Investigating the Role of Exogenous Hydrogen Peroxide in Nanotoxicity Reduction in Maize hybrid and its Parents

By

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The thesis is submitted in the partial fulfilment of the requirement for the degree of Masters of Science

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MS Thesis Work

We hereby recommend that the thesis prepared by **Namra Haq**
titled "Investigating the role of Exogenous Hydrogen Peroxide in Nanotoxicity Reduction in Maize Hybrid and its Parents" be accepted in its present form to satisfy the thesis requirement of MS degree.

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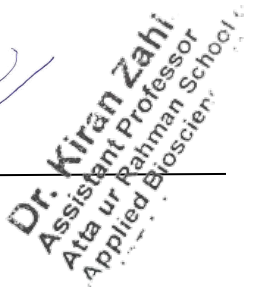

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Dedication

I dedicate my entire research to my beloved prophet Muhammad (SAWW)

Special Thanks

To

My dear Mother and My Supervisor Dr. Kiran Zahid

Acknowledgement

I am thankful to Almighty ALLAH, Who blessed me with countless blessings throughout my life. HE (SWT) made me the believer of HIS (SWT) beloved Prophet Muhammad (SAWW), through HIS (SAWW) teachings; I developed the potential abilities that made me able to face the challenges of life successfully. Through the teachings of Prophet Muhammad (SAWW), I was able to contemplate on the status of a human being in the world and what are ones rights and duties to prosper in this life and hereafter. Verily, Almighty ALLAH says in the holy Quran

"And if Allah touches you with harm, there is none who can lift it but HE, and if HE intends good for you, then none can repel His favour which HE causes to reach whom HE wills among HIS servants, and HE is the Pardoning, the Merciful." (Chapter: 10, Verse: 107)

I am grateful to Engineer Muhammad Asghar, Rector NUST for providing the infrastructure and support for my research. I also take the opportunity here to thank Dr. Peter John, Principal, ASAB and HOD, Plant Biotechnology Department, Dr. Muhammad Tahir for giving the right ambience and feasible environment to make my research possible.

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I pay my sincere gratitude to my parents; my mother really helped me all through my life, every moment that was triumphant, exultant or crowned in my life is the blessing of Almighty Allah Who (SWT) blessed me with such a great mother, who loved me to an extent which cannot be gauged. My mother is the one who always remained steadfast by the blessing of Almighty Lord in every single decision of my life. I do not remember a time when I was in need and she was not there for me or when I asked her for something and she had replied me in negative. She is the greatest blessing of Almighty Lord to me. May Allah Almighty keep her with me all through my life (Amen). I am also grateful to my father for when and where he helped me during the entire course of MS degree.

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All those who helped me were from Almighty ALLAH alone, as ALLAH says in the glorious Quran

"Which is it of the favours of your Lord that you deny." (Chapter: 55)

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List of Abbreviations

Ag	Silver
Al	Aluminium
AOS	Active Oxygen Scavenging
APX	Ascorbate Peroxidase
CAT	Catalase
Cu	Copper
DMSO	DIMETHYL SULPHOXIDE
Hydrogen Peroxide	H_2O_2
NPs	Nanoparticles
Pb	Lead
ROS	Reactive Oxygen Species
TiO_2	Titanium dioxide
XRD	X-ray diffraction
Zn	Zinc

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Abstract

Maize is one of the most important grains of the world, which is considered as an essential constituent of diet of the people of Pakistan and around the globe. Maize is regarded as one of the primeval grain that was cultivated by the early human beings. Maize has always remained in focus by the agricultural scientists and experts. During the 1930's, first hybrid maize was used by the growers. Hybrid maize became popular because of its potential ability to give high yield and tolerance towards various biotic and abiotic stresses. This is because of the hybrid vigour or heterosis depicted by the hybrid plants. One of the important aspects, which requires focus are the various abiotic stresses which the maize crop is susceptible to. These abiotic stresses particularly include drought, salinity, chilling and heat. To overcome these stresses various types of maize hybrids have had been made and exploited. Nanoscience is an upcoming field in which the scientific community is interested because of its perceived advantages. However nanotoxicity is one of the potential issues that require our diligent attention. Nanotoxicity studies in maize and other plants are in their initial stages. The present study emphasised on studying the effects of nanotoxicity and also to analyse the effects of pre-treatment of hydrogen peroxide in nanotoxicity reduction in maize hybrid and its parents, as hydrogen peroxide pre-treatment has been reported to curb various abiotic stresses in maize and other plants. The current study revealed that maize seedlings pre-treated with hydrogen peroxide prior to TiO_2 nanoparticles stress showed nanotoxicity reduction at both physiological and biochemical levels when analysed.

Chapter 1

INTRODUCTION

1.1 Maize

Maize (*Zea Mays*), also known as corn is one of the most abundantly produced crops of the world. It belongs to the family *Poaceae* (Gramineae) and is a constituent member of the order *Poales* (Kellogg 2001; Bolot et al., 2009).

It is a monocotyledonous plant that grows in almost all climatic zones of the world. The nutrient content of maize can be split up as approximately 72% starch, 10%protein and 4% fat and the overall energy density met by maize is 365Kcal/100g (Nuss et al., 2010).

As per gross global production per annum, it even surpasses rice and wheat. Maize has wide range of uses and applications. Maize is considered to be a staple food crop in many parts of the world, it is also used as livestock feed and for ethanol production. Because of such applications there is an ever increasing demand of maize productivity around the globe.

1.2 Theoretical Background

The world literature suggests that the origin of maize can be traced in Americas in ancient times and happened by the efforts of Indians in the Mexican highlands (Piperno et al., 2001).

However, it was soon cultivated by the European settlers of Spanish, English and French origin. From Europe it moved to Asia and Africa, where like other parts of the world was successfully cultivated (Brown and Darrah, 1985; Gibson and Benson, 2002; Vollbrecht and Sigmon, 2005).

1.3 Evolution of Maize

The origin and evolution of the various members of grass family can be traced about 50 to 70 million years ago (Kellogg 2001; Bolot et al., 2009).

Maize is suggested to have been originated in Mexico, some seven to ten thousand years back; nevertheless, there is some evidence that suggests its origin in Himalayas in Asia (Smith, 2001; Piperno and Flannery, 2001).

1.4Development of Commercial Hybrid Maize

Results published by Charles Darwin, as a consequence of the experiments he conducted in native England in a small greenhouse on self and cross pollination stated that inbreeding resulted in decreased plant vigour however cross breading resulted in improved plant vigour (Darwin, 1876).

The hybrid maize was developed by the Scientist named James Beal on studying the results conducted by Charles Darwin with the sole intention of increasing yield, via hybrid vigour.

Dr. G.H. Shull in 1908 reported that self fertilization results in separation and purification of strains however it ends up with reduction in plant vigour, but this vigour can be reinstated again via cross breeding of the inbred lines, through his exclusive experiments (Shull, 1908).

In 1914, Shull introduced the world with the word heterosis to explain the ingrained vitality and luxuriance of hybrid seed. Through explanation to the understanding of the word Heterosis and Hybrid Vigour have had been provided later by Shull (Shull, 1948).

Later in 1917, a relatively new and innovative concept of double cross was introduced by Donald F. Jones, which proved to be a breakthrough and eventually resulted in the development of modern day hybrid corn that was endowed with various favourable characteristics in addition to the increased yields (Jones, 1917).

1.5 Importance of Hybrid Maize

Hybrid vigour (Heterosis) proved to be a game changer in the field of science, though its genetic basis was and is still not known to the scientists but it has been found that the new hybrids are superior compared with their parents and also they are more tenacious and healthy (Duvick, 2001).

Hybrid seeds have had been developed against different biotic and abiotic stresses by various research organizations around the world. In the year 1997, CIMMYT began a program that was meant to improve maize for drought stricken and mid - altitudes of Southern Africa. Other limitations, such as low nitrogen and major diseases of maize leaf and ear were also taken under consideration.

Hybrid seed development thus proved to be productive for this zone and also suggested that the hybrid seed development can play a very important role towards coping up various biotic and abiotic stresses (Banziger et al., 2006).

1.6 Cultivation of Maize in the World

Maize is one of the chief grain crops that are being produced in the world along with wheat and rice. According to an estimate, 875,226,630 tons is the ballpark figure of the global maize gross production in the year 2012 (FAO, 2012). The three main producers of maize in the world are the USA, China and Brazil reaping 31%, 24% and 8% respectively of the entire maize production in the world (Ranum et al., 2014).

It is estimated that by 2050’s the world population will be around 9 billion. Thus it is the need of the hour to work out for the future situation of foreseen aggravated issues of food insecurity in the world.

1.7 Cultivation of Maize in Pakistan

Maize is cultivated on 1.016 million ha of Pakistan's total arable land. Pakistan is self sufficient as per the production of maize is concerned. Maize accounts for about 4.8% of the total crop cover of Pakistan and is responsible for 3.5% of the agricultural value output. In Pakistan 99% of the maize is cultivated in NWFP and Punjab, and the rest of 1% is cultivated in Sindh and Baluchistan. The ballpark figure recorded for average maize production in Pakistan is 2850Kg/ha, following the two cereal grains wheat and rice (Tariq and Iqbal, 2010).

Pakistan maize production by year from 1960 to 2014

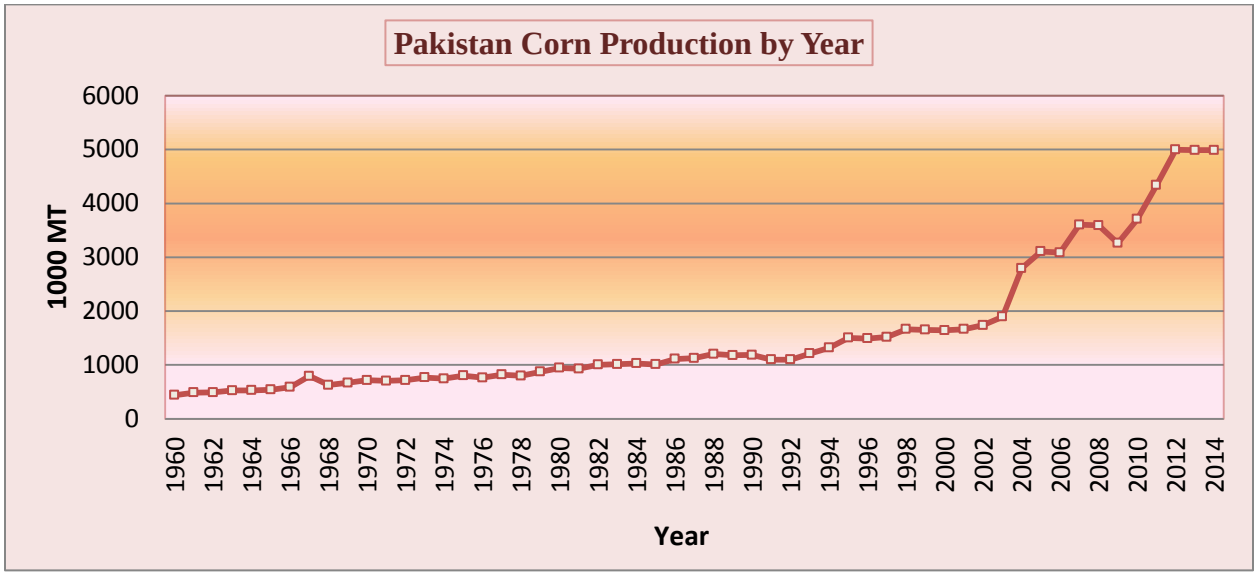

Figure 1.1: From the above graph it can be concluded that there is a continuous increase in the gross production of maize from 1960 to 2014. In 1960 it was 436 MT, which rose to 5100 MT in 2014.

Pakistan Maize Production Annual Growth Rate

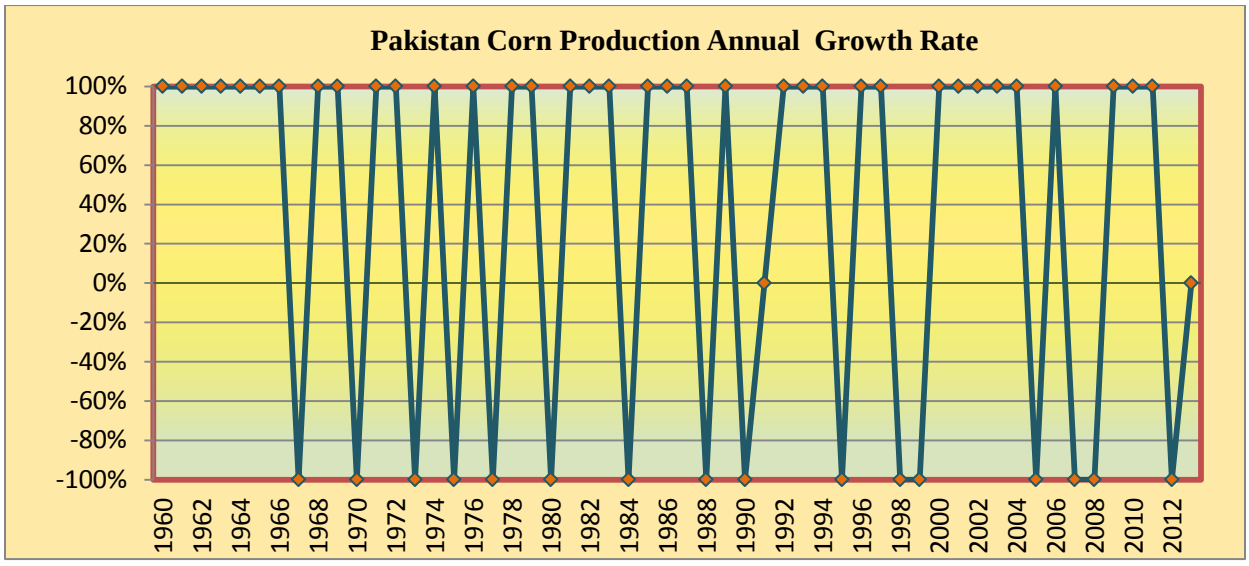

Figure 1.2: The figure shows the Pakistan Maize Production Annual Growth Rate from 1960 to 2015

Pakistan maize area harvested by year in Pakistan from 1960 to 2014

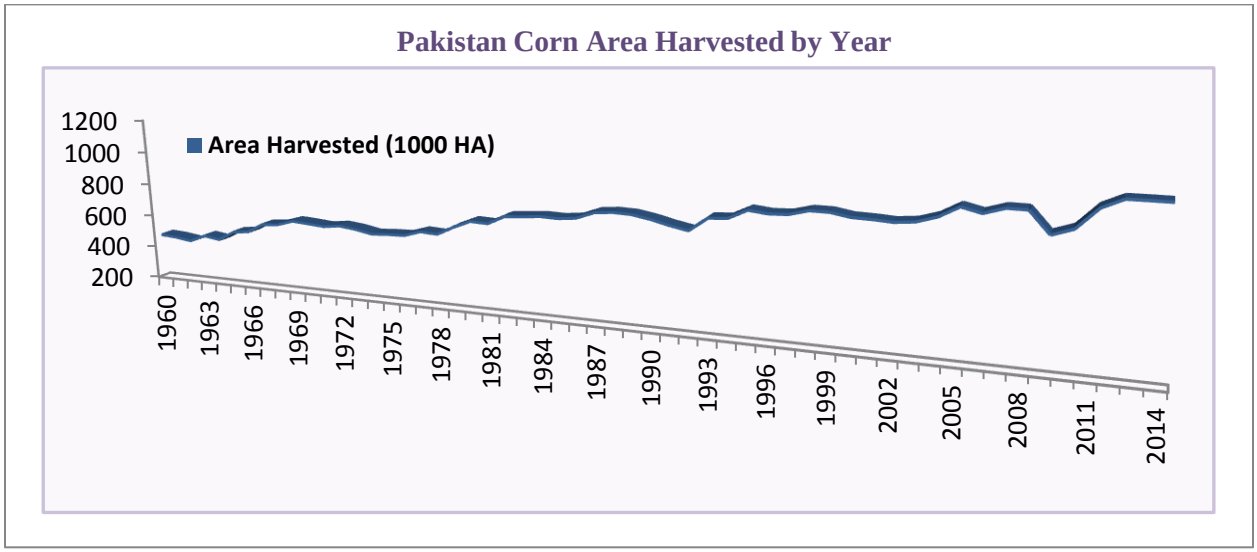

Figure 1.3: The graph above shows that corn area harvested by year started does not show a constant rate. Rather, it shows a random pattern of increase in few year, then decrease and then increase in the next year.

Pakistan Maize Area Harvested Annual Growth rate

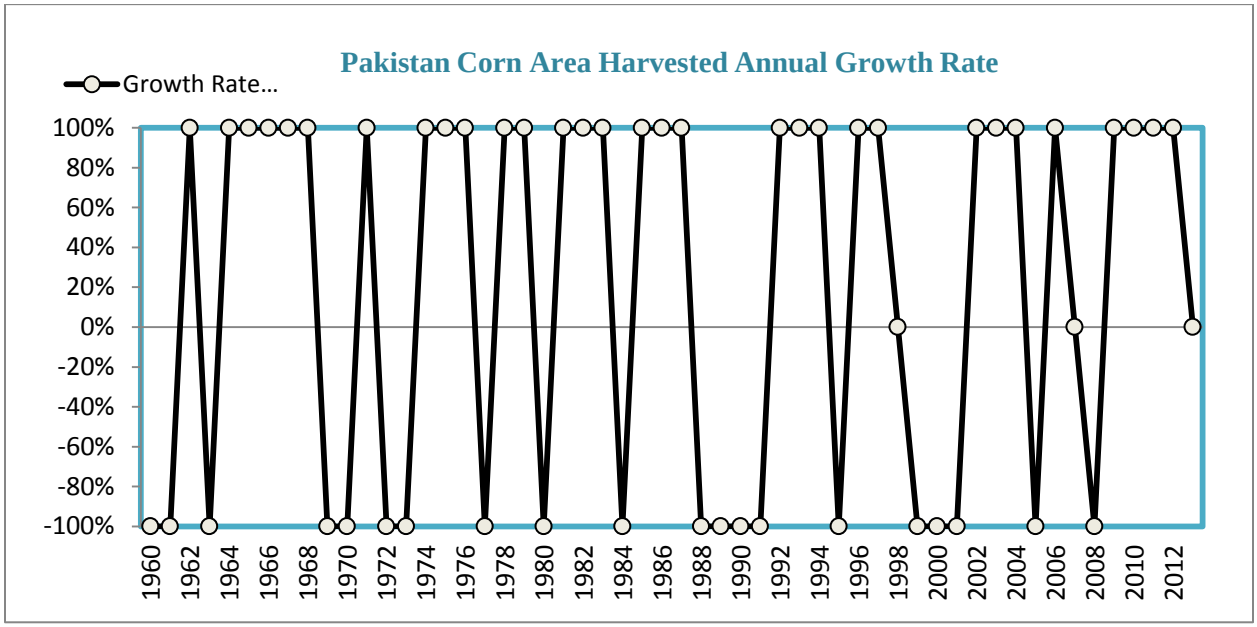

Figure 1.4: The figure shows the Pakistan Maize Area Harvested Annual Growth Rate from 1960 to 2015.

Pakistan maize yield by year from the year 1960 to 2014

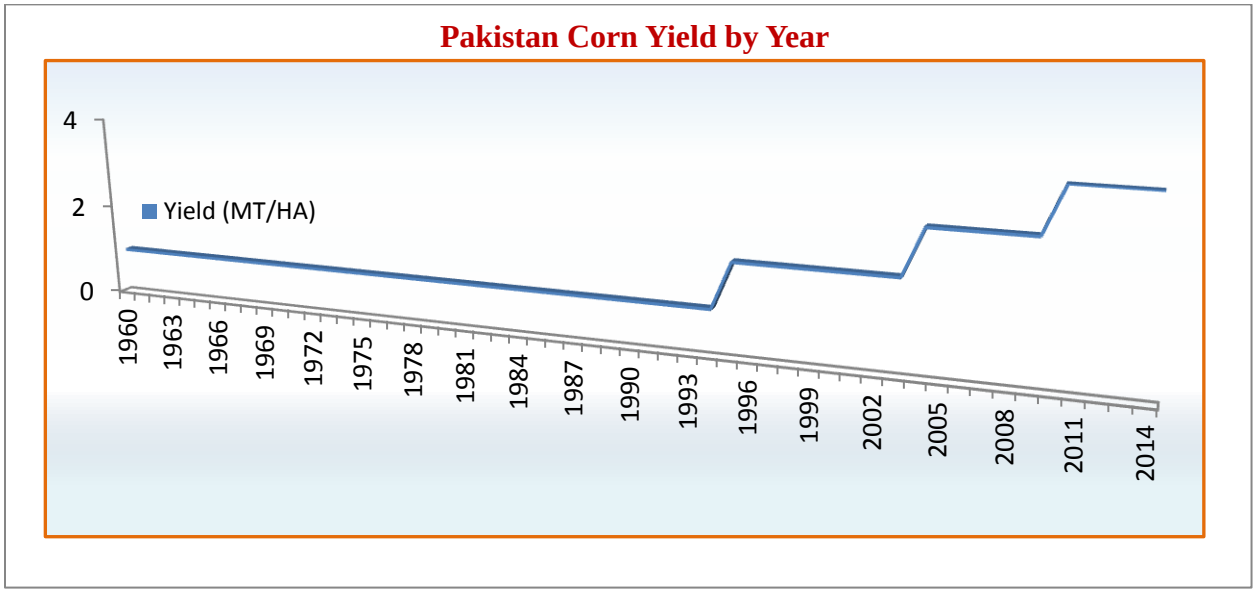

Figure 1.5: Pakistan maize yield by year shows that maize yield remained the same since 1960 till 1994; however a twofold increase in the yield was observed in the year 1995, then another increase of one fold was observed in the year 2004 and in 2010 again an increase in yield was observed.

Pakistan Maize Yield Annual Growth Rate of Pakistan

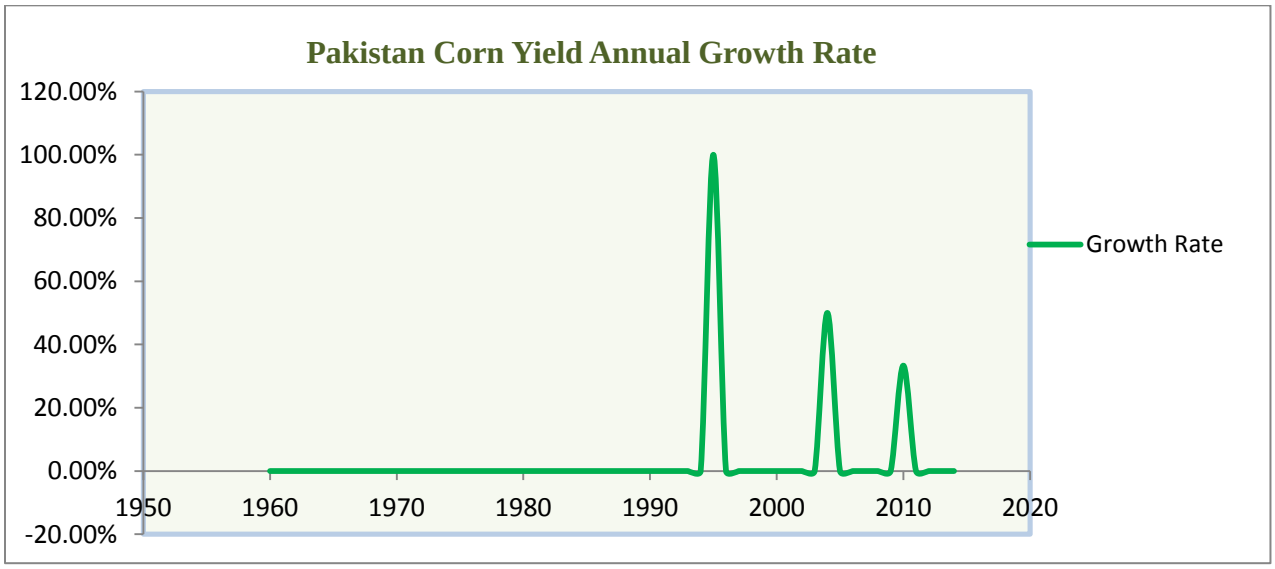

Figure 1.6: The figure shows a cent percent increase in maize yield in the year 1995, then another 50% increase in yield was observed and a further 33.33 % increase in yield was observed in 2010. Thus this graph shows that during the course of time yield has increased in Pakistan.

Pakistan Maize Domestic Consumption by Year from 1960 to 2015

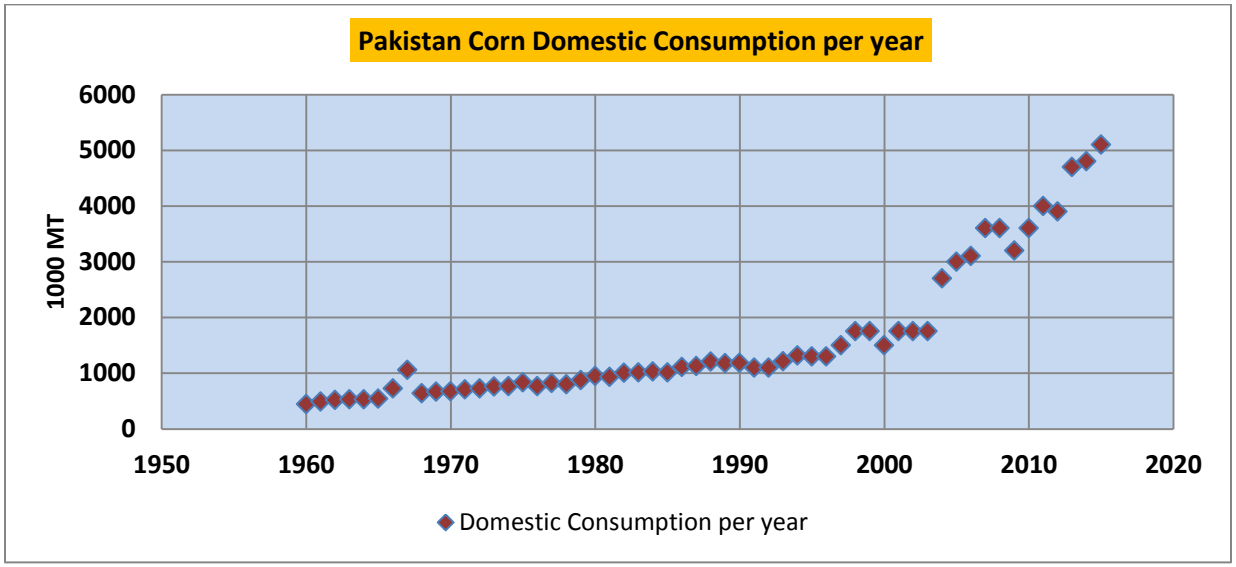

Figure 1.7: The above graph shows the domestic consumption of maize in the year since 1960 till 2015. Domestic consumption includes all possible uses of a commodity: food, feed, seed, waste and industrial processing. However the trend is not constant, rather variations are observed during the course of years.

Pakistan Maize Domestic Consumption Annual Growth Rate

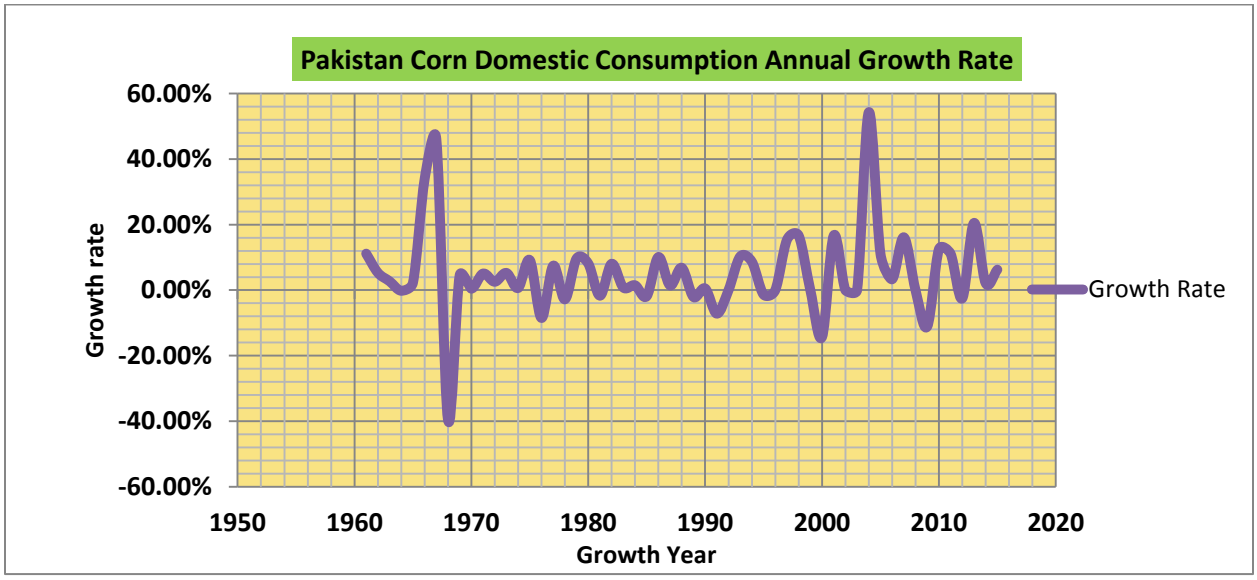

Figure 1.8: The figure shows Pakistan corn domestic consumption annual growth rate since 1960 till 2016. The highest domestic consumption was recorded 46.06% in 1967 and 54.29% in 2004.

1.9 Cultivation of Hybrid Maize in Pakistan

Due to the introduction of hybrid seeds in Pakistan, the maize yield is showing a continuous momentum. Maize hybrids have been, successful because of their two ingrained properties of being superior in terms of management as well as inputs (Tariq and Iqbal, 2010).

The graph precisely depicts the overall area share among both hybrid maize and their synthetic/ local counterparts (Tariq and Iqbal, 2010).

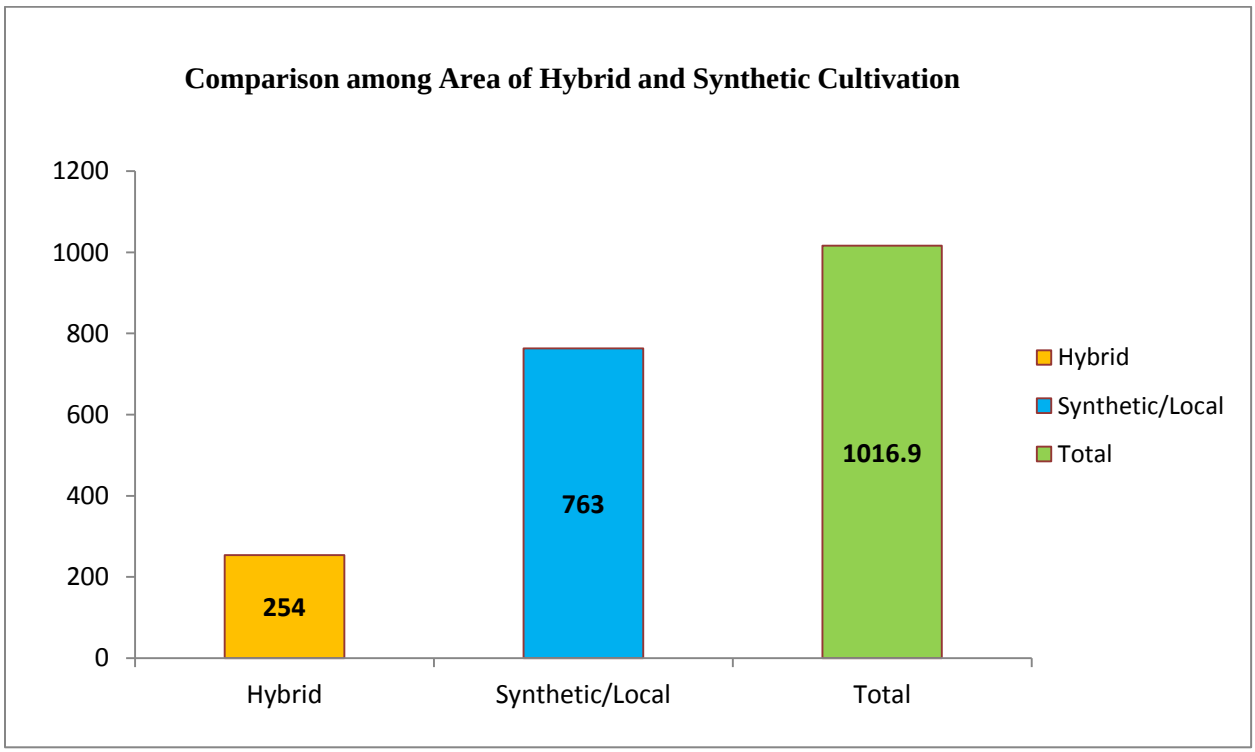

Figure 1.9: This graph shows that though less than synthetic/local counterparts, hybrid maize is still making a good contribution as per the area under cultivation in Pakistan.

The graph depicts the overall percentage share of both hybrid maize and their synthetic /local counterparts (Tariq and Iqbal, 2010).

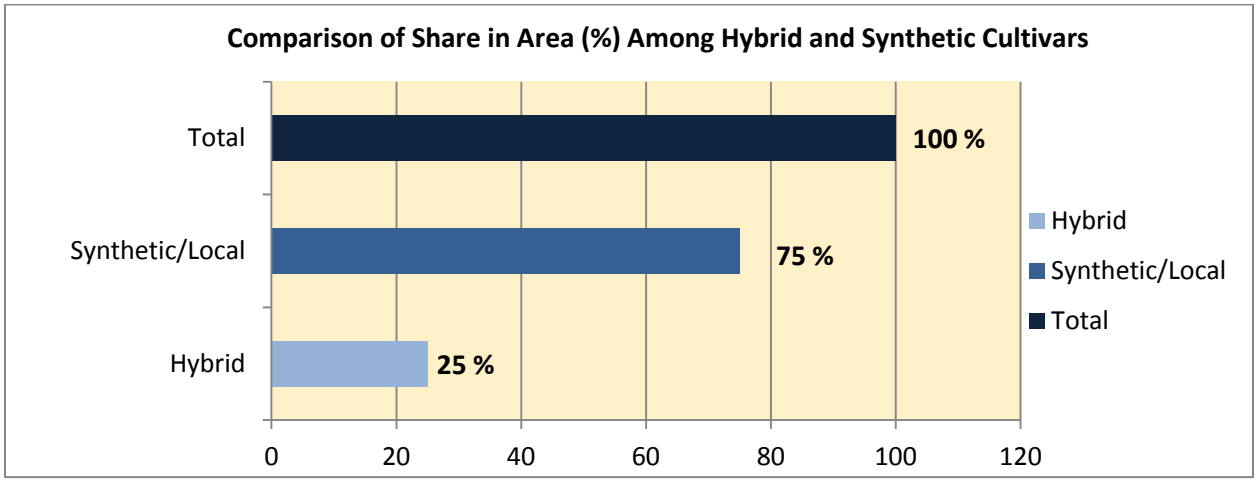

Figure 1.10: The graph shows that one quarter of the area under maize cultivation has been occupied by hybrid maize in Pakistan.

The pie chart provides with the information on production value of both hybrid maize and its synthetic/ local counterparts (Tariq and Iqbal, 2010).

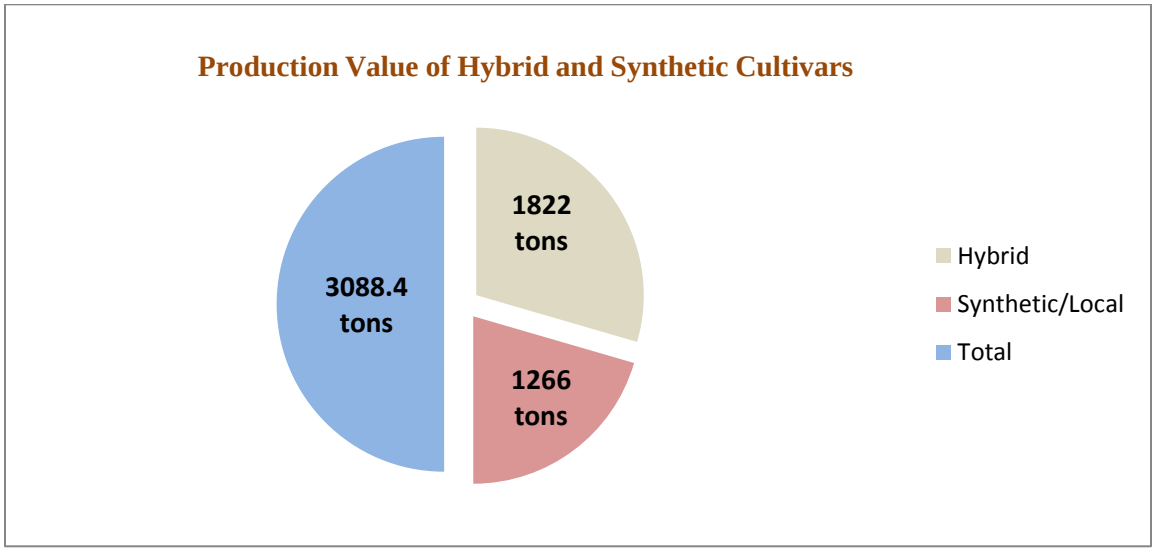

Figure 1.11: As per chart a good production value can be attributed to hybrid maize in Pakistan.

The pie chart provides with the production percentage of both hybrid maize and its synthetic/ local counterparts (Tariq and Iqbal, 2010).

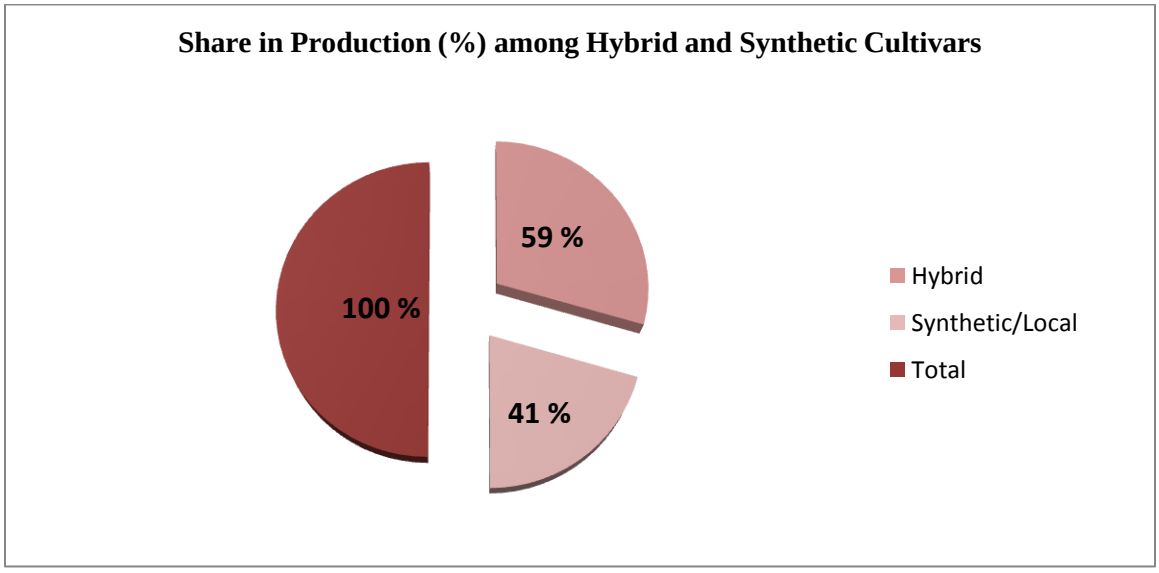

Figure 1.12: The chart shows the production percentage of hybrid maize to be 41%, and synthetic/ local maize to be 59% of the total production percentage of maize in Pakistan. Hybrid maize thus is a biggest competitor of synthetic/ local cultivars.

1.10 Role of Exogenous Hydrogen Peroxide during Abiotic Stresses

Various types of environmental factors serve as a stress to plants; some of the most common environmental conditions which serve as a stress to maize include shortage of water, saline soils, excessive chilling and heat. To counter these stresses various approaches have had been devised by the scientists, one of them being the exogenous application of Hydrogen Peroxide (H_2O_2).

In maize, when the plant gets exposed to chilling stress, H_2O_2 generation within the plant turns out to be plethoric, so when the maize plants were pre-treated with H_2O_2, it was noticed that this pre- treatment actually conferred chilling stress tolerance to it (Prasad et al., 1994).

Gondim et al. (2010) reported that pre- treatment with H_2O_2 of maize seeds influences germination and acclimation of seedlings, thus conferring the plants with salinity tolerance. Paulin et al. (2012) reported that exogenous application of H_2O_2 resulted in the amelioration of some phenological and biochemical features of maize. Guzel and Terzi (2013) reported that H_2O_2 pre- treatment plays role in alleviating the toxic effects of Cu stress and Terzi et al. (2014) also reported that pre- treatment of H_2O_2 confers maize plant with osmotic stress tolerance by regulating osomolyte and abscisic acid levels within the leaves.

1.11 Evaluating the effects Nanotechnology in Agriculture

Nanotechnology is considered as one of the emerging new technologies of the world. The basis of this field is nano sized particles popularly known as nano particles or nano materials (Stern and Mcneil, 2008).

Currently due to the perceived advantages of nanotechnology, its use is constantly increasing; however it is the need of the time to look the negative impact of it as well.

Chapter 2

REVIEW OF LITERATURE

Plants are faced by a number of different environmental stresses – biotic and abiotic in nature that hinder their growth and development processes and cause yield losses.In order to overcome these stresses, plants have developed different mechanisms that enable them to exhibit tolerance and adaptability to such foreign factors. Abiotic stresses include environmental factors such as drought (Farooq et al., 2009), salinity (Farooq et al., 2015), low or high temperature (Crafts – Brandner and Salvucci, 2002) and others.

2.1 Nanotechnology

Nanotechnology is one of the upcoming technologies in the world, that employs nano sized particles or materials in the various different arenas of sciences (Stern and Mcneil, 2008).

Nanoparticles are defined as the materials that are less than 100nm in at least one of the dimensions thet possess with unique physical and chemical properties (Oberdörster et al., 2005; Aitken et al., 2006; Buzea et al., 2007; Nowack and Bucheli, 2007; Handy et al., 2008; Stern and Mcneil, 2008; Walker and Bucher, 2009; Farré et al., 2011).

Nanoparticles are not created by human beings rather they are being produced by nature for centuries. For instance they have been reported to be present in ice cores around ten thousand years (Murr et al., 2004).Naturally, they are being produced by various geological and volcanic activities. In addition, various different biological activities can also result in the production and release of these nano- scaled materials into the environment (Handy et al., 2008).

These materials have attained recognition in the technological world round the globe because of their promising attributes revealed in variety of practical applications, most common of them include electronics, which is characteristically based on energy vitally possessed by electrons, pharmaceutical sciences and the products pertaining to them, cosmetic industry particularly in sun creams, environmental sciences and their associated applications, biomedical sciences and their related practical applications, energy and its related aspects, catalytic properties and its applications, science of materials and their immense utility, (Nowack and Bucheli, 2007) agricultural applications in fertilizers as well as plant protection products (Gogos et al., 2012; Khot et al., 2012; Rai et al., 2012).

2.2 Nanotoxicity

Nanotoxicity is one of the growing concerns among the scientists and regulatory authorities. Currently, in addition to their professed benefits, they have revealed their negative implications as well, it's been reported that these particles raise grave health and environmental concerns. These nano- sized particles have a negative potential because of their greater surface to volume ratio, which endows them with both oxidative and catalytic properties (Mandal, 2012).

Moreover, they are able to penetrate the cell membrane and interact with the biological systems. Nanoparticles that are being used in skin care products can result in serious repercussions thus extensive studies are a pre- requisite to come up with solution (Mandal, 2012).

2.3 Titanium dioxide (TiO_2) nanoparticles induced nanotoxicity

TiO_2 nanoparticles are widely used in consumer products, 10,000 t/a of these were reported to be produced in 2012 in Europe. Even so the production of bulk TiO_2 was even more marking an upper limit of 15, 00,000 t/a as it's been employed in various different industries such as food, paints and cosmetics (Römpp, 2013; Sun et al., 2014).

However, the thing which further worsens the situation are the masked nano- sized TiO_2 being present within the various different pigments, which when released can also exacerbate the existing predicament (Weir et al., 2012).

TiO_2 nanoparticles used in fertilizers, pesticides and fungicides is popular these days, because it is required in lesser amounts than the conventional chemicals, it is less prone to degradation than its bulk counterparts and it also ingrains the fungicides with better attributes to deal with the fungus (Nair et al., 2010).

Additionally, the photo catalytic property of TiO_2 nanoparticles, the pesticides and fungicides are endowed with enhanced potential to deal with the perpetrator. The positive effects were reported for both rice and maize crop plants (Lu et al., 2006).
However, the point to be noted is that nanoparticles like TiO_2 nanoparticles can even penetrate via pores present in the leaves and can affect the growth and development of plants when applied directly through foliar spray. This can even be toxic and can result in Nanotoxicity if it crosses the safety limits (Dhoke et al., 2013).

The toxicity of nanoparticles also depends on variety of dynamics for instance plant species, size and concentration of nanoparticles and also upon the particular developmental stage of the plant that comes across the nanoparticles thrust. The size of the nanoparticles determines their nanotoxicity potential, in plants which can be observed in terms of plants photosynthetic or respiratory ability (Navarro et al., 2008).

One of the studies conducted on algae and daphnids for evaluating the nanotoxicity of TiO_2 nanoparticles revealed that the nanotoxic potential of nano – TiO_2 was by and large dependent on the size, concentration and the organism being exposed to the toxic dose. Both algae and daphnids were exposed to the same toxicity dose but even so it was more palpable in algae (Hund- Rinke and Simon, 2006).

TiO_2 NPs in water was also reported to be toxic for aquatic life. Nano – TiO2 reduced the light capturing potential of algal cell and thus have been found to have caused the reduced algal growth (Sharma, 2009). TiO_2 nanotoxic potential affected Marine Phytoplankton in the presence of light (Miller et al., 2012). TiO_2 NPsnegatively affected the growth of *Lemna paucicostata,* at 500 –ppm. TiO_2 NPs were also reported to reduce biomass of wheat observed to be deleterious for the plant (Du W. et al., 2011). Nano -TiO_2 was reported to have been the cause of DNA damage and lipid per oxidation in onion (Gosh et al., 2010). In *Arabidopsis Thaliana* when TiO_2 NPs were applied to a three week old plant for seven days nanotoxic affects were reported (Garcia et al., 2015).

2.4 Nanotoxicity studies in Plants

Genotoxic affects of AgNPs were reported in *Allium cepa* (Kumari et al., 2009). Genotoxic affects of TiO_2 NPs was reported in *Allium cepa* and *Nicotiana tabacum* (Ghosh et al., 2010). TiO_2 NPs and ZnO NPs are reported to be negatively affecting the growth of wheat plant (Du et al., 2011). Effects of Ag NPs at different concentrations on common beans and corn were also reported (Salama, 2012). TiO_2 NPs are reported to negatively affect the symbiotic relationship between Rhizobium bacteria and legumes, causing a delay in the development of root nodules and thus affecting the process of nitrogen fixation (Fan et al., 2014). Pronounced effects of TiO2 NPs at higher conc. was reported to affect the germination rate, root length, shoot length and chlorophyll content in *Mentha Piperita* (Samadi et al.,2014).

2.5 TiO_2 nanoparticles (TiO_2 NPs) induced nanotoxicity in Maize Plants

It was reported that TiO_2 NPs/ inorganic betonite clay at concentrations of around 300 to 1000mg/L in maize plants resulted in hydraulic conductivity inhibition, and negatively influenced the growth and transpiration (Asli and Neumann, 2009) and Kumar (2014) reported that TiO_2 NPs affect various different growth parameters of maize plants when applied at concentrations 500mg/L, 1000mg/L and 2000mg/L.

However effects were much less with 500mg/L and were more pronounced at 2000mg/L. Nanotoxicity studies in maize and other plants is demonstrated in the table 2.1:

Table 2.1:Nanotoxicity in different plants

Nanoparticles	Plant	Affects	Reference
Ag NPs	*Allium cepa*	Genotoxicity	Kumari et al., 2009
TiO_2 NPs	*Allium cepa* *Nicotiana tabacum*	Genotoxicity	Ghosh et al., 2010
TiO_2 NPs	Wheat	Growth (negative)	Du et al., 2011
ZnO NPs	Wheat	Growth (negative)	Du et al., 2011
TiO_2 NPs		Root nodule development (negative)	Fan et al., 2014
TiO_2 NPs	*Mentha Piperita*	Reduction in germination rate, shoot length, root length & chlorophyll content.	Samadi et al.,2014
ZnO NPs	*Oryza sativa*	Reduction in root growth	Boonyanitipong et al.,2011
Pb NPs	*Kiwi*	Toxic effects on kiwi fruit pollen.	Speranza et al.,2010

Cu NPs	*Phaseolus radiates* *Triticum aestivum*	Growth inhibition	Lee et al.,2008
TiO_2 NPs	Maize	Hydraulic conductivity inhibition, negatively affect growth and transpiration	Asli and Neumann, 2009
TiO_2 NPs	Mize	Reduction in germination rate, shoot length, root length and SVI-I	Kumar et al.,2014
Al_2O_3	Maize	Reduction in germination rate, shoot length, root length and SVI-I	Kumar et al., 2014
Ag NPs	*Phaseolus vulgaris* *Zea mays*	Growth inhibition	Salama, 2012

2.6 Role of Hydrogen Peroxide (H_2O_2) in Abiotic Stress Tolerance in Plants

H_2O_2 is a non radical form of Reactive Oxygen Species (ROS). H_2O_2 is a molecule that has paradoxical roles in life of plants. H_2O_2 on one hand serve as a highly reactive toxicant for plants and when produced during normal metabolic processes is always taken up by the plants as a deleterious by- product that can result in a damage to the plant cell, but on the other hand if produced during abiotic stress by the plant can work

as a signalling molecule that can elicit various stress related genes allowing the plant to cope up with the stress (Foyer and Noctor, 2009).

ROS production rises when plants are under stress in which antioxidant and scavenging enzymes are charged up to protect the plant from the oxidative damage, however if it exceeds the limit then the maximum damage to the plant is oxidative in nature (Kant et al., 2006; Türkan and Demiral, 2009; Geissler et al., 2010).

However, under stress conditions the production of ROS is also responsible for the activation of various stress responsive genes, these genes either encode for enzymes which are responsible for the biosynthesis of various antioxidants or are directly involved in the detoxification of reactive oxidative radicals. For example it has been found that under stress; production of H_2O_2 inside the cell becomes augmented, which in turn enhances the expression of genes of active oxygen scavenging (AOS) enzymes (Zhao et al., 2004).

H_2O_2 plays a very significant role as a signalling molecule and a very vital role in conferring tolerance against abiotic stresses in plants.

It is a known fact that under normal circumstances ROS is a by- product of metabolic activities happening in plant cell, however under stress condition, the production of radicals becomes elevated into the cytosol, that ultimately results in oxidative stress, and radicals are instantaneously transformed into oxygen and H_2O_2. As evident inordinate amounts of H_2O_2 gets accumulated inside the plant cell. H_2O_2 plays role in providing plant with acclimation and cross tolerance abilities, as a consequence the earlier exposure to one type of stress enables plant to cope up with the impending stress either of the same or different kind (Bowler and Fluhr, 2000: Bhattacharjee, 2005).

H_2O_2 survives for considerable period after it has been produced; it has the potential to pass through the biological membranes, diffuse from one cell to another and it can travel to long distances from where it is produced in the plant cell. These properties of it make it an ideal intracellular signalling molecule, and because of these inherent attributes it has gained immense importance in the recent times (Alvarez et al., 1998).

The organelle, where H_2O_2 is produced, also is an indicator that either it is produced under normal circumstances or stress. For example if the point of origin of is chloroplasts, mitochondria or peroxisomes it's a cue that it is being produced under normal circumstances, however if the point of origin are cytosolic membrane bound NADPH oxidases then it's a cue that it is either generated as a consequence of biotic or abiotic stress and if the point of origin are Electron Transport Chain then it's a cue that it is being generated in response to some abiotic stress (Huchzermeyer and Koyro, 2005).

The dual role played by H_2O_2, both as a toxicant and as redeemer in maize plants was delineated during different types of abiotic stresses. The most auspicious delineation of it was during chilling stress in which the pre- treatment with H_2O_2 resulted in the activation of acclimation mechanisms, because of it being recognised as a stress, however due to the cross- tolerance mechanisms it worked out as an elicitor of innate immune system, thus conferring tolerance against chilling stress. Earlier it was reported that the concentration of H_2O_2 becomes elevated when the maize plant is being exposed to chilling stress, however, in non acclimatized maize seedlings, its towering accumulation made it a toxicant (Prasad et al., 1994). Role of H_2O_2 pre-treatment in conferring tolerance against various different abiotic stresses is demonstrated in table 2.2:

Table 2.2:Role of exogenous H_2O_2 in abiotic stress tolerance in different plants

Abiotic stress	Plant	Reference
Cold	Maize Manila grass Mung bean	Prasad et al., 1994 Yu et al., 2002 Wang et al., 2010
Heat Shock	Nodal potato Rice	Lopez-Delgado et al., 1998 Uchida et al., 2002
Salinity	Rice	Uchida et al.,2002
Drought (Osmotic Stress)	Cucumber	Liu et al., 2010
Cadmium	Rice	Hu et al., 2009
Aluminum	Wheat	Xuet al., 2010
Copper	Maize	Guzel and Terzi, 2013
Chromium	Canola	Yildizet al., 2013

Thus it is obvious that H_2O_2 serves as a signalling molecule that indispensably affects the various physiological, biochemical and molecular patterns within the plant cell; It plays role in inducing abiotic stress tolerance in plants, when plants are pre- treated

with H_2O_2, however its toxicity can never be overruled, and therefore application should be paid before applying it to the plants.

2.7 Hybrid vigour

Hybrid vigour, is the tendency of resultant plant to exhibit qualities superior to either of both the parents. It can be in terms of increase in the size or the growth rate of the progeny etc.over its parents. It is also known as heterosis. This has been employed on large scale for production of hybrids of plants and animals for over 75years. Hybrids of crop plants such as maize, sorghum and sunflower are widely grown in the developed countries of the world. Hybrids are also becoming popular in the developing parts of the world because of because of the extraordinary attributes they are endowed with. Similar to China, India has also started to cultivate hybrid rice over wide range of lands (Virmani, 1994). The first and foremost advantage of hybrid seeds perceived by the world was its ability to produce more crops on a lesser area.

Later on hybrid maize were developed against drought stress by CYMMT. Similar attempts were made against other biotic and abiotic stress.

Chapter 3

MATERIALS & METHODS

3.1 Plant Material

The plant material used in the study, comprised of a hybrid (IL-107* IL-168) with its parents 1L-107 and IL-168 respectively. The seeds were collected from Maize, Sorghum, Millet & Fodder Program, National Agriculture Research Centre (NARC) Islamabad, Pakistan.

3.2 Sowing

Initially the pots were filled with composted soil, and were separated and labelled for three individual groups of seeds. Pots were filled with soil and were mildly sprayed with water a day before the sowing of seeds. Then with the help of wooden skewer three holes of approximately 1 inch in each pot was made. The already imbibed and dried seeds were placed and the holes were covered with soil. These pots were tap watered with 8ml of water to each pot.

3.3.1 Growth Room Conditions

Maize was grown in a controlled environment in a growth room. The plants were kept at 28ºC with ± 1ºC and a photoperiod of 16hrs day and 8hrs dark.

3.3.3 Plant Height Measurement

Plant heights were recorded, by holding the leaf in a straight upright position and measuring the height from the bottom of the stalk to the edge of the longest leaf by scale. The height was recorded on the alternate days.

3.3.4 Hydrogen Peroxide (H_2O_2)Treatment

For three consecutive days, 10 days post germination 1 mm H_2O_2 treatment was given to groups 2A, 2B, 2C, 4A, 4B and 4C respectively. Rest of the groups were only watered with tap water. Here it should be noted that groups 2A, 2B and 2C served as H_2O_2 stressed groups and groups 4A, 4B and 4C served as H_2O_2 pre- treatment groups.

3.3.5 TiO2 Nanoparticles Treatment

Following these three days of H_2O_2 treatment, Plants were treated with 1000mg/L TiO_2 nanoparticles solution, for seven consecutive days. This treatment was given to groups 3A, 3B, 3C, 4A, 4B and 4C respectively. Rest of the groups were only watered with tap water. Here it should be noted that groups 3A, 3B and 3C served as TiO_2 NPs stressed groups and 4A, 4B and 4C served as the ones which were pre- treated with H_2O_2. The details of different groups of treatments are illustrated in table 3.1:

Table 3.1: Group Labels and Description

Group Names	Plant Accession No.	Labels	Description
1A	IL-107	Parent A	Water
1B	IL-168	Parent B	Water
1C	IL-107×IL-168	Hybrid	Water
2A	IL-107	Parent A	H_2O_2
2B	IL-168	Parent B	H_2O_2
2C	IL-107×IL-168	Hybrid	H_2O_2
3A	IL-107	Parent A	TiO_2 NPs
3B	IL-168	Parent B	TiO_2 NPs
3C	IL-107×IL-168	Hybrid	TiO_2 NPs
4A	IL-107	Parent A	H_2O_2 + TiO_2 NPs
4B	IL-168	Parent B	H_2O_2 + TiO_2 NPs
4C	IL-107×IL-168	Hybrid	H_2O_2 + TiO_2 NPs

3.3.6 Harvesting

Following the treatment, the leaves of the plants were harvested, and were immediately put in liquid nitrogen container and were then stored at -150°C.

3.3.7 XRD Analysis of Nanoparticles Treated Samples

XRD Analysis of Nanoparticles Treated Samples was performed to determine the accumulation of nanoparticles in nanoparticles treated samples. This technique was used to detect the TiO_2 nanoparticles in plant tissues by examining diffraction peak of sample (Odum, 2007). Treated samples were dried at 70ºC for 72hours. These were then ground to fine powder and were contained in 2ml microfuge tube before XRD analysis.

3.4 Germination Assay

3.4.1 Germination Rate

Germination Rate of the seeds for hybrid and its parents was calculated using the following formula:

$$\textbf{Germination Percentage} = \frac{\textbf{No.ofseedsgerminated}}{\textbf{Totalno.ofseeds}} \times \mathbf{100}$$

3.5 Physiological Parameters

3.5.1Shoot Length

Average shoot length was recorded for the five seedlings for all groups were recorded with the scale on the final day of treatment.

3.5.2 Root Length

Average root length was recorded for the five seedlings for all groups were recorded with the scale on the final day of treatment.

3.5.3 Water Content

Following the harvest, three of random seedlings of all groups were picked up and weighed and then these were oven dried at 70º C for 72 hours. Water content was then calculated by using the following formula:

$$\textbf{Water Content} = \frac{\textbf{Wet Weight} - \textbf{Dry Weight}}{\textbf{Dry Weight}}$$

3.5.4 Vigour Index

Seedling Vigour Index I and II of all groups was calculated using the following formula (Kumar et al., 2012)

$$\textbf{SVI} = \frac{\textbf{Germination Percentage} \times \textbf{mean seedling length}}{\textbf{100}}$$

$$\textbf{SVII} = \frac{\textbf{Germination Percentage} \times \textbf{mean seedling dry mass}}{\textbf{100}}$$

3.6 Biochemical Assays

3.6.1 Chlorophyll Content

The chlorophyll content of the maize leaves was estimated by using three different protocols for chlorophyll extraction:

- **Acetone Extraction Method**

Chlorophyll estimation was conducted using 80% acetone by following the method of Witham et al., (1971) and Bansal et al., (1999). 0.15g of a leaf from all the four groups of three individual groups was weighed, then each one was placed in a sequence in a mortar and then almost 15 ml of 80%acetone was added to it. Then the leaf fragment was very finely ground using pestle. Then each one was successively placed in a 25 ml centrifuge tube respectively. When all the twelve centrifuge tubes were ready, they were then centrifuged at 1000 rpm for 30 minutes at 4ºC. Supernatant was then decanted off carefully in another 25 ml centrifuge tube, and finally the volume was made up to 25 ml. During the entire course of experiment tubes were kept in dark and

acetone used was chilled. The optical density was then measured at 663nm, 645nm, 510nm and 480nm respectively. The chlorophyll content was estimated using the following formula:

$$\textbf{Chlorophyll a} = \frac{(12.7\times A663)-(2.69\times A645)\times V}{1000\times \text{Fresh Shoot Weight}}$$

$$\textbf{Chlorophyll b} = \frac{(22.9\times A645)-(4.69\times A663)\times V}{1000\times \text{Fresh Shoot Weight}}$$

$$\textbf{Total Chlorophyll} = \frac{(22.2\times A645)+(8.02\times A645)\times V}{1000\times \text{Fresh Shoot Weight}}$$

$$\textbf{Total Carotenoid} = \frac{7.6(A480)-1.49(A510)\times V}{1000\times \text{Fresh Shoot Weight}}$$

- **Ethanol Extraction Method**

Chlorophyll estimation was conducted by following the method of Aron (1949). 12 test tubes containing 5ml of 80 % ethanol each were added with 0.15g of a leaf fragment from each group. These test tubes were then tightly screwed and placed in water bath at 80ºC for 10 minutes. Then these test tubes were removed from the water bath and the extract from each test tube was carefully decanted off in another test tube and was then cooled in dark room. Then the optical density was measured at 663nm and 645nm respectively. The chlorophyll content was estimated using the following formula:

$$\textbf{Chlorophyll a} = \frac{(12.7\times A663)-(2.69\times A645)\times V}{1000\times \text{Fresh Shoot Weight}}$$

$$\textbf{Chlorophyll b} = \frac{(22.9\times A645)-(4.69\times A663)\times V}{1000\times \text{Fresh Shoot Weight}}$$

$$\textbf{Total Chlorophyll} = \frac{(22.2\times A645)+(8.02\times A645)\times V}{1000\times \text{Fresh Shoot Weight}}$$

- **DMSO Extraction Method**

Chlorophyll estimation was conducted following the method Hiscox and Tsraelstam (1979). 12 test tubes each containing 7ml of DMSO were added up with 0.15g of a

leaf fragment from each group. These tubes were then screwed tightly and were placed in a water bath at 65ºC for 30 minutes. Subsequently the supernatant was decanted off carefully in a graduated tube and the volume was made up to 10ml with DMSO. Then the optical density was measured at 663nm and 645nm respectively. The chlorophyll content was estimated using the following formula:

Chlorophyll a = 0.0127 × A663 – 0.00269 × A645

Chlorophyll b = 0.0229 × A645 – 0.00468 × A663

Total Chlorophyll = 0.0202× A645 +0.00802 × A663

3.6.2 Total Soluble Sugar Content

Total soluble sugar was estimated by the using the method of Dubois (1951). 12 Test tubes each containing 0.15g of the leaf fragment were each filled up with 5ml of 80% ethanol and were and were tightly screwed up. Then these tubes were placed in a water bath at 80ºC for 60 minutes. Then 1ml of extract from each test tube was placed in another test tube and then 1ml each of 18% phenol and distilled water was added to each tube separately. Then these test tubes were kept at room temperature for 60 minutes and then to each of the test tube 5ml of conc. H_2SO_4 was added. Finally each of the test tubes was vortexed. Then the absorbance was read at 490nm wavelength.

3.6.3 Total Soluble Protein Content

The total soluble protein was estimated using Bradford Assay (Yadegari et al.,2008). Standard curve was made using BSA. 0.15g of leaf fragment was homogenised in 5ml phosphate buffer solution. Then each of the samples was placed in a 25ml centrifuge tube separately and was centrifuged at 1000 rpm for 35 minutes at 4ºC. Then 0.5ml of the sample extract (supernatant) was placed in another set of test tube, to which 0.5ml of distilled water and 3ml of bio- red dye was added to each test tube separately. Finally the test tubes were vortexed and the absorbance was read at 595nm wavelength.

3.6.4 Anti oxidant Enzyme Activity Assays

- **Enzyme Extraction**

0.5 g of leaf tissue was weighed for each sample and was finely crushed in liquid nitrogen using mortar and pestle. Each of these macerated samples was separately suspended in 1.5ml of phosphate buffer and was then centrifuged at 1000rpm for 35 minutes at 4ºC. Then the supernatant was cautiously decanted off in another 2ml microfuge tube separately.

3.6.4.1 Ascorbate Peroxidase (APX) Enzyme Activity

To 100ul of each enzyme extract, 600ul of 50mM phosphate buffer, 100ul of 1mM EDTA, 100ul of 5mM ascorbate and finally 100ul of 1mM H_2O_2 was added to the mixture to initiate the reaction. Reduction in ascorbate concentration was recorded at 290nm wavelength continuously for 3 minutes.

3.6.4.2 Catalase (CAT) Enzyme Activity

Catalase assay was performed by following Aebi (1983).

To 666ul of each enzyme extract, 334ul of 73mM H_2O_2 was added. Absorbance was read at 240nm, for 3 minutes.

Chapter 4

RESULTS

Growth and Maintenance of Plant Lines

Plants were grown under controlled conditions, in growth room at ± 28°C, for 14 days post germination, 16 hrs of day light and 8hrs of dark, as illustrated in table 4.1:

Table 4.1: Conditions and Settings in Growth Room

Conditions	**Settings**
Temperature	± 28°C
Day Light	16hrs
Dark Light	8hrs

Characterisation of Nanoparticles

Characterization of TiO2 NPs was performed using the techniques: X-ray Diffraction (XRD), from School of mechanical Engineering (SCME, NUST).

Characterisation of TiO2 Nanoparticles

XRD patterns of TiO_2 NPs 4.1, the strong diffraction peak was observed at 25°.

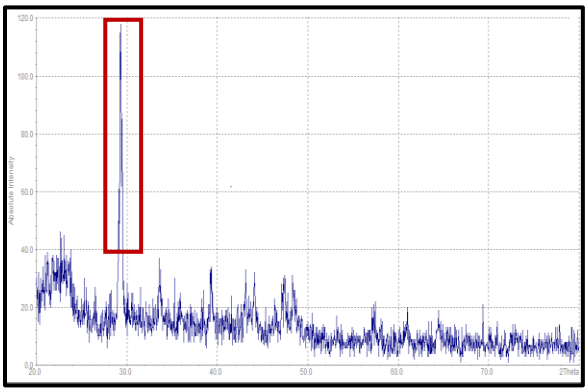

Figure 4.1 TiO_2 Nanoparticles. The peak intensity is represented at 120 (a.u), while the diffraction peak is at 25°.

X – Ray Diffraction (XRD) of TiO_2 Nanoparticles

The peak intensity represented at 120 (a.u) shows that the particle is of small size, while the diffraction pattern peak at 25° shows that nanoparticle is in anatase phase.

The 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 TiO2 on plant cells (Thamaphat et al., 2008).

Plant stage Selection and Treatment

Effects of TiO2 NPs were studied on physiological and biochemical activities of maize seedlings. 10 days old seedlings were stressed with H_2O_2 and TiO_2 NPs and also were given the two in combination to study the effects of pre- treatment of H_2O_2 on TiO_2 nanotoxicity reduction.

4.1 Determination of Nanoparticles Accumulation in Plant Seedlings

X-Ray Diffraction

To determine the accumulation of TiO2 nanoparticles in tomato and wheat seedlings, X-ray Diffraction (XRD) was performed. This technique was used to detect TiO2

nanoparticles in plant tissues by examining diffraction peak of sample (Marquel Odum., 2007).

TiO_2 NPs accumulation in Maize Hybrid and its Parents

Maize seedlings treated with 1000mg/L TiO_2 NPs showed the accumulation of nanoparticles in maize hybrid and its parents. The accumulation of these nanoparticles confirms their presence in TiO_2 NPs treated plants samples.

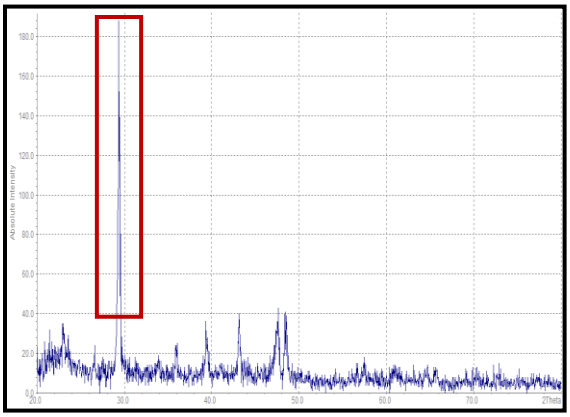

Figure: 4.2 (a) IL-107 – Parent A

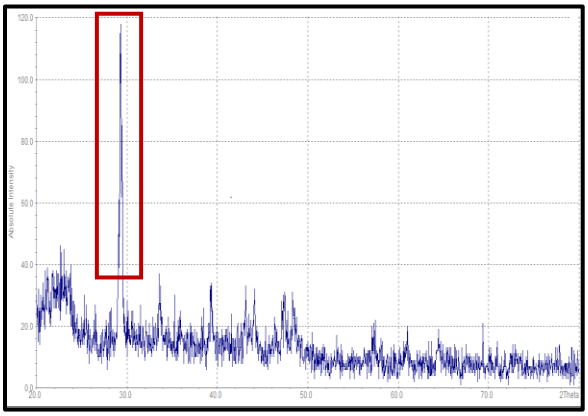

Figure: 4.2 (b) IL-168- Parent B

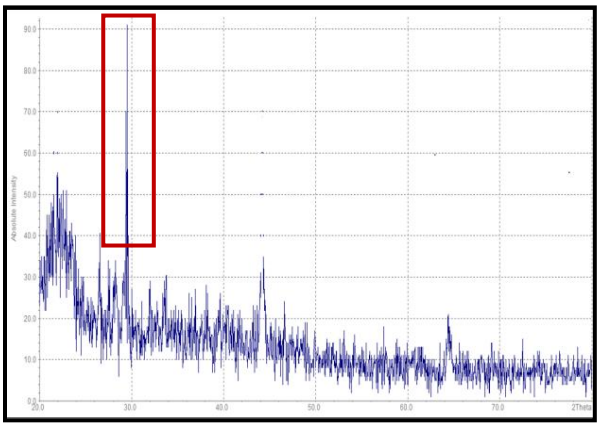

Figure: 4.2 (c) IL-107*IL-168- Hybrid

Figure 4.2 (a, b & c): XRD patterns of TiO_2 are shown in figure 4.1 (a) and (b) and (c) in parents and its hybrid. The strong diffraction peak was observed at 25°. This confirms the presence of TIO_2NPs in parents and its hybrid.

The germination assay primarily deal with hybrid vigour, and the physiological and biochemical parameters deal with H_2O_2 stress, TiO_2 nanoparticles stress and their effects in combination. The results are presented in terms of mean and ± SD calculated using SPSS, 23.

4.2 Germination Assays

Germination Rate

Germination rate provides an estimated germination potential or viability of seeds in a seed lot. This helps us in determining that which of the seeds should be opted for cultivation in the field (Saupe, 2009). The results of our study show that 76.66% of hybrid seeds, 70% of IL-107 seeds and 53.33% of IL-168 seeds germinated. The performance of hybrid was better than either of the parents.

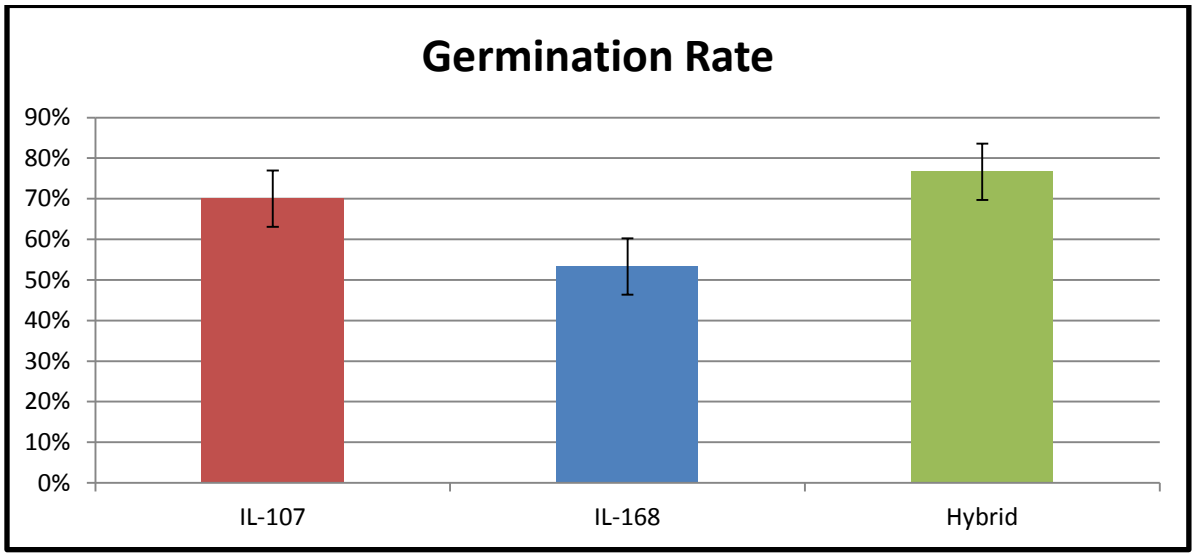

Figure 4.3 Germination Rate of maize seedlings

The germination rate was recorded for a period of 10 days for the hybrid and its parents, in controlled environment, as per the details mentioned in table 2 Hybrid performed the best in this test..

4.3 Physiological Parameters

4.3.1 Effect of H_2O_2 pre- treatment on TiO_2 NPs on Post germinated maize Shoot Length

To study the effect of H_2O_2 pre- treatment in TiO_2 nanotoxicity reduction, shoot length was measured using a scale (cm). Shoot Length was decreased by 3.03% and 16.5% in groups 2A and 3A respectively as compared with control group 1A.However in group 4A, the increase of 8% as compared with group 3A was observed. Similarly in IL-168, decrease in shoot length was observed by 3.13% and 20.36% in group 2B and 3B was observed respectively. However in group 4B, an increase of 16.3% compared with group 3B was observed. Similarly in hybrid shoot length was found to reduce by 5.66% and 20.28% in group 2C and 3C respectively. However an increase of 38.9% in group 4C compared with group 3C was observed. Thus our results show that exogenous application of 1mM H_2O_2 plays a positive role in TiO_2 nanotoxicity

reduction in maize plants. The effect of H_2O_2 pre- treatment on TiO_2 NPs on post germinated maize shoot length is demonstrated in the figure 4.4:

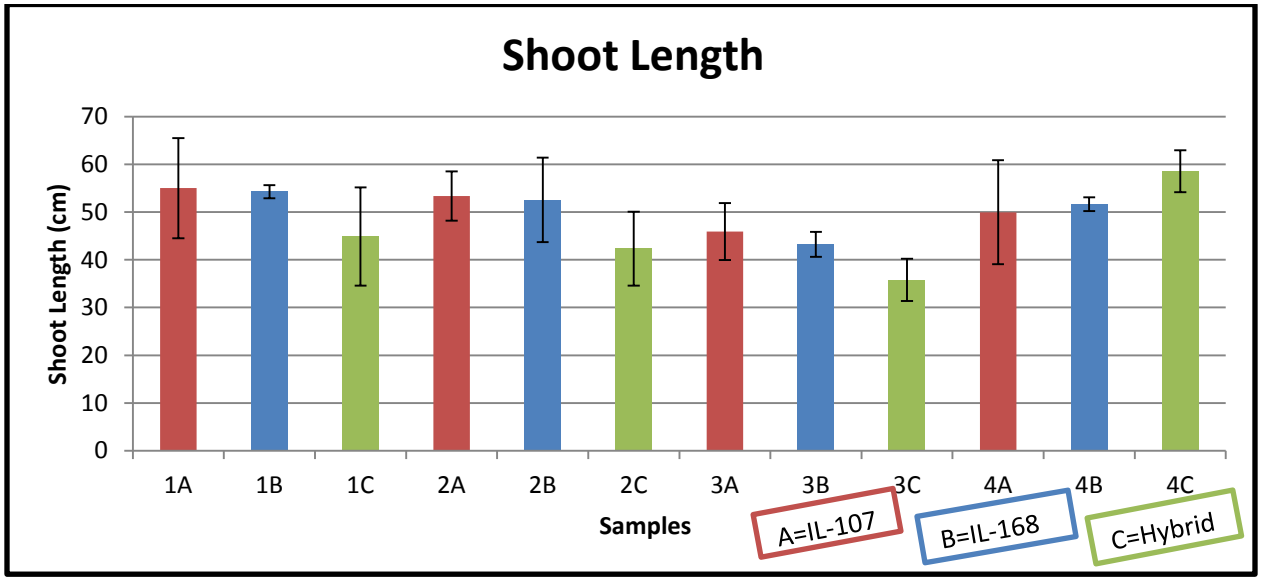

Figure 4.4: Effect of H2O2 pre- treatment on TiO_2NPs treated maize shoot length. Pre-treated groups showed improvement in shoot length.

4.3.2Effect of H_2O_2 pre- treatment on TiO_2 NPs on Post germinated maize Root Length

To study the effect of effect of H_2O_2 pre- treatment in TiO_2 nanotoxicity reduction, root length was measured using a scale (cm). Root Length was decreased by 19% and 27.6% in group 2A and 3A respectively as compared with group 1A. However root length was increased by 51.15% in group 4A as compared with group 3A. Similarly root length was decreased by 7.33% and 14.84% in group 2B and 3B respectively. However an increase of 43.17% was observed in group 4B compared with group 3B. Similarly root length was decreased by 4.02% and 10.8% in group 2C and 3C respectively. However root length was increased by 45.12% in group 4C as compared with group 3C. The effect of H_2O_2 pre- treatment on TiO_2 NPs on post germinated maize root length is demonstrated in the figure 4.5:

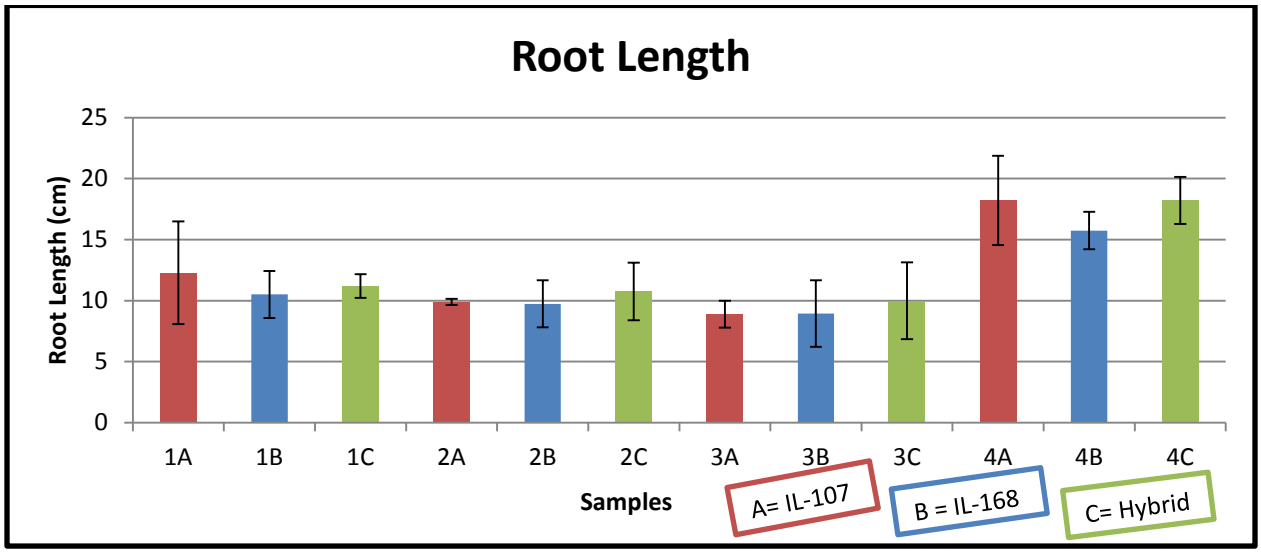

Figure 4.5: Effect of H2O2 pre- treatment on TiO_2NPs treated maize root length. Pre-treated groups showed improvement in root length.

4.3.3 Effect of H_2O_2 pre- treatment on TiO_2 NPs on Post germinated maize seedlingWater Content

To analyse the effect of H_2O_2 pre- treatment on TiO_2 NPs on Post germinated maize water content was taken into account. It describes the sum total of water present in leaf compared with its dry weight. In IL-107, water content was reduced by 14% and 28.44% in group 2A and 3A respectively as compared with group 1A was observed. However an increase of 12.63% in group 4A compared with group 3A was observed. Similarly, in IL-168 a reduction of 15.56% and 30.19% was observed in group 2B and 3B compared with group 1B was observed respectively. However an increase of 13.85% was observed in group 4B compared with group 3B. Similarly in hybrid, a reduction of 7.33% and 38.35%was observed in group 2C and 3C respectively compared with group 1C. However an increase of 4.99% in group 4C compared with group 3C was observed. The effect of H_2O_2 pre- treatment on TiO_2 NPs on post germinated maize seedling water content is demonstrated in the figure 4.6:

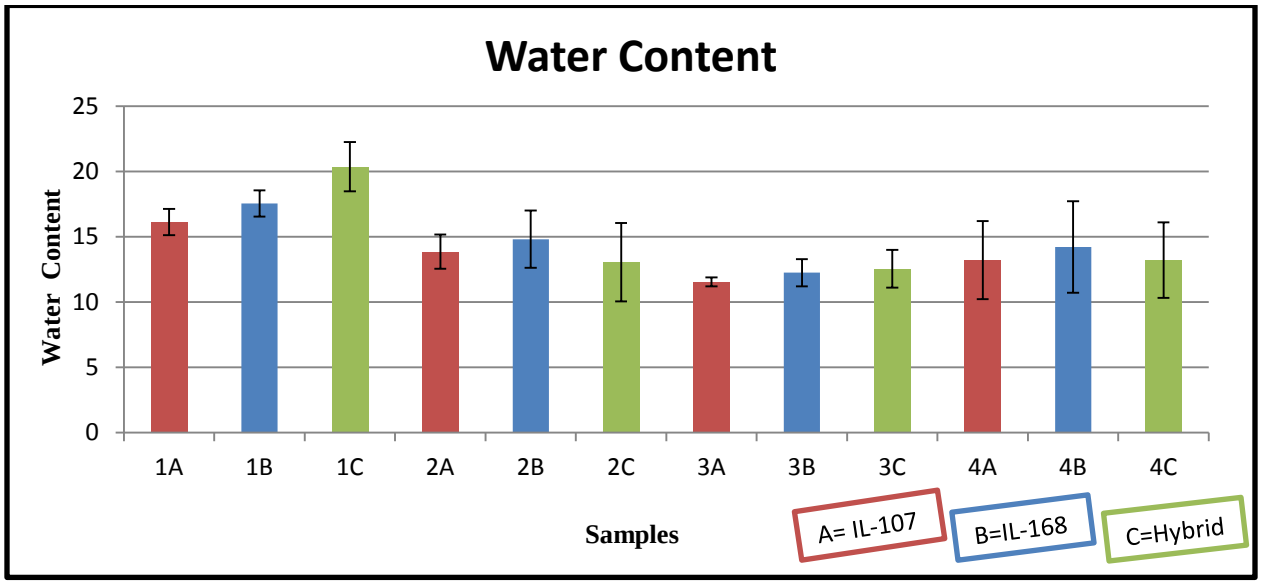

Figure 4.6: Effect of H_2O_2 pre- treatment on TiO_2NPs treated maize seedling water content. H_2O_2 pre – treated groups showed improvement in water content.

4.3.4 Effect of H_2O_2 pre- treatment on TiO_2 NPs on Post germinated maize seedling Vigour Index

Vigour index is one of the most important and reliable method of seed testing when it comes to commercial implications of seeds and selecting the seed with better attributes than others of various different seed lots, and this test is stated to be even better than the germination test, when it comes to the determination and evaluation of seed performance both in context of yield and abiotic stress impact and the potential of a seed to cope up with the stress in a lot of many different varieties (Filho, 2015).

4.3.4.1 Effect of H_2O_2 pre- treatment on TiO_2 NPs on Post germinated maize seedling Vigour Index I (SVI-I)

To analyse the effect of H_2O_2 pre- treatment on TiO_2 NPs on Post germinated maize seedlings seed vigour index I (SVI-I) based on mean seedling length was calculated. In IL-107, the reduction of 5.92% and 11.2% in group 2A and 3A was observed respectively compared with the group IA. However an increase of 19.65% was observed in group 4A compared with group 3A.Similarly, in IL-168, the reduction of

6.08% and 16.97% in group 2B and 3B respectively was observed compared with group 1B. However an increase of 20.82% was observed in group 4B compared with group 3B. Similarly, in hybrid, a reduction of 2.4% and 17.44% was observed in group 2C and 3C respectively. However an increase of 39.1% in group 4C was observed. The effect of H_2O_2 pre- treatment on TiO_2 NPs on post germinated maize seedling vigour index I (SVI-I) is demonstrated in the figure 4.7:

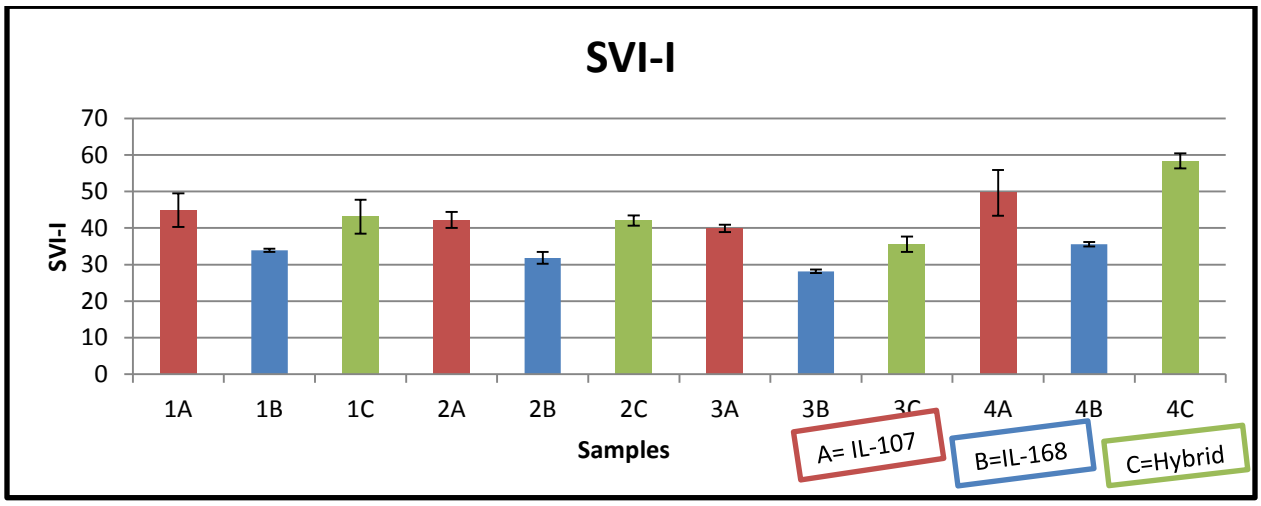

Figure 4.7: Effect of H_2O_2 pre- treatment on TiO_2NPs treated maize seedling vigour index I (SVI-I): H_2O_2 pre- treated groups showed improvement in vigour index I (SVI-I).

4.3.4.2 Effect of H_2O_2 pre- treatment on TiO_2 NPs on Post germinated maize seedling Vigour Index II (SVI-II)

To analyse the effect of H_2O_2 pre- treatment on TiO_2 NPs on Post germinated maize seedlings seed vigour index II (SVI-II) based on dry mass was calculated. In IL-107, a reduction of 12.9% and 37.74% was observed in group 2A and 3A respectively compared with group 1A. However an increase of 67.39% in group 4A was observed compared with group 1A. Similarly in IL-168 a reduction of 4% and 16% was observed respectively compared with group 1A. However an increase of 58.84% in group 4B compared with group IB was observed. Similarly in hybrid, a reduction of 20% and 28.57% in group 2C and 3C was observed respectively. However an increase

of 64.79% in group 4C was observed compared with group 3C. The effect of H_2O_2 pre- treatment on TiO_2 NPs on post germinated maize seedling vigour index II (SVI-II) is demonstrated in the figure 4.8:

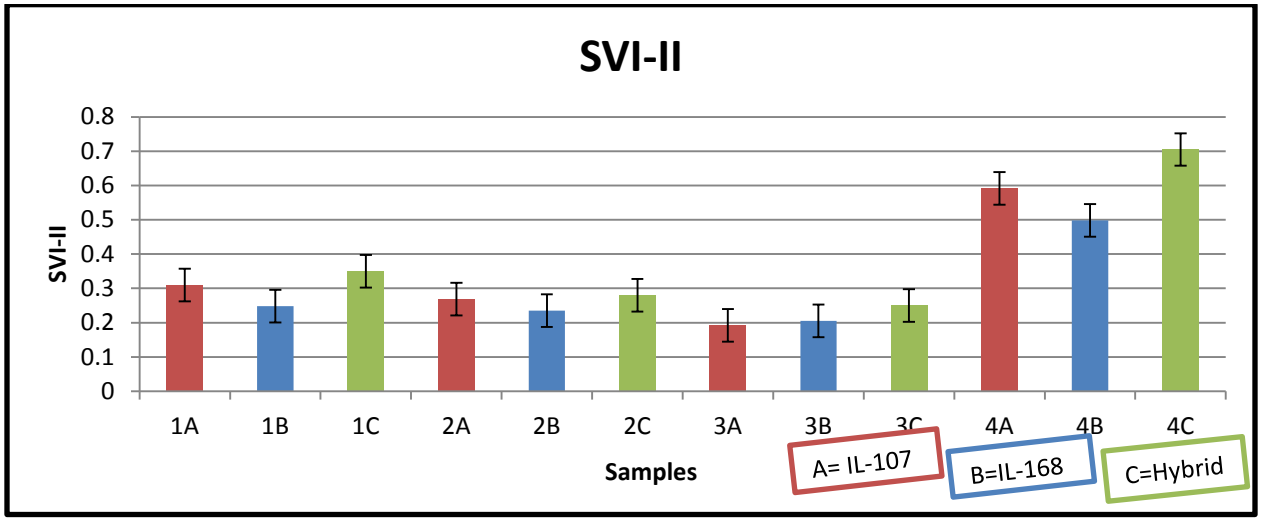

Figure 4.8: Effect of H_2O_2 pre- treatment on TiO_2NPs treated maize seedling vigour index II (SVI-II): H_2O_2 pre- treated groups showed improvement in vigour index II (SVI-II).

4.4 Biochemical Parameters

4.4.1 Effect of H_2O_2 pre- treatment on TiO_2 NPs on Post germinated maize seedling Pigments

4.4.1.1 Effect of H_2O_2 pre- treatment on TiO_2 NPs on Post germinated maize seedling Chlorophyll a, Chlorophyll b and Total Chlorophyll Content

To analyse the effect of H_2O_2 pre- treatment on TiO_2 NPs on Post germinated maize seedling chlorophyll a, chlorophyll b and total chlorophyll content was estimated. In IL-107, a reduction of 23.54%, 48.8% & 33.14% and 34.71%, 62.59% & 45.15% in group 2A and 3A was observed respectively compared with group 1A. However an increase of 27.88%, 33.33% and 29.16% was observed in group 4A compared with group 3A. Similarly in IL-168, a reduction of 25.63%, 17.02% & 23.51% was

observed in group 3B was observed compared with group IB. However an increase of 20.45%, 33.49% and 24.31% in group 4B was observed compared with group 3B. Similarly, in hybrid a reduction of 8.45%, 29% & 15.03% and 4.23%, 11.82% &8.49% was observed in group 2C and 3C respectively compared with group 1C. However an increase of 47.28%, 36.43% and 45.74% in group 4C was observed compared with 3C. The effect of H_2O_2 pre- treatment on TiO_2 NPs on post germinated maize seedling, chlorophyll a, chlorophyll b and total chlorophyll content is demonstrated in figure 4.9:

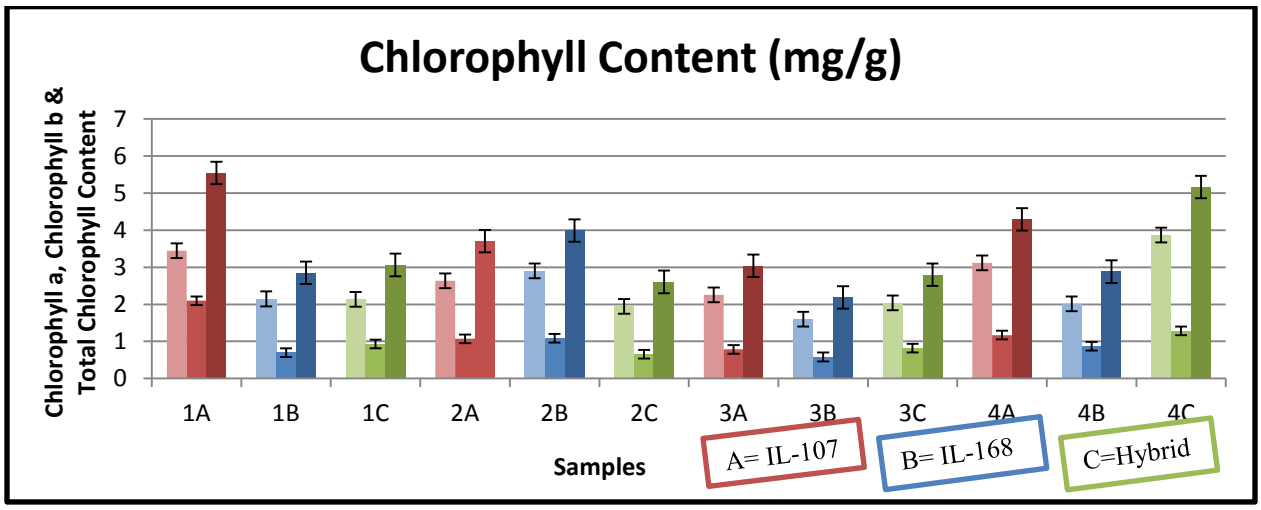

Figure 4.9: Effect of H_2O_2 pre- treatment on TiO_2NPs treated maize seedling chlorophyll a, chlorophyll b and total chlorophyll content. H_2O_2 pre- treated groups showed significant increase in chlorophyll a, chlorophyll b and total chlorophyll content.

4.4.1.2 Effect of H_2O_2 pre- treatment on TiO_2 NPs on Post germinated maize seedling Total Carotenoid Content

To investigate the effect of H_2O_2 pre- treatment on TiO_2 NPs on post germinated maize seedling on total carotenoid content was estimated. In IL-107, a reduction of 52.81%, 59.55% was observed in group 2A and 3A respectively compared with group IA. However an increase of 32.46% was observed in group 4A compared with group

3A. Similarly in IL-168, a reduction of 26.31% and 59.84% was observed in group 2B and 3B respectively compared with group 1B. However an increase of 31.94% was observed in group 4B compared with group 3B. Similarly in hybrid, a reduction of 64.13% and 40.72% in group 2C and 3C respectively compared with group 1C. However an increase of 19.51% was observed in group 4C. The effect of H_2O_2 pre-treatment on TiO_2 NPs on post germinated maize seedling, total carotenoid content is demonstrated in figure 4.10:

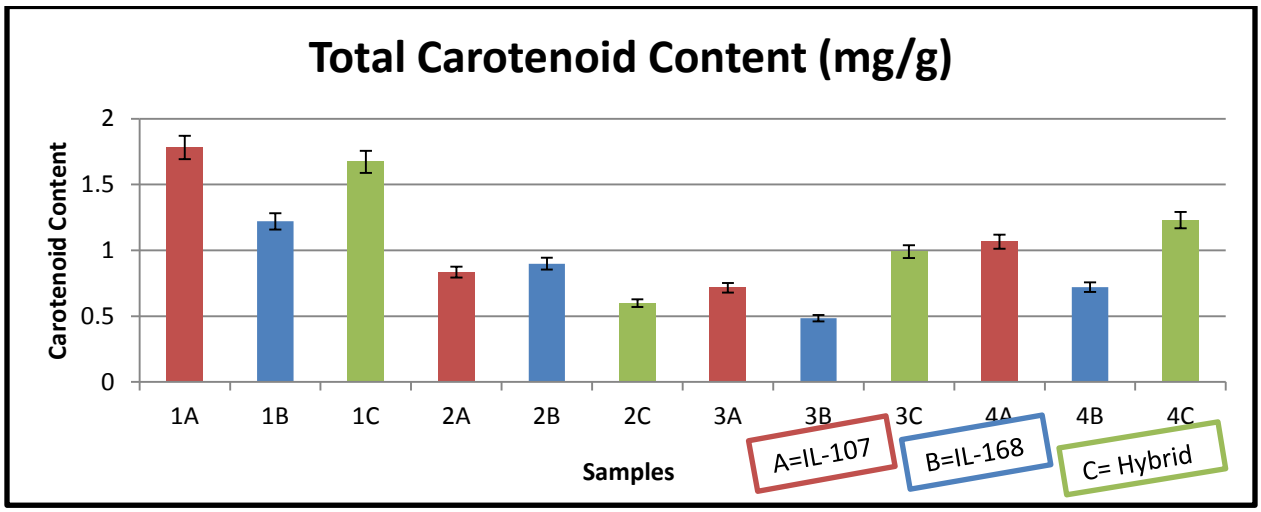

Figure 4.10: Effect of H_2O_2 pre- treatment on TiO_2NPs treated maize seedling total carotenoid content. H_2O_2 pre- treated groups showed improvement in total carotenoid content.

4.4.2Effect of H_2O_2 pre- treatment on TiO_2 NPs on Post germinated maize seedling Total Soluble Sugar Content

To evaluate the effect of H_2O_2 pre- treatment on TiO_2 NPs on Post germinated maize seedling total soluble sugar content was measured by the phenol-sulfuric acid method (Doubois et al., 1956). In IL-107 a reduction of 26.67% was observed in group 3A compared with group 1A. However an increase of 47.62% was observed in group 4A compared with group 3A. Similarly in IL-168, a reduction 14.29% was observed in group 3B compared with group 1B. However an increase of 33.33% in group 4B was

observed compared with group 3B. Similarly in hybrid a reduction of 17.65 % was observed in group 3C compared with group 1C. However an increase of 26.32% in group 4C was observed compared with group 3C. The effect of H_2O_2 pre- treatment on TiO_2 NPs on post germinated maize seedling, total soluble sugar content is demonstrated in figure 4.11:

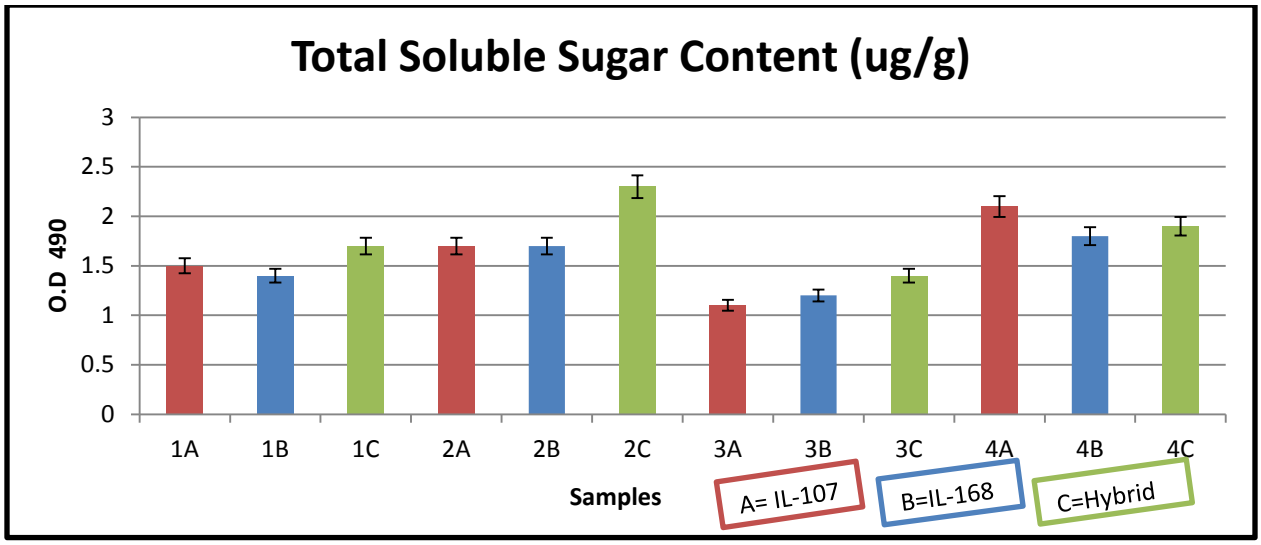

Figure 4.11: Effect of H_2O_2 pre- treatment on TiO_2NPs treated maize seedling total soluble sugar content. H_2O_2 pre- treated groups showed improvement in total soluble sugar content.

4.4.3Effect of H_2O_2 pre- treatment on TiO_2 NPs on Post germinated maize seedling Total Protein Content

To examine the effect of H_2O_2 pre- treatment on TiO_2 NPs on post germinated maize seedling total protein content by Bradford Method. In IL-107, a reduction of 44.22% and 42.31% was observed in group 2A and 3A respectively compared with group 1A. However an increase of 44.77% was observed in group 4A compared with group 3A. Similarly in IL-168, a reduction of 45.59% and 22.08% was observed in group 2B and 3B respectively compared with group 1B. However an increase of 24.69% was observed in group 4B compared with group 3B. Similarly in hybrid a reduction of 21.17% and 22.94% was observed in group 2C and 3C respectively compared with

group 1C. However an increase of 24.55% was observed in group 4C compared with group 3C. The effect of H_2O_2 pre- treatment on TiO_2 NPs on post germinated maize seedling, total protein content is demonstrated in figure 4.12:

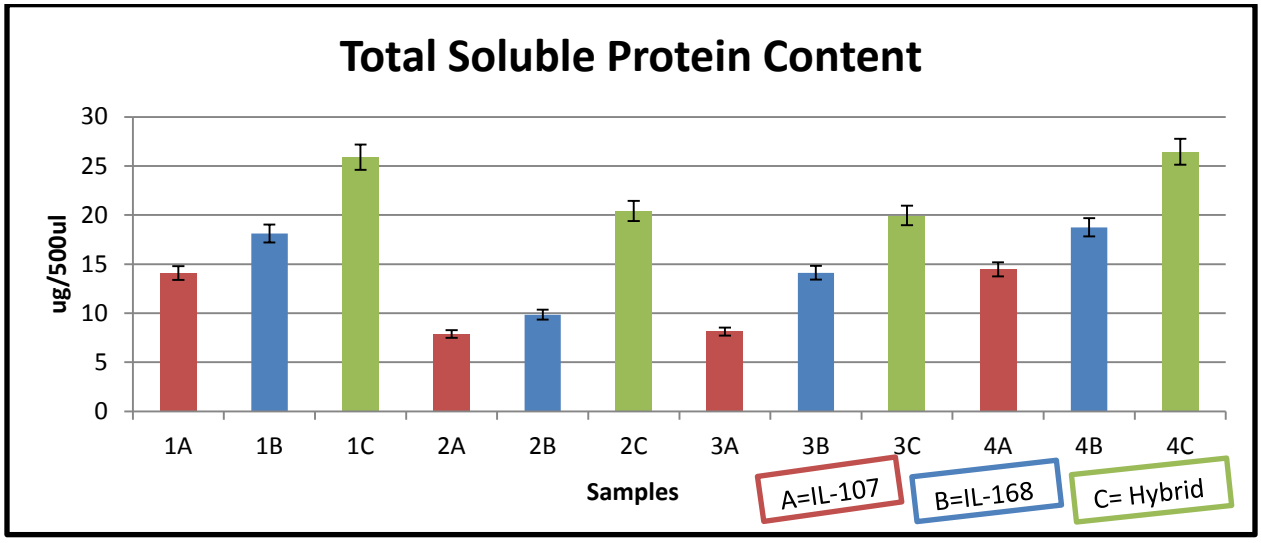

Figure 4.12: Effect of H_2O_2 pre- treatment on TiO_2NPs treated maize seedling total protein content. H_2O_2 pre- treated groups showed improvement in total protein content.

4.4.4Effect of H_2O_2 pre- treatment on TiO_2 NPs on Post germinated maize seedling Anti Oxidant Enzyme Activity Assays

4.4.4.1 Effect of H_2O_2 pre- treatment on TiO_2 NPs on Post germinated maize seedling Ascorbate Peroxidase (APX) Activity

To study the effect of H_2O_2 pre- treatment on TiO_2 NPs on post germinated maize seedling ascorbate peroxidase was measured. In IL-107 a reduction of 5.29% and 76.89% was observed in group 2A and 3A was observed respectively compared with group IA. However an increase of 84.27% was observed in group 4A compared with group 3A. Similarly in IL-168, a reduction of 2.16% and 83.42% was observed in group 2B and 3B respectively compared with 1B. However an increase of 87.73% was observed in group 4B compared with group 3B. Similarly in hybrid a reduction of

12% and 73.33% was observed in group 2C and 3C respectively compared with 1C. However an increase of 67.54% was observed in group 4C compared with group 3C. The effect of H_2O_2 pre- treatment on TiO_2 NPs on post germinated maize seedling, ascorbate peroxidase activity is demonstrated in figure 4.13:

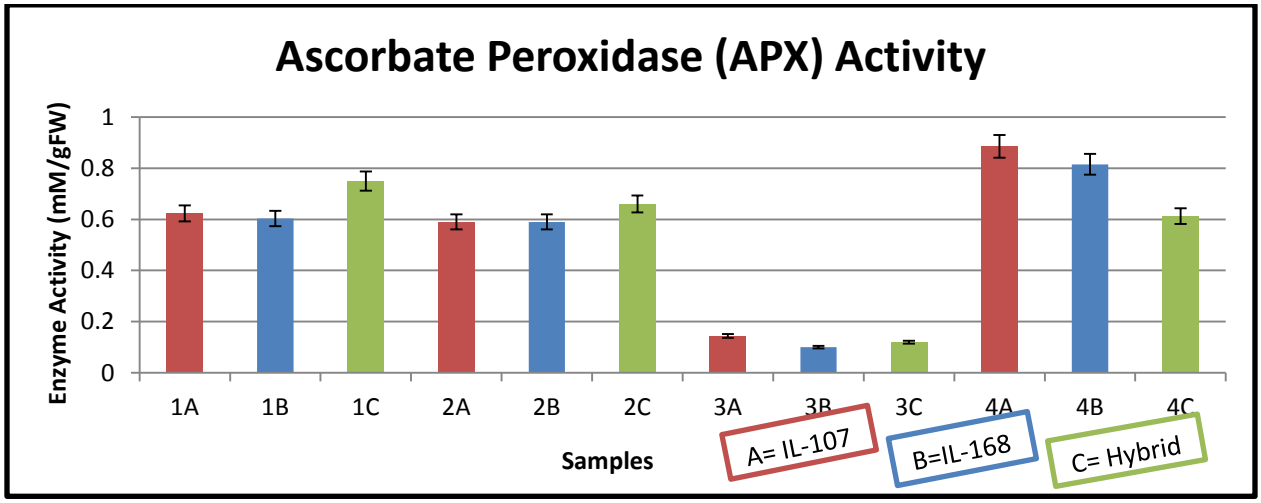

Figure 4.13: Effect of H_2O_2 pre- treatment on TiO_2NPs treated maize seedling ascorbate peroxidase activity. H_2O_2 pre- treated groups showed a significant increase in ascorbate peroxidase activity.

4.4.4.2 Effect of H_2O_2 pre- treatment on TiO_2 NPs on Post germinated maize seedling Catalase (CAT) Activity

To examine the effect of H_2O_2 pre- treatment on TiO_2 NPs on post germinated maize seedling catalase activity was measured (Aebi, 1983). In IL-107, an increase in catalase activity by 8.45% was observed in group 2A and a reduction in catalase activity by 50.35% was observed in group 3A compared with group 1A was observed respectively. However an increase in catalase activity by 48.98% in group 4A compared with group 3A was observed. Similarly in IL-168 an increase in catalase activity by 8.58% was observed in group 2B and a reduction in catalase activity by 75.69% in group 3B was observed compared with group 1B was observed respectively. However an increase in catalase activity by 69.09% was observed in group 4B compared with group 3B. Similarly in hybrid an increase in catalase activity

by 18.18% in group 2C and a reduction in catalase activity by 73.33% in group 3C compared with group 1C was observed respectively. However an increase in catalase activity by 62.85% in group 4C compared with group 3C was observed. The effect of H_2O_2 pre- treatment on TiO_2 NPs on post germinated maize seedling, catalase activity is demonstrated in figure 4.14:

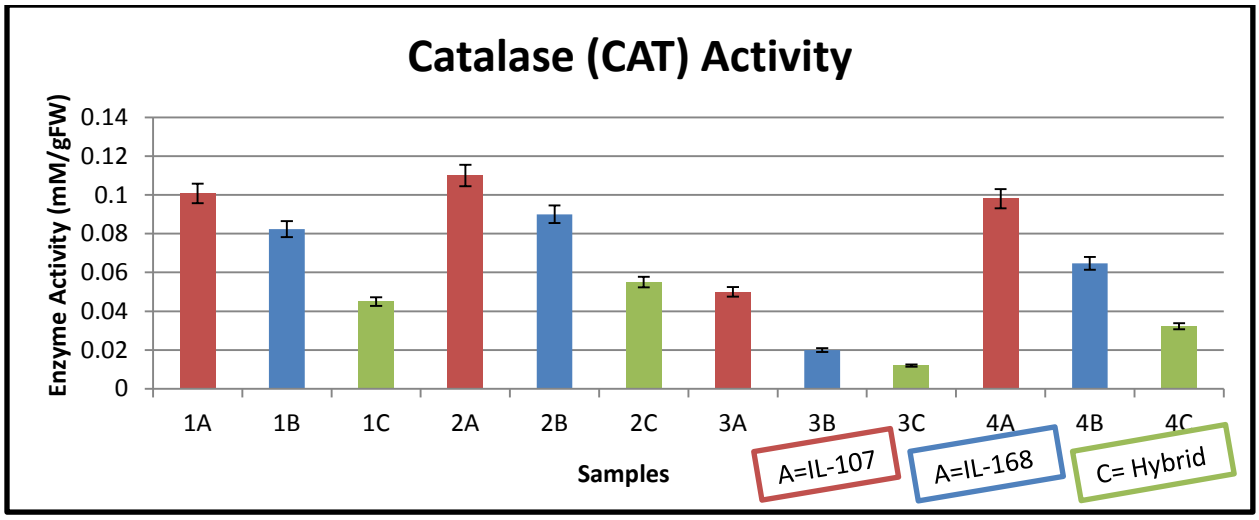

Figure4.14: Effect of H_2O_2 pre- treatment on TiO_2NPs treated maize seedling catalase activity. H_2O_2 pre- treated groups showed an improvement in catalase activity.

Chapter 5

DISCUSSION

Nanotechnology is an emerging field. Its use is increasing day by day. However there are studies which suggest that these nanoparticles enter into the arial, terristrial and marine environment and from there accumulate into the food chain and thus food web. Therefore it is essential to look for the possible ways of reducing their toxicity at different trophic levels, one of them being plants.

The objective of the present study was to analyse the effects of H_2O_2 pre-treatment in TiO_2 nanotoxicity reduction in maize hybrid and its parents. In the present study the highest germination rate was observed in hybrid maize than its parents. This could be attributed to the hybrid vigour in hybrid seeds (Janick, 2004).

To study the effect of H_2O_2 pre –treatment in nanotoxicity reduction, on 10 days post germinated maize seedlings, different physiological parameters were analysed. Results showed reduction in shoot length, root length, water content and SVI- I and SVI-II of hybrid maize and its parents in groups 3A, 3B and 3C respectively. We assume that this reduction in shoot length, root length and water content is because at conc. ranging from 300mg/L to 1000mg/L TiO_2NPs induces nanotoxicity in maize plants thus inhibiting the hydraulic conductivity, leaf growth and transpiration (Asli and Neumann, 2009). Furthermore it has been reported that if the conc. of TiO_2NPs reaches above 10ppm, it causes reduction in shoot length (Feizi et al., 2012). Reduction in shoot length, root length and SVI-I at 1000mg/L TiO_2NPs conc. is in accordance with the previous study reported by Kumar (2014). However in groups 4A, 4B and 4C respectively, an improvement in shoot length, root length water content SVI-I and SVI- II was observed. We assume that this is because of the fact that exogenous application of H_2O_2 sensitizes the plant so that it develops an immunity to cope up with the impending stress situation (Bhattacharjee, 2005).

To examine the effect of H_2O_2 pre –treatment in nanotoxicity reduction, 10 days post germinated seedlings different biochemical parameters were also studied. Results showed decrease in photosynthetic pigments of maize hybrid and its parents in groups 3A, 3B and 3C respectively. We assume that decrease in chlorophyll content is because of chlorophyll degradation in plant leaves at such a high concentration of TiO_2NPs. It has been reported that if a plant comes under stress chlorophyll begins to degrade and ultimately affects the growth, development and survival of the plants (Uvalle et al., 2007). However in groups 4A, 4B and 4C respectively an improvement in these photosynthetic pigments was observed. This could be because H_2O_2 is reported to be an important stress signalling molecule. Thus pre- treatment with H_2O_2 helps plant to cope up with the impending abiotic stress (Neill, 1999).

Results showed reduction in total soluble sugar content of plants, in groups 3A, 3B and 3C respectively. We assume that reduction in total soluble sugar content is because of the fact that various environmental stress factors affect the photosynthetic process occurring in plants. Thus the synthesis of sugars lowers down in plants and thus the movement of sugars from the source tissue to sink tissue. When plants face the conditions of sugar dearth, various physiological and biochemical variations occur to sustain the process of respiration and other metabolic activities in plants. During the early stages of plant development of early seedling establishment, storage sugars are converted to soluble sugars in plants, from seed tissues to other tissues such as stem and radical, where these converted sugars play role in growth and development of plants and also maintains osmotic homeostasis of the cells. Thus germinating seeds and early seedlings are more liable to damage caused by instability in soluble sugar and thus these are the stages which when studies provide valuable information regarding soluble sugar variations (Rosa et al., 2009).

However variations in soluble sugar content also depends on the type of stress the plant is facing and the strategy it employs to cope up with the particular stress it has come across. Generally it has been observed that soluble sugar content increases during drought, salinity, low temperature and flooding stresses and soluble sugar

content decreases during high light irradiance, heavy metal toxicity, nutrient deficiency and ozone caused damage (Strand et al., 1999 and Gill et al., 2001). This also supports our results as in most of the cases heavy metal toxicity are somehow show positive relation with metal caused nanotoxicity. Furthermore results of groups 4A, 4B and 4C showed improvement in total soluble sugar content respectively. This is in accordance with the earlier results as H_2O_2 is reported to be an important stress signalling molecule and is responsible for the induction of cross tolerance in plants (Mazid et al., 2012).

Results showed decrease in total protein content of groups 3A, 3B and 3C respectively. This could be due to the excessive accumulation of ROS, which cause damage to plants at physiological, metabolic and morphological levels, as this has been reported with reference to heavy metal toxicity in plants (Emamverdian, 2015). Similar effects were reported with various different heavy metals in maize plants by (Ghani, 2010). In general abiotic stresses cause decrease in total protein content (Baruah et al., 1998). However an improvement in total protein content was observed in groups 4A, 4B and 4C respectively.

Results showed reduction in ascorbate peroxidase (APX) activity in groups 3A, 3B and 3C respectively. We assume that this decrease in APX activity could be due to considerable level of nanotoxicity induced in maize seedlings. Such reduction in APX activity was reported with reference to drought stress in plants (Sharma and Dubey 2005). However a significant increase in ascorbate peroxidase (APX) activity was observed in groups 4A, 4B and 4C respectively. This is in line with the findings of Hso and Kao (2007), where H_2O_2 pre- treatment of *O. Sativa* seedlings resulted in increased APX activity and thus protected the plant against Cd stress.

Results showed a decrease in catalase (CAT) activity in groups 3A, 3B and 3C respectively. This is in line with the findings to the metal stress, where CAT activity decreases in various plants. Its activity was reported to decrease in *Glycine max* (Balestrasse et al., 2001), *Phragmites australis* (Lannelli et al., 2002), *Capsicum*

annum (Leon et al., 2002) and *Arabidopsis thaliana* (Cho and Seo, 2005) respectively. However results showed significant increase in CAT activity in groups 4A, 4B and 4C respectively. This is in line with the findings of Hso and Kao (2007), where pre-treatment of rice seedlings with H_2O_2 resulted in increased CAT activity and thus conferred tolerance to the plant against Cd stress.

Results showed increase in antioxidant enzyme activity in H_2O_2 pre- treated groups, this is in line with the findings of Kumar et al., (2012), which illustrates that exogenous application of H_2O_2, directly leads to the accumulation of H_2O_2 in plants, thus increasing the activity of various different enzymes. For example in H_2O_2 pre-treated groups, the activity of CAT was reported to increase, thus protecting the plants against heat shock.

Thus our results are in line with the previous findings that suggest H_2O_2 pre – treatment helps the plant to cope up against various different abiotic stresses. Our findings show that H_2O_2 pre – treatment causes reduction in TiO_2 induced nanotoxicity in maize seedlings.

CONCLUSION & RECOMMENDATIONS

Nanotechnology is expanding continuously in the world due to its perceived advantages when used in electronics, energy, medicine and agricultural sciences, however nanotoxicity studies are not at pace with its rapid increasing and extensive use. It is the need of an hour to study its negative affects as well and how to counter these negative effects. The current study attempted to address the issues of TiO_2 induced nanotoxicity in post germinated maize seedlings and how can we effectively reduce the nanotoxicity being induced in post germinated maize seedlings. Maize plants were opted for the reason that it is amongst one of the three major crops grown in the world, it is not only used as food for human beings but it is also used as feed for livestock, as a bio fuel as well as a raw material for industrial applications. In addition to these many advantages maize also serves as a model plant. In the current study, the effects of pre- treatment of H_2O_2 in TiO_2nanotoxicity reduction were studied, as earlier the exogenous application of H_2O_2 is reported to have reduced various abiotic stresses in maize and other plants. Our results are in line with the previous findings and thus H_2O_2 pre – treatment was found to cause reduction in TiO_2 induced nanotoxicity in maize seedlings at both physiological and biochemical levels with the experimental design employed in the current study. However it is recommended to study the effects of H_2O_2 pre – treatment in TiO_2 induced nanotoxicity reduction at various other stages of maize development, because plants respond differently at different developmental stages of their life cycle. Furthermore it is also required to study the effects of TiO_2 induced nanotoxicity and H_2O_2 pre – treatment in TiO_2 induced nanotoxicity reduction in maize plants with different experimental designs. It is imperative to study how TiO_2NPs distribute and accumulate in plants tissues and how they interact with different biological molecules within a plant cell. Besides all these aspects it is important to study the effects of TiO_2 induced nanotoxicity and role of exogenous H_2O_2 in nanotoxicity reduction at molecular level. Before coming to the final conclusion it is important to conduct gene expression profiling in order to obtain a

holistic picture of cellular functions inside the plant cell. This will help us in understanding the regulation of gene expression in maize plants in response to TiO_2 induced nanotoxicity and H_2O_2 pre – treatment in TiO_2 induced nanotoxicity reduction in maize plants.

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Figure 1.1 to 1.8 are based on the data obtained using the following link. http://www.indexmundi.com/.