Effect of *Fusarium oxysporum* on the physiological and molecular responses of cowpea plants

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

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KEYWORKS

Ascorbate peroxidase

Biotic stress

Cell death

Cowpea

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Hydrogen peroxide

Lipid peroxidation

Pathogen infection

Photosynthetic pigments

Reactive oxygen species

Superoxide

Superoxide dismutase

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ABSTRACT

Cowpea is a tropical, drought-tolerant legume crop that is indigenous to Africa. The crop is of nutritional importance to both animals and humans as part of their diet. Stress to crop plants is defined as any factor that negatively influences the growth and reproduction of a plant below the capabilities of the genotype. Due to biotic stress conditions, crops production and its availability is declining, thus threating food security in many parts of the world. Plants are governed by physical and biochemical processes which occur both internally and externally in order to function and sustain life. However, certain external changes results in the plant responding by altering its internal processes for survival, which in turn alters the physiology and biochemistry of the plant. The soil borne pathogen, *Fusarium oxysporum*, is a major cowpea fungal pathogen and it has profound implications on the development of infected cowpea, resulting in *Fusarium* wilt. *F. oxysporum* is soil born and in near perfect conditions infects the plant thus causing an imbalance in nutrients uptake and accumulation of toxic phytochemicals. This causes a cascade of biochemical alteration which led to physiological changes. Therefore, this study investigated the influence of *F. oxysporum* infection on the physiological and molecular responses of cowpea plants.

Cowpea seeds were inoculated with *Fusarium oxysporum* spore suspension (4.9×10^6 cells/ml) and were allowed to germinate for a period of 5 days. Changes in plant growth

parameters (shoot fresh weights and shoot length), relative water content and chlorophyll content were measured after 21 days post infection. In addition, changes in reactive oxygen species accumulation (as seen for superoxide and hydrogen peroxide), the extent of lipid peroxidation and antioxidant enzyme activities (superoxide dismutase, ascorbate peroxidase and glutathione reductase) were measured in the shoots of cowpea plants.

Seed germination (over a 5-day period) was significantly increased when inoculated with *F. oxysporum* (4.9×10^6 cells/ml). However, the opposite response was observed for the various plant growth parameters including biomass production. Water retention and photosynthetic metabolism (as seen for total chlorophyll content) was restricted in the shoots of plants infected with *F. oxysporum* when compared to the controls. A significant increase in O_2^- and H_2O_2 was observed in the shoots of cowpea plants infected with *F. oxysporum*. This increase in reactive oxygen species resulted in an increase in cellular death albeit not due to excessive levels of lipid peroxidation. Antioxidant enzymes (superoxide dismutase, ascorbate peroxidase, and glutathione reductase) were differentially regulated (as seen for individual isoforms) to control the increase in reactive oxygen species accumulated in the shoots of cowpea plants. Given the increase in reactive oxygen species, coupled with reduced photosynthetic metabolism and differential antioxidant capacity we suggest that cowpea plants are sensitive to *F. oxysporum* infection. However, the noted differentially antioxidant activity observed here can be utilized to enhance cowpea defenses against plant pathogens.

DECLARATION

I declare that "Effect of *Fusarium oxysporum* on the physiological and molecular responses of cowpea plants" is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Mihlali Badiwe December 2017

Signed ….. …..

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LIST OF ABBREVIATIONS

µg	Microgram
µl	Microliter
ANOVA	Analysis of variance
APX	ascorbate peroxidase
AsA	ascorbic acid
BSA	bovine serum albumin
CAT	catalase
CBB	coomassie brilliant blue
Cu/Zn – SOD	copper zinc superoxide dismutase
ddH_2O	Double distilled water
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DW	Dry weight
EDTA	ethylenediaminetetraacetic acid
Fe – SOD	iron superoxide dismutase
FW	Fresh weight
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidised glutathione
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
K_2HPO_4	Potassium phosphate dibasic
KCN	Potassium cyanide

KH_2PO_4	Potassium phosphate monobasic
KI	Potassium iodide
MDA	malondialdehyde
mg	Miligram
ml	Mililitre
Mn – SOD	manganese superoxide dismutase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	nitroblue tetrazolium
NOX	NADPH oxidase
PAGE	polyacrylamide gel electrophoresis
PDA	Potato Dextrose Agar
PMS	Phenazine methosulfate
PVP	polyvinylpyrrolidone
ROS	reactive oxygen species
RWC	Relative water content
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TEMED	N,N,N′,N′ - Tetramethylethylenediamine
TW	Turgid weight

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CHAPTER 1

LITERATURE REVIEW

1.1 *Vigna unguiculata* (cowpea)

Vigna unguiculata L. Walp (cowpea) is a tropical legume crop that is deemed drought-tolerant. Cowpea is indigenous to Africa and it has been cultivated in sub-Saharan Africa for centuries. Other countries around the world with warm climates also cultivate the legume (Singh and Rachie, 1985). Legumes are one of the most important crops available, contributing major impacts on agriculture and the environment, due to their ability (with soil-borne bacteria) to fixed atmospheric nitrogen to the soil, making it beneficial for crops plants (Graham and Vance, 2003). Cowpeas are very nutritious crops, providing animals and humans with major nutrients like vitamin B9, iron, copper, phosphorus and tryptophan. The crop also provides various health benefits like intestinal cancer preventions, anemia prevention, healthy metabolism promotion, strong bone development and muscle tissue repair, reduction of cardiovascular disease and diabetes (Trinidad *et al.* 2010).

In South Africa, cowpea is mostly cultivated in Limpopo, Mpumalanga, Gauteng, North-West and Kwa Zulu Natal. Cowpea cultivation requires temperatures above 10 °C for seed germination and between 20 °C and 30 °C for optimal growth and development (van Rensburg *et al.* 2007). The crop is cultivated for many uses whereby the leaves and growth points are picked utilized in vegetable dish, dried leaves are utilized as a meat substitute, and the green seeds can be roasted like peanuts and thus utilized as a substitute for coffee. Seeds can also be dried and ground to be fried in oil with spices and onions (Giami, 1993). Apart from cooking to be eaten, the seeds can

also be used as green manure and can be sown to serve as an intercrop. Cowpea green leaves can be mixed with maize and sorghum to make silage for animal feed (Wood, 2012).

Cowpea has been used as an intercrop in some farms in South Africa and farmers increase their crop production by up to 1.5 t/ha due to the extra nitrogen in the soil fixed by the crop (Segun-Olasanmi and Bamire, 2010). In South Africa the total income and expenditure in agriculture increased by 18.4% and 16.4% respectively, in the 2014/15 season. However, there have been numerous reports on fusarium infection in legumes which are surfacing, which affect production of the crops and increases expenditure because of the numerous cycles of fungicide application and temperature controlled storage facilities. Furthermore this poses a threat to humans consuming the crop and animals grazing on it.

Figure 1.1: *Vigna unguiculata* L. Walp field plant, with purple flower.

1.2 Fungal pathogens

Fungi comprises of spore-forming, eukaryotic, non-chlorophytic organisms which are mostly branched and filamentous in morphology (Petersen, 2013). This is a large kingdom of approximately 100 000 species which are saprophytes, however only above 20 000 known species are parasitic and have the ability to cause disease in crops (Petersen, 2013). Furthermore, parasitic fungi are the most prevalent plant pathogens, taking in to consideration that all plant species are susceptible to attack from at least one or more species of phytopathogenic fungi (Ma and Michailides, 2005). In addition, a single species of fungi is able to parasitize one or more different plant species. This has proven to be a continuous problem in agricultural development whereby certain food crops are infected by phytopathogenic fungi. This has a negative impact on crop yield and the health of people and animals once ingested.

1.2.1 *Fusarium*

Fusarium is a vast genus of a group of filamentous fungi which can be referred to as hyphomycetes. It consists of widely distributed fungi which are soil-borne and are associated with plants. Some species in this group are regarded as harmless and are abundant constituents of the soil microbial community (Howard, 2003). However, other species like (but not limited to) *Fusarium graminearum, Fusarium verticiloides and Fusarium oxysporum* are considered harmful as they are known to produce mycotoxins in cereal crops and legumes, which are secondary metabolites; namely fumonisins and trichothecenes. These mycotoxins can affect human and animal health once introduced to the food chain (Howard, 2003). For the purpose of this thesis literature review, *F. oxysporum* will be the main focus.

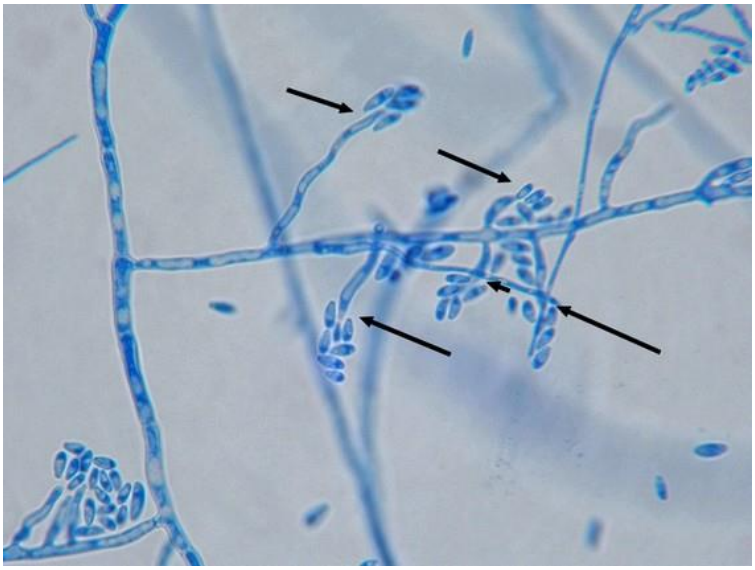

Figure 1.2: *Fusarium oxysporum*, arrows point to tips of phialides where microconidia are produced and accumulate (UWO, 2012).

1.2.1.1 Fusarium oxysporm

Fusarium oxysporum is prominent species in the soil-borne fungi community, available in most soil types around the world. These species appear in fungal communities in the rhizosphere of plants (Burgess, 1981; Gordon and Martyn, 1997). All known *Fusarium oxysporum* strains grow and thrive for long periods in various organic matter including some plant species, making them saprophytic. Furthermore, *Fusarium oxysporum* is known to be pathogenic to different plant species as they infiltrate the roots, and can invade the vascular system (Garrett, 1970). A well-known trait of *Fusarium oxysporum* is to cause *fusarium* wilt, which severely damages crops, thus resulting in heavy economic implications. Synonymous symptoms of *Fusarium* wilt in cowpea plants is noticeable by stunted growth during development, leaf chlorosis, premature defoliation and withering of leaves to leading to a brownish purple colour on the leaves (Singh and Rachie, 1985; Davis *et al.* 1991). Seedlings are prone to rapid wilting which leads to death in the case of severe infection. Furthermore, transmission is mediated through the

soil since *fusarium* is soil borne and sometimes thought the seeds (Singh *et al.* 1997). *Fusarium oxysporum* has a high plant host specificity which depends on the plant cultivar (Armstrong, 1981).

1.2.1.2 Reproduction of *Fusarium oxysporum*

Fusarium oxysporum is one of the fungal species that have sexual stage, however it is known for its production of three kinds of asexual spores; namely chlamydospores, macroconidia and microconidia (Snyder and Hansen, 1940). Chlamydospores are produced in pairs or singly and can occur in clusters in the soil. Their morphology is described as being round thick walled spores which are produced in a mature macroconidia (Sneh *et al.* 1984). Unlike other spores, chlamydospores are able to survive in soil for elongated periods. Macroconida occur on sporodochia, which are found in the surface of infected plants, where they are found in groups of three to five cells and have a morphology which is known to be pointed, with curved edges (Jibril *et al.* 2016). The most common of the produced spores are the microconidia which are produced on aerial mycelia and they have an elliptical shape (Diamond and Cooke, 1997).

1.2.1.3 Disease cycle of *Fusarium oxysporum*

Fusarium oxysporum survives as mycelia and other spore types in the soil, however more commonly as chlamydospores (Snyder and Hansen, 1940). The *fusarium oxysporum* infection spreads in two ways; it can either spread over relatively short distances by rain water flow, and through contaminated planting equipment or over long distances by infected transplants and seeds. Furthermore, once in contact with the specific host crop, it can infect it by germinating the spores and entering the plants roots

through the tip or through root wounds. The germinated mycelium thus progresses intracellularly up the roots and enters the xylem. The mycelium remains in the xylem and produces microconidia which are thus able to translocate up the plant. The accumulation of these mycelia and spores thus restricts nutrient flow and the plant hyper transpires. This leads to stomatal closure and nutrient deprivation. The plant suffers and eventually wilts and dies, and subsequently the fungus degrades the plant tissue and sporolates, thus infecting neighboring crops (Sastry, 2013). In contrast, the oxidative burst known as overproduction of reactive oxygen species (ROS) is the initial cellular response of plants subsequent to successful recognition of pathogen invasion (Auh & Murphy, 1995).

1.3 Reactive oxygen species (ROS)

Molecular oxygen (O_2) known as a free radical, contains two unpaired electrons with the same spin. This phenomena gives O_2 a high affinity for free electrons, thus forming the superoxide radical (O_2^-) which leads to the production of ROS (Gill and Tuteja, 2010). ROS are continuously produced in plants via metabolic pathways which occur in different cellular organelles such as the chloroplast, mitochondria and peroxisomes. The chloroplast house the thylakoid membrane systems for light capturing, whereas the mitochondria is the energy house for cells, producing energy in the form of adenosine triphosphate (ATP) and peroxisomes facilitate the degradation of long chain fatty acids (Luis *et al.* 2006). Thus in non-stress related circumstances ROS molecules function as signaling molecules used to activate various essential pathways, however they are closely regulated by enzymatic and non-enzymatic antioxidative mechanisms. These antioxidative mechanisms function to maintain equilibrium of ROS molecules,

eliminating the risk of oxidative stress (Foyer and Noctor, 2015). The regulated ROS equilibrium can easily be manipulated by biotic stress factors to favour intracellular ROS accumulation which causes severe damage to protein, lipids and DNA, leading to programmed cell death (PCD) (Bhattacharjee, 2005).

Biotic stressors much like *Fusarium oxysporum* infection of plants induce a cascade of metabolic pathways, serving as the earliest cellular response after successful detection of pathogen presence. The apoplastic production of O_2^- and its dismutated counterpart hydrogen peroxide (H_2O_2) have been reported subsequent to the recognition of various pathogen infections in plants (Doke, 1983; Grant *et al*. 2000). During pathogen infection ROS plays multiple important roles between the pathogen and the host. Furthermore during pathogenesis signaling by NADPH oxidase (NOX) induces an oxidative burst together with PCD response. This is the central core of plant defense against infectious pathogens (Apel and Hirt, 2004). The pathogen – plant host interaction can be observed in figure 1.3, whereby various pathways that lead to ROS production are highlighted.

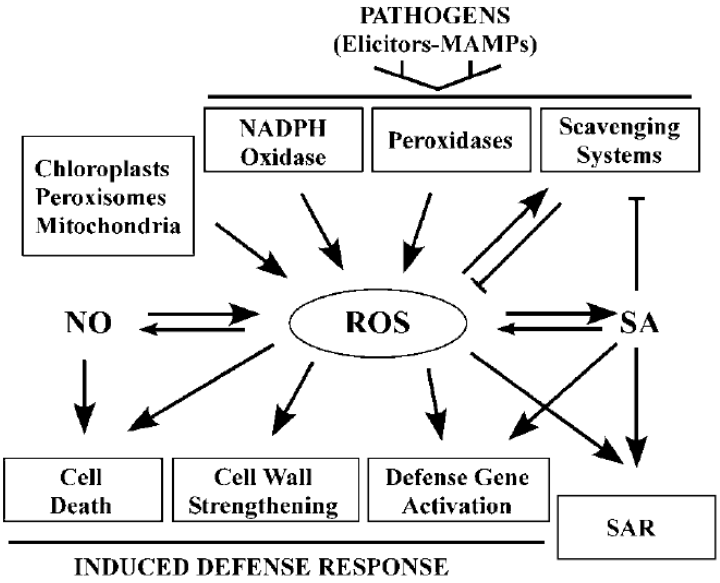

Figure 1.3: ROS production and functions in response to pathogens (Torres *et al.* 2006).

In plants, ROS acts as damaging molecules, protective molecules as well as signaling molecules, totally depending on the obscure equilibrium between ROS production and scavenging at crucial site and certain time (Gratão *et al.* 2005). Thus, ROS accumulation by stress induction is closely regulated by enzymatic antioxidants such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), catalase (CAT) and glutathione reductase (GR), together with non-enzymatic antioxidants such as ascorbic acid (ASH), glutathione (GSH), α-tocopherol, flavonoids, carotenoids and non-protein amino acids (Gill and Tuteja, 2010). These antioxidants are often found in all cellular compartments, illustrating the importance of ROS detoxification for cell survival. Thus, plant stress tolerance can be improved with the enhancement of *in vivo* levels of antioxidants (Gill *et al.* 2011). The mechanism of which these antioxidants scavenge ROS is highlighted in figure 1.4.

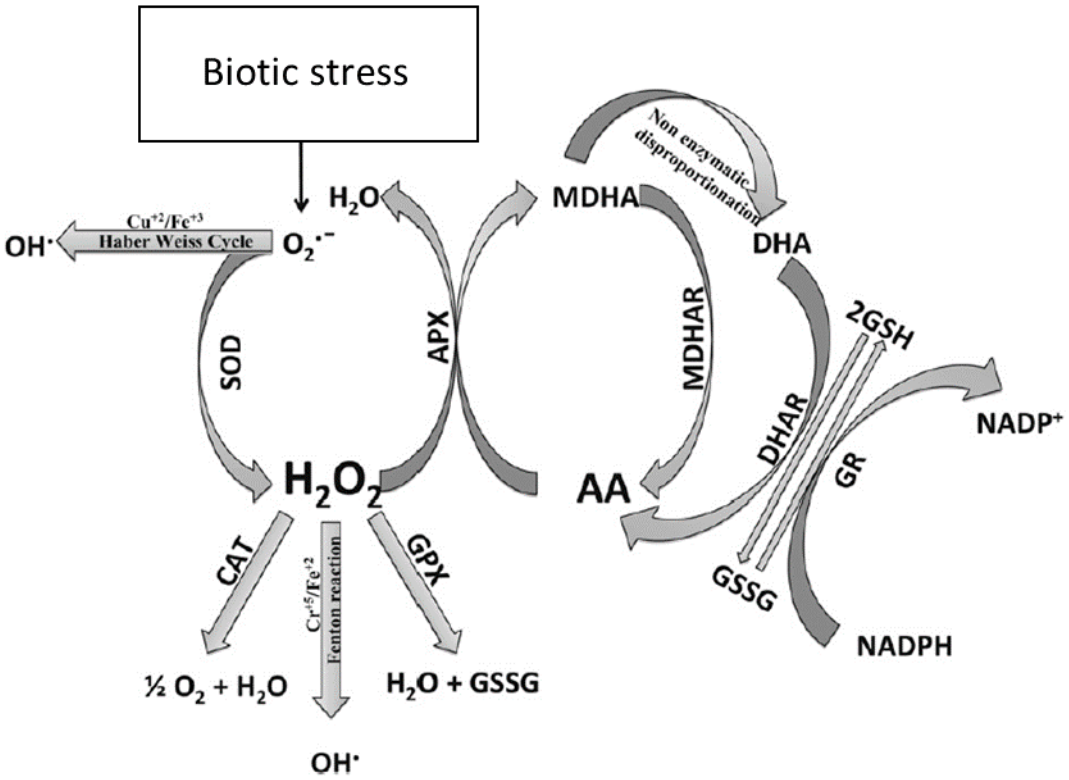

Figure 1.4: ROS and antioxidants defense mechanism (Gill and Tuteja, 2010).

1.4 Antioxidants

Due to their sessile nature, plants are unable to physically evade stress environments thus resulting in the accumulation of ROS (to toxic levels). However, plants have evolved the above mentioned antioxidants to scavenge and maintain ROS at nontoxic levels (Bhattacharjee, 2011). These antioxidants are the first line of defense against oxidative damage and they are imperative to maintaining homeostasis within plant cells (Gill and Tuteja, 2010).

1.4.1 Superoxide dismutase (SOD)

SOD is an effective intracellular enzymatic antioxidant which has been associated with ROS mediated oxidative stress. Thus the antioxidant plays a vital role in plant tolerance to stress as it is the first line of defense once ROS levels escalate to toxic levels (Mittler,

2002). The metalloenzyme SOD is known for catalyzing the dismutation of O_2^- to form H_2O_2 and O_2. The elimination of O_2^- ultimately reduces the risk of OH^- production through the Haber Weiss-type reaction, thus reducing oxidative stress (Mahanty *et al.* 2012). SOD is a metalloenzyme because of its association with metal cofactors, forming three types of SODs namely; copper/zinc SOD (Cu/Zn-SOD), manganese SOD (Mn-SOD) and iron SOD (Fe-SOD) (Wang *et al.* 2005). These enzymes are situated in different cellular compartments and can be differentiated through gel analysis (NATIVE PAGE) they can be differentiated. H_2O_2 and potassium cyanide (KCN) are inhibitors used to inhibit the activity of certain SOD isoforms situated in different organelles. Therefore, the Cu/Zn-SOD is sensitive to both inhibitors, the Mn-SOD is resistant to both inhibitors and Fe-SOD is resistant to KCN but sensitive to H_2O_2 (Gill and Tuteja, 2010). Using this knowledge, one can identify the different SOD isoforms.

1.4.2 Ascorbate peroxidase (APX)

The antioxidant enzyme APX is the main scavenger of H_2O_2, thus facilitating the regulation of ROS accumulation in algae, euglena, plant species and other organisms. (Maruta *et al.* 2012). Like most peroxidases, APX has a heme group which assists the enzyme to assume its role efficiently, thus utilizing H_2O_2 to convert ascorbic acid to form dehydroascorbate, ultimately reducing the chance of H_2O_2 accumulation (Verma and Dubey, 2003; Sinha and Saxena, 2006). This is performed in water-water and ASH-GSH cycles whereby ASH is utilized as an electron donor (Gill and Tuteja, 2010). The enzyme is located in the chloroplast, cytosol, mitochondria and peroxisomes of plant species (Kornyeyev *et al.* 2003; Sinha and Saxena, 2006). APX has been reported to be up-regulated in stress subjected plants due H_2O_2 accumulation. APX activity has been

shown to increase to aid in Cd, heat and salt stress tolerance in various crops (Aravind *et al.* 2003; Aravind and Prasad, 2003; Zlatev *et al.* 2006). However, very limited knowledge is available in the public domain, showing the activity of APX during fungal infections.

1.4.3 Catalase (CAT)

CATs are tetrameric heme enzymes which catalyze the dismutaion of H_2O_2, thus producing H_2O and O_2 as byproducts during ROS accumulation while the plant is under stress (Garg and Manchanda, 2009). H_2O_2 is predominately produced by the β-oxidation of fatty acids, purine catabolism and photorespitation (Bolwell and Wojtaszek, 1997). CAT have the highest turnover rate, with one molecule of the CAT facilitating the conversion of 6 million H_2O_2 molecules per minute. Furthermore, there are multiple isoforms of CAT that have been identified in higher plants, which are located in different chromosomes and are differentially expressed during H_2O_2 dismutation (Willekens *et al.* 1994).

1.4.4 Glutathione reductase (GR)

GR is a flavo-protein oxidoreductase that contains nucleic acid derivatives of riboflavin, which catalyzes the transfer of electrons (Romero-Puertas *et al.* 2006). GR is present in the ASH-GSH cycle, thus facilitating the reduction of GSH in the effort to defend plant cells against ROS accumulation. GSH is involved in metabolic and antioxidative pathways in plants, where GR catalyzes the NADPH dependent reaction forming disulphide bonds to GSSG, essentially maintaining the GSH supply. In addition, GR breaks the disulfide bond linking two GSH molecules in GSSG to form GSH, which is vital for cell systems (Rao and Reddy, 2008). Furthermore, GR is located in chloroplast

but has been localized in the mitochondria and cytosol, albeit in small quantities (Edwards *et al.* 1990). GR and GSH play fundamental roles in achieving tolerance to various stresses in plants.

1.4.5 Glutathione peroxidase (GPX)

GPXs are isozymes from a diverse family, which aid plant cells to reduce oxidative damage, by reducing of H_2O_2 using GSH (Noctor *et al.* 2002). GPX isoforms have been identified in the cytosol, mitochondria, endoplasmic reticulum and the chloroplast (Millar *et al.* 2003). During stress conditions in plants due to biotic factors, GPX activity is increased to reduce oxidative stress and enhance stress tolerance in plants (Light *et al.* 2005).

1.5 Conclusion

Given the above mentioned environment constraints, South Africa and other developing countries need to devise novel solutions to combat the challenges facing agricultural development and economic growth, in order to ensure food security and health for people and animals. There is a desperate need for the production and availability of nutritious foods that will reach people at affordable prices. Scientific research has illustrated a link between many chronic diseases such as cancer, malnutrition, cardiovascular diseases and food intake. Thus, the results obtained in this study serve as a way forward to try and enhance crop tolerance to environmental factors. It provides relevant insight to how cowpea crops response to *F. oxysporum* infection in a physical and biochemical point of view. Future endeavors can employed recombinant DNA technologies to increase antioxidant content to alleviate crops from stresses. This can include the use of small signaling molecules such as nitric oxide (NO), to enhance crop

tolerance to various abiotic and biotic stress conditions, as crops are exposed to multiple stress factors in the field. Thus, the major nutritious food crops like legumes, cereals and pseudocereals need to be extensively studied in order to develop eco-friendly and healthy alternatives to improve crop defenses to stress, which will increase agricultural yield and strengthen food security.

PROJECT AIMS AND OBJECTIVES

This research project aims to analyze the influence of *Fusarium oxysorum* infection on the physiological and molecular responses of cowpea plants.

Objectives:

- Analysis of cowpea seed germination.
- Analysis of plant grown growth and biomass production.
- Evaluation of ROS accumulation (O_2^- and H_2O_2) and the extent of oxidative damage. Detection and analysis of antioxidant activity of superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR).

Thus, given that cowpea-*F. oxysporum* responses are not adversely reported, this research will provide substantial knowledge about the responses of cowpea during this interaction.

CHAPTER 2

METHODOLOGY

2.1 Fungal culture preparation and storage

The *Fusarium oxysporum* strain (PPRI 19027) was obtained from the fungal culture collection at the Agricultural Research Council (ARC) (Pretoria, South Africa). Glycerol stocks were prepared in 20% glycerol and were stored at -80 ºC. The fungal strain was sub-cultured by means of mycelial plug transfer (4 mm) onto potato dextrose agar (PDA) (Merck, Johannesburg), into the center of 90 mm petri dishes. The cultures were then incubated at 30 ºC for 14 days before use. Spores were harvested by pouring sterile ddH_2O into the petri dish, followed by scraping of mycelium with a sterile glass rod. The resulting liquid was transferred into a sterile 50 ml tube, via sterile cheese cloth filtration. The spore suspension was calculated using a hemocytometer as described by Aberkane *et al*. (2002).

2.2 Cowpea seed sterilization

Vigna unguiculata (cowpea) seeds var. Saunders were obtained from Agricol Pty Ldt (Brackenfell, South Africa) were imbibed in sterile ddH_2O 20 minutes at room temperature. Cowpea seeds were heat sterilized in a 49 ºC water bath for 30 minutes to eliminate all endophytic microorganisms. After heat sterilization, seeds were submerged in 10% sodium hyperchloride for 10 minutes and rinsed 5 times with sterile ddH_2O.

2.3 Seed inoculation and plant growth

Sterilized cowpea seeds were infected with *F. oxysporum* as described by Oren *et al.* (2003) with slight modifications. For inoculation, the PDA plates containing the 14 day old fungus were flooded with 20ml of ddH_2O. Conidiospores were scraped off the PDA

plate and quantified using a haemocytometer to a final concentration of 4.9 x 10^6 conidiospores/ml for infection. Cowpea seeds were infected by incubation in spore suspension solution for 2 hours at 30 °C with slight agitation. Seeds (control and infected) were dried for 2hours in a laminar flow and germinated on PDA plates until seed radicle emerged (after 2 days). Germinated seeds were sown in sterile 20 cm pots (one plant per pot) containing sterile pre-soaked (ddH_2O) filtered silica sand (98% SiO_2, Rolfes Silica, Brits, South Africa). Plants were grown in a greenhouse with a 25/19 °C day/night temperature cycle under a 16/8 hours light/dark cycle, at a photosynthetic photon flux density of 300 µmol photons.m-2.s^{-1} during the day phase for 21 days.

2.4 Measuring physiological growth parameters and water retention of cowpea plants

Growth analysis was performed on cowpea plants by measuring shoot length, shoot fresh weight (FW), and dry weight (DW). The DW was determined by drying shoot tissue in an oven at 55°C for 48 h as described by Valentovic *et al.* (2006).

Relative water content (RWC) of leaf tissue was measured as described by Mohammadkhani and Heidari (2007) using the following formula:

$$RWC = \frac{Fresh\ weight\ (FW) - Dry\ weight\ (DW)}{Turgid\ weight\ (TW) - Dry\ weight\ (DW)} \times 100$$

2.5 Evaluate photosynthetic pigments in cowpea plants

The analysis was performed to determine any changes in chlorophyll concentrations between the infected plants and the control plants, thus a modified method by Oancea *et al*. (2005) was utilized. Therefore, 200 mg of ground shoot material was submerged in 80% (v/v) acetone and was mixed thoroughly by vortexing. The mixture was subsequently centrifuged at 10 000 X g for 10 minutes. This processes was repeated until no chlorophyll was observed from the shoot material. The sample had a reading taken using a spectrophotometer at the 662 nm and 644 nm wavelength. The resulting optical readings were thus utilized in a calculation considering specific parameters to determine to chlorophyll concentrations.

Chlorophyll **a** (µg/ml) = 12.21 (A663) – 2.81 (A646)

Chlorophyll **b** (µg/ml) = 20.13 (A663) – 5.03 (A646)

2.6 Protein isolation for biochemical analysis

Cowpea shoots were ground into a fine powder in liquid nitrogen. From this homogenized plant material, 400 mg was mixed with 6% trichloroacetic acid (TCA). The mixture was thus centrifuged at max speed for 20 minutes, followed by supernatant transfer. This TCA extract was used for the measurement of H_2O_2 content and lipid peroxidation in leaves. A polyvinyl pyrrolidone (PVP) extraction was performed on the ground plant material using 200 mg from the leaves, by the addition of 800 µl of PVP buffer [40 mm Dipotassium phosphate (K_2HPO_4) pH 7.4, 1 mm ethylenediaminetetraacetic acid (EDTA), 5% (w/v) PVP (40000 g/mol), 5% w/v) glycerol]. The mixture was centrifuged at max speed for 15 minutes. The resulting supernatant was transferred to new clean 2 ml Eppendorf tubes, to be utilized in the Ascorbate peroxidase (APX) spectrometry assay, Superoxide dismutase (SOD) spectrometry assay, APX in gel

NATIVE page electrophoresis, SOD in gel NATIVE page electrophoresis and Glutathione reductase (GR) in gel NATIVE page electrophoresis.

2.7 Measurement of hydrogen peroxide (H_2O_2) content in cowpea shoots

A modified method was utilized whereby from the TCA extract, 50 µl of each sample was added to 20 mM pH 5 Monopotassium phosphate (KH_2PO_4) and 1M potassium iodide (KI), to a final volume of 200 µl. This mixture was incubated at room temperature for 20 minutes and subsequently had the absorbance read at 390 nm (Velikova *et al.* 2000).

2.8 Measurement of lipid peroxidation (MDA detection) in cowpea shoots

A modified method was used to measure the lipid peroxidation (Zhang *et al.* 2007). From the TCA extract sample, 300 µl was mixed with 600 µl TCA/TBA solution [20% TCA and 0.5% thiobarbituric acid (TBA)]. This was followed by a 30 minute incubation at 95 ºC and a subsequent 10 minute incubation on ice. The samples were thus centrifuged at max speed for 5 mins and 150 µl of the supernatant use used for absorbance acquisition at 532 nm.

2.9 Measurement of cell viability in cowpea shoots

Fresh cowpea leaves from the first trifoliate were obtained and cut into 1x1 cm squares. These were thus submerged in 0.25% Evans blue solution for an hour at room temperature. This was followed by submersion in 1% Sodium dodecyl sulfate (SDS) and incubated at 55 ºC for an hour. The absorbance was thus measured from a 200 µl sample at 600 nm (Baker and Mock, 1993).

2.10 Measurement of superoxide (O_2^-) radical content in fresh cowpea shoots

A modified method of Russo *et al*. (2008) was utilized to quantify the superoxide content. Cowpea first trifoliate leaves from each treated plant were cut into four 1x1 cm squares and the reaction buffer as added [100 mM KCN, 100 mM H_2O_2, 6.4 mM nitro blue tetrazolium chloride (NBT) and 100 mM Potassium phosphate (KPO_4) pH 7.0]. This mixture was thus incubated at room temperature for 20 minutes. The leaf pieces were crushed and thus centrifuged at max speed for 20 minutes. The absorbance was measured to 200 µl of the supernatant at 600 nm.

2.11 Detection and characterization of SOD isoforms

The detection of SOD isoforms in cowpea shoots was performed in a native PAGE at 4 °C, containing 90 µg of the PVP extracted proteins. This was performed in 40% polyacrylamide, in the presence of ice cool running buffer [3.07 g/L Tris and 14.4 g/L Glycerol]. Subsequently, the SOD isoforms were detected by means of staining with a) 50 mM KPO_4 pH 7.8 + inhibitors [5 mM potassium cyanide (KI) or 6 mM H_2O_2], b) 50 mM KPO_4 pH 7.8 + 20 mg NBT, c) 50 mM KPO4 pH 7.8 + 209 µl/50 ml of TEMED + 9.5 mg/ 50 ml Riboflavin. H_2O_2 inhibits both Cu/ZnSOD and FeSOD isoform and KCN inhibits the Cu/ZnSOD isoform (Beauchamp and Fridovich, 1971).

2.12 Detection of APX isoforms

The detection of APX isoforms in cowpea shoots was performed in a native-PAGE at 4 °C, containing 50 µg of the PVP extracted proteins. This was performed in a 40% polyacrylamide, in an ice cold APX running buffer [3.07 g/L Tris, 14.4 g/L Glycerol and 0.352 g/L Ascorbic acid (ASA)]. The APX isoforms were detected by means of staining with a) 0.1 mM KPO_4 pH 7.8, 2 mM ASA, b) 0.1 mM KPO_4 pH 7.8 + 4 mM

ASA + 2 mM H2O2, c) 50 mM KPO_4 pH 7.8 + 209 µl/50 ml TEMED + 15 mg/ 50 ml nitroblue tetrazolium (NBT) (Lee and Lee 2000).

2.13 Detection of GR isoforms

The detection of GR isoforms in cowpea shoots was conducted in a 40% native-PAGE at 4 °C, containing 90µg of the PVP extracted proteins. A modified detection method by Lee and Lee (2000) was utilized, in the presence of an ice cold running buffer [3.07 g/L Tris and 14.4 g/L Glycerol]. GR isoforms were visualized by means of incubation in 0.25 M Tris–HCl buffer (pH 7.9) containing 4.0 mM glutathione disulfide (GSSG), 1.5 mM NADPH, and 2 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) for 20 min. This was followed by negative staining in the absence of light in 1.2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 1.6 mM phenazine methosulfate (PMS) for 10 min at 30 °C.

Table 1.1: Preparation of the stacking and separating gels for native-PAGE. These constituents were used to correctly form one 1.5 mm SDS-PAGE gel.

	Stacking		**Separating**
Gel percentage (%)	5	Gel percentage (%)	12
40% Polyacrylamide	1.25	40% Polyacrylamide	1.5
1 M Tris pH 6.8	1.25	1.5 M Tris pH 8.8	1.25
10% Ammonium persulfate	0.1	10% Ammonium persulfate	0.05
TEMED	0.01	TEMED	0.002
H_2O	7.4	H_2O	2.2
Total volume (ml)	10	Total volume (ml)	5

2.12 Densitometry analysis of antioxidant enzymes

AlphaEase FC imaging software (Alpha Innotech Corp) was utilized to analyze the changes in SOD, APX and GR activity as identified using native-PAGE. The occurring isoforms from the various antioxidant native-PAGE gels had their intensity analyzed and arbitrary values were allocated to them. The average pixel intensity was measured in arbitrary units (Klein, 2012).

2.13 Statistical analysis

The obtained data was analyzed using the one-way analysis of variance (ANOVA) and tested for significance by the Tukey-Kramer test at 5% level of significance.

CHAPTER 3

FUSARIUM OXYSPORUM ALTERS SEED GERMINATION, GROWTH AND PHOTOSYNTHETIC METABOLISM OF COWPEA PLANTS

3.1 INTRODUCTION

Cowpea is a tropical, drought-tolerant legume crop that is indigenous to Africa (Singh and Rachie, 1985). The crop is very nutritious, providing rich protein, phosphorus, tryptophan, vitamin B9 and iron dose to people and animals that consume it. Due to biotic stress conditions, the crop production and its availability is fast declining. In general, stress is defined as any factor that negatively influences the growth and reproduction of a crop below the capabilities of the genotype (Osmond *et al.* 1987). Biotic stresses like *F. oxysporum* infections have profound impacts on the physiological development of infected cowpea, resulting in *fusarium* wilt.

Physiological studies of plants are a subdiscipline of botany which examines the plants physical changes in response to various degrees of environmental changes (Salisbury and Ross, 1992). Plants are governed by physical and biochemical processes which occur internally and externally in order to function and sustain life. However, certain external changes result in the plant responding by altering its cellular processes for survival, which in turn alters the physiological state of the plant (Fosket, 1995). Thus, plant physiological studies focuses on examining crucial processes that take place within the plant such as, seed germination, plant development (stunted growth), photosynthesis and internal water diffusion.

F. oxysporum is a major cowpea fungal pathogen, causing *fusarium* wilt to the crop. This pathogen is soil born and in near perfect conditions infects the plant and results in an imbalanced of nutrient uptake and accumulation of toxic *phytochemicals* (Dita *et al.* 2006). This in turn causes a cascade of biochemical alteration which lead to physiological changes. Studies on cowpea and *Fusarium* interactions showed minimal effects on the shoot and roots of cowpea seedling (Kotze, 2016). Studies conducted on wheat and *F. culmorum* interactions revealed no significant effects to the germination rate of on infected wheat (Caruso *et al.* 1999). However there is limited information on the physiological responses of cowpea plants to *F. oxysporum* infection in the public domain. Therefore, this part of the thesis will focus on the physiological responses of cowpea plants to *F. oxysporum* by closely examining the germination rate, growth parameters, relative water content (RWC) and the chlorophyll content.

3.2 RESULTS

3.2.1 *Fusarium oxysporum* accelerates germination rate of cowpea

Sterilized cowpea seeds (100 seeds per treatment) infected with *F. oxysporum* (4.9×10^6 cells/ml) were germinated for a period of 5 days. The rate of seed germination for control and infected seeds were monitored daily until the end of day 5. The results showed that *F. oxysporum* enhanced the rate of seed germination when compared to the control treatment. After 2 days of germination ± 65% of seeds infected with of *F. oxysporum* were germinated compared to ± 45% of the untreated seeds (Figure 3.1). This gradual increase in the rate of seed germination of infected seeds compared to the untreated seed was maintained until the last day of germination (day 5) when all 100

seeds from each treatment had germinated (Figure 3.1). The results suggest that *F. oxysporum* infection of cowpea seeds improved the rate of germination compared to that of the untreated seeds.

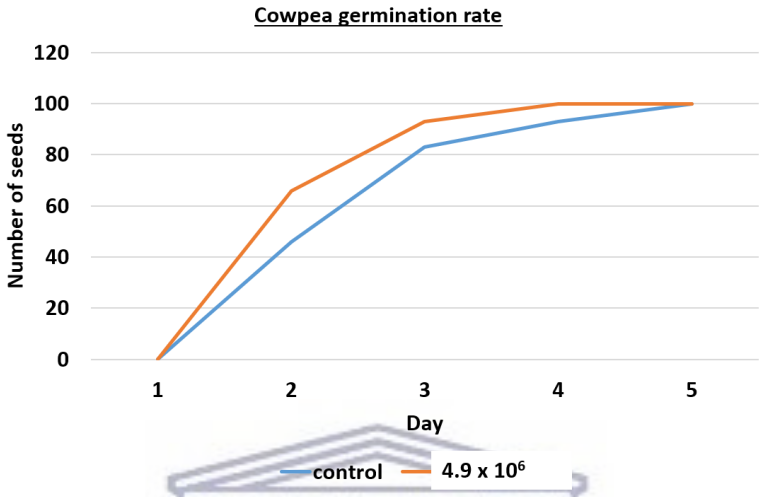

Figure 3.1: The effect of *F. oxysporum* infection on the germination rate of cowpea seeds. The germination rate of cowpea seeds was measured over a period of 5 days using a total of 100 cowpea seeds per treatment. Cowpea seeds (infected and uninfected) were germinated on sterile moist filter paper within petri-dishes.

3.2.2 *F. oxysporum* inhibits plant growth and biomass production

The results showed that *F. oxysporum* infection restricts cowpea growth when compared to the untreated plants (Figure 3.2 A). This inhibition in plant growth was evident when measuring shoot length. Shoot length was reduced by ± 16% as a consequence of *F. oxysporum* infection (Figure 3.2 B). The reduction in shoot growth corresponded with the reduction in biomass production of cowpea plants. The result showed that cowpea biomass (indicated by shoot fresh weight) was reduced by ± 13.2% when compared to the untreated plants (Figure 3.2 C).

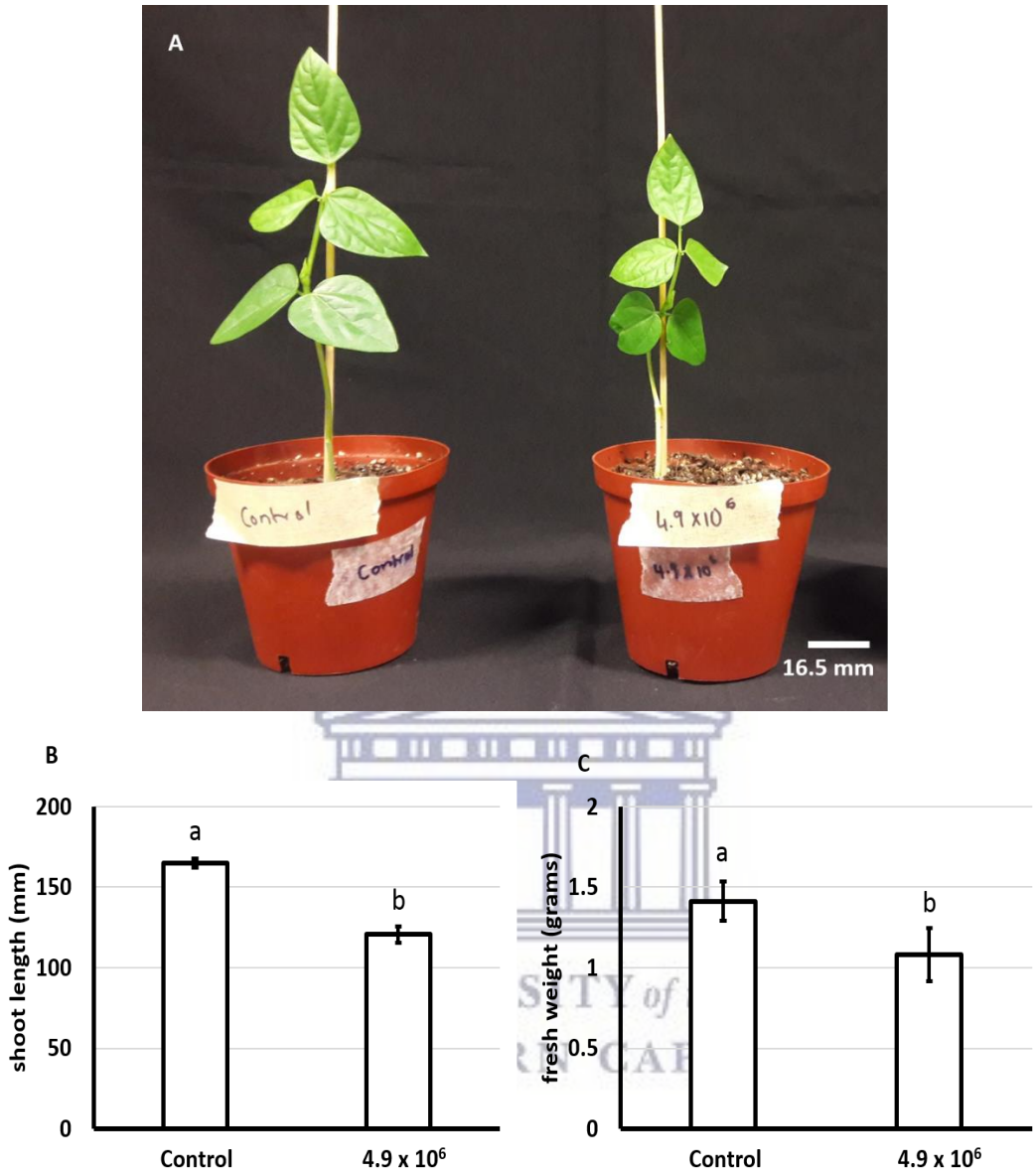

Figure 3.2: *F. oxysporum* inhibits shoot length and fresh weight of cowpea plants. Cowpea plants infected with *F. oxysporum* showed a significant reduction in (A) plant growth, (B) shoot length and (C) shoot fresh weight. The error bars represent the mean of three independent experiments per treatment. Different letters above each error bar indicate significant statistical differences between the infected plant and the control ($p < 0.05$).

3.2.3 *F. oxysporum* infection reduces water retention in cowpea shoots

Higher plants depend on their ability to retain water in order to survive especially during stress conditions. Water retention is critically important during nutrition uptake and

transport. When plants experience stress, relative water content (RWC) is an important stress marker to indicate the water status and how cellular processes are influenced that leads to physiological damage. Changes in water retention (as indicated by relative water content) in cowpea plants (infected and uninfected) was measured as described in as per section 2.4. Relative water content (RWC) in infected shoots was reduced by ± 20% when compared to the uninfected shoots (Figure 3.3).

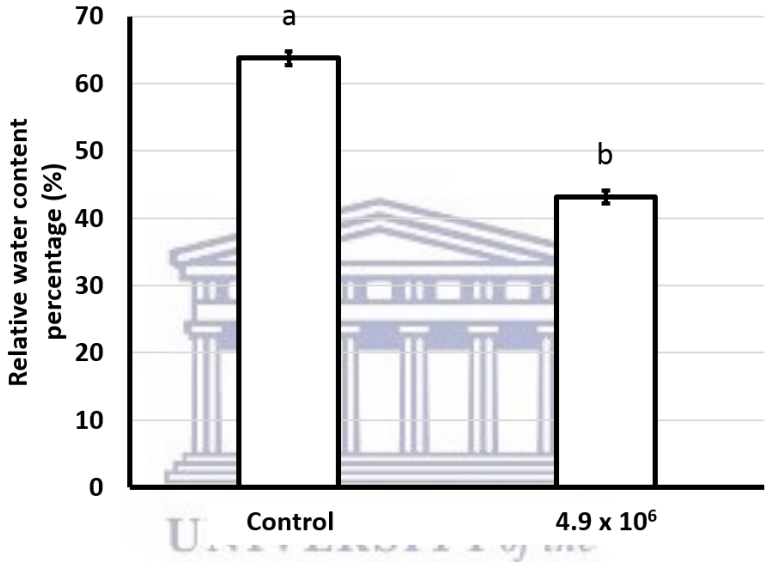

Figure 3.3: *F. oxysporum* water retention in cowpea shoots. Relative water content in the shoots of cowpea plants was significantly reduced when infected with *F. oxysporum.* The error bars represent the mean of three independent experiments per treatment. Different letters above each error bar indicate significant statistical differences between the infected plant and the control ($p < 0.05$).

3.2.4 Photosynthetic pigments in cowpea shoots are significantly influenced by *F. oxysporum infection*

Plant homeostasis is significantly influenced by biotic stress conditions. The disruption in plant homeostasis have been shown to inhibit photosynthetic metabolism in various plant species. Here we have demonstrated that *F. oxysporum* infection significantly

reduces photosynthetic pigments as observed for chlorophyll 'a', 'b' and total chlorophyll content (Figure 3.4). As a consequence of *F. oxysporum* infection, both chlorophyll 'a' and 'b' in the shoots of cowpea plants was reduced by ± 19% compared to the untreated sample (Figure 3.4). However, a more significant decrease in total chlorophyll content ± 20% was observed in the shoots of infected plants compared to the untreated plants.

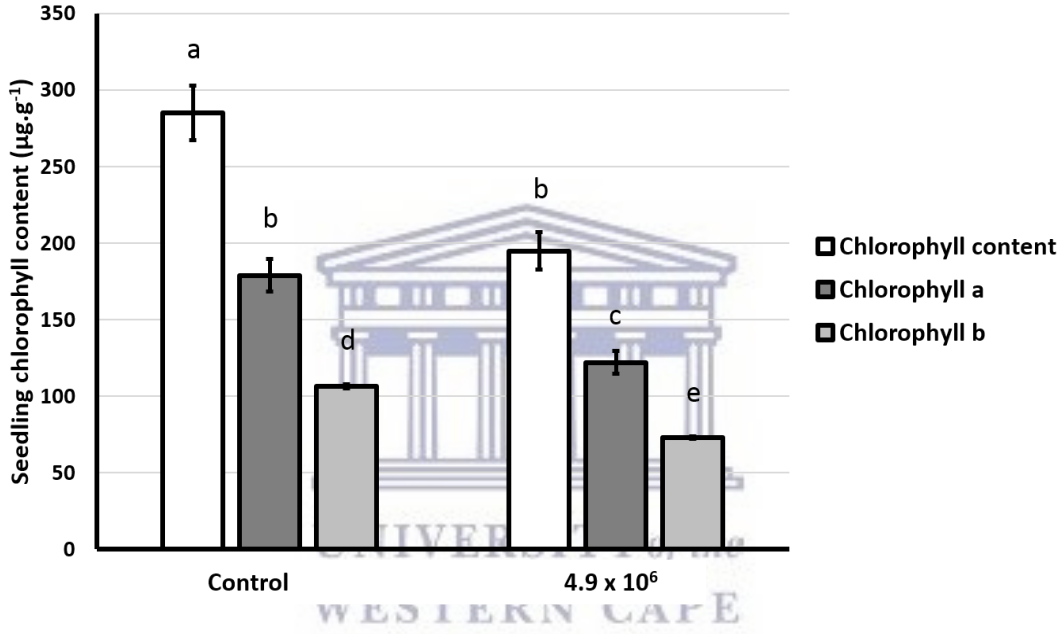

Figure 3.4: *F. oxysporum* infection significantly reduces chlorophyll content of cowpea. Cowpea seedlings infected with *F. oxysporum* showed a significant reduction in chlorophyll content. The error bars represent the mean of three independent experiments per treatment. Different letters above each error bar indicate significant statistical differences between the infected plant and the control ($p < 0.05$).

3.3 DISCUSSION

In this chapter, we analyzed some physiological responses of cowpea plants to *F. oxysporum* infection. The results obtained in this study showed that *F. oxysporum* increased the rate of seed germination but negatively altered plant growth and biomass

production. The decrease in plant growth and biomass was accompanied by significant decrease in water retention and photosynthetic metabolisms.

3.3.1 *F. oxysporum* improves the rate of cowpea seed germination

Seed germination is mostly defined as the growth of a plant from a seed, which results in the formation of a radicle, thus developing into a seedling (Washa, 2015). This is the process of reactivation of the metabolic machinery contained within the seed, due to favourable conditions. Seed germination is dependent on various internal and external factors which ensure successful activation of metabolic pathways, leading to emergence of the radicle (Tweddle *et al.* 2003). Seeds are very vulnerable to disease and injury thus the seed is evolved to accelerate the process to ensure a high germination rate.

The increase in the rate of seed germination under stress conditions have been described to enhance plant survival (Li *et al.* 2013; Sohrabikertabad *et al.* 2013). This was observed in an osmotic pressure germination experiment in barley seeds, where the initial stages of germination were accelerated under stress (Zhang *et al.* 2010). However, minimal literature is available about fungal pathogens affecting seed germination as described in this study. To our knowledge, this is the first study of its kind that describes how pathogen infection accelerates seed germination of cowpea.

Contrary to what was observed in this study, Caruso *et al*. (1999) showed that *Fusarium culmorum* did not influence the rate of germination of wheat seeds. Given the removal of any internal microorganisms including *Fusarium* pathogen/endophytes during the heat sterilization process we can confidently state that the results obtained here was as a consequence of externally applied pathogen. The results suggest that during seed germination, *F. oxysporum* serves as an endophytic microorganism that promotes the

emergence of seed radical. However, the endophytic response of *F. oxysporum* was not present in the seedling stage of development given the suppression of plant growth and biomass production observed in this study.

3.3.2 *F. oxysporum* restricts plant growth and biomass production

The result observed in this study showed that *F. oxysporum* infection significantly reduced plant growth and development. Under severe conditions, this would also result in wilting of leaves. Suppression of plant growth and development including leaf chlorosis and wilting have been previously described (Singh and Rachie, 1985; Davis *et al.* 1991). After 21 days post infection (dpi), no form of leaf wilting and chlorosis was observed although shoot growth was significantly stunted. This could be attributed to the mode of infection that the pathogen utilizes. Since *F. oxysporum* is a soil borne pathogen, it enters host plants through the roots where it can multiply and obstruct nutrient uptake. The pathogen normally migrates up the plant, restricting nutrient uptake, which results in a cascade of events that are detrimental to plant growth (Singh *et al.* 1997). This results in the suppression of metabolic pathways and accumulation of toxic molecules, thus limiting growth and normal functioning of plant cells.

The reduction in growth and biomass due to pathogen infection have been described previously. Soil borne pathogens such as *Fusarium* and *Gibberella* have been shown to suppress plant growth of *Arrhenatherum elatius* (Aguilar-Trigueros and Rillig, 2016). This is in agreement to what we have observed in this study (Figure 3.2). The reduction of biomass due to fungal infection was also observed in tomato plants (Naing *et al.* 2015).

Given the reduction in plant growth coupled with decreased biomass observed in this study we hypothesize that *F. oxysporum* possibly restricted nutrient uptake from the growth medium that resulted in stunted growth. Given the lack of wilting and chlorosis observed in this study, we suggest that *F. oxysporum* was localized in the roots and not transported to the shoots.

3.3.3 *F. oxysporum* infections reduces water retention

F. oxysporum mycelia germinate and progress intracellularly up the roots of infected plants into the vascular system. The mycelia remain in the xylem and produce microconidia, which is transported to the rest of the plant (Sastry, 2013). However, this restricts water uptake and the flow of nutrients. Thus it was necessary to examine the RWC content of *F. oxysporum* infected cowpea in order to identify low relative water content as a stress indicator (Figure 3.3). The RWC of *F. oxysporum* infected cowpea plants was significantly reduced by ± 20% compared to the uninfected (control) plants. This phenomena was also observed in tomato leaves after 31 dpi (Nogués *et al.* 2002). RWC findings pertaining to pathogen-plant interactions are very limited in the public domain, thus the results of this study make a contribution to knowledge relating to the effect of fungal infections on water retention capacity of crop plants. The decreased water retention leads to stress and deterioration of vital plant processes such as photosynthetic metabolism

3.3.4 *F. oxysporum* alters the photosynthetic pigments of cowpea

The term photosynthetic pigments refer to various pigments present in the chlorophyll of plants that are essential for photosynthesis as they capture light (Gitelson *et al.* 2006). These include chlorophyll a and b, among others, which was investigated in this study.

Chlorophyll a and b are the blue - green and yellow – green pigments respectively, which facilitate the absorption solar photons (Kira *et al.* 2015). This energy is processed in the photosystems to provide plants with energy. This process can be affected by biotic stress in plants and in this study it was observed that *F. oxysporum* (4.9×10^6 cell/ml) reduced chlorophyll a and b contents in cowpea shoots, leading to a general reduction of total chlorophyll content.

Total chlorophyll content measured in infected cowpea plants was reduced by ± 31.62%, compared to the control plants. This effect has been noted in a study conducted on lentils infected with *Fusarium solani* (Ahmed and Shahab, 2017). Similarly, a significant decrease in chlorophyll content in chickpea infected by root fungi was observed (Hayat and Gautam, 1995). *F. oxysporum* and *F. salani* also reduced total chlorophyll content in cucumbers (Al-Tuwaijri, 2009).

Given the finding in this study and the supporting findings in literature, it can be said that *F. oxysporum* infection in cowpea directly reduces the chlorophyll content. This could be due to the fact that the pathogen induces a level of inhibition to metabolic pathways which lead to the loss of functionality of these photosynthetic pathways. This results in a physiological change in plants which is indicative of stress and cowpea was indeed under fungal infection stress.

CHAPTER 4

FUSARIUM OXYSPORUM INFLUENCES ROS SCAVENGING CAPACITY IN COWPEA SHOOTS

4.1 INTRODUCTION

Fusarium oxysporum is a well-known pathogenic fungi that is the causative agent of a common disease in cowpea plants, known as fusarium wilt. It is a soil borne pathogen that proliferates efficiently and can survive for long periods as chlamydospores (Berger *et al.* 2016). *F. oxysporum* enters its host plant by the roots and invades the vascular system, thus restricting nutrient uptake and causes biochemical instability. This results in stunted growth, wilting of leaves and ultimately death to the crop (Rodrigues *et al.* 2006). In addition plants have developed defense mechanisms which are activated once the pathogen is detected, in order to limit pathogen growth and damage.

F. oxysporum infection initiates a cascade of metabolic processes once the pathogen is detected. Apoplastic production of superoxide (O_2^-) is the first event that takes place in plants under stress conditions and this O_2^- is subsequently converted to hydrogen peroxide (H_2O_2). These molecules serve as signaling molecules in plants when present in low to moderate levels. However once hyper accumulated, they cause oxidative damage to crop plants (Doke, 1983; Grant *et al.* 2000). This suggest that ROS accumulation causes severe damage to protein, lipids and DNA, leading to programmed cell death (PCD) (Bhattacharjee, 2005). In addition, plants have evolved ROS scavenging capabilities which ensure ROS levels are maintain at levels non-toxic to plants. Thus, ROS accumulation to pathogen infection is regulated by enzymatic antioxidants such as superoxide dismutase (SOD), ascorbate peroxidase (APX),

glutathione peroxidase (GPX), catalase (CAT) and glutathione reductase (GR), together with non-enzymatic antioxidants namely; ascorbic acid (ASH), glutathione (GSH), α-tocopherol, flavonoids, carotenoids and non-protein amino acids (Gill and Tuteja, 2010). However, in this chapter only SOD, APX and GR and their responses to ROS accumulation will be described.

A biochemical analysis of the O_2^- and H_2O_2 content in fusarium infected cowpea plants reveals an insight on the ROS accumulation status of the crop during infection. This information provides evidence that infection was successful and the cowpea plants are subjected to stress. Furthermore, MDA content analysis and cell death quantification serves as indicators of stress as the plant is being negatively affected by the infection. In addition, antioxidant activity analysis (SOD, APX and GR) reveals the defense mechanisms put into place in order to cope with the infection and restrict plant death. The results from this study could provide potential stress biomarkers which can be utilized to screen for cowpea cultivars with high levels of antioxidants, which could be used to breed new cowpea cultivars with tolerance to *F. oxysporum* infection.

4.2 RESULTS

4.2.1 *F. oxysporum* infection induces ROS accumulation in cowpea shoots

Infected cowpea seeds were grown as described in section 2.3. With accordance to most findings in literature, pathogen infections in plants results in high ROS (O_2^- and H_2O_2) accumulation which subsequently leads to oxidative damage and ultimately programmed cell death. Thus, the levels of O_2^- and H_2O_2 in cowpea (both infected and non-infected) was investigated (Figure 4.1). The results showed that *F. oxysporum*

increased O_2^- levels by ±14.7% compared to the uninfected plants (Figure 4.1A). Superoxide levels in plants are controlled by superoxide dismutase (SOD) by reducing the highly reactive compound to form H_2O_2. If not maintained, increased levels of H_2O_2 can result in oxidative stress.

For H_2O_2, a similar trend was observed as seen for O_2^-. *F. oxysporum* infection increased H_2O_2 content by ± 10% when compared to the uninfected plants (Figure 4.1 B). The result presented here suggest that *F. oxysporum* was sufficient to induce some level of stress as shown by the increased levels of O_2^- and H_2O_2.

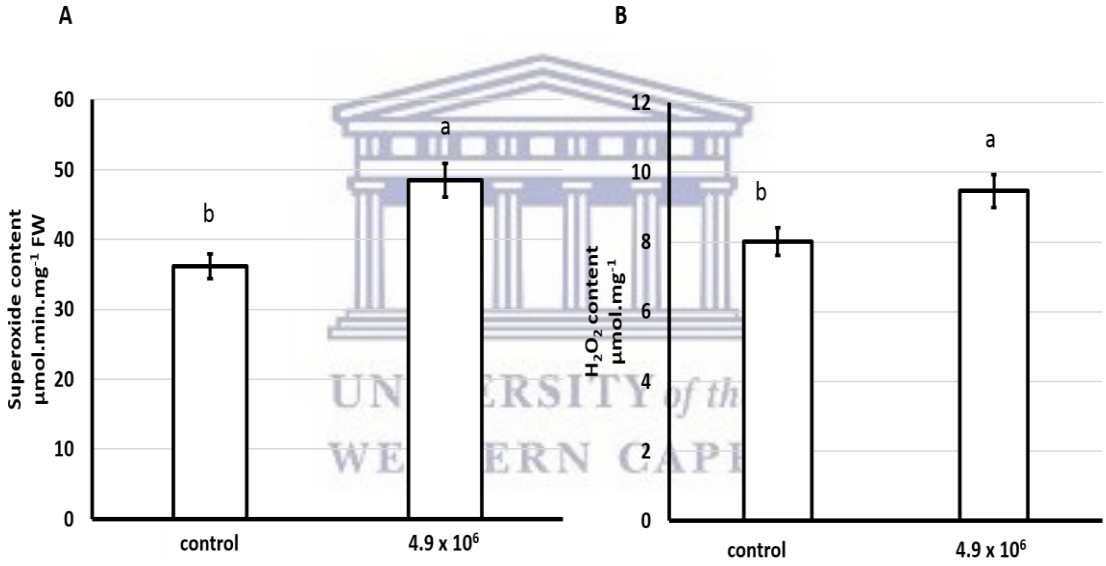

Figure 4.1: *F. oxsporum* increases levels of ROS (O_2^- and H_2O_2) in cowpea. Cowpea plants infected with *F. oxysporum* increased (A) O_2^- and (B) H_2O_2 contents, after 21 dpi. The error bars represent the mean of three independent experiments per treatment. Different letters above each error bar indicate significant statistical differences between the infected plant and the control ($p < 0.05$).

4.2.2 *F. oxysporum* alters the extent of lipid peroxidation manifested as increased cell death

Lipid peroxidation is often a tell sign of oxidative stress occurring in a plant. When lipids are oxidatively degraded, the end product produced is malondialdehyde (MDA), which is a reactive aldehyde. Thus the accumulation of MDA can be regarded as

indicative of lipid peroxidation. Thus, the amount of MDA in *Fusarium oxysporum* (4.9 x 10^6 cells/ml) infected cowpea leaves was measured (Figure 4.2). It was noted that the control (uninfected) cowpea plants showed a ± 13.72% decrease in MDA levels compared to the control (Figure 4.2 A). Following MDA detection, the level of cell death was measured because the increase in ROS due to stress result in oxidative damage and ultimately cell death. Therefore, the level of cell death measured in cowpea plants infected with *Fusarium oxysporum* was ± 8.4% higher than the cowpea control (uninfected) plants (figure 4.2 B).

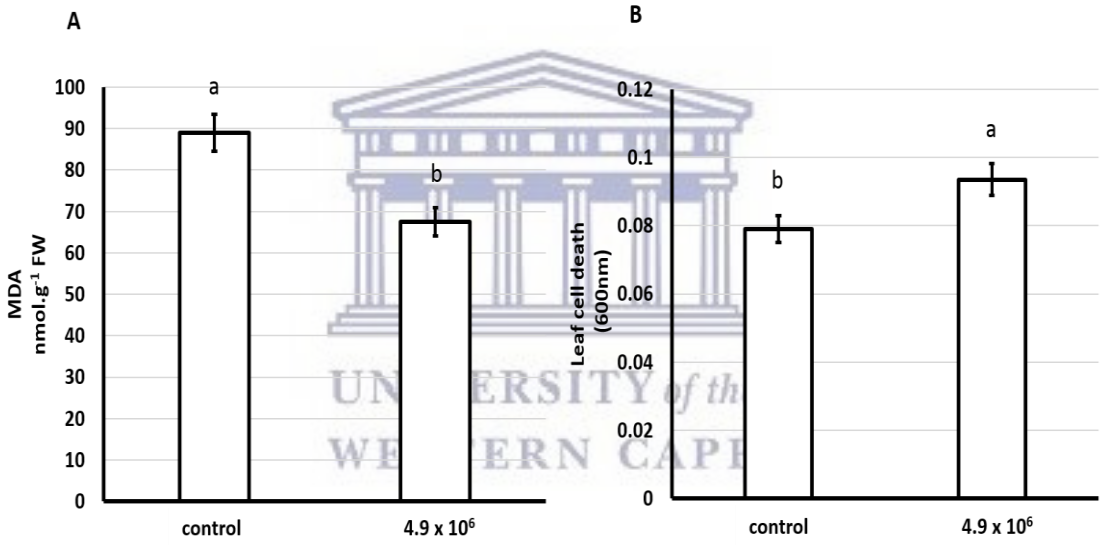

Figure 4.2: The influence of *F. oxysporum* on lipid peroxidation and cellular death. Changes in (A) MDA content and (B) cell death was measured in response to *F. oxysporum* infection 21 dpi. The error bars represent the mean of three independent experiments per treatment. Different letters above each error bar indicate significant statistical differences between the infected plant and the control ($p < 0.05$).

4.2.3 *F. oxysporum* infection differentially alters SOD activity

Superoxide dismutase (SOD) is the first line of defense in controlling ROS accumulation in the Halliwell-Foyer-Asada pathway. SOD catalyzes the reduction of

O_2^-, a highly reactive ROS, to H_2O_2. To understand the role of SOD in regulating ROS accumulation in cowpea plants during *Fusarium* infection we investigated changes in SOD activity by detection and quantification of individual SOD isoforms. SOD activity was identified as described in section 2.11. Seven SOD isoforms were detected using SOD specific staining as shown in Figure 4.3 A. SOD isoforms were functionally characterized using SOD specific inhibitors including potassium cyanide (KCN) and hydrogen peroxide (H_2O_2) (Figure 4.3 B-C). Based on the sensitivity and resistance of isoforms to these inhibitor; three manganese SODs (Mn-SOD), two iron SODs (Fe-SOD) and two copper/zinc SODs (Cu/Zn-SOD) were identified (Figure 4.3 A). The enzymatic activities of these SOD isoforms were differentially regulated by *F. oxysporum* infection. Densitometry analysis was performed on all SOD isoforms and the results document in Table 4.1.

The results showed that the enzymatic activities of all three Mn-SOD (1-3) isoforms were significantly suppressed in response to *Fusarium* infection. Contrary to what was observed for enzymatic activities of Mn-SOD isoforms, both Fe-SODs were significantly increased in response to *Fusarium* infection. The enzymatic activity of Fe-SOD 1 was increased by ± 12% whereas Fe-SOD 2 was increase by ± 10% when compared to the activities observed in the untreated samples. The enzymatic activity of Mn-SOD 1 was reduced by ± 8%. Mn-SOD 2 and Mn-SOD 3 was showed a significant reduction in activity of ± 16% and ± 19% respectively. For Cu/Zn-SOD, no significant changes in enzymatic activities was observed for Cu/Zn-SOD 1 and Cu/Zn-SOD 2 isoform in response to *Fusarium* infection.

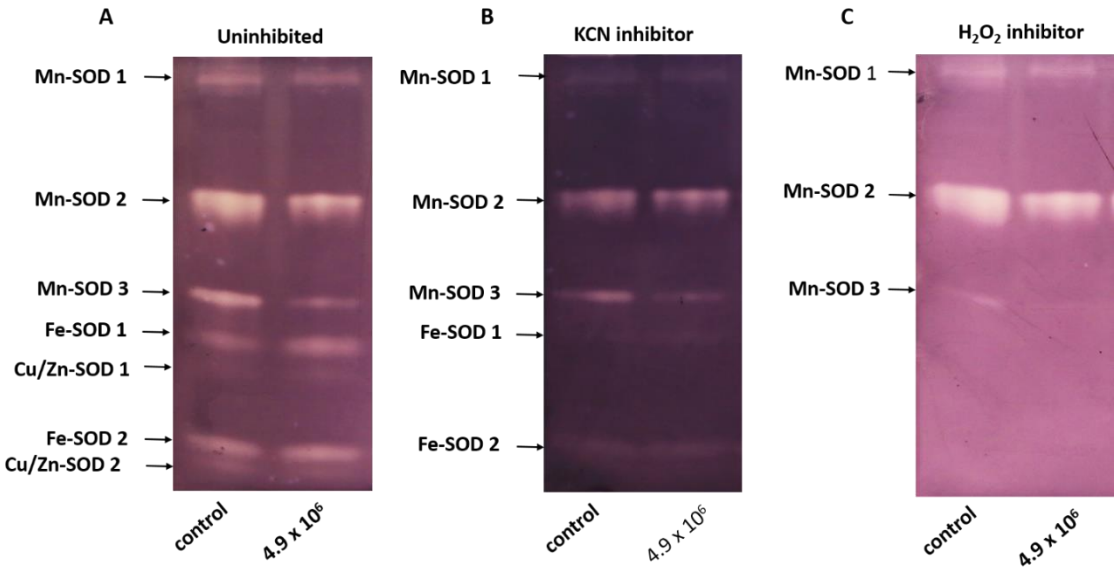

Figure 4.3: *F. oxysporum* infection modulates changes in SOD activity in cowpea shoots. *F. oxysporum* infection differentially regulate SOD isoforms in cowpea shoots. Identification of SOD enzymatic activity in cowpea leaf samples using in-gel activity assays. Cowpea plants were infected with *F. oxysporum* (4.9 x 10^6 cells/ml) and monitored for 21 days. Individual SOD isoforms were identified by incubating native PAGE gels in b) B = KCN and C = H_2O_2.

Table 4.1: Relative activity of individual SOD isoforms after densitometry analysis.

Relative SOD Activity (Arbitrary values)	Cowpea shoot SOD isoforms	Treatments	
		Control	4.9 x 10^6
	Mn-SOD 1	1 ± 0.05^a	0.842 ± 0.042^b
	Mn-SOD 2	1 ± 0.05^a	0.718 ± 0.036^b
	Mn-SOD 3	1 ± 0.05^a	0.638 ± 0.032^b
	Fe-SOD 1	1 ± 0.05^b	1.366 ± 0.068^a
	Fe-SOD 2	1 ± 0.05^b	1.245 ± 0.062^a
	Cu/Zn-SOD 1	1 ± 0.05^b	1.098 ± 0.055^a
	Cu/Zn-SOD 2	1 ± 0.05^a	1.089 ± 0.054^a

Data presented in this table are the means ± standard error of three replicates (n = 3). Means marked with the different letters in the same row for the same isoform indicate significant difference between treatments at 5% level of significance according to Tukey-Kramer test.

4.2.4 *F. oxysporum* infection influences APX activity in cowpea

Ascorbate peroxidase (APX) is the second enzyme involved in ROS scavenging in the Halliwell-Foyer-Asada pathway. APX catalyzes the conversion of H_2O_2 to H_2O using reduced ascorbate as substrate. The role of APX activity in ROS scavenging are well documented (Gill and Tuteja, 2010). Here we describe the influence of *F. oxysporum* (4.9 x 10^6 cell/ml) infection on APX activity as shown for individual isoforms (Figure 4.4 A-B). Densitometry analysis was used to quantify changes in APX activity (individual isoforms) in response to *F. oxysporum* infection (Figure 4.4 B). Five APX isoforms was identified using antioxidant specific staining as described in section 2.12. The results showed that the activities of all five isoforms were altered by *F. oxysporum* infection (Figure 4.4 A). The enzymatic activity of APX 1 was increased by ± 6% in the

F. oxysporum treated sample compared to the untreated sample (Figure 4.4 A-B). A similar trend was observed for APX 2 where the activity was significantly enhanced by ± 14% in the *Fusarium* treatment. The activity of APX 3 was more abundant then the other APX isoforms detected in this study. *Fusarium* infection enhanced APX 3 activity by ± 8% compared to the untreated sample (Figure 4.4 A-B). The activity of APX 4 and 5 was enhanced by ± 6% and ± 2% respectively.

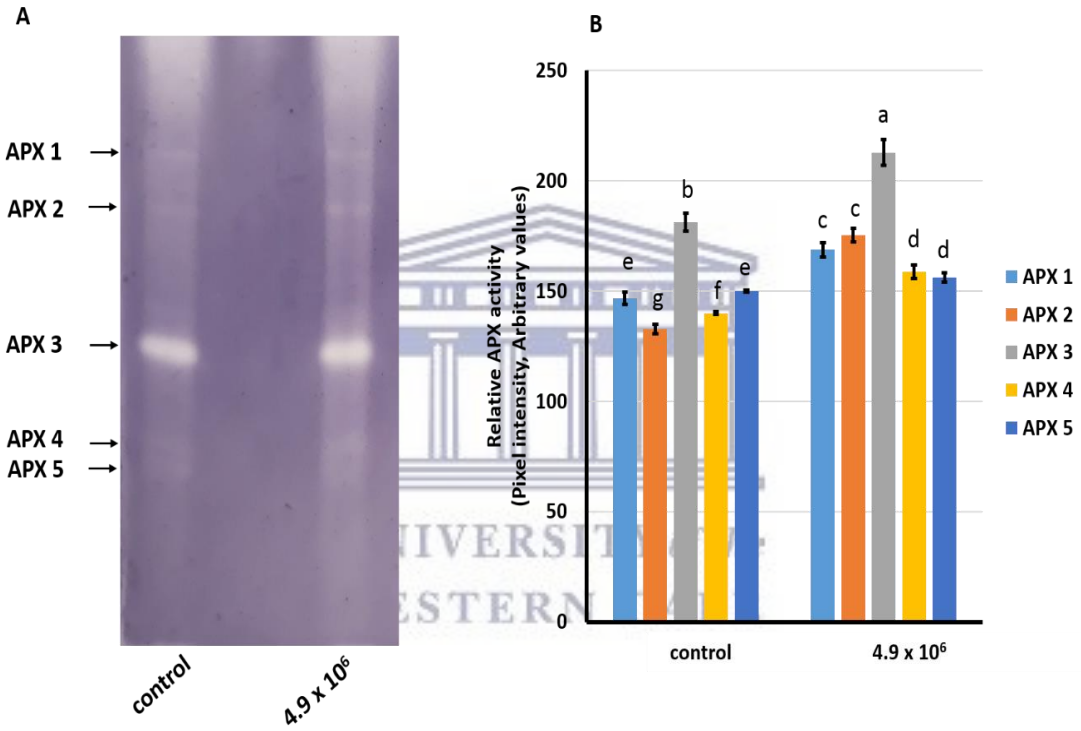

Figure 4.4: *F. oxysporum* enhances APX activity in cowpea shoots (A) APX activity (as individual isoforms) in cowpea plants were identified using native PAGE. (B) The enzymatic activity of each isoforms was quantified using densitometry analysis. The error bars represent the mean of three independent experiments and the letters above each graph indicate significant statistical differences between infected plant and control ($p < 0.05$).

4.2.5 *F. oxysporum* infection alters GR activity in cowpea shoots

As much as APX is important for converting H_2O_2 to H_2O, GR is a flavor-protein oxidoreductase which is also involved in the conversion of H_2O_2. However, GR is essential in the ASH-GSH cycle, by reducing GSSG to form and maintain GSH levels. This is very important in defense systems against ROS accumulation. To analyze the enzymatic changes of GR (as individual isoforms) in response to *F. oxysporum* infection, protein extracts from cowpea shoots were subjected to native polyacrylamide gel electrophoresis (PAGE) and densitometry analysis (Figure 4.5 A-B). The result showed that four GR isoforms (1-4) were identified using antioxidant specific staining as described in section 2.13. The enzymatic activity of each isoform was quantified using densitometry analysis (Figure 4.5 B). The enzymatic activity of GR 1 was enhanced by ± 6% in in response to *F. oxysporum* infection. On the contrary, GR 2 activity was reduced by ± 4% when plants was infected with *F. oxysporum.* However, for GR 3 and GR 4, no significant difference in their activities was observed when cowpea plants was infected with *F. oxysporum.*

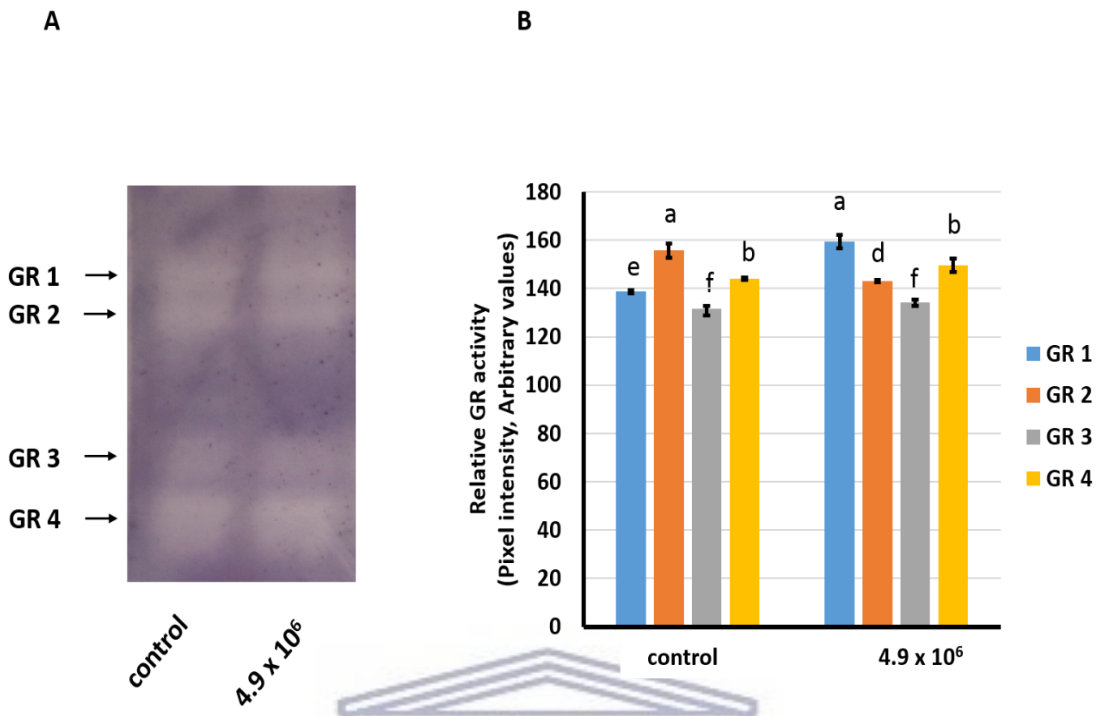

Figure 4.5: *F. oxysporum* infection alters GR activity in cowpea shoots. (A) GR activity was detected in cowpea shoots using native PAGE. (B) Densitometry analysis was used to quantify the activity of each GR isoform. The error bars represent the mean of three independent experiments and the letters above each graph indicate significant statistical differences between infected plant and control ($p < 0.05$).

4.3 DISCUSSION

In this study, we investigated the influence of *F. oxysporum* (4.9 x 10^6 cells/ml) infection on ROS accumulation and antioxidant capacity in cowpea plants by monitoring changes in ROS-induced oxidative damage (manifested as increased cellular death) and ROS-scavenging antioxidant enzyme activities.

4.3.1 *F. oxysporum* infection augments ROS induced oxidative damage in cowpea plants

When investigating plant response to stress by evaluating changes in ROS coupled with changes in antioxidant activity, it is important to understand that ROS function as signaling molecules which facilitate the activation of defense systems/genes in plants (Levine *et al.* 1994). However, oxidative burst is known as the overproduction of ROS from the consumption of oxygen. This is known to be the first response to pathogen interaction whereby, O_2^- from the apoplast of plants is generated and subsequently dismutated into H_2O_2 by the antioxidant SOD (Torres *et al.* 2006). Various studies conducted on pathogen interaction with host plants have reported an increased production in O_2^- levels (Doke, 1983; Auh and Murphy, 1995). *F. oxysporum* infection increased the levels of O_2^- in cowpea more than the non-infected (control) cowpea plants (Figure 4.1 A). As O_2^- is dismutated, the levels of H_2O_2 are expected to increase as it is the product of this pathway (Daub *et al.* 2013). Thus in this study, *F. oxysporum* resulted in increased levels of H_2O_2 in cowpea (Figure 4.1 B). This has been noted as well in a study conducted on *Arabidopsis thaliana* cell suspension cultures responding to an elicitor from *F. oxysporum* (Davies *et al.* 2006). In another study where the association of H_2O_2 with the restriction of *Septoria tritici* proliferation in resistant wheat cultivars revealed an increase in H_2O_2 levels during infection (Shetty *et al.* 2003).

Accumulation of H_2O_2 in plants results in programmed cell death (PCD). It is important to note that PCD is important for developmental processes including responses to pathogen infection (Bethke and Jones, 2001). Therefore, lipid peroxidation was investigated in *F. oxysporum* infected cowpea plants (Figure 4.2 A) and it was noted

that MDA levels were higher in the non-infected (control) plants. This is an obscure finding when considering the knowledge of ROS resulting in high lipid peroxidation. However, a study conducted on the early response of wheat antioxidants to *Fusarium* infection showed that in the initial days of infection the MDA levels were high, however from day 5 to 14 the levels dropped to levels significantly lower than that of the control (Spanic *et al.* 2017). We therefore hypothesized that, during pathogen infection H_2O_2 is hyper accumulated and it is targeted at the infection sites predominately, because H_2O_2 inhibits the growth of fungal pathogens (Shetty *et al.* 2007). In this way, lipid peroxidation is limited. Furthermore in this study, PCD was increased in response to *F. oxysporum* infection lead to higher cell death to cowpea plants. This is due to the nature of increased ROS due to stress, leading to DNA and protein damage and ultimately cell death (Gechev and Hille, 2005; Daub *et al.* 2013; Apel and Hirt, 2004).

4.3.2 *F. oxysporum* infection alters SOD activity in cowpea plants

Fungal infections in plants results in the overexpression of ROS and in this study it has been shown that such an oxidative burst leads the growth limitations and PCD. However, plants have evolved mechanisms involving antioxidants (both enzymatic and non-enzymatic) which scavenge ROS with the intention to alleviate oxidative damage to the plant (Baker and Orlandi, 1995). Therefore, O_2^- levels increase during the infection and SOD needs to be more active in order to maintain O_2^- homeostasis. SOD is the first line of defense involved in ROS metabolism and subsequently *F. oxysporum* infected cowpea plants were analyzed for SOD activity by means of native-PAGE (as per section 2.11). It was noted that the total SOD activity was generally reduced by a

small margin in the *F. oxysporum* infected cowpea plants (Figure 4.3 A). This is normally the case, as the analysis was performed 21 days post infection. A study done on *Mycosphaerella fragariae* infection in strawberry leaves showed an increase in SOD activity, reaching the peak on the 3 day post inoculation, and it was noted that the SOD activity decreased slowly as the number of days proceeded (Ehsani-Moghaddam *et al.* 2006).

In total, 7 SOD isoforms were identified from both infected and non-infected cowpea shoots (Figure 4.3 A) and their sensitivity and resistance to H_2O_2 and KCN inhibitor determined (Figure 4.3 B and 4.3 C). This enabled the characterization of Mn-SODs, Fe-SODs and Cu/Zn-SODs. In addition, these SOD isoforms are located in different cell organelles whereby Mn-SODs are located in mitochondrial and peroxisomal organelles, Fe-SODs are located in the chloroplast and Cu/Zn-SODs are located the cytosol and chloroplasts (Gill and Tuteja, 2010). It was noted that all three Mn-SOD isolated activities from the *F. oxysporum* infected cowpea had reduced activity. There was no statistical differences noted between the activity of Cu/Zn-SOD 1 and 2 isoforms between the infected and control cowpea plants. However, Fe-SOD 1 and 2 showed enhanced activity in the *F. oxysporum* infected cowpea plants. Thus it is hypothesized that Fe-SOD 1 and 2 were the main drivers of the total SOD activity, as the chloroplast (where the isoforms are located) is a heavy electron transport chain house.

Considering these findings, it would be of great benefit to investigate Mn-SODs as they are stress biomarkers that have been negatively influenced by *F. oxysporum* infection in cowpea. Their overexpression would drastically increase the activity of SOD thus

scavenging O_2^- efficiently. This would in turn increase cowpea antioxidant defense against the pathogen.

4.3.3 *F. oxysporum* infection influences APX activity in cowpea plants

The antioxidant APX is the main H_2O_2 scavenging antioxidant which functions in the ascorbate-glutathione cycle (Asada, 1992). The antioxidant is located mainly in the chloroplast and however can be found in the cytosol, mitochondria and peroxisomes (Asada, 1992; Noctor and Foyer, 1998). During stress on a plant, it's generally projected that antioxidant activity increases (Shigeoka *et al.* 2002; Caverzan *et al.* 2012), because O_2^- dismutation to H_2O_2 increases. As H_2O_2 levels increase, APX functions as a scavenger and also contributes to lignification to strengthen plant cell walls as a defense mechanism against fungal pathogens (Mäder and Füssl, 1982).

Thus APX activity was investigated in *F. oxysporum* infected cowpea shoots (as per section 2.12). A total of five APX isoforms were identified in both *F. oxysporum* infected and non-infected cowpea plants (Figure 4.4 A) and the APX activity was enhanced in infected cowpea plants. In another study, it was reported that APX activity had increased after 4 days post inoculation with *Botrytis cenerea* in tomato plants (Kużniak and Skłodowska, 2005). Thus it was quite evident that this had to be investigated in this study as well as the analysis was done 21 days post inoculation. Thus, it was observed that APX 1 and APX 3 - 5 isoforms isolated from *F. oxysporum* infected cowpea shoots had increased activity, however the increased percentage was statistically insignificant. However APX 2 activity was significantly enhanced, indicating that it was the main driver of the total APX activity. This finding is in

accordance with most literature studying pathogen-plant interaction. (Jimenez *et al.* 1997; García-Limones *et al.* 2002; Mandal *et al.* 2008).

4.3.4 *F. oxysporum* infection alters GR activity in cowpea

Located predominantly in chloroplast, GR is an antioxidant that catalyzes the reduction of GSH and it can be found in small amounts in the cytosol and mitochondria (Edwards *et al.* 1990). It is important to note that GSH participates in metabolic regulation and antioxidative pathways, thus GR facilitates the breakdown of disulphide bonds in GSSG by utilizing NADPH. This results in the formation of GSH thus maintaining a steady supply of the molecule (Rao *et al.* 2006; Rao and Reddy, 2008). In addition, GR role is characterized in the Halliwell-Asada pathway and it has been reported to increase during in order to provide adequate GSH for oxidative defense (León *et al.* 2002; Skórzyńska-Polit *et al.* 2003 and Khan *et al.* 2007).

To our knowledge GR activity in *F. oxysporum* infected cowpea plants as unique to this study as there is no literature found about its activity. Therefore, GR activity was investigated in *F. oxysporum* infected cowpea shoots (as per section 2.13), and four GR isoforms were identified in both the infected and non-infected (control) cowpea plants (Figure 4.5 A). It was noted that GR 1 had enhanced activity in response to *F. oxysporum* infection and GR 2 had decreased activity. GR 3 and 4 isoforms showed no statistically significant changes. Therefore GR 1 was the main contributor to total GR activity, however the total GR activity was not significant enough to generate adequate GSH to effectivity defend against oxidative stress in cowpea under *F. oxysporum* infection. This is because ROS levels were quite elevated leading the high cell death levels.

CHAPTER 5

CONCLUSION AND FUTURE WORK

The decline in cowpea production and the health risks to humans and animals due to *F. oxysporum* infection are a serious burden that need much attention. Food security is affected as people have less access to the nutritious crop, the crop is being used less as an intercrop and it is getting more expensive to purchase. Cowpeas response to *F. oxysporum* infection needs to be well understood as this is a beneficial crop.

In chapter 3, the physiology of infected cowpea was analyzed. This is because physiological changes in plants subjected to stress are the first indicator of stress. This makes it is imperative to study the physiological responses of cowpea induced by *F. oxysporum* infection, as this provide vital information about the level of infection with in the plant. Germination of cowpea seeds in the presence of *F. oxysporum* (4.9×10^6 cells/ml) showed no adverse effect on the germination rate after 5 days of germination. However, once the infected plant was allowed to growth for 21 days, *F. oxysporum* infection resulted in the stunting of growth and development of cowpea. This lead to the reduction of infected cowpea biomass, reduction of water retention abilities and to the reduction of the total chlorophyll content.

In chapter 4, the biochemical response of infected cowpea was examined. Pathogen-plant interactions are limiting factors to the development and growth of cowpea. It was shown that this is due to the influence they have by eliciting an oxidative burst, resulting in hyper accumulation of O_2^- and H_2O_2. These ROS molecules initiate oxidative damage to DNA and proteins which then leads to PCD. It was determined that O_2^- and H_2O_2 levels were elevated by *F. oxysporum* in cowpea shoots. This resulted in elevated cell

death as well. However, antioxidants (SOD, APX and GR) function as ROS scavenging enzymes and they were noted to have high activity in infected cowpea shoots. Consequently, to attempt to reduce O_2^- and H_2O_2 accumulation although the increase level of activity was not adequate enough reduce ROS and avoid excess cell death.

For future work, the antioxidants identified in *F. oxysporum* in cowpea can be utilized as biomarker that can be overexpressed to reduce oxidative damage and alleviate cowpea from growth limitation resulting from the infection. The Mn-SODs and Cu/Zn-SODs were isoforms of SOD that contributed the least activity. Therefore, they serve is likely candidates to be overexpressed as it is hypothesized that *F. oxysporum* infection reduces their ability to function optimally. This can firstly be coupled with a proteomic study, whereby differentially expressed proteins can serve as protein biomarkers that can be targeted. Genetic sequence information pertaining cowpea is easily available, thus such a study would be pivotal in order to answer the questions left out in this study and hypotheses.

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