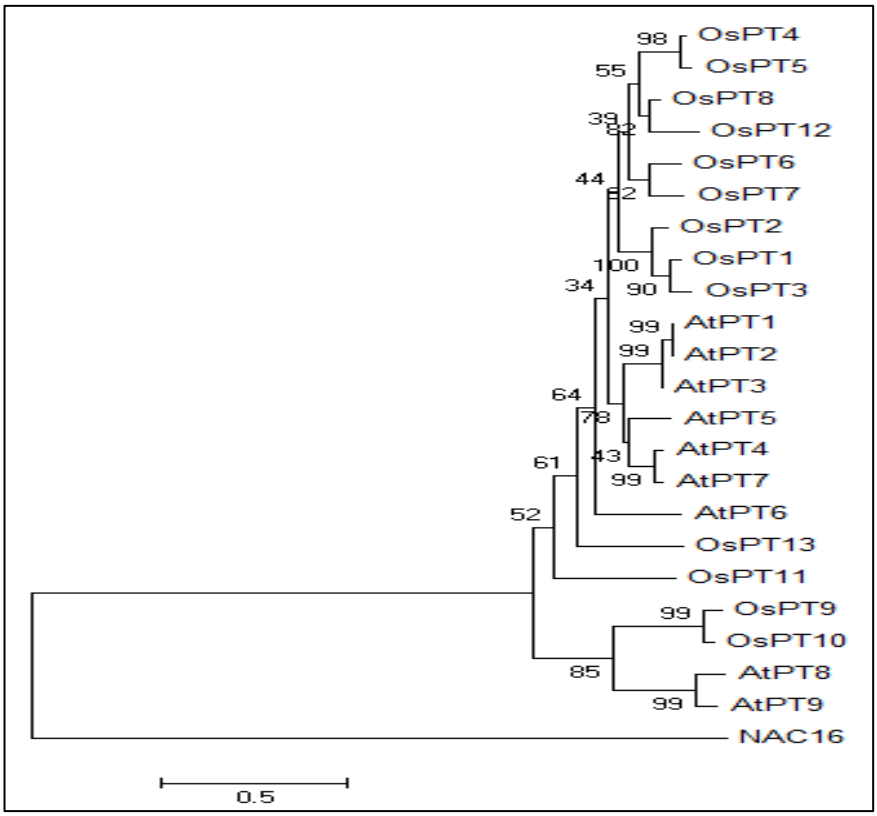

Figure 4.20: Maximum Likelihood Phylogenetic Tree with NAC 16

4.8.3 Conserved Regions

Conserved domains and signature sequences were detected from the literature were compared with the conserved motifs identified by using three computational models (Tusnády & Simon, 2001). *Oryza sativa* has many similar conserved domains and signature motifs which are also present in other cereal crops including wheat, maize, sorghum, barley etc. Signature sequences and motifs were retrieved by using RHYTHM, GLAM2 and MEME.

4.8.4 RHYTHM

RHYTHM is a knowledge based calculation of helix to helix contacts (Rose *et al.,* 2009). The trans-membrane helix sections were predicted in Phosphate Transporter Gene of both *Oryza sativa* and *Arabidopsis* by using RHYTHM. Conserved Pfam domains get differently colored dots. Brown dot is for Sugar Transporters (ST) whereas blue dot represents Major Facilitator Superfamily (MFS). Amino acids in red represent the Helix while in blue color show the membrane contact. Similar structures and Pfam domains were also reported in case of OsPT9 and OsPT10 (Wang *et al.,* 2014).

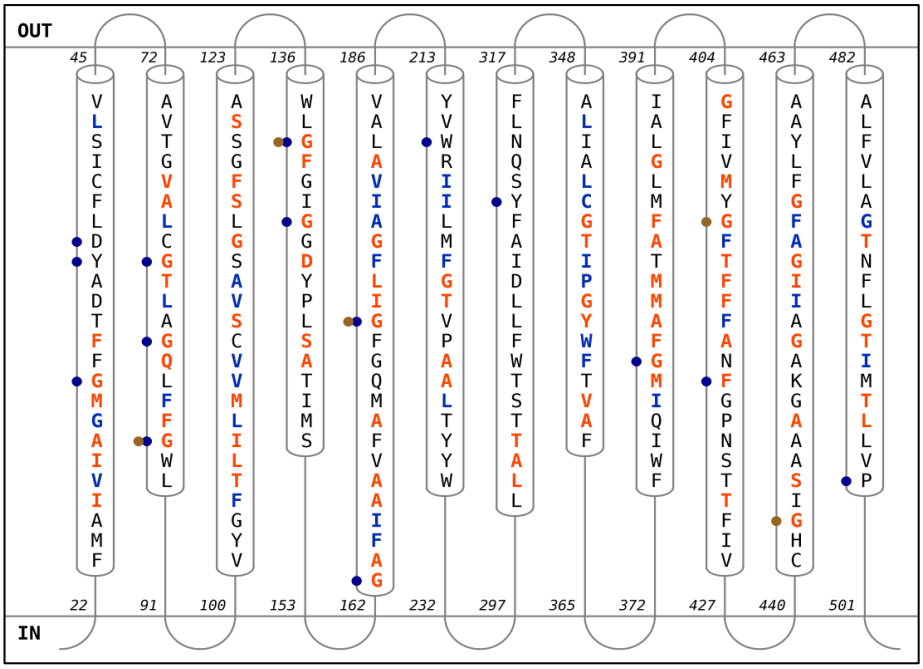

Figure 4.21: Structures of the Predicted Trans-membrane Domains of OsPT1 from RHYTHM

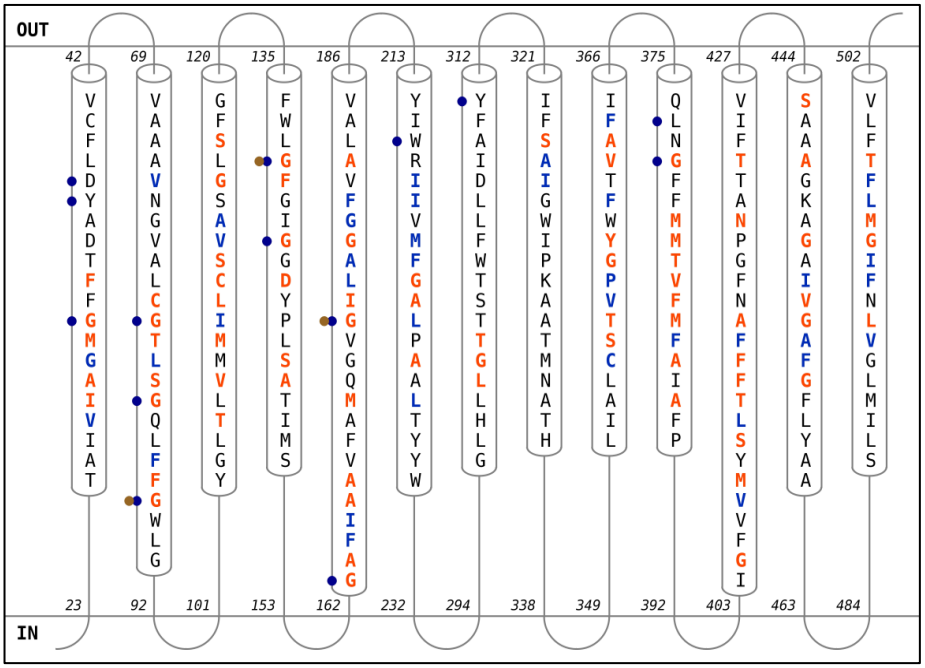

Figure 4.22: Structures of the Predicted Trans-membrane Domains of AtPT1 from RHYTHM

4.8.5 Gapped Local Alignment of Motifs (GLAM 2)

GLAM2 is specially promising for short protein motifs and provide alignment of key segments (Frith *et al.*, 2008). By running the sequences of both *Oryza sativa* and *Arabidopsis thaliana* 10 Motifs were obtained.

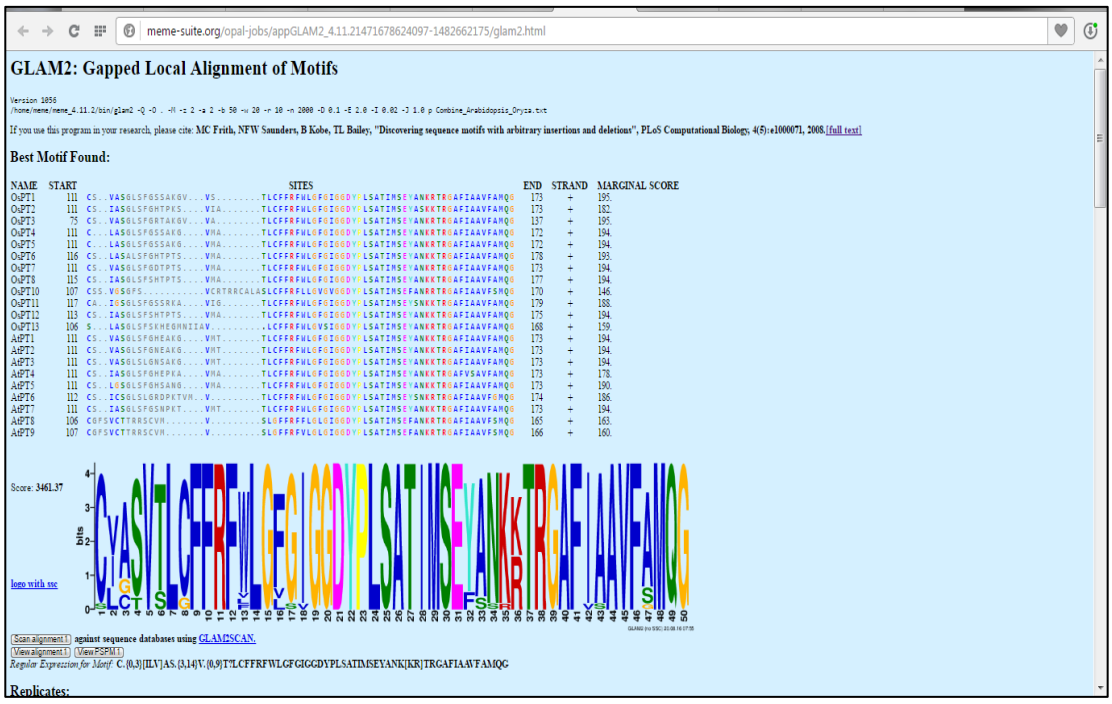

Figure 4.23: Motifs Retrieved from GLAM 2

Gapped Local Alignment of Motifs Scan (GLAM2SCAN) takes a motif found by GLAM2 and scans it against a database of sequences. Sequences were scanned against **Ensembl Plant Genomes and Proteins** including *Arabidopsis thaliana*, *Oryza sativa* and *Zea mays* databases and found that the same motifs are present in all three plant species (Figure 4.24).

Figure 4.24: Similar Motifs in *Arabidopsis thaliana*, *Oryza sativa* and *Zea mays* Retrieved by Using GLAM2SCAN

4.8.6 Multiple Em for Motif Elicitation (MEME)

MEME estimates how many times every motif occurs in each sequence present in datasets (Bailey and Elkan, 1994). About 15 motifs were obtained as depicted in the Figure 4.25. Motif Alignment and Search Tool (MAST) calculates the p-value of the motifs. And it was found that p-value is less than 0.0001 in this case (Figure 4.26).

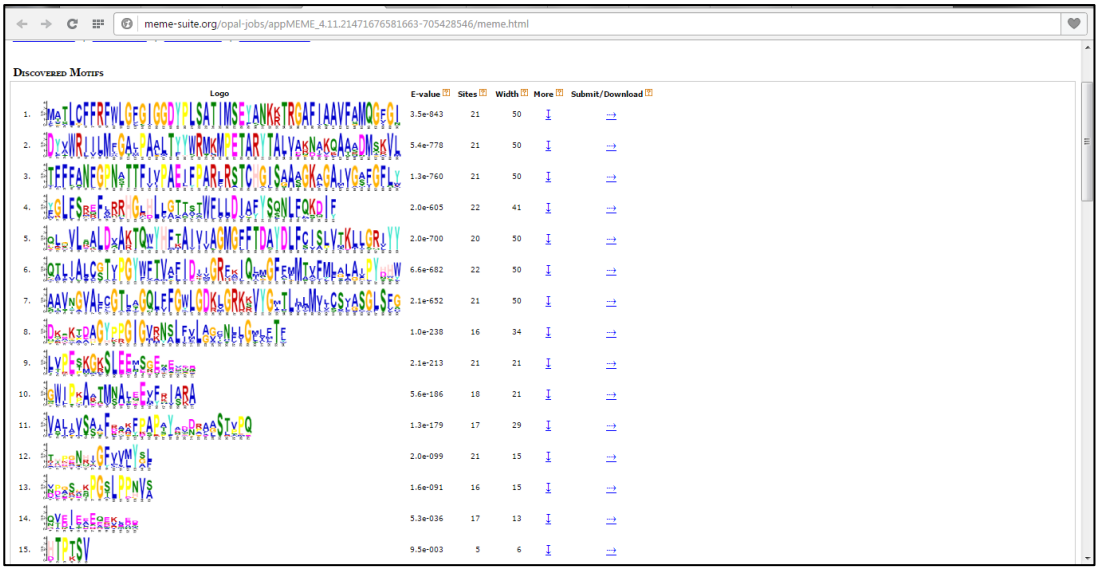

Figure 4.25: Motif discovered by using Multiple Em for Motif Elicitation

Figure 4.26: Results of Motif Alignment and Search Tool (MAST)

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Titania nanoparticles application on *Oryza sativa* significantly affected plant growth in terms of vegetative traits and nutrient availability. Results of germination test showed that genotype *Super Basmati* when treated with TNP found more resistant and showed increase in GP%, SLSI, RLSI, SVI and PHSTI as compared to other genotypes including P.*S.2*, *KSK Basmati*, *IRRI 9* and *Basmati 86.* In comparison with control, *Super Basmati* treated with TNP in soil medium showed improvement in root, shoot length, biomass, chlorophyll content and available phosphorus. Shoot length of plants treated with TNP was increased up to 15% and phosphorus availability enhanced by 38% as compared to control. Chlorophyll and plant biomass also increased at all tested treatment levels. Morphological difference was found among treated and control plants which was confirmed by Scanning Electron Microscopy analysis. *In silico* study was done by using Phosphate Transporter genes of both *Arabidopsis thaliana* and *Oryza sativa* and blasting of (OsPT1) with other cereal crops. Sequences were retrieved from different databases and it was confirmed that there was great similarity between all studied cereal crops in terms of phosphate transporters (PiTs) with rice. Computational models confirmed the presence of similar motifs among *Oryza sativa* and *Arabidopsis thaliana*. The results of the study indicate towards the advantageous characteristics of TNP, which will further encourage the application of NPs in the field of agriculture.

5.2 Future Recommendations

From current study, we found noteworthy effects on the *Oryza sativa* in response to titania nanoparticles. Such studies could be helpful to overcome the issues of nutrient deficiency in soil and help in providing better crop yield by reducing use of pesticides and fertilizers and ultimately helpful in betterment of environment. Following are the recommendations for future work:

- There is need to study the relationship between TNP and disease attacks (e.g. fungal infections, pests' attacks etc.).
- Gene expression studies both *in-vitro* and *in-vivo* is needed.
- Identification of co-relation between nutrient availability and gene expression on nanoparticles application is required.

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ANNEXURES

ANNEXURE I

Table: Seedlings Growth Stress Indices in Response to Titania Nanoparticles

	KSK Basmati		**Super Basmati**		**P. S. 2**		**Basmati 86**		**IRRI 9**	
TNP Conc. (mg L^{-1})	SLSI	RLSI	SLSI	RLSI	SLSI	RLSI	SLSI	RLSI	SLSI	RLSI
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
100	89.88	102.25	97.09	109.02	94.46	88.90	85.25	82.26	102.96	96.97
200	90.06	106.75	94.65	109.18	115.14	93.01	90.80	90.62	94.85	74.05
300	81.22	70.35	97.67	101.14	93.58	77.42	69.70	78.94	80.66	76.89
400	75.23	82	101.98	100.00	108.31	85.47	81.35	79.93	102.35	98.52
500	75.76	71.95	94.88	102.17	104.59	101.19	71.80	80.68	94.95	81.89

TNP: Titania Nanoparticles, SLSI: Shoot Length Stress Index, RLSI: Root Length Stress Index

ANNEXURE II

Figure: Neighbor Joining Tree of OsPT1 with *Arabidopsis* other Cereal Crops