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Using biochemical and nutrient analysis to understand the role of methylglyoxal signalling in soybean exposed to zirconium

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A thesis submitted in partial fulfilment of the requirements for the degree of Magister Scientiae in the Department of Biotechnology, University of the Western Cape.

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TABLE OF CONTENTS

KEYWORDS

APX	-	Ascorbate peroxidase
GLY-I	-	Glyoxalase I
H_2O_2	-	Hydrogen peroxide
HMs	-	Heavy metals
ICP-OES	-	Inductively coupled plasma-optical emission spectroscopy
MDA	-	Malondialdehyde
MG	-	Methylglyoxal
O_2^-	-	Superoxide
ROS	-	Reactive oxygen species
SOD	-	Superoxide dismutase
Zr	-	Zirconium

ABSTRACT

Using biochemical and nutrient analysis to understanding the role of methylglyoxal signalling in soybean exposed to zirconium

By: L.E.G Ndlovu

MSc Thesis, Department of Biotechnology, University of the Western Cape

Soybean have been listed as a priority commodity crop in South Africa (SA) and provide a good source of protein to the population. Therefore, soybean has been earmarked as an important food security crop and strategies are currently being discussed at governmental level to increase and sustain soybean production. However, the SA landscape poses many challenges to the agricultural sector such as prolong drought periods, flooding, nutrient poor soils, saline soils and heavy metal contaminated soils. Heavy metal (HM) contamination is becoming a serious concern and is aggravated by historical mining in SA. Indeed, SA has established itself as the number one ranked mining country in the world and is frequently mining metals such as chromium, vanadium, gold, zirconium, platinum, and antimony. Prolong rainfall near mining areas leads to acid mine drainage which lowers the soil pH to approximately two. These highly acidic soils will solubilize the metals and cause the metals to leach into river systems as well as the water table leading to increase heavy metal contamination in nearby soil sites. This increase metal content negatively affects seed germination and overall plant development. Nonetheless, plants have evolved numerous internal mechanisms that help them to survive HM toxicity; by either avoiding or tolerating the stress. Two stress-activated pathways that help the plant tolerate stress have attracted much interest i.e. the glyoxalase system and reactive oxygen species (ROS) - antioxidant system as they detoxify methylglyoxal (MG) and ROS. These toxic

molecules increase due to biotic or abiotic stress in plants. Recent studies have shown that low concentrations of ROS can signal plant growth and development. In view of these reports the possible novel role of MG as a signalling molecule in the defence (antioxidant-ROS) system is worth investigating. Therefore, this study aims to investigate MG as a possible antioxidant defense triggering agent through exogenous application to Zr exposed Soybean. In addition, part of this project is also to understand the effects of Zr exposure on Soybean. The study started by germinating Soybean seeds and thereafter selecting uniform seedlings. The seedlings were exogenously treated with 6 µM MG, 1 mM Zr and MG+Zr for 21 days. Subsequently, the leaves and roots were harvested, snap frozen and stored for further studies and some were used for biomass determination. Biochemical assays commenced, such as cell viability (Cell death), superoxide (O_2^-) content determination, hydrogen peroxide(H_2O_2) content determination, superoxide dismutase (SOD) activity, ascorbate peroxidase (APX) activity, chlorophyll content determination, malondialdehyde (MDA) content (lipid peroxidation test), methylglyoxal (MG) determination and glyoxalase I (GLY I) activity. A nutrient and mineral uptake analysis also commenced and it was completed by Inductively coupled plasma-optical emission spectrometry (ICP-OES) on both leaves and roots. The biomass of the leaves and roots was attained and MG+Zr treated leaves displayed an improvement in biomass as compared to solely Zr treated leaves with a significant decrease in the biomass of Zr treated plants at 46% in leaves and 23% in roots. These results corresponded with the cell death results were MG reduced cell death by at least 20% in plants exposed to MG+Zr as compared to the Zr treated leaves. Zr treated plants had an increase of 100% in cell death as compared to the control. MDA content results demonstrated an increase in all plants from the

different treatments as compared to the control with Zr exposed roots at above 100% as compared to control. The total chlorophyll content decreased in all the plants as compared to the control. Zr treated plants had the highest decrease of 28% and MG treated plants had a decrease of 8%. The ROS content also increased, there was an increase of more than 100% in H_2O_2 content in the roots and leaves of plants treated with Zr as compared to control. MG also increased ROS scavenging activity i.e. SOD and APX activity in the presence of Zr. This was witnessed from the kinetic assay results performed for the activity of these enzymes respectively. The ICP-OES test showed that MG can lower Zr uptake, figure 4.1 displayed a higher accumulation of Zr in roots of MG+Zr treated plants than in the leaves as compared with the Zr solely treated plants which showed a higher accumulation of Zr in leaves than roots. MG also increased the translocation factor (TF) values of micronutrients and it increased macronutrient accumulation in leaves and showed no significant difference in the roots. These biochemical and mineral studies demonstrated that MG could possibly have signaling properties in low concentrations that trigger the plants defense mechanism against oxidative stress.

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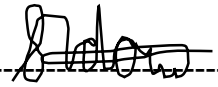

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Signature **Date**

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List of figures

List of tables

CHAPTER ONE

LITERATURE REVIEW

1.1 INTRODUCTION

The unavoidable exposure of plants to heavy metal (HM) toxicity has seen a great rise in the last century and South African crops are not exempted from this dilemma (Cvjetko, et al., 2014; D'Amore, et al., 2005; Khan, et al., 2008; Nicholson, et al., 2003; Satarug, et al., 2003). Waste from industry, agriculture and the domestic sector have been reported as contributors of this exponential growth in HM pollution with mining as the leading source of environmental HM pollution in Southern Africa as a whole (Yabe, et al., 2010). South Africa (SA) has the highest contribution to the worlds platinum group metals (PGM) as displayed in figure 1.1. It produces approximately 59 different minerals such as gold, platinum, titanium, chromium, manganese, vanadium and zirconium from 1115 mines and quarries (Langmi & Bessarabov, 2013) and this heavy mining increases HM pollution (Okedeyi, et al., 2014; Turkdogan, et al., 2002; Rascio & Navari-Izzo, 2011; Fatoki, 1996).

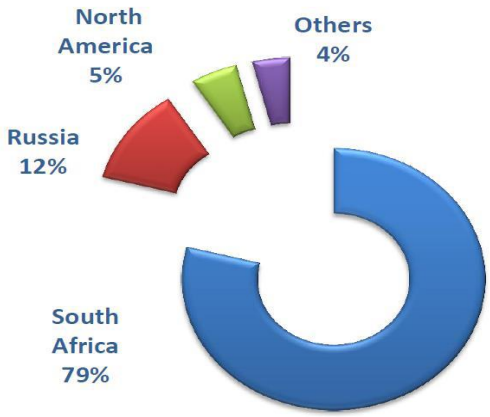

***Figure 0-1 Diagram showing the worlds platinum group metals (PGM) suppliers**. South Africa has the highest contribution to the worlds PGM (Langmi & Bessarabov, 2013).*

HM contamination is not restricted to mining areas solely, HM contamination can spread to neighbouring cities and countries via transboundary water systems like rivers and groundwater systems (Ravengai, et al., 2005; Meck, et al., 2006; Naicker , et al., 2003; Pettersson & Ingri, 2001; Von der heyden & New, 2004). Farming areas may be exposed to the polluted water. Consequently, resulting in decreased crop growth, development and yield (Dalcorso, et al., 2010; Hossain, et al., 2011). HM accumulation in plant tissues interferes with various morphological, physiological, and biochemical processes (Cvjetko, et al., 2014). Inside a cell, these metals may deregulate important processes such as photosynthesis, respiration, mineral nutrition absorption, and enzymatic reactions (Cvjetko, et al., 2014; Oancea, et al., 2005). When mineral absorption is disrupted plant growth and development is disrupted. Aside from being a mining country SA takes pride in its agricultural sector. South Africa is known for producing high yields of wheat, malting barley, canola, sweet lupines in winter and maize,

sorghum, groundnuts, sunflower seed, dry beans and soya-beans in summer (compiled by Directorate statistics & economic , 2013; Hall, 1930).

Agriculture is the foundation of developing economies, as one of these economies, SA needs to ensure a healthy agricultural industry that contributes to the country's gross domestic product (GDP), food security, social welfare, job creation and ecotourism, while adding value to raw materials (Issued by the Directorate Agricultural statisctics, 2006). However, the fate of the agricultural sector is intertwined with factors such as HM pollution.

Nevertheless, plants have developed numerous internal mechanisms that help them survive stresses; by either avoiding or tolerating the stress. When plants avoid the stress they restrict the concentration accumulation within the cells by extracellular precipitation or bio-sorption to cell wall resulting in a controlled influx of the contaminant (Hossain, et al., 2009). Plants can also tolerate certain stresses by activating the activity of several genes from physiological and biochemical pathways within the plant cell (Dalcorso, et al., 2010; Hossain, et al., 2011) these pathways assist the plant to survive in the presence of the contaminant (Hossain, et al., 2011; Sasaki-Sekimoto, et al., 2005). Two stress-activated pathways and their logistics have attracted much interest i.e. the glyoxalase system and reactive oxygen species (ROS) system as they detoxify methylglyoxal (MG) and ROS respectively. These molecules increase due to biotic or abiotic stress (Chen, et al., 2004; Hossain, et al., 2009; Hossain, et al., 2011; Singla-Pareek, et al., 2006; Veena & Sopory, 1999; Yadav, et al., 2005), this accumulation is a result of an imbalance between production and detoxification. High concentrations in plant cells have been proven to be highly toxic as they

interfere with important physiological processes present in plants (Hossain, et al., 2011; Saito, et al., 2011). Lipid peroxidation, biological macromolecule deterioration, membrane dismantling, ion leakage, DNA-strand cleavage, photosynthesis process interference and finally death of the plant result due to ROS and MG accumulation (Dalcorso, et al., 2011; Hossain, et al., 2010; Hossain, et al., 2011; Navari-Izzo, 1998; Rascio & Navari-Izzo, 2011). Studies have proven that when detoxification and production of these molecules are balanced it could lead to plant tolerance to biotic or abiotic stress (Veena & Sopory, 1999; Singla-Pareek, et al., 2003; Saxena , et al., 2011) (Mitler, et al., 2004). Moreover, recent studies have proven that several essential biological processes such as growth and development in plants are signalled by h low concentrations of ROS (Baxter, et al., 2013; Mittler, et al., 2011). In view of this signalling role of exogenous treatment with ROS, the exogenous treatment of plants with MG needs to be investigated.

1.2 SOYBEAN

1.2.1 SOYBEAN PRODUCTION IN SOUTH AFRICA

Soybean (*Glycine max*) a high protein source crop was first introduced into South Africa in 1903, but its production was delayed since South African farmers had no experience with farming it (Du Toit, 1942). In recent years soybean has attracted attention as a crucial crop for human and animal feed and as a growing contributor to the countries agricultural economy (De Beer & Prinsloo, 2013). Thousands of South Africans are poor and live in rural areas they face malnutrition due to increasing food prices caused by various factors, therefore the incorporation of soybean based foods that are

economical into household diets will contribute to huger reduction and increase food security in economically challenged areas (Dlamini, et al., 2014).

In 2010 the crop was added into the country's industrial policy action plan (IPAP), and ever since then national seed companies have been encouraged to develop improved soybean seed varieties that can perform well locally (De Beer, 2012) (Dti, 2010) (Van De Merwee, et al., 2013). In 2013 the South African Bureau for food and Agricultural policy (BFAP, 2013) advised that there be an increase in the total of land set aside for commercial soybean production in South Africa. However, not all the provinces in South Africa have suitable conditions for soybean production. From the nine provinces in South Africa and only three provinces have dominated in soybean production for 25 years as seen in figure 1.2: Mpumalanga (MP) 43%, Free State (FS) 12% and KwaZulu-Natal (KZN) 21% (Blignaut & Taute, 2010; Dlamini, et al., 2014).This is a result of different rainfall patterns in different parts of the country. These provinces are not the only provinces with suitable conditions Gauteng province (GP) and Eastern Cape (EC) are also suitable for soybean production but minimal to no production has happened in the past 25 years (Dlamini, et al., 2014; Joubert & Jooste, 2013).

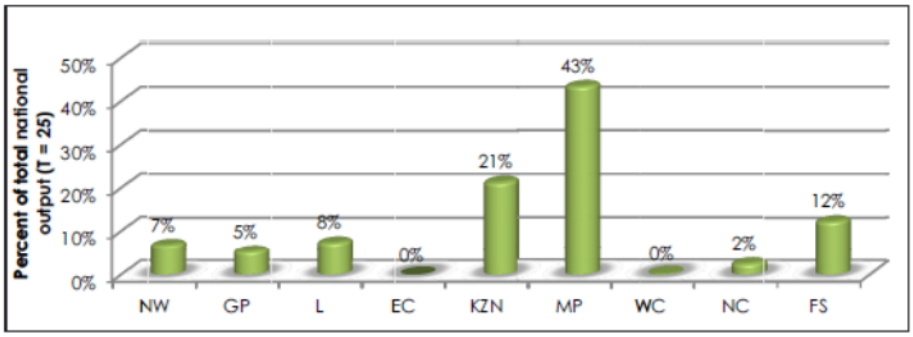

Figure 0-2 Diagram showing soybean production per province in South Africa in 25 years. *Mpumalanga province (MP) is by far the largest producer of soybean in SA in terms of output and area planted, Kwazulu-Natal province (KZN) is second followed by Free State (FS). Western Cape (WC) and Eastern Cape (EC) haven't produced any in the 25 years (Dlamini, et al., 2014).*

According to Opperman and Varia (2011), the demand for soybean is well established in South Africa and throughout the Southern Africa Development Community (SADC). South Africa's population is growing at an estimated 2% per year according to 2014 world bank statistics. The population of 49 million in 2009 is expected to grow to 82 million by the year 2035 (World Bank, 2014). Food production or imports must more than double to feed the expanding population, and production needs to increase using the same or fewer natural resources (Agricultural Statistics, 2009). South Africa can counteract the consequences of this dilemma by increasing their own food production moreover the production of food that have high nutritional value.

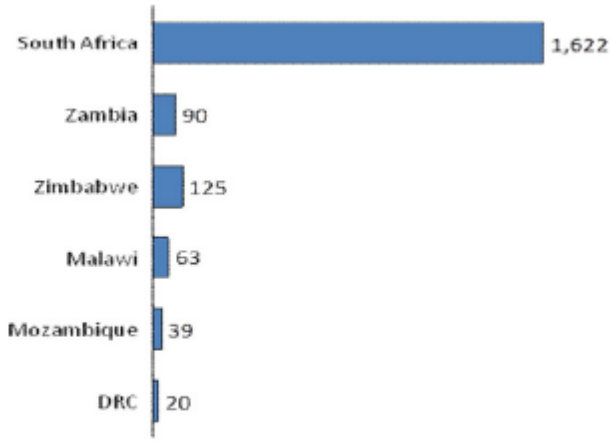

Figure 0-3 Diagram showing soybean oil demand*. This only represent selected Southern African development communities (SADC) countries (Dlamini, et al., 2014).*

High protein meals and soybean oils are the most noticeable soybean products used in South Africa as seen on figure 1.3. The oil is used in the industrial sector for the productions of various products and the high protein meal is and essential ingredient in the manufacturing of feed for the poultry and pork industry (Dlamini, et al., 2014). The South African government also introduced soybean meals into school feeding schemes in the year 2002 to the majority of disadvantaged areas across the country (ETU, 2014). The school feeding is a small part of the integrated Food Security strategy for South Africa, it was introduced by the department of health social development, Land affairs and agriculture. This project was one of the small contributors of soybean production increase in South Africa.

Soybean processed for meal and oil increased by 20% annually from 2005 (Van De Merwe, et al., 2013). Nonetheless, on average the domestic soybean meal production meets only 10% of the domestic soybean meal demand, 90% of the soybean meal consumed domestically is imported from Argentina

(Van De Merwe, et al., 2013). Ideally South Africa ought to be doing its own processing of Soybean given that the potential to do so exists. This is especially important in light of the fact that soybean meal is currently one of South Africa's largest agricultural import products (Opperman & Varia, 2011).

1.3 NUTRIENT UPTAKE IN PLANTS

A plant nutrient is a chemical element that is essential for plant growth and reproduction (Barker & Pilbeam, 2007). A large amount of plant nutrients are produced from the fixation if atmospheric CO_2 into simple sugar using the energy from the sun this process is known as photosynthesis (Wild, et al., 1987), nevertheless plant growth and reproduction is not only dependent on it. Plants also need a number of inorganic nutrients some in large quantities also known as macronutrients and some in smaller quantities also known as micronutrients from their surroundings (Amon & Stout, 1939; Barker & Pilbeam, 2007).

The different categories of nutrients have different functions: macronutrients nitrogen (N) and the minerals potassium (K), calcium (Ca), magnesium (Mg), phosphorous (P) and sulphur (S) are present in plant tissues in relatively large amounts (Marschner, 1995; Maathuis, 2009). By contrast, inorganic macronutrients are usually present at low concentrations in the soil and often need to be accumulated against steep concentration gradients. Although generally low, soil availability can fluctuate greatly in both space and time due to factors such as precipitation, temperature, wind, soil type and soil pH. As sessile organisms, plants therefore have had to develop

adaptive and flexible strategies for the attainment of nutrients and these are mechanically similar for all macronutrients (Barker & Pilbeam, 2007). Micronutrients are elements that are essential for plant growth but are required in much smaller amounts. Micronutrients are involved in virtually all metabolic and cellular functions, like energy metabolism, primary and secondary metabolism, cell protection, gene regulation, hormone perception, signal transduction, and reproduction among others (Romheld & Harschner, 1991; Hansch & Mendel, 2009) as seen on table 1.

Table 0-1: This table displays essential plant nutrients, the form in which plant absorb them and a few examples of their function (Baker & Pilbeam, 2007)

Elements	Principal from in which element is absorbed	Examples of Important function
MACRONUTIERNTS		
Carbon	CO_2	Major component of organic molecules
Oxygen	O_2, H_2O	Major component of organic molecules
Hydrogen	H_2O	Major component of organic molecules
Nitrogen	NO_3^-, NH_4^+	Component of amino acids, proteins, nucleotides, nucleic acids, chlorophyll
Potassium	K^+	Protein synthesis, operation of stomata
Calicium	Ca^{++}	Component of cell walls, maintenance of membrane structure and activates some enzymes
Magnesium	Mg^{++}	Component of chlorophyll molecule, activates many enzymes
Phosphorus	$H_2PO_4^-$, HPO_4^-	Component of ADP and ATP, nucleic acids, phospholipids, several coenzymes
Sulfur	$SO_4^=$	Components of some amino acids and proteins. Coenzyme A
MICRONUTRIENTS		
Chlorine	Cl^-	Osmosis and ionic balance
Iron	Fe^{++}, Fe^{+++}	Chlorophyll synthesis, cytochromes, nitrogenase
Manganese	Mn^{++}	Activator of certain enzymes
Zinc	Zn^{++}	Activator of many enzymes, active in formation of chlorophyll
Boron	BO_3^- or $B_4O_7^-$	Possibly involved in carbohydrate transport, nucleic acids synthesis
Copper	Cu^{++}	Activator or component of certain enzymes
Molybdenum	$MoO_4^=$	Nitrogen fixation, nitrate reduction

Plants acquire these nutrients from the soil through their roots. Once in the roots, the ions, which are plant nutrients, are transported via the xylem or phloemthroughout the plant. The ions may follow the cell walls and the spaces between them or more often go directly through the plasma membranes and the protoplasm of adjacent cells figure 1.4. When mineral ions pass between the cell walls, they do so nonselective. Eventually, on their journey inward, they reach the endodermis and any further passage through

the cell walls is blocked by the Casparian strips. Water and ions must pass through the plasma membranes and protoplasts of the endodermal cells to reach the xylem. However, transport through the cells of the endodermis is selective. The endodermis, with its unique structure, along with the cortex and epidermis, controls which ions reach the xylem (Johnson & Peter, 2010). This system can be hindered by varies factors such as drought. However, when present in elevated concentrations, the same redox properties that make metal ions essential elements lead to the formation of reactive oxygen species with detrimental consequences for the cell. Moreover, metal excess can lead to ectopic binding of metals to proteins thus disturbing protein structures (Lavado, et al., 2001; Richard, 1993; Romheld & Harschner, 1991).This beneficial versus detrimental duality caused the development of precisely tuned homeostatic cellular networks involving metal chaperones (Lavado, et al., 2001; Barker & Pilbeam, 2007; Johnson & Peter, 2010).

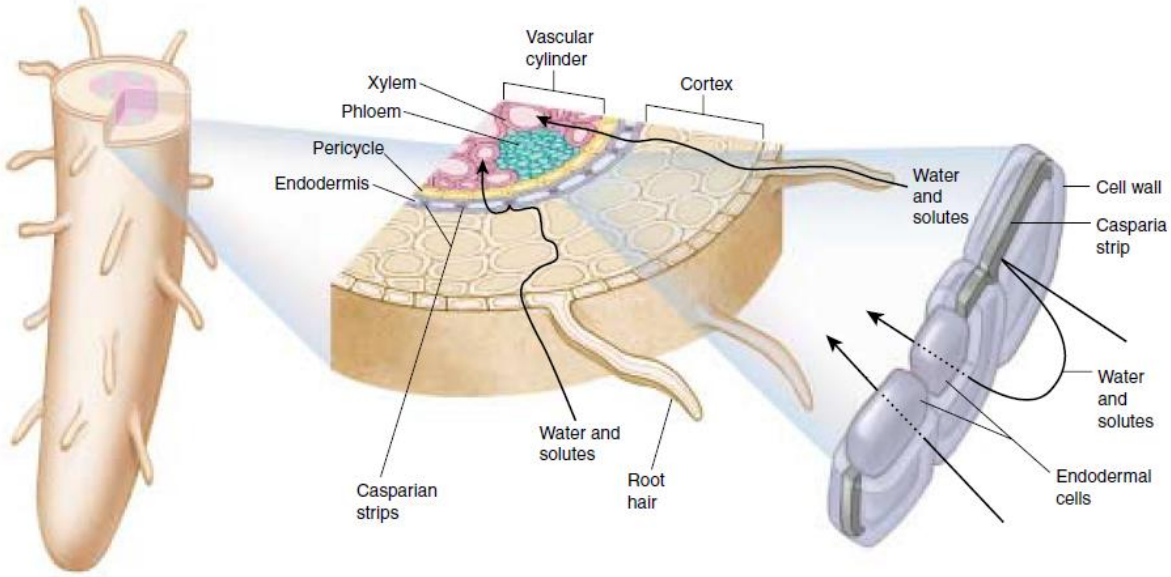

Figure 0-4 The pathways of mineral transport in roots*. Minerals are absorbed at the surface of the root, mainly by the root hairs. In passing through the cortex, they must either follow the cell walls and the space between them or go directly through the plasma membranes and protoplasts of the cells, passing from one cell to the next (Johnson & Peter, 2010).*

1.4 HEAVY METAL STRESS AND PLANT RESPONSE

Heavy metal (HM) soil contamination is a critical environmental concern due to its serious and chronic toxic effects on plants. Biologists use the term HM for metals or metalloids with toxic effects to plants, it is also important to note that there are certain metals that at very low concentrations are quite beneficial for plant growth and development. HM are natural elements that occur naturally in the environment but contamination of soils by toxic metals and metalloids occurs due to human activities and is increasing at an alarming rate (Hossain, et al., 2011). There are two groups of HM's i.e. redox active and redox inactive. Directly involved in the redox reactions in cells are the redox active HM's and

they result in the production of O_2^- and later of H_2O_2 and $^{\cdot}OH$ (Dietz, et al., 1999; Schutzendubel & Polle, 2002). Oxidative stress results in plant cells that interact with inactive redox HM's as a result of indirect mechanisms such as contact with the antioxidant defence system, disruption of the electron transport chain, or initiation of lipid peroxidation (Hossain, et al., 2011). As a result of HM's toxicity MG production and accumulation increases, this is due to the disruption of the glyoxalse system that finally causes oxidative stress by reducing the glutathione (GSH) content (Hossain, et al., 2009; Hossain, et al., 2010; Singla-Pareek, et al., 2006; Hossain, et al., 2011; Hossain, et al., 2011).

A variety of physiological and metabolic changes are activated by HM toxicity (Villers, et al., 2011; Hossain, et al., 2011). HM toxicity causes a decline in plant growth (Sharma & Dubey, 2007; Hossain, et al., 2011) together with leaf chlorosis, necrosis, denaturation of enzymes, disruption of membrane integrity, tugor loss, a decrease in the germination percentage and a cripples photosynthesis (DalCorso, et al., 2008; Dalcorso, et al., 2010; Foy, et al., 1978; Sandalio, et al., 2001; Carrier, et al., 2003; Hossain, et al., 2011).

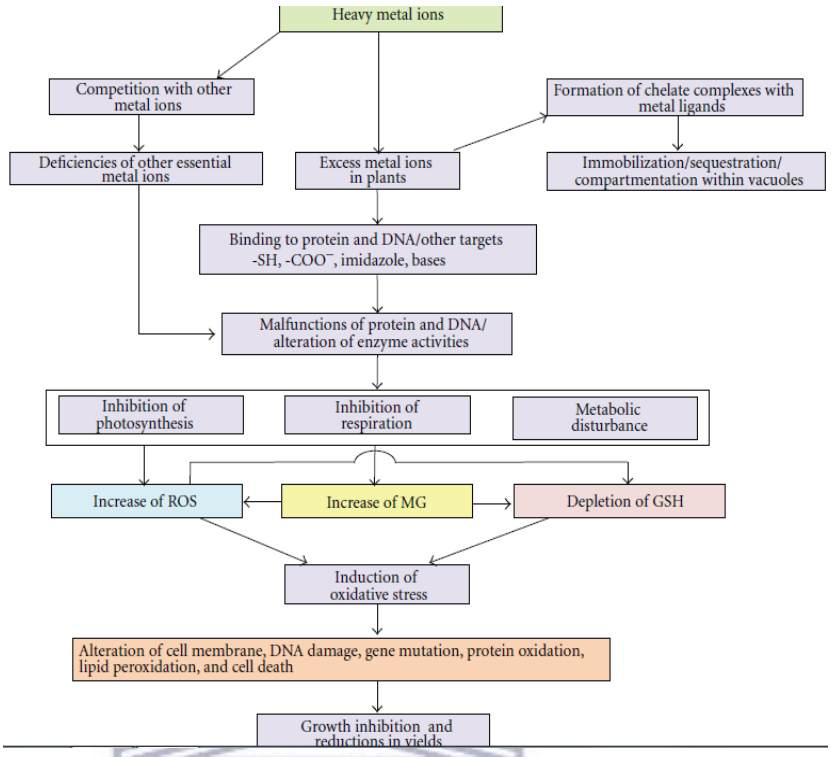

Figure 0-5 *Diagram showing the summary of plant response to HM ion exposure (Hossain, et al., 2011)*

Hossain M.A et.al (2011) concluded that HM toxicity results due to 3 main reasons also displayed in figure 1.5: i) by the stimulation of ROS and MG production or by modification of the antioxidant defence system and the glyoxalase system, ii) the HM targets structural, catalytic and transport sites of the cell due to the direct interaction HMs have with proteins because of their affinity for thioyl-,histidyl-,and carboxyl-groups or finally iii) essential metal ions are displaced from their binding sites this results in function collapse (Hossain, et al., 2011; Sharma & Dietz, 2009; Schutzendubel & Polle, 2002).

1.4.1 HEAVY METAL POLLUTION

As a result of industrial revolution through technological advancement heavy metal pollution is increasing at a high rate. Mining activities are a major source

of HM contamination along with other anthropogenic such as activities linked with fossil fuel, coal combustion, industrial sewages, solid waste disposal, fertilizers, and metal processing (Olande, 1987). Countries such as South Africa, Russia, Australia and Ukraine are known as mining countries and therefore experience HM contamination in high concentrations. Across South Africa a variety of mineral deposits can be found, to date this country is the world's main manufacturer of chrome, platinum group metals, manganese, vanadium, and vermiculite and the world's second largest producer of ilmenite, palladium, rutile, and zirconium (MininginSouthAfrica, 2014). These mining activities affect vegetation and some land sites cannot be used due to prolonged mining leading to serious contamination.

1.4.2 ZIRCONIUM BEHAVIOUR AND EFFECCTS

Zirconium (Zr) is one of the second most produced heavy metals in South Africa. It's a strong, ductile, malleable, transition metal with chemical and physical properties matching to those of titanium but lighter than steel, and as hard as copper (Louvel, et al., 2009). This HM is popular in the debate relating to the rising anthropogenic pressure on the environment and has since gained significant attention as a major pollutant (Shahid, et al., 2013). Zr naturally occurs in the earth's crust and natural levels of Zr in soil were reported to vary from 32 to 850 mg/kg (Kumpiene , et al., 2011) (Shahid, et al., 2013). Major soil and water pollution are caused by its uses in the past and present in anthropogenic activities in industry and nuclear reactors (Muhs, et al., 2010; Mushtaq, 2012) (Shahid, et al., 2013).

Zr is absorbed by the roots and generally more than 90% is absorbed and accumulates in plant roots (Krzeslowska, et al., 2009; Krzeslowska , et al., 2010; Shahid, et al., 2013). A low concentration of Zr has been found in the edible parts of plants even in very contaminated soils (Shahid, et al., 2013). A higher increase of Zr content in nodules and roots of legumes than in aerial parts was report and the same was found for soybean plants (Shahid, et al., 2013). The restriction in metal translocation may result from blockage, accumulation in plasma membrane, precipitation as insoluble Zr or immobilization by negatively charged exchange site within the cell wall, or sequestration in the vacuoles of rhizodermal and cortical cells (Krzeslowska , et al., 2010).

Stimulation or inhibition of enzymes in plant cells were reported after being exposed to Zr. A decrease in ascorbate peroxidase (APX) and glutathione reductase (GR) with an increase in peroxidase (POD) was reported, furthermore after application a marked decrease in the total phenol content of plant tissues was reported. (Mou, et al., 2011). The physiological changes in plants may explain the possible mechanisms responsible in Zr induced inhibition of growth or modification in enzyme activity (Pourrut, et al., 2008; Rascio & Navari-Izzo, 2011). Zr like other HM's in high concentration was found to delay germination and decrease plant biomass. There is still insufficient information about the mechanism of Zr in plants and more research would be worthwhile.

1.4.3 HEAVY METAL TOLERANCE

Physiological and molecular characteristics allow for certain plants to be tolerant to HM, understanding the mechanism is important (DalCorso, et al., 2008; Hossain, et al., 2010; Hossain, et al., 2011). The manipulation of glutathione-

related and phytochelatins (PC's) synthesis genes in plants are of interest (Yadav, 2010) to engineering HM tolerant plants. There are genes who's over expression in various plants has contributed to higher tolerance of HM accumulation, therefore, these genes have been found to be potential candidates for providing HM stress tolerance, genes such as γ-glutamyleysteine synthese (GSH1), glutathione synthetase (GSH2), cystathioine synthase (CTS), ATP sulfurylase (APS), serine acetyltransferase (SAT), glutathione reductase (GR), phytochelatin synthase (PCS) and glyoxalases (*glyoxalase I and II*) their regulation specialize in maintaining GSH and PC's levels (Yadav, 2010).

Different plant species may have evolved different mechanisms to tolerate excess HM's and even more than one mechanism could be in operation in one plant species (Mehag, 1994). Plants have both an adaptive in addition to the constitutive mechanisms to survive excess HM's (Mehag, 1994). Plants that are known to be tolerant to HM stress have evolved their production of specific HM transporters, induction of stress proteins, immobilization, signalling molecules such as salicylic acid and nitric oxide and induction of mechanisms contrasting the effects of ROS and MG (DalCorso, et al., 2008; Hossain, et al., 2009; Hossain, et al., 2010; Sharma & Dietz, 2009; Hossain, et al., 2011; Singla-Pareek, et al., 2006; Hossain, et al., 2011; Dalcorso, et al., 2010; Foy, et al., 1978).

1.5 REACTIVE OXYGEN SPECIES

Reactive oxygen species (ROS) are forms of molecular oxygen normally produced in cells by the transferal of electrons resulting in partially reduced or excited oxygen species and have significant parts in cell signalling and homeostasis (Mehdy, 1994; Davasagayam, et al., 2004). The generation of ROS

is a basic characteristic of any living organism, producing: superoxide radical (O_2-), hydrogen peroxide (H_2O_2), and hydroxyl radical (.OH) (Wojtaszek, 1997). Plants produce ROS in the chloroplast during photosynthesis, via membrane-bound NADPH oxidase complexes and all 7 isoforms found in the cell membranes, in the mitochondria during respiration, in peroxisomes during photorespiration and fatty acid oxidation and in the endoplasmic reticulum (ER) this is the same for plants and animals (Han, et al., 2001) (Wojtaszek, 1997). As displayed in figure 1.6 superoxide (O_2-) is the first ROS produced during the normal process of adenosine triphosphate (ATP) production called oxidative phosphorylation. In this process hydrogen ions are transpired through the inner mitochondrial membrane by the electron transport chain where electrons are passed across a series of proteins via oxidation-reduction reactions, the last destination for an electron along this chain is an oxygen molecule (Xinyan, et al., 2013). This whole process would then result in the oxygen producing water however; the oxygen can instead be prematurely and incompletely reduced to form a superoxide radical due to the electrons passing through the chain (Xinyan, et al., 2013) and this is mainly due to complex I and complex III in the mitochondria.

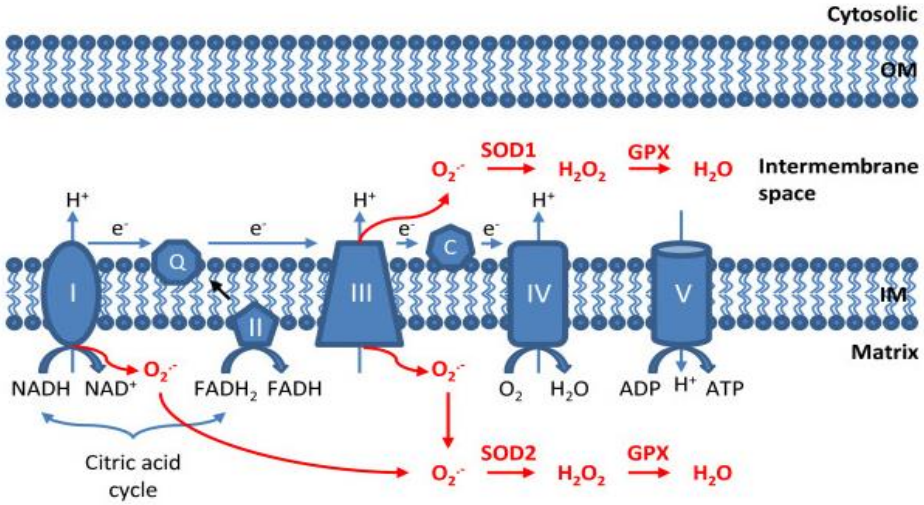

Figure 0-6 *Production and disposal of mitochondrial ROS (mt ROS) generated by Xinyan, L. et al (2013).*

Superoxide on its own is not reactive, but after a variety of reactions superoxide results in the production of hydrogen peroxide, hydroxyl radicals and other ROS which all are capable of causing damage in various ways (Moller, 2010) (Xinyan, et al., 2013). The ROS then react with proteins attacking the backbones, nucleic acids causing mutations and lipids resulting in structural changes (Brooker, 2011).

Early research concerning ROS metabolism was fixated on the toxicity of ROS resulting in cell injury and tissue dysfunction and it also paid close attention to the different ROS-scavenging mechanisms, such as the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), and glutathione reductase (GR), ROS can also be scavenged by low molecular weight antioxidants such as proline (PRO), cysteine (Cys), nonprotein thiol (NPT), ascorbic acid (AsA), and glutathione (GSH), more

current studies have concentrated on the role ROS play as signalling molecules (Moller, 2010) (Wojtaszek, 1997).

To make use of ROS as a signalling molecules, non-toxic levels must be maintained in a balancing act between ROS production, involving ROS-producing enzymes or the unavoidable production of ROS during basic cellular processes, and the metabolic regulatory route involving ROS-scavenging pathways and ROS scavenging. The benefits of ROS at low concentrations have been identified as follows: ROS is able to activate the defence response of plants against abiotic stress, they trigger programmed cell death in plants (PCD), they signal call growth and differentiation, activate defence against pathogens and are secondary messengers involved in signal transduction pathways that regulate plant responses to stress (Baxter, et al., 2013). It has been concluded that a balance between ROS production and scavenging ROS determines stress tolerance.

1.5.1 DEFENCE LINE IN PLANTS AGAINST ROS

As a consequence of HM stress plants over produce ROS and most of the ROS is known to be produced in the mitochondria. It was also discovered that plants have defence lines present to try and limit ROS production, detoxify it after being formed and to repair cellular damages caused by ROS activity.

1.5.1.1 AVOIDANCE OF ROS PRODUCTION

In an attempt to avoid ROS accumulation the electron transport chain is kept in a sufficiently oxidized state by assuring a sense of balance between substrate availability and ATP requirement (Moller, 2001). ROS production in the

mitochondria of various organisms can also be avoided by the activation of the alternative oxidase (AOX), an enzyme that forms part of the ETC (Moller, 2001). The AOX is expressed during stress and is beneficial as it enhances the organism's ability to resist stresses by reducing the level of oxidative stress (Maxwell, et al., 1999; Vanlerberghe & Mclntosh, 1997). The activation of uncoupling proteins (UCPs) is also beneficial, UCPs 1-3 are mitochondrial carrier proteins that are reported to play important roles in minimizing ROS emission from the ETC, they reduce ROS emission by providing a negative feedback loop for mitochondrial ROS production the loop results after the UCPs 2 and 3 are activated by ROS to induce proton leak (Mailoux & Harper, 2011) (Azzu & Brand, 2010) (Moller, 2001). The activation of rotenone-insensitive NAD (P) H dehydrogenases are also vital for the avoiding over production of ROS (Moller, 2001).

1.5.1.2 DETOXIFICATION OF ROS

Under normal conditions, antioxidant systems of the cell minimize the distresses caused by ROS. When ROS generation is increased due to stress to an extent that overcomes the cellular antioxidants, the result is oxidative stress (Mates, 2000). Antioxidants are substances that delay or inhibit the oxidation of cellular oxidizable substrates. The various antioxidants exert their effect by scavenging superoxide O_2^- as it is the first ROS produced after O_2^- is produced superoxide dismutase (SOD) scavenges the superoxide resulting in hydrogen peroxide H_2O_2 which is also a ROS and causes cellular damage. The H_2O_2 is further scavenged by catalase (CAT) resulting in O_2+H_2O, the H_2O_2 could also go through the ascorbate / glutathione cycle resulting in H_2O, or the hydrogen peroxide could

also be reduced to form H_2O via the glutathione peroxidase system or thioredoxin (Trx) reductase system (Moller, 2001).

1.5.1.3 REPAIR OF ROS-MEDIATED DAMAGE

Superoxide is the first mediator of ROS damage because it cannot diffuse easily throughout the cell and has been hypothesized to mostly target mtDNA (Kirkinezos & Moraes, 2001), H_2O_2 being the next key player in mitochondrial derived ROS is not a free radical and due to its small size and relatively benign reactivity compared to the rest of the ROS H_2O_2 can diffuse freely across several cell radii therefore it facilitates toxic effects far from the site of ROS production its role in ROS mediated damage is extremely significant by virtue of chemical versatility and infusibility (Esposito, et al., 1999; Kirkinezos & Moraes, 2001). When H_2O_2 reacts with free transition metals via the Fenton reaction (Fe2+ + H2O2 ↔ Fe3+ + OH + OH•), it produces the extremely reactive hydroxyl radical OH•. OH• has a very short half-life and reacts with almost any molecules in close proximity (Kirkinezos & Moraes, 2001). Nitric oxide (NO) too has been reported in ROS-mediated damage by inhibiting cytochrome oxidase and mitochondrial respiration (Brown, 1997; Kirkinezos & Moraes, 2001). To repair the mitochondria after ROS mediated damage fatty acid hydroperoxides, by glutathione peroxidase proteins are activated for production

1.6 PRODUCTION OF METHYLGLYOXAL

Glycolysis intermediates glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) naturally form MG which can also be called pyruvaldehyde or 2-oxopropanal, which is a cytotoxic compound (Richard, 1993; Hossain, et al., 2009; Yadav, et al., 2005) (Lee, et al., 2005). MG has a

molecular formula of $C_3H_4O_2$, with an average mass of 72.06266 g/mol, and a boiling point of 72°C (Batchelor, et al., 2014).MG production is unavoidable and it increases during stress as the rate of glycolysis increases (Richard, 1993; Hossain, et al., 2009). DHAP is used in the formation of ene-diol(ate) phosphate intermediates in the initial step of this reaction pathway, secondly phosphate is eliminated and breakdown of the ene-diol(ate) to form methylglyoxal as an alternative to forming the isomer GAP (Saadat & Harrison, 1999). Displayed in figure 1.7 is the production process that has been reported to take place in yeast, animals and higher plants.

Through enzymatic reactions of GAP and DHAP, MG can also be formed. Pompliano et al. (1999) showed that the triosephosphate isomerase hydrolyzes G3P and DHAP and the removal of the phosphate results in MG (Pompliano , et al., 1999; Hossain, et al., 2011). MG is also proven to be part of the production of advanced glycation end-products (AGEs) (Shinohara, et al., 1998) which are substances that accelerate the deterioration of a degenerative disease (Vistoli, et al., 2013). The MG forms the AGEs when it attaches to the free amino groups of lysine and arginine and with thiol groups of cysteine, this was reassured when the heat shock protein 27 (Hsp 27) was identified and concluded that it's interaction with MG led to posttranslational modifications in human metastatic melanoma cells (Bair, et al., 2010). The production of MG is natural but its increase during stress needs to be controlled.

1.6.1 METHYLGLYOXAL IN OTHER ORGANISMS

Methylglyoxal is better understood in other organisms than in plants, its levels have been stated to rise due to stress in most animals, mammals, yeast and

bacterial systems (Cooper, 1984; Abordo, et al., 1999; Kalapos , et al., 1992; Hossain, et al., 2009). In yeast (*Saccharomyces cerevisiae*) it was found to signal numerous signal transduction pathways, such as transcription factors Yap1 and Msn2 and triggers Hog1 a mitogen-activated protein (MAP) kinase cascade (Maeta, et al., 2005; Takatsume, et al., 2006; Hossain, et al., 2011).

In humans MG was found to bind to the nerves responsible for pain registration leading to an increased pain sensation (Bierhaus, et al., 2012). MG is believed to have a medicinal property in the Manuka honey which was found to have antibacterial activity in vitro, there is still insufficient information about this honey but it has potential and MG is thought to be the major antibacterial component (Lusby, et al., 2002) (Majtan, et al., 2012). In diabetic patients MG is in higher concentrations the increase causes low-density lipoproteins damage resulting in arterial atherogenesis (Rabbani, et al., 2011).

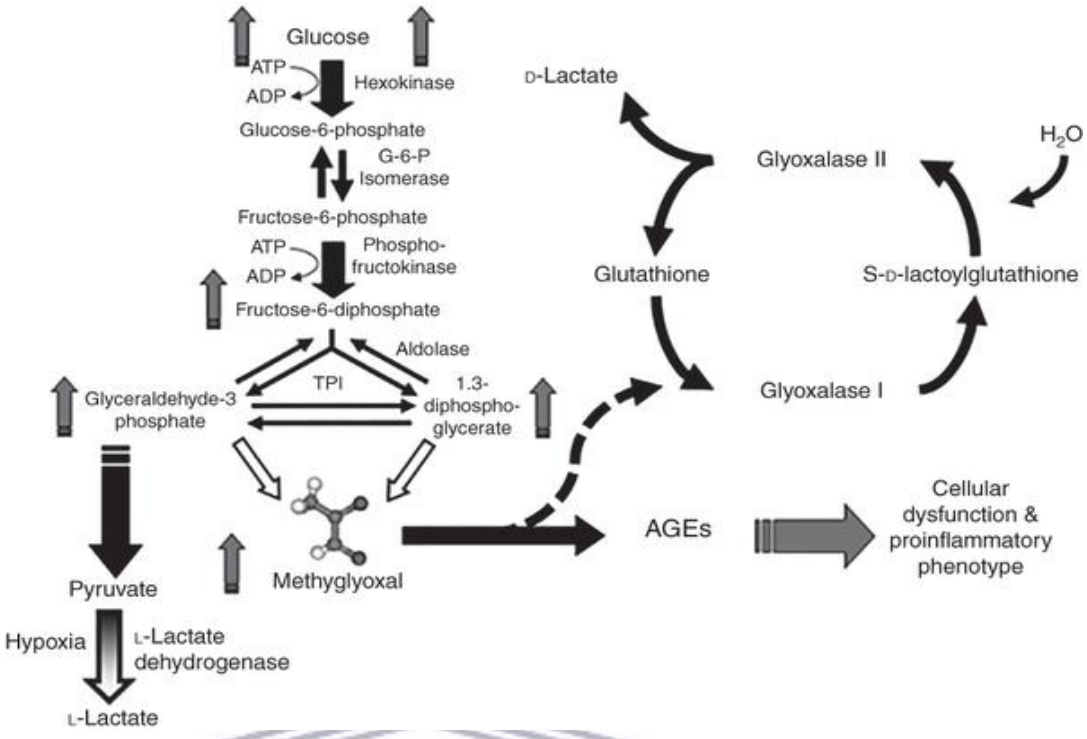

Figure 0-7 *The image displays the formation and breakdown of MG in a biological system (Kiefer, et al., 2014).*

1.6.2 METHYLGLYOXAL IN PLANTS

Insufficient information has been done in plants concerning MG, and the biological significance of the pathway it's involved in has just began to be explored (Espartero, et al., 1995; Singla-Pareek, et al., 2003; Yadav, et al., 2005) . The signalling activities it has in *Saccharomyces cerevisiae* (Maete, et al., 2005; Yadav, et al., 2005), is reason to investigate whether or not it could act as a signal molecule in plants. Yeast belong to the Fungi kingdom and this kingdom is very similar to the plant kingdom in cell structure, reproduction processes, mobility as both are stationary organisms and both their ecological roles are crucial to the function of a healthy ecosystem (Brunus, 2006).

It was proven in 2005 that most plants have their own concentration of MG which ranges from 30-75μM and this concentration increases during stress 2 to 6 fold

(Yadav, et al., 2005). Hossain et al. (2009) proved that the build-up of methylglyoxal in plants under different stressful circumstances is a common phenomenon, in the study 5 day seedlings were stressed with low temp (4°C), high temperature (42°C), heavy metal (1mM $CdCl_{2)}$, drought, salinity (300mM NaCl) MG (25mM), 2,4-D (50µM), ABA (50µM) and white light (60µmol photon m^{-2} s^{-1}) stresses for 24 hours figure 1.8 displays the results (Hossain, et al., 2009). This proved that most abiotic stresses activate an increase in MG production. Investigating MG accumulation during biotic stress would be worthwhile too.

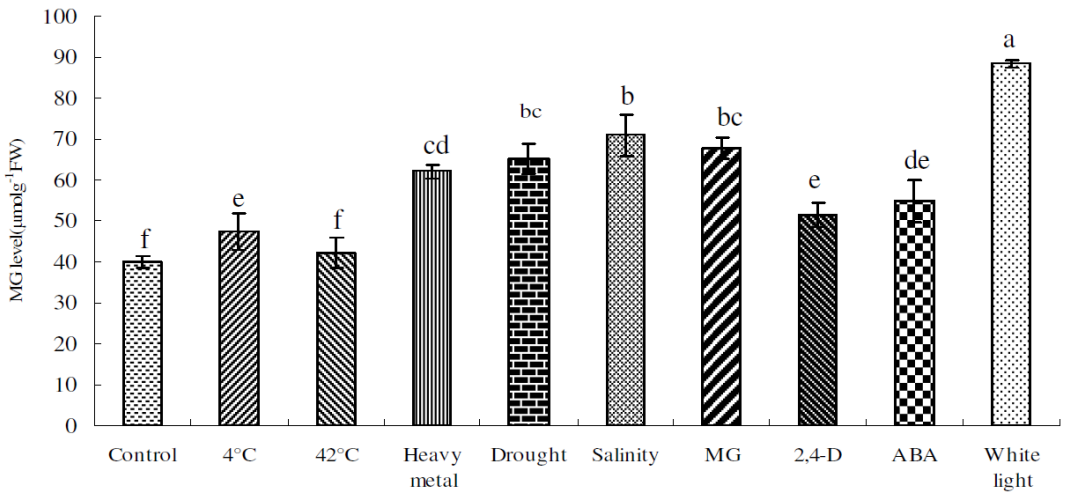

Figure 0-8 *Displays the MG levels of various abiotic stresses on 5 day old pumkin seedlings the stresses are as follow: low temperature (4°C), high temperature (42°C), heavy metal (1 mM CdCl2), drought, salinity (300 mM NaCl), MG (25 mM), 2,4-D (50 µM), ABA (50 µM) and white light (60 µmol photon m-2 s-1) stresses for 24 hours (Hossain, et al., 2009).*

1.6.3 METHYLGLYOXAL DETOXIFICATION SYSTEM

Adapting to a detoxification system for overproduction of MG produced during abiotic or biotic stress is important because its accumulation can cause serious

damage to the organism. Numerous detoxification pathways for methylglyoxal exist; different enzymes are used in these pathways to convert methylglyoxal to a less toxic compound (Caspi, et al., 2014). Included in these enzymes are: glyoxalase enzymes, methylglyoxal reductases, aldose reductases, aldehyde reductases and methylglyoxal dehydrogenases (Caspi, et al., 2014). There are 8 known methylglyoxal degradation pathways from different living organisms. For example in *Cyanobacterium Synechococcus* MG is detoxified by an aldo-keto reductase (Xu, et al., 2006) .

1.6.3.1 GLYOXALSE SYSTEM

The glyoxalse system is known to be the major detoxifying system for MG in plants taking place as displayed in fig. 1.9. It occurs in several cellular organelles such as the mitochondria but it mostly takes place in the cytosol of cells (Hossain, et al., 2011). This pathway has a fundamental importance in biological systems as it is common in living organisms.

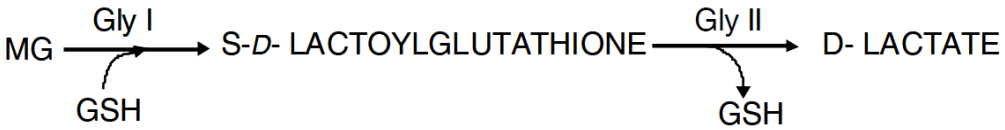

Figure 0-9 *MG detoxification via the glyoxalase system with two enzymes catalysing the production of D-Lactate with reduced glutathione (GSH) as a co-factor.*

The glyoxalase system has two enzymes which work together in two steps to transform MG as well as 2-oxoaldehydes to their 2-hydroxyacids with GSH (Thornalley, 1990) (Hossain, et al., 2011) resulting in irreversible reactions. Glyoxalase I (Gly I; EC 4.4.1.5) with GSH as a cofactor is used in the first step

to convert MG to S-D-Lactoylglutathione (SLG) after forming hemithioacetal, glyoxalase II (Gly II, EC 3.1.2.6) is responsible for catalysing the hydrolysis of SLG to D-lactate releasing GSH back into the system. The metabolites involved in this pathway are also known to be beneficial for plants during stress.

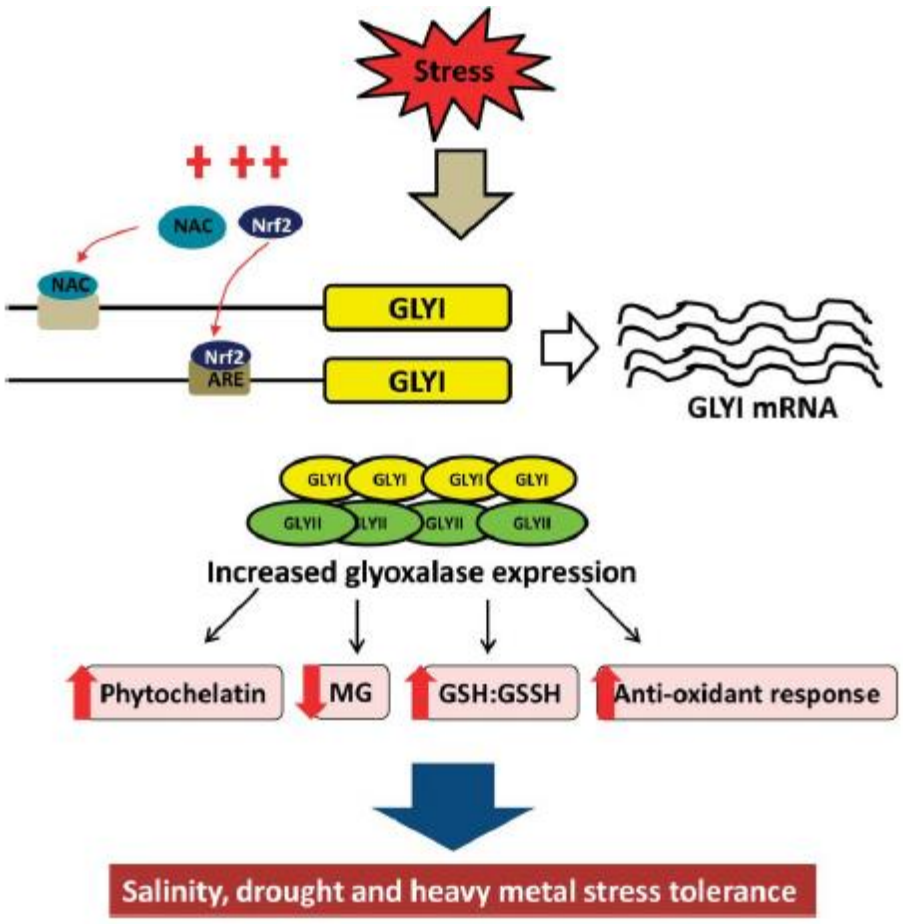

Figure 0-10 Depicts the mechanism of induction of glyoxalase and associated stress tolerance*. Increased glyoxalase expression, as a result of stress and also through over-expression in plants, induces tolerance to various stresses by lowering MG levels and stimulating raising of phytochelatin, GSH: GSSH and anti-oxidant response (Kaur, et al., 2015).*

HM tolerance in plants that involved alteration of the glyoxalase pathway has been reported by Reddy V and Sopory (1999) and Singla-Pareek et.al (2006) they testified to the fitness of this alteration strategy for enhanced HM tolerance in transgenic tobacco. These transgenic tobacco plants grew, flowed and had normal viable seeds in the presence or 5mM $ZnCl_2$ without any yield consequence. MG and MDA accumulation under high levels of Zn was controlled in these transgenic plants, proving that the detoxification system works hand in hand with a HM tolerant plant. Kaur et.al 2015 also displayed that with enhanced GlyI in rice can result in rice that is tolerant to abiotic stress as displayed in figure 1.10.

1.6.3.2 PATHWAY COFACTORS

In most plants glutathione (GSH) is known to be the main source of non-protein thiols because of the chemical reactivity of the thiol groups the GSH can function in plenty biochemical reactions in most organisms (Bergmann & Rennenberg, 1993; Chengbin, et al., 2001). GSH was proven to be a significant metabolite which plays a role in protecting plants from oxidative stress due to various stresses and it's a major cellular antioxidant cofactor. Further analysis to prove that GSH has a role in protection of plants from environmental stress was conducted by Chengbin X et.al (2001) by studying genetically modified *Arabidopsis* with different levels of GSH. The modified plants had GSH levels ranging from 3% to 200% of the parent plants. Hypersensitivity to Cd in plants with low GSH levels resulted and plants with 50% wild-type levels of GSH required higher Cd concentrations to inhibit growth. Chengbin X et.al (2001) also concluded that increasing GSH levels does not increase metal resistance. This

conclusion encourages the focus on MG as a potential agent to trigger growth and development in plants in controlled concentrations.

The availability of GSH strongly affects MG detoxification. Shortage of GSH restricts the process of hemithioacetal production, resulting in the accumulation of MG. It was also discovered through experimental work that exogenous applied GSH lowers MG levels (Yadav, et al., 2005).GSH can aid the plants to resist accumulation of MG but GSH is only useful if the plant has a competitive level of *gly I* (Yadav, et al., 2005).

1.6.3.3 S-D-LACTOYLGLUTATHIONE (SLG)

SLG is an intermediate of the glyoxalase system it was discovered that it can enter the mitochondria and get hydrolysed with the mitochondrial *gly II* enzyme to D-lactate and release GSH. This function is vital for the cell as mitochondrial DNA and its membranes get damaged during oxidative stress. So the ability of SLG to enter the mitochondria from the cytosol is vital in maintaining mitochondrial functionality (Armeni, et al., 2014).

The mitochondria is the main source of (ROS) this is because cellular respiration occurs in this organelle and in turn it is the organelle most exposed to damage by oxygen radicals (Armeni, et al., 2014; Kaelin Jr, 2005), an efficient antioxidant defence system is present in the mitochondria to maintain its normal function in the presence of the oxygen species (Armeni, et al., 2014; Cardenas & Davies, 2000), and in particular the GSH/GSSG couple is considered the most important redox homeostasis (Albrecht, et al., 2011; Reliene & Schiestl, 2006; Yin, et al., 2012) .

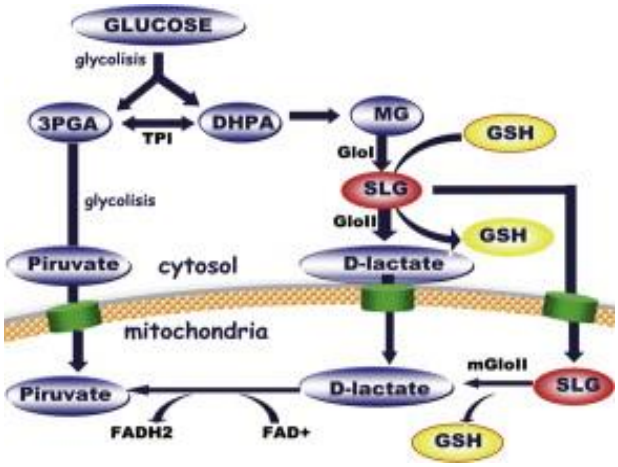

Figure 0-11 Displays a graphical explanation of what Armeni T et.al (2014) proved after a series of experiments*. SLG enters the mitochondria and is hydrolysed by mitochondrial glyII to produce D-Lactate and GSH (Armeni, et al., 2014).*

The disadvantage of the mitochondrion is that it does not have enzymes for GSH synthesis therefore GSH must be transported from the cytosol to the mitochondria this result in a low count of total GSH in the mitochondrion (10-15%) with 80-85% of GSH found in the cytosol (Armeni, et al., 2014; Griffith & Meister, 1985). As displayed on figure 1.11 the SLG is an alternative supplier of GSH in the mitochondria, after SLG is hydrolysed by mitochondrial *gly II* it releases GSH (Armeni, et al., 2014) which increases the GSH content inside the mitochondrion which can then be used to protect the mitochondrial DNA and membranes.

1.6.3.4 D-LACTATE

Lactic acid is produced by many living organism and it has a chiral structure with two visual isomers known as the L-(+)-Lactic acid and its mirror image is D-(-)-Lactic acid. Alan.M et al (1990) reported that the L-lactic acid and its polymers promote plant growth and increase plant biomass the monomeric lactic acid and

polymers of D-Lactic acid displayed no biological activity in plants. Recently in a medical paper for Parkinson's-disease it was concluded that D-lactate supports mitochondrial membrane potential (Toyoda, et al., 2014) and similarly in plant research D-Lactate was found to be beneficial to the plants mitochondria because after SLG enters the mitochondria and is hydrolysed by mitochondrial *glyII* to produce GSH and D-lactate the D-Lactic acid can be oxidized and so generating mitochondrial membrane potential (Armeni, et al., 2014).

1.6.4 THE ENZYMES INVOLVED IN THE GLYOXALASE PATHWAY (GLY I & GLY II)

Glyoxalase I (*gly I*) has a significant role in the detoxification of methylglyoxal which is formed as a sign of stress in plant (Kumar, et al., 2014). It catalyses the production of SLG from MG with the cofactor GSH, plants over producing *glyI* have been proven to tolerate abiotic stresses such as salinity better (Veena & Sopory, 1999) (Yadav, et al., 2005) as it maintains low MG levels within the plant cells. Glyoxalase II (*gly II*) plays a role in maintaining GSH levels in the cell as it returns the GSH used by *gly I*, this GSH is then used by the cell to continue the detoxification. There is an abundance of these two enzymes with in all plant cells especially in the cytoplasm (Armeni, et al., 2014).

Transgenic plants comparison of relative tolerance experiment was performed, where either *gly I* or *gly II* was over expressed or both at the same time, this experiment revealed that both enzymes being over expressed performed better than having just 1 over expressed (Yadav, 2010).

1.6.5 ROLE OF GSH AND PHYTOCHELATINS IN HM STRESS TOLERANCE

GSH plays a role in MG detoxification as a cofactor in the glyoxalase pathway it also plays a role in the ROS detoxification system via the ascorbate-glutathione cycle (Yadav, 2010). GSH is also a precursor for the synthesis of phytochelatins (PC's) which are a set of HM-binding peptides which are found in some eukaryotes, including higher plants (Gekeler , et al., 1989). PC's are synthesised from GSH by PC synthase (PCS) during exposer of the plant to HM. They have been reported to have a role in HM detoxification as well as in the maintenance of ionic homeostasis (Hirata, et al., 2005; Yadav, 2010). It was also proven that transgenic plants over expressing the glyoxalase pathway enzymes individually and both together in the same pant maintained higher levels of GSH and PC's and they were tolerant to HM stress (Singla-Pareek, et al., 2006). Once GSH levels are maintained during heavy metal stress, PCS become active and catalyses the formation of a PC-metal complex. The PC-metal complex can then be transported into the vacuole and form high-M_r complexes which are the ultimate and most stable storage of HM's in plants (Mendoza-Cozalt, et al., 2008; Vatamaniuk, et al., 2000).

1.7 CONCLUSION

This literature study attempts to highlight that Soybean production in South Africa should be increased to improve the country's economy and to help the country meet its domestic demand for the crop. South Africa could then later export its produce to other African or international countries. This review also attempts to draw attention to the possibility of MG a natural plant molecule as a

possible mediator between abiotic stresses faced by South African crops and crop yield in the country. This phenomenon could possibly be achieved with a controlled MG concentration in a plant triggering transduction signalling growth and development. Thorough clarification of MG metabolism by integrating other "omics" such as proteomics and metabolomics, and dissecting its signalling roles by using model plant species would be useful research to improve multiple abiotic stress tolerance. Thorough clarification is also required about whether or not this molecule would block or increase plant nutrient uptake in the presence or absence of HM stress. Crop yield for most African countries is affected by various abiotic stress. Crop yield could drastically increase when this compound is thoroughly investigated and the perfect concentration is attained to enable local farmers to treat their field with it. Further studies on plant's behaviour in response to Zr are needed; these studies should emphasize on the mechanisms that are involved in uptake and phytotoxicity of Zr and should assess the potential health risks associated with Zr-polluted plants that may be consumed by humans. ROS forms a good platform for branching off into further studies for MG and together these previously malicious associated cytotoxins could be the solution for the future taking into perspective the growing rate of humans that need good nutritious food and the fact that SA face many abiotic stresses.

1.8 JUSTIFICATION

The majority of South African landscape is not favourable for crop growth due to high levels of HM pollution after many years of mining. Provinces like Gauteng have been reported to have areas that are unable to support crop growth due to HM pollution (Okedeyi, et al., 2014; Turkdogan, et al., 2002; Rascio &

Navari-Izzo, 2011; Fatoki, 1996). South Africa to date is the world's second largest producer of Zirconium and its toxic effects have recently attracted interest in the mineral and agricultural sector as a major pollutant (Shahid, et al., 2013) therefore developing ways in which plants can be tolerant to Zr stress is worthwhile. Studies have also proven that HM's can spread to surrounding cities and even neighbouring countries via water bodies including underground water systems (Yabe, et al., 2010). Most farms and private households opt to using ground water for water irrigation system, this could unintentionally lead to crops and humans being exposed to HM pollution. This is detrimental to SA's population as it results in the need to import crops for animal and human consumption that could have been home grown. Soybean is one such crop, a vast percentage of consumed soybean in South Africa is imported from Argentina (Dlamini, et al., 2014). Soybean like any another crop has favourable climate conditions that are not found throughout the provinces. In the past 25 years on 3 provinces are leading in soybean production instead of more. Various natural factors cause this setback such as drought and HM pollution. The government too have recognized that SA needs to produce more soybean and plans on improving this protein high crop are being made. One possible solution this study aims to bring fourth is the use of natural molecules that plant naturally produce ie methylglyoxal. Despite the recognized toxic nature of MG accumulation in living organisms the existing reports based on animals and yeast studies could be a suggestion that similar signalling occurs in plants and it is worth investigating (Kaur, et al., 2015). In yeast, MG has been shown to act as a signal initiator during oxidative stress (Maeta, et al., 2005). Current research like the study conducted by Kauer, et al., 2015 gives glimpse of hope for this theory but further studies on

how exogenous treatment could affect plant mechanisms is worth investigating, if MG can be absorbed in the presence of a HM and what effect it has on nutrient uptake is worth looking into. As nutrient uptake is essential for plant growth and development. The world population is increasing at an alarming rate and South Africa is not exempted from this, more so the country is experiencing setbacks that can't be controlled such as HM toxicity as a result of many years of mining and global warming. Having tolerant plants would be highly beneficial for the country, economically as well as human well-being.

1.9 OBJECTIVES

The purpose of the current study is to conclude on the possibility, if any, of MG as a trigger of a signalling cascade, comparable to recent evidence of MG signalling in yeast and comparable to ROS as a vital player in plant stress signalling. A physiological study was conducted where MG treated soybean plants were compared with control to observe if MG promotes growth and whether MG will improve tolerance of plants exposed to Zr stress. Biochemical investigations also commenced exploring the signalling role of MG in the defence (antioxidant – ROS) systems, uncovering the internal effects of exogenously applied Zr, investigating the effects of exogenously applied MG on *Glycine max* in the presence and absence of Zr stress. Furthermore, a mineral analysis took place to disclose the nutrient uptake of *Glycine max* exogenously treated with MG, investigate nutrient uptake of *Glycine max* exposed to Zr stress and investigate nutrient uptake of *Glycine max* exogenously treated with MG during Zr stressed.

2 CHAPTER TWO

MATERIALS AND METHODS

2.1 *GLYCINE MAX* GERMINATION, TREATMENTS, HARVESTING AND STORAGE

Healthy *Glycine max* seeds from 4 °C storage were surface sterilized with sodium hypochlorite (0.35% v/v; for 10 minutes), rinsed thoroughly with distilled water (dH_2O) thereafter the seeds were imbibed in dH_2O for 1 hour. The seeds were then germinated in darkness on wet tissue paper for 3 days. Thereafter plants of uniform phenotypes were selected and transferred to 20cm brown pots. Each pot received 3 seedlings, sown approximately 5 cm deep in a soil mixture consisting of compost-enriched potting soil and filter sand (1:3). The experimental design included a control (water only), 6 µM Methylglyoxal (MG), 1 mM Zirconium (Zr) and 6 µM MG/+1 mM Zr. The concentration of 6 µM MG was selected based on a previous experiment were seeds were germinated in the presence of different concentrations of MG, the chosen concentration had the highest germination percentage and seedling biomass, in that experiment. Treatments were administered twice per week in 200 ml doses. Plants were harvested after 21 days of treatment. Some of the plants were ground to a fine powder in liquid nitrogen using a sterile mortar and pestle. Ground-up material was immediately transferred to new 50 ml Greiner tubes which were stored at -80°C. The frozen ground material was kept for the necessary lab experiments.

2.2 DRY WEIGHT DETERMINATION IN *GLYCINE MAX* SUBJECTED TO MG AND ZR TREATMENTS RESPECTIVELY

Foil envelopes were made in replicates of three per respective treatment. Each envelope contained 4 *Glycine max* leaves or roots the envelops were the placed in an oven overnight at 80°C in order to remove water content within the plant. Thereafter, the dried material was weighed and compared.

2.3 PROTEIN EXTRACTION AND QUANTIFICATION

Frozen ground leaf material (100mg) was added to an eppendorf tubes (1 tube per plant sample). Protein extraction buffer was added per plant sample (0.7ml) [0.004M phosphate buffer, 1mM EDTA, 5% (w/v) PVP]. Afterwards, 4 minutes of vortexing followed, and thereafter the samples were pelleted with a centrifuge at 12000 xg for 10 minutes. The supernatant was transferred into a clean eppendorf tube. The protein was then quantified using the Bradford assay then the protein was stored at -20°C.

2.4 BIOCHEMICAL ASSAYS PERFORMED ON *GLYCINE MAX* SUBJECTED TO MG AND ZR TREATMENTS RESPECTIVELY

2.4.1 Evans blue

The cell death level in leaf material was measured by using the Evans blue stain following a modified method of Sanevas et al. (2007). Into a 2 ml eppendorf tube. 1 ml of 0.25% (w/v) Evans blue solution was added, thereafter the tube with the solution was weighed. The sample was added as 1 cm^3 block of fresh leafs or 4 cm of root material (from the root tip) and the tube was then weighed to determine the weight of the sample. An hour incubation commenced and the Evans blue was then rinsed off with dH_2O. The sample was crushed after the addition of SDS (w/v). Following an hour long incubation at 65°C on a heating block, the sample was

centrifuged for 10 minutes at 13 000 xg to pellet the plant material and obtain the supernatant. The supernatant (200 µl) was then added into a microtiter plate and the absorbance was measured at 600 nm on a spectrophotometer.

2.4.2 Superoxide content determination

The superoxide content was determined by adding a 10 mM KCN, 10 mM H_2O_2, 2% SDS, 80 µM NBT made up to 800 µl with 50 mM potassium phosphate (pH 7.0) into an eppendorf tube with 8 X 1 cm^3 blocks of leaf sample and 4 cm of root material from each treatment. This is a light sensitive experiment so the eppendorf tubes were covered foil and brown tape. The samples in the solutions were incubated for 20 minutes then crushed. Thereafter the samples were centrifuged at 13 000 xg for 5 minutes. Then the supernatant was added into a clean tube and was briefly centrifuged to ensure that all plant material is removed. The clean supernatant was added into the microtiter plate and the absorbance was read at 600 nm.

2.4.3 Malondialdehyde (MDA) determination

A modified method from Zhang et al. (2007) was used to measure the level of lipid peroxidation by estimating malondialdehyde (MDA) content in the leaves and roots of *Glycine max*. The leaf and root samples respectively (100 mg) from each treatment were added into a 1.5 ml eppendorf tube. Thereafter 5 vol of 6% (w/v) Trichoroacetic acid (TCA) was added followed by a short mixing with a vortex. The sample was pelleted at 13 000 xg for 10 minutes and thereafter 400 µl of supernatant was removed and added to a new tube. Into the new tube, 600 µl of 0.5% TBA (which was made up in 20% TCA) acting as the reactive material was added. The sample was then briefly vortexed and thereafter the lid of the eppendorf

tube was wrapped with parafilm. The sealed eppendorf tubes were then placed on a heating block at 90°C for 20 minutes immediately thereafter the sample was incubated on ice for 10 minutes. The sample was then centrifuged for 5 minutes at 13 000 xg. The clean supernatant was added into the microtiter plate and its absorbance was measured at 532 nm and was corrected for nonspecific absorbance at 600 nm, thereafter the OD received at 600 nm was subtracted from the OD received at 532 nm. The concentration of MDA was calculated by using the extinction coefficient of 155 $mM^{-1}.cm^{-1}$ and expressed as nanomoles of MDA per gram fresh weight.

2.4.4 Hydrogen peroxide (H_2O_2) determination

Frozen ground leaf and root material (100 mg) were transferred into a 1.5 ml eppendorf tube. To each tube 5 vol of 6% Trichoroacetic acid (TCA) was added. The tubes were briefly mixed by vortexing followed by centrifugation at 13 000 xg for 10 minutes. Samples were loaded in triplicate into a 96 well microtitre plate by adding 50 µl of TCA extract, 50 µl of 5 mM K_2HPO_4 and 100 µl of 500 mM KI in each well. H_2O_2 dilution standards were prepared and loaded in triplicates in the microtitre plate, absorbencies were measured at 235 nm and the final H_2O_2 concentration was determined from the standard curve.

2.4.5 Superoxide dismutase activity

Protein was extracted and quantified as mentioned in section 2.3. The protein 200 µg was then added into the microtiter plate with phosphate buffer pH 7.5 (20 mM), NBT (0.1 mM), EDTA (0.1 mM), Riboflavin (0.005 mM), methionine (10 mM) and made up to 200 µl with dH_2O. The absorbance was measured at 560 nm and SOD activity for each sample was calculated.

2.4.6 Ascorbate peroxidase (APX) activity determination

Total proteins were extracted and quantified as described in sections 2.3 from frozen ground up *Glycine max* leaves and roots. Aliquots of the protein extracts were decanted to 0.5 ml and incubated with 2 mM ascorbate for 5 minutes. The resulting mixture was used as the sample and 10 µl for each respective treatment was prepared in a 96-well mictrotitre plate in triplicate. To each sample, 71.43 mM K_2HPO_4 and 0.36 mM ascorbate was added, 0.714 mM H_20_2 was added to activate the reaction and before absorbance values were read at 290 nm. The reaction volume was made up to 200 µl. The Ascorbate peroxidase activity was determined using the extinction coefficient of 2.8 $mM^{-1}.cm^{-1}$.

2.4.7 Methylglyoxal determination assay

The methylglyoxal determination assay employed in this study is a modified version of Hossain et al. (2009) experiment which was originally performed on tobacco plants. To an empty 2ml Eppendorf tube 1.5 ml of 0.5 M Phosphoric acid was added onto frozen ground leaf material (250 mg). The tubes were vortexed for 1-2 minute followed by an incubation on ice for 15 minutes. After vortexing a centrifugation step at 13 000 rpm for 10 minutes followed. The supernatant was transferred into 2ml Eppendorf tube with activated charcoal (10mg/ml). Tubes were inverted a few times to mix and incubated at room temperature for 15 minutes after which it was centrifuged at 13 000 rpm for 10 minutes. The supernatant was collected and 400 µl of saturated KOH was added to neutralise the samples. The neutralised supernatant was incubated for 15 minutes at room temperature followed by a centrifugation step at 13 000 rpm for 10 minutes.

Samples were loaded in triplicate into a 96 well microtitre plate in addition to adding 20µl of 5 M Phosphoric acid, 130 µl sample and 50 µl of 7.2 mM Diaminobenzene. Standards were prepared in triplicate as well. The plate was incubated for 40 minutes at room temperature and the absorbance was measured at 405 nm. The final MG concentration was determined from the standard curve.

2.4.8 Glyoxalase 1 (GLY 1) activity in *Glycine max*

This assay was performed in accordance with the method described by Chakravarty and Sopory (1998) to determine the Glyoxalase I activity in *Glycine max.* Sample proteins were extracted as described in sections 2.3 from which 20 µl aliquots were transferred to quartz cuvettes. Each sample cuvette received 5 mM potassium phosphate and 0.92 mM reduced glutathione to a final volume of 2 ml. The reaction was activated by the addition of 1.8 mM MG which directly preceded the absorbance readings taken at 240 nm from time 0 for 2 minute intervals. Thioester formation occurred over the period of 2 minutes.

2.4.9 Chlorophy *ll* assay

Chlorophyll determination of all the plant samples was achieved by using a modified method of Oancea et al. (2005). Frozen ground leaf material (100 mg) was added to a 1.5 ml Eppendorf tube. The Eppendorf tubes were wrapped in foil to prevent the degradation of chlorophyll species. Thereafter 10 vol of 100% (v/v) acetone was added to the same Eppendorf tube and mixed briefly using a vortex. Once mixed, the samples were added to glass cuvette in triplicate and read on a spectrophotometer at the wavelengths 662 nm and 644 nm respectively. The OD readings were used in a calculation to determine chlorophyll A and B concentrations respectively.

2.4.10 Nutrient and mineral uptake of *Glycine max* exposed to Zr and MG by Inductively coupled plasma-optical emission spectroscopy (ICP-OES)

Glycine max leafs and roots of each respective treatment were used for determination of nutrient and mineral uptake by ICP-OES. The plant material was digested and prepared for ICP-OES by the following method. Frozen ground material (200 mg) obtained as described in section 2.1 were transferred to 2 ml Eppendorf tubes. To each sample tube 1.5 ml 65% (v/v) nitric acid was added and homogenized by vigorous shaking and mixing by vortex. This was followed by an incubation at 90ºC for 4 hours to allow for complete digestion. Eppendorf tubes were sealed with parafilm prior to incubation to prevent the loss of sample mixtures with increased temperature and pressure. Sample homogenates were subjected to centrifugation to pellet leaf material and the supernatants were transferred to new Eppendorf tubes. In a Greiner tube (15 ml), samples were diluted (1:10) in a final volume of 10 ml with 2% (v/v) nitric acid and this served as the sample for the subsequent ICP-OES. In total 11 nutrients or minerals were analyzed which include calcium, zirconium, magnesium, molybdenum, iron, nickel, phosphorus, manganese, potassium, copper and zinc.

2.4.11 Statistical analysis

Statistical analysis was performed using the Duncan's multiple range test (DMRT), where significance was represented by a $P < 0.05$ and statistically validated based on standard error (SE). Significance was represented by different alphabetical letters.

3 CHAPTER THREE

BIOCHEMICAL ANALYSIS TO UNDERSTAND THE ROLE OF METHYLGLYOXAL SIGNALLING IN SOYBEAN EXPOSED TO ZIRCONIUM

3.1 INTRODUCTION

The unavoidable exposure of crops to heavy metal (HM) toxicity has seen a great rise in the last century because of human activities such as industrial activity, wastewater treatment and contaminated sewage sludge this then resulted in huge releases of HM's (Cvjetko, et al., 2014; D'Amore, et al., 2005; Khan, et al., 2008; Nicholson, et al., 2003; Satarug, et al., 2003). HM's are metals with an atomic number above 20 and a specific gravity over 5 g cm^{-3}, such as nickel (Ni), iron (Fe), arsenic (As), zirconium (Zr) and copper (Cu) (Cvjetko, et al., 2014).

Studies have shown that plants in South Africa are highly affected by HM toxicity (Baker & Brooks, 1989; Okedeyi, et al., 2014; Rascio & Navari-Izzo, 2011). This factor is a result of heavy mining for many decades (Okedeyi, et al., 2014; Langmi & Bessarabov, 2013). South Africa produces about 59 different minerals such as gold, platinum, titanium, chromium, manganese, vanadium and zirconium from 1115 mines and quarries (Langmi & Bessarabov, 2013). The top soil of many South African cities such as Johannesburg is heavily polluted with HM's (Okedeyi, et al., 2014; Turkdogan, et al., 2002; Rascio & Navari-Izzo, 2011; Fatoki, 1996) thus affecting crop growth, development and yield. With the increasing human population and food demand this is a major problem.

Crop growth, development and yield are heavily affected by HM pollution as plants are non-motile organisms that cannot escape the stress (Dalcorso, et al., 2010; Hossain, et al., 2011). Heavy metal accumulation in plant tissue is toxic to most plants and interferes with various morphological, physiological, and biochemical processes (Cvjetko, et al., 2014). Inside a cell, these metals damage important processes such as photosynthesis, respiration, mineral nutrition, and enzymatic reactions (Cvjetko, et al., 2014; Oancea, et al., 2005).

To help with the possible unavoidable abiotic stress such as HM toxicity plants, have developed numerous internal mechanisms that help them survive these stresses; by either avoiding or tolerating the stress. When plants avoid the stress they restrict the concentration or accumulation within the cells by extracellular precipitation or biosorption to cell wall only resulting in a controlled influx of the contaminant (Hossain, et al., 2009). Plants can also tolerate the stress by activating the activity of several genes from physiological and biochemical pathways within the plant cell (Dalcorso, et al., 2010; Hossain, et al., 2011) these pathways assist the plant to survive in the presence of the contaminant. Understanding the complex association of these biochemical pathways which are altered during stress has attracted high interest (Hossain, et al., 2011; Sasaki-Sekimoto, et al., 2005). Two stress-activated pathways and how they work has attracted much interest i.e. the glyoxalase system and antioxidant-reactive oxygen species (ROS) system as they detoxify methylglyoxal (MG) and ROS which increase due to biotic or abiotic stress in plants (Chen, et al., 2004; Hossain, et al., 2009; Hossain, et al., 2011; Singla-Pareek, et al., 2006; Veena & Sopory, 1999; Yadav, et al., 2005).

In normal conditions ROS appear as an unavoidable by-products formed as a result of one-electron reductions of molecular oxygen (0_2) (Wojtaszek, 1997). In stress conditions however, the protective mechanisms maintaining low levels of ROS inside the cell are overridden by the speedy production of huge amounts of ROS (Jacks & Davidonis, 1996; Wojtaszek, 1997). Earlier research concerning ROS was fixated on the toxicity of ROS resulting in cell injury and tissue dysfunction. Those studies paid close attention to the different ROS-scavenging mechanisms such as the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR), and low molecular weight antioxidants. More current studies have concentrated on the role of ROS as signaling molecules (Moller, 2010; Wojtaszek, 1997). To make use of ROS as a signaling molecule, non-toxic levels must be maintained in a balancing act between ROS production and scavenging. The benefits of ROS at low concentrations have been identified as follows: ROS is able to activate the defense response of plants against abiotic stress, they trigger programmed cell death in plants (PCD), they signal cell growth and differentiation, activate defense against pathogens and are secondary messengers involved in signal transduction pathways that regulate plant responses to stress (Baxter, et al., 2013). It has been concluded that a balance between ROS production and scavenging determines stress tolerance (Hossain, et al., 2011). In view of this and other findings it is worthwhile to investigate whether MG can have the same signaling properties.

Methylglyoxal (MG; CH_3COCHO) is a cytotoxic compound which forms as a by-product of numerous metabolic pathways, such as glycolysis, lipid peroxidation and oxidative degradation of glucose and glycated proteins (Hossain, et al., 2009; Lee,

et al., 2005; Richard, 1993; Yadav, et al., 2005). MG production is unavoidable and it increases during stress as the rate of glycolysis increases (Hossain, et al., 2009; Richard, 1993). The glyoxalse system is known to be the major detoxifying system of MG in plants under normal and stress conditions it takes place in the cytosol of cells (Hossain, et al., 2011; Yadav et al. 2005). MG reacts with DNA, RNA and protein, leading to inactivation of the antioxidant defense systems and consequent disruption of cellular functions just like ROS (Hossain, et al., 2009). In yeast (*Saccharomyces cerevisiae*) it was found to signal numerous signal transduction pathways, such as transcription factors Yap1 and Msn2 and triggers Hog1 a mitogen-activated protein kinase (MAPK) (Hossain, et al., 2011; Maeta, et al., 2005; Takatsume, et al., 2006). In plants, regulatory proteins such as MAPKs and b-ZIP transcription factors are involved in gene expression and signal transduction in stress responses (Shinozaki & Yamaguchi-Shinozaki, 1997). Hoque et al., 2012 found that MG can affect transcription and plant developmental processes in Arabidopsis. In view of this report it would be worthwhile to investigate the mechanism of association of exogenously applied MG on *Glycine max*.

The prime objective of this chapter is to investigate whether exogenously applied MG in low concentration could trigger the antioxidant defense system possibly resulting in growth promotion in *Glycine max* exposed to HM toxicity. To achieve this objective, a low concentration that will favor *Glycine max* growth in the face of an abiotic stress needs to be identified there-after commerce with treatment of young stage plants. Towards this objective, a biochemical study needs to be employed, aiming to assess the effects of exogenously applied MG on *Glycine max*. The study will use optimized biochemical assays for antioxidant enzymes and

reactive oxygen species (ROS) production in soybean leaves and roots to understand the signaling role of MG in the defense (antioxidant-ROS) system.

3.2 RESULTS

3.2.1 DRY WEIGHT DETERMINATION IN *GLYCINE MAX* SUBJECTED TO MG AND Zr TREATMENTS RESPECTIVELY

The biomass of plants can indicate whether plants are growing healthily or not. The results gathered from the dry-weights across the respective treatments were very informative, where the leaves and roots of plants treated with MG did not have any significant difference from the control. Compared to the control Zr and MG+Zr-treated plants showed a decrease in biomass leaves and roots. The leaves of Zr- and MG+Zr treated plants had a decrease of 46% and 32% as compared to the. The roots of Zr- and MG+Zr treated plants showed a decrease of 23% and 22% as compared to control.

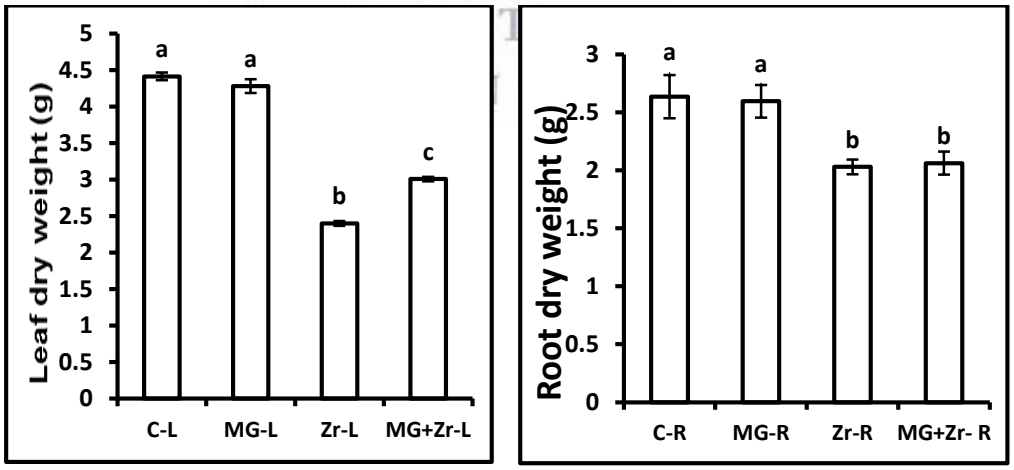

Figure 3-1 The effect of Methylglyoxal on the dry-weights of Glycine max leaves and roots in response to MG treatment and Zr stress. *Glycine max plants were treated with 6 µM MG, 1 mM Zr respectively and a combination of both for 21 days and the biomass of the leaves and roots was determined respectively. The different letters*

indicate a significant change across means at P< 0.05 (DMRT). Values are means ± S.E (N=3)

3.2.2 EVANS BLUE ASSAY (Cell death)

Evans blue dye assay has been used as a viability assay on the basis of its penetration into non-viable cells since 1994 when Baker and Mock developed a spectrophotometric procedure that allowed rapid, reproducible quantification of the stain retained by dead cells. In this experiment a small difference was observed with the leaves and roots treated with 6 µM MG as compared to the control and the the increases were 15% and 12% respectively. Therefore, the MG concentration used in this study showed small signs of cell death increase maybe as a result of MG toxicity. Zr treated plants displayed an enormous increase in cell death with the leaves displaying 90% increase and roots 44% increase as compared to the control, this was expected as Hossain et al., 2012 also reported that at the cellular and molecular level, HM toxicity results in cell death. The suspected ability of MG to reduce cell death was seen in the plants treated with 6 µM MG and 1 mM Zr as they displayed an approximate reduction of 30% as compared to the 1 mM Zr treated plants. However, the roots showed no significant difference. As compared to the control the leaves and the root of the 1 mM Zr treated plants had an increased cell death percentage of 81% and 45%.

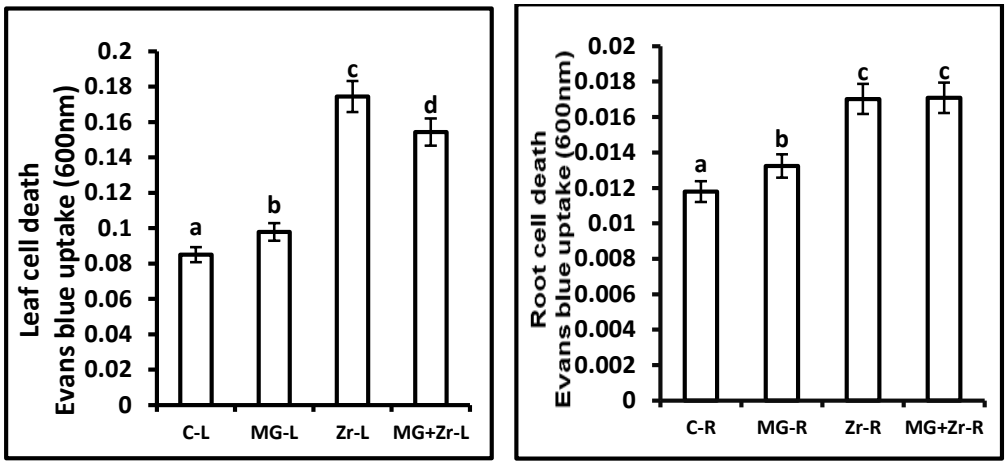

Figure 3-2 The effect of MG and Zr treatment on the cell death of Glycine max leaves and roots. *The cell death within the leaves and roots of Glycine max was determined after the various treatments based on the uptake of Evans blue. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values represent the means of three replicates± S.E.*

3.2.3 CHLOROPHYLL DETERMINATION ASSAY

Chlorophyll synthesis is known to be disrupted by HM toxicity; the accumulation of heavy metals within the plant can disrupt enzymatic steps of chlorophyll production or results in a deficiency of an essential nutrient (Emamverdian, et al., 2014). Chlorophyll reduction in plants is therefore a stress indicator (van Assche & Clijsters, 1990; Kancheva, et al., 2005). In this study plants grown in media containing MG were found to have an increased chlorophyll level (Roy, et al., 2004). A decrease was observed in total chlorophyll content with all treatments when compared to the control, MG had a decrease of 8%, Zr treated plants had a 28% decrease and the MG+Zr treated plants had a 16% decrease. Chlorophyll A content displayed a decrease in MG and Zr treated plants with 32% and 36% respectively and had no significant change in the MG+Zr treated plants when compared to the control. Chlorophyll

B content had a different trend as compared to chlorophyll A. All the plants experienced a decrease, 12% for MG treated plants, 44% for Zr treated plants and 45% for MG+Zr treated plants.

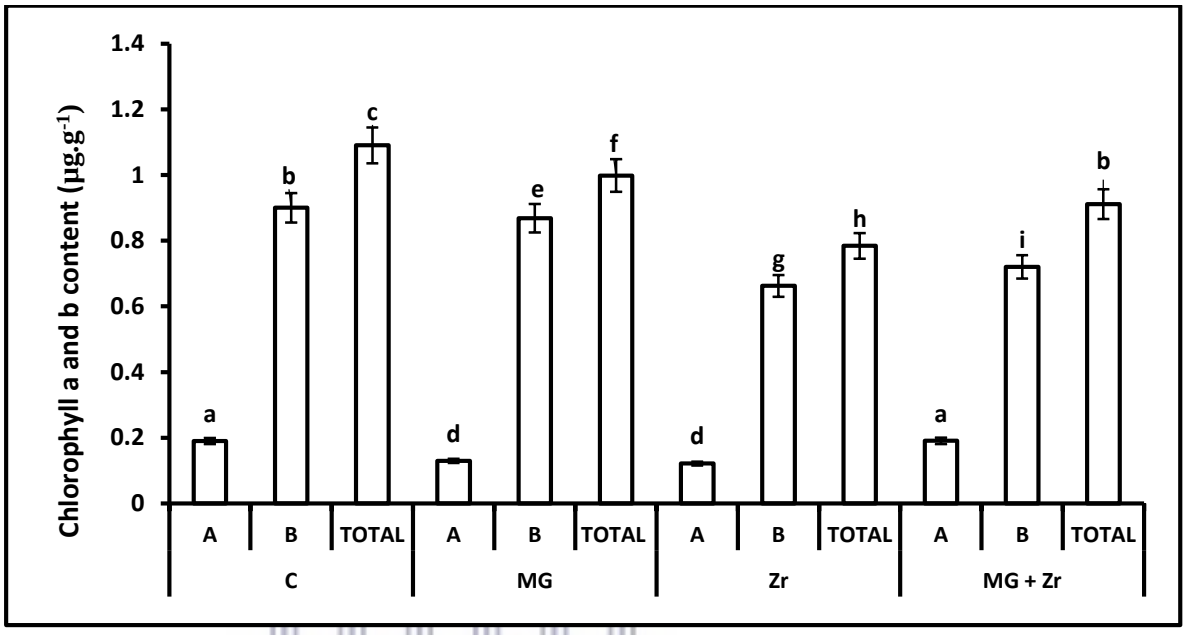

Figure 3-3 The effect of MG and Zr treatment on Glycine max chlorophyll a, b and total content (µg. g^{-1}). *The chlorophyll content within the leaves of Glycine max was determined after the various treatments. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values represent the means of three replicates± S.E.*

3.2.4 LIPID PEROXIDATION DETERMINED BY THE MDA CONTENT IN PLANTS EXOGENOUSLY TREATED WITH MG AND ZR

Malondialdehyde (MDA) is a naturally occurring product of lipid peroxidation. Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals and is used as an indicator of oxidative stress in cells and tissues. The monitoring of MDA levels in biological materials can be used as an important indicator of lipid peroxidation in vitro and in vivo for various diseases. In this study

there was an overall increase in MDA levels in both leaves and roots of plants from the different treatments as compared to the control. Zr treated plants had the highest increase of 44% in leaves and 102% in roots indicating a high levels of lipid peroxidation. The leaves of MG treated plants had no significant increase in the leaves and a contradictory 42% increase in roots, indicating that a lot more lipid peroxidation occurred in the roots. This could be because of a delay in GlyI activation to decrease MG levels or a delay in the activation of SOD and APX to scavenge O_2^- and H_2O_2. The increase in MDA in the leaves of plants exposed to MG+Zr was 14% and 55% in the roots. Overall the roots of all these plants experienced much more lipid peroxidation as compared to the leaves.

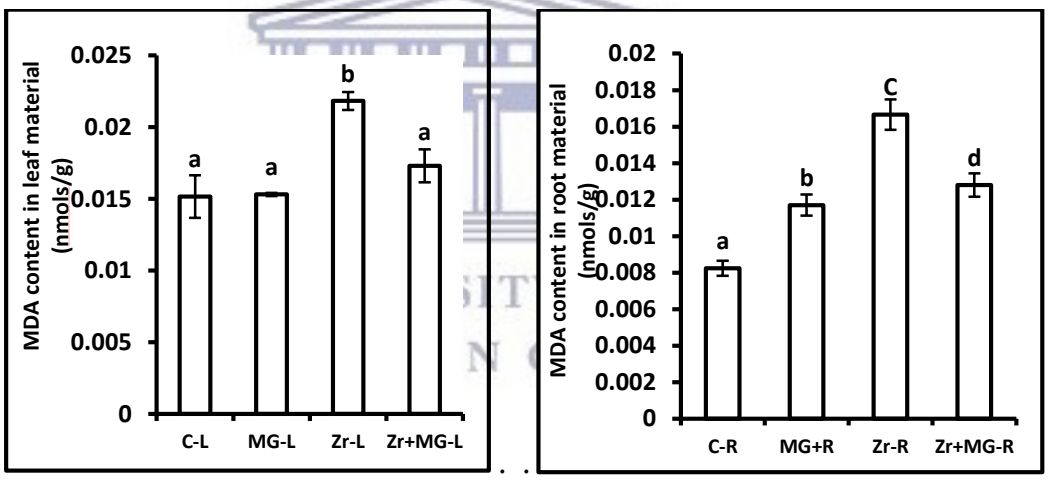

Figure 3-4 The effect of MG and Zr treatment on MDA levels within Glycine max leaves and roots. *The MDA levels provide an indication of lipid peroxidation. This graph displays the MDA content in the leaves and roots of the plants treated with MG, Zr and MG+Zr. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values represent the means of three replicates± S.E.*

3.2.5 REACTIVE OXYGEN SPECIES (ROS) ASSAYS

Further studies were performed to determine ROS i.e. superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) content accumulation within the plants leaves and roots,

as ROS accumulation is a consequence of HM toxicity in plants (Devi and Prasad, 1998). In this study MG treated plants displayed an increase in O_2^- content in their roots and leaves with 81% and 37% respectively. Zr treated plants displayed the highest percentage increase in the leaves at 146% and roots at 56%. The MG+Zr treated plants also had a drastic increase of 117% in the leaves and 50% in the roots. With the H_2O_2 content determination assay there was no significant difference with the MG treated plants as compared to the control in roots and in contrast there was a 68% increase in leaves. The Zr treated leaves and roots had the highest increase 233% and 146% respectively as compared to the control. In the 1mM Zr + 6µM MG treatment we observed a decrease as compared to Zr treated only plants thus showing that MG can be linked to ROS control because we observed a 186% and 45% increase as compared to the control leaves and roots was observed.

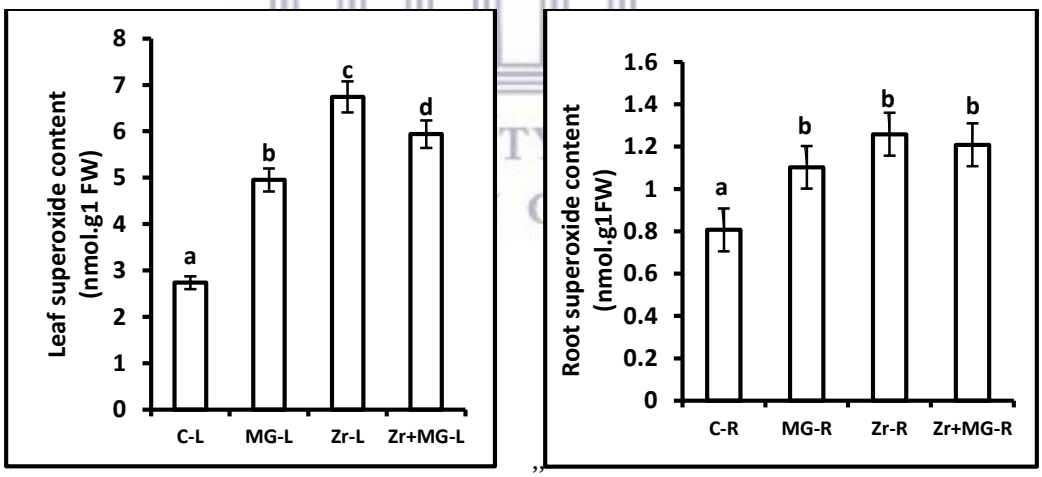

Figure 3-5 The effect of MG and Zr treatments on the superoxide content in Glycine max leafs and roots*. O_2^- content in Glycine max after 21 days of treatment with control (H_2O), MG (6 µM), Zr (1 mM) and MG + Zr was tested. The results are represented in this graph. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values represent the means of three replicates± S.E.*

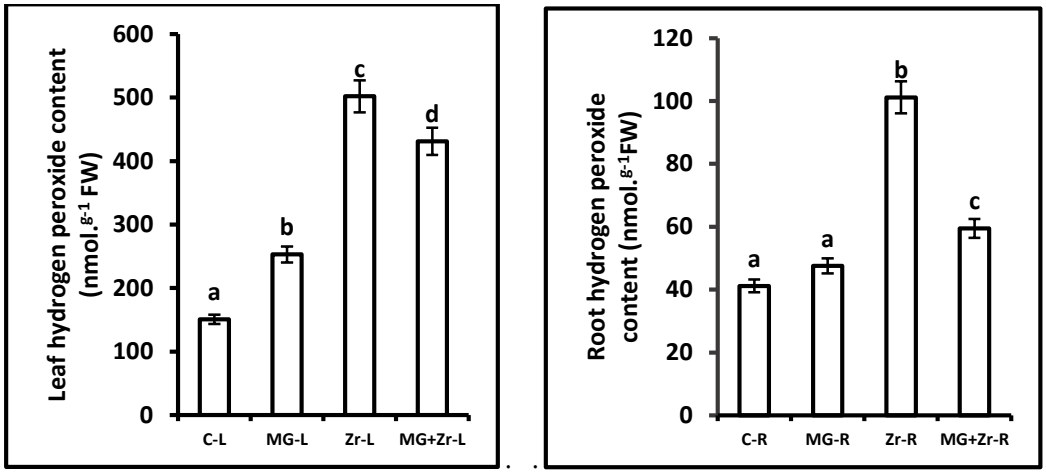

Figure 3-6 The effect of MG and Zr treatment respectively and together on the hydrogen peroxide content in Glycine max leaves and roots*. Glycine max was treated with control (H_2O), MG (6 µM), Zr (1 mM) and MG + Zr for 21 days thereafter the root and leaf material was harvested and stored for various assays. The hydrogen peroxide content within the leaves and roots was then determined and is shown in the graph. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values represent the means of three replicates± S.E.*

3.2.6 ANTIOXIDANT ACTIVITY

The scavenging of ROS is a crucial system in all organisms as ROS accumulation can have detrimental effects on biological cells. An increase in ROS in a plant system needs to be accompanied by an increase in ROS scavenging enzyme activity to ensure the plants survival from potential plant cell damages. In this study the activities of superoxide dismutase (SOD) and ascorbate peroxidase (APX) were investigated to connect antioxidant activity to the ROS content. SOD activity in both roots and leaves decreased as compared to the control. MG treated leaves and roots showed a 36% and 17% decrease respectively. Zr treated plants had the lowest SOD activity with a 55% decrease observed in the leaves and 37% observed in roots. MG+Zr treated plants displayed an increase as compared to Zr-only treated

plants and a 47% and 18% decrease in leaves and roots as compared to the control was observed.

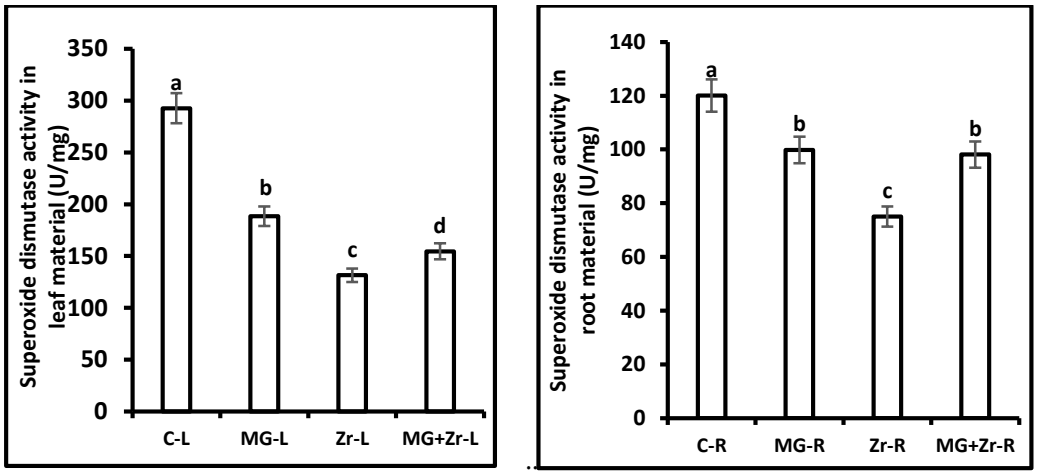

Figure 3-7 The effects of MG and Zr treatment on SOD activity in Glycine max leaves and roots. *The graph represents the SOD activity in units per mg of leaf or root sample from the different treatments. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values represent the means of three replicates± S.E.*

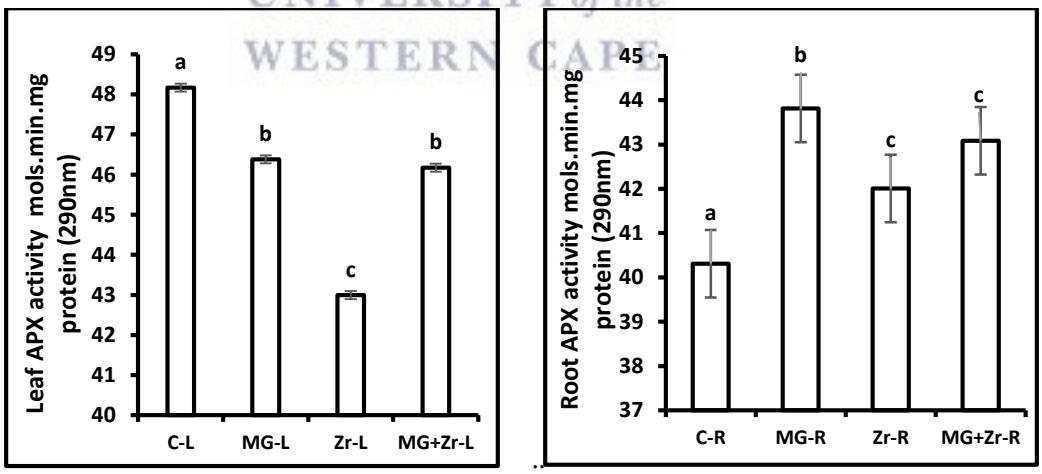

Figure 3-8 The effects of MG and Zr treatment on APX activity in Glycine max leaves and roots. *The graph represents APX activity in mols.min.mg protein of leaf or root sample from the different treatments (Control, 6µM MG, 1mM Zr and 6M MG + 1mM*

Zr. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values represent the means of three replicates± S.E.

3.2.7 MG CONENT AND GLY I ACTIVITY

MG is naturally produced in plants but a balance in production and scavenging is crucial as this compound in high concentrations could result in detrimental results. These levels are mainly maintained by the glyoxalase system. With the exogenous application of MG to soybean as performed in this study it was worthwhile to investigate the levels of MG and activity of GlyI. The content of MG was observed to have increased in both leaves and roots as compared to the control and this was expected. MG treated plants had an increase of 235% and 186% in the leaves and root respectively. The Zr treated plants had the largest increase in MG content with 431% in leaves and 302% in roots. MG+Zr treated plants had 236% increase in leaves and 205% increase in roots respectively as compared to the control.

A pattern complimentary to the MG content was observed with the GlyI activity. In leaves, MG treated plants had the highest activity of 430% higher than the control, second highest GlyI activity was that of MG+Zr treated plants at 192% as compared to the control and Zr treated plants had the lowest with 54% increase as compared to control leaves. In the roots a similar pattern was observed with MG treated roots with the highest GlyI activity at 87% increase and MG+Zr treated roots at 32% and Zr treated roots had a 13% increase respectively as compared to the control.

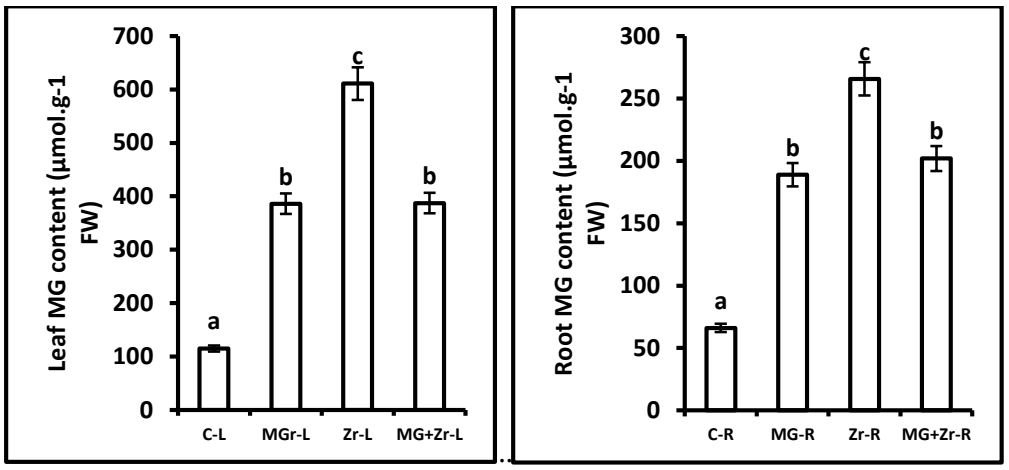

Figure 3-9 The effect of Methylglyoxal application on MG content in Glycine max in response to Zr stress. *High levels of MG are toxic in plants and it also occurs as a by-product of normal metabolism ubiquitously. MG content was determined in Glycine max. after exposure to Zr stress and MG treatments respectively. The different letters indicate a significant change across means at P< 0.05 (DMRT). Values are means ± S.E (N=3).*

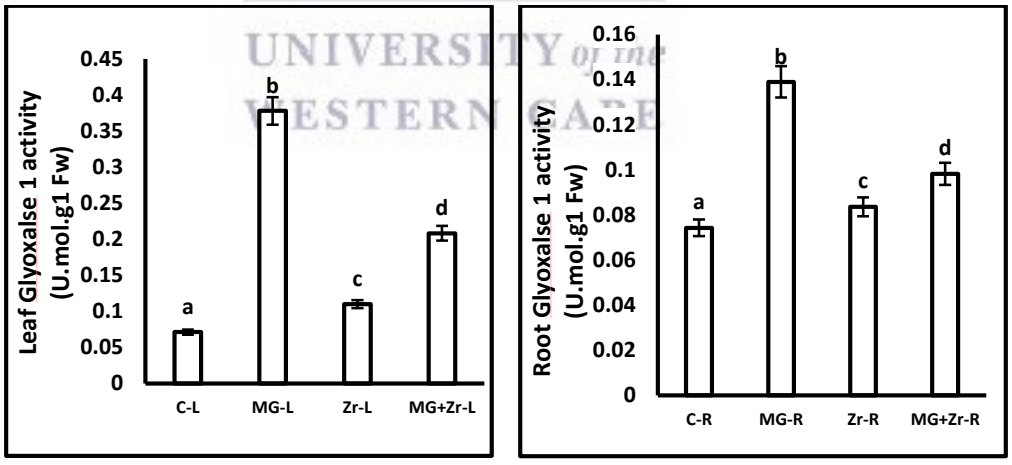

Figure 3-10 The effect of MG and Zr exogenous treatment on Glyoxalase I activity in Glycine max. Glyoxalase I of the glyoxalase system occurs in all plants*. It has proliferative properties but more importantly it effectively scavenges and metabolises >90% of MG produced as well as other oxoaldehydes. Gly-I activity was determined in Glycine max. after exposure to Zr stress and MG treatments respectively. The*

different letters indicate a significant change across means at P< 0.05 (DMRT). Values are means ± S.E (N=3).

3.3 DISCUSSION AND CONCLUSION

This study aimed to investigate whether exogenously applied MG could trigger a plants defense system thereafter resulting in plant growth promotion in *Glycine max*. in the presence or absence of a HM stress. To determine the effects of this application several experiments were conducted to evaluate the effects MG on the biochemistry of *Glycine max* in the presence and absence of Zr.

MG concentration in plants is altered in response to stress (Hoque, et al., 2016) and the accumulation can be very detrimental if not controlled. MG has been defined as a cytotoxin that can react with and modify DNA and protein moieties (Hossain, et al., 2014). MG has also been pointed out as an initiator of stress induced signaling cascades through ROS, resulting in cell death and or growth arrest (Hoque, et al., 2016). In view of the ability of MG to initiate a signaling cascade via ROS which leads to negative results, it is worthwhile to investigate whether low concentrations of MG can trigger the same cascade of a more manageable ROS production and latter scavenging. As studies have proven that plants that maintain a balance between the production and scavenging of ROS or maybe also MG results in stress tolerance (Gill & Tuteja, 2010).

Yadav et al., 2005 proved that seeds exposed to MG in high concentrations experience cytotoxicity and germination blockage, this was proven by engineering a tobacco plant with altered levels of Gly I. In fact, Hoque et al., 2012 showed that 0.1 mM MG did not affect germination but delayed root

elongation, whereas 1.0 mM MG inhibited germination and root elongation and induced chlorosis. Therefore, the ability of the *Glycine max* seeds to germinate in the parameters chosen for this experiment was an indication that the MG concentrations were low enough. Choosing the best low concentration which was 6 µM MG came about after a preliminary study with biomass of the seedlings exposed to MG (data not shown). The Zr concentration used in this experiment was not inhibiting levels and there weren't any significant differences in the germination percentage of the seeds. The success of the germination could be because of the tissues casing the embryo. The casing plays a role in selective diffusion of different heavy metals and chemicals into the seed (Li, et al., 2005).

After identifying the best suited concentration, young plants experiments were conducted after 21 days of treatment. The 21 days will ensure that the plant shows evidence of exposure to MG and Zr. If the period could be prolonged than the plants acclimation stage could be activated and then the results would not be a true reflection also if the treatment period is shortened, then it would too not be a true reflection of HM toxicity and MG effects.

Yeild of crops such as *Glycine max* contribute towards the economy of South Africa (Dlamini, et al., 2013) therefore discovering ways of enhancing their tolerance to any abiotic stress is meaningful. In this experiment there were visible signs of HM stress in the plants treated only with Zr. These plants had a decreased leaf size and yellowing as compared to the control which could be a sign of HM toxicity in plants. The impact of Zr on plant physiology alone shows that Zr has unfavorable effects on plant growth and development.

Furthermore, the dry weight of the Zr treated plants showed a decline as compared to all the other plants. South Africa is the world's second largest producer of Zr (USGS, 2012) contributing to 30% of the world production of Zr. Therefore, understanding how Zr affects plants and how it could possibly change crop physiology and biochemistry can be meaningful.

ROS in low concentrations mediate growth and development in plants (Gill & Tuteja, 2010). Therefore, known low concentrations of MG can be predicted to trigger manageable amounts of ROS which could signal growth. Leave biomass of the plants treated with MG + Zr showed a slight increase as compared to the Zr treated plants but it was still lower than the control or MG only treated plant leaves. Suggesting that MG could possibly alleviate Zr effects but not necessarily maintaining normal conditions similar to the control. There was no significant difference in the mass of the control and MG treated leaves and roots this could possibly mean that the concentration of MG chosen for this study triggered a steady MG detoxification leading to a mass similar to the control.

Towards the main objective of this project which is to uncover the biochemical reactions occuring within *Glycine max* when exposed to Zr, low concentrations of MG as a combination treatment with Zr were examined. Evans blue assay measuring cell death revealed a reduction in cell death with plants treated with MG in the presence of Zr. Therefore, MG induced cell death reduction in the combination treatment. The MG could have triggered a gradual increase in ROS production which in turn triggered a gradual increase in ROS scavenging resulting in a less stressed plant in the presence of Zr stress. Further studies

were performed to assess on ROS generation i.e. superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) content because an increase in ROS generation is normally a consequence of plant stress (Gill & Tuteja, 2010). Increased levels of ROS result in permanent metabolic dysfunction and cell death as a results of it reacting with important biomolecules (Emamverdian, et al., 2014; Gill & Tuteja, 2010). Therefore, the initiation of antioxidant enzymes including SOD, CAT, or APX is an important protective mechanism to minimize oxidative damage in stressed plants.

The O_2^- content in the plants was measured and the plant treated with MG showed an increase in superoxide content. This result was similar to the results in a study done by Hoque, et al., 2016 which proved that MG can trigger O_2^- generation. The MG treated plants had a lower O_2^- content as compared to Zr treated plants in both roots as well as leaves and a similar pattern was observed with H_2O_2 content. The plants treated with MG+Zr showed a slight decrease as compared to the Zr only treated plant leaves with regards to O_2^- and H_2O_2 content. The roots of the MG+Zr treated plants showed no significant difference with regards to O_2^- content and a significant decrease was observed in H_2O_2 content. Therefore, one could assume that in the presence of MG+Zr O_2^- increases and is not readily converted into H_2O_2 in the roots of soybean. The activation of SOD was not triggered fast enough to result in an increase H_2O_2. There was a higher quantity of H_2O_2 than compared to O_2^-. There are benefits and challenges of having high quantities of H_2O_2, the challenge is that it may lead to cell damage and an advantage is that recently Gill in 2010 and colleagues proved that H_2O_2 triggers a signaling cascade resulting in plant

tolerance to abiotic or biotic stress. This observation could suggest that MG could possibly initiate signal transduction pathways even in higher plants through H_2O_2 activation. MG's signalling capabilities were first discovered in yeast (Maeta, et al., 2004) and were not yet fully explored in higher plants therefore findings in this study will contribute to studies aiming to decipjer the signaling mechanisms of MG.

We evaluated the effects of the treatments on SOD activity and APX activity. Zr has been reported to decrease APX and glutathione reductase (GR) activities with an increase in peroxidase (POD) activities (Shahid, et al., 2013) . Additionally, the exogenous application of MG (0.5 to 10 mM) to tobacco was shown to inhibit glutathione S-transferase (GST) activity and APX activity (Hossain, et al., 2011). Gupta, et al., 1993 demonstrated how the overexpression of SOD can protect plants from oxidative stress but in a study conducted by Moirangthem, et al., 2014 it was shown that the overexpression of SOD alone did not result in tolerance but that other antioxidants had to also be overexpressed. This is because O_2^- dismutation results in molecular oxygen and H_2O_2 and an increase in H_2O_2 is known to be harmful therefore a parallel rise in H_2O_2 detoxification needs to be obtained as well as maintained. Therefore, balanced detoxification of both O_2^- and H_2O_2 is necessary to combat oxidative stress effectively. Kim, et al., 2010 stated that a combined overexpression of SOD and APX resulted in oxidative stress tolerance as well as high temperature stress tolerance in potato plants. In this study, in roots there was an increase in the activity of APX and a decrease was observed in leaves as compared to the control.

Another biomarker for assessing the harshness of a stress in plants is to look at the accumulation of MDA, which is mainly produced by ROS degrading membrane lipids. In this study, the MDA content in leaves of *Glycine max.* treated with Zr increased in both roots and leaves, these results were due to the inefficient antioxidant defense system under the HM stress which corresponded with the reduction in SOD and APX activity ultimately resulting in the damage of membrane lipids. The MDA content of the MG treated plants didn't show any significant difference as compared to the control in leaves and an increase of 43% was observed in roots, therefore this could suggest that the exogenous application of MG possibly does not result in a rapid increase of lipid degradation and therefore, in this study, we provide evidence that 6 µM of MG proves to be an appropriate concentration for *Glycine max*. The plants treated with MG and Zr as a combination treatment displayed a significant reduction in MDA content as compared to Zr only treated plants but we observed a slight increase of 14% and 55% in leaves and roots respectively when compared to control samples. This is very important as it suggests that MG could possibly reduce possible membrane damage in the presence of a HM stress.

Chlorophyll content determination can also be used as a stress biomarker because stress is known to hinder photosynthesis resulting in chlorophyll degradation. Roy, et al., 2004 was the first to provide evidence to the possible chlorophyll content increase in the presence of 0.5 mM MG, for their study they used carrot (*Daucus carota*), *Solanum nigrum* and *Dalbergia sissoo*. Furthermore, the concentration used by Roy and colleagues is the concentration Hoque and colleagues (2010) proved to be very stressful for the plant. In this

experiment a decrease in chlorophyll A, B and total chlorophyll content was observed in the MG leaves compared to the control. Additionally, an increase in total chlorophyll content was observed in the plants treated with MG+Zr, as compared to the plants treated solely with Zr. There was a total of 16% reduction in total chlorophyll in plants treated with MG+Zr as compared to the control which is not that significant.

The results found in this study could allow us to conclude that the exogenous application of MG can promote an increase in biomass of soybean in their while experiencing Zr toxicity. Plants exogenously treated with MG in low concentrations can experience a decrease in ROS accumulation even during HM stress by up regulating the activity of ROS scavenging enzymes. Therefore, ultimately balancing production and scavenging of ROS. This conclusion was also supported by the MDA content found in the plants treated with MG. MG can therefore be categorized as a suitable biomarker for plant stress tolerance. The role of MG as a signaling molecule can be further studied in other abiotic stresses and other crop plants. Thorough clarification of MG metabolism by integrating other "omics" tools such as proteomics and metabolomics, to dissect the signaling roles of MG by using model plant species would be useful research to improve plant HM- stress tolerance in crop plants. Crop yield in most African countries is affect by various abiotic stresses. Therefore, crop yield could possibly increase if this metabolite is thoroughly implemented at the perfect concentration to farm lands. With the antioxidant system constantly switched on, cytotoxins would be produced and immediately

removed. Further studies on the possible effects of having this system permanently switched on needs further investigated.

In conclusion, the physiological and biochemical results obtained in this study demonstrated that the application of 6 µM MG to Soybean can regulate cell death, maintain plant biomass, chlorophyll content and lipid peroxidation. MG slightly increases O_2^- and H_2O_2 levels and complementary SOD and APX which possibly results in a balance between production and detoxification of the cytotoxins. Furthermore, in the presence of Zr, MG showed signs of having the potential to reduce cell death in leaves, increase biomass, increase chloropyll content and reduce lipid peroxidation as compared to Zr only treated plants. SOD and APX activity was also increased as compared to the Zr only treated plants. This cascade of events highly suggest that MG has possible signalling capabilities.

4 CHAPTER FOUR

NUTRIENT ANALYSIS TO UNDERSTAND THE ROLE OF MG SIGNALLING IN SOYBEAN EXPOSED TO ZIRCONIUM

4.1 INTRODUCTION

Plants require essential nutrients for normal functioning and growth. Photosynthesis which is the fixation of atmospheric CO_2 into simple sugars using the energy of the sun, is the major contributor in plant nutrition (Marschner, 2012; Fangeria, 2009; Baker & Pilbeam, 2015). However, CO_2 and light energy are not sufficient for the synthesis of all the molecules which plants require for nutrition (Fangeria, 2009). Plants require a number of inorganic nutrients these nutrients are divided into two groups namely macro-nutrients and micro-nutrients; the division is according to the plants requirement for the nutrient (Marschner, 1995). There are nine macronutrients: carbon, hydrogen, and oxygen—the three elements found in all organic compounds—as well as nitrogen (essential for amino acids), potassium, calcium, phosphorus, magnesium (the center of the chlorophyll molecule), and sulfur (Maathius, 2009). Each of these nutrients may greatly exceed 1% of the dry weight of a healthy plant (Miller, 2009; Maathius, 2009). The seven micronutrient elements— iron, chlorine, copper, manganese, zinc, molybdenum, and boron—constitute less than one to several hundred parts per million in most plants (Hansch & Mendel, 2009).

Under natural circumstances, nutrients come from soil. Soils are complex mixtures of minerals, water, air, organic matter, and countless organisms that are the decaying remains of once-living things (Chapin, 1980). A constant balance of macro and micro nutrients needs to be maintained in order for crops to grow and

function properly. In South Africa most soils are contaminated with heavy metals (HM) and these Heavy metals are among the most prominent types of contaminants in the environment (Yabe, et al., 2010). Plants cannot escape from the soil therefore resulting in continuous exposure to heavy metal contamination. Plants have evolved highly specific and very efficient mechanisms to obtain essential micronutrients from the environment, even when present at low ppm levels. Plant roots, aided by plant-produced chelating agents and plant-induced pH changes and redox reactions, are able to solubilize and take up micronutrients from very low levels in the soil, even from nearly insoluble precipitates (Johnson & Peter, 2010). Plants have also evolved highly specific mechanisms to translocate and store micronutrients. These mechanisms are also involved in the uptake, translocation, and storage of toxic elements, whose chemical properties often simulate those of essential elements (Johnson & Peter, 2010).

Amongst metals of concern in South Africa is zirconium (Zr) because South Africa is the second world-ranked producer of this metal (Mining in South Africa, 2014; Langmi & Bessarabov, 2013). Zr has an atomic number of 40 and is a transition metal that resembles titanium in physical and chemical properties (Zaccone, et al., 2008). Zr is the twentieth most common element in the earth's crust (Fodor, et al., 2005; Shahid, et al., 2013) allowing it to be widely used in many chemical industry processes as well as in nuclear reactors (Kamal, et al., 2011; Sandoval, et al., 2011), owing to its useful properties like hardness, corrosion-resistance and permeability to neutrons (Mushtaq, 2012). In soil, Zr is more than twice as abundant as copper and zinc and has ten times the abundance of lead. Hence, the recent increased use

of Zr by industry, results in enhanced environmental levels in soil and waters (Kruglov, et al., 1996; Mosulishvili, et al., 1994; Yirchenko & Agapkina, 1993).

Thus, the purpose of the current chapter is to analyze whole nutrients using ICP-OES on soybean roots and leaves furthermore understand nutrient uptake during zirconium stress as well as the signaling role of MG.

4.2 RESULTS

4.2.1 NUTRIENT AND MINERAL UPTAKE OF *GLYCINE MAX* EXPOSED TO ZR AND MG BY INDUCTIVELY COUPLED PLASMS-OPTICAL EMISSION SPECTROMETRY (ICP-OES)

ICP-OES which was first commercialized in 1983 is a type of spectrometry which is capable of detecting metals and non-metals at very low concentrations (Thomas, 2001). This technique has great speed, precision and sensitivity (Tanner & Baranov, 1999). In this study, there were four macronutrients analyzed, six micronutrients and Zr. Normally, these minerals are taken up by plant roots from the soil solution in ionic form or as cations and distributed throughout the plant. Therefore, this analysis aims to analyze their absorption and distribution throughout the plant which are exposed to the experimental treatments. Consequently, the leaves of the MG treated plants had an increase in macronutrients taken up as compared to the control. Zr treated plants had an increase in accumulation of Ca+ and Mg as compared to the control and the leaves of the MG+Zr treated plants experienced a decrease in all the macronutrients analyzed. There wasn't much difference with the micronutrients found in the roots of the MG treated plants as compared to the control. Zr caused a decrease in Cu, Fe, Mo and Zn as compared to the control. Plants treated with Zr had a higher

accumulation of Zr in leaves than roots and MG+Zr treated plants had a higher Zr accumulation in roots than leaves. Indicating that MG can possibly act as hindrance of Zr translocating to the rest of the plant. The translocation factors (TFs) of the metal ions were calculated as TFs = [Metal concentration in shoots]/ [Metal concentration in roots] (Kovacik, et al., 2014; Zhang, et al., 2014). The TF is explained as the ratio of shoot to root metal levels. The TF value of different elements differs due to plant species.

Table 4-1 Quantitative changes of some macro-nutrients in leaves and roots of Soybean treated with MG and Zr. Data is shown as the mean ± SD (n=3)

		Calcium (mg/kg-1 FW)	Potassium (mg/kg-1 FW)	Magnesium (mg/kg-1 FW)	Phosphorus (mg/kg-1 FW)
Leaves					
	Control	69 ± 6^{c}	311 ± 12^{d}	30 ± 2^{a}	55 ± 1.5^{b}
	MG	113 ± 10^{g}	406 ± 25^{h}	37 ± 0.3^{e}	68 ± 3.5^{f}
	Zr	101 ± 8^{k}	254 ± 20^{l}	44±0.8j	56±0.5i
	MG+Zr	53 ± 5^{b}	207 ± 20^{m}	26 ± 0.5^{n}	33 ± 2.5^{o}
Roots					
	Control	16 ± 1^{a}	450 ± 4^{b}	14 ± 0.5^{c}	65 ± 4^{d}
	MG	105 ± 8^{e}	160 ± 14^{f}	13 ± 0.3^{g}	65 ± 3^{h}
	Zr	30 ± 3^{j}	230 ± 16^{j}	10 ± 0.8^{k}	40 ± 3^{l}
	MG+Zr	35 ± 3.5^{p}	118 ± 10^{o}	11 ± 0.5^{n}	37 ± 1.2^{m}

Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ±S.E (N=3).

Table 4-2 Quantitative changes of some micro-nutrients in leaves and roots of Soybean treated with MG and Zr. Data are shown as the mean ± SD (n=3)

		Copper (mg/kg-1 FW)	Iron (mg/kg-1 FW)	Manganese (mg/kg-1 FW)	Molybdenum (mg/kg-1 FW)	Nickel (mg/kg-1 FW)	Zinc (mg/kg-1 FW)
Leaves							
	Control	0.2±0.004^{a}	0.5±0.06^{e}	0.3±0.01^{i}	0.002±0.00009^{m}	0.02±0.0009^{q}	0.3±0.0001^{u}
	MG	0.3±0.005^{b}	0.6±0.07^{f}	0.4±0.02^{j}	0.004±0.0002^{n}	0.02±0.0006^{r}	0.5±0.0002^{v}
	Zr	0.2±0.002^{c}	0.8±0.04^{g}	0.7±0.03^{k}	0.005±0.0002^{o}	0.02±0.0006^{s}	0.6±0.0001^{w}
	MG+Zr	0.1±0.004^{d}	0.6±0.01^{h}	0.3±0.01^{l}	0.006±0.0003^{p}	0.007±0.0003^{t}	0.4±0.0004^{x}
Roots							
	Control	0.2±0.002^{a}	1±0.09^{c}	0.7±0.02^{e}	0.008±0.0003^{g}	0.01±0.0008^{i}	0.5±0.01^{k}
	MG	0.20.007^{b}	1.2±0.12^{d}	0.6±0.03^{f}	0.007±0.0002^{h}	0.01±0.0007^{j}	0.6±0.02^{l}
	Zr	0.2±0.004^{m}	0.7±0.13^{o}	0.9±0.05^{q}	0.004±0.0001^{s}	0.02±0.001^{u}	0.4±0.03^{w}
	MG+Zr	0.3±0.01^{n}	1.4±0.06^{p}	0.4±0.003^{r}	0.008±0.0005^{t}	0.02±0.0009^{v}	0.6±0.02^{x}

Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ±S. E (N=3).

Table 4-3 Translocation factor(TFs) values for some macro-nutrients in Soybean treated with MG and Zr.

	Calcium (mg/kg-1 FW)	Potassium (mg/kg-1 FW)	Magnesium (mg/kg-1 FW)	Phosphorus (mg/kg-1 FW)
C	4 ± 0.1^{a}	0.7 ± 0.09^{b}	2±0.09^{c}	0.8±0.02^{d}
MG	1.07 ± 0.03^{e}	2.5 ± 0.06^{f}	2.8±0.1^{g}	1.0±0.08^{h}
Zr	3.4 ± 0.11^{i}	1 ± 0.06^{j}	4.4±0.6^{k}	1.4±0.1^{l}
MG+Zr	1.5 ± 0.09^{m}	1.7 ± 0.08^{n}	2.4±0.2^{o}	0.9±0.03^{p}

Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ±S. E (N=3).

Table 4-4 Translocation factor (TFs) values for some micro-nutrients in soybean treated with MG and Zr

	Copper (mg/kg-1 FW)	Iron (mg/kg-1 FW)	Maganese (mg/kg-1 FW)	Molybdenum (mg/kg-1 FW)	Nicel (mg/kg-1 FW)	Zinc (mg/kg-1 FW)
C	1 ± 0.02^{a}	0.5 ± 0.05^{b}	0.4 ± 0.02^{c}	0.25 ± 0.02^{d}	2 ± 0.08^{e}	0.6 ± 0.03^{f}
MG	1.5 ± 0.06^{g}	0.5 ± 0.03^{h}	0.7 ± 0.03^{i}	0.6 ± 0.03^{j}	2 ± 0.06^{k}	0.8 ± 0.05^{l}
Zr	1 ± 0.09^{m}	1.1 ± 0.2^{n}	0.8 ± 0.05^{o}	1.3 ± 0.1^{p}	1 ± 0.2^{q}	1.5 ± 0.05^{r}
MG+Zr	0.3 ± 0.002^{s}	0.4 ± 0.05^{t}	0.8 ± 0.04^{u}	0.8 ± 0.05^{v}	0.4 ± 0.03^{w}	0.7 ± 0.06^{x}

Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ±S. E (N=3).

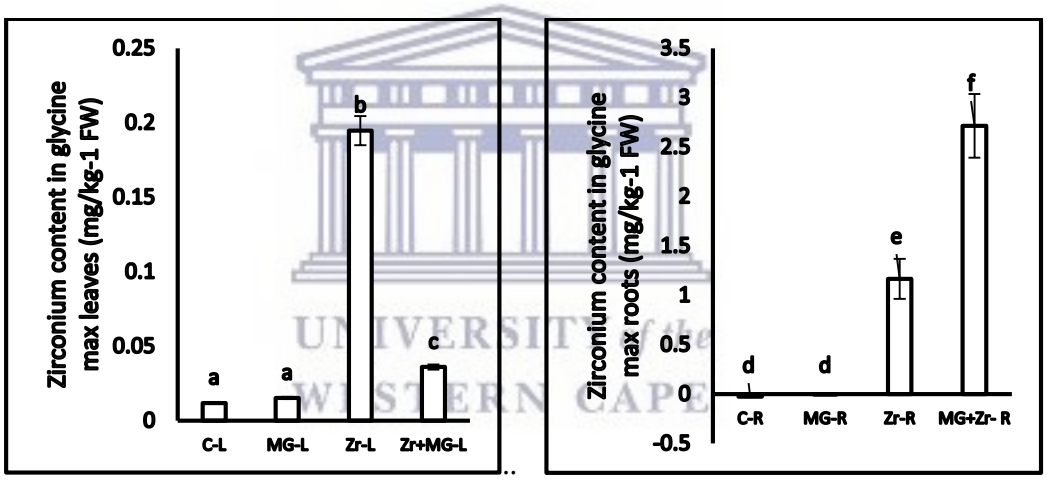

Figure 4-1: Detection of Zr uptake in Soybean exposed to exogenous MG and Zr (mg.kg-1 dry weight)*. Glycine max plants were treated with 6 µM MG, 1 mM Zr respectively and a combination of both for 21 days and the Zr content of the leaves and roots was determined respectively. The different letters indicate a significant change across means at P< 0.05 (DMRT). Values are means ± S.E (N=3)*

Table 4-5 Translocation factor (TFs) values of Zirconium treated soybean

	Zirconium (mg/kg-1 FW)
C	-0.5±0.013 [a]
MG	-4.1± 0.012 [b]
Zr	0.2±0.014 [c]
MG+Zr	0.01±0.0013 [d]

Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ±S. E (N=3).

4.3 DISCUSSION AND CONCLUSION

Plants require a few macronutrients in large amounts and several micronutrients in trace amounts. Sylvestre, et al., 2013 reported that soybean take up 58.46 $mg.kg^{-1}$ of phosphorus (P) and 114.15 $mg.kg^{-1}$ of potassium (K^+) in a field experiment and the site was red sandy loam. In our study, the phosphorus results correspond with up-take found in the control plant of this study which was 55 $mg.kg^{-1}$ and MG treated plants showed a significant increase to 68 $mg.kg^{-1}$. Organic P is mainly bound to hydroxyl groups of sugars and alcohols via esterification (Marschner, 1995; Maathius, 2009). Alternatively, Pi binds to other phosphate groups via pyrophosphate bonds. Formation and disruption of the pyrophosphate bond is one of the central mechanisms in cellular energy homoeostasis (Maathius, 2009). K^+ is an essential macronutrient needed for metabolic reactions because of its capacity to activate a multitude of enzymes (Britto & Kronzucker, 2008; Maathuis & Sanders, 1993) The potassium (K) in the control of our study was higher than that reported by Sylvestre, et al., 2013 and MG treated leaves had an even higher concentration of potassium as compared to the control as displayed in table 4.1. Zr and MG+Zr treated plant leaves showed a decrease. Calcium (Ca) is very abundant in the lithosphere. Severe weathering and leaching of soils may lead to deficiency

in Ca. Ca^{2+} plays an analogous role in cell membranes where Ca^{2+} coordinates with phosphate groups from phospholipids. This complexation occurs predominantly at the external face of the plasma membrane (Karley & White, 2009). Removal of membrane Ca^{2+}, or its replacement with other cations rapidly compromises membrane integrity. In this study we observed no significant difference in Ca^{2+}concentration of leaves of plants treated with a combination of MG+Zr as compared to the control. Moreover, in this study the leaves generally had a higher accumulation of macronutrients than roots. Based on the data in table 4.3 MG showed the potential to increase the TF value as compared to the control with regards to potassium, magnesium and phosphorus.

The Zr treated plants surprisingly displayed a higher accumulation of macronutrients in roots and leaves and showed no significant difference in micronutrients as compared to the control. This could be as a result of the plant survival system being initiated as a result of stress. The plant could be trying to absorb as much nutrients to help it to survive, if the experiment period could be extended from 21 days we could possibly observe this plant rapidly growing and then dying off before all the other plants. Micronutrients are involved in all metabolic and cellular functions. Plants differ in their need for micronutrients, and the focus of this study are some of those elements that are generally accepted as essential for all higher plants such soybean: copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni), and zinc (Zn). Copper is essential for photosynthesis and mitochondrial respiration, for carbon and nitrogen metabolism, for oxidative stress protection, and is required for cell wall synthesis (Santagostini, et al., 2004). Under physiological conditions, copper exists in the two oxidation

states Cu^{1+} and Cu^{2+} and can interchange between these forms (monovalent copper is unstable) (Piol, et al., 2006). This allows copper to function as a reducing or oxidizing agent in biochemical reactions (Piol, et al., 2006; Yamasaki, et al., 2007). As displayed in table 4.2 MG increased leave Cu accumulation and no significant difference was observed in the roots and this element had a TF of 1. The mean TF value obtained by Radulescu, et al., 2013 for Cu uptake by *Brassica oleracea L. var. capital* (cabbage) was 0.8, furthermore various studies have reported various Cu TF values with 0.07 in *P.australis* as the lowest (Deng, et al., 2004), *T. latifolia* was at 1.04 (Deng, et al., 2004; Stoltz & Greger, 2002) and the highest TF was 1.6 in cabbage (Radulescu, et al., 2013). As a redox-active metal, iron is involved in photosynthesis, mitochondrial respiration, nitrogen assimilation, hormone biosynthesis (ethylene, gibberellic acid, jasmonic acid), production and scavenging of reactive oxygen species, osmoprotection, and pathogen defense. Up to 80% of the cellular iron is found in the chloroplasts which is consistent with its major function in photosynthesis. As displayed in Table 4.2 MG displayed the potential to increase iron uptake in MG+Zr treated plant roots and it maintained the same value as the MG solely treated plant leaves. The TF reported in other studies such as the one conducted by Radulescu, et al., 2013 reported the TF of Fe in cabbage to range between 0.05 and 0.2 and in this study the TF value was higher ranging between 0.4 and 1.1. Manganese is essential for plant metabolism and development and occurs in oxidation states II, III, and IV in approximately 35 enzymes of a plant cell (Hebbern, et al., 2009; Kusunki, 2007). Manganese can fulfill two functions in proteins: (1) it serves as catalytically active metal, or (2) it exerts an activating role on enzymes (Hansch & Mendel, 2009). In this study an increase in Mn was observed in plant leaves treated with MG as compared to the control and MG

maintained the same concentration on Mn as the control in MG+Zr treated plant leaves. MG also increased the TF value in MG+Zr treated plants as compared to the control. There were very low levels of molybdenum (Mo) observed in this study ranging from 0.002 and 0.008 in leaves and roots of all the plants. Only a handful of plant proteins are known to contain molybdenum in their active sites (Hille, 1996). These proteins, however, are very important as they are involved in nitrogen assimilation, sulfur metabolism, phytohormone biosynthesis, and stress reactions (Schwarz & Mendel, 2006). Nickel had the highest TF values in the micronutrients. Among plants, Nickel occurs not only in oxidation states II, but also in states I and III. A deficiency symptom in plants is the accumulation of toxic urea that could be explained with the complete loss of urease activity within the cell (Eskew, et al., 1983; Bai, et al., 2006; Eslew , et al., 1984). Zinc was the last micronutrient examined and MG showed signs of potentially being able to increase the translocation of Zinc in Zr exposed plants. Zinc is important as a component of enzymes for protein synthesis and energy production and maintains the structural integrity of biomembranes (Kawagashira, et al., 2001) . More than 1200 proteins in Arabidopsis are predicted to contain, bind, or transport Zn^{2+}, including among others a large numbers of zinc-finger containing proteins and transcription factors, oxidoreductases, and hydrolytic enzymes such as metalloproteases (Kramer & Clemens, 2005; Lin, et al., 2005; Hansch & Mendel, 2009).

Zirconium a glossy silver-gray metal is yet to be better understood in plant science. A TF value equal to 0.01 corresponds to a low metal absorption rate and a TF value of 10 indicates that the plant accumulates the metal and table 4.5 displays that the control and MG treated plant had no Zr absorption, this was expected as the control and MG treated plant were not exposed to Zr. The mean TF value obtained by

Tome, et al., 2003 for Zr uptake by grass-pasture grown in soils near a uranium mine was 0.09. Furthermore, numerous studies have reported that Zr accumulates in various crop parts if the crops were grown on Zr-contaminated soil (Sanzharova & Aleksakhin, 1982; Fodor, et al., 2002). Sanzharova and Aleksakhin (1982) concluded that Zr was taken up by barley, corn, and alfalfa. Gundersen, et al., 2000 observed that Zr was absorbed by *P. sativum* (between 0.425 and 5.29 mg/kg of Zr per fresh weight). Kabata-Pendias, 1993 indicated that the Zr levels found in food plants vary from 0.005 to 2.6 mg/kg. The translocation rate of Zr in higher plants is low (Sanzharova & Aleksakhin, 1982; Kabata-Pendias, 1993; Ferrand, et al., 2006). Kabata-Pendias & Pendias, 1992 reported a higher increase of Zr content in nodules and roots of legumes than in aerial parts. Wang, et al., 2000 observed the same result for soybean plants that were cultivated in contaminated soil, even for a longer growing period (up to 60 days after sowing). Additional studies on Zr that address movement and accumulation in numerous other plant species are required. In particular, data is needed on the distribution of Zr in leafy or root vegetables and should emphasize the consumed parts of the vegetables. Nonetheless, in this study the Zr treated plant had a higher translocation of the metal than the plant with MG+Zr. This indicate that MG has the potential to hinder Zr translocation in Soybean.

In conclusion, the results obtained from this work have demonstrated that the interactive effects of MG and Zr substantially reduced Zr phytotoxicity in Soybean plants. The application of MG increased macronutrient accumulation in leaves and showed no significant difference in the roots. MG can hinder Zr uptake in Soybean. MG increased the TF value of micronutrients. MG also hindered Zr translocation into plant shoot. Zr accumulated in roots and did not move up the plant in MG+Zr

treated plants as compared to Zr solely treated plants that experienced a higher accumulation of Zr in leaves than in roots.

REFERENCES

Abellanosa, A. L. & Pava, H. M., 1987. *Introduction to crop science,* Bukidon: Centre Mindanao University, Musuan.

Abordo, E. A., Minhas, H. S. & Thornalley, P. J., 1999. Accumulation of alpha-oxoaldehydes during oxidative stress: a role in cytotoxicity. *Biochem. Pharmacol,* Volume 58, pp. 641-648.

Alan, M. et al., 1990. Promotion of plant growth by polymers of lactic acid. *Plant growth regulation,* Volume 9, pp. 137-146.

Albrecht, S. C. et al., 2011. In vivo mapping of hydrogen peroxide and oxidized glutathione reveals chemical and reginal specificity of redox homeostasis. *Cell Metab,* Volume 14, pp. 819-829.

Amon, D. J. & Stout, P. R., 1939. The essentiality of certain elements in minute quality for plants with special reference to copper. *Plant Physiol,* Volume 14, pp. 371-375.

Armeni, T. et al., 2014. S-D-Lactoylglutathione can be an alternative supply of mitochondrial glutathione. *Elsevier,* Volume 67, pp. 451-459.

Azzu, V. & Brand, M. D., 2010. The on-off switches of the mitochondrial uncoupling proteins. *Trends Biochem Sci,* Volume 35, pp. 298-307.

Bai, C., Reilly, C. C. & Wood, B. W., 2006. Nickel deficency disrupts metabolism of ureides, amino acids, and organic acids of young pecan foliage. *Plant physiol,* Volume 140, pp. 433-443.

Bair, W. B. 3. et al., 2010. GLO1 overexpression in human malignant melanoma. *Melanoma Res,* 20(2), pp. 85-96.

Baker, A. J. & Brooks, R. R., 1989. Terrestrial higher plants which hyperaccumulate metallic elements-a review of their discription, ecology and phytochemistry. *Biorecovery,* Volume 1, pp. 81-126.

Baker, A. V. & Pilbeam, D. J., 2015. Handbook of plant nutrition,. 2nd ed. New York: CRC Press taylor & Francis group.

Barker, A. V. & Pilbeam, D. J., 2007. Handbook of Plant nutrition. 1st ed. USA: Taylor & Francis Group.

Batchelor, C. et al., 2014. *ChemSpider: Search and share chemistry (Methylglyoxal).* [Online]
Available at: http://www.chemspider.com/Chemical-Structure.857.html [Accessed 14 May 2014].

Baxter, A., Mittler, R. & Suzuki, N., 2013. ROS as a key players in plant stress signalling. *Journal of experimental botany,* 65(5), pp. 1229-1240.

Bergmann, L. & Rennenberg, H., 1993. Glutathione metabolism in plants. In: L. J. De Kok, ed. *Sulfur nutrition and assimilation in higher plants.* The Hague: SPB Academic Publishing, pp. 109-123.

BFAP, 2013. The Bureau for food and policy research , Pretoria: 10th BFAP Baseline Agricultural Outlook 2013-2022.

Bierhaus, A. et al., 2012. Methylglyoxal modification of Nav 1.8 facilitates nociceptive neuron firing and causes hyperalgesia in diabetic neuropathy. *Nature Medicine* , 18(6), p. 926.

Blignaut, C. & Taute, M., 2010. The development of a map showing the soybean production regions and surface areas of the RSA, Pretoria: University of Pretoria.

Britto, D. T. & Kronzucker, H. J., 2008. Celllular mechanisms of potassium transport in plants. *Physiol plant,* Volume 133, pp. 637-650.

Brooker, R. J., 2011. *Genetics: analysis and principles.* 4th ed. s.l.:McGraw-Hill Science.

Brown, G. C., 1997. Nitic oxide inhibition of cytochrome oxidase and mitochondrial respration" implications for inflammatory, neurodegenerative and ischaemic pathologies. *Mol Cell Biochem,* Volume 174, pp. 189-192.

Brunus, T., 2006. Evolutionary biology: a kingdom revised. *Nature,* 443(7113), pp. 758-761.

Cardenas, E. & Davies, K. J., 2000. Mitochondrial free radical generation, oxidative stress and ageing. *Free Radic. Biol Med,* Volume 29, pp. 222-230.

Carrier, P., Baryla, A. & Havaux, M., 2003. Cadmium distribution and microlacalization in oil seed rape (*Brassica napus*) after long-term growth on cadmium-contamination soil. *Planta,* 216(6), pp. 939-950.

Caspi, R., 2014. *METACYC: A member of the BioCyc database collection.* [Online] Available at: http://metacyc.org/META/NEW-IMAGE?type=PATHWAY&object=PWY-5386
[Accessed 14 May 2014].

Chapin, S. F., 1980. The mineral nutrition of wild plants. *Ann.Rev.Ecol.Syst,* Volume 11, pp. 233-60.

Chengbin, X., Bonnie, L. W., E'Lise, M. C. & David, J. O., 2001. The biological functions of Glutathione revisited in Arabidopsis Transgenetic plants with altered Glutathione levels. *American society of plant physiologists* , 126(2), pp. 564-574.

Chen, Z. Y., Brown, R. L., Dam, n. K. & Cleveland, T. E., 2004. Identification of a maize kerel stress-relatedprotein and its effect on aflatoxin accumulation.. *Phytopathology,* 94(9), pp. 938-945.

compiled by Directorate statistics & economic , a., 2013. *Abstract of agricultural statistics,* Republic of South Africa: Agriculture, forestry & fisheries department.

Cooper, R. A., 1984. Metabolism of methylglyoxal in microorganism. *Annu Rev Microbiol,* Volume 38, pp. 49-68.

Cvjetko, P., Mira, Z. & Balen, B., 2014. Proteomics of heavy metal toxicity in plants. *Arh Hig Rada Toksikol,* Volume 65, pp. 1-18.

Dalcorso, G., Bernardini, G. & Santucci, A., 2011. Linking protein oxidation to environmental pollutants: redox proteome approaches. *Journal of Proteomics,* 74(11), pp. 2324-2337.

Dalcorso, G., Farinati, S. & Furini, A., 2010. Regulatory networks of cadmium stress in plants. *Plant signaling and behaviour,* 5(6), pp. 1-5.

Dalcorso, G., Farinati, S. & Furini, A., 2010. Regulatory networks of cadmium stress in plants. *Plant signaling and behavior,* 5(6), pp. 1-5.

DalCorso, G., Farinati, S., Maistri, S. & Furini, A., 2008. How plants cope with cadmium: staking all on metabolism and gene expression. *Journal of integrative plant biology* , 50(10), pp. 1268-1280.

D'Amore, J. J., Al-Adab, S. R., Scheckel, K. G. & Ryano, T. A., 2005. Methods for speciation of metals in soils:a review. *Journal of environmental quality,* 35(5), pp. 1707-1745.

Davasagayam, T. P. et al., 2004. Free radicals and antioxidants in human health : current status and future prospects. *Journal of association of physicians of India,* Volume 52, p. 796.

De Beer, A., 2012. Soybean cultivar recommendations for 2012/2013 , Potchefstroom: ARC - Grain crops institute.

De Beer, A. & Prinsloo, T., 2013. The national soybean cultivar trials in South Africa - 34 years experiences and progress , Potchefstroom: Grain Crops Institute : Agricultural Research Council .

Deng, H., Ye, Z. H. & Wong, M. H., 2004. Accumulation of lead, zinc, copper and cadmium by 12 wetland plant species thriving in metal-cintaminated sites in China. *environmental pollution,* Volume 132, pp. 29-40.

Dietz, K. J., Bair, M. & Kramer, 1999. Free radical and ractve oxygen species as mediators of heavy metal toxicity in plants. In: M. N. Prasad & J. Hagemeyer, eds. *Heavy meatal stress in plants from molecules to ecosystems.* Berlin: Springer-Verlag, pp. 73-79.

Dlamini, T. S., Tshabalala, P. & Mutengwa, T., 2013. Soybeans production in South Africa. *Oilseeds & fats crops and lipids,* pp. 1-11.

Dlamini, T. S., Tshabalala, P. & Mutengwa, T., 2014. Soybeans production in South Africa. *OIlseeds & fats crops and lipids* , 21(2), p. D207.

Dti, 2010. Industrial Policy Action Plan, Pretoria: Department of trade and industry .

Du Toit, J. J., 1942. The cultivation of soybeans., s.l.: Farming in South AFrica.

Emamverdian, A., Ding, Y., Mokhberdoran, F. & Xie, Y., 2014. Heavy metal stress and some mechanisms of plant defense response. *The scientific world journal,* p. 18.

Eskew, D. L., Welch, R. M. & Norvell, W. A., 1983. Mickel: an essential micronutrient for legumes and possibly all higher plants. *Science* , Volume 222, pp. 621-623.

Eslew , D. L., Welch, R. M. & Norvell, W. A., 1984. Nickel in higher plants: futher evidence for an essential role. *Plant physiol,* Volume 76, pp. 691-693.

Espartero, J., Sanchez-Aguayo, I. & Pardo, J. M., 1995. Molecular charecterization of glyoxalase-I from a higher plant; upregulation by stress. *Plant Mol. Bio,* Volume 29, pp. 1223-1233.

Esposito, L. A. et al., 1999. Mitochondrial disease in mouse results in increased oxidative stress. *Proc Natl Acad Sci USA* , Volume 96, pp. 4820-4825.

ETU, 2014. *Education policy: School feeding scheme,* s.l.: www.etu.org.za.

Fangeria, N. K., 2009. *The use of nutrients in crop plants*. Illustrated ed. New York: CRC Press.

Fatoki, S. O., 1996. Trace zinc and copper concentration in roadside surface soils and vegetation-measurment of local atmospheric pollution in Alice, South AFrica. *Environ. Int,* Volume 22, pp. 759-762.

Ferrand, E., Dumat, C., Leclerc-Cessac, E. & Benedetti, M. F., 2006. Phytoavailability of Zr in relation to its initial added form and soil characteristics. *Plant Soil,* Volume 287, pp. 313-325.

Fodor, M., Hegedus, A. & Stefanovitis-Banyai, E., 2002. Examination of effect of Zr ascorbate on wheat seedlings. In: I.Pais (ed) Proceedings of the 10th international trace element symposium. *Budepest,* pp. 76-82.

Fodor, M., Hegedus, A. & Stefanovits-Banyai, E., 2005. Zirconium induced physiological alterations in whear seedlings. *Biol Plantarum,* Volume 49, pp. 633-636.

Foy, C. D., Chaney, R. L. & White, M. C., 1978. Physiology of metal toxicity in plants. *annual review of plant physiology and plant molecular biology,* Volume 29, pp. 511-566.

Gekeler , W., Grill, E., Winnacker, E. L. & Zenk, M. H., 1989. Survey of the plant kingdom for the ability to bind to heavy-metals through phytochelatins. *Zeitschrift fur Naturforschung,* Volume 44, pp. 361-369.

Gill, S. S. & Tuteja, N., 2010. Reactive oxygen speciesand antioxidant machinery in abiotic stress tolerance in crop plants. *Plant physiology and biochemistry,* pp. 909-930.

Griffith, O. W. & Meister, A., 1985. Origin and turnover of mitochondrial glutathione. *Proc.Natl.Acad.Sci.USA,* Volume 82, pp. 4668-4672.

Gundersen, V., Bechmann, I. E., Behrens, A. & Sturup, S., 2000. Comparative investigation of concentrations of major and trace elements in organic and conventional Danish agricultural crops.1. onions (*Allium cepa Hysam*) and Peas (*Pisum sativum ping pong*). *J Agric Food Chem,* Volume 48, pp. 6094-6102.

Hall, W. S., 1930. *Legumes for the summer seasons,* Pretoria: Farming in South Africa.

Han, D., Williams , E. & Cadenas, E., 2001. Mitochondrial respiration chain-dependant generation of superoxide anion and its release into the intermembrane space. *Biochem. J.,* 353(2), pp. 411-6.

Hansch, R. & Mendel, R. R., 2009. Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B, Cl). *Current Opinion in plant biology,* Volume 12, pp. 259-266.

Hansch, R. & Mendel, R. R., 2009. Physiological functions of minerla micronutrients (Cu,Zn,Mn,Fe,Ni,Mo,B,Cl). *Current opinion in plant biology,* Volume 12, pp. 259-266.

Hebbern, C. A. et al., 2009. Latent manganese dificiency increases transpiration in barley (*Hordeum vulgare*). *Physiol Plant,* Volume 135, pp. 307-316.

Hille, R., 1996. The mononuclear molybdenum enzymes. *Chem Rev* , Volume 96, pp. 2757-2816.

Hirata, K., Tsuji, N. & Miyamoto, K., 2005. Biosynthesis regulation of phytochelatins, heavy-metal binding peptides. *Journal of bioscience and bioengineering* , Volume 100, pp. 593-599.

Hoque, T. S. et al., 2016. Methylglyoxal: An emerging signaling molecule in plant abiotic stress responses and tolerance. *Cross mark,* Volume 7, pp. 1-11.

Hossain, A. M., Hossain, M. Z. & Fujita, M., 2009. Stress-induced changes of methylglyoxal level and glyoxalase I activity in pumpkin seedlings and cDNA cloning of glyoxalase I gene. *Australian Journal of crop science,* 3(2), pp. 53-64.

Hossain, M. A., Hasanuzzaman, M. & Fujita, M., 2010. Up-regulation of antioxidant and glyoxalase systems by exogenous glycinebaine and proline in mung bean confer tolerance to cadmium stress. *Physiology and molecular Biology of plants* , 16(3), pp. 259-272.

Hossain, M. A., Jaime, A., Saliva, T. d. & Fujita, M., 2011. Glyoxalase System and Reactive Oxygen Species Detoxification System in Plant Abiotic Stress Response and Tolerance: An intimate Relationship. In: P. A. Shanker, ed. *Abiotic Stress in Plants-Mechanisms and Adaptations.* Europe: Intech, pp. 307-394.

Hossain, M. A., Mostofa, M. G., Burritt, D. J. & Fujiita, M., 2014. Modulation of reactive oxygen species and methylglyoxal detoxification systems by exogenous glycinebetaine and proline improves drought tolerance in mustard (*Brassica Juncea L.*). *Int. J. Plant Biol,* pp. 2-14.

Hossain, M. A., Piyatida, P., Teixeira da Silva, J. A. & Fujita, M., 2011. Molecular mechanism of Heavy Metal Toxicity and tolerance in Plants: Central role of Glutathione

in Detoxification of reactive Oxygen species and methylglyoxal and in Heavy Metal chelation. *Jounal of Botany,* Volume 2012, pp. 1-37.

Issued by the Directorate Agricultural statisctics, 2006. *Crops and markets,* republic of South Africa: Department of agriculture.

Jacks, T. J. & Davidonis, G. H., 1996. Superoxide, hydrogen peroxide, and the respiratory burst of fungally infected plant cells. *Mol. Cell. Biochem,* Volume 158, pp. 77-79.

Johnson, G. B. & Peter, H., 2010. Chapter 39 Nutrition and Transport in plant. In: *Plant form and function.* Washington: McGraw Hill, pp. 777-792.

Johnson, G. B. & Peter, H., 2010. Nutrition and Transport in Plants. In: 6, ed. *Biology.* Washington: McGraw Hill, pp. 777-792, chapter 39.

Joubert, J. C. & Jooste, A. A., 2013. Comparative analysis of the different regions of South African soybean industry, Pretoria: National Agricultural Marketing Council.

Kabata-Pendias, A., 1993. Behavioural properties of trace metals in soils. *Appl Geochem,* Volume 2, pp. 3-9.

Kabata-Pendias, A. & Pendias, H., 1992. Trace elements in soils and plants. 2nd ed. Boca Raton: CRC.

Kaelin Jr, W. G., 2005. ROS: really involved in oxygen sensing. *Cell Metab,* Volume 1, pp. 357-358.

Kalapos , M. P., Garzo, T., Antoni, F. & Mandal, J., 1992. Accumulation of S-D-lactoylglutathione and transient decrease of glutathione level caused by methylglyoxal load in isolated hepatocytes. *Biochim. Biophys. Acta.,* Volume 1135, pp. 159-164.

Kamal, A. et al., 2011. An efficient one-pot synthesis of benzothiazolo-4 beta-anilino-podophyllotoxin congeners: DNA tpopisomerase-II inhibition and anticancer activity.. *Bioorg,* Volume 21, pp. 350-353.

Kancheva, R. H., Borisova, D. S. & Iliev, I. T., 2005. *Chlorophyll florescence as a plant stress indicator,* Bulgarian: Solar-terrestrial influences laboratory, Bulgarian Academy of Sciences.

Karley, A. & White, P. J., 2009. Moving cationic minerals to edible ticcues: potassium, magnesium, calcium. *Curr Opin Plant Biol,* Volume 10, pp. 4-13.

Kaur, C. et al., 2015. Analysis of global gene expression profile of rice in response to methylglyoxal indicates its possible role aas a stress signal molecule. *Plant Science,* Volume 6, pp. 1-13.

Kawagashira, N. et al., 2001. *Multiple zinc finger motifs with comparison of plant and insects.* Tokyo, International conference on Genome informatics.

Khan, S. et al., 2008. Healt risks of heavy metals in containated soils and food crops irrigated with wastewater in Beijing, China. *Environmental pollution,* 152(3), pp. 686-692.

Kiefer, A. S. et al., 2014. Methylglyoxal concentrations differin standard and washed neonatal packed red blood cells. *pediatirc research,* Volume 75, pp. 409-414.

Kim, M. D. et al., 2010. Enhanced tolerance to methyl viologen-induced oxidative stress and high temperature in transgenic potato plants overexpressing the CuZnSOD, APX and NDPK2 genes. *Physiologia Plantarum,* 140(2), pp. 153-162.

Kirkinezos, I. G. & Moraes, C. T., 2001. Reactive oxygen species and mitochondrial diseases. *Cell & development biology,* Volume 12, pp. 449-457.

Kramer, U. & Clemens, S., 2005. Function and homeostasis of zinc, copper, and nickel in plants. *Topics Curr Genet,* Volume 14, pp. 215-271.

Kruglov, S. V., Vasil'yeva, N. A., Kurinov, A. D. & Aleksakhin, A. M., 1996. Distribition of radionuclides from Chernobyl fallout with regard to fractions of the soil-particle distribution of sod-podzolic soils. *Eur soil sci,* Volume 28, p. 2635.

Krzeslowska , M. et al., 2010. Lead deposited in the cell wall of *Funaria hygrometrica* protonemata is not stable - a remobilization can occur. *Environ Pollut,* Volume 158, pp. 325-338.

Krzeslowska, M. et al., 2009. Pectinous cell wall thickenings formation -a response of moss protonemata cells to lead. *Environ Exp Bot,* Volume 65, pp. 119-131.

Kumar, S. M., Swaminathan, K., Clemens, D. L. & Dey, A., 2014. Modulation of GSH with exogenous agents leads to changes in glyoxalase 1 enzyme activity in VL-17A cells exposed to chronic alcohol plus high glucose. *Food function,* Volume 5, pp. 345-358.

Kumpiene , J., Brannvall, E., Taraskeviuus, R. & Aksamitauskas Zinkutus R, 2011. Spatial variability of topsoil contamination with trace elements in preschools in Vilnius, Lithuania. *J Geochem Explor,* Volume 108, pp. 15-20.

Kusunki, M., 2007. Mono-manganese mechanism of the phytosystem II water splitting reaction by a unique Mn4Ca cluster. *Biochim Biophys Acta,* Volume 1767, pp. 484-492.

Langmi, H. & Bessarabov, D., 2013. Hydrogen storage for duel cell applications: Challenges, opportunities and pospects for metal-organic framworks. Korea, Deparment of Science and technology : Republic of South Africa.

Lavado, R. S., Porcelli, C. A. & ALvarez, R., 2001. Nutrient and heavy metal concentration and distribution in corn, soybean and wheat as affected by different tillage systems in the Argentine pampas. *Soil & Tillage research,* Volume 62, pp. 55-60.

Lee, H. L., Howell, S. K., Sanford, R. J. & Beisswenger, P. J., 2005. Methylglyoxal can modify GAPDH activity and structure. *Annals of New York acedemy of sciences,* Volume 1043, pp. 135-145.

Lin, C. W., Chang, H. B. & Huang, H. J., 2005. Zinc induced mitogen-activated protein kinase activation mediated by reactive oxygen species in rice roots. *Plant Physiol Biochem,* Volume 43, pp. 963-968.

Li, W., Khan, M. A., Yamaguchi, S. & Kamiya, Y., 2005. Effects of heavy metals on seed germination and early seedling growth of Arabidopsis thaliana. *Plant growth regulation*, pp. 45-50.

Louvel, M. et al., 2009. *Zr speciation and partitioning in SiO2-rich aqueous fluids ad silicate melts*, s.l.: Goldschmit conference Abstract pg73.

Lusby, P. E., Coombes, A. & Wilkinson, J. M., 2002. Honey: a potent agent for wound healing?. *official publication of The wound, Ostomy and continence Nurses society/ WOCn*, 29(6), pp. 295-300.

Maathius, F. J., 2009. Physiological functions of mineral macronutrients. *Current opinion in plant biology*, Volume 12, pp. 250-258.

Maathuis, F. J., 2009. Physiological functions of mineral macronutrients. *Current opinion in plant biology*, Volume 12, pp. 250-258.

Maathuis, F. J. & Sanders, D., 1993. Energization of potassium uptake in *Arabidopsis thaliana*. *Planta*, Volume 191, pp. 302-307.

Maeta, K., Izawa, S. & Inoue, Y., 2005. Methylglyoxal, a metabolite derived form glycolysis, functions as signal initiatorof the high osomolarity glycerol-motogen-activated protein kinasecascade and calcineurin/Crz1-mediated pathway in *Sacchromyces cerevisiae*. *Journal of biological Chemistry*, Volume 280, pp. 253-260.

Maeta, K. et al., 2004. Activity of the Yap1 transcription factor in *Saccharomyces cerevisiae* is modulated by methylglyoxal a metabolite derived from glycolysis. *Mol.Cell Biol*, pp. 8753-8764.

Maete, K., Izawa, S. & Inoue, Y., 2005. Methylglyoxal, a metabolite derived from glycolysis, functions as a signal initiator of the high osmolarity glyceerol-mitogen-activated protein kinase cascade and calcineurin/Crzl-mediated pathway in *Saccharomyces cerevisiae*. *J.Biol.Chem*, Volume 280, pp. 253-260.

Mailoux, R. J. & Harper, M. E., 2011. Uncoupling proteins and the control of mitochondrial reactive oxygen species production. *Free radic Biol Med*, 51(6), pp. 1106-15.

Majtan, J. et al., 2012. Methylglyoxal-induced modifications of significant honeybee proteinous components in manuka honey:Possible therapeutic implications. *Fitoterapia*, 83(4), pp. 671-7.

Marschner, H., 1995. Mineral nutrition in higher plants , London: Academic Press.

Marschner, H., 1995. Mineral nutrition in higher plants. *London: Academic Press*, Volume 16, pp. 4-12.

Marschner, H., 2012. Marschner's mineral nutrition of higher plants. 3 ed. San Diego: Academic Press.

Mates, J. M., 2000. Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. *Toxicology,* 153(1-3), pp. 83-104.

Maxwell, D. P., Wang, Y. & Mclnotsh, L., 1999. The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. *Proc. Natl. Acad. Sci. U.S.A.,* 96(14), pp. 8271-6.

Meck, M., Love, D. & Mapani, B., 2006. Zimbabwean mine dumps and their impact on river water quality - a reconnaisance study. *Phys. Chem. Earth,* Volume 31, pp. 797-803.

Mehag, A. A., 1994. Integrated tolerance mechanisms: constitutive and adaptive plant responses to elevated metal concentrations in the environment. *Plant, cell and environment,* 17(9).

Mehdy, M. C., 1994. Active oxygen species in plant defense against pathogens. *Plan Physiol,* Volume 105, pp. 467-472.

Mendoza-Cozalt, D. G. et al., 2008. Identification of high leels of phytochelatins glutothone and admium in the phloem sap of *Brassica napus*. A role for thiol-peptides in the long distance transport of cadmium ad the effect of cadmium on iron translocation. *plant journal,* Volume 54.

Miller, A. J., 2009. Freeways in the plant: transporters for N, P and S and their regulation. *Curr Opin Plant Biol,* Volume 4, pp. 10-16.

MininginSouthAfrica, 2014. *Mining in South Africa,* Johanesburg: s.n.

Miyata, S. et al., 2002. Activation of MAP kinase superfamily signaling pathways by methylglyoxal. *Int Congr. Ser,* 12(45), pp. 87-89.

Moirangthem, L. D. et al., 2014. A high constitutive catalase activity confers resistance to methyl viologen-promoted oxidative stress in a mutant of the *Cyanobacterium nostoc* punctiforme ATCC 29133. *Springer Link,* 98(8), pp. 38-59.

Moller, I. M., 2001. Plant mitochondria and oxidative stress: Electron transport, NADPH trnover and metabolism of reactive oxygen species. *Annu. Rev. Plant Physiol. Plant Mol. Biol.,* Volume 52, pp. 561-91.

Moller, I. M., 2010. Chapter 11-Reactive oxygen species (ROS) and plant respiration. In: *Plant physiology*. 5th ed. Denmark: s.n.

Mosulishvili, L. M., Shoniya, N. I., Katamadze, N. M. & Ginturi, E. N., 1994. ENvironmental radionuclide distribution in the republic of Georgia after the Cherobyl catastrophe. *Zh ANal Khim,* Volume 49, pp. 135-139.

Mou, D. et al., 2011. Plant high tolerence to excess manganese related with root growth, manganese distribution and antioxidative enzyme activity in three grape cultivars. *Ecotoxicol Environ Saf,* Volume 74, pp. 776-786.

Muhs, D. R. et al., 2010. The role of African dust in the formation of Quaternary soils in Mallorca, Spain and implications for the genesis of red mediterranean soils. *Quaternary Sci Rev,* Volume 29, pp. 2518-2543.

Mushtaq, A., 2012. Producing radioisotopes in power reactors. *J. Radioanal Nucl Chem ,* Volume 292, pp. 793-803.

Mushtaq, A., 2012. producing radioisotopes in power reactors. *J Radioanal Nucl Chem ,* Volume 292, pp. 793-802.

Naicker , K., Cukrowska, E. & McCarthy, T. S., 2003. Acid mine drainage arising from gold mining activity in Johannesburg, South Africa and environs. *Environ. Pollut,* Volume 122, pp. 29-40.

Navari-Izzo, F., 1998. Thylakoid-bound and stromal antioxidative enzymes in wheat treated with excess copper. *Physiologia Plantarum,* 104(4), pp. 630-638.

Nicholson, F. A. et al., 2003. AN inventory of heav metalsinput to agricultural soils in England and Wales. *Environ,* Volume 311, pp. 205-219.

Oancea, S., Foca, N. & Airinei, A., 2005. Effects of heavy metals on plant growth and photosynthetic activity.

Okedeyi, O. O., Dube, S., Awufolu, O. R. & Nindi, M. M., 2014. Assessing the enrichment of heavy metals in surface soil and plant (Digitara eriantha) around coal-fired power plants in South Africa. *Environ Sci Pollut Res,* Volume 21, pp. 486-4696.

Olande, M. A., 1987. Heavy metal pollution and the need for monitoring: illustrated for developing countries in West Africa. In: T. C. Hutchinson & K. M. Meema, eds. *Lead, Mercury, Cadmium and Arsenic in the Environment.* Ibadan: John Wiley & Sons Ltd.

Opperman , C. & Varia, S., 2011. *Techical report: Soybean value chain.,* s.l.: AECOM International Development.

Pettersson, U. T. & Ingri, J., 2001. The geochemistry of Co and Cu in the Kaufe River as it drains the Copperbelt mining area, Zambia. *Chem. Geol,* Volume 177, pp. 399-414.

Piol, M. et al., 2006. Copper cofactor delivery in plant cells. *Curr Opin Plant Biol,* Volume 9, pp. 256-263.

Pompliano , D. L., Peyman, A. & Knowels, J. R., 1999. stabilization of a reaction intermediate as a catalytic device: definition of the functional role of the flexible loop in triosephosphate isomerase. *Biochemistry,* 29(13), pp. 3186-3194.

Pourrut, B. et al., 2008. Potential role of NADPH-oxidase in early steps of lead-induced oxidative burst in *Vicia faba roots*. *J Plants Physiol* , Volume 165, pp. 571-579.

Rabbani, N. et al., 2011. Glycation of LDL by methylglyoxal increases arterial atherogenicity. A possible contributor to increased risk of cardiovascular disease in diabetes. *Diabetes,* 60(7), pp. 1973-80.

Radulescu, C. et al., 2013. Heavy metal accumulation and translocation in different parts of *Brassica oleracea* L. *Rom.Journ.Phys,* Volume 58, pp. 13337-1354.

Rascio, N. & Navari-Izzo, F., 2011. Heavy metal hyperaccumulating plants: how and why do they do it? And what makes them so interesting?. *Plant Science* , 180(2), pp. 169-181.

Ravengai, S. et al., 2005. Impact of Iron Duke Pyrite Mine on water chemistry and aquatic life - Mazowe valley, Zimbabwe. *Water,* Volume 31, pp. 219-228.

Reliene, R. & Schiestl, R. H., 2006. Glutathione depletion by buthionne sulfoximine induces DNA deletions in mice. *Carcinogenesis,* Volume 27, pp. 240-244.

Richard, J. P., 1993. Mechanism for the formtion of methylglyoxal from trioephosphate. *Biochem. Soc,* Volume 21, pp. 549-553.

Romheld, V. & Harschner, H., 1991. *Function of micronutrients in plants,* Germerny: Institue of Pflanzenernahrung, Stuttgart.

Roy, K., De, S., Ray, M. & Ray, S., 2004. Methylglyoxal can completely replace the requirment of kinetin to induce differentiation of platlets from some plant calluses. *Plant growth Regul,* pp. 33-45.

Saadat, D. & Harrison, D. H., 1999. The crystal structure of methylglyoxal synthase from *Escherichia coli. Elsevier Science,* Volume 7, pp. 309-317.

Saito, R. et al., 2011. Methylglyoxal functions as Hill oxidant and stimulates the photoreductin of O2 at photosystem I: a symptom of plant diabetes. *Plant , cell and environment* , 34(9), pp. 145-1464.

Sandalio, L. M. et al., 2001. Cadmium-induced changes in the growth and oxidative metabolism of pea plants. *Journal of experimental botany,* 52(364), pp. 2115-2126.

Sandoval, R. et al., 2011. Removal of asenic and methylene blue from water by granular activated carbon media impregnated with zirconium dixide nanoparticles. *J Harzard Mater,* Volume 193, pp. 296-303.

Santagostini, L. et al., 2004. Probing the location of the substrate binding site of ascorbate oxidase near type 1 copper: an investigation through spectroscopic, inhibition and docking studies. *Int J Biochem Cell Biol,* Volume 36, pp. 881-892.

Sanzharova, D. I. & Aleksakhin, R. M., 1982. Uptake of 22Na, 32P, 65Zn, "Sr and ""Ru by crops. *Pochvovedeniye,* Volume 9, pp. 59-64.

Sasaki-Sekimoto, Y. et al., 2005. Coordinated activation of metabolic pathways for antioxidants and defence compounds by jasmonates and their roles in stress tolerance in Arabidopsis. *The Plant Journal,* Volume 44, pp. 568-668.

Satarug, S. et al., 2003. A global perspective on cadmium pollution. *Toxicol lett,* Volume 137, pp. 65-83.

Saxena , M. et al., 2011. Overexpression of the Glyoxalase II gene leads to enhanced salinity tolerance in *Brassica juncea*. *The open Plant Sci. J,* Volume 5, pp. 23-28.

Schutzendubel, A. & Polle, A., 2002. Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. *Journal of experimental botany* , Volume 372, pp. 1351-1365.

Schwarz, G. & Mendel, R. R., 2006. Molybdenum cofactor biosynthesis and molybdenum enzymes. *Ann Rev Plant Biol,* Volume 57, pp. 623-647.

Shahid, M., Ferrand, E., Schreck, E. & Dumat, C., 2013. Behaviour and impact of zirconium in the soil-plant system: plant uptake and phytotoxicity. *Rev Envoron Contam Toxicol,* Volume 221, pp. 107-27.

Shahid, M., Ferrand, E., Schreck, E. & Durnat, C., 2013. Behavior and impact of Zirconium in the Soil-plant system: Plant uptake and pytotoxicity. *Reviews of environmntal contamination and toxicity,* Volume 221, pp. 107-127.

Sharma, P. & Dubey, R. S., 2007. Involvement of oxidative stress and role of antioxidative defense system in growing rice seed-lings exposed to toxic concentrations of aluminum. *Plant cell reports,* 26(11), pp. 2027-2038.

Sharma, S. S. & Dietz, K. J., 2009. The relationship between metal toxicity and cellular redox imbalance. *Trends in plant science* , 14(1), pp. 43-50.

Shinohara, M. et al., 1998. overexpression of glyoxalase-I in bovine endothelial cells inhibits intracellular advanced glycation endproduct formation and prevents hyperglycemia-induced increases in macromolecular endocytosis. *j Clin Invest,* 101(5), pp. 1142-1147.

Shinozaki, K. & Yamaguchi-Shinozaki, K., 1997. Gene expression and signal transduction and signal transduction in water-stress response. *Plant physiology,* Volume 115, pp. 327-334.

Singla-Pareek, S. L., Reddy, M. K. & Sopory, S. K., 2003. Genetic engineering of the glyoxalase pathway in tabacco leads to enhanced salinity tolerance. *Proc. Natl. Acad.Sci.USA,* Volume 100, pp. 14672-14677.

Singla-Pareek, S. L. et al., 2006. Transgenic tabacco overexpressing glyoxalase pathway enzymes grow and set viable seeds in zinc-spiked soils. *Plant physiology,* Volume 140, pp. 1532-2548.

Stoltz, E. & Greger, M., 2002. Accumulation properties of As,Cd, Cu, Pd and Zn by four wetland plant species growing on submergeed mine tailings. *Environmental and experimental botany* , Volume 47, pp. 271-280.

Sylvestre, H. et al., 2013. Nutrient uptake and yeild of Soybean (*Glycine max* L.) as influenced by pre and post-emergence herbicides. *Pelagia research library,* 3(5), pp. 33-40.

Takatsume, Y., Izawa, S. & Inoue, Y., 2006. Methylglyoxal as a signal initiator for activation of the stress-activated protein kinase cascade in the fission yeast *Schizosacchararomyces pombe*. *Journal of biological chemistry,* 281(14), pp. 9086-9092.

Tanner, s. & Baranov, V., 1999. A dynamic reaction cell for ICP-MS. Part 2: Reduction of interferences produced within the cell. *Journal of the american society for mass spectrometry,* 10(11), pp. 1083-1094.

Thomas, R., 2001. A beginner's guide to ICP-MS. *Spectroscopy. Advanstar communications,* 05(09), pp. 2005-2014.

Thornalley, P. J., 1990. The glyoxalase system: new developments towards funtional characterization of a metabolic pathway fundamental to biological life.. *Biochemical Journal* , Volume 269, pp. 1-11.

Tome, F. V., Blanco, R. & Lozano, J. C., 2003. Soil-to-plant transfer factors for natural radionuclides and stable elements in a Mediterranean are. *J Environ Radioact,* Volume 65, pp. 161-175.

Toyoda, Y. et al., 2014. Products of the Parkinson's-disease related glyoxalase Dj-1, D-lactae and glycolate, support mitochondrial membrane potential and neuronal survival, Germany:

Turkdogan, M. K. et al., 2002. Heavy metals in soil, vegetables and fruits in the endemic upper gastrointestinal cancer region of Turkey. *Environmental toxicology and pharmacology,* Volume 13, pp. 175-179.

USGS, 2012. *United states geological survey.* [Online] Available at: http://minerals.usgs.gov/minerals/pubs/commodity/ zirconiuM+. Assessed 23

van Assche, F. & Clijsters, H., 1990. Effects of metals on enzyme activity in plants. *Plant, cell & environment,* pp. 195-206.

Van De Merwee, R., Van Biljon, A. & Hugo, A., 2013. *Current and potential usage of soybean products as food in SOuth AFrica,* Bloemfontein: Univerisity of the Free State: Abstract 333.

Van De Merwe, R., Van Biljon , A. & Hugo, A., 2013. *Current and potential usage of soybean products as food in South Africa* , Bloemfontein: University of the Free State Abstract 333.

Vanlerberghe, G. C. & Mclntosh, L., 1997. ALternative oxidase: from gene to function. *Annual review of Plant physiology and plant molecular biology,* Volume 48, pp. 703-734.

Vatamaniuk, O. K., Mari, S., Lu, Y. & Rea, P. A., 2000. Mechanism of heay metal ion activation of phytochelatin synthase. *Jounal of biological chemistry* , Volume 278, pp. 31451-31459.

Veena , R. V. & Sopory, S. K., 1999. GlyoxalaseI from *Brassica juncea*: molecularcloning, regulation and its over-expressionconfer tolerance in transgenictobacco under stress.. *The Plant Journal*, Volume 17, pp. 385-395.

Villers, F., Ducruix, C. & Hugouvieux, V., 2011. Investigating the plant response to cadmium exposure by proteomics and metabolomic approaches. *Proteomics,* 11(9), pp. 1650-1663.

Vistoli, G. et al., 2013. Advanced glycoxidation and lipoxidation end products (AGEs and ALEs): an overview of their mechanisms of formation. *Free Radic Res,* 47(12), pp. 3-27.

Von der heyden, C. J. & New, M. G., 2004. Sediment chemistry: a history of mine contaminant remediation and an assessment of processess and pollution potential. *J. Geochem. Explor* , Volume 82, pp. 35-57.

Wang, H. F., Takematsu, N. & Ambe, S., 2000. Effects of soil acidity on the uptake of trace elements in soybean and tomato plants. *Appl Radiat Isot*, Volume 52, pp. 803-811.

Wang, W., Vinocur, B. & Altam, A., 2003. Plant responses to drought, salinity and extreame temperatures towards genetic engineering for stress tolerance. *Planta*, Volume 218, pp. 1-14.

Wild, A., Sauer, H. & Ruhle, W., 1987. The effects of phosphinothrican (glufosinate) on photosynthesis. I. inhibition of photosynthesis and ammonia accumulation. *C-Biosciences*, Volume 42, pp. 263-269.

Wojtaszek, P., 1997. Mechanisms for he generation of reactive oxygen species in plant defence response. *ACTA physiologiae plantarum,* 19(4), pp. 581-589.

Wojtaszek, P., 1997. Mechanisms for the generation of reactive oxygen species in plant defence response. *APP: Acta physiologiae plantarum,* 19(4), pp. 581-589.

World Bank, 2014. *South Afrian population statistics*, s.l.: World bank.

Xinyan, L. et al., 2013. Targeting mitochondrial reactive oxygen species as novel therapy for inflammatory diseases and cancer. *Journal of hematology and oncology* , Volume 6, pp. 1-19.

Xu, D., Liu, X., Guo, C. & Zhao, J., 2006. Methylglyoxal detoxification by an aldo-keto reductase in the *Cyanobacterium synechococcus* sp.PCC 7002. *microbiology*, Volume 152, pp. 2013-21.

Yabe, J., Ishizuka, M. & Umemura, T., 2010. Current levels of heavy metal pollution in Africa. *J.Vet.Med.Sci,* 72(10), pp. 12657-1263.

Yabe, J., Ishizuka, M. & Umemura, T., 2010. *Current levels of heavy metal pollution in AFrica*, Hokkaido University, Japan: Review - Toxicology.

Yadav, S. K., 2010. Heavy mtals toxixity in plans: An overview on the role of glutathione and phytocheains in heavy metal stress tolerance of plants. *Science direct,* Volume 76, pp. 167-179.

Yadav, S. K. et al., 2005. Methylglyoxal levels in plants under salinity stress are dependent on glyoxalase I and glutathione. *Biochemical and Biophysical research communication* , Volume 337, pp. 61-67.

Yadav, S. K. et al., 2005. Transgenic tabacco plants overexpressing glyoxalas eenzymes resist an increase in methylglyoxal and maintain higher reduced glutathione levels under salinity stress. *FEBS Letters* , Volume 579, pp. 6265-6271.

Yamasaki, H. et al., 2007. Regulation of copper homeostasis by micro-RNA in Arabidopsis. *J Biol Chem*, Volume 282, pp. 16369-16378.

Yin, F., Sancheti, H. & Cadenas, E., 2012. Mitochondrial thiols in the regulation of cell death pathways. *Antioxid.Redox.Signal*, Volume 17, pp. 1714-1727.

Yirchenko, Y. P. & Agapkina, G. I., 1993. Organic radionuclide compounds in soils surrounding the CHernobyl nuclear power plant. *Eur Soil Sci*, Volume 25, pp. 51-59.

Zaccone, C. et al., 2008. Distribution of As, Cr, Ni, Rb, Ti and Zr between peat and its humic fraction along an undisturbed ombrotrophic bog profile (NW Switzerland). *Appl Seochem*, Volume 23, pp. 25-33.