

Oxidative stress in plants may arise as a result of various environmental factors that causes the overproduction of ROS viz. $\bullet\text{OH}$, 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, 2011). 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 (6 μ M) 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

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 O₂ to yield reactive oxygen intermediate; O₂⁻ (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; Iqbal *et al.*, 2010). As a result the antioxidant defence in plants is also induced under HM conditions with SOD,

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 $\bullet\text{OH}$; 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 $\bullet\text{OH}$ 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.

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 μ M 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.

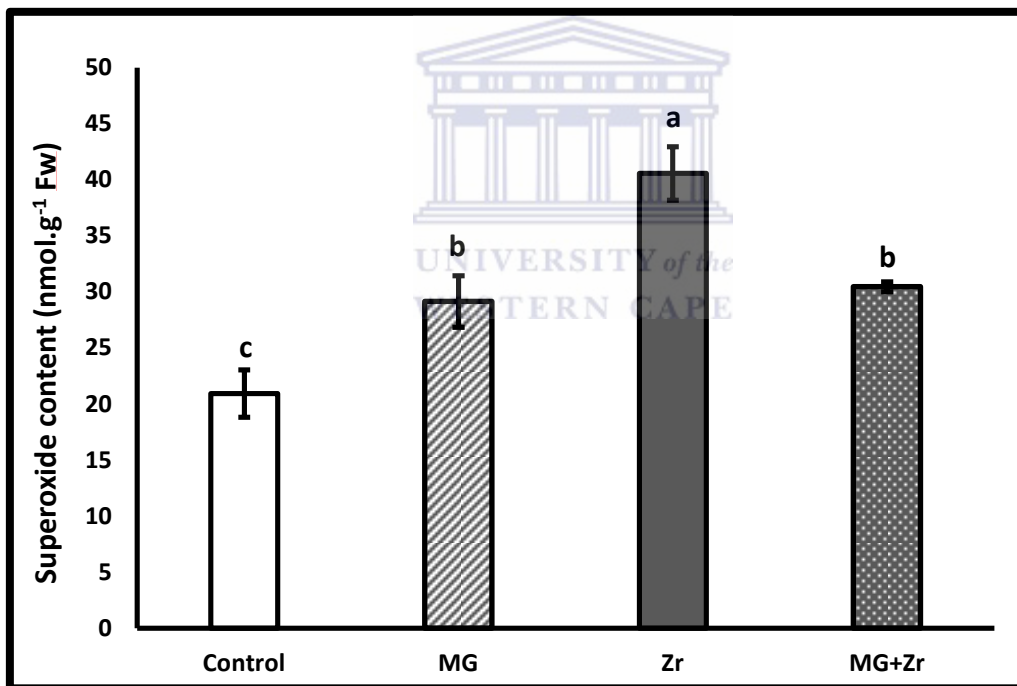


Figure 4.2.1. The effect of Methylglyoxal on Superoxide content in *B. rapa* L. seedlings under Zr stress. Superoxide occurs as a by-product of normal aerobic metabolism when O_2 is reduced. Superoxide content was determined in *B. rapa* L. seedlings after exposure to Zr stress and MG treatments respectively. The different letters indicate a significant change across means at $P < 0.05$ (DMRT). Values are means \pm S.E (N=3).

The production of hydroxyl radicals (\bullet 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 $\bullet\text{OH}$ (Ahsan *et al.*, 2003). Another means to form $\bullet\text{OH}$ is from Haber-Weiss reactions where the interaction of H_2O_2 with O_2^- results in the formation of $\bullet\text{OH}$, H_2O and O_2 (Ahsan *et al.*, 2003; Iqbal *et al.*, 2010). $\bullet\text{OH}$ cannot diffuse across the membrane therefore it is important that it is metabolised to render it less reactive. If $\bullet\text{OH}$ accumulates it is capable of reacting with any biomolecule (Mittler, 2002; Ahsan *et al.*, 2003). $\bullet\text{OH}$ 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 $\bullet\text{OH}$ is capable of causing, it was important to determine the degree to which Zr-stress would affect $\bullet\text{OH}$ 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 ($6\ \mu\text{M}$) in Zr-stressed seedlings (figure 4.2.2). There was no change, or signs of stress, in MG-treated seedlings as the $\bullet\text{OH}$ levels were nearly identical to the control-treated seedlings. Zr-treated seedlings however, were negatively affected by a 29% increase in $\bullet\text{OH}$ content. In MG+Zr-treated seedlings $\bullet\text{OH}$ 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%.

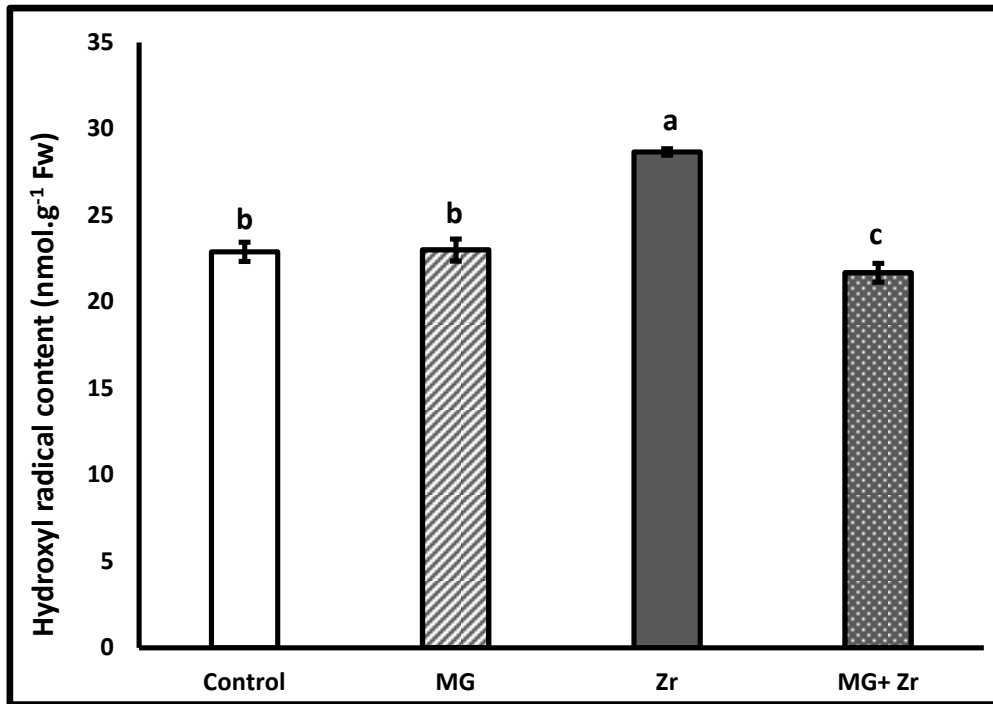
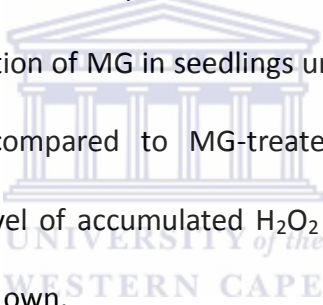


Figure 4.2.2. The effect of Methylglyoxal on Hydroxyl radical content in *B. rapa* L. seedlings in response to Zr stress. •OH is readily produced from commonly occurring species viz. H₂O₂ and O₂ via Fenton-like reaction and due to its high reactivity it is known to cause damage in plant cells. •OH-content 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 (H₂O₂), 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 H₂O₂ (Cheeseman, 2007; Hossain *et al.*, 2011; 2012). Hossain *et al.* (2012) states that maintaining H₂O₂ 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 $\bullet OH$ (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.

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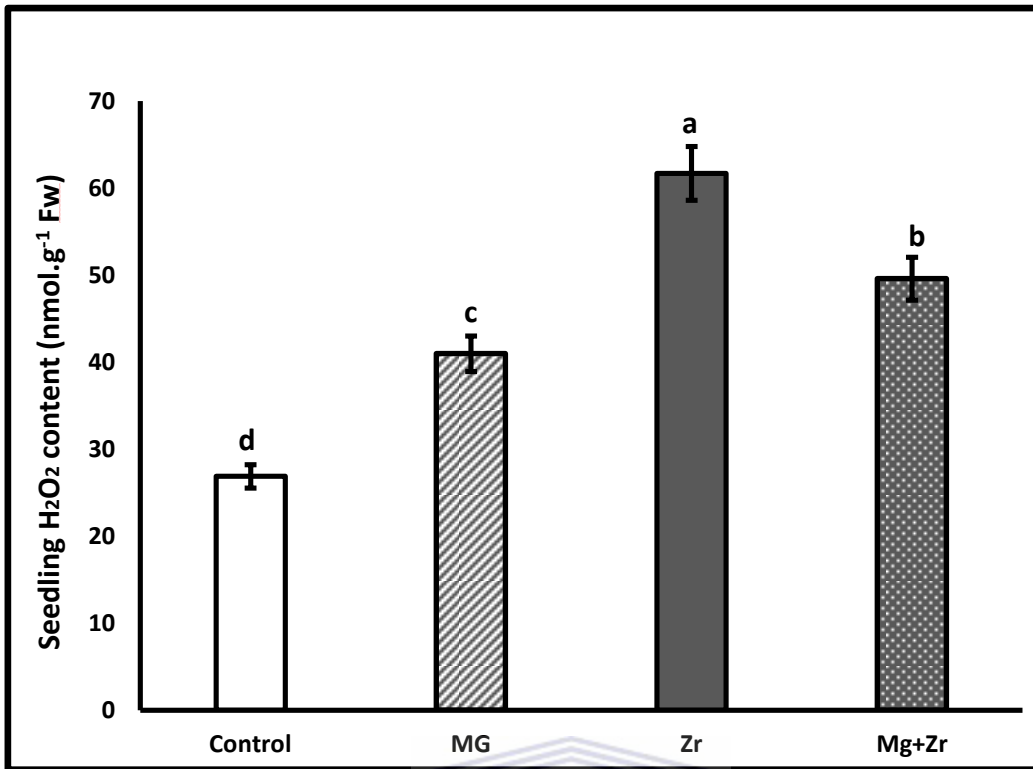


Figure 4.2.3. The effect of Methylglyoxal on H₂O₂ content in *B. rapa* L. seedlings in response to Zr stress. Under perceived stress plants' changes in H₂O₂ levels serves as an indicator of stress. This ROS has a relatively low reactivity however relatively long half-life and often leads to oxidative damage. H₂O₂ 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 O₂⁻, catalysing its dismutation to H₂O₂ and O₂ (table 1.1); two far less reactive and harmful species (Mourato *et al.*, 2012). Especially the ROS by-product; H₂O₂ 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 (Bradbenet *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.

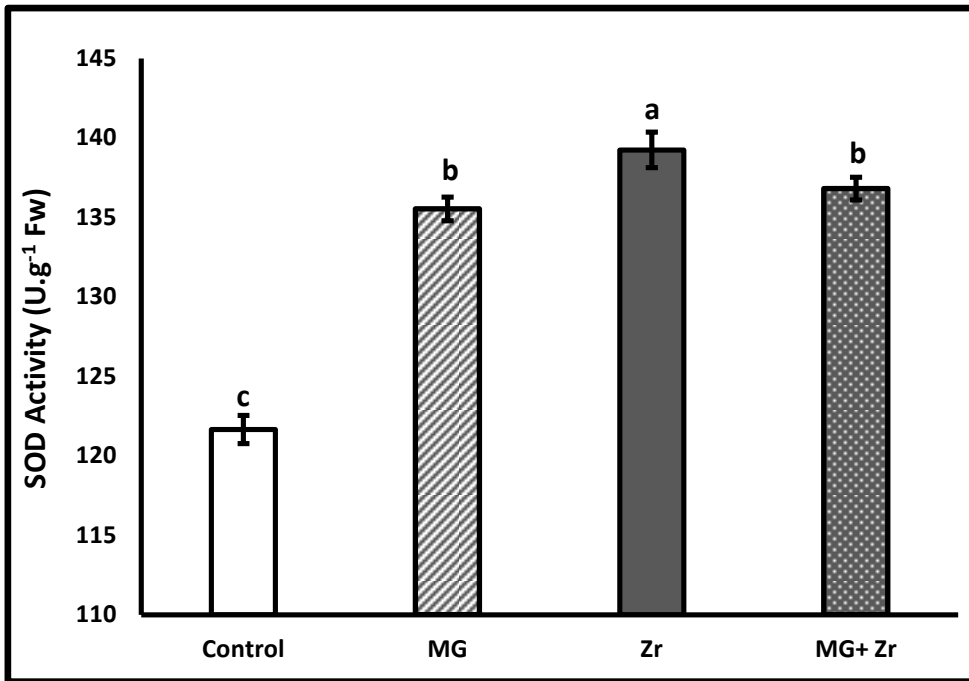


Figure 4.2.4. The effect of Methylglyoxal on SOD activity in *B. rapa* L. seedlings in response to Zr stress. Under perceived stress in plants SOD activity has been known to increase. SOD scavenges harmful radicals and decreases oxidative damage in plants. SOD activity was determined in *B. rapa* L. 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 \pm 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

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.

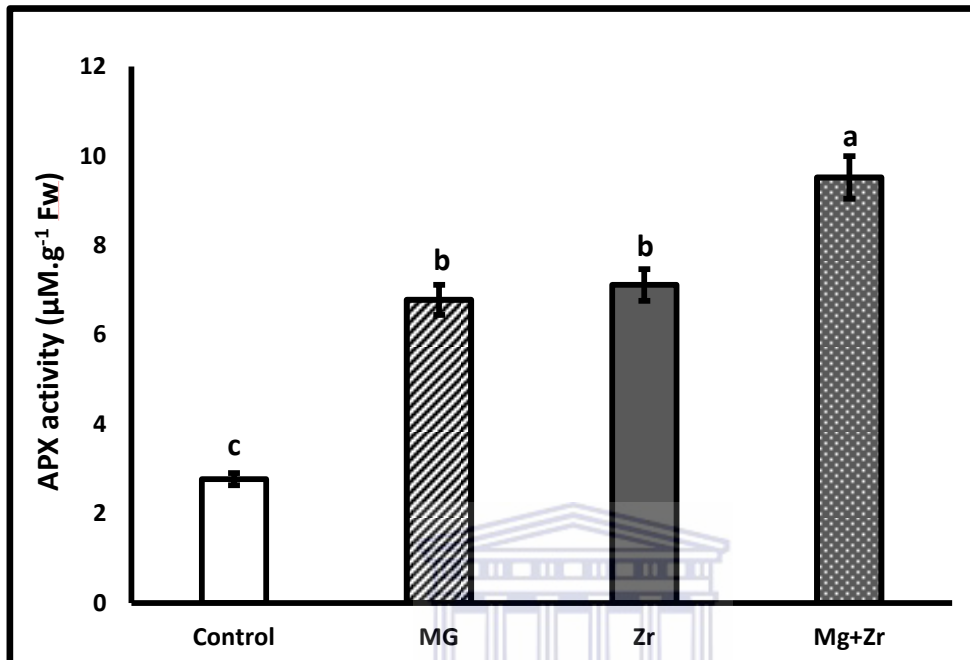


Figure 4.2.5: The effect of Methylglyoxal on Ascorbate peroxidase activity in *B. rapa* L. seedlings in response to Zr stress. In plants APX is foremost in scavenging and metabolising H₂O₂ and is therefore crucial in protecting plants from the damaging effects of H₂O₂ and thus oxidative stress. APX activity 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 H₂O₂ is their major substrates. They scavenge any excess H₂O₂ directly and catalyse the conversion of H₂O₂ molecules (table 1.1) to H₂O and molecular O₂ (Scandalios *et al.*, 1997; Mhamdi *et al.*, 2010). By this mechanism CATs spare the cell from any further damage by removing the excess H₂O₂ 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.

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.

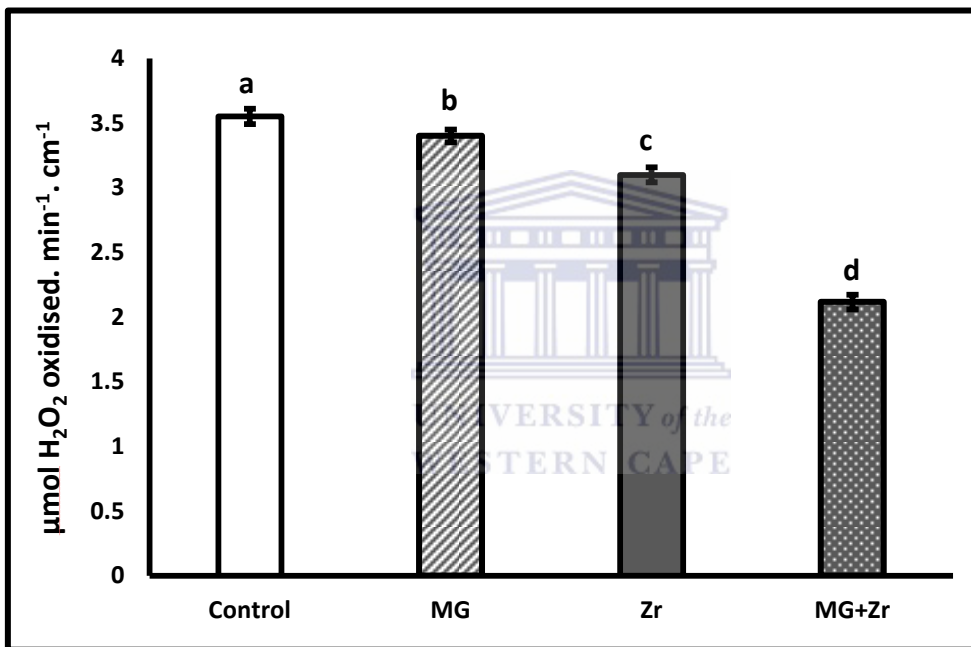


Figure 4.2.6. The effect of Methylglyoxal on Catalase activity in *B. rapa* L. seedlings in response to Zr stress. In plants CAT is known to scavenge excess H_2O_2 and is therefore crucial in alleviating plant stress. Catalase activity was determined in *B. rapa* L. seedlings after exposure to Zr stress and MG treatments respectively. The different letters indicate a significant change across means at $P < 0.05$ (DMRT). Values are means \pm 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 O₂ 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 $\mu\text{mol.g}^{-1}$ 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.

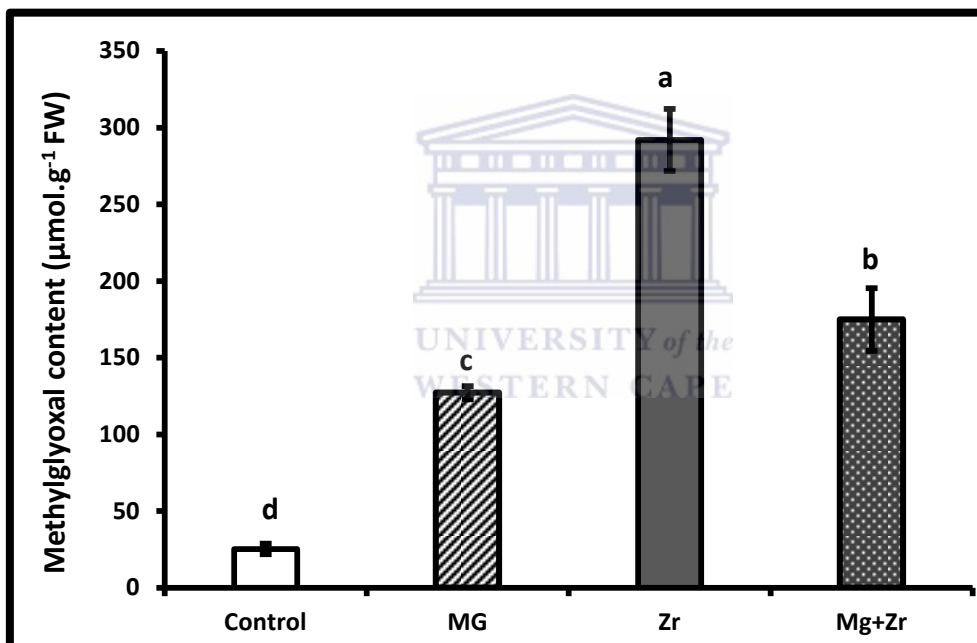
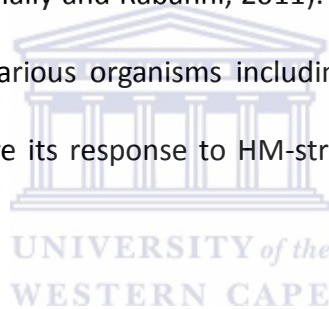


Figure 4.2.7. The effect of Methylglyoxal application on MG content in *B. rapa* L. seedlings in response to Zr stress. MG is cytotoxic at elevated levels in plants and it also occurs as a by-product of normal metabolism ubiquitously. MG content was determined in *B. rapa* L. seedlings after exposure to Zr stress and MG treatments respectively. The different letters indicate a significant change across means at $P < 0.05$ (DMRT). Values are means \pm 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).

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 μ M), 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.

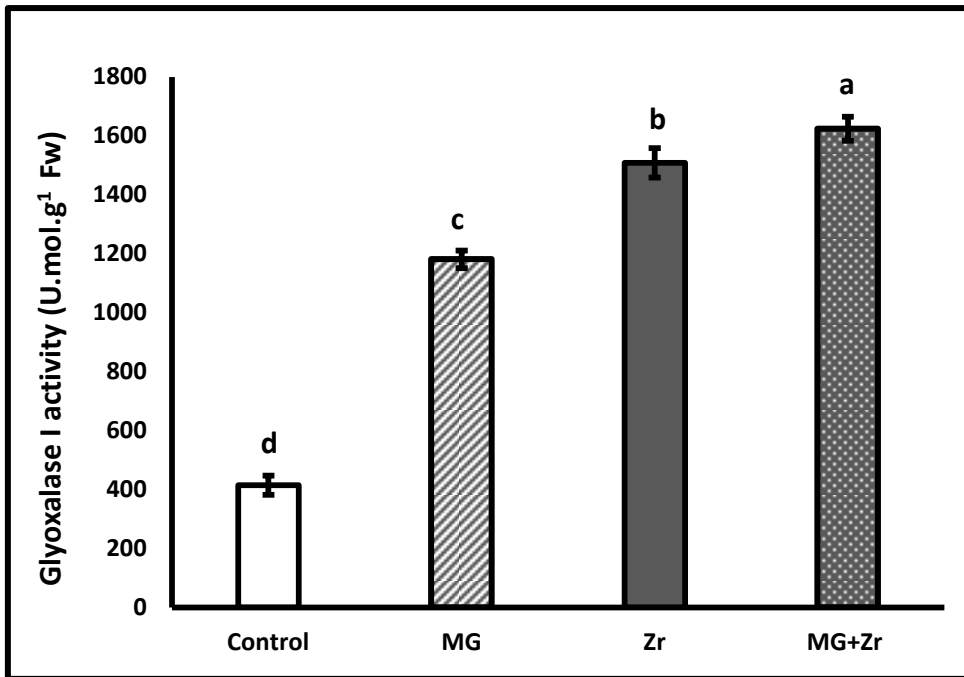


Figure 4.2.8. The effect of Methylglyoxal on Glyoxalase I activity in *B. rapa* L. seedlings in response to Zr stress. Glyoxalase I of the glyoxalase system occurs in all plants. It has proliferative properties but more importantly it effectively scavenges and metabolises >90% of MG produced as well 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 \pm S.E (N=3).

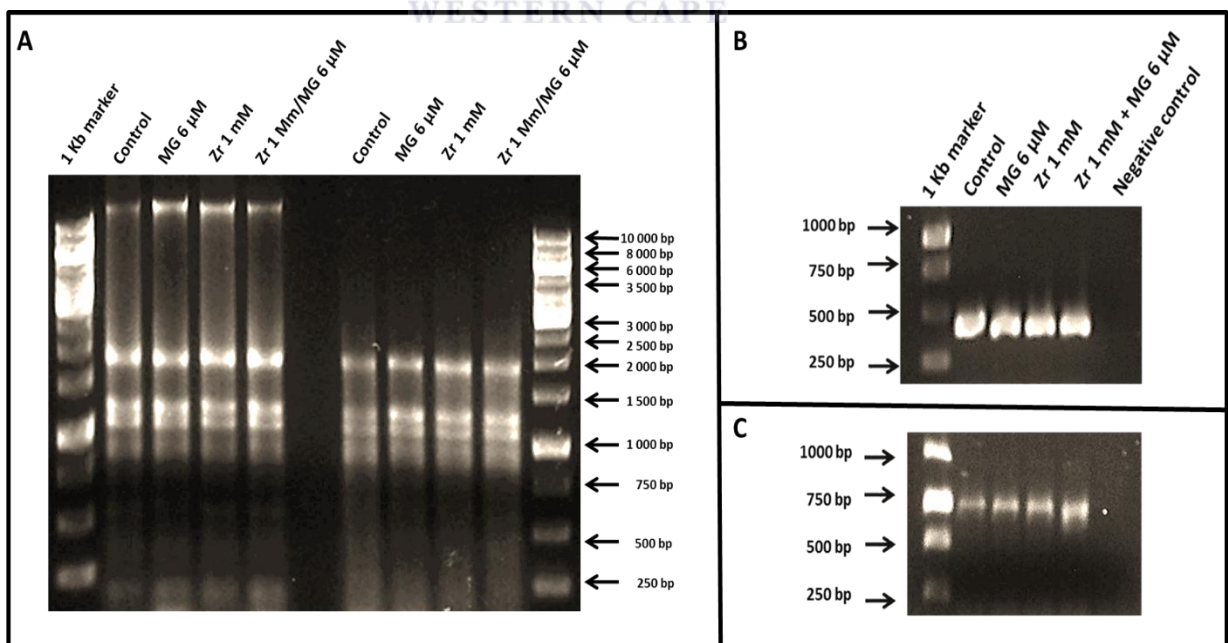


Figure 4.2.9. RNA extractions and Gly-I amplification of *B. rapa* L. seedlings in response to Zr stress and MG treatments respectively. (A) Shows the RNA extracted from seedlings and RNA after DNase-treatment. Lanes 1 - 4 represent 200 ng RNA before DNase-treatment and lanes 5 - 8 represent 200 ng RNA after DNase-treatment. RNA appear alongside a 1 kb molecular weight ladder. **(B)** Shows *BrUBQ* amplification, **housekeeping gene,** **(C)** and *BrGlyI* amplification.

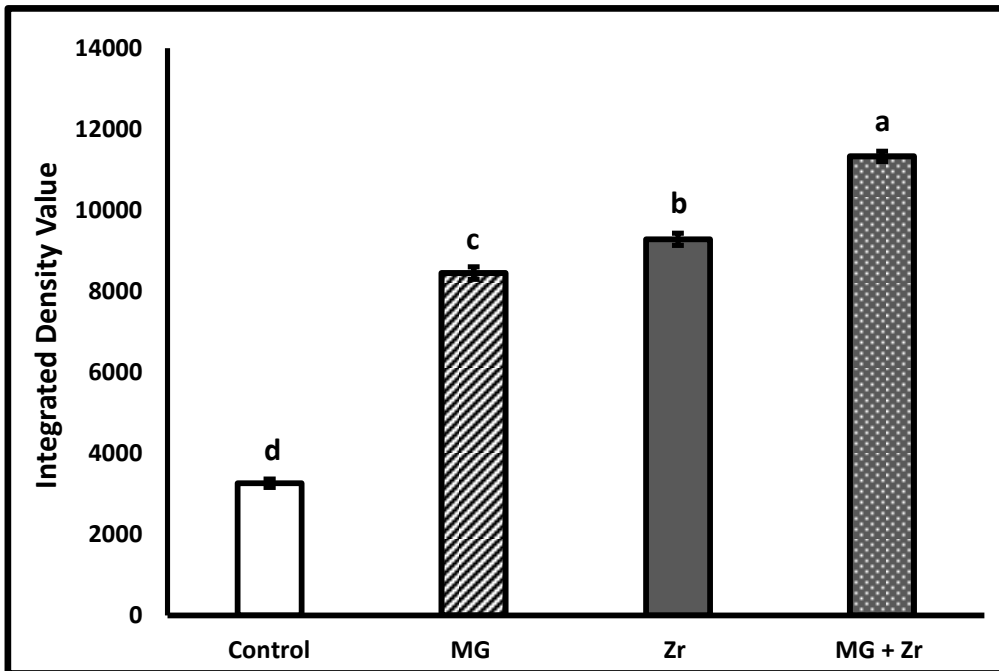


Figure 4.2.10: Densitometry analysis of Glyoxalase I mRNA transcript expression in *B. rapa* L. seedlings subjected to Zr, Methylglyoxal and MG+Zr. BrGly-I expression levels were determined in *B. rapa* L. seedlings after exposure to Zr stress and MG treatments respectively. BrGly-I mRNA transcript bands were subjected to densitometry analysis. Different letters indicate a significant change across means at $P < 0.05$ (DMRT). Values are means \pm 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 (Ferrand *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-adsorption 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 $\bullet OH$ radicals upon contact with transition metal ions (Ahsan *et al.*, 2003). $\bullet OH$ 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 $\bullet OH$ (Ahsan *et al.*, 2003; Mourato *et al.*, 2012). $\bullet OH$, is

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)

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 O₂ to yield toxic ROS and subsequent activation of antioxidants points to a signalling role of MG.

Hydrogen peroxide (H₂O₂) 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 H₂O₂ 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 H₂O₂ 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 H₂O₂ had occurred in MG-treated seedlings than in Zr-treated ones which indicate an oxidative response in the cell in a regulatory sense. H₂O₂ content increased when MG was administered and therefore we believe that the H₂O₂ 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 H₂O₂ concentration to circumvent cell damage. The literature also validates that slightly elevated H₂O₂ 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 H₂O₂ was observed, however this was a

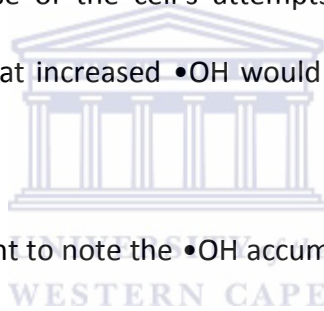
minimal increase in H₂O₂ content compared to Zr-treated seedlings with a 1.3-fold increase.

The effect of exogenous MG appears to be indirectly involved in reducing the accumulation of toxic ROS under stress conditions. The presence of Zr unmistakably caused oxidative stress in seedlings given that H₂O₂ 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 H₂O₂ can lead to more toxic active oxygen species the increase in •OH; and O₂⁻ 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 H₂O₂.

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 O₂, reducing it to O₂⁻. 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-

treated seedlings and the control exhibiting no difference. Given the assumption of MG-induced $\bullet\text{OH}$ 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 $\bullet\text{OH}$ 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 $\bullet\text{OH}$, the conversion of H_2O_2 to $\bullet\text{OH}$ 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 $\bullet\text{OH}$ would indicate drastic metabolic and oxidative distress.



For this reason it was important to note the $\bullet\text{OH}$ accumulation in response to Zr-stress in *B. rapa* L. seedlings. With 30% increase in $\bullet\text{OH}$ 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 $\bullet\text{OH}$ accumulation in MG+Zr-treated seedlings. Remarkably the $\bullet\text{OH}$ accumulation in MG+Zr-treated seedlings does not indicate zero oxidative stress with a reduced $\bullet\text{OH}$ content, but it suggests that the sequestration rate of $\bullet\text{OH}$ and $\bullet\text{OH}$ -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.

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 H₂O₂ in cells and regulates the levels of H₂O₂ by maintaining its low levels. It also has a higher affinity for H₂O₂ than CAT. APX acts with the use of ascorbate to neutralise H₂O₂ to 2H₂O and dehydroascorbate (DHA) (Blokhina *et al.*, 2003). The level of involvement in plants defence against oxidative stress will determine the extent of H₂O₂ 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 H₂O₂ levels to increase as in figure 4.2.3 and this increased H₂O₂ causes increased oxidative stress, and the subsequent formation toxic O₂ 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 H₂O₂ 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 H₂O₂ being scavenged and thus a much lower H₂O₂ content (figure 4.2.3) was observed in MG-treated seedlings. Secondly, Zr interacts with H₂O₂ 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 $\bullet OH$ 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 $\mu\text{mol.g}^{-1}$ 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 ($127 \text{ } \mu\text{mol.g}^{-1}$ 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; glyoxalase 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. H₂O₂ had a 1.5-fold increase in tobacco plants subjected to Zn, similarly H₂O₂ 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 phytoavailability 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 occurred. 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

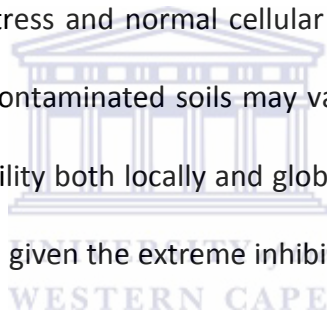
(figure 3.2.1 – 3.2.3). The accumulation of MG also incites the activation of other antioxidants, which occurs as MG initially increases ROS levels; this phenomenon can be described as a direct early-onset activation of antioxidants, or pre-inductive activation of ROS-mitigating enzymes.

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 $\bullet OH$ content had decreased as well. $\bullet OH$ 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 Kohli, 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 glyoxalase-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. Biochem.* 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

- 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., Puchowski, 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, ruthenium 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

- 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 Științifice Ale Universității "AL. I. CUZA" Iași.*
- 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 Zygodlo, 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., Porrou, B., Dumat, C., Nadeem, M., Aslam, M and Pinelli, E.** (2014) "Heavy Metal-induced Reactive Oxygen Species: Phytotoxicity 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 α -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.