# 3,3’ Diindolylmethane mediated signalling and its role in *Brassica napus* L. responses to vanadium stress

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**Dissertation presented for the degree of Doctor of Biotechnology at the University of the Western Cape.**

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## Chapter 1

## Chapter 2



## Chapter 3

## Chapter 4

## Chapter 5

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| EDTA | Ethylenediaminetetraacetic acid |
| SOD | Superoxide dismutase |
| APX | Ascorbate peroxidase |
| TCA | Trichloroacetic acid |
| TBA | Thiobarbituric acid |
| TEMED | Nitro blue tetrazolium chloride |
| NBT | 3, 3 Diindolylmethane |
| DIM | 3,3'Diindolylmethane |
| CAT | Catalase |
| GST | Glutathione-S-Transferase |
| MDA | Malondialdehyde |

| | |
|---|---|
| PCD | Programmed cell death |
| ROS | Reactive oxygen species |
| TBA | Thiobarbituric acid |
| TEMED | N, N, N, N'- Tetramethylethylenediamine |
| Gly | Glyoxalase |
| MG | Methylglyoxal |
| CD | Conjugated dienes |

## LIST OF FIGURES

## LIST OF TABLES

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**Arun Gokul**

## KEYWORDS

Antioxidant enzymes

Ascorbate peroxidase

Biomass

Cell death

Heavy metal

Hydrogen peroxide

Lipid peroxidation

Reactive oxygen species

Superoxide

Superoxide dismutase

Vanadium

3,3’ Diindolylmethane

**3,3' Diindolylmethane mediated signalling and its role in *Brassica napus* L. responses to vanadium**

**Arun Gokul**

**PhD Thesis, Department of Biotechnology, University of the Western Cape**

## ABSTRACT

Anthropogenic activities such as mineral mining, improper watering practices, and the use of heavy metal contaminated fertilizers have caused an influx of heavy metals into arable lands. These heavy metals may have a negative impact on plant growth, as they are able to increase ROS species within plants resulting in plant metabolism deterioration and tissue damage. Heavy metals also have the ability to render important enzymes non-functional or may decrease their activity resulting in poor growth. Vanadium was used as the heavy metal of choice in this study, as South Africa is one of the top producers of this metal worldwide. In an effort to improve growth of crop plants, mechanisms have to be identified to increase growth under vanadium stress. One method to increase growth is the use of exogenously applied signalling molecules. In this study, one such compound 3,3' Diindolylmethane (DIM) was investigated to identify whether it had growth promoting properties. The *Brassica napus* L. plant was used as the plant of choice as it is economically beneficial to the South Africa economy. In addition, *B. napus* have the metabolic machinery to produce DIM *in planta*. The study also aimed at

determining whether DIM could have positive effects on vanadium stressed seedlings. Physiological experiments such as cell death, lipid peroxidation, chlorophyll and biomass was determined to understand how vanadium and DIM affected the seedlings both individually as well as in a combination treatment. The oxidative state of the seedlings in response to the treatment with vanadium and DIM were assessed as well. The oxidative state was assessed by employing spectrophotometric assays for hydrogen peroxide, superoxide and hydroxyl radicals. Due to the changes observed in the oxidative state of the seedlings which were ultimately due to the different treatments, the changes in antioxidant enzymes had to be observed. The antioxidant enzymes assessed in this study were superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione- s- transferase (GST) and glyoxalase (Gly). Both cell death and lipid peroxidation were increased when the seedlings were treated with vanadium, however when the seedlings were treated with the combination of vanadium and DIM the cell death and lipid peroxidation was not as pronounced as observed for vanadium alone. DIM application increased chlorophyll content within the seedling under normal and vanadium stressed conditions. DIM was also observed to increase the antioxidant enzymes such as SOD and APX in the *B. napus* seedlings, which could be an explanation for the improved growth of the seedlings even under vanadium stress. The seedlings treated with DIM displayed a slight increase in ROS molecules hydrogen peroxide and superoxide, but the levels observed were not in the detrimental range, where damage may occur.

This observation lead us to believe that DIM was able to increase ROS concentration for signalling purposes within the seedlings.

To determine the effects of DIM and vanadium on the uptake and accumulation of essential metals including the toxic metal vanadium, Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-OES) was used. DIM had no effect on the uptake of vanadium, but was able to increase calcium, iron and magnesium content under normal and vanadium stress conditions. The ability for DIM to modulate the nutrient profile was interesting as some of the growth promoting properties of DIM could be attributed to the increase in uptake of essential metals.



Here, it is illustrated for the first time that DIM application can improve *B. napus* seedling growth through the induction of hydrogen peroxide and superoxide. DIM also improved the antioxidant capacity of the seedlings, which resulted in less damage observed when the seedlings were exposed to a combination of DIM and vanadium stress. Furthermore, the nutrient profile was affected by both DIM and vanadium but DIM increased the uptake of essential metals, which could explain the increase in growth observed under vanadium stress.

# Chapter 1
# Literature Review

## 1.1. Introduction

Abiotic stresses can be detrimental to plant growth and functioning due to the inability of plants to move away from the stress causing agents (Tuteja *et al*., 2009). Abiotic stresses which affect plants include high and low pH of soils, drought, salinity and heavy metals (Nakashima *et al*., 2012). The latter is especially harmful due to the fact that these metals can cause damage over prolonged periods of time due to their long half-life in soils (Reichman, 2014). Heavy metals at elevated concentrations in soils have the ability to inhibit growths of plants and reduce the functioning of enzymes. This was clearly observed in a study by Iannone et al. (2015) where increased concentrations of cadmium resulted in reduced plant growth and NADPH oxidase activity. Heavy metal contamination is fast becoming one of the leading global agricultural problems as land in all parts of the world are slightly to highly contaminated with heavy metals (Yadav, 2010; Russo *et al*., 2008).

South Africa is one of the foremost suppliers of vanadium worldwide (Moskalyk & Alfantazi, 2003). The metal constitutes about 0.01% of the world's crust and is

therefore naturally distributed widely in the environment (Tian *et al*., 2014). Due to increased mining practices, an even greater concentration of vanadium and other metals should be expected in soils especially at or near the mines as well as the surrounding lands (Saco *et al*., 2013). Heavy metals such as vanadium may get into the ground water or can be swept up by the air were they can be transported far from their site of origin (Bi *et al*., 2014). Vanadium is a heavy metal that is may be required by plants in minute quantities. This was observed in a study by Vachirapatama et al. (2011) where Chinese green mustard plants experienced enhanced growth when treated with vanadium concentrations below 100 µM. However, this hypothesis is considered controversial at the time of our study. Nevertheless, it has been observed that at higher vanadium concentrations in soils, plants experienced lower yields, stunted growth, as well as yellowing of leaves (Vachirapatama *et al*., 2011; Wang & Liu, 1999). Due to the high prevalence of vanadium as well as it deleterious nature it is important to identify methods or mechanisms which will improve the tolerance of plants to this abundant heavy metal.



Methods to increase tolerance of plants to heavy metals include the addition of elements such as silicon, beneficial microorganisms and priming compounds such as sodium chloride (Wu *et al*., 2013; Li *et al*., 2013; Li *et al*., 2011). 3,3' Diindolylmethane (DIM) is a compound which naturally occurs in the plant family

called Cruciferae and is quite abundant in the genus Brassica (Banerjee *et al*., 2011). DIM has been shown to induce reactive oxygen species (ROS) in cancerous tissue in mammals and induce stress activated pathways (Banerjee *et al*., 2011). Therefore, this thesis will aim for the first time according to our knowledge, to unravel novel signaling roles of DIM in plants. Furthermore, the thesis will aim to use DIM as a potential rescuing signaling molecule against vanadium stress. Hence, this literature review will discuss potential signaling pathways for DIM signaling as well as general background of the study.


## 1.2. Reactive oxygen species within plants

Due to all the threats plants face they have evolved and adapted to form a signalling network, which consist of growth regulators to sense and also protect the plant from damage (Bhattacharjee, 2011). One of the primary responses to abiotic or biotic stress is the augmented generation of ROS. Due to the reactive nature of these compounds as well as the ability to cross membranes, they can signal throughout the plant alerting the plant of any danger (Bela *et al*., 2015; Schmitt *et al*., 2014). During normal aerobic metabolism, ROS are produced as by-products (Mai *et al*., 2013; Bhattacharjee, 2011; Gill & Tuteja, 2010). At basal levels ROS are not only signaling molecules in stress conditions but were also found to regulate processes such as polar growth, chloroplast movements and stomatal activity

(Demidchik, 2015; Schmitt *et al*., 2014). Although ROS compounds act as signaling molecules, their concentrations need to be tightly controlled because if they over accumulate, they may cause oxidative damage to the plant (Ueda *et al*., 2013; Zhang *et al*., 2007). The accumulation of ROS can lead to oxidative damage of many molecules in the plant, which include DNA, carbohydrates, lipid and proteins and could ultimately lead to cell death (Talaat *et al*., 2015; Maruta *et al*., 2012; Gill & Tuteja, 2010). The accumulation of ROS is believed to be due to an imbalance in the ROS production and the antioxidant systems (Zhang *et al*., 2007). The balance between the generation of ROS and its removal may be affected by many factors such as pH, salinity, viruses and heavy metals (Li *et al*., 2016). ROS include compounds such as hydrogen peroxide ($H_2O_2$), superoxide ($O_2^-$) and hydroxyl radical (•OH) (Schmitt *et al*., 2014; Sinha & Saxena, 2006).



### 1.2.1. Hydroxyl radicals

The hydroxyl radical (•OH) is highly reactive and can react on contact with other molecules (Gill & Tuteja, 2010; Babbs *et al*., 1989). Its short half-life (1 ns) coupled with its oxidative nature makes it very difficult to detect making it one of the most dangerous reactive molecules in living organisms (Asano *et al*., 2014). Due to its short half-life, the distance which the molecule can travel is often very short (Demidchik, 2015). The proposed reason for the formation of •OH include; its

involvement in regulating oxygen toxicity in living organisms (Kataria *et al*., 2005; Vranova *et al*., 2002). Hydrogen peroxide and superoxide in the presence of transition metals may be converted to •OH through a process known as the Fenton reaction (Gill & Tuteja, 2010). Due to the different metabolic requirements of plants, they allow the constant uptake of transition metals. With the uptake of transition metals comes the accompanied danger of over production of •OH and cell damage. The •OH is extremely reactive and downstream damage results in cells not having enzymatic machinery for the detoxification of the radical. Cells therefore rely on mechanism which prevents the formation of the radical (van Doorn & Ketsa, 2014).



### 1.2.2. Superoxide ($O_2^-$)

Superoxide ($O_2^-$) is generated in plants when oxygen is partially reduced and often occurs during photosynthetic processes (Gill & Tuteja, 2010; Schützendübel & Göttingen, 2001). The half-life of $O_2^-$ is relatively short which only allows for movement of a few micrometers from the site of production (Kavdia, 2006). The predominate function of $O_2^-$ is as a reducing agent and can act on a variety of compounds (Demidchik, 2015). Compounds that can be reduced by $O_2^-$ include $Fe^{3+}$ and $Cu^{2+}$ that are prevalent in plants. It should be noted that although $O_2^-$ may cause oxidative stress, it cannot directly modify macromolecules (Demidchik, 2015). $O_2^-$ is generated as soon as a few seconds after the addition or perception of a stress-

causing agent but it can take hours to reach the amount necessary for detection (Kawano *et al*., 1998; Schraudner *et al*., 1998). The conversion of superoxide to oxygen and hydrogen peroxide is performed by the enzyme superoxide dismutase which acts as one of the oxidation defense mechanisms in plants (del Rio *et al*., 2006).

### 1.2.3. Hydrogen Peroxide ($H_2O_2$)

The univalent reduction of superoxide results in $H_2O_2$ (Gill & Tuteja, 2010). $H_2O_2$ is only moderately reactive but it also has a relatively longer half-life when compared to other ROS (Gill & Tuteja, 2010; Bhattachrjee, 2005). Due to the stability of $H_2O_2$, it is suited and essential for many physiological functions (Foyer & Noctor, 2009). The ability of $H_2O_2$ to oxidize thiol groups on enzymes ultimately resulting in decreased functioning or total inactivity of enzymes, makes $H_2O_2$ at high concentrations, very dangerous to plants (Brou *et al*, 2007). Concentrations of $H_2O_2$ range from 0.03-1 µM at basal levels but under stressful situations $H_2O_2$ may elevate and range from 0.1-10 mM (Demidchik, 2015; Forman *et al*., 2003). $H_2O_2$ is detoxified by the enzyme class peroxidases. Interestingly, proposed negative feedback mechanisms which are thought to regulate the peroxidases may be inhibited by $H_2O_2$. The ability of this ROS to down regulate antioxidant enzymes makes it dangerous to plants (Demidchik, 2015; Kitajima, 2008). It should also be noted that peroxidases are not the only

enzymes which may be affected by $H_2O_2$ but enzymes such as fructose biphosphate may be totally inactivated by this compound (Demidchik, 2015).

### 1.2.4. Methyglyoxal (MG)

Methylglyoxal (MG) is produced during glycolysis or Embden Meyerhof pathway by the degradation of triophospahtes, Glyceraldehyde -3-phosphate (G3P) and Dehydroxyacteone phosphate (DHAP) (Singla- Pareek *et al*., 2006). MG is also produced during cellular respiration (Skipsey *et al*., 2000). MG is a compound that has the ability to react with DNA, RNA and proteins causing changes in their structure (Yadav *et al*., 2005). When MG concentration increases due to accumulation, cell growth is affected and may lead to cell death (Hossain & Fujita, 2010). Normal levels of MG were observed to be in the range of 1- 2 µM (Thornally & Rabanni, 2001). Levels above 200 µM were observed to induce cytotoxic damage within cells (Thornally & Rabanni, 2001). The enzyme glyoxalase I scavenges excess MG to prevent its accumulation (Yadav *et al*., 2005).

## 1.3. ROS and cell biochemistry

### 1.3.1. Lipid peroxidation

Lipid peroxidation is the catalytic change of structure and function of membranes (Yadav, 2010). Lipid peroxidation is widely used as an indicator of oxidative damage. As mentioned before, ROS are over produced or accumulated when plants undergo any type of stress. These radicals may lead to disruption of polyunsaturated fatty acids (which form the lipid membrane) (Sinha *et al*., 2005; Verma & Dubey, 2003). The compound Malondialdehyde (MDA) is a cytotoxic compound that is produced during the process of lipid peroxidation and can be used as an indicator of oxidative damage to lipids (Wahsha *et al*., 2012). The consequences of lipid peroxidation include the damage of the cell membrane, which affects the permeability of the cell, causing leakage of important ions such as potassium (Zhang *et al*., 2007). To protect themselves from the accumulation of ROS molecules, plants use enzymatic and non-enzymatic antioxidants. These antioxidants include the non-enzymatic molecules such as ascorbate, glutathione and cysteines and the enzymes ascorbate peroxidase, superoxide dismutase and glutathione peroxidase (Sinha *et al.*, 2005).

### 1.3.2. Chlorosis induced by stress

The green colour of plants are due to a pigment called chlorophyll, which is one of the most abundant biological pigments found on earth (Hörtensteiner & Krautler, 2011). Chlorosis is the yellowing of a plant especially the leaves due to the decrease of chlorophyll content (Sghaier *et al.*, 2015; Abadia *et al.*, 2011). The decreased in the chlorophyll content may be attributed to the failure to produce chlorophyll or due to damage of existing chlorophyll (Li et al., 2015; Yadav, 2010). Chlorosis may be caused by many abiotic and biotic stress factors. Heavy metals are able to induced ROS molecules, which damage chloroplasts resulting in decreased chlorophyll in plants (Henriques, 2010). Heavy metals at concentrations even as low as 10 mg/L were observed to induce chlorosis in some plants (Fatoba & Emem, 2008). Santos et al. (2014) observed at elevated zinc concentrations that chlorophyll content decreased in the plants. The decrease in chlorophyll content may be detrimental to plants as it restricts photosynthesis resulting in inhibition of energy production and nutrient deficiency (Sghaier *et al*., 2015; Wang *et al*., 2005).



### 1.3.3. DNA damage

Although the genome of plants are reported to be very stable, damage may occur when DNA is exposed to DNA damaging compounds (Gill & Tuteja, 2010). DNA is one of the most susceptible molecules and can be damaged when an organism

undergoes oxidative stress (Saroj Arora & Vig, 2015). One of the reactive oxygen species namely •OH has the ability to damage the purine and pyrimidine bases, which make up part of the structure of DNA (Wiseman & Halliwell, 1996). The singlet oxygen however is only able to target and damage the guanine nucleotides (Wiseman & Halliwell, 1996). Damage to DNA can occur in many ways which include strand cleavage, deletion or addition of nucleotide bases (Tuteja *et al*., 2001). The ROS molecules $H_2O_2$ and $O_2^-$ cannot directly damage DNA strands in plant cells (Tuteja *et al*., 2001). It is well known that elevated concentrations of heavy metals such as copper and lead can lead to DNA damage. The repercussions of the DNA damage include the disruption in the synthesis of certain proteins (Gichner *et al*., 2006).



## 1.4. Antioxidant enzymes and compounds prevalent in plants

### 1.4.1. Superoxide dismutase (SOD)

Superoxide dismutase (SOD) is one of the first antioxidant enzymes, which is relied upon in the defense of the plant against oxidative damage by ROS (Wang *et al*., 2014; Lee *et al*., 2007; Wang *et al*., 2005). SOD can reduce $O_2^-$ levels by catalyzing the conversation of two $O_2^-$ molecules to $H_2O_2$ and oxygen (Wang *et al*., 2014). SODs, being metalo-proteins, requires certain metals to function properly. These metals

include iron, manganese and copper-zinc. SODs are classified according to the metal cofactor, which they require. Copper-zinc SODs are the most prevalent isoforms observed in plants (Mahanty *et al*., 2012). The need for different metals by different SODs were theorized to be due to evolutionary responses to the availability of the different metals (Mahanty *et al*., 2012). It should be noted that if the correct or specific metal cofactor is substituted by another metal the enzyme becomes inactivated (Mahanty *et al*., 2012). Depending on the metal stress, these substitutions may occur, resulting in a decreased antioxidant response and thus an increase in oxidative damage in the plant will be the result. The $H_2O_2$ produced during $O_2^-$ regulation by SODs may be scavenged by peroxidases such as ascorbate peroxidases (Zhang *et al*., 2007).



**1.4.2. Ascorbate peroxidase (APX)**

The isoenzyme ascorbate peroxidase (APX) which is located in the peroxisomes, thylakoid membrane and the cytosol is a regulator of $H_2O_2$ produced by plants (Maruta *et al*., 2012; Li *et al*., 2012). The enzyme regulates $H_2O_2$ concentration by using $H_2O_2$ molecules in a reaction where ascorbic acid is converted to dehydroascorbate (Sinha & Saxena, 2006). APXs were found to be very important in protecting the photochemical machinery used in photosynthesis against oxidative damage (Caverzan *et al*., 2014). Literature has shown that under stressful conditions

gene expression of APX genes as well as the activity of the resulting proteins have increased to help protect the plant (van Doorn & Ketsa, 2014). This fact was observed in a study by Zhang et al. (2013) where the cytosolic APX were knocked out of rice resulting in inhibition of growth under drought, salt and cold stress. A study by Wang et al. (1999) showed that increased resistance to oxidative stress was achieved by over expressing the peroxisomal APX in the tobacco.

### 1.4.3. Glutathione-S-Transferase (GST)

Glutathione-S-Transferases (GSTs) are widely distributed among living organisms and are found in bacteria, yeasts, animals and plants (Cicero *et al*., 2015; Liu *et al*., 2013). GSTs are for the most part soluble and found in the cytosol of cells (Cummings *et al*., 2011). GST have been observed to protect against oxidative stress (Sheehan *et al*., 2001). GSTs have the ability to catalyze glutathione dependent peroxidase reactions to scavenge hydroperoxides as well as $H_2O_2$ resulting in enhanced oxidative protection (Dinler *et al*., 2014; Froza, 2006). GSTs can add glutathione to electrophilic groups on hydrophobic toxic compounds, resulting in inactive compounds which are also water soluble which aids in their removal or segregation into vacuoles (Cicero *et al*., 2015). The segregated compounds may undergo further transformation resulting in promotion of their degradation (Bartholomew *et al*., 2002).

### 1.4.4. Glyoxalase (Gly)

The Glyoxalase system was first observed in the year 1951 (Thornally and Rabanni, 2011). The glyoxalase system consists of two enzymes namely Gly I and Gly II (Veena *et al*., 1999). The two enzymes cooperate to convert 2-oxoaldehydes into 2-hydroxyacids using glutathione as a cofactor (Thornalley, 1993). Methyglyoxal (MG) is the primary substrate for the Gly I enzyme (Hossain *et al*., 2012). Gly I isomerases the hemithioacetal from MG and glutathione, to yield S-D-Lactoylglutathione. Gly II catalyses the conversation of S-D-Lactoylglutathione to D- Lactate (Yadav et al., 2005). An article by Veena et al. (1999) stated that compounds such as auxins and cytokinins which cause cellular growth could increases Gly I activity.



## 1.5. Uptake of metals

Plants interact with soils for a variety of reason, which include anchoring or for stability as well as water and nutrient acquisition. Due to the interaction between the roots and the surrounding soils, plants are also exposed to the pollutants within the soils (Mingorance *et al*., 2007; Kovács *et al*., 1993). These pollutants include inorganic waste and heavy metals and may be taken up by plants (Qian *et al*., 2014; Panagos *et al*., 2013). The uptake of heavy metals are affected by many factors such as the solubilization of the metals within the water found in the soils as well as the

pH of soils. The pH of soils are very important as it has an effect on the leaching ability and mobility of metals. At low pH (acidic), the mobility and leaching rate of metals are increased (Touceda-González *et al*., 2015). Some plants have the ability to secrete chemicals to modify the solubilization of metals thus affecting their bioavailability (Kidd *et al*., 2009). Once the metals have been absorbed by the roots they may leach into surrounding tissues and subsequently be transported through the plant through the transportation systems such as the phloem and xylem (Nyguist & Greger, 2007). Once metals are in contact with cells they can bind to negatively charged sites found on the cell wall (Abadia *et al*., 2011). The high metal concentration outside the cell may cause a gradient across the membrane, which promotes the influx of metals into the cell (Nyguist & Greger, 2007). Plants are able to employ many strategies to protect itself from heavy metal toxicity. One strategy, which a plant may employ, is the immobilization of the heavy metals in different tissues, localizing the metals and minimizing the damage to the plant (Nyguist & Greger, 2007; Tuteja *et al*., 2009). A second strategy plants may employ is the use of transporters to move the metals to an area where it might be removed from the plant cells (Verkleij *et al*., 2009).

## 1.6. *Brassica napus* L.

*Brassica napus* was produced and widely used in Europe in the early 14$^{th}$ century (Grispen *et al*., 2006). *Brassica napus* L. commonly known as Canola is a member of the Brassicaceae family (Elahi *et al*., 2016). The development of Canola occurred during the early 1970's in Canada (Przybylski *et al*., 2005). Through breeding programs, unwanted compounds such as glucosinolates and erucic acid concentrations in Canola were reduced (Miller-Cebert *et al*., 2009; Eskin *et al*., 1996). The major importance of canola is its high nutritive quality, which takes the form of the oil it accumulates during seed development (Miller-Cebert *et al*., 2009). Other uses of Canola include livestock fodder and plant biomass for the production of biofuels (Grispen *et al*., 2006). Due to the different characteristics desired different Canola cultivars have been developed.



## 1.7. Glucosinolates

Plants can produce secondary products that are used in the defense of the plant especially against disease as well as pests (Herr & Buchler, 2010). The concentration of these secondary plant products were observed to be greater when the plants were undergoing stress (Herr & Buchler, 2010). In recent years, it has been observed that these secondary plant products are not only beneficial to the plant but that

these compounds could also be beneficial to humans (Tiwari *et al*., 2015). The one class of these secondary plant products are called glucosinolates. The glucosinolates are the group of compounds that give certain vegetables a bitter taste (Tiwari *et al*., 2015). A high concentration of the glucosinolates were found to be present in cruciferous vegetables, which include cabbage, broccoli, rapeseed and *Brassica napus* L. (canola) (Tiwari *et al*., 2015). When cruciferous vegetables undergo mechanical damage such as chewing or crushing, damage then occurs to the cell wall of myrosin cells leading to the release of the β-thioglucosidase (myrosinase) which hydrolyze glucosinolates forming important products such as Indole-3 carbinol (I3C) and other isothiocyanates (Asad *et al*., 2015; Herr & Buchler, 2010; Tiwari *et al*., 2015; Shapiro *et al*., 1998). Isothiocyanates are produced as toxic compounds to deter insects and animals from further consuming the plant (Asad *et al*., 2015; Textor and Hershenzon, 2009). It should be noted that glucosinolate content will differ according to the type of damage inflicted on the plant as well as the duration of the damage (Textor and Hershenzon, 2009). Glucosinolates have also been observed to have the ability to protect cells against damage by hindering the activation of promutagens or by inducing detoxification enzymes (Saroja Arora & Vig, 2015). One of the indole containing compounds produced from glucosinolates is 3,3' Diindolylmethane (DIM), which has gained notable interest in the past few years as it has shown induction of signalling within cancer cells (Semov *et al*., 2012; Rahimi *et al*., 2010).

### 1.7.1. 3,3' Diindolylmethane (DIM)

Auxins are a group of naturally found compounds that act as phytohormones within plants and play critical roles in growth (Pal *et al*., 2007). Auxins have the ability to promote the division of cells as well as stimulate root formation (Haissig and Davis, 1993). DIM which is a derivative of I3C (and fall in the same structural class as auxins) has been used as dietary supplements and as well as a cancer treatment (Nachshon- Kedmi *et al*., 2003). Scientist have also observed that DIM could have possible radical scavenging abilities (Khan *et al*., 2008; Benabadji *et al*., 2004; Chen *et al*., 1996). Indole containing compounds such as DIM were hypothesized to control the defense system of plants as well as certain hormones (Lee *et al*., 2015). It was observed in a study by Pal et al. (2007) that DIM derivatives can increase germination as well as growth of *Oryza sativa* (rice) when compared to other indole containing compounds such as Indole-3-acetic acid (IAA). The same study also showed that the increase in growth of the rice was dependent on the concentration of DIM derivative administered.



## 1.8. Justification

The world is becoming increasingly more industrialized resulting in the expansion of the fuel and mining sectors to achieve more raw materials. Land suitable for

agriculture is lost to these aforementioned sectors due to their perceived importance to a country's economy. The land surrounding mines are continuously contaminated with heavy metals, which then leach into the soils and ground water flow, resulting in the spread of the metals far from the site of origin. The accumulation of heavy metals in agricultural soils could lead to reduced germination and slower growth. Due to the negative impact of vanadium on crop plants, scientific research is needed to mitigate this problem. One method we would like to investigate is the exogenous application of DIM and its effect on vanadium stress. We would like to identify whether small quantities of DIM could induce enough ROS to induce stress-activated pathways and mechanisms to help the plant against vanadium stress. By elucidating whether DIM can mediate the effect of vanadium stress in canola, this could allow for the increase in tolerance of other economic important crops. Increased metal tolerance would lead to a rise in crop yields, which would ultimately lead to increased food security and economic benefits for the country.



## 1.9. Objectives of this study

The focus of this study is to investigate whether DIM can rescue *Brassica napus* L. seedlings from vanadium stress toxicity, and the effect of DIM on the biochemical and physiological characteristics of the plants. This project will obtain information

pertaining to the toxicity of vanadium and the mechanisms, which DIM, could induce to increase the tolerance of *Brassica napus* L. seedlings to vanadium stress. The aims of this study include observing whether DIM has any growth promoting properties on *Brassica napus* L. seedlings. The second aim will be to identify how ROS concentrations changed in response to vanadium stress and the exogenous application of DIM. The antioxidant profiles will also be observed to understand how DIM could affect their activities. The effect of vanadium on biomass production of the *Brassica napus* L. seedlings will also be investigated. The use of inductively coupled plasma optical emission spectroscopy (ICP-OES) will be utilized to determine the micronutrient, macronutrient and vanadium concentrations in seedlings treated with the different treatments. The ICP-OES results will give us an indication of how metal uptake is affected in *Brassica napus* L. seedlings that underwent the different treatments. This knowledge could lead to the growth promotion of other economically beneficially crop plants. Since South Africa is a major mining country, mechanism to increase heavy metal tolerance of crop plants have to be identified, this study aims to identify for the first time whether DIM could play a role in such mechanisms.

# Chapter 2
# Materials and Methods

**Table 2.1: List of chemicals and suppliers**

| | |
|---|---|
| 2- Thiobarbituric acid | Sigma-Aldrich |
| 2,4- Dinitrochlorobenzene | Sigma-Aldrich |
| 2-Deoxy-D- Ribose | Sigma-Aldrich |
| 3.3'- Diindolylmethane | Sigma-Aldrich |
| 30 % Acrylamide solution 37:5:1 | Sigma-Aldrich |
| Acetone | Sigma-Aldrich |
| Diaminobenzene | Sigma-Aldrich |
| Ethanol 200 proof | Sigma-Aldrich |
| Ethylenediaminetetraacetic acid | Sigma-Aldrich |
| Evans blue | Sigma-Aldrich |
| Filter sand cape silica | Cape Silica |
| AV Garnet | Agricol |
| Glacial acetic acid | Sigma-Aldrich |
| Glycine 99 % | Sigma-Aldrich |
| L- Ascorbic acid | Sigma-Aldrich |
| L- Glutathione reduced | Sigma-Aldrich |

| | |
|---|---|
| Methylglyoxal 40 % in $H_2O$ | Sigma-Aldrich |
| Nitro blue tetrazolium chloride monohydrate | Sigma-Aldrich |
| Phosphoric acid | Sigma-Aldrich |
| Polyvinylpyrrolidone | Sigma-Aldrich |
| Potassium cyanide | Sigma-Aldrich |
| Potassium hydroxide | Sigma-Aldrich |
| Potassium iodide | Sigma-Aldrich |
| Potassium phosphate dibasic | Sigma-Aldrich |
| Potassium phosphate monobasic | Sigma-Aldrich |
| Potting soil | Stodels, South Africa |
| Quick start Bradford dye reagent 1X | Bio-Rad |
| Riboflavin | Sigma-Aldrich |
| Sodium dodecyl sulfate | Bio-Rad |
| Sodium hydroxide | Sigma-Aldrich |
| Sodium metavanadate | Sigma-Aldrich |
| Trichloroacetic acid 99 % | Sigma-Aldrich |
| Tween 80 | Sigma-Aldrich |
| Nitric acid (65 %) | Sigma- Aldrich |

**Table 2.2: List of equipment**

| Instrument | Model | Company |
|---|---|---|
| Centrifuge | 5415D | Eppendorf |
| Freeze drier | Freezone Plus 2.5 Litre | Labconco |
| Heating block | ABHZ | FMH instruments |
| ICP-OES | Varian Vista Pro | Varian |
| Incubator | Quincy Lab economy digitial incubator | ProLab Scientific |
| Mass balance | WTB200 | Radwag |
| Spectrophotometer | Ultrospec 2 | Biochrom |
| Spectrophotometer (Microtitre plate reader) | POLARstar | Omega |



## 2.1. Preparation of DIM

The DIM solution was prepared by adding 10 mg of DIM powder, 235 µl Tween 80 and 10 ml of absolute ethanol in a tube which was then mixed until all the powder was dissolved. The solution was snap frozen using liquid nitrogen and placed in a freeze dryer at -41 °C overnight. The control solutions were made as stated for the DIM solution except no DIM powder was added. Thereafter, 19.765 ml of deionised water was added to the solution. The resulting DIM stock solution was 2030 µM.

## 2.2. Growth Parameters

*B. napus* (AV Garnet) seeds were germinated in pots containing 500 g of soil. The soil makeup was a potting soil to compost mix with a ratio of 1:1. The plants were treated twice a week with 100 ml of 15 µM DIM, 15 µM DIM and 350 µM vanadium, Tween 80 and 350 µM vanadium or deionised water containing Tween 80 (control). The germination percentage was determined by firstly pre-treating the soil with DIM water (control) followed by planting 100 seeds (25 from different seed lots x [n=4] per treatment and observing the number of seeds germinated. Germination was defined as seeds with radical 3 mm or more in length. The plants were harvested once they reached the seedling stage and before entering the vegetative stage. Seedling shoots were harvested by removing them from the soil and snap freezing the material with liquid nitrogen and then grinding the seedling shoots for long term storage. Some seedling shoots were removed from the soil and used for fresh material experiments.



## 2.3. The effect of the different treatments on biomass production in roots and leaves

Once the plants had grown (while treated) for 2 weeks (14 days). Whole seedlings were removed from their pots for the purpose of this experiment. The roots were severed at the interface between the root and the stem. The seedling shoots were

inserted into separate envelopes, holes were poked into the foil so moisture could escape from the envelope. Samples were dried overnight in an incubator at 80 °C. Thereafter, their mass was determined using a fine mass balance or scale.

**2.4. Cell viability assay (Evans blue)**

A modified method of Sanevas et al. (2007) was followed to determine the cell viability of the plant tissues. An aliquot of 1 ml 0.25 % (w/v) Evans blue solution was added to Eppendorf tubes. A whole seedling shoot was inserted into the Eppendorf tubes containing the Evans blue reagent. The samples were incubated in the Evans blue solution for 1 hour at room temperature. After the incubation was completed the unbound Evans blue solution was rinsed from the samples using deionised water. The samples were then incubated in deionised water overnight at room temperature. Following the overnight incubation, the water was decanted and a 1 % (w/v) sodium dodecyl sulfate (SDS) solution was added to all the samples in the Eppendorf tubes before being crushed with a miniature pestle. The samples were then incubated at 65 °C for 1 hour. Following the incubation, the samples were centrifuged at 10000 x *g* to pellet the plant material before removing the supernatant to a new Eppendorf tube. The supernatant served as the sample and was loaded on a 96-well microtitre plate for reading at 600 nm on a spectrophotometer.

## 2.5. Protein extraction

Protein extraction was done from all sample plants. This was done by weighing out 100 mg of frozen ground plant material in three individual Eppendorf tubes (three tubes per sample). Protein extraction buffer (0.5 ml) containing [0.004 M phosphate buffer, 1 mM ethylenediaminetetraacetic acid (EDTA) and 5 % (w/v)] PVP was added to one of the three tubes. The sample tube was then homogenised using a vortex. Then, the sample tube was centrifuged at 9000 x *g* for 5 minutes in order to pellet the material, the resulting supernatant was then removed from the tube and inserted into the next tube containing 100 mg plant material. The previous steps were then repeated for the remaining tubes. The supernatant was recovered from the third tube and inserted into a clean Eppendorf tube. The protein concentrations were then quantified using a Bradford assay. Thereafter, the protein samples were stored for future experiments, at -20 °C.



## 2.6. Determination of lipid peroxidation

A modified method by Zhang et al. (2007) was followed for the lipid peroxidation assay. A mass of 100 mg ground frozen plant material was added to Eppendorf tubes. To the samples in the Eppendorf tubes 5 volumes of 6 % (w/v) Trichloroacetic acid (TCA) was added. The samples were homogenised using a vortex and then

centrifuge at 1000 x *g* for 10 minutes to pellet the plant material. A volume of the supernatants (200 µl) were transferred into new Eppendorf tubes, to these tubes 300 µl of 0.5 % (w/v) thiobarbituric acid (TBA) was added. The samples were briefly mixed once again using a vortex. Parafilm was wrapped around the lids of the tubes to ensure that they remained sealed during the high temperature incubation. The sample tubes were placed in a heating block and was allowed to incubate at 90 °C for 20 minutes. Following the incubation at high temperature the samples were incubated on ice for 10 minutes. Once the incubation on ice was completed the samples were centrifuged at 10000 x *g* for 5 minutes. Samples were then loaded in triplicate on a 96-well microtitre plate. The plate was then read at 532 nm and 600 nm on a spectrophotometer. The absorbance at 600 nm was subtracted from the absorbance at 532 to correct for non-specific turbidity. The extinction co-efficient of 155 $mM.cm^{-1}$ was used to determine MDA concentrations.



**2.7. A spectrophotometric assay to determine conjugated diene (CD) content**

A modified method by Blockhina et al. (1999) was followed for the conjugated diene assay. Frozen ground plant material (50 mg) was homogenised in 2 ml ethyl alcohol. The sample was then centrifuged at 3000 x *g* for 3 minutes. The resulting supernatant was spectrophotometrically read at 234 nm and the extinction coefficient of 26.5 $mM^{-1}. cm^{-1}$ was used to calculate the concentration.

## 2.8. A spectrophotometric assay for hydrogen peroxide content determination

A modified method of Velikova et al. (2000) was followed to determine the $H_2O_2$ content in the plant material. The $H_2O_2$ standards which ranged from 0 nM, 5000 nM, 10000 nM, 15000 nM, 20000 nM and 25000 nM were prepared by diluting an appropriate volume of $H_2O_2$ in deionised water. The TCA extraction which was done on ground frozen material (in section 2.7) served as the sample for $H_2O_2$ analysis. To the microtitre plate, 50 µl of the TCA extract (sample) was added. To the samples as well as the standards, 1.25 mM dipotassium hydrogen phosphate ($K_2HPO_4$) and 250 mM potassium iodide (KI) was added. Once the plate was prepared and the solutions properly mixed, it was incubated for 20 minutes at room temperature. The samples were then read at 390 nm using a spectrophotometer.



## 2.9. A spectrophotometric assay for superoxide content determination

A modified method of Russo et al. (2008) was used as basis to determine superoxide content in plant material. Eppendorf tubes containing 10 mM KCN (to inhibit Cu/Zn SODs), 10 mM $H_2O_2$ (to inhibit Mn and Cu/Zn SODs), 2 % SDS (to inhibit Mn and Fe SODs) and 80 µM NBT were prepared. The solution was made up to a final volume of 10 ml using a 50 mM potassium phosphate buffer solution (pH7.0). The samples used for this analysis were whole seedling shoots. The plant material was then

incubated in 15 ml conical tubes for 20 minutes within the prepared solution. After the incubation the material was ground in the solution with a miniature pestle to release the superoxide present in the sample. The tube was then centrifuged at 13000 *x g* for 5 minutes to pellet the plant material, the resulting supernatant was transferred to a new Eppendorf tube. A volume of 200 µl of the supernatant (sample) was then loaded onto a microtitre plate. The process was repeated for all the treatments as well as repeats. The samples were read at a 600 nm on a spectrophotometer. Once the results were obtained the extinction coefficient of 12.8 mM. $cm^{-1}$ was used to determine the superoxide concentration. The blue coloured formazan formed during the experiment was an indication of superoxide levels.



**2.10. A spectrophotometer assay to determine hydroxyl ion concentration**

The method of Halliwell et al. (1987) was used to determine hydroxyl ion concentrations. Frozen ground plant material (50 mg) was further homogenised in 1 ml of a 10 mM phosphate buffer (pH 7.4) containing 15 mM 2-Deoxy-D-Ribose. The samples was then incubated at 37 °C for 2 hours. A volume of 0.7 ml of the above prepared sample was added to a reaction mixture containing 3 ml of a 0.5 % (w/v) TBA made up in 5 mM sodium hydroxide (2 ml) and 1 ml glacial acetic acid. The sample was briefly mixed using a vortex. The reaction mixture was then heated for

30 minutes at 100 °C. After the heating step was completed the sample was cooled on ice for 5 minutes. The samples were then centrifuged for 5 minutes at 10000 x *g*. The resulting supernatant was read at 532 and 600 nm to determine the concentration using the extinction coefficient of 155 $mM^{-1}$. $cm^{-1}$.

**2.11. A spectrophotometric assay for Methylglyoxal content determination**

A range of standards (0 mM, 6.5 mM, 45.5 mM, 65 mM and 78 mM) were prepared by diluting an appropriate volume of methylglyoxal in distilled water. The standards were loaded in triplicate. Samples were prepared by adding five volumes of phosphoric acid to 250 mg frozen ground material. The samples were homogenized using a vortex for 2 minutes. Samples were incubated on ice for 15 minutes. The samples were then centrifuged at 10000 x *g* for 10 minutes. The resulting supernatants were then transferred to new Eppendorf tubes. To the supernatants 10 $mg.ml^{-1}$ activated charcoal was added, followed by a 15 minute incubation at room temperature. After the completion of the incubation period the samples were subjected to a 10 minute centrifugation step at 10000 x *g*. The supernatants were again transferred to new Eppendorf tubes followed by the addition of 400 µl saturated potassium hydroxide solution to each tube. The mixture was then incubated for 15 minutes at room temperature to neutralize the sample. After neutralization, the samples were centrifuged for 10 minutes at 10000 x *g*. The

resulting supernatants now served as the samples. Each well contained 0.5 M phosphoric acid, 1.8 mM Diaminobenzene and 130 µl (sample or standard) in a final volume of 200 µl. Once the 96-well microtitre plate contained all the necessary reagents it was incubated for 40 minutes at room temperature. The plate was then read at 405 nm using a spectrophotometer.

**2.12. A kinetic spectrophotometric assay to determine total Ascorbate peroxidase activity**



The method of Singh et al. (2007) was used to determine ascorbate peroxidase activity. Protein was extracted and quantified from frozen ground material as in section 2.6. The protein samples were aliquoted into 0.5 ml Eppendorf tubes and incubated with 2 mM ascorbate for 5 min. The protein samples were loaded in triplicate on the microtitre plate. To each well containing 10 µl protein sample, 71.43 mM $K_2HPO_4$ and 0.36 mM ascorbate was added. Just before taking the absorbance readings at 290 nm on a spectrophotometer, 0.714 mM $H_2O_2$ was added to start the reaction. Reactions were made up to a total volume of 200 µl with deionised water. Using the extinction coefficient of 2.8 mM. $cm^{-1}$ the activity was calculated.

## 2.13. A spectrophotometer assay to determine the total Superoxide dismutase activity

The method of Singh et al. (2007) was used to determine superoxide dismutase activity. Protein was extracted and quantified from frozen ground material as in section 26 and the SOD activity was. Samples were diluted to 1 $mg.ml^{-1}$ before a volume of 10 µl protein samples were loaded onto a microtitre plate. To the samples on the plate 20 mM phosphate buffer, 0.1 mM Nitrotrezolium blue choride (NBT), 0.005 mM riboflavin, 10 mM methionine and 0.1 mM (EDTA) was added and made up to a volume of 200 µl using deionised water. The plate was then incubated for 20 minutes at room temperature on a light box. Following the incubation, the samples were spectrophotometrically read at 560 nm. One unit of SOD represented the amount that inhibited NBT photoreduction by 50 %.



## 2.14. A kinetic spectrophotometric assay to determine total Glyoxalase activity

Glyoxalase activity was carried out according to the method of Chakravarty and Sopory (1998). Twenty microliters of the extracted protein from the respective samples were aliquoted into different quartz cuvettes. To each of the protein samples 5 mM potassium phosphate and 0.92 mM reduced glutathione was added and made up to a volume of 2 ml. To initiate the reaction 1.8 mM methylglyoxal was

added to the cuvette followed by observing the increase in absorbance at 240 nm over a period of 2 minutes due to the thioester formation.

### 2.15. A kinetic spectrophotometric assay to determine Glutathione-S-Transferase (GST) activity

The Glutathione-S-Transferase was carried out according to the method of Mannervik (1985). Twenty microliters of the extracted protein from the respective samples were aliquoted into different plastic cuvettes. To each of the protein samples a volume of 980 µl reaction mixture containing 1 mM (CDNB), 1 mM glutathione made up in PBS buffer (pH 6.5) was added. The absorbance was measured at 340 nm for 3 minutes to determine the kinetic rate. Using the extinction coefficient of 0.0096 $\mu M^{-1}$. $cm^{-1}$ the activity was calculated.



### 2.16. Inductively coupled plasma optical emission spectroscopy (ICP-OES) analysis

Plant material of the differentially treated plants were digested for ICP-OES. Frozen ground plant material (150 mg) was inserted into 2 ml Eppendorf tubes followed by the addition of 1 ml of 65 % nitric acid. The resulting solution was then mixed through vigorous shaking on a vortex and then incubated at 90 °C on a heating block for 4 hours. It should be noted that the caps of the Eppendorf tubes have to

properly sealed with Parafilm to make sure the caps do not open as a result of the high temperature. After the digestion the samples were centrifuged to pellet all residual material and the supernatant was transferred to a new tube. A ten times dilution was made in a 5 ml final volume using 2 % nitric acid as a diluent. This was then analysed on the ICP-OES machine.

**2.17. Statistical analysis**

All statistical data was analysed using two –way analysis of variance (ANOVA) and tested for significance using the GraphPAD Prism 5.03 software by applying the Tukey- Kramer test at 5 % level of significance level. All experiments in this study were repeated four times for repeatability and reproducibility purposes.

# Chapter 3
# Exogenous 3,3' Diindolylmethane increases growth of *Brassica napus* L. seedlings by modulating superoxide and hydrogen peroxide content

## 3.1. Introduction

Cruciferous vegetables of the family Brassicaceae include Horseradish, Cabbage, Brussels sprouts and Canola (*Brassica napus* L.). The Brassicaceae family are known to contain indole glucosinolate compounds that are known as glucobrassicins (Naeem *et al*., 2001). Cruciferous plants also contain myrosin cells that contain the enzyme myrosinase. The role of the myrosin cells are to separate the myrosinase enzyme from the glucosinolates which are present in S-cells (Husebye *et al*., 2002). When plants of this family are damaged by action such as being chopped, bruised or chewed by animals or insects, the myrosin and S-cells are damaged allowing the myrosinase enzyme and glucosinolates to interact. The compounds produced from the interaction of the myrosinase enzyme and glucosinolates are made by the plant in an effort to deter animals and insects from further damaging the plant. One of the products of the aforementioned interaction is the compound Idole-3-Carbinol (I3C)

(Wittstock & Halkier, 2002). Under appropriate conditions, two I3C molecules may combine to form a dimer 3,3' diinolylmethane (DIM) (Nachshon-Kedmi *et al*., 2003). DIM is one of the more stable by-products of the glucosinolate breakdown. The role of DIM has been reported in many mammalian systems especially in cancer research (Jin *et al*., 2015; Marques *et al*., 2014), yet very little studies have looked at the signalling roles of DIM within plants. Katz et al. (2015). Reported that exogenous application of I3C inhibits Arabidopsis root elongation by manipulating auxin signalling. That study was able to show that the compounds produced from the breakdown of glucosinolates could have signalling properties in plants. The study presented in this chapter was undertaken to investigate the effect of exogenously applied DIM on the physiological and biochemical characteristics of *Brassica napus* L. seedlings.



## 3.2. Results

### 3.2.1. The exogenous application of 15 µM DIM increases the germination rate of *Brassica napus* L. seedlings

I It is well documented that seed germination and seedling growth are critical stages in the growth of plants (Dodd & Donovan, 1999). The importance of these two stages in particular is due to the increased sensitivity of the plant to abiotic and biotic stress. Due to this increased sensitivity to stress high mortality rates may be

observed leading to decreases in crop yields. Therefore, it was necessary to understand whether DIM could benefit seeds or seedlings during these two stages of growth. Results showed that the rate of germination was increased by ±108 % when DIM was exogenously applied on the *Brassica napus* L. seeds.

**Table 3.1: The effect of DIM on the germination of *Brassica napus* L. seedlings**

| | Control | DIM |
|---|---|---|
| **Germination percentage** | $37 \pm 5^{a}$ | $77 \pm 8^{b}$ |

Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test) values are means ± S.E (N=5)



### 3.2.2. The exogenous application of DIM increases seedlings growth shoot growth

Changes in crop growth is very important as this may affect the productivity and yield of the crop. Abiotic stress, biotic stress and hormones have the ability to decrease plant's growth and reduce yields. It was necessary to understand the effect the exogenous application of DIM had on the *Brassica napus* L. seedling shoot. A ±61 % increase in length was observed in the seedling shoots treated with DIM when compared to the control seedlings refer to figure 3.1.

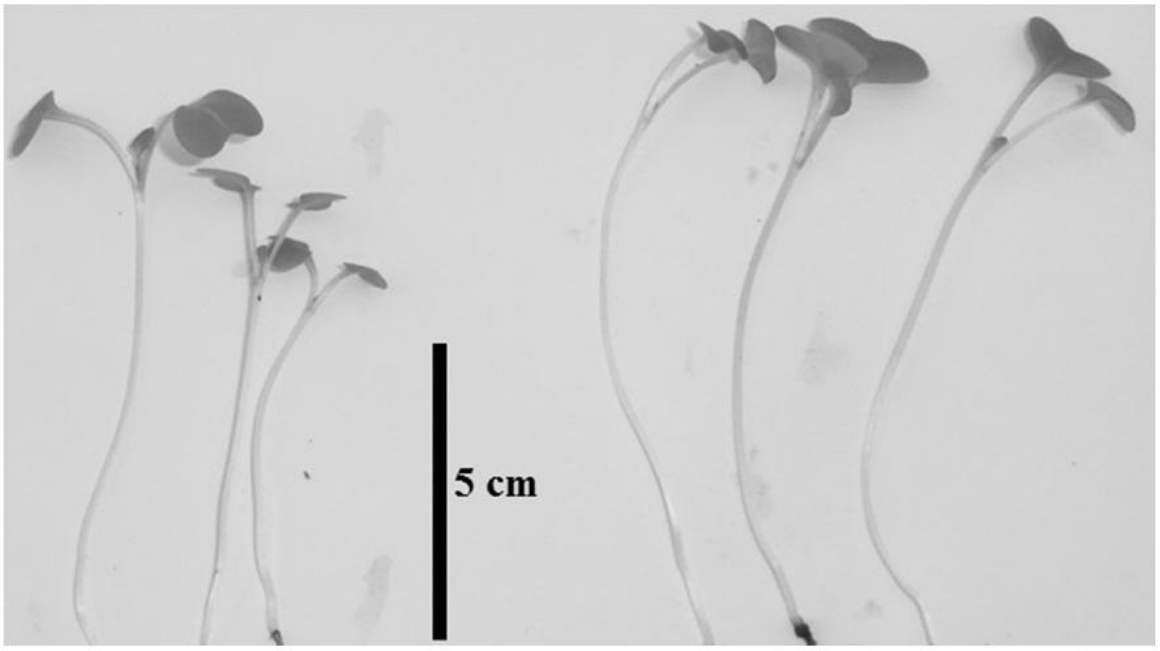


**Figure 3.1: The effect of DIM on the seedling physiology of *Brassica napus* L. seedlings**. The *Brassica napus* L. seedlings were treated with 15 µM DIM for 14 days to determine the effect of the compound on the seeds or seedlings.

### 3.2.3. The exogenous application of DIM increases fresh and dry weight of *Brassica napus* L. seedling shoots

The observation that DIM was able to increase the seedling shoot length prompted us to investigate what effect it may have on the weight of the seedlings. The fresh weight of DIM treated seedling shoots were increased by ±123 % when compared to the control seedlings. A ±64 % increase in dry weight was observed in the DIM

treated seedlings when compared to the control seedlings. The change in biomass is important as this gives us an indication of the seedlings' physiological status.

**Table 3.2: The effect of DIM on the biomass of *Brassica napus* L. seedlings**

| | **Control** | **DIM** |
|---|---|---|
| **Fresh weight** | $32 \pm 4.3^{a}$ | $71.4 \pm 5.7^{b}$ |
| **Dry weight** | $3.26 \pm 0.3^{c}$ | $5.33 \pm 0.23^{d}$ |

Different letters indicate significant differences between means at $P < 0.05$ (Tukey-Kramer test) values are means ± S.E (N=5)



### 3.2.4. The exogenous application of DIM causes an increase in superoxide concentration

It is well documented that ROS can be beneficial to plants growth at low concentrations as well as detrimental to plants at high concentrations. Due to the increases in growth observed in the seedlings treated with DIM it was necessary to quantify the level of DIM in the seedlings, as a possible mechanism for the growth promotion. A ±20 % increase in $O_2^-$ concentration was observed in the DIM treated seedlings when they were compared to the control seedlings.

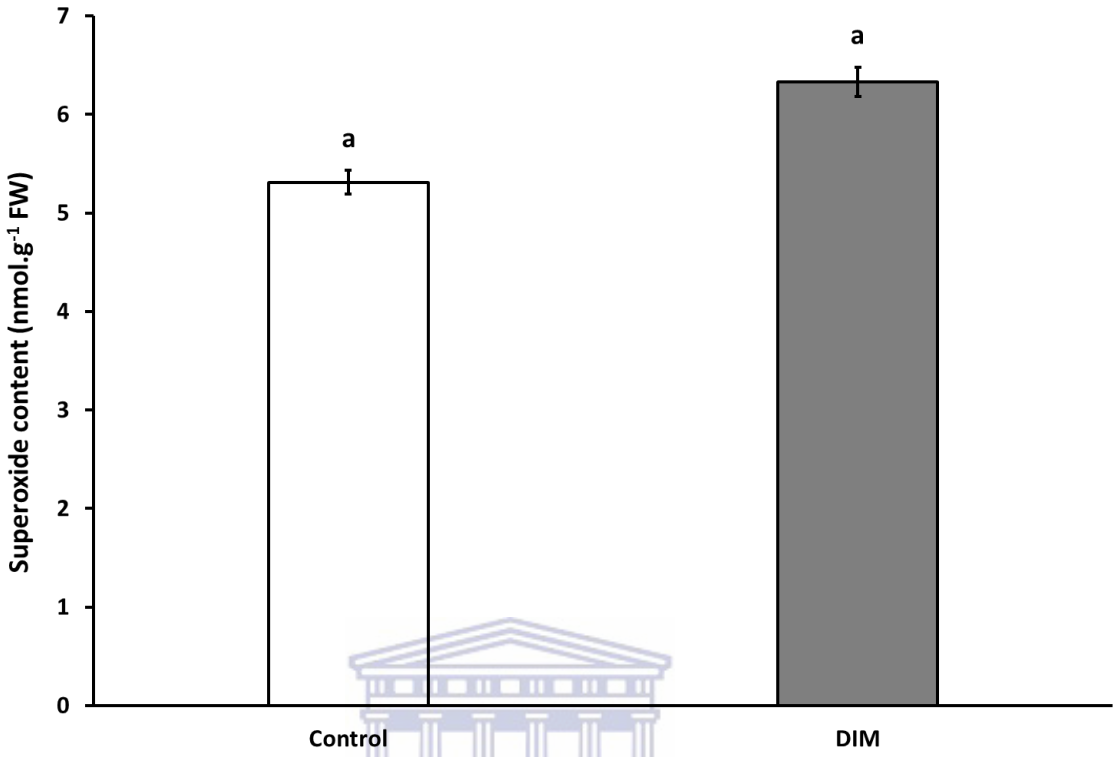


**Figure 3.2: The effect of DIM on the superoxide content in *Brassica napus* L. seedling shoots**. The *Brassica napus* L. seedlings were treated with 15 µM DIM for 14 days to determine the effect of the compound on the superoxide levels in the seedlings. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).

### 3.2.5. DIM causes an increase in hydrogen peroxide in *Brassica napus* L. seedlings

ROS molecules such as $H_2O_2$ have been observed to play critical roles in metabolic process within plants. However, the levels of $H_2O_2$ has to be carefully controlled to avoid oxidative damage occurring in plant tissues. As with $O_2^-$ when the concentrations of $H_2O_2$ are low enough it may perform a signalling role. To determine if $H_2O_2$ played a role in the increase in seedlings growth its concentration

had to be determined. An increase in $H_2O_2$ content of ±49 % was observed in the DIM treated seedlings.

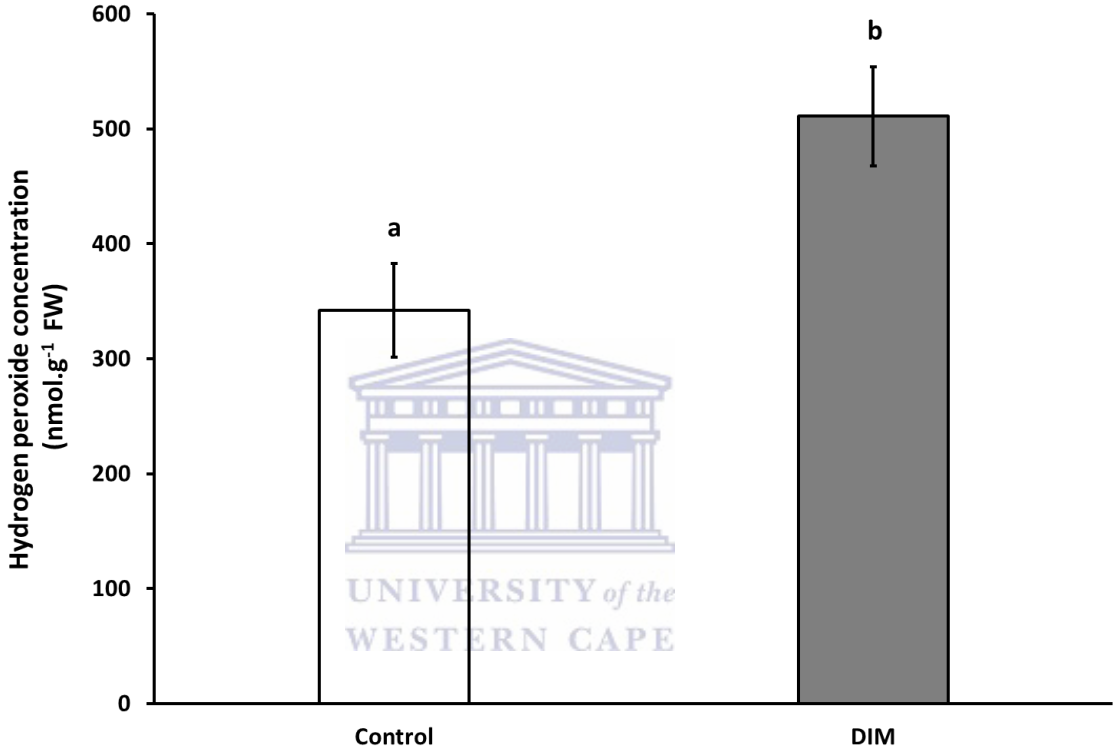




**Figure 3.3: The effect of DIM on the hydrogen peroxide content in *Brassica napus* L. seedling shoots**. The *Brassica napus* L.. seedlings were treated with 15 µM DIM for 14 days to determine the effect of the compound on the hydrogen peroxide levels in the seedlings. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).

### 3.2.6. The exogenous application of DIM does not affect cell viability

Cell viability is an important factor in any organism. Cell viability may be used to assess the health status of an organism. If cells are exposed to agents that decrease cell vitality for a prolonged period, the organism might perish. It was important to assess whether DIM had any negative effects on cell viability. No significant difference in cell death was observed when comparing the DIM and water treated seedlings. Cell viability remained unchanged in the seedlings treated with DIM as no real stress was imposed on the seedlings.

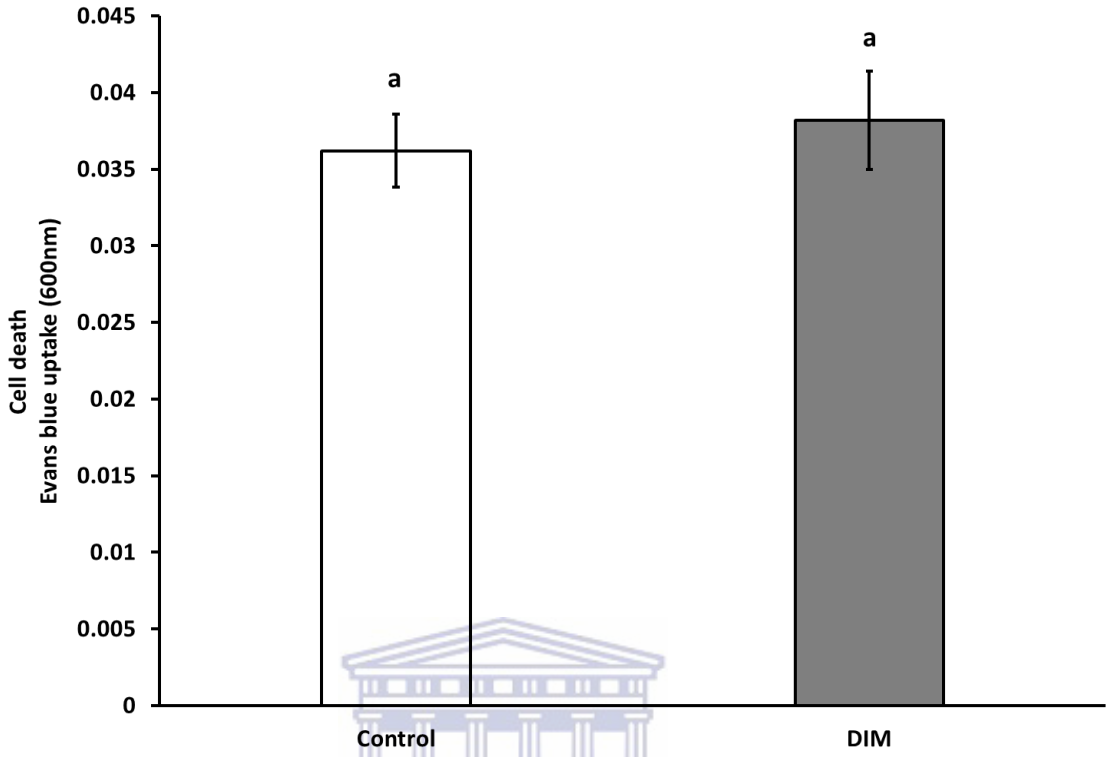


**Figure 3.4: The effect of DIM on the cell viability of *Brassica napus* L. seedling shoots**. The *Brassica napus* L. seedlings were treated with 15 µM DIM for 14 days to determine the effect of the compound on the seeds/ seedlings cell viability. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).

### 3.2.7. The exogenous application of DIM does not affect lipid peroxidation

Lipids are important macromolecules that are important for the formation of certain organelles. One such organ in plants is the cell membrane. Stress, hormones and chemical compounds have the ability to disrupt the cell membrane either directly or through the generation of ROS. The damage done to the cell membrane can be used as an indicator of the stress imposed on the plant. No significant difference in lipid peroxidation was observed when comparing the control and DIM treated seedlings.

The results indicate that DIM did not pose a threat to the seedlings and thus caused no significant damage, when comparing it to the control seedlings. The ROS molecules which under normal conditions cause damage, were controlled by the antioxidant systems.

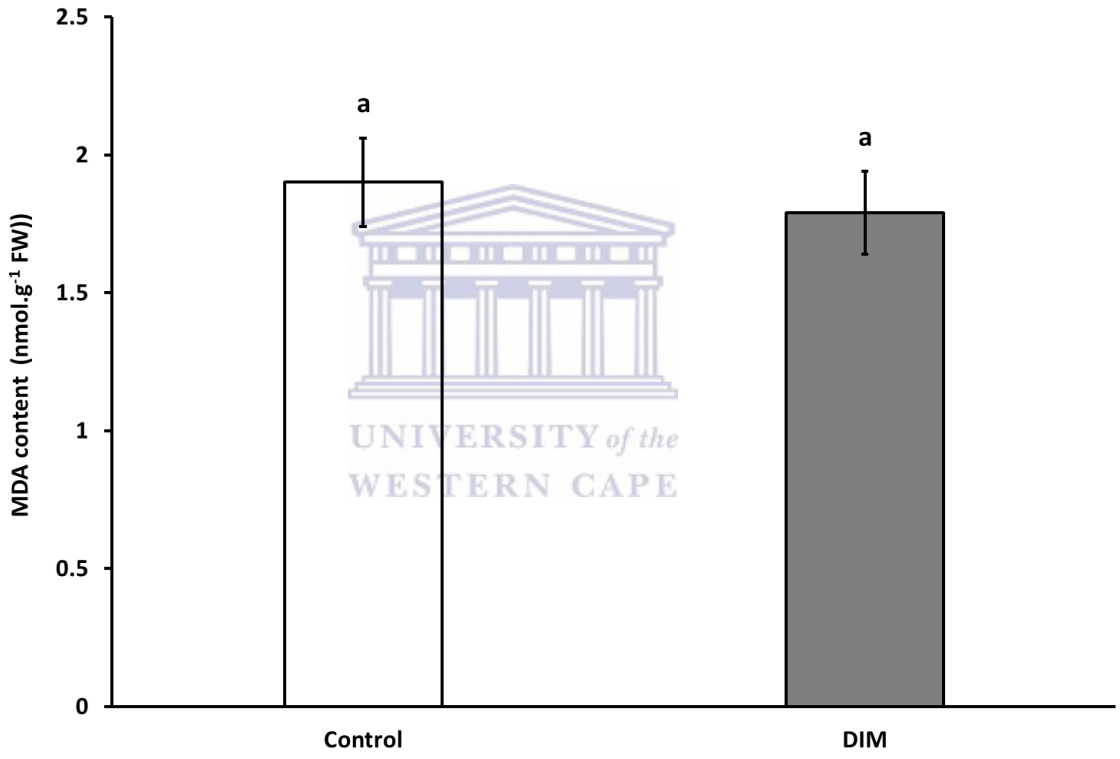



**Figure 3.5: The effect of DIM on the lipid peroxidation in *Brassica napus* L. seedling shoots**. The *Brassica napus* L. seedlings were treated with 15 µM DIM for 14 days to determine the effect of the compound on the MDA levels in the seedlings. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).

### 3.2.8. The exogenous application of DIM leads to an increase in superoxide dismutase activity

Antioxidant enzymes such as SOD play a critical role in maintaining the ROS homeostasis within plants (Lee *et al*., 2007). Damage may occur when there is an imbalance between the generation of ROS and the scavenging ability of the antioxidant enzymes (Jiang & Zhang, 2002). Increases in SOD activity has been observed to improve plant growth under unstressed conditions (Jiang & Zhang, 2002). It was deemed necessary to understand how DIM affected the SOD activity in order to control $O_2^-$ content. An increase of ±412 % in SOD activity was observed when comparing the DIM treated seedlings to the control seedlings.

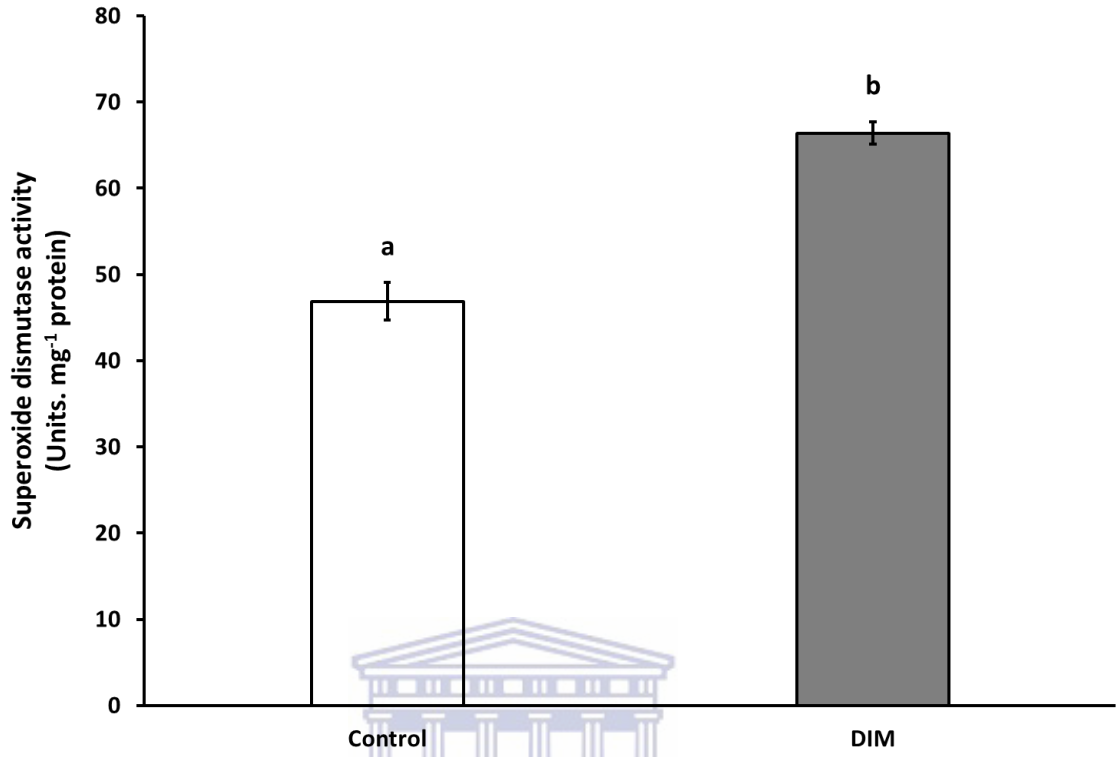


**Figure 3.6: The effect of DIM on the superoxide dismutase activity in *Brassica napus* L. seedling shoots**. The *Brassica napus* L. seedlings were treated with 15 µM DIM for 14 days to determine the effect of the compound on the superoxide dismutase activity in the seedlings. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).

### 3.2.9. DIM increases ascorbate peroxidase activity in *Brassica napus* L. seedlings

When plants undergo stress, it is common to observe an increase in APX activity (Sato *et al*., 2001). Hydrogen peroxide accumulation, which is observed under stress conditions are controlled by enzymes such as APX that convert the ROS molecules into less harmful compounds. Due to the improvement in growth, maybe through the increase in $H_2O_2$, we investigated APX activity. The exogenous application of DIM led to an increase in APX activity of ±23 % when compared to the control seedlings.

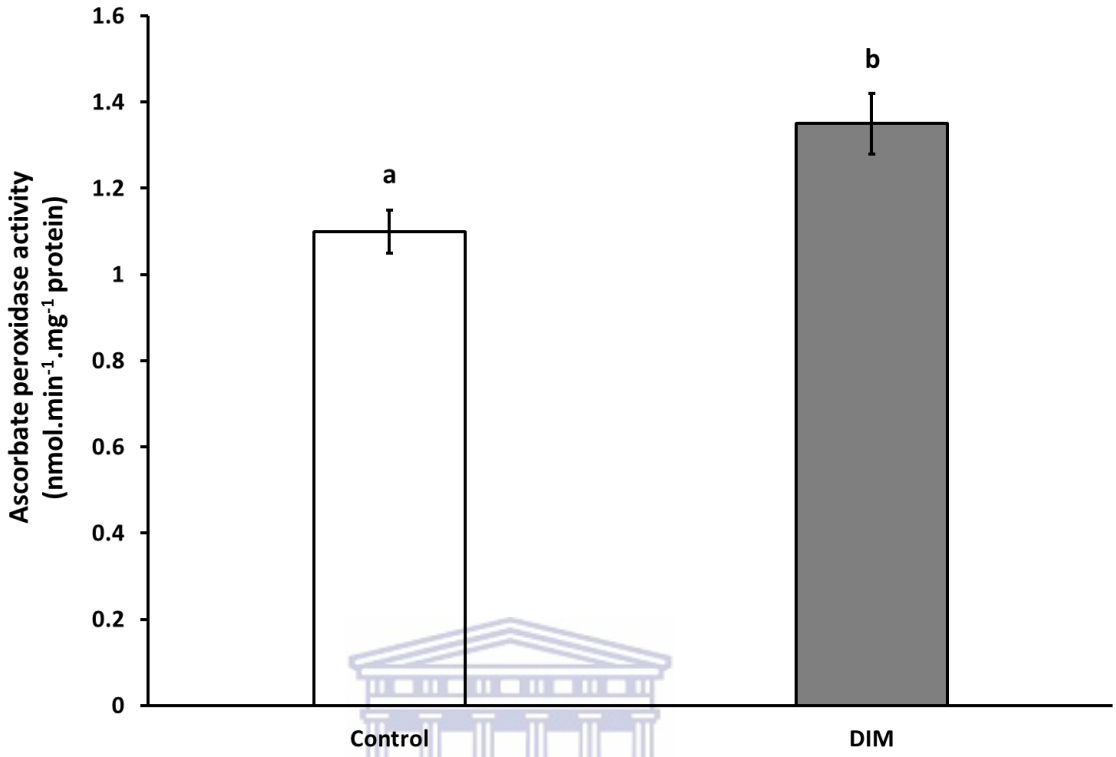


**Figure 3.7: The effect of DIM on the ascorbate peroxidase activity in *Brassica napus* L. seedlings shoots**. The *Brassica napus* L. seedlings were treated with 15 µM DIM for 14 days to determine the effect of the compound on the ascorbate peroxidase activity in the seedlings. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).

## 3.3. Discussion

As stated before herbivore action as well as pathogen attack will cause *Brassica napus* plants to increase their glucosinolate concentrations as deterrents for these threats. As in most biotic and abiotic stresses, when damage is done to plants an increase of ROS molecules ensues. In this study, we investigated whether there was a link between glucosinolate production, ROS production and ROS antioxidant

pathways in *Brassica napus* L. seedlings. DIM which is innately produced in cruciferous plants such as canola is a part of the glucosinolate class of compounds. An increase in seed germination was observed when DIM was exogenously applied to *Brassica napus* L. seeds. A study by Kranner et al. (2010) indicated that ROS accumulation was important for *Pisum sativum* seed germination as well as the growth of the seedlings. Therefore, our hypothesis is that glucosinolates such as DIM could be eliciting minor increases in ROS production thus increasing antioxidant activity, leading to positive seed germination and seed development. One of the positive effects of ROS on seed germination is the breaking of seed dormancy. The results of this study are consistent with a study by Oracz et al. (2007) who observed an increase in germination rate when ROS accumulation increased. The same study hypothesised that ROS may act as cell signalling molecules breaking dormancy. It is clear that ROS plays a critical role in breaking seed dormancy, however the amount of ROS accumulated should not exceed the "signalling threshold" as it would start to cause oxidative damage to the seed or seedlings. *Brassica napus* L. has emerged as an important food source as well as feedstock for biofuels. Due to the number of benefits of this crop, the plant was chosen for subsequent experiments. In pilot studies, different cultivars (Cobbler, Tawriffic, AV Garnet, Agamax and CB Jardee HT) were grown to determine their germination percentage (Results not shown in thesis, Refer to appendix A ). The cultivar with the lowest germination percentage was determined to be AV Garnet. The AV Garnet cultivar was chosen as plant of choice

for this study as it would allow us to observe changes in germination and growth due the exogenous application of DIM. To determine the optimum concentration of DIM, a range of concentrations (0 µM, 2 µM, 5 µM, 10 µM, 15 µM) were applied to seeds and their growth was observed. The optimum concentration was observed to be 15 µM as we observed the highest germination rate and growth at this concentration.

Seedling shoot length was positively affected by the exogenous application of DIM. Indole glucosinolates are sulphur (S) and nitrogen (N) rich compounds which when broken down could provide essential nutrients for the growth of seedlings (Martinez-Ballesta *et al*., 2013). A positive link between glucosinolates and increases in plants and increases in S and N have been reported (Zhao *et al*., 1999). Therefore, changes in S and N in the DIM treated seedlings will be investigated in the near future.

An increase in dry and fresh weight was observed in the seedlings treated with DIM. The increase in dry and fresh weight could be explained by the increase in length observed in the seedling treated with DIM. The improvement in growth and thus weight could be attributed to DIM augmenting the nutrient acquisition of the DIM

treated seedlings. To validate this hypothesis nutrient profiling using Inductively coupled plasma optical emission spectroscopy (ICP-OES) was performed (Chapter 5).

An article by Dickman & Figueiredo (2013) stated that during necrosis cells have no control over apoptotic events yet during programmed cell death (PCD) cell are under direct genetic control. PCD can be used by plants to protect themselves by forcing damaged cells to perform apoptosis before they become necrotic. As stated before no significant difference in cell viability was observed when comparing the DIM and control treated seedlings. From the above-mentioned finding, it is evident that while DIM might promote growth through the production of ROS it does not cause cell death in plants. The exogenous application of DIM was hypothesised to not cause damage to cells, due to DIM being innately produced in cruciferous plants such as *Brassica napus* L. and the plant could identify the increase in DIM as a natural defence to mechanical damage due to herbivore action.



No significant difference in lipid peroxidation was observed when comparing the control and DIM treated seedlings. This observation was expected as no increase in cell death or cell damage was observed in the DIM treated seedlings when conducting the Evans blue uptake assay (cell viability). The hypothesised accumulation of ROS molecules, which had an effect on seedling growth, could have

been adequately controlled by the antioxidant enzymes thus not leading to oxidative damage. The lack of oxidative damage could account for similar cell death and MDA levels in the DIM and control seedlings. A study by Baily (2004) suggested that an increase in ROS levels in seed germination would not always lead to lipid peroxidation and subsequent cell death. To test the hypothesis the ROS and antioxidant levels had to be determined.

A review by El-Maarouf-Bouteau (2008) stated the importance of $O_2^-$ and $H_2O_2$ in plants seed germination and seedling growth. Superoxide was also observed to be involved in root hair formation, cell elongation and cell differentiation (Baily *et al*., 2008). In this study, a slight increase in $O_2^-$ concentration was observed in the DIM treated seedlings. As stated before the DIM compound is produced when plants undergo mechanical damage such as bruising or due to herbivore action. The increase in $O_2^-$ concentration could be due to the plants perception of damage due to the increase in endogenous DIM. This increase in $O_2^-$ could result in ROS accumulation for signalling purposes. Due to the observed increase in $O_2^-$, it was necessary to understand if the increase was due to increased production of the molecule or the down regulation of the SOD enzyme.

The levels of hydrogen peroxide were assessed to understand the effects DIM had on the production of the molecule. In this study, the seedlings treated with DIM showed an increase in $H_2O_2$ accumulation. The finding of this study are consistent with a study by Hara et al. (2013) who observed an increase in $H_2O_2$ content with an increase of glucosinolate concentration. Furthermore, a review by Baxter et al. (2014) stated that ROS molecules such as $H_2O_2$ form a signalling network. Plants use $H_2O_2$ and other ROS molecules to communicate that it is undergoing stress, and thus can mount a defence against the stress in the form of increasing the activity of the antioxidant enzymes (APX. SOD and CAT). Therefore, it should be noted that the increase in $H_2O_2$ was not as high as in an abiotic stress response, but sufficient to play a signalling role. Also, due to the observed increase in $H_2O_2$, it was necessary to understand if the increase was due to increased production of $H_2O_2$ or the down regulation of the APX enzyme.



Superoxide dismutase is a plants' first defence mechanism against ROS molecules that may cause oxidative damage. The SOD enzyme can dismutate $O_2^-$ to produce $H_2O_2$, which is then scavenged by enzymes such as APX and CAT. In this study, an increase in SOD activity was observed in the DIM treated seedlings when comparing them to the control seedlings. From the result observed we concluded that the increase in $O_2^-$ was due to the increased accumulation of the molecule rather than the down regulation of the SOD enzyme. The increase in SOD activity could be a

response to the increase in $O_2^-$ concentration that we observed. The increase in SOD activity would allow for optimal scavenging which could account for the increase in growth due to balance in $O_2^-$ content.

Ascorbate peroxidase in conjunction with SOD and other antioxidant enzymes play a major role in a plants' defence against oxidative damage caused by ROS. Reactive oxygen species is an inevitable by-product of aerobic metabolism but can also be produced as signalling molecules in plants (Sharma *et al*., 2012). A significant increase in APX activity was observed in the DIM treated seedling when they were compared to the control seedlings. The link between glucosinolates such as DIM and APX activity could be explained by the known increase of $H_2O_2$ in response to DIM. Ascorbate peroxidase (APX) requires ascorbic acid for its proper functioning. Bones and Rossiter (1997) suggested that glucosinolates co-localize with ascorbic acid. Therefore, the link between DIM, ascorbic acid and APX will be investigated in the future. The increase in APX activity did however not remove all the $H_2O_2$ from the system that suggests a possible controlling mechanism for $H_2O_2$ levels below the toxic range.

In conclusion, the results of this study suggest that exogenous application of DIM could increase the germination percentage of *Brassica napus* L. seeds. DIM was observed to enhance shoot growth which could have been achieved through the

possible induction of $O_2^-$ and $H_2O_2$. Hydrogen peroxide has previously been observed to play important roles in seed germination and development  Furthermore, the increase in $O_2^-$ and $H_2O_2$ could be for signalling purposes rather than oxidative stress and cell death triggers (no increase in cell death or MDA were observed). The antioxidant enzymes activities (SOD and APX) increased in response to DIM which subsequently was able to adequately regulate the ROS. By regulating ROS accumulation the seed or seedling were able to increase growth without incurring any damage. The findings in this study are important as it is a crucial first step in finding mechanisms to increase crop yields and to tackle problems of food security.

# Chapter 4

# Exogenous 3,3’ Diindolylmethane mediates vanadium stress in *Brassica napus* L. seedlings by modulating antioxidant enzyme activities

## 4.1. Introduction



Anthropogenic activities such as improper irrigation, the use of fertilizers and mining are resulting in the ever-increasing vanadium contamination of our arable soils (Vachirapatama *et al*., 2011; Yang, *et al*., 2011; Yadav, 2010). Vanadium like most heavy metals may cause damage to plants when it is accumulated at high concentrations (Mukherjee *et al*., 2004). The accumulation of vanadium may lead to heavy metal toxicity, which may have an effect on the physiology, biochemistry and overall growth of a plant (Iannone *et al*., 2015; Vachirapatama *et al*., 2011). The physiologic damage can often be observed by the discolouration of the leaves, reduced growth, smaller leave area and poor root formation (Wang & Liu, 1999). The tolerance of any plant to heavy metal stress hinges on the metal uptake, the substances that may complex with the metals and the degree of modification of the antioxidant systems to protect the plant cells from oxidative damage (John *et al*.,

2009). *Brassica napus* L. belongs to the Brassicaceae family and is an important oil seed crop that is used in the production of oil and animal feed (Grispen *et al*., 2006). The Brassicaceae are a family that are able to produce compounds called glucosinolates (Herr & Buchler, 2010). These glucosinolates were observed to be at high concentrations in plants which experiences stress and is hypothesised to play a role in the defence of plants against disease and pests (Herr & Buchler, 2010). The molecule 3,3' Diindolylmethane (DIM) used in this study forms part of the glucosinolate class of compounds. DIM was shown to have signalling roles within animal systems, therefore we hypothesised that it could have signalling effects in *Brassica napus* L. even when vanadium stress is imposed. Therefore, the study presented in this chapter was undertaken to investigate the effect of DIM on *Brassica napus* L. seedlings that were exposed to vanadium stress. To achieve this aim physiological and biochemical assays were employed to understand whether DIM can mediate vanadium stress.



## 4. 2. Results

### 4.2.1. Vanadium toxicity reduces biomass and changes physiological characteristics but is alleviated by the addition of DIM

Scientists use many indicators to assess how well plants cope under different conditions. These indicators include the associated biomass, the leaf area and morphology as well as length (Acosta-Motos *et al*., 2016; Gupta & Sinha, 2009). Some metals at elevated concentrations have the ability to disrupt normal plant metabolism. The disruption in normal metabolism may lead to reduction in plant biomass as well as changes in physiological characteristics such as leaf formation. In this study, when comparing the control seedlings with the seedlings treated with vanadium and the combination of DIM plus vanadium a reduction of ±26 % and ±7 % in length was observed respectively. A marked increase of ±36 % was observed in the DIM treated plants when compared to the control seedling shoots. The DIM treated seedling shoots had a ±84 % increase in length when compared to the vanadium seedlings and ±45 % when compared to those treated with the combination treatment. Furthermore, an increase in dry weight of ±64 % and ±11 % was observed in the DIM and the combination treated seedling shoots respectively when comparing them to the control seedling shoots. A ±16 % decrease in dry weight was observed in the seedlings treated with vanadium when compared to the control seedlings.

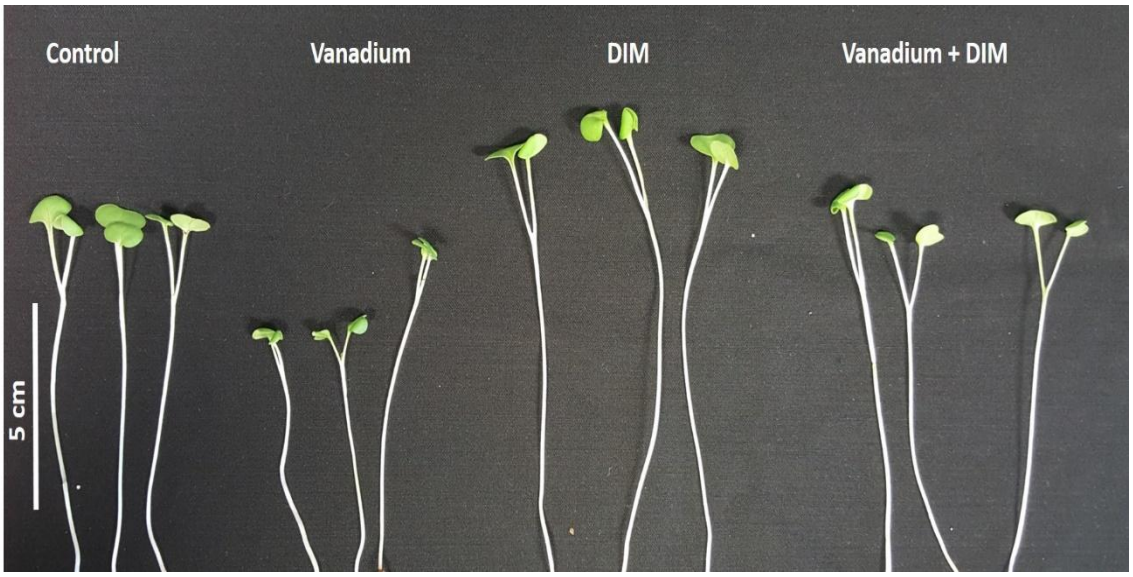


**Figure 4.1: The effect of DIM, vanadium and a combination treatment on the seedling physiology of *Brassica napus* L. seedling shoots**. The *Brassica napus* L. seedlings were treated with 15 µM DIM, 350 µM vanadium and a combination of the two compounds for 14 days to determine the effect of each of the treatments on the seedling shoots.

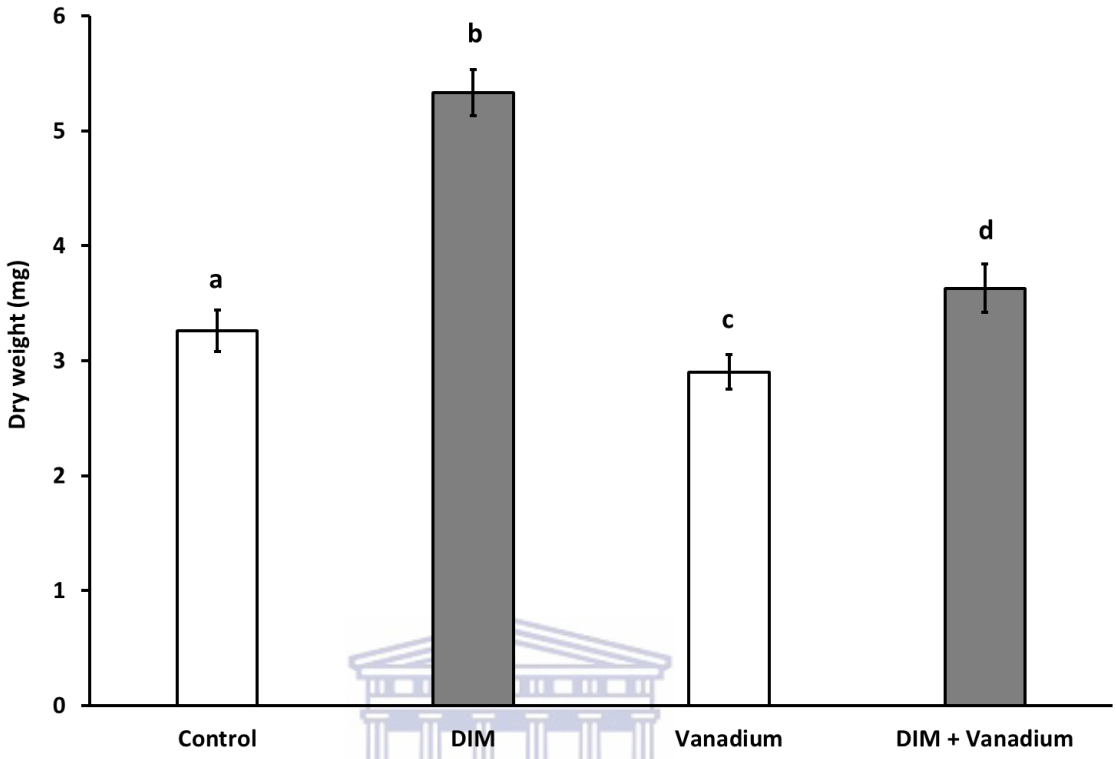


**Figure 4.2: The effect of DIM, vanadium and the combination of the two compounds on the seedling shoot biomass of *Brassica napus* L**. The *Brassica napus* L. seedlings were treated with 15 µM DIM, 350 µM vanadium and a combination of the two compounds for 14 days to determine the effect of each of the treatments on the biomass of the seedling shoots. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).



### 4.2.2. Cell death was exacerbated by vanadium stress but when supplemented with DIM damage was minimized in *Brassica napus* L.

When plants undergo stress they produce ROS and they subsequently alter their metabolic activity as protective mechanisms (Sharma *et al*., 2016). The over production and accumulation of ROS will lead to oxidative damage of DNA, lipids and proteins ultimately resulting in cell death (Sharma *et al*., 2016; Maruta *et al*.,

2012). Due to the relationship between cell death and stress, cell death can be used as an indicator for the damage in plants. Cell death can be analysed by using an Evans blue assay, which works on the principle that the Evans blue reagent will only enter and be retained in dead cells (Baker & Mock, 1994). In this study, the *Brassica napus* L. seeds and seedlings were exposed to vanadium, DIM or DIM and vanadium for a period of 2 weeks (14 days). No significant difference in cell death was observed when comparing the control seedlings (treated with deionised water containing tween-80) with the seedlings treated with DIM. The seedlings treated with vanadium showed an increase in cell death of ±41 % and ±39 % when compared to the control and DIM treated seedlings respectively. The seedlings treated with the combination treatment had a decrease in cell death of ±25 % when compared to the seedlings treated with vanadium. A decrease in cell death of ±25 % was observed in the seedlings treated with the combination treatment when compared to the seedlings just treated with vanadium only.

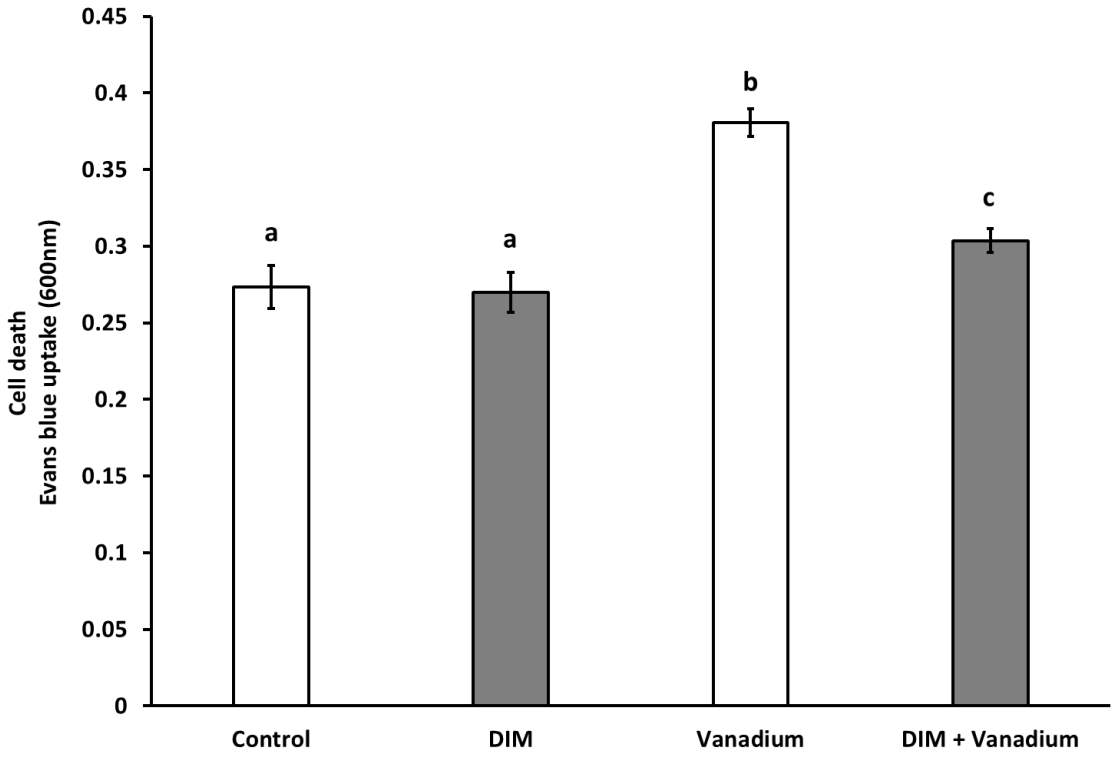


**Figure 4.3: The effect of DIM, vanadium and a combination treatment on cell death within *Brassica napus* L. seedling shoots**. Seedlings were treated with one of four treatments and their effect on cell death was determined. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).

### 4.2.3. Vanadium decreases chlorophyll *a* and *b* but when supplemented with DIM chlorophyll content is increased in *Brassica napus* L. seedlings

Chlorophyll content can be used as a tool to evaluate how heavy metals influence the physiological state of plants (Zurek *et al*., 2014). Heavy metals are known to effect photosynthetic pathways and as a result effect metabolic pathways (Borek *et al*., 2013). A decrease in chlorophyll species could indicate a disruption of important pathways and could indicate sensitivity to the particular metal. An increase in chlorophyll species could potentially mean an enhanced effect on metabolic and

photosynthetic pathways. In this study, a reduction of ±23 % in Chlorophyll a, ±35 % in chlorophyll b and ±25 % in total chlorophyll was observed in the seedlings treated with vanadium when compared to the control seedlings. An increase of ±35 % and ±25 % in chlorophyll a and total chlorophyll respectively was observed in the DIM treated samples when compared to the control seedlings. No significant difference in chlorophyll b was observed when comparing the control and DIM treated seedlings. No significant difference was observed in chlorophyll a and total chlorophyll when comparing the combination treated seedlings to the control seedlings. A decrease of ±21 % was observed in chlorophyll b when comparing the combination treated seedlings to the control seedlings treated with deionised water (containing tween-80). An increase in concentration of ±36 %, ±23 % and ±32 % was observed in chlorophyll a, chlorophyll b and total chlorophyll respectively when the seedlings treated with the combination and vanadium treatments were compared.

**Table 4.1: The effect of DIM, vanadium and their combination on plant chlorophyll (µg.g-1) a and b**

| | Chlorophyll a | Chlorophyll b | Total Chlorophyll |
|---|---|---|---|
| **Control** | 143.36 ± 15.1 [a] | 58.81 ± 3[d] | 202.18 ± 18.4[g] |
| **DIM** | 194.15 ± 16.7[b] | 61.18 ± 3.56[d] | 255.33 ± 20.2[h] |
| **Vanadium** | 112.39 ± 10.45[c] | 38.07 ± 2.6[e] | 150.47 ± 13.06[i] |
| **DIM + Vanadium** | 152.50 ± 11.7[a] | 46.61 ± 2.09[f] | 199.12 ± 13.05[g] |

Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).



### 4.2.4. Vanadium increases lipid peroxidation (MDA) and decreases conjugated dienes (CD) in *Brassica napus* L. but this damage is mitigated with the supplementation of DIM.

There are many compounds which can contribute to disruption of the polyunsaturated fatty acids which form part of the lipid membrane of plants cells. One class of these compounds are ROS molecules (Wahsha *et al*., 2012). The ROS molecules can strip electrons from lipids resulting in damage of the cell membrane. The damage done to the cell membrane can be used as an indicator of ROS production and damage to a perceived stress. The principle of the lipid peroxidation assay hinges around the reaction between thiobarbituric acid and malodialdehyde, the reaction yields a fluorescent product which can be measured using a

spectrophotometer (Sinha & Saxena, 2006). Contrastingly, the concentration of CD decrease when plants undergo damage. In this study, no significant difference in MDA and CD content was observed when the control seedlings were compared to the DIM treated seedlings. The MDA levels displayed in the vanadium and combination treated plants were ±99 % and ±71 % higher respectively when compared to the MDA levels in the control seedlings. The CD content decreased in the vanadium and combination treated plants when compared to the control seedlings. The decrease in CD content were ±16 % and ±7 % for the vanadium and combination treated seedlings respectively. The comparison between the combination and vanadium treated seedlings displayed a ±11 % increase in CD content.

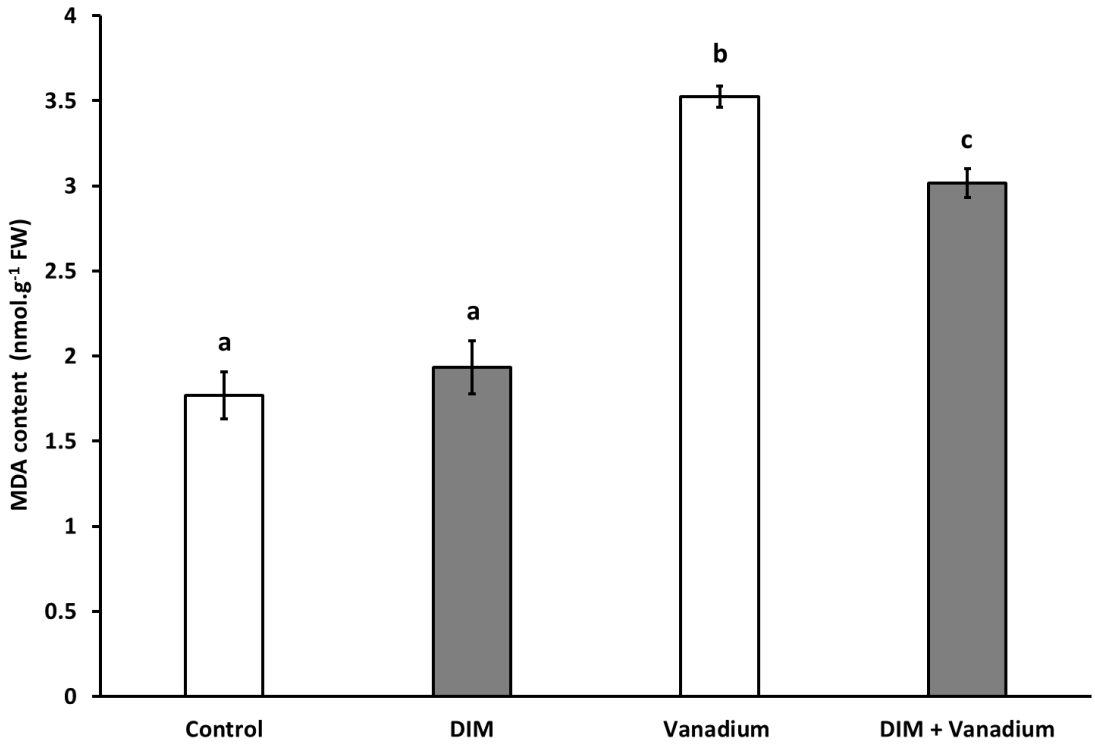


**Figure 4.4: The effect of DIM, Vanadium and their combination on lipid peroxidation within *Brassica napus* L. seedling shoots**. Seedlings were treated with one of four treatments and their effect on MDA levels was determined Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).

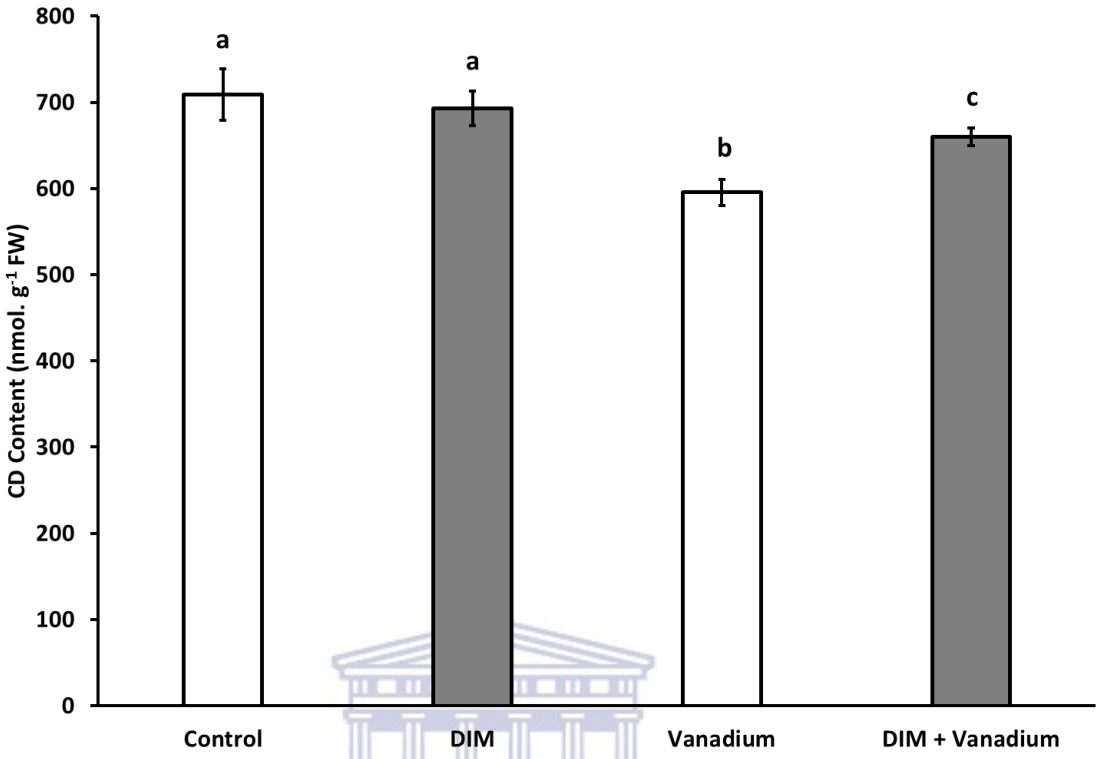


**Figure 4.5: The effect of DIM, Vanadium and their combination on conjugated dienes within *Brassica napus* L. seedling shoots**. Seedlings were treated with one of four treatments and their effect on conjugated diene content was determined. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).

### 4.2.5. The application of DIM and vanadium results in an increase in superoxide content in *Brassica napus* L. seedling shoots

It is widely reported that heavy metal toxicity causes an increase in ROS such as superoxide in plants (Mustafa & Komatsu, 2016). When oxygen is partially reduced then the result is $O_2^-$ (Gill & Tuteja, 2010). The electron transport system which is part of the photosystem l is a major area of $O_2^-$ formation (Gill & Tuteja, 2010). Due to its high reactivity, this compound is able to reduce a myriad of compounds

(Demidchik, 2015). Due to the disruptive nature of $O_2^-$ to normal plant metabolism it was necessary to understand how the addition of vanadium and DIM affected the accumulation of $O_2^-$. In this study, increases of ±23 %, ±146 % and ±87 % in superoxide concentration were observed in the seedlings treated with DIM, vanadium and the combination of the two when compared to the control seedlings. Increases in superoxide concentration was observed in both the vanadium and combination treated seedlings when compared to the DIM treated seedlings. The increases were ±106 % and ±57 % in the vanadium treated and combination treated seedling shoots respectively. A decrease of ±24 % was observed when comparing the combination treatment with the vanadium-only treated plants.

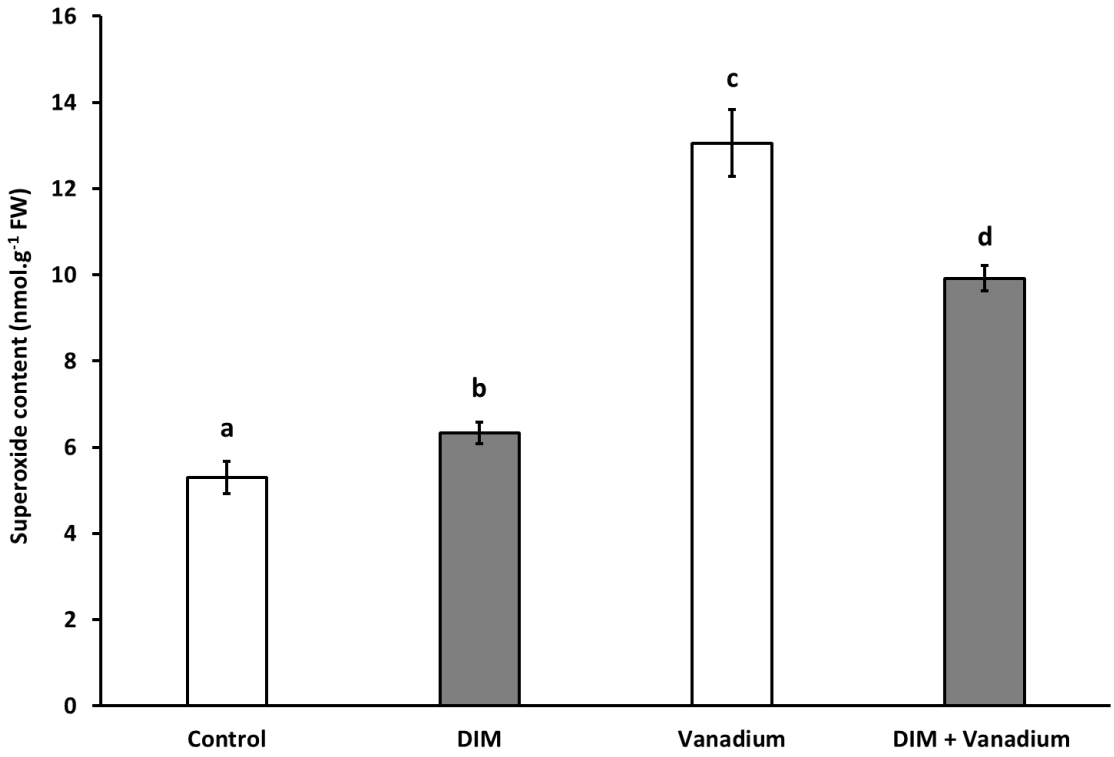


**Figure 4.6: The effect of DIM, vanadium and a combination treatment on the Superoxide concentration within *Brassica napus* L. seedling shoots**. Seedlings were treated with one of four treatments and their effect on Superoxide concentrations were determined. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).

### 4.2.6. Hydrogen peroxide content increases in response to vanadium and DIM treatments but the combination treatments lowers hydrogen peroxide content

The production of ROS is often seen as an indicator of stress and subsequent damage to cellular metabolism and structures (Wang *et al*., 2016; Talaat *et al*., 2015). However, new evidence suggests that these ROS molecules such as superoxide and hydrogen peroxide have signalling roles as well within plants (Wang *et al*., 2016; Demidchik, 2015). It is evident that both superoxide and hydrogen

peroxide production has to be tightly controlled to allow for regulation of plant process without the damaging effects observed when ROS is over accumulated. Therefore, it was necessary to understand how vanadium and DIM impacted the production of hydrogen peroxide. In this study, increases in hydrogen peroxide content in all treated plants (DIM, vanadium and DIM plus vanadium) were observed when compared to the control seedlings. The increases were ±62 %, ±149 % and ±91 % in the seedlings treated with DIM, vanadium and their combination respectively. When comparing the vanadium and combination treated seedlings to the DIM seedlings, increases in hydrogen peroxide concentrations of ±54 % and ±18 % were observed respectively. A decrease of ±23 % in hydrogen peroxide content was observed in the seedlings treated with the combination treatments when compared to those treated with vanadium only.

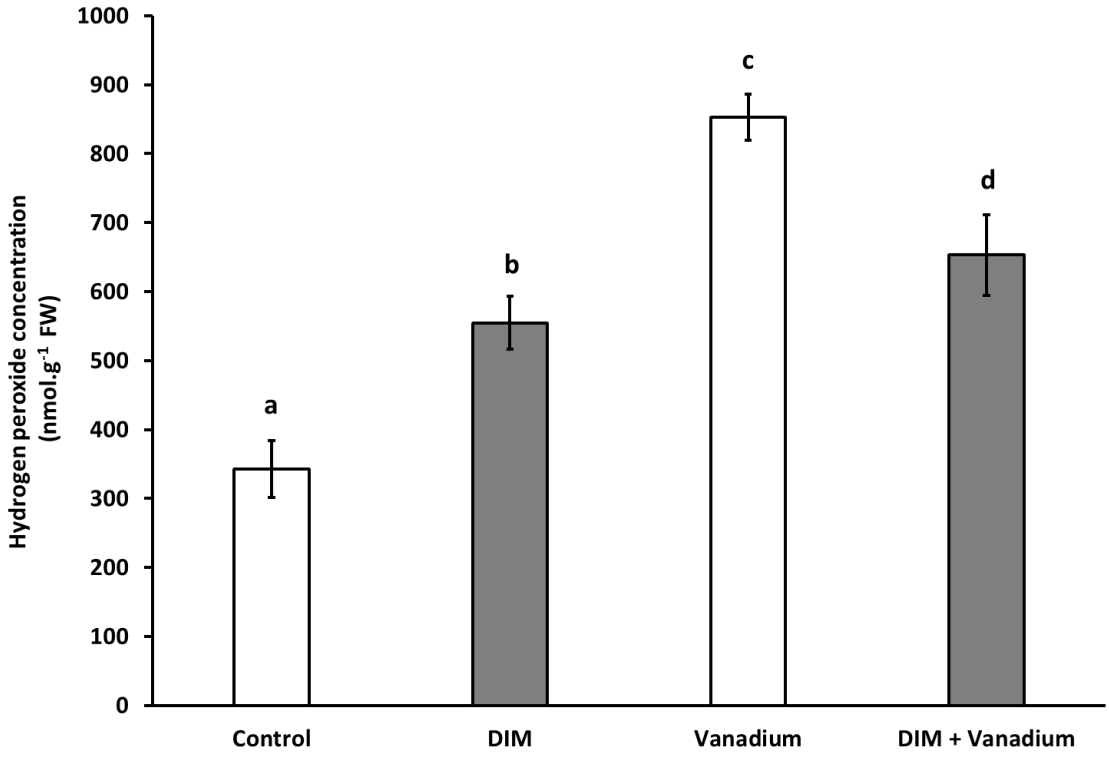


**Figure 4.7: The effect of DIM, vanadium and their combination on Hydrogen peroxide content within *Brassica napus* L. seedling shoots**. Seedlings were treated with one of four treatments and their effect on hydrogen peroxide content was determined. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).



### 4.2.7. Vanadium drastically increases hydroxyl radical concentration in *Brassica napus* L. seedling shoots

The hydroxyl radical is a compound which is produced during normal plant metabolism (Li *et al*., 2016). Hydroxyl radicals are produced at basal levels under normal conditions but increase significantly when plants undergo stress (Bhattacharjee, 2011). The overproduction and subsequent accumulation of hydroxyl radicals may lead to serious damage within plants. Hydroxyl radicals may

react with the cell walls of plants causing severe damage (Schopfer, 2001). Another problem associated with accumulation of hydroxyl radicals, is the oxidation of intracellular compounds (Gill & Tuteja, 2010). Thus, it is evident that hydroxyl radicals are highly damaging molecules, therefore the accumulation of these molecules should be monitored to understand the state of the plants when stress induced treatments are administered. In this study, no significant difference was observed when comparing the hydroxyl radical concentration within the control and DIM treated seedling shoots. Increases in hydroxyl radical concentrations of ±143 % and ±15 % was observed for the vanadium and combination treated seedlings respectively when comparing them to the control seedlings. After comparing the vanadium and combination treated seedlings, a ±53 % decreases in hydroxyl radicals concentration was observed in the combination treated plants.

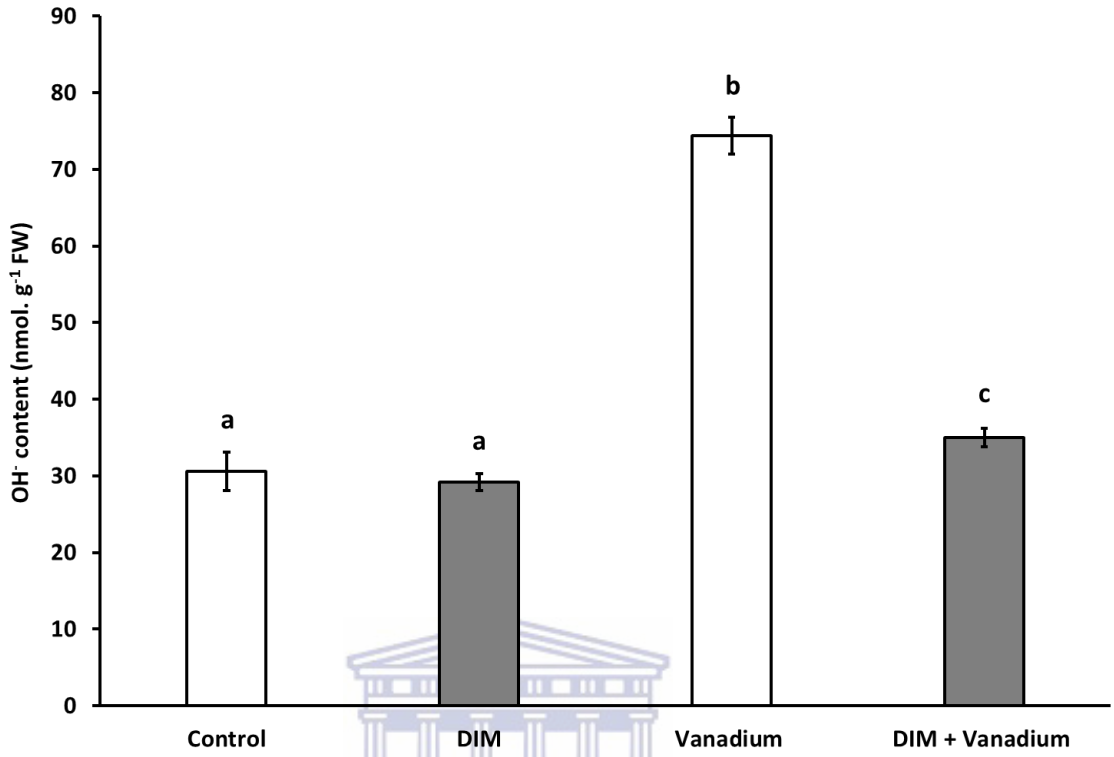


**Figure 4.8: The effect of DIM, vanadium and their combination on Hydroxyl radical concentrations within *Brassica napus* L seedlings**. Seedlings were treated with one of four treatments and their effect on hydroxyl radical concentrations were determined. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).

### 4.2.8. Methylglyoxal levels increases in response to vanadium stress in *Brassica napus* L. seedling shoots

Methylglyoxal is a highly reactive compound which has many deleterious effects such as causing the modification of proteins as well as causing weakness to cellular integrity (Jiménez-Aspee *et al*., 2016). Methylglyoxal may be produced as an intermediate chemical compound during the glycolysis pathway as well as by direct synthesis by the methylglyoxal synthase (Yadav *et al*., 2005). Due to the damaging

nature of methylglyoxal, it is an indicator of the biochemical state of a plant. In this study, no significant difference was observed in the methylglyoxal concentration when the control and DIM seedlings were compared. A significant increase of ±277 % in methylglyoxal concentrations were observed when comparing the seedlings treated with vanadium to the control seedlings. The seedlings treated with the combination treatment also showed an increase of ±75 % when compared to the control seedlings. A decrease of ±54 % in methylglyoxal levels was observed in the seedlings treated with the combination of the two compounds when compared to the seedlings treated with vanadium only.

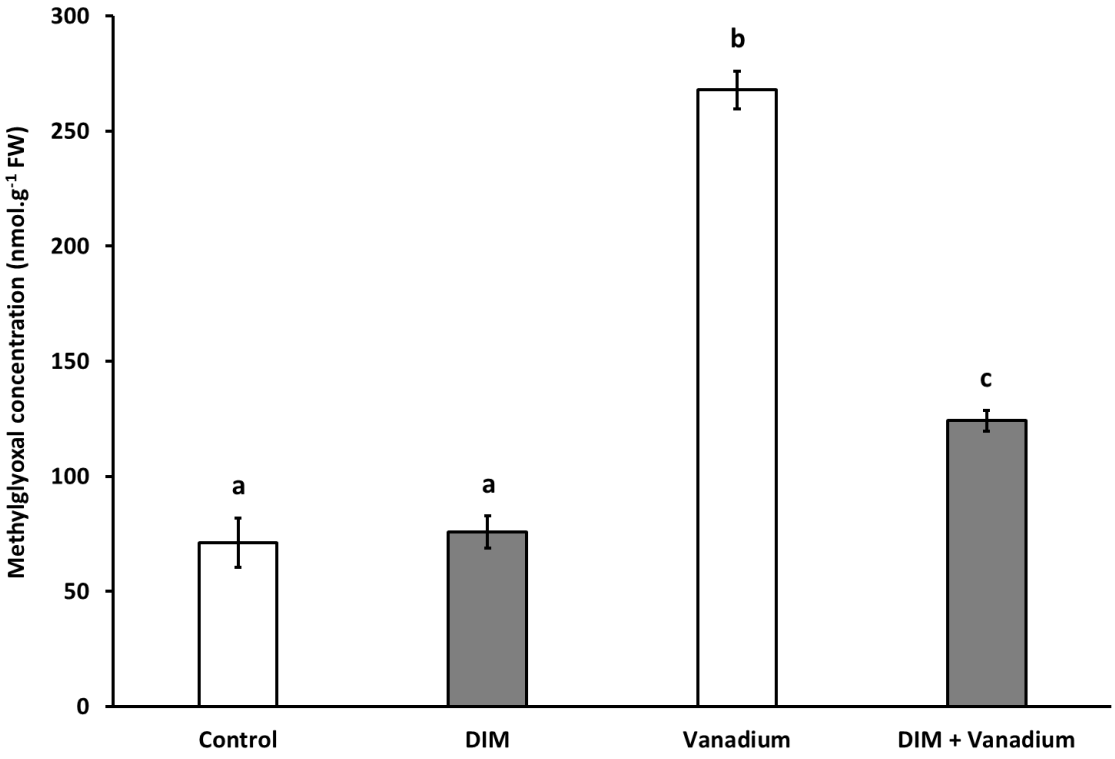


**Figure 4.9: The effect of DIM, vanadium and their combination on methylglyoxal within *Brassica napus* L. seedling shoots**. Seedlings were treated with one of four treatments and their effect on methyglyoxal content was determined. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).



### 4.2.9. Superoxide dismutase activity increases in *Brassica napus* L. in response to DIM and vanadium treatments

Superoxide dismutase is the first line of defence against ROS, which is associated with oxidative damage (Liu *et al*., 2016). Superoxide dismutase has the ability to convert $O_2^-$ into oxygen and $H_2O_2$ (Wang *et al*., 2016). Cu/ZnSODs are generally found within the cytoplasm of organisms that allow for scavenging of superoxide radicals (Wang *et al*., 2016). It was therefore deemed necessary to understand the antioxidant state of the plant in response to the different treatments. In this study,

increases of ±21 %, ±36 % and ±21 % was observed in superoxide dismutase activity in the vanadium, DIM and combination treated seedlings respectively when compared to the control seedlings.. Superoxide dismutase activity was decreased by ±6 % in the combination treated seedlings when compared to the vanadium only treated seedlings.

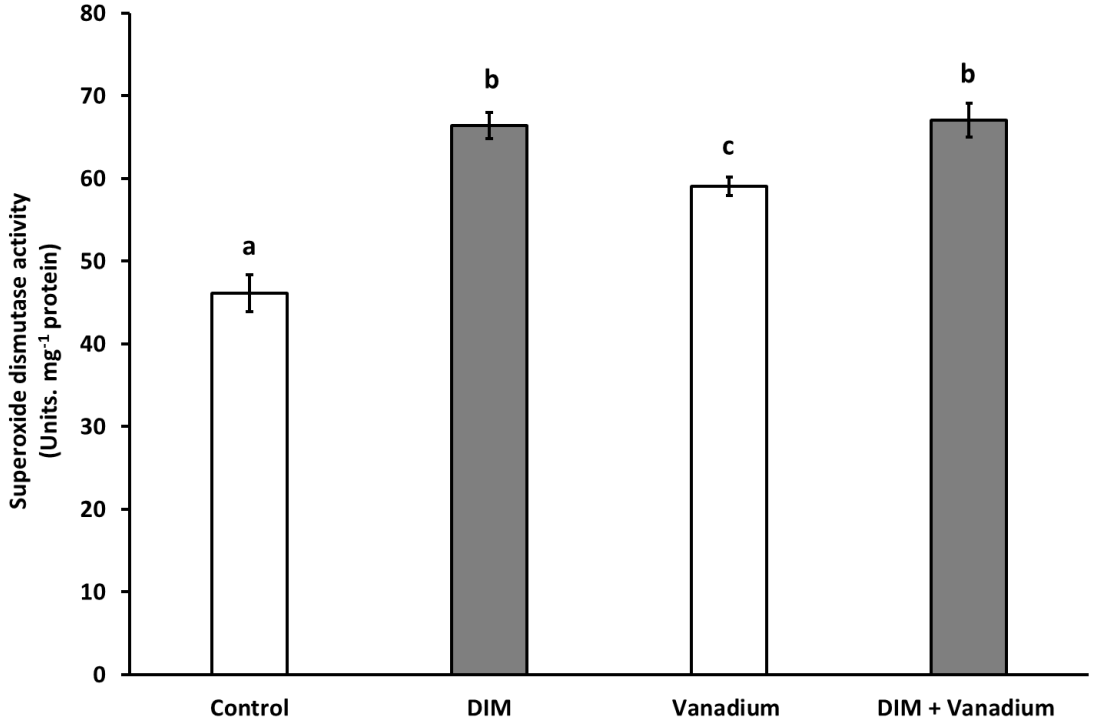


**Figure 4.10: The effect of DIM, vanadium and their combination on SOD activity within *Brassica napus* L. seedling shoots**. Seedlings were treated with one of four treatments and their effect on SOD activity was determined. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).

### 4.2.10. Ascorbate peroxidase activity is modified in *Brassica napus* L. seedling shoots in response to application of Dim and vanadium

The APX enzyme scavenges $H_2O_2$ that is produced during biotic and abiotic stress (Sarowar *et al*., 2005). The APX enzyme uses ascorbate as an electron donor to reduce $H_2O_2$ to water (Ullah *et al*., 2016). It was observed that the APX activity increased in tomatoes exposed to cold stress (Duan *et al*., 2021). In this study, the APX activity was observed in plants exposed to vanadium and DIM to understand how they affected the plant individually and in combination. The APX activity increased in all treatments when compared to the control seedlings. The increases observed were ±19 %, ±69 %, and ±152 % for DIM, vanadium and the combination treatment respectively. The APX activity increased by ±42 % in the vanadium treated seedlings when compared to the DIM treated seedlings. An increase of ±112 % in APX activity was observed when comparing the combination treated seedling shoots to the DIM treated seedling shoots. The APX activity decreased by ±33 % in the vanadium treated samples when compared to the activity in the combination treated seedlings.

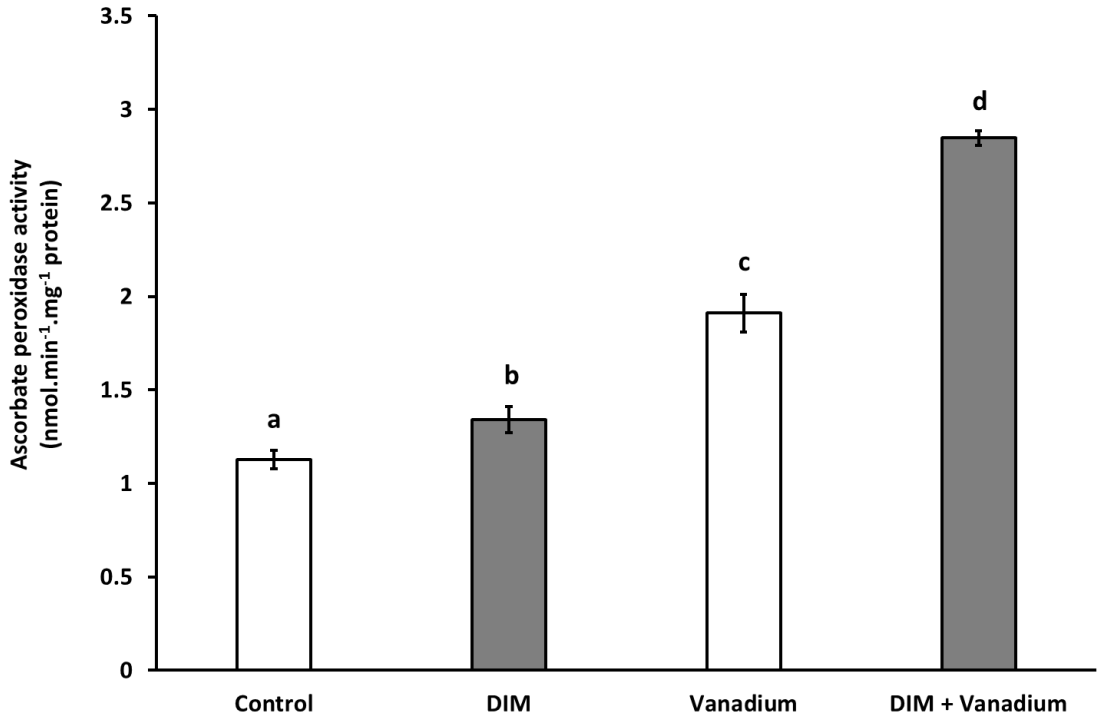


**Figure 4.11: The effect of DIM, vanadium and their combination on APX activity within *Brassica napus* L. seedling shoots**. Seedlings were treated with one of four treatments and their effect on APX activity was determined. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).

### 4.2.11. Vanadium decreases the glyoxalase I activity in *Brassica napus* L. seedling shoots

The glyoxalase I enzyme protects cells against methylglyoxal accumulation and reactive dicarbonyls (Peculis *et al*., 2013). The enzyme reduces the methyglyoxal levels by transforming the methyglyoxal into non-reactive compounds (Xue *et al*., 2011; Thornalley, 2003). The methylglyoxal concentration in living cells need to be tightly regulated as accumulation may cause damage to the proteome and genome (Xue *et al*., 2011). In this study, the glyoxalase I activity was observed to understand

the effect of the addition of vanadium and DIM on the enzyme within the differentially treated seedlings. No significant difference in glyoxalase I activity was observed when comparing the control and the DIM treated seedlings. The vanadium and combination treated seedlings both displayed a decrease in glyoxalase I activity when compared to the control seedlings. The decreases were ±67 % and ±30 % for the vanadium and combination treated seedlings respectively. The vanadium treated seedlings displayed a ±53 % decrease in glyoxalase I activity when compared to the combination treated seedlings.

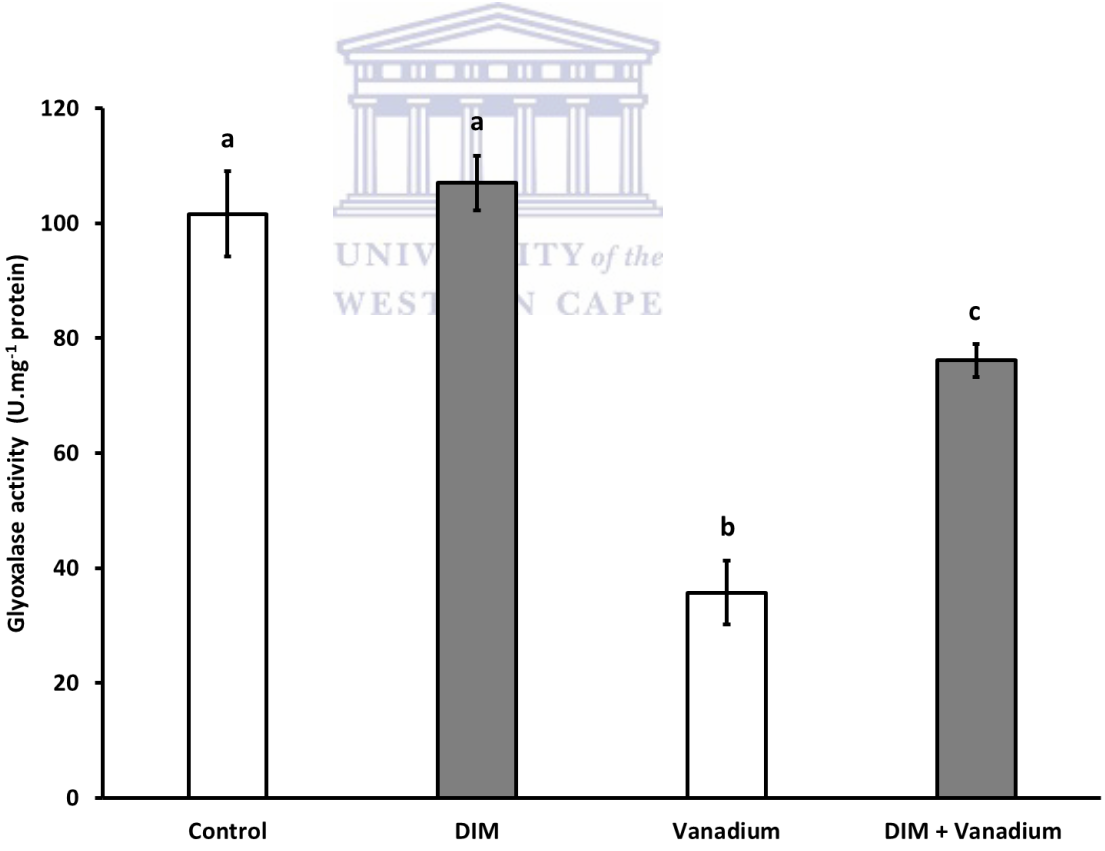


**Figure 4.12: The effect of DIM, vanadium and their combination on glyoxalase I activity within *Brassica napus* L. seedling shoots**. Seedlings were treated with one of four treatments and their effect on glyoxalase I activity was determined. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).

### 4.2.12. The exogenous application of DIM and Vanadium increase Glutathione-S-Transferase activity (GST)

Glutathione-S-Transferase forms part of the defence system used by plants to combat the accumulation of ROS (Roxas *et al*., 2000). The improved activity of enzymes such as GST have been observed to improve growth by improving ROS removal and decreasing oxidative damage. In this study, the GST activity was observed to understand the effect of the addition of vanadium and DIM on the enzyme within the differentially treated seedlings. The DIM treated seedlings displayed a small but significant increase in GST activity when compared to the control seedlings. The vanadium and combination treated seedlings both showed significant increases in GST when compared to the control treated seedlings.

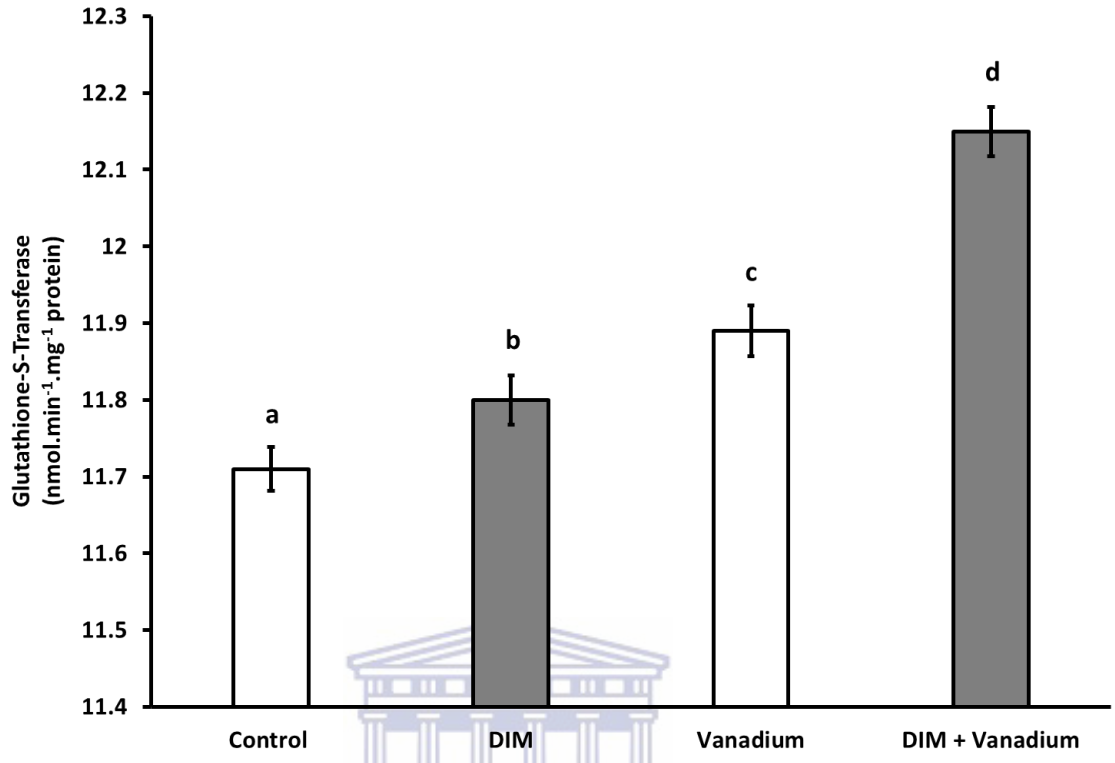


**Figure 4.13: The effect of DIM, vanadium and their combination on the GST activity within *Brassica napus* L. seedling shoots**. Seedlings were treated with one of four treatments and their effect on GST activity was determined. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).

## 4.3. Discussion

The work reported here investigated the mediating effects of DIM on *Brassica napus* L. seedlings, which were exposed to vanadium stress. Experiments were conducted to determine the oxidative state and antioxidant profile of the seedlings when exposed to DIM and vanadium. To be able to understand the oxidative state of the seedlings, when exogenously applying DIM and vanadium, reactive oxygen species such $H_2O_2$, $O_2^-$, hydroxyl radical as well as other toxic by-products such as

methylglyoxal levels had to be observed. Because vanadium stress was imposed on certain seedlings, damage had to be assessed by investigating cell death, lipid peroxidation (MDA), conjugated dienes and chlorophyll levels. The antioxidant enzymatic profile of the seedlings exposed to the different treatments were assessed by observing the changes in activity of the SOD, APX, Glyoxalase I and GST enzymes.

It is well known that under abiotic stress such as heavy metal stress, plants may experience physiological changes such as decreases in growth and biomass. In this study, an expected significant decrease in biomass and length was observed in the seedlings, which were treated with vanadium. The decrease was attributed to vanadium disrupting the normal metabolism of the seedlings thus causing slower and weaker growth. The results of this study are consistent with a study by Pan et al. (2011) who observed that *Zea mays* plant lengths were decreased when antimony was applied to the plants. A study by Gür et al. (2016) observed that with an increase in the heavy metal boron concentration the antioxidant enzymes activates and dry weights of *L. minor* were negatively affected. Heavy metals have a detrimental effect on plants due to their contribution to the generation of ROS molecules and thus causes subsequent oxidative damage.

The reduction in biomass of the vanadium treated seedlings could be attributed to the decrease in nutrient and water uptake due to poor root growth under vanadium toxicity. The decrease in root length and root volume causes a reduction in surface area needed for the acquisition of water and nutrients. The results of this study are consistent with a study by Saco et al. (2013) which observed that at high concentrations of vanadium (above 240 µM) the root and leaf morphology of *Phaseolus vulgaris* L. were greatly reduced. The results are also consistent with a study by Vachirapatama et al. (2011) which observed a decrease in leaf, root and stem lengths of Chinese green mustard when exposed to high concentrations of vanadium.

The third factor that could have influenced the growth of the seedlings could be the effect of the high vanadium concentration on the microbial population in the soil. The microbial population within soils are very important as they have the ability to solubilise nutrients which may then be taken up by plants. It is therefore evident that any disruption to the microbial population could lead to the decrease in plant growth. A study by Xiao et al. (2014) observed that an increase in vanadium concentration in the soil leads to a decrease in microbial enzyme activities as well as reductions in their respiration.

The use of microorganisms and secondary metabolites to promote plant growth has become popular in the last few years (Abhilash *et al*., 2016). In this study, when DIM was applied to the *Brassica napus* L. seedlings, a significant increase in both biomass

and length were observed when compared to the control and vanadium treated seedlings. Furthermore, an increase in biomass was also observed in the seedlings, which were treated with DIM. The results of this study are consistent with a study by Pal et al. (2007) which showed that an increase in root and shoot length of *Oryza sativa* was observed when DIM derivatives were applied in a hydroponic experiment. The results observed in this study were also consistent with a study by Kumar et al. (2016) who observed an increase in biomass production in *Hordeum vulgare* L. when 10 $mg.L^{-1}$ Tricyclazole (TCZ) was applied. Tricyclazole is a fungicide which was thought to induce stress in barley but when low doses were administered plants experienced increases in growth, length and biomass. The increases in growth was attributed to TCZ inducing stress resistance by augmenting the activity of enzymatic and non-enzymatic antioxidant capacities. Due to DIM increasing the ROS in this study, it could also be inducing stress resistance in the seedlings, resulting in increases in the length and biomass. An increase in length was observed when the combination treated seedlings were compared to the vanadium treated seedlings. A possible reason for the seedlings performing better could be that DIM can reduce the uptake of vanadium by the seedlings. To test this hypothesis inductively coupled plasma optical emission spectroscopy had to be performed to understand how much vanadium entered the plants in the different treatments (Chapter 5).

When a biological cell stops performing its specific function and loses cell integrity it undergoes apoptosis and ultimately cell death occurs (Lam *et al*., 1999). When plants undergo abiotic stress, ROS concentrations increase resulting in the disruption of metabolic pathways as well as increasing oxidative damage, and the subsequent damage may cause cells to perish. The amount of cell death observed can be an indicator to assess the amount of damage a particular stress is causing to a plant. The principle of the Evans blue assay hinges around the fact that the Evans blue dye cannot enter intact cells, so an increase in Evans blue uptake would indicate damage to the cell membrane and essentially the cell. In this study, the cell death observed in the seedlings treated with vanadium were significantly higher than the control and DIM treated seedlings. This observation was expected because when the seedlings were treated with a heavy metal such as vanadium oxidative damage to cell membrane, DNA and lipid damage through the over production of ROS may occur. The observation of this study were consistent with a study done by Iannone et al. (2015) who observed a similar increase in cell death when cadmium was administered to tobacco plants. The results are also consistent with a study by Basset and Matsumoto (2008) which observed a significant increase in cell membrane damage when the plants were treated with aluminium. To determine the cause of the deterioration of the cell membrane and the increase in cell death the homeostasis of the plant cells had to be observed. This included monitoring the production of ROS and the scavenging ability of the antioxidant enzymes. The DIM

treated seedlings exhibited the same level of cell death as the control and a reduced level of cell death when compared to the seedlings treated with vanadium only. It should be noted that DIM does increase ROS but not to levels were oxidative damage occurs as observed in the vanadium treated seedlings. Thus, a lower cell death would be expected in the DIM treated seedlings as there is less oxidative damage to the cell membranes. A reduction in cell death was observed when comparing the combination treated seedlings to the vanadium treated seedlings. Taking into consideration the decrease in cell death coupled with the increase in growth could indicate that the seedlings were benefiting from the application of DIM and somehow this agent was mitigating some of the stress caused by vanadium. One of the possible reasons could be the priming affects that DIM may induce. DIM concentrations increase when certain plants are physically damaged. Therefore, the seedlings may be increasing their antioxidant activities to compensate for the ROS associated with the assumed stress by the DIM release. Thus, when the stress of vanadium is imposed, the seedlings are better equipped to deal with the stress resulting in better growth and less cell death.



A difference in green colour intensity were observed in the seedlings treated with the different treatments. The vanadium treated seedlings also showed slight yellowing. The suggested reason for the yellowing could be attributed to the chlorophyll production being hindered or the destruction of the chlorophyll present.

The phenomenon whereby a reduction of chlorophyll species is observed is call chlorosis (Hörtensteiner & Krautler, 2011). To ascertain whether the proposed hypothesis was true, a chlorophyll assay had to be performed. A drastic decrease in Chlorophyll species (a and b) were observed in the seedlings treated with vanadium. The findings of this study are consistent with a study done by Zengin and Munzuroglu (2005) which observed that increasing concentrations of lead decreases the chlorophyll species. In the same study Zengin and Munzuroglu (2005) used proline as a stress indicating amino acid and found that under increasing lead concentrations plants increased there proline levels, which indicted increased stress experienced by the plants. It was interesting to note that the findings of our study were not only in agreement with studies applying metals to plants but also to other abiotic stresses such as salt. A study by Taïbi et al. (2016) showed that by adding salt in excess of 50 mM on *Phaseolus vulgaris* L., a decrease in chlorophyll a, b and total carotenoids were observed. The proposed reason for the decrease in chlorophyll in that study was the activation of chlorophyllase as well as the inhibition of chlorophyll synthesis. The proposed reason for the decrease in chlorophyll species in the vanadium treated seedlings in this study is the over production and accumulation of $O_2^-$ and $H_2O_2$ molecules causing oxidative damage to Photosystem I and chloroplasts respectively. Chlorophyll a, b and total carotenoids were observed to be the highest in the seedlings treated with DIM. The increase in chlorophyll could be due to the increased production in chlorophyll by the enhanced ROS

signalling. The increase in the activity of the antioxidant enzymes (APX and SOD) when DIM is applied would allow for improved ROS scavenging thus protecting the photosystems and subsequently chlorophyll from oxidative damage (as observed in Chapter 3). DIM could also be inhibiting the chlorophyll degrading enzymes chlorophyllase resulting in seedlings with more chlorophyll. This hypothesis was observed in a study by Shi et al. (2016) who observed a decrease in chlorophyllase activity when nitric oxide was exogenously applied, thus a higher chlorophyll content was observed in those plants. The seedlings treated with the combination of DIM plus vanadium showed an increase in all species of chlorophyll when compared to the seedlings treated with only vanadium. It should also be noted that when DIM was exogenously applied the decrease in chlorophyll due to vanadium stress was reverted back to control levels. The proposed reason for the improvement in chlorophyll concentrations include; DIM was able to induce better antioxidant potential in the seedlings thus protecting the chlorophyll and the associated organelles from oxidative damage. Zhu et al. (2004) observed that silicone was able to alleviate an abiotic stress resulting in decreased damage and increased chlorophyll concentrations. The study by Zhu et al. (2004) observed that the silicone induced higher antioxidant enzyme activates which lead to increased protection against oxidative damage.

Taking into consideration the changes in growth and chlorophyll content of the seedlings in response to the different treatments, a lipid peroxidation test was deemed necessary to understand the damage of the vanadium stress imposed on the seedlings. Lipid peroxidation is termed as the change in structure and function of a membrane (Yadav, 2010). As previously stated when ROS molecules are over produced and accumulated these molecules may cause oxidative damage and disrupt polyunsaturated fatty acids which is a component of membranes (Li *et al*., 2013; Verma & Dubey, 2003). A TBARS assay was used to determine the malondialdehyde (MDA) concentration, which is an estimation of lipid peroxidation. The MDA levels present in the vanadium treated seedlings were higher than in any other treatment. The high concentration of MDA was an indication that a large amount of lipid peroxidation and thus damage occurred. This was further echoed by the vanadium treated seedlings, which showed the lowest CD content. The damage in the vanadium treated seedlings were attributed to the uncontrolled production of $O_2^-$ and $H_2O_2$, which caused oxidative damage due to the vanadium stress. The results obtained by this study are in concurrence with a study by Li et al. (2013) who observed an increase in lipid peroxidation in *Hibiscus cannabinus* L. when exposed to cadmium stress. That same study showed that $H_2O_2$ concentrations significantly increased in the cadmium treated seedlings that would provide evidence to the fact that ROS molecules cause lipid peroxidation. The concentration of MDA was statistically the same when comparing the DIM and control seedlings. The lipid

peroxidation results coincided with the CD content results as no significant difference in the CD content was observed. This observation could mean that the stress imposed by DIM and control seedlings were the same, and the effects imposed on the two treatments were similar. Although an increase in ROS molecules ($O_2^-$ and $H_2O_2$) was observed in the DIM treated seedlings, no damage was observed due to the level of ROS being high enough for hypothetical signalling but low enough to not cause oxidative damage. A reduction in MDA concentration was observed when comparing the DIM plus vanadium to the vanadium only treated seedlings. As the MDA concentration decreased, we expected that the CD content should increase, and this phenomenon was observed in the combination treated seedlings. The reduction in MDA and thus lipid peroxidation was attributed once again to DIM alleviating the vanadium stress by inducing the antioxidant mechanisms (SOD, APX and CAT) and reducing oxidative damage. DIM might be able to induce the antioxidant mechanisms by slightly increasing the ROS molecules at an early stage in the plants' development thus priming the plant for true stressful situations.



The Foyer-Halliwell- Asada pathway states that superoxide ($O_2^-$) is the first ROS molecule produced in plants (Yadav, 2010). $O_2^-$ like other ROS molecules are produced at low concentrations to allow for signalling events within plants. However, when plants are exposed to stresses such as heavy metal toxicity, the levels of $O_2^-$ increases to toxic levels which subsequently increases the production of

other ROS molecules as well as causing downstream oxidative damage. In this study, it was observed that the seedlings treated with vanadium had the highest $O_2^-$ content when compared to the other treated seedlings. A proposed reason for the large increase in $O_2^-$ content was the up-regulation of the synthesis of $O_2^-$ in an effort for the plant to signal that it is under stress from the applied vanadium. The second reason was that vanadium played a role in down regulating the superoxide dismutase enzyme (SOD) which enables the removal of the $O_2^-$, thus resulting in an accumulation of this ROS molecule. A slight increase in $O_2^-$ was observed in the DIM treated seedlings when compared to the control seedlings. DIM is released as a deterrent when certain plants undergo mechanical damage such as herbivoric action by animals. The increase in $O_2^-$ by DIM could be for signalling purposes and be attributed to the seedlings assuming they are undergoing mechanical damage due to the increase in DIM concentrations. A decrease in $O_2^-$ content was observed when comparing the combination seedlings and those treated with vanadium only. The decrease in $O_2^-$ content was attributed to the DIM in the combination treatment, which possibly promoted the SOD activity which resulted in more efficient scavenging of $O_2^-$. As was proposed before, the increase in the concentration of DIM could be giving the seedlings a pseudo sense of mechanical damage thus promoting an increase in antioxidant enzymes such as SOD. A journal article by León et al. (2000) stated that when plants undergo wounding, ROS concentrations increase for

signalling purposes and that the system becomes stimulated to further oxidative bursts.

The conversion of $O_2^-$ by the SOD enzyme results in the formation of $H_2O_2$ and oxygen (Gill & Tuteja, 2010). Due to the relative long half-life and stability, $H_2O_2$ is capable to move large distances from its point of origin (Gill & Tuteja, 2010). These characteristics make it an ideal signalling molecule in plants. However, in the same way as other ROS molecules, when the $H_2O_2$ levels increase to high levels, it may cause modification to certain enzymes as well as cause oxidative damage. From the results obtained from the lipid peroxidation and chlorophyll assays we observed that certain seedlings experienced more damage than others. To determine the cause of this damage, ROS assays such as for $H_2O_2$ content had to be performed. In a similar trend to the $O_2^-$, the $H_2O_2$ levels were observed to be the highest in the vanadium treated seedlings. One reason for the sudden increase in $H_2O_2$ was the increase in dismutation of $O_2^-$ by the SOD enzyme, which produced more $H_2O_2$. $H_2O_2$ may be formed for signalling purposes to relay a message that the plant is undergoing vanadium stress, but through consistent signalling the production levels may increase and cause accumulation, which further results in higher $H_2O_2$ concentrations. A study by Schützendübel et al. (2001) observed an increase in $H_2O_2$ content in cadmium treated plants which coincide with the finding of the current study. Nonetheless, in our study an increase in $H_2O_2$ was observed when comparing

the seedlings treated with DIM to the control. One proposed mechanism through which DIM might have increased $H_2O_2$, is by up-regulating the $O_2^-$ concentrations which was then further converted into $H_2O_2$ by the SOD enzyme. It should be noted that the increase in $H_2O_2$ levels were below levels which would induce oxidative damage. A study by Sun et al. (2016) observed that when $H_2O_2$ levels were slightly increased in cucumber, its growth was increased and its antioxidant abilities were improved. The results of this study are consistent with the result in Sun et al. (2016) as we also observed improvement in growth and antioxidant abilities when DIM increased $H_2O_2$ concentrations. A decrease in $H_2O_2$ content was observed when comparing the combination treated seedlings to those treated with vanadium only. A proposed reason for the decrease in $H_2O_2$ content was attributed to the DIM in the combination treatment promoting the induction of APX activity resulting in more efficient scavenging of $H_2O_2$ and thus a reduction in its accumulation. To further corroborate the above mentioned statement the APX activity was observed to increase in the combination treated seedlings. The amount of $O_2^-$ present in the vanadium treated seedlings were greater than that present in the seedlings treated with the combination treatment. Taking into consideration the fact that $O_2^-$ is dismutated to $H_2O_2$ it should be logical that the vanadium treated seedlings would contain higher levels of $H_2O_2$.

The hydroxyl radical (•OH) has been observed to have an effect on cell wall loosening and has impacts on elongation and growth (Schopfer, 2001). The same study suggested that •OH may be generated from $H_2O_2$ and $O_2^-$. As stated before, •OH is highly reactive and may damage DNA as well as cause oxidative damage (Richards *et al*., 2014; Chen & Schopfer, 1999). In our study, the seedlings treated with vanadium had the largest increase in •OH content. This observation should be expected as the increase in $O_2^-$ and $H_2O_2$ concentrations in the vanadium treated seedlings would allow for increased production of •OH. Due to the damaging nature of •OH, the damage observed in the vanadium treated seedlings can be attributed to the high concentrations of •OH present in the seedlings and to a lesser extent due to $H_2O_2$ and $O_2^-$. No statistical difference was observed between the control and DIM treated seedlings. This observation indicated that •OH was not increased when DIM was exogenously applied. One would expect an increase in •OH concentration in the DIM treated seedlings due to the increase in $O_2^-$ and $H_2O_2$ as seen in the vanadium treated seedlings. The major difference in the two treatments were the level of increase of the two ROS species. The ROS levels in the DIM treated seedlings only slightly increased when compared to the vanadium treated seedlings. We proposed that the ROS produced in the DIM treated seedlings were well in range of the "oxidative window" whereas the ROS in the vanadium treated seedlings were not. A drastic decrease in •OH concentration was observed in the combination treatment when compared to the vanadium-only treated seedlings. When comparing the ROS

production in the vanadium and combination treated seedlings, we observed significantly lower $O_2^-$ and $H_2O_2$ levels in the combination treated seedlings. This lower concentration of $O_2^-$ and $H_2O_2$ would lead to lower levels of •OH in the combination treated seedlings. The decrease in •OH concentration coincides with the decrease in MDA concentration in the combination treated seedlings.

Methylglyoxal (MG) is an intermediate of the glycolysis pathway within eukaryotic cells (Yadav *et al*., 2005). MG may also be produced by an enzyme called MG synthase (Martins *et al*., 2001). The accumulation of MG may cause toxic effects such as the inhibition of cell proliferation as well as modification and degradation of proteins (Ray *et al*., 1994). Observing the decrease in growth and increase in damage in certain seedlings in this study, it was necessary to investigate the levels of methylglyoxal under the different treatments. The vanadium treated seedlings were observed to have an increase of more than 1.5 times the levels found in the seedlings treated with other treatments. A study by Yadav et al. (2005) observed that under abiotic stress methyglyoxal levels increased within roots and leaves of *O. Sativa*, *N. tabacum* and *B. juncea* that is consistent with what was observed in our study. The proposed reason for the increase in methyglyoxal concentrations within the vanadium treated seedlings in this work could be due to the increase in glycolysis under stressful conditions. Glyoxalase the enzyme that controls the degradation of methyglyoxal might be inhibited in the presence of vanadium stress

resulting in an increase in methyglyoxal. To determine whether this hypothesis was true, a glyoxalase I assay had to be performed. The methyglyoxal levels observed in the DIM and control treated seedlings were statistically the same. No increase in methyglyoxal was observed in the DIM treated seedlings because DIM did not induce stress in the plants thus glycolysis would remain unchanged. The combination treated seedlings showed a major decrease in methyglyoxal concentration when compared to the seedlings that were only treated with vanadium. The decrease in methyglyoxal concentration is consistent with the decrease in damage observed in the combination treated seedlings when they were compared to the vanadium treated seedlings. The decrease in methyglyoxal concentration in the combination treated seedlings could be due to DIM inducing a more active glyoxalase I system, thus lowering methyglyoxal concentrations. Due to the decrease in damage observed in the combination treated seedlings it could be expected that the glycolysis pathway would not be as up-regulated as in the vanadium treated seedlings, leading to a lower methyglyoxal concentration. The results observed in this study are consistent with a study by Hossain and Fujita (2010) who observed that an increase in glyoxalase activity lead to a decrease in methyglyoxal concentrations and thus a reduction in damage.



Plants use the SOD enzyme to control the concentration of $O_2^-$ within their tissues. Due to the increases in $O_2^-$ observed in all the treated seedlings, the superoxide

dismutase activity had to be determined. Proteins had to be extracted from the differentially treated seedlings to assess the SOD activity. It has to be noted that the extracted proteins were in their native form and not denatured to allow the SOD activity to be assessed. In this study it was observed that the seedlings treated with vanadium had increased SOD activity when compared to the control seedlings. This observation was unexpected, as the contrary was believed before performing the assay, due to the large increase in superoxide concentrations. In a study by Li et al. 2016, it was observed that a cadmium concentration of 100 µM resulted in a decrease in SOD activity this result was contrary to what was observed in our study. The difference in the SOD activity between the two studies could be the different metals that the plants were treated with as well as the species of plants used for the experiments. The increase in SOD activity was attributed to the plant trying to control the $O_2^-$ concentration but due to the continuing vanadium stress, this was not possible. The seedlings treated with DIM had the highest SOD activity when compared to the control and vanadium treated seedlings. The enhancement of SOD activity in the DIM treated seedlings was attributed to the increase in $O_2^-$ concentration due to the application of DIM. The SOD activity increased to maintain the oxidative homeostasis within the plants. It should be noted that when DIM was administered, the increase in superoxide content was not in the detrimental range where oxidative damage occurs. The results observed in this study are consistent with a study by Kishorekumar et al. (2008). In that study the authors observed that

when triazole compounds (Triadimefon and Hexaconazole) which have been observed to have growth promoting properties were administered to *S. rotundifolius* the SOD activity increased. The authors attributed the increase in SOD activity to an increase in $O_2^-$ concentration when triazole compounds were applied to the plants. The seedlings treated with the combination of DIM plus vanadium had statistically the same activity as the seedlings treated with DIM only, which were higher than the activity observed in the control and vanadium treated seedlings. The increase in the SOD activity in the combination treatment was again attributed to the DIM increasing the antioxidant enzymes such as SOD. In our opinion, the vanadium had very little effect on the SOD activity in the seedlings treated with the combination treatment, because if it did, then the activity should have been much higher as the activity observed in the DIM (only) and vanadium (only) treated seedlings both showed increases in SOD when compared to the control seedlings.



The effect of the different treatments on the APX activity of the seedlings was determined through a kinetic spectrophotometric assay. The proteins extracted from the seedlings again had to be in their native form to allow APX activity to be determined. The seedlings treated with vanadium had a significant increase in their APX activity when compared to the control seedlings. A study by Iannone et al. (2015) observed that when tobacco plants were treated with high concentrations of cadmium the APX activity increased which is consistent with the results observed in our study. Hydrogen peroxide content increased in the seedlings treated with

vanadium, the increase in APX activity could be attributed to the plant trying to reduce the $H_2O_2$ concentration in order to reduce the oxidative damage which the plant may be experiencing. The APX activity increased in the DIM treated seedlings when compared to the control seedlings. The increase in APX activity was attributed to DIM activating non-enzymatic and enzymatic antioxidant systems by enhancing the production of $H_2O_2$ and $O_2^-$. Similar observations were made by Egbichi et al. (2014) who observed and increase in APX activity when nitric oxide (NO) a known growth promoter was applied to soybean. The study by Egbichi et al. (2014) attributed the increase in APX activity to the application of NO. The combination treated seedlings were observed to have the highest APX activity. As stated before, DIM and vanadium both had the ability to elicit and increase in APX concentrations. The increase in APX activity in the combination treated seedlings was attributed to the compounded increases in APX due to DIM and vanadium separately. The results of this study are consistent with a study by Egbichi et al. (2014) who observed a compounded increase in APX activity when NaCl and nitric oxide was applied to the plants.



Plants use the Gly I enzyme to control the methyglyoxal concentrations within tissues. Due to the difference in concentrations of methyglyoxal observed in the vanadium and combination treated seedlings, the Gly I activity had to be determined. The effect of the different treatments on the Gly I activity of the

seedlings was determined through a kinetic spectrophotometric assay. It has to be noted that the extracted proteins were in their native form and not denatured to allow the Gly I activity to be assessed. The vanadium treated seedlings had the lowest Gly I activity. Vanadium was able to decrease the activity of the enzyme thus resulting in an increase in methyglyoxal concentration as hypothesised before. The generation of ROS due to vanadium stress could be causing modification and therefore damage to the glyoxalase I enzyme and in this way the activity was decreased. An article by Maruta et al. (2012) stated that ROS has the ability to cause oxidative damage to cellular components. In our study, no significant difference in Gly I activity was observed between the DIM and control treated seedlings. The methyglyoxal levels in the DIM and control seedlings were statistically the same therefore the plants did not need to increase the glyoxalase activity. The combination treated seedlings was observed to have lower Gly I activity than the control but higher activity than the vanadium treated seedlings. As observed before the vanadium in the combination treatment caused a reduction in Gly I activity. What was interesting to observe, was the combination of the vanadium stress and the signalling from the application of DIM, was able to increase the Gly I activity in the combination treated seedlings. The priming effect of DIM hypothesised previously could be responsible for the increase in Gly I activity. The plants defences preparing to combat the apparent mechanical damage due to the application of DIM

could be priming the Gly I enzyme thus performing better when exposed to the vanadium stress.

An increase in GST activity has been observed to improve germination and growth (Roxas *et al*., 1997). Under abiotic stresses such as low temperature and salt, plants with high GST activity retained a good growth rate (Roxas *et al*., 2000). Seedlings were observed to accumulate more GSSG in times of stress, which could indicate a dependency on GST to help reduce oxidative damage. In this study, an increase in GST activity was observed in the seedlings that were treated with vanadium. The increase in activity was attributed to the seedlings trying to protect themselves from oxidative damage caused by vanadium toxicity. The results of this study are consistent with a study by Roxas et al. (2000) who observed an increase in GST activity under salt stress conditions. The seedlings treated with DIM also showed an increase in GST activity. It was no surprise when an increase in GST activity was observed in the DIM treated seedling as the other antioxidant enzymes SOD and APX also increased under the DIM treatment. As stated before DIM concentration could have increased after mechanical damage occurs in some plants. The exogenous application of DIM could signal a message to the seedlings that they are undergoing mechanical stress. Therefore, in an effort to protect themselves from the oxidative damage which accompanies the stress, it might increase the activity of the GST enzyme. The combination treated seedlings were observed to have the highest GST

activity. The increase in GST activity was attributed to the compounded effects of the DIM and vanadium in the combination treated seedlings, as both were observed to increase GST activity separately.

In conclusion, in this chapter, V had detrimental effects on the biochemical and physiological characteristics of the *Brassica napus* L. seedling (shoots). The physiological damage could be observed by the reduction in growth and chlorophyll content. The negative impact of V could also be observed by the decrease in cell viability and increases in lipid peroxidation. Contrastingly, DIM was able to increase chlorophyll content as well as seedling growth by inducing slight increases in $H_2O_2$ and $O_2^-$. The slight increases in the two ROS molecules resulted in enhanced APX, SOD and GST activity. The enhancement of the antioxidant enzymes due to DIM was able to partially mitigate the oxidative damage due to the V stress in the seedlings treated with the combination of the two chemicals. The results observed in this chapter show that DIM was not only able to improve the physiological ad biochemical characteristics of *Brassica napus* L. seedlings but also increase V stress tolerance in these seedlings. The aforementioned observations is very important as increasing crop yield by increasing stress tolerance is one method to tackle South Africa's food security problem.

# Chapter 5

# The application of 3,3' Diindolylmethane, Vanadium and the combination treatment modifies the nutrient profile of *Brassica napus* L. seedlings

## 5.1. Introduction

The accumulation of heavy metals in arable soils is fast becoming a serious threat to South Africa (Moskalyk & Alfantazi, 2003; Yadav 2010). It is a known fact that high concentrations of heavy metals have an impact on the physiology, growth and yields of plants (Russo *et al.,* 2008). The above mentioned fact has far-reaching implications as reduced yields have an effect on the economic status of the country as well as food security for the South African population. The introduction of high concentrations of heavy metals into arable soils also raises questions about the nutritional quality and safety of South African food crops. Plants obtain much of their required essential macro and micro nutrients from the soil surrounding their roots (Jungk, 2001). Due to this mode of nutrient acquisition, it is no surprise that plants in contaminated soils would have higher heavy metal concentrations in both roots and shoots. Plants do not require most heavy metals for metabolism and therefore their uptake may result in

inadequate uptake of other essential metals. The study presented in this chapter was undertaken to investigate the effect of DIM application on the essential metals as well as the downstream effect of nutrient regulation under vanadium stress in *Brassica napus* L. seedlings.

## 5.2. Results

### 5.2.1. Vanadium (V) uptake in differentially treated *Brassica napus* L. seedlings

Vanadium is the 22$^{nd}$ most abundant element found in the earth's crust (Qian *et al*., 2014). South Africa is one of the primary producers of V, mining ±28 % of the total V produced every year (Kamika & Momba, 2014). Due to its versatile nature, V may be used to produce shock resistant alloys as well as catalysts. Due to the drive to mine this metal, South African soils have been enriched with V. This lead to the uptake of large quantities of vanadium in crop plants resulting in crop damage and lower yields. Due to the lower damaged observed in the seedlings treated with the combination treatment (DIM + V) (Figure 4.4) it was deemed necessary to understand how DIM affected the uptake of different metals such as V. No significant difference in V content was displayed when the control and DIM treated seedlings were compared. Increases in V content of ±153 % and ±113 % was observed in the V and combination treated seedlings respectively when compared to the control seedlings.

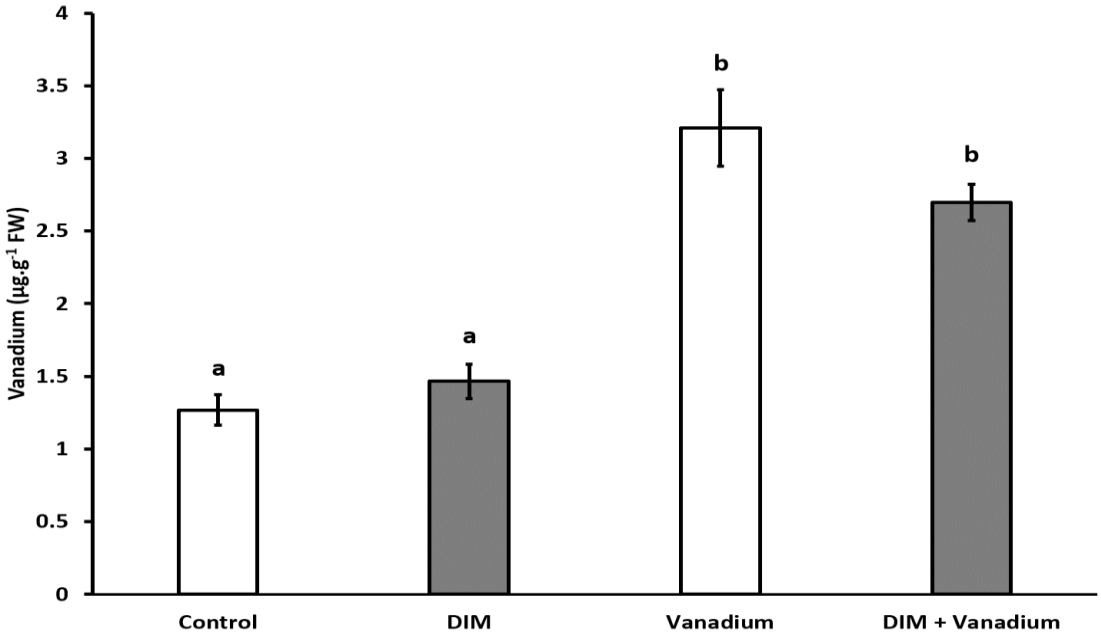


**Figure 5.1: The effect of DIM, Vanadium and their combination on the vanadium content within *Brassica napus* L. seedling shoots**. Seedlings were treated with one of four treatments and their effect on vanadium content was determined. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).



### 5.2.2. The addition of the compounds DIM and vanadium increase iron (Fe) content in *Brassica napus* L. seedlings

Iron is abundant in the earth's crust and predominantly exist in the form of Fe (III) (Cohen *et al*., 1998). At neutral to alkaline pH's Fe (III) may form precipitates and be unaccessible for plant uptake (Cohen *et al*., 1998). Iron is an essential metal for plants, as they require it for the functioning of their photosystem I (Pätsikkä *et al*., 2002). Iron is also used as a cofactor for enzymes such as the FeSODs. Due to the physiological damage and slight chlorosis in some of the seedlings, the iron content of all the treated seedlings had to be analysed. All treatments showed an increase in Fe concentration when compared to the

control seedlings. The increases in Fe concentration was ±78 %, ±115 %, and ±265 % in the DIM, V and combination treatment respectively.

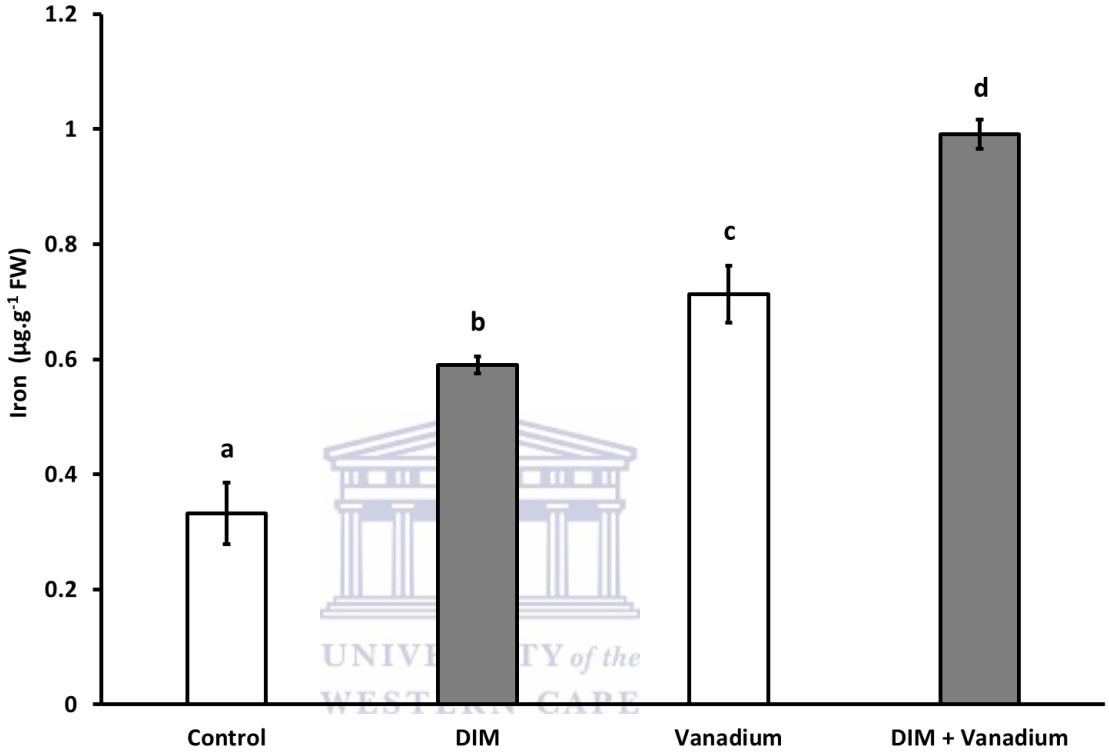


**Figure 5.2: The effect of DIM, Vanadium and their combination on the Fe content within *Brassica napus* L. seedling shoots**. Seedlings were treated with one of four treatments and their effect on Fe content was determined. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).

### 5.2.3. The addition of vanadium increases copper (Cu) content in *Brassica napus* L. seedlings

Copper is an essential metal in plants as they act as cofactors for enzymes such as Cu/Zn SODs (Kabata-Pendias, 2010; Alscher *et al*., 2002). In this study, no significant difference in Cu content was displayed between the control and DIM treated seedlings. The V and combination treated seedlings both showed a

marked increase in Cu content when they were compared to the control seedlings. The increases in copper content was ±376 % and ±118 % for the V and combination treated seedlings respectively.

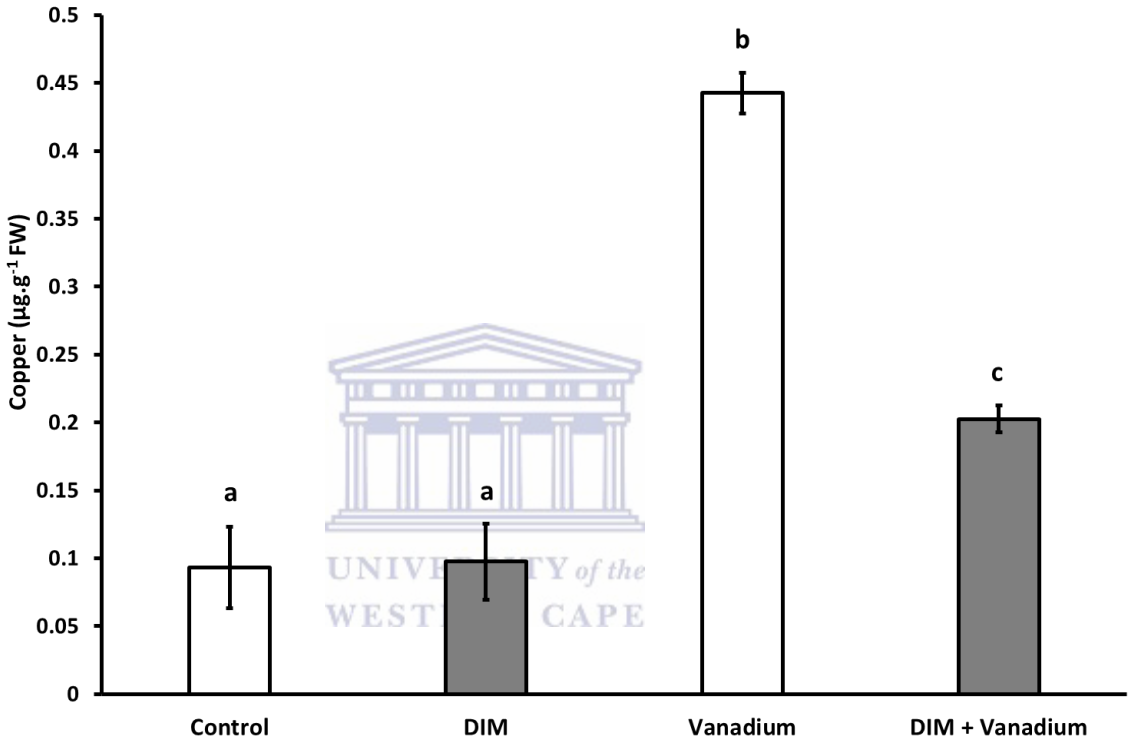


**Figure 5.3: The effect of DIM, Vanadium and their combination on the Cu content within *Brassica napus* L. seedling shoots**. Seedlings were treated with one of four treatments and their effect on Cu content was determined. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).

### 5.2.4. Vanadium blocks the uptake of calcium (Ca) in *Brassica na*pus L. seedlings

Calcium is an essential metal for most life forms (Hepler, 2005). The role of Ca as a secondary messenger in animal and plants has been observed (Poovaiah, 1988). Calcium channels are present within plant cell membranes that facilitate

the movement of Ca ions (White, 2000). Defects associated with low levels of Ca include water soaking, leaf necrosis and poor root development (Hepler, 2005). Poor root formation was observed in this study when the seedlings were treated with V therefore it was necessary to determine the Ca concentrations in the differentially treated seedlings. The seedlings treated with DIM showed an increase in Ca content of ±39.5 % when compared to the control seedlings. Seedling treated with V showed a reduction in Ca content. The reduction in Ca content was ±99 % and ±58 % for the V and combination treatment respectively when compared to the control seedlings.

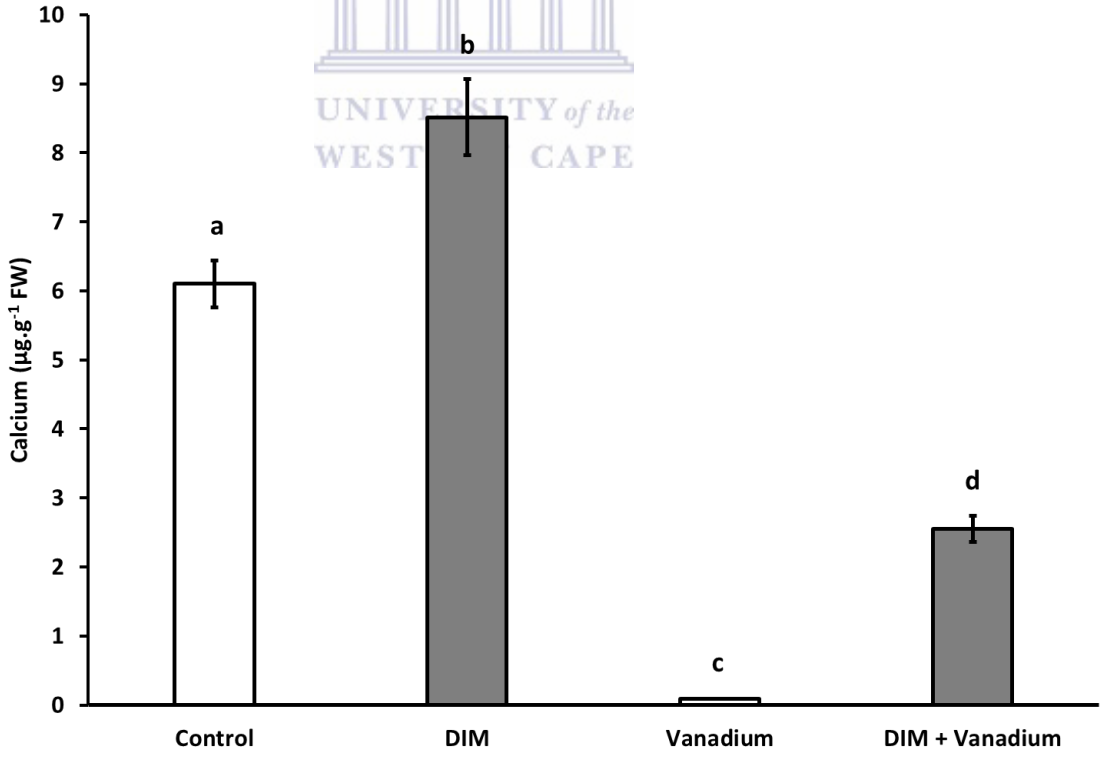


**Figure 5.4: The effect of DIM, Vanadium and their combination on the Ca content within *Brassica napus* L. seedling shoots**. Seedlings were treated with one of four treatments and their effect on Ca content was determined. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).

### 5.2.5. DIM increases magnesium (Mg) content in *Brassica napus* L. seedlings

By the year, 1925 Mg was officially recognised as an essential nutrient (Schonewille, 2013). Magnesium plays many roles within animal and plant systems. One of Mg's roles is the stabilization of macromolecules such as proteins and cell membranes (Joy *et al*., 2013). Due to its osmotically active nature, Mg in conjunction with potassium helps to regulate the turgor pressure within cells (Gerendás & Führs, 2013). One of the most notable roles of Mg s in plants is acting as a cofactor involved in the photosynthesis process (Cakmak & Kirkby, 2008). Due to changes in chlorophyll levels in the differentially treated seedlings in this study, it was necessary to observe the Mg levels in those seedlings. An increase in Mg content of ±7 % was observed in the seedlings treated with DIM when compared to the controls. A decrease in Mg content was observed in both the V and combination treated seedlings when they were compared to the control seedlings. The decreases were ±15 % and ±5 % in the V and combination treatment respectively.

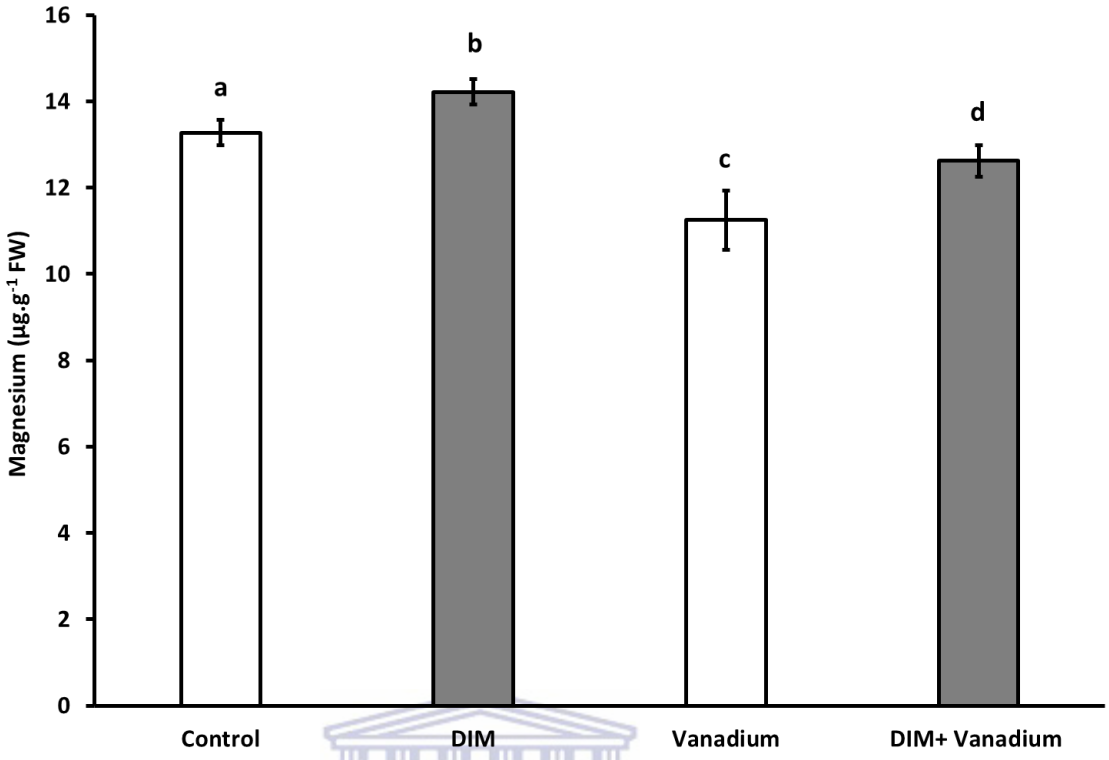


**Figure 5.5: The effect of DIM, Vanadium and their combination on the Mg content within *Brassica napus* L. seedling shoots**. Seedlings were treated with one of four treatments and their effect on Mg content was determined. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).



### 5.2.6. The addition of vanadium and DIM decrease the potassium (K) content in *Brassica napus* L. seedlings

In certain plants, K is required for the regulation of transpiration, respiration and lipid synthesis (Rashid *et al*., 2016). Potassium was observed to have effects on the distribution of primary metabolites such as amino acids and sugars (Amtmann *et al*., 2008). A number of enzymes have been found to be dependent on K and the deficiency of the nutrient lead to the total loss in enzyme activity (Rashid *et al*., 2016). Due to K, being such an important nutrient its concentration

had to be ascertained in the seedlings treated with the different compounds. All the treated seedlings displayed a decrease in K content when compared to the control seedlings. The decrease in K content were ±29 %, ±11 % and ±13 % for the V, DIM and combination treatment respectively. When comparing the DIM and combination treated seedlings no statistical difference was observed.

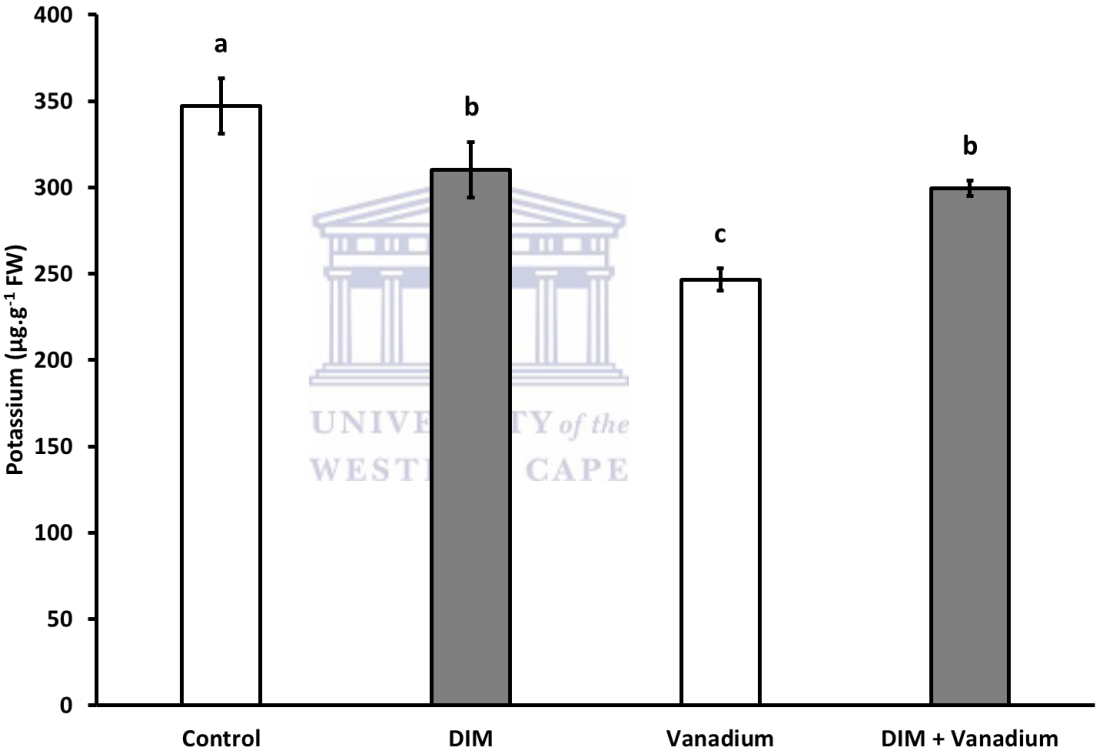


**Figure 5.6: The effect of DIM, Vanadium and their combination on the K content within *Brassica napus* L. seedling shoots**. Seedlings were treated with one of four treatments and their effect on K content was determined. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).

### 5.2.7. The addition of vanadium and DIM increases phosphorus (P) content in *Brassica napus* L. seedlings

The six essential macronutrients include N, K, Ca, Mg, S and P (Vance *et al*., 2003). Plants acquire phosphorus through their roots in the form of dihydrogen phosphate (Hammond *et al*., 2004). Concentrations of P is often low in soils which results in slow uptake of the element through diffusion (Fitter & Hay, 2012). When plants undergo P deficiency, they will expand their root system to maximize their root surface area to obtain nutrients (Hammond *et al*., 2004). No statistical difference in the phosphorus content was observed between the control and the DIM treated seedlings. The vanadium and combination treated seedlings both showed an increase in P concentration when compared to the control seedlings. The increases in P concentrations were ±15 % and ±18 % for the V and combination treated seedlings respectively. When comparing the V and combination treated seedling no statistical difference was observed.

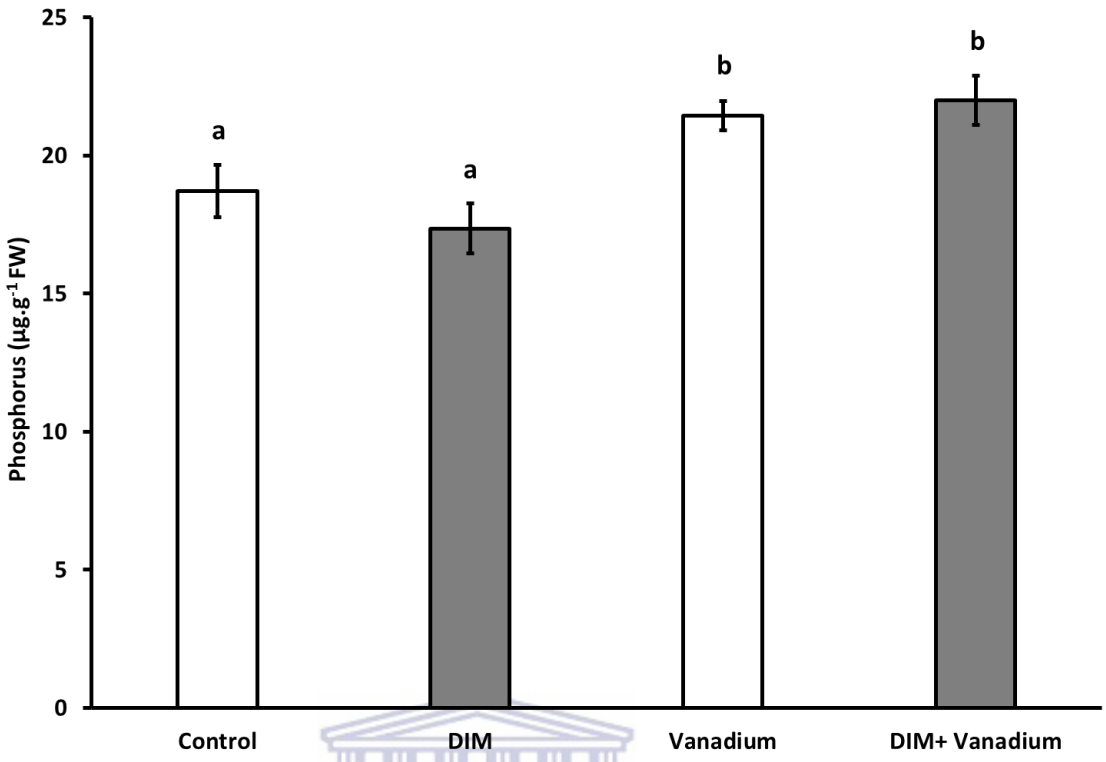


**Figure 5.7: The effect of DIM, Vanadium and their combination on the P content within *Brassica napus* L. seedling shoots**. Seedlings were treated with one of four treatments and their effect on P content was determined. Different letters indicate significant differences between means at P< 0.05 (Tukey-Kramer test). Values means ± S.E (N=4).



## 5.3. Discussion

Due to the physiological and biochemical damage observed in Chapter 4 of this study, it was necessary to understand how much V was present in the seedlings after treatment. The seedlings which were treated with V were observed to have the highest concentration of V. Due to the increase in V in the soil, the natural acquisition of nutrients could lead to a greater uptake of V. Hall (2002) stated that high concentrations of zinc and Cu would lead to increased uptake of these metals in the plant and exacerbate ROS production after exogenous application.

Therefore, that study clearly demonstrated that high exogenous application of metals to plants would lead to higher accumulation of those metals inside the plants which eventually leads to increase in ROS and damage. Thus, in this study, the seedlings with the highest V content were also the seedlings that experienced the most damage, which is consistent with the hypothesis of Hall (2002). When comparing the control and DIM treated seedlings no significant difference in V content was observed. Vanadium was present in the potting soil used for these experiments therefore the DIM and treated seedlings were observed to have low levels of the metal. A decrease in V content was observed in the combination treated seedlings when compared to the V treated seedlings. The decrease in V uptake in the combination treated seedlings was interesting as these plants were exposed to the same amount of V as the V-only treated seedlings. One hypothesis for the decrease in V content could be that the plant perceived the V stress and somehow used DIM to signal for the over production of pectin and hemicellulose thus creating a physical barrier limiting the amount of V which could be taken up. Therefore, in addition to the plants natural ability to increase pectin and hemicellulose under metal stress the presence of DIM could lead to even more production of the two molecules. A Study by Yang et al. (2008) observed that when rice was exposed to aluminium the pectin, hemicellulose 1 and 2 concentrations increased, limiting the uptake of aluminium. Another hypothesis is that the seedlings under the exogenous application of DIM was able to excrete chemicals into the soil to reduce the uptake of V. A study by Zhu et al. (2011) observed that when oxalate was

secreted from the roots of *Lycopersicon esulentum* cadmium uptake was reduced, leading to tolerance in the plants.

Iron is an essential metal for most life due to its numerous functions. Due to its importance, its deficiency creates many problems for living organisms. Due to the insolubility of Fe, plants may struggle to take up this essential metal (Cohen *et al*., 1998). A second problem plants may face is the toxicity if the concentration of the metal is too high (Hell & Stephan, 2003). In this study, V caused an increase in Fe uptake in both the V and DIM combination treated seedlings. Vanadium is known to produce ROS that may affect the photosystems of plants (MacFarlane & Burchett, 2001). In this study, the V treated seedlings experienced a large spike in ROS production that would have affected the photosystem I. Therefore, the seedlings may have increased their uptake of Fe to help improve the photosynthesis process in order to survive the toxicity. A similar result was observed by Reboredo (1994) after applying excessive concentrations of Zinc onto *Halimione portulacoides* plants. In that study, toxic levels of Zinc lead to a drastic increase in Fe however, this increase in Fe could not rescue the chlorophyll content. Indeed, similar to results observed in the current study (chlorophyll content under V stress), chlorophyll degradation was still observed by Reboredo (1994) in the Zn treated plants. Furthermore, in this study, the seedlings treated with DIM also showed an increase in Fe content when compared to the control seedlings. The increase in Fe could have

attributed to the improved chlorophyll production observed in Chapter 4. DIM could be inducing the complexion of the Fe metal increasing the seedlings ability to take it up. Similar results were observed in a study by Graziano et al. (2002) who observed an increase in Fe content when nitric oxide was applied to plants. Graziano and colleagues proposed that the increase in Fe was due to the interaction between nitric oxide with the metals forming metal-nitrosyl complexes, making the metal more accessible to plants. Nonetheless, in this study, the combination treated seedlings had the highest concentration of Fe within their tissue. Although less V was present in the combination treated seedlings, the combined effects mentioned before of the V and DIM that was present could have caused the increase in Fe uptake in the seedlings.



The seedlings treated with V had more than four times the Cu content when compared to the control and DIM treated seedlings. As stated before, Cu is essential for the proper functioning of certain antioxidative enzymes. The increase in Cu uptake and thus the Cu content in the seedling tissue could be attributed to the seedlings trying to reduce ROS damage. The seedlings may have accumulated the Cu to allow increased Cu/Zn SOD activity to reduce the increasing $O_2^-$ levels due to V toxicity. A study by Demirevska-Kepova et al. (2004) observed that an increase in Cu content resulted in an increase in SOD activity. At high concentrations of Cu, the metal can also cause damage to plants, the damage observed in (chapter 4) could be due to the combined effect of V and Cu

toxicity on the seedlings. No significant difference in Cu content between the control and DIM treated seedlings was observed. The observation meant that the seedlings treated with DIM were able to acquire and maintain the same amount of Cu as the control seedlings. The seedlings treated with DIM may have "perceived" mechanical damage but no damage/stress was induced therefore there was no change in Cu uptake in the seedlings. A slight increase in Cu content in the combination treated seedlings was observed. The reduction in V content in the combination treated seedlings would lead to decreased V toxicity in these seedlings. The perceived lower V toxicity (in the combination treatment) could have led to the seedlings not acquiring and accumulating as much Cu in their tissue as in the V-only treated seedlings. The lower V and Cu content in the combination treated seedlings could be the reason for the reduction in damage when compared to the seedlings treated with just V only.



Plants use Ca ions to strengthen their cell walls and to provide stress protection (Hirschi, 2004). Calcium is a very important element to plants, which is evident by its storage within plant vacuoles (Hepler, 2005). In this study, the seedlings treated with V showed an almost complete lack of Ca content in their tissues. The decrease in the Ca content could be a result of the V disrupting the Ca membrane transporters. Without transporters many essential nutrient cannot enter cells. Vanadium could also be competing for the same membrane channels as Ca. A study by Perfus- Barbeoch et al. (2002) observed that heavy metals such

as cadmium were able to enter plants using the Ca channels leading to disruption to the plant's water status and metabolism. Our results show an increase in Ca content in the DIM treated seedlings. The increase of Ca could be attributed to the seedlings perceiving mechanical damage due to the increase in DIM concentration and therefore upregulating the uptake of the metal ion to allow for increased stress tolerance. An article by Xiong et al. (2002) stated that under abiotic stresses such as cold, drought and salinity, Ca content increased due to an influx of the metal. Plants may use the metal ion as a signalling entity for processes such as phospoprotein cascades and in this way plants try to overcome the damage caused by abiotic stresses. A second hypothesis is that ROS activates downstream processes using Ca ions therefore promoting the uptake of Ca by the plant. A study by Bennet-Clark (1956) suggested that auxins such as Indole acetic acid (IAA) had antagonistic effects on Ca. This idea was later challenged by Cleland (1960) who observed no enhancement of loss or redistribution of Ca in the presence of IAA. We observed an increase in Ca content in the combination treated seedlings when compared to the V-only treated seedlings. The V in the combination treatment again down regulated the uptake of Ca ions, but DIM was able to increase Ca content through an unknown mechanism (at the time of this study). A journal article by Knight (1999) stated that Ca content increased in response to mechanical damage. Therefore, in our study the release of DIM could be perceived as mechanical damage by the seedlings as DIM and other glucosinolates are only release upon tissue damage due to herbivore action etc. The increase in Ca content in the combination treated seedlings could play

signalling roles thus resulting in the combination treated seedlings not incurring as much damage as the V-only treated seedlings.

Magnesium is important for enzyme activities such as kinases and polymerases (Cowan, 2002). More than 75 % of leaf Mg is used in the synthesis of proteins and are also associated with chlorophyll pigments (White & Broadley, 2009). Characteristics of Mg deficiency is the early onset of ageing, poor growth and reduction in crop yields (Maguire & Cowan, 2002). In this study, the seedlings treated with V had the lowest Mg content. The decrease in Mg content in the V treated seedling could be contributing to the chlorosis observed in these seedlings (chapter 4). A study by Tewari et al. (2006) observed that the deficiency of Mg induced oxidative stress in mulberry plants. Therefore, the absence of the required Mg content coupled with the oxidative damage would explain some of the physiological and biochemical damages observed in the V treated seedlings, in this study. The oxidative damage due to ROS produced in response to the V toxicity could have altered root morphology resulting in impaired uptake of essential ions such as Mg. A study by Chou et al. (2011) also observed that under Mg deficiency (i.e low magnesium concentration) the uptake of cadmium was increased. The difference in Mg and V concentration in the soil could be so vast resulting in the uptake of V over Mg in the V treated seedlings, in this study. The contrary was observed in the seedlings treated with DIM. Under abiotic stress, plants tend to increase their uptake of essential

nutrients in an effort to increase their chances of survival. Plants may increase biomass to the organs responsible for the uptake of these nutrients in an effort to increase their internal concentration in the plant (Herman *et al.*, 2006). The perceived mechanical stress due to the increase in DIM concentration in the seedlings could have triggered the plants survival mechanism to increase uptake of essential nutrients as previously stated. The increased uptake of Mg (under DIM application) could have led to the increase of Mg content in the seedling tissue. An increase in Mg content was observed in the combination treated seedlings when compared to the V treated seedlings. The biochemical difference between the V and combination treated seedlings was the high ROS concentration present in the V treated seedlings. The DIM in the combination treated seedlings was able to induce higher antioxidant capacity in the seedlings under V stress. The higher antioxidant capacity thus lead to less ROS accumulation and therefore less damage to organs such as the roots system. The increase in Mg content in the combination treated seedlings could be attributed to a better functional root system.



Potassium is the most abundant positively charged ion found in higher plants (Ashley *et al*., 2006; Schachtman & Schroeder, 1994). Plants use this metal ion for growth, enzyme homeostasis and osmoregulation of cells (Maathuis & Sander, 1994). Plants can only acquire K ions when they are in solution (Yanai *et al*., 1996). In this study, a decrease in K content was observed in the V treated

seedlings. Plants use one of two strategies to acquire nutrients. The first strategy is for the roots to grow into an area containing the desired nutrient or to transport the nutrient to the surface of the root (Jungk & Claasen, 1997). Therefore, one hypothesis for the decrease in K content is that when the V solution was added to the soil it increased the concentration of V in a certain layer, the root system might have been localised to this layer of the soil thus promoting the uptake of V instead of essential nutrient like K. A second hypothesis is that the seedlings might have tried to exclude the V from the roots depositing the V in the soil surround the roots thus inhibiting the access of essential nutrient to the roots surface. A third hypothesis is that V and K competes for the same transporters therefore we observed lower K concentration in the V treated seedlings. A decrease in K content was also observed in the seedlings treated with DIM. Mg and K have antagonistic effects on each other (Horie *et al*., 2011). In fact, Horie et al. (2011) concluded that K and Mg might compete for the same unidentified transporters. Thus, in this study, the increase in Mg content observed in the DIM treated seedlings could have resulted in a lower uptake of K ions. The seedling treated with the combination treatment was observed to have a similar K uptake as the DIM treated seedlings. Therefore the V in the combination treated seedlings had little effect on the K uptake in the combination treated seedlings.

Although soils often contain a high amount of P, only a small portion may be available for uptake by plants (Schachtman *et al*., 1998). To increase the availability of usable P, some plants have the ability to secrete organic acids into the soil thus lowering the pH and this results in the increased mobilization of metal ions including P (Marschner, 1995). A study by Lopez-Bucio et al. (2000) observed that an increase in citric acid production resulted in an increase in growth even in P deficient experiments. The proposed idea in that study was that even in P deficient soil the plants which secreted more citric acid were able to use the P that was present in the soil more effectively than those which secreted the normal amount of citric acid. An increase in P content was observed in the seedling treated with V, in this study. As stated before heavy metals such as V may lead to metal toxicity within the microbial population of soils. Due to the metal stress, the microbial population would change and bias the growth of metal tolerant microorganisms (Pennanen *et al*., 1996). Heavy metal tolerant bacteria have the ability to produce organic acids as well, thus an increase in these organism may lead to a decrease in soil pH (Pennanen, 2001). Plants under stress are also known to excrete compounds which are able to change the pH of the soils surrounding the roots of the plants as part of a defence mechanism. A decrease in soil pH would favour the solubility and mobility of heavy metals in the soil, leading to increased uptake of these metals. No significant difference in P content was observed between the control and DIM treated seedlings. This observation indicated that the uptake and accumulation of the P ions remained the same in the seedlings and the exogenous application of DIM had no effect on

P content. The application of DIM may not have affected the secretion of organic acids into the soil thus the pH remained the same and the mobilization of the metals were not affected. The combination treated seedlings showed a similar increase in P content as the V treated seedlings. Due to P content in the combination treated seedlings being almost identical to that observed in the V treated seedlings, similar uptake mechanisms might be at play. Vanadium and P are structurally similar and therefore V might be transported through P transporters in the plant. Due to the high concentration of V outside the plant this could cause a concentration gradient causing an influx of the two ion into the cells of the plants.




In conclusion, in this Chapter, it was observed that the different treatments had an effect on the uptake and accumulation of heavy metals and essential metal ions in the *Brassica napus* L. seedlings. DIM displayed mostly positive effects on uptake of essential metal ions. Which can be observed by the increase in metal ions such as Mg, Ca and Fe. The seedlings treated with V however showed a decrease in essential metals such as Mg, Ca and K but a vast increase in the heavy metal V. The combination treated seedlings showed a higher accumulation of essential metal ions when compared to the V-only treated seedlings. From the results obtained it is evident that DIM application modifies the metal ion composition within plants. In the combination treatment, DIM was able to slightly compensate for the decreases in essential metals due to V. The increase

in uptake and accumulation of essential metals and conversely the decrease in uptake of heavy metals would result in plants growing better under normal and stressful conditions.

# Conclusion and Future work

This study has established that DIM has positive effects on *Brassica napus* L. seed/seedlings when exogenously applied. The germination rate of the *Brassica napus* L. seeds where greatly increased when exposed to the exogenous application of DIM. DIM was observed to increase biomass of the seedlings. Although the ROS ($O_2^-$ and $H_2O_2$) content increased in response to DIM their levels did not accumulate to detrimental levels, this could be observed by no significant increase in cell death or lipid peroxidation. The slight increase in ROS could be attributed to the seedlings perceiving undergoing mechanical damage due to an increase in DIM concentration and therefore signals through ROS. The low accumulation of ROS was attributed to an increase in activity of antioxidant enzymes (APX and SOD).



This study then went further to determine the effect DIM could have on vanadium stressed *Brassica napus* L. seedlings. Vanadium at concentrations of 350 $\mu M$ was observed to be detrimental to *Brassica napus* L. seedlings. The negative effects could be observed by the reduction in germination percentage of the seeds. Vanadium was not only detrimental to the germination but also increased damage to the seedlings. The damage could be observed by an increase in cell death and lipid peroxidation. The damage was further showed when a decrease in conjugated diene content was observed in the vanadium

treated seedlings. However, when DIM and vanadium were applied in conjunction with one another a reduction in damage was observed as lower levels of cell death and lipid peroxidation was observed. Both vanadium and the combination treated seedling displayed an increase in ROS ($O_2^-$ and $H_2O_2$) concentrations. However, the increase in ROS concentrations in the vanadium treated seedling were far more pronounced than the combination treated seedlings. The reason for the lower ROS accumulation was due to the increased antioxidant enzymes namely SOD, APX, GST and Gly.

In an effort to further, elucidate the mechanisms involved in DIM mediating vanadium stress in *Brassica napus* L., Ionomic analysis was conducted. As expected, the seedlings treated with vanadium had the highest vanadium content but the seedlings treated with the combination treatment displayed a decrease in vanadium accumulation. Both DIM and vanadium increased the accumulation of iron in the seedlings, which then lead to the combination treated seedlings having the highest iron content. The accumulation of calcium in the seedlings were inhibited by the addition of vanadium. However, DIM was able to increase the accumulation of calcium within the DIM treated seedlings which was also observed in the seedlings treated with the combination treatment. As stated before copper is an essential metal and in this study vanadium increased the uptake of this metal in the seedlings. Although the combination treated seedling displayed an increase in Cu, it was not as

pronounced as in the vanadium treated seedlings. The increase in copper content in the combination treated seedlings was not as pronounced as the stress was relatively less than in the vanadium treated seedlings. It was also observed that DIM was able to increase the Mg content within the brassica seedlings. Even when we observed a decrease in Mg content under vanadium stress, the combination treatment showed an increase in Mg content when compared to the vanadium only treatment. Therefore, from this work it was clear that DIM was able to modify the metal uptake and accumulation with the *Brassica napus* L. seedlings. Subsequently, from the results obtained we can attribute some of the growth promoting properties of DIM to DIM's ability to induce the uptake and accumulation of essential nutrients.



Future work will include, using 2D PAGE analysis to identify potential proteins involved in improving growth under exogenous DIM treatment. In an effort to identify the proteins their amino acid sequence will be obtained using matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF). Using bioinformatics tools the gene sequences of the proteins will be determined followed by the synthesized of primers for the genes. Once the primers are produced semi-quantitative analysis of the related gene expression will be analysed to observe these gene responses to DIM and vanadium. The full length genes will then be isolated and inserted into appropriate vectors followed by the expression of these recombinant proteins. Once the recombinant proteins

are purified, relevant functional assays will be performed. These candidate DIM signalling proteins can then be used to make plants more tolerant to vanadium stress in the near future.

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# Appendix A: *Published article*

# Exogenous 3,3’ diindolylmethane increase *Brassica napus* L. seedlings shoot growth through modulation of superoxide and hydrogen peroxide content

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Short communication

# Exogenous 3,3′-diindolylmethane increases *Brassica napus* L. seedling shoot growth through modulation of superoxide and hydrogen peroxide content

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Superoxide radical

ABSTRACT

*Brassica napus* L. (cv. AV Garnet) seeds were pre-treated with 15 μM 3,3′-diindolylmethane (DIM) to investigate whether DIM could enhance seed germination. Further treatment of seedlings with 15 μM DIM for 14 days explored the effects on seedling shoot growth. Exogenous DIM led to improved germination percentage, increased seedling shoot lengths, and increased fresh and dry weights. Furthermore, DIM triggered induction of superoxide radical ($O_2^-$) and hydrogen peroxide ($H_2O_2$) content however, no change in malondialdehyde (MDA) content and cell death (assessed with Evans Blue assay) was detected for both the control and DIM treated seedling shoots. We also observed increases in superoxide dismutase (SOD) activity and ascorbate peroxidase (APX) activity in response to exogenous DIM, two fundamental enzymes in the control of reactive oxygen species (ROS) in plants. These results indicate that exogenous DIM treatment enhances seed germination and improves seedling shoot growth through possible activation of a reactive oxygen species signalling pathway involving $O_2^-$ and $H_2O_2$ in *B. napus*.




## 1. Introduction

Cruciferous vegetables, including broccoli, brussels sprouts, canola (*Brassica napus*) and cabbage, are good sources of sulphur-containing indole glucosinolate compounds known as glucobrassicins (Jongen, 1996; Fahey et al., 2001). Myrosinase, an enzyme that catalyzes the hydrolysis of glucosinolates, is located in myrosin cells which physically separate the enzyme from glucosinolates in intact plant cells (Bones et al., 1991; Andréasson et al., 2001). When plant cells are damaged, as when cruciferous vegetables are chopped or chewed by insects or herbivore action, the interaction of myrosinase and glucobrassicin results in the formation of indole-3-carbinol (I3C) (Halkier and Gershenzon, 2006). Under appropriate conditions (mostly acidic), two I3C molecules can combine to form the dimer 3,3′-diindolylmethane (DIM) (Pilipczuk et al., 2015). 3,3′-diindolylmethane (DIM) is one of the more stable by-products of indole glucosinolate breakdown and have been extensively studied in mammalian research especially in cancer biology. Roles for DIM have been reported in breast cancer (Marques et al., 2014), colon cancer (Lee et al., 2014), liver cancer (Harrill et al., 2015), gastric cancer (Jin et al., 2015), bone metabolism (Yu et al., 2015) and prostate cancer (Cho et al., 2011; Zhang et al., 2014) to name a few. Furthermore, Bradlow and Zeligs (2010) studied the breakdown of I3C in tissue culture media and concluded that DIM was the predominant spontaneous breakdown product of I3C therefore hypothesizing that the anticancer properties of I3C could be contributed to DIM. This theory was supported by Beaver et al. (2012), who reported that DIM and not I3C is responsible for anticancer events in prostate cancer in a concentration dependent manner. Even though the dietary importance of cruciferous vegetables is now widely studied using mainly glucosinolate products such as I3C and DIM, plant research with regards to these glucosinolates have not enjoyed the same attention. However, some of the potential roles of glucosinolates in Brassicaceae as secondary metabolite in various physiological and stress responses have been reviewed by Zukalová and Vasak (2002) as well as Del Carmen Martínez-Ballesta et al. (2013). Furthermore, Ahuja et al. (2011) confirmed the importance of glucosinolate production in *B.*

*Abbreviations:* ABA, abscisic acid; APX, ascorbate peroxidase; ANOVA, analysis of variance; Cat, catalase; DIM, 3,3′-diindolylmethane; GA, gibberellic acid; GC, gas chromatograph; HPLC, high-performance liquid chromatography; HR, hypersensitive response; I3C, indole-3-carbinol; IAA, indole-3-acetic acid; MDA, malondialdehyde; NBT, nitro blue tetrazolium chloride; ROS, reactive oxygen species; SA, South Africa; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

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*napus* by producing transgenic plants with much lower myrosinase activity and subsequent lower acid condensation glucosinolates than wild type plants. These transgenic plants had a slower growth and development pattern than wild type plants therefore highlighting an important role for glucosinolate hydrolysis products through the myrosinase system in Brassicaceae. In contrast, Katz et al. (2015) reported that exogenous I3C inhibits Arabidopsis root elongation in a concentration dependent manner by manipulating auxin signalling and therefore concluded that glucosinolate hydrolysis products like I3C are signalling molecules with roles other than only in herbivore attack. Conversely, derivatives of DIM have been shown to increase plant growth of *Oryza sativa* in liquid medium after a five day treatment period (Pal et al., 2007). This study highlighted the potential role of DIM hydrolysis products as growth promoting signalling molecules in plants that do not produce glucosinolates or specifically DIM. Knowing that a link exists between biotic stress (herbivore action and pathogen attack), the triggered response [for example the hypersensitive response (HR)] and the Foyer-Halliwell-Asada cycle [especially reactive oxygen species (ROS) generation and scavenging by antioxidant enzymes] (Mur et al., 2006; Tan et al., 2013), we thus hypothesize, in this study, that exogenous application of DIM onto *B. napus* seeds can stimulate HR-like responses to produce ROS (superoxide and hydrogen peroxide). In addition, this ROS could be under careful control of increased antioxidant enzymes (triggered in response to the increased ROS) to levels that does not cause injury or plant cell death and therefore ultimately control positive seed germination and further seedling development.

## 2. Materials and methods

### 2.1. Preparation of DIM solution

The DIM [sigma; ≥98% (HPLC)] stock solution was prepared as follows: 10 mg of DIM was homogenized in 235 μl tween 80 (sigma; viscous liquid, cell culture tested) and made up to 10 ml using 99.8% (v/v) ethanol [sigma; absolute, (GC)]. The solution was then snap frozen using liquid nitrogen and freeze dried at −41 °C overnight in a Labconco benchtop freeze drier (FreeZone 1). Thereafter 19.77 ml deionised water was added to DIM resulting in a 2 mM stock solution [containing 1.18% (v/v) Tween 80]. The control was prepared in the exact same manner as mentioned for DIM preparation but did not contain the DIM powder (Tween 80 final concentration of 1.18%).

### 2.2. Plant growth, treatments and determination of growth parameters

Plant growth experiments were performed in 15 cm diameter plastic pots containing a nutrient rich potting mix [Stodels Nurseries South Africa; 1 part Double grow weed-free compost and 1 part Double grow potting soil] under a 25/19 °C day/night temperature cycle with a 16/8 h light/dark cycle, at a photosynthetic photon flux density of 300 μmol photons.$m^{-2}$ $s^{-1}$ (during the day phase), in a randomized design. Finally, the seeds [Agricol South Africa; AV Garnet cultivar] and subsequent seedlings were treated with 100 ml of 15 μM DIM solution or deionised water (as control) both containing 0.009% (v/v) Tween 80, twice a week for 14 days. The germination percentage was determined by firstly pre-treating (100 ml) the pots (no seeds) with DIM or the control followed by planting 100 seeds (25 from different seed lots x [n = 4]) per treatment and observing the number of seeds germinating (defined as seeds with radical 3 mm or more in length) up until no further seeds germinated. For subsequent experiments [until the end of the seedling stage (14 days)], we removed the seedling roots to prevent erroneous data interpretation caused by possible root damage from seedling removal from the soil. Dry weight analysis was performed by drying 25 seedling shoots (individually) per treatment at 80 °C for 20 h and the weights were subsequently recorded. Furthermore, the lengths of 25 seedling shoots were also measured.

### 2.3. Biochemical assays

Cell viability was assessed by using a modified method of Sanevas et al. (2007). Briefly, the undamaged seedling shoot was immersed in 0.25% (m/v) Evans Blue (sigma; Dye content ≥75%) and dye uptake was assessed by spectrophotometry at 600 nm. Lipid peroxidation by monitoring malondialdehyde (MDA) production in seedling shoots was quantified using the thiobarbituric acid reactive substances (TBARS) assay as described by Zhang et al. (2007). Superoxide concentrations were determined by submerging intact seedling shoots in a solution containing; 10 mM KCN (to inhibit Cu/Zn SODs), 10 mM $H_2O_2$ (to inhibit Mn and Cu/Zn SODs), 2% (m/v) SDS (to inhibit Mn and Fe SODs), 80 μM nitro blue tetrazolium chloride (NBT) (sigma; powder, for molecular biology) and 50 mM potassium phosphate (pH 7.0). The seedling shoots were incubated for 20 min within the solution after which the seedling was homogenized (in solution), centrifuged (10,000*g* for 5 min) and the supernatant was spectrophotometrically analysed at 600 nm. The superoxide concentration was calculated using the NBT extinction coefficient of 12.8 mM $cm^{-1}$. Hydrogen peroxide was quantified in the control and DIM treated plants using the method of Velikova et al. (2000). Seedling proteins were extracted as described in Egbichi et al. (2014) and the protein concentrations were determined using the RC DC Protein Assay Kit 11 (Bio-Rad Laboratories). Superoxide dismutase (SOD) assays were performed on seedling protein extracts using the method of Stewert and Bewley (1980). Seedling ascorbate peroxidase (APX) activities were measured in extracts using a method previously described by Asada (1984).

### 2.4. Statistical analysis

All statistical data was analysed using two-way analysis of variance (ANOVA) and tested for significance using the GraphPad Prism 5.03 software by applying the Tukey-Kramer test at 5% level of significance. All experiments in this study were repeated four times (independently) for repeatability and reproducibility purposes.

## 3. Results and discussion

### 3.1. Exogenous DIM increases seed germination percentage of B. napus

During herbivore action as well as pathogen attack, members of the Brassicaceae family releases soluble as well as insoluble glucosinolates as deterrents (Kim and Jander, 2007) and antimicrobials (Tierens et al., 2001; Sanchez-Vallet et al., 2010). Furthermore, wounding also triggers hypersensitive responses (HRs) often accompanied by ROS production as well as responses to the Foyer-Halliwell-Asada cycle for removal of the toxic ROS molecules (Brederode et al., 1990; Conklin, 2001). Therefore in this study, we investigated whether a link exists between glucosinolate production and ROS-antioxidant enzyme pathways in *B. napus* seedlings. Furthermore, Kranner et al. (2010) indicated that ROS accumulation is important for *Pisum sativum* seed germination as well as seedling development therefore we hypothesized that if exogenously applied glucosinolates can trigger minor increases in ROS production coupled with increased antioxidant activity (for ROS control), we could observe positive seed germination as well as seedling development. Recently, 3,3′-diindolylmethane (DIM) have emerged as an important glucosinolate molecule in cancer

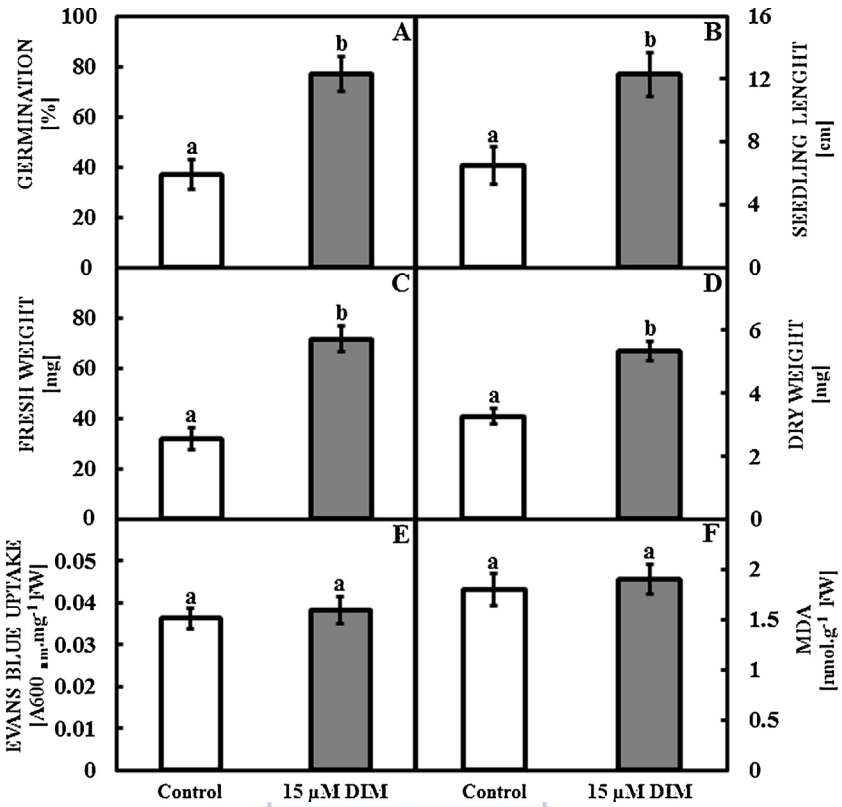


**Fig. 1.** The effects of control or 15 μM DIM treatments on *B. napus*. (A) seed germination percentage, (B) seedling shoot length, (C) seedling shoot fresh weight, (D) seedling shoot dry weight, (E) seedling shoot cell viability (as Evans blue dye uptake), (F) and seedling shoot MDA content. All evaluations were performed immediately at the end of the seedling stage (14 days). Means ± SE of three replicates from four independent experiments are represented and the same letter do not differ significantly at $P < 0.05$ according to the Tukey-Kramer test.

studies however, little is known about its role in plants. To date, only Pal et al. (2007) studied the effects of DIM derivatives on *O. sativa* growth in liquid medium after exposure for five days and concluded that exogenous application of DIM derivatives increased shoot and root length. It is important to note that unlike the Brassicaceae family *O. sativa* does not have the molecular machinery to produce DIM and therefore in this study we used *B. napus* as our plant of choice. *B. napus* or canola have emerged as an important plant in the production of edible oils for human consumption, food sources for livestock and oil for biodiesel. Therefore, in this study, we tested the germination rates of five commercial *B. napus* cultivars (Cobbler, Tawriffic, AV Garnet, Agamax and CB Jardee HT) in order to identify the cultivar with the lowest germination percentage. This cultivar would allow us to properly study increases in germination percentage under exogenous DIM treatment. Under our growth conditions AV Garnet was identified to be the lowest germinating *B. napus* cultivar (±40% seed germination) (Supplementary Fig. S1. A). AV Garnet seeds were pre-treated with different concentrations of DIM (0 μM, 2 μM, 5 μM, 10 μM and 15 μM) in greenhouse pot-experiments in order to identify the concentration at which the highest germination increase occurred. Under our experimental conditions, pre-treatment with 15 μM DIM increased the germination percentage of AV Garnet whereas no statistical difference in germination percentage was observe when seeds were pre-treated with 2 μM, 5 μM or 10 μM (Supplementary Fig. S1. B). We observed a ±100% increase in germination percentage when compared to the control for seeds pre-treated with 15 μM DIM (Fig. 1A). This result served as the basis for selecting 15 μM DIM as our concentration of choice to complete the study. Many authors have studied the endogenous levels of glucosinolates in Brassicaseae seeds (McGregor, 1988; Ciska et al., 2008; Feng et al., 2012) and have hinted of a link between endogenous changes in glucosinolate content and seed germination. However, no data currently exists for the indole glucosinolate DIM in plant emergence and development. Nevertheless, the available data suggests that glucosinolate content is highest in seeds and seedlings which subsequently decline with further development (Kondo et al., 1985; Palmer et al., 1987; McGregor, 1988). In addition, Ciska et al. (2008) reported that indole glucosinolate content increased from ±17% to ±50% during rape seed germination and correlated the glucosinolate increase with positive seed germination. Furthermore, a link exists between increase indole glucosinolate hydrolysis and formation of the plant hormone indole-3-acetic acid (IAA) (Bak et al., 2001). Roychowdhury et al. (2012) observed a germination percentage increase of ±75% upon IAA application of *Dianthus caryophyllus* seeds and concluded that IAA is an important hormone for overcoming seed dormancy. In this study, exogenous DIM increased seed germination of *B. napus* possibly via IAA production however this hypothesis is currently under investigation.

### 3.2. Exogenous DIM application increases B. napus seedling growth

To study the effect of exogenous DIM on seedling development and growth, we continued the treatment of germinated seeds (with control and DIM treatments) until the end of the seedling stage (14 days). We observed much taller seedlings when plants were exposed to DIM treatments (Fig. 2). Furthermore, the increase in seedling shoot growth correlated with increases in seedling shoot length (Fig. 1B, ±50% increase), seedling shoot fresh weights (Fig. 1C, ±100% increase) and seedling shoot dry weights (Fig. 1D, ±50% increase) when compared to the control plants. Indole glu-

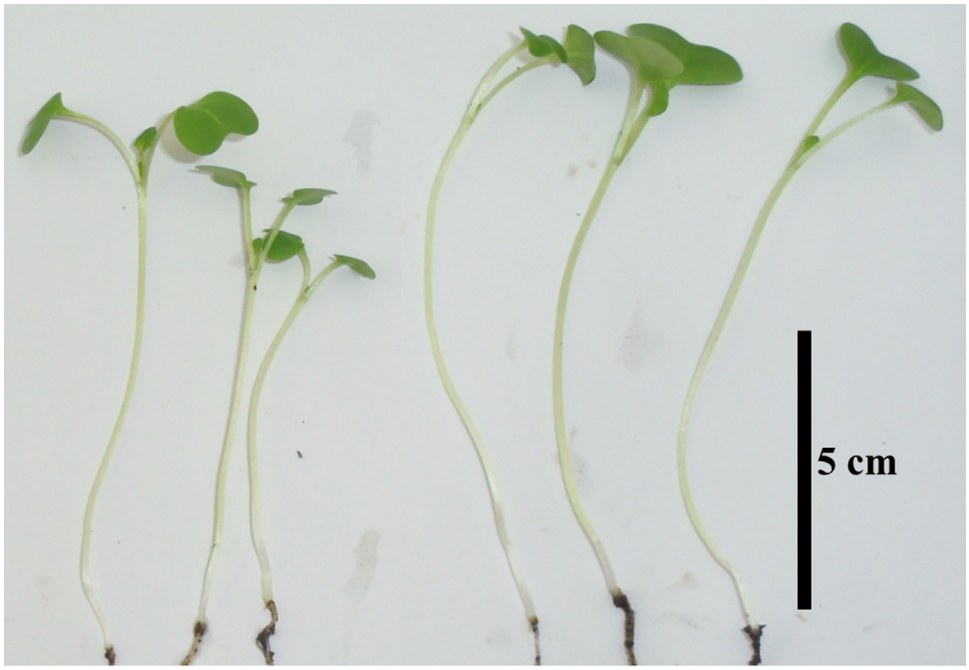


**Fig. 2.** Responses of *B. napus* seedling shoot to control or 15 µM DIM treatments after a 14 day growth period. Both treatments were prepared in deionized water and contain 0.009% Tween 80.

cosinolates are sulphur (S) and nitrogen (N) rich compounds which upon further breakdown could provide essential nutrients for plant growth (Del Carmen Martínez-Ballesta et al., 2013). Therefore, a positive link between indole glucosinolate increases *in planta* and an increase ratio of S:N have been reported (Zhao et al., 1999; Falk et al., 2007). In this study, exogenous DIM could have contributed to a higher level of S and N supply which is important for seedling shoot development as suggested by Nad et al. (2001) hence the production of seedling shoots with increase biomass.

### 3.3. An increase in superoxide and hydrogen peroxide contents in response to DIM application does not lead to increases in lipid peroxidation and cell death

To answer the hypothesis that exogenous DIM application may trigger responses in the Foyer-Halliwell-Asada cycle for signalling purposes in seed development we measured the levels of superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$) in *B. napus* seedlings. We observed an increase of ±23% for $O_2^-$ as well as an increase of ±49% for $H_2O_2$ in the DIM treated seedlings (Table 1) when compared to the controls. Research by Kranner et al. (2010) as well a review by El-Maarouf-Bouteau and Bailly (2008) highlighted the importance of $O_2^-$ and $H_2O_2$ in plant seed germination and seedling growth. More specifically, $O_2^-$ plays a role in root hair formation, cell division, cell elongation and cell differentiation whereas the more stable $H_2O_2$ plays a role in seedling growth and controlling the interplay between ROS and various hormones such as abscisic acid (ABA), gibberilic acid (GA) and ethylene (Bailly et al., 2008). Hara et al. (2013) observed an increase in $H_2O_2$ with an increase in glucosinolates which supports the findings in our study however, data on the interplay between indole glucosinolates and $O_2^-$ production is currently non-existent. Nevertheless, in our study the DIM induced ROS did not lead to enhance lipid peroxidation and subsequent cell death because we observed no statistical differences in MDA production (Fig. 1F; which indicates lipid peroxidation) as well as Evans Blue uptake (Fig. 1E; which indicates cell death) between the DIM treated seedlings and controls. Bailly (2004) suggested that increase ROS levels in seed germination will not always lead to lipid peroxidation and subsequent cell death if accompanied by increases in antioxidant enzymes for tight control. Therefore, in this study, we measured the enzymatic activity of two important plant antioxidant enzymes namely Superoxide dismutase (SOD, EC 1.15.1.1) as well as Ascorbate peroxidase (APX, EC 1.11.1.11). Our results indicate an increase of ±42% in SOD activity (Table 1) when exposing seedlings to DIM treatment. Furthermore, the link between SOD and increase indole glucosinolates is not yet studied. However, in our study, the SOD increase did not lead to complete dismutation of $O_2^-$ which possibly suggests a consistent production of $O_2^-$ under DIM exposure coupled with a steady increase in SOD activity. Moreover, the balance between $O_2^-$ production and SOD activity under DIM treatment does not only highlight the importance of $O_2^-$ as a signalling molecule but additionally reiterate the hypothesis of Bailly (2004) that antioxidant enzymes such as SOD play crucial roles in seed growth and development.

**Table 1**
The effects of control or 15 µM DIM treatments on biochemical responses of *B. napus* seedling shoots. All assays were performed immediately at the end of the seedling stage (14 days). Both treatments were prepared in deionized water and contain 0.009% Tween 80. Means ± SE of three replicates from four independent experiments are represented and the same letter do not differ significantly at $P < 0.05$ according to the Tukey-Kramer test.

| Biochemical assays | Control | 15 µM DIM |
|---|---|---|
| *ROS* | | |
| Superoxide (nmol $g^{-1}$ FW) | $5.31 \pm 0.121^a$ | $6.33 \pm 0.110^b$ |
| Hydrogen peroxide (nmol $g^{-1}$ FW) | $342 \pm 41^c$ | $511 \pm 43^d$ |
| *Antioxidant enzymes* | | |
| Superoxide dismutase (U $mg^{-1}$ protein) | $46.87 \pm 2.2^e$ | $66.38 \pm 1.3^f$ |
| Ascorbate peroxidase (nmol $min^{-1}$ $mg^{-1}$ protein) | $1.10 \pm 0.05^g$ | $1.35 \pm 0.07^h$ |

### 3.4. Exogenous DIM application increases superoxide dismutase and ascorbate peroxidase activities to regulate superoxide and hydrogen peroxide levels

Superoxide dismutase (SOD) enzymes dismutate $O_2^-$ to produce $H_2O_2$ and $H_2O$ which triggers responses in peroxide scavenging enzymes such as APX (to remove the $H_2O_2$). In this study, we observed an increase of ±23% in seedling APX activity (Table 1) when comparing the DIM treatment with the controls. The link between indole glucosinolates and APX activity could be explained by the known increase of $H_2O_2$ in response to glucosinolates. Ascorbate peroxidase (APX) oxidizes ascorbic acid for enzyme activation in order to convert $H_2O_2$ into $O_2$ and $H_2O$. Therefore, our observation of an increase in APX activity in response to DIM treatment was expected because indole glucosinolates co-localize with ascorbic acid as suggested by Bones and Rossiter (1997) and correlates to $H_2O_2$ production in plants. Furthermore, the increase observed in APX activity under DIM treatment did not remove all the $H_2O_2$ from the system which suggests a possible controlling mechanism for the more stable $H_2O_2$ to levels below the toxic range.

## 4. Conclusion

Our results suggest that exogenously supplied DIM could increase the germination percentage of *B. napus* ultimately leading to seedling shoot growth enhancement through possible induction of $O_2^-$ and $H_2O_2$. In fact, ROS such as $O_2^-$, $H_2O_2$ and hydroxyl ion ($OH^-$) play important roles in seed germination and seedling development. However, we did not observe any significant changes in $OH^-$ when comparing the control to the DIM treated seedlings (Supplementary Fig. S2. A). Furthermore, the increase in $O_2^-$ and $H_2O_2$ could be for signalling purposes rather than a cell death trigger response (no increase in cell death or MDA content were observed) with important contributions of SOD and APX activities which subsequently increased to regulate $O_2^-$ and $H_2O_2$ levels. This result was further supported by the observation that DIM treatments increased $O_2^-$ and $H_2O_2$ contents as well as SOD and APX activity in seedling shoots assayed at different time-points after germination (Supplementary Fig. S3). In our study, APX is the candidate enzyme in controlling $H_2O_2$ levels which increase during DIM treatment and not catalase (Cat). This result was supported by the fact that we did not observe any significant differences in Cat enzymatic activity when comparing the control and DIM treated seedlings (Supplementary Fig. S2. B). To our knowledge, this is the first report which indicates positive growth responses following exogenous DIM treatment in plants using pot-experiments and highlights the potential signalling role of DIM in early seedling development.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2016.03.013.

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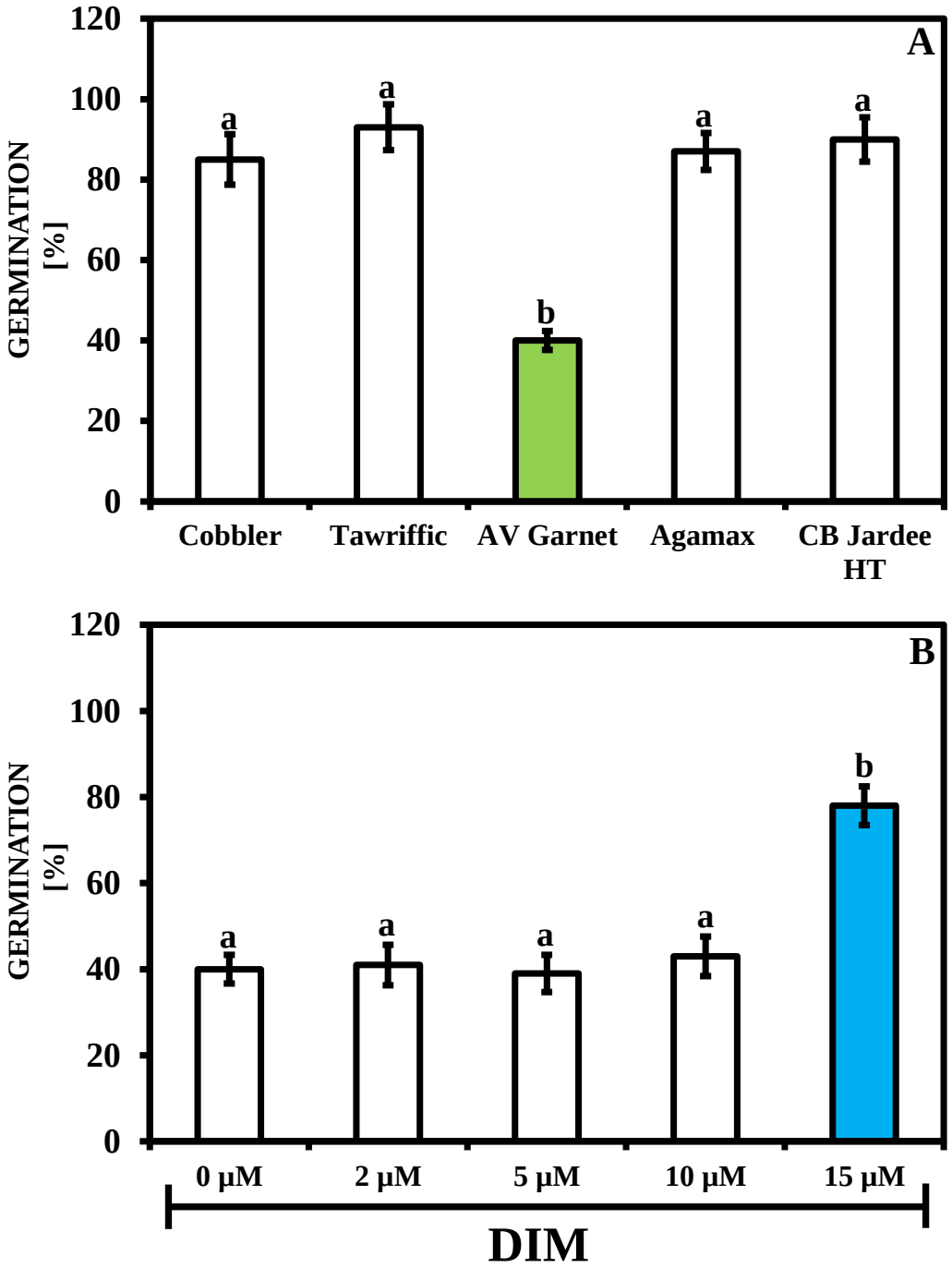
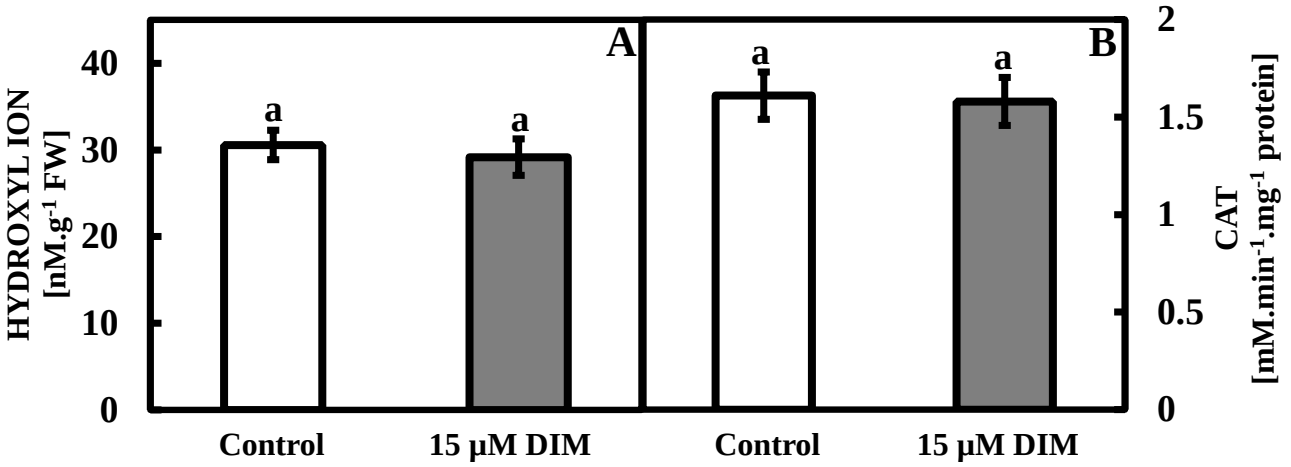
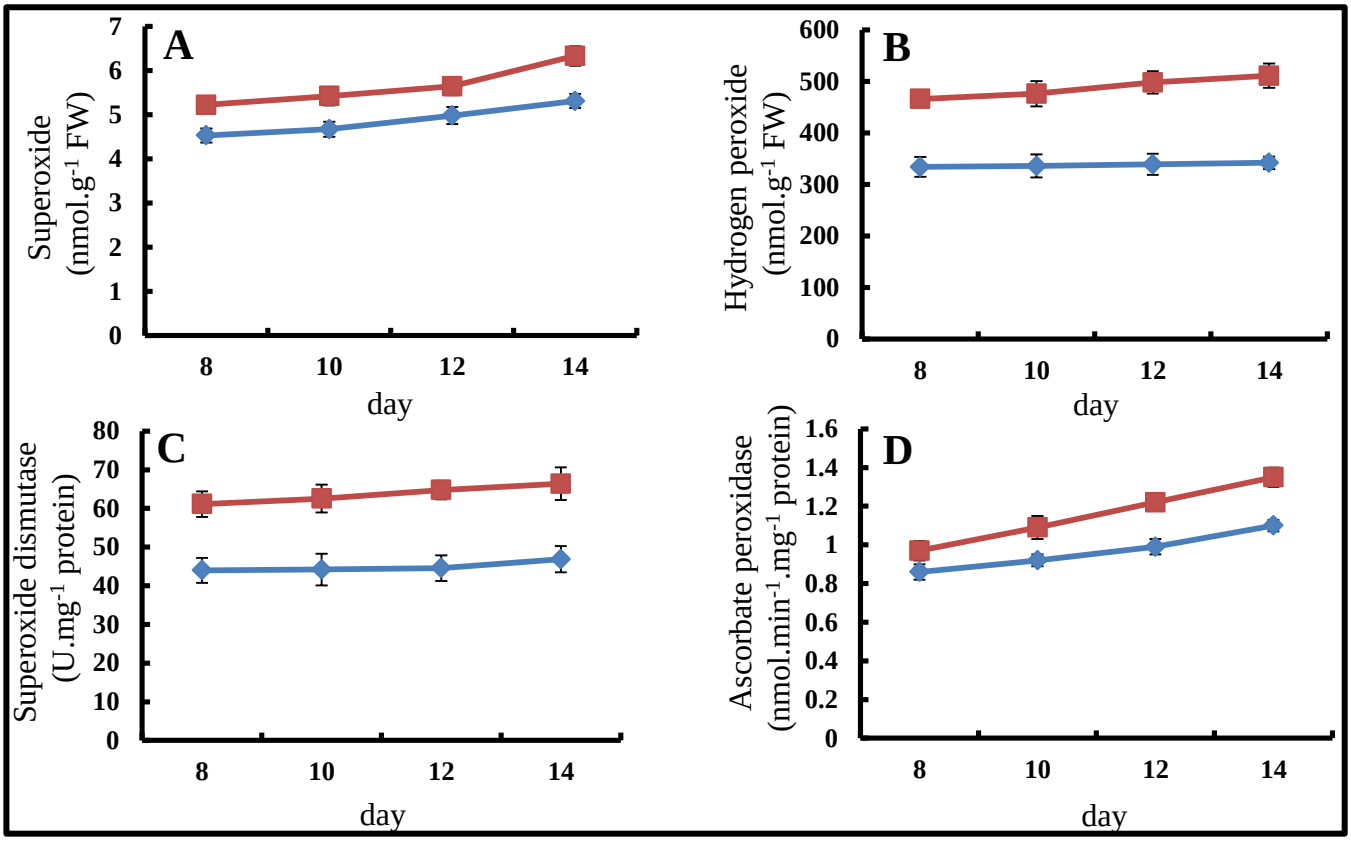
## Supplementary data




Supplementary Fig. S1. Measurement of *B. napus* seed germination percentage (%) in response to control and various concentrations of DIM treatments. (A) The responses of a variety of *B. napus* seeds to the control treatment. (B) The responses of AV Garnet seeds to different DIM concentration (0 µM - 15 µM). All treatments were prepared in deionized water and contain 0.009% Tween 80. Means ± SE of three replicates from four independent experiments are represented and the same letter do not differ significantly at $P < 0.05$ according to the Tukey-Kramer test.




Supplementary Fig. S2. Exogenous DIM application does not affect hydroxyl ion content and catalase activity in *B. napus* seedling shoots. (A) Hydroxyl ion content was measured in control and DIM treated plants according to Halliwell et al. (1987). (B) Catalase activity was measured in control and DIM treated plants according to Aebi (1984). All treatments were prepared in deionized water and contain 0.009% Tween 80. Means ± SE of three replicates from four independent experiments are represented and the same letter do not differ significantly at $P < 0.05$ according to the Tukey-Kramer test.




Supplementary Fig. S3. (A) superoxide content, (B) hydrogen peroxide content, (C) SOD activity and (D) APX activity in *B. napus* seedling shoots assessed 8 to 14 days after germination in control (blue line) and DIM (red line).

Supplementary Table 1. The effects of control or 15 µM DIM treatments on *B. napus* seed germination percentage.

| | Germination [%] | |
|---|---|---|
| **Genotypes** | **Control** | **15 µM DIM** |
| **Cobbler** | **81** | **100** |
| **Tawriffic** | **95** | **100** |
| **AV Garnet** | **40** | **81** |
| **Agamax** | **82** | **100** |
| **CB Jardee HT** | **82** | **100** |

## Supplementary Information

The AV Garnet seed lots used to complete this study were produced in January 2014 according to the manufacturer Agricol South Africa. Experiments commenced in April 2014.

The DIM emulsion becomes unstable at concentrations higher than 15 µM when using Tween 80 at 1.18%.

## Supplementary references

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# Appendix B: *Published article*
# 3,3’-diindolylmethane is a novel signalling molecule in Canola plants

Figure 1: Chemical compound and antioxidant enzyme production under herbivore feeding (e.g. animal bite).

Figure 2: A) Chemical compound (DIM, superoxide and hydrogen peroxide) and antioxidant enzyme (superoxide dismutase and ascorbate peroxidase) production under normal growth conditions. B) Chemical compound and antioxidant enzyme production under exogenous DIM application.

# 3,3'-diindolylmethane is a novel signalling molecule in Canola plants

Glucosinolates are organic compounds which exist in the Brassica family of plants and which are derived from glucose and amino acids and consists of sulphur and nitrogen in their structure. Glucosinolates exist as many different compounds in plants which can be distinguished by the different chemical side group linked to a central carbon. In the Brassica family of plants, glucosinolate precursors are often localized in myrosin cells for separation from the enzyme myrosinase. When plant cells are damaged, the myrosin cells gets damaged allowing the myrosinase enzyme to interact with glucosinolates. During this interaction, myrosinase removes the glucose from the glucosinolate and the remaining molecule spontaneously converts into thiocyanate, isothiocyanate or a nitrile. The final conversion in the catalysis, is often dependent on the given physiological conditions such as pH and the presence or absence of cofactors. The final products of the reaction are often toxic and therefore glucosinolates are released as defence molecules against herbivore action.

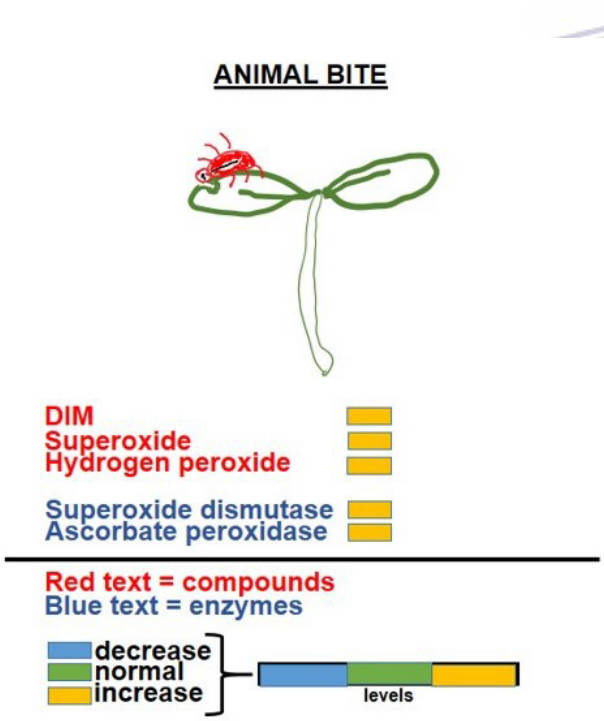


Fig. 1. Chemical compound and antioxidant enzyme production under herbivore feeding (e.g. animal bite).

One isothiocyanate compound namely 3,3’-diindolylmethane (DIM) have been extensively studied in mammalian systems especially in cancer research. This molecule effectively inhibit the

progression of a wide variety of cancers when tested against cell lines. However, knowledge about the role of DIM in Brassica plants are non-existent. Therefore, we developed an experiment to test the responses of *Brassica napus* (Canola) to DIM. Our hypothesis was based on what is known to happen under herbivore action (e.g. animal feeding) (Fig. 1) where the damage of the plant cell will result in DIM release. Furthermore, during this damaging process, a burst of reactive oxygen species (ROS) is normally expected. The production of ROS will often trigger changes in antioxidant enzyme activity in order to control the levels of ROS to below toxic levels. ROS does not only function in plant cell senescence responses but also perform key signalling roles in plants. Two ROS compounds namely superoxide and hydrogen peroxide have known signalling roles in seed germination as well as plant cell growth. However, to control the levels of these two ROS compounds within the range for signalling rather than cell death, antioxidant enzymes such as superoxide dismutase (which breakdown superoxide) and ascorbate peroxidase (which breakdown hydrogen peroxide) must be activated. Therefore, we hypothesized that a possible link could exist between DIM release and ROS production and if we could control the level of DIM through exogenous application then we should trigger ROS responses possibly within the signalling range. Furthermore, to control the range of ROS production we expected activation of antioxidant enzymes. This signalling cascade could lead to an increase in canola seed germination as well as seedling shoot growth.

In our study we observed that under normal conditions superoxide and hydrogen peroxide remain at levels below the toxic range (Fig 2.A). Subsequently, superoxide dismutase and ascorbate peroxidase activities remained at levels as previously described for canola seedling shoots. However, when we applied DIM to our canola seeds, we observed increased seed germination when compared to the control plants. When we allowed our germinated seeds to grow into seedlings, we observed that the DIM treated seedlings were bigger than the control seedlings (Fig 2.B). When we performed biochemical assays for ROS production we observed an increase in superoxide content as well as hydrogen peroxide content (Fig 2.B). Furthermore, we observed increases in superoxide dismutase and ascorbate peroxidase activity (Fig 2.B).

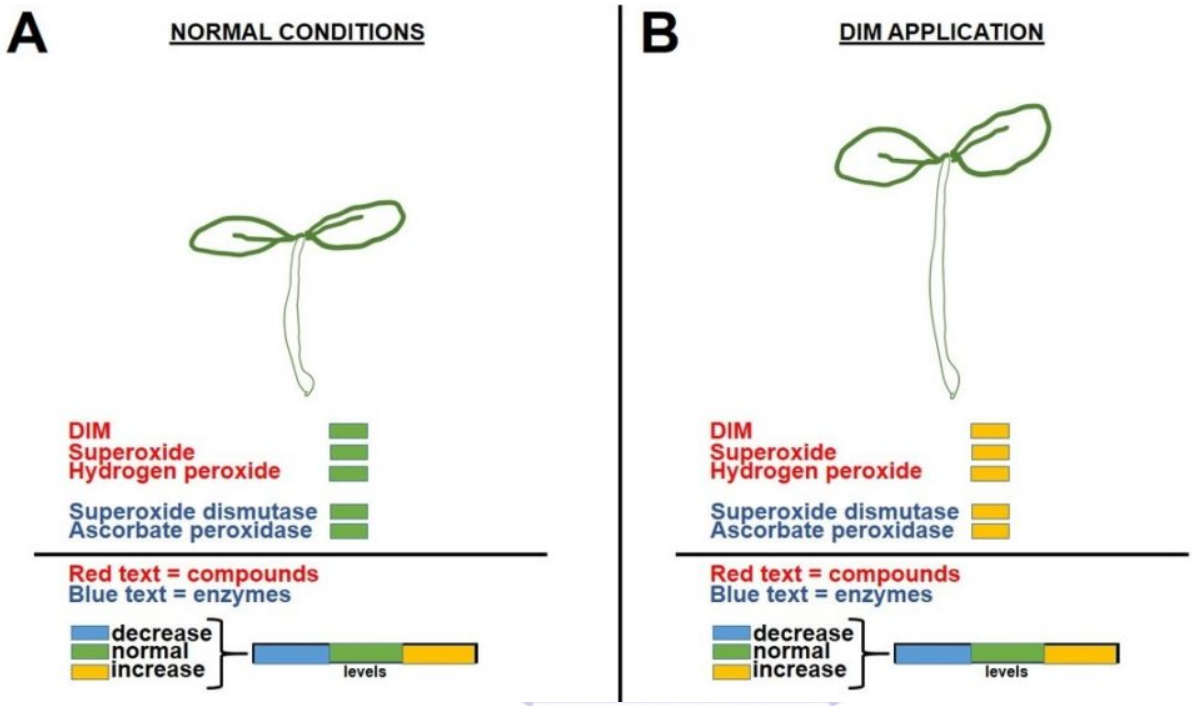


Fig. 2. A) Chemical compound (DIM, superoxide and hydrogen peroxide) and antioxidant enzyme (superoxide dismutase and ascorbate peroxidase) production under normal growth conditions. B) Chemical compound and antioxidant enzyme production under exogenous DIM application.



We concluded, that DIM stimulate production of superoxide as well as hydrogen peroxide which is regulated by increases in superoxide dismutase and ascorbate peroxidase activities. Furthermore, in our study, superoxide dismutase and ascorbate peroxidase activities is key in controlling superoxide and hydrogen peroxide to within levels for signalling. Thus, DIM is a potential novel signalling molecule in canola which controls superoxide and hydrogen peroxide content to increase seed germination as well as seedling shoot growth.

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## Publication

Exogenous 3,3'-diindolylmethane increases Brassica napus L. seedling shoot growth through modulation of superoxide and hydrogen peroxide content.
Gokul A, Roode E, Klein A, Keyster M

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