Physiological and molecular characterization of wheat cultivars to *Fusarium oxysporum* infection

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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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KEYWORDS

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ABSTRACT

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Biotic stress is one of the main causes for agricultural loss of economically important cereal crops. The increasing prevalence of biotic stress inflicted by fungal species such as *Fusarium* has significantly reduced yields and quality of cereals, threatening sustainable agriculture and food security worldwide. Interactions between wheat and *Fusarium spp.* such as *Fusarium oxysporum* promotes the accumulation of reactive oxygen species (ROS). Overproduction of ROS can become toxic to plants depending on the scavenging ability of antioxidant systems to maintain redox homeostasis. This study investigated the effects of *F. oxysporum* on the physiological and biochemical response of three wheat cultivars namely, SST 056, SST 088 and SST 015. Physiological responses were monitored by measuring changes observed in plant growth parameters including shoot and root growth and biomass, relative water content as well as photosynthetic metabolism and osmolyte content in all three wheat cultivars. Downstream biochemical analysis involved monitoring the accumulation of ROS biomarkers (superoxide and hydrogen peroxide) as well as the detection of enzymatic activity of superoxide dismutase (SOD), ascorbate peroxidase (APX) and peroxidase (POD). These biochemical responses were only monitored on the two wheat cultivars which presented contrasting responses to *F. oxysporum* infection.

Results showed that *F. oxysporum* significantly reduced plant growth, biomass, chlorophyll pigments and relative water content of all three cultivars, with the highest reduction observed

for SST 088 relative to SST 015 and SST056. On the other hand, proline content was significantly enhanced in all three wheat cultivars, with the highest increase observed for SST 015 relative to SST 056 and SST 088. Based on the contrasting physiological results observed for these three cultivars, downstream biochemical analysis was focused on SST 015 and SST 088. *F. oxysporum* trigged an increased in superoxide and hydrogen peroxide contents in both cultivars, with the highest increase observed for SST 088. A similar trend was observed for the extent of lipid peroxidation, manifested as enhanced MDA levels. Furthermore, *F. oxysporum* differentially altered antioxidant enzyme activity relative to the control of both wheat cultivars. A Significant increase in SOD activity was observed for both cultivars in response to *F. oxysporum*. However, contrasting responses in APX and POD activity (as seen for the band intensities of individual isoforms) was observed in these wheat cultivars in response to *F. oxysporum*.

Based on the results obtained in this study we suggest that *F. oxysporum* infection has varying degrees of severity in different wheat cultivars. In light of the significant reduction of plant development coupled with enhanced ROS accumulation and differential antioxidant capacity for SST 015 relative SST 088, we suggest that SST 015 is more resilient to *F. oxysporum*. We thus conclude that a direct relationship exists between ROS accumulation and antioxidant scavenging in regulating plant tolerance against *F. oxysporum* pathogens.

DECLARATION

I declare that "Physiological and molecular characterization of wheat cultivars to *Fusarium oxysporum* infection" 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.

Danielle Andrea Davids **December 2019**

Signed  ……..

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
APX	Ascorbate peroxidase
AsA	Ascorbic acid/ascorbate
BSA	Bovine serum albumin
CAT	Catalase
Cu/ZnSOD	Copper zinc superoxide dismutase
DHAsA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
DTT	Dithiothreitol (Cleland's reagent)
EDTA	Ethylenediaminetetraacetic acid
FeSOD	Iron superoxide dismutase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
MDA	Malondialdehyde
MnSOD	Manganese superoxide dismutase
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitrotetrazolium blue chloride
PVP	Polyvinylpyrrolidone
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
TEMED	N,N,N′,N′ - Tetramethylethylenediamine
RBD	Random block design
MDHA	Monodehydroascorbate reductase
POD	Peroxidase
PDA	Potato dextrose agar
PAGE	Polyacrylamide gel electrophoresis
QAC	Quaternary ammonium compounds

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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

The importance of sustainable agriculture is driven by the continuously increasing human population. It is estimated that by 2050 the global population size will exceed 9 billion. This makes the production of good quality nutritious food required to feed the growing population challenging. Agricultural commodities such as cereal crops form an integral part of the human and animal diets and plays a significant role in feeding the masses. Cereal grains represent a fundamental element of the human diet for thousands of years and contributed to shaping human civilization as we know it today. Cereal crops such as rice, wheat and maize, are some of the most important staple foods critical for the daily survival of billions of people. The world is currently experiencing a diverse range of challenges that affect the production of economical important cereal crops to sustainably feed its people. Challenges include the impact of climate change, land degradation and persistent environmental stress as well as rapid population growth. These challenges, in conjunction with inadequate or weak environmental policies and limited financial resources, makes it difficult for countries worldwide (specifically developing countries) to meet projected demands for food. As a result, developing countries such as South Africa are under pressure to produce enough staple foods to meet the basic nutritional requirements of the population.

It is estimated that the global agricultural production is required to increase by between 60% to 110% in order to meet the increasing demands by 2050, ensuring food security to 10.8% of the world's population, currently suffering from undernourishment (FAO *et al.* 2019; Tilman *et al.* 2011). As it stands 26.4% of the world's population is regarded as food insecure, with

the African continent presenting the highest level of food insecurity relative to the total population (Figure 1.1). Based on the statistics compiled by the Food and Agricultural Organization of the United Nations (FAO) for the year 2018, more than half of the continent suffers from food insecurity. Different regions of Africa presented varying prevalence of food insecurity with 29.5% in Northern Africa, 47.9% in Western Africa, 53.6% in Southern Africa and 62.7% in Eastern Africa (FAO *et al.* 2019).

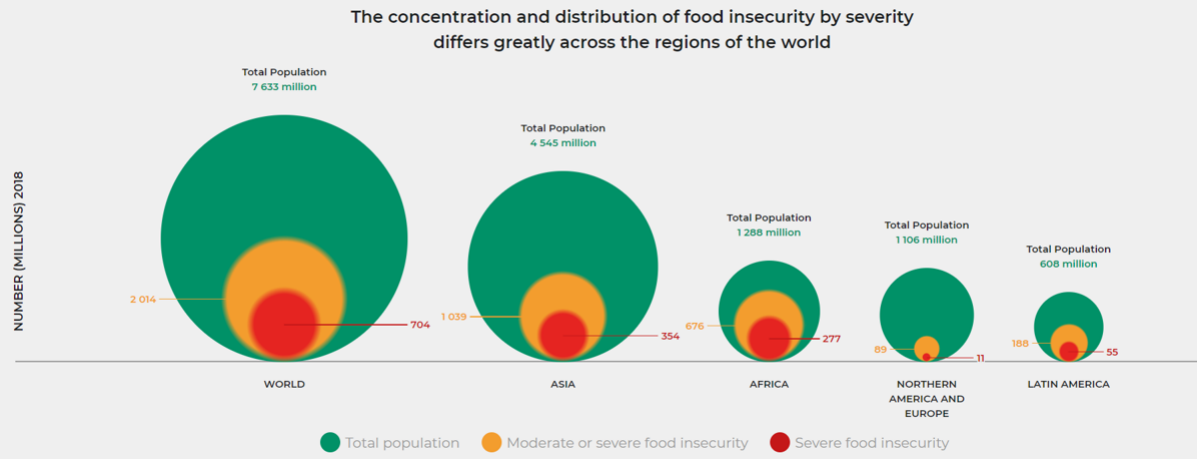

Figure 1.1: The concentration and distribution of food security by severity differs greatly across regions worldwide. This image was adapted from FAO *et al.* (2019).

According to the US Department of Agriculture (USDA), food insecurity is defined as the economic and social condition of households with limited and no access to adequate, nutritional food (Ers.usda.gov, 2019). This has become a fundamentally critical issue in various developing countries including sub-Saharan Africa (Drimie and McLachlan, 2013). In 2017, Sub-Saharan Africa was found to have the highest prevalence of food insecurity (55%) in relation to the rest of the world (Figure 1.2) (Smith and Meade, 2019).

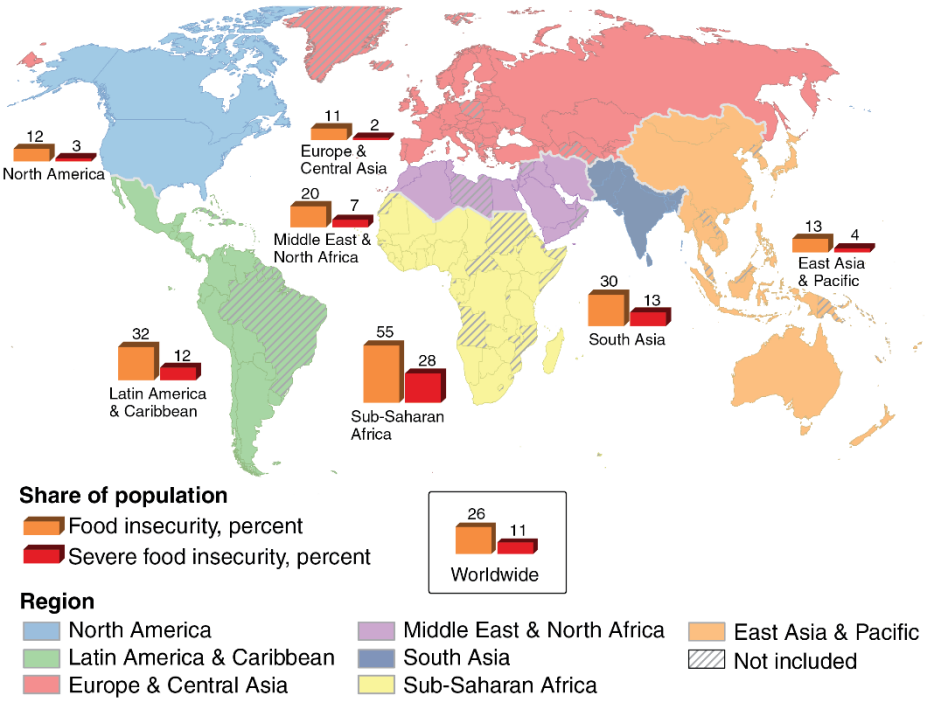

Figure 1.2: Geographic representation of food insecurity and severe food insecurity worldwide in 2015. This map was adapted from Smith and Meade (2019).

South Africa was deemed a medium food secure nation that produces sufficient staple foods and has the capacity to import foods essential to meet basic nutritional requirements for the population. However, Hart *et al.* (2009) concluded that although the country is food secure at a national level, this is not the case of households in rural areas. A survey conducted by Statistics South Africa (Aliber, 2009), reported that approximately 20% of South African households have inadequate or severely inadequate access to food. Despite the political and economic advances seen in South Africa since 1994, the country remains one of the fastest growing populations (Larkins *et al.* 2008). The country is overwhelmed by the increasing poverty and unemployment rates, in addition to the global economic crisis, which led to the significant increase in food and fuel prices as well as high-energy tariffs and increasing interest rates (Drimie and McLachlan, 2013; Labadarios *et al.* 2011; Altman *et al.* 2009). In addition to the economic challenges faced by countries like South Africa, meeting the required crop

yields has been severely affected and influenced by environmental stresses including salt, drought, water excess, UV-B radiation, severe changes in temperature, pathogens, insects, chemicals, ozone, and oil nutrient deprivation (Mahajan and Tuteja, 2005). These abiotic and biotic stresses have shown to influence both plant development and reproduction at varying severity levels and is often maximized in combinations (Caverzan *et al.* 2016).

In addition to environmental adversities due to increasing abiotic and biotic stresses, the world is faced with a fast approaching and significantly increasing agricultural crisis (Curtis and Halford, 2014). To combat the increase in demand, several authors have suggested that promoting crop production through more efficient uses of the available arable lands (Foley, 2011), with the most suitable management practices in place will reduce yield gaps worldwide (Gregory and George, 2011; Godfray *et al.* 2010). Increases in crop yield was shown to be an effective tool in reducing the global poverty and undernourishment (Pingali, 2012). In contrast, several studies have shown that between 24% to 39% of the most important croplands in various regions worldwide are not improving yields (Ray *et al.* 2013). These areas represent many of the top crop producing nations, with significant increasing populations, increasing affluence and the combination of these factors (Tilman *et al.* 2011). Alternatively, additional strategies targeted at plant-based diets and reducing food waste (Foley *et al.* 2011) could potentially also reduce the expected demand in crop growth for food (Tilman *et al.* 2011) as a result of food security being shown to be highly influenced by plant-based food sources such as cereal crops.

1.2 Cereal crops

Cereal crops, more commonly known as cereal grains belong to the grass family (*Gramineae*) and are grown in large quantities, providing more food energy than any other crop. These crops

are classified into two main groups namely true cereals and pseudo-cereals (Klinck, 2015). True cereals include crops from the botanical family (*Poaceae*) which includes wheat, oats, rice, maize (corn), barley, sorghum, rye and millet. These groups are further categorized into different varieties. Alternatively, pseudo-cereals such as amaranth, buckwheat and quinoa do not belong to the same botanical family, however, they are nutritionally similar and used in similar ways.

1.2.1 The importance of cereal crops

Cereal crops have been one of the principal components of human diet for thousands of years. Due to their valuable nutritional and medicinal properties, they have played a major role in shaping human civilization (Awika, 2011). Current studies suggest that food security is dependent on the production of three main cereals that are widely cultivated. These include wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.) and maize (*Zea mays* L.) which belong to the grass family (*Gramineae*) (Curtis and Halford, 2014). These cereals are more important in comparison to other food sources due to their role as staple food crops in many parts of the world. In addition to playing a vital role in the human diet, these cereals are also used to produce animal feed, oils, starch, flour, sugar, syrup, processed foods, malt, alcoholic beverages, gluten and renewable energy, thus making them high in demand. Approximately 50% of the world's calories are provided by wheat, rice and maize. Of the three, wheat is the leading sources of vegetal proteins in human food, with a protein content of approximately 13% and provides 19% of global dietary energy (Ray *et al.* 2013).

1.3 Triticum aestivum L. (Wheat)

Triticum asetivum L. (wheat) is suggested to have been first cultivated in regions of the Fertile Crescent in the Near East areas, presently occupied by Syria, Turkey, Afghanistan, Iraq and

Iran. However, archaeological remains of the domesticated wheat grains were found in Ali Koshi (Iranian Khusistan) around 6500 BC and in Anatoliain (Turkey) approximately 5500 BC, suggesting that the crop originated from these regions (Gustafson *et al.* 2009). The cultivation of wheat then spread from its origin to India, Pakistan and China in the east and the Mediterranean countries in the west. In addition, cultivation spread to the Union of Soviet Socialist Republic (USSR) as well as several other countries in the north. It is suggested that wheat was introduced to Cape Town in South Africa during the middle of the 17th century and its cultivation spread throughout the country shortly thereafter (Gustafson *et al.* 2009). To date, wheat is cultivated in more areas than any other food crop, occupying more than 220.4 million hectares, with the annual wheat production reaching approximately 749 million tonnes in 2016, making it the second most-produced cereal after maize (Ray *et al.* 2013).

1.3.1 Classification and uses

Wheat is classified as one of the main cereal crops belonging to the *Gramineae* family including several important cereals such as rice, maize, oats and sorghum. Wheat production represents an integral part of a low input agricultural system that provides food, income and supports the platform for the productivity of other crops. As a result, it is high in demand specially in countries such as South Africa where wheat is one of the main food sources. Due to reduction in growth experienced, the country has become more dependent on imports to meet the local demand of approximately 3.3 million tons. Wheat is most commonly used for production of consumables, with approximately 75% of the yield being used to produce wheat flour, required for the production of bread, cakes and biscuits. A smaller portion of the yield is used for the manufacturing of industrial products such as starch, gluten and dextrose whereas the lower quality grains including the by-products of the refinery process is used in the animal feed industry (Mason *et al.* 2015).

1.3.2 Structural characteristics

The wheat plant is described as herbaceous annual plant that grows up to 1.2 m in height and consists of hollow cane-like stems. The growth of the stems occurs through the stretching of tissue above the nodes (meristem). The development of leaves as depicted in Figure 1.3 A, occurs from these nodes and like several grasses, they consist of two main parts namely the petiole sheath that surrounds and functions to protect the meristem and the growth zone as well as the limbo that is elongated in shape and has parallel veins (ScienceAid, 2019). Flowers are grouped in spikes, whereby each spike consists of a main axis on to which several spikelets are distributed laterally. The main axis from which some filaments arise are terminated by the glumes that enclose the flowers until maturity (Figure 1.3 B). In addition, the flowers are protected by two bracts known as the palea (inner) and lemma (outer). The latter is covered with beard, giving the ear of the wheat a feathery appearance. Female wheat flowers consist of an ovary from which two styles emerge with two feathery sticky stigmas each. In contrast, the male flowers possess three stamens that are either gold, green or violet in colour.

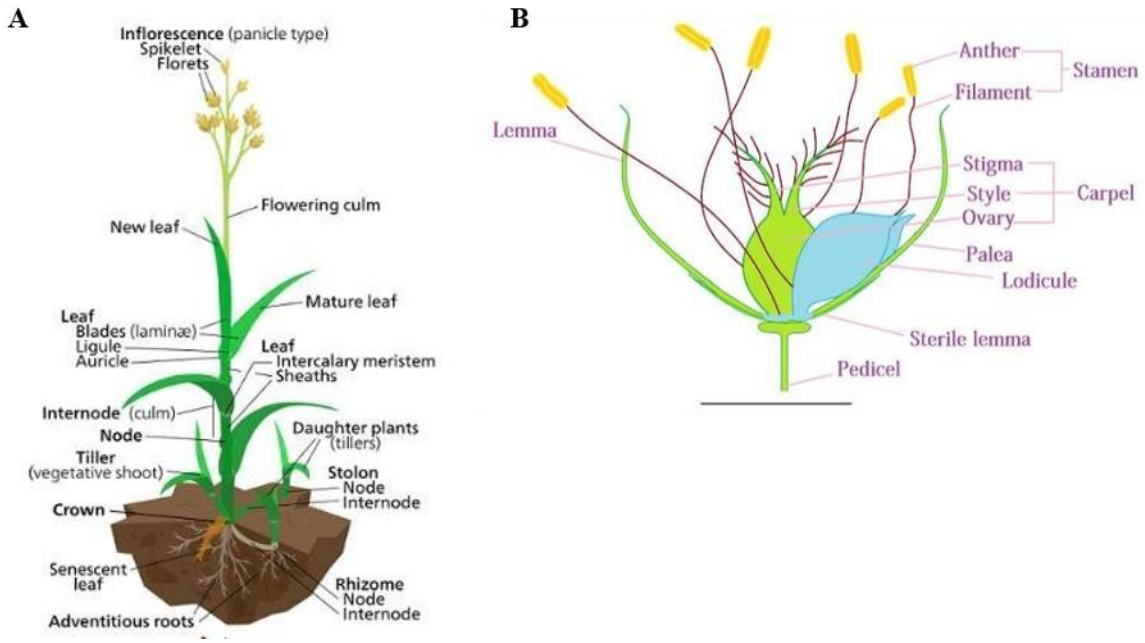

Figure 1.3: Wheat plant and flower structural characteristics. The plant structure (A) and flower structure (B) images were adapted from ScienceAid (2019).

1.3.3 Nutritional and medicinal values

Wheat represents one of the most important cereals in the world. Approximately 85% and 82% of the global population is dependent on wheat for the basic calories and protein, respectively (Chaves *et al.* 2013). In addition, whole wheats are shown to be rich in nutrients, minerals and vitamins, which play a vital role in reducing and improving various medical issues and diseases. These include reducing the risk of type 2 diabetes, improves metabolism, aids in lowering cholesterol and reduces the risk of cardiovascular diseases as well as breast and colon cancer (Drankham *et al.* 2003). These benefits are as a result of the high levels of minerals such as potassium, magnesium, iron, zinc and phosphorous in addition to being rich in vitamins B6, vitamin E and folate. Wheat contains high levels wheat germ, phytosterols and nutrients such as calories and carbohydrates (Kumar *et al.* 2011). The global demand for wheat continues to grow due to the unique viscoelastic and adhesive properties of gluten proteins. These proteins play a vital role in the production of processed foods such as breads, pasta, biscuits, noodles, couscous and beer (Curtis and Halford, 2014), that are increasingly being consumed as a result of the adaptation to a westernized diet (Scherm *et al.* 2013). Apart from its use for human consumption, wheat is also used in the development of non-food products such as biofuel (Chaves *et al.* 2013).

1.4 Influence of biotic stress on cereal crops

Due to the increasing demand for crop production, the occurrence of several environmental stresses continues to threaten agricultural productivity and is one of the major causes of pre- and postharvest loss (Dresselhaus, 2018; Zhao *et al.* 2017; Singla and Krattinger, 2016). Environmental stresses play a vital role in the productivity, survival and reproduction of many crops and plants (Redondo-Go´mez *et al.* 2010). These stresses are grouped into two main types namely abiotic stress which refers to the changes in the physical environmental and biotic stress

caused by living organisms (Redondo-Go´mez, 2013). In contrast to abiotic stresses caused by environmental factors, plants encounter several biotic agents including bacteria, fungi, nematodes, viruses, viroids, insects and protists (Onaga and Wydra, 2016) that directly deprive the host plant of nutrients which result in reduced plant vigour and increased plant death (Singla and Krattinger, 2016). Several publications have shown that these biotic stress agents cause various diseases, infections and damage to crops, ultimately leading to significant reductions in crop productivity, although many biotic agents may co-exist symbiotically or synergistically with plants (Onaga and Wydra, 2016; Gull *et al.* 2019). One of the major problems we are faced with is that various cereal crops such as wheat, grown and used in the production of staple foods are severely affected by adverse biotic stresses (Rahaie *et al.* 2013). These include biotic agents such as disease-causing fungi, bacteria and viruses that can significantly damage various crops. These agents also affect annual yields influencing the production, quality and accounts for persistent yield losses of up to 20% of the world's harvest, with a further 10% loss post-harvest (Oerke, 2006; Flood, 2010).

1.5 Fungal pathogens affecting cereal crops

Disease-causing fungi are one of the most dominant groups of pathogens affecting cereal crops and can cause disease at each level of the plant's physiology (Dean *et al.* 2012; Doehlemann *et al.* 2017). The variation of fungal infections results in a diverse range of symptoms that contribute to significant yield losses. Fungal infections often result in the formation of necrotic lesions on plant leaves and stems that ultimately lead to leaf senescence in addition to the reduction in grain quality. A study by Lule *et al.* (2014) demonstrated a 40% reduction in yield of the globally important finger millet as a result of infection by the fungal pathogen *Magnaporthe oryzae*. In addition, pathogenic soil-borne fungal species invade plant roots primarily causing root rot from the base of the plant upwards, whilst weakening the host plants

by utilizing the plants nutrients as shown in a study conducted on wheat infected with *Gaeumannomyces graminis* (Cook, 2003). Other fungi including *Fusarium spp*. do not necessarily cause senescence; however, the infection negatively impacts the yield as a result of the significant reduction in grain quality, which can significantly increase in response to the production of high mycotoxin concentrations (Antonissen *et al.* 2014; Dean *et al.* 2012).

1.6 Fusarium interaction with plants

Fusarium is a highly diverse genus of filamentous ascomycete fungi (*Sordariomycetes: Hypocreales: Nectriaceae*) and consists of phytopathogenic and toxigenic species. The genus was first described by Link in 1809 as Fusisporum and later renamed *Fusarium*. The genus consists of twenty monophyletic species complexes that are further classified into nine species (Rana *et al.* 2017) that encompass all the economically important *Fusarium species* of approximately sixty different types (Husaini *et al.* 2018). These species are present in a wide range of plants, soils and water, acting as endophytes, parasites or saprophytes (Rana *et al.* 2017). The pathogenic *Fusarium species* are causative agents to many plant diseases that affect a variety of plant species (Figure 1.4).

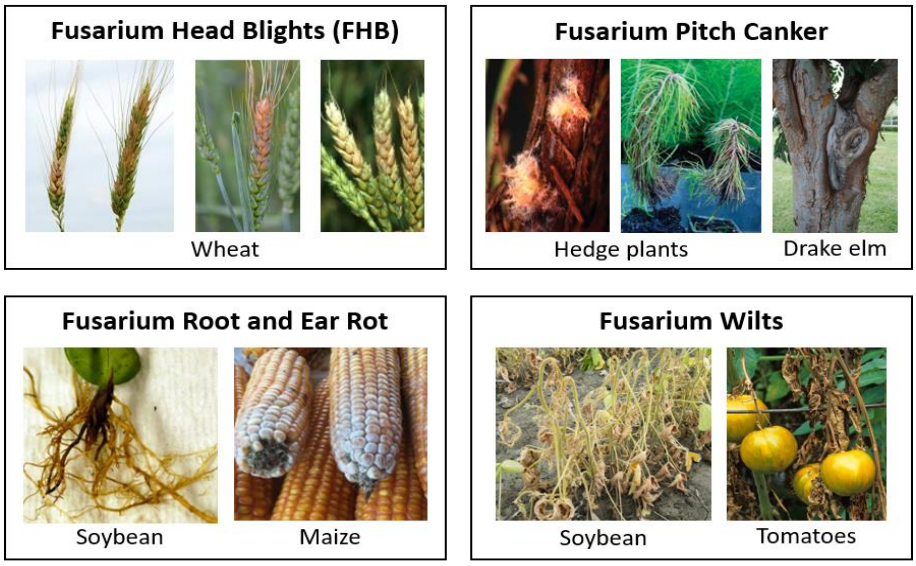

Figure 1.4: Common plant diseases caused by *Fusarium species.* Plant diseases include Fusarium head blight (FHB), pitch canker, root and ear rot and wilts.

1.7 Fusarium oxysporum

Fusarium oxysporum (*F. oxysporum*) represents one of the most abundant soil-borne species that comprises all varieties and forms within the infrageneric group in the Elgans section (Rana *et al.* 2017), belonging to the family *Nectriaceae*. The fungus (*F. oxysporum*) consists of more than 100 strains (formae speciales: ff. spp) and inhabits several hosts ranging from soils, herbaceous plants to trees (Gordon, 2017). They are predominantly harmless in native soils, acting as beneficial plant endophytes or soil saprophytes. The saprophytic species are known to degrade lignin (Rodriguez *et al.* 1996; Sutherland *et al.* 1983) and complex carbohydrates that are associated with soil debris (Christakopoulos *et al.* 1996; 1995). The endophytic species are highly pervasive and can colonize plant roots in addition to protecting the plants by initiating the basis of disease suppression (Rana *et al.* 2017). However, many strains within the *F. oxysporum* complex are pathogenic to many plants and can be detrimental to plant health especially those of great agricultural importance. Pathogenic *F. oxysporum* species are further classified into opportunistic parasites, true pathogens and obligate pathogens, which are dependent on the specialization of fungal-plant interactions. Opportunistic parasites colonize the weakened host plants in comparison to true pathogens that require living plants from their developments and growth, and obligate pathogens that essentially require live host plants to complete their life cycle production (Scheffer, 1991).

1.7.1 Reproduction

Fusarium pathogens have diverse life cycles and can produce meiotically (sexually) or mitotically (asexually) with a minimum of three types of spores. *F. oxysporum* reproduces asexually and produces all three spore types namely; microconidia, macroconidia and chlamydospores (Figure 1.5).

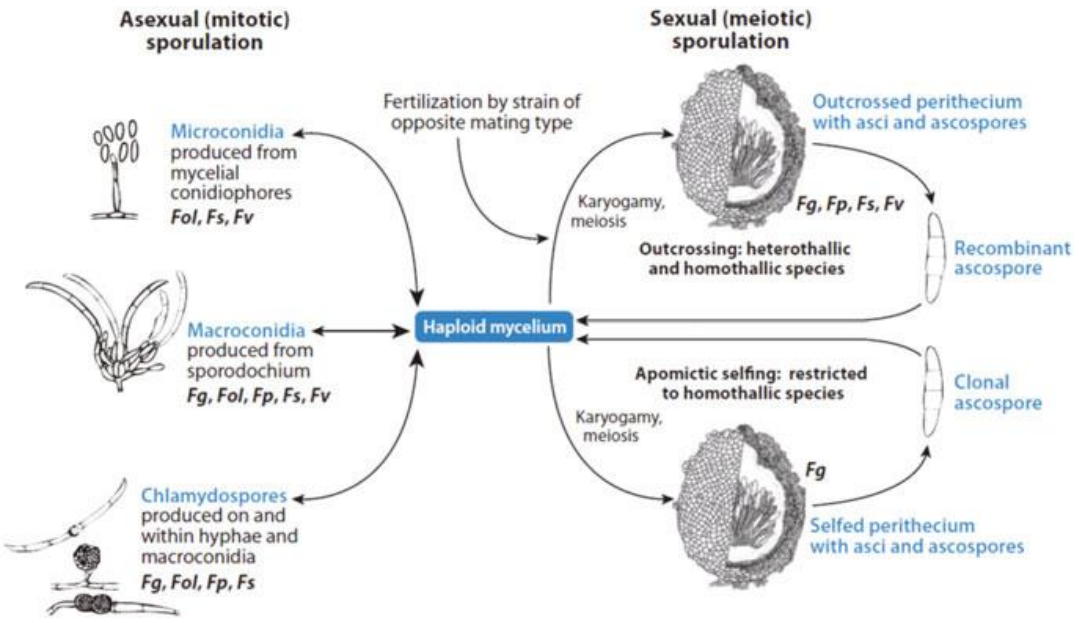

Figure 1.5: Generalized life cycle of Fusarium depicting varying reproduction strategies. Adapted from Rana *et al.* (2017).

Microconidia produced from the mycelial conidiophores are uninucleate which results in poor gemination efficiency ranging between 1% to 20% (Ebbole and Sachs, 1990) in comparison to macroconidia produced from sporodochium, which are multinucleate and germinate rapidly. The third spore type, chlamydospores are produced on and within the hyphae and macroconidia (Schippers and van Eck, 1981). *F. oxysporum* was found to be diversified with regards to the macroconidia shape, micro-conidiophores structure and the formation of chlamydospores (Beckman, 1987). Spores produced by the fungus can remain dormant in soil for up to 30 years limiting adequate control and management (Rana *et al.* 2017). Due to the various dispersal methods of these fungal spores, there is a high prevalence of *F. oxysporum* infection and plant disease across its hosts.

1.7.2. Plant infection and disease management

The ubiquitous, soil-borne *F. oxysporum* species represents one of the most destructive disease-causing pathogens and causative agents of various plant diseases. According to a study

conducted by Gordon (2017), *F. oxysporum* strains have more than 100 host-specific species (formae speciales: ff. spp.) which are distributed worldwide. It is also an emerging pathogen on immune compromised patients (O'Donnell *et al.* 2004) and mammals (Ortoneda *et al.* 2004). *F. oxysporum* can infect both monocotyledonous and dicotyledonous plants (Armstrong and Armstrong, 1981). From a commercial aspect, *F. oxysporum* strains infect a wide range of plants including tomatoes, potatoes, peppers, legumes, cereals, banana and watermelon (Altinok, 2013). From the various plant diseases caused by the species, the common fusarium wilt caused by *F. oxysporum* is a lethal vascular syndrome that affects many plant species, thus posing a threat to a wide range of economically and commercially important plants and crops (Husaini *et al.* 2018; Joshi, 2018). Visual symptoms include wilting, stunted growth, leaf chlorosis in addition to internal symptoms that affect vascular tissue resulting in a brown/black colour (Chung, 2012). The pathogen significantly reduces field crop yields and presents an increasing prevalence in infection. Between 2008 and 2009, South African maize yields suffered a significant loss as a result of approximately 12 million metric tonnes being infected by *Fusarium* species including *F. oxysporum* (Gerber, 2010).

There are several strategies in place to aid in reducing the prevalence and occurrence of infection by *Fusarium species*. However, due to the pathogens ability to remain dormant for an extended period in addition to the existence of perennial host plants, control and eradication methods are not as effective. Several methods involving field sanitation are available and suggested to be the most suitable form of control. Other control measures such as certified and pathogen free seeds are used alongside modified cultural practices, which involve complete removal and destruction of infected plants (Renard and Quillec, 1983). However, scientists suggest that the use of resistant wheat breeding lines will be the most effective control method that will ensure reductions in infection and plant loss due to of *Fusarium* species.

1.8 Pathogen-induced ROS accumulation

Reactive Oxygen Species (ROS) are natural by-products of cellular metabolism that can result in significant damage to membrane systems and DNA. When plants are subjected to biotic and abiotic stresses, they result in characteristic increases in the production of ROS such as single oxygen ($^1O_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl ($OH^\bullet$) (Mittler *et al.* 2004). Of these ROS, O_2^- is commonly the first to form and is constantly produced due to the partial reduction of oxygen (O_2). This ROS undergoes transformation into more reactive and toxic ROS in addition to causing lipid peroxidation (Halliwell, 2006). When O_2^- undergoes both univalent reduction as well as protonation, the moderately reactive H_2O_2 is formed. H_2O_2 is known for its multiple functions in plant defence against pathogen attack. These functions include direct microbicidal activity at sites of pathogen invasion, cell wall reinforcement by lignification and oxidative cross-linking of hydroxyproline-rich proteins and other cell wall polymers (Kuzniak and Urbanek, 2000). In addition, H_2O_2 was found to be necessary for phytoalexin synthesis and may trigger programmed cell death (PCD) in cells during hypersensitive response that restricts the spreading of infection (Kuzniak and Urbanek, 2000). However, due to H_2O_2 being moderately reactive, the extent of damage is only observed when it is converted to subsequent ROS.

1.8.1 Mechanisms of ROS scavenging

Like many cereal crops, wheat plants have a specialised mechanism to combat biotic stress such as pathogenic infections. The continuous evolution of different wheat lines has allowed them to adapt and survive in stressful environmental conditions (Rejeb *et al.* 2014). Under these stress conditions, plants tend to control the over production of ROS such as $O_2^-$and H_2O_2 (Torres, 2010; Fujita *et al.* 2006). This is achieved through the activation and initiation of various defence mechanisms to manage ROS levels, ensuring less toxicity in plants. One such

mechanism is the activation of ROS-scavenging pathways for the strict control of these ROS, to maintain redox homeostasis in plant cells. To ensure the protection of the cell from oxidative damage caused by oxidative stress, the plant will activate its antioxidant defence systems, which include both enzymatic and non-enzymatic systems (Dietz, 2003). Both antioxidant systems play a vital role in the balancing and preventing oxidative damage (Foyer,, 1994). The antioxidants in these systems function involves delaying, inhibiting or preventing the effects of oxidative stress by scavenging free radicals (Dai and Mumper 2010).

1.8.1.1 Enzymatic mechanisms

Enzymatic components consist of several antioxidant enzymes that form part of the Halliwell – Asada pathway also known as the Ascorbate-Glutathione (AsA-GSH) pathway. This pathway represents the main H_2O_2 detoxifying system in the cytosol, chloroplasts and mitochondria in plant cells (Noctor and Foyer,, 1998) and involves five enzymatic ROS scavenging antioxidant enzymes. These enzymes include superoxide dismutase (SOD; EC 1.15.1.1), ascorbate peroxidase (APX; EC 1.11.1.11), monodehydroascorbate reductase (MDHAR: EC 1.6.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1) and glutathione reductase (GR; EC 1.6.4.2) (Inze and Van Montagu 1995) as depicted in Figure 1.6. Each enzyme in the pathway has various subcellular isoforms, which differs in spatial and temporal expression and are differentially regulated based on biotic and abiotic stresses. Although there are alternate forms of ROS scavenging, literature suggests that the enzymes from the AsA-GSH pathway are more effective as they cover a greater surface area within the plant and have a greater affinity for H_2O_2 and O_2^-. In this study, the focus was mainly on SOD, APX and guaiacol peroxidase (POD; EC 1.11.1.7) as these enzymes work together by initiating the relevant cycles which aid in reducing the detrimental effects of the overproduced ROS.

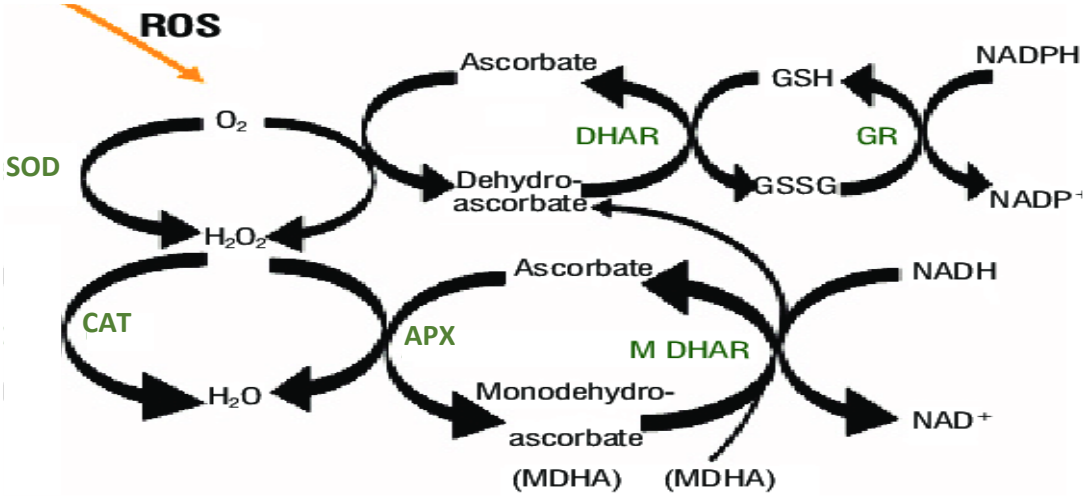

Figure 1.6: The redox cycling of ROS and Brassinosteroids-regulated in plants as described by the Asada-Halliwell pathway. This pathway involves the use of enzymatic antioxidants catalase (CAT), superoxide dismutase (SOD), Ascorbate peroxidase (APX) and glutathione reductase (GR) to assist in the scavenging of accumulated ROS molecules in response to stress. This image was modified after Arora *et al.* (2010).

SOD is present in all aerobic organisms and represents the first line of defence against ROS induced damage. This is due to gene expression that occurs when plants are exposed to biotic stress, indicated by the increase in O_2^- (Cheng and Song 2006; Lee and Lee 2000). SOD can be further classified into three isoenzymes MnSOD (mitochondria), FeSOD (chloroplast) and Cu/ZnSOD (cytosol, peroxisome, chloroplast) dependent on their localization and the metal ion they bind to (Mittler *et al.* 2004). The antioxidant enzyme's primary function is to catalyse the removal of O_2^- by dismutation yielding the less toxic H_2O_2 and H_2O.

$$O_2^- \xrightarrow{SOD} H_2O_2 + H_2O$$

The reduction of O_2^- by SOD into H_2O_2, results in the activation of the AsA-GSH (Halliwell-Asada) scavenging enzymes such as APX. APX represents the first enzyme of AsA-GSH pathway and prevents the accumulation of toxic levels of H_2O_2. This is achieved through the detoxification of H_2O_2 to H_2O and monodehydroascorbate reductase (MDHA) by utilizing AsA

as an electron donor (Asada, 1992). To date, five APX isoenzymes namely; cytosol, mitochondrial, peroxisomal/glyoxysomal and chloroplastic have been identified in plants (Dabrowska *et al.* 2007).

$$\mathbf{AsA + H_2O_2 \xrightarrow{APX} MDHA + H_2O}$$

In addition to SOD and APX, POD is found to be widely distributed in higher plants and is suggested to be an indicator of stress, however it also functions to protect cells against oxidative damage (Lin and Kao, 2002; Sudhakar *et al.* 2001; Dionisio-Sese and Tobita, 1998). The enzyme's key role involves the biosynthesis of lignin and defends the plant against biotic stresses such as pathogen attack, by consuming H_2O_2 present in various cell compartments including the cell wall, cytosol, vacuole and extracellular space (Karuppanapandian *et al.* 2011).

$$\mathbf{2\ GSH + H_2O_2 \xrightarrow{POD} GS\text{-}SG + 2\ H_2O}$$

1.9 The role of osmoprotectants against pathogen infection in plants

Under stressful conditions, the production of free radicals often exceeds the total cellular antioxidative potential, which leads to oxidative stress, which in turn has detrimental effects on plant growth. To combat the generation of these free radicals, plants accumulate high levels of different types of compatible solutes. These solutes protect plants from stress by contributing to osmotic adjustment, ROS detoxification and the protection of membrane integrity as well as the stabilization of enzymes and proteins (Ashraf and Foolad, 2007; Yancey, 1994). These solutes include proline, trehalose and quaternary ammonium compounds (QACs) such as glycine betaine (GB), proline betaine (PB), alinine betaine (AB) as well as pipecolate betaine (PCB) (Ashraf and Harris, 2004; Hare *et al.* 1998; Rhodes, 1993). From the solutes mentioned,

proline and GB represent two of the major organic osmolytes that accumulate in a wide variety of plant species in response to stress.

Proline is an efficient OH and $^1O_2$ scavenger and inhibits the damage caused by lipid peroxidation (LPO). Plants exposed to biotic stresses such as pathogen attack present elevated levels of proline, which has the potential to improve synthesis and reduce the occurring degradation (Verbruggen and Hermans, 2008). In addition, GB is considered one of the most powerful osmoprotectants in plants and play a vital role in enhancing stress tolerance. GB is responsible for osmotic adjustment through controlling water absorption as well as the scavenging of ROS (Chen and Murata, 2011).

Although the role of proline and GB as osmoprotectants in plants is considered controversial, literature has shown that both compounds have positive effects on both enzyme and membrane integrity in addition to playing adaptive roles in mediating the osmotic adjustment in plants under stress (Hayat *et al.* 2012).

Project aims and objectives

This research aimed to comparatively analyse the effect of *Fusarium oxysporum* on the physiological and biochemical responses for *Triticum aestivum* L. (wheat) cultivars. This was achieved by:

i. Monitoring changes in plant growth parameters (shoot and root length and biomass) and relative water content (RWC).
ii. Measuring changes in photosynthetic pigments and osmolyte content.
iii. Quantifying the levels of ROS biomarkers (O_2^- and H_2O_2) and the extent of lipid peroxidation.
iv. Detecting changes in ROS scavenging antioxidant enzymes.

CHAPTER 2

METHODS AND MATERIALS

2.1 General chemicals, kits and suppliers

Table 2.1: List of reagents and chemicals used in this study

Reagents/Chemicals	**Supplier**
Acetone	Merck Millipore
Acrylamide/Bis (40%)	BIO-RAD
Agarose D – 1 LE	White Scientific
Ammonium Persulfate (APS)	BIO-RAD
Ascorbic acid / Ascorbate	Sigma Aldrich
Ammonium acetate	Sigma Aldrich
Bovine Serum Albumin (BSA) Fraction V	Roche
Bradford Reagent (1X)	BIO-RAD
Coomassie® brilliant blue (CBB) R-250	BIO-RAD
3,3'-Diamobenzidine	Sigma Aldrich
Ethanol 99.9%	Kimix
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich
Evans Blue	Sigma Aldrich
Glacial acetic acid	Merck Millipore
Glucose	Merck Millipore
Glycerol	Merck Millipore
Glycine	BIO-RAD
Hydrochloric acid (HCl)	Merck Millipore
Hydrogen peroxide (H_2O_2)	Merck Millipore
(L)-Proline	Sigma Aldrich
Ninhydrin	Sigma Aldrich
Nitrotetrazolium blue chloride powder (NBT)	Sigma Aldrich
Nitrisol	Builders Warehouse
Methanol (MeOH)	Sigma Aldrich
Polyvinylpyrrolidone (PVP) MW: 40 000	Sigma Aldrich
Potassium cyanide (KCN)	Sigma Aldrich

Potassium iodide (KI)	Sigma Aldrich
Potassium phosphate monobasic (KH_2PO_4)	Sigma Aldrich
Potassium phosphate dibasic (K_2HPO_4)	Sigma Aldrich
Potato dextrose agar (PDA)	Sigma Aldrich
Unstained Protein Standard Broad range (10-200kDa) (P7704S)	New England BioLabs
Potting soil	Stodels Garden Centre
Propan-2-ol (isopropanol)	Merck Millipore
Riboflavin	Sigma Aldrich
Silica sand	Silica South Africa
Sodium dodecyl sulphate (SDS)	BIO-RAD
Sodium hydroxide (NaOH)	Merck Millipore
5-Sulfosalicylic acid hydrate	Sigma Aldrich
N,N,N',N'-Tetramethylethylenediamine (TEMED)	BIO-RAD
Thiobarbituric acid (TBA)	Sigma Aldrich
Thiourea	Sigma Aldrich
Trichloroacetic acid (TCA)	Merck Millipore
Tris(hydroxymethyl)-aminoethanes	BIO-RAD
Trypan blue	Sigma Aldrich
Urea	Sigma Aldrich

2.2 Stock solutions and buffers

Table 2.2: Preparation and calculations of buffers and stock solutions

Acetone (70%)	70% (v/v) acetone in distilled water.
APS (10%)	10% (w/v) APS in distilled water. The solution was freshly prepared before use.
BSA stock solution (10 mg/ml)	10 mg/ml BSA in PVP extraction or IEF buffer
Ethanol (70%)	70% (v/v) ethanol in distilled water.
Evans blue stock solution (0.25%)	0.25% (w/v) Evans blue in distilled water.
Hydrogen peroxide (H_2O_2) reaction buffer	5 mM K_2HPO_4 (pH5.0) and 0.5 M KI
HCl (1 M)	1 M HCl in distilled water.

KH_2PO_4 (1M) stock solution	1 M KH_2PO_4 in distilled water.
K_2HPO_4 (1M) stock solution	1 M K_2HPO_4 in distilled water.
KPO_4 (0.1 M), pH 7	1 M KH_2PO_4 (9.625 ml), 1 M K_2HPO_4 (15.373 ml) in distilled water.
KI (0.5 M) stock solution	0.5 M KI in distilled water.
Native gel running buffer stock solution (1 X)	20 mM Tris-base; 186 mM glycine in distilled water.
Native gel loading dye (5 X)	50% (v/v) glycerol; 0.02% (w/v) bromophenol blue in distilled water and add 3 X GelRed.
Proline reaction buffer	3% Sulfosacylic acid (200 µl); glacial acetic acid (100 µl); ninhydrin (200 µl).
PVP extraction buffer	40 mM K_2HPO_4 at pH 7.4; 1 mM EDTA; 5% PVP MW = 40 000; 5% glycerol in distilled water.
Superoxide (O_2^-) reaction buffer	0.1 mM KPO_4 (pH 7); 6.4 mM NBT; 100µM H_2O_2 and 100 mM KCN in distilled water.
TCA (5%) extraction buffer	5% (w/v) TCA in d.H_2O
TCA (20%) / TBA (0.5%)	0.5% (w/v) TBA in 20% (v/v) TCA stock solution.
Tris-HCl (1 M)	0.1 M Tris in distilled water.
Tris-HCl (1 M), pH 6.8	0.1 M Tris in distilled. Adjusted the pH to 6.8 with concentrated HCl.
Tris-HCl (1.5 M), pH 8.8	1.5 M Tris in distilled water. Adjusted the pH to 8.8 with concentrated HCl.

2.3 Fungal culture collection

Fusarium oxysporum isolate (PRI 10138) was collected from Plant Protection Institute, Agricultural Research Council (ARC), Pretoria, South Africa. The isolated fungus was cultured on potato dextrose agar (PDA) media and grown under 12-hour light and 12-hour dark

conditions at 30°C. A *Fusarium oxysporum* spore suspension for plant inoculation was prepared in distilled water containing 0.1% Tween 20 and adjusted to two working concentrations (1×10^5 and 1×10^7 spores/ml) using a hemocytometer.

2.4 Seed inoculation, germination and plant growth

Wheat (*Triticum aestivum* L.) seeds from three cultivars (SST 015, SST 056 and SST 088) (received from the Research and Technology Development services at the Department of Agriculture, Cape Town, South Africa) were washed with sterile distilled water and heat sterilised at 49 °C for 20 minutes, soaked in 1% ethanol for 10 minutes. Following sterilisation, the seeds were washed with sterile distilled water several times. Seeds were germinated on moist sterile filter paper in a dark environment for 2-3 days. Germinated seeds were transferred to sterile greiner tubes containing *F. oxysporum* inoculum of different concentrations (1×10^5 and 1×10^7 spores per/ml) and incubated for 3-4 hours at room temperature (Imathiu *et al.* 2014).

Control (uninfected) and experimental (infected) wheat seedlings were transplanted (6 seeds per pot) into a moist soil mixture of silica sand and potting soil in a 3:1 ratio. The seedlings were grown in a conditioned growth room with a 16-hour light and 8-hour dark cycle at 22 °C using a Random Block Design (RBD). Each pot was watered with 200 ml of distilled water every second day until the V1 stage. At this stage, each pot was supplemented with 200 ml of 0.5 X Nitrisol solution (Builders Warehouse, Cape Town, South Africa) twice a week until the end of the experiment. Watering and supplementation of the nutrient solution (200 ml per pot) was applied to each plant directly at the base of the stem in the pot three times a week for 21 days after pot rotations occurred according to RBD.

2.5 Analysis of plant growth

After 21 days of plant growth, wheat plants were carefully removed from the soil mixture to avoid damaging or loss of shoots and roots material. The following physiological parameters were measured namely, shoots length (SL), root length (RL), shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW) and root dry weight (RDW). The DW for each cultivar was determined using a modified method described by Valentovic *et al.* (2006), where shoot and root material were dried in an oven at 55 °C for 72 hours.

2.6 Measuring relative water content (RWC)

Relative water content (RWC) was measured and calculated using the method described by Mohammadkhani and Heidari (2007) using the following formula:

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

2.7 Measurement of chlorophyll content

Estimation of chlorophyll content was based on a modified method previously described by Wildermuth *et al.* (1992). Shoot material (0.1 g) from each plant was ground into a fine powder with liquid nitrogen and homogenised in 1 ml of 80% acetone. The extracts were vortexed and centrifuged at 13 2000 rpm for 10 minutes. The supernatant was collected in a sterile tube and the process was repeated until a clear pellet was observed. The absorbances of the various fractions were recorded at 663 nm and 646 nm respectively. The chlorophyll content was calculated using the following formulas:

$$Chlorphyll\ \boldsymbol{a}\ (\mu g/ml) = 12.21\ (A663) - 2.81\ (A646)$$

$$Chlorphyll\ \boldsymbol{b}\ (\mu g/ml) = 20.13\ (A663) - 5.03\ (A646)$$

2.8 Measurement of proline content

The proline content was estimated using a modified method described by Carillo and Gibon, (2011). Fresh shoot material from each treatment (0.1 g) were homogenized in 500 µl of 3% (w/v) sulphosalicylic acid using a mortar and pestle. About 200 µl of each homogenate was mixed with 200 µl of glacial acetic acid to which 200 µl of ninhydrin was added. The reaction mixture was boiled in a water bath at 100 °C for 30 minutes and immediately cooled in an ice bath. After cooling, 400 µl of toluene was added to the reaction mixture. After thorough mixing, the chromophore containing toluene was separated and the absorbance of the red colour developed was read at 520 nm against the toluene blank on FLUOstar Omega UV-visible spectrophotometer (BMG LabTech GmbH, Ortenberg, Germany).

2.9 Measurement of cell viability

The cell viability of shoots from all wheat cultivars and their respective treatments were measured using a modified method described by Sanevas *et al.* (2007). Fresh shoot material (approximately 0.1 g) were selected from three different plants from each of the respective treatments per cultivar and stained with 0.25% (w/v) Evan's Blue for 1 hour at room temperature. The stained samples were washed with distilled several times at room temperature to remove the access dye still present on the surface. The extraction of the Evans Blue stain (taken up by dead cells) from shoot tissue was achieved by incubating the sample in 1% (w/v) SDS for 1 hour at 55°C. The level of cell death (reflected by the amount of Evans Blue taken up by the extracts) was determined by measuring the absorbance of the extracts at 600 nm.

2.10 Protein extraction for biochemical assays

The shoots tissue from each plant was harvested and ground into a fine powder using liquid nitrogen. Approximately 0.2 g of the ground shoot material were homogenized in 1ml of 5%

trichloroacetic acid (TCA) for the analysis of H_2O_2 and lipid peroxidation (MDA). For the analysis and measurements of antioxidant enzymatic activities, shoot material (0.2 g) was homogenized in 800 µl PVP buffer (see Section 2.2). The protein concentrations for all extracts were measured using Bradford's method (Bradford, 1976).

2.11 Measuring ROS accumulation using spectrometric assays

2.11.1 Superoxide detection

The detection of superoxide (O_2^-) in wheat shoots was achieved using a modified method by Able *et al.* (1998). Approximately 1-2 cm^3 of leaf material was cut from the third youngest leaf and homogenized in 800 µl of reaction buffer (section 2.2) and incubated at room temperature in the dark for 20 minutes. The leaf was crushed in the tube with a sterile micro pestle and the sample was centrifuged at 13 000 rpm for 20 minutes. The supernatant was collected and the absorbance measured at 600 nm as described by Sutherland and Learmonth (1997).

2.11.2 Hydrogen peroxide detection

The H_2O_2 content was determined in the shoots of wheat plants using a method described by Velikova *et al.* (2000), whereby 50 µl of the TCA extract (section 2.11) was added to H_2O_2 reaction buffer (section 2.2). Samples were incubated at room temperature for 10 minutes and the absorbance was measured at 390 nm. The H_2O_2 content was calculated based on the standard curve constructed from the H_2O_2 standard absorbance reading at 390 nm.

2.12 Measuring the extent of lipid peroxidation

Lipid peroxidation (malondialdehyde; MDA) in wheat shoots were measured according to a modified method previously described by Buege and Aust (1978). Using the TCA extracts, 200 µl from each sample was added to 300 µl of 0.5% TBA (dissolved in 20% TCA). The sample

mixture was incubated at 95°C for 30 minutes in a water bath and immediately transferred to ice for 10 minutes. The sample was centrifuged at 13 000 rpm for 5 minutes at room temperature and the absorbance of each supernatant was measured at 532 nm and 600 nm respectively. The concentration of MDA was calculated using a molar extinction coefficient 155 mM^{-1} cm^{-1}.

2.13 Detection of ROS scavenging antioxidants

2.13.1 Superoxide dismutase (SOD)

The SOD activity in the shoots of wheat plants were detected using native polyacrylamide gel electrophoresis (PAGE) according to Laemmli (1970). For each sample, 50 µg PVP protein extract were separated on a native PAGE gel for 3-4 hours at 90 V. The detected SOD isoforms were further identified using specific inhibitors namely, potassium cyanide (KCN; which was shown to inhibit the copper/zinc – containing Cu/ZnSOD) and H_2O_2 (which inhibits both Cu/ZnSOD and the iron – containing FeSOD). The identification of manganese – SODs (MnSOD) may be evident as a result of the resistance to both KCN and H_2O_2. Staining of the gels were carried out as depicted in Table 2.3.

Table 2.3: SOD native gel staining

Stain	Uninhibited gel	KCN inhibited gel	H_2O_2 inhibited gel	Time (Minutes)
1	50mM KPO_4 (pH 7.8)	50mM KPO_4 (pH 7.8); 5mM KCN	50mM KPO_4 (pH 7.8); 5mM H_2O_2	15-20 Minutes (In the dark)
2	50mM KPO_4 (pH 7.8); 10 mg NBT			20 Minutes (In the dark)
3	50mM KPO_4 (pH 7.8); 209 µL TEMED; 9.5 mg Riboflavin			Until bands are visible (In light)

2.13.2 Ascorbate peroxidase (APX)

APX isoforms were detected using 50 µg of PVP protein extract, separated on an 8% PAGE gel as described by Mittler and Zilanskas (1993). Individual APX isoforms were detected by incubating the native PAGE gels with 50 mM phosphate buffer (KPO_4; pH 7.0) for 20 minutes in the dark followed by 50 mM phosphate buffer (KPO_4; pH 7.8) containing 4 mM ascorbate and 2 mM H_2O_2 for 20 minutes. The gel was then stained with 50 mM KPO4 (pH 7.8) containing 209 µl TEMED and 15 mg NBT until the APX isoforms were visible.

2.13.3 Peroxidase (POD)

POD isoforms were detected using a modified method described by Zhang *et al.* (2004) where, 30 µg of PVP protein extract was separated on an 8% native PAGE gel for 2-3 hours at 90 V. The detection of POD isoforms was visualised by staining the native PAGE gel in 50 mM phosphate buffer (KPO_4; pH 7.0) containing 50 µl of 32% H_2O_2 for 10 minutes in the dark. The gel was then incubated in 50 mM KPO_4 (pH 7.0) containing 0.02% 3,3′-Diaminobenzidine (DAB) until the POD isoforms were visible.

2.14 Optical density analysis of ROS scavenging enzymes

The in-gel images for SOD, APX and POD were analysed using the Spot Denso tool of AlphaEase FC imaging software (Alpha Innotech Corporation). The enzymatic activity (for SOD, APX and POD) were observed for each isoform detected on the gels of the scavenging enzymes. The average measurement of the relative pixel intensities and expressed in arbitrary values (relative enzymatic activity) of three independent gels were determined. The isoform with the lowest pixel intensity was assigned a value of 1 and the rest of the pixel intensities for the enzymatic isoforms in the other plants were expressed relative to this isoform.

2.15 Statistical analysis

All experiments described were performed independently in triplicates, with measurements taken from six plants (for physiology/plant growth analysis) and three plants (for biochemistry analysis) for each treatment respectively. One-way analysis of the variance (ANOVA) test was conducted for all data and means for three independent experiments.

CHAPTER 3

F. OXYSPORUM ALTERS THE PHYSIOLOGICAL RESPONSES OF THREE WHEAT CULTIVARS

3.1 Introduction

The increase in crop production and increasing prevalence of biotic stress is a major threat to the agricultural productivity and one of the main causes in crop loss both pre- and post-harvest (Dresselhaus *et al.* 2018). Biotic stress such as pathogen attack can be detrimental to various crop species as they directly deprive plants of its nutrients which in turn reduces plant growth and increases plant death (Singla and Krattinger, 2016). Disease causing pathogens significantly influence annual yields as well as crop quality and accounts for approximately 20% of yield loss worldwide (Oeke, 2006; Flood, 2010).

Pathogen attack activates various plant defence mechanisms (Ferrigo *et al.* 2015). Due to the nature of the physiological changes in plants under biotic stress, changes in the primary and secondary metabolism are expected as a result of the stress induced plant defence mechanism (Berger *et al.* 2007). *Fusarium species* have been shown to cause several plant diseases in many cereal crops, altering the physiological appearance, development and quality of the plant (Kosiak *et al.* 2003). Furthermore, other studies on plant-pathogen interactions showed that pathogenic fungi also alter metabolic pathways like photosynthesis (Yang *et al.* 2016). In addition, the detection of osmolytes such as proline, can be used as a marker to investigate the impact of stress conditions in plants (Hayat *et al.* 2012). In this study, changes in chlorophyll and proline content of wheat in response to *F. oxysporum* infection was investigated to determine the severity of infection induced by the pathogen.

This chapter focuses on determining the effects of *F. oxysporum* on the physiological responses of three wheat cultivars as an indication of the plants' health. These characteristics were determined by monitoring various growth parameters (length and biomass of shoots and roots), the extent of water loss as well as photosynthetic pigment (chlorophyll) and osmolyte content.

3.2 Results

3.2.1 *F. oxysporum* alters the physiological responses of SST 015

3.2.1.1 F. oxysporum infections reduces plant growth for SST 015

Significant differences in plant growth was observed for cultivar SST 015 in response to *F. oxysporum* and results suggest that these differences are concentration dependent. (Figure 3.1).

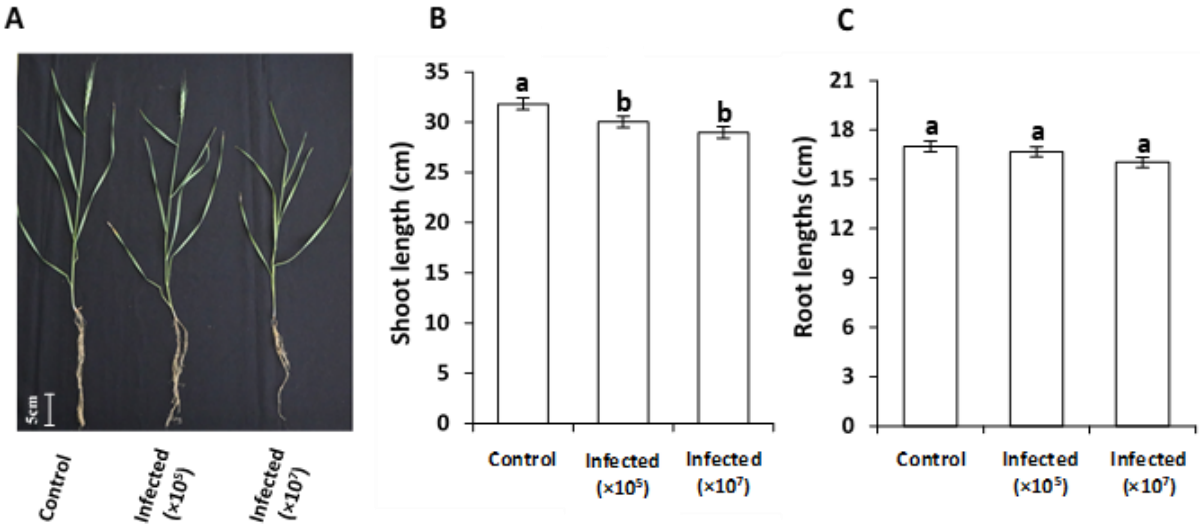

Figure 3.1: The effect of *F. oxysporum* infection on wheat cultivar SST 015 growth. Plant growth parameters include individual representatives of each treatment (A), shoot length (B) and root length (C) after 28 days of growth. The error bars are representative of the mean (±SE) of 3 independent experiments from 6 plants per treatment in each experiment. Means with different letters are significantly different from each other.

SST 015 plants infected with 1×10^5 spores/ml showed a similar profile to that of the control where spike formation was clearly visible, whereas no spike formation was observed in plants infected with 1×10^7 spores/ml (Figure 3.1 A). Interestingly, the rate at which SST 015 plants developed was reduced when infected with 1×10^7 spores/ml relative to the control and the

lower *F. oxysporum* treatment. A minimal but significant decrease in shoot growth was observed in both *F. oxysporum* treatments relative to the control (Figure 3.1 B). Statistically, no significant difference in root growth was observed in response to *F. oxysporum* treatment when compared to the control (Figure 3.1 C).

3.2.1.2 The effects of F. oxysporum on SST 015 biomass

F. oxysporum infection differentially alters the biomass of SST 015 as indicated by the results presented in Figure 3.2. No significant change in shoot fresh weight (SFW) was observed in plants treated with 1×10^5 spores/ml, whereas a 19.36% reduction was recorded in the 1×10^7 spores/ml treatment. (Figure 3.2 A). It is interesting to note, that given the reduction in SFW (Figure 3.2 A), no change in shoot dry weight (SDW) was observed in both treatments (Figure 3.2 B).

In contrast to what was observed for shoot growth, root growth was significantly altered by both treatments. In response to *F. oxysporum* at 1×10^5 spores/ml root fresh weight (RFW) was significantly reduced by 29.89%, whereas an even higher reduction (51.34%) was observed in the 1×10^7 spores/ml treatment relative to the control (Figure 3.2 C). A similar trend was observed for root dry weight (RDW), where both concentrations of *F. oxysporum* resulted in a significant reduction relative to the control (Figure 3.2 C-D). Plants treated with 1×10^5 spores/ml reduced RDW by 31%, whereas those treated with 1×10^7 spores/ml showed a 55% reduction relative to the control (Figure 3.2 D).

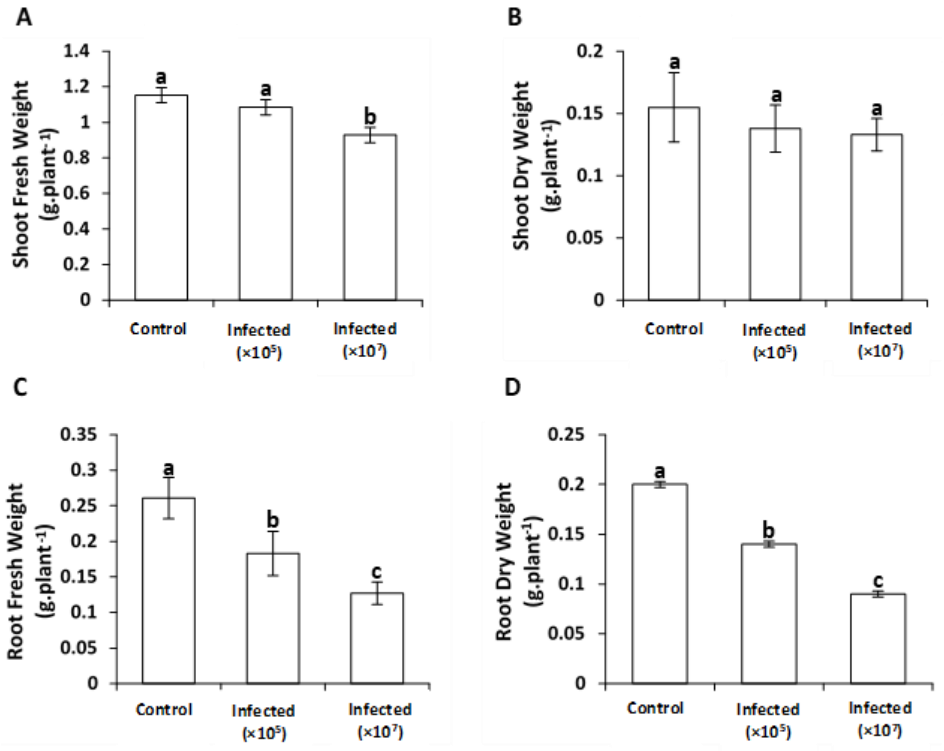

Figure 3.2: The effect of *F. oxysporum* infection on SST 015 plant biomass. Plant biomass is represented by the shoot (including leaf) Fresh (A) and Dry (B) weights as well as the root Fresh (C) and Dry (D) weights. The error bars are representative of the mean (±SE) of 3 independent experiments from 6 plants per treatment in each experiment. Means with different letters are significantly different from each other.

3.2.1.3 F. oxysporum alters water retention, chlorophyll and proline content in SST 015 plants

F. oxysporum restricts water retention of SST 015 as seen for reduced relative water content (RWC). SST 015 plants treated with 1×10^5 spores/ml reduced RWC by 11.35%, whereas a slight significant change (of 5.48%) in RWC was observed in plants treated with 1×10^7 spores/ml (Figure 3.3 A). In addition, the osmolyte content and photosynthetic metabolism in SST 015 plants were significantly influenced in response to *F. oxysporum* infection (Figure 3.3 B-C). Total proline content in the shoots of SST 015 was enhanced by both *F. oxysporum* concentrations with the highest increased observed in the 1×10^7 spores/ml treatment (Figure 3.3 B). The accumulation of proline in the infected plants presented a significant increase of

10.53% and 45.49% with the increasing concentration of *F. oxysporum* infection relative to the uninfected control plants (Figure 3.3 B). The results obtained for the photosynthetic metabolism represented by chlorophyll content depicted a significant reduction of 41.03% and 33.82% respectively, in SST 015 plants relative to the control (Figure 3.3 C).

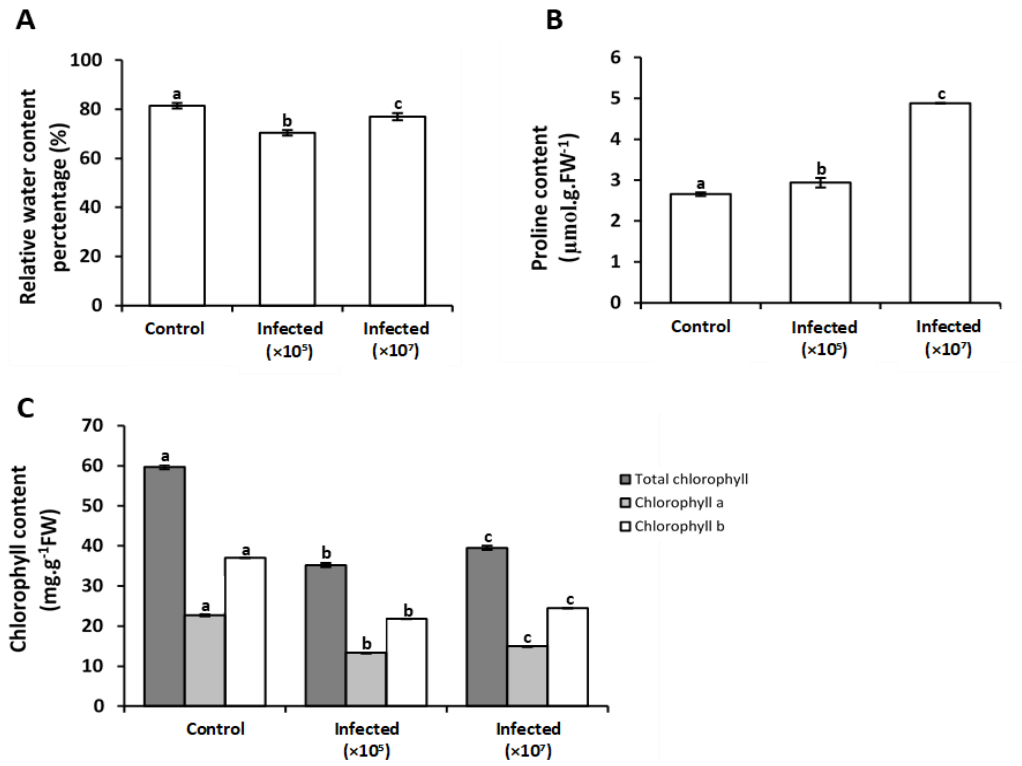

Figure 3.3: The effect of *F. oxysporum* infection on the Relative Water content, Photosynthetic and Osmolyte content in wheat cultivar SST 015. Relative water content (A), Proline content (B) and Chlorophyll content (C) measured includes individual representatives of each treatment for cultivar SST 015. The error bars are representative of the mean (±SE) of 3 independent experiments from 3 plants per treatment in each experiment. Means with different letters are significantly different from each other.

3.2.2 *F. oxysporum* alters the physiological responses of SST 056

3.2.2.1 F. oxysporum infections restricts SST 056 growth

Based on the data presented in Figure 3.4, *F. oxysporum* reduced the growth and development of SST 056. The shoot length (SL) of SST 056 was reduced by 10.82% and 8.16% respectively, for the different *F. oxysporum* concentrations (Figure 3.4 B) In addition, no significant change in root length (RL) was observed in both treatments relative to the control (Figure 3.4 C). Based

on visual inspection, root volume (not depicted) was reduced with an increase in *F. oxysporum* concentration.

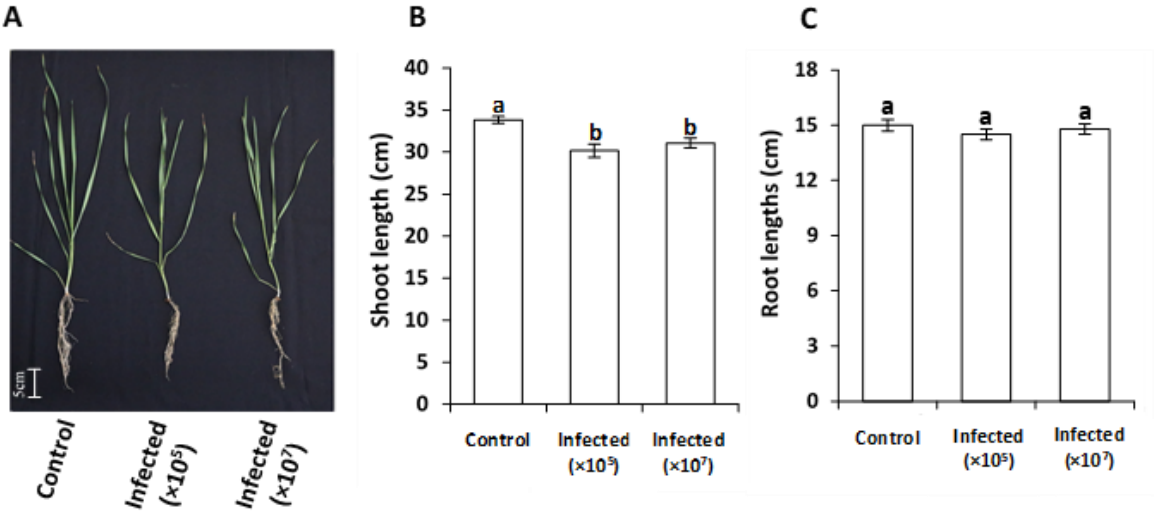

Figure 3.4: The effect of *F. oxysporum* infection on wheat cultivar SST 056 growth. Plant growth parameters include individual representatives of each treatment (A), shoot length (B) and root length (C) after 28 days of growth. The error bars are representative of the mean (±SE) of 3 independent experiments from 6 plants per treatment in each experiment. Means with different letters are significantly different from each other.

3.2.2.2 Effects of F. oxysporum on SST 056 biomass

Here we monitored changes in shoot and root biomass of SST 056 in response to *F. oxysporum.* The data shows that the biomass of SST 056 was significantly influenced by *F. oxysporum* (Figure 3.5). SST 056 plants treated/infected with 1×10^5 spores/ml reduced SFW by 25.07% whereas treatment with 1×10^7 spores/ml resulted in a reduction of 34.68% relative to the control (Figure 3.5 A). For SDW, no significant change was observed in response to 1×10^5 spores/ml, whereas a 40.35% reduction was observed in response to 1×10^7 spores/ml. Similar to what was observed for SFW, *F. oxysporum* restricted RFW, with the highest reduction observed in plants treated/infected with 1×10^7 spores/ml (Figure 3.5 C). *F. oxysporum* at 1×10^5 spores/ml reduced RFW by 27.44% whereas 1×10^7 spores/ml reduced RFW by 55.26% relative to the control (Figure 3.5 C). Although a significant reduction in RDW was observed in SST 056 plants treated/infected with *F. oxysporum* relative to the control, no significant difference

was observed between the two *F. oxysporum* treatments (Figure 3.5 D). In response to 1×10^5 spores/ml, RDW was reduced by 35%, whereas 1×10^7 spores/ml reduced RDW by only 30% relative to the control (Figure 3.5 D).

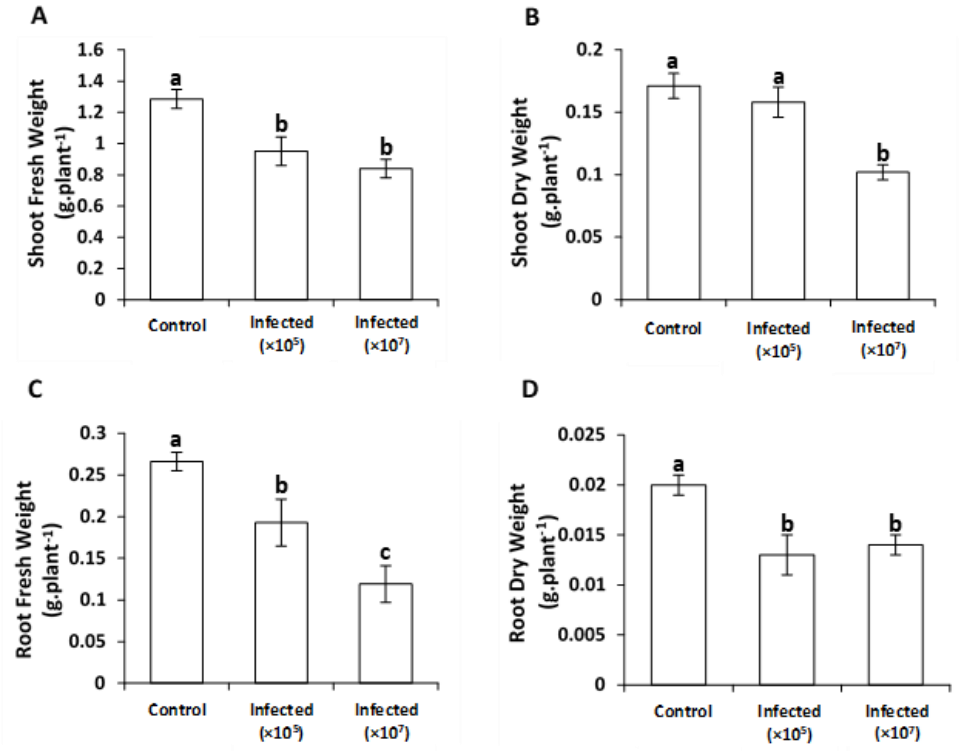

Figure 3.5: The effect of *F. oxysporum* infection on SST 056 plant biomass. Plant biomass is represented by the shoot (including leaf) Fresh (A) and Dry (B) weights as well as the root Fresh (C) and Dry (D) weights. The error bars are representative of the mean (±SE) of 3 independent experiments from 6 plants per treatment in each experiment. Means with different letters are significantly different from each other.

3.2.2.3 F. oxysporum alters water retention, chlorophyll and proline content in SST 056 plants

F. oxysporum reduced water retention in SST016 relative to the control. RWC (an indicator of water retention) was reduced by 11.14% in response to 1×10^5 spores/ml whereas an even higher reduction (22.78%) was observed in the higher concentration (1×10^7 spores/ml) (Figure 3.6 A).

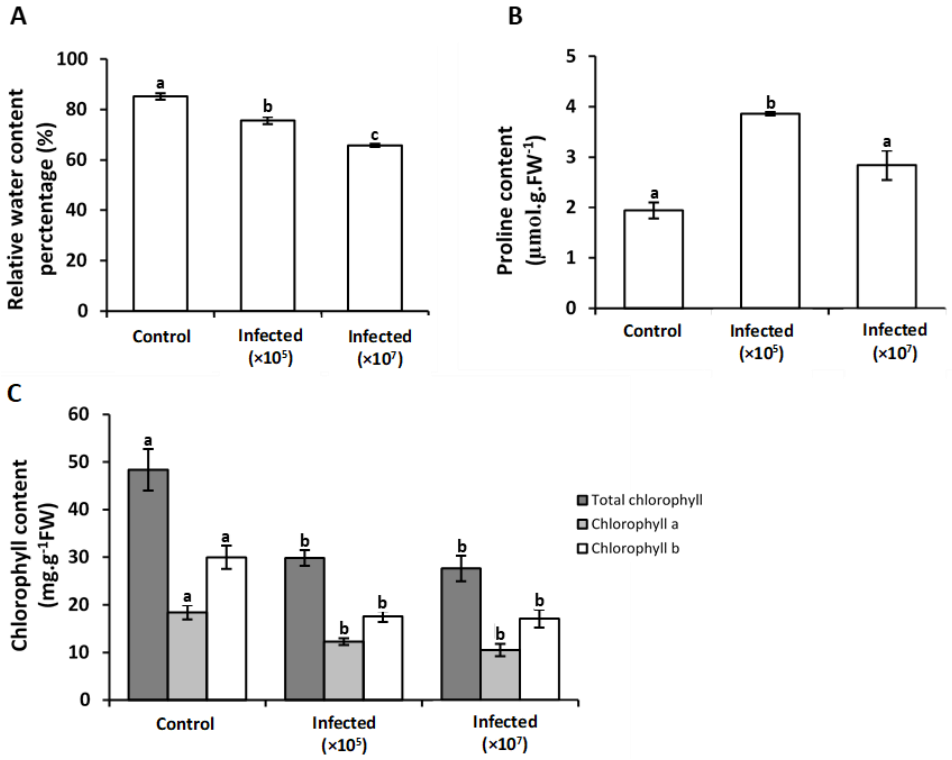

Figure 3.6: The effect of *F. oxysporum* infection on the Relative Water content, Photosynthetic and Osmolyte content in wheat cultivar SST 056. Relative water content (A), Proline content (B) and Chlorophyll content (C) measured includes individual representatives of each treatment for cultivar SST 056. The error bars are representative of the mean (±SE) of 3 independent experiments from 3 plants per treatment in each experiment. Means with different letters are significantly different from each other.

Total proline was significantly enhanced by *F. oxysporum* in relation to the control (Figure 3.6 B) with $\times 10^5$ spores/ml presenting the highest increase (98.97%). *F. oxysporum* at $\times 10^7$ spores/ml also increase (46.39%) proline content relative to the control albeit not to the level of $\times 10^5$ spores/ml. In addition, chlorophyll content was reduced by *F. oxysporum* relative to the control, with significant difference observed between the two treatments. On average, a 40% decrease in chlorophyll content was observed in the *F. oxysporum* treatments relative to the control (Figure 3.6 C).

3.2.3 *F. oxysporum* alters the physiological responses of SST 088

3.2.3.1 F. oxysporum restricts SST 088 plant growth

Wheat cultivar SST 088 plants presented varying susceptibility to *F. oxysporum* infection at increasing concentrations and resulted in differential responses in plant growth and development (Figure 3.7 A).

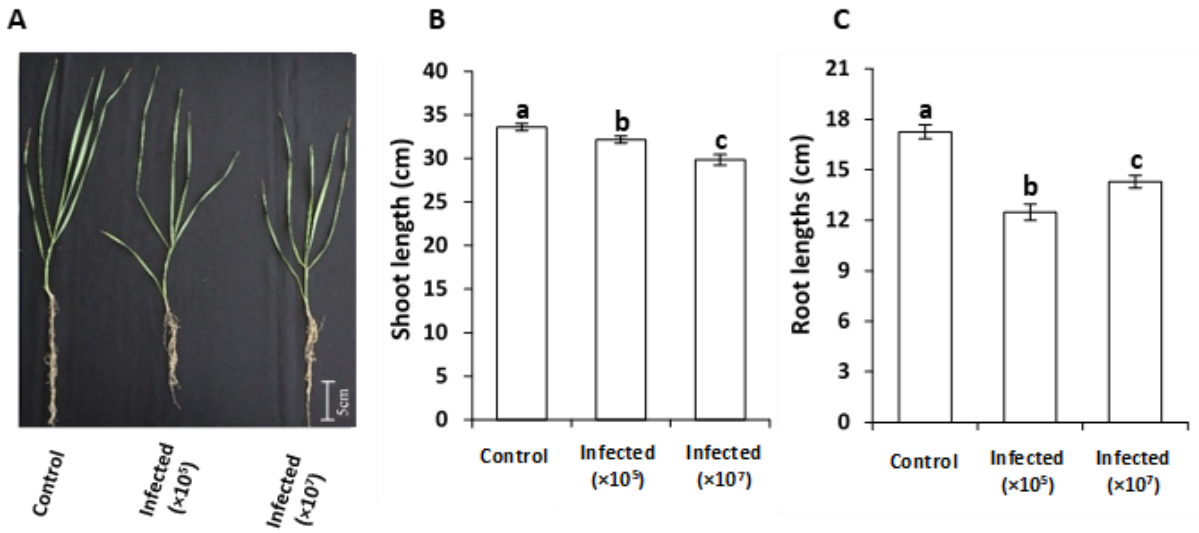

Figure 3.7: The effect of *F. oxysporum* infection on wheat cultivar SST 088 growth. Plant growth parameters include individual representatives of each treatment (A), shoot length (B) and root length (C) after 28 days of growth. The error bars are representative of the mean (±SE) of 3 independent experiments from 6 plants per treatment in each experiment. Means with different letters are significantly different from each other.

Shoot length of infected plants were found to be significantly reduced by 4.26% at 1×10^5 spores/ml *F. oxysporum* infection and 11.14% at 1×10^7 spores/ml (Figure 3.7 B). In addition, root lengths presented a similar trend in reduction for plants infected with a low concentration (1×10^5 spores/ml) resulting in a reduction of 9.16%, however, the reduction for plants infected with 1×10^7 spores/ml of 0.12% was insignificant (Figure 3.7 C) relative to the uninfected control plants.

3.2.3.2 Effects of F. oxysporum on SST 088 biomass

Wheat cultivar SST 088 shoot and root development was found to be differentially influenced by increasing concentrations of *F. oxysporum* infection (Figure 3.8).

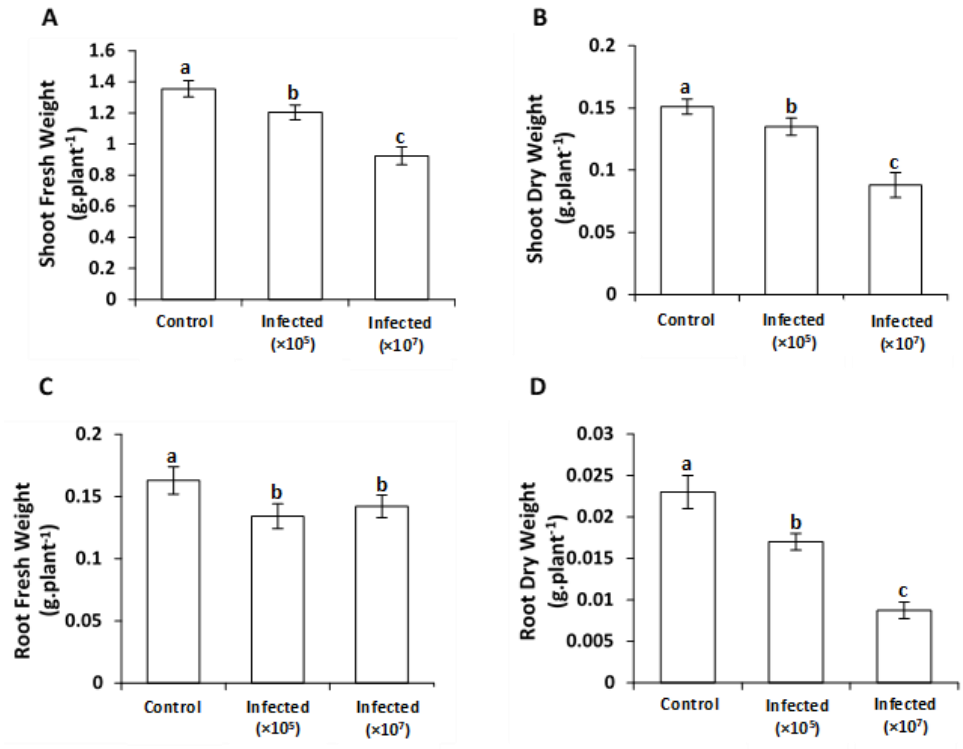

Figure 3.8: The effect of *F. oxysporum* infection on SST 088 plant biomass. Plant biomass is represented by the shoot (including leaf) Fresh (A) and Dry (B) weights as well as the root Fresh (C) and Dry (D) weights. The error bars are representative of the mean (±SE) of 3 independent experiments from 6 plants per treatment in each experiment. Means with different letters are significantly different from each other.

Biomass results for shoots depicted in Figure 3.8 A indicated a significant reduction of 11.14% in fresh weights and 10.60% in dry weights (Figure 3.8 B) for plants infected with *F. oxysporum* at 1×10^5 spores/ml. In comparison, a significant reduction of 31.88% (fresh weight) and 41.72% (dry weight) relative to the respective uninfected control plants were observed for plants infected with 1×10^7 spores/ml. The root development of SST 088 plants was also influenced by *F. oxysporum* infection at both concentrations tested. Root fresh weights presented significant changes with reductions of 17.79% and 12.88% respectively. Root dry weights of infected plants followed a similar trend with significantly decreases of 26.09% and

62.17% in response to the increasing concentration of *F. oxysporum* infection (Figure 3.8 C and D).

3.2.3.3 F. oxysporum alters water retention, chlorophyll and proline content in SST 088 plants

The RWC of *F. oxysporum* infected SST 088 plants was found to be significantly reduced by 9.36% in plants infected with 1×10^5 spores/ml and 9.95% in plants infected with a higher concentration (1×10^7 spores/ml) of *F. oxysporum* in comparison to the control plants (Figure 3.9 A). In addition, the osmolyte content represented by the proline content depicted in Figure 3.9 B was found to have significantly increased in all infected plants, with the accumulation increasing up to 18.24% in plants infected with 1×10^5 spores/ml and a significant increase of 131.45% was observed in plants infected with 1×10^7 spores/ml relative to the uninfected control plants. *F. oxysporum* infection at increasing concentrations was also found to significantly influence the photosynthetic metabolism of wheat plants from cultivar SST 088 (Figure 3.9 C). The results obtained for the photosynthetic metabolism represented by chlorophyll content depicted a significant reduction of 73.52% and 61.94% in total leaf chlorophyll content of wheat plants infected with increase *F. oxysporum*. The most significant reduction was observed in plants affected with the lower concentration of *F. oxysporum* in respect to the control plants (Figure 3.9 C).

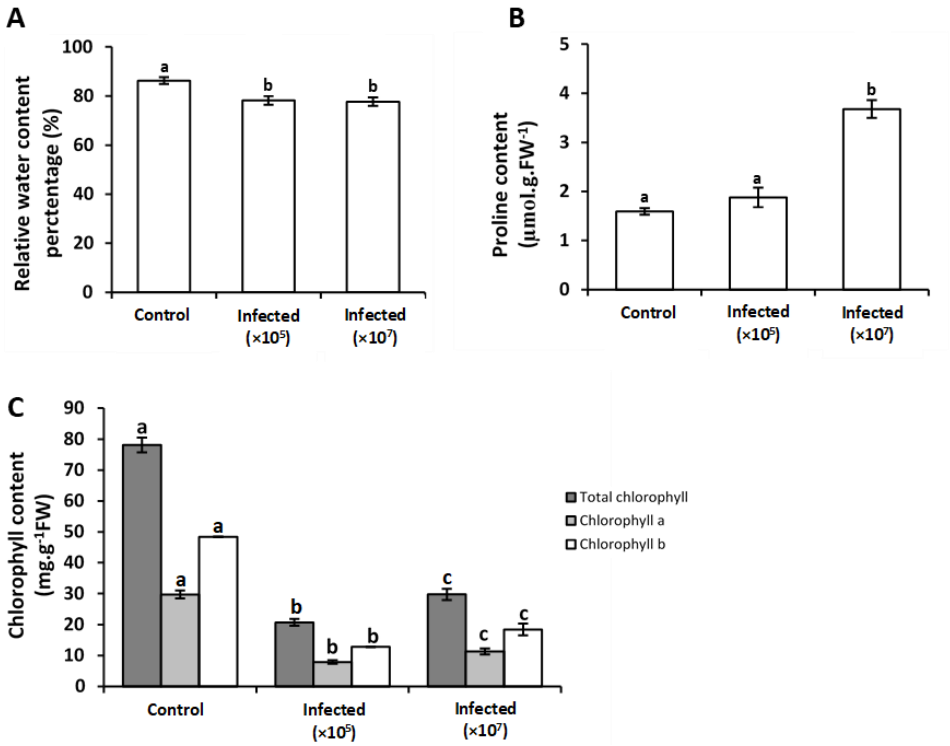

Figure 3.9: The effect of *F. oxysporum* infection on the Relative Water content, Photosynthetic and Osmolyte content in wheat cultivar SST 088. Relative water content (A), Proline content (B) and Chlorophyll content (C) measured includes individual representatives of each treatment for cultivar SST 088. The error bars are representative of the mean (±SE) of 3 independent experiments from 3 plants per treatment in each experiment. Means with different letters are significantly different from each other.

3.3 Discussion

Fugal pathogens like *F. oxysporum* are known to survive in soil for long periods and are problematic to eradicate. Several studies have shown that *Fusarium* induces stress in plant tissues resulting in several physiological and biochemical changes (Warzech *et al.* 2015). In this study we analysed the influence that *F. oxysporum* has on the physiological responses of three wheat (*Triticum aestivum* L.) cultivars (SST 015, SST 056 and SST 088). The results show that *F. oxysporum* differentially alters plant growth and development of three wheat cultivars. Each cultivar presented varying levels of susceptibility to the fungal pathogen and comparison of concentrations used for infection indicated that *F. oxysporum* at higher

concentrations (1×10^7 spores/ml) was found to be more detrimental to plant growth and development in comparison to the lower concentration of (1×10^5 spores/ml).

3.3.1 *F. oxysporum* reduces growth and biomass

Plant growth and development results indicated that each wheat cultivar presented differential responses to *F. oxysporum* infection. Several studies have shown that plant diseases caused by *Fusarium species* significantly reduce the emergence of seedlings coupled with, reduced plant growth and yield (Wang *et al.* 2006; Berger *et al.* 2007; Joshi, 2018). This study presented similar results for wheat cultivars SST 015, SST 056 and SST 088 which presented significant reductions in plant growth and biomass in response to *F. oxysporum* infection (Figure 3.1; Figure 3.4; Figure 3.7). Plant growth analysis (represented by the shoot and root lengths) shows that *F. oxysporum* significantly restricts shoot and root length. This observation was made for all three wheat cultivars. Interestingly, the most notable reduction in plant growth for SST 015 and SST 088 was observed in response to 1×10^7 spores/ml, whereas the opposite was observed for SST 056. Based on the results presented in this study, SST 015 appears to be an early flowering cultivar when compared to SST 056 and SST 088 (Sensako.co.za, 2019). The presence of the spikes in plants infected with *F. oxysporum* at 1×10^5 spores/ml shows that these plants have a higher tolerance infection at this concentration as the formation of spikes in plants infected with the higher concentration of *F. oxysporum* were absent (Al-Abdalall, 2010). Strikingly, is that *F. oxysporum* at high concentration (1×10^7 spores/ml) delays the formation of the spikes to initiate early flowering. However, at low concentration (1×10^5 spores/ml) the rate at which these spikes formed were not altered. A study by Banerjee and Mittra (2018) showed that *F. oxysporum* infection resulted in the inhibition of plant growth and development of wheat (Banerjee and Mittra, 2018). The reduction in root lengths across all three cultivars (Figure 3.1 C; Figure 3.4 C; Figure 3.7 C) was in agreement to what was observed in a study

conducted by Al-Abdalall (2010), whereby wheat presented significant reduction in root length and development as a consequence of *F. oxysporum, Rhizoctonia solani and F. culmorum* infection (Al-Abdalall, 2010).

The development of the infected plants reinforced the visual reduction in plant growth by the significant decrease in the shoot and root biomass (Figure 3.2; Figure 3.5; Figure 3.8). In this study, the reduction in biomass observed in wheat plants for all three cultivars in response to *F. oxysporum* imitates similar findings described by Scherm *et al.* (2013) where *Fusarium* reduced yield of wheat plants due to the deterioration of the plants' health. Based on the biomass data presented in this study SST 056 and SST 088 was most severely affected by *F. oxysporum* when compared to the SST 015 plants. The results obtained for plant growth and biomass corresponds to several studies that shows *Fusarium species* significantly alter the primary metabolism and activity responsible for plant growth and development as a result of stress induced by the pathogen (Berger *et al.* 2007; Chávez-Arias *et al.* 2019).

3.3.2 Water retention is reduced in response to *Fusarium oxysporum*

Several studies suggest that plants undergo water translocation and severe water loss when subjected to biotic stresses such as pathogen infections (Taiz, 2010; Bishop and Copper, 1983). In this study, we show the effects that *F. oxysporum* infection had on the relative water content (RWC) of three wheat cultivars (Figure 3.3 A; Figure 3.6 A; Figure 3.9 A). Results indicated significant reductions in RWC for infected plants across all three cultivars. Based on the reductions, SST 056 and SST 088 were most severely affected by *F. oxysporum* (1×10^7 spores/ml) in comparison to SST 015. This phenomenon has been demonstrated by Warzecha *et al.* (2010), where pathogenic *Fusarium species* that thrive in moist and warm conditions were shown to significantly alter the relative water content (RWC) in cereal crops by

obstructing of the plant's xylem and restricting photosynthetic metabolism in leaves (Chávez-Arias *et al.* 2019). Therefore, the results observed in this study in correlation to previous studies conducted suggests that at increasing concentrations of *F. oxysporum*, the RWC in wheat plants are expected to increase (Chávez-Arias *et al.* 2019; Warzecha *et al.* 2010).

3.3.3 *F. oxysporum* differentially alters photosynthetic metabolism and osmolyte content

While plant growth is controlled by a multitude of physiological, biochemical, and molecular processes, the physiological traits have been considered a powerful tool for detecting and identifying the effects of various disease-causing pathogens (Debona *et al.* 2014). Among these relevant physiological traits, photosynthesis is primarily affected by fungal diseases (Debona *et al.* 2014), as infection of plant tissue by these pathogens was shown to be closely linked to changes in metabolic pathways (Yang *et al.* 2016). A study conducted by Wagner *et al.* (2006) showed that the disease index of tomato plants infected with *F. oxysporum* correlated with photosynthetic activity. In this study *F. oxysporum* infection reduced photosynthetic metabolism in three wheat cultivars (Figure 3.3 C; Figure 3.6 C; Figure 3.9 C). The results indicated a significant decrease in the chlorophyll-a and chlorophyll-b content, ultimately leading to the reduction observed in the overall total chlorophyll content in infected wheat plants in comparison to their respective controls. Cultivar SST 088 presented the most excessive reduction in total chlorophyll content when compared to SST 015 and SST 056, suggesting a higher susceptibility to *F. oxysporum*. The adverse effect of photosynthesis in response to *Fusarium* induced stress was described by Berger *et al.* (2007) and Zhang *et al.* (2009). They showed that the infection process significantly influenced the pigment production and activity in wheat (Zhang *et al.* 2009) and reduced the photosynthesis represented by the total chlorophyll content in several cereal crops (Berger *et al.* 2007). Although it is not

uncommon that stressful environments can substantially damage photosynthetic pigments (Ashraf and Harris, 2013), the decreasing levels of total chlorophyll content observed is a common phenomenon. Studies suggest that stress induced changes in leaf chlorophyll content are as a result of impaired biosynthesis or accelerated pigment degradation (Perveen *et al.* 2010).

In addition, this study investigated the effects *F. oxysporum* on proline content in different wheat cultivars. Several studies have shown that the accumulation of an important compatible osmolyte and osmoprotective compound such as proline, in stressed plants represents a primary defence response which maintains the cellular osmotic pressure and protects cellular structures, proteins and membranes (Desingh and Kanagaraj, 2007; Koca *et al.* 2007). The capacity to accumulate proline under stress conditions was shown to be closely correlated with the stress tolerance of several plant species (Maggio *et al.* 2002; Claussen, 2005) and therefore, its accumulation is used as an indicator of stress present in plants (Fujita *et al.* 2006). The results obtained in this study showed the excessive accumulation of free proline in *F. oxysporum* infected plants for all three cultivars (Figure 3.3 B; Figure 3.6 B; Figure 3.9 B). Comparison of the cultivars indicated that cultivars SST 056 and SST 088 were most severely affected by the *F. oxysporum* relative to SST 015. The accumulation of proline observed in this study is in support of previous research reported by Hassanein *et al.* (2016) and Gherbawy *et al.* (2012). These outcomes are also supported by Rathod and Vakharia (2011), where proline content presented significant increases in chickpeas and cotton (Jiang and Huang, 2001) in response to fungal infection.

In summary, based on the physiological responses observed in this study, it is evident that the growth and development of SST 088 was most severely compromised by *F. oxysporum*

infection in comparison to SST 015 which presented a higher degree of tolerance. As a result of the variation in susceptibility observed, only SST 015 and SST 088 were investigated further as they presented the most significant but contrasting results in response to *F. oxysporum* infection.

CHAPTER 4

EFFECTS OF *F. OXYSPORUM* ON ROS METABOLISM AND ANTIOXIDANT CAPACITY IN WHEAT CULTIVARS

4.1 Introduction

Wheat production represents an integral part of a low input agricultural system that provides food, income and supports the platform for the productivity of other crops making it high in demand and is one of the main export crops (Scherm *et al.* 2013). One of the major problems we are faced with, is that various wheat cultivars are shown to be susceptible to biotic stresses such as fungal, bacterial and viral pathogens. These stresses influences the production and grain quality and can account for persistent yield losses of up to 20% of the world's harvest, with a further 10% loss post-harvest (Oerke, 2006; Flood, 2010). Pathogenic fungal infections in wheat cultivars are highly common, with some of the main fungi being *Puccinia* ssp., *Blumeria graminis* and *Fusarium* ssp., among others. *Fusarium* is one of the major fungal genera that is associated with African cultivated cereals and is multifaceted as it causes yield and quality loss due to the sterility of florets and the formation of discolouration which reduces kernel size and weight (D'Mello *et al.* 1999). Infections by fungal pathogens such as Fusarium often lead to the induction of oxidative stress and activation of plant defence systems to protect the plant against the reactive oxygen species (ROS).

ROS is considered as the main cause of cell damage under biotic and abiotic stresses (Bor *et al.* 2003; Candan and Tarhan, 2003). The production of ROS commonly occurs in all aerobic organisms under normal metabolic activity. Changes in normal cellular metabolism has been shown to result in the oxidative stress which causes lipids peroxidation and damages proteins, pigments and nucleic acids, are vital indicators of ROS (Imlay, 2003). When under biotic stress

conditions such as pathogen attack the production of ROS is induced in plants (Ali *et al.* 2018). ROS intermediates such as hydrogen peroxide (H_2O_2) is essential for the signalling and defence mechanism against pathogen infection in plants through the interaction with superoxide (O_2^-) radical or additional metal ions for the formation of hydroxyl radial (OH•). Overproduction of ROS can initiate the peroxidation of membrane lipids which is an indicator of oxidative stress. This in turn will induce cellular damage as it is directly related to the plant's response to the pathogen infection (Mandal *et al.* 2008). The overproduction of ROS occurs when the endogenous antioxidant defence system is unable to maintain homeostasis between the generation and the removal of ROS (Gülçin and Küfrevioglu 2002; Duh *et al.* 1999).

The removal of excess ROS generated during the host–pathogen interaction in plants involves non-enzymatic and enzymatic mechanisms (Scandalios, 2011). The enzymatic mechanism involves the antioxidant defence systems in plants for protection against stress-induced oxidative damage through partial suppression of ROS production or scavenging ROS present within the plant through a series of complex reactions (Cavalcanti *et al.* 2007; Torres *et al.* 2006; Ye *et al.* 2006). These complex reactions include the dismutation of superoxide radical (O_2^-) by superoxide dismutase (SOD) into molecular hydrogen peroxide (H_2O_2) which constitutes the first line of defence against oxidative stress. The accumulated H_2O_2 is further detoxified by various enzymes including ascorbate peroxidase (APX) and peroxidase (POD) and catalase (CAT) (Debona *et al.* 2012; Scandalios, 2005; Noctor and Foyer,, 1998). Several studies have shown that pathogen attack of wheat plants resulted in increased activity of antioxidant enzymes in addition to elevated levels of ROS and lipid peroxidation. This phenomenon was observed in cucumber (*Cucumis sativus* L.) roots in response to *Fusarium* infection (Mandal *et al.* 2008; El-Khallal, 2007; Ye *et al.* 2006). The aim of this study was to comparatively analyse the impact of *F. oxysporum* on the molecular responses of two wheat

cultivars (SST 015 and SST 088) due to the significant contrast in physiological responses observed (Chapter 3) in response to infection.

4.2 Results

4.2.1 *F. oxysporum* differentially alters ROS accumulation in wheat cultivars

The effect of *F. oxysporum* on ROS production in the shoots of two wheat cultivars (SST 015 and SST 088) were investigated. *F. oxysporum* significantly increased O_2^- and H_2O_2 contents for both SST 015 and SST 088. The increase in these ROS biomarkers is concentration dependent. For SST 015, *F. oxysporum* (as seen for both concentrations) increased O_2^- content relative to the control with highest increased observed in response 1×10^7 spores/ml (33.13%). A similar trend was observed, for SST 088 plants infected with *F. oxysporum* although the increase was significantly higher than observed for SST 015. SST 088 plants infected with 1×10^5 spores/ml increased O_2^- content by 37.98% whereas plants infected with 1×10^7 spores/ml increased O_2^- content by 43.46% (Table 4.1).

Table 4.1: ROS accumulation of two wheat cultivars in response to *F. oxysporum* infection

ROS intermediates	SST 015			SST 088		
	Control	Infected (1×10^5 spores/ml)	Infected (1×10^7 spores/ml)	Control	Infected (1×10^5 spores/ml)	Infected (1×10^7 spores/ml)
O_2^- (μmol.min.mg^1 FW)	3.43 ± 0.23 [a]	4.69 ± 0.2 [b]	5.13 ± 0.32 [b]	4.80 ± 0.38 [a]	7.74 ± 0.35 [b]	8.49 ± 0.25 [b]
H_2O_2 (μmol.g^1 FW)	4.56 ± 0.27 [a]	4.64 ± 0.34 [a]	6.01 ± 0.22 [b]	3.59 ± 0.21 [a]	4.71 ± 0.32 [b]	6.17 ± 0.25 [c]

The data in the above table presents the means ± standard error of three replicates (n=3) for each indicator. Means marked with different letters in the same row for the same indicator indicates significant difference between infections based on the standard error calculated for each. Infection concentration labels represent *F. oxysporum* infection at 1×10^5 spores/ml and *F. oxysporum* infection at 1×10^7 spores/ml.

Similarly, changes in H_2O_2 content for both cultivars were concentration dependent. No significant changes in H_2O_2 content was observed in SST 015 plants infected with 1×10^5 spores/ml, whereas a significant increase of 24.08% was observed in response to 1×10^7 spores/ml. For SST 088, *F. oxysporum* (as seen for both concentrations) significantly enhanced H_2O_2 content relative to the control. Similar responses were observed for H_2O_2 content, with no significant changes at the lower concentration (1×10^5 spores/ml) of the infection. In addition, a substantial increase of 32.31% in plants infected with a higher concentration (1×10^7 spores/ml) of *F. oxysporum* in comparison to the uninfected control plants from SST 015 (Table 4.1). These results demonstrate that *F. oxysporum* infection induced oxidative damage in wheat plants through the accumulation of ROS biomarkers (O_2^- and H_2O_2).

4.2.2 Characterization of oxidative damage and the extent of cell death in wheat cultivars

In addition, the extent of malondialdehyde (MDA) accumulation used as an indicator of lipid peroxidation in the wheat plants, was measured as an estimate of the oxidative damage in response to *F. oxysporum.* The MDA content of SST 015 in response to *F. oxysporum* was significantly increased with the highest increase observed in response to 1×10^7 spores/ml (50.78%) (Table 4.2). Similar to what was observed for SST 015, the MDA content of SST 088 was increased by *F. oxysporum*. SST 088 plants infected with 1×10^5 spores/ml showed an increase of 45.90%, whereas 1×10^7 spores/ml increased MDA content by 83.61%. The increase in MDA content for SST 088 was significantly higher than that observed for SST 015 (Table 4.2). The increase in MDA in response to *F. oxysporum* was manifested as an increase in cell death (indicated by the uptake of Evans blue). For SST 015, *F. oxysporum* increase cell death by 45.90% (1×10^5 spores/ml) and 83.61% (1×10^7 spores/ml) respectively. For SST 088, *F. oxysporum* increased cell death by 14.86% in response to 1×10^5 spores/ml, whereas an even

higher increase of 184.03% in cell death was observed in response to 1×10^7 spores/ml (Table 4.2).

Table 4.2: Extent of oxidative damage of two wheat cultivars in response to *F. oxysporum* infection

Indicators	SST 015			SST 088		
	Control	Infected (1×10^5)	Infected (1×10^7)	Control	Infected (1×10^5)	Infected (1×10^7)
Lipid peroxidation (MDA nmol.g^1 FW)	21.95 ± 0.45 [a]	29.94 ± 0.38 [b]	33.10 ± 0.41 [c]	36.42 ± 0.31 [a]	54.28 ± 0.51 [b]	66.82 ± 0.37 [c]
Cell death (Evans Blue A_{600nm})	0.61 ± 0.35 [a]	0.89 ± 0.26 [b]	1.12 ± 0.22 [c]	0.698 ± 0.24 [a]	0.79 ± 0.31 [b]	1.96 ± 0.46 [c]

The data in the above tables presents the means ± standard error of three replicates (n=3) for each indicator. Means marked with different letters in the same row for the same indicator indicates significant difference between infections based on the standard error calculated for each. Infection concentration labels represent *F. oxysporum* infection at 1×10^5 spores/ml (1×10^5) and *F. oxysporum* infection at 1×10^7 spores/ml (1×10^7).

4.2.3 *F. oxysporum* differentially alters superoxide dismutase activity in wheat cultivars

We investigated the effect of *F. oxysporum* on the enzymatic activity (as individual SOD isoforms) in two wheat cultivars namely SST 015 and SST 088. Using polyacrylamide gel electrophoresis, a total of four SOD isoforms was detected in the shoot extracts of SST 015 and SST 088 respectively. These isoforms were characterised using KCN and H_2O_2 as different inhibitors. For SST 015, two MnSOD, one FeSOD and one Cu/ZnSOD were detected and *F. oxysporum* was found to differentially regulate the activity of these isoforms (Figure 4.1).

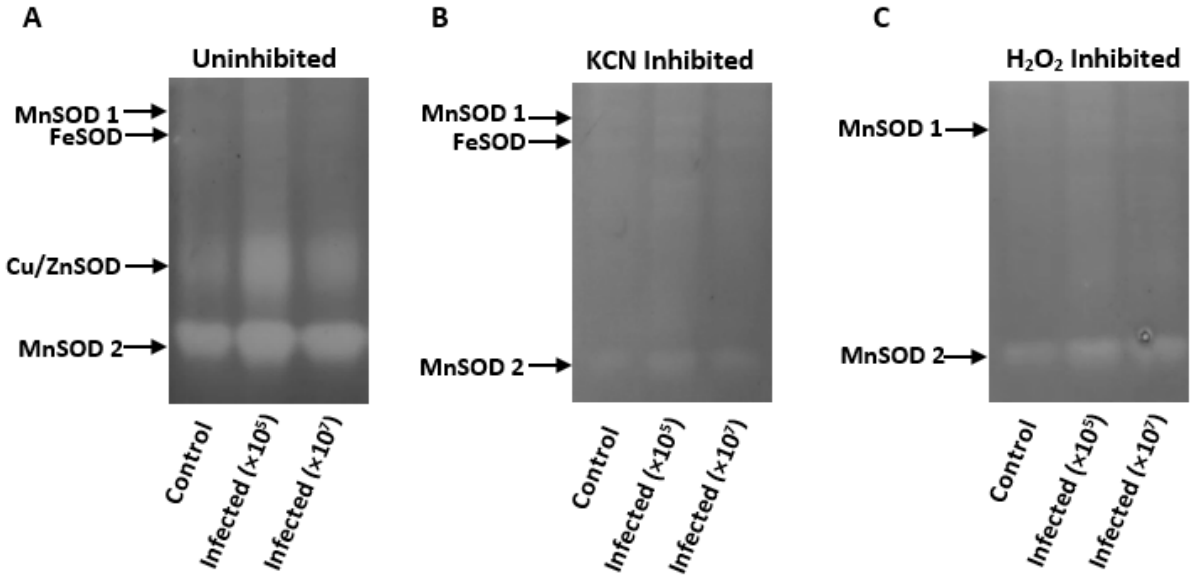

Figure 4.1: *F. oxysporum* infection differentially regulate SOD isoform activity of SST 015. In-gel activity assays were performed on wheat shoots from cultivar SST 015 infected with *F. oxysporum* and grown for 24 days. The native polyacrylamide gels present the detection of SOD isoforms with no inhibitors (A), in the presence of 5 mM KCN (B) and in the presence of 6 mM H_2O_2 (C) in wheat cultivar SST 015 response to the infection.

Based on the densitometry analysis, *F. oxysporum* infection was shown to differentially alter the activity of each SOD isoform (Table 4.3). For MnSOD 1, treatment/infection with 1×10^5 spores/ml increased activity by 23.99% relative to the control. No significant changes in the activity of MnSOD 1 was observed in response to treatment with 1×10^7 spores/ml (Table 4.3). A similar trend was observed for MnSOD 2, where the highest increase in activity (33.68%) was observed in response to 1×10^5 spores/ml, whereas 1×10^7 spores/ml increase the activity by 32.12% relative to the control (Figure 4.1; Table 4.3). The activity detected for FeSOD is relatively low and very difficult to quantify using visual inspection and densitometry analysis. For Cu/ZnSOD, the activity presented here was concentration dependent. In response to treatment with 1×10^5 spores/ml, Cu/ZnSOD activity increased by 57.45% relative to the control. Although an increase in Cu/ZnSOD activity was observed in response treatment with 1×10^7 spores/ml, this increase (31.2%) was not as pronounced as observed for treatment with 1×10^5 spores/ml (Figure 4.1; Table 4.3).

Table 4.3: Relative SOD activity of individual isoforms for SST 015

Relative SOD Activity (Arbitrary Units)	SOD isoforms	Control	Infected ($\times 10^5$)	Infected ($\times 10^7$)
	MnSOD1	1.167 ± 0.07^a	1.447 ± 0.05^b	1 ± 0.11^a
	MnSOD2	1 ± 0.13^a	1.337 ± 0.09^b	1.321 ± 0.07^b
	FeSOD	1.09 ± 0.06^a	1.03 ± 0.09^a	1 ± 0.11^a
	Cu/ZnSOD	1 ± 0.12^a	1.5745 ± 0.11^b	1.312 ± 0.11^b

Table 4.3 represents the integrated pixel density values of the superoxide dismutase isoforms activity for SST 015 plants determined using the Alpha Ease FC software and expressed as arbitrary units, as observed on 12% native acrylamide gel (Figure 4.1). The data presents the means ± standard error of three replicates (n=3). Means marked with different letters in the same row for the same isoform indicates significant difference between treatments based on the standard error for each isoform.

For SST 088, four SOD isoforms (two MnSODs, one FeSOD and one Cu/ZnSOD) were detected based on their resistance and sensitivity to KCN and H_2O_2 (Figure 4.2).

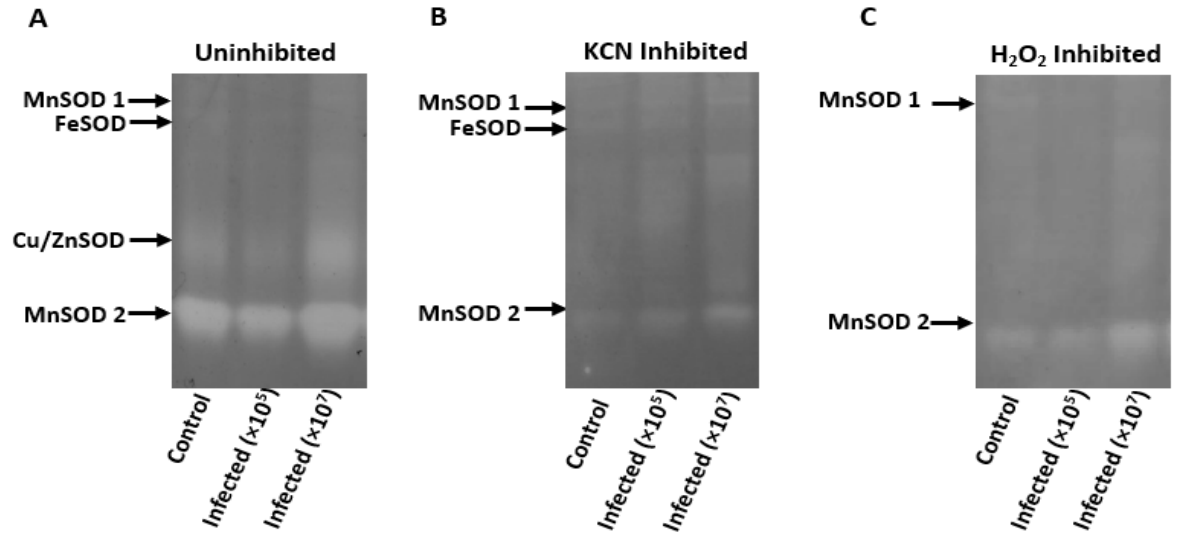

Figure 4.2: *F. oxysporum* infection differentially regulate SOD isoform activity of SST 088. In-gel activity assays were performed on wheat shoots from cultivar SST 088 infected with *F. oxysporum* and grown for 24 days. The native polyacrylamide gels depict the detection of SOD isoforms with no inhibitors (A), in the presence of 5 mM KCN (B) and in the presence of 6 mM H_2O_2 (C) in wheat cultivar SST 088 response to the infection.

The enzymatic activity of these isoforms was quantified using densitometry analysis (Table 4.4). Based on the densitometry analysis, these isoforms were differentially regulated by *F.*

oxysporum and its regulation is concentration dependent. MnSOD1 presented a similar trend as observed in SST 015 with very low abundance/absence in the control and 1×10^5 spores/ml treatment plants, however a significant increase of 27.5% was observed in plants infected with the 1×10^7 spores/ml treatment (Figure 4.2; Table 4.4). For MnSOD2, no significant change in activity was observed in response to 1×10^5 spores/ml, whereas an increase 51.2% was observed in the 1×10^7 spores/ml treatment when compared to the control (Figure 4.2; Table 4.4). FeSOD isoform was present in low abundance across all the plants for SST 088 and was difficult to quantify using visual inspection and densitometry analysis. For Cu/ZnSOD, the activity in response to the infection was found to be similar to that of MnSOD2 with no significant changes in plants infected at 1×10^5 spores/ml infection. However, a significant increase of 83.7% was observed in response to *F. oxysporum* infection (at 1×10^7 spores/ml) as depicted in Figure 4.2 and Table 4.4.

Table 4.4: Relative SOD activity of individual isoforms for SST 088

Relative SOD Activity (Arbitrary Units)	SOD isoforms	Control	Infected ($\times10^5$)	Infected ($\times10^7$)
	MnSOD1	1 ± 0.14^a	1.15 ± 0.09^a	1.28 ± 0.09^b
	MnSOD2	1.14 ± 0.07^a	1 ± 0.08^a	1.72 ± 0.05^b
	FeSOD	1.15 ± 0.12^a	1.13 ± 0.09^a	1 ± 0.13^a
	Cu/ZnSOD	1.06 ± 0.18^a	1 ± 0.09^b	1.95 ± 0.10^c

Table 4.4 represents the integrated pixel density values of the superoxide dismutase isoforms activity for SST 088 plants determined using the Alpha Ease FC software and expressed as arbitrary units, as observed on 12% native acrylamide gel (Figure 4.1B). The data presents the means ± standard error of three replicates (n=3). Means marked with different letters in the same row for the same isoform indicates significant difference between treatments based on the standard error for each isoform.

4.2.4 APX activity is altered by *F. oxysporum* infected wheat cultivars

The accumulation of H_2O_2 as a by-product of scavenging of O_2^- by SOD is detoxified further by various enzymes like ascorbate peroxidase (APX) which detoxify H_2O_2. Here we

demonstrate the influence of *F. oxysporum* infection at different concentrations on the enzymatic activities of individual APX isoforms detected in two wheat cultivars (SST 015 and SST 088). For each cultivar, two APX isoforms (labelled from top of the gel) were detected (Figure 4.3 A and Figure 4.4 A). For SST 015, *F. oxysporum* differentially alter (in a concentration dependent manner) APX activity as detected on the polyacrylamide gel and quantified using densitometry analysis (Figure 4.3 A-C). *F. oxysporum* increased APX 1 activity by 27.78% in response to treatment with 1×10^5 spores/ml, whereas treatment with 1×10^7 spores/ml increase the activity by 38.89% relative to the control plants (Figure 4.3 B). For APX 2, *F. oxysporum* increase the enzymatic activity by 20% and 58% respectively in a concentration dependent manner relative to the control (Figure 4.3 C).

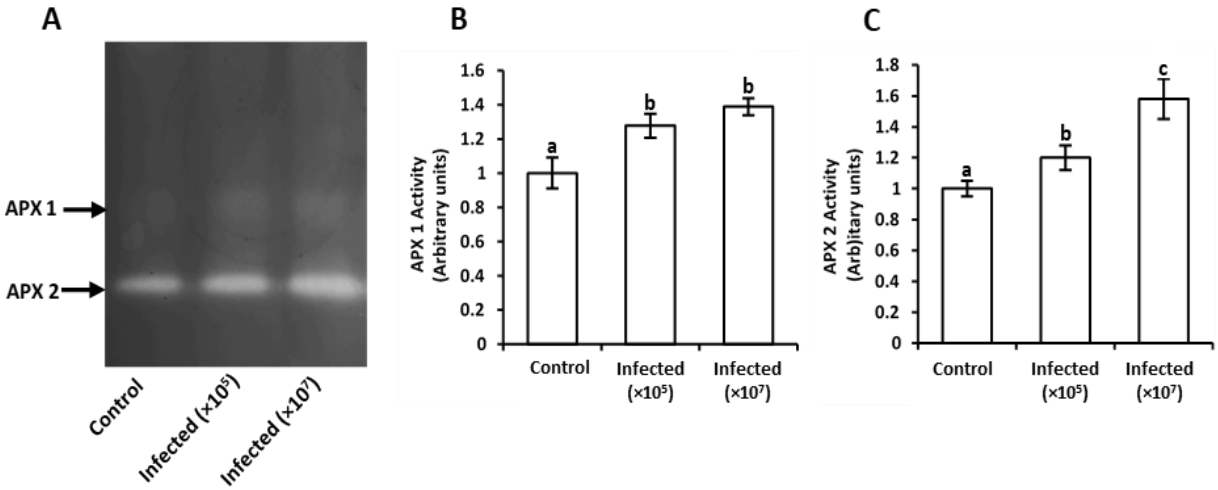

Figure 4.3: The effect of *F. oxysporum* infection on APX activity of SST 015. In-gel activity assays were performed on wheat shoots of SST 015 infected with *F. oxysporum* and grown for 24 days. Data represent the mean (± SE) of three independent experiments from 3 plants per treatment in each experiment. Means with different letters are significantly different from each other.

Contrasting to what was observed for SST 015, APX activity for SST 088 remained unaltered or was reduced by *F. oxysporum* (Figure 4.4 A-C). No significant changes in APX 1 and APX 2 activity was observed in response to treatment with 1×10^5 spores/ml relative to the control plants (Figure 4.4). However, a significant reduction in activity for both isoforms was observed

in response to treatment with 1×10^7 spores/ml. For APX 1, this reduction was measured at 23.54% whereas for APX 2 the reduction was measured at 41.51% relative to the controls (Figure 4.4 C).

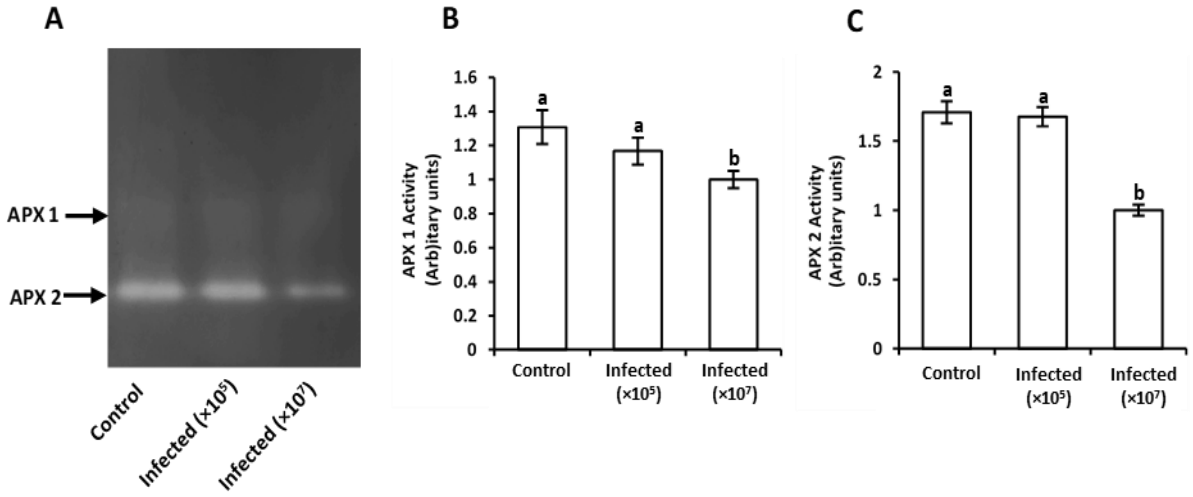

Figure 4.4: The effect of *F. oxysporum* infection on APX activity of SST 088. In-gel activity assays were performed on wheat shoots of SST 088 infected with F. oxysporum and grown for 24 days. Data represent the mean (± SE) of three independent experiments from 3 plants per treatment in each experiment. Means with different letters are significantly different from each other.

4.2.5 *F. oxysporum* infected wheat exhibited differential POD isoform activity.

In addition to APX, peroxidases (POD) scavenges H_2O_2, to protect cells from oxidative damage. We comparatively analyse the response of *F. oxysporum* on the POD isoforms detected in two wheat cultivars, SST 015 and SST 088 (Figure 4.5). A total of seven POD isoforms were detected for SST 015 and only five were clearly detected for SST 088 plants (Figure 4.5). For SST 015, *F. oxysporum* increase the enzymatic activities of all seven POD isoforms (POD1-7). Interestingly, the more pronounced increased in activity occurred in the lower *F. oxysporum* concentration in comparison to the increase in activity observed for the higher *F. oxysporum* concentration (1×10^7 spores/ml) relative to the controls (Figure 4.5 A).

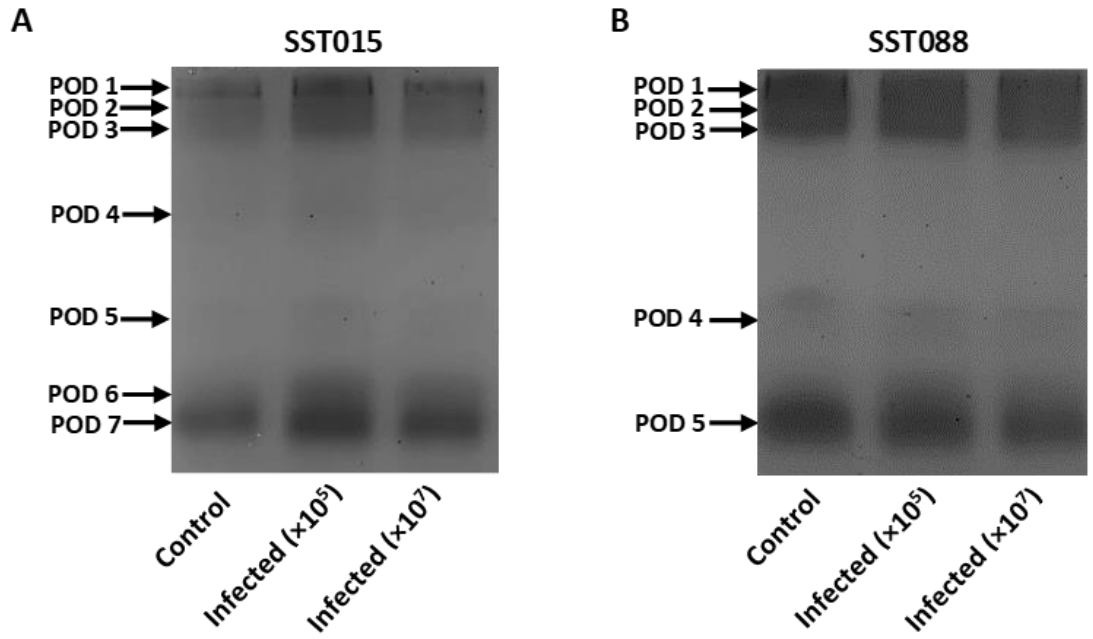

Figure 4.5: The effect of *F. oxysporum* infection on POD activity. In-gel activity assays were performed on wheat shoots of SST 015 (A) and SST 088 (B) infected with *F. oxysporum* and grown for 24 days. Data represent the mean (± SE) of three independent experiments from 3 plants per treatment in each experiment.

Based on the densitometry analysis for SST 015, POD isoform activity of POD 1, POD 2, POD 3, POD 6 and POD 7 presented the highest increase in activity at 1×10^5 spores/ml treatment of 64%, 56%, 68%, 45% and 65% respectively. In addition, POD4 and POD5 presented minimal increases of 28% and 23% for the same treatment. POD isoform activities in plants infected with the higher infection treatment (1×10^7 spores/ml) presented a similar trend observed in 1×10^5 spores/ml plants. The increase in activity of POD 1, POD 2, POD 3 and POD 6 exceeded 30%, with POD 7 increasing by a mere 27%. In addition, POD 4 and POD 5 again had shown a slight increase in activity at 1×10^7 spores/ml relative to the controls (Figure 4.5 A; Table 4.5).

Table 4.5: Relative POD activity for each isoform of SST 015

Relative POD Activity (Arbitrary Units)	POD isoforms	Control	Infected ($\times 10^5$)	Infected ($\times 10^7$)
	POD 1	1 ± 0.07^a	1.64 ± 0.05^b	1.35 ± 0.09^c
	POD 2	1 ± 0.05^a	1.56 ± 0.09^b	1.36 ± 0.08^c
	POD 3	1 ± 0.09^a	1.68 ± 0.07^b	1.31 ± 0.11^c
	POD 4	1 ± 0.15^a	1.28 ± 0.11^b	1.17 ± 0.13^a
	POD 5	1 ± 0.08^a	1.23 ± 0.07^b	1.13 ± 0.6^a
	POD 6	1 ± 0.08^a	1.45 ± 0.12^b	1.33 ± 0.1^c
	POD 7	1 ± 0.09^a	1.65 ± 0.04^b	1.27 ± 0.05^c

Table 4.5 represents the integrated pixel density values of the peroxidase (POD) isoforms activity for SST 015 plants determined using the Alpha Ease FC software and expressed as arbitrary units, as observed on 12% native acrylamide gel (Figure 4.5). The data presents the means ± standard error of three replicates (n=3). Means marked with different letters in the same row for the same isoform indicates significant difference between treatments based on the standard error for each isoform.

In contrast to what was observed for SST 015, only five POD isoforms were successfully identified for SST 088 and presented significant reductions in activity (Figure 4.5 B). Based on the densitometry results, reductions in activity were more prominent in the higher *F. oxysporum* concentration 1×10^7 spores/ml relative to the control plants (Table 4.6).

Table 4.6: Relative POD activity for each isoform of SST 088

Relative POD Activity (Arbitrary Units)	POD isoforms	Control	Infected ($\times 10^5$)	Infected ($\times 10^7$)
	POD 1	1.255 ± 0.07^a	1.13 ± 0.07^b	1 ± 0.09^c
	POD 2	1.25 ± 0.05^a	1.24 ± 0.06^b	1 ± 0.11^c
	POD 3	1.20 ± 0.13^a	1.20 ± 0.09^a	1 ± 0.08^b
	POD 4	1.16 ± 0.11^a	1.16 ± 0.1^a	1 ± 0.11^a
	POD 5	1.21 ± 0.18^a	1.21 ± 0.12^a	1 ± 0.12^b

Table 4.6 represents the integrated pixel density values of the peroxidase (POD) isoforms activity for SST 015 plants determined using the Alpha Ease FC software and expressed as arbitrary units, as observed on 12% native acrylamide gel (Figure 4.5). The data presents the means ± standard error of three replicates (n=3). Means marked with different letters in the same row for the same isoform indicates significant difference between treatments based on the standard error for each isoform.

At 1×10^7 spores/ml, POD activity of POD 1, POD 2 and POD 3 indicated significant decreases of 25.5%, 25% and 24% respectively. Furthermore, POD 4 presented no significant changes in activity, in addition to POD 5 that presented reduction of 19% (Table 4.4). For 1×10^5 spores/ml plants, less pronounced reductions ranging between 13% and 24% were observed for POD 1, POD 2, POD 3 and POD 5, with POD 4 presenting no significant change (Table 4.4).

4.3 Discussion

4.3.1 *F. oxysporum* alters ROS accumulation and induces oxidative damage wheat cultivars

When plants are exposed to fungal induced stress, fungal components such as chitin and several other elicitors activate plant immune responses, employing ROS alongside other molecules that are responsible for fighting off the pathogen and restrict it from spreading throughout the plant (Ali *et al.* 2018). Several studies have shown that when subjected to stress conditions such as pathogen attack, plants' present an increase in the production of ROS (Magbanua *et al.* 2007). In this study we investigated the effect of *F. oxysporum* infection in two wheat cultivars (SST 015 and SST 088) and monitored changes in ROS accumulation and ROS scavenging antioxidant enzymes. According to a study by Debona *et al.* (2012) on the biochemical changes that occur in wheat plants in response to *Pyricularia oryzae*, an increase in H_2O_2 and O_2^- in response to the infection was detected. A similar observation was made in this study where *F. oxysporum* induced ROS accumulation (Table 4.1) and subsequent oxidative damage manifested as enhanced cell death (Table 4.2).

In addition to the increase in ROS, the increase in the extent of lipid peroxidation observed in both wheat cultivars demonstrated the severity of oxidative damage as a result of the pathogen induced stress, ultimately contributing to the increase cell death that was observed (Table 4.2).

This phenomenon was reported by Mandal *et al.* (2008), where peroxidation of membrane lipids is used as an estimate of the extent of the cellular damage caused by the oxidative stress related to the plant response against the pathogen infection. Comparison of the two cultivars indicated that SST 088 presented more significant increases in ROS induced oxidative damage, thus suggesting that this cultivar was more sensitive to the infection by *F. oxysporum*. This view was supported by several studies that have shown how ROS-mediated cell death in plants can potentially be triggered by the increase in oxidative damage manifested by the increased levels of MDA in response to pathogen infection (Debona *et al.* 2012; Aziz and Larher, 1998).

4.3.2 *F. oxysporum* differentially alters antioxidant enzymatic activity in wheat plants

The production of ROS is an important plant defence mechanism against pathogen infection (Ali *et al.* 2018). The scavenging of ROS in wheat plants infected with *Fusarium species* represents a fundamental defence mechanism to combat the over production of ROS. Enzymatic activity of SOD, APX and POD were differentially regulated in response to *F. oxysporum* in wheat cultivars (SST 015 and SST 088) compared to the uninfected controls. A study conducted by Gherbawy *et al.* (2012) presented differential regulation of several antioxidants in wheat plants in response to *Fusarium* infection. The wheat cultivars tested in this study presented contrasting results in the differential expression in activity in response to the infection. SST 015 plants presented increases in enzymatic activity with SST 088 plants presenting fluctuation/reductions in activity. This result could suggest that the wheat cultivars have varying degrees of resistance to the infection by *F. oxysporum* which could affect the efficiency of the scavenging antioxidant enzymes.

4.3.2.1 Changes in SOD enzymatic activity in response to F. oxysporum

In this study we looked at the effect of fungal pathogen (*F. oxysporum*) infection on the enzymatic activity of various SOD isoforms in wheat shoots detected by native PAGE analysis. Based on the native gel, the SOD activity consisted of four individual SOD isoforms including two MnSODs isoforms, one FeSOD and one Cu/ZnSOD detected in both wheat cultivars (SST 015 and SST 088) shown in Figure 4.1 and Figure 4.2 respectively. The individual SOD isoform activities detected in this study were differentially regulated, with both cultivars producing a gradual increase in MnSOD1 activity in contrast to MnSOD2 and Cu/ZnSOD activity which was found to significantly increase response to the infection by *F. oxysporum* (Table 4.3 and Table 4.4). The results obtained in this study are in agreement with a study conducted by Debona *et al.* (2012), where SOD activity significantly increased in two wheat cultivars (BR18 and BRS 229) in response to wheat blast caused by *Pyricularia oryzae*. An additional study on strawberries infected by *Mycosphaerella fragariae* showed a similar increase in SOD as a result of the infection (Ehsani-Moghaddam *et al.* 2008).

4.3.2.2 F. oxysporum differentially regulates APX activity in wheat plants

Ascorbate (ASA) is known as a major metabolite in plants and is associated with several components of the antioxidant system that protects plants against oxidative damage as a result of biotic and abiotic stresses (Pandey *et al.* 2015; Smirnoff, 1996;). According to Pandey *et al.* (2015), APX is considered one of the key enzymes that form part of the ascorbate-glutathione (Halliwell-Asada) pathway that detoxifies H_2O_2 to less toxic forms (H_2O_2 and O_2) using ASA as an electron donor. APX has been detected in all plant cell compartments including mitochondria, cytosol, chloroplasts and microbodies (Mittler *et al.* 2004; Shigeoka *et al.* 2002 Asada, 1999; Jimenez *et al.* 1997) making it extremely valuable. Caverzan *et al.* (2012) demonstrated that in addition to other enzymatic antioxidants, APX activity is found to increase

in response to various biotic and abiotic stresses as it is one of the main scavengers of H_2O_2. In this study we comparatively analysed APX activity (as individual isoforms) in two wheat cultivars (SST 015 and SST 088) in response to *F. oxysporum*. Contrasting responses was observed in both cultivars where the APX activity for SST 015 was enhanced whereas the opposite was observed for SST 088. The response in APX activity for SST 015 plants was in accordance with Mandal *et al.* (2008), who showed significant increases in APX activity among other enzymatic antioxidants during the oxidative burst of *Solanum lycoperiscum and F. oxysporum f. sp. Lycopersici* interactions in response to the pathogen inoculation. In addition, a study conducted by Gherbawy *et al.* (2012) showed both the increase and decrease in APX activity of wheat shoots infected with various *Fusarium species* including *F. oxysporum, F. poae, F. merismoides and F. moniliforme, F. poae and F. sambucinum.* The interaction between all of the *Fusarium species* apart from *F. poae* presented the significant increase in APX activity which was similar to that observed in SST 015 plants in this study. Furthermore, the interaction between wheat and *F. poae* presented the decrease in APX activity which was the case for SST 088 plants in this study Gherbawy *et al.* (2012).

4.3.2.3 POD activity is differentially altered by F. oxysporum

In addition to SOD and APX, peroxidase (POD) is found to be widely distributed in higher plants and functions to protect cells against oxidative damage (Lin and Kao, 2002; Sudhakar *et al.* 2001; Dionisio-Sese and Tobita, 1998). The enzyme's key role involves the biosynthesis of lignin and defends the plant against biotic stresses such as pathogen attack, by utilizing the H_2O_2 present in various cell compartments including the cell wall, cytosol, vacuole as well as in extracellular space (Karuppanapandian *et al.* 2011). In this study we were able to detect seven POD isoforms for SST 015 and five isoforms for SST 088 using native PAGE analysis. The increase in POD activity of SST 015 infected observed for all POD isoforms (Figure 4.4A;

Table 4.3A) was in agreement with the study conducted by El-Khallal (2007) that showed the significant increase in POD activity of tomato plants in response to *F. oxysporum* (El-Khallal, 2007). In addition, Mohammadi and Kazemi (2002) reported the increase in POX activity wheat plants in response to *F. graminearum* infection which was seen for the case of SST 015 in response to *F. oxysporum* in this study. Furthermore, the contrasting reduction in POD activity for all isoforms apart from POD 5, observed for SST 088 plants was supported by Gherbawy *et al.* (2012) who showed the reduction of POD activity of wheat in response to *F. sambucinum.*

In summary both cultivars presented significant increases in ROS induced oxidative damage, however, SST 088 presented the most significant increases suggesting that it had higher levels of susceptibility to *F. oxysporum* infection in comparison to SST 015. The initiation of ROS scavenging for redox homeostasis indicated contrasting responses from the two wheat cultivars. SST 015 plants presented increases in enzymatic activity with SST 088 plants presenting fluctuation/reductions in activity. This result could suggest that the wheat cultivars have varying degrees of resistance to the infection by *F. oxysporum* which could affect the efficiency of the scavenging antioxidant enzymes.

CHAPTER 5

CONCLUDING REMARKS AND FUTURE PROSPECTS

The study was set out to investigate the effects of *Fusarium oxysporum* on the physiological and biochemical responses of wheat cultivars due to the detrimental effects of *Fusarium* species (including *F. oxysporum)* on economically important food crops. The increasing prevalence of infections by *Fusarium species* continues to threaten agricultural productivity and sustainability of valuable cereal crops such as wheat. Wheat represents one of the most important cereal crops worldwide and plays a fundamental role in sustaining food security in developing countries such as South Africa. As a result, this study focused on comparatively analysing changes in physiological responses of wheat cultivars (SST 015, SST 056 and SST 088) through the measurement of growth parameters (such as shoot and root growth and biomass as well as relative water (RWC), chlorophyll pigmentation and osmolyte content) in response to *F. oxysporum*. In addition, this study also explored the changes in biochemical responses, by measuring the accumulation of ROS, extent of lipid peroxidation and antioxidant enzyme activity for SST 015 and SST 088 in response to *F. oxysporum*. The results presented in this thesis were separated into two research chapters (Chapter 3 and Chapter 4).

Chapter 3 described the effect of *F. oxysporum* on the growth, biomass, relative water content (RWC), chlorophyll metabolism and osmolyte content of three wheat cultivars (SST 015, SST 056 and SST 088). The study established that *F. oxysporum* negatively alters plant growth (Figures 3.1; 3.4 and 3.7) and biomass (Figures 3.2; 3.5 and 3.8) as well as RWC and chlorophyll pigmentation (Figures 3.3; 3.6 and 3.9). In addition, proline content of all three wheat cultivars increased in response to *F. oxysporum* indicating the stress induced by the pathogen. Interestingly, these results showed that SST 088 was more susceptible to *F.*

oxysporum as it presented the most pronounced physiological changes. Although, SST 015 and SST 056 presented significant changes in response to *F. oxysporum*, SST 015 presented the biggest contrast to those observed for SST 088. Therefore, the biochemical responses for only SST 015 and SST 088 were further investigated.

In chapter 4, the effects of *F. oxysporum* on ROS metabolism and antioxidant capacity of two wheat cultivars (SST 015 and SST 088) were described. *F. oxysporum* significantly enhanced the accumulation of ROS biomarkers in both cultivars, a common response previously described in literature. Furthermore, *F. oxysporum* promoted the extent of lipid peroxidation, which led to the increase in cell death for SST 015 and SST 088. The regulation of ROS by enzymatic antioxidant scavengers (SOD, APX and POD) were differentially altered by *F. oxysporum* in SST 015 (Figures 4.1; 4.3 and 4.5) and SST 088 (Figures 4.2; 4.2 and 4.5). This study showed that the activity of some isoforms was increased in response to *F. oxysporum*, however, this increase did not ensure sufficient scavenging of ROS biomarkers as a consequence of *F. oxysporum*. The contrasting responses in enzymatic activity seen for SST 015 and SST 088 suggest that wheat cultivars have varying degrees of tolerance to *F. oxysporum*. Therefore, suggesting that there is a direct relationship between ROS accumulation and scavenging by antioxidant enzymes for the regulation of plant tolerance against *F. oxysporum*.

In this study, *F. oxysporum* was not only found to significantly reduce plant growth and development of wheat cultivars investigated, the infection also enhanced the accumulation of ROS and differentially altered antioxidant capacity for SST 015 relative to SST 088. These results ultimately suggested that SST 015 was more resilient to *F. oxysporum*. This study provides significant insight on the effects of *F. oxysporum* on the physiological and

biochemical responses during early stages of wheat development. Many *Fusarium*-wheat interaction studies in the public domain primarily focus on the changes that occur during late stages of plant development/pre-harvest and post-harvest.

Furthermore, the knowledge gained from this study has significant potential for future investigation of the total protein abundance for the identification of differentially expressed proteins using proteomic approaches. Subsequent analysis would involve the identification of differentially expressed proteins that could be used as potential biomarkers. These biomarkers would be used in the development of treatment strategies that would prevent or suppress the onset of plant diseases caused by fungal pathogens such as *F. oxysporum* during early plant-pathogen interaction. In addition, genes from the candidate proteins could be isolated and based on their expression, could potentially be transformed back into the plant under a stress responsive promoter to drive its expression. This genetic engineering strategy may pave the way for improving the resilience of wheat and other crops to pathogen infection, ultimately reducing crop losses and improving the sustainability of crop production required for food security.

REFERENCES

1. Able, A.J., Guest, D.I. and Sutherland, M.W. (1998). Use of a new tetrazolium-based assay to study the production of superoxide radicals by tobacco cell cultures challenged with avirulent zoospores of *Phytophthora parasitica varnicotianae*. *Plant Physiology*, 117, 491-499.
2. Al-Abdalall, A. (2010). Assessment of yield loss caused by root rots in wheat and barley. *Journal of Food, Agriculture and Environment*, 8, 638-641.
3. Ali, M., Cheng, Z., Ahmad, H. and Hayat, S. (2018). Reactive oxygen species (ROS) as defenses against a broad range of plant fungal infections and case study on ROS employed by crops against *Verticillium dahliae* wilts. *Journal of Plant Interactions*, 13(1), 353-363.
4. Aliber, M. (2009). Exploring statistics South Africa's National Household surveys as sources of information about household-level food security. *Agrekon*, 48(4).
5. Altinok, H. (2013). *Fusarium Species* Isolated from Common Weeds in Eggplant Fields and Symptomless Hosts of *Fusarium oxysporum f. sp.melongenae* in Turkey. *Journal of Phytopathology*, 161(5), 335-340.
6. Altman, M., Hart, T. and Jacobs, P. (2009). Household food security status in South Africa. *Agrekon*, 48(4), 345-361.
7. Antonissen, G., Martel, A., Pasmans, F., Ducatelle, R., Verbrugghe, E., Vandenbroucke, V., Li, S., Haesebrouck, F., Van Immerseel, F. and Croubels, S. (2014). The impact of *Fusarium Mycotoxins* on human and animal host susceptibility to infectious diseases. *Toxins*, 6(2), 430-452.
8. Apel, K. and Hirt, H. (2004). Reactive Oxygen Species: Metabolism, Oxidative Stress, and Signal Transduction. *Annual Review of Plant Biology*, 55(1), 373-399.
9. Arias, M., Leandro, L. and Munkvold, G. (2013). Aggressiveness of *Fusarium Species* and impact of root infection on growth and yield of soybeans. *Phytopathology*, 103(8), 822-832.
10. Armstrong, G. and Armstrong, J.K. (1978). Formae Speciales and Races of *Fusarium oxysporum* causing wilts of the cucurbitaceae. *Phytopathology*, 68(1), 19.
11. Arora, P., Bhardwaj, R. and Kanwar, M. (2010). 24-epibrassinolide regulated diminution of Cr metal toxicity in *Brassica juncea L*. plants. *Brazilian Journal of Plant Physiology*, 22(3), 159.

12. Asada, K .(1992). Ascorbate peroxidase–a hydrogen peroxide-scavenging enzyme in plants. *Physiologia Plantarum*, 85, 235-241.
13. Asada, K. (1999). The water-water cycle in chloroplasts: Scavenging of active oxygens and dissipation of excess photons. *Annual Review of Plant Physiology and Plant Molecular Biology*, 50(1), 601-639.
14. Ashraf, M. and Foolad, M. (2007). Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environmental and Experimental Botany*, 59(2), 206-216.
15. Ashraf, M. and Harris, P. (2004). Potential biochemical indicators of salinity tolerance in plants. *Plant Science*, 166(1), 3-16.
16. Ashraf, M., and Harris, P. J. C. (2013). Photosynthesis under stressful environments: an overview. *Photosynthetica*, 51, 163-190.
17. Awika, J. (2011). Major Cereal Grains Production and Use around the World. *ACS Symposium Series*, 1-13.
18. Aziz, A. and Larher, F. (1998). Osmotic stress induced changes in lipid composition and peroxidation in leaf discs of *Brassica napus L. Journal of Plant Physiology*, 153(5-6), 754-762.
19. Banerjee, A. and Mittra, B. (2018). Morphological modification in wheat seedlings infected by *Fusarium oxysporum*. *European Journal of Plant Pathology*, 152(2), 521-524.
20. Beckman, C.H. (1990). Host responses to the pathogen. In: Ploetz, R.C. (Ed.), Fusarium wilt of banana. APS Press, St Paul, Minnesota, USA, 93-105.
21. Berger, S., Sinha, A. and Roitsch, T. (2007). Plant physiology meets phytopathology: plant primary metabolism and plant pathogen interactions. *Journal of Experimental Botany*, 58(15-16), 4019-4026
22. Bishop, C. and Cooper, R. (1983). An ultrastructural study of root invasion in three vascular wilt diseases. *Physiological Plant Pathology*, 22(1), 15-31.
23. Bor, M., Özdemir, F. and Türkan, I. (2003). The effect of salt stress on lipid peroxidation and antioxidants in leaves of sugar beet *Beta vulgaris L*. and wild beet *Beta maritima L*. *Plant Science*, 164(1), 77-84.
24. Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, 248-254.
25. Buege, J.A. and Aust, S.D. (1978). Microsomal lipid peroxidation. *Methods in Enzymology* 52, 302-310.

26. Candan, N. and Tarhan,, L. (2003). The correlation between antioxidant enzyme activities and lipid peroxidation levels in Mentha pulegium organs grown in Ca2+, Mg2+, Cu2+, Zn2+ and Mn2+ stress conditions. *Plant Science*, 165(4), 769-776.
27. Carillo, P. and Gibon, Y. (2011). Protocol: extraction and determination of proline. [online] PrometheusWiki. Available at: http://prometheuswiki.publish.csiro.au/tiki-index.php?page=PROTOCOL%3A+Extraction+and+determination+of+glycine+betaine [Accessed 19 Apr. 2019].
28. Cavalcanti, F., Resende, M., Lima, J., Silveira, J. and Oliveira, J. (2006). Activities of antioxidant enzymes and photosynthetic responses in tomato pre-treated by plant activators and inoculated by Xanthomonas vesicatoria. *Physiological and Molecular Plant Pathology*, 68(4-6), 198-208.
29. Caverzan, A., Casassola, A. and Brammer, S. (2016). Antioxidant responses of wheat plants under stress. *Genetics and Molecular Biology*, 39(1), 1-6.
30. Caverzan, A., Passaia, G., Rosa, S., Ribeiro, C., Lazzarotto, F. and Margis-Pinheiro, M. (2012). Plant responses to stresses: role of ascorbate peroxidase in the antioxidant protection. *Genetics and Molecular Biology*, 35(4), 1011-1019.
31. Chaves, M., Martinelli, J., Wesp-Guterres, C., Graichen, F., Brammer, S., Scagliusi, S., da Silva, P., Wiethölter, P., Torres, G., Lau, E., Consoli, L. and Chaves, A. (2013). The importance for food security of maintaining rust resistance in wheat. *Food Security*, 5(2), 157-176.
32. Chávez-Arias, C., Gómez-Caro, S. and Restrepo-Díaz, H. (2019). Physiological, biochemical and chlorophyll fluorescence parameters of *Physalis Peruviana L*. seedlings exposed to different short-term waterlogging periods and Fusarium wilt infection. *Agronomy*, 9(5), 213.
33. Chen, T. and Murata, N. (2010). Glycinebetaine protects plants against abiotic stress: mechanisms and biotechnological applications. *Plant, Cell and Environment*, 34(1), 1-20.
34. Cheng, H. and Song, S. (2006). Species and organ diversity in the effects of hydrogen peroxide on superoxide dismutase activity in vitro. *Journal of Integrative Plant Biology*, 48(6), 672-678.
35. Christakopoulos, P., Kekos, D., Macris, B., Claeyssens, M. and Bhat, M. (1995). Purification and mode of action of a low molecular mass endo-1,4-β-d-glucanase from *Fusarium oxysporum*. *Journal of Biotechnology*, 39(1), 85-93.

36. Christakopoulos, P., Nerinckx, W., Kekos, D., Macris, B. and Claeyssens, M. (1996). Purification and characterization of two low molecular mass alkaline xylanases from *Fusarium oxysporum* F3. *Journal of Biotechnology*, 51(2), 181-189.
37. Chung, G. (2012). Effect of Pests and Diseases on Oil Palm Yield. *Palm Oil*, 163-210.
38. Claussen, W. (2005). Proline as a measure of stress in tomato plants. *Plant Science*, 168(1), 241-248.
39. Curtis, T. and Halford, N. (2014). Food security: the challenge of increasing wheat yield and the importance of not compromising food safety. *Annals of Applied Biology*, 164(3), 354-372.
40. D'Mello, J., Placinta, C. and Macdonald, A. (1999). Fusarium mycotoxins: a review of global implications for animal health, welfare and productivity. *Animal Feed Science and Technology*, 80(3-4), 183-205.
41. Dabrowska, G., Kata, A., Goc, A., Szechy´nska-Hebda, M. and Skrzypek, E. (2007). Characteristics of the plant ascorbate peroxidase family. *Acta biologica Cracoviensia / Series botanica*, 49, 7-17.
42. Dai, J. and Mumper, R. (2010). Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules*, 15(10), 7313-7352.
43. Dean, R., Van Kan, J., Pretorius, Z., Hammond-Kosack, K., Di Pietro, A., Spanu, P., Rudd, J., Dickman, M., Kahmann, R., Ellis, J. and Foster, G. (2012). The Top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology*, 13(4), 414-430.
44. Debona, D., Rodrigues, F., Rios, J. and Nascimento, K. (2012). biochemical changes in the leaves of wheat plants infected by *Pyricularia oryzae*. *Phytopathology*, 102(12), 1121-1129.
45. Desingh, R. and Kanagaraj, G. (2007). Influence of salinity stress on photosynthesis and antioxidative systems in two cotton varieties. *Applied Plant Physiology*, 33, 3-4.
46. Dietz, K. (2003). Plant peroxiredoxins. *Annual Review of Plant Biology*, 54(1), 93-107.
47. Dionisio-Sese, M. and Tobita, S. (1998). Antioxidant responses of rice seedlings to salinity stress. *Plant Science*, 135(1), 1-9.
48. Doehlemann, G., Okmen,, B., Zhu, W. and Sharon, A. (2017). Plant pathogenic fungi. *Microbiology Spectrum*, 5, 5-17.
49. Drankham, K., Carter, J., Madl, R., Klopfenstein, C., Padula, F., Lu, Y., Warren, T., Schmitz, N. and Takemoto, D.J. (2003). Antitumor activity of wheats with high orthophenolic content. *Nutrition and Cancer*, 47, 188-194.

50. Dresselhaus, T. and Hückelhoven, R. (2018). Biotic and abiotic stress responses in crop plants. *Agronomy*, 8(11), 267-273.
51. Drimie, S. and McLachlan, M. (2013). Food security in South Africa—first steps toward a transdisciplinary approach. *Food Security*, 5(2), 217-226.
52. Duh, P., Du, P. and Yen, G. (1999). Action of methanolic extract of mung bean hulls as inhibitors of lipid peroxidation and non-lipid oxidative damage. *Food and Chemical Toxicology*, 37(11), 1055-1061.
53. Ebbole, D. and Sachs, M. (1990). A rapid and simple method for isolation of Neurospora crassa homokaryons using microconidia. *Fungal Genetics Reports*, 37(1), 21-30.
54. Ehsani-Moghaddam, B., Charles, M., Carisse, O. and Khanizadeh, S. (2008). Regulation of superoxide dismutase isoforms in resistant and susceptible strawberry cultivars subjected to leaf spot disease. *Archives Of Phytopathology And Plant Protection*, 41(7), 492-500.
55. El-Khallal, S. (2007). Induction and modulation of resistance in tomato plants against Fusarium wilt disease by bioagent fungi (arbuscular mycorrhiza) and/or hormonal elicitors (Jasmonic acid and Salicylic acid): 2-Changes in the antioxidant enzymes, phenolic compounds and pathogen related- proteins. *Australian Journal of Basic and Applied Sciences*, 1(4), 717-732.
56. Ers.usda.gov. (2019). *USDA ERS* - Definitions of Food Security. [online] Available at: https://www.ers.usda.gov/topics/food-nutrition-assistance/food-security-in-the-us/definitions-of-food-security.aspx [Accessed 28 Nov. 2019].
57. Ferrigo, D., Raiola, A., Bogialli, S., Bortolini, C., Tapparo, A. and Causin, R. (2015). In Vitro Production of Fumonisins by *Fusarium verticillioides* under Oxidative Stress Induced by H2O2. *Journal of Agricultural and Food Chemistry*, 63(19), 4879-4885.
58. Flood, J. (2010). The importance of plant health to food security. *Food Security*, 2(3), 215-231.
59. Foley, J., Ramankutty, N., Brauman, K., Cassidy, E., Gerber, J., Johnston, M., Mueller, N., O'Connell, C., Ray, D., West, P., Balzer, C., Bennett, E., Carpenter, S., Hill, J., Monfreda, C., Polasky, S., Rockström, J., Sheehan, J., Siebert, S., Tilman, D. and Zaks, D. (2011). Solutions for a cultivated planet. *Nature*, 478(7369), 337-342.
60. Foyer,, C., Lelandais, M. and Kunert, K. (1994). Photooxidative stress in plants. *Physiologia Plantarum*, 92(4), 696-717.
61. Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2006). Crosstalk between abiotic and biotic stress responses: a

current view from the points of convergence in the stress signalling networks. *Current Opinion in Plant Biology*, 9(4), 436-442.

62. Gerber, J. (2010). Yield response of Fusarium infected maize seed treated with biological control agent formulations. *Dissertation*.
63. Gherbawy, Y., El-Tayeb, M., Maghraby, T., Shebany, Y. and El-Deeb, B. (2012). Response of antioxidant enzymes and some metabolic activities in wheat to Fusarium spp. Infections. *Acta Agronomica Hungarica*, 60(4), 319-333.
64. Godfray, H., Beddington, J., Crute, I., Haddad, L., Lawrence, D., Muir, J., Pretty, J., Robinson, S., Thomas, S. and Toulmin, C. (2010). Food Security: The Challenge of Feeding 9 Billion People. *Science*, 327(5967), 812-818.
65. Gordon, T. (2017). Fusarium oxysporum and the Fusarium Wilt Syndrome. *Annual Review of Phytopathology*, 55(1), 23-39.
66. Gregory, P. and George, T. (2011). Feeding nine billion: the challenge to sustainable crop production. *Journal of Experimental Botany*, 62(15), 5233-5239.
67. Gülçin, İ., Oktay, M., Küfrevioğlu, Ö. and Aslan, A. (2002). Determination of antioxidant activity of lichen Cetraria islandica (L) Ach. *Journal of Ethnopharmacology*, 79(3), 325-329.
68. Gull, A., Ahmad Lone, A. and Ul Islam Wani, N. (2019). Biotic and abiotic stresses in plants. *Abiotic and Biotic Stress in Plants*, Alexandre Bosco de Oliveira, IntechOpen, Available from: https://www.intechopen.com/books/abiotic-and-biotic-stress-in-plants/biotic-and-abiotic-stresses-in-plants [Accessed on 12 Sept. 2019].
69. Gustafson, P., Raskina, O., Ma, X. and Nevo, E. (2009). Wheat evolution, domestication, and improvement. *Wheat Science and Trade*, 3-30.
70. Halliwell, B. (2006). Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiology*, 141(2), 312-322.
71. Hare, P., Cress, W. and Van Staden, J. (1998). Dissecting the roles of osmolyte accumulation during stress. *Plant, Cell and Environment*, 21(6), 535-553.
72. Hart, T.G.B. and Aliber, M. (2009). Should subsistence agriculture to be supported as a strategy to address rural food insecurity? *Agrekon*, 48(4).
73. Hassanein, R.A., Hashem, H.A., Fahmy, A.H., Ibrahim, A.S., El Shihy, O.M. and Qaid, E.A., 2016. Protection against fusarium head blight: important defense mechanisms studied in three regenerated Egyptian wheat cultivars. *Journal of Stress Physiology and Biochemistry*, 12(3), 18-43.

74. Hayat, S., Hayat, Q., Alyemeni, M., Wani, A., Pichtel, J. and Ahmad, A. (2012). Role of proline under changing environments. *Plant Signaling and Behavior*, 7(11), 1456-1466.
75. Husaini, A., Sakina, A. and Cambay, S. (2018). Host–pathogen interaction in Fusarium oxysporum infections: Where do we stand?. *Molecular Plant-Microbe Interactions*, 31(9), 889-898.
76. Imlay, J. (2003). Pathways of oxidative damage. *Annual Review of Microbiology*, 57(1), 395-418.
77. Inze, D. and Van Montagu, M. (1995). Oxidative stress in plants. *Current Opinion in Biotechnology*, 6, 153-158.
78. James Cook, R. (2003). Take-all of wheat. *Physiological and Molecular Plant Pathology*, 62(2), 73-86.
79. Jiang, Y. and Huang, B. (2001). Drought and heat stress injury to two cool-season turfgrasses in relation to antioxidant metabolism and lipid peroxidation. *Crop Science*, 41(2), 436-447.
80. Jimenez, A., Hernandez, J., del Rio, L. and Sevilla, F. (1997). Evidence for the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea leaves. *Plant Physiology*, 114(1), 275-284.
81. Jiménez-Ruiz, J., Leyva-Pérez, M., Schilirò, E., Barroso, J., Bombarely, A., Mueller, L., Mercado-Blanco, J. and Luque, F. (2017). Transcriptomic analysis of L. roots during the early infection process. *The Plant Genome*, 10(1), 7-10.
82. Joshi, R. (2018). A review of *Fusarium oxysporum* on its plant interaction and industrial use. *Journal of Medicinal Plants Studies*, 6(3), 112-115.
83. Karuppanapandian, T., Moon, J.C., Kim, C., Manoharan, K., Kim, W. (2011). Reactive oxygen species in plants: their generation, signal transduction, and scavenging mechanisms. *Australian Journal of Crop Science*, 5, 709–725.
84. Klinck, H. (2019). *Cereal Crops | The Canadian Encyclopedia*. [online] Thecanadianencyclopedia.ca. Available at: https://thecanadianencyclopedia.ca/en/article/cereal-crops [Accessed 1 Aug. 2019].
85. Koca, H., Bor, M., Özdemir, F. and Türkan, İ. (2007). The effect of salt stress on lipid peroxidation, antioxidative enzymes and proline content of sesame cultivars. *Environmental and Experimental Botany*, 60(3), 344-351.
86. Kosiak, B., Torp, M., Skjerve, E. and Thrane, U. (2003). The prevalence and distribution of *Fusarium species* in Norwegian Cereals: a Survey. *Acta Agriculturae Scandinavica, Section B - Soil and Plant Science*, 53(4), 168-176.

87. Kumar, P., Yadava, R., Gollen, B., Kumar, S., Verma, R. and Yadav, S. (2011). Nutritional contents and medicinal properties of wheat: A Review. *Life Sciences and Medicine Research*, 22, 12-16.
88. Kuźniak, E. and Urbanek, H. (2000). The involvement of hydrogen peroxide in plant responses to stresses. *Acta Physiologiae Plantarum*, 22(2), 195-203.
89. Labadarios, D., Mchiza, Z., Steyn, N., Gericke, G., Maunder, E., Davids, Y. and Parker, W. (2011). Food security in South Africa: a review of national surveys. *Bulletin of the World Health Organization*, 89(12), 891-899.
90. Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-685.
91. Larkins, B., Bringgs, S., Delmer, D., Dick, R., Flavell, R., Gressel, J., Habtemariam, T., Lal, R., Pell, A.N., St Leger, R.J. and Wall, R.J. (2008). Emerging technologies to benefit farmers in sub-Saharan Africa and South Asia. *National Academy Press*, 269, 1-293.
92. Lee, D. and Lee, C. (2000). Chilling stress-induced changes of antioxidant enzymes in the leaves of cucumber: in gel enzyme activity assays. *Plant Science*, 159(1), 75-85.
93. Lin, C.L., Kao, C.H. (2002). Osmotic stress-induced changes in cell wall peroxidase activity and hydrogen peroxide level in roots of rice seedlings. *Plant Growth Regulation* 37, 177–184.
94. Link, H.F. (1809). Observationes in Ordines plantarum naturales. *Dissertatio 1ma. Magazin der Gesellschaft Naturforschenden Freunde Berlin*, 3, 3-42.
95. Liu, H., Xin, Z. and Zhang, Z. (2011). Changes in activities of antioxidant-related enzymes in leaves of resistant and susceptible wheat inoculated with Rhizoctonia cerealis. *Agricultural Sciences in China*, 10(4), 526-533.
96. Liu, Y., Shi, J., Feng, Y., Yang, X., Li, X. and Shen, Q. (2012). Tobacco bacterial wilt can be biologically controlled by the application of antagonistic strains in combination with organic fertilizer. *Biology and Fertility of Soils*, 49(4), 447-464
97. Lule, D., de Villiers, S., Fetene, M., Bogale, T., Alemu, T., Geremew, G., Gashaw, G. and Tesfaye, K. (2014). Pathogenicity and yield loss assessment caused by Magnaporthe oryzaeisolates in cultivated and wild relatives of finger millet (Eleusine coracana). *Indian Journal of Agricultural Research*, 48(4), 258.
98. Magbanua, Z., De Moraes, C., Brooks, T., Williams, W. and Luthe, D. (2007). Is catalase activity one of the factors associated with maize resistance to aspergillus flavus?. *Molecular Plant-Microbe Interactions*, 20(6), 697-706.

99. Maggio, A., Miyazaki, S., Veronese, P., Fujita, T., Ibeas, J., Damsz, B., Narasimhan, M., Hasegawa, P., Joly, R. and Bressan, R. (2002). Does proline accumulation play an active role in stress-induced growth reduction?. *The Plant Journal*, 31(6), 699-712.
100. Mahajan, S. and Tuteja, N. (2005). Cold, salinity and drought stresses: An overview. *Archives of Biochemistry and Biophysics*, 444(2), 139-158.
101. Mandal, S., Mitra, A. and Mallick, N. (2008). Biochemical characterization of oxidative burst during interaction between Solanum lycopersicum and *Fusarium oxysporum* f. sp. lycopersici. *Physiological and Molecular Plant Pathology*, 72(1-3), 56-61.
102. Mason, N., Jayne, T. and Shiferaw, B. (2015). Africa's Rising Demand for Wheat: Trends, Drivers, and Policy Implications. *Development Policy Review*, 33(5), 581-613.
103. Mehdy, M., Sharma, Y., Sathasivan, K. and Bays, N. (2008). The role of activated oxygen species in plant disease resistance. *Physiologia Plantarum*, 98(2), 365-374.
104. Miller, G., Sukuki, N., Ciftci-Yilmaz, S. and Mittler, R. (2010). Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant, Cell and Environment*, 33(4), 453-467.
105. Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science*, 7, 405-410.
106. Mittler, R. and Zilinskas, B.A. (1993). Detection of ascorbate peroxidase activity in native gels by inhibition of the ascorbate-dependent reduction of nitroblue tetrazolium. *Analytical Biochemistry*, 212, 540-546.
107. Mohammadi, M. and Kazemi, H. (2002). Changes in peroxidase and polyphenol oxidase activities in susceptible and resistant wheat heads inoculated with *Fusarium graminearum* and induced resistance. *Plant Science*, 162(4), 491-498.
108. Mohammadkhani, N. and Heidari, R. (2007). Effects of water stress on respiration, photosynthetic pigments and water content in two maize cultivars. *Pakistan Journal of Biological Sciences*, 10, 4022-4028.
109. Noctor, G. and Foyer,, C. (1998). Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Physiology and Plant Molecular Biology*, 49(1), 249-279.
110. O'Donnell, K., Sutton, D., Rinaldi, M., Magnon, K., Cox, P., Revankar, S., Sanche, S., Geiser, D., Juba, J., van Burik, J., Padhye, A., Anaissie, E., Francesconi, A., Walsh, T. and Robinson, J. (2004). Genetic Diversity of Human Pathogenic Members of the Fusarium oxysporum Complex Inferred from Multilocus DNA sequence data and Amplified Fragment Length Polymorphism analyses: evidence for the recent dispersion

of a geographically widespread clonal lineage and nosocomial origin. *Journal of Clinical Microbiology*, 42(11), 5109-5120.

111. Oerke, E. (2005). Crop losses to pests. *The Journal of Agricultural Science*, 144(1), 31-43.
112. Onaga, G. and Wydra, K. (2016). Advances in plant tolerance to biotic stresses. *Plant Genomics*, 2, 1-12.
113. Ortoneda, M., Guarro, J., Madrid, M., Caracuel, Z., Roncero, M., Mayayo, E. and Di Pietro, A. (2004). Fusarium oxysporum as a multihost model for the genetic dissection of fungal virulence in plants and mammals. *Infection and Immunity*, 72(3), 1760-1766.
114. Pandey, P., Singh, J., Achary, V. and Reddy, M. (2015). Redox homeostasis via gene families of ascorbate-glutathione pathway. *Frontiers in Environmental Science*, 3, 12-19.
115. Perveen, S., Shahbaz, M., and Ashraf, M. (2010). Regulation in gas exchange and quantum yield of photosystem II (PSII) in salt stressed and non-stressed wheat plants raised from seed treated with triacontanol. *Pakistan Journal of Botany,* 42, 3073-3081.
116. Pingali, P. (2012). Green Revolution: Impacts, limits, and the path ahead. *Proceedings of the National Academy of Sciences*, 109(31), 12302-12308.
117. Rahaie, M., Xue, G.P. and Schenk, P.M. (2013) The role of transcription factors in wheat under different abiotic stresses. In: Vahdati, K and Leslie, C (eds) Abiotic Stress - Plant Responses and Applications in Agriculture. InTech, Rijeka, Croatia, 367-385.
118. Rana, A., Sahgal, M. and Johri, B. (2017). Fusarium oxysporum: Genomics, Diversity and Plant–Host Interaction. *Developments in Fungal Biology and Applied Mycology*, 1, 159-199.
119. Ray, D., Mueller, N., West, P. and Foley, J. (2013). Yield Trends Are Insufficient to Double Global Crop Production by 2050. *PLoS ONE*, 8(6), p.e66428.
120. Redondo-GÃ³mez, S., Mateos-Naranjo, E., Figueroa, M. and Davy, A. (2010). Salt stimulation of growth and photosynthesis in an extreme halophyte, Arthrocnemum macrostachyum. *Plant Biology*, 12(1), 9-87.
121. Rejeb, B.K., Abdelly, C. and Savouré, A. (2014). How reactive oxygen species and proline face stress together. *Plant Physiology and Biochemistry*, 80, 278-284.
122. Renard, J.L. and Quillec, G. (1983). Fusarium and replanting. Elements to be considered when replanting oil palm in a Fusarium zone in West Africa [Ivory Coast]. *Oléagineux*, 38, 421-427.

123. Rhodes, D. and Hanson, A. (1993). Quaternary ammonium and tertiary sulfonium compounds in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, 44(1), 357-384.
124. Rodriguez, A., Perestelo, F., Carnicero, A., Regalado, V., Perez, R., Fuente, G. and Falcon, M. (1996). Degradation of natural lignins and lignocellulosic substrates by soil-inhabiting fungi imperfecti. *FEMS Microbiology Ecology*, 21(3), 213-219.
125. Sanevas, N., Sunohara, Y. and Matsumoto, H. (2007). Characterization of reactive oxygen species-involved oxidative damage in Hapalosiphon species crude extract-treated wheat and onion roots. *Weed Biology and Management*, 7, 172-177.
126. Scandalios, J. (1993). Oxygen stress and superoxide dismutases. *Plant Physiology*, 101(1), 7-12.
127. Scandalios, J. (2005). Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. *Brazilian Journal of Medical and Biological Research*, 38(7), 995-1014.
128. Scheffer, R. (1991). Role of toxins in evolution and ecology of plant pathogenic fungi. *Experientia*, 47(8), 804-811.
129. Scherm, B., Balmas, V., Spanu, F., Pani, G., Delogu, G., Pasquali, M. and Migheli, Q. (2012). Fusarium culmorum: causal agent of foot and root rot and head blight on wheat. *Molecular Plant Pathology*, 14(4), 323-341.
130. Schippers, B. and van Eck, W.H. (1981). Formation and survival of chlamydospores in Fusarium: In Nelson PE, Toussoun TA, Cook RJ (eds) Fusarium: diseases, biology and taxonomy. Penn State University Press, University Park, 250–260.
131. ScienceAid. (2019). *Cereal Grains: The Structure and Uses of Wheat - ScienceAid*. [online] Available at: https://scienceaid.net/Economic_Botany [Accessed 25 Oct. 2019].
132. Shigeoka, S. (2002). Regulation and function of ascorbate peroxidase isoenzymes. *Journal of Experimental Botany*, 53(372), 1305-1319.
133. Singla, J. and Krattinger, S. (2016). Biotic stress resistance genes in wheat. *Encyclopedia of Food Grains*, 388-392.
134. Smirnoff, N. (1996). Botanical briefing: The function and metabolism of ascorbic acid in plants. *Annals of Botany*, 78(6), 661-669.
135. Smith, M. and Meade, B. (2019). *USDA ERS* - Who are the world's food insecure? Identifying the risk factors of food insecurity around the world. [online] Ers.usda.gov. Available at: https://www.ers.usda.gov/amber-waves/2019/june/who-are-the-world-s-

food-insecure-identifying-the-risk-factors-of-food-insecurity-around-the-world/ [Accessed 25 Sep. 2019].

136. Sudhakar, C., Lakshmi, A. and Giridarakumar, S. (2001). Changes in the antioxidant enzyme efficacy in two high yielding genotypes of mulberry (*Morus alba* L.) under NaCl salinity. *Plant Science,* 161(3), 613-619.
137. Sutherland, M.W. and Learmonth, B.A. (1997). The tetrazolium dyes MTS and XTT provide new quantitative assays for superoxide and superoxide dismutase. *Free Radical Research,* 27, 283-289.
138. Sutherland, R., Viljoen, A., Myburg, A. and van den Berg, N. (2013). Pathogenicity associated genes in *Fusarium oxysporum f. sp. cubense* race 4. *South African Journal of Science*, 109(5/6), 1-10.
139. Taiz, L. and Zeiger, E. (2010). Plant Physiology. 5th Edition, Sinauer Associates Inc., *Sunderland*, 782, 12-17.
140. Thecanadianencyclopedia.ca. (2019). *Cereal Crops | The Canadian Encyclopedia*. [online] Available at: https://thecanadianencyclopedia.ca/en/article/cereal-crops [Accessed 25 May. 2019].
141. Tilman, D., Balzer, C., Hill, J. and Befort, B. (2011). Global food demand and the sustainable intensification of agriculture. *Proceedings of the National Academy of Sciences*, 108(50), 20260-20264.
142. Torres, M. (2010). ROS in biotic interactions. *Physiologia Plantarum*, 138(4), 414-429.
143. Torres, M., Jones, J. and Dangl, J. (2006). Reactive Oxygen Species signaling in response to pathogens. *Plant Physiology*, 141(2), 373-378.
144. Valentovic, P., Luxova, M., Kolarovic, L. and Gasparikova, O. (2006). Effect of osmotic stress on compatible solutes content, membrane stability and water relations in two maize cultivars. *Plant Soil and Environment,* 52, 186-191.
145. Velikova, V., Yordanov, I. and Edreva, A. (2000). Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. *Plant Science,* 151, 59-66.
146. Verbruggen, N. and Hermans, C. (2008). Proline accumulation in plants: a review. *Amino Acids*, 35(4), 753-759.
147. Wagner, A., Michalek, V. and Jamiolkowska, A. (2006). Chlorophyll fluorescence measurements as indicators of fusariosis severity in tomato plants. *Agronomy Journal*, 4, 461-464.

148. Wang, W., Vignani, R., Scali, M. and Cresti, M. (2006). A universal and rapid protocol for protein extraction from recalcitrant plant tissues for proteomic analysis. *Electrophoresis*, 27, 2782-2786.
149. Warzecha, T., Adamski, T., Kaczmarek, Z., Surma, M., Goliński, P., Perkowski, J., Chełkowski, J., Wiśniewska, H., Krystkowiak, K. and Kuczyńska, A. (2010). Susceptibility of hulled and hulless barley doubled haploids to *Fusarium culmorum* head blight. *Cereal research communications*, *38*(2), 220-232.
150. Warzecha, T., Skrzypek, E. and Sutkowska, A. (2015). Effect of *Fusarium culmorum* infection on selected physiological and biochemical parameters of barley (Hordeum vulgare L.) DH lines. *Physiological and Molecular Plant Pathology*, 89, 62-69.
151. Wildermuth, G., Tinline, R. and McNamara, R. (1992). Assessment of yield loss caused by common root rot in wheat cultivars in Queensland. *Australian Journal of Agricultural Research*, 43(1), 43.
152. www.fao.org. (2019). *SOFI 2019 - The State of Food Security and Nutrition in the World*. [online] Available at: http://www.fao.org/state-of-food-security-nutrition/en/ [Accessed 9 Oct. 2019].
153. Yancey, P.H. (1994). Compatible and counteracting solutes. In: Strange, K. (Ed.), Cellular and molecular physiology of cell volume regulation. CRC Press, Boca Raton, FL, 81-109.
154. Yang, S., Li, X., Chen, W., Liu, T., Zhong, S., Ma, L., Zhang, M., Zhang, H., Yu, D. and Luo, P. (2016). Wheat resistance to Fusarium head blight is associated with changes in photosynthetic parameters. *Plant Disease*, 100(4), 847-852.
155. Ye, S., Zhou, Y., Sun, Y., Zou, L. and Yu, J. (2006). Cinnamic acid causes oxidative stress in cucumber roots and promotes incidence of Fusarium wilt. *Environmental and Experimental Botany*, 56(3), 255-262.
156. Zhang, F., Guo, J.K., Yang, Y.L., He, W.L., and Zhang, L.X. (2004). Changes in the pattern of antioxidant enzymes in wheat exposed to water deficit and rewatering. *Acta Physiologiae Plantarum*, 26(3), 345–352.
157. Zhao, C., Wang, P., Si, T., Hsu, C., Wang, L., Zayed, O., Yu, Z., Zhu, Y., Dong, J., Tao, W. and Zhu, J. (2017). MAP kinase cascades regulate the cold response by modulating ICE1 protein stability. *Developmental Cell*, 43(5), 618-629.