Biocontrol potential of fungal endophytes against *Fusarium proliferatum* in maize

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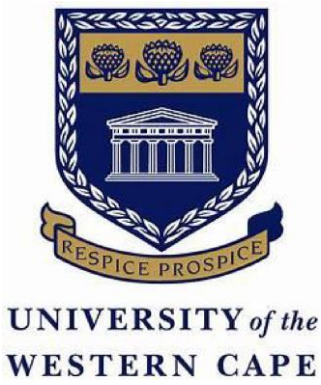

A thesis submitted in partial fulfilment of the requirements for the degree of Masters of Science, in the Department of Biotechnology, University of the Western Cape.

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Co-supervisor: Prof Marshall Keyster and Dr Arun Gokul
2022

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Stacey Fisher

KEYWORDS

1D SDS PAGE

Antioxidant enzymes

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Oxidative stress

Peroxidase

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Protein identification

Reactive oxygen species

Superoxide

Superoxide dismutase

ABSTRACT

Biocontrol potential of fungal endophytes against *Fusarium proliferatum* in maize

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MSc Thesis, Department of Biotechnology, University of the Western Cape

Fusarium proliferatum is a fungal pathogen that is the cause of numerous diseases in various crops of fruit and vegetables. About 25% of the maize crops harvested annually are affected by mycotoxins produced by *F. proliferatum* which causes huge economic fatalities to the agricultural and industrial services. Fungal endophytes are naturally occurring and ever-present in various host plants. Fungal endophytes exist symbiotically with host plants; thus, they gain nutrients whilst providing benefits to the host plant. Due to the harmful effects of fungicides, an alternative eco-friendly method is required to protect crops from pathogenic fungi such as *F. proliferatum*, this includes the use of fungal endophytes, however, its effects have not been elucidated in literature.

This study investigated the *in vitro* biological control of 12 fungal endophytes against the pathogenic fungi, *F. proliferatum*. Additionally, the study investigated the physiological effect of *F. proliferatum* on maize seed root growth and the priming of maize seeds with fungal endophytes (B3 and B4) to control *F. proliferatum in planta*. Moreover, this study investigated the biochemical responses of maize roots infected with *F. proliferatum* and roots primed with fungal endophytes (B3 and B4) and subsequently infected with *F. proliferatum*, respectively. Furthermore, this study also investigated how pathogenic infection and fungal endophytic priming influenced protein changes in maize roots using gel based proteomic analysis coupled with liquid chromatography mass spectrometry.

The results showed that the 12 fungal endophytic isolates showed an antagonistic effect (with different degrees) against *F. proliferatum*.

The results showed that infection with *F. proliferatum* significantly reduces root length. Contrary to what was observed for infected seeds, two fungal endophytes (B3 and B4) showed biocontrol of *F. proliferatum* whilst improving root length *in planta*. When pathogen-stressed plants were primed with the fungal endophytes, the negative effects observed in the infected treatment were reversed albeit not to the level of the untreated control.

A similar trend was observed for ROS accumulation as denoted by hydrogen peroxide content, superoxide levels and the extent of lipid peroxidation. The pathogenic infection and priming with fungal endophytes differentially altered antioxidant enzyme activity to control ROS metabolism. The results showed a low activity of superoxide dismutase and ascorbate peroxidase in infected roots and a higher activity in the roots primed with the fungal endophytes prior to infection, however, it showed a higher activity of superoxide dismutase and ascorbate peroxidase in roots primed with the fungal endophytes prior to infection and a decrease in activity in roots infected with the pathogen. Additionally, the results show that catalase, guaiacol peroxidase and peroxidase with catalase activity showed a differential activity in infected roots and roots primed with fungal endophytes and subsequently infected with *F. proliferatum*.

In the proteomic analysis we identified 127 proteins associated with roots across all treatments. The unique proteins identified in each of the treatments were functionally characterised to various subcellular compartments, molecular functions, and biological processes. The proteomics analysis revealed that there was an absence of antioxidant enzymes in seed infected with *F. proliferatum* relative to the biocontrol treatment which showed the presence of aldehyde dehydrogenase and superoxide dismutase. Additionally, it revealed that heat shock proteins and ferritin proteins were present in the infected seeds. Moreover, pathogen defence-related proteins such as the indole-3-acetaldehyde oxidase, bowman-birk type wound-induced proteinase inhibitor, isoflavone reductase homolog, non-specific lipid-transfer protein, and aquaporin PIP2-6 were present in seeds primed with B3 prior to infection with *F. proliferatum*. Furthermore, the protein biomarkers identified in this study are putative candidates for genetic improvement of pathogenic tolerance in maize plants.

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“Science never solves a problem without creating ten more.”

-George Bernard Shaw

“Don’t stop until you’re proud.”

“Surely God is my salvation; I will trust and not be afraid. The Lord, the Lord, is my strength and my song; He has become my salvation.”

-Isaiah 12:2

LIST OF ABBREVIATIONS

1D SDS PAGE	one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis
$^1O_2$	singlet oxygen
ABA	abscisic acid
ANOVA	one-way analysis of variance
APX	ascorbate peroxidase
AQP	aquaporin
BBI	bowman-birk inhibitor
BCA	biological control agents
CBB	coomassie brilliant blue
CO_2	carbon dioxide
Cu/Zn SOD	copper zinc superoxide dismutase
dH_2O	distilled water
DHAR	dehydroascorbate reductase
DTT	dithiothreitol (Cleland's reagent)
EDTA	ethylenediaminetetraacetic acid
ETI	effector-triggered immunity
F. proliferatum	*Fusarium proliferatum*
FCRR	*Fusarium* crown and root rot
Fe-SOD	iron superoxide dismutase
FORL	*Fusarium oxysporum* f. sp. *radicis-lycopersici*
GA	gibberellin

GPOX	guaiacol peroxidase
GPX	glutathione peroxidase
GST	glutathione-S-transferase
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HR	hypersensitive response
HSP	heat shock protein
HSR	heat shock response
IAA	indole-3-acetic acid
IAAId	Indole-3-acetaldehyde oxidase
IBBWP	bowman-birk wound-induced proteinase inhibitor
ITS	internal transcribed spacer
KCN	potassium cyanide
KI	potassium iodide
LC-MS	liquid chromatography mass spectrometry
MDA	malondialdehyde
MDHAR	monodehydroascorbate reductase
Mn-SOD	manganese superoxide dismutase
MRL	maximum residue limits
NaCl	sodium chloride
NaOH	sodium hydroxide
NBT	nitrotetrazolium blue chloride
ns-LTP	non-specific lipid-transfer protein

O^{2-}	superoxide
O_2	molecular oxygen
OH•	hydroxyl radical
PAGE	polyacrylamide gel electrophoresis
PCC	protein containing complex
PCD	programmed cell death
PDA	potato dextrose agar
PIP	plasma membrane intrinsic protein
PLTP	phospholipid-transfer protein
POD	peroxidase
PRR	pattern recognition receptor
PTI	PAMP-triggered immunity
PTM	post translational modification
PVP	polyvinylpyrrolidone
PVPP	polyvinylpolypyrrolidone
R	resistance protein
RO•	alkoxyl
ROO•	peroxyl
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
SIP	small basic intrinsic protein
SOD	superoxide dismutase
TBA	thiobarbituric acid

TCA	trichloroacetic acid
TEMED N,N,N',N'	tetramethylethylenediamine
TFA	trifluoroacetic acid
TIP	tonoplast intrinsic protein

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Chapter 1

Literature Review

1 Introduction

Maize has been established as the second greatest crop and most produced cereal worldwide (Santpoort, 2020; International Plant Biotechnology Outreach, 2017). In excess of 300 million people, in Africa, depends on maize as their primary food source. Almost 1 billion tons of maize is grown in 170 countries on approximately 180 million hectares of land (International Plant Biotechnology Outreach, 2017). Globally, 90 % of the maize produced is yellow, however, the production of white maize is over 90 % in Africa alone (Ekpa *et al*., 2018).

According to the Organisation for Economic Co-operation and Development and the Food and Agriculture Organisation Agricultural Outlook (OECD/FAO), the rapid increase in the African population drives the increase in the need for food. Approximately 90 % of the increase in cereal crop production is due to the growth in population (Santpoort, 2020).

Maize is generally grown for food and the requirement for maize is intimately associated with the increase in population (Santpoort, 2020). Although it is grown worldwide, the season and area where maize is produced causes fluctuation in yield. The typical yield per hectare in many African countries (~2 tons per hectare) is extremely low compared to the rest of the world (5.5 tons per hectare) (International Plant Biotechnology Outreach, 2017). This is mainly due to various abiotic and biotic stress factors (Shiferaw *et al*., 2011). Abiotic stresses include extreme environmental temperatures, drought, ineffective soil fertility, and inadequate post-harvest management strategies whereas biotic stresses include bacteria, viruses, stem borers, nematodes, and fungi (Shiferaw *et al*., 2011). The most often deemed principal reason of grain loss is insects, with fungi ranking as second as the causative agent for maize crop losses (Fandohan *et al*., 2003). There are various pathogenic fungal species that contaminate grain, from which the *Aspergillus*, *Penicillium*, and *Fusarium* species are the most prominent contaminants of maize and producers of mycotoxins. (Fandohan *et al*., 2003; Krnjaja *et al*., 2017). The contamination of maize crops by pathogenic fungi and their respective mycotoxins may occur in the field throughout growth, harvest, and during storage until consumption (Krnjaja *et al*., 2017). A study by Ekwomadu *et al*. (2018) analysed 100 maize samples for fungal contamination and found a predominance of *Fusarium* (82 %), *Penicillium* (63 %), and *Aspergillus* (33 %) species in comparison to other genera.

Fusarium spp. are significant plant pathogens that affect a variety of hosts including cereal crops, which is suggestive of its astonishing ability to adapt to various climate conditions and nutrient circumstances, to negatively impact food safety by the reduction in crop yield (Isack *et al*., 2014; Jian *et al*., 2019). *Fusarium proliferatum* is a toxigenic species that produces a variety of mycotoxins such as fumonisins, moniliformin beauvericin, fusaproliferin and fusaric acid (Armengol *et al*., 2005). Mycotoxins are secondary metabolites that are made by several fungi in essential foodstuffs (Fandohan *et al*., 2003). These mycotoxins cannot be removed during procedures such as pasteurization, baking, cooking, roasting pasteurization (Kamle *et al*., 2019). Mycotoxins impact the marine and terrestrial ecosystems as well as animal and plant biodiversity (Mahmood *et al*., 2016; Meftaul *et al*., 2020).

About 25% of the maize crops harvested annually are affected by mycotoxins which causes huge economic losses to the agricultural and industrial services therefore it is important to control the growth of these disease-causing pathogens as well as prevent the production of mycotoxins which influences a number of downstream processes (Agriopoulou *et al*., 2020; Kamle *et al*., 2019). Fungi have developed resistance to a number of conventional chemical treatments and thus a more efficient method would be to incorporate microorganisms in the treatment that is able to control fungal growth (Deepthi *et al*., 2016). Biological control agents (BCA's) offer a better alternative to manage soilborne illness than chemical pesticides (Wei *et al*., 2019). Bio-pesticides are natural products derived from plants, animals, microorganisms, and other particular materials (Saad *et al*., 2019). These products are an eco-friendly way to manage agricultural pests and are specific for target organisms (Saad *et al*., 2019; Wei *et al*., 2019). Fungal endophytes possess the ability to enhance host defence against diseases and decrease the damages caused by infection of pathogenic microorganisms (Fadiji and Babalola, 2020).

The aim of this review is to investigate the impact of *F. proliferatum* on maize growth and explore the biocontrol potential of endophytic fungi against these disease-causing pathogens.

2 Maize seed germination, growth, and development

Maize (*Zea mays L*.) is the utmost essential grain crop in South Africa and is grown all over the country in varied environments (Du Plessis, 2003). It is a socioeconomically significant crop used as animal feed, industrial resources as well as in human diets (Shin *et al*., 2014).

The effective production of maize is reliant on the accurate use of production inputs that supports the environment and agricultural production (Du Plessis, 2003). These inputs include but are not limited to financial capital, disease and insect control, method of harvesting, plant population, marketing, fertilisation, soil cultivation, adapted cultivars, and weeding (Du Plessis, 2003).

There are approximately 50 diverse variations of maize that are grown worldwide. Classification of maize can be achieved based on the size, shape of the kernel, taste and colour. The two major kernel shapes are tooth shaped (dent maize) and round (flint maize) (International Plant Biotechnology Outreach, 2017). The most common colours are yellow, red, and white; however, light red, red-brown, pale yellow, black, and orange kernels can also be produced (Ranum *et al*., 2014).

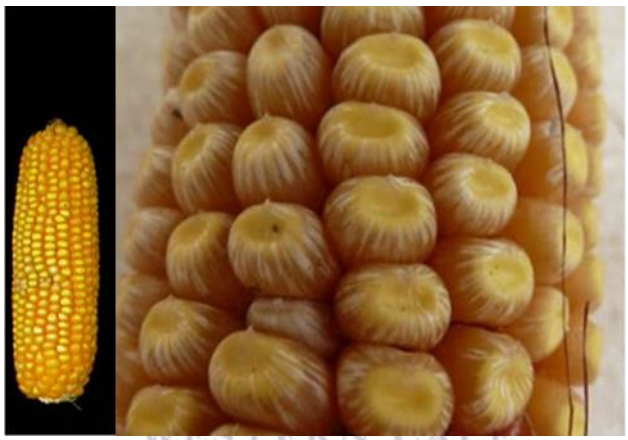


Figure 1.1 An example of yellow maize kernels. Adapted from Maschietto *et al*. (2017).

The maize seed goes through various stages throughout its life cycle, such as seed germination and emergence, early vegetative development, late vegetative development, and the reproductive stage to produce fruits (Montgomery and Brown, 2008).

2.1 Seed germination

Seed germination is an essential phase in crop production, and directly impacts maize grain quality and yield (Han *et al*., 2020). Seed germination begins with the hydration of the seed and ends with the emergence of the embryonic axis (commonly the radicle) from the seed coat (Figure 1.2; Figure 1.3) (Srivastava, 2002).

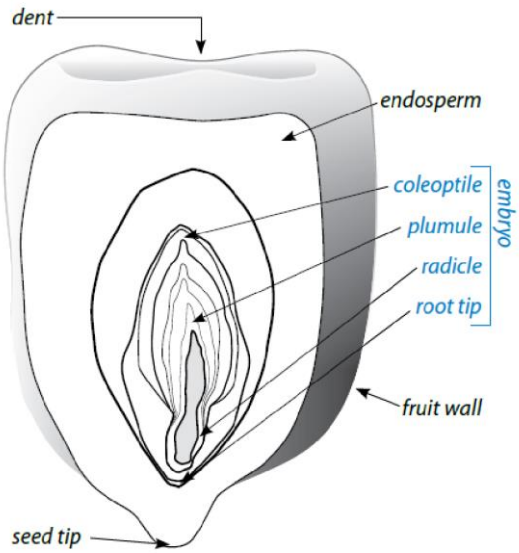


Figure 1.2 The anatomy of a maize seed. Adapted from Montgomery and Brown (2008).

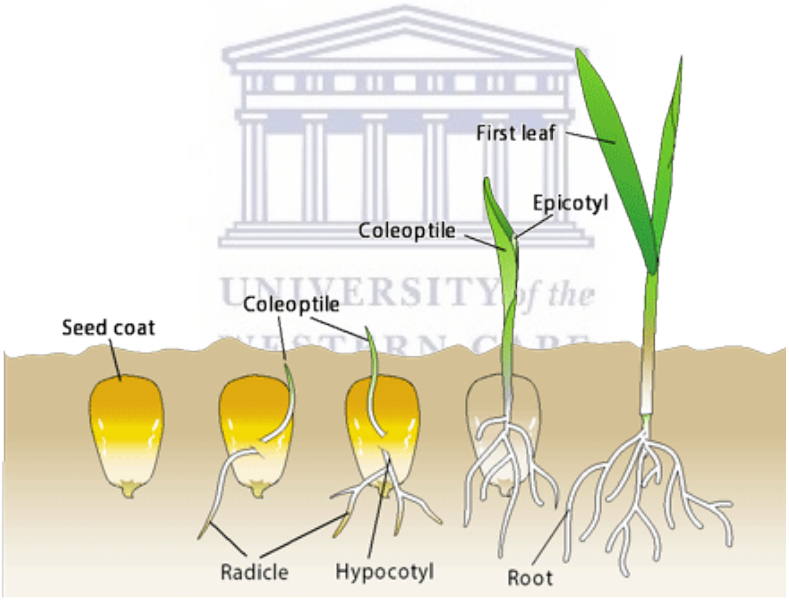


Figure 1.3 The germination and root emergence process in maize seedlings. Adapted from Agronomy eUpdates (2018).

The basic components of seeds include the endosperm, embryo, and the seed coat. The vital elements needed for the new plant are encapsulated within the embryo that develops after germination of the seed (Locascio *et al*., 2014). Seed germination represents a developmental transition from dormancy to an active metabolic state, where the reserves within the seed are used for seedling establishment (Han *et al*., 2020). The germination of seeds is initiated with the uptake of water, known as imbibition, and ends when the radicle emerges from the seed

coat, followed by the introduction of translation, transcription, cellular division, and energy metabolism (Rajjou *et al*. 2012; Weitbrecht *et al*., 2011). The progression of seed germination is regulated by several environmental and genetic factors (Han *et al*., 2020).

At the molecular and physiological levels, the process of germination involves the regulation of transcription factors (TF's) and hormones in order to guarantee the consistent interchange of signals between the various seed compartments (Gutierrez *et al*., 2007; Locascio *et al*., 2014) The optimum concentration of phytohormones, such as auxin, gibberellins (GA), ethylene, and abscisic acid (ABA), promote the improvement of germination performance as well as the growth and yield of the crop under various growth conditions (Han *et al*., 2020).

The availability of water and its movement into the seeds is incredibly important in order to stimulate germination, initial root growth, the elongation of shoots and the establishment of a uniform stand. The imbibition process occurs due to distinctive differences in levels of osmotic potential between the dry seed and the water in the substrate of germination. Nevertheless, the seeds require a satisfactory level of hydration in order to reactivate seed metabolic processes. Ordinarily, the seed water content of cereal crops has to reach at least 35 to 45% of its dry mass in order for the germination process to begin (Queiroz *et al*., 2019).

Reactive oxygen species (ROS) play a significant role in a number of events throughout seed development and are typically produced from embryogenesis to germination. Though ROS have mainly been deemed detrimental to the seeds, they also act as a positive signal in the release of seed dormancy. The 'oxidative window for germination' hypothesis expects that high or low levels of ROS obstruct seed germination (Zhang *et al*., 2018).

Seed germination involves three distinct phases: (1) imbibition/priming, (2) lag phase, and (3) radicle emergence and growth (Fu *et al*., 2011; Tian *et al*., 2014). Priming prolongs the lag phase, which permits pre-germinative biochemical and physiological processes to occur but prevents germination. Tian *et al*. (2014), showed that seed priming considerably increases the rate of germination in maize. The increase in seed germination by priming was previously reported by Murungu *et al*. (2004) and Basra *et al*. (2006). There is an increase in evidence which suggests that seed priming can alter crop performance from a molecular, physiological, and biochemical viewpoint (Tian *et al*., 2014).

Seed germination begins with the absorption of water and with this process starts a series of complex chemical and physical processes which includes activation of a number of enzymatic

systems, degradation of storage substances and membrane repair activities. Therefore, the soluble protein and small molecular products levels increase and provide nutrients, energy and protein that are specific for seedling growth (Cao *et al*., 2019).

Additionally, soluble proteins act as vital osmotic regulatory substances in adverse environments (Cao *et al*., 2019). The rise in and accumulation of soluble proteins acts as a defence strategy, where the water-holding capacity of the cells are improved and plays a role in protecting the cells important biological material and cell membranes. In a study by Cao *et al*. (2019), high soluble protein accumulation was observed after seed priming which suggest that high concentrations of soluble proteins in primed waxy corn seedlings can protect and stabilize membranes from damage-associated to functions pertaining to membrane formation and repair under low temperature stress.

The lag phase is an important period for the kernel development in maize. The fundamental capacity of the endosperm to accumulate dry matter, known as kernel sink capacity, is established in the lag phase and is a function of the number of endosperm cells formed by cell division and the amount of starch granules that are formed via amyloplast biogenesis. Environmental factors are able to disrupt the kernel sink capacity and therefore the kernel development and grain yield (Cheikh and Jones, 1994).

During the lag phase, there is very little net gain of water; there is a uniform state between the amount of water taken up from the seed's environment and the amount of water lost by the seed due to evaporation (Shen-Miller *et al*., 1995). The seed coat then ruptures and the radical and plumule begins to emerge from the seed. Thereafter, the seed activates its internal physiology and begins to respire (Xue *et al*., 2021). The expansion of the embryo completes germination, and the radical begins to emerge (Shen-Miller *et al*., 1995).

2.2 Vegetative growth

In the early stages of growth, the leaves and stems are not distinguishable due to the fact that the growing point (whorl) stays underground until the first five leaves emerge (Montgomery and Brown, 2008; Xue *et al*., 2021). The adventitious root system grows from the initial stem node below the surface of the soil and takes over the function of the main root approximately 10 days after emergence occurs (Cao *et al*., 2019; Montgomery and Brown, 2008; Xue *et al*., 2021). All the leaves the maize plant will produce occurs by a single growing point below the surface of the ground throughout the first two to three weeks of growth (Montgomery and

Brown, 2008). Once the three weeks after emergence is complete, the growing point is at the surface of the soil and develops an embryonic tassel (Cao *et al*., 2019; Montgomery and Brown, 2008).

The late vegetative developmental stage is the most important stage in the maize plant's development (Montgomery and Brown, 2008; Xue *et al*., 2021). The stem rapidly elongates, and the plant grows, and it therefore has a high demand for nutrients such as phosphorous, nitrogen and potassium as well as water (Cao *et al*., 2019). After the tassel develops, the ears begin to form and the ear size is determined (Montgomery and Brown, 2008; Xue *et al*., 2021). First, the number of rows per ear is determined and then the kernels per row. Any damage to ear structure and pollen at this stage will be permanent (Montgomery and Brown, 2008).

2.3 Reproductive growth

Maize is a monoecious plant, meaning that each plant possesses both female and male flowers (Cao *et al*., 2019; Montgomery and Brown, 2008; Xue *et al*., 2021). The male flowers are responsible for the production of pollen and are found on the tassel (Cao *et al*., 2019). When the tassel is visible, the innermost leaf in the growing point will be the last leaf produced. The female flowers receive the pollen and is carried in the ears of the plant (Cao *et al*., 2019; Xue *et al*., 2021). The resultant pollinated female flower develops into kernels (Montgomery and Brown, 2008).

2.4 Biotic stress factors that influences maize growth

Seeds harbour bacteria and phytopathogenic fungi that can develop during the seed's germination period, which delays germination and therefore kills the seed (Santos *et al*., 2021). Among the major pathogens transmitted by seeds are the fungi from the *Penicillium* genus, which can infect a wide variety of economically important crop plants (Santos *et al*., 2021).

Seed borne pathogens can cause germination failure as well as the reduction of seedling vigour, which ultimately causes a decrease in the overall yield. A study done by Shetty (1988) showed that fungi accounted for 75% of seed-borne pathogens which cause diseases such as blight, rot, necrosis, and discolouration (Alabi *et al*., 2005).

Fungi from the genera *Fusarium, Aspergillus, Penicillium* and *Rhizoctonia* are known to produce mycotoxins which impacts maize seed quality and decrease seed viability. The etiological agents of disease can enter host plants by indirect penetration by natural openings such as the stomata, lenticels and hydathodes and via wounds, and by direct penetration using

mechanical force. As a substitute to direct penetration, fungi infect foliage and develop infectious structures that consider the stomata as their penetration route. The stomata's dysfunction therefore affects the physiological processes of the host seedling (Garuba *et al*., 2014).

Maize seedlings and seeds are vulnerable to seed and soilborne diseases as a large number of seeds may decay before or after germination. Additionally, infected plants may suffer from reduced ear size, stunted growth, and in severe conditions may die due to poor root system. (Vincelli, 2008). Control of seedborne fungal pathogens with chemical fungicides are not only costly but are extremely hazardous to the environment (Debnath *et al*., 2012).

3.1 The impact of *Fusarium* spp. on maize growth and development

The genus *Fusarium* belongs to the family *Nectriaceae* and are known as saprophytes in plants and soil around the world. *Fusarium* spp. can inhabit the rhizospheres of these plants and are consequently able to penetrate the plant system (Kamle *et al*., 2019). *Fusarium* spp. are septate hyaline moulds that are found in soil and on plants in hot and moderate environments (Palmore *et al*., 2010).

3.1.1 *Fusarium proliferatum*

Fusarium proliferatum (teleomorph *Gibberella* intermedia) is a filamentous ascomycete saprophytic pathogenic fungus that is distributed worldwide and has been related to an assortment of diseases in vital economical florae (Figure 1.4 A and B) (Gao *et al*., 2017; Li *et al*., 2012; Sun *et al*., 2018; Sun *et al*., 2019). The various plant families that are infected by the *F. proliferatum* fungus include but are not limited to garlic, asparagus, onion, tomato, maize, rice, banana, date palm and soybean (Gao *et al*., 2017; Kamle *et al*., 2019). *F. proliferatum* resides on the plant and yields a great amount of conidia that can live in the soil for many years (Gao *et al*., 2017). As the weather and environment gets warmer, the conidia germinate and are therefore able to spread via the movement of rainwater and atmospheric dust, and subsequently able to infect seeds, soil, and plant material (Gao *et al*., 2017; Isack *et al*., 2014).

Figure 1.4 (A) Macroscopic characteristics of *Fusarium proliferatum*. (B) Microscopic characteristics of *Fusarium proliferatum*. Adapted from Masratul Hawa *et al*. (2013); Husain *et al*. (2017).

Fusarium spp. can cause ear rot, stalk rot, seedling blight and root rot (Okello *et al*., 2019; Wang *et al*., 2021). In addition to causing the physiological deformities (Figure 1.5 A and B). *F. proliferatum* is a producer of mycotoxins that, if not controlled, can be detrimental to human and animal health (Fandohan *et al*., 2003).

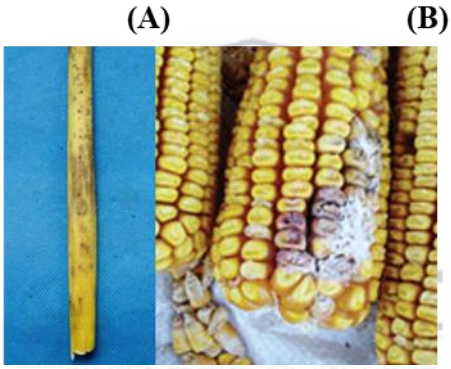


Figure 1.5 (A) Maize sheaths inoculated with *Fusarium proliferatum*. (B) Maize ear rot caused by *Fusarium proliferatum*. Adapted from Ncube (2012); Wang *et al*. (2021).

Mycotoxins are secondary metabolites produced by several fungi in essential products (Fandohan *et al*., 2003). Many of them are deemed to be imperative worldwide, but the five most common and well documented include zearalenone, aflatoxins, ochratoxin, deoxynivalenol/nivalenol, and fumonisins (Fandohan *et al*., 2003). *F. proliferatum* produces mycotoxins such as fumonisins, moniliformin and fusaproliferin (Isack *et al*., 2014; Jeney *et al*., 2004). The toxic secondary metabolites known as mycotoxins produced include moniliformin, fusaproliferin, fusaric acid, beauvericin, and fumonisins (FB1 and FB2) (Gao *et al*., 2017; Li *et al*., 2012). Fusaric acid is a familiar phytotoxin that causes wilt disease in a variety of plants. Furthermore, this mycotoxin has low to moderate synergistic properties with other mycotoxins (Li *et al*., 2012).

About 25% of the maize crops harvested annually are affected by mycotoxins, which results in huge economic losses to the agricultural and industrial services (Kamle *et al*., 2019). These mycotoxins cannot be removed during procedures such as pasteurization, baking, cooking, and roasting (Kamle *et al*., 2019).

3.1.2 Reactive oxygen species (ROS) accumulation and antioxidant capacity

In the case of the interactions between plants and pathogens, programmed cell death (PCD) is a welcomed event for the host plant, as pathogen-triggered cell death is detrimental for the plant (Zhang *et al*., 2019). Numerous necrotrophic fungal pathogens, such as *F. proliferatum*, produce mycotoxins that can induce ROS accumulation which eventually causes PCD (Gechev *et al*., 2004; Stone *et al*., 2000). ROS are naturally produced during respiration and photosynthesis via the electron transport chain (Zhang *et al*., 2019). Moreover, biotic stresses such as the infection with pathogenic fungi can drastically increase the accumulation and production of ROS (Zhang *et al*., 2019).

ROS are vital for signalling in various developmental and growth-related processes as well as in comprehending abiotic and biotic stresses along with PCD (Bailey-Serres and Mittler, 2006). However, when ROS are in excess amounts, they cause severe harm to macromolecules and cellular structure (Halliwell, 2006). The scavenging systems within the cell, which consist of a number of enzymes and non-enzymatic antioxidants which counter the ROS and alter them into less-toxic products (Pandey *et al*., 2017).

Under stress conditions, the redox homeostasis is hastily disrupted which results in the excessive accumulation of ROS (Pandey *et al*., 2017). The overall level of ROS in a cell is controlled by their synthesis and scavenging by antioxidants (Das and Roychoudhury, 2014). When ROS levels surpass the requirement of the plant's metabolic processes, it becomes toxic to the cell and causes significant damage to the macromolecules of the cell, such as the lipids, proteins, and nucleic acids (Pandey *et al*., 2017).

Plants possess many antioxidant systems that can protect them against prospective cytotoxic effects (Berwal and Ram, 2018). Antioxidative enzymes are the vital parts of the scavenging system of ROS. Non-enzymatic antioxidants include glutathione (GSH), ascorbic acid (ASA), alkaloids, non-proteinaceous amino acids, phenolic compounds, and α-tocopherols (Berwal and Ram, 2018). Enzymatic antioxidants include superoxide dismutase (SOD), ascorbate peroxide (APX), glutathione reductase (GR), peroxidase (POX), monodehydroascorbate reductase (MDHAR), guaiacol peroxidase (GOPX), dehydroascorbate reductase (DHAR), and

glutathione -S- transferase (GST) (Berwal and Ram, 2018). The significant ROS-scavenging enzymes found within plants include ascorbate peroxide (APX), superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), and peroxiredoxin (PrxR). Alongside the antioxidant's glutathione and ascorbic acid, these enzymes can provide the plant cells with significant processes to detoxify superoxide and hydrogen peroxide (Yousuf *et al*., 2012).

In the agricultural and natural environments, plants are impacted by biotic stresses (Zipfel and Oldroyd, 2017). Plants have evolved molecular processes to adapt to these environmental changes such as ROS accumulation and changes in their proteins (Liu *et al*., 2019 a). The roles of proteins are significant in the plant's response to biotic stress because proteins directly participate in the production of plant phenotypes by the regulation of physiological traits in order to adapt to the environment; and proteins are significant facilitators of cellular mechanisms that maintains cellular homeostasis (Liu *et al*., 2019 a).

In fact, the plant's immune system responds to biotic stresses via an intricate system of interactions between various signals and numerous stress-tolerance related proteins (Liu *et al*., 2019 a).

4 Strategies to Control *Fusarium proliferatum*

4.1 The use of fungicides to control pathogenic fungi

Fungicides are biological or chemical compounds used to defend agricultural products from harmful fungi or fungal spores (Dias, 2012). The unrestrained and continued use of fungicides has serious impacts on the environment, human and animal health (Kara *et al*., 2020). Due to the effectiveness, straightforward usage and low cost, fungicides have become the primary means to control fungi (Dias, 2012). However, the widespread use of fungicides has caused fungal strains to become resistant to these commercial products (Dias, 2012). Fungal diseases are difficult to eliminate as outbreaks can endure throughout the various seasons, which often originates from spores that were dormant through winter (Wightwick *et al*., 2010). The use of fungicides can cause carcinogenic, teratogenic, and mutagenic effects on various organisms' reproductive systems. Various types of fungicides utilize different molecular pathways to exert neurotoxic action (Kara *et al*., 2020).

Product spoilage due to fungal infection may occur during the pre-harvest, harvest, or post-harvest phases due to inadequate storage, unfavourable environmental conditions, and non-

scientific practices (Deepthi *et al*., 2016). The continuous use of fungicides to protect plants against fungal infection causes a long-term deposit in the environment and foods such as vegetables and fruits (Dias, 2012).

4.1.1 Impact of fungicides on the environment

Contamination of surface water in a specific region is dependent on various factors including the proximity of the crops to surface water, characteristics of the fields in surrounding areas (distance to water bodies, grassland, and slope), and climatic conditions (humidity, precipitation, wind and temperature) (Szekacs *et al*., 2015).

The consistent use of fungicides may present a threat to the environment, predominantly if the residues are persistent in the soil or enters the waterways due to run-offs or spray-drifts (Wightwick *et al*., 2010; Zubrod *et al*., 2019). If this run-off occurs, it may lead to antagonistic impacts to the health of aquatic and terrestrial ecosystems (Wightwick *et al*., 2010; Zubrod *et al*., 2019). Fungicides are repeatedly applied for a specified period of the year, and thus poses a bigger risk to the environment when compared to other pesticides (Wightwick *et al*., 2010).

4.1.2 Impact on human and animal health

Neurological dysfunctions and neurotoxicity may be partially attributed to fungicides, herbicides, carbamates, pytheroids, organophosphates, organochlorines, and fumigants (Kara *et al*., 2020). Livestock are accidently poisoned by fungicides that are applied to agricultural material (Oruc, 2010). Overall, the newly developed fungicides have low-to-moderate toxicity. However, teratogenic, mutagenic, reproductive, and carcinogenic effects or organ toxicity may occur due to fungicide exposure (Gupta, 2018; Oruc, 2010). An example of a fungicide used for the protection of maize is Azole fungicides (Gupta, 2018; Oruc, 2010). Once these fungicides enter the environment they are dispersed into various environmental compartments (Chen and Ying, 2015). The residues of these fungicides may have antagonistic effects on non-target organisms such as fish and algae (Chen and Ying, 2015).

An additional example is the use of carbendazim (Fang *et al.*, 2010). The various toxic effects of this ingredient include down-regulated humoral immunity, germ cells sloughing, reproductive toxicity, and spermatogenic failure (Fang *et al*., 2010). The exposure to carbendazim has shown to damage liver function, reproduction, and haematopoiesis in various mammals (Selmanoglu *et al*., 2001).

4.1.3 Commercial fungicides to control pathogenic fungi

Fungicides are applied before or prior to an infection in order to protect the plant from fungal infections (Dias, 2012). They are classified as either contact, systemic, or translaminar fungicides (Singh *et al*., 2019). Contact fungicides are not absorbed by the plant and provide topical protections to plants tissues, whereas systemic fungicides are able to enter the plants tissue and are then distributed throughout the plant via the xylem vessels to the site of infection (Dias, 2012; Singh *et al*., 2019). On the other hand, translaminar fungicides are not truly systemic but can move from the upper leaf that has been sprayed to the lower leaf surface which has not been sprayed (Dias, 2012; Singh *et al*., 2019). A list of commercial fungicides with its maximum residue limits (MRL) used to protect maize crops are documented in Table 1.1.

Table 1.1 Registered fungicides in South Africa and its chemical ingredients, classification and MRL for maize crop fungal infections

Fungicide	Chemical ingredient	Type of Fungicide	South African MRL (mg kg-1) for maize	Adapted from
Azole	Propiconazole	Systemic Fungicide	0.05 - 0.5	Cools *et al*., 2013; Quinn *et al*., 2011.
Benzimidazole	Benomyl	Systemic Fungicide	0.05 - 0.1	Dias, 2012; Quinn *et al*., 2011
Benzimidazole	Carbendazim	Systemic Fungicide	0.01 - 0.1	Dias, 2012; Quinn *et al*., 2011
Fungicide	Epoxiconazole	Systemic Fungicide	0.01 - <0.05	Ferreira *et al*., 2008; Quinn *et al*., 2011
Strobin	Azoxystrobin	Systemic Fungicide	0.01 - 0.05	Vincelli, 2002; Quinn *et al*., 2011
Strobin	Trifloxystrobin	Systemic Fungicide	0.05 - 0.5	Han *et al*., 2012; Quinn *et al*., 2011

4.2 Endophytes as Biological Control Agents

Biological control, using beneficial microorganisms, has been commercially used in agriculture for the past 120 years, however, it has only recently gained attention due to its ability to control diseases in an environmentally safe manner (Latz *et al*., 2018; Wei *et al*., 2019).

Endophytes are globally abundant; they form associations with various groups of organisms throughout the plant kingdom and they offer plants indirect defence against herbivores (Bamisile *et al*., 2018; Kaur, 2020). Endophytes are able to exist in its host plant as a mutualistic root endophyte or as a plant-associated endophyte (Bamisile *et al*., 2018; Kaur, 2020). These plant-connected microorganisms can inhabit and exist a portion of their life cycle within the plant without initiating any harm (Bamisile *et al*., 2018, Hardoim *et al*., 2015). The organs and tissue of the host plant including branches, stems, flowers, leaves, and fruits, are frequently colonized devoid of any visible symptoms (Amatuzzi *et al*., 2017; Bamisile *et al*., 2018).

There are various challenges that exist when studying endophytic biocontrol agents' mechanisms (Latz *et al*., 2018). These challenges include four types of control principles namely, 1) the competition for nutrients and space, 2) antibiosis causing direct inhibition, 3) mycoparasitism and 4) induced resistance of the plant due to the plants defence system. Some of these mechanisms may be active at the same time (Latz *et al*., 2018).

4.2.1 Fungal Endophytes

Fungal endophytes are biologically and taxonomically diverse, however, they all share the characteristic of colonizing plant tissue without causing visible harm to the host plant (Mejia *et al*., 2008; Ting *et al*., 2011). A single part of the plant, for example its roots, leaves and stem may have diverse endophytic species (Bamisile *et al*., 2018; Kaur, 2020). Higher vascular plants have been identified to host fungal endophytes in a symbiotic fungus-plant relationship, where the endophytic fungi offer benefits to the host in exchange for the nutrients that the host plant offers (Bamisile *et al*., 2018; Kaur, 2020).

Endophytic fungi may be broadly classified into different ecotypes based on their functional roles and/or diversity (Bamisile *et al*., 2018). Based on these classifications, endophytic fungi have been grouped into two main categories known as non-clavicipitaceous and clavicipitaceous fungal endophytes (Bamisile *et al*., 2018; Gautam and Avasthi, 2019). Non-clavicipitaceous fungal endophytes are present in non-vascular and vascular plant species whereas clavicipitaceous endophytes are typically found in grasses (Bamisile *et al*., 2018; Gautam and Avasthi, 2019). Several authors have specified the necessity to classify these fungal endophytes based on various requirements including their transmission mode, host range, reproduction mode, nutrition source, part of the plant been colonized and their capacity to cause symptoms in the host plant (Brem and Leuchtmann, 2001; Purahong and Hyde, 2011; Rodriguez *et al*., 2009; Saikkonen *et al*., 2002; Varma *et al*., 1999). Endophytic fungi are

classified based on the following criteria (Bamisile *et al*., 2018; Lugtenberg *et al*., 2016; Tintjer *et al*., 2008):

- their mode of reproduction; asexual or sexual,
- their manner of transmission in the host; horizontally or vertically transmitted,
- their nutrition source; obtains nutrients from dead or living matter,
- their expression of infection; asymptomatic or symptomatic.

Endophytes induce the production/synthesis of chemicals that hinder the development and growth of competitors including pathogenic organisms and assist host plants to tolerate abiotic stresses like heat stresses, drought and salt, and biotic stresses, for example pathogenic fungi, nematodes, and other root-feeding insects (Bamisile *et al*., 2018; Singh *et al*., 2011).

5 Aims and objectives

To our knowledge, there has been no previous study on the impact of *F. proliferatum* infection on maize seeds as well as the usage of fungal endophytes to biologically control the growth of *F. proliferatum* and assist maize seeds in the recuperation of plant growth pertaining to physiology, biochemistry, and proteins. This study aimed to control *F. proliferatum in vitro* using fungal endophytes and explored the impact of *F. proliferatum* on maize seed germination. In addition, the study further explored the biocontrol potential of fungal endophytes against *F. proliferatum* in maize by monitoring the root length of seeds, biochemical and molecular responses.

An *in vitro* assay will be done to investigate the biocontrol potential of the 12 fungal endophytes against the growth of *F. proliferatum*. Additionally, an *in planta* assay will be done to determine the detrimental effects of *F. proliferatum* on maize seed root length and the effect of priming seeds with fungal endophytes prior to infection with *F. proliferatum*. Moreover, biochemical analysis will be done to determine the oxidative stress and antioxidant enzyme content in roots infected with *F. proliferatum* and roots primed with fungal endophytes prior to infection with *F. proliferatum*, respectively. Furthermore, a proteomics analysis will be done via LC/MS to determine the presence and absence of proteins in roots infected with *F. proliferatum* and roots primed with fungal endophyte *Penicillium griseofulvum* prior to infection with *F. proliferatum*, respectively.

6 Conclusion

F. proliferatum is a widely distributed fungal pathogen, which produces mycotoxins that are responsible for the infection of economically important crops such as maize. Conventional methods to eradicate fungal infections using commercial fungicides are harmful to the environment and the health of humans and animals. An eco-friendly approach is the biological control of *F. proliferatum* which has a less detrimental impact on the sustainability of the environment and animal and human health. Biological control of these infectious fungi is the use of fungal endophytes, which can assist plant growth and the inhibition of pathogenic degradation. The use of fungal endophytes would therefore assist in the improvement of maize crop yield and quality under stress conditions.

Chapter 2

Materials and Methods

2.1 Chemicals and Suppliers

Table 2.1 List of chemicals and reagents

Chemical / Reagent	Supplier
Acetone	Merck
Acrylamide/Bis (35 %)	BIO – RAD
Ammonium acetate ($C_2H_3O_2NH_4$)	Merck
Ammonium bicarbonate (AmBic)	Merck
Ammonium nitrate (NH_4NO_3)	Merck
Ammonium persulfate (APS)	BIO – RAD
Ascorbic acid / Ascorbate	Merck
Bleach	BioSmart
Bradford Reagent (1X)	BIO – RAD
Bromophenol blue	Merck
β-mercaptoethanol	Merck
Coomassie® brilliant blue (CBB) R-250	BIO – RAD
3,3′-Diaminobenzidine	Merck
Dithiothreitol (DTT) Cleland's reagent	Merck
Ethanol 99.9%	B&M Scientific
Ethylenediaminetetraacetic acid (EDTA)	Merck
Ethylenediaminetetraacetic acid ferric sodium salt	Merck
Evans Blue	Merck
Ferric chloride	Merck
Guaiacol	Merck
Glacial acetic acid	Merck
Glycerol	Merck
Glycine	BIO – RAD
HCL	Merck
Hydrogen peroxide (H_2O_2)	Merck
Iodoacetamide	Merck
Lactophenol blue	Merck
L-Ascorbic acid	Merck

MagResyn HILIC magnetic particles	Resyn Biosciences
Magnesium chloride ($MgCl_2$)	Merck
Methanol 99.9%	Merck
Methionine	Merck
Nitrotetrazolium blue chloride powder (NBT)	Merck
Norgen DNA extraction kit	Merck
PageRulerTM pre-stained protein ladder	Fermentas
Potato dextrose agar (PDA)	Merck
Polyvinylpyrrolidone (PVP) MW: 40 000	Merck
Potassium cyanide (KCN)	Merck
Potassium ferricyanide	Merck
Potassium iodide (KI)	Merck
Potassium phosphate monobasic (KH_2PO_4)	Merck
Potassium phosphate dibasic (K_2HPO_4)	Merck
Propan-2-ol (isopropanol)	Merck
Proteinase K	Merck
Riboflavin	Merck
Sodium chloride (NaCl)	Merck
Sodium dodecyl sulfate (SDS)	BIO – RAD
Sodium hydroxide (NaOH)	Merck
Sucrose	Merck
Taq Amplicon Mastermix	Merck
N,N,N',N'-Tetramethylethylenediamine (TEMED)	BIO – RAD
Thiobarbituric acid (TBA)	Merck
Thiourea	Merck
Trichloroacetic acid (TCA)	Merck Millipore
Trifluoroacetic acid (TFA)	Merck
Tris(hydroxymethyl)-aminethane	BIO – RAD
Tryphan Blue	Merck
Tween 20	Merck

2.2 Stock solutions and buffers

Table 2.2 List of buffers and stock solutions prepared for this study

Buffer/Stock solution	Composition
Acetone (80 %)	80 % (v/v) acetone in dH_2O.

APS (10 %)	10 % (w/v) APS in dH_2O. The solution was freshly prepared prior to use.
BSA stock solution I (10 mg/ml)	10 mg/ml BSA in PVP extraction buffer.
BSA stock solution II (10 mg/ml)	10 mg/ml BSA in IEF buffer.
Destaining solution	10 % (v/v) acetic acid and 1 % (v/v) glycerol in dH_2O.
Ethanol (70 %)	70 % (v/v) ethanol in dH_2O.
Equilibration buffer	6 M urea; 2 % (w/v) SDS, 50 mM Tris – HCl, pH 8.8 and 20 % (v/v) glycerol in dH_2O.
IEF buffer	7 M Urea; 2 M thiourea; 4 % (w/v) CHAPS; 20 mM DTT; 1 % (w/v) bromophenol blue in dH_2O.
Methanol Buffer	80 % (v/v) methanol; 0.1 M ammonium acetate in dH_2O.
Native gel running buffer (5 X)	25 mM Tris - base; 192 mM glycine in dH_2O.
Native PAGE loading dye (6 X)	375 mM Tris-HCl at pH 6.8; 50 % (v/v) glycerol; 0.02 % (w/v) bromophenol blue in dH_2O.
Phosphate buffer	1M K_2HPO_4; KH_2PO_4
Protein binding buffer	200 mM sodium acetate; 30 % acetonitrile, pH 4.5.
Protein solubilisation buffer	50 mM Tris containing 2 % SDS and 4 M urea.
PVP extraction buffer	40 mM K_2HPO_4 at pH 7.4; 1 mM EDTA; 5 % PVP MW = 40 000; 5 % glycerol in dH_2O.
SDS buffer	0.1 M Tris - HCl, pH 8.0; 2 % (w/v) SDS; 5 % (v/v) β-mercaptoethanol; 30 % (w/v) sucrose and 1 mM PMSF in dH_2O.
SDS gel loading dye	100 mM Tris - HCl at pH 6.8; 4 % (w/v) SDS; 0.2 % (w/v) bromophenol blue; 20 % (v/v) glycerol; 200 mM DTT in dH_2O.
SDS running buffer (5 X)	25 mM Tris - base; 192 mM glycine; 0.1 % (w/v) SDS in dH_2O.
SDS (10 %) stock solution	10 % (w/v) SDS in dH_2O.
SOD assay buffer	50 mM KPO_4 at pH 7.4; 13 mM methionine; 75 µM NBT; 0.1 mM EDTA; 2 µM riboflavin in dH_2O.
TCA (6 %) extraction buffer	6 % (w/v) TCA in dH_2O.

TCA/Acetone (10 %)	10 % (w/v) TCA in acetone.
TCA (20 %) / TBA (0.5 %)	0.5 % (w/v) TBA in 20 % (v/v) TCA stock solution.
Tris-HCl (0.5 M), pH 6.8	0.5 M Tris in dH_2O adjusted to pH 6.8 with concentrated HCl.
Tris-HCl (1.5 M), pH 8.8	1.5 M Tris in dH_2O adjusted to pH 8.8 with concentrated HCl.
Washing buffer	95 % acetonitrile, pH 4.5.
Yeast lysis buffer	2 % Triton X-100, 1 % SDS; 100 mM NaCl; 10 mM Tris- CL, pH 8.0; 1 mM EDTA, pH 8.0.

2.3 Maize seeds and *Fusarium proliferatum* isolate

Maize seeds were sponsored by Agricol (PTY) LTD (Brackenfell, South Africa).

The *Fusarium proliferatum* strain was obtained from the Plant Protection Research Institute (Agricultural Research Council). Maintenance of *F. proliferatum* was achieved by cutting out 1 cm x 1 cm plugs grown on PDA media (Potato Dextrose Agar) (potato extract; dextrose; agar; distilled water) and incubated at 30 °C for a period of 7 days as seen in Supplementary Figure 3.4. Thereafter, glycerol stocks were made by placing 5 mm x 5 mm plugs in 80% glycerol, stored at -80 °C.

2.4 Isolation of endophytic fungi

A modified method by Mia (2018) was used to isolate a total of 12 fungal endophytes from leaves, stems, and roots of six *Echium plantaginium* weed plants collected in Durbanville, South Africa. The plant material was stored in polyethene bags and processed in the research laboratory of Prof Marshall Keyster (Environmental Biotechnology Laboratory, Department of Biotechnology, University of the Western Cape, Cape Town, South Africa). Following the incubation period, the fungal colonies were visually analysed, and pure cultures were made in a sterile laminar flow hood by cutting 1cm x 1cm plugs and transferring them to PDA plates (Supplementary Figure 3.1; Supplementary Figure 3.2; Supplementary Figure 3.3) The fungal isolates were incubated for 1-2 weeks at 30 °C and were grouped based on their morphological appearance. The isolates were grouped as either **A** (A4, A7, and A8), **B** (B1, B2, B3, and B4), and **C** (C1, C2, C4, C5, and C9).

2.5 Species Identification

2.5.1 DNA Extraction

Genomic DNA was extracted from mycelium using the method previously described by Jin *et al*. (2004) with modifications of the Norgen plant/fungi DNA extraction kit after the lysis buffer was added.

The DNA concentration was determined using a Thermo Scientific Nanodrop 2000 Spectrophotometer (Installation version 1.6.198). DNA was run on a 1 % Agarose gel at 80 V for a period of 45 minutes to determine DNA quality.

2.5.2 PCR amplification and DNA-sequencing of the Internal Transcribed Spacer region (ITS) regions

The ITS1 and 4 regions from the fungal DNA were amplified using the primer pair ITS1 (5' CTTGGTCATTTAGGGAAGTAA 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3'). The PCR reaction consists of 1 µL of template DNA, 1 X Taq Amplicon Mastermix, 25 mM $MgCl_2$, 0.5 µL of each primer, in a final volume of 25 µL with Nuclease-free water. The PCR amplification procedure included an initial denaturation at 95 °C for 5 minutes, followed by 25 cycles of denaturation at 95 °C for 30 seconds, primer annealing at 55 °C for 30 seconds, and primer extension at 72 °C for 30 seconds, followed by final extension at 72 °C for 5 minutes. Thereafter, the PCR solution was stored at 4 °C. Amplification was determined by size fractionating 5 µL of the PCR products on a 1 % agarose gel.

The DNA was sent to the Central Analytical Facility at the University of Stellenbosch for sequence analysis.

2.5.3 DNA sequence analysis

The resultant sequencing data was edited using BioEdit (BioEdit, version 7.2.5) and searched against the GenBank database via a BLAST search.

The multiple sequence analysis of sequence and related strains were performed using BioEdit and Paup star (PAUP*, version 4.0a169). The phylogenetic trees were constructed using the bootstrap neighbour-joining method to determine the taxonomy of the sequenced strains.

2.5.4 Microscopy analysis of fungal hyphal structures

Microscope slides were prepared using a modified version of the slide culture technique by Johnson (1946) where hyphal structures from *F. proliferatum* and fungal endophytes were

prepared by placing 1 cm x 1 cm PDA plugs on a sterile glass microscope slide. Five microlitre fungal cell suspension was placed on the two opposite sides on the plug and covered with a glass coverslip. The fungi were grown for 4 days. Thereafter, the glass coverslip was removed from the plug, stained with 10 µL of lactophenol blue and viewed under the Zeiss Primo Star Binocular Microscope at 40 X magnification.

2.6 Evaluation of antagonistic activity of fungal endophytes

2.6.1 Dual-culture assay (*in vitro* assay)

An *in vitro* antagonistic assay was performed using a dual culture method on PDA medium to determine which fungal endophytes had the potential to inhibit the growth of *F. proliferatum*.

Mycelial plugs (1cm x 1cm) from each of the fungal endophytes were co-cultured with *F. proliferatum* at 30 °C for 7-10 days. The antagonistic activities of each endophyte against *F. proliferatum* were assessed qualitatively. For statistical purposes, this experiment was repeated three times independently.

The percentage inhibition of *F. proliferatum* mycelial growth was calculated using a formula previously described Bivi *et al*. (2010),

$$Percentage\ inhibition\ (PI) = \frac{C-T}{C} X\ 100$$

where, PI is the percent of *F. proliferatum* growth inhibition. C represents the growth of the pathogenic fungi in the absence of the antagonist (cm), T represents the growth of the pathogenic fungi in the presence of the antagonist (cm).

2.7 Quantification of *Fusarium proliferatum* and endophyte concentration using Hemocytometer

2.7.1 Preparation of cell suspension and quantification of fungal concentration

A method previously described by Kumar *et al*. (2016) with slight modifications was used to prepare the cell suspension of *F. proliferatum*, where spores were harvested from PDA plates (cultured for 7 days at 30 °C) using a sterile microscope glass cover with the addition of 14 ml sterile dH_2O. The resulting suspension was filtered through a sterile cheesecloth into a 50 ml Greiner tube and labelled accordingly. Each step was repeated for the cell suspension preparation for each of the respective endophytes namely B3 and B4. The concentrations of each fungal isolate were enumerated using a hemocytometer chamber.

The concentration of viable cells was then calculated using the following calculations:

1) $\text{Percentage of viable cells} = \frac{\text{Number of viable cells}}{\text{Total number of cells}} \times 100$

2) $\text{Average number of cells} = \frac{\text{Number of viable cells}}{\text{Number of squares}}$

3) $\text{Dilution factor} = \frac{\text{Final volume}}{\text{Volume of cells}}$

4) $\text{Concentration of viable cells} = \text{Average number of cells} \times \text{Dilution factor} \times 10^4$

The quantification steps were repeated to determine the concentration for each respective endophyte (B3 and B4) to be used for seed inoculation. Once the concentration of *F. proliferatum* and endophytes were calculated, serial dilutions were performed using sterile dH_2O to reach a final concentration of 10^8 cells/ml for *F. proliferatum*, and 10^8 cells/ml for B3 and B4.

2.8 *In planta* assay

Of the 12 fungal endophytes isolated, the two best performing isolates showing the best growth inhibition percentage of *F. proliferatum* mycelia, were selected for the *in planta* assay. The selected endophytes were B3 and B4. Maize seeds were germinated on sterile autoclaved paper in petri dishes.

2.8.1 Seed sterilization

Maize seeds were sterilized at 49 °C in a water bath for 20 minutes and surface sterilized with 5 % bleach in an Erlenmeyer flask. The seeds were incubated at room temperature for 10 minutes and rinsed with sterile dH_2O to remove all traces of bleach.

2.8.2 Seed inoculation with *Fusarium proliferatum*

Two independent experiments were set up for each of the following treatments: the control (dH_2O), and 10^8 cells/ml. A total of 38 seeds were used for each treatment.

Maize seeds were imbibed in the respective concentrations of *F. proliferatum* for 2 hours with slight agitation. Seeds used in the control treatment were imbibed in distilled water. Imbibed seeds were aseptically transferred (using tweezers) to petri dishes containing moist tissue paper and incubated in the greenhouse at a temperature range of 20 - 26 °C for a period of 7 days.

2.8.3 Seed priming with endophytes and infection with *Fusarium proliferatum*

Maize seeds were primed with B3 and B4 inoculum (at final concentration of 10^8 cells/ml) for 2 hours with slight agitation. Bio-primed seeds were aseptically transferred to petri dishes containing sterile tissue paper totalling 38 seeds. The primed seeds were allowed to air dry and inoculated with 100 µl of corresponding *F. proliferatum* concentration. Inoculated seeds were allowed to air dry for 2 hours, tissue paper moistened. Plates incubated in the greenhouse at a temperature range between 20 °C and 26 °C for a period of 7 days. Plant tissue was harvested and stored at -80 °C until further use.

2.9 Sample preparation for biochemical analysis

Root tissue from all treatments were harvested and ground to a fine powder using liquid nitrogen. Root material (0.2 g/ 200 mg) from each treatment was homogenized in 1 ml 6 % (w/v) trichloroacetic acid (TCA) to analyse lipid peroxidation and H_2O_2 content and 1 ml PVP extraction buffer (Table 2.2) to detect superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPOX), and peroxidase with catalase activity (POD with CAT activity), respectively. The protein concentration of each treatment was determined using the Thermo Scientific Nanodrop 2000 Spectrophotometer (Installation version 1.6.198) and Alpha Ease using the "invert" option.

2.9.1 Measurement of superoxide content

A method previously described by Gokul *et al*. (2016) with slight modifications was used to measure and calculate the superoxide content in the roots of maize seeds. Superoxide content was determined by submerging 2 cm of the root for each treatment in a phosphate buffer (Table 2.2) containing 100 mM KCN (to inhibit Cu/Zn SODs), 100 mM H_2O_2 (to inhibit Mn and Cu/Zn SODs), 6.4 mM nitro blue tetrazolium chloride (NBT), and 50 mM potassium phosphate (pH 7.0) and incubated at room temperature for 20 minutes in the dark. Root samples were crushed with a miniature pestle. The samples were then centrifuged at 13 000 X g for 20 minutes and the respective absorbances measured at 600 nm. The superoxide concentration was calculated using the molar extinction coefficient of 12.8 mM cm^{-1}.

2.9.2 Measurement of hydrogen peroxide content

A method previously described by Velikova *et al*. (2000) was used to determine the hydrogen peroxide content in maize roots. The reaction mixture containing 50 µl TCA extract, 5 mM K_2HPO_4 (pH 5.0) and 0.5 M KI was incubated at 25 °C for 20 minutes and absorbances

measured at 390 nm. The hydrogen peroxide concentration was determined using a standard curve based on the absorbance readings (390 nm) of H_2O_2 standards.

2.9.3 Measuring the extent of lipid peroxidation

A method previously described by Vos *et al*. (1991) was used to determine the extent of lipid peroxidation by measuring malondialdehyde (MDA) content. TCA protein extracts from each treatment (200 µl) were mixed with 400 µl of 0.5 % TBA (dissolved in 20 % TCA) and incubated at 95 °C for a total of 30 minutes, and subsequently incubated on ice for 10 minutes. The samples were then centrifuged at 13 000 X g for 5 minutes. The absorbances of each treatment's supernatant were measured at 532 nm and 600 nm, respectively. The concentration of MDA was calculated using the molar extinction coefficient of 155 mM^{-1} cm^{-1}.

2.9.4 Measuring total superoxide dismutase activity

A method previously described by Samantary (2002) was used to determine the total superoxide dismutase activity in maize roots. The reaction mixture contained 10 µl PVP protein extract and 190 µl SOD assay buffer (Table 2.2) to a final volume of 200 µl. The reaction was initiated by exposing the samples to light for 15 minutes or until a colour change was observed and absorbances measured at 560 nm. The SOD activity was calculated based on the amount of enzyme needed to reduce 50 % NBT to blue formazan.

2.9.5 Measuring total of ascorbate peroxidase activity

A method previously described by Asada (1992) was used to detect the ascorbate peroxidase activity in maize roots where each reaction mixture contained 10 µl PVP protein extract and 180 µl of APX assay buffer (Table 2.2). The reaction was initiated by the subsequent addition of 10 µL of 90 µM H_2O_2 and thereafter the absorbance readings were measured at 290 nm. The APX activity was calculated using the molar extinction coefficient 2.8 mM^{-1} cm^{-1}.

2.9.6 Detection of peroxidase isoforms

Guaiacol peroxidase activity was detected in maize roots using native polyacrylamide gel electrophoresis using a previous method by Kim *et al*. (1994). The protein extracts of each treatment (40 µg) were separated on a 10 % native polyacrylamide gel, washed with dH_2O followed by an incubation in 5 mM H_2O_2 for 10 minutes. The gels were then stained with 1 mg/ml 3,3′-Diaminobenzidine for 1 hour.

2.9.7 Detection of peroxidase isoforms with catalase activity

Peroxidase with catalase activity was detected in maize roots using native polyacrylamide gel electrophoresis using a previous method by Kim *et al*. (1994) with modifications. The protein extracts of each treatment (40 µg) were separated on a 10 % native polyacrylamide gel, washed with dH_2O followed by an incubation in 5 mM H_2O_2 for 10 minutes. The gels were then stained with 1 $mg.ml^{-1}$ guaiacol for 24 hours.

2.9.8 Densitometry analysis of antioxidant enzymes

The Native PAGE gels for GPOX and POD with CAT activity antioxidant enzymes were analysed by densitometry analysis using the Alpha Ease FC imaging software (Alpha Innotech Corporation). The enzymatic activity of each isoform from three independent gels were measured according to Klein (2012). The average of the pixel intensities was expressed as relative arbitrary units. This was achieved by assigning the isoforms in the untreated plants to a value of 1 and expressing the pixel intensities of the rest of the isoforms in the various treatments relative to this isoform.

2.10 Sample preparation for liquid chromatography mass spectrometry (LC/MS)

2.10. Protein Extraction

Maize root (1 g) from each treatment was ground to a fine powder with liquid nitrogen and homogenised with 3 ml TCA/Acetone extraction buffer (10% TCA in 100 % acetone) with the addition of 0.5 g polyvinylpolypyrrolidone (PVPP). The sample extracts were transferred to sterile eppendorf tubes and centrifuge at 16 000 X g for 3 minutes at 4 °C. The supernatant was discarded, and pellets washed with 2 ml 80 % (v/v) methanol/ 0.1 M ammonium acetate (Table 2.2), vortexed for 1 minutes and centrifuged at 13 000 X g for 10 minutes. The supernatant was discarded, and the pellet re-suspended in 1 ml 80 % acetone, vortexed and centrifuged at 13 300 X g for 5 minutes. This step was repeated until a clear supernatant was observed. The supernatant was discarded, and the respective pellets were air-dried at room temperature for 1 hour. The pellets were suspended in 800 µl of sodium dodecyl sulfate (SDS) buffer (Table 2.2). The samples were vortexed and a 1:1 ratio of phenol was added (800 µl). Thereafter, the samples were vortexed and centrifuged at 13 500 X g for 15 minutes. The upper aqueous layer was transferred to new eppendorf tubes and precipitated with 4 volumes of 80 % (v/v) methanol/0.1 M acetate (Table 2.2) at -20 °C overnight.

The samples were then centrifuged at 13 000 X g for 10 minutes and the supernatant discarded. The pellets were washed with 1 ml methanol briefly vortexed and centrifuged at 13 000 X g

for 5 minutes and supernatant discarded. Acetone (1 ml) was added to each sample, briefly vortexed and centrifuged at 13 000 X g for 5 minutes. The supernatant was discarded, and the resulting pellet was left to air dry at room temperature for 1 hour. The air-dried pellet was dissolved in a 30 µl 7 M Urea buffer, vortexed for 15 minutes and stored at -20 °C for downstream analysis. A method previously described by Bradford (1976) was used to determine the protein concentration of each sample.

2.10.2 1D SDS polyacrylamide gel electrophoresis

Protein samples (15 µg), prepared in an SDS gel loading dye (Table 2.2), and boiled at 95 °C for 3 minutes were subjected to a 1D SDS PAGE analysis to separate proteins based on their molecular weight.

A method previously described by Brunelle and Green (2014) with slight modifications was used to separate proteins based on molecular weight, where 30 % polyacrylamide (37:5:1) was used. The gel was electrophoresed at 120 V for approximately 90 minutes, until the dye had reached the bottom of the gel.

2.10.3 Protein pellet solubilisation

All protein pellets were solubilised in the protein solubilization buffer (50 mM Tris containing 2 % SDS and 4 M urea) and vortexed for 30 minutes. Samples were quantified using the Pierce microplate BCA protein assay kit (Thermo Scientific) according to the manufacturer's instructions with ovalbumin as positive control. Approximately 50 µg of protein for each treatment was used for trypsin digestion.

2.10.3.1 On-bead HILIC digest and solid-phase extraction

All reagents are analytical grade or equivalent. Samples were re-suspended in 50 mM ammonium bicarbonate before reduction with 10 mM dithiothreitol (DTT) for 30 minutes at room temperature. This step was followed by an alkylation with 30 mM iodoacetamide at room temperature in the dark. After reduction and alkylation of the protein samples, the samples were diluted with an equal volume of binding buffer (200 mM sodium acetate; 30 % acetonitrile, pH 4.5).

The protein solution was added to MagResyn HILIC (Resyn Biosciences) magnetic particles prepared according to manufacturer's instructions and incubated overnight at 4 °C. After binding, the supernatant was removed, and the magnetic particles were washed twice with washing buffer (95 % acetonitrile). After washing, the magnetic particles were suspended in

50 mM ammonium bicarbonate containing trypsin to a final ratio of 1:50. After a 4-hour incubation at 37 °C, the peptides were removed from the beads and collected in a fresh tube. The adsorbed peptides were removed by incubating it for 3 minutes at room temperature in 20 µl 1% TFA.

Residual digest reagents were removed using an in-house manufactured C_{18} stage tip (Empore Octadecyl C_{18} extraction discs; Supelco). The samples were loaded onto the stage tip after activating the C_{18} membrane with 30 µl methanol and equilibration with 30 µl 2 % acetonitrile: water; 0.05 % TFA. The bound sample was washed with 30 µL 2 % acetonitrile: water; 0.1 % TFA before elution with 30 µL 50 % acetonitrile: water 0.05 % TFA. The eluate was evaporated to dryness. The dried peptides were dissolved in 2 % acetonitrile: water; 0.1 % FA for LC-MS analysis.

2.10.4 Liquid chromatography mass spectrometry (LC-MS) analysis

LC-MS was performed on a Thermo Scientific Ultimate 3000 RSLC equipped with a 5mm x 300 µm C_{18} trap column (Thermo Scientific) and a CSH 25cm x 75 µm 1.7 µm particle size C_{18} column (Waters) analytical column. The solvent system employed was loading: 2 % acetonitrile: water; 0.1 % FA; Solvent A: 2 % acetonitrile: water; 0.1 % FA and Solvent B: 100 % acetonitrile: water. The samples were loaded onto the trap column using loading solvent at a flow rate of 2 µL/minutes from a temperature controlled autosampler set at 7 °C. Loading was performed for 5 minutes before the sample was eluted onto the analytical column. Flow rate was set to 250 nl/minute and the gradient generated as follows: 5.0 % -35 %B over 60 minutes and 35-50 %B from 60-75 minutes. The outflow delivered to the mass spectrometer through a stainless-steel nano-bore emitter.

Data was collected in positive mode with spray voltage set to 1.8 kV and ion transfer capillary set to 280 °C. Spectra were internally calibrated using polysiloxane ions at m/z = 445.12003 and 371.10024. MS1 scans were performed using the orbitrap detector set at 120 000 resolutions over the scan range 350-1650 with AGC target at 3 E5 and maximum injection time of 50 ms. Data was acquired in profile mode.

MS2 acquisitions were performed using monoisotopic precursor selection for ion with charges +2-+7 with error tolerance set to +/- 10 ppm. Precursor ions were excluded from fragmentation once for a period of 60 seconds. Precursor ions were selected for fragmentation in HCD mode using the quadrupole mass analyser with HCD energy set to 30 %. Fragment ions were detected

in the orbitrap mass analyser set to 30 000 resolutions. The AGC target was set to 5E4 and the maximum injection time to 80 ms. The data was acquired in centroid mode.

2.10.5 Data analysis

The raw files generated by the mass spectrometer were imported into Proteome Discoverer v1.4 (Thermo Scientific) and processed using the Sequest and Amanda algorithms. Database interrogation was performed using the *Zea Mays* database concatenated with the common repository of adventitious proteins (cRAP) contaminant database (https://www.thegpm.org/crap/). Semi-tryptic cleavage with 2 missed cleavages was allowed for. Precursor mass tolerance was set to 10 ppm and fragment mass tolerance set to 0.02 Da. Deamidation (NQ), oxidation (M) and acetylation of protein N-terminal was allowed as dynamic modifications and thiomethyl of C as static modification. Peptide validation was performed using the Target-Decoy PSM validator node. The search results were imported into Scaffold Q+ for further validation (www.proteomesoftware.com).

2.11 Functional classification, transmembrane domain identification and subcellular localization

Proteins were functionally characterized using the data available in the UniProt database (www.uniprot.org), Scaffold_5.0.1 (www.proteomesoftware.com) as well as literature sources.

2.12 Statistical analysis

All experiments were performed as three independent studies. For seed germination experiments, 38 seeds per treatment was analysed. For all other experiments, 38 seeds per treatment were used. For statistical purposes, the one-way analysis of variance (ANOVA) test was used for all data and means (for three independent experiments) were compared according to the Tukey–Kramer test at 5 % level of significance.

Chapter Three

Species identification and biocontrol potential of fungal endophytes against *Fusarium proliferatum*

3.1 Introduction

Fusarium species is the causative agent for various plant diseases including the root rot of maize seedlings, vascular wilt of banana and tomatoes, collar rot of seedlings as well as stalk rot of maize (Ares *et al*., 2004; White, 2000). Some pathogenic *Fusarium* species produce secondary metabolites, which are harmful to animal and human health (Armengol *et al*., 2005; Kamle *et al*., 2019). Although various chemical treatment strategies exist to control *Fusarium* species, they can be harmful to human and animal health as well as the environment (Chen and Ying, 2015; Zubrod *et al*., 2019). There is an urgent need to use an eco-friendy approach to manage the spread of *Fusarium* that is less toxic to the environment; this includes the use of biological agents such as fungal endophytic organisms that are beneficial to plant growth and development (Fadiji and Babalola, 2020).

Endophytic fungi are located in the internal tissues of plants and shapes the integral portion of the microbial community that is associated to numerous types of plants, which includes trees, crop plants, shrubs, herbs, grasses, lichens, ferns, and moss (Zhang *et al.* 2006). The complex interaction between the host plant and endophyte can be mutualistic, parasitic, and symbiotic (Sieber, 2007). The relationship is dependent on the genetic disposition between the host plant and endophyte, nutritional status, developmental stage, and environmental factors (Redman *et al*. 2001; Schulz and Boyle 2005).

The use of fungal endophytes to control the growth and development has been previously reported in a study by Calistru *et al*. (1997) where *Trichoderma* fungal species controlled the phytopathogenic *Fusarium* species. A similar response was reported Rivas-Franco *et al*. (2019) where the incidence of *Fusarium* symptoms was decreased in seeds coated with the *Beauveria* fungal species.

For the identification of endophytes, the Internal Transcribed Spacer (ITS) region is mostly used as this region is regarded as the universal DNA marker for fungal identification (Schoch *et al*. 2012; Sun and Guo, 2012). The use of the ITS region is beneficial due to the availability of primers and databases and high success rates for amplifications (Nilsson *et al*., 2009;

Vilgalys, 2003). The ITS region has been previously used to identify *Penicillium* species (Visagie *et al*., 2014), *Fusarium oxysporum* (Abd-Elsalam *et al*., 2003), *Fusarium proliferatum* (Seyfarth *et al*., 2008), *Fusarium falciforme* (Bailey *et al*., 2018), and *Aspergillus alliaceus* (Ozhak-Baysan *et al*., 2010).

ITS sequencing was used to identify and classify fungal endophytes isolated from *E. plantaginium* and their antagonistic potential against *F. proliferatum* using *in vitro* bioassays were determined.

3.2 Results

Fungal endophytes were isolated from the host plant *E. plantaginium*. DNA was extracted from all isolated fungal endophytes and the pathogenic fungi *F. proliferatum* and run on an agarose gel (Figure 3.1) to determine the presence of DNA prior to PCR amplification using ITS primers (Figure 3.2).

A total of 12 isolates of endophytic fungi were isolated from the tissue of the host plant *E. plantaginium*. All the isolates sporulated and were identified into various taxa based on their morphological characteristics and hyphal structures. The results of this study showed that *Penicillium* species were the most prevalent species isolated from the host plant.

3.2.1 Isolation and identification of endophytic fungi from *Echium plantaginium*

The crude DNA agarose gels showed the DNA bands corresponding to each fungal endophyte and the pathogen (Figure 3.1). Distinct bands can be seen for each fungal endophyte, however, the concentration of the DNA for B4 (Figure 3.1; Table 3.1) was very low and thus the faintness of the band. The agarose gel for the ITS PCR amplicons products showed distinct bands for all samples (Figure 3.2). All the fungal endophytes and the pathogen showed similar band sizes, except for A4 which showed a bigger band size (Figure 3.2).

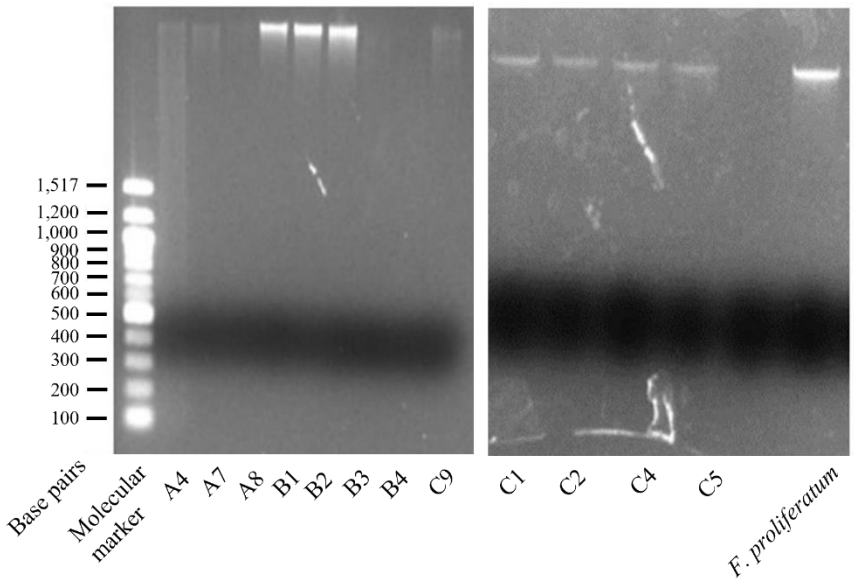


Figure 3.1. Agarose gel with the crude DNA banding patterns of fungal endophytes and *Fusarium proliferatum*.

Table 3.1. DNA concentration of fungal endophytes and *Fusarium proliferatum*.

Fungal isolate	DNA Concentration (ng/µl)
A4	81.0
A7	71.6
A8	54.4
B1	97.0
B2	90.8
B3	93.6
B4	38.8
C1	82.5
C2	87.0
C4	91.2
C5	80.1
C9	40.4
Fusarium proliferatum	120.5

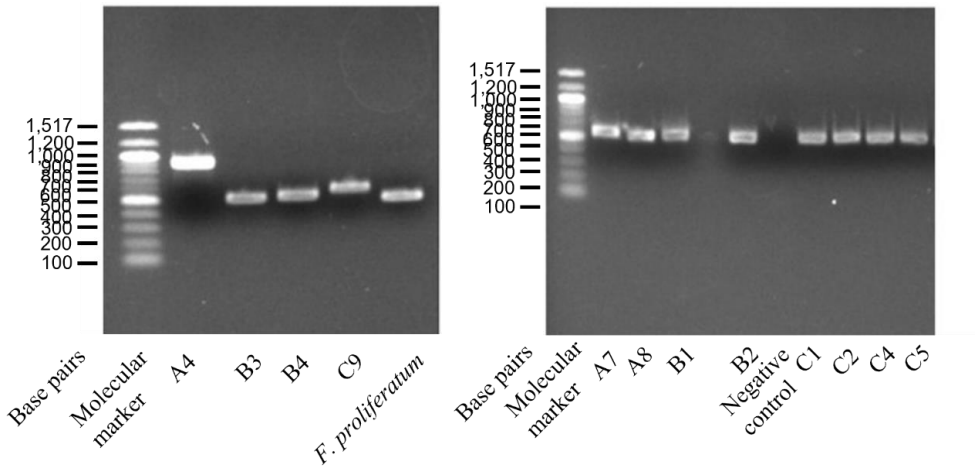


Figure 3.2. Agarose gel with the ITS PCR amplicons of fungal endophytes extracted from *Echium plantaginium* and the fungal pathogen *Fusarium proliferatum*.

Table 3.2 shows the number of different endophytes isolated from *E. plantaginium* as well as their most similar species-based sequence similarity arising from blast search results. The results show that 6 of the 12 extracted fungal endophytes were from the *Penicillium* species, 5 were from the *Fusarium* species and 1 was from the *Aspergillus* species (Table 3.2).

Table 3.2. Species identification of fungal endophytes extracted from *Echium plantaginium*.

Sample name/code	Species Name	Accession Number	Query Cover (%)	Max Identity (%)	e-value
A4	*Penicillium simplicissimum*	KY315584.1	93	99.18	0.0
A7	*Penicillium expansum* DUCC5734	MT582774.1	100	100	0.0
A8	*Fusarium falciforme* DTO 422-H8	MT251175.1	100	100	0.0
B1	*Penicillium steckii* K23	MK179265.1	100	100	0.0
B2	*Penicillium griseofulvum* VIBENF4	MN545450.1	100	100	0.0
B3	*Penicillium griseofulvum* MPR1	MH006592.1	100	100	0.0
B4	*Penicillium expansum* DUCC5734	MT582774.1	100	100	0.0
C1	*Fusarium thapsinum* CBS 771.96	MH862612.1	100	100	0.0
C2	*Fusarium thapsinum* W_KSSO2_4_16	MK595260.1	100	100	0.0
C4	*Fusarium thapsinum* isolate W_KSSO2_4_16	MK595260.1	100	100	0.0
C5	*Fusarium thapsinum* CBS 130176	MH865738.1	100	100	0.0

C9	*Aspergillus alliaceus* CBS 132161	MH865971.1	100	99.81	0.0
FP	*Fusarium proliferatum* BL4	MW995863.1	100	100	0.0

3.2.1.1 Morphological characterization of endophytic isolates and pathogenic fungi

3.2.1.1.1 Morphological characterization of endophytic isolates related to *Penicillium*

The *Penicillium* species that were previously identified using ITS sequencing were further characterised by visualising their hyphal structures microscopically. The microscopic view of the *Penicillium* endophytes provided a better insight to its identification. Figure 3.3 A- F shows the conidiophores, phialides, matulaes, and stipes of each fungal endophyte pertaining to each *Penicillium* species.

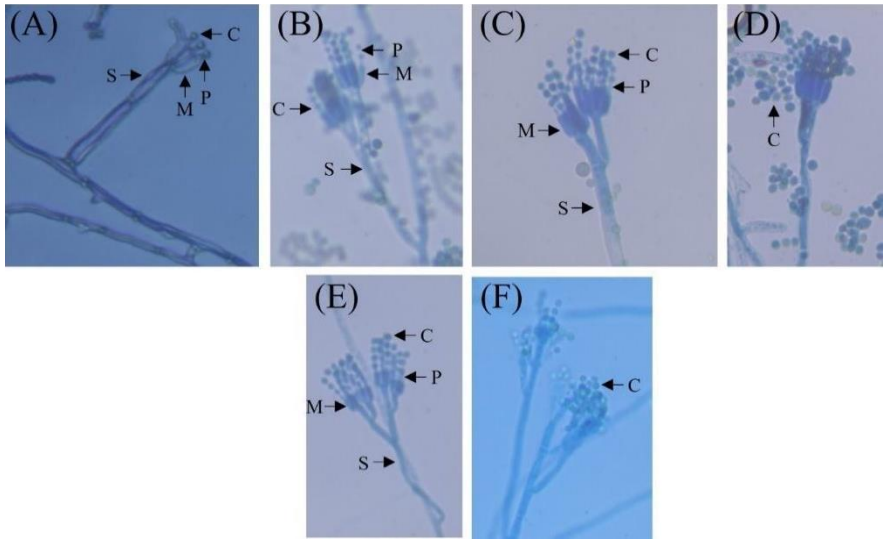


Figure 3.3 The microscopic view of the hyphal structures of the *Penicillium* fungal endophytes. (A) Monoverticillate hyphal structure of the A4 fungal endophyte. (B) Divaricate hyphal structure of the B1 fungal endophyte. (C - D) Divaricate hyphal structure of the A7 and B4 fungal endophyte. (E - F) Divaricate hyphal structure of the B2 and B3 fungal endophyte. C, conidiophores; P, phialide; M, metulae; and S, stipe.

3.2.1.1.2 Morphological characterization of endophytic isolates and pathogenic fungi related to *Fusarium*

Hyphal structures from each *Fusarium* endophyte and the pathogenic fungi *F. proliferatum* was microscopically viewed to assist in the morphological characterization and species identification. The microscopic view of the *Fusarium* endophytes provided a better insight to its identification. Figure 3.4 A-D shows the microconidia and macroconidia of each fungal endophyte, respectively, pertaining to each *Fusarium* species. Figure 3.5 shows the microconidia of the pathogen *F. proliferatum*.

The BLAST search and hyphal structures showed that the fungal endophytes C1, C2, C4 and C5 were in fact all *F. thapsinum*.

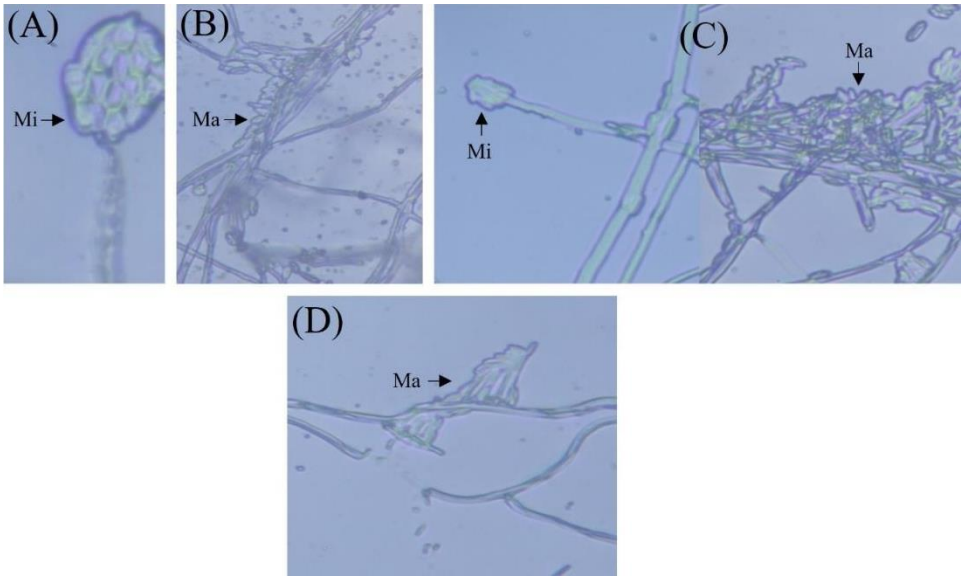


Figure 3.4 The microscopic view of the hyphal structures of the *Fusarium* fungal endophytes. (A) Hyphal structure of the C1 fungal endophyte. (B) Hyphal structure of the C2 fungal endophyte. (C) Hyphal structure of the C4 fungal endophyte. (D) Hyphal structure of the C5 fungal endophyte. Mi, microconidia in false head on monophialide. Ma, macroconidia.

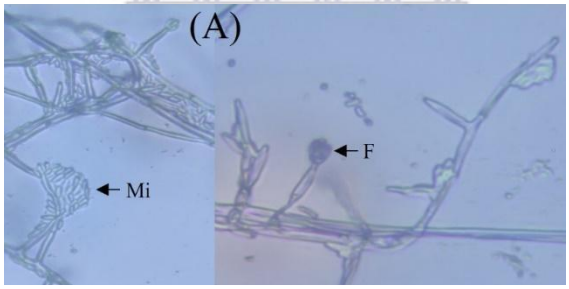


Figure 3.5 The microscopic view of the hyphal structures of the *Fusarium proliferatum*. Mi, microconidia; F, false head on monophialide.

3.2.1.1.3 Morphological characterization of endophytic isolates related to *Aspergillus*

Hyphal structures from the *Aspergillus* endophyte and was microscopically viewed to assist in the morphological characterization and species identification. The microscopic view of the *Aspergillus* fungal endophyte provided a better insight to its identification. Figure 3.6 shows that conidia used to positively identify *A. alliaceus*.

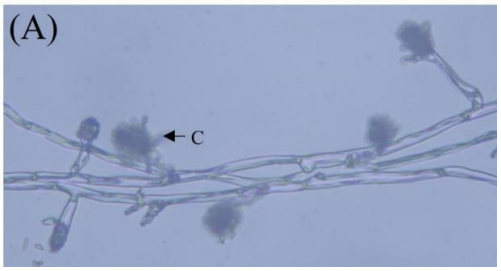


Figure 3.6 The microscopic view of the hyphal structures of the *Aspergillus* fungal endophyte. (A) Hyphal structure of the C9 fungal endophyte. C, conidia.

3.2.2 *In vitro* evaluation of antagonistic activity of endophytes against *Fusarium proliferatum*

The antagonistic activity of all 12 isolates of endophytic fungi isolated from *E. plantaginium* was tested against the pathogenic fungi *F. proliferatum* under *in vitro* conditions using dual-cultures. The percentage growth inhibition was calculated using the diameter of growth inhibition by each endophyte vis-à-vis the pathogen. All fungal endophytes isolated from the *E. plantaginium* host showed antagonistic activity against *F. proliferatum* (Table 3.3). The 12 fungal endophytes were capable of significant inhibition on the mycelial growth of the pathogen. The mycelial growth of *F. proliferatum* was significantly different based on each of the 12 endophytes, however, the 4 most effective fungal endophytic species were *P. simplicissimum*, *P. griseofulvum*, *P. expansum*, and *A. alliaceus*.

Table 3.3 Antagonistic interaction between endophytic fungi and *Fusarium proliferatum* causal agent of wilt of maize

Endophyte number	Host plant	Fungal endophyte	Growth inhibition against *F. proliferatum* (%)
A4	*Echium plantaginium*	*Penicillium simplicissimum*	78,65
A7	*Echium plantaginium*	*Penicillium expansum*	56,73
A8	*Echium plantaginium*	*Fusarium falciforme*	51,57
B1	*Echium plantaginium*	*Penicillium steckii*	41,53
B2	*Echium plantaginium*	*Penicillium griseofulvum*	51,70
B3	*Echium plantaginium*	*Penicillium griseofulvum*	67,33
B4	*Echium plantaginium*	*Penicillium expansum*	74,41
C1	*Echium plantaginium*	*Fusarium thapsinum*	40,27
C2	*Echium plantaginium*	*Fusarium thapsinum*	39,98

C4	*Echium plantaginium*	*Fusarium thapsinum*	46,02
C5	*Echium plantaginium*	*Fusarium thapsinum*	40,64
C9	*Echium plantaginium*	*Aspergillus alliaceus*	73,22

The control plate showing the growth of *F. proliferatum* on PDA media showed that the pathogen had grown over an average of 62.83 % of the plate and had surpassed the denoted middle line of the petri dish (Figure 3.7 A).

The dual-culture plates between *F. proliferatum* and endophyte A4 showed that A4 significantly restricted the growth of *F. proliferatum* when compared to the control (Figure 3.7 B). The growth of the A4 endophyte had shown an average of 78.65 % inhibition of mycelial growth of *F. proliferatum* (Table 3.3).

When compared with the control endophyte A7 restricted the growth of *F. proliferatum* with an average of 56.73 % (Figure 3.7 C; Table 3.3). Endophyte A8 had impacted the growth of *F. proliferatum*, with an 51.7% average inhibition (Figure 3.7 D; Table 3.3). Endophyte A4 had shown the greatest antagonistic effect on the growth of *F. proliferatum* of all the group A isolated endophytes (Table 3.3). The dual-culture results for trial one and three for the group A fungal endophytes can be seen in the Supplementary Figure 3.8; Supplementary Figure 3.11.

The percentage inhibition of the 12 fungal endophytes on the growth of *F. proliferatum* and the average percentage inhibition of the three trials can be accessed in Supplementary Table 3.1.

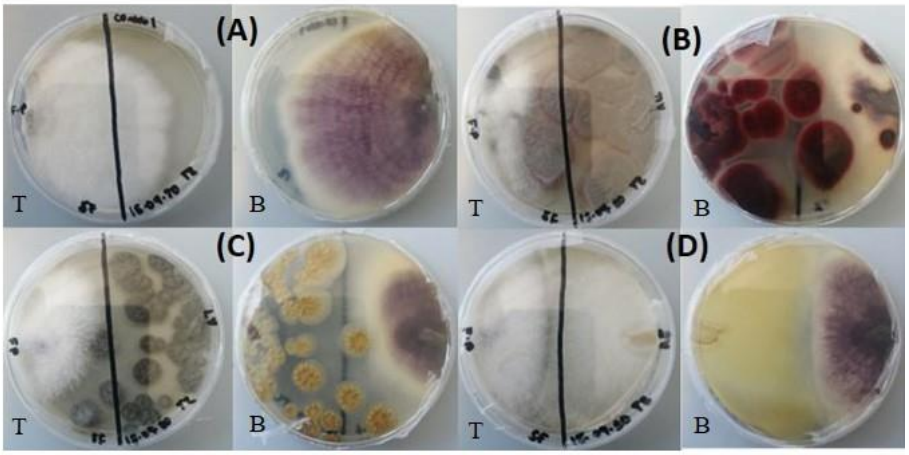


Figure 3.7 The *in vitro* antagonistic assay of fungal endophytes on the growth of *Fusarium proliferatum* on PDA media. (A) The growth of *F. proliferatum* on PDA media (control). (B) Dual culture of *F. proliferatum* and fungal endophyte A4. (C) Dual culture of *F. proliferatum* and fungal endophyte A7. (D) Dual culture of *F. proliferatum* and fungal endophyte A8. Each dual culture was grown for 7-10 days. T - Top of plate; B - Bottom of plate.

When compared to the control in Figure 3.8 A, the growth of *F. proliferatum* was significantly impacted by endophytes B1, B2, B3 and B4 which showed similar growth patterns. The B1 endophyte had reduced the growth of *F. proliferatum* by an average inhibition of 41.53 % (Table 3.3) whereas the growth of the B1 endophyte was widely distributed across the rest of the plate, far exceeding the line denoting the middle of the plate as shown in Figure 3.9 B. On average, endophytes B2 and B3 restricted the growth of *F. proliferatum* by 51.70 % and 67.33 % respectively (Figure 3.8 C and D; Table 3.3). B4 suppressed the growth of the pathogen by an average of 74.41 %, showing a substantial antagonistic impact of *F. proliferatum* (Figure 3.8 E; Table 3.3). The B3 and B4 endophytes had shown the greatest antagonistic effect on the growth of *F. proliferatum* of all the group B isolated endophytes (Table 3.3). The dual-culture results for group B fungal endophytes can be accessed in Supplementary Figure 3.9; Supplementary Figure 3.12.

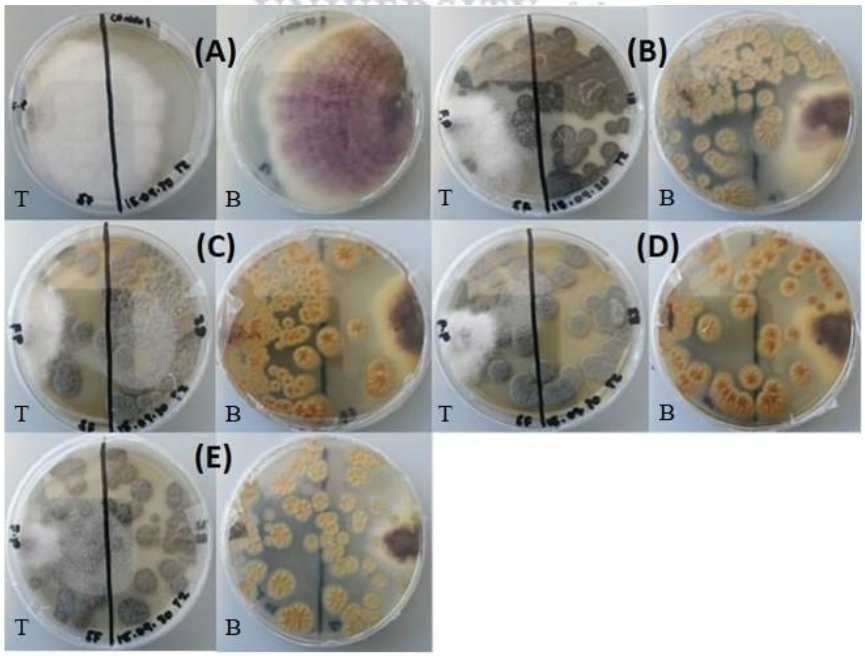


Figure 3.8 The *in vitro* antagonistic assay of group B fungal endophytes against *Fusarium proliferatum*. (A) The growth of *F. proliferatum* on PDA media. (B) Dual culture of *F. proliferatum* and fungal endophyte B1. (C) Dual culture of *F. proliferatum* and fungal endophyte B2. (D) Dual culture of *F. proliferatum* and fungal

endophyte B3. (E) Dual culture of *F. proliferatum* and fungal endophyte B4. Each dual culture was grown for 7-10 days. T - Top of plate; B - Bottom of plate

When endophytes were compared to the control (Figure 3.9 A) the growth of *F. proliferatum* was partially impacted by endophytes C1, C2, C4, and C5 with a similar growth pattern. The C1 had inhibited the growth of the pathogen by an average of 40.27 % whereas C2 showed an average growth inhibition of 39.98 % (Figures 3.9 B and C; Table 3.3). Endophyte C5 restricted the mycelial growth of *F. proliferatum* by 40.64% when compared to the growth of the control (Figure 3.9 E; Table 3.3). Although the endophytes C1, C2 and C5, as well as *F. proliferatum* had a similar growth percentage, the two fungal species had not grown over each other at the middle line. A similar pattern was observed in the dual culture between C4 and *F. proliferatum* (Figure 3.9 D), however, the growth of endophyte C4 had shown an average inhibition of 46.02 % (Table 3.3). Endophyte C9 had shown an increased antagonistic effect on the growth of *F. proliferatum* where its growth was restricted by 73.22 % (Figure 3.9 F; Table 3.3). An overlap of growth between each respective C-grouped endophyte and *F. proliferatum* had not been observed. The C9 endophyte had shown the greatest antagonistic effect on the growth of *F. proliferatum* of all the group C isolated endophytes (Table 3.3). The dual-culture results for trial one three for the group C fungal endophytes can be accessed in the Supplementary Figure 3.10; Supplementary Figure 3.13

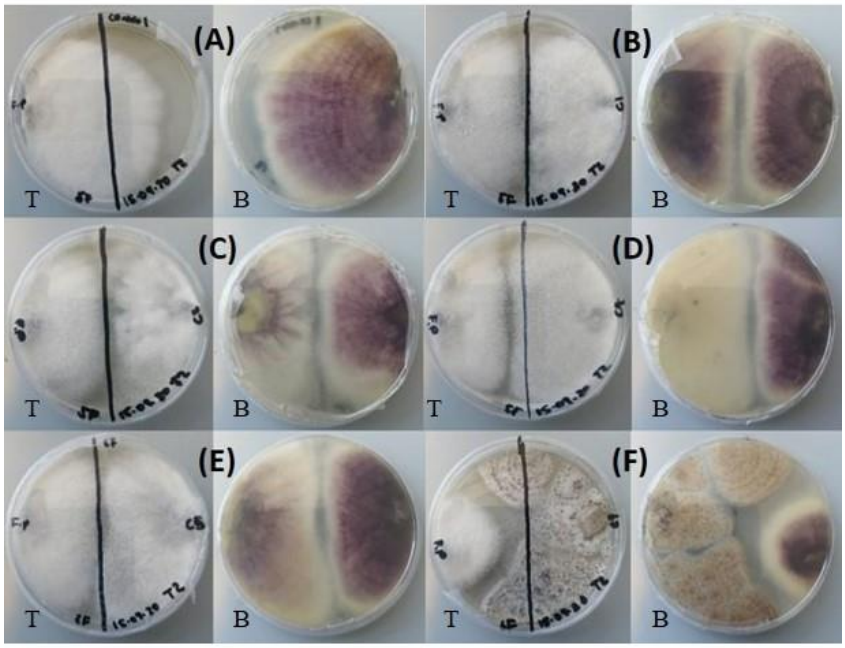


Figure 3.9 The *in vitro* antagonistic assay of fungal endophytes on the growth of *Fusarium proliferatum* on PDA media. Each showing the top and bottom of the plate. (A) The growth of *F. proliferatum* on PDA media. (B) Dual culture of *F. proliferatum* and fungal endophyte C1. (C) Dual culture of *F. proliferatum*

and fungal endophyte C2. (D) Dual culture of *F. proliferatum* and fungal endophyte C4. (E) Dual culture of *F. proliferatum* and fungal endophyte C5. (F) Dual culture of *F. proliferatum* and fungal endophyte C9. Each dual culture was grown for 7-10 days. T - Top of plate; B - Bottom of plate

Based on the *in vitro* antagonistic results observed in this study, endophytic isolates B3 and B4 were selected downstream *in planta* assay (Table 3.3).

3.3 Discussion

Numerous fungal endophytes have been successfully tested as biological control agents against pathogenic fungi *in vitro* (Medina-Córdova *et al*., 2016; Worapong and Strobel, 2009). The strategy is significant in that it improves crop productivity and is an alternative to chemical fungicides which are harmful to the environment and to human and animal health. (Wang *et al*., 2016; Wu *et al*., 2016). However, the use of various fungal endophytes isolated from *E. plantaginium* as biocontrol agents against the fungal pathogen *F. proliferatum in vitro* conditions has not been reported. The novelty and special interest in this study is that the endophytic fungi were isolated from host plants collected in South Africa.

Numerous authors have used endophytic fungi such as *Alternaria, Curvularia, Cladosporium, Phaeoacremonium,* and *Trichoderma* isolated from various host plants such as tomato, star anise, agarwoor and mangrove, and were used to biologically control pathogenic fungi (Debbab *et al*., 2013; Premalatha and Kalra, 2013).

In the present study, 12 endophytic fungi were evaluated for their antagonistic potential against *F. proliferatum.* The results showed that among the 12 isolates, *Penicillium* and *Aspergillus* species reduced the mycelial growth of *F. proliferatum* with a growth inhibition percentage that ranged up to 78,65 % (Table 3.3), however, the antagonistic effect was endophyte dependent. Similar responses were previously reported by Saravanakumar *et al*. (2016); Zhang *et al*. (2014); Xiang *et al*. (2016), who isolated fungal endophytes from cucumber and other medicinal plants. Fungal endophytes can control plant pathogens under *in vitro* conditions using diverse mechanisms (Zhang *et al*., 2014). Similarly, in a study by Worapong and Strobel (2009), endophytic fungi, *Muscodor albus*, controlled *Pythium ultimum* under *in vitro* conditions with the production of volatile compounds.

3.3.1 Species identification and morphological characterization of isolated fungal endophytes

The main fungal genera associated with maize include *Aspergillus*, *Fusarium*, and *Penicillium* (Fandohan *et al*., 2003). The *Penicillium* species is one of the most common fungi found in a range of diverse habitats (Visagie *et al*., 2014). Certain *Penicillium* species is known for its production of Penicillin which has revolutionized the treatment of bacterial infections (Houbraken and Samson, 2011). However, the key function of *Penicillium* is to decompose organic material in natural habitats, and this may ultimately damage important food crops (Assaf *et al*., 2020). *P. expansum* is commonly found on tomatoes, pears and apples and is known as one of the most common pathogenic fungi which causes blue mold (Song *et al*., 2020). This may result in serious economic loss for the aforementioned produce worldwide (Song *et al*., 2020). *P. expansum* can produce resistant asexual spores that increases the difficulty of elimination. The species is also responsible for the production of patulin which is a secondary metabolite that threatens consumer health (Ma *et al*., 2020; Song *et al*., 2020).

The phylogenetic tree based on bootstrap neighbour-joining showed that the A4 fungal endophyte was distantly related to all the other isolated *Penicillium* fungal endophytes (Supplementary Figure 3.1). Additionally, the phylogenetic tree confirms the species identification that showed that the fungal endophytes A7 and B4 are the same species, and thus they are closely related (Supplementary Figure 3.1). Furthermore, the phylogenetic tree confirms the species identification that showed that the fungal endophytes B2 and B3 are the same species, and thus they are closely related (Supplementary Figure 3.1).

The A4 fungal endophytes hyphal structures were positively identified using previous studies by Oh *et al*. (2011) and Visagie *et al*. (2014). Based on these studies the conidiophores, phialide, metulae and the stipe were positively identified (Figure 3.3 A). Although the A4 fungal endophyte was sequenced, the percentage identity was only 99.18 % related to *P. simplicissimum* (Table 3.2).

The B1 fungal endophytes hyphal structures were positively identified using previous studies by Houbraken *et al*. (2011); Visagie *et al*. (2014). The conidiophores, phialide, metulae and the stipe were positively identified as the structures belonging to *P. steckki* (Figure 3.3 B) which was positively identified with a 100 % identity (Table 3.2). The A7 and B4 fungal endophytes hyphal structures were positively identified using previous studies by Errampalli (2014); Visagie *et al*. (2014). Based on these studies the conidiophores, phialide, metulae and the stipe were positively identified as the structures belonging to *P. expansum* (Figure 3.3 C) which was positively identified with a 100 % identity (Table 3.2). The B2 and B3 fungal endophytes

hyphal structures were positively identified using studies by Moslem *et al*. (2010); Visagie *et al*. (2014). The conidiophores, phialide, metulae and the stipe were positively identified as the structures belonging to *P. griseofulvum* (Figure 3.3 D) which was positively identified with a 100 % identity (Table 3.2). In a study by Yao *et al*. (2021), the genome of the coral-derived fungus *P. steckii was* found to encode 28 secondary metabolite gene clusters. The major mycotoxin observed in the study was citrinin which exhibited antibacterial activities. According to Xing *et al*. (2021), *P. steckii* is the main cause of mangoes' postharvest infection and deterioration which significantly impacts the quality of the fruit and shortens the shelf-life. *P. griseofulvum* can produce the antifungal agent known as griseofulvin which is industrially produced (Rana *et al*., 2019).

The interaction between an endophytic fungus and a host can change from neutral to pathogenic, mutualistic, or saprophytic depending on genetic dispositions, environmental factors, and time (Zerroug *et al*., 2018). Wakelin *et al*. (2004), showed that a total number of 223 endophytes were isolated and identified as *Talaromyces flavus*, *P. griseofulvum*, *Penicillium radicum*, *Penicillium bilaiae* and *P. simplicissimum*.

Members of the *Fusarium solani* complex accounts for almost two-thirds of all *Fusarium* species (O'Donnell *et al*., 2008*). Fusarium falciforme* appears to be one of the most common and genetically diverse mycosis-associated species in the *F. solani* complex (Edupuganti *et al*., 2011; Palmore *et al*., 2010;).

The phylogenetic tree based on bootstrap neighbour-joining showed that the C1, C2, C4 and C5 fungal endophytes were more closely related to each other (Supplementary Figure 3.2). Therefore, the phylogenetic tree confirms the species identification that showed that the endophytes were the same species (Supplementary Figure 3.2). Furthermore, the phylogenetic tree showed a sequence divergence between the C,1 C2, C4, and C5 fungal endophytes and *F. proliferatum* (Supplementary Figure 3.2).

The C1, C2, C4, and C5 fungal endophytes hyphal structures were positively identified using previous studies by Kim *et al*. (2016). Based on these studies the microconidia, macroconidia and microconidia in false head on monophialide was positively identified as the structures belonging to *Fusarium thapsinum* (Figure 3.4 A-D) which was positively identified with a 100 % identity (Table 3.2). The *F. proliferatum* hyphal structures were positively identified using previous studies by Masratul Hawa *et al*. (2013) and based on these studies the microconidia,

and false head in monophialide was positively identified as the structures belonging to *F. proliferatum* (Figure 3.5) which was positively identified with a 100 % identity (Table 3.2). In a study by Medeiros Araujo *et al*. (2021), it was observed that *F. falciforme* was the most commonly occurring species.

The phylogenetic tree based on bootstrap neighbour-joining showed that the C9 fungal endophyte was closely related to the species identified using the BLAST search (Supplementary Figure 3.3). The C9 fungal endophytes hyphal structures were positively identified using a previous study by Ozhak-Baysan *et al*. (2010) and based on these studies the conidia were positively identified as the structures belonging to *A. alliaceus* (Figure 3.6) which was positively identified with a 100 % identity (Table 3.2).

Aspergillus is a well-known saprotroph and is easily isolated from plant material. Taxonomic similarities and classification of *Aspergillus* species are complex and controversial due to morphological flexibility among the various strains at an intraspecies level.

3.3.2 Evaluation of antagonistic activity of fungal endophytes

3.3.2.1 Dual-culture assay (*in vitro* assay)

In a previous study conducted by Orole and Adejumo (2009), fungal endophytes were observed for their impact on the growth of *Fusarium oxysporum, Fusarium pallidoroseum, Fusarium verticillioides* and *Cladosporium herbarum*, each isolated from maize plants infected with seedling blight and root and stalk rot. The fungal endophytes used in the study included *Beaveria bassiana, Trichoderma koningii, Alternaria alternata, Phoma sp., Acremonium strictum*. The studies *in vitro* assay had shown that *T. koningii* and *A. alternata* reduced the growth of the pathogens by 25 -75 % and 53 -80 %, respectively. Similarly, in a study conducted by Nefzi *et al*. (2019), the endophyte *Withania somnifera* was utilized as a BCA of *Fusarium* crown and root rot (FCRR) in tomato which is initiated by the species *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*FORL*). The endophytic isolates used in the study had enhanced the growth of tomato plants by 21.5 - 90.3 % relative to the control.

Additionally, in a study conducted by Gonzalez *et al*. (2020), 7 fungal endophytes were examined on their antagonistic impact on diseases such as *Fusarium wilt, Monosporascus cannonballus* and *Macrophomina phaseolina*, in melon and watermelon plants. Percentage inhibitions of up to 67 % were observed. Moreover, in a study done by Fierro-Cruz *et al*.

(2016), five isolated endophytes had shown an inhibition on the growth of *F. oxysporum* via the production of metabolites, to a percentage inhibition of approximately 40 %.

The results in this study suggest that *E. plantaginium* harbours diverse endophytic fungi, which can be used as possible biocontrol agents against disease causing pathogens like *F. proliferatum*. The 12 fungal endophytes used in the study had all shown an antagonistic effect on the growth of *F. proliferatum* (Table 3.3). The fungal endophytes, *P. griseofulvum* (B3) and *P. expansum* (B4), were used *in planta* and downstream analysis.

Chapter 4

Penicillium griseofulvum and *Penicillium expansum* modulates root growth and biochemical responses in maize under *Fusarium proliferatum* infection

4.1 Introduction

Fusarium proliferatum (teleomorph Gibberella intermedia) is a filamentous ascomycete saprophytic pathogenic fungus that is distributed worldwide and has been related to an assortment of diseases in vital economical florae (Gao *et al*., 2017; Sun *et al*., 2018; Sun *et al*., 2019). As a representative of the section Liseola, the microconidia of *F. proliferatum* are shaped as an ovoid and obovoid. In addition, the microconidia are situated on lined chains and polyphialides (Isack *et al*., 2014).

The various plant families that *F. proliferatum* fungus are pathogenic to include but are not limited to garlic, asparagus, onion, tomato, maize, rice, banana, date palm, and soybean (Gao *et al*., 2017; Kamle *et al*., 2019). *F. proliferatum* yields a great amount of conidia that can live in the soil for many years (Gao *et al*., 2017). As the weather and environment get warmer due to season change, the conidia germinate and are therefore able to spread via the movement of rainwater and atmospheric dust, and subsequently able to infect seeds, soil, and plant material (Gao *et al*., 2017; Isack *et al*., 2014). Maize seed infected with pathogenic fungi such as *F. proliferatum* causes significant problems such as the loss of the seeds capacity to germinate and ultimately leads to the reduction in yield (Galli *et al*., 2005; Kaur *et al*., 2020; Masiello *et al*., 2021). The infected seed that can germinate possesses changes in their phenotypes, and this may be caused by an imbalance in redox homeostasis. A study by Klein and Bangani (2019) showed that *F. proliferatum* causes a restriction in wheat seed germination, plant growth and biomass.

Endophytes are plant-based organisms that are globally abundant; they form associations with various groups of organisms through the plant kingdom and offer an indirect defence against pathogenic fungi (Bamisile *et al*., 2018; Kaur, 2020). Endophytes are able to exist in its host plant as a mutualistic root endophyte or as a plant-associated endophyte (Bamisile *et al*., 2018;

Kaur, 2020). Fungal endophytes reside in the internal tissues of living plants and do not cause detrimental effects on the host plant (Aly *et al*., 2011).

Fungal endophytes possess numerous characteristics which makes them suitable biological control agents (BCA's), including their ability to directly penetrate the insect's cuticle, increase the mortality rates of the pest population and the potential for epizootics (Amatuzzi *et al*., 2017). Thus, these fungi are able to colonize plants as endophytes (Amatuzzi *et al*., 2017). A single part of the plant, for example, its root, leaf, and stem may have diverse endophytic species (Bamisile *et al*., 2018; Kaur, 2020).

Endophytic fungi may be broadly classified into ecological categories or based on their functional roles or diversity (Bamisile *et al*., 2018). Based on these classifications, endophytic fungi have been placed into two main groups known as non-clavicipitaceous and clavicipitaceous fungal endophytes (Bamisile *et al*., 2018; Gautam and Avasthi, 2019). Non-clavicipitaceous fungal endophytes are found in non-vascular and vascular plant species and clavicipitaceous endophytes are typically found in grasses (Bamisile *et al*., 2018; Gautam and Avasthi, 2019).

Endophytic microorganisms can improve plant growth via the secretion of phytohormones that consequently aids in the nutritional improvement of the host plant using bidirectional nutrient transfer which also hinders the development and growth of competitors including pathogenic organisms (Andreozzi *et al*., 2019; Shen *et al*., 2019). Endophytic microorganisms protect their host plant via indirect and direct mechanisms; where the indirect mechanisms induce the plants resistance to pathogens, stimulates plant secondary metabolites, promotes plant physiology and growth and direct mechanisms includes the production of phytohormones, the secretion of lytic enzymes, the solubilization of phosphate, and the production of siderophore and indolic compounds (Fadiji and Babalola, 2020).

Maize seeds inoculated with *F. proliferatum* caused a reduction in seed root length but when primed with fungal endophytes prior to inoculation the rate/percentage of seed germination and plant growth increased (Chang *et al*., 2015; Mastouri *et al*., 2010). The priming of seeds with endophytes also reversed the ROS-induced oxidative damage as opposed to seeds infected with *F. proliferatum*.

Therefore, this study aimed to investigate the impact of *F. proliferatum* on maize seed root growth and biochemical responses and how biopriming with fungal endophytes *P. griseofulvum* (B3) and *P. expansum* (B4) can modulate these changes.

4.2 Results

Two fungal endophytes that showed the best antifungal activity against *F. proliferatum* under *in vitro* conditions were tested against *F. proliferatum* infected maize seeds under greenhouse conditions.

4.2.1 *Fusarium proliferatum* restrict maize root growth

Maize seeds inoculated with *F. proliferatum* showed reduction in maize root growth (Figure 4.1 A and B). In comparison to the controls average root length (14.65 cm), when maize seeds were infected with *F. proliferatum* at a concentration of 10^8 cells/ml, the root length significantly decreased by 49 % (Figure 4.1 A). The results showed that high concentrations of the pathogen significantly decreased root length in comparison to the control (Figure 4.1 B). Given the response of infection with *F. proliferatum* at a concentration of 10^8 cells/ml on maize root growth all downstream analysis will only focus on this concentration.

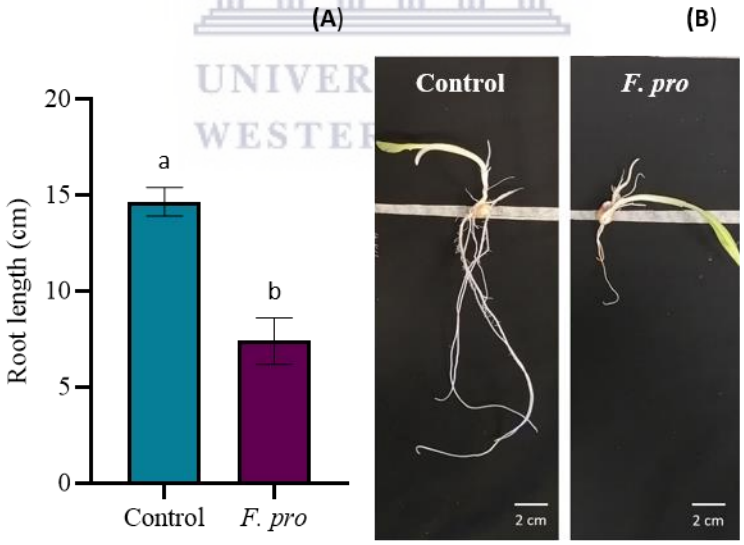


Figure 4.1 The effect of *Fusarium proliferatum* on the maize root growth. (A) Average root length of white maize seeds infected with *F. proliferatum* at a concentration of 10^8 cells/ml was measured. (B) Root length of white maize seeds infected with *F. proliferatum* at a concentration of 10^8 cells/ml. Seeds were germinated for 7 days' post inoculation. Data presented are means (±SE) of two independent experiments (n=2). Error bars denote standard deviation, where bars with the same letters are statistically similar and varying letters indicate statistical differences where $P < 0.05$.

4.2.2 Biopriming with *Penicillium griseofulvum* (B3) *and Penicillium expansum* (B4) improves maize root growth under *Fusarium proliferatum* infection

Maize seeds primed with *P. griseofulvum* and inoculated with *F. proliferatum* at a final concentration of 10^8 cells/ml showed a significant increase in root length (65 %) when compared to those inoculated with *F. proliferatum* treatment alone, albeit not to the level of the control (Figure 4.2 A and B). Interestingly, more lateral root formation was observed in the B3-primed experiments as opposed to the *F. proliferatum* inoculated experiments.

Maize seeds primed with *P. expansum* and inoculated with *F. proliferatum* at a final concentration of 10^8 cells/ml showed a significant increase in root length (72 %) when compared to those inoculated with *F. proliferatum* alone, albeit not the level of the control (Figure 4.2 A and B).

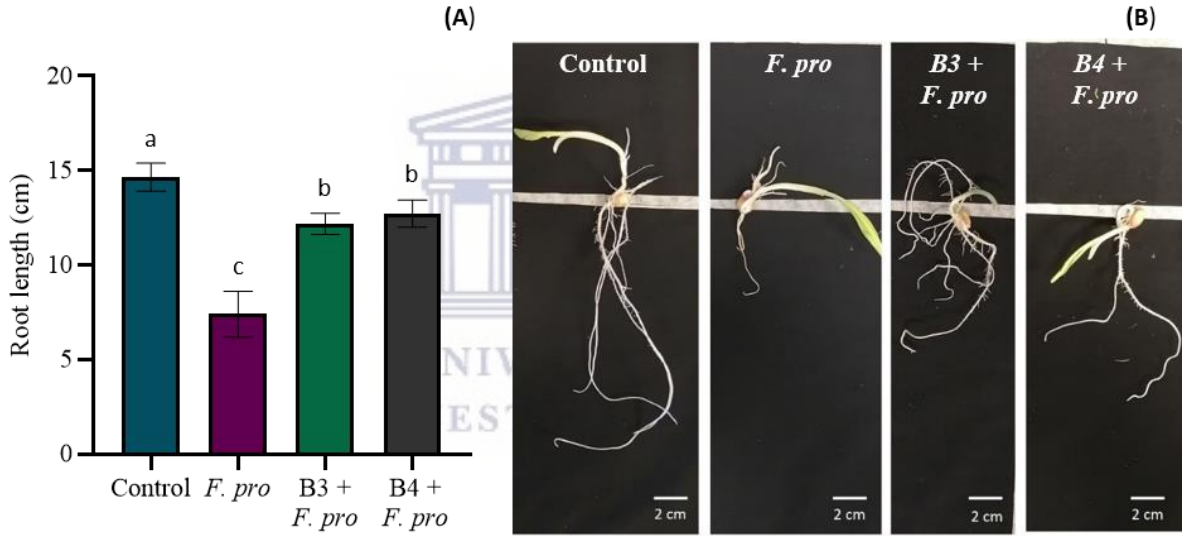


Figure 4.2 Effect of *Penicillium griseofulvum* (B3) and *Penicillium expansum* (B4) on root growth under *Fusarium proliferatum* infection at a concentration of 10^8 cells/ml. (A) Average root length of maize seeds primed with B3 and B4 fungal endophytes (10^8 cells/ml) and infected with *F. proliferatum* at a concentration of 10^8 cells/ml. (B) Root length of white maize seeds primed with B3 and B4 fungal endophytes and infected with *F. proliferatum* at a concentration of 10^8 cells/ml. Seeds were germinated for 7 days' post priming/inoculation. Data presented are means (±SE) of two independent experiments (n=2). Error bars denote standard deviation, where bars with the same letters are statistically similar and varying letters indicate statistical differences where $P < 0.05$.

4.2.3 *Penicillium griseofulvum* (B3) and *Penicillium expansum* (B4) restricts *Fusarium*-induced ROS accumulation in maize roots

In response to *F. proliferatum*, superoxide content was increased by 91 % when compared to the untreated control (Figure 4.3 A). However, when maize seeds were primed with *P. griseofulvum* prior to inoculation with *F. proliferatum*, superoxide content was reduced by 14 % relative to the *F. proliferatum* treatment, albeit still higher than that of the control (Figure 4.3 A). Interestingly, when maize seeds were primed with *P. expansum* prior to inoculation with *F. proliferatum*, superoxide content was increased to levels significantly higher than observed for both *F. proliferatum* and untreated control. This increase was measured at 91 % higher than the *F. proliferatum* treatment (Figure 4.3 A).

Maize seeds inoculated with *F. proliferatum* increased hydrogen peroxide content by 550 % relative to the control (Figure 4.3 B). Maize seeds primed with *P. griseofulvum* prior to infection with *F. proliferatum* showed a reversal in hydrogen peroxide content to levels observed for the untreated control. The reduction in hydrogen peroxide content for *P. griseofulvum* primed seeds was measured at 87 % relative to the *F. proliferatum* treatment only (Figure 4.3 B). Likewise, to what was observed for *P. griseofulvum* primed seeds, *P. expansum* primed seeds showed a significant reduction in hydrogen peroxide accumulation in the roots relative to the *F. proliferatum* treatment. Hydrogen peroxide content in the roots of B4-primed seeds inoculated with *F. proliferatum* was reduced by 82 % relative to the *F. proliferatum* (Figure 4.3 B).

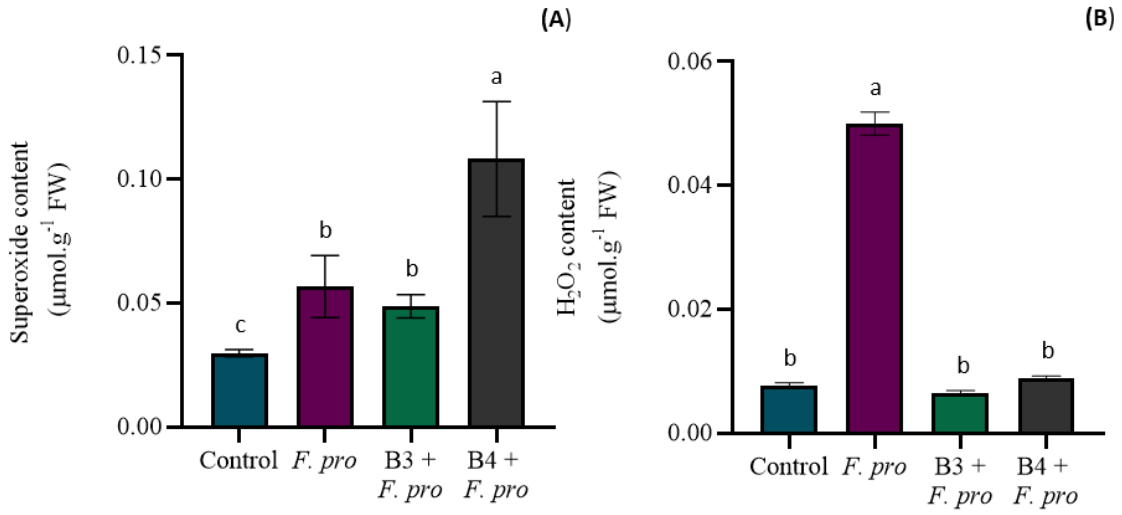


Figure 4.3 *Penicillium griseofulvum* (B3) and *Penicillium expansum* (B4) alters superoxide and hydrogen peroxide contents in maize roots infected with *Fusarium proliferatum* at a concentration of 10^8 cells/ml. (A)

Superoxide content in maize roots primed with *P. griseofulvum* and *P. expansum* and infected with *F. proliferatum*. (B) Hydrogen peroxide content in maize roots primed with *P. griseofulvum* and *P. expansum* and infected with *F. proliferatum*. Data presented are the means (±SE) of three independent experiments (n=3). Error bars denote standard deviation, where bars with the same letters are statistically similar and varying letters indicate statistical differences where $P < 0.05$.

4.2.4 *Penicillium griseofulvum* (B3) and *Penicillium expansum* (B4) restricts the extent of lipid peroxidation in maize roots under *Fusarium proliferatum* infection

The MDA levels in *F. proliferatum* inoculated and *Penicillium*-primed (B3 and B4) maize roots are shown in Figure 4.4. Maize seeds inoculated with *F. proliferatum* increased in MDA content by 213 % relative to the untreated control.

Maize seeds primed with *P. griseofulvum* and inoculated with *F. proliferatum* showed a reduction (45 %) in root MDA content relative to the *F. proliferatum* treatment alone, whereas no significant changes were observed when compared to the control (Figure 4.4). A similar response was observed in *P. expansum* primed roots inoculated with *F. proliferatum*. Relative to the *F. proliferatum* treatment, maize seeds primed with *P. expansum* and inoculated with *F. proliferatum* restricted root MDA content by 31%, albeit not the level of the control (Figure 4.4).

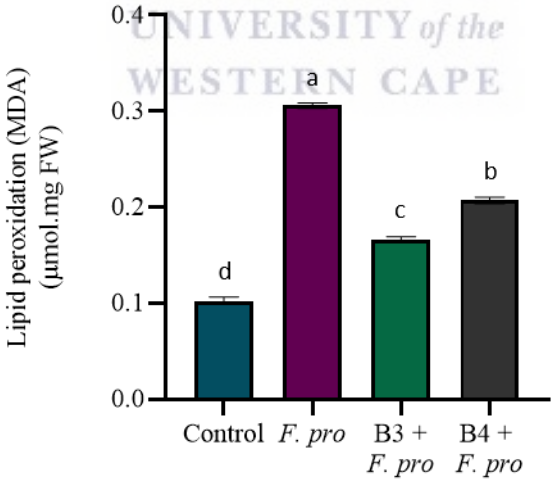


Figure 4.4 *Penicillium griseofulvum* (B3) and *Penicillium expansum* (B4) reduces malondialdehyde (MDA) content in maize roots infected with *Fusarium proliferatum* at a concentration of 10^8 cells/ml. MDA content in maize roots primed with *P. griseofulvum* and *P. expansum* and infected with *F. proliferatum*. Data presented are means (±SE) of three independent experiments (n=3). Error bars denote standard deviation, where bars with the same letters are statistically similar and varying letters indicate statistical differences where $P < 0.05$.

4.2.5 *Penicillium griseofulvum* (B3) and *Penicillium expansum* (B4) augments the activity of antioxidants in maize roots under *Fusarium proliferatum* infection

4.2.5.1 *Penicillium griseofulvum* (B3) and *Penicillium expansum* (B4) augments the superoxide dismutase activity in maize roots under *Fusarium proliferatum* infection

The superoxide dismutase content in *F. proliferatum* infected and *Penicillium*-primed (B3 and B4) maize roots are shown in Figure 4.5. Maize seeds inoculated with *F. proliferatum* decreased in SOD activity by 92 % relative to the untreated control.

Maize seeds primed with *P. griseofulvum* and inoculated with *F. proliferatum* showed an increase (1195 %) in root SOD content relative to the *F. proliferatum* treatment alone, whereas no significant changes were observed when compared to the control (Figure 4.5). A similar response was observed in *P. expansum* primed roots inoculated with *F. proliferatum*. Relative to the *F. proliferatum* treatment, maize seeds primed with *P. expansum* and inoculated with *F. proliferatum* increased root SOD content by 1595 %, albeit not the level of the control (Figure 4.5).

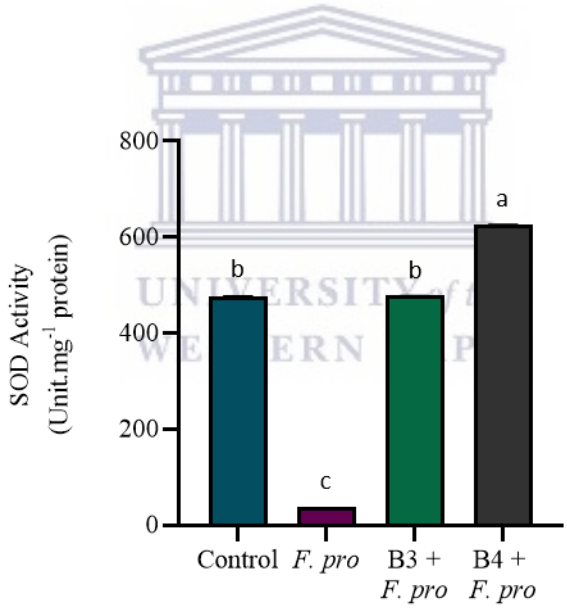


Figure 4.5 *Penicillium griseofulvum* (B3) and *Penicillium expansum* (B4) augments superoxide dismutase content in maize roots infected with *Fusarium proliferatum* at a concentration of 10^8 cells/ml. Data presented are means (±SE) of three independent experiments (n=3). Error bars denote standard deviation, where bars with the same letters are statistically similar and varying letters indicate statistical differences where $P < 0.05$.

4.2.5.2 *Penicillium griseofulvum* (B3) and *Penicillium expansum* (B4) augments the ascorbate peroxidase activity in maize roots under *Fusarium proliferatum* infection

The ascorbate peroxidase content in *F. proliferatum* infected and *Penicillium*-primed (B3 and B4) maize roots are shown in Figure 4.6. Maize seeds inoculated with *F. proliferatum* increased in APX activity by 79 % relative to the untreated control.

Maize seeds primed with *P. griseofulvum* and inoculated with *F. proliferatum* showed an increase (377 %) in root APX content relative to the *F. proliferatum* treatment alone (Figure 4.6). A similar response was observed in *P. expansum* primed roots inoculated with *F. proliferatum*. Relative to the *F. proliferatum* treatment, maize seeds primed with *P. expansum* and inoculated with *F. proliferatum* increased root APX content by 541 % (Figure 4.6).

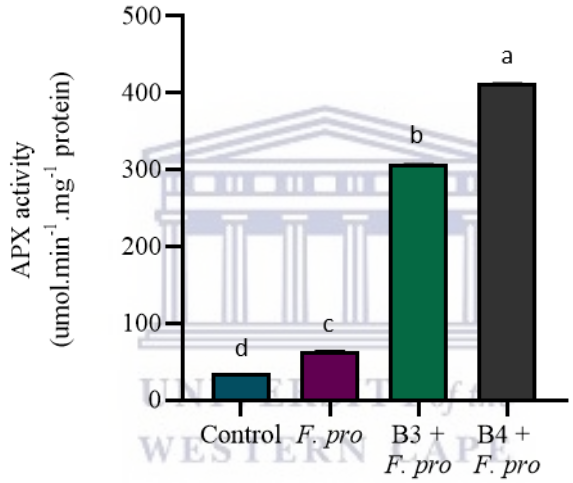


Figure 4.6 *Penicillium griseofulvum* (B3) and Penicillium *expansum* (B4) augments ascorbate peroxidase content contents in maize roots infected with *Fusarium proliferatum* at a concentration of 10^8 cells/ml. Data presented are means (±SE) of three independent experiments (n=3). Error bars denote standard deviation, where bars with the same letters are statistically similar and varying letters indicate statistical differences where $P < 0.05$.

4.2.5.3 *Penicillium griseofulvum* (B3) and *Penicillium expansum* (B4) changes the guaiacol peroxidase activity in maize roots under *Fusarium proliferatum* infection

Maize seeds inoculated with *F. proliferatum* increased guaiacol peroxidase activity by 13 % relative to the control for GPOX 1 (Figure 4.7 A and B) and 17 % for GPOX 2 (Figure 4.7 A and C).

Maize seeds primed with *P. griseofulvum* prior to inoculation with *F. proliferatum* showed a reversal in guaiacol peroxidase activity to levels observed for the untreated control. The

reduction in guaiacol peroxidase activity for *P. griseofulvum* primed seeds was measured at 12 % relative to the *F. proliferatum* treatment only for GPOX 1 (Figure 4.7 A and B) and 13 % for GPOX 2 (Figure 4.7 A and C). Contrary to what was observed for *P. griseofulvum* primed seeds, *P. expansum* primed seeds showed a minor increase in guaiacol peroxidase activity in the roots relative to the *F. proliferatum* treatment. Guaiacol peroxidase activity in the roots of B4-primed seeds inoculated with *F. proliferatum* was increased by 1 % for GPOX 1 (Figure 4.7 A and B) and decreased by 4 % for GPOX 2 relative to the *F. proliferatum* (Figure 4.7 A and C).

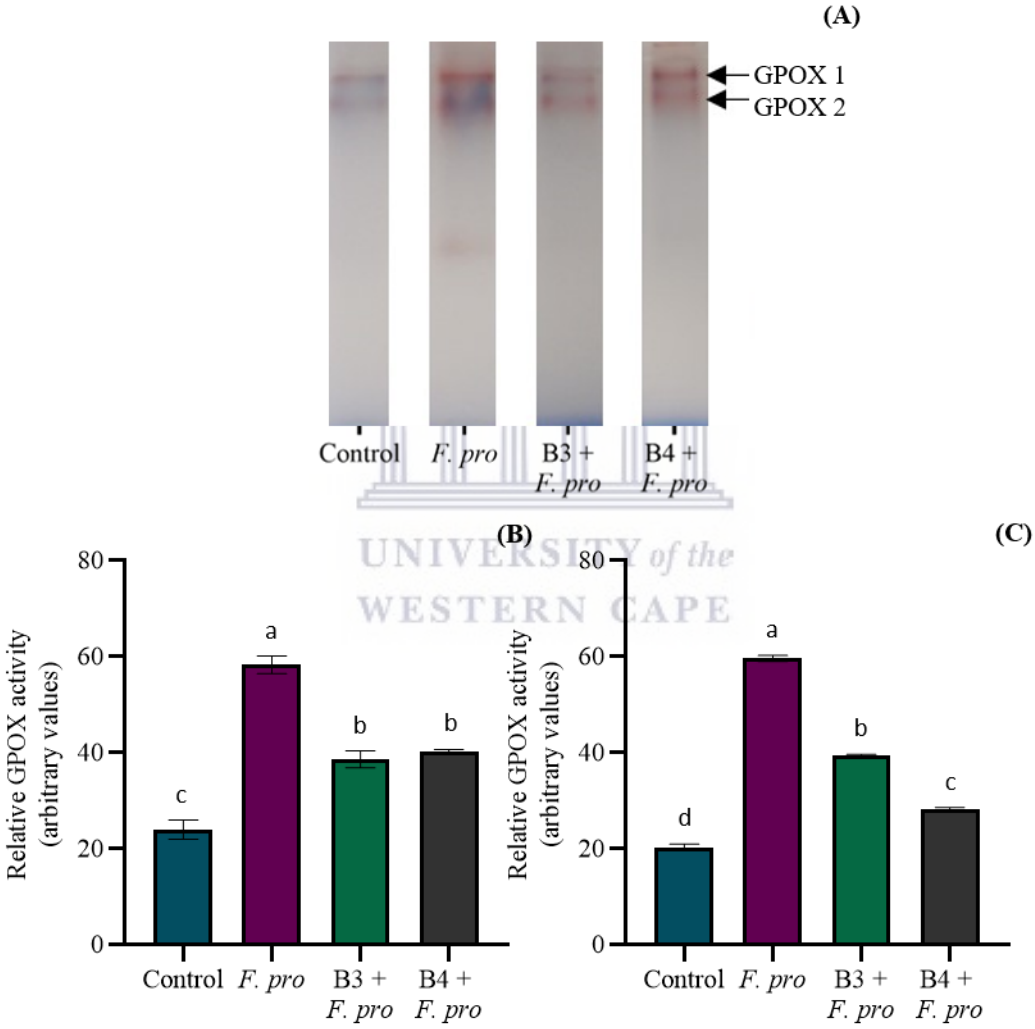


Figure 4.7 *Penicillium griseofulvum* (B3) and *Penicillium expansum* (B4) changes guaiacol peroxidase activity in maize roots infected with *Fusarium proliferatum* at a concentration of 10^8 cells/ml. The in-gel activity assay of GPOX isoforms in response to the various treatments is represented in (A), from which pixel intensities of GPOX 1 (B) and GPOX 2 (C) were determined. Data represents the means (±SE) of three independent experiments (n=3). Error bars denote standard deviation, where bars with the same letters are

statistically similar and varying letters indicate statistical differences where $P < 0.05$. Band intensities were evaluated using Alpha Ease with the "invert" function.

4.2.5.4 *Penicillium griseofulvum* (B3) and *Penicillium expansum* (B4) changes the peroxidase with catalase activity in maize roots under *Fusarium proliferatum* infection

The peroxidase with catalase activity in *F. proliferatum* infected and *Penicillium*-primed (B3 and B4) maize roots are shown in Figure 4.8 A- C. Maize seeds infected with *F. proliferatum* increased in peroxidase with catalase activity by 66 % for POD with CAT activity 1 (Figure 4.8 A and B) and 40 % for POD with CAT activity 2 (Figure 4.8 A and C), respectively, relative to the untreated control.

Maize seeds primed with *P. griseofulvum* and inoculated with *F. proliferatum* showed a decrease (16 %) in root peroxidase with catalase activity relative to the *F. proliferatum* treatment for POD with CAT activity 1 (Figure 4.8 A and B) and increased by 3 % for POD with CAT activity 2 (Figure 4.8 A and C). A similar response was observed in *P. expansum* primed roots inoculated with *F. proliferatum*. Relative to the *F. proliferatum* treatment, maize seeds primed with *P. expansum* and inoculated with *F. proliferatum* decreased root peroxidase with catalase activity by 29 % for POD with CAT activity 1 (Figure 4.8 A and B), however, it decreased by 17 % for POD with CAT activity 2 (Figure 4.8 A and C).

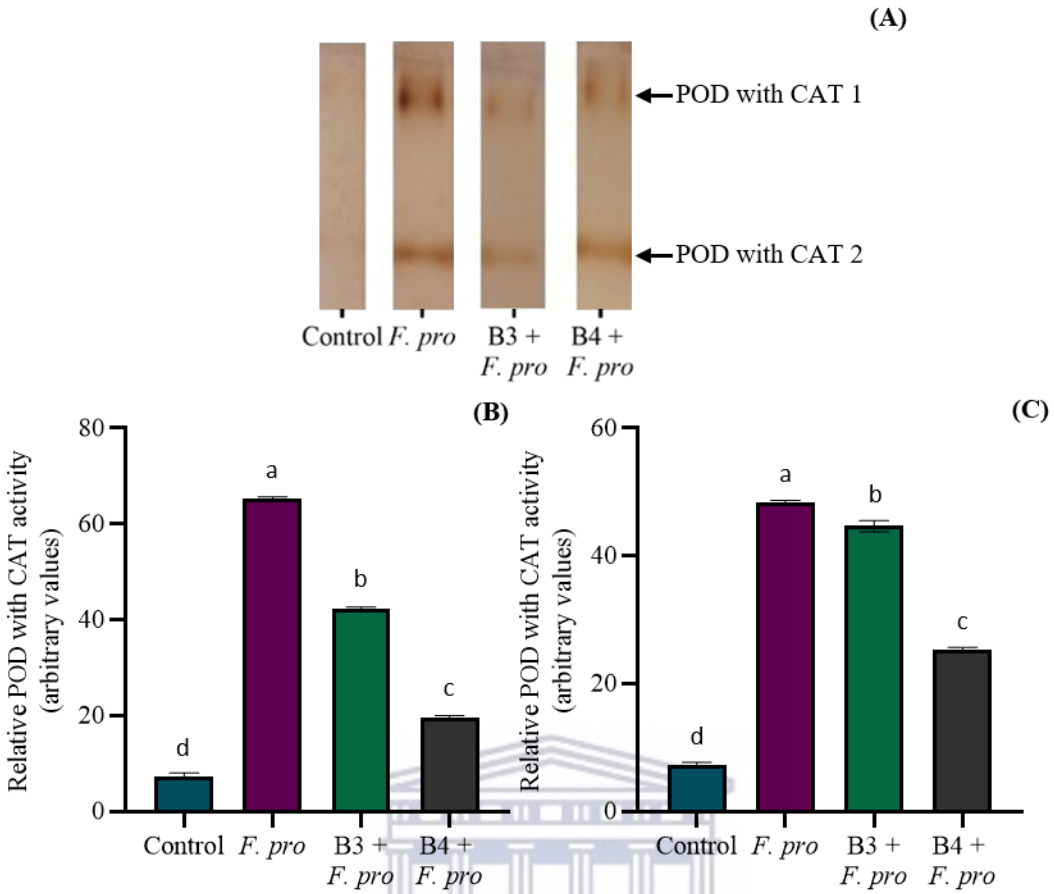


Figure 4.8 *Penicillium griseofulvum* (B3) and *Penicillium expansum* (B4) changes peroxidase with catalase activity in maize roots infected with *Fusarium proliferatum* at a concentration of 10^8 cells/ml. The in-gel activity assay of POD with CAT activity isoforms in response to the various treatments is represented in (A), from which pixel intensities of POD with CAT 1 (B) and POD with CAT 2 (C) were determined. Data represents the means (±SE) of three independent experiments (n=3). Error bars denote standard deviation, where bars with the same letters are statistically similar and varying letters indicate statistical differences where $P < 0.05$. Band intensities were evaluated using Alpha Ease with the "invert" function.

4.3 Discussion

The influence of *F. proliferatum* on the physiological responses of maize seeds have been analysed and the results indicate that *F. proliferatum* induces stress on the host maize seed and thus restricts the growth and development of the plant. However, when the maize seeds are primed with the fungal endophytes *P. griseofulvum* and *P. expansum* prior to infection, there is an increase in seed root growth and development relevant to the infected seeds.

A single inoculation of endophytes to plants or seeds that are able to simultaneously assist with resistance to biotic stresses would be of significant importance in the agricultural food production industry (Mastouri *et al*., 2010).

Despite the numerous current studies on the diseases caused by *F. proliferatum* in plants, very little is known about the impact of seed infection with *F. proliferatum* as well as the use of *Penicillium griseofulvum* and *Penicillium expansum* to control its infection in maize.

4.3.1 Maize seed infection with *Fusarium proliferatum* reduces the root lengths

A study by Chang *et al*. (2015) showed that the infection of *F. proliferatum* on soybean plants had produced the greatest root rot and had reduced the emergence of seeds out of four *Fusarium* species that had been identified in the study such as *Fusarium culmorum, F. oxysporum, Fusarium avenaceum* and *F. proliferatum*. The study showed that on a scale of 0-4 for root rot severity, where 4 is the highest severity and 0 is the lowest level of severity; *F. proliferatum* had a severity score of 4. Additionally, in a previous study by Argyris *et al*. (2003), elevated levels of *Fusarium graminearum* during the development and maturation of seeds resulted in the decline in seed germination and vigour. In cereals, *Fusarium* seedling blight causes a significant decline in the plant's formation. The disease commonly begins during seed germination due to the sowing of the seeds that are infected by *Fusarium* spp. (Argyris *et al*., 2003).

Infection by *F. culmorum* shows that it can efficiently penetrate the seedling roots. Thereafter, the fungi travel from the hypocotyl towards the upper stems' internodes and leaves, colonizes the cells and tissues of the host plant, blocks the vascular bundles, and disrupts the supply of nutrients and metabolic processes which all aids in the significant decline in seedling growth. (Beccari *et al*., 2011; Kang and Buchenauer, 2000). *F. proliferatum* may travel through the maize plant in the same way and thereby cause infection (Figure 4.1 A and B), however, the interaction between maize plants and *F. proliferatum* from germination to the development of seedlings is largely unknown and to our knowledge, there appears to be no report on physiological responses in maize elicited by *F. proliferatum* during seed germination.

Likewise, to these results, in a study conducted by Brodnik (1975), where the influence of toxic products of *F. graminearum* and *Fusarium moniliforme* was examined on the germination of seed and embryo growth of maize seeds, it was shown that the *F. graminearum* infection causes

a decline in the rate of germination in cereal crops. Contrast to these results, a study by Yang *et al*. (2011) observed the effect of *F. graminearum* on barley seed germination and vigor. The study showed that the rate of germination was not significantly different relative to the control from day 1 to day 3. However, a vigor test, which indicates the development of normal seedlings under field conditions in a more precise manner, showed that there was a significant decline in the *F. graminearum*-inoculated embryos at day 3. A similar observation was made by Galli *et al*. (2005), where there was no decline in seed germination but the seed vigor declined under laboratory conditions when maize seeds were colonized by *F. graminearum* for a period of 32 h. Additionally, Munkvold and O'Mara (2002) observed that the majority of the *Fusarium* spp. does not cause a reduction in the rate of seed germination, however, it does cause the decay of the seeds and the arrest of the development of radicals which is similar to the results depicted in Figure 4.1 A and B in this study.

The delivery of entomopathogenic fungi was evaluated in seed coating to deduce whether the fungal presence impacts maize performance (Rivas-Franco *et al*., 2019). Additionally, the biocontrol ability of the fungi was evaluated with respect to the resistance to the larvae of *Costelytra giveni* (Coleoptera: Scarabaeidae) and *F. graminearum* (Nectriaceae). The maize seeds were coated with the conidia from the *Metarhizium* spp. or *Beauveria Bassiana* and the performance of the plant was determined based on the plants dry weight and seed germination. The presence of both stresses was proven to be detrimental to the performance of maize. The plants that were infected with *Fusarium* were classified by the presence of a necrotic area which impacts the mesocotyl, close to the root crown, where the roots were dark brown to black, decaying, discoloured or completely rotted (Rivas-Franco *et al.*, 2019). A similar response can be seen in Figure 4.1 A and B in this study where *F. proliferatum* caused discolouration and decay of the roots of the infected maize seeds. The growth of maize was negatively impacted by the presence of *C. giveni* or *F. graminearum* and was expectedly worse when they were both present (Rivas-Franco *et al*., 2019).

4.3.2 Priming with *Penicillium griseofulvum* (B3) and *Penicillium expansum* (B4) endophytes and infection with *Fusarium proliferatum*

Contrary to the results seen in Figure 4.2 A and B, in a previous study by Orole and Adejumo (2009), the endophytic *Phoma* species could not control the wilting of maize seeds in the greenhouse, where the seeds primed with *P. griseofulvum* and *P. expansum* pre-infection with *F. proliferatum* showed an increase in root length relative to the roots that were infected with

F. proliferatum. Maize seeds primed with *P. griseofulvum* and *P. expansum* (Supplementary Figure 4.1 A and B) shows that the length of the roots had not drastically decreased when primed with the endophyte, even though it showed no growth promoting activities, respectively. In a previous study by Mastouri *et al*. (2010) it was shown that seeds treated with *Trichoderma harzianum* have the ability to alleviate physiological and abiotic stresses within seedlings and seeds. Similarly, a previous study by Calistru *et al*. (1997) showed that *Trichoderma* fungal species possesses the potential to control phytopathogenic fungi such as *Fusarium* species.

The coating of seeds with biological control agents has the potential to reduce the usage of fertilizers and pesticides applied for the protection of the natural environment (Mastouri *et al*., 2010). Similar to the results of this study shown in Figures 4.2 A-B and Figures 4.3 A-B; a study by Kthiri *et al*. (2021) evaluated the impact of seed coating with *Meyerozyma guilliermondii*, strain INAT (MT731365) on the germination of seeds, the growth and photosynthesis of plants, as well as the plants resistance to the fungal pathogen *Fusarium culmorum* in wheat plants. The results showed that when compared to the control, seeds coated with *M. guilliermondii* had improved wheat growth in terms of its roots and shoot length as well as biomass. At 21 days' post infection, *M. guilliermondii* caused a reduction in disease severity and incidence, where the reduction rates were up to 30.4 % and 31.2 %, respectively.

Additionally, the study by Rivas-Franco *et al*. (2019) also showed that the coating of seeds with the conidia of *Metarhizium* or *Beauveria* can be used as a delivery system for the control of pests and pathogens. The incidence of *Fusarium* symptoms increased in the plants from seeds without the fungal coating and the incidence decreased in plants from seeds that were coated with the various endophytic treatments. Additionally, the entomopathogenic fungi had caused the death of soil-dwelling larvae and the decline in root rot symptoms that is caused by the plant pathogenic fungi. The entomopathogenic fungi has caused an often-beneficial effect on the growth of the plants in the presence of the pathogenic fungi and insect pest. Furthermore, a study by He *et al*. (2019) used an artificial inoculation assay to show that maize stalk rot and ear rot caused by *Fusarium* infection and mycotoxin accumulation was controlled with the introduction of the biological control agent *Trichoderma asperellum* granules by 49.83 % and 39.63 %, respectively.

4.3.3 *Penicillium griseofulvum* (B3) and *Penicillium expansum* (B4) restricts *Fusarium*-induced ROS accumulation in maize roots

The decline in plant growth is caused by a large number of biological processes, where the damage caused by ROS accumulation is most reported in literature (Smirnoff, 2008). Pathogens have been shown to stimulate the production of ROS by NADPH oxidases (Smirnoff, 2008). The improved production of ROS during stressful conditions may be detrimental to the cells, but it can act as a signal for the activation of defense pathways or stress-responses, therefore ROS are viewed as cellular indicators of stress as well as messengers involved in the stress-response signal transduction pathway (Berwal and Ram, 2018). ROS are produced in various cellular compartments such as the chloroplast, peroxisomes, mitochondria, and cytoplasm or in the apoplast via various enzymes. ROS are produced via the reduction of molecular oxygen (O_2) that contains free radicals such as hydroxyl radicals (OH•), superoxides (O^{2-}), peroxyl (ROO•), alkoxyl (RO•) as well as nonradical products such as singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) (Berwal and Ram, 2018).

Like animals, plants produce ROS by oxidase enzymes when they are infected with pathogens via oxidative burst. This may have a direct role in defence by allowing peroxidative cross-linking of cell wall polysaccharides and proteins and by damaging the pathogen (Smirnoff, 2008). ROS plays a role in the initiation of the hypersensitive response which is a form of programmed cell death (PCD) which is induced by incompatible pathogens (Smirnoff, 2008).

Several studies have reported on the roles of ROS in plant development without reporting its source. In a study by Shorning *et al*. (2000), it was shown that superoxide was present in the morphogenesis of wheat seedlings. Additionally, the study showed the seedlings incubated in antioxidants had decreased the superoxide content and distorted the development.

Hydrogen peroxide (H_2O_2) is a reactive oxygen species that is able to initiate damage to a number of cellular structures (Nurnaeimah *et al*., 2020; Mhamdi *et al*., 2010).

H_2O_2 has been noted as the central component of the signal transduction pathway in plant-adaptation to changes in the environment as it is the only ROS with a high penetrability across membranes (Mhamdi *et al*., 2010; Ozyigit *et al*., 2016). In contrast, when the balance between the creation and scavenging of H_2O_2 is unhinged, it results in considerable damage to cellular structures or even programmed cell death (PCD) (Nurnaeimah *et al*., 2020; Ozyigit *et al*., 2016). In plants, H_2O_2 and superoxide radicals (O_2) are produced during cell metabolism and

in general cell compartments especially within the chloroplast which is well equipped with defense enzymes against H_2O_2 and O_2 (Nurnaeimah *et al*., 2020).

Programmed cell death has been previously studied during plant-pathogen interactions and environmental stresses. The creation and disproportionate accumulation of ROS is associated with cell death in plants. A previous study by Dat *et al*. (2003) has shown that PCD was triggered by a modified H_2O_2 homeostasis, thereby linking high H_2O_2 production to PCD in transgenic tobacco plants. The disconcertion of H_2O_2 homeostasis in catalase deficient transformants had stimulated signalling that led to an oxidase-dependent burst and consequently cell death.

In contrast to this study where H_2O_2 content decreased in maize seed roots primed with *P. griseofulvum* and *P. expansum* (Figure 4.3 B), a previous study by Yang *et al*. (2011) showed that the albumin content, absorbed water, and H2O2 concentration had increased throughout the imbibement regardless of whether or not the pathogen was added when barley was infected with *F. graminearum*. This increase is due to the fact that seed germination begins with the uptake of water which is followed by the elongation of the radical (Bønsager *et al*., 2007). Once imbibition begins, the rate of cellular respiration and the synthesis of metabolic enzymes and compounds which includes ROS for signaling significantly increases for the growth of the seed (Fincher, 1989). H_2O_2 accumulation in infected seeds strongly suggests that there is an interaction between the host and the pathogen and that the defence responses are active due to infection (Yang *et al*., 2011).

4.3.4 *Penicillium griseofulvum* (B3) and *Penicillium expansum* (B4) restricts the extent of lipid peroxidation in maize roots under *Fusarium proliferatum* infection

An excessive increase of H_2O_2 can induce lipid peroxidation, which harms cell membranes and is seen as a marker for the inactivation of membrane proteins and membrane perturbation which ultimately leads to cell death (Zhang *et al*., 2019). Malondialdehyde (MDA) content is commonly used as an indicator for lipid peroxidation (Jajic, *et al*., 2015; Zhang *et al*., 2019).

An increase of H_2O_2 destroys the cell bio-membrane lipid layers, which induces the production and increase of MDA and causes a loss in intracellular water (Zhang *et al*., 2019). Likewise, to this study (Figure 4.4), it was observed that the increase in H_2O_2 concentration caused an increase in MDA content in roots infected with the fungal pathogen. Additionally, the MDA content decreased with the decline in H_2O_2 concentration in roots primed with the fungal endophytes prior to infection. This therefore shows that there is a relationship between H_2O_2

concentration and lipid peroxidation (Figure 4.3 B; Figure 4.4). Due to the lack of research like the kind in this study, we can only suggest that the increase in lipid peroxidation, indicated by the MDA levels (Figure 4.4), was caused due to the increase in ROS production. Likewise, to these results, Harrach *et al*. (2013) observed that barley roots infected with *F. culmorum* showed an increase in MDA content and a decline in MDA content in roots primed with the endophytic fungus *Piriformospora indica*.

4.3.4 *Penicillium griseofulvum* (B3) and *Penicillium expansum* (B4) augments the activity of antioxidants in maize roots under *Fusarium proliferatum* infection

The change in the activity of antioxidant enzymes in response to oxidative damage caused by ROS accumulation is well documented in numerous plant species.

4.3.4.1 *Penicillium griseofulvum* (B3) and *Penicillium expansum* (B4) augments the superoxide dismutase activity in maize roots under *Fusarium proliferatum* infection

Superoxide dismutase (SOD) is an abundant metalloenzyme that forms the initial defence against ROS (Kandhari, 2004). It forms one of significant enzymatic components of the detoxification of superoxide radicals that is produced in biological systems via catalysing its dismutation to hydrogen peroxide and lastly H_2O and O_2 by the enzymes peroxide and catalase (Berwal and Ram, 2018).

Until its initial study in plants by McCord and Fridovich (1969) SOD was known as a group of metalloproteins that possessed no function. SOD isoforms differ based on their active site metal ions, such as Fe-SOD, Cu/Zn-SOD and Mn-SOD (Berwal and Ram, 2018). SOD plays a significant role in the protection of the cells against toxic effects of superoxide radicals that are made in various compartments of the cell (Berwal and Ram, 2018).

Fe-SOD's are found within the plasmids, Cu/Zn-SOD in the plastids, cytosol, peroxisomes and extracellular space, and the Mn-SOD's are located in mitochondrial matrix, peroxisomes and some have also been found in cell walls (Bowler *et al*., 1992; Kukavica *et al*., 2009; Miller, 2012).

We observed that SOD activity increased in maize seed roots primed with *P. griseofulvum* or *P. expansum* (Figure 4.5) prior to infection. This was also observed in a study by Cheng *et al*. (2020) where they showed that banana plants infected by *Fusarium oxysporum* f. sp. *cubense* and colonized by *Serendipita indica* increased superoxide dismutase activity. Additionally, Harrach *et al*. (2013) also showed an increase in SOD activity in roots primed with *P. indica*

prior to infection with *F. culmorum*. Furthermore, a study by Bagheri *et al.* (2013), showed an increase in SOD activity in rice plants primed with the endophyte *P. indica*.

4.3.4.2 *Penicillium griseofulvum* (B3) and *Penicillium expansum* (B4) augments the ascorbate peroxidase activity in maize roots under *Fusarium proliferatum* infection

Ascorbate peroxide belongs to the family of heme-containing peroxidases which catalyses the oxidation of a number of organic molecules based on the availability of H_2O_2 (Pandey *et al*., 2017; Shigeoka *et al*., 2002). APX plays a major role in the regulation of growth within plant species. The ascorbate-glutathione (ASC-GSH) or the Foyer-Halliwell-Asada Pathway, is one which is connected by the APX enzymes which is a linking molecule for the maintenance of the redox balance under stresses (Pandey *et al*., 2017). Previous studies by Zhang *et al*. (2014) have shown that APX increases in the presence of other antioxidant enzymes such as glutathione reductase and superoxide dismutase, which indicates a relationship between the antioxidant enzymes. Ascorbate peroxidase has been studied in the chloroplast and was found to be associated with NADPH (Foyer and Halliwell, 1976). Therefore, the APX ascorbate link regulates the NADPH/NAD ratio when the plant is under stress. This pathway is significant due to its presence within the mitochondria, chloroplast (thylakoid and stromal), and peroxisomes (Mittler *et al*., 2004; Pandey *et al*., 2017).

We observed that APX activity increased in maize seed roots primed with *P. griseofulvum* or *P. expansum* (Figure 4.6) prior to infection. Cheng *et al*. (2020) showed that banana plants infected by *Fusarium oxysporum* f. sp. *cubense* and colonized by *S. indica* increased that ascorbate peroxidase activity. The study by Harrach *et al*. (2013) also showed an increase in APX activity in roots primed with *P. indica* prior to infection with *F. culmorum*. Furthermore, the study by Bagheri *et al.* (2013), showed an increase in APX activity in rice plants primed with the endophyte *P. indica*.

The total APX activity showed a slight increase in infected roots and a major increase in roots that were primed with the fungal endophytes prior to infection (Figure 4.6). However, the peroxidase that uses guaiacol (GPOX) instead of ascorbate showed an increase in the intensity in infected roots and a decline in intensity in roots primed with the fungal endophytes prior to infection (Figure 4.7 A-C).

4.3.4.3 *Penicillium griseofulvum* (B3) and *Penicillium expansum* (B4) changes the guaiacol peroxidase and peroxidase with catalase activity in maize roots under *Fusarium proliferatum* infection

Peroxidases participate in a number of physiological processes in plants pertaining to responses to abiotic and biotic stresses as well as the biosynthesis of lignin (Abadi and Sepehri, 2016). Lignin is a polymer that makes the plant more rigid and thus stronger and also makes the cell walls hydrophobic (Abadi and Sepehri, 2016; Vicuna, 2005). Peroxidases are able to scavenge ROS. Although peroxidases are sources of H_2O_2, they are also able to scavenge it (Vicuna, 2005). In this study, an increase in GPOX and POD with CAT activity was observed in the infected roots (Figure 4.7 A-C; Figure 4.8 A-C) relative to the increase in H_2O_2 content in infected roots (Figure 4.3 B) and thus we hypothesise that the levels of GPOX and POD with CAT activity increased to scavenge the H_2O_2 in infected roots. However, the GPOX and POD with CAT activity increased (Figure 4.7 A-C; Figure 4.8 A-C) even though the H_2O_2 content was low in primed roots prior to infection (Figure 4.3 B). We therefore hypothesized that this increase in GPOX activity was caused by the production of lignin.

Contrast to what was observed in this study where GPOX and POD with CAT activity declined when maize seed roots were primed with *P. griseofulvum* and *P. expansum* (Figure 4.7 A-C; Figure 4.8 A-C), respectively, prior to infection with *F. proliferatum*, a study by Abadi and Sepehri (2016) showed that the peroxidase activity increased when wheat plants were primed with the fungal endophyte *Piriformospora indica*.

Chapter 5

Seed biopriming with *Penicillium griseofulvum* alters Maize root protein abundance under *Fusarium proliferatum* infection

5.1 Introduction

Maize is one of the most important crops worldwide (Bai *et al*., 2021; Wu *et al*., 2011; Yue *et al*., 2018). In Africa, in excess of 300 million people depend on maize as their primary food source (International Plant Biotechnology Outreach, 2017). Almost 1 billion tons of maize is grown in 170 countries on approximately 180 million hectares of land (International Plant Biotechnology Outreach, 2017). During the growth of maize, it is susceptible to a variety of pathogens that negatively impacts its seed germination, growth, and development (Bai *et al*., 2021; Wu *et al*., 2011). *F. proliferatum* is an example of such a pathogenic fungus that impacts the growth of maize (Ncube, 2012; Wang *et al*., 2021).

F. proliferatum (teleomorph *Gibberella* intermedia) is a filamentous ascomycete saprophytic pathogenic fungus that is distributed worldwide and has been related to an assortment of diseases in vital economical florae (Gao *et al*., 2017; Li *et al*., 2012; Li *et al*., 2017; Sun *et al*., 2018; Sun *et al*., 2019). *Fusarium* spp. can cause ear rot, stalk rot, seedling blight, and root rot (Okello *et al*., 2019; Wang *et al*., 2021). In addition to causing the physiological deformities, *F. proliferatum* is a producer of mycotoxins that, if not controlled, can be detrimental to human and animal health (Fandohan *et al*., 2003; Li *et al*., 2017).

Seeds ability to germinate is an important factor that influences seedling performance, and consequent crop growth and development. Seed germination is regulated by both internal and external factors, including seed structure and chemistry, humidity, temperature, and biotic factors, including pathogenic fungi, viruses, and bacteria (Lei *et al*., 2013; Shiferaw *et al*., 2011).

A single treatment of endophytes on plants or seeds that are able to simultaneously assist with resistance to biotic stresses would be of significant importance in the agricultural food production industry (Mastouri *et al*., 2010). Seed germination and emergence can be improved by priming seeds with fungal endophytes (Kthiri *et al*., 2021; Mastouri *et al*., 2010; Rivas-

Franco *et al*., 2019). Fungal endophytes are biologically and taxonomically diverse, however, they all share the characteristic of colonizing plant tissue without causing visible harm to the host plant (Mejia *et al*., 2008; Ting *et al*., 2011). Endophytes induce the production/synthesis of chemicals that assists host plants to tolerate stresses and hinders the development and growth of competitors including pathogenic organisms (Bamisile *et al*., 2018; Singh *et al*., 2011).

To comprehensively determine how pathogens, manipulate the infection process in maize, the investigation of protein changes under infection will be useful to explore the pathogenic mechanism of fungal pathogens. Therefore, the proteomic analysis and protein mechanisms should be understood in order to develop new measures for seed germination, crop production and yield.

Mass spectrometry-based proteomics provides scientists with a remarkable ability to study plants in a more precise way (Liu *et al*., 2019 b). Presently, proteomics is being transformed from a sequestered field into a comprehensive tool for biological research which may be used to explain biological functions (Liu *et al*., 2019 b). Various studies have successfully utilized proteomics as a discovery tool to understand the plants resistance mechanisms (Liu *et al*., 2019 b). Presently, most of the knowledge about the plant's responses to biotic stress has been acquired via genomic, genetic, or transcriptomic approaches (Meena *et al*., 2017).

Therefore, this study aimed to investigate the protein responses in maize seed roots infected with *F. proliferatum* and how biopriming with fungal endophyte *P. griseofulvum* (B3) can modulate these changes.

5.2 Results

Given the positive response of priming maize seed roots with *P. griseofulvum* prior to infection with *F. proliferatum* pertaining to its growth and biochemical responses, the downstream proteomics analysis will focus solely on the fungal endophyte *P. griseofulvum*.

5.2.1 Separation, visualization, and quantification of maize root proteins

A fraction (15 µg) of each treatment was separated on a 1D SDS polyacrylamide gel prior to identification using liquid chromatography mass spectrometry (LC-MS). The results showed that the separated proteins were of high quality with no visible streaking or protein degradation. Distinct difference in protein band intensities was observed between the treatments (see black

arrows). The separated proteins covered a molecular weight range between 10 to 250 kDa (Figure 5.1).

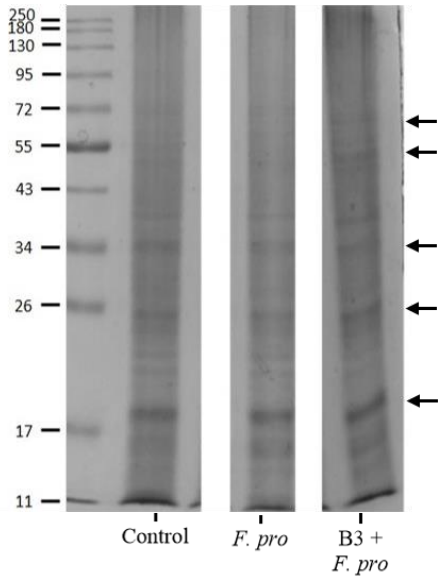


Figure 5.1 One – dimensional sodium dodecyl sulphate (SDS) gel electrophoresis of total soluble maize root proteins under different treatment conditions. Protein extracts (15 µg) from different treatments were size fractionated on a 12 % denaturing 1D SDS polyacrylamide gel. The black arrows indicate differences that were visually observed between the different treatments.

Given the quality and integrity of visualised proteins from each treatment, protein pellets from each sample were digested with sequence grade trypsin (Section 2.10.3.1). The protein content/concentration for each sample was quantified as described in Section 2.10.3 and the results documented in Table 5.1.

Table 5.1 Maize root protein concentrations for the three different treatments

Maize tissue	Treatment	Protein concentrations (mg/ml)
Root	Control	7.84
Root	*F. proliferatum*	8.08
Root	*P. griseofulvum* + *F. proliferatum*	18.53

5.2.2 Identification of maize roots proteins using LC-MS/MS

The data generated for system suitability samples shows that all individual samples were analysed using LC/MS and depicted as individual TICs (Figure 5.2 A-C). The peaks detected for the control and infected samples on a total ion chromatogram (TIC) show uniformity and no contamination (Figure 5.2 A-C).

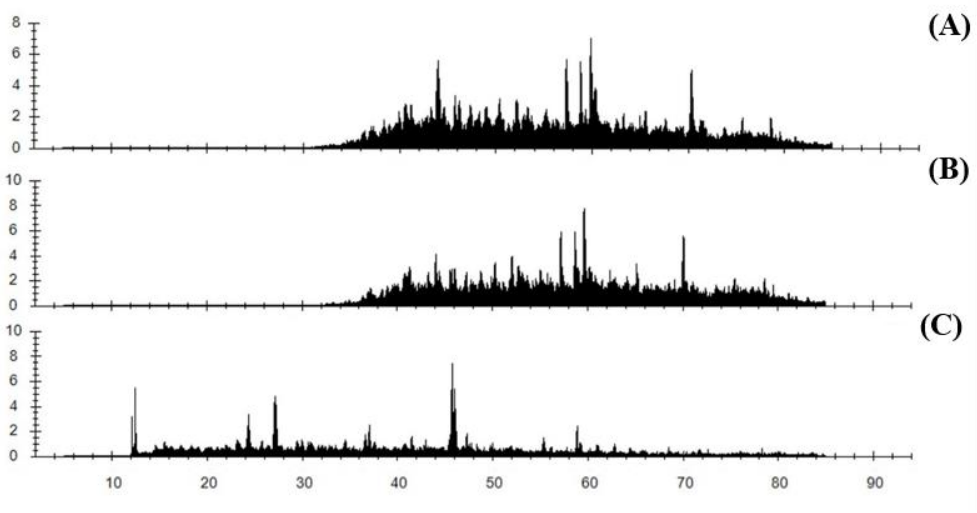


Figure 5.2 The total ion chromatography LC/MS analysis of maize seeds infected with *Fusarium proliferatum,* and seeds primed with *Penicillium griseofulvum* prior to infection with *Fusarium proliferatum,* respectively. A) Control, B) Seeds infected with *F. proliferatum* at a concentration of 10^8 cells/ml, and C) Seeds primed with *P. griseofulvum* and infected with *F. proliferatum* at a concentration of 10^8 cells/ml.

The total ion chromatography for all treatments were analysed using the Uniprot Plant database where 290 proteins were identified with an FDR of 0.9 %. However, to ensure quality and correct identification of proteins, a threshold criterion was set for the identified proteins. To be considered as a positive identification, the protein exclusive unique peptide count was above 2, protein identification probability was above 95 % and the percentage sequence coverage was greater than 10 %. Using these parameters, a total of 380 proteins were identified across all treatments, where 126 proteins were in the control, 127 proteins were in infected roots, and 127 proteins were in roots primed with B3 prior to infection (Figure 5.3).

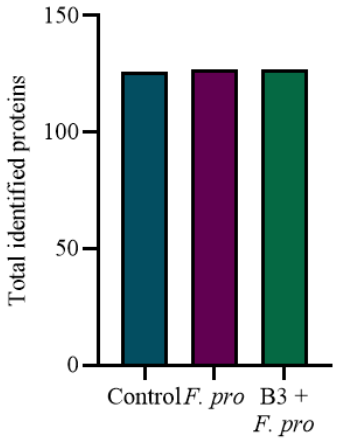


Figure 5.3 Total number of proteins identified in maize seeds infected with *Fusarium proliferatum* and seeds primed with *Penicillium griseofulvum* prior to infection with *Fusarium proliferatum* at a concentration of 10^8 cells/ml, respectively.

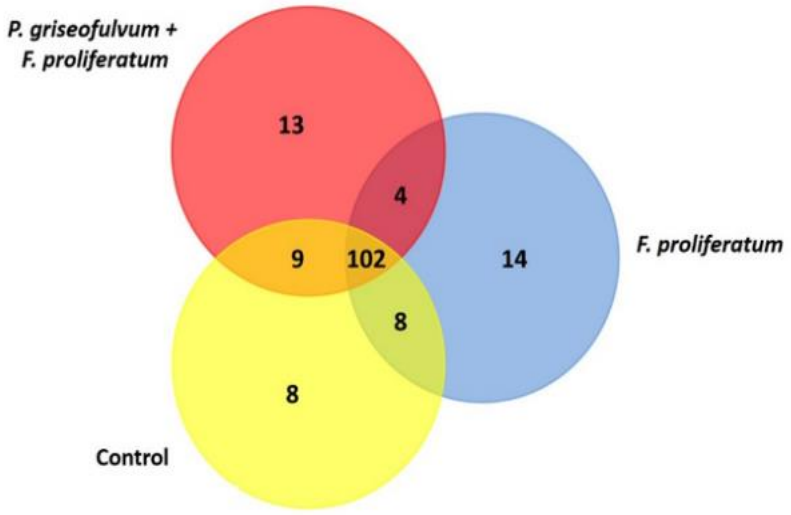
5.2.3 *Penicillium griseofulvum* priming alters root protein regulation under *Fusarium proliferatum* infection

The control had 8 unique proteins (Figure 5.4 A; Table 5.2), and the roots infected with *F. proliferatum* at concentration of 10^8 cells/ml had 14 unique proteins (Figure 5.4 A; Table 5.2). Roots primed with B3 and infected with *F. proliferatum* at a concentration of 10^8 cells/ml, respectively, had 13 unique proteins (Figure 5.4 A; Table 5.2). Additionally, the control and roots infected with *F. proliferatum* at a concentration of 10^8 cells/ml shared a total of 8 proteins, the control and roots primed with B3 and infected with *F. proliferatum* at a concentration of 10^8 cells/ml, respectively shared a total of 9 proteins and the roots infected with *F. proliferatum* at concentrations 10^8 cells/ml and roots primed with B3 and infected with *F. proliferatum* at a concentration of 10^8 cells/ml, respectively, shared a total of 4 proteins (Figure 5.4 A; Table 5.2).

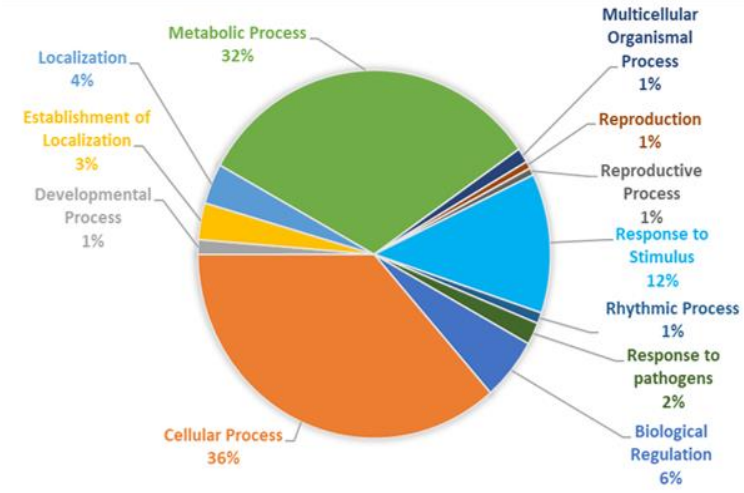
Proteins were functionally characterized using the data available in the UniProt database (www.uniprot.org), Scaffold 5.0.1 (www.proteomesoftware.com) as well as literature sources. The biological characterisation of the identified proteins (Figure 5.4 B) revealed that a large proportion of these proteins were involved in cellular processes (36 %). The second largest group of proteins were involved in metabolic processes (32 %). Other functional categories included proteins involved in response to stimulus (12 %), biological regulation (6 %), localization (4 %), establishment of localization (3 %), response to pathogens (2 %), developmental process (1 %), multicellular organismal process (1 %), reproduction (1 %), reproductive process (1 %), and rhythmic process (1 %). The subcellular localization of the identified proteins (Figure 5.4 C) revealed that the identified proteins were mostly located in cytoplasm (31 %), intracellular organelle (20 %), organelle part (13 %), membrane (9 %), ribosome (5 %), mitochondrion (5 %), nucleus (4 %), plasma membrane (4 %), organelle membrane (3 %), golgi apparatus (1 %), cytoskeleton (1 %), and endoplasmic reticulum (1 %). The molecular characterisation of the identified proteins (Figure 5.4 D) revealed that a large proportion of these proteins were involved in molecular function (36 %). The second largest group of proteins were involved in binding (32 %). Other functional categories included proteins involved in response to catalytic activity (25 %), structural molecule activity (4 %),

antioxidant activity (3 %), transport activity (3 %), enzyme regulator activity (1 %), electron carrier activity (1 %), and defence (1 %).

(A)



(B)



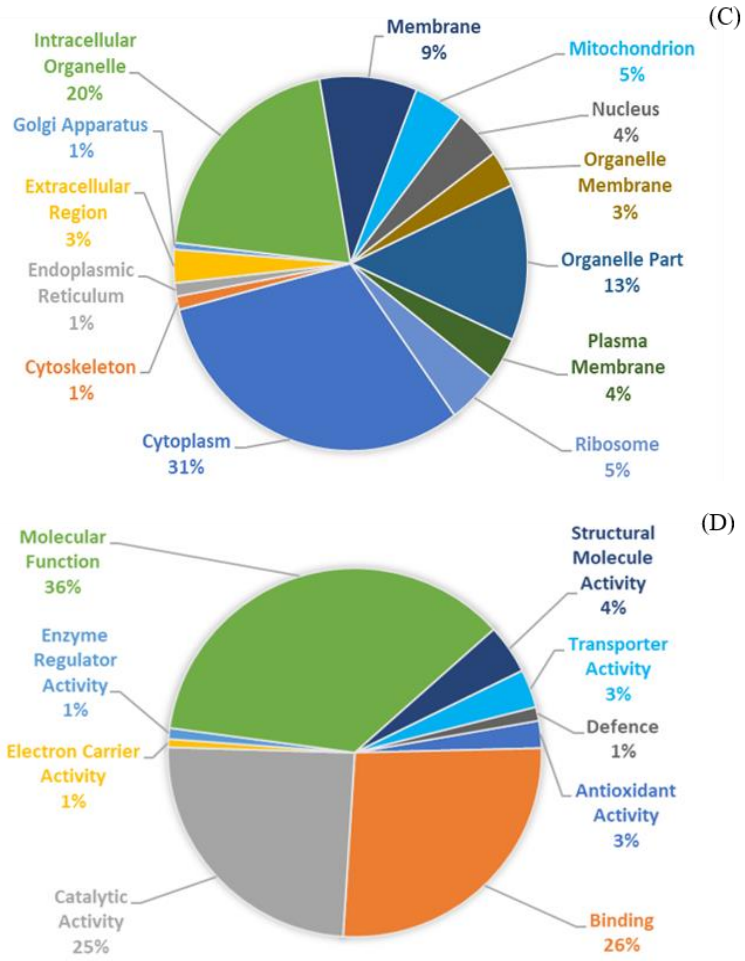


Figure 5.4 *Penicillium griseofulvum* (B3) alters the presence of proteins in maize roots infected with *Fusarium proliferatum* at a concentration of 10^8 cells/ml. (A) The identified proteins in maize seeds primed with distilled water (control), infected with *F. proliferatum* at concentrations 10^8 cells/ml, and primed with *P. griseofulvum* prior to infection with *F. proliferatum* at a concentration of 10^8 cells/ml, respectively. (B) Biological characterisation of proteins in maize seeds primed with distilled water (control), infected with *F. proliferatum* at concentrations 10^8 cells/ml, and primed with *P. griseofulvum* prior to infection with *F. proliferatum* at a concentration of 10^8 cells/ml, respectively. (C) Subcellular localisation of identified proteins in maize seeds primed with distilled water (control), infected with *F. proliferatum* at concentrations 10^8 cells/ml, and primed with *P. griseofulvum* prior to infection with *F. proliferatum* at a concentration of 10^8 cells/ml, respectively. (D) Molecular functions of proteins in maize seeds primed with distilled water (control), infected with *F. proliferatum* at concentrations 10^8 cells/ml, and primed with *P. griseofulvum* prior to infection with *F. proliferatum* at a concentration of 10^8 cells/ml, respectively.

Table 5.2. Identified proteins in maize seed roots primed with water (control), infected with *Fusarium proliferatum,* and primed with *Penicillium griseofulvum* prior to infection with *Fusarium proliferatum,* respectively. Proteins marked with an "A" were unique to the control, Proteins marked with a "B" were unique to the infected treatment, and proteins marked with an "C" were unique to the biocontrol treatment.

Protein accession numbers	Protein name	Scaffold percentages (%)			Protein molecular weight (Da)	PI	Function
		Control	*F. proliferatum*	*P. griesofulvum + F. proliferatum*			
14331_MAIZE	14-3-3-like protein GF14-6	100%	100%	100%	29,663.7	4,81	Associated with a DNA binding complex to bind to the G box, a well-characterized cis-acting DNA regulatory element found in plant genes.
ACT1_MAIZE	Actin-1	100%	100%	100%	41,617.9	5,39	Highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells. Essential component of cell cytoskeleton; plays an important role in cytoplasmic streaming, cell shape determination, cell division, organelle movement and extension growth.
ADF3_MAIZE	Actin-depolymerizing factor 3	100%	100%	100%	15,900.5	5,67	Severs actin filaments (F-actin) and binds to actin monomers.
ADH1_MAIZE	Alcohol dehydrogenase 1	100%	100%	100%	40,980.5	6,73	Plays a role in the formaldehyde catabolic process
ADH2_MAIZE	Alcohol dehydrogenase 2	100%	100%	100%	41,054.0	6,06	Plays a role in the formaldehyde catabolic process

ADHX_MAIZE	Alcohol dehydrogenase class-3	100%	100%	100%	40,770.5	6,81	Plays a role in the formaldehyde catabolic process
AL2B4_ARATH (C)	Aldehyde dehydrogenase family 2 member B4, mitochondrial	100%	100%	100%	58,590.4	7,46	Possesses activity on acetaldehyde and glycolaldehyde *in vitro*.
ALDO1_MAIZE	Indole-3-acetaldehyde oxidase	100%	100%	100%	146,684.8	6,05	In higher plants aldehyde oxidases (AO) appear to be homo- and heterodimeric assemblies of AO subunits with probably different physiological functions.
ALDO2_MAIZE (C)	Indole-3-acetaldehyde oxidase	100%	100%	100%	145,177.2	6,55	Involved in the biosynthesis of auxin.
ALF_MAIZE	Fructose-bisphosphate aldolase, cytoplasmic isozyme	100%	100%	100%	38,605.0	7,61	Plays a role in the fructose 1,6-bisphosphate metabolic process, glycolytic process, sucrose biosynthetic process.
AMYB_MAIZE	Beta-amylase	100%	100%	100%	55,180.6	5,03	Plays a role in the polysaccharide catabolic process.
AP2S1_MAIZE (B)	AP-2 complex subunit sigma	93%	100%	79%	16,015.0	7,09	Component of the adaptor complexes which link clathrin to receptors in coated vesicles.
ARF_MAIZE	ADP-ribosylation factor	100%	100%	100%	20,661.5	6,8	GTP-binding protein involved in protein trafficking; may modulate vesicle budding and uncoating within the Golgi apparatus.
ARGJ_MAIZE (A)	Arginine biosynthesis bifunctional protein ArgJ, chloroplastic	100%	100%	100%	47,952.1	6,89	Catalyzes two activities which are involved in the cyclic version of arginine biosynthesis.

ATPAM_MAIZE	ATP synthase subunit alpha, mitochondrial	100%	100%	100%	55,181.6	6,1	Mitochondrial membrane ATP synthase (F(1)F(0) ATP synthase or Complex V) produces ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain.
ATPBM_MAIZE	ATP synthase subunit beta, mitochondrial	100%	100%	100%	59,104.1	6,42	Mitochondrial membrane ATP synthase (F(1)F(0) ATP synthase or Complex V) produces ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain.
BIP2_MAIZE	Luminal-binding protein 2	100%	100%	100%	73,086.8	5,21	Plays a role in facilitating the assembly of multimeric protein complexes inside the ER.
BIP3_MAIZE	Luminal-binding protein 3	100%	100%	100%	73,158.8	5,24	Plays a role in facilitating the assembly of multimeric protein complexes inside the ER.
BX6_MAIZE	DIBOA-glucoside dioxygenase BX6	100%	100%	100%	41,369.4	6,62	2-oxoglutarate-dependent dioxygenase required for hydroxylation in position C-7 of the benzoxazinoids.
BX7_MAIZE	TRIBOA-glucoside O-methyltransferase BX7	100%	100%	100%	42,131.4	5,73	O-methyltransferase involved in the benzoxazinoid glucoside biosynthesis.
BX8_MAIZE	DIMBOA UDP-glucosyltransferase BX8	100%	100%	100%	49,469.7	5,71	Glucosyltransferase involved in the last step of benzoxazinoid glucoside biosynthesis.
BX9_MAIZE	DIMBOA UDP-glucosyltransferase BX9	100%	100%	100%	50,017.1	5,31	Glucosyltransferase involved in the last step of benzoxazinoid glucoside biosynthesis.

C71C1_MAIZE (B)	3-hydroxyindolin-2-one monooxygenase	100%	100%	100%	59,717.0	8,16	Catalyzes the conversion of 3-hydroxyindolin-2-one to 2-hydroxy-1,4-benzoxazin-3-one (HBOA).
C71C3_MAIZE	Cytochrome P450 71C3	100%	100%	100%	60,717.4	7,31	Catalyzes the conversion of 2-hydroxy-1,4-benzoxazin-3-one (HBOA) to 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA).
CADH_MAIZE	Probable cinnamyl alcohol dehydrogenase	100%	100%	100%	38,737.2	6,39	Involved in lignin biosynthesis.
CALR_MAIZE (A)	Calreticulin	100%	100%	100%	47,941.8	4,69	Molecular calcium-binding chaperone promoting folding, oligomeric assembly and quality control in the ER via the calreticulin/calnexin cycle.
CAPP2_MAIZE	Phosphoenolpyruvate carboxylase 2	100%	100%	100%	110,003.4	6,04	Through the carboxylation of phosphoenolpyruvate (PEP) it forms oxaloacetate, a four-carbon dicarboxylic acid source for the tricarboxylic acid cycle.
CATA1_MAIZE	Catalase isozyme 1	100%	100%	100%	56,877.8	7,68	Serves to protect cells from the toxic effects of hydrogen peroxide.
CATA3_MAIZE	Catalase isozyme 3	100%	100%	100%	56,796.6	6,96	Serves to protect cells from the toxic effects of hydrogen peroxide. Its levels are highest in the light period and are lowest in the dark period, hence it may be important for scavenging hydrogen peroxide at night, rather than during the day.
CFI_MAIZE (A)	Chalcone--flavonone isomerase	100%	100%	100%	24,249.7	6,06	Catalyzes the intramolecular cyclization of bicyclic chalcones into tricyclic (S)-flavanones.
CH61_MAIZE	Chaperonin CPN60-1, mitochondrial	100%	100%	100%	61,212.1	5,85	Implicated in mitochondrial protein import and macromolecular assembly.
CH62_MAIZE	Chaperonin CPN60-2, mitochondrial	100%	100%	100%	60,935.6	5,85	Implicated in mitochondrial protein import and macromolecular assembly.

CHIA_MAIZE	Endochitinase A	100%	100%	100%	29,124.4	7,94	Defense against chitin-containing fungal pathogens.
CHIB_MAIZE	Endochitinase B (Fragment)	100%	100%	100%	28,164.9	8,56	Defense against chitin-containing fungal pathogens.
COMT1_MAIZE	Caffeic acid 3-O-methyltransferase	100%	100%	100%	39,567.7	5,78	Catalyzes the conversion of caffeic acid to ferulic acid and of 5-hydroxyferulic acid to sinapic acid.
COX2_MAIZE	Cytochrome c oxidase subunit 2	100%	100%	100%	29,680.2	4,87	Component of the cytochrome c oxidase, the last enzyme in the mitochondrial electron transport chain which drives oxidative phosphorylation.
CYC_MAIZE	Cytochrome c	100%	100%	100%	12,014.7	9,19	Electron carrier protein.
CYNS_MAIZE	Cyanate hydratase	100%	100%	100%	18,526.5	5,87	Catalyzes the reaction of cyanate with bicarbonate to produce ammonia and carbon dioxide.
CYPH_MAIZE	Peptidyl-prolyl cis-trans isomerase	100%	100%	100%	18,348.4	8,69	PPIases accelerate the folding of proteins. It catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides.
CYSK_MAIZE	Cysteine synthase	100%	100%	100%	34,207.5	6,15	
CYSP1_MAIZE	Cysteine proteinase 1	100%	100%	100%	40,347.5	6,35	Involved in the degradation of the storage protein zein. May play a role in proteolysis during emergencies.
CYSP2_MAIZE (A)	Cysteine proteinase 2	100%	100%	100%	39,199.0	7,28	Involved in the degradation of the storage protein zein. May play a role in proteolysis during emergencies.
DHE3_MAIZE	Glutamate dehydrogenase	100%	100%	100%	44,023.1	6,54	Plays a role in the cellular response to nitrogen starvation and in the glutamate catabolic process.
DHN1_MAIZE (B)	Dehydrin DHN1	100%	100%	100%	17,160.2	9,11	Plays a role in defence against pathogens, cold acclimation, response to abscisic acid, and response to water deprivation.

DRE2_MAIZE	Anamorsin homolog	100%	100%	100%	27,487.4	5,29	Component of the cytosolic iron-sulfur (Fe-S) protein assembly (CIA) machinery.
E134_MAIZE	Endo-1,3;1,4-beta-D-glucanase	100%	100%	100%	33,159.8	7,23	Plays a role in control of plant growth. Mediates specific degradation of cell wall (1,3)(1,4)-beta-D-glucans and is related to auxin-mediated growth and development of cereal coleoptiles.
EF1A_MAIZE	Elongation factor 1-alpha	100%	100%	100%	49,233.7	9,11	This protein promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis.
EFTS_MAIZE (B)	Elongation factor Ts, mitochondrial	100%	100%	100%	41,214.3	7,53	Associates with the EF-Tu.GDP complex and induces the exchange of GDP to GTP. It remains bound to the aminoacyl-tRNA.EF-Tu.GTP complex up to the GTP hydrolysis stage on the ribosome.
EIF3A_MAIZE	Eukaryotic translation initiation factor 3 subunit A	100%	100%	100%	111,568.4	9,26	RNA-binding component of the eukaryotic translation initiation factor 3 (eIF-3) complex, which is involved in protein synthesis of a specialized repertoire of mRNAs and, together with other initiation factors, stimulates binding of mRNA and methionyl-tRNAi to the 40S ribosome.
ENO1_MAIZE	Enolase 1	100%	100%	100%	48,064.3	5,33	Plays a role in the glycolytic process.
ENO2_MAIZE	Enolase 2	100%	100%	100%	48,163.1	5,97	Plays a role in the glycolytic process.

FPPS_MAIZE	Farnesyl pyrophosphate synthase	100%	100%	100%	40,015.1	5,27	Catalyzes the sequential condensation of isopentenyl pyrophosphate with the allylic pyrophosphates, dimethylallyl pyrophosphate, and then with the resultant geranylpyrophosphate to the ultimate product farnesyl pyrophosphate.
FRI1_MAIZE	Ferritin-1, chloroplastic	100%	100%	100%	28,024.7	5,81	Stores iron in a soluble, non-toxic, readily available form. Important for iron homeostasis. Has ferroxidase activity. Iron is taken up in the ferrous form and deposited as ferric hydroxides after oxidation.
FRI2_MAIZE (B)	Ferritin-2, chloroplastic	98%	100%	88%	27,749.3	6,11	Stores iron in a soluble, non-toxic, readily available form. Important for iron homeostasis. Has ferroxidase activity. Iron is taken up in the ferrous form and deposited as ferric hydroxides after oxidation.
G3PC1_MAIZE	Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic	100%	100%	100%	36,523.0	6,96	Key enzyme in glycolysis that catalyzes the first step of the pathway by converting D-glyceraldehyde 3-phosphate (G3P) into 3-phospho-D-glyceroyl phosphate.
G3PC2_MAIZE	Glyceraldehyde-3-phosphate dehydrogenase 2, cytosolic	100%	100%	100%	36,542.1	6,89	Key enzyme in glycolysis that catalyzes the first step of the pathway by converting D-glyceraldehyde 3-phosphate (G3P) into 3-phospho-D-glyceroyl phosphate.
G3PC3_MAIZE	Glyceraldehyde-3-phosphate dehydrogenase 3, cytosolic	100%	100%	100%	36,448.4	7,47	Key enzyme in glycolysis that catalyzes the first step of the pathway by converting D-glyceraldehyde 3-

							phosphate (G3P) into 3-phospho-D-glyceroyl phosphate.
G6PI_MAIZE	Glucose-6-phosphate isomerase, cytosolic	100%	100%	100%	62,237.8	7,4	Plays a role in the glucose 6-phosphate metabolic process and the glycolytic process.
GLNA1_MAIZE	Glutamine synthetase root isozyme 1	100%	100%	100%	39,251.1	5,9	Plays a role in the flow of nitrogen into nitrogenous organic compounds.
GLNA2_MAIZE (B)	Glutamine synthetase root isozyme 2	98%	100%	100%	40,094.9	5,9	Plays a role in the flow of nitrogen into nitrogenous organic compounds, and defence against pathogens.
GLNA3_MAIZE	Glutamine synthetase root isozyme 3	100%	100%	100%	39,169.8	5,36	Plays a role in the flow of nitrogen into nitrogenous organic compounds.
GLNA4_MAIZE (C)	Glutamine synthetase root isozyme 4	100%	100%	100%	38,981.5	5,35	Plays a role in the flow of nitrogen into nitrogenous organic compounds, and defence against pathogens.
GRPA_MAIZE	Glycine-rich RNA-binding, abscisic acid-inducible protein	100%	100%	100%	15,438.7	5,6	Possibly has a role in RNA transcription or processing during stress.
GSTF1_MAIZE	Glutathione S-transferase 1	100%	100%	100%	23,821.9	5,62	Conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles. Involved in the detoxification of certain herbicides.
GSTF3_MAIZE	Glutathione S-transferase 3	100%	100%	100%	23,849.7	6,52	Conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles. Involved in the detoxification of certain herbicides
GSTF4_MAIZE	Glutathione S-transferase 4	100%	100%	100%	24,570.1	6,15	Conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles. Involved in the detoxification of certain herbicides.

H2A_MAIZE	Histone H2A	100%	100%	100%	16,427.8	10,58	Core component of nucleosome.
H2B1_MAIZE (C)	Histone H2B.2	96%	97%	100%	16,421.1	9,99	Core component of nucleosome.
H2B2_MAIZE (C)	Histone H2B.2	97%	99%	100%	16,174.7	10,08	Core component of nucleosome.
H32_MAIZE	Histone H3.2	100%	100%	100%	15,268.6	11,3	Core component of nucleosome.
HGGL1_MAIZE	4-hydroxy-7-methoxy-3-oxo-3,4-dihydro-2H-1,4-benzoxazin-2-yl glucoside beta-D-glucosidase 1, chloroplastic	100%	100%	100%	64,240.0	6,7	Is implicated in many functions such as ABA metabolism, hydrolysis of conjugated gibberellins, conversion of storage forms of cytokinins to active forms. Also acts in defense of young plant parts against pests via the production of hydroxamic acids from hydroxamic acid glucosides.
HMGYA_MAIZE	HMG-Y-related protein A	100%	100%	100%	19,825.8	10,51	Binds A/T-rich DNA (e.g. present in the storage gamma-zein gene promoter) with a highly dynamic distribution into the nucleus.
HSP70_MAIZE	Heat shock protein	100%	100%	100%	70,574.8	5,33	
HSP82_MAIZE (B)	Heat shock protein 82	100%	100%	100%	81,894.1	5,11	Molecular chaperone that promotes the maturation, structural maintenance and proper regulation of specific target proteins involved for instance in cell cycle control and signal transduction.
IBBWP_MAIZE (C)	Bowman-Birk type wound-induced proteinase inhibitor WIP1	100%	100%	100%	10,975.8	7,96	Plays a role in defence and possess serine-type endopeptidase inhibitor activity
IF4A_MAIZE	Eukaryotic initiation factor 4A	100%	100%	100%	46,537.0	5,59	ATP-dependent RNA helicase which is a subunit of the eIF4F complex involved in cap recognition and is required for mRNA binding to ribosome.

IF5_MAIZE (B)	Eukaryotic translation initiation factor 5	100%	100%	100%	48,913.5	5,69	Catalyzes the hydrolysis of GTP bound to the 40S ribosomal initiation complex (40S.mRNA.Met-tRNA[F].eIF-2.GTP) with the subsequent joining of a 60S ribosomal subunit resulting in the release of eIF-2 and the guanine nucleotide.
IF5A_MAIZE	Eukaryotic translation initiation factor 5A	100%	100%	100%	17,496.5	5,99	The precise role of eIF-5A in protein biosynthesis is not known but it functions by promoting the formation of the first peptide bond.
IFRH_MAIZE (C)	Isoflavone reductase homolog IRL	100%	100%	100%	32,852.2	5,99	Plays a role in defence and cellular response to sulfur starvation
IN21_MAIZE (B)	Protein IN2-1	100%	100%	100%	26,989.8	4,98	Plays a role in the glutathione metabolic process and protein glutathionylation.
IN22_MAIZE	IN2-2 protein	100%	100%	100%	33,829.0	8,69	Possesses D-threo-aldose 1-dehydrogenase activity, aldo-keto reductase (NADP) activity
IPYR_MAIZE	Soluble inorganic pyrophosphatase	100%	100%	100%	24,370.2	5,69	Plays a role in the phosphate-containing compound metabolic process.
ITPK1_MAIZE (A)	Inositol-tetrakisphosphate 1-kinase 1	100%	100%	100%	37,312.3	6,47	Kinase that can phosphorylate various inositol polyphosphate such as Ins(3,4,5,6)P4 or Ins(1,3,4)P3 and participates in phytic acid biosynthesis in developing seeds.
LDH_MAIZE	L-lactate dehydrogenase	100%	100%	100%	38,551.2	8,87	
LPAT_MAIZE (B)	1-acyl-sn-glycerol-3-phosphate acyltransferase PLS1	100%	100%	100%	42,572.8	9,91	Converts lysophosphatidic acid (LPA) into phosphatidic acid by incorporating acyl moiety at the 2 position.
MASY_MAIZE	Malate synthase, glyoxysomal	100%	100%	100%	61,637.1	6,64	Plays a role in the glyoxylate cycle and tricarboxylic acid cycle.

MDHC_MAIZE	Malate dehydrogenase, cytoplasmic	100%	100%	100%	35,589.4	6,09	Malate dehydrogenase; catalyzes a reversible NAD-dependent dehydrogenase reaction involved in central metabolism and redox homeostasis.
MEG5_MAIZE (C)	Protein MATERNALLY EXPRESSED GENE 5	100%	100%	100%	17,813.8	6,51	Plays a role in mRNA binding.
MI25_MAIZE	ATP synthase protein MI25	100%	100%	100%	24,372.2	8,38	This is one of the chains of the nonenzymatic component (CF(0) subunit) of the mitochondrial ATPase complex.
MMT1_MAIZE	Methionine S-methyltransferase	100%	100%	100%	120,311.8	5,94	Catalyzes the S-methylmethionine (SMM) biosynthesis from adenosyl-L-homocysteine (AdoMet) and methionine.
MNB1B_MAIZE (B)	DNA-binding protein MNB1B	100%	100%	100%	17,146.6	5,95	Recognizes an AAGG motif at the MNF1-binding site.
MTBC_MAIZE	Probable bifunctional methylthioribulose-1-phosphate dehydratase/enolase-phosphatase E1	100%	100%	100%	56,970.8	6,19	Plays a role L-methionine salvage from S-adenosylmethionine and L-methionine salvage from methylthioadenosine
MTHR1_MAIZE	Methylenetetrahydrofolate reductase 1	100%	100%	100%	66,430.6	5,91	The probable reversibility of the MTHFR reaction in plants suggests that they can metabolize the methyl group of 5,10-methylenetetrahydrofolate to serine, sugars, and starch.
MTNA_MAIZE	Methylthioribose-1-phosphate isomerase	100%	100%	100%	38,623.1	6,05	Catalyzes the interconversion of methylthioribose-1-phosphate (MTR-1-P) into methylthioribulose-1-phosphate (MTRu-1-P).
NLTP_MAIZE (C)	Non-specific lipid-transfer protein	100%	95%	100%	11,705.0	8,72	Plant non-specific lipid-transfer proteins transfer phospholipids as

							well as galactolipids across membranes.
OBP1A_MAIZE (B)	Oil body-associated protein 1A	100%	100%	99%	26,223.6	6,3	Lipid particle.
PALY_MAIZE	Phenylalanine/tyrosine ammonia-lyase	100%	100%	99%	74,927.4	6,99	Catalyzes the non-oxidative deamination of L-phenylalanine and L-tyrosine to form trans-cinnamic acid and p-coumaric acid respectively with similar efficiencies.
PCNA_MAIZE	Proliferating cell nuclear antigen	100%	100%	99%	29,343.6	4,75	This protein is an auxiliary protein of DNA polymerase delta and is involved in the control of eukaryotic DNA replication by increasing the polymerase's processibility during elongation of the leading strand.
PDI_MAIZE	Protein disulfide-isomerase	100%	100%	99%	57,098.3	5,41	Participates in the folding of proteins containing disulfide bonds, may be involved in glycosylation, prolyl hydroxylation and triglyceride transfer.
PER1_MAIZE	Peroxidase 1	100%	100%	99%	38,354.2	7,14	Removal of H_2O_2, oxidation of toxic reductants, biosynthesis and degradation of lignin, suberization, auxin catabolism, response to environmental stresses such as wounding, pathogen attack and oxidative stress.
PER2_MAIZE	Peroxidase 2	100%	100%	99%	35,750.3	5,66	Removal of H_2O_2, oxidation of toxic reductants, biosynthesis and degradation of lignin, suberization, auxin catabolism, response to environmental stresses such as wounding, pathogen attack and oxidative stress.

PER42_MAIZE	Peroxidase 42	100%	100%	100%	33,011.5	6,07	Removal of H_2O_2, oxidation of toxic reductants, biosynthesis and degradation of lignin, suberization, auxin catabolism, response to environmental stresses such as wounding, pathogen attack and oxidative stress.
PER66_MAIZE	Peroxidase 66	100%	100%	100%	33,419.5	8,07	Removal of H_2O_2, oxidation of toxic reductants, biosynthesis and degradation of lignin, suberization, auxin catabolism, response to environmental stresses such as wounding, pathogen attack and oxidative stress.
PER70_MAIZE	Peroxidase 70	100%	100%	100%	33,481.5	8,9	Removal of H_2O_2, oxidation of toxic reductants, biosynthesis and degradation of lignin, suberization, auxin catabolism, response to environmental stresses such as wounding, pathogen attack and oxidative stress.
PGMC1_MAIZE	Phosphoglucomutase, cytoplasmic 1	100%	100%	100%	63,098.0	5,72	This enzyme participates in both the breakdown and synthesis of glucose.
PGMC2_MAIZE	Phosphoglucomutase, cytoplasmic 2	100%	100%	100%	63,042.2	5,71	This enzyme participates in both the breakdown and synthesis of glucose.
PIP15_MAIZE	Aquaporin PIP1-5	100%	100%	100%	30,725.3	8,16	Water channel required to facilitate the transport of water across cell membrane.
PIP21_MAIZE	Aquaporin PIP2-1	100%	100%	100%	30,215.7	7,84	Water channel required to facilitate the transport of water across cell membrane. Active as homomers. Increased activity when heteromerization with PIP1-2.

PIP22_MAIZE	Aquaporin PIP2-2	100%	99%	100%	30,260.2	8,15	Aquaporins facilitate the transport of water and small neutral solutes across cell membranes.
PIP25_MAIZE	Aquaporin PIP2-5	100%	100%	100%	29,836.1	7,85	Water channel required to facilitate the transport of water across cell membrane. Its function is impaired by Hg (2+). May play a role in water uptake from the root surface. Active as homomers. Increased activity when heteromerization with PIP1-2.
PIP26_MAIZE (C)	Aquaporin PIP2-6	100%	100%	100%	30,191.5	8,25	Aquaporins facilitate the transport of water and small neutral solutes across cell membranes.
PLDA1_MAIZE	Phospholipase D alpha 1	100%	100%	100%	92,243.9	5,6	Hydrolyzes glycerol-phospholipids at the terminal phosphodiesteric bond. Plays an important role in various cellular processes.
PMGI_MAIZE	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	100%	100%	100%	60,620.2	5,53	Catalyzes the interconversion of 2-phosphoglycerate and 3-phosphoglycerate.
PSAN_MAIZE ©	Photosystem I reaction center subunit N, chloroplastic (Fragment)	100%	95%	100%	12,621.8	8,13	May function in mediating the binding of the antenna complexes to the PSI reaction center and core antenna.
PURA_MAIZE	Adenylosuccinate synthetase, chloroplastic	100%	100%	100%	51,913.6	7,33	Plays an important role in the de novo pathway and in the salvage pathway of purine nucleotide biosynthesis.
REHY_MAIZE	1-Cys peroxiredoxin PER1	100%	100%	100%	24,905.5	6,8	Thiol-specific peroxidase that catalyzes the reduction of hydrogen peroxide and organic hydroperoxides to water and alcohols, respectively (By similarity).
RL10_MAIZE	60S ribosomal protein L10	100%	100%	100%	24,919.5	10,26	Plays a role in ribosomal large subunit assembly.

RL17_MAIZE	60S ribosomal protein L17	100%	100%	100%	19,506.7	10,26	Plays a role in ribosomal large subunit assembly.
RL19_MAIZE	60S ribosomal protein L19 (Fragment)	95%	100%	100%	7,176.0	10,49	Plays a role in ribosomal large subunit assembly.
RL30_MAIZE	60S ribosomal protein L30	100%	100%	100%	12,491.1	9,58	Plays a role in ribosomal large subunit assembly.
RLA0_MAIZE	60S acidic ribosomal protein P0	100%	100%	100%	34,505.4	5,3	Ribosomal protein P0 is the functional equivalent of *E.coli* protein L10.
RLA1_MAIZE	60S acidic ribosomal protein P1	100%	100%	100%	11,097.1	4,51	Plays an important role in the elongation step of protein synthesis.
RLA2A_MAIZE	60S acidic ribosomal protein P2A	100%	100%	100%	11,363.0	4,28	Plays an important role in the elongation step of protein synthesis.
RLA3_MAIZE (C)	60S acidic ribosomal protein P3	100%	100%	100%	12,219.0	4,56	Plays an important role in the elongation step of protein synthesis.
RS11_MAIZE	40S ribosomal protein S11	100%	100%	100%	17,690.4	10,58	Plays a role in translation.
RS13_MAIZE	40S ribosomal protein S13	100%	100%	100%	17,059.3	10,45	Plays a role in translation.
RS141_MAIZE	40S ribosomal protein S14	100%	100%	100%	16,258.0	10,7	Plays a role in translation.
RS21_MAIZE	40S ribosomal protein S21	100%	100%	100%	9,020.4	8,18	Plays a role in translation.
RS27A_MAIZE	Ubiquitin-40S ribosomal protein S27a	100%	100%	100%	17,682.2	9,8	Ubiquitin exists either covalently attached to another protein, or free (unanchored). When covalently bound, it is conjugated to target proteins via an isopeptide bond either as a monomer (monoubiquitin), a polymer linked via different Lys residues of the ubiquitin (polyubiquitin chains), or a linear polymer linked via the initiator Met of the ubiquitin (linear polyubiquitin

							chains). Polyubiquitin chains, when attached to a target protein, have different functions depending on the Lys residue of the ubiquitin that is linked: Lys-48-linked is involved in protein degradation via the proteasome.
RS28_MAIZE	40S ribosomal protein S28	100%	100%	100%	7,410.5	10,37	Plays a role in maturation of SSU-rRNA, ribosomal small subunit assembly, and translation.
RS4_MAIZE	40S ribosomal protein S4	100%	100%	100%	30,018.5	10,15	Plays a role in translation.
RS8_MAIZE	40S ribosomal protein S8	100%	100%	100%	25,057.1	10,37	Plays a role in maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA), ribosome assembly, and translation.
SCRK1_MAIZE	Fructokinase-1	100%	100%	100%	34,691.6	5	May play an important role in maintaining the flux of carbon towards starch formation in endosperm. May also be involved in a sugar-sensing pathway.
SCRK2_MAIZE	Fructokinase-2	100%	100%	100%	35,482.4	5,58	May play an important role in maintaining the flux of carbon towards starch formation. May also be involved in a sugar-sensing pathway.
SIR_MAIZE	Sulfite reductase [ferredoxin], chloroplastic	100%	100%	100%	70,016.6	8,69	Essential protein with sulfite reductase activity required in assimilatory sulfate reduction pathway during both primary and secondary metabolism and thus involved in development and growth.

SODC5_MAIZE (C)	Superoxide dismutase [Cu-Zn] 4AP	100%	100%	100%	15,070.2	6,1	Destroys radicals which are normally produced within the cells, and which are toxic to biological systems.
SODM4_MAIZE (A)	Superoxide dismutase [Mn] 3.4, mitochondrial	100%	100%	90%	25,239.0	7,27	Destroys superoxide anion radicals which are normally produced within the cells, and which are toxic to biological systems.
SPP1_MAIZE (B)	Sucrose-phosphatase 1	100%	100%	100%	47,214.6	5,73	Catalyzes the final step of sucrose synthesis. Inactive with fructose 6-phosphate as substrate.
SUI1_MAIZE	Protein translation factor SUI1 homolog	100%	100%	100%	12,704.6	8,79	Probably involved in translation.
SUS1_MAIZE	Sucrose synthase 1	100%	100%	100%	91,736.0	6,38	Sucrose-cleaving enzyme that provides UDP-glucose and fructose for various metabolic pathways. Most active in the sink tissues where it is responsible for the breakdown of the arriving sucrose.
SUS2_MAIZE	Sucrose synthase 2	100%	100%	100%	92,941.5	6,48	Sucrose-cleaving enzyme that provides UDP-glucose and fructose for various metabolic pathways.
TBA1_MAIZE	Tubulin alpha-1 chain	100%	100%	100%	49,731.1	5,02	Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha chain.
TBA2_MAIZE	Tubulin alpha-1 chain	100%	100%	100%	49,731.1		Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha chain.
TBA5_MAIZE	Tubulin alpha-5 chain	100%	100%	100%	49,624.8	5,1	Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on

							the beta chain and one at a non-exchangeable site on the alpha chain.
TBB7_MAIZE	Tubulin beta-7 chain	100%	100%	100%	50,094.3	4,82	Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha chain.
TBB8_MAIZE (B)	Tubulin beta-8 chain	100%	100%	100%	49,944.0	4,84	Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha chain.
TCTP_MAIZE	Translationally-controlled tumor protein homolog	100%	100%	100%	18,690.5	4,67	Involved in calcium binding and microtubule stabilization.
THI42_MAIZE (A)	Thiamine thiazole synthase 2, chloroplastic	100%	100%	100%	37,233.2	5,88	Involved in biosynthesis of the thiamine precursor thiazole.
TIP22_MAIZE	Aquaporin TIP2-2	100%	100%	100%	25,043.9	6,02	Aquaporins facilitate the transport of water and small neutral solutes across cell membranes.
TIP23_MAIZE	Aquaporin TIP2-3	100%	100%	100%	25,132.5	6,67	Water channel required to facilitate the transport of water across cell membrane.
TKTC_MAIZE	Transketolase, chloroplastic	100%	100%	100%	72,994.8	5,72	Catalyzes the reversible transfer of a two-carbon ketol group from fructose-6-phosphate or sedoheptulose-7-phosphate to glyceraldehyde-3-phosphate to yield xylulose-5-phosphate and erythrose-4-phosphate or ribose-5-phosphate, respectively.
TPIS_MAIZE	Triosephosphate isomerase, cytosolic	100%	100%	100%	27,023.7	5,68	Plays a role in the glyceraldehyde-3-phosphate biosynthetic process, glycerol catabolic process, and glycolytic process.

UCRI_MAIZE	Cytochrome b-c1 complex subunit Rieske, mitochondrial	100%	100%	100%	29,835.4	8,88	Component of the ubiquinol-cytochrome c oxidoreductase, a multisubunit transmembrane complex that is part of the mitochondrial electron transport chain which drives oxidative phosphorylation.
UPTG_MAIZE	Alpha-1,4-glucan-protein synthase [UDP-forming]	100%	100%	100%	41,206.1	6,13	Probable UDP-L-arabinose mutase involved in the biosynthesis of cell wall non-cellulosic polysaccharides (By similarity).
VATA_MAIZE	V-type proton ATPase catalytic subunit A (Fragment)	100%	100%	100%	61,953.6	6,2	Catalytic subunit of the peripheral V1 complex of vacuolar ATPase.
VDAC_MAIZE	Outer plastidial membrane protein porin	100%	100%	100%	22,476.0	8,13	Forms a channel through the cell membrane that allows diffusion of small hydrophilic molecules.
YPTM2_MAIZE	GTP-binding protein YPTM2	100%	100%	100%	22,476.0	6,2	Protein transport. Probably involved in vesicular traffic.
ZB14_MAIZE (A)	14 kDa zinc-binding protein	100%	100%	100%	14,300.9	6,68	Plays a role in the purine ribonucleotide metabolic process, and sulfur compound metabolic process.

5.3 Discussion

Plants have evolved various responses to defend themselves against pathogens which are elicited throughout their life cycle in response to developmental signals and pathogen attack (Huynh *et al*., 1992). An example of such a response is the alteration of the pattern of protein synthesis in which a number of enzymes made function is to inhibit the growth of parasitic invaders (Verburg *et al*., 1992).

Proteins that were present in response to fungal infection included proteins participating in biosynthetic processes, pathways, signalling responses, anti-pathogenic proteins, and proteins with other functions (Figure 5.4 B-D; Table 5.2). Proteins such as sucrose-phosphatase 1 and glutamine synthetase were also present which suggests an increase in the requirement of energy in the early stages of root growth. Proteins that were present in response to priming with the *P. griseofulvum* (B3) prior to infection included proteins that are involved in anti-pathogen defence as well as antioxidant activity (Figure 5.4 B-D; Table 5.2).

5.3.1 Infection with *Fusarium proliferatum* and biocontrol with *Penicillium griseofulvum* effects the protein regulation in maize roots

5.3.1.1 Unique proteins involved in biological regulation in maize seed roots

Proteins involved in biological regulation in the infected maize seed roots included the elongation factor, 1-acyl-sn-glycerol-3-phosphate acyltransferase, and heat shock protein 82 and in the biocontrol maize seed roots, the protein involved in biological regulation was the 60S acidic ribosomal protein P3.

In this study, the elongation factor was involved in mitochondrial translational elongation (Figure 5.4 B and D; Table 5.2) and localized in the mitochondrial matrix (Figure 5.4 C; Table 5.2). Translational elongation factors (EFT's) are proteins that play significant roles in the elongation cycle of protein biosynthesis localized in the ribosome (Maloy and Hughes, 2013). Firstly, EFT's bring aminoacyl-transfer RNA to the ribosome during the synthesis of proteins and secondly, EFT's are involved in translocation where peptidyl-tRNA is transferred to various ribosomal sites whilst the messenger RNA moves through the ribosome (Maloy and Hughes, 2013). The mitochondria are the main site for the supply of ATP and cellular respiration. They play significant roles in redox homeostasis and provide molecules which act as metabolic intermediates in important biosynthetic pathways (Bahaji *et al*., 2019).

The 1-acyl-sn-glycerol-3-phosphate acyltransferase protein was involved in the CDP-diacylglycerol biosynthetic process (Figure 5.4 B and D; Table 5.2) and is an integral component of membrane (Figure 5.4 C; Table 5.2). 1-acyl-sn-glycerol-3-phosphate acyltransferase is involved in the CDP-diacylglycerol biosynthetic process where it produces CDP-diacylglycerol, CDP-1,2-diacylglycerol (Blunsom and Cockcroft, 2020). CDP-diacylglycerol is required for the synthesis of phosphatidylinositol via the reaction of the nucleotide lipid with free inositol (Kent, 2004). Phosphatidylinositol is an important lipid due to the fact that it is a constituent of membranes, and it participates in important metabolic processes in plants (Ridgway, 2021).

In this study, heat shock protein 82 was involved in protein stabilization, cellular response to heat, and protein folding (Figure 5.4 B; Table 5.2), possesses ATP binding, unfolded protein binding and ATPase activity (Figure 5.4 D; Table 5.2), and is localized in the cytosol (Figure 5.4 C; Table 5.2). Heat shock proteins (HSP's) are molecular chaperones which regulates the accumulation, localization degradation, and folding of protein molecules in plants which plays a role in a number of cellular processes, which therefore may impart a generalized role in the plant's tolerance to environmental stresses (Abou-Deif *et al*., 2019; Baniwal *et al*., 2004; Li *et al*., 2021; Park and Seo, 2015; Piterková *et al*., 2013; Swindell *et al*., 2007). Members of the HSP gene families have been found to be expressed at certain developmental stages in several organisms in the absence of heat (Marrs *et al*., 1993). Besides the specific protein production, plants are able to respond to a variety of stresses via the production of HSP's which is indicative of the plant's adaptive mechanism where HSP's are used to protect the cells against many stresses (Abou-Deif *et al*., 2019).

Plants respond to infection by pathogens by using two innate immune responses that are facilitated by pattern recognition receptors (PRR's) or resistance (R) proteins. HSP's play a significant role as a molecular chaperone in quality control of plasma membrane-resident PRR's as well as intracellular proteins against potential pathogenic invaders (Park and Seo, 2015). HSP's in plants have received substantial attention due to their function in innate immunity in studies done by Li *et al*. (2009); Liu and Howell (2010); Nekrasov *et al*. (2009). Plants are able to respond to pathogenic infection by using a two-branched innate immune system which consists of PAMP-triggered immunity (PTI) and effector-triggered immunity (EIT). HSP's play a protective role in plants experiencing biotic and abiotic stress. As observed in this study where HSP's were present in the infected treatment, Campo *et al*. (2004) also

showed that HSP's were up-regulated in maize embryos that were infected with *Fusarium verticillioides.*

The 60S acidic ribosomal protein P3 was involved in translational elongation and protein synthesis (Figure 5.4 B and D; Table 5.2) and is localized in the cytosolic large ribosomal subunit (Figure 5.4 C; Table 5.2). Ribosomes contain two subunits which are both required for translation. The small subunit (40S) is responsible for decoding the genetic messages and the large subunit (60S) is responsible for catalysing the peptide bond formation (Gregory *et al*., 2019).

5.3.1.2 Unique proteins involved in response to pathogens in maize roots

Proteins involved in response to pathogen infection included the eukaryotic translation initiation factor 5, sucrose-phosphatase 1, AP-2 complex subunit sigma, dehydrin, ferritin-2 in infected roots. The protein glutamine synthetase root isozyme was found in infected roots, whereas the glutamine synthetase root isozyme 4 was present in the biocontrol roots.

In this study, the eukaryotic translation initiation factor 5 was involved in the response to pathogen infection (Figure 5.4 B; Table 5.2), and possesses GTP binding, eukaryotic initiation factor eIF2 binding, and translation initiation factor activity (Figure 5.4 D; Table 5.2). It is localized in the cytosol (Figure 5.4 C; Table 5.2). The eukaryotic translation initiation factor 5 (eIF5) plays significant roles in initiating protein synthesis. It forms a complex with eIF2 via the interaction with the β subunit of eIF2. This interaction is vital for the eIF5-promoted hydrolysis of GTP bound to the 40 S initiation complex (Das *et al*., 2001). Likewise, to this study where maize seeds were infected with *F. proliferatum*, previous studies by Campo *et al*. (2004); Chivasa *et al*. (2005); Huang *et al*. (2009) (a and b); Mohammadi *et al*. (2011); Pechanova *et al*. (2011); Wu *et al*. (2013) showed that proteins involved in pathogen-infected maize consisted of eukaryotic translation initiation factor 5A (eIF-5A), various HSPs, peptidylprolyl cis-trans isomerases, cyclophilin, and chaperonins. The eukaryotic translation factor 5A (eIF5) has been well documented for its ability to initiate eukaryotic cellular protein biosynthesis, however, it has recently been found to be a pathogen-response protein (Pechanova and Pechan, 2015). The exact role of eIF5 in the interactions between maize and pathogens have not been solved, however, its role in other plant species have been well documented. An example is (Hopkins *et al*., 2008) where the eIF-5A serves as a pivotal element in the induction of programmed cell death due to infection by a virulent pathogen.

In this study, sucrose-phosphatase 1 was involved in the sucrose biosynthetic process and response to pathogen infection (Figure 5.4 B; Table 5.2) and possesses magnesium ion binding and sucrose-phosphate phosphatase activity (Figure 5.4 D; Table 5.2). Sugars constitutes the primary substrate for energy provision and structural material for defence-responses in plants, and they act as signalling molecules which interact with hormonal signalling networks which regulates the plants immunity (Rolland *et al*., 2006; Ruan, 2012; Smeekens *et al*., 2010). Additionally, sugars are able to enhance the oxidative burst during the early stages of infection which increases the lignification of cell walls and induces PR proteins (Morkunas and Ratajczak, 2014). In most interactions between plants and pathogens, a high level of sugars within the plant tissues increases the plants resistance to infection (Morkunas and Ratajczak, 2014). Owing to its regulatory functions in plants, sugar impacts all the phases within its life cycle and interacts with phytohormones and controls its development and growth (Stokes *et al*., 2013; Wind *et al*., 2010). To date, there have been various reports on the importance of sugars in a plants resistance to disease caused by fungal pathogens, however, their role as signalling molecules in the defence responses have only been recently examined (Bolouri Moghaddam and Van den Eden, 2012; Doehlemann *et al*., 2008; Morkunas *et al*., 2011).

Various issues are faced when interpreting the dependence of sugar levels in plants that are infected with pathogenic fungi. Pathogens can interfere with host metabolism not only via the uptake of sugars but also via other metabolites to benefit themselves, however, this may disturb the plants metabolism (Morkunas and Ratajczak, 2014). Plants and pathogens engage in an evolutionary tug-of-war, where the plant limits the access of nutrients from the pathogen and induces immune responses and the pathogen evolves mechanisms in which to gain access to the plants nutrients and suppress host immunity (Boller and He, 2009; Chen *et al*., 2010). Like this study, pathogens have developed various mechanisms to compete with their host plants for sucrose. Wahl *et al*. (2010) showed that *Ustilago maydis* utilizes the sucrose transported *UmSRT1* which possesses a higher affinity than the host sucrose transporters. On the other hand, Voegele *et al*. (2006) observed that *Uromyces fabae* first hydrolyses sucrose to hexose which is then taken up by fungi via a hexose transporter.

In this study, AP-2 complex subunit sigma was involved in clathrin-dependent endocytosis, protein transport, and vesicle-mediated transport and response to pathogen infection (Figure 5.4 B; Table 5.2), and clathrin adaptor activity (Figure 5.4 D; Table 5.2). Clathrin-mediated

endocytosis (CME) is significant in the morphogenesis of plants (Chen *et al*., 2011). It regulates and constitutes the recycling and uptake of plasma membrane (PM) proteins that are crucial for the uptake of nutrients, transport of auxin, cellular processes involved in the production of cell plates during cytokinesis as well as the signalling of hormones and pathogens (Chen *et al*., 2011; McMichael and Bednarek, 2013). The heterotetrameric adaptor protein 2 (AP-2) plays a role in the CME adaptor complex which interacts with the CME accessory proteins and cargo, membrane and clathrin (Brodsky, 2012; Di Rubbo *et al*., 2013; Kitakura *et al*., 2011; McMahon and Boucrot, 2011; Wang *et al*., 2013; Yamaoka *et al*., 2013).

To infect and colonize host plants, fungal pathogens secrete effectors such as hydrolytic enzymes that are able to kill plant tissue (Souibgui *et al*., 2021). The secreted proteins are transported from the golgi apparatus (GA) and the endoplasmic reticulum (ER) to the extracellular space via intracellular vesicles (Souibgui *et al*., 2021). In various multicellular and unicellular eukaryotes, the role of clathrin coated vesicles (CCV) in CME is well documented where they are involved in the intake of membrane and extracellular compounds (McMahon and Boucrot, 2011). In connection to this, they are also exploited by pathogens to gain access into plant cells (Latomanski and Newton, 2019; Robinson *et al*., 2018; Souibgui *et al*., 2021; Yang and Shen, 2020). Various clathrin mutants in several pathogenic microorganisms have been produced to determine its importance in virulence. In a study by Allen *et al*. (2003), the depletion of clathrin by antisense RNA in *Trypanosoma brucei* resulted in rapid lethality in the bloodstream of the parasite. Interestingly, a study by Bairwa *et al*. (2019) showed that the encapsulated yeast, *Cryptococcus neoformans*, which lacked the clathrin heavy chain-encoding gene was defective in the uptake of haemoglobin, a main source of iron for the fungal pathogen, as well as in the synthesis of two main virulence factors, namely melanin and capsule.

To date, not much is known about clathrin in filamentous fungi despite the relevance of vesicular trafficking in fungal growth (Shoji *et al*., 2014), however, clathrin is an essential part of the infectious process of the phytopathogenic fungi *Botrytis cinerea* (Souibgui *et al*., 2021). Additionally, it showed that the protein plays a role in the secretion of cell death-inducing proteins (CDIPs), which are associated with ROS production and the degradation of cell walls. Furthermore, the study showed that clathrin is important in the development of infectious cushions, which are structures dedicated to the penetration and the early destruction of host tissue by the pathogen.

In this study, dehydrin was involved in cold acclimation, response to abscisic acid, response to water deprivation, and pathogen infection (Figure 5.4 B; Table 5.2), and phosphatidic acid binding, phosphatidylglycerol binding, phosphatidylinositol binding, and phosphatidylserine binding (Figure 5.4 D; Table 5.2). The dehydrin protein is localized in the vesicle membrane (Figure 5.4 C; Table 5.2). Plants have developed complex systems that allow them to respond to stresses (Liu *et al*., 2017). Late embryogenesis (LEA) proteins are a diverse family that plays significant roles in stress tolerance in plants (Liu *et al*., 2019). Dehydrins (DHN's) belong to the group II LEA proteins which are considered to be stress proteins (Liu *et al*., 2017). DHN's play a significant role in a plant's adaptation and response to abiotic stress (Hanin *et al*., 2011). They typically accumulate in maturing seeds or vegetative tissues in response to dehydration, freezing, cold, and salinity stress (Hanin *et al*., 2011; Liu *et al*., 2013, Liu *et al*., 2016).

Dehydrins possess the ability to bind metal ions (Hara *et al*., 2005, Hara *et al*., 2011; Mittler, 2002), bind DNA (Hara *et al*., 2009; Wise and Tunnacliffe, 2004), bind phospholipids (Koag *et al*., 2009; Kooijman *et al.*, 2007; Petersen *et al*., 2012), prevent denaturation of proteins by binding them (Drira *et al*., 2013; Drira *et al*., 2015; Hara *et al*., 2016; Yang *et al*., 2015), and scavenge free radicals (Hara *et al*., 2003; Hara *et al*., 2013, Hara *et al*., 2016). N'Guyen *et al*. (2019) showed the importance of dehydrin proteins in *Alternaria brassicicola* to effectively accomplish key mechanisms in its pathogenic life cycle. The study showed that single-deletion mutants portrayed that dehydrin-like proteins have impacts on conidial survival when exposed to freezing and high temperatures as well as oxidative stress tolerance. Additionally, double-dehydrin mutants showed an increase in compromised pathogenicity with a decline in aggressiveness on *Brassica oleracea* leaves and a decline in its capacity to be transmitted to *Arabidopsis* seeds via siliques (N'Guyen *et al*., 2019).

In this study, ferritin-2 was involved in intracellular sequestering of iron ion, in iron ion transport and in the response to pathogen infection (Figure 5.4 B; Table 5.2), possesses ferric iron binding, ferrous iron binding and ferroxidase activity (Figure 5.4 D; Table 5.2), and is localized in the chloroplast (Figure 5.4 C; Table 5.2). Iron (Fe) is an important micronutrient used hormonal and chlorophyll synthesis and photosynthesis; therefore, plants tightly regulate its iron uptake, transport, localization, and storage (Briat *et al*., 2010; Camprubi *et al*., 2017; Lobreaux *et al*., 1992). Iron homeostasis needs to be strictly controlled to avoid toxicity and deficiency, which are both known to significantly damage the physiology of plants which ultimately affects their growth and development (Briat *et al*., 2010). Among the molecules that

are needed for important processes to occur, ferritins are a class of ubiquitous iron storage proteins that are found in all living kingdoms (Briat *et al*., 2010).

In plants, the ferritin gene expression is regulated by a number of environmental factors including cold, light intensity, drought, and pathogen attack (Camprubi *et al*., 2017). Therefore, it is likely that plant ferritins, by buffering iron, assists the plant to cope with these adverse environments (Briat *et al*., 2010). Plants use two strategies to maintain homeostasis and increase iron solubility in low-iron environments (Chaney *et al*., 1972; De Vos *et al*., 1986; Marschner *et al*., 1986; Palmgren, 2001). Strategy I plants include all non-Poaceae angiosperms which primarily acquire iron by the acidification of the rhizosphere in order to increase its iron solubility and thereafter reduce iron in the soil prior to direct uptake (Kobayashi *et al*., 2019). Strategy II plants include Poaceae plants such as maize and rice (Kobayashi *et al*., 2019). Strategy II plants secrete phytosiderophores to bind ferric iron in the rhizosphere for transport into the root (Kobayashi *et al*., 2019; Marschner *et al*., 1986). Like this study, Supplementary Figure 5.3 shows the siderophore activity of *F. proliferatum*, siderophores are iron bearers; they include pathogenic microbes which acquires iron and facilitates its uptake (Andrews *et al*., 2003; Haas *et al*., 2008; Niehus *et al*., 2017).

Bacteria and fungi use methods similar to strategy I and II for iron acquisition (Andrews *et al*., 2003; Philpott, 2006; Sandy and Butler, 2009). The most studied method used by pathogenic fungi and bacteria is based on the secretion of a high-affinity iron-binding siderophores which is analogous to strategy II (Aznar and Dellagi, 2015; Franza and Expert, 2013; Haas *et al*., 2008; Khan *et al*., 2018; Neilands, 1995). Albarouki and Deising (2013) studied the importance of ferroxidase (FET)-mediated iron uptake in maize pathogen *Colletotrichum graminicola*. The fungi lacking the iron-deficiency induced FET protein had and the wild type fungi had grown well on the iron-sufficient media, however, the mutant fungi exhibited a decline in virulence on maize as well as an abnormal morphology. The authors showed that iron assimilation is vital for the pathogen *in planta*. Iron sequestration methods have not been well described in plants; however, some evidence suggests that iron restriction is an important component of plant immunity. Plant iron-binding ferritins (FER) have been shown to be up-regulated in various plants when infected. Examples of such studies include the *Arabidopsis* plants infected with *Dickeya dadantii* and the potato tubers infected with *Phytophthora infestans* (Dellagi *et al*., 2005; García Mata *et al*., 2001).

In this study, the glutamine synthetase root isozyme in the infected and biocontrol treatments is involved in response to pathogen infection and in the glutamine biosynthetic process (Figure 5.4 B; Table 5.2), specifically in ATP binding and glutamate-ammonia ligase activity (Figure 5.4 D; Table 5.2) and is localized in the cytoplasm (Figure 5.4 C; Table 5.2). Nitrogen (N) is an important mineral required for the development of plants (Lam *et al*., 1996; Prinsi and Espen, 2015). Glutamine synthetase (GS) isozymes play critical roles in nitrogen (N) metabolism (Bernard and Habash, 2009; Harper *et al*., 2010; Martin *et al*., 2006; Thomsen *et al*., 2014; Wei *et al*., 2018; Wei *et al*., 2020). In higher plants, the important physiological assimilation is carried out by the enzyme glutamine synthetase (GS) via the glutamine synthetase and glutamine-oxoglutarate aminotransferase (GOGAT) cycle (Cren and Hirel 1999; Forde and Lea 2007). Glutamate metabolism (GM) in plants play an important role in the metabolism of amino acids and metabolic functions, with key roles in the plants defence against pathogens (Seifi *et al*., 2013).

The changes in the host GM in the response to various pathogenic infections functions in two ways, where it either backs the ongoing defence strategy to shape an effective resistance response or it is exploited by the pathogen in order to facilitate and promote infection (Seifi *et al*., 2013). During interactions with pathogens, the glutamate metabolism in the host is altered, and leads to either a metabolic state known as “evasion” where cell death is facilitated or “endurance” where cell viability is maintained (Seifi *et al*., 2013). Pathogens, however, have evolved mechanisms such as hemibiotrophy, toxin secretion, and the utilization of selective amino acid to exploit the plant's GM in order to benefit itself (Seifi *et al*., 2013).

A study by Kretschmer *et al*. (2017) examined whether disease caused by *Ustilago maydis* will be influenced by the inhibition of glutamine synthetase by glufosinate which affects the biosynthesis of amino acids. The study showed that increased glutamine formation in the infected maize tissue was beneficial to the plant and not to *U. maydis*, suggesting that the glutamine levels contributed to the plants defence rather than functioning as a nitrogen source for the pathogen. These results are similar to the result of this study as well as several other studies regarding amino acid homeostasis in plant-pathogen interactions (Asai *et al*., 2008; Bolton, 2009; Guillet and De Luca, 2005; Kim *et al*., 2013; Köllner *et al*., 2010; Liu *et al*., 2010; Walters, 2003; Wang *et al*., 2006).

5.3.1.3 Unique proteins with antioxidant activity in maize roots

Proteins with antioxidant activity present in the maize seed roots primed with *P. griseofulvum* prior to infection included aldehyde dehydrogenase and superoxide dismutase [Cu-Zn].

In this study, aldehyde dehydrogenase possessed aldehyde dehydrogenase (NAD) activity, (Figure 5.4 D; Table 5.2) and was localized in the cytosol (Figure 5.4 C; Table 5.2). Several studies have shown the up regulation of antioxidants in plants infected by pathogens (Chivasa *et al*., 2006; Geddes *et al*., 2008; Yang *et al*., 2010; Zhou *et al*., 2006;). Contrast to this study where aldehyde dehydrogenase was present in the cytosol of seed roots primed with B3 prior to infection with *F. proliferatum* (Figure 5.4 A; Table 5.2), Yang *et al*. (2010) found that the aldehyde dehydrogenase protein was up regulated in infected barley during grain filling. Additionally, gene expression of aldehyde dehydrogenase was up regulated in an interaction between barley and the pathogen *Pyrenophora teres* (Bogacki *et al*., 2008). Aldehyde dehydrogenase is responsible for the detoxification of aldehydes produced by lipid peroxidation due to ROS- accumulation under stress (Bogacki *et al*., 2008). This study showed that lipid peroxidation decreased in roots primed with B3 prior to infection with *F. proliferatum*, relative to roots infected with *F. proliferatum* (Figure 4.4).

Bilal *et al*. (2017) used RT-PCR analysis to portray the expression of aldehyde dehydrogenase for indole-acetic acid, indole-3-acetamide hydrolase, and geranylgeranyl-diphosphate synthase for the synthesis of gibberellins. They showed that inoculation with the endophytic fungi *Paecilomyces formosus* significantly improved the growth of soybean in nickel (Ni) polluted soils. A previous study by Yuan *et al*. (2019) observed that the endophytic fungus *Gilmaniella sp.* AL12 plays important roles in the metabolism of *Atractylodes lancea*, where the endophyte-inoculated plants portrayed an enhanced carbon metabolism and an upregulation in genes involved in the transport and metabolism of carbon. Genes encoding for aldehyde dehydrogenase (ALDH), lactate dehydrogenase (LDHD), and pyruvate decarboxylase (PDC), were upregulated suggesting that the endophyte assisted the plant in the conversion of lactate into pyruvate and ultimately the biosynthesis of acetic acid.

In this study, superoxide dismutase (SOD) [Cu-Zn] is responsible for the removal of superoxide radicals, it possesses superoxide dismutase activity and copper ion binding (Figure 5.4 D; Table 5.2) and is localized in the cytoplasm (Figure 5.4 C; Table 5.2). SOD is an enzyme that is responsible for the conversion of superoxide radicals into hydrogen peroxide and oxygen molecules (Thirach *et al*., 2007). The iron and manganese SODs are genetically similar;

however, the Cu-Zn SOD displays no significant homology with the other two enzymes (Culotta, 2001; Gralla and Kosman, 1992; Lynch and Kuramitsu, 2000). Likewise, to this study the up regulation of proteins in plants that are implicated in oxidative stress (Figure 5.3 A; Table 5.2) was observed in barley and wheat that was infected with *F. graminearum* during grain filling (Geddes *et al*., 2008; Yang *et al*., 2010; Zhou *et al*., 2006). In contrast to this study, which showed a decline in SOD activity in infected roots (Figure 4.5), Yang *et al*. (2010) showed *F. graminearum* had caused the production of SOD in mature infected barley seeds which suggested that the pathogen was exposed to and attempted to overcome the plants defence-related ROS.

As observed where the SOD [Cu-Zn] protein was present in the biocontrol treatment (Figure 5.4 A; Table 5.2), the Cu-Zn SOD is predominantly located in the cytoplasm where the peripheral location suggests that it may protect the surface of the fungi against the extracellular superoxides that are produced by the host cells (Figure 5.4 A; Table 5.2) (Thirach *et al*., 2007). ROS acts as a signalling molecule that activates a number of downstream plant defence responses, which includes the production of pathogenesis-related proteins, as well as changes to the cell wall (Segal and Wilson, 2018).

5.3.1.4 Unique proteins involved in defence in maize roots

Proteins involved in defence present in the maize seed roots primed with *P. griseofulvum* prior to infection included indole-3-acetaldehyde oxidase, bowman-birk type wound-induced proteinase inhibitor, isoflavone reductase homolog, non-specific lipid-transfer protein, and aquaporin PIP2-6.

In this study, indole-3-acetaldehyde oxidase is involved in defence, abscisic acid biosynthetic process and auxin biosynthetic process (Figure 5.4 B; Table 5.2), possesses 2 iron, 2 sulfur cluster binding, and iron ion binding activity (Figure 5.4 D; Table 5.2), and is localized is in the cytoplasm (Figure 5.4 C; Table 5.2). Indole-3-acetaldehyde oxidase (IAAId oxidase) is an important enzyme involved in the synthesis of indole-3-acetic acid (IAA) (Takei *et al*., 2019). The plant hormone, IAA, is a significant signal in the communication between the endophyte and host (Jahn *et al*., 2021). Endophytes can produce IAA to alter the IAA homeostasis in plants, however, there is little information on the pathway of IAA production in endophytes (Jahn *et al*., 2021). Together with other phytohormones, IAA is responsible for plant development and growth (Davies, 2010).

Several studies have investigated the role that IAA plays in the interaction and communication between host plants and endophytes (Rai and Varma, 2005; Suebrasri *et al*., 2020) and plant-associated microorganisms (Contreras-Cornejo *et al*., 2009; Ortiz-Castro *et al*., 2011; Rai and Varma, 2005). Endophytic fungi can regulate IAA levels in plants and produce their own, thus they can influence the plant metabolism for their own benefit (Jahn *et al*., 2021; Suebrasri *et al*., 2020). Therefore, microbial IAA may act as a mediator between microbes and plants by playing a role in communication between them (Jahn *et al*., 2021).

The methods endophytic fungi use to produce IAA is important to understand the interaction between host and endophyte (Mehmood *et al*., 2019; Mehmood *et al*., 2020). Similar to what was observed in this study where IAAId oxidase was present in maize roots primed with *P. griseofulvum* (Supplementary Figure 5.1; Supplementary Table 5.2), and in roots primed with *P. griseofulvum* prior to infection (Figure 5.4 A; Table 5.2), Contreras-Cornejo *et al*. (2009) showed that *Trichoderma virens* interacted with *Arabidopsis thaliana* by its own IAA production. Additionally, Pons *et al*. (2020) and Vadassery *et al*. (2008) observed IAA production in *Rhizophagus irregularis* and *Serendipita indica.*

In this study, bowman-birk type wound-induced proteinase inhibitor possessed defence and serine-type endopeptidase inhibitor activity (Figure 5.4 D; Table 5.2) and was localized in the extracellular region (Figure 5.4 C; Table 5.2). Plant proteinase inhibitors are small proteins which are prevalent in both dicotyledons and monocotyledons and function as a part of the defence system against pathogen attack (Eckelkamp *et al*., 1993; Filiz *et al*., 2014; Qu *et al*., 2003). The Maize Wip1 gene encodes a wound-induced Bowman-Birk inhibitor (BBI) protein which is a serine protease inhibitor expressed as a result from wounding or infection, and thereby confers resistance against pathogens and pests (Qi *et al*., 2005).

Although plants do not have specialized cells that are equivalent to the immune system in animals, they can respond to pathogenic infection or physical injury. One of these systems is wound response, where wounding may cause a change in gene expression of several proteins which includes glycine-rich proteins, proteinase inhibitors, or hydroxyproline-rich glycoproteins (Rohrmeier and Lehle, 1993). Similar to what was observed in this study where bowman-birk type wound-induced proteinase inhibitor was present in maize roots primed with *P. griseofulvum* (Supplementary Figure 5.1; Supplementary Table 5.2), and in roots primed with *P. griseofulvum* prior to infection (Figure 5.4 A; Table 5.2), Qu *et al*. (2003) showed that

there are seven BBI genes in rice and the overexpression of the rice BBI2-2 had conferred resistance to fungal pathogens in transgenic rice plants.

In this study, isoflavone reductase homolog was involved in defence and the cellular response to sulfur starvation (Figure 5.4 B; Table 5.2), possessed NADPH binding and oxidoreductase activity (Figure 5.4 D; Table 5.2), and was localized is in the cytoplasm (Figure 5.4 C; Table 5.2). In plants the biosynthetic pathway of isoflavonoid phytoalexin requires the enzyme Isoflavone reductase (IFR). IFR's are crucial in a plant's response to several biotic and abiotic stresses (Cheng *et al*., 2015). Isoflavones are a class of flavonoids which play important roles in the interactions between plants and microorganisms, such as defence responses and rhizobia-legume symbiosis (Sugiyama, 2019). Isoflavones also act as phytoalexins which are compounds produced by plants during pathogen attack and stress (Rípodas *et al*., 2013; Sohn *et al*., 2021). Flavonoids also act as antioxidant agents which neutralizes ROS and maintains normal functionality in the plant cellular membrane (Khan *et al*., 2013). Previous studies (Graham *et al*., 1990; Yu *et al*., 2003) have shown that IFR is an important enzyme required for the synthesis of glyceollins from daidzein.

Daidzein and genistein are important chemicals that plants utilize to combat pathogenic infection and disease (Catford *et al*., 2006; Dakora and Phillips, 1996). These chemicals can induce physiological changes in plants which leads to nitrogen fixation and nodule morphogenesis. When this happens, isoflavanoids are synthesised to create positive feedback cycles between the microorganism and the plant (Dakora and Phillips, 1996; Yu and McGonigle, 2005). This feedback response is crucial in creating a symbiotic relationship because some plants can also secrete isoflavanoids from their roots (Yu and McGonigle, 2005). Numerous studies have shown that abiotic stress and the presence of fungal and bacterial endophytes may change the expression of enzymes involved in flavonoid biosynthesis (Sharifi *et al*., 2007; Shaw *et al*., 2006; Subramanian *et al*., 2006).

Similar to what was observed in this study where isoflavone reductase homolog was present in maize roots primed with *P. griseofulvum* (Supplementary Figure 5.1; Supplementary Table 5.2), and in roots primed with *P. griseofulvum* prior to infection (Figure 5.4 A; Table 5.2), Waqas *et al*. (2017) reported an enhanced production of isoflavanoids in a biochar treatment as well as a treatment with the fungal endophyte *Geotrichum candidum* which showed similar findings as that of Hao *et al*. (2010), Khan *et al*. (2013) and; Waqas *et al*. (2014). Isoflavonoids reduce the growth of pathogenic fungal development by inhibiting spore germination and

mycelial growth. Additionally, it inhibits the respiration of pathogenic fungi and inhibits its nutrient uptake (Morkunas and Ratajczak, 2014). Therefore, we hypothesise that the isoflavanoids produced in the presence of *P. griseofulvum* inhibits the growth of *F. proliferatum*.

In this study, non-specific lipid-transfer protein was involved in defence and lipid transport (Figure 5.4 B; Table 5.2) and possesses lipid binding activity (Figure 5.4 D; Table 5.2). The intracellular transport of phospholipids requires the participation of phospholipid-transfer proteins (PLTP) mainly located in the cytosol in organs such as the ovaries, germinating and maturing seeds, roots, anthers, leaves, stems, and pollens (Han *et al*., 2001; Petit *et al*., 1994; Tchang *et al*., 1988; Wei and Zhong, 2014). These PLTPs play important roles in the renewal and biosynthesis of membranes as well as in the transport of hydrophobic compounds (Arondel *et al*., 1991; Tchang *et al*., 1988). The functions of plant non-specific phospholipid-transfer proteins (nsLTP's) include the transport of cuticular components that are needed for the inhibition of fungal and bacterial pathogens on plants (Molina *et al*., 1993; Terras *et al*., 1992). Investigations into the innate immunity of plants have shown that endophytic fungi possess the ability to protect plants against pathogens by the production of toxic- secondary metabolites, elicitors and enzymes, which induces systemic resistance in plants (Choudhary *et al*., 2007; Gozzo, 2003; Li *et al*., 2008; Wei and Zhong, 2014). These compounds are used as biocontrol agents that are alternatives to the chemical methods of disease management (Odintsova *et al*., 2019).

Similar results to these where nsLTP's were present in maize roots primed with *P. griseofulvum* (Supplementary Figure 5.1; Supplementary Table 5.2), and in roots primed with *P. griseofulvum* prior to infection (Figure 5.4 A; Table 5.2) which assisted the seed in its resistance to *F. proliferatum* infection, several studies have shown the overexpression of lipid transfer protein genes in transgenic plants enhances pathogen resistance. Sun *et al*. (2008) showed that the overexpression of wheat nsLTP genes in transgenic plants possessed enhanced disease resistance where *in vitro* antifungal assays were done with eight wheat nsLTPs against three non-wheat and eight wheat pathogens. They also showed differential inhibition of spore germination and mycelial growth. Additionally, all the wheat nsLTPS portrayed activity against *Fusarium graminearum*. Moreover, Zhu *et al*. (2012) observed that the overexpression of wheat lipid transfer protein TaLTP5 gene enhanced the resistance to *F. graminearum* and *Cochliobolus sativus.* Moreover, Safi *et al*. (2015) reported that *A. thaliana* plants expressing

the TdLTP4 gene showed an enhanced resistance to *Alternaria solani* and *B. cinerea* (Odintsova *et al*., 2019). Furthermore, Jayaraj and Punja (2007) showed that the combined expression of a barley chitinase chi-2 and wheat lipid transfer-protein in transgenic carrots possessed an increased resistance to fungal pathogens *Alternaria radicicola* and *B. cinerea*.

In this study, aquaporin PIP2-6 possessed defence and water channel activity (Figure 5.4 D; Table 5.2) and is an integral component of membrane (Figure 5.4 C; Table 5.2). Aquaporins (AQP's) are a family of channel proteins that are responsible for transporting water and neutral metabolites across biological membranes (Chaumont *et al*., 2001; Zhang *et al*., 2019). In plants, the movement of water is important for various physiological processes such as the opening of the stomata guard cells and cell elongation as well as in the transport of hormones and nutrients (Lopez *et al*., 2003) Aquaporins are generally localized within the plasma membrane (Lopez *et al*., 2003). The PIP-subfamily is divided into two subgroups known as PIP1 and PIP2 (Fetter *et al*., 2004; Lopez *et al*., 2003; Zhang *et al*., 2019). PIP1 proteins assessed for water transport activity in *Xenopus laevis* showed low or a lack of activity and some are responsible for the facilitation of the diffusion of small neutral solutes, whereas all PIP2 proteins showed a high water-transport activity (Lopez *et al*., 2003; Fetter *et al*., 2004).

In previous studies by Chaumont *et al*. (2000), Dixit *et al*. (2001), Marin-Olivier *et al*. (2000); and Moshelion *et al*. (2002), it was shown that the PIP2 proteins have high water channel activity in *Xenopus oocytes.* AQP's of the PIP subfamily face a persistent risk of hijack by pathogens that are trying to infect the plant (Fetter *et al*., 2004). Similar to what was observed in this study where PIP2-6 was present in maize roots primed with *P. griseofulvum* (Supplementary Figure 5.1; Supplementary Table 5.2), and in roots primed with *P. griseofulvum* prior to infection, PIP's may also possess a function in the plant's immunity against pathogenic infection (Zhang *et al*., 2019).

Partida-Martínez and Heil (2011) observed that when a plant is supplemented with a beneficial microorganism, the benefit can only be seen in the presence of biotic or abiotic stresses. In the absence of the stressor, the other beneficial microorganism generally has a detrimental impact on the plant in terms of the loss of nutrients. The plant therefore tries to control the growth of the microorganism, which could ultimately cause a decline in the plant's growth, as seen in (Supplementary Figure 4.1 A) where the length of maize roots was slightly decreased when primed with B3. However, when an external negative factor is present, the supplemented

microorganism supplies the plant with the ability to cope (Figure 4.2 A and B), and therefore the detriment between harbouring the microorganism and reducing the negative effect of the stress should result in no differences in the plants performance. This response was seen by Kabaluk and Ericsson (2007), where the biocontrol activity of entomopathogenic fungi against wireworm pests had a positive impact on maize plant growth.

Additionally, Cosme *et al*. (2016) showed that there were no variances between rice plants inoculated with fungal endophytes with or without larvae and endophyte-free plants without root feeding larvae. Therefore, the decline in plant growth promotion in the absence of stressors is not a lack of efficiency of the endophytic treatment. A similar response can be seen in Supplementary Figure 5.1; Supplementary Table 5.2 where pathogenic-defence proteins were present in seeds that were primed with *P. griseofulvum*. The aforementioned pathogen-defence proteins assisted the plant in the recuperation of its growth in the seeds primed with *P. griseofulvum* prior to infection with *F. proliferatum* relative to the seeds infected with *F. proliferatum* (Figure 4.2 A and B).

Chapter 6

Conclusion and Future Work

Fusarium proliferatum is a widely distributed fungal pathogen which produces mycotoxins responsible for the infection of economically important crops such as maize. Conventional methods to eradicate fungal infections using commercial fungicides are harmful to the environment and the health of humans and animals. An eco-friendly approach is the biological control of *F. proliferatum* which has a less detrimental impact on the sustainability of the environment and animal and human health. The two chosen endophytes showing the greatest antagonistic effect in the *in vitro* assay, had controlled the growth of *F. proliferatum* after priming and subsequent infection and had improved the growth of root length. The endophytic fungi used in this study possess antagonistic effects on the growth of *F. proliferatum* in the *in vitro* and *in planta* assays and can therefore be concluded to be good candidates for the biological control of this disease-causing pathogen.

Taken together, the accumulation signalling, anti-pathogenic and stress-related proteins correlated to the infection of *F. proliferatum* and priming with the fungal endophyte B3 which suggests that the maize-seeds oxidative and defence response is active. The proteomics analysis revealed that there was an absence of antioxidant enzymes in seeds infected with *F. proliferatum* relative to the primed treatment which showed the presence of aldehyde dehydrogenase and superoxide dismutase. Additionally, it revealed that HSP's and ferritin proteins were present in the infected seeds. Moreover, there were defence-related proteins in seeds primed with B3 prior to infection with *F. proliferatum*, such as indole-3-acetaldehyde oxidase, bowman-birk type wound-induced proteinase inhibitor, isoflavone reductase homolog, non-specific lipid-transfer protein, and aquaporin PIP2-6. The results can be used to develop a maize cultivar that is resistant to infection by *F. proliferatum* for the increase in seed emergence as well as yield.

To our best knowledge, this is the first study investigating the response of germinating maize seeds to infection by the pathogenic fungus *F. proliferatum*, which can cause the decline in germination, as well as the biocontrol capabilities of *P. griseofulvum* and *P. expansum* against *F. proliferatum* which can assist the plants recuperation during infection. By conducting experiments on physiological, biochemical, and the presence of proteins it clearly indicated

that the maize seeds were infected by *F. proliferatum*, and the defence responses were triggered in the presence of the pathogen as well as the fungal endophytes.
An increase in the understanding of maize defence mechanisms and how they assist the plant in overcoming infection by *F. proliferatum* is essential for the development of commercial hybrids that possess an improved resistance to disease.

Future work could entail the gene knock out or overexpression of anti-pathogen proteins that were present when seed roots were primed with B3 prior to infection with *F. proliferatum*, to determine why the maize plant was producing these proteins and how it may impact yield in hybrid plants. The gene knock-out study will assist in understanding whether the plant will still survive when primed with *P. griseofulvum* (B3) prior to infection with *F. proliferatum* present and the gene overexpression studies will assist in understanding the genes and other proteins that are expressed in the absence of B3 in the presence of the pathogen.

Chapter 7

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Appendix

Table of Content

Chapter Three

Species identification and biocontrol potential of fungal endophytes against *Fusarium proliferatum*

3.1 Phylogenetics of isolated endophytic fungi

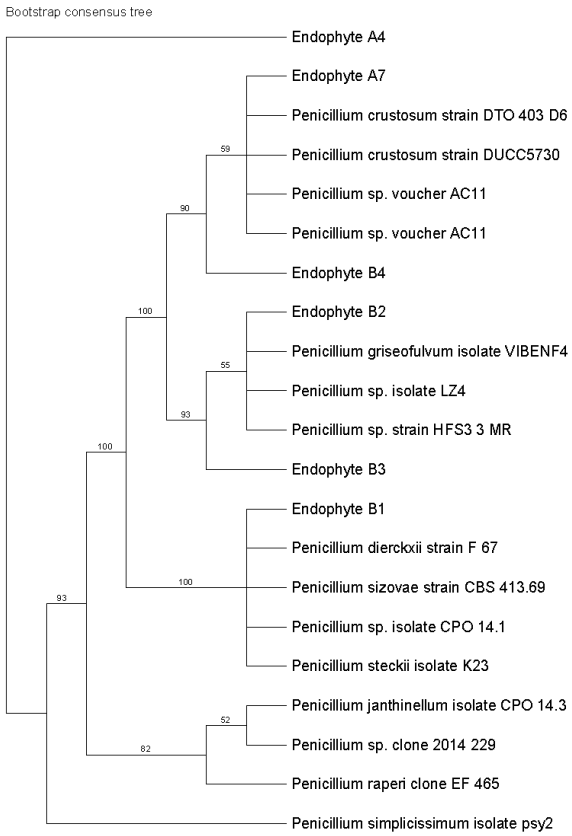


Figure 3.1 Phylogeny of identified *Penicillium* fungal endophytes obtained from bootstrap neighbour-joining analysis based on ITS sequence dataset from 21 BLAST hits. Distance based on bootstrap neighbour-joining where 538 characters were included, all characters had equal weight and the tree was unrooted.

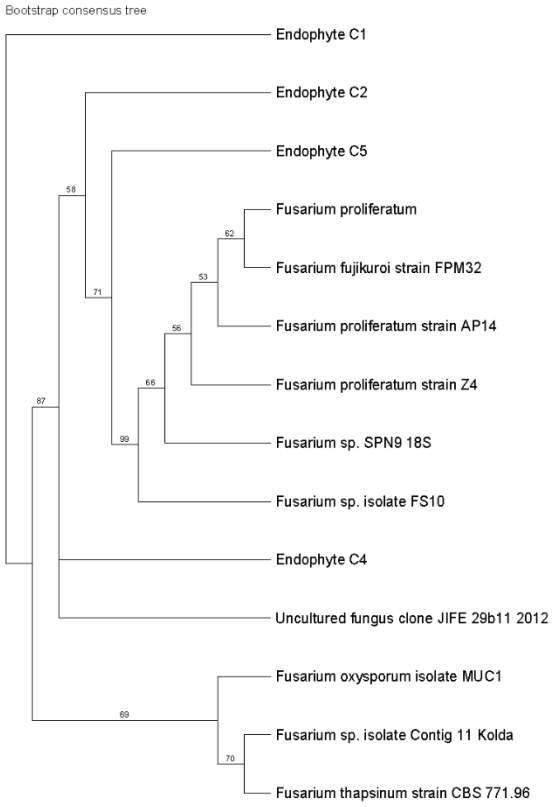


Figure 3.2 Phylogeny of identified Fusarium fungal endophytes and *Fusarium proliferatum* obtained from bootstrap neighbour-joining analysis based on ITS sequence dataset from 14 BLAST hits. Distance based on bootstrap neighbour-joining where 538 characters were included, all characters had equal weight and the tree was unrooted.

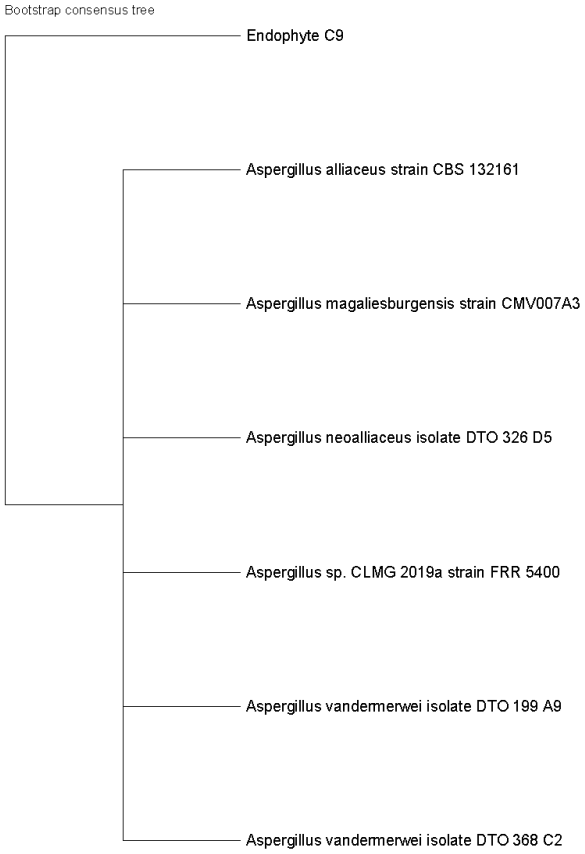
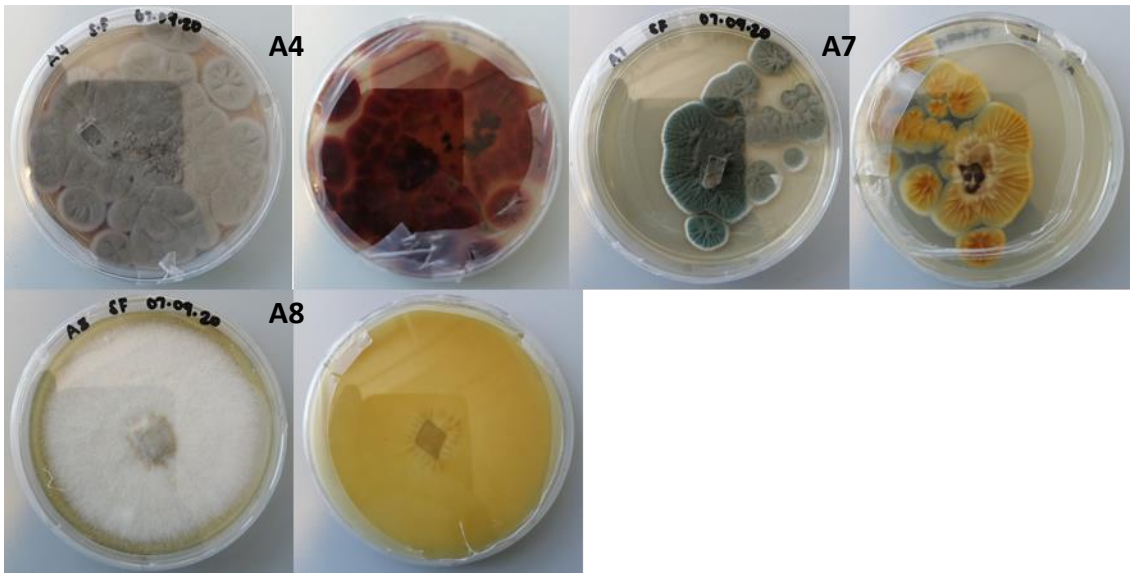


Figure 3.3 Phylogeny of identified *Fusarium* fungal endophytes and *Fusarium proliferatum* obtained from bootstrap neighbour-joining analysis based on ITS sequence dataset from 7 BLAST hits. Distance based on bootstrap neighbour-joining where 538 characters were included, all characters had equal weight and the tree was unrooted.

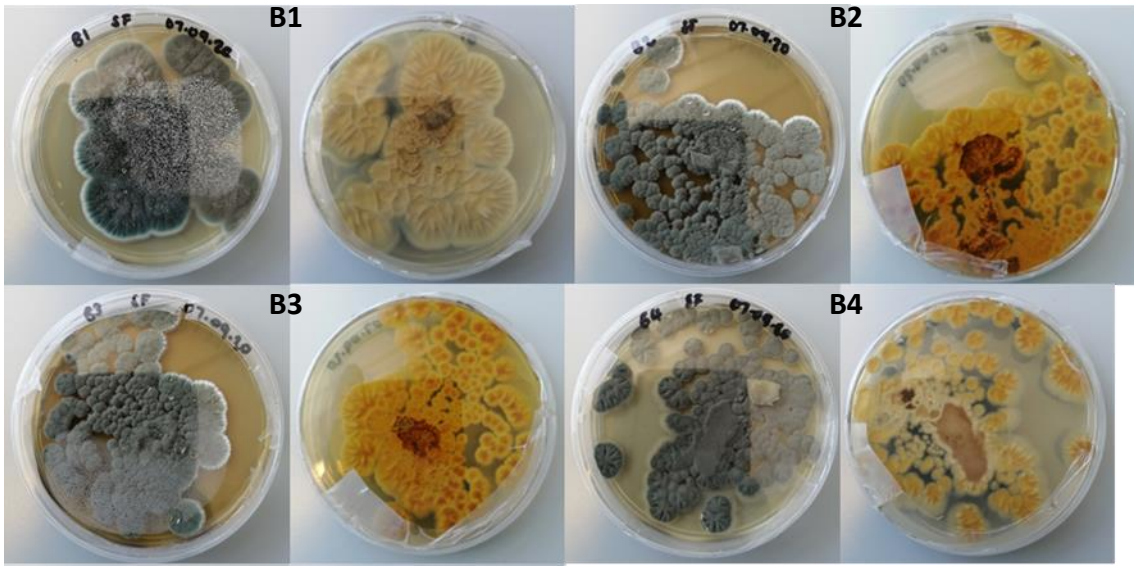
3.2.1 PURE CULTURES

3.2.1 Group A fungal endophytes



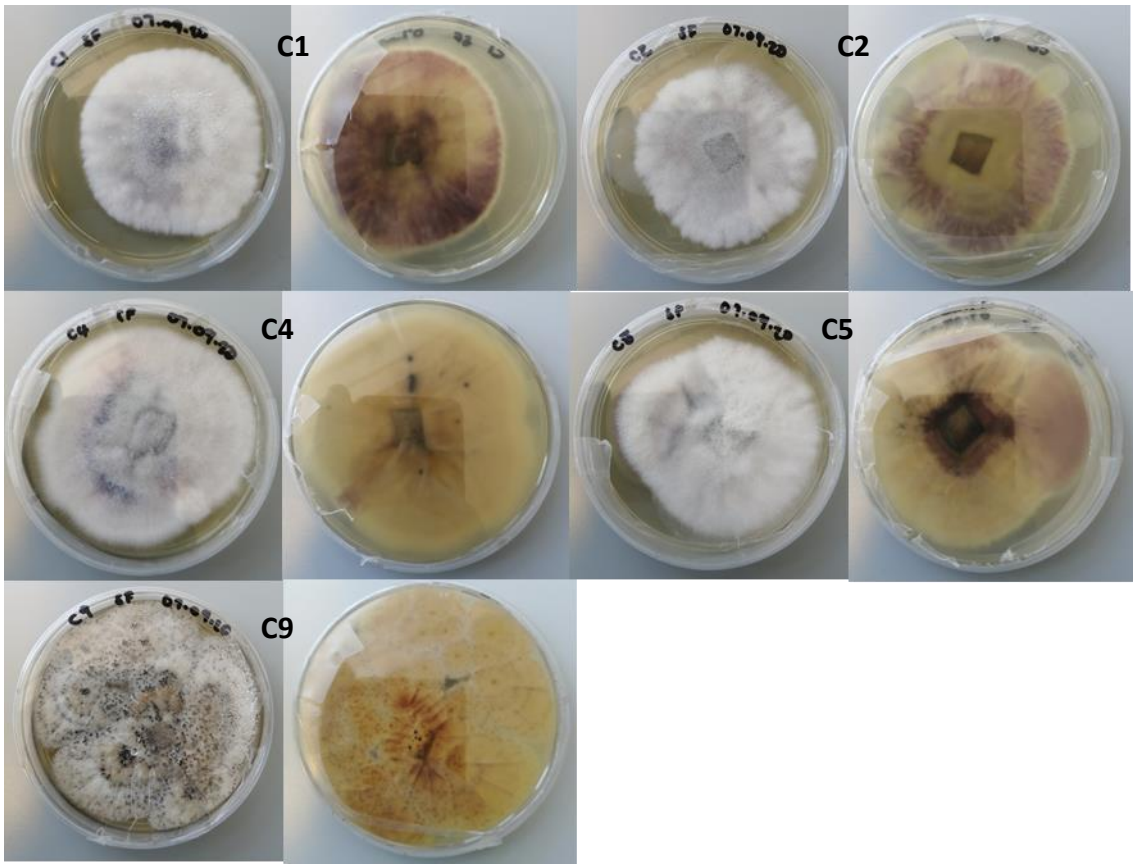
Supplementary Figure 3.4 Pure cultures of the group A isolated fungal endophytes used in *the in vitro* assay.

3.2.2 Group B fungal endophytes



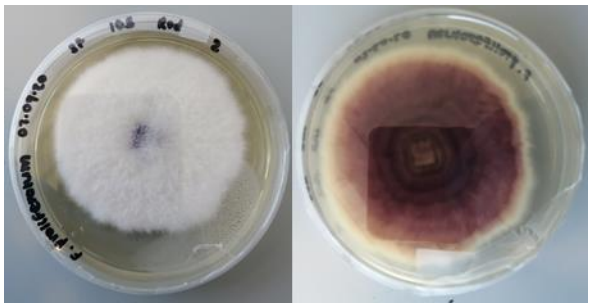
Supplementary Figure 3.5 Pure cultures of the group B isolated fungal endophytes used in *the in vitro* assay.

3.2.3 Group C fungal endophytes



Supplementary Figure 3.6 Pure cultures of the group C isolated fungal endophytes used in *the in vitro* assay.

3.2.4 *Fusarium proliferatum*

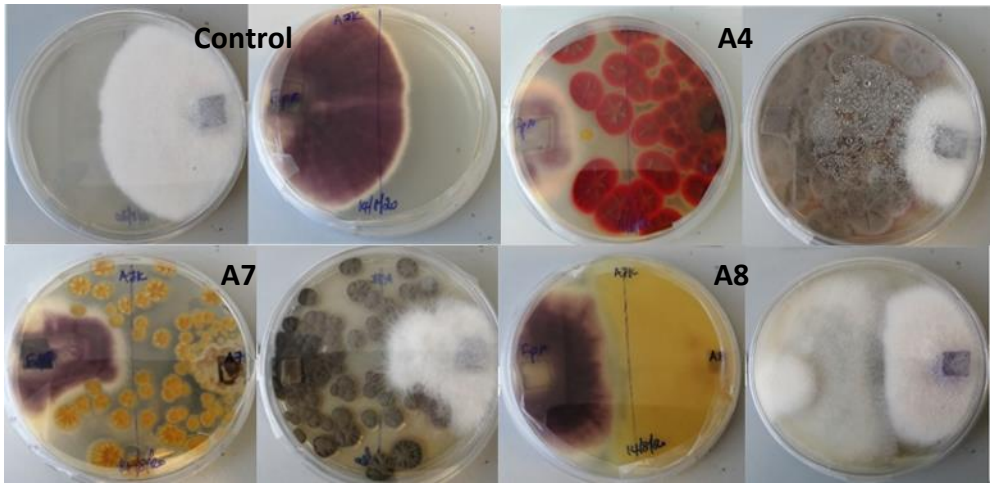


Supplementary Figure 3.7 Pure culture of *Fusarium proliferatum* used in *the in vitro* assay.

3.2.2 *In vitro* evaluation of antagonistic activity of endophytes against *F. proliferatum*

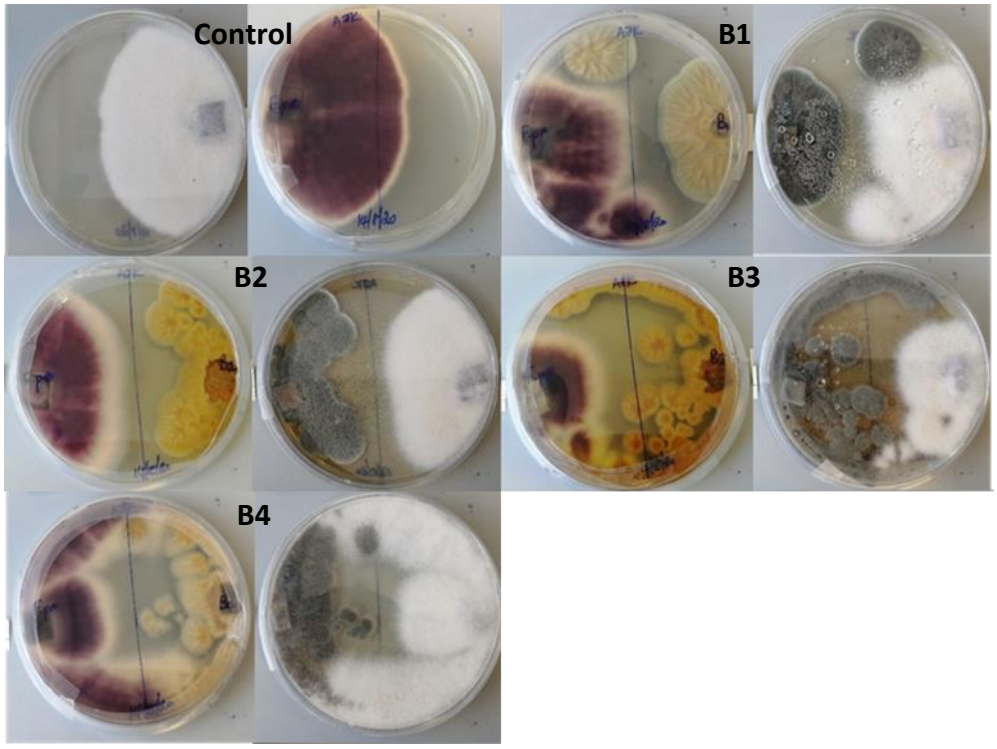
3.2.2.1 Dual-Culture Trial One

3.2.2.1.1 Group A fungal endophytes



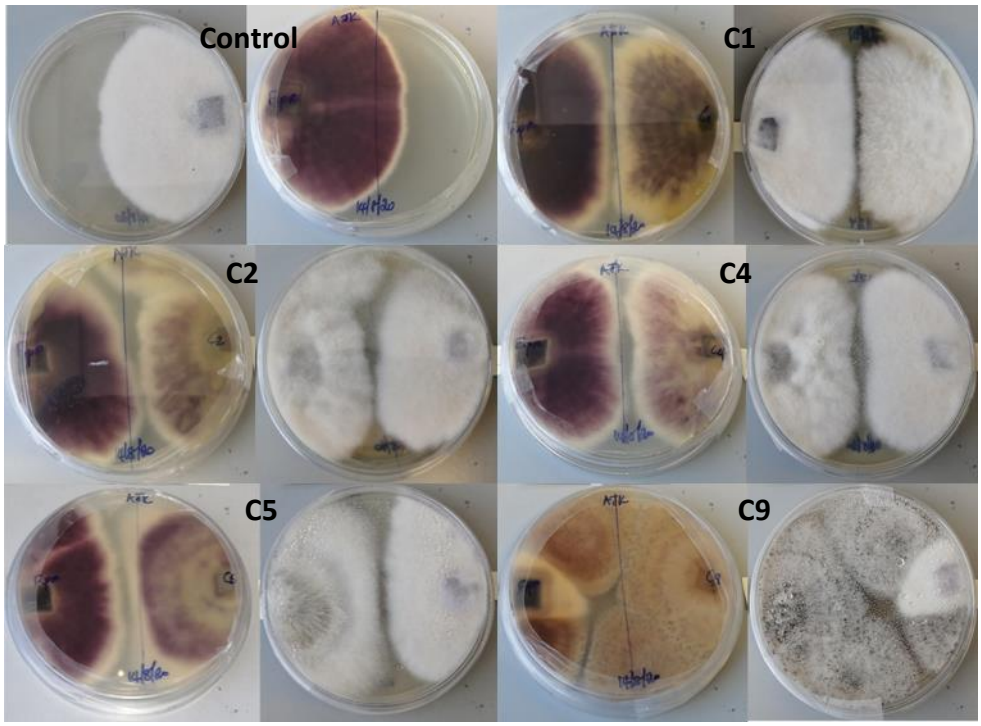
Supplementary Figure 3.8 Dual- cultures of the group A isolated fungal endophytes against the growth of *F. proliferatum* (trial one).

3.2.2.1.2 Group B fungal endophytes



Supplementary Figure 3.9 Dual- cultures of the group B isolated fungal endophytes against the growth of *F. proliferatum* (trial one).

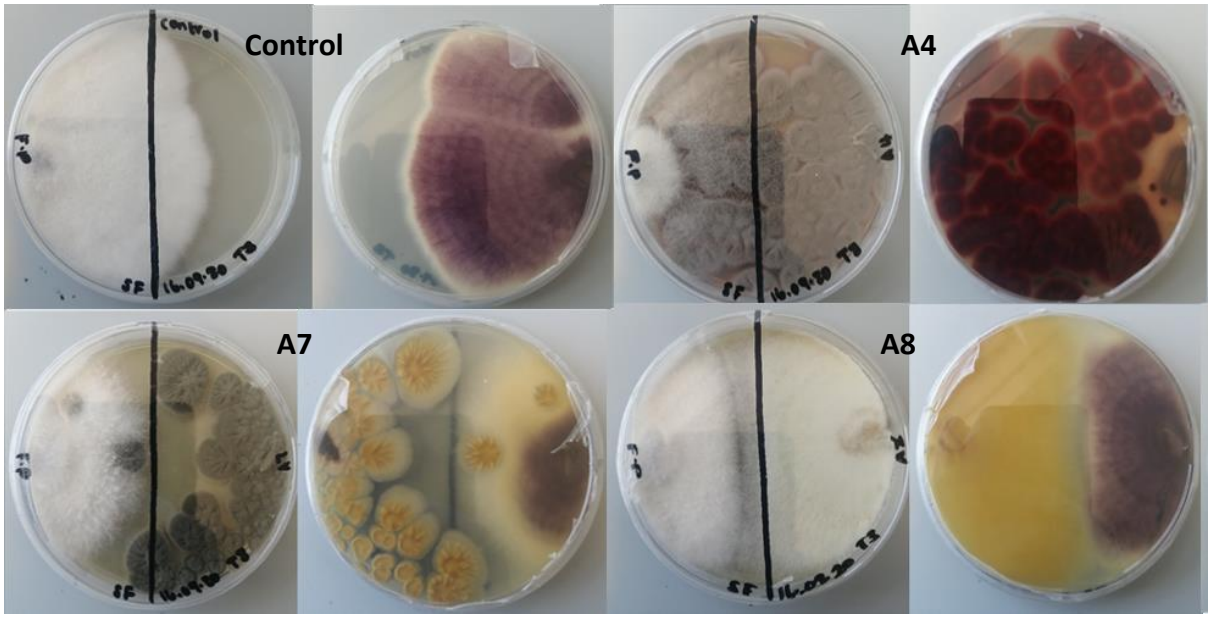
3.2.2.1.3 Group C fungal endophytes



Supplementary Figure 3.10 Dual- cultures of the group C isolated fungal endophytes against the growth of *F. proliferatum* (trial one).

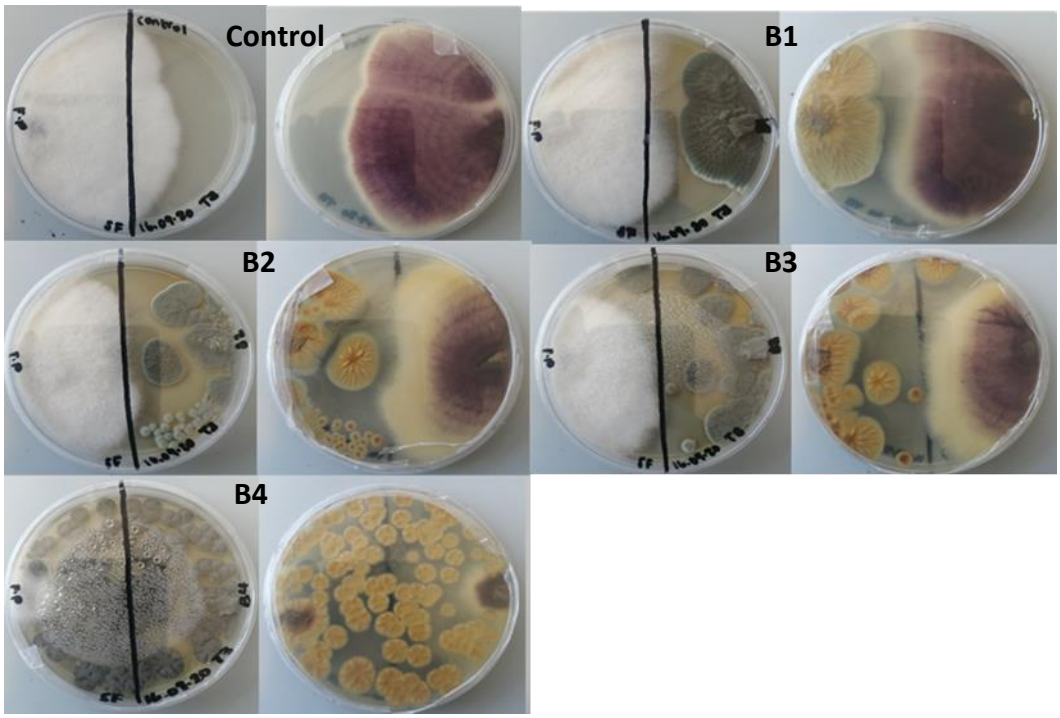
3.2.2.2 Dual-Culture Trial Three

3.2.2.2.1 Group A fungal endophytes



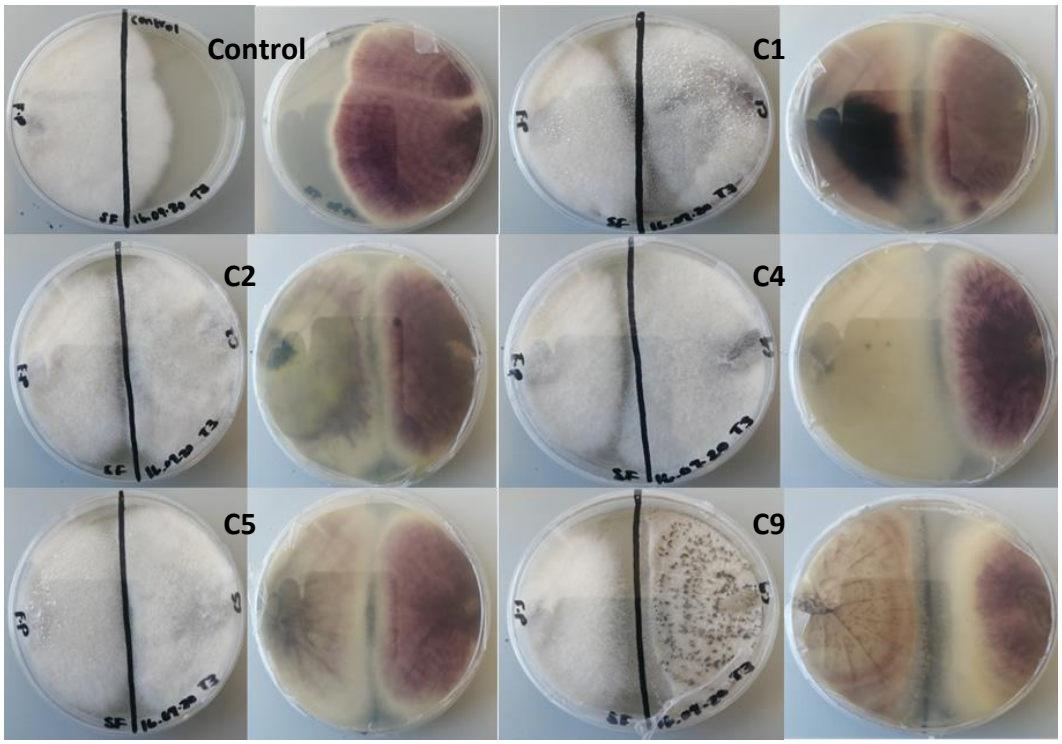
Supplementary Figure 3.11 Dual- cultures of the group A isolated fungal endophytes against the growth of *F. proliferatum* (trial three).

3.2.2.2.2 Group B fungal endophytes



Supplementary Figure 3.12 Dual- cultures of the group B isolated fungal endophytes against the growth of *F. proliferatum* (trial three).

3.2.2.2.3 Group C fungal endophytes



Supplementary Figure 3.13 Dual- cultures of the group C isolated fungal endophytes against the growth of *F. proliferatum* (trial three).

Supplementary Table 3.1: The percentage inhibition of the 12 fungal endophytes on the growth of *F. proliferatum* for three trials and the average percentage inhibition of the three trials, grown on PDA media for a period of 10 days.

Fungal isolate	Trial one (percentage inhibition) (%)	Trial two (percentage inhibition) (%)	Trial three (percentage inhibition) (%)	Average percentage inhibition (%)
Control	56,16	64,19	68,13	62,83
A4	70,73	74,42	90,82	78,65
A7	48,92	61,09	60,19	56,73
A8	42,11	55,59	56,99	51,57
B1	21,77	71,39	31,42	41,53
B2	31,55	79,99	43,55	51,70
B3	65,01	84,18	52,79	67,33
B4	40,49	88,63	94,10	74,41
C1	27,75	44,31	48,76	40,27
C2	26,11	44,98	48,85	39,98
C4	26,48	54,81	56,78	46,02
C5	29,40	46,37	46,15	40,64
C9	86,45	75,18	58,04	73,22

Chapter 4

Penicillium griseofulvum and *Penicillium expansum* modulates root growth and biochemical responses in maize under *Fusarium proliferatum* infection

4.2.2 Biopriming with *Penicillium griseofulvum* (B3) and *Penicillium expansum* (B4)

Supplementary Figure 4.1 Effect of B3 and B4 fungal isolate on root growth. (A) Root radical length of maize seeds primed with B3 fungal endophyte (10^8 cells/ml). (B) Root radical length of maize seeds primed with B4 fungal endophyte (10^8 cells/ml).

Chapter 5

Seed biopriming with *Penicillium griseofulvum* alters Maize root protein abundance under *Fusarium proliferatum* infection

5.3.1 Maize seed infection with *Fusarium proliferatum*

Figure 5.1 *Penicillium griseofulvum* (B3) **alters the presence of proteins in maize roots.**

Table 5.1 Unique proteins in maize seed roots primed with distilled water (control).

Protein names	Gene names	Mass	Entry name
Alcohol dehydrogenase 2	ADH2	41,054	ADH2_MAIZE
Ferritin-1, chloroplastic	FER1	28,025	FRI1_MAIZE
Luminal-binding protein 2	BIPE2	73,085	BIP2_MAIZE
Calreticulin	CRT	47,940	CALR_MAIZE
DIBOA-glucoside dioxygenase BX6	BX6	41,369	BX6_MAIZE
Arginine biosynthesis bifunctional protein ArgJ, chloroplastic		47,952	ARGJ_MAIZE
Chalcone--flavanone isomerase (Chalcone isomerase)	CHI CHI1	24,250	CFI_MAIZE
HMG-Y-related protein A	HMGIY2 GRMZM2G106133 Zm.66288	19,825	HMGYA_MAIZE
Protein translation factor SUI1 homolog (Protein GOS2)	TIF	12,704	SUI1_MAIZE
Proliferating cell nuclear antigen (PCNA)	PCNA	29,343	PCNA_MAIZE
Glutamine synthetase root isozyme 1	GLN6 GS1-1	39,250	GLNA1_MAIZE
14 kDa zinc-binding protein	ZBP14 PKCI	14,301	ZB14_MAIZE
Soluble inorganic pyrophosphatase	IPP	24,370	IPYR_MAIZE
Thiamine thiazole synthase 2, chloroplastic	THI1-2	37,234	THI42_MAIZE

Inositol-tetrakisphosphate 1-kinase 1	ITPK1 LPA2	37,313	ITPK1_MAIZE
Superoxide dismutase [Mn] 3.4, mitochondrial	SODA.3 SOD3.4	25,239	SODM4_MAIZE
Cysteine proteinase 1	CCP1	40,347	CYSP1_MAIZE
Cyanate hydratase (Cyanase)	CYN	18,526	CYNS_MAIZE
Chaperonin CPN60-2, mitochondrial (HSP60-2)	CPN60II CPNB	60,935	CH62_MAIZE
Peroxidase 2	PER2 POX2 PRX2	35,750	PER2_MAIZE
Indole-3-acetaldehyde oxidase (IAA oxidase)	AO1	146,683	ALDO1_MAIZE

Table 5.2 Unique proteins in maize seed roots primed with *P. griseofulvum*.

Protein names	Gene names	Mass	Entry name
1-acyl-sn-glycerol-3-phosphate acyltransferase PLS1	PLS1	42,571	LPAT_MAIZE
Indole-3-acetaldehyde oxidase (IAA oxidase)	AO2	145,176	ALDO2_MAIZE
60S ribosomal protein L19 (Fragment)	RPL19	7,176	RL19_MAIZE
Isoflavone reductase homolog IRL	IRL	32,852	IFRH_MAIZE
Aquaporin PIP2-6 (Plasma membrane intrinsic protein 2-6) (ZmPIP2-6) (ZmPIP2;6)	PIP2-6	30,191	PIP26_MAIZE
DNA-binding protein MNB1B	MNB1B	17,146	MNB1B_MAIZE
Acetolactate synthase 1, chloroplastic	ALS1 AHAS108	68,930	ILVB1_MAIZE
Tubulin alpha-5 chain (Alpha-5-tubulin)	TUBA5 TUA5	49,625	TBA5_MAIZE
Endochitinase A (EC 3.2.1.14) (ChitA)	CHIA	29,125	CHIA_MAIZE
60S acidic ribosomal protein P3 (P1/P2-like) (P3A)	RPP3A	12,219	RLA3_MAIZE
Bowman-Birk type wound-induced proteinase inhibitor WIP1	WIP1	10,976	IBBWP_MAIZE
Non-specific lipid-transfer protein (LTP)		11,705	NLTP_MAIZE
Glutamine synthetase root isozyme 5	GS1-5	39,259	GLNA5_MAIZE
Protein MATERNALLY EXPRESSED GENE 5	MEG5	17,814	MEG5_MAIZE
Aquaporin TIP2-1 (Tonoplast intrinsic protein 2-1)	TIP2-1 TIP2A	24,870	TIP21_MAIZE
Histone H2B.2		16,174	H2B2_MAIZE
Histone H2B.1		16,420	H2B1_MAIZE

Superoxide dismutase [Cu-Zn] 4AP	SODCC.2 SOD4AP	15,071	SODC5_MAIZE
Aldehyde dehydrogenase family 2 member B4, mitochondrial (ALDH2a)	ALDH2B4 ALDH2 At3g48000 T17F15.130	58,589	AL2B4_ARATH
Glutamine synthetase root isozyme 4	GLN5 GS1-4	38,981	GLNA4_MAIZE

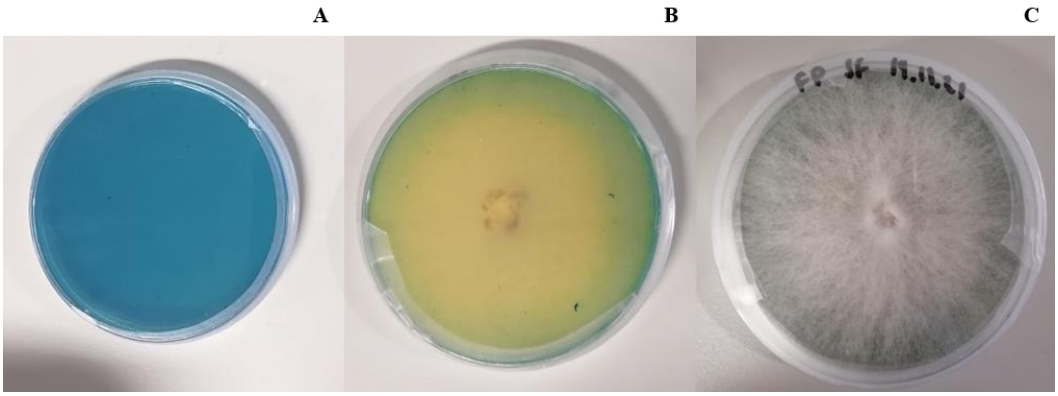


Figure 5.3 The growth of *Fusarium proliferatum* on siderophore media. (A) Control siderophore plate with no growth. (B) Bottom of siderophore plate showing the growth of *F. proliferatum*. (C) Top of siderophore plate showing the growth of *F. proliferatum.* The culture was grown for 7-10 days.