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Effects of exogenous myristic acid on growth and germination of *Brassica napus* L. under zirconium toxicity

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

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LIST OF ABBREVIATIONS

APX — ascorbate peroxidase

Ca — calcium

Cd — cadmium

CAT — catalase

EDTA — ethylenediaminetetraacetic acid

Fe — iron

HMs — heavy metals

H_2O_2 — hydrogen peroxide

ICP- OES — inductively coupled plasma-optical emission spectrometry

K — potassium

KCN — potassium cyanide

Mg — magnesium

MDA — malondialdehyde

MA — myristic acid

NR — nitrate reductase

NO — nitric oxide

NBT — nitroblue tetrazolium

O_2^- — superoxide

$\cdot OH^-$ — hydroxyl radical

ONOO⁻ - peroxynitrite

PA - phosphatidic acid

PM - plasma membrane

PUFA - poly unsaturated fatty acids

PVP - polyvinylpyrrolidone

ROS - reactive oxygen species

SDS - sodium dodecyl sulphate

SOD - superoxide dismutase

TBA - thiobarbituric acid

TCA - trichloroacetic acid

Zn - zinc

Zr - zirconium
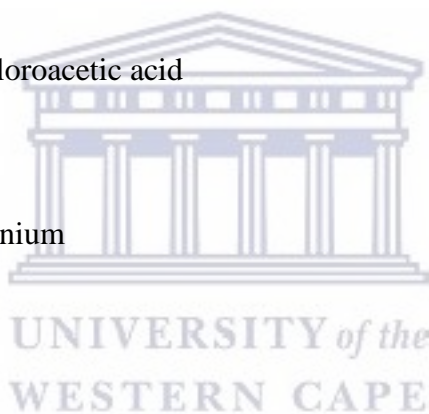
ABSTRACT

Lipids when exogenously applied are known to cause various changes in ROS levels produced within plants. They can either be beneficial to the plant when not stimulating the overproduction of ROS thus resulting in improved germination and development or on the contrary, increasing the level of ROS produced, causing oxidative stress and thus leading to cell death of the plant. In this study, we report that a saturated fatty acid known as MA increased the germination percentages of *Brassica napus* L. seedlings when applied at a low concentration. When applied at higher concentrations, it was shown that elevated levels of ROS within the seedlings occurred therefore leading to a decrease in germination percentage as well as stunting of seedling growth. Physiological experiments such as biomass and cell death determination were conducted to further elucidate the effects of MA on the seedlings. Biochemical assays were performed to determine the oxidative state of specific ROS such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2). It was observed that MA at a low concentration had low levels of both O_2^- and H_2O_2, thus leading to an improved germination rate. At higher concentrations; the level of both the aforementioned ROS were significantly high thus leading to oxidative stress and therefore causing a decrease in germination percentage as well as leading to cell death. Due to increasing anthropogenic activities, such as excessive mining especially in South Africa, the overproduction of heavy metals will have an impact on the surrounding crop yield. According to statistics, globally South Africa accounts for a large portion of Zirconium (Zr) production. The effects of the optimal MA concentration combined with Zr were therefore investigated. We

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observed an increase in germination rate and improved seedling shoot growth in the combination treatment (MA+Zr) when compared to the Zr only treatment. Moreover, a reduction in ROS (O_2^- and H_2O_2) in response to MA+Zr treatment aswell as improved cell viability compared to the Zr treatment were observed. In addition, the nitric oxide (NO) levels were stimulated in the MA and MA+Zr treatments, indicating a signaling role of MA via elevating NO production. NO is known to be beneficial in a dose-dependent manner, therefore when observing its effects in response to MA, a reduction in the antioxidant enzymes were observed. This indicates a possible role of MA in modulating antioxidant pathwhays via NO induction and thus subsequently alleviating Zr stress. Furthermore, the effects of MA on the mineral nutrient composition within seedlings were observed by conducting ICP-OES analysis. MA was observed to elevate certain macronutrients aswell as regulate the micronutrient content while under Zr stress. The findings of this study have provided for the first time, much needed insight on the functions of MA at various concentrations and signaling roles in plants as well as aiding in the alleviation of Zr toxicity.

CHAPTER ONE

LITERATURE REVIEW

1.1. INTRODUCTION

Lipids are biomolecules which are known to have a low solubility in water and high solubility in organic solvents (Smith *et al*., 2007). They are essential cellular constituents which contribute largely to cell membrane structure, as well as yielding large quantities of energy toward metabolic function (Wang, 2004).

Differences in the structural-chemistry of lipids allow them to be separated into classes of distinct groups. Fatty acids are amphiphatic biomolecules which are composed of a long hydrocarbon chain as well as a hydrophilic head group. Unsaturated fatty acids can be further divided into mono-unsaturated and polyunsaturated (Garret and Grisham, 2006). It has been established that polyunsaturated fatty acids (PUFA) have crucial roles with regards to biological functions which include membrane fluidity, cellular signaling and regulation of various genes. Fatty acids have the ability to regulate the expression of various genes which is involved in modulating the activity of signaling molecules as well as roles in lipid metabolism. While it is known that the expression and activity of lipogenic enzymes are suppressed due to PUFA, saturated fatty acids and their effects within plants have not been fully elucidated (Lindsey *et al*., 2003).

1.2.   THE ROLE OF LIPID SIGNALING IN PLANTS

Cellular signaling can be divided into two sectors, intercellular signaling and intracellular signaling. Intercellular signaling occurs when a message is sent between two or more cells and could involve the excretion of a compound by cells that can interact with a cell surface receptor protein. When the interaction between the signaling molecule and the cell surface protein occur, the result is a conformational change in the structure of the protein, thereby resulting in the message being delivered inside the cell. Intracellular signaling occurs when molecules such as reactive oxygen species (ROS) and lipids, carry messages in and out of cells (Weinberg, 2007).

Lipid signaling plays a significant role in cellular signaling such as cell repair, senescence and cell growth. There has been increasing evidence in recent years, which have indicated that lipids may act as mediators in various plant processes such as cytoskeleton rearrangements and signal transduction which are crucial for growth and differentiation as well as for responses to water, temperature, salinity and pathogens. Lipid signaling in plants includes various molecules such as glycerolipids, sphingolipids, oxylipins and sterols (Lindsey *et al.*, 2003). Various lipid signaling enzymes such as phospholipases, lipid kinases and phosphatases control the production of lipid mediators. The spatial and temporal production of lipid mediators in response to specific biotic or abiotic signals are regulated by the activities of the above mentioned enzymes. In order to understand lipid signaling, it is crucial to study the enzymes as well as the genes which are involved in lipid mediator production, due to the fact that multiple signaling reactions can result from lipid mediators such as Phosphatidic acid (PA) and lysophospholipids. Factors

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which are crucial to lipid signaling are spatial and temporal regulation, as lipid mediators in general have a low rate in terms of mobility within the cell. As more information becomes available with regards to lipid signaling, our knowledge with regards to the signaling that mediates the stress responses as well as growth and development will improve (Meijer and Munnik, 2003; Wang *et al*., 2002).

1.3. PHOSPHOLIPIDS AND IMPACTS ON PLANT GROWTH

Phospholipids play a major role in plant cell signaling and this is evident due to the fact that when plants are under stress the levels of phospholipids can increase or decrease. The two main types of phospholipids which play a role in plant cell signaling include inositol hexakisphosphate and PA. PA is an important lipid mediator with regard to the regulation of cellular functions. In mammalian systems, PA regulates various processes such as signal transduction, respiratory burst and secretion. Recent studies have shown in plants that PA promotes pollen-tube growth; thereby decreasing H_2O_2 induced cell death which in turn mediates the polymerization of the actin cytoskeleton (Munnik, 2001).

1.4. MYRISTIC ACID AND NITRIC OXIDE (NO) STIMULATION

Myristic acid is a saturated fatty acid consisting of 14 carbons as well as having a carboxyl group at its end. It is present at high concentrations in many oils extracted from coconut, nutmeg and palm kernel. Myristic acid is a component of both animal and vegetable fats thus playing an important part of the diet of various animals. It has various commercial applications, such as being involved in the production of shaving creams and cosmetics (Garret and Grisham, 2006).

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According to a study conducted by Isenberg et al. (2007), it was observed in mammalian systems that myristic acid and TSP 1 modulated nitric oxide signaling in endothelial cells in a CD 36 dependent manner. The link between MA and NO in plants has not been established to date. Therefore, could myristic acid play the same role via a specific metabolic pathway in increasing the production of NO above basal levels in plant cells? Also could these effects within the plant be concentration dependant?

1.4.1. NITRIC OXIDE (NO) PRODUCTION IN PLANTS

Nitric oxide (NO) has been identified to be a gaseous signaling molecule which plays a vital role with regards to maintaining plants redox homeostasis (Neill *et al*., 2003). NO signaling has various functions within plant cells such as being involved in inducing cell death and by interacting with ROS during abiotic stress. NO has the ability to mediate molecules such as hormones as well as mediating ABA-induced stomatal closure (Neill *et al*., 2003). During biotic stress it has been observed that NO plays a role in signaling a hypersensitive response (Delledonne, 2005; Durner *et al*., 1998). It has been shown that during abiotic stress such as water stress, the production of NO increases suggesting that NO is linked and plays a part in abiotic stress (Leshem and Haramaty, 1996). The NO molecule has an unpaired electron thereby allowing it to interact with ROS such as the superoxide radical (O_2^-) which leads to the formation of peroxynitrite which is toxic in animal cells but less toxic to plant cells (Tada *et al*., 2004; Kopyra and Gwozdz 2003). The production of NO in plants occurs via enzymatic and non-enzymatic processes (Rockel *et al*., 2002).

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1.4.2. ENZYMATIC PRODUCTION OF NO IN PLANTS

There are two pathways which results in the production of NO in plants. The first being the arginine-dependant pathway which is mediated by NO synthase (NOS) utilizing the substrates L-arginine, O_2 and NADPH thereby produces L-citrulline and NO (Figure 1.2). According to studies conducted by Cueto et al. (1996), Ninnemann and Maier, (1996) and Barroso et al. (1999) NOS activity was present in higher plants and peroxisomes. However, there has not been a gene to date that is homologous to the mammalian NOS that have been identified in higher plants. Studies shown by Foresi et al. (2010), showed that a novel NOS gene has been characterized from *Osterococcus tauri* which is the smallest known single celled photosynthetic eukaryote belonging to the group chlorophyta found in the plant kingdom. This important discovery of a NOS enzyme is vital, as it provides a basis to discover the NOS enzyme in higher plants (Foresi *et al*., 2010).
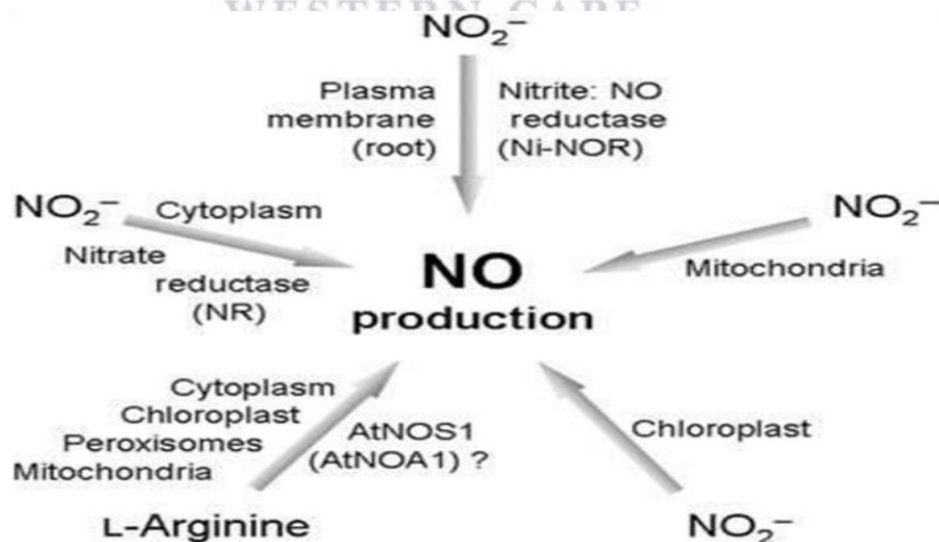
Figure 1.1. Various routes of NO production in plant cells. NO is enzymatically synthesized from nitrite (NO_2^-) by nitrate reductase (NR). It is also shown that there is L-arginine-dependent NOS activity in plants even though the AtNOS1 protein is no longer a NOS adapted from (Wilson *et al*., 2008).

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The second pathway is the nitrite-dependant pathway, which is catalyzed by nitrate reductase (NR) which is the only enzyme to date and currently known to be responsible for the production of NO in plants (Dean and Harper, 1988). It has two forms, in the plasma membrane (PM-NR) as well as occurring in cytosolic regions (cNR) (Figure 1.2). Studies have reported that the generation of nitrous oxide is a pre-cursor of peroxynitrite and NO. Under aerobic conditions in agreement with these findings is the fact that NR activity utilizing nitrites as a substrate produces NO and peroxynitrite (Yamasaki and Sakihama, 2000).

It has been established that higher plants do not produce NO from NR in the mitochondria of leaves but only in the roots, which suggests that there are other sources of NO which remain to be discovered which are responsible for the production of NO in leaves (Gupta *et al*., 2005).

1.4.3. NON-ENZYMATIC PRODUCTION OF NO IN PLANTS

There are several non-enzymatic reactions which have also been known to produce NO in plants, such as Nitrous acid and Ascorbate which reacts under acidic conditions to produce NO and dehydroascorbic acid. Studies conducted by Stohr and Ullrich (2002), have shown that the non-enzymatic reduction of nitrite can lead to NO being produced. According to studies conducted by Beligni et al. (2002) it has been shown that by reducing nitrite using ascorbate at an acidic pH can lead to the synthesis of NO.

1.4.4. NO SIGNALING IN PLANTS

 It has been established that there is evidence of NO function in plant systems (Crawford and Guo 2005; Lamotte *et al*., 2005). NO has shown to be an important

signaling molecule in various signal transduction pathways, where its downstream mediator may be cyclic guanosine monophosphate (cGMP). NO has the ability to interact either directly or indirectly with other molecules such as H_2O_2 which is also involved in signaling (Lamotte *et al*., 2004; Wendehenne *et al*., 2004). An example of NO interacting with other signaling molecules would be when it reacts with O_2^- radical to form peroxynitrite and thiol groups (Neill *et al*., 2008). However even though the downstream targets of NO during signaling have not been elucidated, the research that has been conducted with regards to NO signaling has progressed over the years and even though the identification of NOS enzyme in plants are uncertain there is enough evidence suggesting that NO plays an important part in plant signaling (Neill *et al*., 2003).

1.4.5. CYCLIC GUANOSINE MONOPHOSPHATE (cGMP)

A secondary messenger molecule known as cGMP is involved in NO signaling (Neill *et al*., 2008). The soluble guanylyl cyclase (sGC) enzyme gets activated by NO thereby stimulating an increase in the level of cGMP which can occur via both dependant and independent pathways. Due to an increase in the level of cGMP, an increase in the calcium levels in the cytosol (Durner *et al*., 1998) occurs as well as having the ability to activate intracellular protein kinases. The main targets of NO which occur via the cGMP independent pathway are metals including copper, zinc and iron (Wendehenne *et al*., 2004).

1.5. HEAVY METALS AND THEIR INTERACTION WITH PLANTS

It has been well established that abiotic stresses pose to be one of the biggest threats to plants due to the fact that they are unable to move away from the stress related

area (Tuteja *et al*., 2001; Bhatnagar-Mathur *et al*., 2008). According to modern statistics, abiotic stress is currently responsible for 50% of crop losses worldwide (Wang *et al*., 2003). Due to the constant exposure to these stressful conditions, plants have evolved over time thus being able to alter their metabolic processes and thereby have developed protective mechanisms (Ciarmiello *et al*., 2011; Vickers *et al*., 2009). The various factors of abiotic stress amongst others include high salinity, drought, extreme temperatures and high levels of heavy metal concentrations (Nakashima *et al*., 2012; Tao *et al*., 2011). Heavy metals are elements that are present in nature at different concentrations, and at trace amounts, some are crucial for the plants survival (Rascio and Navari-Izzo, 2011; Vachirapatama *et al*., 2011). However, elevated levels of these metals due to industrial processes and excessive mining become toxic to surrounding plant life thus resulting in a reduction in the uptake of nutrients and eventually lead to low crop yield (Yadav, 2010; Wei *et al*., 2008). It has also been observed that high concentrations of these metals have the ability to alter the activities of plant growth promoting microorganisms (Wani *et al*., 2007). It has thus become evident that due to heavy metal contaminated soils, the end result is large losses of agricultural yield as well as having a hazardous effect on life in general once entering the food chain (Schickler and Caspi, 1999).

South Africa (SA) and Australia collectively are responsible for the production of 80% of the world's Zirconium (Zr) giving an indication that the mining industry within SA serves as a significant factor within SA's economy (Emsley, 2001). The mining industry plays a huge role within South Africa's economy. Therefore, large quantities of Zr are produced thus resulting in high concentrations of Zr within SA soils especially surrounding mining areas. Previous studies have demonstrated that

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heavy metals such as vanadium at high concentrations within soils may lead to a dramatic decrease in plant yield thus posing a huge threat to farmers (Feng *et al*., 2009; Xuan Tham *et al*., 2001).

As plants adapt to the conditions where they grow, the presence of heavy metals can induce oxidative stress thus activating several defence factors within plants (Prasad, 2004). It is important to understand how certain plants possess the ability to withstand high concentrations of heavy metals, in order to produce crops capable of growing on contaminated soils to aid in environmental cleanup via phytoremediation (Adriano *et al*., 2004).

1.5.1. HEAVY-METAL HYPER-ACCUMULATIING PLANTS

Hyperaccumulating plants are notorious for tolerating as well as accumulating high heavy metal concentrations within plant cellular components (Rascio & Navari-Izzo, 2011; Verbruggen *et al*., 2009; Ozturk *et al*., 2003). They are usually identified by having characteristics such as, taking up large quantities of metals from the soil, efficient root to shoot translocation of metals and detoxifying high quantities of metals within the leaves (Rascio & Navari-Izzo, 2011).

1.5.1.1. STRATEGIES OF METAL UPTAKE BY PLANTS

As aforementioned, soils contaminated with high concentrations of heavy metals pose a huge threat to animal and human health. Some plants therefore employ strategies to limit the entrance of heavy metals and thus limit damage caused by lipid peroxidation (Tuteja *et al*., 2009).

It has been observed that certain plants grow on soils which contain high concentrations of heavy metals thus having various survival coping mechanisms to

thrive under these toxic conditions. The majority of the heavy metals are detoxified in their roots which minimize translocation to the leaves as they are highly sensitive to phytotoxic effects. These plants can be categorized as being either hypertolerant hyperaccumulators or hypertolerant non-hyperaccumulators (Rascio & Navari-Izzo, 2011).

1.5.1.2. HEAVY METAL UPTAKE BY HYPERTOLERANT HYPERACCUMULATOR PLANTS

These are plants which possess the ability to tolerate high heavy metal concentrations while showing no sign of phytotoxicity due to an important property called hypertolerance. They are also able to take up large quantities of heavy metals, which are stored in organs such as the leaves. These plant species which are capable of taking up large amounts of heavy metals and can be used for phytoremediation thus aiding the removal of these heavy metal contaminants from the soil (Rascio & Navari-Izzo, 2011; Verbruggen *et al*., 2009; Ozturk *et al*., 2003).

According to modern statistics approximately 25% of hyperaccumulators belong to the Brassicaceae family. According to Smith and Raskin, (1998) *Brassica juncea* (L.) demonstrated a high capacity to take up extremely large amounts of Cd in their roots as well as being able to have a high removal efficiency of metals such as Pb (Smith & Raskin; 1998). Therefore, it has become evident that plants from the *Brassicaceae* family possess a high potential for heavy metal bioaccumulation, thus being beneficial as aforementioned in the process of phytoremediation and therefore can benefit environmental cleanup as a whole (Verbruggen *et al*., 2009; Verkleij *et al*., 2009).

1.5.1.3. HEAVY METAL UPTAKE BY HYPERTOLERANT NON-HYPERACCUMULATOR PLANTS

A phenomenon known as exclusion is employed by non-hyperaccumulator plants to slow down the accumulation of these heavy metals. This principle involves plants excreting organic acids which bind to these heavy metals, thus hindering the uptake of heavy metals into plant tissues (Hossain *et al*., 2012; Rascio & Navari-Izzo, 2011).

1.6. ROS AND THEIR ANTIOXIDANT DEFENSE SYSTEM

One of the protective mechanisms as aforementioned that enable plants to survive these abiotic stresses is through an antioxidant pathway. It has been shown that under stressful conditions, plants overproduce molecules which are highly toxic known as ROS which include mainly the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\bullet OH$) causing oxidative damage to proteins, nucleic acids and lipids (figure 1.3) (Shao *et al*., 2008). Although when under abiotic stress these ROS molecules are detrimental to the plant, it has been confirmed that under normal conditions these molecules are involved in signaling and regulating plant growth and development (Bailly *et al*., 2008; Mittler *et al*., 2004). A study by Bailly et al. (2008) showed that favourable environmental conditions known as the 'oxidative window' occur when ROS at their optimal levels are able to promote seed germination and development (figure 1.2).

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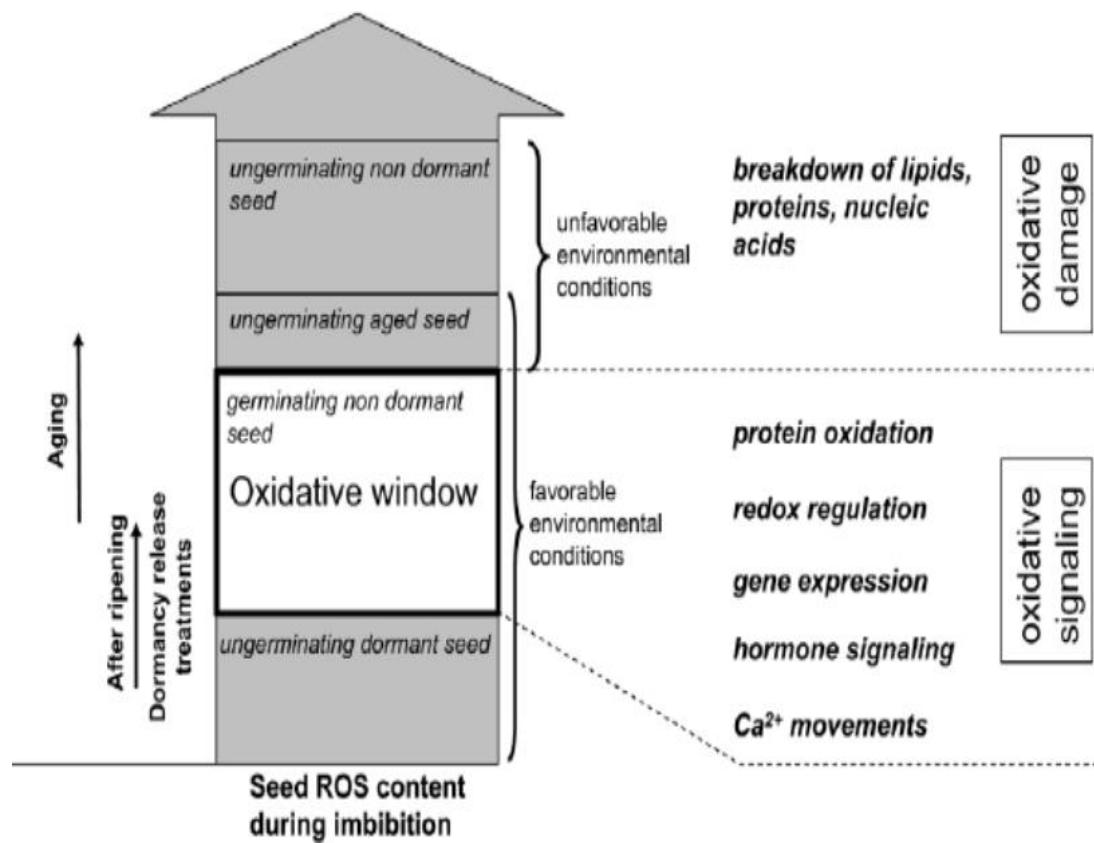
Figure 1.2. Favourable environmental conditions promote seed germination. ROS when present at their optimal levels provide various cellular signaling in plants (Bailly *et al*., 2008).

When the amount of ROS are in excess plants have evolved and thus have developed an antioxidant defense system which help reduce these specific ROS molecules, which include superoxide dismutase (SOD) that is responsible for scavenging O_2^- as well as ascorbate peroxidase (APX) which scavenges H_2O_2. These antioxidant enzymes involve a network of pathways as well as cofactors to assist the plant in alleviating these toxic molecules (Dombrowski, 2003). ROS can be divided into two groups, mainly free radical and non-free radical ROS. Free radical ROS are molecules which have a charge such as $\bullet OH^-$ and are highly reactive forms of ROS, whereas non-free radical molecules do not possess a charge such as H_2O_2 (Apel and Hirt, 2004).

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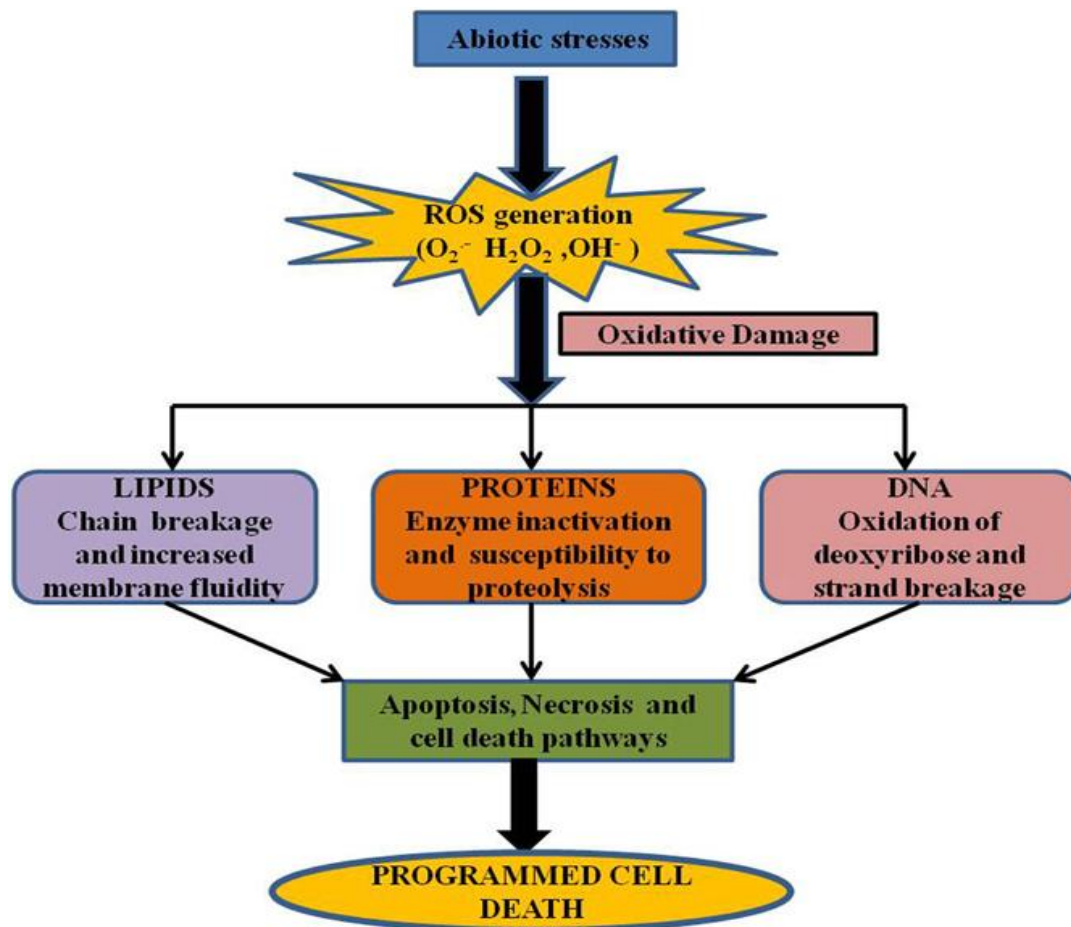
Figure 1.3. Abiotic stress in plants which may lead to cell death. Abiotic stress and organelles which produce ROS leading to oxidative damage and cell death adapted from (Rashmi *et al*., 2015).

1.6.1. HYDROXYL RADICALS (•OH)

One of the free radicals includes the hydroxyl radical which is known to be one of the most reactive compounds among the ROS (Gill and Tuteja, 2010; Babbs *et al*., 1989). When the OH^- radical comes into contact with macromolecules such as lipids, proteins and DNA, it tends to react with these macromolecules therefore damaging the cell structures which eventually results in the death of the cell if not removed (Gill and Tuteja, 2010). According to literature, the OH^- compound is produced in order to regulate the oxygen toxicity in plants (Vranova *et al*., 2002).

1.6.2. SUPEROXIDE ANION (O_2^-)

The major production site of the O_2^- radical occurs within the thylakoid membrane. When a plant experiences stress, the O_2^- is typically the first form of ROS to be produced. The O_2^- radical has been known to be moderately reactive, having a maximum half-life of 4μs (Mourato *et al*., 2012; Yang *et al*., 2011). Typically upon the generation of O_2^-, other forms of ROS are produced which result in lipid peroxidation of the plasma membrane due to the weakening of the poly-unsatuarted fatty acid (PUFAs) double bonds (Ahsan *et al*., 2003).

1.6.3. HYDROGEN PEROXIDE (H_2O_2)

Hydrogen peroxide is moderately reactive in comparison to other ROS. It also has a significant long half-life and when coupled with its high stability, it can permeate across membranes (Gill and Tuteja, 2010). Along the ascorbate peroxidase pathway, H_2O_2 gets detoxified into H_2O and is therefore known as the water-water cycle (Asada, 1999). It is known that when the level of H_2O_2 is at low concentrations, it has the ability to act as a signaling molecule which induces abiotic stress tolerance in plants (Gill and Tuteja, 2010; Quan *et al*., 2008). At high concentrations which are above basal levels, it initiates programmed cell death in plants (Quan *et al*., 2008). It has also been identified to regulate processes such as photosynthesis in plants (Gill and Tuteja, 2010). Furthermore, it has also been shown that H_2O_2 plays a role in root gravitropism. This phenomenon was proven when roots were exposed to the H_2O_2 radical and the root gravitropism increased (Joo *et al*., 2001).

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1.6.4. IMPACT OF ROS SCAVENGING ENZYMES

ROS scavenging occurs as a result of antioxidant enzymes that work together. These enzymes include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and catalase (CAT) (Vranová *et al*., 2002). Various major peroxidases which are present in plants include APX and GPX which plays an important part in the detoxification of hydrogen peroxide thus producing water as the final product (Apel and Hirt, 2004; Mittler, 2002). When the accumulation of ROS exceeds basal levels, it has various detrimental effects. The balance between the production of ROS as well as its removal by antioxidant defense systems determines the amount of damage caused by ROS within plant cells. In order to reduce these effects, plants have an antioxidant defense system which consists of both enzymatic and non-enzymatic antioxidants (figure 1.4) (Khan and Panda, 2008; Demiral and Turkan, 2005).

1.6.4. ENZYMATIC ANTIOXIDANTS
1.6.4.1. SUPEROXIDE DISMUTASE (SOD)

SOD is a metalloenzyme which is known to be a major scavenger of superoxide, as it has the ability to catalyse the conversion of superoxide (O_2^-) to hydrogen peroxide (H_2O_2) which is eliminated by ascorbate peroxidase (Lee *et al*., 2007). The SOD antioxidant acts as the first line of defense against the toxic effects of ROS above basal levels. There are various types of metal SOD cofactors which include Cu/Zn, Mn and Fe SODs, each of which is located in different organelles (Mittler, 2002). The organelles in which the various SODs are located are as follows; the Mn SOD is located in the mitochondria and peroxisomes, the Cu/Zn SOD in the chloroplast and cytosol and lastly the Fe SOD is situated in the

chloroplast (Tuna *et al*., 2008). By conducting negative staining, the activity of the SOD isozymes can be identified due to their sensitivity to inhibitors such as KCN and H_2O_2. It has been discovered that the Mn SOD shows resistance to both of the inhibitors, while the Cu/Zn SOD is sensitive to both of the inhibitors. However the Fe SOD has proven to be resistant to KCN and sensitive to H_2O_2 (del Rio *et al*., 2003). The level of SOD is increased or upregulated in order to reduce the oxidative stress resulting from various types of abiotic and biotic stress, such as the increase of SOD activity when plants such as *lycopersicon esculentum* are under salinity stress (Gapinska *et al.*, 2008). It is therefore evident that SOD plays a crucial role in reducing the levels of ROS (Tuna *et al*., 2008).

1.6.4.2. ASCORBATE PEROXIDASE (APX)

It is believed that APX plays the most important role in ROS scavenging and is located in various plant organelles such as the mitochondria, peroxisomes, chloroplasts and cytosol (Maruta *et al*., 2012; Sinha and Saxena, 2006). APX is known to have five isoforms, each in different organelles which include the thylakoid, cytosol and stroma (Miyake and Asada, 1996). It is responsible for the enzymatic conversion of H_2O_2 to H_2O thereby lowering the concentration of H_2O_2 during the ascorbate-gluthathione cycle, by using ascorbate as an electron donor. The APX then becomes monodehydroascorbate (MDHA) with the use of ascorbate as a substrate. In order for the plant to keep regenerating ascorbate, the enzyme monodehydroascorbate reductase (MDHAR) reduces MDHA into ascorbate by oxidizing NADPH (Kangasjarvi *et al*., 2008). During various stress conditions, high expression of APX activity levels resulted thus renderring the plant being more tolerant to that specific stress and therefore plays a key role in the ROS scavenging

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mechanism (Kornyeyev *et al.*, 2003). It has been reported that tobacco plants with high chloroplast APX activity are much more tolerant to salinity stress (Hamid-Badawi *et al.*, 2004).
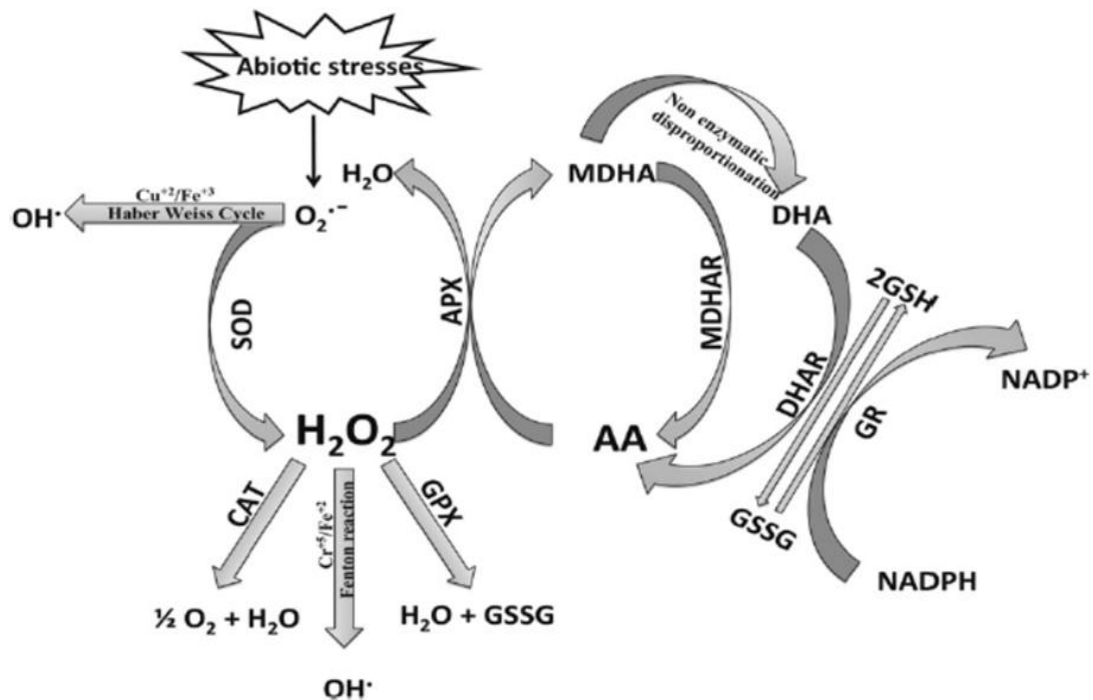
Figure 1.4. ROS and its antioxidant defense mechanism. Plant cells as well as its organelles have antioxidant defense mechanisms which include SOD, APX, MDHAR, DHAR and GR from (Gill and Tuteja, 2010).

1.6.4.3. CATALASE (CAT)

The catalase antioxidant enzyme was the first to be discovered in the 1800s. It converts H_2O_2 to O_2 and H_2O (Mhamdi *et al.*, 2010). It has been observed that one CAT molecule has the ability to convert six million molecules of H_2O_2 to H_2O per minute, thereby proving to have the highest turnover rates in comparison to other enzymes. CAT also functions in removing H_2O_2 which is formed in peroxisomes during the process of oxidases (Polidoros and Scandalios, 1999). There are around

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12 isozymes of CAT found in Brassica which have shown both to be spatially and temporally regulated as well as showing different responses in the presence of light (Skadsen *et al*., 1995; Willekens *et al*., 1994).

1.6.4.4. GLUTATHIONE PEROXIDASE (GPX)

GPX has an important role in assisting the plant in relieving the amount of oxidative stress present by utilising GSH in order to reduce the amount of H_2O_2 present in plant cell into H_2O as well as having the ability to reverse lipid peroxidation (Noctor *et al.,* 2002). It has been observed that high GPX activity renders the transgenic tobacco tolerant to salinity stress (Yoshimura *et al*., 2004).

1.6.4.5. GLUTATHIONE REDUCTASE (GR)

GR is an oxidoreductase which has been found in organelles such as the chloroplasts as well as being present in small amounts in cytosolic and mitochondrial regions (Edwards *et al*., 1990; Creissen *et al*., 1994). GR has an active role in the glutathione-ascorbate cycle as it is plays a role in the enzymatic reaction by utilizing NADPH in catalysing the formation of disulphide bonds of GSSG. It has been shown that in some abiotic stress experiments such as in drought stressed rice seedlings, the activity of GR has increased (Leterrier *et al*., 2005).

1.7. NO AND ALLEVIATION OF HEAVY METAL- INDCUED OXIDATIVE STRESS

According to literature it has been observed that NO is associated with regulating various biological processes within plant systems (Neill *et al*., 2003). Over recent years, there has been a significant interest in the effects of exogenous NO on potentially alleviating heavy metal stress, namely aluminium and arsenic (Xiong *et*

al., 2009). It has been observed that when soybean cells were treated with the metal cadmium (Cd), there was a rapid increase in the production of NO suggesting that NO is involved in signaling during the alleviation of heavy metal toxicity (Kopyra and Stachon-Wilk, 2006). According to Gould et al. (2003), in tobacco leaf suspensions when exposed to high temperature and salinity conditions, there was an increase in NO production. It has been established that NO has the ability to protect plants undergoing oxidative stress by regulating various antioxidative enzymes (Laspina *et al*., 2005). It has been demonstrated that exogenous application of NO on *Lupinus luteu* reduces the toxic effects caused by heavy metals on root growth, suggesting that the protective nature of NO can be potentially attributed to an increase in antioxidant activity mainly to the SOD enzyme (Kopyra & Gwozdz, 2003). NO interacts with ROS metabolism thus interacting with ROS itself directly (Terwari *et al*., 2008). It has thus been established that the interaction between NO and ROS, depending on their respective concentrations can be either detrimental or protective to the plant (Beligni & Lamattina, 1999).

1.8. MINERAL NUTRIENTS AND THEIR ROLES IN ALLEVIATING HEAVY-METAL TOXICITY

In order for plants to grow optimally, the essential nutrients required, need to be within a sufficiency range in order to maximize plant growth (figure 1.6). When these nutrients are present in excess quantities, they become highly toxic resulting in a reduction in overall plant growth and development (Shanmugam *et al*., 2011; Williams and Pittman, 2010). Similarly, a decline in growth occurs when nutrients are not available in sufficient quantities in order to meet the plants requirements, renders the plant to be nutrient deficient (Schmidt, 1993).

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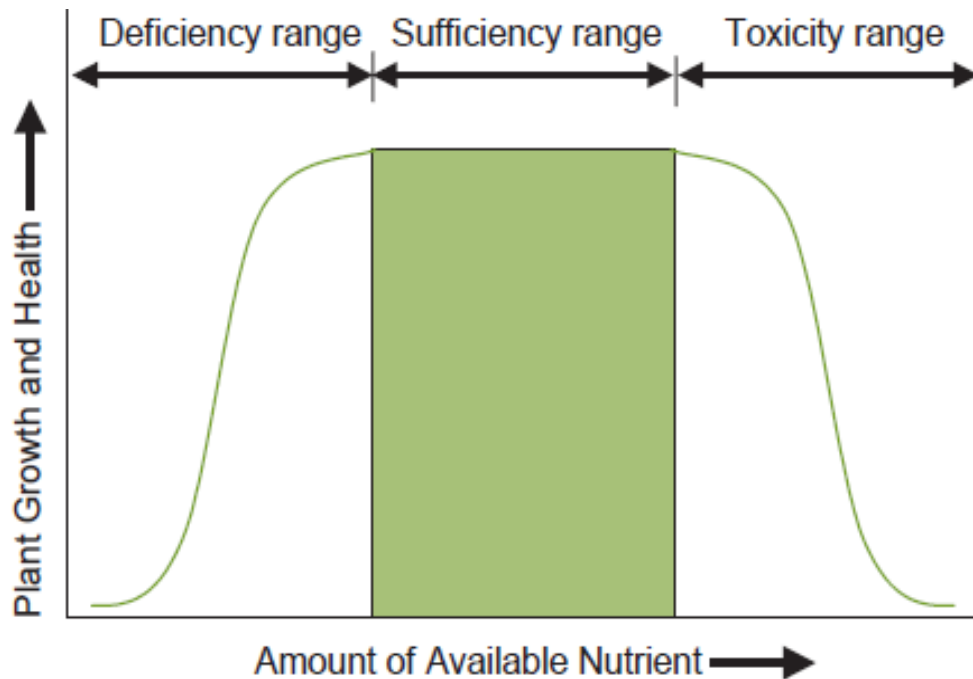
Figure 1.5. Relationship between plant growth and nutrient availability. Plant growth is determined by a specific threshold in which optimal nutrient functioning occurs (Brady and Weil, 1999).

Over the years, high concentrations of heavy metals have contaminated large areas of lands due to various anthropogenic activities (Jarup, 2003). As angiosperms evolved, macro and micronutrients at basal concentrations played significant roles with regards to plant growth and alleviating heavy metal toxicity (figure 1.5). One of the major nutrients is nitrogen (N), as it is the major constituent of nucleic acids, vitamins and proteins. It increases chlorophyll synthesis by enhancing the photosynthetic capacity thus enhancing antioxidant enzymes and eventually alleviating toxicity caused by heavy metals (Lin *et al*., 2011; Sharma and Dietz, 2006). It has been observed that other nutrients such as phosphorous (P) which forms major parts of cellular membranes as well as being required for the process of phosphorylation is involved in alleviating heavy metal toxicity by dilution of the metals (Sarwar *et al*., 2010). Elements known as trace elements at low

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concentrations are crucial for biological systems. They have been known to play important roles in the alleviation of heavy metal toxicity, in which the effects on the metal are either direct or indirect (Sarwar *et al*., 2010). The trace elements and heavy metals compete for the same membrane transporters (Qiu *et al*., 2005; Baszynski *et al*., 1980). An increase in the plants biomass provides the ability for dilution of the heavy metal concentration to take place thus resulting in alleviation of the heavy metal toxicity by increasing the antioxidant system (Jalloh *et al*., 2009; Hassan *et al*., 2005; Suzuki, 2005).
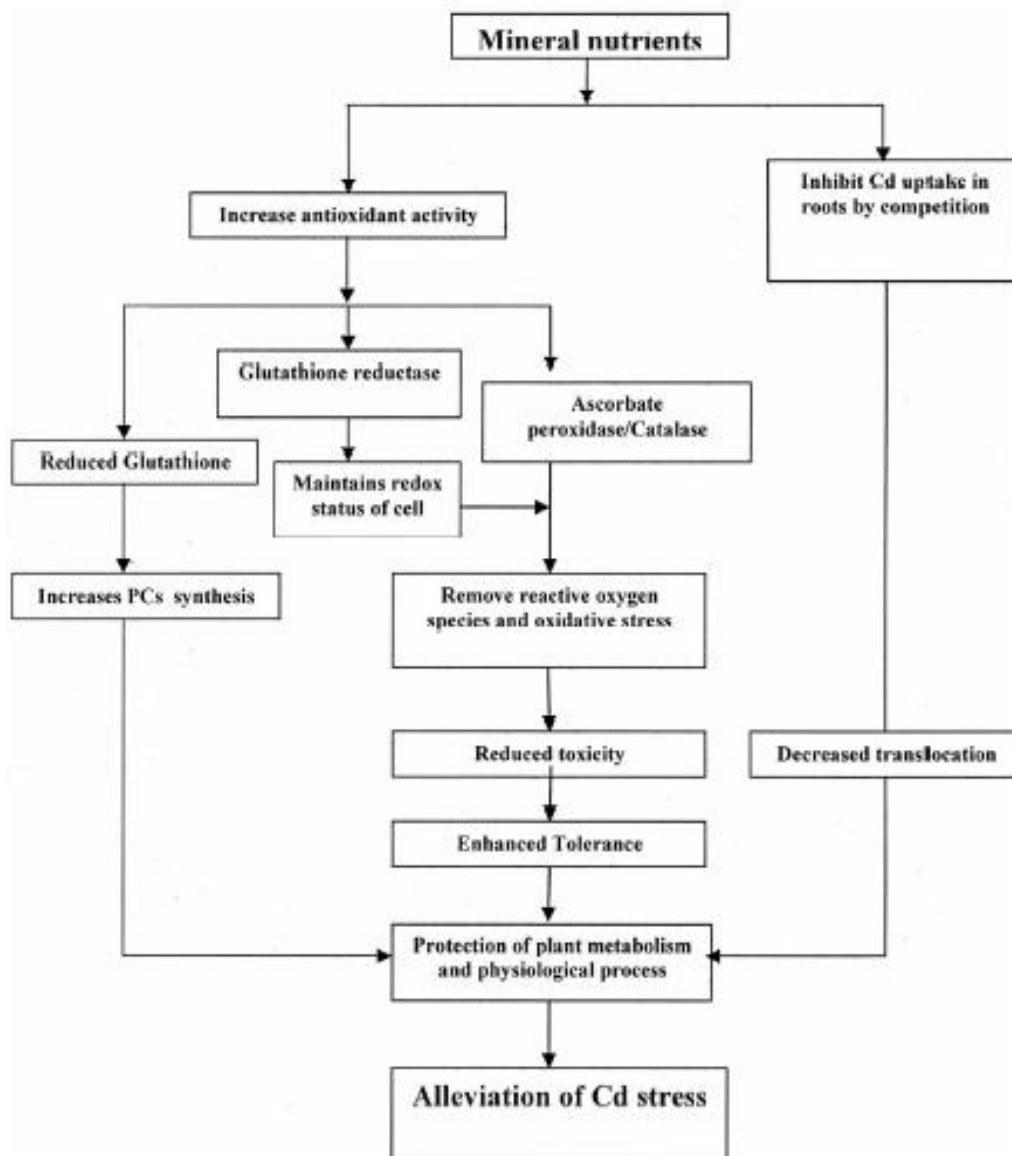
Figure 1.6: Role of mineral nutrients in antioxidant defense system. The Physiological and metabolic processes of mineral nutrients associated with the alleviation of Cd stress in plants (Nazar *et al*., 2012).

It is therefore essential to use analytical tools such as Inductively coupled plasma optical emission spectrometry (ICP- OES) in order to quantify and analyze the elemental composition within plants, in response to heavy metal conditions (Salt *et al*., 2008).

1.9. OBJECTIVES

The aims of this study were firstly to determine, if any, the effects (beneficial/detrimental) of MA at a range of concentrations in *B*. *napus* L. seedlings. Seedling physiology, lipid peroxidation and cell viability in response to MA concentrations will be investigated. The quantification of ROS and their respective antioxidant enzymes will also be analyzed by means of spectrophotometric assays in response to various MA concentrations, with the goal of obtaining the optimal MA concentration for enhanced seedling growth and development.

Due to the scarcity of research conducted on the effects of Zr toxicity in plants; the same tests will be performed using the optimal MA concentration combined with Zr stress, thereby establishing whether the supplementation of MA can decrease the toxicity caused by Zr.

Furthermore, the effect of MA on NO activity within seedlings will be determined in order to establish if by exogenously applying MA, possible enhancement of NO activity would occur. Inductively coupled plasma-optical emission spectrometry (ICP- OES) will be performed in order to analyse seedling mineral nutrient content in response to MA and Zr treatments respectively.

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CHAPTER TWO

MATERIALS AND METHODS

2.1. PREPARATION OF MA

MA (0.05 µM) was prepared by adding an amount of 10 mg to a 50 ml Greiner tube, followed by the addition of 235 µl of Tween 80 and 10 ml of 100% ethanol which was mixed by vortexing. The solution was then snap frozen in liquid nitrogen followed by placing the Greiner tubes in a lypholizer overnight, to remove the ethanol. An amount of 2 µM MA was added to deionised water to make up a stock with a final volume of 20 ml. The stock was diluted to make up a working concentration of 0.05 µM and 15 µM MA containing 0.0001% Tween 80.

2.2. GERMINATION, TREATMENTS AND HARVESTING OF *B. NAPUS* L. SEEDLINGS

Steam sterilized potting soil was mixed with filter sand in a 1:2 ratio. Some pots were pre-treated with a concentration of 0.05 µM and others with 15 µM MA and the rest were pre-treated with Tween 80 which served as a control. A total of five seeds were sown per pot the following day. Furthermore, a volume of 100 ml of 0.05 µM, 15 µM MA and Tween 80 solutions were used to treat the seeds twice per week (every third day). The germinated seedlings were further treated until reaching a two leaf stage, followed by harvesting for dry weights and for conducting biochemical assays. Each seedling was removed from the pots following the treatment. For the biochemical assays which required ground material, seedlings were ground into a fine powder using a mortar and pestle as

well as liquid nitrogen to prevent thawing of material. For biochemical assays which required fresh plant material, the entire seedling was used.

2.3. DETERMINATION OF SEEDLING DRY WEIGHTS EXPOSED TO MA AND ZR TREATMENTS RESPECTIVELY

In order to determine dry weights of seedlings, a total of four seedlings per treatment were placed in foil envelopes. To calculate statistical significance, a total of three foil envelopes (n=3) (each containing four seedlings) were evaluated. All the foil envelopes were then incubated at 80 °C for three days, followed by determining the dry weights using a fine balance.

2.4. PROTEIN EXTRACTION OF SEEDLINGS

Proteins were extracted from both the untreated as well as from the MA treated seedlings. A triple extraction was performed to extract the protein from each sample by adding approximately 100 mg of each sample into three clean Eppendorf tubes. A volume of 500 μl of protein extraction buffer (40 mM phosphate buffer, pH 7.4, 1 mM EDTA and 5% (w/v) PVP) was added to one of the three tubes. The tubes were then mixed vigorously using a vortex, followed by pelleting the plant material using a centrifuge at 13,000 xg for 5 minutes and then transferring the supernatant into the second tube containing 100 mg of ground frozen plant material. The procedure was then repeated for the next two tubes (2 and 3) followed by transferring the supernatant from the third tube into a final clean tube. The final protein extract was then used for quantification using a Bradford assay. The protein extract were then stored at -20 °C until used.

2.5.   PROTEIN EXTRACTION (TCA)

Approximately 100 mg of ground seedling material was obtained, transferred to an Eppendorf tube followed by the addition of 5X the volume of Tricholoroacetic acid (TCA) (6%). The tube was then vortexed for 5 minutes followed by centrifugation at 13 000 x g for 12 minutes. The supernatant was then transferred to a clean Eppendorf tube for further analysis.

2.6.   PROTEIN CONCENTRATION DETERMINATION

The concentration of the extracted proteins was determined as specified by the manufacturer of Biorad Quick Start 1x Bradford's reagent.

2.7.   SUPEROXIDE (O$_2^-$) CONTENT DETERMINATION

A modified method of Russo et al. (2008) was used to determine the superoxide content. A total of six eppendorf tubes were prepared each containing 10 mM potassium cyanide, 10 mM H$_2$O$_2$, 2% SDS and 80 µM NBT made up to a final volume of 800 µl with 50 mM potassium phosphate pH 7.0. From the germination experiment, the entire seedling shoot were used as material to perform the assay which determines superoxide levels within the seedling shoots. The material was then inserted into six different eppendorf tubes which were then incubated at room temperature for 20 minutes, followed by crushing the material with a miniature pestle. The tubes were then centrifuged at 13,000 x g for 5 minutes in order to obtain a pellet, followed by transferring the supernatant to clean eppendorf tubes and then centrifuge again at 13,000 x g for 5 minutes to obtain a pellet from the remaining material. A volume of 200 µl of each sample was loaded in triplicate into

a Greiner 96 well flat bottom plate. The absorbances were recorded at a wavelength of 595 nm using the OMEGA spectrophotometer.

2.8. HYDROGEN PEROXIDE (H$_2$O$_2$) CONTENT DETERMINATION

A modified method of Velikova et al. (2000) was followed to determine H$_2$O$_2$ content within seedling shoots. In order to determine the amount of H$_2$O$_2$ present within the seedling shoots following treatment, an H$_2$O$_2$ assay was performed using an amount of approximately 100 mg of grounded material. The 0.05 µM, 15 µM MA treated and 0.0001% Tween 80 treated seedling material was each added to separate 1.5 ml Eppendorf tubes. A volume of 500 µl of 6% TCA was added to each Eppendorf tube, followed by vigorously vortexing the tubes. The tubes were then centrifuged at 13,000 x g for a further 5 minutes. The supernatant was then transferred to clean 1.5 ml Eppendorf tubes. The reaction mixture consisted of 50 µl of 0.5 M KI and 5 mM of K$_2$HPO$_4$ in a final volume of 200 µl. A 96 well microtitre plate containing the reaction mixtures were incubated at room temperature for a period of 20 minutes. The absorbances were recorded at a 390 nm wavelength using the OMEGA spectrophotometer. The H$_2$O$_2$ levels were calculated by means of a standard curve which was constructed using known H$_2$O$_2$ levels.

2.9. SUPEROXIDE DISMUTASE (SOD) ACTIVITY ASSAY

A modified method of Beauchamp and Fridovich, (1971) was followed in order to determine the level of SOD activity within the seedling shoot. The reaction mixture contained 20 mM phosphate buffer (pH 7.5), 0.1 mM NBT, 0.1 mM EDTA, 0.005 mM riboflavin and 10 mM methionine. The samples of the various treatments 0.05

μM, 15 μM MA and 0.0001% Tween 80 treated were then diluted, followed by loading 10 µl of the sample in triplicate to a 96 well microtitre plate. A volume of 190 µl of the mastermix was then loaded to each of the sample wells followed by covering the microtitre plate with foil and then placing it on a shaking incubator for 5 minutes. The microtitre plate was then placed on a light box for 10 minutes to allow the reagents to react thus producing O_2^-. The absorbance was ultimately recorded at a wavelength of 560 nm using the OMEGA spectrophotometer.

2.10. ASCORBATE PEROXIDASE (APX) ACTIVITY ASSAY

In order to determine the total ascorbate peroxidase (APX; EC 1.11.1.11) activity within the seedling shoots, a modified method from Asada (1984) was followed where the reaction mixture contained 10 µl PVP protein extract and 180 µl buffer (50 mM K_2HPO_4 (pH 7.0), 0.2 mM EDTA and 0.25 mM ascorbic acid) in a final volume of 190 µl. A volume of 10 µl H_2O_2 (90 μM) was added in order to initiate the reaction followed by the absorbance measured at a wavelength of 290 nm. Based on the change in absorbance, APX activity was determined using the extinction coefficient of 2.8 mM $^{-1}$ cm^{-1}.

2.11. LIPID PEROXIDATION DETERMINATION BY MALONDIALDEHYDE (MDA) QUANTIFICATION

A modified method of Zhang et al. (2007) was followed for the lipid peroxidation assay. An amount of approximately 100 mg of seedling shoot material was ground using liquid nitrogen, and was added into separate 1.5 ml Eppendorf tubes. To each of the Eppendorf tubes, 5 volumes of 6% (w/v) TCA were added. The tubes were mixed by vortexing followed by centrifugation at 13,000 xg for 10 minutes in order

to pellet the seedling material. A volume of 200 µl of the supernatant was removed and added to a clean Eppendorf tube in which a volume of 300 µl of 0.5% (w/v) TBA and 20% (w/v) TCA was added. The tubes were then placed on a heating block at 90 °C for a period of 20 minutes, followed by incubating the tubes on ice for 10 minutes. The samples were then centrifuged at 13,000 x g for 5 minutes followed by loading in triplicate onto a 96 well microtitre plate which was read on a spectrophotometer at wavelengths 532 nm and 600 nm. The absorbance obtained at 600 nm was subtracted from the absorbance at 532 nm to correct for non-specific turbidity. The MDA concentration was calculated using the extinction coefficient of 155 mM^{-1}.cm^{-1}.

2.12. ESTIMATION OF CELL VIABILITY (EVANS BLUE UPTAKE) ASSAY

A modified method of Sanevas et al. (2007) was followed when conducting the cell death assay. A cell death assay was performed in order to measure the amount of Evans blue dye retained by non-viable cells within the seedling shoot. The entire seedling shoot was placed into a 15 ml greiner tube containing a volume of 10 ml of 0.25% Evans blue respectively followed by incubation for 1 hour at room temperature. The Evans blue solution was then removed from seedlings by rinsing with distilled water. In order to wash off all the unbound dye, the seedlings were incubated in distilled water overnight at room temperature. The distilled water was discarded followed by inserting the seedlings into a volume of 1 ml of 1% (w/v) SDS solution. The seedlings were then crushed using a small pestle and incubated at 65 °C for a period of 1 hour in order to release the trapped Evans blue from the cells. Following incubation, the material was centrifuged at 13,000 x g for 5 min in

order to pellet the plant material. The supernatant was then transferred to clean Eppendorf tubes followed by centrifuging to pellet the excess debris. A volume of 200 µl of each sample was transferred to a 96 well Greiner microtitre plate and loaded in triplicate. The absorbances were recorded by spectrophotometrical analysis using an OMEGA spectrophotometer at a wavelength of 600 nm.

2.13. NITRIC OXIDE (NO) CONTENT DETERMINATION BY SPECTROPHOTOMTERIC QUANTIFICATION

A modified method of Murphy and Noak (1994) was followed when conducting the NO assay. Protein extracts were obtained from plant seedling shoots (± 100 mg) by grinding the seedling shoots into fine powder in liquid nitrogen followed by homogenizing the powder with 1ml of homogenizing buffer [40 mM K_2HPO4, pH7.4, 1 mM EDTA, 5% (w/v) polyvinylpyrrolidone (PVP)]. The homogenates were then centrifuged at 13 200 x g for 30 min and incubated with 100 Units of catalase and superoxide dismutase respectively for 10 min, followed by addition of oxyhaemoglobin to a final concentration of 10 µM. The mixture was incubated for 2 min, followed by spectrophotometric measurement of NO content by following the conversion of oxyhaemoglobin to methaemoglobin at 401 and 421 nm.

2.14. QUANTIFICATION OF MINERAL ELEMENTS BY INDUCTIVEY COUPLED PLASMA-OPTICAL EMISSION SPECTROMETRY (ICP- OES)

In order to quantify K^+, P, Mg^{2+}, Ca^{2+}, Fe^{2+}, Zn and Zr in seedlings of *B. napus* .L, an amount of ± 200 mg of seedling shoot material were digested in 1ml of 65% HNO_3 at 75°C followed by incubation for 1 hour. Each sample, following the

incubation, was diluted (1:10, v/v) in 4% HNO_3 and analysed by ICP- OES (Varian Vista Pro).

2.15. STATISTICAL ANALYSIS

Statistical analysis for all assays conducted was performed using the Duncan's Multiple Range Test (DMRT) at $P < 0.05$ and validated statistically on standard error (SE). The significance was represented by different alphabetical letters.

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CHAPTER THREE
EFFECT OF EXOGENOUS MA ON GROWTH AND GERMINATION OF *B. NAPUS* L. SEEDLINGS

3.1. INTRODUCTION

Lipids can be classified into various groups such as fatty acids which can be further divided into unsaturated and saturated fatty acids (Garret and Grisham, 2006). According to Isenberg et al. (2007) it was observed in mammalian systems, that myristic acid (MA) modulated endothelial nitric oxide (NO) signaling. NO has many key roles in plant processes such as improving plant growth as well as having the ability to maintain cellular redox homeostasis during abiotic stress (Shingles *et al*., 1996). It has also been observed that phosphatidic acid (PA) which is a lipid can modulate stress responses in plants. During abiotic stress conditions the intracellular PA levels have been noted to increase (van der Luit *et al*., 2000; Young *et al*., 1996). It has therefore been suggested that PA has the ability to act as a secondary messenger in various stress-signaling pathways as well as mediating responses to these stresses (Munnik, 2001). It has been reported that this lipid also functions in regulating various cellular processes mainly the regulation of ROS production (Sang *et al*., 2001). A study conducted by Park et al. (2004) showed that PA induced cell death in leaves as well as increasing the levels of ROS in the entire leaf. Thus, we hypothesised that by exogenously adding MA to plants at various concentrations the level of ROS could either remain steady thereby promoting

germination, growth and development or promote oxidative stress thus decreasing germination and growth, leading to cellular death. One of the mechanisms that enable plants to survive abiotic stresses is through an antioxidant pathway. It has been shown that under stressful conditions, plants overproduce molecules which are highly toxic known as reactive oxygen species (ROS) which include mainly the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\bullet OH$) causing oxidative damage to proteins, nucleic acids and lipids (Shao *et al*., 2008).

Although, when under abiotic stress these ROS molecules are detrimental to the plant, it has also been identified that under normal conditions these molecules are involved in signaling and regulating plant growth and development (Mittler *et al*., 2004). When the amount of ROS are in excess, plants have evolved or developed an antioxidant defense system which help reduce these specific ROS molecules, which amongst others include superoxide dismutase (SOD) which is responsible for scavenging O_2^- and ascorbate peroxidase (APX) which scavenges H_2O_2. These antioxidant enzymes involve a network of pathways as well as cofactors to assist the plant in alleviating these toxic molecules (Dombrowski, 2003).

It has been established that oil seeds have an important role in food security and industry globally. A crucial factor for the cultivation of oil seeds under various environmental conditions is a seed product which has a high rate of germination. Seed germination and the early stages of seedling growth are the most stress sensitive stages. It has been identified that oil seeds are very sensitive to lipid peroxidation of the plasma membrane, which are caused by the oxidation of lipids (Dell-Aquilla & Beweley, 1989). As previously noted, ROS at exceedingly high levels can damage essential membrane lipids, proteins and nucleic acids (Noctor

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and Foyer, 1998). Some authors have however shown that the production of ROS during seed germination may be a beneficial biological reaction coupled with high germination capacity, seedling development and protective function against parasitic organisms during germination (Schopfer *et al*., 2001).

Canola and rapeseed are the second largest oilseed crops in the world and currently contribute close to 14% of the total world production of oilseeds. Canola oil is known for its health benefits and recently being most commonly used as cooking oil as well as in manufacturing of margarines. When comparing other vegetable oils, canola oil has the highest amount of omega-3 fatty acids which have been proven scientifically to reduce LDL cholesterol levels. Due to the fact that the canola crop is high in nutritional value, it can make a valuable contribution to the needs of people in South Africa (De Kock and Agenbag, 2009). There have been few studies focusing on canola, specifically *B. napus* L. as it is the only species of canola that is commercially cultivated in South Africa, and the effects of oxidative stress on its germination capacity. The objective of this study is to determine the effect of MA at various concentrations on the germination, growth and development of seedlings following treatments.

3.2. RESULTS

3.2.1. THE EFFECTS OF MA ON THE GERMINATION RATE OF *B. NAPUS* L. SEEDS

Seeds were subjected to various concentrations of MA to investigate potential germination enhancement properties, thus various MA concentrations were used in order to determine the optimal concentration at which germination enhancement occurs (result not shown). The results (figure 3.1) indicated a ≈74% increase in

germination percentage when comparing the 0.05 µM MA treated seeds to the control; however a reduction of ±35% was observed when comparing the 15 µM MA treatments to the control. Furthermore, a significant increase of ≈152% was observed when comparing the germination percentage of 0.05 µM to the 15 µM MA treatment.
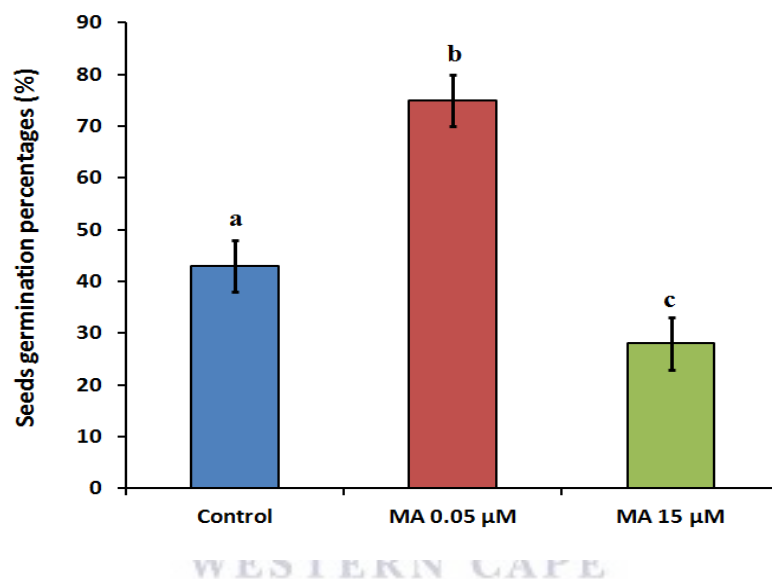
Figure 3.1. Effect of MA treatments on germination of seeds. Control has been treated with 0,0001% Tween 80, experiment has been treated with 0.05 µM and 15 µM MA. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ≈ S.E (N=5).

3.2.2. THE INCREASE IN SEEDLING BIOMASS IN RESPONSE TO EXOGENOUS APPLICATION OF MA

Biomass determination of seedlings was investigated by measuring their respective dry weights in response to the various MA concentrations. For seedling dry weights (figure 3.2) there was an increase of ≈45% when comparing the 0.05 µM MA treatment to the control. A reduction of ≈47% in seedling dry weights was observed when comparing the 15 µM treatment to the control. An increase of ≈176% in

seedling dry weight was observed when comparing the 15 µM to the 0.05 µM MA treatment.
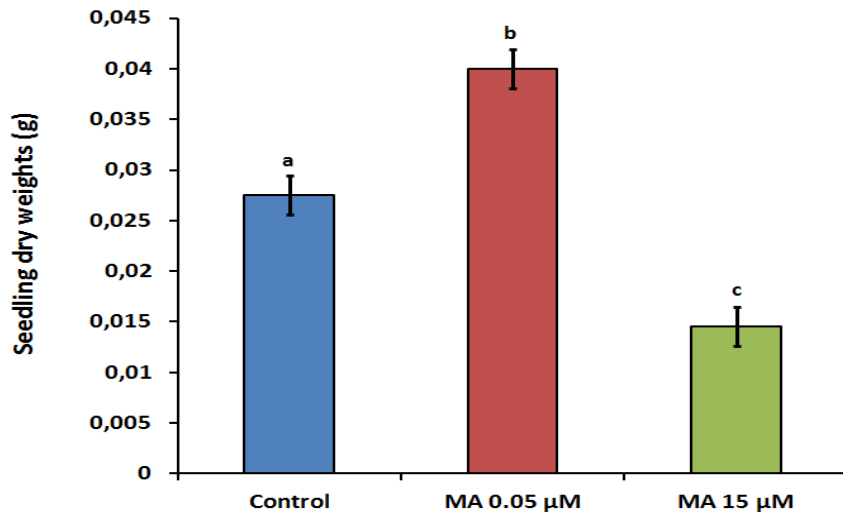
Figure 3.2. The dry weights of seedlings exposed to various MA treatments. Control has been treated with 0,0001% Tween 80 and experiment has been treated with 0.05 µM and 15 µM MA respectively. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ≈ S.E (N=3).

3.2.3. EXOGENOUS MA CONFERS AN INCREASE IN SEEDLING LENGTH

After exposing seedlings to various concentrations of MA any physiological changes which might have occurred were observed. The results (figure 3.3) show an increase in seedling shoot length of ≈20% when comparing the 0.05 µM MA treatment to the control. A reduction of ≈20% in seedling shoot length was observed when comparing the 15 µM MA treatment to the control. Furthermore an increase of ≈33% in the length of the seedling shoots was observed when comparing the 15 µM to the 0.05 µM MA treatment.
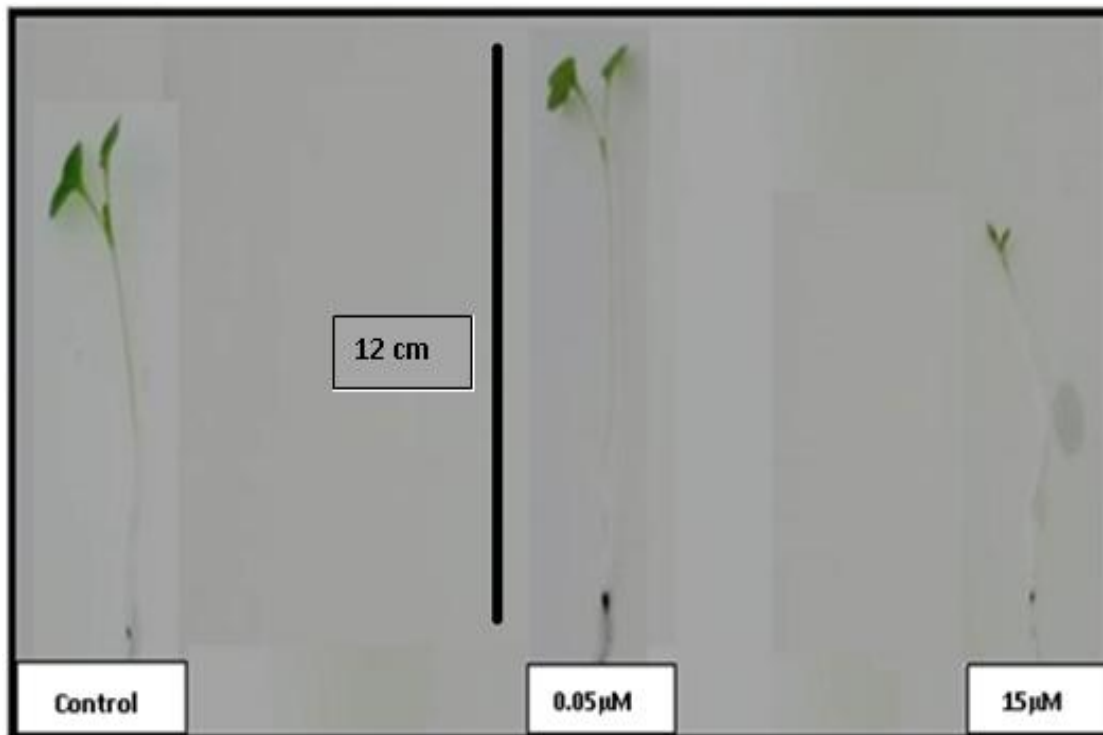
Figure 3.3. The effects of various MA concentrations on *B. napus* L. seedling shoots. Control has been treated with 0,0001% Tween 80 and experiment has been treated with 0.05 μM and 15 μM MA. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ≈ S.E (N=3).

3.2.4. ROS QUANTIFICATION BY SPECTROPHOTOMETRIC ASSAYS

According to previously obtained results, it is evident that MA at high concentrations may lead to an increase in the level of ROS above basal levels within the plant, therefore decreasing its germination percentage; stunting its growth and eventually causing cell death. Therefore, by measuring the levels of O_2^- and H_2O_2 the ROS status within the seedlings can be achieved and observed.

3.2.5. DETERMINATION OF SUPEROXIDE (O_2^-) CONTENT

The results following the O_2^- assay indicate that when comparing the 0.05 μM MA treatments to the control there was a ≈25% increase in superoxide content within

the seedlings. Furthermore, when comparing the 15 µM treatment to the control the results showed an increase of ≈117% of O_2^- within the seedling shoots.
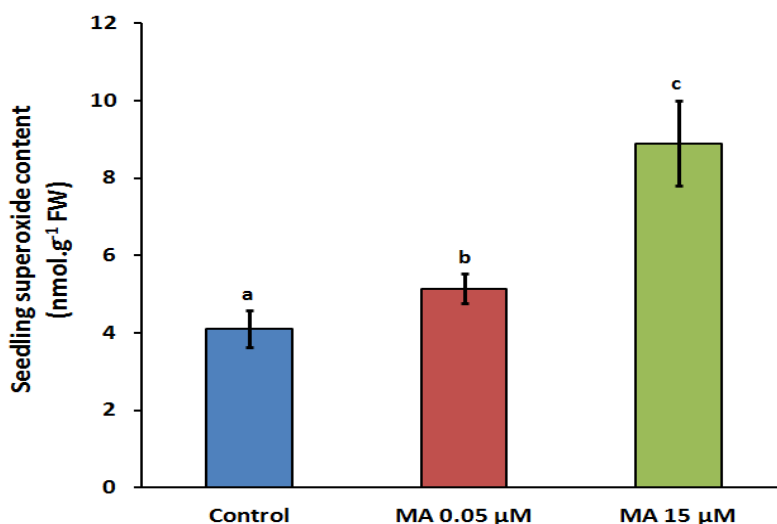
Figure 3.4. The effect of MA treatment on seedling superoxide content. Seedlings were treated with 0,0001% Tween 80, 0,05µM and 15µM MA. Superoxide levels were determined when seedlings formed two true leafs. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ≈ S.E (N=3).

3.2.6. DETERMINATION OF HYDROGEN PEROXIDE (H_2O_2) CONTENT WITHIN SEEDLINGS

In order to determine the level of H_2O_2 within seedlings following the treatment period, a H_2O_2 assay was performed. The results obtained in figure 3.5 indicated that when comparing the 0.05 µM treatment to the control, the level of hydrogen peroxide content increased by ≈16%. After treatment with 15 µM MA, the level of hydrogen peroxide increased by ≈31% when compared to the control.

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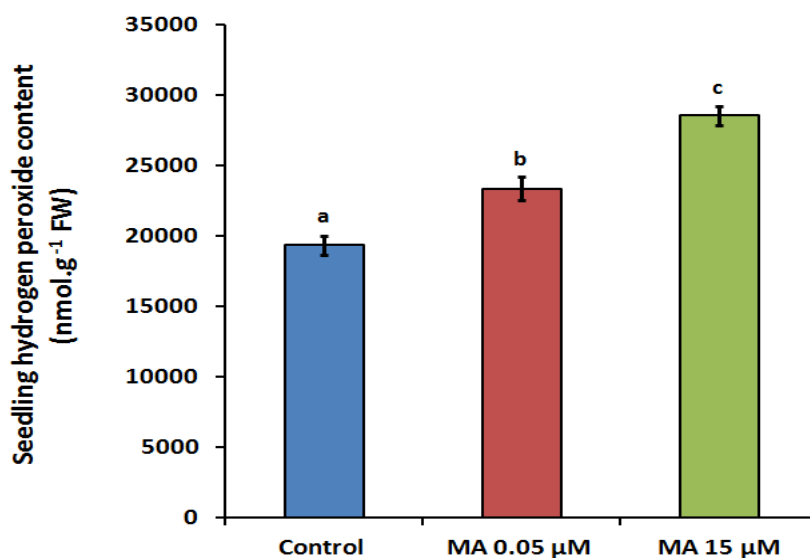
Figure 3.5. The effect of MA treatment on seedling hydrogen peroxide content. Seedlings were treated with 0,0001% Tween 80, 0.05 μM and 15 μM myristic acid. Hydrogen peroxide levels were determined when seedlings formed two true leafs. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ≈ S.E (N=3).

3.2.7. ANTIOXIDANT RESPONSE TO EXOGENOUS MA IN *B. NAPUS* L. SEEDLINGS

3.2.7.1. SUPEROXIDE DISMUTASE (SOD) ACTIVITY ASSAY

A SOD assay was performed in order to determine the amount of SOD activity occurring within the seedlings following the treatment period. The results indicated an increase of ≈46% in SOD activity when comparing the 0.05 μM treatment to the control. When comparing the 15 μM treatment to the control, the level of SOD activity increased further by ≈72%.
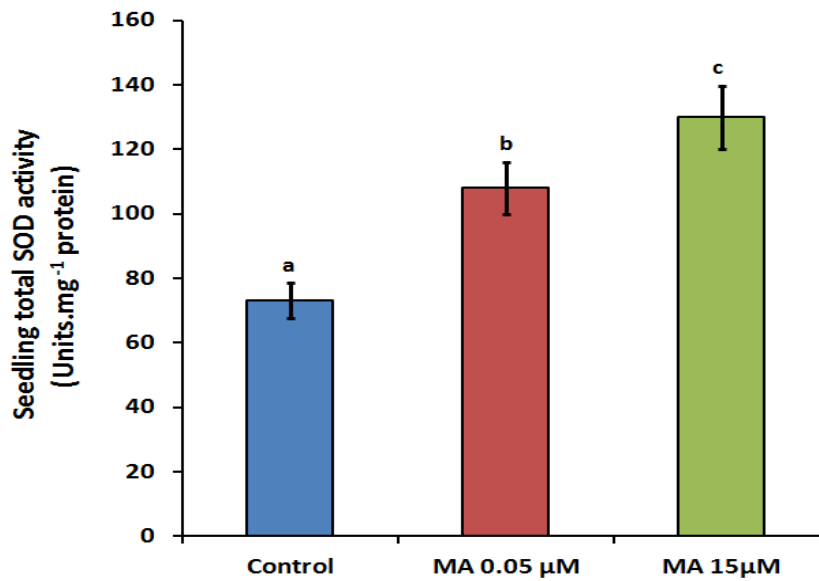
Figure 3.6. The effect of MA treatment on SOD activity of seedlings. Seedlings were treated with 0,0001% Tween 80, 0.05 μM and 15 μM MA. SOD activity was determined when seedlings formed two true leafs. Different letters indicate significant differences between means at $P< 0.05$ (DMRT). Values are means ≈ S.E (N=3).

3.2.8. LIPID PEROXIDATION WITHIN SEEDLINGS IN RESPONSE TO MA CONCENTRATIONS

The amount of lipid peroxidation occurring within the seedlings following the treatment period was measured by the amount of Malondialdehyde (MDA) present, which is produced as a product of lipid peroxidation (Zhang *et al*., 2007). The 0.05 μM treatment showed a ≈35% increase in MDA content when compared to the control. It was observed that when comparing the 15 μM treatment to the control, there was a ≈75% increase in MDA content.
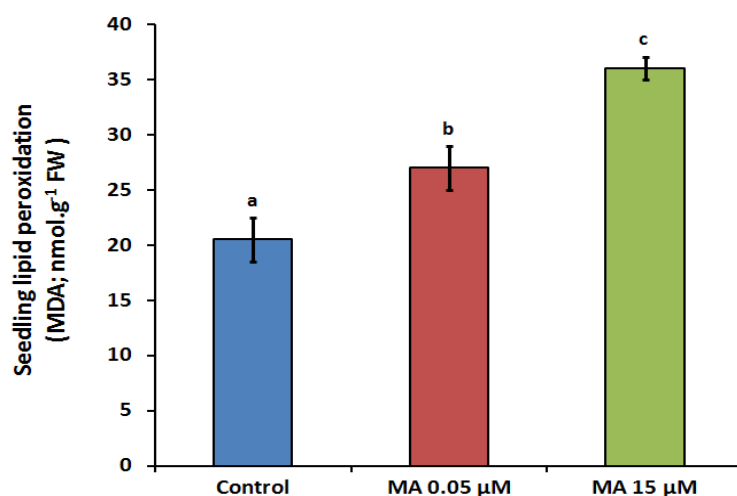
Figure 3.7. The effect of MA on lipid peroxidation within seedlings. Control has been treated with 0,0001% Tween 80 and experiment has been treated with 0.05 µM and 15 µM MA. Different letters indicate significant differences between means at $P< 0.05$ (DMRT). Values are means ≈ S.E (N=3).

3.2.9. CELL VIABILITY WITHIN SEEDLINGS IN RESPONSE TO EXOGENOUS MA

Literature states that excess levels of ROS within plants rapidly destroy biomolecules such as proteins and lipids, thus leading to cellular death (Gill & Tuteja, 2010). It was therefore necessary to investigate whether MA increased the level of ROS to the extent where cell death would occur. Cell death was measured by means of Evans blue uptake of entire seedling shoots. The results (figure 3.8) indicate that there was no statistical difference between the level of cell death between the 0.05 µM MA treatment and the control, however when comparing the 15 µM treatment to the control, there was a significant increase of ≈66% in the level of cell death within seedlings.

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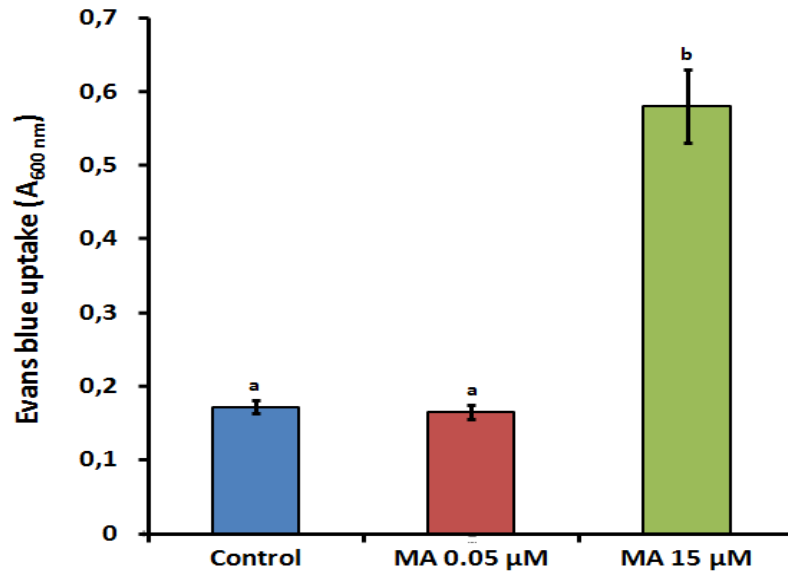
Figure 3.8. The effect of MA on the level of cell death occurring within *B. napus* L. seedling shoots. Cell death was determined by the amount of Evans blue uptake by non-viable cells. The amount of Evans blue uptake was determined within seedlings after MA treatments. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ≈ S.E (N=3).
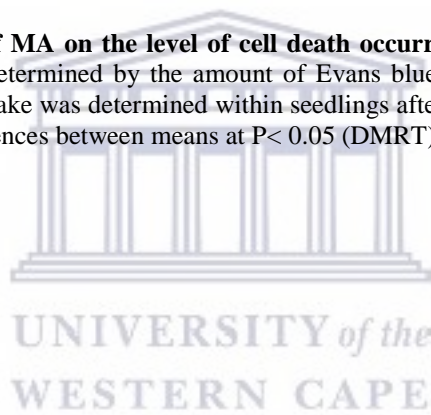
3.3. DISCUSSION

The effects of the exogenous application of fatty acids have been studied in various plant species; however influences of lipids on seed germination in canola are not known (Park *et al*., 2004). The content aforementioned, investigated the effect of the exogenous application of MA on the physiology and morphology of the seedlings of *Brassica napus* L. Various biochemical assays were conducted in order to determine the amount of ROS produced within the seedlings as well as the viability of the cells when exposed to various concentrations of MA.

When exogenously applying MA to *B. napus* L. seeds, a variation was observed in the germination percentages in response to the concentrations of MA. A study by Wanner and Stocker, (1977) showed that the germination percentage of *Nicotiana tabacum* was significantly lower when treated with fatty acids C12, C18, C20, C22 and C24; however following treatment with MA (C14) the seed germination percentage increased. In this study, it has been identified according to (figure 3.1) that MA at 0.05 μM had the highest germination percentage of seeds. This is a clear indication that by applying MA at lower concentrations, it proves to be beneficial to seeds germination. A study by Bailly et al. (2008) showed that favourable environmental conditions known as the 'oxidative window', which is when ROS are at their optimal levels and capable of promoting seed germination and development. These observations are consistent with results in our study which shows that MA at low concentrations stimulates ROS production within the 'correct' range thus being beneficial in seed germination. When MA was applied at high concentrations such as 15 μM the germination percentage decreased. A study by Ferrarese et al. (1998) suggested that fatty acids containing chains of up to 24

carbons at a specific concentration had inihibitory effects on seed germination of *Lycopersion esculentum*. The results of this study were consistent with our findings which showed that MA (C14) at high concentrations decreased seed germination significantly. An explanation for the decrease in germination percentage could be due to the fact that MA at high concentrations leads to the overproduction of ROS above basal levels which ultimately causes oxidative stress therefore having a detrimental effect on seed germination.

Various forms of abiotic stress such as drought and salt stress are known to cause endogenous changes such as increasing the level of ROS produced within the plant therefore resulting in changes in the plants morphological characteristics and thus decreasing the plants biomass. The dry weights of the seedlings were determined by depleting all the moisture present within the seedlings. According to (figure 3.2) the dry weight results indicated that seedlings treated with 0.05 μM MA showed a slight increase in biomass. There was however a dramatic decrease in seedling biomass after comparing the 15 μM MA treatment to the control. Factors contributing to a decrease in seedling biomass could be due to the fact that seedlings utilize the energy needed for growth and development to alleviate the high levels of ROS thus causing oxidative stress. The morphological structure of the seedlings such as the root, shoot and leaves were much smaller when treated with a high concentration of MA, which also contributes to a decrease in seedling biomass. Another reason which can be attributed to a reduction in seedling biomass is the reduction in mineral and water uptake by the seedling due to impaired growth and width of roots. It has also been shown by Liu et al. (2005) that the antioxidant defense mechanism of plants during their seedling stage is underdeveloped and

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therefore incapable of reducing sufficient levels of ROS in order to alleviate the oxidative stress.

After treating seedlings with various concentrations of MA the results indicated variations in growth. The results obtained (figure 3.3) indicated that when comparing the lengths of the 0.05 μM treatment to the control, there was an increase in seedling length from 10 cm to 12 cm.

A reduction from 10 cm to 8 cm was observed when comparing the control to the 15 μM treatment. The leaf, stem and root morphology of the seedlings treated with 15 μM were highly underdeveloped in comparison to the 0.05 μM treatment, due to the elevated concentration of MA. This induced the level of ROS thus initiating oxidative stress and having a detrimental impact on the growth and development of these essential structures which play crucial roles in the survival of seedlings. It can therefore be deduced from the results obtained that MA at the optimal concentration of 0.05 μM increases seedling length.

Plants have a well developed antioxidant defense system protecting them against ROS, involving both limiting the formation of ROS as well as instituting its removal. According to Yadav, (2010) superoxide (O_2^-) is the first ROS which is produced in the Foyer-Halliwell-Asada pathway in plants. The O_2^- radical is produced during the reduction of O_2 produced by the electron transport chain along the non-cyclic pathway in plant cells. In our study, according to (figure 3.4) when comparing the 0.05 μM treatment to the control, there was an increase in the level of O_2^- within the seedling. It was noted that when comparing the 15 μM treatment to the control, there was an elevated level of O_2^- content within the seedlings. This

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was an expected result, as MA at high concentrations induces an increase in the levels of ROS. This result was consistent with a study done by Park et al. (2004) who observed that the exogenous application of phosphatidic acid (PA) to *Arabidopsis thaliana* at high concentrations induces the level of O_2^- within the plant. According to Gill and Tuteja, (2010) the dismutation of O_2^- molecules is carried out by the enzymatic antioxidant superoxide dismutase (SOD). Therefore in our study it was necessary to determine the level of SOD activity within the seedlings after treatment with MA. According to the results obtained (figure 3.6), when comparing the 0.05 µM treatment to the control, there was an increase in SOD activity indicating that the level of O_2^- increased slightly following the treatment. It was noted when comparing the 15 µM MA treatment to the control; there was a significant increase in SOD activity. This may be attributed to the fact that high concentrations of MA increased the O_2^- levels significantly more within the seedlings therefore requiring SOD to perform at a higher level of efficiency in order to reduce the excess levels of O_2^- hence resulting in elevated levels of SOD activity within seedlings.

According to literature, excess levels of H_2O_2 within plant cells lead to the initiation of oxidative stress. It has been reported by Quan et al. (2008) that H_2O_2 has the ability to play a dual role in plants, at low concentrations it acts as a signaling molecule involved in triggering tolerance to various biotic and abiotic stresses and at high concentrations induces cell death. Similarly, a study conducted by Bailley et al. (2008) showed that low levels of H_2O_2 promote seed germination due to its relatively long half-life and high permeability across membranes, however when present at high levels, it prevents seeds to germinate effectively. These studies

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coincides with results obtained in our study, as the results indicate (figure 3.5) that when comparing the 0.05 μM treatment to the control, there was a slight increase in the level of H_2O_2, however when comparing the 15 μM treatment to the control the level of H_2O_2 content increased dramatically. This is a clear indication that the germination percentage of the seedlings improved when MA was applied at low concentrations due to low quantities of H_2O_2 being produced therefore improving seedling germination. At high concentrations of MA (15 μM) there was an excess level of H_2O_2 above the threshold, resulting in a significant decrease in seedling germination. It has been reported by Bhattachrjee, (2011) that H_2O_2 has a longer half-life (1ms) compared to that of O_2^- which has a half-life of 2-4 μs, thus resulting in H_2O_2 being more detrimental to the seedlings due to the fact that it remains present for a longer period of time within the seedling thus resulting in greater damage. According to Oracz et al. (2007) the ability of seeds to germinate is linked to the accumulation of a certain level of H_2O_2, as this ROS molecule is produced at a higher level in non-dormant imbibed seeds compared to dormant imbibed seeds.

According to literature, Zhang et al. (2007) stated that lipid peroxidation is one of the downstream effects resulting from excess levels of ROS which are believed to be a good indicator of tissue damage. Due to the fact that ROS has an extremely short half-life, they cannot be easily measured; however several by-products of ROS damage resulting from oxidative stress can be measured, such as thiobarbituric acid reactive substances (TBARS). In our study, a TBARS assay was employed which measures malondialdehyde (MDA) concentrations present in seedlings and thus is used to estimate the level of lipid peroxidation and damage caused by high concentrations of ROS. The results displayed (figure 3.7) that the

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0.05 μM treatment increased the MDA content slightly within the seedling when compared to the control, however it was evident that the 15 μM treatment showed the highest level of lipid peroxidation as the MDA content increased significantly when compared to the control. This result coincides with the results obtained for O_2^- and H_2O_2 establishing that MA when applied at 15 μM is detrimental inducing oxidative stress within seedlings resulting in cell death. The results were consistent with a study by Gavino et al. (1981) who observed an increase in MDA concentration within tissue cultured cells when exposed to high concentrations of fatty acids. The overall effects of lipid peroxidation are to decrease membrane fluidity significantly aswell as to increase the leakiness of the membrane to substances that do not normally cross it other than through specific channels and damaged membrane proteins (Gill and Tuteja, 2010). Another study conducted by Moller et al. (2007) observed that by increasing the amount of polyunsaturated fatty acids in plants lead to decreases in the fluidity of the membrane thus increasing leakiness and results in secondary damage to membrane proteins. Furthermore, it can be deduced thus far that saturated fatty acids such as MA has an effect on the physiological changes that occur within the plant and depending on the concentration can either be beneficial or detrimental.

It was observed by Apel and Hirt, (2004) that when seedlings were under oxidative stress it results in the damage to lipids, DNA and proteins which eventually lead to the death of the seedlings. The Evans Blue viability assay is based on the fact that cell membranes which remain intact will not take up the Evans blue reagent, therefore increasing the uptake of Evans blue indicates that damage has occurred to the cellular membrane of assayed plants. In our study, according to (figure 3.8) it

was observed that when comparing the 0.05 µM treatment to the control, there was no significant difference in the level of cell death within the seedlings which is a clear indication that MA at 0.05 µM was not detrimental to seedlings and therefore did not result in cell death. On the contrary, when comparing the 15 µM treatment to the control, the level of cell death increased significantly. This is an expected result as the previous results indicated increased levels of both O_2^- and H_2O_2 which results in higher levels of oxidative stress thus increasing the amount of cell death occurring after the 15 µM MA treatment. A proposed reason for this phenomenon is that MA at high concentrations as aforementioned increases ROS to high enough levels to cause oxidative stress thus resulting in a high level of lipid peroxidation and therefore causing cell death. A study conducted by Park et al. (2004) showed that the exogenous application of phosphatidic acid (PA) at high concentrations, lead to an increase in the levels of cell death within *Arabidopsis thaliana* leaves.

The results obtained from our study indicates that low concentrations of MA (0.05 µM) treatments only increase ROS levels to where signaling can occur in germination enhancement and not to where it can result in oxidative stress.

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CHAPTER FOUR
ROLES OF MA AND NO IN ALLEVIATING ZR TOXICITY IN *B. NAPUS* L. SEEDLINGS

4.1.   INTRODUCTION

Upon the duration of a plants' life span, they are exposed to various abiotic stress factors such as drought, lack of nutrients and heavy metals thus altering their metabolism and reducing their growth and development (Mourato *et al*., 2012). The genus *Brassica* is one of the largest commercially cultivated crop plants in SA which includes over 30 different species (Rakow, 2004). Most of these species are used for animal fodder, biofuel, vegetables, condiments and oil production (Schmidt and Bancroft, 2010).

It has been established that heavy metals (HMs) have contaminated arable lands worldwide (Neilson *et al*., 2015; Nagajyoti *et al*., 2010). Due to being highly toxic in environmental conditions, HM contamination affects plants in the following ways; disruption of the plant plasma membrane causing membrane leakage and overproduction of ROS affecting nutritional composition leading to growth impairment among other deleterious effects (Emamverdian *et al*., 2015; Hossain *et al*., 2011). Amongst a vast number of HMs, Zirconium (Zr) has been the element of choice in this study due to a lack of research being conducted specifically on this metal. Zr is mined in excessive quantities in South Africa and has been established in literature to be detrimental in plants (Shahid *et al*., 2013; Fodor *et al*., 2005; Wang, 2000).

Zr when present in the soil has the ability to form stable complexes particularly with inorganic ligands such as Cl^- and SO_4^{2-}. The use of Zr over recent years, have resulted in a significant increase in environmental pollution, mainly through soil and water contamination. Generally Zr possesses a low mobility in soil and the type of media also influences its behaviour (Hao *et al*., 2010; Aznar *et al*., 2009; Muhs *et al*., 2007). Although having a low mobility in soil, quantities of Zr are absorbed by plants mainly via the root system which ultimately enters the food chain (Shahid *et al*., 2014; Fodor *et al*., 2005). It has been established that although Zr to date does not serve a functional role in the metabolic processes of plants or animals, a significant reduction in plant growth does occur due to phytotoxicity which may also impact enzymatic activity (Ferrand *et al*., 2006).

MA is a medium-chained saturated fatty acid which in plant systems is present in minute quantities. According to previous findings (chapter 3) obtained, exogenous MA has been observed to be concentration dependant, at higher levels proved to be detrimental and at much lower concentrations beneficial to plants. Literature has established that free fatty acids have the ability to regulate heavy metal tolerance (Kachroo and Kachroo, 2009; Upchurch, 2008). There has been a strong correlation between saturated fatty acids and NO stimulation in plants. A study by Mandal et al. (2012) reported that Oleic acid demonstrated to modulate NO-associated proteins in Arabidopsis which in turn regulated NO-mediated defense signaling.

The role of NO in alleviating heavy metal toxicity has been well established in literature, as NO is involved in scavenging via the Fenton-reaction (Fe^{2+}) as well as regulating antioxidant enzymes which protect plants against the deleterious effects of oxidative stress (Arasimowicz and Floryszak-wieczorek, 2007; Laspina *et al*.,

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2005; Wink *et al*., 1995). NO is also actively involved in ROS metabolism and may be detrimental or beneficial depending on the relative concentration between the interactions of ROS/NO (Beligni and Lamattina, 1999).

It was therefore necessary to investigate the effects of exogenous MA at its optimal concentration on Zr stressed *B*. *napus* L. seedlings. Furthermore, we want to investigate whether the potential alleviation may be through the stimulation of NO production or by influencing the antioxidant defense system.

4.2. RESULTS

4.2.1. EXOGENOUS MA CONFERS AN INCREASE IN THE GERMINATION RATE OF *B. NAPUS* L. SEEDS UNDER ZR-TOXICITY

As aforementioned SA together with Australia constitutes 80% of the worlds Zr thus resulting in possibly high concentrations of Zr being present within SA soil and essentially decreasing surrounding crop yield. It was therefore important to investigate the effects of exogenously applied MA on the germination percentage of seeds while under Zr toxicity. The seeds were exposed to 0.05 μM MA, 1 mM Zr and 0.05 μM MA + 1 mM Zr respectively. The results (figure 4.1) indicate that there was a significant reduction of ≈42% in the germination percentage of Zr treated seeds in comparison to the control, however an increase in germination percentage of ≈70% was observed when comparing the control to the MA treated seeds. In the MA+Zr treatments, despite the presence of Zr stress, there was an increase of ≈37% compared to the control. Furthermore, there was a drastic increase of ≈136% in seed germination when comparing MA+Zr to Zr only treated seeds.
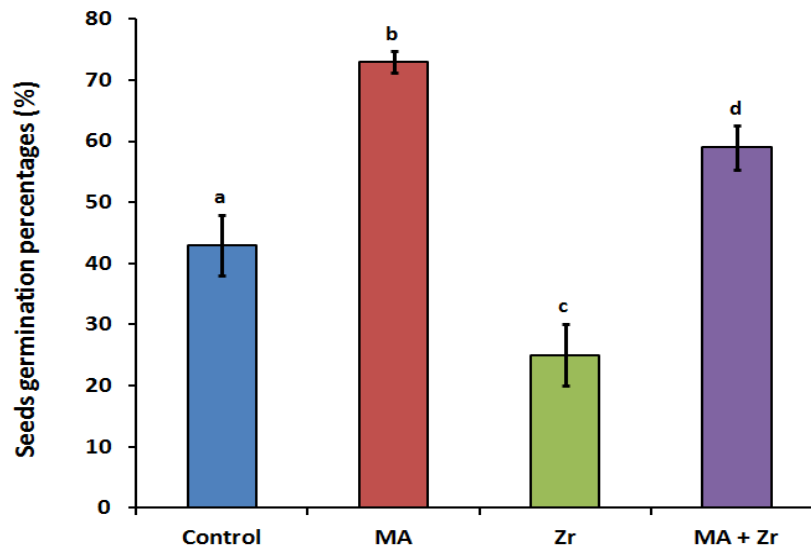
Figure 4.1. The effect of MA and Zr treatments on the germination percentages of *B. napus* L. seeds. Control has been treated with 0,0001% Tween80, experiment has been treated with 0.05 μM MA and (0.05 μM) MA + (1 mM) Zr respectively. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ≈ S.E (N=5).

4.2.2. THE EFFECT OF MA ON THE BIOMASS OF *B. NAPUS* L. SEEDLINGS UNDER ZR TOXICITY

The seedling biomass was determined by measuring the dry weights in response to their respective treatments. The results (figure 4.2) indicate an increase of ≈27% in seedling biomass when comparing the control to the MA treated seedlings, however a reduction of ≈28% was observed in response to Zr stress. When comparing the control to seedlings exposed to MA+Zr, there was a reduction of ≈12 % in seedling biomass. Furthermore, an increase of ≈22% was observed when comparing the Zr to the MA+Zr treated seedlings.
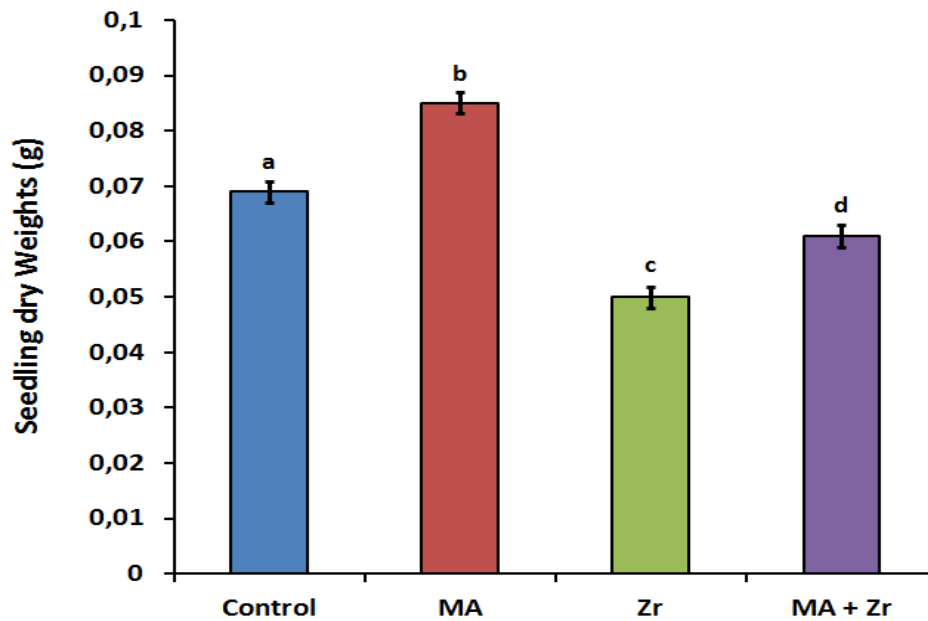
Figure 4.2. The effect of MA on the biomass of *B*. *napus* L. seedlings under Zr toxicity. The control has been treated with 0,0001% Tween 80 and experiment has been treated with 0.05 μM MA and 1 mM Zr respectively. The different letters indicate a significant change across means at P< 0.05 (DMRT). Values are means ≈ S.E (N=3).

4.2.3. THE EFFECT OF MA ON GROWTH AND DEVELOPMENT OF *B*. *NAPUS* L. SEEDLINGS UNDER ZR TOXICITY

The results (figure 4.3) indicated that when comparing the control to the MA treated seedlings, there was an increase of ≈62% in seedling length. The seedlings exposed to Zr showed a ≈35% reduction in seedling length compared to the control indicating that the toxicity of Zr leads to stunting of seedling growth. When comparing the control to seedlings exposed to MA+Zr an increase in length of ≈15% was observed. Furthermore, when comparing the Zr treated seedlings to the MA+Zr treatments, an increase of ≈26% was evident.

Figure 4.3. The effect of MA on seedling length of *B*. *napus* L. under Zr toxicity. The control has been treated with 0,0001% Tween 80, the experiments treated with 0.05 µM MA and 1 mM Zr respectively. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ≈ S.E (N=4).

4.2.4. THE QUANTIFICATION OF ROS BY SPECTROPHOTOMETRIC ANALYSIS

4.2.4.1. EFFECT OF MA ON SUPEROXIDE (O_2^-) CONTENT WITHIN SEEDLINGS UNDER ZR TOXICITY

The O_2^- content (figure 4.4) within *B*. *napus* L. seedlings showed a distinct difference when comparing seedlings exposed to Zr and MA+Zr respectively. The lowest amount of O_2^- content accumulated was observed in the control treated seedlings. There was a slight increase of ≈23% in O_2^- content when comparing the control to the MA treated seedlings; however those seedlings exposed to Zr stress displayed a further ≈108% increase thus being the highest amount observed of O_2^-

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content within seedlings compared to the control. When comparing the control to the MA+Zr treatment, there was an increase in O_2^- content of $\approx71\%$. Furthermore the MA+Zr treatment exhibited a reduction of $\approx18\%$ in O_2^- content compared to the Zr treatment, indicating the potential alleviating properties of MA when combined with Zr stress.
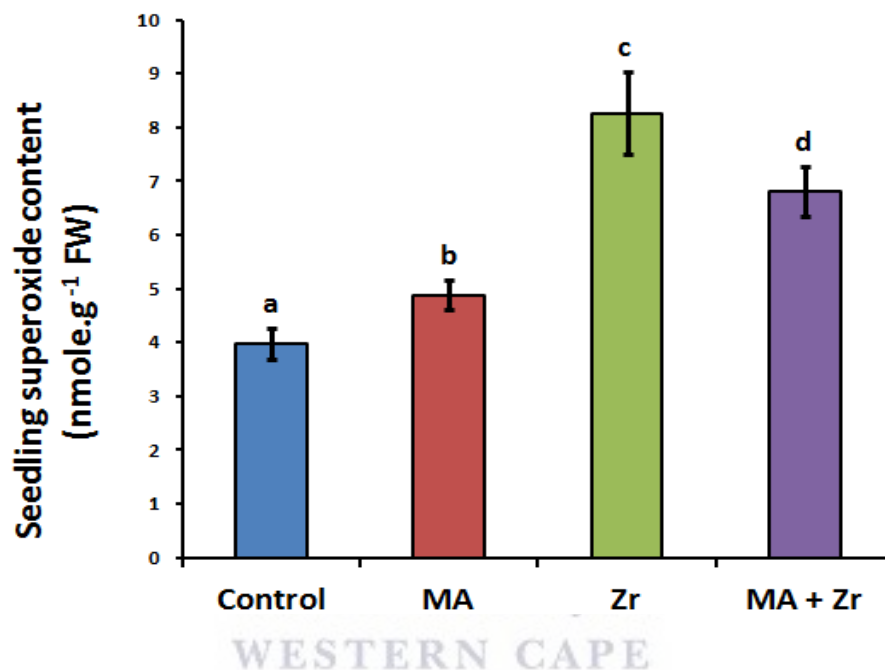
Figure 4.4. The effect of exogenous MA on the O_2^- content within seedlings under Zr toxicity. The amounts of superoxide present within *B*. *napus* L. seedlings were determined following the MA and Zr treatments. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means $\approx$ S.E (N=3).

4.2.5. EFFECT OF MA ON HYDROGEN PEROXIDE (H_2O_2) CONTENT WITHIN SEEDLINGS UNDER ZR- STRESS

It was observed (figure 4.5) that the amount of H_2O_2 increased by $\approx30\%$ in the MA treated seedlings when compared to the control. It was also observed that compared to the control, there was a dramatic increase of $\approx130\%$ in H_2O_2 content in the Zr stressed seedlings. A $\approx55\%$ increase in H_2O_2 levels was apparent in the MA+Zr

treated seedlings when compared to the control. Furthermore it was evident that the combination of MA+Zr treated seedlings showed a reduction in H_2O_2 content by ≈32% when compared to the Zr stressed seedlings thus indicating that the presence of MA decreases the accumulation of H_2O_2 within seedlings while combined with Zr stress.
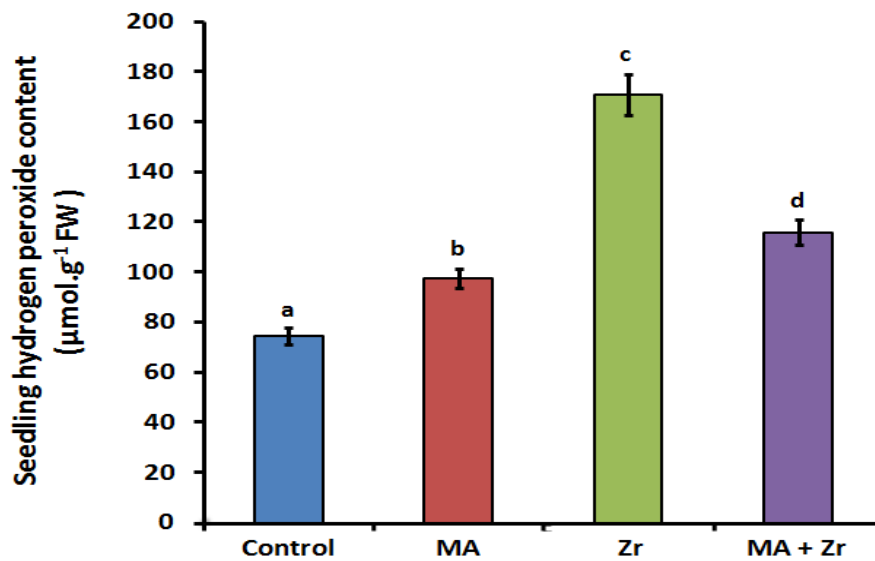
Figure 4.5. The effect of exogenous MA on H_2O_2 content within seedlings under Zr toxicity. Seedling H_2O_2 content was determined after exposure to MA and Zr treatments respectively. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ≈ S.E (N=3).

4.2.6. ANTIOXIDANT PROFILE DETERMINATION OF SEEDLINGS IN RESPONSE TO EXOGENOUS MA UNDER ZR- STRESS

4.2.6.1. SUPEROXIDE DISMUTASE (SOD) ACTIVITY ASSAY

The SOD activity was determined within seedlings subjected to the respective treatments. The results (figure 4.6) displayed an increase of ≈48% in the MA treatment compared to the control. A dramatic increase of ≈129% was observed in the Zr treatment, which represented the highest amount of SOD activity within

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seedlings in comparison to the control. An increase of ≈77% was observed in the MA+Zr treated seedlings compared to that of the control seedlings. A reduction ≈23% in SOD produced was observed in the MA+ Zr treatment compared to the Zr stressed seedlings.
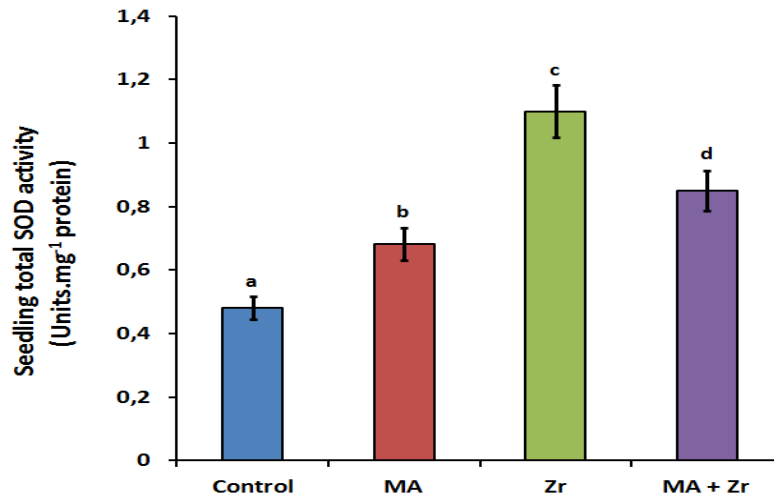
Figure 4.6. The effect of MA on the SOD activity within *B. napus* L. seedlings under Zr toxicity. The SOD antioxidant enzyme acts as the first line of defense against the harmful effects of ROS molecules. The level of SOD activity was measured by spectrophotometric analysis. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ≈ S.E (N=3).

4.2.6.2. ASCORBATE PEROXIDASE (APX) ACTIVITY ASSAY

Literature states that APX is deemed to be one of the most crucial antioxidants involved in the scavenging of ROS, thus making it a crucial indicator of oxidative stress within plants. The APX levels were therefore investigated in response to the various treatments. It was observed in seedlings exposed to MA that there was a ≈60% increase in APX activity compared to the control. In response to the Zr treatment, there was a significant increase of ≈234%. The MA+Zr treated seedlings showed an increase of ≈155% in APX activity, however when compared to the Zr treatment, a reduction of ≈24% was observed.
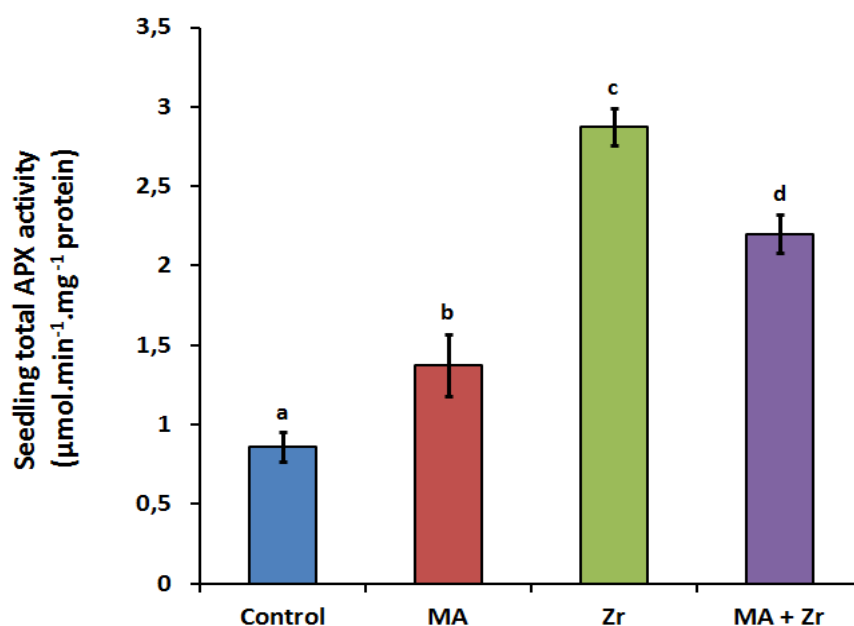
Figure 4.7. The effect of MA on the APX activity within *B*. *napus* L. seedlings under Zr toxicity. The level of APX activity was measured by spectrophotometric analysis. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ≈ S.E (N=3).

4.2.7. THE EFFECT OF MA ON THE LIPID PEROXIDATION (MDA) CONTENT WITHIN *B*. *NAPUS* L. SEEDLINGS UNDER ZR TOXICITY

MDA is a by-product of lipid peroxidation taking place within plants under stress. The amount of MDA was measured (figure 4.8) in response to respective treatments. There was a ≈21% increase in MDA content in response to the MA treatment compared to the control. Compared to the control, a drastic increase of ≈100% was observed in the Zr treated seedlings. The MA+Zr treatment showed a ≈43% increase compared to the control, however compared to the Zr treatment, a reduction in MDA content of ≈29% was observed.
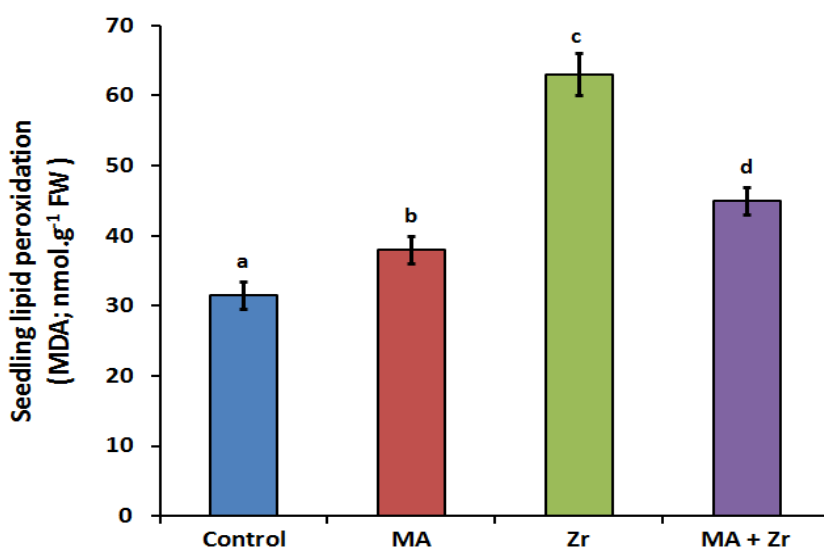
Figure 4.8. The effect of MA on the MDA content within *B*. *napus* L. seedlings under Zr toxicity. MDA is produced as a consequence of lipid peroxidation and thus acts as a marker for oxidative stress. The MDA content can therefore be used as an indicator in the level of lipid peroxidation occurring within seedlings after exposure to MA and Zr respectively. Different letters indicate significant differences between means at $P < 0.05$ (DMRT). Values are means $\approx$ S.E (N=3).

4.2.8. EXOGENOUS MA AMELIORATES THE EFFECTS OF CELL DEATH WITHIN SEEDLINGS UNDER ZR TOXICITY

Cell death was measured within seedlings in response to MA and Zr treatments. The control and the MA treated seedlings had unchanged levels of cell death, however in comparison to the Zr treatment; there was an increase of $\approx$68%. There was a slight increase of $\approx$11% observed in the MA+Zr treated seedlings compared to the control. Furthermore, when comparing the MA+Zr to the Zr treatment there was a reduction of $\approx$34% observed.

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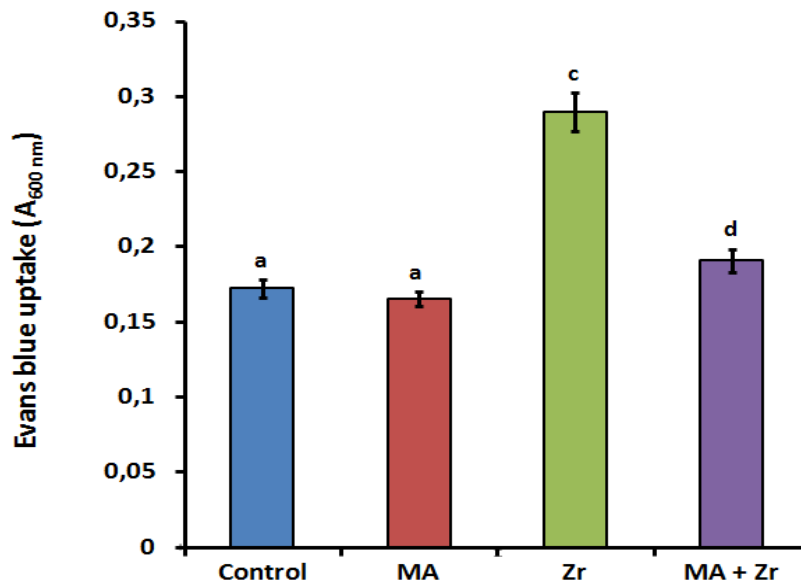
Figure 4.9. The effect of MA on the level of cell death occurring within *B*. *napus* L. seedlings under Zr toxicity. Cell death was determined by the amount of Evans blue uptake by non-viable cells. The amount of Evans blue uptake was therefore determined within seedlings after MA and Zr treatments. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ≈ S.E (N=3).

4.2.9. EXOGENOUS MA CONFERS AN INCREASE IN NITRIC OXIDE (NO) CONTENT WITHIN SEEDLINGS UNDER ZR TOXICITY

NO plays various fundamental roles within mammalian and plant systems. NO has many key roles in plant processes such as improving plant growth as well as having the ability to maintain cellular redox homeostasis during abiotic stress (Shingles *et al*., 1996). It has also been stated that the application of exogenous NO at optimal dosages has the ability to alleviate heavy metal toxicity in plants, namely arsenic and aluminium.

It was thus necessary to determine whether MA, when exogenously applied at the optimal concentration could increase NO content thus alleviating Zr toxicity within seedlings. The results (figure 4.10) indicate that there was an increase of ≈36% in

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NO content in the MA treated seedlings compared to the control. In response to the Zr treatment, NO activity increased by ≈19% compared to the control. An increase of ≈40% was observed in the MA+Zr treatment compared to the control, and no statistical difference was observed between the MA+Zr and the Zr treatment.
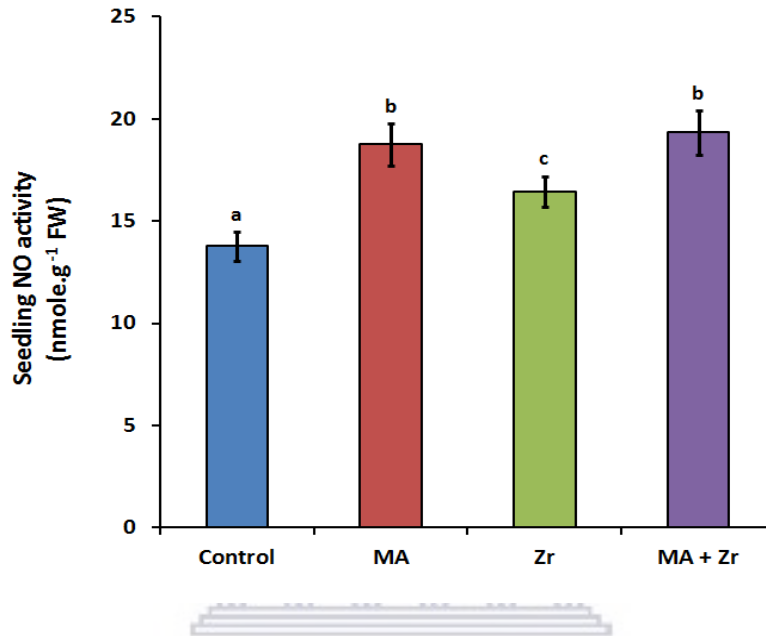
Figure 4.10. The effect of MA on the NO content within *B. napus* L. seedlings under Zr toxicity. NO at optimal levels are able to alleviate heavy metal toxicity within plants aiding in the protection against oxidative stress. The NO content within seedlings was determined after being exposed to MA and Zr treatments respectively. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ≈ S.E (N=3).

4.3. DISCUSSION

Heavy metal contamination has been known to be prominent in arable land worldwide (Neilson and Rajakaruna, 2015), therefore increasing the urgency to enhance the production of crops under heavy metal contamination. This may lead to the utilization of contaminated soil, hence resulting in contaminated crops which ultimately affect human health (He *et al*., 2005). A study by Zhang and colleagues (2002) reported that the germination and growth of wheat seedlings were stunted in response to elevated As concentrations. The heavy metal Zr has received considerable attention over the past decade as being a soil pollutant due to increasing anthropogenic activities. However, there has been limited research conducted in terms of the effects Zr on plant metabolic activities. It has however been established that as a result of Zr toxicity, a significant reduction in plant growth occurs which in turn affects plant enzymatic activity (Ferrand *et al*., 2006; Shahid *et al*., 2013). Initially, in this study, the response of seed germination to Zr stress was investigated; as literature suggests, that by observing seedling germination will provide a good indication of the plants' response to heavy metals (Di Salvatore *et al*., 2008).

According to the results obtained, it was observed that when subjecting *B*. *napus* L. seeds to MA at the optimal concentration (figure 4.1), a substantial increase in seed germination occurred. A study by Haluk Çağlar Kaymak (2012) reported that MA at low or high amounts may play a crucial role in the germination of the cucurbit species. However, a significant reduction in seed germination was observed in response to Zr. This correlates with findings obtained by Fodor and colleagues (2005) where the germination percentage of wheat seedlings were reduced when

exposed to Zr stress. A reduction in germination when exposed to heavy metal toxicity can be attributed to many factors which tend to act together and may include direct effects caused by metal damage or through the induction of oxidative stress. As aforementioned (Chapter 3) MA at the optimal concentration proved to be beneficial to *B. napus* L. seedlings in terms of producing a greater yield. However, attempts were made to investigate whether this result would remain consistent while under Zr toxicity. Remarkably, seedlings treated with MA+Zr displayed an increase in germination percentage which was higher compared to that of both the control and Zr only treated seedlings despite the presence of Zr stress. As a result of heavy metal toxicity, the cellular membranes but more specifically the plasma membrane experiences significant damage (Harwood 1995, Janicka *et al*., 2008). This correlates with a study conducted by Sfaxi-Bousbih and colleagues (2010) who observed that Cadmium (Cd) toxicity inhibited germination as well as to cause induction of damage to cellular membranes which lead to the leakage of minerals and ultimately a loss of nutrients. This loss of essential nutrients has detrimental effects such as inhibiting seed germination. A study by Szollosi and colleagues (2009) identified that large amounts of Cd was absorbed in *Brassica juncea* seeds when exposed to Cd toxicity, confirming that early in plant development oxidative stress occurs. In this study, when supplemented with MA the toxic effects of Zr was not significant, which may be attributed to the fact that exogenous MA results in maintaining of the membrane integrity while under heavy metal toxicity. This concurs with a study by Morsy and colleagues (2012), where it was reported that MA increased in both species of *Zygophyllum* under heavy metal polluted soils. This provides a good indication that MA which is naturally present

within plant systems, possibly attempts to prevent plasma membrane damage when subjected to heavy metal stress. The concentration of MA however is at very minute levels which might not be sufficient in the prevention of membrane damage. By the exogenous application of MA, the germination results are evidently promising when under heavy metal stress.

When observing seedling dry weights (figure 4.2), the results indicated an increase in dry weight when exposed to the MA treatment. A significant reduction was observed in response to Zr stress, which correlates with a study by Gupta and colleagues (2016) where elevated lead (Pb) concentrations resulted in a reduction in dry weights of soybean seedlings. Interestingly, when supplementing MA to the Zr treatment, the dry weights of the MA+Zr treated seedlings surpassed that of the Zr treated seedlings thus demonstrating that MA improves seedling dry weight whilst undergoing Zr stress. It is well established that seeds undergoing germination tend to be more sensitive to metal toxicity compared to that of plants at a mature stage, due to less developed defense mechanisms (Liu *et al*., 2005). This however establishes whether seedlings can thrive under heavy metal conditions.

Seedling shoot length was investigated to determine the effects of the respective treatments on the seedlings (figure 4.3). Literature mentions that the evaluation of seedling shoot length could provide a good indication of seedling vigour as observed in rice (Zhang *et al*., 2005). The results obtained indicated a significant increase in shoot length in MA treated seedlings compared to the control seedlings. It was also evident that a reduction of seedling shoots was observed in response to Zr stress. This coincides with findings of Keunen and colleagues (2011) where a decrease in growth and yield was observed when growing plants in a heavy metal

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contaminated environment. Similarly, literature mentions that the first symptoms caused by the onset of heavy metals include stunted growth (Feigl *et al*., 2013). This reduction in growth may lead to a loss of plant yield which may ultimately result in food insecurity (Chibuike and Obiora, 2014). In our study, in seedlings treated with MA+Zr it was evident that the effects of Zr were alleviated as the shoot length elevated above that of the control and Zr-treated seedlings. This indicates that MA prevents the inhibition or stunting of shoots when in contact with Zr stress and still improved the shoot length regardless.

The earliest effects of heavy metals within plant cells are the overproduction of ROS. This generation of ROS caused by the onset of heavy metals can only be produced via different indirect mechanisms compared to that of redox-active metals (Iron and Copper); which include the stimulation of NADPH oxidase activity and the displacement of cations from enzyme binding sites (Shahid *et al*., 2014). In this study, the first ROS molecule investigated in response to the respective treatments was the accumulation of Superoxide; O_2^- (figure 4.4). Abiotic stress factors are reported to stimulate the overproduction of O_2^- (Hossain *et al*., 2011). The results exhibited a minor increase in O_2^- with respect to the MA treated seedlings. It is known that heavy metals induce oxidative stress in plants; therefore results observed for the Zr treated seedlings remains consistent with literature on the increase in ROS accumulation within plants under heavy metal stress environments (Hossain *et al*., 2009; Singla- Pareek *et al*., 2006; Yadav *et al*., 2005). Interestingly, when investigating the level of O_2^- in the MA+Zr treatment, a reduction in the level of O_2^- was observed indicating that the addition of MA aided in the alleviation of O_2^- caused by the onset of Zr stress.

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Another crucial ROS investigated was hydrogen peroxide (H_2O_2) accumulation, which is known to be detrimental to plants when present in excessive amounts but also beneficial in playing a role in cellular signaling at lower levels (Cheeseman, 2007). According to the results obtained (figure 4.5), similarly to that of O_2^- levels (figure 4.4) a slight increase in H_2O_2 was observed in MA treated seedlings which was not detrimental to the plant. This slight increase in H_2O_2 content as a result of exogenous MA may be involved in further downstream signaling. Literature also validates that H_2O_2 at slightly enhanced levels protects plants against oxidative damage (Hossain *et al*., 2011; Hernandez *et al*., 2001). However, a significant increase was evident in response to Zr stress indicating oxidative stress. This concurs with results observed in literature where H_2O_2 levels have been reported to typically increase in relation to toxicity by various heavy metals (Blasco *et al*., 2015). Furthermore, a study by Yusuf and colleagues (2012) demonstrated that the application of Ni and NaCl to germinating seeds of *Brassica nigra* resulted in an increase in H_2O_2 levels as well as a reduction in membrane stability. Strikingly, as observed for O_2^-, a reduction in H_2O_2 content was evident when supplementing Zr with MA (MA+Zr) in *B*. *napus* L. seedlings. This indicates that not only does MA attempt to alleviate the ROS caused by Zr toxicity, but also that it maintain membrane stability. Literature mentions that an increase in fatty acid saturation occurs due to environmental stress therefore adaptive changes within membrane lipids take place in order to maintain the membrane properties (Salama *et al*., 2007). Through the supplementation of MA to Zr stressed seedlings, membrane stability can be maintained as well as the alleviation of oxidative stress.

As previously established, generally plants undergo oxidative stress when exposed to heavy metals thus affecting the metabolic processes within the plant (Mittler, 2002). The effects of various heavy metals on plants have not been fully elucidated, thus due to the lack of research being conducted on Zr in plants it was deemed necessary to investigate the effects of Zr toxicity on the antioxidative defensive system within *B. napus* L. seedlings. The enzymatic component of plants antioxidant defense system includes Superoxide Dismutase (SOD, EC 1.15.1.1) which is typically known to serve as the first line of defense. Therefore, the effects of Zr on SOD activity within *B. napus* L. was determined (figure 4.6). The results yielded a slight increase in SOD activity in response to exogenous MA. However, more drastic increase was observed in response to Zr stressed seedlings. This significant increase in response to heavy metals correlates with updated literature, where findings by Mourato and colleagues (2015) observed a general increase in SOD activity within *Brassica* species in response to various heavy metals. More specifically, a similar result was observed by John and colleagues (2009), where SOD activity was reported to increase in response to Cd and Pb stress. The enhanced levels of SOD are related to an increase in stress factors caused by elevated levels of ROS production, more specifically O_2^- as seen in (figure 4.4). Another crucial antioxidant enzyme responsible for the detoxification of H_2O_2 into H_2O is Ascorbate peroxidase (APX, EC 1.11.1.11).

According to the findings obtained (figure 4.7), MA increased the APX activity slightly above that of the control seedlings. This increase may be attributed to the fact that MA attempts to act as a signaling molecule which prepares or prime the plant for abiotic stress, thereby increasing the level of APX activity. A study

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conducted by Keyster et al. (2011) observed that in soybean root nodules, the APX activity increased in response to exogenous NO which according to literature can be stimulated in response to MA in mammalian systems. Therefore, if MA can stimulate NO in plants, then a similar trend is observed then MA may enhance APX activity via the production of NO.

Fatty acids have been reported to function as signaling lipids involved in plant stress responses (Kachroo and Kachroo, 2009). In order to alleviate environmental stress factors, enhanced synthesis of lipids which accumulate in trace amounts are usually observed under stress conditions (Okazaki and Saito, 2014). This correlates with properties of MA, as the quantity of MA is very minute and naturally increases their production in response to stress. Therefore, when exogenously applied may alleviate the toxicity produced by heavy metals.

In response to Zr a significant increase in APX was observed as a possible attempt to alleviate the high level of H_2O_2 toxicity. This concurs with findings of Wang and colleagues (2004) where *Brassica juncea* reported to display an increase in APX activity under Cu toxicity. Similarly, other studies have identified an increase in APX activity when plants were exposed to aluminium stress (Sharma and Dubey, 2007). However, the application of MA to the Zr treated seedlings (MA+Zr) displayed a reduction of APX activity, similarly to that observed for SOD (figure 4.6). It has become evident that MA is indirectly capable of alleviating Zr toxicity by means of various mechanisms. One of which may be through the stimulation of Nitric Oxide (NO) production (figure 4.10). NO has been identified to have two mechanisms by which it aids against oxidative damage. The first is to detoxify ROS such as O_2^- to form the less toxic peroxynitrite ($ONOO^-$) therefore limiting

oxidative damage (Martinez *et al*., 2000). Secondly, would be to act as a signaling molecule by activating the antioxidant defense system (Lamattina *et al*., 2003) (figure 4.6 – figure 4.7).

Typically free radical or non-radical species tend to pursue lipids consisting of carbon double bonds such as PUFAs during a process described as lipid peroxidation (Yin *et al*., 2011). The last product produced by lipid peroxidation is malondialdehyde (MDA) being indicative of physiological stress (Quariti *et al*., 1997). The MDA content was therefore observed in *B*. *napus* L seedlings in response to their respective treatments (figure 4.8). In response to MA, a slight increase in the MDA content was observed. Minute quantities of MA are present within the plasma membrane; therefore by further addition of MA above the concentration found within seedlings could be the reason for the slight increase in MDA content which as observed in this study, did not pose any threat to the plant.

Previous research by Weckx and Clijsters (1997) has established that MDA levels were the highest in comparison to the control due to heavy metal exposure. This collaborates with findings of our study, where significantly elevated levels of MDA were observed in response to Zr stress. A study by Elloumi and colleagues (2014) reported that changes in fatty acid profiles were observed suggesting an alteration in the plants membrane fluidity due to Cd toxicity. Similarly, as a consequence of Zr stress the accumulation of ROS was inevitable causing the peroxidation of PUFA's. This lead to the damaging of membrane lipids causing a loss of membrane integrity which increased the MDA levels within *B*. *napus* L. seedlings.

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Remarkably, when determining the MDA content in response to the MA+Zr treatment, a marked reduction in lipid peroxidation was evident. This decrease in the level of lipid peroxidation demonstrates the mitigating effects of exogenously applied MA despite the presence of Zr. According to literature, the presence of heavy metals causes the disruption of cellular membranes thus resulting in unsaturated fatty acids being converted into hydrocarbon fragments such as MDA as previously observed with regards to Zr-treated seedlings (Kappus, 1985; Tappel, 1973). However, when supplemented with MA a reduction of MDA levels was observed due to the fact that saturated fatty acids provide a membrane rigidifying effect on the plants' plasma membrane which is known to be a mechanism against stress while also preventing an increase in membrane fluidity which is known to be extremely detrimental to cells. This corroborates with findings from Morsy *et al.*, 2012, where fatty acid saturation maintains membrane integrity under heavy metal toxicity.

In our study, the level of cell death occurring within *B. napus* L. seedlings was investigated. The results obtained (figure 4.9) indicated that no significant difference was observed between the control and MA treatment, thus indicating that cell death was not induced in response to MA. Saturated fatty acids such as MA maintain cellular membrane integrity where optimal functioning of the plasma membrane can take place. These levels which are usually close to that of the plants basal levels. Therefore no significant differences in cell death levels are expected to occur in response to MA. In response to Zr, a drastic increase in cell death was evident. This may be attributed to various factors, one of which is the increase in ROS production (figure 4.4 – figure 4.5). Xiong and Wang (2005) reported that

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electrolyte leakage and cell death levels were escalated in response to heavy metals due to the overproduction of ROS in *Brassica pekinensis* species. Literature has established that upon heavy metal exposure, metal ions bind to sites on the plasma membrane which causes a disruption in membrane functionality and ultimately leading to cell death (Janicka- Russak *et al*., 2008). However, a striking observation was made when supplementing Zr seedlings with MA as observed in the MA+Zr treatment. A reduction in cell death levels was evident despite the presence of metal toxicity. This finding correlates with literature, where saturated fatty acids have been observed to increase membrane rigidity or stability which protects cells from injury caused by abiotic stress factors (Van Blitterswijk *et al*., 1981). It can therefore be deduced that although the harmful effects of Zr on membrane structure occurred, the exogenous MA prevented the deleterious effects of Zr by preventing ion leakage by maintaining the integrity of the plasma membrane. This observation agrees with observations in literature where it was established that the maintenance of membrane integrity by the saturation effect of saturated fatty acids under heavy metal toxicity is a crucial element in plant tolerance to heavy metal stress (Ben Ammaar *et al*., 2007; Demidchick *et al.,* 1997).

In this study, apart from the ability of MA to alleviate ROS through the elevation of antioxidant enzymes as well as providing rigidity to the plasma membrane as a defense mechanism, it has become evident that MA also had a prominent effect on the levels of Nitric Oxide (NO) within seedlings. Literature has reported that NO may be beneficial to plants by having a protective impact or detrimental by being toxic depending on its concentration (Wink and Mitchell, 1998).

When investigating the effect of MA on NO content (figure 4.10), a remarkable finding was obtained where MA had drastically increased the NO levels. It can be deduced that this increase in NO proved to be beneficial to *B. napus* L. seedlings according to previous findings obtained in this study. This correlates with literature where it was observed in *Arabidopsis* that fatty acids such as Oleic acid had the ability to module NO-associated proteins thus enabling the regulation of NO defense signaling (Mandal *et al*., 2012). Upon exposure to Zr, the NO content also increased but was lower than that observed in MA. Typically in response to heavy metal stress, NO levels have been observed to increase above the plants basal levels. Similarly, Bartha et al. (2005) noted that in response to Cu stress, NO production in *Brassica juncea* L. increased. Due to the overproduction of ROS (figure 4.4 - figure 4.5) induced by Zr, endogenous NO reacts with this high levels of O_2^- forming peroxynitrite known to have deleterious effects on plants (Beckman *et al*., 1990). When observing the effects of the MA+Zr treatment on NO levels, an astonishing finding was obtained. Under Zr stress, when supplemented with MA the NO levels increased to the same level as seen in the MA treatment. This indicates that although the plant is experiencing heavy metal toxicity, MA has the ability to maintain its stimulation of NO production without being affected by Zr stress.

CHAPTER FIVE

EXOGENOUS MA ALTERS THE MINERAL NUTRIENT CONTENT WITHIN *B. NAPUS* L. SEEDLINGS IN RESPONSE TO ZR TOXICITY

5.1. INTRODUCTION

Heavy metal contamination has become a major concern over recent years with regards to being highly toxic and effecting crop quality and yield. These metals are naturally occurring within the earth's crust. However, due activities such as excessive mining especially in SA these heavy metals are released in excess amounts into the environment (Jarup, 2003). This affects the distribution of essential plant nutrients which ultimately impacts plant growth (Singh *et al*., 2016). Mineral nutrients such as Phosphorous (P), Potassium (K), Magnesium (Mg) and Calcium (Ca) are classified as macronutrients. Those nutrients which are present in minute quantities are known as trace elements namely Copper (Cu), Iron (Fe) and Zinc (Zn) which are classified as micronutrients essential for plant growth and development (Lin *et al*., 2011; Sharma and Dietz, 2006).

Recent statistics have identified that, two-thirds of the world population are potentially at risk of being deficient in essential mineral nutrients. This lack of nutrients occurs within many staple crops consequently resulting in human malnutrition (Williams and Salt, 2009). Often these lack of nutrients are due to many abiotic stress factors such as heavy metal toxicity, where these metal ions out-compete nutrients for the same membrane transporters ultimately reducing the

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mineral content in plants (Qui *et al.*, 2005; Baszynski *et al.*, 1980). Apart from a lack of nutrients being detrimental, concentrations exceeding that of the required amount become toxic to the plant. Increasing the availability of these essential nutrients while remaining within the threshold aids in the alleviation of heavy metal toxicity (Singh *et al.*, 2016).

Plant lipids form part of the major cellular constituents of biological membranes as they can sense extracellular conditions, as well as being an essential component in membrane structure. Over the past decade, increasing evidence identifies that not only does lipids play a role as defense signaling molecules but also has roles in mitigating stress within plants (Moellering *et al.*, 2010; Munnik and Testerink, 2009; Nakamura *et al.*, 2009; Gaude *et al.*, 2008; Wang *et al.*, 2006; Wang, 2004). Among a wide range of signaling lipids, more specifically saturated fatty acids are of particular interest. As an environmental defense mechanism against heavy metal toxicity, medium-chained saturated fatty acids were observed to increase in order to restore the physical properties of the membrane as well as enhancing stability (Kamel *et al.*, 2012). This occurs due to the fact that the plasma membrane is the first target when exposed to toxic metals which usually increases the fluidity of the plasma membrane resulting in a loss of membrane integrity and function (Nouairi *et al.*, 2006). By increasing the exposure of plants to exogenous saturated fatty acids while under heavy metal stress researchers can potentially ameliorate the toxicity levels caused by heavy metals.

In this study, the effects of exogenous MA on *B. napus* L. nutritional content while under Zr stress were investigated. We hypothesised a potential benefit of MA in

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improving the bioavailability of essential nutrients (macro and micro) and in the process alleviate Zr toxicity.

5.2. RESULTS

5.2.1. EFFECT OF EXOGENOUS MA ON THE MACRO AND MICRONUTRIENT CONTENT WITHIN *B*. *NAPUS* L. SEEDLINGS UNDER ZR TOXICITY

The results (figure 5.1 a) indicate that when comparing the control to the MA treatment there was an increase of approximately ≈22% in P content. It was observed that there was a ≈15% decrease in P when comparing the control to the Zr treated sample. However, it was observed that when comparing the control to the MA combined with Zr there was a ≈11% increase in P content regardless of Zr being present. According to (figure 5.1 b), with regards to K^+ content when comparing the control to the MA sample there was a slight decrease of ≈10% in K^+ content. However, it was observed that the Zr treatment showed a ≈20% decrease in K^+ content when compared to that of the control. Furthermore, it was shown that the combination of MA and Zr provided a similar result to that of the Zr treatment in comparison to the control for K^+ content. The macronutrient Mg^{2+} forms an integral part in the biosynthesis of chlorophyll (Chou *et al*., 2011). The results (figure 5.1 c) indicated that the MA treatment displayed a slight decrease in Mg^{2+} content of approximately ≈12% compared to the control, however in comparison to the control sample the Zr treatment showed an increase in Mg^{2+} content of ≈27%. In the MA+Zr treated seedlings the Mg^{2+} content increased slightly by ≈10% compared to the control sample. However, interestingly when compared to the Zr treated sample there was a slight decrease of ≈13% indicating that MA when

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present tends to lower Mg^{2+} content with or without Zr in the treatment. As aforementioned, Ca^{2+} is an essential macronutrient actively involved in enzyme activation as well as in the regulation of the plant metabolism. The results (figure 5.1 d) showed that there was a ≈20% increase in Ca^{2+} content in the MA treatment compared to that of the control; however it was observed that in comparison to the control there was an even larger increase of ≈92% in the Zr treatment. Furthermore, in comparison to the control, the level of Ca^{2+} content increased by ≈36% in the MA+Zr treatment.

The results (figure 5.2 a) indicated that in the MA treatment, the amount of Fe^{2+} was reduced by ≈10% when compared to the control seedlings. No differences were observed between the Zr and MA+Zr treated samples as statistically they both showed a similar reduction in Fe content compared to the control. The results (figure 5.2 b) indicated a ≈32% decrease in Zn in the MA treated sample compared to the control. When comparing the Zr treated sample to the control, there was a significant increase of ≈65% in the level of Zn present within seedlings, however according to the MA+Zr treatment a reduction of ≈40% in Zn content was observed, which was ≈25% less compared to the Zr treatment.

When observing the amount of Zr present (figure 5.3) within the respective treatments, it was observed that both the control and MA treated samples contained a low and statistically similar amount of Zr. However, the Zr treatment displayed a significant increase of approximately ≈207% when compared to the control. Furthermore, it was observed that the MA+Zr treated sample had an increase of ≈158% compared to the control.

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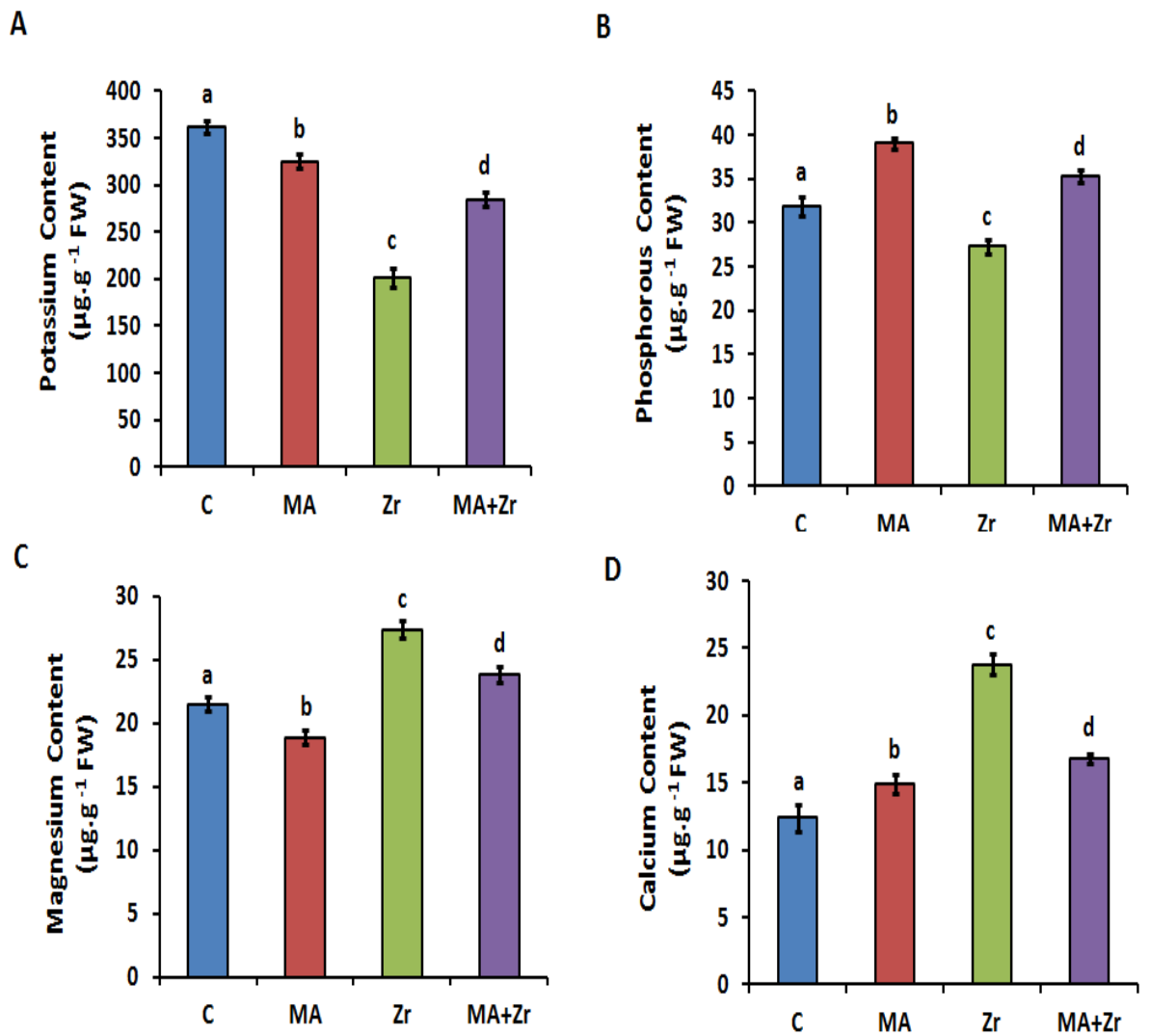
Figure 5.1. The effect of MA and Zr on the macronutrient content within *B. napus* L. seedlings. Macronutrients were (A) P content, (B) K^+ content, (C) Mg^{2+} content and (D) Ca^{2+} content. The macronutrient content was determined within seedlings subjected to MA and Zr treatments respectively. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ≈ S.E (N=3).
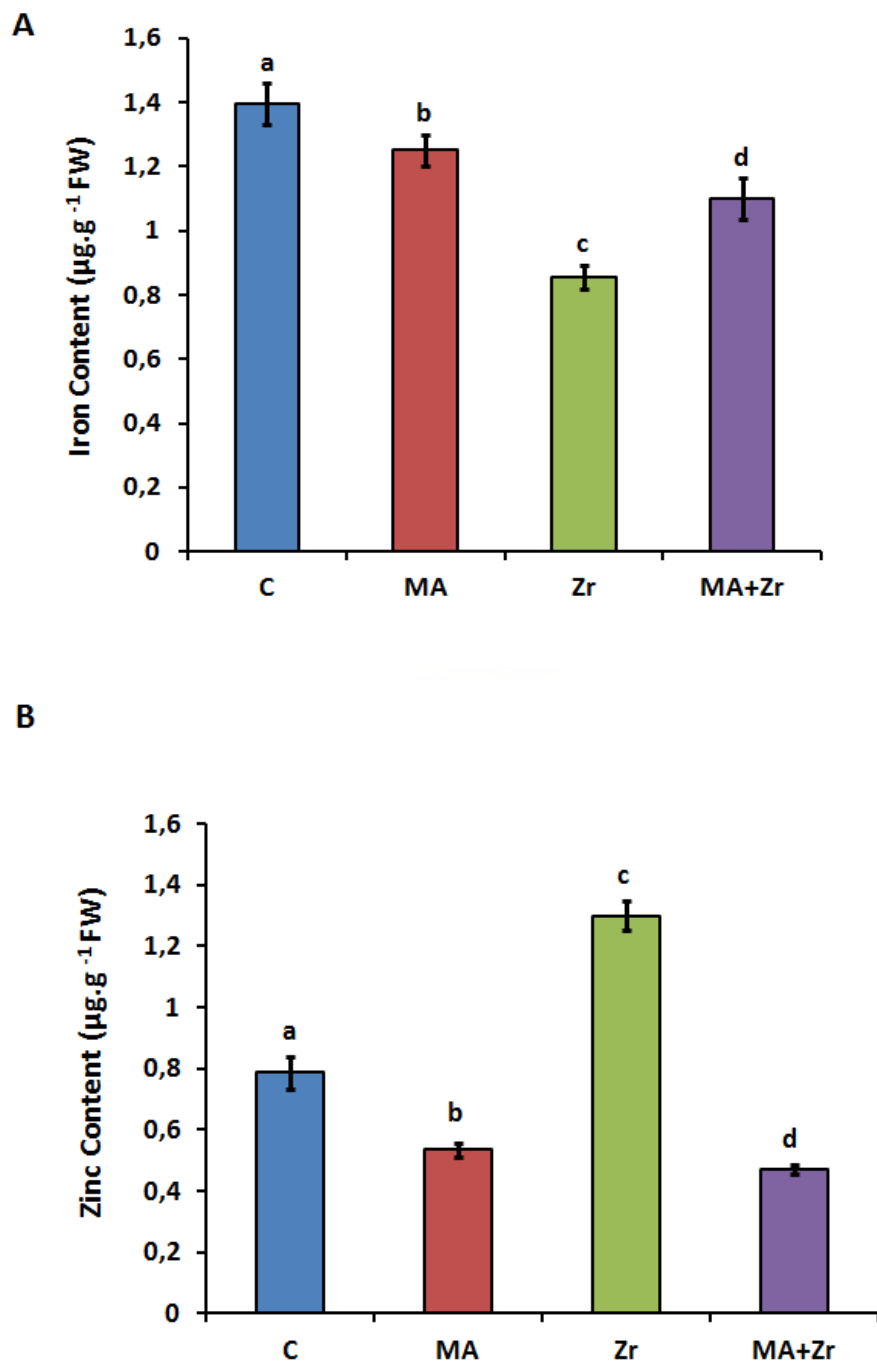
Figure 5.2. The effect of MA and Zr on the micronutrient content within *B. napus* L. seedlings. The micronutrient content was determined within seedlings subjected to MA and Zr respectively. Micronutrients were (A) Fe content and (B) Zn content. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ≈ S.E (N=3).
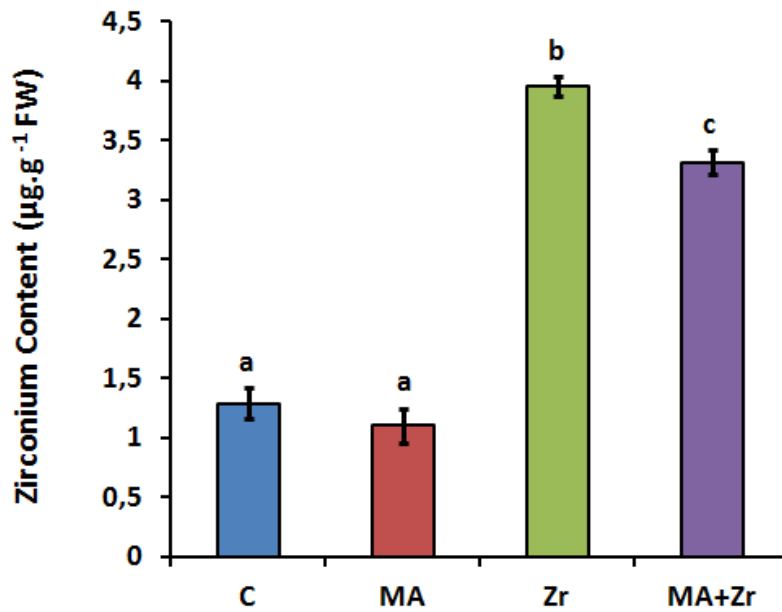
Figure 5.3. The effect of MA on the Zr content within *B. napus* L. seedlings under Zr toxicity. The Zr content within seedlings was determined after exposure to MA and Zr respectively. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ≈ S.E (N=2).
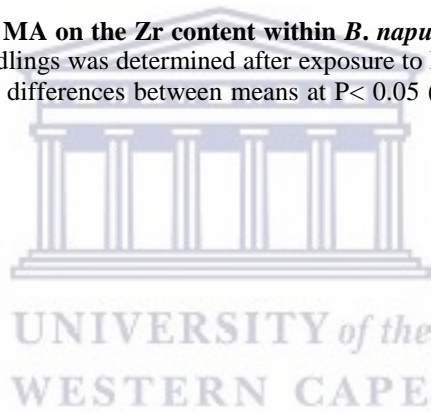
5.3. DISCUSSION

Apart from the harmful impacts imposed on plants by high levels of heavy metals (HMs) through oxidative damage, literature states that HMs influence mineral nutrient content within plants. It was theferfore significant to investigate the effects of exogenous MA on the mineral nutrient content within *B. napus* L. seedlings. One of the major plant macronutrients is phosphorous (P). Apart from being an integral part in the cell membrane, it is also essential for phosphorylation events (Sarwar *et al*., 2010).

In this study, it was observed that seedlings exposed to MA displayed an increase in P content, indicating that exogenous MA stimulates an increase in the production of P. According to Theodorou and Plaxton, (1993) P is essential in controlling important enzymes as well as being involved in regulating metabolic pathways. A study conducted by Hossain et al. (2010) suggests that Cadmium (Cd) accumulation inhibits stomatal opening, disturbs photosynthesis and alters the plants antioxidant metabolism and mineral nutrients. This was confirmed in (figure 5.1 a) which displayed that in response to Zr toxicity; the P content goes down below the basal levels of the seedlings. A study done by Fodor et al. (1995) stated that the functions of a plants' plasma membrane is altered due to the toxic effects caused by the onset of heavy metals at high concentrations. The first sign of membrane damage is an increase in the membrane permeability which therefore leads to an ionic imbalance within the cell. This correlates with the findings obtained in this study where the Zr ions may have outcompeted the P ions therefore causing a lower absorption of P content within seedlings. It can also be observed in a study by Nazar et al. (2012)

where it was demonstrated that nutrients and the Cd heavy metal tend to compete for the same membrane transporters ultimately resulting in mineral deficiency. This coincides with the findings of the current study, which shows that the Zr treated seedlings displayed P content levels lower than that observed in the seedlings basal levels which gives a clear indication of competition taking place.

Plants respond to abiotic stress factors by altering the fluidity of the plasma membrane thus causing the α-linolenic (18:3) fatty acid to be released from membrane lipids. Heavy metal toxicity leads to the accumulation of ROS which causes the peroxidation of polyunsaturated fatty acids namely (18:2) and (18:3) thus leading to deleterious effects in membrane integrity. The results obtained in this study indicated that MA+Zr displayed an increase in P content above the seedlings' basal levels but slightly lower than that seen in the MA treatment.

As aforementioned, Zr when present at high levels out-competes the P ions for the same membrane transporters. However, when supplemented with MA which is a saturated fatty acid, it was observed that MA alleviates the level of Zr within seedlings thus allowing the P nutrients to out-compete the Zr ions which ultimately results in an increase in P content. This correlates with the findings by Nazar et al. (2012) where it was reported that an increase in nutrient availability may reduce the accumulation of heavy metals in plants. Furthermore, Nazar and colleagues demonstrated that elevated levels of P reduced the accumulation of Cd in *Trema micrantha* plants as well as triggering an increase in antioxidant ezyme activities such as peroxidases in *Elodea nuttallii*.

In order for the plant to maintain the balance of anion-cation within cells, the macronutrient Potassium (K^+) is required. The oxidative stress which occurs within plants can be reduced by increasing the nutritional level of K^+ (Shen *et al*., 2000). The K^+ content (figure 5.1a) for *B. napus* L. seedlings were therefore determined in response to the above mentioned treatments. The MA treatment showed a slight decrease in K^+ below basal levels. However, this reduction was possibly not below the deficiency threshold. This slight reduction may be attributed to the fact that MA may signal a priming effect to be activated only when the plant encounters any form of stress. This correlates with a study by Jung et al. (2009) where Azelaic acid much like MA (also a saturated fatty acid) have been observed in priming plants to produce salicylic acid (SA), known to act as a signal of defense against pathogen infection.

The Zr treatment showed a more drastic reduction in K^+ content below that of the control and MA treatments. This may be due to the Zr ions interfering with the nutrient uptake by altering the plasma membrane resulting in a leakage of nutrients thus affecting nutrient transportation. It has been reported by Llamas et al. (2000) that the application of Cd competes with other essential macronutrients for the same transporters, thus lowering its content within plants. This competition occurring between heavy metals and nutrients for binding sites within various cellular compartments namely the cell wall and plasma membrane influences the distribution of the metal ions which ultimately leads to a reduction in nutritional content. This lowering of K^+ could also lead to a deficiency of K^+ thus causing the onset of ROS production which may result in degradation of cellular membranes.

With regards to seedlings subjected to MA+Zr, there was no significant difference observed in K^+ content compared to the Zr treatment, however the beneficial effects of MA on seedling growth (figure 4.3.3) was still evident. Literature states that when subjecting mustard plants to Cd stress, the K^+ availability was reduced thus resulting in a reduction in H_2O_2 and MDA content, leading to an increase in antioxidant enzyme activity (Umar *et al*., 2008). This observation goes parallel with the results obtained in this study which indicates that MA lowers the K^+ content which ultimately resulted in alleviating Zr toxicity due to the antioxidant enzymes such as SOD and APX (figure 4.6 - 4.7).

It has been well established that the Mg^{2+} nutrient is essential for various enzymatic processes as well as regulating ion transport in plants (Hailes *et al*., 1997). Literature also reports on the ameliorating properties of Mg^{2+} when exposed to aluminium toxicity. In this study the effects of the above mentioned treatments (figure 5.1 c) on Mg^{2+} content within *B*. *napus* L. seedlings was investigated. The seedlings treated with MA displayed a slight decrease in Mg^{2+} content, however possibly not enough to cause damage. The exogenous MA being a saturated fatty acid may have an impact on the plasma membrane permeability as it is known to increase membrane rigidity thus preventing some of the Mg^{2+} ions to access their respective transporters situated within the plasma membrane.

Seedlings treated with Zr demonstrated an increase in Mg^{2+} content exceeding that of the seedlings basal levels. This result remains consistent with findings of Rezvani et al. (2012) where an increase in macronutrient content within *Aeluropus littoralis* was evident when exposed to high concentrations of Cd stress. Typically Mg^{2+} is an essential nutrient for plant growth and development, thus a balanced

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supply of this macronutrient is required. A study by Gao et al. (2015) reported that high levels of Mg^{2+} may be detrimental to plants as it promotes ROS formation amongst other deleterious effects. This remains consistent with the results obtained in this study, where Zr stress possibly promoted the accumulation of Mg^{2+} in excess quantities above that required by the plant. The MA+Zr treatment showed a decrease in Mg^{2+} content. It is evident that the supplementation of MA is responsible for the decline in Mg^{2+} content, as MA attempts to bring the level of Mg^{2+} towards that of the control where the optimal supply of Mg^{2+} can be utilized. This observation is in agreement with a study done by Hermans et al. (2011) who have reported that Mg^{2+} at lower concentrations close to that of basal levels yielded a protective effect in plants against Cd stress.

Mineral nutrients such as calcium (Ca^{2+}) have various crucial functions within plants and have been well established in literature. These roles include enzyme activation as well as the regulation of metabolic activities (Lauer-Junior *et al.*, 2008). The level of Ca^{2+} content (figure 5.1 d) within *B. napus* L. seedlings was determined in response to exogenous MA and Zr toxicity. The results yielded an increase in Ca^{2+} content in response to MA. This increase may be due to the fact that MA and Ca^{2+} both has an impact on the cell membrane. MA provides membrane stability and Ca^{2+} provides structure in the cell membrane. Therefore, MA may signal an increase in Ca^{2+} to protect the plant from possible stress factors.

A study conducted by Sanz et al. (2009) reported that when nutrients are present at higher metabolic concentrations than that required by the plant, phytotoxicity often occurs. This phenomenon was observed where beneficial nutrients such as Nickel at low concentrations stimulates seed germination and growth, however when present

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in excess levels resulted in the impairment of many physiological processes (Chen *et al*., 2009; Seregin and Kozhevnikova, 2006). Early studies conducted indicated that Ca^{2+} at elevated levels led to the inhibition in shoot growth, however lower levels enhanced cell and tissue elongation (Tagawa and Bonner, 1957; Bennet-Clark, 1956). Nutrients in excessive quantities promote the overproduction of ROS often resulting in membrane degradation, loss of membrane functionality and eventually causing cell death (Wang *et al*., 2011). The aforementioned literature correlates with findings obtained in this study, where the amount of Ca^{2+} was seen to be drastically higher compared to that of the seedlings' basal levels in response to Zr toxicity. However, when supplemented with MA (MA+Zr) a reduction in Ca^{2+} was evident. This indicates that MA attempts to regulate Ca^{2+} levels when combined with Zr, enabling the Ca^{2+} levels to remain below that of the threshold while at the same time proving to be beneficial by increasing the levels within the safety parameters of the plant.

Previous literature has established that Fe is essential for cell division, photosynthesis and required only in minute quantities (Marenco *et al*., 2005). A common disorder often observed in many crop plants is Fe deficiency which results in poor yield and stunted growth (Schmidt, 1993). The results obtained in this study (figure 5.2 a) indicated a slight reduction in Fe in response to the MA treatment. This reduction was possibly not significant enough to cause Fe deficiency hence posing no threat to the plant. This was confirmed by the low antioxidant levels (figure 4.6- figure 4.7) as well as increased seedling shoot length (figure 4.3).

In response to the Zr treatment it was evident that a reduction in Fe content occurred below that of the control. It was evident that the Zr ions possibly

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outcompetes the Fe ions for the same membrane transporters thus causing the reduction in Fe content. Literature also states that the transporters which are involved in the translocation of nutrients such as Fe are inhibited by Cd stress (Papoyan and Kochian *et al*., 2004). The Zr ions may also interfere with the binding of Fe ions by altering the permeability of the membrane thus resulting in a leakage of nutrients. This was confirmed by Sun and Shen *et al*., 2007 where it was shown that a decrease in Fe content resulted in response to Cd stress. This reduction in Fe content caused by the onset of Zr toxicity results in overproduction of ROS (figure 4.4 - 4.5) which in turn elevates the antioxidant enzymes (figure 4.6 - 4.7) in attempt to alleviate the metal-induced toxicity. This reduction in Fe content may also be a contributing factor in the inhibition of seedling growth (figure 4.3).

Due to a lack of Fe caused by the onset of Zr stress, many deleterious effects within seedlings will occur. This can be observed in literature where Fe deficiency in dicotyledenous species induced oxidative stress which resulted in the disturbance of antioxidant enzyme expression (Ranieri *et al*., 2001). Similarly, according to Msilini et al. (2014) Fe deficiency resulted in a marked increase in MDA content as well as electrolyte leakage which suggested a detrimental impact on the membrane stability. However, in this study, in response to the MA+Zr treatment, the level of Fe increased above that of the Zr treated seedlings. MA maintains membrane integrity and stability, therefore although being in the presence of Zr, the deleterious effects were less impactful when supplemented with MA.

The interaction between heavy metals and micronutrients can be either antagonistic or synergistic. This phenomenon can be seen in a study by Yang et al. (1998) where a reduction in Fe content in maize and cabbage was observed. However, there was

an increase in P content when subjected to Cd stress. This correlates with findings observed in this study, where the Fe content may have decreased when supplemented with MA but as a consequence the P content (figure 5.1 b) significantly increased. It can therefore be elucidated that although MA might have reduced the Fe content, other macronutrients are increased as a result of a decrease in micronutrients which are required only in trace amounts.

The results obtained in this study (figure 5.2 b) indicated a reduction in Zn content in response to the MA treatment. This reduction was similar to that observed in Fe content which was not detrimental. The reduction observed in the Zr treatment was drastically lower compared to the control and MA treated seedlings, due to the impairment of transporters inhibiting Zn ions to be transported successfully. This correlates with a study by Sarwar et al. (2010), where it was suggested that when plants are exposed to Cd stress membrane permeability was indeed affected which resulted in an alteration in the plants nutrient composition.

The MA+Zr treatment showed a significant reduction in Zn content indicating again, that MA when present reduces the micronutrients to a level not harmful to the plant. As previously stated, plants lower the availability of certain nutrients in order to increase the activity of antioxidant enzymes (Umar *et al*., 2008). The affects of MA coincides with that mentioned in literature, as it is evident that MA reduces the availability of micronutrients by allowing the increased accumulation of other essential macronutrients as well as enhancing the antioxidant system (figure 4.6 - 4.7) to protect the plant against oxidative damage. MA alleviates the excess levels of Zn closer to that of the plants basal levels where the optimal Zn requirement can be utilized by the plant.

Increasing exposure toward Zr has occurred due to the ever increasing demand for the Zr-alloy and Zr-derived products as it possess anti-corrosion properties (Fodor *et al*., 2005). Excessive mining activities have lead to elevated levels of Zr toxicity within soils thus reducing crop yield and ultimately affecting the economy as a whole. It was therefore of immense importance to establish its content in *B*. *napus* L. as well as determining its potential alleviation through the supplementation of MA. The results (figure 5.3) yielded no change in Zr content in response to MA, indicating that exogenous MA did not elevate the toxicity levels of Zr within *B*. *napus* L. seedlings hence establishing that MA poses no threat in terms of promoting Zr uptake within seedlings. A drastic increase in Zr content was observed with respect to the Zr treated seedlings, which correlates with previous findings where increased antioxidant levels (figure 4.6 - 4.7) was observed as well as stunted seedling growth (figure 4.3). Interestingly, when supplemented with MA a reduction in Zr content was observed, therefore confirming that although a slight reduction was observed in certain nutrients, it may have served as a priming effect as it was evident that MA alleviates Zr toxicity within seedlings through various signaling mechanisms. This result may also demonstrate that exogenous MA hinders the interaction between the binding of Zr to their respective transporters through remodelling of the plasma membrane. A remarkable finding by Morsy et al. (2012) observed that in two different cultivars of the *Zygophyllum* species, an increase in saturated fatty acids such as palmitoleic (16:0) and myristic acid (14:0) was identified in response to heavy metal polluted soils. This provides a firm foundation where MA levels were observed to increase in attempt to restore the membrane properties which have been degraded due to metal toxicity. Increasing

the concentration of MA may prevent the degradation of membranes in response to heavy metals thus maintaining the membrane integrity. This concurs with literature where it has been reported that changes in lipids of the plasma membrane may be favourable to restore optimal membrane functionality (Salama *et al*., 2012).

CONCLUSION AND FUTURE PROSPECTIVES

The initial goal of this study was to determine the effects of various concentrations of MA on *B. napus* L. seedlings. Remarkable findings were obtained, which included MA at high concentrations being detrimental to seedlings by stunting germination and growth (figure 3.1 - 3.3) and promoting ROS (figure 3.4 - 3.5). The increase in ROS increased the level of lipid peroxidation (figure 3.4.2) and cell death (figure 3.4.3). However through optimization, the optimal concentration was obtained which increased the growth and yield of seedlings while reducing the level of ROS hence proving to be beneficial. Furthermore, the optimal MA concentration was combined with the heavy metal Zr (MA+Zr) to observe whether MA had any alleviating properties. Striking evidence was obtained as MA alleviated Zr toxicity as depicted through biochemical analyses (figure 4.6 - 4.7). Similarly, the growth and germination improved even under exposure to Zr.

Apart from modulating ROS through an increase in the antioxidant response, other mechanisms may have been triggered through lipid signaling which could also play a role in the detoxification of ROS. The level of NO in response to MA was therefore also determined, to observe whether MA had any pronounced effects. NO has been reported in several studies to alleviate ROS when at optimal concentrations as well as having growth enhancement properties. Increased levels of NO (figure 4.10) was observed in both MA and MA+Zr treatments, implicating that MA has the ability to act as a signaling molecule involved in stimulating NO production while in the presence of Zr. The elevated level of NO regulates the

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antioxidant enzymes aswell as reducing the toxicity of ROS molecules during their interaction.

Recently, the concept of mineral nutrition of crops has become an integral component within agriculture; having a major impact on food security. The mineral nutrient composition was therefore investigated in response to MA through the analyses of the seedling ionome. MA elevated essential macronutrients while being subjected to Zr toxicity, and when reducing certain micronutrients, a priming effect was evidently clear as this reduction was partly responsible for the elevation of specific macronutrients. Evidence obtained demonstrated that exogenous MA was capable of elevating essential plant nutrients while under Zr toxicity which is a breakthrough in plant biofortification.

This recent advancement will enable us to develop crops for biofortification of essential nutrients thus preventing the issue of nutrient deficiency in both adults and children. The findings obtained in this study provided the foundation to which exogenous MA can be applied; however extensive research is required in other disciplines such as lipidomics (study the pathways and networks of cellular lipids), proteomics (identify stress-inducible proteins), transcriptomics (identify transcription factors) and metabolomics (role of metabolites) in order to fully understand the mechanisms through which MA mitigates oxidative stress while displaying improvements in overall yield and nutritional composition.

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