Investigating the role of exogenous Hydrogen Peroxide in nanotoxicity reduction in *Capsicum annuum L.* and its hybrids

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بسم الله الرحمن الرحيم

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Dedication

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Abstract

Concentration of nanoparticles in environment is increasing day by day due to their increasing use in consumer products that causes pollution and adverse effects on the environment. Nanoparticles adverse effects on ecosystem leading to toxicity in crop plants when released through: manufacturing goods, usage and disposal. Further the transfer of nano sized particles into the food chain by eatable plants is very significant. Nanoparticles enter the plant cells and cause imbalance and disturbance in anti-oxidant processes and induce oxidative stress leading to cell damage. Hence it is important to study effects of nanoparticles on plants because they are stationary organisms and cannot move away from environmental stresses like animals. To date, very few nanoparticles and plant species have been studied, mainly at early growth stages of the plants; whereas there has been no comparative research on role of hydrogen peroxide (H_2O_2) when given in combination with nanoparticles on hybrid plants and its parents. H_2O_2 generate Reactive Oxygen Species (ROS) that trigger scavenging antioxidant enzymes in plant cells to repair the impairment. This strategy helps plant to survive the biotic and abiotic stress. In this research we investigated effects of various doses of H_2O_2 in combination with Titanium dioxide nanoparticles on *Capsicum annuum L*. hybrid and the parents and their effects are studied at physiological, and biochemical levels such as chlorophyll content, sugar content and anti-oxidant enzyme activities of plants. We find out the H_2O_2 can act as oxidative tool to reduce biochemical loss in plants.

Key words: *Nanoparticles, H_2O_2, Capsicum annuum, ROS, antioxidant enzymes*

LIST OF ACRONYMS

%	Percentage
µl	Micro liter
µmol	Micro mole
AOS	Active oxygen scavenging
APX	Ascorbate peroxidase
BSA	Bovine Serum Albumin
C. annuum	*Capsicum annuum L*
ca.	Approximately
CAT	Catalase
EDTA	Ethylenediamine Tetraacetic Acid
ETC	Electron transport chain
gFW	gram per fresh weight
H_2O_2	Hydrogen Peroxide
Hr	Hours
MC	Moisture Content
MDA	Malondialdehyde
mg	Milligram
min	Minutes
ml	Milliliter
mM	Mili mole
MWCNTs	multiwalled carbon nanotubes
NaOCl	Sodium hypochlorite
NBT	Nitro Blue Tetrazolium
nM	Nano mole
nm	Nano meter
$\bullet O_2$	Superoxide radical
O^-	Superoxide ion

LIST OF ACRONYMS

OD	Optical density
OH	Hydroxyl ion
pmol	Pico mole
POX	Peroxidases
PCD	Programmed cell death
QD	Quantum dots
ppb	Parts per billion
ROS	Reactive Oxygen Species
rpm	revolutions per minute
SOD	Superoxide Dismutase
TiO_2	Titanium dioxide
UV	Ultra Violet
XRD	X-ray Diffraction

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1 INTRODUCTION

Hydrogen peroxide (H_2O_2) is generally known as signaling molecule for plants, facilitating the attainment of resistance against abiotic stresses and acquired tolerance (Slesak et al., 2007, Ahmad et al., 2013, Wahid et al., 2007). The increased cellular concentrations of Reactive Oxygen Species (ROS) and the subsequent conversion of ROS into H_2O_2 is one of the consequences among numerous other stresses. It was concluded through extensive research that oxidative stress is induced due to high concentration of H_2O_2 when applied exogenously whereas if low concentration of H_2O_2 is applied, it increase the tolerance to abiotic stress by improving the antioxidant activities in maize (Chen et al., 2009b, Wang et al., 2010, Ahmad et al., 2012) and *Brassica juncea* (Ahmad et al., 2013) and the resistance against salt stress was induced by boosted actions of antioxidants and by decreasing the peroxidation of membrane lipids present in the roots and leaves of the maize (Ahmad et al., 2013, de Azevedo Neto et al., 2005).

In future the chances of manifestation of abiotic stresses will be increased due to fluctuating climatic factors. Plants are exposed to multiple biotic and abiotic stresses simultaneously. Plants bearing two or more autonomously arising stresses, essentially do not need to tolerate them when they arise instantaneously (Collins et al., 2008, Mittler, 2006, Mittler and Blumwald, 2010, Nostar et al., 2013, Atkinson and Urwin, 2012, Pandey et al., 2015). The damage is occurred in stress due to activation of reactive oxidative species for instance when the temperature is reduced to 10 ^{0}C in *Capsicum annuum L.* (*C. annuum*), oxidative damage to numerous cellular structures and macromolecules were caused due to activation of ROS (Apel and Hirt, 2004, Marocco et al., 2005) as a result poor seedlings were produced (Guan et al., 2009). Numerous endogenous resistance mechanisms against ROS comprise of enzymes, such as Catalase (CAT), Peroxidase (POD) and Superoxide Dismutase (Noctor and Foyer, 1998, Ahmad et al., 2013)

Fundamentally, all abiotic and biotic stresses are induced or involved in oxidative stress to some extent and the capability of plants to regulate oxidant intensities is greatly

associated with the stress tolerance. It is also recognized that oxidative metabolism, and principally H_2O_2, is convoluted to an extensive diversity of responses and signaling cascades essential for all phases of plant development and the integration of action, fluctuating from the growth of individual root hairs, to lignification and differentiation of xylem, to cross-linking and loosening of wall, to coordination of shoot/root and to control stomata. Consequently, the contribution of H_2O_2 in stress conditions is very significant, it certainly must be deliberated in the perspective of, and even as a distinct case; the involvement of H_2O_2 in "standard" development in metabolism (Cheeseman, 2007)

H_2O_2 is basically not a free radical but it is hypothetically reactive oxygen and the product of two electron reduction molecules of superoxide radical ($\bullet O_2$) (Halliwell et al., 2000, Cheeseman, 2007). H_2O_2 is moderately "harmless" as compared to hydroxyl radical, (•OH) and $\bullet O_2$, when the transition metals are not present, it is unreactive and nontoxic even at increased concentrations than a biotic system would ever produce. Practically, this imparts on it more movement within the tissues, and the possible utilization as a substrate in different reactions and also as a molecule for the activation of ROS (Cheeseman, 2007).

Conversely, H_2O_2 is possibly somewhat reactive with other transition metals or molecules comprising Fe^{2+} through the Fenton reaction (Becana et al., 1998). The toxicity of H_2O_2 is due to the bond dissociation of hydrogen peroxide into •OH, that's why it is the worst drawback of this reaction. For instance, Rubisco is inhibited by the exogenous hydrogen peroxide because it causes the disintegration of the LSU at glycine present in the site of catalysis (Ishida et al., 1999). The direct reaction of the –SH groups with H_2O_2 has been reported in previous studies as the system by which the inactivation of fructose bisphosphatase occur due to H_2O_2 in chloroplasts (Charles and Halliwell, 1980, Charles and Halliwell, 1981). The generation of •OH is prevented by enzymatically (by using ascorbate peroxidase or catalase) removing the H_2O_2 in order to reduce the toxicity of H_2O_2 (Toda, 2005, Andrade et al., 2006).

It is reported by Hernández et al. (2001) that at the lower end, levels of tissue that range from 10-150 pmol/gFW in the apoplast of pea leaf with the variation (induced by salt) is

adequate to cause oxidative abrasions. On the other hand, He et al. (2005) stated that in the leaves of *Poa pratensis* the concentration is 1.3% of the dry weight of leaves. The data on which their report was based is ca. 100 mM or 60 μmol/gFW on the basis of water present in leaves. By contrast, the plants grown in fields were analyzed with care to justify the probable interventions as well as sustained metabolism of H_2O_2 after harvesting and it was proposed that values that range from 1-5 μmol/gFW may be normal (Cheeseman, 2006). The tobacco leaf (cv. Xanthi) when critically exposed to ozone (200 ppb/2 hr), the levels of H_2O_2 had raised ca. 4x (in the range of 100 nmol/gFW) reported by Chen and Gallie (2005).

In an aerobic atmosphere ROS and H_2O_2 are usually very important elements of life (Møller, 2001). Main sources involve backfires in the ETC (electron transport chain) of mitochondria and chloroplasts, a wide diversity of limited substrate oxidases for example type III peroxidases, oxidases of NADPH and glycolate oxidase present in peroxisomes as well as the Mehler reaction (Halliwel and Gutteridge, 1999). H_2O_2 is directly produced by some of these substances for example; limited substrate oxidases while others require some additional reactive intermediates (i.e. $\bullet O_2$) in order to produce H_2O_2. Generally, these events that result in production of H_2O_2 are boosted by various biotic and abiotic stresses (Alscher et al., 1997, Bolwell, 1999), even though they are reported as an essential part of several plant developmental processes.

H_2O_2 is in general rapidly utilized by catalase as it is produced in glyoxisomes and peroxisomes, while isoforms of APX3 (ascorbate peroxidase) confined to the organelles might also play role in detoxification of H_2O_2 (Wang et al., 1999). The association between signaling network and H_2O_2 has been broadly recognized for various stress responses, containing wounding, insect feeding, high temperature, pathogen elicitors and closing of stomata related to ABA (Larkindale and Knight, 2002, Apel and Hirt, 2004, Peng et al., 2004, Mateo et al., 2006).

In several recent studies many new characteristics and properties of H_2O_2 are discussed in various crops i.e. seedlings of maize when exposed to chilling stress, results in endogenous H_2O_2 production in plant tissues and cells, but the resistance and tolerance of maize seedlings is enhanced by exogenously applied H_2O_2 against the chilling stress.

In seedlings of maize this rapid defense is generated as a result of improved anti-oxidative system and the enzymes of anti-oxidant system don't let accumulation of ROS against this stress (Prasad et al., 1994). The capability of rice seedlings is improved by pre-treating with different concentrations of H_2O_2 against combined stress of heat and salt (Uchida et al., 2002). Anti-oxidative enzyme is directly or indirectly activated by hydrogen peroxide against the salt stress (Liang et al., 2003). Hence, the pre-treatment of H_2O_2 behaves as a signal molecule and responsible for generating the resistance against various stresses i.e. heat, salt and chilling sensitive genotype of maize, drought (Gong et al., 2001, Prasad et al., 1994), and in rice against heat stress (Uchida et al., 2002).

Nanoparticles also act as abiotic stress in several plants depending on size, type, physical and chemical properties of nanoparticles as well as plant species. The astonishing biological effects i.e. toxicity could be due to the distinctive properties of nanoparticles. Certain previous studies have revealed that nanoparticles have poisonous effects on microorganisms (Jones et al., 2008, Rai et al., 2009, Song et al., 2013), animals (Griffitt et al., 2007, Bar-Ilan et al., 2009) and plants (Lee et al., 2008, Castiglione et al., 2011, Geisler-Lee et al., 2012). Several nanoparticles such as nanoparticles of silver are even synthesized for their lethal properties (e.g. antibacterial activity). Conversely, in spite of latest advancement in understanding the environmental and health concerns of nanoparticles, the toxicity of some nanoparticles is still argumentative and contradictory results are often described. Consequently, for upcoming research certain challenges are still needed to be accomplished (Wiesner et al., 2006, Song et al., 2013). Moreover, while there are numerous studies on the toxic effects of nanoparticles in bacteria and animals, studies evaluating the toxic effects of nano-materials in advanced plants are inadequate (Lin and Xing, 2007, Castiglione et al., 2011, Song et al., 2013).

The nanoparticles of titanium dioxide (TiO_2) are produced globally in large amounts in order to produce cosmetics, particularly sun block, that contains TiO_2 nanoparticles which help to guard the skin from ultra violet rays (Song et al., 2013, Trouiller et al., 2009). TiO_2 nanoparticles are also extensively used for the production of antibacterial products and to decompose organic material in waste water (Castiglione et al., 2011,

Song et al., 2013). Numerous articles described the toxicity of TiO_2 nanoparticles in animals, i.e. cytotoxicity (Kang et al., 2008) DNA damage (Trouiller et al., 2009, Song et al., 2013) and neurotoxicity (Long et al., 2006). Conversely, several studies, inspecting the genotoxicity, cytotoxicity, sensitization and acute toxicity have stated reverse consequences, discovered no toxicity (Nohynek et al., 2008) and no indication of substantial penetration of TiO_2 nanoparticles was found (Newman et al., 2009). The toxic effects of nanoparticles of titanium dioxide in animals are still controversial. Therefore, the biological effects of TiO_2 nanoparticles are further need to be analyzed (Newman et al., 2009). In recent studies only few articles are investigating the properties of TiO_2 nanoparticles on plants (Castiglione et al., 2011, Hong et al., 2005, Zheng et al., 2005, Yang et al., 2006a, Song et al., 2013).

An increased demand to engineer nanoparticles is generated due to several applications of nanotechnology. It is estimated that by 2020, the value for the products of nanotechnology will exceed to 3 trillion U.S. dollars (Roco, 2011, Servin et al., 2013). The transfer of nano sized particles into the food chain by eatable plants is very significant. The vegetables that can be grown in gardens may be accompanying with nanoparticles by direct application of agrichemicals and bio solids (Servin et al., 2013).

It is indicated by latest studies that TiO_2 is the most extensively used nanoparticle with up to 10,000 tons per year of universal production (Piccinno et al., 2012). Almost 50−80% of the overall production of nanoparticles of TiO_2 is utilized in sunscreen industries and cosmetics industries (Kaida et al., 2003, Klaine et al., 2008). Among the various other applications, the nanoparticles of TiO_2 have been utilized in plastics, coatings, cement, paints (Kaida et al., 2003) and as a photocatalyst (Nakata and Fujishima, 2012).

It was investigated in earlier studies that TiO_2 nanoparticles can be associated with leading toxicological harms (Rothen-Rutishauser et al., 2006). Experiments were conducted on lung cells of humans *in vitro* and was revealed that cell growth was inhibited by nanoparticles of TiO_2 in anatase crystalline phase and oxidative stress was induced (Gurr et al., 2005), intracellular contents of cytokine was enhanced (Val et al., 2009) and cell death was occurred due to intrinsic apoptotic pathway (Shi et al., 2010).

Several other reports have also exposed that plants and bacterial species can also be affected by the TiO_2 nanoparticles (Servin et al., 2013).

TiO_2 nanoparticles improved the malondialdehyde level, which acts as an indicator of lipid peroxidation in *Nicotiana tabacum* and *Allium cepa*, signifying that DNA damage could be occurred (Ghosh et al., 2010). Larue et al. (2012) stated that nanoparticles of TiO_2 were accumulated in shoots and roots of wheat (*Triticum aestivum*) grown in hydroponic conditions. Likewise, in a prior research, we verified that plants of cucumber (*Cucumis sativus*) uptake nanoparticles of TiO_2 from the roots and transport them to the leaf trichomes (Servin et al., 2013).

Nanoparticles are specific particles and the size of the nanoparticles ranges between 1-1000 nm. There are several applications of nanotechnology in various research fields i.e. medicine, agriculture, etc. Nowadays, nanoparticles of TiO_2 are broadly used in industries (Mohanraj and Chen, 2006, Samadi et al., 2014). These nanoparticles generally exist in crystalline and non-crystalline forms, among crystalline forms these particles exist with three titles: anatase, rutile and brukite. Nanoparticles of TiO_2 are applied in layered surfaces, disinfectant sprays, dipole electron tubes, sporting goods, optical module etc. The physiologic parameters of plants and biological systems are affected by these particles. Antioxidant system which can enhance the water absorbing and utilizing abilities can be stimulated by nanoparticles of TiO_2 (Lin and Xing, 2007, Samadi et al., 2014).

The 9^{th} most abundant element found in the earth is titanium (Feizi et al., 2013). TiO_2 belongs to the transition metal oxide family and is the oxide of the titanium metal (Li et al., 2010). TiO_2 is thermally stable but it is not soluble in water (Samadi et al., 2014). Recently the discipline of nanotechnology has started taking its roots in agriculture and plant biotechnology (Pérez-de-Luque and Rubiales, 2009). Nano-sensors are a good example; they are indeed very promising for the delivery of small materials into the plants. More over nano-fertilizres have been developed which promises new and efficient method for nutrient delivery to the fields (Torney et al., 2007). But with all this glory the technology has not been tested in case of animals and plants.

It is suspected and has been understood that these small although beneficial nano-

materials can accumulate in plants and animals and pass on into humans (Lin and Xing, 2007). Studies conducted on translocation of these elusive nano-materials on various crops and plants helps us to understand the route taken by these chemicals. If they accumulate in our food chain then consequences could be chaotic (González-Melendi et al., 2008). It has been reported that magnetic nanoparticles have translocated into the leaves of cucumber plant (Corredor et al., 2009). Both in animal or plant the risk exists and thorough studies are needed (González-Melendi et al., 2008).

The compulsive force behind this huge investment is the small size of these nanoparticles, moreover, surface structures also play important role in alluring huge sums of money (Corredor et al., 2009). Some materials with dimensions less than 5 nm show various properties like electrical conductivity optical and magnetic properties and different reactivates which big size particles of the same materials does not possess (Rawat et al., 2006). Due to small size, these nanoparticles show large surface area, which in turn gives more reactivity (Yih and Al-Fandi, 2006). Besides this study on fish, rats and nematodes have also shed light on toxicity (Testillano et al., 2002). In terms of toxicity and its effect a great deal of work has been done in aquatic organisms. Others have also repotted data and study on the effects of these nanoparticles on roots and germination levels of plants (Lam et al., 2004). As plants face these toxic particles ROS species are produced.

In current study we selected *Capsicum annuum L. (C. annuum)* as it is a significant and efficient economical vegetable. In 2013, the genome of *C. annuum* is completely sequenced and the size of the genome is approximately 3.26 Gb (Qin et al., 2014). It can be used as fresh vegetable as well as numerous processing industries to produce products such as ground pepper, pepper sauce, dried pepper and pickled pepper. The genus *Capsicum* belongs to *Solanaceae* family and it has 2n=24 number of chromosome. After tomato and potato *Capsicum annuum* L. is the third most significant crop of *sloanaceae* family. In various areas across the world only 5 species (*C. chinense, C. pubescens, Capsicum annuum, Capsicum baccatum and C. frutescens*) are cultured and grown among all the other 20 to 27 (Tong and Bosland, 1999, Bosland and Votava, 2000). Commonly only two species of *Capsicum* i.e. *Capsicum annuum* L (hot pepper) and *Capsicum frutescens* L (sweet pepper) are cultivated in Pakistan. *Capsicum*

annuum L. being a summer vegetable is the most widely and commonly cultivated species in Sindh and southern Punjab. In Pakistan, the area under chili production is 62.7 thousand hectares and the yield harvested from this area 150.3 thousand tons while the average yield obtained is 2.7 tons/ha (Farooq, 2013)

The hybrids of plants are developed by adopting selective breeding methods that makes them heritably more vigorous and superior characters than both the parent plants. The phenomenon in which F_1 generation exhibit diverse or superior characters than the parent plants is termed as hybrid vigor. The uniformity, advancement and crop efficiency can be exploited by using hybrid vigor as an influential tool. Refaat and Elgarhy (2007) described that the yield obtained from hybrid pepper is improved 200 to 300 percent. Fruit color, fruit shapes and the quality of fruit were also enhanced. Enhancement in quality of fruit by hybridization of chili was stated by Milerue et al. (2000). It was revealed by Ganesh Reddy et al. (2010) that resistance against diseases i.e. caused by viruses was increased due to hybridization of chili. It was observed that F_1 hybrid pepper was competitively superior and more fruitful than parents. Till date there has been no comparative research on role of H_2O_2 when given in combination with nanoparticles on hybrid plants and its parents. In current research project we investigated, either ROS is produced endogenously by exogenously applied H_2O_2? If so is it enabling the plant to tolerate stress caused by nanoparticles? In this research we take a step forward to find solutions for reducing the emerging nanotoxicty in plants and help us to understand the mechanisms used by hybrid to perform better and survive in abiotic stress conditions. The objective of this research is to find the role of pretreated H_2O_2 in overcoming nanotoxicity on *C. annuum* hybrid and its parents.

The objectives of the study are to determine if (1) hybrids, in general, are more resistant to nanoparticles induced toxicity than its inbred parents, (2) pre-treatment of H_2O_2 could increase resistance against nanotoxicity in plants and hybrids; and (3) if hybrids exhibit more resistance to nanotoxicity on pre-treatment with H_2O_2 than both the parents.

The methodology of the study comprises of selection and genetic crossings of two inbred lines of chilli plant to produce hybrids. Both the hybrids and the parents would

be pre-treated with H_2O_2 four weeks-post germination. Following the pre-treatment, the hybrids and parents will be treated with TiO_2 nanoparticles. Samples will be taken from both the treated and control groups and a number of physical and biochemical assays will be performed to determine if the pre-treatment with H_2O_2 has facilitated resistance against the established nanotoxicity. Molecular analyses will also be performed to determine the differential expression of various stress related genes in H_2O_2 pre-treated hybrids and the parents in comparison to the control group that is not pre-treated with H_2O_2.

Outcomes of this study would help us to understand if signalling molecules like H_2O_2 could play positive roles in overcoming the stress caused by the nanoparticles in plants; and if the hybrids are more resistant to stress caused by nanotoxicity than the inbred lines.

2 REVIEW OF LITERATURE

2.1 Applications of Nano Biotechnology:

Nano biotechnology has numerous applications among them the most renowned and frequently used application is delivery of materials (Rieux et al., 2006). These particles were evolved around 1970 originally used and intended for therapeutic use against cancer and as vaccines carriers (Kumar, 2000). Now these tiny particles are used extensively for delivery of materials like drugs into living tissues of organisms. As a result these items and this technology have turned out to be a leading edge in the research (Hans and Lowman, 2002). Nanoparticles generally have a nano size ranging from 10 to 100 nm. The compass of nanotechnology is not restricted only to health associated components but has also been broaden to commercial and industrial products (Franklin et al., 2007). Consequently, structure of particles when contrasted with bulk of the same materials, contributes in changing physical-chemical properties of nanoparticles (Nel et al., 2006). Agricultural sector is also improving by nanotechnology through range of applications, such as nanocides that are used for the treatment of plant diseases and producing energy from food and agricultural waste (Moraru et al., 2003).

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 are easily absorbed by plant roots and translocated to different tissues of plants. Zinc oxide (ZnO) nanoparticles are used as nano-fertilizer in the form of colloidal solutions. Nano-fertilizers are used in very small quantity in comparison to 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).

2.2 Engineering of Nanoparticles in Industry:

Engineered nanoparticles are well-known and extensively used as they are prepared specifically for a particular task and have discrete chemical properties that make them different from others, hence appealing for commercialization (Medina et al., 2007). Engineered nanoparticles established their way into food, agriculture, health and technology. Their usage is influenced by different surface, size and chemical properties. Due to the minute size of less than 100 nm, here is a likelihood that they can transfer from lungs to systemic areas, piercing natural barriers and resulting in toxic effects (Medina et al., 2007). As these particles can cause problems to the health, studies are required to perceive their effects. In the field of medicine, their application is increasing and because of being proficient delivery systems, there is no other option available. Besides this, cellular toxicity needs to be determined in order to validate health and other field applications (Ngoy et al., 2011).

2.3 Toxicity Caused by Nano-Materials in Living Organisms:

2.3.1 Nanoparticles in Humans and Animals:

Some studies have reported accumulation of Titanium Dioxide (TiO_2) nanoparticles in the liver, lungs and kidneys in mice when injected with TiO_2 nanoparticles (Chen et al., 2009a). In other studies, inhalation was the main source and exposed rats showed phagocytosis in lungs as a result of nanoparticles. (Wang et al., 2007)reported that 5g/Kg dose of TiO_2 nanoparticles with size of 80 nm could cause hepatic injury. Accumulation of TiO_2 nanoparticles in liver lungs and kidney was observed at a dose of 5mg/kg (Fabian et al., 2008). Studies by Chen et al. (2006) have shown that copper nanoparticles cytotoxicity can trigger injuries on the lymph, payer's patches, liver, spleen and kidney of the experimental animals. Other symptoms associated with the toxicity were related to alimentary canal such as loss of appetite, vomiting and diarrhea. Others included tremor and arching of the back (Chen et al., 2006).

2.3.2 Nanoparticles in Plants:

Plant transformation by bombardment with gold coated with DNA is a good example of nano scale materials application (Taylor and Fauquet, 2002). Other studies of Torney et

al. (2007) showed Mesoporus silca nanoparticles can control the release of certain particles into the protoplast. Carbon coated nano-materials have translocated in plants. The purpose for this is to release certain chemicals to control the effect of certain infections (González-Melendi et al., 2008).

Agriculture has observed great use of nanoparticles in few years, it has been reported that CeO_2 particles are easily taken up by plants and are translocated. Presence of these nanoparticles has been confirmed in tomato, corn, soybean and cucumber (Zhang et al., 2012). Nanoparticles interactive with plants and can become the component of the ecosystem (Battke et al., 2008). As a result, this interaction can be an issue and cause accumulation of nanoparticles leading to biomagnifications. These particles can attach themselves with the roots when dilevered to soil. As a result toxic effects can be created (Battke et al., 2008). There is increase trend of phyto toxicity occurrence with increasing use of nanoparticles (Lin et al., 2009). Moreover it was reported that Zinc oxide (ZnO) inhibited several developmental stages including seed germination (Lin and Xing, 2007). Copper nanoparticles were toxic for two different crops, wheat and mung bean, evident by retarded growth of the seedlings (Lee et al., 2008).

2.4 Toxicity Caused by Nanoparticles in Plants:

2.4.1 Uptake, Translocation and Accumulation of Nanoparticles in Plants:

Nanoparticles have different range of sizes and shapes; it can range from 1 to 100 nm. They may be in gas, liquid or powder form (Witzmann and Monteiro-Riviere, 2006). The penetration of copper and gold nanoparticles of different sizes was investigated by Boyer and Proseus in algal cells (Proseus and Boyer, 2005). While it was demonstrated that wheat cells can take up Single Walled Carbon Nano Tubes (SWCNT) (Liu et al., 2009). Another study showed internalization of silica mesoporous nanoparticles into the plant cells. In this study protoplast was used to investigate translocation of nanoparticles (Torney et al., 2007). Others have shown that 40 nm size can be taken up easily; sycamore cultured cells were used and polystyrene nanospheres along with 20 nm Cadmium “Quantum Dots” (QD) were noted to translocate into these cultured cells (Etxeberria et al., 2006). It was demonstrated that the entry into cells was through certain plasma proteins in a similar fashion as it’s is performed in animal cells through

endocytic pathway. 40 nm nanoparticles were seen to be stored in the vacuole while QD remained in the cytoplasm (Etxeberria et al., 2006).

If any micro or macro molecules enter a plant cell it can be translocated via plasmodesmata into the cell and eventually make its way into the organelles. A size of 20 to 50 nm can easily be up taken by different cell organelles (Lucas and Lee, 2004). There are many pathways through which these particles can move in a selective or non-selective manner. These pathways go through plasmodesmata and are known to transport regulatory proteins (Kim, 2005). It was observed that silver nanoparticles of size 20 nm were taken up by plants and were transported through plasmodesmata (Crawford and Zambryski, 2000).

Uptake and translocation of nanoparticles is of very importance, as this is increasing day by day. It was demonstrated that copper nanoparticles can be taken up by bean and wheat plants in tissue culture media. It was reported that increase in the concentration of these particles could facilitate their uptake (Lee et al., 2008).

2.4.2 Internalization of Nanoparticles in Plants:

Many examples of cellular toxicity have been reported against carbon nanoparticles in plants and animals (Cotae and Creanga, 2005). As there are many barriers which a plant cell possesses like characteristics of cell wall, different protective waxes and the cuticle, the entry of the particles can be through stomata. It was reported that hydrophilic particles of size 43 nm have been seen to translocate inside the cell through stomata (Eichert et al., 2008).

A study was conducted by Corredor and his team to see the translocation of these nanoparticles aggregates inside the plant cell. It was noted that they have travelled far from the application point only after 48 hours of application. The size of the particles was 46 nm on average while variable sizes of these particles were found to reside in the pith cavity and cells that were close towards the application point. This suggests that only particles of small size can further translocate inside the cell (Corredor et al., 2009).

2.4.3 Entry of Nanoparticles in Plant Cells:

Models of nanoparticles entry inside the cells can be helpful in understanding potential risks and benefits (Dausend et al., 2008). The size and shape of these particles plays important role in translocation and movement inside the cell (Nel et al., 2006). Moreover it has been found that this movement can be further enhanced when these particles binding to specific proteins creating protein corona. This further improves the interaction with other proteins found inside the cell (Gessner et al., 2003). However the size of 200 nm or greater is found not as effective as smaller ones; therefore it can be assumed that larger size nanoparticles cannot easily translocate inside the cells (Costa et al., 2011).

2.4.4 Accumulation of Nanoparticles in Plant Cells:

A study conducted on tobacco plant using cell suspension method showed the translocation of carbon nanotubes inside the vacuoles. While a study on Alfafa showed silver particles accumulation on the surface of root cell organelles. Other study by Gardea-Torresdey et al. (2003) showed that silver nanoparticles can translocate into stem. Another issue of upmost importance is the transmission of these particles to the next generation (Lin et al., 2009). If these particles are found in gametic cells then there is a possibility that they can be transferred to next generation the cycle will move on and accumulation will increase with each generation.

The transmission to next trophic levels is also a concern. A study conducted on algae and tobacco plants confirmed the movement from one level to another (Judy et al., 2010). With so many industries manufacturing nanoparticles the chance of accumulation has greatly increased (Pan and Xing, 2010). Studies are conducted on various crops like rape, lettuce, radish, corn and cucumber and have reported the presence of these particles (Lin and Xing, 2007). Previous research by Kurepa et al. (2010) showed that TiO_2 anatase nanoparticles of 3 nm in size penetrates Arabidopsis cells where in root cells these particles are accumulated in nuclei and vacuoles while in leaves, hypocotyl and cotyledons TiO_2 tend to accumulate in vacuole or in endosomes.

Different nanoparticles have been found to make complexes with the membranes of the cell; therefore they can be easily translocated inside the cell. Once they have moved

inside, they can further translocate via apoplastic pathway or symplastic pathway. They can also go from one plant cell to next by plasmodesmata. Some of the plants can take up these particles very readily while others can take time. While single wall carbon nanotubes are very large to go inside the cell wall. It was observed in Arabidopsis that endocytosis can help to translocate these materials inside the cell of leaf (Shen et al., 2010). The hydrophobic properties if these nonmaterials able those to react with organic substances, thus making them move inside the cell easily (Yang et al., 2006b).

2.4.5 Toxic Effects of Nanoparticles in Edible Plants:

The size and toxicity of nano sized particles can also be related. It was observed that smaller seed species like onion, tomato, *Capsicum* and lettuce have high toxicity rates (Cañas et al., 2008). It can be due to surface area to volume ratio (Lin and Xing, 2007). Small seeds have higher surface area compared with larger ones (Lee and Sedlak, 2008). Plants with more roots have higher toxicity rates as compared to the ones with fewer roots. Dicots have larger primary root system as compared to the monocots (Ma et al., 2010). A study conducted on monocots and dicots showed that mung bean a dicot was more sensitive to nanoparticles of copper than wheat which is monocots (El-Temsah and Joner, 2012). However this study cannot confirm that all monocots will have less toxicity when nanoparticles are given (Ma et al., 2010). Solutions of nano sized particles are more effective as compared with nanoparticles themselves as they tend to dissolve and can easily be translocate inside the cell and plant. The size and surface characterization can also play an important role in the toxic effects created by nanoparticles (Yang and Watts, 2005). The size and surface characterization can also play an important role in the toxic effects created by nanoparticles (Yang and Watts, 2005). Another factor effecting toxicity is the concentration of the given material. Copper nanoparticles reduced the growth of wheat plants at the concentration of 200 mg/L (Shah and Belozerova, 2009).

2.4.6 Effects of Nanoparticles on Plant Physiology:

Nanoparticles act as catalysts and dissolved in solution form active metal ions that interact physically and chemically, affecting plants either in positive or negative manner (Dietz and Herth, 2011). Khodakovskaya et al. (2012) confirmed the penetration of

Multi Walled Carbon Nanotubes (MWCNTs) through seed coat of tomato after several days of incubation. These results suggest that carbon nano-tubes puncture the seed coat and allow the movement of oxygen and water into the seed, enhancing metabolic processes and accelerating seed germination. However, nanoparticles in large quantities agglomerate on seed coat, thereby blocking the exchange of oxygen and water into the seed, which ultimately reduces seed germination.

2.4.7 Biotransformation of Nanoparticles in Edible Plants:

Biotransformation is the upmost concern regarding nanoparticles in agriculture and plant delivery mechanisms. In one case it was found that zinc oxide (ZnO) in soya bean plants was in oxidized state. It was concluded that it transformed inside the plant and in root surface areas. In other study Ni $(OH)_2$ NPs were given to mesquite plants and it was seen with the help of XAS spectra that Ni+ was located in roots and shoots of the plants. The ionic form can be associated with transformation of the particles. As in this form it can react with other particles and can be transformed (Parsons et al., 2010)

2.4.8 Nanoparticles induced Stress in Plants:

A nanoparticle enter the plant cells and causes imbalance and disturbance in anti-oxidant processes and induces oxidative stress leading to cell damage (Pulskamp et al., 2007, Yang et al., 2009b). As the level of oxidative stress increased it caused modification of proteins, nucleic acids and lipids resulting in activation of antioxidant defense system to neutralize the environment leading to cell death. Meanwhile, ROS increases the gene expression of death receptors and DNA damage elevates (Yang et al., 2009a). Nanoparticles are further reported to cause point mutations or single or double strand break in lysosomes as a result of ROS (Singh et al., 2009). Previous studies have reported chromosomal aberration in onion root tips as a result of ZnO nanoparticles treatment (Raskar and Laware, 2014). Treating maize with Ag nanoparticles decreased root elongation in response to high and low concentration, at high concentration seedling die within a week of treatment (Nair et al., 2010).

2.5 Titanium Dioxide Nanoparticles and its Effects on Plants:

It was observed that TiO_2 nanoparticles due to their distinctive properties altered the photosynthetic ability, dry weight, and some other features of metabolisms in autotrophic organisms (Mohammadi et al., 2014). The second most abundant element of the world is TiO_2 and as they are including in number of marketable goods so their oxides is produced in a great deal. Their improper disposal may cause their releases into environment because of the wide spread production, usage or recycling of nano-TiO_2 (Gottschalk et al., 2009).

The cells of plant are composed of thick and inflexible wall that is hydrophobic. This wall could be punctured with nanoparticles having diameter of 5 nm (Carpita et al., 1979). TiO_2 anatase (2.8 nm) combined with alizarin red S was congregated in *Arabidopsis thaliana* in hydroponics (Kurepa et al., 2010). Because these TiO_2 nano-conjugates pierce through cell wall they were translocated to discrete subcellular compartments (Wang et al., 2011). These TiO_2 nanoparticles by passing the pore of cell wall assimilate in plant roots. Nevertheless, the nanoparticles size larger than pore of cell wall was also stated (Corredor et al., 2009). Thus, many studies determined that nanoparticles can enter and assimilate into leaves even if the plant cell wall diameter is smaller, because plant species, and physiochemical parameters of nanoparticles determines the efficiency of nanoparticles accumulation (size, composition, agglomeration and dissolution) (Larue et al., 2012). Though TiO_2 nanoparticles (mixture of anatase and rutile) of 100 nm hindered the germination process in *Zea mays* and blocked in *Vicia narbonensis* (Zheng et al., 2005). TiO_2 nanoparticles (rutile) of 2.5% boosted the germination process in seeds with encouraging effects on young seedlings as nitrogen uptake was improved in spinach (Yang et al., 2006b). It also guarded spinach chloroplast from aging as it diminished the rate of free radicals production (Hong et al., 2005). In contrast, contact of TiO_2 to *Salix* for a short time period was found unsuccessful in displaying major deviations in transpiration, water use efficiency and growth. This result reveals that woody species are not sensitive to acute toxic effects of TiO_2 (Feizi et al., 2013).

TiO_2 nanoparticles with primary diameter lesser or equal to 36 nm assimilate in wheat roots. TiO_2 nanoparticles above 140 nm failed to access parenchyma and their movement is restricted to root epidermis. On contrary nanoparticles with primary diameter of 36 nm reached and accumulated in parenchyma but were inhibited to reach stele; as the movement of nanoparticles is blocked by casparian strip. However, diameter less than 36 nm acquire the access to cross casparian strip, reaching vascular cylinder, and accumulating into wheat root parenchyma (Chafe and Wardrop, 1972).

Lu et al. (2002) demonstrated that SiO_2 and TiO_2 in combination enhanced the seed germination and biochemical activity of soybean like superoxide dismutase, catalase, and nitrate reduce reductase and peroxidase. However in maize seedlings, no change in root growth was observed in response to different concentrations of TiO_2 (Song et al., 2013).

2.6 Role of Reactive Oxygen Species in Stress:

Reactive oxygen species (ROS) are produced at high rates in plants under stress conditions. ROS accumulation leads to severe injuries because of their extraordinary oxidizing potential, comprising degradation of polysaccharides, denaturation of proteins and enzymes, and the scission, cross linking, and nicking of DNA strands (Gill and Tuteja, 2010). Consequently, the cells either acclimatize by producing anti-oxidant enzymes to these variations or may die due to inadequate responses.

Antioxidant enzymes are generated in the organelles which tackle oxidative stress (Foreman et al., 2003). Antioxidants are also called as oxidation inhibitors as they inhibit oxidation of molecules by inhibiting propagation of initiation of oxidizing chain reaction (Pokorný and Korczak, 2001). There is a large class of these enzymes like peroxidase, catalase NADPH oxidase (Foyer and Noctor, 2005b, Foyer and Noctor, 2005a). One antioxidant scavenges and neutralizes single free radical by donating electron and ending carbon stealing reaction to further prevent tissue damage. There are variety of components inside cells that neutralize free radicals of both exogenous and endogenous origin (Jacob, 1995). Having such a wide range of arsenal, plants can adapt to any biotic and abiotic stress situations (Mittler et al., 2004). Antioxidants are classified as first \ second line or third line of defense. Primary antioxidants terminate

chain reaction by donating electron and producing stable products, while secondary antioxidants are chelating agents or oxygen scavengers. Antioxidants—are important inhibitors of lipid peroxidation: a reaction produced by free radicals that cause oxidative damage to cells (Vimala and Adenan, 1999).

2.6.1 Scavenging of ROS:

ROS is a collective term used for oxygen derived species specifically oxygen radicals: non radicals like superoxide (O_2•), hydroxyl (OH^-), hydrogen peroxide (H_2O_2), and lipid peroxide (LOOH) that increases their reactivity (Pham-Huy et al., 2008). Accumulation of these ROS is the major cause of loss in yields worldwide. There are many enzymatic scavengers that plants posses like, Superoxide Dismutase (SOD), Glutathione S-Transferase **(**GST) and Catalase (CAT) to counter the threat of toxicity (Mittler et al., 2004). Catalase—a tetrahedral protein is comprised of four heme groups that actively catalyze dismutation of H_2O_2 to H_2O and O_2. Ascorbate peroxidase (APX) detoxifies H_2O_2 and regenerates glutathione via ascorbate-gluthathione pathway, as APX particularly scavenges H_2O_2 produced by superoxide dismutase by using ascorbate as electron donor. To combat oxidative stress plant triggers antioxidant enzymes system such as superoxide di-mutases (SODs), ascorbate peroxidases (APXs), and catalases (CATs) that defend the plant efficiently against these oxidative stresses (Gill and Tuteja, 2010). Zhao et al. (2012) showed that CeO_2 nanoparticles induced the antioxidant enzymes in maize (*Z. mays*). These enzymes are involved in defense system against stresses by scavenging the reactive oxygen ions and radicals. Therefore, plants have active system to counter free radicals. A free radical is a molecule or molecular fragment comprised of unpaired electrons in outermost orbital, and is also capable of existing independently (Gill and Tuteja, 2010)

2.6.2 Production of ROS:

ROS are produced mostly in the mitochondria during the cellular oxidative reaction as by products (Pung et al., 2012). During cell signaling and mitogenic induced responses, ROS play a crucial and advantageous role (Valko et al., 2006, Pung et al., 2012). In addition to these reactions there are many reactions in which ROS are produced such as transition metals like iron and copper can also produce ROS (Pung et al., 2012). ROS

are generally produced during normal biological functions but their over-production can lead to the oxidative stress (Meng et al., 2009).

Hydroxyl radicles may be produced because of extracellular 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 becomes high (Xia et al., 2006). Metal nanoparticles produce ROS and may lead to oxidative stress in plants (Xia et al., 2008).

A study carried out by Levine (1999) introduced number of stressors to the cells of soybean and determined that H_2O_2 produced due to these introductions bring rapid influx of calcium ions, a variation that give rise to apoptosis.

2.7 Role of Exogenous Hydrogen Peroxide in Stress:

H_2O_2 is a significant ROS molecule. Depending upon its concentration, it has a dual role in plant cells, as it can work as an indicator of oxidative stress and initiate a signaling cascades following early response of plants to abiotic stress where at very high concentration itself act as stress. This response to abiotic stress can also be established by exogenously applying elicitor molecules, that trigger the activation of transduction cascades and hormonal pathways, which recruit induced resistance to environmental stress by activating antioxidant system (Mejía-Teniente et al., 2013)

The purpose of elicitor-induced plant signaling is to assist as a guide to a number of intracellular events that finish in triggering of transduction cascades and hormonal pathways, which activate induced resistance making plant immune to abiotic stress (Mejía-Teniente et al., 2013). Different factors describe role of elicitors, such as the amount of the elicitor, plant stage in which elicitor is given as well as time of elicitation, and stage in which elicitor is applied (Mejía-Teniente et al., 2013).

Peroxisomes act as a main site for reactions, producing H_2O_2 containing photorespiration, β oxidation of fatty acids, and further oxidation of other substrates (Dat et al., 2000a, Dat et al., 2000b, George, 2014). As there are several pathways for synthesis, there are also numerous pathways for removal or scavenging of H_2O_2. For scavenging of peroxide and other free radicals present in cells, numerous enzyme

systems are involved as presented in schematic diagram. A study conducted on *C. annuum* showed that exogenous application of 6.7 and 10 mM salicylic acid concentration, and, 14 and 18 mM H_2O_2 concentrations brought an endogenous H_2O_2 production and gene expression. It was observed that plants were able to attain a high degree control over ROS toxicity, through a highly balanced and strongly organized network of minium152 genes, which translate both ROS-producing genes and ROS-scavenging enzymes (Mejía-Teniente et al., 2013). Consequently, ROS species have been applied as signaling molecules and therefore the relationship among the ROS-producing and ROS-scavenging pathways will define the localization, duration and intensity of the ROS signals (Mejía-Teniente et al., 2013). Exogenous application of H_2O_2 assisted in surviving chilling conditions in field crops (Kumar et al., 2010, Ahmad et al., 2013). A general pathway for production of H_2O_2 has been shown in fig. 2.1.

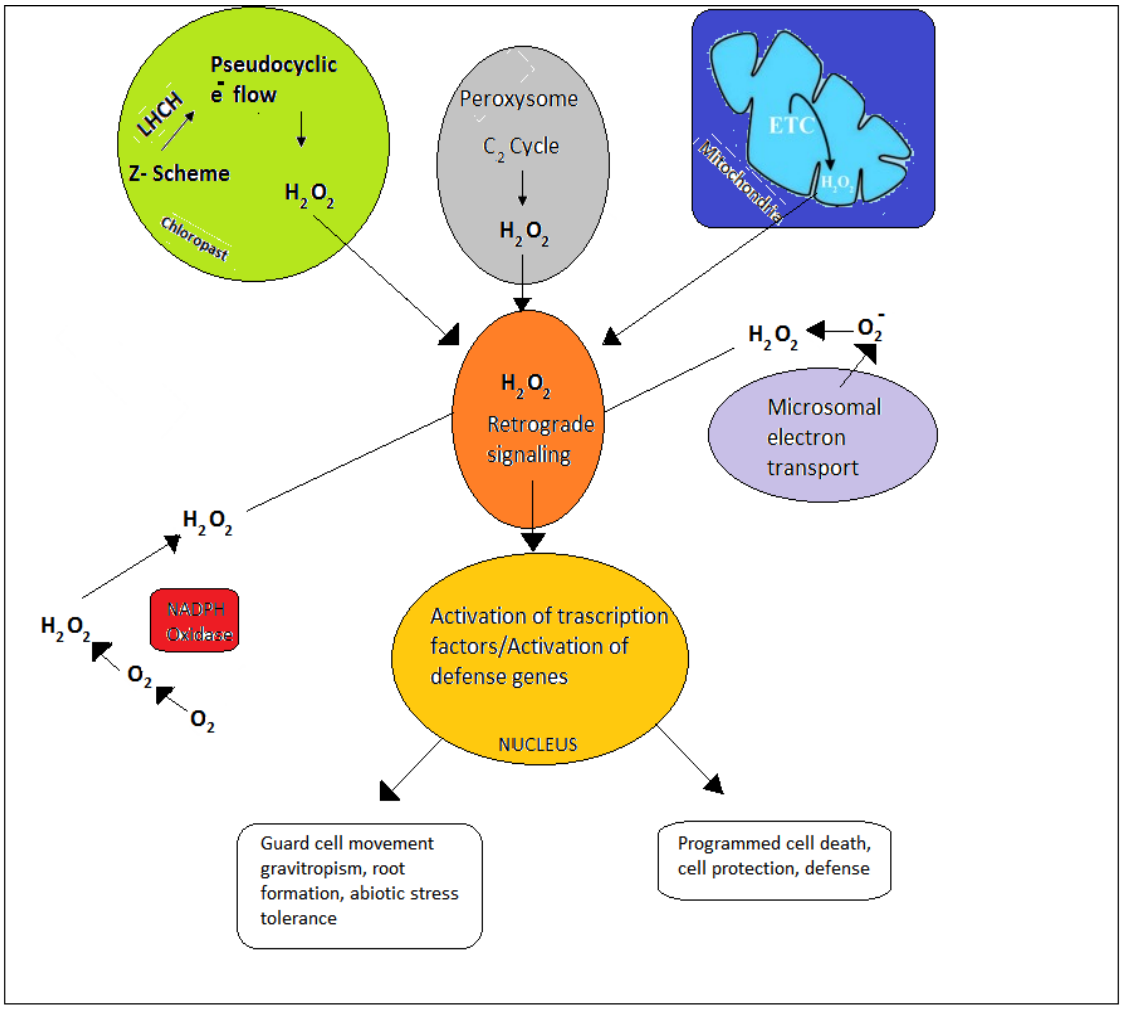

Figure 2.1 Schematic diagram representing production of H_2O_2 in a cell
H_2O_2 production in various intra- and extra-cellular sites and the resulting signaling cascade coupled with the regulation of expression of defense genes in plant cells. Figure modified from Hossain et al. (2015).

3 METHODOLOGY

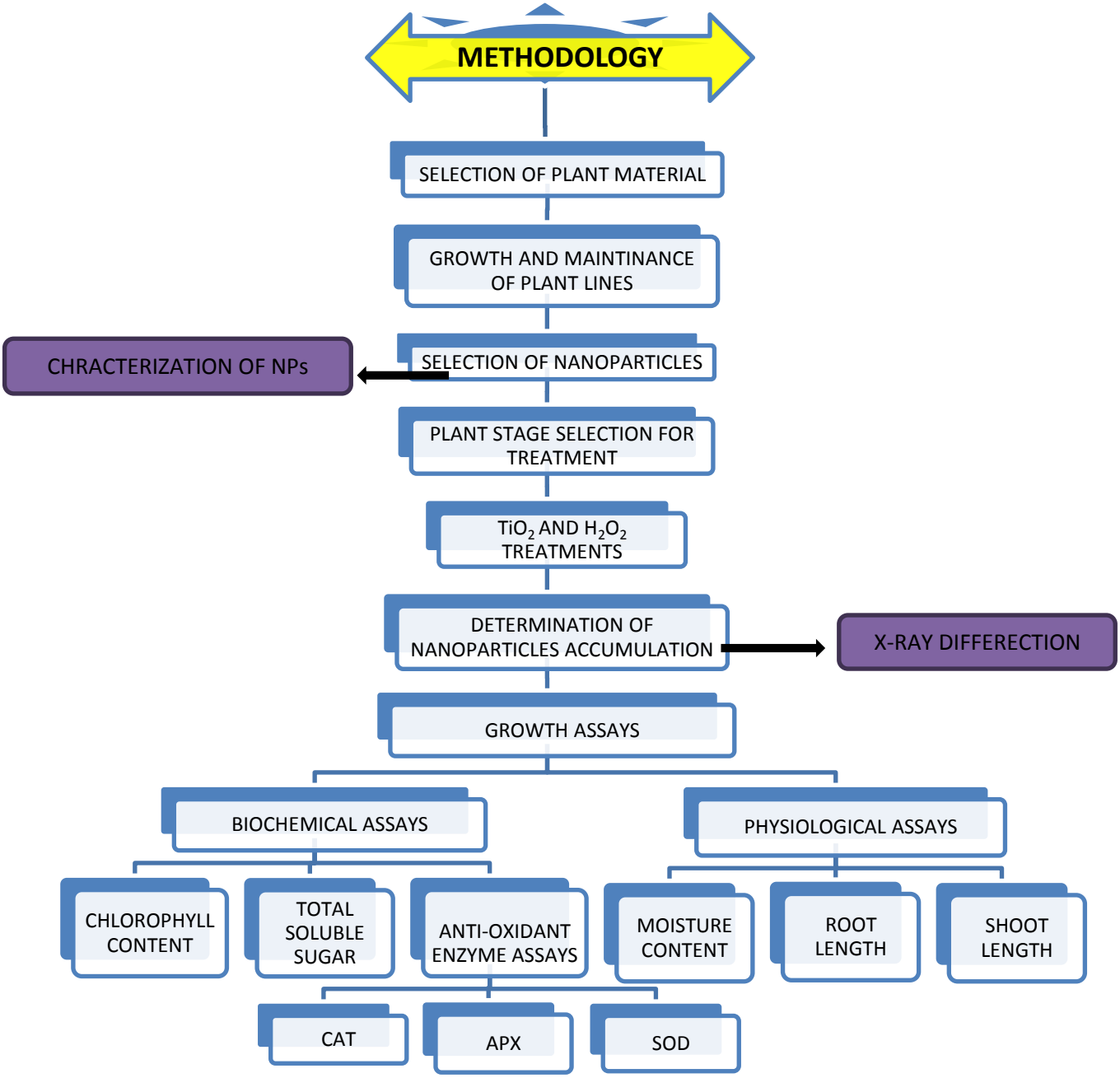

Figure 3.1 Flow chart of overview of methodology

3.1 Plant Material:

Seeds of two cultivars of *Capsicum annuum L.* (*C. annuum*) with code no. 16/8 and 14/9 and their hybrid (produced by cross breeding of homozygous parental lines) were obtained from Vegetable Department, Horticulture Research institute, National Agriculture Research Center (NARC), Islamabad. 16/8 was termed as Parent 1 (P1), 14/9 as Parent (P2) and hybrid as H for the study.

3.2 Growth and Maintenance of Plant Lines:

Petri dishes were lined with Whatmann filter papers and seeds of *C. annuum* including Parent 1 (P1), Parent 2 (P2) and their Hybrid (H) were distributed on Whatmann filter papers with the same distance in separate petri dish. The petri dishes were placed in growth room at 26±1 ^{0}C in dark conditions until germination of seeds. Only 18 seeds for each cultivar were implanted in tray after germination. The soil mixture used in trays for growing *C. annuum* seedlings was prepared by mixing 1:1:1 ratio of soil, peat moss and sand respectively. The trays containing 32 pots were placed at 26±1 ^{0}C under 16 hours of daylight (provided by fluorescent tube lights) and 8 hours of dark period. *C. annuum* seedlings were allowed to grow for 30 days post-germination. The plants were only watered with tap water during this period.

3.3 Selection of Nanoparticles:

Titanium dioxide nanoparticles (TiO_2) were used in this research. TiO_2 nanoparticles were obtained from Santa Cruz Biotechnology.

3.3.1 Characterization of Nanoparticles:

Nanoparticles differ in their chemical characteristics and physical properties and these properties affect their ability to assimilate into plant roots and cells and ultimately on plant physiology (Feizi et al., 2012). Here, we used X-ray Diffraction (XRD) technique to characterize TiO_2 particles and its uptake into plant tissues to understand its impact on plant physiology, and biochemical activity. This technique was executed to detect phase, and crystalline state of TiO_2 nanoparticles. It's a multipurpose and non-destructive technique that describes crystallographic and chemical structure of nanoparticles consists of different phases. These phases are assessed by studying

subsequent diffractogram formed via addition of distinct patterns (Karan et al., 2009, Aromal et al., 2012).

3.4 TiO_2 Nanoparticles and H_2O_2 Treatments:

TiO_2 nanoparticles suspension were prepared to determine the combined effects of nanoparticles and exogenously provided H_2O_2 on *C. annuum*. Nanoparticles suspension was prepared by adding 1 g TiO_2 nanoparticles in 1 L double distilled autoclaved water in the regent bottle, covered with aluminum foil to protect from light. The bottle was placed in shaker for 15 min on high speed to ensure the proper mixing of nanoparticles. They were further placed in ultra-sonicator for 60 min to ensure their proper dispersion. Hydrogen Peroxide (H_2O_2) treatment was performed using 1 mM and 10 mM concentrations also prepared in distilled water.

Table 3.1: To determine the effects of pretreated H_2O_2 and TiO_2 nanoparticles in *C. annuum* parental lines; Parent 1(P1), Parent 2, (P2) and their Hybrid (H), following group codes were assigned to treated groups.

S. No.	Group Code	Treatment Description
1	a	Water
2	b	1 mM H_2O_2
3	c	10 mM H_2O_2
4	d	1 g/L TiO_2
5	e	1 mM H_2O_2 + 1 g/L TiO_2
6	f	10 M H_2O_2 + 1 g/L TiO_2

3.5 Plant Stage Selection for Treatment:

Capsicum annuum L. (*C. annuum*) seedlings were grown for 30 days in a tray containing soil, sand and peat. Treatment of H_2O_2 was given for two days followed by a gap day when water was given to all groups of both parents and their hybrid to help withstand and survive the stress and uphold its rigidity. To determine the effects of H_2O_2 pretreatment on seedlings TiO_2 treatment was provided for 10 days. Group "a" was used as control treated with distilled water in both parents and their hybrid; Group "b" was used as control for H_2O_2 with 1 mM concentration in both cultivars and their hybrid, Group "c" as control for 10 mM H_2O_2 concentration in parental and hybrid lines. Group "d" was treated with TiO_2 nanoparticles to determine the effect of nano sized particles on plant physiology. Group "e" was pretreated with 1 mM H_2O_2 for 2 days followed by a gap day and then 1 g/L TiO_2 for 10 days. While, group "f" was treated with 1000 mg/L of TiO_2 for 10 days along with 2 days pretreatment of 10 mM H_2O_2. Plant seedlings were uprooted on 44^{th} day post germination from the tray to determine physiological, and biochemical parameters.

3.6 Determination of Nanoparticles Accumulation:

3.6.1 X-Ray Diffraction (XRD):

Nanoparticles uptake analysis was performed by X-Ray Diffraction (XRD) technique to determine crystal structure and chemical composition of a nanomaterial assimilated by plant from soil. The measurement for standard peak was performed on amorphous and crystal structures of TiO_2 nanoparticles in *C. annuum* seedlings. Uprooted seedlings treated with TiO_2 were selected. The soil was removed by washing with running tap water; further roots were washed with distilled water 2-3 times. The seedlings were placed in dried oven at 70 ^{0}C for 72 hours, and the seedlings were grounded into fine powder to get homogenized mixture using pestle and mortar (Odum, 2007). Powder was collected in autoclaved eppendroff tubes and conveyed for XRD (to SCME, NUST) to detect the accumulation and presence of TiO_2 in plant samples via software.

3.7 Physiological Parameters:

3.7.1 Seed Germination:

To analysis how treatment of H_2O_2 can affect the germination rate in combinations with TiO_2 nanoparticles the germination assay was carried out. To analyze changes in physiological and biochemical behavior of plant seeds exposed to TiO_2 nanoparticles, concentration of 1 g/L, germination rate of *C. annuum* was determined. A total of 10 seeds for each group were used in this germination assay as replicates. Healthy seeds were chosen and seeds that float in water were discarded. After that seeds of *C. annuum* were surface sterilized using 5% concentration of Sodium hypochlorite (NaOCl) and washed thrice for 3-4 min with tap water to ensure complete removal of NaOCl. Whatmann filter paper 1 was saturated by spraying 3 ml of distilled water and used as control for each cultivar. Petri dishes containing whatmann filter paper were soaked with TiO_2 solution (1 g/L concentration) prepared by sonication and used as nanoparticles control for each cultivar. Two other sets of petri dishes were prepared containing 1 mM and 10 mM concentrations of H_2O_2 separately for both parents and their hybrid. But for combination treatments, seeds of *C. annuum* were imbibed in 2 ml H_2O_2 of 1 mM and 10 mM concentrations independently for 2 hour and were spread on filter papers presoaked with 1 g/L concentration of TiO_2 solution separately for each cultivar (Pokhrel and Dubey, 2013). The seeds were placed at a distance of 1cm, closed with parafilm and wrapped in aluminum foil. Petri dishes containing *C. annuum* seeds were placed in growth room at 26±1 ^{0}C for 8 to 10 days (until germination). The germination percentage was evaluated using the following formula (Pokhrel and Dubey, 2013).

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

3.7.2 Root and Shoot Length Measurements:

Effects of H_2O_2 and TiO_2 treatment on plant growth and development was determined by measuring root and shoot lengths. The germinated seedlings were transferred to

trays containing soil mixture. Each treatment contained 3 replicates. 30 days post-germination, treatments of H_2O_2 were given for two days followed by nanoparticles treatment for 10 days. The plants of each cultivar and each treatment group were uprooted on 44th day for measuring root and shoot length. The soil was removed from roots and measurements were taken with the help of scale as shown in figure no. 3.2.

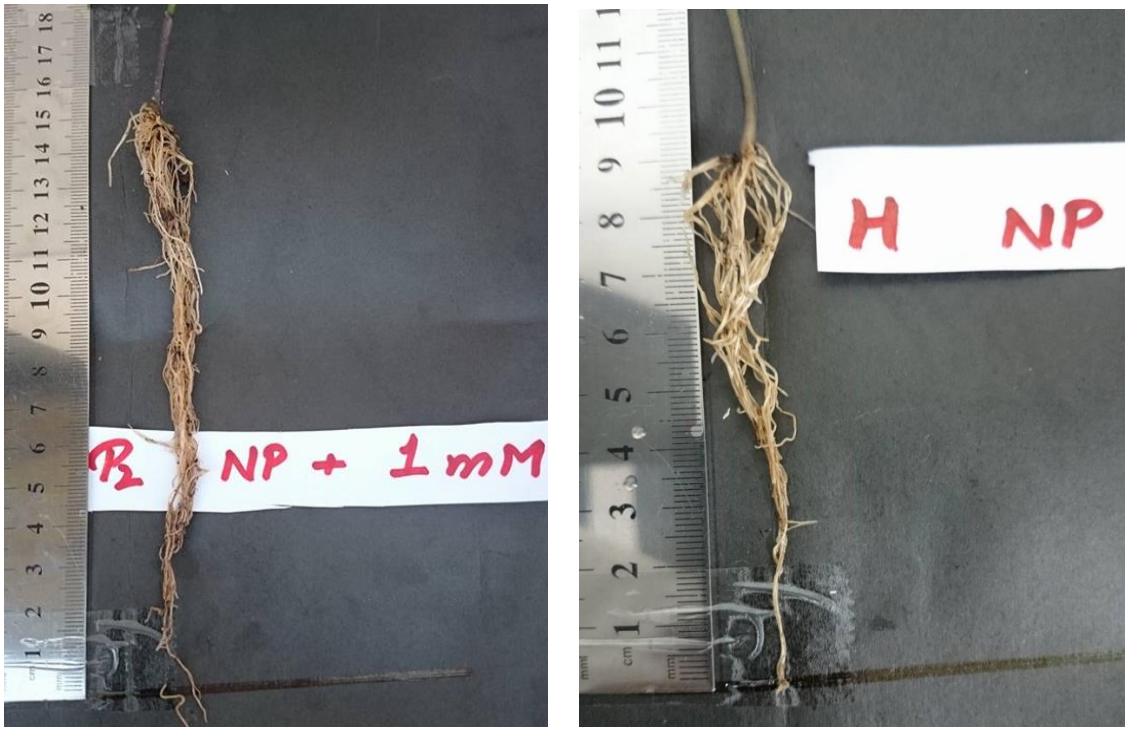

Figure 3.2 Measurement of root length
Root length was measured with the help of scale on 44th day in presence of light.

Similarly measurement of shoot length was also taken with scale as shown in figure 3.3.

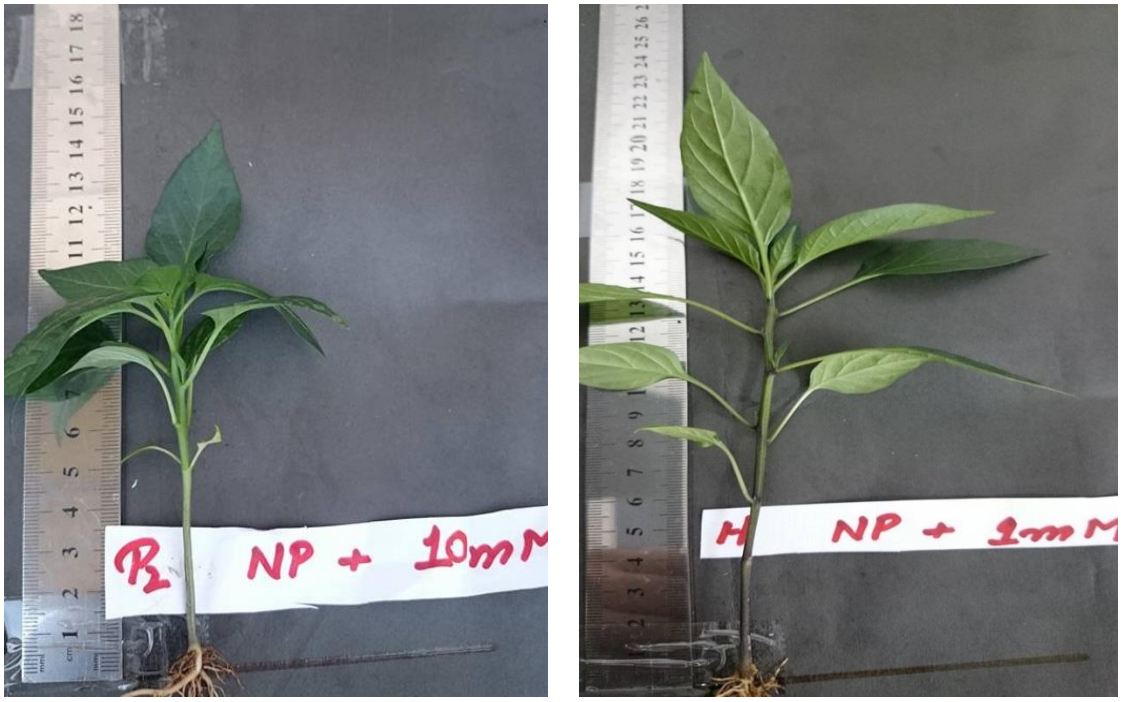

Figure 3.3 Measurement of shoot length
Shoot length was measured with the help of scale on 44th day in presence of light.

3.7.3 Moisture Content:

Moisture content (MC) of young seedlings provides understanding on water content retained in groups "a, b, c, d, e and f" as mentioned in section 1 of this chapter. Seedlings were weighted and fresh weight was recorded, the seedlings were then kept in oven at 70 ^{0}C for 3 days so that all the moisture retained is evaporated. The dry weight was determined subsequently. The difference of wet weight and dry weight of seedling were calculated as water content and expressed in percentage (%) (Pokhrel and Dubey, 2013).

The relative change in water content was determined using the following formula:

$$\text{MC \%} = \frac{\text{Fresh Weight} - \text{Dry Weight}}{\text{Fresh Weight}} * 100$$

3.8 Biochemical Assays:

To understand the effects of nanoparticles and H_2O_2 pretreatment on anti-oxidant enzymes activity of *C. annuum* seedlings, biochemical assays were performed. This technique let us know the up-regulation or down-regulation of antioxidant enzymes due to treatments.

3.8.1 Chlorophyll Content:

Chlorophyll content in leaves will help us to investigate photosynthesis of respective *C. annuum* groups affected as a result of various treatments. *C. annuum* seedlings from parental and hybrid lines were treated with TiO_2 nanoparticles solution (1g/L concentration) after H_2O_2 pretreatment. The chlorophyll content of TiO_2 treated seedlings was determined using ethanol method. Fresh leaves of from each treated group were weighted 0.25 mg and were soaked in 5 ml of 80% of ethanol. The test tubes were capped and placed in water bath at 80 ^{0}C for 10 min. This extract was cooled at 4 ^{0}C in dark. Chlorophyll content was measured by taking Optical Density (OD) value at 666 nm through spectrophotometer. Quantification of photosynthetic pigment was carried out in triplicates. Chlorophyll content was determined using following formula:

$$\textbf{Chlorophyll (mg/g fresh weight)} = \frac{\textbf{(Chlorophyll -0.01) x 10}}{\textbf{92.6474 x fresh weight of sample}}$$

3.8.2 Soluble Sugar Content:

Soluble sugar content from *C. annuum* fresh leaves was determined using the method of Dubois et al. (1956) known as phenol and sulfuric acid method. 500 mg of Fresh leaves of 44 days old plants from each group (a, b, c, d, e and f as mentioned in section 1 of this chapter) of hybrid and the parents, were dipped in 80% ethanol in a test tube closed with cap and placed in water bath at 80 ^{0}C for 1 hour to make sample extract. After 1 hour, in another set of test tubes 250 µL of this sample extract was poured together with 500 µL of 18 % phenol. This solution was incubated at 4 ^{0}C for 1 hour in absence of light. After incubation, 1.25 mL of concentrated sulfuric acid (H_2SO_4) was

added to each sample and test tubes were vortexed. Amount of sugar of each sample was measured by evaluating absorbance at 420 nm via UV-Vis spectrophotometer. Standard glucose curve was used to determine concentration of sugar content.

3.8.3 Enzyme Extraction:

250 mg of fresh leaves were taken and stored immediately at -80 ^{0}C. For total protein extraction, the frozen leaves from group "a, b, c, d, e and f" of *C. annuum* hybrid and the parents were used and grounded to fine powder with liquid nitrogen using pre-chilled pestle mortar separately. The leaf powder was homogenized with 1.2 mL potassium phosphate buffer with molarity 0.2 M (pH 7.8 with 0.1 mM EDTA) and collected in autoclaved eppendorf tubes. These homogenized samples from were centrifuged at 14,000 rpm for 30 min at 4 ^{0}C. The supernatant was stored in separate vials at -80 ^{0}C and used to perform anti-oxidant enzyme assays: Catalase (CAT), Ascorbate Peroxidase (SOD), and Superoxide Dismutase (SOD) content (Elavarthi and Martin, 2010).

3.8.4 Total Soluble Protein Estimation:

Total soluble protein content was estimated via Bradford assay (Yadeghari et al., 2008). Standard curve was produced by using Bovine Serum Albumin (BSA) test (Bradford, 1976). However, for total protein content measurement, 0.5 mL of Bradford reagent, 2 mL distilled water and 20 µL of crude protein sample from group "a, b, c, d, e and f" of *C. annuum* hybrid and the parents was added separately. The samples were vortex and incubated for 10 min at room temperature. Absorbance was measured at wavelength of 595 nm on UV/Vis spectrophotometer. Total soluble protein concentration was estimated by using standard curve generated through BSA test.

3.8.5 Catalase:

The enzyme assay according to Aebi (1984) method with few modifications was performed which gives a measure of catalase activity based on detection of decomposition of H_2O_2 (Elavarthi and Martin, 2010). 3 mL reaction mixture containing 2 mL of leaf extract that is diluted 200 times in extraction buffer (50 mM potassium phosphate buffer with pH 7), and 1mL of 10 mM H_2O_2 was prepared. For dilution of

leaf extract 10 uL protein extract was added in 1990 uL extraction buffet. The absorption was measured at 240 nm via UV spectrophotometer. The reduction in absorbance was observed for 30 seconds and reading was recorded after every 5 seconds. Catalase reactivity was measured using extinction coefficient of H_2O_2 (39.4 mM/cm at 240 nm) that was stated in millimole per minute per gram of sample fresh weight (FW).

$$\textbf{CAT (mM/gFW) activity} = \frac{\textbf{Absorbance change * Total volume of reaction mixture}}{\textbf{Sample Volume * EC * minutes * gFW of sample}}$$

3.8.6 Ascorbate Peroxidase:

Ascorbate Peroxidase (APX) enzyme activity of water controls and treatment groups (treated with TiO_2 and H_2O_2) of each cultivar of *C. annuum* seedlings was estimated using Nakano and Asada (1981) method with few modifications. Decline in optical density due to oxidative reaction of ascorbate in reaction mixture was observed for 30 seconds at 290 nm after every 5 sec using UV-Vis spectrophotometer. 3000 uL of reaction mixture was prepared containing 600 uL 50 mM potassium phosphate buffer (pH 7.0), 100 uL (1 mM) EDTA, 100 uL (5 mM) Ascorbate, 100 uL (0.1 mM) H_2O_2 and 100 uL crude protein extract. For blank, 700 uL of potassium phosphate buffer was used however blank did not contain protein sample. The 2.8 mM/cm extinction coefficient was applied to calculate ascorbate in millimole per minute per gram of sample fresh weight.

$$\textbf{APX (mM/gFW) activity} = \frac{\textbf{Absorbance change * Total volume of reaction mixture}}{\textbf{Sample Volume * EC * minutes * gFW of sample}}$$

3.8.7 Superoxide Di-mutase (SOD) activity:

To calculate SOD catalytic activity SOD buffer was manufactured according to Beauchamp and Fridovich (1971). 3 mL of SOD buffer was taken for each sample in

10 mL falcon tube whereas 100 µL riboflavin stock and 100 µL of total protein extract was added from each sample to 3 mL SOD buffer in same falcon tube. All the falcon tubes containing resultant mixture of SOD buffer and protein 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 falcon tubes prepared containing same reaction mixture for each sample but placed under dark for the same time as mentioned above. Mixture of 3 ml of SOD, 100 µL of riboflavin stock and 100 µL of distilled water was used as blank. At the wavelength of 560 nm, OD was measured of both sets on UV/Vis spectrophotometer.

Riboflavin stock was prepared by adding 0.0016 gms of riboflavin to 5 mL of distilled water and SOD buffer was synthesized by adding following chemicals:

Reagent	**Quantity (per 100 mL)**
Methionine	0.194 gm
ETDA (Ethylenediamine Tetraacetic Acid)	0.0367 gm
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

4 RESULTS

4.1 Growth and Maintenance of Plant Lines:

Capsicum annuum L. (C. annuum) plants of both parents and their hybrid were grown and maintained at 26 ±1 ^{0}C for 44 days post-germination, in a soil trays with 16 hours of light and 8 hours of dark period. These seedlings were further tested for physiological, biochemical activities and nanoparticle accumulation

4.2 Characterization of Nanoparticles:

Characterization of TiO_2 particles was performed using X-ray Diffraction (XRD) technique from School of mechanical Engineering (SCME, NUST). This technique was used to determine phase and assimilation of TiO_2 nanoparticles in plant tissues by examining diffraction peak of sample (Odum, 2007). XRD pattern of TiO_2 is shown in figure. In figure strong diffraction peaks were observed at 26.3^0 and 29^0, whereas the data analysis showed 16% anatase, and 54.8 nm crystalline size of the nanoparticles. The XRD analysis showed that nanoparticles possessed orthorhombic crystal system and 3.93 g/cm^3 calculated density. Figure shows diffraction peak at 140 absorbance intensity (a.i) and 87 a.i. Additionally, size and phase of nanoparticles are parameters that influence physiology and biochemical activity of plants; thereby XRD result gives relevant information regarding crystalline size of nanoparticles in order to further formulate effects of TiO_2 on plant cells (Thamaphat et al., 2008).

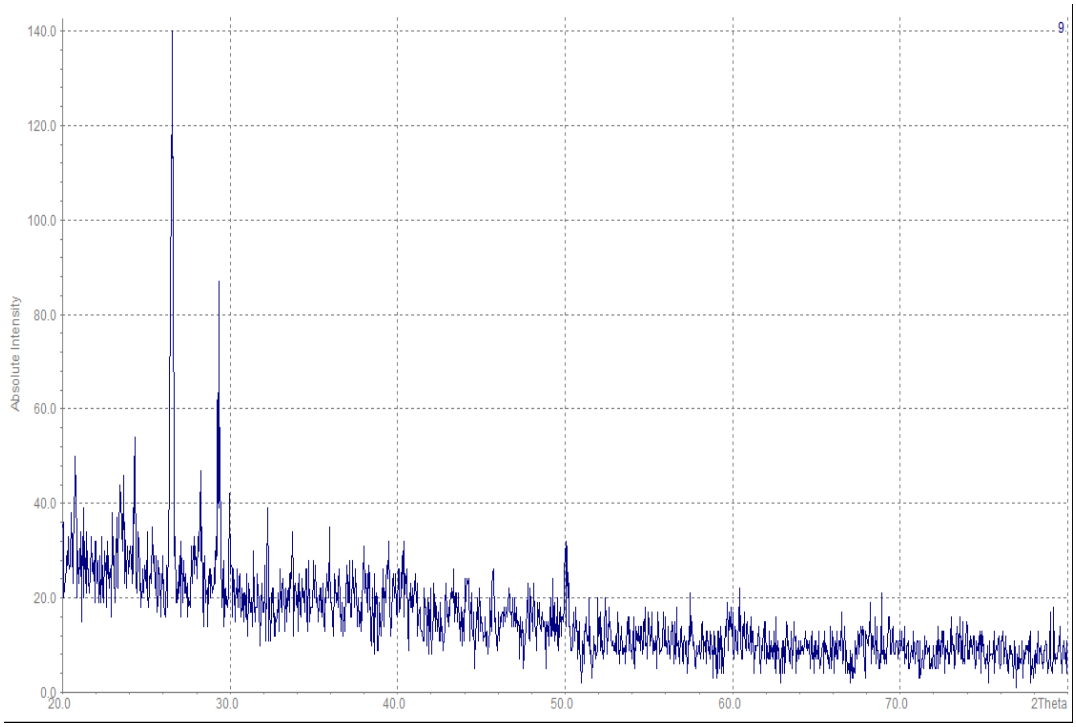

Figure 4.1 X-Ray Diffraction of TiO_2 nanoparticles
The peaks show that nanoparticles assimilate into *C. annuum* seedlings. The peak intensity at 140 absorbance intensity shows that particles are of small size, while the diffraction pattern peaks at 26.30 and 290 show that nanoparticles are in anatase phase and in polycrystalline form. The nanoparticles show tetragonal and orthorhombic crystalline forms.

4.3 Physiological Parameters:

4.3.1 Effects of TiO_2 and H_2O_2 Treatments on Plant Growth/Physiology:

Treatments of Titanium Dioxide (TiO_2) nanoparticles and H_2O_2 had significant effects on the growth of the treated plants. Water treated control plants showed normal growth but TiO_2 treated plants showed stunted growth. Hybrid plants of *Capsicum annuum L. (C. annuum)*, pretreated with H_2O_2 showed comparatively better growth as compared to its parental lines indicating resistance towards nanotoxicity caused by TiO_2 nanoparticles treatments.

It was observed that 1 mM H_2O_2 played positive role in plant growth in both the parents while it caused minor increase in root and shoot length in the hybrids. However 10 mM H_2O_2 showed positive effects on physiology of hybrid plants. But

this concentration of H_2O_2 was itself found toxic to parental lines as indicated by retarded growth of parents at high concentration of H_2O_2.

4.3.2 Effect of TiO_2 and H_2O_2 on Germination of *C. annuum* Seeds:

To examine the effects of TiO_2 and H_2O_2 treatments on *C. annuum* germination, seeds of Parent 1 (P1), Parent 2 (P2) and their Hybrids (H) were soaked in distilled water (Group a), 1 mM H_2O_2 (Group b), 10 mM H_2O_2 (Group c) and 1 g/L TiO_2 (Group d) in separate petri plates for each treatment. For combined treatments of H_2O_2 and TiO_2, seeds from both parents and their hybrids were pretreated with 1 mM H_2O_2 for 2 hours and transferred to separate petri dishes containing 1 g/L TiO_2 (Group e) likewise another group was pretreated with 10 mM H_2O_2 also transferred to petri dishes containing 1 g/L TiO_2 nanoparticles (Group f).

For group P2a (P2 for "parent 2" and "a" denotes "water control group" as described above), 48% germination was observed that is higher from Ha and P1a which were 43% and 37%, respectively. Interestingly 1 mM H_2O_2 increased germination in group Hb with 46 %, group P1b with 40% and P2b with 52% as compared to their respective water controls. For both parents and hybrids, we observed highest germination with 62% for Hc group and 49% for P1c group while P2c showed 66% germination. It showed that 10 mM concentration of H_2O_2 is highly effective for increasing germination percentage in parental and hybrid lines in *C. annuum*. This may be due to rupturing of seed coat by oxidative effect of H_2O_2. Group d showed decreased germination percentage as compared to water control group with germination 41%, 33% and 47% for Hd, P1d and P2d groups respectively This result could be due to small size of nanoparticles and phase composition of nanoparticles which enters plants cells and creates oxidative stress. Similarly in group e, pretreatment of 1 mM H_2O_2 concentration in parental and hybrid lines showed significant decrease in germination rate with 38% for He, P1e showed 32% germination and 44% was observed for group P2e. The Same trend was observed in group f, where pretreatment of seeds from parental and hybrid lines with 10 mM concentration of H_2O_2 showed more decrease in germination with 35%, 30% and 41% for Hf, P1f and P2f respectively. This may be due to the

fact that H_2O_2 break the seed coat and more nanoparticles enter into the seeds and destroyed the embryo cells. All the three lines of *C. annuum* plants used for study revealed highest germination rate when used 10 mM H_2O_2 concentration, hence we conclude that 10 mM H_2O_2 can be used as a tool to increase germination rate.

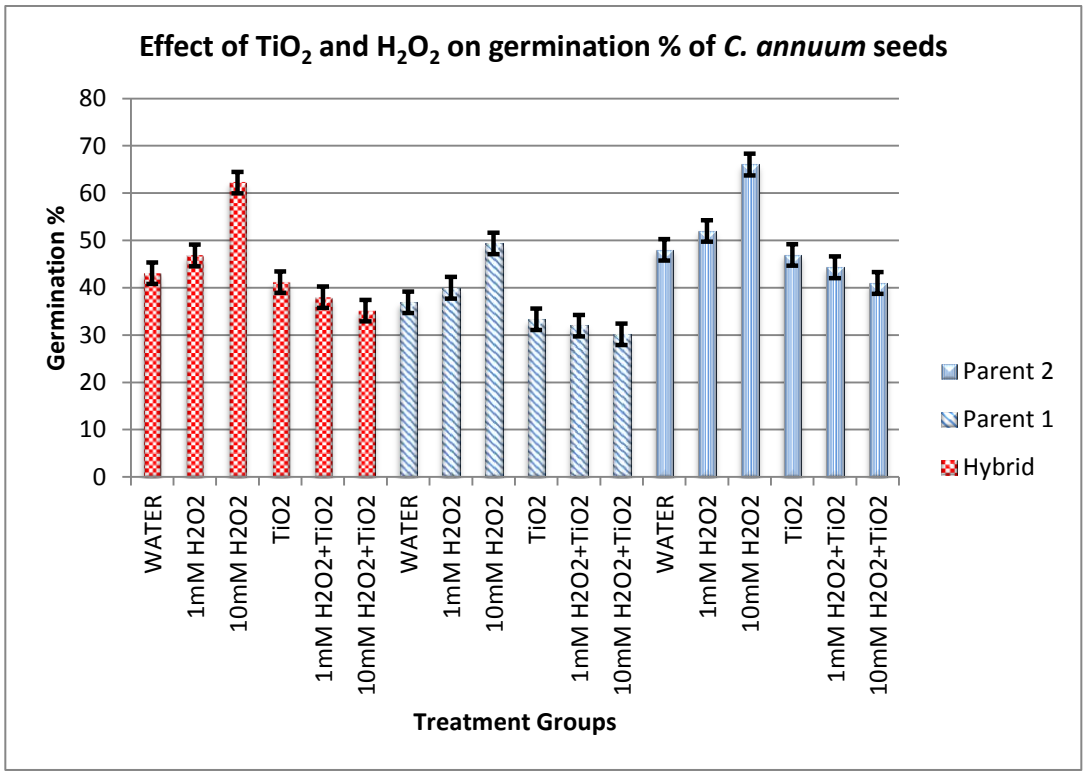

Figure 4.2 Germination percentages of *C. annuum* hybrids and parents seeds of H_2O_2 and TiO_2 treatment groups
Pretreatment with H_2O_2 increased nanotoxic effects of TiO_2 by decreasing the germination percentage of *C. annuum* hybrid and the parents. However 10 mM H_2O_2 showed highest germination rate in each group. Data represents the means of three independent experiments with 3 plants for each treatment in each experiment. Error bars are standard error (SE) for three replicate reactions.

4.3.3 Comparison on effects of H_2O_2 and TiO_2 on *C. annuum* germination:

Parent 2 showed highest germination rate in all treated and water controlled groups while for parent 1 least germination rate was observed in each treated as well as in water control group as presented in figure 4.3. Hence hybrid possessed moderate germination rate in comparison to both parents. The observed differences are may

be due to difference in genetic make-up of both parents and also in their hybrids. All the three lines of *C. annuum* plants used for study revealed highest germination rate when used 10 mM H_2O_2 concentration, hence we conclude that 10 mM H_2O_2 can be used as a tool to increase germination rate.

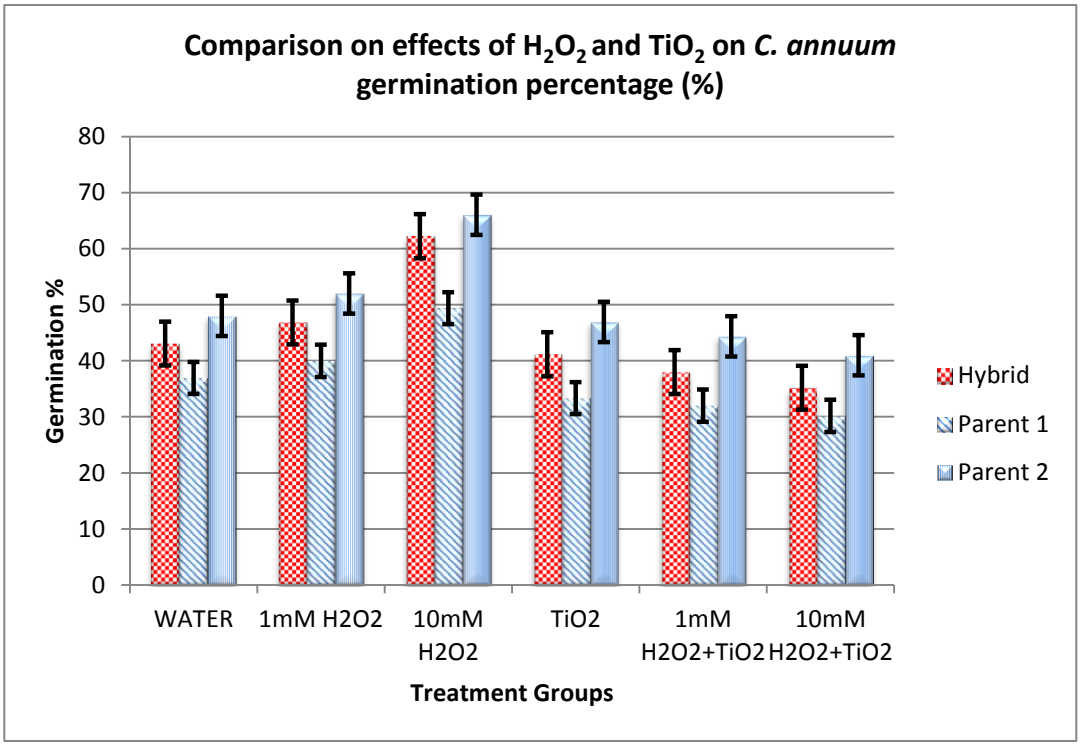

Figure 4.3 Comparison of germination rate between hybrids and their parents Highest germination rate for parent 2 was observed for each treatment and control and least germination rate was revealed by parent 1 in each treatment. Data represents the means of three independent experiments with 3 plants for each treatment in each experiment. Error bars are standard error (SE) for three replicate reactions.

4.3.4 Effects of H_2O_2 and TiO_2 on Post-Germinated Root Lengths in *C. annuum* Seedlings of Hybrids and the Parents:

The effects of pretreatment of H_2O_2 for 2 days and subsequent 10 days treatment of TiO_2 nanoparticles on root length of *C. annuum* seedlings was observed by measuring root length of *C. annuum* seedlings on 44th day post-germination. In group Hb 2.8% increase in root length was observed as compared to water control

in group Ha, while for group Hc that is treated with 10 mM H_2O_2. 13.2% increase in root length was observed. The hybrid nanoparticles control group "Hd" showed decrease in root length as compared to respective water control "Ha" group by 23.5%. Hence we can conclude that nanoparticles possessed phototoxic effects on growth of roots in hybrid plants. However when nanoparticles were given to "He" group pretreated with 1 mM H_2O_2, the decrease in root length was reduced to 9.1% as compared to Ha group. It showed that 1 mM concentration of H_2O_2 can reduce the phototoxic effect of nanoparticles. For plants in group Hf, that were pretreated with 10 mM H_2O_2, the root lengths were observed to increase by 3.6%, showing that 10 mM H_2O_2 is more effective in reducing nanotoxicity in hybrids. We also concluded that 10 mM H_2O_2 treatment in Hc group showed highest root length in all treated plants as shown in figure 4.4.

In group "P1b", 5.6% increase in root length was observed as compared to control "P1a" that is highest for all treated groups of parent 1. Hence, it could be assumed that 1 mM H_2O_2 is found effective in initiating the mechanisms involved in increasing root length of parent 1. Contrary to that, when H_2O_2 concentration was increased to 10 mM, a reduction in root length of 11.1% was shown as compared to water control group. It may be due to that fact that high concentration of H_2O_2 acted as a stress to plants. The TiO_2 treated group of parent 1 showed further decrease in root length to 21% as compared to control. These results suggested phototoxic role of TiO_2 on root length. However pretreatment with 1 mM H_2O_2 decreased the phototoxic effect of TiO_2, because increase in root length of 2% was observed as compared to water. Pretreatment with high concentration of H_2O_2 also decreased the phototoxic effects of TiO_2 but to a less extent. This pretreatment showed a decrease of 16% in root length as compared to water control.

Parent 2 also showed similar behavior to parent 1of increased in root length with 12.4% when 1 mM H_2O_2 was applied as compared to water control. Increased concentration of H_2O_2 had a minor positive role in increasing the root length by 2% while 20% decrease in root length was observed for TiO_2 treated group. Group pretreated with 1 mM H_2O_2 showed 17% increase and 10 mM H_2O_2 pretreated group showed 8% increase in root length as compared to water control plants.

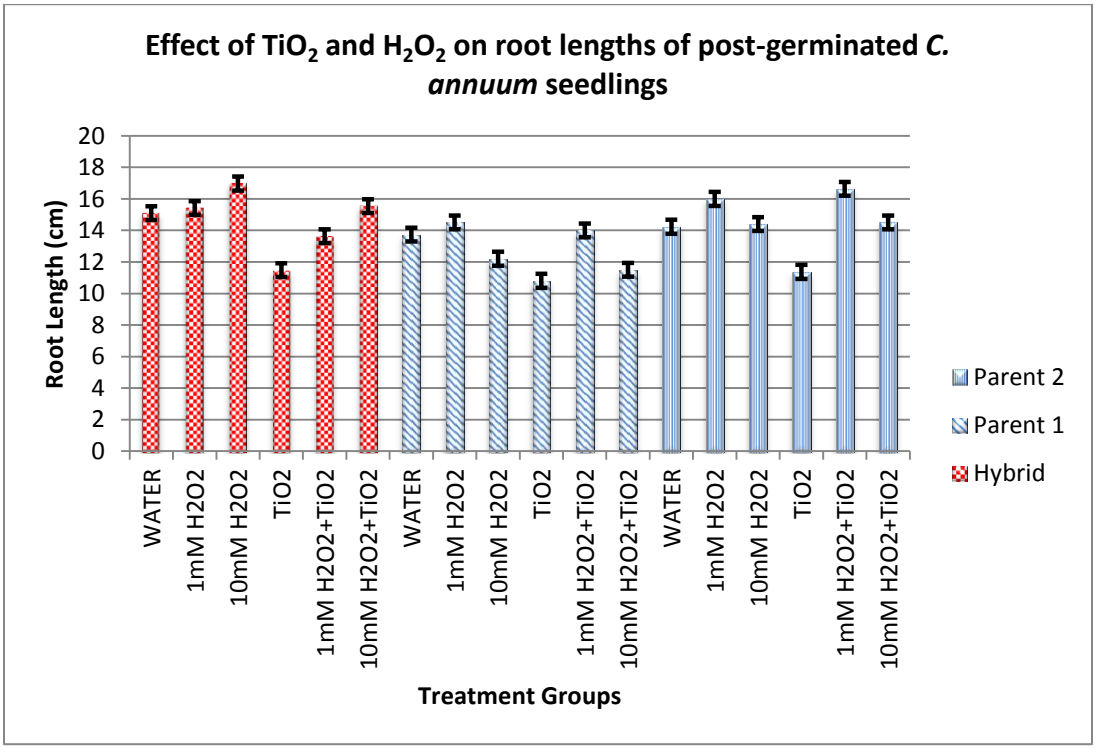

Figure 4.4 Effects of pre-treated H_2O_2 and TiO_2 on post-germinated root lengths of *C. annuum* seedlings of hybrid and the parents
10 mM H_2O_2 was determined a better candidate in reducing photo toxic effects of nanoparticles in hybrids and 1 mM H_2O_2 showed same results in parental lines. Nanoparticles treated plants reduced root lengths in each group of *C. annuum*. Data represents the means of three independent experiments with 3 plants for each treatment in each experiment. Error bars are standard error (SE) for three replicate reactions.

4.3.5 Comparison on Effects of H_2O_2 and TiO_2 on Root Length of *C. annuum* Seedlings of Hybrids and the Parents:

1 mM H_2O_2 increased root length in hybrid and its parents while 10 mM caused stress in parent 1 but increased root length in hybrid and parent 2 as presented in figure 4.5. This difference is may be due to difference in genotype of these groups. So we concluded that 1 mM played positive role in reducing nanotoxicity in parental lines while 10 mM played the same role for hybrid.

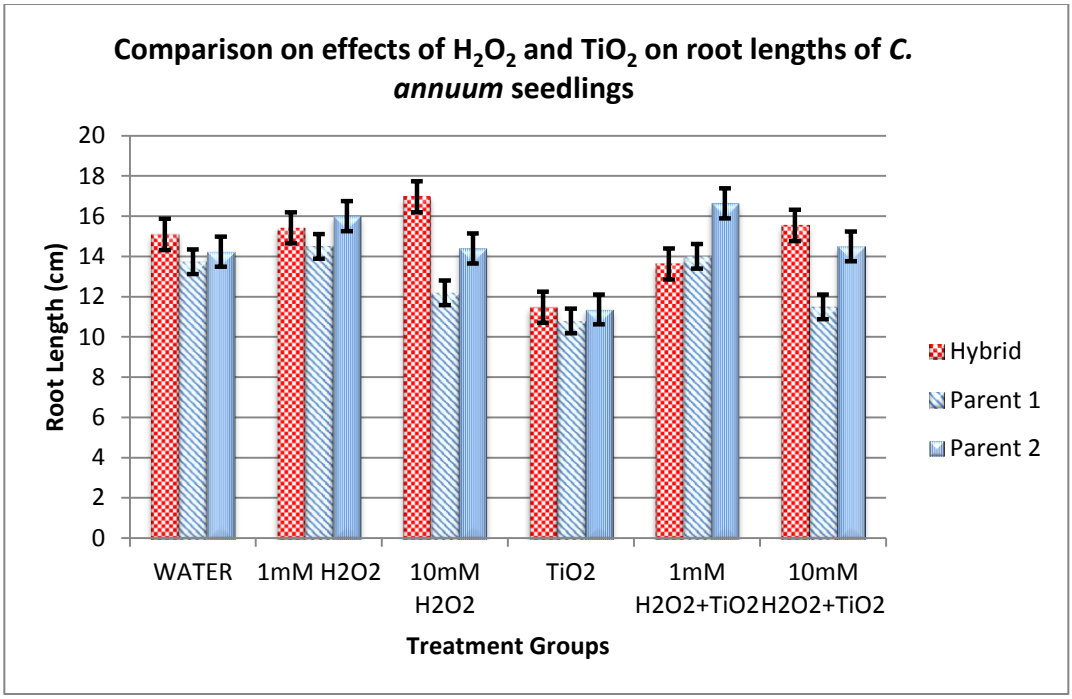

Figure 4.5 Comparison on effects of pretreated H_2O_2 and TiO_2 on root lengths of *C. annuum*

Nanoparticles have negative effects on root lengths which were reduced by 1 m M H_2O_2 in parental lines and 10 mM H_2O_2 in hybrid. Nanoparticles reduced root lengths in each group. However, 10 mM H_2O_2 enhanced root length in hybrid only. Data represents the means of three independent experiments with 3 plants for each treatment in each experiment. Error bars are standard error (SE) for three replicate reactions.

4.3.6 Effects of H_2O_2 and TiO_2 on Post-Germinated Shoot Lengths in *C. annuum* Seedlings of Hybrids and the Parents:

The shoot length was also measured on 44th day post-germination. From measurement it was investigated that 1 mM H_2O_2 increased shoot length by 1.2 %, 8.8% and 8.6% in hybrid, parent 1 and parent 2 respectively as compared to their respective water controls. The increased concentration of H_2O_2 to 10 mM also increased 12.4% shoot length but decreased shoot length by 9.5% and 14% in parent 1 and parent 2 respectively. Nanoparticles have shown to effect negatively and cut the shoot growth to 9.7% in hybrid, 17% in parent 1 and 10% in parent 2. Pretreatment of 1 mM H_2O_2 reduced the toxicity caused by nanoparticles as reduction in decrease of shoot length was detected as compared to TiO_2 control

with 7.7% reduction in shoot length compared to water control. Conversely, 6% increase in shoot length was noticed for parent 1 and reduction in shoot length was decrease to 1.3% in parent 2 as displayed in figure 4.6. Pretreatment with 10 mM H_2O_2 caused 9.4% growth increase in hybrid and decreased 7.7% shoot length in parent 1 and 22% in parent 2.

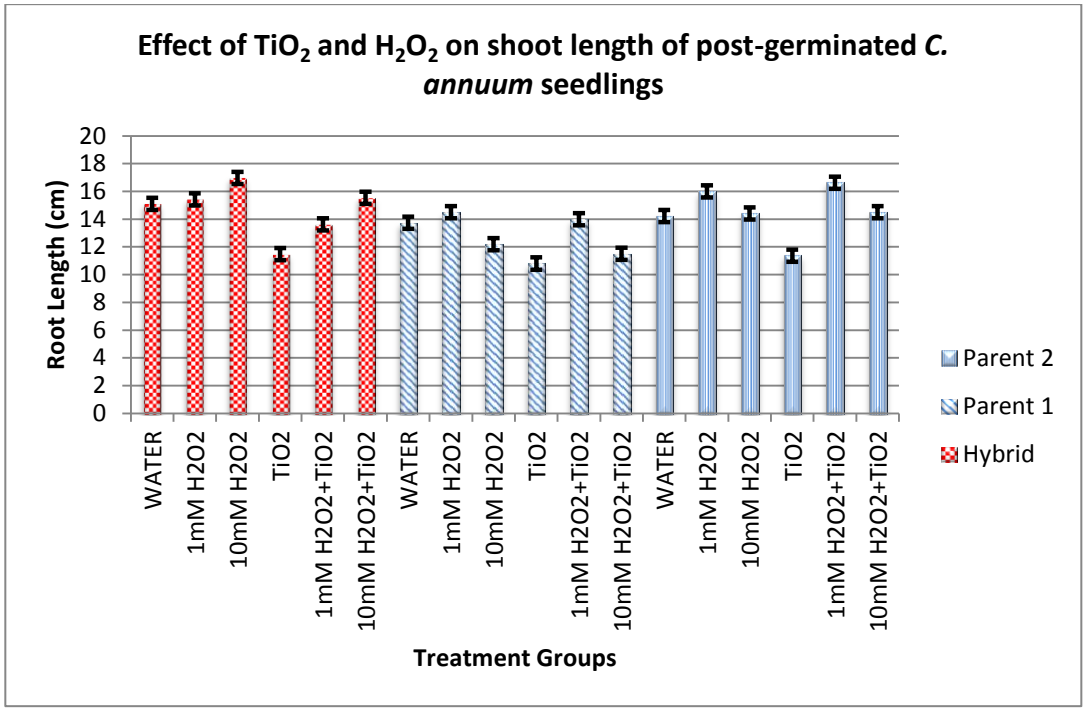

Figure 4.6 Effects of pre-treated H_2O_2 and TiO_2 on post-germinated shoot lengths of *C. annuum* seedlings

TiO_2 negatively effects shoot growth of *C. annuum* hybrid and the parents as compared to water control, while 1 mM H_2O_2 reduced the phototoxic effect of TiO_2 in parental lines and 10 mM H_2O_2 do the same in hybrid as revealed by shoot lengths in these groups. Nanoparticles reduced shoot growth in each group. Data represents the means of three independent experiments with 3 plants for each treatment in each experiment. Error bars are standard error (SE) for three replicate reactions.

4.3.7 Comparison on Effects of H_2O_2 and TiO_2 on Shoot Lengths of *C. annuum* Seedlings of Hybrids and the Parents:

Hence, it is concluded that in controlled conditions when only water is applied hybrid showed enhanced shoot growth relative to its parents. 1 mM H_2O_2 elongated

shoots in all the three groups but the increase was more pronounced in parents. Boosted concentration of H_2O_2 to10 mM also revealed further improvement in shoot length contrary to both parents in which this treatment acts as a stress. TiO_2 exhibited toxic property towards shoot growth in *C. annuum* which discovered to be diminished with pretreatment of 1 mM H_2O_2 in parents and with 10 mM H_2O_2 in hybrid as represented in figure 4.7.

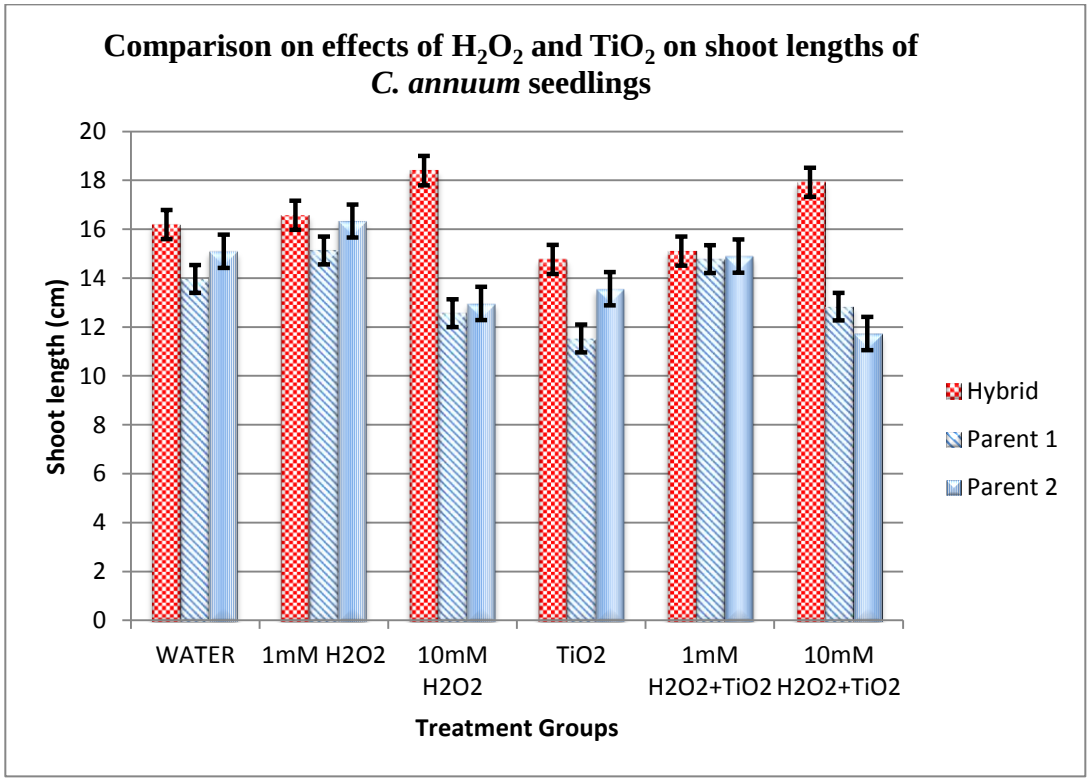

Figure 4.7 Comparison on effects of pre-treatment of H_2O_2 and TiO_2 on shoot lengths of *C. annuum* seedlings

10 mM H_2O_2 increased shoot length in hybrid however this concentration showed growth retardation in parental lines. 1 mM H_2O_2 pretreatment reduced toxic effect of TiO_2 in parents and 10 mM H_2O_2 do the same in hybrid. Data represents the means of three independent experiments with 3 plants for each treatment in each experiment. Error bars are standard error (SE) for three replicate reactions.

4.3.8 Effects of H_2O_2 and TiO_2 on Moisture Content of *C. annuum* Seedlings of Hybrids and the Parents:

The amount of moisture that plant contains, points towards physio-chemical assets of plant tissues and effect of environment. To pinpoint probable impact of pretreated H_2O_2 and TiO_2 on moisture content, fresh weight and dry weight of *C. annuum* hybrid and parents seedlings was recorded, and moisture content was calculated. In hybrid plants, exposed to 10 mM H_2O_2, the moisture content was found 3.3% more as compared to water control that is highest for all the groups of hybrid plants. Treatment with 1 mM H_2O_2 also enhanced 0.6% moisture content of hybrid plants contrary to TiO_2 which lessened moisture value by 2.3% relative to control group. The maximum decrease in moisture content was noticed in plants exposed to 1 mM pretreatment of H_2O_2 showing 4% decrease in moisture level of tissues. Likewise, decreasing trend is also observed with 10 mM pretreatment of H_2O_2 which showed 3% decrease in the moisture content.

In parent 1, highest moisture content was noted with 1 mM H_2O_2 treatment, while a decrease of 0.2% and 0.9% in moisture content was recorded in plants exposed 10 mM H_2O_2 and TiO_2 treatment. The highest decrease of 2.6% in moisture level was detected with 1 mM pretreatment. However 10 mM pretreatment showed 1% lessened moisture relative to the water control. A similar trend was noticed for parent 2, where highest moisture content of 0.2% was found in plants with 1 mM H_2O_2 treatment, while lowest (6.8%) is observed when 1 mM H_2O_2 was given as pretreatment to nanoparticles treated groups. Among the rest of the groups, plants treated with 10 mM H_2O_2, TiO_2 and pretreated with 10 mM H_2O_2 showed a decrease of 1%, 2.5%, and 5.3 respectively as shown in figure 4.8.

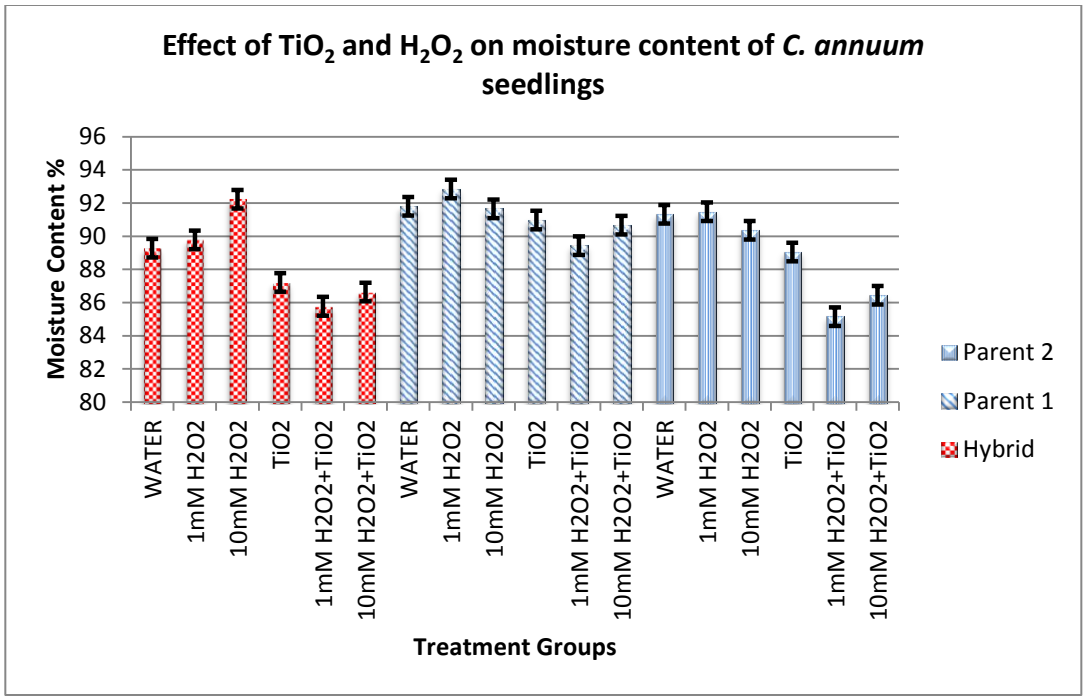

Figure 4.8 Effect of TiO_2 and H_2O_2 on moisture content of *C. annuum* seedlings

10 mM H_2O_2 in hybrid and 1 mM H_2O_2 in both parents showed highest moisture level. However, H_2O_2 treatment prior to nanoparticles decreased moisture level in all groups. So, least moisture content was observed in pretreatment groups. Data represents the means of three independent experiments with 3 plants for each treatment in each experiment. Error bars are standard error (SE) for three replicate reactions.

4.3.9 Comparison on effects of H_2O_2 and TiO_2 on Moisture Content of *C. annuum* Seedlings of Hybrids and the Parents:

In controlled conditions highest moisture content was observed in parent 1, and least was present in hybrid. Highest increased in moisture content was noticed at 1 mM H_2O_2 concentration while slight increase was observed at 10 mM H_2O_2 in all groups. TiO_2 lessened moisture level in both parents but hybrid was most affected. Pretreatment with 1 mM H_2O_2 indicated least moisture level in all groups while 10 mM pretreatment exhibited slight increase in moisture level of tissues as compared to 1 mM H_2O_2 pretreatment as displayed in figure 4.9.

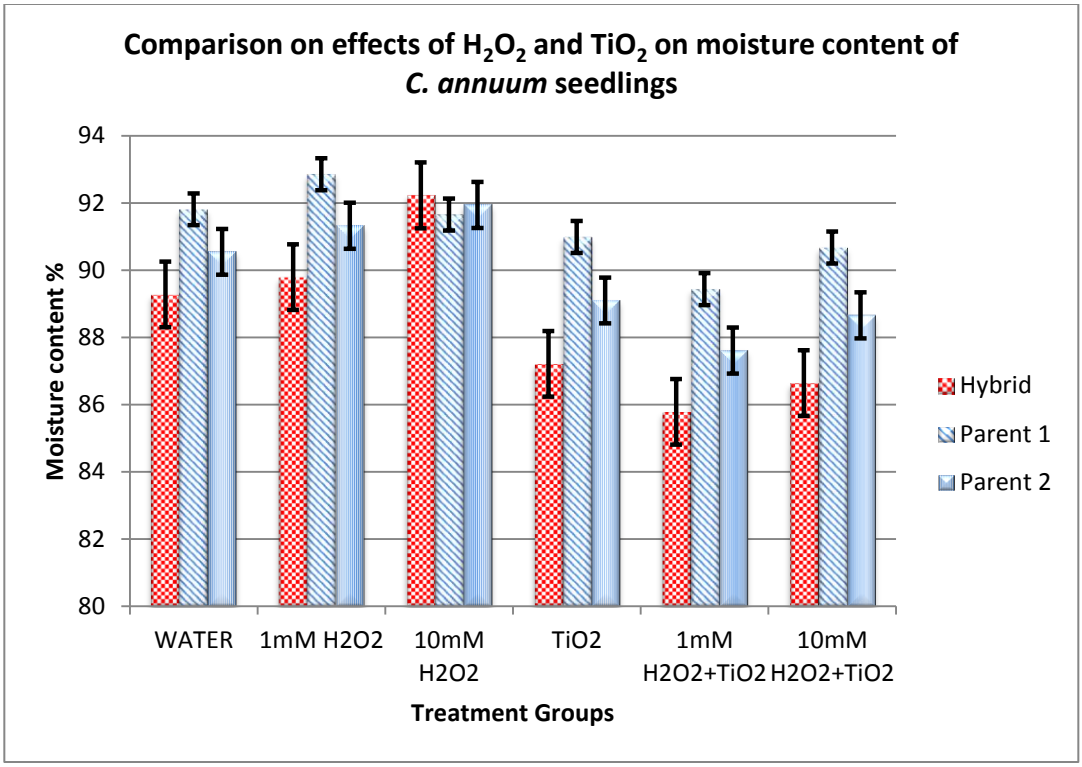

Figure 4.9 Comparison on effects of H_2O_2 and TiO_2 on moisture content of *C. annuum* seedlings
1 mM H_2O_2 was found remarkable in increasing moisture level while treatment of H_2O_2 at same concentration prior to nanoparticles application has negative effect on moisture content as compared to nanoparticle control groups. Parent 1 has highest water content in its tissues while hybrid has least moisture level. Data represents the means of three independent experiments with 3 plants for each treatment in each experiment. Error bars are standard error (SE) for three replicate reactions.

Hence we conclude that both combinations of exogenous H_2O_2 when applied prior to TiO_2 nanoparticles decrease the water content of tissues. This may be used as a protective mechanism because decreasing water content of cell increase solute potential of cell that consequently prevent the nanoparticles to enter into the cell but without nanoparticles both combinations of exogenous H_2O_2 increased water level of cell, that witnessed a positive role of exogenous H_2O_2 to modify a plant internal mechanisms so that it can retain high water level in tissues to combat stress.

4.4 Biochemical Assays:

4.4.1 Effects of H_2O_2 and TiO_2 on Chlorophyll Content of *C. annum* Hybrid and Parents Seedlings:

TiO_2 have controversial effects on physiology and biochemical activities of plants. Plants grown for 30 days post-germination were applied with H_2O_2 for 2 days and afterward provided with TiO_2 for 10 days were harvested and leaves were used to analyze chlorophyll content.

Exogenous application of 1 mM H_2O_2 in hybrid caused an increase of 1.7% in chlorophyll while increasing H_2O_2 concentration to 10 mM enhanced 3.7% photosynthetic pigments. Contrary to that, 5.5% decrease in green pigment was recorded by application of nanoparticles. However when nanoparticles were applied after 1 mM and 10 mM H_2O_2 treatment, chlorophyll level enhanced by 11.9% and 5.8% respectively relative to control group. Hence these results describe role of H_2O_2 in increasing photosynthetic ability of hybrid plants. Similar results were revealed by parent 1 where 1 mM and 10 mM boosted 29% and 10.5% green pigment in leaf tissues where as 6% decrease was recorded for nanoparticles as compared to water control. In pretreatment groups with less H_2O_2 and high H_2O_2, 16.5 and 21.7% respective increase in light absorbing pigment was recorded as shown in figure 4.10. But in parent 2 a slight different behavior was noticed. 1 mM like previous 2 groups intensify chlorophyll level (10%) but 10 mM displayed negative role and lessened 24% pigment level of cell as compared to control. Nanoparticles application also demonstrated 10% declining ability of plants to synthesize chlorophyll comparative to control group. Pretreatment with 1 mM and 10 mM improved the pigment level by 10.5% and 3% respectively.

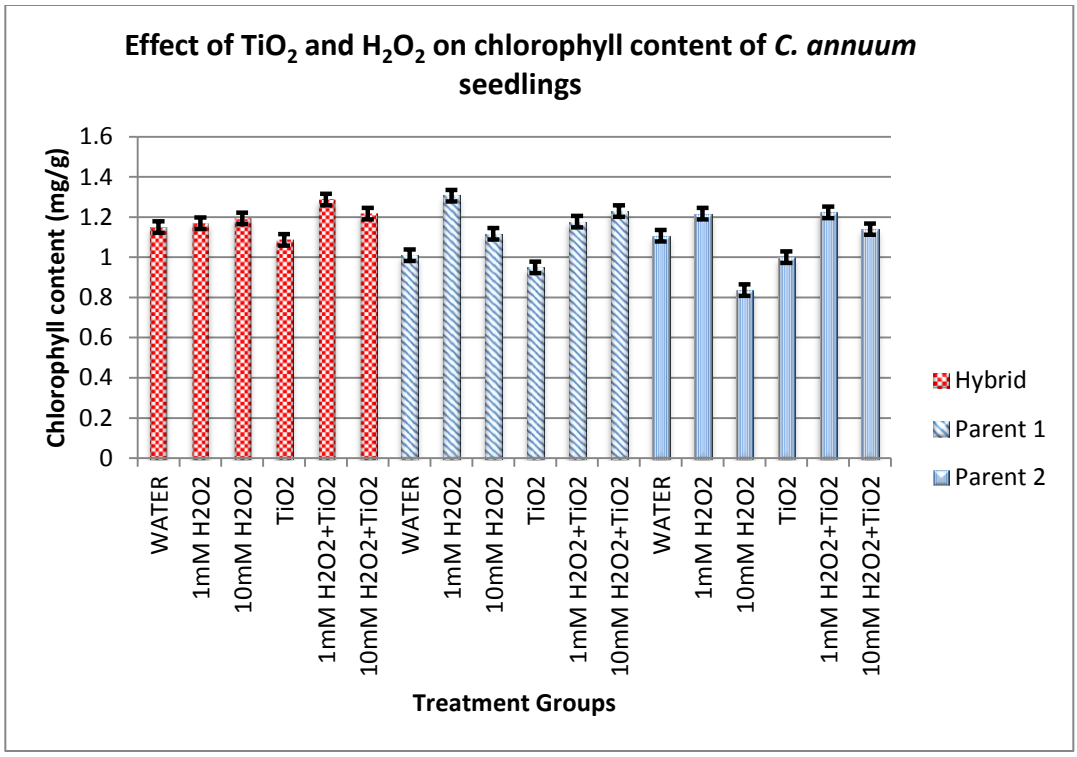

Figure 4.10 Effect of TiO_2 and H_2O_2 on chlorophyll content of *C. annuum* seedlings

Exogenous application of H_2O_2 in combination with TiO_2 enhanced photosynthesizing ability of *C. annuum* plants tissues, while nanoparticles alone showed negative affect on level of green pigment. Data represents the means of three independent experiments with 3 plants for each treatment in each experiment. Error bars are standard error (SE) for three replicate reactions.

4.4.2 Comparison on effects of H_2O_2 and TiO_2 on Chlorophyll Content of *C. annuum* Hybrid and Parents Seedlings:

Comparative analysis on green pigment among parental and hybrid lines was performed, which revealed that hybrid has maximum photosynthesizing pigment whereas parent 1 has the least under controlled conditions but 1 mM exogenous application of H_2O_2 boosted light absorbing ability of leaves in parent 1 although hybrid was least affected. Contrary to that, use of 10 mM H_2O_2 enhanced chlorophyll content in hybrid however this concentration has negative effect on chlorophyll in parent 2. This difference is may be due to difference in genetic makeup. TiO_2 has minor negative effects on photosynthesis as compared to respective control of all three groups.

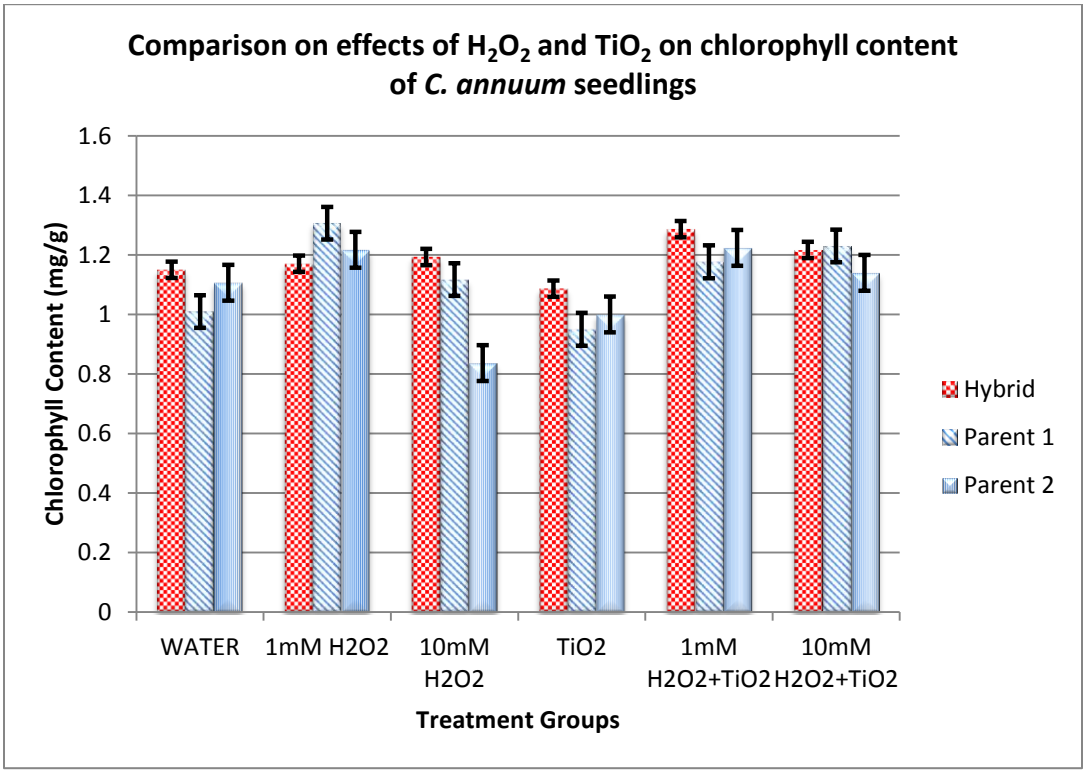

Figure 4.11 Comparison on effects of H_2O_2 and TiO_2 on chlorophyll content of *C. annuum* hybrid and parents seedlings
1mM H_2O_2 improved photosynthesizing pigment in parental lines relative to water respective water control whereas this concentration also showed increasing effect along with 10 mM H_2O_2 on chlorophyll when given in combination with nanoparticles to hybrid and its parents as compared to respective nanoparticle control groups. Data represents the means of three independent experiments with 3 plants for each treatment in each experiment. Error bars are standard error (SE) for three replicate reactions.

A unique trend was observed with 1 mM pretreatment of H_2O_2 as it enhance chlorophyll in hybrid (which was least affected when 1 mM H_2O_2 alone was applied), whereas decrease chlorophyll pigment in parent 1 (which had most positive effect when 1 mM H_2O_2 alone was applied) as indicated in figure 4.11. 10 mM H_2O_2 when applied in combination improved photosynthesizing pigment in parental lines as compared to their 10 mM control whereas hybrid indicated no remarkable change.

4.4.3 Effects of H_2O_2 and TiO_2 on Soluble Sugar Content of *C. annuum* Hybrid and Parents Seedlings:

High sugar content is required by young seedlings to fulfill their energy demand especially in actively growing regions(Moghaddam and Van den Ende, 2012, O'Hara et al., 2013). Sugar is synthesized in leaves by the process of photosynthesis and help plant not only to perform normal physiological process but also combat stress conditions, so the level of sugar in plant tissues can be used as an indicator of stress on plants. To measure amount of sugar present in leaves, standard curve was generated from known concentrations of sugar as presented in figure 4.12.

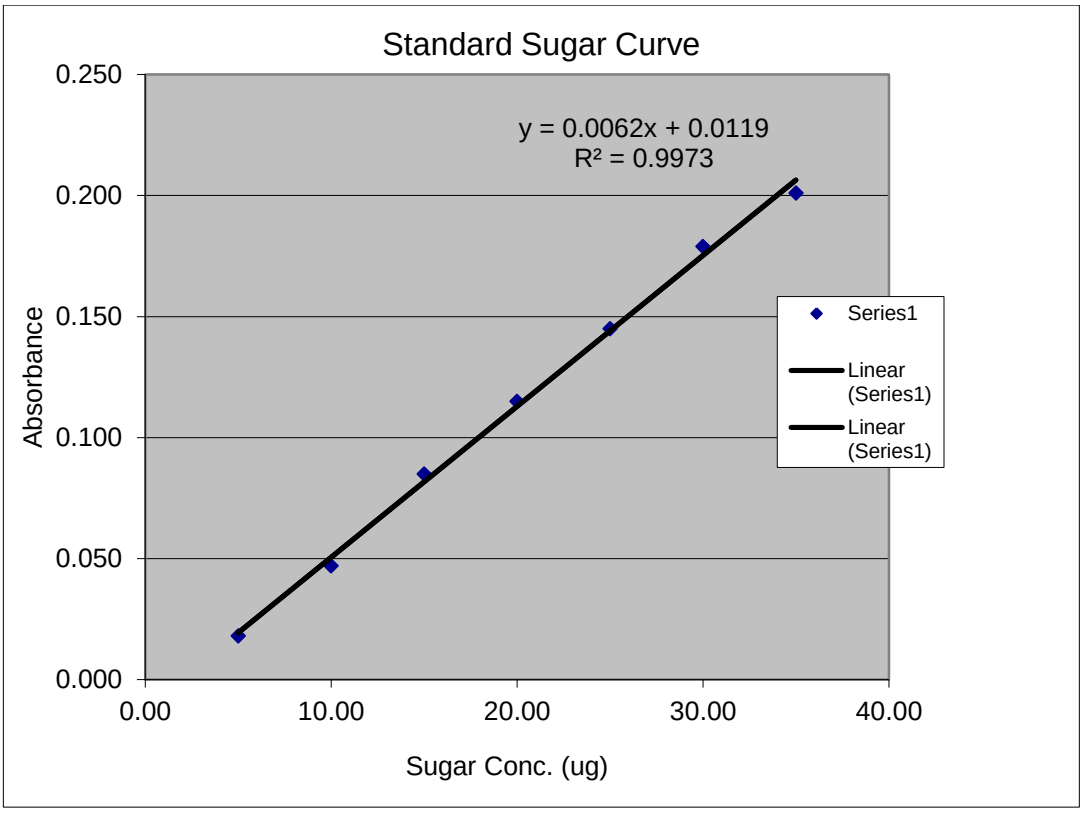

Figure 4.12 Standard curve for sugar content
The straight line indicated direct relation in absorbance and sugar concentration.

Absorbance of experimental samples when plotted on standard sugar curve graph provided a measure of sugar concentrations of the samples under examination.

Sugar content in *C. annuum* hybrid increased with increasing concentration of H_2O_2 as 4.6% and 23.6% increase in sugar was detected in group "b" and group "c" plants respectively. So it was concluded that increasing stress caused increase in sugar level as application of TiO_2 also enhanced 21% photosynthate level as compared to group "a". Pretreatment with 1 mM H_2O_2 reduced total soluble sugar by 5% relative to water control indicating stress tolerance role of 1 mM H_2O_2 in hybrid plants. However, 10 mM H_2O_2 improved toxic role of TiO_2 and increased stress as 34% escalation in photosynthate was recorded. A similar trend was noted in parent 1 where increasing H_2O_2 concentration increased 20% and 33% in sugar content as compared to its water control. Application of nanoparticles in root zone proved to be highly toxic as 166% rise in photosynthate was noticed.

Contrary to that, when nanoparticles were used after 1 mM and 10 mM H_2O_2 pretreatment, the increase in sugar level relative to water control was reduced to 8% and 83% respectively. It again displayed positive role of H_2O_2 in reducing stress imposed by TiO_2. Use of 1 mM H_2O_2 in parent 2 reduced 4.6% sugar content instead of increasing relative to its control, though its 10 mM concentration caused 17% increase in leaf sugar. Again it was displayed that enhancing H_2O_2 concentration, increased stress on plants that in turn rise the sugar level of leaves. Likewise, use of nanoparticles further improved sugar content to 60% as compared to its control. However, prior application of 1mM and 10mM H_2O_2 to TiO_2 reduced the increase in sugar level to 23% and 2% respectively as displayed in figure 4.13.

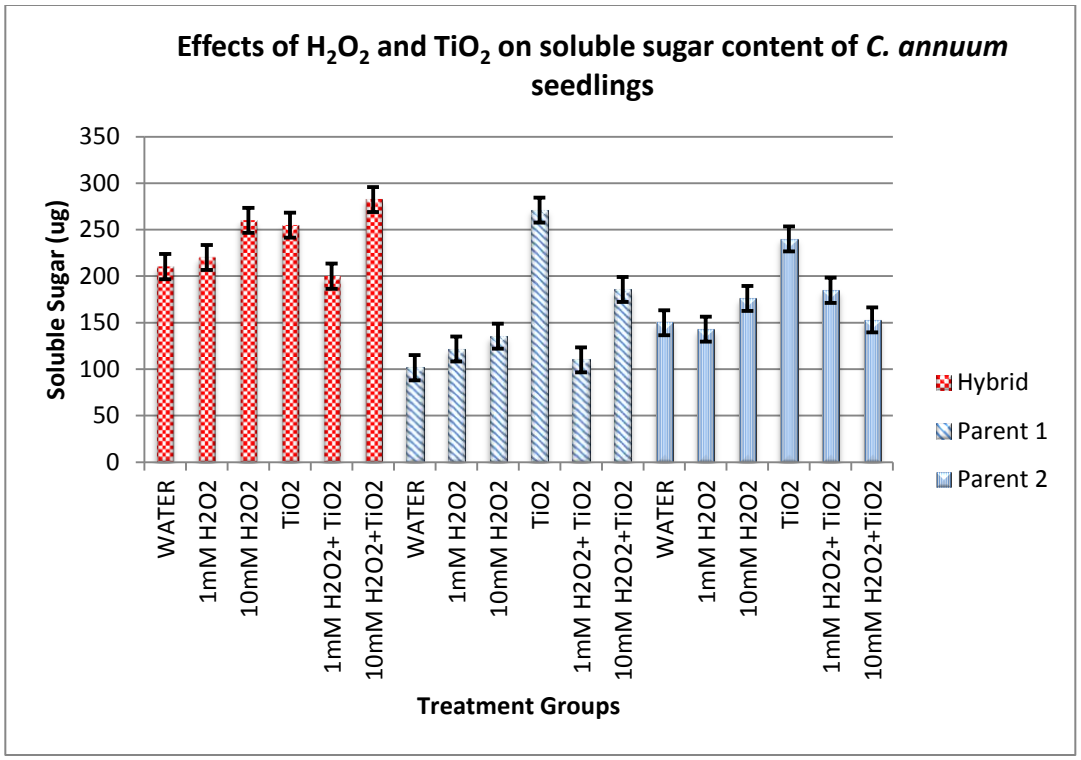

Figure 4.13 Effects of H_2O_2 and TiO_2 on soluble sugar content of *C. annuum* seedlings

Increasing the oxidative stress with exogenous H_2O_2 application led to increase in sugar level as compared to respective water controls in *C.annuum* seedlings of hybrid and its parents. Nanoparticles also enhanced sugar content, however pretreatment of H_2O_2 reduced the increase in sugar level. Data represents the means of three independent experiments with 3 plants for each treatment in each experiment. Error bars are standard error (SE) for three replicate reactions.

Under controlled conditions, highest sugar level was observed in hybrid and least was present in parent 1. Use of 1 mM and 10 mM H_2O_2 increased photosynthate level in all groups. Similarly, nanoparticles also improved sugar level of plants in parental and hybrid lines. With the exception of pretreatment of 10 mM H_2O_2 in hybrid, prior treatment of H_2O_2 either 1mM or 10 mM reduced sugar level by reducing stress in all groups. From comparison of results, it is witnessed that 1 mM H_2O_2 prior application can be used as tool to reduce stress imposed by subsequent application of TiO_2 as represented in figure 4.14.

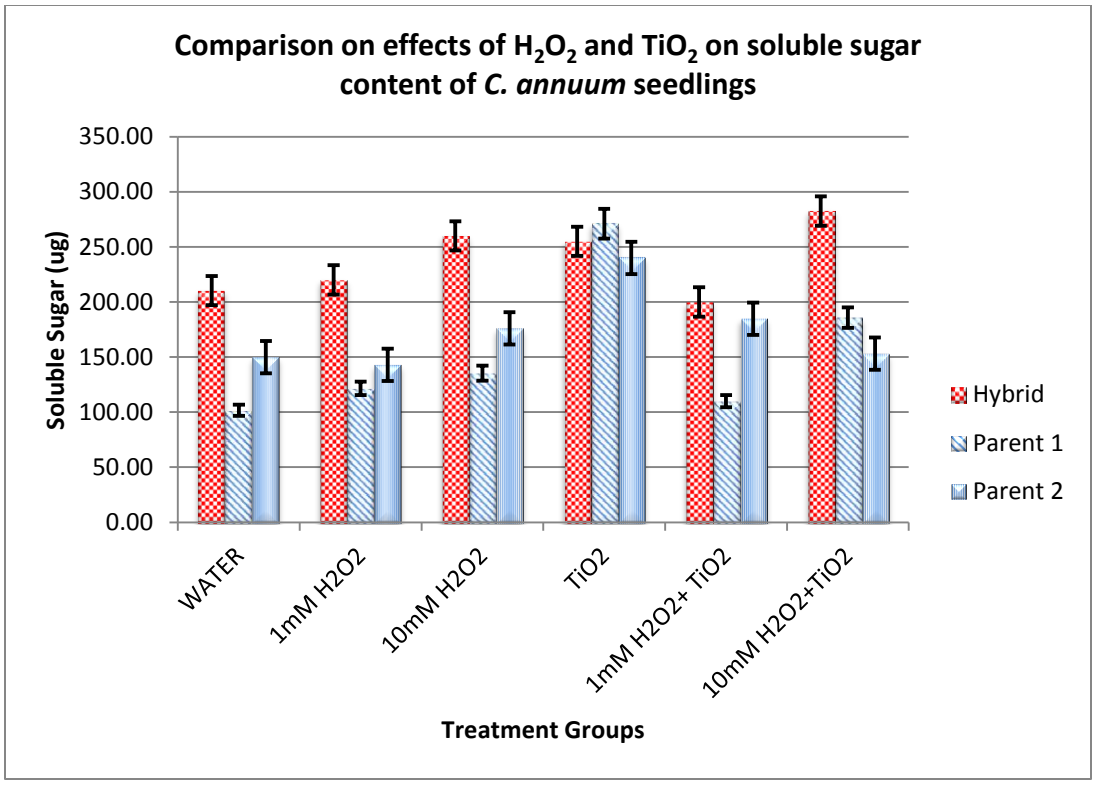

Figure 4.14 Comparison on effects of H_2O_2 and TiO_2 on soluble sugar content of *C. annuum* seedlings
Treatment with 10 mM H_2O_2 and nanoparticles increased sugar content in each group of *C. annuum* while 1mM pretreatment of H_2O_2 reduced sugar level in all groups. Data represents the means of three independent experiments with 3 plants for each treatment in each experiment. Error bars are standard error (SE) for three replicate reactions.

4.5 Antioxidant enzyme assays:

When plants encountered any biotic stress like pathogen attack or abiotic stress like drought and salinity, they react by triggering multiple defense mechanisms, one of them is the immediate manufacturing and accumulation of ROS (reactive oxygen species) including hydrogen peroxide (H_2O_2) (Király et al., 2008). These ROS can interact with lipids, nucleic acids and proteins modifying the biological potential of these biomolecules (Halliwell and Gutteridge, 2007, Mateos et al., 2013). Cells from these plants have an antagonistic mechanism that comprises antioxidant systems that are capable to rummage ROS, thus permitting the cell to survive with diverse stress conditions. The key antioxidant enzymatic systems are ascorbate

peroxidase, superoxide dismutase and catalase (Mittler et al., 2004, Halliwell and Gutteridge, 2007, Mateos et al., 2013). Here, we assessed total soluble protein and investigated the activity of antioxidant enzymes in *C. annuum* in response to exogenous H_2O_2 and TiO_2.

4.5.1 Total Soluble Protein of *C. annuum* Hybrid and Parents Seedlings:

To study the effect of pretreatment of H_2O_2 and TiO_2 on total proteins of *C. annuum* cells, total soluble protein was measured by Bradford method. Hybrid plants exposed to 1 mM and 10 mM H_2O_2 treatment possessed 5% and 25% rise in protein content respective to water control. This may be due to stress conditions imposed by these concentrations and plant responded by increasing protein level in tissues to survive the stress. When protein content for nanoparticles treated hybrid plants was detected, 12.5% increase was found in leaf tissues, which again indicated stress, caused by nanoparticles. However, when nanoparticles were given after 1 mM application of H_2O_2, increase in protein content was reduced to 7% relative to nanoparticle control but 10 mM pretreatment has opposite role to 1 mM pretreatment and increase protein content to 14.2% as compared to water control which is higher than nanoparticle control but less than respective H_2O_2 control.

Plants from parent 1 applied with 1 mM and 10 mM H_2O_2 showed 64% and 31% increase in protein level whereas nanoparticles increase protein content to 38%. When nanoparticles were given after 1 mM and 10 mM H_2O_2 as prior treatment, 40.5% and 2.5% rise in protein content were measured. On the other hand, 57.5% and 47.5% increase was detected in plants from parent 2 applied with 1 mM and 10 mM H_2O_2 respectively as compared to respective water control. Contrary to that, when nanoparticles were used as treatment, decrease of 12% was noticed in protein content of leaf tissues. Combination groups in parent 2 again exhibited 45% and 35% increase in protein level as presented in figure 4.15.

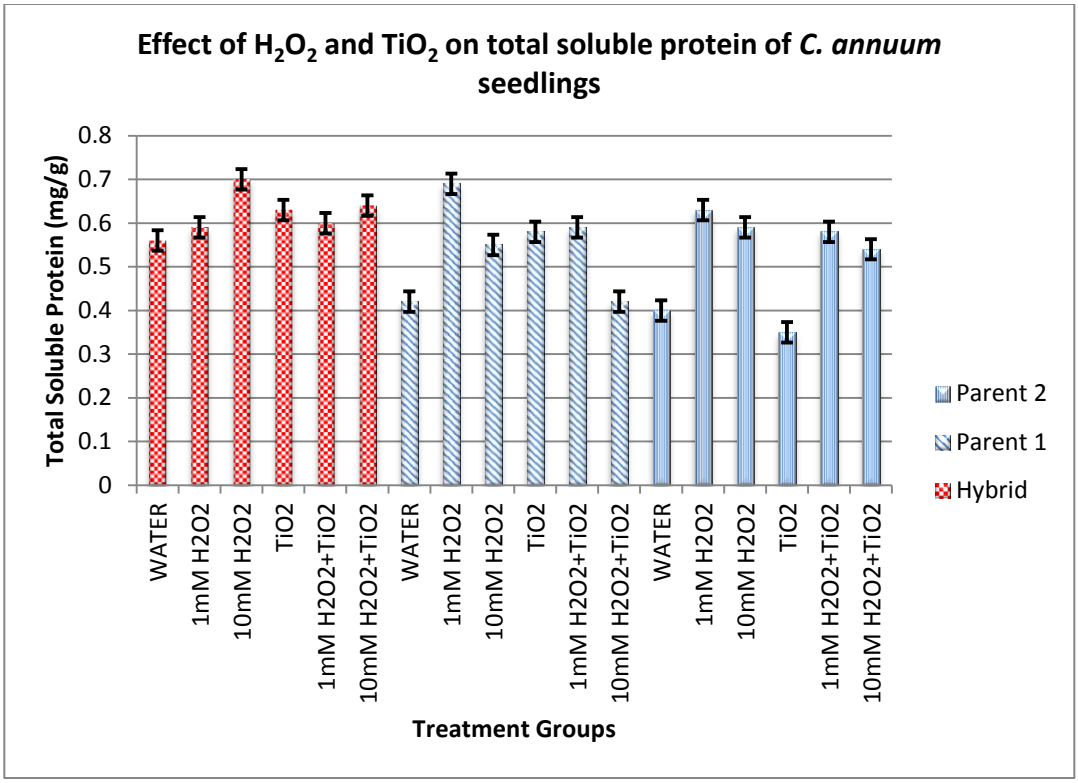

Figure 4.15 Effect of H_2O_2 and TiO_2 on total soluble protein of *C. annuum* seedlings

10 mM H_2O_2 in hybrid, 1 mm H_2O_2 in parent 1 and parent 2 have shown an increase protein content which may be an indication of stress to which plant responded by increasing protein level, whereas pretreatment with 1 mM and 10 mM H_2O_2 in hybrid and parent 1 respectively, decrease the protein level which may be due to the fact that these concentrations of H_2O_2 decrease the stress imposed by nanoparticles when given in combination. Data represents the means of three independent experiments with 3 plants for each treatment in each experiment. Error bars are standard error (SE) for three replicate reactions.

In controlled conditions, hybrid showed more protein level as compared to parental lines whereas 1 mM H_2O_2 enhanced protein level in all groups but least in hybrid. It revealed that 1 mM act as stress in parental lines while hybrid possessed resistance towards exogenous oxidative stress at 1 mM. The difference in response may be due to difference in genotype. On the other hand, 10 mM acted as oxidative stress in hybrid although it also increased protein content in parental lines. Nanoparticles decrease protein content in parent 2 whereas 1 mM pre-application of H_2O_2 showed its protective role against nanotoxicity. Likewise, 10

mM pretreatment was found more effective in reducing nanotoxicity in parent 1 as shown in figure 4.16.

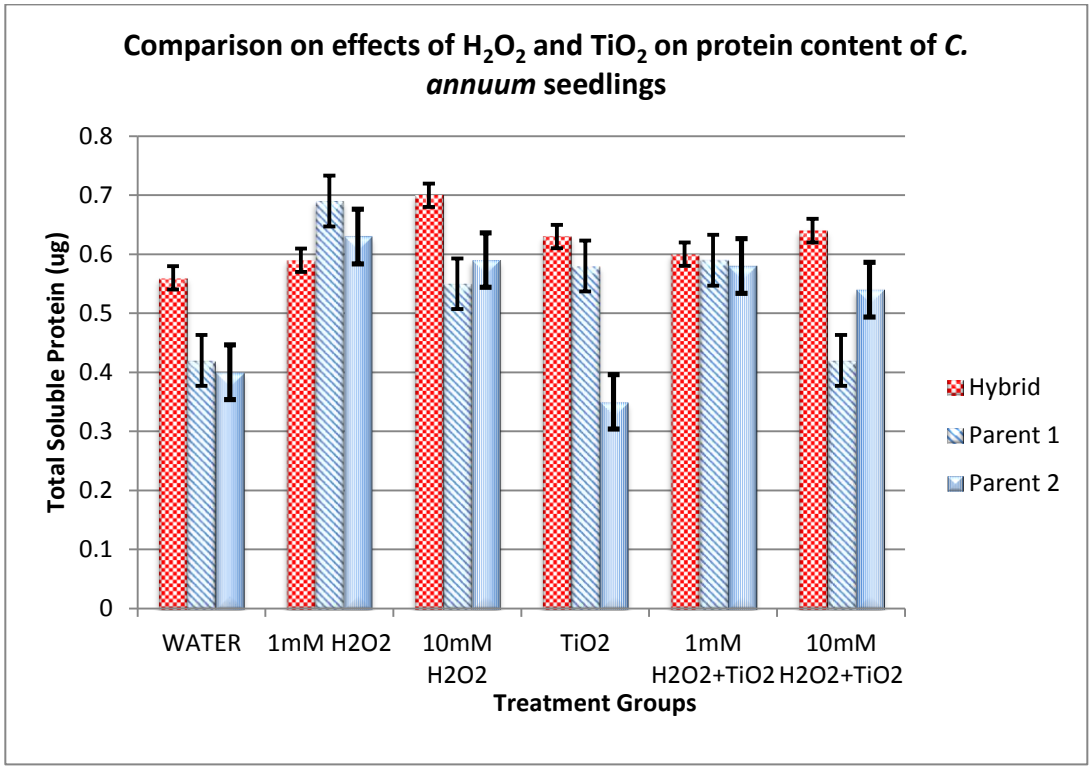

Figure 4.16 Comparison on effects of H_2O_2 and TiO_2 on protein content of *C. annuum* seedlings
H_2O_2 acted as oxidative stress in *C. annuum* hybrid and its parent's seedlings and increased total protein concentration in each group at both concentration. Data represents the means of three independent experiments with 3 plants for each treatment in each experiment. Error bars are standard error (SE) for three replicate reactions.

4.5.2 Effect of H_2O_2 and TiO_2 on Catalase Activity of *C. annuum* Hybrid and Parents Seedlings:

CAT is present in all organisms and helps to maintain endogenous H_2O_2 by decomposing it into H_2O and O_2 (Tanaka et al., 1999). We observed 8% and 101% increase in CAT activity in group "Hb and Hc" respectively as compared to group "Ha" of *C. annuum*. Group "Hd" also showed increase in CAT activity (86%) as compared to group "Ha" of *C. annuum*. This could be explained due to the toxic effects induced by TiO_2, which in turn increases ROS, consequently anti-oxidant

system activate to scavenge ROS molecules. However, in group "He and Hf" 12% increase and 30% decrease in CAT activity was determined respectively. This increase in enzyme activity is lower that nanoparticles control "Hd" group. Similarly in P1, group "b and c" revealed 29% and 100% improvement in CAT activity respectively as compared to group "a". Likewise, group P1d also exhibited enhanced activity of CAT by 61% as compared to "P1a" group. Group "e and f" in P1 revealed 35% and 2% decline in enzyme activity as compared to "P1a" group. Correspondingly, in P2 group "b and c" displayed 21% and 105% increase in enzyme activity whereas group P2d exhibited 68% improvement in enzyme activity. However, group P2e and P2f showed 55% and 29% decline in enzyme activity as compared to group P2a. The results support our hypothesis that pretreatment of H_2O_2 reduce toxicity induced by TiO_2 nanoparticles as displayed in figure 4.17.

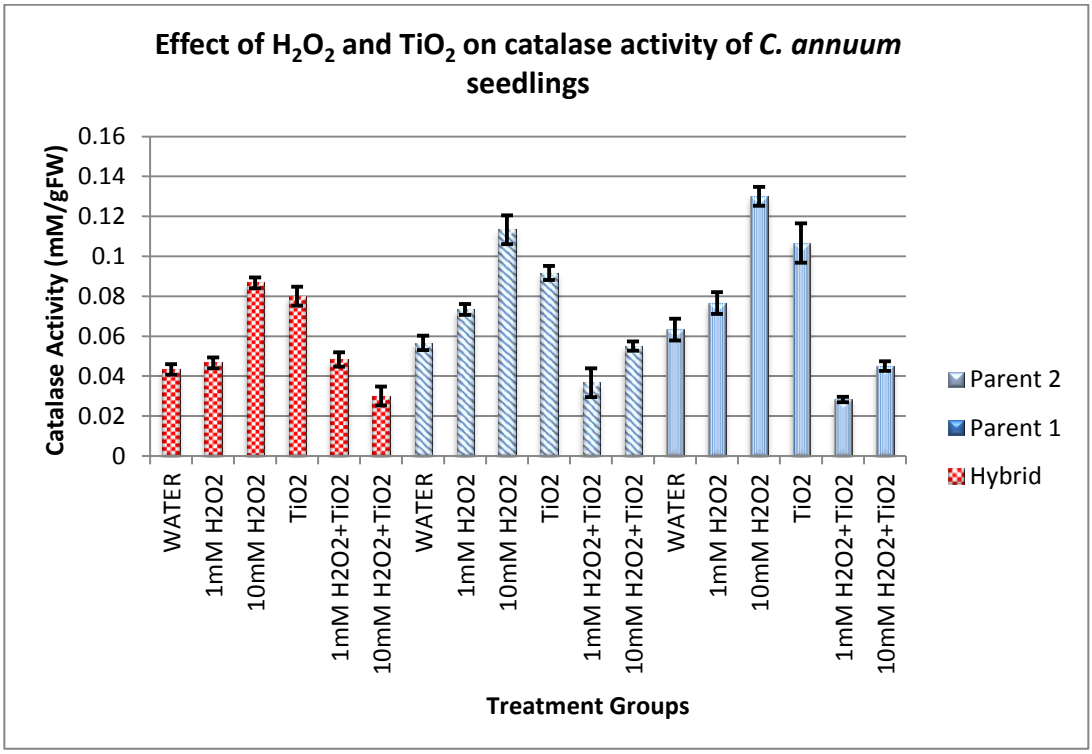

Figure 4.17 Effect of H_2O_2 and TiO_2 on catalase activity of *C. annuum* seedlings

H_2O_2 acted as oxidative stress in *C. annuum* hybrid and its parent's seedlings and increased catalase activity in both parents and the hybrid at 1 mM H_2O_2 and 10 mM H_2O_2 concentration. TiO_2 treated groups also exhibited increase in enzyme activity. However, pretreated groups showed decrease in enzyme activity. Data represents the means of three independent experiments with 3 plants for each treatment in each experiment. Error bars are standard error (SE) for three replicate reactions.

The hybrid possessed lowest CAT activity in groups Ha, Hb, Hc, Hd and Hf (as mentioned in section I of chapter 3) as compared to its parents whereas in group He, hybrid exhibited improved enzyme activity as compared to P1 and P2. Group "e and f" of *C. annuum* hybrid and the parents displayed reduced enzyme activity relative to group "d". Hence we conclude that, pretreatment of H_2O_2 reduced oxidative stress as well as nanotoxicity induced by TiO_2 nanoparticles as shown in figure 4.18.

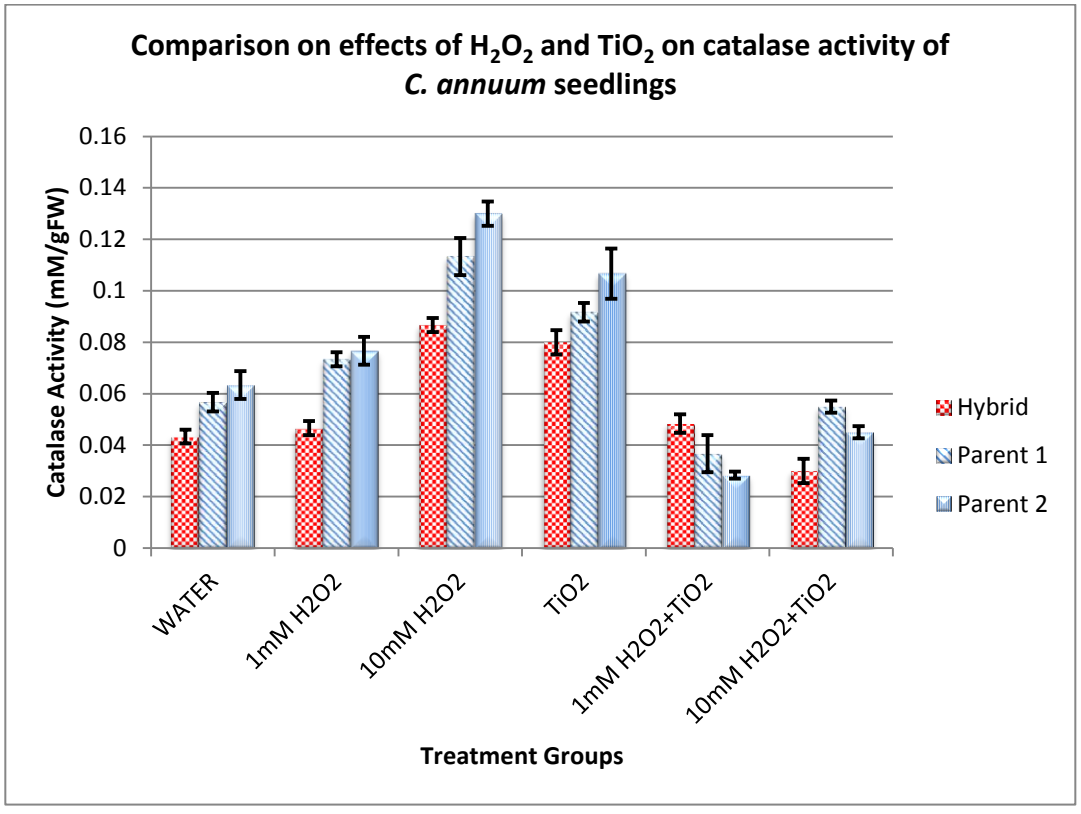

Figure 4.18 Comparison on effects of H_2O_2 and TiO_2 on catalase activity of *C. annuum* seedlings

Parent 2 exhibited highest activity of CAT in 1 mM H_2O_2, 10 mM H_2O_2 and TiO_2 nanoparticles treated groups. However, lowest activity was revealed in hybrid.

4.5.3 Effect of H_2O_2 and TiO_2 on Ascorbate Peroxidase Activity of *C. annuum* Hybrid and Parents Seedlings:

Oxidative stress causes ROS production which activates plants' internal antioxidant system to reduce the damage caused by free radical. High concentration of H_2O_2 in hybrid plants, enhanced ascorbate peroxidase (APX) activity 3 folds, whereas 1 mM concentration caused 1 fold increase in enzyme activity. When nanoparticles were applied alone or in combination with H_2O_2, increased in APX activity was found. The same trend was observed in parent 2 except in 1 mM pretreatment group, where decrease in APX activity was noticed. In parent 1, use of 10 mM H_2O_2 and nanoparticles alone and when given in combination, cause reduction in APX activity as demonstrated in figure 4.19.

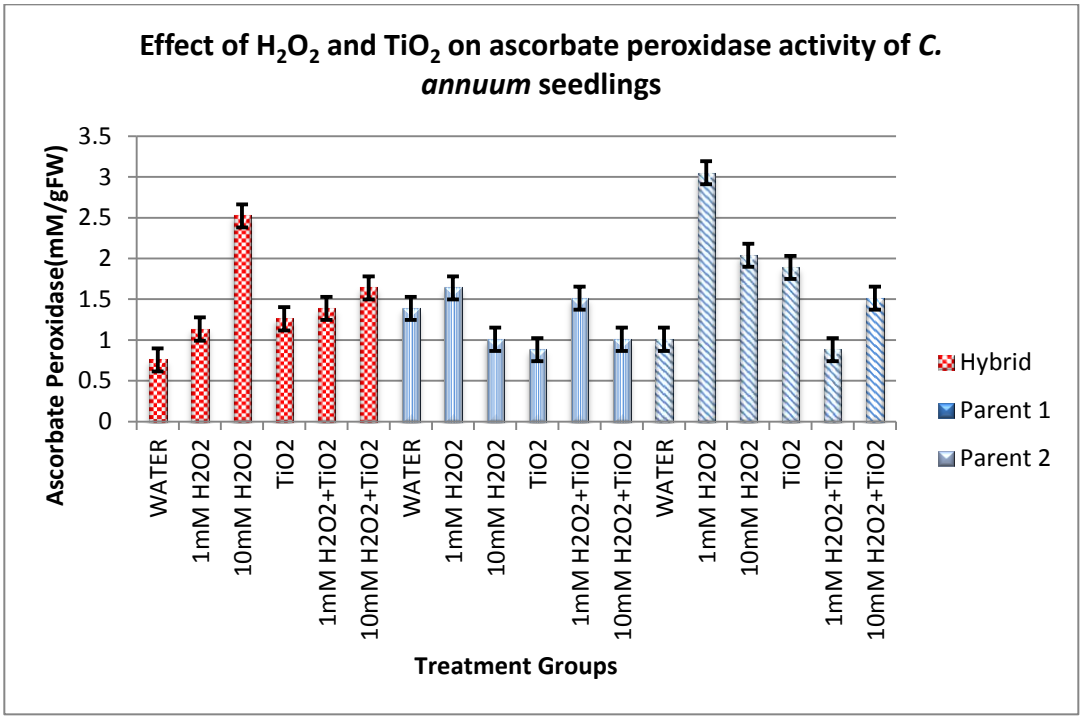

Figure 4.19 Effect of H_2O_2 and TiO_2 on ascorbate peroxidase activity of *C. annuum* seedlings

10 mM H_2O_2 enhanced APX activity in hybrid and parent 2 relative to respective water control and nanoparticles reduced enzyme action in parent 1, however hybrid and parent 2 exhibited increased enzyme action as compared to water control. Data represents the means of three independent experiments with 3 plants for each

treatment in each experiment. Error bars are standard error (SE) for three replicate reactions.

In controlled conditions, parent 1 possessed high APX activity while 1 mM, 10 mM H_2O_2 and TiO_2 application enhanced peroxidase activity in parent 2. The use of 1 mM H_2O_2 and 10 mM H_2O_2 prior to nanoparticles reduced APX activity in parent 2 and parent 1 respectively. In hybrid, 10 mM H_2O_2 was observed to enhance enzyme action at most among other treatments as demonstrated in figure 4.20.

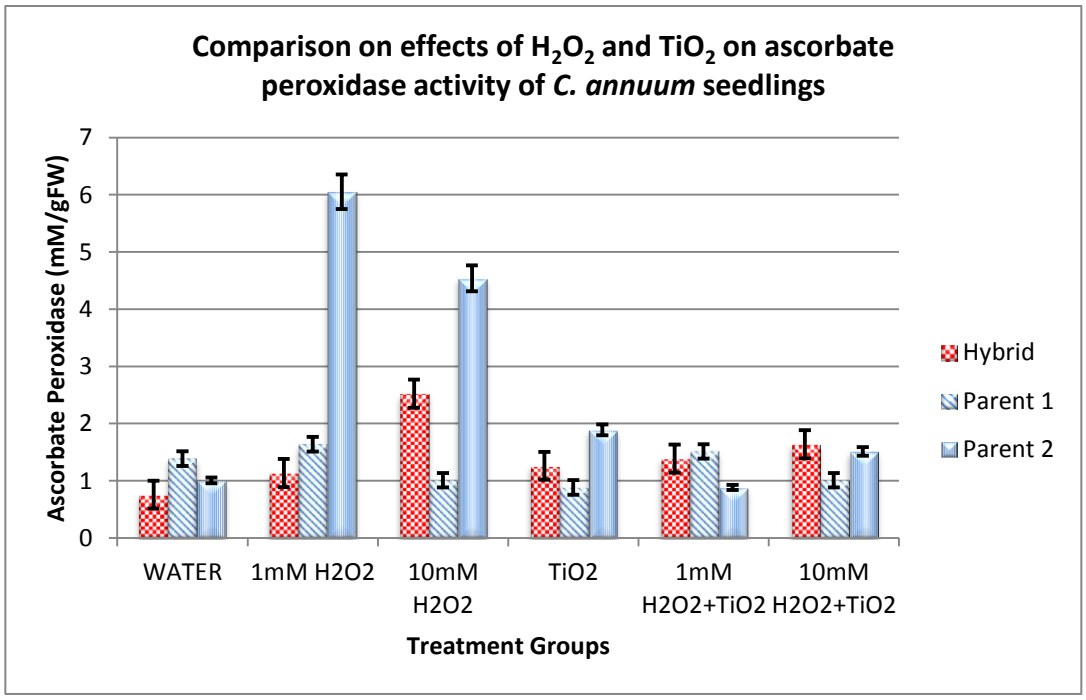

Figure 4.20 Comparison on effects of H_2O_2 and TiO_2 on ascorbate peroxidase activity of *C. annuum* seedlings
1 mM displayed highest ascorbate activity in all groups. Data represents the means of three independent experiments with 3 plants for each treatment in each experiment. Error bars are standard error (SE) for three replicate reactions.

4.5.4 Effect of H_2O_2 and TiO_2 on Superoxide Dismutase Activity of *C. annuum* Hybrid and Parents Seedlings:

Superoxide anion (O_2^-) is dismutated by antioxidant enzymes superoxide dismutases (SODs) to H_2O_2 that is further catalyzed to H_2O by catalase. Contrary to that, high dose and/or inadequate elimination of ROS, particularly superoxide anion, results in oxidative stress, so SODs are the major line of defense against oxidative stress (Alscher et al., 2002). The content of superoxide dismutase (SOD) in TiO_2 nanoparticle treated *C. annuum* hybrid and its parents was calculated using Beauchamp and Fridovich (1971) method with some modifications.

44 days old seedlings of *C. annuum* treated with H_2O_2 and TiO_2 were harvested and for SOD measurement. In hybrid highest SOD activity was recorded in 10 mM H_2O_2 treated group where 100.7% rise in enzyme reaction was recorded as compared to water control. 1 mM H_2O_2 treatment also displayed 13.2% increase whereas nanoparticles application in root zone caused 53% escalation in SOD catalysis in hybrid relative to its water control. This upsurge in enzyme action is an indication of oxidative stress, hence exogenous 10 mM H_2O_2 application in root zone is acting as signal to initiate production of SOD to overcome oxidative stress. That's why, use of 1 mM and 10 mM H_2O_2 (exogenously) before nanoparticles application, reduced SOD activity to 8% and 17% respectively relative to their water control. This decrease in SOD activity presented that H_2O_2 may have overcome the nanotoxicty caused by nanoparticles as displayed in figure 4.21.

Similarly, in parent 1, SOD activity was found maximum with 10 mM H_2O_2 application contrary to 1 mM H_2O_2 treatment where 5% less dismutase reaction was recorded. Use of 1 g/L TiO_2 also enhanced enzyme reaction by 26.3%. Correspondingly, use of TiO_2 after 1 mM H_2O_2 in parent 1 instigated 27% rise in SOD activity in leaves. It may be due to the fact that 1 mM H_2O_2 was unable to act as a signaling molecule in parent 1 and incapable to initiate pathway leading to production of SOD, hence it was found inept to reduce stress make happened by nanoparticles. Conversely, exogenous pretreatment of 10 mM H_2O_2 reduced 10% SOD activity relative to its water control. This reduction in dismutation reaction

pinpoint supporting role of 10 mM H_2O_2 in stress survival. For parent 2, highest SOD activity was measured in TiO_2 treated group, where 34.7% upsurge in dismutation reaction was recorded relative to water control in leaf samples. This result again suggested toxic behavior of TiO_2 due to which plant undergo in stress condition. A similar rise (34.3%) in enzyme action was also noticed in 10 mM H_2O_2 treated group but 1 mM only caused 3% increase in SOD activity. On the other hand, when TiO_2 was applied after 1mM and 10 mM H_2O_2 treatment, than upsurge in enzyme reaction was reduced to 7.7% and 4.8% separately relative to its water control. The result again displayed positive role of 10 mM H_2O_2 in reducing nanotoxocity.

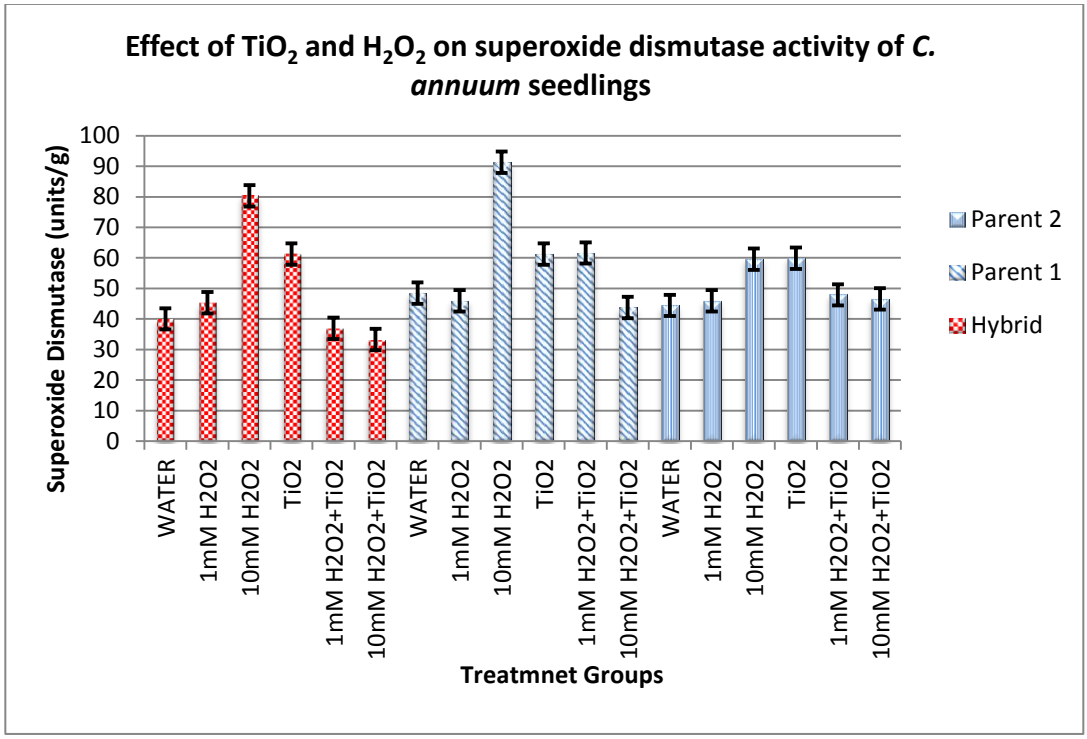

Figure 4.21 Effect of H_2O_2 and TiO_2 on superoxide dismutase activity of *C. annuum* seedlings

Nanoparticles enhanced SOD activity in each group but prior use of 10 mM H_2O_2 reduced dismutase activity. Data represents the means of three independent experiments with 3 plants for each treatment in each experiment. Error bars are standard error (SE) for three replicate reactions.

Hybrid possessed relatively low SOD activity as compared to parental lines while parent1 exhibited high dismutase reaction. Similarly parent 1 is highly affected by 10 mM H_2O_2 but parent 2 is least affected. On the other hand, 1 mM H_2O_2 is found incapable of reducing stress when applied after nanoparticles in parent 1, however it was found effective in decreasing stress in parent 2 and hybrid as presented in figure 4.22. This difference in response to same treatment is may be due to difference in genotype.

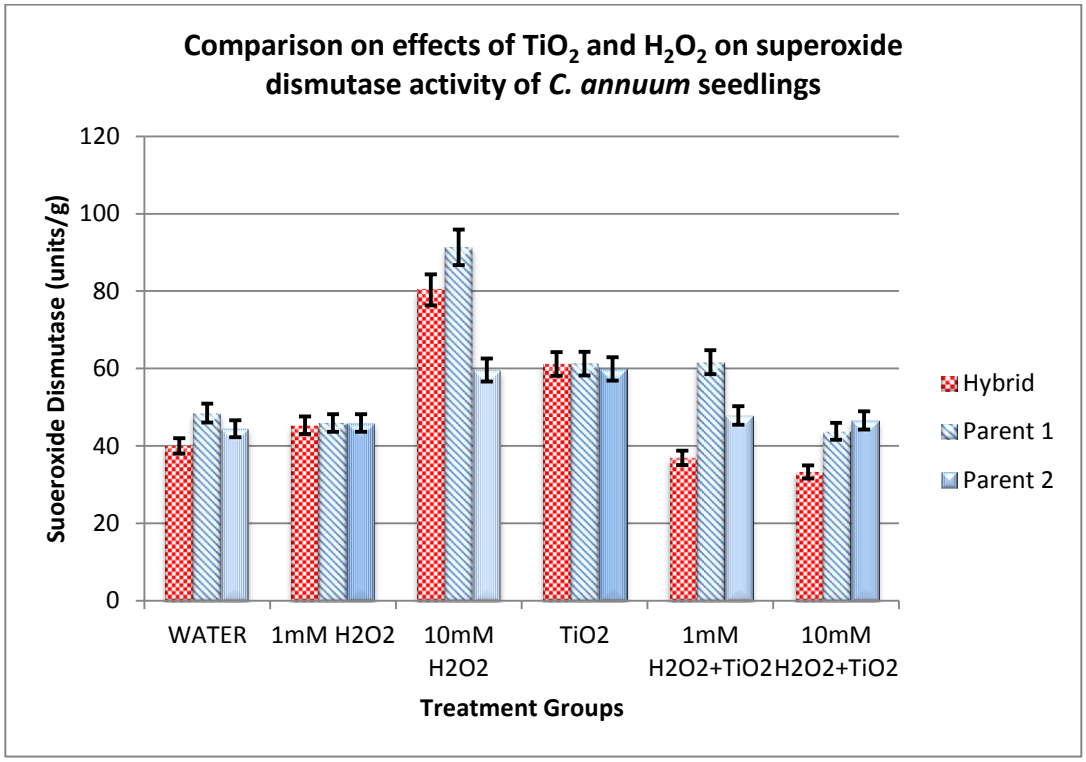

Figure 4.22 Comparison on effects of TiO_2 and H_2O_2 on superoxide dismutase activity of *C. annuum* seedlings

Parent 1 showed slight different response to 1 mM pretreatment**.** Data represents the means of three independent experiments with 3 plants for each treatment in each experiment. Error bars are standard error (SE) for three replicate reactions.

5 DISCUSSION

Nanotechnology has created many advancements in the field of science and the products generated by this technology facilitates in medicine, food, cement, textile health, agriculture and in many other fields. Engineered nanoparticles have been included in many industrial materials and this has raised the threat of accumulation in the food chain and can cause toxicity and stress. Plants cannot move from one place to another like animals, and are always exposed to different types of stresses. These stresses affect the plants: they disturb their metabolism, quality and quantity (Navarro et al., 2008). Plants normally produce endogenous ROS in response to external stresses, which trigger anti-oxidant enzyme system that helps the plant to combat external biotic or abiotic stresses. The issue under discussion in our study was to find the role of pretreated H_2O_2 in nanotoxicity reduction in *C. annuum* hybrid and its parents and on the basis of that we give a comparative analysis in behavior of hybrid and non-hybrid lines towards nanoparticles induced abiotic stress and role of H_2O_2 in resistance against such stress.

Many studies have reported the translocation of these nanoparticles from industries to aquatic and terrestrial environments. These investigations help to tell us the path taken by these materials which directly lead them into food chain. The important aspect that needs attention in this field is the effect created by such particles both in humans and in plants, whether it is positive or negative (Theerakarunwong and Chouychai, 2013). In this study we chose *C. annum* plant, which is used as a model for investigating role of secondary metabolite in plant development as well as a popular crop that is used in cooking and as a flavoring agent (Martínez-López et al., 2014). Moreover being consumed as fresh vegetable, it also find an increase in its demand in several processing industries such as dried pepper, pickled pepper, pepper sauce and ground pepper (Aza-González et al., 2012). This research project facilitated us to apply H_2O_2 as oxidative tool in overcoming abiotic stress indorsed by nanoparticles in *C. annuum* hybrids and its parents. One of the key approaches for enhancing animal and crop productivity since last few years is the utilization of hybrid vigor. Hybrid vegetable varieties produced in last few decades played a key role in increasing their production worldwide (Hua et al.,

2003). India ranked largest *C. annuum* producer in world due to access to improved hybrid cultivars. The principal approach in hybrid breading is to utilize heterosis effect expressing in F_1 generation (Tembhurne and Rao, 2012).

5.1 Accumulation of Nanoparticles:

XRD results for *C. annuum* seedlings in group "d" (TiO_2 control groups), group "e" and group "f" (pretreated groups) of both parents and their hybrid showed presence and accumulation of TiO_2 nanoparticles. We assume that TiO_2 enters plant cell through roots from soil. The uptake of fullerene C70 in rice was confirmed in previous studies. Adsorption of nanoparticles on roots causes chemical reactions on surface, precipitation and ion exchange that causes changes in functional groups (Mazumdar and Ahmed, 2011). Cell wall pores with small diameter can be affected by surface area of nanoparticles that creates large pores causing nanoparticles to enter protoplast (Navarro et al., 2008). The presence of nanoparticles provides details on expected adverse effects, once these particles act as transponders and targets specific organelles (Hoshino et al., 2004), or bound to proteins or RNA molecule in cell (Suzuki et al., 2007) inside organisms (Åkerman et al., 2002). Nanoparticles are highly catalytic and reactive due to high surface to volume ratio; as they tend to pass through cell membranes (Yin et al., 2011).

5.2 Germination Rate:

In plant life, seed germination is considered as the most essential phase of life cycle affecting its development and growth (Wojtyla et al., 2016). *C. annuum* seeds showed highest germination percentage when treated with 10 mM H_2O_2 alone (group "c") in each cultivar. However, lowest percentage was observed in group "f" of both parents and the hybrid as compared to group "a". The results revealed that seed germination depends primarily on stored reserves to carry out anabolic process which is activated on water penetration into seed coat (Liu et al., 2005). The significance of crosstalk between H_2O_2 and various signaling molecules including different hormones of plants playing role in plant development such as ethylene, gibberellins, abscisic acid and reactive molecules such as hydrogen sulphide and nitric oxide on signaling and cell communication has been highlighted in previous studies (Bentsink and Koornneef,

2008). We assume that penetration of H_2O_2 into the germinating seeds increases germination rate as low concentration of H_2O_2 (group "b") showed less increase in germination whereas high concentration (group "c") showed more increase in germination as compared to group "a" in each *C. annum* cultivar. This may be due to the fact that molecules of H_2O_2 cause pores in seed coat which allow more water penetration into the seeds that reaches embryonic tissue, hence facilitating germination (Akinci and Akinci, 2010, El-Maarouf-Bouteau and Bailly, 2008). The role of H_2O_2 has been acknowledged in seeds during imbibition and germination during the early stages, primarily as a result of a noticeable enhancement in production in their intracellular and extracellular environments (Schopfer, 2001, Kranner et al., 2010, Zhang et al., 2014, Kubala et al., 2015). On the other hand TiO_2 nanoparticles in group "d" decreased germination rate as compared to group "a". Nanoparticle slowly penetrates into seed coat and changes its metabolism *in vivo* (Navarro et al., 2008). In a research conducted on *Mentha piperita*, when seeds were exposed to TiO_2 nanoparticles, they showed decrease in germination percentage. This has been reported that TiO_2 nanoparticles slowly penetrate through the pores in the seed coat and agglomerate and accumulate inside the seed coat near the pores, hence closing the passage for water uptake and oxygen by the seed (Samadi et al., 2014). Hence the toxic effect of TiO_2 on seed germination indicates that seeds were stressed by TiO_2 nanoparticles (Mushtaq, 2011). Yet, it is also reported that TiO_2 nanoparticle showed positive impact on seed germination by enhancing water absorption (Feizi et al., 2012, Feizi et al., 2013). This difference in results is due to difference in nanoparticles concentration, as low concentration of nanoparticles increase germination rate whereas high concentration of nanoparticles reverses the effects (Zheng et al., 2005). Nanoparticles induced toxicity also depend on plant species as cucumber and lettuce are not sensitive to metal oxides nanoparticles (Barrena et al., 2009). Pretreatment with H_2O_2 further decreases germination rate in all cultivars of *C. annuum* when nanoparticles were given. This may be due to the fact that H_2O_2 increases pore size, which ultimately allows more nanoparticles to enter into the cells and accumulate in the seed. So pretreatment of H_2O_2 increases toxic effects of nanoparticles.

5.3 Root Length:

Root growth in post-germinated *C. annuum* seedlings was similar to germination rate except in groups that are pretreated with H_2O_2. Roots are the first organs to counter nanoparticles, therefore, the growth of roots were directly affected by nanoparticles. Nanoparticles decreased root growth in group "d" of *C. annuum* of hybrid and the parents that revealed toxic effect of nanoparticles which is consistent with previous results of Castiglione et al. (2011). However TiO_2 also showed positive results on roots according to Zheng et al. (2005). Toxic effects of nanoparticles on root elongation, analogous with conclusions of our experiments, were reported through applying various types of nanoparticles by Yang and Watts (2005) in corn, carrot, cucumber, cabbage and soybean. The negative effects on root growth is may be due to toxicity caused by TiO_2 which reduced mitotic ability of plant cells at root apex as reported by Castiglione et al. (2011) by examining mitotic behavior of cells through cytological analysis at root apex. However, our results contradict the data presented by Răcuciu and Creangă (2009) who observed that, effects of magnetic nanoparticles in *Zea mays* was normally positive, enhancing plant root proliferation. But this is not unanticipated, bearing in mind that nanoparticles can demonstrate their actions based on the concentrations applied, the shape and/or the size of the particles, the precise conditions of research experiment, the plant species and their pathways of assimilation (Brunner et al., 2006), (Lin and Xing, 2007, Eichert et al., 2008, Castiglione et al., 2011). *C. annuum* hybrid seedlings treated with 1 mM H_2O_2 (group "Hb") and 10 mM H_2O_2 (group "Hc") showed increase root lengths as compared to group "Ha" but group "Hc" showed maximum increase in root growth. However in parents, increase in root growth was shown at 1 mM (group "b"), but 10 mM H_2O_2 (in group "c") showed toxic effect towards root growth. The results are consistent with previous results of Renew et al. (2005) that described that ROS is required for root elongation. The increase in root growth was linked with the oxidative breakdown of polyamines by H_2O_2, as it was observed in soybean and cucumber (Li et al., 2007).

The decrease in root growth in group "c" in parental lines describes toxic role of H_2O_2 as it is evident that high concentration lead to programmed cell death (PCD) (Gechev et

al., 2006) and stomatal closure (Bright et al., 2006) (Deng et al., 2012). It is well established that oxidative molecules particularly H_2O_2, are key molecules in signaling pathways involved in all phases of plant growth, extending from the growth of individual root hairs, to xylem differentiation and lignification, and stomatal control at low concentrations (Cheeseman, 2007). It has been reported by Ahmad et al. (2013) that application of H_2O_2 on leaves considerably enhanced root and shoot growth in maize.

So we can conclude that role of H_2O_2 as stress or as a signaling molecule is concentration dependent. Pretreatment of H_2O_2 decrease toxic effects of nanoparticles as displayed by increase in root growth in group "e and f" as compared to group "d". It is reported in a previous study that the pretreatment of H_2O_2 intricate in oxidation reaction signaling, which triggers particular transcription factors to express, resulting in activation of antioxidative enzymes. Certainly, it has been reported that pretreatment of plants with exogenous H_2O_2 induces chilling tolerance (Yu et al., 2002) (Yu et al., 2003). We assume a similar mechanisim may be happened in *C. annum* seedlings when combined treatments of H_2O_2 and TiO_2 were given hence reducing the nanoparticles stress.

5.4 Shoot Growth:

C. annuum Hb and Hc groups seedlings showed increased shoot lengths as compared to Ha group but group Hc showed maximum increase in shoot growth as compared to the water control (group Ha) in hybrid, whereas in parents, increase in shoot growth was observed with in group "b". However, group "c" in parental lines showed toxic effects and reduced shoot growth compared to group "a". So our results show that 10 mM H_2O_2 cause oxidative stress in parental lines but causes resistance to nanotoxicity in hybrids. Thus, with respect to shoot growth, hybrid exhibits more resistance to nanotoxicity with the aid of H_2O_2 than parental lines. The exogenous use of H_2O_2 in root zone of plants has been verified promising in successfully enhancing the shoot growth of numerous plants and vegetables, modifying the biochemical activities inside and outside the plant cell (Ishibashi et al., 2011, Deng et al., 2012, Ashfaque et al., 2014). It has been reported by Ahmad et al. (2013) that application of H_2O_2 on leaves considerably enhanced root and shoot growth in maize. So we can claim that our results are

consistent with earlier published data. The negative effect of 10 mM H_2O_2 concentration can be described on the basis of fact that H_2O_2 has dual role. At low concentration it acts as signaling molecule while at high concentration it causes oxidative stress (Gechev et al., 2006).

TiO_2 induced nanotoxicity decreased shoot growth in all groups of *C. annuum* that revealed toxic effects of nanoparticles, which is consistent with previously published results as most of research on nanoparticles to date exploit their toxicity in plant growth (Siddiqui et al., 2015). The results could be explained if we focus on the findings that nanoparticles assimilate into the plants producing numerous variations in morphology and physiology based on characteristics of nanoparticles. These modifications are deleterious to the plant development (Khodakovskaya et al., 2012). Contradictory results to our data are also reported, for instance Zheng reported that combination of SiO_2 and TiO_2 nanoparticles when given in low concentrations, improved nitrate reductase catalytic activity in the rhizosphere of leguminous plants and thus accelerated germination and shoot growth in soybean (Zheng et al., 2005) and *Larix Olgensis* (Bao-shan et al., 2004, Siddiqui et al., 2015). However, pretreatment of H_2O_2 in TiO_2 treated groups decreased the nanoparticles induced toxicity as displayed by increase in shoot growth as compared to group "d". Treatment of 10 mM H_2O_2 in hybrid and 1mM H_2O_2 in parental lines prior to TiO_2 application, were observed to increase shoot length as compared group "d".

Similar results were presented by Li et al. (2011) that verified role of exogenous H_2O_2 in reducing salt stress. This tolerance is conferred by the plant because pretreatment of H_2O_2 inhibits production of malondialdehyde (MDA), decreases the concentration of superoxide radicals ($\bullet O_2$) and enhance the scavenging activities of ascorbate peroxidase (APX), superoxide dismutase (SOD), catalase (CAT) and the production of glutathione (GSH). Consequently, participation of H_2O_2 in anti-oxidant enzyme system induces tolerance to the abiotic (nanoparticles) stress. The increased concentration of MDA, (that is produced as a result of lipid peroxidation) is taken as an indicator for oxidative damage (Meloni et al., 2003). Exogenous H_2O_2 application could inhibit lipid peroxidation and protect the *C. annuum* cells from the toxic effects of TiO_2 induced

toxicity. That's why, H_2O_2 signaling possesses important significance to any research project intended to improve crop tolerance to stress.

5.5 Chlorophyll:

The toxicity of nanoparticles depends on the environment in which they are dispersed, their fate, shape and hydrodynamic diameters. Solubility and agglomeration properties of nanoparticles in soil affect its absorption from the surrounding environment (Song et al., 2013). We found that chlorophyll content of *C. annuum* leaves was increased in all groups treated with H_2O_2 except in parent 2 (P2) in which 1 mM H_2O_2 (group "P2b") showed increase in photosynthetic pigment but 10 mM H_2O_2 (group "P2c") exhibited decrease in chlorophyll level. The group "d" in all cultivars of C. *annuum* showed minimum chlorophyll content that explains phytotoxic effect of TiO_2. Group "e and f" showed increase in chlorophyll content as compared to their respective group "d" that support our hypothesis that H_2O_2 can play positive role in reducing nanotoxicity. Small size of nanoparticles contributes in its high uptake by plants to the stem and leaves. This toxicity of nanoparticles is supported from literature as reported earlier that Zn causes damages to chlorophyll by replacing central atom in the stressed plant (Mukherjee et al., 2014). As a result of this substitution, photosynthetic light-harvesting is prevented that reduces photosynthesis by damaging the chlorophyll molecule (Küpper et al., 1996). It is known that TiO_2 nanoparticles when assimilate into plant tissues cause imbalance in ROS that causes DNA damage via oxidation of purine molecules (Afaq et al., 1998). While another study states that TiO_2 nanoparticles as compared to Ag nanoparticles showed no significant difference in chlorophyll content or very little phytotoxicty, as Ag nanoparticles cause potential stress as indicated by high content of SOD released on exposure to heavy metals (Song and Lee, 2010), thereby we can conclude that depending on size and concentration, nanoparticles can cause physiological stress (Song et al., 2013). Previous study also shows opposing results that TiO_2 nanoparticles tested to improve chlorophyll and total soluble protein content significantly, and is promising nutrient for plant physiology and development (Raliya et al., 2015). This difference in results is may be due to difference in size and crystalline phase of TO_2 nanoparticles.

The plant researchers have increased plants' capability to absorb and use more sunlight energy by transferring nanotubes of carbon into chlorophyll containing organelle in plant cells, and carbon nanotubes can also assist as artificial light capturing antennae that allows chloroplast to harvest those wavelengths of light which is not in their ordinary range, such as green, UV, and near-infrared (Cossins, 2014, Giraldo et al., 2014, Siddiqui et al., 2015). It was also noticed in a previous study that treatment with higher concentrations of nano sized TiO_2 (2000 mgL^{-1}) improved leaf photosynthetic pigment as compared to water treated plants. This increased chlorophyll accumulation may be the consequent of corresponding effects of other essential nutrients like iron, magnesium and sulfur. Similar data was published by Zheng et al. (2005) when *Spinacia oleracea* was given nanoscale TiO_2 particles. These results from previous study is confusing as the plant showing more chlorophyll in high TiO_2 nanoparticles concentration did not exhibited enhanced growth. Nano sized TiO_2 might pass through the membranes of chloroplast and accelerate redox reaction that speeds up electron transport and release of oxygen (Mahmoodzadeh et al., 2013).

5.6 Sugar Content:

High sugar content is required by young seedlings to fulfill their energy demand especially in actively growing regions of the plants. Sugar is synthesized in leaves by the process of photosynthesis and help plant not only to perform normal physiological processes but also combat stress conditions, so the level of sugar in plant tissues can be used as an indication of stress on plants (Alcaraz-López et al., 2004).

TiO_2 is considered as growth stimulator when provided in small amounts; however the rapid built up of TiO_2 in environment increases toxicity in soil, affecting the plants at a large scale (Mushtaq, 2011). Under this scenario, pretreatment of H_2O_2 can work better in keeping up the growth of seedlings. *C. annuum* seedlings showed high sugar content in TiO_2 (group "d"): (this is somewhat how you refer to the groups) controls of both parents and their hybrid. The data is supported by the previous experimentation of Khater (2015), which revealed that application of TiO_2 nanoparticles in coriander plant resulted in a substantial increase with total sugars content relative group 'a' (water control). While, H_2O_2 1 mM pretreatment in hybrid (H) and "P1" and 10 mM

pretreatment in "P2" drastically reduced total sugar levels in pretreated groups while all other treatment increased chlorophyll levels as compared to group "a". Previous study reveals that low concentration of TiO_2 (10 to 40 μg ml^{-1}) on onion roots increases activity of enzymes like amylase and protease to produce soluble sugar and amino acid for early germination and seedling growth, along with sufficient amount of oxygen and increased water uptake (Haghighi and da Silva, 2014).

5.7 Enzyme Assays:

We determine biochemical activity of *C. annuum* in response to nanoparticles induced toxicity along with pretreatment of H_2O_2. ROS are actively produced in plants' chloroplast, peroxisome, and mitochondria as a result of normal metabolic activities (Sharma et al., 2012). High activity of enzymes in groups "b and c" are indicative of stimulation of anti-oxidant enzymes, which are found less in group "a" and group "d". Plant cells are equipped by antioxidant system that scavenges the oxidative burst causing radicals. CAT, APX, and SOD are among important enzymes that scavenges and quenches H_2O_2 in tissues and oxidize its substrate (Sharma et al., 2012). Peroxidase enzymes are localized in peroxisomes where it catalyzes degradation of H_2O_2 into H_2O and O_2—in stressful conditions. The effective removal of H_2O_2 from cell reduces formation of highly reactive hydroxyl ion (OH^-). Foyer et al. (1994) suggested that exogenously applied H_2O_2 increases intracellular activity of various iso-enzymes of CAT, and SOD. Furthermore, defense genes are sensitive to respond to exogenously applied H_2O_2, by up regulating the expression of proteins and enzymes under abiotic stresses (Kumar et al., 2012). Our results also suggested increase in enzyme activity in H_2O_2 treated groups. Similarly, *C. annuum* seedlings of group "d" also exhibited slight improvement in anti-oxidant enzymes activity. However, Hawthorne et al. (2014) suggested that CeO_2 at high concentration causes decrease in enzyme activities and reduction in protein concentration. Rice root exposed to CeO_2 were reported to decrease CAT activity at low concentration (Rico et al., 2013). Root of mesquite (*Prosopis juliflora-velutina*) exposed to ZnO increased enzyme activity due to production of ROS (Hernandez-Viezcas et al., 2011). Previous studies indicated that exposure to low stress help plant cells to combat oxidative burst through scavenging processes. Whereas, high

protein content indicated active expression of stress related proteins to counter damages created by oxidative burst in tissues for long time (Ashfaque et al., 2014).

Nanoparticles are toxic in ion form, and causes toxicity while they accumulate and produces ROS upon contact, causing subsequent damages to the cells (Stampoulis et al., 2009). CAT is present in all organisms and helps to maintain endogenous H_2O_2 by decomposing it into H_2O and O_2 (Tanaka et al., 1999). We found minor increase in CAT activity in group "d" as compared to group "a" of *C. annuum* of hybrid and the parents. This is due to the toxic effects of TiO_2, which in turn increases ROS, consequently anti-oxidant system activate to scavenge ROS molecules. All H_2O_2 pretreated groups (e and f groups) showed reduced level of CAT activity, as compared to group "d" of *C. annuum* hybrid and the parents which support our hypothesis. Previous studies support our results that exogenous H_2O_2 can help in reducing the abiotic stress. Similar results were observed in Wheat. With increase in concentration of H_2O_2 and TiO_2, high level of CAT activity was observed. Abiotic stress increases the production of H_2O_2 that further stimulates expression of active oxygen scavenging (AOS) enzymes gene (Tanaka et al., 1999). In previous study, high rate of H_2O_2 accumulated in leaves during high temperatures; indicative of tolerance in plants as they act as signaling molecules to activate stress induced genes that scavenge the free oxygen radical and protect plant's innate proteins from denaturation. Previous studies showed that intercellular H_2O_2 accumulation due to exogenously applied H_2O_2 was 10 mM/L (Kumar et al., 2012). Ozden et al. (2009) suggested that high concentration of H_2O_2 is a result of oxidative stress. Whereas, in groups "e and f" CAT activity was reduced as compare to group "d" explicitly showing low level oxidative stress in pretreated groups of *C. annuum* hybrid and the parents. This result depicts that H_2O_2 boosts plants internal immunity to fight against oxidative stress better than untreated plants.

APX plays vital role in reducing oxidative stress, by converting H_2O_2 into H_2O and oxygen, similar to previous results, very high activity of APX was recorded in response to exogenous application of H_2O_2 and TiO_2 controls in *C. annuum* parents and their hybrid. However, among pretreated groups hybrid with 10 mM and parent 1 with 1 mM H_2O_2 showed increased level of APX as compared to nanoparticles control. Kumar et

al. (2012) shows that exogenously applied H_2O_2 causes direct accumulation of H_2O_2 which increases the activity of various enzymes

Superoxide radicals (O_2^-) are lethal derivatives of oxidative metabolism and can interfere with H_2O_2 to produce extremely reactive hydroxyl radicals (OH^-) that are supposed to be first and foremost accountable for oxidative toxicity in the plant cells (Bowler and Fluhr, 2000, Vaidyanathan et al., 2003). The dismutation of O_2^- resulting in production of oxygen and H_2O_2 is an essential event that helps in defending the cell, and is carried out by SOD. Among the control groups (b, c and d), group "c" showed highest increase in SOD activity where pretreated groups showed decrease in SOD activity as compared to group "d" in *C. annuum* hybrid and its parents. This also shows that H_2O_2 can help in reducing the oxidative stress by TiO_2. Similar results to our data was reported by Li et al. (2011) who published enhanced SOD activity in wheat, applied with exogenous H_2O_2. Consequently, plants displayed improved $\bullet O_2$ radical scavenging capability. It has also been described that tolerance to salt is directly linked to enhance in SOD catalytic activity (Shalata et al., 2001, Badawi et al., 2004, Li et al., 2011). In previous studies high response of SOD due to exogenous spray of H_2O_2 indicates protective role of SOD to prevent damage of key enzymes involved in metabolic pathway from denaturation due to ROS (Kumar et al., 2012). CAT enzyme maintains balance of H_2O_2 in cells as high accumulation of H_2O_2 may lead to cell death. Exogenously provided H_2O_2 protects plants from heat shock, by increasing the activity of CAT enzyme (Kumar et al., 2012). The SOD content in *C. annuum* increases with increase in H_2O_2 concentration. In group "e and f" high toxicity of H_2O_2 decreases SOD content in leaves. However, due to limited information the exact mechanism of nanoparticle tolerance in plants is yet to be explored.

6 CONCLUSION

We conclude that pretreatment of H_2O_2 can aid in decreasing the oxidative radical stress and toxicity caused by TiO_2 nanoparticles in parental lines and their hybrids. However, in parental lines, 10 mM H_2O_2 itself cause oxidative stress but in hybrid it plays better role in reducing stress as compared to low dose of H_2O_2. Hence, overall $H_2O_2$was observed to enhance the plant tolerance against nanotoxicity in both parents and their hybrid at physiological and biochemical levels. However the results were noted to be dependent on exogenous H_2O_2 concentrations that differ for parents and hybrid. Although exact mechanism of H_2O_2 and its role in reducing toxicity and activating anti-oxidative defense is still unclear however results revealed that exogenous H_2O_2 application is capable of raising endogenous APX, CAT and SOD. So, we assume that release of oxidative radical could be eradicated rapidly and prevented lipid peroxidation by oxidative radicals and thus secured *C. annuum* cells from nanoparticles stress and damage. The outcomes support the hypothesis that H_2O_2 applied exogenously can improve tolerance of *C. annuum* hybrid and its parents against nanotoxicity by increasing the activity of enzymatic antioxidants. Furthermore, genetic studies are required to develop understanding of the exact mechanism used by H_2O_2 in reducing nanoparticles stress.

7 REFERENCES

AEBI, H. 1984. [13] Catalase in vitro. *Methods in enzymology,* 105**,** 121-126.

AFAQ, F., ABIDI, P., MATIN, R. & RAHMAN, Q. 1998. Cytotoxicity, pro-oxidant effects and antioxidant depletion in rat lung alveolar macrophages exposed to ultrafine titanium dioxide. *Journal of Applied Toxicology,* 18**,** 307-312.

AHMAD, I., AHMAD, T. K. A., BASRA, S. M., HASNAIN, Z. & ALI, A. 2012. Effect of seed priming with ascorbic acid, salicylic acid and hydrogen peroxide on emergence, vigor and antioxidant activities of maize. *African Journal of Biotechnology,* 11**,** 1127-1137.

AHMAD, I., BASRA, S. M., AFZAL, I., FAROOQ, M. & WAHID, A. 2013. Growth improvement in spring maize through exogenous application of ascorbic acid, salicylic acid and hydrogen peroxide. *International Journal of Agriculture and Biology,* 15**,** 95-100.

ÅKERMAN, M. E., CHAN, W. C., LAAKKONEN, P., BHATIA, S. N. & RUOSLAHTI, E. 2002. Nanocrystal targeting in vivo. *Proceedings of the National Academy of Sciences,* 99**,** 12617-12621.

AKINCI, I. E. & AKINCI, S. 2010. Effect of chromium toxicity on germination and early seedling growth in melon (Cucumis melo L.). *African Journal of Biotechnology,* 9**,** 4589-4594.

ALCARAZ-LÓPEZ, C., BOTIA, M., ALCARAZ, C. F. & RIQUELME, F. 2004. Effect of foliar sprays containing calcium, magnesium and titanium on peach (Prunus persica L) fruit quality. *Journal of the Science of Food and Agriculture,* 84**,** 949-954.

ALSCHER, R. G., DONAHUE, J. L. & CRAMER, C. L. 1997. Reactive oxygen species and antioxidants: relationships in green cells. *Physiologia Plantarum,* 100**,** 224-233.

ALSCHER, R. G., ERTURK, N. & HEATH, L. S. 2002. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *Journal of experimental botany,* 53**,** 1331-1341.

ANDRADE, R. G., GINANI, J. S., LOPES, G. K., DUTRA, F., ALONSO, A. & HERMES-LIMA, M. 2006. Tannic acid inhibits in vitro iron-dependent free radical formation. *Biochimie,* 88**,** 1287-1296.

APEL, K. & HIRT, H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.,* 55**,** 373-399.

AROMAL, S. A., BABU, K. D. & PHILIP, D. 2012. Characterization and catalytic activity of gold nanoparticles synthesized using ayurvedic arishtams. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy,* 96**,** 1025-1030.

ASHFAQUE, F., KHAN, M. I. R. & KHAN, N. A. 2014. Exogenously applied H2O2 promotes proline accumulation, water relations, photosynthetic efficiency and growth of wheat (Triticum aestivum L.) under salt stress. *Annual Research & Review in Biology,* 4**,** 105.

ATKINSON, N. J. & URWIN, P. E. 2012. The interaction of plant biotic and abiotic stresses: from genes to the field. *Journal of experimental botany,* 63**,** 3523-3543.

AZA-GONZÁLEZ, C., NÚÑEZ-PALENIUS, H. G. & OCHOA-ALEJO, N. 2012. Molecular biology of chili pepper anthocyanin biosynthesis. *Journal of the Mexican Chemical Society,* 56**,** 93-98.

BADAWI, G. H., KAWANO, N., YAMAUCHI, Y., SHIMADA, E., SASAKI, R., KUBO, A. & TANAKA, K. 2004. Over-expression of ascorbate peroxidase in tobacco chloroplasts enhances the tolerance to salt stress and water deficit. *Physiologia Plantarum,* 121**,** 231-238.

BAO-SHAN, L., CHUN-HUI, L., LI-JUN, F., SHU-CHUN, Q. & MIN, Y. 2004. Effect of TMS (nanostructured silicon dioxide) on growth of Changbai larch seedlings. *Journal of Forestry research,* 15**,** 138-140.

BAR-ILAN, O., ALBRECHT, R. M., FAKO, V. E. & FURGESON, D. Y. 2009. Toxicity assessments of multisized gold and silver nanoparticles in zebrafish embryos. *Small,* 5**,** 1897-1910.

BARRENA, R., CASALS, E., COLÓN, J., FONT, X., SÁNCHEZ, A. & PUNTES, V. 2009. Evaluation of the ecotoxicity of model nanoparticles. *Chemosphere,* 75**,** 850-857.

BATTKE, F., LEOPOLD, K., MAIER, M., SCHMIDHALTER, U. & SCHUSTER, M. 2008. Palladium exposure of barley: uptake and effects. *Plant biology,* 10**,** 272-276.

BEAUCHAMP, C. & FRIDOVICH, I. 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Analytical biochemistry,* 44**,** 276-287.

BECANA, M., MORAN, J. F. & ITURBE-ORMAETXE, I. 1998. Iron-dependent oxygen free radical generation in plants subjected to environmental stress: toxicity and antioxidant protection. *Plant and Soil,* 201**,** 137-147.

BENTSINK, L. & KOORNNEEF, M. 2008. Seed dormancy and germination. *The Arabidopsis Book,* e0119.

BOLWELL, G. P. 1999. Role of active oxygen species and NO in plant defence responses. *Current opinion in plant biology,* 2**,** 287-294.

BOSLAND, P. & VOTAVA, E. 2000. Peppers: Vegetable and spice capsicums. Crop production science in Horticulture Series No. 12. *204Wallingford,, UK: CABI Publishing*.

BOWLER, C. & FLUHR, R. 2000. The role of calcium and activated oxygens as signals for controlling cross-tolerance. *Trends in plant science,* 5**,** 241-246.

BRADFORD, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry,* 72**,** 248-254.

BRIGHT, J., DESIKAN, R., HANCOCK, J. T., WEIR, I. S. & NEILL, S. J. 2006. ABA-induced NO generation and stomatal closure in Arabidopsis are dependent on H2O2 synthesis. *The Plant Journal,* 45**,** 113-122.

BRUNNER, T. J., WICK, P., MANSER, P., SPOHN, P., GRASS, R. N., LIMBACH, L. K., BRUININK, A. & STARK, W. J. 2006. In vitro cytotoxicity of oxide nanoparticles: comparison to asbestos, silica, and the effect of particle solubility. *Environmental science & technology,* 40**,** 4374-4381.

CAÑAS, J. E., LONG, M., NATIONS, S., VADAN, R., DAI, L., LUO, M., AMBIKAPATHI, R., LEE, E. H. & OLSZYK, D. 2008. Effects of functionalized and nonfunctionalized single-walled carbon nanotubes on root elongation of select crop species. *Environmental toxicology and chemistry,* 27**,** 1922-1931.

CARPITA, N., SABULARSE, D., MONTEZINOS, D. & DELMER, D. P. 1979. Determination of the pore size of cell walls of living plant cells. *science,* 205**,** 1144-1147.

CASTIGLIONE, M. R., GIORGETTI, L., GERI, C. & CREMONINI, R. 2011. The effects of nano-TiO2 on seed germination, development and mitosis of root tip cells of Vicia narbonensis L. and Zea mays L. *Journal of Nanoparticle Research,* 13**,** 2443-2449.

CHAFE, S. & WARDROP, A. 1972. Fine structural observations on the epidermis. *Planta,* 107**,** 269-278.

CHARLES, S. A. & HALLIWELL, B. 1980. Effect of hydrogen peroxide on spinach (Spinacia oleracea) chloroplast fructose bisphosphatase. *Biochemical Journal,* 189**,** 373-376.

CHARLES, S. A. & HALLIWELL, B. 1981. Light activation of fructose bisphosphatase in isolated spinach chloroplasts and deactivation by hydrogen peroxide. *Planta,* 151**,** 242-246.

CHEESEMAN, J. M. 2006. Hydrogen peroxide concentrations in leaves under natural conditions. *Journal of experimental botany,* 57**,** 2435-2444.

CHEESEMAN, J. M. 2007. Hydrogen peroxide and plant stress: a challenging relationship. *Plant stress,* 1**,** 4-15.

CHEN, J., DONG, X., ZHAO, J. & TANG, G. 2009a. In vivo acute toxicity of titanium dioxide nanoparticles to mice after intraperitioneal injection. *Journal of Applied Toxicology,* 29**,** 330-337.

CHEN, J., PATIL, S., SEAL, S. & MCGINNIS, J. F. 2006. Rare earth nanoparticles prevent retinal degeneration induced by intracellular peroxides. *Nature nanotechnology,* 1**,** 142-150.

CHEN, X. Y., DING, X., XU, S., WANG, R., XUAN, W., CAO, Z. Y., CHEN, J., WU, H. H., YE, M. B. & SHEN, W. B. 2009b. Endogenous hydrogen peroxide plays a positive role in the upregulation of heme oxygenase and acclimation to oxidative stress in wheat seedling leaves. *Journal of Integrative Plant Biology,* 51**,** 951-960.

CHEN, Z. & GALLIE, D. R. 2005. Increasing tolerance to ozone by elevating foliar ascorbic acid confers greater protection against ozone than increasing avoidance. *Plant Physiology,* 138**,** 1673-1689.

COLLINS, N. C., TARDIEU, F. & TUBEROSA, R. 2008. Quantitative trait loci and crop performance under abiotic stress: where do we stand? *Plant Physiology,* 147**,** 469-486.

CORREDOR, E., TESTILLANO, P. S., CORONADO, M.-J., GONZÁLEZ-MELENDI, P., FERNÁNDEZ-PACHECO, R., MARQUINA, C., IBARRA, M. R., DE LA FUENTE, J. M., RUBIALES, D. & PÉREZ-DE-LUQUE, A. 2009. Nanoparticle penetration and transport in living pumpkin plants: in situ subcellular identification. *BMC plant biology,* 9**,** 1.

COSSINS, E. A. 2014. One-carbon metabolism. *The biochemistry of plants,* 2**,** 365-418.

COSTA, C., CONTE, A., BUONOCORE, G. & DEL NOBILE, M. 2011. Antimicrobial silver-montmorillonite nanoparticles to prolong the shelf life of fresh fruit salad. *International Journal of Food Microbiology,* 148**,** 164-167.

COTAE, V. & CREANGA, I. 2005. LHC II system sensitivity to magnetic fluids. *Journal of magnetism and magnetic materials,* 289**,** 459-462.

CRAWFORD, K. M. & ZAMBRYSKI, P. C. 2000. Subcellular localization determines the availability of non-targeted proteins to plasmodesmatal transport. *Current Biology,* 10**,** 1032-1040.

DAT, J., VANDENABEELE, S., VRANOVÁ, E., VAN MONTAGU, M., INZÉ, D. & VAN BREUSEGEM, F. 2000a. Dual action of the active oxygen species during plant stress responses. *Cellular and Molecular Life Sciences CMLS,* 57**,** 779-795.

DAT, J. F., LOPEZ-DELGADO, H., FOYER, C. H. & SCOTT, I. M. 2000b. Effects of salicylic acid on oxidative stress and thermotolerance in tobacco. *Journal of plant physiology,* 156**,** 659-665.

DAUSEND, J., MUSYANOVYCH, A., DASS, M., WALTHER, P., SCHREZENMEIER, H., LANDFESTER, K. & MAILÄNDER, V. 2008. Uptake mechanism of oppositely charged fluorescent nanoparticles in HeLa cells. *Macromolecular bioscience,* 8**,** 1135-1143.

DE AZEVEDO NETO, A. D., PRISCO, J. T., ENÉAS-FILHO, J., MEDEIROS, J.-V. R. & GOMES-FILHO, E. 2005. Hydrogen peroxide pre-treatment induces salt-stress acclimation in maize plants. *Journal of plant physiology,* 162**,** 1114-1122.

DENG, X.-P., CHENG, Y.-J., WU, X.-B., KWAK, S.-S., CHEN, W. & ENEJI, A. E. 2012. Exogenous hydrogen peroxide positively influences root growth and exogenous hydrogen peroxide positively influences root growth and metabolism in leaves of sweet potato seedlings. *Australian Journal of Crop Science,* 6**,** 1572.

DIETZ, K.-J. & HERTH, S. 2011. Plant nanotoxicology. *Trends in plant science,* 16**,** 582-589.

DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. & SMITH, F. 1956. Colorimetric method for determination of sugars and related substances. *Analytical chemistry,* 28**,** 350-356.

EICHERT, T., KURTZ, A., STEINER, U. & GOLDBACH, H. E. 2008. Size exclusion limits and lateral heterogeneity of the stomatal foliar uptake pathway for aqueous solutes and water-suspended nanoparticles. *Physiologia Plantarum,* 134**,** 151-160.

EL-MAAROUF-BOUTEAU, H. & BAILLY, C. 2008. Oxidative signaling in seed germination and dormancy. *Plant Signaling & Behavior,* 3**,** 175-182.

EL-TEMSAH, Y. S. & JONER, E. J. 2012. Impact of Fe and Ag nanoparticles on seed germination and differences in bioavailability during exposure in aqueous suspension and soil. *Environmental toxicology,* 27**,** 42-49.

ELAVARTHI, S. & MARTIN, B. 2010. Spectrophotometric assays for antioxidant enzymes in plants. *Plant Stress Tolerance: Methods and Protocols***,** 273-280.

ETXEBERRIA, E., GONZALEZ, P., BAROJA-FERNANDEZ, E. & ROMERO, J. P. 2006. Fluid phase endocytic uptake of artificial nano-spheres and fluorescent quantum dots by sycamore cultured cells: evidence for the distribution of solutes to different intracellular compartments. *Plant Signaling & Behavior,* 1**,** 196-200.

FABIAN, E., LANDSIEDEL, R., MA-HOCK, L., WIENCH, K., WOHLLEBEN, W. & VAN RAVENZWAAY, B. 2008. Tissue distribution and toxicity of intravenously administered titanium dioxide nanoparticles in rats. *Archives of toxicology,* 82**,** 151-157.

FAROOQ, O. 2013. Pakistan economic survey 2012-13: Agriculture. Ministry of Finance, Islamabad, Pakistan.

FEIZI, H., KAMALI, M., JAFARI, L. & MOGHADDAM, P. R. 2013. Phytotoxicity and stimulatory impacts of nanosized and bulk titanium dioxide on fennel (Foeniculum vulgare Mill). *Chemosphere,* 91**,** 506-511.

FEIZI, H., MOGHADDAM, P. R., SHAHTAHMASSEBI, N. & FOTOVAT, A. 2012. Impact of bulk and nanosized titanium dioxide (TiO2) on wheat seed germination and seedling growth. *Biological trace element research,* 146**,** 101-106.

FOREMAN, J., DEMIDCHIK, V., BOTHWELL, J. H., MYLONA, P., MIEDEMA, H., TORRES, M. A., LINSTEAD, P., COSTA, S., BROWNLEE, C. & JONES, J. D. 2003. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature,* 422**,** 442-446.

FOYER, C. H., LELANDAIS, M. & KUNERT, K. J. 1994. Photooxidative stress in plants. *Physiologia Plantarum,* 92**,** 696-717.

FOYER, C. H. & NOCTOR, G. 2005a. Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. *Plant, Cell & Environment,* 28**,** 1056-1071.

FOYER, C. H. & NOCTOR, G. 2005b. Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *The Plant Cell,* 17**,** 1866-1875.

FRANKLIN, N. M., ROGERS, N. J., APTE, S. C., BATLEY, G. E., GADD, G. E. & CASEY, P. S. 2007. Comparative toxicity of nanoparticulate ZnO, bulk ZnO, and ZnCl2 to a freshwater microalga (Pseudokirchneriella subcapitata): the importance of particle solubility. *Environmental science & technology,* 41**,** 8484-8490.

GANESH REDDY, M., MOHAN KUMAR, H. & SALIMATH, P. 2010. Heterosis studies in chillies (Capsicum annuum L.). *Karnataka Journal of Agricultural Sciences,* 21.

GARDEA-TORRESDEY, J. L., GOMEZ, E., PERALTA-VIDEA, J. R., PARSONS, J. G., TROIANI, H. & JOSE-YACAMAN, M. 2003. Alfalfa sprouts: a natural source for the synthesis of silver nanoparticles. *Langmuir,* 19**,** 1357-1361.

GECHEV, T. S., VAN BREUSEGEM, F., STONE, J. M., DENEV, I. & LALOI, C. 2006. Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. *Bioessays,* 28**,** 1091-1101.

GEISLER-LEE, J., WANG, Q., YAO, Y., ZHANG, W., GEISLER, M., LI, K., HUANG, Y., CHEN, Y., KOLMAKOV, A. & MA, X. 2012. Phytotoxicity, accumulation and transport of silver nanoparticles by Arabidopsis thaliana. *Nanotoxicology,* 7**,** 323-337.

GEORGE, M. P. 2014. The Role of Hydrogen Peroxide in Controlling Plant Cell-Signaling and Gene-Expression Patterns Related to Stress and Defense Responses. *Student Pulse,* 6.

GESSNER, A., LIESKE, A., PAULKE, B. R. & MÜLLER, R. H. 2003. Functional groups on polystyrene model nanoparticles: influence on protein adsorption. *Journal of Biomedical Materials Research Part A,* 65**,** 319-326.

GHOSH, M., BANDYOPADHYAY, M. & MUKHERJEE, A. 2010. Genotoxicity of titanium dioxide (TiO 2) nanoparticles at two trophic levels: plant and human lymphocytes. *Chemosphere,* 81**,** 1253-1262.

GILL, S. S. & TUTEJA, N. 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant physiology and biochemistry,* 48**,** 909-930.

GIRALDO, J. P., LANDRY, M. P., FALTERMEIER, S. M., MCNICHOLAS, T. P., IVERSON, N. M., BOGHOSSIAN, A. A., REUEL, N. F., HILMER, A. J., SEN, F. &

BREW, J. A. 2014. Plant nanobionics approach to augment photosynthesis and biochemical sensing. *Nature materials,* 13**,** 400-408.

GONG, M., CHEN, B., LI, Z.-G. & GUO, L.-H. 2001. Heat-shock-induced cross adaptation to heat, chilling, drought and salt stress in maize seedlings and involvement of H 2 O 2. *Journal of plant physiology,* 158**,** 1125-1130.

GONZÁLEZ-MELENDI, P., FERNÁNDEZ-PACHECO, R., CORONADO, M. J., CORREDOR, E., TESTILLANO, P., RISUEÑO, M. C., MARQUINA, C., IBARRA, M. R., RUBIALES, D. & PÉREZ-DE-LUQUE, A. 2008. Nanoparticles as smart treatment-delivery systems in plants: assessment of different techniques of microscopy for their visualization in plant tissues. *Annals of botany,* 101**,** 187-195.

GOTTSCHALK, F., SONDERER, T., SCHOLZ, R. W. & NOWACK, B. 2009. Modeled environmental concentrations of engineered nanomaterials (TiO2, ZnO, Ag, CNT, fullerenes) for different regions. *Environmental science & technology,* 43**,** 9216-9222.

GRIFFITT, R. J., WEIL, R., HYNDMAN, K. A., DENSLOW, N. D., POWERS, K., TAYLOR, D. & BARBER, D. S. 2007. Exposure to copper nanoparticles causes gill injury and acute lethality in zebrafish (Danio rerio). *Environmental science & technology,* 41**,** 8178-8186.

GUAN, Y.-J., HU, J., WANG, X.-J. & SHAO, C.-X. 2009. Seed priming with chitosan improves maize germination and seedling growth in relation to physiological changes under low temperature stress. *Journal of Zhejiang University Science B,* 10**,** 427-433.

GURR, J.-R., WANG, A. S., CHEN, C.-H. & JAN, K.-Y. 2005. Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells. *Toxicology,* 213**,** 66-73.

HAGHIGHI, M. & DA SILVA, J. A. T. 2014. The effect of N-TiO2 on tomato, onion, and radish seed germination. *Journal of Crop Science and Biotechnology,* 17**,** 221-227.

HALLIWEL, B. & GUTTERIDGE, J. 1999. Free radicals in biology and medicine.(3rdedn). Oxford.

HALLIWELL, B., CLEMENT, M. V. & LONG, L. H. 2000. Hydrogen peroxide in the human body. *FEBS letters,* 486**,** 10-13.

HALLIWELL, B. & GUTTERIDGE, J. 2007. Cellular responses to oxidative stress: adaptation, damage, repair, senescence and death. *Free radicals in biology and medicine,* 4**,** 187-267.

HANS, M. & LOWMAN, A. 2002. Biodegradable nanoparticles for drug delivery and targeting. *Current Opinion in Solid State and Materials Science,* 6**,** 319-327.

HAWTHORNE, J., DE LA TORRE ROCHE, R., XING, B., NEWMAN, L. A., MA, X., MAJUMDAR, S., GARDEA-TORRESDEY, J. & WHITE, J. C. 2014. Particle-size dependent accumulation and trophic transfer of cerium oxide through a terrestrial food chain. *Environmental science & technology,* 48**,** 13102-13109.

HE, Y., LIU, Y., CAO, W., HUAI, M., XU, B. & HUANG, B. 2005. Effects of salicylic acid on heat tolerance associated with antioxidant metabolism in Kentucky bluegrass. *Crop Science,* 45**,** 988-995.

HERNANDEZ-VIEZCAS, J., CASTILLO-MICHEL, H., SERVIN, A., PERALTA-VIDEA, J. & GARDEA-TORRESDEY, J. 2011. Spectroscopic verification of zinc absorption and distribution in the desert plant Prosopis juliflora-velutina (velvet mesquite) treated with ZnO nanoparticles. *Chemical engineering journal,* 170**,** 346-352.

HERNÁNDEZ, J. A., FERRER, M. A., JIMÉNEZ, A., BARCELÓ, A. R. & SEVILLA, F. 2001. Antioxidant Systems and O2.−/H2O2 production in the apoplast of pea leaves. Its relation with salt-induced necrotic lesions in minor veins. *Plant Physiology,* 127**,** 817-831.

HONG, F., YANG, F., LIU, C., GAO, Q., WAN, Z., GU, F., WU, C., MA, Z., ZHOU, J. & YANG, P. 2005. Influences of nano-TiO2 on the chloroplast aging of spinach under light. *Biological trace element research,* 104**,** 249-260.

HOSHINO, A., FUJIOKA, K., OKU, T., NAKAMURA, S., SUGA, M., YAMAGUCHI, Y., SUZUKI, K., YASUHARA, M. & YAMAMOTO, K. 2004. Quantum dots targeted to the assigned organelle in living cells. *Microbiology and immunology,* 48**,** 985-994.

HOSSAIN, M. A., BHATTACHARJEE, S., ARMIN, S.-M., QIAN, P., XIN, W., LI, H.-Y., BURRITT, D. J., FUJITA, M. & TRAN, L.-S. P. 2015. Hydrogen peroxide priming modulates abiotic oxidative stress tolerance: insights from ROS detoxification and scavenging. *Frontiers in plant science,* 6.

HUA, J., XING, Y., WU, W., XU, C., SUN, X., YU, S. & ZHANG, Q. 2003. Single-locus heterotic effects and dominance by dominance interactions can adequately explain the genetic basis of heterosis in an elite rice hybrid. *Proceedings of the National Academy of Sciences,* 100**,** 2574-2579.

ISHIBASHI, Y., YAMAGUCHI, H., YUASA, T., IWAYA-INOUE, M., ARIMA, S. & ZHENG, S.-H. 2011. Hydrogen peroxide spraying alleviates drought stress in soybean plants. *Journal of plant physiology,* 168**,** 1562-1567.

ISHIDA, H., MAKINO, A. & MAE, T. 1999. Fragmentation of the large subunit of ribulose-1, 5-bisphosphate carboxylase by reactive oxygen species occurs near Gly-329. *Journal of Biological Chemistry,* 274**,** 5222-5226.

JACOB, R. A. 1995. The integrated antioxidant system. *Nutrition research,* 15**,** 755-766.

JONES, N., RAY, B., RANJIT, K. T. & MANNA, A. C. 2008. Antibacterial activity of ZnO nanoparticle suspensions on a broad spectrum of microorganisms. *FEMS microbiology letters,* 279**,** 71-76.

JUDY, J. D., UNRINE, J. M. & BERTSCH, P. M. 2010. Evidence for biomagnification of gold nanoparticles within a terrestrial food chain. *Environmental science & technology,* 45**,** 776-781.

KAIDA, T., KOBAYASHI, K., ADACHI, M. & SUZUKI, F. 2003. Optical characteristics of titanium oxide interference film and the film laminated with oxides and their applications for cosmetics. *Journal of cosmetic science,* 55**,** 219-220.

KANG, S. J., KIM, B. M., LEE, Y. J. & CHUNG, H. W. 2008. Titanium dioxide nanoparticles trigger p53-mediated damage response in peripheral blood lymphocytes. *Environmental and molecular mutagenesis,* 49**,** 399-405.

KARAN, N., AGRAWAL, A., PANDEY, P., SMITHA, P., SHARMA, S., MISHRA, D. & GAJBHIYE, N. 2009. Diffusion flame synthesis of hollow, anatase TiO 2 nanoparticles. *Materials Science and Engineering: B,* 163**,** 128-133.

KHATER, M. 2015. Effect of Titanium Nanoparticles (TiO 2) on Growth, Yield and Chemical Constituents of Coriander Plants. *Arab Journal of Nuclear Sciences and Applications,* 48**,** 187-194.

KHODAKOVSKAYA, M. V., DE SILVA, K., BIRIS, A. S., DERVISHI, E. & VILLAGARCIA, H. 2012. Carbon nanotubes induce growth enhancement of tobacco cells. *ACS nano,* 6**,** 2128-2135.

KIM, J.-Y. 2005. Regulation of short-distance transport of RNA and protein. *Current opinion in plant biology,* 8**,** 45-52.

KIRÁLY, L., HAFEZ, Y., FODOR, J. & KIRÁLY, Z. 2008. Suppression of tobacco mosaic virus-induced hypersensitive-type necrotization in tobacco at high temperature is associated with downregulation of NADPH oxidase and superoxide and stimulation of dehydroascorbate reductase. *Journal of General Virology,* 89**,** 799-808.

KLAINE, S. J., ALVAREZ, P. J., BATLEY, G. E., FERNANDES, T. F., HANDY, R. D., LYON, D. Y., MAHENDRA, S., MCLAUGHLIN, M. J. & LEAD, J. R. 2008. Nanomaterials in the environment: behavior, fate, bioavailability, and effects. *Environmental toxicology and chemistry,* 27**,** 1825-1851.

KRANNER, I., MINIBAYEVA, F. V., BECKETT, R. P. & SEAL, C. E. 2010. What is stress? Concepts, definitions and applications in seed science. *New Phytologist,* 188**,** 655-673.

KUBALA, S., WOJTYLA, Ł., QUINET, M., LECHOWSKA, K., LUTTS, S. & GARNCZARSKA, M. 2015. Enhanced expression of the proline synthesis gene P5CSA in relation to seed osmopriming improvement of Brassica napus germination under salinity stress. *Journal of plant physiology,* 183**,** 1-12.

KUMAR, M. 2000. Nano and microparticles as controlled drug delivery devices. *J. Pharm. Pharm. Sci,* 3**,** 234-258.

KUMAR, M., SIRHINDI, G., BHARDWAJ, R., KUMAR, S. & JAIN, G. 2010. Effect of exogenous H2O2 on antioxidant enzymes of Brassica juncea L. seedlings in relation to 24-epibrassinolide under chilling stress.

KUMAR, R. R., SHARMA, S. K., GADPAYLE, K. A., SINGH, K., SIVARANJANI, R., GOSWAMI, S. & RAJ, D. R. 2012. Mechanism of action of hydrogen peroxide in wheat thermotolerance-interaction between antioxidant isoenzymes, proline and cell membrane. *African Journal of Biotechnology,* 11**,** 14368-14379.

KÜPPER, H., KÜPPER, F. & SPILLER, M. 1996. Environmental relevance of heavy metal-substituted chlorophylls using the example of water plants. *Journal of experimental botany,* 47**,** 259-266.

KUREPA, J., PAUNESKU, T., VOGT, S., ARORA, H., RABATIC, B. M., LU, J., WANZER, M. B., WOLOSCHAK, G. E. & SMALLE, J. A. 2010. Uptake and distribution of ultrasmall anatase TiO2 Alizarin red S nanoconjugates in Arabidopsis thaliana. *Nano letters,* 10**,** 2296-2302.

LAM, C.-W., JAMES, J. T., MCCLUSKEY, R. & HUNTER, R. L. 2004. Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation. *Toxicological sciences,* 77**,** 126-134.

LARKINDALE, J. & KNIGHT, M. R. 2002. Protection against heat stress-induced oxidative damage in Arabidopsis involves calcium, abscisic acid, ethylene, and salicylic acid. *Plant Physiology,* 128**,** 682-695.

LARUE, C., LAURETTE, J., HERLIN-BOIME, N., KHODJA, H., FAYARD, B., FLANK, A.-M., BRISSET, F. & CARRIERE, M. 2012. Accumulation, translocation and impact of TiO 2 nanoparticles in wheat (Triticum aestivum spp.): influence of diameter and crystal phase. *Science of the total environment,* 431**,** 197-208.

LEE, C. & SEDLAK, D. L. 2008. Enhanced formation of oxidants from bimetallic nickel− iron nanoparticles in the presence of oxygen. *Environmental science & technology,* 42**,** 8528-8533.

LEE, W. M., AN, Y. J., YOON, H. & KWEON, H. S. 2008. Toxicity and bioavailability of copper nanoparticles to the terrestrial plants mung bean (Phaseolus radiatus) and wheat (Triticum aestivum): plant agar test for water-insoluble nanoparticles. *Environmental toxicology and chemistry,* 27**,** 1915-1921.

LEVINE, A. 1999. Oxidative stress as a regulator of environmental responses in plants. *Plant responses to environmental stresses: from phytohormones to genome reorganization. New York: M. Dekker***,** 247-265.

LI, J.-T., QIU, Z.-B., ZHANG, X.-W. & WANG, L.-S. 2011. Exogenous hydrogen peroxide can enhance tolerance of wheat seedlings to salt stress. *Acta Physiologiae Plantarum,* 33**,** 835-842.

LI, S., XUE, L., XU, S., FENG, H. & AN, L. 2007. Hydrogen peroxide involvement in formation and development of adventitious roots in cucumber. *Plant growth regulation,* 52**,** 173-180.

LIANG, Y., CHEN, Q., LIU, Q., ZHANG, W. & DING, R. 2003. Exogenous silicon (Si) increases antioxidant enzyme activity and reduces lipid peroxidation in roots of salt-stressed barley (Hordeum vulgareL.). *Journal of plant physiology,* 160**,** 1157-1164.

LIN, D. & XING, B. 2007. Phytotoxicity of nanoparticles: inhibition of seed germination and root growth. *Environmental Pollution,* 150**,** 243-250.

LIN, S., REPPERT, J., HU, Q., HUDSON, J. S., REID, M. L., RATNIKOVA, T. A., RAO, A. M., LUO, H. & KE, P. C. 2009. Uptake, translocation, and transmission of carbon nanomaterials in rice plants. *Small,* 5**,** 1128-1132.

LIU, Q., CHEN, B., WANG, Q., SHI, X., XIAO, Z., LIN, J. & FANG, X. 2009. Carbon nanotubes as molecular transporters for walled plant cells. *Nano letters,* 9**,** 1007-1010.

LIU, X., ZHANG, S., SHAN, X. & ZHU, Y.-G. 2005. Toxicity of arsenate and arsenite on germination, seedling growth and amylolytic activity of wheat. *Chemosphere,* 61**,** 293-301.

LONG, T. C., SALEH, N., TILTON, R. D., LOWRY, G. V. & VERONESI, B. 2006. Titanium dioxide (P25) produces reactive oxygen species in immortalized brain microglia (BV2): implications for nanoparticle neurotoxicity. *Environmental science & technology,* 40**,** 4346-4352.

LU, C., ZHANG, C., WEN, J. & WU, G. 2002. Effects of nano material on germination and growth of soybean. *Soybean Sci,* 21**,** 168-171.

LUCAS, W. J. & LEE, J.-Y. 2004. Plasmodesmata as a supracellular control network in plants. *Nature Reviews Molecular Cell Biology,* 5**,** 712-726.

MA, X., GEISER-LEE, J., DENG, Y. & KOLMAKOV, A. 2010. Interactions between engineered nanoparticles (ENPs) and plants: phytotoxicity, uptake and accumulation. *Science of the total environment,* 408**,** 3053-3061.

MAHMOODZADEH, H., AGHILI, R. & NABAVI, M. 2013. Physiological effects of TiO2 nanoparticles on wheat (Triticum aestivum). *Technical Journal of Engineering and Applied Sciences,* 3**,** 1365-1370.

MAROCCO, A., LORENZONI, C. & FRANCHEBOUD, Y. 2005. Chilling stress in maize. *Maydica,* 50**,** 571.

MARTÍNEZ-LÓPEZ, L. A., OCHOA-ALEJO, N. & MARTÍNEZ, O. 2014. Dynamics of the chili pepper transcriptome during fruit development. *BMC genomics,* 15**,** 1.

MATEO, A., FUNCK, D., MÜHLENBOCK, P., KULAR, B., MULLINEAUX, P. M. & KARPINSKI, S. 2006. Controlled levels of salicylic acid are required for optimal

photosynthesis and redox homeostasis. *Journal of experimental botany,* 57**,** 1795-1807.

MATEOS, R. M., JIMÉNEZ, A., ROMÁN, P., ROMOJARO, F., BACARIZO, S., LETERRIER, M., GÓMEZ, M., SEVILLA, F., DEL RÍO, L. A. & CORPAS, F. J. 2013. Antioxidant systems from pepper (Capsicum annuum L.): Involvement in the response to temperature changes in ripe fruits. *International journal of molecular sciences,* 14**,** 9556-9580.

MAZUMDAR, H. & AHMED, G. 2011. Phytotoxicity effect of silver nanoparticles on Oryza sativa. *IJ ChemTech. Res,* 3**,** 1494-1500.

MEDINA, C., SANTOS-MARTINEZ, M., RADOMSKI, A., CORRIGAN, O. & RADOMSKI, M. 2007. Nanoparticles: pharmacological and toxicological significance. *British journal of pharmacology,* 150**,** 552-558.

MEJÍA-TENIENTE, L., DURÁN-FLORES, F. D. D., CHAPA-OLIVER, A. M., TORRES-PACHECO, I., CRUZ-HERNÁNDEZ, A., GONZÁLEZ-CHAVIRA, M. M., OCAMPO-VELÁZQUEZ, R. V. & GUEVARA-GONZÁLEZ, R. G. 2013. Oxidative and molecular responses in Capsicum annuum L. after hydrogen peroxide, salicylic acid and chitosan foliar applications. *International journal of molecular sciences,* 14**,** 10178-10196.

MELONI, D. A., OLIVA, M. A., MARTINEZ, C. A. & CAMBRAIA, J. 2003. Photosynthesis and activity of superoxide dismutase, peroxidase and glutathione reductase in cotton under salt stress. *Environmental and Experimental Botany,* 49**,** 69-76.

MENG, J.-Y., ZHANG, C.-Y., ZHU, F., WANG, X.-P. & LEI, C.-L. 2009. Ultraviolet light-induced oxidative stress: effects on antioxidant response of Helicoverpa armigera adults. *Journal of insect physiology,* 55**,** 588-592.

MILERUE, N., NIKORNPUN, M. & KASETSART, J. 2000. Studies on heterosis of chili (Capsicum annuum L.). *Kasetsart J.(Nat. Sci.),* 34**,** 190-196.

MITTLER, R. 2006. Abiotic stress, the field environment and stress combination. *Trends in plant science,* 11**,** 15-19.

MITTLER, R. & BLUMWALD, E. 2010. Genetic engineering for modern agriculture: challenges and perspectives. *Annual review of plant biology,* 61**,** 443-462.

MITTLER, R., VANDERAUWERA, S., GOLLERY, M. & VAN BREUSEGEM, F. 2004. Reactive oxygen gene network of plants. *Trends in plant science,* 9**,** 490-498.

MOGHADDAM, M. R. B. & VAN DEN ENDE, W. 2012. Sugars and plant innate immunity. *Journal of experimental botany***,** ers129.

MOHAMMADI, R., MAALI-AMIRI, R. & MANTRI, N. 2014. Effect of TiO2 nanoparticles on oxidative damage and antioxidant defense systems in chickpea seedlings during cold stress. *Russian Journal of Plant Physiology,* 61**,** 768-775.

MOHANRAJ, V. & CHEN, Y. 2006. Nanoparticles–a review. *Trop J Pharm Res,* 5**,** 561-573.

MØLLER, I. M. 2001. Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. *Annual review of plant biology,* 52**,** 561-591.

MORARU, C. I., PANCHAPAKESAN, C. P., HUANG, Q., TAKHISTOV, P., SEAN, L. & KOKINI, J. L. 2003. Nanotechnology: a new frontier in food science. *Food Technology,* 57**,** 24-29.

MUKHERJEE, A., PERALTA-VIDEA, J. R., BANDYOPADHYAY, S., RICO, C. M., ZHAO, L. & GARDEA-TORRESDEY, J. L. 2014. Physiological effects of nanoparticulate ZnO in green peas (Pisum sativum L.) cultivated in soil. *Metallomics,* 6**,** 132-138.

MUSHTAQ, Y. K. 2011. Effect of nanoscale Fe3O4, TiO2 and carbon particles on cucumber seed germination. *Journal of Environmental Science and Health, Part A,* 46**,** 1732-1735.

NAIR, R., VARGHESE, S. H., NAIR, B. G., MAEKAWA, T., YOSHIDA, Y. & KUMAR, D. S. 2010. Nanoparticulate material delivery to plants. *Plant Science,* 179**,** 154-163.

NAKANO, Y. & ASADA, K. 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant and Cell Physiology,* 22**,** 867-880.

NAKATA, K. & FUJISHIMA, A. 2012. TiO 2 photocatalysis: design and applications. *Journal of Photochemistry and Photobiology C: Photochemistry Reviews,* 13**,** 169-189.

NAVARRO, E., PICCAPIETRA, F., WAGNER, B., MARCONI, F., KAEGI, R., ODZAK, N., SIGG, L. & BEHRA, R. 2008. Toxicity of silver nanoparticles to Chlamydomonas reinhardtii. *Environmental science & technology,* 42**,** 8959-8964.

NEL, A., XIA, T., MÄDLER, L. & LI, N. 2006. Toxic potential of materials at the nanolevel. *science,* 311**,** 622-627.

NEWMAN, M. D., STOTLAND, M. & ELLIS, J. I. 2009. The safety of nanosized particles in titanium dioxide–and zinc oxide–based sunscreens. *Journal of the American Academy of Dermatology,* 61**,** 685-692.

NGOY, J. M., IYUKE, S. E., NEUSE, W. E. & YAH, C. S. 2011. Covalent functionalization for multi-walled carbon nanotube (f-MWCNT)-folic acid bound bioconjugate. *Journal of Applied Sciences,* 11**,** 2700-2711.

NOCTOR, G. & FOYER, C. H. 1998. Ascorbate and glutathione: keeping active oxygen under control. *Annual review of plant biology,* 49**,** 249-279.

NOHYNEK, G., DUFOUR, E. & ROBERTS, M. 2008. Nanotechnology, cosmetics and the skin: is there a health risk? *Skin pharmacology and physiology,* 21**,** 136-149.

NOSTAR, O., OZDEMIR, F., BOR, M., TURKAN, I. & TOSUN, N. 2013. Combined effects of salt stress and cucurbit downy mildew (Pseudoperospora cubensis Berk. and Curt. Rostov.) infection on growth, physiological traits and antioxidant activity in cucumber (Cucumis sativus L.) seedlings. *Physiological and molecular plant pathology,* 83**,** 84-92.

O'HARA, L. E., PAUL, M. J. & WINGLER, A. 2013. How do sugars regulate plant growth and development? New insight into the role of trehalose-6-phosphate. *Molecular plant,* 6**,** 261-274.

ODUM, L. M. 2007. *Effect of Silver nanoparticles on tomato plants and Development of a Plant Monitoring System (PMS).* Auburn University.

OZDEN, M., DEMIREL, U. & KAHRAMAN, A. 2009. Effects of proline on antioxidant system in leaves of grapevine (Vitis vinifera L.) exposed to oxidative stress by H 2 O 2. *Scientia Horticulturae,* 119**,** 163-168.

PAN, B. & XING, B. 2010. Chapter three-manufactured nanoparticles and their sorption of organic chemicals. *Advances in agronomy,* 108**,** 137-181.

PANDEY, P., RAMEGOWDA, V. & SENTHIL-KUMAR, M. 2015. Shared and unique responses of plants to multiple individual stresses and stress combinations: physiological and molecular mechanisms. *Frontiers in plant science,* 6.

PARSONS, J. G., LOPEZ, M. L., GONZALEZ, C. M., PERALTA-VIDEA, J. R. & GARDEA-TORRESDEY, J. L. 2010. Toxicity and biotransformation of uncoated and coated nickel hydroxide nanoparticles on mesquite plants. *Environmental toxicology and chemistry,* 29**,** 1146-1154.

PENG, J., DENG, X., JIA, S., HUANG, J., MIAO, X. & HUANG, Y. 2004. Role of salicylic acid in tomato defense against cotton bollworm, Helicoverpa armigera Hubner. *Zeitschrift für Naturforschung C,* 59**,** 856-862.

PÉREZ-DE-LUQUE, A. & RUBIALES, D. 2009. Nanotechnology for parasitic plant control. *Pest management science,* 65**,** 540-545.

PHAM-HUY, L. A., HE, H. & PHAM-HUY, C. 2008. Free radicals, antioxidants in disease and health. *Int J Biomed Sci,* 4**,** 89-96.

PICCINNO, F., GOTTSCHALK, F., SEEGER, S. & NOWACK, B. 2012. Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. *Journal of Nanoparticle Research,* 14**,** 1-11.

POKHREL, L. R. & DUBEY, B. 2013. Evaluation of developmental responses of two crop plants exposed to silver and zinc oxide nanoparticles. *Science of the total environment,* 452**,** 321-332.

POKORNÝ, J. & KORCZAK, J. 2001. Preparation of natural antioxidants. *Antioxidants in food: practical application. Cambridge England: Woodhead Publishing Limited***,** 311-341.

PRASAD, T. K., ANDERSON, M. D., MARTIN, B. A. & STEWART, C. R. 1994. Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. *The Plant Cell,* 6**,** 65-74.

PROSEUS, T. E. & BOYER, J. S. 2005. Turgor pressure moves polysaccharides into growing cell walls of Chara corallina. *Annals of botany,* 95**,** 967-979.

PULSKAMP, K., DIABATÉ, S. & KRUG, H. F. 2007. Carbon nanotubes show no sign of acute toxicity but induce intracellular reactive oxygen species in dependence on contaminants. *Toxicology letters,* 168**,** 58-74.

PUNG, Y. F., ROCIC, P., MURPHY, M. P., SMITH, R. A., HAFEMEISTER, J., OHANYAN, V., GUARINI, G., YIN, L. & CHILIAN, W. M. 2012. Resolution of mitochondrial oxidative stress rescues coronary collateral growth in Zucker obese fatty rats. *Arteriosclerosis, thrombosis, and vascular biology,* 32**,** 325-334.

QIN, C., YU, C., SHEN, Y., FANG, X., CHEN, L., MIN, J., CHENG, J., ZHAO, S., XU, M. & LUO, Y. 2014. Whole-genome sequencing of cultivated and wild peppers provides insights into Capsicum domestication and specialization. *Proceedings of the National Academy of Sciences,* 111**,** 5135-5140.

RĂCUCIU, M. & CREANGĂ, D.-E. 2009. Biocompatible magnetic fluid nanoparticles internalized in vegetal tissue. *Rom Journ Phys,* 54**,** 115-124.

RAI, M., YADAV, A. & GADE, A. 2009. Silver nanoparticles as a new generation of antimicrobials. *Biotechnology advances,* 27**,** 76-83.

RAIKOVA, O., PANICHKIN, L. & RAIKOVA, N. Studies on the effect of ultrafine metal powders produced by different methods on plant growth and development.

Nanotechnologies and information technologies in the 21st century. Proceedings of the International Scientific and Practical Conference, 2006. 108-111.

RALIYA, R., BISWAS, P. & TARAFDAR, J. 2015. TiO 2 nanoparticle biosynthesis and its physiological effect on mung bean (Vigna radiata L.). *Biotechnology Reports,* 5**,** 22-26.

RASKAR, S. & LAWARE, S. 2014. Effect of zinc oxide nanoparticles on cytology and seed germination in onion. *Int. J. Curr. Microbiol. App. Sci,* 3**,** 467-473.

RAWAT, M., SINGH, D., SARAF, S. & SARAF, S. 2006. Nanocarriers: promising vehicle for bioactive drugs. *Biological and Pharmaceutical Bulletin,* 29**,** 1790-1798.

REFAAT, M. & ELGARHY, H. A. 2007. Relationship between hybrid performance and genetic diversity based on ISSR-PCR markers in pepper (Capsicum annuum. L.). *Annals of Agric. Sci., Moshtohor,* 45**,** 1565-1579.

RENEW, S., HEYNO, E., SCHOPFER, P. & LISZKAY, A. 2005. Sensitive detection and localization of hydroxyl radical production in cucumber roots and Arabidopsis seedlings by spin trapping electron paramagnetic resonance spectroscopy. *The Plant Journal,* 44**,** 342-347.

RICO, C. M., HONG, J., MORALES, M. I., ZHAO, L., BARRIOS, A. C., ZHANG, J.-Y., PERALTA-VIDEA, J. R. & GARDEA-TORRESDEY, J. L. 2013. Effect of cerium oxide nanoparticles on rice: a study involving the antioxidant defense system and in vivo fluorescence imaging. *Environmental science & technology,* 47**,** 5635-5642.

RIEUX, A., FIEVEZ, V., GARINOT, M., SCHNEIDER, Y.-J. & PRÉAT, V. 2006. Nanoparticles as potential oral delivery systems of proteins and vaccines: a mechanistic approach. *Journal of controlled release,* 116**,** 1-27.

ROCO, M. C. 2011. The long view of nanotechnology development: the National Nanotechnology Initiative at 10 years. *Journal of Nanoparticle Research,* 13**,** 427-445.

ROTHEN-RUTISHAUSER, B. M., SCHÜRCH, S., HAENNI, B., KAPP, N. & GEHR, P. 2006. Interaction of fine particles and nanoparticles with red blood cells visualized with advanced microscopic techniques. *Environmental science & technology,* 40**,** 4353-4359.

SABIR, S., ARSHAD, M. & CHAUDHARI, S. K. 2014. Zinc oxide nanoparticles for revolutionizing agriculture: synthesis and applications. *The Scientific World Journal,* 2014.

SAMADI, N., YAHYAABADI, S. & REZAYATMAND, Z. 2014. Effect of TiO2 and TiO2 nanoparticle on germination, root and shoot Length and photosynthetic pigments of Mentha piperita. *International Journal of Plant & Soil Science,* 3**,** 408-418.

SCHOPFER, P. 2001. Hydroxyl radical-induced cell-wall loosening in vitro and in vivo: implications for the control of elongation growth. *The Plant Journal,* 28**,** 679-688.

SELIVANOV, V. & ZORIN, E. 2001. Sustained Action of ultrafine metal powders on seeds of grain crops. *Perspekt. Materialy,* 4**,** 66-69.

SERVIN, A. D., MORALES, M. I., CASTILLO-MICHEL, H., HERNANDEZ-VIEZCAS, J. A., MUNOZ, B., ZHAO, L., NUNEZ, J. E., PERALTA-VIDEA, J. R. & GARDEA-TORRESDEY, J. L. 2013. Synchrotron verification of TiO2 accumulation in cucumber fruit: a possible pathway of TiO2 nanoparticle transfer from soil into the food chain. *Environmental science & technology,* 47**,** 11592-11598.

SHAH, V. & BELOZEROVA, I. 2009. Influence of metal nanoparticles on the soil microbial community and germination of lettuce seeds. *Water, air, and soil pollution,* 197**,** 143-148.

SHALATA, A., MITTOVA, V., VOLOKITA, M., GUY, M. & TAL, M. 2001. Response of the cultivated tomato and its wild salt-tolerant relative Lycopersiconpennellii to salt-dependent oxidative stress: The root antioxidative system. *Physiologia Plantarum,* 112**,** 487-494.

SHARMA, P., JHA, A. B., DUBEY, R. S. & PESSARAKLI, M. 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Journal of Botany,* 2012.

SHEN, C.-X., ZHANG, Q.-F., LI, J., BI, F.-C. & YAO, N. 2010. Induction of programmed cell death in Arabidopsis and rice by single-wall carbon nanotubes. *American Journal of Botany,* 97**,** 1602-1609.

SHI, Y., WANG, F., HE, J., YADAV, S. & WANG, H. 2010. Titanium dioxide nanoparticles cause apoptosis in BEAS-2B cells through the caspase 8/t-Bid-independent mitochondrial pathway. *Toxicology letters,* 196**,** 21-27.

SIDDIQUI, M. H., AL-WHAIBI, M. H., FIROZ, M. & AL-KHAISHANY, M. Y. 2015. Role of nanoparticles in plants. *Nanotechnology and Plant Sciences.* Springer.

SINGH, S., PATEL, P., JAISWAL, S., PRABHUNE, A., RAMANA, C. & PRASAD, B. 2009. A direct method for the preparation of glycolipid–metal nanoparticle conjugates: sophorolipids as reducing and capping agents for the synthesis of water re-dispersible silver nanoparticles and their antibacterial activity. *New Journal of Chemistry,* 33**,** 646-652.

SLESAK, I., LIBIK, M., KARPINSKA, B., KARPINSKI, S. & MISZALSKI, Z. 2007. The role of hydrogen peroxide in regulation of plant metabolism and cellular signalling in response to environmental stresses. *ACTA BIOCHIMICA POLONICA-ENGLISH EDITION-,* 54**,** 39.

SONG, U., JUN, H., WALDMAN, B., ROH, J., KIM, Y., YI, J. & LEE, E. J. 2013. Functional analyses of nanoparticle toxicity: a comparative study of the effects of TiO 2 and Ag on tomatoes (Lycopersicon esculentum). *Ecotoxicology and environmental safety,* 93**,** 60-67.

SONG, U. & LEE, E. J. 2010. Ecophysiological responses of plants after sewage sludge compost applications. *Journal of Plant Biology,* 53**,** 259-267.

STAMPOULIS, D., SINHA, S. K. & WHITE, J. C. 2009. Assay-dependent phytotoxicity of nanoparticles to plants. *Environmental science & technology,* 43**,** 9473-9479.

SUZUKI, K. G., FUJIWARA, T. K., EDIDIN, M. & KUSUMI, A. 2007. Dynamic recruitment of phospholipase Cγ at transiently immobilized GPI-anchored receptor clusters induces IP3–Ca2+ signaling: single-molecule tracking study 2. *The Journal of cell biology,* 177**,** 731-742.

TANAKA, Y., HIBINO, T., HAYASHI, Y., TANAKA, A., KISHITANI, S., TAKABE, T. & YOKOTA, S. 1999. Salt tolerance of transgenic rice overexpressing yeast mitochondrial Mn-SOD in chloroplasts. *Plant Science,* 148**,** 131-138.

TAYLOR, N. J. & FAUQUET, C. M. 2002. Microparticle bombardment as a tool in plant science and agricultural biotechnology. *DNA and cell biology,* 21**,** 963-977.

TEMBHURNE, B. & RAO, S. 2012. Heterosis and combining ability in CMS based hybrid chilli (Capsicum annuum L.). *Journal of Agricultural Science,* 4**,** 89.

TESTILLANO, P. S., RAMIREZ, C., DOMENECH, J., CORONADO, M.-J., VERGNE, P., MATTHYS-ROCHON, E. & RISUENO, M. C. 2002. Young microspore-derived maize embryos show two domains with defined features also present in zygotic embryogenesis. *International Journal of Developmental Biology,* 46**,** 1035-1047.

THAMAPHAT, K., LIMSUWAN, P. & NGOTAWORNCHAI, B. 2008. Phase characterization of TiO2 powder by XRD and TEM. *Kasetsart J.(Nat. Sci.),* 42**,** 357-361.

THEERAKARUNWONG, C. & CHOUYCHAI, W. 2013. Efficiency of Titanium Dioxide on Mungbean Seed Sterile and their Nanotoxicity to Mungbean Growth In Vitro. *International Journal of Agriculture & Biology,* 15.

TODA, S. 2005. Antioxidative effects of polyphenols in leaves of Houttuynia cordata on protein fragmentation by copper-hydrogen peroxide in vitro. *Journal of medicinal food,* 8**,** 266-268.

TONG, N. & BOSLAND, P. W. 1999. Capsicum tovarii, a new member of the Capsicum baccatum complex. *Euphytica,* 109**,** 71-77.

TORNEY, F., TREWYN, B. G., LIN, V. S.-Y. & WANG, K. 2007. Mesoporous silica nanoparticles deliver DNA and chemicals into plants. *Nature nanotechnology,* 2**,** 295-300.

TROUILLER, B., RELIENE, R., WESTBROOK, A., SOLAIMANI, P. & SCHIESTL, R. H. 2009. Titanium dioxide nanoparticles induce DNA damage and genetic instability in vivo in mice. *Cancer research,* 69**,** 8784-8789.

UCHIDA, A., JAGENDORF, A. T., HIBINO, T., TAKABE, T. & TAKABE, T. 2002. Effects of hydrogen peroxide and nitric oxide on both salt and heat stress tolerance in rice. *Plant Science,* 163**,** 515-523.

VAIDYANATHAN, H., SIVAKUMAR, P., CHAKRABARTY, R. & THOMAS, G. 2003. Scavenging of reactive oxygen species in NaCl-stressed rice (Oryza sativa L.)—differential response in salt-tolerant and sensitive varieties. *Plant Science,* 165**,** 1411-1418.

VAL, S., HUSSAIN, S., BOLAND, S., HAMEL, R., BAEZA-SQUIBAN, A. & MARANO, F. 2009. Carbon black and titanium dioxide nanoparticles induce pro-inflammatory responses in bronchial epithelial cells: need for multiparametric evaluation due to adsorption artifacts. *Inhalation toxicology,* 21**,** 115-122.

VALKO, M., RHODES, C., MONCOL, J., IZAKOVIC, M. & MAZUR, M. 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-biological interactions,* 160**,** 1-40.

VIMALA, S. & ADENAN, M. I. 1999. Malaysian tropical forest medicinal plants: a source of natural antioxidants. *Journal of Tropical Forest Products,* 5**,** 32-38.

WAHID, A., PERVEEN, M., GELANI, S. & BASRA, S. M. 2007. Pretreatment of seed with H 2 O 2 improves salt tolerance of wheat seedlings by alleviation of oxidative damage and expression of stress proteins. *Journal of plant physiology,* 164**,** 283-294.

WANG, J., ZHANG, H. & ALLEN, R. D. 1999. Overexpression of an Arabidopsis peroxisomal ascorbate peroxidase gene in tobacco increases protection against oxidative stress. *Plant and Cell Physiology,* 40**,** 725-732.

WANG, J., ZHOU, G., CHEN, C., YU, H., WANG, T., MA, Y., JIA, G., GAO, Y., LI, B. & SUN, J. 2007. Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration. *Toxicology letters,* 168**,** 176-185.

WANG, P., ZHAI, Y., WANG, D. & DONG, S. 2011. Synthesis of reduced graphene oxide-anatase TiO 2 nanocomposite and its improved photo-induced charge transfer properties. *Nanoscale,* 3**,** 1640-1645.

WANG, Y., LI, J., WANG, J. & LI, Z. 2010. Exogenous H2O2 improves the chilling tolerance of manilagrass and mascarenegrass by activating the antioxidative system. *Plant growth regulation,* 61**,** 195-204.

WIESNER, M. R., LOWRY, G. V., ALVAREZ, P., DIONYSIOU, D. & BISWAS, P. 2006. Assessing the risks of manufactured nanomaterials. *Environmental science & technology,* 40**,** 4336-4345.

WITZMANN, F. A. & MONTEIRO-RIVIERE, N. A. 2006. Multi-walled carbon nanotube exposure alters protein expression in human keratinocytes. *Nanomedicine: Nanotechnology, Biology and Medicine,* 2**,** 158-168.

WOJTYLA, Ł., LECHOWSKA, K., KUBALA, S. & GARNCZARSKA, M. 2016. Different Modes of Hydrogen Peroxide Action During Seed Germination. *Frontiers in plant science,* 7.

XIA, T., KOVOCHICH, M., BRANT, J., HOTZE, M., SEMPF, J., OBERLEY, T., SIOUTAS, C., YEH, J. I., WIESNER, M. R. & NEL, A. E. 2006. Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm. *Nano letters,* 6**,** 1794-1807.

XIA, T., KOVOCHICH, M., LIONG, M., MÄDLER, L., GILBERT, B., SHI, H., YEH, J. I., ZINK, J. I. & NEL, A. E. 2008. Comparison of the mechanism of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties. *ACS nano,* 2**,** 2121-2134.

YADEGHARI, L. Z., HEIDARI, R. & CARAPETIAN, J. 2008. The influence of cold acclimation on proline, malondialdehyde (MDA), total protein and pigments contents in soybean (Glycine max) seedlings. *Res J Biol Sci,* 3**,** 74-79.

YANG, F., HONG, F., YOU, W., LIU, C., GAO, F., WU, C. & YANG, P. 2006a. Influence of nano-anatase TiO2 on the nitrogen metabolism of growing spinach. *Biological trace element research,* 110**,** 179-190.

YANG, H., WANG, X., LIU, X., WU, J., LIU, C., GONG, W., ZHAO, Z., HONG, J., LIN, D. & WANG, Y. 2009a. Antioxidant peptidomics reveals novel skin antioxidant system. *Molecular & Cellular Proteomics,* 8**,** 571-583.

YANG, K., ZHU, L. & XING, B. 2006b. Adsorption of polycyclic aromatic hydrocarbons by carbon nanomaterials. *Environmental science & technology,* 40**,** 1855-1861.

YANG, L. & WATTS, D. J. 2005. Particle surface characteristics may play an important role in phytotoxicity of alumina nanoparticles. *Toxicology letters,* 158**,** 122-132.

YANG, X., ZHANG, X., MA, Y., HUANG, Y., WANG, Y. & CHEN, Y. 2009b. Superparamagnetic graphene oxide–Fe 3 O 4 nanoparticles hybrid for controlled targeted drug carriers. *Journal of Materials Chemistry,* 19**,** 2710-2714.

YIH, T. & AL-FANDI, M. 2006. Engineered nanoparticles as precise drug delivery systems. *Journal of cellular biochemistry,* 97**,** 1184-1190.

YIN, L., CHENG, Y., ESPINASSE, B., COLMAN, B. P., AUFFAN, M., WIESNER, M., ROSE, J., LIU, J. & BERNHARDT, E. S. 2011. More than the ions: the effects of silver nanoparticles on Lolium multiflorum. *Environmental science & technology,* 45**,** 2360-2367.

YU, C.-W., MURPHY, T. M. & LIN, C.-H. 2003. Hydrogen peroxide-induced chilling tolerance in mung beans mediated through ABA-independent glutathione accumulation. *Functional Plant Biology,* 30**,** 955-963.

YU, C.-W., MURPHY, T. M., SUNG, W.-W. & LIN, C.-H. 2002. H2O2 treatment induces glutathione accumulation and chilling tolerance in mung bean. *Functional Plant Biology,* 29**,** 1081-1087.

ZHANG, P., MA, Y., ZHANG, Z., HE, X., ZHANG, J., GUO, Z., TAI, R., ZHAO, Y. & CHAI, Z. 2012. Biotransformation of ceria nanoparticles in cucumber plants. *ACS nano,* 6**,** 9943-9950.

ZHANG, Y., CHEN, B., XU, Z., SHI, Z., CHEN, S., HUANG, X., CHEN, J. & WANG, X. 2014. Involvement of reactive oxygen species in endosperm cap weakening and embryo elongation growth during lettuce seed germination. *Journal of experimental botany,* 65**,** 3189-3200.

ZHAO, L., PERALTA-VIDEA, J. R., VARELA-RAMIREZ, A., CASTILLO-MICHEL, H., LI, C., ZHANG, J., AGUILERA, R. J., KELLER, A. A. & GARDEA-TORRESDEY, J. L. 2012. Effect of surface coating and organic matter on the uptake of CeO 2 NPs by corn plants grown in soil: insight into the uptake mechanism. *Journal of hazardous materials,* 225**,** 131-138.

ZHENG, L., HONG, F., LU, S. & LIU, C. 2005. Effect of nano-TiO2 on strength of naturally aged seeds and growth of spinach. *Biological trace element research,* 104**,** 83-91.