Effect of Nanoparticles on *Triticum aestivum* Under Phosphorus Stress

By
Zehra Abbas

Department of Biochemistry
Faculty of Biological Sciences
Quaid-i-Azam University
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"In (or with) the name of Allah, the Beneficent, the Merciful"

This work is dedicated to my one and only Beloved Brother, Mohammad Ali and my Parents who are my whole world and without whom nothing would have been possible.

CERTIFICATE

This thesis, submitted by **Ms. Zehra Abbas** to the Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the thesis requirement for the Degree of Master of Philosophy in Biochemistry/Molecular Biology.

Supervisor:

Dr. Samina Shakeel
Supervisor
Associate Professor

External Examinar:

Dr. Iftikhar Ahmed
Principal Scientific Officer
NARC Islamabad

Chairman:

Dr. Muhammad Rashid Khan
Professor

Dated: **July 1, 2020**

DECLARATION

I hereby declare that the work presented in the following thesis is my own effort and that this thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Zehra Abbas

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List of Abbreviations

µL	Microlitre
ABA	Abscisic Acid
ACC	1-Amino cyclopropane-1-carboxylic Acid
ATP	Adenosine Tri Phosphate
cDNA	Complementary Deoxyribonucleic Acid
CKs	Cytokinin
CTAB	Cetyl Tri Ammonium Bromide
CTR	Constitutive Triple Response
DEPC	Diethyl pyro carbonate
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra Acetic Acid
ETR	Ethylene Receptor
mM	Millimolar
NARC	National Agriculture Research Centre
PCR	Polymerase Chain Reaction
Ppm	Parts Per Million
PR	Primary Root
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Specie
RPM	Revolution Per Minute
TBS	Tris Buffer Saline

ABSTRACT

Cereals are essential part of human diet comprising about 50 % of the consumed calories. Wheat is an important crop and staple food for both the developing and developed countries but its currently global warming and abrupt changes in the environmental conditions are leading to changes in our agricultural systems, which interns effect the crop production though plants usually develop morphological and physiological changes to cope up with the environmental and nutrient. Phosphorous deficiency is the major limiting factor of wheat production in many areas of Pakistan especially where farmers cannot afford the high cost of soil analysis and fertilizers. Considering the food security under such threats, we can utilize advance nanotechnology for boosting wheat production by assuring its sustainability. Nanomaterials offer a wider specific surface area to fertilizers and pesticides. In addition, nanomaterials as unique carriers of agrochemicals facilitate the site-targeted controlled delivery of nutrients with increased crop protection. Different nanomaterial have different effects on growth and development of plants depending upon their varieties, therefore we used novel zinc oxide and silicon dioxide nanoparticles synthesized in our lab by a former student named, Asma Noor, to study the wheat roots in terms of changes in root architecture, anatomy and morphology playing role in phosphorous acquisition and under the presence of nanoparticles

Root system architecture (RSA) of two of the local wheat varieties of Pakistan SKD-1 and PAK-81 have been studied along with molecular expression of ethylene and cytokinin signaling genes in response to phosphorous deficiency in the presence of nanoparticles. Our results showed that phosphorous deficiency has significant effect on root architecture. There was significant difference in root architecture of SKD-1 and PAK-81 varieties under different phosphorous concentrations and in presence of ZnO, SiO_2 nanoparticles. These were compared to control having no nanoparticles exhibiting that roots under control had a moderate effect under phosphorus stress while roots within nanoparticles showed high and low contrasting effects at both physical and molecular level. Different root traits were studied in both varieties of at physiological, cellular and molecular levels. We tried to analyze the factors,

conditions and components that can have a substantial role to identify phosphorous and nanoparticles efficient varieties like SKD-1. Our data of root architecture showed significant changes in response to both the stresses. Similarly, microscopic data of apical meristem showed decrease in cell divisions in phosphorous deficient conditions which results in inhibition of primary root. Transcript analysis showed that unavailability of phosphorous enhances the expression of ethylene biosynthesis pathway genes which supports ethylene signaling role in regulation of phosphorous deficiency with increasing expression under silicon dioxide nanoparticles and a decreasing expression under the presence of zinc oxide nanoparticles making SKD-1 to be opted as one of the finest variety that can be grown in areas with phosphorous deficiency and its growth can be facilitated in the presence of appropriate nanoparticles under optimized conditions. Further research is required to study ethylene signaling pathway and its interactions with other pathways to regulate the nutrient deficiencies in the presence of nanoparticles.

1. INTRODUCTION

Wheat is one of the most widely grown cereal crop in the world, covering about 237 million hectares annually, accounting for an aggregate of 420 million tonnes (Isitor etal., 1990) (Olabanji etal., 2007) and for at least one-fifth of human calorie consumption (Ohiagu etal.,1987). It is the prevailing crop in temperate regions that is being utilized for human food and livestock feed. Its triumph as per productivity rate depends not only on its adaptability and high yield potential but also on the gluten protein segment which includes the viscoelastic properties that permit dough to be processed into noodles, bread, pasta and other food products. This cereal crop contributes to crucial and essential vitamins, amino acids, minerals, valuable phytochemicals and dietary fiber components to the human diet, and these are predominantly enriched in whole-grain products (Shewry 2009).

Although wheat is adjusted to climate between the latitudes of 30^0 and 60^0N and $27^0$and 40^0S, however, it is grown under extensive range of climatic circumstances within the Arctic circle to higher altitudes near the equator. During the past two decades research by the International Maize and Wheat Improvement Center (CIMMYT) has shown that wheat production is more feasible in warmer areas. In altitude, the crop is grown from sea level to < 3,000 m.a.s.l., and it has also been reported at 4,570 m.a.s.l in Tibet. The optimal growing temperature is about 25°C, with minimum growth temperature at 3° to 4°C and maximum at 30° to 32°C, respectively. Wheat is adapted to an extensive range of moisture conditions and can be grown in most localities where precipitation ranges from 250 to 1750 mm. For winter wheat, heading is deferred until the plant experiences a range of cold winter temperatures (0° to 5°C), Spring wheat, as the name indicates, is usually planted in the spring (can be sown in autumn in states like Pakistan that experience mild winters) and matures during summer(Dr. M. Shahid Masood 2013).

1.1 Origin and evolution of wheat:

The first cultivation of wheat occurred about 10,000 years ago, as part of the 'Neolithic Revolution', which saw a transition from hunting and gathering of food to

settled agriculture. The genetic transitions during domestication exhibits that modern day wheat is impotent to survive wild in competition with enhanced adapted species. This was sophisticatedly validated by John Bennet Lawes in the 1880s when he decided to permit part of the famous long-term Broadbalk experiment at Rothamsted to return to its natural state (Dyke GV. 1993). Hence, he left part of the wheat crop unharvested in 1882 and examined the growth in consecutive years. After a good crop was produced in 1883 the weeds dominated and in 1885 the limited remaining wheat plants (which were spindly with small ears) were collected and photographed.

1.2 Wheat (*Triticum aestivum*) Taxonomy:

Wheat belongs to the genus *Triticum,* for which there are 10 species. Among these 10 species, six are the ones which are cultivated and four of the species are not cultivated. The most economically significant species, *T. aestivum,* has five subspecies (USDA, 2005).

Table 1.1: Wheat Taxonomy

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Lilioposida
Subclass	Commelinidae
Order	Cyperales
Family	Poaceae
Genus	*Triticum*

Species	*Triticum aestivum*

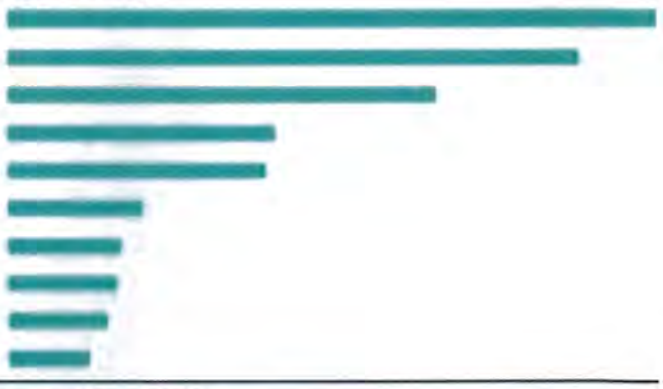
1.3 Wheat Production World Wide:

Wheat is produced on the scale of about > 240 million ha, larger as compared to any other crop, with a grand world trade than for all other crops combined. FAO has put forward the world wheat output in the year 2013 at 704 million tonnes, an increase of 6.8 percent from the previous years has been observed, which would infer to more than full recovery from the previous year's reduction and bringing world production of wheat to its highest level in the overall historic time zone.

World Wheat Production (million tones)

Growing season	2009-10	2010-11	2011-12	2012-13	
				estimate	current
Production	685.7	655.4	701.5	659.3	704.1

Rank	Country	Production (1000 MT)
1.	EU-27	137,443.00
2.	China	121,000.00
3.	India	92,000.00
4.	United States	56,613.00
5.	Russian Federation	54,000.00
6.	Canada	29,000.00
7.	Australia	24,500.00
8.	Pakistan	24,330.00
9.	Ukraine	19,500.00
10.	Turkey	17,600.00



Source: FAO Cereal supply and demand brief July, 2013 and USDA

	Wheat			Coarse grains			Rice (paddy)			Total cereals			
	5-yr Avg.	2018 estim.	2019 f'cast	5-yr Avg.	2018 estim.	2019 f'cast	5-yr Avg.	2018 estim.	2019 f'cast	5-yr Avg.	2018 estim.	2019 f'cast	Change: 2019/2018 (%)
Far East	**257.0**	**260.9**	**266.3**	**371.6**	**378.1**	**374.5**	**681.0**	**697.9**	**703.0**	**1 309.6**	**1 336.8**	**1 343.7**	**0.5**
Bangladesh	1.3	1.1	1.3	2.6	3.2	3.3	52.6	54.5	54.6	56.5	58.8	59.2	0.6
Cambodia	0.0	0.0	0.0	0.8	1.2	1.2	10.0	10.9	11.0	10.8	12.1	12.2	0.7
China (Mainland)	132.1	131.4	134.0	268.6	267.3	264.3	211.5	212.1	210.6	612.3	610.9	608.9	-0.3
India	94.6	99.9	102.2	43.6	45.4	43.7	164.4	173.4	176.3	302.6	318.7	322.2	1.1
Japan	0.9	0.8	0.8	0.2	0.2	0.2	10.9	10.8	11.0	12.0	11.8	12.0	1.8
Myanmar	0.1	0.1	0.1	2.5	2.8	2.9	28.9	30.4	30.5	31.5	33.3	33.5	0.6
Nepal	1.9	2.0	2.2	2.7	3.0	3.0	5.0	5.3	5.4	9.6	10.3	10.6	2.5
Pakistan	25.7	25.1	25.2	6.2	6.8	6.8	10.6	10.8	10.9	42.5	42.7	42.9	0.5
Philippines	0.0	0.0	0.0	7.6	7.8	8.0	18.7	19.0	19.4	26.3	26.8	27.4	2.4
Republic of Korea	0.0	0.0	0.0	0.2	0.2	0.2	5.5	5.2	5.2	5.7	5.4	5.4	0.2
Sri Lanka	0.0	0.0	0.0	0.3	0.3	0.3	3.8	3.9	4.5	4.0	4.2	4.8	14.3
Thailand	0.0	0.0	0.0	4.9	5.2	5.2	31.2	32.0	33.3	36.1	37.3	38.5	3.4
Viet Nam	0.0	0.0	0.0	5.2	4.9	4.9	44.0	44.0	43.8	49.2	48.9	48.7	-0.4

Figure 1.1: Production of wheat showing the growth rate worldwide (2009-2019)

The above figure shows the highest production rate in Europe and the lowest in Turkey while Pakistan lies on 8th rank from the scale of 1 to 10.

1.4 Wheat Production in Pakistan:

In the Islamic Republic State of Pakistan, wheat being the chief staple food is cultivated on the largest acreages. Pakistan comes under the category in ten major wheat-producing countries of the world in the aspects of area under wheat cultivation, yield per hectare and total production. Wheat is the crucial diet of population as it constitutes 60% of the routinely diet of a common man in Pakistan and average per capita consumption is about 125 kg and lodges a central position in agricultural strategies of the government. The government announced wheat support price of Rs. 1200 which created interest on the part of farming community. Wheat contributes 10.1 % to the value added in agriculture and 2.2 % to GDP (Gross Domestic Product).

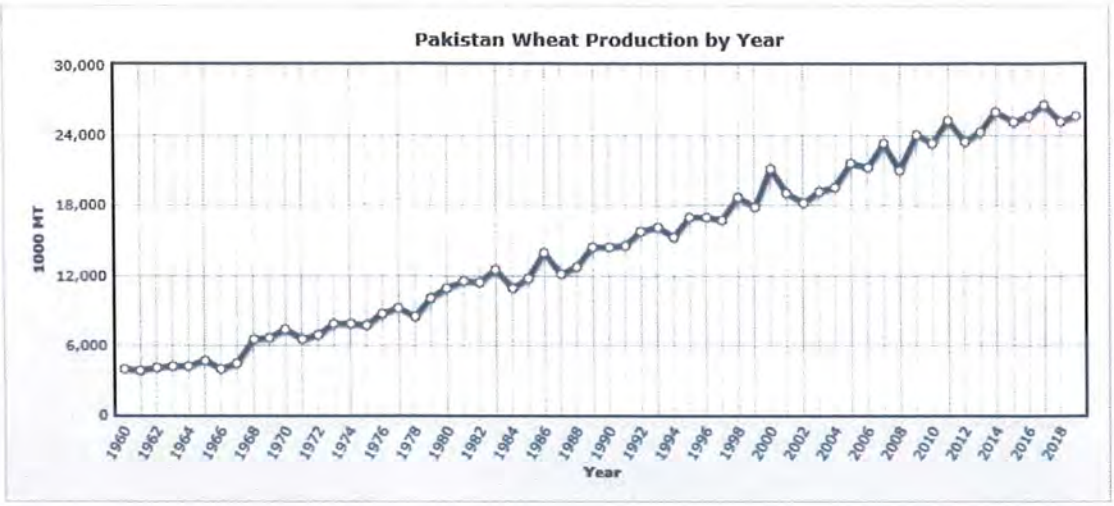


Figure 1.2: Graphical representation of wheat production (1960-2018)

Table 1.2 Wheat Production in Pakistan by Year

Market Year	Production	Unit of Measure	Growth Rate
2008	20959	(1000 MT)	-10.03 %
2009	24033	(1000 MT)	14.67 %
2010	23311	(1000 MT)	-3.00 %
2011	25214	(1000 MT)	8.16 %
2012	23473	(1000 MT)	-6.90 %
2013	24211	(1000 MT)	3.14 %
2014	25979	(1000 MT)	7.30 %
2015	25086	(1000 MT)	-3.44 %

2016	25633	(1000 MT)	2.18 %
2017	26600	(1000 MT)	3.77 %
2018	25100	(1000 MT)	-5.64 %
2019	25600	(1000 MT)	1.99 %

(United States Department of Agriculture PSD database 2019- IndexMundi)

1.5 Stresses in Plants:

A variable condition that can alter the plant developmental processes by disrupting its equilibrium is termed as stress. Strain or pressure induced form is one of the condition under stress (Gaspar et al. 2002). Plants are sessile species and exposed to environmental stress factors that enhances their chances of being vulnerable to these stresses. Precise mechanism in plants has been established that permits them to respond to environmental variations and minimize damage (Rizhsky 2004). Plants can survive in stressed induced environment by utilizing their tolerance and defense mechanisms (Sung et al. 2003). Over the past years, Plants have shown evolution in these mechanisms to detect external stress factors and produce responses more effectively. There are a variety of Internal and External factors involved in producing stress in plants i.e. nutrient deficiencies, oxygen radicals, temperature ,water and salinity (Balestrasse et al. 2010). Roots of the plant exhibit morphological and physical changes in structure in order to survive in stressed environment (Oono et al. 2013). The major production losses in wheat are caused more by abiotic stresses such as nutritional deficiencies drought, high temperature and salinity as compared to damage by biotic stress factors (Kumar et al., 2018).

1.5.1 Classification of Stress Factors

Stress factors in plants are divided into two main categories

- Biotic stress factors
- Abiotic stress factors

1.5.2 Biotic Stress Factors

Living Organisms are the causative agents of stress in plant species. These causative agents comprises of viruses, fungi, weeds, bacteria, parasites and insects. Biotic stress results in pre and post harvested fatalities in agriculture (Angessa and Li 2015). As Plant species are deprived of adaptive immune responses and are unable to memorize infection, plants have evolved certain tactics against biotic stresses. Genetic basis of defense mechanism deposited in plants as genetic codes. Hundreds of biotic stress resilient genes are encoded by genome of the plants. Genome sequencing of important cash crops e.g. Wheat, rice and maize has been completed (Singla and Krattinger 2015).

1.5.3 Abiotic Stress Factors

Numerous abiotic stresses caused by non-living causative agents can adversely affect plants. E.g. Elevated or depleted temperature ranges, salinity, nutrient deficiencies, drought, light intensity, oxygen depletion, and high winds (Wang *et al.*,2003). Abiotic stress is a foremost restraint in yield of crops. These stresses in plants results in the production of ethylene. In response to these stresses plant shows variety of responses (Stearns and Glick 2003). Reactive oxygen species (O^2, O^{2-}, H_2O_2) accumulates in plants as result of abiotic stresses and results in numerous cytotoxic effects (Pastori 2002).

1.5.4 Stress induced by Nutritional Deficiency:

Nutrients are the components that are essentially needed for proper development, growth, metabolism and production of the plants. Each nutrient in the soil plays has a key role in growth of the plant. Phosphorous are one of the macronutrient and is a vital requirement for plants as it strengthens plant roots system and involved in formation of the seeds too. Deficiencies of these nutrients results in inadequate plant life cycles, reduced metabolism causing effects on roots and leaves (Knight *et al.*, 2001). These are the components that are present under the soil and used in form of

ions by the plants. Plants undergo stunted growth, chlorosis and necrosis due to nutrient deficiency. Ethylene is a gaseous hormone produced in plants and involved in germination of the seeds, root and shoot development, development of lateral buds, pollination, flowering initiation, differentiation of the tissues and ripening of the fruits (Jonathan Lynch and Brown 1997). Deficiency of phosphorous (P) and nitrogen (N) results in the production of ethylene hormone. As phosphorous deficiency is mainly involved in the morphological changes in roots architecture, root hair development and aerenchyma formation (Jonathan Lynch and Brown 1997) and even minimum phosphorus deficiency has a major influence on plant growth and development. It has been estimated that 30% of the world's arable soils are deficient in phosphorus and require its fertilization to improve yields (MacDonald et al., 2011). Plants have evolved numerous responses to stabilize the fluctuating phosphorus deficiency levels that occur at the tissue level in natural environments, such as (1) changing root morphology and initiating mycorrhizal symbiosis to improve soil exploration for P; (2) stimulating exudation processes in the rhizosphere to mobilize Pi from the soil; (3) inducing Pi transporter proteins and regulating Pi uptake kinetics; and (4) remobilizing Pi in source organs to meet the necessities and requirements of the developing sink tissue (Ramaekers et al., 2010).

1.6 Symptoms of Nutrient Deficiency in Plants

Symptoms commonly caused due to nutritional deficiency are enlisted below:

- Stunted Growth
- Chlorosis
- Intervienal Chlorosis
- Purple-red Coloration
- Necrosis

As nutrient perform their role in numerous plants functions e.g. stem elongation, protein synthesis, photosynthesis, and several other cellular processes. This eventually effects the growth of plant which causes reduced stature in them. If a plant is deficient in nutrients involved in chlorophyll production or in photosynthesis then it leads to

chlorosis and intervienal chlorosis. Chlosrosis is yellowing of plants due to absence of chlorophyll and intervienal chlorosis is yellowing in between the leaf veins. Necrosis is the condition of plant tissue death due to injury. Deficiencies turn tissue brown and results in death of the plant (Yu and Bell 1998).

1.7 Nanotechnology and Nanoparticles

Nanotechnology is a pioneering pitch with innovative tactics and one of the most promising fields of interdisciplinary studies that deals with nanoparticles. It unlocks an extensive range of potential usages in numerous areas of science and industry, such as medicine, electronics, biology, pharmacology, and breeding of plants (Begum *et al.*, 2014). Nanoparticles range in magnitudes between 1 and 100 nanometers with one or more dimensions and are made up of carbon, metal oxides, metals or organic matter (Hasan, 2015).

Nanoparticles are manufactured by numerous methods that are categorized into bottom-up or top-down method. Bottom-up or constructive method is the build-up of material from atom to clusters to nanoparticles whereas Top-down or destructive method is the reduction of a bulk material to nanometric particles (Ealias *et al.*, 2017). A variety of techniques are used for the synthesis of ZnO. These techniques generally can be divided into three types including physical, chemical and biological methodologies. Chemical synthesis can further be divided into liquid phase synthesis and gas phase synthesis. Liquid phase synthesis compromises of precipitation, coprecipitation method, sol-gel processing etc. Gas or vapor phase fabrication includes pyrolysis and inert gas condensation methods (Naveed Ul Haq *et al.*, 2017). Physical methods of ZnO nanoparticles synthesis include high energy ball milling, melt mixing, physical vapor depositionetc. A number of natural moieties such as plants, fungi, algae, bacteria, and viruses can also be used to synthesize the ZnO as a biological method(Naveed Ul Haq *et al.*, 2017).Silica nanoparticles have been reported to be synthesized by several methods like reverse microemulsion, flame synthesis, high temperature flame decomposition and the most widely used sol-gel procedure(Rahman & Padavettan, 2012).

1.7.1 Nanoparticles usage in Plants

Traditionally fertilizers were utilized for plant growth and development and they still have a significant role but most of the applied fertilizers are unavailable to plants due to various factors, such as hydrolysis, leaching, degradation by photolysis, and decomposition. In order to overcome this shortcoming it is essential to minimize nutrient losses in fertilization, and to escalate the crop yield and revenue through the exploitation of novel applications with the help of nanotechnology and nanoparticles (Siddiqui *et al.*, 2015). The high surface to volume ratio of nanoparticles makes them tremendously reactive with prominent physical properties. Nanotechnology facilitates agricultural sciences and lessens environmental pollution by production of chemical fertilizers and pesticides via the usage of nanoparticles and nano capsules with the capability to regulate delivery, absorption with the utmost effective and environmental friendly production of nano-crystals to intensify the efficacy of pesticides with lower dose (Sharon *et al.*, 2010).

1.7.2 Zinc Oxide (ZnO) Nanoparticle

Zinc as a plant micronutrient has a significant role in growth and physiological functions of plants and deficiency of zinc can result in stunted growth and less crop yield. It is also critical for human immune system and its deficit level is related to numerous problems including skin problems, memory loss, and weakened body muscles (Hafeez etal., 2013). ZnO is recently listed as a "generally recognized as safe (GRAS)" material by the Food and Drug Administration and also used as food additive. ZnO nanostructures demonstrate high catalytic efficiency, as well as strong adsorption ability, and have been used frequently in the manufacture of sunscreens (Huang *et al.*, 2001). Zinc oxide nanoparticles have certain potential role in central nervous system (CNS) during the development processes of diseases through facilitating neuronal excitability and even in the release of neurotransmitters. Some studies have specified that these nanoparticles affect functions of different cells, tissues and neural tissue engineering (Rasmussen *et al.*, 2010). As these nanoparticles have the targeting potential so they have possible utility in the treatment of cancer and/or autoimmunity (Hanley *et al.*, 2008). Zinc oxide nanoparticles have the capability to boost the growth and yield of food crops. Peanut seeds were treated with

variable concentrations of zinc oxide nanoparticles. Zinc oxide Nano scale treatment (25 nm mean particle size) at 1000 ppm concentration was used which endorsed seed germination, seedling vigor, and plant growth. Also these zinc oxide nanoparticles have also proved to be effective in increasing stem and root growth in peanuts (Prasad *et al.*, 2012). Zinc interacts with various nutrients in soil mostly with nitrogen and phosphorus. High phosphorus concentrations are linked to Zinc deficiency while zinc depressed amount of Cu resulted in less grain yield in wheat (Loneragan & Webb, 2011). So, optimum concentration of Zinc might have a beneficial effect on the growth of wheat crop.

The excessive usage of nanoparticles has been demonstrated to be toxic as low concentration of ZnO nanoparticles exhibited beneficial effects on plant growth and development while higher concentrations compromised seed germination (Siddiqui *et al.*, 2015).

1.7.3 Silicon Dioxide (SiO_2) Nanoparticle

Silicon is favorable for plant growth as it increases the photosynthetic rate and aids in fighting several biotic and abiotic stresses such as disease, cold, heat heavy metal and salinity stress though not considered as an essential element (Ma & Yamaji, 2004).

SiO_2 nanoparticles improved the germination of seeds in maize by enhancing availability of the nutrients (Suriyaprabha *et al.*, 2012). Exogenous application of SiO_2 nanoparticles enhanced growth of Changbai larch (*Larixolgensis*) seedlings mainly by improving root growth and enhanced synthesis of chlorophyll (Bao-shan *et al.*, 2004). The application of Silicon dioxide in agriculture might also lead to global food security by facilitating in the development of improved varieties with high productivity rate (Parisi *et al.*, 2015).

Silicon nanoparticles are promising and have agricultural inferences alongwith several new applications that are being investigated for plants. Due to the distinctive physical and chemical properties of silicon nanoparticles, they can easily enter into plant cells and affect the plant growth and development by altering their metabolism through varied interactions, thus eliciting the potential to combat under stress conditions. SiO2

nanoparticles can act as an agent for target-specific delivery of herbicides and fertilizers (Wanyika *et al.*, 2012).

1.8 Classification of Plant Nutrients

On planet earth approximately 92 elements have been found and out of these seventeen are critical elements that are mandatory by plants for survival and growth. Numerous inorganic fertilizers contain nutrients like potassium, phosphorous, and nitrogen used to obtain higher crops yield by replenishing soil nutrients. However, fertilizers application to crops is costly and also have adverse side effects on ecosystem and soil (Garnett *et al.*, 2009). These elements are divided into two main groups called as non-mineral and mineral nutrients.

1.8.1 Non-Mineral Nutrients

Non-Mineral nutrients comprise of oxygen (O), carbon (C) and hydrogen (H). These are available in gaseous form mostly and plants take these nutrients by air or water. In the process of photosynthesis plant take energy from sun and convert CO_2 and H_2O into sugars and starch.

Table 1.3: Essential Nutrients Taken Up by Plants (Mengel 1982)

Elements	Symbol	Form(s) Taken Up by Roots
Macronutrients		
Carbon	C	CO_2
Hydrogen	H	H_2O
Oxygen	O	O_2 , H_2O
Nitrogen	N	NH_4^+ , NH_3^+

Potassium	K	K^+
Phosphorous	P	HPO_4^{2-} , $H_2PO_4^-$
Calcium	Ca	Ca^{2+}
Magnesium	Mg	Mg^{2+}
Sulphur	S	SO_4^{2-}
Micronutrients		
Iron	Fe	Fe^{2+} , Fe^{3+}
Zinc	Zn	Zn^{2+} , $Zn(OH)_2$
Manganese	Mn	Mn^{2+}
Copper	Cu	Cu^{2+}
Boron	B	$B(OH)_3$
Molybdenum	Mo	MoO_4^{2-}
Sodium	Na	Na^+
Chlorine	Cl	Cl^-
Nickel	Ni	Ni^{2+}

1.8.2 Mineral Nutrients

Plants acquire mineral nutrients dissolved in water from soil by roots. At all times, mineral nutrients are not fully enough for plants to grow healthy. To overcome this deficiency farmer utilize fertilizers to add enough nutrients to soils. Further mineral nutrients are divided into two groups:

- Macronutrients

- Micronutrients

1.8.3 Macronutrients Required by Plants

Macronutrients are required by the plants in greater quantities (> 0.1% of dry mass) and it comprises primary macronutrients and secondary macronutrients. Nitrogen (N), Potassium (K) and Phosphorous (P) are primary macronutrient. Plants mostly require primary macronutrients in large amounts because soils are deficient of these nutrients and as NPK required for several processes of plants survival and growth. Secondary macronutrients include Calcium (Ca), Magnesium (Mg) and Sulphur (S). Secondary macronutrients are accessible in soils in sufficient amounts as in acidic soils Magnesium (Mg) and Calcium (Ca) are added in the form of Lime. In process of decomposition of organic matter soil gets adequate amounts of sulphur (Williams and Salt 2009).

1.9 Importance of Roots System Architecture in Plants

Roots are a significant part of plant as helps in uptake of water and nutrients that is required by the plant to grow and survive. Roots are also needed for anchoring, support and storage purposes. Roots of some plants serve as food for humans like turnips and carrots. To overcome challenges faced by plants being sessile, roots alter their architecture in response to different stress factors (Waines and Ehdaie 2007). The roots are interface between plants and biotic abiotic stress factors in soil (Smith and de Smet 2012). Root architecture varies in different species and it also varies within species sue to genetic makeup and environmental effects (J. Lynch 1995).

1.9.1 Basic Root Systems

Root system is downward portion of plant axis. After the germination of seed, first part that comes out is radical. It elongates and form roots either primary root or tap root. It also forms lateral roots and branches thus results in formation of root system of plants. There are mainly two types of root systems.

1.9.1.1 Tap Roots systems

In this system root consist of one main root alongwith with small branches that grows from it. Tap root is a main root which is deep rooted and grows vertically downward. It gives plant strong anchorage. It is dicot form of root system (Morita and Yamazaki 1993).

1.9.1.2 Fibrous Roots Systems

Fibrous root system consists of several thin roots appear like bushes and have short primary root. Several fibers like roots arise from radical base which are shallow and horizontally placed. Hence can't provide strong anchorage and support to plants. This system of roots called as monocot root system as it is present in rice, maize and wheat (Morita and Yamazaki 1993) .

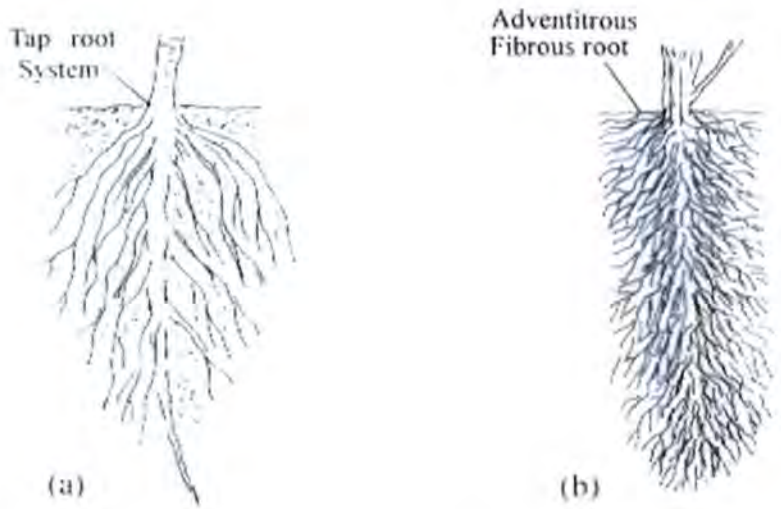


Figure 1.3 : Tap Root System (A) and Adventitious Root System (B) (Morita and Yamazaki 1993)

1.9.1.3 Root Apical Meristem

Root apical meristem is the region at root tip where cells are capable of repeated cell division and protected by root cap at tip (Jiang and Feldman 2005). Root tip divided into three zones:

- Cell division zone
- Cell elongation zone
- Cell maturation/differentiation zone

Cell division zone is the closest to the root tip and consist of cells of apical meristem which are actively dividing cells. In cell elongation zone length of cell increases which eventually increases root length. The area where formation of root hairs takes place called as cell maturation zone where first root hair develops and starts differentiating which contributes in formation of special cell types. Meristem cells in root tip have ability to regenerate and it is due to asymmetric cell division in these cells (Ohlstein et al. 2004).

In plants root growth occurs by elongation of cells which are root apical meristem (RAM) descendants. A specialized tissue called as root apical meristem present at tip of root and produces cells of all root tissues that formed during postembryonic development (Dinneny and Benfey 2008). At center of meristem and behind root cap group of cells present which rarely divide and known as quiescent center that is the inactive region in root apical meristem. Its function is in organizing primary root growth pattern (Clowes 1976).

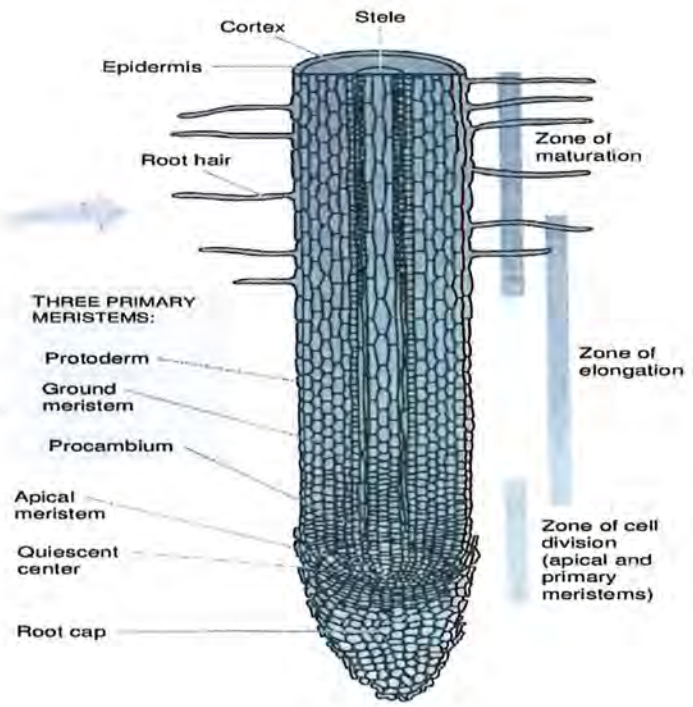


Figure 1.4: Schematic Diagram of Plant Root Apical Meristem Showing Zones of Cell division, Maturation and Elongation (Janice E Thies, 2006).

1.10. Phosphorus Deficiency in Soil and Roots Response

Phosphorous deficiency in soils is limiting factor for crops production and has an effect on crop growth. It is an important abiotic stress that limits production of crops 30-40% (von Uexküll *et al.*, 1995). It is majorly present in soils where phosphorous bound to other particles in soils. This interaction of phosphorous with soil particles limits phosphorous availability. Due to phosphate highest capacity to absorbed by soil particles which limits its uptake via roots to plant. It shows that phosphorous uptake is correlated with plant ability to explore soil. Adaptation of root system architecture in nutrient deficient conditions makes plant capable of exploring soil and ensure uptake of phosphorous (Sattelmacher *et al.*, 1994). Plant roots undergo changes in response to P deficiency in several ways. Lateral roots development, lateral roots branching, growth of root hairs and primary root elongation occurs in p deficiency (J. P. Lynch and Brown 2001). Another important root adaptation is increase in density and number of root hairs which eventually help in phosphorous uptake by expanding surface area of roots to explore more soil (Gahoonia and Nielsen 1997).

Phosphorous is an essential macronutrient required to sustain quality and production in plants (Zapata and Zaharah 2002). It plays important role in different processes of plants e.g. Metabolism, Glycolysis, membrane synthesis, important signaling pathways and in synthesis of cellular components (Raghothama 1999). Particularly its role is in root physiology and morphology including root branching and lateral roots development (Jin et al. 2005). Plants evolved strategies to cope in phosphorous starved conditions which results in several changes in root system architecture, biochemistry and morphology of plant (Linkohr et al. 2002). In comparison with other essential nutrients mostly phosphorous is least mobile and least available in soils which makes it main limiting factor in plants growth. Almost 5.7 billion hectares land worldwide has phosphorous deficiency (Batjes 1997). And 30-40% of arable land gives poor crop yield due to phosphorous deficiency (Runge-Metzger 1995). It is

available in two forms; organic and inorganic in soils. Plants root uptake inorganic phosphorous (P_i) and it is the least available phosphorous form in soil (Teng eal. 2013). Low availability of phosphorous in soil is due to its phosphate ion (P) reactivity to the other soil constituents which limit its availability in inorganic phosphorous (P_i) form (Ozanne 1980).

1.11 Plant Hormones

Plant hormones are naturally occurring substances that can affect several physiological processes like differentiation, plant growth, development and stomatal movements at very low concentrations. Plant hormones are also known as "Phytohormones". There are five major plant hormones Auxins, Gibberellins, ethylene, abscisic acid and cytokinin (Davies 2010).

Below is the brief description of the ones being involved in this experiment at molecular level.

1.11.1 Cytokinin (CKs)

Cytokinins are derivatives of adenine and are involved in cell division in plants in auxin presence. CKs found in seeds and root tip involve in cell division hence help in root elongation and seed germination. Other functions of CKs are leaf expansion, lateral buds formation and morphogenesis (Davies 2010). Metabolism and signaling of cytokinin has been explicated in *Arabidopsis thaliana*. Cytokinins are synthesized initially through a cascade of enzymes encoded by a set of three gene families, comprising of isopentenyltransferase (IPT), cytokinin trans-hydroxylase (CYP735A) and cytokinin nucleoside 5-monophosphate phosphoribohydrolase (LOG) genes (Sakakibira 2006). The process of degradation of cytokinin is catalysed by cytokinin oxidase/dehydrogenases encoded by CKX genes (Schmülling et al., 2003).

The chief function of leaves is to provide assimilates for growth of the plants through the process of photosynthesis. CKs affect the functional as well as the structural characteristics of photosynthesis at numerous levels. CKs induce cell division and differentiation even in the primary stages of leaf development. The effect of CKs has been studied within whole leaf and found that they altered the leaf structure in order

to have a greater number of cells per leaf area (Chernyad'ev 2000a) and a greater number of vascular bundles comprising xylem and phloem elements (Chernyad'ev 2000b).

1.11.2 Ethylene

Ethylene is a gaseous hormone and controls multiple physiologic activities in plants. It plays its role in plant growth and development. It also involved in roots development and seed germination by breaking dormancy. In high concentration ethylene also inhibit PR elongation (Abeles *et al.*, 1992). Ethylene affect size of cell as it mostly reduces cell elongation in other words it act as a signal for cell expansion. It also controls growth of plants through cell division. Ethylene is considered as a major modulator in response to environmental stresses (Road 2007). Ethylene also known as an "aging hormone" as it is involved in several developmental processes e.g. ripening, abscission and senescence. Genetic analysis on Arabidopsis model plant identified certain key elements act to mediate responses to ethylene (Alonso and Stepanova 2009). The enzyme that is directly involved in ethylene biosynthesis is 1-aminociclopropane-1-acid carboxylic oxidase (ACO or EFE - ethylene forming enzyme; EC 1.14.17.4), which transforms ACC into ethylene hormone (Hegg EL, 1997).

Ethylene signal transduction activates considerable variations in the gene expression of plant cells. Analysis of Promoter region of the genes induced by ethylene directed to the identification of cis-acting elements and the trans-acting protein EREBP (ethylene responsive element binding protein) family, which interacts with ERFs (ethylene response factors) DNA (Leubner-Metzger et al., 1998). The enhanced transcript accumulation for various ERF genes have also been reported under drought, salt and cold stresses (Miao Z et al., 2015).

1.12 Ethylene Biosynthesis and Signaling Pathway

Ethylene produced in all plant tissue and its production varies with tissue type, developmental stage and species. Its biosynthesis occurs by methionine pathway which is a 3 step processes and required ATP. Methionine added with ATP and H_2O with which loss of three phosphate groups synthesize S-adenosylmethionine (S-

AdoMet) and reaction catalyzed by an enzyme SAM synthetase (McKeon *et al.*, 1995). S-adensylmethionine (AdoMet) then converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by enzyme called as 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and it this step requires oxygen for oxidation. Ethylene production occurs in result of ACC oxidation in presence of an enzyme ACC oxidase (ACO) (Song and Liu, 2015).

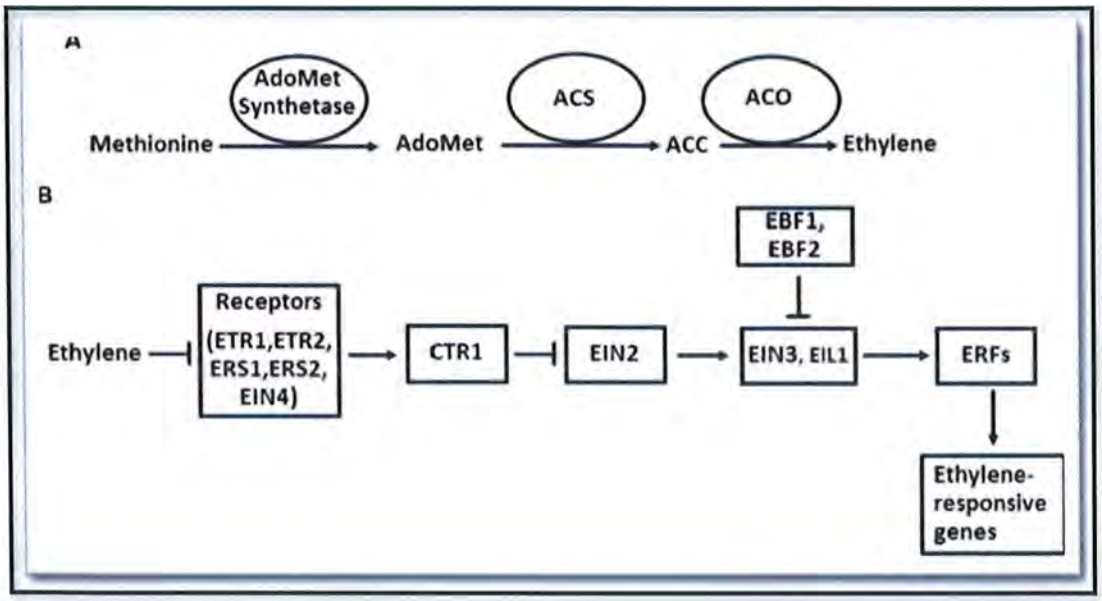


Figure 1.5: Ethylene Biosynthesis Pathway in Plants (A) and Ethylene Signaling Pathway in *Arabidopsis* (B). Arrows indicates promotion while perpendicular lines shows inhibition (Song and Liu, 2015).

1.13 Objective of the study:

Phosphorous is an important nutrient involved in several metabolic pathways in plants like glycolysis, membrane synthesis and respiration and also present in phospholipids, nucleic acids and ATP. It is required for better plant growth and has its effect on root architecture of plants.

Nanoparticles have variable positive and negative effects on the growth and development of plants depending upon the nature, composition, reactivity and their dose administered in the plant species (Khodakovskaya etal., 2012). Plants uptake

phosphorous and nanoparticles by their roots immersed in the Hoagland nutrient solution containing considerable amount of nanoparticles. Low availability of phosphorous along with incorporated ZnO and SiO2 nanoparticles can have differing effects on plant growth and yield.

In this study, we aimed to reveal the effects on root system architecture of local wheat varieties of Pakistan along with molecular expression of ethylene and cytokinin signaling in response to phosphorous deficiency and the role of zinc oxide and silicon dioxide nanoparticles in overcoming this phosphorus deficiency by observing and analyzing the

1- Root system architecture (RSA) of SKD-1 and PAK-81 at the physiological level.
2- Transcript level analysis of ethylene biosynthesis and signaling pathway genes along with cytokinin related genes to infer the level of stress induced under phosphorus deficiency and combating effects of nanoparticles facilitating the growth of plants with adaptable nanoparticle efficacy rate at molecular level.

2. MATERIALS AND METHODS

2.1 Seeds Collection

In this study, two varieties of wheat *(Triticum aestivum)* genotypes (SKD-1 and Pak-81) seeds were collected from National Agriculture Research Centre (NARC) Islamabad.

2.1.1 Seeds Sterilization and Stratification

Wheat *(Triticum aestivum)* seeds were sterilized using 30 % of bleach solution and subsequently washed with distilled water for several times until the bleach was fully removed and placed on autoclaved filter paper in a petri plate. For Stratification petri plates were covered with aluminum foil and placed inside refrigerator at 4^0C.

2.2 Preparation of Nanoparticle solution

To make Zinc oxide and silicon dioxide solution, 100ppm of ZnO and similarly 100ppm of SiO_2 were used and the flasks containing these nanoparticles solutions were covered with Aluminum foil. For proper mixing, these flasks were placed for about 3-4 hours in Jeken Ps-10 Ultrasonido Limpiador 2l 80w Sonicator.

2.3 Preparation of Hoagland Solution

Macro and micro nutrient salt solutions were used to prepare Hoagland solution to grow seedlings hydroponically. A required concentration of macro and micro nutrients in known quantity was added to distilled water to prepare Hoagland solution. 1M KH_2PO_4, 1M $MgSO_4$, 1M K_2HPO_4, 1M $CaCl_2$, 40mM H_3BO_3, 1mM Na_2MoO_4, 71mM Fe-DTPA/Fe-HEDTA, 20mM $CuSO_4$, 60mM $MnCl_2$, 6mM $Ca(NO_3)_2$, 20mM $ZnSO_4$ and KNO_3 are concentrations of stock to prepare Hoagland solution. These salts are the source of various macro and micro nutrients like Mg, Ca, Fe, Mn, P, B, Cu, Zn, Mo, N, K.

In this experiment 1L Hoagland solution prepared in two 1L bottles each and 333ml respective nanoparticle solution was added to make final concentration of 1000ml. We used zero (Po) and full phosphorus concentrations (P_F), which were further divided into three subgroups as Control (zero nanoparticles), ZnO (333ml Hoagland solution + 33ml ZnO) and SiO_2 (333ml Hoagland solution + 33ml SiO_2) as shown in the table below.

Table 2.1: Solution Division-Phosphorus and Nanoparticles

Number of Bottles with Hoagland Solution	**Phosphorous**	**Nanoparticles**
1L Bottle A	P_0 No Phosphorus Added Under Phosphorus Stress	Control (No Nanoparticles)
		Zinc Oxide (33ml added)
		Silicon Dioxide (33ml added)
1L Bottle B	P_F Phosphorus Added Under No Phosphorus Stress	Control (No Nanoparticles)
		Zinc Oxide (33ml added)
		Silicon Dioxide (33ml added)

Table 2.2: Hoagland solution composition

Hoagland nutrient solution				
Nutrients		Stock Solution	Hoagland Concentration Solution	For 1 L
Mg	$MgSO_4$	1M	2mM	2ml
K	KH_2PO_4	1M	1mM	1ml
P	K_2HPO_4	1M	1mM	1ml
Ca	$Ca(NO_3)_2$	1M	2mM	2ml
Fe	Fe-DTPA/FeHEDTA	71mM	71μM	1ml
Mn	$MnCl_2$	60mM	10μM	0.166ml
B	H_3BO_3	40mM	50μM	1.25ml
Cu	$CuSO_4$	20mM	6μM	0.3ml
Zn	$ZnSO_4$	20mM	6μM	0.3ml
Mo	Na_2MoO_4	1mM	0.1μM	0.1ml
N	KNO_3 $Ca(NO3)_2$	6mM	5-6μM	3ml
Cl	$CaCl_2$	1M	2mM	5ml
NH_4	NH_4Cl	6mM	5-6μM	5ul

2.4 Wheat Growth Conditions within Test tubes

Etiolated growth of wheat was setup within the Electrothermal Constant Temperature Drying Incubator to provide optimum conditions of temperature. Autoclaved test tubes, filter papers, forceps and scissors were used to minimize any possible contamination. The experiment was done in triplicates, for all the treatments as described earlier to check root architecture; ethylene and cytokinin responses under Po and P_F concentrations alongwith the presence of ZnO and SiO_2 NPs. Optimum temperature of around 25°C and complete dark photoperiod timings were maintained during experimental setup.

2.5 Sample Collection

Wheat plants were taken after 5-6 days of growth. For each variety of wheat *(Triticum aestivum)* root samples were divided into two parts, to use samples for both physiological and molecular analysis.

2.6 Physiological Analysis of Wheat Varieties

After one week of growth, wheat plants were taken for physiological analysis. To check the root architecture, roots were scanned via scanner and then images taken were used for further analysis via ImageJ (ij152-win-java8) software. ImageJ software analyzed different parameters of roots e.g. specific roots length (cm/cm^3) and average root width diameter (cm). In Order to analyze root apical meristem and count number of cells, microscopy of root tips was done by using chloral hydrate solution.

2.6.1 Analysis of Root Architecture of Wheat Plant

Scanned images of roots were taken to analyze root architecture via ImageJ (ij152-win-java8) software. ImageJ Software organized images automatically and is a Java-based image processing program established at the National Institutes of Health and the Laboratory for Optical and Computational Instrumentation. Preprocessing of images were done e.g. Scale set, scale bar, segmentation, rotation, and calibration. As rotation and segmentation of images are standard transformation for images while scale calibration is the most important tool that allows setting of scale in pixels/cm.

These transformations were applied to all images that were earlier imported to software.

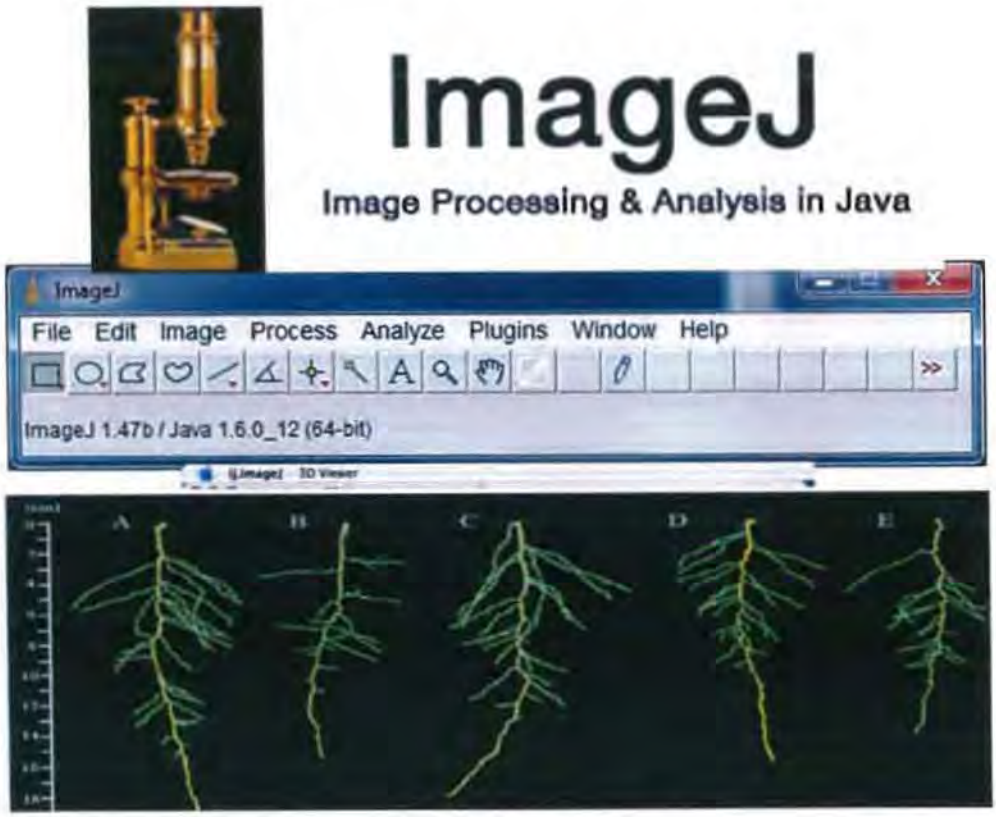


Figure 2.1: ImageJ Software for High Throughput Analysis of Root Architecture System in Plants

Software tools were used to analyze images of root networks. Parameters were set to enable software to identify roots relative to background e.g. Image segmentation, Scale calibration, trait selection. Interested traits were selected for measurement which includes specific roots length and average root width diameter (cm). Software recorded traits estimation in proper units which is the advantage of ImageJ. All of these methods were used separately to analyze images as each algorithm has default values of parameters. ImageJ was directed to compute traits after setting all parameters.

2.6.2 Microscopy of Root Apical Meristem in Wheat

Root apical meristem of wheat roots were examined and analyzed by using chloral hydrate solution. It contains chloral hydrate water and glycerol in (8: 3: 1) ratio. Cortical layer cells were counted in line from quiescent center till length of cell increased using (Olympus, CX41) 10X objective lens was used (Perilli and Sabatini, 2010).

2.6.3 Molecular Analysis of Wheat Varieties

SKD-1 and Pak-81 variety samples were taken for molecular analysis. Relative expressions of ethylene and cytokinin related genes were checked in roots samples of wheat plants under ZnO, SiO_2 nanoparticles and phosphorous stress via PCR (RT-qPCR) technique. For gene expression analysis RNA extraction and quantification was done to synthesize cDNA. Roots cDNA samples were then used for quantitative Real-time PCR (RT-qPCR) to check expression levels of genes.

2.7 Primer Designing

For all primers same procedure was used. Sequences of target genes were obtained from ncbi GeneBank (https://www.ncbi.nlm.nih.gov/genbank/collab/) and then sequences were aligned using ClustalW programme (https://www.ebi.ac.uk/Tools/msa/clustalw2/). Obtained sequences for each target reported in table 2.2.

Table 2.2: List of Genes and Primers Used For This Study

Gene	Forward Primer Sequence	Reverse Primer Sequence	T_m°C
Ta Ub	GGACTACAACATCCA GAAGGA	TTGTGAACCCAGAGA CAGAAG	54.6°C
Ta ACO2	GAGGAACGAGGGCGA GGAG	TCAGTTATCAGGCGG TGG	57.5°C
Ta ERF	GACAGGGAATGGGAC	GCTCAACCAGAGTAG	55°C

	TGATA	TCTTTA	
Ta ERS2	GGAGTCGTCCTTCTTC CCATA	GTGGTGGAAAGTCCA GAGGTTT	54.8°C
Ta CKX2	TGGAGCGGAAGAGAA GTATG	CAGTTGGGCGGGCG TCAATTGATATA G	54.3°C
Ta CKX8	CCAGGAGCTGCTCAT TTCTAA	GAGAAGAGGGGGCG TTGACTT	54.6°C

2.8 RNA Extraction

In this experiment RNA was extracted from wheat (*Triticum aestivum*) roots samples by using Trizole reagent (Cat. No 15596026; Ambion Life Technologies, USA) method. RNA extraction was performed in sterilized environment. 1g of root was taken and grinded in autoclaved pestle mortar using liquid nitrogen. Roots were grinded until it turned into fine powdered form. 1ml of Trizole reagent poured on fine powdered sample in pestle mortar. Sample was then kept for 2-3min until it thaws. After proper mixing sample was poured in 3 eppendorf and 200 µl of chloroform (Cat. No 1.02445.2500;EMSURE) added in each eppendorf which then preceded to vortex for 30 sec. Samples were centrifuged at 12000rpm for 15mins and at 4°C. Supernatant was taken into another eppendorf and 500-700µl of chilled isopropanol added in it. These eppendorfs were then placed horizontally on clean desk for 10 mins at room temperature and preceded to centrifugation at 12000rpm for 8 mins at 4°C. Supernatant was discarded and pellet obtained at bottom of eppendorf which was washed with 70% ethanol twice and then kept for few minutes to get it air dried. Pellet then dissolved in 30 µl of DEPC (Diethylpyrocarbonate) water which is RNAse free water that has the capability to inactivate RNAses and protects RNA. RNA stored at -80°C after extraction (Yin *et al.*, 2016). RNA presence and its quality were checked by running on a 1% agarose gel as mentioned previously. The used conditions were 400mA, 80 V for 45 minutes.

2.8.1 DNAse Treatment

DNAse treatment was done to remove DNA contamination and kit utilized for this treatment was RNAse-free kit (Cat No. EN0521, Fermantas, USA). 8µl of RNA sample was taken then 1µl DNAse I and 1 µl of 10X buffer (Lot No. 00065923, Fermentas, USA) were added to make final volume of 10µl. Solution was incubated at 37°C for 30mins in PCR machine (Biometra, Germany). Then PCR was paused and 1µl of EDTA (25mM) (Lot No. 00058815, Fermentas, USA) was added and PCR was resumed at 65°C for 10 minutes. After this, RNA was quantified.

2.8.2 RNA Quantification

The extracted RNA from Roots sample was quantified by using Nanodrop (Titertek Berthold, Germany) to check purity and concentration of RNA in extracted mixture. Specified programme for RNA extraction was selected on Nanodrop. Nanodrop works on common spectrophotometer principle. Nanodrop first given DEPC treated water to remove contamination on lens. Nanodrop was then blanked with 1µl of DEPC treated water water at 260nm. Nanodrop calibration was done with DEPC treated water as RNA was also stored in DEPC treated water. Blank reading was taken to remove zero error. To get RNA concentration 1µl of sample was placed over Nanodrop lens. RNA concentration was recorded in ng/µl. Single peak was observed which showed pure RNA in extraction solution. RNA concentration values and (A) Ratios noted at 260nm/280nm.

2.8.3 First Strand cDNA Synthesis

For cDNA synthesis Revert aid first strand cDNA synthesis kit (Cat No. K1622, Fermentas, USA) was used. Reagents include 1µg of RNA, 4µl of 5X reaction buffer (Lot No. 00515206; Thermoscientific, USA), 1µl of 100mM oligo dT primer (Lot No. 00525000; Thermoscientific, USA, 2µl of 10mM dNTP mix (Cat. No. R0181, Thermoscientific, USA), 1µl of 200U/L Revert Aid and the volume of reaction mix was made up to 20µl by adding nuclease free water. After DNAse treatment RNA sample volume having 50mM EDTA was added with oligo dT primers making the total volume of 13µl. PCR tubes were used to prepare master mix having 4µl of 5X reaction buffer, 2µl of dNTP mix, 1µl of Revert Aid and 1µl of Ribolock making the

total volume of reaction mix 8µl now. Reaction mix was added to PCR tubes having the previously treated RNA samples making the total volume of 20µl. Then reaction mixture incubated in PCR at 42°C for 60 min. Again, mixture was heated at 70°C for 5 min. Incubation were given in Thermocycler (Biometra-2005, Germany). Then cDNA was stored at -20°C.

2.8.4 Quantitative (qRT) PCR Amplification

For relative genes quantification real time PCR was performed on cDNA samples. My Go pro real-time PCR Thermo cycler (IT-IS International Ltd, UK) machine was used for this amplification. For each primer set qRT PCR efficiency was determined by using pure 2µl cDNA. Each dilution was amplified against each primer with conditions and reagents in table 2.3 and 2.4 respectively. Then, qRT PCR was performed against different primer set with Zero and full phosphorous concentration alongwith ZnO and SiO_2 nanoparticles samples with conditions and reagents of Table 2.3 and 2.4. Reagents used for (qRT) PCR include Maxima Cyber green/ROX PCR master mix (Cat No. K0221; Thermoscientific, USA), primers (forward+reverse), nuclease free water and template cDNA. Reaction cocktail 1 of cyber green and nuclease free water made in 1.5ml PCR tube. Reaction cocktail 2 includes nuclease free water and sample diluted primers. Then a master mix of 8 µl of these both cocktails and 2 µl of cDNA was added in each PCR strip tubes (Cat No. P-01X8-F; Extragene, Thaiwan). Tubes then were closed and placed in MyGo pro Thermocyler.

Table 2.3: Reagents used in qRT-PCR

	Serial no	Reagents Used un (qRT) PCR	Quantities (µl)
Cocktail 1	1	Syber green maxima	5 µl
	2	Nuclease free water	2 µl
Cocktail 2	3	Gene specific forward+reverse primer	1 µl

	4	Cdna	2 µl
		Total reaction volume	**10 µl**

Table 2.4: Conditions for qRT-PCR

S/No.	Steps		Incubation Temperature	Time	Ramp (°C/s)	acquire	Cycles
1	Hold		95°C	600 sec	4	-	-
2	3 Step amplification		95°C	10sec	5	-	45
			58 °C	45 sec	4	-	
			72°C	17 sec	5	Yes	
3	Pre-melt hold		95°C	15sec	5	-	-
4	High Resolution melting	Initial	58°C	60 sec	4	-	-
		Final	95°C	15sec	0.05	-	

2.8.4.1 Data Analysis Delta Ct Method

To analyze qrt-PCR data pfafti method (Pfaft1. 2001) were used. To check the changes in Fold change of gene expression following formula were used:

$$Ratio = \frac{(E_{target})^{\Delta CP_{target(control-sample)}}}{(E_{ref})^{\Delta CP_{ref(control-sample)}}}$$

Δ= delta= represent difference between two values

CP=PCR cycles= represent no of cycles where fluorescent signal density detectable

The REST-384 version 2 (Qiagen, USA) was used for this analysis.

3. RESULTS

Nutrients are essentially required by plants for proper development, growth and nourishment. Phosphorous is one of the basic nutrients that play a significant role in plants growth, root development, ripening, early flowering and its deficiency in wheat is one of the growth limiting factors. Phosphorous deficiency influenced the growth of roots as it reduces the primary root length, root density and root biomass. Appropriate concentration of phosphorous in considerable amount elongates primary root length that has been observed in two varieties of wheat. Nanoparticles also have different effects on wheat with ZnO showing a positive effect in deficiency elongating root length. We investigated phosphorous stress alongwith ZnO and SiO_2 nanoparticles effects on root architecture of wheat varieties. At microscopic levels effect of phosphorous deficiency, effect of Zinc oxide and Silicon dioxide nanoparticles via ethylene suppression on division zone of apical meristem of root was analysed in wheat varieties.

Additionally, transcript analysis of ethylene biosynthesis and signaling pathway genes in selected varieties were carried out to observe interactions of ethylene signaling pathway with phosphorous deficiency and nanoparticles efficacy. Our data provided a comprehensive comparison at physiological as well as at molecular level. Two wheat varieties (SKD-1 and Pak-81) were taken from National Agriculture Research Center (NARC) Islamabad. Seeds of both SKD-1 and Pak-81 varieties were grown hydroponically in nutrient solution with no or full concentrations of phosphorous alongwith nanoparticles as tabulated and shown in Table 2.1

We observed and recorded physiological parameters of roots growth under given conditions i.e. Specific root length and average root width diameter. These root parameters were analysed by ImageJ Software tool that facilitated root architecture analysis. Molecular analysis was carried out on two of the wheat varieties and they were graphically analysed for further authentication.

3.1 Differences in Wheat Root Architecture

Both the aspects of Nanoparticles and phosphorus stress were observed in the variety of wheat crop plant.

3.1.1 SKD-1

Wheat (SKD-1) variety seedlings showed significant differences in primary root length (cm/cm3) and root width (cm) etc. Figure 3.1 illustrates the effect of ZnO and SiO_2 nanoparticles on SKD-1 seedlings i.e. A, B, C control having no nanoparticles , D, E, F Zinc oxide nanoparticles and G, H, I Silicon Dioxide nanoparticles. All this effect is under phosphorus deficiency with no phosphorus added (Po). An increase in length with efficient growth was observed in the roots grown under zinc oxide nanoparticle. Control showed a moderate effect with median root length under the absence of nanoparticles. However, under the addition of silicon dioxide nanoparticles, the roots showed a contrasting behavior with decrease in their length at physiological level.

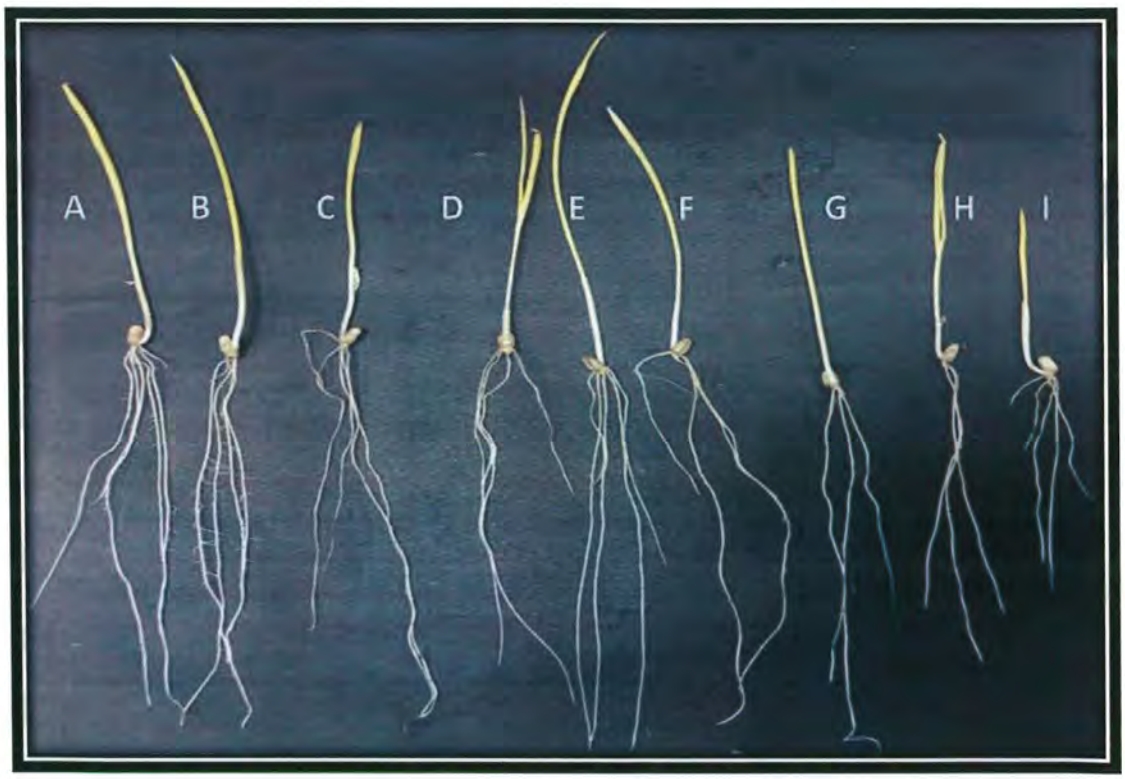


Figure 3.1: Effect of Nanoparticles on Wheat (SKD-1) Variety Root Architecture under Po (Phosphorus absent) with increase in length under zinc oxide and decrease in length under Silicon Dioxide nanoparticles.

Similarly Figure 3.2 below shows the condition in which phosphorus is present (P_F). When a comparison was observed in both conditions for P_o and $P_{F,\ it}$ was found that root length decreased in Po while increased under P_F . However, seedlings grown with Zinc oxide nanoparticles have shown increase in length in both P_o and P_F while Silicon Dioxide has shown a decrease in length.

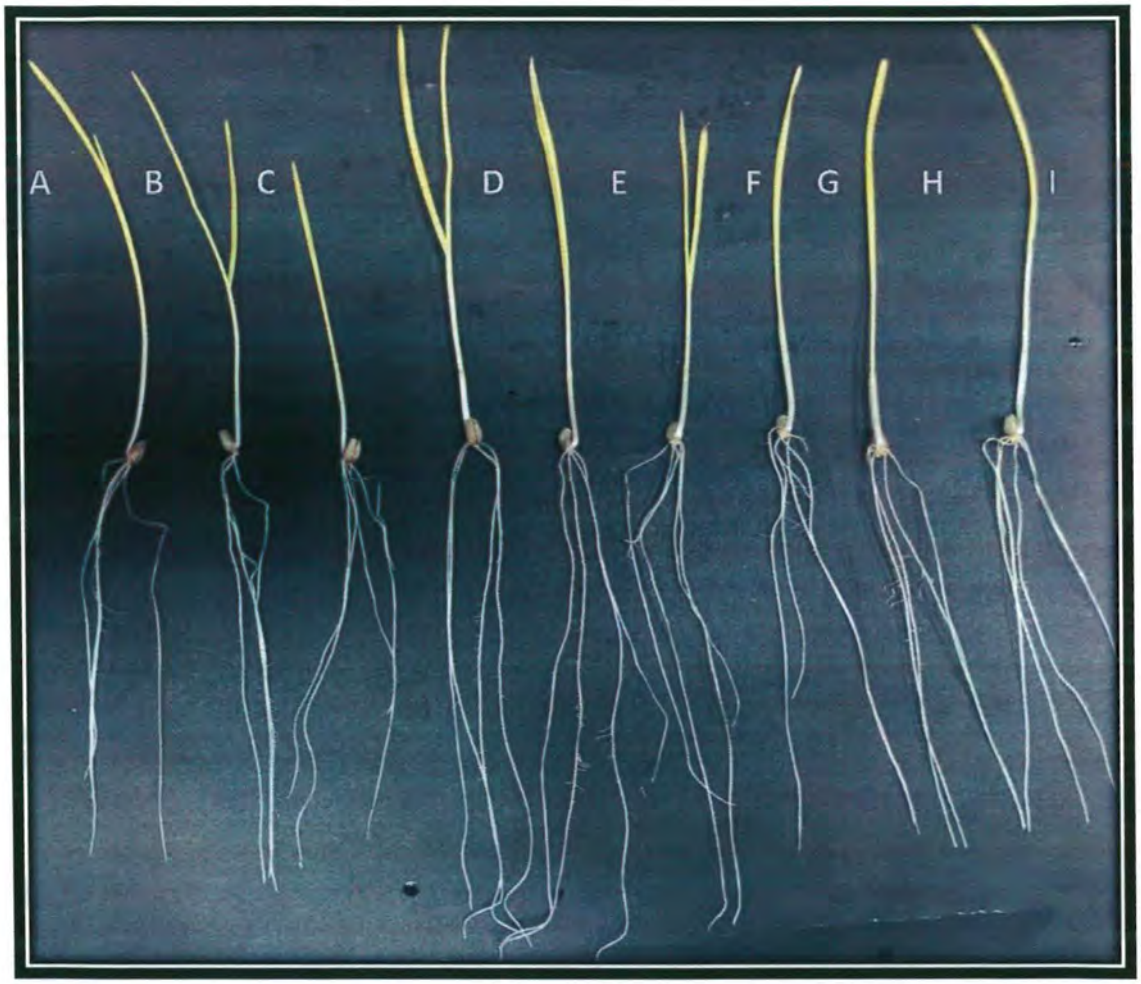


Figure 3.2: Effect of Nanoparticles on Wheat (SKD-1) Variety Root Architecture under P_F (Phosphorus present). More increase in length was observed as compared to Po however nanoparticles showed a similar effect under both stresses.

3.1.2 PAK-81

Wheat (PAK-81) variety seedlings exhibited important alterations in primary root length (cm/cm3) and root width (cm) etc. In below figures A, B, C is control having no nanoparticles. D, E, F Shows Zinc oxide nanoparticles and G, H, I show Silicon dioxide nanoparticles. It was observed that seedlings grown in presence of ZnO NPs showed increase in root lengths while SiO_2 nanoparticles displayed decrease in length.

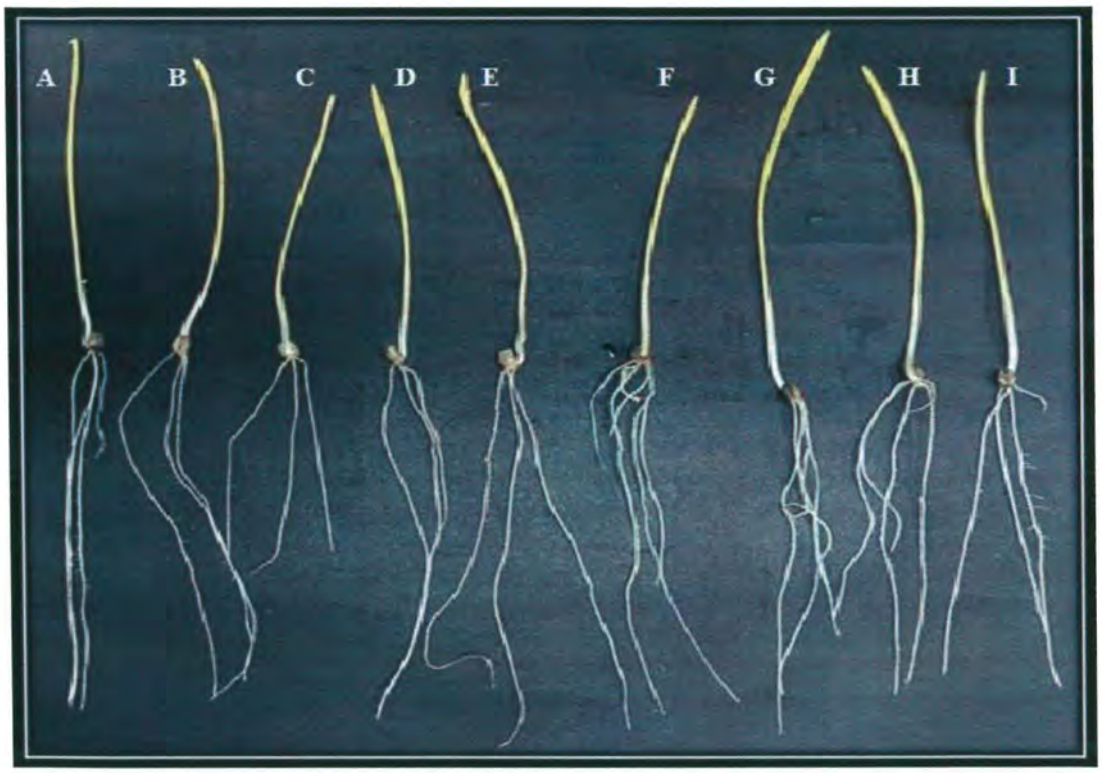


Figure 3.3: Effect of Nanoparticles on Wheat (PAK-81) Variety Root Architecture under Po (Phosphorus absent) with increase in length under zinc oxide and decrease in length under Silicon Dioxide nanoparticles.

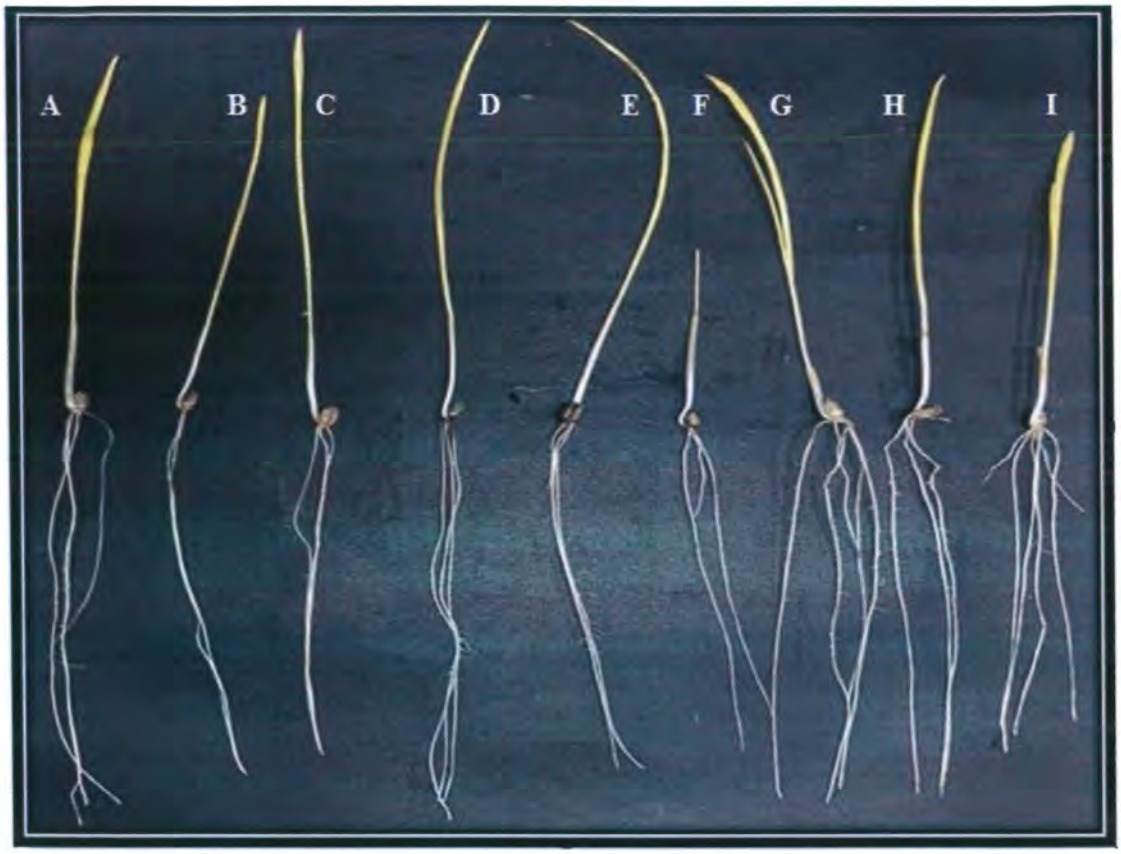


Figure 3.4: Effect of Nanoparticles on Wheat (PAK-81) Variety Root Architecture under P_F (Phosphorus present). More increase in length was observed as compared to Po however nanoparticles showed a similar effect under both stresses.

When a comparison was observed in both conditions for P_o and P_F it was found that root length decreases in Po while increases when phosphorus is available . However, the sample with Zinc Oxide nanoparticles has shown increased in length in both P_o and P_F and Silicon Dioxide has shown a decrease in length. That will be validated further with graphical analysis in the section 3.3.

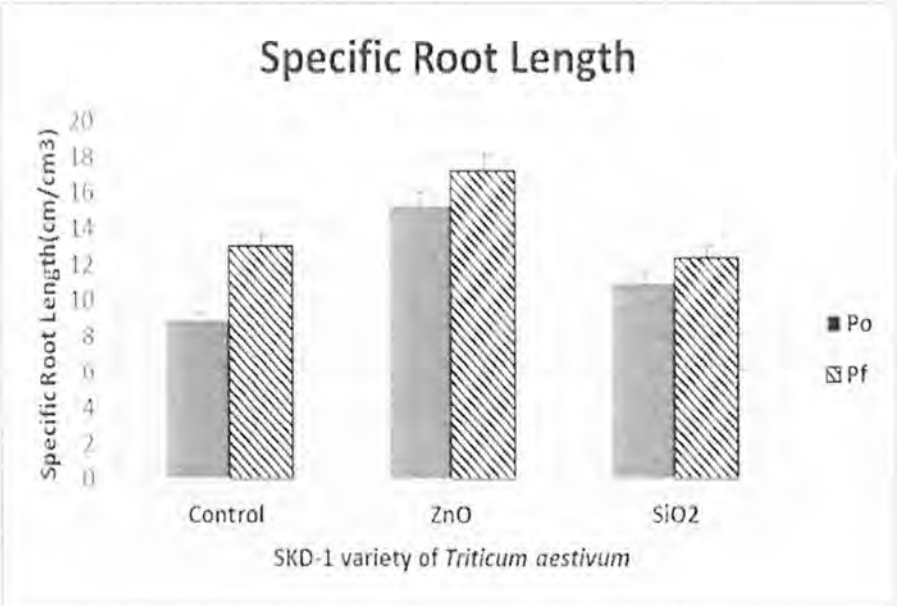
3.2 Intervarietal Differences of Wheat Root Architecture in Response to Nanoparticles and Phosphorous Deficiency

ImageJ software used for statistical analysis of roots images grown on control, phosphorous stress, ZnO and SiO_2 nanoparticles. Different parameters of roots e.g. specific roots length (cm/cm3) and average root width diameter (cm) were

analyzed. ImageJ analysis of roots shows significant changes in root architecture of treated samples in comparison with control samples.

3.3 Specific Root Length

Data analyzed by ImageJ software tool showed changes in specific root length of varieties used in this study under ZnO, SiO_2 nanoparticles and Po, P_F phosphorous treatments. Both varieties responded to phosphorous starvation differently. Specific root length (cm/cm^3) of SKD-1 showed inhibition of primary root (PR) length in phosphorous starved (P_o) plants while other variety Pak-81 showed slight decreases in root length under phosphorous concentration stress. It shows that both varieties adapt themselves to the environment by making changes in root architecture which includes changes in specific root length (cm/cm^3). Under nanoparticles inclusion, ZnO Showed the most increase in length in both the varieties and specific root length shows significant decrease in absence of phosphorous as compared with roots of plants grown in P_F under full phosphorous concentration. The highest recorded value of specific root length was recorded in SKD-1 variety under phosphorus and in the presence of zinc oxide nanoparticles (16.91 cm/cm^3) grown under full phosphorous (P_F) concentration while lowest value recorded was observed under control in phosphorus stress (8.10 cm/cm^3) as shown in figures below.



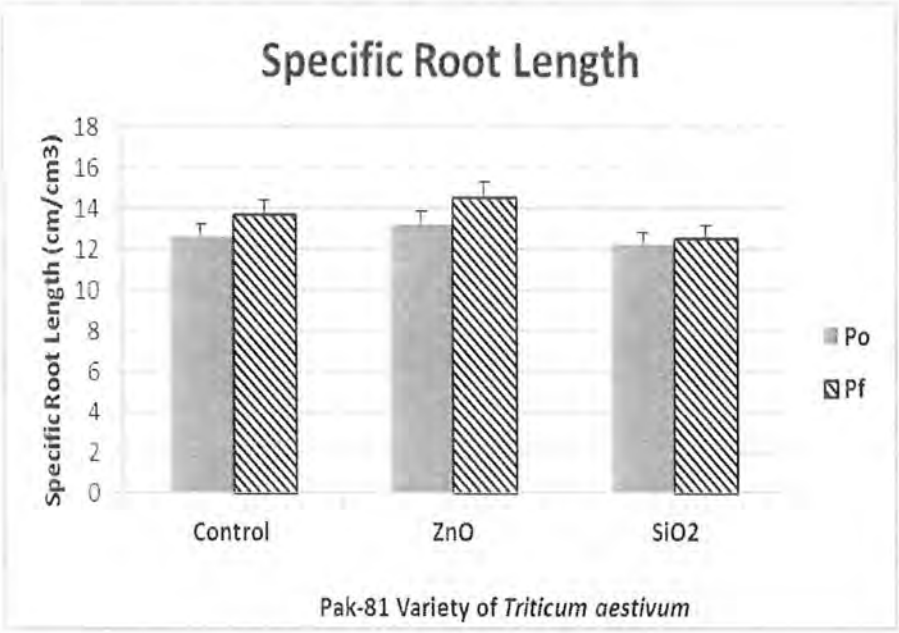
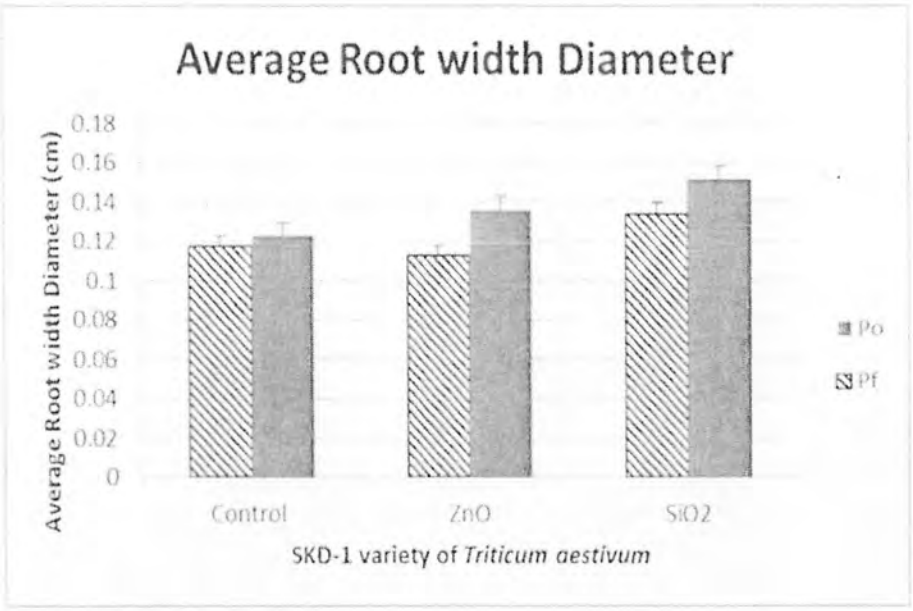


Figure 3.5 Intervarietal Differences of Specific Root Length (cm/cm^3) In Wheat (*Triticum aestivum*) Varieties Grown under Different Phosphorous Concentrations in the presence of nanoparticles. In above figure lined and grey bars represent wheat varieties grown under Full Phosphorus (P_F) and no or zero phosphorous (P_0) concentration. In both of the above figures, Y-axis represents values for specific root length in cm/cm^3 while X-axis represents wheat varieties (SKD-1 and Pak-81). Graph representing mean ± SE values for twice replicates of each treatment.

3.4 Average Root Width Diameter

Average root width diameter is the mean value of root width. Data obtained by ImageJ Software analysis of root images showed that root width increases in absence of phosphorous. Roots of seedlings grown on zero or no phosphorous (P_0) showed increase in root width as compared with seedlings grown in full phosphorous (P_F) concentrations. Width of seedlings grown on phosphorous starved (P_0) conditions was more than seedlings grown in full phosphorous (P_F) concentration or in normal phosphorous concentrations. The Highest recorded average value for root width was from SKD-1 variety (0.15 cm) grown under phosphorous full (P_F) conditions in the presence of SiO_2 Nanoparticles. The lowest recorded average value of root width diameter was from PAK-81 variety (0.085 cm) under Po in the presence of ZnO Nanoparticles. (Figure 3.5).



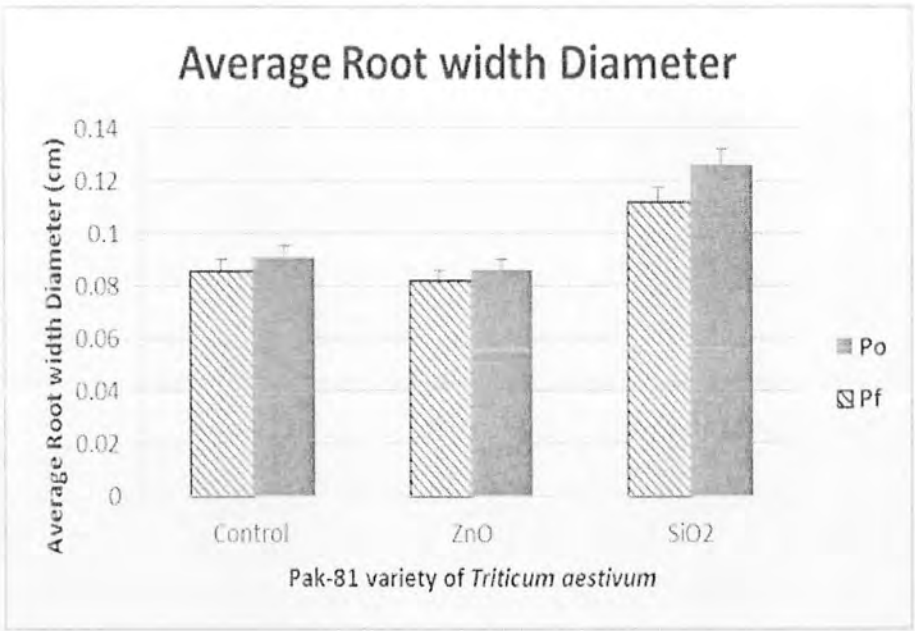


Figure 3.6 Intervarietal Differences Of Average Root Width Diameter (cm) In Wheat (*Triticum aestivum*) Varieties Grown under Different Phosphorous Concentrations in the presence of nanoparticles In above figure lined and grey bars

represent wheat varieties grown under Full Phosphorus (P_F) and no or zero phosphorous (P_0) concentration. In both of the above figures, Y-axis represents values for Average root width diameter (cm) while X-axis represents wheat varieties (SKD-1 and Pak-81). Graph representing mean ± SE values for twice replicates of each treatment.

3.5 Microscopic Analysis of Wheat Root Apical Meristem

Modifications in root architecture in response to phosphorous deficiency occur due to changes at cellular level in apical meristem of primary root. These modifications influenced by interactions of several hormones and nutrients. Microscopic analysis of cell count in the root apical meristem of PR (primary root) showed that cell number decreases in roots apical meristem of both wheat varieties grown under phosphorous starved (P_0) conditions as compared with plants grown under full phosphorous (P_F) concentration. As root elongation occurs due to flux of newly formed cell from division zone to elongation zone in root apical meristem. Phosphorous starvation reduces the elongation of root by decreasing the rate of cell division hence reducing the cell number in elongation zone. Numbers of cells were observed under both the above mentioned phosphorus conditions alongwith presence of control, zinc oxide and silicon dioxide nanoparticles.

3.5.1 SKD-1

Figure 3.7 Effect of Nanoparticles and Phosphorus stress on Apical Meristem of Root in SKD-1: Microscopic analysis of cell count in wheat variety (SKD-1) grown under full phosphorous (P_F) and Po or zero phosphorous concentrations. A: Wheat seedlings grown under control without any nanoparticles and the number of cells were the average as compared to presence of nanoparticles. B: Wheat seedlings grown under Zinc oxide nanoparticles concentration showed increase in cell count in roots. C: Wheat seedlings grown under Silicon dioxide concentration showed the minimum number of cell count among the three conditions. When an analysis was done on the basis of stress under phosphorus concentrations it was observed that the root cells grown under phosphorus stress had decrease number of cell count as compared to when phosphorus was fully available.

3.5.2 Pak- 81

Figure 3.8 Effect of Nanoparticles and Phosphorus stress on Apical Meristem of Root in Pak-81: Microscopic analysis of cell count in wheat variety (Pak-81) grown under full phosphorous (P_F) and Po or zero phosphorous concentrations. A: Wheat seedlings grown under control without any nanoparticles and the number of cells were the average as compared to presence of nanoparticles. B: Wheat seedlings grown under Zinc oxide nanoparticles concentration showed increase in cell count in roots. C: Wheat seedlings grown under Silicon dioxide concentration showed the minimum number of cell count among the three conditions. When an intervarietal comparison was made it was found that SKD-1 had an increase number in cell count in comparison to Pak-81. Analysis was also done on the basis of stress under phosphorus concentrations and it was observed that the root cells grown under phosphorus stress had decrease number of cell count as compared to when phosphorus was fully available.

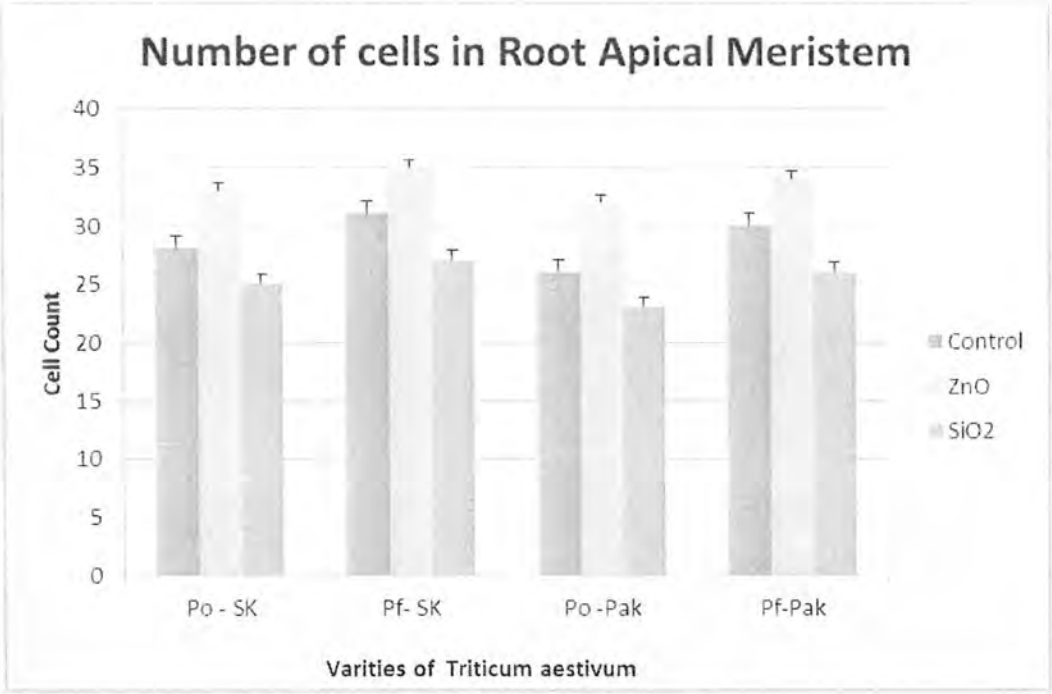


Figure 3.9 Cell Count Trend in Root Apical Meristem under Phosphorous and Nanoparticles stress in Wheat (*Triticum aestivum*) Varieties Bar graph shows cell count of wheat varieties grown under different phosphorous conditions alongwith control, zinc oxide and silicon dioxide nanoparticles. As graphically analyzed, cell counts was increased in zinc oxide and decreased under silicon dioxide nanoparticles. While in repose to phosphorus, Number of cells were low under phosphorus stress (Po) as compared to when phosphorus was fully available (P_F). Results are presented in mean ± SEM. Error bars.

3.6 Extraction of Good Quality RNA

In order to check the expression of ethylene related and cytokinin related genes, Wheat root samples were preceded for RNA extraction. RNA was extracted from 12 samples of both the wheat varieties under control, zinc oxide and silicon dioxide nanoparticles alongwith different phosphorus concentrations (Po and P_F). These samples were then run on 1% agarose gel. RNA bands were observed by Gel documentation system.

Figure 3.10 Agarose Gel of Wheat Varieties RNA from Roots Samples: Gel image shows RNA bands extracted from Wheat (*Triticum aestivum*) roots.

3.7 Transcript Analysis of Ethylene Biosynthesis and Signalling Pathway Genes

Ethylene is a gaseous hormone and plays an important role in plants development and growth. In roots samples relative expression of ethylene biosynthesis and signaling genes including TaACO2, TaERF and TaERS2 were checked under phosphorous treatments for Po and P_F. Also the inclusion of zinc oxide and silicon dioxide nanoparticles compared to control (having no nanoparticles) under phosphorus stress were analyzed for ethylene related genes. The enzyme that is directly involved in ethylene biosynthesis is 1-aminociclopropane-1-acid carboxylic oxidase (ACO or EFE - ethylene forming enzyme; EC 1.14.17.4), which transforms ACC into ethylene hormone (Hegg EL, 1997). Ethylene signal transduction activates considerable variations in the gene expression of plant cells. Analysis of Promoter region of the genes induced by ethylene directed to the identification of cis-acting elements and the trans-acting protein EREBP (ethylene responsive element binding protein) family, which interacts with ERFs (ethylene response factors) DNA (Leubner-Metzger et al., 1998).

Transcript levels of ethylene biosynthesis genes TaACO2, TaERF and TaERS2 were increased significantly under phosphorus deficiency i.e. Po concentration. However lower expression of ethylene biosynthesis genes were observed when phosphorus was available in full concentration without any deficiency i.e. P_F condition. Relative expression of TaACO2 increases 2.5 fold under the presence of silicon dioxide nanoparticles in root samples as compared to control. While expression level for TaERF and TaERS2 increases greater than 2 fold under the presence of silicon

dioxide nanoparticles as compared to control. Relative expression of TaACO2 and TaERF genes decreases in the presence of zinc oxide nanoparticles under both Po and Pf concentrations compared to control as an intermediate between the expressions of genes increasing under the presence of silicon dioxide nanoparticles. When relative intervarietal expression of genes was observed it was found that the genes in SKD-1 wheat variety showed more response to the stress under phosphorus deficiency and in the presence of nanoparticles as compared to PAK-81 wheat variety (Figure 3.11-3.16).

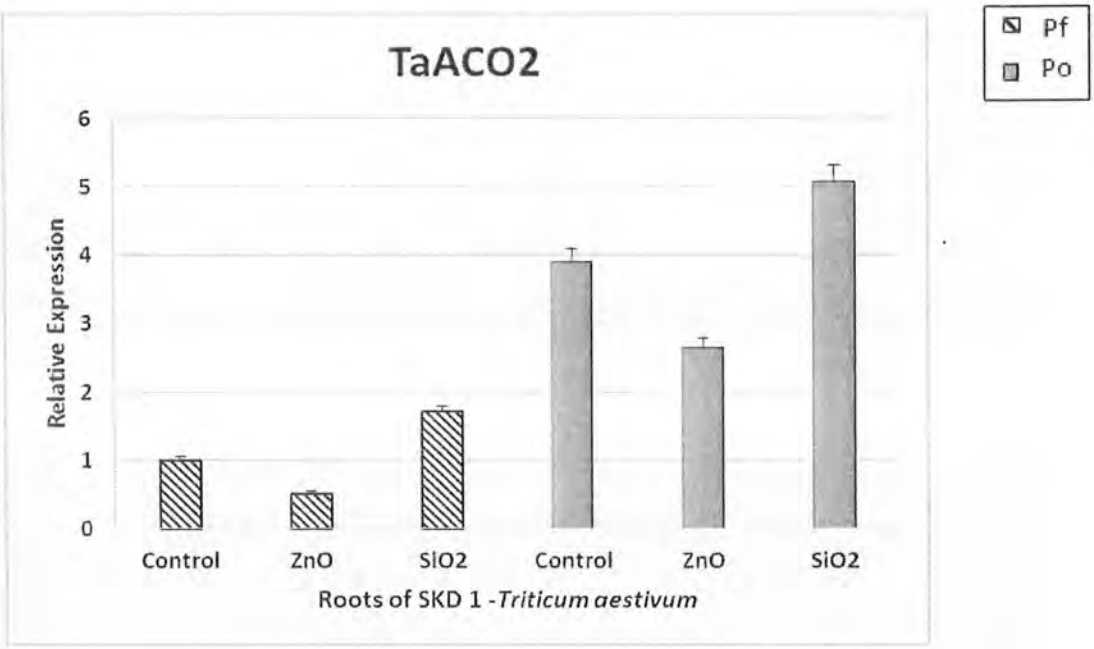


Figure 3.11: Relative Expression of TaACO2 gene in Wheat (SKD-1) Roots Grown under Phosphorous (Po and P_F) Concentrations alongwith presence of Nanoparticles. Data was normalized with housekeeping gene Ta Ubiquitin. Results show almost 2 fold increase in the TaACO2 gene expression under phosphorous starved (Po) condition as compared to P_F. With the most expression observed under silicon dioxide nanoparticles and least in zinc oxide nanoparticles. Results are presented in mean ± SEM shown with error bars.

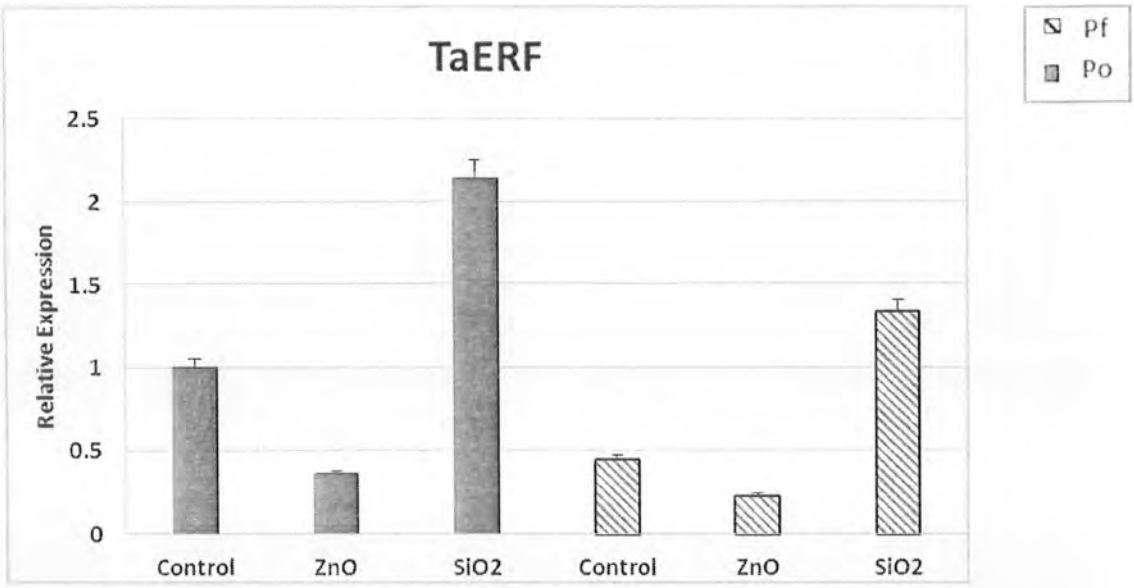


Figure 3.12: Relative Expression of TaERF gene in Wheat (SKD-1) Roots Grown under Phosphorous (Po and P_F) Concentrations alongwith presence of Nanoparticles. Data was normalized with housekeeping gene Ta Ubiquitin. Results show almost 2.5 fold increase in the TaERF gene expression under phosphorous starved (Po) condition as compared to P_F. With the most expression observed under silicon dioxide nanoparticles and least in zinc oxide nanoparticles. Results are presented in mean ± SEM shown with error bars.

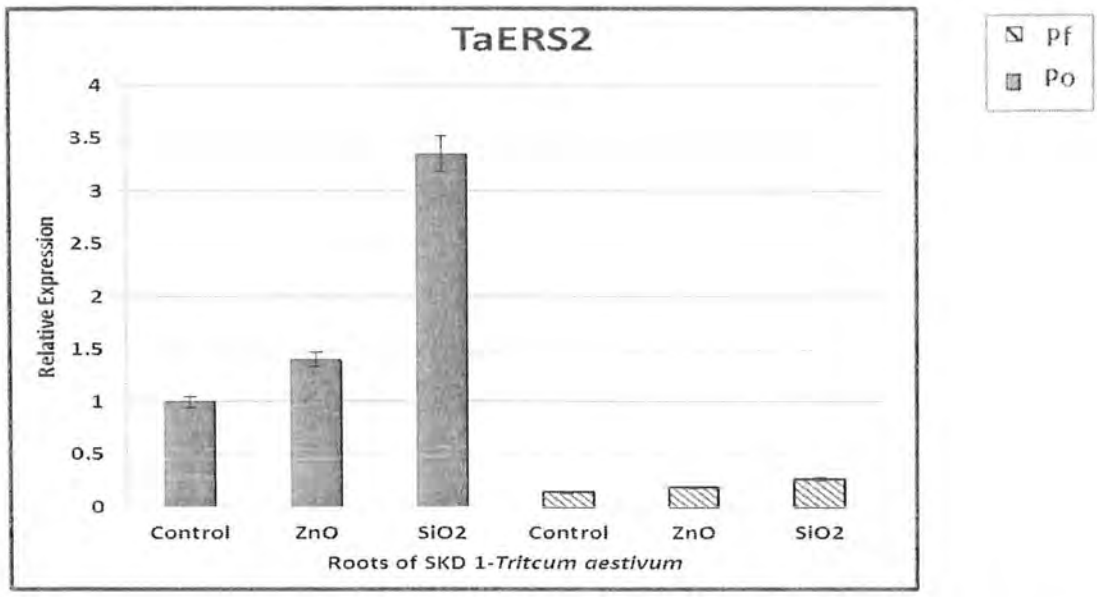


Figure 3.13: Relative Expression of TaERS2 gene in Wheat (SKD-1) Roots Grown under Phosphorous (Po and P_F) Concentrations alongwith presence of Nanoparticles. Data was normalized with housekeeping gene Ta Ubiquitin. Results show almost 3-4 fold increase in the TaERS2 gene expression under phosphorous starved (Po) condition as compared to P_F. With the most expression observed under silicon dioxide nanoparticles and least in control. Results are presented in mean ± SEM shown with error bars.

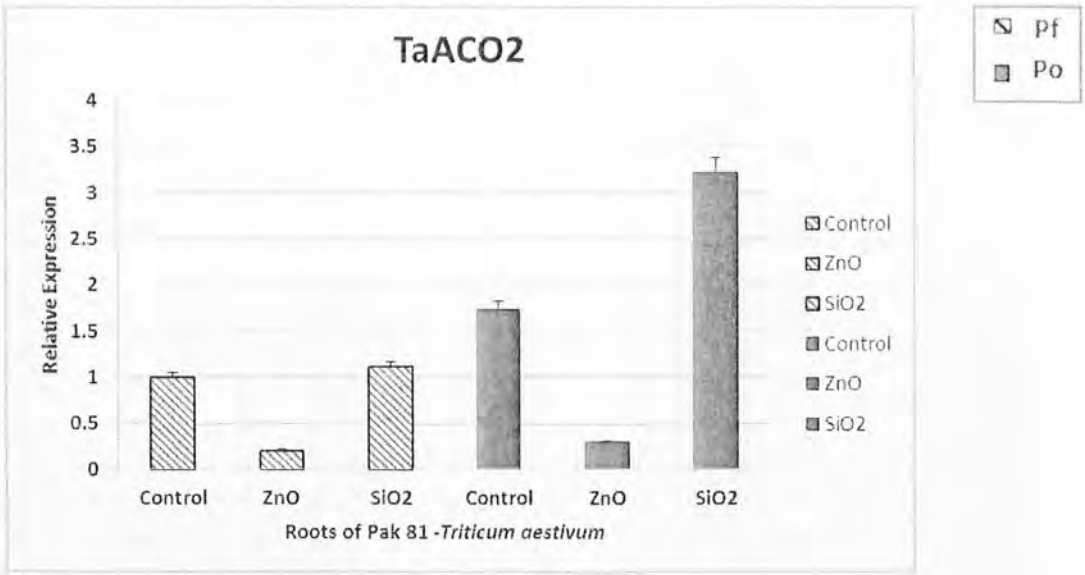


Figure 3.14: Relative Expression of TaACO2 gene in Wheat (Pak-81) Roots Grown under Phosphorous (Po and P_F) Concentrations alongwith presence of Nanoparticles. Data was normalized with housekeeping gene Ta Ubiquitin. Results

show almost 2.5 fold increase in the TACO2 gene expression under phosphorous starved (Po) condition as compared to P_F. With the most expression observed under silicon dioxide nanoparticles and least in zinc oxide. Results are presented in mean ± SEM shown with error bars.

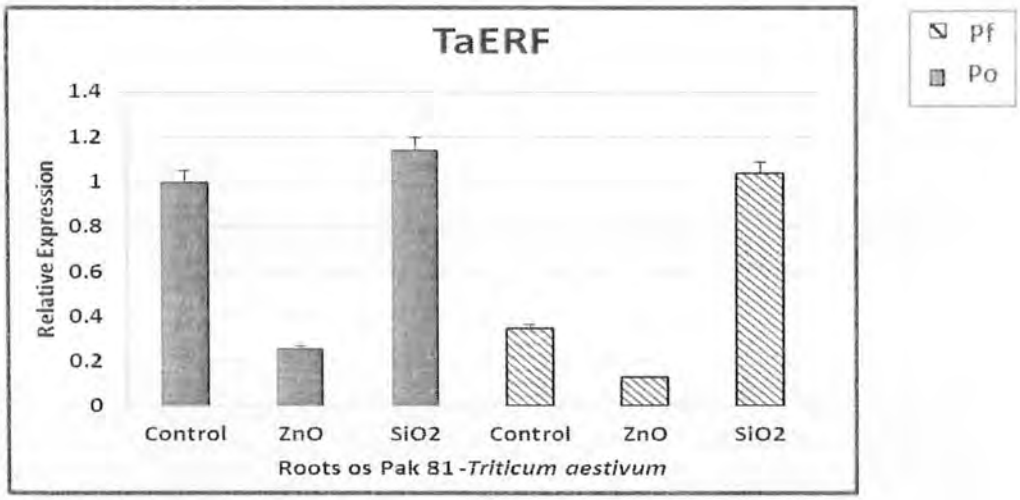


Figure 3.15: Relative Expression of TaERF gene in Wheat (Pak-81) Roots Grown under Phosphorous (Po and P_F) Concentrations alongwith presence of Nanoparticles. Data was normalized with housekeeping gene Ta Ubiquitin. Results show almost 0.5 fold increase in the TaERF gene expression under phosphorous starved (Po) condition as compared to P_F. With the most expression observed under silicon dioxide nanoparticles and least in zinc oxide. Results are presented in mean ± SEM shown with error bar.

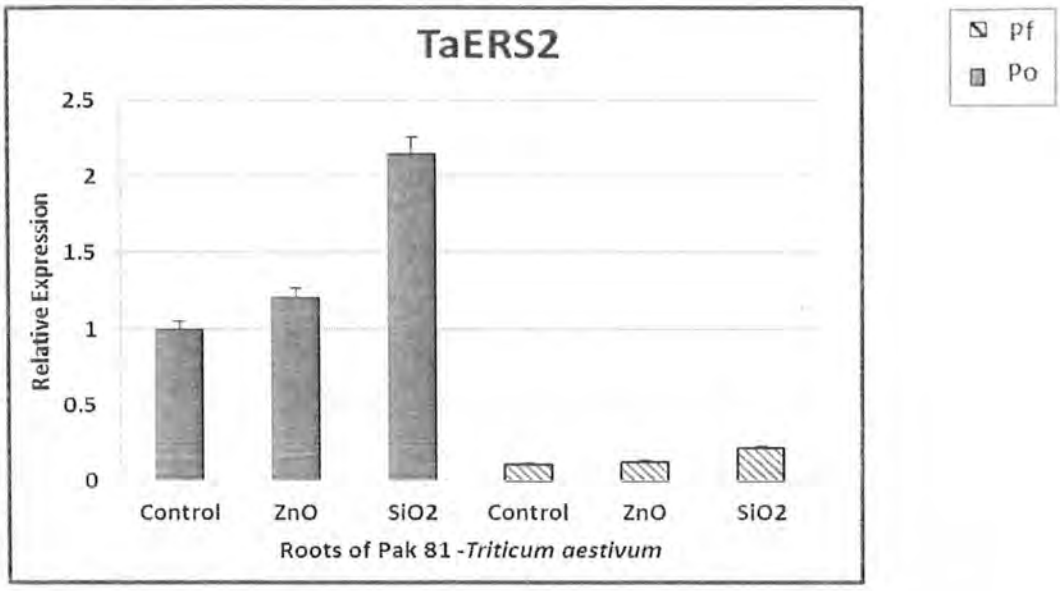


Figure 3.16: Relative Expression of TaERS2 gene in Wheat (Pak-81) Roots Grown under Phosphorous (Po and P_F) Concentrations alongwith presence of Nanoparticles. Data was normalized with housekeeping gene Ta Ubiquitin. Results show almost 3-4 fold increase in the TaERS2 gene expression under phosphorous starved (Po) condition as compared to P_F. With the most expression observed under silicon dioxide nanoparticles and least in control. Results are presented in mean ± SEM shown with error bar.

3.8 Transcript Analysis of Cytokinin Signalling Pathway Genes

Expression levels of Cytokinin genes TaCKX2 and TaCKX8 were checked under phosphorous treatments for Po and P_F. Also the inclusion of zinc oxide and silicon dioxide nanoparticles in response to control (having no nanoparticles) under phosphorus stress were analyzed for ethylene related genes. Transcript levels of TaCKX2 and TaCKX8 were decreased significantly under phosphorus deficiency in Po concentration. However higher expression of ethylene biosynthesis genes were observed when phosphorus was available in full concentration without any deficiency in P_F condition in both the varieties. Whereas under the inclusion of nanoparticles; both the genes showed different trends. TaCKX2 gene expression was increased in the presence of zinc oxide nanoparticles and showed least expression under control

(without nanoparticle) whereas TaCKX8 gene expression was increased when no nanoparticles was available and decreased in the presence of silicon dioxide nanoparticles whereas silicon dioxide showed an intermediate response (Figure 3.17-3.20).

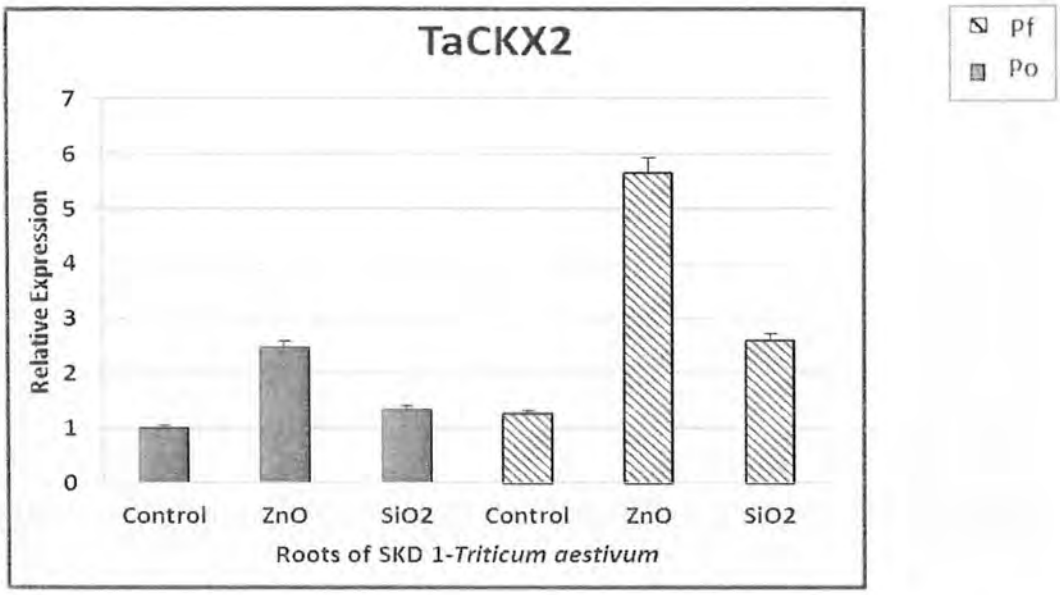


Figure 3.17: Relative Expression of TaCKX2 gene in Wheat (SKD-1) Roots Grown under Phosphorous (Po and P_F) Concentrations alongwith presence of Nanoparticles. Data was normalized with housekeeping gene Ta Ubiquitin. Results show decrease in the TaCKX2 gene expression under phosphorous starved (Po) condition as compared to P_F. With the most expression observed under zinc oxide nanoparticles and least in control. Results are presented in mean ± SEM shown with error bar.

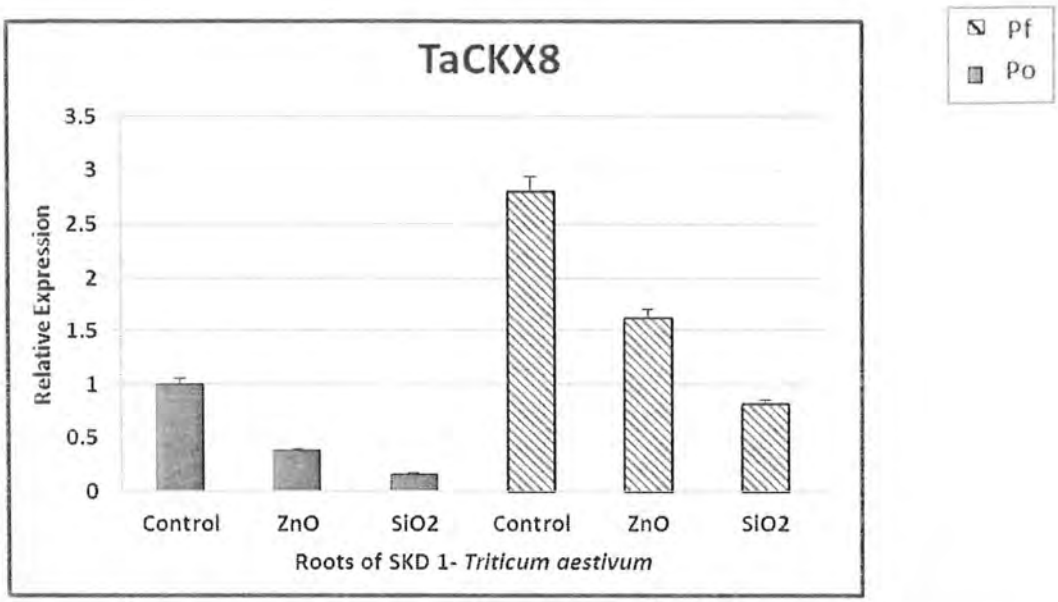


Figure 3.18: Relative Expression of TaCKX8 gene in Wheat (SKD-1) Roots Grown under Phosphorous (Po and P_F) Concentrations alongwith presence of Nanoparticles. Data was normalized with housekeeping gene Ta Ubiquitin. Results show almost 2.5 fold decrease in the TaCKX8 gene expression under phosphorous starved (Po) condition as compared to P_F. With the most expression observed under control and least in silicon dioxide. Results are presented in mean ± SEM shown with error bar.

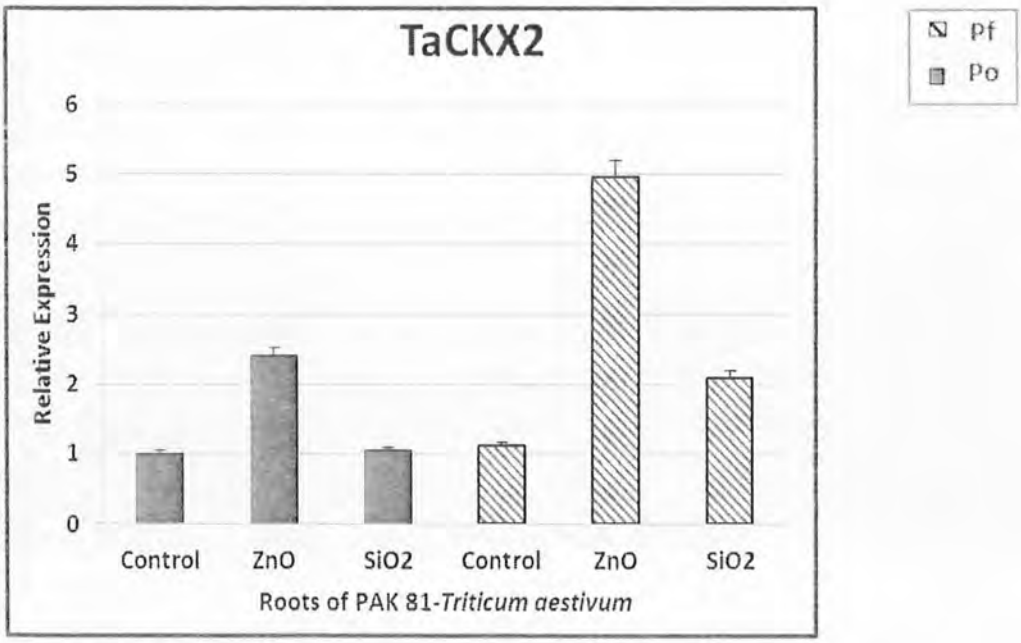


Figure 3.19: Relative Expression of TaCKX2 gene in Wheat (PAK-81) Roots Grown under Phosphorous (Po and P_F) Concentrations alongwith presence of Nanoparticles. Data was normalized with housekeeping gene Ta Ubiquitin. Results show decrease in the TaCKX2 gene expression under phosphorous starved (Po) condition as compared to P_F. With the most expression observed under zinc oxide nanoparticles and least in control. Results are presented in mean ± SEM shown with error bar.

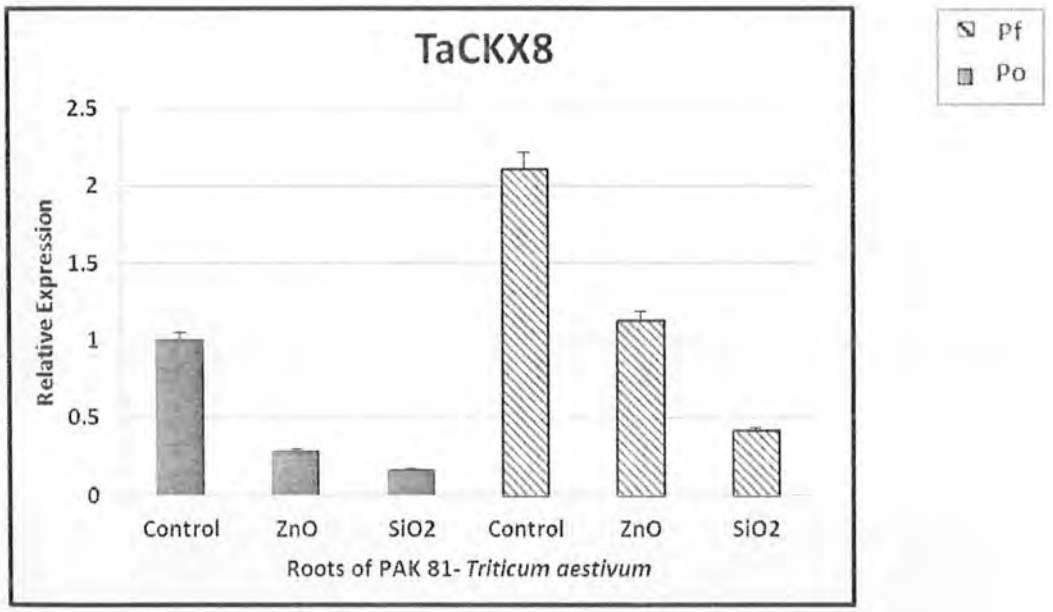


Figure 3.20: Relative Expression of TaCKX8 gene in Wheat (PAK-81) Roots Grown under Phosphorous (Po and P_F) Concentrations alongwith presence of Nanoparticles. Data was normalized with housekeeping gene Ta Ubiquitin. Results show almost 2 fold decrease in the TaCKX8 gene expression under phosphorous starved (Po) condition as compared to P_F. With the most expression observed under control and least in silicon dioxide. Results are presented in mean ± SEM shown with error bar.

4. DISCUSSION

Wheat is one of the world's most important cereal crops and as it contributes to crucial and essential vitamins, amino acids, minerals, valuable phytochemicals and dietary fiber components to the human diet so it's productivity holds an utmost importance providing all the essential nutrients. Due to costly fertilizers and overgrowing population it will be beneficial for farmers to select varieties that are more stress tolerant and can survive in nutrient deficient soil. The present study was conducted to check root architecture of wheat (*Triticum aestivum)* varieties (SKD-1 and PAK-81) and expression of ethylene biosynthesis and signaling related genes under phosphorous deficient conditions in the presence of nanoparticles. In addition to this expression level of cytokinin related genes were also analyzed in the conditions mentioned above. Seeds were grown in hydroponic systems with Po (phosphorus absent) and P_F (phosphorus present) alongwith zinc oxide and silicon dioxide nanoparticles.

After 5-7 days of growth *Triticum aestivum* mature plant roots were taken and analyzed by ImageJ (ij152-win-java8) software. As the roots are present beneath the soil surface and has an important character in structure and function of plants so they hold a significant importance. Plant root requires inorganic phosphate (Pi), which is not easily accessible. Soil phosphates are the only source of phosphorous for proper growth and development of roots. Less availability of phosphorous effects the plant health and crop yield. To cope up with nutrient deficient environment plant adapts several strategies by changing root architecture. These morphological and physiological changes in root architecture make plant to explore more soil. Soil exploration in search of phosphorous helps plant to survive (Jiang *et al.*, 2007). Roots proper development is important to fulfill the requirements of water, nutrients and to provide anchorage in the soil. They provide lifelong biological interaction between plant and microbiota either by facilitating the plant or assisting the chemical defenses underneath (Galkovskyi *et al.*, 2012).

ImageJ software tool is used in the current study for analysis of root architecture. Primary root length is an important parameter to study the changes in root architecture

under phosphorous deficient environment. Phosphorous deficiency effect the length of primary root. In *Arabidopsis* roots showed inhibition of primary root (PR) under phosphorous starved conditions (Niu *et al.*, 2013). Inhibition of primary root occurs due to decrease in cell number at apical meristem. Roots grown under phosphorous (P_F) concentration showed that root length increased while phosphorous deficient (Po) plant roots were shortened. ImageJ analysis of roots confirmed that phosphorous deficient plant roots have more lateral roots and root hairs increases surface area of roots so they can explore the soil more in order to cope up with the phosphorous deficient environment. Two grown varieties behave differently to phosphorous deficiency under both zinc oxide, silicon dioxide nanoparticles but overall pattern was same. Under Phosphorus stress, similar type of adaptations were reported in other cultivated plants including common bean (*Phaseolus vulgaris*), maize (*Zea maize*), tomato (*Solanum lycopersicum*), white lupin (*Lupinus albus*) and *Brassica nigra.* Unlike Arabidopsis, other plant species showed longer PR under phosphorus deficient conditions. In previous studies, Primary root (PR) inhibition was observed in phosphorous deficient *maize* plant while control plant root length of was elongated (Li *et al.*, 2012). Other modifications of roots in response to phosphorous deficiency were observed in the number of roots (n), root width (cm) and root elongation etc.

In previous studies on *Arabidopsis thaliana* showed that roots has ability to modify under nutrient deficient condition. Root parameters respond to phosphorous deficiency differently among species. Like low availability of phosphorus in *Arabidopsis thaliana* showed reduced primary root length and increase in number of lateral roots and root hairs (Martin *et al.*, 2000). Plants undergo several stresses like nutrient deficiency stress, drought stress, salinity stress and temperature stress. To survive under stressed environment plants adopt certain strategies to survive in those conditions. By changing root architecture plants increase chances to explore more soil so to get sufficient amount of nutrients.

Microscopic analysis of root apical meristem of Phosphorus deficient plant showed that primary root inhibition correlates with reduced cell differentiation and cell proliferation in the elongation zone (Ticconi *et al.*, 2004; Ricaud *et al.*, 2007). Number of cells in meristematic and elongation zone decreases in Phosphorus deficient plant roots as compared with roots grown under full phosphorous

concentration (Ma, 2003). Our study showed significant decrease in number of cells at meristematic region of root apical meristem of Phosphorus deficient plant root. This indicates cell division decreases in Phosphorus deficiency and results in inhibition of primary root. Phosphorous plays major role in cell division at apical meristem which eventually results in elongation of roots. Microscopic examination of apical meristem showed that under phosphorous deficiency cell division decreases which results in inhibition of primary root. However, number of cells in the apical meristem was increased in zinc oxide and decreased under silicon dioxide nanoparticles. Ethylene is involved in primary root inhibition by affecting cell elongation in root apical meristem (Swarup *et al.*, 2007).

Nutrient deficiencies also affect the biosynthesis of phytohormones. Researchers are investigating phytohormones role in response to phosphorous (P), nitrogen (N), Boron (B) and potassium (K) deficiencies. Role of ethylene in response to phosphorous deficient elucidated (Wang *et al.*, 2006). Ethylene is gaseous plant hormone involve in several metabolic and physiological processes. It is also involve in seed germination, abscission of petal and leaves, cell division and elongation. Ethylene plays a prominent role throughout plant life cycle. From seedlings endogenous ethylene formed an apical hook that protect hypocotyl and seed then pushes soil to come out of the soil. Ethylene production is interrelated with phosphorous starvation. Whenever there is phosphorous deficiency ethylene production increases which enhances the expression of RSL4 gene that is involved root hair development in plants (McKeon *et al.*, 1995). Transcript analysis of ethylene biosynthesis and signaling genes showed that ethylene production increased under stressed condition which then enhances expression of other genes involved in root architecture. Cytokinin genes expression decreases in absence of phosphorous and it is possible that this decrease in root cytokinin levels in response to phosphorus deficiency might release the inhibition of root growth and act as a negative regulator for Phosphorus induced root growth (Martín *et al.*, 2000). Cytokinins are recognized to have an impact on the acquirement of several macronutrients, such as nitrogen, phosphorus and sulfur. Precisely, the expression of genes encoding multiple macronutrient transporters, including phosphate, sulfate and nitrate transporters were decreased by CKs (Brenner *et al.*, 2003). In present study TaACO2, TaERF and TaERS2 genes expression were checked in wheat roots grown under different concentrations of

phosphorus which indicates that under phosphorous deficient conditions expression of genes involved in ethylene biosynthesis increased as compared with plants grown under full (P_F) phosphorous concentration. In the absence of ethylene EIN2 (ethylene insensitive 2) is phosphorylated by CTR1 that prevents translocation of EIN2 C-terminal to the nucleus. Where, it is required for stabilization of downstream gene activation (Wang *et al.*, 2010; Shakeel *et al.*, 2013). Both varieties (SKD-1 and PAK-81) showed similar effect in activation of ethylene signaling by up regulating genes involved in biosynthesis and signaling of ethylene hormone. Thus, indicating that phosphorus deficiency activates stress response by up regulating ethylene signaling.

In previous studies of *Arabidopsis* plant, same results were showed which confirmed that less availability of phosphorous changes root architecture and makes plant able to explore more soil to survive under stressed environment (Borch *et al.*, 1999).

Limiting availability of phosphorus in wheat results in shortening of primary roots length. Transcript analysis of ethylene biosynthesis genes under limited phosphorus availability showed over expression of genes which increased production of ethylene. Ethylene facilitates throughout the life cycle of plant by stimulating or regulating the ripening of fruit, the opening of flowers and the shedding of leaves. Expression analysis was different under zinc oxide and silicon dioxide nanoparticles in both the P_F and Po conditions within intervarietal and intravarietal two selected species of wheat. Transcription factors involved in ethylene biosynthesis pathway have been identified under phosphorus deficiency however; the contrasting expression of these transcription factors under the presence of different nanoparticles need to be explored more to have better understanding of the revolutionizing effects of nanoparticles in overcoming the phosphorus deficiency alongwith variable effects of different growth regulatory hormones produced under stress.

5. CONCLUSION

Based on above discussion and previous studies it is stated that phosphorous deficiency regulates root architecture by different modifications as it is an important nutrient and its deficiency is major threat to crop productivity. In this study two types of nanoparticles (zinc oxide and silicon dioxide) were analyzed for comparison purpose both at physiological and molecular level in two different wheat varieties under phosphorus deficiency. Root architecture analysis showed significant changes in response to no availability of phosphorous which included inhibition of primary root length and increase in root width diameter. Increased in length under Zinc oxide in comparison to silicon dioxide nanoparticles showed that the former one have more beneficial effects in wheat aiding it to cope with phosphorus stress. Transcript analysis at molecular level showed that limited availability of phosphorous enhances the expression levels of ethylene biosynthesis and signaling genes while decreases the expression level of cytokinin genes. Under silicon dioxide nanoparticles, the expression was elevated during phosphorus stress to help the plant to cope up with stress conditions and cytokinin genes showed an up regulation of expression with zinc oxide nanoparticles under phosphorus stress. SKD-1 variety appeared to be the best adapted variety at phosphorous deficient condition and has ability to cope up under phosphorous deficiency with ethylene genes increasing expressional response to silicon dioxide nanoparticles and cytokinin genes increasing the expression in response to zinc oxide nanoparticles. Thus, depicting the combating effects of nanoparticles in overcoming phosphorus deficiency stress with the contrasting expressional response of ethylene signaling and cytokinin related genes under the presence of nanoparticles. It is predicted therefore that SKD-1 variety can be grown in areas with phosphorous deficiency and the growth can be facilitated in the presence of appropriate nanoparticles under optimized conditions.

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