Investigating the Role of Exogenous Hydrogen Peroxide in Nanotoxicity Reduction in Monocot (Maize) and Dicot (Tomato) Plants

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Dedication

ALL MY ACHIEVEMENTS ARE DEDICATED TO MY MOTHER, MY BROTHER AND MY SISTER FOR THEIR ENDLESS LOVE, SUPPORT AND ENCOURAGEMENT.

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Sandal Sheikh

Table of Contents

List of Abbreviations

%	Percentage
µg	Micro gram
µl	Micro liter
Ag	Silver
Al	Aluminum
APX	Ascorbate peroxidase
CAT	Catalase
CNTs	carbon nanotubes
DNA	Deoxyribonucleic acid
ENPs	Engineered nanoparticles
g/L	Gram per liter
GR	Glutathione reductase
H2O2	Hydrogen Peroxide
Kg	Kilogram
mg	Milligram
Min	Minutes
mM	Millimolar
MWCNTs	Multiwalled carbon nanotubes
NPs	Nanoparticles
O^{-}	Superoxide

OH	Hydroxyl ion
POX	Peroxidases
ROS	Reactive Oxygen Species
rpm	Revolution per minute
SOD	Superoxide Dismutase
TiO2	Titanium Dioxide
UV	Ultra Violet
XRD	X-ray Diffraction
Zn	Zinc
ZnO	Zinc Oxide

List of Figures

List of Tables

ABSTRACT

Use of nanoparticles in consumer products increases the concentration of nanoparticles in environment that causes pollution and adverse effects on the environment. It is important to study effects of nanoparticles on plants because they are stationary organisms and cannot move away from environmental stresses like animals. Therefore, they must overcome these stresses by non-enzymatic and enzymatic defense such as antioxidant enzyme activities. Very few nanoparticles and plant species have been studied, mainly at early growth stages of the plants. Currently, there is limited research in the field of Nano toxicity in plants; uptake and bioaccumulation of nanoparticles in food crops are still not well understood. But there is no research on combined effect of nanoparticles with hydrogen peroxide. Hydrogen peroxide is the stable and major non-radicle reactive oxygen species that regulate defense, development and acclimation. The purpose of this study was to evaluate the impact of nanoparticles on hydrogen peroxide pretreated plants and to determine if hydrogen peroxide pretreated plants were better in resisting nanoparticles. Tomato (dicot) and maize (monocot) were germinated under controlled condition in the growth room to conduct the experiment. Plants were exposed to different concentrations of hydrogen peroxide (H_2O_2) in combination with different concentrations of nanoparticles and their effects will be studied at physiological, and biochemical levels such as chlorophyll content, sugar content and enzyme activities of plants. Pretreatment of hydrogen peroxide showed positive results this means that Hydrogen peroxide can reduce the oxidative damage at physiological level and biochemical level which is caused by nanoparticles stress. The results were depends on the concentrations of nanoparticles and plant species.

Chapter 1

INTRODUCTION

Nanomaterials are substances that have dimensions less than 100nm (Buzea *et al*., 2007; Walker and Bucher, 2009; Nowack and Bucheli, 2007; Oberdörster *et al*., 2005; Stern and Mcneil, 2008; Handy *et al*., 2008; Aitken *et al*., 2006; Farré *et al*., 2011). The nanometer derived from the word "Nano" means "dwarf" which is a Greek word and represent the particles size less than 1 μm or 1000nm (Buzea *et al*., 2007). In nanotechnology, nanoparticles are considered as the building blocks (Stern and Mcneil, 2008). Nanoparticles have applications in variety of consumer products and areas for example electronics, pharmaceuticals, environmental, biomedical, energy, catalytic, cosmetic and material uses (Nowack and Bucheli, 2007). However, these nanomaterials have attractive and unique properties but these attractive properties could lead to unpredicted and sudden environmental and health hazards (Maynard *et al*., 2006; Dowling *et al*., 2004). Greater than 300 nano technological claimed products are present in the market (Maynard *et al*., 2006; Maynard and Michelson, 2006). The global investment in nanotechnology fields exceeded $4 billion in 2005 and it is estimated that by 2005 the annual values could reach to $1 trillion (Roco, 2005). In 2004 the global production of engineered nanoparticles was 10^3 tons per year but after 2010, it is estimated to increase in annual production is 10^4 to 10^5 tons (Dowling *et al*., 2004).This expansion in nano technology field can lead to release of nanoparticles directly or indirectly in to aquatic and terrestrial ecosystems during their synthesis, consumption and removal (Navarro *et al*., 2008; Nel, *et al*., 2006; Lee and An, 2010).

In environment, there are two main sources of nanoparticles: natural sources and anthropogenic sources which includes incidental or unwanted nanoparticles and manmade or engineered nanoparticles, respectively (Nowack and Bucheli, 2007; Biswas and Wu, 2005; Lidén, 2011; Tervonen *et al*., 2009). Natural processes for

the production of nanoparticles are shedding of hairs and skin of animals, erosion, forest fires, photochemical reactions and volcanic eruptions (Buzea *et al*., 2007). Nanoparticles are also found on earth in the form of aerosols, salts and sulfates from the sea (Motzer, 2008.). In Anthropogenic sources, incidental and unwanted nanoparticles are produced in the form of byproduct of chemical manufacturing industries like welding and smelting processes, combustion processes for generation of power from burning of coal and fuel oil, cooking food, combustion in airplane and vehicles engines and from sewage treatment (Nowack and Bucheli, 2007).

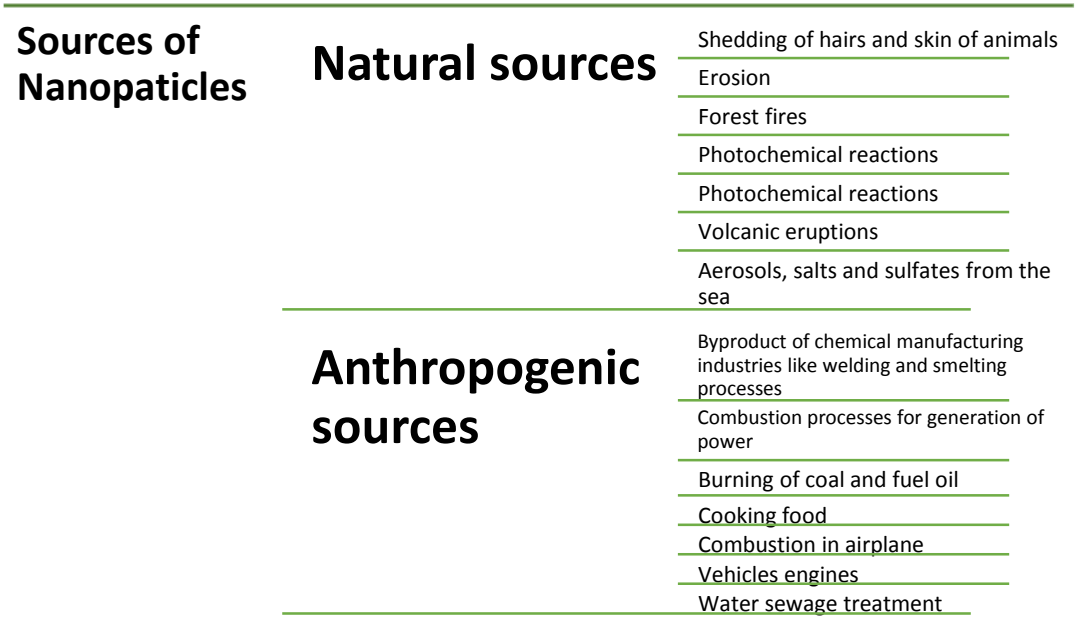

Figure 2.1: Sources of nanoparticles. *Two main sources of nanoparticles through which nanoparticles enter in environment and effect the environment.*

Engineered nanoparticles are produced in different way by man, and classified in different categories according to their properties, such as carbon nanomaterials, dendrimers, quantum dots and metal nanoparticles, which include zero valence metal nanoparticles and metal oxides (Farré, *et al*., 2011).

Unlike bulk and large particles of the similar material, unique physiochemical characteristics such as tremendously small size, surface properties and unique structure are observed in nanoparticles Nanomaterial which have less

than 5nm dimensions demonstrate unique properties that are different from the bulk particles of the same matter, such as magnetic and optical properties, catalytic reactivities, electronic state (Handy *et al*., 2008) and particle size less than 100nm. These enhance the solubility of the substances that are insoluble. These particles have exclusive structure with higher proportions of atoms on the exterior surface of the structure as compared to the interior, thus enhance the surface reactivity due to the larger surface area equivalent to mass (Auffan *et al*., 2009). It has been observed that the nanoparticles of 10nm has 40% atoms located on its surface of while a nanoparticles with 20nm in size has 20% atoms on its surface (Auffan, 2008.). Due to unique composition, nature and multiple conformation, nanoparticles have almost infinite applications and functions .Thus, nanotechnology is an emerging field of technology particularly, nanoparticles have great interest due to its ability to get synthesized and manipulated.

Engineered nanoparticles are released as waste (solid and liquid) from manufacturing industries and atmospheric emissions into the ecosystem by intentional and unintentional means (Dutschk *et al*., 2014) .These nanoparticles enter ecosystem through several ways like incidental and direct emission from the processes and products of industries, sewerage waste water treatment plants (Grieger *et al*., 2009; Zhang and Fang 2010) .Waste water emitted nanomaterials would be accumulated relatively higher in the water bodies and terrestrial environment than the nanomaterials emitted from the diesel. These nanoparticles have potential to contaminate the soil and cause land pollution when they reach the terrestrial environment. From land, they transfer to the water bodies either through the surface or through the rain and wind. Once they enter into the food web or interact with living organism, it is estimated that these nanomaterials show the behavior of colloidal solutions (Ostrowski *et al*., 2009). Colloidal suspensions are generally engineered nanoparticles that are unstable but when they come close enough to each other, van der Waals forces that are attractive forces overcome the electrostatic force of repulsion (Holsapple *et al.*, 2005; Thomas *et al*., 2006). This cause aggregation of particles followed by their sedimentation (Linkov *et al*., 2011).

Thus, release of nanoparticles confirm the involvement and presence of nanoparticles in the ecosystem and this involvement have suggested that nanoparticles enter into the food chain and can accumulate into the organisms that are present in the food chain at the top place by the process of bioaccumulation. Plants are at the base of food chain, these are the producers in the food chain and they are an important part of ecosystem and food chain. According to this idea, there may be possible pathway for the transport and transfer of nanoparticles in the food chain. So, in the ecosystem plants are the route for the bioaccumulation (Zhu *et al*., 2008).

Presently, it is reported that the nanoparticles have beneficial and adverse effects on plants. In order to increase the use of nanoparticles in agriculture, the researchers and scientists have studied the effect and role of nanoparticles on germination, growth and development of plants. Though, many reports on the adverse and phytotoxic effect of nanoparticles on the plants have been confirmed but at the same time the nanoparticles have also been found to have positive effects on the germination of seed and crops and plants due to their unique properties (Khot *et al*., 2012).

To date , there are only few studies on the toxicity of nanoparticles in higher plants showing that nanoparticles are up taken by plants , bio accumulate (Gardea-Torresdey *et al*., 2003; Rico *et al*., 2011; Khodakovskaya *et al*., 2009; Lin and Xing, 2007, 2008; Judy *et al*., 2010; Yin *et al*., 2011) and change the physiology of plants for example root and shoot length and biomass (Rico *et al*., 2011; Khodakovskaya *et al*., 2009; Lin and Xing, 2007) and damage DNA (Atha *et al*., 2012) Then these nanoparticles are distributed to the ecosystem through plants (Khodakovskaya *et al*., 2009; Lin and Xing, 2008).

At the present time, metal oxides have gained attention and are used in extensive range of areas since they have optical, ultraviolet shielding, semiconducting and piezoelectric properties (Meulenkamp, 1998; Serpone *et al*., 2007; Becheri *et al*., 2008). It is estimated that in 2004, the production of metal and

metal oxide nanoparticles is two thousand tons, which is increased from more than 58 thousand tons per year from 2011 to 2020 (Niederberger, 2007; Franke *et al*., 2006; Kolmakov and Moskovits, 2004; Stoimenov *et al*., 2002).

In metal oxide nanoparticles, zinc oxide nanoparticles are one of most common and highly used nanoparticles. Zinc oxide have been used in a verity of consumer products, which are available commercially like titanium oxide. Zinc oxide is an inorganic ultra violet blockers, when exposed to high temperature and ultraviolet radiations. Due to these properties, zinc oxide is used in transparent ultra violet- protection screens and in sunscreens as ultraviolet filter (Meulenkamp, 1998; Serpone *et al*., 2007; Becheri *et al*., 2008). Zinc (Zn) is a transition metal most abundant transition metal in environment, after iron, and .Only zinc (Zn) metal is present in all enzyme classes which are as follow: transferase lyases, ligases, oxidoreductases, hydrolase and isomerase (Auld, 2001). It is a micronutrient that is essential nutrient for the plants, animals and humans. In higher plants zinc functions as structural, functional and metal part of enzymes or as a cofactor that regulates a large number of enzymes. For this purpose, the plants absorb this nutrient in divalent form (Camp, 1945; Chapman, 1966; Viets,, 1966; Anderson, 1972; Fageria *et al*., 2002; Brown *et al*., 1993; Marschner, 1993; Mengel and Kirkby, 1987). Zinc is necessary for the germination, production of chlorophyll, fertilization, pollen functions (Pandey *et al*., 2006; Cakmak, 2000; Kaya and Higgs, 2002) and production of biomass (Kaya and Higgs, 2002). Demanding research has emphasize the importance of zinc oxide nanoparticles and is focused on discovery and finding of these nanoparticles, indicated by current reports that zinc oxide nanoparticles are used in the area of bio imaging (Senthilkumar *et al*., 2009) and in the printing electronic devices production (Bubel *et al*., 2009). For the cancer treatment, zinc nanoparticles are also used in medicines because nanoparticles favorably killed cancer cells (Hanley *et al*., 2008). As a result it increases the consumption of zinc oxide nanoparticle products, the zinc oxide nanoparticles released into both aquatic and terrestrial environment intentionally or intentionally (Sharma *et al*., 2009, Adamson *et al*., 2000, Hussein *et al*., 2002, Wang *et al*., 2004); where they effect the organisms that live in that environment.

Nevertheless, biotransformation of nanoparticles has been focused very less in plants. It is highly important to determine the process of nanoparticles biotransformation because they interact with the components of ecosystem. It has been directed to determine the biotransformation of metal nanoparticles in plants. Biotransformation has not been reported in tomato (*Lycopersicon esculentum*), corn (*Zea mays*), alfalfa (*Medicago sativa*), and cucumber (*Cucumis sativus*), when exposed to Ceria (CeO2) NPs (López-Moreno *et al*., 2010) while , in *Salsola tragus, Parkinsonia florida and pjuliflora-velutina* plants nanoparticles were not noticed when they were grown in the presence of zinc oxide nanoparticles (De La Rosa *et al*., 2011).

In ecosystem, plants are the base components (Dwivedi and Randhawa, 1974). Many studies indicate the beneficial effects of zinc oxide on plants, such as higher growth and development of plants. Prasad *et al*. (2012) ,Sedghi *et al.* (2013) Ramesh *et al*. (2014) and Raskar and Laware (2014) in peanut, soybean, wheat and onion, respectively, stated that advantageous effects on seed germination are demonstrated when exposed to the lower concentration of ZnO-NPs. However, zinc oxide in higher concentrations cause toxic effects. The effect on zinc oxide nanoparticles depend on the two factors; 1) concentration of particles 2) variety of plants (Siddiqui *et al*., 2015; Prasad *et al*., 2012; Ramesh *et al*., 2014; Raskar and Laware, 2014; Sedghi *et al*., 2013). The experiments related to phytotoxic effects on plants that are caused by zinc and zinc oxide nanoparticles remain unrevealed. Two different approaches of action may be involved: one is that they cause toxicity by release of chemicals like production of toxic ions, which is chemical composition based; and second is due to shape surface and size of the particles that cause stress (Parthasarathi, 2011). Zinc oxide and other nanoparticles (aluminum, zinc, alumina and carbon nanotubes) inhibited the root growth of rye grass, radish corn, cucumber, rape and lettuce (Lin and Xing, 2007). In 2008, Lin and zing studied transport and effect of zinc oxide nanoparticles on *Lolium perenne*. (Rye grass). Biomass reduction, shrunk root tips, distorted and extremely vacuolated cortical and epidermal cells of roots were observed but transport of zinc oxide was detected to

be very little (Lin and Xing, 2008). In the crop plants, when *Oryza sativa* were exposed to zinc oxide nanoparticles, reduction in root growth was observed (Boonyanitipong *et al.*, 2011). When *C. sativus* plant was exposed to zinc oxide nanoparticles, antioxidant enzyme such as CAT, POD and SOD activities increased in plant root tissues (Kim *et al*., 2012). Kumari *et al.* (2011) investigated the phytotoxicity in *Allium cepa* (onion) at genotoxic and cytotoxic level. Zinc oxide inhibited the root growth which was related to the mitosis inhibition. This study shows that direct relationship between the mutations in chromosomes and concentration of the nanoparticles. The phytotoxicity is also observed at molecular level and at the level of reactive oxygen species (ROS) activation. The biological membranes are disrupted due to production and release of ROS. During the production and release of ROS, toxic lipid peroxidases are produced from the conversion of fatty acids (Gratão *et al*., 2005). As a result, nanoparticles and metals easily enter and disrupt the cell, producing thiobarbituric acid ROS that cause the damage to the permeability of membrane (Arruda *et al*., 2015). ROS might be produced when engineered nanoparticles interact with agents such as UV- radiations and other organisms in the environment. When engineered nanoparticles are exposed to ultraviolet radiation, ROS are produced due the photocatalytic properties of these particles (Kus *et al*., 2006). So, it may be possible that the defense mechanism of ROS, which depends on activated oxygen scavenger enzymes for example catalases, peroxidases, and superoxide dismutase (SOD) (Asada, 1992; Noctor, and Foyer, 1998), might protect the organism from the engineered nanoparticles effects (Navarro *et al*., 2008).

The ROS, for example hydrogen peroxide (H2O2), superoxide anion ($\bullet O2^-$), hydroxyl radical (•OH) and singlet oxygen (Ślesak *et al*., 2007; Bray *et al*., 2000) are extremely reactive and cause oxidative damage to the cell and cell contents like nucleic acid, carbohydrates and proteins that changes the usual metabolism of cell and reason for the peroxidation process of lipids present in the membrane (Azevedo Neto *et al*., 2008). To inhibit the oxidative impairment of (ROS) plants develop an antioxidative system that includes antioxidant defenses, which are enzymatic and non-enzymatic (Azevedo Neto *et al*., 2008; Asada, 1992). Defenses include

glutathione, carotenoids. α-tocopherol and ascorbate are non-enzymatic while guaiacol peroxidase (GPX), dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) are enzymatic (Azevedo Neto *et al*.,2008; Chinnusamy *et al*., 2005). Hydrogen peroxide is the stable and major ROS, which regulates basic processes like development, defense and acclimation. (Ślesak *et al*., 2007). It has no net charge and it is a non-radical ROS (Halliwell, 2006). Because of its relatively high ability to diffuse in membrane and stability, it is involved in long distance signaling (Vranová *et al*., 2002). Damage to cell membranes and macromolecules that is induced by ROS, is regulated by the signaling of hydrogen peroxide (Dat *et al*., 2000; Yu *et al*., 2002; Overmyer *et al*., 2003; Hung *et al*., 2005). Hydrogen peroxide is directly involved in the defense system by the regulation of various hypersensitive response and defense genes (Kovtun *et al*., 2000), defensive and signaling protein like phosphatase, and kinase, transcription factors and antioxidants (Hung *et al*., 2005; Forman, 2007). Thus, H2O2 signaling is a possible implication to improving tolerance of crops against environmental stresses (Li *et al*., 2011).

1.1 Objective of Research

Currently, zinc oxide nanoparticles have been widely used in many consumer products, entered the ecosystem and ultimately reached the agriculture land which have raised concern about the effect of these nanoparticles on plants. Since it is known fact that zinc oxide nanoparticles are most toxic among the metal oxide nanoparticles. The aims of this research were:

- To investigate whether zinc oxide nanoparticles can be absorbed by plants.
- If zinc oxide nanoparticles are absorbed by plants , whether they effect the plants at physiological and biochemical level
- To observe the effect of different concentrations of zinc oxide in combination with different concentrations of hydrogen peroxide on monocot (maize) and dicot (tomato) and to compare their effects.

- To determine the effect between zinc oxide and combination of hydrogen peroxide and zinc oxide nanoparticles.

Chapter 2

REVIEW OF LITERATURE

2.1 Properties of Zinc Oxide (ZnO) Nano Particles

Zinc oxide (ZnO) belongs to inorganic compounds and have molecular formula "ZnO". It is white powder which has very low solubility in water. Zinc oxide powder mostly used in many products and materials like glass, rubber, ceramics, batteries, pigments, plastics, ointment, lubricants, cement, fire retardants, adhesives paints sealants, ferrites and as a zinc source in foods. Naturally zinc oxide found as mineral called zinicite in the earth crust and also synthesize for the commercial uses. (Wang *et al*, 2004). In the periodic table, due to presence of zinc in 2^{nd} and oxygen in 6^{th} group, Zinc oxide is called as "II-VI semi-conductor" (Neumark *et al.*, 2007). Because of many of its unique properties like better transparency, broad bad gap, extraordinary electron mobility and high luminescence at room temperature, zinc oxide uses in electronic applications such as heat protecting or energy saving windows, translucent electrodes in crystal display, and other applications: because of many of its unique properties like better transparency, broad bad gap, extraordinary electron mobility and high luminescence at room temperature. At room temperature, Zinc oxide have 3.3eV band gap energy and is recognized as "wide band gap semiconductor" (Wang *et al*., 2004). Zinc oxide is easily exploited at Nano-level among all metal oxides. All these properties have made the zinc oxide important for industrial and scientific applications (Wang *et al*., 2004).

2.2 Effects of Zinc Oxide (ZnO) on Plants

2.2.1 Beneficial effect

Nanotechnology has a potential to transform agriculture by modifying the conventional methods of agricultural practices. Nanoparticles are the effective means of distributing the fertilizer and chemicals like pesticides herbicides and fungicides. Nano sensors are developed for the purpose to detect the quantity required for the chemicals and fertilizers in a land and also sense the level of nutrient and moisture in the soil. (Sabir *et al*., 2014).

Nano fertilizers easily absorb by plants. Zinc oxide (ZnO) nanoparticles are used as Nano fertilizer in the form of colloidal solutions. Nano fertilizers has a significant effect in agriculture; supply nutrients to the plants without harmful effects like other chemical fertilizers, used as pesticides and recovers the organic state of soil. Nano fertilizers are used in very small quantity then other fertilizers, the quantity of Nano fertilizer is 40 – 50 kg while 150 kg of conventional fertilizer is required for a tree (Selivanov and Zorin, 2001; Raikova *et al*., 2006). The seeds of wheat treated with metallic nanoparticles were shown the 20- 25 percent higher average yield (Batsmanova *et al*., 2013).

According to some studies, Zinc oxide nanoparticles have beneficial effect on plant growth and yield. The impact of Zinc oxide nanoparticles on Peanut seeds were studied and different concentration were used on the seeds. At the concentration of 1000ppm zinc oxide nanoparticles were showed positive effects on germination of seed, plant growth and seedling vigor. Root and shoot growth of peanuts effectively increased by the zinc oxide nanoparticles (Prasad *et al*., 2012). Another study on the effect of zinc oxide nanoparticles on the growth parameters and seed yield was investigated by Laware and Raskar (2014). The results revealed that zinc oxide nanoparticles at 20 and 30 µg/ ml concentrations have positive effects on the plants growth and seeds then control. Seed yield per umbel and seed weight was significantly increased and reduced the flowering period by 10 to 12 days in the onion plants.

2.2.2 Toxicity of zinc oxide (ZnO) nanoparticles

Metal oxide nanoparticles have a vital role in plants to improve their growth, germination and yield but it is also important to know the toxic effect of nanoparticles. The studies on the toxic effect of nanoparticles increase in numbers with time but there are few researches conducted on the toxic effect on plants of zinc oxide nanoparticles (Lin and Xing, 2007; 2008; Stampouliet *et al*., 2009; López-Moreno *et al*., 2010).

Many reports on the toxicity of nanoparticles on the macro organism as well as on the microorganism have been stated. López-Moreno *et al*., in 2010, first time reported the biotransformation of zinc oxide and cerium oxide nanoparticles by the edible plants and genotoxic effects on the soybean plants. Synchrotron x ray absorption spectroscopy results shown cerium oxide found in roots while zinc oxide are not found in soya bean.

In 2009, the effects of Zinc oxide and four other nanomaterials; multi walled carbon nanotubes (MWCNT), copper (Cu) silicon (Si) and silver (Ag) on zucchini (*Cucurbita pepo*) were studied. There were no effect on seed germination of any nanoparticles. Reduction in biomass were observed in Ag, Cu and MWCNT treated plants and shorter root length was detected in plants that exposed to cu nanoparticles as compared to their corresponding bulk materials and control. In this study, no significant differences were observed in biomass and root length between, ZnO nanoparticle, ZnO powder and control (Stampoulis *et al*., 2009).

In 2013, Lee *et al*. studied the effect of zinc oxide nanoparticles on the buckwheat. Plants were grown under hydroponic conditions and bioaccumulation, growth and antioxidant enzyme activity of plant were investigated. Reduction in bio mass was 7.7- 26.4 % at 10-2000mg/l and scanning electron microscopy and transmission electron microscopy analysis showed the presence of nanoparticles on roots surface and in roots cells. Moreover reduction in the catalases and glutathione activity level were observed (Lee *et al*., 2013).

Hernandez-Viezcas *et al*. (2011) investigated the effect of zinc oxide nanoparticles, 10nm in size, by using the concentrations 500 to 4000mg/l in hydroponic culture. The velvet mesquite (*Prosopis julifloravelutina*) plants were grown for 15 days hydroponically with different conditions of nanoparticles. The presence of ZnO nano particles were determined in leaves stem and roots by the ICP-OES spectroscopy (inductively coupled plasma optical emission spectroscopy). Ascorbate peroxidases and catalases enzyme activity assays were conducted to estimate the stress on plants which showed the increase in the activity of these enzyme under stress conditions. There were no physiological symptoms like necrosis, chlorosis, wilting and stunting of growth were observed even in 30 days treated plants. It was evaluated that the plants has certain level of tolerance against the zinc oxide nanoparticles.

2.2.3 Effect of zinc oxide (ZnO) on monocot

Kumara *et al*. (2011) investigated the phyto toxicity of nano particles on the root cells of onion (*Allium cepa).* The phyto-toxicity of zinc oxide nanoparticles are evaluated by the investigating the genotoxic effect at cytogenetic stage. Transmission electron microscope (TEM) and scanning electron microscope (SEM) have used to examine the morphology of cell, changes in chromosomes and presence of micronucleus. The cytotoxic and genotoxic effect of ZnO nanoparticles was depended on the concentration of nanoparticles .and the chromosomal abbreations have direct relationship with the concentration of zinc oxide nanoparticles.

Effects of ZnO nanoparticles on the growth parameters and seed yield were investigated by Laware and Raskar (2014). The results revealed that ZnO nanoparticles have positive effects on the plants growth and seeds then control at 20 and 30 µg/ ml. Seeds yield per umbel and seeds weight significantly increased and reduced the flowering period by 10 to 12 days in the onion plants.

Ghodake *et al.* (2011) examined the phytotoxic effect of zinc oxide and cobalt nanoparticles on onion bulbs (*Allium cepa).* This study evaluated that the inhibition of roots elongation of onion and nanoparticle concentration have direct

relation with each other. The cobalt oxide nanoparticles caused toxicity by their huge adsorption in roots, while zinc oxide NPs damaged the plant cell and chromosomes.

Dimkpa *et al*. (2012) studied the comparative analysis of copper oxide (CuO) and zinc oxide (ZnO) bulk size particles with CuO and ZnO nanoparticle on the wheat in sandy soil. There was no difference present between the two types of particles.

2.2.4 Effect of zinc oxide (ZnO) on Maize

Maize is grown worldwide and the 3rd most important crop globally (Guzel and Terzi, 2013). It is staple food crop of various countries and in 2006, maize account for about 712 million metric tons. . Many abiotic stress like drought, heat and metal stresses etc. have adverse effect on maize. Abiotic stresses are major cause to reduce the yield of maize like other crops by 50 percent. (Bray *et al.*, 2000) nanotechnology is the emerging field of science. Due to use high use in consumer industry, nanoparticles have adverse effect on environment as well as on plants and also include in abiotic stress.

In 2013, a study on the effect of zinc oxide (ZnO) and citrate-coated silver (Citrate–nAg) nanoparticles compared with their ionic salts ($ZnSO_4$ and $AgNO_3$) on moisture content, germination rate ,root length and uptake of metal of two crops plants maize (*Zea mays L*) and cabbage (*Brassica oleracea var. capitata L*.) was done. The experiments on roots growth and germination indicated that the nanoparticles caused low toxicity then their free salts ions (Pokhrel and Dubey, 2013).

2.2.5 Effect of zinc oxide on dicot

Yoon *et al.* (2014) reported that zinc oxide have negative effect on growth, development and reproduction of soybeans. Plants that are grown on the soil that were treated with the 50 mg/kg of zinc oxide nanoparticles, showed reduction in

shoots, root, volume and surface area. These plants also produced no seeds compared to control plants.

Zinc oxide (ZnO) nanoparticle had no effect on the germination but had impact on the development of the garden pea that were prolonged expose to zinc oxide. Reduced surface area, stem length, transpiration and number of lateral roots were observed in treated plants (Huang *et al.*, 2014).

In 2014, Rao and Shekhawat conducted experiments on Brassica juncea to know effect of ZnO on the bioaccumulation, growth and antioxidant enzyme activity. Plants were treated with 0 200 500 100 and 500mg/l concentration of zinc oxide nanoparticles for 96 hours under hydroponic conditions. Results showed the negative effect on biomass and alter the activities of SOD, CAT, GR and APX antioxidant enzymes.

2.2.6 Effect of zinc oxide (ZnO) on Tomato

Tomato (*Solanum lycopersicum*) is the main model system for development of fruit and major plant in crops, belongs to solanum genera which is the one of the largest genera of angiosperm, comprises perennial and annual plants and grow in different habitats (Frodin, 2004). In agriculture crops tomato products used worldwide. The amount of Tomatoes used in products is more than 80% tomato mostly used in commercially processed products like in ketchup, paste, tomato juices, salsa, puree and sauces. Main characteristic of tomato is deep red color of mature fruit which is due to a compound lycopene (Helyes *et al*., 2009). Presence of lycopene and other natural compounds in high concentration which are beneficial to health and promote the health, increase the use of tomato in human nutrition. To meet all these needs a large part of this crop is grown in greenhouses, using special substrates and fertilization techniques involving reutilization of water, therefore implying an increased risk of heavy metal or metal concentration increases (Gil *et al*., 2004). Therefore, there is a need to study the responses of food crops such as

tomato to nano toxicity. Many researches have been conducted on tomato plant at different level.

Faisal *et al*. (2013) studied phyto-toxicity of nickel oxide nanoparticles in roots of tomato (*Lycopersicon esculentum*) seedling. in this study translocation of nickel nanoparticles, ultra-structural alteration in the organelles of cells, nickel ion released by nickel oxide nanoparticles, ROS production, mitochondrial dysfunction induction ,oxidative stress, activity of enzymes like CAT, GSH, SOD and LPO, changes in cell cycles and necrosis and apoptosis analysis were evaluated. This broad and well-designed study has proved the progress in phyto toxic mechanism in tomato plant by nickel oxide.

De La Rosa *et al*. (2013) observed the effect and uptake of ZnO np and zinc on the root elongation and seed germination in tomato (*Solanum lycopersicum*), cucumber (*Cucumis sativus*) and alfalfa (*Medicago sativa*). Germination of the seeds of cucumber was increased while seed germination was reduced in tomato and alfalfa at 1600mg/l concentration of ZnO nanoparticles. The results of X-ray Absorption spectroscopy showed that plants were probably bio transformed the zinc oxide nanoparticles.

2.3 Nanoparticles and Reactive Oxygen Species (ROS)

Molecular oxygen reduces to water by a series of combined electron and proton transfer reactions during the production of ATP in the mitochondria. But all the molecular oxygen convert is not reduced into water. A small portion of oxygen is not reduced entirely resulting the production of ions and radical of super oxides and oxygen, respectively. Thus ROS produces mostly in the mitochondria during the cellular oxidative reaction as the by product. ROS include hydroxyl ions hydrogen peroxide superoxide anion radicles and singlet oxygen (Yin *et al.*, 2012).

During cell signaling and mitogenic induced responses, ROS play a crucial and advantageous role (Valko *et al*., 2006; Yin *et al*, 2012). In addition to these reaction there are many reaction in which ROS are produced such as transition metals like iron and copper can also produce ROS (Yin *et al.*, 2012).

ROS produces during the normal biological functions but the over production of ROs can lead to the oxidative damage. As a result cell fails to regulate the physiological function which are regulate by the redox reactions (Halliwell and Gutteridge, 1989; Meng *et al*., 2009). The impairment of the cell growth and functions comprises production of protein radical from the oxidative alteration (Stadtman and Berlett,1997) , DNA damage by modification and breakage of strands of DNA and nucleic acid (Evans et al.,2004)and initiation of lipid peroxidation production (Butterfield and Kanski, 2001; Poli *et al.*, 2004; Poon *et al*.,2004) leads to genotoxic effect and caused cell death(Chiang *et al*., 2012; Fu *et al*., 2012; Xia *et al*., 2011, 2012, 2006, 2007).Toxicity of metal nanoparticles may be depends on the followings :chemical toxicity that is related to chemical composition like discharge of metal ion which are toxic and stimuli or stress which caused due to size shape and surface of nanoparticles (Xia *et al*., 2011). Hydroxyl radicles may be produced because of extracellular reactive oxygen species (ROS) in result of metal nanoparticle toxicity. Extracellular reactive oxygen species may change the permeability of membrane leading to cell membrane damage. As a result the possibility of entrance of metal nanoparticles become high (Xia *et al*., 2006).

Phytotoxicity of ZnO have also described on the base of activation of reactive oxygen species (ROS).fatty acid convert to toxic lipid peroxide during the production and release of ROS and leading to the destruction of biological membranes of cells (Gratão *et al*., 2005).

A study on cell line showed the toxic effect zinc oxide nanoparticles which are higher calcium level in cell, damage of DNA, lipid peroxidation, and leakage of cell membrane. Then further studies confirmed these toxic effect and mechanism. it has been demonstrated the zinc oxide nanoparticles induced ROS that cause

oxidative stress and damage which further cause inflammation by the release of mediators and consequently, leading to death of cells and cause cyto-toxicity in RAW 264.7 phygicytotic cell BEAS_2B Epithelial bronchial cells also transformed due to zinc oxide nanoparticles (Xia *et al.*, 2008). Karlesson *et al*. (2008) studied the effect of metal oxide and investigate the DNA damage, oxidative stress and cytotoxic effect of metal oxides such as CuO, TiO2, ZnO, CuZnFe2O4, Fe3O4, and Fe2O3. Study shows that zinc oxide in A549 epithelial cell line in human. Zinc oxide nanoparticles cause DNA damage and cytotoxicity.

2.4 Zinc Oxide Nanoparticles (ZnO-NPs) and Antioxidant Enzyme

Metal nanoparticles produce reactive oxygen species and may lead to oxidative damage plants. Antioxidant enzymes such as Superoxide di-mutases (SODs), peroxidases, and catalases are efficiently defend against these oxidative stresses (Gill and Tuteja, 2010). Zhao *et al.* showed that CeO2 nanoparticles induced the antioxidant enzymes in maize (*Z. mays*). These enzymes are involved in defense system against stresses via the formation of ROS.

Nerkoasova *et al*. (2011) investigate the effect of cu nanoparticles and copper ions on planch plant (*Elodea densa),* in copper ions treated plants lipid peroxidation was enhanced by 120% while in copper nanoparticles the level of lipid per oxidation was enhanced by 180% with respect to control or non-treated plants. Nanoparticles were accumulated in plants more than the other plants and increased the activities of SOD and CAT.

In another study, *C. sativus* treated with cu14 mg/dm3 (Cu2+), and zinc 262 mg/dm3 (Zn2+), ions and copper 333 mg/dm3 (Cu NPs), 376 mg/dm3 (CuO NPs), and zinc oxide1700 mg/dm3 (Zn NPs), 629 mg/dm3(ZnO NPs), nanoparticles. In treated plants tissues there is high concentration of zinc oxide nanoparticles. ZnO nanoparticles treated plants indicated high defense system due to accumulation of zinc oxide particles higher than the other nanoparticles. It might be due to root

exudates that changes the behavior and properties of ZnO nanoparticles and increase its uptake by the plant. And antioxidant enzymes like SOD, POD and CAT activities increased in roots of plants when they exposed to zinc oxide and copper oxide nanoparticles (Kim *et al*., 2012).

2.5 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide (H_2O_2) is the produced by the reduction of two electron of oxygen. It is not a free radicle but a reactive oxygen species (Halliwell *et al*., 2000). It is safe, when transitions metals are absent, then the other ROS like super oxide and hydroxyl radicles. But in the presence of transition metals, H_2O_2 became reactive and resulting the 2 OH radicles (Becana *et al*., 1998) and cause toxicity. The toxicity of hydrogen peroxide is removed by enzyme such as catalase (CAT) and ascorbate peroxidase (APX) (Toda, 2005; Andrade *et al*., 2006).

Many studies demonstrated that H_2O_2 has vital role in transductions of signals which are related to the tolerance of biotic and abiotic stresses. In plants cross tolerance of stress is observed due to H_2O_2. A numbers of studies showed that H_2O_2 induced tolerance in different stress conditions such as drought, heat, salinity, chilling and metal stresses. All these stress increase the production of H_2O_2 (Gong *et al*., 2001; Uchida *et al*., 2002; de Azevedo-Neto *et al*., 2005; Chao *et al*., 2009; Liu *et al*., 2010a; Ishibashi *et al.*, 2011; Gondim *et al*., 2012, 2013; Hossain and Fujita, 2013; Wang *et al*., 2010a, 2014a).

Prasad *et al*. (1994) stated that in maize, seedling production of endogenous hydrogen peroxide has been increased when exposed to cold stress and if exogenous hydrogen peroxide is applied to maize plants, tolerance is increased. Hydrogen peroxide was the cause of enhancing the antioxidant system by avoiding the accumulation of ROS (Prasad *et al*., 1994). Nodal potato, which are sub-cultured from micro-plants that are treated with hydrogen peroxide, remained resistant to a 4 week lethal treatment of heat shock (Lopez-Delgado *et al*, 1998). Exogenous

hydrogen peroxides improves the root system in wheat (Hameed *et al*., 2004) and also effect on the young leaves and on the growth of coleoptile in the wheat seedlings which were etiolated (Amjad *et al*., 2003). Hydrogen peroxide in low concentration induce tolerance while in high concentration causes oxidative stress (Uchida *et al.,* 2002, Chen *et al*., 2009). Exogenous hydrogen peroxide increase tolerance in manila grass and mung bean, when exposed to cold treatment (Yu *et al*., 2002; Wang *et al*., 2010) and under heat and salt stresses in rice (Uchida *et al*., 2002). It also increases the multi-resistance in rice seedlings against heat, drought, salt and cold stresses (Gong *et al*., 2001). Pretreatment of hydrogen peroxide enhances the tolerance in *Oryza sativa* when exposed to cadmium metal (Hu *et al*., 2009) and increase aluminum tolerance in wheat seedlings (Xu *et al.,* 2010). Guzel and Terzi (2013) studied the effect of Cu metal toxicity on the hydrogen peroxide acclimated maize plants and observed enhancement in soluble protein contents, mineral concentration, growth, proline content and mineral concentration than the control plants. In 2013, Yildiz *et al*. investigated the effect of chromium (Cr) metal on the canola plants in combination with hydrogen peroxide and observed the higher activity of antioxidant enzymes like APX and POD. It has been found that hydrogen peroxide also increases tolerance against the metal stress but to date, there is no research found on the effect of hydrogen peroxide in combination with nanoparticles. Role of exogenous hydrogen peroxide with abiotic stresses are demonstrated in table 2.1:

Table 2.1: Role of exogenous hydrogen peroxide with abiotic stresses in different plants

Abiotic stresses	Plants	References
Cold	Maize	Prasad *et al*., 1994
	manila grass	Yu *et al*., 2002;
	mung bean	Wang *et al*., 2010
Heat shock	Nodal potato	Lopez-Delgado *et a.l*, 1998
	Rice	Uchida *et al*., 2002

Salinity	Rice	Uchida *et al.*, 2002
Cadmium	Rice	Hu *et al.*, 2009
Aluminum	Wheat	Xu *et al*., 2010
Copper	Maize	Guzel and Terzi, 2013
Chromium	Canola	Yildiz *et al*., 2013
Drought (Osmotic Stress)	Cucumber	Liu *et al*., 2010

Chapter 3

MATERIALS AND METHODS

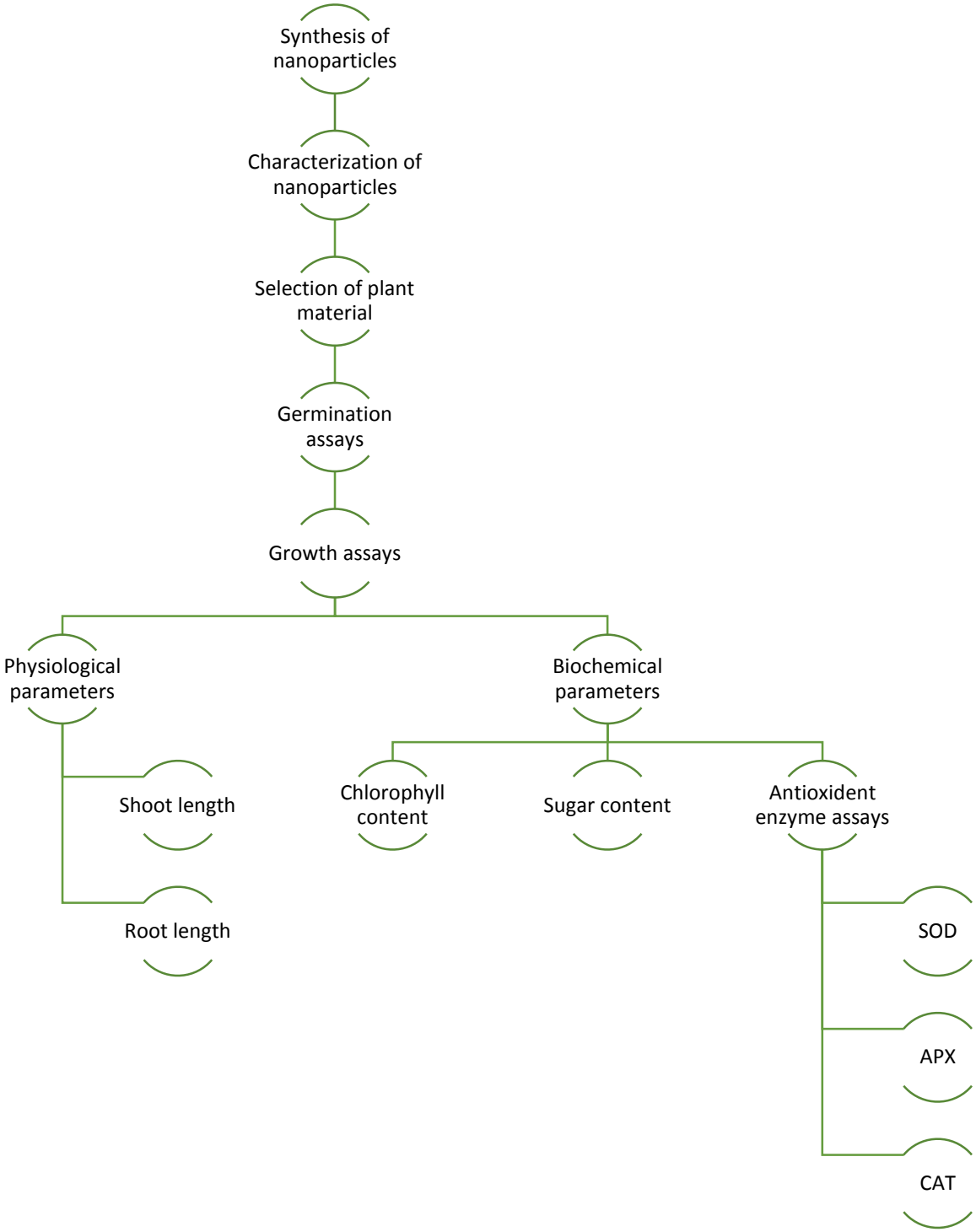

Figure 3.1: Flow chart of overview of methodology

3.1 Synthesis of Nanoparticles

The Chemical co- precipitation method was used to prepare the ZnO in the laboratory. To prepare the ZnO by this method, molar ratio is measured for the chemical concentrations. Precursor used for this method was Zinc Nitrate ($ZnNO_3$). Sodium Hydroxide (NaOH) is a strong base and used to maintain the solution pH value. For the regulating, and controlling the distribution of particle size, Acetic Acid (CH_3COOH) was used that work as capping agent as well. Distilled water was used for this reaction. The solution was stirred vigorously at continuous heating. Reagents were used in this process as follow:

Reagents	**Quantity (per 200ml)**
Zinc Nitrate ($ZnNO_3$)	5.9498 gm
Sodium Hydroxide (NaOH)	7.9994 gm
Acetic Acid (CH_3COOH)	2 ml

In a beaker, 5.9498 gm of Zinc Nitrate was added to 200 ml of distilled water and the solution was put on the magnetic stirrer machine for 20 min at 800 rotation per minute (rpm). After continuous stirring for 20 min 2mL of acetic acid (CH_3COOH) was added to the solution on the stirrer machine and stirred for 20 min again. Sodium Hydroxide (NaOH) solution was prepared by adding 7.9994 gm of Sodium Hydroxide (NaOH) to 200ml of distilled water. To increase the value of pH Sodium Hydroxide (NaOH) solution was poured in the above solution drop by drop until the pH value of 8.5 was reached and at this point precipitation was formed due to nucleation (precipitate formation phenomenon in chemical co-precipitation). The solution was sonicated for 20 min then centrifuged at 3000rpm for ten min and washed from distilled water five times. Supernatant was discarded and distilled water was added to the pallet to make the solution, this solution was kept for drying in the oven at 70^oC for overnight. The solution was dried and grinded in to powder form. Characterization of these nanoparticles was done by XRD.

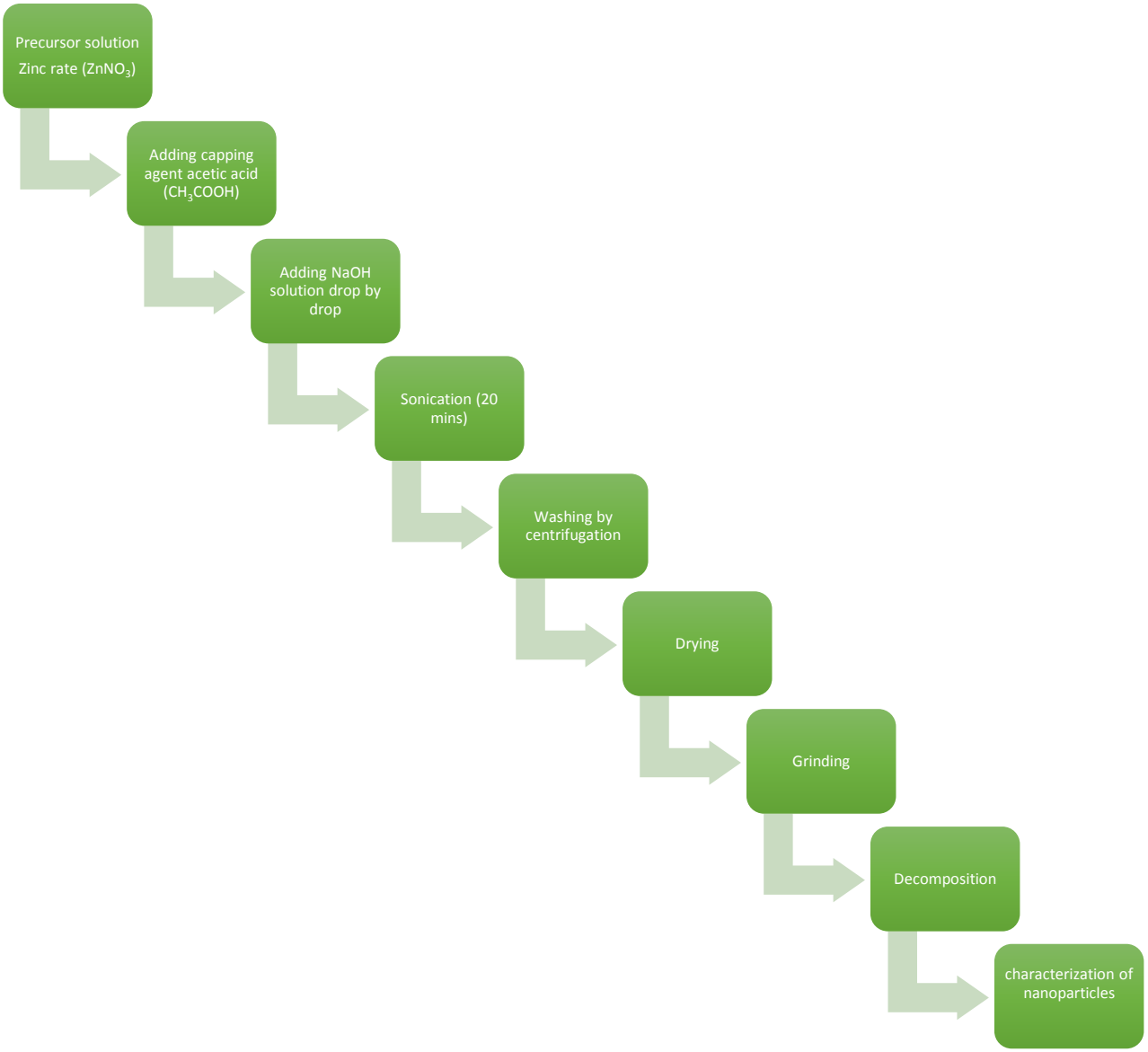

Figure 3.2: Flow chart illustrating synthesis of nanoparticles

3.2 Plant Material

In this study, maize (*Zea mays*) and tomato (*Solanum lycopersicum*) seeds were selected as monocot and dicot, respectively, for the experiments. Seeds of maize accession no. 19200 were obtained from Plant Genetic Resources Institute (PGRI), National Agricultural Research Center, Pakistan and seeds of Roma variety of tomato were acquired from Horticulture Research Institute (HRI), National Agricultural Research Center, Pakistan.

3.3 Preparation of ZnO Nanoparticles Suspensions and Hydrogen Peroxide Concentration

Zinc oxide nanoparticles suspensions were made by using water bath sonicator machine. Three concentrations of ZnO nanoparticles were prepared by adding ZnO nanoparticles in autoclaved distilled water for 30 minutes. Three concentrations used in the experiments were 500 mg/l, 1000 mg /l and 2000 mg /l.

1mM and 10mM solutions of hydrogen peroxide were prepared by using distilled water.

3.4 Germination Assays

To see the effect on seed germination the assays were performed on petri dishes. Tomato and maize seeds were surface sterilized by using 1% sodium hypochlorite then washed thoroughly with distilled water. Maize and tomato seeds were imbibe for 24hr by soaking in distilled water (control) or in 1mM and 10mM solution of H_2O_2 at room temperature. After 24hr, the solutions were discarded and seeds were transferred to 12 Petri dishes. These Petri dishes contained filter paper moisten with water and act as control and three concentrations of ZnO nanoparticles.

After seeds were transferred petri dishes were sealed from Para film and transferred to the growth room. The germination percentage was recoded for one week for maize and 2 weeks for tomato and calculated by using the following formula:

$$\textbf{Germination}\ \% = \frac{\textbf{no. of germinated seeds} - \textbf{total no. of seeds}}{\textbf{total no. of seeds}} \times \mathbf{100}$$

3.5 Growth Assays

Growth assay was performed in the soil. Maize and tomato seeds were grown in growth room in trays. The temperature of growth room was maintained at 28±2 ^{o}C with 16h/8h light dark period. Seeds started to germinate after one week. Treatment was given to the plants 14 days after post germination.

3.5.1Treatment of plants with H_2O_2 and nanoparticles

Maize and tomato Plants were divided into three groups for the control (pre-treated with distilled water) and two concentrations of H_2O_2 and distilled water. 1st group was treated with distilled water, 2nd group was treated with 1mM hydrogen peroxide and 3rd group was treated with 10mM hydrogen peroxide.

After 48hr of the hydrogen peroxide treatment, each group was then divided into four groups further and each group treated with distilled water and three concentrations, 500mg/l, 1000mg/l and 2000mg/l, of ZnO nanoparticles. Distilled water (control) and ZnO nanoparticles treatment was given to plants for one week. Group names and description of each group is demonstrated in table 3.1

Table 3.1: Groups names of hydrogen peroxide and zinc oxide treated plants and their description

Group Numbers	Description of groups
Group 1	Water treated and act as control
Group 2	1mM H_2O_2 treated
Group 3	10mM H_2O_2 treated
Group 4	0.5 g/l nanoparticles treated
Group 5	1 g/l nanoparticles treated
Group 6	2 g/l nanoparticles treated
Group 7	Combination of 1mM H_2O_2 and 0.5 g/l nanoparticles treated
Group 8	Combination of 1mM H_2O_2 and 1 g/l nanoparticles treated
Group 9	Combination of 1mM H_2O_2 and 2 g/l nanoparticles treated
Group 10	Combination of 10mM H_2O_2 and 0.5 g/l nanoparticles treated
Group 11	Combination of 10mM H_2O_2 and 1 g/l nanoparticles treated
Group 12	Combination of 10mM H_2O_2 and 2 g/l nanoparticles treated

3.5.2 Collection of plant materials

Plant materials were collected after one week of ZnO nanoparticles and distilled water (control) treatment.

3.6 Physiological Parameters

Three healthy plants from each group were taken for the measurement of root and shoot length. Root length and shoot length of each plant was measured by scale in centimeters (Pokhrel and Dubey 2013) and three replicates of each group was used to validate the data.

3.7 Elemental Analysis

Nanoparticles uptake analysis was done by using x ray diffraction (*STOE Stadi MP Germany; Software: WinXPOW*) Plant samples with their root and shoot were collected from three plant groups, oven dried for three days at 70c. These samples were grounded into fine powder and were used for the analysis.

3.8 Biochemical Analyses

Biochemical analysis were performed on fresh leaves of the plants.

3.8.1 Chlorophyll content

0.25g of fresh leaves were taken from the plants of all 12 groups). Leaves were put into 12 test tubes containing 80% ethanol and capped immediately. These test tubes were placed on the water bath for 10 min at 80c. After 10 min, extract in the test tubes was cooled in dark room. Optical density (OD) was measured at 666nm wavelength on spectrophotometer. Chlorophyll content was calculated by using the following formula:

$$\boldsymbol{Chlorophyll\ (mg/mg\ DW)} = \frac{(\boldsymbol{chlorophyll - 0.01}) \times \mathbf{10}}{\mathbf{92.6474} \times \boldsymbol{Dwt\ of\ sample}}$$

3.8.2 Soluble sugar content

Soluble sugars were determined by method of phenol sulphuric acid which is described by Doubois *et al*. (1956). O.25g of fresh weight of leaves were taken from the 12 groups and put into 5ml of 80% ethanol in the test tubes. These test tubes were heated on the water bath at 80c for 1h. 0.25ml of extract from each test tube was transferred to the new test tubes, 500µl of 18% phenol was added to it and

incubated for 1h at room temperature.1.25ml of conc.H_2SO_4 was added to each test tube. Then vortexed and optical density (OD) of each sample was measured at 420nm by using UV-Spectrophotometer. Soluble sugar content was measured by standard glucose curve.

3.8.3 Antioxidant enzyme assays

To perform the enzyme activity, whole protein was extracted from the plant leaves. For this purpose, 200 mg (approximately) of fresh leaf samples were collected from each plant groups and then grounded in liquid nitrogen to fine powder in pre chilled mortar and homogenized thoroughly in 1.2 ml of pre-cooled protein extraction buffer(containing 0.2mM potassium phosphate buffer with pH7.8 and 0.1mM EDTA). Centrifugation was done of these samples at 14000 rpm at 4^{o}C for 20 minutes. The supernatant of each tube was transferred to another Eppendorf of 2ml and the pellet again suspended in 0.8ml of the extraction buffer this new suspension again centrifuged at 14000rpm for 12 min at 4^{o}C. Supernatants were combined and stored at -80 ^{o}C till further use (Elavarthi and Martin, 2010).

3.8.3.1 Total soluble protein estimation

Total soluble protein was determined by using Bradford assay (Yadegari *et al.,* 2008). Standard curve was generated by using Bovine Serum Albumin (BSA) (Bradford, 1976). 0.25ml of Bradford reagent, 1ml distilled water and 10µl of crude protein sample was added for each sample. Then samples were vortex and incubate for 10 min. Absorbance was measured at 595nm wavelength on UV/Vis spectrophotometer. Total soluble protein concentration was estimated by using standard curve.

3.8.3.2 Catalysis (CAT) specific activity

The activity of catalases was measured by the method described by Aebi and Lester (1984). For each sample 3ml of reaction mixture was prepared in test tube, each for each sample, which contain 2ml of leaf extract which is diluted 200 times in extraction buffer (50mM potassium phosphate buffer with pH7.0) and 1 ml of 10mM H2O2. The optical density (OD) was measured for each sample at 240nm by using UV/Vis spectrophotometer. Catalases activity was calculated by using the following formula:

$$\boldsymbol{catalases\ activity}\ (\mu\boldsymbol{mol/ml/min}) = \frac{(\Delta \boldsymbol{A_{240}/min}) \times \boldsymbol{V(ml)} \times \boldsymbol{dil})}{\boldsymbol{\varepsilon_{mM}} \times \boldsymbol{V_{enz}(ml)}}$$

3.8.3.3 Ascorbate Peroxidase (APX) specific activity

APX activity was determined by modified method used by Nakano and Asada (1981). The 1 mL of substrate mixture was prepared for each treated and non- treated samples. Containing potassium phosphate buffer (50mM) with pH 7.0, 0.5mM H2O2, 0.5mM ascorbate, and 10µL of total leaf extract for monocot and 100µL for dicot. Optical density (OD) was measured at 290nm and was recorded after every 30 sec for 3 min. Ascorbate peroxidases activity was calculated by using the following formula:

$$\boldsymbol{Ascorbate\ peroxidases\ activity}\ (\mu\boldsymbol{mol/ml/min}) = \frac{(\Delta \boldsymbol{A_{290}/min}) \times \boldsymbol{V(ml)} \times \boldsymbol{dil})}{\boldsymbol{\varepsilon_{mM}} \times \boldsymbol{V_{enz}(ml)}}$$

3.8.3.4 Superoxide Di-mutase (SOD) activity

SOD buffer was prepared (Beauchamp and Fridovich, 1971). 3ml of SOD buffer was taken for each sample.100µL of riboflavin stock and 100µL of sample extract was added to SOD buffer. All the samples were placed under florescent lamp of 40 watts on the shaker for half an hour. Yellow color, in the test tubes, was change

to brown color. One more set of same samples were prepared and placed them under dark for the same time as mention above. Mixture of 3ml of sod, 100µL of riboflavin stock and 100µL of distilled water was used as blank. At the wavelength of 560nm Optical density was measured of both sets on UV/Vis spectrophotometer. Mixture of 3ml of SOD 100.

Riboflavin stock was prepared by adding 0.0016gms of riboflavin to 5ml of distilled water. SOD buffer containing the following chemicals:

Reagent	**Quantity (per 100 ml)**
Methionine	0.194gm
ETDA (Ethylenediamine tetraacetic Acid)	0.0367gm
NBT (Nitro Blue Tetrazolium)	0.006132 mg

The activity of SOD was calculated by using the following calculations:

SOD (units/g) = R4/A

R4=R3-R2,

And A=50% control= 1unit of enzyme= R1 (50/100).

Where: R1=absorbance of control

R2=absorbance of blank R3=absorbance of sample

Chapter 4

RESULTS

4.1 Zinc Oxide Nanoparticles XRD Analysis

In order to examine the crystalline structure and phase of ZnO nanoparticles have been analyzed by XRD (X-ray diffraction) at wave length (λ) 1.5405Å. The range was from 20˚ to 70˚ at angle and step size was taken 0.02. The XRD pattern of ZnO is shown in Fig.4.1

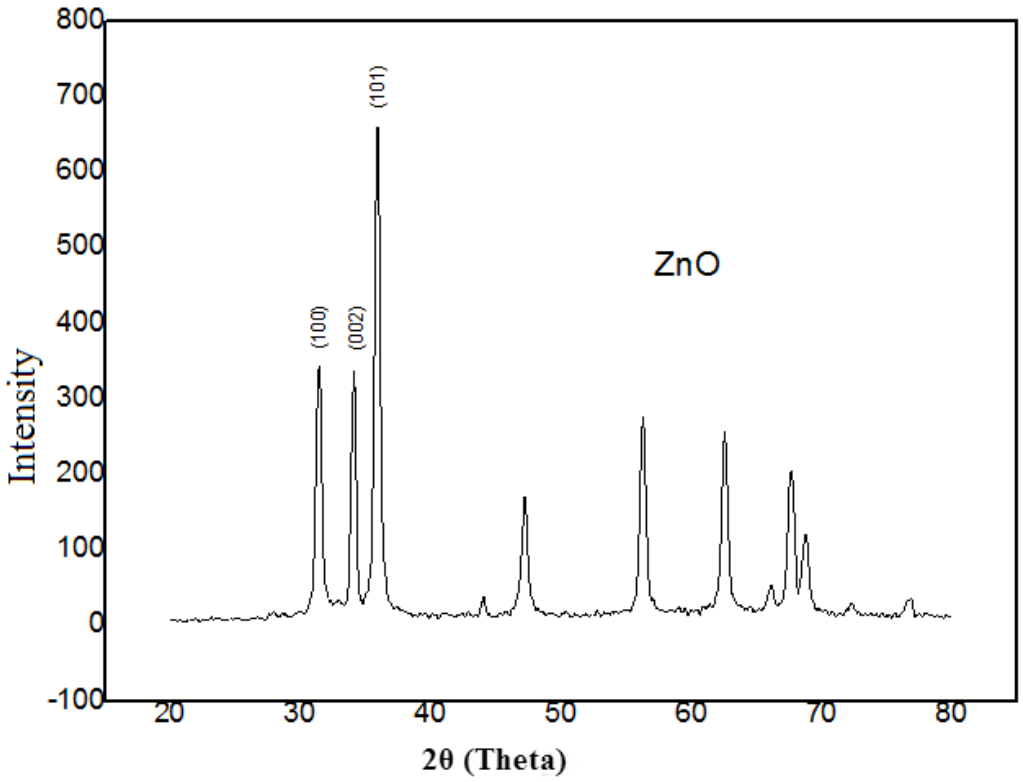

Figure 4.1: X- Ray diffraction (XRD) pattern of zinc oxide (ZnO) nanoparticles

The presence of broad peaks in these figures confirms the nano crystalline nature of these samples. The values of d spacing, major peak angle, crystallite size and corresponding plane for each sample are given in the Table 4.1

Table 4.1: d spacing, major peak angle, crystallite size of ZnO nanoparticles

Sample	**Major reflection**	**d (Å)**	**Crystallite size (nm)**	**(h k l)**
ZnO	36.048	2.4916	34.32	(101)

d spacing is determined by the Bragg's law

$2d\sin\theta = n\lambda$ …… (1)

Taking n = 1

$$d = \lambda/2\sin\theta \text{……..... (2)}$$

The crystallite sizes are calculated by Scherer formula;

$$D = k\lambda/\beta\cos\theta \text{……. (a)}$$

Where K and λ are shape constant and wavelength of the X rays: having values 0.9 and 1.54 Å respectively while β is full width at half maximum (FWHM).

This pattern indicates the successful synthesis of ZnO nanoparticles, as all the main peaks of the pattern satisfy the standard card of the ZnO. There are no peaks belonging to the secondary phase impurity.

4.2 Seed Germination Rate

Effects pretreatment of two different concentrations (1mM and 10mM) of H_2O_2 on ZnO nanoparticles stress was observed on the germination of tomato and maize. Results showed no effect on the germination % of maize of pretreatment of H_2O_2 on zinc oxide (ZnO) nanoparticles stress. Maize seeds showed 100%

germination in all groups. But In tomato, group2 and group 3 showed 11.1% increase with respect to group 1 (control). Group 7, group8, group 9, group 10, group 11 and group 12 showed relatively better growth than group 4, group5 and group 6. Effect of H_2O_2 pretreatment and ZnO nanoparticles on germination rate of tomato showed in figure 4.2:

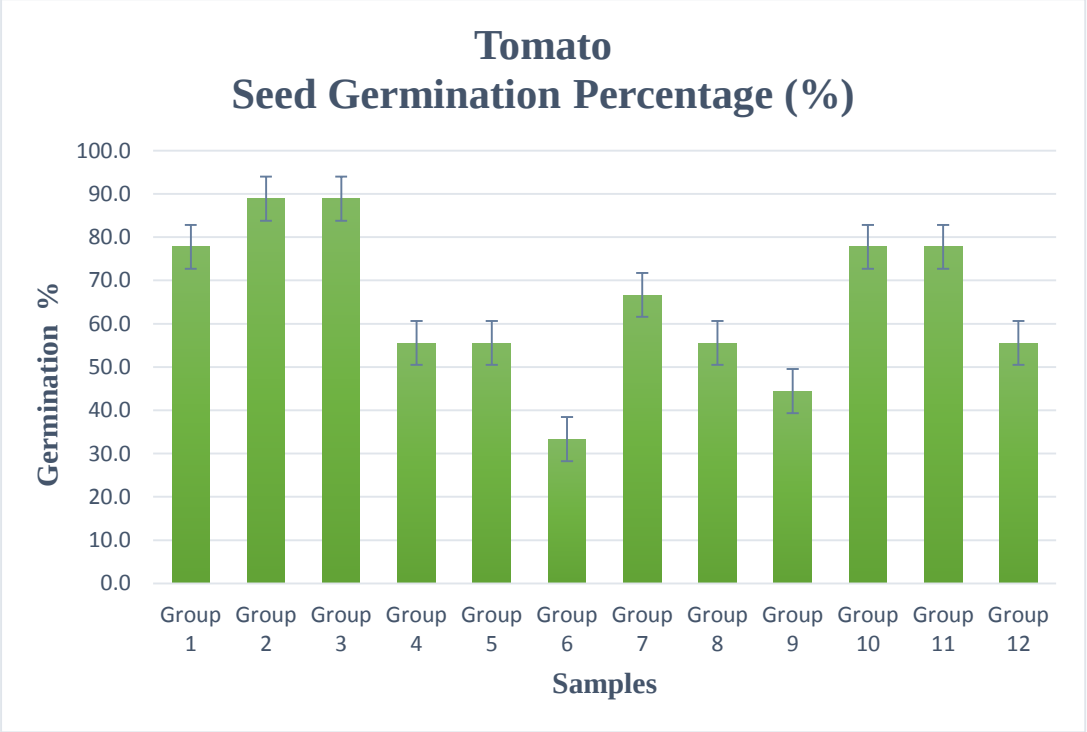

Figure 4.2: Effect of hydrogen peroxide (H_2O_2) pretreatment on ZnO treated tomato seed germination. *Pretreatment of 1mM and 10mM H_2O_2 increase germination % in ZnO treated plants.*

4.3 Physiological Parameters

4.3.1 Shoot length

To analyze the effect of pretreatment of H_2O_2 and ZnO nanoparticles on physiology of plants, shoot length was measured in centimeter (cm). The toxic and non-toxic effect on the above ground parts of the plants, can be analyzed by the decrease and increase in shoot length, respectively. Maize shoot length was

measured to analyze physiology of maize. Shoot length of maize group 2 elevated to 3.5 % and group 3 increased by 11.2% with respect to control. Group 4 and group 5 increased by 14.1% and 2.3 % while in group 6 there was 1.2 % decrease in shoot length as compared to control. Group 7 increased by 36.9 % which is the highest percentage of increase in shoot length while group 8 and group 9 showed 7.1 and 3.8% increase in comparison to group 1 (control). 32.2 % and 10% increase of shoot length in group 10 and 11 and in group 12 there was 28.3% decrease in length. 1mM H_2O_2 and 10mM H_2O_2 increased shoot length when treated with ZnO except at 10mM H_2O_2 with highest concentration of ZnO as compared to group 4, 5 and 6. Higher shoot length was observed at low concentration. Effect of hydrogen peroxide pretreatment on ZnO treated maize shoot length are demonstrated in figure 4.3:

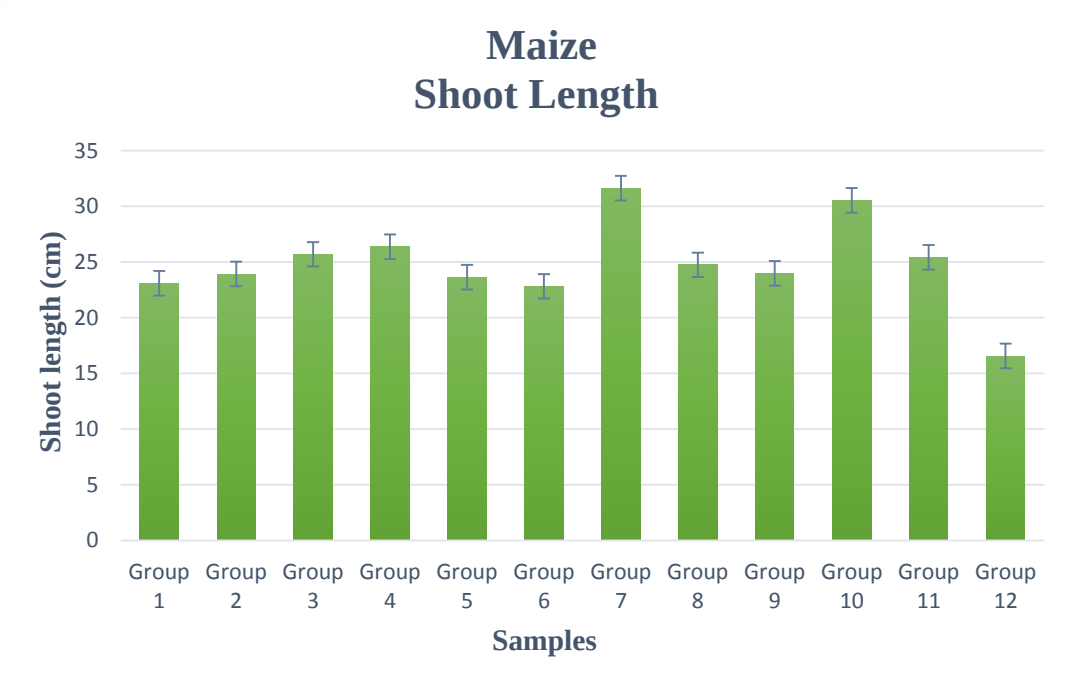

Figure 4.3: Effect of hydrogen peroxide (H_2O_2) pretreatment on ZnO treated maize shoot length. *1mM and 10mM H_2O_2 increase shoot length when treated with ZnO. Shoot length was higher at low concentration of ZnO nanoparticles.*

In tomato, plants of group 2 and group 3 have decreased shoot length by 2.2% and 9.5% with respect to control. Group 4 have increased in value 9.9 %, group

5 and group 6 have 2.6% and 9.9 % decrease in shoot length. Group 7 and group 8 showed decrease in root length with 8.2% and 5.2%. In group 9 shoot length increased by 8.6 % . Group 10 showed 0.4% increase but in group 11 and group 12, 7.8% and 4.3 % decrease with respect to group 1 (control). These results showed pretreatment of 1mM H_2O_2 decreased shoot length at low concentration and increased shoot length at high concentration of ZnO with respect to group 4, 5 and 6. Pretreatment of 10mM H_2O_2 lower shoot length at low concentrations and higher the shoot length at high concentration of ZnO with respect to group 4, 5 and 6. Effect of H_2O_2 pretreatment on ZnO treated shoot length of tomato plants are showed in figure 4.4:

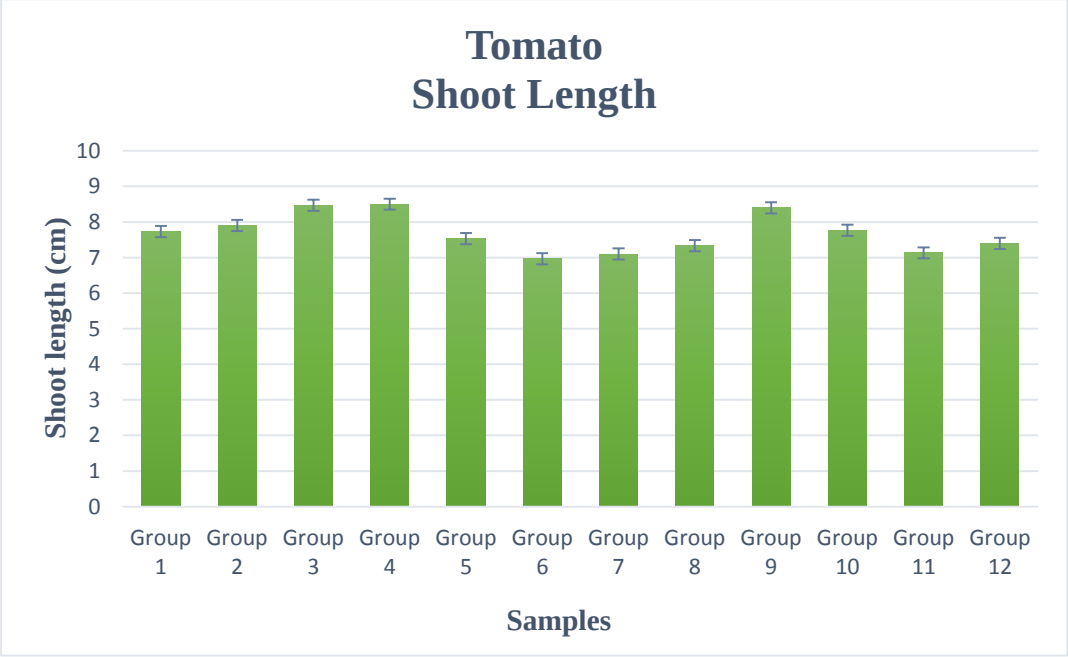

Figure 4.4: Effect of hydrogen peroxide (H_2O_2) pretreatment on ZnO treated tomato shoot length. *Pretreatment of 1mM H_2O_2 decrease shoot length at low concentration and increases shoot length at high concentration of ZnO treated seedlings. Pretreatment of 10mM H_2O_2 lower shoot length at low concentrations and higher the shoot length at high concentration in ZnO treated seedlings.*

4.3.2 Root length

To inspect the effect of pretreatment of H_2O_2 and ZnO nanoparticles on physiology of plants, root length of plant was measured in cm with a scale. The toxic and non-toxic effect on the underground parts of the plants, can be analyzed by the decrease and increase in root length, respectively. Root length of maize was observed to analyze physiology of maize Group 2 and group 3 have decreased root length by 33.3% and 20.3% with respect to group 1 (control). Group 4, group 5and group 6 have 7.2%, 39.1% and 2.9% increase in root length. .Group 7 and group8 have 23.2%and 44.9% increase in root length with respect to group 1(control).Root length was in group 9 showed highest value of root length with 75.4% increase. Group 10 showed 0% effect but group 11 and group 12 presented high value with 52.2 % and 2.9 % increase with respect to group 1(control). Pretreatment of 1mM H_2O_2 increased root length in ZnO treated seedlings while Pretreatment of 10mM H_2O_2 showed no significant differences, only at medium concentration, there was slightly increase in root length in ZnO treated seedlings. Effect of H_2O_2 pretreatment on ZnO treated Maize root length are presented in figure 4.5:

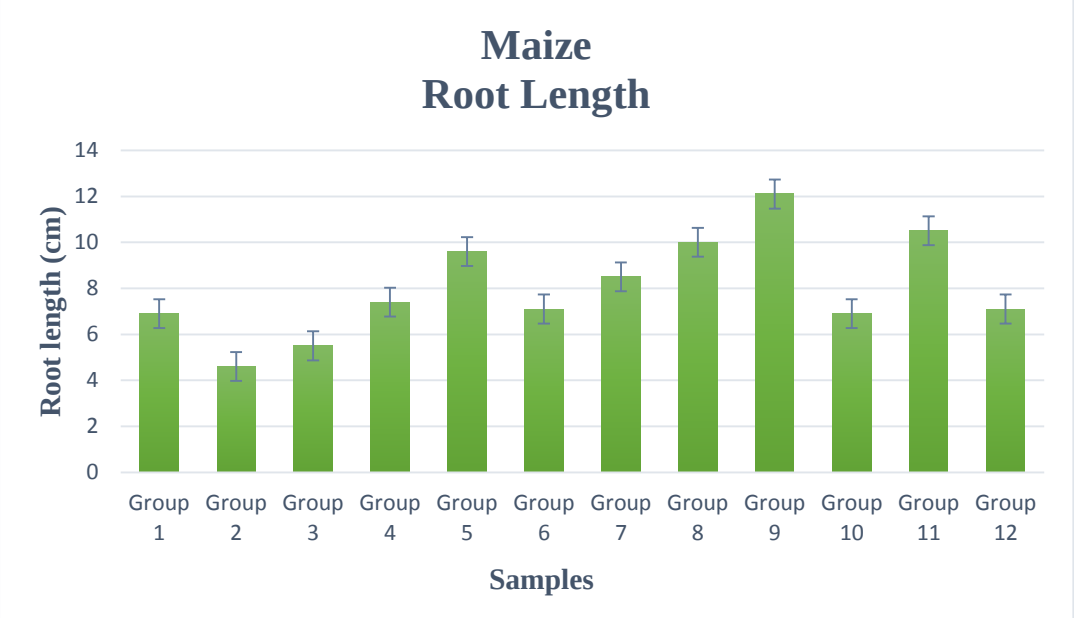

Figure 4.5: Effect of hydrogen peroxide (H_2O_2) pretreatment on ZnO treated maize root length. *Pretreatment of 1mM H_2O_2 increases root length in ZnO treated seedlings while Pretreatment of 10mM H_2O_2 shows no significant differences , only*

at medium concentration, there is slightly increase in root length in ZnO treated seedlings.

In dicot (tomato), plants of group 2 and group 3 have increased root length by 1.2% and 22.6% with respect to group 1 (control). Group 4, group5 and group 6 have 48.2%, 8.3% and 6.0 % increased value and group 5 have highest value. Root length was in group 8 showed 8.3 % decrease in value of root length, group 7 and group 9 have 21.4 and 11.9% increase. Group11 showed 0% increase or decrease but in group 10, 27% increase and group 12 11.9% decrease occurred with respect to group (control). Pretreatment of 1mM and 10mM H_2O_2 decreased root length in ZnO treated seedlings. Effect of H_2O_2 pretreatment on ZnO treated tomato root are presented in figure 4.6

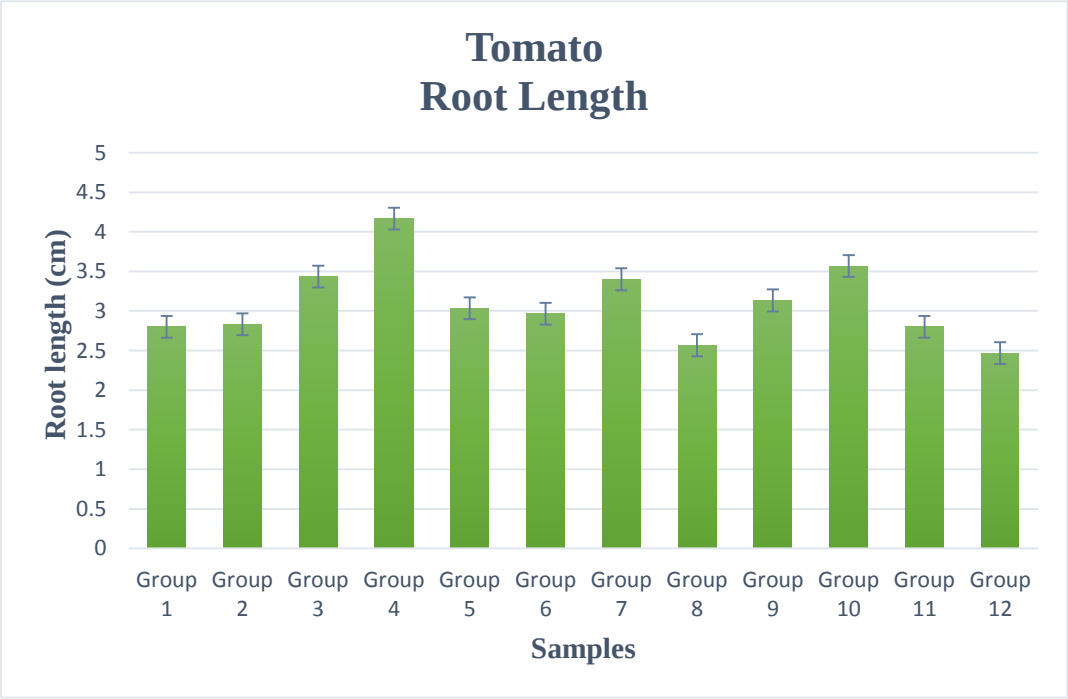

Figure 4.6: Effect of hydrogen peroxide (H_2O_2) pretreatment on ZnO treated Tomato root length. *Pretreatment of 1mM and 10mM H_2O_2 decreases root length in ZnO treated tomato seedlings.*

4.4 Biochemical Parameters

4.4.1 Sugar content

To study the effect of pretreatment of H_2O_2 and ZnO nanoparticles on biochemical parameters like sugar concentration of plants, total soluble sugar content of plants was measured by the phenol-sulfuric acid method (Doubois *et al*., 1956). The toxic and non-toxic effect on plants, can be analyzed by the increase and decrease in sugar content, respectively. Effects of H_2O_2 pre-treatment on soluble sugar content in maize leaves were investigated. Plants of group 2 have 9.5 % decrease and group 3 have36.6% increase in sugar content with respect to group1 (control). Group 4, group 5 and group 6 have 25.9%, 19% and 24.6 % increased value. Root length of group 7, group 8 and group 9 have 13.4, 6.5 and 6.9% increase in sugar content. Group10 and group 11 showed 8.6and 11.6% decrease but plants of group 12 have increased 93.5% with respect to group 1(control). Group 12 demonstrated the highest value of sugar content. 1mM H_2O_2 activity showed no significant difference but slightly reduce at all concentrations and 10mM H_2O_2 increased sugar content at highest concentration while reduced at low concentrations of ZnO nanoparticles. Effects of hydrogen peroxide pretreatment on total soluble sugar content of ZnO treated maize seedling are displayed in figure 4.7:

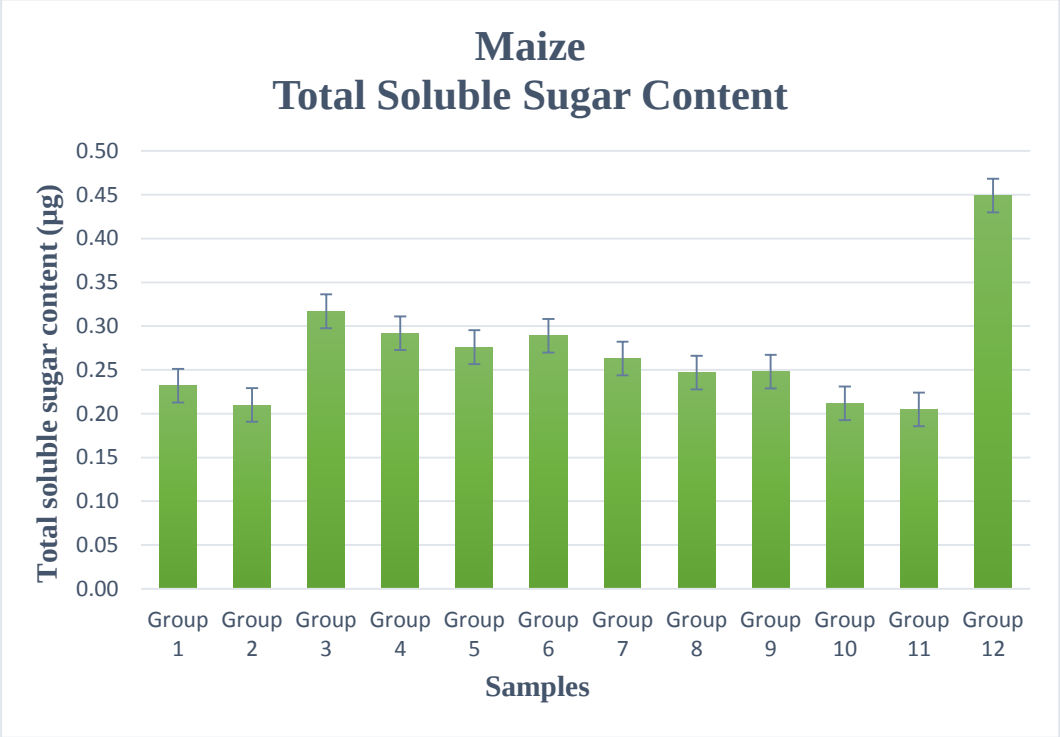

Figure 4.7: Effect of hydrogen peroxide (H_2O_2) pretreatment on total soluble sugar content of ZnO treated maize seedling. *1mM H_2O_2 activity shows no significant difference but slightly reduce at all concentrations of ZnO.10mM H_2O_2 increases sugar content at highest concentration while reduces at low concentrations of ZnO.*

In tomato, Plant of group 2 and group 3 have 6 % and 22% decreased in sugar content with respect to group1 (control). Group 4 and group 5 have 5.3 %, and 24 % decreased value and group 6 have 6% increased value. Group 7 and group 9 showed 8 %and 26.7% increase in value of sugar content, group 8 have 2% decrease in sugar content. Group 10 and group 12 showed 20% and 16.7% increase but in group 11 32.7% decrease occur with respect to group 1 (control). 1mM H_2O_2 pretreatment increased total soluble sugar content, high at highest concentration of ZnO nanoparticles. 10mM H_2O_2 increased the sugar content at high and low concentration while reduces at medium concentration. Effect of hydrogen peroxide pretreatment on total soluble sugar content of ZnO treated tomato seedling are demonstrated in figure 4.8:

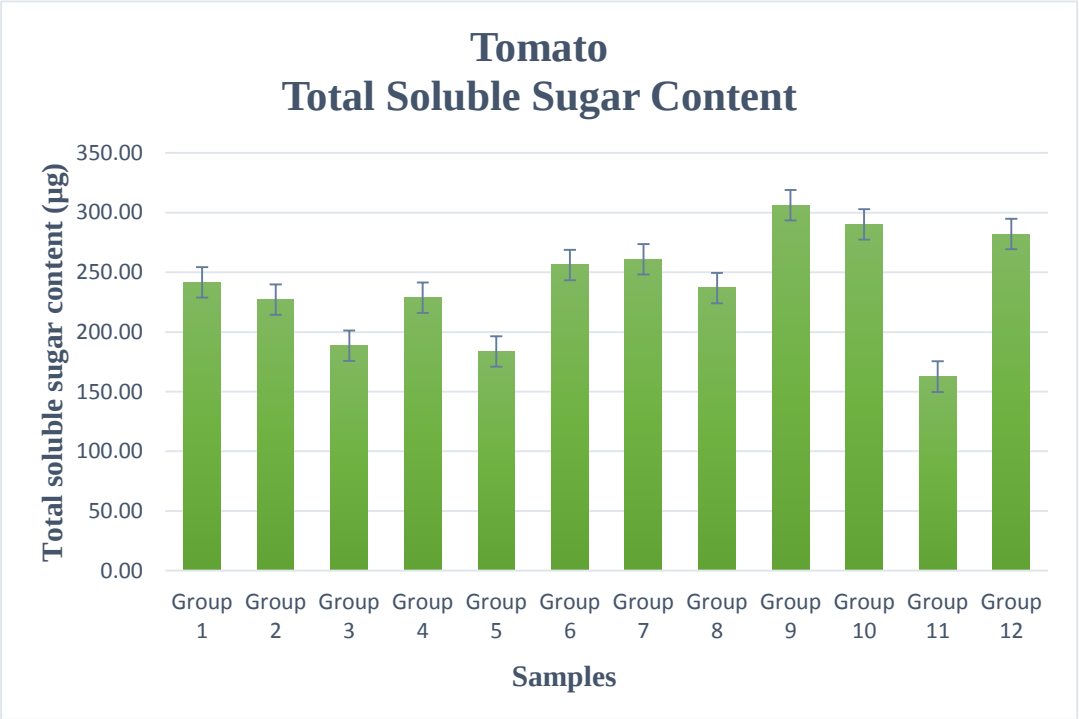

Figure 4.8: Effect of hydrogen peroxide (H_2O_2) pretreatment on total soluble sugar content of ZnO treated tomato seedling*. 1mM H_2O_2 pretreatment increases total soluble sugar content, high at highest concentration and 10mM H_2O_2 increases the sugar content at high and low concentration while reduces at medium concentration of ZnO.*

4.4.2 Chlorophyll content

To investigate the effect of pretreatment of H_2O_2 and ZnO nanoparticles on biochemical parameters of plants, total chlorophyll content was measured by ethanol method. The toxic and non-toxic effect on the photosynthetic behavior of the plants, can be analyzed by the decrease and increase in chlorophyll content, respectively. Chlorophyll content of maize was measured to investigate the biochemical parameters of maize. Plant of group 2 and group 3 have decreased value by23.5% and 63.7% with respect to group 1 (control). Chlorophyll content of Group 4 group 5 and group 6 decreased with 56.9%, 16.7 % and 43.1 % chlorophyll content of group 7, 8and 9 have decreased value with 65.7% , 5.9% and 13.7% , respectively. Group 10 showed 36.3% increase but group 11 and group

12 presented 50% and 80.4 %decrease with respect to group1 (control). 1mM H_2O_2 pretreatment increased chlorophyll content and dependent on concentration, high concentration showed high chlorophyll content.10mM H_2O_2 pretreatment increased chlorophyll content in low concentration, while reduced significantly in high concentration. Effects of hydrogen peroxide pretreatment on chlorophyll content of ZnO treated maize seedling are presented in figure 4.9:

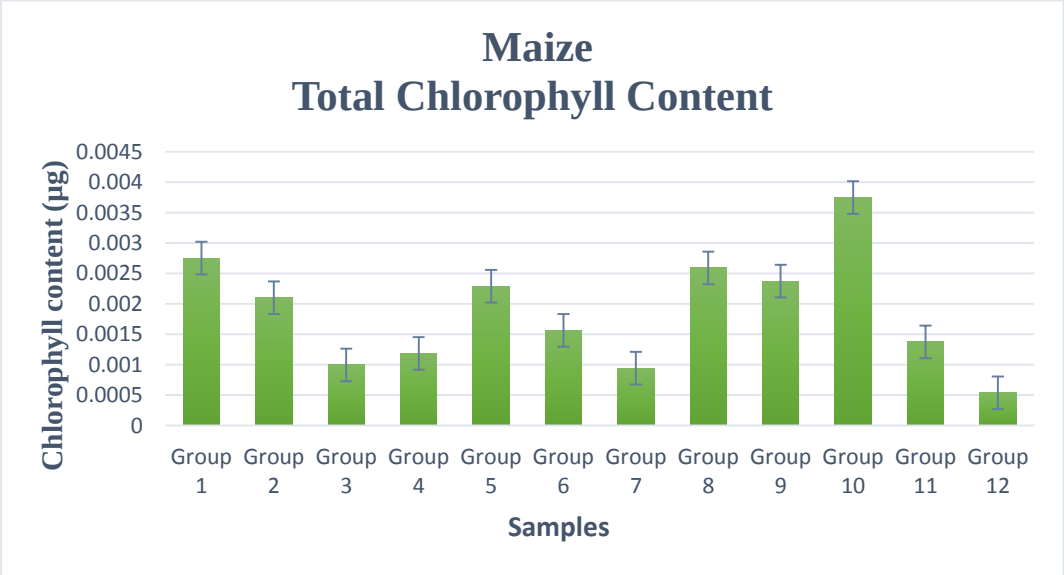

Figure 4.9: Effect of hydrogen peroxide (H_2O_2) pretreatment on total chlorophyll content of ZnO treated maize seedling. *1mM H_2O_2 and 10mM H_2O_2 pretreatment increases chlorophyll content at low concentrations of ZnO, while 10mM H_2O_2 pretreatment reduces significantly at high concentration of ZnO.*

In tomato, chlorophyll content was measured. Plant of group 2 have increased value with 11.4% and group 3 have decreased value by 9.2% with respect to group 1 (control). Chlorophyll content of Group 4 and group 5 have increased value by 15.3% and 0.1% but group 6 have 56.9 % decrease in chlorophyll content. Chlorophyll content of group 7, group 8 and group 9 have increased value with 24.9%, 16.9% and 1.6%, respectively. Group 10 and group 12 showed 7.9% and 16.3 % increase but group 11 presented 1.7% decrease with respect to group1 (control). Pretreatment of 1mM and 10mM H_2O_2 increased chlorophyll content in ZnO treated seedlings. Pretreatment of 1mM H_2O_2 with 0.5g/l ZnO nanoparticles showed highest

value of chlorophyll content. Effects of hydrogen peroxide pretreatment on chlorophyll content of ZnO treated tomato seedling are presented in figure 4.10:

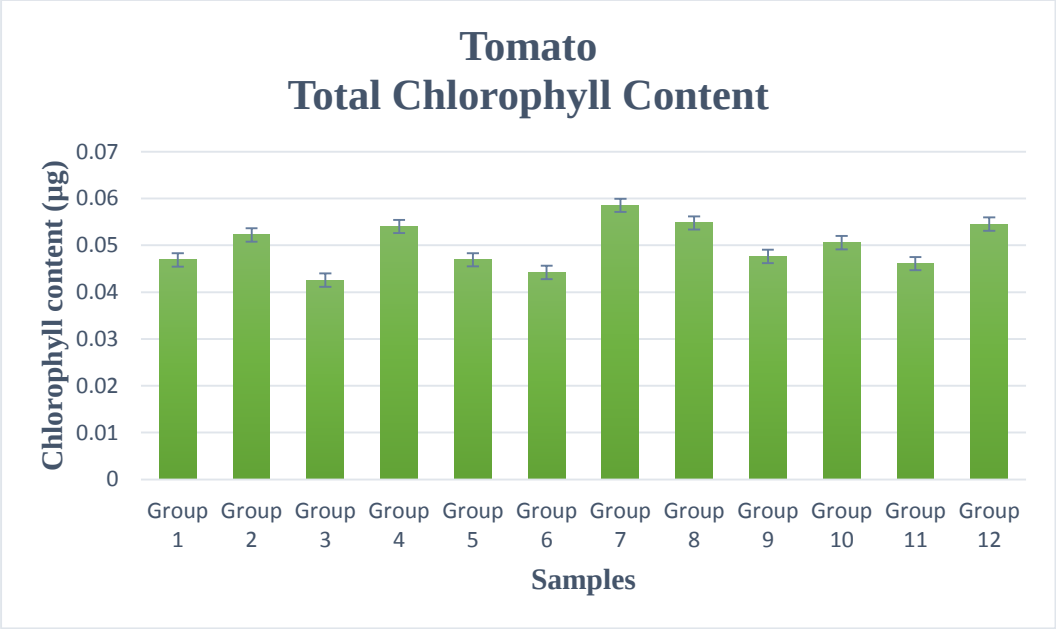

Figure 4.10: Effect of hydrogen peroxide (H_2O_2) pretreatment on total chlorophyll content of ZnO treated tomato seedling*. Pretreatment of 1mM and 10mM H_2O_2 increases total chlorophyll content in ZnO treated tomato seedlings.*

4.4.3 Enzyme activities

4.4.3.1 Total soluble protein

To examine the effect of pretreatment of H_2O_2 and ZnO on proteins of plants, total soluble protein was measured by Bradford method. The effect on the proteins of the maize seedlings can be analyzed by the decrease and increase in protein concentration. Plants of group 2 have no effect with respect to group 1 (control) and group 3 have increased protein concentration by 22.2 % with respect to control. Group 4 and group 5 have increased protein value by 27.8% and 47.2% but group 6 with 13.9% decrease. Protein value of group 7 and group 8 have increased with 5.6%, 27.8 %, respectively and group 9 have decrease 80.3%. Group 10, group 11

and group 12 showed 5.6, 27.8 and 19.4 % increase with respect to group 1 (control). Pretreatment of 1mM and 10mM H_2O_2 decreased total soluble protein content in ZnO treated maize seedlings. Effects of hydrogen peroxide pretreatment on total soluble protein content of ZnO treated maize seedling are presented in figure 4.11:

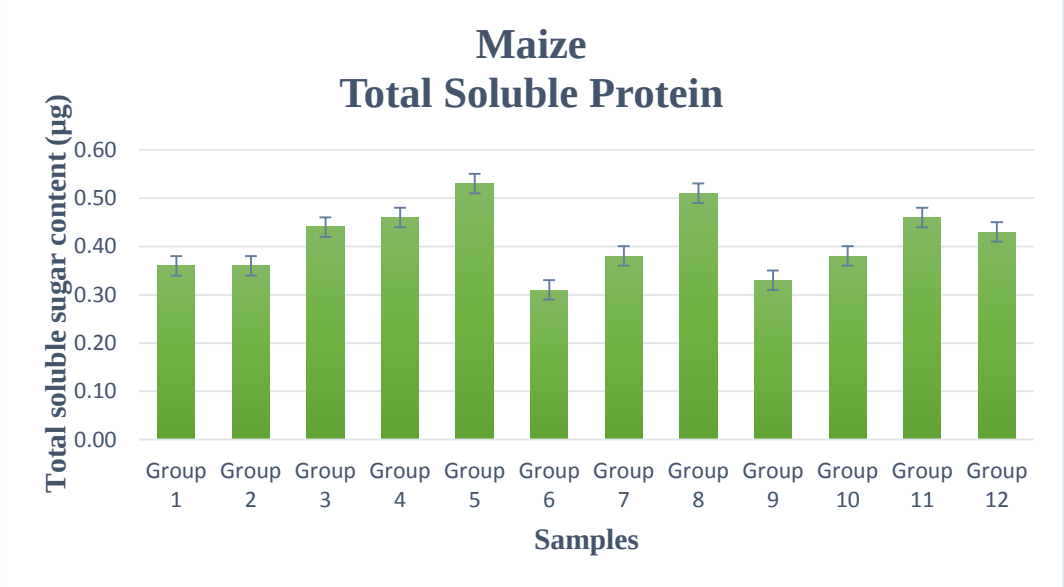

Figure 4.11: Effect of hydrogen peroxide (H_2O_2) pretreatment on total soluble protein content of ZnO treated maize seedling*. Pretreatment of 1mM and 10mM H_2O_2 decreases total soluble protein content in ZnO treated seedlings.*

To analyze the effect on proteins of dicot (tomato), total soluble was measured .Plants of group 2 and group 3 have decreased protein concentration by 4.8 % and 28.6% with respect to group 1(control). Group 4 and group 5 g have increased protein value by 11.9% and16.7% but group 6 with 97% decrease. Group 7, group 8 and group 9 have increased with 61.9%, 52.4 %and 50%, respectively. Group 10, group 11 and group 12 showed 38.1, 52.4 and 47.6 % increase with respect to control. . Pretreatment of 1mM and 10mM H_2O_2 increased total soluble protein content in ZnO treated seedlings. Effects of hydrogen peroxide pretreatment on total soluble protein content of ZnO treated tomato seedling are showed in figure 4.12

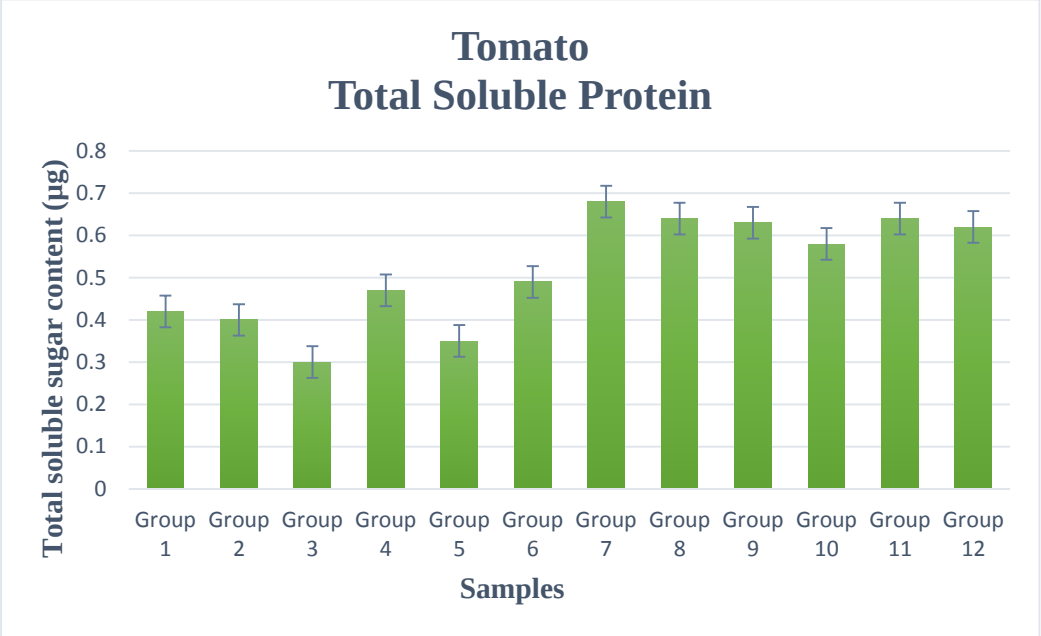

Figure 4.12: Effect of hydrogen peroxide (H_2O_2) pretreatment on total soluble protein content of ZnO treated tomato seedling. *Pretreatment of 1mM and 10mM H_2O_2 increase total soluble protein content in ZnO treated seedlings.*

4.4.3.2 Catalases (CAT) activity

To examine the effect of pretreatment of H_2O_2 and ZnO nanoparticles on antioxidant enzymes of plants. Catalases was measured by the method described by Aebi and Lester (1984). The effect on the activity of enzyme on the plants, can be analyzed by the decrease and increase in enzyme activity. In maize, plant of group 2 have increased by 16 % and group 3 have decreased protein concentration by 45.2 % with respect to control. Group 4 and group 6 have increased protein value by 3.2% and 52.6% but group 5 with 32.3% decrease. Catalases activity of group 7 and group 9 have increased with 10.6 %and 9.5%, respectively and group 8 decrease by 29.7%. Group 10, group 11 and group 12 showed 6.3%, 9.2% and 17 % decrease with respect to control. 1mM H_2O_2 increased CAT activity at low concentration, remain same at medium and decreased on highest concentration of ZnO nanoparticles and10mM H_2O_2 showed no effect on CAT activity at low concentration, slightly increased at medium and decreased on highest concentration of ZnO nanoparticles as compared to group

4, 5 and 6. Effects of hydrogen peroxide pretreatment on catalases activity of ZnO treated maize seedling leaves are presented in figure 4.13:

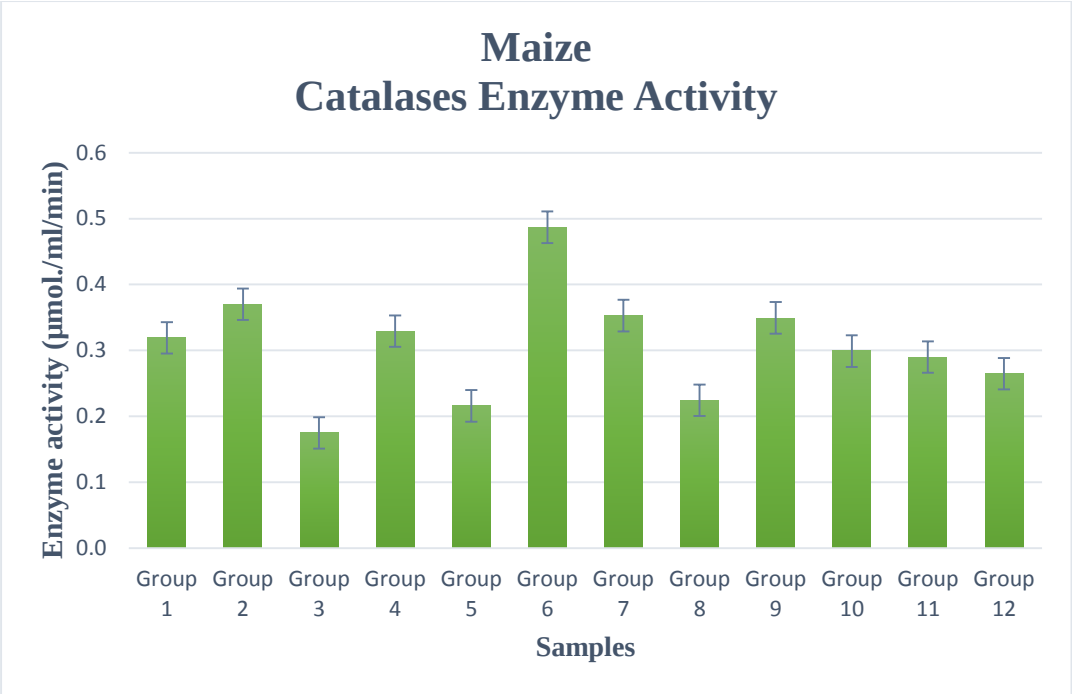

Figure 4.13: Effects of hydrogen peroxide (H_2O_2) pretreatment on catalases (CAT) activity of ZnO treated maize seedling leaves. *1mM H_2O_2 increases CAT activity at low concentration, remain same at medium and decrease on highest concentration and10mM H_2O_2 shows no effect on CAT activity at low concentration, slightly increase at medium and decrease on highest concentration of ZnO.*

Activity of catalases in tomato leaves decreased in all treated groups when compared to control. Group 2 decreased by 10%, group 3 decrease by 20%group 4 decrease by 36.2 %, group 5 by 48.6%, group 6 by51%, group 7 by 47.1%, group 8 by 43.7%, group 9 by 33.3%, group 10 by 69%group 11 by 25% and group 12 by 51.6 % with respect to control. 1mM H_2O_2 increased CAT activity with increase in concentration of ZnO and10mM H_2O_2 decreased CAT activity at low concentration, increases at medium and had no effect on highest concentration of ZnO nanoparticles when compared to the same concentrations of ZnO treated groups. Effects of

hydrogen peroxide pretreatment on catalases activity of ZnO treated tomato seedling leaves are presented in figure 4.14

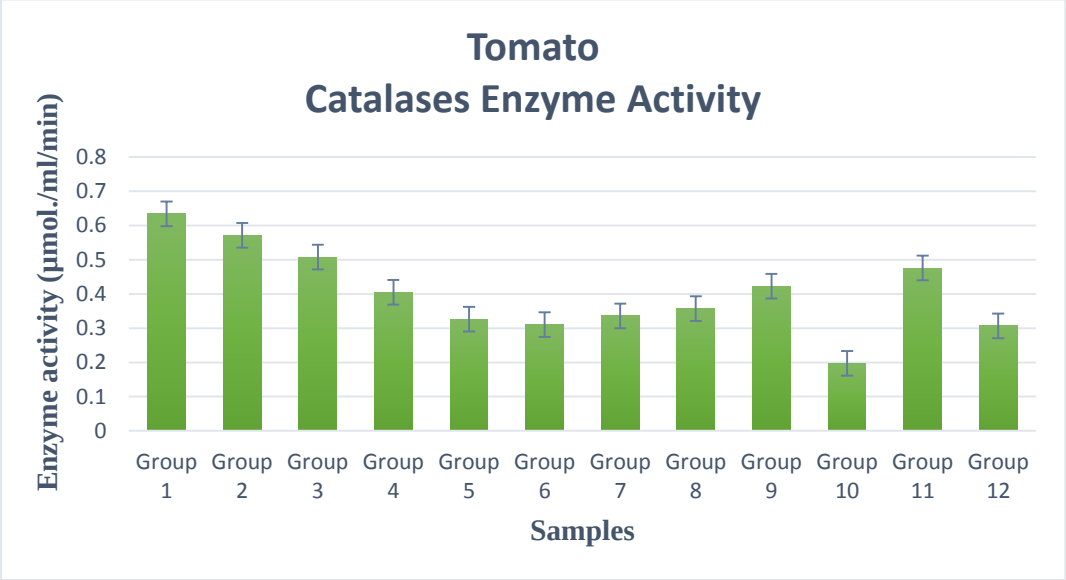

Figure 4.14: Effects of hydrogen peroxide (H_2O_2) pretreatment on catalases (CAT) activity of ZnO treated tomato seedling leaves. *1mM H_2O_2 increases CAT activity with increase in concentration and10mM H_2O_2 decreases CAT activity at low concentration, increases at medium and has no effect on highest concentration of ZnO.*

4.4.3.3 Ascorbate peroxidases (APX) activity

To scrutinize the effect of pretreatment of H_2O_2 and ZnO nanoparticles on biochemical parameters of plants on antioxidant enzymes of plants , ascorbate peroxidases was measured by the method described by Nakanoand Asada (1981). The effect on the activity of enzyme on the plants, can be analyzed by the decrease and increase in enzyme activity. Effects of H_2O_2 Pre-treatment on Ascorbate Peroxidase (APX) Activity of Maize Leaves was observed to analyze the enzyme activity. Plant of group 2 and group 3 have increased by 28.8 % and 14.6 % with respect to group 1 (control). Group 4 and group 5 have decreased activity value by 10.6% and 43.8% but group 6 with 9.6 % increased. Ascorbate peroxidases activity of group 7 has increased with 37.5%. Group 8 and group 9 decrease by 39.6% and

36.8 %. Group 10, group11 and group 12 showed 57.8%, 30.9% and 27.5% decrease with respect to group 1 (control). 1mM H_2O_2 increased ascorbate peroxide activity when treated with low concentration of ZnO, slightly increase in medium concentration and low at highest concentration of ZnO. 10mM H_2O_2 decreased ascorbate peroxide activity when treated with low and high concentrations of ZnO and slightly increase in medium concentration of ZnO. Effects of hydrogen peroxide pretreatment on Ascorbate peroxidases activity of ZnO treated maize seedling leaves are presented in figure 4.15

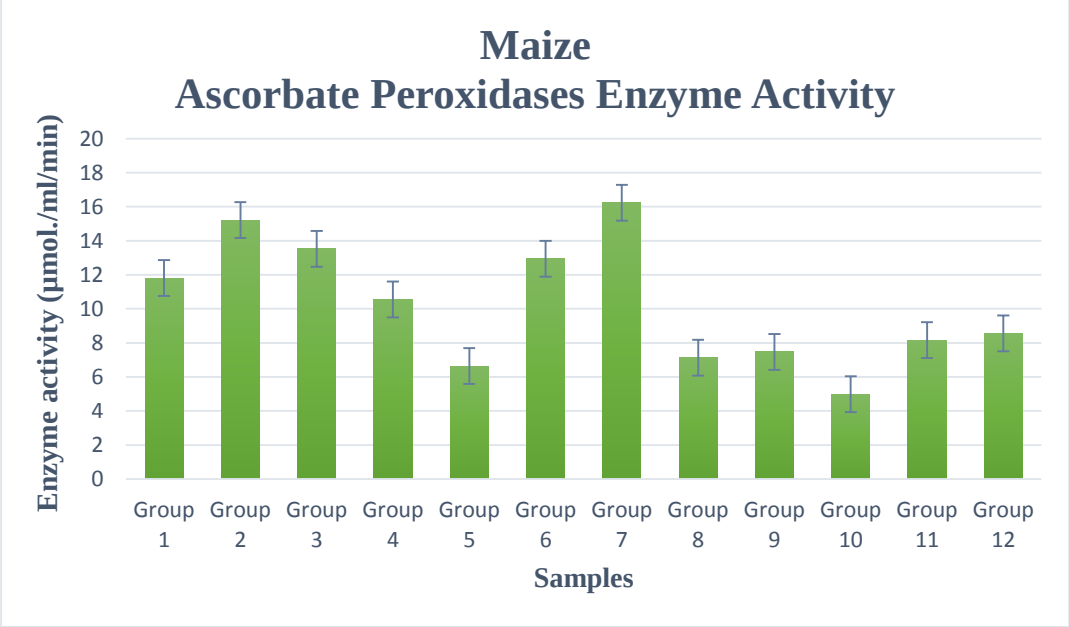

Figure 4.15: Effects of hydrogen peroxide (H_2O_2) pretreatment on Ascorbate peroxidases (APX) activity of ZnO treated maize seedling leaves*. 1mM H_2O_2 increases ascorbate peroxide activity when treated with low concentration of ZnO, slightly increase in medium concentration and low at highest concentration of ZnO. 10mM H_2O_2 decreases ascorbate peroxide activity when treated with low and high concentrations of ZnO and slightly increase in medium concentration of ZnO.*

In tomato, all the groups showed decrease in activity but only group 5 have increased activity. Activity of Group 2 decreased by 14.9%, group 3 decrease by 39.5 %group 4 decrease by 15.5 %, group 6 by28.2%, group 7 by 53.3%, group 8

by 52.1%, group 9 by 53.2%, group 10 by 53%group 11 by 61% and group 12 by 35.9 % with respect to control. Group 5 have 26.5% increased activity. 1mM H_2O_2 increased ascorbate peroxide activity when treated with low concentration of ZnO, slightly increased in medium concentration and low at highest concentration.10mM H_2O_2 decreased ascorbate peroxide activity when treated with low and high concentrations of ZnO and slightly increase in medium concentration. Effects of hydrogen peroxide pretreatment on Ascorbate peroxidases activity of ZnO treated tomato seedling leaves are presented in figure 4.16:

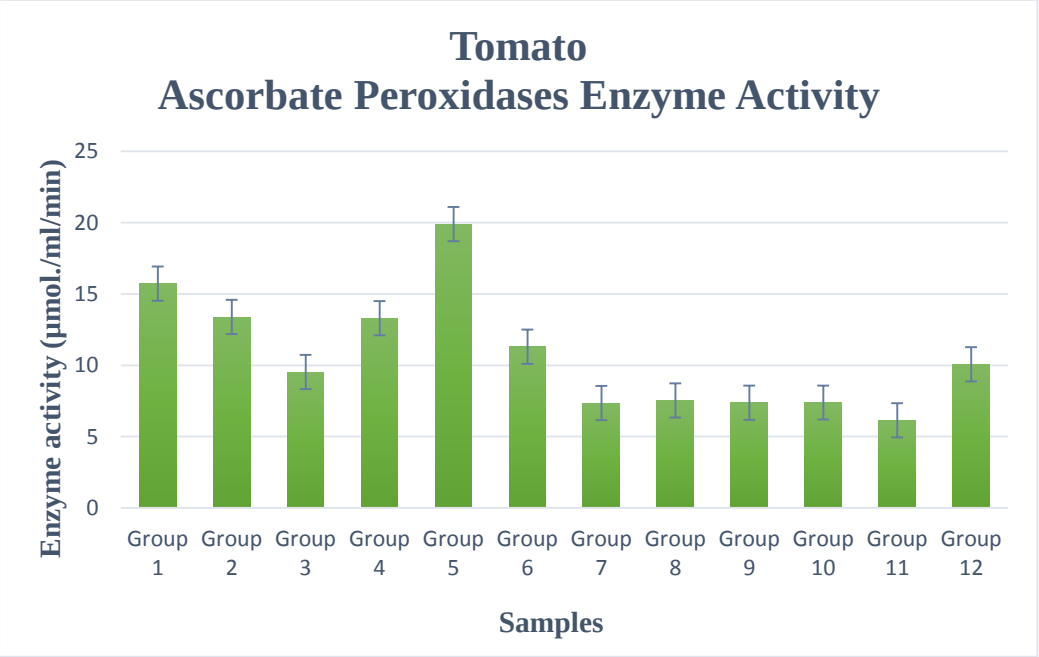

Figure 4.16: Effects of hydrogen peroxide (H_2O_2) pretreatment on Ascorbate peroxidases (APX) activity of ZnO treated tomato seedling leaves*. 1mM H_2O_2 increases ascorbate peroxide activity when treated with low concentration of ZnO, slightly increase in medium concentration and low at highest concentration.10mM H_2O_2 decreases ascorbate peroxide activity when treated with low and high concentrations of ZnO and slightly increase in medium concentration.*

4.4.3.4 Superoxide dismutase (SOD) activity

To examine the effect of pretreatment of H_2O_2 and ZnO nanoparticles on antioxidant enzymes of plants of maize , SOD was measured by the NBT method (Beauchamp and Fridovich, 1971).The effect on the activity of enzyme on the plants, can be analyzed by the decrease and increase in enzyme activity. Activity of SOD in maize plants decreased in all treated groups when compared to control. Group 2 decreased by 46.5%, group 3 decrease by 37.2%group 4 decrease by 45.7 %, group 5 by 65.6%, group 6 by55.9%, group 7 by 38%, group 8 by 34.8%, group 9 by 47.5%, group 10 by 45.2%group 11 by 50.7% and group 12 by 46.6 % with respect to control. 1mM H_2O_2 and 10mM H_2O_2 showed slightly increase in superoxide dismutase activity when treated with ZnO as compared to same concentrations of ZnO nanoparticles groups. Effects of hydrogen peroxide pretreatment on catalases activity of ZnO treated maize seedling leaves are presented in figure 4.17:

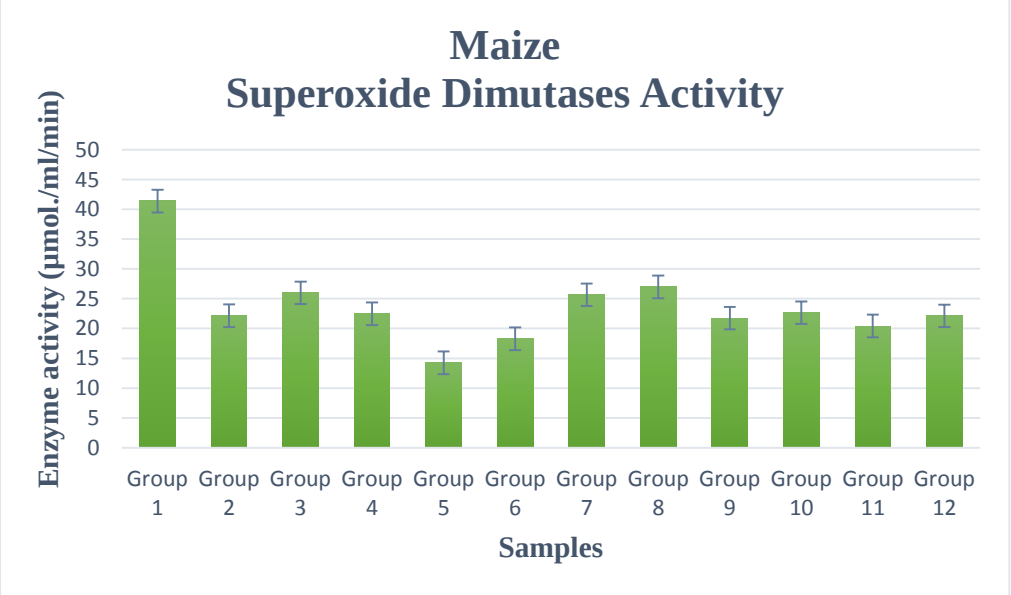

Figure 4.17: Effects of hydrogen peroxide (H_2O_2) pretreatment on superoxide dismutase (SOD) activity of ZnO treated maize seedling leaves. *1mM H_2O_2 and 10mM H_2O_2 shows slightly increase in superoxide dismutase activity when treated with ZnO*

In tomato, all the groups showed decrease in activity but only group 5 have increased activity. Activity of Group 2 decreased by 46%, group 3 decrease by 40.7 %group 4 decrease by 64.4 %, group 6 by71.2%, group 7 by 72.2%, group 8 by 76.2%, group 9 by 21.8%, group 10 by 42%group 11 by 79% and group 12 by 65 % with respect to control. Group 5 have 13% increased activity. 1mM H_2O_2 and 10mM H_2O_2 decreased superoxide dismutase activity when treated with ZnO as compared to their ZnO nanoparticles respective groups. Effects of hydrogen peroxide pretreatment on super oxide dismutase (SOD) activity of ZnO treated tomato seedling leaves are presented in figure 4.18

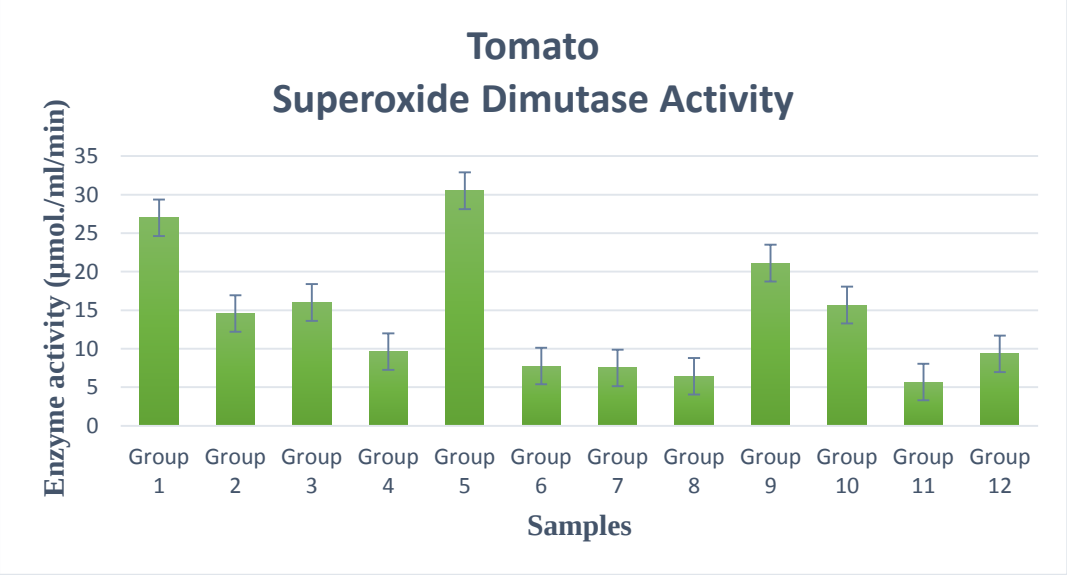

Figure 4.18: Effects of hydrogen peroxide (H_2O_2) pretreatment on superoxide dismutase (SOD) activity of ZnO treated tomato seedling leaves. *1mM H_2O_2 and 10mM H_2O_2 decreases superoxide dismutase activity when treated with ZnO.*

Chapter 5

DISCUSSION

Nanotechnology is an emerging field and due to production of unique and new products and materials provide development in all areas. Nanoparticles are being used in many consumer products and in many fields like medicine, cosmetics, fabrics, textile, paints, cement, and furniture industry and in many foods. Despite their use in many industries, research on nanoparticles is still going on the use of nanoparticle for the welfare of man and called engineered nanoparticles.

Engineered nanoparticles which are used in consumer industries are now released in the environment by direct and indirect means. By entering in the atmosphere, water bodies and terrestrial environment they enter in the food chain and then into the food web. Now it is important to know that these nanoparticles enter in the food chain, which are the ways they used to enter the food chain and whether they are harmful or beneficial to the environment and if they are harmful then how to prevent them or minimize their effect in the environment and to know about crop plants (monocot and dicot) which are more suitable in the presence of nanoparticles.

In the present study we selected the one monocot plant and one dicot plant. Monocot crop plant used for this study is maize, due to its importance in edible crops. It is the staple crop in many countries and widely used in many food products. And third most important crop which is growing globally (Guzel and Terzi, 2013).Among dicot, we picked the tomato plant because its importance in the research as main model system, and commercially used in food products (Helyes *et al.,* 2009). It is also beneficial health due to the presence of a compound lycopene (Gil *et al*., 2004). Plants cannot move from one place to another place like animals, and are re exposed to different stresses. These stress affect the plants, disturbed their

metabolism and affect their quality and quantity. Therefore it is important to investigate the effect of nanoparticle stress on plants and study the mechanism involved in response to these stresses and also the role of different chemical stress like H_2O_2 when combined to nanoparticles stress.

To date, no study on the combined effect of nanoparticles stress and chemical stress like H_2O_2 are present in literature. But there are some studies found in the literature on the effect of ZnO nanoparticles and H_2O_2, separately. This study scrutinized the effect of ZnO combined with the hydrogen peroxide. Results showed that hydrogen peroxide pretreatment to the plants decrease the toxicity of ZnO nanoparticles. Increase in root length, shoot length, chlorophyll content and sugar content and decrease in antioxidant enzyme activities were observed.

When ZnO combined with different concentration of hydrogen peroxide showed different results then the ZnO only. Results showed that the hydrogen peroxide have dual effect (positive and negative) on the plants. And there is a narrow range of hydrogen peroxide concentration in which hydrogen peroxide work as secondary messenger but at higher concentration it may be cytotoxic (Christman *et al*., 1985; Greenberg *et al.*, 1990).

5.1 Germination Rate

Results showed that there is no effect on seed Germination of maize and in tomato higher germination showed in hydrogen peroxide treated groups. This is because the hydrogen peroxide may break the dormancy of seeds and the dormancy of seed which is caused by the environment and also called as secondary dormancy. Fontaine *et al*. (1994) showed that the oxidants like hydrogen peroxide can be used to break the dormancy of the seeds.

5.2 Physiological Parameters

To know the effects of ZnO nanoparticles, study of physiology is important and primary indicator for the toxicity because it gives the visual clue whether plants are under stress or not. In this study results showed that the ZnO oxide have non-toxic effect on plants at low concentration but toxic effect increased with the increase in concentration. The toxicity of ZnO depends on the concentration. Results showed no specific differences in monocot (maize) and dicot (tomato). At low concentration ZnO nanoparticles showed positive effect on the plants. Because zinc is the essential element, plays a crucial role in the plant growth and development and is a vital element for enzyme to perform their activities (Vallee, 1976). Required concentration of zinc for the growth of leaves is 15 to 20 mg per kg (Broadley *et al*., 2007). But when zinc exceed the required quantity can be lead to the toxicity of plants. The intensity of toxicity related to the concentration of nanoparticles and uptake of zinc by the plants (Takkar and Mann, 1978; Fang and Kao, 2000; Vaillant *et al*., 2005; Broadley *et al*., 2007). In the combination of zinc oxide with low concentration of hydrogen peroxide, plants showed high growth rate then the control and group 4 5 and 6. At highest concentration of hydrogen peroxide and zinc oxide plant growth became reduced due to high stress.

5.3 Biochemical Parameters

5.3.1 Chlorophyll content

Chlorophyll is the key component of light harvesting complex and an index of those components present in the chloroplast membrane of electron transport complex (Terry, 1983). In stress chlorosis starts due to decrease in chlorophyll content. Result showed that when maize and tomato plants exposed to the high concentration of ZnO chlorophyll content reduced as the concentration became high with respect to control.in combination groups (group 7 8, 9, 10, 11 and 12). Chlorophyll content increased except group 12 compared to the group 4, group 5 and 6. Group 12 is under stress due to highest concentration of ZnO and hydrogen peroxide (10mM). In tomato all combination group showed highest concentration which means that the hydrogen peroxide reduced the ZnO stress.

5.3.2 Sugar content

Plants showed accumulation of different metabolites such as soluble sugar under stress conditions Study showed the Sugar content, proline content, and amines (Seki *et al.,* 2007). In maize, increase in various groups showed that the plants were under stress while in tomato sugar content decrease in ZnO nanoparticles groups with respect to control. And increase in combinations groups except group 11 with respect to group 4, group 5 and group 6.

5.3.3 Antioxidant enzymes activities

Reactive oxygen species is the foremost defense system when biotic and abiotic stresses applied to plants. During stresses, over production of reactive oxygen species occur and antioxidant enzymes protect the plants from over produced reactive oxygen species which cause cellular and oxidative damage (Fu *et al.*, 2014). In antioxidant defense systems Catalases (CAT), ascorbate peroxidases (APX), and superoxide mutases (SOD) are vital enzyme which eliminate H_2O_2 (hydrogen peroxide) and other ROS from the plants (Wang, 1988; Scandalios, 1993).

Catalase (CAT) is a hydrogen peroxide scavenging, tetrameric and, heme-containing enzyme and present in the cell cytosol peroxisomes, mitochondria, glyoxisome, and root nodules of the plants. Hence, catalase remove the hydrogen peroxide to protect the cell. At high concentration of ZnO catalases have high activity then control. In tomato catalases activity decrease with the increase in concentration may be due long duration stress of nanoparticles. When ZnO applied to the plants for a long time reactive oxygen produced and this reactive oxygen inhibits the protein of enzyme and may decrease the catalases activity (Cakmak and Marschner, 1988). Wheat (*Triticum aestivum*) plants showed higher CAT activity when exposed to CuO than the control plants. While, when plants exposed to zno nanoparticles showed reduced catalases activity (Dimkpa *et al*., 2012). In maize

combination showed low activity of catalases then group 4 5 and 6. In stress condition catalases showed high activity. In maize catalases showed low activity in combination groups then the ZnO stress groups only. But in tomato high activity in combinations groups except in group 10 and group 12 was observed. Catalases have a vital role in reduce the ROS effect. In stress conditions catalases shows high activity then the normal.

Ascorbate peroxidase (APX)belongs to algae and plants only that is plays an important role to defense the chloroplast and other cell organelles from destruction caused by hydrogen peroxide (H_2O_2) and hydroxyl radical (OH). Maize have high activity of ascorbate peroxidases at high concentration then the control. In combination groups, group 7 showed highest APX activity and all other groups showed low activity of APX enzyme as compared to control and group 4, group 5 and group 6. In tomato ascorbate peroxidases activity increase in group 5. This is the threshold for the enzyme activity and high amount of ZnO cause decrease in activity. All combinations showed the low activity of APX then control and group 4, group 5 and group 6.

Superoxide dismutase (SOD) belongs to the group of metalloisozymes. It plays an important role by protecting the cell against oxidative stress. Highly reactive O_2 neutralized by SOD enzyme into O_2 and H_2O_2. Sod have low activity in maize then control plants this is because of long term stress effect may cause inhibit the enzyme protein and decrease the activity of SOD. But in tomato results showed the highest activity in group 5 with respect to control. Combination groups showed low activity of SOD with respect to group 4, group 5 and group 6. SOD detoxify the reactive oxygen and covert it to the hydrogen peroxide which is toxic. It is necessary to remove hydrogen peroxide by changing it to water (De Azevedo Neto *et al*., 2005).

Results showed different results at different concentration due to the beneficial effects of ZnO , high concentration cause stress to the plants and sometimes more high concentration exceed the threshold level of the ZnO which

may cause the low activity of enzyme. Result displayed the positive effect on the pretreated hydrogen peroxide plants when exposed to ZnO nanoparticles. Results also showed no specific differences between monocot and dicot.

5.4 Conclusion

This study was intended to know the combined effect of ZnO nanoparticles and hydrogen peroxide and the comparative analysis of their effect on the monocot and dicot. The hydrogen peroxide reduced the toxicity of ZnO peroxide on the physiology and biochemical parameters. Germination rate, shoot length and root length indicate that the toxicity of ZnO depends on the concentration of nanoparticles plants exposed to different concentration showed different results. At high concentration reduction in root length occur and when combined to hydrogen peroxide, plants show better results than ZnO nanoparticles only. In the presence of ZnO nanoparticles antioxidant enzyme activity increase but when combined to the hydrogen peroxide antioxidant enzymes showed low activity. Results showed no significant differences between monocot plants and dicot plants.

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