The effect of exogenous DIM on *Brassica napus* and its role in response to heavy metal stress

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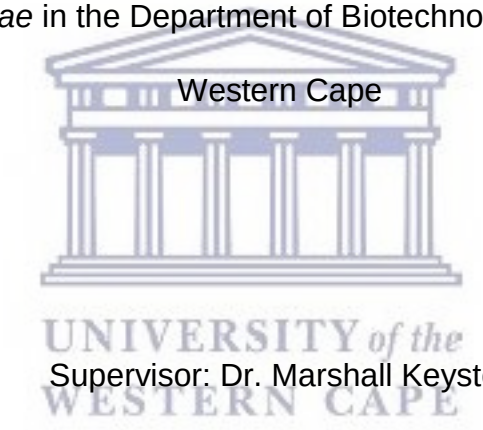
A thesis submitted in partial fulfilment of the requirements for the degree of *Magister Scientiae* in the Department of Biotechnology, University of the Western Cape



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List of Abbreviations

ALA -	Aminolevulinic acid
APX -	Ascorbate peroxidase
ATP -	Adenosine triphosphate
BHT -	Butylated hydroxytoluene
BRCA 1 -	Breast cancer protein
BSO -	Buthionine sulfoximine
CA -	Citric acid
cAPX -	Cytosol ascorbate peroxidase
CdPSC1 -	*Ceratophyllum demersum* phytochyletin synthase 1
DEDTC -	Diethyl dithiocarbamate
DIM -	3,3-Diindolylmethane
DNA -	Deoxyribose nucleic acid
DPI -	Diphenylene iodonium
DPPH -	2,2-diphenyl-1-picrylhydrazyl
FB 1 -	Fumonisin

GLS -	Glucosinolates
gmAPX -	Glyoxisome ascorbate peroxidase
GPX -	Glutathione peroxidase
GSH -	Glutathione
HM -	Heavy metal
HM-PC -	Heavy metal - phytochyletins complex
I3C -	Indole-3-carbinol
I3M-GS -	Indol-3-ylmethylglucosinolate
IAA -	Indole-3-acetic acid
IAN -	Indole-3-acetonitrile
IG -	Indole glucosinolate
IOAx -	Indole-3-acetaldoxime
MDA -	Malonaldehyde
mRNA -	Messenger ribonucleic acid
MT's -	Metallothionins
NADPH -	Nicotinamide adenine dinucleotide phosphate
NO -	Nitric oxide
Pb-PC -	Lead-phytochyletin complex

PC'(s) -	Phytochyletin(s)
PCS -	Phytochyletin synthase
PEITC -	2-phenylethyl isothiocyanate
PGR -	Plant growth regulator
ROS -	Reactive oxygen species
RP-UHPLC/MS -	Reversed phased - Ultra high pressure liquid chromatography/ Mass spectrometry
sAPX -	Chloroplast stroma ascorbate peroxidase
SNP -	Sulphide nitroprusside
SOD -	Superoxide dismutase
tAPX -	Thylakoid Ascorbate peroxidase
TBARS -	Thiobutaric acid reactive substances
UGTB74B1 -	thio-glucosyltransferase

Abstract

Brassica napus is a plant that is used for human and animal consumption. This plant is also used for phytoremediation due to its relatively higher level of heavy metal tolerance. In South Africa, mining is one of the main drivers of the economy. One of the major negative environmental impacts of mining is heavy metal contamination. Soil metal content can rise to levels that are quite high and can even have a negative impact on the yields of *B. napus* crop. The glucosinolate-myrosinase system of *B. napus* is a system that is used as defence against biotic stressors. Indole glucosinolate breakdown products have been proven to enhance the antioxidant capacity of plants. Some have also shown growth promoting properties in plants.

We studied the effect of exogenous DIM on *B. napus* and it role in Zr induced heavy metal stress. Germination percentages revealed that DIM increased germination, Zr application decreased germination and the DIM-Zr treatment reversed the negative impact of Zr application on *B. napus*. The effect of treatments on the biomass of *B. napus* was assessed by determining the dry weights. Results show that exogenous DIM improves biomass. Zr application decreased biomass and DIM-Zr treatment ameliorated the effect of Zr application. ROS content was determined by using spectrophotometric assays. DIM increased ROS content slightly, while Zr caused a massive increase in both superoxide and hydrogen peroxide content. The DIM-Zr co-treatment lowered the content of both superoxide and hydrogen peroxide content in comparison with Zr only treated seedlings. Enzymatic antioxidant activity of APX and SOD was

determined by making use of spectrophotometric assays. The results revealed that enzymatic activity of DIM treated seedlings increased relative to the control. Zr treated samples had activities that was higher than DIM treated samples and the SOD and APX activities of the DIM-Zr co-treated samples was the highest. MDA content was determined by using a TBARS assay to assess the level of oxidative stress. DIM treated seedlings showed no difference in comparison to the control. Zr treated seedlings had an increase in MDA content and the MDA content of DIM-Zr treated seedlings is lowered in comparison with Zr only treated. Cell death was determined by the Evans blue dye uptake assay. Results revealed a similar trend to MDA results i.e. no increase for DIM treated seedlings in comparison with control, increase for Zr treated samples in comparison with control and a decrease for DIM-Zr treated seedlings in comparison with Zr treated samples. The effect of treatments on nutrient contents of *B. napus* was assessed by ICP-OES. All elements increased in DIM treated seedlings. The opposite was observed for Zr treated seedlings, all elements decreased with the exception of Ca that also increased.

It was concluded that DIM have a growth promoting effect on *B. napus*. DIM slightly increased ROS content because DIM is a stress related secondary metabolite. The slight increases in ROS content might have played a role in the better germination and growth promotion. The ROS content of DIM treated seedlings was regulated by the enzymatic antioxidants. MDA and cell death results revealed that there was no evidence of oxidative stress. ROS content only increased slightly to play a positive signalling role. DIM

played an ameliotory role for Zr stressed seedlings. It decreased ROS content of the stressed seedlings by increasing the activity of enzymatic antioxidants. It also increased all elements that were decreased by Zr application. As a result of improved antioxidant defences and better nutrient uptake germination and growth of DIM-Zr seedlings improved in comparison with Zr only treated seedlings.

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CHAPTER 1- LITERATURE REVIEW

1.1 Introduction

Humans use canola as an oil source. This crop is also used for animal feed, biofuel production and phytoremediation. Mining and the use of mined heavy metals (HM) in industry can lead to soil HM contamination. High soil metal content may place plants under HM stress. Heavy metal stress can result in reactive oxygen species (ROS) accumulation that may lead to cell death and ultimately crop loss. Plants however have defences to guard them against the effect of HM stress. Plants first prevent entry of HM by blocking it in the cell walls. When HM pass the first line of defence chelators bind these metals to prevent the damaging effects of HM. When metal cause excessive ROS production, the antioxidant system will activate in order to prevent excessive plant damage. Besides these abovementioned defences canola also has another defence system known as the glucosinolate-myrosinase system. When plants are under biotic stress glucosinolates will be broken down to produce the biologically significant and reactive compounds. These compounds have different roles; some of these compounds deter insects while others have been shown to trigger the antioxidant response. In this review, the negative effects of HM stress like excessive ROS production on canola and closely related plants are discussed. The defence systems like chelation and the antioxidant response are also discussed. Special attention is paid to the glucosinolate-myrosinase system and the signalling role of indole glucosinolate breakdown products.

1.2 Production and uses of Canola

1.2.1 Production of *Brassica napus*

Brassicaceae is a family that is part of the order of capperales. The family contains about 350 genara and about 3500 species are found collectively in those genera. Species include the cruciferous vegetable group that contain among other cabbage, broccoli, brussel sprouts and *Brassica napus* (canola). *B. napus* was cultivated as early as the 14th century in Europe (Lagercrantz, 1998). Variants of these species contain high concentrations of both euric acid and glucosinolates that give these plants an unpleasant taste. Plant breeding of different canola cultivars resulted in variants that are more suitable for human and animal consumption. Canola contains low euric acid (less than 2 % in its oil) and low glucosinolate content (30 µmol/g of oil cake). There are also herbicide resistant canola variants that are among other TT canola (resistant to triazine), CL canola (resistant to imazamox) and RR canola (resistant to glyphosphate). According to a report by De Kock and Agenbag (2009), canola is the second largest oil crop in the world. Canola contributes almost 14 % to the world's oilseed crop production. The main countries in production of this crop are England, France and Germany, Canada, India and China. Collectively these countries produced almost 88 % of canola for the 2009/2010 year. Official cultivation of canola in South Africa started in 1992 due to low profit margins of cereal crops. South Africa is not one of the major producers of canola as it produced only 0.04 tons during the 2009/2010 year.

1.2.2 Canola for human and animal use

Humans mainly use canola as a cooking oil source. Canola oil contains low levels of saturated fatty acids and it is high in oleic and linolenic acid (CANSA). In some African countries, canola is being consumed as a leafy green vegetable. A comparative mineral content analysis done by Miller-Cebert et al. (2009) of cabbage, collard greens, kale and canola cultivars has shown that canola is a perfect substitute for cabbage, collard greens and kale as a green leafy vegetable. The crop is also used to feed livestock. Canola oilcake (material after removal of oil) contains about 37 % protein, which makes it suitable to be used as an animal feed (De Cock and Agenbag, 2009).

1.2.3 Canola for environmental use

Canola has also made waves in the green economy. Canola oil is being used to produce biofuel (fatty acid methyl esters) in a transesterification process (Dizge and Keskinler, 2008). Production of biofuel with canola as a source is being done on a commercial scale in European countries. Commercial production of biodiesel using canola as an oil source in South Africa is however still being considered. Trials are also being done to use *B. napus* in the field of phytoremediation of heavy metal contaminated sites (Grispen *et al*., 2006). The topic will be discussed in more detail in later section of this review.

1.3 Mining of Zirconium and the use of this metal in industries

Zirconium has an atomic number of 40 and it is similar to titanium. The properties of zirconium make it a useful metal (Zaccarone *et al*., 2008).

Zirconium is used in high technology devices such as transducers in audio equipment, oxygen sensors and television screens. Zircon is used to give a shiny finish to tiles, crockery and bath ware. The zircon coating makes these products more durable (Tyler and Minnett, 2004). According to U.S. Geological Survey, thousands of metric tons of zirconium are mined each year (see figure 1). The mining of this metal and subsequent industrial use of this metal may lead to increased zirconium content in soil. Metal contamination of soil has been shown to cause problems for agriculture and other industries.

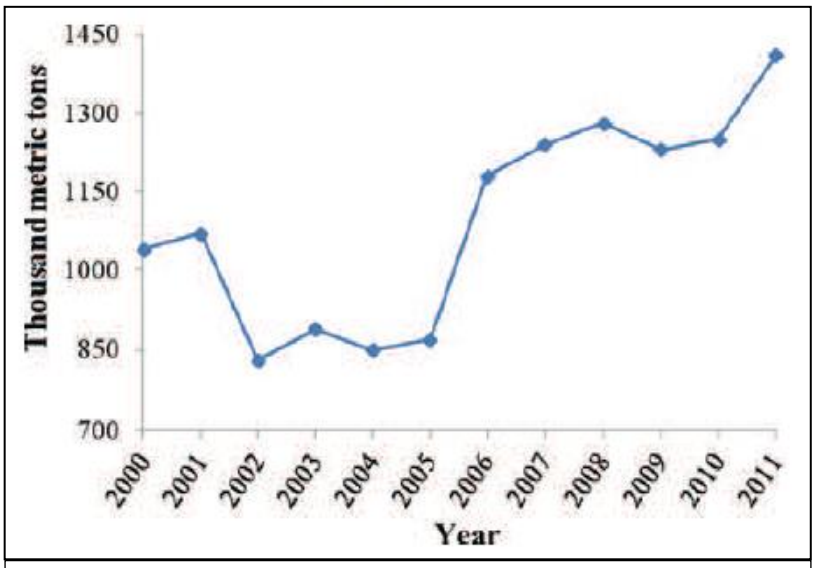

Figure 1.3: World-wide production of zirconium. The graph depicts that zirconium production (adopted from USGS, 2012)

1.4 Abiotic stress

Plants are susceptible to many environmental stresses. The stresses can be classified into two groups namely biotic and abiotic stresses. Biotic stress results when plants are under attack by living things. This can range from

bacteria to insects and large herbivores (Apel and Hirt, 2004). Abiotic stress is stress inflicted on plants by non-living things these include extreme temperature (chilling and heat stress), drought, salinity and heavy metal stress (Gill and Tutetja, 2010). Metals can damage plants by producing ROS directly or indirectly, by activating signalling cascades, inhibiting photosynthesis, inhibiting enzymes or by inducing expression of ROS producing enzymes.

1.5 HM triggered ROS production

Reactive oxygen species are reduced or partially reduced forms of oxygen. They include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) (Gill and Tutetja, 2010). ROS are produced during normal metabolic processes of living organisms. Plants produce O_2^- in chloroplast during the process of photosynthesis (Scarpeci *et al*., 2008). Plants usually deal with these concentrations effectively. However, when plants are under stress an excess of ROS are produced. Sometimes the plant defence against excessive ROS production, which is the antioxidant system, cannot effectively remove ROS. This leads to the situation that is termed oxidative stress. When plants are under oxidative stress these highly abundant ROS react with essential biomolecules like DNA, protein and lipids (see figure 1.2) (Foyer and Noctor, 2005). The result of these reactions is usually cell death. This section of the review will focus on the effect of HM induced ROS production and oxidative stress.

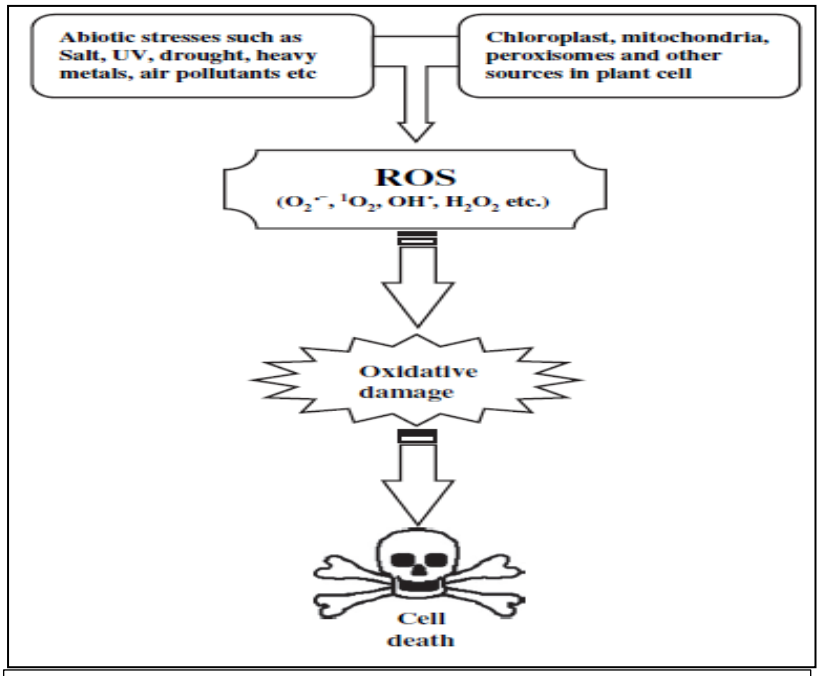

Figure 1.5: The effect of abiotic stress on plant cells. Abiotic leads to ROS production, which may eventually lead to cell death (adopted from Gill and Tutetja, 2010)

1.5.1 Superoxide (O_2^-)

Superoxide is produced during photosynthesis inside chloroplast. It is produced in one electron transfer reaction during electron transport chain (Scarpeci *et al*., 2008). Superoxide is also produced during reaction between NADP oxidase and molecular oxygen. The half-life of O_2^- is about 2-4 µseconds (Bhattachrjee, 2005). O_2^- is the first ROS produced in plant cells, all other ROS can be produced using O_2^- as a precursor. This ROS molecule can react with Fe^{3+} to produce singlet oxygen and a reduced form of iron (Fe^{2+}) (Elstner, 1987). Hydrogen peroxide can be formed by the dismutation of superoxide by the enzymatic antioxidant, superoxide dismutase (SOD). The H_2O_2 and Fe^{3+} can then react to form two hydroxyl molecules and Fe^{2+}, which is known as the Fenton reaction (Gill and Tutetja, 2010).

1.5.2 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is produced in cells by the dismutation of O_2^- by SOD. Unlike superoxide, H_2O_2 has a long half-life of one millisecond. When plants are faced with heavy metal stress H_2O_2 levels increase. This is shown by a study of Schutzendubel et al. (2001), where the exogenous application of 50 µM Cd inactivated H_2O_2 targeted antioxidants and that subsequently led to the accumulation of H_2O_2. The increase of hydrogen peroxide led to inhibition of root growth and increased cell death levels. In a similar study by Mobin and Khan (2005), cadmium application resulted in an increase in hydrogen peroxide content. The increase in hydrogen peroxide resulted in lipid peroxidation and cell membrane leakage of *Brassica juncea* plants. Another study by Eleftherio et al. (2015), revealed that the exogenous application of chromium ($K_2Cr_2O_7$) had deleterious effects on the model plant *Arabidopsis thaliana*. Root growth decreased in a concentration dependant manner and cell death and H_2O_2 increased in a time and concentration dependant manner. There are structural damage to plastids, mitochondrion and Golgi bodies, which was caused by an increase in H_2O_2 and other reactive oxygen species. A report by Shakoor et al. (2014), indicated that Pb increased H_2O_2 in a concentration dependant manner. Consequently, it led to increased chlorosis, electrolyte leakage and MDA content.

1.6 Plant defence systems to heavy metal stress (chelators)

The first line of plant defence against HM is to try and prevent HM from crossing biophysical barriers like the cell wall and trichomes (Emamverdian

et al., 2015). The second line of defence that plants use against heavy metals is chelation. Cytoplasmic chelators can be divided into two groups. One group being glutathione based chelators, which include phytochyletins, metallothionins and glutathione. The non-glutathione based chelators are among other organic acids, glycinebetaine and nicotinamide (Hall, 2015). The next section focuses on the role that chelators play during HM stress.

1.6.1 Phytochyletins

Phytochyletins (PC's) are non-protein, sulphur rich peptides. They have the general structure of [γ-glutamyl (Glu) - cysteinyl (Cys)] n–X, where n = 2–11 and X is glycine (Gly), serine, b-alanine, glutamate or glutamine. They are synthesized enzymatically by phytochelatin synthase (PCS) in response to metals (Vatamaniuk *et al*., 2004). Findings of Vatamaniuk et al. (2001), have shown that PC's are required for heavy metal detoxification in animals. In this study the PCS gene has been knocked out in *C. elegans* and it made the organism more sensitive to cadmium stress. A study by Thangaval et al. (2007), have shown that plants produce phytochyletins upon heavy metal exposure. PC's can also make plants more tolerant to heavy metals. This is shown by Fernandez et al. (2012), that Pb-PC complexes increased the tolerance of *Melilotus alba* and *Melilotus officinalis* to Pb. A report by Shukla et al. (2012), have shown that the heterologous expression of *CdPSC1* (phytochelatin synthase gene) of *Ceratophyllum demersum* in *Nicotania tabacum* improved cadmium and arsenic accumulation in the tobacco plant. Metals bind the sulphur atom of cysteine residues of PC and then transport

the PC-HM complexes to vacuoles by using ABC-type transporters (Solanki and Dhankhar, 2011).

1.6.2 Metallothionins

Another thiol containing metal chelator are metallothionins (MT's). Metallothionins are found in fungi, animals, plants and even in some prokaryotes. Unlike PC, metallothionins are synthesized by the translation of an mRNA transcript (Cobbet and Goldsborough, 2002). Metallothionins are produced in response to osmotic, drought, temperature, heavy metal and nutrient stress (Sekhar *et al*., 2011). Besides the role of a heavy metal chelator in plants, MT's are also believed to play a role in ROS homeostasis, cell division and DNA repair. For the purpose of this review, there will be a focus on the role of metallothionins and its role of heavy metal chelation and ROS homeostasis. According to a study by Xia et al. (2012), the heterologous expression of an *Elsholtzia haichowensis* metallothionin in tobacco resulted in an improved copper tolerance. Metallothionins also led to decreased hydrogen peroxide accumulation and it increased the activity of an enzymatic hydrogen peroxide scavenger. In a similar study Zhou et al. (2014), showed that expression of a *Tamarix androssowii* MT in tobacco led to better tolerance to cadmium stress and that an increase of SOD activity played a role in the tolerance.

1.7 Antioxidant defence system of plants against HM triggered ROS production

Plants defend themselves against the damaging effect of ROS by making use of antioxidants. The antioxidant system consists of both enzymatic and

non-enzymatic antioxidants. These antioxidants are used together to ward off the effects of oxidative stress on plants (Gill and Tutetja, 2010). This section focusses on the antioxidant response to abiotic stress and more in particular heavy metal stress.

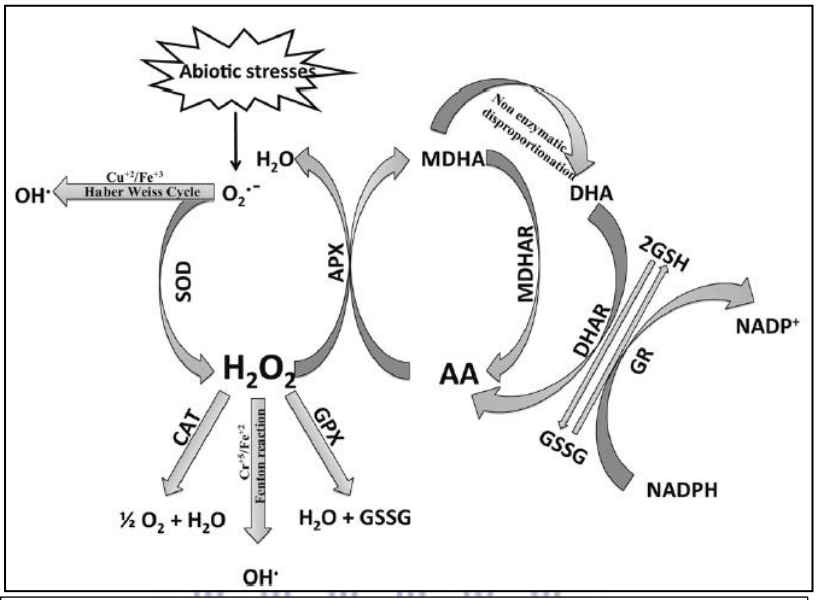

Figure 1.7: The antioxidant system of plants. This show how ROS molecules are scavenged by enzymatic and non-enzymatic antioxidants. It also depicts the regeneration of non-enzymatic antioxidants AA and GSSG (adopted from Gill and Tutetja, 2010)

1.7.1 Enzymatic antioxidants

1.7.1.1 Superoxide dismutase

Superoxide dismutase catalyses the dismutation of O_2^- to H_2O_2 and O_2. Superoxide dismutase is found in all aerobic organisms. They are metalloenzymes and they are classified according to the metal used as co-factor. Superoxide dismutase isoenzymes include Fe-SOD, Mn-SOD and Cu/Zn-SOD (Mitler, 2007). In plants, Fe-SOD are localized in the chloroplast, Mn-SODs in the mitochondria and peroxisomes and Cu/Zn-

SODs in the chloroplast and cytosol. Superoxide dismutase plays an important role in keeping a redox homeostasis of plants during abiotic and biotic stress situations. Superoxide dismutase activity usually increases when plants are under HM stress. This was shown in a study by Mobin and Khan (2007), where *Brassica juncea* plants subjected to Cd stress resulted in significant increased SOD activity. In a similar study Li et al. (2009), found that the exogenous application of Cu on *B. juncea* plants also resulted in an increase of SOD activity. A study by Lee et al. (2007), revealed that the overexpression of both SOD and APX (of *Nicotiana tabacum* origin) into *Festuca arundinacea Schreb* cv. Kentucky-31 led to the transgenic plant being more tolerant to various heavy metals like Cd, Cu and As.

1.7.1.2 Catalase

Catalases are enzymes that catalyse the reaction of H_2O_2 to H_2O and O_2 (Garg and Manchanda, 2009). Catalases have a somewhat broader specificity in that they can react with methyl hydrogen peroxide (Ali and Alqurainy, 2006). These enzymes are heme-containing enzymes and they exist as tetramers. Another characteristic of this enzyme is its high catalytic rates. Catalases can catalyse the conversion of 6 million molecules of hydrogen peroxide to H_2O and O_2 per minute (Garrett and Grisham, 2010). Catalases, like other antioxidant enzymes play a protective role against oxidative stress. A report by Nouairi et al. (2009), has shown that there is an increase of CAT activity in response to Cd treatment on *B. juncea* leaves. Contrary to the previous study, John et al. (2007), found that there is a decrease in catalase activity when *B. juncea* is subjected to cadmium

stress. Findings of Gichner et al. (2004), suggested that a catalase deficient transgenic *N. tabacum* plant showed more DNA damage when exposed to Cd compared to the wild type plant. In a study by Guan et al. (2009), a catalase gene of *B. campestris* was expressed heterologously in *N. tabacum*. The transgenic tobacco plant had a higher catalase activity and tolerance to cadmium stress was enhanced.

1.7.1.3 Ascorbate peroxidase

Another antioxidant enzyme that is responsible for scavenging hydrogen peroxide is ascorbate peroxidase. As its name may suggests, this enzyme uses ascorbate as an electron donor in the catalytic reaction. The reaction will then result in formation of water and monodehydroascorbate (Apel and Hirt, 2004). Ascorbate peroxidases are more efficient at scavenging hydrogen peroxide than CAT and other peroxidases due to a higher affinity for hydrogen peroxide (Gill and Tutetja, 2010). This enzyme exists in several sub-cellular locations, which are the cytosol (cAPX), chloroplast stroma (sAPX), thylakoid (tAPX) and glyoxisome membrane (gmAPX) (Noctor and Foyer, 1998). Changes in APX activity when plants are under HM have been noted in several studies. A study by Fodor et al. (2005) has shown that there is an increase of APX activity when wheat seedlings were treated with high doses (550 mM) of Zr. This trend is also seen in a study by Schutzendubel et al. (2001), where cadmium exposure to scots pine seedlings led to an increase of APX activity in the seedling roots. Findings by Malar et al. (2014), suggest that APX activity increased when hyacinth plants are placed

under Pb stress. The APX activity increased as the concentration of the metal increased.

1.7.1.4 Glutathione peroxidase

Glutathione peroxidases also scavenge hydrogen peroxide. Unlike APX, GPX use glutathione instead of ascorbate as a coenzyme to scavenge hydrogen peroxide (Apel and Hirt, 2004). Glutathione peroxidases like proteins have been discovered in plant species. It was established that the model plant, *A. thaliana* has up to seven GPX-like proteins (Millar *et al*., 2003). Like the other antioxidant enzymes that has been mentioned afore, GPX activity also changes when plant find themselves in HM stress situations. A study by Haluskova et al. (2009), has shown that GPX activity in root tips increased when barley plants are under cadmium stress. Findings of Hossain et al. (2010), have shown that exposure of mung bean seedlings to 1 mM Cd led to an increase of GPX activity. On the contrary, a study by Vestena et al. (2011), revealed that the exogenous application of cadmium on water hyacinth resulted in a decrease of GPX activity.

1.7.2 Non-enzymatic antioxidants

1.7.2.1 Glutathione

Glutathione is a tripeptide that consists of glutamine, cysteine and glycine (Jozefczak *et al*., 2012). Two enzymes namely GSH 1 and GSH 2 synthesize this antioxidant molecule. The synthesis of glutathione is a two-step process from its amino acid precursors. In the first step GSH 1 will link γ-glutamate and cysteine using ATP. Then GSH 2 will add glycine in another

ATP dependant step to form glutathione (May *et al*., 1998). Glutathione can directly scavenge hydrogen peroxide and it will result in the formation of GSSG. De Vos et al. (1992), exemplified the importance of GSH during HM stress in a study that revealed that BSO pre-treatment (GSH 1 inhibitor) resulted in higher lipid peroxidation levels when plants were exposed to Cu stress. Findings of Chen et al. (2010), showed that the exogenous application of GSH ameliorates the negative effects caused by Cd HM stress of barley.

1.7.2.2 Ascorbic acid

Ascorbic acid, which is also known as vitamin C, is an antioxidant. It is found in most plant cell types and organelles (Smirnoff, 2000). The site of ascorbate synthesis is in the mitochondria. Ascorbate is transported from the mitochondria to other cell locations by a proton-electrochemical gradient and facilitated diffusion (Horemans *et al*., 2000). This antioxidant also serves as a substrate of APX. Ascorbic acid can scavenge singlet oxygen, superoxide and H_2O_2 on its own. Ascorbate plays a role in HM stress. A study by Pandey et al. (2009), revealed that when spinach is under Ni, Cu, Zn and Cd stress it resulted in an increase of ascorbate. Work by Al-Khami and Hamada (2011), showed that exogenous ascorbic acid alleviated the effects of Cu stress on wheat plants.

1.8 The defence related glucosinolate-myrosinase system

Another defence related system of plants in the order of Capperales is the glucosinolate-myrosinase system (Halkier and Gershenzon, 2006). When plants of this order are faced with biotic stress factors these plants will

increase their production of glucosinolates and the breakdown products thereof (Fahey *et al*., 2001). These compounds deter insects to protect plants against biotic stress and some have secondary signalling roles in plants (Katz *et al*., 2015). The next section focuses on the synthesis of indole glucosinolates (IG), the organization of glucosinolate-myrosinase system (mustard bomb) and the signalling roles of the IG breakdown products in plants.

1.8.1 Glucosinolates

Glucosinolates (GLS) are S and N rich anionic compounds that are partly responsible for the bitter taste of brassica vegetables (Rodman *et al*., 1996). Over 120 different glucosinolates have been identified and plenty more of their breakdown products (Fahey *et al*., 2001). These secondary metabolites have a general structure of a β-D-glucopyranose ring that is linked to a (Z)-N-Hydroximinosulfate ester via a S atom, with a variable R group attached. Glucosinolates are classified based on these R groups that are derived from amino acids (Daxenbichler *et al*., 1991). Glucosinolates with an R group derived from leucine, isoleucine, alanine and valine are called aliphatic glucosinolates, those with R groups from phenylalanine and tyrosine are called aromatic glucosinolates and lastly those with R groups derived from tryptophan are called indole glucosinolates (Fahey *et al*., 2001). For the purpose of this review there will be a focus on the synthesis of IG only.

1.8.2 Synthesis of indole glucosinolates in plants

As mentioned previously indole glucosinolates are synthesised from tryptophan (Halkier and Gershenzon, 2006). CYP 79B3 and CYP 79B2 are the main enzymes that are responsible for the conversion of tryptophan to indole-3-acetaldoxime (IOAx). However, CYP 79B3 is used to convert Trp to IOAx, when IOAx is used as a precursor for IG synthesis (Hansen and Halkier, 2005). In a study by Brader et al. (2001), where *A. thialana* was exposed to the bacterial pathogen *Erwinia carotorova,* the CYP 79B3 gene was induced instead of CYP 79B2. To further prove that CYP 79B3 is used for indole glucosinolate production, gene knockout studies by Zhao et al. (2002), showed that CYP 79B2 knockout mutants retain their ability to produce IG. CYP 79B3 mutants on the other hand presented significant decreased IG synthesis. In *A. thaliana,* four enzymes are then responsible for the conversion of IOAx to IG. The first one is CYP 83B1 which is an oxime metabolising enzyme. CYP 83B1 will oxidize IOAx to an activated oxime believed to be aci-nitro or nitrile oxide. The aci-nitro/nitrile oxide metabolite will then be conjugated to a sulphur donor. The second enzyme is a C-S lyase that will cleave a C-S bond to yield thiohydroximic acid (Mikkelsen *et al*., 2004). The final two enzymes in the pathway are a thio-glucosyltransferase (UGTB74B1) (Grubb *et al*., 2004) and an indole specific sulfotransferase (STa 5) (Piotrowski *et al*., 2004).

1.8.3 Myrosinases

Myrosinases are enzymes that belong to the glycoside hydrolase family. The three-dimensional structure and properties of myrosinases are similar

to that of O-glucosidases. This is seen in a study by Burmeister et al. (1997), where the three-dimensional structure of myrosinases of *Sinapsis alba* was determined. There are plenty of S-S bridges, salt bridges and H-bonding in the structure of myrosinases. These bonds aid in the stability of myrosinase that can catalyse reactions in an extracellular environment when tissue damage has occurred. The main post-translational modification of myrosinases is glycosylation. Glycosylation enhance the stability of the proteins by making it more tolerant to hydrolytic reactions (Halkier and Gershenzon, 2006). Ascorbic acid is an essential co-factor for myrosinase activity. Studies by Wilkinson et al. (1984), showed that ascorbic acid is needed for myrosinase activity but only at low concentrations. According to Bones and Rossiter (1996), high amounts of ascorbic acid inhibit myrosinase activity. In a X-ray crystallography study by Burmeister et al. (1997), it was again proven that myrosinase use ascorbate due to the presence of an ascorbate binding site. The number of functional copies of myrosinase genes differs among the different species. In *A. thaliana,* for example, 4 genes have been identified (Xu *et al*., 2004). Whereas in *B. napus* 20 putative myrosinase genes have been identified to date (Rask *et al*., 2000). The locations of expression of these genes are also somewhat different among species, but this will be explained in the next section, which deals with the mustard bomb theory.

1.8.4 The mustard bomb Theory

The mustard oil bomb theory is a term that was coined by Luthy and Matile (1984). This theory states that there is a spatial separation between the

components of the mustard oil bomb (aforementioned GLS and myrosinases). However, when plants are under conditions that cause tissue damage like biotic stress for example this barrier becomes demolished and the components of the mustard bomb can then come in contact (Kissen *et al*., 2009). Glucosinolate molecules will become hydrolysed when they come into contact with myrosinases to produce the more reactive and biologically significant glucosinolate breakdown molecules (Brader *et al*., 2006). The breakdown products will depend on the type of glucosinolate molecule, the chemical conditions, and the presence/absence of certain proteins. Based on mustard oil bomb theory, GLS can be separated from myrosinases in the following way; GLS and myrosinases are kept in separate cells, they can be in the same cell but in different sub-cellular locations or the GLS molecules can be together with myrosinases but these enzymes are inactivated (Kissen *et al*., 2009). The arrangement of the GLS-myrosinase system differs from species to species. The next section will focus on the arrangement of the myrosinase-glucosinolate system.

1.8.5 Localization of glucosinolates

Glucosinolates are found in all plant organs. The type and concentration of these compounds differ in the different organs (Shelton, 2005). The development stages (Brown *et al*., 2003) and environmental factors (abiotic and biotic stress) have an effect on GLS content. A few studies have been done to determine the cellular localization of GLS in plant species. Work done by Kelly et al. (1998), suggests that GLS exist in all cells of *B*. *juncea* cotyledons. This was determined by immunohistochemically localizing the

GLS molecule, singirin. In another study by Koroleva et al. (2000), it was shown that GLS was localized in a specific cell type. The GLS content in these cells was inferred by the sulphur content in these cells. Myrosinase enzyme assay of these S-cells extracts also confirmed that there is indeed high GLS content in these cells. A study by Thangstad et al. (2001), where radiolabelled GLS precursor was used revealed that GLS may localize in myrosin cells or in very close proximity to myrosin cells.

1.8.6 Localization of myrosinases

Myrosinases are localized in myrosin cells. Myrosin cells are protein-accumulating idioblast and it has been discovered more than 125 years ago. Most of the studies performed to localize myrosinases were based on antibody detection techniques. A study by Thangstad et al. (1991), revealed that myrosinases is localized in myrosin cells of *B*. *napus*. Hoglund et al. (1991), detected myrosinases in myrosin cells of *B*. *napus* embryos by using the 3D7 and K505 myrosinase antibodies. Most evidence points to the vacuoles when it comes to the subcellular location of myrosinases. A study by Bones et al. (1991), suggested that myrosinases localize in protein bodies. A different study by Hoglund et al. (1991), suggested that myrosinases is localized in the cytoplasmic space. Thangstad et al. (1991), revealed that myrosinases are localized in vacuoles using immunogold-EM and the polyclonal antibody K089. The findings of Geshi et al. (1998), also support the idea that *B*. *napus* myrosinases are localized in vacuoles of myrosin cells.

1.8.7 Synthesis and signalling roles of IG breakdown products

Indol-3-ylmethylglucosinolate (I3M-GS) is one of the main indole glucosinolate found in *Brassica* species (Kim *et al*., 2008). When this GLS is cleaved by myrosinases, unstable products will be formed and this will lead to the formation of Indole-3-Actetic acid (IAN) and Indole-3-Carbinol (I3C). Indole-3-carbinol can react with several cellular metabolites to give rise to several different products.

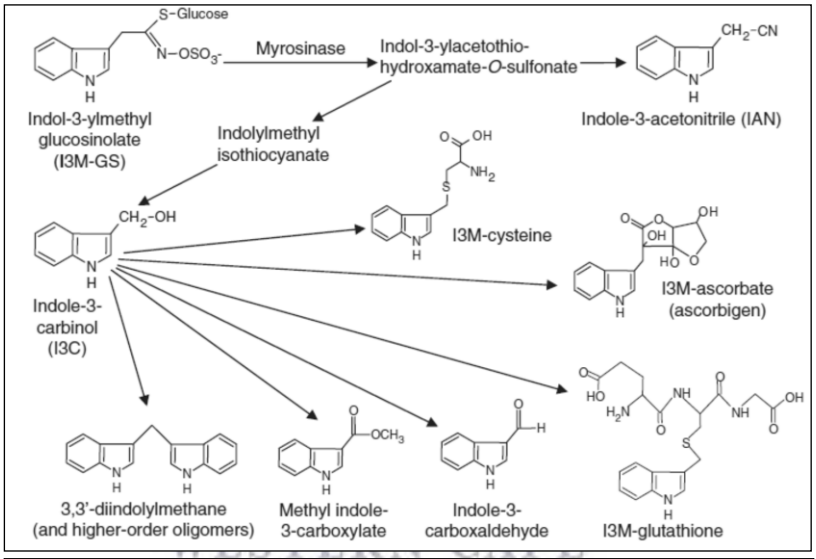

Figure 1.8.6: IG breakdown products derived from I3M-GS. I3C can react with a variety of plant metabolites to form various products (adopted from Kim *et al*., 2008).

Now that the production of indole glucosinolates and their breakdown products have been discussed, this next section will focus on the signalling roles of these breakdown products. The emphasis will be placed on DIM since it is more significant to this study. In a study by Zhao et al. (2015), *A. thaliana* was subjected to mimicked biotic stress using FB1 (fungal phytotoxic compound). Exposure of the plant to FB1 led to ROS

accumulation and eventually resulted in cell death. Plants pre-treated with I3C and IAN however showed a decrease in ROS accumulation and cell death levels similar to the control. Antioxidant activity of I3C and IAN pre-treated plants also increased. A study by Katz et al. (2015), revealed that I3C inhibits root growth of *A. thaliana* seedlings by acting as an auxin antagonist. Diindolylmethane consists of two indole rings. This molecule is synthesized in plants by an acid condensation reaction of two I3C molecules. Diindolylmethane is an antioxidant due to the two N-H group. Findings of Benebadji et al. (2004), showed that DIM is a better antioxidant than vitamin E using the DPPH test assay. To the best of my knowledge only one study of exogenous DIM on plants have been performed. In the study by Pal et al. (2007), DIM and other derivatives of DIM were tested for growth promoting effects on *Orizia savita*. According to this study, DIM was shown to be a growth enhancer of *O. savita* seedlings. The effect of DIM and its role in cancer treatment has been studied intensely in recent times. Andromycin is used as a cancer treatment drug, but it causes an increase in cellular ROS content that eventually leads to cardiovascular fibrosis. A study by Yao et al. (2013), revealed that the co-treatment of Andromycin and DIM lead to a decrease in cardiovascular fibrosis. This was due to DIM, which increased the antioxidant capacity of cells in a BRCA 1 dependant manner.

1.9 Justification

The mining sector in South Africa makes up a very big part of the South African economy. It creates jobs and brings wealth to the country. However,

there is a negative side to mining and that is in some instances mining may lead to water and land pollution. Some mining practices leads to the contamination of land with HM's. South Africa is the second largest producer of zirconium in the world. As a result it can be expected that the soil will get contaminated with zirconium. It has been stressed in this review that exposure of plants to HM's may lead to plant death/crop loss. Soil HM contamination thus leads to less arable land. In a country with an ever-growing population, land for agricultural practices is always sought after. One solution to soil HM contamination is phytoremediation. Canola is one of the suitable crops for use in phytoremediation purposes. This plant can grow in relatively high metal concentrations, it does take metals up from soil and it grows relatively fast as well. However, canola is not immune to very high HM content. One strategy of enhancing the tolerance of canola to HM stress is to increase the antioxidant capacity of this plant. Plants naturally produce compounds that increase its antioxidant capacity. A study by Zhao et al. (2015), revealed that two indole glucosinolate breakdown products namely I3C and IAN increase the antioxidant capacity of *A. thaliana*. Several animal studies have been undertaken to determine the effect DIM on cancer tissues. These studies revealed that DIM increase the antioxidant capacity of these cells. Diindolylmethane also has been proven to increase growth of rice seedlings. No studies have been done to date to show if DIM can increase the antioxidant capacity and growth of canola. If this chemical can do both then a plant producing more DIM would surely be advantageous to be used in the field of phytoremediation.

1.10 Objectives

The main aims of the project are to determine the effect of DIM treatment on seedling samples and its effect on HM stress seedlings (triggered by Zr application). Since DIM is insoluble in water, a protocol will be designed to solubilise DIM in water for exogenous application on seedlings. The effect of treatments (Control, DIM only, Zr treated, DIM-Zr treated) on seedlings growth will be assessed by doing physiological studies like seedling length, fresh weight and dry weight. Heavy metal stress and stress related secondary compounds (like DIM) have an effect on cellular ROS levels. For this reason, ROS content (superoxide and hydrogen peroxide) will be analysed by spectrophotometric biochemical assays. The extent of lipid peroxidation and cell death will be studied by using TBARS and Evans blue assay respectively. The effect of treatments on antioxidants (SOD, APX and CAT) will also be determined by spectrophotometric assays. The treatments may also have an effect on micronutrient content and therefore it will be analysed by ICP-OES.

CHAPTER 2 - METHODS

2.1 Solubilisation of DIM

Diindolylmethane stock solution was prepared as follow: 10 mg of DIM powder was weighed using a fine balance and transferred to a 50 ml centrifuge tube. Then 235 µl of Tween 80 was added to the tube containing DIM. The mixture was dissolved in 10 ml absolute ethanol. The mixture was snap frozen using liquid nitrogen and the ethanol was removed by lyophilizing overnight at -50 °C (Labconco benchtop freeze-drier).

2.2 Treatments

A 1:3 mixture of soil (Stodels Nursery South Africa double grow potting soil) and filter sand (Cape Silica Suppliers cc.) was added in pots with diameter of 19 cm. Then the pots were pre-treated with 100 ml of 1.18 % (v/v) Tween 80 (control), 15 µM DIM, 1 mM Zirconium Chloride or 15 µM DIM-1mM Zirconium Chloride. Five seeds were sown per pot the following day. Seedlings were treated every third day for a period of three weeks. The seedlings were grown in a greenhouse with a constant temperature of 25 °C and light/dark cycle of 16/8 hours.

2.3 Dry weight and germination percentage determination

Dry weights were determined by placing four seedlings in a pre-weighed foil envelope. The envelopes were then placed in an oven at 80 °C for three days. The dry weights of the seedlings were determined using a fine balance. Twenty-five foil packets were made for each treatment and the dry weight experiment was repeated four times to ensure validity of results.

Germination percentages were recorded on the first day after sowing and every second day thereafter. Germination percentages were calculated based on the percentage germination of each pot.

2.4 Quantification of ROS content

2.4.1 Superoxide assay

Superoxide content of seedlings were determined by using a modified protocol of Russo et al. (2008). Entire seedlings were placed in 800 µl of 50 mM potassium phosphate pH 7.0, 10 mM potassium cyanide, 10 mM hydrogen peroxide and 80 µM NBT. Seedlings were incubated for 20 minutes. Following incubation, seedling material was crushed using a miniature pestle and pelleted by centrifugation at 13 000 *g* for 5 minutes. Then 200 µl of each sample was loaded into a 96 well flat-bottom microtitre plate and the absorbance readings were recorded at 560 nm using the POLAstar Omega microplate reader spectrophotometer (BMG Labtech).

2.4.2 Hydrogen peroxide assay

A modified method of Velikova (2000) was used to determine hydrogen peroxide levels of samples. Approximately 100 mg of grounded seedling material was mixed with 500 µl of 6% (w/v) TCA using a vortex. Plant material was pelleted by centrifuging at 13 000 *g* and 4 °C for 5 minutes. The supernatant was collected and used to determine the hydrogen peroxide content of each sample. Reaction mixture contained 0.25 M KI, 1.25 mM K_2HPO_4, pH 5.0 and 50 µl of TCA extract in a final volume of 200 µl. The microtitre plate containing the reaction mixtures was incubated for

20 minutes at room temperature on a shaker. Absorbance readings at 390 nm were recorded using an OMEGA microplate reader. Hydrogen peroxide content of samples was calculated using a hydrogen peroxide standard.

2.5 Determination of MDA content

The level of lipid peroxidation was determined by the estimation of MDA content using the TBARS method by Beuge and Aust (1978). Approximately 100 mg of finely grounded plant material was added to microcentrifuge tubes. A TCA extraction was performed by adding 500 µl of 6% TCA to plant material and mixing by vortexing. The plant material was pelleted by centrifuging for 5 minutes at 13 000 *g* and then the supernatant was recovered. Then 300 µl of the extract and 200 µl of 20% TCA/0.5% TBA solution were mixed together in a microcentrifuge tube using a vortex. The mixture was incubated in a waterbath at 95°C for 20 minutes for the MDA-TBA adduct to form. Samples were incubated on ice for 10 minutes immediately after the reaction. Then the samples were centrifuged at 13 000 *g* for 10 minutes. All samples were loaded in triplicate on a 96-well flat-bottom microtitre plate. Absorbance readings at wavelengths of 532 nm and 585 nm were recorded using the OMEGA microplate reader. The reading of 532 nm was subtracted from 585 nm to account for non-specific turbidity. MDA content was calculated using a molar extinction coefficient of 155 $mM^{-1}.cm^{-1}$.

2.6 Chlorophyll content analysis

Ground cotyledons material was weighed and about 100 mg were placed in 15 ml centrifuge tubes. Chlorophyll was extracted by adding 10 ml of ice cold 80% (v/v) acetone to the cotyledons and vortexing vigorously. The plant material was then pelleted by centrifugation at 13 000 *g* for 10 minutes at 4 °C. Samples were loaded in triplicate on a 96-well flat bottom micro-titre plate. The absorbance readings at 663 nm and 645 nm for chlorophyll *a* and *b,* respectively were recorded using a spectrophotometer. Total chlorophyll content was determined by using this formula [(12.7× (A_{654}) - 2.96× (A_{663})) + (22, 9× (A_{663}) - 4, 68 × (A_{654}))] ×V / W, where W= amount of fresh weight plant material used and V= the extraction volume used (Roychoudhury, 2010).

2.7 Cell death assay

Cell death of seedlings was determined using the Evans blue dye uptake method by Baker and Nock (1994). Fresh whole seedlings were placed in 15 ml centrifuge tubes containing 10 ml of 0.25% (w/v) Evans blue dye and incubated for an hour. Seedlings were carefully removed after incubation in Evans blue and placed in 15 ml centrifuge tubes containing 10 ml dH_2O overnight at 25 °C. The seedlings were then placed in 1.5 ml microcentrifuge tubes containing 1 ml of 1% SDS and finely ground with a miniature pestle. An incubation step for 1 hour at 65 °C in a heating block followed to extract Evans blue dye. The plant material was pelleted by centrifugation at 13 000 *g* for 5 minutes. Then 200 µl of each sample was loaded in triplicate

into a 96-well flat bottom micro-titre plate. Absorbance reading at 600 nm was recorded using the OMEGA microplate reader.

2.8 Protein extraction and concentration determination

A triple extraction with ice cold protein extraction buffer (4 mM Potassium phosphate buffer pH 7.4 containing 4% (w/v) PVP and 1 mM EDTA) was performed to obtain protein from all treatment samples. In brief, 100 mg of plant material was homogenised with 500 µl of ice cold protein extraction buffer. The plant material was pelleted at 13 000 *g* and 4 °C for 5 minutes. The supernatant containing the protein was collected and the procedure in the sentence above was repeated twice to obtain enough protein. Then 100 µl aliquots were made and protein was stored at -20 °C until required. Protein was quantified by means of a Bradford assay using the RC DC Protein Assay Kit 11 (Bio-Rad Laboratories).

2.9 Enzymatic antioxidant activity determination

2.9.1 Superoxide dismutase activity

Superoxide dismutase activity of samples was determined based on the ability of SOD to inhibit the photochemical reduction of NBT (Beaucamp and Fridorich, 1971). Protein amounts of 1 µg of each treatment sample were added in triplicate to a microtitre plate. Then 190 µl of the reaction master mix (5 mM potassium phosphate buffer pH 7.8, 2 µM riboflavin, 1 mM Methionine, 10 µM EDTA, 0.1 mM NBT) was added making a final volume of 200 µl. The plate was covered in foil and placed on a shaker for a minute. Then the plate was placed on a light box with a 70 Watt lamp for 10 minutes.

The absorbance reading was recorded at a wavelength of 560 nm. The units of each reaction was then calculated, where one unit equals the amount to cause a 50 % inhibition of the reaction. The reaction was designed where all samples inhibit the reaction by less than 50 % to ensure accuracy of the result.

2.9.2 Ascorbate peroxidase activity

Protein extracts used for APX activity was supplemented with ascorbate to a final concentration of 2 mM and incubated on ice for 5 minutes. The reaction mixture consisted of 5 µg of enzyme extract, 50 mM potassium phosphate buffer pH 7.0, 0.5 mM ascorbate, 0.1 mM EDTA and 0.1 mM H_2O_2, that was added last in a final volume of 200 µl. The depletion of ascorbate was monitored at a wavelength of 290 nm for 15 minutes as it was oxidized. Ascorbate peroxidase activity was calculated using the extinction co-efficient of 2.8 $mM^{-1}.cm^{-1}$ (Asada, 1984).

2.10 Assessing the effect of treatments on the ion content by ICP-OES analysis

Samples were prepared for ICP by wet acid digestion. In brief, 200 mg of plant material was homogenised with 1.8 ml of 65 % (w/w) nitric acid. The samples were then placed in a heating block at 85 °C for 4 hours to complete digestion. The digest was diluted 1:10 using 2 % (v/v) nitric acid as diluent and passed through a 0.4 µm nylon filter. The samples were then analysed for ion content (K, P, Mg, Ca, Fe, Zn and Zr) by ICP-OES.

CHAPTER 3 – THE PHYSIOLOGICAL AND BIOCHEMICAL EFFECTS OF EXOGENOUS DIM ON *BRASSICA NAPUS* SEEDLINGS

3.1 Introduction

Brassica napus belongs to the family of brassicaceae, which include crops like cabbage, broccoli and lettuce (Jongen, 1996). *Brassica napus* is a crop that has many uses. The seeds of this crop are used to make canola oil that is used for human consumption. The waste product after the oil has been extracted (oil cake) is used for animal feed (De Cock and Agenbag, 2009). This crop is also used for the production of biofuel (Dizge and Keskinler, 2008). *Brassica napus* has a higher tolerance for heavy metals when compared to other common crops and therefore this crop is also used for phytoremediation (Grispen *et al*., 2006). Crops of this family have a characteristic bitter taste and it is partly due to the glucosinolate content of these crops. Glucosinolates are classed into three groups that is aliphatic, aromatic and indole glucosinolates. When plants are under biotic stress, enzymes called myrosinases cleave these glucosinolates and this results in the formation of glucosinolate breakdown products (Halkier and Gershenzon, 2006). When indole glucosinolate is cleaved it will lead to the formation of I3C, which is very unstable and it can react with other metabolites in the cell to form various different products. Indole-3-carbinol can also react with itself and form a dimer of I3C, which is DIM. These glucosinolate breakdown products have a deterrent effect on herbivores

(Kim *et al*., 2008). Studies of the signalling role of these compounds in plants are however scarce. A study by Zhao et al. (2015) revealed that I3C and IAN trigger the antioxidant response of *A. thaliana*. Work by Katz et al. (2015) show that I3C inhibits the growth of *A. thaliana* seedlings. A study by Pal et al. (2007), showed that DIM enhance the growth of rice seedlings. It has been showed that hydrogen peroxide content increases when indole glucosinolate content increases (Hara *et al*., 2013). There are numerous reports that suggest that slight increases in ROS content can result in positive signalling such as cell division, germination etc. (El-Maarouf-Bouteau and Bailly (2008); Kranner *et al*., 2010). There is no data on the effects of exogenous DIM on *B. napus*. To date no studies revealed how DIM improves the growth of plants. We hypothesize that DIM will increase growth and improve seed germination by slightly altering ROS content.

3.2 DIM application leads to changes in germination percentage

Seed germination is influenced by various factors. Various studies in literature show that ROS, particularly hydrogen peroxide has an influence on seed dormancy release (El-Maarouf-Bouteau and Bailly (2008); Kranner *et al*., 2010). Diindolylmethane is a compound that is produced during biotic stress (Kim *et al*., 2008). Treatment of seeds with this compound might signal that the plant is under stress and it could perhaps change seed ROS content. A study was performed to determine if the exogenous application of DIM has an effect on seed germination. The results show that DIM treatment improves germination by 120 %.

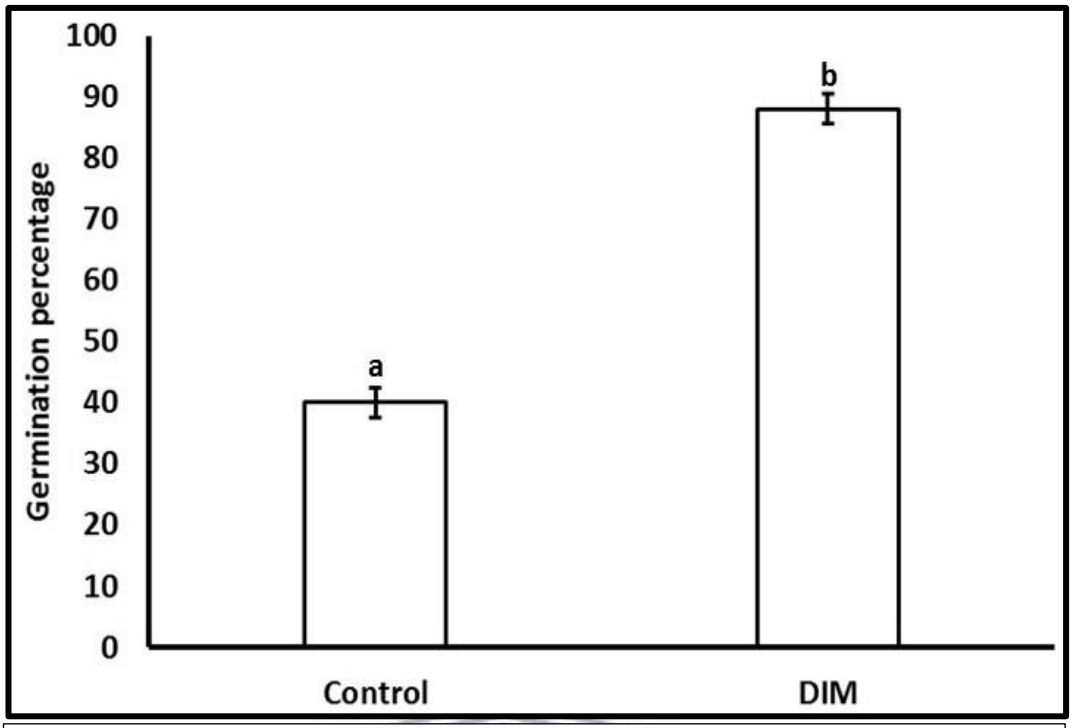

Figure 3.2: The effect of DIM treatment on seed germination of *B. napus*. Soil was pre-treated and treated with DIM or Tween (Control). Germination percentages were calculated at the end of treatment period. The different letters indicate significant changes across means at P< 0.05 (DMRT). Values are means ± S.E. (N=5).

3.3 Exogenous DIM increases biomass

As mentioned previously, DIM is a secondary metabolite that increases in concentration when plants are under stress (Kim *et al*., 2008). Reactive oxygen species molecules when at slightly elevated levels can trigger growth promotion (Bailly, *et al*., 2008; Bakeeva, 2010). If DIM application can change ROS concentrations slightly it might have a growth promoting effect. A study has shown that DIM and some of its derivatives have a growth promoting effect on rice seedlings (Pal *et al*., 2007). Based on this information, a study on the effect of exogenous DIM application on canola seedling growth was done. Results show that DIM treatment indeed has a

growth promoting effect on canola seedlings. The biomass increased by 21 %.

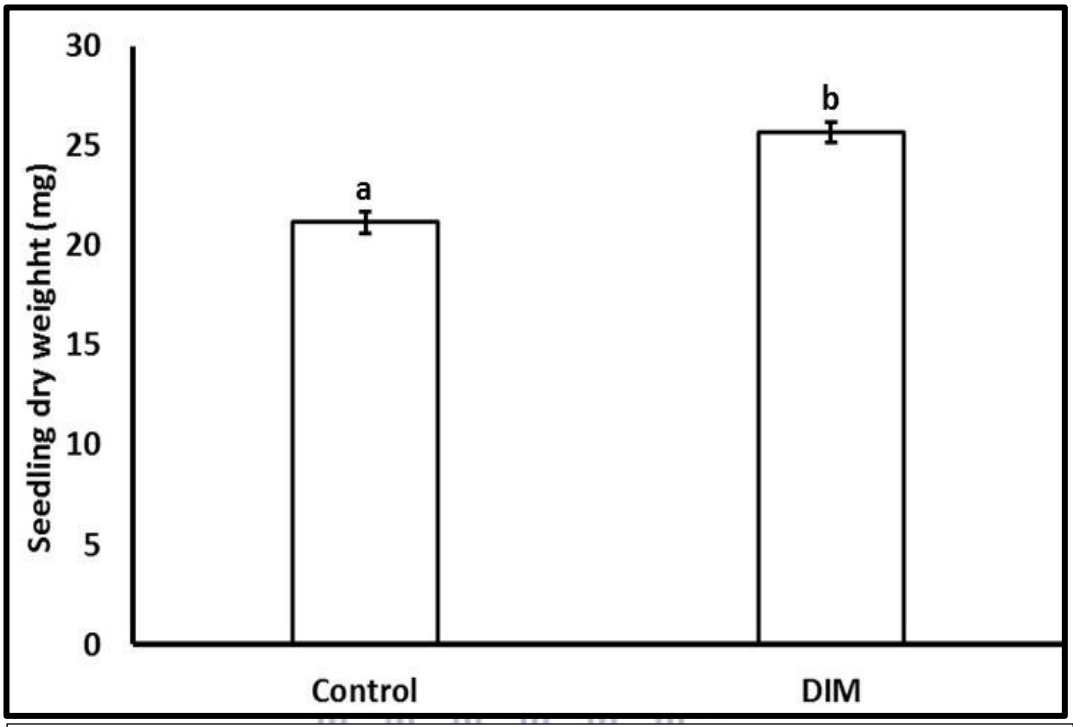

Figure 3.3: The effect of DIM treatment on seedling biomass of *B. napus*. Seedlings treated with either DIM or Tween (Control) until the end of cotyledon stage (approximately 14 days). Dry weights were then determined. The different letters indicate significant changes across means at P< 0.05 (DMRT). Values are means ± S.E. (N=4).

3.4 The exogenous application of DIM changes both ROS content and antioxidant activity of seedlings

A) Changes in superoxide content

Diindolylmethane is a secondary metabolite that is produced during biotic stress (Kim *et al*., 2008). Some secondary metabolites play a role in the hypersensitive response. Reactive oxygen species molecules like superoxide have been shown to have an effect on plant growth and germination (El-Maarouf-Bouteau and Bailly (2008); Kranner *et al*., 2010). It

is for these reasons that effect of exogenous DIM on seedling superoxide levels has been examined. The results reveal that exogenous DIM application increases superoxide content overtime. On day fourteen, there was a 19 % increase in superoxide content of DIM treated *B. napus* seedling shoots sample when compared to the control as shown in figure 3.4 A.

B) Changes in hydrogen peroxide content

Superoxide is a precursor to the more stable but also reactive hydrogen peroxide molecule. An increase in superoxide is usually accompanied by an increase in hydrogen peroxide (Gill and Tutetja, 2010). Increases in hydrogen peroxide levels also results in improved growth and better germination (Singh *et al*., 2014) and therefore we decided to examine *B. napus* seedling shoots for hydrogen peroxide content. Results indicate exogenous DIM also results in the increase of hydrogen peroxide content overtime. On day fourteen, there is a 41 % increase in hydrogen peroxide content of DIM treated *B. napus* seedling shoots samples when compared to the control (Figure 3.4 B).

C) Changes in SOD activity

When ROS increase, it can have deleterious effects on plants. Plants have defence mechanisms against these ROS molecules, which are enzymatic and non-enzymatic antioxidants. The role of SOD is to scavenge superoxide (Gill and Tutetja, 2010). The superoxide content result indicated that there is a significant increase in plant superoxide content. A study was undertaken to establish if changes in superoxide content changed SOD activity. The results revealed that there was quite a significant increase in SOD activity.

On day fourteen, there is a 19 % increase in SOD activity of DIM treated *B. napus* seedling shoots sample when compared to the control (Figure 3.4 C).

D) Changes in APX activity

When superoxide levels increase and SOD activity increases then an increase in hydrogen peroxide levels can be expected. H_2O_2 like any other ROS molecule can cause damage to plants when levels get to high. Plants have defence mechanisms to prevent oxidative damage due to H_2O_2 accumulation. Ascorbate peroxidase is a ROS scavenging enzyme that keeps H_2O_2 levels normal (Gill and Tutetja, 2010). Results show that exogenous DIM application results in an increase in H_2O_2 levels. This prompted the study of APX activity upon exogenous DIM application. The results show that the exogenous application of DIM resulted in a 36 % increase in APX activity on day fourteen.

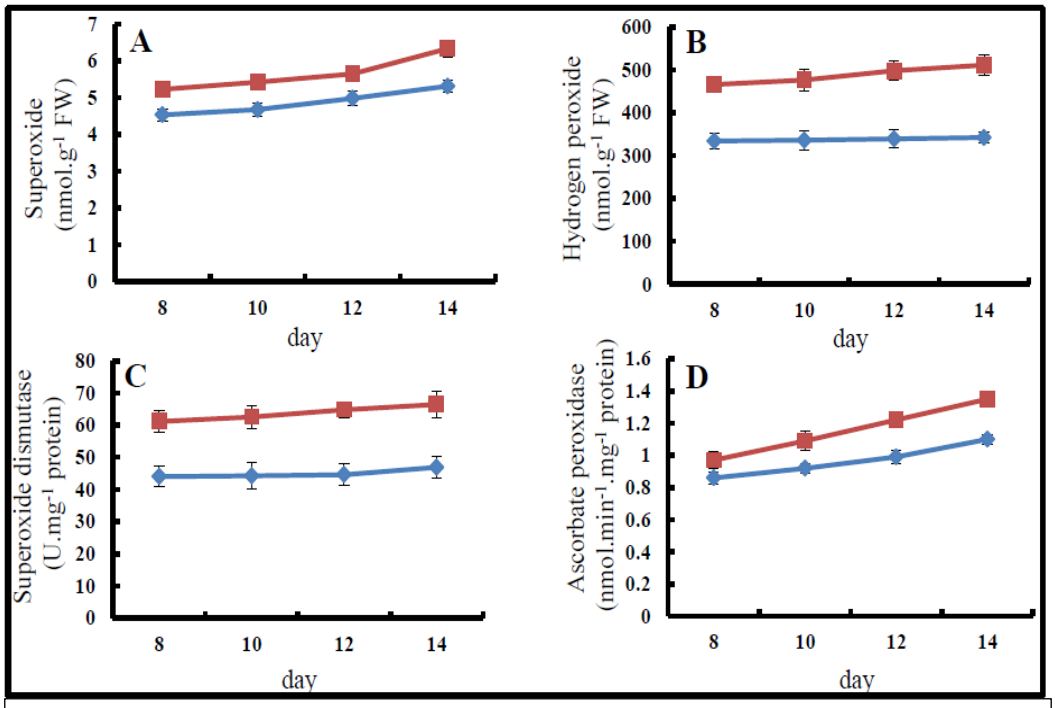

Figure 3.4: ROS content and antioxidant enzyme activity in response to DIM treatment. Seedlings treated with either DIM (Red line) or Tween (Blue line) until the end of cotyledon stage (approximately 14 days).Superoxide (A), Hydrogen peroxide (B), SOD (C) and APX (D) were determined 8 to 14 days after germination. Values are means ± S.E. (N=5).

3.5 DIM application does not result in lipid peroxidation

Lipid peroxidation can occur in plant cell when ROS levels are too high. The cell membrane, which is a very important component of the cell, contains lipids. Reactive oxygen species molecules can react with membrane lipids for example, which will cause membrane damage (Montillet *et al*., 2005). This study has revealed that DIM application results in an increase in both superoxide and hydrogen peroxide molecules. This prompted the study of cellular lipid peroxidation levels. Lipid peroxidation was estimated using the TBARS assay. The results revealed that the exogenous application of DIM did not cause any changes in MDA content (Figure 3.5).

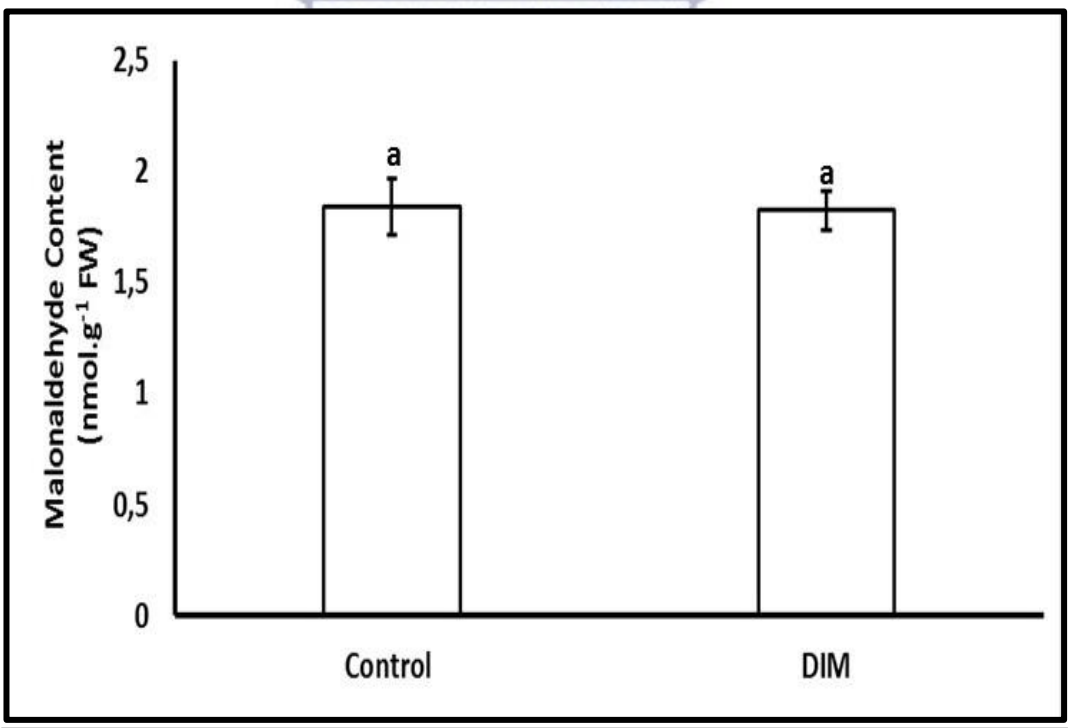

Figure 3.5: MDA levels in response to DIM application. Seedlings treated with either DIM or Tween (Control) until the end of cotyledon stage (approximately 14 days). MDA content of seedlings was determined using TBARS assay as an indicator of lipid peroxidation. The different letters indicate significant changes across means at P< 0.05 (DMRT). Values are means ± S.E (N=5).

3.6 Exogenous DIM has no effect on cell death levels of seedlings

When cells are damaged irreparably, cell death occurs. Cell death can be either necrotic or apoptotic. Oxidative stress is one of the processes that can trigger cell death. ROS react with essential cellular biomolecules and then it eventually lead to cell death (Wang *et al*., 2005). The results show that there is an increase ROS content and therefore the level of cell death was determined in this study. The Evans blue assay was used for this study. The assay is based on the premise that the Evans blue dye will only penetrate dead cells (extremely damage cells to be precise) and stain the cellular content (Baker and Nock, 1994). The cells are then lysed and the lysate is then measured spectrophotometrically. Results indicated that DIM application does not alter cell death levels.

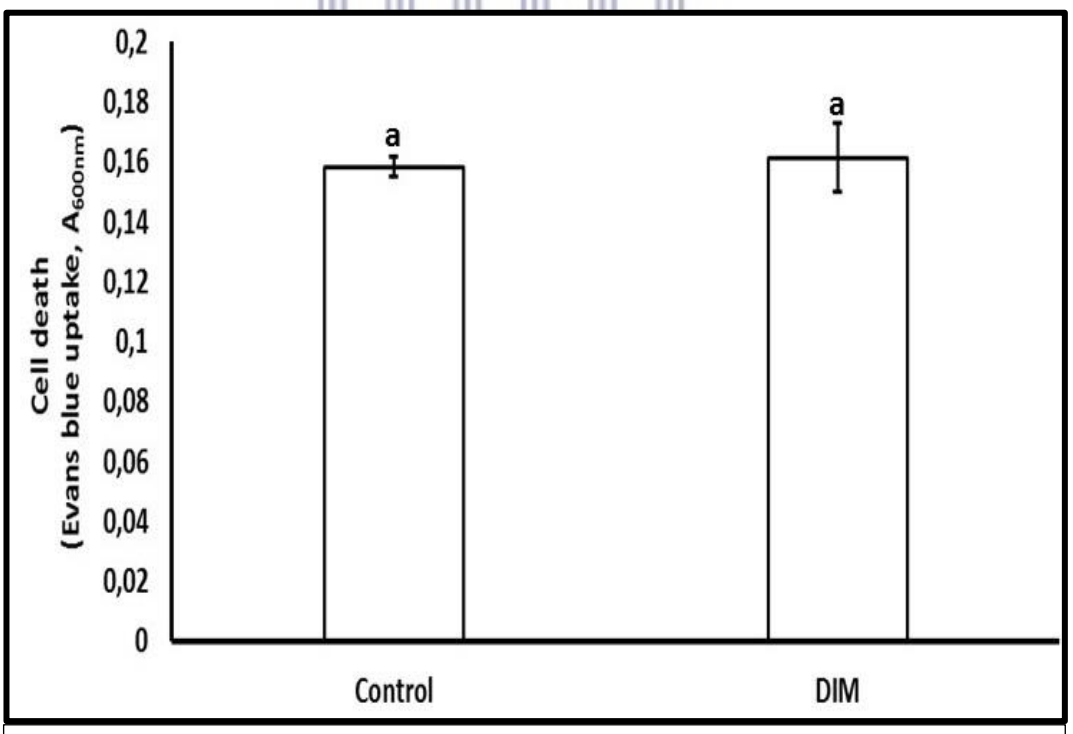

Figure 3.6: Cell death levels in response to DIM application. Seedlings treated with either DIM or Tween (Control) until the end of cotyledon stage (approximately 14 days). Fresh seedling material was used in an Evans blue assay to determine cell death levels. The different letters indicate significant changes across means at P< 0.05 (DMRT). Values are means ± S.E. (N=5).

3.8 Discussion

Indole glucosinolates are molecules that play important roles in biotic stress. A study by Brader et al. (2001), showed that IGS content increase when *A. thaliana* plants are exposed to the plant pathogen, *Erwinia carotovora*. A study by Kim et al. (2008), showed that indole glucosinolate breakdown products also increase when *A. thaliana* is exposed to a herbivore attack by green peach aphid *Myzus persicae*. Some of the breakdown products in their study act as a deterrent to insects. It is therefore clear that IGS and its breakdown products are important in biotic stress defence. Our results reveal that both superoxide and hydrogen peroxide content increase when *B. napus* seedlings are exposed to DIM. When plants are under biotic stress, ROS content seem to increase (Mendoza, 2011). A study by Orozco-Cardenas and Ryan (1999), revealed that plants produce hydrogen peroxide content immediately after wounding. Findings of Hara et al (2013) also revealed that hydrogen peroxide content increased together with an increase of indole glucosinolates. Thus, the findings of Hara et al. (2013), support our results which show that an indole glucosinolate breakdown product increase hydrogen peroxide content. A study by Doke (1983), showed that superoxide content of potato tuber tissue increases when infected. There are no results that show a relationship between an increase in superoxide content and an increase IGS or DIM. It can be argued that DIM, the stress related compound, increases ROS content.

According to figure 3.2 in the results section, DIM application increases the germination percentage of *B. napus*. Studies suggest that GLS molecules

other than IGS molecules might play a role in seed germination. These compounds are at their highest in seeds and tend to decline during the later stages of growth. A study by Auger et al. (2012), revealed that 2-phenylethyl isothiocyanate [2-PEITC], root exudate of *B. napus* act as a germination stimulant of a *B. napus* associated weed *Phelipanche ramosa*. In a study by Ciscka et al. (2008), indole glucosinolate content of *B. napus* increased during the germination process, suggesting that IGS could play a positive role in seed germination. In the previously mentioned studies, an increase in glucosinolate content led to the germination of the seeds of aforementioned plants. Similarly, in this study, the exogenous application of DIM increased the DIM content of seeds and this 'increase' of DIM had the same effect on the germination of the *B. napus* seeds in our study.

Biotic stress conditions can trigger the hypersensitive response i.e. ROS production and subsequently trigger the Foyer-Halliwell-Asada cycle (Tan *et al*., 2013). Diindolylmethane is a molecule that is produced when plants are under biotic attack and because of this reason, exogenous DIM application stimulated ROS production. Studies in literature have shown that ROS play a very important role in germination and seedling growth (El-Maarouf-Bouteau and Bailly, 2008). A study by Ishibashi et al. (2010), has shown that superoxide is needed for germination. This study shows the inhibition of NADPH oxidase (a superoxide producing enzyme) by Diphenylene iodonium (DPI) decreases the production of superoxide. The decrease in superoxide resulted in the decrease of germination percentage of *Hordeum vulgare* L. Work done by Kranner et al. (2010), showed that

superoxide increased during the last stages of germination and it plays an important role in radical elongation. A study by Singh et al. (2014), also supports the idea that superoxide is needed for seed germination. The results show that superoxide scavengers $CuCl_2$ and Tiron inhibited and retarded the germination of *Vigna radiata* (L.). In our study, a similar trend occurs so it is reasonable to say that an increase in superoxide can result in the improvement of germination percentage of *Brassica napus L*. seeds.

A study by Ogawa and Iwabuchi (2001), has shown that the exogenous application of hydrogen peroxide increased germination of *Zinnia elegans* seeds. The exogenous application of hydrogen peroxide increased the endogenous concentration of hydrogen peroxide in those seeds and thereby improved germination. Work by Singh et al. (2014), showed that treatment of seeds with DEDTC (SOD inhibitor) resulted in inhibition of germination. Hydrogen peroxide application reversed the inhibitory effect of DEDTC on germination. This proves that hydrogen peroxide serves as a signalling molecule in seeds to trigger germination. Our study also supports the idea that slight increases in hydrogen peroxide content improve germination.

It has been established that there is cross talk between hydrogen peroxide and phytohormones (Wojtyla *et al*., 2016). In a study by Ye et al. (2012), the exogenous application of the germination suppressor signalling molecule, ABA, caused a decrease in ROS content in rice. Liu et al. (2010), revealed that the addition of hydrogen peroxide increased gibberellic acid (GA) content, which is considered to be a germination enhancer. A study by

Bahin et al. (2011), suggested that increase in hydrogen peroxide content increases the content of the positive germination regulator, GA and GA in turn decreases ABA content by triggering its catabolism. This was proven again in a study by Wang et al. (1995), which showed that exposure of barley seeds to hydrogen peroxide decreases the intracellular level of ABA. The reduction of the endogenous ABA levels favoured germination. These studies clearly highlight the interplay between ROS and phytohormones involved in the germination/dormancy process. The effect of DIM and its influence on GA and ABA content remains to be done in our study.

Another downstream effect of how ROS regulate germination is through mRNA oxidation (El-Maarouf-Bouteau *et al*., 2013). A study by Bazin et al. (2011), revealed that 24 specific mRNA of sunflower seeds have been oxidized during the germination process. The oxidation of mRNA can lead to truncated proteins, decreased protein expression or altered proteins and each of these can cause a signalling effect (Tanaka *et al*., 2007; Chang *et al*., 2008) The effect of DIM on mRNA oxidation caused by DIM triggered ROS increase is still to be investigated in our study.

The increase in ROS content also results in the oxidation of specific proteins and these oxidised proteins then play a role in germination (El-Maarouf-Bouteau *et al*., 2013). There are studies that suggest that hydrogen peroxide might activate transcription factors (Vandenabeele *et al*., 2003). These activated transcription factors will then lead to the transcription of genes that partake in seed dormancy release and germination. Oxidation of amino acyl groups of Lys, Arg, proline and Thr results in the formation of

irreversible carbonyl groups. Work by Barbara-Espin (2011), showed that carbonylation of vicilins and albumin occurred in pea seeds. A study revealed that alcohol dehydrogenase was carbonylated in all actively germinating seeds to promote germination (Corbineau et al., 1991). In a review by El-Maarouf-Bouteau et al. (2013), it is hypothesized that oxidation of glycolytic enzymes can result in the activation of the pentose phosphate pathway that is needed for germination.

Figure 3.3 show that the exogenous application of DIM on *B. napus* seedlings increases the biomass of the seedlings. Our finding is supported by the study of Pal et al. (2007). The study by Pal et al. (2007), showed that DIM and some derivatives of DIM have a growth promoting effect on rice seedlings under certain concentrations. The concentration of exposure to rice seedlings is however different to our concentration of 15 µM DIM, but it must be mentioned that they are working with rice seedlings in their study and not canola seedlings as in our case and that may contribute to the reason why these growth promoting concentration are different. Rice seedlings do not possess the glucosinolate-myrosinase system that canola and other plants of the order capperales have and therefore differences can be expected. A study by Katz et al. (2015), revealed that a precursor molecule of DIM, I3C inhibits growth of Arabidopsis seedlings. Indole-3-carbinol inhibits the growth in a concentration dependant manner. It was hypothesized by the researchers that I3C antagonize the growth hormone IAA. It does so by binding to the binding site of IAA on the TIR1 protein and thereby blocking further signalling for growth to take place. Our results are

in contrary with the findings of Katz et al. (2015). This can be explained by the fact that DIM though similar to I3C in the fact that they are both indoles but there are also differences between these two chemicals. DIM consists of two indole rings instead of one that results in a size difference. In addition, DIM does not contain a hydroxyl group. These differences can prevent DIM to bind to the protein and thereby DIM cannot act as an IAA antagonizer.

Reactive oxygen species also play a role in the growth and development of seedlings. As mentioned previously, DIM application resulted in slight increases in seedling hydrogen peroxide and superoxide content. A study by Bakeeva et al. (2001), revealed that superoxide plays an important role in the growth of wheat seedlings. This study shows that there are bursts of superoxide content that coincide with important events inside cell that lead to growth. Treatment of these wheat seedlings with the antioxidant BHT resulted in retarded growth and what is also interesting is that BHT effected growth negatively in a concentration dependant manner. Findings of the Bakeeva et al. (2001), study support the findings of our study and that is superoxide increases growth of seedlings.

As discussed previously ROS molecules can trigger growth of seedlings. There is however evidence in literature to show that these very same ROS molecules can also retard seedling and plant growth. In a study by Zulfugarov et al. (2014), the gene, *PsbS 2* has been knocked out. This resulted in the mutant producing more superoxide and hydrogen peroxide, which in turn caused retarded growth of rice seedlings. In a study by Fahnenstich et al. (2008), *A. thaliana* plants overexpressing glyoxylate

oxidase produced more hydrogen peroxide and glyoxylate under 75 µmol quanta $m^{-2}s^{-1}$ in their chloroplast. This then resulted in chlorophyll loss in rosettes and more importantly retarded growth. These findings are not in agreement with results obtained in our study. A possible explanation for the contradiction in results could be the level of ROS increase. It has been mentioned in literature that reactive oxygen species can act as signalling molecules that does not result in any deleterious effects on plants like growth and development. This only happens when ROS levels are low and under the control of antioxidants. When ROS levels are high and not under control of antioxidant system then it led to the deleterious effects like chlorophyll loss and retarded growth. Bailey et al. (2008), proposed the concept of the oxidative window and it states when ROS levels are slightly increased it can result in 'positive signalling' like germination for instance. It seems that in our study the concept of the oxidative window also applies for increased seedling growth.

Figure 3.4 (C) show that SOD activity of *B. napus* seedlings increase in response to DIM application. Figure 3.4 (A) show that the superoxide content of *B. napus* increases in response to DIM treatment. Superoxide is a ROS molecule that plays an important role in cellular signalling processes such as germination, growth and development as discussed previously. However, when the concentration of this ROS molecule start to increase to levels where it cannot be effectively scavenged then it can result in dire consequences to plants. It can result in damage to important biomolecules such as DNA and proteins (Gill and Tutetja, 2010). This in turn can impair

normal cellular function and may even cause cell death depending on the extent of damage caused by ROS molecules like superoxide. It is therefore important to control the levels of superoxide. The antioxidant system is responsible for keeping ROS levels at bay and SOD is the main enzyme that is responsible for keeping superoxide below toxic levels. Superoxide dismutase catalyses the conversion of superoxide to hydrogen peroxide and oxygen. Several studies show that SOD activity increases with the increase in superoxide content. In a study by Shah et al. (2001), Cd was applied to rice seedlings. This led to an increase of superoxide content of rice seedlings. SOD activity also increased in their study and the authors reasoned that SOD activity increased to defend the plant from the harmful effects of HM induced superoxide production. The findings of the previous study are in agreement with the results found in our study. The study together with our study shares the idea that SOD activity increases to keep superoxide content below toxic levels.

According to figure, 3.4 (D) DIM treatment results in an increase of APX activity of *B. napus* seedlings. Figure 3.4 (B) show the hydrogen peroxide content of seedlings increase in response to DIM application. Similar to superoxide, hydrogen peroxide also partakes in signalling events that will not cause harm to plants when at normal or slightly elevated concentrations. When hydrogen peroxide levels get high it leads to negative effects like lipid peroxidation, DNA damage, chlorosis and cell death (Gill and Tutetja, 2010). Plants therefore have mechanisms in place to try to prevent these negative effects. Ascorbate peroxidase is one of the main enzymes that scavenge

hydrogen peroxide and it uses ascorbate in the reaction. The reason why DIM application results in increased APX activity is because DIM application, increases hydrogen peroxide content. The hydrogen peroxide then needs to be kept at non-toxic levels and therefore APX activity increases. This result is also seen in the work of Tavallali et al. (2010), where salt stress increased hydrogen peroxide content of barley seedlings. Ascorbate peroxidase activity also increased to keep hydrogen peroxide at non-toxic levels.

Lipid peroxidation occurs when the levels of ROS molecules like hydroxyl radical is high and reacts with lipids. One of the end products of lipid peroxidation is MDA (Zhang *et al*., 2007). The results in our study show that there is no detectable increase in MDA content of DIM treated *B. napus* seedlings when compared to the control. This might seem odd because there is an increase of hydrogen peroxide content in DIM treated seedlings, which usually results in an increase in hydroxyl radical and then result in lipid peroxidation. A study by Tavallali et al. (2010), show that zinc application to barley seedlings increase hydrogen peroxide content and this subsequently leads to an increase in lipid peroxidation which is measured by MDA content. A study by Hossain et al. (2010), revealed that application of 1 mM cadmium to mung bean plants also increased hydrogen peroxide content and MDA content increased as a result which supports the findings of the previous study. The findings of the previous two studies are not in agreement with the results found in our study, which show that MDA levels remain the same despite the hydrogen peroxide increase. This again can

be explained by the fact that the increase of ROS molecules in our study caused by DIM application is not as high as the increases caused by oxidative stress situations. The increase in ROS content in our study is a slight increase that results in normal signalling such as cell division, cell elongation, germination etc. (El-Maarouf-Bouteau and Bailly, 2008). The hydrogen peroxide and superoxide content in our study is controlled by APX and SOD activity, respectively.

Cell death occurs because of extensive damage to cells. High ROS levels can cause cell death by damaging DNA, proteins and cell membranes (Wang *et al*., 2005). Cell death levels can be established using the Evans blue uptake assay. Dead cells seem to retain the dye and based on this the cell death levels can be determined (Baker and Nock, 1994). Based on figure 3.6 DIM treatment of *B. napus* seedlings did not have any effect on cell death levels. This result is expected when compared to the findings of the MDA result in this study, which also show that MDA levels remained the same. The MDA result means that there is no extensive lipid damage and that serves as an indication that ROS levels did not increase to levels where it can cause damage. A study by Keyster et al. (2012), revealed that salt stressed maize plants suffered an increase in hydrogen peroxide content and this led to an increase in lipid peroxidation and subsequently an increase in cell death levels. The increase in hydrogen peroxide and superoxide content in our study makes the findings of the cell death levels somewhat unexpected. This can be explained with the same explanation used for unchanged levels in MDA content. The increase of ROS levels in

our study is not as high as in other studies where oxidative stress is taking place. The ROS levels are only increased for positive signalling and it is tightly regulated by the two antioxidant enzymes, APX and SOD.

In conclusion, exogenous DIM application improves germination percentage of *B. napus* seeds. Diindolylmethane also enhanced the growth of *B. napus* seedlings. There are slight increases in both superoxide and hydrogen peroxide content of seedlings caused by DIM application. It was argued that DIM trigger these increases in ROS content because it is a biotic stress related compound. The improved germination and enhanced growth of seedlings was because of the slight increases of both superoxide and hydrogen peroxide radical. The ROS molecules were at levels where they do not cause damage but results in positive signalling and this falls in line with the oxidative window concept. There was no increase in MDA content and subsequently cell death levels remained the same. It was argued that ROS levels increased only slightly that allowed better germination and improved growth. The activities of SOD and APX increased to prevent the increase in superoxide and hydrogen content.

CHAPTER 4 – EXOGENOUS DIM HAS AN AMELIORATORY EFFECT ON ZIRCONIUM STRESSED *BRASSICA NAPUS* SEEDLINGS

4.1 Introduction

Heavy metals are metals that have a relatively high density. These metals are found throughout the earth's crust (Fergusson, 1990). Abnormally high levels of HM can be found in the environment mainly due to human activities such as mining, use of HM in refineries, coal burning, electronic manufacturing etc. (Tchounwou *et al*., 2012). Natural environment processes like volcanic eruptions and erosions can also contribute to high HM levels in human environments (Nriagu, 1989). Some HM are proven toxic to humans at very low concentrations (Tchounwou *et al*., 2012). Plants are also susceptible to heavy metals. The exposure of plants to heavy HM's can cause oxidative stress. This will stunt growth, cause chlorosis, decrease germination, affect mineral uptake and cause plant death which will eventually lead to decreases in crop production (Mourato *et al*., 2015). Some plants are better than others when it comes to growth in soil with a relatively high metal content. These plants are also able to extract more HM from the soil and live with considerably high metal content within them. They also tend to store more HM in shoot and roots (Kramer, 2010). They are known as the hyper-accumulating plants. *B. napus* is one such plant. This plant can accumulate metals and it has a relative fast growth time (Ghnaya *et al*., 2007). To make the process of phytoremediation a more efficient and

feasible one, there are studies to make plants with a good phytoremediation ability more tolerable to heavy metals. As mentioned previously metals exposure results in oxidative stress and eventually leads to plant death. Therefore, one of the current strategies is aimed at improving the antioxidant capacity of plants by applying molecules that increase the plant enzymatic antioxidant activity. Various molecules like NO, ALA, CA etc. have been proven to play an ameliotary role for plants under HM stress (Hu *et al*., 2007; Shakoor *et al*., 2014; Ali *et al*., 2013). DIM like these 'HM stress ameliotary signalling compounds' also show the ability to increase some enzymatic antioxidants in *B. napus* as seen in the previous chapter of this thesis. We thus hypothesize that exogenous DIM will negate or at least ameliorate the negative effects of Zr induced heavy metal stress.

4.2 Addition of DIM reverses the negative effect of Zr application on seed germination

Heavy metal stress has an impact on many development stages of a plant and one such stage is germination. Studies have shown that heavy metal exposure to seeds can result in poor germination (Sethy and Ghosh, 2013). Results of the prevoius chapter revealed that DIM improves germination of canola. The effect of zirconium stress on canola germination and the effect of DIM on heavy metal stressed canola seeds have not been studied before and therefore we chose to undertake this study. Results show that zirconium application decreased the germination percentage by 31 %. Germination of the co-treated DIM-Zr seed improved by 97 % in comparison with the control

and it also increased germination by 184 % in comparison with Zr-treated seeds.

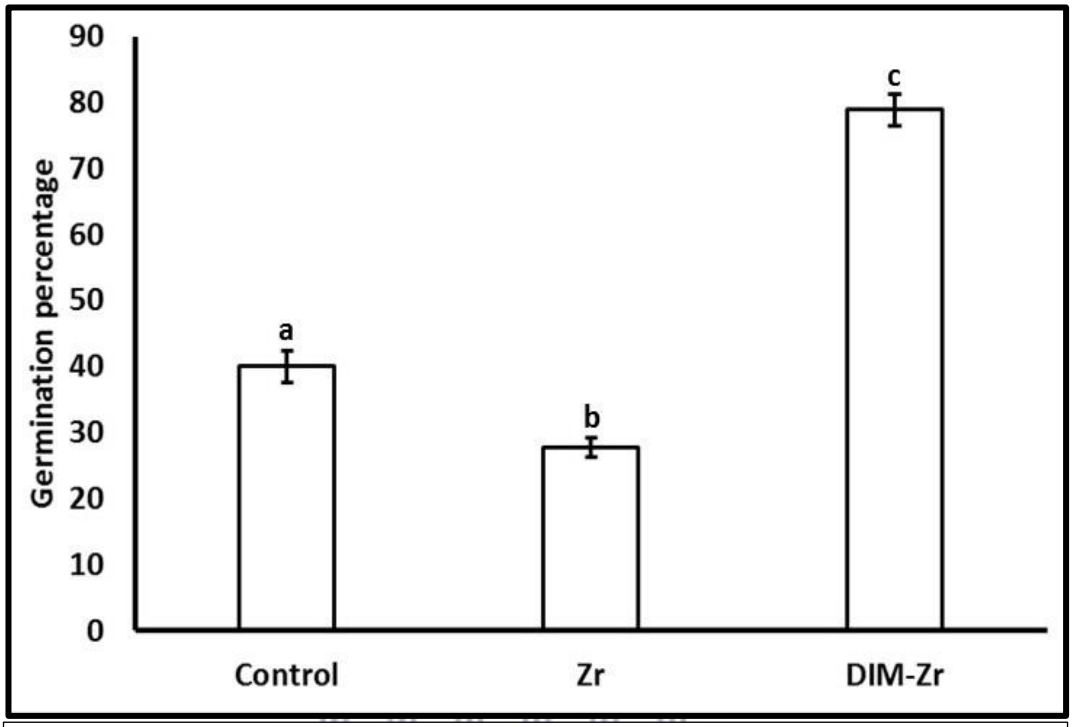

Figure 4.2: The effect of Zr treatment and DIM-Zr co-treatment on seed germination of *B. napus*. Soil was pre-treated and treated with either Tween (Control), Zr only or DIM-Zr (co-treatment). Germination percentages were calculated at the end of treatment period. The different letters indicate significant changes across means at P< 0.05 (DMRT). Values are means ± S.E. (N=5).

4.3 Exogenous DIM negates the negative effects on growth caused by Zr application

It has been proven that heavy metal stress has an effect on plant growth. A study by Pal et al. (2007), has shown that DIM show growth promoting properties. Results of chapter 3 also show that the exogenous application of DIM have growth promoting effects on canola. The effect of Zr stress on canola seedlings was investigated. A study was also performed to see if DIM could improve growth of HM stressed canola seedlings. Results show that Zr application inhibits the growth of seedlings. Zirconium stress

seedlings show a 18 % reduction in biomass. The DIM-Zr treated samples however do not show a decrease in comparison with the control. There is a slight increase of 11 % in comparison w.r.t control. The comparison between Zr and DIM-Zr treated samples show DIM-Zr treated samples increased biomass by 35 % in comparison with Zr treated samples.

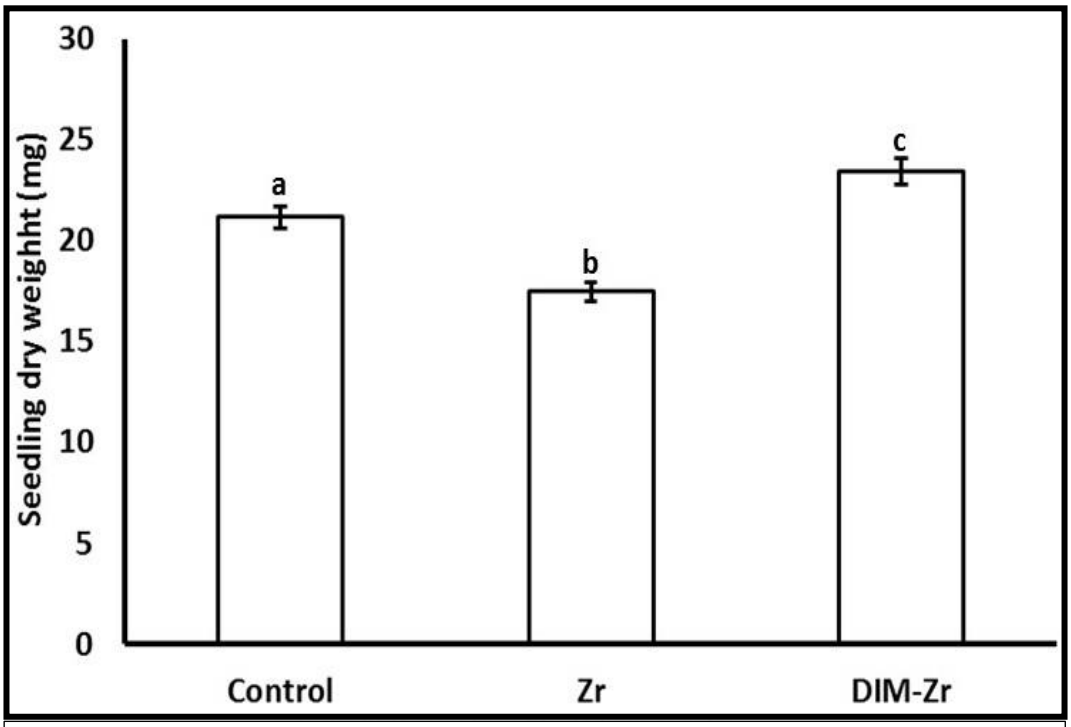

Figure 4.3: The effect of Zr treatment and DIM-Zr co-treatment on seedling biomass of *B. napus*. Seedlings treated with either Tween (Control), Zr only or DIM-Zr (co-treatment) until the end of cotyledon stage (approximately 14 days). Dry weights were then determined. The different letters indicate significant changes across means at P< 0.05 (DMRT). Values are means ± S.E. (N=5).

4.4 Addition of DIM lowers the ROS content of Zr-stressed seedlings

4.4.1 Superoxide

A common trend among all the different types of abiotic stress on plant is an increase in ROS content (Gill and Tutetja, 2010). These ROS molecules increase to levels where they cannot be effectively scavenged and lead to the condition called oxidative stress. Superoxide is one of the first ROS

molecules produced during stress (Gill and Tutetja, 2010). The secondary metabolite, DIM, produced during biotic stress also increase cellular superoxide as results in the previous chapter reveal (Kim *et al*., 2008). The effect of Zr stress on seedling superoxide content has been studied. The role of DIM on the superoxide content of HM stressed canola seedlings has also been studied. Zirconium application led to an increase of 70 % in comparison to control. DIM-Zr treated seedling samples showing a 26 % decrease in superoxide content w.r.t Zr only treated samples.

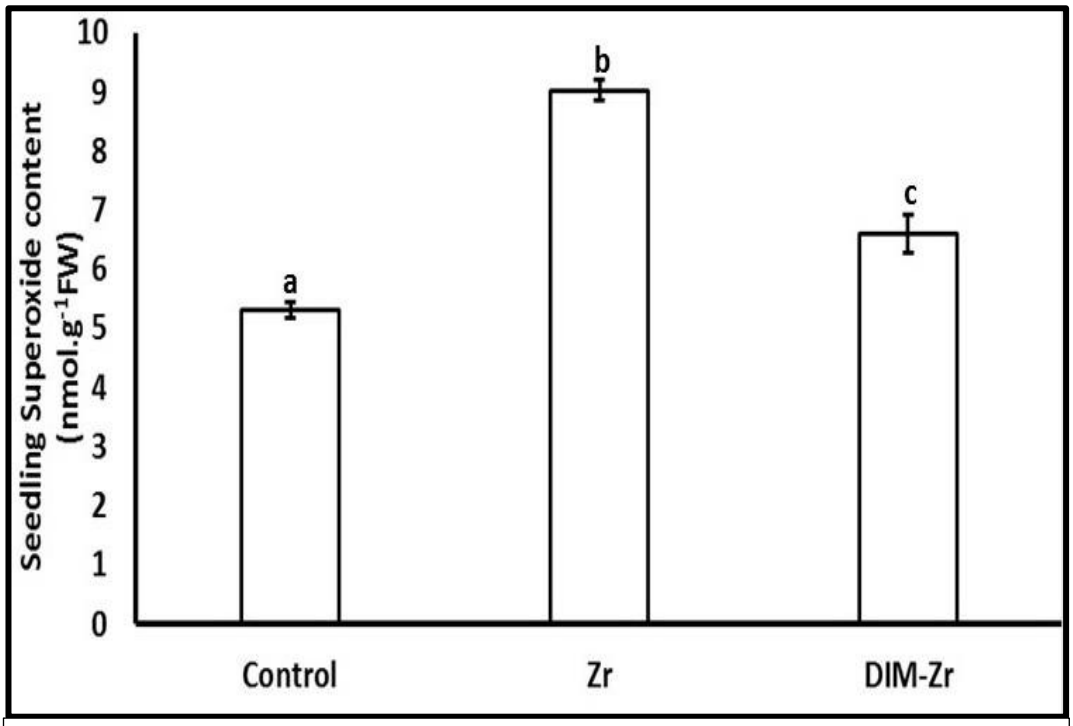

Figure 4.4.1: The effect of exogenous Zr and DIM-Zr on *B. napus* seedling superoxide levels. Seedlings treated with either Tween (Control), Zr only or DIM-Zr (co-treatment) until the end of cotyledon stage (approximately 14 days). Superoxide content was then determined. The different letters indicate significant changes across means at P< 0.05 (DMRT). Values are means ± S.E. (N=5).

4.4.2 Hydrogen peroxide

Hydrogen peroxide has many cellular signalling functions. These include signalling in germination and growth (Singh *et al*., 2014). It is known that

HM stress alter plant H_2O_2 content (Shahid *et al*., 2014). Exogenous application of DIM also alters H_2O_2 as seen in the previous chapter results. Hydrogen peroxide is synthesised from superoxide and the superoxide levels changed in our study due to different treatments. It was decided to study hydrogen peroxide content using a spectrophotometric assay. Results show that Zr treatment induced a huge increase in H_2O_2 content; it raised H_2O_2 content by 130 % in comparison to the control. The DIM-Zr treatment also increased by 56 %, which is not as high as Zr only treated samples. The DIM-Zr H_2O_2 content decreased by 32 % in comparison to Zr only treated samples.

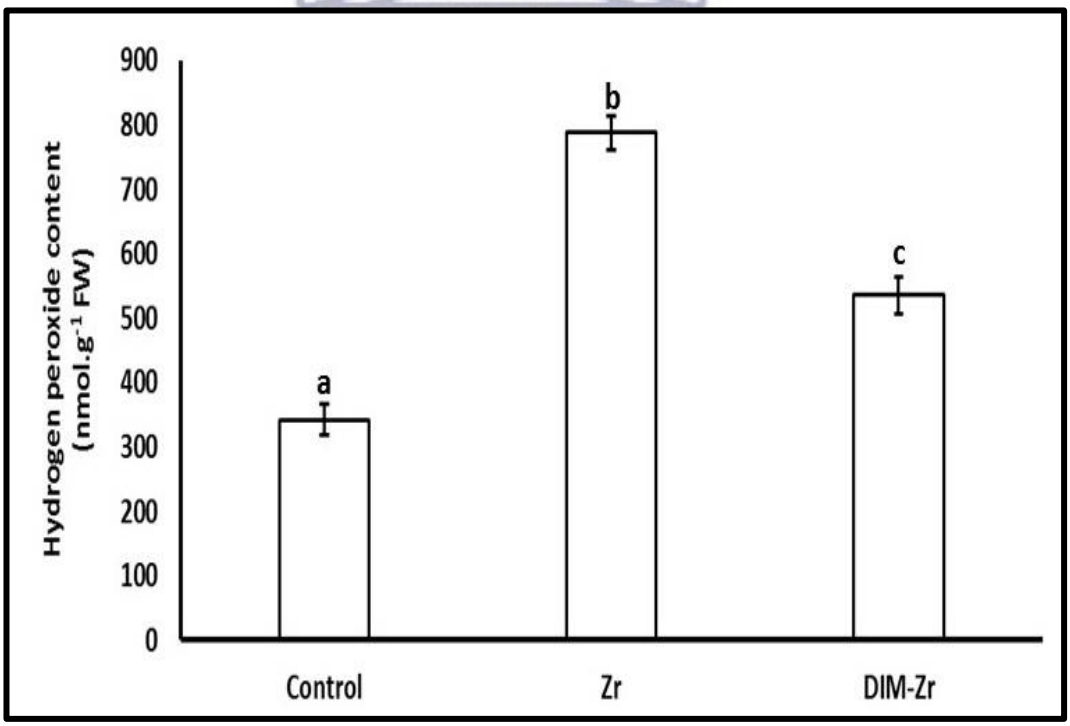

Figure 4.4.2: The effect of exogenous Zr and DIM-Zr on *B. napus* seedling hydrogen peroxide levels. Seedlings treated with either Tween (Control), Zr only or DIM-Zr (co-treatment) until the end of cotyledon stage (approximately 14 days). Hydrogen peroxide content was then determined. The different letters indicate significant changes across means at P<0.05 (DMRT). Values are means ± S.E. (N=5).

4.5 Both DIM and Zr application altered the antioxidant enzymatic activities of seedlings

4.5.1 SOD

Scavenging of harmful oxygen radicals is of utmost importance for plant survival during abiotic stress. The role of SOD is to remove excess superoxide radicals by enzymatically converting it to hydrogen peroxide and oxygen (Gill and Tutetja, 2010). Results show that both zirconium and DIM-Zr treated seedling samples show an increase in superoxide content. The increase in the latter treated samples are not as high as that of the former. A study of SOD activity was done by means of a SOD inhibition assay to determine if SOD activity changed. Results show that there is an increase of SOD activity of Zr treated samples by 35 % in comparison with controls. The SOD activity increase is even higher for DIM-Zr treated samples. DIM-Zr treatments show a 27 % increase in comparison with Zr treated samples and 71 % in comparison with control samples.

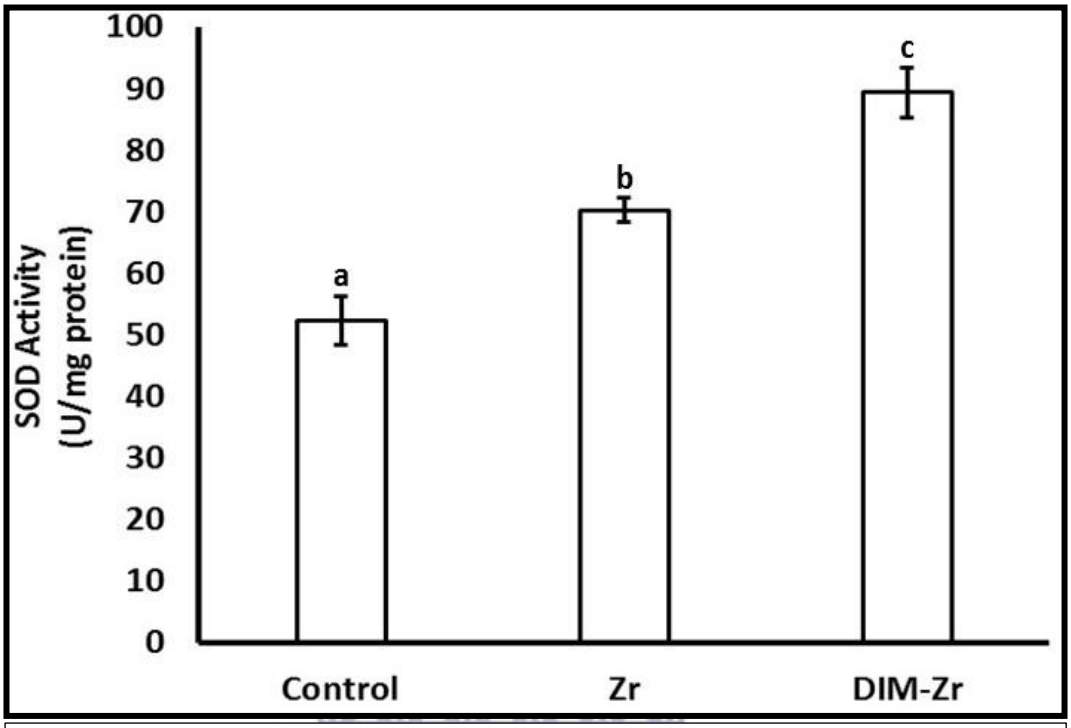

Figure 4.5.1: The effect of exogenous Zr and DIM-Zr on SOD activity of *B. napus* protein extracts. Seedlings treated with either Tween (Control), Zr only or DIM-Zr (co-treatment) until the end of cotyledon stage (approximately 14 days). Protein was extracted and SOD activity of extracts was determined spectrophotometrically. The different letters indicate significant changes across means at P< 0.05 (DMRT). Values are means ± S.E. (N=5).

4.5.2 APX

Hydrogen peroxide is another ROS molecule that can cause damage to plants when concentrations are too high (Apel and Hirt, 2004). One enzyme that can prevent oxidative damage due to high hydrogen peroxide levels is APX. This enzyme can be located at various subcellular locations scavenging hydrogen peroxide using its coenzyme, ascorbate (Gill and Tutetja, 2010). It is known that heavy metal stress has an effect on APX activity (Farid *et al*., 2015). More so, hydrogen peroxide levels changes may also result in APX activity changes. The APX activity was determined in our study by monitoring ascorbate depletion. The APX activity results show that

Zr treatments results in an increase APX activity and the co-treatment of DIM-Zr results in an even higher increase. The Zr treated and DIM-Zr treated samples increased by 117 % and 163 % respectively.

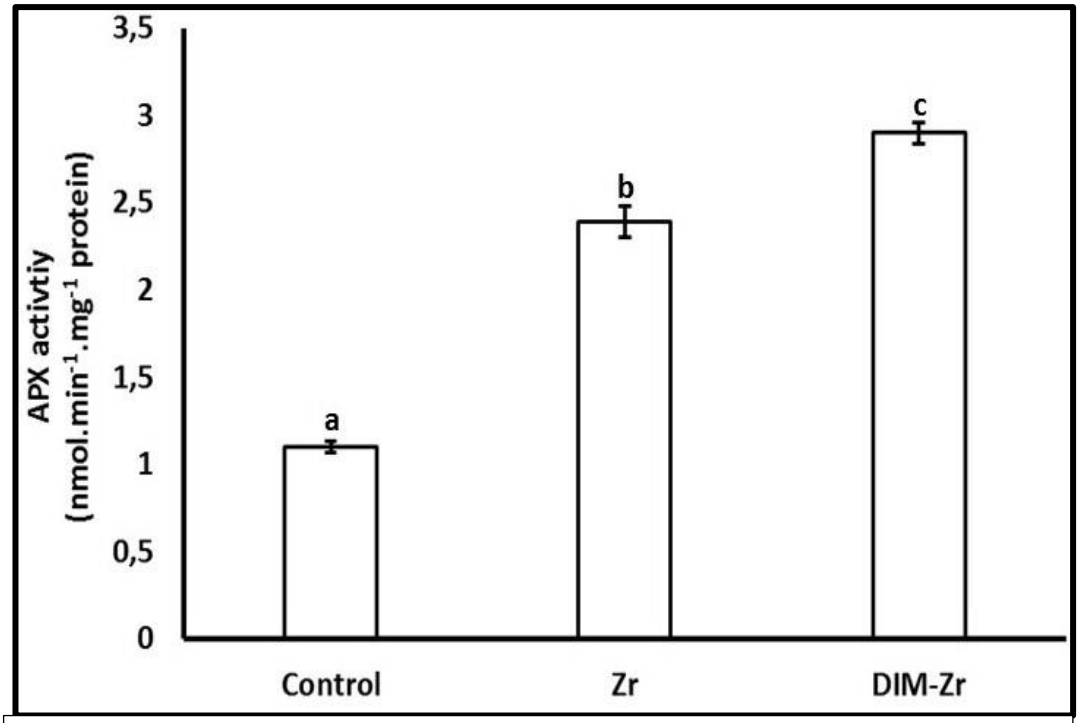

Figure 4.5.2: The effect of exogenous Zr and DIM-Zr on APX activity of *B. napus* protein extracts. Seedlings treated with either Tween (Control), Zr only or DIM-Zr (co-treatment) until the end of cotyledon stage (approximately 14 days). Protein was extracted and APX activity of extracts was determined spectrophotometrically. The different letters indicate significant changes across means at P< 0.05 (DMRT). Values are means ± S.E. (N=5).

4.6. DIM lowered MDA content of Zr-stressed seedlings

Reactive oxygen species react with many other molecules and biomolecules, one of which is lipids (Das and Roychoudhury, 2014). It is shown in literature that heavy metal stress can cause lipid peroxidation. This is due to the increase in ROS content as a result of heavy metal exposure (Mobin and Khan, 2006). Results in this chapter show that there is an increase in ROS content of treated samples. This prompted the study of lipid peroxidation of samples by using the TBARS method to determine MDA

content. Results reveal that there is an increase of 46 % of the Zr stressed samples MDA content in comparison with the control seedlings. The DIM-Zr treatment shows no significant difference to the control sample. The MDA content of DIM-Zr decreased by 28 % when compared to Zr stress samples.

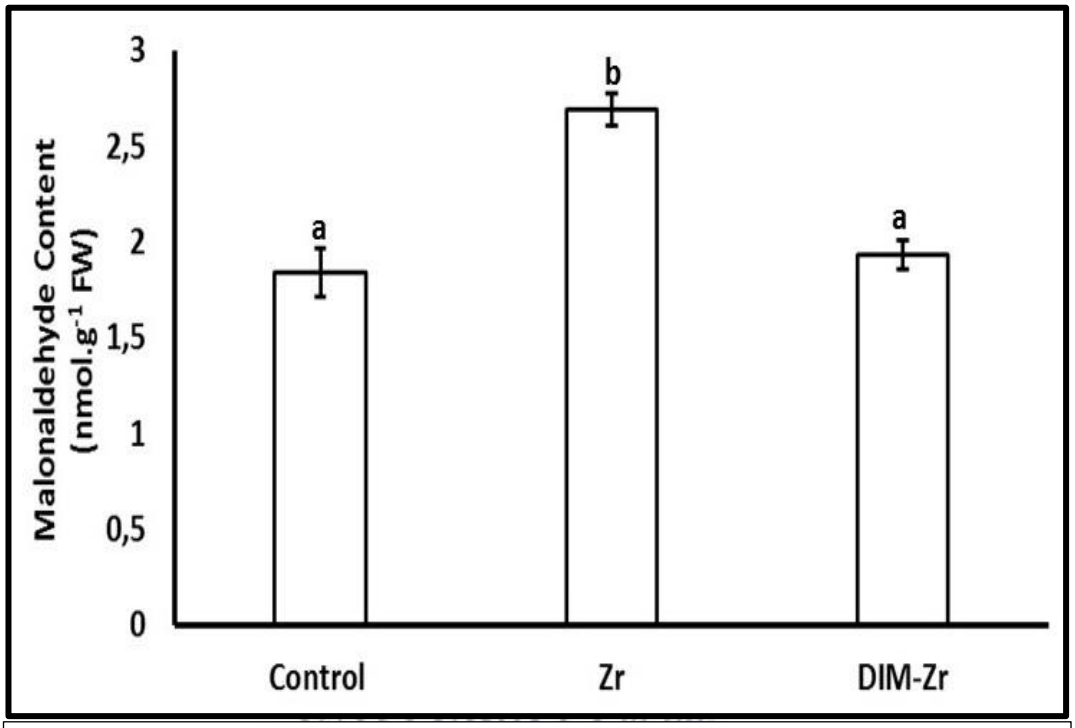

Figure 4.6: MDA levels in response to Zr and DIM-Zr application. Seedlings treated with either Tween (Control), Zr only or DIM-Zr (co-treatment) until the end of cotyledon stage (approximately 14 days). MDA content of seedlings was determined using TBARS assay as an indicator of lipid peroxidation. The different letters indicate significant changes across means at P< 0.05 (DMRT). Values are means ± S.E (N=5).

4.7 Exogenous DIM application reversed cell death caused by Zr-stress

High ROS content can cause cell death in plants (Gill and Tutetja, 2010). The results in this chapter show that ROS molecules increased to the point where they cause lipid peroxidation (inferred by MDA results) for Zr treated seedlings. The DIM-Zr co-treated samples also had an increase in ROS content but to a lesser degree and MDA content were unchanged. The

effect of Zr and DIM-Zr treatments on the seedling cell death was investigated by doing an Evans blue assay. The cell death assay results show that Zr treatment caused cell death. The cell death levels of the Zr treated sample increased by 69 % in comparison to control. Cell death results also show that there is no significant change in cell death levels between control and DIM-Zr treated samples.

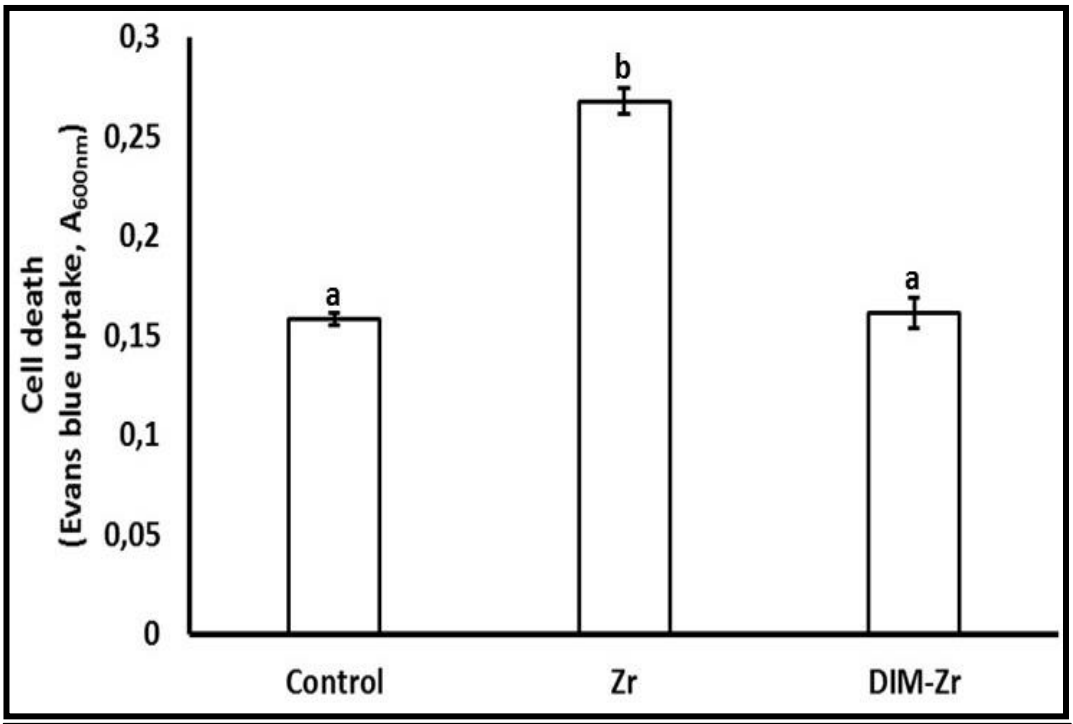

Figure 4.7: Cell death levels in response to Zr and DIM-Zr application. Seedlings treated with either Tween (Control), Zr only or DIM-Zr (co-treatment) until the end of cotyledon stage (approximately 14 days). Fresh seedling material was used in an Evans blue assay to determine cell death levels. The different letters indicate significant changes across means at P< 0.05 (DMRT). Values are means ± S.E (N=5).

4.8 The addition of DIM led to unchanged chlorophyll content in Zr-stressed seedlings

Chlorosis can be defined as the decrease in chlorophyll pigments in plants (Hörtensteiner & Krautler, 2011). Chlorophyll pigments play an important

role in the plant energy synthesis process called photosynthesis (Garret and Grisham, 2010). It is therefore needless to mention that a decrease in these pigments could hold dire consequences for the plant. It is well published in literature that abnormal levels of soil HM content could result in chlorosis (Burzynski, 1984). The effect of Zr only and DIM-Zr treatment on seedlings was evaluated in this study. The results show that treatment of seedlings with Zr only led to a decrease in chlorophyll content by 19 %. According to the results, the co-treatment of DIM-Zr did not cause any change in total chlorophyll content.

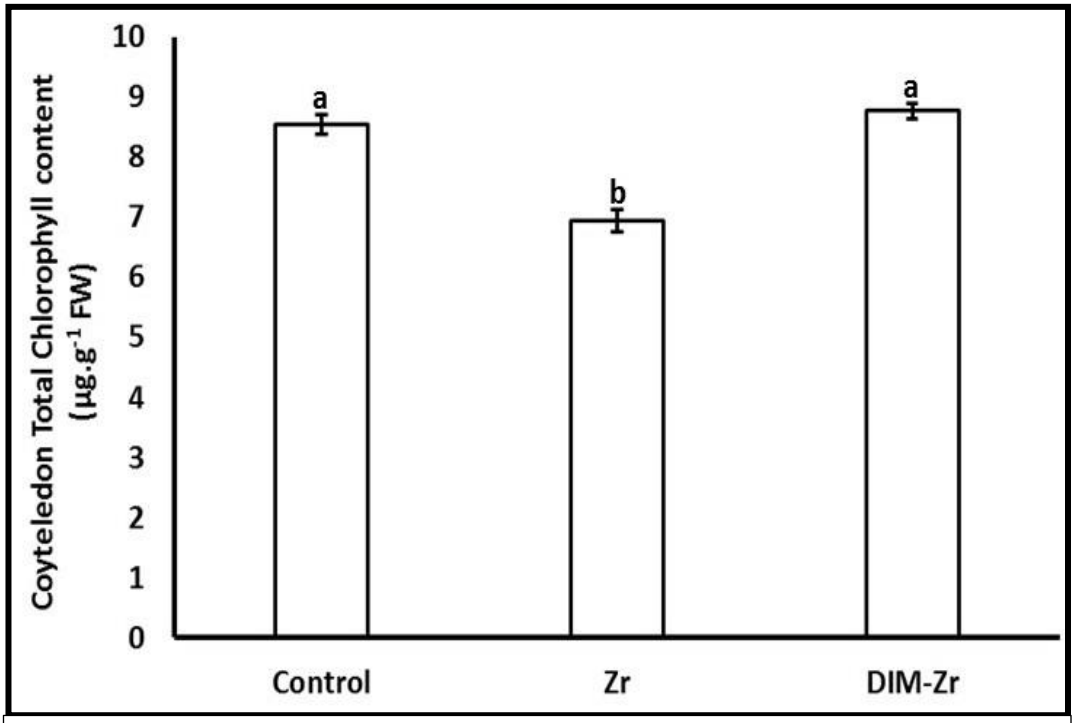

Figure 4.8: The effect of exogenous Zr and DIM-Zr on *B. napus* cotyledon chlorophyll content. Seedlings treated with either Tween (Control), Zr only or DIM-Zr (co-treatment) until the end of cotyledon stage (approximately 14 days).Chlorophyll content was then determined. The different letters indicate significant changes across means at P< 0.05 (DMRT). Values are means ± S.E. (N=5).

4.8 Treatments caused changes in nutrient content of *B. napus*

Elements are what plants are made of, it is used to make biomolecules and organelles of cells and are involved in chemical reactions and some have the ability to signal. Calcium plays an important role in cell division and growth and stress signalling. Potassium is involved in over 50 enzyme catalysis reactions. Phosphorus makes an important part of the backbone of nucleic acids. Magnesium is an important element in the photosynthesis process of plants and together with Fe and Zn being important co-factors of various enzymes (Singh *et al*., 2016). The results show that Zr application reduced all element content with the exception of Ca. DIM increased the content of all measured nutrients both alone and in the combinatory treatment.

Table 4.1: The effect of DIM and Zr induced HM stress on *B. napus* seedling nutrient content (µg/g of fresh weight)

Treatments				
Elements	**Control**	**DIM**	**Zr**	**DIM-Zr**
Ca	32,491 ± 1,325^{a}	43,7408 ± 1,25^{b}	61,1263 ± 1,08^{c}	44,2218 ± 2,12^{b}
K	391,611 ± 5,23^{a}	410,35 ± 4,865^{b}	350,069 ± 7,38^{c}	408,293 ± 6,81^{b}
P	22,124 ± 0,84^{a}	26,6267 ± 0,48^{b}	18,1338 ± 0,321^{c}	26,3361 ± 0,518^{b}
Mg	22,9029 ± 0,14^{a}	28,9481 ± 0,12^{b}	18,886 ± 0,147^{c}	27,26705 ± 0,14^{d}
Fe	1,31706 ± 0,02^{a}	1,7004 ± 0,013^{b}	0,86855 ± 0,04^{c}	1,67684 ± 0,01^{d}
Zn	0,52584 ± 0,01^{a}	0,605208 ± 0,01^{b}	0,39887 ± 0,01^{c}	0,57279 ± 0,01^{d}
Zr	ND*	ND*	0,24001 ± 0,01^{a}	0,238421 ± 0,01^{a}

Different letters indicate significant differences between means at P< 0.05 (DMRT) and analysis was performed per row. Values are means ± S.E (N=3). * None Detected (ND).

Discussion

Germination is the first stage of plant development. The germination process itself is then dived into different stages (Canola Council, 2016). At the germination stage, plants are most vulnerable to biotic and abiotic stress factors (Canola Council, 2016). Heavy metal stress also has an impact on the germination of seeds (Sethy and Ghosh, 2013). According to our germination results, the application of 1 mM Zr to *B. napus* seeds resulted in a decrease in germination percentage. It has been shown that HM stress inhibit germination by preventing the mobilization of carbohydrates that is needed for germination to take place (Sethy and Ghosh, 2013). A study by Ashraf et al. (2011), revealed that the exposure of *Helianthus annuus* L. to nickel resulted in poor germination. Nickel application reduced α-amalyse and protease activity in these seeds. This resulted in less starch available for energy to fuel the germination and less free amino acids for protein synthesis that eventually hampered the germination process. A study by Rahoui et al. (2010), also revealed that cadmium restricted the mobilization of sugars and amino acids in germinating pea seeds. Cadmium application also resulted in membrane damage, possibly due to oxidative stress. High HM content in germinating seeds results in high levels of ROS production and this also has a negative impact on germination. A study by Hassan and Mansoor (2014), revealed that both lead and cadmium application to mung bean seeds resulted in a decreased germination percentage. Results of their study also revealed that HM application increased ROS content to levels where it caused oxidative stress. The effect of exogenous zirconium

on mobilization of carbohydrate reserves and both amylase and protease activity is yet to be determined in our study. Therefore, our results cannot be compared to studies of HM on carbohydrate mobilisation and protease and amylase activity. However, our study support the findings of Hassan and Mansoor (2014). Our results also reveal that Zr application increases ROS content in *B. napus*. The increase of ROS content caused oxidative stress. The oxidative stress condition then results in a decrease of germination percentage.

Our results revealed that DIM-Zr treated *B. napus* seeds have a higher germination percentage than Zr only treated seeds. A possible explanation for this result is that DIM improved germination percentages by increasing the antioxidant capacity of seeds. Results from the previous chapter revealed that DIM, when applied to *B. napus* has the ability to regulate ROS content by increasing SOD and APX activities. Hu et al. (2007), revealed that copper stressed wheat seeds suffered a decrease in germination. Wheat seeds pre-treated with the nitric oxide donor molecule, sulphide nitroprusside (SNP) did not experience a decrease in germination. This was attributed in part to the ability of NO to increase the antioxidant capacity of seeds that prevented the accumulation of ROS molecules to levels where it can inhibit germination. In a study by Kopyra and Gwozdz (2003) exogenous NO also played an ameliotary role for HM stressed *Lupinus luteus* seeds in the process of germination. Like NO in both the studies of Kopyra and Gwozdz (2003) and Hu et al. (2007), DIM also improves germination of HM stressed seeds seemingly through a similar mechanism.

An increase in ROS content is a common occurrence when plants are exposed to high concentration of heavy metals (Yadav, 2010). Metals can increase ROS content directly by reacting with oxygen containing molecules or indirectly by activating ROS production pathways (Shahid *et al*., 2014). Our results reveal that the exposure of *B. napus* seedlings to zirconium increased its superoxide anion content. A study by Gill et al. (2014), showed that chromium increased the superoxide content of four *B. napus* cultivars in a concentration dependant manner. In another study by Shah et al. (2001), cadmium application increased the superoxide anion content of rice seedlings. The findings of the previous studies support the findings of our study that heavy metals tend to increase superoxide anion content in plants.

The results of our study indicate that there is a decrease in superoxide anion content when DIM-Zr treated seedlings are compared to Zr only treated seedlings. A possible explanation for this result could be that DIM-Zr seedlings have a better superoxide scavenging ability than Zr only treated seedlings. In support of this idea, our results show that DIM-Zr seedlings have a higher SOD activity than Zr only treated seedlings. As mentioned previously SOD catalysis the removal of superoxide. It can be assumed that the higher SOD activity gave the seedlings the ability to keep superoxide content at lower levels. Our results are consistent with the findings of Zhao et al. (2015). Their study revealed that treatment of *A. thaliana* with the mycotoxin, FB1 increased superoxide content of this plant. Pre-treatment of *A. thialana* with the indole, I3C increased SOD activity and led to decreased

superoxide content when FB1 stressed plants are compared to FB1 stressed plants pre-treated with I3C.

According to our results, zirconium application increased the hydrogen peroxide content of *B. napus* seedlings. This result is expected because our results also show that there is an increase in superoxide content. SOD converts superoxide to hydrogen peroxide and oxygen and this usually leads to an increase in hydrogen peroxide content. There is plenty of evidence to suggest that heavy metals increase hydrogen peroxide content to levels where it can cause oxidative stress (Shahid *et al*., 2014). A study by Shakoor et al. (2014), revealed that the exposure of *B. napus* seedlings to lead resulted in a concentration dependant increase in hydrogen peroxide content. Gill et al. (2011), showed that the application of chromium also resulted in a concentration dependant increase of hydrogen peroxide content in four different *B. napus* cultivars. The findings of our study are in agreement with most studies suggesting that HM tend to increase hydrogen peroxide content.

Our results also reveal that the co-treated DIM-ZR seedling samples show a decrease in hydrogen peroxide content when compared to Zr only treated samples. This observation can be explained by comparing the activity of hydrogen peroxide scavenging enzyme, APX between the two samples. The APX activity of DIM-Zr treated samples is much higher than that of the Zr only treated samples. The DIM-Zr samples had a better scavenging ability than Zr treated samples and hence less hydrogen peroxide content. A study by Shakoor et al. (2014), support our findings, it shows the

combination of lead and citric acid treatment of *B. napus* seedlings had a higher APX activity than the lead only treated seedlings. This then resulted in *B. napus* seedlings treated with both citric acid and lead having a lower hydrogen peroxide content than lead only treated seedlings. DIM treatment in HM stressed plant shares a similar story with the citric acid study HM stress plants of *B. napus*. In both of these studies, these molecules lowered hydrogen peroxide content by increasing APX activity.

As discussed previously, Zr application increased superoxide anion content of *B. napus* seedlings. Changes in superoxide anion content have an influence on the activity of superoxide dismutase enzymes. Our results show that exogenous zirconium increased SOD activity of *B. napus* seedlings. Reports in literature vary when it comes to HM and its effect on SOD activity. A study by Gallego et al. (2002), shows that three different heavy metals cadmium, chromium and aluminium result in an increase of SOD activity in sunflower cells. The authors suspected that the increase in SOD was due to an increase of superoxide anion content. In a different study by Gallego et al. (1996), it also revealed that SOD activity of sunflower leaves increased when placed under copper stress. Findings by Qureshi et al. (2007), revealed that lead induced oxidative stress also increased the SOD activity of *Cassia angustifolia*. Our study supports the findings of studies that show that HM exposure to plants increase SOD activity. The reasoning behind the increase of SOD activity in these plants is that SOD increase to decrease the superoxide anion content in an attempt to protect the plant against the harmful effects of high superoxide content. A study by

Youssef and Azooz (2013), revealed that the exposure of Okra to Zn and lead HM stress resulted in a decrease in SOD activity. This finding is however contrary to the idea that metals increase SOD activity. The authors explained that SOD activity was inactivated due to a very high ROS content. Our results do not show a decrease in overall SOD activity, so it is obviously clear that the high ROS contents in our study did not affect overall SOD enzymes to an extent where it can cause a decrease in overall SOD activity. Native PAGE studies can be done in future to see if Zr application effects the activity of individual isoforms.

According to our results, Zr application increases APX activity of *B. napus* seedlings. In our study Zr application, increased superoxide content and SOD activity also increased and therefore hydrogen peroxide content increased. As discussed previously hydrogen peroxide can do damage if concentrations get too high. Ascorbate peroxidases activity then tends to increase to lower the concentration of this ROS molecule in an attempt to protect the plant from oxidative stress (Caverzan *et al*., 2012). Various studies show that HM stress increase APX activity. A study by Farid et al. (2015), revealed that application of 10 µM cadmium to hydroponically grown *B. napus* plants increased its APX activity. Shakoor et al. (2014), showed that the exogenous application of lead also increased the activity of *B. napus* APX. Findings of Wang et al. (2004), showed that copper application increases the APX activity of *B. juncea* in a concentration dependant manner. These results reveal clearly that ascorbate peroxidases are very responsive to heavy metals. The findings of these studies are also in

agreement with our study that show HM increases APX activity. All of these authors argued that APX activity increase to decrease hydrogen peroxide content in an effort to protect the plant from the harmful consequences of highly increased hydrogen peroxide content.

The result of the TBARS assay in our study indicates that malonaldehyde content of Zr treated *B. napus* seedlings increased. Lipid peroxidation can occur when ROS molecules react with lipids (Sharma *et al*., 2012). Malonaldehyde is a by-product of lipid peroxidation and an increase in MDA therefore suggest an increase in lipid peroxidation (Haliwell and Gutteridge, 1989). This result is totally expected because our results on hydrogen peroxide content as well as superoxide content show increases. The increase in these ROS molecules then caused increased lipid peroxidation. Our finding is supported by various studies that show that the exposure of plants to heavy metals can cause ROS levels to increase and subsequently lead to heightened levels of lipid peroxidation. In a study by Mobin and Khan (2006), two B. *juncea* cultivars were subjected to cadmium stress. The RH-30 cultivar had a greater increase in hydrogen peroxide content than the Varuna cultivar. Their results also showed that the MDA content of the RH-30 cultivar was higher than the Varuna cultivar. This finding is in accordance with our results, showing that higher ROS increases lead to higher MDA content. Work done by Pandey et al. (2009), also supports our results. Their study revealed that exposure of spinach plants to high levels of Ni, Co, Cu and Zn led to increased TBARS levels. The authors suggested that the

increase in hydrogen peroxide content of spinach plants caused the increase in TBARS content.

Our results show that treatment of *B. napus* seedlings with DIM-Zr resulted in a decrease in MDA content when compared to the Zr only treated seedlings. As mentioned previously the DIM-Zr treated samples had a lower ROS content when compared to Zr treated *B. napus* seedlings. This was due to the effect of DIM increasing the antioxidant capacity by increasing APX and SOD activity of *B. napus* seedlings. It can be assumed that the decrease in ROS content resulted in the decrease in MDA content because increasing ROS levels results in increasing MDA content. A study by Shakoor et al. (2014), revealed the co-treatment of *B. napus* plants with citric acid-lead resulted in a decrease in ROS when compared to lead only treated *B. napus* plants. This was because citric acid improved the antioxidant capacity of *B. napus*. Subsequently the MDA levels of citric acid-lead treated plants were lower than the lead only treated *B. napus* plants. The same result is seen in a study by Hossain et al. (2010), that deals with mung bean plants, cadmium stress and exogenous glycine betaine. Pre-treatment of mung bean plants with glycine betaine resulted in increased antioxidant activity, lower ROS content and consequently lower MDA levels than cadmium stressed only mung bean plants.

It is obvious that the exposure of *B. napus* seedlings to 1 mM Zr results in oxidative stress. Our results that show an increase in both ROS and MDA content are in agreement with this observation. Increase in ROS content results in damage to important biomolecules like DNA, protein and

importantly cellular membranes (Sharma *et al*., 2012). When irreparable damage has occurred then it will result in cell death. We have undertaken a study to determine if exposure of Zr to *B. napus* seedlings resulted in cell death. Based on our results, it shows that Zr has indeed caused an increase in cell death. This observation is very common when it comes to exposure of heavy metals to plants. A study by Basset and Matsumoto (2008), revealed that exposure of tobacco cells to aluminium resulted in cell death. Work by Iakimova et al. (2008), is also in agreement with the findings of our study. According to their study cadmium application to tomato cells increased the hydrogen peroxide to levels where it resulted in oxidative stress. This then resulted in programmed cell death. Eleftheriou et al. (2015), showed that hexavalent chromium [Cr (VI)] caused changes in ROS content and it eventually resulted in cell death in the roots of *A. thaliana* seedlings.

Our results also reveal that cell death levels of DIM-Zr co-treated samples are lower than the levels of Zr only treated samples. This result is expected because our results show that Zr increase ROS content, but co-treatment of DIM-Zr lowers the ROS content in comparison with Zr only treated samples. As discussed previously high levels of ROS content can result in cell death but normal levels does not. Thus, our observation of cell death results is not surprising. Findings of Zhang et al. (2015), is in agreement with results of our study. Their study revealed that cadmium causes cell death by increasing ROS content of *B. rapa* roots to high levels where it caused cell death. Pre-treatment with hydrogen sulphide however

prevented Cd from causing cell death. The authors proved that exogenous hydrogen sulphide improved the antioxidant capacity of *B. rapa* roots and therefore it was able to maintain low ROS levels and reduce cell death levels.

Chlorophyll is a pigment that is found in algae, cyanobacteria and plants. Chlorophyll is responsible for giving the leaves of plants and trees its green colour (Mader, 2006). In plants there are two types of chlorophyll molecules named chlorophyll a and chlorophyll b. The main function of chlorophyll is to absorb light and transfer the energy obtained from light to power the photosynthetic process (Garret and Grisham, 2010). Chlorosis occurs when there is a decrease in plant chlorophyll content (Hörtensteiner & Krautler, 2011). Chlorosis usually occurs when plants are under biotic or abiotic stress. Our results show that exposure of Zr to *B. napus* seedlings resulted in a decrease in total chlorophyll content of *B. napus* cotyledons. The finding of our results is supported by Shakoor et al. (2014), which showed that lead application also decreases the total chlorophyll content of *B. napus*. A study by Meng et al. (2009), also revealed that exposure of *B. napus* seedlings to cadmium results in a decrease in chlorophyll content. The way in which Zr decrease chlorophyll content has not been published to date. However, there are a few studies of how Pb decreases chlorophyll content. A study by Burzynski (1984), showed that exposure of lead to cucumber seedlings decreased chlorophyll content inhibiting the synthesis of aminolevulinic acid, which is a precursor of chlorophyll. According to Liu et al. (2008),

chlorophyllase activity increase when lead is exposed to plants and it leads to a decrease in chlorophyll content.

Our results also indicate that the DIM-Zr co-treated samples show a greater chlorophyll content than Zr treated samples and it is equal to the control. Shakoor et al. (2014), found a similar trend when, lead decreased chlorophyll content but when co-treated with citric acid (Pb-CA) chlorophyll content raised higher than lead only treated. The authors of the Shakoor et al. (2014), argued that CA increased the chlorophyll content of lead stressed pants, because CA application on its own increase chlorophyll content of *B. napus*. Similar results were observed by Ali et al. (2013), when *B. napus* was exposed to cadmium stress and then co-treated with 5-ALA. A possible mechanism by which DIM can maintain high chlorophyll content is by inhibiting chlorophyllase activity but this is yet to be determined in our study. It must also be noted that in our study that ROS levels of DIM-Zr seedlings cotyledons are not as high as Zr only stressed samples. This may also have an impact on chlorophyll content, because oxidative stress and chlorosis commonly occur together.

Our results show that the exposure of Zr to *B. napus* seedlings results in a decrease in biomass. Our results also show that exposure of *B. napus* to this heavy metal results in oxidative stress. Retarded or stunted growth is a common feature of plants that suffers oxidative stress. A study by Meng et al. (2009), showed that growth of *B. napus* seedlings in the presence of cadmium also result in a decrease of seedling dry and fresh weight. The malonaldehyde results of their study reveal that cadmium application to

seedlings results in increased MDA, which is indicative of oxidative stress. A study by Jayakumar et al. (2007), is in agreement with the findings in our study. It showed that HM stress (induced by cobalt) results in the decrease in growth of radish seedlings. The authors argued that Co exposure blocked cell elongation and cell growth, which resulted in less root exposure to soil that in turn caused a decrease in nutrient uptake. Work by Fodor et al. (2005) revealed that exposure of high concentration of Zr (500 µM) to wheat resulted in decreased root and shoot of wheat seedlings. Their results also showed similar to our study that Zr was causing oxidative stress that can be inferred by increase in antioxidant activity and decrease in chlorophyll content in their study. A study by Aidid and Okamato (1993), suggested that Cd induced heavy metal stress can also stunt plant growth by interfering with the photosynthetic process. In our study there is a decrease in chlorophyll content, so it can be assumed that HM in our study also hampered the photosynthetic process.

According to our results the co-treated (DIM-Zr) seedlings does not suffer the same fate as Zr only treated seedlings when it comes to biomass. It shows that DIM-Zr seedlings has a higher biomass than Zr stressed seedlings. If a comparison is drawn between the ROS content, enzyme antioxidant activity, MDA content and chlorosis results, then the biomass results is not much of a surprise. A high ROS content when alleviated to levels where it can cause oxidative stress is known to effect the growth and biomass of plants. In the DIM-Zr samples, ROS content is not as high as the Zr only treated samples and this is brought about by the increased SOD

and APX activities. DIM-Zr treated seedlings do not experience oxidative stress and the unchanged MDA content (compared to control), no chlorosis and no negative impact on growth prove this. The observation where the exogenous application of a molecule ameliorates the negative effect of HM stress on growth has been reported in several studies in literature. A study by Ali et al. (2013), revealed that the plant growth regulator (PGR), ALA improved the growth of *B. napus* seedlings under Cd stress. Their results show similar to ours that ALA application lowers ROS content by increasing enzymatic antioxidant activity and thereby preventing Cd to cause oxidative stress which can lead to decrease in biomass. Findings of Najeeb et al. (2011), are in agreement with the results found in our study. According to their study Cd application caused oxidative stress in *Juncus effusus* by increasing ROS content and this then lowered the biomass. Co-treatment with citric acid alleviated oxidative stress by increasing enzymatic antioxidant activity of the plant. The biomass of their plants then increased under the co-treatment.

It can be concluded that Zr application had a negative impact on *B. napus*. The decrease in both germination and biomass served as prove. The exposure of *B. napus* to Zr caused oxidative stress. ROS content was very high and this led to an increase in lipid peroxidation as measured by MDA content. Oxidative stress also resulted in cell death. Chlorophyll content also decreased due to exogenous Zr. Co-treatment of Zr and DIM (DIM-Zr) alleviated all of the negative effects that the Zr only treatment had on *B. napus*. Reactive oxygen species content decreased significantly under DIM-

Zr treatment. This was due to DIM that increased the activity of enzymatic antioxidants. The DIM-Zr treated seedlings did not suffer under oxidative stress which is revealed by an unchanged MDA content to control. Germination percentage, seedling biomass, chlorophyll content recovered due to DIM addition.

Calcium is an element that plays important signalling roles in various cellular processes. These processes among others are plant growth, development and response to biotic and abiotic stress. Our data show that the Ca content of Zr stressed *B. napus* seedlings increased in comparison with control. It is a common observation for calcium content to increase in plants that are under oxidative stress. A study by Price et al (1994), revealed that oxidative stress increase cytosolic Ca of *Nicotania plumbagifolia* seedlings. Findings of Islam et al. (2015), showed that exposure of jute plants to the heavy metal arsenic resulted in increased root Ca content. The authors explained that Ca content increased as a protective response against heavy metal toxicity because Ca can trigger enzymes and to keep cells stable. Our results are in agreement with the findings of Islam et al. (2015), and it can possibly be that Ca increase in our plants for the same reason, i.e. activate enzymes and keep cells stable as a defence against Zr induced heavy metal stress. Lamhamdi et al. (2013), showed that both wheat and spinach seedlings suffer decreases in calcium content in response to lead treatment. This finding is contrary to our results. Lamhamdi and authors explained that their observation could be due to the similarities of Pb^{2+} and Ca^{2+} that could result in competition between these two elements for uptake. It could be that in

our study there is no competition between Zr and Ca and thus allow Ca to enter cells unhindered to the point where the seedling Ca content can increase.

Our Ca content results show that both DIM and DIM-Zr treatments also result in an increase in seedling Ca content but the increase is not as striking like the Zr only treated *B. napus* seedling samples. It is also interesting to note that the trend that is seen in ROS content data is also seen in the Ca content data (slightly increased ROS for DIM and DIM-Zr samples and very high increase in ROS content for Zr only treated samples). This observation is also presented in literature, which shows that there is a strong correlation between ROS and Ca content. A study by Pei et al. (2000), revealed that ROS activate plasma membrane Ca channels (I_{Ca}) to allow cytosolic Ca increases in *Arabidopsis* cells. It could be that ROS levels also had an impact on Ca content in our study and therefore similar trends are observed. Work by Siddiqui et al. (2010), showed that exogenous Ca increases the enzymatic antioxidant activity of wheat APX, SOD and CAT. This helped wheat plants to overcome Ni stress. Calcium can thus play a similar role in our study.

Potassium is another one of the macro-elements found in plants. This element plays important roles during the process of protein synthesis. Potassium is also an important cofactor and activator of over 50 enzymes as discovered to date. Our results reveal that the potassium content of Zr-stress *B. napus* seedlings decreases. The decrease of potassium content is a common occurrence in HM stress plants. A study by Islam et al. (2015),

revealed that both As and Cr resulted in a decrease in plant potassium uptake. Sundaramoorthy et al. (2010), revealed that chromium decrease potassium content of rice. The authors concluded the decrease might be due to binding of chromium to a potassium carrier and thereby outcompetes K in cell entry. A study by Islam (2014), also revealed that Cr decrease K content in jute plants. The authors reasoned that it might be because Cr decrease root growth. The decrease in root growth results in impaired soil penetration and this leads to decreased nutrient uptake. It is probable that the decrease of K content in *B. napus* Zr-stressed seedlings could be due to poor root growth as our results show that Zr application effects seedling growth. The possibility of Zr binding to K carriers and thereby influencing K content can also be true but further studies needs to be done to determine it.

Our results reveal that DIM increases K content of *B. napus* as seen in both DIM only and DIM-Zr combination treatments. A study by Siddiqui et al. (2012), application of potassium alone improved the growth *Vicia faba*. Our study shows that DIM increase K and by so doing also improves growth. The authors reasoned that the increase in K content acted as a catalyst to activate enzyme that play a role in plant growth and development. The study by Siddiqui et al. (2012), also revealed that K application plays an ameliotory role when under Cd stress. In our study, DIM also played an ameliotary role for *B. napus* under Zr stress. Siddiqui et al. (2012), reasoned that the ameliotary role of K was due to the ability of K to activate enzymes that play a role in growth even in HM stress conditions, improve enzymatic

antioxidant activity and to increasing photosynthetic pigments by preventing the degradation of newly formed chlorophyll and its precursor ALA. In our study, DIM also played an ameliotary role of Zr stressed *B. napus* by increasing enzymatic antioxidant activity, improving chlorophyll content relative to Zr stressed samples and improving growth. As mentioned previously, exogenous DIM increased K content in *B. napus* so it would be reasonable to believe that K also had a hand in reversing negative effects caused by Zr stress i.e. DIM could be signalling through K.

The abundance of phosphorus also makes it a macronutrient of plants. Phosphorus is used for the formation of the backbone of DNA and RNA, it also forms part of phospholipid membranes and phosphorylation reactions. Our ICP data show that Zr treatments decrease the content of phosphorus. Work by Piechelak et al. (2011), showed that lead decreases the phosphorus content of pea plants. In that same study, it shows that elevated levels of Cu also decreased phosphorus content. Piechelak and authors reasoned that plant phosphorus content decrease might be due to damaged membranes that can result in mineral leakage. Work by Azmat and Akhter (2010) revealed that Cr exposure to mung bean also decreased phosphorus content. The authors reasoned that Cr was competing with phosphorus to bind uptake proteins and the fact that Cr caused stunted root growth to the point where it impaired mineral uptake from the soil also contributed to a decrease in phosphorus content. The finding of our study correlates with the results of the other two studies. We can also use the reasoning of Azmat and Akhter (2010) that Zr (HM used in our study) like Cr stunted root growth

and lead to poor soil manifestation and subsequent impaired nutrient uptake that caused a decrease in seedling P content.

Our results reveal that DIM treatments increase P content when compared to the control. The manner in which DIM achieves this is still unknown and further studies needs to be conducted in order to determine how the increase in P content occurs. The increase in P content can however shed some light on how DIM-Zr seedlings are able to perform better than Zr only treated seedlings. Wang et al. (2009), revealed that increase in P content leads to increase in GSH content which can give plants a better chance to reduce oxidative stress. Glutathione is also a known chelator of metals that can also give plants protection against metals. Phosphate also improves membrane stability to help the plant overcome HM stress. In our study GSH and phytochyletin content was not measured so it cannot be concluded that increase in P content helped DIM-Zr seedlings in this way to overcome stress. It is however reasonable to believe that the increase in P content might have contributed to membrane stability as our cell death assay result (somewhat of a indicator of cell membrane damage) show that is no significant reduction in cell death levels of DIM-Zr seedlings.

The macronutrient, magnesium plays an important role in the synthesis of the photosynthetic pigment chlorophyll and henceforth it is an important element in the process of photosynthesis. Extreme decreases in this element can thus hold dire consequences for plants. Our ICP data reveal that Zr application to *B. napus* decreases Mg content. Work by Lamhamdi et al (2013) revealed that lead decreased Mg content of both wheat and

spinach in a concentration dependant manner. Their studies also showed that there is a decrease in both chlorophyll a and b content of both treated seedlings. The authors attributed it to the decrease in Mg content. We found similar results in our study, Zr application decreased Mg content and it also decreased chlorophyll content. Our also revealed that DIM application increase Mg content in comparison with control as seen in both DIM and DIM-Zr treatments. The increase in Mg content might have played a role in increasing chlorophyll content of DIM-Zr treated seedlings when compared to Zr only treated seedlings. A study by Shen et al. (2016), revealed that exogenous Mg application reversed the negative effects Pb stress inflicted on chlorophyll content. Organic acids are known chelators and play a protective role for plants against HM. It is reviewed by Rengel et al. (2015), that increased Mg also increase the activity of organic acid synthesizing enzymes and thereby increasing organic acids. It could be that DIM increased organic acid content by increasing Mg content and thereby increasing tolerance to Zr. However, organic acid content is yet to be determined in our study.

Micronutrients or trace elements (Cu, Fe, Zn, Mn, and Co) are found in low levels in plants and higher levels it can be toxic. These elements play very important roles in plant metabolism that include among others functioning as enzyme co-factors. Our ICP data reveal that all measured trace elements in our study (Fe, Mn and Zn) decreased when exposed to Zr. A study by Lamhamdi et al. (2014), revealed that lead also decreased all measured trace elements in their study. The authors reasoned that it might be due to

a combination of the HM preventing entry of these minerals into the plant and leakage from these ions from the plant. The decrease of Fe, Mn and Zn content in our study could also be due to ion leakage and Zr preventing entry of these ions in our study.

A study by Wu and Zhang (2002), showed that Zn protected barley plants from Zn stress by improving the plants antioxidant activity. Work by Qureshi et al. (2010), revealed that increased of plant Fe content by exogenous application protected Indian mustard from Cd toxicity by stabilizing protein-pigment membrane complexes. Nada et al. (2007), revealed that Fe application ameliorated growth retardation of almond seedlings because of cadmium exposure. In our study DIM also increased these micronutrients and it the increase in micronutrient content that could have played a part in the ameliotary effect of DIM on Zr induced HM stress.

Conclusion and Future work

Based on the findings of chapter 3 it can be safely concluded that the exogenous application of DIM has been beneficial for *B. napus*, the improved growth and increase in germination percentage proves it. The biochemical results revealed that exposure of *B. napus* to DIM resulted in slight increases in both superoxide and hydrogen peroxide content. It was concluded that the increase in both superoxide and hydrogen peroxide content did not cause the seedlings any harm. The MDA content as assayed by TBARS method of DIM treated seedlings remained unchanged w.r.t control seedlings. This served as an indication that these seedlings did not suffer lipid peroxidation, which is a hallmark of oxidative stress. Diindolylmethane treatment also did not affect cell death levels of *B. napus* seedlings thus further proving that DIM treatment did not cause oxidative stress. It was argued that the slight increases in the superoxide and hydrogen peroxide played a positive signalling role rather than causing destruction. The increase in O_2 and H_2O_2 content is believed to be behind the improved seedling growth and increased germination percentages. Treatment with DIM also led to increased SOD and APX activity. It was argued that the activities of these antioxidant enzymes increased to control O_2 and H_2O_2 content. The tight control of O_2 and H_2O_2 content by the antioxidant enzymes kept it in a range where it could trigger positive signals i.e. kept ROS content in the 'oxidative window'.

Application of Zr resulted in high O_2 and H_2O_2 increases. Based on MDA and cell death results, it is very clear that Zr treated *B. napus* seedlings were

under oxidative stress. Both SOD and APX activity increased in an attempt to decrease ROS levels. Germination percentages, biomass and chlorophyll decreased under Zr induced HM stress. The co-treatment of DIM-Zr produced different results to Zr only treated results. In DIM-Zr treated samples, both O_2 and H_2O_2 content was reduced in comparison to Zr only treated samples. Subsequently, lipid peroxidation and cell death levels decreased to levels of the control indicating that DIM relieved *B. napus* from oxidative stress. Germination percentages, biomass and chlorophyll content also recovered as a result of the co-treatment. Based on these results it is clear that DIM played an ameliotary role for seedlings under Zr stress. The SOD and APX activity was highest in the co-treatment (higher than control, DIM and Zr treatments). Therefore, it is most likely that the increase in enzymatic antioxidant activity played an important role in making *B. napus* more tolerant to Zr stress. The increase in both SOD and APX activity lowered O_2 and H_2O_2 content and thereby prevented oxidative stress. Treatment with Zr also decreased all measured micro- and macro-nutrients with the exception of Ca. This clearly had a negative impact on the biomass of the seedlings. Application of DIM on the other hand led to increase in macro- and micro-nutrient content. The increase in nutrient content under DIM-Zr treatments could also have played a role in the ameliotary effect of DIM during Zr induced HM stress.

This study has given some insight on the effect of DIM on B. napus and its role in Zr induced HM stress but it also raised a few unanswered questions. Diindolylmethane increases ROS content of *B. napus* seedlings and thereby

triggering plant developmental parameters. No studies have been conducted to determine exactly how DIM increase ROS content. One possible way could be that DIM application results in the increase of ROS producing enzymes like NADPH oxidase. Spectrophotometric enzyme assays can be done to determine if DIM triggered ROS increase is brought about this way. When molecules are applied exogenously to plants it is usually taken up and the endogenous concentration of this compound increase. The uptake of DIM in *B. napus* has not been confirmed in our study. Quantification of DIM in plant extracts can be achieved by RP-UHPLC/MS. Exogenous application of Zr resulted in an increase in Zr content in both Zr and DIM-Zr treatments. A common manner in which these ameliotary compounds ameliorate stress is by reducing HM uptake, this is however not the case in our study. As discussed previously, DIM could be increasing HM chelator content in addition to the already confirmed increased antioxidant capacity. Sulphur containing chelators such as glutathione, metallothionins and phytochyletins and non-sulphur chelators like citric acid and glycine betane can be quantified by LC/MS methods to establish if DIM also protects plants in this manner. Omics studies such as transcriptomics, proteomics and metabolomics can be done to get a deeper insight and to paint a much clearer picture of the effect of DIM on *B. napus* and its role in Zr stress.

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