2015

UNIVERSITY *of the*
WESTERN CAPE

Yo-Neal Bless

Modulation of *Brassica rapa* L. antioxidant activities by exogenous Methylglyoxal under Zirconium stress

Contents

UNIVERSITY *of the*
WESTERN CAPE

FACULTY OF NATURAL SCIENCE

GENERAL PLAGIARISM DECLARATION

Name: Yo-Neal Bless

Student number: 2849848

1. I hereby declare that I know what plagiarism entails, namely to use another's work and to present it as my own without attributing the sources in the correct way. (Refer to University Calendar part 1 for definition)

2. I know that plagiarism is a punishable offence because it constitutes theft.

3. I understand the plagiarism policy of the Faculty of Natural Science of the University of the Western Cape.

4. I know what the consequences will be if I plagiarise in any of the assignments for my course.

5. I declare therefore that all work presented by me for every aspect of my course, will be my own, and where I have made use of another's work, I will attribute the source in the correct way.

Y. Bless   -11 March 2016-

Signature   Date

ACKNOWLEDGEMENTS

v

Lastly, to **Arun Gokul**, mere words are inadequate to express the gratitude I feel. In these four years of knowing you, I regard you as my mentor and friend. Thank you for the unfailing support and assistance academically, and for the hours you spent helping me and facilitating this whole process. Your continuous support deserves an award. Your guidance and prep talks helped me remain calm when the alternative was an easy route. Thank you for being the best friend I could ask for and for always being there for me.

LIST OF FIGURES

viii

LIST OF TABLES

UNIVERSITY *of the*
WESTERN CAPE

LIST OF ABBREVIATIONS

AGEs - advanced glycation end products

APX - Ascorbate peroxidase

CAT - catalase

CD - conjugated dienes

cDNA - copy deoxyribonucleic acid

DCP - dicarbonyl proteome

DNA - deoxyribonucleic acid

ETC - electron transport chain

GLY-I - glyoxalase I

GLY-II - glyoxalase II

H_2O_2 - hydrogen peroxide

HMs - heavy metals

ICP-OES - inductively coupled plasma-optical emission spectroscopy

MDA - malondialdehyde

MG - methylglyoxal

O_2^- - superoxide

•OH - hydroxyl radical

PUFA - poly unsaturated fatty acids

RNA - ribonucleic acid

ROS - reactive oxygen species

SOD - superoxide dismutase

VLCMFAs - very long chain monounsaturated fatty acids
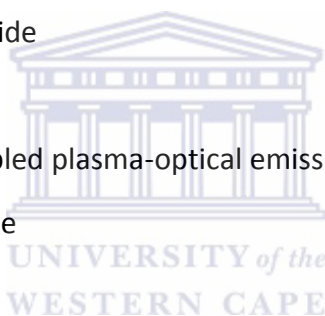
ABSTRACT

With a decrease in water availability and arable land, and the ever-increasing reports of toxic chemical pollutants, it is crucial to elucidate plants' mechanisms of adaptability to these abiotic stressors. South Africa alone accounts for approximately 30% of global Zirconium (Zr) production. However, reports on Zr-induced stress in plants are sparse. Increased mining activity leads to soil contamination which subsequently has harmful effects on crop plants. Under normal conditions *B. rapa* crop plants flourish, they are rapid in their cycling and circumvent the seed dormancy stage which enables them to have high yields over relatively short periods. However, when unfavourable conditions arise, such as exposure to toxic chemicals and metal ions like Zirconium, the development and growth of *B. rapa* L., much like other crop plants is affected. More specifically, the damaging effects of Zr is not only attributed directly; as with substitutions of biometals [like Iron (Fe)] in various biomolecules rendering them inactive, but more as a consequence of the production of toxic molecules such as reactive oxygen species (ROS) and methylglyoxal (MG). ROS such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are known to have signalling roles in plants with reports on their involvement in alleviating seed dormancy and seedling development. However, the signalling roles of MG are not known with regards to plant cells and have been reported more so in animal cells; playing vital roles in fat signalling in diseases such as diabetes. Furthermore MG, in plant and animal cells, directly converts oxygen (O_2) to O_2^- and thus increases the cell's oxidative imbalance, leading to cell damage if O_2^- is not rapidly dismutated to H_2O_2 and H_2O by superoxide dismutase (SOD). In turn, H_2O_2 is more stable than O_2^- and consequently is more toxic to cells over time. Therefore, H_2O_2, must be removed as well by a collection of enzymes, such as ascorbate peroxidase (APX) and catalases (CAT). In this study, possible stress-signalling of MG in seedlings under normal conditions and Zr-stress were investigated to establish whether MG at a low dose (6 µM) would benefit

seedling growth and development, via a proposed preinduction of the *B. rapa* L. antioxidant system. Therefore, it was proposed that ROS accumulation due to the exogenous application of MG, would incite the activation of antioxidants and thus mitigate the effects of Zr stress. Physiological tests to determine dry weights (figure 3.2.3) and germination percentage (figure 3.2.2) revealed that MG-treated seedlings yielded an improved biomass and early development compared to Zr-treated seedlings and the control. Membrane damage as assessed by lipid peroxidation viz. Malondialdehyde [MDA] (figure 3.2.4) and conjugated dienes [CD] (figure 3.2.5) also indicated less damage in MG-treated seedlings compared to the Zr-treated set. The chlorophyll content observed was prominent (table 3.1). MG-treated seedlings exhibited a 40% and 15.5% increase compared to Zr-treated seedlings and the control respectively. Moreover cell viability had improved in MG-treated seedlings compared to the control, and in MG+Zr-treated seedlings only a slight increase in cell death occurred despite Zr being present. O_2^-, H_2O_2 and •OH (figure 4.2.1 – 4.2.3) were investigated in *B. rapa* L. seedlings in response to Zr and MG by spectrophotometric biochemical assays, as well as their scavenging enzymes, MG accumulation and Gly-I activity. Furthermore, *BrGLY1* gene expression and Zr-uptake by ICP-OES were performed. Seedlings treated with MG and Zr respectively showed an increase in ROS. However, all of the ROS observed in MG+Zr-treated seedlings were markedly lower compared to Zr-treated seedlings. SOD and CAT activity observed in MG+Zr-treated seedlings had decreased compared to Zr-treated seedlings, whereas APX activity had increased. Gly-I activity and *BrGlyI* gene expression had increased across all treatments, showing an elicited response to oxidative stress, due to the observed upregulation, as a result of the accumulated MG. The observed Zr-uptake in MG+Zr-treated seedlings was inhibited by 5-fold compared to Zr-treated seedlings. Clear signs of stress were evident in seedlings treated with Zr compared to the control and MG-treated seedlings, the MG-supplemented (MG and MG+Zr) seedlings displayed a vast improvement comparatively. Modulation of antioxidant activity observed in this study is

indicative of an incited response to oxidative stress (figure 4.2.4 – 4.2.6). MG revealed distinct involvement in stress-signalling, ROS levels had increased, although not as severely as with Zr-treated seedlings, but seemingly enough to activate antioxidants without eliciting damage. Furthermore, the proposed early-onset activation of antioxidants has been observed in *B. rapa* L. seedlings of this study, and as such has resulted in improved growth, development and seed germination. The results of this study has therefore negated the previous reports on MG-toxicity (at high concentrations), and has shed light on further properties of this ubiquitous and inevitably-occurring metabolite at low levels.

UNIVERSITY *of the*
WESTERN CAPE

CHAPTER ONE

LITERATURE STUDY

1.1. INTRODUCTION

In recent years, both locally and globally, abiotic stress in plants has been exacerbated by natural environmental factors such as drought, accumulated salts in soil, flooding, acidic precipitation as well as anthropogenic activities such as mining (Yang *et al.,* 2011). The latter, having caused an accumulation of toxic metal ions and chemical wastes in soils, has been under scrutiny, however only a few common Heavy metals (HMs) have been considered; Cd, Cu, Pb and Zn (Hossain *et al.,* 2012). On a global scale mining activity viz. mining, production and purification or treatment of metal products, has been on the rise for the past few decades (Yang *et al.,* 2011; Yadav, 2010).

South Africa is one of the top producers of HMs and precious metals such as Gold, Platinum and Vanadium, and it accounts for nearly a third of the global production of Zr (StatsSA, 2012; USGS, 2012). With its broad range of applications from ceramics to metal-alloys, due to its high heat- and corrosive-resistant properties, the demand for production of Zr will increase imminently (Fodor *et al.,* 2005; USGS, 2012).

Therefore, a gap in information regarding the damage to plants by HMs such as Zr, whose secondary mining products leech into soils in surrounding areas, has been identified (Shahid *et al.,* 2013; Fodor *et al.,*2005; USGS, 2012). It is crucial to

1

understand and determine the extent of damage to plants by HM exposure since it is known that once water has reached the water table it traverses hundreds to thousands of kilometres from its origin (Gill and Tuteja, 2010). Plainly, this means that the soil from farms, rural towns and cities surrounding mining activities are wide-open to the toxic run-off.

Consequently crop plants that grow in affected areas are exposed to uncertain levels of toxic HMs that may or may not be taken up, but that definitely influence plant growth, development, seed dormancy, germination, biological function, DNA and protein integrity and nutritional profiles (Hossain *et al.,* 2012; Yadav *et al.,* 2005a; Mourato *et al.,* 2012) .

The literature supports the notion that HMs are detrimental to plants and a range includes Cd, Ni, Zn, B, Cu and V (Mourato *et al.,* 2012; Yang *et al.,* 2011). These metals have proven to be toxic at low levels in plants and exclude biometals like Fe, Se, and Mg. Zr has reportedly played a role in DNA damage and interference with proteins (Ghosh, 1992; Shahid *et al.,* 2013, 2014), however scant evidence is available, thus making it crucial that new insights are gained to improve crop security and sustainability (Hossain *et al.,* 2012). It is also important since HM's cause oxidative stress and increases ROS to cytotoxic levels (Yadav *et al.,* 2005, Hossain *et al.,* 2009).

Oxidative stress is often caused by abiotic stress; an umbrella term for unfavourable conditions caused by extreme heat, light, cold, chemical wastes, salinity, drought, and heavy metals. HMs occur naturally and sometimes as a result of industrialization, and despite some of them having biological function; biometals, there are those HMs that have the potential to wreak havoc on cell viability and metabolic processes, if not for

2

antioxidants. Certain antioxidants are ROS-scavenging enzymes that serve to detoxify ROS that are unavoidably accumulated, either naturally or due to the onset of cellular stress. Their increased activity therefore also indicates oxidative stress within the cell. The accumulation of ROS may lead to growth and developmental issues and often causes cell death and hampered photosynthesis. Because HMs have been associated with oxidative stress and damage in plants, and due to the lack of information regarding the effects of Zr, stringent efforts to mitigate the damage to plants is of critical importance.

Previously arable soils are now contaminated to the extent that cultivation is impossible in certain parts of South Africa. Because soils that surround mining activity have not been spared, plants such as economically important *Brassicaceae* species have been gravely impacted (Cardoza and Stewart, 2004; Pua and Douglas, 2004; Cartea *et al.,* 2011). The Brassicas (*Brassicaceae*) yield high antioxidant and nutritious levels over a range of products including seed oils, feedstock, biofuels and vegetative crops (Reiner *et al.,* 1995; Cardoza and Stewart, 2004). With the decline in crop security and an increase in soil contamination as the literature has shown, the concern is no longer if HMs cause damage to plants, but the extent to which plants are impacted regarding their physiology, biochemistry and molecularly. We believe that elucidating possible MG-associated pathways that alleviate stress in plants, is one step towards to improving crop security.

1.2. *BRASSICA RAPA* L.

1.2.1. ECONOMIC IMPORTANCE AND APPLICATIONS

The history of *Brassica rapa*, of the *Brassicaceae* family, dates back to the 1300's as reported by Reiner et al. (1995). Cardoza and Stewart (2004) describe the Brassicas with its vast umbrella of subspecies to be the most important genus, economically (Mussury and Fernandes; 1999; Cardoza and Stewart, 2004; Pua and Douglas, 2004).

B. rapa L. in its own right is an important species with *Brassica* oil seeds contributing most significantly on an economical base and as a healthier substitute to saturated oils, with the added benefit of genetically enhanced HILO (High linoleic acid) and HERO (High erucic acid) properties (King, 2005). Brassica oilseed production is the most important source of vegetable oils in the world, next to soybean and cotton seed (Pua and Douglas, 2004). *B. rapa* also has a wide range of other uses which includes vegetables such as Pak Choi, turnip and Chinese cabbage (Celucia *et al.,* 2009; Cartea *et al.,*2011), feedstock, and biofuel crops, that provide rich genomic variety, produce phenolic compounds and have medicinal properties (Reiner *et al.,* 1995; Mussury and Fernandes, 1999; Cardoza and Stewart, 2004; King, 2005).

Brassica species have been bred to have high heat, or low water adaptability (Cardoza and Stewart, 2004; King, 2005), and very long chain monounsaturated fatty acids, or VLCMFA'S (Cardoza and Stewart, 2004) which is not only suitable to grow in the harsh Sub-Saharan climate, but are also suitable for low fatty-acid, low cholesterol and strict low lipid-profile dietary requirements (King, 2005; Cartea *et al.,* 2011). Their uses have been exploited since the 14th century by the Dutch (Reiner *et al.,* 1995), and has in the last few centuries been firmly incorporated into human diets worldwide (Cartea *et al.,*

4

2011). As recently as the last few years there has been a new application called Brassiodol, from which rapeseed oil is used as a vector for iodination (King, 2005). Another key advantage is farming Brassica plants as they grow well with little to no cultivation which vastly reduces labour costs, and this is especially advantageous since post-processing costs far outweigh the cost of farming and harvesting (Cardoza and Stewart, 2004; King, 2005).

1.2.2. *B. RAPA* L.: BIOTECHNOLOGICAL SIGNIFICANCE

B. rapa L. is a 'fast plant' and although it grows well in high temperatures, it adapts very well to cold conditions too, it circumvent the seed dormancy stage which allows for a rapid life-cycle and experience high female fertility as well as rapid seed maturation (Miller and Schemske, 1990; Cardoza and Stewart, 2004; Pua and Douglas, 2004). Although the flavour or phenotypic profiles will differ directly due to environmental factors, Brassica plants can assure higher yields due to their rapid life cycle (Miller and Schemske, 1990; Mussury and Fernades, 1999; Cardoza and Stewart, 2004; Celucia *et al.,* 2009). On a molecular level *B. rapa* species are advantageous since they have a relatively small genome of 500 - 550 Mbps which is also genetically diverse (King, 2005). Plants naturally have their own unique glycoproteins; signalling proteins that signal the abortion of self-fertilized ovaries. This "self-incompatibility makes these 'fast plants' dependent on pollinators like the honey bee that not only contributes to, but also ensures *B. rapa's* rich diversity (Adler *et al.,* Cardoza and Stewart, 2004; Celucia *et al.,* 2009; 1993; King, 2005).

Nutritionally, the Brassicas are well-known for their vegetables having a high nutrient content with high fibre, folate and vitamin A, C, E and potassium, in addition to the presence of iron and phenolic compounds (Cardoza and Stewart, 2004, King, 2005; Cartea *et al.,* 2011). High folate levels are crucial to unborn babies and the health of expecting mothers and it has been linked with prevention of *Spina bifida*, megaloblastic anemia, neuropsychiatric disorders and certain forms of cancer in humans (King, 2005; Pua and Douglas, 2004).

Studies have shown that phytochemicals in brassica plants can up-regulate detoxifying systems in consumers of Brassica vegetables and oilseeds (Cardoza and Stewart, 2004). *B. rapa* and the Brassicas have been affiliated with alleviating or preventing aging, cataracts, cardiovascular disease, Alzeimer's disease and respiratory illnesses in humans (Miller and Schemske, 1990; Cardoza and Stewart, 2004; Celucia *et al.,* 2009). With that in mind, the importance of Brassica-geared research on various topics is evident especially since it is the closest relative of *Arabidopsis thaliana* from which much information has been gained in recent years that can further lend insight into *B. rapa* L. (Miller and Schemske, 1990; King, 2005).

A host of stress-inducible genes have been discovered and researched since *A. thaliana's* genome has been sequenced (Mourata *et al.,* 2012) and with the recently sequenced *B. rapa* genome (Cheng *et al.,* 2013) many opportunities for genetic and molecular studies remain untapped. *Brassica* crops were some of the first targeted for traits such as herbicide resistance and modification of male sterility and for commercial transgenic genetic modification (King, 2005; Cartea *et al.,* 2011). Furthermore there have been many research initiatives and patents associated with

modification of fatty acid pathways for the production of novel oils (Cardoza and Stewart, 2004).

1.3. HEAVY METALS [HMs] AND THEIR IMPACTS ON PLANTS

In nature there are metals known as bioelements or biometals whose presence allow biological function at relatively low levels (Masarovicova and Kralova, 2012). There are also industrially used metals that may be used in pesticide and herbicide manufacturing as well as anthropological by-products and waste products due to mining and production as well as paper milling and so forth. (Hossain *et al.,* 2012; Masarovicova and Kralova, 2012). The mining metals, being predominantly heavy metals (HMs), occurring in even the minutest doses may result in physiological damage to plants and affect the health of its consumers. Even humans may be affected through the food chain (Hossain *et al.,* 2012; Ryzhenko *et al.,* 2008). These toxic metals and biometals at high concentrations form a group of elements, in part, that make up the xenobiotics, and are also known as HMs (Marasovicova and Kralov, 2012).

Despite certain HMs occurring naturally in soils, the increase in mining activities and industrialization has resulted in toxic levels of HMs present in soils in recent years (Sanchez *et al.,* 2010; Ryzhenko *et al.,* 2008; Marasovicova and Kralov, 2012). This is of global and serious concern since plants are unable to physically avoid them and suffer oxidative stress when exposed to HMs even over short or varying periods (Hossain *et al*., 2012). The stress caused can be damaging as they interfere with plants' biological, physiological and biochemical processes (Fones *et al.,* 2010; Guala *et al.,* 2010; Hossain *et al.,* 2012).

7

HM effects on plants have been widely studied, therefore a platform for comparison to zirconium (Zr) toxicity exists. The effects of HM's in Alfalfa seedlings and mature plants were studied at concentrations above 100 mg.L^{-1}. Cr, Cd, Ni, and Cu, drastically affected seedlings to the point of lethality (Perata-Videa *et al*., 2004), whereas Zn had a lesser effect in the same study. Despite this, all metals from this study affected alfalfa growth and development.

1.3.1. SIGNIFICANCE, PRODUCTION AND APPLICATIONS OF ZIRCONIUM [ZR]

Zr is extensively mined globally and particularly in South Africa; the world's second largest producer (USGS, 2012; StatsSA, 2012). In 2010 there was an estimated 390 000 metric tons produced according to USGS (2010). Mining processes and the use of Zr in nuclear reactors results in production wastes, that if not bioremediated or properly disposed of leads to soil contamination (Shahid *et al.,* 2014) and further results in physiological stress in plants and detriments cell viability (Mourato *et al.,* 2012; Fodor *et al.,* 2005). Shahid et al. (2013) have shown the increase in Zr production by 90 thousand metric tons annually. They have also shown the increase across industrially used HMs including Zr, with As alone having a decrease in the production percentage from 2007 – 2011 (figure 1.3.1) obtained from USGS (2012). This firmly establishes the importance of determining the extent of Zr damage to arable lands and crop plants.
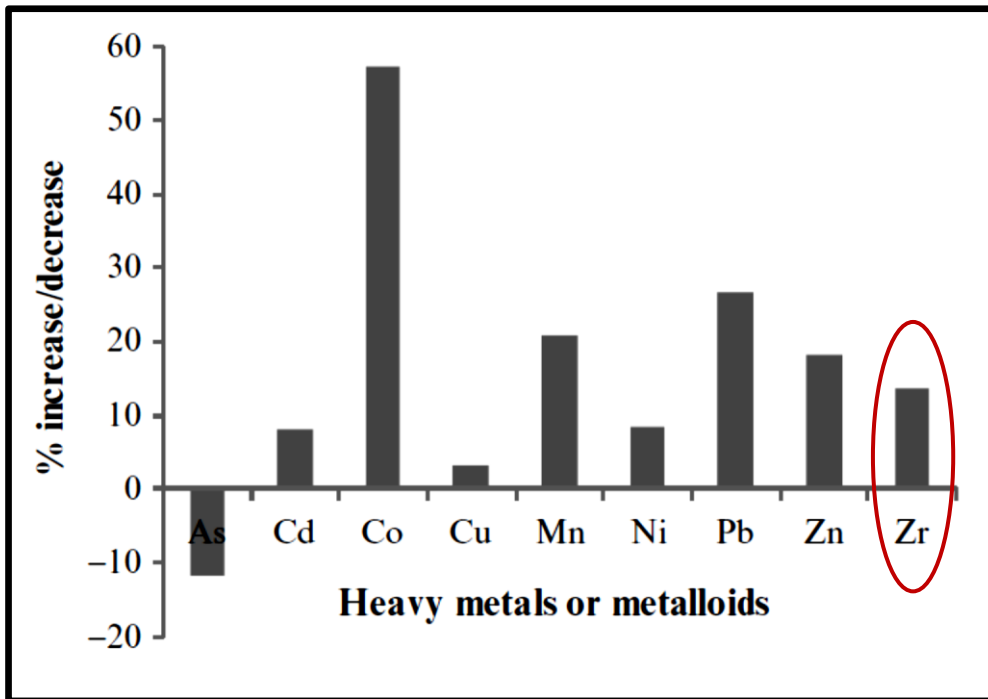
Figure 1.3.1. Heavy metal and metalloid production (%) for 2007 – 2011. (USGS [2012])

Zirconium is one of nature's most abundant elements (Ghosh *et al.,* 1992) it is the twentieth most abundant element in the earth's crust (Fodor *et al.,* 2005) and it has been found in marine sediments; 132 ppm, rocks; 170 ppm and in seawater; 4 ppm (Ghosh *et al.,* 1992). This semiprecious gem; zircon, that was discovered in 1789 by Klaproth is ubiquitous in nature and is sometimes found in higher concentrations than trace elements and metals like Cu (Ghosh *et al.,* 1992; Fodor *et al.,* 2005). Zr is especially popular due to its high heat-, corrosion- and acid-resistant properties (Ghosh *et al.,* 1992; Ferrand *et al.,* 2006). Zr's permeability to neutrons, its ability to form stable complexes and its relatively low biological toxicity further adds to its popularity (Ghosh *et al.,* 1992; Fodor *et al.,* 2005; Shahid *et al.,* 2013, 2014).

Because Zr melts at 1850 °C and boils at 4377 °C it has become widely used in industry for refractory metals, jewellery, dental supplies as well as coatings of metal alloys and

ceramics (Ghosh *et al.,* 1992; Ferrand *et al.,* 2006). Zirconium contamination and a resulting increase in anthropogenic emissions in the environment can be attributed to ceramic dust, mining and mining run-offs (improper dumping of waste) mostly from industrial sites that have been deserted (Chow *et al.,* 2003; Schulin *et al.,* 2007). Heavy metals are a potential risk to plants and may result in chlorosis and increased susceptibility to disease in plants as well as inhibition of plant growth and thus lower yields. In addition HMs can alter the ability for nutrient absorption and affect leguminous plants' ability to fix nitrogen (Chaudri *et al,* 2000; Fodor *et al.,* 2005; Guala *et al.,* 2010).

1.3.2. PHYTOAVAILIBITY OR BIOAVAILIBILITY AND ABSORPTION OF ZIRCONIUM

Zr is not known to be very mobile in soil since it complexes with soil compounds by the use of zirconcene dichloride or zirconium dioxide and it does not readily solubilise in water (Yang *et al.,* 2011), however factors like tropical weather conditions can influence mobility (Shahid *et al.,* 2013; Yang *et al.,* 2011). Apart from metal concentration other factors influence the ultimate toxicity of metals in plants (Yang *et al.,* 2011). Additional factors include mineral composition, organic substances present, other competing HMs in the soil, pH and redox potential (Marasovicova Kralova, 2012). Many different plants species have been observed to contain Zr from lichens to conifers and ferns and even pasture plants. The main transport route into plants is via root and leaf absorption (Navari-Izzo *et al.,* 2011). The toxicological effects of Zr to plants depend greatly on the absorption and desorption processes inherent in the soil surrounding their roots (Masarovicova and Kralova, 2012). Therefore, the

10

bioavailability or phytoavailability of Zr determines its effect on plants (Shahid *et al.,* 2013). Growth stage, plant type, soil environment and the form of Zr species must all be considered, as the impact of Zr to plants will be determined accordingly (Ferrand *et al.,* 2006).

The presence of Zr in maize, barley and alfalfa was shown by Sanzharova and Aleksakhin (1982) who reported that the Zr accumulation in plants had increased three fold when the soil moisture increased and Zr was absorbed mostly as a complex hydrous oxide (Ghosh *et al.,* 1992). Ryzhenko and colleagues (2008) also observed that absorption and mobility in soils is greatly influenced by pH levels.

Further similar reports have been made by workers in the refractory industry especially those who have been exposed to Zr for extended periods (Yang *et al.,* 2011). In human studies, salt complexes with Zr showed that it induced cell proliferation to weakly mitogenic. As Zr concentrations increased in higher organisms more drastic effects were observed such as turbagenic and limited clastogenic effects, this relates to affecting the spindle and signs of genetic aberrations (Masarovicov and Klarov, 2012). Because very little is known about the direct impact of Zr and with the little evidence of deposition in the human brain, it is crucial that further investigation and studies be performed especially since Zr shares properties with Al: a metal with known association to Alzheimers disease (Yang *et al.,* 2011).

1.3.3. ZIRCONIUM DETRIMENTS PLANTS

The role of metals in plants has met much debate in the past, but some of the trace elements (Fe, Mn, Zn and Cu) have crucial roles in the functioning of antioxidants and other protective molecules such as polyphenols and glutathione (GSH) (Yadav *et al.,*

11

2005; Hossain *et al.,* 2012). These metals are required by plants for regulation of physiological processes and metabolism and are used as co-factors (Yadav *et al.,* 2005; Hossain *et al.,* 2012; Marasovicova and Kralov, 2012). This however is only one aspect of metals as they are also known to be involved with the generation of free radicals and ROS (Fones *et al.,* 2010; Ahsan *et al.,* 2010). This is due to the nature of these transition metals having unpaired electrons (e$^-$) and are thus catalysts for the reduction of molecular O_2 (Fones *et al.,* 2010; Guala *et al.,* 2010).

Zirconium has no known function in the metabolism of plants or animals (Shahid *et al.,* 2013), in plants it mostly accumulates in root cells (Ferrand *et al.,* 2006); and as the literature reports the Zr associated with plant roots accumulate more on the roots' exterior than in cell walls (Ferrand *et al.,* 2006). Zirconium phytotoxicity has previously been found to be relatively low in plants (Ferrand *et al.,* 2006). However, Zr phytotoxicity does have detrimental impacts on plant growth as Ferrand and colleagues (2006) reports that dry weights were reduced after exposure to high dose of Zr in *P. sativum, H. vulgare* and *S. lycopersicum* (Ferrand *et al.,* 2006; Shahid *et al.,* 2013).

The link between soil to plant transfer impacts consumers (Vachirapatama *et al.,* 2011; Ryzhenko *et al.,* 2008) and because there is a shortage of this type of study, with regards to Zr, it is important that interest and time be invested in order to sustain the demand for plant products.

1.3.4. HEAVY-METAL HYPER-ACCUMULATING PLANTS

Since it is vital to enable plants to grow in environments contaminated with heavy metals and other pollutants, it is also important to attempt to ensure that such pollutants are only present in the parts of plants that are not edible (Marasovicova and Kralov, 2012). For this to be achieved, it is necessary to determine the levels of metals that plants accumulate while still being able to develop and produce crops and to determine the threshold of such metals in plants, for important purposes like phytoremediation or genetically modifying crops to produce a higher nutrient content yield (Guala *et al.,* 2010; Marasovicova and Kralov, 2012).

Metal hyperaccumulation is described as the excessive accumulation of HMs in aerial parts of plants (Fones *et al.,* 2011; Visioli and Marmiroli, 2012). Hyperaccumulators are organisms that are able to grow in metalliferous soils with much greater amounts of metals than non-hyperaccumulators, whilst evading the phytotoxic effects of present metals (Fones *et al.,* 2010; Verbruggen *et al.,* 2009). HM hyperaccumulators have the ability to extract HM ions far greater than non-hyperaccumulators. Their added benefits are the ability to metabolise and sequester HMs in their shoots and root-to-shoot translocation is markedly more effective than their counterparts (Visioli and Marmiroli, 2012). Since all plants are affected, even hyper-accumulators, it is important to establish the cytotoxicity and phytotoxicity of HMs with relatively little supporting literature and focus viz. Zr.

Hyperaccumulation is believed to have evolved due to several hypotheses that are non-mutually exclusive (Rascio and Navarri-Izzo, 2011). These include the defence

against predators and pathogens, an increased tolerance to metals and drought and an unintended uptake of metals (Rascio and Navari-Izzo, 2011; Verbruggen *et al.,* 2009). Many *B. rapa* species are known hyperaccumulators of heavy metals. There are three distinguishing factors that set these plants apart from related non-hyperaccumulators, these include: faster root-to-shoot translocation events, the ability to take up more heavy metals and the ability to detoxify more metals present in leaves than non-hyperaccumulators (Rascio & Navari-Izzo, 2011).

Over 450 species of plants have been recognized as hyperaccumulators, 0.2% of which are angiosperms (hyperaccumulators of trace elements, non-metals and metalloids) and most of these are hyperaccumulators of Ni (Verbruggen *et al.,* 2009; Rascio and Navarri-Izzo., 2011; Visioli and Marmiroli, 2012). Zn and Cd hyperaccumulators occur within the family *Brassicaceae*; species include *Arabidopsis halleri* and the genus *Thlaspi* (Verbruggen *et al.,* 2009).
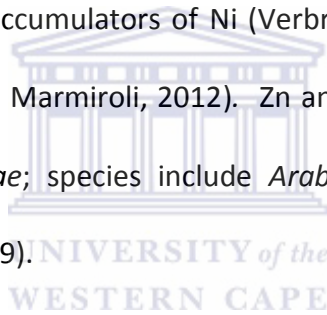
1.4. ROS AND THEIR ROLE IN PLANTS

The formation of reactive oxygen species (ROS) from molecular oxygen occurs when O_2 accepts four e^-'s during respiration, this reaction (figure 1.4.1) results in the formation of two H_2O molecules (Scandalios *et al.,* 1997; Halliwell and Gutteridge, 1984). However O_2 is incapable of accepting these e^-'s simultaneously, due to spin restrictions, and as such is reduced with a single electron at a given time (Halliwell and Gurtteridge, 1984). As a consequence of this univalent reduction of O_2, intermediates that are stable which include some forms of ROS are formed in a series of stepwise reactions similar to Fenton and Fenton-like reactions (Mourato *et al.,* 2012). These intermediates are capable of reacting with many different types of biomolecules, upon

which they may experience biochemical modifications (Held *et al.,* 2012). This attack on biomolecules by ROS results in their impeding function and activity where sometimes the activity and function of biomolecules is completely lost (Hossain *et al.,* 2011). The implications of ROS molecules reacting with biomolecules impacts plants in a plethora of ways and can be described overall by a single term known as 'oxidative stress' (Gutteridge and Halliwell, 1984; Scandalios *et al.,* 1997; Held *et al.,* 2012; Mourato *et al.,*2012).
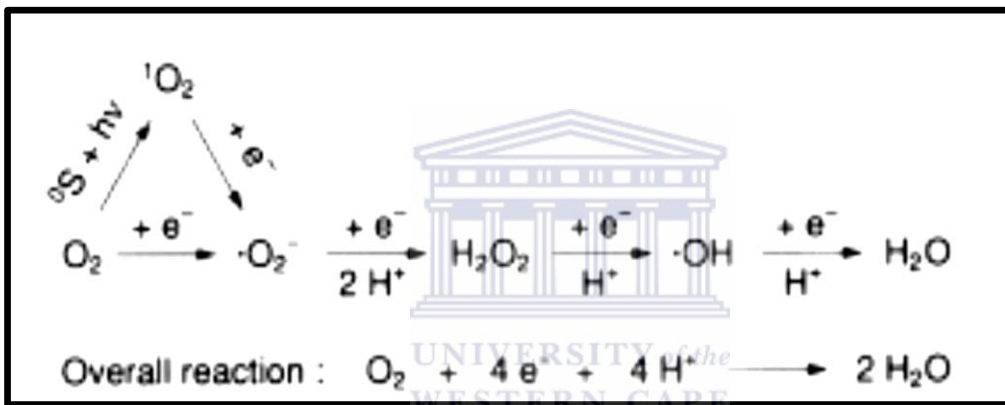
Figure 1.4.1. Pathways in the single electron (univalent) reduction of oxygen which results in the formation of several intermediate ROS. Reactive oxygen species are produced as by-products of cellular respiration when oxygen becomes reduced in a series of stepwise reactions (Scandalios *et al.,* 1997).

With or without anthropogenic pressures, plants are exposed to stresses that include high heat and cold stress, salt stress, drought and desiccation, ultra violet radiation, mechanical stress, air pollutants and the ever increasing HM stress (Hossain *et al.,* 2011, 2012). Increased reactive oxygen species, or ROS production, is a common consequence of such stress in plants (Yadav *et al.,* 2011). When ROS production surpasses plants' defence and protective mechanisms' capacity, oxidative stress and damage occurs (Mourato *et al.,* 2012). Oxidative stress is simply defined by

15

Vangronsveld and Clijsters (1994) as physiological changes that results from an excess production of ROS.

ROS as illustrated in figure (1.4.2), occur naturally in aerobic living systems and in plants they occur as by-products and sometimes products of aerobic metabolism (Mourato *et al.,* 2012). ROS is produced via pathways that involve mitochondrial- and photo-respiration and the photosynthetic apparatus (Almeselmani *et al.,* 2006). The inevitable reduction of molecular oxygen (O_2) to more highly reactive oxygen species; superoxide anion (O_2^-), hydroxyl radical ($\bullet OH$), hydrogen peroxide (H_2O_2) and singlet oxygen (O^-), is indicative that through natural biological processes ROS is always present (Mourato *et al.,* 2012). However, at persistently increased levels, these potentially toxic molecules can easily overwhelm plants' antioxidant systems that sets off signal cascades (Hossain *et al.,* 2012). This signalling overly excites antioxidant pathways whose end products may be less reactive, but might have a longer half-life which may be more harmful than more reactive radicals (Almselmani *et al.,* 2006; Mourato *et al.,* 2012). This is seen with SOD which metabolizes O_2^- to H_2O_2 and H_2O (Mourato *et al.,* 2012). The over-excitement of these pathways also leads to an imbalance to cellular homeostasis which leaves plants susceptible to DNA damage, non-specific oxidation of proteins and membrane lipids (Hossain *et al.,* 2009; Mourato *et al.,* 2012; Yadav *et al.,* 2007).

Under normal conditions plants are equipped to withstand ROS by being able to control rates of removal and production (Buettner, 1998). However, when the environment causes stress to plants the level of ROS production may become overwhelming for antioxidant enzymes to function optimally (Yang *et al.,* 2011; Yadav,

16

2005; 2010). An imbalance in one of the antioxidant systems potentially causes susceptibility to pathogenic organisms, inhibited development, seed dormancy, chlorosis or cell death (Cartea *et al.,* 2011; Hortensteiner and Krautner, 2011). The removal of a single hydrogen at the unsaturation point of a fatty acid molecule, to which an oxygen can easily bind may form a lipid peroxyl radical (Held *et al.,* 2012; Mourato *et al.,* 2012). As there may be further unsaturation points a series of these reactions often occurs (Gill and Tuteja, 2010).

Although ROS at elevated levels relative to a given organism is generally toxic and detrimental, it is important to note that ROS are essential to plants' defence against biotic pathogens where they are involved in formation of distinguishable features of development and reproduction. In addition they serve as signalling molecules for the regulation of genes (Held *et al.,* 2012; Yang *et al.,* 2011). Therefore, in light of the significance of ROS, it is pertinent for plants to strictly regulate it, but not do away with ROS completely (Mourato *et al.,* 2012).
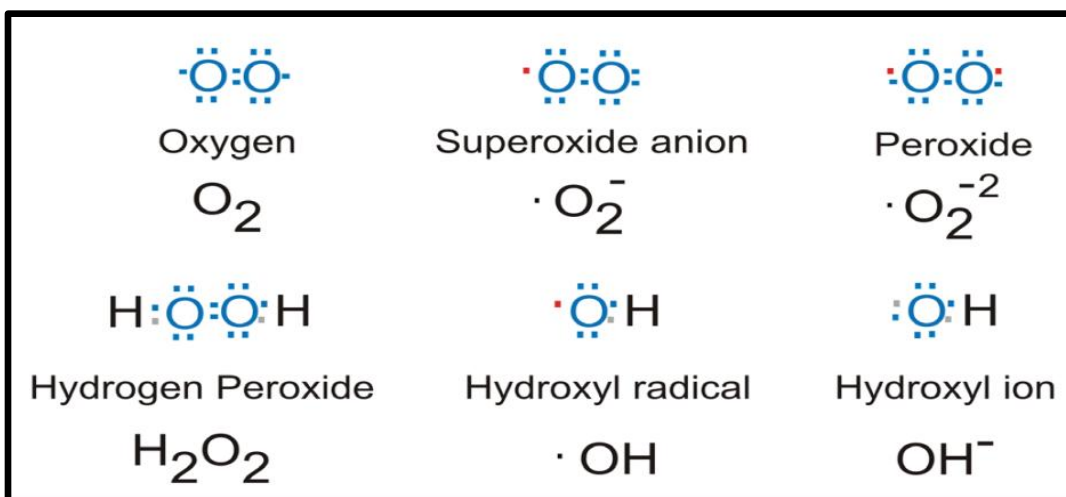
Figure 1.4.2. Electron structures and formulas of a few familiar reactive oxygen species. • Indicates an unpaired electron (Held, 2012)

17

1.4.1. SUPEROXIDE ANION [O_2^-]

The superoxide (O_2^-) radical is predominantly produced in chloroplasts and mitochondria, by NADPH oxidase in the plasma membrane and seldom in the peroxisome (Hossain *et al.,* 2011; Mourato *et al.,* 2012). Mourato and colleagues (2012) categorises O_2^- as having a medium relative reactivity. Because this radical cannot diffuse across the cell there needs to be mechanisms for effective scavenging, which is the function of SOD (Held *et al.,* 2012). SOD metabolizes this ROS by converting it to H_2O_2 (Table 1.1). O_2^- is a highly reactive radical, with a half-life of 4μs in water, is primarily produced at PII and PI in the chloroplast and it often also results from lipoxygenase activity (Mourato *et al.,* 2012; Yang *et al.,* 2011). Poly-unsaturated fatty acids (PUFAs) are its main target as it prefers the conjugated double bonds (Ahsan *et al.,* 2003). This reaction can be detected by aldehydes like malondialdehyde (MDA) that form due to PUFA peroxidation (Mourato *et al.,* 2012).
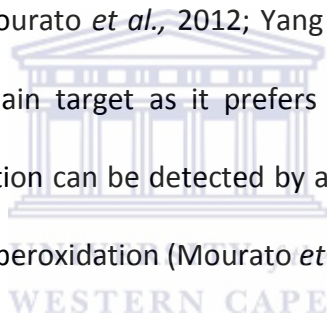
1.4.2. HYDROGEN PEROXIDE [H_2O_2]

H_2O_2 which is mainly produced in peroxisomes and mitochondria, often occurs as a by-product from the dismutation of superoxide via superoxide dismutase (SOD) activity (Held *et al.,* 2012; Mourato *et al.,* 2012). Unlike the radicals, which H_2O_2 is not, it can cross the cellular membrane and has a half-life of 1 ms (Hossain *et al.,* 2011; Mourato *et al.,* 2012). This also makes it the least reactive of the ROS species (Held *et al.,* 2012; Mourato *et al.,* 2012). Hydrogen peroxide and superoxide radical have the lowest reactivity levels however, it is important that they are produced more abundantly than singlet oxygen and the hydroxyl radical (Hossain *et al.,* 2012). Due to their abundance

in the cell H_2O_2 and O_2^- are capable of triggering reactions that result in more toxic species (Hossain *et al.,* 2012).

H_2O_2, with its notoriety for being detrimental to cells and causing oxidative damage at high levels, can be detoxified by several enzymes as part of the cell's defence mechanism (Ahsan *et al.,* 2003). There are several mechanisms for H_2O_2 detoxification in cells; H_2O_2 is the main substrate of enzymes like CAT and peroxidases that catalyse the conversion of H_2O_2 to H_2O and O_2. Secondly, it can be converted to hypochlorous acid (HOCL) in neutriphills by myeloperoxidase (MPO) (Ahsan *et al.,* 2003). This product is involved with phagocytic events to guard against harmful bacteria and is a strong oxidant, and when more H_2O_2 diffuses across cell membranes the excess H_2O_2 can then react with HOCL to yield O_2^- (Ahsan *et al.,* 2003). Lastly there is the reaction of H_2O_2 with transition metal ions to yield the highly reactive •OH (Ahsan *et al.,* 2003).

Despite the reputation for damaging plant cells H_2O_2 also serves as a signal molecule. Hossain and colleagues (2013) reports that maintaining H_2O_2 concentrations at relatively low to intermediate levels may reinforce plants' defence against environmental stresses and stimulate plant development through modulation of gene expression and redox signalling pathways (Singla-Pareek *et al.,* 2006; Hossain *et al.,* 2012).

1.4.3. HYDROXYL RADICAL [•OH]

The Hydroxyl radical is the most reactive of the active oxygen species that causes serious damage to plants if not metabolised (Cartea *et al.,* 2011; Held *et al.,* 2012). This very highly reactive free radical, for which there is no known enzyme system to degrade it, can be produced from H_2O_2 and O_2^- via Fenton and Fenton-like reactions

(Ahsan *et al.,* 2003; Mourato *et al.,* 2012). The •OH exerts damage upon photosynthetic pigments, proteins, DNA and lipids (Broadbent *et al.,* 1995; Hossain *et al.,* 2009; 2012). Given the extent of destruction that can be attributed to •OH, the prevalence of H_2O_2 as a consequence of •OH metabolism is a means of ensuring its sustainability in cells even if H_2O_2 itself is harmful (Almeselmani *et al.,* 2005; Broadbent *et al.,* 1995). Polyunsaturated fatty acids (PUFAs) are one of •OH radical's biggest targets (Ahsan *et al.,* 2003). DNA is especially prone to •OH-induced damage as nitrogenous bases largely prefer the reaction with •OH rather than with sugar moieties (Ahsan *et al.,* 2003).

1.4.4. CELL AND DNA DAMAGE IN PLANTS DUE TO ROS

1.4.4.1. CHLOROSIS SUFFERED TO PLANTS

Chlorosis may be defined as the lack of the green pigment (chlorphyll) in plant leaves and stems that is synonymous with the term "plants". Chlorosis may occur as a result of interference with the photosynthetic pathway, a lack of direct sunlight, the lack of or destruction of chlorophyll molecules in plants (Cartea *et al.,* 2011; Hortensteiner and Krautler, 2011). Although the function of chlorophyll molecules is limited to photosynthesis, chlorophyll is immensely important given that its presence provides plants with their energy source. The occurrence of chlorosis is often a common precursor to oxidative stress in plants. Common inducers of such stress include salinity and drought, but also include HM stress (Hossain *et al.,* 2012; Peralta-videa *et al.,* 2004).

Often Chlorosis is one of the first visible or phenotypic indicators of abiotic stress in plants, others include stunted growth and development of plant anatomical structures (Yadav *et al.,* 2005; Hortensteiner and Krautler, 2011; Goud and Kachole, 2011). Without sufficient chlorophyll *a* and *b* in plants, photosynthesis will be hindered as will the electron transport chain (ETC) and cellular respiration. Because of the essential nature of these metabolic processes, the presence of chlorophyll is of immense importance to the survival and functioning of plants (Pua and Douglas, 2004; Singla-Pareek *et al.,* 2004).

1.4.4.2. LIPID PEROXIDATION DUE TO PERCEIVED STRESS

Prevalent MDA is often indicative of oxidative stress since it is a decomposition product of fatty acid membrane degradation. Thus, MDA levels can be measured as one way of determining whether a plant is under stress (Yadav, *et al.,* 2010). Unsaturated fatty acids and PUFAs from lipid membranes are particularly susceptible to oxidation by ROS which causes an increased leakage of the membranes. Lipid peroxidation occurs as PUFAs and lipids are attacked by accumulated free radicals in plants under stress (Ahsan *et al.,* 2003; Held *et al.,* 2012).

One of the most common determinants of oxidative stress is the levels of MDA, a product of lipid peroxidation, in stressed plants (Hossain *et al.,* 2011). Higher levels of H_2O_2 and MDA have been linked with the disruption of certain metabolic functions and a diminished cellular integrity (Yadav *et al.,* 2005). Hossain et al. (2013) reports on the damaging effects of accumulated ROS in plants, such as ion leakage, inactivation of antioxidant molecules, inhibition of photosynthesis, membrane dismantling, DNA strand cleavage and lipid peroxidation (Hossain *et al.,* 2009; Yadav *et al.,* 2005).

One of the main, and deleterious, effects of oxidative stress provoked by heavy metals is lipid peroxidation which may lead to bio-membrane corrosion in addition to the other damaging effects (Hossain *et al.,* 2009; Yadav, 2010).

1.4.4.3. DNA AND RELATED CELLULAR DAMAGE

Cellular components most at risk to ROS are lipids, proteins, carbohydrates and nucleic acids (Blokhina *et al.,* 2003; Moller *et al.,* 2007). Proteins are affected upon ROS oxidation by causing a loss of catalytic activity in some enzymes, an increase in proteolysis and the production of carbonyl groups (Moller *et al.,* 2007 and Palma *et al.,* 2002). Proteins may also be affected by observing a change in protein content and the profile of such proteins. This may occur as the production of certain defence or chaperone proteins may be induced or activated causing a higher concentration to be observed (Hossain *et al.,* 2009; 2012). Thiol groups are particularly susceptible, oxidation often leads to loss of function conformation and denaturation (Cartea *et al.,* 2011; Hossain *et al.,* 2012; Mourato *et al.,* 2012).

Thornally et al. (1998; 2008) have reported on MG-induced DNA modifications; it reacts with guanyl residues to form imidazopurinone adducts and leads to a decrease in cell viability. ROS also impacts DNA; it damages nucleotide bases and leads to degraded DNA that may cause genetic defects and genetic mutations (Hossain *et al.,* 2009; Mourato *et al.,* 2012). Fortunately carbohydrates are highly reactive with the HO⁻, this is revered as a defence mechanism, by reacting with the radical before it attacks more biologically important molecules (Moller *et al.,* 2007). This however does not mean that DNA is often spared, as the biochemical nature of DNA, with sugar moieties forming its backbone makes it susceptible to substitutions of reduced oxygen species at thiol groups and H's (Held *et al.,* 2012; Hortensteiner and Krautner, 2011).

1.5. METHYLGLYOXAL [MG]: SYNTHESIS, CELLULAR DAMAGE AND REGULATION

MG, or 2-oxoaldehyde, is regarded as a ROS since it not only reacts with DNA, RNA and proteins and is capable of modifying them, but also because it stunts growth and development in plants at elevated levels similar to the previously mentioned ROS (Hossain *et al.,* 2012).

1.5.1. MG SYNTHESIS

MG synthesis (figure 1.5.1) is an inevitable fate of the Glycolysis or Embden Myerhoff pathway, formed spontaneously by degradation of triosephosphates. Triosephosphates are very unstable metabolites, and removal of the phosphoryl group by elimination from 1, 2-enediolate of these trioses leads to the formation of MG (Hossain *et al.,* 2009), through the enzymatic activities of Glyceraldehyde -3-phosphate (G3P) (Sipsey *et al.,* 2000; Firestone *et al.,* 2007). It is also formed by other metabolic reactions including cellular respiration, catabolism of threonine and acetone and enzymatically from dihydroxyacetone phosphate (DHAP) which is catalysed by MG synthase (Skipsey *et al.,* 2000; Yadav *et al.,* 2012). To date, no genetic or protein homolog of MG synthase has been discovered in plants.
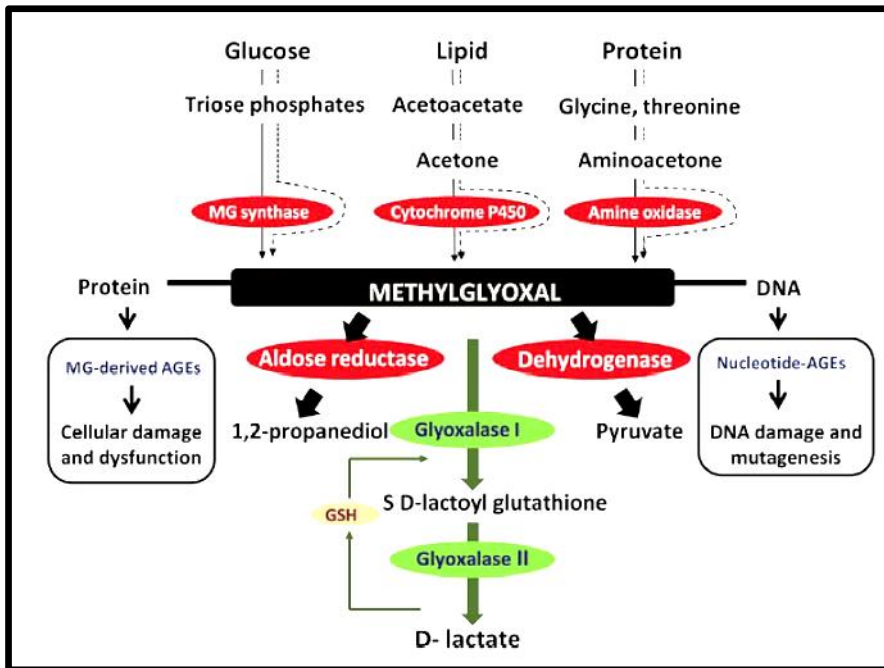
Figure 1.5.1. Schematic illustration of pathways involved in MG synthesis, possible consequences of MG reacting with biological molecules and its metabolism by the glyoxalase system. MG is formed inevitably by the glucose, protein and lipid metabolism and is capable of interacting with all three to form AGEs (advanced glycation end products). MG in the cell is detoxified by various pathways involving the action of aldose reductase, aldose dehydrogenase and the glyoxalase pathway. Perforated lines indicate the non-enzymatic reactions and solid lines indicates enzymatic ones (Kaur *et al.,* 2014).

MG spontaneously reacts with GSH in the GHS-dependent Glyoxalase pathway, which leads to it being detoxified enzymatically and converted to D-lactate by Glyoxalase I (plant Gly-I) and Glyoxlase II (plant Gly-II) in tandem (Hossain *et al.,* 2012; Skipsey *et al.,* 2000; Veena and Sopory, 1999). This highly reactive metabolite forms adducts with sugars and lipids to form advanced glycation end-products (AGEs) of nucleotides and other biomolecules (Freire *et al.,* 2003; Pua and Douglas, 2004;Thornally and Rabanni, 2011) and can cause damage to cells in varying degrees (Hossain *et al.,* 2012; Firestone *et al.,* 2007; Skipsey *et al.,* 2000). Furthermore reducing capability of molecular O_2 by MG that leads to the formation of toxic O_2^- implicates this metabolite in causing oxidative stress in plants (Saito *et al.,* 2011).

MG is present under normal growth conditions, but toxic accumulation occurs as a result of a range of stresses that include HM-stress (Yadav *et al.,* 2005a; Kumar and Yadav, 2009; Hossain *et al.,* 2009). Therefore, it is imperative that MG be metabolised to prevent the inhibition of cell growth and cell death by its accumulation (Yadav *et al.,* 2005; Kaur *et al.,* 2014). Although MG is cytotoxic at higher concentrations it is hard to firmly establish values at which it is either cytostatic or cytotoxic in various organisms. However it has been established that the normal cellular levels of MG are 1 - 20 µM in yeast and bacterial cells (Firestone *et al.,* 2007) and 30 – 75 µM in plants (Hossain *et al.,* 2009), and although MG is typically considered to be cytotoxic at 200 µM - 1 mM, it has been observed to induce cytotoxicity at 10 µM concentration in human osteoblasts (Thornally and Rabanni, 2011). Yadav et al. (2005) has shown that in plants, upon MG assays, MG levels under normal conditions are species-specific. In higher plant species viz. *Pennisetum glaucum*, rice, *Brassica juncea* and tobacco, MG was assayed in leaf material of seedlings to establish MG levels under normal conditions. In *B. juncea* 55 µM was observed, in other organisms concentrations varied between 50 µM for both *P. glaucum* and tobacco, and 75 µM in rice.

1.5.2. GLYOXALASE I [GLY-I] AND THE GLYOXLASE SYSTEM

In 1951 it was observed that the terminal product of the Glyoxylase system is metabolised by the two-step process catalysed by Glyoxylase I (Gly-I) and Glyoxylase II (Gly-II) and that the end-product was D-lactate; the end-product of glycolysis (Thornally and Rabanni, 2011). Since its discovery, over a century ago an active glyoxalase system has been observed during the cell's vital stages that include tissue

maturation, and from embryogenesis right up until cell death and apoptosis (Deswal and Sopory, 1991). Gly-I therefore plays a role in cell proliferation with a host of functions in plant systems such as a detoxification pathway against damaging free radicals (Thornally, 1990; Yadav *et al.,* 2005; Deswal and Sopory, 1991; Thornally and Rabanni, 2011).

Gly-I, also known as Lactoyl-glutathione lyase, is as ubiquitous in nature as MG, being studied in animals, yeasts, microorganisms and in higher plants such as the Brassica family (Hossain *et al.,* 2012; Deswal and Sopory, 1991; Yadav *et al.,* 2005). The significance of this antioxidant pathway is exhibited by its involvement in various essential biological functions (Thornally, 1990; Thornally and Rabanni, 2011). Although it is known that Gly-I is a metal-binding enzyme, there has been evidence of a lack of specificity for its catalytic metal ion (Skipsey *et al.,* 2000). Gly-I is dependent on cationic metals such as Ni and Zn for its activity. Mammalian and animal Glyoxalase I use divalent Zn cations and in animals a Zn is tightly bound to each subunit, however its affinity and metal dependence in plants has not yet been defined (Skipsey *et al.,* 2000).

The glyoxalase system is the primary catabolic pathway of MG in eukaryotes that comprises two co-functioning enzymes; Gly-I and Gly-II, the first of which isomerises the non-enzymatically formed hemithioacetal, from MG and GSH, to yield S-D-lactoylglutathione (Hossain *et al.,* 2012; Yadav *et al.,* 2005 and 2007). Gly-II then catalyses the hydrolysis reaction to D-lactate and puts GSH back into the system (Yadav *et al.,* 2005; Singla-Pareek *et al.,* 2006).

Alpha-oxoaldehydes, glyoxals and methylglyogal being the main targets of Gly-I for metabolism makes it a very important enzyme, especially since it is found ubiquitously

and has been implicated with alleviating the signs of oxidative stress in various organisms including plants, yeasts, animals and humans (Singla-pareek *et al.,* 2006; Hossain *et al.,* 2009; Kaur *et al.,* 2014). Gly-I activity and expression have been investigated in literature (Yadav *et al.,* 2005; Hossain *et al.,* 2009; Kaur *et al.,* 2014) and its upregulation has been associated with various cellular stresses modifications viz. salinity, drought, heavy metals and Gly-I-overexpressing transgenic plants respectively. Gly-I has therefore been designated as having a function in plants defence against oxidative stress especially due to increased levels having been associated with accumulated MG; a cytototic metabolic by-product whose accumulation leads to the production of ROS (Hossain *et al.,* 2009; 2011; Kaur *et al.,* 2014). Given its upregulation upon stress in plants (figure 1.5.2) it has been the topic of research in drought, salinity and HM-induced stress in plants (Kaur *et al.,* 2014) and its mechanism of action has therefore been proposed in relation to salinity, drought and heavy metals stress by Kaur and colleagues (2014).
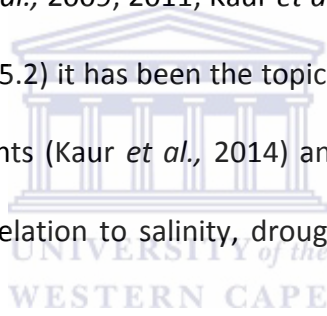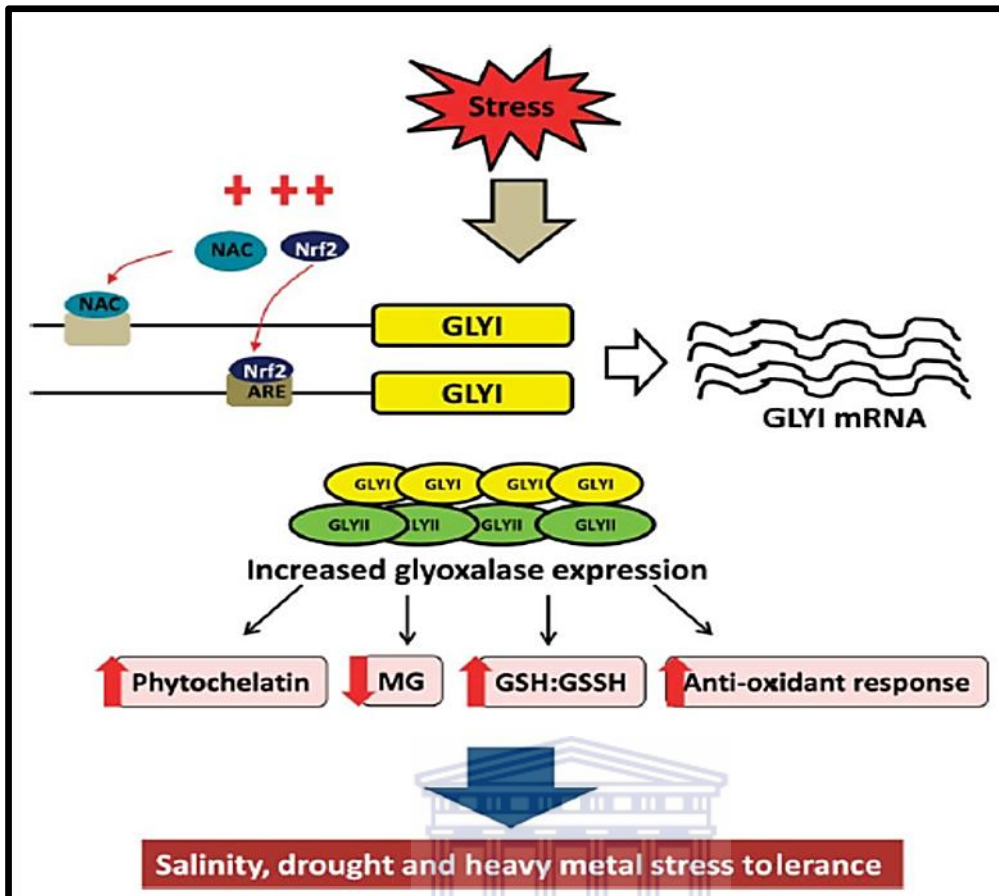
Figure 1.5.2. Schematic depiction of the mechanism of Gly-I activation and its associated tolerance to abiotic stresses. Stress inducible factors such as NAC and Nrf2 become induced by stress, they bind to Gly-I promoter gene to induce gene expression. Resulting Gly-I upregulation and overexpression confers tolerance to a range of stresses in plants through decreasing MG levels and increasing the antioxidant response (Kaur *et al.,* 2014).

1.6. ANTIOXIDANT ENZYMES AND COMPOUNDS FOR PLANTS' DEFENCE AND PROTECTION

The generation of ROS is a consequence of cellular metabolism as well as downstream effects of abiotic and biotic stress (Mourato *et al.,* 2012). These stresses are not limited to heavy metals, but include other forms such as salt and drought, excessive cold, chemical pollutants as well as predatory and pathogenic organisms (Hossain *et al.,* 2012). All of which require the interference of antioxidants to counteract and mitigate

the damage caused, often directly, by ROS (Held *et al.,* 2012; Mourato *et al,*2012; Hossain *et al.,* 2012).

Table 1.1 <u>**ROS scavenging and detoxifying enzymes and the reactions they catalyse**</u> **(Blokhina *et al.,* 2003)**

ENZYME	EC NUMBER	REACTION CATALYSED
Superoxide dismutase	1.15.1.1	$2O_2^- + 2O_2/ 2H^- \leftrightarrow 2H_2O_2 + O_2$
Catalase	1.11.1.6	$2H_2O_2 \leftrightarrow O_2 + H_2O$
Glutathione peroxidise	1.11.1.12	$2GSH + PUFA\text{-}OOH \leftrightarrow GSSG + PUFA + 2H_2O$
Glutathione S-transferases	2.5.1.18	$RX + GSH \leftrightarrow HX + R\text{-}S\text{-}GSH$
Phospholipid-hydroperoxide glutathione peroxidise	1..1111.1.9	$2GSH + PUFA\text{-}OOH \ (H_2O) \leftrightarrow GSSG + 2H_2O$
Guaicol type peroxidise	1.11.1.7	$Donor + H_2O_2 \leftrightarrow$ oxidized donor $+ 2H_2O$
Monodehydroascorbate reductase	1.6.5.4	$NADH + 2MDHA \leftrightarrow NAD^+ + 2AA$
Dehydroascorbate reductase	1.8.5.1	$2GSH + DHA \leftrightarrow GSSG + AA$
Glutathione reductase	1.6.4.2	$NADPH + GSSG \leftrightarrow NADP^+ + 2GSH$
Ascorbate peroxidise	1.11.1.11	$AA + H_2O_2 \leftrightarrow DHA + 2H_2O$

Plants' strategic defence against free radical-induced oxidative stress make use of antioxidant defences, preventative- and repair-mechanisms (Yadav *et al.,* 2005; Mourato *et al.,* 2012). A few well-known antioxidants that metabolise and detoxify ROS, either directly or as components of a multi-step system, will be described below.

29

1.6.1. SUPEROXIDE DISMUTASE [SOD]

SOD is the catalytic component for the dismutation of superoxide radicals to H_2O_2 and H_2O (Held *et al.,* 2012) and it employs several metal ions as cofactors viz. Cu, Zn, Mn and Fe respectively (Almeselmani *et al.,* 2006; Mourato *et al.,* 2012). Its localization has been reported in different cellular components which include FeSODs in chloroplasts, MnSODs in mitochondria and peroxisomes, Cu/ZnSOD in chloroplasts and the cytosol. Other isozymes have been reported to be in the matrix mitochodrion as tetramers (Beuttner *et al.,* 1995; Braodbent *et al.,* 1998; Mourato *et al.,* 2012).

Buettner (1998) describes SOD as the only enzyme known to act directly on a radical and is therefore known as a primary antioxidant. SOD is therefore important in detoxifying superoxide into H_2O_2 especially since H_2O_2 is abundant and despite its lower rate of reactivity, it has a longer half-life than the other O_2 radicals (Broadbent *et al.,* 1995; Buettner *et al.,* 1998). This action normally triggers the induction of H_2O_2 metabolising enzymes in plants.

1.6.2. ASCORBATE PEROXIDASE [APX] AND OTHER PEROXIDASES

Ascorbate peroxidase (APX) is an antioxidant enzyme responsible for the decomposition of H_2O_2 as well as the maintenance of H_2O_2 at low levels (Almeselmani *et al.,* 2006; Cartea *et al.,* 2011). APX uses the available cellular ascorbate as its cofactor. It is found in chloroplasts, the cytosol, vacuoles and apoplasts and is one of the most abundant antioxidants in the plant cell (Mourato *et al.,* 2012). Although APX is not the only H_2O_2 - scavenging enzyme, it does however have a greater affinity for H_2O_2 than catalase (CAT) which possibly indicates its significance in the role of H_2O_2

detoxification in the cell (Mourato *et al.,* 2012). APX also forms an integral part of the Ascorbate-Glutathione cycle that involves other scavenging antioxidant molecules to form various reactions including compartmentalization and metabolism of ROS in the cell which attempts to alleviate and prevent oxidative damage (Mourato *et al.,* 2012). There are several other peroxidases; Guaicol peroxidase which mainly uses phenols as substrates and Glutathione peroxidase which uses glutathione (GSH) as an activator. These cell-wall peroxidases not only scavenge H_2O_2, but are also involved with ROS production, only in minimal doses, and aid in signalling for perceived stress as a defence (Mika *et al.,* 2004). Two less commonly known antioxidant enzymes; polyphenol oxidase (PPO) and peroxiredoxin (PRX) produce quinones through catalysing the oxidation of polyphenols and reduces H_2O_2 to peroxide respectively, these functions are imperative since they have both been known to increase under heavy metal stress (Barranco-Medina *et al.,* 2007 and Gerdemann *et al.,* 2001).

1.6.3. CATALASE [CAT]

CAT (catalase) catalyses the conversion of H_2O_2 (table 1.1) to water and O_2. CAT and other molecules scavenge H_2O_2. However, CAT acts merely when there is an excess of free H_2O_2 (Mourato *et al.,* 2012). Various isozymes have been identified viz. CAT-1, which occur in peroxisomes, and CAT-2 in the cytosol and glyoxisomes and CAT-3 in the cytosol and mitochondria (Held *et al.,* 2012). The H_2O_2 that SOD produces is converted to water by CAT and peroxidases which make them vital components of the detoxification system. Catalase and peroxidases are the two main enzyme groups to regulate the intracellular levels of H_2O_2 (Blokhina *et al.,* 2003; Held *et al.,* 2012). CATs occur primarily in peroxisomes with their mechanism of action having been described as a 'ping-pong' effect, whereupon a particular CAT's cofactor; either Fe or Mn,

becomes oxidised by a single H_2O_2 molecule (Mhamdi *et al.,* 2010). CAT's cofactor then transfers the newly bound H_2O_2 to the next substrate to come along (Mhamdi *et al.,* 2010; Held *et al.,* 2012). Because of the inverse relationship between APX and CAT, since they both scavenge H_2O_2, CAT comes into action once APX has already been exposed to the present H_2O_2 in the cell. Without CATs present in the cell excess H_2O_2 would wreak havoc given its longer lifespan as a ROS and its ability to cross cellular membranes.

Calcium-binding proteins such as calmodulins binds calcium, Bowler and Fluhr (2000) reports however, that in the process plant CATs become activated as well. This seemingly dual function of Ca to regulate H_2O_2 homeostasis makes CATs crucial to plants' antioxidant defence and serves as a co-contributor to CAT levels in H_2O_2 detoxification.
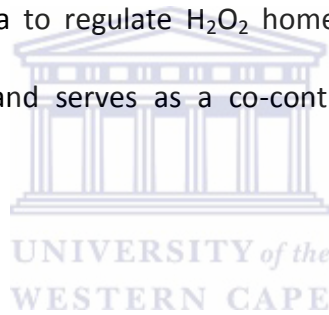
1.7. JUSTIFICATION

Due to the immotile nature of plants, there are many stresses, threats and potential threats that they need to either adapt to or face to avoid certain death (Hossain *et al.,* 2012; Rascio and Navarri-Izzo, 2011). Fortunately plants have inherent survival mechanisms that perform like a network of different, but equally important, components (Yadav *et al.,* 2005). ROS serve as signalling molecules and their metabolizing gene products and cofactors such as antioxidants and polyphenols have detoxifying capabilities (Held *et al.,* 2012; Thornally *et al.,* 2011). With the help of these antioxidants and signalling molecules plants are sometimes able to withstand and even flourish in the presence of abiotic and biotic factors such as pesticides and herbicides, anthropological waste from industrial sites and HMs (Mourato *et al.,* 2012). HMs affect plants and humans differently (Hossain *et al.,* 2012). The extent of potential damage caused, depends on the ability of the affected organism to metabolise or digest the metal as part of its metabolism, and also whether the accumulated compound exceeds the threshold for detoxification (Yadav *et al.,* 2010; Chaudri *et al.,* 2000). It is known that HMs cause oxidative stress, however they are also involved with activation of defence systems in plants (Chaudri *et al.,* 2000). Therefore it is crucial to elucidate the effects of HMs on plants; and also to establish the levels that impact plant growth and development.

Many of the heavy metals have been studied in the past including **Cd, Zn, Ni, Pb, Bo** while other metals like **Zr**, have sparse reports (Shahid *et al.,* 2013). However in light of the harmful effects supported by literature and because of its significance in the metals industry and the South African economy, further insights are crucial especially concerning the already affected crop plants locally (StatsSA, 2010; Yadav *et al.,*2012).

33

Our in-house data along with literature suggests the possible signalling function of MG which possibly causes a delay in abiotic stress effects (Hossain *et al.,* 2011).This is presumably due to MG's relation to Gly-I, where during abiotic stress MG levels increase and this incites the activation and further production of Gly-I across different organisms and in plants (Hossain *et al.,* 2011; Rabanni and Thornally, 2012). Given the antioxidant capabilities of the glyoxalase system, this signal pre-induces a defence response in plants and could possibly occur more rapidly, in tandem or possibly before other antioxidant mechanisms become alerted as MG levels increase.

Plants, including *B. rapa* L., naturally possess a host of secondary metabolites that make them highly beneficial along with antioxidant effects from flavonoids to antimicrobial effects, however most striking was the ability of plants' secondary metabolites to modulate detoxification enzymes; also known antioxidants (King, 2005; Marasovicova and Kralov, 2012). Hossain and colleagues (2012) reports that maintaining H_2O_2 concentrations at relatively low to intermediate levels may reinforce plants' defence against stresses and may stimulate plant development through modulation of gene expression and redox signalling pathways. In light of this the potential modulation of antioxidant pathways by the exogenous application of MG, at low levels, to mitigate Zr's damaging effects, has been proposed for this study.

1.8. OBJECTIVES

The aims of this study were to determine the possible evidence, if any, in support of MG as a signalling molecule, similar to common ROS such as H_2O_2. Physiological and biochemical evidence was elucidated through noting the morphological response to MG being applied at micromolar concentrations and assaying various ROS and their scavenging antioxidant enzymes spectrophotometrically. Furthermore, semi-

34

quantitative methods were employed for further insight to the role of MG in plants, with regards to the activation of glyoxalase I. *BrGlyI* gene was amplified in *B. rapa* L. experimental and control samples with the use of densitometry and statistical tools for analyses.

Because so little is understood about the cytotoxicity of Zr in plants, similarly the possible functions of the ubiquitous by-product (MG), both Zr and MG were administered on their own as well as in combination. This was done to establish whether the presence of added MG would influence Zr uptake and cellular stress in *B. rapa* L. as well as possible activation of the antioxidant-ROS pathways. Seedlings from this study were also assessed for trace amounts of Zr through inductively coupled plasma-atomic emission spectrometry, ICP-OES.

This study undertook to determine the extent of Zr-toxicity in *B. rapa* L. seedlings, but moreover to elucidate the signalling properties of MG; whose cytotic effects in plants have been reported at high concentrations. Due to MG increasing under stress conditions, it suggests involvement during cellular distress. MG at 6 µM was therefore applied exogenously to *B. rapa* L. seedlings under normal and HM-stressed conditions.

CHAPTER TWO
MATERIALS AND METHODS

2.1. *BRASSICA RAPA* L. GERMINATION, TREATMENTS, HARVESTING AND STORAGE

B. rapa L. seeds were chilled at 4 °C prior to sowing them. Seeds were propagated into 1 litre brown pots containing a pre-treated potting mixture consisting of compost-enriched potting soil and filtrate sand (1:2). Each pot received 5 seeds, sown approximately 5 cm deep. Treatments consisted of a (water only) control, 6 µM Methylglyoxal (MG), 1 mM Zirconium (Zr) and 6 µM MG/1 mM Zr. Treatments were administered twice per week in 200 ml doses. Seedlings were harvested 2 weeks after germination. Some of the seedlings were ground to a fine powder in liquid nitrogen using a sterilized mortar and pestle. Ground up material was immediately decanted to new 50 ml Greiner tubes which were stored at -80°C for. The frozen ground material was for subsequent lab experiments.

2.2. DRY WEIGHT DETERMINATION IN *B. RAPA* L. SEEDLINGS SUBJECTED TO MG AND ZR TREATMENTS RESPECTIVELY

Sterilized foil envelopes in replicates of four per respective treatment were used. Envelopes were punctured to facilitate desiccation. Each envelope containing three *B. rapa* L. seedlings were placed in a desiccator overnight at 80°C in order to remove any water content within the seedlings. Envelopes were weighed prior to and after

desiccation in order to compare the biomass from seedlings of each respective treatment.

2.3. CHLOROPHYLL ASSAY

Ground material obtained as in section (2.1) was used to determine the chlorophyll *a* and *b* content in *B. rapa* L. seedlings. This assay was adapted from the methods described by Oancea et al. (2005). To foil-wrapped Eppendorf tubes (to prevent chlorophyll degradation) 100 mg ground material was added per treatment. Ten X the volume of 100% (v/v) acetone was added to sample tubes. Tubes were mixed briefly by vortex and the resulting homogenates were loaded to a microtitre plate in triplicate per sample. The absorbance values were read at 662 nm and 644 nm respectively by a spectrophotometer.

2.4. PROTEIN EXTRACTION WITH TCA

Ground leaf material (100 mg per sample) as obtained in section (2.1), was transferred to a chilled Eppendorf tube to which 5X the volume of [6%] Trichloroacetic acid (TCA) was added. The sample tube was mixed by vortex for 5 minutes to homogenise followed by centrifugation at 13 000 x g for 10 minutes. The resulting supernatant, which served as the sample in selected analyses, was transferred to a new Eppendorf tube.

2.5. MALONDIALDEHYDE [MDA] ASSAY TO DETERMINE THE DEGREE OF LIPID PEROXIDATION

This spectrophotometric assay was adapted from the one described by Zhang et al. (2007). The TCA extracts (200 µl), obtained as in section (2.4.), were decanted to clean

37

Eppendorf tubes containing 300 µl 0.5% (w/v) thiobarbituric acid (TBA) and mixed briefly by vortex. Sample tubes were incubated at 90°C for 20 minutes and sealed with Parafilm to prevent the loss of liquid during incubation. This was followed by a 10 minute incubation on ice before subjecting samples to centrifugation at 13 000 x g for 5 minutes. Following the sample preparation 200 µl of each sample was loaded to a 96-well microtitre plate. Absorbance readings were taken at 532 nm and at 600 nm. Non-specific turbidity was adjusted by subtracting the value at 600 nm from that of the reading at 532 nm. The MDA levels in respective samples were determined using the extinction coefficient of 155 mM^{-1}.cm^{-1}.

2.6. CONJUGATED DIENES [CD] ASSAY TO DETERMINE THE DEGREE OF LIPID PEROXIDATION

Ground Seedling material as described in section (2.1) was used for the determination of conjugated dienes content in *B. rapa* L. seedlings. Frozen seedling material (50 mg) was homogenised in an Eppendorf tube containing 2 ml ethyl alcohol by vortex for 2 minutes. Each sample tube was subjected to centrifugation at 3000 x g for 3 minutes. The resulting supernatant was used as the sample and loaded to a 96-well microtitre plate from which absorbance values were read at 234 nm. Conjugated dienes content was determined using the extinction coefficient of 265 mM^{-1}.cm^{-1}.

2.7. CELL VIABILITY [EVANS BLUE UPTAKE] ASSAY

This assay was adapted from the one described by Sanevas et al. (2007) and used to determine the cell viability within *B. rapa* L. seedlings. One whole seedling was added to an Eppendorf tube containing 1 ml Evan's Blue solution [0.25% (w/v)]. Sample tubes were incubated at room temperature for 60 minutes. Seedlings were removed from

Evan's blue reagent and rinsed with distilled water to remove unbound Evan's blue reagent. Seedlings were incubated overnight in water at room temperature after which the water was removed and samples were then immersed in a 1% (w/v) sodium dodecyl sulphate (SDS) solution inside the Eppendorf tubes. Seedlings were crushed using a miniature pestle following an incubation at 65°C for 60 minutes. Next sample tubes were subjected to centrifugation at 13 000 $x\ g$ for the effective removal of the resulting supernatant. The supernatant was decanted to a clean Eppendorf tube and a volume of it was loaded onto a 96-well microtitre plate where absorbance values were read at 600 nm.

2.8. EXTRACTION OF TOTAL PROTEINS

A 5x total protein extraction was performed for an optimal concentration. For the extraction five 2 ml Eppendorf tubes were cooled in liquid nitrogen along with sterilized spatulas. Using a cooled spatula 100 mg ground up seedlings (as in section 2.1) were added to each Eppendorf tube. To the first of five tubes 500 µl extraction buffer, containing [0.004 M phosphate buffer, 1 mM ethylenediamenetetraacetic acid (EDTA) and 5% (w/v)] was added and vigorously homogenised by vortex for 2 minutes. The sample homogenate was subjected to separation of the leaf matter from the liquid phase by centrifugal force at 12 000 $x\ g$ for 5 minutes. The liquid phase from tube 1 was removed, leaving behind the leaf matter, and was added to tube 2 containing 100 mg ground material. This was again homogenised by vortex, subjected to centrifugation and decanted to tube 3, and so forth, up until the last tube. Total proteins were stored at -20°C for further use.

2.9. PROTEIN QUANTIFICATION

Total proteins were quantified by Bradford assay using a 1x Quick start Bradford dye reagent. Total proteins were diluted to 1:10 with deionised water. In a 96-well microtitre spectrophotometric plate a protein standard of BSA was prepared ranging from 0 mg.ml^{-1} to 10 mg.ml^{-1}. Furthermore 10 µl extracted proteins (1:10) were loaded to the microtitre plate. Lastly Bradford reagent was added to protein standards and samples respectively to a final volume of 200 µl. Both samples and standards were loaded in triplicate and incubated on a shaker at room temperature for 5 minutes before taking a reading. Protein absorbance values were taken at 595 nm and the sample concentrations were extrapolated from the standard curve.

2.10. SUPEROXIDE [O$_2^-$] ASSAY

This assay was adapted from the one described by Russo et al. (2008) to determine the superoxide accumulation in *B. rapa* L. seedlings. Eppendorf tubes (2 ml) were prepared with 10 mM potassium cyanide [(KCN) for inhibition of Cu/Zn SODs], 10mM H$_2$O$_2$ (for inhibition of Cu/Zn- and Mn-SODs), 2% SDS (for inhibition of Fe- and Cu/Zn-SODs) and 80 µM NBT. The prepared mixture was made to a final volume of 800 µl with 50 mM potassium phosphate buffer (pH 7.0). The resulting solution was used for incubation of whole seedlings at room temperature for 20 minutes. The seedlings within the solution were then crushed with a miniature pestle to release the superoxide present within them. This was followed by centrifugation at 13 000 x *g* for 5 minutes to pellet the leaf matter. The resulting supernatant was loaded to a 96-well microtitre plate from which absorbance value were read at 600 nm. Superoxide content was determined using the

extinction coefficient of 12.8 $mM^{-1}.cm^{-1}$. The colorimetric reaction indicating O_2^- levels was observed by the blue colour change in solution.

2.11. HYDROXYL RADICAL [•OH] ASSAY

This assay was performed according the method described by Halliwell et al. (1987) to determine the hydroxyl ion (•OH) accumulation in *B. rapa* L. seedlings. Frozen ground up material (50 mg) was homogenised in 1 ml phosphate buffer (10 mM at pH 7.4) containing 15 mM 2-deoxy-D-ribose briefly by vortex. Next, samples were incubated at 37°C for 2 hours. To 0.7 ml of the prepared sample a TBA solution containing 3 ml of 05% (w/v) TBA prepared in 5 mM sodium hydroxide (NaOH) and 1 ml glacial acetic acid was added and subsequently mixed briefly by vortex. The resulting sample mixture was incubated at 100 °C for 30 minutes followed by an incubation period of 5 minutes on ice to cool. Next, samples were pelleted by centrifugation at 10 000 x g for 5 minutes. The supernatant was loaded in triplicate to a microtitre plate and absorbance values were read at 532 nm and 600 nm. •OH content was determined using the extinction co-efficient of 155 $mM^{-1}.cm^{-1}$.

2.12. HYDROGEN PEROXIDE [H_2O_2] ASSAY

This assay was adapted from the one described by Velikova et al. (2000) to determine the H_2O_2 accumulation in *B. rapa* L. seedling material. The standards, ranging from 0 nm to 2 500 nm were prepared by diluting an appropriate volume of H_2O_2 in distilled water. The sample preparation involved the steps described in section (2.4.). The resulting extract served as the sample for the analysis to follow. Both the samples (50 µl) and the standards were loaded onto a 96-well microtitre plate, and to each well 1.25 mM dipotassium hydrogen phosphate (K_2HPO_4) and 250 mM potassium iodide

41

(KI) was added. The plate was incubated at room temperature for 20 minutes after which the absorbance values were read at 390 nm.

2.13. SUPEROXIDE DISMUTASE [SOD] ACTIVITY IN *B. RAPA* L. SEEDLINGS

Sample proteins were extracted and quantified as described in sections (2.8.) and (2.9.) from frozen ground *B. rapa* L. seedlings. Aliquots of 10µl of protein extracts were loaded in triplicate onto a 96-well microtitre plate subsequent to a dilution to 1 mg. ml^{-1}. Each sample well received 20 mM phosphate buffer, 0.1 mM nitrozoliumblue chloride (NBT), 0.005 mM riboflavin, 10 mM methionine and 0.1 mM EDTA which was made to a final volume of 200 µl with distilled water. The sample plate was set on a light box and incubated at room temperature for 20 minutes. Absorbance values were read at 560 nm and the superoxide activity was subsequently determined by the measure of the amount of superoxide required to inhibit a 50% decrease in reducing NBT to formazan.

2.14. ASCORBATE PEROXIDASE [APX] ACTIVITY IN *B. RAPA* L. SEEDLINGS

Total proteins were extracted and quantified as described in sections (2.8.) and (2.9.) from frozen ground up *B. rapa* L. seedlings. 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_2O_2 was added to activate the reaction 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.cm^{-1}.

2.15. CATALASE [CAT] ACTIVITY IN *B. RAPA* L. SEEDLINGS

This assay was adapted from the methods described by Aebi (1984) to determine the catalase (CAT) activity in *B. rapa* L. seedlings. The principle of this assay was based on the dissociation of H_2O_2 and observed by a decrease in absorbance of CAT. The reaction mixture (1 ml) containing 100 mM K_2HPO_4 (pH 7.0), 0.5 mM EDTA, 1 mM H_2O_2 and 20 µl protein extract was combined and loaded to a microtitre plate. Absorbance values were read at 240 nm using the extinction coefficient of 39.4 mM^{-1}.cm^{-1} to determine the catalase activity.

2.16. METHYLGLYOXAL [MG] ASSAY

An appropriate volume of Methylglyoxal was diluted with distilled water in preparation of the standards for this assay, to determine the MG accumulation in *B. rapa* L. seedlings. The standards ranged from 0 mM to 78 mM in concentration and were loaded to a 96-well microtitre plate in triplicate. Sample preparation involved aliquots of 250 mg of ground seedling material for each respective sample added to an Eppendorf tube to which 5 x volume of phosphoric acid was added. Sample tubes were homogenised by vortex for 2 minutes. The homogenates were incubated on ice for 15 minutes after which they were subjected to centrifugation at 13 200 *x g* for 10 minutes to pellet all leaf particulates. The resulting supernatant was transferred to a fresh Eppendorf tube to which 10 mg.ml^{-1} activated charcoal was added for filtration, and

followed by a further incubation at room temperature for 15 minutes. Sample tubes were then subjected to centrifugation at 13 200 *x g* for 10 minutes and the resulting supernatant was transferred to a new tube. Saturated potassium hydroxide (400 µl) was added to sample tubes to neutralize the samples and incubated at room temperature for 15 minutes. Neutralized samples were separated from the resulting precipitate by centrifugation at 13 00 *x g* for 10 minutes and the supernatant was subsequently transferred to a new tube which served as the sample. To 130 µl samples and standards plated respectively, 0.5 M phosphoric acid and 1.8 mM diaminobenzene were added to a final volume of 200 µl. The plate was incubated for 40 minutes at room temperature on a shaker and absorbance values were subsequently read at 405 nm.

2.17. GLYOXALASE I [GLY-I] ACTIVITY IN *B. RAPA* L. SEEDLINGS

This assay was performed in accordance with the method described by Chakravarty and Sopory (1998) to determine the Glyoxalase I activity in *B. rapa* L. seedlings. Sample proteins were extracted as described in sections (2.8.) and (2.9.) 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 minutes. Thioester formation occurred over the period of 2 minutes.

2.18. RNA EXTRACTION AND PURIFICATION

Frozen ground *B. rapa* L. seedlings obtained as in section (2.1) from each respective treatment were used as starting material. Total RNA was extracted from seedlings subjected to various treatments according to manufacturer's guidelines using the Plant DNA/RNA purification kit from Norgen Biotek Corp. RNA concentrations were determined by Nanodrop spectrophotometer at 260 nm. Subsequent RNA purification was performed using 1 000 ng RNA with the Fermentas DNase I kit after which the resulting RNA was quantified by Nanondrop spectrophotometer once more. The RNA extraction and purification was performed under stringent RNAse-free conditions with surfaces prepared with DEPC solution for the prevention of undue RNAse activity.

2.19. cDNA ISOLATION BY REVERSE TRANSCRIPTION- POLYMERASE CHAIN REACTION [PCR]

First strand cDNA synthesis was performed by reverse transcription using the Fermentas cDNA synthesis reagents on 400 ng DNase-treated RNA. In a 20 µl reaction, 5X RT buffer (375 mM KCl, 50 mM DTT, 50 mM Tris-HCL pH 8.3 and 15 mM MgCl$_2$), 20 U Ribolock^{TM} RNase inhibitor(Fermentas), 200 U Revertaid^{TM} Reverse Transcriptase (Fermentas), 125 mM dNTP mix (Fermentas), 1 µM Oligo(dT) primer and 100 ng RNA were added. The reaction mixture was incubated at 42°C for 60 minutes for cDNA synthesis, the reaction was subsequently terminated at 70 °C for 5 minutes. The cDNA was stored at -20°C for subsequent reactions.

2.20. SEMI-QUANTITATIVE GENE EXPRESSION ANALYSIS OF *BRASSICA RAPA* UBIQUITIN [*BrUBQ*] BY POLYEMRASE CHAIN REACTION [PCR]

BrUBQ (*B. rapa* L. housekeeping gene) was amplified using gene specific primers. Primers specific to BrUBQ were designed. *BrUBQ* served as the reference gene to standardise subsequent gene expression data. For the amplification of *BrUBQ* the Velocity Taq DNA amplification kit was used. Primer sequences for *BrUBQ* were, BrUBQF: **5'-ATT CGT GAA GAC GCT GAC G-3'** and BrUBQR: **5'-GGC CAC ACT TCT TCT TCC TG-3'**. A 25 µl reaction, containing 1 µl of cDNA, 0.4 µM of each primer, 250 µM dNTPs, Velocity Taq 5X HiFi buffer, 3% DMSO and nuclease-free water, was prepared in a microfuge tube. Cycle parameters: 98°C for 2 minutes, 30 cycles of 98°C for 30 seconds, 63°C for 30 seconds and 72°C for 30 seconds. The final extension was performed at 72°C for 10 minutes. The reaction was catalysed by 0.5 U Velocity Taq DNA (Celtic Diagnostics). Amplicons were loaded alongside a 1 KB O'GeneRuler molecular weight marker onto a 1% agarose gel and subjected to electrophoresis at 80V for approximately 90 minutes. Semi-quantitative analysis was based on the resulting gel electrophoretogram.

2.21. SEMI-QUANTITATIVE GENE EXPRESSION ANALYSIS OF *BRASSICA RAPA* GLYOXALASE I (*BrGLY1*) BY PCR

BrGly-I was amplified using gene specific primers. For the amplification of *BrGLY-I* the Celtic Diagnostics Velocity Taq DNA amplification kit was used. Primer sequences for

the putative *BrGly-I* (Phytozome Accession number: Bra018654) were, BrGLY1F: **5'-GTTGAGGAATTCATGGGTTCATACTCAATAG-3'** and BrGLY1R: **5'-GTCAATGCGGCCGCTCAAGCTGCGTTTCCGGC-3'.** A 25 µl reaction mixture, containing 1 µl of cDNA, 0.4 µM of each primer, 250 µM dNTP mix, Velocity 5X HiFi buffer, 3% DMSO and nuclease-free water, was prepared in a microfuge tube. Cycle parameters: 98°C for 30 seconds, 30 cycles of 98°C for 30 seconds, 53°C for 30 seconds and 72°C for 30 seconds. The final extension was performed at 72°C for 10 minutes. The reaction was catalysed by 0.5 U Velocity DNA polymerase (Celtic Diagnostics). Amplicons were loaded alongside a 1 Kb O'GeneRuler molecular weight marker onto 1% agarose gel and subjected to electrophoresis at 80V for approximately 90 minutes.

2.22. DENSITOMETRY ANALYSIS

Analysis of gene expression data was performed using the AlphaEase FC Imaging software by Alpha Innotech Corporation for densitometry analysis. The software was used according to the manufacturer's specifications.

2.23. ZR UPTAKE IN *B. RAPA* L. SEEDLINGS BY INDUCTIVELY COUPLED PLASMA-OPTICAL EMISSION SPECTROSCOPY [ICP-OES]

B. rapa L. seedlings of each respective treatment were used for determination of Zr uptake by ICP-OES. Seedlings were digested and prepared for ICP-OES by the following method. Frozen ground seedlings (150 mg) obtained as described in section (2.1) were transferred to 2 ml Eppendorf tubes. To each sample tube 1 ml 65% nitric acid was added and homogenised by vigorous shaking and mixing by vortex. This was followed

by an incubation at 90 °C for 4 hours to allow for digestion. Eppendorf tubes were sealed with Parafilm prior to incubation to prevent the loss of sample mixtures with increased pressure. Sample homogenates were subjected to centrifugation to pellet leaf matter and the supernatants were transferred to new Eppendorf tubes. In a Greiner tube, samples were diluted (1:10) in a final volume of 5 ml with 2% nitric acid, this served as the sample for the subsequent ICP-OES analysis on the ICP-OES machine.

2.24. STATISTICAL ANALYSIS

Values obtained for all assays and experiments described herein were subjected to the Duncan's Multiple Range Test, or DMRT at P<0.05 and statistically validated based on SE, or standard error. Significance was represented by different alphabetical letters.

UNIVERSITY of the
WESTERN CAPE

CHAPTER THREE

PHYSIOLOGICAL STRESS RESPONSE IN ZR-STRESSED *B. RAPA* L. SEEDLINGS WITH EXOGENOUS MG

3.1. INTRODUCTION

Plants, being immotile organisms constantly face environmental factors such as abiotic stress which puts strain on metabolic functioning and frequently leads to impaired growth and development (Yadav *et al.,* 2005; Mourato *et al.,* 2012). The growth and development of plants is crucial especially concerning crop plants such as the, economically important and locally cultivated *Brassicaceae* species, whose crops give us condiments, vegetables, fodder, feedstock, and biofuels and can even be used as medicinal sources (Reiner, 1995; Cardoza and Stewart, 2006; Pua and Douglas , 2004). Furthermore *Brassica rapa* plants have rapid life cycles and as such farmers and the economy rely on them (USGS, 2012). However when these crop plants face adverse conditions, yields are affected which can be attributed, in part, to anthropological wastes in the form of toxic chemicals and metal ions that reach plants through the soil (Pua and Douglas, 2004; Cardoza and Stewart, 2006).

HMs have been known to cause damage to plants in a host of different ways viz. disruption of essential metabolic functioning, membrane dismantling due to accumulated active oxygen species, diminished chlorophyll content, impaired cell viability, and impeding growth and development (Yadav *et al.,* 2005; Hossain *et al.,*2011). The list is extensive and so in this report Zirconium (Zr) was chosen as a stressor because of the scant reports on its impacts on plants in literature (Fodor *et al.,*

49

2005). Furthermore this element was chosen because it is extensively mined and produced globally; South Africa alone accounts for nearly 30% of the global Zr production (USGS, 2012) and because the literature indicates Zr as a harmful element in plants (Ghosh *et al.,* 1992; Wang, 2000; Shahid, 2014; Shahid *et al.,* 2013).

Zr as an element is not readily soluble in water and so its phytoavailability depends on the soil composition (Ferrand *et al.,* 2006). Because of the minerals and chemicals available in soil such as chlorides, O_2 and POH^{3+}, Zr is able to easily form species with them (Ghosh *et al.,* 1992; Ferrand *et al.,* 2006). Once this occurs Zr is more mobile and readily absorbed through plants' root systems (Ghosh *et al.,* 1992; Fodor *et al.,* 2005; Shahid *et al.,* 2014). Once Zr has passed the root cell membrane, translocation to aerial parts of the plant may occur which negatively affects biochemical pathways and cellular functioning becomes impeded (Ferrand *et al.,* 2006; Shahid *et al.,* 2014). Zr has no known function in biological systems, however Ferrand et al. (2006) have reported the phytotoxicity of Zr and the diminished growth and development in three different higher plant species viz. *S. lycopersicum, P. vulgare* and *P. minor.* Fodor et al. (2005) observed drastic developmental and physiological effects of Zr in wheat seedlings, whereas similarly Shahid (2013) also observed the growth-impeding effects of Zr exposure in plants.

MG is a cytotoxic by-product of metabolic functioning, it occurs ubiquitously in nature and has therefore been investigated in plants, yeast and bacteria. However, its impacts in biological systems have been looked at previously using high doses (Yadav *et al.,* 2005; Hossain *et al.,* 2013). MG has been established as a reducing agent and impacts the *dicarbonyl proteome*, or DCP, upon which advanced glycation ends also known as

AGE's are formed which causes irreversible damage to cells (Freire *et al.,* 2003; Korybalska *et al.,* 2003; Firestone *et al,* 2007). In addition MG is capable of reducing molecular oxygen (Saito *et al.,* 2011), which results in more highly reactive oxygen species being formed and thus causes oxidative damage (Hossain *et al.,* 2013). Due to its harmful effects and the ROS-induced oxidative stress caused by MG, it is often considered a ROS (Hossain *et al.,* 2009; 2011). Because ROS are known signalling molecules at low levels, the possible signalling capability of MG was explored in this study and its possible growth-promoting properties at a low concentration of 6 µmol.L^{-1}.

Plant physiology, which most frequently is noted by morphology, is often the first indicator of perceived stress in plants (Ahuja *et al.,* 2015). Mhamdi et al. (2010) describes stress in plants as "a physiological condition caused by any environmental constraint that limits growth, reproductive success, yield, quality, or other traits desirable to humans". Plants under stress may exhibit visible signs or alterations in their features that include leaf yellowing and leaf rolling, stunted root and shoot development, structural augmentation and necrosis (Cardoza and Stewart, 2006; Hortensteiner *et al.,* 2011; Ahuja *et al.,* 2015). The above mentioned symptoms are indicative of oxidative stress in plants, the extent of which is measurable through physiological testing by determining germination percentage, lipid peroxidation, chlorophyll content, dry weights and cell death. These tests were employed in this study as a means to determine the impact of exogenous MG in Zr stressed *B. rapa* L. seedlings for the possible benefits of MG at a low concentration to alleviate physiological stress and improve seedling growth and development.

3.2. RESULTS

3.2.1.   MG CONFERS AN INCREASE IN BIOMASS, GERMINATION RATE AND SEEDLING LENGTHS IN *B. RAPA* L. SEEDLINGS UNDER ZR-STRESS

Due to the extent that Zr is produced, with an upward trend in production projected by USGS (2012) from 2007 − 2012 (figure 1.3.1), it is evermore concerning that crop fields are being exposed to Zr wastes and thus plants growing in these contaminated soils face immense environmental pressures. *B. rapa* L. being one such organism, is related to canola plants and other Brassica crops that include broccoli and cabbage (Reiner *et al.,* 1995; Cardoza and Stewart, 2004). Therefore it was important to note the effects of toxic mineral waste such Zr on *B. rapa* L. given the significance of this crop family. Seeds were exposed to 1 mM Zr, continuous Zr was applied until seedlings were ready for harvesting. Germination percentage and seedling biomass were noted under these conditions. Additionally the possible growth-promoting or signalling properties of MG were investigated. MG, similar to ROS has been observed to cause oxidative damage with cytotoxic responses in plants having been reported, at high concentrations (Hossain *et al.,* 2012). Furthermore, given the fact that MG is capable of converting O_2 directly to O_2^- (Yadav *et al.,* 2005; Saito *et al.,* 2011), it was proposed that MG, similar to other ROS such as H_2O_2, may at low levels have signalling properties that could incite growth promotion in young stage *B. rapa* L.

Seedlings of this study were subjected to MG (6 µM), Zr (1 mM) and MG plus Zr (6 µM+ 1 mM). In Zr-treated seedlings (figure 3.2.1.) a 20% reduction in seedling length was

observed as compared to the control. MG-treated seedlings showed a 30% increase in shoot length and in seedlings treated with MG+Zr there was approximately 40% increase in seedling length compared to those under Zr-stress only.

For germination (Figure 3.2.2.) no differences were observed in the control and MG-treated seeds however, a decrease of 10% was observed in the Zr-treated seed germination percentage. For the MG+Zr-treatments there was an increase observed in germination percentage by 5% compared to the Zr-treated seeds.

More drastic results were observed with regards to the biomass of the seedlings (figure 3.2.3) where a 60% increase compared to the control was observed in the MG-treated seedlings. A staggering 50% reduction was observed in Zr-treated seedlings as compared to the control which indicates the drastic effects that Zr has on initial growth and development in *B. rapa* L. seedlings. In seedlings treated with MG+Zr a 15% increase in yield was observed as compared with the Zr-treated seedlings.
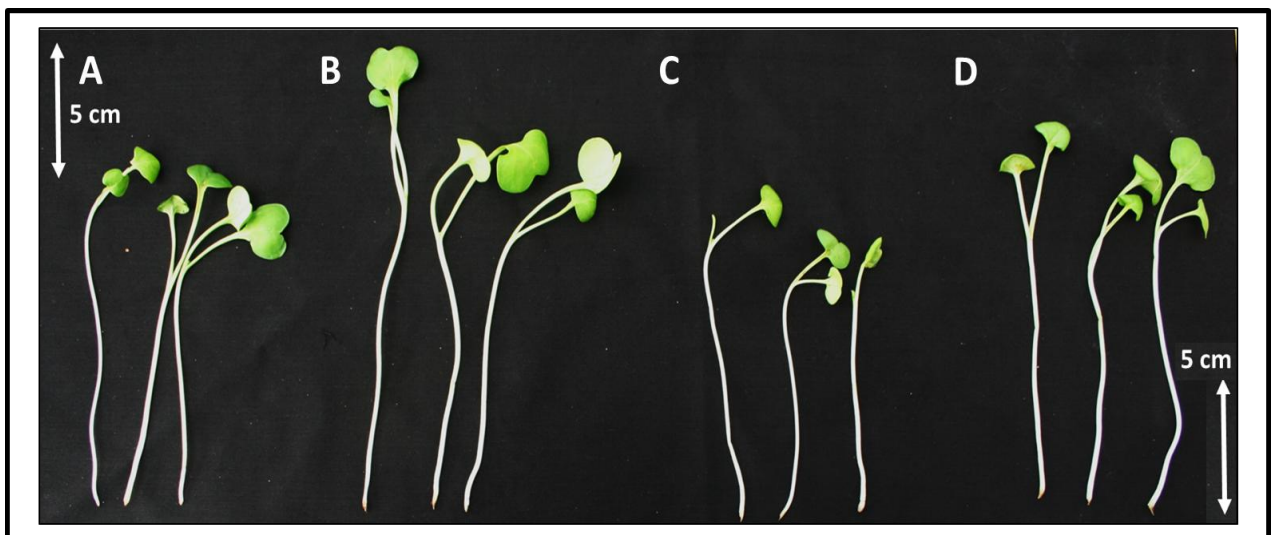
Figure 3.2.1. The effect of Methylglyoxal in Zr stress responses in *B. rapa* L. seedling length. *B. rapa* L. seeds were sown onto soil pre-treated with the respective treatments. Once germinated the seedlings were treated every 3 days for 14 days from the day of sowing. Seedlings were harvested 2 weeks after germination. Treatments were **(A)** water-fed control, **(B)** 6 μM MG, **(C)** 1 mM Zr, **(D)** 6 μM MG + 1 mM Zr.
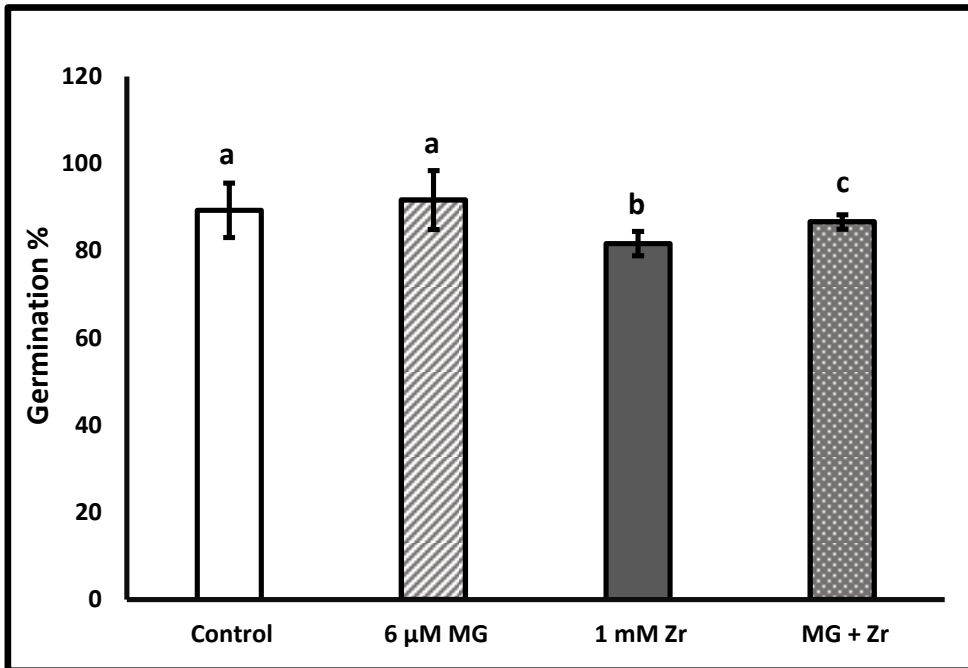
Figure 3.2.2. The effect of Methylglyoxal on germination percentage of *B. rapa* **L. seedlings in response to Zr stress.** Seeds were placed in soil that was treated with the respective treatments shown in the figure. Once seeds had germinated a germination percentage was calculated statistically. The different letters indicate a significant change across means at P< 0.05 (DMRT). Values are means ± S.E (N=3).

Figure 3.2.3. The effect of Methylglyoxal on the dryweights of *B. rapa* **L. seedlings in response to Zr stress.** *B. rapa* L. seedlings were subjected Zr stress and MG treatments respectively as well as in combination (MG+Zr). Post-harvest, the dry weights were determined. The different letters indicate a significant change across means at P< 0.05 (DMRT). Values are means ± S.E (N=3).

3.2.2. MG INCREASES THE CHLOROPHYLL CONTENT IN ZR-STRESSED *B. RAPA* L. SEEDLINGS

All plants have a light-absorbing pigment that give them their unmistakeable green colour; chlorophyll (Hortensteiner and Krautler, 2011). Chlorophyll absorbs blue and red wavelengths that are then visualised as the green pigment we find in plant stems and leaves (Pua and Douglas, 2004; Cartea *et al*., 2011; Hortensteiner and Krautler, 2011). When chlorophyll content decreases, essential biological processes are hindered (Ahsan *et al.,* 2003). Chlorophyll, also known as a photoreceptor, is localized in chloroplasts. Its function is limited to photosynthesis, however this is no menial or inconsequential task since chloroplasts are the "food producers" or energy generators of the cell (Pua and Douglas, 2004; Hortensteiner and Krautler, 2011). Because chlorophyll is essential for photosynthesis to take place, diminished chlorophyll content has been associated with oxidative stress due to environmental factors viz. exposure to chemicals, drought and salt stress (Halliwell and Gutteridge, 2007; Hortensteiner and Krautler, 2011).

The chlorophyll content (table 3.1) across the respective treatments was very informative, where MG seedlings had an increase in chlorophyll *a* of 32.4% compared to the control, the highest chlorophyll *a* content across all seedlings in this study. Compared to the control, Zr- and MG+Zr-treated seedlings had an 11.4% decrease and a 20% increase respectively. A similar trend was observed with chlorophyll *b* where a 15% increase was seen in MG-treated seedlings, a 26% decrease and 10.3% increase in Zr and MG+Zr-treated seedlings respectively compared to the control. Moreover the total chlorophyll content showed that in MG-treated seedlings the highest chlorophyll

content was observed with a 27.5% increase compared to the control. In MG+Zr-treated seedlings a 16.5% increase was shown and in the Zr-treated set a decrease in total chlorophyll content by 15.5% was measured with respect to the control.

Table 3.1. <u>The Effect of Methylglyoxal and Zirconium on Chlorophyll *a* and *b* (μg.g^{-1}) *B. rapa* L. seedlings</u>

Metabolite	Treatments			
	Control	6 μM MG	1 mM Zr	MG+Zr
Chlorophyll *a*	124.5 ± 0.03[c]	164.9 ± 0.324[a]	110.2 ± 0.11[d]	148.5 ± 0.193[b]
Chlorophyll *b*	48.3 ± 0.023[e]	55.7 ± 0.153[f]	35.9 ± 0.26[g]	53.3 ± 0.103[h]
Total Chlorophyll	172.9 ± 0.018[i]	220.6 ± 0.276[j]	146.1 ± 0.16[k]	201.5 ± 0.165[l]

Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ± S.E (N=4).

3.2.3. ZR-INDUCED LIPID PEROXIDATION MITIGATED BY EXOGENOUS MG IN *B. RAPA* L. SEEDLINGS

As Malondialdehyde (MDA) indicates lipid peroxidation and membrane damage, Singh et al. (2009), similar to other reports (Halliwell and Gutteridge, 2007; Ahuja *et al.,* 2015), have found that upon increased MDA content an observable decrease in conjugated dienes (CD) content is observed as a result of perpetuating oxidative damage (Halliwell and Gutteridge, 2007; Singh *et al.,* 2009; Ahuja *et al.,* 2015). CD and MDA; by-products of lipid peroxidation can be related in an inversely proportional manner, where CD is the primary product of lipid peroxidation, and when more ROS accumulates, they naturally react or collide with CD, and MDA is the secondary

product formed from this collision (Halliwell and Gutteridge, 1984; Ahuja *et al.,* 2015). This then means that the more ROS present due to oxidative stress, the more CD will react with ROS to form MDA and the less resulting CD will be present. Ahuja et al. (2015) further supports this observation in their reports where a decrease in CD content is indicative of excess ROS production which causes oxidative stress. They have reported that this decrease in CD content upon ROS formation and collision is indicative of ROS attack on plant membranes that will ultimately cause membrane disruption and leakage (Ahuja *et al.,* 2015).

In this study, MDA and CD were measured (figure 3.2.4 and 3.2.5). Both being by-products of lipid peroxidation, having been associated with membrane disruption as a result of oxidative stress due to increased ROS (Singh *et al.,* 2009; Shahid, 2013; Ahuja *et al.,* 2015). Control and MG-treated seedlings had unchanged MDA levels. Seedlings exposed to Zr stress however showed an 18% increase compared to the control, and seedlings exposed to MG+Zr had MDA levels slightly higher than the control, but still lower than the Zr-treated seedlings by 13%.

Conversely the CD content observed in both MG- and control-treated seedlings showed a 20% higher CD content than the Zr-treated seedlings, and a 26% increase in the MG+Zr-treated seedlings.
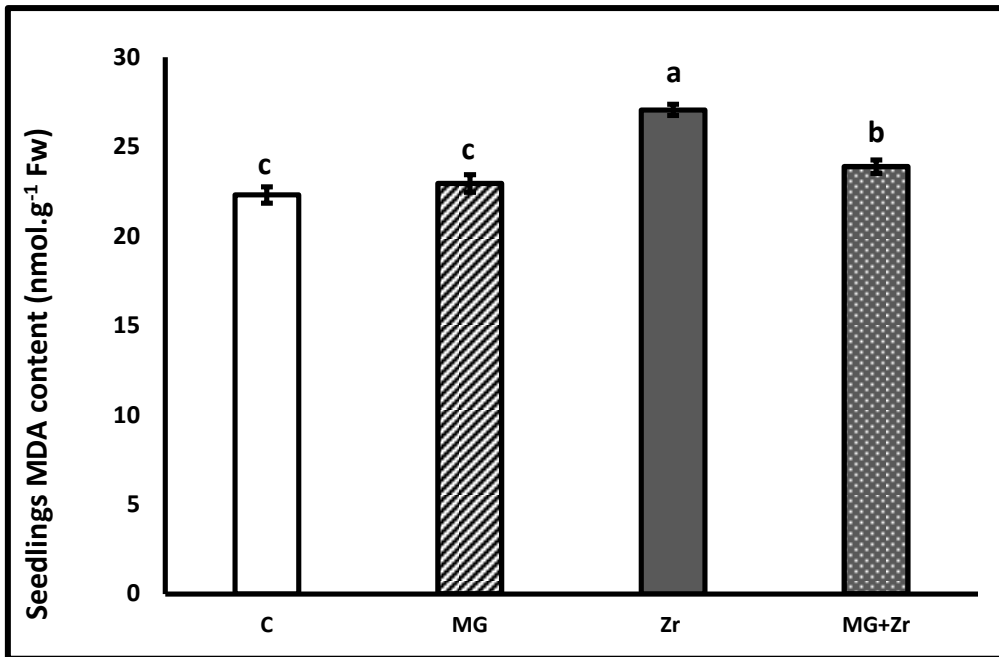
Figure 3.2.4. The effect of Methylglyoxal on MDA content in *B. rapa* L. seedlings in response to Zr stress. Under perceived stress in plants MDA is often used as a stress marker as it indicates lipid peroxidation. MDA content was determined in *B. rapa* L. seedlings after exposure to Zr stress and MG treatments respectively. The different letters indicate a significant change across means at P< 0.05 (DMRT). Values are means ± S.E (N=3).
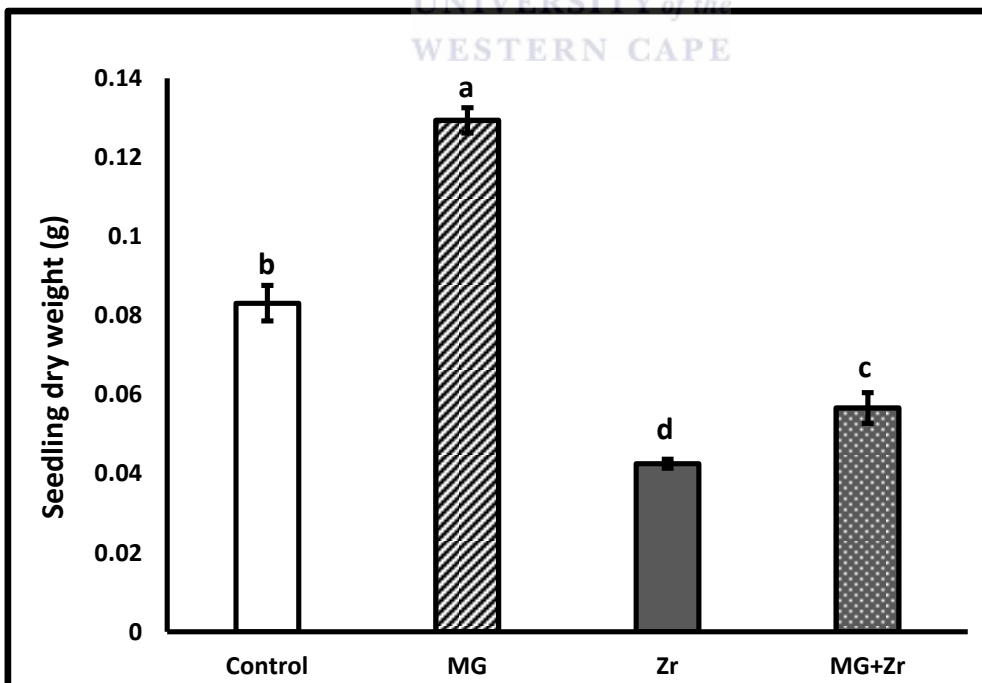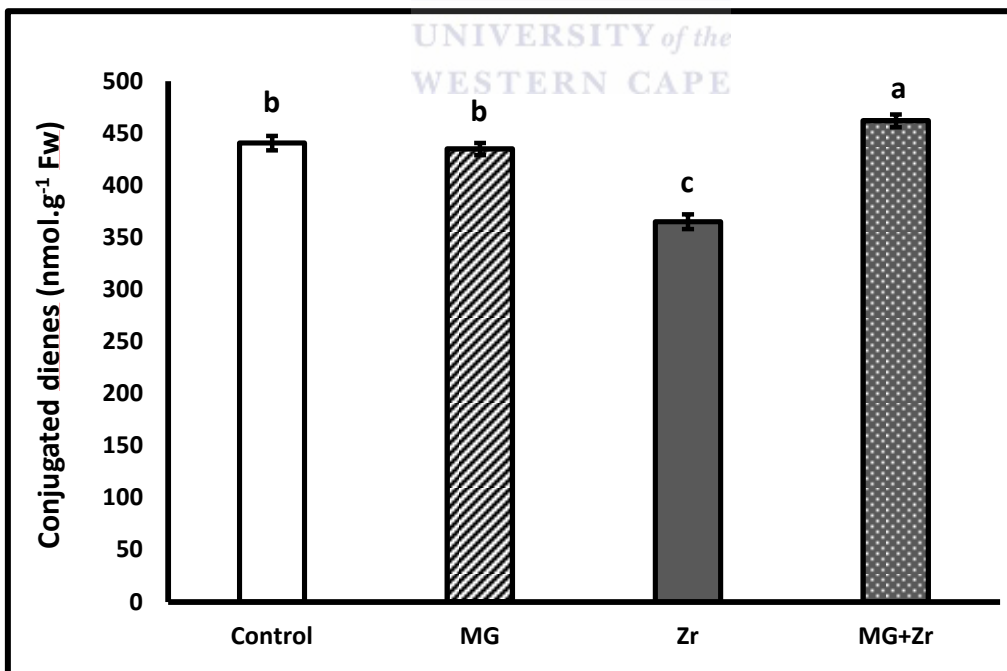
Figure 3.2.5. The effect of Methylglyoxal on the content of conjugated dienes in *B. rapa* L. seedlings in response to Zr stress. CD content is often measured in plants subjected to oxidative stress as it is the primary product upon degradation of lipid molecules. The level of conjugated dienes was determined in *B. rapa* L. seedlings 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).

3.2.4. IMPROVED CELL VIABILITY WHEN MG IS PRESENT IN *B. RAPA* L. SEEDLINGS SUBJECTED TO ZR-STRESS

Cell survivability, also known as cell viability, is important to plants as sessile organisms that cannot physically avoid environmental factors causing damage and distress. Therefore the measure of cell death in *B. rapa* L. seedlings was determined by spectrophotometric assaying, in light of reports by Halliwell et al. (2012) and Singh et al. (2009) having observed that cell viability decreased when exposed to oxidative stress.

Cell death rate in *B. rapa* L. seedlings in this study (figure 3.2.6) show that the control and MG-treated seedlings had unchanged absorbance's at 600 nm. With the Zr-treated seedlings however, a 56% increase was observed. While in MG+Zr-treated seedlings a 41.1% lower cell death rate was observed compared to seedlings treated only with Zr.
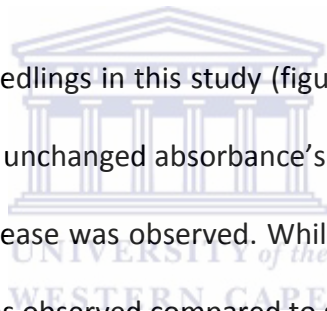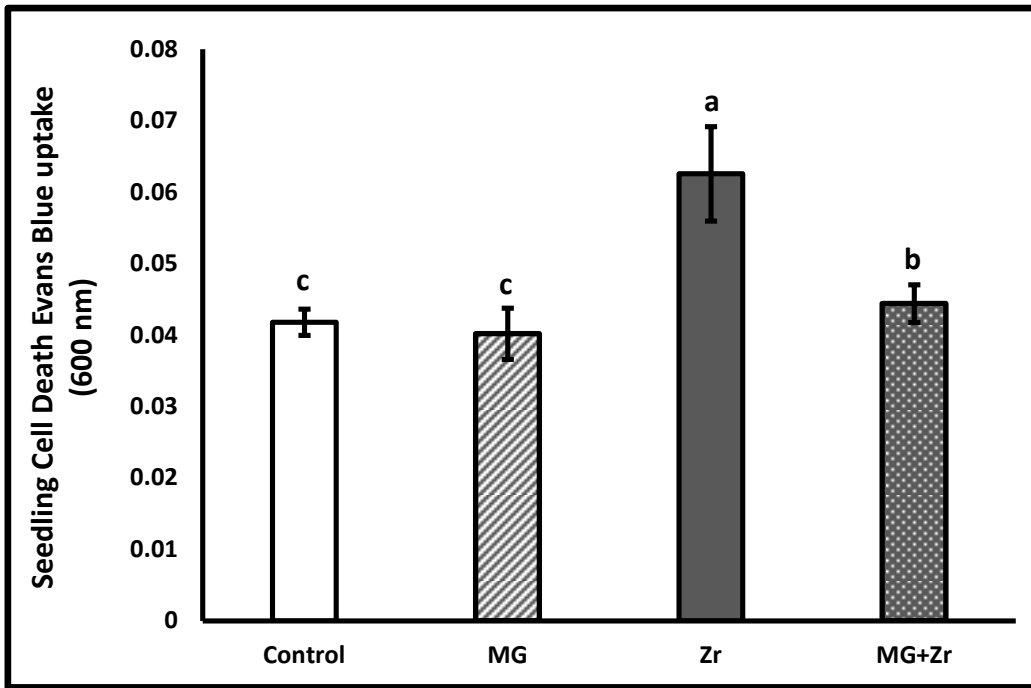
Figure 3.2.6. The effect of Methylglyoxal on *B. rapa* L. seedlings' cell death response under Zr stress. Cell death was determined in *B. rapa* L. seedlings after exposure to Zr stress and MG treatments respectively. The different letters indicate a significant change across means at P< 0.05 (DMRT). Values are means ± S.E (N=3).

3.3. DISCUSSION

Although the function of Zr in plant metabolism has never been established and the literature on this topic is minimal (Ferrand *et al.,* 2006; Shahid *et al.,* 2013), there have been reported cytotoxic and damaging effects of Zr in various higher plants (Ghosh *et al.,* 1992; Wang, 2000; Shahid, 2013, 2014). Shahid (2013) reports on the toxic effects of Zr observed in yeasts, algae, bacteria, fish and higher plants. Slight toxic effects were noted by Davis et al. (1978) in *Hordeum vulgare* seedlings with a decrease in biomass after Zr exposure. Similarly, Ferrand and colleagues (2006) observed a reduction in *P. sativum* and *S. lycopersicum* dry weights after exposure to Zr. Ferrand et al. (2006) also observed a difference in the rate of Zr-uptake between pea and tomato plants and that Zr is fast absorbed by roots, with a higher Zr concentration found in the root cells than in other cells.

Although Zr is not readily absorbed, its tendency for speciation with other chemicals in soils make it harder to firmly establish its toxicity, due to soil components having great variation (Ghosh *et al.,* 1992; Ferrand *et al.,* 2006). This tendency to form species with other soil chemicals such as Cl-, OH- and POH^{3+} allows for easy absorption in plants through their roots (Wang *et al.,* 2000; Ferrand *et al.,* 2006). Jones (1998) reports that low molecular weight organic molecules like root exudates is a favoured means of mobilisation of Zr in plants. This shows that despite the low solubility of Zr in solution and its bioavailability being low for uptake into plants, Zr has the ability to readily form compounds with organic chemicals already present in soils and in this manner it can be taken up by roots and cause negligible to extensive damage (Jones, 1998; Ferrand *et al.,* 2006; Shahid *et al.,* 2014). The extent of damage will depend on the species of

plant, the composition of the soil surrounding the roots and the age or maturity of the plant that Zr comes into contact with (Jones, 1998; Wang, 2000). This is of great concern because it makes the reliable determination of toxic levels of Zr challenging, and raises the alarm for more such research to be undertaken.

Because MG and Zr are known to have toxic effects in plants, and that accumulation of either one will result in impaired growth and development, and oxidative stress. This study therefore investigated the extent of damage that results from Zr exposure in *B. rapa* L. seedlings, as well as the impacts on plant overall health and coping mechanisms in the presence of a low dose of MG when under Zr-stress.

Based on the results obtained, it is evident that Zr affects growth and development of young plants (Figure 3.2.1-3.2.3); seedling germination rate had a significant reduction (Figure 3.2.2). Seed dormancy can be indicative of perceived stress in plants and demonstrates early developmental impediments, which in the case of *B. rapa* L. is unusual as they are known to circumvent seed dormancy and typically germinates at close to 100% under normal conditions (Reiner *et al.,* 1995; Pua and Douglas *et al.,* 2004; Cardoza and Stewart, 2004). Seedling biomass (Figure 3.2.3) had decreased drastically by 0.6-fold and 0.4-fold after exposure to Zr compared to MG-treated seedlings and the control respectively. The presence of HMs in plants have been reported to cause physiological distress and oxidative stress (Shahid *et al.,* 2014) thus indicating the effects that Zr has in the development and yield of *B. rapa* L.. Seedling lengths were noticeably reduced by Zr exposure as well (Figure 3.2.1C) similar to reports by Fodor et al. (2005) and Shahid et al. (2014) who reported reduced shoot and root lengths after exposure to Zr-Ascorbate at over 100 µM and Zr respectively. This

further supports that Zr at 1 mM detriments plants physiologically, with respect to germination percentage, dry weights and morphology in this study. Singh et al. (2009) reported that in *A. fatua, P. minor* and *C. rotundus* minor to significant reductions in their dry weights and seedling growth, and seed germination was affected by oxidative stress. Further supporting the evidence of Zr-toxicity and its effects on seedling physiology in this study, with an observed reduction in *B. rapa* L. seedling biomass and seed germination (figure 3.2.2 and 3.2.3) respectively in response to Zr.

In plants treated with MG+Zr the effects of Zr were alleviated in each case (figure 3.2.1 – 3.2.3), where seedling length surpassed the Zr-treated set (figure 3.2.1C) and slightly surpassed the control (figure 3.2.1A), the dry weights (figure 3.2.3) and germination (figure 3.2.2) were improved compared to Zr-treated seedlings. Even more striking was the effect of MG on its own in seedlings of this study. The germination (figure 3.2.2) rates were unchanged, but there was a drastic increase in the dry weights (figure 3.2.3) and seedling length (figure 3.2.1B) when subjected to 6 µM MG. This indicates that MG does influence *B. rapa* L. growth and development at the seedling stage.

Chlorophyll in plants is essential, it gives plants their iconic green pigment and in healthy plants greener leaves will be observed as opposed to their stressed counterparts (Hortensteiner and Krautler, 2011). Because chlorophyll is responsible for the breakdown of energy in plant cells, they are localized within and around the photosystems. Upon excitation of PII at P650 and the subsequent excitation of PI at P700, sunlight becomes converted to a useable chemical energy form; the process we know as photosynthesis (Peralta-Videa *et al.,* 2004; Cartea *et al.,* 2011; Hossain *et al.,* 2011).

In this study a significant decrease in total chlorophyll (table 3.1) was observed in Zr-treated seedlings and as a result slight leaf yellowing; Chlorosis, was observed (figure 3.2.1C). This reduction in chlorophyll molecules by 15.5% was a significant one and will disturb photosynthesis, and thus cell biochemistry will be impacted because plants obtain their chemical energy as a result of photosynthesis (Goud and Kachole, 2011). In MG+Zr-treated seedlings a noteworthy increase in chlorophyll content was observed at 16.5%, indicating that despite the presence of Zr that chlorophyll degradation diminished and its production was induced when MG was added. MG-treated seedlings above all exhibited the highest chlorophyll levels by 0.3-fold increase which is very significant and beneficial to plants since chlorophyll is so vital to plants' continued growth and metabolic function. This finding clearly designates MG as a beneficial additive at low levels for improved chlorophyll production and subsequent photosynthesis.
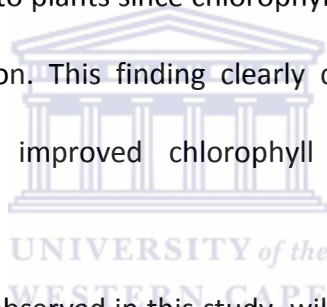
The detrimental impact of Zr observed in this study, will only cause the stress in plants to perpetuate as the available energy in plants becomes depleted, and given the largely reduced seedling dry weights (figure 3.2.3), length (figure 3.2.1), and reduced germination rate (figure 3.2.2) observed, Zr toxicity in plants could have far-reaching impacts and detriment plant physiology tremendously. This may then cause serious oxidative stress and damage, impaired growth and thus the reduction in yield observable in this study, similar to other toxic HMs; Cd, Zn and Ni (Yadav *et al.,* 2005).

Goud and Kachole (2011) observed that upon accumulation of ROS, after exogenous H_2O_2 under oxidative stress, that the stress response in pigeon pea plants caused a decrease in chlorophyll molecules, and that it immediately preceded the breakdown of proteins during leaf senescence. This has also been reported by Hung and Kao (2007),

64

who associates the depletion of chlorophyll molecules and protein breakdown with leaf senescence. Reduced chlorophyll levels directly causes the inefficient conversion of energy. As plants then face unfavourable amounts of energy, the already limited energy must be used during photosynthesis, making even less energy available for essential metabolic activities (Ahsan *et al.,* 2003; Cartea *et al.,* 2011; Hortensteiner and Krautler, 2011). This causes great adversity to plants especially when chlorophyll becomes damaged (Cartea *et al.,* 2011).

Singla-Pareek et al. (2006) observed increased antioxidant activity in wild type tobacco plants exposed to 5 mM Zn, as well as an extreme decline in biomass and morphological signs of stress (Singla-Pareek *et al.,* 2006). Furthermore they observed a steep decline in the chlorophyll content of tobacco plants subjected to Zn, Cd and Pb. This was also evident from the extreme yellowing and white appearance in the leaves, with Cd having the harshest impact among the three HMs (Singla-Pareek *et al.,* 2006). With reduced chlorophyll levels, plants' biological function will become hindered due to limited or rapidly declining available energy within cells (Pua and Douglas, 2004; Hortensteiner and Krautler, 2011).

Hung and Kao (2007) reports on the pre-indication of senescence and protein degradation upon observing reduced chlorophyll levels. They also regard the loss of chlorophyll molecules the main criterion or the primary indicator of leaf senescence in plants, the first visible signs being yellowing of leaves, commonly known as chlorosis (Hortensteiner and Krautler, 2011). Since leaf yellowing occurs directly as a result of the loss of chlorophyll, it is evident that chlorophyll molecules are indirectly involved with a host of metabolic processes, and once chlorophyll levels diminish stress is

65

experienced and is often irreversible (Hortensteiner and Krautler, 2011). Because leaf senescence is also associated with lipid peroxidation, knowing the status of chlorophyll levels in plants under stress can be regarded as a predetermining factor for membrane disruption and damage which can be determined by a measure of lipid peroxidation (Ahuja *et al.,* 2015). Lipid peroxidation is determined by measuring the levels of MDA and conjugated dienes (CD) in plant cells in response to environmental stress as observed in figures (4.2.5) and (4.2.6) (Cartea *et al*., 2011; Hortensteiner and Krautler, 2011).

Oxidative damage is often determined by a measure of lipid peroxidation since it directly results in the formation of free radicals and H_2O_2 (Cherif *et al.,* 1997). Cherif et al. (1997) investigated plant root-pathogen interaction in poorly aerated soil, stress was determined by measuring lipid peroxidation (conjugated dienes content). In literature lipid peroxidation is reported to be a precursor to ROS production and accumulation via peroxidation of membrane lipids (Cherif *et al.,* 1997; Ahuja *et al.,* 2015). Since damage to, and weakening of lipid membranes can be detected by determining the CD and MDA content, it was therefore observed in this study in *B. rapa* L. seedlings under Zr-stress, MG-treatments, and in seedlings subjected to a combination of MG+Zr. Because CD is the primary product of oxidation of PUFAs and forms MDA as a secondary product, under oxidative stress it would be expected that MDA content would increase, and with ongoing stress CD content would decrease respectively to MDA levels.

In our study the increase in lipid peroxidation in *B. rapa* L. seedlings under Zr stress indicates oxidative stress. A decrease in CD (figure 3.2.5) content and an increase in MDA (figure 3.2.4) were observed, both indicative of the oxidative stress response and

66

damage to membrane lipids. This was similar to other reports (Ahuja *et al.,* 2015) where CD and MDA decreased and increased respectively, relative to one another upon ROS-mediated oxidative stress. In seedlings subjected to MG+Zr there was a response similar to the control whereby MDA levels had only slightly increased but show a marked lower level of lipid peroxidation than in Zr-stressed seedlings. This decrease in lipid peroxidation indicates the mitigating effects of MG at low levels, despite exposure to Zr. Given that MG at low levels caused the level of lipid peroxidation to remain unchanged, and the fact that it caused little negative response despite the same levels of Zr present, it indicates that MG is possibly involved with signal transduction upon stress perception in plants. Furthermore, this signalling allows the effects of HM stress to become diminished despite the toxicity of Zr when it is administered on its own. In MG-treated seedlings the change in MDA indicated no difference, an interesting observation since MG-toxicity in plants is reported to be high. These findings point to a stress-relieving function in MG; a known cytotoxic by-product of aerobic metabolism which has been focussed on previously, but only at high doses in the past (Hossain *et al.,* 2012).

An increase in MDA of nearly 50% was observed by Hung and Kao (2007) in rice leaves subjected to oxidative stress. Ahuja and colleagues (2015) noted a similar increase and decrease in MDA and CD respectively, amidst ROS-induced oxidative stress in *A. fatua.* MDA was measured in response to Zn stress in tobacco plants where a 3-fold increase in MDA was observed in the presence of Zn, indicating the harsh and toxic effects of HMs in plants (Singla-Pareek *et al.,* 2006).

Oxidative stress in plants may arise as a result of various environmental factors that causes the overproduction of ROS viz. •OH, H_2O_2, O_2^-, all which have an impact on plant metabolism and essential biological function (Hossain *et al.,* 2011). Often lipid membranes suffer damage as well as cell viability amidst the inevitable reduction of cellular O_2 (Hossain *et al.,* 2011; Hortensteiner and Krautler, 2011). The accumulation of excessive ROS in plants causes damage in most, if not all, cellular components viz. chloroplasts, pigments, membrane lipids, enzymes and nucleic acids (Goud and Kachole, 20110. Vulnerability of said cellular components on their own or in combination may put enough pressure on cell viability to cause cell death (Verma and Dubey, 2003; Goud and Kachole, 2011; Ahuja *et al*., 2015). Goud and Kachole (2011) describes cell death as a result of hydrolysis of proteins, chlorophyll molecules, lipids, polysaccharides and DNA.
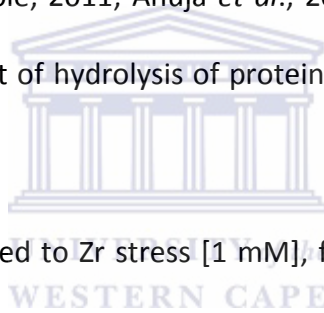
In *B. rapa* L. seedlings subjected to Zr stress [1 mM], from seed germination up until harvesting, early developmental difficulties were observed. The decreased seedling length (figure 3.2.1C) indicated oxidative and physiological stress. Additionally cell viability (figure 3.2.6), otherwise referred to as cell survivability or conversely cell death, was negatively impacted.

MG causes damage to plants, and ROS accumulation has been associated when high doses were administered (Hossain *et al.,* 2013). However, MG at low levels in this study showed no change in *B. rapa* L. cell death levels (figure 3.2.6). Again this indicates no additional oxidative stress when MG is administered. But when Zr was applied seedlings were detrimentally affected by a significant increase in cell death of 0.56-fold (figure 3.2.6). A notable difference in seedling biomass (figure 3.2.3),

increased lipid peroxidation (3.2.4) and a reduction in total chlorophyll content (table 3.1) had occurred after exposure to Zr, all of which have been associated with cell death by Goud and Kachole (2011) and Gepstein (2004). Fortunately, seedlings treated with a low dose of MG (6µM) in tandem with Zr indicated that the cytotoxic effects of Zr were muted or countered that resulted in only a slight increase in cell death by 6% (figure 3.2.6) compared to the control; a dramatic improvement from seedlings exposed to Zr on its own. This indicates that although Zr serves no useful purpose in cellular functioning, it is harmful to plant cells and at 1 mM is toxic in young stage *B. rapa* L.. Despite their ability to grow and survive amidst toxic chemicals, damage still occurs in the presence of 1 mM Zr.

Cell death was detrimentally impacted, upon induction of oxidative stress in higher plants. Up to 24% cell death was observed in a dose-dependent study by Singh et al. (2009). This along with our findings in figure (3.2.6) not only further supports the evidence of physiological stress, but also firmly establishes that Zr causes an increase in cell death (figure 3.2.6) and affects plant cells similar to another study where ROS-induced oxidative stress is reported (Shahid, 2013). Furthermore membrane damage and plant biomass was affected similar to observations in plants with accumulated ROS (Singh *et al.,* 2009; Shahid, 2014) which means that the physiological signs of stress exhibited in *B. rapa* L. seedlings is indicative of rapid ROS accumulation under Zr-stress.

Singh et al. (2009) along with Batish et al. (2007) correlates cell viability indirectly with cellular respiration in plants, where the TTC in viable healthier cells absorbs electrons from mitochondrial ETC and is subsequently reduced. Thus positively relating cell viability to respiration, and so upon increased cell death a decrease in respiratory

69

activity should also be expected (Batish *et al.,* 2007; Singh *et al.,* 2009). These authors simultaneously relate this decrease in respiratory activity with either a reduction or an interference in energy metabolism during synthesis of macromolecules like proteins, lipids and nucleotides which can cause impaired growth in plants, just as our observations of impeding growth (figure 3.2.1) in *B. rapa* L. seedlings relative to the increase in cell death (figure 3.2.6).

To conclude this chapter, stark evidence of the harmful impact of short-term Zr-stress in *B. rapa* L. seedlings was observed in this study. Not only from a morphological aspect, with a vast reduction in seedling length (figure 3.2.1) and yellowing of the young leaves, but also the firm evidence indicated by the physiological tests with reduced biomass (figure 3.2.3), reduced chlorophyll (table 3.1) and CD content (figure 3.2.5), and an increase in MDA levels (figure 3.2.4). These findings all designate Zr as a cytotoxic elemental compound, whose low solubility in water and resulting low phytoavailability have little to no bearing on its adverse effects in *B. rapa* L. seedlings. Since lipid peroxidation, cell death and diminished chlorophyll levels have all been associated with oxidative stress and irreversible damage, there can be no doubt that if oxidative stress in *B. rapa* L. seedlings is validated (Chapter 4), that it can be attributed to the Zr-stress induced from the results of this study.

Similarly striking was our observation of the impact of exogenous MG when applied in tandem with Zr. Seedlings to whom MG+Zr were administered escaped the fate that Zr-treated seedlings suffered. Changes in physiological responses were often negligible and only slightly worse than in the control seedlings across the compilation of tests (figure 3.2.2 – 3.2.3; figure 3.2.4 – 3.2.6). More telling was the fact that the MG+Zr-

treated seedlings had increased chlorophyll content (table 3.1) and increased seedling length (figure 3.2.1) in comparison with control seedlings, again illustrating the countered response to physiological stress observed, as well as growth-signalling properties when a low dose of MG was administered.

Even without noting the marked physiological response in MG-treated seedlings, the damning correlation between Zr-treated seedlings and MG+Zr-treated seedlings indicates the alleviating and growth-promoting capability of MG at a low administered dose. In seedlings where MG alone was applied, the beneficial effects of MG were evident where seedling biomass (figure 3.2.3), length (figure 3.2.1 B and D) and total chlorophyll (table 3.1) were largely improved which further establishes MG as a growth-promoting agent.

Despite reports on MG as cytotoxic, at a minimal dose it was shown in this study that MG is capable of mitigating the toxicity of HM Zr, and that when applied on its own it resulted in seedlings that were healthier, with an increased dry weights (figure 3.2.3) and, most importantly showed, no sign of increased oxidative stress. Furthermore MG can be regarded as a signalling molecule because it confers tolerance to HM stress as shown by the increased chlorophyll content (table 3.1) and reduced lipid peroxidation (figure 3.2.4 - 3.2.5) in seedling subjected to MG+Zr, two critically important aspects of plant physiology both known to indicate the onset and occurrence of oxidative stress (Ahsan *et al.,* 2003; Hossain *et al.,* 2011). Based on these findings, the next chapter (MODULATION OF ROS AND THE ANTIOXIDANT RESPONSE VIA EXOGENOUS MG IN *B. RAPA* L. SEEDLINGS UNDER ZR STRESS) will be used for validation of MG's capability to

mitigate oxidative stress due to Zr, by investigating antioxidant activity and the toxic

ROS they metabolise.

CHAPTER FOUR

MODULATION OF ROS AND THE ANTIOXIDANT RESPONSE VIA EXOGENOUS MG IN *B. RAPA* L. SEEDLINGS UNDER ZR STRESS

4.1. INTRODUCTION

Plants' antioxidant defence system act as a network to scavenge and effectively prevent the accumulation of toxic intermediates of molecular O_2 known as ROS, under both stressed and normal conditions (Ahsan *et al.,* 2003; Mourato *et al.,* 2012). ROS increases upon perceived stress which in turn activates these ROS-scavenging enzymes such as Superoxide dismutase (SOD), Ascorbate peroxidase (APX) and Catalases (CAT). SOD scavenges O_2^- and converts it to H_2O_2 (Cheeseman, 2007). H_2O_2 is then scavenged and removed by APX in chloroplasts and the cytosol and by CAT in peroxisomes and glyoxisomes (Ahsan *et al.,* 2010; Held *et al.*, 2011; Mourato *et al.,* 2012). Ahuja et al. (2015) describes oxidative stress as a result of the rate of ROS accumulation surpassing the rate the of ROS sequestration. From this notion it is fair to infer that upon ROS accumulation, either rapidly or over a period of time, in plants whose antioxidant enzymes cannot metabolise all the present ROS, that cell damage will occur.

It is known that when ROS increases so will the their scavenging antioxidant enzymes, in defence against oxidative damage (Mourato *et al.,* 2012) and therefore increased ROS and antioxidants are indicators of oxidative stress in plants. However, increased antioxidant activity might not always be effective in mitigating the inevitable damage caused by ROS (Ahsan *et al.,* 2003), since the damage to tissue and vital cellular

structures may be irreversible despite ROS being removed (Ahsan *et al.,* 2003; Hossain *et al.,* 2011).

Therefore, it was imperative to determine the accumulation of ROS as well as the activity of their scavenging enzymes; SOD, APX and CAT, in *B. rapa* L. seedlings under Zr-stress. More importantly, this investigation was themed with observing the mitigation of HM-induced oxidative stress (caused by Zr), through the induced modulation of the antioxidant response with exogenous Methylglyoxal (MG). MG is a ubiquitous by-product of aerobic metabolism whose accumulation in cells is inevitable (Hossain *et al.,* 2009). Its formation occurs by a number of pathways such as glycolysis and photorespiration. Moreover, more critical is MG's involvement as a reducing agent where it reacts directly with molecular O_2 to yield reactive oxygen intermediate; O_2^- (Saito *et al.,* 2011; Hossain *et al.,* 2011). MG has been suggested as a signalling molecule in plants (Kaur *et al.,* 2014). However, in literature it has only been extensively researched at high concentrations, often exceeding normal basal levels (Yadav *et al.,* 2005; Kaur *et al.,* 2014). Because the accumulation is ever-present in plants under different stresses, the possible signalling function of MG under normal and HM-stressed conditions was considered in this study.

HM-stress is a known inducer of free radical accumulation in plants (Ahsan *et al.,* 2003; Yadav *et al.,* 2005; Hossain *et al.,* 2011). These ROS are known to cause considerable damage especially if not metabolised efficiently, and will subsequently lead to serious metabolic constraints (Ahsan *et al.,* 2003; Cheeseman, 2007; Igbal *et al.,* 2010). As a result the antioxidant defence in plants is also induced under HM conditions with SOD,

74

APX and CAT having increased activity under both HM- and oxidative stress conditions as reported (Wang *et al.,* 2000; Hossain *et al.,* 2011).

Many metal ions viz. Zn, Ni, Cu and Mn, although toxic at elevated levels are required by plants for their biochemical processes, either directly or indirectly (Hossain *et al.,* 2011). These metals accumulate mostly in the cytosol of plants and once a level of toxicity is reached, vital functions like transpiration and carbohydrate metabolism initiates responses to oxidative stress which can be very harmful to plants (Yhang and Chu, 2011). Sequestration of metal ions prevents the formation of toxic •OH; formed by Haber-Weiss and Fenton reactions (Ahsan *et al.,* 2003). Therefore, SOD, APX and CAT are crucial, not only in preventing the formation of more toxic radicals such as •OH and H_2O_2, but also in preventing extensive oxidative damage by inhibiting the accumulation of ROS (Wang *et al.,* 2000; Ahsan *et al.,* 2003; Iqbal *et al.,* 2010). If MG confers improved tolerance to harmful HMs in plants, through activation of antioxidants, then a crucial role of MG negating oxidative damage will be established.

75

4.2. RESULTS

4.2.1.   MITIGATION OF ROS ACUMULATION IN ZR-STRESSED *B. RAPA* L. SEEDLINGS UPON THE ADDITION OF MG

The generation of ROS is a consequence of biotic and abiotic stresses (Rabanni and Thornally, 2012). The latter includes salt stress and drought, along with other forms such as extreme cold, toxic chemicals and heavy metals (Yadav *et al.,* 2005). All of which require the interference of antioxidants to counteract and alleviate the damage caused by ROS (Yadav *et al.,* 2005). In a similar fashion to salinity stress, HMs induce a host of stress and damage in plants (Yadav *et al.,* 2005; Hossain *et al.,* 2009) that may be studied and quantified by performing assays and antioxidant activity tests.

In cells superoxide (O_2^-) is one of the first active oxygen species to occur from the reduction of molecular O_2 (Pua and Douglas, 2004; Hung and Kao, 2007). Oxygen upon its journey to being converted to H_2O requires the interaction of four e^-, however this reduction occurs in a stepwise manner that sees only a single e^- acting upon it (O_2), and so through this single-electron reduction, mainly via the mitochondrial ETC, O_2^- is formed (Ahsan *et al.,* 2003). O_2^- is a harmful and reactive molecule known to cause oxidative damage observable by increased lipid peroxidation and increased physiological stress (Ahsan *et al.,* 2003; Yadav *et al.,* 2005; Held *et al.,* 2012; Ahuja *et al.,* 2015). This may lead to damage of cell components and a loss of chlorophyll molecules which eventually affects photosynthesis (Igbal *et al.,* 2010; Hortensteiner and Krautler, 2011).

In *B. rapa* L. the O_2^- content (figure 4.2.1) was observed in seedlings subjected to 6 μM MG (Methylglyoxal), 1 mM Zr (Zirconium) and MG+Zr-treatments with which a clear discrepancy was observed between seedlings subjected to Zr and MG+Zr respectively. The control set exhibited the lowest O_2^- levels at 21 nmol.g^{-1} fresh weight, whereas the Zr-stressed seedlings showed an increase of 1-fold. This was the highest observed O_2^- content. The O_2^- levels in MG-treated seedlings showed a 39% increase from control *B. rapa* L. seedlings. Furthermore O_2^- content between MG-treated seedlings and those subjected to MG+Zr-treatments was unchanged. Despite the presence of Zr-stress, MG+Zr-treated seedlings had a 25% decrease in O_2^- compared to Zr-treated seedlings.
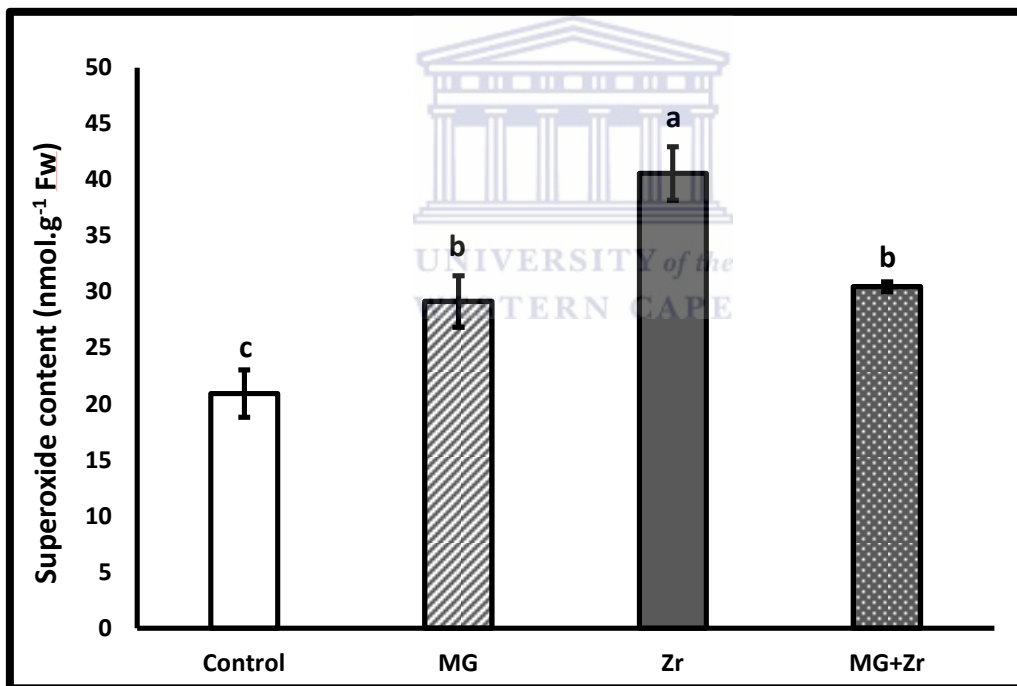
Figure 4.2.1. The effect of Methylglyoxal on Superoxide content in *B. rapa* L. seedlings under Zr stress. Superoxide occurs as a by-product of normal aerobic metabolism when O_2 is reduced. Superoxide content was determined in *B. rapa* L. seedlings after exposure to Zr stress and MG treatments respectively. The different letters indicate a significant change across means at P< 0.05 (DMRT). Values are means ± S.E (N=3).

The production of hydroxyl radicals (•OH) in cells is an unavoidable fate that occurs when hydrogen peroxide (H_2O_2) is not efficiently metabolised and traverses cell membranes to react with metal ions such as Fe^{2+} and Cu^+ (Iqbal *et al.,* 2010). Once this

occurs H_2O_2 is readily converted to •OH (Ahsan *et al.,* 2003). Another means to form •OH is from Haber-Weiss reactions where the interaction of H_2O_2 with O_2^- results in the formation of •OH, H_2O and O_2 (Ahsan *et al.,* 2003; Iqbal *et al.,* 2010). •OH cannot diffuse across the membrane therefore it is important that it is metabolised to render it less reactive. If •OH accumulates it is capable of reacting with any biomolecule (Mittler, 2002; Ahsan *et al.,* 2003). •OH is known to have an avid affinity for PUFAs and it readily interacts with RNA, proteins and nucleotides that results in fragmented DNA, base changes and single- and double-strand breaks (Hossain *et al.,* 2011; Held *et al.,* 2012). Given the extent of damage to cells that accumulated •OH is capable of causing, it was important to determine the degree to which Zr-stress would affect •OH levels in *B. rapa* L. seedlings (figure 4.2.2) of this study, and also to establish the impact that exogenous MG elicits on the production of this radical when under Zr-stress.

Noteworthy was the effect of MG-supplementation at a low dose (6 µM) in Zr-stressed seedlings (figure 4.2.2). There was no change, or signs of stress, in MG-treated seedlings as the •OH levels were nearly identical to the control-treated seedlings. Zr-treated seedlings however, were negatively affected by a 29% increase in •OH content. In MG+Zr-treated seedlings •OH levels were not only lower than in Zr-stressed seedlings, by 23.75%, but also lower than in unstressed control sample set by 5.75%.
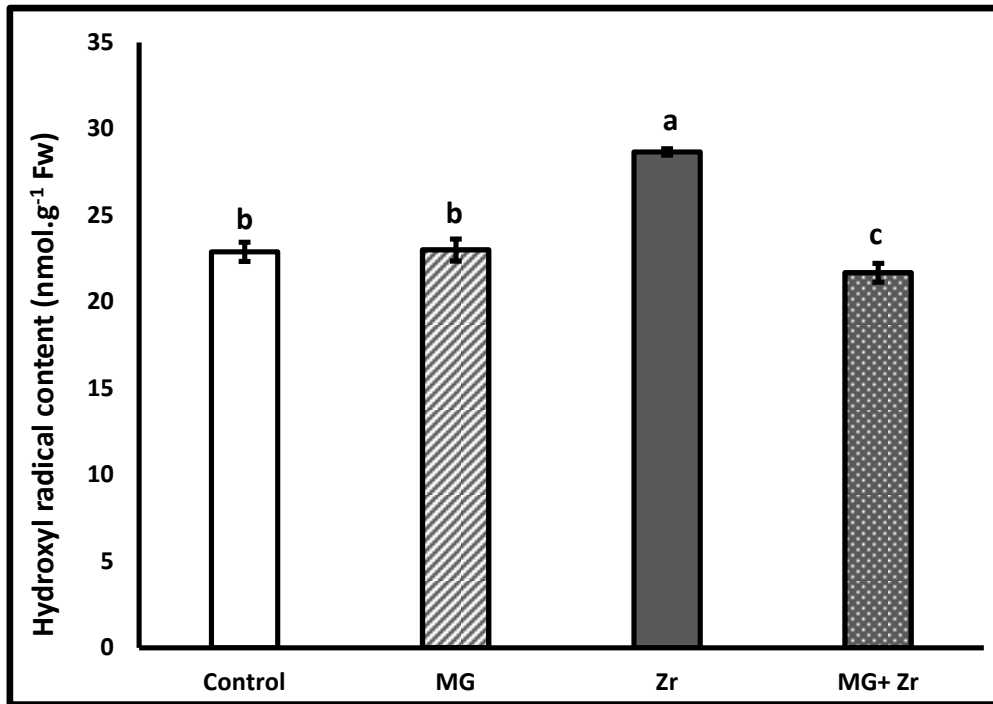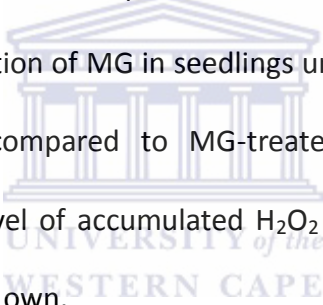
Figure 4.2.2. The effect of Methylglyoxal on Hydroxyl radical content in *B. rapa* L. seedlings in response to Zr stress. •OH is readily produced from commonly occurring species viz. H_2O_2 and O_2^- via Fenton-like reaction and due to its high reactivity it is known to cause damage in plant cells. •OH-content was determined in *B. rapa* L. seedlings 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).

Hydrogen peroxide (H_2O_2), the least reactive and damaging of the active oxygen species, is known to be involved in cell-signalling, but has also been reported to alleviate ROS-induced oxidative damage in plants (Singla-Pareek *et al.,* 2006; Hossain *et al.,* 2012). This has been reported to occur through its involvement in environmental responses and plant development (Singla-Pareek *et al.,* 2006; Cheeseman, 2007; Hernandez *et al.,* 2010; Mhamdi *et al.,* 2010). However this is achievable only at maintained relatively low to moderate levels of H_2O_2 (Cheeseman, 2007; Hossain *et al.,* 2011; 2012). Hossain et al. (2012) states that maintaining H_2O_2 levels at low concentrations may reinforce the defence mechanisms in plants, it may also stimulate plant development by modulation of gene expression and signalling pathways. In

young stage *B. juncea* L. H_2O_2 content under normal conditions was observed to be at 50 - 60 nmol.g-1 fresh weight in young stage plants (Iqbal *et al.,* 2010).

H_2O_2 content (figure 4.2.3) in *B. rapa* L. seedlings was measured in this study and as with the previous two ROS assays; O_2^- (figure 4.2.1) and •OH (figure 4.2.2), MG-, Zr- and MG+Zr-treatments were administered. Seedlings subjected to exogenous MG had accumulated lower levels of H_2O_2 than in Zr-stressed seedlings. A 51% increase in H_2O_2 levels were measured in MG-treated seedlings was observed compared to the control. A far more drastic change was noted in Zr-stressed seedlings with a 1.3 fold increase in accumulated H_2O_2. In MG+Zr-treated seedlings, despite experiencing apparent stress, H_2O_2 content decreased by 26% compared to Zr-treated seedlings by the mere presence of MG. Supplementation of MG in seedlings under Zr-stress resulted in a 21% and 84.5% increase when compared to MG-treated seedlings and the control respectively. A much lower level of accumulated H_2O_2 than the increase observed in seedlings treated with Zr on its own.

Figure 4.2.3. The effect of Methylglyoxal on H_2O_2 content in *B. rapa* L. seedlings in response to Zr stress. Under perceived stress plants' changes in H_2O_2 levels serves as an indicator of stress. This ROS has a relatively low reactivity however relatively long half-life and often leads to oxidative damage. H_2O_2 content was determined in *B. rapa* L. seedlings 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).

4.2.2. MODULATION OF PLANTS' DEFENCE SYSTEM OBSERVED IN ZR-STRESSED *B. RAPA* L. SEEDLINGS WITH EXOGENOUS ADDITION OF MG

SOD is the primary defence against O_2^-, catalysing its dismutation to H_2O_2 and O_2 (table 1.1); two far less reactive and harmful species (Mourato *et al.,* 2012). Especially the ROS by-product; H_2O_2 which although has a longer viability within the cell, is far less reactive with cell components, and thus less damaging (Broadbent *et al.,* 1995*;* Held *et al.*, 2012). For its catalytic function SODs make use of metal cofactors viz. Cu/Zn, Mn and Fe (Buettner *et al.,* 1998; Mourato *et al.,* 2012). Cu/Zn SOD is localized in the

cytosol and in chloroplasts; Mn-SOD occurs in the mitochondria and peroxisomes and Fe-SODs are localized in chloroplasts (Bradbent *et al.,* 1995; Almeselmani *et al.,* 2006). SODs' widespread occurrence in cells is indicative of a vital role in cell viability and against the effects of ROS.

In *B. rapa* L. seedlings SOD activity was determined (figure 4.2.4), in MG-, Zr- and MG+Zr-treated seedlings, as a measure of its unit of activity per gram of fresh weight. Where one unit (U) is designated as the amount of enzyme activity required to catalyse the conversion of one micro mole of substrate in one minute (NC-IUB, 1979). In seedlings subjected to MG- and MG+Zr-treatments an increase of 15.05 U.g^{-1} fresh weight was observed. No change was observed between seedlings subjected to MG and MG+Zr. SOD's response in MG+Zr-treated seedlings was reduced by 2% in comparison with Zr-treated seedlings. Zr-treated seedlings exhibited the highest SOD activity in response to HM stress with a change of 14.45%, or an increase of 18 U of SOD activity.gram^{-1} of fresh weight in comparison with the control.
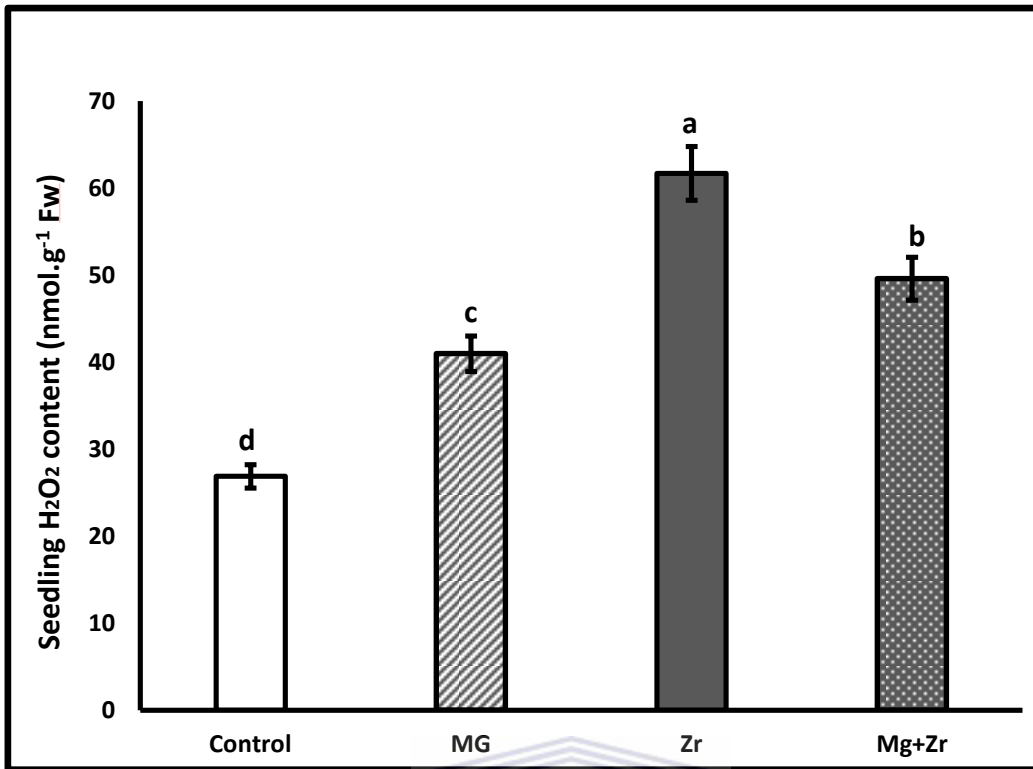
Figure 4.2.4. The effect of Methylglyoxal on SOD activity in *B. rapa* L. seedlings in response to Zr stress. Under perceived stress in plants SOD activity has been known to increase. SOD scavenges harmful radicals and decreases oxidative damage in plants. SOD activity was determined in *B. rapa* L. seedlings 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).

APX is described as one of the most important ROS-scavenging antioxidants. Like CAT, APX is involved in plants' defence mechanisms whereby it detoxifies H_2O_2 to dehydroascorbate and H_2O using ascorbate (table 1.1). APX has a much higher affinity for H_2O_2 than CATs and along with its ubiquitous localization in the cell; cytosol, mitochondria, apoplast, peroxisome and chloroplasts, its significance in the cell's biological function and metabolic efficiency cannot be mistaken (Mittler, 2003; Mourato *et al.,* 2012; Hossain *et al.,* 2011). In literature APX has been observed to increase upon HM stress by up to 4-fold (Iqbal *et al.,* 2010; Hossain *et al.,* 2011). Because the increase in APX is indicative of oxidative stress, and because of the crucial role of APX in H_2O_2 detoxification, its levels in *B. rapa* L. seedlings were investigated.

B. rapa L. seedlings were subjected to MG, Zr, and MG+Zr treatments. In seedlings subjected to MG a 1.45-fold increase was observed (figure 4.2.5). Zr-treated seedlings

83

showed the same response in APX activity. However, in MG+Zr-treated seedlings the combination of stress treatment had a drastic impact on APX activity with a 2.5-fold increase being observed.
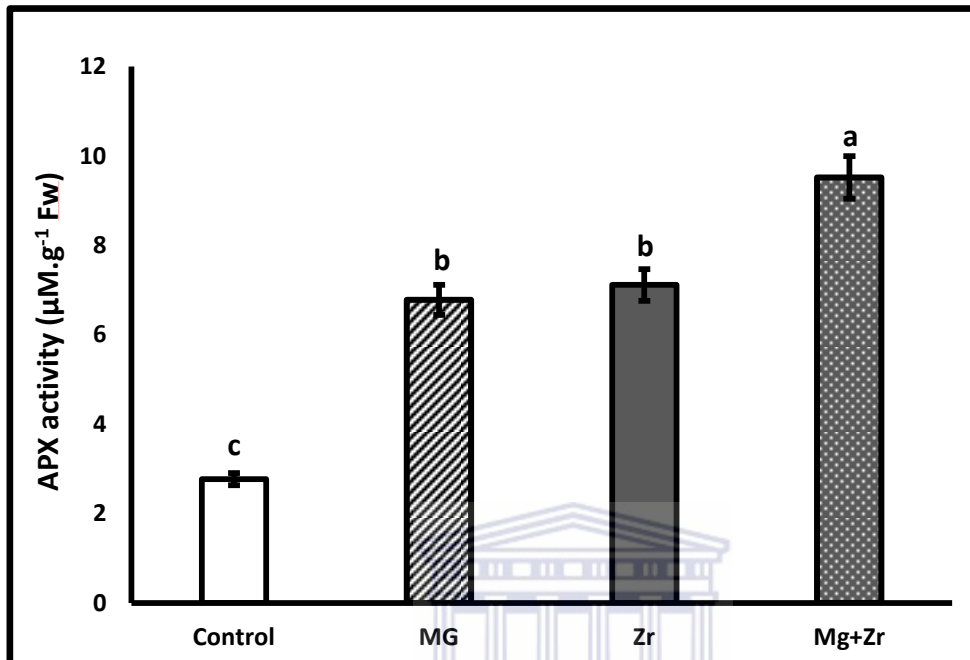
Figure 4.2.5: The effect of Methylglyoxal on Ascorbate peroxide activity in *B. rapa* L. seedlings in response to Zr stress. In plants APX is foremost in scavenging and metabolising H_2O_2 and is therefore crucial in protecting plants from the damaging effects of H_2O_2 and thus oxidative stress. APX activity was determined in *B. rapa* L. seedlings 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).

Catalases (CATs), whose action was first observed in plant and animal tissues in 1818 (Scandalios *et al.,* 1997), serve an important function in plants where H_2O_2 is their major substrates. They scavenge any excess H_2O_2 directly and catalyse the conversion of H_2O_2 molecules (table 1.1) to H_2O and molecular O_2 (Scandalios *et al.,* 1997; Mhamdi *et al.,* 2010). By this mechanism CATs spare the cell from any further damage by removing the excess H_2O_2 that APX have not. Given the localization of CATs is in several cellular components, its biological function and importance is of significance similar to the case of SODs.

84

In this study CAT activity (figure 4.2.6) was determined, by the rate at which H_2O_2 was oxidised in *B. rapa* L. seedlings subjected to MG-, Zr-stress and MG+Zr-stress treatments. *B. rapa* L. seedlings treated with MG exhibited a 4.2% decrease in H_2O_2 oxidation when compared to the control. In the Zr-treated seedlings a decrease in the rate of CAT activity by 12.7% was observed when compared to control. In MG+Zr-treated seedlings, with the lowest CAT activity, an even lower rate of H_2O_2 oxidation was observed compared to the control with a 40.7% decrease.
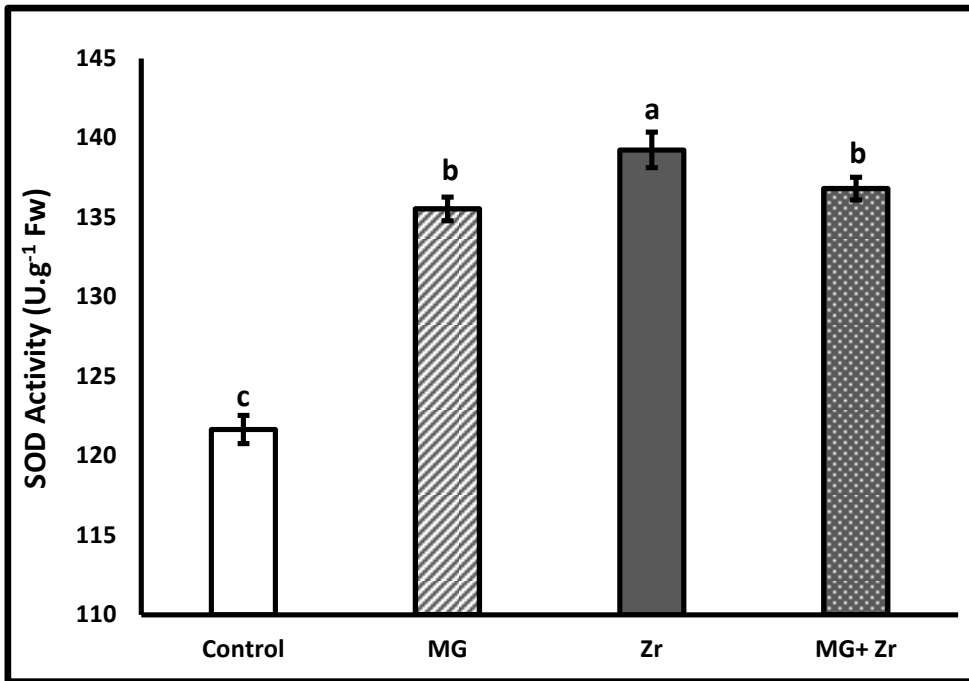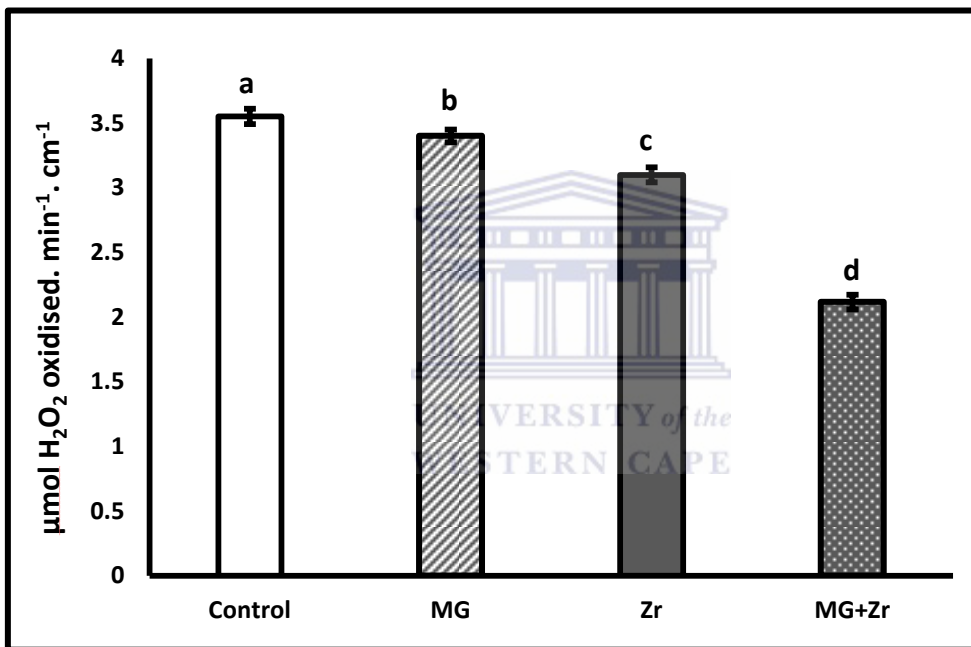

Figure 4.2.6. The effect of Methylglyoxal on Catalase activity in *B. rapa* L. seedlings in response to Zr stress. In plants CAT is known to scavenge excess H_2O_2 and is therefore crucial in alleviating plant stress. Catalase activity was determined in *B. rapa* L. seedlings after exposure to Zr stress 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).

4.2.3. INCREASED MG AND GLY-I REGULATION IN RESPONSE TO INCREASED MG LEVELS IN *B. RAPA* L. SEEDLINGS UNDER ZR-STRESS

Methylglyoxal; MG, is a known cytotoxic oxo-aldehyde capable of causing irreversible damage in plant cells by reducing molecular O_2 to form activated oxygen species known as ROS (Firestone *et al.,* 2007; Hossain *et al.,* 2009; Saito *et al.,* 2011). MG, a by-product of cellular respiration and glycolysis, is also known as 2-oxo-aldehyde and is often thought of as a ROS due to its ability to interfere with biomolecules such as proteins, RNA and DNA (Himo and Siegbahn, 2001; Hossain *et al.,* 2009). MG forms advanced glycation ends (AGE's) in glycation events that render the affected sugar and lipid molecules irreversibly changed (Rabanni and Thornally, 2012). It is this attack on lipids and sugars that sees macromolecules such as DNA and proteins, irrevocably damaged and degraded by MG (Freire *et al.,* 2003; Hossain *et al.,* 2012). Furthermore MG at high concentrations can inhibit cell proliferation, it interacts with *R, C* and *K* residues to spur the degradation of proteins, it interacts with guanyl nucleotides in DNA and MG is capable of inactivating antioxidants upon its accumulation (Yadav *et al.,* 2005). MG occurs as a result of the spontaneous degradation of triose phosphates, Glyceraldehyde 3-phosphate, DHAP and during catabolic metabolism (Skipsey *et al.,* 2000; Yadav *et al.,* 2012). Through its interactions and interference with important cellular components that lead to cell death, inhibition of cell growth and the accumulation of ROS, MG causes notable damage that render its effective metabolism an essential occurrence in cell physiology (Skipsey *et al.,* 2000; Himo and Siegbahn, 2001; Hossain *et al.,* 2012).

MG content (figure 4.2.7) was determined in *B. rapa* L. seedlings subjected to MG-, Zr-stress and MG+Zr-stress treatments. In control seedlings 22 $\mu mol.g^{-1}$ fresh weight was measured, a concentration that conforms to the cellular levels of MG in plants under normal conditions, at a range of 30 – 75 µM; species-dependent (Yadav *et al.,* 2005). In *B. rapa* L. seedlings treated with 6 µM MG a 4-fold increase was observed. In Zr-stressed seedlings a 10.5-fold increase was observed compared to the control and in MG+Zr-treated seedlings the addition of exogenous MG resulted in a 0.4-fold decrease in accumulated MG compared to Zr-treated seedlings.
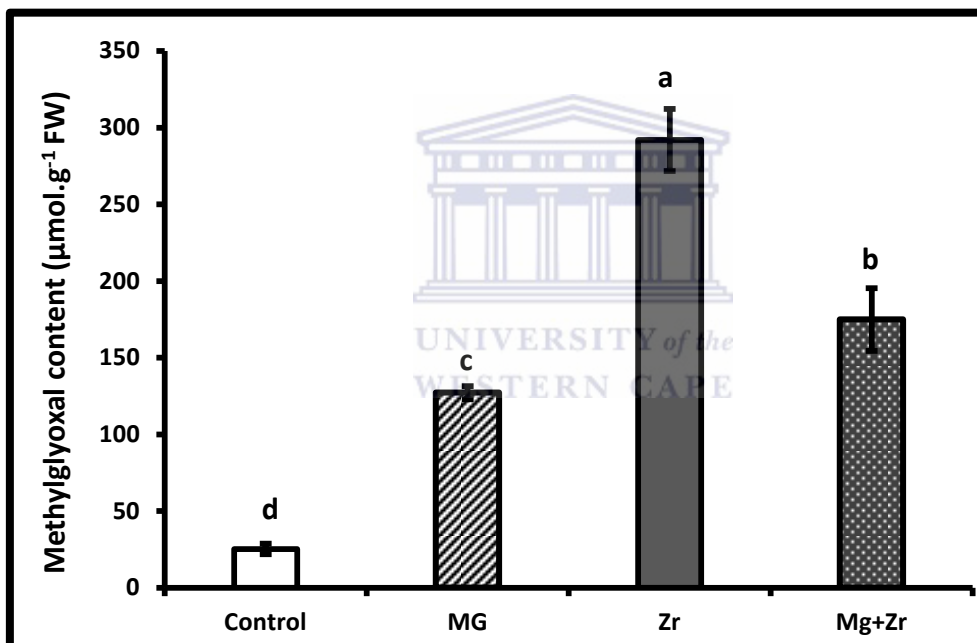
Figure 4.2.7. The effect of Methylglyoxal application on MG content in *B. rapa* L. seedlings in response to Zr stress. MG is cytotoxic at elevated levels in plants and it also occurs as a by-product of normal metabolism ubiquitously. MG content was determined in *B. rapa* L. seedlings after exposure 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).

Plants' defence system comprises of a host of networks, working alongside and sometimes in tandem. Their attempts serve to metabolise accumulated ROS and harmful metabolic by-products, and subsequently to mitigate ROS' detrimental biochemical effects (Cartea *et al.,* 2003; Mourato *et al.,* 2012; Held *et al.,* 2012).

87

Glyoxalase I, otherwise referred to as Gly-I (in plants), forms part of a two-enzyme system; the glyoxalase system, wherein it catalyses the conversion of MG to S-D-lactoyl glutathione, using GSH as its cofactor (Hossain *et al.,* 2007; Veena and Reddy *et al.,* 2006). MG is its primary substrate, others include glyoxals and other α-oxoaldehydes (Firestone *et al.,* 2007). In nature Lactoyl-glutathione lyase is equally as ubiquitous as MG (Himo and Siegban, 2001), this points to its vital function of detoxifying MG upon its accumulation. Active Gly-I has been detected during vital stages of the cell cycle such as tissue maturation, embryogenesis and cell death, further attesting to the significance of Gly-I and the glyoxalase system in cell development (Deswal and Sopory, 1991; Yadav *et al.,* 2005; Thornally and Rabanni, 2011). Gly-I has also been reported to alleviate oxidative stress in various organisms including plants (Singla-Pareek *et al.,* 2006; Kaur *et al.,* 2014). There its response to HM-stress and MG accumulation was investigated.
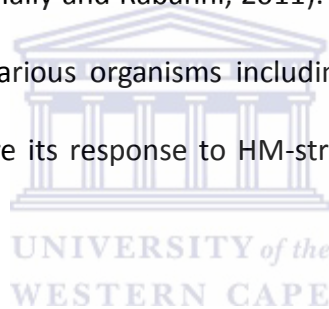
In our study Gly-I activity (figure 4.2.8) was determined in MG-, Zr- and MG+Zr-treated *B. rapa* L. seedlings. Additionally Gly-I expression levels (figure 4.2.9) under Zr-stress and MG treatments were investigated by semi-quantitative analysis. This was implemented in order to establish on two separate levels; biochemically and molecularly, the impact of Zr on Gly-I activity. Furthermore, the next objective was to determine the effects of exogenous MG on Gly-I expression and activity, as well as observing a change, if any, when the two stressors (MG and Zr) were applied in tandem in *B. rapa* L. seedlings.

Gly-I activity (figure 4.2.8) in MG-treated seedlings exhibited a nearly 2-fold increase in comparison with the control. In Zr-treated seedlings a 2.5-fold increase in Gly-I activity was measured and in MG+Zr-stressed seedlings, the highest Gly-I activity was observed with a 3-fold increase. Additionally Gly-I expression (figure 4.2.9.C) was observed in MG-, Zr- and MG+Zr-treated *B. rapa* L. seedlings.

Since Gly-I is the primary scavenger of MG in cells the expectation was for Gly-I expression levels to increase with the increased accumulation of MG, since both increased MG and oxidative stress has been observed here. However, this could only be known once the semi-quantitative (figure 4.2.9) and subsequent densitometry (figure 4.2.10) and statistical analyses were performed. With the exogenously applied MG (6 µM), Gly-I expression increased to over 1.6-fold. An even greater increase of over 2-fold was observed in Zr-treated seedlings, and greater yet was the Gly-I expression observed in MG+Zr-treated seedlings with a 2.5-fold increase observed. Gly-I expression in *B. rapa* L. seedlings increased not only with increased MG levels, but also upon Zr-exposure. The highest measure of Gly-I expression being found in seedlings subjected to MG+Zr.
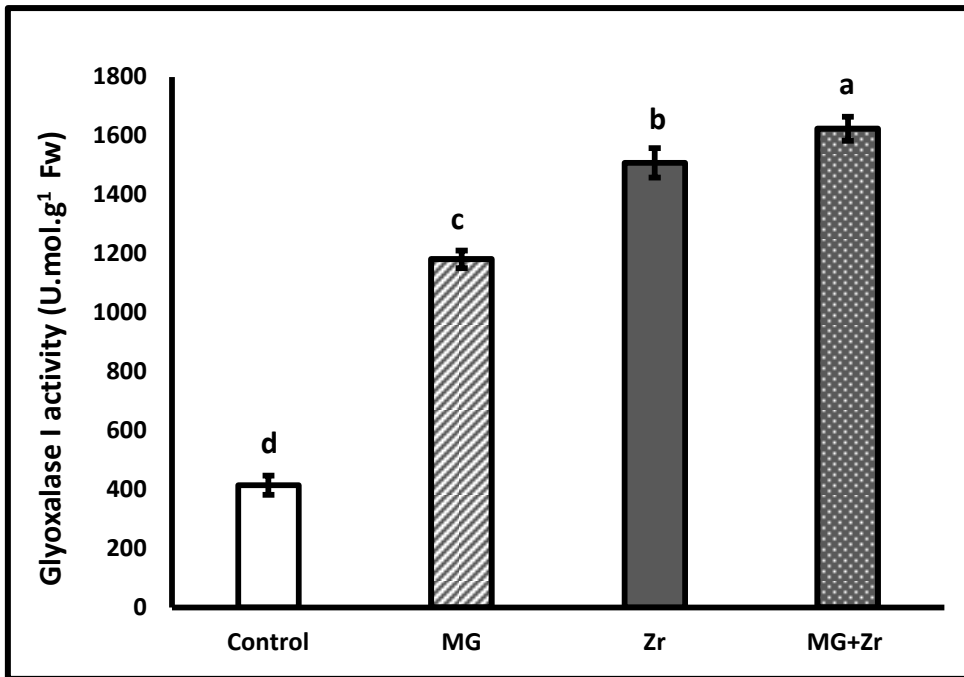
Figure 4.2.8. The effect of Methylglyoxal on Glyoxalase I activity in *B. rapa* L. seedlings in response to Zr stress. Glyoxalase I of the glyoxalase system occurs in all plants. It has proliferative properties but more importantly it effectively scavenges and metabolises >90% of MG produced as well as other oxo-aldehydes. Gly-I activity was determined in *B. rapa* L. seedlings 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).
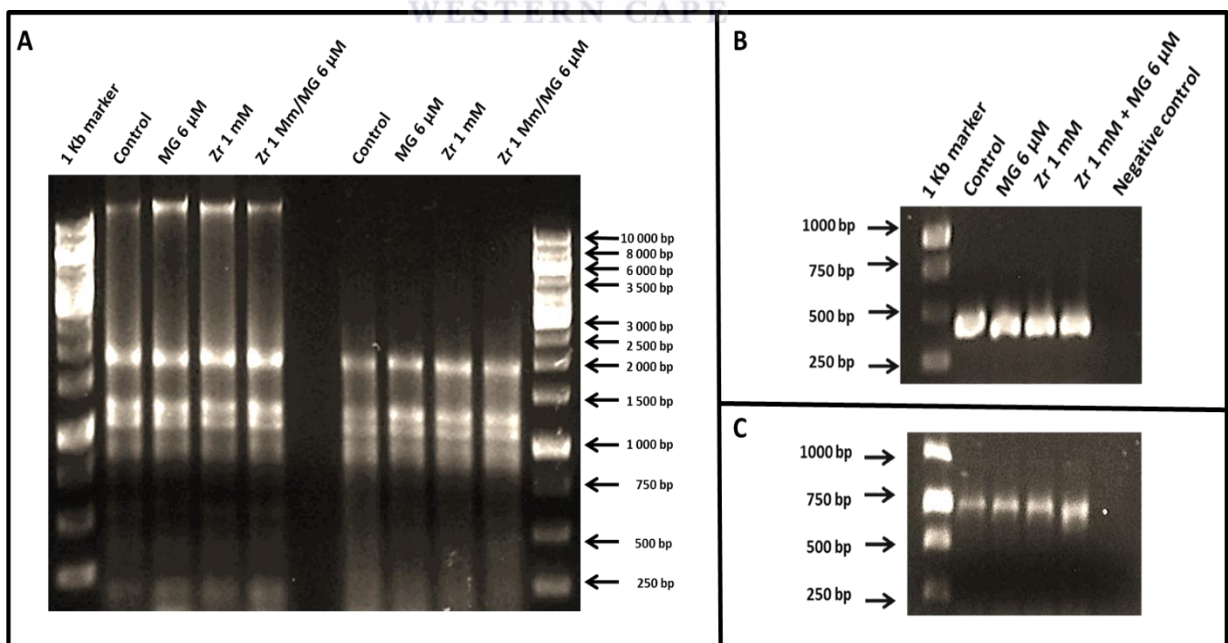
Figure 4.2.9. RNA extractions and Gly-I amplification of *B. rapa* L. seedlings in response to Zr stress and MG treatments respectively. (A) Shows the RNA extracted from seedlings and RNA after DNase-treatment. Lanes 1 - 4 represent 200 ng RNA before DNase-treatment and lanes 5 - 8 represent 200 ng RNA after DNase-treatment. RNA appear alongside a 1 kb molecular weight ladder. **(B)** Shows *BrUBQ* amplification, **housekeeping gene**, **(C)** and *BrGlyI* amplification**.**
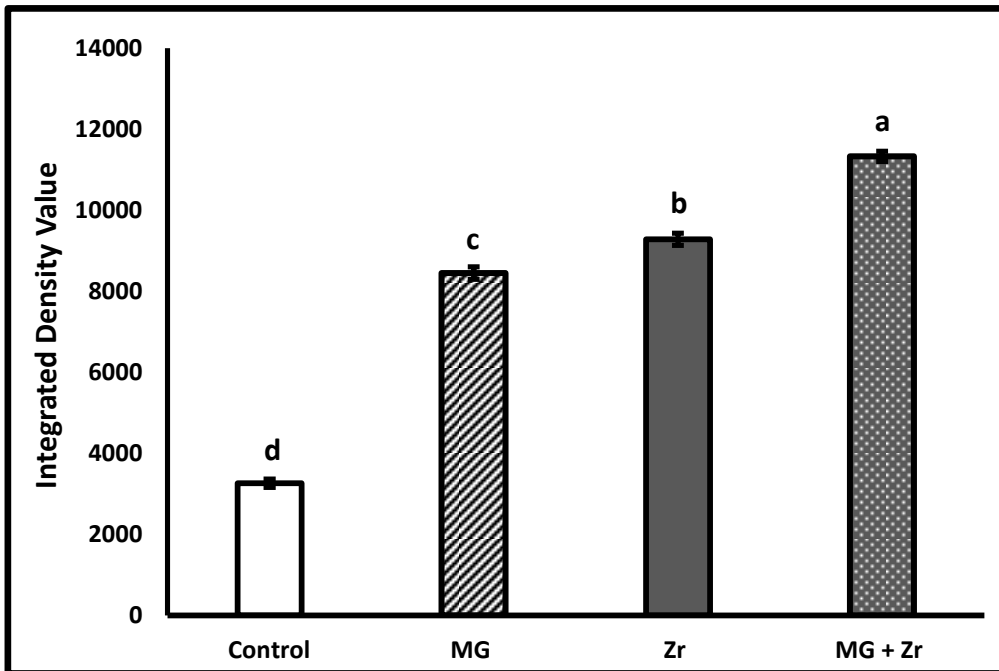
Figure 4.2.10: Densitometry analysis of Glyoxalase I mRNA transcript expression in *B. rapa* L. seedlings subjected to Zr, Methylglyoxal and MG+Zr. BrGly-I expression levels were determined in *B. rapa* L. seedlings after exposure to Zr stress and MG treatments respectively. BrGly-I mRNA transcript bands were subjected to densitometry analysis. Different letters indicate a significant change across means at P< 0.05 (DMRT). Values are means ± S.E (N=3).

Zr being used broadly; in metal, refractory, jewellery, nuclear reactors and catalysis industry, has enjoyed favour with its demand at ever-increasing levels due to its anti-corrosion and high heat resistance properties (Ferrrand *et al.,* Fodor *et al.,*2005). This growing popularity for Zr increases the risk of environmental pollution (Fodor *et al.,* 2005). Zr readily forms stable complexes with compounds and minerals already present in soil which then increases the phytoavailability of this highly insoluble element (Ferrand *et al.,* 2006). Ferrand and Dumat (2006) have reported that Zr-absorption and desorption is highly influenced by soil components, plant age, organ as well as the environmental pH. Due to the scant evidence of Zr toxicity, it is hard to establish toxic levels. However, Zr is known to interact with DNA and other biomolecules and so it was important to establish the Zr content under normal

conditions and then investigate the uptake rate in the presence of added MG in Zr-stressed *B. rapa* L. seedlings in this study.

B. rapa L. seedlings in this study were treated with Zr, MG and MG+Zr. The Zr concentration was determined by Inductively Coupled Plasma – Atomic Emission Spectrometry, or ICP-OES, and correlated to mg.kg^{-1} dry weight. Seedlings exposed to MG had a slightly higher Zr uptake compared to the control. However, in seedlings treated with Zr an 81-fold increase in Zr concentration was observed. Seedlings subjected to a combination of MG and Zr exhibited a severe decrease in measured Zr; a decrease of 0.8-fold was observed compared to Zr-treated seedlings despite the same level of Zr exposure.

Table 4.1. Detection of Zr uptake in *B. rapa* L. seedlings exposed to exogenous MG and Zr (mg.kg^{-1} dry weight)

Element	Treatments			
	Control	6µM MG	1 mM Zr	MG+Zr
Zr	2.65 ± 0.03^{c}	2.86 ± 0.03^{d}	241.56 ± 0.02^{a}	54.18 ± 0.04^{b}

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

4.3. DISCUSSION

The generation of ROS occurs naturally as a consequence of cellular metabolism and due to environmental factors (Pua and Douglas, 2004; Thornally and Rabanni, 2011). Fortunately cells have defence mechanisms in place that attempt to metabolise ROS and in effect detoxify them (Mourato et $al.,$ 2012; Held et $al.,$ 2012). ROS are generated primarily from the electron transport chain (ETC) which is driven by mitochondria, and this occurs as a consequence of oxidative phosphorylation which yield the toxic by-products known collectively as ROS (Ahsan et $al.,$ 2003).

Typically in cells O_2 is metabolised to H_2O upon complete reduction with 4 e$^-$, but because O_2 is reduced univalently due to spin restrictions, several free radicals occur (Ahsan et $al.,$ 2003; Yadav et $al.,$ 2007). Superoxide anion; O_2^-, is thought to be the first of the active oxygen species. Once O_2^- has been detected, SOD activity increases and converts O_2^- quickly to yield the less reactive and less harmful hydrogen peroxide (H_2O_2) (Halliwell and Gutteridge, 1984). H_2O_2 on its own does not cause extensive damage (Ahsan et $al.,$ 2003), however upon its accumulation, H_2O_2 has been established as a significant factor in oxidative stress (Mourato et $al.,$ 2012).

H_2O_2 is capable of diffusing across the cell membrane, unlike free radicals, to form toxic and highly reactive •OH radicals upon contact with transition metal ions (Ahsan et $al.,$ 2003). •OH radical is the most reactive of the ROS, its generation occurs as a result of two commonly known reactions in cellular physiology and biochemistry; the Fenton reaction; where reduction of H_2O_2 occurs as it interacts with metal ions. Secondly, it is generated in the Haber-Weiss reaction where O_2^- and H_2O_2 react; H_2O_2 is reduced to yield H_2O and •OH (Ahsan et $al.,$ 2003; Mourato et $al.,$ 2012). •OH, is

93

capable of reacting with all kinds of biological macromolecules such as lipids, proteins, carbohydrates and nucleic acids and leads to both single- and double-strand breaks, base changes and conformational alterations in DNA (Ahsan *et al.,* 2010; Yadav *et al.,* 2005). Furthermore •OH can lead to formation of DNA-protein cross-links, protein fragmentation and lipid peroxidation (Ahsan *et al.,* 2003; Yadav *et al.,* 2007).

In this study the impact of a low dose of exogenous MG was investigated in *B. rapa* L. seedlings under normal and in Zr-stressed conditions.

Superoxide; O_2^-, was the first ROS whose accumulation was measured in this study (figure 4.2.1). O_2^- is known to be produced as a result of mitochondrial respiration when molecular O_2 is reduced (Hossain *et al.,* 2011). It is also reported to increase in plants under abiotic stress (Hossain *et al.,* 2011). *B.* rapa L. seedlings were subjected to both MG and Zr respectively and exhibited increased O_2^-. MG and Zr both known to induce oxidative stress and having increased the O_2^- content therefore indicates oxidative stress. However, Zr-treated seedlings exhibited a more drastic increase in O_2^- levels by 1-fold compared to MG-treated *B. rapa* L. seedlings with a 0.4-fold increase. These findings are consistent with literature on MG-induced ROS accumulation (Hossain *et al.,* 2011; 2012), as well as increased ROS in plants under HM-stress (Yadav *et al.,* 2005; Singla-Pareek *et al.,* 2006; Hossain *et al.,* 2009). Most interesting was the observation of the level of O_2^- accumulation in seedlings under Zr-stress with added MG (MG+Zr).

Not only did the low administered dose of MG cause O_2^- content to decrease, but it lowered the O_2^- content to the same level as seedlings treated with MG only. This extreme inhibition of ROS formation whilst exposed to a known toxic element (Zr)

94

indicates why MG occurs ubiquitously at low molecular concentrations in plants. Furthermore it demonstrates biological importance of MG in defence against active oxygen species, and whose mechanistic reduction of O_2 to yield toxic ROS and subsequent activation of antioxidants points to a signalling role of MG.

Hydrogen peroxide (H_2O_2) was investigated in *B.* rapa L. seedlings in this study. It is known as a toxic ROS at elevated levels and also conversely aids in plants' defence against abiotic stress at low to intermediate levels, thus serving a signalling function (Cheeseman, 2007). Therefore it was important to establish the H_2O_2 accumulation in response to MG and Zr respectively in *B. rapa* L. seedlings. Seedlings were subjected to MG, Zr and MG+Zr respectively, an increase was observed in MG and Zr-treated sets respectively. However the H_2O_2 content in Zr-treated seedlings was drastically higher than in MG-treated seedlings indicating the Zr-induced oxidative stress. Because the stress-alleviating properties of MG were investigated here as well, it was interesting to see that a lower accumulated H_2O_2 had occurred in MG-treated seedlings than in Zr-treated ones which indicate an oxidative response in the cell in a regulatory sense. H_2O_2 content increased when MG was administered and therefore we believe that the H_2O_2 levels were maintained in such a way that it enabled improved growth, development and the antioxidant response. This indicates possible signalling of MG through inciting the activation of antioxidants in plants by a slight enough increase in H_2O_2 concentration to circumvent cell damage. The literature also validates that slightly elevated H_2O_2 concentration aids in plants defence against oxidative stress (Hernandez *et al.,* 2010; Hossain *et al.,* 2011). In order to better determine the effects of exogenous MG to mitigate oxidative stress, it was applied along with Zr (MG+Zr) in *B. rapa* L. seedlings; a 84.5% increase in H_2O_2 was observed, however this was a

minimal increase in H_2O_2 content compared to Zr-treated seedlings with a 1.3-fold increase.

The effect of exogenous MG appears to be indirectly involved in reducing the accumulation of toxic ROS under stress conditions. The presence of Zr unmistakeably caused oxidative stress in seedlings given that H_2O_2 at high levels is harmful to plants, causing oxidative damage and is cytotoxic for prolonged periods (Cheeseman, 2007; Yadav *et al.,* 2005; Wang *et al.,* 2000; Hossain *et al.,* 2011). Because accumulated H_2O_2 can lead to more toxic active oxygen species the increase in •OH; and $O_2^{-\prime}$ is expected. This means that despite an increase in the antioxidant response of *B. rapa* L. seedlings, that the sequestration rate of ROS was lower than the rate of accumulation especially with this high an increase in H_2O_2.

Hydroxyl radicals; •OH, have obtained their notoriety by being highly reactive in cells, interacting with every kind of macromolecule viz. lipids, DNA, RNA, protein and carbohydrates. There is an interesting correlation between the impact of accumulated •OH and increased MG in cells. MG is a metabolite whose presence leads to irrevocable AGEs being formed (Hossain *et al.,* 2011). MG attacks PUFAs, lipids and acts directly on O_2, reducing it to O_2^-. Similarly •OH readily interacts with biomolecules causing irreversible damage in DNA, proteins and RNA (Ahsan *et al.,* 2003; Hossain *et al.,* 2011). If upon MG-treatment, and the resulting accumulation of MG in cells, the •OH levels drastically increases with the MG increase, it may have devastating consequences that impact plants in a plethora of ways.

In this study •OH content was determined in *B. rapa* L. seedlings subjected to MG, Zr, and MG+Zr treatments. The •OH content observed was quite unexpected with MG-

96

treated seedlings and the control exhibiting no difference. Given the assumption of MG-induced •OH accumulation due to an increase in other ROS this finding defies the expectation. This was beneficial for the seedlings especially knowing the toxicity that is normally observed in plants under oxidative stress. Because •OH is formed from essential metal ions interacting with the infiltrating H_2O_2, it is inevitable in its accumulation even under normal conditions. Therefore, upon excessive O_2 reduction, either when cellular respiration is hindered or perturbed, or as a result of MG reducing O_2 to O_2^- and the subsequent formation of •OH, the conversion of H_2O_2 to •OH could have only been prevented by APX and CAT activity, to restrict the excessive accumulation of H_2O_2. Because of the cell's attempts to destroy as much H_2O_2 as possible, it can be inferred that increased •OH would indicate drastic metabolic and oxidative distress.
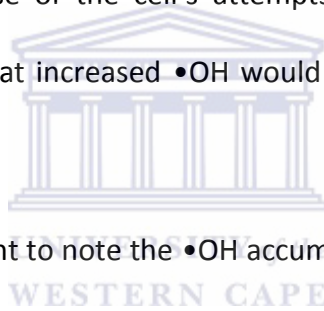
For this reason it was important to note the •OH accumulation in response to Zr-stress in *B. rapa* L. seedlings. With 30% increase in •OH content when exposed to Zr, this is one of the most telling findings and evidence implicating Zr as an inducer of serious oxidative stress and certain damage to cells. Given the increase noted when *B. rapa* L. was subjected to Zr, it was crucial to observe the indication of oxidative stress by •OH accumulation in MG+Zr-treated seedlings. Remarkably the •OH accumulation in MG+Zr-treated seedlings does not indicate zero oxidative stress with a reduced •OH content, but it suggests that the sequestration rate of •OH and •OH-precursors such as H_2O_2 was improved beyond that of *B. rapa* L. seedlings under normal conditions too. This, of all the ROS in this study negates MG as only being harmful to plants, and it further attests to the signalling and stress-mitigating effects of MG despite the evident stress that Zr imposes.

97

It is known that in plants where higher levels of activity in antioxidant system is observed, be it induced or constitutive, that a greater resistance to oxidative damage will occur (Hernandez *et al.,* 2010). In this study, the effects of exogenous MG on antioxidant enzymes; SOD, CAT and APX, were investigated. *B. rapa* L. seedlings were exposed to MG, Zr and MG+Zr treatments respectively in an attempt to observe the modulating effects of MG to the oxidative system in normal and Zr-stressed plants.

SOD is believed to be the most important of the ROS-scavenging enzymes, because it directly acts upon O_2^- in cells. SOD activity increases as soon as O_2^- is detected in the cell and without sufficient SOD activity O_2^- accumulation would increase (Ahsan *et al.,* 2003). O_2^- whose main targets are lipid molecules and particularly PUFAs cause lipid peroxidation and results in deteriorated membrane integrity and membrane leakage (Ahsan *et al.,* 2003; Hossain *et al.,* 2011; Mourato *et al.,* 2012).

Given the enormous task of SODs in the cell (metabolising O_2^- directly, as it is frequently formed by the unavoidable univalent reduction of O_2), it was important to observe the change in SOD activity, given the known toxicity of both MG and Zr. Seedlings under Zr-stress exhibited the highest increase in SOD activity by 15%, clearly indicating the accumulation of O_2^- and thus indicative of oxidative stress. Furthermore the SOD activity observed in MG-treated and in MG+Zr-treated seedlings exhibited no change and had increased by 10% compared to seedlings under normal conditions. The increased SOD activity observed, illustrates that increased MG levels induces SOD in defence against cellular damage. This finding was reasonably unexpected, but points to the alleviation of oxidative stress due to less O_2^- accumulating in MG- and MG+Zr-treated seedlings than the Zr-treated set. This is supported by the decrease in O_2^-

content in MG+Zr-treated seedlings also having no change compared to MG-treated seedlings.

APX in plants form an essential part of the defence network against ROS-induced oxidative damage (Ahsan *et al.,* 2003; Held *et al,,* 2011). APX is the first response to the accumulation of H_2O_2 in cells and regulates the levels of H_2O_2 by maintaining its low levels. It also has a higher affinity for H_2O_2 than CAT. APX acts with the use of ascorbate to neutralise H_2O_2 to $2H_2O$ and dehydroascorbate (DHA) (Blokhina *et al.,* 2003). The level of involvement in plants defence against oxidative stress will determine the extent of H_2O_2 accumulation which can be observed by a measure of increased APX activity.

B. rapa L. seedlings were exposed to Zr, MG and MG+Zr. In seedlings subjected to Zr and MG respectively a significant increase of 1.45-fold, was observed compared to seedlings under normal conditions. Because no change was observed between seedlings treated with the individual stressors this is indicative that Zr causes H_2O_2 levels to increase as in figure 4.2.3 and this increased H_2O_2 causes increased oxidative stress, and the subsequent formation toxic O_2 intermediates if not scavenged effectively (Ahsan *et al.,* 2003). Similarly, MG-treated seedlings showed the same significant increase in APX activity, it indicates that the accumulation of MG results in a rapid increase in the activation of APX. However, the H_2O_2 content in MG-treated seedlings were markedly lower than in Zr-treated seedlings. This allows two inferences: Firstly, that a swift increase in APX activation led to far more H_2O_2 being scavenged and thus a much lower H_2O_2 content (figure 4.2.3) was observed in MG-treated seedlings. Secondly, Zr interacts with H_2O_2 molecules or other biomolecules

that then interfered with the effective scavenging of H_2O_2, since a far greater H_2O_2 accumulation (figure 4.2.3) was observed in Zr-treated seedlings. Remarkably, the APX activity observed in seedlings exposed to a combination of the treatments; MG+Zr, showed a drastic increase in enzymatic activity of 2.5-fold. This falls in line with not only the extreme reduction in H_2O_2 accumulation in MG+Zr- seedlings, but also corroborated the low level of CAT activity observed in MG+Zr-treated seedlings in this study, since CAT scavenges excess H_2O_2 in the cell.

Catalases (CAT), the first antioxidant to be described in 1818 (Scandalios *et al.,* 1998) are a group of enzymes whose only substrate is H_2O_2 and serve only to scavenge and metabolise excess H_2O_2. The mechanism of action (table 1.1) oxidises its cofactors; Fe or Mn, by forming adducts with H_2O_2 to yield H_2O and O_2 (Cheeseman, 2007; Held *et al.,* 2011; Hossain *et al.,* 2011). There are several enzymes and antioxidant molecules involved with the detoxification of H_2O_2 due to its tendency to traverse the cell membrane whereupon it forms highly toxic •OH radicals, when it reacts with biometals such as Cu (Ahsan *et al.,* 2003). The activity of CAT, reduced or induced, was therefore important in establishing the extent of oxidative stress as well as the efficacy of APX in metabolising H_2O_2, in response to Zr and exogenous MG.

In *B. rapa* L. seedlings subjected to MG-treatment and Zr respectively both exhibited a decrease in CAT activity, this agrees with the increase in APX activity observed for these two experimental sets. Again congruent to APX activity, there was a significant decrease in CAT activity in MG+Zr-treated seedlings with a 0.4-fold decrease. This corresponds with the extreme increase in APX activity and a reduced H_2O_2 content in MG+Zr-treated seedlings which results in less excess H_2O_2 for CAT to metabolise

MG which forms adducts with many biological compounds like sugars and lipids, causes considerable damage in plants (Hossain *et al.,* 2009; Kaur *et al.,* 2014). All of the mechanisms and pathways involved with MG synthesis is yet undiscovered (Yadav *et al.,* 2005; Kaur *et al.,* 2014). However it is known that MG reacts with biological molecules like DNA and proteins (Kaur *et al.,* 2014) and is capable of modifying these biomolecules and therefore MG (at high concentrations) impedes growth and development in plants (Yadav *et al.,* 2005). MG is sometimes regarded as a ROS due to its harmful impacts directly on biomolecules (Hossain *et al.,* 2011). MG has been observed to increase in plants upon a plethora of stresses including salinity, drought, cold stress and heavy metals (Hossain *et al.,* 2009).

Given the noticeable accumulation of MG in plants under different stresses in literature (Hossain *et al.,* 2009; 2011; 2012; Yadav, 2005), MG was therefore investigated in order to establish its role as a signalling molecule to induce growth and mitigate oxidative stress (Hossain *et al.,* 2011; Kaur *et al.,* 2014). The effects of exogenously applied MG at a low dose (6 µM), 1 mM Zr and MG+Zr in *B. rapa* L. seedlings was investigated. Control Seedlings exhibited MG levels; 25.25 µmol.g^{-1} fresh weight, that conforms to the normal basal MG levels in plants; 30 - 75 µM (Yadav *et al.,* 2005; Hossain *et al.,* 2011). In seedlings subjected to MG treatments a 4-fold increase in accumulated MG was observed. This is 69% higher than the reported basal levels of MG in plants under normal conditions. Because MG is known to cause oxidative stress (Yadav *et al.,* 2005a; Kaur *et al.* 2014) an increase in the formation of MG was expected given the accumulated ROS observed in figures (4.2.1 – 4.2.3). However since oxidative stress leads to increased MG accumulation and with the further addition of MG, the

level of MG observed in this study is justifiable. Hossain et al. (2009) also observed an increase in MG accumulation in response to MG treatments in pumpkins seedlings.

An upward change in MG was also noted in this study in Zr-treated seedlings where a sharp increase; 10.55-fold, in MG content was observed. Hossain and colleagues (2009) observed an increase in MG after Cd was administered in pumpkin seedlings. Fodor et al. (2005) similarly investigated the effects of Zr in wheat which exhibited higher accumulated MG. Thus affirming that the presence of HMs in plants leads to an induction of MG accumulation which then leads to an oxidative response since MG is known to increase ROS and is directly involved with formation of O_2^- (Saito et $al.,$ 2011). MG+Zr-treated seedlings in this study, though also showing an increase in accumulated MG this showed an improvement in maintaining low cellular levels of MG with a 40% lower MG content than observed in Zr-treated seedlings. This drastic decrease in MG content similar to MG found in MG-treated $B.$ $rapa$ L. seedlings shows that the exogenous MG reduced the MG accumulation in cells under HM stress and this can be directly associated with diminished oxidative stress observed across seedlings treated with MG+Zr where the ROS content (figure 4.2.1 4.2.3) decreased compared to Zr-treated seedlings.

MG is able to indirectly incite mitigation of oxidative damage in plants by the following mechanisms: (1) MG on its own elicits a stress response and causes the accumulation of ROS, this is justifiable since MG is capable of directly converting molecular O_2 to O_2^-. This then incites SOD activity because SOD activity immediately increases upon the formation of O_2^-. The dismutation of O_2^- leads to the formation of H_2O_2 and thus we see an increase in H_2O_2 upon increased MG levels, this suggestion is validated by a

similar trend in O_2^- (figure 4.2.1) and H_2O_2 (figure 4.2.3). (2) Exogenous MG increased MG levels in seedlings (figure 4.2.7) to an optimal level of MG (figure 4.2.7) for H_2O_2 accumulation to increase (figure 4.2.3) but remain low enough (41 nmol.g^{-1} fresh weight) to avoid oxidative stress and damage, and high enough (127 µmol.g^{-1} fresh weight) to activate the antioxidant response (figure 4.2.3 – 4.2.6) that allows the increased protection against any oxidative stress before ROS is generated. This preinduction of antioxidant activity results in the alleviation of plant cells under stress amidst toxic levels of Zr.

There needs to be a finely balanced rate of removal of accumulated MG to evade its toxic overproduction in order to prevent the cytotoxic effects that leads to cell damage. This ROS homeostasis, to incite the antioxidant response in plants is a brilliant strategy that relies heavily on Gly-I.

Glyoxalase I plays a major role in the detoxification of MG, a metabolite that has been implicated in many human diseases like diabetes and various types of cancers (Raju *et al.,* 1998; Himo *et al.,*2001 Korybalska *et al.,*2003; Hossain *et al .,* 2011), MG is equally as ubiquitous in nature as Gly-I, being studied in animals, yeasts and microorganisms and has been discovered in higher plants by Yadav et al (2005), which further highlights its importance in maintaining cell viability.

The glyoxalase system is the primary catabolic pathway of MG in eukaryotes, it comprises two co-functioning enzymes; glyoxlase I and glyoxalase II, the first of which isomerises the non-enzymatically formed hemithioactal from MG and GSH; S-D-lactoylglutathione. Gly-II then catalyses the hydrolysis reaction to D-lactate and puts GSH back into the system (Yadav *et al.,* 2005). Because Gly-I has been upregulated in

response to HM stress in plants (Lin *et al,.*2001; Hossain *et al.,*2009; Hasanuzzaman and Fujita, 2011) and in response to accumulated MG in plants (Hossain *et al.,* 2009), its activity and gene expression was investigated in this study.

The seedlings in this study were subjected to MG-, Zr- and MG+Zr- treatments in order to observe an elicited response of antioxidants in the presence of exogenous MG while under Zr-stress. In *B. rapa* L. seedlings the Gly-I response under Zr-stress showed a significant increase for Gly-I activity (figure 4.2.8) and its corresponding encoding mRNA transcript (figure 4.2.9 and 4.2.10). Because Gly-I has been up-regulated in response to Zr-treatments it is evident that cellular damage and stress was experienced given the increased toxic ROS content (figure 4.2.1 - 4.2.3) and the subsequent increases in antioxidant activity (figure 4.2.4, 4.2.6; figure 4.2.8) and MG content (figure 4.2.7). Both ROS and antioxidant increases have been reported as indicators of oxidative stress and cell damage (Kaur *et al.,* 2014). MG-treated seedlings exhibited Gly-I activity nearly 2-fold increase (figure 4.2.8) and a 1.6-fold increase in Gly-I mRNA transcripts (figure 4.2.9). This observed Gly-I upregulation at transcript and protein level shows its efficient response in cells at the onset of MG accumulation. In MG+Zr-treated seedlings an even more significant activation of Gly-I was observed with a 2.5-fold and 1.85-fold increase in Gly-I enzyme activity and Gly-I gene expression respectively.

Although Gly-I is constitutively expressed under normal cellular conditions, this congruent increase between Gly-I expression and enzyme activity indicates that MG incites promoter activation which results in the increased expression observed in this study. Because Gly-I expression increased further with the two stressors combined, it

is probable that MG's mechanism of action when mitigating ROS-induced stress occurs directly through Gly-I, where it maintains low levels of MG which allowed the accumulation of ROS to occur at a lower rate which then also prevented SOD, CAT and APX from increasing too drastically. Determining the mechanism of MG-regulation is the most crucial aspect in elucidating the role of MG in alleviation of oxidative damage in plants.

Gly-I increases in response to oxidative stress in plants (Esparto *et al.,* 1995; Veena and Reddy, 1999; Yadav *et al.,* 2005) and upon MG accumulation (Yadav *et al.,* 2005), and because both instances were observed in this study it can be stated that oxidative stress occurred in the presence of Zr. MG content was also observed in *B. rapa* L. seedlings subjected to the same stresses. H_2O_2 had a 1.5-fold increase in tobacco plants subjected to Zn, similarly H_2O_2 levels increased by 1-fold in response to Zn exposure (Chaudri *et al.* 2000). Hossain and colleagues (2009) investigated the Gly-I and MG response in 30 day old pumpkin seedlings subjected to a range of stresses including MG, HM and salt stress. Gly-I expression increased significantly in response to HM stress in pumpkin seedlings subjected to 1 mM Cd (Hossain *et al.,* 2009). Similarly a marked increase in Gly-I activity was reported with a 1.34-fold and 1.19-fold increase in pumpkin seedlings subjected to MG and HM-stress respectively. In tomato and *Brassica*, Gly-I levels were upregulated in response to HM, salt and water stress (Hossain *et al.,* 2009).

Due to the increasing demand for Zr-alloy and Zr-derived products in the metals, refractory and nuclear reactor industry (Ghosh *et al.,* 1992; Shahid *et al.,* 2014; Fodor *et al.,* 2005), increasing exposure to Zr will occur (Fodor *et al.,* 2005; Shahid *et al.,*

2014). Zr is ubiquitous in nature and sometimes found in higher concentrations than trace elements and metals like Cu. Zr is the 20th most abundant element (Fodor *et al.,* 2005) and as such it occurs naturally in soil and land sediments. However, because it has been reported to cause toxicity in plants it was important to establish not only its uptake in *B. rapa* L. seedlings as observed in these results (table 4.1), but also to determine possible mitigating effects of Zr-uptake through the application of MG.

In this study uptake of Zr from soil exposed to the respective treatments; MG, Zr, MG+Zr were measured. The seedlings treated with MG exhibited no change in Zr-uptake thus indicating that exogenous MG did not increase or accelerate Zr entering *B. rapa* L. seedlings. However in seedlings treated with Zr a substantial increase was observed. A high uptake of Zr was observed in seedlings treated with Zr where an 81-fold increase in Zr was measured. This indicated that less Zr is present in seedlings exposed to exogenous MG and that it hinders the uptake of Zr. This could occur by MG interfering with Zr-speciation by itself forming adducts with biomolecules and macromolecules. Given its low phytoavailabilty as reported in literature (Ghosh *et al.*, 1992 Ferrand *et al.,* 2006; Fodor *et al.,* 2005) the uptake rate of Zr observed here demonstrates that it readily forms complexes with soil components for uptake by root cells.

This substantial uptake should not be overlooked especially since evident signs of oxidative stress (figure 4.2.1 – 4.3 9) along with growth and developmental impediments (figure 3.2.1 – 3.2.6) were observed in Zr-stressed seedlings of this study. Most conspicuous was the extreme reduction of Zr-uptake in *B. rapa* L. seedlings treated with Zr plus MG, the added MG reduced the uptake of Zr, and it did so by a

large margin [where 241.56 mg.kg^{-1} was observed in Zr-treated seedlings vs. 54.18 mg.kg^{-1} in MG/Zr-treated seedlings (table 4.1)]. Although the same amount of Zr was administered in MG+Zr treated seedlings a striking 77% lower Zr uptake had occured. This finding suggests either a priming effect or an inhibitory effect of MG on root cells at which point MG may interact with them before Zr can form complexes thus enabling cells to avert the uptake of toxic Zr.

The presence of Zr in maize, barley and alfalfa was reported by Sanzharova and Aleksakhin (1982) who also found that the concentration of this metal had increased with a soil moisture increase by as much as three times and that Zr was absorbed mostly as a complex hydrous oxide. Ryzhenko and colleagues (2008) also observed that absorption and mobility in soils is greatly influence by pH levels. Because the literature on Zr is sparse and the demand for it is increasing, it leads to increasing nuclear fall-out, mining wastes and pollution. This clearly demonstrates a need for more research geared towards understanding and establishing the extent of (Zr) toxicity and toxic levels in plants. Trace amounts of Zr measured in pea and tomato plants show a low uptake of Zr in aerial parts of these plants (Fodor *et al.,* 2005). However *B. rapa* L. are known HM hyperaccumulators capable of faster root-to-shoot translocation of HMs thus allowing a higher Zr-uptake observed in the leaves and shoots of seedlings of this study.

CONCLUSION AND FUTURE PROSPECTIVES

Stark evidence of the signalling capability of MG was observed due to the apparent MG-induced activation of ROS-detoxifying molecules viz. APX (figure 4.2.5), CAT (figure 4.2.6) and SOD (figure 4.2.4), and Gly-I (figure 4.2.9). It is the responsibility of Gly-I to maintain it at moderate levels in the cell. MG levels seem to have been maintained at a concentration that is optimal for improved growth (figure 3.2.1), chlorophyll synthesis (table 3.1) and antioxidant activation (figure 4.2.4 - 4.2.6) in MG-treated seedlings. Moreover the alleviating effects of MG was exhibited in Zr-stressed seedlings supplemented with MG (MG+Zr-treatments). All of the ROS and antioxidant activity measured have implicated MG in reducing the level of toxic ROS (O_2^-, $\bullet OH$, H_2O_2) in figure (4.2.1 - 4.2.3), and even MG levels (figure 4.2.7). This increased detoxification of ROS and MG was made possible through an increase in the antioxidant response, for all the antioxidant molecules investigated in this study and Gly-I, as there was an increase compared to the control. SOD, CAT and APX levels (figure 4.2.4 – 4.2.6) in MG+Zr-treated seedlings were significantly lower than in Zr-stressed seedlings, the only exception being Gly-I activity and expression (figure 4.2.8 and 4.2.9c) because it had to be upregulated to restrict MG accumulation.

This evidence could indicate the pathway involved with the modulation of ROS-induced oxidative stress in plants. This also implicates MG indirectly in activation of the oxidative response by inciting Gly-I activity (figure 4.2.8), and thus conferring a mitigation of ROS accumulation. MG can therefore be described as a signalling molecule since it activates Gly-I upon its production. Gly-I then metabolises the MG accumulated in the cell, but not before the occurrence of MG-induced ROS formation

(figure 3.2.1 – 3.2.3). The accumulation of MG also incites the activation of other antioxidants, which occurs as MG initially increases ROS levels; this phenomenon can be described as a direct early-onset activation of antioxidants, or pre-inductive activation of ROS-mitigating enzymes.

Increased Gly-I levels (figure 4.2.9C and figure 4.2.10), where activation was also induced by MG, was observed by the extent of MG detoxification in *B. rapa* L. seedlings treated with MG+Zr, compared to Zr-treated seedlings. A reduction in MG content was observed in MG-Zr-treated seedlings, a decrease similar to the MG accumulation in MG-treated seedlings. Since MG has been reported as a reducing agent, the significantly lower MG content in MG+Zr-treated seedlings, as a direct consequence of the extremely elevated Gly-I activity observed, indicates that less O_2 was reduced to O_2^-. This decrease in O_2^- levels also resulted in a lower H_2O_2 accumulation, and thus •OH content had decreased as well. •OH accumulation was lower in Zr-treated seedlings as well as the control which again validates MG as a stress-alleviating molecule.

The clear evidence of MG's capability to modulate the antioxidant response through activation of Gly-I activity, is a breakthrough since previously MG's notoriety stemmed from its toxicity and cytotoxic effects in plant, animal and bacterial cells. ROS are not all bad, they are involved in different biological processes that are important such as apoptosis, biotic and abiotic stress responses in the cell, and includes signalling and maintaining a state of redox balance only when unfavourable conditions do not persist (Mourato *et al.,* 2012). For this reason it is crucial that plants maintain a balanced state

of ROS sequestration vs. accumulation. Through these findings MG is now known to facilitate this defence mechanism in *B. rapa* L.

With the new role of MG elucidated in stress-signalling, further proteomics and molecular research will give further insights to the role of MG. Molecular cloning of Gly-I may shed light on its mechanism of action against oxidative damage under normal and stressed conditions and further support the findings of this study. Since MG is intrinsically a part of a number of biochemical pathways, a metabolomics approach may be useful in finding other pathways involved with MG's ability to mitigate oxidative stress and improve growth, development and seed germination. This can be studied in both stress and normal cellular conditions. Facilitating growth and inhibiting HM-uptake in contaminated soils may vastly improve the current state of food security and sustainability both locally and globally. This may be accomplished if MG is applied to crop plants, given the extreme inhibition of Zr-uptake by MG shown in this study.
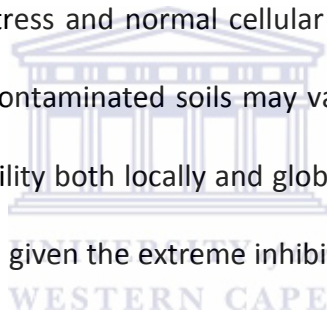
CITATIONS

Adler, L. S., Wikler, K., Wyndham, F. S., Linder, C. R. and Schmitt, J. (1993) "Potential for persistence of genes escaped from canola: germination cues in crop, wild, and crop-wild hybrid Brassica rapa" *Functional Ecology* Vol. 7(6): 736-745

Aebi, H. (1984) "Catalase *in-vitro*" *Methods Enzymol* Vol. 105: 121-126

Ahsan, H., Ali, R. and Ali, A. (2003) "Oxygen free radicals and systemic autoimmunity" *Clin Exp Immunol* Vol. 131: 398–404

Ahuja, N., Singh, H. P., Batish, D. R. and Kholi, R. K. (2015) "Eugenol-inhibited root growth in *Avena fatua* involves ROS-mediated oxidative damage" *Pesticide Biochemistry and Physiology* Vol. 118: 64–70

Almeselmani, M., Deshmukh, P. S., Sairam, R. K, Kushwaha, S. R. and Singh, T. P. (2006) "Protective role of antioxidant enzymes under high temperature stress" *Plant Science* Vol. 171: 382-388

Batish, D. R., Lavanya, K., Singh, H. P., and Kohli, R. K. (2007) "Phenolic allelochemicals released by Chenopodium murale affect the growth, nodulation and macromolecule content in chickpea and pea" *Plant Growth Regul* Vol. 51:119–128

Broadbent, P., Creissen, G.P., Kular, B., Wellburn, A.R. and Mullineaux, P. (1995) "Oxidative stress responses in transgenic tobacco containing altered levels of glutathione reductase activity" *Plant J*. Vol. 8: 247–255.

Buettner, G. R. (1998) "Antioxidant enzymes" *Antioxidants Enzymes and Function* P1-20

Cardoza, V. and Stewart, C. N. (2004) "Invited Review: Brassica Biotechnology: Progress in Cellular and Molecular Biology" *In Vitro Cell Development Biology-Plants* Vol. 40: 542-551

Cartea, M. E., Francisco, M., Soengas, P. and Velasco, P. (2011) "Phenolic Compounds in *Brassica* Vegetables" *Molecules* Vol. 16: 251-280

Celucia, S. C., de la Peña, R. C. and Villa, N. O. (2009) "Genetic Characterization of *Brassica rapa chinensis* L., *B. rapa parachinensis*, and *B. oleracea alboglabra*

Using Simple Sequence Repeat Markers" *Philippine Journal of Science* VOL. 138 (2): 141-152

Chakravarty, T. N. and Sopory, S. K. (1998) "Blue light stimulation of cell proliferation and glyoxalase I activity in callus cultures of *Amaranthus paniculatus*" *Plant Sci.* Vol. 139: 63-69

Chaudri, A.M., Allain, C.M., Barbosa-Jefferson, V.L., Nicholson, F.A., Chambers, B.J. and McGrath, S.P. (2000) "A study of the impacts of Zn and Cu on two rhizobial species in soils of a long term field experiment" *Plant Soil* vol. 22: 167–179.

Cheng, F., Mandakova, T., Wu, J., Xia, Q., Lysak, M. A. and Wang, X. (2013) "Deciphering the Diploid Ancestral Genome of the Mesohexaploid *Brassica rapa*" *American society of biological research* Vol. doi: http://dx.doi.org/10.1105/tpc.113.110486

Chow, J. C., Watson, J. G., Ashbaugh, L. L. and Magliano, K. L. C. N. (2003) "Similarities and differences in PM10 chemical source profiles for geological dust from the San Joaquin Valley, California." *Atmospheric Environment* vol. 37:1317–1340

Deswal, R. and Sopory, S. K. (1991) "Purification and partial characterization of glyoxalase I from a higher plant Brassica juncea" *Federation of European and Biochemical Societies* Vol. 282(2): 277-280

Drew, M. C. and Lynch, J. M. (1980) "Soil anaerobiosis, microorganisms and root function" *Annu Rev Phytopathol* Vol.18: 37–66

Espartero, J., Sanchez-Aguayo, I. and Pardo, J. M. (1995) "Molecular characterization of glyoxlase-I from a higher plant; up-regulation by stress" *Plant Molecular Biology* Vol. 29: 1223 – 1233

Ferrand, E., Dumat, C., Leclerc-Cessac, E. and Benedetti, M. F. (2006) "Phytoavailability of zirconium in relation to its initial added form and soil characteristics" *Plant Soil* Vol. 287:313–325

Fodor, M., Hegedus, A. and Stefanovits-Banyai, E. (2005) "Zirconium induced physiological alterations in wheat seedlings" *Biologia Plantarum* Vol. 49: 633–636

Fones, H., Davis, C. A. R., Rico, A., Fang, F., Smith, J. A. C. and Preston, G. M. (2010) "Metal Hyperaccumulation Armors Plants against Disease" *PLos Pathogens* Vol. 6(9): e1001093. doi:10.1371/journal.ppat.1001093

Freire, A. P, Ferreira, A. G. R. and Cordeiro, C. (2003) "Anti-glycation defences in yeast*" Biochem. Soc. Trans.* Vol.31: 1409–1412.

Ghosh, S., Sharma, A. and Talukder, G. (1992) "Zirconium: An abnormal trace element in biology" *Biological Trace Element Research* Vol. 35: 247-271

Goud, P. B and Kachole, M. S. (2011) *"*Effect of hydrogen peroxide on protein content and catalase activity in *Cajanus cajan* (l.) Millsp. Leaves" *International Journal of Current Research* Vol. 33 (4): 268-271

Guala, S. D., Vega, F. A. and Covelo, E. F. (2010) "The dynamics of plant-soil interactions in heavy metal contaminated soil." *Ecological modelling* Vol. 221: 1148 – 1152

Halliwell, B. and Gutteridge, J.M.C. (1984) "Oxygen-derived species and herbicide action".

Halliwell, B. H. and Gutteridge, J. M. C. (2007). *Free Radicals in Biology and Medicine*, 4th edn. Oxford: Oxford University Press.

Halliwell, B., Gutteridge, J. M., Aruoma, O. I. (1987) "The deoxyribose method: a sample test tube assay for determination of rate constants for reaction of hydroxyl radicals" *Anal. Biochme.* Vol. 165: 215-219

Held, P. (2012) "An introduction to reactive oxygen species: Measurement of ROS in cells" *Biotek Application guide* BioTek Instruments, Inc., P.O. Box 998, Highland Park, Winooski, Vermont 05404-0998 USA.

Himo, F. and Siegbahn, P. E. M. (2001) "Catalytic Mechanism of Glyoxalase I: A Theoretical Study" *J. Am. Chem. Soc.* Vol**.** 123: 10280-10289

113

Hörtensteiner, S. and Krautler, B. (2011) "Chlorophyll breakdown in higher plants" *Biochim Biophys Acta*. Vol. 1807: 977-988.

Hossain, A., Teixeira da Silva, J., A. and Fujita, M (2011) "Glyoxalase System and Reactive Oxygen Species Detoxification System in Plant Abiotic Stress Response and Tolerance: An Intimate Relationship, (Abiotic Stress in Plants - Mechanisms and Adaptations)" *InTech* ISBN: 978-953-307-394-1

Hossain, M. A. and Fujita, M. (2013) "Hydrogen Peroxide Priming Stimulates Drought Tolerance in Mustard (Brassica juncea L.) Seedlings" *Plant Gene and Trait* 4. doi: 10.5376/pgt.2013.04.0020.

Hossain, M. A., Hossain, M. Z. and 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 Vol. 3(2):53-64

Hossain, M. A., Piyatida, P., Teixeira da Silva,J. A. and Fujita, M. (2012) "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," Journal of Botany, Vol. 2012: 37 pages,. doi:10.1155/2012/872875

Hung, K.T., Kao, CH. (2007) "Hydrogen peroxide, calcium, and leaf senescence in rice" *Crop, Environment & Bioinformatics* Vol. 4: 145-150

Iqbal, N., Masood, A., Nazar, R., Syeed, S. and Khan, N. A. (2010) "Photosynthesis, Growth and Antioxidant Metabolism in Mustard (*Brassica juncea* L.) Cultivars Differing in Cadmium Tolerance" *Agricultural Sciences in China* Vol. 9(4): 519-527 doi: 10.1016/S1671-2927(09)60125-5

Jackson, M. B., Davies, D. D. and Lambers, H. (1991) "Plant Life under Oxygen Deprivation. Ecology" *Physiology and Biochemistry*, SPB Academic Publishing bv, The Netherlands. 326

Jones, D. L. (1998) "Organic acids in the rhizosphere – a critical review" *Plant Soil* Vol. 205:25–44

Kaminski, P., Koim-Puchowska, B., Puchowsi, P., Jerzak, L., Wieloch, M. and Bombolewska, K. (2012) "Enzymatic Antioxidant Responses of Plants in Saline Anthropogenic Environments" *Plant Science* dx.doi.org/10.5772/51149

Kaur, C., Singla-Pareek, S. L. and Sopory, S. K. (2014) "Glyoxalase and Methylglyoxal as Biomarkers for Plant Stress Tolerance" *Critical Reviews in Plant Sciences* Vol. 33(6): 429-456.

King, G. J. (2005) *"Brassica*: Harvesting the Genome, Diversity and Products. The multinational *brassica* genome project" Vol. 1 ed. 10*, Rothamsted Research*

Klechkovsky, V. M. and Guliakin, I. V. (1950) "Behavior of trace amounts of strontium, caesium, ruthernium and zirconium in soils and plants according to the date of investigations with radioisotops of these elements. USSR Ministry of Agriculture, Moscow, USSE.

Kocsy, G., Galiba, G. and Brunold, C. (2001) "Role of glutathione in adaptation and signalling during chilling and cold acclimation in plants" *Physiol. Plant.* Vol. 113: 158–164.

Korybalska, K., Wisniewska–Elnur, J., Trómin´ska, J., Jörres, A., Borowicz, A. B. and Witowski, J. (2003) "The role of the glyoxalase pathway in reducing mesothelial toxicity of glucose degradation products" *Peritoneal Dialysis International* Vol. 26: 259–265

Kumar, V., and Yadav, S. K. (2009) "Proline and betaine provide protection to antioxidant and methylglyoxal detoxification systems during cold stress in Camellia sinensis (L.) O. Kuntze" *Acta Physiol*. *Plant.*Vol. 31: 261–269.

Lin, F., Xu, J., Shi, J., Li, H., and Li, B. (2010) "Molecular cloning and characterization of a novel glyoxalase I gene TaGly-I in wheat (Triticum aestivum L.)" *Mol. Biol. Rep.* Vol. 37: 729–735.

Masarovicova, E. and Kralova, K. (2012) "Plant-heavy metal interaction: phytoremediation, biofortification and nanoparticles" *Advances in plant physiology aspects.*Dr. Giuseppe Montanaro (Ed.) ISBN: 978-953-51-0557-2, InTech doi: 10.5772/33722

115

Mhamdi, A., Queval, G., Chaouch, S., Vanderauwera, S., Van Breusegem, F. and Noctor, G. (2010) "Catalase function in plants: a focus on Arabidopsis mutants as stress-mimic models" *Journal of Experimental Botany* Pg: 1-24 doi:10.1093/jxb/erq282

Miller, T. E. and Schemske, D. W. (1990) "An Experimental Study of Competitive Performance in Brassica rapa (Cruciferae)" *American Journal of Botany*, Vol. 77(8): 993-998

Mourato, M., Reis, R. and Martins, L. L. (2012) "Characterization of Plant Antioxidative System in Response to Abiotic Stresses: A Focus on Heavy Metal Toxicity", in Montanaro, G. and Dichio, B., Advances in Selected Plant Physiology Aspects, Janeza Trdine 9, 51000 Rijeka, Croatia: Intech, 23-44

Mussury, M. S. and Fernandes, W. D. (2000) "Studies of the Floral Biology and Reproductive System of *Brassica napus* L. (Cruciferae)" arch. biol. technol. Vol. 43(1)

Nomenclature Committee of the International Union of Biochemistry (NC-IUB) (1979). "Units of Enzyme Activity". *Eur. J. Biochem.* Vol. 97 (2): 319–20. doi:10.1111/j.1432-1033.1979.tb13116.x.

Nouairi, I., Ammar, W. B., Youssef, N. B., Miled, D. D. B., Ghorbal, M. H. and Zarrouk, M. (2009) "Antioxidant defence system in leaves of Indian mustard (*Brassica juncea*) and rape (*Brassica napus*) under cadmium stress," *Acta Physiologiae Plantarum*, Vol. 31 (2): 237–247

Oancea, S., Foca, N. and Airinei, A. (2005) Effects of heavy metals on plant growth and photosynthetic activity. *Analele Ştiintifice Ale Universititajii "AL. I. CUZA" Iasi*.

Peralta-Videa, J. R., de la Rosa, G., Gonzalez, J. H. and Gardea- Torresdey, J. L. (2004) "Effects of the growth stage on the heavy metal tolerance of alfalfa plants". *Advanced Environmental Research* Vol. 8: 679-685

Pua, E.C and Douglas, C.J (2004) "Biotechnology in Agriculture and Forestry", *Brassica* Vol. 54 (Springer-Verlag), Berlin Heidelberg.

Rabanni, N. and Thornally, P. J. (2012) "Mehtyglyoxal, glyoxalase I and the dicarbonyl proteome" *Amino Acids* vol. 42: 1133–1142 *doi: 10.1007/s00726-010-0783-0*

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

Reiner, H., Holzner W. and Ebermann, R. (1995) "The development of turnip-type and oilseed-type *Brassica rapa* crops from the wild type in Europe. - An overview of botanical, historical and linguistic facts. - Rapeseed today and tomorrow" Vol. 4: 1066-1069, 9th International Rapeseed Congress, Cambridge, UK 4 - 7 July 1995

Russo, M., Sgherri, C., Izzo, R. and Navari-Izzo, F. (2008) "*Brassica napus* subjected to copper excess: phospholipases C and D glutathione system in signalling" *Environ. Exp. Bot* Vol. 62(1): 128-132

Ryzhenko, B. N., Kovalenko, N. I., Prisyagina, N. I., Starshinova, N. P. and Krupskaya, V. V. (2008) "Experimental determination of zirconium speciation in hydrothermal solutions" *Geochemistry International* Vol. 46:328–339

Saito, R., Yamamoto, H., Makino, A., Sugimoto, T., and Miyake, C. (2011) "Methylglyoxal functions as Hill oxidant and stimulates the photoreduction of O(2) at photosystem I: a symptom of plant diabetes" *Plant Cell Environ.* Vol. 34: 1454–1464.

Sánchez, M. S., Torrenegra, R. D., Químico, H. M., Salazar, C. E. and Barahona R. (2010) "biomass production and heavy metal absorption by four plants grown at the Moravia dump, Medellín, Colombia" *Acta biol. Colomb.* Vol. 15 (2): 271 – 288

Sanevas, N., Sunohara Y. and Matsumoto, H. (2007) "Characterization of reactive oxygen species-involved oxidative damage in *Hapalosiphon* species crude extract-treated wheat and onion roots" *Weed Biol Manag* Vol.7: 172-177

Scandalios, J. G., Guan, J. and Polidoros, A. N. (1997) "Oxidative Stress and the Molecular Biology of Antioxidant Defense" © Cold Spring Harbor Laboratory Press 0-87969-502-1/97 Catalases in Plants: Gene Structure, Properties, Regulation, and Expression" Department of Genetics North Carolina State University Raleigh, North Carolina 27695-7614

Schulin, R., Curchod, F., Mondeshka, M., Daskalova, A. and Keller, A. (2007) "Heavy metal contamination along a soil transect in the vicinity of the iron smelter of Kremikovtzi (Bulgaria)" *Geoderma* vol. 140:52–61

Scrivanti, L.R. Zunino, M.P. and Zygadlo, J.A. (2003) "*Tagetes minuta* and *Schinus areira* essential oils as allelopathic agents" *Biochem. Syst. Ecol.* Vol. 31: 563–572.

Shahid, M., Ferrand, E., Schreck, E. and Dumat, C. (2013) "Behaviour and Impact of Zirconium in the Soil–Plant System: Plant Uptake and Phytotoxicity" *Reviews of Environmental Contamination and Toxicology* Vol. 221: 107-127

Shahid, M., Porrout, B., Dumat, C., Nadeem, M., Aslam, M and Pinelli, E. (2014) "Heavy Metal-induced Reactive Oxygen Species: Phytotoxity and Chemical Changes in Plants" *Reviews of Environmental Contamination and Toxicology* Vol. 232: 1 – 44.

Singh, H.P. Kaur, S. Mittal, S. Batish, D.R. and Kohli, R.K. (2009) "Essential oil of *Artemisia scoparia* inhibits plant growth by generating reactive oxygen species and causing oxidative damage" *J. Chem. Ecol.* Vol. 35: 154–162.

Singla-Pareek, S.L., Yadav, S. K. Pareek, A. Reddy M. K., and Sopory, S. K. (2006) "Transgenic tobacco overexpressing glyoxalase pathway enzymes grow and set viable seeds in zinc-spiked soils," *Plant Physiology*, Vol. 140 (2): 613–623

Skipsey, M., Andrews, C. J., Townson, J, K., Jepson, I. and Edwards, R. (2000) "Cloning and characterization of Glyoxalase I from soybean" *Archives of Biochemistry and Biophysics* Vol. 374 (2): 261-268

Statistics South Africa (2012) Mid-year population estimates

Thornally, P. J. (1990) "The glyoxalase system: new developments towards functional characterization of a metabolic pathway fundamental to biological life" *Journal of Biochemistry* Vol. 269: 1-11.

Thornally, P. J. (1998) "Glutathione-dependent detoxification of a-oxoaldehyde by the glyoxalase system: involvement in disease mechanisms and anti-proliferative activity of glyoxalase I inhibitors" *Chem. Biol. Interact*. Vol. 111–112: 137–151.

Thornally, P. J. (2008) "Protein and nucleotide damage by glyoxal and Methylglyoxal in physiological systems - role in ageing and disease" *Drug Metabol. Drug Interact*. Vol. 23: 125–150.

Thornally, P. J. and Rabbani, N. (2011) "Glyoxalase in tumourigenesis and multidrug resistance" *Seminars in Cell & Developmental Biology* Vol. 22: 318–325

USGS (United States Geological Survey) (2012) http://minerals.usgs.gov/minerals/pubs/commodity/ zirconiuM+. Assessed 23 Apr 2012

Vachirapatama, N., Jirakiattikul, Y., Dicinoski, G., Townsend, A. T. and Haddad, P. R. (2011) "Effect of vanadium on plant growth and its accumulation in plant tissues" *Journal of Science Technology* Vol. 3(3): 255-261.

Veena, Reddy, V.S. and Sopory, S.K. (1999) "Glyoxalase I from *Brassica juncea*: molecular cloning, regulation and its over-expression confer tolerance in transgenic tobacco under stress" *The Plant Journal* Vol. 17: 385–395

Velikova, V., Yordanov, I. and Edreva, A. (2000) "Oxidative stress and some antioxidant systems in acid rain treated bean plants: protective role of exogenous polyamines" *Plant Sci* Vol. 151: 59-66.

Verma, S. and Dubey, R.S. (2003) "Lead toxicity induces lipid peroxidation and alters the activities of antioxidant enzymes in growing rice plants" *Plant Science* Vol. 164: 645-655

Visioli, G. and Marmiroli, N. (2013) "The proteomics of heavy metal hyperaccumulation by plants" *Journal of Proteomics* Vol. (79): 133-145

Wang, H. F. Takematsu, N. and Ambe, S. (2000) "Effect of soil acidity on the uptake of trace elements in soybean and tomato plants" *Appl Radiat Isotopes* Vol. 52: 803–811

Wingsle, G. and Karpinski, S. (1996) "Differential redox regulation by glutathione of glutathione reductase and CuZn-superoxide dismutase gene expression in *Pinus sylvestris L.* needles" *Planta* Vol. 198: 151–157

Wu, C., Ma, C., Pan, Y., Gong, S., Zhao, C., Chen, S. and Li, H. (2013) "Sugar beet M14 *glyoxalase I* gene can enhance plant tolerance to abiotic stresses" *Journal of Plant Research* Vol. 126(3): 415 – 425

Yadav, S. (2010) "Heavy metals in plants: an overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants" *South African Journal of Botany* Vol. 76: 167-179

Yadav, S. K., Singla-Pareek, S, L., Kumar, M., Pareek, A., Saxena, M., Sarin, S. B. and Sopory, S. K. (2007) "Characterization and functional validation of glyoxalase II from rice" *Protein expression and purification*, Vol. 51: 126-132.

Yadav, S. K., Singla-Pareek, S, L., Reddy, M.K. and Sopory, S. K. (2005) "Transgenic tobacco plants overexpressing glyoxalase enzymes resist an increase in methylglyoxal and maintain higher reduced glutathione levels under salinity stress" *FEBS Letters* Vol. 579: 6265 – 6271

Yadav, S. K., Singla-Pareek, S. L., Ray, M., Reddy, M, K. and Sopory, S, K. (2005a) "Methylglyoxal levels in plants under salinity stress are dependent on glyoxalase I and glutathione" *Biochemical and Biophysical Research Communications* Vol. 337: 61–67

Yang, Z. and Chu, C. (2011) "Towards Understanding Plant Response to Heavy Metal Stress" *Abiotic Stress in Plants – Mechanisms and Adaptations* Prof Arun Shanker (Ed.) doi: 10.5772/24204

Zhang, F., Wang, Y., Lou, Z. and Dong, J. (2007) "Effect of heavy metal stress on antioxidative enzymes and lipid peroxidation in leaves and roots of two mangrove plant seedlings (*Kandelia candel* and *Bruguiera gymnorrhiza*)" *Chemosphere* Vol. 67: 45-50.

Zunino, M.P. Zygadlo, J.A. (2004) "Effect of monoterpenes on lipid oxidation in maize" *Planta* Vol. 219: 303–309.