Comparative analysis of 3, 3'- diindolylmethane and indole-3-carbinol respective treatments in *Brassica napus* L.

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

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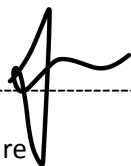
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List of Abbreviations

O_2^-	Superoxide
APX	Ascorbate peroxidase
AsA	Ascorbic acid
CAT	Catalase
DIM	3, 3'- diindolylmethane
EDTA	Ethylenediaminetetraacetic acid
H_2O_2	Hydrogen peroxide
I3C	Indole-3-carbinol
MDA	Malondialdehyde
NBT	Nitro blue tetrazolium
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid

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Keywords

3, 3'-diindolylmethane

Antioxidant enzymes

Ascorbate peroxidase

Catalase

Hydrogen peroxide

Indole-3-carbinol

Macronutrient

Micronutrient

Lipid peroxidation

Reactive oxygen species

Superoxide

Superoxide dismutase

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Mogamat Fahiem Carelse

MSc Thesis, Department of Biotechnology, University of the Western Cape

Abstract

Lately, there has been serious contamination of soils and ground water caused by mineral mining and environmental pollution leading to heavy metal accumulation within soils and over time rendering the soil infertile. The contaminated soil affects crop; yield, germination percentage and leads to seed dormancy. Additionally, as plants are sessile organisms, exposure to environmental stress involves different defense cycles and signalling molecules. Oxidative stress, increases the production of reactive oxygen species (ROS) at greater rates than that of the metabolism. Moreover, oxidative damage leads to the loss in cellular function and eventually death. Nonetheless, plants have adapted ROS-scavenging systems driven by reduction-oxidation reactions as defense mechanisms. In this way, cellular homeostasis is an essential defending process and finding means to optimize these reactions would benefit in the development of plants. Hence, environmentally friendly solutions are being reported such as using glucosinolates, a secondary plant metabolite found in relatively high concentrations within crucifers such as *Brassica napus* L. (*B. napus*) which has the ability to promote plant development. Once plant cells are injured, for instance, when crucifers are cut or crushed by herbivore or insects' action, this interaction of myrosinase and glucobrassicin results in the development of indole-3-carbinol (I3C). Furthermore, under-fitting conditions most likely acidic conditions two I3C molecules are capable of combining, creating the dimer 3, 3'- diindolylmethane (DIM). Nonetheless, DIM is seen as the more stable by-product of indole glucosinolate breakdown. Moreover, the breakdown of glucosinolates may also lead to further

signalling in plant defense responses. For this reason, using secondary plant metabolites such as glucosinolates to stimulate plant development seems promising. However, there are limitations in this field of research as most of the reviews currently available are based on the function of glucosinolates within mammalian cancer research. Little scientific knowledge for its dynamics in plant science is known. Thus, investigating the interplay of glucosinolate by-products; DIM and I3C on *B. napus* will help us understand how these secondary metabolites assist in plant defense mechanisms. Furthermore, *B. napus* is an important food crop and is used for animal feed in South Africa but low levels of it are produced due to lower utilization levels, seed dormancy and poor germination percentage.

In this study, we investigated the effects of exogenous 15 µM DIM or I3C treatment on *B. napus* plants. This was done to investigate how each treatment comparatively affects seed germination percentage, plant morphology, oxidative stress levels, antioxidant activities and the nutrient profile of the *B. napus* plants under each treatment. Our study revealed that the exogenous application of DIM was advantageous to *B. napus* plants because it improved growth, biochemical activities and the morphology of the plants possibility through the initiation of the reactive oxygen species (ROS) signalling pathway involving hydrogen peroxide (H_2O_2) and superoxide (O_2^-). This study further proves that DIM is used for signalling purposes and for maintaining ROS below toxic levels. Additionally the increase in the ROS signaled the DIM treated *B. napus* plants to increase their antioxidant capacity to help accommodate the increase in ROS. Contrastingly, I3C was toxic to *B. napus* plants because we observed increased ROS to detrimental levels. Moreover, it critically disturbs the balance of the reduction-oxidation reactions occurring in the Foyer-Halliwell-Asada cycle therefore the increase H_2O_2 and O_2^- levels triggered cell death in the I3C treated plants. Hence the increase in ROS under the I3C treatment down regulated the antioxidant enzymes with further resulted in the poor functioning of the I3C treated *B. napus* plants. Furthermore, DIM increased the uptake of essential nutrients which possibly aided in the steadfast development of the *B. napus* plants compared to the nutrient profile of I3C which plants as most of the major macro and micro nutrients was significantly decreased under the I3C treatment. The decrease in these nutrients further elucidated why the exogenous I3C treatment was so crippling to the *B. napus* plants.

Chapter 1

Literature Review

Introduction

Food prices are rising and will continue to rise (Graham *et al*., 2005). As early as 2006, global food prices for staples like wheat, corn, rice, and soybeans were increasing significantly (Holt-Giménez *et al*., 2008). Statistics released by Food and Agricultural Organization of the United Nations in 2011 showed that food prices are now at their highest since the 2008 crisis.

Agriculture is one of the main engines of economic growth in South Africa. However, feeding the vastly increasing population of this country has become a daunting task. While South Africa can be charactersised by a state of food security nationally, at the household level for much of the population, a trend to the contrary is apparent (Barrios *et al*., 2008,). It has been estimated that approximately 14 million people in South Africa are at risk of becoming part of the food insecurity division. Recent reports have shown that more than 1.5 million children mostly under age 6 suffer from malnutrition (Koch, 2010).

The government realized the importance of enabling the public to feed themselves and when this is not possible, safeguarding and providing strategic plans to assist. Furthermore, as much as the agricultural sector influences economic growth within the country, the mining sector is also considered one of the biggest contributors to economic growth within the country. However, the negative effects of climate change and mining create water shortage which destroys crops. Early seasonal changes makes it difficult to plant and harvest and increases desertification which reduces the amount of arable land available and ultimately disrupting food supply (FAO, 2010). Therefore, sustainable growth of crops in South Africa is needed.

Hence, in recent years plant research within South Africa has grown significantly to help the country overcome these obstacles. The "five per day" nutrition campaign encourages populace to include at least five or more servings of fruits and vegetables per day for better health which is supported by governmental, health and economical organizations (Meyer and Adam, 2008).

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Among the vegetable families the Brassica research has managed to maintain a greater pace for the past 15 years (Devi and Thangam, 2010). Cruciferous vegetables in the Brassica genus include broccoli, cabbage, cauliflower, and canola. Cruciferous vegetables have received much attention due to their anti-carcinogenic and antitoxic properties (Aggarwal and Ichikawa, 2005). Cruciferous vegetables are important sources of glucosinolates (Ugolini *et al.*, 2008) which is an anionic, hydrophilic plant secondary metabolites that plays a role in preventing cancer and other chronic diseases (Fahey *et al.*, 2003). Since glucosinolates affects the quality of human nutrition, understanding their natural biosynthetic pathway has become an area of interest as well as the manipulation of their levels in plants (Mitten *et al.*, 2000). Once plant cells are injured for instance when crucifers are cut or crushed by insects or herbivore action, the interaction of myrosinase and glucobrassicin results in the development of indole-3-carbinol (I3C) (Halkier and Gershenzon, 2006). I3C is one of the most widely spread phytochemicals that is specifically produced from the breakdown of indole-3-methylglucosinolate (Agerbirk *et al.*, 2009). I3C has the ability to form conjugates by reacting with many other plant metabolites. Moreover, due 0to their toxic effects the breakdown of glucosinolates may also lead to further signalling in plant defense responses (Clay *et al.*, 2009). In 2015, Katz et al. showed that the exogenous application of I3C to *Arabidopsis thaliana* plays a role in directly competing with auxins, such as the major plant growth hormone indole-3-acetic acid (IAA) in fine tuning plant growth and development. This showed that I3C inhibits plant growth.

Furthermore, under fitting conditions most likely acidic conditions two I3C molecules can combine to create the dimer 3,3'-diindolylmethane (DIM) (Pilipczuk *et al.*, 2015). Nonetheless, DIM is seen as the more stable by-product of indole glucosinolate breakdown and has been investigated significantly in mammalian research in cancer biology. The functions of DIM have been reported in many cancers including, colon cancer, liver cancer, breast cancer, gastric cancer and prostate cancer to name a few. According to Zhao et al. (2005), the key functions of glucosinolates in plants are for protection against insects, herbivores, and pathogens. In plants, the accumulation of phytochemicals frequently occurs as a result of stress including various signalling molecules or Reactive oxygen species (ROS) (Dörnenburg and Knorr, 1995). According to Shanker et al. (2005), ROS, toxicity occurs to be one of the main causes of low crop

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productivity worldwide. Recently, Gokul et al. (2016), showed that DIM increases seedling shoot lengths and seed germination through possible activation of the ROS signalling pathway involving H_2O_2 and O_2^- in *Brassica napus* (*B. napus*). This showed that DIM promotes plant growth.
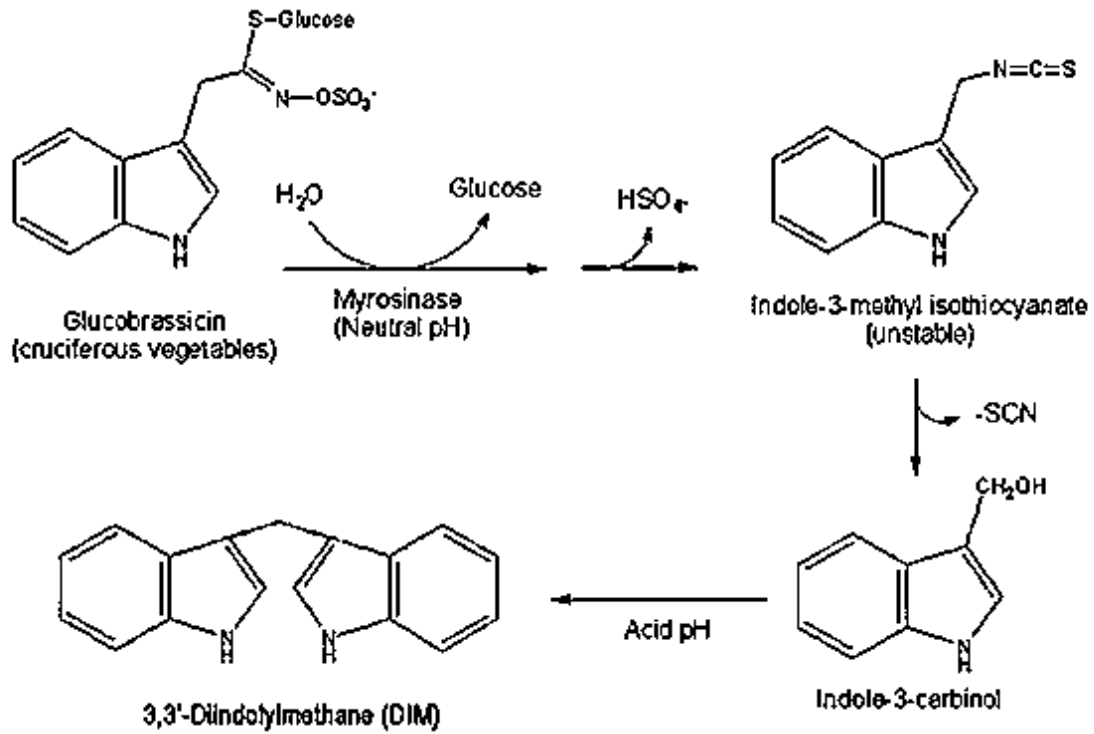
Figure1.1: Diagram showing the hydrolysis of glucobrassicin from cruciferous vegetables by myrosinase action. At neutral pH results in an unstable indole isothiocyanate that degrades to form indole-3-carbinol (I3C) and under acidic conditions the condensation of two I3C molecules combines to form the dimer 3, 3'-diindolylmethane (DIM) adapted from (Shertzer and Senft , 2002).

Production and signalling roles of Reactive Oxygen Species in plants

Plants have developed and adapted a signalling network to survive abiotic stresses which consist of various growth regulators to not only detect on the contrary also offer protection to the plant when stressed (Bhattacharjee, 2011). The increase of Reactive oxygen species (ROS) are important cues used by plants to respond to environmental stress (Bhattacharjee, 2011).ROS includes free radicals, alkoxyl radicals and non-radicals which are comprised of compounds such as hydroxyl radicals (OH^-), hydrogen peroxide (H_2O_2) and superoxide (O_2^-) (Sinha and Saxena, 2006). In unstressed plants ROS are produced at basal levels as by-products, however, the level of ROS is enhanced during stressful conditions and results in the leakage of electrons from electron transport activities (Gill and Tuteja, 2010). The production of ROS thus, has dual functions within plants, at basal levels it acts as a stress indicator whereas, the accumulation of these compounds may be damaging to the plant (Zhang *et al*., 2007). The build-up of ROS could potentially damage proteins, lipids, carbohydrates and DNA which would lead to cellular death (Maruta *et al*., 2012). In plants, ROS molecules can be generated within the mitochondria, cytosol, peroxisomes, chloroplasts and the apoplastic space (Mittler, 2002). Furthermore, as the chloroplast contains a high concentration of oxygen involved within the photosynthetic electron transfer system this makes them more susceptible to an increase in ROS. According to Zhang et al. (2007), the accumulation of ROS is believed to be as a result of the disturbance in the balance of the ROS production and the antioxidation systems. There are many developmental and metabolic processes that are controlled by cross-talk involving ROS and hormones (Zhang *et al*., 2007).

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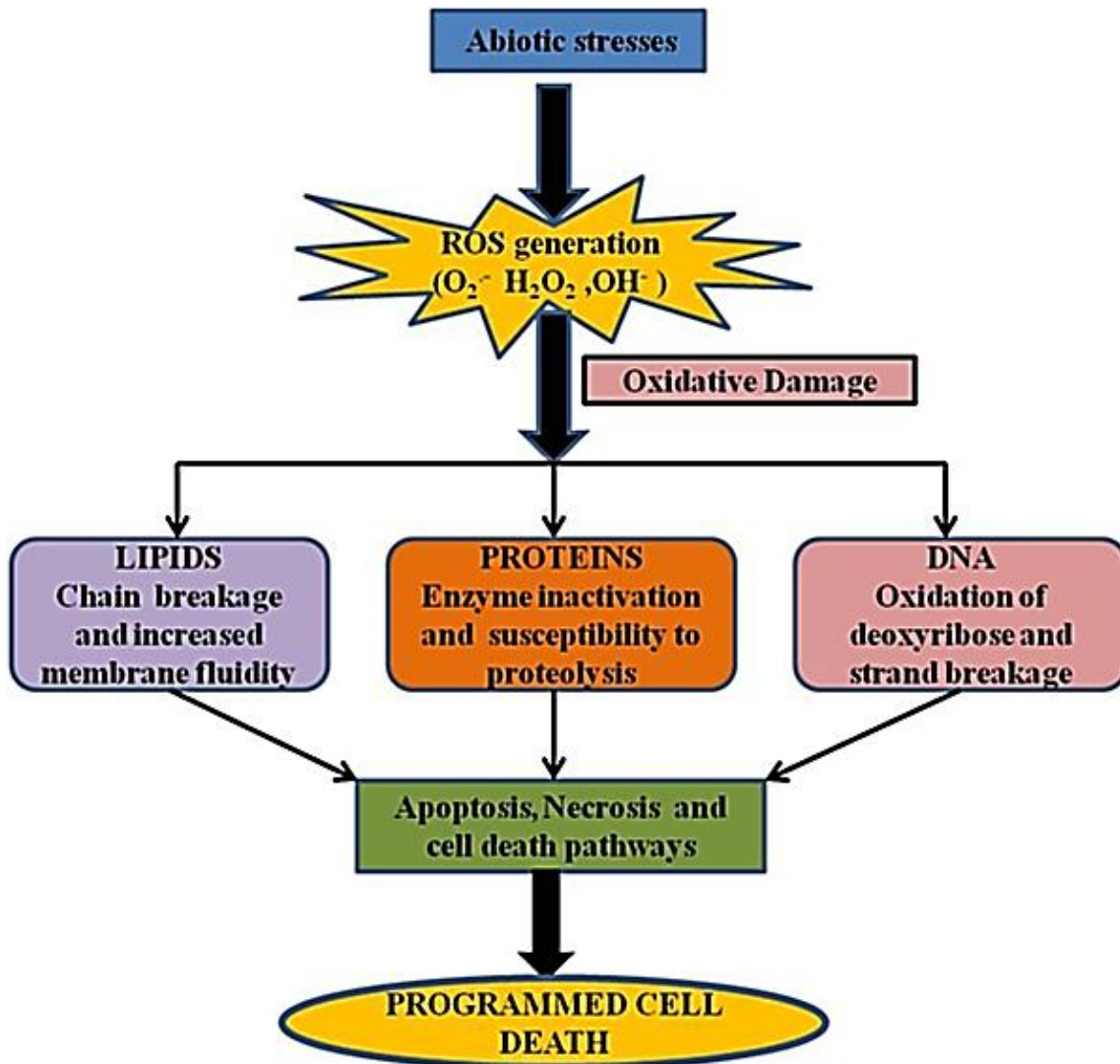
Figure 1.2: Diagram showing how stress in plants may lead to the development of Reactive Oxygen Species. How induced oxidative abiotic stress leads to organelle damage to lipids, proteins and DNA adapted from (Awasthi *et al*., 2015).

The production of Hydroxyl radicals

The most reactive compounds amongst the ROS in the presence of iron are the hydroxyl radical (OH^-) (Babbs *et al*., 1989; Gill and Tuteja, 2010). H_2O_2 and O_2^- may be converted into OH^- through the Fenton reaction (Gill and Tuteja, 2010). Plants permit access <u>to</u> a variety of different transition metals considering that they are needed in metabolic processes. However, once these metals have contained its own presence it can lead to the overproduction of radicals which results in cellular damage.

$$H_2O_2 + O_2 \longrightarrow OH\text{-} + O_2 + OH\bullet$$

Fe $^{2+}$, Fe^{3+} or (Other transition metals)
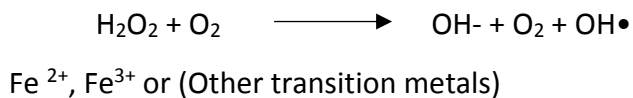
Figure 1.3: Hydroxyl radicals produced through a Fenton reaction using iron as the transition metal adapted from (Gill and Tuteja, 2010).

According, to Vranova et al. (2002), the proposed reason for the production of radicals is their involvement in regulating oxygen toxicity within plants. Furthermore, as radicals are naturally reactive they are able to react with most of the macromolecules that they come into contact with. These macromolecules include proteins, lipids and DNA which eventually leads to cellular damage and if they are not removed in time, it will lead to cell death (Gill and Tuteja, 2010).

The production of Superoxide

The production superoxide (O^{2-}) occurs through the incomplete reduction of oxygen within plants frequently during photosynthesis (Gill and Tuteja, 2010). It has been shown that up to 2% of the total oxygen consumption within plants will be due to the production of O^{2-} (Gill and Tuteja, 2010). The electron acceptor bound to the thylakoid of the photosystem I is seen as a major site for the development of O^{2-} (Boveris and Puntarulo, 1998; Gill and Tuteja, 2010). The half-life of O^{2-} is 2-4 microseconds. Furthermore, O^{2-} is also one of the first ROS produced and usually results in the production of other ROS (Halliwell, 2006). When these other ROS are initiated this leads to the preceding production of these ROS which leads to damage within plant organelles such as lipid peroxidation and cell structure.

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The production of Hydrogen Peroxide

The reduction of O_2^- may result in the formation of Hydrogen peroxide (Gill and Tuteja, 2010). Furthermore, although H_2O_2 is said to be moderately reactive it has a longer half-life at 1 millisecond when compared to other ROS (Gill and Tuteja, 2010). Thus, due to its high stability and longer half-life it is seen as a competent signalling molecule as it is able to migrate moderately large distances along with permeating across membranes. Even though H_2O_2 is moderately constant, at higher concentrations it has the ability to inactivate enzymes through oxidizing thiol groups (Cheeseman, 2007; Tewari *et al*., 2006). Considering this, it can be seen why H_2O_2 is an extremely dangerous compound to many organisms. Therefore, at low concentrations H_2O_2 is used as a signalling molecule but at high concentrations, it initiates programmed cell death in plants (Gill and Tuteja, 2010; Quan *et al*., 2008). In addition O^{2-} to being used as a signalling molecule H_2O_2 has been identified to be a regulator for processes such as photosynthesis and senescence in plants.

Downstream effects of Reactive Oxygen Species in plants

As previously stated ROS performs dual functions in plants; acting either as signalling cues or in the development of toxic by-products which leads to organelle damage. This results in lipid peroxidation leading to an increase in lipid fluidity, protein damage causing proteolysis, chlorosis damage to chlorophyll species and DNA strand breakage as a result of deoxyribose oxidation. These damaging effects eventually results in necrosis, apoptosis and ultimately programmed cell death.

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Lipid peroxidation

Lipid peroxidation is defined as the catalytic alteration of the structure and function of a membrane (Verma and Dubey, 2003; Yadav, 2010). As stated before ROS is over produced by the plant when it undergoes stress. Moreover, these free radicals have the ability to disturb the polyunsaturated fatty acids and eventually lead to lipid peroxidation (Verma and Dubey, 2003). Malondialdehyde (MDA) is a cytotoxic compound that is produced during lipid peroxidation and can thus be utilized as an indication of lipid peroxidation production, radical production, and oxidative damage caused to an organism (Zhang *et al*., 2007). The consequences of lipid peroxidation consists of the weakening of the cell membrane thus affecting the permeability of the cell and results in the loss of essential ions for instance potassium ions (Sinha and Saxena, 2006). The production of MDA and thiobarbituric acid is used as an indication of the degree of lipid peroxidation within the tissue (Verma and Dubey, 2003).

Chlorosis occurring within plant material

During photosynthesis chlorophyll is the most important pigment involved in the absorption of light energy (Taiz and Zeiger, 2010). Chlorophyll is also the most abundant pigment and is responsible for the iconic green colour associated with plants in general (Hörtensteiner and Krautler, 2011). However, when the plant fails to produce chlorophyll and/or any disruption of the chlorophyll occurs, this leads to chlorosis which is the abnormal yellowing of plant tissue (Abadia *et al*., 2011; Fatoba and Emem, 2008). It is also important to note that the chloroplast is sensitive to oxidative stress. Therefore an increase in ROS can lead to chloroplast damage and will lead to a decrease in the production of the chlorophyll eventually leading to chlorosis (Mourato *et al*., 2012; Yadav, 2010). Then, if the plant is unable to reverse chlorosis then its ability to photosynthesize will be hindered which will lead to nutrient deficiency and in due course death (Wang *et al*., 2005).

Damage to DNA

The plant genome is conveyed to be greatly stable however, damage can occur if it comes into contact with DNA damaging compounds (Gill and Tuteja, 2010). Damage to the DNA may be caused by OH^- or singlet oxygen under stressful conditions. OH^- has the ability to attack the primary structure of the DNA including the pyrimidines and purines. Furthermore, the guanine nucleotides within the DNA are damaged by the singlet oxygen molecules (Wiseman and Halliwell, 1996). The damage caused by these reactive molecules involve the modification and deletion of bases and strand cleavage (Tuteja *et al*., 2001). It is important to understand that the more stable ROS such as H_2O_2 and unstable 0_2^- does not directly cause damage to the DNA within in plant cells however, the consequences of DNA damage leads to a decrease in protein synthesis.

Antioxidant enzymes prevalent in plants

As a result of the disparaging nature of reactive compounds, plants uses ROS-scavenging pathways as control measures (Bhattacharjee *et al.*, 2011). The ROS-scavenging pathways has the ability to metabolize ROS and thus reduce their concentration within plants decreasing the toxic effects caused by ROS (Mahanty *et al*., 2012). It has been found that ROS-scavenging enzymes such as catalase (CAT), ascorbate (APX) and superoxide dismutase (SOD) is very important in remediating these toxic levels within plants. SOD is one of the first enzymes scavenged in this pathway SOD converts O_2^- to H_2O_2, the H_2O_2 is then removed by APX by oxidizing ascorbate to monohydroascorbate (Lee *et al*., 2007; Wang *et al*., 2005).

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Superoxide dismutase

Superoxide dismutase (SOD) consist of a group of metallo-proteins that has the ability to dismutase the O_2^- to and H_2O_2. Furthermore, SOD produces H_2O_2 that is scavenged further down through ascorbate peroxidases and catalases. Once plants are subjected to stress they react by producing increased levels of ROS and SOD offers the primary line of defense and thus is essential in plant stress tolerance (Chaparzadeh *et al*., 2004; Mittler, 2002).

$$O_2^- + O_2^- + 2H \longrightarrow 2H_2O_2 + O_2$$

Figure 1.4: Superoxide dismutation reaction using Superoxide as a substrate adapted from (Gill and Tuteja, 2010).

SOD eliminates O_2^- therefore, reducing the risk of OH^- formation faster than spontaneous dismutation through the Habber-Weiss-type reaction. Superoxide dismutases are classified according to their metal cofactors it needed for proper functioning (Mahanty *et al*., 2012; Wang *et al*., 2005). These metal cofactors includes: manganese (Mn-SOD), copper/zinc (Cu/Zn-SOD) and the iron (Fe-SOD) along with nickel localized in different cellular compartments (Mittler, 2002). Eukaryotic organisms have certain metal superoxide dismutases that are located inside their specific organelles, for instance Mn-SOD is located in the peroxisomes and mitochondria; in higher plants Cu/Zn-SOD can be found in the cytosolic fractions and the chloroplast (Del Rio *et al*., 2003; Lee *et al*., 2007). Even though Fe-SOD isozymes are hardly ever found in higher plants, when they are found, they localize in the chloroplast. SOD isozyme activity can be detected by their sensitivity to KCN and H_2O_2. Cu/Zn-SOD is sensitive to both inhibitors whereas, Mn-SOD is resistant to both inhibitors. Furthermore, Fe-SOD is resistant to KCN but sensitive to H_2O_2. (Alscher *et al*., 2002).

Catalase

Catalases (CAT) are crucial tetrameric heme containing enzymes involved in ROS detoxification when plants are under stress. The CAT detoxification reaction is regarded by the direct dismutation of two H_2O_2 molecules into O_2 and H_2O. This reaction decreases the H_2O_2 concentration and result in the protection of cells from oxidative damage (Mhamdi *et al*., 2010). The majority of plants have either one of two isoforms of CAT enzymes present. These isozymes localize either in the cytosol or peroxisomes (Mhamdi *et al*., 2010; Petrova *et al*., 2004).

Ascorbate peroxidase

Ascorbate peroxidase (APX) localizes in the chloroplast and is considered as a vital antioxidant enzyme. APX scavenges H_2O_2 in a process forming H_2O as a by-product and dehydroascorbate (DHA) as the final product using ascorbic acid which serves as an enzyme co-factor and directly scavenge O_2, OH^- and singlet oxygen (Kornyeyev *et al*., 2003; Maruta *et al*., 2012). The enzymes are found in the cytosol, chloroplasts and within the peroxisomes of plants (Sinha and Saxena, 2006). A particular level of ascorbate has to be kept within the cell. This is done by dehydroascorbate reductase by means of recycling DHA back into ascorbate by oxidizing reduced glutathione (GSH) (Moller, 2001). Ascorbate peroxidase scavenges ROS and thus offers a way of protecting plants (Gill and Tuteja, 2010). Ascorbate peroxidase uses ascorbate as an electron donor which is involved in H_2O_2 scavenging (Noctor and Foyer, 1998). However, it is important to note that according to an experiment done by Lee et al. (2007), it was observed that when one antioxidant increases it may not always result in an increase in ROS-scavenging or even lead to the tolerance in stress. Furthermore, in contrast with other antioxidant enzymes, APX has a superiority in affinity for H_2O_2 and therefore might have an important role in ROS management during stress.

Antioxidant compounds in plants

There are many non-enzymatic antioxidant compounds produced within plants. This includes ascorbic acid and glutathione, which exists either in its reduced or oxidized form depending on the reaction. According to the Foyer- Halliwell- Asada cycle proposed in 1976 reduced gluthione (GSH) scavenges OH^-, O_2^- and H_2O_2 and restores ascorbic acid (AsA) through the AsA-GSH cycle, which provides an antioxidative defense system to compensate for the increase in oxidative stress.

Ascorbic acid

Ascorbic acid (AsA) found in plants serves as a key contributor to the cellular redox state (Smirnoff, 2000). The major role of AsA is protecting plants against stress caused by pathogenic attack. AsA is produced at different sites indirectly to respond against pathogenesis (Pastori *et al*., 2003; Pavet *et al*., 2005). During attack ascorbic acid's antioxidant role, is to act as a detoxification substrate against ROS. Ascorbic acid is used with APX to converts H_2O_2 into H_2O (Traber and Stevens, 2011). Furthermore, AsA has the ability to serve as an enzyme co-factor and directly scavenge O_2^-, OH^- and singlet oxygen (Eskling *et al*., 1997). Other roles of AsA include progression through cell cycle and regulation of cell elongation and (Horemans *et al*., 2000). A study done by Zhang et al. (2007), proved this by demonstrating that an increase in H_2O_2 can be induced by exogenous application of ascorbate and thus acts as a scavenger for H_2O_2.

Glutathione

Glutathione has been discovered to be relatively abundant within plant cells and has been considered as one of the crucial intercellular defense metabolites against ROS induced oxidative damage (Mittler and Zilinskass 1992; Herna *et al*., 1998). In plant tissues including the mitochondria, vacuoles and the chloroplast it is found ubiquitously in its reduced form (Yadav, 2010). Moreover, it plays an essential part during physiological processes, for example sulphate transport regulation, expression of stress reactive genes, and detoxification of xenobiotics, signal transduction as well as metabolite conjugation (Xiang *et al*., 2001; Mullineaux and

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Rausch, 2005). Reduced glutathione (GSH) is also important during plant development such as cell differentiation, pathogen resistance and enzymatic regulation (Rausch and Wachter, 2014). Furthermore, GSH is vital for upholding a normal reduced cell state in this manner lessening inhibitory effects caused by induced oxidative stress (Meyer, 2008). GSH scavenges OH^-, O_2^- and H_2O_2 and restores AsA through the AsA-GSH cycle, which provides an antioxidative defense system (Foyer and Halliwell, 1976). It has been proven that when there is a decline in GSH concentrations the redox state becomes more oxidized when stress levels are raised. Consequently resulting in the deterioration of the system (Tausz *et al.*, 2004).
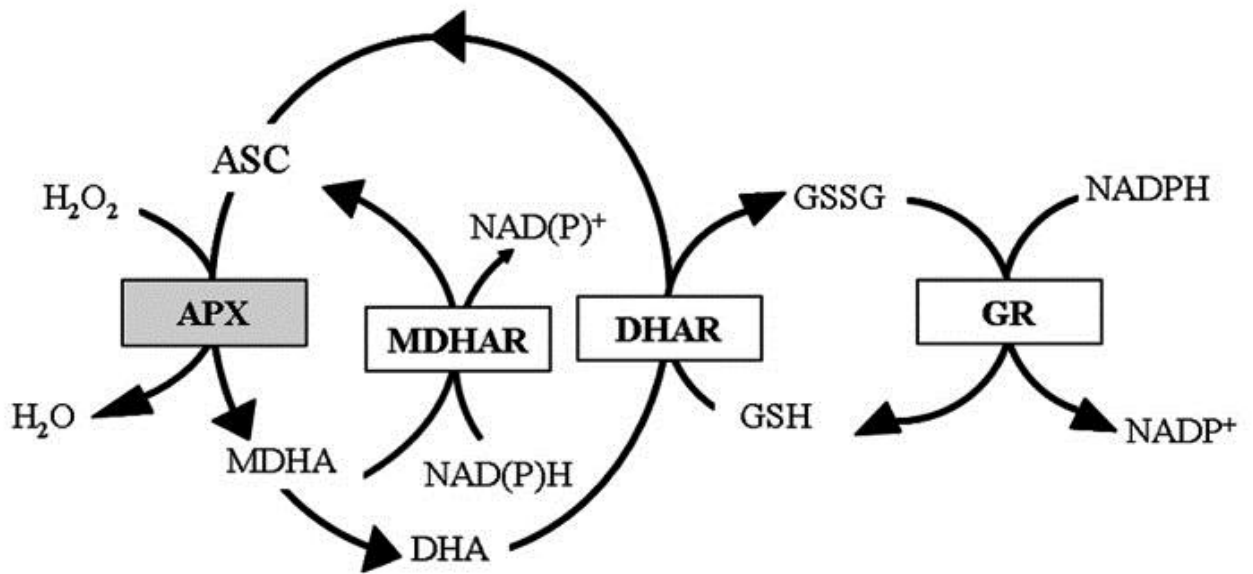
Figure 1.5: The Enzyme and intermediates involved in the Foyer- Halliwell- Asada cycle. In this cycle the white boxes represents enzymes involved both animal and plant cells whereas APX in the grey box is only involved in plant cells adapted from (Foyer and Halliwell, 1976).

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Secondary metabolites as signalling molecules and their functions in plants

As plants are sessile organisms they require a large number of defense compounds which includes primary and secondary metabolites. During normal plant growth and development, secondary metabolites are produced and play pivotal roles in plant defense mechanisms. They act as, for instance, phytoalexins and phytoanticipins collectively defined as phytoanticipins. Secondary metabolites are categorized in three groups.

1.   Flavonoids and allied phenolic and poly phenolic compounds

2.   Terpenoid compounds

3.   Nitrogen and sulphur containing alkaloid compounds

Role of Glucosinolates as secondary metabolites within plants

Glucosinolates are classified as sulphur-and-nitrogen containing plant secondary metabolites. (Fenwick *et al*., 1983). They are common in the *Capparales* order consisting of the *Brassicaceae* family that is composed of agriculturally important crops such as *Brassica* vegetables. All glucosinolates has similar core structures consisting of a B-D-thioglucose group linked to a sulfonated aldoxime moiety with a variable side chain that is derived from amino acids and their precursors. Additionally, glucosinolates are divided into three separate groups according to their different amino acid precursors; aliphatic glucosinolates which derived from Methionine, Isoleucine, Leucine or Valine aromatic glucosinolates consisting of Phenylalanine or Tyrosine and lastly indole glucosinolates derived from Tryptophan (Wittstock and Halkier, 2002). A plant that has the ability to accumulate glucosinolates continuously has a thioglucosidase that is known as myrosinase. Myrosinase has the ability to catalyse the hydrolysis glucosinolates to various compounds possessing a range of biological compounds. However, this reaction can only occur once plant tissues are disrupted as a result of physical damage or pathogenic attack because this is the time the myrosinase enzymes comes into contact with its glucosinolate substrates (Bones and Rossiter, 1996; Rask., *et al.*, 2000).Thus, glucosinolates and their

hydrolysis products are often examined as a means of plant defense systems when they are faced with abiotic and biotic stress (Fahey *et al.*, 2001).

Indole glucosinolates and their biological function in agriculture and nature

Indole glucosinolates are derived from tryptophan and possess a variable R group side chain found in cruciferous plants. Modifications of indole glucosinolates occurs via hydroxylations and methoxylations that are catalysed from a variety of different enzymes (Petersen *et al.*, 2002). The content and types of indole glucosinolates within different organs found in plants are dependent on the environmental stimuli. Four different indole glucosinolates are most commonly found in *Brassica* species which includes: glucobrassicin, 4-methyoxyglucobrassicin, neoglucobrassicin and 4-hydroxyglucobrassicin (Brown *et al.*, 2003; Petersen *et al.*, 2002).

Nutritional status of the plant and its impact on glucosinolate-myrosinase system

High amounts of sulphur are needed by most brassica species and it has been suggested that glucosinolates themselves contains high amounts of sulphur which can be mobilized through the hydrolysis of myrosinases and can provide the sulphur needed by the brassica species (Ceynowa and Schnug, 1990). Furthermore, glucosinolates also contains relatively high amounts of nitrogen. According to an experiment done by Zhao et al. (1999), there were a clear influence of both sulphur and nitrogen supply on glucosinolates in *Brassica napus* (B .*napus*) (Mailer, 1989). Their results indicated that the increase in nitrogen supply favoured the hydrolysis step which coverts 3-butenyl to (2R)-2-hydroxyl-3-butenyl glucosinolates. Furthermore, aliphatic glucosinolates seemed to be more sensitive to sulphur deficiency when compared to indole glucosinolates caused by the already sulphur containing precursor Methionine. Moreover, a number of studies has proved that with an increase in sulphur availability there was an increase in glucosinolate content, while a decrease resulted in a decrease of free sulphate and glucosinolates, but there was an increase in myrosinase activity (Chisholm *et al.,* 1980; Gupta, 1990). However, recent studies has proven that sulphate is the primary source containing the sulphur compound which is stored in the vegetative tissue (Zhao *et al.*, 1999). Furthermore, sulphur measurements within *B. napus* showed that glucosinolates contained only but a small amount of the crops over-all sulphur content (Fieldsend and Milford,

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1997). In addition, more recently, a combination consisting transcript and metabolite profiling showed coordinated repression of most glucosinolate pathway genes in response to sulphate limitation (Hiraimy *et al.*, 2005).

Plant hormone Indole-3-acetic acid

Plant hormones control or influence a wide selection of physiological and cellular processes. This includes bud dormancy, cell division, leaf abscission, flowering, cell enlargement, seed germination, seed dormancy and fruit ripening. One of the most essential plant hormones is auxins. It is responsible in root formation and in the differentiation between xylem and phloem (Kelen *et a*l., 2004). Furthermore, auxins also mediates the tropism (bending), response to light and gravity. A common natural auxin occurring in plants is indole-3-acetic acid (IAA) which is one of the most physiologically active auxins derived from tryptophan dependent and independent pathways in plants. IAA plays important roles in root elongation as well as the formation of hair and lateral roots which is crucial in nutrient uptake (Datta and Basu, 2000).IAA also promotes cell elongation by the modification of osmotic contents within the cell, water permeability is increased into the cell, and wall pressure is decreased. Furthermore, IAA also increase the synthesis of proteins. According to Zhao, 2010 IAA promotes embial activity, by the inhibition or delay in abscission of leaves and promotes fruiting and flowering.

Glucosinolate metabolism in growth regulation

It has been proposed that indole glucosinolates are precursors for the plant hormone indole-3-acetic acid (IAA). Moreover, indole glucosinolates are allegedly hydrolyzed to indole acetonitrile (IAN) that can be further hydrolyzed to IAA via nitrilases (Bartel and Fink, 1994; Searl et al., 1982). During indole glucosinolate biosynthesis the first intermediate, Indol-3-acetaldoxine which is also a product of the catalyses by CYP79B2 and CYP79B3 has been shown to be a precursor of IAA and as well as being the branch point between the two metabolic pathways (Bak *et al.*, 2001). It has been proven that if plants overexpress CYP79B2 there was an increase in their levels of glucosinolates and IAA. However, another partway was suggested for *Arabidopsis* as cyp79B2 and cyp79B3 was strongly deficient indole glucosinolates and partially deficient in IAA.

Justification

The main reason for low crop productivity in South Africa is caused by three devastating factors which includes; seed germination percentage, seed dormancy and infertile soil. Therefore, finding means to overcome these challenges would be a great benefit to the agricultural industry in South Africa Furthermore, as plants are sessile organisms, exposure to environmental stress involves different defense cycles and signalling molecules. Oxidative stress enhanced ROS production consists of the uncontrolled oxidation of ROS leading to cellular death. Nonetheless, plants have adapted ROS-scavenging systems as defense mechanisms. Along these lines, cellular homeostasis is an essential defense process and finding means to assist in these reactions would benefit in plant growth and development. Hence, using secondary plant metabolites such as glucosinolates to stimulate plant development seems promising. However, there are limitations in this field of research as most of the reviews currently available are based on the function of glucosinolates within mammalian cancer research. Little scientific knowledge for its dynamics in plant science is known. The only work available is the work done by Katz et al. (2015), which proved that I3C acts as an auxin antagonist in *Arabidopsis thaliana.* Whereas, research done by Gokul et al. (2016), contradicts this showing that DIM increases seedling shoot length and seed germination by means of modulating H_2O_2 and O_2^- content. Thus, investigating the interplay of glucosinolate by-products; DIM and I3C on *B. napus* will help us understand how these secondary metabolites assist in plant defense mechanisms. Furthermore, *B. napus* is classified as one of the second largest cruciferous oilseed, crops cultivated in the world. Canola meal, the by-product of canola oil processing that contains high levels of proteins is used for animal feed in South Africa. However, in South Africa large quantities of this animal feed has to be imported each and every year due to lower levels of production caused by lower utilization levels, seed dormancy and poor germination levels for canola in the country. Therefore, sustainable growth of *B. napus* is needed to help increase sustainable canola production to thus reduce the quantity of canola imports, help combat malnutrition in the country as *B. napus* is a protein rich crop and reduce food insecurity

Objectives and Aims of study

The objective of this study is to investigate and compare what effects the exogenous application of DIM and I3C has on the morphology, biochemistry and nutrient profile of *B. napus* plants that will be treated with either compound. The aims of this project includes observing whether DIM and/ or I3C has any growth promoting properties on *B. napus* plants after treatments. This set of aims includes investigating germination percentage, plant biomass, lipid peroxidation levels and Chlorophyll damage. The next set of aims will be to identify how ROS and the antioxidant concentrations changed in response to the exogenously applied DIM or I3C treatments. Then inductively coupled plasma optical emission spectroscopy (ICP-OES) will then be employed to investigate how the macronutrient and micronutrient concentrations in the plants differ according to their respective treatments. By investigating the interplay of these glucosinolate by-products on *B. napus* the information obtained could help us understand how these secondary metabolites assists in plants defense mechanisms and bridge the scientific gap pertaining the lack DIM and I3C knowledge in plant science.
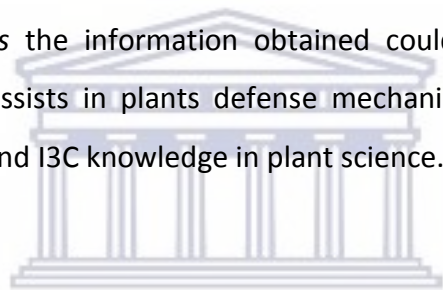
Chapter 2

Material and methods

Table 2.1: List of chemical supplies and suppliers

3,3'-diindolylmethane	Sigma- Aldrich
2- Thiobarbituric acid	Sigma- Aldrich
30% acrylamide solution 37.5:1	Sigma- Aldrich
Acetone	Sigma- Aldrich
Dipyridyl	Sigma- Aldrich
Ethanol 200 proof	Sigma- Aldrich
Filter Sand	Cape Silica
Garnet Seeds	Agricol
Phosphoric Acid	Sigma- Aldrich
Indole-3-carbinol	Sigma-Aldrich
Iron(ii)Chloride	Sigma- Aldrich
L- Ascorbic acid	Sigma- Aldrich
L- Glutathione reduced	Sigma- Aldrich
Nitro blue tetrazolium chloride Monohydrate	Sigma- Aldrich
Nitric Acid	Sigma-Aldrich
Potassium hydroxide	Sigma- Aldrich
Potassium phosphate dibasic	Sigma- Aldrich
Potting soil	Pick 'n Pay

Quick start Bradford dye reagent 1x	Bio-Rad
Sodium dodecyl sulphate	Bio-Rad
Trichloroacetic acid 99%	Sigma- Aldrich
Tris (hydroxymethyl) amino-methane	Sigma- Aldrich

Preparation of DIM and I3C solution

The DIM and I3C [sigma; ≥98% (HPLC)] stocks was prepared as follows: 10 mg and 6 mg of DIM and I3C was homogenized in 235 µl Tween 80 and was made up to a final volume of 10 ml using 99.8% (v/v) ethanol respectively. The solutions was then snap frozen using liquid nitrogen and freeze dried at -41°C over a 72 hour period in a Lyophilizer. After that 19.77 ml of nuclease free water was added to the DIM and I3C solutions respectively this resulted in a 2 mM stock of each solution [containing 1.18% (v/v) Tween 80]. The control was prepared in the exact same manner as mentioned for the preparation of DIM and I3C but did not contain DIM or I3C powder (Tween 80 final concentration 1.18%).

Plant growth, parameters and treatments

Brassica napus L. seeds were germinated in plastic cups containing a nutrient rich potting mix [Stodels South Africa; 1 part Double grow weed-free compost and 3 parts Double grow silica sand] under a 30/25 °C day and night temperature cycle with a 16/8 hour light cycle. Then, the seeds [Agricol South Africa; AV Garnet cultivar] were treated with 100 ml of 15 µM DIM, I3C solution or deionised water (as control) all solutions containing 0.009% (v/v) Tween 80, twice per week for a duration of 21 days until the first sign of true leaves The plants were then left to grow for another 21 days making the full duration of a 42 day growth period. Complete seed germination was recorded at day 14.

Biomass Determination

At the end of the treatments some of the plants were carefully removed from their respective cups and rinsed. Foil packing were then prepared to the appropriate diameters and the weight of the foil packing were then reordered using a fine mass balance. The entire plant were then placed into separate foil packing according to their respective treatments and holes were poked into the foil packages to allow moisture to seep out. The samples were dried in an incubator at

80°C for 12 hours. After the incubation period concluded the foil packages were weighed, once again, using a fine mass balance and the readings were recorded.

Determination of chlorophyll a and b content

A modified method of Oancea et al. (2005), was followed to determine the chlorophyll concentrations within the DIM, I3C and control treated plants. 100 mg of frozen ground plant material was added to respective 1.5 ml Eppendorf tubes. The Eppendorf tubes was then wrapped in foil to prevent the degradation of any chlorophyll species. Ten volumes of 100% (v/v) acetone was then added to the same Eppendorf tube and mixed briefly using a vortex. Once mixed, the samples was added into separate cuvettes and read on a spectrophotometer at the wavelengths 662 nM and 644 nM respectively. All samples was tested in triplicates. The optical readings was used in a calculation to determine the different chlorophyll species concentrations.

A spectrophotometric assay for the determination of lipid peroxidation by quantifying MDA

A modified method of Zhang et al. (2007), was done to quantify the MDA content within the DIM, I3C and control treated plants. 100 mg of plant material, was added into different 1.5 ml Eppendorf tubes. To the Eppendorf tubes, 5 volumes of 6% (w/v) Trichloroacetic acid (TCA) was be added. The tubes was vortexed and centrifuged at 13000 x g for 10 minutes to pellet the plant material. A volume of 200 µl of the supernatant was removed from the tube and added to a new Eppendorf tube, to this tube 300 µl 0.5% (w/v) thiobarbituric acid (TBA) was added. The solution was then briefly mixed by vortexing. Parafilm was wrapped around the lid of the Eppendorf tubes to ensure they don't open during heating. The tubes was then placed in a heating block at 90 °C to allow the samples to boil for 20 minutes. Once the samples were taken from the heating block they were incubated on ice for 10 minutes. Once the incubation step was completed the samples was centrifuged at 13000 x g for 5 minutes. The samples was then loaded in triplicate onto a microtitre plate and read on a spectrophotometer at wavelengths

532 nM as well as 600 nM. The absorbance at 600 nM was then subtracted from the absorbance at 532 nM to correct for non-specific turbidity. The MDA values was then calculated using the extinction coefficient of 155 mM.cm^{-1}.

A Spectrophotometric assay for hydrogen peroxide (H_2O_2) content determination

A modified method of Velikova et al. (2000), was followed to determine H_2O_2 content in the plant material. The standards for this assay included (0 nM, 5000 nM, 10000 nM, 15000 nM, 20000 nM and 25000 nM) that was prepared by diluting an appropriate volume of H_2O_2 in distilled water. The standards was then loaded in triplicate onto a microtitre plate. Samples was then prepared by using TCA extraction on frozen ground plant material. 50 µl of the TCA extraction was added onto the plate. 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 all the reagents were added to the appropriate wells, the plate was incubated on a shaker for 20 minutes at room temperature. The samples was then read at a wavelength of 390 nM.

A spectrophotometric assay to determine the superoxide content

A modified method of Russo et al. (2008), was followed to determine superoxide content. An Eppendorf tube 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 was prepared, the solution in the tube was then made up to a final volume of 800 µl using a solution of 50 mM potassium phosphate (pH 7.0). This was followed by cutting eight 1 cm^3 squares of fresh leaf material and carefully inserted into the above prepared solution in the Eppendorf tube. The plant material was then incubated for 20 minutes in the solution. Once the incubation is completed the plant material was then crushed using a miniature pestle. The tube was then centrifuged at 13000 x g for 5 minutes to pellet the crushed plant material and the supernatant was then removed carefully and added to a clean Eppendorf tube. Once the supernatant from the sample was free of plant material it was then loaded onto the microtitre plate by adding 200 µl into each well.

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The samples were then read at a wavelength of 600 nM. A calculation taking into consideration the extinction coefficient of 12.8 mM.cm^{-1} was used to determine the superoxide content. The intensity of the blue colour produced by the reaction was an indication of superoxide levels.

Protein extraction

Protein extractions was done in triplicate from DIM, I3C and control plant material by adding 100 mg of frozen ground plant material to three individual Eppendorf tubes (three tubes per plant sample). Protein extraction buffer (0.5 ml) [0.004 M phosphate buffer, 1 mM EDTA and 5% (w/v) PvP] was added to one of the tubes. The homogenate in the tube was then further mixed by vortexing. After the mixture had been adequately mixed, the plant material was then pelleted in a centrifuge at 12000 x g for 5 minutes. The supernatant was removed and inserted into the second tube containing another 100 mg plant material. The previous steps was then repeated for the second and third tube. The supernatant was then removed from the third tube and inserted into a clean Eppendorf tube. The protein concentrations was then quantified using a Bradford assay. Thereafter, the protein samples was stored at -20 °C for future analysis.

A kinetic spectrophotometric assay to determine the catalase activity

The catalase activity was determined using a modified method of Aebi (1984). The protein concentrations were quantified using a Bradford assay. A 1 ml reaction mixture containing 100 mM K_2HPO_4 (pH 7.0), 0.5 mM EDTA, 1 mM H_2O_2 and 20 µl protein extract was prepared. The absorbance of the reaction mixture was read at 240 nM and an extinction coefficient of 39.4 mM^{-1}.cm^{-1} was then used to calculate the activity.

A kinetic spectrophotometric assay to determine the ascorbate peroxidase activity

The ascorbate peroxidase (APX) activity was determined by following a modified method of Nakano and Asada, (1981). Protein samples were aliquoted into 0.5 ml Eppendorf tubes and incubated with 2 mM ascorbate for 5 minutes. The protein samples were then 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 then added. Just before taking the absorbance readings at 290 nM on the spectrophotometer 0.714 mM H_2O_2 was then added to start the reaction. The reactions were made up to a total volume of 200 µl with deionised water. By using the extinction coefficient of 2.8 mM. cm^{-1} the APX activity was then calculated.

A spectrophotometer assay to determine the superoxide dismutase activity

The superoxide dismutase (SOD) activity was determined by using a modified method of Beauchamp and Fridovich, (1971). The protein concentrations were then quantified using a Bradford assay. The samples were then diluted to 1 mg.ml^{-1} before a volume of 10 µl protein samples was loaded onto a microtitre plate. Then to the samples on the plate 20 mM phosphate buffer, 0.1 mM Nitrotrezolium blue chloride (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 distilled water. The sample plate was then incubated for 20 minutes at room temperature on a light box. Following the incubation the samples was then read at 560 nM on a spectrophotometer. The SOD activity was then calculated by determining how much SOD was needed to inhibit a 50 % decrease in the reduction of NBT to formazan.

A spectrophotometer assay to determine Ascorbic acid activity

The procedure for the determination of AsA was based on the formation of the red chelate between dipyridyl-EtOH and the ferrous ion reduced from ferric ion by AsA in acid solution. A stoichiometric relationship observed between AsA in the sample and formation of the chelate

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compound is available for the determination of AsA. 100mg of frozen ground plant material was transferred into a 2mL Eppendorf tube. To this tube 1mL 5 % TCA was added and vortexed for 1 min. To the respective wells 50 uL of the sample extract and 5 % TCA was added. The following reagents was added into the respective wells in this sequence; 50 uL of 100% EtOH, 0.8 uL of 0.4% H_3PO_4-EtOH, 10 uL of 0.5% dipyridyl-EtOH and 0.6 uL of 0.03% $FeCl_3$-EtOH. After adding the 0.03% $FeCl_3$-EtOH immediately incubate this solution at 30˚C for ~90 mins. A pink colour change should occur during this incubation period and similarly cherry smell should be observed. The absorbance of the colour solution was then read at 534 nm. By using the extinction coefficient of 0.2132 mM. cm^{-1} the AsA activity was then calculated.

Elemental analysis using ICP-OES

The elemental profile of the different treatments was determined using a modified method for from Zarcinas and colleagues (1987), for the acid digestion of the plant materials. Frozen ground plant material (200mg) was transferred into 2 mL Eppendorf tubes. 65% Nitric acid was added to the tubes. The tubes was then wrapped using parafilm, this was done to prevent the cap from opening during the incubation period. Then incubation proceeded for 3 hours on a heating block at 90˚C this was done to ensure proper digestion of the plant material. After the digestion, filters and syringes was set up. 1mL of the digested sample and 9mL of 2% Nitric acid was added to the respective syringes for each sample digested. The dilution was then filtered into a 15mL greiner tube. These samples were then subjected to ICP analysis (ICP-OES).

Statistical analysis

The statistical analysis was carried out using the Duncan's multiple range test. Significance differences were represented by a P<0.05.

Chapter 3

Physiological and morphological changes of exogenously applied DIM and I3C treatment to *Brassica napus* L. plants.

The work reported in this chapter investigated the effect of two glucosinolate compounds DIM and I3C on the morphology of the *Brassica napus L. (B. napus)* plants that were treated with either compound for 21 days but the plants were grown for 42 days. The experiment was carried out by treating 15 out of 45 *B. napus* plants with 15 µM of either treatment as a previous study done by Gokul et al. (2016), showed that the exogenous application of 15 µM of DIM to *B. napus* plants promotes growth whereas in another study done by Katz et al. (2015), showed that the exogenous application of 100 µM of I3C inhibits the growth of *Arabidopsis thaliana (A. thaliana)*. Furthermore, it is important to note that we choose *B. napus* as our plant of choice because unlike previous work done by Katz et al. (2015), and Pal et al. (2007), on *A. thaliana* using I3C and *Oryza sativa* using DIM, *B. napus* has the molecular machinery to naturally produce DIM. Moreover, recently *B. napus* plants has surfaced as important plants as it can be used to feed livestock, for the production of biodiesel and edible oils that can be consumed by humans. Additionally, to date, there are few studies available that are based on the function of glucosinolates such as DIM within plants. Most of the scientific knowledge available is based on the functions of DIM within mammalian cancer research. For this reason most of the justification based on the function of DIM within plants was limited.

Results

Responses of *Brassica napus* L*.* plants treated with 15 µM of DIM, I3C and control treatments after a 42 day growth period. The roots were then excised and the effect of either treatment on the leaves and stems were then assessed and photographed.

Figure 3.1: The effect of control 15 µM I3C or 15 µM DIM treatments on the leaf and stem physiology of B. napus.

In this study 45 *B. napus* plants were treated with either treatment for a 21 day period consisting of control, 15 µm of DIM or I3C. Seed germination was recorded for the full duration until maximum seed germination percentages was reached for all treatments. According to figure 3.2 DIM treated *B. napus* plants had a higher germination percentage was observed it was when compared to both the I3C and control treated plants. A ~35% increase in germination percentage was observed for the *B. napus* plants treated with 15 µM of exogenous DIM when it was compared to the controls. Whereas a ~20% increase in germination percentage was observed for I3C treated plants when it was compared to the controls.
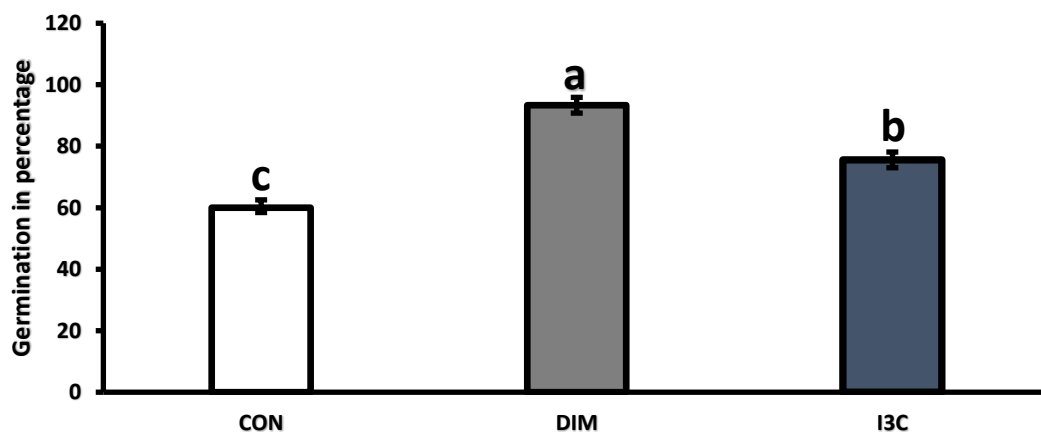
Figure 3.2: The germination percentage of control, 15 µM DIM or 15 µM I3C treatments in B. napus. Means ~ SE of three replicates from either treatment are represented by different letters if they differ significantly at (P < 0.05) according to the Tukey-Kramer test.

A ~50% increase in plant biomass was observed for the DIM treated *B. napus* plants compared to the control treatment. Furthermore, a ~44% decrease in plant biomass was observed for the I3C treated *B. napus* plants compared to the DIM treated *B. napus* plants.
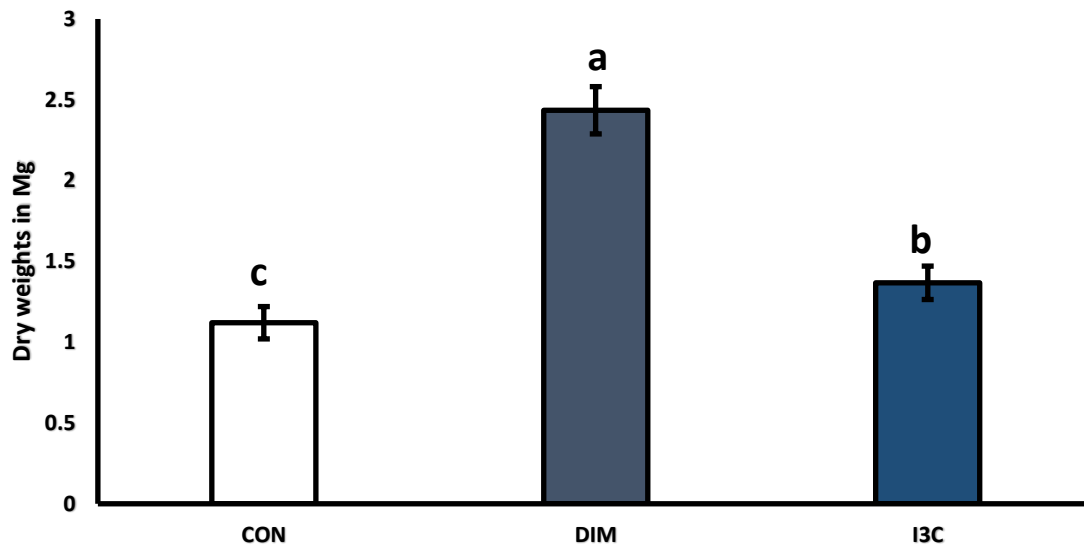
Figure 3.3: The effect of control, 15 µM DIM or 15 µM I3C on the biomass on B. napus plants.
Means ~ SE of three replicates from either treatment are represented by different letters if they differ significantly at (P < 0.05) according to the Tukey-Kramer test.
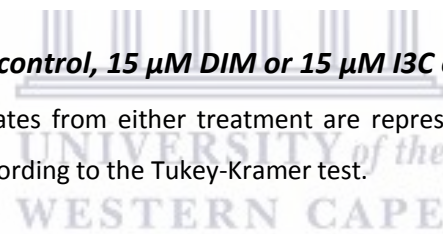
Lipid peroxidation assay was followed to determine the damage done to the lipid membranes of either treatment by measuring the amount of MDA levels present. Statistically there was no difference observed in the MDA levels of the DIM treated plants compared to the controls treated plants. However, more lipid membrane damage was observed for the I3C treated *B. napus* plants ~15% increase when it was compared to the control and DIM treated plants.
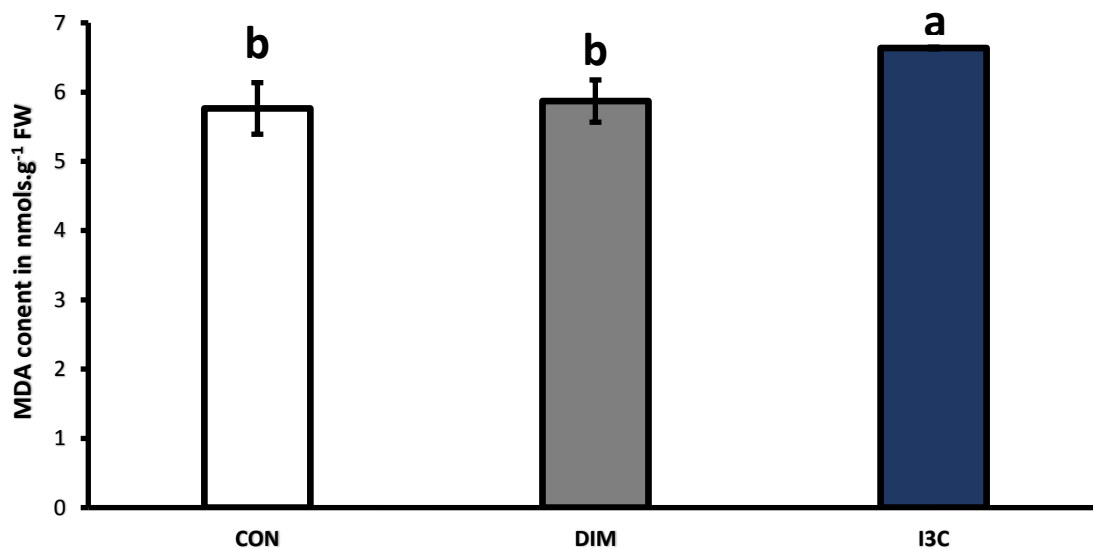
Figure 3.4: The effect of control, 15 μM DIM or 15 μM I3C on MDA levels within B. napus plants. The MDA levels yields an indication of lipid peroxidation. This figure displays the MDA content in the plants after 21 days of treatment. Means ~ SE of three replicates from either treatment are represented by different letters if they differ significantly at (P < 0.05) according to the Tukey-Kramer test.

Chlorophyll damage leads to chlorosis with plant material, nutrient deficiency and ultimately death. Table 3.1 shows a ~25% decrease in the total chlorophyll content within the I3C treated *B. napus* plants when compared to the control plants. Whereas, there is a 5% increase in total chlorophyll content for DIM treated plants when compared to the controls.

Table 3.1: The effect of 15 μM DIM, I3C and control treatments on the chlorophyll a and b and total chlorophyll content of B. napus plants in (mg.g-1). Means ~ SE of three replicates from either treatment are represented by different letters if they differ significantly at (P < 0.05) according to the Tukey-Kramer test.

	Chlorophyll a	Chlorophyll b	Total Chlorophyll
CON	0.1339 ~ 0.07 [a]	0.2450 ~ 0.11 [d]	0.3788 ~ 0.02 [g]
DIM	0.1389 ~ 0.13 [b]	0.20540 ~ 0.08 [e]	0.3929 ~ 0.03 [h]
I3C	0.1013 ~ 0.12 [c]	0.1860 ~0.06 [f]	0.2873 ~ 0.04 [I]

Discussion

In the present study, the exogenous application of both 15 µM of DIM and I3C increased seed germination percentage when compared to the controls. However, the DIM treated plants displayed a greater increase in seed germination percentage when compared to the I3C treated plants. These results corroborates with previous studies done by Gokul et al. (2016); Roychowdhury et al. (2012), and Brown et al. (2003), As stated by Gokul et al. (2016), the exogenous application of DIM to *B. napus* seeds increased seed germination percentage through possible production of Indole-3-acetic acid (IAA) an important hormone needed in plant growth and development. Additionally, a study done by Katz et al. (2015), on *A.thaliana* showed that the exogenous application 100 µM I3C treatment acted as an antagonist in directly competing with IAA in plant development and growth, thus decreasing seed germination. On the contrary, in our study only 15 µM of I3C was applied, this could explain the possible increase in seed germination percentage detected. Roychowdhury et al. (2012), observed a ~75% increase in seed germination percentage upon application of IAA to *Dianthus caryophyllus* seeds and concludes that IAA is crucial in overcoming seed dormancy. Moreover, a study done by Brown et al. (2003), observed that plants naturally producing glucosinolates store it in their vegetative and reproductive parts and had higher concentrations of indole glucosinolate compounds in their seeds with an exception to very young leaves. For this reason according to Brown et al. (2003), high concentrations of indole glucosinolates in seeds is needed for maximum defensive potential and as precursors for plant hormone development.

Improper formation in the morphology of plants would lead to a negative effect in the biochemistry of the plant, especially improper root formation as this would lead to the inability of the plant to take up certain macro and micro nutrients needed by the plant for survival. Specifically leading to the over production of ROS. Hence in this study, the roots, stems and leaves were assessed after the plants were grown up for 42 days as previously described in the methods and material section. I3C treatment caused yellowing of the leaves in the *B. napus* plants when compared to both the DIM and controls. The yellowing of the I3C treated leaves lead to the suspicion of chlorosis and initiated us to perform a chlorophyll assay to confirm our suspicion. Furthermore, the leaves of the DIM treated plants also covered a greater surface area than both the I3C and control plants. Moreover, once the stems of the DIM treated plants was measured it was longer, thicker and more rigid when compared to both the I3C and control treated plants. Additionally, wilting of the stems and leaves was observed for the I3C treated plants even though all plants were kept under the same conditions. The roots is not displayed in figure 3.1 but a deformed root system was clear in the I3C treated plants as few lateral roots and hair roots developed putting the plant at a disadvantage when compared to both the DIM and control plants. Both DIM and control plants had a well-developed lateral, hair and tap root system which could have led to good nutrient absorption however, further analysis such as Inductively coupled plasma spectroscopy is needed to confirm this assumption. Once the plant biomass was investigated we observed a significant increase in plant biomass for the DIM treated *B .napus* plants compared to the I3C treated plants. The increase in biomass obtained for the DIM treated plants can be explained by the increase in shoot length observed under the DIM treated *B. napus* plants. Similar results were recorded in a study done by Gokul et al. (2016), they showed a significant increase in both fresh and dry weights for *B. napus* plants treated with exogenous DIM and linked this increase to higher levels of S and N supply which is important for seedling shoot development hence the production of seedling shoot with increase biomass. Furthermore, the decrease in biomass obtained for the I3C treated *B. napus* plants can be attributed to the decrease in shoot length under the I3C treatment. This

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observation is in alignment with a study done by Katz et al. (2015), who observed a decrease in the biomass of *Arabidopsis thaliana* when it was treated with exogenously applied I3C.

The chlorophyll content was tested to help examine whether the yellowing in the leaves was indeed caused by chlorosis. Chlorophyll damage may lead to chlorosis within plant material and heavily affects the photosynthetic pathway as chlorophyll is the key pigment responsible in the photosynthetic process. Furthermore, chlorophyll damage leads to nutrient deficiency and ultimately death (Hörtensteiner & Krautler, 2011). DIM treated plants showed significant increases in chlorophyll a, b and total chlorophyll content when compared against the control and I3C treated plants. This observation is in alliance with figure 3.1 showing that the DIM treated plants had broader and greener leaves when compared to the I3C and control treated plants. Additionally a cold-stressed study done by Hola (2011), observed that the exogenous application of Brassinosteroids (a plant hormone) slowed down the degradation of chlorophyll, associated proteins and reduced oxidative damage thus acting as a signalling molecule. Furthermore, the results of our study is in confirmatory with a study done by Shi et al. (2016), they noticed a decrease in chlorophyll degrading enzymes, chlorophyllase when nitric oxide was applied exogenously which resulted in seedlings with more chlorophyll present. In addition, Kaushal et al. (2011), showed that the exogenous application of proline in chickpea plants were used as molecules to help plants develop and grow when they are under abiotic stress moreover, their study showed less injury to membranes and improved chlorophyll and water contents. Nonetheless, noteworthy decreases were seen in total chlorophyll content as well as in the chlorophyll a and b species for the I3C treated plants when compared to the controls and DIM treated plants. Additionally, this decrease in chlorophyll content is evident in figure 3.1 as the results showed that chlorosis occurred in the leaves of the I3C treated plants. Moreover, our results are in correlation with a previous study done by Costa et al. (2005), who showed that yellowing of broccoli florets is accompanied by a loss in fresh weight, chlorophyll content, sugars and proteins. This was done by increasing in the chlorophyll degradation enzymes.

To boot, in our study, no significant difference in MDA production between the DIM and control treated plants was observed even though there was a significant increase in the ROS production. This observation is in corroboration with a study done by Bailly (2004), as they concluded that lipid peroxidation and cell death will not always occur with an increase in ROS, if the antioxidant system is kept under tight control. Additionally Gokul et al. (2016), observed similar results in their study showing that an increase in H_2O_2 and O_2^- did not lead to an increase in lipid peroxidation as antioxidant enzymes (APX and SOD) was increased to accompany the increase in ROS. Moreover, a study done by Wu et al. (2014), showed that exogenously applying Nitric oxide (NO) to broccoli decreased lipid peroxidation levels, NO acted as a protector against various stressful impacts scavenging free oxygen radicals and counteracts oxidative damage by regulating the cellular redox balance and accelerating the transformation of O_2^- anion and enhancing the activities of antioxidant enzymes. Therefore, DIM could be using the same mechanisms as NO against oxidative stress, hence leading to a decrease in lipid peroxidation as experienced in the DIM treated *B. napus* plants in our study. However, significant increases in MDA content was observed for I3C treated plants. Zhu (2002), showed that with a higher increase in H_2O_2 concentration cellular components such as lipids, proteins and nucleic acids can be oxidized. This will eventually result in apoptosis, which further explains the poor germination and lipid destruction obtained for the I3C treated plants in this study.

Chapter 4

The biochemical effects of exogenously applied DIM and I3C on *Brassica napus* L.

Plants have developed and adapted a signalling network to survive abiotic stresses which consist of various growth regulators to not only detect but also offer protection to the plant when stressed (Bhattacharjee, 2011). The increase of Reactive oxygen species (ROS) are important cues used by plants to respond to environmental stress (Bhattacharjee, 2011).ROS includes free radicals, alkoxyl radicals and non-radicals which are comprised of compounds such as hydroxyl radicals (OH^-), hydrogen peroxide (H_2O_2) and superoxide (O_2^-) (Sinha and Saxena, 2006). In unstressed plants ROS are produced at basal levels as by-products, however, the level of ROS is enhanced during stressful conditions and results in the leakage of electrons from electron transport activities (Gill and Tuteja, 2010). The production of ROS thus, has dual functions within plants, at basal levels it acts as a stress indicator whereas, the accumulation of these compounds may be damaging to the plant (Zhang *et al*., 2007). The build-up of ROS could potentially damage proteins, lipids, carbohydrates and DNA which would lead to cellular death (Maruta *et al*., 2012). In plants, ROS molecules can be generated within the mitochondria, cytosol, peroxisomes, chloroplasts and the apoplastic space (Mittler, 2002). Furthermore, as the chloroplast contains a high concentration of oxygen involved within the photosynthetic electron transfer system this makes them more susceptible to an increase in ROS. According to Zhang et al. (2007), the accumulation of ROS is believed to be as a result of the disturbance in the balance of the ROS production and the antioxidation systems. There are many developmental and metabolic processes that are controlled by cross-talk involving ROS and hormones (Kocsy *et al*., 2013). (Zhang *et al*., 2007). As a result of the disparaging nature of reactive compounds, plants uses ROS-scavenging pathways as control measures (Bhattacharjee *et al.*, 2011). The ROS-scavenging pathway has the ability to metabolise ROS and thus reduce

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their concentration within plants decreasing the toxic effects caused by ROS (Mahanty *et al.*, 2012). It has been found that ROS-scavenging enzymes such as catalase (CAT), ascorbate (APX) and superoxide dismutase (SOD) is very important in remediating these toxic levels within plants. SOD is one of the first enzymes scavenged in this pathway SOD converts O_2^- to H_2O_2, the H_2O_2 is then removed by APX by oxidising ascorbate to monohydroascorbate (Lee *et al.*, 2007; Wang *et al.*, 2005).

Results

O_2^- has the ability to cause oxidative damage and the photosystem I is seen as the major site for the development of O_2^-. An increase O_2^- content in the leaves was observed for both DIM and I3C treated *B. napus* plants when compared to the controls represented in figure 4.1 A. The O_2^- content in the leaves increased by ~21% for the DIM treated plants and ~35% for the I3C treated plants when compared to the control. However, when comparing the O_2^- content of the stems in figure 4.1 B a ~92% increase in the I3C was observed when it was compared to the control and DIM treated plants.
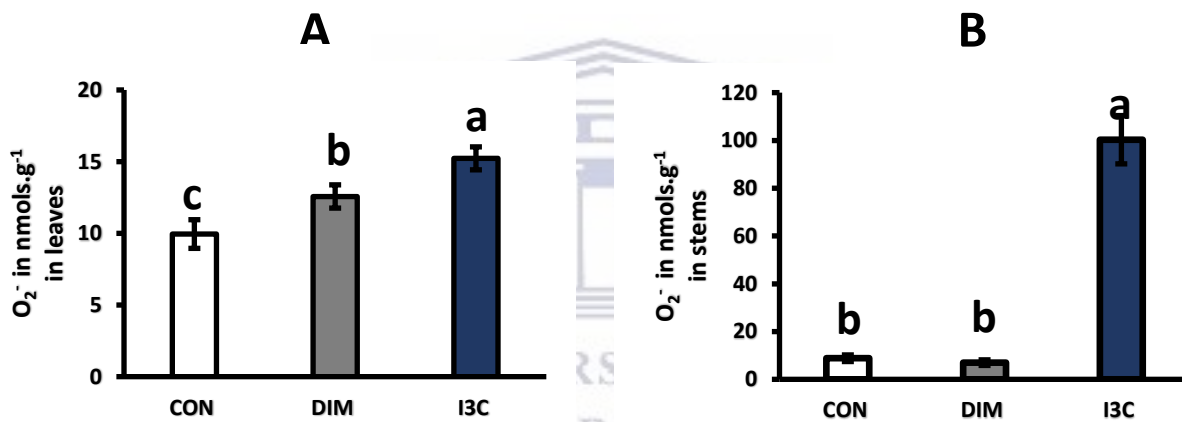
Figure 4.1: The effects of control 15 µM DIM or 15 µM I3C treatments on the superoxide content in the leaves and stems in B. napus. For each treatment the error bars represents the mean (~SE) per experiment. Different letter are representative of means differing significantly from one another (p<0.05) according to the Tukey-Kramer test.

H_2O_2 is seen as an extremely dangerous ROS and increases in its content would lead to cell damage. Figure 4.2 shows a ~10% increase in H_2O_2 content for the DIM treated plants compared to the control treated *B. napus* plants. Whereas, a ~17% increase in H_2O_2 was observed for the I3C treated *B. napus* plants when compared to the control.
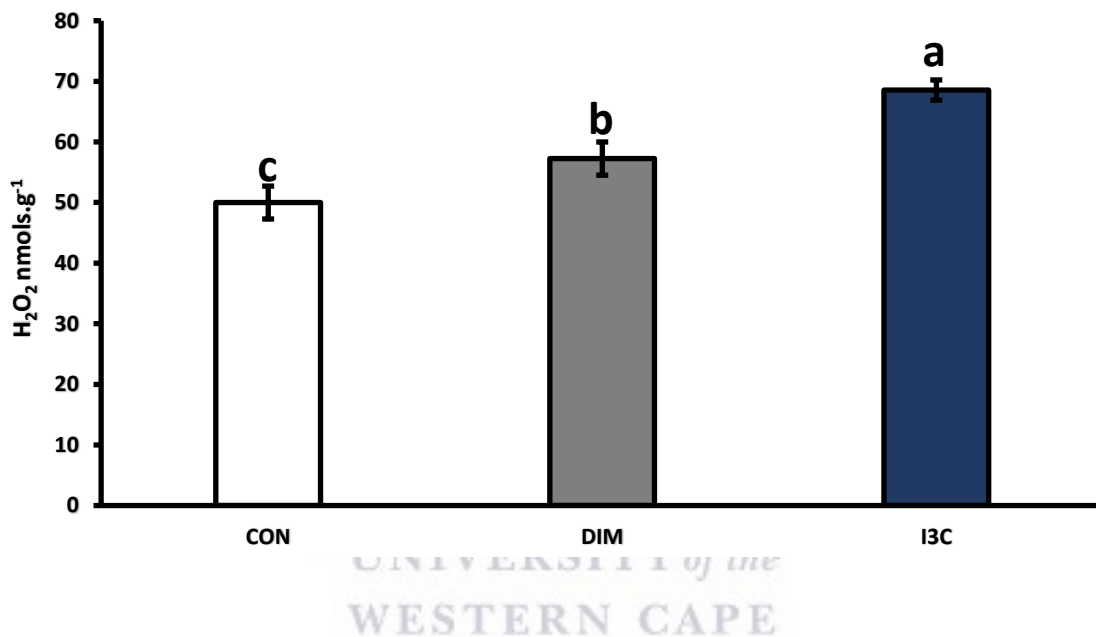
Figure 4.2: The effects of control 15 μM DIM or 15 μM I3C treatments on the hydrogen peroxide content in B. napus. For each treatment the error bars represents the mean (~SE) per experiment. Different letter are representative of means differing significantly from one another (p<0.05) according to the Tukey-Kramer test.

Plants have developed a ROS-scavenging system to defend themselves against oxidative stress, this includes the production of antioxidant enzymes and compounds. One of these antioxidant enzymes produced includes Ascorbate peroxidase (APX) represented in figure 4.3. In figure 4.3 a ~37% increase in the APX content of the DIM treated *B. napus* plants was observed when it was compared to the control and I3C treated plants. Furthermore, no statistical difference in APX activity was observed between the APX content of I3C treated plants when it was compared to the controls.

Figure 4.3: The effect of control, 15 µM DIM or 15 µM I3C treatments on the APX activity in B. napus plants. For each treatment the error bars represents the mean (~SE) per experiment. Different letter are representative of means differing significantly from one another (p<0.05) according to the Tukey-Kramer test.

In figure 4.4 no significant difference in the CAT content of the DIM treated *B. napus* plants was observed when it was compared to the control treated plants. Furthermore, ~40% decrease in CAT content was observed for the I3C treated plants when it was compared to the DIM and control treated plants.

Figure 4.4: The effect of control 15 µM DIM or 15 µM I3C treatments on the CAT activity in B. napus plants. *For each treatment the error bars represents the mean (~SE) per experiment. Different letter are representative of means differing significantly from one another (p<0.05) according to the Tukey-Kramer test.*

In figure 4.5 a ~17% increase in the SOD content of the DIM treated *B. napus* plants was observed when it was compared to the control treated. Furthermore, ~30% increase in SOD content was observed for the I3C treated plants when it was compared to the controls.

Figure 4.5: The effect of control, 15 µM DIM or 15 µM I3C treatments on the SOD activity in B. napus plants. For each treatment the error bars represents the mean (~SE) per experiment. Different letter are representative of means differing significantly from one another (p<0.05) according to the Tukey-Kramer test.

In figure 4.6 a no statistical difference was observed between the DIM and control treated *B. napus* plants. However, a ~22% decrease in AsA concentration was observed for the I3C treated plants once compared to both the DIM and control treated *B. napus* plants.
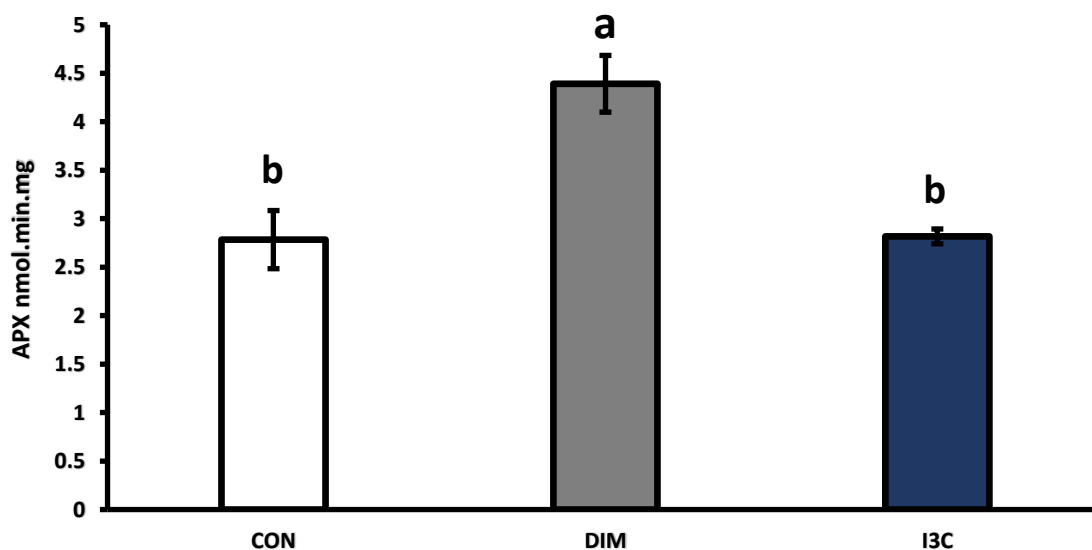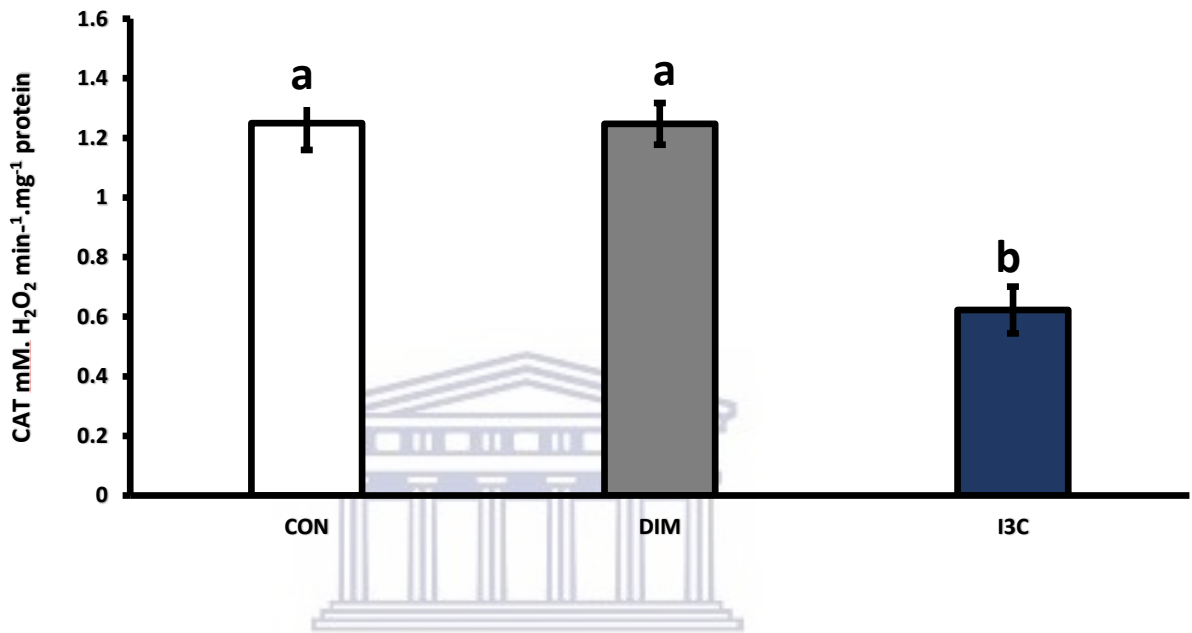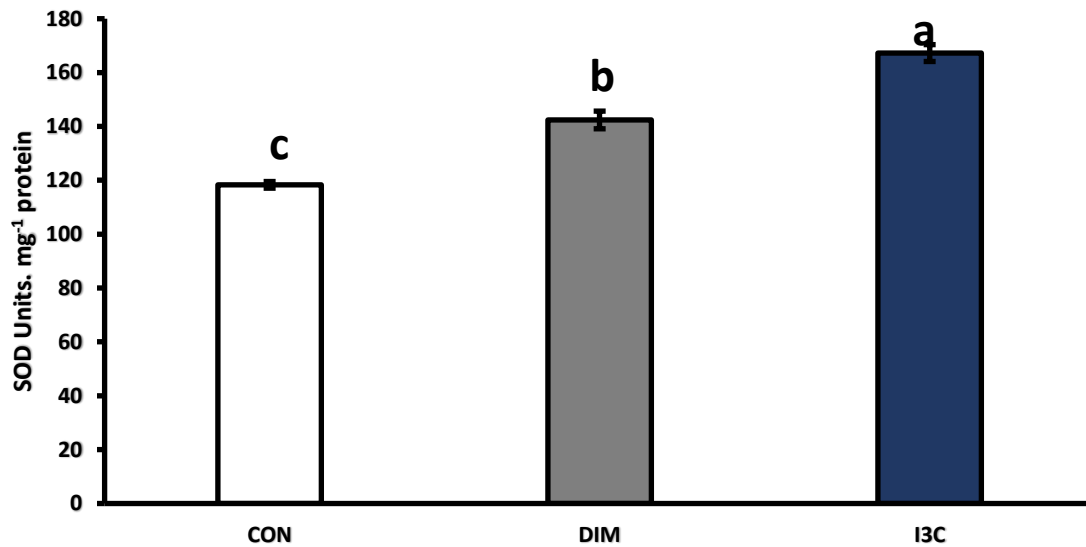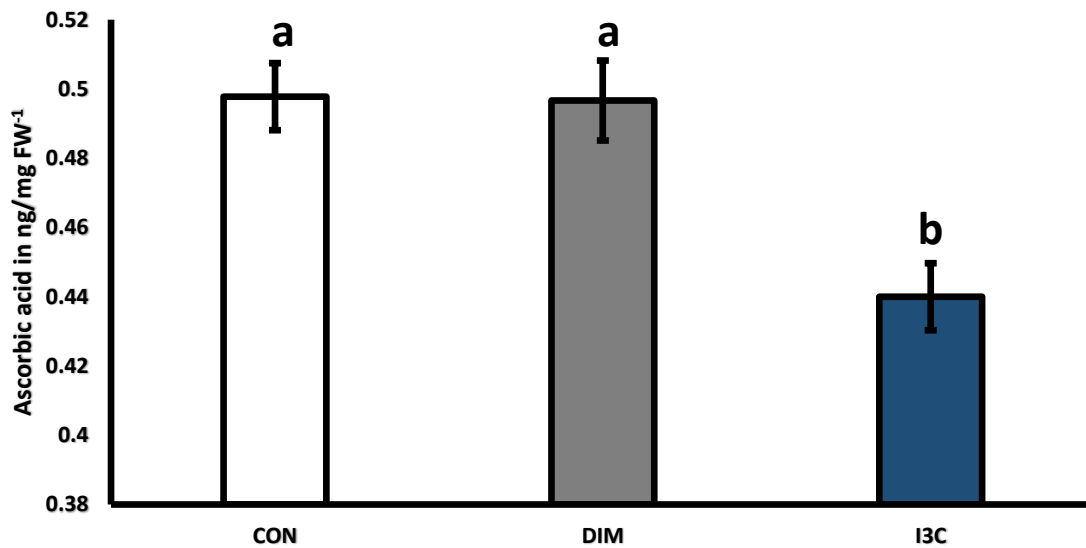

Figure 4.6: The effect of control, 15 µM DIM or 15 µM I3C treatments on the AsA activity in B. napus plants. For each treatment the error bars represents the mean (~SE) per experiment. Different letter are representative of means differing significantly from one another (p<0.05) according to the Tukey-Kramer test.

Discussion

To examine whether the exogenous application of DIM And I3C to the *B. napus* plants disturbed the signalling pathway of the *B. napus* plants, thus reduction-oxidation reactions occurring in the Foyer-Halliwell-Asda cycle the change in the levels of O_2^- and H_2O_2 was measured. We detected a notable increase in O_2^- content in the leaves for the DIM plants whilst being compared to the controls. This increase could be used for signalling purposes as according to Gokul et al. (2016), who showed that DIM treated *B. napus* seedlings showed an increase in O_2^- content but did not result in any plant damage. Furthermore, a more significant increase in O_2^- content was detected for the I3C treated plants when it was compared against the controls. This increase however did result in plant damage as seen in figure 3.1 by the deformed leaf and stem system and by the lipid damage observed in figure 3.4 after measuring the increase in MDA levels which is an indication of lipid peroxidation. Moreover, when the O_2^- content of the stems were assessed only the I3C treated plants showed a significant increase in the O_2^- content whereas, both DIM and the controls revealed lower levels. These results draw attention that the exogenous application of 15 µM of DIM does increase O_2^- content. However not to a detrimental level on the contrary, it leads to an increase in seed germination and development. Likewise, these findings is supported by previous studies done by (Schopfer *et al.*, 2002; Müller *et al.*, 2009; Duan *et al.*, 2014 and Smirnova *et al.*, 2014) as all have concluded that this radical has a positive effect on reproduction, germination and eventually growth. According to Oracz et al. (2007), the accumulation of ROS particularly H_2O_2 and O_2^- anions was needed in the seeds of *Helianthus annuus L.* to assist in the oxidation of certain embryo proteins involved in overcoming seed dormancy. Furthermore, these results are supported by Gokul et al. (2016), as they proved that an increase in O_2^- and H_2O_2 content does not lead to an increase in cell death and lipid peroxidation but rather improves the growth of *B. napus* plants when treated with 15 µM of DIM. To further support these results, a study done by Rachel and Dolan (2006), emphasized the significance of ROS especially O_2^- required for the formation of hair root growth involved in controlling the activity of the calcium channels needed for polar growth. Additionally we can hypothesize with an increase in O_2^- content observed in the DIM treated *B. napus* plants

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an increase in SOD should be observed as SOD could regulate the O_2^- radical below toxic levels and suggesting a possible signalling mechanism.

Once the H_2O_2 content of the DIM, I3C and control treated plants were compared to each other a significant increase in the H_2O_2 content was observed for I3C treated plants when compared to both controls and DIM treated plants. Whereas a slight increase in H_2O_2 content for the DIM treated plants was observed when compared to the controls. These observations are in conformity with a study done using *B. napus* seedlings by Gokul et al. (2016), they observed significant increases in H_2O_2 contents in DIM treated plants and concluded that H_2O_2 is used for signalling purposes as the increase in H_2O_2. did not result in plant damage as it was controlled by APX. Nonetheless, the slight increase in H_2O_2 production observed in our study could possibly be due to the fact that the plants in our study were grown up for a longer period of time. Moreover, this could have led to an increase in antioxidant activity such as the APX activity needed to compensate for the increase in H_2O_2 content under the DIM treatment to thus regulate and scavenge the excess H_2O_2 being produced.

As previously mentioned, oxidative stress caused by an increase in ROS will lead to cellular death eventually if the plant is unable to produce certain metabolites to help alleviate the stress caused. For this reason plants has adapted ROS-scavenging systems driven by reduction-oxidation reactions as defense mechanisms consisting of certain antioxidant molecules aiding in plant protection. Within a cell, SOD is the first line of defense against ROS; therefore in our study we investigated the levels of SOD for the treated plants to answer the hypothesis whether the increase in O_2^- resulted in an increase in SOD levels. A significant increase in the SOD levels was observed for the DIM treated plants when it was compared against the control. In addition APX levels of the DIM treated plants also increased significantly when it was compared to the control and I3C treated plants. These increases were expected as our study revealed significant increases in H_2O_2 and O_2^- levels. Hence antioxidant enzyme activities increased in an effort to control the increase in ROS levels. These results are supported by a study done by Gokul et al. (2016), in their study they showed that the exogenous application of DIM increased the ROS levels which stimulated the production of antioxidants; SOD and APX to regulate the increase in H_2O_2 and O_2^- levels explaining the link between APX activity and indole glucosinolates.

Furthermore, this increase in APX was expected for the DIM treated plants as indole glucosinolates co-localize with ascorbic acid as stated by Bones and Rossiter (1996), and in addition correlates to H_2O_2 production in plants. Nonetheless, even though there was an increase in APX activity for the DIM treated plants, not all H_2O_2 was removed from the system. This suggests that the APX is produced as a possible controlling mechanism for keeping the H_2O_2 levels below the toxic range.

Additionally we saw no statistical difference in the AsA content between the DIM and control treated *B. napus* however; our results displayed a noteworthy decrease in Ascorbic acid (AsA) content under the I3C treatment compared to both the control and DIM treated *B. napus* plants. According to Hernandez et al. (2000), AsA is small plant molecule and is a key substance in the network of antioxidants including ascorbate, glutathione, a-tocopherol and a series of other antioxidant enzymes. Furthermore, AsA has multiple roles partaking in plant growth, cell division, cell wall expansion and other developmental processes (Pignocchi and Foyer, 2003). A study done by Noctor and Foyer (1998), showed that AsA detoxifies H_2O_2 which is formed by the dismutation of O_2^-. This could possibly explain why we detected a significant increase in H_2O_2 levels under the I3C treated *B. napus* plants. Maybe too little AsA was being produced to help detoxify the increased levels of H_2O_2 produced under the I3C treated plants. This could have led to the detrimental increase of H_2O_2 levels being produced under the I3C treatment. According to Noctor and Foyer (1998), AsA is a substrate needed by APX for the catalysis reaction of H_2O_2. Therefore since APX works concurrently with AsA and we observed lower levels of AsA activity under the I3C treatment this could be a possible explanation for the decrease in APX observed under the I3C treatment.

Furthermore, no significant difference for the CAT activity was observed once the DIM and control treated plants were compared to each other. This suggests that the exogenous application of DIM promoted ROS levels which then stimulated the increased production of SOD and APX but not CAT activity whereas APX activity was significantly upregulated under DIM treatment once compared to I3C and control treated plants. Therefore we can conclude that APX is the candidate enzyme in controlling H_2O_2 levels under DIM treatment. This increase in H_2O_2 levels under the DIM treatment then signaled the DIM treated *B. napus* plants to

upregulate their APX activity to accommodate for the increase in H_2O_2 levels in an effort to eliminate the threat at hand. On the other hand the I3C treated plants displayed the highest increase in SOD activity. However this seems reasonable as SOD is the first line of plant defense and as the I3C treatment was crippling to the plant, the primary defense antioxidant compound SOD, should have been signaled by the increase in ROS activity to alert the plant of the stress it is faced with. In spite of this CAT and APX levels for I3C treated plants did not display an accompanying pattern for the increase observed in SOD on the contrary the reduction in APX and CAT levels rather aided in the poor functioning of the I3C treated plants. Additionally, the reduction in CAT observed could be due to that the fact that I3C treatment inhibits CAT activity and suggests that CAT plays a significant role in the toxicity of H_2O_2 when under I3C treatment. This implies that the increase in ROS activity under I3C treatment resulted in the down regulation of CAT and APX. These results are in corroboration with a previous report done by Luna et al. (1994), who demonstrated that Cu ions decrease APX and CAT activity in oat leaf segments due to a direct effect of Cu induced ROS on the enzyme protein. In their study high concentrations of Cu treatment in light to oat seedlings caused the breakdown of chlorophyll and increased membrane permeability rates. Moreover, their study showed that Cu_2^+ ions enhanced SOD levels especially in young leaves but in contrast decreased CAT and APX levels in both younger and older leaves which indicated that an excess of Cu_2^+ ions caused rapid senescence in plant leaves via oxidative reactions in the light

Chapter 5

The Macro and Micro elemental profile of exogenously applied control, DIM and I3C treated *Brassica napus* L. plants using ICP-OES.

According to White and Brown (2010), plants require at least 14 essential mineral elements in order to complete their life-cycles. Based on these requirements, the nutrient ions can be divided into three categories: macronutrients, micronutrients and beneficial elements. However, in this chapter we only focused on two of these categories and in these categories we only investigated the important macro and micro nutrient profiles of the control, DIM and I3C treated plants. The macro elements required by plants we investigated included Nitrogen (N), Phosphorus (P), Potassium (K), Calcium (Ca), Magnesium (Mg) and Sulphur (S). Likewise, the micro elements we investigated included Chlorine (Cl), Baron (B), Iron (Fe), Manganese (Mn), Zinc (Zn), Copper (Cu), Nickle (Ni) and Molybdenum (Mo). Plants take up nutrients from the soil via their roots, these nutrients are water soluble taken up as ions in the form of a solution. The successive uptake and exploitation of these mineral ions into plants are regulated by traits that affects transport, translocation and sequestration (White and Brown, 2010). Furthermore, these elements move across the root via apoplastic and symplastic pathways to the stele in both its chelated and free-ionic form (White and Brown, 2010). With this in mind, the primary goal of this chapter was to measure the nutrient content of the *B. napus* treated plants. The estimation of the nutrient content is immensely important as poor nutrient absorption of required nutrients at their specific concentrations would lead to improper functioning and growth of the plant.

Results

In addition to oxygen and carbon dioxide macro nutrients are required in larger amounts by the plant for survival as they are involved in essential plant functions such as plant growth, photosynthesis and energy transfer to name a few. In the proceeding study the composition of four essential macro nutrient elements required was examined. This included K, Mg, Ca and P. Figure 5.1 represents the outcome of these results. A ~ 15% increase in P content was observed for DIM treated plants compared to the controls and a ~ 20% increase in P content was observed for I3C treated plants compared to the controls. Moreover, ~ 25% increase in Mg content was observed for DIM treated plants compared to the controls and a ~ 65% decrease in Mg content observed for I3C treated plants compared to the controls. Furthermore, a ~ 20% increase in K content was observed for DIM treated plants compared to the controls and a ~ 10% increase in K content observed for I3C treated plants compared to the controls. Lastly a ~ 20% increase in Ca content observed for DIM treated plants compared to the controls and a ~ 15% increase in Ca content was observed for I3C treated plants compared to the controls.
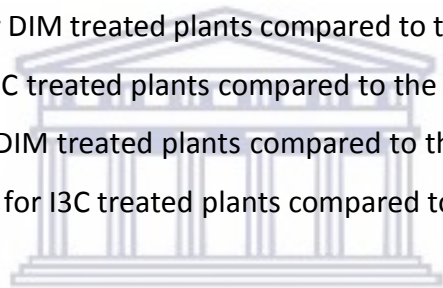
Figure 5.1: The effect of control 15 μM DIM, I3C treatments on the Macronutrient profile of the B. napus plants. For each treatment the error bars represents the mean (~SE) per experiment. Different letters represents the mean differing significantly from one another (p<0.05) according to the Tukey-Kramer test. The letters above the graph represents the different Macronutrients analysed with A representing Potassium (K), B representing Phosphorus (P), C representing Magnesium (Mg) and D representing Calcium (Ca).

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Many studies confirmed that micronutrients are needed in trace amounts and in our study we analysed four of these micronutrients which included Iron (Fe), Manganese (Mn), Copper (Cu) and Zinc. Even though these nutrients are required in much smaller dosages by the plant any deficiency or for that matter over exposure of these nutrients can have detrimental effects on the growth and development of plants. Our study reveal trends supporting this statement. In Figure 5.2 we saw a ~ 80% increase in Cu content was observed for DIM treated plants compared to the controls furthermore, ~ 75% increase in Cu content was observed for I3C treated plants compared to the controls. A ~ 46% increase in Fe content was observed for the DIM treated plants once it was compared to the control treated plants statistically there was no significant difference in Fe content observed between the control and I3C treated plants. Statistically there was no significant difference in Zn content observed between the control and DIM treated plants whereas ~ 13% increase Zn content was observed for the I3C treated plants compared to both the control and DIM treated plants. A ~ 57% increase in Mn content was observed for DIM treated plants when compared to the control treated plants and a ~ 44% increase in Mn content was observed for the I3C treated plants when compared to the control treated plants.
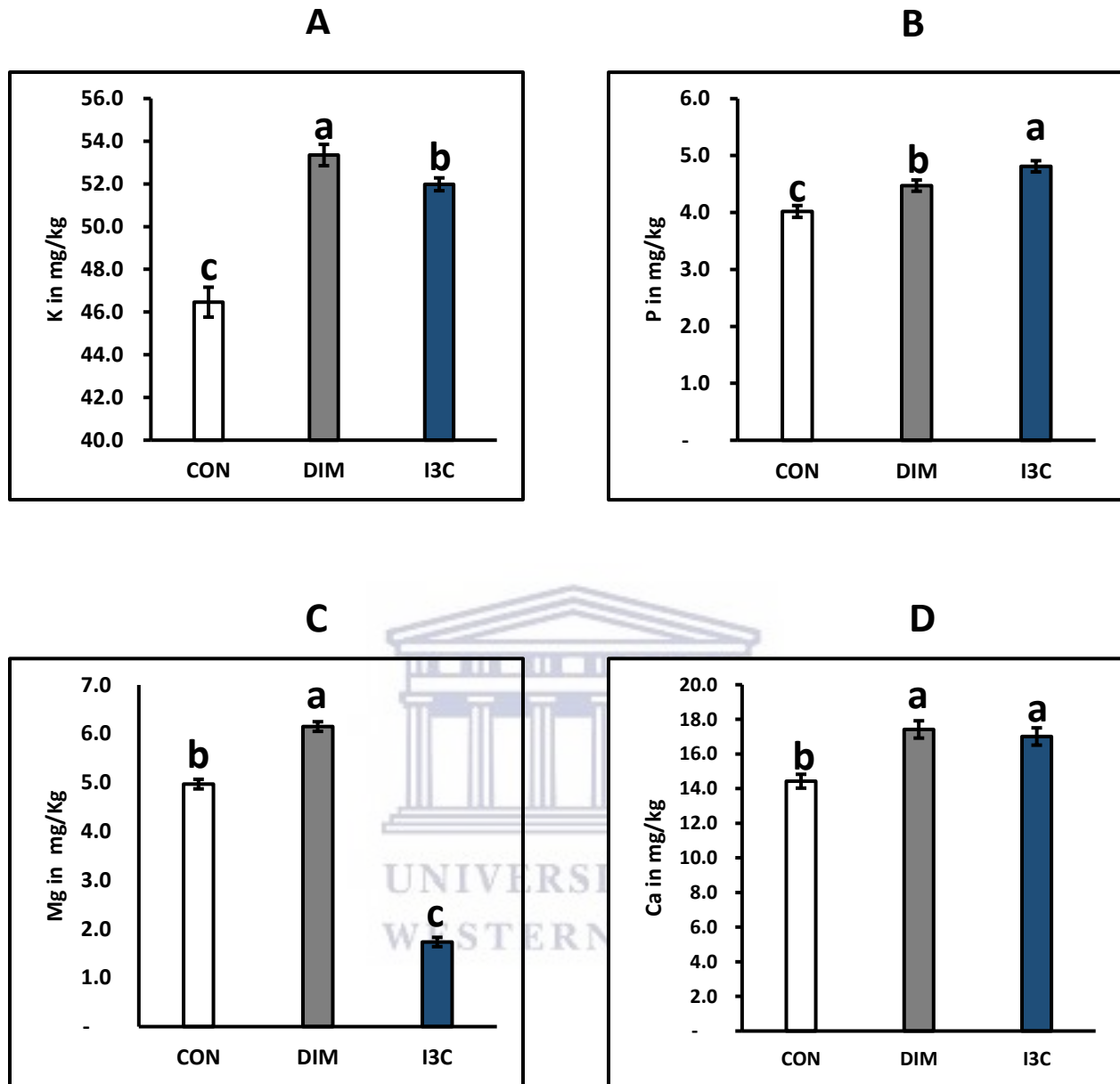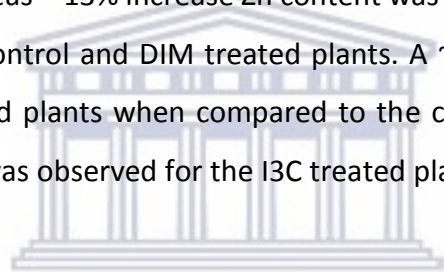
A

B

C

D
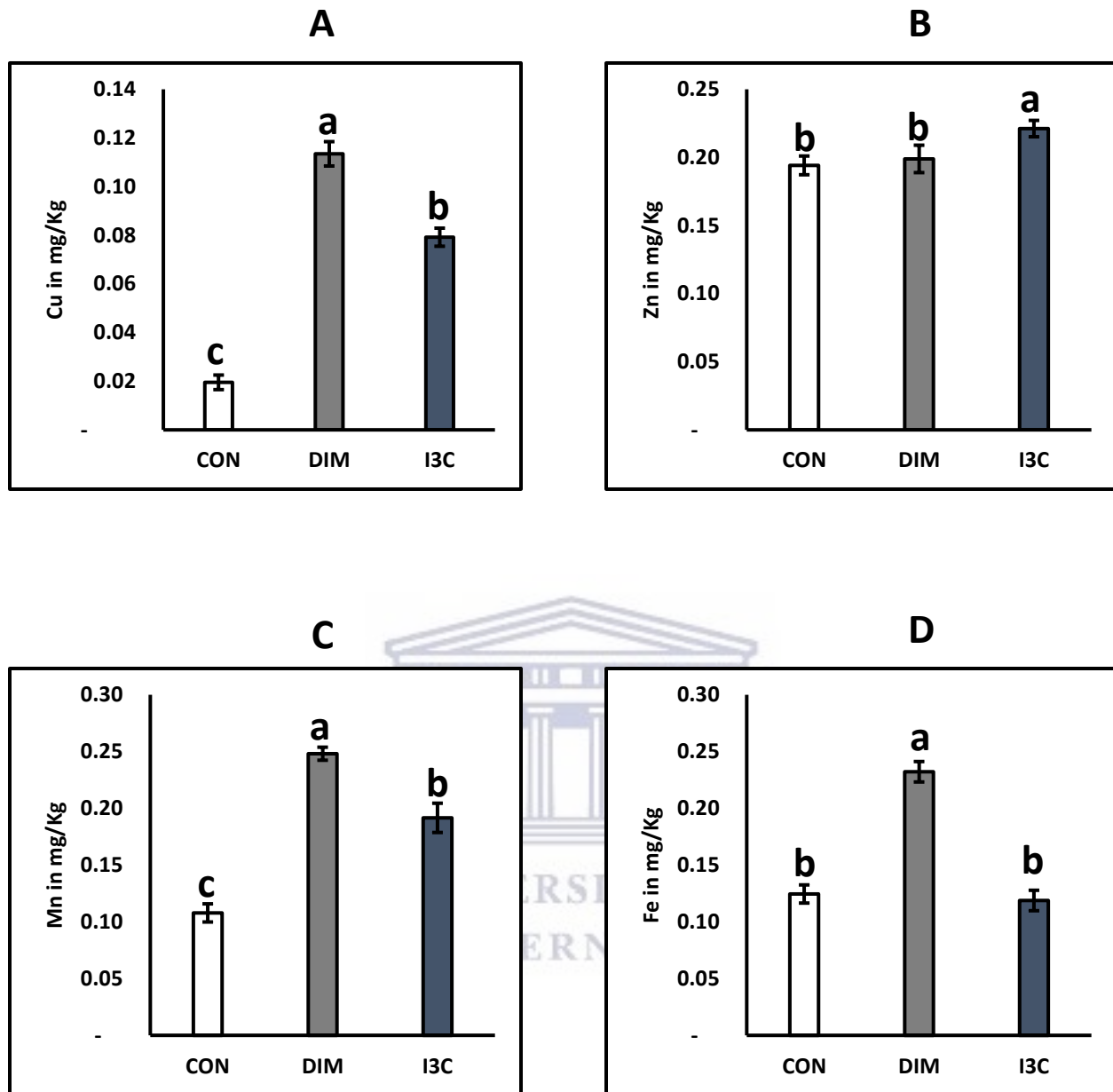
Figure 5.2: The effect of control 15 µM DIM, I3C treatments on the Micronutrient profile of the B. napus plants. For each treatment the error bars represents the mean (~SE) per experiment. Different letters represents the mean differing significantly from one another (p<0.05) according to the Tukey-Kramer test. The letters above the graph represents the different Micronutrients analysed with A representing Copper (Cu), B representing Zinc (Zn), C representing Manganese (Mn) and D representing Iron (Fe).

Discussion

The analysed *B. napus* treated plants differed significantly with respect to the content of most of the eight elemental nutrients analysed using the ICP analyses technique. Regarding the macro elemental profile of the *B. napus* treated plants a significant increase in the Mg, Ca and K content was depicted for the DIM treated *B. napus* plants once compared to both the control and I3C treated plants. However, once the P content was analysed the I3C treated *B. napus* plants displayed the highest increase in P content.

According to Cakmak and Yaziei (2010), magnesium (Mg) in its ionic form has a number of particular metabolic functions within plants which includes partaking in processes such as photophosphorylation (such as ATP formation in chloroplasts), protein synthesis, chlorophyll formation, phloem loading and generation of ROS. Consequently Mg ions also influence critical physiological and biochemical processes within plants and thus a deficiency of it would lead to serious growth and yield impairments. The decrease in the Mg content experienced within the I3C treated *B. napus* plants could possibly explain the onset of chlorosis as discussed Chapter 3, Table 3.1. This observation is in alliance with a study done by Jezek et al. (2015), their results clearly demonstrated that Mg deficiency markedly reduced chlorophyll concentrations and the leaves showed deficiency symptoms like interveinal yellowing, chlorosis and necrosis. Consequently the increase in Mg content depicted for the DIM treated plants could have led to the increase in chlorophyll species which lead to the vibrant green plants observed under the DIM treated *B. napus* plants as shown in Figure 3.1. Likewise, the decrease in Mg concentration experienced in the I3C treated *B. napus* plants could also be linked to the decrease in Mn observed and the increase in K observed. These results are confirmed by the a study done by Mengel and Kirby (2001), who demonstrated that Mg re-supply does not only affect metabolic processes but also affects cations such as K and Mn moreover, they showed that the variations in one nutrient is compensated by changing the uptake of other cations instead. Additionally, Kleiber et al. (2012), demonstrated that antagonistic interactions exists between Mg, Ca and K in onion plants. Furthermore the detrimental increase in ROS experienced under the I3C treatment can be correlated to the decrease in Mg content observed and its associated cations

under the I3C treatment. These results is supported by Cakmak and Kirby (2008), they found that the accumulation of carbohydrates and the impairment in photosynthetic CO_2 fixation in Mg-deficient leaves appears to proceed to ROS generation. Moreover, another study done by Farhat et al. (2015), reported that Mg-deficiency induced ROS scavengers such as Ascorbic acid (AsA) and SH containing compounds this further explains why there was such a significant decrease in AsA observed under the I3C treated plants. Therefore as the exogenous application of I3C is detrimental to the *B. napus* plants thus imposing stress and Mg deficiency influences ROS and antioxidant levels it can be depicted that Mg deficiency could possibly influence plant signalling within the *B. napus* treated plants. This observation is in alliance with a study done by Hermans et al. (2010), they showed that the plant hormone Ethylene which is responsible for ripening is increased significantly under Mg deficiency and leads to premature ripening.

As stated by Saidi in (2009), calcium (Ca^{2+}) is an extremely important mineral to plants. One of the primary roles assigned to Ca in plants is the key role it plays in cell wall development. Low Ca can make the cell wall more pliable and this can make it rupture easily. Furthermore, Ca is also important in the development of roots and it has been observed that short roots are developed in Ca deficient plants. A study done by White and Broadly in (2003), stated that Ca^{2+} influx occurs when plants are under pathogen attack. Moreover, in our study we observed an increase in Ca content under both the DIM and I3C treated *B. napus* plants. According to Halkier and Gershenzon (2006), the production of glucosinolates such as DIM and I3C are increased when the *Brassica* spp. are under mechanical or pathogenic damage as a protection mechanism to fend off for e.g. pests. Therefore this observation can possibly be due to the fact that the *B. napus* plants perceived these treatments as mechanical damage as another study done by Knight in 1999, proved that the Ca content had increased when *A. thaliana* was exposed to mechanical damage. Furthermore, another study done by Rentel et al. (2004), on *A. thaliana* showed a rise in Ca content when *A. thaliana* plants was treated with H_2O_2, moreover, they suggested that the Ca channels might be triggered by the imbalance in the ROS production state. This further explains the increase in Ca experienced under the I3C treated *B. napus* plants as discussed in chapter 4 we also detected a significant increase in H_2O_2 activity under the I3C

treated plants. Moreover, in our study there was a significant increase in APX and CAT activity displayed for the DIM treated *B. napus* plants and this could be linked to the increase in Ca content detected under the DIM treated *B. napus* plants. This observation can be confirmed by a study done by Zouari et al. (2017), who showed that the antioxidant activity such as SOD, POD, APX, CAT and GR increased when sesame seeds were under cadmium stress once proline was added. Additionally, a study done by Sagi and Fluhr (2001), showed that ROS is required to stimulate the influx of Ca^{2+}. Therefore, in our study we saw a significant increase in both the O_2^- and H_2O_2 content under the I3C treatment and this could have possibly lead to the increase in Ca content observed for the I3C treated *B. napus* plants.

Out of all the mineral nutrients potassium (K) plays the most critical role in plant development and metabolism (Wang *et al.*, 2013). Potassium helps plants survive during exposure to biotic and abiotic stress including drought, disease, pest, salinity and frost exposure. In our study we saw a notable increase in K content for the DIM treated *B. napus* plants and this could have helped the DIM treated plants excel in growth and development compared to the I3C treated plants. Moreover, Wang et al. (2013), also demonstrated that plants with a sufficient supply of K tend to produce increased amounts of defensive antioxidant compounds and this was observed for the DIM treated plants in our study. Higher APX and CAT activities was obtained under the DIM treated plants. Ashley et al. (2006), reported that low K induced the formation of ROS and phytochromes such as auxins and this was observed for the I3C treated *B. napus* plants as our results yielded low K content for the I3C treated plants and this could have possibly contributed to the significant increase in ROS levels observed under the I3C treatment. Egilla et al. (2001), discovered that plants with an inadequate amount of K can lead to a decrease in the plants biomass. This could possibly be an explanation for the significant decrease in plant biomass observed for the I3C treated *B. napus* plants. Furthermore, according to Sinha and Saxena (2006), an increase lipid peroxidation levels affects the permeability of the cell and can result is the loss of essential ions such as K ions for instance. In our study we saw a significant increase in the MDA levels under the I3C treated plants and this could have possibly lead to the decrease in K content we observed. Horie et al. (2011), confirmed that K and Mg could possibly

compete for the same unidentified transporters and this could explain why we saw a significant decrease in Mg content but saw an increase in K for the DIM treated *B. napus* plants. The latter was seen for the I3C treated *B. napus* plants with lower uptake of K but much higher Mg content was observed.

Phosphorus (P) is vital to plant growth and is found in every living plant cell. It is involved in several key plant functions, including energy transfer, photosynthesis, transformation of sugars, starches, nutrient movement within the plant and transfer of genetic characteristics from one generation to the next (Wang *et al*, 2013). The uptake of P is dependent on the pH of the soil as stated by Marschener (1995). In our study we detected a significant increase in P content for the DIM treated *B. napus* plants. However, an even more significant uptake of P content was detected for the I3C treated *B. napus* plants and this could have triggered the onset of chlorosis we observed in the I3C treated plants. De Kock and Strmecki (2009), obtained results that showed a higher ratio of P in mustard plants resulted in the formation of chlorosis and this was observed in our study. Furthermore, in our study we observed lower levels of Zn for the DIM treated *B. napus* plants when compared to the I3C treated plants and this could be due to the high levels of P detected under the DIM treated plants. These results are in conclusion with a study done by Singh et al. (1986), they observed a lower content of Zn when higher amounts exogenous P was applied to Wheat plants.

When we analysed the Cu content we observed a significant increase in Cu content under both the DIM and I3C treated *B. napus* plants once compared to the control treated plants. As stated by Raven et al. (1999), Cu^{2+} is seen as an essential redox-active transition metal involved in many physiological processes within plants including; acting as a structural element in regulatory proteins and participates in the photosynthetic electron transport system, mitochondrial respiration, oxidative stress responses, cell hormone and hormone signalling and furthermore, acts as cofactors in many enzymes such as Cu/Zn SODS. The significant increase in Cu observed under the I3C treated plants could have contributed to the increased SOD levels observed in the I3C treated plants and could thus have signaled the I3C treated plants that it is under stress.

This observation is in alliance with a study done by Karimi et al. (2012), they showed that excess Cu on *Astragalus neo-mobayenii* increased the activities of SOD, POD, and CAT activities once they compared it to the control group. Furthermore, they detected a gradual decrease in chlorophyll amounts with the increase in Cu and this observation is in agreement with our study for the I3C treated *B. napus* as we to detected a decrease in chlorophyll amounts with the increase in Cu content we observed. Moreover, they also saw increases in the MDA levels with the increase in Cu activity observed. This again supports our results for the I3C treated *B. napus* plants as we to saw an increase in MDA under the I3C treatment in conjunction with the increase in Cu content observed once compared to the control treated plants. However, even though our study revealed a greater increase in Cu content for the DIM treated *B. napus* plants this still did not negatively affect the MDA levels and SOD content suggesting that the exogenously applied DIM treated *B. napus* seedling somehow have a coping mechanism in place, through its signalling to compensate for the increase in Cu under the DIM treated *B. napus* plants. Therefore, we hypothesize that the influx in Cu under the DIM treated *B. napus* plants could possibly have been to compensate for the increase in SOD activity, since Cu is most commonly used as a co-factor for SOD and the SOD activity had increased under the DIM treated *B. napus* plants. Therefore, an influx in Cu would need to occur to compensate for the increase in SOD activity.

The term heavy metal refers to any metallic element which has a relatively high specific gravity and is often toxic or poisonous even at low concentrations (Rout and Sahoo, 2015). However, some of the heavy metals for e.g. Iron (Fe) are known to be essential for plants and animals (Wintz *et al.*, 2002). Fe is an essential micronutrient playing critical roles in metabolic processes such as respiration, DNA synthesis and photosynthesis. Once the Fe content of our plants was investigated we observed a significant increase in Fe content for the DIM treated *B. napus* plants whereas no statistical difference between the Fe content of the I3C and control plants was observed. Even though there was a significant increase in Fe content observed for the DIM treated *B. napus* plants this did not have a negative effect on the DIM treated *B. napus* plants.

Since Fe is needed in the formation of Fe SOD's the Fe uptake under the DIM treated *B. napus* plants would need to increase to compensate for the increase in SOD activity observed under the DIM treated *B. napus* plants. A study done by Lee et al. (2007), showed that certain metal co-factors for example such as Fe is needed for the formation of Fe-SODs in Eukaryotic organisms. Furthermore, Fe is used is needed in the chlorophyll a metabolic process and we detected an increase in chlorophyll a species under the DIM treated plants again suggesting an increase in Fe would be needed to support the increase in chlorophyll a species. This observation is supported by a study by Del Rio et al. (2003) who stated that in higher plants Fe-SODs, Cu/Zn-SODs can be found in the cytosolic fractions and the chloroplasts of plants and is needed in the process of photosynthesis. The increase in Fe content for the DIM treated *B. napus* plants could possibly have been triggered by the increase in ROS observed. The DIM treated *B. napus* plants could have done this to help increase for e.g. the photosynthesis process in order to help the plant survive the increased levels of DIM which is signalling the plant that it is under 'mechanical stress'. These results is in cahoots with a study done by Graziano et al. (2002), who showed that when nitric oxide was applied to plants the increase in Fe was observed therefore with nitric oxide being a signalling molecule and DIM is a known signalling molecule, this might explain the increase in Fe observed in our for the DIM treated *B. napus* plants.

According to Mousavi et al. (2011), Manganese (Mn) can be classified as one the main micronutrients and is involved in photosynthesis and many other processes. In our study we observed a significant increase in Mn activity for the DIM treated *B. napus* plants compared to both the control and I3C treated *B. napus* plants. This increase in Mn activity observed under the DIM treatment could possibly explain why we saw a significant increase in SOD activity under the DIM treated plants as a study done by Mousavi et al. (2011), showed that Mn is an important component needed in the structural formation of SOD. As previously stated we saw a significant increase in Mg content for the DIM treated *B. napus* plants and this could have contributed to the increase in Mn content observed under the DIM treatment. This can be confirmed by a study done by Mengel and Kirby (2001), showed that an increase in Mg activity

could possibly lead to an increase in cations such as K and Mn. Moreover, we saw an increase in Mn content under the I3C treatment compared to control but this increase was much lower compared to the DIM treated *B. napus* plants. This could be due to I3C already negatively affecting the Mg content.

According to Hafeez et al. (2013), Zinc (Zn) is a plant micronutrient involved in many physiological functions and an inadequate supply of it will not only reduce crop yields but also result in chlorosis, development of smaller leaves and increasing crop maturity period to mention a few. Moreover, Zn is involved in various enzymatic reactions, metabolic processes, is needed for nitrogen metabolism, energy transfer and protein synthesis. In our study we saw no statistical difference between the Zn content of the DIM treated *B .napus* plants compared to the controls. However, we observed a noteworthy increase in Zn content of the I3C treated *B. napus* plants compared to the control and DIM plants. Although Zn is an essential micronutrient, increased amounts of it can be detrimental to the plants health affecting the development, enzymatic reactions, metabolic processes and cause inadequate absorption of other essential micronutrients such as Cu and Zn of the plant. This might explain why we observed lower levels of Fe and Cu under the I3C treatment as the excessive absorption of Zn suppressed the adequate absorption of Fe and Cu ions. Nevertheless, excessive Zn has been shown to generate oxidative stress (Rao and Sresty, 2000 and Millan *et al.*, 2005) by producing toxic levels O_2^- and H_2O_2. This could have exacerbated the increase in ROS we detected in our study for the I3C treated *B. napus* plants. Additionally, studies done by Jain et al. (2010), Cui and Zhao (2011), showed that excessive Zn caused disruptions in the antioxidant defense system and the photosynthetic electron transport system. Moreover, their results showed reductions in namely SOD, APX, GR and AA activity and reduced levels of both chlorophyll a and b species under excessive Zn. Therefore, it can be deduced that the increase in the Zn content we detected for the I3C treated *B. napus* plants could have aided in the poor functioning of the I3C treated plants in terms of chlorosis, MDA levels, increased ROS levels and stunting the growth of the plants.

Conclusion and Future Prospectives

This investigation has ascertained that the exogenous application of I3C negatively effects the growth and development of *B. napus* plants. Contrastingly, the exogenous application of DIM has proven to have positive effects on growth and development of *B. napus* plants. The results reported here concludes that both DIM and I3C treatments increased seed germination percentage of *B. napus* plants, as both of these compounds are glucosinolates and helped the seeds overcome seed dormancy. However, DIM did not only increase seed germination but also enhanced seedling shoot growth whereas I3C stunted growth. I3C treatment inhibited the growth of the *B. napus* plants as it acted as an antagonist by directly competing with the plant growth hormone IAA in fine tuning plant growth and development. I3C treatment also negatively affected the morphology of the plants leading to improper leaf, stem, and root formation eventually poor nutrient absorption. Moreover, I3C treatment stimulated the increase of ROS which lead to the destruction of many cellular components. Therefore focusing specifically on the chloroplast, I3C treatment caused chlorosis in addition chloroplast damage resulted in detrimental increases in lipid peroxidation. On the other hand DIM treatment lead to increased levels of chlorophyll species by reducing the amount of chlorophyll degrading enzymes thus improving photosynthesis and ultimately growth. Additionally well-developed leaves, stems and roots were present aiding in the steadfast growth and development of the DIM treated plants. Nonetheless, the I3C treatment increased O_2^- and H_2O_2 concentrations to levels that was detrimental to the plants leading to deformities in the plants morphology and stagnant functioning in the biochemistry of the plants. Furthermore, I3C treatment increased SOD levels but decreased CAT, APX and AsA levels which facilitated in the inadequate functioning and development of the I3C treated plants. Nevertheless, DIM treated plants also lead to an increase in SOD levels but not as high as for the I3C treated plants in addition APX levels was also upregulated accommodating for the increase in O_2^- and H_2O_2 experienced by the

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DIM treated plants. In all I3C was damaging to the *B. napus* plants stunting growth, increasing ROS to toxic levels and decreasing vital antioxidants. Furthermore we now know that the significant increase in H_2O_2 and O_2^- levels triggered cell death in the I3C treated plants. Whereas the significant increase experienced in H_2O_2 and O_2^- production for the DIM treated plants was used for signalling purposes as it promoted growth, increased ROS but below detrimental levels and increased antioxidant levels to help alleviate the oxidative stress caused. Furthermore, DIM increased the uptake of essential nutrients with the most notable increase being magnesium which possibly aided in the steadfast development of the *B. napus* plants compared to the nutrient profile of I3C plants as most of the major macro and micro nutrients was significantly decreased under the I3C treatment. The decrease in these nutrients further elucidated why the exogenous I3C treatment was so crippling to the *B. napus* plants.

Future work will include, using 2D PAGE analysis to identify potential proteins involved in improving growth under exogenous DIM treatment and identifying proteins involved in the poor functioning under exogenous I3C treatment. Furthermore, HPLC analysis would be done to determine the concentration of Indole acetic acid, DIM and I3C to investigate the endogenous levels of DIM and I3C in the *B. napus* plants. Investigate what genes are affected by Magnesium to find out whether Magnesium and Indole acetic acid uses similar transporters, because Magnesium was one of the major macro nutrients that was upregulated in our investigation.

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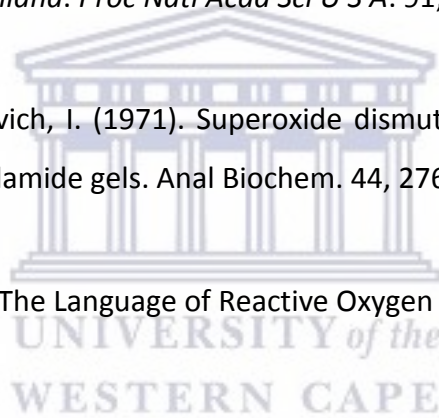
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