Physiological and biochemical characterization, of antimony stress, responses in *Phaseolus vulgaris*

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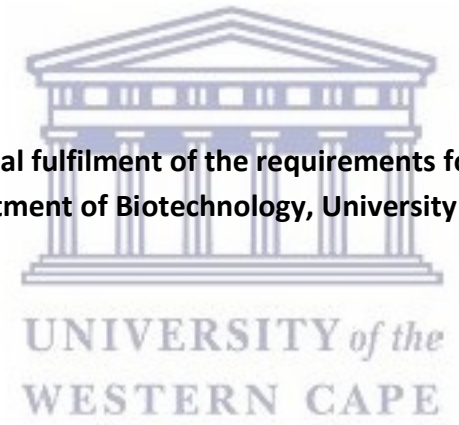

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CONTENTS

LIST OF ABBREVIATIONS

APX	Ascorbate peroxidase
CAT	Catalase
Cont	Contender
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ETC	Etcetera
GDP	Gross Domestic product
ICP	Inductively coupled plasma atomic emission spectroscopy
MDA	Malondaldehyde
NBT	Nitro blue tetrazolium chloride
ROS	Reactive oxygen species
Sb	Antimony
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
Tim	Timbavati
WHO	World Health Organisation

LIST OF FIGURES AND TABLES

Chapter 4

Physiological and biochemical characterization, of antimony stress, responses in *Phaseolus vulgaris.*

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Master's Thesis, Department of Biotechnology, University of the Western Cape

ABSTRACT

The mining industry in South Africa is of immense importance as this sector contributes largely to the countries income. In the Limpopo province, a large production of antimony (Sb) is generated per year. Antimony (Sb) is a trace element, which accumulates in the environment through anthropogenic activities, such as mining and smelting industries. Antimony is toxic to all living organisms and plants, and it is found to increase the peroxidation of membrane lipids and encourage an antioxidant response. Sb contamination in plants also accounts for DNA damage. The reduction in yield is due to the disruption of plant metabolism by reactive oxygen species (ROS).

To combat abiotic stresses, plants have generated a signalling network that utilises multiple growth regulators that would offer protection against the stress. An increase in ROS is one of the responses to abiotic stresses. ROS is generated in response to the pants interaction with heavy metals, through the Harber-Weiss reaction. ROS compounds include: superoxide, hydrogen peroxide and hydroxyl radicals. Under normal conditions ROS molecules are produced as by-products, however, under stressful conditions the production of ROS molecules are increased to levels where they are detrimental to the plants. Therefore, the accumulation of ROS results in damage to proteins, lipids, carbohydrates and DNA which would lead to cellular death. ROS accumulation is thought to be a result of the disruption in the balance of ROS production and the anti-oxidation systems. The anti-oxidative system is thus introduced to restore the balance of ROS molecule production and to combat oxidative damage caused by the ROS molecules. The anti-oxidative system consists of various enzymes: superoxide dismutase, catalase, and ascorbate peroxidase and glutathione reductase. Each antioxidant scavenges one or two ROS molecules.

In the present investigation, both cultivars of the *Phaseolus vulgaris* family; the Contender cultivar and the Timbavati cultivar was analysed and compared to their respective controls (0 µM antimony). The two *Phaseolus vulgaris* cultivar's physiology (biomass, chlorophyll

and cell death) were analysed using the fresh weights obtained after the commencement of the growth period. Their biochemical characteristics were also analysed to obtain information of how each cultivar (Contender and Timbavati) response to antimony stress. In the investigation, both cultivars of the *Phaseolus vulgaris* family; Contender and Timbavati experienced an effect under antimony stress, however, the significance was observed in the degree of effect experienced between the cultivars. The Contender and the Timbavati cultivars were selected from a preliminary screening of five cultivars of the *Phaseolus vulgaris* family, and these two cultivars illustrated various degrees of effects under antimony stress. The Timbavati cultivar illustrated the largest increase in its ROS molecule production, correlating to the oxidative damage levels seen in the lipid peroxidation results (~24.55% in leaves and ~14.1% in roots) and the increase in cell death (~28.68% in leaves and 67.67% in roots). Even though the Contender cultivar illustrated an effect, by increase of ROS molecules the increase were not as significant as the Timbavati cultivar. The slightly higher degree of tolerance could be attributed to an efficient defence mechanism employed by the Contender cultivar.

The Contender cultivar represented an efficient antioxidant system, as the enzymes sufficiently scavenged the ROS molecules. Timbavati, however, did not illustrate and effective antioxidant system, thus explaining why the cultivar experienced the damage observed. *Phaseolus vulgaris*, common beans, is known as a staple food, which serves as an excellent source of carbohydrates, proteins for low population areas, and a main source of vitamins and minerals, for instance iron, for population in Eastern Africa and Latin America. Thus, it was of great importance to understand how antimony stress influenced the overall nutrient profile of the two cultivars. This was conducted, using ICP-OES analysis. Once again, even though Contender illustrated a degree of susceptibility the degree was not as severe as that observed in Timbavati cultivar.Reviewing the results obtained it can be stated that under high concentrations of antimony the Contender cultivar is better equipped with dealing with antimony stress than the Timbavati cultivar.

Keywords: Antimony, *Phaseolus vulgaris L.,* heavy metals, micronutrient, macronutrient

CHAPTER 1

1.1. Introduction

In the natural environment, plants are constantly exposed to numerous environmental stresses (Ramegowda and Senthil-Kumar, 2015). The problem arises, due to plants existing as sessile organisms; consequently this hinders them from evading the stress imposed upon them. In order to respond efficiently to these adverse environmental conditions, plants have adapted particular mechanisms. These mechanisms are employed to elicit complex cellular and molecular responses to avoid damage and ultimately ensure plant survival, however, at the detriment of growth and yield (Atkinson and Urwin, 2012). Ramegodwa and Senthil-Kumar (2015) specified that two stresses occur in the environment: biotic stress and abiotic stress. Plants are simultaneously confronted with numerous biotic and abiotic stresses. Biotic stresses are living organisms or their materials (organic matter), which directly or indirectly affect the organism in its environment (predation, disease and parasitism). Abiotic stresses are known to vary in the environment; these stresses determine the types and quantity of organisms that exist in that environment (upsurge in heavy metal concentrations, drought, high salinity, pH levels and annual temperature). Owing to alterations in climate, numerous factors are escalating the possibility of abiotic stress occurrence. Schützendübel and Polle (2001) realized that environmental changes are resultant from anthropogenic activities, including industrial manufacturing processes, domestic refuse and waste material, which contributes to excess pollution of heavy metals into the environment initiating disruptions of the natural terrestrial ecosystems (Guala *et al*, 2010). Hence, it is of immense importance to understand the mechanisms participating in stress tolerance (Schützendübel and Polle, 2001).

One of the leading pressures on plants is abiotic stresses. Vandegehuchte et al (2010) detailed that Abiotic factors can exert neutral, positive or negative effects on plants. Positive effects exhibited could be an improvement in the nutritional qualities of the leaves of plants and negative effects could be exhibited as damage to the production site of the defence molecules in the root systems of plants. Generally, abiotic stresses imposed upon a plant produces a negative effect on the growth and productivity of plants, which initiates morphological, physiological, biochemical and molecular alterations (Bhatnagar-Mathur *et*

al, 2008). The general response to abiotic stress imposed upon a plants, are the generation of Reactive Oxygen Species, ROS (Tuteja *et al*, 2009). In the agricultural sector, abiotic stresses are the main motive behind crop failure, decreasing the average yields, for most major crops, by more than 50% (Tuteja *et al*, 2009).

Extensive loss of agricultural production worldwide is prompted by abiotic stresses (Mittler, 2005). Plants are known to require trace amounts of particular metals to survive (Rascio and Navari-Izzo, 2011). In spite of this, the problem arises when plants are exposed to high concentrations of heavy metals, which consequently leads to the disruption in the biochemistry of plants and physiological effects (Vachirapatama *et al*, 2011). Heavy metal stress on plants often result in the production of reactive oxygen species (ROS), which are relatively reactive compared to molecular oxygen therefore are potentially toxic (Gjorgeiva *et al*, 2013). The anti-oxidative system of plants usually balances the production and destruction of oxygen radicals (Gjorgeiva *et al*, 2013). Anti-oxidative systems include enzymatic molecules: superoxide dismutase, non-specific peroxidases, ascorbate peroxidases and catalases. The tolerance to heavy metals has been correlated with efficient anti-oxidative defence systems shown by (Van Assche and Clijsters 1990). A lot of information is available on the effects of metals on various antioxidant processes in plants. One possible mechanism, in which elevated concentrations of heavy metals may damage plant tissues, is by oxidative stress (Gjorgeiva *et al*, 2013). Heavy metals such as copper and iron can be toxic because of their participation in redox cycles producing hydroxyl radicals (OH) which are extremely toxic to living cells.

All metals, introduced at high concentrations, are considered environmental pollutants, since they exhibit strong toxic properties (Guala *et al*, 2009). Heavy metals are potentially toxic towards plants, since phytotoxicity advances into chlorosis, plant development. In beans it can result in the reduced ability to fixate molecular nitrogen. Due to the beans nitrogen-fixing proficiencies, they are proficient enough to improve the soils nutrient status. Soils that are highly contaminated with heavy metals produce problems for crop production in agricultural sectors as well as hazardous health consequences as they enter into the food chain (Guala *et al*, 2009). Plant growth, in heavy metal polluted soils, are inhibited by metal absorption. Nevertheless, certain plants are capable of accumulating large concentrations of heavy metals before a stress is imposed upon them (Guala *et al*,

2009). However, this could pose a potential threat to animal and humans, as crops growing in contaminated soils may well transfer the heavy metals up into the food chain (Guala *et al*, 2009). An added concern is the transfer of heavy metals into the natural ecosystem. The toxicity of the heavy metals within the soil significantly varies, due to soil characteristics and the duration of contamination (Guala *et al*, 2009).

Antimony (Sb) is known as a non-essential, trace element by means of it is studied to exhibit no known role as nutrient (Schützendübel and Polle, 2002), which accumulates in the environment by means of anthropogenic activities, for instance mining industries, through the smelting of antimony ores or the burning of fossil fuels (Vaculik *et al*, 2015). Shooting ranges are another source of antimony being introduced into soil by means of Sb being exercised in the hardening of bullets (Vaculik *et al*, 2015). Antimony can be toxic towards all living organisms, including humans (Vaculik *et al*, 2015). Sb is present in two oxidative states in the environment, namely; antimonate (which possess more toxic effects on plants) and antimonite (Vaculik *et al*, 2015). Ren et al indicated that plants are more efficient in absorbing SbIII compared to SbV, and that Sb accumulated typically in the root systems of plants. Feng *et al* found that most of the Sb accumulated within the cytosol whereas Ren *et al* discovered that in rice plants Sb accumulated typically in the cell walls and less in the organelles and cytosol. Furthermore, Ren *et al* discovered that Sb increased the peroxidation of membrane lipids and stimulated a defence antioxidant system in plants. Increased Sb concentrations in plants are known to provoke DNA damage and bring about further toxic effects (Vaculik *et al*, 2015). Antimony has a tendency to disturb numerous biochemical activities by displacing essential metals from their respective binding sites (Mendoza-Cózatl *et al*, 2014). It was estimated that the average crustal abundance of Sb is 0.2 mg/kg (Smith and Huyck, 1999). Its environmental concentrations have been increasing due to human activities, such as mining, smelting, fossil fuel combustion and waste incineration. The maximum permissible pollutant concentration of Sb recommended by World Health Organisation, WHO is 36 mg/kg (Chang *et al*, 2002). It was reported that high Sb concentrations at some sites far exceeded the standard. In Spain, the Sb concentrations in three abandoned Sb mining areas in Extremadura reached up to 225–2449.8 mg/kg (Murciego *et al*, 2007). China is a major producer of Sb, and most Sb mines are in Hunan and Guangxi Provinces (Huang *et al*, 2011). The Sb concentration in paddy soils near

Xikuang Mountain Sb mining area in Hunan Province has reached 1565 mg/kg (He and Yang, 1999). Plant grown on Sb contaminated soils can accumulate high levels of Sb (Murciego *et al*, 2007), posing a great threat to human health through food chain (Cai *et al*, 2016).

The state of food security is developing into a more undesirable state, mainly owing to the changes in the environment. The changes in the environment are resulting in the destruction of land and thus reduction in crop yield. Overall, this causes a major impact on our food prices, increase the prices to such an extent that the lower populations are unable to afford them (Rosegrant and Cline, 2003). Roughly 795 million people are stated to be malnourished, by the Food and Agricultural Organisation of the United Nations (McGuire, 2015). In South Africa the agricultural sector contributes about 2.6% to the Gross Domestic Product, GDP, of the country, however, many of the South African population is still recorded to live in poverty and found to be malnourished. One of the most well-known crop plants, the common bean, is of great importance globally as it is considered the most important grain of legume for human consumption (Beebe *et al*, 2013). They are an essential source of nutrients and for that reason, it would be of great devastation if these crop plants were susceptible to heavy metal concentration.

The literature review will discuss the importance of *Phaseolus vulgaris* as a staple crop plant, the major problem faced by the accumulation of heavy metals within the environment, how plants respond to the heavy metal accumulation (paying attention to ROS molecules and antioxidant enzymes) and finally justify why the current study is important.

1.2. *Phaseolus vulgaris* as an essential crop plant

Phaseolus vulgaris, common bean, is presently estimated to be one of the important legumes known worldwide. They are usually used as a model plant (Gjorgeiva *et al*, 2013). Estimates indicate that 300 million people in Eastern Africa and Latin America consider beans to be one of the most essential sources of nutrients. This information correlates with the data collected about that 65% of the proteins consumed and 32% of the energy is transferred (Petry *et al*, 2011). The common bean is the most important grain of legume for human consumption (Beebe et al, 2013). They are of great importance for they are protein-rich and they supply a large proportion of carbohydrates. Beans are also suitable source of

vitamin C and minerals such as calcium, iron, folic acid and riboflavin (vitamin B2). It is known as a tropical crop and is consequently sensitive to low temperatures. In general, farmer's plant both bush bean as well as runner varieties. Green beans are of tropical origins.

To understand the mechanisms of heavy metal uptake in *Phaseolus vulgaris*, it will enable scientist to discover this crop plants potential tolerance to heavy metal contamination.

1.3. Accumulation and translocation of heavy metals within plants

Hyper-accumulators are distinguished from non-hyperaccumulators by three factors; hyper-accumulators possess a greater proficiency for heavy metal uptake, they conduct root-to-shoot translocation of the heavy metals (transport the heavy metals) and finally, hyperaccumulators possess the ability to detoxify or sequest heavy metals (Singh *et al*, 2016). Hyperaccumulators possess the ability to absorb heavy metals from the soil and this is conducted under various concentrations of the heavy metals (Singh *et al*, 2016). On the other hand, the heavy metal uptake by hyperaccumulators is affected by various factors, namely; pH, water content and organic substances. Hyperaccumulators contains a suitable transporting systems required for heavy metal uptake. The pH of the soil surrounding the plants is of great importance as pH can influence the proton secretion by roots (Singh *et al*, 2016). These in terms lead to the acidification of the rhizosphere, thereby, allowing for metal-accumulating plants to increase its ability to flourish.

In the soil, heavy metals are merely only provided entrance to the initial millimetres of the beginning of the root tip. Conferring to the concentration gradient that exist heavy metals is carried into the apoplastic space. One other location the heavy metals are inclined to accumulate in is the cell walls of the root systems of the plants (Petry *et al*, 2011), due to these occurrences a toxic effect is exhibited at the plasma membranes within the cell. In the plants, it has been established that two routes are available for the uptake of heavy metals; the passive uptake, which is propelled by the concentration gradient that exists at that time across the membrane, and the inducible substrate-specific and energy-dependent uptake. Petry et al (2011) reported that there was a mutual transmembrane transporter that was discovered for Cadmium, Copper and Nickel and these particular compounds are competitively hindered by Potassium, Calcium and Magnesium (Petry *et al*, 2011).

In view of the fact that plants are sessile organisms and merely contain the limited number of mechanisms to attempt to circumvent stress, it is required of plants to possess an adaptable way for acclimation to the changing environmental conditions, thus suggested by Schützendübel and Polle (2002), to enhance a plant's protection, it is of great importance to understand the mechanisms contributing to stress tolerance.

1.4. Reactive oxygen species within plants

Reactive oxygen species (ROS) are generally produced as by-products (Gill *et al*, 2011) in various organelles, for instance the chloroplast, mitochondria and peroxisomes, whilst a plant is experiencing aerobic metabolism, under favourable conditions (Das and Roychoudhury, 2014). ROS molecules exhibit the capability to regulate the expression of genes, by means of functioning as intermediate signalling molecule. Therefore, it is of great importance for cells to strictly regulate the levels of reactive oxygen molecules, and no tot eliminate these molecules completely (Schützendübel and Polle, 2002). Under encouraging conditions, ROS molecules are produced at their basal levels which do not instigate detrimental consequences. Detrimental consequences do not occur, considering that these ROS molecules are efficiently scavenged by various antioxidant components (Das and Roychoudhury, 2014).

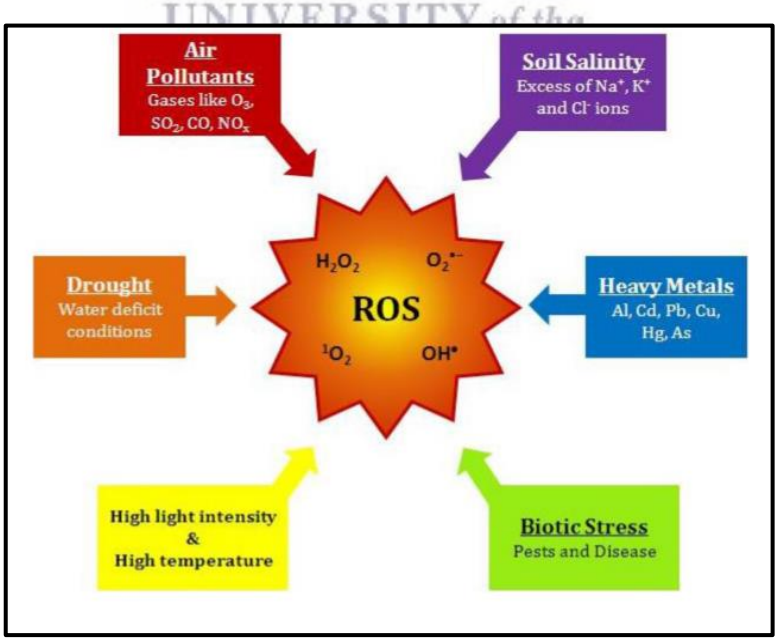

Figure 1.1: Diagram illustrating multiple elements liable for the production of reactive oxygen species (ROS). Mentioned are only a few elements that lead to production of various ROS molecules in various organelles of plant (Das and Roychoudhury, 2014).

Adverse environments trigger the disruption of the delicate balance that exists between the ROS production and ROS scavenging, and the endurance of the plants depends on their proficiency to adapt to variations, likewise depends on their growth patterns. Research approximates that only 1- 2% of oxygen absorption by plants, advances to ROS production (Das and Roychoudhury, 2014). An upsurge in ROS production activates a series of damaging outcomes, for instance extensive damage caused to lipids, DNA and proteins. Reactive oxygen species, such as superoxide, hydrogen peroxide, and singlet oxygen in elevated intracellular concentrations precedes to cellular death due to oxidative stress (membrane lipid peroxidation, protein oxidation, enzyme inhibition and damage to nucleic acids) (Gill *et al*, 2011). The chloroplast in plants exhibits a high sensitivity to ROS molecules largely owing to elevated concentrations of oxygen reacting within the photosynthetic electron transfer system (Wang *et al*, 2005).

1.4.1. Superoxide (O_2^-)

The chloroplast is a major source of ROS production, largely owing to partial reduction of oxygen molecules or owing to the transfer of energy to oxygen (Das and Roychoudhury, 2014). Superoxide radicals (O_2^-) are produced in various energy producing cellular sections, such as the mitochondria, chloroplast, peroxisomes, cytosol and apoplastic space (Agrawal *et al*, 2003). In spite of this, the key development of superoxide radical is exhibited in the thylakoid-localized PSI during the non-cyclic electron transfer chain (ETC). O_2^- is formed when the oxygen molecules reacts with different ETC components (Das and Roychoudhury, 2014). O_2^- is commonly the first ROS to be produced, and further reaction of this radical gives rise to other members of ROS (Das and Roychoudhury, 2014). O_2^- is moderately reactive and has a very short half-life of 2-4 μs. Their moderately short half-life leads to their incompetence to transverse the phospholipid bilayer since it possesses a charge; consequently, their reaction is limited to the close proximity of their production (Bhattacharjee, 2005). Hence, superoxide's inability to initiate extensive damage, as an alternative it undergoes transformation into more toxic hydroxyl radicals (OH^-) and singlet oxygen molecules (O_2) that results in membrane lipid peroxidation (Das and Roychoudhury, 2014).

1.4.2. *Hydrogen Peroxide (H_2O_2)*

Hydrogen peroxide (H_2O_2) is a reasonably reactive ROS molecule. This molecule is produced after O_2^- is reduced (Das and Roychoudhury, 2014). It is also produced in a reaction catalysed by superoxide dismutase (SOD) or the procedure of dismutase to H_2O_2 under low pH conditions. H_2O_2 is mostly produced in peroxisomes during photorespiration (Foyer and Noctor, 2005). The major locations of H_2O_2 production in plants are situated in the ETC in the chloroplast, mitochondria, ER, cell membrane, β-oxidation of fatty acids and photorespiration (Das and Roychoudhury, 2014). The H_2O_2 produced in the mitochondria and chloroplast, is mostly generated from SODs that dismutate O_2^- generated by the electron leakage from the ETCs (Foyer and Noctor, 2005). As all ROS molecules, at low concentrations H_2O_2 is beneficial to the plants, then again at high concentrations it is damaging to the cell. At low intracellular concentration it functions as a regulatory signal that is vital for physiological processes (senescence, photorespiration and photosynthesis, stomatal movement, cell cycle and growth and development). H_2O_2 operates as an effective signalling molecule mainly due to it exhibiting a lengthy half-life of 1 ms and is highly stable (Cheeseman, 2007; Tewari *et al*, 2006). The coupling of its half-life and stability permits this ROS molecule to cross membranes via the aquaporin and to extend considerable lengths within the cell (Cheeseman, 2007; Tewari *et al*, 2006). Consequently at low intracellular concentrations, H_2O_2 is a signalling molecule which induces abiotic stress tolerance in plants (Gill and Tuteja, 2010; Quan *et al*, 2008). At high intracellular concentrations, H_2O_2 oxidises both cysteine (-SH) and methionine ($-SCH_3$) residues and inactivates the Calvin cycle enzymes: Cu/Zn SOD and Fe-SOD, by oxidising the thiol groups within the enzyme structure (Cheeseman, 2007; Tewari *et al*, 2006). High H_2O_2 concentrations in plants, are responsible for programmed cell death (Das and Roychoudhury, 2014). Similar to O_2^-, H_2O_2 is moderately reactive, therefore their damage is only fully realised when converted into more reactive species, OH^- (Das and Roychoudhury, 2014).

1.4.3. *Hydroxyl radicals (OH^-)*

Hydroxyl radicals (OH^-) are small, highly mobile, water-soluble molecules (Ayala *et al*, 2014). OH^- exhibits the greatest toxicity in plants (Das and Roychoudhury, 2014). OH^- is produced at neutral pH levels by means of the Fenton reaction that transpire between H_2O_2 and O_2^- which is catalysed by transition metals; iron (Fe) (Gill and Tuteja, 2010). The OH^- radical

could also be produced from H_2O_2 and O_2^- in the presence of copper ions via the Haber-Weiss reaction (Halliwell and Gutteridge, 1999). A cell produces about 50 hydroxyl radicals every second, amounting to about 4 million produced per day (Ayala *et al*, 2014). These OH^- radicals could be neutral or attack biomolecules (Ayala *et al*, 2014). Plants uptake various transition metals, which aid in metabolic processes, however, the uptake of these metals could lead to the overproduction of OH^-. This ROS molecule has the proficiency to damage various cellular components by lipid peroxidation (LPO), protein damage and membrane destruction (Das and Roychoudhury, 2014). OH^- radicals exhibit the most devastating consequences, mainly owing to no existing enzymatic system that aids in scavenging this toxic radical and excess accumulation of OH^- reacts with macromolecules (proteins, lipids and DNA). This ROS molecule has this ability to react with all biological molecules due to its charge, causing it to be strongly oxidizing ROS (Bhattacharjee, 2005) which leads to cellular death (Gill and Tuteja, 2010). OH^- has a short half-life of 2-4 µs (Vreeburg and Fry, 2004).

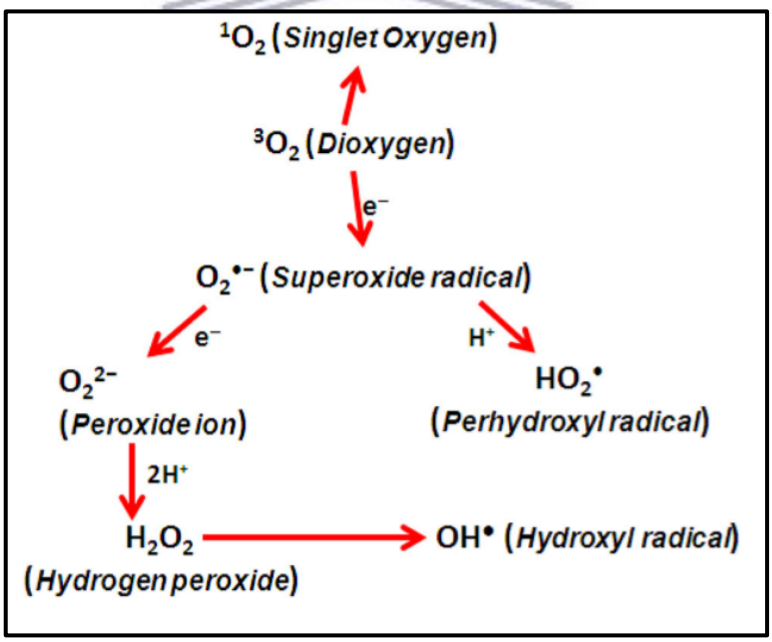

Figure 1.2: Diagram illustrating the production of reactive oxygen species by the transference of energy. All reactive oxygen species and how their production leads to production of other ROS molecules (Das and Roychoudhury, 2014).

To establish preservation of redox homeostasis in plant in adverse environments and essential regulate oxidant levels (Schützendübel and Polle, 2002), the anti-oxidative system is introduced, comprising of antioxidant components (Schützendübel and Polle, 2002). The antioxidant defence system is comprised of enzymatic components (and non-enzymatic

components): superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione-S-transferase (GST) (Das and Roychoudhury, 2014). It is of great necessity that a plant is detoxified of ROS, to ensure cellular survival (Das and Roychoudhury, 2014).

1.5. Causes of oxidative stress in plants

1.5.1. Lipid Peroxidation

Untamed oxidative stress instigates oxidative damage and elevated levels of free radicals or ROS molecules impose damages onto lipids (Ayala *et al*, 2014). The mitochondria, plasma membrane, ER and peroxisomes are the main locations of endogenous ROS. The two highly important ROS molecules, in terms of lipid destruction, are hydroxyl radicals (OH^-) and hydroperoxyl (OH^-_2). OH^- performing a crucial function in the chemistry of lipid peroxidation (Ayala *et al*, 2014). Lipid peroxidation is a process under which free radicals affect the lipid containing carbon-carbon double bonds (polyunsaturated fatty acids, PUFAs). Other lipid victims consist of glycolipids, phospholipids and cholesterol, which undergoes lethal peroxidation modification (Ayala *et al*, 2014).An upsurge in carbonylated proteins and malondialdehyde (MDA) and additionally the escalation of the production of eythlene, consequently transpires due to tissue cells coming about damage by oxidative stress, such as lipid peroxidation (Schützendübel and Polle, 2002). Cell death or to ensure cell endurance is experienced in a cell, in response to lipid peroxidation. Antioxidant defence systems or signalling pathway activations are conducted under low lipid peroxidation conditions, to allow maintenance of cells. This procedure is conducted to up-regulate antioxidant proteins resulting in an adaptive stress response (Ayala *et al*, 2014). In occurrence of medium or high lipid peroxidation, the damage overpowers the repair ability, and consequently a cell will induce apoptosis or necrosis programmed cell death, both which results in pathological states and accelerated aging. The lipid peroxidation process consist of three steps; initiation, propagation and termination. Malondialdehyde, MDA, is one of the main end-products of LPO. MDA production is straightforwardly associated with membrane destabilisation and fusion. The determination of MDA content is a widely used reliable tool to detect oxidative stress hazard, this by estimating the formation of lipid peroxides (Wahsha *et al*, 2012). MDA is the most mutagenic product of lipid peroxidation; consequently it is the most convenient biomarker for lipid peroxidation (Ayala *et al*, 2014).

1.5.2. Damage to DNA

The nuclear DNA within plants are tightly secured by histones and associated proteins, conversely, the mitochondrial and chloroplast DNA does not contain this line of defence, and are therefore open for attack. The mitochondrial and chloroplast DNA stands the brunt of ROS attack. The other cause for these organelle DNA being most susceptible to ROS attack, is due to their relatively closeness to the ROS production machinery (Das and Roychoudhury, 2014). The oxidative damage of DNA does not only happen at one level, it happens at numerous levels. Levels including; oxidation of deoxyribose sugar residue, modification of the nucleotide bases, abstraction of a nucleotide, breaks in either DNA strand or cross-linking of the DNA and protein. OH^- poses several channels of attack, as they damage deoxyribose sugar backbone (extracting H-atom), they possess the ability to react with double bonds of purines and pyrimidine bases (Das and Roychoudhury, 2014). When ROS molecules attack the deoxyribose sugar backbone, they abstract the C-4-H atoms of the deoxyribose sugar to produce deoxyribose radicals. These deoxyribose radicals react to cause single strand breaks in DNA or associated proteins. In terms of cross-linking, this occurrence is not easily reparable and possess the ability to be lethal to the plant cells. (Das and Roychoudhury, 2014).

1.5.3. Chlorosis in plants

Work performed by Pfannschimdt (2003) mentioned that the chloroplast is defined by a methodical system of thylakoid membranes which are of immense significance, considering that they are comprised of the light capturing photosynthetic machinery and contain the necessities for efficient light harvesting (Pfannschimdt, 2003). The photosystems (PSI and PSII), housed within the chloroplasts, are the key locations of ROS productions, by means of o_2^- production which was triggered by abiotic stress. This occurrence usually results in water limitations, carbon dioxide concentrations and productions of additional light (Pfannschimdt, 2003). O_2^- production is brought about by the leaking of electrons from the electron transport chain, ETC, of the PSI. Pfannschimdt (2003) mentioned that the reasoning behind the leakage of electrons is due to the 2Fe-2S and 4Fe-4S clusters. On the other hand, in the PSII, the production of O_2^- is brought about by the seeping of electrons, via the QA and QB electron acceptors (Pfannschimdt, 2003).

The O_2^- produced from either routes mentioned, is converted into OH^- through the H_2O_2 intermediate in the process of the Fenton reaction at the Fe-S centres. Pfannschimdt (2003) stated that the Singlet oxygen molecules are produced in two ways: Environmental conditions, disrupting the delicate balance between light harvesting and energy utilization. This occurrence will essentially lead to the formation of triplet Chl. Singlet oxygen is released when triplet Chl reacts with dioxygen (Karuppanapandian *et al*, 2011). Secondly, singlet oxygen is produced, in the instance when the ETC is over reduced, leading to the light harvesting complex (LHC) at the PSII (Das and Roychoudhury, 2014). Tseng et al (2007) mentioned that the peroxidation of the lipid membrane in the chloroplast is based on the accumulation of singlet oxygen molecules. The P680, which is considered the reaction centre of the PII, is placed in jeopardy, mainly due to damage of membrane proteins. Thus it is of great importance to control and scavenge reactive oxygen species in the chloroplast, to protect the plant cells and ensure survival.

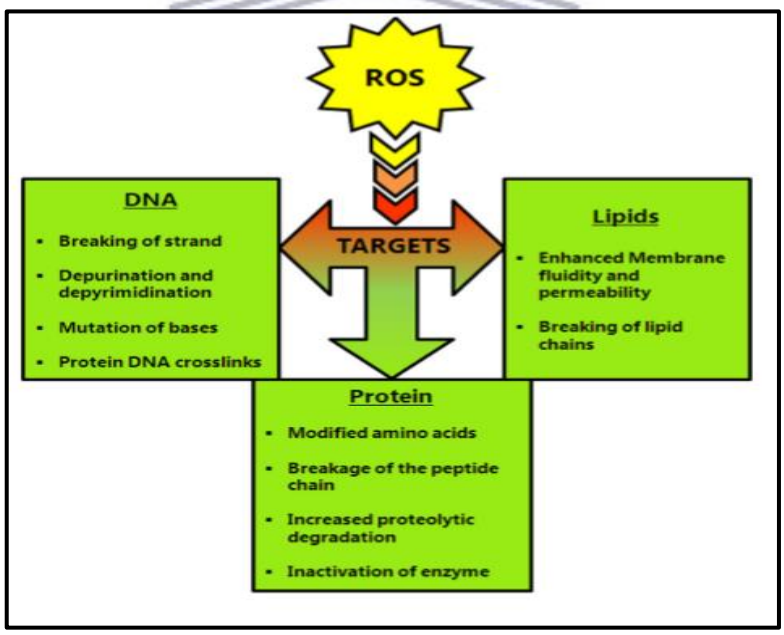

Figure 1.3: Diagram illustrating the multiple focuses of reactive oxygen species damage in plants. ROS molecules target many components of the cell to ultimately result in oxidative damage (Das and Roychoudhury, 2014).

1.6. Antioxidant enzymes in plants

Plants have acquired important control measures, ROS-scavenging pathways, to combat the destructive nature of reactive compounds (Bhattacharjee, 2005). The reactive oxygen species defence system is comprised of antioxidant machineries, assisting in the reduction

of oxidative stress-induced damages (Das and Roychoudhury, 2014). The pathways employed are able to metabolise ROS and thereby decreasing the concentration within plants (Gill and Tuteja, 2010; Mahanty *et al*, 2012). Superoxide dismutase, ascorbate peroxidase and catalase are three of the most important anti-oxidative enzymes, superoxide being the first scavenging enzyme in the antioxidant pathway.

1.6.1. Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) is an anti-oxidative enzyme that belongs to the family metalloenzymes, omnipresent in all aerobic organisms (Das and Roychoudhury, 2014). This anti-oxidative enzyme forms the first line of defence against ROS induced-damages. SOD catalyses the removal of O_2^- by dismutating this ROS molecule to produce O_2 and H_2O_2 (Das and Roychoudhury, 2014). The H_2O_2 molecules produced by the SODs are possibly scavenged by peroxidases; this interaction thus reduces the concentration of ROS (Zang *et al*, 2007). SOD also possesses the possibility to remove OH^- formation via the Haber-Weiss reaction. Superoxide dismutase groups consist of three isozymes, based on the metal ion it binds to or metal cofactor, Mn-SOD (localized in the mitochondria), Fe-SOD (localized in the chloroplast) and Cu/Zn-SOD (localized in the cytosol, peroxisomes and chloroplast). The metal cofactors are essential for proper functioning (Mahanty *et al*, 2012; Wang *et al*, 2005). The Cu/Zn-SOD is discovered to be the most prevalent SOD isoform (Mahanty *et al*, 2012). SOD enzymes exhibit up-regulation in abiotic stress conditions (Das and Roychoudhury, 2014).

1.6.2. Ascorbate Peroxidase (APX)

The anti-oxidative enzyme ascorbate peroxidase (APX) is a fundamental element of the ascorbate-glutathione cycle. Similar to all peroxidases, APX encompasses a heme group which assist the enzymes to perform their respective functions (Verma and Dubey, 2003). Catalase is recognized to primarily scavenge hydrogen peroxide in the peroxisomes; conversely, the same role is accomplished in the cytosol and chloroplast by means of APX (Das and Roychoudhury, 2014). Explained by Schützendübel and Polle (2002) the ascorbate glutathione pathway is of great importance as this pathway is responsible for maintaining ascorbate in its reduced form. Two other approaches are by use of monodehydroascorbate radical reductase (MDAR) and NAD (P)H and by making use of ferredoxin as a reductant.

What occurs in the pathway is the reduction of dehydroascorbate in conjunction with the oxidation of glutathione (GSH), which is sequentially reduced b glutathione reductase by the oxidation of NADPH (Schützendübel and Polle, 2002).

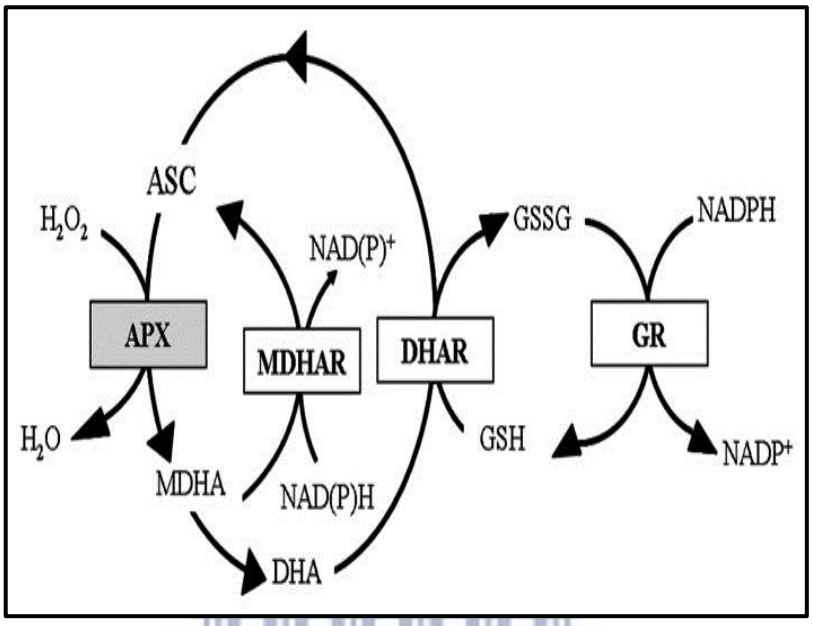

Figure 1.4: Diagram illustrating the Ascorbate-glutathione pathway. Indicates the process of how ascorbate peroxidases scavengers' hydrogen peroxides to reduce the detrimental impacts of hydrogen peroxides on plants (reference).

APX reduces H_2O_2 to form H_2O and converts ascorbic acid to dehydroascobate (DHA) (Sarowar *et al*, 2005; Sinha and Sexena, 2006). Ascorbate peroxidase group consist of five isoforms and these are based on the amino acid and location. APX is known to have a higher affinity for H_2O_2 than catalase has, consequently, being a more efficient scavenger of H_2O_2 in times of stress (Das and Roychoudhury, 2014). An up-regulation of APX in plants accompanies tolerance to abiotic stresses in plant (Kornyeyev *et al*, 2003).

1.6.3. Catalase (CAT)

Catalase (CAT) is a tetrameric, heme-containing enzyme that exercises dismutation reactions to catalyse the reaction, hence, the absence of a reductant molecule (Mhamdi *et al*, 2010). CAT is liable for catalysing the dimutation of H_2O_2 to produce H_2O and O_2 (Das and Roychoudhury, 2014). CAT was one of the original antioxidant enzymes discovered (Mhamdi *et al*, 2010). CAT possesses a high affinity for H_2O_2 and a low affinity for organic peroxides. Due to particular normal reactions, H_2O_2 is highly produced in the peroxisomes

(Das and Roychoudhury, 2014). Catalase is also observed to be located in various other cellular organelles. Catalase aids in the removal of hydrogen peroxide in an energy efficient manner (Das and Roychoudhury, 2014)

1.6.4. Glutathione Reductase (GR)

Glutathione Reductase (GR) is a flavoprotein oxidoreductase. The GR utilizes NADPH as a reductant to reduce GSSG to GSH (Das and Roychoudhury, 2014). The problem arises, when reduced glutathione (GSH) is depleted to restore AA from MDHA and DHA, thus resulting in its conversion to its oxidized form (GSSG) (Das and Roychoudhury, 2014). In the ascorbate glutathione pathway, the anti-oxidant enzyme glutathione is of great importance as it is essential for the catalysing of the disulphide bond to uphold an elevated GSH/GSSH ratio. This anti-oxidant enzyme is localised mainly in the chloroplast and in minute concentration, in the mitochondria and cytosol (Yadav, 2010). The most essential function of all is that is a reductant that prevents the thiol groups from oxidizing. Once these oxidize they are able to react with reactive oxygen species (O_2^- and OH^-) (Yadav, 2010).

1.7. Justification

Despite the fact that the industrial sector continues to expand, the anthropogenic activities currently employed are adversely influencing agricultural lands and the ecosystems, which are vastly being wasted. Escalation of heavy metals being introduced into agricultural lands and additionally the lands surrounding these areas are of particular danger as this provides a mode of entry into the food chain, thus putting human's health at risk. For that reason it is of great importance to obtain information on stress-tolerant plants, to aid in the re-cultivation of tainted land and the recovery of industrial sites, for instance mines, which are contaminated by heavy metals. In South Africa, especially the province of Limpopo, it is of great importance that knowledge of plants tolerant to antimony is obtained. In the present day, biotechnological advances are being conducted to enhance the plants stress tolerance and in conjunction to remove contaminants from the soil. The aim, in the end, is using these plants for soil clean-up. It is of immense importance to apprehend the fundamental principles as regards to how the contaminants are absorbed and, especially, at how the plants respond to this absorption on a cellular and tissue level. This information obtained

will aid in the guidance of developing novel plans for phytoremediation and increased tolerance of plants.

Five cultivars of *Phaseolus vulgaris* will be screened on five different concentrations of antimony to establish a cultivar illustrating a degree of tolerance and another cultivar illustrating a degree of susceptibility to antimony. Beans being an essential crop plant are of great importance in South Africa as this plant is concentrated in proteins and carbohydrates that are essential in developing countries, such as South Africa. Beans are high in minerals and vitamins, and are an especially good source of Iron in comparison to other staples. It is for that reason that this investigation on *Phaseolus vulgaris* is of immense importance, as evidence obtained will be beneficial in providing food security of beans for human consumption, and to gain knowledge for plant breeding strategies, to produce highly tolerant bean crops.

CHAPTER 2

MATERIALS AND METHODS

Abiotic stresses, such as high concentrations of heavy metals within the environment, are one of the severest threats experienced by plants, mainly owing to the plants inability to evade the stress imposed upon them. The plants are, however, able to recognize the abiotic stress and thereby respond by altering their metabolism and growth rate. It was discovered that agricultural land was slightly contaminated with metals. This discovery was of no concern, as plants needed trace amounts of certain metals to survive. The problem developed when plants were exposed to high concentrations of heavy metals.

The first objective of this study was to fully understand the effects of antimony on both cultivar's physiology and biochemistry and thus utilized to understand each cultivar's defence systems towards this abiotic stress. This was conducted to ultimately distinguish which cultivar illustrated a degree of tolerance and which cultivar illustrated susceptibility to antimony stress. Once the cultivars ROS production were analysed, acquiring the antioxidant activity was beneficial in understanding how effective each cultivar's (Contender and Timbavati) defence mechanisms were, under antimony stress.

The nutrient and elemental composition of common beans are of immense importance, because that is what makes it especially important for low-income populations. Therefore, conducting ICP and nutrient analysis on the various treated plants was conducted to achieve in obtaining profiles (of each cultivar, in each treatment) to understand exactly how the treatments influenced the uptake, translocation of elements and nutrients. Thus the ultimate objective was to fully understand what made certain cultivars more tolerant than others.

2.1. List of chemicals and suppliers

Table 2.1: The list of all chemicals utilized in the current investigation.

Chemical/ Reagents	Supplier
2-Deoxy-D-Ribose	Sigma-Aldrich
2-Thiobarbituric acid	Sigma-Aldrich
Acetone	Sigma-Aldrich
Contender seeds	Starke Ayres
Dipotassium hydrogenphosphate	Sigma-Aldrich
EDTA	Sigma-Aldrich
Eppendorf Tubes (1.5 ml)	Sigma-Aldrich
Ethanol 200 Proof	Sigma-Aldrich
Evans Blue	Sigma-Aldrich
Filter Sand (Silica sand)	Cape Silica
L- Ascorbic acid	Sigma-Aldrich
Liquid Nitrogen	University of the Western Cape, Chemistry department
Nitric Acid	Sigma-Aldrich
Nitro blue tetrazolium chloride monohydrate	Sigma-Aldrich
Opaque Cups (~500 ml)	Crazy Plastics, Bellville (Cape Town)
Potassium antimony tartrate trihydrate	Sigma-Aldrich
Potassium iodide	Sigma-Aldrich
Potting soil	Stodels, South Africa
Quick start Bradford's dye reagent 1X	Bio-Rad
Sodium dodecyl sulphate (SDS)	Bio-Rad
Sodium Hydroxide	Sigma-Aldrich
Timbavati seeds	MayFord
Trichloroacetic acid 99%	Sigma-Aldrich

2.2. Preliminary Results

In preparation for this study, a preliminary assessment was conducted on five cultivars of *Phaseolus vulgaris L.* plants, namely Lazy Housewife, Witsa, Contender, Timbavati and Bush beans. The five cultivars were treated with five different antimony concentrations [0 µM, 50 µM, 150 µM, 350 µM and 500 µM] and treatments were conducted twice a week, for three weeks (21 days). The results obtained from the preliminary screening indicated no significant difference between the concentrations (50 µM, 150 µM, 350 µM), when in comparison to the control (0 µM). The only difference significant enough was experienced between the control (0 µM) and 500 µM of antimony solution, thus resulting in the treatments selected for further analysis. Studying the cultivars, two cultivars (Contender and Timbavati) was selected to be studied further, as Contender fared better and Timbavati showed a greater degree of damage, when compared to the Contender cultivar. Therefore, to study why they react differently they were further analysed and their response to antimony stress recorded.

2.3. Growth parameters

In this study, two cultivars of *Phaseolus vulgaris L.* (Contender and Timbavati) were planted separately, within opaque plastic cups (~500 g of soil capacity) with efficient drainage. Prior to the planting, the seeds were not sterilized (as there were some complications with sterilization). Each cup housed one seed, planted 3 cm deep, in soil containing 1:3 ratio of Silica sand to Potting soil respectively. The plants were all treated with 100 ml of deionised water and allowed to grow until the two leaf phase (~ 21 days). In preparation for the antimony treatment, the potassium antimony tartrate trihydrate powder was dissolved an appropriate volume of water, to produce a 500 µM antimony solution. Plants illustrating similar morphology (height, leaf size and leaf distribution (two leaf phase)) were selected for further antimony treatment. At the two leaf phase plants were ready to be treated with the antimony concentration. Half of the plants were treated with 100 ml of 500 µM antimony whilst the others continued to be treated with 100 ml of deionised water and served as the control plants. Treatments were conducted twice a week for a period of 3 weeks (21 days).

2.4. Biomass determination

At the completion of the treatments, some of the plants (four plants of each cultivar, per treatment) were carefully removed from their respective cups and rinsed. The plants were sectioned off for their separate plant biomass determination. The roots samples of the plants were acquired by cutting at the location where the root ends and the stem begins. A leaf sample from each trifoliate (three trifoliate) on the plant was acquired by cutting at the foot of the leaf, where it is attached to the branch. The sections acquired were enclosed into separate foil packages. The foil packages were weighed using a fine mass balance before the sections were placed in, and these readings were recorded. Appropriate drainage, to allow moisture to seep out, was provided by prodding holes into the foil packages. The samples were placed into an incubator and dried at 80°C for 12 hours. Once the drying period concluded the foil packages were weighed using a fine mass balance and the readings were readings were recorded.

2.5. Harvesting of cultivars

Some of the other plants were carefully removed from their respective cups and placed into water to thoroughly wash off all soil materials that were adhering to the plant. The plants were dried and sectioned off (three trifoliate, roots and stems). Each section from a cultivar was separately ground in liquid nitrogen using a mortar and pestle. The spatula used to transfer the frozen ground up plant materials, was frozen in liquid nitrogen to assist in conserving plant material during transfer into greiner tubes. The frozen ground up plant material was placed in frozen 50 ml greiner tubes and stored at -80°C for future use.

2.6. Cell Death assay (Evans Blue assay)

A method adopted from Gokul, 2013 was employed for the analysis of the Evans blue assay. To several Eppendorf tubes, 1 ml of 0.25% (w/v) Evans blue solution was added. Using some of the remaining fresh plant material, 1 cm^3 blocks were exercised from the leaves of each trifoliate of the two cultivars. These blocks were placed separately into Eppendorf tubes containing Evans blue solution. The root samples were exercised by cutting 2 cm lengths from the tip of the roots and these were placed into Eppendorf tubes containing Evans blue solution. The plant samples were incubated within the Evans blue solution for an hour at room temperature (±21°C). After the incubation period, the Evans blue solution was

carefully rinsed off with water and the samples were incubated in water, for 12 hours. After water incubation, the water was decanted and 1 ml of 1% SDS solution was added into the Eppendorf tubes. The samples were crushed in the SDS solution using a miniature pestle. Once thoroughly crushed the samples were incubated on a heating block at 65°C for an hour. After the incubation period, the samples were subjected to centrifugal force to pellet the plant material and to acquire a supernatant. The supernatants acquired were loaded into the wells of a microtitre plate and read at a wavelength of 600 nm on a spectrophotometer.

2.7. Chlorophyll a and b determination

A method adopted from Gokul, 2013 was employed to conduct the chlorophyll assay. Frozen ground leaf material (100 mg) was used from both antimony treated plants and the control plants of both cultivars. This material was placed into 1.5 ml Eppendorf tubes. To provide protection to the light sensitive chlorophyll in the plant material, the Eppendorf tubes were concealed in foil. To the Eppendorf tubes, ten volumes (1 ml) of 100% (v/v) acetone were added and the contents of the tubes were vortex to mix. Once mixed, the samples were loaded into the wells of a microtitre plate, in triplicates, and read on a spectrophotometer at the wavelengths 662 nm and 644 nm respectively. The readings obtained were then used to determine the chlorophyll species concentration by using a specific calculation.

2.8. Superoxide content determination (Spectrometric assay)

A method adopted from Gokul, 2013 was employed to determine and analyse the superoxide content within the plants. The remaining plants were used as fresh weights for this assay. In preparation for the assay, a solution was prepared in an Eppendorf tube, to a final volume of 800 µL with 50 mM potassium phosphate (pH 7.0), containing 10 mM KCN, 10 mM H_2O_2, 2% SDS and 80 µM NBT. Into Eppendorf tubes containing the solution, eight 1 cm^3 fresh leaf materials were carefully placed. Into separate Eppendorf tubes containing the solution, 4 cm of the roots were placed. All the plant samples were then incubated for 20 minutes at room temperature (±21°C). After the incubation period, the plant material was crushed using a miniature pestle. The contents of the tube were then subjected to centrifugal force of 13 000 rpm's for 5 minutes to pellet the plant material. The supernatant

acquired was transferred into a clean Eppendorf tube. On a clean microtitre plate, 200 µL of the sample was loaded into the wells. The samples were read at a wavelength of 600 nm. Observing the microtitre plate, this alone could be used as an estimation of which cultivar contained the highest superoxide levels. The blue tint aids in quantifying superoxide radicals, and this tint is generated when formazan is produced from the reduction of NBT. Through the use of the extinction coefficient, 12.8 mM. cm^{-1}, a calculation was used to determine the superoxide content.

2.9. Hydrogen peroxide content determination (Spectrometric assay)

A method adopted from Gokul, 2013 was employed to determine and analyse the H_2O_2 content of the plant materials. The standards were prepared for the assay by diluting calculated volumes of H_2O_2 in distilled water (0 nM, 5000 nM, 10000 nM, 15000 nM, 20000 nM and 25000 nM). After the standards were prepared, they were loaded into the wells of a microtitre plate, in triplicate. The samples used in this assay, were prepared by using a TCA extraction completed on frozen ground material (section 2.10.). Onto the microtitre plate, 50 µL of the TCA extraction was loaded into the wells. To both the samples and the standard, 1.25 mM dipotassium hydrogenphosphate (K_2HPO_4) and 250 mM potassium iodide (KI) were added. Once all the reagents were added to the appropriate wells, the plate was incubated on a shaker for 20 minutes at room temperature (±21°C). The samples were read at the wavelength of 390 nm.

2.10. Hydroxyl Radical determination (Spectrometric assay)

A method adopted from Halliwell and colleagues in 1987, was employed to determine and analyse the hydroxyl radical content within the second trifoliate of both *Phaseolus vulgaris L.* cultivars. 50 mg of frozen ground up leaf material were used of both cultivars and placed into separate 1.5 ml Eppendorf tubes. To these tubes 1 ml of 10 mM phosphate buffer (pH 7.4), containing 15 mM 2-Deoxy-D-Ribose., was added. The tubes, containing the samples, were then incubated for 2 hours at 37°C. After the incubation period, 0.7 ml of the sample was transferred into another tube containing 3ml of 0.5% (w/v) Thiobarbituric acid, which was made up in 5 mM sodium hydroxide. The samples were vortex and then incubated for 30 minutes at 100°C. After the incubation period has commenced, the samples were incubated for 5 minutes on ice. The tubes were then centrifuged for 5 minutes at 10000

rpm's and the supernatant was recovered and 200 µL was loaded into the wells of a microtitre plate. The samples were read at wavelengths of 532 nm and 600 nm. The absorbance at 600 nm was subtracted from the absorbance obtained at 532nm. The hydroxyl radical content was then calculated using the extinction co-efficient of 155 $mM.cm^{-1}$.

2.11. Determination of Lipid peroxidation by quantifying MDA

A method adopted from Gokul, 2013 was employed for the analysis of the lipid peroxidation. Into 1.5 ml Eppendorf tubes, 100 mg of the frozen ground leaf materials were placed. Five volumes (500 µL) of 6% Trichloroacetic acid (TCA) were added into the Eppendorf tubes. The Eppendorf tubes were vortex and then subjected to centrifugal force of 13 000 rpm's for 10 minutes to pellet the leaf material. The supernatant (200 µL) was transferred into a new Eppendorf tube and 300 µL of 0.5% (w/v) thiobarbituric acid (TBA) was added. The contents of the Eppendorf tubes were vortex and parafilm was wrapped around the lids of the tubes to ensure the cap would not open during incubation. The tubes were incubated on a heating block at 90°C for 20 minutes. After the incubation period, the samples were incubated on ice for 10 minutes. Once this period was completed, the samples were subjected to centrifugal force of 13 000 rpm's for 5 minutes. The supernatants were then loaded into the wells of a microtitre plate, in triplicates, and read on a spectrophotometer at wavelength of 532 nm as well as 600 nm. The absorbance at 600 nm was subtracted from the absorbance obtained at 532 nm to correct for non-specific turbidity. The MDA values were then calculated using the extinction co-efficient 155 $mM.cm^{-1}$.

2.12. Protein extraction

The protein extraction was done in triplicates (technical replicates) from both controls and antimony treated leave and root materials of plants. Leaf material and root material (200 mg) were placed separately into three individual Eppendorf tubes. A 0.5 ml protein extraction buffer [0.004 M phosphate buffer, 1 mM EDTA and 5% (w/v) PvP] was added to one of the three Eppendorf tubes. The contents in the Eppendorf tube were vortex and then subjected to centrifugal force at 12 000 rmp's for 5 minutes. The supernatant was transferred into the second Eppendorf tube containing 200 mg of frozen plant material

(either frozen ground leaf material or frozen ground root material) and the previous steps were repeated for the second and third tubes. The supernatant obtained from the third tube was transferred into a clean Eppendorf tube and the protein concentrations were then quantified using Bradford's assay. Finally, these protein samples were stored at -20°C, until further use.

2.13. Superoxide dismutase assays

2.13.1. Determination of Superoxide dismutase activity

A method adopted from Gokul, 2013 was employed in the analysis of the SOD activity, within the leaves of the Contender and Timbavati cultivars. The protein samples obtained were quantified using the Bradford's assay (section 2.11.). These quantified protein samples were diluted to 1 $mg.ml^{-1}$ and 10 µL of this dilution was loaded into the wells of a microtitre plate. To the samples in the wells, 20 mM phosphate buffer, 0.1 mM NBT, 0.005 mM riboflavin, 10 mM methionine and 0.1 mM EDTA were added and this was prepared to a final volume of 200 µL with distilled water. The samples on the microtitre plate were incubated for 20 minutes at room temperature (±20°C), on a light box. Once the incubation period passed, the microtitre plate was then read at 560 nm on a spectrophotometer. Using a calculation the SOD activity was then determined. This calculation indicated what quantity of SOD vital to produce the inhibition of a 50% decrease in the decline of NBT to formazan.

2.13.2. In-gel determination of Superoxide Dismutase isoforms

2.13.2.1. In-gel preparation

10% Acrylamide gels were prepared to determine the isoforms of Superoxide Dismutase. After gel preparation, the gels were stored in water at 4°C until future use.

2.13.2.2. Running of the gels

The gels were set up and 80 µg/ml protein was loaded of each sample. The gel was run at a voltage of 65 V for 45 minutes and then the voltage was increase to 80 V until the gel front has reached the bottom of the gel. The gels were then stained and viewed under light to expose the bands.

2.14. Determination of Ascorbate peroxidase activity

A method adopted from Gokul, 2013 was employed in the analysis of the APX activity, within the leaves of the Contender and Timbavati cultivars. The protein samples (section 2.11.) were transferred into separate 1.5 ml Eppendorf tubes and along with an Eppendorf tube containing 2 mM ascorbate they were incubated for 5 minutes in a waterbath. Protein samples (10 µL) were loaded into the wells of a microtitre plate, in triplicate, and 71.43 mM K_2HPO_4 and 0.36 mM ascorbate was added to the samples within the wells of the microtitre plate. Finally, 0.714 mM H_2O_2 was added to the samples and the microtitre plate and the solution in each well was made to a final volume of 200 µL with distilled water. The microtitre plate was immediately read at 290 nm on a spectrophotometer. The APX activity was determined by using a calculation and the extinction coefficient 2.8 $mM.cm^{-1}$.

2.15. Determination of Catalase activity

A modified method adopted from Aebi, 1984 was exercised in the analysis of the CAT activity, within the leaves of the Contender and Timbavati cultivars. The protein samples (section 2.11.) were quantified using Bradford's assay. A 1 ml reaction mixture was prepared [100 Mm K_2HPO_4 (pH 7.0), 0.5 mM EDTA, 1 Mm H_2O_2 and 20 µL quantified protein sample] and the absorbance of the reaction mixture was read at 240 nm. Employing an extinction coefficient of 39.4 $mM.cm^{-1}$, the CAT activity was determined.

2.16. Elemental analysis using ICP-OES

2.16.1. Sample digestion

An approach adopted from Zarcinas and colleagues, 1987 was applied for the acid digestion of the frozen, ground plant material. Frozen ground leaf (200 mg) and root material (200 mg) were weighed and placed in separate 1.5 ml Eppendorf tubes. To these tubes 65% Nitric acid was added and the caps of the Eppendorf tubes were wrapped in parafilm, to prevent cap opening during incubation period. The tubes were incubated on the heating block at 90°C for 3 hours, with 15 minute check-ups, to digest the leaf and root material.

2.16.2. Digest filtration

Syringes with filters were placed at the opening of 50 ml greiner tubes, and 9 ml of 2% nitric acid was added to the syringe, followed by 1 ml of the digested sample mentioned in section 2.16.1. The digest was filtered into the greiner tubes and these samples were then subjected to ICP-OES analysis.

2.17. Statistical analysis

All experiments in this study were repeated three times for repeatability and reproducibility purposes. All the plant material mentioned in the study was collected from four plants per treatment. In this study, excluding the biomass determination, growth periods and superoxide dismutase in-gel assays (for which only biological replicates were conducted), all other assays conducted in this studies consists of three biological and technical repeats.

CHAPTER 3

The effects of antimony concentrations on the physiology and biochemical characteristics of two *Phaseolus vulgaris L.* cultivars

Abiotic stresses, such as high concentrations of heavy metals within the environment, are one of the worst threats experienced by plants, mainly due to the plants inability to evade the stress imposed upon them (Tuteja *et al*, 2009; Bhatnagar-Mathur *et al*, 2008). The plants are, however, able to recognize the abiotic stress and thereby respond by changing their metabolism and growth rate (Wani *et al*, 2007). It was discovered that agricultural land was slightly contaminated with heavy metals. This discovery is of no concern, as plants need trace amounts of certain metals to survive (Rascio and Navari-izzo, 2011; Vachirapatama *et al*, 2011). The problem arises when plants are exposed to high concentrations of heavy metals.

The mining industry in South Africa is of great importance as it is responsible for a major part of the countries income (Gzik *et al*, 2003). In South Africa, the Limpopo province is recognised to produce the largest quantity of antimony. The production of antimony happens to be profitable for the economy of the country, however, environmentally it is detrimental. Antimony accumulation within the environment may conceivably be lethal to plants cultivated within these areas (Saco *et al*, 2013; Vachirapatama *et al*, 2011). *Phaseolus vulgaris L.* plants are of great importance, worldwide as it is the second most consumed legume crop, following soybean (Gjorgieva *et al,* 2013). Thus, it was essential to assess how these crops react to antimony stressed environments within South Africa (Gjorgieva *et al*, 2013. In plants, Sb is found to increase the peroxidation of membrane lipids and encourage the antioxidant systems in plants (Vaculik *et al*, 2015). Antimony contamination in plants also accounts for DNA damage (Vaculik *et al*, 2015). The reduction in yields is due to the disruption of plant metabolism by reactive oxygen species (ROS).

To combat abiotic stresses, plants have generated a signalling network that makes use of multiple growth regulators that would offer protection to the stress. An increase in ROS is one of the responses to abiotic stresses (Bhattacharjee, 2011). ROS is generated in response to the pants interaction with heavy metals, through the Harber-Weiss reaction

(Yadav, 2010). ROS compounds include: superoxide, hydrogen peroxide and hydroxyl radicals (Sinha and Saxena, 2006). Under normal conditions ROS are produced as by-products, however, under stressful conditions the production of ROS is increased to levels where they are detrimental to the plants (Bhhattacharjee, 2011; Gill and Tuteja, 2010). The reactive molecules in plants are used as signalling molecules; however, accumulation is detrimental as they promote cellular damage (Zhang *et al*, 2007; Wang *et a*l, 2005). Therefore, the accumulation of ROS results in damage to proteins, lipids, carbohydrates and DNA which would lead to cellular death. Chloroplast is said to be very sensitive to ROS, as high concentrations of oxygen interferes with the photosynthetic electron transfer system (Wang *et a*l, 2005). ROS accumulation is thought to be a result of the disruption in the balance of ROS production and the antioxidation systems (Zhang *et al*, 2007).

RESULTS

3.1. The effects of antimony concentrations on the physiological characteristic and the biomass of two *Phaseolus vulgaris L.* cultivars

In this study, plant materials were acquired after a three week treatment period (section 2.3). A reduction in leaf and root length was observed for both *Phaseolus vulgaris L.* cultivars (Figure 3.1.1, 3.1.2 and 3.1.3). The leaves of the antimony treated Contender cultivars illustrated that the biomass measurements were significantly the same, when compared to their controls. In the Timbavati cultivar, a significant reduction of ~51.97% was recorded in the biomass, when comparing to the controls (Figure 3.1.4). The roots for the Contender cultivar represent the same findings as seen in the leaves, where there is no significant difference in their biomass (Figure 3.1.4). The opposite is once again observed in Timbavati, in which this cultivar experiences a drastic reduction (~74%) in the root biomass, in comparison with its control.

Figure 3.1.1: The influences of antimony concentration on the Contender cultivar. The three plants on the left were treated with antimony and the three on the right served as the controls.

Figure 3.1.2: The influences of antimony concentration on the Timbavati cultivar. The three plants on the left were treated with antimony and the three on the right served as the controls.

Figure 3.1.3: Physiological comparison of two cultivars of Phaseolus vulgaris L. under antimony concentration. (Cont) Signify the Contender cultivars treated and (Tim) signify the Timbavati cultivars. The antimony treated plants are depicted on the left and the controls are depicted on the right.

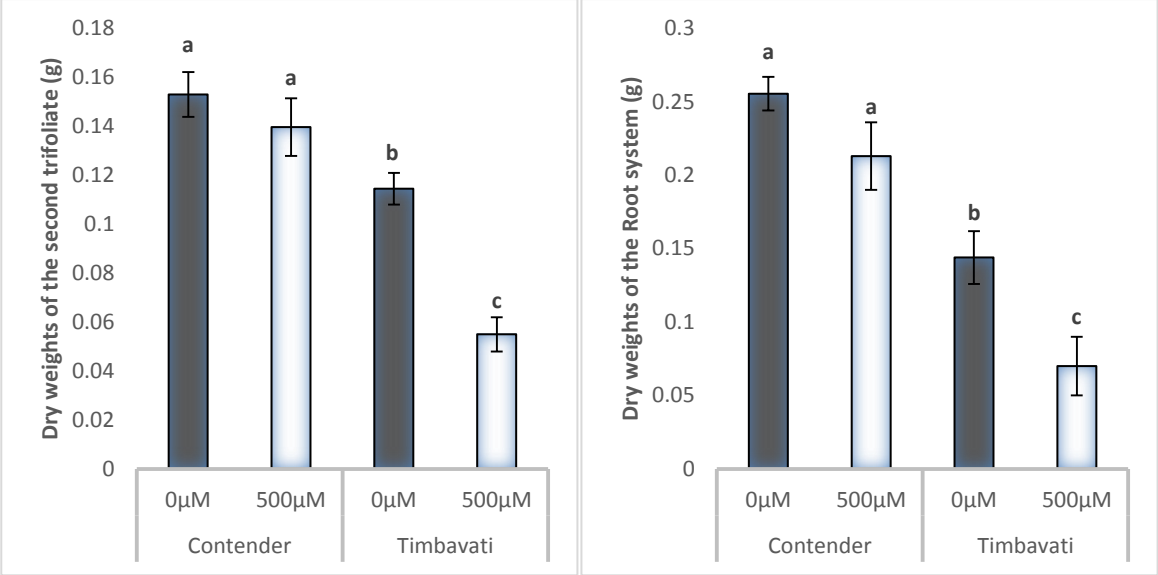

Figure 3.1.4: The influences of antimony on the biomass of two *Phaseolus vulgaris L.* cultivars. Antimony was administered to the two cultivars and the biomass, of the leaves and roots, were determined. The Different letters indicate the significant differences *between the means at P< 0.05 (DMRT). The values are means ± S.E (N=12).*

3.2. The effects of antimony concentration on the cell death of two *Phaseolus vulgaris L.* cultivars

Plants experiencing heavy metal stress have been recorded to respond to these adverse conditions by increasing the generation of ROS molecules. Owing to this response mechanism, excessive production of these reactive molecules tend to damage essential components of the plant cells (for instance; lipids, proteins and nucleic acid molecules) which ultimately could result in cell death (Maruta *et al*, 2012; Wang *et al*, 2005). On a whole, this main outcome of cell death could be used as a marker for metal toxicity. In the cell viability assay (section 2.6), Evans blue solution was used as an indicator to detect non-viable cells. The reasoning behind this was because Evans blue solution could only be taken up by plants whose membranes were disrupted (dead cells). The two *Phaseolus vulgaris L*. cultivars were exposed to three week of antimony treatments and further processed (Section 2.3 and 2.6). The leaves from the Contender plants, treated with antimony, showed an increase of ~21.51% in cell death, whereas the leaves from the Timbavati plants, treated with antimony, showed an increase of ~28.68%, when compared to their controls (Figure 3.2). Within the roots, Contender illustrated an ~18.17% increase in cell death, when compared to the control and Timbavati experienced a ~67.67% increase in the roots when compared to the control.

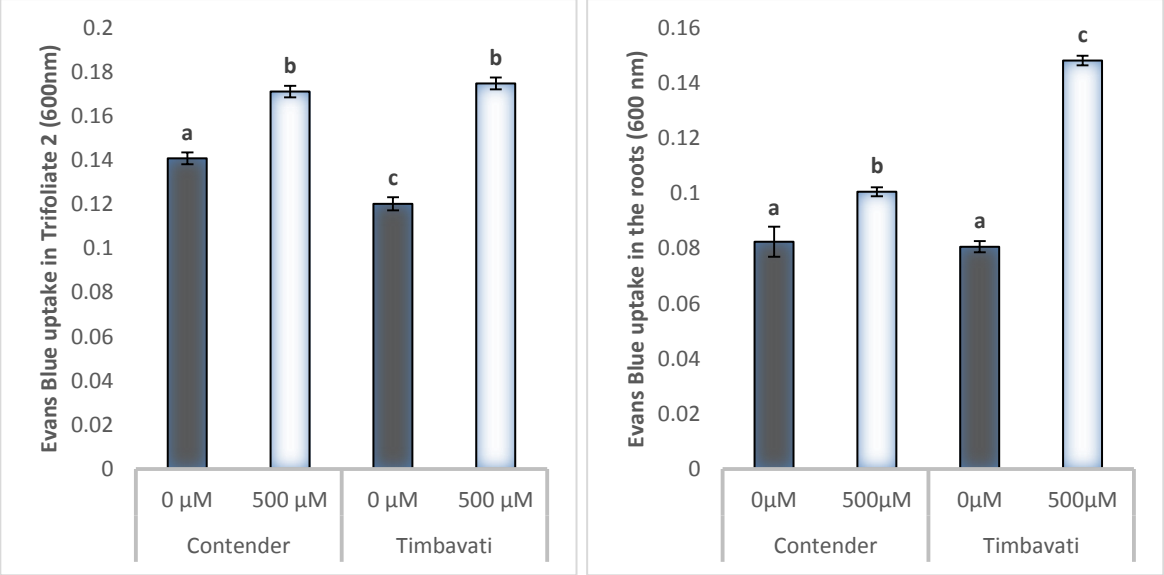

Figure 3.2: The influence of antimony on the cell death of two *Phaseolus vulgaris L.* cultivars. Antimony was administered to two *Phaseolus vulgaris L.* cultivars and the cell death, within the leaves and roots, were determined. The Different letters indicate the significant differences between the means at P< 0.05 (DMRT). The values are means ± S.E (N=12).

3.3. The effects of antimony concentration on the chlorophyll a and b content in two *Phaseolus vulgaris L.* cultivars

Within plants, the photosynthetic system is recognised to be highly sensitive, and literature provides evidence to show that heavy metals tend to inhibit this process by varying degrees depending on the metal (Sheetal *et al*, 2015). Wang and colleagues, 2005 reported that under high concentrations of heavy metals, an inhibition of different species of chlorophyll (*a* and *b*) as well as an effect on the total chlorophyll occurred. A decrease in chlorophyll could affect plant growth and as well as be an indicator of sensitivity to a particular stress, therefore it is important to study the chlorophyll content of plants under antimony stress (Table 3.1). In both cultivars a decrease was experience in both chlorophyll *a* and *b* species and total chlorophyll content. Chlorophyll *b* species exhibited the largest decrease. The Contender cultivar experienced a ~60% decrease in total chlorophyll content and the Timbavati cultivar experience ~88% decrease, when comparing each of the plants to their controls (Table 3.1).

Table 3.1: The effects of antimony on the plants chlorophyll *a* and *b* species as well as total chlorophyll content ($mg.g^{-1}$).

Trifoliate 2 (Fresh weight)			
	Chlorophyll *a*	**Chlorophyll *b***	**Total Chlorophyll**
Timbavati 0 µM	0.225 ± 0.008^{a}	0.3999 ± 0.020^{d}	0.634 ± 0.022^{f}
Timbavati 500 µM	0.178 ± 0.008^{b}	0.325 ± 0.053^{d}	0.546 ± 0.065^{g}
Contender 0 µM	0.232 ± 0.001^{a}	0.421 ± 0.002^{e}	0.654 ± 0.002^{f}
Contender 500 µM	0.211 ± 0.016^{c}	0.359 ± 0.026^{d}	0.594 ± 0.044^{g}

The different letters indicate the significant differences between means at P< 0.05 (DMRT). The values are means S.E (N=4).

3.4. The effects of antimony concentrations on the superoxide content *within Phaseolus vulgaris L.* cultivars

As previously stated, superoxide, has the ability to bring about oxidative damage within both plants and animals (Gokul, 2013). O_2^- molecules are important, as they possess the ability to promote the generation of other ROS molecules, especially H_2O_2 that is extremely damaging and toxic if it accumulates within plants (Gokul, 2013). Due to the location of O_2^- production (electron transport systems), this ROS molecule is of utmost importance. Thus it is of great necessity to determine how antimony stress affected the O_2^- content within the *Phaseolus vulgaris L*. cultivars (Gokul, 2013). Two *Phaseolus vulgaris L.* cultivars were subjected to antimony for 21 days. After this period the fresh material of the leaves were used for O_2^- assays (Section 2.8). There was a ~25.44% increase in superoxide content in the Contender cultivar leaves, while there was a ~45.77% increase in the superoxide content of the Timbavati cultivar leaves (Figure 3.3) when compare to their respective controls. Within the roots Contender illustrated a ~35.94% increase in superoxide content when compared to the control and in Timbavati roots a ~33.50% increase was observed when compared to the control.

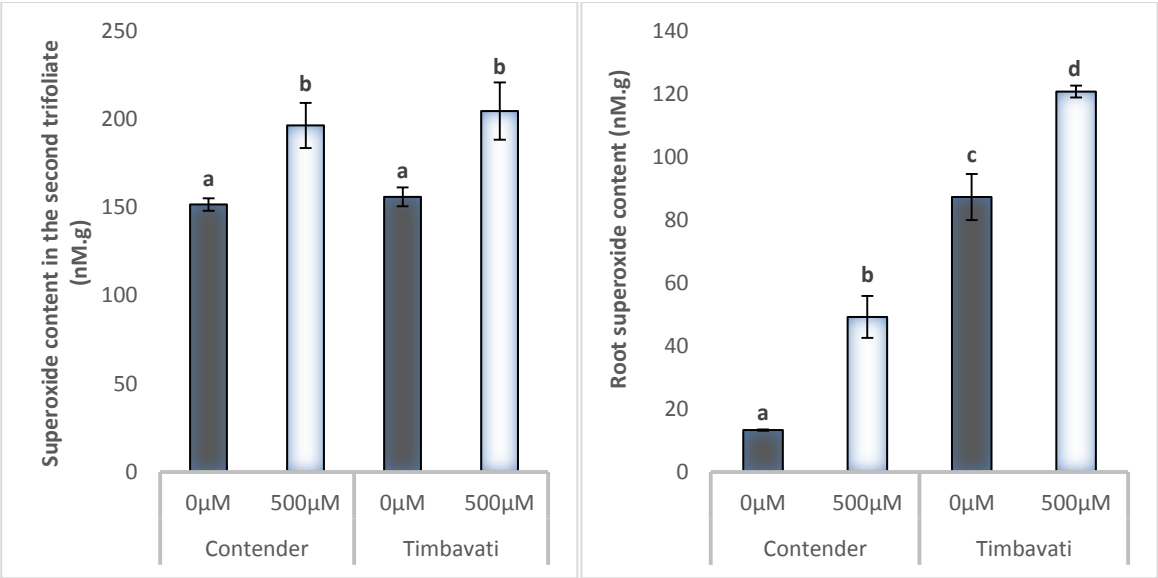

Figure 3.3: The influences of antimony on the superoxide content of two *Phaseolus vulgaris L.* cultivars. Antimony was administered to two cultivars and the superoxide content, within the leaves and roots, were determined. The Different letters indicate the significant differences between the means at P< 0.05 (DMRT). The values are means ± S.E (N=12).

3.5. The effects of antimony concentrations in hydrogen peroxide content within *Phaseolus vulgaris L.* cultivars

Hydrogen peroxide (H_2O_2) is recognized to serve as an essential signalling molecule. This molecule has several characteristics that explains its importance, such as, relatively low toxicity levels, a relatively long life span and has the seamless ability to cross cellular membranes (Cuypers *et al*, 2016). However, when the balance between the production and scavenging of H_2O_2 is disturbed, the plant will experience a stress (Cuypers *et al*, 2016). It is essential to understand the effects of antimony on H_2O_2 generation, because increased the H_2O_2 content is understood to be in conjunction with the increase in oxidative damage in plants. The cultivars were exposed to antinomy treatments for three weeks and sampled by grinding to a powder in liquid nitrogen. The frozen ground plant materials where further processed (Section 2.9). The Contender cultivar treated with antimony exhibited no difference in H_2O_2 content within the leaves, compared to its control (Figure 3.4). The Timbavati cultivar exhibited a ~24.55% increase in H_2O_2 content within the leaves (Figure 3.4), when compared to the controls. Within the roots of the Contender cultivar, their H_2O_2 content was remained unchanged, when compared to the control. In the Timbavati roots a ~14.1% increase was experienced when compared to the control.

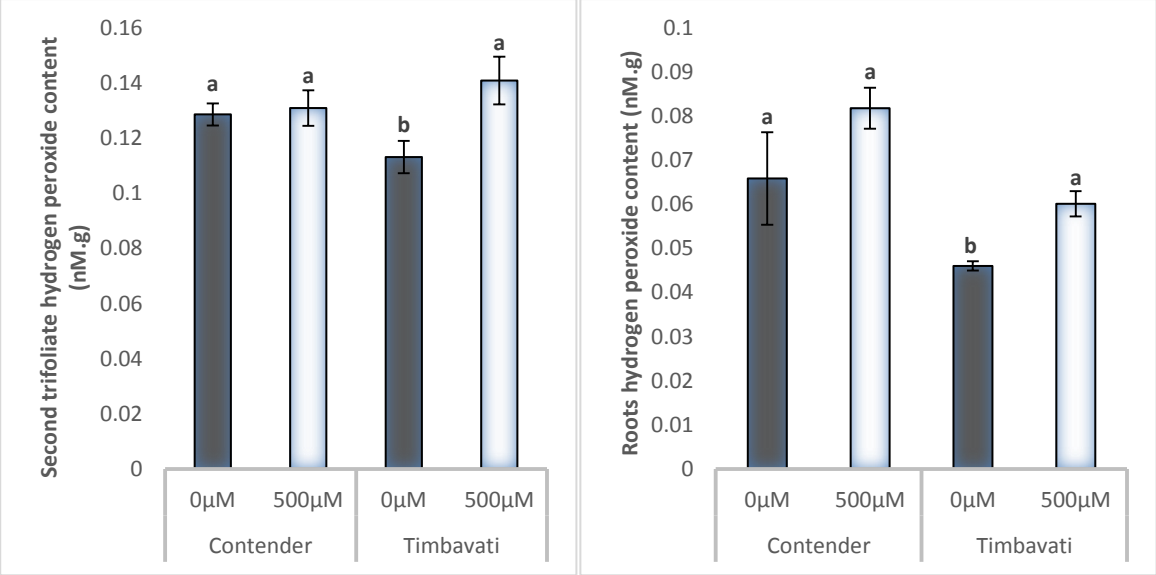

Figure 3.4: The influences of antimony on the hydrogen peroxide content of two *Phaseolus vulgaris L.* cultivars. Antimony was administered to two cultivars and the H_2O_2 content, within the leaves and roots, were determined. The Different letters indicate the significant differences between the means at P< 0.05 (DMRT). The values are means ± S.E (N=12).

3.6. The effects of antimony concentration on the Hydroxyl radical content of two *Phaseolus vulgaris L.* cultivars

Under normal conditions, hydroxyl radicals are produced by the Fenton reaction and are highly reactive, as a result this leads to the oxidation of biomolecules (Ravet and Pilon, 2013).The problem arises when there is a significant increase in hydroxyl radicals, when a plant is subjected to stress (Bhattacharjee, 2011). An overproduction of hydroxyl radicals within plants has been seen to have a detrimental effect, causing damage within plants. Schopfer, 2001 stated that these molecules are known to react with cell walls, causing major damage. In conjunction with this problem, accumulation of hydroxyl radicals tends be involved in the oxidation of intracellular compounds (Gill and Tuteja, 2010). Thus, it is of great importance that the content of these radicals are observed, especially in understanding their regulation under abiotic stress, such as antimony stress. The Contender cultivar treated with antimony exhibited a ~5.143% decrease in the hydroxyl radical content within the leaves (Figure 3.5). The Timbavati cultivar exhibited a ~24.593% increase in hydroxyl radical content within the leaves (Figure 3.5), when compared to the controls.

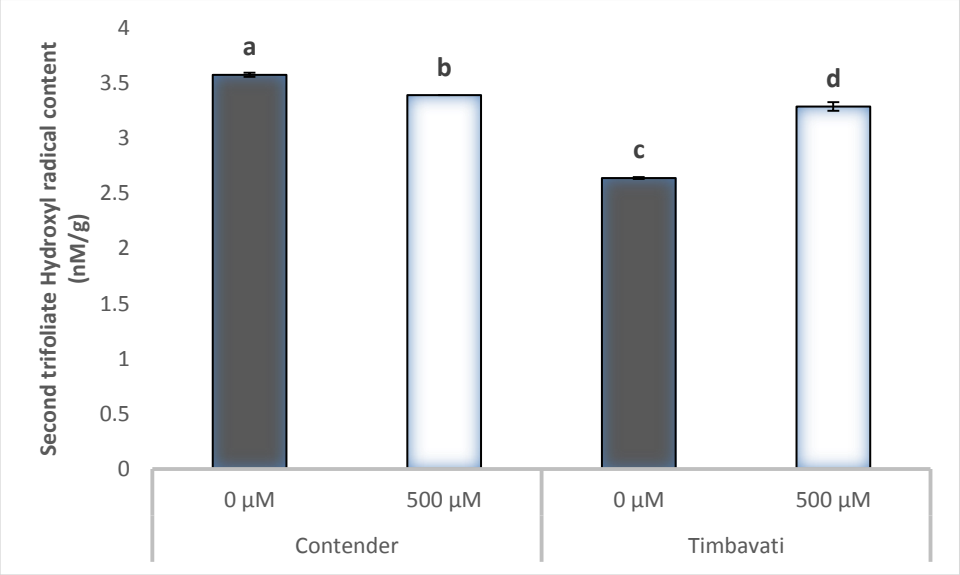

Figure 3.5: The influences of antimony on the hydroxyl radical content of two Phaseolus vulgaris L. cultivars. Antimony was administered to two cultivars and the hydroxyl radical content, within the leaves and roots, were determined. The different letters indicate the significant differences between the means at P< 0.05 (DMRT). The values are means ± S.E (N=12).

3.7. The effects of antimony concentration on the MDA content of two *Phaseolus vulgaris L.* cultivars

Fatty acids are the main constituent that comprises the lipid membranes. Under stressful conditions, ROS molecules accumulate and damage these polyunsaturated fatty acids within the membranes. Hence this damage is identified as lipid peroxidation (Gokul, 2013). The main product of the lipid peroxidation process is Malondialdehyde, MDA. Thus, MDA is used as an indicator to assess the extent of lipid peroxidation within plant tissues (Singh and Sexena, 2006; Verma and Dubey, 2003). MDA is quantified by a reaction involving Thiobarbituric acid (TBA). The lipid peroxidation assay (section 2.11) was conducted to determine the damage done to the membranes of two *Phaseolus vulgaris L.* cultivars, under normal and antimony stress conditions. As stated previously MDA makes for the perfect indicator for lipid peroxidation, as it is produced as a by-product of this process (Zang *et al*, 2006). An increase in heavy metal concentration is said to increase the MDA content. This occurrence indicated a concentration-dependent free radical generation (Zang *et al*, 2006). The leaves of Contender plants, treated with antimony, indicated a ~17.57% increase in MDA content when compared to its control (Figure 3.6). The Timbavati leaves, treated with antimony, indicated a ~37.91% increase in MDA content when compared to its control

(Figure 3.6).Within the roots the Contender cultivar experienced a ~47.25% increase in MDA content when compared to its control and Timbavati cultivar experienced a ~78.29% in its MDA content within the roots.

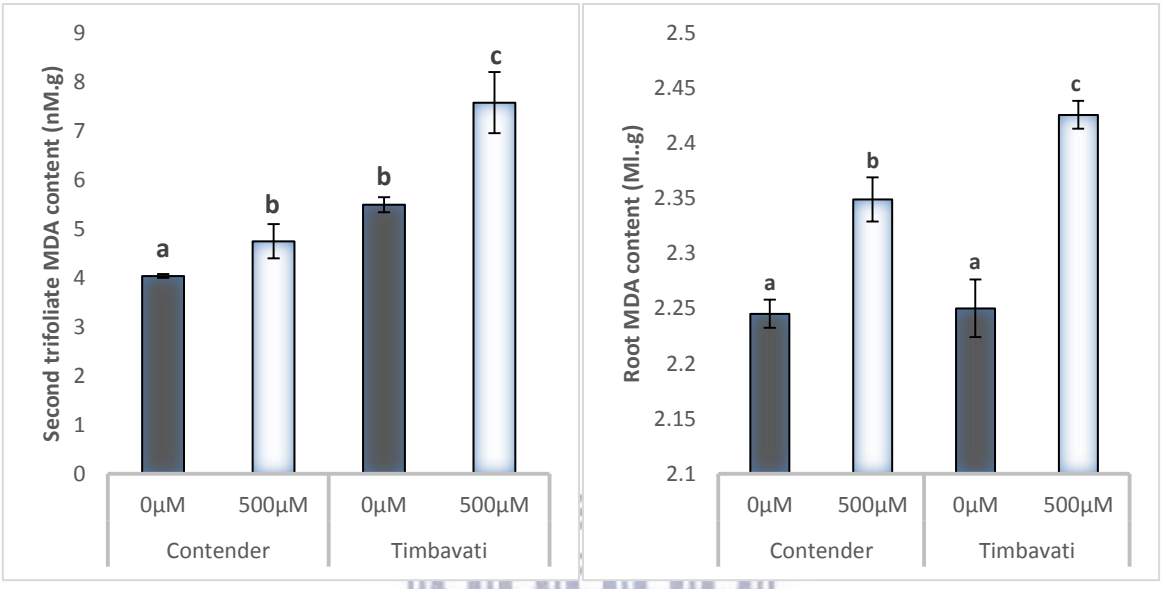

Figure 3.6: The influences of antimony on the MDA content of two *Phaseolus vulgaris L.* cultivars. Antimony was administered to two cultivars and the MDA content, within the leaves and roots, were determined. The Different letters indicate the significant differences between the means at P< 0.05 (DMRT). The values are means ± S.E (N=12).

3.8. The effects of antimony concentration on the SOD activity of two *Phaseolus vulgaris L.* cultivars

The antioxidant enzyme SOD is of immense importance as it is first enzyme in the cellular defence mechanism (Zang *et al*, 2006). This enzyme modulates the quantity of O_2^- , thus producing H_2O_2, which can then be scavenged by ascorbate peroxidase. SOD furthermore diminishes the chance of OH^- radical formation, which is extremely reactive and may bring about server damage to the lipids, proteins and DNA biomolecules (Zang *et al*, 2006). The SOD activity assay (Section 2.13) was conducted to determine the SOD activity of two *Phaseolus vulgaris L.* cultivars, under normal and antimony stress conditions. Leaves of Contender plants, treated with antimony, indicated no significant difference in SOD activity when compared to its control (Figure 3.7). The Timbavati leaves, treated with antimony, indicated a ~5.79% decrease in SOD activity when compared to its control (Figure 3.7).

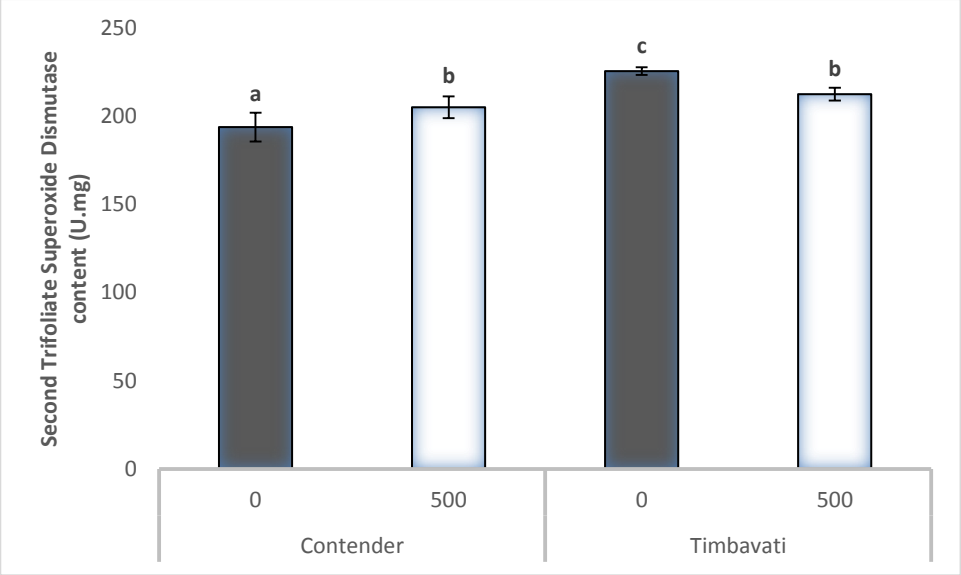

Figure 3.7: The influences of antimony on the SOD activity of two *Phaseolus vulgaris L.* cultivars. Antimony was administered to two cultivars, and the SOD activities, within the leaves, were determined. The Different letters indicate the significant differences between the means at P< 0.05 (DMRT). The values are means ± S.E (N=12).

3.9. The effects of antimony concentration on the SOD isoforms of two *Phaseolus vulgaris L.* cultivars

Superoxide Dismutase Native PAGE gels were conducted firstly, to establish and determine how many and which isoforms of SOD, *P.vulgaris* plants contained and to obtain a profile of SOD isoforms. Secondly, this assay was conducted to determine which isoforms were mostly affected under antimony stress. In total both cultivars, Contender and Timbavati, indicated four isoforms in the profile, within the roots and leaves. Figure 3.8 illustrates that the cultivars contained a MnSOD, FeSOD, and two Cu/ZnSOD. These were determined by conducting inhibition tests using H_2O_2 and KCN. A Total of 4 SODs were identified in both Contender and Timbavati cultivars. The SOD NATIVE PAGE gel illustrates four isoforms present in both Contender and Timbavati cultivars (Figure 3.8). A visual decrease in intensity of the lowest Cu/ZnSOD is experienced in both cultivars in the roots and a slight decrease within the leave and roots of the treated plants (Figure 3.8 (A)). Figure 3.8 (B) illustrates a total inhibition of the FeSOD, to H_2O_2, of all samples loaded and Figure 3.8 (C) illustrates that only The MnSOD was not inhibited by the KCN.

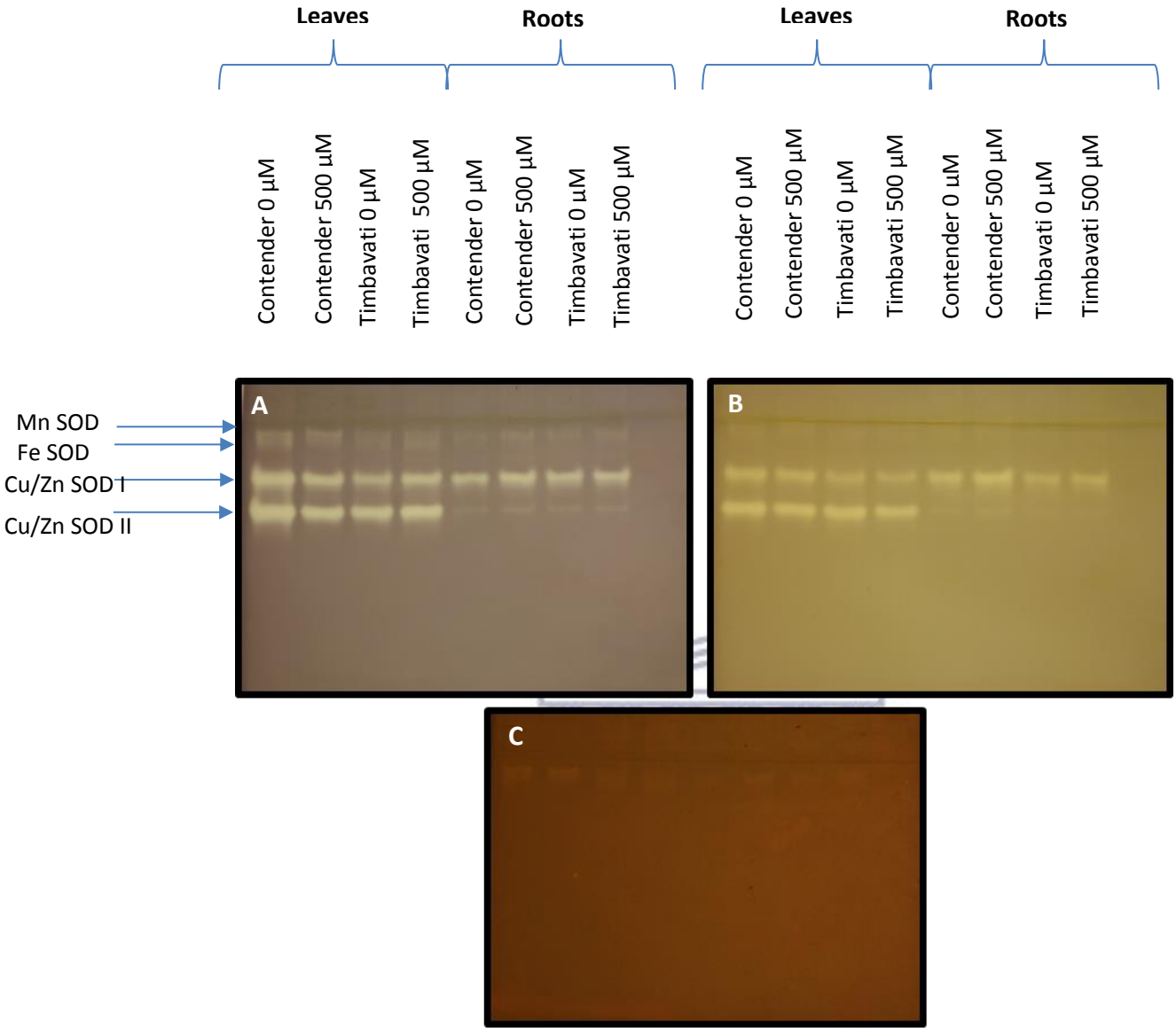

Figure 3.8: The SOD profiles of two cultivars of Phaseolus vulgaris L. The influences of antimony on the activity profile of superoxide dismutase in two *Phaseolus vulgaris L.* cultivars. The lanes were loaded as follows; Contender control leaves (lane 1), Contender 500 µM leaves (lane 2), Timbavati control leaves (Lane 3), Timbavati 500 µM leave (lane 4), Contender control roots (lane 5), Contender 500 µM (lane 6), Timbavati control roots (lane 7), Timbavati 500 µM roots (lane 8). (A) Signifies the full SOD profile in the control gel. (B) Signifies the H_2O_2 inhibited gels and (C) Signifies the KCN inhibited gels for both cultivars.

3.10. The effects of antimony concentration on the APX activity of two *Phaseolus vulgaris L.* cultivars

Ascorbate peroxidase, APX, is a vital antioxidant enzyme implicated in the ascorbate-glutathione cycle. The main purpose of this enzyme is to react with H_2O_2 to transform this molecule into H_2O and in addition to this purpose it also converts ascorbic acid to dehydroascorbate, DHA (Sarowar *et al*, 2005; Sinha and Sexena, 2006). APX is recognized to

possess a higher affinity for H_2O_2 more so than catalase, consequently, being a more efficient scavenger of H_2O_2 in times of stress (Das and Roychoudhury, 2014). The APX activity assay (Section 2.14) was conducted to determine the APX activity of two *Phaseolus vulgaris L.* cultivars under normal and antimony stress conditions. The leaves of Contender plants, treated with antimony, indicated no significant difference in APX activity when compared to its control (Figure 3.9). The Timbavati leaves, treated with antimony, indicated a ~69.72% increase in APX activity when compared to its control (Figure 3.9).

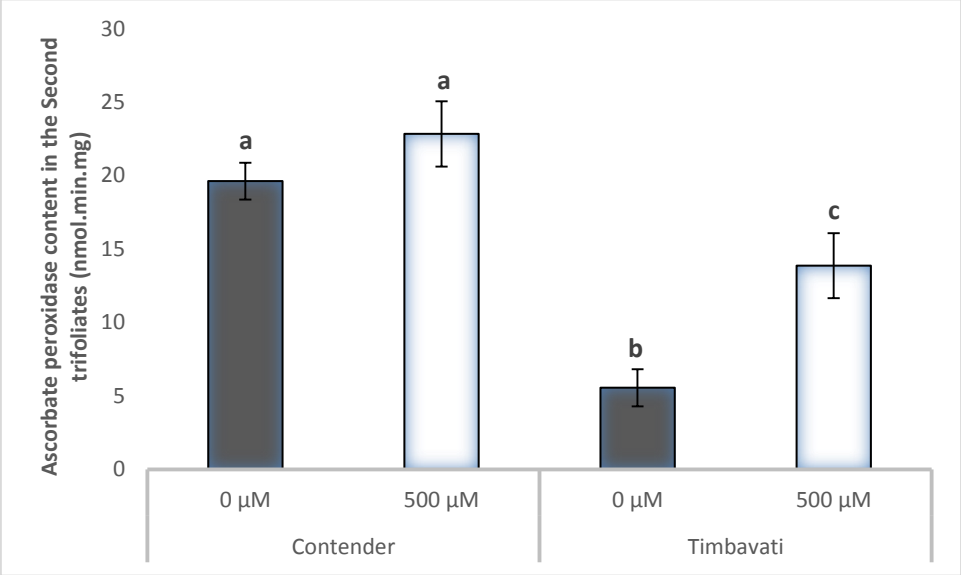

Figure 3.9: The influences of antimony on the APX activity of two *Phaseolus vulgaris L.* cultivars. Antimony was administered to two cultivars, and the APX activities, within the leaves, were determined. The Different letters indicate the significant differences between the means at P< 0.05 (DMRT). The values are means ± S.E (N=12).

3.11. The effects of antimony concentration on the CAT activity of two *Phaseolus vulgaris* L. cultivars

The antioxidant enzyme, known as CAT, is accountable for catalysing the dismutation of H_2O_2, to produced H_2O and O_2 and ultimately reducing the effects of H_2O_2 within plants (Das and Roychoudhury, 2014). Contributing to their optimal scavenging of H_2O_2 is their high affinity to the said ROS molecule. CAT successfully eliminates H_2O_2 in an energy efficient manner, mainly because stressful conditions demand greater energy generation and expenditure of cell (Das and Roychoudhury, 2014). The CAT activity assay (Section 2.15) was conducted to determine the CAT activity of two *Phaseolus vulgaris L.* cultivars, under normal and antimony stress conditions. The leaves of Contender plants, treated with

antimony, indicated a ~14.71% increase in CAT activity when compared to its control (Figure 3.10). The Timbavati leaves, treated with antimony, indicated an ~8.59% increase in CAT activity when compared to its control (Figure 3.10).

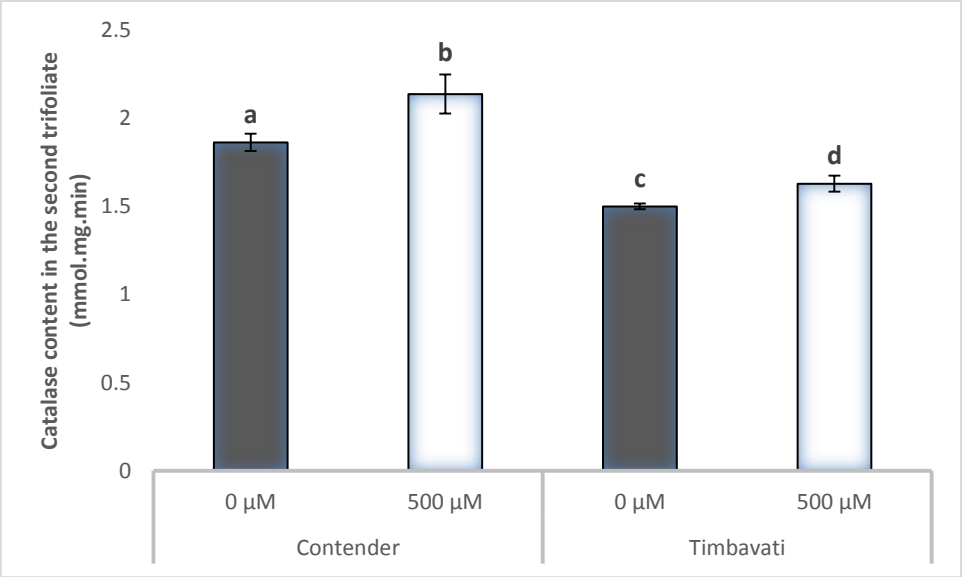

Figure 3.10: The influences of antimony on the CAT activity of two *Phaseolus vulgaris L.* cultivars. Antimony was administered to two cultivars, and the CAT activities, within the leaves, were determined. The Different letters indicate the significant differences between the means at P< 0.05 (DMRT). The values are means ± S.E (N=12).

DISCUSSION

3.12. Physiological Characteristics

3.12.1. The decrease in plant growth and biomass of both Phaseolus vulgaris L. cultivars, under antimony stress

Figure 3.1.1, 3.1.2 and 3.1.3 revealed exactly how the antimony concentrations affected the growth of both *Phaseolus vulgaris* cultivars, Contender and Timbavati. Decreases in total plant growth within the plants were observed, in both cultivars that underwent antimony treatment. Studying the Contender cultivars growth rate (Figure 3.1.1), it was observed that antimony had a minimal effect on the growth of the plant, however a slight effect was observed when compared to the control. Studying the Timbavati cultivars growth (Figure 3.1.2), it was observed that the antimony had a significant effect on the growth rate of the plant. Figure 3.1.3 perfectly depicts the differences in how the antimony treatments affected each cultivar. As recorded, under normal conditions the Contender cultivar

produced larger plants, growing taller than the Timbavati cultivar. With an overview of the results, it was observed that a greater negative effect was recorded in the Timbavati cultivar. This result correlated with the biomass results obtained in Figure 3.1.4 where Timbavati experienced the largest decrease in its leaf and root biomass, when compared to the Contender cultivar.

Observing the natural growth of the Contender and Timbavati plants, it was noted that the Contender plants are much taller, with elongated stems, complex root systems and larger leaf surface area compared to the Timbavati plants, which were much smaller. Hence it was hypothesized that antimony would have a greater effect on the Timbavati cultivar's growth. A decrease in biomass observed in the Timbavati cultivar could have been attributed to the plants exercising vital energy and resources that was essentially designated for growth. The decrease could also be attributed to a decrease in water and mineral absorption into the plants, thus investigating the mineral absorption would provide more insight to this possibility. The minimal effect of antimony on the Contender cultivar's growth could have been attributed to this cultivar possessing a more advanced defence system that provided them with a better tolerance to abiotic stresses, than that of the Timbavati cultivar.

The physiological results in this study (Figure 3.1.1-3.1.3) correlated to a study conducted by Rubio and colleagues, 1994 wherein they recorded that the root and shoot lengths were considerably lower in the metal treated plants, when compared to their controls. Likewise, in Chibuike and Obiora, 2014 they discussed a study where a decrease in growth rate was recorded in cluster beans, under Zinc contamination. These results may perhaps be because of inhibition of growth due to the heavy metal interactions with enzymes involved in protein synthesis, photosynthetic systems and essentially cell division. These interactions could have led to abnormal cell division, protein synthesis in the roots and chromosomal.

The reduction in growth under metal accumulation could have been owed to the metal disrupting essential channels, not allowing essential elements to be absorbed into the plant. Concerning the biomass results (Figure 3.1.4), similar outcomes were recorded by Cook and colleagues, 1997 in which they investigated the effects of copper on *Phaseolus vulgaris L.* They recorded a 56% decrease in leaf biomass, which was similar to what was observed in the Timbavati cultivar leaves. These effects could have been the result of this cultivar translocating antimony up into the leaves where it possibly disrupted the balance

of the production of reactive oxygen species, ROS, leading to oxidative stress. Many other studies depicted similar biomass results as observed in this study. Studies such as one conducted by Vaculik and colleagues, 2015 studying the effects of antimony on sunflower seed and Saco and colleagues, 2013 studying how vanadium decreased the biomass of *Phaseolus vulgaris L. plants*. The changes in growth was mainly affected by the changes in the oxidative balance and functioning of the plants, therefore, investigation of these molecules within the plants were crucial.

3.12.2. The decrease different chlorophyll species of both Phaseolus vulgaris L. cultivars, under antimony stress

Table 3.1 indicated the effects of antimony on the chlorophyll content of two *Phaseolus vulgaris L.* cultivars. In the chlorophyll *a* and *b* species, both *Phaseolus vulgaris L.* cultivars experienced a decrease in the chlorophyll levels. However, a higher decrease was experienced in the Chlorophyll *b* species. The impairment in these photosynthetic systems could have been due to antimony interacting with enzymes essential in the proper functioning of these systems. It was also proposed that antimony might be averting chlorophyll synthesis either by direct inhibition of an enzymatic step or by inhibition of a major nutrient.

Yurekli and Porgeli, 2005 reported the similar results. These authors illustrated that under excess copper a decrease was observed in different chlorophyll species, in *Phaseolus vulgaris L.* and deduced that the copper molecules were interrupting the enzymes within these systems. Sheetal and colleagues, 2015 likewise indicated that a reduction in chlorophyll links with the inhibition of enzymes or the interaction of heavy metals with some essential nutrients. Table 3.1 indicated that the chlorophyll b content, in both cultivars, was dramatically affected. This result could have been attributed to antimony affecting the photochemical efficiency of the photosystem II, which contains majority of chlorophyll b. A study conducted by Campanharo and colleagues, 2010 presented that at high levels of nickel in beans reflected negative results in chlorophyll. In this study they also stated that chlorophyll *b* was more susceptible than chlorophyll *a*, similar results were observed in this study (Table 3.1) and this effect led to photosynthetic apparatus impairment in plants.

3.12.3. The increase in cell death in both Phaseolus vulgaris L. cultivars, under antimony stress

Figure 3.2 revealed the effects of antimony on two *Phaseolus vulgaris L.* cultivars. In both cultivars, an increase in cell death was experienced in the plants that underwent antimony treatments. A slightly larger amount of non-viable cells were recorded in the Timbavati cultivar. The Contender cultivar depicted an increase in cell death, and this was expected, seeing as the cell was being subjected to stress, however, the increase in cell death was not as pronounced as observed in the Timbavati cultivar. The reasoning behind these dead cells could probably have been due to damage to cell structure because of possible oxidative stress, however further assessment of ROS molecules production and lipid peroxidation needed to be conducted to analyse the oxidative stress.

Similar results were recorded in a study conducted by Basset and Matsumoto, 2008 where they observed and upsurge in cell membrane disruption when the Tobacco plants were exposed to aluminium. Non-viable cells, because of cell membrane damage, DNA and lipid molecules damage, were detected by Evans blue, a non-permeating dye, which seeped through ruptured membranes and stained the contents of dead cells (Baker and Mock, 1994). Cell death is the most useful assay in depicting damage in plants caused by stress. Less damage was experienced by the Contender cultivar (Figure 3.2) because their plant cells might have possibly consisted of more intact cell membranes, therefore explaining why the Evans blue solution couldn't be taken up as much. The possible reasoning for Contender having more viable cells could have been attributed to this cultivars possible efficient antioxidant defence system. A system (consisting of APX and CAT) that possibly sufficiently scavenged H_2O_2 thereby reduced the chances of cell membrane damage, by lipid degradation (lipid peroxidation) by the H_2O_2 molecule. Hence, ensuring the viability of the cells. However, to elucidate this possibility, investigations needed to be conducted on ROS molecule concentrations.

3.13. Biochemical responses of *Phaseolus vulgaris L.* cultivars to antimony stress

When plants are exposed to high heavy metal concentrations, this stimulate oxidative stress within the plants, through the generation of superoxide (O_2^-), hydrogen peroxides

(H_2O_2) and hydroxyl radicals (OH^-). The collective term provided for these molecules are reactive oxygen species, ROS molecules (Zang *et al*, 2006). Over decades of research, the role of these molecules has been of great importance mainly owing to their well-known roles as signalling molecules under normal conditions (Cuypers *et al*, 2016). Nevertheless, their destructive nature cannot be disregarded. ROS molecules, in high concentrations, are toxic to the plants, causing damage to various cellular structures, focussing their effects on biomolecules such as nucleic acids, lipid molecules, proteins and amino acids (Cuypers *et al*, 2016; Zang *et al*, 2006). As stated, the accumulation of ROS molecules can bring about an imbalance in the oxidative state of plants, in so doing cause damage to the plants metabolisms and organelles (Gill and Tuteja, 2010).

Previously in this study the physical effects of the cultivars were observed (Figures 3.1.1-3.1.4). It was only proper, to understand which pathways and mechanisms were affected by the antimony that brought about these negative physiological effects. To understand the biochemical effects, investigations on O_2^- and H_2O_2 in plants needed to be observed. This was done to determine the extent of the damage caused by the antimony. Lipid peroxidation was also assessed in both cultivars to establish the extent of the damage.

3.13.1. The increase in superoxide content in both Phaseolus vulgaris L. cultivars, under antimony stress

Figure 3.3 revealed exactly how the antimony concentration affected the production of the ROS molecule, O_2^-, in the leaves and roots. An increase was observed in O_2^- molecules, in both cultivars that underwent antimony treatment. The Timbavati cultivar showed a slightly higher production of O_2^-, compared to the Contender cultivar. This observation could suggest the ineffectiveness of the Timbavati antioxidant defence system employed.

The results obtained (Figure 3.3) could also have been due to antimony stress that led to an overproduction of reactive oxygen species (ROS) in plants. High levels of O_2^- molecules were usually observed in leaves, owing to the production site of most O_2^- molecules were located in the chloroplast, photosystems (PSI and PSII) (Gill and Tuteja, 2010).

Observing the results obtained in this study (Figure 3.3), similar results were obtained in Gokul, 2013. The author recorded that under vanadium concentrations the leaves of two

Brassica napus L. cultivars, namely Garnet and Agamax, the O_2^- content increased when compared to their respective controls. The increase in O_2^- levels within antimony treated Contender and Timbavati plants were possibly attributed to antimony playing a role in the down regulation of the ROS scavenging enzymes. The increase in O_2^- content within the leaves indicated that the roots are not the only organ of the plant being affected, and that antimony is using a plant mechanism to be translocated within the plant. Even though, the O_2^-content (Figure 3.3) was increased, the cell death (Figure 3.2) and damage experience in Contender was not highly detrimental. This outcome could possibly be indicating that the antioxidant enzyme, SOD, was efficiently scavenging O_2^- molecules. By observing the O_2^- results it could be hypothesized that a similar trend would be observed in the H_2O_2 results, as previously stated O_2^- molecules promote generation of H_2O_2 molecules, therefore an increase in O_2^- could have essentially resulted in an increase in H_2O_2.

3.13.2. The increase in hydrogen peroxide content in both Phaseolus vulgaris L. cultivars, under antimony stress

Figure 3.4 depicted the effects that antimony concentrations had on both the Contender and Timbavati cultivars. One observation was that in both cultivars, that underwent antimony treatment, the plant's leaves and roots illustrated and increase in the H_2O_2 content. With closer inspection (Figure 3.4) it was observed that the Timbavati cultivar represented the greatest H_2O_2 production, when compared to the Contender cultivar. When observing the roots, it was observed that a higher degree of H_2O_2 content was recorded at this location. The increase in H_2O_2 could have been attributed to the increased levels of superoxide, O_2^-, produced being dimutated to H_2O_2. These levels in Contender could also have been attributed to its relatively longer life-span therefore being observed in the plant.

Literature explained that higher H_2O_2 would have been observed in the leaves. This owning to the mitochondria, chloroplast and xylem are all organelles that produce H_2O_2, as a signalling molecule. (Cheeseman, 2007). Owing to the location of these organelles in the leaves, it could provide an explanation as to why under high antimony concentrations, high concentrations of H_2O_2 is observed in the leaves. However, the same was not seen in this study. The results obtained in Figure 3.4 could have been attributed to the roots being the

primary site of entrance of antimony into the plant and the roots were more in contact with higher concentrations of antimony than the leaves, therefore it was expected that more H_2O_2 production and ultimately more damage would have been experienced within the roots.

Cuypers and colleagues, 2016 explained that granting H_2O_2 itself is weakly reactive, different studies have demonstrated that oxidative DNA damage and protein oxidation was accompanied with an upsurge in H_2O_2 levels in various plant species, under heavy metal stress. The results attained in the current study (Figure 3.4) corresponded to a study conducted by Gorska-Czekaj and Borucki, 2013 wherein they revealed that when treated with mercury and copper, the H_2O_2 levels increased substantially within the plants. The large increase in H_2O_2 content in Timbavati plants exposed to antimony was suggested to be due to the antimony playing a role in down regulating the ROS scavenging enzymes thus disturbing the oxidative homeostasis.

An explanation for the high content of H_2O_2 in Timbavati, under antimony treatments could have been due to a higher oxidative stress imposed upon this cultivar by the antimony. Similar results were recorded in Singh and colleagues, 2011 in the RH30 plant. A reason to obtaining these results in the Timbavati cultivar was that this cultivar most possibly possessed an inefficient signalling mechanism. However, Contender cultivar indicated similar levels of H_2O_2 when comparing the control to the treated plants. This could have been because the generation of H_2O_2 was reduced by an efficient antioxidative mechanism, therefore resulted in the rapid removal of ROS by antioxidants.

Cuypers and colleagues, 2016 indicated that lipid peroxidation, along with an increase in H_2O_2 levels, was shown to occur in many different plant species exposed to Aluminium, Cadmium, Copper, Mercury, Nickle, Lead and Zinc. High levels of H_2O_2 could have possibly led to lipid degradation, mainly due to H_2O_2 ability to cross through cellular membranes. Hence, an increase in H_2O_2 should have essentially led to an increase in MDA levels. It was essential to understand the effects of antimony on H_2O_2 generation, because Guo and colleagues, 2005 illustrated how stress environments increased the H_2O_2 content and in conjunction with the increase in oxidative damage in plants.

3.13.3. Changes in hydroxyl radical content in both Phaseolus vulgaris L. cultivars under antimony stress

Figure 3.5 depicted the effect that of antimony concentration had on the hydroxyl radical content in the leaves of two *Phaseolus vulgaris L.* cultivars. Studying Figure 3.5, it was noted that the antimony treated Contender cultivar experienced a slight decrease in hydroxyl content when compared to the control however the opposite was observed for Timbavati which experienced a large increase in the hydroxyl content when compared to the control. Essential under normal conditions hydroxyl radicals are found in plants at basal levels. However with an increase as seen in Timbavati it had been observed to have had an effect on cell wall loosening (Schopfer, 2001).

The results collected here correlated with those found in O_2^- and hydrogen peroxide, in which not much difference was seen in Contender antimony treated plants and a significant increase was observed in Timbavati treated plants when compared to its control. This increase in O_2^- and H_2O_2 could have explained the increase in hydroxyl radicals in Timbavati, as these two molecules contributed to the production of hydroxyl radicals. The damaging effect of hydroxyl radicals could have explained the various oxidative effects observed within the plants under antimony stress. The general lower production of ROS molecule in Contender explained why this cultivar did not experience an increase in hydroxyl radicals, as the antioxidants efficiently scavenged these molecules to prevent production of more detrimental molecules such as hydroxyl radicals. Schopfer, 2001 stated that these molecules are known to react with cell walls, causing major damage. In conjunction with this problem, accumulation of hydroxyl radicals tends to be involved in the oxidation of intracellular compounds (Gill and Tuteja, 2010).

3.13.4. The increase in MDA content in both Phaseolus vulgaris L. cultivars, under antimony stress

Figure 3.6 depicted the effect that antimony concentrations had on the MDA content in the leaves and roots of two *Phaseolus vulgaris L.* cultivars. Studying Figure 3.6, it was noted that the antimony treated Contender cultivar experienced much less lipid peroxidation, when compared to the Timbavati cultivar leaves. Essentially under normal conditions the

Contender cultivar did not experience high lipid peroxidation, which was noted in the Timbavati cultivar. This could have aided in explaining why the Contender fared much better than the Timbavati cultivar under both normal and stressed environments. The results shown in Figure 3.4 and 3.5 correlates to the results shown in Figure 3.6, when concerning the roots of the Contender plants.

In the current study, an upsurge in ROS molecules were found in both cultivars of *Phaseolus vulgaris L.* under antimony stress (Figure 3.4, 3.5 and 3.6), which affected MDA production (Zang *et al*, 2006). The information suggested that the toxic effects of heavy metals were probably exerted through ROS generation (Zang *et al*, 2006). O_2^- undergoes transformations to produce hydroperoxyl radicals (OH^- and H_2O_2) which convert fatty acids to toxic lipid peroxides.

In a study conducted by Yurekli and Porgeli, 2005 they recorded that the MDA contents increase in *Phaseolus vulgaris L*. leaves, under excess copper. These results correlated with the results obtained in the current study (Figure 3.6). A reason to obtaining these particular results could have been explained, in literature, in which evidence had been shown that particular heavy metal brought about an increase in the lipoxygenase activity which catalysed lipid peroxidation, especially of unsaturated fatty acids and due to this reaction various radicals were formed which led to the degradation of membrane structures (Yurekli and Porgeli, 2005). Singh and colleagues, 2011 stated in their research that previous studies indicated that Cd increased lipid peroxidation in plants due to excessive ROS production. It had been reported that metal ions tended to block the electron flow in PS II, which resulted in the formation of excited Chl causing the production of free oxyradicals (Singh *et al*, 2011).

3.14. Antioxidant defence systems in response to antimony stress

ROS molecules contain the ability to swiftly attack biomolecules and essentially cause metabolic dysfunction and cell death (Zang *et al*, 2006) and for that reason, plants have adopted particular important defensive mechanisms to assist in reducing the oxidative

damage under various stresses (Gill and Tuteja, 2010). The defensive mechanisms consist of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) (Zang *et al*, 2006). The responsibility of the antioxidant defence system is to regulate the cascade of uncontrollable oxidation, thus by protecting the plant cells from oxidative damage by scavenging of ROS molecules (Gill and Tuteja, 2010). The inherent ability of a plant to efficiently deal with increasing ROS levels, due to heavy metals, would play a vital role in the tolerance of plants to antimony. To protect against antimony-induced oxidative damage, plant cells and its organelles like chloroplast, mitochondria and peroxisomes employ antioxidant defence systems (Singh *et al*, 2011).

3.14.1. The level of SOD activity in both Phaseolus vulgaris L. cultivars, under antimony stress

Figure 3.7 depicted the effects of antimony concentrations on the SOD activity within the leaves of two *Phaseolus vulgaris L.* cultivars. Observed was an increase in SOD activity within the Contender cultivar under antimony treatment. Overall the Contender cultivar illustrated a lower SOD activity in both control and treated plants, when compared to the Timbavati cultivar. Strangely, in the Timbavati cultivar, a decrease was observed in the SOD activity of the antimony treated plants (Figure 3.7).

SOD, is an essential protective enzyme of the antioxidant systems, and exists in various parts of the cell. This antioxidative enzyme catalyse the dismutation of O_2^- molecules to produce H_2O_2 and O_2 (Yurekli and Porgeli, 2005). Research indicates that the amounts of antioxidant enzymes, like SOD, increase in plants highly exposed to heavy metals (Yurekli and Porgeli, 2005). In a study conducted by Yurekli and Porgeli, 2005 it was observed that the leaves of *Phaseolus vulgaris L.* plants, in excess Cu, illustrated increase in SOD activity, however, this increase was not as significant as observed in the roots of these plants. Similar results were obtained in the current study (Figure 3.7), within the Contender cultivar's leaves. This result could suggest that there was less accumulation of antimony within the leaves of the Contender cultivars, in which further analysis on antimony content was conducted. Singh and colleagues, 2011 reported high SOD activity within the RH30 cultivar, similar results were obtained for the Timbavati cultivar leaves. Timbavati might

have had an inherent higher SOD activity when compared to Contender but when the stress was imposed this level was seen to drop. This drop in SOD activity under stress could have possibly depicted an inefficient antioxidant defence mechanism, possessed by Timbavati. Observing the high levels obtained for SOD activity in both plants, it could have been said that this level was probably high owing to the high superoxide (O_2^-) levels within the leaves and the plants attempting to combat the O_2^- molecule effects (Figure 3.4). The Contender cultivar illustrated a larger increase in SOD activity under stress, when compared to Timbavati. The increase in SOD activity within Contender could have been attributed to the plant registering an upsurge in ROS molecules (O_2^-), thus responding to this upsurge by producing SOD which was known to scavenge O_2^- molecules. Zang and colleagues, 2006 suggested that plants experiencing an increase SOD activity have better protection against oxidative damage. Thus, helping to explain that Contender cultivars were better protected against ROS molecules. This statement helped explain Timbavati's susceptibility to antimony stress, because observed was a decrease in SOD activity in the treated plants (Figure 3.7), a decrease in SOD opened the plant to more oxidative damages (Zang *et al*, 2006). Decreases in SOD could have been attributed to decreases in essential elements (Iron, Copper, Zinc and Manganese) that form co-factors with this antioxidant enzyme. Ravet and Pilon, 2013 validated this as they stated that the superoxide dismutase enzymes located in the chloroplast were all dependent on Iron or Copper co-factors. Hence it could have been predicted that the Contender cultivar would have been more tolerant to antimony stress than the Timbavati cultivar, owing to its higher antioxidant capacity. The main product of SOD is H_2O_2, which is a toxic substance and therefore must be eliminated by conversion to H_2O in a subsequent reaction (Zang *et al*, 2006). Therefore as long as the stress is not too strong for the plant's defence capacity, the main response to heavy metals is an increase in SOD and POD activities (Zang *et al*, 2006).

SOD enzymes are known for their various abilities ranging from removing O_2^- and decreasing peroxidation of membrane lipids, to essentially maintain cell membrane stability (Zhang *et al*, 2007). The decrease in SOD activity experienced in the Timbavati cultivar correlated with the results obtained in the MDA content (Figure 3.6). Less SOD enzymes were present in order to decrease O_2^- content. This failure in reducing O_2^- content possibly led to the peroxidation of membrane lipids, thus an increase in lipid peroxidation was

experienced in the Timbavati cultivar. Therefore, the combined effects of reduced activity of SOD and increase production of H_2O_2 (which causes lipid degradation, as H_2O_2 had the ability to cross through cellular membranes) all led to the significant increase in MDA exhibited in the Timbavati cultivar (Cuypers *et al*, 2016). A similar decrease in SOD was reported in a study conducted by Zhang and colleague, 2007 in leaves of *K.candel* plants, this decline could indicate that the oxygen scavenging function of SOD was impaired.

The SOD NATIVE PAGE gels were conducted to validate the results obtained for the SOD activity assay, to obtain the SOD profile of *P.vulgaris L*. cultivars and observe how antimony concentrations affect the SOD profiles of the two cultivars in this study. The gels were conducted to deduce the class of each isoform using compounds such as hydrogen peroxide (H_2O_2) and potassium cyanide (KCN), which have been recorded to inhibit different classes of SODs. H_2O_2 inhibit the Cu/Zn SODs and MnSODs, while KCN has been shown to inhibit Cu/ZnSODs.

Figure 3.8 illustrated that both cultivars possessed four SOD isoforms, which were identified using two inhibition gels (H_2O_2 and KCN). Talukdar and Talukdar, in 2016, illustrated that that *P.vulgaris* contained four isoforms of SOD, the results obtained in this investigation slightly correlated to the profile shown in Talukdar and Talukdar, 2016. However the inhibitory effect of H_20_2 on the MnSOD and Cu/ZnSODs were not as potent as expected. However, the intensity of the bands under H_2O_2 inhibition did decrease showing a degree of inhibition. In the KCN inhibition gel, only the top two bands were visible and the two lowest bands completely disappeared, showing inhibition of these bands. KCN is known to inhibit Cu/ZnSODs, thus combination of the three gels could be used to deduce the profile of the SOD isoforms. It was interesting to observe (Figure 3.8 (A)) that the roots generally had less of the Cu/ZnSOD II than that compared to the leaves. The MnSOD and FeSOD bands were very faint when compared to the Cu/ZnSODs. When comparing the controls to the treated samples there was a clear decrease in isoforms under stress and the roots has less isoforms when compared to the leaves. Overall, Timbavati had less intense bands illustrating that this cultivar had lower SOD activity under normal condition and especially under stressful conditions.

3.14.2. The increase in APX activity in both Phaseolus vulgaris L. cultivars, under antimony stress

Figure 3.9 depicted how antimony concentrations affect the APX activity within the leaves of two *Phaseolus vulgaris L.* cultivars. Analysing the results brought about the discovery that the APX activity in both cultivar's leaves increased under antimony treatments. The increase in APX could possibly have been in response to increases in hydrogen peroxide (H_2O_2) molecules within the plants, and as a defence mechanism, both cultivars promoted the increase in production of APX, to scavenge H_2O_2. Even though Timbavati illustrated an increase in APX activity, the damage experienced by the cultivar was still significant. This could have been due to Timbavati still not producing sufficient amounts of APX to scavenge the accumulation of hydrogen peroxide. On the other hand, Contender exhibited an efficient scavenging system as this plant coped better than Timbavati, under antimony stress.

Singh and colleagues, 2011 in their study reported a significant increase in the APX activity within all the cultivars exposed to Cadmium. This result correlated with the results obtained in the current study (Figure 3.9), affirming that an increase in APX activity exemplified an efficient functioning of the ascorbate-glutathione cycle, therefore increased tolerance to antimony (Singh *et al*, 2011). Xue *et al*, 2015 suggested that the enhanced activities of APX were an important tolerance mechanism in antimony tolerant plants. APX is an important enzyme for the scavenging of H_2O_2 in the chloroplast and cytosol. Therefore the results in Figure 3.9 correlated with the results in Figure 3.4. Contender's APX increased to such an extent that it efficiently maintained the H_2O_2 levels to normal levels, thus conferring increased tolerance to heavy metal stress and an up-regulation of APX in plants accompanied tolerance to abiotic stresses in plant (Kornyeyev *et al*, 2003).

3.14.3. The increase in CAT activity in both Phaseolus vulgaris L. cultivars, under antimony stress

Studying the results obtained Figure 3.10 an increase was recorded in both cultivars, with an increase in antimony concentration, within the leaves. The Contender cultivar leaves

however, experienced a much greater increase in the CAT content, when compared to the Timbavati cultivar. A greater increase in the CAT content within the Contender leaves could have possibly been linked to its antioxidant systems ability to efficiently scavenge H_2O_2 by producing high concentrations of CAT. Yurekli and Porgeli, 2005 revealed that in excess of Cu there was an increase in CAT enzyme activity within the roots, stem and leaves of the *Phaseolus vulgaris L.* plants, however, the highest CAT recording was in the roots. An additional study conducted by Singh and colleagues, 2011 reported a significant increase in CAT activity in all cultivars used in the study, exposed to Cadmium. In a study conducted by Yurekli and Porgeli, 2005 excess copper slightly increased activity of SOD and CAT in the leaves of *Phaseolus vulgaris L.* plants. The higher SOD and CAT activities in Contender (Figures 3.7 and 3.10) indicated that the H_2O_2 scavenging mechanism was more effective than in Timbavati, since CAT activity synchronised with SOD activity, play a central protective role in the O_2^- and H_2O_2 scavenging process, thus the reasoning behind stating that the Contender cultivar was more tolerant to antimony stress than Timbavati cultivar (Zang *et al*, 2006).

In South Africa, it is of immense importance that the economy of the country flourishes, however, this should not be at the expense of the environment. The current anthropogenic activities exercised are adversely affecting the environment, especially the agricultural lands within the country. Heavy metal concentrations have become increasingly accentuated within the environment, and as a consequence plants are absorbing and accumulating these non-essential elements within their cells. Attributable to this occurrence, a mode entry of heavy metals, into the food chain, is established, consequently placing humans at risk. For this reason, it was of grave importance to understand the plants response systems concerning abiotic stresses and similarly recognise the various degrees of tolerance to antimony stress.

Considering all results obtained within this study, a conclusion was reached that the Contender cultivar was much more tolerant to the antimony effects, when compared to the Timbavati cultivar of the *Phaseolus vulgaris L*. plants. It was stated that the Contender cultivars tolerance was owed to the cultivars excellent antioxidative defence system employed within these adverse conditions.

CHAPTER 4

The effects of antimony concentrations on the elemental content of two *Phaseolus vulgaris L.* cultivars

This chapter presents the nutrient analysis of both cultivars of *Phaseolus vulgaris,* the Contender cultivar and the Timbavati cultivar. The second most important grain of legume for human consumption, *Phaseolus vulgaris L.* (the common bean), is recognized per se owing to its great source of protein beneficial for low-income populations. Furthermore, its rich source of carbohydrates, ideal sources of vitamin C, minerals and especially their valuable source of iron (in comparison to other staple crops) results in common beans being of immense importance in South Africa (Castro-Guerrero *et al*, 2016). An additional important sector in South Africa is the mining sector, which contributes significantly to the country's income.

In the past few centuries, the factors of increase population growth, consumption of natural resources and the increase in improper waste disposal techniques have brought about a dramatic increase in pollution and degradation of our environment (Clark *et al*, 2015). In South Africa, anthropogenic practices, such as the mining sector, are one of the major contributors of heavy metal accumulation within the country's environment. The accumulation of heavy metals within the soils are dramatically becoming a dangerous threat to the country, not only in our mining industry but mostly in our agricultural sector (Moskalyk and Alfantazi, 2003; Yadav, 2010). The heavy metal contamination has been seen to not only disturb the intermediate region but also have an effect on a greater region. This effect is mainly due to the major mechanism through which heavy metals can be transported from mining sites to the atmosphere, ground water sources and land (Kamunda *et al*, 2016). This results in the loss of arable land due to the high levels of toxic heavy metal accumulation within the soil, affecting the yield and quality of various crop species. These plants are particularly susceptible to abiotic stresses, and resting on the gravity of the stress and the degree of the plant's tolerance, the harvest could be harshly affected (Castro-Guerrero *et al*, 2016).The presence of heavy metals in soils may affect the availability of other elements within the soil and hence the plant (Chibuike and Obiora,

2014). The ability of plants to accumulate essential metals equally enables them to acquire other non-essential metals, in which, excessive amounts of these non- essential metals are toxic to plants (Chibuike and Obiora, 2014). Metals cannot be broken down, when concentrations within the plant exceed optimal levels, therefore they adversely affect the plant both directly and indirectly (Chibuike and Obiora, 2014).

Nutrient elements are of great importance to plants as they form part of many different key plant processes. The elements within the soil occur in a natural balance, which when absorbed the balance internally serves an essential role in the plants' metabolism. However, this natural balance within the soil and the overall composition of the soil is being transformed by chemical substances produced by humans. Increasing evidence suggests that mineral nutrients play a crucial role in plant stress resistance. (Wang et al, 2013).

It is for that reason that this investigation was of immense importance, to help understand the effects that these metals had on a staple crop, such as the common bean. The effects on the elemental content of common beans needed to be reviewed. The investigation was crucial as negative effects on nutrients of a staple crop, could affect a large percentage of the world's population as these essential nutrients are required in several metabolic reactions.

RESULTS

4.1. Nutrient analysis in *Phaseolus vulgaris L*. cultivars using Inductively Coupled Plasma Mass Spectrometry (ICP) in leaves and roots

Nutrient molecules are of immense importance to plants as they form part of many different key roles in plant processes. Plants and their seeds serve as important sources of Iron, Zinc, Manganese and Copper, however this also provides multiple modes of entry for toxic elements to utilize (Mendoza-Cózatl *et al*, 2014).The variations in the soil and water composition are observed largely based on multiple factors such as the geological locations, environmental conditions and climate change of a particular area. These ecosystems are where the plants mainly absorb all their micro- and macronutrient molecules, which are vital for their growth and development, as some nutrient molecules act as essential co-factors in a variety of biological processes. The molecules within the soil occur in a natural balance, which when absorbed the balance internally serves an essential role in the plants

metabolism (Liu *et al*, 2015). However, this natural balance within the soil and the overall composition of the soil is being transformed by chemical substances produced by humans (Liu *et al*, 2015). The ICP-OES analysis (Section 2.16) was conducted to determine the elemental content of two *Phaseolus vulgaris L.* cultivars, under normal and antimony stress conditions. Comparing the leaves of both cultivars, Contender and Timbavati, both of the cultivars experienced an increase in Iron, Phosphorus and Magnesium uptake when compared to their respective controls (Table 4.1). A decrease was recorded in all the other elements with an exception of an increase in Zinc in Contender treated plants leaves and an increase in Manganese in the Timbavati treated plants leaves (Table 4.1). In the roots of the two cultivars and increase was observed in Iron and Calcium in both cultivars, when compared to their respective controls (Table 4.2). All other elements indicated a decrease in uptake under antimony stress, in both cultivars (Contender and Timbavati) (Table 4.2). Table 4.3 depicts how antimony stress influences the translocation of elements from the roots to leaves. The translocation of Manganese, Potassium and Phosphorus increases in both cultivars under antimony treatment and the other elements all indicates a decrease.

Table 4.1: Full nutrient profile of two *Phaseolus vulgaris* L. cultivar leaves (mg/kg).

		Leaf			
		Contender		Timbavati	
		0 µM	500 µM	0 µM	500 µM
Micronutrients	Iron	0.009^{a}	0.018^{b}	0.002^{c}	0.007^{a}
	Copper	0.002^{a}	0.001^{a}	0.004^{a}	0.001^{a}
	Zinc	0.049^{a}	0.0241^{b}	0.063^{c}	0.019^{d}
	Manganese	0.136^{a}	0.097^{b}	0.112^{a}	0.117^{a}
Macronutrients	Calcium	11.462^{a}	7.511^{b}	8.844^{c}	8.315^{d}
	Potassium	52.954^{a}	50.637^{b}	64.472^{c}	53.897^{d}
	Phosphorus	1.774^{a}	2.259^{b}	3.104^{c}	3.424^{d}
	Magnesium	3.269^{a}	3.051^{b}	2.486^{c}	3.244^{a}

The different letters indicate the significant difference between means at P<0.05 (DMRT). The values are means S.E (N=4).

Table 4.2: The full nutrient profile of two *Phaseolus vulgaris* L. cultivar roots (mg/kg).

		Roots			
		Contender		Timbavati	
		0 µM	500 µM	0 µM	500 µM
Micronutrients	Iron	0.017[a]	0.034[a]	0.003[a]	0.004[a]
	Copper	0.008[a]	0.004[a]	0.007[a]	0.003[a]
	Zinc	0.236[a]	0.183[b]	0.319[c]	0.151[b]
	Manganese	0.149[a]	0.083[b]	0.149[a]	0.079[b]
Macronutrients	Calcium	3.818[a]	5.370[b]	2.694[c]	2.831[d]
	Potassium	23.175[a]	16.623[b]	32.693[c]	18.493[d]
	Phosphorus	4.127[a]	2.726[b]	4.071[c]	2.779[b]
	Magnesium	0.930[a]	0.917[a]	1.134[b]	0.850[b]

The different letters indicate the significant difference between means at P<0.05 (DMRT). The values are means S.E (N=4).

Table 4.3: Translocation factors, between the roots and leaves, of all the elements of two *Phaseolus vulgaris L.* cultivars.

		Translocation Factor			
		Contender		Timbavati	
		0 µM	500 µM	0 µM	500 µM
Micronutrients	Iron	0.559[a]	0.541[a]	0.697[b]	2.064[c]
	Copper	0.302[a]	0.144[b]	0.533[c]	0.372[d]
	Zinc	0.207[a]	0.131[b]	0.197[a]	0.123[b]
	Manganese	0.910[a]	1.176[b]	0.753[c]	1.485[d]
Macronutrients	Calcium	3.002[a]	1.399[b]	3.28[c]	2.937[a]
	Potassium	2.285[a]	3.046[b]	1.972[c]	2.914[b]
	Phosphorus	0.430[a]	0.829[b]	0.763[c]	1.232[d]
	Magnesium	3.514[a]	3.328[b]	2.192[c]	3.816[d]

The different letters indicate the significant difference between means at P<0.05 (DMRT). The values are means S.E (N=4).

4.2. Antimony content

Antimony (Sb), as an analog of arsenic (As), has been listed as a priority pollutant by the United States Environmental Protection Agency and the European Union (Cui *et al*, 2015). Sb has no known biological function (Filella *et al*, 2002) and is toxic, even in trace amounts. The ICP-OES analysis (Section 2.16) was conducted to determine the elemental content of two *Phaseolus vulgaris L.* cultivars, under normal and antimony stress conditions as well determine the antimony content of leaves and roots within each cultivar. Figure 4.1 depicted that the Contender cultivar absorbed ~2 folds and ~6 folds of excess antimony in the leaves and roots respectively, when compared to their controls. The Timbavati cultivar on the other hand absorbed ~8 folds and ~10 folds of excess antimony in the leaves and roots respectively, when compared to their controls.

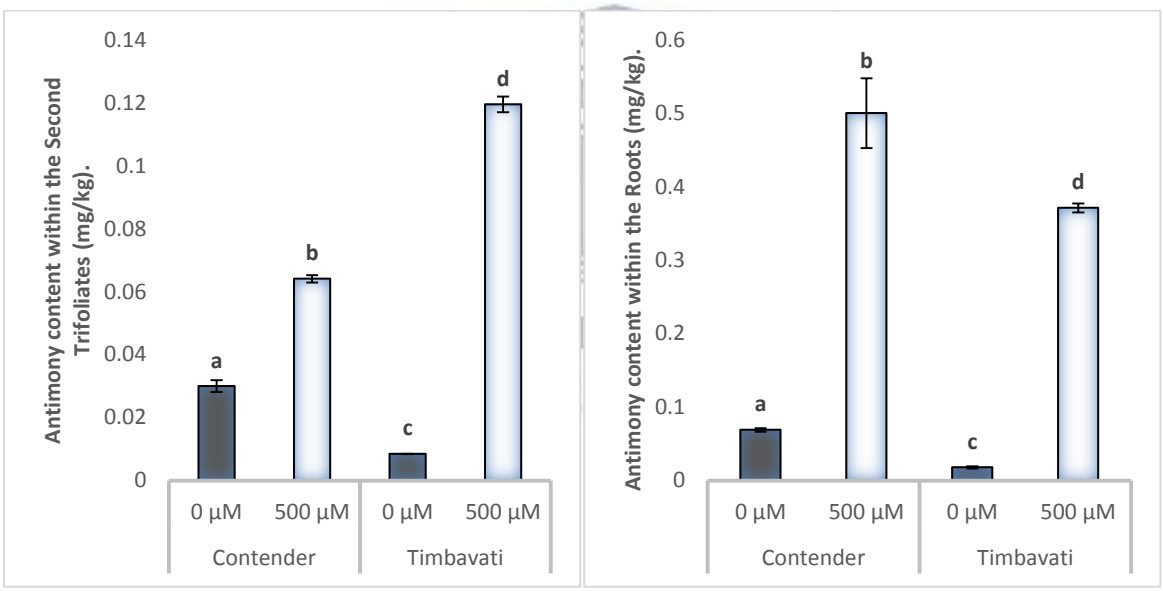

Figure 4.1: The uptake of antimony into two *Phaseolus vulgaris L.* cultivars. Antimony was administered to two cultivars, and the antimony content were analysed within the leaves and roots. The Different letters indicate the significant differences between the means a P< 0.05 (DMRT). The values are means ± S.E (N=10).

DISCUSSION

4.3. Nutrient analysis using ICP-OES

The work expressed here investigated the effects of antimony on the elemental absorption and translocation profiles, of two *Phaseolus vulgaris L.* cultivars, Contender and Timbavati. Experiments were conducted to determine and analyse how antimony affects the mineral content of the plants and to determine a possible route of entry for antinomy into the plant system, by comparing the changes in uptake of micro- and macro-nutrients, using Inductively Coupled Plasma Mass Spectrometry (ICP). Finally, the results collected were used to determine the degree of tolerance of each cultivar to antimony stress.

Micronutrients are vital in the control of the activity of many cellular organelles; these molecules are required only in minute quantities within the plants (da Silva Lobato *et al*, 2016).

Examining Table 4.1 and Table 4.2 it was shown that with an introduction of 500 µM of antimony, to both *Phaseolus vulgaris L.* cultivars, an increase in Iron content was recorded within the leaves and roots. A higher fold increase of Iron uptake was recorded in the Contender cultivar under stress when compared to its control and compared to the treated Timbavati cultivar. This increase in Iron content, observed in both cultivars, could be observed as the cultivar attempting to maintain a sense of homeostasis or possibly counteracting the antimony stress, by increasing its Iron uptake. Iron is of great importance in the chlorophyll pathway, mainly since this element promotes the development of chlorophyll molecules. The results obtained in Table 4.1 and 4.2 could correlate to the chlorophyll results in Table 3.1. as even though a decrease was observed in both chlorophyll species under antimony stress, the overall Contender cultivar exhibited a lesser degree of decrease in its chlorophyll content, when compared to the Timbavati cultivar. This result indicated that the increase in Iron uptake aided in reducing the effects of antimony on the chlorophyll content and possibly promoting the formation of chlorophyll.

Iron molecules additionally act as oxygen carrier and are involved in reactions involved in cell division and plant growth (Ravet and Pilon, 2013). Naturally, Contender was a larger plant when compared to Timbavati (Figure 3.1.1-3.1.3) however the increase Iron uptake

could have explained the result of almost no physiological change in the Contender cultivar biomass (Figure 3.1.4). Contrary, Timbavati did not represent the same mechanisms, because even with an increase in iron uptake, under antimony stress, this cultivar still exhibited a significant decrease in plant biomass (Figure 3.1.4), which could have indicated that other factors were inhibiting the growth of Timbavati plants under stress. Surprisingly, neither one of the cultivars experienced chlorosis; this could be attributed to the result of increase Iron uptake. Ravet and Pilon, 2013 validated this claim, as they stated that photosynthesis and the photosystem rely greatly on essential metals, such as Iron and Copper. They recorded that there was a larger concentration of Iron and Copper located in chloroplast. Thus, the increase in Iron possibly maintained enough chlorophyll production to not result in chlorosis.

Observing the Copper content within both cultivars leaves and roots (Table 4.1 and 4.2) it was illustrated that both cultivars experienced a significant decrease in Copper content within the leaves and roots. The degree of reduction was much higher in roots when compared to the leaves, of both cultivars. Copper is of immense importance within plants as it catalyses several plant processes and serves a major function within photosynthesis and the reproductive stages within plants (Ravet and Pilon, 2013). da Silva Lobato and colleagues, 2016 indicated that a decrease in Copper could account for the structural changes observed in leaves. In both cultivars this deficiency could be observed within their treated plants leaves when compare to the controls. Figure 3.1.1, 3.1.2 and 3.1.3 illustrated these effects, as the leaves appeared smaller when compared to their control plants. A decrease in Copper could have attributed to the ineffectiveness of the Timbavati antioxidant defence system, especially in relation to superoxide dismutase, SOD, enzymes. Copper is a co-factor for SOD (Cu/ZnSODs) and a decrease in this nutrient possibly affected the functioning of the SOD enzymes, thus could possibly be the reason why Timbavati's SOD scavenging abilities were not effective in scavenging superoxides, ultimately resulting in significant damage to the cultivar. A study conducted by Yurekli and Porgeli, 2005 indicated that in the leaves of *Phaseolus vulgaris L.* plants, in excess Copper, depicted an increase in SOD activity and decrease in damage was observed, thus validating that Copper and SOD levels correlated with each other.

Another element uptake that was significantly affected by antimony stress, was Zinc (Table 4.1 and 4.2). Generally from the tables, it was observed that both cultivars absorb high amounts of Zinc, especially Timbavati under normal conditions. Timbavati exhibited higher translocation of Zinc to aerial components of plant. However, this cultivar also illustrated the largest decrease in Zinc uptake and translocation under antimony stress. Zinc is very important in plant growth hormones and enzyme systems, and also essential for chlorophyll production. The significant reduction in Zinc content under stress in Timbavati could possibly be another factor, which could be affecting the growth of plants. Zinc serves as an activator of enzymes systems; therefore the significant decrease in Zinc, in Timbavati, could possibly explain the inadequate functioning or production of SOD, APX and CAT enzymes, these enzymes that are of great importance in the plants defence system. A decrease in Zinc content in both cultivars under stress could represent a possible use of this channel as a mode of entry, of antimony, within the plants, thereby significantly decreasing Zinc. Ravet and Pilon, 2013 indicated the importance of Zinc in SOD enzymes. Zinc is a co-factor for SOD enzymes, Cu/ZnSODs. Thus, the decrease in Zinc coupled with the decrease observed in Copper could have drastically reduced the efficiency of SOD enzymes ability to scavenge superoxide. Thereby, explaining the results observed in Figure 3.4 in which superoxide were increased.

In Table 4.1 and 4.2, Manganese which was recorded to be involved in chlorophyll synthesis and also involved in certain enzyme systems, showed a decrease in both Contender and Timbavati roots and Contender leaves. Interestingly, an increase was recorded in the Timbavati leaves which can be observed in Table 4.3, illustrating the translocation factor of each element. An increase in this case could be possibly one of Timbavati's defence mechanisms or a response to the reduction in chlorophyll content experienced by the plant. Timbavati could be trying to combat the adverse effects experienced in the chlorophyll system by increasing the translocation of manganese to the leaves. Manganese also plays a role in increasing the availability of Phosphorus, and the results in Table 4.1 and 4.2 in the macronutrients correlated to this statement, as the Phosphorus content was not greatly affected by the antimony stress.

Macronutrients are a collection of nutrients required in sizable quantities and are essential for the development and the physiological activity of plants. They are of very crucial in the maintenance of the plant structure (da Silva Lobato *et al*, 2016).

Observing Table 4.1 and 4.2, all macronutrients (except phosphorus in leaves) experienced a decrease in uptake and translocation within the plants. The Calcium content in the leaves of both cultivars illustrated a decrease in content. Calcium is important as this element is involved in strengthening cell walls and is involved in stimulating root tip elongation (Van Martins *et al*, 2013). Interestingly, Calcium in Contender roots, under stress, increased. This was of importance as this could be a possible mechanism that Contender cultivars implemented under antimony stress to avoid root damage. This could also have been a mechanism employed to strengthen root walls when in contact with contaminated soil, possibly by means of reducing the uptake of the heavy metal. Ahmad and colleagues, 2015 illustrated that under Cadmium stress the increase in Calcium reduced the negative effects cadmium had on root length and development, in mustard plants. The results obtained in this investigation could possibly illustrate that Contender cultivar adopted the same mechanisms to try and alleviate the effects of antimony on root development, by strengthening root cell walls.

The increase of Calcium recorded in Timbavati could explain why the MDA levels (Figure 3.7) in the leaves were not significant. Calcium exhibited important roles in stabilizing membranes, thus strengthening these walls from lipid peroxidation caused by accumulation of ROS molecules. Ahmad and colleagues, 2015 validated this result as they recorded a decrease in their MDA levels with an increase in calcium, under Cadmium stress.

In both cultivars roots and leaves an increase in Phosphorus and Magnesium content is experienced, in the leaves. Phosphorus is essential as it forms part of the protein molecule and is essential for the transport of energy (Song and Liu, 2015). Magnesium on the other hand forms part of the chlorophyll molecule, therefore this element is essential on the photosynthesis process (Niu *et al*, 2016).Increases in these elements, in the leaves could possibly have been a defence mechanism adopted by *P. vulgaris* against metal stress. Timbavati's result in decrease in root growth and development could be attributed by the decrease in Phosphorus in roots. Khan and colleagues, 2016 recorded that a deficiency in

Phosphorus led to reduce in growth in primary root elongation. The decrease levels of Magnesium in leaves could have been attributed to the reduction in chlorophyll content in the leaves of Contender cultivar, however this effect was not as significant as the increase in Iron content recued the decrease in Magnesium in the chloroplast. Niu and colleagues, 2016 illustrated similar results in which in Arabidopsis thaliana a decrease in chlorophyll was observed under decrease Magnesium content. The increase in Magnesium content in Timbavati could have been a defence against antimony stress, as it could have been seen as the plants trying to combat the effects antimony on plants.

The results obtained in this study (Table 4.1 and 4.2) indicated that both cultivars had the largest Potassium content when compared to the other elements. In literature it is shown that plants require more potassium than any other nutrient except nitrogen. With overall observation, Timbavati illustrated the largest decrease in Potassium in the treated plant when compared to the control plants and Contender cultivar. This decrease in Potassium could be due to Antimony using the Potassium channel as a means of entering the plants or it could be due to Timbavati trying to reduce uptake of Antimony so it reduces the functioning of the Potassium channel. The Contender treated plants also indicated a decrease in Potassium, but not as great as observed in Timbavati, when compared to its control. This reduction could be indicative that the cultivar still experiences a stress.

The possibility of Timbavati using the Potassium channel in trying to reduce antimony absorption and translocation could be detrimental to the cultivar. Potassium is an essential plant nutrient and required in large amounts for proper growth and reproduction of plants. This element is known to affect plant shape, size, colour and taste. Potassium has various roles within plants. In photosynthesis, Potassium regulates the opening and closing of stomata, thus regulating CO2 uptake. This element also triggers the activation of enzymes and is an essential element for the production of Adenosine Triphosphate (ATP). Thus by the possibility of Timbavati using this mechanism, it is disrupting all these processes in the plant.

Furthermore, Potassium has a broad range of roles in many growth related enzymes in plants. Deficiency in Potassium in plants results in slow or stunted growth, owning to Potassium being an important growth catalyst in plants, a deficiency will result in slower or

stunted growth. Poor Potassium uptake will result in less water circulation in the plant. This will make the plant more susceptible to drought and temperature changes. If the Potassium deficiency is left unattended it will result in the plants losing their leaves sooner than they should.

Table 4.3 illustrated the translocation of element in both cultivars. Table 4.3 indicated that in the Contender cultivar 5 elements exhibited a decrease in translocation further up into the plant, whilst Timbavati illustrated 3 elements decrease in aerial part. A possible reasoning behind why Contender exhibited more decrease in translocation could be owed to its possible defence mechanisms, reducing the possible modes of translocation of antimony to the edible, aerial components of the plants. Timbavati retaining its translocation abilities could be the possible reason why this plant fairs worse than Contender, as the normal translocation of elements in the plants introduced multiple modes that antimony could have utilize to enter the aerial components.

Within all plants the cell membrane serves a vital role in metal homeostasis, these enzymes can inhibit or diminish entry into the cell. Hence, plants that are experiencing heavy metal stress can adopt particular uptake mechanisms, in doing this the plants inhibit entry of toxic ions into the cells (Hall, 2001). In literature there is a study conducted on arsenic toxicity, in which this study indicated an example of low uptake as an adapted tolerance mechanism. The study was conducted on *Holcus lanatus* roots and the authors suggested that the phosphate and arsenate was taken up by the same system. They recorded a reduced rate of uptake of both anions; therefore suggesting that the suppression of high affinity transport system was a good possibility to tolerance (Meharg and Macnair, 1992). Therefore the decrease in the Contender cultivar could possibly have been observed as a defence mechanism employed under antimony stress, to help the plant avoid the possible uptake and translocation of antimony into the plant (into aerial parts of the plant), the plant essentially decreases the uptake of all nutrients into the plant. The reverse could be happening in the Timbavati cultivar where an increase in the uptake of nutrients could possibly expose the plant to excessive antimony uptake as well, however, further studies needs to be conducted, such as the antimony uptake and translocation within the *Phaseolus vulgaris L.* cultivars.

4.4. Antimony absorption and content within plants

Conducting the full elemental content was of great importance to aid in understanding what exactly was occurring in the plants and with the elements. Thus, it was essential, with the full elemental content, to incorporate the investigation of antimony uptake, accumulation and translocation within plants, especially the two *Phaseolus vulgaris L.* cultivars. Figure 4.1 recorded levels of antimony in the controls, in the leaves and roots of both cultivars. This finding was important, as it represented exactly how serious the problem of antimony contamination is within South African soils, that antimony has to be incorporated in the silica sand to mimic the conditions of South African soils. Smith and Huyck, 1999 mentioned that the crustal abundance of antimony was 0.2mg/kg. The amount of antimony absorbed by the cultivars roots were about 0.1mg/kg in Contender and less in the Timbavati cultivar, within their controls. This finding was significant because in potting soil alone the amount of antimony could possibly be more than 0.2mg/kg, this soil which is a scale down version that of the natural environment (which should be much higher concentration). This was significant as it represented just how much the antimony pollution has increased over the course of 18 years that potting soil alone possibly contained 0.1mg/kg of antimony. Thus it would be beneficial in analysing the concentration of antimony within the soil mixture (Silica sand: potting soil) before the commencement of the antimony treatments and at the end of the treatment period.

Figure 4.1 depicted that the Contender cultivar absorbed ~2 folds and ~6 folds of excess antimony in the leaves and roots respectively, when compared to their controls. The Timbavati cultivar on the other hand absorbed ~8 folds and ~10 folds of excess antimony in the leaves and roots respectively, when compared to their controls. Overall, it appeared that the Contender cultivar absorbed more antimony from the soil, indicating that it had an active mode of entry into the plants. The large content of antimony coupled with low effect on Contender cultivar could have been due to the antimony recorded in these plants could be possibly located mostly in the vacuoles for degradation, rather than the other organelles. The Contender cultivar could have been using this mechanism in order to establish homeostasis in the plant.

The uptake of antimony into the two cultivars showed a pattern with the changes in uptake in Copper, Zinc and Potassium, leading to the conclusion that perhaps one of these, or some of these, channels were being utilized by antimony for the uptake into the plants. However, further analysis needs to be conducted to support this. Timbavati also illustrated a large uptake of antimony within the plants, in the treated roots. Larger concentrations of antimony was expected in the roots since this was the part of the plant that was in constant contact with the soil and antimony, therefore, higher accumulation of antimony was recorded. The high absorption rate of antimony into the Timbavati plants, when compared to the Contender cultivar, could possibly explain the reasoning behind why the growth of the Timbavati cultivar was drastically reduced, when compared to the control (Figure 3.1.3).

Figure 4.2 illustrated that Contender absorbed more antimony however less antimony was translocated further up into the plant when compared to Timbavati. This could possibly have been one of the reasons why Contender fared better than Timbavati, as it represented a sufficient antioxidant defence system and also showed promise of a good hyper-accumulator. On the other hand, Timbavati illustrated a large translocation ratio.

In the near future it would be interesting and important to analyse whether Contender cultivars accumulates any antimony within its stem, to avoid the translocation further into the aerial parts, such as the seeds and flowers (edible parts). This is important as the molecular mechanisms of loading of essential elements and non-essential elements into the seed are still largely unknown. Therefore, looking at metal accumulation and distribution would be important and interesting. Investigating these topics, would allow for the development of strategies to omit metals from seeds and thus also improving nutrient value of seeds. Another approach would be to record the concentration of antimony within the soils to observe whether the increase in uptake of antimony by Contender aids in decreasing the concentration of antimony within the soils. Finally to establish the transport mechanisms used for the uptake and translocation of antimony within the *Phaseolus vulgaris L.* cultivars.

Chapter 5

CONCLUSION AND FUTURE WORKS

In South Africa, it is important that the economy of the country flourishes, however, this should not be at the expense of the environment. The current anthropogenic activities exercised are adversely affecting the environment, especially the agricultural lands within the country. Heavy metal concentrations have become increasingly accentuated within the environment, and as a consequence plants are absorbing and accumulating these non-essential elements within their cells. Attributable to this occurrence, a mode of entry of heavy metals, into the food chain, is established, consequently placing humans at risk. For this reason, it is of great importance to understand the plants response systems concerning abiotic stresses and similarly recognise the various degrees of tolerance to antimony stress.

The current study ascertained that under excessive concentrations of antimony, the two *Phaseolus vulgaris L.* cultivars exhibited detrimental effects. These effects were witnessed in the physiology, of the two cultivar leaves, in which a decline in the growth and biomass was noted. The Contender cultivar, however, experienced fewer effects to physiology and biomass, when compared to the Timbavati cultivar. Reviewing the overall oxidative stress on the two cultivars, it was noted that in the presence of high antimony concentrations; there was an upsurge in O_2^- and H_2O_2, which ultimately led to an upsurge in lipid peroxidation within the leaves. The oxidative stress was greatly experienced in the Timbavati cultivar. Therefore, to understand how the plants defend against oxidative stress, the antioxidative system was also investigated. Reviewing the Contender cultivar's production of SOD, APX and CAT, in response to ROS molecule production, this cultivar exhibited a proficiently operated antioxidative system which presented them with a slightly higher tolerance to antimony, when compared to the Timbavati cultivar. To further understand the effects of antimony on these two cultivars, an elemental analysis was conducted to observe how the uptake and concentrations of micro- and macronutrients were affected.

Considering all results obtained within this study, it was concluded that the Contender cultivar was much more tolerant to the antimony effects, when compared to the Timbavati

cultivar of the *Phaseolus vulgaris L.* plants. It can be stated that the Contender cultivars tolerance is owed to the cultivars excellent antioxidative defence system employed within these adverse conditions.

Future work, on this study, will encompass a proteomic approach in revealing, on a molecular level, how excessive concentrations of antimony affect the *Phaseolus vulgaris L.* cultivars. This work will be conducted to observe which particular antioxidant enzymes are up-regulated or down-regulated within the plants under antimony stress. Further analysis of isoforms of the various antioxidative enzymes will also be established to support this analysis. The antimony uptake and translocation will also be assessed to aid in explaining why particular damage is highly experienced in specific sections of the two *Phaseolus vulgaris L.* cultivars, Contender and Timbavati. Furthermore, evidence obtained will be beneficial in providing food security of beans for human consumption, gain knowledge for plant cultivation strategies and to produce highly heavy metal tolerant bean crops.

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