Impact of vanadium stress on physiological and biochemical characteristics in heavy metal susceptible and tolerant Brassicaceae

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

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LIST OF ABBREVIATIONS

APX	Ascorbate peroxidase
AU	Agamax untreated (control)
AV	Agamax treated with vanadium
CAT	Catalase
DNA	Deoxyribonucleic acid
GPX	Glutathione peroxidase
GU	Garnet untreated (control)
GV	Garnet treated with vanadium
MDA	Malondialdehyde
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazollium Bromide)
NBT	Nitro blue tetrazolium chloride
PAGE	Polyacrylamide gel electrophoresis
PCD	Programmed cell death
PMS	Phenazine methosulfate
POD	Guaiacol Peroxidase

RNA	Ribonucleic acid
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SSA	5-Sulfosalicyclic acid
TBA	Thiobarbituric acid
TEMED	N,N, N', N'- Tetramethylethylenediamine

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Impact of vanadium stress on physiological and biochemical characteristics in heavy metal susceptible and tolerant Brassicaceae

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KEYWORDS

Antioxidant enzymes

Ascorbate peroxidase

Biomass

Cell death

Heavy metal

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Hypertolerant

Lipid peroxidation

Reactive oxygen species

Superoxide

Superoxide dismutase

Vanadium

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ABSTRACT

There is an influx in heavy metals into soils and ground water due to activities such as increased mineral mining, improper watering and the use of heavy metal contaminated fertilizers. These heavy metals are able to increase the ROS species within plants which may result in plant metabolism deterioration and tissue damage. Heavy metals may also directly damage plants by rendering important enzymes non-functional through binding in metal binding sites of enzymes. The heavy metal focused on in this study was vanadium due to South Africa being one of the primary produces of this metal. Two related *Brassica napus* L cultivars namely Agamax and Garnet which are economically and environmentally important to South Africa were exposed to vanadium. Physiological experiments such as cell death, chlorophyll and biomass determination were conducted to understand how these cultivars were affected by vanadium toxicity. A low cost, sensitive and robust vanadium assay was developed to estimate the amount of vanadium in samples such as water, soils and plant material. The oxidative state as well as the antioxidant profile of the two cultivars were also observed under vanadium stress. A chlorophyll assay which was conducted on the two cultivars

exposed to vanadium showed a marked decrease in chlorophyll A in the suspected sensitive cultivar which was Garnet. However, the suspected tolerant cultivar Agamax fared better and the decrease in chlorophyll A was much less. A similar trend was observed for the two cultivars when the cell death assay was conducted. The vanadium assay showed that Garnet had higher concentrations of vanadium within its leaves and lower concentrations in its roots when compared to Agamax. This observation displayed that Agamax had inherent mechanisms which it used to localize vanadium in its roots and which assisted in its tolerance to the vanadium stress.

The oxidative state was determined by doing assays for the specific reactive oxygen species namely hydrogen peroxide and superoxide. It was observed that vanadium treated Garnet leaves had higher reactive oxygen species (ROS) production when compared to the Agamax treated leaves. In-gel native PAGE activity gels were conducted to determine the antioxidant profile for the two cultivars which were exposed to vanadium. The antioxidant enzymes which were under investigation were ascorbate peroxide (APX), superoxide dismutase (SOD) and glutathione-dependent peroxidases (GPX-like) as these enzymes are known to be responsible for controlling the ROS produced in the plants. The GPX-like profile consisted of three isoforms. No isoforms were inhibited by vanadium treatments but one isoform had increased activity in both the Garnet and Agamax treated samples. The SOD profile for Garnet consisted of six isoforms

and Agamax had seven isoforms. One isoform which was visualized in both Agamax as well as Garnet was inhibited by vanadium treatments. Agamax also had two isoforms which were up-regulated however the corresponding isoforms in Garnet showed no change. The Ascorbate peroxidase profile consisted of seven isoforms for both Garnet and Agamax. No isoforms were inhibited by vanadium treatment. Three isoforms were up-regulated in Garnet and Agamax under vanadium treatments.

Here, it is illustrated that Garnet lacked certain mechanisms found in Agamax (and thus experienced more cell death, yield and chlorophyll loss) and performed worst under high vanadium concentrations. Although Garnet increased the activity of some of its antioxidant isoforms in response to increasing ROS levels it was not adequate to maintain a normal oxidative homeostasis. This disruption in oxidative homeostasis lead to plant damage. Agamax was observed to produce less ROS than Garnet and was able to control the ROS produced more effectively than Garnet and thus less damage was observed in Agamax.

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Chapter 1

Literature review

1.1. Introduction

Abiotic stresses are among the most dangerous threats to plants due to a plants inability to move away from a stress affected area (Tuteja *et al*., 2009; Bhatnagar-Mathur *et al*., 2008) Plants are able to perceive abiotic stresses and may respond by altering their metabolism and growth processes (Wani *et al*., 2007). This response was observed in white clover, which showed decreased nitrogen fixation when exposed to heavy metals (Wani *et al*., 2007). Abiotic stresses includes: extreme temperature, drought, high salinity, extreme pH as well as elevated heavy metal concentrations (Nakashima *et al*., 2012; Tao *et al*., 2011).

Agricultural land was found found to be slightly to moderately contaminated with heavy metals such as Zinc, Nickel, Cobalt and Arsenic (Yadav, 2010). Plants need certain metals in trace amounts to survive (Rascio & Navari-Izzo, 2011; Vachirapatama *et al*., 2011). However, when plants are exposed to high concentrations of heavy metals they often experience disruption to their biochemistry as well as retarded growth (Vachirapatama *et al*., 2011; Wang & Liu, 1999). Plants are not always directly affected by heavy metals, elevated heavy metal concentrations have the ability to hinder the activity of certain

microorganisms within the soil. These microorganisms are often beneficial to plants and a reduction in their growth leads to poor plant growth (Guala *et al.,* 2010; Chehregani *et al.,* 2005). The contamination of soils can be attributed to the use of phosphatic fertilizers, bad watering, industrial waste and mining activities (Vachirapatama *et al*., 2011; Yang *et al*., 2011; Yadav, 2010).

The mining industry within South Africa is a major supplier of heavy metals such as vanadium (Moskalyk & Alfantazi, 2003). It is therefore expected that there would be a high concentration of vanadium as well as other heavy metals within South African soils in particular surrounding these mining activities (Saco *et al*., 2013). Vanadium at elevated concentrations similar to other heavy metals may pose serious problems to plants as well as animals when consumed (Mukherjee *et al*., 2004). It is therefore important to determine the mechanisms which afford some plants elevated tolerance to high vanadium concentrations (Mahanty *et al*., 2012; Mourato *et al*., 2012).

Vanadium is one of the 17 metals which have been proven to be a potential benefit to plants in trace amounts (Shyam & Aery, 2012). It was observed by Vachirapatama et al. (2011) that Chinese green mustard plants experienced enhanced growth when given vanadium at concentrations lower than 10 mg/kg (100 µM). The most toxic vanadium species is vanadium with five valence electrons (V) which has been found to be present in soil as well as water

(Vachirapatama *et al*., 2011; Panichev *et al*., 2006). Phosphate fertilizers as well as soils were found to have concentrations of vanadium which was higher than 180 mg/kg (Vachirapatama *et al*., 2011). It was also noted that at just 30 mg/kg of vanadium in soils, plants experienced significantly reduced yields as well as yellowing of leaves (Vachirapatama *et al*., 2011; Wang & Liu, 1999). The reduced yields were attributed to the disruption of plant metabolism by reactive oxygen species (ROS) (Vachirapatama *et al*., 2011).

1.2. Reactive Oxygen Species within plants

To survive abiotic stresses plants have evolved and adapted a signalling network which involves different growth regulators to not only sense but also offer protection to the plant (Bhattacharjee, 2011). One of the possible responses to environmental stresses is the increased generation of (ROS) (Bhattacharjee, 2011). ROS may also be directly generated from the interaction with heavy metals through the Haber-Weiss reaction (Yadav, 2010). ROS include compounds such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) (Sinha & Saxena, 2006). During normal aerobic metabolism ROS are produced as by-products, but under stressful conditions the production is increased (Bhattacharjee, 2011; Gill & Tuteja, 2010). Although reactive compounds are used as stress signalling molecules within the plant, when these compounds are accumulated it may be detrimental to the cells as they promote cellular damage (Zhang *et al*., 2007; Wang *et al*., 2005). The accumulation of ROS may damage

proteins, lipids and carbohydrates as well as DNA which would lead to cellular death (Maruta *et al*., 2012; Gill & Tuteja, 2010; Tsai *et al*., 2005). Organelles such as the chloroplast are particularly sensitive to ROS due to the high concentration of oxygen which reacts within the photosynthetic electron transfer system (Wang *et al*., 2005). The accumulation of ROS is thought to be as a result of the disruption in the balance of ROS production and the antioxidation systems (Zhang *et al*., 2007).

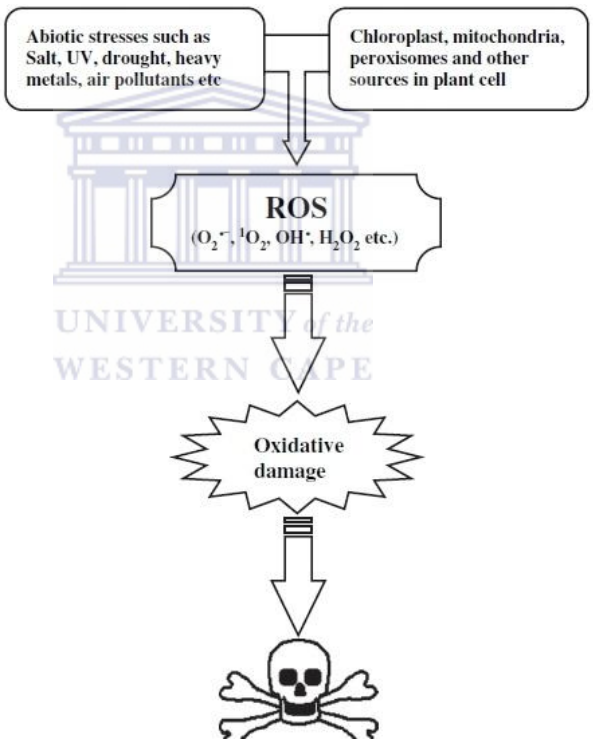

Figure 1.1: Diagram showing how stress in plants may lead to cell damage. Stresses and organelles which produce ROS leading to oxidative damage and cell death (adapted from Gill & Tuteja., 2010).

1.2.1. Hydroxyl radicals (OH^-)

The hydroxyl radical is one of the most reactive compounds among the ROS (Gill & Tuteja, 2010; Babbs *et al*., 1989). In the presence of transition metals such as iron. H_2O_2 and O_2^- may be converted into OH^- through the fenton reaction (Gill & Tuteja, 2010). Plants allow uptake of many different transition metals as they are needed for metabolic processes. Once taken up these metals could lead to the overproduction of hydroxyl radicals which would result in cell damage.

$$H_2O_2 + O_2^- \xrightarrow{Fe^{2+},\ Fe^{3+}\ \text{or (Other transition metals)}} OH^- + O_2 + OH\bullet$$

Figure 1.2: Hydroxyl radicals produced through a Fenton reaction using iron as the transition metal

One suggested reason for the production of OH^- compounds is the proposed involvement in regulating oxygen toxicity within plants (Vranova *et al*., 2002). Due to the reactive nature of this compound it is able to react with most of the macromolecules that it comes in contact with; these include proteins, lipids and DNA (Gill & Tuteja, 2010). Thus OH^- is able to damage many cell structures and if not removed will lead to cell death.

1.2.2. Superoxide (O_2^-)

Superoxide is produced by the partial reduction of oxygen within plants often during photosynthesis (Gill & Tuteja, 2010). Up to 2% of the total oxygen consumption within a plant will be due to the production of O_2^- (Gill & Tuteja, 2010). A major site of O_2^- formation is the electron acceptor of the photosystem I that is bound to the thylakoid membrane (Gill & Tuteja, 2010; Boveris & Puntarulo, 1998). O_2^- has a short half-life of 2-4 microseconds. O_2^- is one of the first ROS which is produced and can initiate the production of other reactive species (Gill & Tuteja, 2010; Halliwell, 2006). The initiation and subsequent production of these ROS may then lead to damage within the plant cells such as peroxidation and weakening of the cell structure (Gill and Tuteja, 2010; Halliwell, 2006).

1.2.3. Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide may be produced in plants by the reduction of O_2^- (Gill & Tuteja, 2010). When compared to other ROS, H_2O_2 was found to be only moderately reactive but also has a longer half-life at 1 millisecond (Gill & Tuteja., 2010). Due to its long half-life coupled with its high stability H_2O_2 is an efficient signalling molecule as it is able to travel relatively large distances as well as permeate across membranes. Although H_2O_2 is relatively stable, at high concentrations it is able to inactivate enzymes by oxidizing thiol groups within

the enzyme structure (Cheeseman, 2007; Tewari *et al*., 2006). Taking into consideration the above mentioned fact it can be understood why H_2O_2 can be seen as a very dangerous compound to many organisms. At low concentrations H_2O_2 is a signalling molecule which induces abiotic stress tolerance in plants (Gill & Tuteja, 2010; Quan *et al*., 2008). At high concentrations however H_2O_2 is able to initiate programmed cell death in plants (Quan *et al*., 2008). Besides its ability to signal, H_2O_2 was identified as a regulator for processes such as senescence and photosynthesis in plants (Gill & Tuteja, 2010; Peng *et al*., 2005). It is interesting to note that a study by Tanoua et al. (2009) where roots were pre-treated with H_2O_2, led to increase SOD, APX and CAT activities when the plants were under salt stress.

1.3. ROS and cell biochemistry

1.3.1. Lipid peroxidation

Lipid peroxidation is the catalytic change of the structure and function of a membrane (Yadav, 2010; Verma & Dubey, 2003). As was previously mentioned ROS are over produced when a plant undergoes stress. These radicals are able to disrupt the polyunsaturated fatty acid (components which make up membrane lipids) and cause lipid peroxidation (Verma & Dubey, 2003). Malondialdehyde (MDA) is one of the cytotoxic compounds which are produced during lipid peroxidation and is therefore used as an indicator of lipid peroxide production,

radical production and oxidative damage to an organism (Wahsha *et al*., 2012; Zhang *et al*., 2007). The implications of lipid peroxidation include the destabilization of the cell membrane which affects the permeability of the cell and leads to a loss of important ions such as potassium ions (Zhang *et al*., 2007; Sinha & Saxena, 2006). The reaction between thiobarbituric acid and MDA is used as an indication to the degree of lipid peroxidation within a tissue (Wahsha *et al*., 2012; Verma & Dubey, 2003).

1.3.2. Chlorosis within plant material

Chlorophyll is one of the most abundant pigments found on earth, and gives plant material their iconic green colour (Hörtensteiner & Krautler, 2011). Chlorosis is the abnormal yellowing of plant tissue due to failure to produce chlorophyll and/or the destruction of the chlorophyll that is present (Abadia *et al*., 2011; Yadav, 2010; Fatoba & Emem, 2008). A study by Fatoba and Emem (2008) showed that metal concentrations as low as 10 mg/L were able to negatively affect chlorophyll in less than 3 weeks as the plant could be seen to be physically weaker. As previously stated chloroplasts are sensitive to oxidative stress (Wang *et al*., 2005). Therefore at high concentrations of ROS chloroplast might experience oxidative damage and the production of chlorophyll will decrease leading to chlorosis (Mourato *et al.,* 2012; Yadav, 2010; Wang *et al*., 2005). If chlorosis is not reversed the plant will lack the ability to perform

photosynthesis which will lead to nutrient deficiency and ultimately death (Wang *et al*., 2005).

1.3.3 Damage to DNA

The genome of plants is reported to be highly stable but damage can occur if it comes into contact with DNA damaging compounds (Gill & Tuteja, 2010). DNA may be damaged by OH^- and singlet oxygen under stressful conditions (Gill & Tuteja, 2010). (OH^-) are able to attack and damage both the purine and pyrimidine which make up the primary structure of DNA (Wiseman & Halliwell, 1996). The singlet oxygen molecule however damages the guanine nucleotides within DNA structures (Wiseman & Halliwell, 1996). The damage which could ensue due to these reactive molecules includes strand cleavage, and the modification and deletion of bases (Tuteja *et al*., 2001). It is interesting to note that the more stable ROS H_2O_2 and unstable O_2^- cannot directly damage DNA within plant cells. The repercussions of DNA damage include a reduction in protein synthesis. Gichner et al. (2006) reported that DNA damage occurred in tobacco and potato leaves when these plants were exposed to soils containing elevated levels of heavy metals such as cadmium, copper, lead and zinc and attributed this to either necrotic or apoptotic DNA fragmentation.

1.4. Antioxidant enzymes and compounds prevalent in plants

Due to the destructive nature of reactive compounds special control measures such as the ROS-scavenging pathways are present in plants (Bhattacharjee, 2011). These pathways are able to metabolise ROS and therefore decrease their concentration within plants (Mahanty *et al*., 2012; Gill & Tuteja, 2010). Among the anti-oxidative enzymes within the plant ascorbate peroxidase (APX), superoxide dismutase (SOD) and catalase (CAT) were found to be very important (Lee *et al*., 2007; Sinha & Saxena, 2006; Blokhina *et al*., 2003). SOD is one of the first scavenging enzymes in the antioxidant pathway and converts O_2^- to H_2O_2 (Lee *et al*., 2007; Wang *et al*., 2005).

1.4.1. Superoxide dismutase (SOD)

Superoxide dismutases are classified by the metal cofactor which is needed for its proper function (Mahanty *et al*., 2012; Wang *et al*., 2005). Certain metals which are required by SODs include iron, manganese, copper-zinc as well as nickel (Mahanty *et al*., 2012; Wang *et al*., 2005). The requirement of different metals by SOD enzymes could be due to the evolutionary response to the availability of certain metals (Mahanty *et al*., 2012). Amino acid sequence information obtained suggests that the manganese SODs as well iron SODs could have a common ancestor enzyme (Mahanty *et al*., 2012). Within eukaryotic organisms the different metal SODs are located within particular organelles, such

as the copper-zinc SODs which are found within chloroplasts as well as the cytoplasm (Mahanty *et al*., 2012; Lee *et al*., 2007). The defensive action of the copper-zinc SOD was investigated by over expressing the enzyme in a plant and observing the degree of oxidative stress tolerance afforded to the plant (Mahanty *et al*., 2012; Lee *et al*., 2007). Copper zinc SODs were found to be the most prevalent SOD isoforms within plants (Mahanty *et al*., 2012). The substitution of copper with other metals within the copper-zinc SOD was observed to inactivate the enzyme, which highlights the importance of the correct metal being incorporated into the SOD (Mahanty *et al*., 2012). The H_2O_2 molecules produced by the SODs may be scavenged by peroxidases, this interaction thus reduces the concentration of ROS (Zhang *et al*., 2007).

1.4.2. Ascorbate peroxidase (APX)

Ascorbate peroxidase (APX) is an enzyme which scavenges H_2O_2 and in this way plays a role in the regulation of ROS within plants (Maruta *et al*., 2012; Kornyeyev *et al*., 2003).The APX much like other peroxidases have a heme group which helps the enzymes to perform their respective functions (Verma & Dubey, 2003). The enzyme uses H_2O_2 and converts ascorbic acid to dehydroascorbate and in this way the concentration of H_2O_2 is lowered (Sinha & Saxena, 2006; Sarowar *et al*., 2005). The enzyme is found within the mitochondria, peroxisomes, cytosol as well as the chloroplasts of plants (Maruta *et al*., 2012; Sinha & Saxena, 2006). It was observed that when APX activity was up-regulated

in plants they were more tolerant to abiotic stresses (Kornyeyev *et al*., 2003). It should however be noted that the increase in activity of only one antioxidant enzyme might not always lead to more efficient ROS scavenging or increased tolerance to abiotic stresses (Lee *et al*., 2007). The peroxidase has also been observed to be involved in the synthesis of lignin which can form a physical barrier to heavy metal poisoning (Sinha & Saxena, 2006).

1.4.3. Catalase (CAT)

The catalase was one of the first antioxidant enzymes to be discovered (Mhamdi *et al*., 2010). Due to the ubiquitous nature of catalases it was established that this enzyme was very important for the preservation of life (Kirkman & Gaetani, 2007). The catalase class of enzymes do not require reductant molecules as the reaction which they catalyse are dismutation reactions (Mhamdi *et al*., 2010; Scandalios *et al*., 1997). The aforementioned characteristic distinguishes the catalase enzymes from other antioxidant enzymes (Mhamdi *et al*., 2010). Another characteristic which distinguishes catalase as well as APXs from other peroxides are their high specificity for H_2O_2 (Mhamdi *et al*., 2010; König *et al*., 2002). The catalase reaction often consists of converting two molecules of H_2O_2 to oxygen and water (Mhamdi *et al*., 2010). The H_2O_2 concentrations within cells are decreased by means of the aforementioned process and protects the cells from oxidative damage (Mhamdi *et al*., 2010). Most plants have been observed to have two isoforms of the catalase enzyme (Mhamdi *et al*., 2010). One of the

isoforms was found to be present within the cytosol of the cells and the other was localized within the plant's peroxisomes (Mhamdi *et al*., 2010; Petrova *et al*., 2004).

1.4.4. Glutathione as an antioxidant compound

Glutathione has been found to be abundant in plant cells, being present in many organelles such as the chloroplast, vacuole and mitochondria (Yadav, 2010). The ratio of reduced versus oxidised glutathione has been suggested to be a redox balance indicator which helps with ROS perception in plants (Yadav, 2010). The compound has also been found to help detoxify heavy metals as well as decrease ROS such as H_2O_2 (Yadav, 2010). Glutathione plays a pivotal role in the production of phytochelatins which allow for the removal of heavy metals from plants (Yadav, 2010). Glutathione is stable, reactive and highly soluble and these characteristics allow the compound to perform its many functions (Yadav, 2010).

1.5. Transport and storage of heavy metals within plant systems

1.5.1. Metal uptake and transport within plant cells

Plants are able to take up many pollutants through their roots. These pollutants may include heavy metals that are subsequently taken up by the root system (Mingorance *et al*., 2007; Kovács *et al*., 1993). Once metals have entered through

the root system they have the ability to leak out into other tissues and subsequently be transported to other parts of the plant (Nyguist & Greger, 2007). Metals are able to bind to negatively charged sites on the cell wall which results in a high concentration outside of the cell (Abadia *et al*., 2011; Nyguist & Greger, 2007; Cutler & Rains, 1974). This high metal concentration may cause a gradient across the membrane and promote the transport of metals into the cell (Nyguist & Greger, 2007). It was identified that certain proteins act as transporters for heavy metals such as the NRAMP which transports cadmium (Verkleij *et al*., 2009). Plants employ many strategies and may respond by immobilizing the metals in different tissues and restricting entry into the cell (Nyguist & Greger, 2007). These strategies are employed to limit the damage from process such as lipid peroxidation which could be induced by high metal concentrations (Tuteja *et al*., 2009). Plants which can survive high concentrations of heavy metals employ different survival mechanism and are either termed hypertolerant hyperaccumulators or hypertolerant non-hyperaccumulators (Rascio & Navari-Izzo, 2011).

1.5.2. Heavy metal hypertolerant hyperaccumulating plants

Hyperaccumulators are plants which developed the ability to not only tolerate high concentrations of heavy metals but also to accumulate these metals within plant cells (Rascio & Navari-Izzo, 2011; Verbruggen *et al*., 2009; Ozturk *et al*., 2003). Characteristics which are used to identify potential hyperaccumulators

include: a greater uptake of heavy metals from the soil; the efficient transport of heavy metals from root to shoot; and the ability to detoxify and deposit high amounts of heavy metals within the leaves (Rascio & Navari-Izzo, 2011). Plants such as *Serbertia acuminate* were reported to be able to accumulate up to 26% (w/w) of the heavy metal nickel which indicates the extent of tolerance of these plants (Verbruggen *et al*., 2009). This phenomenon could be beneficial as these plants could be used as tools for heavy metal phytoremediation (Verbruggen *et al*., 2009; Verkleij *et al*., 2009). The ability to hyperaccumulate heavy metals was found to be present in more than 34 plant families. A high occurrence of the hyperaccumulator trait was found to be present within the Brassicaceae family (Rascio & Navari-Izzo, 2011; Verbruggen *et al*., 2009). Hyperaccumulation of certain metals such as zinc and cadmium were found to only be present in the Brassicaceae family (Verbruggen *et al*., 2009; Ozturk *et al*., 2003). The hypothesised factors which lead to plants evolving and acquiring this trait include; increased metal tolerance, protection against pathogens and herbivores, inadvertent uptake and drought tolerance (Rascio & Navari-Izzo, 2011; Verbruggen *et al*., 2009). One reason why plants would use heavy metals as part of their defence is due to the uptake of the metals from the soil being free and not synthesised from the plant making the defence more metabolically cost effective to the plant (Rascio & Navari-Izzo, 2011). Meerts and Van Isacker (1997) observed that populations of hyper-accumulators which lived on soils rich in heavy metals would accumulate less metal than their counterparts which live on nonmetallicolous soils when grown on the same substrate. It was also observed

in the same study that the nonmetallicolous populations had reduced performance which indicates that these plants were ill equipped to function maximally in soils with high heavy metal concentrations.

1.5.3. Heavy metal hypertolerant non-hyperaccumulating plants

Whereas hyperaccumulators accumulate large amounts of heavy metals within plant material, non-hyperaccumulators tend to use a method of exclusion to reduce the amount of heavy metals entering the plant (Ozturk *et al*., 2003). Non-hyperaccumulators try to hinder the entry of metals into the plant by excreting organic acids which then bind to the heavy metals (Hossain *et al*., 2012; Rascio & Navari-Izzo, 2011). The heavy metals which do however enter the plant are bound to organic acids, amino acids or phytochelatins and then removed from the plant (Rascio & Navari-Izzo, 2011; Verkleij *et al*., 2009). Another mechanism used by hypertolerant plants is to produce metal binding ligands to segregate heavy metals and reduce damage to the plants (Verkleij *et al*., 2009). Two types of metal binding peptide ligands which are produced are the phytochelatins and metallothioneins (Hossain *et al*., 2012). Non-accumulators may also deposit heavy metals within less active tissue such as the epidermal cells (Hossain *et al*., 2012). Most plants are able to use at least one of these strategies when faced with heavy metal stress, hypertolerant plants however fair better because they are able to use more strategies.

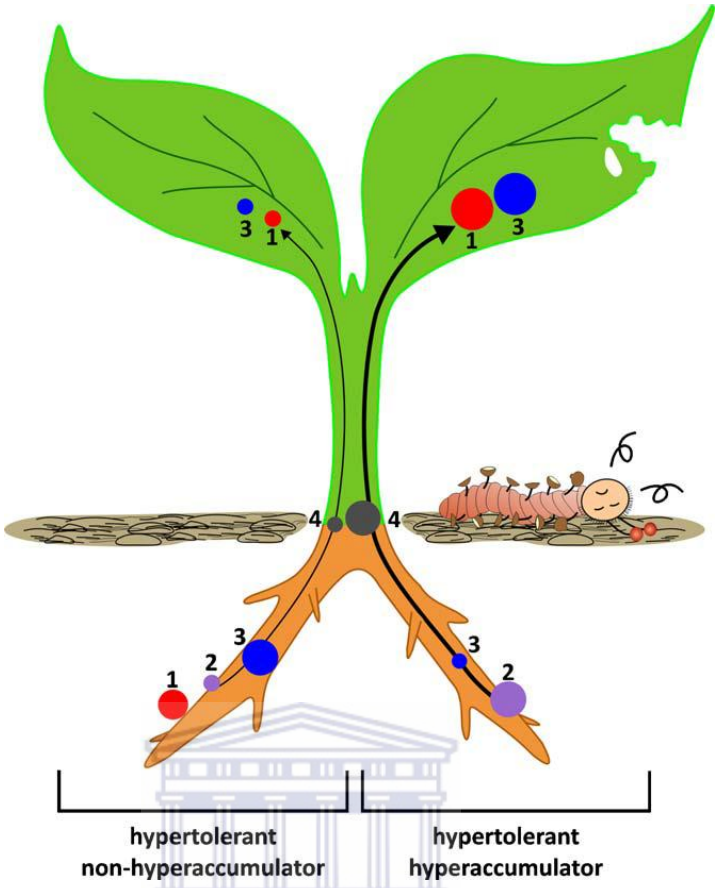

Figure 1.3: Two types of hypertolerance found in plants and how heavy metals are translocated within plants for each system. The left indicates non-hyperaccumulating plants which limit most of the heavy metal intake to the roots. The right indicates hyperaccumulating plants which are able to transport and store heavy metals throughout the plants. The colours indicate heavy metals, the bigger circles indicating high metal concentrations (Adapted from Rascio and Navari-Izzo (2011).

1.6. *Brassica napus*

The use and production of *Brassica napus* (Canola) started in Europe in the early 14th century (Grispen *et al*., 2006; Lagercrantz, 1998). Canola which is associated

with the family Brassicaceae was bred to remove components such as erucic acid and glucosinolates which were not nutritionally valuable and safe for human consumption (Miller-Cebert *et al*., 2009). Canola produces seeds which are rich in proteins as well as oils with the latter having concentrations of up to 40% within the seeds (Miller-Cebert *et al*., 2009; Pass and Pierce, 2002). Due to the aforementioned facts, canola has the ability to lower the risk of coronary heart disease and has been endorsed by many organisations (Miller-Cebert *et al*., 2009; Van Duyn & Pivonka, 2000). Canola may also be used as animal feed as well as biomass for biofuel production (Grispen *et al*., 2006). The interest generated by the benefits associated with this crop has led to the development of cultivars with different characteristics. It would therefore be beneficial to understand how heavy metal toxicity will influence this crop.

1.7. Justification

As the world becomes more industrialised and industries such as the mining sector are always expanding. Not only is land lost to these mining activities but the surrounding land becomes toxic for plants and animals due to the leaching and spreading of heavy metals such as gold, silver, lead, copper, cadmium, chrome and vanadium within the soil and air. It is therefore important to identify plants which are tolerant to these heavy metals especially vanadium. One reason for this is that plants which have vanadium stress resistance as well as the ability to absorb vanadium may be used in phytoremediation to improve soil quality. By

identifying plants with improved characteristics when exposed to vanadium stress, plant breeding strategies can be used to increase the tolerance of other plants. Countries such as Canada have legislation on the maximum concentrations of different heavy metals which are allowed in soils. As of yet South Africa has no such legislation. This could be detrimental to our food supply as heavy metal contamination could not only decrease food yields but may also be consumed by humans leading to health complications.

1.8. Objectives of this study

The focus of this study is to understand how vanadium stress may affect the biochemical and physiological characteristics of *Brassica napus* cultivars. This project will obtain information pertaining to the toxicity of vanadium and the survival mechanisms which the *B. napus* cultivars may employ. The aims of this study include developing a robust, sensitive and cost effective method for quantifying vanadium concentrations in plant material. To identify vanadium tolerant and susceptible *B. napus* cultivars. It also includes determining the antioxidant profiles for the *B. napus* cultivars to identify why certain cultivars fair better under vanadium stress. The effect of vanadium on biomass production of the *B. napus* cultivars will also be investigated. Biochemical tests will be used to determine the damage done by ROS on the *B. napus* cultivars. This knowledge could be used to develop high throughput methods to determine vanadium concentrations within South African soils and plants. The information obtained

could also be used as the basis for legislation for lower heavy metal levels in these soils.

Chapter 2

Materials and Methods

2.1. List of chemicals and suppliers

(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazollium Bromide)	Sigma- Aldrich
2- Thiobarbituric acid	Sigma- Aldrich
30 % acrylamide solution 37.5: 1	Sigma- Aldrich
5- sulfosalicylic acid dehydrate	Sigma- Aldrich
Acetone	Sigma- Aldrich
Agamax Seeds	Agricol
Ethanol 200 proof	Sigma- Aldrich
Evans blue	Sigma- Aldrich
Filter Sand	Cape Silica
Garnet Seeds	Agricol
Glycine 99%	Sigma- Aldrich
L- Ascorbic acid	Sigma- Aldrich

L- Glutathione reduced	Sigma- Aldrich
Nitro blue tetrazolium chloride monohydrate	Sigma- Aldrich
Phenazine methosulfate	Sigma- Aldrich
Ponceau xylidine	Sigma- Aldrich
Potassium Bromate	Sigma- Aldrich
Potassium hydroxide	Sigma- Aldrich
Potassium phosphate dibasic	Sigma- Aldrich
Potassium phosphate monobasic	Sigma- Aldrich
Quick start bradord dye reagent 1x	Bio-Rad
Sodium dodecyl sulfate	Bio-Rad
Sodium metavanadate	Sigma- Aldrich
Trichloroacetic acid 99%	Sigma- Aldrich
Tris (hydroxymethyl) amino-methane	Sigma- Aldrich

2.1. Growth parameters

B. napus L (Agamax and Garnet) seeds were germinated in a foil pan containing a soil mix with a ratio of 1:2 of soil and filter sand respectively. Once plants had grown to the seedling stage, each plant was carefully removed and re-planted into a pot with 500 g of the same soil mix. A solution of 350 µM sodium metavanadate was prepared by dissolving an appropriate mass of powder in tap water. Treatment of plants commenced when the plants were at the four leaf stage. A group of these plants were then treated with 100 ml of 350 µM sodium metavanadate solution and the other group which were the controls were treated with 100 ml of water. Treatments were given twice a week to both sets of plants. These plants were grown for three weeks (21 days).

2.2. The effect of vanadium on biomass production in roots and leaves

Once the plants had grown (while treated) for three weeks (21 days) they were removed from their individual pots. It is important to note not all the plants were harvested as some were kept in the pot for assays which would need fresh plant material. The roots were removed by cutting them at the interface between the root and stem. Four leaves from each plant were also removed by cutting at the base of the leaf. The roots and four leaves of a plant were inserted into separate foil envelopes, holes were then poked into the foil so that moisture could escape the envelope. The samples were then dried overnight in an incubator at 80°C.

Once dry, the samples were weighed using a fine mass balance and the values were recorded.

2.3. Evans blue assay (cell death)

A modified method of Sanevas et al. (2007) was followed for the cell death assay. A 0.25% (w/v) Evans blue solution was prepared and 1 ml of the solution was then aliquoted into Eppendorf tubes. A 1 cm^3 block was excised from fresh leaf material an inserted into an Eppendorf containing the Evans blue solution. Roots were assayed by cutting a 2 cm length from the tip of the root and inserted into the Evans blue solution. The samples were then incubated for 1 hour at room temperature in the Evans blue solution. After the incubation period the Evans blue was rinsed from the samples. These samples were incubated in water overnight. The water was decanted and 1 ml of a 1% (w/v) SDS solution was added to the sample. The samples were then crushed in the SDS solution and incubated at 65°C on a heating block for 1 hour. After incubation the samples were centrifuged to pellet the plant material and obtain the supernatant. The supernatants were added to a microtitre plate and read at a wavelength of 600nm on a spectrophotometer.

2.4. Determination of chlorophyll A and B

A modified method of Oancea et al. (2005) was followed for the chlorophyll assay. This assay was done to determine the chlorophyll concentrations within the leaves from both vanadium treated and untreated plants. One hundred milligram of frozen ground leaf material was added to a 1.5 ml Eppendorf tube. The Eppendorf tubes were wrapped in foil to prevent the degradation of 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 were added to wells on a glass microtitre plate in triplicate and read on a spectrophotometer at the wavelengths 662 nm and 644 nm respectively. All samples were tested in quadruplicate (four tubes per plant tissue sample). The optical readings were used in a calculation to determine the different chlorophyll species concentrations.

2.5. Protein extraction

Protein extraction was done in triplicate from untreated as well as the vanadium treated plant leaf tissue was done by adding 100 mg of frozen ground leaf 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 then added to one of the three tubes. The homogenate in the tube was then further mixed using a vortex. 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 then removed and inserted in to the second tube containing another 100 mg leaf material. The previous steps were 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 were then quantified using a Bradford assay. Thereafter, the protein samples were stored at -20°C.

2.6. Ascorbate peroxidase in-gel PAGE activity assay

A modified method of Seckin et al. (2010) was used to determine the activity of the APX isoforms in the two *Brassica napus* cultivars. A 5% (v/v) stacking and 13% (v/v) resolving native PAGE (polyacrylamide gel electrophoresis) gel was prepared and allowed to equilibrate in native PAGE running buffer containing 192 mM Glycine, 24 mM Tris base and 2 mM ascorbate for 30 minutes at 4°C. A volume containing 200 µg of protein was mixed with 30 µl of 4x orange G loading dye. The protein/loading dye mixture was then loaded onto a native PAGE gel. The gel was then electrophoresed at 80V until the loading dye reached the bottom edge of the gel. The gel was then removed from the tank and the casting plates and placed into a small container and then washed using water. All incubations henceforth were done in the dark. A solution containing 50 mM potassium phosphate buffer (pH 7.0) and 2 mM ascorbate was added onto the gel. The gel was then allowed to incubate on a shaker for 20 minutes. After the

incubation the first solution was discarded and a second solution containing 50 mM potassium phosphate buffer (pH 7.8), 4 mM ascorbate and 2 mM H_2O_2 was added to the gel. The gel was allowed to incubate again for 20 minutes on a shaker. After the incubation the second solution was discarded and a third solution was added to the gel containing 50 mM potassium phosphate buffer (pH 7.8), 28 mM TEMED and 0.5 mM NBT. The gel was then allowed to incubate in the third solution on a shaker for approximately 20 minutes. After conclusion of the incubation steps the third solution was discarded and the gel was washed with water. The gel was then exposed to light on a light box and allowed to develop so that activity bands could be visualized.

2.7. GPX-like peroxidase in-gel PAGE activity assay

A modified method of Seckin et al. (2010) was used to determine the activity of the GPX-like isoforms in the two *Brassica napus* cultivars. A 5% (v/v) stacking and 13% (v/v) resolving native PAGE gel was prepared and allowed to equilibrate in native PAGE running buffer 192 mM Glycine, 24 mM Tris base and 2mM glutathione for 30 minutes at 4°C. A volume containing 200 µg of protein was mixed with 30 µl of 4x orange G loading dye. The protein/loading dye mixture was then loaded onto a native PAGE gel. The gel was then allowed to run at 80V until the loading dye reached the bottom edge of the gel. The gel was then removed from the tank and the casting plates and placed into a small container and then washed with water. All incubations that follow were done in the dark. A

solution containing 50 mM potassium phosphate buffer (pH 7.0) and 2 mM glutathione was added onto the gel. The gel was allowed to incubate on a shaker for 20 minutes. After this incubation the first solution was discarded and a second solution containing 50 mM potassium phosphate buffer (pH 7.8), 4 mM glutathione and 2 mM cumin hydroperoxide was added onto the gel. The gel was allowed to incubate again for 20 minutes on a shaker. After the incubation the second solution was discarded and a third solution was added to the gel containing 50 mM potassium phosphate buffer (pH 7.8), 1.2 mM (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazollium Bromide)(MTT).and 1.6 mM phenazine methosulfate (PMS) .The gel was then allowed to incubate in the third solution on a shaker for approximately 10 minutes at 30 °C. After conclusion of the incubation steps the third solution was discarded and the gel was washed with water. The gel was then exposed to white light on a light box and allowed to develop so that activity bands could be visualized.

2.8. Superoxide dismutase in-gel PAGE activity assay

A modified method of Beauchamp and Fridovich (1971) was used to determine the SOD activity profile in the two *Brassica napus* cultivars. A 5% (v/v) stacking and 13% (v/v) resolving native PAGE gel was prepared. A volume containing 200 µg of protein was mixed with 1 x orange G loading dye. The protein/ dye mixture was then loaded onto the native PAGE gel. The gel was electrophoresed until the loading dye reached the bottom end of the gel. The gel was then removed from

the tank and the casting plates and inserted into a container where it was washed with distilled water. All the incubation steps to follow were done in darkness. The PAGE gel was then washed with 50 mM potassium phosphate buffer (pH 7.0) on a shaker for 20 minutes. After the wash step was completed the wash solution was discarded. A second solution containing potassium phosphate buffer 50 mM (pH 7.8) and 0.5 mM NBT was added to the gel thereafter it was allowed to incubate for 20 minutes. The second solution was then discarded and a third solution containing 50 mM potassium phosphate buffer (pH7.8), 35.5 mM TEMED and 0.5 mM riboflavin was added to the gel. After the addition of the third solution the gel was allowed to incubate on a shaker for 20 minutes. The third solution was then discarded and the gel was washed with distilled water. The gel was then exposed to light on a light box and allowed to develop so activity bands could be visualized.

2.9. Class determination of superoxide isoforms

A modified method of Hernandez et al. (2001) was used to determine the class of the SOD isoforms in the *Brassica napus* cultivars. Four native PAGE gels were prepared and run as in the protocol for the superoxide dismutase in gel assays (Section 2.9). The gels were then washed with water and inserted into different containers. The gels were then washed using a 50 mM potassium phosphate buffer (pH 7.0) for 10 minutes on a shaker. All the incubation steps to follow were conducted in the dark. After the wash step was completed the wash

solution was discarded from the four gels. A solution containing 50 mM potassium phosphate buffer (pH 7.8) was added to the first gel and 5 mM H_2O_2 was added to the second gel. To the third gel a 50 mM potassium phosphate buffer containing 5 mM potassium cyanide (KCN) was added. To the fourth gel a solution containing 50 mM potassium phosphate buffer and 2% Sodium dodecyl sulfate (SDS) was added. All four gels were allowed to incubate on a shaker for 20 minutes. Potassium cyanide, H_2O_2 and SDS are compounds which inhibit the activity of different types of SODs (KCN inhibits Cu/Zn SODs, H_2O_2 inhibits Cu/Zn and Mn SODs and SDS inhibits Mn and Fe SODs). The solutions from the four gels were discarded and a solution containing 50 mM potassium phosphate buffer (pH 7.8) and 0.5 mM NBT was added to the gels separately. The gels were then allowed to incubate on a shaker for 20 minutes. After incubation, the solutions from the gels were discarded and a final solution containing 50 mM potassium phosphate buffer, 35.5 mM TEMED and 0.5 mM riboflavin was added to all the individual gels. The gels were allowed to incubate in this solution for 20 minutes. Using the gel with no inhibitor as a reference and studying the three inhibitor gels for the absence or presence of an activity band, the class of SOD could be identified.

2.10. Densitometry analysis

Densitometry analysis was done using the AlphaEase FC Imaging software (Alpha Innotech Corporation). Software was utilised as described in the manufactures specification manual.

2.11. Determination of lipid peroxidation by quantifying MDA

A modified method of Zhang et al. (2007) was followed for the lipid peroxidation assay. This assay was done on both untreated and treated plants. A mass of 100 mg of leaf material, which was ground using liquid nitrogen, was added into different 1.5 ml Eppendorf tubes. To the Eppendorf tubes, 5 volumes of 6% (w/v) Trichloroacetic acid (TCA) were added. The tubes were then mixed using a vortex followed by a 13000 x *g* centrifugation for 10 minutes to pellet the leaf 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 also added. The solution was then briefly mixed using a vortex. Parafilm was then wrapped around the lid of the Eppendorf tubes to ensure they would not open during heating. The tubes were then placed in a heating block at 90°C to allow the samples to boil for 20 minutes. After the samples were taken from the heating block they were incubated on ice for 10 minutes. Once the incubation was completed the samples were centrifuged at 13000 x *g* for 5 minutes. The samples were 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 600nm was subtracted from the absorbance at 532 nm to correct for non-specific turbidity. The MDA values were then calculated using the extinction coefficient of 155 $mM.cm^{-1}$.

2.12. Determination of vanadium concentration using a spectrophotometry kinetic assay

The vanadium assay was carried out as a novel method based on a method of Ulusoy and Gürkan (2009). Standards for the assay were prepared by diluting an appropriate mass of sodium metavanadate in nitric acid which was neutralized using potassium hydroxide. The standard stock concentrations used were 32.8 µM, 65.6 µM, 98.4 µM, 131.2 µM and 164 µM. Samples for this assay were prepared by weighing and adding approximately 100 mg of material into an Eppendorf tube. The material was then digested in 5 volumes 65% (v/v) nitric acid for 1 hour or till the material was totally digested. After digestion the nitric acid in the Eppendorf tube was diluted to 32.5% using deionized water. The solution in the Eppendorf tube was then neutralized by adding 3.71 M potassium hydroxide solution. A 100 times dilution of the sample was prepared using a neutralized nitric acid/potassium hydroxide solution as the diluent. From the diluted stock 50 µl of the sample was added to the microtitre plate. A reaction mastermix containing 150 mM phosphoric acid, 0.06 mM ponceau xylidine and 0.6 mM 5-Sulfosalicyclic acid dihydrate was prepared. To the sample on the plate

an adequate volume of the reaction master mix was added. Once the standards and samples were prepared and added to the wells on the microtitre plate the reaction was started by adding 16 mM Potassium bromate. After the addition of the potassium bromate the plate was inserted into a spectrophotometer and read at a wavelength of 500 nm. Readings were taken every minute to produce kinetic readings for each well. Using the readings, the reaction kinetics of each well was calculated using Microsoft excel software. The rate of the reaction was catalyzed by the presence of vanadium therefore the concentration of vanadium could be calculated.

2.13. A Spectrophotometric assay for superoxide content determination

A modified method of Russo et al. (2008) was used 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 volume of 800 µl using a solution of 50 mM potassium phosphate (pH 7.0). Eight 1 cm^3 squares were cut from fresh leaf material and carefully inserted into the above prepared solution in the Eppendorf tube. The root samples were prepared by making 4 cm cuttings from the tip of the root and inserted into a tube with the above prepared solution. The plant material was then incubated for 20 minutes within the solution. Once the incubation was completed the plant

material was 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 removed carefully and added to a clean Eppendorf tube. Once the supernatant (sample) was free of plant material it was loaded onto the microtitre plate by adding 200 µl into a well. This process was repeated for untreated as well as treated plant samples. The samples were then read at a wavelength of 600 nm. A calculation taking into consideration the extinction coeffient of 12.8mM. cm^{-1} was used to determine the superoxide. The intensity of the blue colour produced by the reaction was an indication of superoxide levels.

2.14. A Spectrophotometric assay for hydrogen peroxide 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 (0 nM, 5000 nM, 10000 nM, 15000 nM, 20000 nM and 25000 nM) were prepared by diluting an appropriate volume of H_2O_2 in distilled water. The standards were then loaded in triplicate onto a microtitre plate. Samples were prepared by using TCA extraction on frozen ground plant material (as in section 2.12). Fifty microliters of the TCA extraction was added onto the plate. To the samples as well as the standards 1.25 mM dipotassium hydrogenphosphate (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 were then read at a wavelength of 390 nm.

2.15. Statistical analysis

Statistical analysis was performed using the Duncan's multiple range test, where significance was represented by a $P< 0.05$.

Chapter 3

Effect of vanadium toxicity on two contrasting *Brassica napus* L cultivars

3.1. Abstract

In South Africa, it is becoming more common to see vanadium soil concentrations exceeding 30 mg/Kg, which is the concentration at which damage often occurs to plants and their growth. This study investigated the effect of vanadium on two *Brassica napus* L (Agamax and Garnet) cultivars that are important to the agricultural industry in South Africa. Physiological experiments such as biomass determination, cell viability (Cell death), chlorophyll determination and lipid peroxidation were conducted. A robust and cost effective vanadium assay was developed to determine vanadium levels within plant material and the uptake and deposition of vanadium within the two *Brassica napus* L cultivars were observed. Physical observations showed that when treated with vanadium, Garnet had significantly shorter roots and smaller leaves when compared to Agamax. Garnet also showed yellowing of the leaves. The biomass (dry weight) of Garnet was also lower than Agamax when exposed to vanadium. The vanadium assay showed that Garnet had higher concentrations of vanadium within its leaves and lower concentrations in its roots when compared to Agamax. This chapter shows that Agamax may employ mechanisms which localize vanadium to its roots thus protecting the aerial parts of the plants

from much of the damage caused by ROS. It also shows that Agamax is the more tolerant cultivar as it performed better, physiologically, than Garnet when exposed to vanadium.

3.2. Introduction

The use of improper irrigation, water contaminated with sewage sludge and fertilizers containing vanadium increases the vanadium content within soils, which results in an increase in vanadium concentrations in plants (Vachirapatama *et al*., 2011; Yang *et al*., 2011; Yadav, 2010). Vanadium is a heavy metal and when accumulated at high concentrations in plants, may lead to toxicity (Mukherjee *et al*., 2004). The toxicity in some plants may lead to physiological damage and poor growth. The physiological damage includes the discoloration of leaves, morphological changes in leaf structure, poor root development, and decrease in plant biomass (Vachirapatama *et al*., 2011; Wang & Liu, 1999). The toxicity of vanadium may extend to the microbial population within the soils and this might be detrimental to plants as the potential benefits associated with these populations will be negated (Guala *et al.,* 2010; Chehregani *et al.,* 2005). The tolerance of plants to heavy metal stress depend on mechanisms such as the uptake of metals into the plant, the degree of complexation of metals with substances within the cells and the degree of modification or damage to metabolic pathways (John *et al*., 2009).

Brassica napus L belongs to the family Brassicaceae and is an important oil seed crop but also has other uses such as animal feeds and feedstock for biofuels (Grispen *et al*., 2006). Certain species of the Brassicaceae family such as the Indian mustard is able to produce high biomasses even in the presence of high concentrations of heavy metals. This suggests that members of this family may be potential candidates for phytofilters and could be used for phytoremediation (John *et al*., 2009). The mechanisms which allow plants to tolerate high metal concentration could be used in plant transgenics to afford other plants the same tolerance. Two cultivars that are related may share many characteristics but the way they have evolved to tolerate stresses such as high metal concentrations may be different, thus leading to one cultivar being more tolerant than its counterpart. It would therefore be beneficial to study how metal toxicity influences these cultivars as well as their defence responses. The study presented in this chapter was undertaken to investigate the effect of vanadium on the physiology of two *Brassica napus* L cultivars namely Agamax and Garnet and their inherent sensitivity/tolerance as well as their adaptation to vanadium stress.

3.3. Results

3.3.1. Vanadium stress reduces the biomass and changes physiological characteristics of two Brassica napus L *cultivars*

It is important to note that South africa is one of the primary producers of vandium in the world and that the metal at elevated concentration may cause toxcity to plants (Saco *et al*., 2013; Vachirapatama *et al*., 2011). Also, very few studies have been done focusing on this metal and its toxic effects on plants. In this study, the roots and leaves samples were obtained after plants were grown and treated for 21 days as described in chapter two (section 2.1). Vanadium treatment caused yellowing of the leaves in the Garnet cultivar as well as reduced leaf and root area in both cultivars (Figure 3.1.1 and 3.1.2).Treament with vanadium also caused a 15% decrease in biomass in Agamax (AV) leaves and a 47% decrease in Garnet (GV) leaves when compared to their untreated controls (Figure 3.1.3 A). The roots showed a similar trend with a 24% decrease in Agamax (AV) root biomass and a 53% decrease in Garnet (GV) root biomass when compared to the untreated controls [Agamax (AU) and Garnet (GU)] plants (Figure 3.1.3 B).

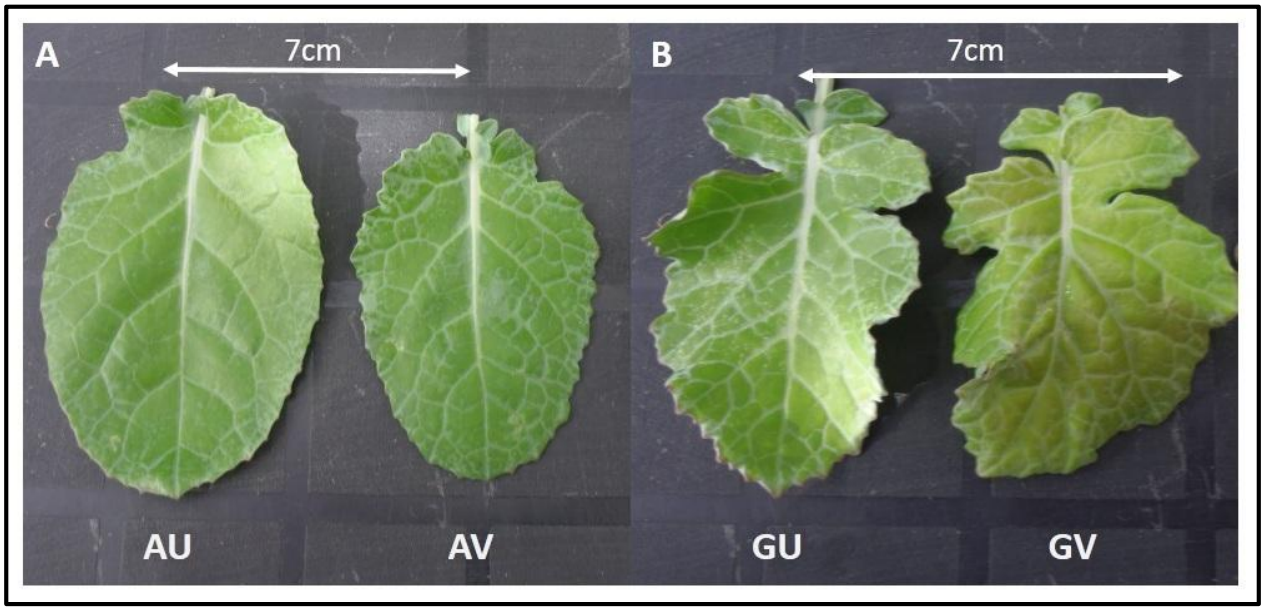

Figure 3.1.1: The effect of vanadium on the leaf physiology of two *Brassica napus* L cultivars. The two *Brassica cultivars* L (Agamax and Garnet) were treated with vanadium for 21 days, leaves were then cut from treated and control plants to determine the effects of vanadium treatment on the physiology of the leaves. Figure A shows the leaves cut from the Agamax cultivar both untreated (AU) and treated (AV) respectively. Figure B shows the leaves cut from the Garnet cultivar for both untreated (GU) and treated (GV) respectively.

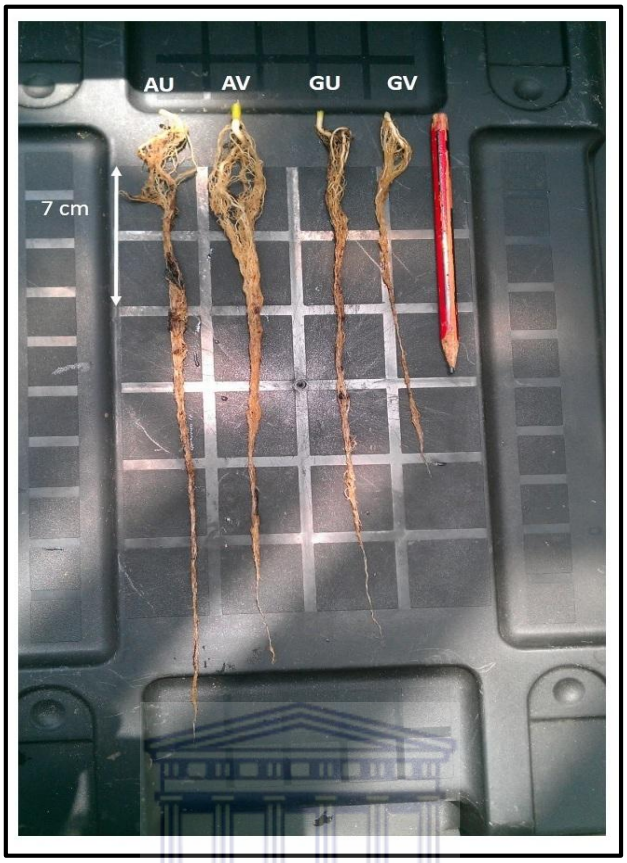

Figure 3.1.2: The effect of vanadium on the root physiology of two *Brassica napus* L cultivars. The two *Brassica cultivars* L (Agamax and Garnet) were treated with vanadium for 21 days, roots were then cut from treated and control plants to determine the effects of vanadium treatment on the physiology of the roots. The figure shows the roots cut from the Agamax cultivar for both untreated (AU) and treated (AV respectively. The figure also shows the roots cut from the Garnet cultivar for both untreated (GU) and treated (GV).

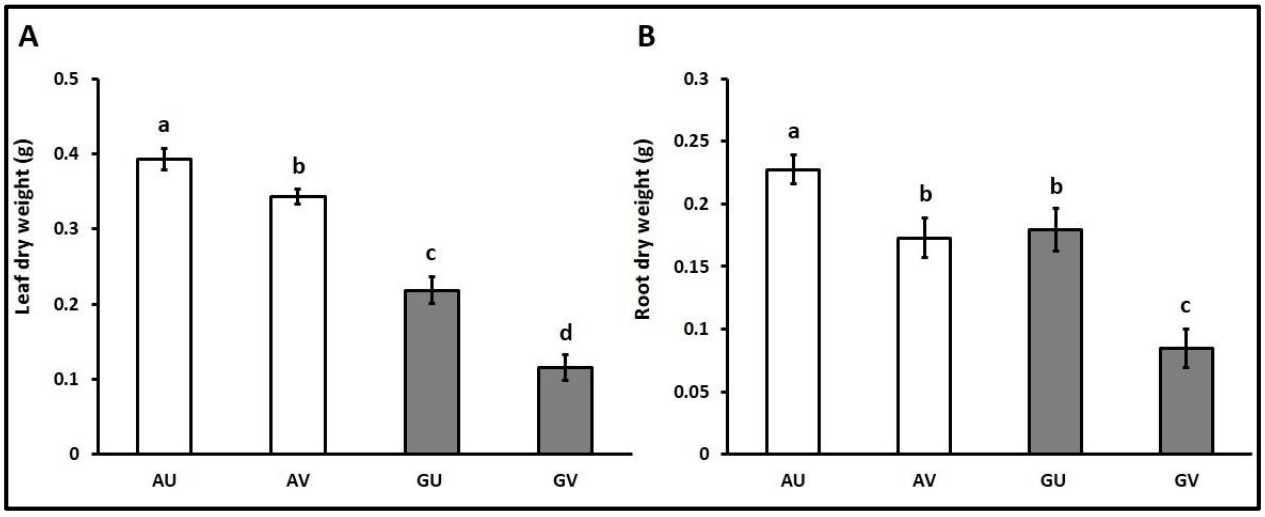

Figure 3.1.3: The effect of vanadium on the biomass of two canola cultivars. Vanadium was applied to the two cultivars and the biomass of the leaves (A) and roots (B) were determined. A and G denote Agamax and Garnet whereas U and V denote untreated and vanadium treated.Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ± S.E (N=10).

3.3.2. Vanadium stress exacerbates cell death within Brassica napus L *cultivars*

The gereration of ROS within plants due to stress may damage important components of plants cells such as lipids, proteins and DNA which ultimately leads to cell death (Maruta *et al*., 2012; Wang *et al*., 2005). Cell death can therefore be used as a proxy indicator of metal toxicity. The cell death in this study was analysed using Evans blue reagent which penetrates dead cells only. This method was also used in a study done by Takahashi et al. (2012) to determine cell viabilty in *Arabidopsis thaliana*. The two *Brassica napus* L cultivars

were exposed to vanadium treatements for a period of 21 days and further processed as in section 2.3. The controls of Garnet and Agamax that were treated with water showed no significant difference in cell death when comparing their leaves and roots (Figure 3.2). Leaves from Agamax and Garnet plants which were treated with vanadium showed an increase in cell death of 17% and 109% respectvily when compared to their controls (Figure 3.2 A). The roots of the two cultivars treated with vanadium also showed an increase in cell death of 31% in Agamax and 132% in Garnet roots respectively when compared to the control plants treated with water (Figure 3.2 B).

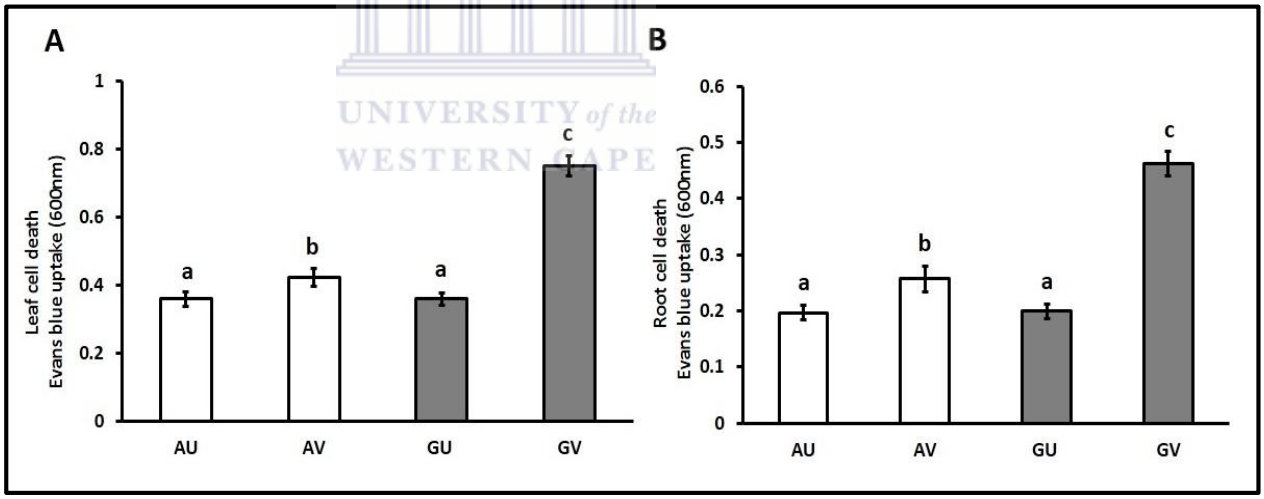

Figure 3.2: The effect of vanadium on the cell death of two *Brassica napus* L cultivars. Vanadium was applied to two cultivars (Agamax and Garnet), the cell death within the leaves (A) and roots (B) were determined. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ± S.E (N=3).

3.3.3. Vanadium stress decreases chlorophyll a and b in Brassica napus L *cultivars*

Heavy metals at elevated concentrations have been observed to have an effect on total chlorophyll production as well as the inhibition of different types of chlorophyll (a and b) (Wang *et al*., 2005). The decrease of chlorophyll could have an effect on plant growth as well as well as be an indicator of sensitvity to a particular stress. It was therefore necessary in this study to evaluate the chlorophyll content of plants under vanadium stress (Table 3.1). Agamax plants treated with vanadium showed no difference in chlorophyll a and b content when compared to the untreated Agamax control. The total chlorophyll content within Agamax also showed no difference when treated with vanadium. Garnet plants which were exposed to vanadium showed a reduction of 43% in chlorophyll a, 27% in chlorophyll b and 39% in total chlorophyll content.

Table 3.1: The effect of vanadium on plant chlorophyll ($\mu g.g^{-1}$) a and b

	Chlorophyll a	Chlorophyll b	Total chlorophyll
AU	0.489 ± 0.004^{a}	0.184 ± 0.004^{b}	0.673 ± 0.002^{c}
AV	0.495 ± 0.003^{a}	0.177 ± 0.003^{b}	0.672 ± 0.006^{c}
GU	0.498 ± 0.005^{a}	0.173 ± 0.003^{b}	0.671 ± 0.005^{c}
GV	0.284 ± 0.002^{d}	0.127 ± 0.002^{e}	0.411 ± 0.002^{f}

Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ± S.E (N=4).

3.3.4. Vanadium uptake within two Brassica napus L *cultivars*

Vanadium which is a heavy metal has been suggested as being essential for higher plant growth (Mukherjee *et al*., 2004; Wang & Liu, 1999). However, higher concentrations of vanadium may be toxic and can lead to a reduction in plant growth and increase cell death (Vachirapatama *et al*., 2011). It was therefore impotant to understand the mechanisms which allow plants to reduce heavy metal uptake as well as the transport of metals which do enter the plants. The control plants, Garnet and Agamax that were treated with water showed no significant difference in their vanadium levels when comparing their leaves and roots (Figure 3.3). The Agamax cultivar treated with vanadium displayed an increase in vanadium concentrations of 53% and 117% in the leaves and roots, respectively, when compared to the control Agamax plants. The Garnet cultivar treated with vanadium displayed an increase in vanadium concentrations of 151% and 62% in the leaves and roots respectively when compared to the control Garnet plants

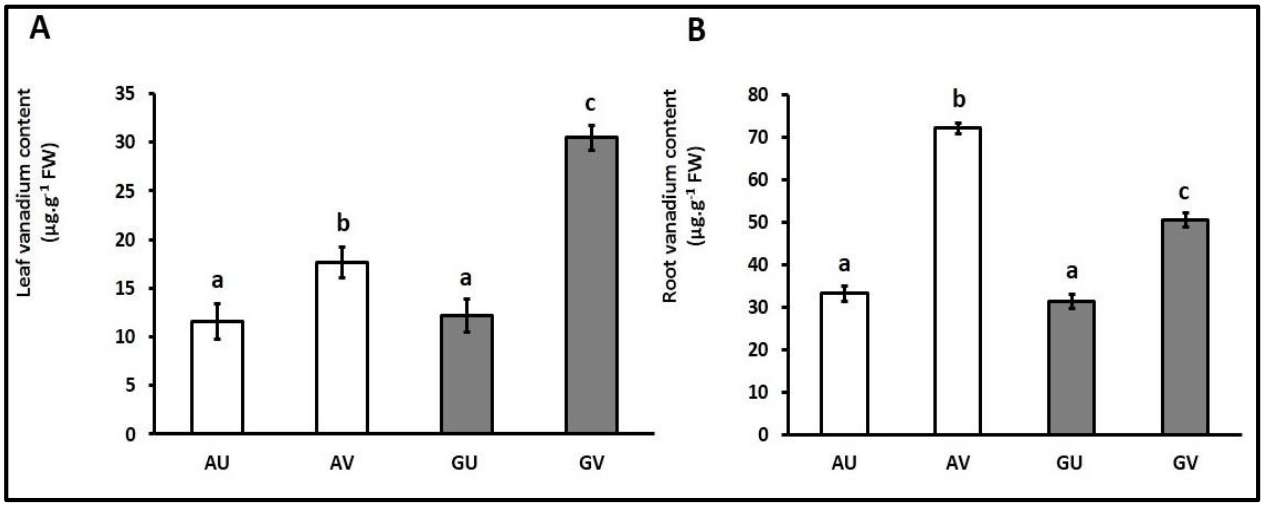

Figure 3.3: The uptake of vanadium in *Brassica napus* L cultivars. Vanadium was applied to two cultivars, the vanadium content in the leaves of the two cultivars are displayed by (A). The vanadium content in the roots of the two cultivars are displayed in (B). Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ± S.E (N=3)

3.4. Discussion

The work reported here investigated the effect of vanadium on the physiology and morphology of two *Brassica napus* L cultivars. Experiments to determine the affect of vanadium on the biomass production, chlorophyll content and cell viability were undertaken to observe how these important plant systems were affected by the toxic effects of high concentrations of vanadium. An assay was also developed to determine the vanadium concentration in plant material, to gain insight in how much of the metal was taken up by the plant as well as metal transport.

Abiotics stresses such as drought, salt and heavy metals are known to cause changes in the physiological characteristics and decrease the biomass of plants.

The Agamax cultivar when exposed to vanadium showed a slight decrease in leaf and root area. Although Garnet naturally had smaller leaf and root areas it was observed that vanadium caused reductions in Garnet leaf and root areas (Figure 3.1.1 and 3.1.2). From the dry weight results it was observed that Agamax and Garnet plants treated with vanadium showed a decrease in leaf and root biomass. The reduction in the biomass of Agamax plants was however much lower when compared to the reduction in biomass of Garnet plants (Figure 3.1.3). A decrease in biomass could be attributed to the plant using valuable energy and resources meant for growth to alleviate the stress that it may be experiencing. The reduction in biomass could also be attributed to the reduction in water and mineral uptake by plants due to impaired growth of the roots due to vanadium stress. A study by Saco et al. (2013) showed that the generation of biomass of *Phaseolus vulgaris* L was decreased when the plants were exposed to elevated concentrations of vanadium. The same study by Saco et al. (2013) also showed that vanadium concentrations above 240 µM caused leaves and roots of the plants to be smaller and led to changes in their morphology which is consistent with the results observed in this study. The results from this study are also consistent with the results of a study done by Vachirapatama et al. (2011) where Chinese green mustard plants were exposed to vanadium, the plants showed a reduction in biomass of the leaves, roots and stem. The same study by Vachirapatma et al. (2011) also observed that the growth of the lateral roots of the Chinese green mustard plants were impaired when exposed to vanadium. Agamax could be able to control and manage its growth and biomass production

much more efficiently than Garnet under vanadium stress therefore reducing its loss in biomass.

Cell death may occur due to a programmed cell death-like pathway or the complete destruction of a cell due to damage caused by stress (Lam *et al*., 1999). Cell death can be used as a useful indicator in assessing the amount of damage caused by stress on plants. Cell death includes the damage to the cell membrane, DNA and lipid molecules. The Evans blue viability assay works on the basis that cell membranes which are intact will not allow for the uptake of the Evans blue reagent and the increased uptake of Evans blue is an indication of damage to the cell membrane. The cell death in the leaves and roots of both Agamax and Garnet treated plants were observed to have higher cell death than unreated controls. This observation showed that vanadium did have a detrimental affect on the plants as well as caused cell death. The results of this study are consistent with a study done by Basset and Matsumoto (2008) which observed increased cell membrane disruption when tabacco plants were expossed to aluminium. It was interesting to note that the cell death in the leaves and roots of Agamax treated plants were much lower than the cell death in the leaves and roots of the Garnet treated plants. Agamax plants may have mechansims which allow it to be tolerant to vanadium toxicity and the resulting damage. The suggested ability of Agamax to continue with its normal nutrient and water transport under

vanadium stress, could also be a reason for lower cell death values in Agamax treated plants when compared to Garnet treated plants.

Yellowing of the leaves of the Garnet cultivar was observed (Figure 3.1.1). The proposed reason for the yellowing, was the reduction of chlorophyll within the leaves and therefore the chlorophyll content was determined. A process known as chlorosis leads to the yellowing of plant leaves (Hörtensteiner & Krautler, 2011). The Agamax untreated, Agamax treated and Garnet untreated plants were all observed to have similar total chlorophyll content and similar values for chlorophyll a and b. Garnet treated plants however showed much lower chlorophyll a and b levels which lead to an overall lower total chlorophyll concentration (Table 3.1). This observation shows that the yellowing of the leaves was due to the decrease in the chlorophyll content of the Garnet treated plants. It was also observed that both chlorophyll a and b were affected by the vanadium toxicity and its downstream effects. Heavy metals such as copper, lead and zinc have been observed to interfere with the chlorophyll production by either directly inhibiting enzymatic steps in the production or by reducing the nutrients needed for chlorophyll production (Chettri *et al*., 1998). It is important to note that metals may also increase the production of reactive oxygen species like hydrogen peroxide which can damage chloroplasts (Perez *et al*., 2002). Cadmium (ii) was reported to affect photosystem 1 (Peralta-Videa *et al*., 2004).The results in this study are consistent with a study by Henriques (2010)

which observed that metals such as chromium had a negative effect on the photosynthetic molecules and systems within tomato plants. The results observed in our study were interesting as they showed that Agamax plants treated with vanadium had a mechansims to protect their chlorophyll production and obtain the relevant nutrients whereas Garnet plants treated with vanadium could not. A study by Chettri et al. (1998) exposed lichens to metals such as zinc and lead, although the plants had high concentrations of metal within their cells there was no reduction in total chlorophyll content. The proposed reasons were that the metals were bound to the cell wall and remained inactive and the metals which made there way into the cells were localized to the mycobiont cells rather than the photobiont cells were they could do damage. Hence, we suggest that Agamax could be using similar mechanisms as described by Cehttri et al. (1998) to avoid any damage to chloroplasts when it comes in contact with vanadium.

For the scope of this study it was necassary to be able to determine the vanadium concentration within plant material. Although there are methods such as inductively coupled plasma mass spectrometry (ICP) and atomic absorption spectrometry (AAS), which are able to determine vanadium concentrations within plant material, these methods are often expensive to use and labour intensive. Due to the simiplicity and lower cost of reagents and equipment, spectrophotometric methods were looked at as an alternative to determine

vanadium concentrations. During the scope of this study a robust and cost effective method to determine vanadium concentrations in plant material was developed. Work done by Ulusoy and Gürkan (2009) was used as the basis for the development of the vanadium determination method. This method was a kinetic assay based on the ability of vanadium to catalyse the oxidation of a dye called ponceau xylydine by potassium bromate in the presence of 5-sulfosalicyclic acid (SSA) as the activator, the faster the rate of the reaction the more vanadium was present in the samples. The oxidation reaction of ponceau xylydine and potassium bromate gave rise to a change in colour from pink to yellow which was measured using a spectrophotometer. The colour change meant that the absorbance decreased over time. Many problems were encountered while developing the spectrophotometic method. It was observed that the method was sensitive to pH, for example when the pH of the reaction was higher than required a precipitate of ponceau xylidine would form and if the pH was lower than required the rate of the reaction was to fast to properly determine the vanadium concetration in the samples. Work done by Melwaki et al. (2001) using phenothiazine derivatives observed that the stability and sensititvity of red radical cations were dependant on the type of acid medium and the pH of the reaction. The wavelength of 640 nm was used to measure the absorbance of the reaction in the work done by Ulusoy and Gürkan (2009) however, when the same wavelength was used in this study very little discrimination between standards with different vanadium concentrations could be observed. A range of wavelengths were tested to determine an adequate

wavelength to use for this method. The wavelength which was finally chosen was 500 nm as proper discrimination was observed between samples with different vanadium concentrations. The discrepancy in the wavelengths could be due to this study using microtitre plates and the study by Ulusoy and Gürkan (2009) using cuvettes. The range of the standards also had to be adjusted as the concentrations of vanadium in the samples were to high. The samples and standards were made up to the required volume by adding a solution containing nitric acid which was neutralized with potassium hydroxide instead of water. When water was used it lead to the rate of the reaction being to fast and thus not allowing for proper determination of vanadium concentrations. The first observation was that our modified method was senstive enough to determine minute concentrations of vanadium. The modified method was also robust enough to analyse diverse sample types such as soil, liquids and plant material.

The leaves and roots of Garnet and Agamax plants which were untreated (treated with water) all showed the presence of vanadium. This was due to the commercial soil containing vanadium and this was also identified when the vanadium assay was conducted on the soil. It was observed that Garnet and Agamax untreated and treated plants showed a higher vanadium concentration in their roots than their leaves. The aformentioned result was expected in the untreated samples due the vanadium which was in the commercial soil and in the case of the treated samples the vanadium treatments were added as a

solution to the soils where the roots were located. Our results were consistent with a study by Fitzgerald et al. (2003) which observed a higher copper concentration in the roots of *A. tripolium* than the shoots and leaves. A study by Vachirapatama et al. (2011) observed that more vanadium was located within the roots of Chinese mustard plants than in their stems and leaves. The vanadium concentration within the leaves of the Garnet treated plants were much higher than the vanadium concentration found in the leaves of the Agamax treated plants. A similar result was observed in a study by Fitzgerald et al. (2003) where *P. maritima* was found to transport and accumalate more lead in its shoots than in its roots. This suggests that Garnet treated plants transport more vanadium to its leaves than Agamax treated plants. Interestingly, the Agamax treated plants showed a higher accumalation of vanadium in its roots when compared to the Garnet treated plants. The higher vanadium concentrations in the leaves of the Garnet treated plants could have played a role in the reduction in chlorophyll in the leaves. It appears that Agamax protects itself from high vanadium concentrations by localizing the vanadium to its roots to prevent damage to the aerial parts of the plant thus protecting systems such as photosynthesis and nutrient transport. The results also show that Agamax is not a vanadium hyper accumulator but that it may be a vanadium hypertolerant plant. Garnet has the characteristics of a hyperaccumalor as it can take up large amount of vanadium from the soil. It can also translocate the vanadium through the shoots and deposit a large amount of vanadium in its leaves, but due to the

damage vanadium ultimately caused to the Garnet plants, Garnet was not categorised as a vanadium hyperaccumulator.

In conclusion it was observed that Agamax plants were more tolerant to vanadium stress. Garnet plants exposed to vanadium however do not exhibit the same level of tolerance and therefore experienced more damage. Garnet plants treated with vanadium also showed damage to the chlorophyll where as the Agamax treated plants did not. Overall biomass reduction was much lower in the Agamax plants than the Garnet plants. It is evident that Agamax has several mechanisms which allows for tolerance to vanadium stress.

Chapter 4

Vanadium toxicity induces oxidative stress and reactive oxygen species scavenging pathways in *Brassica napus* L

4.1. Abstract

It was observed in chapter 3 that vanadium had a negative effect on the physical characteristics of the *Brassica napus* L cultivars. It was therefore necessary to understand which pathways and mechanisms were affected by vanadium that lead to the negative effects observed in chapter 3. In this chapter, this study observed how vanadium affected the oxidation state, antioxidant enzyme profiles and the damaged caused to the two *Brassica napus* L cultivars. To determine the oxidation state of Agamax and Garnet which were exposed to vanadium, the hydrogen peroxide (H_2O_2) and superoxide (O_2^-) levels were assessed in leaves and roots of the plants. To determine the extent of the damage caused by vanadium, lipid peroxidation was assessed in both cultivars. The enzyme profiles (APX, SOD and GPX-like) of the two *Brassica napus* L cultivars were identified both under normal conditions as well as under vanadium stress. The types of SODs were also identified using in-gel activity inhibition PAGE gel assays. The Garnet cultivar was observed to have higher H_2O_2 and O_2^- concentrations when compared to the Agamax cultivar. The degree of lipid peroxidation within the Garnet cultivar was also higher than in the Agamax

cultivar. There were seven APX and SOD isoforms observed to be present in the Agamax plants. The Garnet plants however showed seven APX and six SOD isoforms respectively. The GPX-like in gel assay showed three isoforms to be present in both Agamax and Garnet. Both Garnet and Agamax increased certain antioxidant isoforms to cope with the increase in ROS concentrations. Garnet was not as effective as Agamax in controlling the ROS, which is probably what resulted in Garnet sustaining more damage.

4.2. Introduction

Due to the inability to move, plants are subjected to many biotic and abiotic stresses (Tuteja *et al*., 2009). Abiotic stresses that affect plants include extreme temperatures, salinity and heavy metals (Nakashima *et al*., 2012). High concentrations of heavy metals such as cadmium, nickel, and vanadium were observed to increase the production of ROS in plants (Yadav, 2010). The increase and accumulation of ROS within plants often leads to an imbalance in the oxidative state of a plant, which results in damage to its metabolism and organelles (Gill & Tuteja, 2010). Due to the aforementioned fact, plants need mechanisms to not only sense ROS but also mechanisms to signal for removal of ROS (Bhattacharjee, 2011). The corrective action for many of these stresses come in the form of antioxidant scavenging pathways. The antioxidant enzymes and molecules used in plants include APX, SOD, glutathione-dependent peroxidases, ascorbate and glutathione (Bhattacharjee, 2011, Zhang *et al*., 2007).

The inherent ability of a plant to efficiently deal with increasing ROS levels, due to heavy metals such as vanadium, would play a vital role in the tolerance of plants to vanadium.

The oxidative scavenging profile of a plant might allow it to perform better when compared to a species from the same family with a different profile. The oxidative scavenging profile could also give an indication to how certain plants deal with different stresses. The mechanisms or profiles found in a vanadium tolerant plant could be used to enhance the tolerance of a vanadium sensitive plant through plant breeding or transgenic programs. The antioxidant profiles can also be scanned for novel biomarkers, which may be used to determine the viability of certain cultivars in soils contaminated with vanadium.

4.3. Results

4.3.1. Hydrogen peroxide content increases within Brassica napus L *cultivars due to vanadium stress*

The primary response of plants to any stress is the generation of ROS (Gill & Tuteja, 2010; Yadav, 2010). In a study by Guo et al. 2005 it was observed that under cold and water stress that H_2O_2 content increased as well as antogether with an increase in oxidative damage within plants. It was therefore necessary to understand how the stress of vanadium impacted the generation of H_2O_2 within

the two *Brassica napus* L cultivars. The two *Brassica napus* L cultivars were exposed to vanadium treatements for a period of 21 days and then ground to a fine powder using liquid nitrogen and were further processed as described in 2.15. The control Garnet and Agamax that were treated with water only showed no significant difference in the concentration of H_2O_2 within their leaves and roots (Figure 4.1). The Agamax cultivar treated with vanadium showed a 36% increase in H_2O_2 content within its leaves (Figure 4.1 A). The Garnet cultivar treated with vanadium showed a 104% increase in H_2O_2 content within its leaves (Figure 4.1 A). Agamax and Garnet roots exposed to vanadium showed an increase in H_2O_2 content of 87% and 188% respectvily when compared to the control plants (Figure 4.1 B).

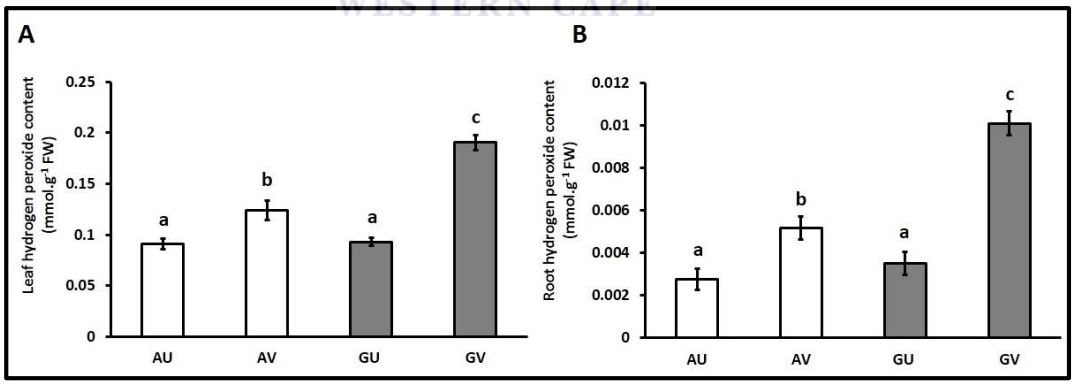

Figure 4.1: The effect of vanadium treatments on hydrogen peroxide content in two *Brassica napus* L cultivars. Two *Brassica napus* L cultivars (Agamax and Garnet) were treated with vanadium. The hydrogen peroxide content within the leaves and roots were then determined and shown in graph A and B respectively. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ± S.E (N=3).

4.3.2. Vanadium stress results in an increase in superoxide content within two Brassica napus L *cultivars*

Superoxide (O_2^-) as one of the ROS has the ability to cause oxidative damage within animals and plants (Gill & Tuteja, 2010). The electron transport system which forms part of the photosystem I within plants is one of the major locations where O_2^- is formed (Gill & Tuteja, 2010). The damage O_2^- could impose on plants is high due to the location of its formation. It was therefore nessecary to determine how vanadium stress affected the O_2^- content within the *Brassica napus* L cultivars. The two *Brassica napus* L cultivars were exposed to vanadium treatements for a period of 21 days, therafter fresh material of the roots and leaves of the plants were used for the O_2^- assay as described in section 2.13. The Garnet and Agamax controls that were treated with water only showed no significant differences in their O_2^- concentration when comparing leaves and roots (Figure 4.2). The O_2^- content within the leaves of Agamax treated with vanadium increased by 21% when compared to the leaves of the Agamax control plants (Figure 4.2 A). The O_2^- content in the leaves of Garnet treated with vanadium increased by 69% when compared to the leaves of the Garnet control plants (Figure 4.2 B). Agamax and Garnet roots which were treated with vanadium showed an increase in O_2^- content of 32% and 158% respectively when compared to the roots of the control plants (Figure 4.2 B)

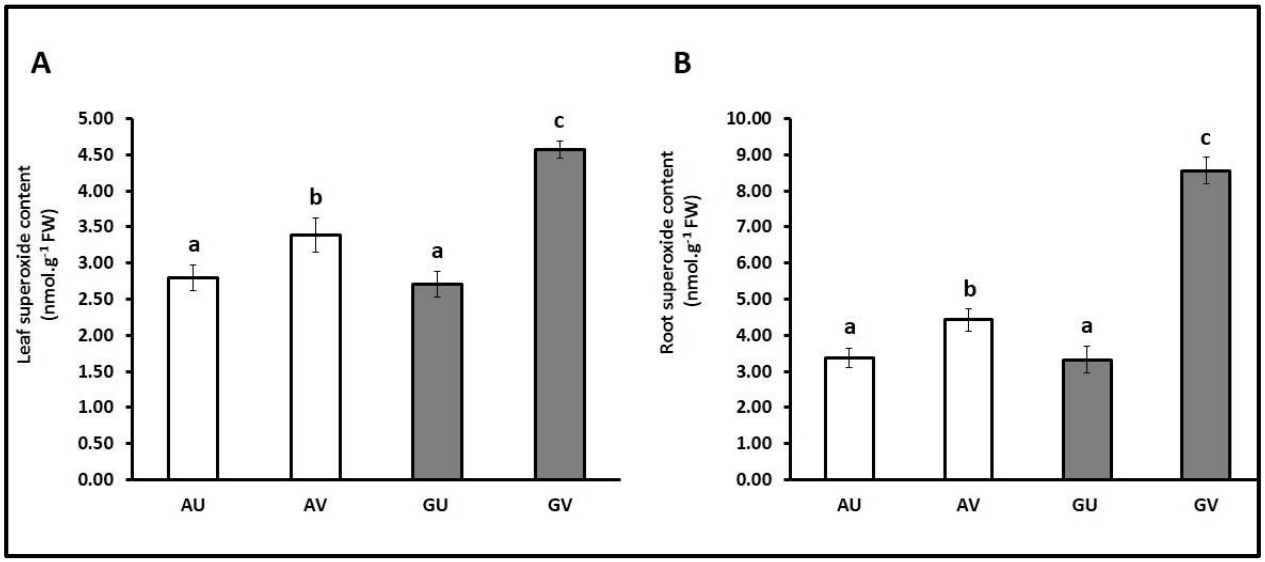

Figure 4.2: The effect of vanadium treatments on the superoxide content within two *Brassica napus* L cultivars. Agamax and Garnet cultivars were treated with vanadium for 21 days, therafter the O_2^- content was determined in the two cultivars. The O_2^- content within the leaves and roots are shown in graph A and B respectively. A and G denote Agamax and Garnet , U and V denote untreated and vandium treated plants. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ± S.E (N=3).

4.3.3. Lipid peroxidation increases in Brassica napus L *culitvars exposed to vanadium stress*

ROS can damage the polyunsaturated fatty acids which form the lipid membrane and this leads to degradation of the lipid membranes through a process known as lipid peroxidation (Wahsha *et al*., 2012; Verma & Dubey, 2003). The level of MDA, which is a product of lipid peroxidation, can be used as an indicator to assess the extent of lipid peroxidation within plant tissue (Sinha & Saxena, 2006; Verma & Dubey, 2003). The MDA content can be measured through a reaction

with thiobarbituric acid (TBA) (Sinha & Saxena, 2006). The lipid peroxidation assay (section 2.11) was used to determine the damage done to themembranes of the two cultivars due to vanadium. The Garnet and Agamax controls that were treated with water showed no significant difference in their MDA levels when comparing their leaves and roots (Figure 4.3). The leaves of Agamax plants treated with vanadium displayed an increase in MDA levels of 26% when compared to control Agamax plants (Figure 4.3 A). The Garnet cultivar displayed an increase of MDA levels of 234% within the leaves of the treated plants when compared to the control plants (Figure 4.3 A). The roots of Agamax and Garnet plants which had been treated with vanadium showed an increase in MDA levels of 88% and 235% respectively when compared to their respective control plants (Figure 4.3 B).

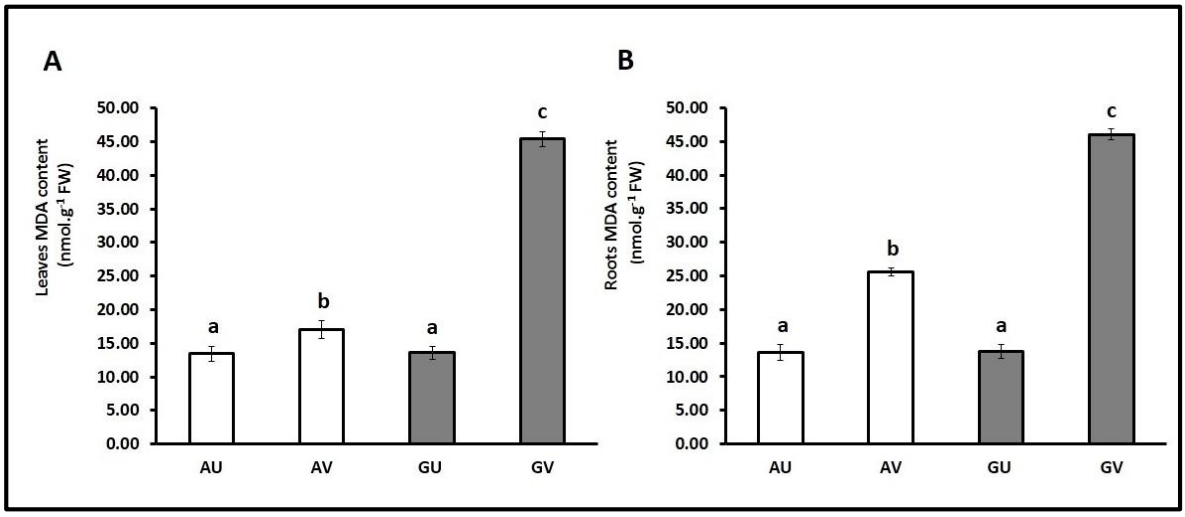

Figure 4.3: The effect of vanadium treatment on MDA levels within two *Brassica napus* L cultivars. The MDA levels give an indication of lipid peroxidation. (A) and (B) displays the MDA content in the leaves and roots respectively of the two *Brassica napus* L cultivars. A and G denote Agamax and Garnet plants respectively and U and V denote untreated and vanadium treated plants respectively. Different letters indicate significant differences between means at P< 0.05 (DMRT). Values are means ± S.E (N=3).

4.3.4. Vanadium has an effect on the antioxidant profiles of the two Brassica napus L *cultivars*

Antioxidant enzymes such as APX, SOD and GPX play a critical role in maintaining the ROS homeostasis within plants and as well as protecting them from oxidative damage (Lee *et al*., 2007; Sinha & Saxena, 2006). Damage may occur when there is an imbalance in ROS production and antioxidant enzymes concentrations. A plant may contain a number of isoforms of the same antioxidant enzyme (Sarowar *et al*., 2005). The antioxidant enzymes may be affected by stresses such as drought, salinity and heavy metals, either down regulating, up regulating or

completely inhibiting an isoforms' activity. Investigating how vanadium affects the antioxidant enzymes could give an indication to why certain plants are sensitive to heavy metal stress and others are more resistant to the stress.

Antioxidant enzymes such as SODs are known to have different types (Wang *et al*., 2005). The type of SOD depends on the metal cofactor, which is required for the enzyme to function (Mahanty *et al*., 2012). Metals such as iron, manganese, copper and zinc can be associated with a particular SOD. A metal required by a SOD can be substituted by another metal, which may result in the inactivation of the SOD (Mahanty *et al*., 2012). It was important to determine the SOD types found in the SOD profiles for the two cultivars in our study, to understand if a type of SOD is up or down regulated when the plants were exposed to vanadium.

4.3.4.1. SOD class identification and the effect vanadium has on the activity of certain SOD isoforms in two Brassica napus L *cultivars*

Agamax was observed to have an extra manganese superoxide dismutase (MnSOD) (Figure 4.4. B). A total of seven and six SODs were identified in the Agamax and Garnet cultivars respectively (Figure 4.4). The SOD profile of Agamax included two MnSODs, two copper/zinc superoxide dismutases (Cu/ZnSODs) and three iron superoxide dismutases (FeSODs) (Figure 4.4. B). The SOD profile of Garnet included one MnSODs, two Cu/ZnSODs and three FeSODs (Figure 4.4. A).

The SOD native PAGE gels also showed seven isoforms present in the Agamax cultivar and six isoforms in the Garnet cultivar (Figure 4.5). A decrease in the SOD 6 band intensity was observed in both Agamax and Garnet treated plants when compared to the band intensity of the respective untreated plants (Figure 4.5). Densitometry was used to determine the change in isoform activity caused by vanadium treatment. There was no change in activity of SOD 1 in the Agamax cultivar (Table 4.1). SOD 1 was not present within the Garnet cultivar. SOD 2 displayed no change in activity for the Agamax vanadium treated plants when compared to the control plants. No change in activity was observed for SOD 2 when the treated and untreated Garnet plants were compared. Garnet had a 38% higher activity for SOD 2 when compared to the activity displayed by Agamax (Table 4.1). The activity of SOD 3 was similar for all plants (Agamax, Garnet treated and untreated). No change in activity of SOD 4 was observed in any of the plants. The activity of SOD 5 remained the same for all plants. The activity of SOD 6 decreased by 70 % in Agamax treated plants when compared to the Agamax control plants. The activity of SOD 6 decrease by 73% in the Garnet treated plants when compared to the Garnet control plants. No change in activity of SOD 7 was observed in control and vanadium treated Agamax as well as Garnet. Agamax had a 9% higher SOD 7 activity when compared to the Garnet cultivar for both control and vanadium treatments.

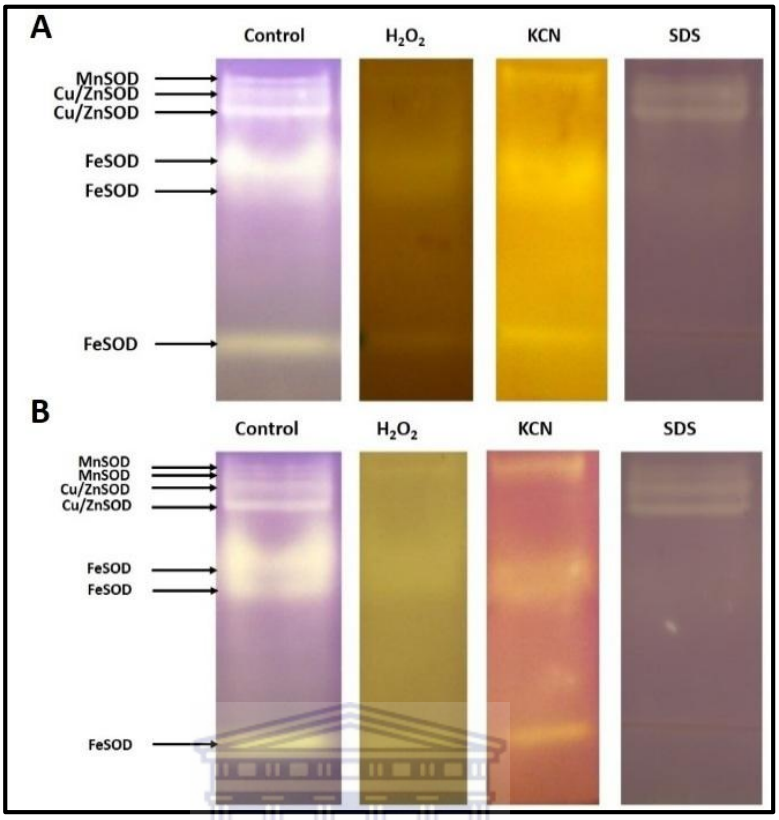

Figure 4.4: Identification of SOD classes. SODs were classified according to which compounds were able to inhibit their activity, the compounds which were used are hydrogen peroxide (H_2O_2), potassium cyanide (KCN) and Sodium dodecyl sulphate (SDS). (A) is the SOD profile for the Garnet cultivar and (B) the SOD profile for the Agamax cultivar.

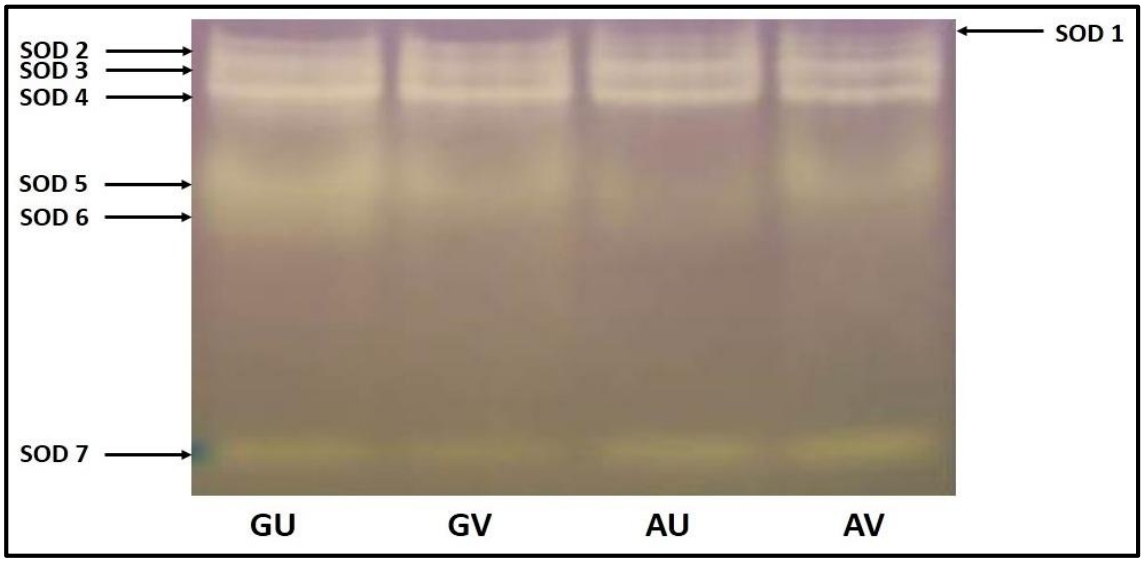

Figure 4.5: The effect of vanadium on the activity profile of superoxide dismutase in two *Brassica napus* L cultivars. SOD activity assays were conducted on plant material which was treated with vanadium for 21 days. This assay was conducted to understand how vanadium affected the seven isoforms of SODs The gel presented displays the effect of vanadium on seven SOD isoforms in two *Brassica napus* L cultivars.

Table 4.1. Densitometry readings for SOD native PAGE activity gels

	Proposed SOD type	**AU**	**AV**	**GU**	**GV**
SOD 1	MnSOD 1	50 ± 1.26^{a}	49 ± 1.31^{a}		
SOD 2	MnSOD 2	48 ± 1.31^{a}	49 ± 1.25^{a}	66 ± 1.41^{b}	66 ± 1.37^{b}
SOD 3	Cu/ZnSOD 1	83 ± 1.50^{c}	82 ± 1.33^{c}	85 ± 1.29^{c}	84 ± 1.55^{c}
SOD 4	Cu/ZnSOD 2	90 ± 1.33^{d}	91 ± 1.39^{d}	92 ± 1.49^{d}	92 ± 1.51^{d}
SOD 5	FeSOD 1	59 ± 1.57^{e}	61 ± 1.49^{e}	56 ± 1.57^{e}	61 ± 1.52^{e}
SOD 6	FeSOD 2	43 ± 1.27^{f}	13 ± 1.35^{g}	45 ± 1.41^{f}	12 ± 1.29^{g}
SOD 7	FeSOD 3	70 ± 1.57^{h}	73 ± 1.49^{h}	64 ± 1.54^{b}	62 ± 1.56^{b}

Different letters indicate significant difference between means at P<0.05 (DMRT). Values are means±S.E (n=3).

4.3.4.2. Vanadium increases the activity of certain APX isoforms

On the APX native PAGE activity gel, seven isoforms were observed to be present in both the Agamax and Garnet cultivars (Figure 4.6). APX 2 and 3 both showed an increase in band intensity in Garnet treated plants when compared to all the other samples. APX 4 and 5 in both Agamax and Garnet treated plants showed an increase in band intensity when compared to the respective controls. APX 1, 6 and 7 displayed no change in band intensity between the different samples. To determine the change in isoform activity caused by vanadium treatment densitometry was conducted. There was no change in activity of APX 1 in both the Agamax and Garnet cultivars (Table 4.2). The Agamax cultivar showed no change in activity for APX 2, but the Garnet treated plants for the same isoform displayed an increase of 102% when compared to the untreated Garnet sample.

APX 3 displayed no change in activity for the Agamax cultivar between the control and treated plants, but an increase in activity of 102% was observed in the Garnet treated plants. APX 4 in the Agamax cultivar showed an increase in activity of 71% in Agamax treated plants when compared to control plants. APX 4 displayed an increase of 108% in Garnet treated plants when compared to Garnet control plants. APX 5 in the Agamax cultivar showed an increase in activity of 43% in Agamax treated plants when compared to control plants. APX 5 displayed an increase of 43% in the Garnet treated plants when compared to the Garnet control plants. There was no change in activity of APX 6 and APX 7 in both the Agamax and Garnet cultivars.

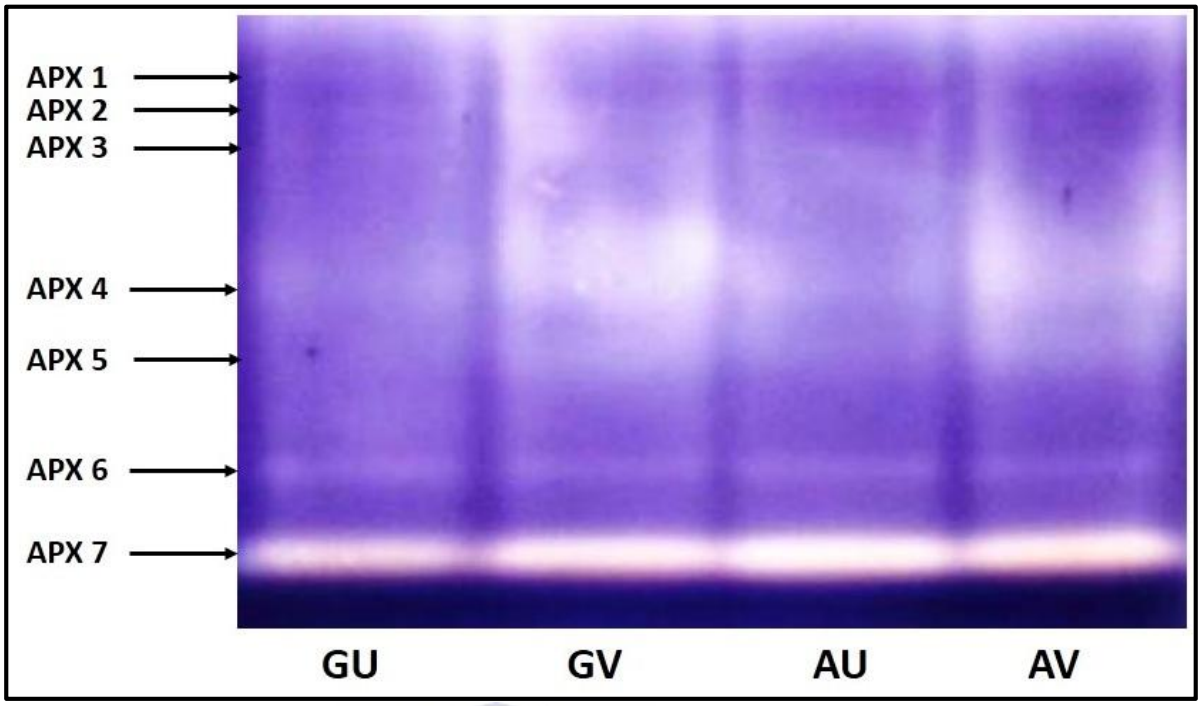

Figure 4.6: The effect of vanadium on the activity profile of ascorbate peroxidase in two *Brassica napus* L cultivars. Two *Brassica napus* L cultivars were treated with vanadium for 21 days therafter proteins were extracted and native PAGE activity gels were conducted. The gel presented displays the effect of vanadium on the seven isoforms in the two *Brassica napus* L cultivars.

Table 4.2. Densitometry readings for APX native PAGE activity gels

	AU	AV	GU	GV
APX 1	49 ± 1.56^{a}	47 ± 1.69^{a}	51 ± 1.54^{a}	51 ± 1.65^{a}
APX 2	48 ± 1.61^{a}	47 ± 1.65^{a}	47 ± 1.55^{a}	95 ± 1.57^{b}
APX 3	48 ± 1.60^{a}	47 ± 1.66^{a}	48 ± 1.65^{a}	97 ± 1.63^{b}
APX 4	65 ± 1.63^{c}	111 ± 1.60^{d}	65 ± 1.59^{c}	135 ± 1.61^{e}
APX 5	49 ± 1.35^{a}	65± 1.50^{c}	47 ± 1.38^{a}	67± 1.61^{c}
APX 6	105 ± 1.52^{f}	106 ± 1.46^{f}	104 ± 1.55^{f}	104 ± 1.63^{f}
APX 7	253 ± 1.65^{g}	257 ± 1.61^{g}	255 ± 1.62^{g}	256 ± 1.58^{g}

Different letters indicate significant difference between means at P<0.05 (DMRT). Values are means±S.E (n=3).

4.3.4.3. Vanadium increases the activity of some GPX-like isoforms

The GPX-like native PAGE activity gel displayed three isoforms present within the Agamax and Garnet cultivars (Figure 4.7). The GPX-like 1 isoform displayed an increase in activity for both the treated Agamax and Garnet plants when compared to their respective controls (Figure 4.7). To determine the change in isoform activity caused by vanadium treatment densitometry analysis was conducted. The activity of GPX-like 1 increased by 28% in the Agamax treated plants when compared to the Agamax control plants. The activity of GPX-like 1 increased by 30% in the Garnet treated plants when compared to the Garnet control plants. No increase in activity of GPX-like 2 was observed when comparing the treated and untreated plants of the Agamax cultivar. The activity of GPX-like 2 increased by 40% in the Garnet treated plants when compared to

the Garnet control plants. No increase in activity of GPX-like 3 was observed when comparing the treated and untreated plants of the Agamax and Garnet cultivars. Garnet plants had a 16% higher GPX-like 3 activity when compared to the GPX-like 3 activity in Agamax plants.

Figure 4.7: The effect of vanadium on the activity profile of Glutathione peroxide-like enzyme in two *Brassica napus* L cultivars. Two *Brassica napus* L cultivars were treated with vanadium for 21 days therafter proteins were extracted and native PAGE activity gels were conducted. The gel presented displays the affect vanadium had on the 3 isoforms of the GPX-like enzyme.

Table 4.3: Densitometry readings for GPX-like native PAGE activity gels

	AU	**AV**	**GU**	**GV**
GPX 1	44 ± 1.26^{a}	54 ± 1.29^{b}	43 ± 1.34^{a}	56 ± 1.27^{b}
GPX 2	26 ± 1.31^{c}	24 ± 1.40^{c}	25 ± 1.24^{c}	35 ± 1.27^{d}
GPX 3	38 ± 1.23^{d}	37 ± 1.31^{d}	43 ± 1.32^{a}	44 ± 1.29^{a}

Different letters indicate significant difference between means at P<0.05 (DMRT). Values are means±S.E (n=3).

4.4. Discussion

The work reported here investigated the effect of vanadium on the oxidation state and antioxidant profiles of two *Brassica napus* L cultivars. Experiments were conducted to determine the affect of vanadium on the H_2O_2 levels, O_2^- levels and the degree of lipid peroxidation (MDA) as a result of ROS production when the cultivars were exposed to high vanadium concentrations. The profiles of the antioxidant enzymes were investigated to understand how they were influenced when plants were exposed to vanadium and if the antioxidant capabilites were affected.

Hydrogen peroxide (H_2O_2) in excess is associated with programmed cell death (PCD) and the hypersensitive response of plants (Grant & Loake, 2000). It was observed that there was much less H_2O_2 present in the roots of the untreated and treated Garnet and Agamax plants when compared to their leaves. Literature states that H_2O_2 is produced by organelles such as the mitochondria,

chloroplasts and xylem as a signaling molecule (Cheeseman, 2007). Due to the amount of these organelles in the leaves, it could explain why higher concentrations were observed in the leaves than the roots. The results observed in this study were consistent with a study done by Bian and Jiang (2009) which showed that Kentucky bluegrass had a higher hydrogen peroxide concentration in their leaves than their roots. This study also showed the levels of H_2O_2 in the leaves and roots of the treated Agamax and Garnet plants were higher than the control plants that were treated with water. The results of this study are consistent with a study done by Gorska-Czekaj and Borucki (2013) which showed that H_2O_2 levels increased in plants treated with metals such as mercury and copper. A study by Cho and Seo (2005) showed that seedlings which were exposed to cadmium incurred a two fold increase in H_2O_2 concentration. The H_2O_2 content in the leaves and roots of the Garnet treated plants were higher when compared to the leaves and roots of the Agamax treated plants. The large increase in H_2O_2 content in the Garnet plants exposed to vanadium was suggested to be due to the vanadium playing a role in down regulating the ROS scavenging enzymes thus disturbing the oxidative homeostasis. It was therefore necessary to determine the profile and activity of ascorbate peroxidase and glutathione dependent peroxidase, which are associated with H_2O_2 scavenging. The increase of H_2O_2 in the plant tissue was suggested to be due to the increase of respiratory activities (due to the stress) and the associated antioxidant systems which remained the same thus proper oxidant scavenging could not occur Gorska-Czekaj and Borucki (2013).

Superoxide (O_2^-) is the first ROS which is produced in the Foyer-Halliwell-Asada pathway in plants (Yadav, 2010). Although O_2^- has a relatively short life span it has the ability to promote the production of other ROS such as H_2O_2 which is very dangerous and toxic to plants if accumulated. The leaves and roots of the untreated (control) Garnet and Agamax plants showed similar levels of O_2^-. The leaves and roots of the vanadium treated Garnet and Agamax plants were observed to have higher O_2^- levels when compared to their respective controls. The leaves and roots of the Garnet treated plants showed higher levels of O_2^- when compared to the leaves and roots of the Agamax vanadium treated plants. It has to be noted that the O_2^- levels in the roots of vanadium treated Garnet plants were much higher than the levels in the vanadium treated Agamax plants. It was also observed that overall higher levels of O_2^- were present in the roots rather than the leaves of the plants. The increase in O_2^- levels within the vanadium treated Agamax and Garnet plants were again attributed to the vanadium playing a role in the down regulation of the ROS scavenging enzymes. It was therefore necessary to determine the profile and activity of superoxide dismutase (SOD) which is associated with O_2^- scavenging and removal.

The TBARS assay, which is used to determine MDA concentrations, was employed to estimate the lipid peroxidation and therefore to assess the damage caused by vanadium on the membranes of Garnet and Agamax plants. The

results observed showed that Agamax and Garnet had similar concentrations of MDA in their roots and leaves when they were not stressed with vanadium. Lipid peroxidation was higher in the leaves and roots of the treated Garnet and Agamax plants when compared to their respective controls as more MDA was present in the treated plants than the control plants. The results were consistent with a study by Ammar et al. (2008) who observed an increase in MDA concentration within the leaves and roots of tomato plants that were exposed to 50 µM cadmium. Due to the increase in vanadium concentrations within the plant tissue of the treated Garnet and Agamax plants (chapter 3) the aforementioned result was similar to Ammar et al. (2008). In this study, when compared to Agamax treated plants, the Garnet treated plants showed the highest concentrations of MDA within their roots and leaves which coincides with the damage seen to the leaves and roots observed in chapter 3. The Agamax treated plants showed a slight increase in MDA in their leaves and a larger increase in MDA in their roots. Due to Agamax plants, localizing most of the vanadium to its roots it was expected that the MDA levels and therefore damage would be higher in the roots than the leaves. This shows that Agamax has mechanism to limit the amount of damage when exposed to vanadium. The mechanisms could include limiting the amount of ROS produced, as Agamax plants exposed to vanadium were observed to have lower levels of H_2O_2 and O_2^- (Figure 4.1 and 4.2) when compared to Garnet plants that were exposed to vanadium.

During the scope of this study problems were encountered when performing the in-gel activity PAGE assays for the SOD, APX and GPX-like enzymes. The discrimination between different isoforms was very poor. Initially, only two SOD and APX activity bands were visualized for the APX and SOD enzymes while only one activity band was visualized for the GPX-like enzyme. The percentage of the gel (percentage N,N-Methylenebisacrylamide) and the migration times at which the proteins were electrophoresed on the gel were changed. These changes increased the resolution and discrimination between different isoforms could be observed. The concentrations of the constituents that made up the activity stains were also changed as the PAGE gels were often to dark and masked the lower activity isoform bands. The time the PAGE gels were exposed to light had to be altered because when the gels were exposed to light for too long they would become too dark and also mask low intensity bands.

The SOD isoform profile (to identify the SOD type) was determined and using proteins extracted from plants which were not exposed to vanadium as seen in (control) (figure 4.4). It was interesting to observe that the Garnet plants had one less SOD isoform than the Agamax plants which contained seven SOD isoforms (figure 4.4). Abedi and Pakniyat (2010) observed that *Brassica napus* L cultivars had 5 SOD isoforms present. Due to Agamax containing an extra SOD isoform it was necessary to determine the class of each isoform. Compounds such as hydrogen peroxide (H_2O_2), potassium cyanide (KCN) and sodium dodecyl sulfate

(SDS) are able to inhibit different classes of SODs using this information the class of SOD could be identified. Potassium cyanide inhibits Cu/ZnSODs, H_2O_2 inhibits Cu/ZnSOD and MnSODs, and SDS inhibits FeSODs and MnSODs. A study by Hernandez et al. (2001) used a similar method using KCN and H_2O_2 to identify the classes of three SODs present in Pea leaves when stressed with salt. In this study, the profiles for Garnet and Agamax were comprised of the same isoforms except for the extra MnSOD, which was found in the Agamax plants. It was interesting to note that the Abedi and Pakniyat (2010) study observed that the 5 SODs were all classified as Cu/Zn SODs which was not consistent with our study as both MnSODs and FeSODs were observed. A study by Brou et al. (2007) incubated their SOD activity native PAGE gel in 2% SDS to inhibit the activity of FeSODs and MnSODs so as to increase their discrimination capacity between different SOD classes. The classes of SODs identified in our study do not correspond with the results in the study by Abedi and Pakniyat (2010) although *Brassica napus* L plants were used, this could be due to SDS being used in our study as an extra step to confirm the identity of the classes of SODs. It is important to note that at times the inhibition of a SOD isoform might be only partial and therefore may not be observed leading to a miss identification of that particular isoform. The activity gels were complented by desometric analyses using AlphaEase FC imaging software. The class of every SOD isoform was identified as seen in figure 4.4. It was observed that SOD 6 which was a FeSOD was negatively affected by vanadium.

Once the SOD profile was determined we needed to understand how these SOD isoforms would be affected when the plants were exposed to vanadium and the resulting stress. No differentiation in activity was observed between Agamax and Garnet for SOD 3, 4 and 5 (Table 4.1). The Garnet plants, both untreated and treated, were observed to have a higher activity for SOD 2 but lower activity for SOD 7 when compared to the Agamax untreated and treated plants. In a study by Abedi and Pakniyat (2010) differences in activities were observed for particular SODs when comparing different *Brassica napus* L cultivars. The observation in our study suggests that Garnet has an inherent higher activity for SOD 2 but lower activity for SOD 7 when compared to Agamax. The suggested reasons for the difference in activity of these two isoforms in these two cultivars include evolution and plant breeding. The increase in activity of SOD isoform 2 was not adequate to control the concentrations of O_2^- being produced in response to vanadium stress. The activity of SOD 6 was observed to be lower in the treated Garnet and Agamax plants. The vanadium that these plants were exposed to could have decreased the activity of SOD 6. Vanadium could have influenced the synthesis of the protein thus changing its structure and not allowing it to function normally or vanadium could be interacting with one of the active sites on the enzyme thus not allowing O_2^- to interact with the SOD. A study by Romero-Puertas et al. (2002) observed oxidative modifications to proteins such as SOD, glutathione reductase (GR) and catalase (CAT) when Pea plants were exposed to cadmium. The same study by Romero-Puertas et al. (2002) observed that the activity of the isoforms of the associated antioxidant enzymes

decreased as well as the degradation of the proteins increased when oxidatively modified. The observations made from the SOD in-gel activity in our study suggest that the increase in O_2^- levels observed in the treated Garnet and Agamax plant samples (Figure 4.2) could be due to the negative effect which vanadium had on SOD 6 (Table 4.1). The decrease in activity of SOD 6 in the treated Garnet and Agamax plants could have led to a reduction in their O_2^- scavenging capabilities.

The effect of vanadium on the APX isoforms was investigated by electrophoreses of total protein of untreated and treated Garnet and Agamax plants on a native PAGE gel and staining specifically for APX activity. The Garnet and Agamax profiles were comprised of the same seven isoforms. No difference in activity was observed between Agamax and Garnet for APX 1, 6 and 7. An increase in activity of APX 2 and 3 was observed in the Garnet treated plants compared to the Garnet control plants. An increase in activity of APX 4 and 5 were observed in the Garnet and Agamax vanadium treated plants. A suggested reason for the increase in the activity of APX 2 and 3 in the Garnet treated plants and APX 4 and 5 in the Garnet and Agamax treated plants could be associated with the plants trying to increase their ROS scavenging abilities in response to the increased H_2O_2 levels caused by vanadium stress. A study by Abedi and Pakniyat (2010) observed that the activity of certain guaiacol peroxidase (POD) isoforms was upregulated under drought stress. The results of this study show that the

increase in the H_2O_2 concentration in the Garnet and Agamax treated plants may be attributed to the vanadium stress but could not be attributed to a decrease in activity of the APX isoforms because we do not observe any decreases in APX isoforms. The Garnet plants were able to increase the activity of four of their APX isoforms but still fared worse (as seen in figures 3.1.1 and 3.1.2) than the Agamax plants which increased the activity of only two isoforms. The results observed suggest that the amount of vanadium taken up by the two cultivars may be important when the plants activate APX in response to the stress.

The effect of vanadium on GPX-like isoforms were investigated by electrophoresis of total protein of untreated and treated Garnet and Agamax plants on a native PAGE gel and staining specifically for GPX activity. It is important to note that we call the enzyme GPX-like and not GPX as controversy exists around the true identity and existence of GPXs in plants. The untreated and treated Garnet and Agamax plants were all comprised of three GPX-like peroxidase isoforms (Figure 4.7). To validate the observed results densitometry analysis was conducted. Densitometry results showed that the activity of GPX-like 1 increased in the treated Garnet and Agamax plants when compared to the untreated plants. The results of this study were consistent with a study by Haluskova et al. (2009) who observed an increase in GPX activity when barley was exposed to cadmium stress. The activity of GPX-like 2 showed that the increase in activity only occurred in the Garnet treated plants. The increase in

activity of the two isoforms was again attributed to the treated plants trying to improve their ROS scavenging capabilities to control the ROS (in this case H_2O_2) production and accumulation. The Agamax treated plants showed similar activities for GPX-like 2 when compared to its control plants. The suggested reason was that Agamax was equipped to control the production and accumulation of H_2O_2, which negated the need to increase the GPX-like 2 isoform. The H_2O_2 result (Figure 4.1) lends evidence to this suggestion, as the H_2O_2 concentration was lower in the Agamax treated plants than the Garnet treated plants. No differentiation in activity of GPX–like 3 was observed between treated and untreated Garnet and Agamax plants. The Garnet plants did however have an inherently higher activity for GPX-like 3 isoform when compared to the Agamax plants.

In conclusion, it was observed that when treated with vanadium Garnet plants produced higher ROS (H_2O_2 and O_2^-) concentrations than the controls and the untreated and treated Agamax plants. The damage in the treated Garnet plants were also higher and was attributed to the high ROS concentrations. The isoform profile for SOD, APX and GPX-like enyzymes of both Garnet and Agamax were succesfully identified. It was observed that vanadium was able to affect certain isoforms of the antioxidant enzymes. Even though Garnet upregulated many of the antioxidant enzymes it was not able to control the concentrations of ROS adequately. The results of the study showed that Agamax performed better than

Garnet under vanadium stress which was atrributed to Agamax controlling and limiting ROS accumulation better than Garnet.

Conclusion and Future Work

This study has established that vanadium at elevated concetrations is detrimental to two *Brassica napus* L cultivars namely Garnet and Agamax. The negative effects of vanadium on the two cultivars were observed by the reduction of the biomass and the area/volume of the roots and leaves. However, Agamax was observed to be more tolerant to vanadium stress than Garnet. This was observed as the reduction in the biomass and area/volume of the roots and leaves of Agamax were lower than that of Garnet when exposed to vanadium. The leaves of the Garnet plants treated with vanadium also showed signs of chlorosis which was illustrated by the yellowing of the leaves and a decrease in chlorophyll where as the Agamax plants did not show the same trend. The amount of cell death was far more pronounced in Garnet when compared to Agamax. An assay to determine vanadium concetrations within water, soil and plant material was developed to determine the vanadium levels in different plant tissues, to assess the uptake of vanadium by these plants and how vanadium could contribute to the reduction in plant growth. It was observed that Garnet did not only take up a large amount of vanadium but also translocated much of the vanadium to the leaves through the shoots. However, in Agamax it was observed that most of the vanadium was localized within the roots of the plants and very little was transported to the leaves or other plant tissues. The ability of Agamax to limit the transport of vanadium from the roots to the shoots and

leaves is one mechanism which allowed for increased tolerance of Agamax to vanadium stress.

The study also established that vanadium induced oxidative stress and affected the antioxidant scavenging pathways of two *Brassica napus* L cultivars (Garnet and Agamax). To observe how the oxidation state of the two cultivars change when exposed to vanadium the H_2O_2 and O_2^- levels were determined. The H_2O_2 levels in the leaves and roots of vanadium treated Agamax plants were lower than the levels in the leaves and roots of vanadium treated Garnet plants. The O_2^- levels in the leaves and roots of the vanadium treated Garnet plants were also higher than in the vanadium treated Agamax plants. A vast increase in lipid peroxidation was observed in the leaves and roots of the Garnet plants treated with vanadium. This increase in lipid peroxidation was attributed to the increase in reactive oxygen species in the Garnet treated plants. The extent of the increase in lipid peroxidation in the Garnet treated plants might explain why cell death was so pronounced in the Garnet treated plants. Due to the increase in ROS the antioxidant pathways were investigated. During this study one (Garnet) and two (Agamax) MnSODs, two Cu/Zn SODs and three FeSODs were identidied to be present in the two *Brassica napus* L cultivars. The aformentioned SOD isoforms to our knowledge were not classifed before. Agamax and Garnet both had one SOD isoform of which they had an inherent increased activity for, evolutionary selection or plant breeding two of the suggested reasons. The

activity of one SOD (SOD 6) was down regulated in both Agamax and Garnet when exposed to vanadium. The high levels of O_2^- accumulated by both cultivars can be attributed to an increased production rate of O_2^- and the lower activity of certain isoforms of the SOD enzymes. The isoform profile of the SOD enzymes showed that Agamax had an extra isoform when compared to Garnet which would explain why the levels of O_2^- were lower in the Agamax vanadium treated plants when compared to the Garnet vanadium treated plants. It was also observed that under vanadium stress, the activity of two APX and one GPX-like enzyme in Agamax and four APX and two GPX-Like enzyme in Garnet were upregulated. The aformetioned observations shows that vanadium does have an effect on APX and GPX-like enzymes in the two *Brassica napus* L cultivars. Garnet was identified as the sensitive cultivar and therefore increased the activity of certain APX and GPX-like enzyme isoforms but could not reduce the H_2O_2 levels as efficiently as the Agamax cultivar when exposed to vanadium. According to literature heavy metals decrease total enzymes activity and therefore should inhibit the activity of many isoforms of antioxidant enzymes however, our study showed that the activity of some isoforms of the antioxidant enzymes may be upregulated.

This study also shows the importance of screening different cultivars (using antioxidant profiling is one method) as even closely related family members may respond very differently when exposed to different stresses. Applying the

information obtained in our study could allow farmers to determine the vanadium concentrations in their soils and in so doing they may make informed desicions to what cultivar or plants will be suited to them. This will lead to an increased crop yield and profit to the farmer and an increase in food security for South Africa.

Future work will include, using of 2D PAGE analysis to confirm that the isoforms that we have identified are all single isoforms. Also, to identify the amino acid sequence of the up/down regulated isoforms of the antioxidant enzymes using matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF). These sequences will be used to produce primers and therafter semi-quantitative analysis of the gene expression (mRNA) will be conducted to confirm responses to vanadium. The full length genes of the interesting isoforms will also be isolated and then inserted into appropriate expression vectors. The genes will then be expressed and recombinant proteins will be purified followed by relevant assays to confirm activity. The antioxidant genes can also be inserted into an appropriate plant transformation vector for subsequent plant transgenic studies to identify whether increased vanadium tolerance can be transferred to other valuable crop plants using the antioxidant genes identified in this study.

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