Physio-biochemical characterization of two wheat cultivars to *Fusarium proliferatum* infection

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

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KEYWORDS

Biotic stress

Fusarium

Fusarium proliferatum

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Plant growth parameters

Photosynthetic metabolism

Osmoprotectants

Reactive oxygen species

Lipid peroxidation

Cell death

Antioxidant enzymes

Seed germination

Mineral nutrients

ABSTRACT

Wheat is a key global commodity in terms of acreage and tradeable value and as a staple in household diets. Many factors including biotic stress conditions have detrimental effects on global wheat production and yield. The increasing prevalence of biotic stress inflicted by fungal species such as *Fusarium* has significantly reduced yield and quality of cereal crops thus, threatening sustainable agriculture and food security. Interactions between wheat and *Fusarium* spp. such as *Fusarium proliferatum* triggers the accumulation of reactive oxygen species (ROS) to levels toxic to the plant thus leading to oxidative damage and ultimate cellular death. In order to maintain redox homeostasis, plants rely on ROS-scavenging antioxidants (enzymatic and non-enzymatic) to control ROS molecules to levels less toxic to plants. This study investigated the impact of *F. proliferatum* on the physio-biochemical responses of two wheat cultivars (SST 015 and SST 088). Changes in seed germination, growth, biomass, chlorophyll and mineral contents were monitored. Furthermore, changes in ROS accumulation and antioxidant enzyme activity was measured in the shoots of both wheat cultivars.

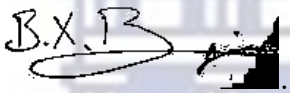
The results showed that *F. proliferatum*-induced stress restricted seed germination, plant growth, biomass, and chlorophyll metabolism of both cultivars with the most significant reduction observed for SST 015. An increase in ROS accumulation (as seen for superoxide and hydrogen peroxide) was observed in the shoots and roots of both cultivars. The increase in ROS resulted in an increase in lipid peroxidation (indicated by increased MDA content). However, the increase in ROS-induced oxidative damage were more pronounced in SST 015 relative to SST 088. Furthermore, *F. proliferatum* differentially altered the antioxidant enzyme activity of both cultivars. However, contrasting responses in antioxidant enzyme activities was observed for both wheat cultivars.

Based on the results presented here we suggest that SST 088 to be more resilient to *F. proliferatum* infection compared to SST 015. Interestingly, wheat responses to *F. proliferatum* were proven to be concentration dependent, which suggest that different pathogen concentrations trigger different pathways and defence mechanisms, which bring about the differential responses observed in the physiology, mineral nutrient status and biochemistry of the plants.

DECLARATION

I declare that "***Physio-biochemical characterization of two wheat cultivars to Fusarium proliferatum 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.

Bonke Xolisa Bangani **December 2019**

Signed……  ……..

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LIST OF ABBREVIATIONS

µg	Microgram
µl	Microliter
ANOVA	Analysis of variance
APX	ascorbate peroxidase
AsA	ascorbic acid
BSA	bovine serum albumin
CAT	catalase
Cu/Zn – SOD	copper zinc superoxide dismutase
dH_2O	distilled water
EDTA	ethylenediaminetetraacetic acid
Fe – SOD	iron superoxide dismutase
FW	Fresh weight
H_2O_2	Hydrogen peroxide
K_2HPO_4	Potassium phosphate dibasic
KCN	Potassium cyanide
KH_2PO_4	Potassium phosphate monobasic

KI	Potassium iodide
MDA	malondialdehyde
mg	Miligram
ml	Mililitre
Mn – SOD	manganese superoxide dismutase
NBT	nitroblue tetrazolium
PAGE	polyacrylamide gel electrophoresis
PDA	Potato Dextrose Agar
PVP	polyvinylpyrrolidone
ROS	reactive oxygen species
SOD	superoxide dismutase
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TEMED	N,N,N′,N′ - Tetramethylethylenediamine

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CHAPTER ONE

Literature Review

1.1. Introduction

An active debate on new farming practices suggests that sustainable agriculture could meet the current challenges faced by conventional agricultural practices. These challenges such as production that is simultaneously economically beneficial, environmentally friendly and socially fair, organic fertilisation, drip irrigation, split fertilisation, biological pest control, reduced tillage, cultivar choice and producing larger food quantities (Wezel *et al.*, 2013). Sustainable agriculture is thus not only important but necessary to meet the above challenges and to minimize or eliminate heavy use of agrochemicals for fertilization and pest management, which are environmentally destructive and lead to loss of biodiversity (Lithourgidis *et al.*, 2011). Examples of crop species that are put forward as the driving force to sustainable agriculture include cereals such as wheat, corn and barley and legumes such as peas, common vetch, climbing beans, common bean and lathyrus (Hauggaard-Nielsen *et al.*, 2001; Ghanbari-Bonjar and Lee, 2002; Schmidt *et al.*, 2003; Chen *et al.*, 2004; Hauggaard-Nielsen *et al.*, 2006; Lithourgidis *et al.*, 2007; Geren *et al.*, 2008; Javanmard *et al.*, 2009; Lithourgidis and Dordas, 2010). Other examples of crops species include shrubs and trees or annuals and perenials (Lithourgidis *et al.*, 2011). The above examples are taken from intercropping, which is a method of sustainable agriculture, which could meet the challenges mentioned above.

Lesk *et al.*, (2016) showed through estimation that the national agricultural production had decreased by an average of (9-10%) worldwide. In Africa, agricultural production was disturbed and thus decreased. Examples of countries showing this decline include Ethiopia (Food Security Information Network, 2018) and Uganda (FAO, 2018a; FAO, 2018b). South Africa is no exception to this global trend, the estimated volume of agricultural production

decreased by 2.3% compared to the previous year of 2016/17. Furthermore, the latest report on Trends in the Agricultural Sector (2018) indicate that field crop production decreased by 9.8%. To counteract this decline in agricultural productivity, good practice should be guided by the need for sustainable intensification of agricultural production to raise productivity while conserving natural resources (Regional overview of food security and nutrition, 2018), which is the idea behind sustainable agriculture.

The decline in agricultural production is due to a number of factors, which can be summed up as biotic and abiotic factors. The abiotic factors include drought and temperature extremes while the biotic factors include threats such as parasites, pests and pathogens, which usually have significant impacts on the crop yields (Fandohan *et al.,* 2003; Pereira *et al.,* 2011). When plants are affected by any of the previously mentioned-factors that can lead to plant stress and disease, which have a negative impact on plant growth and development and will ultimately lead to economic loses as a result of reduced yield production (Pereira *et al.,* 2011), as reported by the sources above. Therefore, to mitigate the prevalence of undernourishment in almost all sub-regions of Africa, which has reached levels of 22.8%, and is double the global level at 11%, and food insecurity caused by the growing population estimated to be 9.1 billion in 2050, an increase in food production is necessary (Wezel *et al.,* 2013).

Numerous contrasting agricultural production practices exist (Wezel *et al.,* 2013), which can increase food production as necessary. However, the underpinning science which informs these practices must be understood. For example, how pathogens cause stress, disease and death in plants, which will give light to which crop varieties should be favoured over others. Therefore, in this chapter, the interaction and impact of disease-causing pathogens on crop production will be reviewed to provide a comprehensive study that will offer or form the basis to offer plant protection solutions and choice of cultivar to cultivate.

1.2. The host: *Triticum aestivum* L.

Wheat is a cereal grain which is believed to have evolved in the Near East, in the geographical regions occupied by countries such as Syria, Turkey, Afghanistan, Iraq and Iran. To be more specific, the grain originated from wild grasses native to the arid regions between Armenia and the Caspian Sea in Western Asia, between 8 000 and 10 000 years ago. There is archaeological evidence of remains of domesticated wheat in Ali Koshi in Iranian Khusistan, dating back to 6 500 BC ("Production guideline for wheat," 2018; "Wheat", 2018).

In South Africa wheat was introduced in the 17th century by Jan van Riebeeck in 1652, in the Cape and over time spread all over the country. It is currently cultivated in three distinct climatic regions as follows (Figure 1.1); in the Mediterranean climate of the Western Cape Province (dryland spring wheat), adjacent to major rivers in summer rainfall areas (irrigated spring wheat) and dryland conditions with stored soil moisture from summer and autumn rains (winter/intermediate wheat).

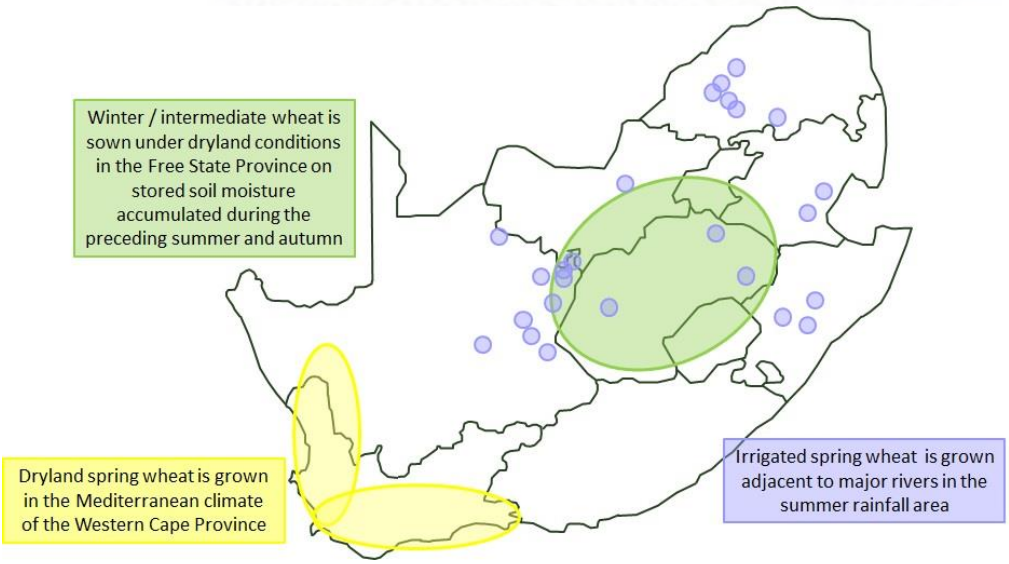


Figure 1.1. The wheat map of South Africa ("Wheat", 2018)

1.2.1 The importance of wheat

Wheat is the most cultivated and dominant food crop globally, as it grows in moderate climatic regions of the world. Furthermore, wheat contributes largely to food security, especially in sub-Saharan countries like South Africa. Several billion people in the world rely on wheat for a substantial part of their diet. Moreover, wheat provides nutrients that promote human health, including 10 of the 20 amino acids found in proteins, and minerals such as iron and zinc (Shewry, 2009; Wheat Initiative, 2013). To date iron deficiency affects about two billion people in developing countries (Stoltzfus and Dreyfuss, 1998) with more than 800 000 child deaths in parts of Africa and Asia (Micronutrient Initiative, 2006). Wheat products include cereals, couscous, bread, spaghetti, etc. Therefore, wheat is an essential food and plays a major role in food security, human health and disease prevention, not only in South Africa but the rest of the world. Despite its nutritional and health benefits, wheat is prone to environmental constraints such as biotic and abiotic stresses that negatively affect crop production and yield.

1.3 The pathogen: *Fusarium proliferatum*

1.3.1. *Fusarium*

Although *Fusarium* species are soil-borne organisms, they are also air-borne, water-borne and present in other substrates. The genus is responsible for causing wilts, rots, blights and cankers of many crops including field, forest, ornamental and horticultural crops in natural ecosystems and agricultural systems. A countless number of species of this genus can be recovered from plants and soils worldwide where they act as endophytes, saprophytes and pathogens (Geiser *et al.*, 2004). Some species (*F. graminearum and F. verticilloides*) mostly infect cereals and have a narrow host range; while some species infect both dicotyledonous and monocotyledonous plants (*F. oxysporum*) and have a broad host range. Many of the species in this genus are known to be phytopathogens and they produce toxic secondary metabolites

known as trichothecens and fumonisins. The effect of the pathogens on plants and their toxins have a negative impact on plant growth, development and yield production (Matny, 2015).

1.3.2. *Fusarium proliferatum*

Fusarium proliferatum is a polyphagous (Gorna *et al.,* 2016; Gorna *et al.,* 2017) and globally occurring plant-infecting soil fungal pathogen (Galvez *et al.,* 2017). It is a fungal species within the *Fusarium* genus, which has had numerous changes in its taxonomy, due to complexities and diversity of the species within this genus. This problem led to a difficulty in finding a precise taxonomic system for this genus (Watanabe *et al.,* 2011). The genus was first classified as Fusisporium by Link in 1909. Then in 1935, Wollenweber and Reinking (1935) published a study which became the standard of the *Fusarium* genus taxonomy. This study introduced "sections" to classify the species and the sections were divided according to the presence or absence of chlamydospores, the location of the chlamydospores-intercalary or terminal, the shape of macroconodia and the shape of basal foot cells on the macroconodia, the presence or absence of microconidia and the shape of microconidia. In 2011, a study which classified the genus into "clades" was published by Watanabe *et al.,* (2011) and the clades were based on maximum likelihood (ML) trees deduced from rDNA cluster (including 18S rDNA, ITS1, 5.8S rDNA and 28S rDNA) (Figure 1.2); b-tub (Figure 1.3); EF-1α (Figure 1.4) and lys2 (Figure 1.5).

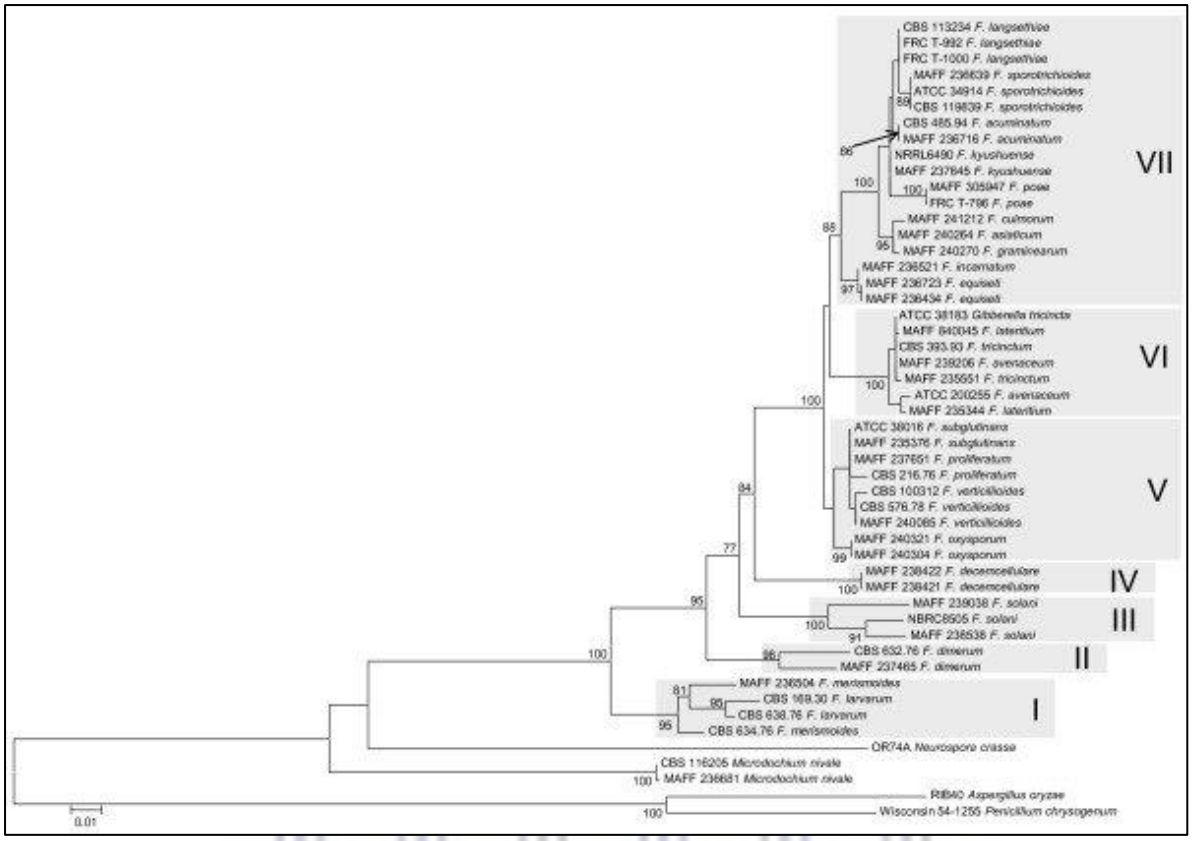


Figure 1.2. ML trees for the *Fusarium* genus and related genera inferred from the rDNA cluster including 18S rDNA, ITS1, 5.8S rDNA and 28S rDNA. The GTR + I + Γ model was used as the model for nucleotide substitution. Branch lengths are proportional to the estimated number of nucleotide substitutions. The BP values over 75% are displayed on the nodes (BP; 1000 replicates) (Watanabe *et al.*, 2011).

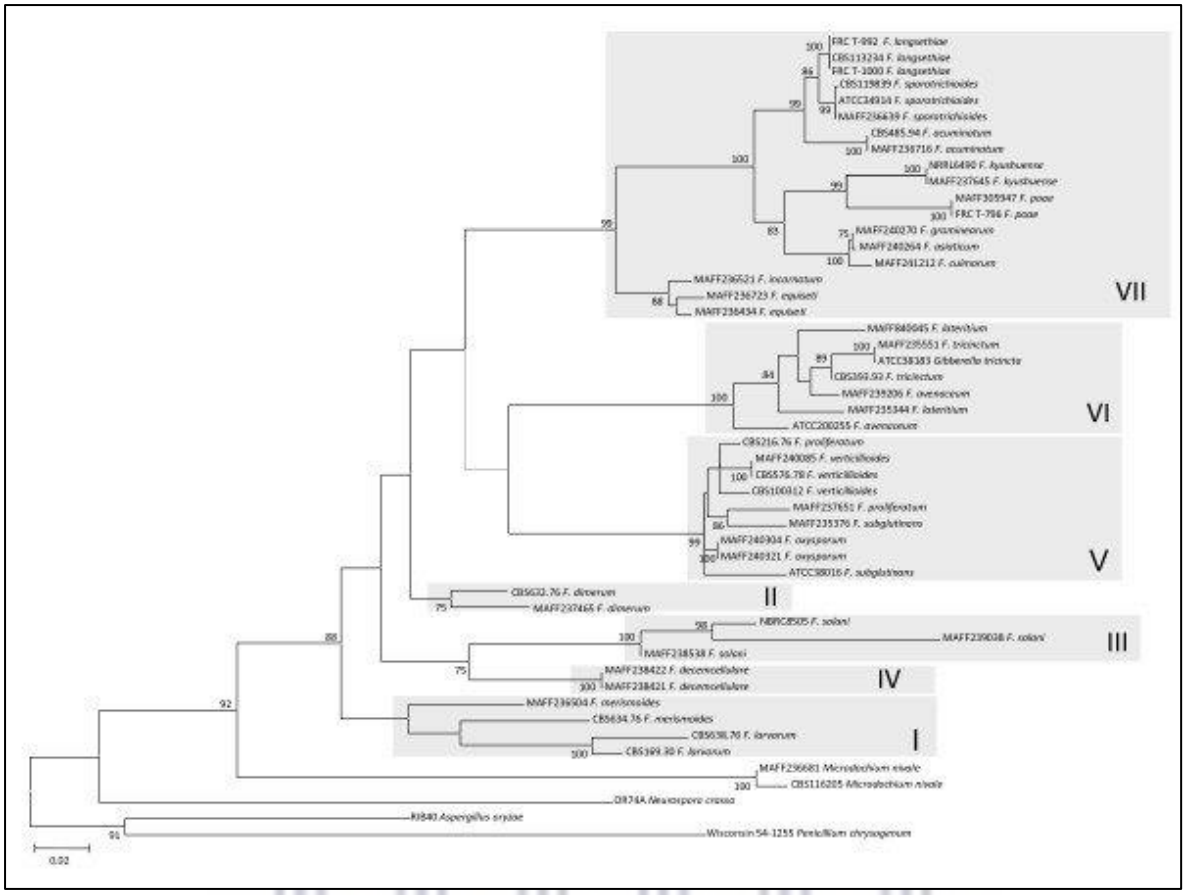


Figure 1.3. Maximum likelihood trees for the *Fusarium* genus and related genera inferred from b-tub. The GTR + I + Γ model was used as the model for nucleotide substitution. Branch lengths are proportional to the estimated number of nucleotide substitutions. Each codon position was analysed separately. The BP values over 75% are displayed on the nodes (BP; 1000 replicates) (Watanabe *et al.*, 2011).

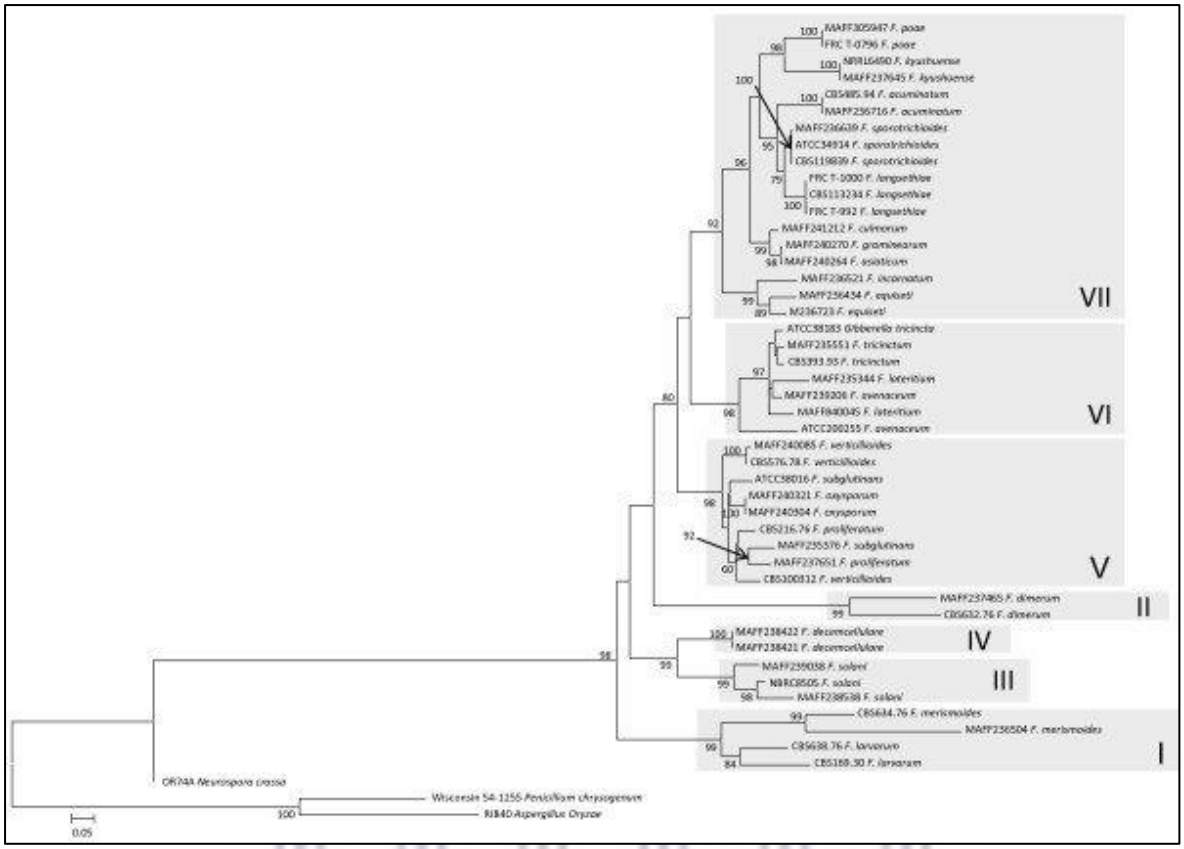


Figure 1.4. Maximum likelihood trees for the *Fusarium* genus and related genera inferred from EF-1α. The GTR + I + Γ model was used as the model for nucleotide substitution. Branch lengths are proportional to the estimated number of nucleotide substitutions. Each codon position was analysed separately. The BP values over 75% are displayed on the nodes (BP; 1000 replicates) (Watanabe *et al.*, 2011).

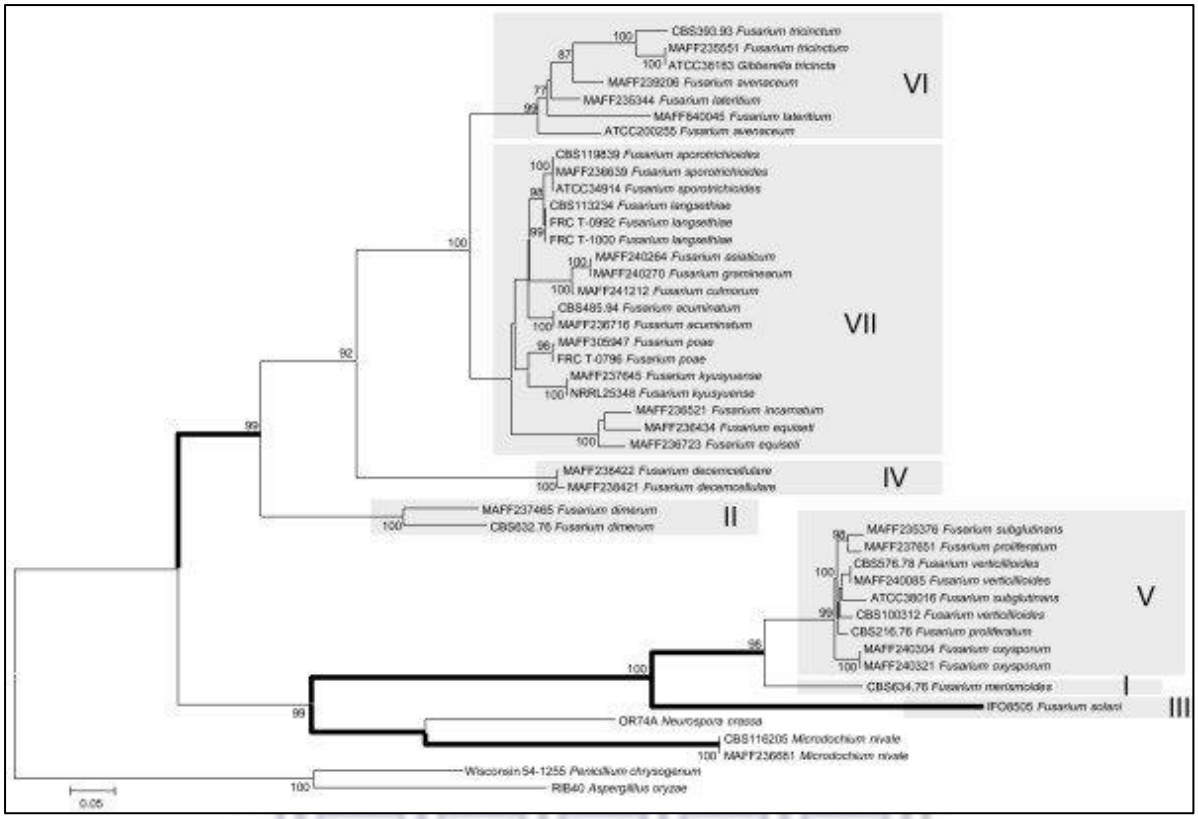


Figure 1.5. Maximum likelihood trees for the *Fusarium* genus and related genera inferred from lys2. The GTR + I +Γ model was used as the model for nucleotide substitution. Branch lengths are proportional to the estimated number of nucleotide substitutions. Each codon position was analysed separately. The BP values over 75% are displayed on the nodes (BP; 1000 replicates). The branches with bold lines indicate the lineages in which positive selection has occurred with the p-value under the null hypothesis that the ω (synonymous substitutions per synonymous site/non-synonymous substitutions per non-synonymous site) of the positively selected sites is equal to 1.0. ($p < 0.001$) (Watanabe *et al.,* 2011).

F. proliferatum is a fungal species within the Liseola section or Gibberella fujikuroi species complex, located within Clade 5, within the genus *Fusarium*. The genus is part of the family Nectriaceae, within the Ascomycota phylum of the Fungi kingdom.

Moreover, *F. proliferatum* also produce mycotoxins and biologically active metabolites. These biologically active metabolites include enniatin, fusarin, moniliformin, beauvericin, fusaproliferin, and fusaric acid (Marasas *et al.,* 1986; Ritieni *et al.,* 1995; Bacon *et al.,* 1996; Herrmann *et al.,* 1996; Moretti *et al.,* 1996; Desjardins *et al.,* 2000; Leslie *et al.,* 2005a; Leslie *et al.,* 2005b).

1.4. Wheat and *Fusarium* interactions

There are numerous *Fusarium* species that can infect wheat. Hence wheat interaction with *Fusarium* species is well documented as it induces plant stress responses, which involves many reactions including the activation of stress-related proteins involved in plant stress responses to pathogen infection. In particular, these reactions include the activation and/or production of hormones, reactive oxygen species (ROS), kinases and transcription factors, which ultimately lead to plant responses to stress (Parry *et al.,* 1995; Battalico, 1998; Desjardins *et al.,* 2007; Amato *et al.,* 2015).

1.4.1 Activation of phytohormones

Primary reactions in response to biotic stresses include the activation and/or synthesis of phytohormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) during pathogen infection (Fujita, *et al.,* 2006; Rejeb, Pastor and Mauch-Mani, 2014). These phytohormones play a remarkable role in response to stresses and plant growth and development. Furthermore, phytohormones accumulate and activate pathways, which synergistically or antagonistically interact with each other and a reprogramming of the genetic machinery results in adequate defence reactions and an increase in plant tolerance in order to minimize the biological damage caused by the stress (Fujita, *et al.*, 2006). Moreover, SA provides resistance against the biotrophic pathogens (Durrant and Dong, 2004) while JA and ET provide resistance against necrotrophs. (Glazebrook, 2005).

Under infection, JA and ET provided resistance against *Fusarium* Head Blight (FHB) in the wheat cultivar "Sumai 3". Furthermore, ET's role in plant defence depends on the pathogen; ie. ET may promote resistance or susceptibility of the plant (Bent *et al.,* 1992, as cited in Hammerschmidt & Nicholson, 2000; Hoffman *et al.,* 1999; Thomma *et al.,* 1999). For example, ET can trigger cell death by initiating plant organ senescence which leads to cell wall softening or dissolving (Li and Yen, 2008). Contrary to this, ET was observed to probably

reduce disease development caused by necrotrophic pathogens (Wang *et al.*, 2002). SA on the other hand is a known Systemic Acquired Resistance (SAR) Trigger (Li and Yen, 2008). In addition, SA induces the expression of genes encoding pathogenesis-related (PR) proteins (Duba *et al.*, 2018), for plant defence-related reactions.

1.4.2 Production of reactive oxygen species

With regards to ROS generation, this response is regarded as a shared response between biotic and abiotic stress (Apel and Hirt, 2004; Torres and Dangl, 2005). The stress-induced reactions occur either independently (reactions specific to either biotic or abiotic stress only) or they overlap between biotic and abiotic stress. Furthermore, the reactions can also be contrasting between biotic and abiotic stress.

Since plants are eukaryotes, they have mitogen-activated protein (MAP) kinase (MAPK) which is involved in sundry cellular processes including stress responses. In particular, in plants, MAPK is involved in abiotic and biotic stress responses, hormone responses and ROS signalling (Nakagami *et al.*, 2005; Jonak, *et al.*, 2002). Therefore, when a plant is subjected to such stress, the gene(s) modulated by this kinase will be more expressed compared to their expression under normal conditions.

Plant cellular metabolism normally produce many products, including reactive oxygen species (ROS). ROS are produced as a result of aerobic metabolism. In plant cells, ROS are formed when electrons leak from the mitochondria, plasma membranes or chloroplast into oxygen, or ROS may be formed in different cellular compartments, as a by-product of numerous metabolic pathways (Foyer and Harbinson, 1994; Foyer, 1997; Heyno *et al.*, 2011). However, when a plant suffers from biotic and/or abiotic stress, ROS are produced in excessive amounts. Then the accumulation of ROS causes progressive oxidative damage and ultimately cell death (Figure 1.7). In spite of their destructive functions, ROS are nevertheless secondary messengers

playing a role in a number of cellular processes (Figure 1.6). Therefore, the function of ROS depends on the equilibrium between ROS production and their scavenging; i.e. whether ROS will function as the cause of oxidative damage or signalling molecules (Sharma *et al.*, 2012).

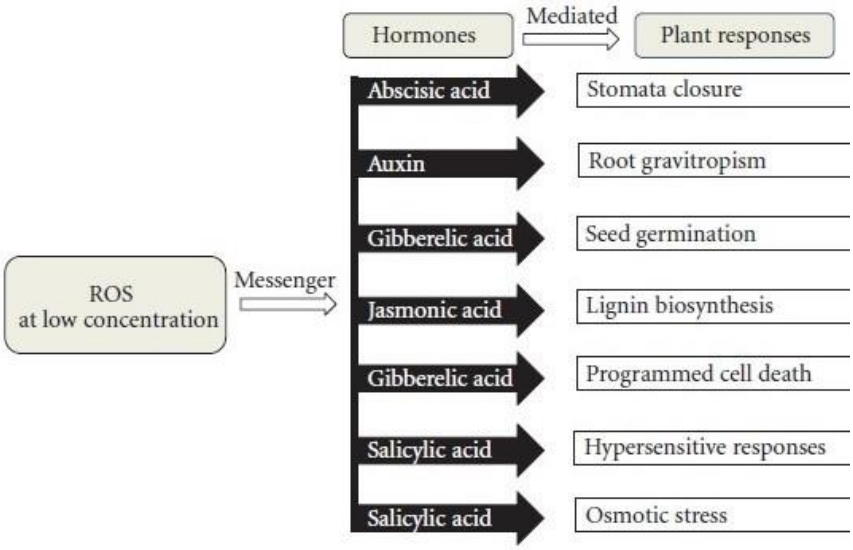


Figure 1.6. ROS at low concentrations functioning as second messengers in various plant responses (Sharma *et al.*, 2012).

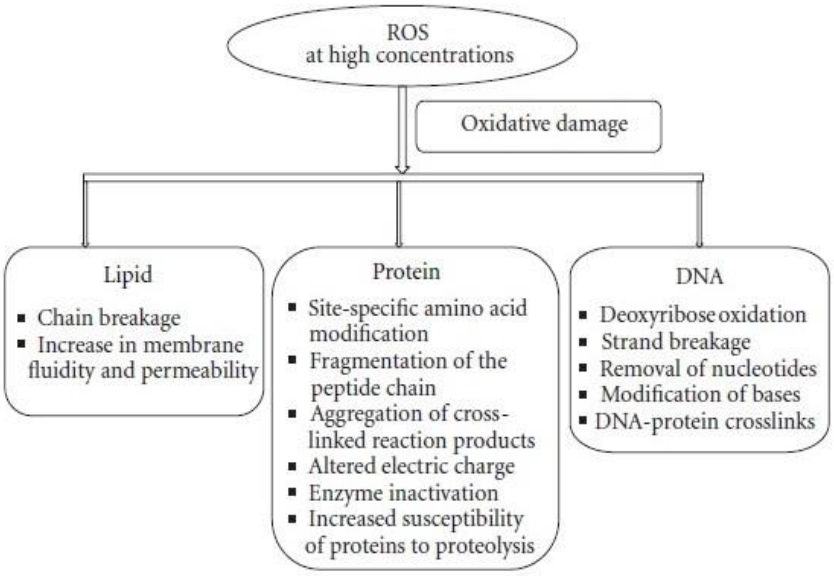


Figure 1.7. ROS at high concentrations causing oxidative damage to DNA, proteins and lipids (Sharma *et al.*, 2012).

1.5. Pathogen-induced plant diseases

Plants exist in the open/natural environment and thus are constantly confronted with different kinds of stresses, which can be categorised as biotic and abiotic stresses. Stress is when the plant is not healthy; i.e. not able to perform its physiological functions to the best of its genetic potential. Furthermore, stress can lead to disease and a plant is said to be diseased when its cells or part of the plant cannot perform its function, due to biological or environmental factors that inhibit, alter or disrupt cell function, which leads to cell malfunction or death (Agrios, 2005).

Fusarium species are known to be causal agents of cereal diseases (Bottalico, 1998). In particular, *Fusarium proliferatum* is known to infect a wide range of plants including cereals, fruits, legumes and ornamental plants. These include head blight and kernel black point disease in wheat (Conner *et al.*, 1996; Desjardins *et al.*, 2007; Amato *et al.*, 2015), crown and root rot

in asparagus (Elmer, 1990; Elmer, 2015), leaf spot disease in *Aloe vera* (Avasthi *et al.*, 2018), bulb rot in garlic (Dugan *et al.*, 2003; Galvez *et al.*, 2017), rice bakanae disease in rice (Desjardins *et al.*, 1997; Rong *et al.*, 2018), root rot in soybean (Hwang *et al.*, 2015), rapid death and complete dryness of the palm in date palm (Abdalla *et al.*, 2000; Ibrahim *et al.*, 2016) and leaf spot in tomato (Gao *et al.*, 2016).

1.6. Plant defence strategies against ROS-induced oxidative stress

Plant defence strategies include enzymatic and non-enzymatic defence systems. When a plant is exposed to infection by a pathogen and is thus under biotic stress, the stress will induce an expedited production of reactive oxygen species (ROS). These include superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH•), alkoxy radical (RO•), peroxy radical (ROO•), singlet oxygen (O^2) and organic hydroperoxide (ROOH) (Torres, 2010; Fujita *et al.*, 2006).

The over amassment of ROS molecules in plant cells results in oxidative stress, which ultimately cause cellular and molecular damage (Blokhina *et al.*, 2003). To counter this plants activate ROS-scavenging mechanisms that metabolize ROS to levels less toxic to plants. Furthermore, for plants to effectively counteract ROS toxicity, a balance between the generation and catabolism of ROS needs to be established as an imbalance will result in physiological challenges that lead to oxidative stress. Therefore, plants protect their cells from oxidative damage resulting from oxidative stress by activating their antioxidant defence systems. These defence systems include non-enzymatic antioxidant molecules (such as glutathione and ascorbic acid), and enzymatic antioxidant molecules such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) (Dietz, 2003).

Moreover, upon stress the plant triggers sensory molecules, which in turn activate downstream responses including changes in cytoplasmic $Ca^{2+,}$ protein signalling and lipid signalling pathways leading to stress-responsive gene expression, protein changes and physiological changes (Apel and Hirt, 2004). However, when these downstream responses are not sufficient to activate antioxidative mechanisms inside plant cells to eliminate or detoxify harmful molecules such as ROS, the plant cells will be destroyed leading to overall loss of cellular function (Lamb and Dixon, 1997).

1.6.1. Non-enzymatic antioxidant defence systems

The non-enzymatic defence systems involve the following molecules: glutathione (γ-glutamyl-cysteinyl-glycine, GSH) and ascorbate (AsA), including tocopherol, phenolic compounds, and carotenoids (Munne-Bosch and Alegre, 2002). AsA and GSH are found in the cytosol, chloroplast, peroxisomes, apoplast and mitochondria. Their basic role is the detoxification of H_2O_2, however, they also can scavenge $^1O_2$, O^{2-} and OH• (Arora *et al.*, 2002; Asada, 2006). Furthermore, tocopherols are synthesized in the plastids, specifically in the envelopes of plastids and then stored in the plastoglobuli of the stroma (Lichtenthaler *et al.,* 1981). Moreover, tocopherols and β-carotene are lipid-soluble molecules that play a role in the membrane structure stability by removing lipid peroxyl radicals and oxygen free radicals (Arango and Heise, 1998; Triantaphylides *et al.,* 2008).

Tocopherols can diffuse laterally into the plane of the membrane, which allows them to react with peroxyl radicals that form during lipid peroxidation to form tocopheroxyl radicals (Munne-Bosch and Alegre 2002). Tocopheroxyl radicals can be reduced by the water-soluble metabolites, ascorbate and glutathione in a tocopherols regeneration process (Munne-Bosch and Alegre, 2002).

1.6.2. Enzymatic antioxidant defence systems

These enzymatic antioxidant molecules detoxify or inactivates ROS into harmless or less harmless substances. Superoxide dismutase (SOD) detoxifies O_2^- to produce H_2O_2. Ascorbate peroxidase (APX) and catalase (CAT) then detoxify H_2O_2 to produce water. These biological responses to stress are not singular pathways, they are complex circuits involving multiple pathways which involve additional cofactors and signalling molecules that regulate specific responses to environmental stimuli (Sewelam *et al.,* 2016).

In this review, not all the antioxidant molecules will be discussed. The only antioxidant molecules that will be covered are those used in the scope of the study; i.e. SOD and two peroxidases (e.g. POD and APX)

1.6.2.1 Superoxide dismutase (SOD)

SOD is a metalloenzyme and it catalyses the dismutation of superoxide to oxygen and hydrogen peroxide. SOD plays a central role in defence against oxidative stress in all aerobic organisms (Scandalios, 1993). In plants there are three reported izoenzymes of SOD, namely; iron SOD (Fe-SOD), copper/zinc SOD (Cu/Zn-SOD) and manganese SOD (Mn-SOD) (Fridovich, 1989; Racchi *et al.,* 2001). Fe- SOD is localized in chloroplasts, while MnSOD is localized in mitochondria (Jackson at al., 1978). On the other hand Cu/Zn-SOD occurs in three isoforms, localised in the cytosol, chloroplast, peroxisome and mitochondria (Bowler, Van Montagu and Inz´e, 1992; Bueno *et al.,* 1995).

In plants exposed to stress, SOD activity has been reported to increase (Sharma and Dubey, 2005; Mishra, Jha and Dubey, 2011). Furthermore, increased activity of SOD is often correlated with increased tolerance of the plant against environmental stresses. Hence an overproduction of SOD has been reported to result in enhanced oxidative stress tolerance in plants (Gupta *et al.,* 1993),

1.6.2.2. Ascorbate peroxidase (APX)

APX is a constituent of Class I of the super family of heme peroxidases (Welinder, 1992) and is modulated by H_2O_2 and redox signals (Patterson and Poulos, 1995). This antioxidant molecule plays a crucial role in the control of intracellular ROS levels and is a key constituent of the AsA-GSH cycle (Welinder, 1992). In higher plants, it has been reported that APX has five enzymatically and chemically different isoenzymes (based on amino acid sequences) localised in subcellular compartments. These are stromal, mitochondrial, cytosolic, peroxisomal, and thylakoidal isoforms (Nakano and Asada, 1987; Madhusudhan *et al.*, 2003; Sharma and Dubey, 2004). APX has been observed to have a much higher affinity for H_2O_2 than CAT, making it a much more efficient scavenger of H_2O_2 when plants are suffering from stress. (Wang, Zhang and Allen, 1999). Under stressful conditions, a more enhanced activity of APX has been reported (Sharma and Dubey, 2005; Sharma and Dubey, 2007; Han, Liu and Yang, 2009; Maheshwari and Dubey, 2009).

1.6.2.3 Peroxidase (POD)

Peroxidases are metalloproteins containing a heme prosthetic group called heme proteins that catalyse the oxidation of phenolic substrates through the associated reduction of hydrogen peroxide in the peroxidase cycle (Pourcel *et al.*, 2006). PODs are classified according to their sub-cellular localisation. Class I POD is intracellular while calls III POD is localised in the apoplast (Takahama, 2004; Passardi *et al.*, 2004).

PODs are involved in numerous physiological processes throughout the life cycle of the plant, possibly due to the high number of enzymatic isoforms (isoenzymes) and to the versatility of their enzyme-catalysed reactions (Passardi *et al.*, 2005). Furthermore, plant PODs play a role in auxin metabolism, lignin and suberin formation, cross-linking of cell wall components, phytoalexin synthesis, and the metabolism of ROS (Almagro *et al.*, 2008).

1.7. Nutrient uptake and availability (effect of the pathogen on available nutrients)

In general, plants that lack an adequate amount of nutrients will have the least resistance to pathogens. However, that tolerance can be improved by providing the deficient nutrients. Furthermore, an adequate supply of nutrients will have a relatively small impact on plant resistance in highly tolerant and intolerant cultivars (extremes). Whereas in moderate (moderately susceptible and partially resistant) cultivars, there will be a substantial impact on plant resistance. For example, the use of fertilisers at different concentrations of constituents directly affects the development, composition and growth of plants. At the same time, the fertilisers also affect microbial activity in the soil and rhizosphere, which indirectly induces cascading and secondary effects on plant resistance and tolerance to pathogens that affect the roots and shoots (Huber *et al.*, 2012). Therefore, the use of nutrients in plant-pathogen interaction has a huge impact in disease tolerance, resistance and susceptibility and thus has a big impact on agriculture at large.

Traditional agricultural systems as practiced today have many problems that raise serious concerns such as environmental degradation, food safety and pesticide resistance. These problems are due to the techniques used to counteract pathogen infection, which significantly reduce the yield of crops and thus result in economic losses and food security problems. These techniques include but are not limited to the use of environmentally harmful chemicals to eliminate pathogens in crops (Dordas, 2008). The above problems have dictated investigations on alternative methods to combat plant diseases caused by pathogens. One of these alternative methods is the supply of adequate nutrients to crops/plants. Nutrients play a significant role in plant diseases. Plants can develop resistance or tolerance to disease when they receive a balanced diet of sufficient and adequate nutrients (Gupta *et al.*, 2017).

According to Graham and Webb (1991), plant disease resistance is the ability of the host to limit the penetration, development and reproduction of the invading pathogens. While tolerance

is defined as the ability of the host plant to maintain its normal growth and development under pathogen-influenced attack (Huber *et al.*, 2012).

Although plant diseases can reduce nutrient distribution, availability, uptake and utilisation by the host plant, through soil-borne pathogens inhibiting root growth and development and thus impairing root activity which leads to an inability of roots to acquire nutrients from the soil. Nutrients on the other hand can make a plant tolerant, resistant or susceptible to pathogens. There are three main mechanisms that can increase plant resistance to pathogens. Firstly, it is the restriction of nutrient transfer to the pathogen which it requires for growth and development. Secondly, the biochemical and physiological changes that lead to higher production of inhibitory or repelling substances and lastly, the changes in anatomy (e.g. a higher degree of lignification and/or silification) (Huber *et al.*, 2012).

The relationship between host and fungal pathogen in plant-pathogen interaction is that of parasitism, defined as a condition where one organism benefits while the other suffers. To fully understand this relationship, an understanding of how the fungus secures its nutrients is necessary. However, most studies in plant-pathogen interactions have been focusing more on understanding pathogenicity, virulence factors, avirulence factors, toxins, cell wall-degrading enzymes and other mechanisms with a direct link on how the pathogen gains access to its host (Divon and Fluhr, 2006). Therefore, it is one of the points of this study to contribute to and expand present knowledge on this less studied aspect of plant-pathogen interaction.

Plant pathogens are classified according to their pathogenic lifestyle, which is based on their metabolic dependency on their host. They are classified into biotrophic, necrotrophic and hermibiotrophic. Biotrophic pathogens are those that are totally dependent on extracting nutrients on living cells of the host to complete their life cycle. The extraction process is done by means of haustoria (specialised infection structures) through differentiation (Mendgen and

Hahn, 2002). In contrast, necrotrophic pathogens are organisms that infect living cells and kill them (sometimes via the plant's programmed cell death) then extract nutrients from the dead cells. Necrotrophs possess no haustoria (Lewis, 1973). Lastly, hermibiotrophic pathogens are pathogens that begin with a biotrophic phase and follows up with a necrotrophic phase to complete their life cycle (Perfect & Green, 2001). These pathogens interact with the plant in three stages of infection, namely; germination, proliferation and sporulation (Solomon *et al.*, 2003).

Although plant disease tolerance and resistance are genetically controlled (Agrios, 2005), nutrient deficiencies, availability, toxicities and the environment can influence plant tolerance and resistance (Marschner, 1995; Krauss, 1999). Plant resistance or tolerance actually depends on three factors; the genotypes of the host and pathogen, the plant's growth stage and changes in the environment. Furthermore, these three factors are influenced by the availability of nutrients in the plant, as nutrients have well understood physiological functions; in spite of the lack of knowledge regarding dynamic interaction between nutrients and the plant-pathogen system (Huber, 1996).

Nutrients are an important aspect of disease control, as they are important for development and growth of plants and also microorganisms (Agrios, 2005). However, there is no general rule regarding their effects and influence in plant-pathogen interaction. A certain nutrient can lower the severity of a disease, while the same nutrient can raise the severity of the disease incidence of other diseases. Furthermore, that same nutrient can have a completely opposite effect on the same diseases in a different environment (Huber, 1980; Graham and Webb 1991; Marschner, 1995).

In the current study, nutrient uptake was evaluated, to see how *F. proliferatum* affects nutrient uptake in the wheat cultivars. The following macro and micro nutrients were studied: Calcium

(Ca), Magnesium (Mg), Sodium (Na), Potassium (K), Copper (Cu), Zinc (Zn), Molybdenum (Mo), Cobalt (Co), Nickel (Ni), Manganese (Mn) and Iron (Fe).

1.7.1. Macro nutrients

Calcium (Ca)

Calcium is a macro nutrient, important for its role in cell membranes. Calcium plays a role cell membrane functioning and stability and thus a deficiency of Ca results in membrane leakage of low molecular biomolecules such as amino acids and sugars (Marschner, 1995; Thor, 2019). Furthermore, calcium is required in the middle lamella as calcium polygalacturonates for the stability of the cell wall. In this way, Ca plays a structural role and is thus a component of the cell wall. In both cases Ca deficiency increases the susceptibility of the plant to fungal pathogens (Gupta *et al.,* 2017). The leakage of low molecular biomolecules will stimulate the infection by the pathogen by providing nutrients to the pathogen. Whereas in plants, Ca deficiency will enable the fungus to invade the xylem and dissolve the cell walls and thus result in wilting (Dordas, 2008). Moreover, plant tissues with low calcium levels are known to be more susceptible to fungal pathogens than tissues with normal calcium level. Lastly, Ca has been observed to make plants more resistant against Pythium, Sclerotinia, Botrytis and *Fusarium* (Graham, 1983).

Potassium (K)

Potassium plays an important role in biochemical and physiological functions (Marschner 1995; Wang *et al.,* 2013). K lowers the host's susceptibility to pathogens by up to the optimal level for growth, of which beyond that point, it offers no further increase in resistance. However, more resistance can be achieved by increasing the K supply (Huber and Graham, 1999). Potassium plays a role in the synthesis of high molecular weight biomolecules such as starch, cellulose and proteins. Furthermore, the development of thicker outer walls in epidermal

cells may be promoted by potassium, which thus decreases the plants susceptibility to pathogen attack. K deficiency results in impaired synthesis of these high molecular weight biomolecules such as proteins leading to the formation of nitrogen compounds such as amides. These nitrogen compounds are used up as nutrients by the pathogens (Marschner 1995; Amtmann *et al.,* 2008; Zörb *et al.,* 2014).

Magnesium (Mg)

The accessibility of magnesium to the plant depends on environmental conditions such as the soil pH, microbial activity and land use, especially agricultural or horticultural activities. Furthermore, Mg accessibility also depends on the ratios with other nutrients, especially Mn, Ca and K, whose high levels may inhibit the uptake of Mg (Persson and Olsson, 2000; Debnath *et al.,* 2015). With an increase in Mg supply, it has been observed that Mg had an variable effect on increasing or decreasing the plants resistance or tolerance of six diseases, depending on the environment. Moreover, Mg decreased the tolerance and resistance of 17 diseases and increased the tolerance and resistance of 22 diseases. Therefore, magnesium's effect on disease is either the direct result of this macro nutrient's role on the physiological functions of the plant or an indirect result due to magnesium's effect on the overall health of the plant (with consideration of how Mg is affected by the environment) (Huber and Jones, 2012).

1.7.2. Micro nutrients

Manganese (Mn)

Manganese is said to be instrumental in controlling various diseases (Huber and Graham 1999; Heckman *et al.,* 2003). Mn plays a role in photosynthesis, lignin biosynthesis and phenol biosynthesis. Mn controls the biosynthesis of lignin and suberin by activating various enzymes of the phynylpropanoid and shikimic acid pathways (Marschner, 1995). Suberin and lignin form biochemical barriers that obstruct fungal pathogen invasion (Hammerschmidt and Nicholson, 2000; Vidhyasekaran, 2004). Suberin and lignin are important because they are

thought to contribute to the resistance of wheat against powdery mildew and other diseases caused by *Gaeumanomyces graminis* (Krauss, 1999). Mn also plays a role in inhibiting the introduction of aminopeptidase into the plant cells. Aminopeptidase is an enzyme which nourishes the fungus by supplying it with essential amino acids for its growth. Furthermore, Mn also inhibits pectin methylesterase, a fungal enzyme that digests cell walls of the host. Soil applications of Mn have been observed to reduce infections by *Fusarium* spp. and other fungi (in cotton) (Graham and Webb, 1991; Agrios, 2005).

Iron (Fe)

Iron is an important micro nutrient required by most living organisms including pathogens (Kieu *et al.,* 2012; Aznar *et al.*, 2015). Unlike other micronutrients such as Mn and Cu, Fe on the other hand is required in high concentrations by plant pathogens (e.g. *Fusarium*) than its host plant, which may give the plant an advantage over the pathogen in Fe deficient soils (Dordas, 2008). Fe plays a dual role in plant pathogen interaction. Firstly, plant hosts may use Fe to increase oxidative stress in defence response against pathogens. Secondly, Fe catalyses the formation of harmful reactive oxygen species. Therefore, due to this duality, iron plays a complex role in plant disease resistance and tolerance. Moreover, Fe deficiency and plant defence strategies share various features such as using common hormone signalling pathways and phenolic compound secretion (Aznar *et al.,* 2015).

The application of Fe has been observed to supress various plant pathogens including *Fusarium oxysporum,* reduce or control the severity of smut in wheat, rust in wheat leaves and other diseases. Nevertheless, Fe may increase or decrease the host's resistance and tolerance to disease (Graham, 1983; Graham and Webb, 1991).

Zinc (Zn)

Zinc plays a role in activating the Cu/Zn SOD (Dordas, 2008) which is responsible for scavenging the superoxide radical in plant cells (Gill and Tuteja, 2010). Thus as an activator of Cu/Zn SOD, Zn protects the membrane from oxidative damage by detoxifying superoxide radicals (Cakmak, 2000). Furthermore, Zn plays a role in starch and protein synthesis and thus low Zn concentrations results in an accumulation of starch and protein constituents (reducing sugars and amino acids respectively) (Römheld and Marschner, 1991; Marschner, 1995; Genc et al., 2006). When it comes to plant disease resistance and tolerance, Zn was observed to increase, decrease or have no effect on the host's susceptibility to disease (Grewal *et al.*, 1996). Therefore, actual effect of Zn in plant disease is not well understood.

Copper (Cu)

A number of enzymes require Cu as a component to function. These include polyphenol oxidase, diamine oxidase, etc. Cu also plays an important role in the synthesis of lignin which provides rigidity and strength to cell wall (Marschner 1995; Broadley *et al.*, 2012). Thus low Cu levels results in reduced lignification which ultimately results in a higher disease incidence. Furthermore, the lipid structure in cell membranes gets altered when Cu is deficient and this negatively affects the membrane's resistance to biotic stress (Broadley *et al.*, 2012). Small grains suffering from Cu deficiency have been reported to suffer from stem melanosis, take-all root rot, and ergot resulting from fungal infection (Marschner 1995). Cu has been shown to decrease leaf infections of wheat when it is applied to the soil. Examples are powdery mildew and ergot in wheat (Evans *et al.*, 2007).

1.7.3. Other nutrients

There was not much information in the literature on nutrients such as Na, Mo, Co and Ni. However, it is important to note that plants do take up these elements and they are required for

growth and development of the plant and disease resistance and control (Datnoff *et al.*, 2007). Particular nutrients have different impacts on different diseases and one nutrient can have different impacts on different diseases and on the same disease in different environments. Moreover, the impact of nutrients on plant disease and plant-pathogen interaction is influenced by the environment and plant genetics (Agrios, 2005).

1.8. Aims and objectives

The aim of this study was to investigate the influence of *Fusarium proliferatum* on the physiological and biochemical responses of two wheat cultivars (SST 015 and SST 088).

The specific objectives of this study was to:

- Monitor the physiological changes in *Triticum aestivum* (wheat) using fresh weights, shoot and root length.
- Measure changes in chlorophyll pigments and osmolyte content.
- Quantify changes in ROS accumulation (superoxide and hydrogen peroxide) and the extent of lipid peroxidation
- Detect changes in antioxidant enzyme activities (SOD, APX and POD) using native PAGE gel electrophoresis.
- Monitor nutrient uptake in roots and shoots using ICP-OES

CHAPTER TWO

Materials and Methods

2.1 List of reagents

Acetone

Acrylamide/Bis (40 %)

Agarose D – 1 LE

Ammonium Persulfate (APS)

Ascorbic acid / Ascorbate

Borric acid (H3BO3)

Bovine Serum Albumin (BSA) Fraction V

Bradford Reagent (1X)

Bromophenol blue

3-[(3-Cholamidopropyl)dimethylammonio]1Propanesulfonate CHAPS

Coomassie® brilliant blue (CBB) R-250

Dithiothreitol (DTT) Cleland's reagent

Ethanol 99.9%

Ethylenediaminetetraacetic acid (EDTA)

Evans Blue

Glacial acetic acid

Glycerol

Glycine

Hydrochloric acid (HCl)

Hydrogen peroxide (H2O2)

2-(N-Morpholino)ethanesulfonic acid (MES) hydrate

β-mercaptoethanol Amresco Methanol

Nitrotetrazolium blue chloride powder (NBT)

PageRuler^{TM} unstained protein ladder

Phenylmethylsulfonyl fluoride (PMSF)

Polyvinylpyrrolidone (PVP) MW: 40 000

Potassium cyanide (KCN)

Potassium iodide (KI)

Potassium phosphate monobasic (KH_2PO_4)

Potassium phosphate dibasic (K_2HPO_4)

Potassium sulfate (K_2SO_4)

Promix Organic Cypress House Trading Propan-2-ol (isopropanol)

Riboflavin

Sodium chloride (NaCl)

Sodium dodecyl sulfate (SDS)

Sodium hydroxide (NaOH)

Sodium molybdate (Na_2MoO_4)

N,N,N',N'-Tetramethylethylenediamine (TEMED)

Thiobarbituric acid (TBA)

Trichloroacetic acid (TCA)

Tris(hydroxymethyl)-aminethane

Trypsin

2.2 Fungal culture collection and inoculum preparation

Fusarium proliferatum isolate (MRC 2059) was collected from the Plant Protection Institute, Agricultural Research Council (ARC), Pretoria, South Africa. The isolated fungi were cultured on potato dextrose agar (PDA) media and grown under 12 hour light and 12 hour dark conditions at 30 °C. A *F. proliferatum* 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.

2.3. Seed sterilization and fungal inoculation

Seeds from two wheat cultivars (SST 015 and SST 088) (received from the Research and Technology Development services at the Department of Agriculture, Cape Town, South Africa) were heat sterilized at 49°C (waterbath) for 20 minutes, and incubated in 3% ethanol for 10 minutes at room temperature. Following sterilisation, the seeds were washed with sterile distilled water (three times) to remove residual ethanol. Sterilized seeds were transferred into sterile greiner tubes containing *F. proliferatum* 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).

2.4 Measuring of the rate of seed germination

Inoculated seeds (100) were germinated on moist sterile filter paper in a dark environment for a period of 5 days. The number of seeds germinated for each treatment were recorded (daily) at the same time for a period of five days. Seed germination was regarded successful with radical emergence of approximately 2 mm. The following formula was used to calculate the daily rate of seed germination and overall germination after 5 days:

[Rate of germination (%) = (germinated seeds / total number of seeds) x 100]

2.5 Plant growth

Germinated seeds (six seeds per pot) from each treatment were transferred to 19 cm plastic pots containing a moist growth mixture of silica sand and potting soil in a 2:1 ratio. The seedlings were grown in a conditioned growth room with a 16-hour light and 8-hour dark cycle at 22°C for a period of 21-days. 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 0.5X Nitrosol solution (Builders Warehouse, Cape Town, South Africa) twice a week until the end of the experiment.

2.6 Analyses of plant growth parameters

The plants were carefully removed from the soil to avoid any loss of root or shoot material. The rate of plant survival for each treatment was recorded at the end of the experiment using the formula described below:

[No. of surviving plants = (No. of plants harvested / total number of plants planted) x 100]

Furthermore, growth analysis was performed by measuring root length, shoot length, root fresh weight (FW), and shoot fresh weight (FW) for each wheat cultivar.

2.7 Measurement of shoot chlorophyll and carotenoid content

A modified method of Lichtenthaler and Wellburn (1983) was followed to estimate the chlorophyll (chlorophylls a and b) and carotenoid content in the shoots of both wheat cultivars.

Ground up shoot material (100 mg) was homogenised in 1 ml of 80% acetone. Shoot extracts were vortexed and centrifuged at 10 000 rpm for 10 minutes. The supernatant was transferred to a new 2 ml centrifuge tube. This process was repeated until a clear pellet was observed. The absorbance of each supernatant fractions (200 µl) was recorded at 470 nm, 663 nm, and 646 nm in a 96-well microtiter plate. Chlorophyll and carotenoid content was calculated using the following formulae describe below:

Chlorphyll a (µg/ml) = 12.7 (A663) – 2.69 (A646)

Chlorphyll b (µg/ml) = 22.9 (A663) – 4.68 (A646)

Carotenoids (µg/ml) = (1000A470 – 3.27[Chl a] – 104[Chl b]) / 227

2.8 Measurement of the proline content

Freshly harvested shoot material from each cultivar was homogenized in 0.5 ml of 3% (w/v) sulphosalicylic acid using a mortar and pestle. About 0.2 ml of each homogenate was mixed with 0.2 ml of glacial acetic acid to which 0.2 ml of ninhydrin was added. The reaction mixture was boiled in a water bath at 96 °C for 30 minutes and immediately cooled in an ice bath. After

cooling, 0.4 ml of toluene was added to the reaction mixture. After thorough mixing, the chromophore containing toluene was separated and absorbance of red colour developed was read at 520 nm against a toluene blank on a UV–visible spectrophotometer (ChemitoSpectrascan, UV 2600).

2.9 Measurement of the superoxide content

Plant material (roots and shoots) was weighed and placed in sterile 2 ml tubes and 800 µL of the reaction buffer (100 mM potassium cyanide, 100 mM hydrogen peroxide, 100 mM potassium phosphate and 6.4 mM NBT) was added to the material and incubated in the dark for 20 minutes. The material was crushed using a plastic pestle and centrifuged at 13 000 rpm for 20 minutes. A fraction of the supernatant (200 µl) was transferred into a microtiter plate and the absorbance was recorded at 600 nm.

2.10 Measurement of hydrogen peroxide

Approximately 100 mg of shoot and root material was pulverised in liquid nitrogen and homogenized in 500 µl of cold 5% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 13 000 rpm for 30 minutes at 4°C to obtain the TCA extract.

Hydrogen peroxide (H_2O_2) content was determined using a method adapted from Velikova *et al.*, (2000). The reaction mixture consisted of 75 µl TCA extract, 5 mM K_2HPO_4, pH 5.0 and 0.5 M KI, and samples were incubated at 25°C for 20 minutes, and the absorbance measured for each sample at 390 nm. The H_2O_2 content was then calculated based on a standard curve constructed from the absorbance (390 nm) of the H_2O_2 standards.

2.11 Measurement of the malondialdehyde (MDA) content

MDA content in the shoots of wheat cultivars was measured using a method previous described by Velikova *et al.*, (2000). A 100 µl aliquot of the TCA extract was mixed with 400 µl of 0.5% TBA (prepared in 20% TCA). Then the mixture was incubated for 30 minutes at 95°C in a

water bath. After 30 minutes, the reaction was terminated by placing the mixture on ice for 5 minutes. After which the reaction mixture was centrifuged at 12 000 rpm for 5 minutes at 4°C. The absorbance of each extract was then measured at 532 nm and 600 nm respectively. After subtracting the non-specific absorbance (600 nm) from each sample, the MDA concentration was determined using the extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as $nmol.g^{-1}FW$.

2.12 Preparation of protein extracts

Shoot material (200 mg) was ground to a fine powder using liquid nitrogen and homogenised in 1 ml of extraction buffer [40 mM K_2HPO_4, pH 7.4, 1 mM ethylenediaminetetra acetic acid (EDTA), 5% (w/v) polyvinylpyrrolidone (PVP) molecular weight = 40 000]. The resulting PVP extracts were centrifuged at 12 000 rpm for 15 minutes and the supernatants used to detect individual isoforms of antioxidant enzymes. Protein concentrations were determined according to the Bradford (1976) method, using bovine serum albumin (BSA) as a standard.

2.13 Quantification of the antioxidant enzyme activity within wheat shoots

Proteins were separated electophoretically on a 12% polyacrylamide native gel according to Laemmli (1970).

2.13.1. Superoxide dismutase (SOD)

SOD activity was detected by staining with 0.5 mM riboflavin and 2.5 mM nitroblue tetrazolium, as described by Beauchamp and Fridovich (1971), using 0.1 mg of total protein extract from each sample. The associated SOD isoform patterns were determined by incubating gels in 5 mM H_2O_2 (to inhibit both Cu/ZnSOD and FeSOD), and in 5 mM KCN (to inhibit only Cu/ZnSOD) (Archibald and Fridovich 1982). The identification of manganese SOD (MnSOD) are evident via its resistance to both KCN and H_2O_2.

2.13.2. Ascobate peroxidase (APX)

APX isoforms were detected using 0.05 mg of the total PVP protein extract, and was separated on a 12% polyacrylamide PAGE gel as a modified method previously described by Mittler and Zilanskas (1993). Individual APX isoforms detection was achieved by incubating the native PAGE gels within 50 mM phosphate buffer (pH 7.0) for 20 minutes in the dark followed by 50 mM phosphate buffer (pH 7.8) containing 4 mM ascorbate and 2 mM H_2O_2 for 20 minutes. The gel was then stained with 50 mM phosphate buffer (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 30 µg of PVP protein extract that was separated on an 8% native PAGE gel for 2-3 hours at 90 V. The POD isoforms were visualised by staining the native PAGE gel in 50 mM phosphate buffer (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 phosphate buffer (pH 7.0) containing 0.02% 3,3′-Diaminobenzidine (DAB) until the POD isoforms were visible.

2.14 Quantification of mineral elements by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP - OES)

Shoot and root material (100 mg) was digested using nitric acid as described by Vachirapatama and Jirakiattikul. (2008). The elemental concentrations was determined using a Varian Vista Pro CCD Simultaneous ICP-OES (Varian, Australia) with certified standards (sigma; TraceCERT®).

2.15 Statistical analysis

All experiments described were performed three times independently. The obtained data was analyzed using the one-way analysis of variance (ANOVA) and tested for significance by the Tukey-Kramer test at 5% level of significance.

CHAPTER THREE

The effect of *Fusarium proliferatum* on seed germination and growth parameters in wheat plants

3.1 Introduction

Wheat is the second most important cereal in the world and is a source of calories as well as source of protein for humans (Cendoya *et al.,* 2018). This cereal is ground into semolina and flour which provides the basic ingredient for bakery products and pasta (Chandrika and Shahidi, 2006). Countries such as Egypt, Algeria, Israel, numerous countries in the Middle East and Eastern Europe, which rely more on wheat consumption compared to other cereals, have wheat flour per capita consumption rates that vary widely by region but nevertheless often exceeds 150 kg per year. In other countries which rely more on other cereals compared to wheat, the wheat flour per capita consumption averages 35 kg per year in Central America and 17 kg in Sub Saharan Africa. Moreover, developing countries use more than 80% of their wheat supply for food, compared to developed countries that use less than 50% (Cendoya *et al.*, 2018).

Fusarium proliferatum is a disease causing fungal pathogen that affect corn (Mohd Zainudin *et al.,* 2017), soybean (Chang *et al.,* 2015), allium plants (Shin and Kim, 2001) and wheat (Cendoya *et al.,* 2017). These interactions disrupt or alter physiological processes and thus the physiology of the plant and cell function (Agrios, 2005).

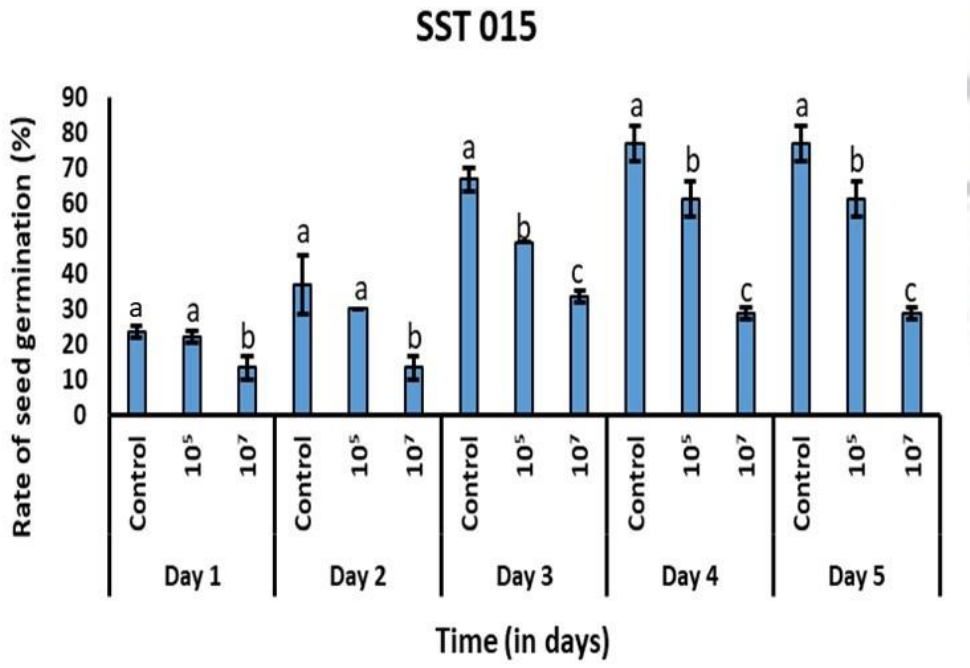
In this chapter, the aim was to observe the changing physiology of the plants due to pathogen infection. In particular, this chapter will focus on how the infection affects the rate of seed germination, plant viability, root and shoot length and weight, chlorophyll pigments, carotenoids and the proline content. Therefore we comparatively analysd the changes in physio-biochemical parameters of two wheat cultivars with contrasting responses to *F. proliferatum* infection.

3.2 Results

3.2.1 *F. proliferatum* reduces the rate of germination and seed viability both wheat cultivars

Seeds were imbibed in different concentrations of *F. proliferatum* inoculum as explained in section 2.2. The results show two distinct germination patterns for these wheat cultivars: (1) For SST 015, the control showed the highest rate of germination followed by the 10^5 spores/ml treatment and 10^7 spores/ml treatment (Figure 3.1A). (2) For SST 088, seeds infected with 10^5 spores/ml showed a higher germination percentage than the control on the first two days, after which the control assumes the highest germination percentage until the end of the experiment (Figure 3.1B). Seeds inoculated with 10^7 spores/ml showed the lowest rate of germination throughout the germination experiment.

A



B

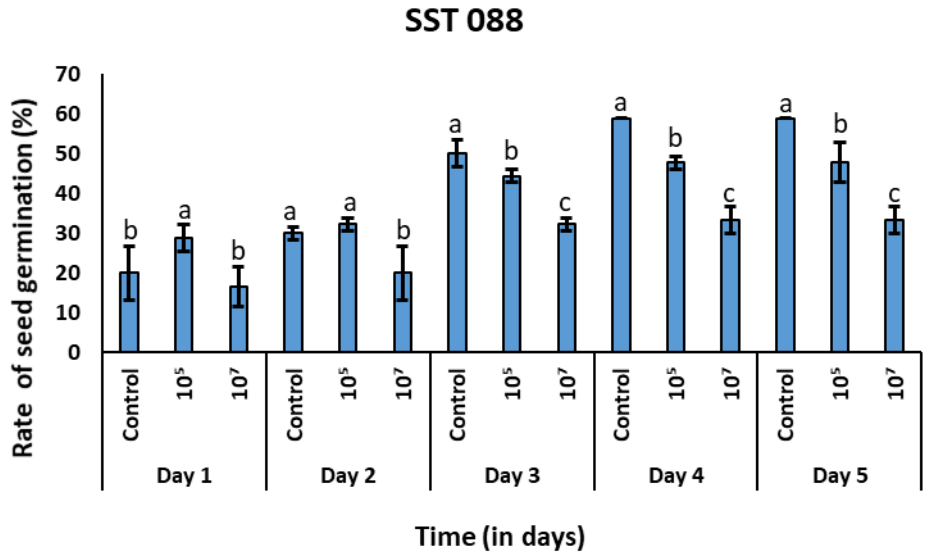


Figure 3.1. The effect of *F. proliferatum* on the germination of two wheat cultivars. The graphs represent the rate of seed germination (as a percentage) under different concentrations of pathogen infection. A represents SST 015 and B represents SST 088. Error bars are representative of the mean (± SE) of three independent experiments from three plants per treatment in each experiment.

Overall, the rate of seed germination of both cultivars to *F. proliferatum* infection seem comparable: Under control conditions, SST 015 showed an overall germination rate of 46% relative to the 42% observed for SST 088. A similar trend was observed in response to 10^5 spores/ml where SST 015 showed a germination of 37% compared to the 34% of SST 088. However, in response to 10^7 spores/ml the rate of germination was higher for SST 088 (24%) compared to SST 015 (17%) (Figure 3.2 A and B).

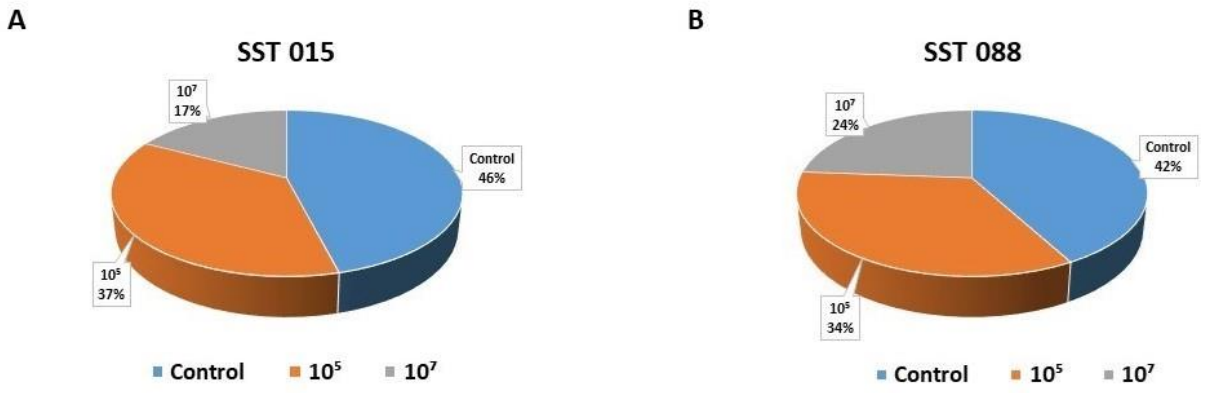


Figure 3.2. Overall rate of seed germination of wheat cultivars in response to *F. proliferatum*. The charts are showing the overall rate of germination for (A) SST 015 and (B) SST 088.

3.2.2. *F. proliferatum* reduces the number of surviving infected plants

The survival of wheat plants infected with *F. proliferatum* was concentration dependent. For SST 015, plant survival was significantly reduced with an increase in *F. proliferatum* concentration (Figure 3.3 A). For SST 088, both concentrations of *F. proliferatum* reduced plant survival, however no significant change in plant survival was observed between the two *F. proliferatum* concentrations (Figure 3.3 B).

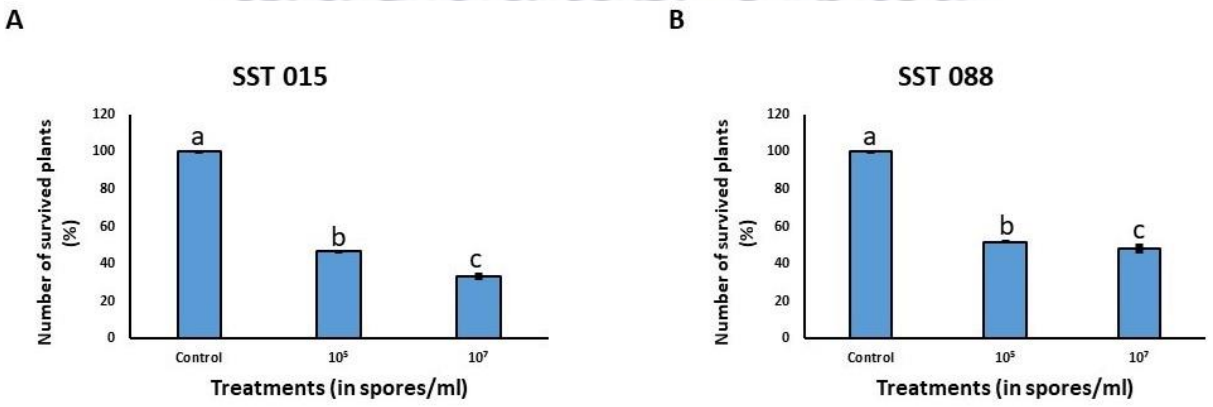


Figure 3.3. The impact of *F. proliferatum* on wheat plant survival. The graphs represent the number of plants for (A) SST 015 and (B) SST 088 that survived for 3 weeks post infection. Error bars are representative of the mean (± SE) of three independent experiments from three plants per treatment in each experiment.

3.2.3. *F. proliferatum* alters the physiological parameters of two wheat cultivars

F. proliferatum restrict plant growth of both wheat cultivars in a concentration dependent manner. Here we measured changes in plant growth (shoot and root length) and biomass (shoot and root fresh weight and shoot and root dry weight) of two wheat cultivars to *F. proliferatum* infection. For SST 015, shoot length was significantly reduced in both treatment relative to the control, with the highest reduction observed in the 10^7 spores/ml treatment (Figure 3.4 A). However, the reduction in shoot length for SST 088 was not concentration depended as observed for SST 015. Both *F. proliferatum* concentrations caused a significant reduction in shoot length relative to the control although no difference in the extent was observed between the two concentrations (Figure 3.4 B).

Similarly to what was observed for shoot length, *F. proliferatum* reduced root length in both cultivars (Figure 3.4 C-D). For SST 015, *F. proliferatum* restricted root length with the highest reduction observed in 10^7 spores/ml (Figure 3.4 C). The same pattern was observed for length of SST 088 (Figure 3.4 D).

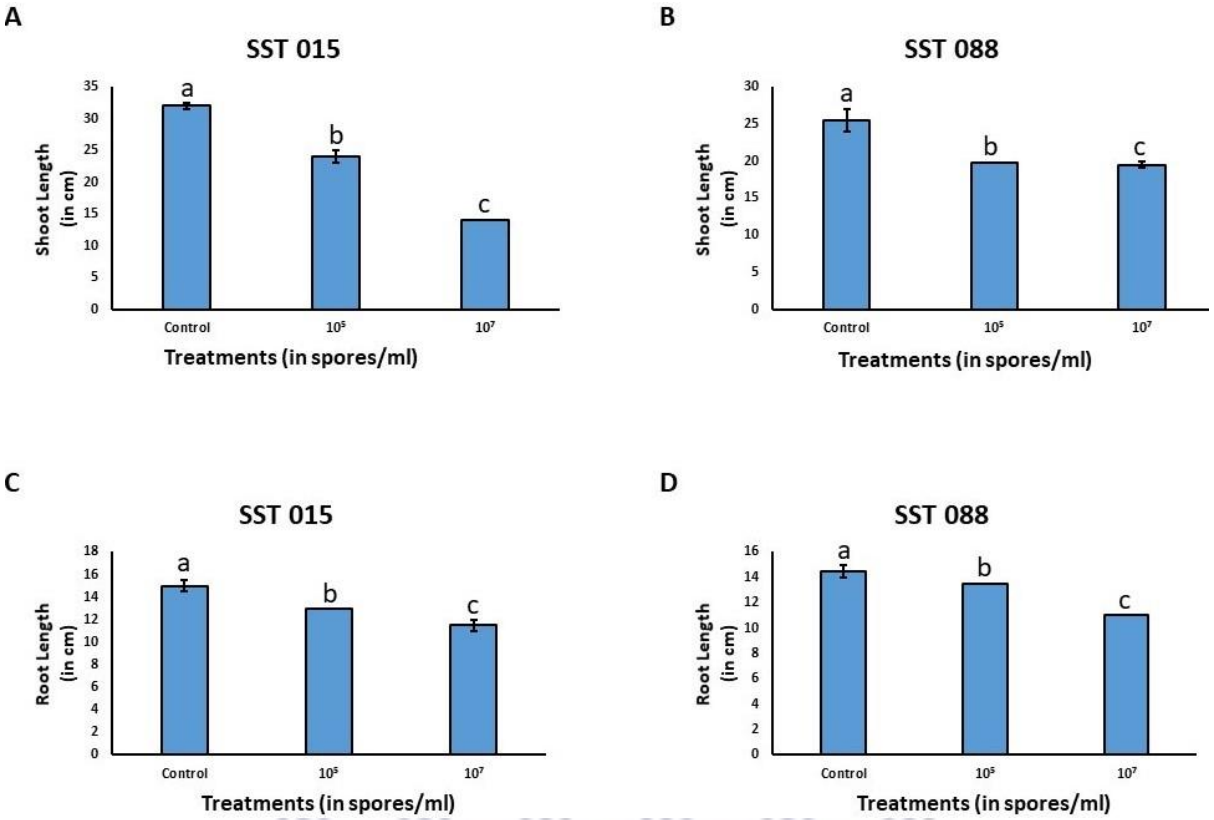


Figure 3.4. The effect of *F. proliferatum* on shoot and root length of two wheat cultivars. Shoot and root length of SST 015 (A, C) and SST 088 (B, D) was measured in response to *F. proliferatum*. Error bars are representative of the mean (± SE) of three independent experiments from three plants per treatment in each experiment.

Based on the results obtained here, it is evident that *F. proliferatum* restricted wheat biomass as seen for changes in fresh weigh of shoots and roots. (Figure 3.5 A-D). For SST 015, *F. proliferatum* significantly reduced shoot fresh weigh with the highest reduction observed in the 10^7 spores/ml treatment (Figure 3.5 A). Although *F. proliferatum* reduced shoot fresh weight of SST 015, the most significant reduction was observed in the 10^5 spores/ml treatment relative to the control.

In contrast to what was observed for shoot fresh weight, the root fresh weights of both cultivars was differentially altered by *F. proliferatum* (Figure 3.5 C-D). For SST 015, contrasting

responses in fresh weight was observed to *F. proliferatum*. SST 015 plants treated with 10^5 spores/ml significantly increased shoot fresh weight, whereas a reduction was observed in response to treatment with 10^7 spores/ml relative to the control (Figure 3.5 C). On the other hand, both *F. proliferatum* concentration increase the root weight of SST 088 relative to the control (Figure 3.5 D).

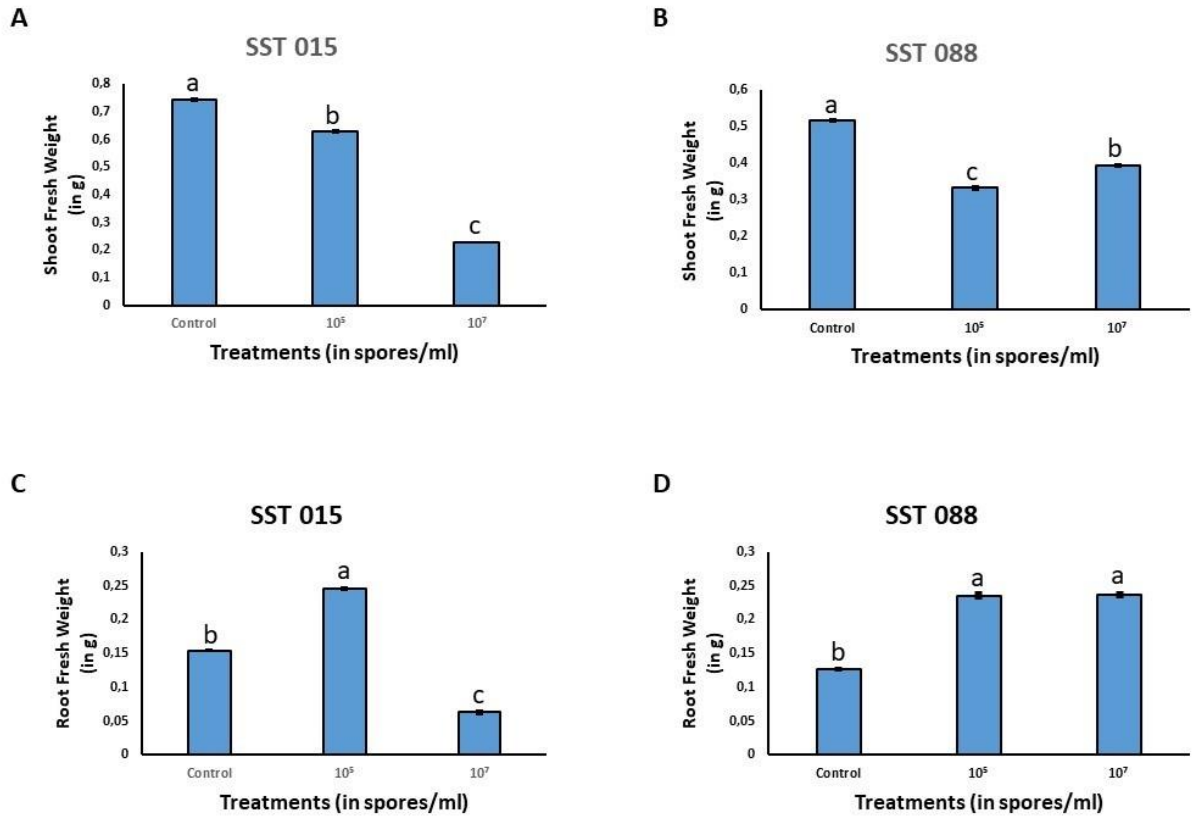


Figure 3.5. The effect of *F. proliferatum* on shoot and root biomass in wheat plants. Shoot and root weight of SST 015 (A, C) and SST 088 (B, D) was measured in response to *F. proliferatum*. Error bars are representative of the mean (± SE) of three independent experiments from three plants per treatment in each experiment.

3.2.4. *F. proliferatum* decreases chlorophyll pigments and carotenoid content in wheat plants

F. proliferatum significantly affected total chlorophyll content in SST 015 (Figure 3.6 A). Both treatments reduced pigment concentrations compared to the control, with the highest decrease -in pigment concentration observed in plants treated with 10^7 spores/ml (Figure 3.6 A). For

SST 088, no significant differences in chlorophyll content was observed in the 10^5 spores/ml treatment relative to the control (Figure 3.6 B). However, a slight but significant decrease in total chlorophyll content was observed in the 10^7 spores/ml treatment.

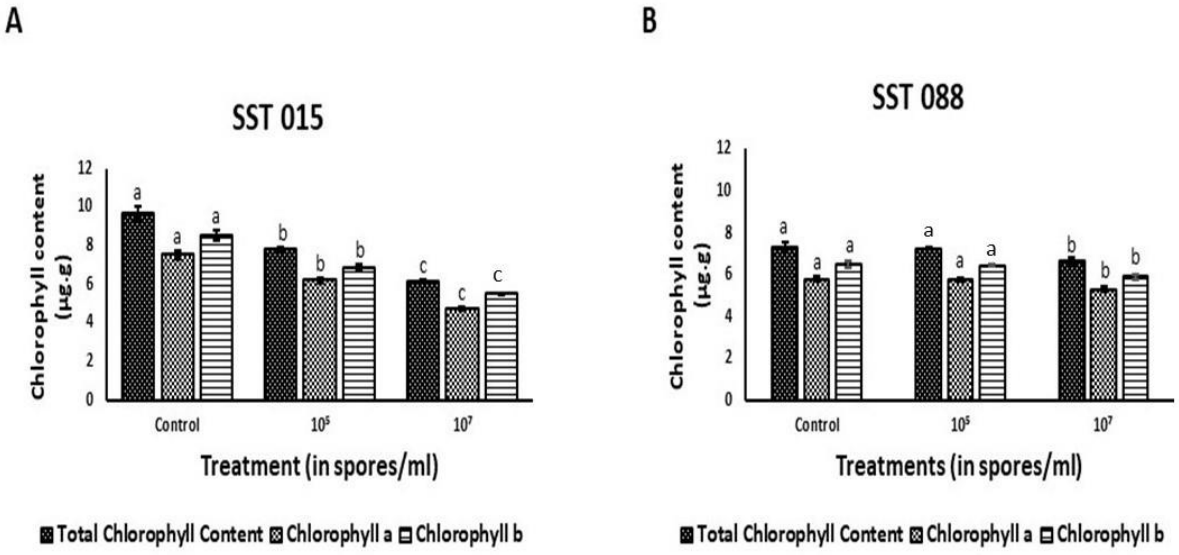


Figure 3.6. ***F. proliferatum*** **alters chlorophyll content in wheat plants.** Chlorophyll content in (A) SST 015 and (B) SST 088 was measured in response to *F. proliferatum*. Error bars are representative of the mean (± SE) of three independent experiments from three plants per treatment in each experiment.

For carotenoid content, the pathogen seems to be affecting SST 015 more than SST 088. For SST 015, both *F. proliferatum* concentration reduced carotenoid content relative to the control with the highest reduction observed in the 10^7 spores/ml treatment (Figure 3.7 A). For SST 088, a significant reduction in carotenoid content was observed in the 10^7 spores/ml treatment whereas no change was observed in the 10^5 spores/ml treatment relative to the control (Figure 3.7 B).

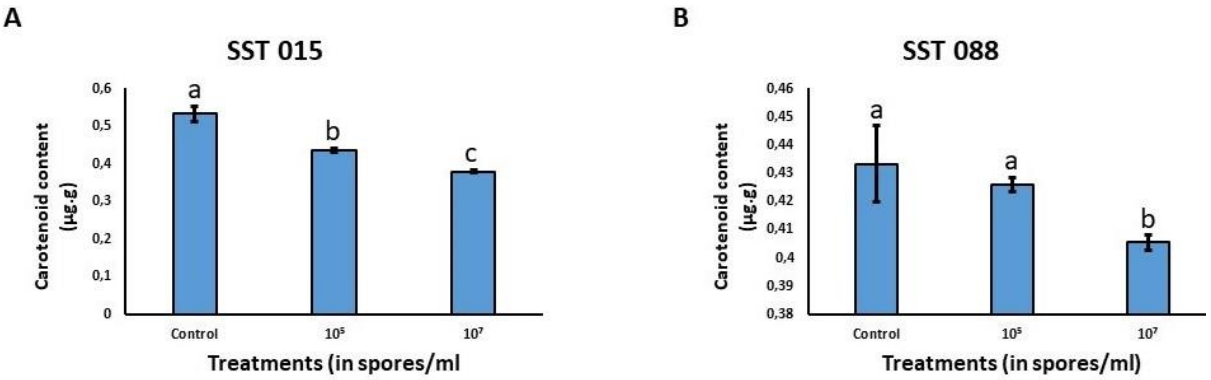


Figure 3.7. Carotenoid content in wheat plants in response to *F. proliferatum* infection. Carotenoid content in (A) SST 015 and (B) SST 088 was measured in response to *F. proliferatum*. Error bars are representative of the mean (± SE) of three independent experiments from three plants per treatment in each experiment

3.2.5. *F. proliferatum* increases the proline content in wheat plants

A significant increase in proline accumulation was observed in both wheat cultivars. For SST 015, both concentrations of *F. proliferatum* increase proline content to levels much higher than the control (Figure 3.8 A). Interestingly, the highest increase in proline as observed in the 10^5 spores/ml treatment (Figure 3.8 A). A similar trend was observed for SST 088, albeit not the same level as observed for SST 015.

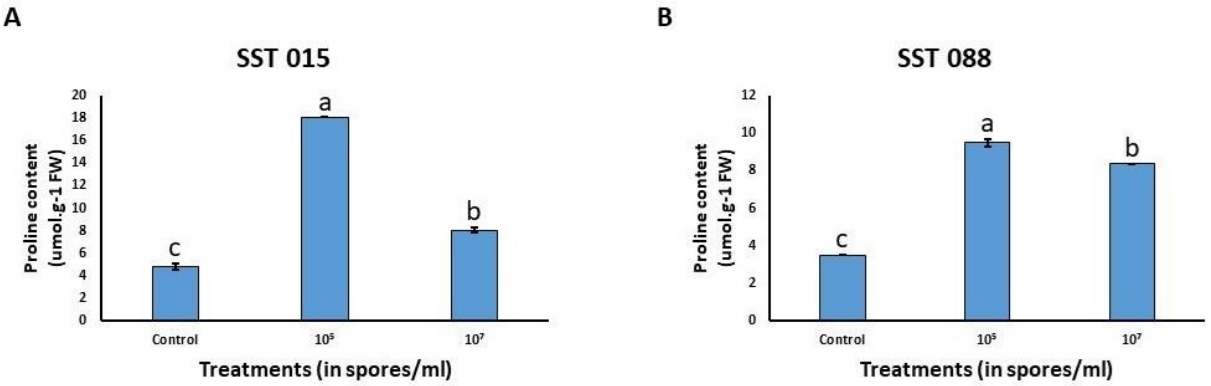


Figure 3.8. Effect of *F. proliferatum* on the proline content of wheat cultivars. Changes in proline content for (A) SST 015 and (B) SST 088 was measured in response to *F. proliferatum*. Error bars are representative of the mean (± SE) of three independent experiments from three plants per treatment in each experiment.

3.3. Discussion

The influence of *F. proliferatum* on the physiological responses of *Triticum aestivum* have been analysed and the results indicate that *F. proliferatum* induces stress on the host wheat plants and thus restrict the growth and development of the plant. Furthermore, in general, the results indicate that the degree of alteration (caused by the pathogen) of the physiology or physiological processes is concentration dependent.

3.3.1. *F. proliferatum* reduces the rate of germination, seed viability and plant survival

The rate of germination occurred following two trends (Figure 3.1. A and B). A comparison between the control and the treatment 10^5 spores/ml in SST 088 (Figure 3.1 B) shows approximately the same germination rate with no significant differences up until the 4th day, when significant differences begin to show. While in SST 015, there were no significant differences between the control and treatment with 10^5 spores/ml up until the third day, after which significant changes were observed. For Trend 1 (Figure 3.1B) this may be due the pathogen activating a condition known as "the oxidative window", where ROS levels within the seed are produced to be at their optimal levels to promote seed germination and development (Bailly *et al.*, 2008).

To explain this further, it is well established that biotic stress normally promotes the production of ROS such as superoxide (Suzuki and Mittler, 2006). Furthermore, when ROS accumulate within the plant, they result in plant stress, which causes cell damage and ultimately death. However, the same ROS can promote germination and development when they are at an optimal level for germination and development. This optimal level is called the oxidative window (Bailly *et al.*, 2008). Therefore, Trend 1 (where the treatment 10^5 spores/ml germinates at a higher rate than the control for the first two days) is probably a result of this oxidative window. Trend 2 (Figure 3.1A) on the other hand illustrate normal germination, where germination is reduced as a result of the pathogen, as hypothesised. Therefore, the results

obtained in the current study are similar to those observed by Bailly *et al.*, (2008), since the pathogen is present at a lower concentration and are showing the oxidative window.

The effect of *F. proliferatum* infection seed germination is detrimental and thus affects seed viability. In a study by Burmeister and Plattner (1987), a species of *Fusarium* (*F. Iricinelwn)* decreased the rate of germination of wheat seeds. Here we presented a similar phenomenon.

Plant survival under pathogen infection can be equated to cell death or reduction of yield due to infection of the plants by the pathogen. Many studies have reported that *Fusarium* infection of cereals lead to cell death, yield loss, economical loss or loss of an entire plant (Chang *et al.*, 2015; Al Hatmi, 2016; Ray *et al.*, 2017)

3.3.2. *F. proliferatum* alters the physiological characteristics of two wheat cultivars

Fusarium species are soil borne pathogens that primarily infect the roots of a plant (Geiser *et al.*, 2004; Galvez *et al.*, 2017). Therefore, the changes that are observed for the root lengths (Figure 3.4 C and D) and weights (Figure 3.5 C and D) for both cultivars in both treatments is a result of the pathogen influencing the roots. However, the differences in how the roots are affected by the pathogen; i.e the roots of SST 015 under the treatment 10^7 spores/ml of pathogen compared to all the other treated roots (both SST 015 under 10^5 spores/ml and SST 088 both treatments) could be due to each cultivars ability to resist the infection, depending on the concentration of the pathogen.

The shoots depend on the roots to grow, as water and nutrients are absorbed by the roots and transported to the shoots. Therefore, the shoots weights (Figure 3.5 A and B) and heights (Figure 3.4 A and B) reduction compared to the control can be attributed to the alteration in their roots, which influences how the nutrients and water will be absorbed by the plant and transported into the shoots, which is ultimately a result of the pathogen altering the roots. Furthermore, a study by Burmeister and Plattner (1987) investigated the influence of *Fusarium*

iricinclum on wheat showed that *Fusarium* restricted leaf and root growth, whilst also inhibiting root elongation This study support the data presented in our investigation where *F. proliferatum* restricted plant growth and development as seen for reduced plant height and biomass.

Hormones are important biomolecules that regulate plant growth and development. For example, gibberellins control plant growth, specifically the height and width of a plant (Tanimoto, 2012). *Fusarium* is known to directly affect the plant's hormone homeostasis by affecting the biosynthesis or metabolism of hormones in the plant or by synthesising and producing the hormone itself, which then inhibits the hormone's biosynthesis by the plant. The pathogen can produce auxins, cytokinins and gibberellins (Vrakba *et al.*, 2019). Therefore, the retardation of root and shoot growth could be as a result of the pathogen influencing the plants hormone homeostasis.

3.3.3. *F. proliferatum* decreases chlorophyll pigments and carotenoid content in wheat plants

Photosynthesis is a primary metabolic process (Rojas *et al.*, 2014) of crucial importance to plant physiology (Pérez-Bueno *et al.*, 2019) and there is a direct link between photosynthetic pigments (chlorophylls a and b and carotenoids) and the plant's physiological status, more specifically the leaves (Gitelson *et al.*, 2006). Therefore, one of the markers used to assess the plant's physiological status under infection by *F. proliferatum* was to evaluate photosynthetic activity under stressed conditions by determining chlorophyll and carotenoid contents.

Chlorophyll a and b are the green pigments whose function is to absorb of solar energy for the purpose of transferring it into the photosynthetic apparatus and thus enabling photosynthesis to occur (Gitelson *et al.*, 2006; Kira *et al.*, 2015). Chlorophyll a and b together with their combined content (total chlorophyll content) were evaluated in this study. The results obtained showed a significant decrease of the chlorophylls a and b and thus overall total chlorophyll

content in both treatments and in both cultivars (Figure 3.6), with the exception of SST 088 treated with 10^5 spores/ml of pathogen (Figure 3.6 B). In SST 088 treated with 10^5 spores/ml, there were no significant differences between the infected plants and the control plants. Overall, the results show that photosynthetic activity was negatively affected by the stress caused by *F. proliferatum*. This is shown by the diminution in chlorophyll content. The diminution in chlorophyll content and thus in the photosynthetic process itself is related to changes in carbon partitioning between the roots and the stem (McCutchan and Shackel, 1992; Massacci *et al.*, 1996; DaCosta and Huang, 2006). Previously, Rajeswari *et al.*, (2014) reported that *F. oxysporum* restricted chlorophyll content in *Arachis hypogaea*. A similar trend was observed in our study where *F. proliferatum* significantly reduced chlorophyll content in both wheat cultivars (Figure 3.6).

Carotenoids are a group of natural tetraterpenoid pigments distributed widely in plants and other organisms and they are synthesised and stored in the plastids (Sun *et al.*, 2018). They range from colourless to yellow, orange, and red, with variations reflected in many fruits, flowers, and vegetables (Nisar *et al.*, 2015). Carotenoids play a necessary role in photosynthesis and photoprotection (Domonkos *et al.*, 2013; Niyogi and Truong, 2013; Hashimoto *et al.*, 2016). In addition, carotenoids form apocarotenoids, their oxidative and enzymatic cleavage products. The apocarotenoids play a role in the assembly of photosystems and light harvesting antenna complexes for photosynthesis and photoprotection, and regulation of growth and development of the plant (Cazzonelli and Pogson, 2010; Ruiz-Sola and Rodrı´guez-Concepcio´na, 2012; Havaux, 2014). In the current study, carotenoids were observed to decline in infected plants compared to the controls (Figure 3.7). This decline was observed in both treatments for each cultivar. The decline was greatest in the 10^7 spores/ml treatment, compared to the 10^5 spores/ml treatment.

The decline is an indicator that the *F. proliferatum* negatively affects the carotenoid content in wheat. In a similar study by Rajeswari *et al.,* (2014), it was reported that pathogen infection of *Arachis hypogaea* by *F. oxysporum* resulted in a decline in carotenoid content as observed in the current study.

3.3.4. *F. proliferatum* increases the proline content in wheat plants

Proline is an osmoprotective compound, which plays a significant role in stress signalling responses (Hare and Cress, 1997; Hare *et al.,* 1998). In addition, proline also plays a role in the stabilisation of the redox balance. In this study, infection of wheat with *F. proliferatum* resulted in the accumulation of proline (Figure 3.8). For SST 088 (Figure 3.8 B), the results showed an increase in proline content of the infected plants compared to the control and the same trend is observed for SST 015 (Figure 3.8 A). Furthermore, the biggest increase was observed in the 10^5 spores/ml treatment in both cultivars, although there was a slight difference between the treatments in SST 088 and a significant difference between the treatments of SST 015. In a similar study using another plant host (*Cucumis sativus* L.), which was inoculated with *F. proliferatum*, similar results were obtained, where the proline content increased as a result of the inoculation (Hao *et al.*, 2005).

3.4 In summary

The rate of germination under pathogen influence has been reduced, with the treatment 10^5 spores/ml having the lowest decline and the treatment 10^7 spores/ml having the highest decline. The final germination percent (FGP) reveals that the *Fusarium proliferatum* has a negative effect on the germination of *Triticum aestivum*. Furthermore, the FGP also reveals that the seeds treated with 10^5 spores/ml of the inoculum have a slightly higher germination rate in SST 015 compared to SST 088. While those treated with 10^7 spores/ml showed a much higher

germination rate in SST 088 compared to SST 015. The number of plants that survived under pathogen influence showed that the pathogen was killing the plants, and SST 088 had more surviving plants compared to SST 015, especially in the plants treated with 10^7 spores/ml, where there was a significant difference between the cultivars. The shoot and root lengths and shoot fresh weights were also negatively affected by the pathogen. The plants treated with 10^5 spores/ml stunted growth compared to the control with those plants treated with 10^7 spores/ml showing an even higher reduction in plant growth. Stunted growth was, in general, observed in the roots. However, the root weights did not follow the same trend as the shoot weights, as it was expected. For example, the roots of SST 015 that were treated with 10^7spores/ml were short and very few and this led to this cultivar and treatment having the lowest weights. The analysis of chlorophyll content showed that for SST 015 the chlorophyll content declined with increasing pathogen concentration. Whereas in SST 088 there were no significant differences between the treatment 10^5 spores/ml and the control. For SST 088, differences were observed in the treatment with 10^7 spores/ml relative to the control, where the chlorophyll content declined. When comparing the two cultivars for chlorophyll content, it was observed that there were no significant differences in chlorophyll content of the cultivars per treatment, except for SST 088 which seemed not to be affected by the treatment 10^5 spores/ml whereas SST 015 was affected. The carotenoid content showed the same results as observed for the chlorophyll content, in all aspects. Lastly, the proline content increased in both treatments compared to the control, with the highest increase observed in the plants treated with 10^5 spores/ml of *F. proliferatum*.

These results clearly suggest that *F. proliferatum* negatively affects the physiology and physiological processes of wheat plants. However, the responses of the plants are not consistent per treatment; i.e. the results disprove the hypothesis that the higher pathogen concentration will affect the plant more compared to the lower pathogen concentration. This probably means

that the different treatments trigger different pathways and mechanisms within the plant, whose initiation depends on the amount of pathogen presented to the plant. Thus, more research in required that investigate different aspects of the *F. proliferatum*-wheat interaction, in order to understand why the responses of the plant seem to depend on the concentration of the pathogen.

CHAPTER FOUR

Fusarium proliferatum infection alters the mineral nutrient content in wheat plants

4.1 Introduction

Plant and microbial interactions such as the plant-fungi interaction, is an evolutionary step that enabled the plants to form relationships or associations with the fungus to ensure healthy growth and development (Evangelisti *et al.*, 2014). However, not all microorganisms benefit the plant. Some pathogens and/or their products can disrupt the plant plasma and organellar membranes or interfering with enzyme activity (Junker and Tholl, 2013). In fact, plant-microbe interaction can be classified in three ways; namely, beneficial, detrimental, or unknown based on their effects on plant growth, health and development (Ntoukakis & Gifford, 2019). *F. proliferatum* can be classified as a detrimental microbe to wheat, based on the pathogen's effect on the physiology and biochemistry of the wheat cultivars. Therefore, investigating the plant mineral nutrient content will provide more insight on how the *F. proliferatum* affects wheat plants.

Mineral nutrients in plants is classified into micro-nutrients and macro-nutrients. Micronutrients are the elements, which are present within plants in minute quantities. These minute elements are also known as trace elements. Macronutrients on the other hand are elements required by the plant in large quantities. Examples of macronutrients include Potassium (K), Magnesium (Mg), Sodium (Na) and Calcium (Ca). On the other hand micronutrients include Copper (Cu), Iron (Fe), Manganese (Mn) and Zinc (Zn). These elements are essential for plant growth and development (Lin *et al.*, 2011; Sharma and Dietz, 2006).

Plant mineral nutrients are often simply understood as plant food necessary for growth and development. However, mineral nutrition also plays a role in plant resistance or susceptibility to pathogens. Furthermore, mineral nutrients may also indirectly affect plant resistance and susceptibility via the pathogen immobilising nutrients in the soil, which makes them unavailable to the plant. Moreover, pathogens can infect plant tissues and thus interfere with the utilisation or translocation of nutrients, which induces nutrient deficiencies or toxicities (Schumann *et al.,* 2017).

In this chapter, the effects of *F. proliferatum* on the accumulation of macro- and micronutrients in wheat plants were investigated. The chosen concentration of the pathogen was 1 x 10^7 spores/ml because it is the highest concentration. It was hypothesised that, the higher the concentration, the greater the effect of the pathogen will be on the host plant's mineral nutrient status.

4.2 Results

4.2.1. *F. proliferatum* alters the macronutrient content in wheat plants

Calcium (Ca)

For SST 015, contrasting responses in calcium accumulation in shoot and roots was observed in response to pathogen infection. Calcium content in the shoots of SST 015 was reduced by *F. proliferatum* whereas an increase was observed in the roots, relative to the control. For SST 088, calcium accumulation in both shoots and roots was significantly reduced by *F. proliferatum* relative to the control (Table 4.1 A and B).

Potassium (K)

The results showed that K content in the shoots and roots of SST 015 was reduced by *F. proliferatum* relative to the control. A similar trend was observed for SST 088 where a significant reduction in shoot and root K content (Table 4.1 A and B). Therefore, *F*

.proliferatum caused a significant reduction in K content in the shoot and roots of both cultivars. However, the reduction in K content was much higher in SST 088 compared to SST 015 shoots.

Magnesium (Mg)

F. proliferatum restricted Mg accumulation in the shoots of SST 015 when compared to the control. Similarly, Mg content in the shoot of SST 088 was reduced in response to *F. proliferatum* albeit not to the level as observed for SST 015 (Table 4.1 A and B).

Table 4.1A. Effect of *F. proliferatum* on the element content within the shoots of SST 015 and SST 088.

	Shoots of SST 015 Control and Infected		**Shoots of SST 088 Control and Infected**	
Element	Av. Conc. (in ppm)	Av. Conc. (in ppm)	Av. Conc. (in ppm)	Av. Conc. (in ppm)
Ca	6,66 ± 0,04	5,68 ± 0,05	9,39 ± 0,04	6,28 ± 0,03
K	81,01 ± 0,30	75,30 ± 0,05	103,97 ± 0,33	74,25 ± 0,31
Mg	3,51 ± 0,06	2,80 ± 0,01	4,16 ± 0,04	2,80 ± 0,01

Table 4.1B. Effect of *F. proliferatum* on the element content within the roots of SST 015 and SST 088.

	Roots of SST 015 Control and Infected		**Roots of SST 088 Control and Infected**	
Element	Av. Conc. (in ppm)	Av. Conc. (in ppm)	Av. Conc. (in ppm)	Av. Conc. (in ppm)
Ca	28,40 ± 0,07	30,82 ± 0,09	24,66 ± 0,14	19,54 ± 0,04
K	50,45 ± 0,08	48,24 ± 0,02	45,24 ± 0,01	41,66 ± 0,08
Mg	3,56 ± 0,03	4,29 ± 0,03	3,56 ± 0,03	3,38 ± 0,01

4.2.2. *F. proliferatum* alters the micronutrient content of wheat plants

Copper (Cu)

Copper (Cu) content in the shoots and roots of SST 015 and SST 088 was measured in response *F. proliferatum* (Table 4.2 A and B). The results presented in Table 4.2 (A and B) shows that *F. proliferatum* did not alter Cu accumulation in the roots of SST 015, whereas there was a slight increase in the shoots. For SST 088, a slight decrease in Cu was observed in both the shoots and roots when compared to the controls. The more pronounced reduction in Cu was observed in the shoots of SST 088.

Iron (Fe)

The Fe content was observed to slightly decrease in the shoots of both cultivars. However, in the roots of SST 015 there was an increase in Fe content, compared to the control. For the roots of SST 088, a significant decrease in Fe content was observed in response to *F. proliferatum* (Table 4.2 A and B).

Manganese (Mn)

The results indicate a decline in the Mn content of the infected roots of both cultivars compared to their respective controls (Table 4.2 A and B). Furthermore, the SST 088 showed a larger decrease in Mn content compared to SST 015, which had a very slight decrease. The shoots showed a similar trend. Both cultivars Mn content within the infected plants had decreased significantly, with that of SST 088 more pronounced.

Zinc (Zn)

A significant decrease in Zn content in the shoots of SST 088 was observed in the *F. proliferatum* treatment relative to the control. The Zn content in the roots of SST 015 plants had no difference compared to their control. The roots of both cultivars showed significant decreases in their Zn content compared to their respective controls, with SST 088 having a more pronounced decrease (Table 4.2 A and B).

Table 4.2A. *F. proliferatum* alters the element content within the shoots of SST 015 and SST 088.

	Shoots of SST 015 Control and Infected		Shoots of SST 088 Control and Infected	
Element	Av. Conc. (in ppm)	Av. Conc. (in ppm)	Av. Conc. (in ppm)	Av. Conc. (in ppm)
Cu	0,10 ± 0,00	0,12 ± 0,00	0,16 ± 0,00	0,10 ± 0,00
Fe	0,20 ± 0,00	0,19 ±0,00	0,29 ± 0,00	0,20 ± 0,00
Mn	0,25 ± 0,00	0,21 ± 0,00	0,34 ± 0,00	0,22 ± 0,00
Zn	0,27 ± 0,00	0,27 ± 0,00	0,29 ± 0,00	0,18 ± 0,00

Table 4.2B. *F. proliferatum* alters the element content within the roots of SST 015 and SST 088.

	Roots of SST 015 Control and Infected		Roots of SST 088 Control and Infected	
Element	Av. Conc. (in ppm)	Av. Conc. (in ppm)	Av. Conc. (in ppm)	Av. Conc. (in ppm)
Cu	0,08 ± 0,00	0,08 ± 0,00	0,11 ± 0,00	0,09 ± 0,00
Fe	1,60 ± 0,02	1,64 ± 0,03	2,11 ± 0,03	1,44 ± 0,00
Mn	0,68 ± 0,00	0,61 ± 0,00	0,94 ± 0,01	0,81 ± 0,00
Zn	0,90 ± 0,01	0,73 ± 0,01	1,06 ± 0,01	0,76 ± 0,00

4.3 Discussion

The effect of mineral nutrients on plant disease is based on the following. Firstly, the observed effects of fertilization on a specific disease's incidence or severity. Secondly, the comparison of mineral concentrations in healthy or resistant tissues compared with diseased or susceptible tissues and thirdly conditions influencing the availability of a specific nutrient with disease (Gupta *et al.,* 2017). This method of studying the effect of mineral nutrients on diseases excludes other factors such as environmental conditions, biological activity and the growth stage of the plant, which also play a role (Meena *et al.,* 2017). Mineral nutrients play a role of either making a plant more resistant or susceptible to disease by their participation in

physiological and biochemical processes within the plant, which play a role in how the plant responds to pathogens (Marschner 1995).

4.3.1. *F. proliferatum* alters the mineral macronutrient content in wheat

Calcium (Ca)

Ca inhibits the formation of pectolytic enzymes produced by pathogens (Gupta *et al.*, 2017). Furthermore, Ca affects the susceptibility of plants in two ways. Firstly, adequate Ca levels within the plant play a role in the functioning and stability of plant membranes and thus when Ca levels drop, there is membrane leakage of low-molecular weight compounds, e.g. sugars and amino acids, from the cytoplasm to the apoplast, which promotes infection by pathogens (Marschner, 1995). Secondly, Ca is present as calcium polygalacturonates within the cell wall structure, which are essential within the middle lamella for cell wall stability (Dordas, 2008). Therefore, Ca plays a role in the formation of rigid cells in plants (Gupta *et al.*, 2017) which prevents the dissolution of the middle lamella by pathogens which thus enable penetration and infection by the pathogens (Marschner 1995).

When Ca levels drop as observed in the infected roots of SST 088 and the shoots of both SST 015 and SST 088, this decline indicates an increased susceptibility to various fungi (Dordas, 2008). Furthermore, Dordas (2008) explained that the fungus preferentially intrudes the xylem and cause cell wall disintegration of the conducting vessels which results in symptoms such as wilting. Therefore, the above information suggests that increased Ca levels under infection promotes the tolerance of the host plant to their respective pathogen, as observed in Table 4.1B.

Potassium (K)

Potassium plays a role in physiological and biochemical processes within the plant (Marschner 1995; Wang *et al.*, 2013). K act as a catalyst in ion transportation in plants. Furthermore, in plants K plays an essential role in the synthesis of starch, cellulose and proteins. Therefore,

when potassium levels are low or have decreased within a plant (as observed in the current study where K levels have decreased in both cultivars and in both roots and shoots), the cellulose becomes leaky, sugars and amino acids accumulate within the apoplast (Shumann, 2017).

Schumann (2017) showed that low K levels results in shorter root length and accumulation of amino acids, among other things. These defined characteristics were observed in the physiological data presented in this study. Therefore, a plant needs adequate levels of K under infection, so as to acquire an increased resistance towards parasites or pathogens (Schumann, 2017). Moreover, it has been reported that increased levels of K lowers the severity of *F. oxysporum* (Dordas, 2008). So perhaps increased levels of K would enhance the resistance of wheat to *F. proliferatum*. Since plants with decreased levels of K have high susceptibility to parasitic diseases because of the metabolic functions of K in plant physiology (Dordas, 2008).

Magnesium (Mg)

Magnesium is a component of chlorophyll as its central atom and thus plays a major role in photosynthesis by capturing light (Marschner 1995). In addition, Mg plays a critical role in transporting the phloem export of photosynthates. When Mg levels drop or are lower than normal (as also indicated by the results obtained in this study which show that Mg levels decreased in the roots of SST 088 and the shoots of both cultivars), amino acids and sucrose get deposited in the leaves of the plant and thus create a conducive environment for disease-causing pathogens to attack the plant (Huber and Jones 2013). On the other hand, some studies have concluded that the effect of Mg within the plant is to reduce disease severity of crops like wheat citrus (Debona *et al.,* 2016).

Mg-induced resistance and susceptibility depends on the factors that govern the uptake of Mg from the soil by the host plant. One of these factors is K-rich soils, which induce Mg deficiency

because of antagonistic interactions (Debnath *et al.*, 2015). Therefore, the availability of Mg within the plant was not only influenced by the pathogen, but also other elements that were present in the soil the plant grew in.

4.3.2. *F. proliferatum* alters the mineral micronutrient content of wheat plants

Zinc (Zn)

Zinc has a crucial function of activating various enzymes involved in metabolic pathways, especially in starch and protein synthesis (Gupta *et al.*, 2017). In addition, Zn also plays a role in maintaining the integrity of biomembranes (Marschner 1995; Huber *et al.*, 2012). Furthermore, lower Zn levels may result in the leakage of low-molecular-weight compounds, which create an environment suitable for the creation of a feeding substrate for the pathogen, because of this leakage (Graham and Webb 1991; Marschner 1995; Huber *et al.*, 2012).

The results obtained in this study shows that Zn content within the plant decreased in the infected plants compared to their respective controls. Marschner (1995) discusses these results and state that reduced Zn concentrations resulted in reduced sugars and amino acid accumulation. Therefore, adequate or elevated Zn concentrations within the plant results in increased tolerance to pathogens, while low or decreased concentrations lead to increased susceptibility of the host plant to that particular pathogen. An example of this is the application of Zn on wheat, which increased the plant's tolerance to *Fusarium solani* (Bolle-Jones and Hilton, 1956; Khoshgoftarmanesh *et al.*, 2010; Gupta *et al.*, 2017).

Manganese (Mn)

Manganese plays a role in the biosynthesis of phenol compounds and lignin (Graham and Webb 1991; Marschner 1995; Broadley *et al.*, 2012). Therefore, the ability of the roots to inhibit or resist fungal hyphae penetration into the roots depends on the level of Mn within the plant (Graham and Webb 1991). Thus, when Mn levels are low within the plant, as observed in the

results obtained in this study (for both cultivars and in both infected roots and shoots), such a plant has increased susceptibility to the invading pathogen.

Manganese enhances plant resistance to disease by activating plant peroxidases in the apoplast (Gupta *et al.,* 2017). Furthermore, Mn is said to control a number of pathogenic diseases (Huber and Graham 1999; Heckman *et al.,* 2003). Therefore, higher or increased Mn levels within the plant promote resistance to disease, while decreased or low levels of Mn promote increased susceptibility to pathogens.

Iron (Fe)

Iron is an interesting micromolecule in that it is required by both host plant and pathogens (Kieu *et al.,* 2012; Aznar *et al.,* 2015). Therefore, Fe has a complex role in plant-pathogen interaction. To elaborate, ROS formation is catalysed by Fe and at the same time the host plant may use Fe to increase oxidative stress in defence responses against pathogens. Therefore, plant response to iron deficiency and plant defence against pathogens share numerous features such as the use of common hormone signalling pathways and the secretion of phenolic compounds (Aznar *et al.,* 2015). Fe therefore has negative and positive effects on the host disease resistance and the host alone (Gupta *et al.,* 2017). The results obtained for Fe concentration within the plants indicated that Fe concentration increased in the infected roots of SST 015 compared to the control, while the infected roots of SST 088 and the shoots of both cultivars had decreased Fe concentrations (Table 4.2 A and B).

Due to this dual function of Fe, the effect of increased or decreased iron concentration is unclear and thus remains a contentious issue. However, what is clear is the effect of the pathogen on the Fe accumulation in the plant. To elaborate more on the uncertainty of the effect of reduced or increased Fe concentration, with reference to Fe susceptibility or resistance of wheat to *F. proliferatum,* the Fe increase observed could be either for fuelling cells with Fe to reach

metabolic needs that could be increased by pathogen attack or to accumulate ROS at the site of infection (Azna *et al.*, 2015)

Copper (Cu)

Decreased Cu levels in plants result in the alteration of lipid structure within the cell membrane, which is essential for resistance to biotic stress (Broadley *et al.,* 2012). In addition, Cu is a component of numerous enzymes crucial for the synthesis of lignin, which ensures membrane rigidity and stress of the cell wall (Marschner 1995; Broadley *et al.,* 2012). The results obtained in this study shows that, there was an increase in Cu accumulation of both infected roots and shoots of SST 015, compared to the controls. While there was a decrease of Cu accumulation in the infected roots and shoots of SST 088, compared to their controls (Table 4.2 A and B).

Low Cu levels within the plant results in reduced lignification and thus less resistant cell walls. Hence, making the plant more susceptible to disease. While the opposite is true with increase Cu levels (Marschner, 1995; Evans *et al.,* 2007; Gupta *et al.*, 2017).

4.4 In summary

Most studies of mineral nutrients focus on the application of these minerals in soils or the exogenous application of a particular element on a specific part of the host plant, in order to test the influence of that element in plant-pathogen interactions. However, despite the fact that an element is applied in the soil or exogenously, that element will be absorbed by the roots or leaves of the plant and its effect will be within the plant, influencing the susceptibility or tolerance of the host plant to its pathogen. In the current study, the effect of *F. proliferatum* on the mineral nutrient status of the plant was investigated to demonstrate which elements play a role in plant defence against the pathogen. Therefore, in a way this chapter provides integrated information, on how the pathogen affects the nutrient status of the host plant and the role these

mineral elements play on the susceptibility and/or tolerance of the plant during pathogen infection (using literature).

Based on the results obtained here, SST 015 appears to be more resilient towards the presence of *F. proliferatum* when compared to SST 088 given the accumulation of Ca, Mg, and Fe in the roots of SST 015, with enhanced levels of Cu in both roots and shoots. However, all mineral elements decreased in the root and shoots of SST 088 during infection.

CHAPTER FIVE

F. proliferatum differentially alters ROS accumulation and antioxidant capacity in wheat plants

5.1 Introduction

Plant protection strategies need to be enhanced in order to protect wheat from pathogens, as wheat is the second most important cereal in the world (Cendoya *et al.*, 2018). These pathogens include fungi, if not controlled can lead to various plant diseases (Choi *et al.*, 2018) and significant field losses (Singh and Sunder, 2012). Exposure to disease causing pathogens activate stress signalling, which disrupt essential metabolic processes such as photosynthesis, resulting in phytotoxic reactions such as lipid peroxidation (Apel and Hirt, 2004) and ultimate cell death (Gill and Tuteja, 2010).

Plants have numerous defence mechanisms to counteract the stress signals due to infection (Ferrigo *et al*., 2015; Andersen *et al.*, 2018). These defence mechanisms are aimed at inhibiting or restricting the damage that will result, should the infection progress without intervention. This plant resistance can be characterised by the formation of incomplete reduction of oxygen to water molecules, which are the basis for the formation of superoxide, hydrogen peroxide and other ROS markers (Magbanua *et al.*, 2007; Kumar *et al.*, 2009). Therefore, these ROS act as second messengers in many plant responses such as stomatal closure, lignin biosynthesis and programmed cell death, which play a role in inhibiting or restricting fungal pathogen infection. However, for ROS to act as second messengers, they must be at low concentrations (Sharma *et al.*, 2012). At high concentrations, ROS cause oxidative damage to fungal and/or plant cells (Demidchik *et al.*, 2014). Thus, the accumulation of ROS is mitigated by antioxidant enzymes which include superoxide dismutase (SOD), ascorbate peroxidase (APX),

peroxidases (POD) among others, that are known to play a role in defence against various stresses (Kumar *et al.,* 2009). Debona at al. (2012), showed an increase in antioxidant enzymes in both the susceptible and partially resistant wheat cultivars in response to *Pyricularia oryzae* infection. Therefore, quantifying the antioxidant enzyme activity in this study may provide insight into the effects of *F. proliferatum* infection in wheat plants.

Therefore, the aim of this chapter was to comparatively analyse ROS accumulation and antioxidant capacity of ROS metabolising enzymes in two wheat cultivars (SST 015 and SST 088), to *F. proliferatum* infection.

5.2. Results

5.2.1. *F. proliferatum* increases superoxide content in wheat plants

The results showed that *F. proliferatum* increased superoxide content in the shoots of both wheat cultivars (Figure 5.1). Superoxide content in the shoots of SST 015 was significantly augmented by *F. proliferatum* relative to the control, with the highest increased observed in the 10^5 spores/ml treatment (Figure 4.1 A). For SST 088, *F. proliferatum* increased (in a concentration dependent manner) superoxide content in the shoots relative to the control. With an increase in *F. proliferatum*, an increase in superoxide content was observed (Figure 4.1 B).

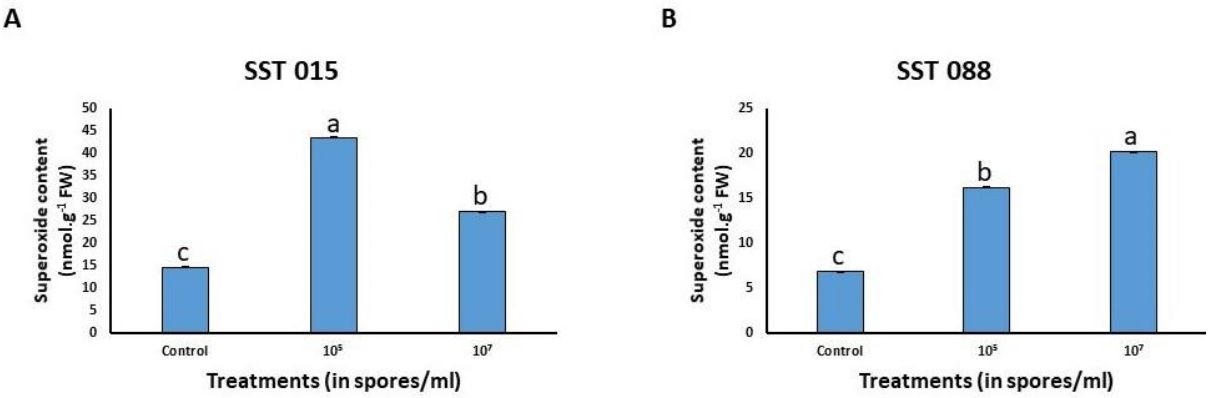


Figure 5.1. The effect of *F. proliferatum* on superoxide content in wheat shoots. Superoxide content for (A) SST 015 and (B) SST 088 was measured in response to *F. proliferatum*. Error bars are representative of the mean (± SE) of three independent experiments from three plants per treatment in each experiment.

The results obtained for the roots show the same trend of increasing superoxide content as the shoots for both cultivars. The highest increase is observed for plants infected with the lowest pathogen concentration of 10^5 spores/ml, while the lowest increase was observed in the highest pathogen concentration of 10^7 spores/ml. This was observed in both cultivars. More specifically, for SST 015, the increase observed in plants treated with 10^5 spores/ml was approximately twice as high when compared to those treated with 10^7 spores/ml, while the increase observed for 10^7 spores/ml was approximately twice as high when compared to the control (Figure 5.2 A). For SST 088, the concentration of superoxide observed in plants treated with 10^5 spores/ml was also twice as high as in those treated with 10^7 spores/ml and the plants treated with 10^7 spores/ml had an increase that was almost ten times higher when compared to the control (Figure 5.2 B). However, SST 088 has a much higher superoxide content compared to SST 015; i.e. SST 088 had an increment that was five times higher and four and a half times higher for the treatments 10^5 spores/ml and 10^7 spores/ml, respectively (Figure 5.2)

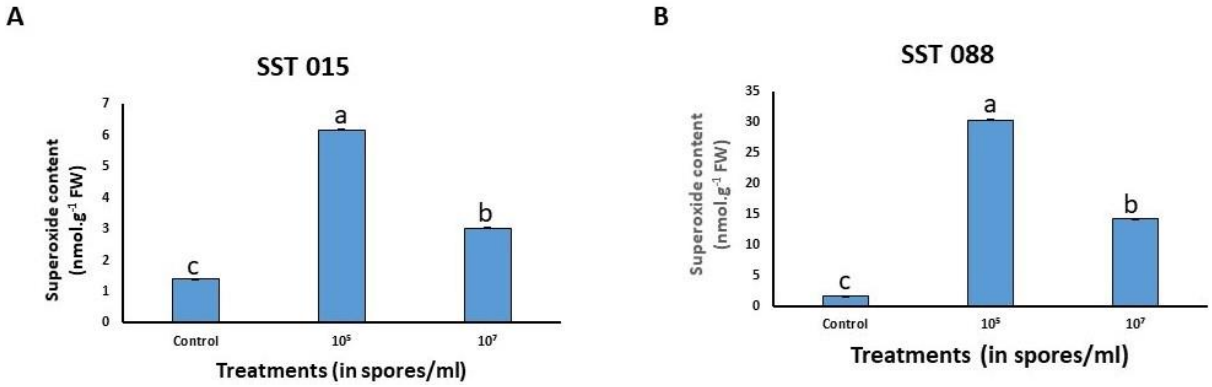


Figure 5.2. The effect of *F. proliferatum* infection on the superoxide content in wheat roots. Superoxide content for (A) SST 015 and (B) SST 088 was measured in response to *F. proliferatum*. Error bars are representative of the mean (± SE) of three independent experiments from three plants per treatment in each experiment.

5.2.2. Changes in hydrogen peroxide content in response to *F. proliferatum* infection

For SST 015 the results indicate an increment in hydrogen peroxide content of the shoots (Figure 5.3A), with the highest increment observed in the 10^5 spores/ml treatment and the lower increment in the 10^7 spores/ml treatment. The shoots of SST 088 showed a similar trend (Figure 5.3B). However, by comparing hydrogen peroxide accumulation in both cultivars, the results shows that the highest increment for 10^5 spores/ml treatment was observed in SST 088. While the highest increment in SST 015 was observed in 10^7 spores/ml treated plants.

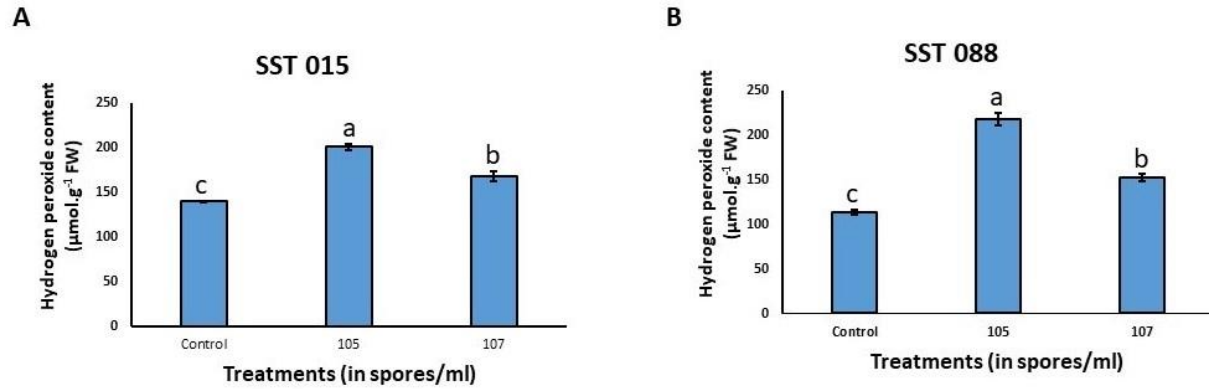


Figure 5.3. The effect of *F. proliferatum* infection on the hydrogen peroxide content in the shoots of *Triticum aestivum* L. Hydrogen peroxide content for (A) SST 015 and (B) SST 088 was measured in response to *F. proliferatum*. Error bars are representative of the mean (± SE) of three independent experiments from three plants per treatment in each experiment.

The results obtained for the roots showed an increase in the hydrogen peroxide content in both treatments for both cultivars (Figure 5.4 A and B). Contrary to the shoots, the trend of increase is as expected. The plants inoculated with 10^7 spores/ml showed the highest increase in hydrogen peroxide content, while those inoculated with 10^5 spores/ml have the lowest increment. When comparing the cultivars, SST 015 treated with 10^5 spores/ml show a much higher increase when compared to SST 088 of the same treatment. While SST 015 treated with 10^7 spores/ml show a lower increment as compared to SST 088 of the same treatment. These results indicate that there are contrasting responses, per treatment, in the hydrogen peroxide content in response to *F. proliferatum*.

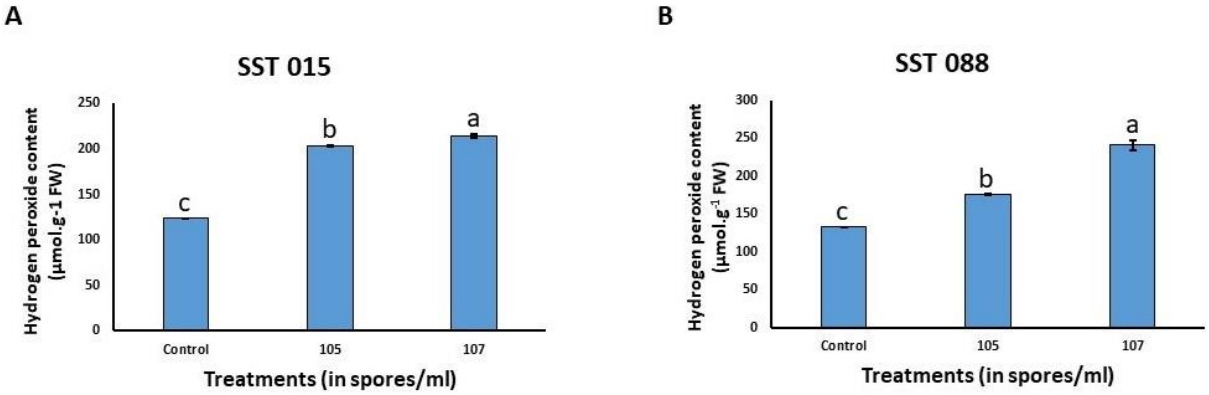


Figure 5.4. The effect of *F. proliferatum* infection on the hydrogen peroxide content in the roots of wheat plants. Hydrogen peroxide content for (A) SST 015 and (B) SST 088 was measured in response to *F. proliferatum*. Error bars are representative of the mean (± SE) of three independent experiments from three plants per treatment in each experiment.

5.2.3. Changes in malondialdehyde (MDA) content in response to *F. proliferatum* infection

The extent of lipid peroxidation was measured in the shoots and roots of wheats plants infected with *F. proliferatum*. The results showed that *F. proliferatum* increased MDA contents in shoots of both wheat cultivars relative to their controls (Figure 5.5). For SST 015, both concentrations of *F. proliferatum* enhanced MDA content with the highest increased observed in the lower pathogen concentration (Figure 5.5 A). For SST 088, *F. proliferatum* also

increased the shoot MDA levels relative to the control albeit not to the level observed for SST 015 (Figure 5. B). Contrasting to what was observed for SST 015, the increase in MDA was directly linked to an increase in pathogen concentration for SST 088.

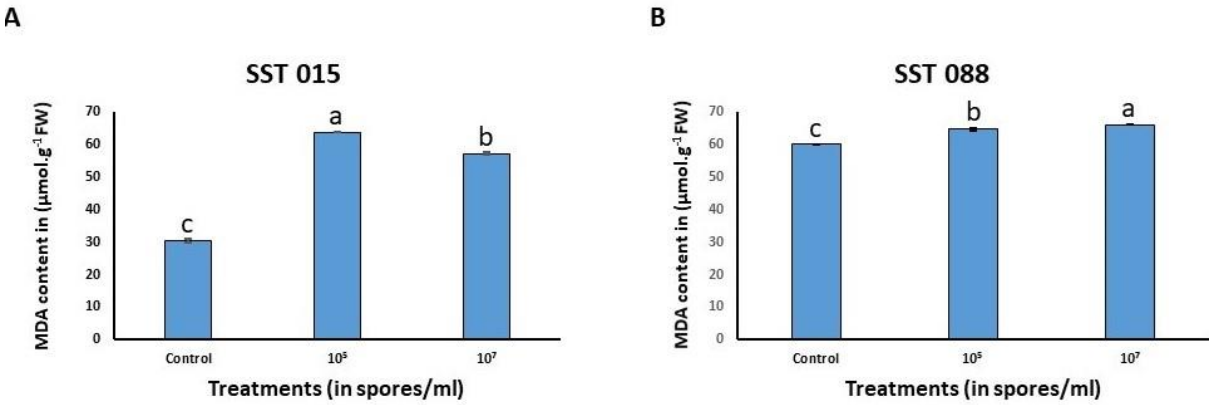


Figure 5.5. Changes in wheat MDA content in response to *F. proliferatum* infection. Shoot MDA contents for (A) SST 015 and (B) SST 088 was measured in response to *F. proliferatum.* Error bars are representative of the mean (± SE) of three independent experiments from three plants per treatment in each experiment.

Similar to what was observed in the shoots, *F. proliferatum* increased the MDA content in the roots of both cultivars (Figure 5.6). A gradual increase in MDA content was observed with an increase in pathogen concentration. For SST 015 (Figure 5.6A), a slight increase in MDA content was observed in the 10^5 spores/ml treatment relative to the control. SST 015 plants treated with 10^7 spores/ml showed a more pronounced increase in MDA content (in roots) relative to the control. For SST 088, an increase in MDA content was observed with an increase pathogen concentration (Figure 5.6B). When comparing the two cultivars, there were no significant changes between the cultivars. The same trend of increasing MDA content with increasing pathogen concentration was observed.

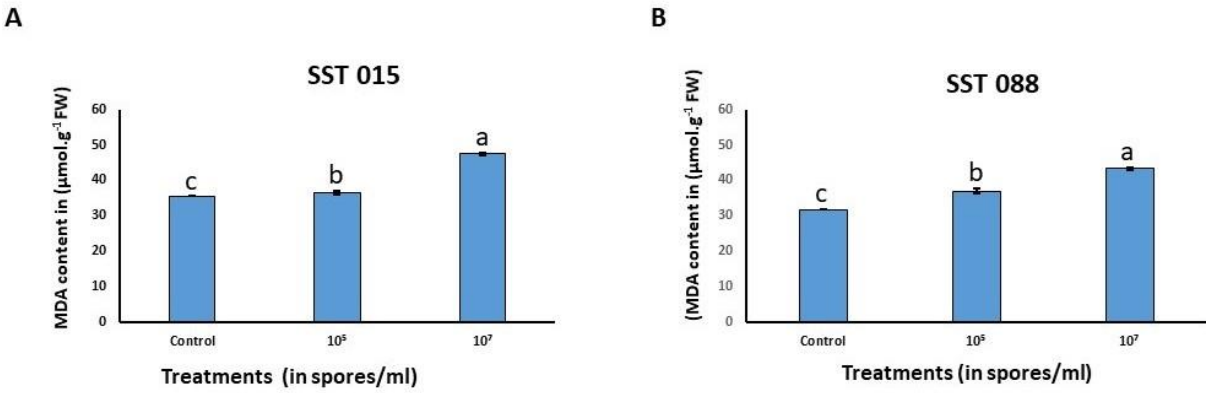


Figure 5.6. Changes in root MDA content in response to *F. proliferatum* infection. Root MDA contents for (A) SST 015 and (B) SST 088 was measured in response to *F. proliferatum*. Error bars are representative of the mean (± SE) of three independent experiments from three plants per treatment in each experiment.

5.2.4. *F. proliferatum* differentially alters SOD activity in two wheat cultivars

SOD enzymatic activity was detected using native polyacrylamide gel eletrophoresis and individual isoforms characterised using potassium cyanide and hydrogen peroxide. Due to the lack of protein obtained from the root tissues of both cultivars, SOD was only detected in the shoot material.

For SST 015, three distinct SOD isoforms were detected (Figure 5.7 A). These isoforms were characterised as manganese SOD (MnSOD), copper zinc SOD (Cu/ZnSOD) and iron SOD (FeSOD) respectively. The results showed that *F. proliferatum* differentially regulates the expression (band intensity) of each isoform. The activity of MnSOD diminished in response to *F. proliferatum* (at a concentration of 10^5 spores/ml) whereas the high concentration (10^7 spores/ml) did not alter MnSOD activity when compared to the control (Figure 5.7A). Similar trends were observed for Cu/ZnSOD and FeSOD. For SST 088, an increase in MnSOD activity was observed in response to *F. proliferatum* (10^5 spores/ml) compared to the control (Figure 5.7B). *F. proliferatum* at 10^5 spores/ml did not alter MnSOD activity when compared to the control. No significant change in activity was observed for Cu/ZnSOD and FeSOD relative to

the control. It is interesting to note the contrasting responses of two wheat cultivars to *F. proliferatum* infection. The high concentrations of *F. proliferatum* (in both cultivars) did not alter SOD activity when compared to the control whereas the lower concentration differentially regulated SOD activity.

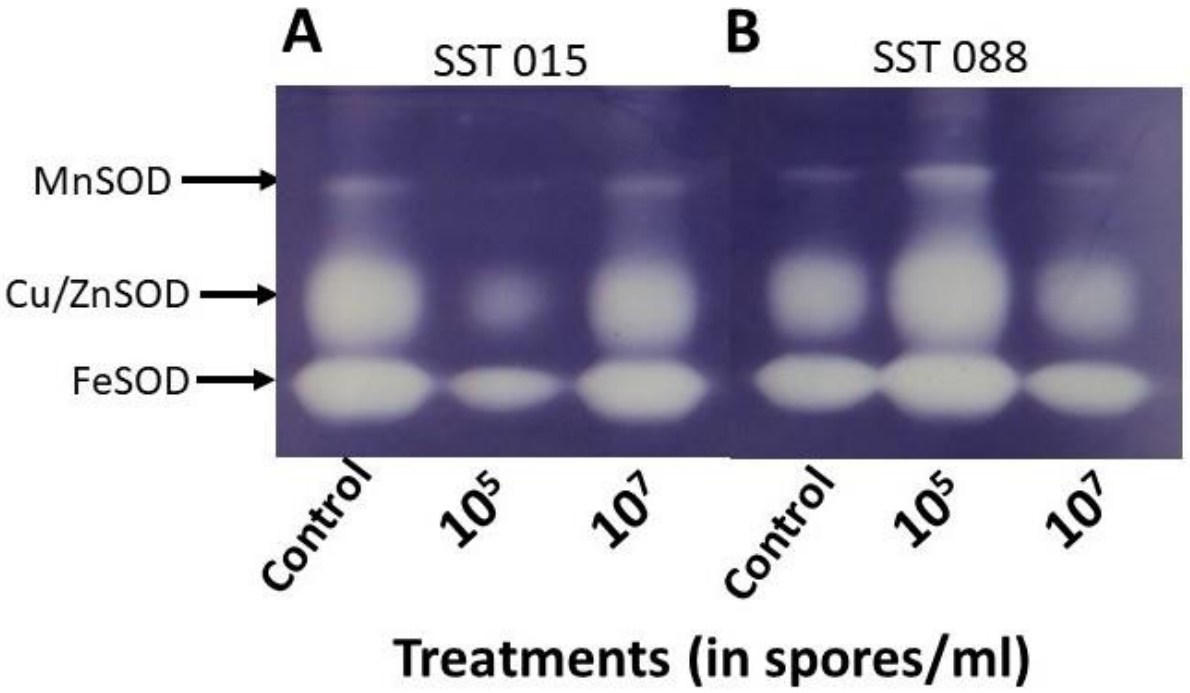


Figure 5.7. Changes in SOD enzymatic activity in the shoots of two wheat cultivars. The in-gel detection assays show the presence of individual SOD isoforms for SST 015 (A) and SST 088 (B).

5.2.5. The effect of *F. proliferatum* on ascorbate peroxidase (APX) activity in wheat plants

APX activity was detected in the shoots of two wheat cultivars (SST 015 and SST 088). In total four APX isoforms was detected in the shoots of SST 015 and SST 088 (Figure 5.8). For SST 015, APX 1 activity was significantly reduced in the 10^5 spores/ml treatment, with no change observed in the 10^7 spores/ml treatment relative to the control (Figure 5.8A). APX 2 activity was only detected in the control and 10^7 spores/ml treatment. The activity for APX 2 in the 10^7 spores/ml treatment was slightly reduced when compared to the control. A similar trend was

observed for APX 3. For APX 4, SST 015 plants treated with 10^5 spores/ml showed reduced activity when compared to the control whereas the opposite was observed in the 10^7 spores/ml treatment. For SST 088, the enzymatic activities of all four APX isoforms (APX 1-4) was significantly increased in response to 10^5 spores/ml when compared to the control (Figure 5.8B). On the other hand, APX activity of the 10^7 spores/ml treatment (as seen for all four isoforms) was decreased when compared to the control.

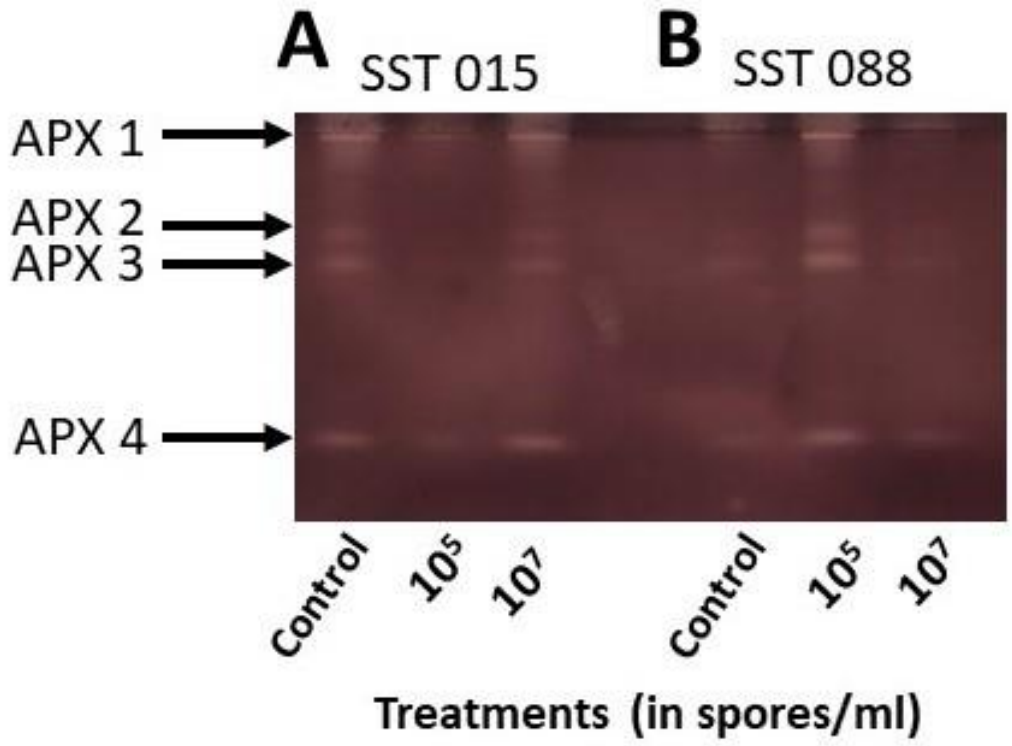


Figure 5.8. *F. proliferatum* differentially alters APX activity in two wheat cultivars (SST 015 and SST 088). The in-gel detection assays show the presence of individual APX isoforms for SST 015 (A) and SST 088 (B) in response to *F. proliferatum*.

5.2.6. The effect of *F. proliferatum* on peroxidase (POD) activity in wheat plants

POD activity was detected in the shoots of two wheat cultivars (SST 015 and SST 088). For each cultivar, five isoforms were detected (Figure 5.9). For SST 015, *F. proliferatum* (both concentrations) increase POD activity relative to the control (Figure 5.9A). A slight but significant increase in POD activity was observed in both treatments relative to the control for

POD 1. For POD 2, *F. proliferatum* increase the enzymatic activity with 10^5 spores/ml showing the highest activity relative to the control. A similar trend was observed for POD 3, POD4 and POD 6. Interestingly, POD 5 was only detected in the *F. proliferatum* treatments and not the control.

In contrast to what was observed for SST 015, the POD enzymatic activity for SST 088 decreased in response to *F. proliferatum* (Figure 5.9B). POD 1 was significantly reduced in the *F. proliferatum* treatments. A similar trend was observed for POD 2 and POD 3. Interestingly, POD 4 and POD 5 was only detected in the control and not the *F. proliferatum* treatments. Although a decrease in activity was observed for POD 6 in both *F. proliferatum* treatments, with 10^5 spores/ml showing the highest level of reduction. When comparing the two cultivars, in SST 015 showed an overall increase in POD activity, while SST 088 showed an overall decrease in POD activity.

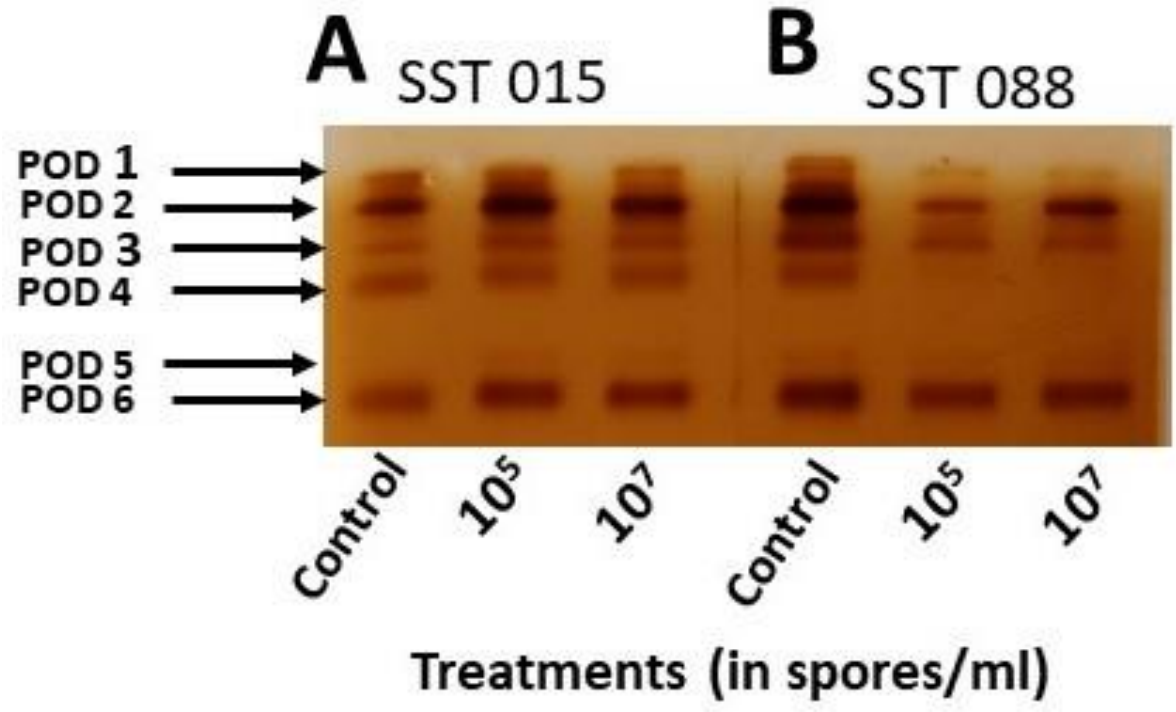


Figure 5.9. *F. proliferatum* differentially alters POD activity in two wheat cultivars (SST 015 and SST 088). The in-gel detection assays show the presence of individual POD isoforms for SST 015 (A) and SST 088 (B) in response to *F. proliferatum*.

5.3 Discussion

The results presented above investigated the effect of *F. proliferatum* on the oxidation state and antioxidant capacity of two wheat cultivars. Changes in ROS biomarkers (superoxide, hydrogen peroxide) and the extent of lipid peroxidation was measured in the shoots and roots of two wheat cultivars (SST 015 and SST 088). To measure the extent of oxidative damage caused by these ROS biomarkers due to pathogen infection, we detected and characterised differential changes in antioxidant enzyme activity to determine which isoforms contribute to total antioxidant capacity in wheat plants.

5.3.1. *F. proliferatum* augments ROS accumulation in wheat plants

5.3.1.1. *F. proliferatum* infection increases the concentration of superoxide

Biotic stress promotes the production of ROS such as superoxide (Suzuki and Mittler, 2006). ROS production is one of the first responses of plant tissues to elicitors and attack by pathogens (Chen *et al.*, 2015). According to the Foyer-Halliwell-Asada pathway superoxide (O_2^-) is the first ROS marker that is produced in plants (Yadav, 2010) and it has the ability to stimulate the production of other ROS molecules such as H_2O_2.

The results obtained here shows an increase in the O_2^-content of the infected plants compared to their respective controls. The highest increase was observed in the plants treated with the lowest concentration of *F. proliferatum*, with the exception of the shoots of SST 088, where the highest increase of $O_2^-$was observed in plants treated with the highest concentration of *F. proliferatum*. When comparing the cultivars, the highest increase of O_2^-in the roots was observed in SST 088, while in the shoots it was observed in SST 015. The results obtained in this study are similar to those reported by Waśkiewicz *et al.*, (2014) where susceptible and resistant wheat cultivars were infected with *Fusarium graminearum*. The results obtained in that study showed increased levels of superoxide after infection. Furthermore, the results showed fluctuating levels of O_2^- in the resistant cultivars. In the current study, SST 088 also

showed fluctuating levels of O_2^-. These fluctuations were attributed to the capturing of electrons originating from O_2^- by semiquinones (Waśkiewicz *et al.,* 2014). In order to understand the trends of O_2^- increments in infected plants versus the uninfected plants, it was logically inevitable to determine the activity and profile of superoxide dismutase (SOD) because it is associated with superoxide scavenging and removal.

5.3.1.2. Hydrogen peroxide (H_2O_2) accumulation in wheat plants in response to *F. proliferatum* infection

H_2O_2 is known to have a dual function in plants. Its function depends on its concentration within the plant tissues. Firstly, at low concentrations it acts as a signalling molecule (Cheeseman, 2007) involved in triggering tolerance to various biotic and abiotic stresses and at high concentrations induces cell death (Grant and Loake, 2000; Quan *et al.,* 2008). The results obtained in this study show an increased concentration of H_2O_2 in the roots of each cultivar. Thus, the highest increment was observed in the plants treated with 10^7 spores/ml, while the lowest increment is observed in plants treated with 10^5 spores/ml in both cultivars. When comparing the cultivars, SST 015 had the highest H_2O_2 concentration when treated with 10^5 spores/ml compared to SST 088. While SST 088 showed the highest increase in H_2O_2 when treated with 10^7 spores/ml of the pathogen, compared to SST 015. The shoots however had an interesting trend, although both treatments had higher H_2O_2 concentrations compared to the control. The highest increment of H_2O_2 was observed in plants treated with 10^5 spores/ml for both cultivars. Furthermore, SST 015 showed the highest H_2O_2 concentration when treated with 10^7 spores/ml compared to SST 088. While SST 088 showed the highest concentration when treated with 10^5 spores/ml of the pathogen, compared to SST 015. Various studies of wheat interacting with different fungal pathogens also observed an increase in H_2O_2

concentration after inoculation. (Waśkiewicz *et al.*, 2014; Chen *et al.*, 2015). The results obtained in these studies are consistent with those obtained in the current study.

5.3.1.3. *F. proliferatum* infection enhanced the extent of lipid peroxidation in wheat plants

Malondialdehyde (MDA) is a secondary product resulting from the oxidation of polyunsaturated fatty acids and it is an indicator of lipid peroxidation (Smirnoff, 1993). Lipid peroxidation is considered to be the most damaging process known to occur in every living organism (Gill and Tuteja, 2010). Thus, increased levels of lipid peroxidation are indicated by increased levels of MDA, as a result of ROS accumulation which induces cell death in plants. The results obtained in this study shows an increase in MDA levels of the infected plants compared to their respective controls, in both roots and shoots. These results are consistent with the results obtained in a study of wheat stripe rust, where MDA levels were increased due to fungal infection (Chan *et al.*, 2015). Furthermore, Kouadioa *et al.*, (2006), in a study of *Fusarium* wilt, recorded an increase in MDA levels post infection. The increase in the MDA levels post infection could be as a result of the generation and accumulation of ROS within the plant (Naguib, 2018).

5.3.2. *F. proliferatum* differentially alters the antioxidant enzyme activity in wheat plants

5.3.2.1. The effect of *F. proliferatum* on superoxide dismutase (SOD) activity in

The first line of plant defence against ROS in the list of enzymes whose function is to protect the plant against oxidative stress is superoxide dismutase (SOD). In the current study, the effect of *F. proliferatum* infection on the enzymatic activity of various SOD isoforms in wheat was investigated. SOD is one of the most abundant enzymatic antioxidants and it contributes majorly to the cellular redox state of the plant by protecting plants against oxidative damage (Smirnoff, 2000). The results obtained here showed that in SST 015 there was a decrease of

SOD activity in the plants inoculated with 10^5 spores/ml of pathogen compared to the control and there were no significant changes between the plants treated with 10^7 spores/ml and the control (Figure 4.8). However, in SST 088 there was an increase in SOD activity in plants treated with 10^5 spores/ml compared to the control and a slight decrease in the plants treated with 10^7 spores/ml compared to the control. In a similar study by Chen *et al.,* (2015), SOD activity was decreased after infection. This is consistent with the results obtained for SST 015. On the other hand Xingfu (1995) and Sorahinobar *et al.,* (2015) recorded an increase in SOD activity after inoculating various cucumber and wheat with downy mildew and F. *graminearum*, respectively. Furthermore, Alguacila *et al.,* (2003) also observed an increase in SOD activity post infection of mycorrhizal shrub species with arbuscular mycorrhizal (AM) fungi. These results are consistant with the results observed in 10^5 spores/ml treated plants in SST 088. The different concentrations of the pathogen seem to activate different mechanisms and pathways within the plant. Hence, the different responses of the plants to the pathogen. Therefore, the activated defence mechanisms and pathways are dependent on the concentration of the pathogen affecting or infecting the plant.

5.3.2.2 *F. proliferatum* differentially alters APX and POD activity in wheat plants

According to the Halliwell–Asada pathway, ascorbate peroxidase is a crucial enzyme, which converts H_2O_2 to water and oxygen. The enzyme uses ascorbate (AsA) as an electron donor in this reduction of H_2O_2 (Caverzan *et al.,* 2012; Pandey *et al.,* 2015). Ascobate is an antioxidant metabolite used by certain enzymes to scavenge ROS and it thus plays an indirect or direct role in H_2O_2 scavenging (Noctor and Foyer, 1998). Caverzan *et al.,* (2012) further explains that APX activity, along with other enzymatic antioxidants, increases in response to various environmental stress. This explanation is consistent with what was observed in the current study for SST 088 in response to 10^5 spores/ml of the pathogen. Moreover, the decrease in

APX activity in SST 015 in agreement with results of Chen *et al.,* (2015) that also indicated a decrease in APX activity infected wheat plants.

PODs are classified into three distantly related structural classes, found within the superfamily of fungal, bacterial and plant PODs (Welinder, 1992).. Class I is composed of mitochondrial yeast cytochrome c PODs, Class II are grouped according to all secretory fungal (manganese) PODs and Class III are of a glycoprotein nature, and are located in vacuoles and cell walls (Passardi *et al.,* 2005). The key role of PODs is to oxidize phenolic substrates at the expense of H_2O_2. Furthermore, PODs also play a role in auxin metabolism, lignin and suberin formation, cross-linking of cell wall components, phytoalexin synthesis, and the metabolism of ROS (Almagro *et al.,* 2009).

POD activity increased in SST 015 with the highest increase observed in the treatment (10^5 spores/ml). While for SST 088, a decrease in POD activity was observed, with the highest decrease observed in the 10^5 spores/ml treatment. Therefore, the results indicate that SST 015 shows an increase in POD activity while SST 088 shows a decrease in POD activity. Chen *et al.,* (2015) observed a similar trend of increasing POD activity after infecting wheat cultivars that were resistant to *Puccinia striiformis.* The increase in POD activity could also be as a result of overproduction of APX, which strengthen the ROS scavenging system and leads to oxidative stress tolerance (Sarowar *et al.,* 2005).

5.4 In summary

F. proliferatum differentially alter ROS accumulation in two wheat cultivars as seen for changes in superoxide, hydrogen peroxide and the extent of lipid peroxidation. Interestingly, the changes for ROS–induced oxidative damage caused by *F. proliferatum* is concentration dependent. While superoxide accumulation in the roots was highest in SST 088 in both

treatments, the opposite was observed in the shoots. H_2O_2 accumulation in the roots showed contrasting responses between the treatments. For the 10^5 spores/ml treatment, H_2O_2 was highest in SST 015, while for 10^7 spores/ml-treated plants, H_2O_2 accumulation was observed in SST 088. On the other hand the shoots showed the exact opposite. Moreover, there were no significant differences in the roots MDA content between the cultivars treated with 10^5 spores/ml. Whereas 10^7 spores/ml treatment had the highest accumulation in SST 015. However, in the shoots MDA accumulation was highest in SST 088 for both treatments.

Antioxidant capacity as seen for SOD, APX and POD activities as also differentially regulated by *F. proliferatum*. *F. proliferatum* reduced SOD activity in SST 015 but increased it in SST 088. A similar trend was observed for APX activity. However, *F. proliferatum* increased POD activity of SST 015 and the opposite was observed for SST 088.

CHAPTER SIX

Conclusion and future prospective

The aim of this study was to investigate the effect of *Fusarium proliferatum* on the physiological and biochemical responses of two wheat cultivars. We hypothesised that the higher concentration of the pathogen will result in enhanced oxidative damage to these plants. Thus, the highest pathogen concentration will be more detrimental to the plant growth and development than to the lower pathogen concentration. Interestingly, the hypothesis was proved wrong and instead we observed that the responses of the wheat cultivars was concentration dependent. To elaborate more on this, it seemed that the different concentrations of the pathogen stimulated and activated different pathways within the plant. This suggests that, the activated pathways within the plant do not just activate because of the presence of the pathogen but they also activate because of how much of the pathogen is present within the plant.

Physiological data, in particular: the overall seed germination, plant survival under infection, shoot and root lengths and the alteration of chlorophyll pigments, indicate that SST 088 displayed more traits of tolerance compared to SST 015. Furthermore, mineral nutrient accumulation indicated that SST 015 was more resistant to the pathogen compared to SST 088. However, Nutrient accumulation in a plant depends on many factors, such as element interaction within the soil, the regulation elements by plant hormones and the soil-borne pathogen, among others, which were outside the scope of this study. Therefore, the mineral nutrient data does not prove conclusively that SST 015 has more tolerance qualities compared to SST 088. Moreover, despite the contrasting nature of the results obtained in the biochemical responses of wheat to *F. proliferatum*, this study suggests that SST 088 is more tolerant compared to SST 015.

Therefore, this recent advancement will enable us to develop disease management strategies, which incorporate these findings. Furthermore, this study forms the basis for future and more detailed studies investigating the plant-pathogen interactions. Future studies could focus on the effect of the different concentrations of the pathogen at different growth stages of the plant, to investigate the extent to which the pathogen has affected the plant. Moreover, the effect of the different nutrients on the pathogen at different growth stages of the host plant could be studied as well, as opposed to the effect of the pathogen on the nutrients and incorporate the role of plant hormones in plant-pathogen interactions.

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