Whole genome sequencing and the biocontrol potential of *Penicillium simplicissimum* A4 against *Fusarium proliferatum* in maize

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3266314

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

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2025

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KEYWORDS

Whole genome sequencing and the biocontrol potential of *Penicillium simplicissimum* A4 against *Fusarium proliferatum* in maize

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1D SDS PAGE
Antioxidant enzymes
Ascorbate peroxidase
Biological control
Biological control agent
Chitin
Endo- β-1,4-glucanase activity
Exo-β-1,4-glucanase activity
Fungal endophyte
Fungal endophyte
Fungal pathogen
Hydrogen peroxide
Intracellular lipase activity
ITS sequencing
LC-MS
Lipid peroxidation
Maize
Maize
Next generation sequencing
Oxidative stress
Peroxidase
Plant proteomics
Polysaccharide
Protein identification

Reactive oxygen species

Superoxide

Superoxide dismutase

Superoxide dismutase

Untargeted metabolome sequencing

Whole genome sequencing

ABSTRACT

Whole genome sequencing and the biocontrol potential of *Penicillium simplicissimum* A4 against *Fusarium proliferatum* in maize

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Crop diseases caused by fungal pathogens pose a significant threat to global food safety and security, particularly in staple crops such as maize. Among these pathogens, *Fusarium proliferatum* causes substantial yield losses and produce harmful mycotoxins. While chemical fungicides are effective, their extensive use negatively affect the environment and can lead to fungicide resistance. Biological control agents (BCAs) offer an eco-friendly alternative, using the natural antagonistic properties of microorganisms. Therefore, this study investigated the biocontrol potential of *Penicillium simplicissimum* A4 against *F. proliferatum* using a combination of genomic, metabolomic, and proteomic approaches. *P. simplicissimum* A4 was identified using morphological and phylogenetic analysis, and its genome was sequenced with SMRT technology from PacBio. Whole genome sequence analysis revealed a genome size of 39 Mb, with 4.3 % dedicated to biosynthetic gene clusters (BGCs). Among the 38 identified BGCs, 60.53 % were novel, while 39.47 % aligned to known clusters, with 33.33 % showing more than 80 % similarity to established clusters. In addition, the *P. simplicissimum* A4 genome was translated to 2615 proteins, with 0.42 % linked to biocontrol of fungal pathogens. Among these biocontrol-related proteins, 36.37 % were associated with mycoparasitism, while 63.64 % were involved in detoxification processes. Metabolomic profiling of *P. simplicissimum* A4 revealed the presence of 5497 secondary metabolites with 0.4 % of the metabolites associated with antifungal activity against phytopathogens. *In vitro* antagonistic activity of *P. simplicissimum* A4 showed 78.65 % inhibition of *F. proliferatum*. Polysaccharide and chitin contents, hydrolytic enzymes, reactive oxygen species accumulation and antioxidant activity of *F. proliferatum* were altered in the presence of *P. simplicissimum* A4. *In planta* assay of infected maize roots bio-primed with *P. simplicissimum* A4 shows changes in the physiological and biochemical markers. Lastly, proteomic analysis revealed significant changes in maize root protein profiles under different treatment conditions. A total

of 126 proteins were identified in both the control and infected roots, while 111 proteins were identified in bio-primed roots. The unique proteins in infected roots were predominantly associated with stress responses and fungal infection regulation, whereas bio-primed roots showed proteins linked to antioxidant activity and enhanced defence mechanisms. These proteins included, superoxide dismutase associated with antioxidant activity and aquaporin channel proteins, non-specific phospholipid-transfer proteins and wound-induced bowman-birk inhibitor (BBI) proteins associated with defence. These proteomic changes highlight the role of *P. simplicissimum* A4 in strengthening maize root resilience and defence against *F. proliferatum*. The results from this study suggest that *P. simplicissimum* A4 employs a multifaceted antagonistic mechanism against *F. proliferatum*, involving cell wall degradation, enzyme inhibition, and oxidative damage, establishing its potential as an effective biocontrol agent in maize agriculture. This research has shed light on new possibilities in the discipline of agriculture as well as mycology and delivers a significant basis for further investigation into biochemical and molecular biomarkers for genetic improvement of pathogen tolerance in maize plants.

DEDICATION

Whole genome sequencing and the biocontrol potential of *Penicillium simplicissimum* A4 against *Fusarium proliferatum* in maize

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I dedicate this dissertation to the three strongest women I have ever known, Charmaine Brandt, Margeret Ruth Brandt, and Millicent Brandt. You have taught me to be resilient in every situation life throws at me.

ACKNOWLEDGEMENTS

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Firstly, I thank my Heavenly Father, for without Him I am nothing.

I would like to thank my supervisor, Prof. Klein and my co-supervisors, Prof. Keyster and Dr. Gokul for their help and patience. You have assisted me through every single high and low with guidance, a smile and a kind word.

A special thanks to Dr. Augustine Daniel and Dr. Husselmann, for your consistent support; and all the students in the Plant Omics Laboratory for their patience, inspiration and support throughout the duration of my project; I would like to extend my gratitude.

Thank you to my mother, Charmaine Brandt for your sacrifices and love, and to my grandmother Margaret Brandt and aunt, Millicent Brandt for your love – I will always miss you.

A special thank you to Adam Frank Burt for his support, patience, guidance, and love. Your joyfulness and encouragement have brought me through the toughest of times; and for that I will always be eternally grateful.

To my family and friends, Nadia Kolbe, Anton Kolbe, Adrian Fisher, Roxanne Walters, Adele Barker, aunty Tania, Uncle Pierre, and Kamva, I appreciate your constant prayers and support.

Additionally, I would like to thank Maize Trust and NRF for financially assisting in the completion of my project.

Lastly, I would like to thank Agricol for their donation of Maize seeds for the completion of my project.

LIST OF ABBREVIATIONS

1D-SDS PAGE	One-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis
$_1O^2$	Singlet oxygen
2-DE	Two-dimensional electrophoresis
ABA	Abscisic acid
ABC transporter	ATP binding cassette transporter proteins
ACC-deaminase	1-aminocyclopropane-1-carboxylate-deaminase
ANOVA	One-way analysis of variance
APS	Ammonium persulfate
APX	Ascorbate peroxidase
AQP	Aquaporin
ASA	Ascorbic acid
BBI	Bowman-birk inhibitor
BCA	Biological control agents
BGC	Biosynthetic gene clusters
CAT	Catalase
CCV	Clatherin-coated vesicles
CDIPs	Cell death-inducing proteins
CMC	Carboxy methyl cellulase
CME	Clathrin-mediated endocytosis
COS	Choline-*O*-sulfate
CuAO	Copper amine oxidase

CW	Cell wall
CWDE	Cell wall degrading enzyme
DGE	Differential gene expression
dH2O	Distilled water
DHN	Dehydrins
DNS	di-nitro salicylic acid
EDTA	Ethylenediaminetetraacetic acid
EFT’s	Translational elongation factors
ER	Endoplasmic reticulum
ETP	Epipolythiodiketopiperazine
F. proliferatum	*Fusarium proliferatum*
FCW	Fungal cell wall
GA	Gibberellin
GB	Glycine betaine
GM	Glutamate metabolism
GPOX	Guaiacol peroxidase
GPX	Glutathione peroxidase
GS	Glutamine synthetase
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HSP	Heat shock protein
HSPs	Heat shock proteins
IAA	Indole-3-acetic acid
ITS	Internal transcribed spacer

KCN	Potassium cyanide
KI	Potassium iodide
LC-MS	Liquid chromatography mass spectrometry
LC-QTOF-MS/MS	Liquid chromatography-quadrupole time-of-flight tandem mass spectrometry
LPAAT	Lysophosphatidic acid acyltransferase
MDA	Malondialdehyde
N	Nitrogen
NaCl	Sodium chloride
NADPH	Nicotinamide adenosine dinucleotide phosphate
NaOH	Sodium hydroxide
NBT	Nitrotetrazolium blue chloride
NGS	Next-generation sequencing
NRPs	Nonribosomal peptides
NRPS-terpenoids	Non-ribosomal peptide synthetase-terpenoids
ns-LTP	Non-specific lipid-transfer protein
nsLTP's	Non-specific phospholipid-transfer proteins
O_2^-	Superoxide radical
O_2	Oxygen
OH•	Hydroxyl radical
P. simplicissimum A4	*Penicillium simplicissimum* A4
PCD	Programmed cell death

PDA	Potato dextrose agar
PDB	Potato dextrose broth
PDR	Pleiotropic drug resistance
PIP	Plasma membrane intrinsic protein
PKs	Polyketides
PLTP	Phospholipid-transfer proteins
POD	Peroxidase
PRR	Pattern recognition receptor
PRR’s	Pattern recognition receptor
PVP	Polyvinylpyrrolidone
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SM	Secondary metabolite
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TC	Terpene cyclase
TCA	Trichloroacetic acid
TEMED	N,N,N’,N’ tetramethylethylenediamine
TF	Transcription factor
TFA	Trifluoroacetic acid
TIP	Tonoplast intrinsic protein
Trr	Thioredoxin reductases
Trxs	Thioredoxin
Tsa	Thiol-specific antioxidant protein

GENERAL INTRODUCTION

Background of the study

Fusarium proliferatum is a filamentous pathogenic fungus distributed in soil and plants worldwide. *F. proliferatum* produces various mycotoxins, such as fumonisins and moniliformins (Vismer et al., 2019) and has been associated with various diseases in economically important crops, including tomato and rice (Gao et al., 2017; Prabhukarthikeyan et al., 2021). In addition, *F. proliferatum* is the causative agent of various plant diseases including root and sheath rot (Okello et al., 2019; Prabhukarthikeyan et al., 2021). Each year, approximately 25 % of maize crops are contaminated with mycotoxins, leading to major economic losses in both the agricultural and industrial sectors (Kamle et al., 2019). Maize (*Zea mays L.*) has been established as the most produced cereal worldwide (Andorf et al., 2019; Du Plessis, 2003). In Africa maize is the primary staple food for approximately 300 million people (Santpoort, 2020). Maize is generally grown for human consumption, and as such, the requirement for maize is intimately associated with the increase in population (Santpoort, 2020).

Maize production is constantly impacted by various biotic and abiotic stresses (Vaughan et al., 2018). Fungal and bacterial pathogens are some of the main biotic stresses facing global crop production and accounts for about 20-40 % crop losses annually. Fungal pathogens account for approximately 80 % of the 20-40 % loss which contributes to high global food insecurity (Daniel et al., 2023; Kim et al., 2019). One of the main fungal groups associated with maize disease belong to the *Fusarium* genus (Santos et al., 2021).

Among the numerous methods available to address plant diseases, the utilization of synthetic chemical fungicides for the control of fungal pathogens continues to be the most effective and economically viable strategy (Pandey et al., 2017). However, the continuous use of these chemical fungicides has detrimental effects on the environment as well as human and animal health (Mosquera-Sánchez et al., 2020). Biological control agents (BCAs), such as fungal endophytes, is an alternative to the use of chemical fungicides. Endophytic fungi can inhabit the internal tissues of host plants where they reside in the intracellular spaces without inducing any disease symptoms (Hutauruk & Pinem, 2020). The use of fungal endophytes are an eco-friendlier method to inhibit the growth of fungal pathogens in economically important crops (Alghuthaymi et al., 2022).

Statement of research problem

Fusarium spp. are significant plant pathogens that affect a variety of hosts, which is suggestive of its astonishing ability to adapt to various climate conditions and nutrient circumstances, to negatively impact food safety (Jian et al., 2019). *Fusarium proliferatum* is a filamentous fungal pathogen capable of infecting numerous host plants, such as rice (Prabhukarthikeyan et al., 2021). Although the use of chemical fungicides is beneficial for the regulation of fungal infection, it poses major risks on the ecosystem (Zhang et al., 2023). In addition to eradicating fungal pathogens, it is also toxic to various other organisms, such as non-target plants, beneficial insects, pets, fish, and birds (Araújo et al., 2023; Naz et al., 2023). Moreover, they have detrimental effects on terrestrial and marine ecosystems including plant and animal biodiversity (Puglisi, 2012; Van Zwieten et al., 2007). Lastly, several fungal species have developed resistance to chemical fungicides which has resulted in their ineffectiveness (Lucas et al., 2015).

Justification of the study

Although chemical fungicides are effective in controlling fungal pathogens, their use has been associated with various detrimental effects (Araújo et al., 2023; Lucas et al., 2015; Naz et al., 2023). Therefore, a more efficient and eco-friendly approach would be to incorporate BCAs, such as fungal endophytes, to management of fungal disease-causing pathogens (Kaur, 2020). BCAs offer a sustainable alternative to chemical fungicides for plant disease control (Palmieri et al., 2022; Tariq et al., 2020). In particular, fungal endophytes belonging to the genus *Penicillium* have been isolated and identified for their potential to inhibit the growth of fungal plant pathogens (Kaur & Saxena, 2023; Oses-Pedraza et al., 2020; Urooj et al., 2018).

Aim and objectives

Aim of the study

The study aimed to evaluate the biological control potential of *Penicillium simplicissimum* A4 against *Fusarium proliferatum*.

Objectives of the study

- To identify and classify *P. simplicissimum* A4, isolated from *E. plantaginium*, using ITS sequencing and hyphal morphology.

- To sequence the whole genome of *P. simplicissimum* A4 to identify genes associated with fungal biocontrol and biosynthetic gene clusters (BGCs).
- To analyse the metabolome of *P. simplicissimum* A4 via LC-QTOF-MS/MS, with emphasis on compounds relevant to fungal pathogen control.
- To assess the antagonistic activity of *P. simplicissimum* A4 against *F. proliferatum* (PPRI 31301) using dual culture assays.
- To evaluate changes in polysaccharide and chitin content of *F. proliferatum* in response to *P. simplicissimum* A4.
- To investigate enzymatic and biochemical responses of *F. proliferatum* upon interaction with *P. simplicissimum* A4.
- To determine the impact of *F. proliferatum* infection on maize root growth and biochemical responses.
- To assess the ability of *P. simplicissimum* A4 bio-priming to mitigate the physiological and biochemical effects of *F. proliferatum* in maize roots.
- To compare protein expression in maize roots infected with *F. proliferatum* with and without prior bio-priming using *P. simplicissimum* A4.

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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Recently, there is an increasing concern in the scientific community regarding the effect of pollutants released into the environment via various human activities such as industrial, agricultural, and mining activities (Matúš et al., 2023). Contamination of soil and water is a major concern for ecological balance (Gouma et al., 2019). Agricultural practices that involve the application of pesticides is one of the major anthropogenic activities contributing to soil pollution (Matúš et al., 2023; Oliveira et al., 2015). Fungicides are chemical or biological compounds used to defend agricultural products from pathogenic fungi or fungal spores (Dias, 2012). The continued unrestrained use of fungicides has serious impacts on human and animal health as well as the environment (Kara et al., 2020). Due to the effectiveness, straightforward methods of usage and low cost, fungicides have become the primary means to control fungi (Dias, 2012). However, the widespread and misuse of these chemical fungicides have caused fungal strains to become resistant to these commercial products (Abd-Elsalam et al., 2023; 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 including humans, plants, animals, aquatic biota, pollinators and beneficial organisms (Gupta, 2022; Hashimi et al., 2020; Zubrod et al., 2019). Various types of fungicides utilize different molecular pathways to exert neurotoxic action on humans and animals (Kara et al., 2020).

The use of biological control agents (BCAs) as a substitution for synthetic chemical fungicides is being increasingly developed with the increase in recognition of the detrimental impacts of synthetic fungicides. BCAs possess numerous advantages, which includes the lack of detrimental effects on the environment, controlling plant diseases, their ability to synthesize compounds that are beneficial to host plants, and its effectiveness during the host plants' life span (Amiri & Tibuhwa, 2021; Figueiredo et al., 2016; Marwah et al., 2007; Silva et al., 2004; Zhang & Yang, 2007).

The aim of this literature review was to investigate the impact of *F. proliferatum* on maize growth and investigate the application of fungal endophytes of the *Penicillium* genus and their roles in the biological control of plant pathogenic fungi reported from 2017 to 2024.

1.2 Maize production

Maize (*Zea mays L.*) has been one of the most important crops worldwide for many decades (Andorf et al., 2019; Du Plessis, 2003). It is an important grain crop in South Africa where it is grown all over the country in various environments (Andorf et al., 2019; Du Plessis, 2003). Maize has the biggest production and it covers the greatest cultivated area (Otegui et al., 2021). It is a significant crop in that it is used as an industrial source, animal feed as well as for human consumption (Shin et al., 2014).

Maize plants can be classified based on their shape and size of kernel, colour and taste (Otegui et al., 2021). The two main kernel shapes are round shaped which are flint maize and tooth shaped which are dent maize (Ranum et al., 2014). Moreover, the main colours are yellow, white, and red, however, various other colours can be produced such as red-brown, black, orange, light red, and pale yellow (Ranum et al., 2014).

For the successful production of maize to occur, numerous inputs are required, such as effective disease and insect control, plant population, adapted cultivars, financial capital, harvesting methods, fertilisation, and soil cultivation (Du Plessis, 2003).

1.3 Maize seed germination

Seed germination is an important phase in the production of crops as it has a direct effect on grain quality and yield (Xue et al., 2021). Seed germination is a developmental process where the seed transitions from dormancy to an active metabolic state in which all seed reserves are utilized for the establishment of seedlings (Han et al., 2020). Germination involves three phases namely: (1) imbibition/priming; (2) lag phase; and (3) radicle emergence and growth (Figure 1.1) (Tian et al., 2014).

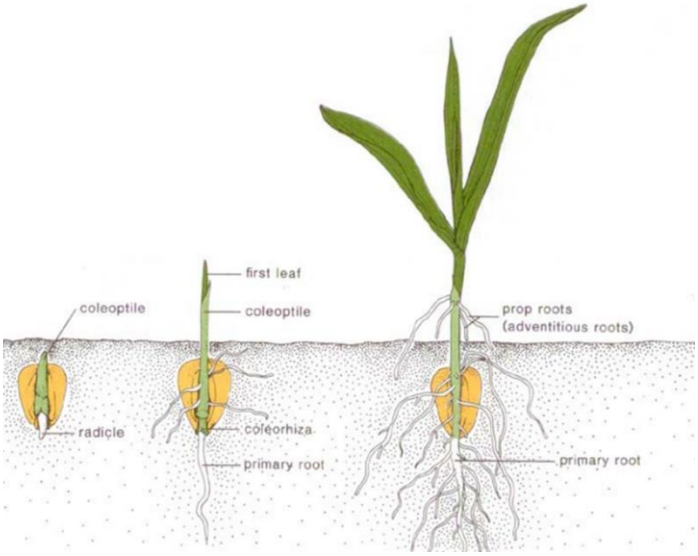


Figure 1.1 Maize seed germination and root emergence. Adapted from Maree (2008).

Phase 1 of seed germination, known as imbibition, starts with the uptake of water (Han et al., 2020; Xue et al., 2021). Phase 2, or the lag phase, is essential for maize kernel development as the endosperms capacity to accumulate dry matter, known as kernel sink capacity, is established in this phase (Xue et al., 2021). Phase 3, which involves the ultimate rupturing of the seed coat allows for radicle and plumule emergence (Figure 1.1; Figure 1.2) which in turn activates the internal physiology and respiration begins (Gallardo et al., 2001; Xue et al., 2021).

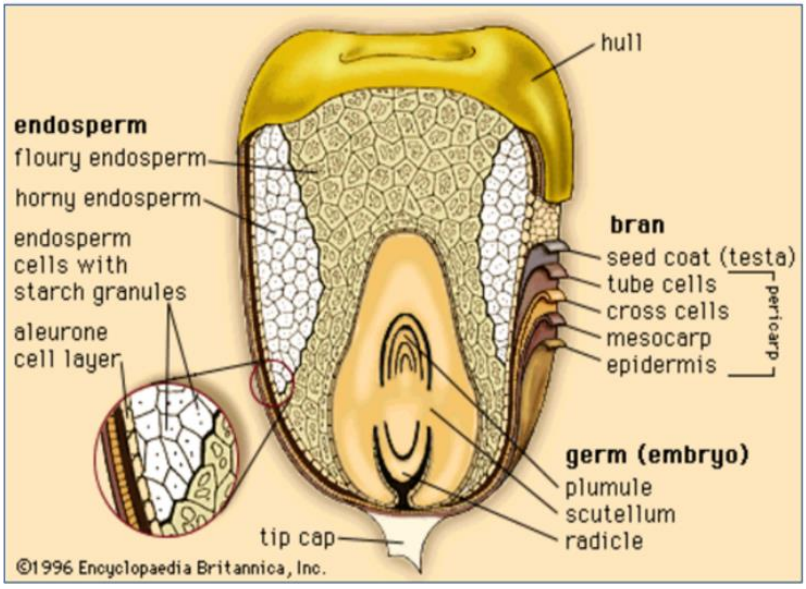


Figure 1.2 The anatomy of a maize seed. Adapted from Suleiman et al. (2013).

At the physiological and molecular level, seed germination involves hormones and transcription factors (TFs) to ensure a consistent signal interchange between the seed compartments (Figure 1.2) (Locascio et al., 2014). Germination performance and subsequent growth and crop yield is improved when concentrations of phytohormones such as abscisic acid (ABA), auxin, ethylene, and gibberellins (GA) are at an optimal level (Han et al., 2020).

Seeds can contain phytopathogenic fungi that can halter germination and development, which ultimately impacts the overall yield (Santos et al., 2021). Examples of these phytopathogens are from the genera *Fusarium, Aspergillus,* and *Rhizoctonia* which are known produces of mycotoxins which impacts maize seed quality and viability (Santos et al., 2021). These disease-causing phytopathogens can enter host plants via direct penetration using mechanical force and via indirect penetration though wounds or natural openings such as the stomata (Santos et al., 2021). Maize plants infected with these pathogens suffer from stunted growth, reduced ear size, and in harsh conditions, they may die due to an infected root system (ur Rehman et al., 2021).

1.4 *Fusarium* spp. adversely affects plant growth and development

The *Fusarium* genus belonging to the *Nectriaceae* family is one of the most significant plant-pathogenic fungi worldwide as it is found in soil, numerous host plants, and climatic zones worldwide (Crous et al., 2021; Summerell, 2019). *Fusarium* spp. are able to penetrate host plant tissues by inhabiting the rhizospheres of host plants (Kamle et al., 2019). Additionally, *Fusarium* spp. are human pathogens as they secrete mycotoxins in food products which are deleterious to human and animal health (Summerell, 2019).

1.4.1 The pathogenic fungus *Fusarium proliferatum*

F. proliferatum is a pathogenic filamentous ascomycete which is distributed worldwide and has been associated with various diseases of economically important crops such as maize, soybean, garlic, onion, banana, date palm, tomato, rice, and asparagus where it is the causative agent of numerous diseases such as stalk, root, ear, and sheath rot as well as seedling blight (Gao et al., 2017; Kamle et al., 2019; Okello et al., 2019; Prabhukarthikeyan et al., 2021; Sun et al., 2019; Sun et al., 2018a; Wang et al., 2021; Yang et al., 2020). *F. proliferatum* resides in soil and within host plants where it produces a great amount of micro-and-macro conidia which can survive for many years (Gao et al., 2017). At optimum temperatures and under moist conditions, the conidia germinate and spreads via rainwater and atmospheric dust where it subsequently infects seeds, soil, and plant tissue (Gao et al., 2017; Isack et al., 2014; Kamle et al., 2019).

Approximately 25 % of maize crops are infected with mycotoxins annually, which results in economic losses to the industrial and agricultural industries (Kamle et al., 2019). These mycotoxins are extremely detrimental to human and animal health and cannot be removed during processes such as cooking, baking, roasting, or pasteurization (Kamle et al., 2019).

1.4.2 Pathogenicity factors of *Fusarium* spp.

Plant pathogens produce different types of extracellular enzymes that enable them to infect/penetrate host tissue. *Fusarium* spp. produces various types of enzymes that degrade cell walls (CWs) and thus they are considered pathogenicity factors (Paccanaro et al., 2017; Sharafaddin et al., 2019; Yang et al., 2015). Due to the distribution and wide host range of *F. proliferatum*, it produces numerous cell wall degrading enzymes (CWDEs) which play important roles in the colonization of host plant tissues such as that of maize and wheat plants (Khaledi et al., 2017; Kikot et al., 2009; Marın et al., 1998; Paccanaro et al., 2017). The plant CW is the main barrier that protects host plants against fungal pathogens (Kikot et al., 2009). When fungal pathogens come into contact with the host plants, they encounter epicuticular waxes and cuticles encompassing the host epidermal cells (Kikot et al., 2009). *Fusarium* pathogens possess no specialized structures to penetrate host cells, instead it enters host plants via natural openings (Pritsch et al., 2000), or it directly penetrates epidermal CWs via the production of enzymes (Mary Wanjiru et al., 2002). Extracellular hydrolytic enzymes have been isolated from *Fusarium* spp. including that of *F. proliferatum* and *Fusarium oxysporum* (Chang et al., 2016; Dar et al., 2013; Kubicek et al., 2014; Saha, 2002). An example of these enzymes are glucanases and lipases which assists the fungal pathogens in the digestion of plant CW polymers in order to acquire nutrients as well as penetration for their ultimate spread via host tissue where glucanase degrades the polysaccharide glucan and lipase degrades the cuticle of plant host CWs (Kikot et al., 2009). Feng (2007); Feng et al. (2005) observed the importance of the lipase enzyme in the pathogenicity of *Fusarium graminearum*. Jenczmionka and Schäfer (2005) observed the regulation of endoglucanase of *F. graminearum*.

1.4.3 *Fusarium*-induced reactive oxygen species accumulation and antioxidant capacity

F. proliferatum produces numerous mycotoxins that results in the accumulation of reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (·OH), which eventually results in programmed cell death (PCD) (Gechev et al., 2004; Zhang et al., 2019b). Although ROS are produced during normal processes such development, growth, photosynthesis, and respiration, when accumulated they can be extremely detrimental

to the cell. Biotic stress such as infection with fungal pathogens can results in the drastic accumulation of ROS (Gechev et al., 2004; Zhang et al., 2019b).

ROS is scavenged by a number of non-enzymatic and enzymatic antioxidants which alters them into less-toxic byproducts (Pandey et al., 2017). Non-enzymatic antioxidants include ascorbic acid (ASA), phenolic compounds, glutathione, alkaloids, α-tocopherols, and non-proteinaceous amino acids, whereas enzymatic antioxidants include superoxide dismutase (SOD), ascorbate peroxide (APX), guaiacol peroxidase (GPOX), and peroxidase (POD) (Berwal & Ram, 2018). Under stressed conditions, the antioxidant enzymes are disrupted and thus the ROS cannot be detoxified which results in damage to cell macromolecules such as lipids, nucleic acids, and proteins (Pandey et al., 2017).

1.5 Strategies to control fungal plant pathogens

1.5.1 The use of chemical fungicides

Chemical fungicides are synthetic compounds that are used to protect important crops from being infected with fungal pathogens (Ul Haq et al., 2020). Due to its effectiveness, general low cost and ease of use, fungicides are the primary means to control pathogenic fungi in the agricultural industry (Ayesha et al., 2021). However, their continued usage has detrimental effects on the environment, as well as human and animal health (Kara et al., 2020), and has caused pathogenic strains to develop resistance (Ayesha et al., 2021). Commonly used chemical fungicides includes but are not limited to carboxamide, organophosphate, azole fungicides containing propiconazole, benzimidazole fungicides containing compounds such as benomyl and carbendazim, respectively, strobin fungicides containing azoxystrobin and trifloxystrobin, respectively, and triazole fungicides containing compounds such as penconazole, difenoconazole and myclobutanil (Cools et al., 2013; Quinn et al., 2011); Zubrod et al. (2019).

1.5.1.1 Impact of chemical fungicides on human and animal health

Livestock are poisoned by chemical fungicides that are applied to agricultural material which may contribute to neurological dysfunctions and neurotoxicity (Hasan, 2010). Although newly developed chemical fungicides have low-to-moderate toxicity, carcinogenic, teratogenic, reproductive, mutagenic, and organ toxicity may occur as a result of its continuous use (Gupta, 2018; Hansen et al., 2011; Rani et al., 2021). An example of a chemical fungicide is carbendazim, which has resulted in various toxic effects such as germ cells sloughing,

spermatogenic failure, liver function damage, reproductive toxicity, and down-regulated humoral immunity (Sharma et al., 2022; Singh et al., 2016b).

1.5.1.2 Impact of chemical fungicides on the environment

The contamination of surface water with chemical fungicides is dependent on numerous factors such as the distance to grassland, water bodies, and slope, climatic conditions, as well as the proximity of the crops to surface water (Tudi et al., 2021). The continuous use of chemical fungicides poses a threat to the environment in that it is persistent in soil or it enters waterways due to spray-drifts and run-offs (Syafriana et al., 2014; Zubrod et al., 2019). When this run-off occurs, it leads to antagonistic effects on the health of terrestrial and aquatic ecosystems (Syafriana et al., 2014; Wightwick et al., 2010; Zubrod et al., 2019).

1.5.2 The use of biological control agents (BCAs)

Biological control involves the employment of microorganisms to control other microorganisms, including pathogenic microorganisms, that influences disease progression in a plant (Abd-Elsalam et al., 2023; Stangarlin et al., 2011). An example of such microorganisms exhibiting biological control influences are fungal endophytes (Adeleke et al., 2022). In recent years, endophytic organisms have been researched as BCAs as well as plant growth promoters (Abdallah et al., 2016; Devi et al., 2023; Mahdi et al., 2014; Mejdoub-Trabelsi et al., 2022; Nefzi et al., 2018). Endophytic fungi grow in internal plant tissue, where they are present in the intercellular spaces without causing disease symptoms in host plants (Hutauruk & Pinem, 2020; Strobel, 2003; Taghinasab & Jabaji, 2020). They contribute to the growth of host plants, induce defence resistance against pathogens, facilitate plant nutrient uptake, and modulate the synthesis of plant secondary metabolites (SMs) (Taghinasab & Jabaji, 2020). Additionally, they are able to adapt to extreme environments and may assist the host in its defence against stressful conditions (Amiri & Tibuhwa, 2021; Wu et al., 2018). Moreover, endophytic fungi can supress the growth of pathogens via the secretion of toxic compounds such as volatiles and via the competition for plant tissue, nutrients and infection sites, etc (Devi et al., 2023; Fontana et al., 2021).

Fungal endophytes can be characterized into 2 categories, namely clavicipitaceous which are associated with grasses, and nonclavicipitaceous which are not found in grasses (Baron & Rigobelo, 2022; Hyde & Soytong, 2008; Singh et al., 2023). They are further classified into 4 classes where: class 1 are clavicipitaceous fungi specific to fungal endophytes that colonize grasses, they can be isolated from the roots or aerial sections of the host plants, and they are

vertically and horizontally transmitted. Class 2 are fungal endophytes that are capable of colonizing aerial sections and roots of the host plant and are transmitted vertically and horizontally. Class 3 fungal endophytes are commonly found in leaves of tropical trees and are only transmitted horizontally. Class 4 endophytes include dark septate fungi that possesses melanin in their septa. They are exclusively found in roots, and they are horizontally transmitted. Horizontal transmission takes place when the endophyte produces spores or vegetative propagules and spreads it to the plant population via the air or some other vectors, whereas vertical transmission is the transference of the fungal endophytes to the host plants via the transference of seeds (Aly et al., 2011; Lugtenberg et al., 2016). Fungal endophytes are also classified based on their function, ecology and diversity (Bamisile et al., 2018b). They can be asexual or sexual according to their mode of reproduction via asexual or sexual spores. With relevance to the expression of infection, endophytic fungi can be characterized as asymptomatic or symptomatic as well as foliar or root endophytes which is dependent on the part of the host plant it colonizes. Lastly, they can be necrotrophic or biotrophic based on their mode of nutrition, where necrotrophic fungal endophytes promote necrosis in order to grow from dead tissue and biotrophic fungal endophytes obtain their nutrients from living tissue (Kemen & Jones, 2012).

Among microbial species, the genus *Penicillium* is one of the biggest fungal groups which is comprised of over 429 recognized species (Ashtekar et al., 2021). *Penicillium* species is more ubiquitous than other fungal species in the environment and some have been found to exist endophytically within plant tissue whilst others are saprophytes involved in the breakdown of organic matter (Liang et al., 2021; Rashmi et al., 2019). The *Penicillium* genus are ubiquitous fungi due to the fact that they have undemanding nutritional necessities, and they possess the ability to grow and survive in a wide range of environments and conditions (Toghueo & Boyom, 2020). Some *Penicillium* species have been isolated as endophytes from various species of plants (Nicoletti et al., 2014).

This endophytic nature has given the *Penicillium* species the protective ability towards plants under stress, they promote the growth of host plants and protect host plants from pathogenic attack via the synthesis of enzymes and antagonistic compounds (Hassan, 2017; Waqas et al., 2015).

1.5.2.1 The biological control of fungal plant pathogens using a multi-omics approach

Next-generation sequencing (NGS) is a nucleotide sequencing method that is much cheaper and faster than the traditional Sanger method which has opened a new era in molecular biology and genomics (Bielecka et al., 2022; Okoń et al., 2024). These new NGS methods for sequencing genomes provides three major advantages, including (1) instead of cloning DNA fragments, the NGS libraries are developed within a cell-free system; (2) NGS sequencing parallelly produces thousands-to-millions of sequencing reactions; and (3) NGS output is distinguished without the use of electrophoresis due to the fact that base interrogations are performed in parallel (Bielecka et al., 2022; Okoń et al., 2024). NGS methods have evolved throughout the past decade via the use of revolutionary innovations in order to tackle the many complications of genome sequencing (Bielecka et al., 2022; Goodwin et al., 2016; Okoń et al., 2024). Short-read sequencing methods, including sequencing by ion semiconductor, synthesis, and nanoball sequencing, maximizes the amount of bases that are sequenced within a short period of time, which generates a wealth of data that assists in understanding complex phenotypes (Bielecka et al., 2022). Whereas long -read sequencing methods sequences longer-continuous DNA sections which are required to resolve structurally complex sections of genomes (Bielecka et al., 2022; Okoń et al., 2024).

This advancement in the technologies used to sequence genomes have enabled researchers to sequence entire genomes of numerous organisms (Bielecka et al., 2022). At present, the modern and efficient NGS technologies have been used to study endophytes due to its sensitivity, precision, and specificity (Bielecka et al., 2022). With the use of metabolomics, proteomics, metagenomics, and bioinformatics, researchers may be able to fill the gap in research of fungal endophytes (Bielecka et al., 2022; Roxo et al., 2024). To date, NGS have been utilized for the identification of fungal endophytes taxonomies and the study of their ecologies such as the resistance to environments of high salinity, and heavy metals as well as its interactions with other fungal species and bacteria (Bullington & Larkin, 2015; Diale, 2022; Liu et al., 2017a; Peršoh, 2013; Roxo et al., 2024). Additionally, it has been used to observe the interactions between hosts and fungal endophytes, as well as their functional heterogeneity and lifestyle (Knapp et al., 2018; Schmid et al., 2017; Xu et al., 2018; Ye et al., 2019; Zhou et al., 2017; Zuccaro et al., 2011). Furthermore, NGS technologies have been utilized to identify biosynthetic pathways of SMs (Bhargavi et al., 2018; Bhargavi et al., 2014; Byers et al., 2023; Carro et al., 2018; Cheng et al., 2020; Figueiredo et al., 2022; Savitha et al., 2016; Vignolle et al., 2020; Wang et al., 2022c; Wang et al., 2015; Wei et al., 2021; Zhao et al., 2021). The whole

genomes of various *Penicillium* species have been sequenced including but not limited to *Penicillium citrinum* DSM 1997 (Schmidt-Heydt et al., 2019), *Penicillium brasilianum* LaBioMMi 136 (Fill et al., 2018), *Penicillium polonicum* hy4 (Kang et al., 2019a), *Penicillium aurantiogriseum* NRRL 62431 (Yang et al., 2014), and *Penicillium janthinellum* P1 (Chi et al., 2021) where they focused on biosynthetic gene clusters (BGCs) and SMs.

Fungi are able to synthesise diverse SMs that are essential for the interactions with their environment and other organisms (Roxo et al., 2024). These SMs are as a results of BGCs which are made up of regulators, enzymes and transporters which all contribute to the biosynthetic pathway (Roxo et al., 2024). Various hurdles need to be overcome when mining the genomes and discovering BGCs of fungal endophytes (Sagita et al., 2021). The problem of low predictive value of known BGCs exists when annotating BGCs of fungal origin using the bioinformatic tools with standard rule-based approaches (Sagita et al., 2021). Fungal BGC prediction has proven to be problematic for researchers as majority of the bioinformatics tools were produced to annotate BGC architecture of bacterial genomes which are different to fungal BGCs which are often divided over multiple loci in bidirectional orientations (Sagita et al., 2021). Additionally, fungal species possesses many BGCs which lacks the homologs of the scaffolding enzymes which are more difficult to annotate (Sagita et al., 2021).

Various endophytic fungi can produce SMs which may possess antibacterial and antifungal activity which majorly inhibits the growth of pathogens (Mejdoub-Trabelsi et al., 2022). SMs synthesized by fungal endophytes for example, flavonoids, diterpenes, terpenoids, alkaloids, steroids, volatiles, isocoumarins, phenolics, and chromones possess antifungal activity against phytopathogenic (Baron & Rigobelo, 2022; Kumar & Kaushik, 2012; Mejdoub-Trabelsi et al., 2022). Antifungal compounds produced by fungal endophytes can cause cellular changes in the morphology of hyphae including the swelling, lysis, cytoplasm aggregation, and distortion of hyphal structures (Firdausi et al., 2020). Fungal endophytes may produce important bioactive secondary compounds and metabolites, exclusive to those produced by the host plant, which contribute to the stimulation of novel SM synthesis as well as plant fitness (Zhang et al., 2006b). In return, the endophytes are able to access nutrients, a particular ecological niche, dissemination to hosts next generation via vertical transmission in seeds, and the protection from desiccation and abiotic stress (Faeth & Fagan, 2002; Furtado et al., 2019).

The SMs produced by *Penicillium* species to inhibit fungal pathogens growth is documented in literature (Al-Rashdi et al., 2022; Azar et al., 2023a; Cheong et al., 2017; Chowdhary & Kaushik, 2019; Khan & Javaid, 2022; Luo et al., 2019; Lykholat et al., 2022; Lykholat et al., 2021; Mejdoub-Trabelsi et al., 2022; Miao et al., 2019; Rojas et al., 2022; Shafique et al., 2023; Urooj et al., 2021). Research by Mejdoub-Trabelsi et al. (2022) isolated *Penicillium chrysogenum* and *Aspergillus niger* from *Solanum tuberosum* L., and tested its antagonism against the pathogen *Rhizoctonia solani*. Cell-free culture filtrates of *P. chrysogenum* and *A. niger* had inhibited *R. solani* growth by above 60 % relative to the control. The authors attributed the control of *R. solani* due to the biologically stable and active SMs produced by the endophytes such as antibiotics and/or lytic enzymes (Table 1.1; Figure 1.3).

Table 1.1 The production of secondary metabolites (SMs) by endophytic *Penicillium* species for the inhibition of fungal plant pathogens.

Species	Host	Metabolite	Pathogen	Reference
Penicillium chrysogenum	*Solanum tuberosum L.*	Crude cell-free culture	*Rhizoctonia solani*	Mejdoub-Trabelsi et al. (2022)
Penicillium chrysogenum	*Chaenomeles speciosa*	11-hexadecyn-1-ol, 2,2-dihydroxymalonic acid, 4-butoxy-2-butanone, 4-hexyl-2,5-dioxo-2,5-dihydro-3-furanyl acetic acid, diisooctyl phthalate 11-hexadecyn-1-ol, and hexadecanoic acid	*Fusarium culmorum*, and *F. oxysporum*	Lykholat et al. (2022)
Penicillium oxalicum	*Aloe dhufarensis*	2,3-butanediol, 2-furanmethanol, 4-hydroxyphenylacetamide, dodecanoic acid, hexadecanoic acid, methyl ester, octadecanoic acid, and tetradecanoic acid	*Cladosporium* sp., and *Fusarium* sp.	Al-Rashdi et al. (2022)
Penicillium sp.	*Urginea maritima*	Mycophenolic acid	*Sclerotinia sclerotiorum, Phoma tracheiphila, Alternaria alternata, Botrytis cinerea, R. solani,* and *F. oxysporum*	Azar et al. (2023a)

Penicillium brefeldianum	*Cucumis melo L.*	Brefeldin A	*Fusarium oxysporum f. sp. Melonis*	Miao et al. (2019)
Penicillium olsonii ML37	*Triticum aestivum*	Asperphenamate	*Fusarium graminearum*	Rojas et al. (2022)
Penicillium citrinum BTF08	*Musa* sp.	Metabolites with callogenesis activity	*Ganoderma boninense*	
Penicillium italicum	-	9,12-octadecadienoic acid (Z,Z), decane, dodecane, benzene,nitro-, benzene,1,3,5-trimethyl, benzene,1,4-diethyl, 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester, and 1-nonadecene	*Macrophomina phaseolina*	Khan and Javaid (2022)
Penicillium janczewskii, Penicillium digitatum, Penicillium verrucosum, and *Penicillium crustosum*	-	One-month old aqueous culture filtrated extracellular SMs	*Phoma herbarum*	Shafique et al. (2023)
Penicillium sp. *ARDS-2.3*	*Asparagus racemosus*	Crude ethyl acetate extracts	*Fusarium oxysporum, S.*	Chowdhary and Kaushik (2019)

			sclerotiorum, and *Rhizoctonia solani*	
Penicillium decumbens, Penicillium nigricans, Penicillium rugulosum, and Pseudomonas monteilii	-	Aqueous suspensions	*Fusarium* spp., *Rhizoctonia solani, and Macrophomina phaseolina*	Urooj et al. (2021)
Penicillium citrinum DBR-9	*Stephania kwangsiensis*	Citrinin, and emodin	*Alternaria citri, Alternaria oleracea, Bipolaris maydis, Colletotrichum capsica, Ceratocystis paradoxa, Cochliobolus miyabeanus, Diaporthe citri,*	Luo et al. (2019)

			Exserohilum turcicum, Pestalotiopsis theae, and *Phytophthora parasitica var. nicotianae*	

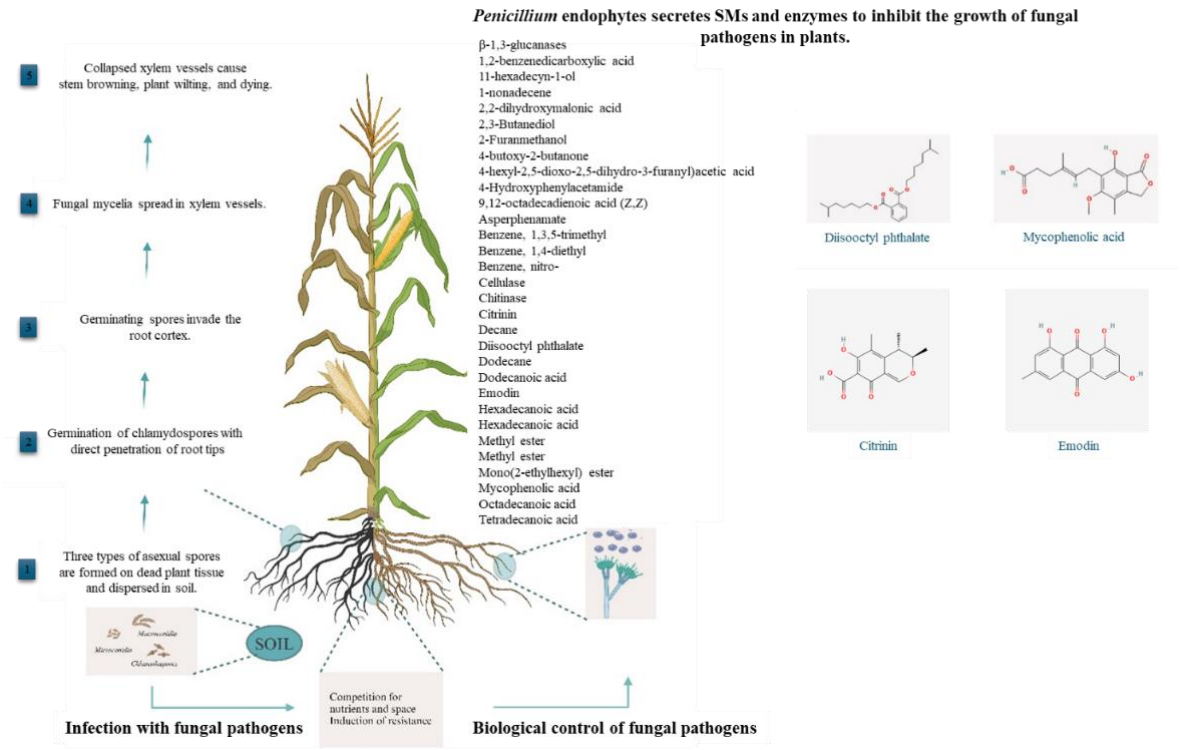


Figure 1.3 Possible modes of action of endophytic fungi as BCAs against plant fungal pathogens. Fungal pathogens infect plant tissue throughout its life cycle from the production of asexual spores to its movement through the xylem causing the subsequent root rot, stem browning and plant wilting. Fungal pathogens and fungal endophytes compete in the plant tissue for nutrients and space. Endophytic *Penicillium* species can exhibit direct antagonism against plant pathogens via the production of metabolites and enzymes which ultimately assists the host plant in its disease resistance and growth promotion. The figure was created with BioRender.com (accessed on 10 March 2024).

Lykholat et al. (2021) observed that *Penicillium expansum* produces antimicrobial metabolites diisooctyl phthalate, 4-hexyl-2,5-dioxo-2,5-dihydro-3-furanyl)acetic acid, 2,2-dihydroxymalonic acid and 11-hexadecyn-1-ol and *P. chrysogenum* synthesized numerous eicosyl, isopropyl ethers, and phthalates of hexadecanoic acid, and 4-butoxy-2-butanone (Table 1.1; Figure 1.3). Furthermore, Al-Rashdi et al. (2022) isolated *P. oxalicum* isolate SQUCC-F3-1 from the leaf tissue of *A. dhufarensis* Lavranos and observed that it exhibited antifungal activity toward phytopathogens *Fusarium* sp. and *Cladosporium* sp. The investigation of the SMs produced by *P. oxalicum* (SQUCC-F3-1) identified antifungal compounds such as 2-furanmethanol, dodecanoic acid and tetradecanoic acid, 2,3-butanediol, octadecanoic acid, methyl ester, hexadecanoic acid, methyl ester and 4-hydroxyphenylacetamide (Table 1.1; Figure 1.3). The authors also showed that the fungal endophytes caused pit formation,

shrivelling and hyphal structure disintegration of the pathogenic fungi. Moreover, a study by Azar et al. (2023b) isolated a biologically active fungal endophyte isolated from squill (*U. maritima*) leaf belonging to the genus *Penicillium*. The SM mycophenolic acid was identified as the main contributor to its antifungal activity against *S. sclerotiorum* and *P. tracheiphila* (30-70 % inhibition) and *A. alternata*, *R. solani*, *B. cinerea*, and *F. oxysporum* (0 – 30 % inhibition) (Table 1.1; Figure 1.3).

Research by Rojas et al. (2022) used RNA-seq and liquid chromatography with tandem mass spectrometry (LC-MS/MS) to understand the metabolic interactions of *P. olsonii* ML37, wheat, and *F. graminearum* under greenhouse conditions. They showed that *P. olsonii* colonised wheat spikes and activated the plants defence mechanisms. The authors showed that the increase in *Penicillium*-produced metabolite asperphenamate confirmed the colonisation of the endophyte (Table 1.1; Figure 1.3). Moreover, a study by Khan and Javaid (2022) screened five *Penicillium* species, namely *P. expansum*, *P. simplicissimum*, *P. citrinum*, *P. oxalicum*, and *P. italicum* for their antagonistic effect on the pathogen *M. phaseolina* using a dual culture method. Among the investigated strains, *P. italicum* exhibited the highest inhibition of *M. phaseolina* by 57 %, followed by *P. citrinum* showing an inhibition of 42 %, *P. simplicissimum* (21 %), *P. expansum* (11 %), and lastly *P. oxalicum* (9 %). Thereafter, the authors exposed the DNA of *M. phaseolina* to SMs of *P. italicum* to determine the mode of action of *P. italicum*. Their findings showed that the metabolites resulted in the complete degradation of fungal DNA after an incubation period of 48 hours. Furthermore, the authors explored the antifungal mechanisms of *P. italicum* by subjecting the chloroform and ethyl acetate fractions of SMs to gas chromatography–mass spectrometry (GC-MS) analysis. The authors showed that the major compounds produced were 9,12-octadecadienoic acid (Z,Z), decane, dodecane, benzene,nitro-, benzene,1,3,5-trimethyl, benzene,1,4-diethyl, 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester, and 1-nonadecene (Table 1.1; Figure 1.3) and could be the cause of the inhibition of *M. phaseolina*. Moreover, a study by Chowdhary and Kaushik (2019) isolated *Penicillium* sp. isolate ARDS-2.3 from the plant tissue of *A. racemosus* and evaluated its antifungal effect on the causal agents of four distinctive plant diseases such as grey mould caused by *B. cinerea*, stem rot caused by *S. sclerotiorum*, root rot caused by *R. solani* and wilting caused by *F. oxysporum*, respectively, using dual-culture bioassays. The authors observed that the crude ethyl acetate extract of *Penicillium* sp. isolate ARDS-2.3 exhibited antifungal activity against *B. cinerea*, *F. oxysporum, S. sclerotiorum*, and *R. solani* with IC50 values of 0.499, 0.381, 2.843, and 0.955 mg/ml, respectively (Table 1.1; Figure 1.3). Moreover, a study by Luo et al. (2019) isolated

two polyketides (PKs), namely citrinin and emodin, from the fungal endophyte *P. citrinum* isolate DBR-9 isolated from *S. kwangsiensis* root tubers. The authors used *in vitro* antifungal assays and observed that the two PKs possessed significant inhibitory activity on the tested phytopathogens, namely *A. citri*, *B. maydis*, *C. paradoxa*, *C. capsica*, *C. miyabeanus*, *D. citri*, *A. oleracea*, *P. theae*, *E. turcicum*, and *P. parasitica* var. *nicotianae*. The PK citrinin (Figure 1.3) exhibited antagonism with IC50 values ranging from 3.1 to 123.1 µg/mL and showed highest antagonism toward *A. citri*, whereas the PK emodin (Figure 1.3) showed antagonism with IC50 values ranging from 3.0 to 141.0 µg/mL, showing the highest antagonism toward *B. maydis* (Table 1.1). Furthermore, the authors showed that emodin is able to affect colony morphology, protein synthesis of the tested fungal cell, and destroy cell membrane integrity.

1.6 Conclusion

This investigation into the current trend of knowledge concerning the multiple agricultural applications of endophytic *Penicillium* species showed that this genus has been researched against a wide array of plant pathogens. However, to our knowledge there have been no recent research on the genomic mining of *Penicillium* species for genes associated with the biological control of fungal plant pathogens. Therefore, this study will provide a baseline data on future genomic and metabolomic profiling on the antifungal activity of *Penicillium* species.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and Suppliers

Table 2.1 List of chemicals and reagents.

Chemical / Reagent	**Supplier**
Acetone	Merck
Acetonitrile (CH_3CN)	Merck
Ammonium acetate ($C_2H_3O_2NH_4$)	Merck
Ammonium bicarbonate (AmBic)	Merck
Ammonium chloride (NH_4Cl)	Merck
Ammonium persulfate (APS) ($(NH_4)_2S_2O_8$)	BIO-RAD
Ammonium sulfate ($(NH_4)_2SO_4$)	Merck
Anthrone	Merck
Ascorbic acid / Ascorbate	Merck
Bromophenol blue	Merck
β-mercaptoethanol	Merck
Calcium chloride ($CaCl_2$)	Merck
Carboxy methyl cellulase (CMC)	Merck
Cobalt (II) chloride hexahydrate ($CoCl_2.6H_2O$)	Merck
Cellulose	Merck
3,3′-Diaminobenzidine	Merck
di-nitro salicylic acid (DNS) ($C_7H_4N_2O_7$)	Merck
Dipotassium phosphate (H_2HPO_4)	Merck
Dithiothreitol (DTT) Cleland's reagent	Merck
Ethanol 99.9%	Reflecta laboratory supplies
Ethylenediaminetetraacetic acid (EDTA)	Merck
Ferrous sulphate heptahydrate ($FeSO_4.7H_2O$)	Merck
Fumaric acid ($C_4H_4O_4$)	Merck
Guaiacol	Merck
Glacial acetic acid	Merck

Glycerol	Merck
Glycine	BIO – RAD
Guaiacol	Merck
Gum acacia	Merck
Hydrochloric acid (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
Magnesium sulfate ($MgSO_4.7H_2O$)	Merck
Manganous sulfate ($MnSO_4.7H_2O$)	Merck
Methanol 99.9 %	Merck
Methionine	Merck
Nitrotetrazolium blue chloride powder (NBT)	Merck
Olive oil	Reflecta laboratory supplies
Oxalic acid ($C_2H_2O_4$)	Merck
Peptone	Merck
Phenol	Merck
Phenolphthalein ($C_{20}H_{14}O_4$)	Merck
Potato dextrose agar (PDA)	Lasec
Potato dextrose broth (PDB)	Merck
Polyvinylpyrrolidone (PVP)	Merck
Potassium cyanide (KCN)	Merck
Potassium iodide (KI)	Merck
Potassium permanganate ($KMnO_4$)	Merck
Potassium phosphate (KPO_4)	Merck
Potassium phosphate monobasic (KH_2PO_4)	Merck
Potassium phosphate dibasic (K_2HPO_4)	Merck
Potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$)	Merck
Riboflavin	Merck

Sodium chloride (NaCl)	Merck
Sodium dodecyl sulfate (SDS)	BIO-RAD
Sodium hypochlorite	BioSmart
Sodium hydroxide (NaOH)	Merck
Sucrose	Merck
Sulfuric acid	Reflecta Laboratory Supplies
Taq Amplicon Mastermix	Merck
Thiobarbituric acid (TBA)	Merck
Trichloroacetic acid (TCA)	Merck Millipore
Trifluoroacetic acid (TFA)	Merck
Trypsin	Promega
Urea	Merck
Yeast extract	Merck
Zinc sulfate ($ZnSO_4.7H_2O$)	Merck
Zymo Research Quick-DNA Fungal-Bacterial Miniprep kit	Inqaba biotec

2.2 Stock solutions and buffers

Table 2.2 List of buffers and stock solutions prepared.

Buffer/Stock solution	**Composition**
Acetone (80 %)	80 % (v/v) acetone in dH_2O.
Acetonitrile (2 %)	2 % (v/v) acetonitrile in dH_2O.
Acetonitrile (50 %)	50 % (v/v) acetonitrile in dH_2O.
Alcohol: acetone	100 % (v/v) in 100 % (v/v) acetone.
Anthrone/Sulfuric acid	0.2 % (w/v) anthrone in 96.7 % sulfuric acid.
APX assay buffer	50 mM KPO_4 (pH 7.4); 0.2 mM EDTA; 0.25 mM ascorbic acid in dH_2O.
Binding buffer	200 mM sodium acetate; 30 % acetonitrile (pH 4.5).
$CaCl_2$	0.6 % (w/v) $CaCl_2$ in dH_2O.

Citrate phosphate buffer	18.15 g (w/v) sodium phosphate dihydrate; 9.605 g (w/v) citric acid in dH_2O (pH 4.8)
Diaminobenzidine	1 mg/ml (w/v) 3,3′-diaminobenzidine in dH_2O.
DNS solution	30 % potassium sodium tartrate; 1 % of DNS; 20 % of 2 N NaOH in dH_2O.
Ethanol (70 %)	70 % (v/v) ethanol in dH_2O.
Glycerol	80 % (v/v) glycerol in dH_2O.
Guaiacol	1 $mg.ml^{-1}$ (w/v) guaiacol in dH_2O.
H_2O_2 reaction mixture	5 mM K_2HPO_4 (pH 5.0); 0.5 M KI in dH_2O.
Mendel's medium	0.1 % peptone; 0.01 % $CaCl_2$; 0.03 % urea; 0.2 % KH_2PO_4; 0.0016 % $MnSO_4.7H_2O$; 0.14 % $(NH_4)_2SO_4$; 0.0014 % $ZnSO_4.7H_2O$; 0.03 % $MgSO_4.7H_2O$; 0.05 % $FeSO_4.7H_2O$; 0.0029 % $CoCl_2.6H_2O$; and 1 % cellulose (pH 5.5) in dH_2O.
Methanol Buffer	80 % (v/v) methanol; 0.1 M ammonium acetate in dH_2O.
NaCl	0.9 % (v/v) NaCl in dH_2O.
O_2^- buffer	100 mM KCN; 100 mM H_2O_2; 6.4 mM NBT; in 50 mM potassium phosphate (pH 7.0)
Olive oil in gum acacia (10 %)	10 % gum acacia (w/v) dissolved in 10 (v/v) olive oil.
Oxalic acid (1 %)	1 % (w/v) $C_2H_2O_4$ in dH_2O.
Phosphate buffer	1M K_2HPO_4; 1M KH_2PO_4 (pH 7.0).
Potato dextrose agar (PDA)	Potato extract; dextrose; agar in dH_2O.
Potato dextrose broth (PDB)	Potato extract; dextrose in dH_2O.
Potassium permanganate (1 %)	1 % (w/v) $KMnO_4$ in dH_2O.

Production medium	0.1 % glucose; 0.5 % NH_4Cl; 3 % olive oil; 0.36 % yeast extract; 0.01 % $MgCl_2$; 0.1 % H_2HPO_4; 0.04 % $CaCl_2$ in dH_2O.
Protein solubilisation buffer	50 mM Tris containing 2 % SDS and 4 M urea.
PVP extraction buffer	40 mM K_2HPO_4 (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.
SOD assay buffer	50 mM KPO_4 (pH 7.4); 13 mM methionine; 75 µM NBT; 0.1 mM EDTA; 2 µM riboflavin in dH_2O.
Sodium acetate buffer	42.9 g (w/v) sodium acetate trihydrate; 10.4 mL (v/v) glacial acetic acid in dH_2O (pH 5)
Sodium hypochlorite	5 % (v/v) sodium hypochlorite in dH_2O.
Sodium perchlorate	2.5 % (v/v) sodium perchlorate in dH_2O.
TBA/TCA buffer	0.5 % (w/v) TBA dissolved in 20 % (v/v) TCA.
TCA (6 %) extraction buffer	6 % (w/v) TCA in dH_2O.
TCA (20%)	20 % (w/v) TCA in dH_2O.
TCA/Acetone extraction buffer (10 %)	10 % (w/v) TCA in 100 % acetone.
TFA (0.05 %)	0.05 % (v/v) TFA in dH_2O.
TFA (1 %)	1 % (v/v) TFA in dH_2O.
Urea buffer	7 M (w/v) urea in dH_2O.
Washing buffer	95 % acetonitrile, pH 4.5.

2.3 Isolation of isolate A4 from *Echium plantaginium*

Isolate A4 was isolated from the roots of six *E. plantaginium* plants collected in Durbanville, Western Cape, South Africa using a method previously described by Mia (2018). Root 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). Plant root material was surface sterilized with 70 % ethanol for 3 minutes, 2.5 % sodium perchlorate for 5 minutes, followed by 70 % ethanol for 1 minute and washed 5 times with sterile dH_2O. Roots were homogenized in 0.9 % saline (NaCl) using a sterile mortar and pestle. The homogenate was diluted (1:100), plated onto PDA (Table 2.2) containing 100 µg/mL chloramphenicol and incubated at 25 °C for 7 days. Following incubation, fungal colonies were visually analysed, and pure cultures were made under a sterile laminar flow hood by cutting 1 cm x 1 cm plugs and transferring them to PDA plates. The pure fungal isolate was incubated for 2 weeks at 30 °C and glycerol stocks were made by placing 1 cm x 1 cm plugs in sterile 80 % glycerol and stored at -80 °C until further use.

2.4 Identification of isolate A4 from *E. plantaginium*

Isolate A4 was grown on PDA media at a temperature of 30 °C for 7-10 days. Approximately 100 mg of biomass was collected, and total genomic DNA was extracted using the Zymo Research Quick-DNA Fungal-Bacterial Miniprep kit (Zymo Research, catalog number D6005). DNA purity and concentration were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific). The ITS1 and 4 regions were amplified using the primer pair ITS1 (5' CTTGGTCATTTAGGGAAGTAA 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3'). The PCR reaction consisted of 1 µL of template DNA, 1 X Taq Amplicon Mastermix, 25 mM MgCl2, 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. The DNA was sequenced at the Central Analytical Facility at the University of Stellenbosch for sequence analysis.

2.5 Phylogenetic analysis of *Penicillium simplicissimum* A4 using ITS markers

The phylogenetic analysis on *P. simplicissimum* A4 was done according to a method previously described by Mkumbe et al. (2018) with modifications. A similarity search was done using BLAST search tool at National Center of Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the species with a similarity to *P. simplicissimum* A4 was selected, including *P. simplicissimum* isolate LN1 (accession OP797625.1), *P.*

simplicissimum culture CBS:280.39 (accession MH856014.1), *P. simplicissimum* strain HF3P23 (accession OP179058.1), *P. simplicissimum* culture CBS:134.63 (accession MH858239.1), and *P. simplicissimum* strain HF2P20 (accession OP178993.1). Additionally, other *Penicillium* species were selected such as *Penicillium janthinellum* CBS 340.48 (accession NR_111504.1), *P. janthinellum* strain NRRL 2016 (accession AF033434.1), *Penicillium steckii* CBS 260.55 (accession NR_111488.1), and *Alternaria alternata* strain YJY-3 (accession OL958426.1) was selected as an outgroup. The multiple sequence alignment was performed using ClustalW using the Molecular Evolutionary Genetic Analysis software (MEGA) (version 11.0.13). Phylogenetic analysis was achieved using the neighbour-joining method on MEGA with 1000 bootstraps runs setting selected, and the evolutionary distances were estimated using maximum composite likelihood.

2.6 Preparation of *P. simplicissimum* A4 spore suspension

A method previously described by Kumar et al. (2016) with slight modifications was used to prepare the cell suspension of *P. simplicissimum* A4 where spores were harvested from 7-day old colonies incubated at 25±2 ℃ in 12 hours light and 12 hours dark conditions. Spores were harvested by adding 14 mL of sterile dH_2O on to the plates and scrapping the mycelia off using a sterile microscope glass slide. Spores were filtered using a sterile cheesecloth, enumerated using a haemocytometer and the concentrations calculated using the calculations below.

1) Percentage of viable cells= $\frac{\text{Number of viable cells}}{\text{Total number of cells}}$ x 1002) Average number of cells = $\frac{\text{Number of viable cells}}{\text{Number of squares}}$

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

4) Concentration of viable cells =Average number of cells × dilution factor $\times 10^4$

Serial dilutions were made using sterile dH_2O to reach a final concentration of 10^8 spores/mL.

2.7 Microscopic analysis of *P. simplicissimum* A4 hyphal structures

Microscope slides of *P. simplicissimum* A4 were prepared using a modified version of the slide culture technique by Nchu et al. (2022) where hyphal structures were prepared by placing 1 cm^2 PDA plug on a sterile glass microscope slide. Five microlitre *P. simplicissimum* A4 spore suspension (Section 2.6) was placed on the two opposite sides on the plug and covered with a glass coverslip and incubated at 30°C for a period of 4 days. The glass coverslip was

subsequently stained with 10 µL of lactophenol blue and viewed under the Zeiss Primo Star Binocular Microscope (Carl Zeiss (Pty) Ltd., Cape Town, South Africa) at 40 X magnification.

2.8 Whole genome sequencing of *P. simplicissimum* A4

2.8.1 DNA extraction and whole genome sequencing

The DNA extraction and whole genome sequencing of *P. simplicissimum* A4 was described by Fisher et al. (2022). High molecular weight (MW) DNA of *P. simplicissimum* A4 was extracted using the Zymo Research Quick-DNA Fungal-Bacterial Miniprep kit (Zymo Research, catalog number D6005) according to the manufacturer's instructions. The extracted DNA was quantified using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) and sent for whole genome sequencing at Inqaba Biotechnical Industries (Pretoria, South Africa) with the single-molecule real-time (SMRT) technology from PacBio.

2.8.2 DNA library construction

Genomic DNA sequencing of *P. simplicissimum* A4 was performed at Inqaba Biotechnical Industries (Pretoria, South Africa) with the SMRT technology from PacBio (Faino et al., 2015). The genomic DNA of *P. simplicissimum* strain A4 (~1 µg) was size selected using the AMPure PB bead size selection kit (Pacific Biosciences, Menlo Park, CA) (Yoshinaga et al., 2018). The HiFi SMRTbell library was constructed using the SMRTbell template preparation kit (version 3.0 and SMRTlink software (version 11) according to the manufacturer's instructions. The HiFi SMRTbell library was sequenced at Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, South Africa) using the PacBio Sequel IIe system in circular consensus sequencing (CCS) mode with 110× coverage per the manufacturer's instructions, resulting in a total read length of 531,767 bp and an N_{50} read of 5,000 kb.

2.8.3 Whole genome assembly and gene prediction analysis of *P. simplicissimum* A4

The primary genome assembly of *P. simplicissimum* A4 was performed using Canu (Version 2.2) (Koren et al., 2017) with default parameters and a genome size setting at 35 Mb. The genome of *P. simplicissimum* A4 assembly was validated using BUSCO (version 5.4.2) (https://busco.ezlab.org/) (Manni et al., 2021) with default parameters.

2.8.4 Gene prediction of the *P. simplicissimum* A4 genome

Fungal genes were predicted using the Augustus prediction tool (version 3.5.0) to delineate genes for the fungal assemblies (Stanke et al., 2004). The gene prediction was achieved using the standard parameters, however, the reference genome for prediction was set to *Aspergillus nidulans*.

2.8.5 Biocontrol protein prediction of *P. simplicissimum* A4 and data analysis

Protein models of *P. simplicissimum* A4 genome was manually curated using all *Penicillium*, *Aspergillus* and *Talaomyceyes* genomes translated into proteomes available in the National Center of Biotechnology Information (NCBI) database (Index of /genomes/genbank/fungi (nih.gov)) as evidence for protein prediction using BLAST+ on the Ubuntu interface where protein hits were stringently filtered to above 95% identity (Yang et al., 2014). Predicted proteins were functionally annotated using BLASTP against the non-redundant database of the NCBI (BLAST: Basic Local Alignment Search Tool (nih.gov)) and classified using the UniProt Knowledgebase (UniProt) (Madeira et al., 2024). The Uniprot service provides access to sequences from multiple sources, such protein sequences nucleotide translations and derived from structures in the Protein Data Bank (PDB) and Structural Genomics Consortium (SGC) initiative.

2.8.6 Biosynthetic gene cluster (BGC) prediction of *P. simplicissimum* A4

Secondary metabolite gene clusters were predicted using Antibiotics & Secondary Metabolite Analysis Shell (AntiSMASH) (version 7.0) (antiSMASH fungal version (secondarymetabolites.org)) fungal version with the "strict" strictness and all extra features selected (Blin et al., 2023).

2.9 Metabolomic profiling of *P. simplicissimum* A4

2.9.1 Extraction of crude secondary metabolites (SMs) from *P. simplicissimum* A4

Crude SMs were extracted from *P. simplicissimum* A4 using a method previously described by Tapfuma et al. (2022) with modifications. *P. simplicissimum* A4 was cultured on PDA medium and incubated at room temperature for a period of 28 days. A total of two cultures were dried in a vented oven at 55 °C for a period of 7 days. Dried cultures were cut into 2 cm cubes using a sterile blade and added to methanol at a ratio of 100 ml of solvent for every fungal plate used for metabolite extraction. The mixtures were incubated at room temperature on a shaker for 4-5 days, the solvent extracts were filtered through a Whatman No. 1 filter paper and concentrated using a rotary evaporator at 60 °C under reduced pressure. The concentrated extracts were air-dried at room temperature for a period of 5 -7 days until a dry and sticky extract was formed. The subsequent dried extracts were stored at −80 °C until further use.

2.9.2 Untargeted metabolomics analysis using LC-QTOF-MS/MS

The untargeted metabolite analysis on *P. simplicissimum* A4 was done according to a method previously described by Nyambo et al. (2024). High-resolution mass spectra were acquired using an AB Sciex® X500R QTOF coupled to an AB Sciex® Exion LC system. The spectral data were acquired using an information-dependent acquisition (IDA) at a mass range of 50–1500 Da. All batches, methods, and data were then processed using the OS Sciex® v3.1. Delustering potential was set at 80 V, ion spray voltage was 5500 V, curtain gas (N2) was at 25 pounds per square inch (psi), and source temperature was set at 450 °C. Ion source gas 1 and 2 were at 45 and 55 psi, respectively and the collision energy 10 eV for the MS scans and 20–50 eV for MS/MS scans. IDA intensity threshold was set at 50 cycles per second, the aqueous mobile phase used was 1 mM ammonium formate in H_2O, and organic mobile phase was 0.5 % formic acid dissolved in methanol. Gradient elution program for organic mobile phase was set to begin at 2 % and stop at 98 % between 0 and 25 minutes, holding for a period of 5 minutes before returning to 2 % over 5 minutes to re-equilibrate for the next injection. Flow rate was set at 700 µL/minute, and run time was 35 minutes and a Kinetex® C18 column (5 µm, 100 Å, 150 mm × 6 mm) with a column protector was utilized. All the solvents were sonicated for a period of 10 minutes before use to remove bubbles.

2.9.3 Data processing and annotation

The raw metabolomic data of *P. simplicissimum* A4 was converted to a ".abf" format by ABF converter software (http://www.reify.cs.com/AbfConverter) and annotated using metabolic workflow on MS-DIAL (version 4.24) (https://systemsomicslab.github.io/compms/msdial/main.html). The parameters for processing the data were set where the MS1 and MS2 tolerance was 0.01 and 0.025 Da, respectively, and [M + H] adducts ions were set at a peak height of 1000 amplitude. The tentative prediction of molecular formula and structure elucidation of the fungal metabolites were processed using MS-FINDER (version 3.50) using the following parameters: MS1 tolerance was set to 5 and MS2 tolerance was set to 15; the formula calculation with isotopic ratio tolerance was 20% and the in-silico MS/MS fragmenter tree depth was set to 2. The databases selected were COCONUT (Natural product), UNDP (Natural product), ChEBI (Biomolecules), KNApSAcK (Natural product), PubChem (Biomolecules). The molecular formulas, structure elucidation, species and function were manually validated and selected based on its known antifungal activity against phytopathogens using databases such as LOTUS (natural products), COCONUT (Natural product), Atlas (natural products), UNDP (Natural product), ChEBI

(Biomolecules), KNApSAcK (Natural product), PubChem (Biomolecules) and published literature.

2.10 Maintenance of *F. proliferatum* isolate

The *F. proliferatum* strain (PPRI 31301) was isolated from diseased maize plants and deposited at the National Collection of Fungi at the Plant Protection Research Institute (Agricultural Research Council). *F. proliferatum* was grown on PDA media (Table 2.2) for 7 days at 30 °C, and 1 cm x 1 cm plugs were stored in 80 % glycerol at −80 °C for further use.

2.11 Evaluation of antagonistic activity of *P. simplicissimum* A4 using a dual-culture assay

An *in vitro* antagonistic assay was done using a dual culture method on PDA (Table 2.2) to determine the biocontrol potential of *P. simplicissimum* A4 against *F. proliferatum* (PPRI 31301). Agar plugs (1 cm x 1 cm) of 7-day old actively growing *P. simplicissimum* A4 and *F. proliferatum* mycelia were co-cultured (on opposite ends of the petri dish) at 26 °C for 7-10 days. The inhibitory response of *P. simplicissimum* A4 against *F. proliferatum* (as mycelial growth inhibition) was qualitatively assessed. For statistical purposes, this experiment was repeated three times independently with five replicates per treatment. The percentage inhibition of *F. proliferatum* mycelial growth was calculated using a formula previously described by Bivi et al. (2010).

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

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

2.12 Enzymatic and biochemical assays of *F. proliferatum*

2.12.1 Preparation of *F. proliferatum* biomass

The biomass of *F. proliferatum* was isolated using the method previously described by Pacios-Michelena et al. (2023) with modifications. *F. proliferatum* was maintained on PDA (Table 2.2) for 7 days at 26 °C. The mycelia were harvested from the plates and stored at -80 °C for downstream analysis.

2.12.2 Estimation of polysaccharide content of *F. proliferatum*

The intracellular and extracellular polysaccharide content of *F. proliferatum* in the presence of *P. simplicissimum* A4 was estimated using the method previously described by Dhiman et al. (2022).

For the estimation of intracellular polysaccharides (IPs), 25 mg *F. proliferatum* biomass (Section 2.12.1) was added to 50 mL PDB (Table 2.2) for the control. For the treatment sample, a spore suspension of *P. simplicissimum* A4 at a final concentration of 10^8 spores per/mL (Section 2.6) was added to 50 mL PDB (Table 2.2) containing 25 mg *F. proliferatum* biomass. All samples were incubated at 26 °C at 150 rpm for 5 days. After incubation, samples were filtered from the culture broth using sterile cheesecloth. A fraction (5 mg) of the resulting fungal mat of each treatment was homogenised in 10 mL phosphate buffer (pH 6.5) and centrifuged at 13 000 rpm for 10 minutes. One mL of the supernatant was mixed with 20 mL of 20 % TCA and incubated at room temperature for 10 minutes. Subsequently, 1 mL of each TCA-treated sample was added to 5 mL of anthrone digested in H_2SO_4 and incubated at 100 °C for 10 minutes.

For the estimation of extracellular polysaccharides (EPs), 25 mg *F. proliferatum* biomass (Section 2.12.1) was added to 50 mL PDB (Table 2.2) for the control. For the treatment sample, a spore suspension of *P. simplicissimum* A4 at a final concentration of 10^8 spores per/mL (Section 2.6) was added to 50 mL PDB (Table 2.2) containing 25 mg *F. proliferatum* biomass. All samples were incubated at 26 °C at 150 rpm for 5 days. After incubation, samples were filtered from the culture broth using sterile cheesecloth. One mL of the PDB filtrates (supernatant) were mixed with 20 mL of 20 % TCA and incubated at room temperature for 10 minutes. Subsequently, 1 mL of each TCA-treated sample was added to 5 mL of anthrone digested in H_2SO_4 (Table 2.2) and incubated at 100 °C for 10 minutes. The IPs and EPs content of each treatment was determined using the UV-vis spectrophotometer at 620 nm.

2.12.3 Estimation of chitin content of *F. proliferatum* biomass

The chitin content of *F. proliferatum* in response to *P. simplicissimum* A4 was estimated using the method previously described by Dhiman et al. (2022). For the estimation of chitin content, 25 mg *F. proliferatum* biomass (Section 2.12.1) was added to 50 mL PDB (Table 2.2) for the control. For the treatment sample, a spore suspension of *P. simplicissimum* A4 at a final concentration of 10^8 spores per/mL (Section 2.6) was added to 50 mL PDB (Table 2.2) containing 25 mg *F. proliferatum* biomass. All samples were incubated at 26 °C at 150 rpm for 5 days. The procured fungal mats were dried overnight in a 40 °C oven. Twenty-five mg of

each treatment was dissolved in 1 M NaOH and incubated in a water bath at 40 °C for 2 hours. The NAOH was discarded, and the samples were washed with sterile dH_2O, and incubated in 1 % of potassium permanganate at room temperature for 1 hour. The potassium permanganate was discarded, and the samples were incubated in 1 % of oxalic acid for the discolouration of the fungal samples. The oxalic acid was discarded, and the resultant chitin was dried at 40 °C overnight and weighed.

2.12.4 Estimation of exo-and-endo-β-1,4-glucanase activity of *F. proliferatum* in response to *P. simplicissimum* A4

The exo-and-endo-β-1,4-glucanase activity of *F. proliferatum* biomass in response to *P. simplicissimum* A4 was estimated using the method previously described by Dhiman et al. (2022).

For the estimation of exo-and-endo-β-1,4-glucanase activity, 25 mg *F. proliferatum* biomass (Section 2.12.1) was added to 50 mL of Mendel's medium (Table 2.2) for the control. For the treatment sample, a spore suspension of *P. simplicissimum* A4 at a concentration of 10^8 spores/mL (Section 2.6) was added to 50 mL of Mendel's medium (Table 2.2) containing 25 mg *F. proliferatum* biomass and incubated at 26 °C at 150 rpm for 5 days. The fungal samples were filtered using sterile cheesecloth and centrifuged at 13 000 rpm at 4 °C for 10 minutes.

For exo-β-1,4-glucanase activity, 1 mL of the resultant supernatant was incubated with 1 mL of 0.5 % cellulose in 0.1 M of citrate phosphate buffer (Table 2.2) and 2 mL of di-nitro salicylic acid (DNS) solution (Table 2.2) at 50 °C for 30 minutes.

For the endo-β-1,4-glucanase activity, 1 mL of the resultant supernatant was incubated with 1 mL of 1% CMC in 0.1 M of sodium acetate buffer (Table 2.2) and 2 mL of DNS (Table 2.2) at 50 °C for 30 minutes.

The resultant reducing sugars for each treatment was measured using the DNS method where one unit (U/mL) of exo-and-endo-β-1,4-glucanase activity is defined as the amount of cellulase required to liberate 1 µmol of reducing sugar (D-glucose) per minute. Exo-and-endo-β-1,4-glucanase activity was calculated using the formula.

$$\text{Enzyme activity} = \frac{\Delta\epsilon \text{ x Vf}}{\text{Vs x } \Delta\text{t x } \sum\text{xd}}$$

where ΔE is the absorbances read at 540 nm, Vf is the final volume of reaction mixture including DNS, Vs is the crude supernatant (mL) containing cellulase used, Δt is the incubation time for hydrolysis, $\sum$ is the extinction coefficient of glucose (0.0026), and d is the diameter of cuvette.

2.12.5 Estimation of intracellular and extracellular lipase activity of *F. proliferatum* biomass in response to *P. simplicissimum* A4

The intracellular and extracellular lipase activity of *F. proliferatum* biomass in response to *P. simplicissimum* A4 was estimated using the method previously described by Dhiman et al. (2022).

For the estimation of intracellular and extracellular lipase activity, 25 mg *F. proliferatum* biomass (Section 2.12.1) was added to 50 mL of production medium (Table 2.2) for the control. For the treatment sample, a spore suspension of *P. simplicissimum* A4 at a concentration of 10^8 spores/mL (Section 2.6) was added to 50 mL of production medium (Table 2.2) containing 25 mg *F. proliferatum* biomass and incubated at 26 °C at 150 rpm for 5 days. After incubation, the fungal samples were filtered using sterile cheesecloth and centrifuged at 13 000 rpm at 4 °C for 10 minutes. The resultant pellet was used for intracellular lipase activity estimation and the supernatant was used for extracellular lipase activity estimation.

For intracellular lipase activity, the fungal pellet was homogenised in 10 mL of phosphate buffer (Table 2.2), filtered using sterile cheesecloth and the supernatant was used for downstream intracellular lipase activity estimation. For intracellular and extracellular lipase enzyme estimation, 1 mL of each respective filtrate (phosphate buffer and production medium, respectively) was incubated in 10 mL of 10 % olive oil in 10 % gum acacia, 5 mL of 1 M phosphate buffer, and 2 mL of 0.6 % $CaCl_2$ at room temperature at 150 rpm for 1 hour. Twenty mL of alcohol: acetone (1 :1) was added to each treatment to stop the reaction. The liberated fatty acid was titrated using 0.1 N NaOH with 100 µL of phenolphthalein as an indicator. The end point was light pink in colour and the extracellular lipase and intracellular lipase activity was calculated using the formula.

$$\text{Enzyme activity} = \frac{(\text{NaOH})(\text{Molarity of NaOH})(1000)(2)(\text{df})}{(1)}$$

where NaOH is the volume of NaOH (mL) used for the treatment – the volume of NaOH (mL) used for control; 1000 is the conversion factor from *m*equivalent to µequivalent; 2 is the time conversion factor from 30 min to 1 h; df is the dilution factor; and 1 is the volume of enzyme (mL) used.

2.12.6 Reactive oxygen species (ROS) profiling of *F. proliferatum* in response to *P. simplicissimum* A4

For the estimation of ROS content, 25 mg *F. proliferatum* biomass (Section 2.12.1) was added to 50 mL PDB (Table 2.2) for the control. For the treatment sample, a spore suspension of *P. simplicissimum* A4 at a final concentration of 10^8 spores per/mL (Section 2.6) was added to 50

mL PDB (Table 2.2) containing 25 mg *F. proliferatum* biomass. All samples were incubated at 26 °C at 150 rpm for 5 days.

The procured fungal mats was used to determine the superoxide (O_2^-) levels using the method described by Gokul et al. (2016). O_2^- content was determined by submerging 200 mg fungal mat from each treatment in O_2^- buffer (Table 2.2) and incubated at room temperature for 20 minutes in the dark. Fungal samples were then homogenized with a miniature pestle, the samples were centrifuged at 13 000 rpm for 20 minutes and the respective absorbances measured at 600 nm. The O_2^- content was calculated using the molar extinction coefficient of 12.8 mM cm^{-1}.

For the determination of hydrogen peroxide (H_2O_2) content in *F. proliferatum*, a method previously described by Velikova et al. (2000) was used where 200 mg fungal material was homogenized in 6 % TCA. The H_2O_2 reaction mixture (Table 2.2) was added to 50 µL TCA extract and incubated at 25 °C for 20 minutes and absorbances were measured at 390 nm. The H_2O_2 concentration was determined using a standard curve based on the absorbance readings (390 nm) of H_2O_2 standards.

2.12.7 Measurement of lipid peroxidation of *F. proliferatum*

Lipid peroxidation by measuring malondialdehyde (MDA) production in *F. proliferatum* biomass was determined using the thiobarbituric acid reactive substances (TBARS) assay described by Zhang et al. (2007) where 200 mg fungal material (Section 2.12.6) was homogenized in 6 % TCA (Table 2.2) and the resultant extracts (200 µl) was mixed with 400 µL of 0.5 % TBA/TCA buffer (Table 2.2) and incubated at 95 °C for a total of 30 minutes, and subsequently incubated on ice for 10 minutes. The samples were centrifuged at 13 000 rpm for 5 minutes and the absorbances of each treatment was 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}.

2.12.8 Measurement of superoxide dismutase (SOD) activity of *F. proliferatum* in response to *P. simplicissimum* A4

Total SOD activity of *F. proliferatum* was determined using a method previously described by Stewart and Bewley (1980). Fungal material (200 mg) (Section 2.12.6) was homogenized in 1 mL of polyvinylpyrrolidone (PVP) extraction buffer (Table 2.2). A fraction of the PVP extract (10 µL) was added to 190 µL SOD assay buffer (Table 2.2) and the reaction was initiated by exposing the samples to light for 15 minutes or until a colour change was observed. The

absorbances were measured at 560 nm and the SOD activity was calculated based on the amount of enzyme needed to reduce 50 % NBT to blue formazan.

2.12.9 Measurement of ascorbate peroxidase (APX) activity of *F. proliferatum* in response to *P. simplicissimum* A4

Total APX activity of *F. proliferatum* was determined using a method previously described by Asada (1984) where the fungal material (200 mg) (Section 2.12.6) was homogenized in 1 mL of PVP extraction buffer (Table 2.2). A fraction PVP extract (10 µL) was added to 180 µL of APX assay buffer (Table 2.2) and the reaction was initiated by the subsequent addition of 10 µL of 90 µM H_2O_2. The absorbances were measured at 290 nm and the APX activity was calculated using the molar extinction coefficient 2.8 mM^{-1} cm^{-1}.

2.13 *In planta* biocontrol potential of *P. simplicissimum* A4 against *F. proliferatum*

2.13.1 Measurement of the antifungal activity of *P. simplicissimum* A4 in maize roots

An *in planta* assay under controlled greenhouse conditions was developed to evaluate the antagonistic activity of *P. simplicissimum* A4 against *F. proliferatum*. Prior to use, maize seeds (sponsored by Agricol (PTY) LTD, Brackenfell, South Africa), were placed in a greiner tube and sterilized at 49 °C in a water bath for 20 minutes and surface sterilized with 5 % sodium hypochlorite and finally rinsed three times with sterile dH_2O. Spore suspensions of *F. proliferatum* and *P. simplicissimum* A4 was done according to Section 2.6 and made up to a final concentration of 10^8 spores per/mL. Three independent experiments were set up for each of the following treatments: the control (dH_2O), the infection with *F. proliferatum* at 10^8 spores/mL, and the priming with *P. simplicissimum* prior to infection at 10^8 spores/mL, respectively. A total of 15 seeds were used for each treatment. Maize seeds were imbibed in the respective suspensions for 2 hours with slight agitation. Imbibed seeds were aseptically transferred (using sterile tweezers) to petri dishes containing moist sterile tissue paper. Seeds primed with *P. simplicissimum* A4 was allowed to air for 30 minutes and infected with 100 uL of *F. proliferatum* spore suspension. Seeds were incubated in the greenhouse at a temperature range of 26±1 °C for a period of 14 days. Plant tissue was harvested using liquid nitrogen and stored at -80 °C until further use.

2.13.2 Measurement of ROS accumulation in maize roots

The detection of O_2^- of maize roots was done according to a method previously described by Gokul et al. (2016) with slight modifications where 2 cm of the root from each treatment was

placed in O_2^- buffer (Table 2.2) and incubated in the dark at room temperature for 20 minutes. The root samples were crushed with a miniature pestle and centrifuged at 13 000 rpm for 20 minutes. The respective absorbances were measured at 600 nm and the O_2^- content was determined using the molar extinction coefficient of 12.8 $mM^{-1}cm^{-1}$. The detection of hydrogen peroxide (H_2O_2) of maize roots was done according to a method previously described by Velikova et al. (2000) with slight modifications where 200 mg root tissue from each treatment was homogenized in 6 % TCA. The H_2O_2reaction mixture (Table 2.2) was added to 50 µL of the resultant TCA extract and incubated at 25 °C for 20 minutes and the absorbances were measured at 390 nm. The H_2O_2 concentration was determined using a standard curve based on the absorbance readings (390 nm) of H_2O_2 standards.

2.13.3 Measurement of lipid peroxidation in maize roots

Lipid peroxidation by measuring MDA production in maize roots was determined using the thiobarbituric acid reactive substances (TBARS) assay described by Zhang et al. (2007) where 200 mg root tissue was homogenized in 6 % TCA (200 µl) (Table 2.2) and mixed with 400 µL of 0.5 % TBA/TCA buffer (Table 2.2). The samples were incubated at 95 °C for 30 minutes and incubated on ice for 10 minutes. The samples were centrifuged at 13 000 rpm for 5 minutes and the absorbances of each treatment was measured at 532 nm and 600 nm, respectively. The concentration of MDA was measured using the molar extinction coefficient of 155 $mM^{-1}cm^{-1}$.

2.13.4 Measurement of SOD activity in maize roots

Total SOD activity of maize roots from each treatment was determined using a method previously described by Stewart and Bewley (1980). Root tissue (200 mg) was homogenized in 1 mL of PVP extraction buffer (Table 2.2). Ten µL PVP extract was added to 190 µL SOD assay buffer (Table 2.2) and the reaction was initiated by exposing the samples to light for 15 minutes or until a colour change was observed. The absorbances were measured at 560 nm and the SOD activity of each treatment was measured based on the amount of enzyme needed to reduce 50 % NBT to blue formazan.

2.13.5 Measurement of APX activity in maize roots

Total APX activity of maize roots from each treatment was determined using a method previously described by Asada (1984). Maize roots (200 mg) was homogenized in 1 mL of PVP extraction buffer (Table 2.2) and 10 µL PVP extract was added to 180 µL of APX assay buffer (Table 2.2). The reaction was initiated by the addition of 10 µL of 90 µM H_2O_2. The

absorbances were measured at 290 nm and the APX activity of each treatment was calculated using the molar extinction coefficient 2.8 $mM^{-1}cm^{-1}$.

2.13.6 Measurement of guaiacol peroxidase (GPOX) and peroxidase (POD) activity in maize roots

GPOX and POD activity was detected in maize roots using a method previously described by Kim et al. (1994) with slight modifications. For GPOX, protein extracts of each treatment (40 µg) were separated on a 10 % native polyacrylamide gel, washed with dH_2O followed by a 10-minute incubation with 5 mM H_2O_2. Thereafter, the gels were stained with 1 mg/ml 3,3′-diaminobenzidine for 1 hour. For POD, protein extracts of each treatment (40 µg) were separated on a 10 % native polyacrylamide gel and the polyacrylamide gels were washed with dH_2O followed by a 10-minute incubation in 5 mM H_2O_2. The gels were stained with 1 $mg.ml^{-1}$ guaiacol for 1 hour. The respective Native PAGE gels were analysed by densitometry analysis using the Alpha Ease FC imaging software (Alpha Innotech Corporation) using the "invert" function. The enzymatic activity of each isoform from three independent gels were measured according to Klein (2012).

2.14 Proteome profiling of maize roots

2.14.1 Protein extraction from maize root material infected with *F. proliferatum* and bio-primed with *P. simplicissimum* A4

Maize root samples (1 g) from each treatment was ground to a fine powder in 0.5 g polyvinylpolypyrrolidone (PVPP) using liquid nitrogen and homogenised with 3 mL TCA/acetone extraction buffer (Table 2.2). The sample extracts were transferred to sterile eppendorf tubes and centrifuged at 16 000 rpm for 3 minutes at 4 °C. The supernatant was discarded, and the pellets were washed with 2 mL 80 % methanol buffer (Table 2.2), vortexed for 1 minute and centrifuged at 13 000 rpm for 10 minutes. The supernatant was discarded, and the pellet was re-suspended in 1 mL 80 % acetone, vortexed and centrifuged at 13 000 rpm 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). The samples were vortexed and centrifuged at 13 000 rpm for 15 minutes and the upper aqueous layer was transferred to new eppendorf tubes and precipitated with 4 volumes of 80 % methanol buffer (Table 2.2) at -20 °C overnight. The samples were centrifuged at 13 000 rpm for 10 minutes and the supernatant discarded. The pellets were washed with 1 mL methanol, briefly vortexed and centrifuged at

13 000 rpm for 5 minutes and supernatant discarded. Acetone (1 mL) was added to each sample, briefly vortexed and centrifuged at 13 000 rpm 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 30 µL urea buffer (Table 2.2), 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.

2.14.2 One-dimensional SDS-polyacrylamide gel electrophoresis (ID SDS-PAGE) analysis of maize root proteins

A fraction of each protein sample (15 µg) was size fractionated using a method previously described by Brunelle and Green (2014).

2.14.3 Protein pellet solubilisation and liquid chromatography mass spectrometry (LC-MS) analysis

Protein pellets were solubilized using the protocol described previously by Mahlare et al. (2023). Protein pellets were solubilised in protein solubilisation buffer (Table 2.2) 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 proteins from each treatment was used for trypsin digestion.

2.14.4 On-bead HILIC digest and solid-phase extraction

The on-bead HILIC digestion and solid-phase extraction was done using the protocol described previously by Mahlare et al. (2023), where all reagents were analytical grade or equivalent. Samples were suspended in 50 mM ammonium bicarbonate before reduction with 10 mM dithiothreitol (DTT) for 30 minutes at room temperature. Samples were alkylated 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 (Table 2.2). The protein solution was added to MagResyn HILIC (Resyn Biosciences) magnetic particles prepared according to manufacturer's instructions and incubated overnight at 4 °C. The supernatant was discarded, and the magnetic particles were washed twice with washing buffer (Table 2.2). The magnetic particles were suspended in 50 mM ammonium bicarbonate containing trypsin to a final ratio of 1:50 and incubated for 4 hours at 37 °C. The peptides were removed from the beads and collected in a fresh tube, and the adsorbed peptides were removed via an incubation in 20 µl 1% TFA for 3 minutes at room temperature. Residual digest reagents were removed using an in-house manufactured C18 stage tip (Empore Octadecyl C18 extraction discs; Supelco). The samples were loaded onto the stage tip after activating the C18

membrane with 30 µl methanol and equilibration with 30 µl 2 % acetonitrile: water; 0.05 % TFA (Table 2.2). The bound samples were washed with 30 µL 2 % acetonitrile: water; 0.1 % TFA (Table 2.2) before elution with 30 µL 50 % acetonitrile: water; 0.05 % TFA (Table 2.2). The eluate was evaporated to dryness. The dried peptides were dissolved in 2 % acetonitrile: water; 0.1 % FA (Table 2.2) and used for LC-MS analysis.

2.14.5 Liquid chromatography mass spectrometry (LC-MS) analysis

LC-MS analysis was done using the protocol described previously by Mahlare et al. (2023). LC-MS was performed on a Thermo Scientific Ultimate 3000 RSLC equipped with a 5mm x 300 µm C18 trap column (Thermo Scientific) and a CSH 25cm x 75 µm 1.7 µm particle size C18 column (Waters) analytical column. The solvent system employed was as follows: 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 achieved for 5 minutes and the sample was eluted onto the analytical column. The flow rate was set to 250 nl/minute and the gradient was generated as follows: 5.0 % -35 % B over 60 minutes and 35-50 % B from 60-75 minutes. The outflow was delivered to the mass spectrometer via a stainless-steel nano-bore emitter. The data was collected in positive mode with spray voltage set to 1.8 kV and ion transfer capillary set to 280 °C. The spectra were internally calibrated using polysiloxane ions at m/z = 445.12003 and 371.10024. MS1 scans were done 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. The data was acquired in profile mode. MS2 acquisitions were done using monoisotopic precursor selection for ion with charges +2-+7 with error tolerance set to +/- 10 ppm. The precursor ions were excluded from fragmentation once for a period of 60 seconds and selected for fragmentation in HCD mode using the quadrupole mass analyser with HCD energy set to 30 %. The 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.

2.14.6 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 (https://www.proteomesoftware.com/). The total ion chromatography for all treatments were analysed using the Uniprot plant database with an FDR of 4.0 %. To ensure that protein identification was correct and of good quality, a threshold criterion was set for the identified proteins, where to be considered a positive identification, the protein exclusive unique peptide count was above 2, protein identification probability was set at above 95 % and the percentage sequence coverage was greater than 10 %. Unique proteins were identified using the data available in the Scaffold_5.0.1 (www.proteomesoftware.com), Funrich (version 3.1.1) (http://www.funrich.org/), and literature souces. Additionally, identified unique proteins were functionally characterized using the data available in the UniProt database (www.uniprot.org) and the protein-to-protein interactions were analysed using STRING (STRING: functional protein association networks (string-db.org)).

2.15 Statistical analysis

All experiments were performed as three independent experiments with multiple (up to 10) replications per treatment. For seed germination experiments, 15 seeds per treatment was analysed. 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 Brown-forsythe test at 5 % level of significance.

CHAPTER 3

GENOMIC AND METABOLOMIC ANALYSIS OF THE ENDOPHYTIC FUNGUS *PENICILLIUM SIMPLICISSIMUM* A4 ISOLATED FROM *ECHIUM PLANTAGINIUM*

3.1 Introduction

Whole genome sequencing of various fungal isolates is currently rising due to reduced sequencing costs and faster sequencing methods (Requena et al., 2023). This has allowed for the expansion in comparative whole genome studies of microorganisms with important roles in biotechnology, health, and agriculture. Amid these important and interesting organisms are the genus *Penicillium*. Genome-wide research has proven to be a great method for establishing the genetic basis behind biocontrol processes in various organisms (Piombo et al., 2018; Sharma et al., 2017), and thus it has opened a door to a comprehensive assessment of the biosynthetic potential of the *Penicillium* genus. With the advancement in long read sequencing technologies, it has provided researchers with the opportunity to generate inexpensive high-quality draft genomes (Petersen et al., 2023; Petersen et al., 2022). These high-quality genomes causes an increase in the comparative power to assess the divergence of pathways, genes, and evolutionary development, which allows for a more accurate identification of orthologous genes, species, and gene clusters, and thus an improved premise for genome mining of unknown enzymes and compounds, as well as an enhanced understanding of evolutionary relationships (Petersen et al., 2023).

In recent years, research on fungal secondary metabolites (SMs) has extensively benefited from the expansion of bioinformatics tools which has thus equipped researchers to identify genes involved in the production of SMs via the mining of genomes (Nielsen et al., 2017). These tools have been exploited and work on the basis that the genes implicated in the biosynthetic pathways of SMs typically cluster closely together within the genome in what is known as biosynthetic gene clusters (BGCs) (Liu et al., 2021; Nielsen et al., 2017). BGCs can encode for the production of a various SM classes including nonribosomal peptides (NRPs), polyketides (PKs), aminoglycosides, terpenes, lantibiotics, siderophores, indolocarbazoles, nucleosides, bacteriocins, and β-lactams (Byers et al., 2023; Weber et al., 2015; Ziemert et al., 2016).

SMs synthesised by fungal endophytes, include flavonoids, diterpenes, steroids, terpenoids, phenolics, alkaloids, volatiles, isocoumarins, and chromones exhibit antifungal activity against phytopathogens (Baron & Rigobelo, 2022; Kumar & Kaushik, 2012; Mejdoub-Trabelsi et al., 2022). Antifungal compounds produced by endophytic fungi can cause cellular changes in hyphal morphology such as hyphal sweeling, distortion, lysis, and cytoplasm aggregation (Firdausi et al., 2020). Fungal endophytes may produce important bioactive secondary compounds and metabolites, exclusive to those fabricated by the host plant, which promote the stimulation of the production of novel metabolites as well as plant fitness (Zhang et al., 2006b). In return, the endophytes gain access to nutrients, a specific ecological niche, dissemination to the next generation of hosts via vertical transmission via seeds, and protection from desiccation and abiotic stress (Faeth & Fagan, 2002; Furtado et al., 2019). The antifungal SMs produced by *Penicillium* species are well established in literature (Shafique et al., 2023). The genus *Penicillium* forms part of the phylum Ascomycota, which is one of the most common groups of fungi in the environment. The genus *Penicillium* is classified into two subgenera which contains 26 sections within the *Aspergillaceae*, with 536 recognized species (Houbraken et al., 2020; Visagie et al., 2014; Visagie et al., 2023). Mycologists as well as the pharmaceutical and agricultural industries have been interested in structurally diverse SMs isolated from *Penicillium* species (Ashtekar et al., 2021). Over the past two decades, *Penicillium* species has been comprehensively studied for its agricultural, biotechnological, and pharmaceutical potential (Toghueo & Boyom, 2020). Alkaloids (Deng et al., 2020; Qi et al., 2019), lactones (Ali et al., 2019; Liu et al., 2019a), PK (Guo et al., 2020; Ying et al., 2021), and terpenoids (Feng et al., 2018; Qin et al., 2020) are among the diverse metabolites produced by *Penicillium* species.

Here we used ITS sequencing to identify and classify *P. simplicissimum* A4 isolated from *E. plantaginium*. Additionally, the study also aimed to sequence the whole genome of the fungal endophyte *P. simplicissimum* A4 and to determine the presence of genes involved in the biological control of fungal plant pathogens as well as BGCs. Lastly, the study aimed to investigate the complete metabolome of *P. simplicissimum* A4 using liquid chromatography-quadrupole time-of-flight tandem mass spectrometry (LC-QTOF-MS/MS) with specific relevance to its potential to biologically control phytopathogens. This work will aid in the advancement of knowledge about this species as well as its biocontrol capabilities in the agricultural industry.

3.2 Results

3.2.1 Molecular Identification of the fungal endophyte fungus

The ITS region of isolate A4 was sequenced for species identification (Table 3.1). The isolate A4 was isolated from roots of *E. plantaginium* plants which showed 99.18 % identity to *P. simplicissimum (*KY315584.1) with a 93 % query cover.

Table 3.1 Species identification of *P. simplicissimum* isolated from *E. plantaginium*.

Sample Name/Code	Species Name	Accession Number	Query Cover (%)	Max Identity (%)	e-value
A4	*P. simplicissimum* isolate psy2	KY315584.1	93	99.18	0.0

3.2.2 Phylogenetic and morphological characterization of *P. simplicissimum* A4

3.2.2.1 Phylogenetic identification of P. simplicissimum isolate A4

From the phylogenetic analysis, I observed that *Penicillium simplicissimum* A4 grouped within a well-supported main clade (bootstrap value of 100%) along with several other *P. simplicissimum* strains. A4 was most closely related to *P. simplicissimum* isolate LN1, with a bootstrap support of 58%, suggesting a moderate level of relatedness. This larger clade also included reference strains such as CBS:134.63, HF3P23, HF2P20, and CBS:280.39, all of which showed high levels of similarity (bootstrap values ranging from 91% to 97%). These results indicate that strain A4 is genetically consistent with other *P. simplicissimum* strains and fits well within the species grouping (Figure 3.1).

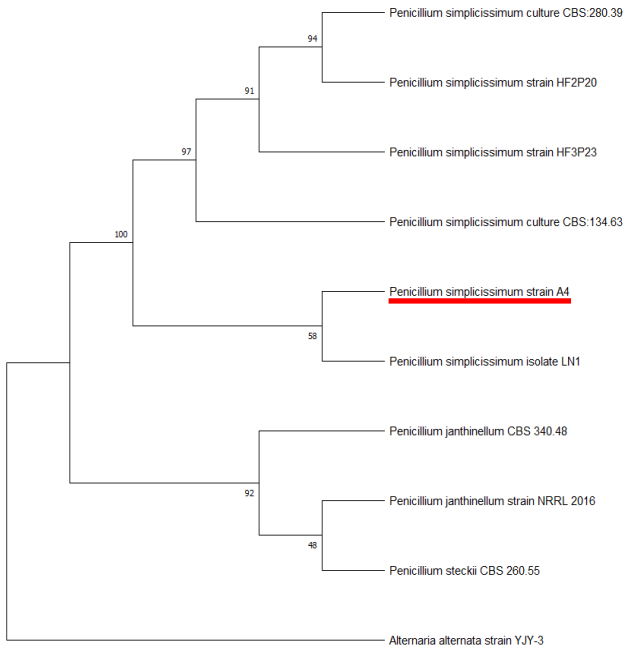


Figure 3.1 Phylogenetic analysis of *P. simplicissimum* isolate A4. The analysis was performed using the Maximum Likelihood method in MEGA, with 1000 bootstrap replications. The tree was rooted with *Alternaria alternata* strain YJY-3. Isolate A4 clustered with *P. simplicissimum* isolate LN1, forming a moderately supported clade with a bootstrap value of 58%.

3.2.2.2 Hyphal structure identification of P. simplicissimum isolate A4

The hyphal structures of *P. simplicissimum* A4 were observed under a light microscope at 40x magnification to study its main morphological features. The hyphae were septate and branched, showing typical asexual reproductive structures. Conidiophores were clearly visible, growing from the hyphae and acting as stalks that supported the spore-forming parts. These ended in brush-like groups of phialides (flask-shaped cells) that produce conidia (spores). The phialides were attached to metulae, which are smaller branches that helped form the characteristic brush shape. At the base of the conidiophores were stipes, anchoring the structures to the mycelium. These features match what is commonly described for *P. simplicissimum*, supporting the identification of the strain (Figure 3.2).

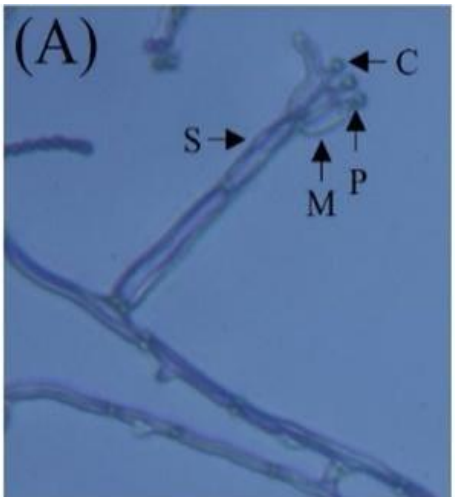


Figure 3.2 The microscopic view of the hyphal structures of the fungal endophyte *P. simplicissimum* isolate A4. (A) Monoverticillate hyphal structure of the A4. C, conidiophores; P, phialide; M, metulae; and S, stipe.

3.2.3 Whole genome assembly and gene prediction of *P. simplicissimum* A4

P. simplicissimum A4 genome consists of 39 Mb assembled in 196 contigs with a 50.61 % GC content. The contig N50 was 3,965.722 kb, with a maximum contig length of 6,478,589 bp (Table 3.2). The BUSCO completeness values for *P. simplicissimum* A4 were estimated to be 96 % with a hit against 4,191 fungal genes. AUGUSTUS predicted the occurrence of 11,862 protein coding genes (Table 3.2).

Table 3.2 The main assembly and annotation features of *P. simplicissimum* A4.

Total genome size	39 Mb
GC content	50.61%
Number of contigs	196
N_{50} contig length	3,965.722 kb
Maximum contig size	6,478,589 bp
Hits against fungal genes	4,191
Number of protein-coding genes	11,862

3.2.4 Biocontrol gene and protein prediction of *P. simplicissimum* A4

Genome mining of *P. simplicissimum* A4 genome showed that 0.42 % of the translated proteins were associated with biological control of fungal pathogens. Out of the proteins associated with biological control, 36.37 % were associated with mycoparasitism which included β-N-acetylglucosaminadase (*PMG11_03254*), β-glucanase (*PMG11_01697*), and glucosidase (*N7496_009503* and *N7496_009908*) (Table 3.3), and 63.64 % were associated with detoxification such as such as ABC transporter (*PMG11_03182*, *IFM46972_11441*, and *N7496_003423*), copper amino oxidase (*PENSUB_4772*), and thioredoxin peroxidase (*N7539_001448*, *PRDX5 PEBR_01141*, and *TRR1 PEBR_18176 PMG11_04671*) (Table 3.3). Protein characterization of the genome of *P. simplicissimum* A4 showed that the proteins possessed numerous biological and molecular functions and were located in various organelles (Table S3.1).

Table 3.3 Biocontrol proteins from *P. simplicissimum* A4 associated with mycoparasitism and detoxification.

Protein Contig Number	Gene Name	Protein Name	Identity (%)	e-value	Blast ID	Uniprot ID	Organism	Length (aa)	Mass (Da)
Proteins associated with mycoparasitism									
g4032	PMG11_03254	Putative β-N-acetylglucosaminidase	95.811	0.0	CEO58537	A0A0F7VGY8_PENBI	*P. brasilianum*	932	101689
g8370	PMG11_01697	AA9 family lytic polysaccharide monooxygenase (Endo-β-1,4-glucanase)	95.582	4.78×10^{-177}	CEJ55439	A0A0F7TF60_PENBI	*P. brasilianum*	249	26185
g5610	N7496_009503	Glucosidase	96.947	1.52×10^{-85}	KAJ5363790.1	A0A9W9V277_9EUROO	*Penicillium cataractarum*	664	76454
g5662	N7496_009908	α-glucosidase	96.891	0.0	KAJ5364195.1	A0A9W9V2K5_9EURO	*P. cataractarum*	579	67042
Proteins associated with detoxification									
g4103	PMG11_03182	Putative ABC transporter (Eurofung)	95.203	0.0	CEO58458	A0A0F7V973_PENBI	*P. brasilianum*	278	30682
g6902	IFM46972_11441	ABC transporter G family member 14	97.413	0	GFF59643	A0A8H3XSB0_9EUROO	*Aspergillus udagawae*	1468	163184
g948	N7496_003423	ABC transporter ATP-binding protein	97.470	0.0	KAJ5380995.1	A0A9W9VIT8_9EUROO	*P. cataractarum*	751	83285
g312	PENSUB_4772	Amine oxidase	97.365	0.0	OKP09848	A0A1Q5UBI9_9EUROO	*Penicillium subrubescens*	806	90045

g6805	N7539_001448	Thioredoxin-dependent peroxiredoxin (Thioredoxin peroxidase)	96.078	9.19×10^{-97}	KAJ5492702.1	A0A9W9XGN9_9EURO	*Penicillium diatomitis*	214	23396
g7629	PRDX5PEBR_01141	Peroxiredoxin-5, mitochondrial	96.648	4.95×10^{-127}	OOQ91118	A0A1S9S1Q1_PENBI	*P. brasilianum*	179	19089
g7742	TRR1PEBR_18176PMG11_04671	Thioredoxin reductase	95.588	0.0	OOQ87236	A0A0F7VDD1_PENBI	*P. brasilianum*	340	36317

3.2.5 Biosynthetic gene cluster (BGC) prediction of *P. simplicissimum* A4

P. simplicissimum A4 genome consists of 39 Mb, of which, 4.3 % are genes encoding the respective BGCs with a total of 1,675,882 bases. The genome of *P. simplicissimum* A4 showed the presence of 38 BGCs with varying degrees of similarity and biological activity, where 15 were aligned to known clusters and 23 are unknown. The similarities to known clusters ranged from high to low; where clavaric acid, alternapyrone, and choline showed 100 % similarity, flavunoidine showed 87 % similarity, and acetylaranotin showed 80 % similarity (Table 3.4). BGCs with low similarities included nidulanin A with a 75 % similarity, followed by squalestatin S1, waikikiamide A, patulin, penigainamide A, shearinine D, iijiquinone, huperzine A, aurofusarin, and paraherquamide ranging from 60 % to 13 % similarity (Table 3.4).

Table 3.4 Biosynthetic gene cluster (BGC) predictions of *P. simplicissimum* A4 using antiSMASH.

Metabolite Class	Metabolite Name	Contig Number	Similarity (%)	From	To
Isocyanide	Unknown	22	-	370,800	412,962
Terpene	Clavaric acid		100	1,334,577	1,358,655
T1PKS	Aurofusarin		18	1,541,271	1,587,068
Terpene	Unknown		-	2,305,648	2,327,171
T1PKS	Unknown		-	4,460,573	4,508,079
Terpene	Squalestatin S1		60	4,932,936	4,954,544
NRPS-like	Unknown	58	-	77,049	121,034
T1PKS, NRPS	Unknown		-	294,822	347,121
T1PKS	Unknown		-	1,081,222	1,129,378
NRP-metallophore, NRPS	Unknown		-	1,363,741	1,423,998
T1PKS	Unknown		-	1,792,219	1,843,120
T1PKS, Indole	Unknown		-	3,401,985	3,462,620
NRPS-like	Unknown		-	3,487,802	3,531,092
NRPS	Unknown		-	3,534,821	3,580,708
T1PKS	Alternapyrone	65	100	43,350	91,768
Indole, NRPS	Paraherquamide		13	120,173	170,695
Terpene	Unknown		-	469,784	490,868
βlactone	Unknown		-	1,560,122	1,591,321
Terpene, Indole	Shearinine D	93	27	1,318,424	1,347,156
T1PKS	Unknown		-	2,991,473	3,039,580
T1PKS	Waikikiamide A	104	54	27,620	74,094

T1PKS	Patulin		46	479,711	525,780
NRPS, T1PKS	Penigainamide A		30	3,162,833	3,232,176
NRPS-like	Unknown	123	-	928,914	974,570
T1PKS	Unknown		-	1,731,707	1,778,822
Terpene	Unknown	183	-	176,578	197,709
NRPS-like	Unknown		-	1,612,894	1,657,552
NRPS-like	Unknown		-	2,775,431	2,818,758
NRPS	Acetylaranotin		80	3,375,727	3,419,960
NRPS	Unknown		-	3,884,819	3,935,040
βlactone	Unknown		-	5,880,084	5,904,532
T1PKS	Unknown		-	5,920,718	5,981,994
NRPS-like	Choline	194	100	995,266	1,039,099
NRPS-like	Unknown		-	2,131,625	2,174,930
NRPS	Nidulanin A		75	2,278,129	2,336,884
Terpene, NRPS	Flavunoidine		87	2,373,249	2,417,293
T1PKS	Iijiquinone		25	3,475,104	3,532,903
T1PKS	Huperzine A	245	23	1,495,339	1,543,388

The genome of *P. simplicissimum* A4 showed the presence of 5 BGC with similarities to known clusters above 80 %, where clavaric acid, alternapyrone, and choline showed 100 % similarity, flavunoidine showed 87 % similarity, and acetylaranotin showed 80 % similarity (Table 3.5).

Table 3.5 Biosynthetic gene cluster (BGC) predictions of *P. simplicissimum* A4 using antiSMASH.

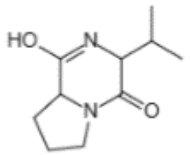
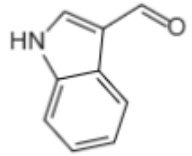
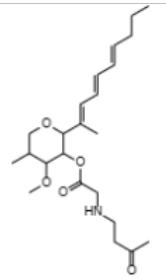
Region	Metabolite Class	Metabolite Name	Gene Contig Number	Similarity to Known Clusters (%)	From	To
14.2	Terpene	Clavaric acid	22	100	1,334,577	1,358,655
44.1	PK	Alternapyrone	65	100	43,350	91,768
188.4	NRP	Acetylaranotin	183	80	3,375,727	3,419,960
129.1	NRP	Choline	194	100	995,266	1,039,099
29.4	Terpene+NRP	Flavunoidine		87	2,373,249	2,417,293

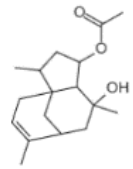
3.2.6 Untargeted metabolome sequencing of *P. simplicissimum* A4 using LC-QTOF-MS/MS

The investigation of the methanol extract of *P. simplicissimum* A4 using LC-QTOF-MS/MS identified a total of 5497 spectra (Figure S3.1). After filtering, the following compounds were identified: 4 SMs previously isolated from *Penicillium* species with antifungal activity against phytopathogens, 22 were previously isolated from various fungal species with antifungal activity against phytopathogens (Table S3.2). Additionally, a total of 16 metabolites previously extracted from various fungal species with known antifungal activity against human fungal pathogens (Table S3.3) and 476 metabolites were previously isolated from numerous fungal species with different functions including anti-diabetic, anti-cancer, and antibacterial activity (Table S3.4). Furthermore, 994 identified metabolites were from different organisms such as plants, cyanobacteria, proteobacteria, and archaeplastida, and compounds with no specific species information. Lastly, a total of 3985 compounds had no information.

The untargeted metabolomic analysis of *P. simplicissimum* A4 showed the presence of 26 metabolites previously isolated from various fungal species which showed antifungal activity against phytopathogens. Compound 1 was detected at a retention time (Rt) of 8.09 and 8.31 (Table 3.6) with a m/z 197.1281 and 197.1282 daltons, respectively, a chemical formular of $C_{10}H_{16}N_2O_2$, a MW of 196.2466 g/mol and was found to be produced by *Penicillium* species previously identified as 3-(propan-2-yl)-octahydropyrrolo[1,2-a]pyrazine-1,4-dione (Table 3.6). Compound 2 was detected at a Rt of 8.43 (Table 3.6) with a m/z 146.0599 daltons, a chemical formular of C_9H_7NO, a MW of 145.1583 g/mol and was found to be produced by fungal species such as *Penicillium vinaceum, Epichloe festucae*, and *Lactarius deliciosus* previously identified as 3-formylindole. Compound 3 was detected at a Rt of 13.15 (Table 3.6) with a m/z 408.2741 daltons, a chemical formular of $C_{23}H_{37}NO_5$, a MW of 407.5445 g/mol and was found to be produced by *Penicillium* species and classified as (+)-Ro 09-1545 (Table 3.6). Compound 4 was detected at a Rt of 14.52 (Table 3.6) with a m/z 279.1956 daltons, a chemical formular of $C_{17}H_{26}O_3$, a MW of 278.3872 g/mol and was previously extracted *Penicillium bilaiae* and identified as penicibilaene B (Table 3.6).

Table 3.6 Biocontrol-related secondary metabolites from *P. simplicissimum* A4 identified by LC-QTOF-MS/MS.

Compound ID	Compound Name	MW (g.mol)	Chemical Formulae	Error ppm	Metabolite Class	Precasor m/z [M+H]+	Associated Fungal Species	Chemical Structure	Associated Species Reference
1	3-(propan-2-yl)-octahydropyrrolo[1,2-a]pyrazine-1,4-dione	196.2466	$C_{10}H_{16}N_2O_2$	5.566; 6.4405	αamino acids and derivatives	197.1281; 197.1282	*Penicillium; Penicillium cluniae; Alternaria alternata; Aspergillus* species*; Cladobotryum varium; Penicillium herquei*		Oliveira et al. (2009); TEZUKA et al. (1994)
2	3-formylindole	145.1583	C_9H_7NO	5.555	Indoles	146.0599	*Penicillium vinaceum; Epichloe festucae; Lactarius deliciosus*		Yue et al. (2000)
3	(+)-Ro 09-1545;Ro 09-1545	407.5445	$C_{23}H_{37}NO_5$	5.2742	αamino acid esters	408.2741	*Penicillium*		Matsukuma et al. (1992)

4	Penicibilaene B	278.3872	$C_{17}H_{26}O_3$	5.7183	Tertiary alcohols	279.1956	*Penicillium bilaiae*		Meng et al. (2014)

3.3 Discussion

3.3.1 Species identification and morphological characterization of *P. simplicissimum* A4

Fungal endophytes are rich reservoirs of numerous phytohormones and phytochemicals (Suresh et al., 2020). Endophytes residing within plants contribute to the synthesis of various novel metabolites that possess major bioactive potential (Ferreira et al., 2017; Suresh et al., 2020; Toghueo, 2019). Several bioactive compounds are produced under abiotic and biotic stressed conditions during the mutualistic interactions between host plants and endophytic fungi causes an increase in the survival of both organisms. The Internal Transcribed Spacer (ITS) region is the most used region to identify endophytes as it is regarded as the universal DNA marker for fungal identification (Mkumbe et al., 2018; Schoch et al., 2012). From this study, phylogenetic analysis showed that the fungal endophyte clustered with *P. simplicissimum* thus confirming its identity (Table 3.1; Figure 3.1). Ezeonuegbu et al. (2022) isolated multiple *Penicillium* species including *P. simplicissimum*, *Penicillium citrinum*, and *Penicillium shearii* and used ITS sequencing to phylogenetically analyse and characterize them for species-level identification. Moreover, Ghosh and Pal (2021) observed the species-level identification of *P. simplicissimum* strain Bar2 using ITS sequencing. Furthermore, Ezeobiora et al. (2023) isolated and identified endophytic *Penicillium* species from medicinal plants from Nigeria based on ITS regions. Anelli et al. (2018) isolated *Penicllium gravinicasei* from cave cheese in Italy and observed that it formed a clade with *Penicillium parvulum* and *Penicillium cinnamopurpureum* with 92 % bootstrap support. Lastly, Kamil et al. (2021) isolated *Penicillium* isolates from India and used morphology and ITS sequencing to phylogenetically analyse them. These authors have shown that ITS sequencing can be successfully utilized for species identification in the *Penicillium* genus.

The reproductive structure of *Penicillium* species may be monoverticillate, biverticillate, terverticillate or quarterverticillate and it encompasses fertile hyphae, rami, stipes, metulae, ramuli, conidia, and phialides. The shape of phialides may be acerose which are cylindrical, or ampulliform which are flask-shaped with short and long collula. Its conidia are ornamented, variable in size and shape and may be pigmented blue or green in colour (Kamil et al., 2021). The hyphal structures of *P. simplicissimum* A4 was used for positive species identification using previous studies by Oh et al. (2011); Visagie et al. (2014). Based on these studies the conidiophores, phialide, metulae and the stipe were positively identified (Figure 3.2). Similarly to our study, Ezeonuegbu et al. (2022) microscopically observed that the *Penicillium* species

namely *P. simplicissimum*, *P. citrinum*, and *P. shearii* possessed septate hyphae with unbranched chains of circular conidia. Furthermore, Ghosh and Pal (2021) microscopically showed that *P. simplicissimum* strain Bar2 possessed distinct septate, mycelium, hyphae, conidia, and condiophores belonging to the genus *Penicillium*.

3.3.2 *P. simplicissimum* A4 proteins associated with biological control of fungal pathogens

Fungal endophytes possess sophisticated mechanisms to inhibit the growth of fungal pathogens (Deshmukh et al., 2018). A good example of this sophisticated mechanism is the production of proteins and metabolites (Latz et al., 2018). From this study, different proteins associated with mycoparasitism, and detoxification activity were identified (Table 3.3). For example, β-N-acetylglucosaminadase (*PMG11_03254*), β-glucanase (*PMG11_01697*), and glucosidase (*N7496_009503* and *N7496_009908*) were associated with mycoparasitism, and ABC transporter (*PMG11_03182*, *IFM46972_11441*, and *N7496_003423*), copper amino oxidase (*PENSUB_4772*), and thioredoxin peroxidase (*N7539_001448*, *PRDX5 PEBR_01141*, and *TRR1 PEBR_18176 PMG11_04671*) were associated with detoxification.

3.3.2.1 P. simplicissimum A4 proteins associated with mycoparasitism

3.3.2.1.1 β-N-acetylglucosaminadase facilitates chitin degradation of fungal pathogens' cell walls (CWs)

The fungal cell wall (FCW) is a dynamic and important fungal organelle which confers plasticity, facilitate alterations in morphology, protects the cell against deleterious environmental factors, and anchors proteins of diverse functions (Brauer et al., 2023). Thus, the FCW is vital in facilitating the interaction between the fungus and its environment and contains numerous molecules that perform hydrolysis which play significant roles in specific niche colonization (Brauer et al., 2023; Garcia-Rubio et al., 2020). From this study, *PMG11_03254* gene encoding for β-N-acetylglucosaminidase was identified in *P. simplicissimum* A4 (Table 3.3). Zhang et al. (2009) reported that NAGase gene produced by *C. cupreum* was overexpressed during a mycoparasitic interaction with *Rhizoctonia solani*. Additionally, Mamarabadi et al. (2009) isolated the *Cr-nag1* gene from *C. rosea* and showed that it possessed antagonistic activity against *Fusarium culmorum*. The authors also observed NAGase activity of *C. rosea* was expressed on medium with *F. culmorum* CW or chitin as its sole carbon source and supressed on medium with high glucose content. Roberti et al. (2002) recommended that NAGase activity may play a role in the explanation of parasitism of the antagonists against *Fusarium* species.

3.3.2.1.2 Glucanase facilitates glucan degradation of fungal pathogens' CWs

β-glucan is one of the main constituents of the FCW and is the main structural component of fungal extracellular sheath and CWs (Geoghegan et al., 2017; Jiang et al., 2024). Due to glucan being a major component of the CW, cell wall degrading enzymes (CWDE) are mainly glucanases (Daguerre et al., 2014). β-glucanases are divided into β-1,3, β-1,4, β-1,6, β-1,3, and other types depending on the glycosidic bonds of glucan (Jiang et al., 2017). They are further divided into exo-β-glucanases and endo-β-glucanase due to the cleavage sites located within the carbohydrate chains. From this study, *PMG11_01697* gene encoding for glucanase enzyme was identified in *P. simplicissimum* A4 (Table 3.3), Chatterton and Punja (2009) identified *glu1* gene encoding for β-1,3-glucanase produced by *C. rosea* f. f. *catenulate* possessed biocontrol activity against *Pythium aphanidermatum* and *Fusarium oxysporum* f. sp. *radicis-cucumerinum*. The authors showed that the enzyme inhibited *F. oxysporum* germination and growth of conidia as well as the growth of *P. aphanidermatum*. Jiang et al. (2024) identified two novel glucanases denoted *Cgglu17A* and *Cgglu16B* from the supernatant of *C. globosum* and observed that it possessed antifungal activity against *Fusarium sporotrichioides* MLS-19 via the hydrolysis of its CW.

3.3.2.1.3 Glucosidase facilitates glucose degradation

β-glucosidases are among the most important enzymes responsible for the degradation of glucose via hydrolysis (Almeida et al., 2015). β-glucosidases are produced by yeasts, animals, fungi, and bacteria (Molina et al., 2018; Singh et al., 2016a), however, β-glucosidases produced by fungi are more desirable due to their high activity, and thermal and pH stability (Syafriana et al., 2014). The genes, *N7496_009503* and *N7496_009908* encoding for glucosidase enzymes was identified in *P. simplicissimum* A4 (Table 3.3), Yoon et al. (2007) examined 106 *Penicillium* species for their ability to degrade cellobiose. The study showed that β-glucosidase activity of all tested *Penicillium* species was generally strong, however, *P. citrinum*, *Penicillium charlesii*, *Penicillium manginii* and *Penicillium aurantiacum* exhibited higher production of β-glucosidase activity. Chen et al. (2020) observed that magnoflorine based on α-glucosidases may be a potential antifungal target for *Candida albicans* and it showed no significant toxicity to human cells. Joo et al. (2010) isolated and identified *Penicillium pinophilum* KMJ601 with high β-glucosidase activity. The authors showed that the internal amino acid sequences of β-glucosidase from *P. pinophilum* exhibited major homology with hydrolases from the glycoside hydrolase family 3.

A wide range of compounds produced by antagonistic fungi have been isolated and identified from various mycoparasitic fungal species. These virulence compounds include various enzymes such as cellulases, chitinases, proteases, and glucanases (Hutauruk & Pinem, 2020; Molla et al., 2022; Puig & Cumagun, 2019; Win et al., 2021; Zhao et al., 2021). However, a successful interaction between mycoparasitic or antagonistic organisms with plants is one which activates defence mechanisms in plants for the protection of fungal structures and metabolisms as well as cell detoxification. These genes associated with mycoparasitism is the conjectured mode of action that *P. simplicissimum* A4 uses for the biological control of fungal pathogens.

3.3.2.2 P. simplicissimum A4 *produces proteins associated with detoxification*

3.3.2.2.1 ATP binding cassette (ABC) transporter proteins facilitate detoxification in the presence of fungal pathogens

Effective biocontrol interactions often demand that the beneficial microorganisms involved are not affected by the antibiotics produced by phytopathogens or themselves, plant antimicrobial compounds, or synthetic compounds (Ruocco et al., 2009). There are limited reports regarding the isolation and characterization of ABC transporter genes in biocontrol *Penicillium* species. From this study, two ABC transporter genes, *PMG11_03182* and *N7496_003423*, and one characterized ABC transporter-G gene *IFM46972_11441* were identified (Table 3.3). Shen et al. (2023) reported 45 ABC transporter protein family members identified in *Penicillium digitatum*. A bioinformatics analysis of the *P. digitatum* genome showed that when comparted to yeast, its genome and ABC members has undergone major expansion and that the ABC transporter proteins were distributed between seven subfamilies namely B-G and I. Interestingly, the study showed that the *PdABCG3*, *PdABCG6*, and *PdABCG9* genes were involved in drug detoxification. Trigui-Lahiani et al. (2021) characterized genes encoding ABC proteins by cloning a gene encoding an ABC transporter from *Penicillium occitanis* using a PCR based approach followed by a genomic library screening. The study showed that most of the ABC transporter proteins in *P. occitanis* belonged to the ABCC subfamily (41%) followed by ABCG (26%) and ABCB (20.7%) subfamilies. The authors isolated a novel gene encoding one of the ABC transporters of *P. occitanis*. Using a comparison to other ABC genes, the authors observed that the isolated gene named *Podr1* belongs to the PDR subfamily associated with antifungal resistance of filamentous fungi.

Biocontrol agents/mycoparasites utilizes pleiotropic drug resistance (PDR) proteins in their fight against plant fungal pathogens. The PDR transporter encoded by the gene abcG5 from *C.*

rosea is involved in its tolerance to zearalenone which is a mycotoxin produced and secreted by the fungal pathogen *Fusarium graminearum*. This transporter is also involved in xenobiotic tolerance (Dubey et al., 2014). This result suggested that the ABCG transporter present in the genome of *P. simplicissimum* A4 also possess detoxification functions (Table 3.3). Ruocco et al. (2009) suggested that ABC transporters are important proteins in numerous interactions by *Trichoderma* biocontrol strains with other microorganisms and an antagonistic environment. The ABC transporter protein in the study was shown to possess pathogen toxin resistance and may avoid autointoxication of the biocontrol agent via the production of inhibitory substances during the antagonistic interaction.

3.3.2.2.2 Copper amine oxidase (CuAO) assists in the detoxification of ROS in P. simplicissimum A4

CuAOs participates in the regulation and breakdown of biologically active amines and play critical roles in various organisms (Shepard & Dooley, 2015). In this study, *PENSUB_4772* gene encoding amine oxidase with copper binding activity was identified in *P. simplicissimum* A4 (Table 3.3). The enzyme is involved in detoxification and is responsible for the reduction of O_2^- and produces H_2O_2 which is involved in signalling (Rhee, 2006). This detoxification may assist the growth and survival of *P. simplicissimum* A4 in the presence of fungal pathogens.

3.3.2.2.3 Thioredoxin peroxidase assists in the detoxification of ROS in P. simplicissimum A4

The thioredoxin system one of the key thiol antioxidant systems in cells (Ma et al., 2018), and it comprises of thioredoxins (Trxs), thioredoxin peroxidases also known as the thiol-specific antioxidant protein (Tsa), thioredoxin reductases (Trr), and nicotinamide adenosine dinucleotide phosphate (NADPH) as a proton donor (Hazra et al., 2024). Three genes, *N7539_001448*, *PRDX5 PEBR_01141*, and *TRR1 PEBR_18176 PMG11_04671*, encoding thioredoxin peroxidase, peroxiredoxin, and thioredoxin reductase, respectively, identified in *P. simplicissimum* A4 (Table 3.3). These genes are involved in detoxification, and numerous biochemical and physiological processes including protein folding, synthesis of deoxyribonucleotides, protein and DNA repair, sulfur metabolism and the detoxification of ROS via the reduction of peroxides such as H_20_2 to harmless products (Cintra et al., 2017; Kang et al., 2019b; Ma et al., 2018). Additionally, Trxs are necessary for genome stability and cell longevity (Ma et al., 2018). Thon et al. (2007) characterized the thioredoxin system of *A. nidulans* and observed the presence of thioredoxin A (*AnTrxA*) with a thioredoxin active site motif (WCGPC) which is encoded by the *trxA* gene, as well as a corresponding thioredoxin reductase (*AnTrxR*) which is encoded by the *trxR* gene. The authors observed that when

combined with *E. coli*, the recombinant proteins *AnTrxA* and *AnTrxR* reduced insulin and oxidized glutathione in an NADPH-dependent manner which indicated that the redox system was functional.

3.3.3 Biosynthetic gene clusters (BGCs) associated with *P. simplicissimum* A4

Fungi are known to synthesize highly diverse SMs which are bioactive molecules that are necessary for growth or other important processes. These SMs possess critical roles in the potential interactions and ecological characteristics of fungi (Frisvad, 2018; Rokas et al., 2020).

AntiSMASH analysis predicted putative BGCs which showed low similarities to known gene clusters, with the exception of terpene which showed 100% similarity to clavaric acid, PK with 100% similarity to alternapyrone, a non-ribosomal peptide (NRP) with 80% and 100% similarity to acetylaranotin and choline, respectively, and Terpene+NRP with 87% similarity to Flavunoidine (Table 3.4; Table 3.5). Zhao et al. (2021) used quantitative real time-PCR analysis and observed that when *Penicillium bilaiae* isolate 47M-1 was co-cultured with *F. oxysporum* in sesame plants, it had resulted in the upregulation of response-related genes such as *NPR1*, *Coi1*, *PR1*, *PR2* and *PR3*. The *NPR1* and *Coi1* marker genes were observed to be involved in disease resistance signalling pathways, and the *PR1*, *PR2* (β-1,3-glucanase) and *PR3* (chitinase) genes were pathogenesis-related proteins in sesame plants. Roxo et al. (2024) sequenced the draft genome of *Penicillium pancosmium* MUM 23.27 isolated from Portuguese raw honey. They observed that *P. pancosmium* possessed 26 BGCs with 4 showing significant similarities to A/chaetoglobosin C, chaetoglobosin, nidulanin A, squalestatin S1, and YWA1. Byers et al. (2023) identified 12 BGC classes in four *Penicillium* strains. These classes included T1PKS, T3PKS, terpenes, NRPS, β-lactones and indoles. They reported that the BGCs in each *Penicillium* genome was 39, while 44 BGCs were identified in *Penicillium* ks20 F15 and *Penicillium* ks20 F20. These studies agreed with our findings where the BGC for clavaric acid was identified in the genome of *P. simplicissimum* A4 (Table 3.4; Table 3.5). Additionally, Byers et al. (2023) observed the BGC identified shared 100 % similarity to known BGCs encoding for the production of clavaric acid. The common SM-BGCs detected in the genomes of the *Penicillium* strains were PKSs, NRPSs and terpenes—a finding that agreed with our study as well as previous research by Carro et al. (2018); Figueiredo et al. (2022); Wang et al. (2022c). Furthermore, the BGCs responsible for the production of clavaric acid has also been detected in *Trichoderma* isolates which showed antagonism towards *Erwinia mallotivora* a pathogen responsible for papaya dieback (Tamizi et al., 2022).

Alternapyrone was also identified in the genome of *P. simplicissimum* A4 (Table 3.4; Table 3.5). Courtial et al. (2022) reported that the genome of *Alternaria dauci* contained genes responsible for the production of SMs such as alternapyrone, tentoxin, alternariol, aslaniol, melanin, 6-methylsalicylic acid, ferricrocin, and extracellular siderophore. Li et al. (2022) isolated five new compounds from the plant-derived fungus *Alternaria* sp. HM 134 namely alternafurones A (1) and B (2), alternapyrones M-O. Additionally, Fujii et al. (2005) cloned five PKS gene alt1-5 from *Alternaria solani* into the fungal host *A. oryzae*. The authors showed that *alt1*, *2*, and *3* genes encode for the cytochrome P450s, the *alt4* gene encodes for the FAD-dependent oxygenase/oxidase, and the *alt5* gene encodes for the type I reduced-type PKS, named PKSN, with a *C*-methyltransferase domain. The authors observed that the *alt5* gene is responsible for the production of alternapyrone under an α-amylase promoter. Since there are genes for three cytochrome P450s (*alt1-3*) and an oxidase (*alt4*) adjacent to the *alt5* gene in the *A. solani* genome, this may modify alternapyrone to create a different metabolites (Noar & Daub, 2016). Li et al. (2018) also reported that alternapyrones B–F from *Parastagonospora nodorum* showed antifungal, antibacterial, antitumor, antiparasitic, anti-germination activities. Interestingly, genes that are similar to *alt5* gene from *A. solani* are found in several fungal genomes (Phakeovilay et al., 2022).

Acetylaranotin which was identified in our present study is an epipolythiodiketopiperazine (ETP) SM possessing a wide range of bioactivities (Sun et al., 2018b). ETPs range of bioactivities including anticancer, antibacterial, antiviral, antimalarial, antiallergic, and cytotoxic effects are due to their unique disulphide bridge via two reported mechanisms. These mechanisms are: (1) production of ROS via the redox cycling between the dithiol and disulphide forms; and (2) the inactivation of free thiol-containing proteins due to the production of intermolecular disulfide bonds and disulphide exchange (Boyer et al., 2013; Nicolaou et al., 2012). Acetylaranotin has been shown to possess many bioactivities such as the SM produced by *Nectria haematococca* Berk which showed antifungal activity (Suzuki et al., 2000), the antiproliferative activity of the acetylaranotin produced by *Aspergillus* sp. KMD 901 (Choi et al., 2011), and antiviral activity of the *A. aureus* (Neuss et al., 1968), and its BGC was observed in the genome of *P. simplicissimum* A4 (Table 3.4; Table 3.5).

Flavunoidine also identified in this study is a new alkaloidal terpenoid possessing a unique tetracyclic cage structure first isolated and identified from the heterologous host *A. nidulans* which expressed a NRPS/TC *flv* gene cluster (Yee et al., 2019). Non-ribosomal peptide synthetase-terpenoids (NRPS-terpenoids) are produced by NRPS and terpene cyclase

(TC) belonging to the class of non-PK meroterpenes (Yee et al., 2019). The tetracyclic core of flavunoidine is synthesized by the TC P450 *FlvD* and *FlvE* genes and is connected to dimethylcadaverine via a second TC *FlvF* via an axial C–N bond (Han et al., 2021). When *Aspergillus flavus* was cultured under general cultivation systems it had resulted in no production of flavunoidine and no bioactivities antifungal activity, cytotoxicity, and antibacterial activity, were identified (Hur et al., 2023; Tararina et al., 2022).

Finally, choline was also identified as part of the BGCs from this study. Choline is a secondary metabolite critical for the growth of filamentous fungi (Pokharel et al., 2022). Choline forms part of the membrane phospholipid, phosphatidyl choline (lecithin), and plays a key role in sulphur metabolism in various species in the form of choline-o-sulphate (Markham et al., 1993). It is generally endogenously synthesized, however, choline can be exogenously absorbed by fungi for the compensation of metabolic deficiencies in choline-requiring mutants for example *A. nidulans* and *Neurospora crassa* (Markham et al., 1993). Park and Gander (1998) reported that choline-*O*-sulfate (COS) and glycine betaine (GB) accumulated inside *Penicillium fellutanum* cultured in a low-phosphate medium. Phosphocholine of extracellular peptidophosphogalactomannan is a precursor of intracellular choline derivatives such as the COS and GB. The authors reported that exogenous choline, and intracellular COS and GB are involved in the osmoprotection of *P. fellutanum* under high-osmolarity and low-phosphate conditions.

3.3.4 Secondary metabolites (SMs) identified in *P. simplicissimum* A4 associated with antifungal activity against plant pathogens

The antifungal of SMs identified in *Penicillium* species are well documented in literature (Shafique et al., 2023). In the present study, *P. simplicissimum* A4 was evaluated for its potential to produce antifungal compounds against plant pathogens, and our results clearly showed that *P. simplicissimum* A4 produced different metabolites known for their antifungal activity (Table 3.6). Similar findings was previously reported by Oliveira et al. (2009); Yue et al. (2000) who isolated antifungal compounds from *Penicillium* species to control the growth of phytopathogens. The compound 3-(propan-2-yl)-octahydropyrrolo[1,2-a]pyrazine-1,4-dione, or cyclo(L-Pro-L-Val) was previously isolated from an endophytic *Penicillium* species associated with *Alibertia macrophylla* (Rubiaceae) (Oliveira et al., 2009). The SMs showed very weak antifungal activity against *Cladosporium cladosporioides* and *Cladosporium sphaerospermum*, respectively. Similarly, 1, 3-(propan-2-yl)-octahydropyrrolo[1,2-a]pyrazine-

1,4-dione, or cyclo(L-Pro-L-Val), was extracted from *P. simplicissimum* A4 in our study (Table 3.6).

Another SM, 3-formylindole also known as indole-3-carboxaldehyde was identified in this study. The SM was previously isolated from *Epichloe festucae* in a study by Yue et al. (2000) where it was shown to possess antifungal activity against *Cryphonectria parasitica;* a causative agent of chestnut blight. Also, (+)-Ro 09-1545 identified in this study is a colourless oil with antifungal antibiotic activity previously isolated from the fermentation broth of *Penicillium* sp. NR6564 (Matsukuma et al., 1992). Their study agreed with compound 3 isolated from *P. simplicissimum* A4 (Table 3.6). Matsukuma et al. (1992) tested the antifungal activity of the (+)-Ro 09-1545 compound and reported that the compound inhibited the growth of *S. cerevisiae.*

The genomic and metabolomic characterization of *P. simplicissimum* A4 fungal endophyte isolated from *E. plantaginium* and the subsequent identification of genes and metabolites associated to the biological control of fungal plant pathogens has not been seen. The novelty and special interest in our study is that the genes and metabolites associated to antagonism has been identified in *P. Simplicissimum* A4 in the *Penicillium* genus, which is known for its pathogenicity. The whole genome sequencing revealed proteins associated with biological control, including enzymes involved in mycoparasitism and detoxification. Furthermore, the untargeted metabolomic analysis identified several metabolites that were previously isolated from *Penicillium* species known to exhibit antifungal activity, confirming the potential biocontrol potential of *P. simplicissimum* A4. *P. simplicissimum* A4 represents a promising fungal endophyte with potential antifungal properties, supported by both its genomic and metabolomic profiles. Future studies will involve antifungal assays to determine the inhibitory potential of *P. simplicissimum* A4 against a known fungal plant pathogen, *Fusarium proliferatum*, *in vitro*.

CHAPTER 4

EVALUATING THE BIOCONTROL POTENTIAL OF *PENICILLIUM SIMPLICISSIMUM* A4 AGAINST *FUSARIUM PROLIFERATUM*

4.1 Introduction

Fusarium proliferatum is a ubiquitous fungal pathogen causing wilt and tissue rot disease in a wide range of economically important crops including maize (Gaige et al., 2020), rice (Lei et al., 2019), wheat (Cendoya et al., 2018), and garlic (Chretien et al., 2020). The presence of this pathogen causes a significant crop loss to farmers particular in low income countries where access to grants and farm inputs such as fungicides and pesticides is limited (Chang et al., 2020). These challenges have resulted in food insecurity globally particularly in Africa where the amount of food production is not sufficient to meet the human population requirements. In addition, *F. proliferatum* produces mycotoxins such as fumonisins, fusaproliferin, beauvericin, moniliformin, and fusarins that have been reported to negatively impact human and animal health (Masiello et al., 2021). To ameliorate the challenges caused by the pathogens, different control strategies such as chemical and cultural methods have been adopted by farmers over the years. Chemical control, which involves the use of agrochemical such as pesticides/fungicides, is limited due to challenges such as pesticides residues in crops which have detrimental health impact in human and animals (Chen & Ying, 2015; Zubrod et al., 2019). Secondly, prolonged or overuse of these chemicals have resulted in resistance in most of the pathogens and environmental pollution (Hawkins et al., 2019; Lykogianni et al., 2021). These chemical fungicides accumulates in the soil and disturbs the biological and physiochemical components which has detrimental effects on fauna and flora and overall soil health which in turn has critical impacts on the ecosystem and crop quality (Masiello et al., 2021; Zhou et al., 2024). Finally, the cost of these pesticides makes them unaffordable for most farmers hence this method of control is unsustainable (Syafrudin et al., 2021). Thus, there is an urgent need to use an eco-friendly approach to control the spread of plant pathogens such *F. proliferatum* to avoid the harmful effect of agrochemicals on human, animals and the environment (Fadiji & Babalola, 2020).

Biological control, which involves the use of an organisms or its products/metabolites to control other harmful organisms, has gained more attention over the years due to its benefits

on human and animal health and the environment (Tariq et al., 2020). Biological control is an extremely dependable approach for disease management and control, and it is exceptionally valuable to make an eco-friendly environment. Biological control plays a significant role to manage plant disease without disturbing flora and fauna, it likewise increments the soil fertility (Tariq et al., 2020). Examples of biological control agents (BCAs) include endophytic fungi or bacteria which exist in a commensal or symbiotic relationship with the plants providing protection against pathogens while deriving nutrients and shelter from the plants. They colonize the internal tissues of plants and shape the integral portion of the microbial community that is associated to different types of plants (Zhang et al., 2006a). Various endophytic fungi can produce secondary metabolites (SMs) which may possess antibacterial and antifungal activity that strongly inhibits the growth of pathogenic microorganisms, including but not limited to polyketides (PKs), non-ribosomal peptides (NRPs) as well as enzymes such as chitinases, glucanases *Penicillium* is the most common species of fungi and some exists as endophytes in plant tissues (Liang et al., 2021). Endophytic *Penicillium* species can control economically important plant pathogens via the induction of the host plants systemic resistance or direct mechanisms (Azar et al., 2023a; Jeya et al., 2010; Urooj et al., 2021; Wang et al., 2022a; Yuan et al., 2017; Zhao et al., 2021). Hence, the aim of this study is to investigate the biological control potential of *Penicillium simplicissimum* A4 against *F. proliferatum*.

4.2 Results

4.2.1 *In vitro* antagonistic activity of *P. simplicissimum* A4 against *F. proliferatum*

The antagonistic activity of *P. simplicissimum* A4 against *F. proliferatum* was assayed using the dual culture technique. There was a significant inhibition (78.65 %) of mycelial growth of *F. proliferatum* by *P. simplicissimum* A4 following co-culturing of the pathogen and fungal endophyte for 7 days when compared with the control cultured in the absence of the endophytes (Figure 4.1 A-E).

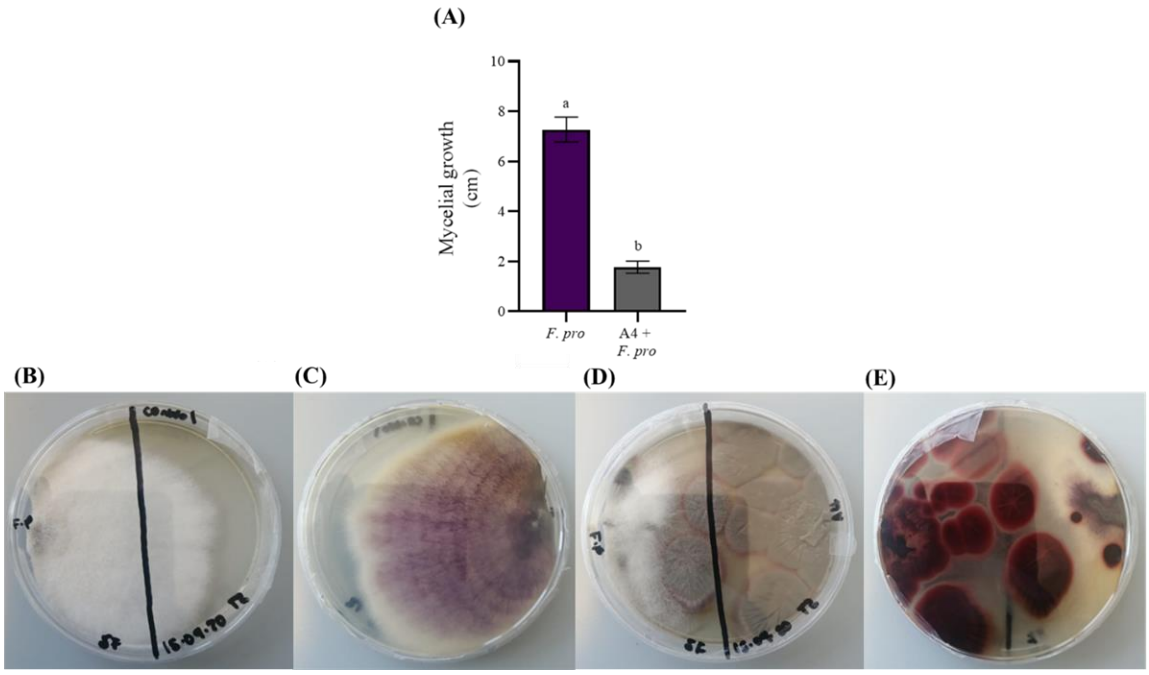


Figure 4.1 Antagonistic effect of *P. simplicissimum* A4 on mycelial growth of *F. proliferatum*. (A) Mycelial growth diameter of *F. proliferatum* in response to *P. simplicissimum* A4 (in cm). (B and C) *F. proliferatum* on PDA media (control). (D and E) Dual- culture of *F. proliferatum* and *P. simplicissimum* A4. Bars with different alphabets are significantly different at $p<0.05$ of triplicate determination.

4.2.2 Effect of *P. simplicissimum* A4 on the polysaccharides and chitin contents of *F. proliferatum*

There was a significant decrease ($P < 0.05$) in the intracellular polysaccharide (47 %) and extracellular polysaccharide (55 %) contents of *F. proliferatum* in the presence of *P. simplicissimum* A4 % relative to the control (Figure 4.2 A and B). Similarly, *P. simplicissimum* A4, decreased the chitin content (35 %) ($P < 0.05$) of *F. proliferatum* following co-inoculation of the organisms for 5 days in PDB (Figure 4.2 C).

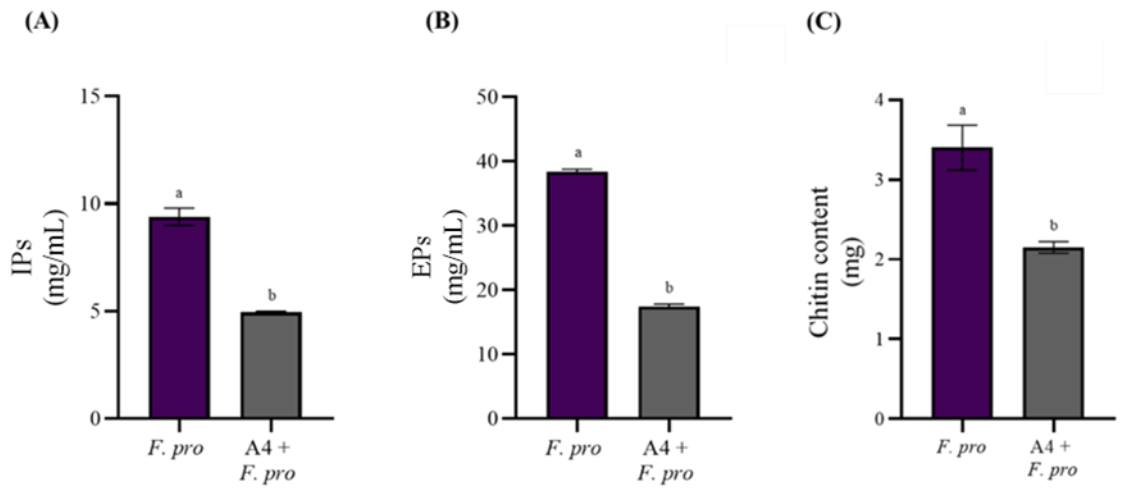


Figure 4.2 Effect of *P. simplicissimum* A4 on the polysaccharides and chitin content of *F. proliferatum*. (A) Intracellular polysaccharide (IPs) content, (B) extracellular polysaccharide (EPs) content, and (C) chitin content of *F. proliferatum* in the presence of *P. simplicissimum* A4. Error bars represent standard error. Bars with different letters indicate statistically significant differences at $P < 0.05$.

4.2.3 Effect of *P. simplicissimum* A4 on the enzymatic activities of *F. proliferatum*

In response to *P. simplicissimum* A4, there was a significant decrease ($P < 0.05$) in endo-β-1,4-glucanse activity (89 %) and exo-β-1,4-glucanase activity (40 %) of *F. proliferatum* relative to the control (Figure 4.3 A and B). Furthermore, there was a significant reduction ($p<0.05$) in the intracellular and extracellular lipase activities of *F. proliferatum* by 63 % and 96 % respectively in the presence of *P. simplicissimum* A4 (Figure 4.3 C and D).

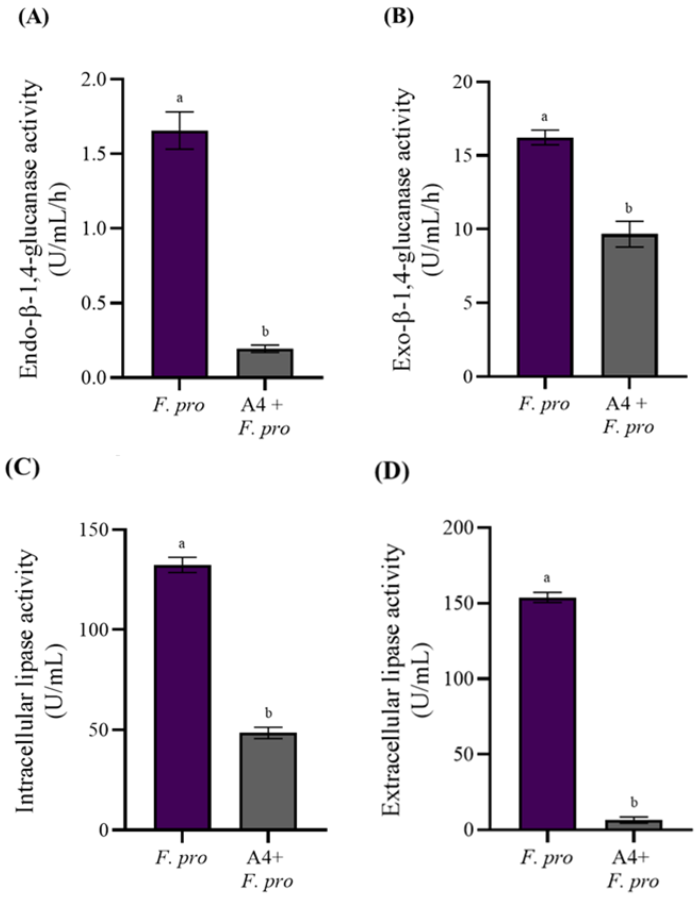


Figure 4.3 Effect of *P. simplicissimum* A4 on the enzymatic activity of *F. proliferatum*. (A) Endo-β-1,4-glucanase activity, (B) exo-β-1,4-glucanase activity, (C) intracellular lipase activity, and (D) extracellular lipase activity of *F. proliferatum* in response to *P. simplicissimum* A4. Error bars represent standard error. Bars with different letters indicate statistically significant differences ($P < 0.05$).

4.2.4 Effect of *P. simplicissimum* A4 on reactive oxygen species accumulation and lipid peroxidation of *F. proliferatum*

In the presence of *P. simplicissimum* A4, there was a significant increase ($p<0.05$) in superoxide (O_2^-) content in *F. proliferatum* relative to the control (Figure 4.4 A). However, there was no significant difference ($P>0.05$) in the hydrogen peroxide (H_2O_2) and MDA content of *F. proliferatum* in the presence of *P. simplicissimum* A4 (Figure 4.4 B and C).

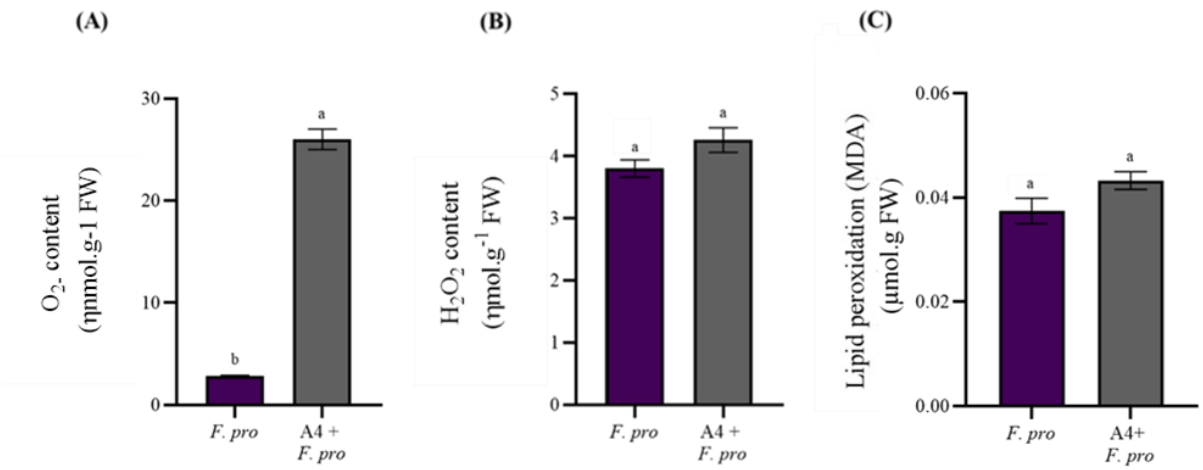


Figure 4.4 Effect of *P. simplicissimum* A4 on ROS-accumulation and lipid peroxidation of *F. proliferatum*. (A) Superoxide (O_2^-) content, (B) hydrogen peroxide (H_2O_2) content, and (C) malondialdehyde (MDA) levels in *F. proliferatum* in the presence of *P. simplicissimum* A4. Error bars represent standard error. Bars with different letters indicate statistically significant differences ($P < 0.05$).

4.2.5 Effect of *P. simplicissimum* A4 on superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities of *F. proliferatum*

The effect of *P. simplicissimum* A4 on the SOD and APX activities of *F. proliferatum* was measured using spectrophotometric assay. In comparison to the control, there was a significant decrease ($p<0.05$) in total SOD and APX activities of *F. proliferatum* following treatment with *P. simplicissimum* A4 (Figure 4.5 A and B).

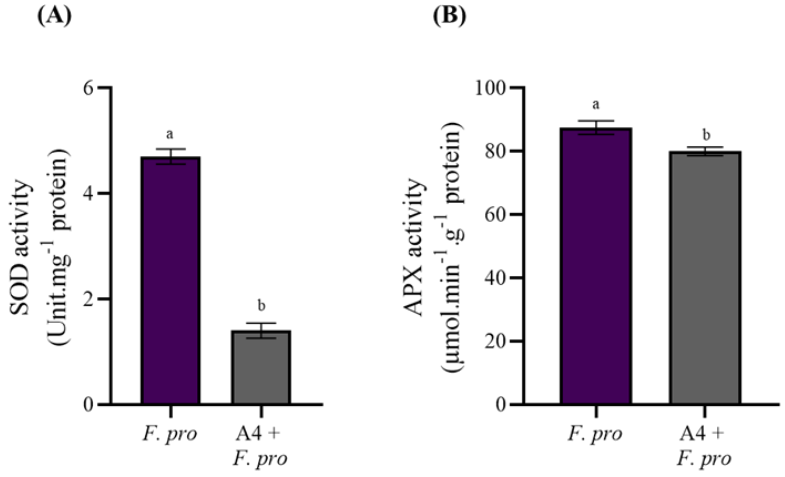


Figure 4.5 Effect of *P. simplicissimum* A4 on total SOD and APX activity of *F. proliferatum*. (A) Changes in SOD activity of *F. proliferatum* induced by *P. simplicissimum* A4. (B) Changes in APX activity of *F. proliferatum* induced by *P. simplicissimum* A4. Bars denote standard error, where bars with different letters indicate statistically significant differences ($P < 0.05$).

4.3 Discussion

4.3.1 *P. simplicissimum* A4 inhibit the mycelia growth of *F. proliferatum*

Plant pathogens affect growth and production of crops resulting in significant yield loss annually (Grote et al., 2021). *Penicillium* sp. has been used for *in vivo* and *in vitro* biocontrol of different phytopathogens Damasceno et al. (2019); Urooj et al. (2018), in this study, the *in vitro* antagonistic activity of *P. simplicissimum* A4 against *F. proliferatum*, a pathogenic fungus that affect a wide range of economically important crops, was evaluated. Fungal endophytes have been successfully used as BCAs against phytopathogens (Damasceno et al., 2019; Mejdoub-Trabelsi et al., 2022; Wang et al., 2022b). The antagonistic assay showed that *P. simplicissimum* A4 inhibited 78.65 % of *F. proliferatum* mycelial growth (Figure 4.1 A-E). Similarly, studies by Damasceno et al. (2019); Urooj et al. (2018); Wang et al. (2022b) have reported on the antagonistic activity of endophytic *Penicillium* against different plant pathogens. Furthermore, Lykholat et al. (2022) isolated six fungal endophytes (*Penicillium expansum*, *Penicillium viridicatum*, *Penicillium hirsutum*, *Penicillium chrysogenum*, *Penicillium cyclopium*, and *Penicillium purpurogenum*) from *Chaenomeles speciosa* fruits and evaluated their antagonistic activity against *Fusarium culmorum* and *Fusarium oxysporum*. Their report showed that all the *Penicillium* sp. inhibited over 50 % of *F. culmorum* and *Fusarium oxysporum* mycelia. Also, Zhao et al. (2021) explored the biocontrol of *F. oxysporum* using *Penicillium bilaiae* isolated from the rhizosphere soil samples of tobacco plants. The results showed that *P. bilaiae* inhibit the growth of *F. oxysporum* mycelia by 81.3 %. Therefore, the report from this study showed that *P. simplicissimum* A4 can be an effective biocontrol candidate against *F. proliferatum.*

4.3.2 *P. simplicissimum* A4 alters the polysaccharide, chitin contents and enzymatic activities of *F. proliferatum*

Antagonistic activities of BCAs can be achieved through various means such as the inhibition or alteration of the growth phase, nutrient source or destruction/disarmament of the pathogens cell wall. From this study, treatment of *F. proliferatum* with *P. simplicissimum* A4 significantly decreased the polysaccharide and chitin contents of the pathogen relative to the control (Figure 4.2 A-C). The ability of *P. simplicissimum* A4 to degrade chitin or polysaccharides depends on the amount of chitinase or glucanase enzymes it produces. Chitin is present in different groups of fungi and constitute an integral components of their cell wall (CW), mycelia and spore membrane structure (El Kady, 2019). The fungal cell wall (FCW) is a vital component of fungal pathogens'interaction or communication with biotic and abiotic environments and thus its

degradation of one of its constituent compounds becomes a controlling strategy in the field (Ehren et al., 2020; Parlindo et al., 2023). During contact with pathogens, endophytic fungi will degrade the pathogen's CW by involving the hydrolytic enzymes it produces, such as chitinase and glucanase. According to Parlindo et al. (2023), besides improving the plant defence system because it degrades chitin as the main component of the CW of fungal pathogens, chitinase also increases plant growth and yield without any negative impact on plants.

Plant pathogenic fungi are able to produce and secrete various extracellular enzymes which enables them to infect/penetrate host tissue. Majority of the enzyme can degrade CW and are therefore considered pathogenicity factors (Paccanaro et al., 2017; Sharafaddin et al., 2019; Yang et al., 2015). Many pathogenic *Fusarium* spp. produces an assortment of these cell wall degrading enzymes (CWDEs) which plays critical roles in their pathogenesis (Chang et al., 2016; Marın et al., 1998; Sharafaddin et al., 2019).

In this study there was a significant decrease ($p<0.05$) in β-1,4-glucanase activity of *F. proliferatum* in the presence of *P. simplicissimum* A4 (Figure 4.3 A and B). Our findings agreed with the report by Li et al. (2016) who observed a suppression of pathogenic factors such as the CWDEs of *F. proliferatum* when an antioxidant inhibitor, butylated hydroxyanisole, was present which had resulted in the decline in the pathogenicity of the fungal pathogen. Additionally, Marei et al. (2012) observed that thymol and (*S*)-limonene strongly inhibited cellulase activity of *F. oxysporum.*

When fungal pathogens come into contact with the host plants, they encounter epicuticular waxes and cuticles encompassing the host epidermal cells (Kikot et al., 2009). Pritsch et al. (2000) observed that lipase degrades the cuticle of plant host cells. Feng (2007); Feng et al. (2005) observed the importance of the lipase enzyme in the pathogenicity of *Fusarium graminearum*. Additionally, Perincherry et al. (2021) observed that several *Fusarium* strains were capable of producing CWDEs, including *F. proliferatum*. The authors showed that the *Fusarium* isolates produced lipase enzymes when infecting resistance and susceptible pea plants. From this study, lipase activity of *F. proliferatum* significantly decreased in the presence of *P. simplicissimum* A4 (Figure 4.3 A and B). Therefore, it can be deduced from this study that *P. simplicissimum* A4 decreased the virulence or pathogenicity of *F. proliferatum* by altering the cellulase, lipase and glucanase activity of the fungi.

4.3.3 *P. simplicissimum* A4 augments oxidative stress and cellular membrane damage in *F. proliferatum*

Interactions between pathogens and microbes causes increase in oxidative stress responses within the susceptible organism resulting in changes in the biochemical processes involves in the survival of the organism (Mattila et al., 2022). In fungi, ROS are critical for intracellular and metabolic homeostasis (Mattila et al., 2022). from this study, the presence of *P. simplicissimum* increases ROS accumulation within *F. proliferatum* (Figure 4.4 A and B). These changes might be the antagonistic effect of the endophyte which resulted in significant inhibition of the pathogen growth, changes in chitin and polysaccharides contents as well as the enzymatic activities of the organism. This study agrees with the report of Kalagatur et al. (2018) who observed a significant increase in ROS accumulation in *F. graminearum* when treated with essential oil extract from *Cymbopogon martini.* The increase in H_2O_2 content of *F. proliferatum* in this study (Figure 4.4 B) agreed with the previous report by Wu et al. (2022) who observed an increase in H_2O_2 content in *Botrytis cinerea* mycelia following treatment with juniper essential oil.

The resultant effect of ROS accumulation in the biological system can lead to deleterious effects such as lipid peroxidation. From this study, treatment of *F. proliferatum* with *P. simplicissimum* A4 resulted in significant increase in lipid peroxidation (Figure 4.4 C). Kalagatur et al. (2018) observed that *F. graminearum* treated with *C. martinii* essential oils had shown an increase in MDA content. Additionally, Wu et al. (2022) observed a major increase in MDA content in *B. cinerea* mycelia that has been treated with juniper essential oil. The increase in lipid peroxidation is proportional to the increase in O_2^- and H_2O_2 content of *F. proliferatum* in the presence of *P. simplicissimum* A4 (Figure 4.4 A and B), which in turn caused membrane damage. It can therefore be postulated that this membrane damage causes cell deformity as well as the loss of interior macromolecules, for example the decrease in polysaccharide and chitin content of *F. proliferatum* (Figure 4.2 A-C). These losses of macromolecules cause unsuitable functioning of the cell membrane (Kalagatur et al., 2018; Ma et al., 2020; Pramila et al., 2012; Tao et al., 2019; Yao et al., 2023).

4.3.4 *P. simplicissimum* A4 interrupts the oxidative defence of *F. proliferatum*

Fungal pathogens are eukaryotic and therefore requires SOD enzymes to detoxify O_2^- present within their intracellular organelles (Mattila et al., 2022; Valenzuela-Cota et al., 2019). Treatment of *F. proliferatum* with *P. simplicissimum* A4 resulted significant decrease in SOD activity of the pathogen (Figure 4.5 A). Antioxidants are scavengers of free-radicals within the

cells and is responsible for the prevention of free-radical mediated processes which includes SOD, as well as numerous peroxidases such as catalase (CAT), glutathione peroxidase (GPX), APX, peroxiredoxin (Kgang et al., 2023; Mattila et al., 2022; Valenzuela-Cota et al., 2019). The observed level of SOD activity in this study may not be sufficient to arrest all the free radicals in the pathogen therefore making them susceptible to the endophytes and ultimately resulting in their dead. A previous study by Kumari et al. (2019) reported a decline in SOD activity of *Alternaria brassicicola* following treatment with silver nanoparticles from cell free filtrates of *Trichoderma viride*. Also, the presence of *P. simplicissimum* A4 resulted in the significant decrease ($p<0.05$) in APX activity of *F. proliferatum* (Figure 4.5 B). This report agreed with the findings of Kumari et al. (2019) who observed a decline in APX activity of *A. brassicicola* treated with silver nanoparticles from cell free filtrates of *T. viride.*

This research study showed that *F. proliferatum* was significantly inhibited by *P. simplicissimum* A4 in that its polysaccharide and chitin content was decreased in response to *P. simplicissimum* A4. The study also showed that the pathogenic factors, such as glucanase and lipase activity, of *F. proliferatum* had significantly declined in response to *P. simplicissimum* A4. The study also showed that in response to *P. simplicissimum* A4, ROS accumulated in *F. proliferatum,* and the antioxidant systems were not able to detoxify the oxidative species within the cells. These results showed that *P. simplicissimum* A4 is a good biological control candidate for the inhibition of fungal plant pathogens. Future studies will focus on the biological control capabilities of *P. simplicissimum* A4 *in planta*.

CHAPTER 5

PENICILLIUM SIMPLICISSIMUM A4 REGULATES MAIZE ROOT GROWTH AND BIOCHEMICAL RESPONSES UNDER *FUSARIUM PROLIFERATUM* INFECTION

5.1 Introduction

Maize (*Zea mays*) is ranked as the second most significant crop and is the most produced cereal crop worldwide (Santpoort, 2020). In addition to its role as a vital food source and a key component in livestock feed, maize is also used in various industrial applications. However, its widespread cultivation and storage make it susceptible to contamination by pathogenic fungal species, including *Aspergillus*, *Penicillium*, and *Fusarium* species being the most prominent contaminants, known for producing harmful 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.

Disease caused by *Fusarium proliferatum* has very high significance due to its economic importance where in excess of 300 million people, in Africa, depend on maize as their primary food source (Santpoort, 2020). *F. proliferatum* 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., 2019; Sun et al., 2018a). As a representative of the section Liseola, the microconidia of *F. proliferatum* are shaped as an ovoid and obovoid. In addition, the microconidia are borne on lined chains and polyphialides (Isack et al., 2014). Maize seed infection with pathogenic fungi causes significant effects such as the loss of the seeds capacity to germinate; thus, the infection with *F. proliferatum* affects the plant seed germination and ultimately leads to the reduction in yield (Galli et al., 2005; Kaur et al., 2020; Masiello et al., 2021). The infected seed that are able to 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 throughout the plant kingdom and offer a direct and indirect defence against pathogenic fungi (Bamisile et al., 2018a; Kaur et al., 2020). They form interactions with host plants in a variety of ways, and each interaction brings about numerous alterations in both the plant and endophyte. Mutualistic fungi possess the ability to enhance the plants pathogen defence system and enhances plant nutrient uptake, therefore, plant-endophyte interactions are important in understanding the potential to prevent plant diseases (Zeilinger et al., 2016). Due to the abundance of endophytic fungi, they may be present in every plant species and can be isolated from various plant organs, thus they represent a dependable and abundant source of chemically novel and bioactive compounds with the potential to be used in the agricultural industry (Strobel, 2003). Host plant-associated endophytes are natural sources of biological control agents (BCAs) as these fungal endophytes may produce novel bioactive substances (Bamisile et al., 2018a; Kaur et al., 2020; Radić & Štrukelj, 2012). Extensive research has been conducted on fungal endophytes ability to inhibit the growth of fungal plant pathogens and their potential as BCAs against various plant diseases (Crozier et al., 2015; Hanada et al., 2010; Mejía et al., 2008).

Endophytic fungi can also enhance plant growth via the secretion of phytohormones that consequently aids in host plant nutrition improvement via bidirectional nutrient transfer which also hinders the development and growth of competitors including pathogenic organisms due to the fact that the improved nutrients acquisition for endophytes results in a decrease in the amount available for the pathogenic organisms (Andreozzi et al., 2019; Shen et al., 2019). Maize seeds inoculated with *F. proliferatum* caused a decline in seed root length but when primed with *Penicillium simplicissimum* A4 prior to infection with *F. proliferatum*, the rate/percentage of seed germination and plant growth increased (Chang et al., 2015). The priming of seeds with endophytes also reversed the reactive oxygen species (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 bio-priming with *P. simplicissimum* A4 can regulate these changes.

5.2 Results

5.2.1 Effect of *P. simplicissimum* A4 bio-priming on root length of maize seeds infected with *F. proliferatum*

Maize seeds infected with *F. proliferatum* showed a concentration dependent reduction in root length (Figure 5.1 A and B). In comparison to the control with an average root length of 14.65 cm, maize root length was significantly decreased ($p<0.05$) by 49 % when infected with *F. proliferatum* (Figure 5.1 A). The results showed that the high concentration (10^8 spores/ml) of the pathogen significantly decreased root length in comparison to the control. However, maize seeds bio-primed with *P. simplicissimum* A4 prior to infection with *F. proliferatum* showed a significant increase ($p<0.05$) in root length (59 %) when compared to those infected with *F. proliferatum* treatment (Figure 5.1 A and B). Interestingly, a significant increase in root hair and an increase in lateral root growth was also observed in the *P. simplicissimum* A4-primed seeds compared to the *F. proliferatum* infected seeds.

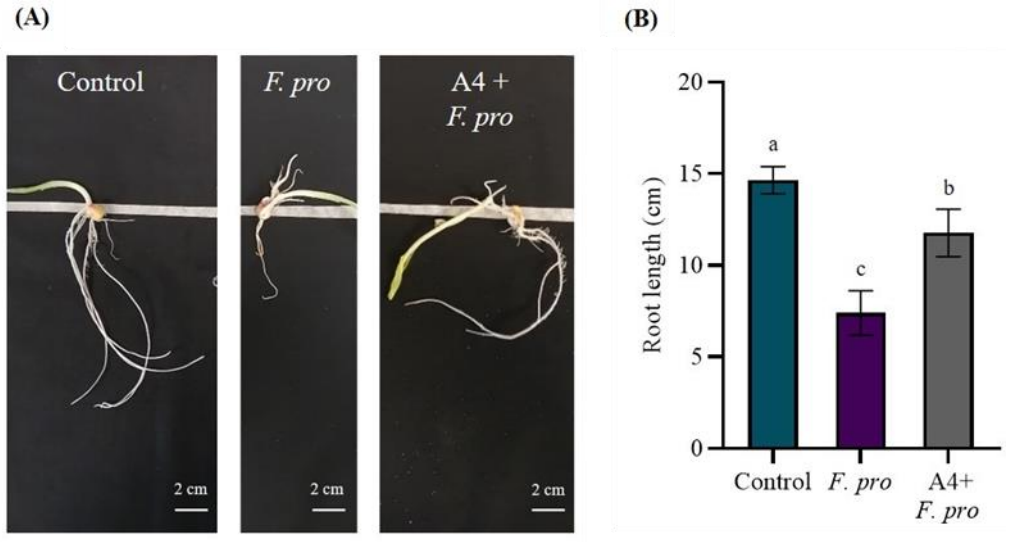


Figure 5.1 Effect of *P. simplicissimum* A4 on maize root growth under *F. proliferatum* infection. (A-B) Changes in maize root length bio-primed with *P. simplicissimum* A4 and infected with *F. proliferatum*. Bars denote standard error, where bars with different letters indicate statistical differences where $P < 0.05$.

5.2.2 Effect of *P. simplicissimum* A4 bio-priming on *F. proliferatum*-induced ROS accumulation and lipid peroxidation in maize roots

The effect of bio-primed maize seeds with *P. simplicissimum* A4 and infected with *F. proliferatum*-induced ROS accumulation was evaluated. In response to *F. proliferatum* infection, there was a significant increase ($p<0.05$) in superoxide (O_2^-) content by 90 % relative

to the control (Figure 5.2 A). However, when maize seeds were primed with *P. simplicissimum* A4 prior to infection with *F. proliferatum*, there was a significant decrease ($p<0.05$) in O_2^- content by 68 % relative to the *F. proliferatum* treatment (Figure 5.2 A). Furthermore, maize seeds infected with *F. proliferatum* showed a significant increase ($p<0.05$) in hydrogen peroxide (H_2O_2) content by 549 % relative to the control, while seeds primed with *P. simplicissimum* A4 prior to infection with *F. proliferatum* showed a 70 % reversal ($P < 0.05$) in H_2O_2 content relative to the infected roots (Figure 5.2 B). On the other hand, maize seeds infected with *F. proliferatum* showed an increase ($p<0.05$) in MDA content by 212 % relative to the control, while bio-priming of the seeds with *P. simplicissimum* A4 prior to infection with *F. proliferatum* showed a significant decrease ($p<0.05$) by 20 % in root MDA content relative to the infected seeds (Figure 5.2 C).

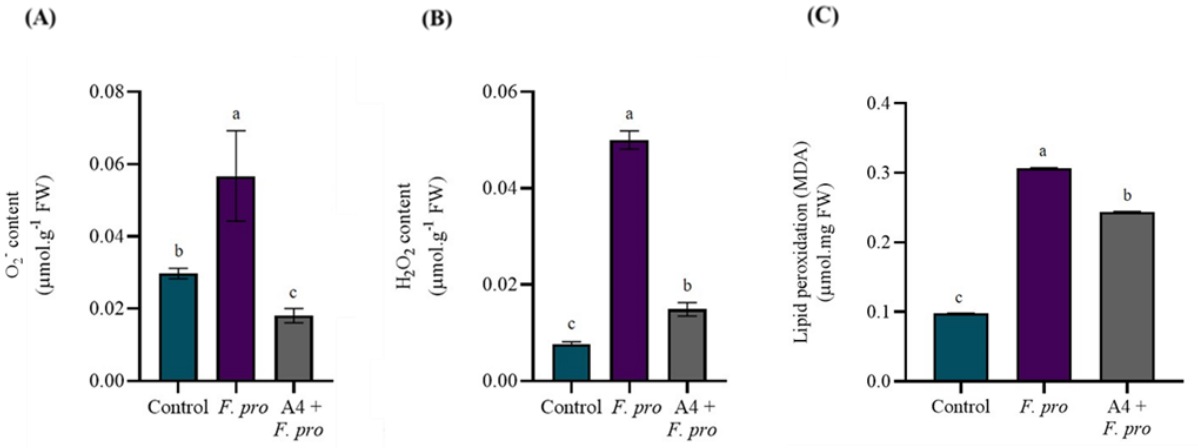


Figure 5.2 Effect of *P. simplicissimum* A4 on ROS-accumulation and lipid peroxidation of maize roots infected with *F. proliferatum*. Changes in (A) superoxide (O_2^-) content, (B) hydrogen peroxide (H_2O_2) content, and (C) malondialdehyde (MDA) levels in maize roots bio-primed with *P. simplicissimum* A4 and infected with *F. proliferatum*. Error bars represent standard error. Different letters indicate statistically significant differences ($P < 0.05$).

5.2.3 Effect of *P. simplicissimum* A4 on superoxide dismutase and ascorbate peroxidase activities in maize roots infected with *F. proliferatum*

The effect of *P. simplicissimum* A4 on superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities of maize roots infected with *F. proliferatum* was measured using spectrophotometric assay. Total SOD activity in maize seeds significantly decreased ($p<0.05$) by 92 % when infected with *F. proliferatum* relative to the control (Figure 5.3 A). However, bio-priming of maize seeds with *P. simplicissimum* A4 showed a significant increase in SOD activity by 172 % ($p<0.05$) compared to *F. proliferatum* treatment alone (Figure 5.3 A). Maize

seeds infected with *F. proliferatum* showed a significant increase ($p<0.05$) in APX activity (79 %) relative to the control. Bio-priming maize seeds with *P. simplicissimum* A4 showed a significant decrease (33 %) in APX activity relative to the *F. proliferatum* infected seeds (Figure 5.3 B).

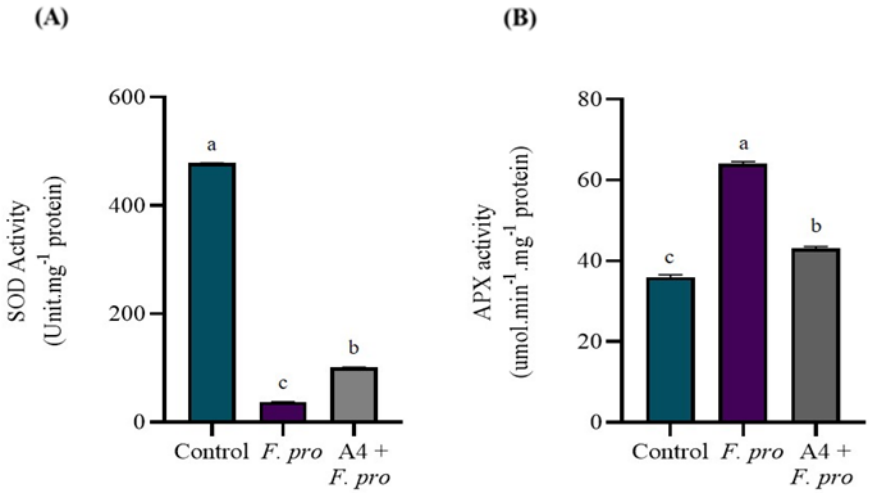


Figure 5.3 Effect of *P. simplicissimum* A4 on SOD and APX activities of maize roots infected with *F. proliferatum*. (A) Total SOD activity in maize roots bio-primed with *P. simplicissimum* A4 and infected with *F. proliferatum*. (B) Total APX activity in maize roots primed with *P. simplicissimum* A4 and infected with *F. proliferatum*. Different letters indicate statistically significant differences ($P < 0.05$).

5.2.4 Effect of *P. simplicissimum* A4 on guaiacol activity (GPOX) in maize roots under *F. proliferatum* infection

Maize seeds infected with *F. proliferatum* showed an increase ($P < 0.05$) in GPOX activity by 80 % relative to the control for GPOX 1 (Figure 5.4 A and B) and 98 % for GPOX 2 (Figure 5.4 A and C). Maize seeds bio-primed with *P. simplicissimum* A4 prior to infection with *F. proliferatum* showed a reversal in GPOX activity. The reduction ($P < 0.05$) in GPOX activity for *P. simplicissimum* A4 primed seeds was measured at 35 % for GPOX 1 (Figure 5.4 A and B) and 57 % for GPOX 2 (Figure 5.4 A and C) relative to the *F. proliferatum* treatment only.

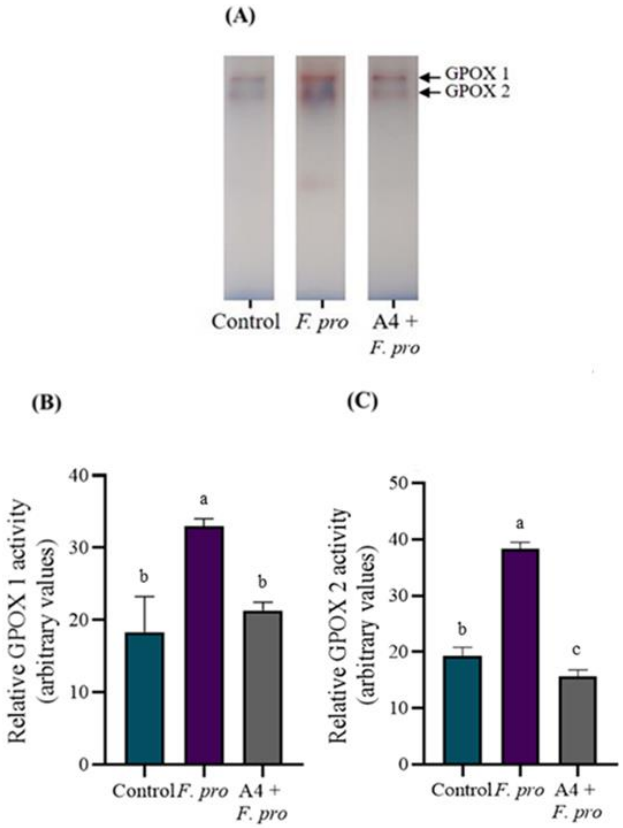


Figure 5.4 Effect of *P. simplicissimum* A4 on GPOX activity of maize roots infected with *F. proliferatum*. The in-gel activity assay of GPOX isoforms in response to the various treatments is represented in (A), from which pixel intensities of GPOX 1 (B) and GPOX 2 (C) were determined. Bars denote standard error, where bars with different letters indicate statistical differences where P < 0.05.

5.2.5 Effect of *P. simplicissimum* A4 on peroxidase activity (POD) in maize roots infected with *F. proliferatum*

Maize seeds infected with *F. proliferatum* showed a significant increase ($p<0.05$) in POD activity by 86 % for POD 1 (Figure 5.5 A and B) and 93 % for POD 2 (Figure A and C) relative to the control. Maize seeds bio-primed with *P. simplicissimum* A4 and infected with *F. proliferatum* showed a significant decrease ($P < 0.05$) (40 %) in root POD activity relative to the *F. proliferatum* infected seeds for POD 1 (Figure 5.5 A and B) and 10 % for POD 2 (Figure 5.5 A and C).

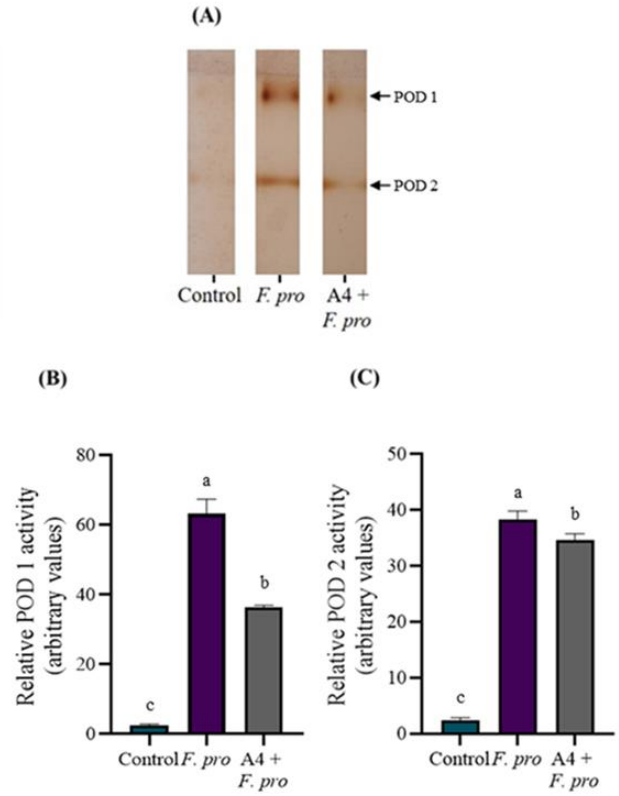


Figure 5.5 Effect of *P. simplicissimum* A4 on POD activity of maize roots infected with *F. proliferatum*. The in-gel activity assay of POD isoforms in response to the various treatments is represented in (A), from which pixel intensities of POD 1 (B) and POD 2 (C) were determined. Bars denote standard error, where bars with different letters indicate statistical differences where $P < 0.05$.

5.3 Discussion

Root rot caused by *Fusarium proliferatum* is regarded as one of the most destructive diseases affecting crop growth and development, leading to significant yield losses in many regions around the world (Farooq et al., 2019; Paravar et al., 2023). Microbial seed treatment involves the exogenous application of microbes or their metabolites on to the surface of the seed to enhance seed quality and characteristics such as seed size and weight. It also enhance the delivery of bioactive compounds including plant growth regulators and micronutrients which can protect seeds from phytopathogens while promoting germination and early plant growth (Paravar et al., 2023). In this study, we evaluated the biocontrol potential of *P. simplicissimum* A4 against *F. proliferatum* in maize.

5.3.1 Bio-priming of *F. proliferatum* infected maize seeds with *P. simplicissimum* A4 influences root lengths of maize seedlings

Bio-priming of maize seeds with *P. simplicissimum* A4 prior to infection with *F. proliferatum* showed a significant increase ($p<0.05$) in root lengths compared to infected treatment (Figure 5.1 A and B). The significant increase in root lengths of the seeds can be attributed to the protective role of the BCA (*P. simplicissimum* A4 against the pathogen (*F. proliferatum*). Root length is essential especially during the growth and development of plants and enable them to absorb nutrients and water from the soil or the surrounding environment (Hodge et al., 2009; Paravar et al., 2023). Also, Zhang et al. (2019c) reported that BCAs promote plants growth through increased in certain enzymes activity and plant growth hormones. Therefore, the significant increase in the root length of the plant can be attributed to the increase in level of these growth hormones or the activities of the enzymes in the apical or growth region of the root.

The use of microorganisms as BCAs in plant disease management is gaining more attention as eco-friendly alternatives to chemical fungicides due to their promising properties such as specificity, cost-effectiveness, ease to use and easily available at all seasons (Baard et al., 2023). Furthermore, BCAs are specific to pathogens and do not kill useful organisms present in the soil (Adeleke et al., 2022). In this study, we evaluated the biocontrol potential of *P. simplicissimum* A4 against *F. proliferatum* in maize. Bio-priming of maize seeds with *P. simplicissimum* A4 prior to infection with *F. proliferatum* showed a significant increase ($p<0.05$) in root lengths of the seeds compared to the infected seedlings alone (Figure 5.1 A and B). The detrimental effects of *F. proliferatum* on the physiological and biochemical responses of maize seeds showed that *F. proliferatum* induced stress on the host maize seed and thus restricted the growth and development of the plant, induced ROS production and influenced the antioxidant enzymes of the seeds. However, post infection of bio-primed seeds with *P. simplicissimum* A4 showed an increase in root lengths and a significant reduction in ROS production and antioxidant content relative to the infected seeds. According to Zhang et al. (2019c), BCAs promote plant growth through the increase in certain enzyme activity such as 1-aminocyclopropane-1-carboxylate-deaminase (ACC-deaminase) activity and plant growth hormones such as indole acetic acid (IAA) production. Furthermore, BCAs also increases ROS scavenging potential of plants to withstand stress from the pathogens. Therefore, the significant increase in root length of the maize seeds (Figure 5.1 A and B) can be attributed to the protective ability of *P. simplicissimum* A4 which enhanced ROS scavenging

potentials (Figure 5.2 A - C) of the plant caused by *F. proliferatum.* A study by McAllister et al. (1994) showed that the interaction between plant pathogens and BCAs showed a significant decrease in the activities of the pathogens in the rhizosphere leading to significant increases in the growth of maize seedlings. Furthermore, co-inoculation of plant pathogens with BCAs was reported to significantly increase the plant biomass and plant growth (Chandanie et al., 2009). Finally, the result from this study agrees with the report that seed priming with BCAs triggers the catabolism of phytohormones and nutrient uptake hence improving seedlings growth (Adhikari et al., 2020).

5.3.2 Bio-priming maize seeds with *P. simplicissimum* A4 influenced ROS-accumulation in roots under *F. proliferatum* infection

To study the effect of bio-priming of the infected seeds with *P. simplicissimum* A4, biochemical assays such as O_2^-, H_2O_2 and lipid peroxidation contents were carried out on the roots of maize seeds. Bio-primed seeds showed a significant decrease ($p<0.05$) in O_2^- and H_2O_2 contents compared to the infected seeds (Figure 5.2 A and B). Furthermore, there was a significant accumulation ($p<0.05$) of MDA content in the infected seeds compared to seeds bio-primed with *P. simplicissimum* A4 prior to infection. ROS have been reported to be involved in plant pathogen interaction (Shetty et al., 2008). The initial phase of ROS production by the host is basically for defence against the pathogen (Graves, 2012). It has been reported that O_2^- accumulation negatively impact the integrity of cells, while H_2O_2 act as a signalling molecule that induces cell growth and defence or detoxification system (Adhikari et al., 2020). Another study has shown that H_2O_2 signalling cascade has cross-link and differential impacts on the physiological and metabolic process of cells (Singh et al., 2014). The growth of axes during seed germination is primarily due to cell extension rather than cell division. This growth persists throughout the growth stage of the seedling. The elongation of plant cells is mostly dependent on the relaxation of the cell wall (CW), which is facilitated by the production of ROS and CW remodelling proteins in the extracellular space (Oracz & Karpiński, 2016). From this study, it was observed that *F. proliferatum* triggered the generation of O_2^- and H_2O_2 in the roots of the seeds compared with the control (Figure 5.2 A and B) which subsequently decreased in the bio-primed seeds with *P. simplicissimum* A4, thus it can be hypothesized that *P. simplicissimum* A4 triggered phytohormone catabolism and quenched the generation of O_2^- and H_2O_2 resulting in the germination of the seeds (Bazin et al., 2011). When the disproportionate accumulation of H_2O_2 results in the disruption of cell bio-membrane lipid layers, it induces the formation of MDA which causes a loss in intracellular water (Zhang et

al., 2019b). This is comparable to our study where the accumulation of H_2O_2 content caused an increase in MDA content in maize roots infected with *F. proliferatum*. Additionally, the decline in H_2O_2 content caused a decline in MDA content in maize roots bio-primed with *P. simplicissimum* A4 prior to infection with *F. proliferatum* (Figure 5.2 C). Similarly to our study where MDA content increased in maize roots infected with *F. proliferatum* (Figure 5.2 C), a study by Harrach et al. (2013) observed that barley roots infected with *Fusarium culmorum* resulted in the increase in MDA content and a decrease in MDA content in roots primed with the fungal endophyte *Piriformospora indica*. Furthermore, Mandal et al. (2008) observed that lipid peroxidation increased in tomato plants infected with *Fusarium oxysporum* f. sp. *lycopersici*.

5.3.3 Bio-priming maize seeds with *P. simplicissimum* A4 increased the enzymatic antioxidant capacity of maize roots under *F. proliferatum* infection

SOD, APX, GPOX and POD are of the most important enzymes involved in plants defence against ROS-mediated oxidative damages (Paul & Rakshit, 2022). From this study, there was a significant increase ($p<0.05$) in SOD activity (Figure 5.3 A) and significant decreases ($p<0.05$) in APX, GPOX and POD activities in the bio-primed roots compared to the infected treatment (Figure 5.3 A; Figure 5.4 A-C; Figure 5.5 A-C). These results suggest an increase resistance against *F. proliferatum* infection by the seedlings following bio-priming with *P. simplicissimum* A4 by increasing the antioxidant activities hence minimizing the ROS-induced cell death caused by pathogen infection. These enzymes are involved in the conversion of ROS into H_2O_2 and if not arrested, H_2O_2 will oxidize the membrane lipid. Lipid peroxidation refers to a sequence of oxidative breakdown events that impact lipids. The process entails the removal of electrons from lipids in cell membranes by free radicals, resulting in harm to the cells. The chain reaction events are propelled by free radicals. Nevertheless, the measurement of lipid peroxidation is determined by assessing the amount of malondialdehyde (MDA) present in the plant cells (Kumar et al., 2024). The increase in the antioxidant enzymes in the infected seeds and a corresponding increase in SOD activity and decrease in APX, GPOX and POD activities (Figure 5.3 A-B; Figure 5.4 A-C; Figure 5.5 A-C) can be correlated with the level of MDA in the respective treatments (Figure 5.2 C). It was reported that MDA content is proportional to the level of infection by pathogens, which can directly kill the host cells and produce toxins that could affect the membrane integrity (Chhabra et al., 2022; Das et al., 2023). Different studies have reported on the role of BCAs in enhancing production of plant defence enzymes against pathogens (Paul & Rakshit, 2022; Singh et al., 2014b; Singh et al., 2011). The

significant increase in the activities of these enzymes can be attributed to the systemic response of the plants to defend themselves against the pathogen. Therefore, *P. simplicissimum* A4 can be said to be effective in reducing the impact of root rot caused by *F. proliferatum* by decreasing the level of O_2^-, H_2O_2 and MDA (Figure 5.2 A – C) contents and altering the activities of SOD, APX, GPOX and POD enzymes in the roots of the plants (Figure 5.3 A and B; Figure 5.4 A – C; Figure 5.5 A - C).

In this study, *P. simplicissimum* A4 was found to be highly effective in suppressing Fusarium infection and enhancing maize plant growth. Moreover, these results confirmed that *P. simplicissimum* A4 elicited biochemical responses that assisted maize plants in the recouperation of its growth and development in the presence of the fungal pathogen. Future studies will focus on the protein changes in maize roots infected with *F. proliferatum* and roots bio-primed with *P. simplicissimum* A4 prior to infection with *F. proliferatum*.

CHAPTER 6

BIO-PRIMING WITH *PENICILLIUM SIMPLICISSIMUM* A4 MODULATES MAIZE ROOT PROTEINS UNDER *FUSARIUM PROLIFERATUM* INFECTION

6.1 Introduction

Maize constitutes one of the staple crops worldwide and a significant nutrient source in animal feed (Bai et al., 2021; Pechanova & Pechan, 2015; Wu et al., 2011; Yue et al., 2018). In Africa, in excess of 300 million people depend on maize as their primary food source (Pechanova & Pechan, 2015). In addition to its importance as a food source, maize is also used in industrial applications for the production of biofuels, textiles and adhesives (Pechanova & Pechan, 2015). Despite its enormous genetic diversity, during the various stages of maize growth, it is vulnerable to an assortment of pathogens that has detrimental effects on seed germination, growth, and development (Bai et al., 2021; Pechanova & Pechan, 2015; Wu et al., 2011).

Crop protection is essential for enhancing productivity in contemporary agriculture (Mohammadbagheri et al., 2021; Tehrani et al., 2020). The significant rise in the human population and the escalating demand for crops have resulted in intensified agricultural practices, which amplify the severity of pathogen attacks (Gholamaliyan et al., 2021; Soheili-Moghaddam et al., 2023). Chemical approaches of disease control with chemical pesticides frequently negatively affect nutrient cycles, natural biocontrol agents (BCAs), and beneficial soil flora and fauna (Haghani et al., 2014; Hönig et al., 2023; Kasem et al., 2019). Thus, the preferable approach is the use of biocontrol organisms to control pathogens. The use of BCAs has been reported to be beneficial because of their numerous advantages. They are environmentally friendly and easy to use (Hajji-Hedfi et al., 2023). Different endophytic organisms have been reported to be useful biocontrol candidates with high ability to inhibit plant pathogens and promote plant growth. For instance, Alfonzo et al. (2009) reported that different strains of *Bacillus subtilis* inhibited the growth of four phytopathogenic fungi of the vine (*Phaeoacremonium aleophilum, Phaemoniella chlamydospora, Fomitiporia mediterranea* and *Lasiodiplodia theobromae*). Also, studies have shown that *B. subtilis* pruning wounds of the vine inhibited over 80 % *Eutypa lata* mycelia growth *in vitro*. Another study also showed that diffusible compounds from *Pseudomonas* sp. I2R21 and Burkholderia

sp.W6R12A and W4R11 inhibited over 50 % of *Botryosphaeriaceous* species (Kassam et al., 2023; Wicaksono et al., 2017).

Recent advancements in omics technologies and next-generation sequencing (NGS) have expanded our understanding of the molecular mechanisms by which biocontrol agents interact with their targets, potentially enhancing their efficacy (Kassam et al., 2023). For instance, transcriptomic analysis elucidated the distinct lifecycle characteristics of endoparasitic fungus *Hirsutella minnesotensis* (Lai et al., 2014). Similarly, comparative transcriptomics demonstrated distinct parasitic strategy of *Trichoderma* sp. (Atanasova et al., 2013). In plant pathogens, proteomic methods can be a useful tool for identification of differentially expressed proteins in fungal species (Manikandan et al., 2018). For instance, cysteine-rich effector protein was first identified as root invader in tomato *Fusarium oxysporum* (Rep et al., 2004). Quantitative and qualitative proteomic analysis of two *Botrytis cinerea* strains showed differential expression in their virulence as well as toxin production level. Among the differentially expressed protein expressions, dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and a cyclophilin proteins involved in virulence were identified (Fernández-Acero et al., 2010). Another study also showed that using two-dimensional electrophoresis (2-DE) reference maps for identification of proteins, mycelial proteomes of *Sclerotinia sclerotiorum* were fully annotated (Yajima & Kav, 2006). Also, Böhmer et al. (2007) used a proteomic approach to identify proteins involved in the transition from budding to filamentous growth in *Ustilago maydis* which is responsible for its pathogenicity. Finally, Pandit et al. (2017) elucidated the fundamental cause of the predatory behaviour of *Arthrobotrys conoides* by differential gene expression (DEG) profiling in the presence and absence of nematode extracts. These investigations indicate that contemporary omics methodologies can effectively facilitate the investigation of the mechanisms responsible for soil fungus stasis. This study aimed to investigate the protein responses in maize roots infected with *F. proliferatum* and how biopriming with fungal endophyte *P. simplicissimum* A4 can alter these changes.

6.2 Results

6.2.1 Separation, visualization, and quantification of maize root proteins

A fraction (15 µg) of each treatment was size fractionated on a 1D SDS-PAGE (Figure 6.1). The results showed that the separated proteins were of high quality with no visible streaking or protein degradation. Distinct differences in protein band intensities were observed between the

treatments (denoted by the black arrows). The separated proteins have a molecular weight between 11 and 72 kDa (Figure 6.1).

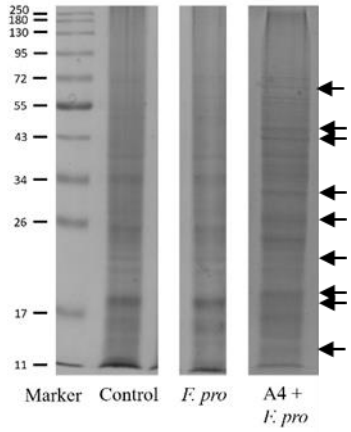


Figure 6.1 One - dimensional sodium dodecyl sulphate (SDS) gel electrophoresis of total soluble maize root proteins under different treatment conditions. Protein extracts (15 µg) were size fractionated on a 12 % denaturing 1D SDS polyacrylamide gel. The black arrows indicate visual differences observed between the different treatments.

6.2.2 Effect of biopriming with *P. simplicissimum* A4 on maize roots proteins under *Fusarium* infection

A total of 126 proteins were identified in the control and infected roots while 111 proteins were identified in roots bio-primed with *P. simplicissimum* A4 (Figure 6.2 A). Additionally, the control and the roots bio-primed with *P. simplicissimum* A4 each contains 11 unique proteins while the infected roots contain 15 unique proteins (Figure 6.2 C; Table 6.1). Seventeen proteins were shared between the control and the infected roots (Table S6.1), 6 proteins between the control and roots bio-primed with *P. simplicissimum* A4 (Table S6.2) and 2 proteins were shared between infected and bio-primed roots (Table S6.3). Lastly, all treatments shared a total of 93 proteins (Table S6.4).

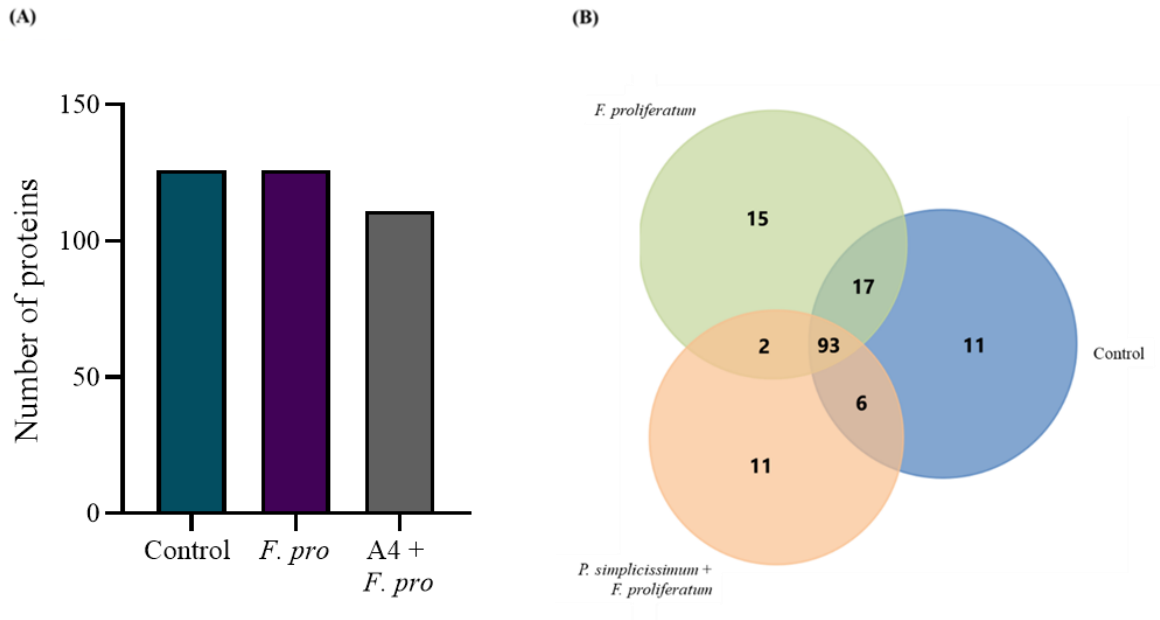


Figure 6.2 Effect of *P. simplicissimum* A4 on the presence of proteins in maize roots infected with *F. proliferatum*. (A) Number of proteins identified in each treatment. (B) Proteins identified in maize seeds primed with distilled water (control), infected with *F. proliferatum*, and primed with *P. simplicissimum* A4 prior to infection with *F. proliferatum*, respectively.

Proteins associated to biological regulation present in maize roots infected with *F. proliferatum* included LPAT_MAIZE and EFTS_MAIZE. Additionally, proteins involved in the plant's response to pathogen infection included AP2S1_MAIZE, CHIA_MAIZE, DHN1_MAIZE, GLNA2_MAIZE, FRI2_MAIZE, HSP82_MAIZE IF5_MAIZE, and SPP1_MAIZE (Table 6.1). The protein involved in biological regulation in roots bio-primed with *P. simplicissimum* A4 prior to infection was RLA3_MAIZE. Additionally, the presence of SODC5_MAIZE protein in the root of maize primed with *P. simplicissimum* A4 enhance the antioxidant activity thereby detoxifying the ROS generated due to the infection by the pathogen. This provides a protective shield to the maize plant against the invading pathogen. Lastly, proteins involved in defence against pathogen attack were present in maize roots primed with *P. simplicissimum* A4 prior to infection included TBA3_MAIZE, TIP21_MAIZE, IBBWP_MAIZE, and NLTP_MAIZE (Table 6.1).

Table 6.1 Identified unique proteins in maize seed roots infected with *F. proliferatum.*

Protein accession numbers	Protein name	Protein MW (Da)	Isoelectric point (PI)	Function
Identified unique proteins in maize seed roots primed with water (control)				
ARGJ_MAIZE	Arginine biosynthesis bifunctional protein ArgJ, chloroplastic	47,952.1	6,89	Catalyzes two activities which are involved in the cyclic version of arginine biosynthesis.
BIP3_MAIZE	Luminal-binding protein 3	73,158.8	5,24	Plays a role in facilitating the assembly of multimeric protein complexes inside the ER.
CALR_MAIZE	Calreticulin	30,260.2	8,15	Aquaporins facilitate the transport of water and small neutral solutes across cell membranes.
CFI_MAIZE	Chalcone--flavonone isomerase	24,249.7	6,06	Catalyzes the intramolecular cyclization of bicyclic chalcones into tricyclic (S)-flavanones.
CYSP2_MAIZE	Cysteine proteinase 2	39,199.0	7,28	Involved in the degradation of the storage protein zein. May play a role in proteolysis during emergencies.
ITPK1_MAIZE	Inositol-tetrakisphosphate 1-kinase 1	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.
PIP21_MAIZE	Aquaporin PIP2-1	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	30,260.2	8,15	Aquaporins facilitate the transport of water and small neutral solutes across cell membranes.
SODM4_MAIZE	Superoxide dismutase [Mn] 3.4, mitochondrial	25,239.0	7,27	Destroys superoxide anion radicals which are normally produced within the cells, and which are toxic to biological systems.
THI42_MAIZE	Thiamine thiazole synthase 2, chloroplastic	37,233.2	5,88	Involved in biosynthesis of the thiamine precursor thiazole.
ZB14_MAIZE	14 kDa zinc-binding protein	14,300.9	6,68	Plays a role in the purine ribonucleotide metabolic process, and sulfur compound metabolic process.
Identified unique proteins in maize seed roots infected with *F. proliferatum.*				
AP2S1_MAIZE	AP-2 complex subunit sigma	16,015.0	7,09	Component of the adaptor complexes which link clathrin to receptors in coated vesicles.
C71C1_MAIZE	3-hydroxyindolin-2-one monooxygenase	59,717.0	8,16	Catalyzes the conversion of 3-hydroxyindolin-2-one to 2-hydroxy-1,4-benzoxazin-3-one (HBOA).
CHIA_MAIZE	Endochitinase A	29 000	7.94	Defense against chitin-containing fungal pathogens. Hydrolyzes glycol chitin and tetra-N-acetylchitotetraose in vitro
DHN1_MAIZE	Dehydrin DHN1	17,160.2	9,11	Plays a role in defence against pathogens, cold acclimation, response to abscisic acid, and response to water deprivation.
EFTS_MAIZE	Elongation factor Ts, mitochondrial	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.

FRI2_MAIZE	Ferritin-2, chloroplastic	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.
GLNA2_MAIZE	Glutamine synthetase root isozyme 2	40,094.9	5,9	Plays a role in the flow of nitrogen into nitrogenous organic compounds, and defence against pathogens.
HSP82_MAIZE	Heat shock protein 82	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.
IF5_MAIZE	Eukaryotic translation initiation factor 5	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.
IN21_MAIZE	Protein IN2-1	26,989.8	4,98	Plays a role in the glutathione metabolic process and protein glutathionylation.
LPAT_MAIZE	1-acyl-sn-glycerol-3-phosphate acyltransferase PLS1	42,572.8	9,91	Converts lysophosphatidic acid (LPA) into phosphatidic acid by incorporating acyl moiety at the 2 position.
MNB1B_MAIZE	DNA-binding protein MNB1B	17,146.6	5,95	Recognizes an AAGG motif at the MNF1-binding site.
RLA2A_MAIZE	60S acidic ribosomal protein P2A	11,363.0	4,28	Plays an important role in the elongation step of protein synthesis.
SPP1_MAIZE	Sucrose-phosphatase 1	47,214.6	5,73	Catalyzes the final step of sucrose synthesis. Inactive with fructose 6-phosphate as substrate.

TBB8_MAIZE	Tubulin β-8 chain	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 β chain and one at a non-exchangeable site on the αchain.
Identified unique proteins in maize seed roots primed with *P. simplicissimum* A4 prior to infection with *F. proliferatum.*				
GLNA4_MAIZE	Glutamine synthetase root isozyme 4	38,981.5	5,35	Plays a role in the flow of nitrogen into nitrogenous organic compounds, and defence against pathogens.
H2B1_MAIZE	Histone H2B.1	16,421.1	9,99	Core component of nucleosome.
H2B2_MAIZE	Histone H2B.2	16,174.7	10,08	Core component of nucleosome.
IBBWP_MAIZE	Bowman-Birk type wound-induced proteinase inhibitor WIP1	10,975.8	7,96	Plays a role in defence and possess serine-type endopeptidase inhibitor activity
MEG5_MAIZE	Protein MATERNALLY EXPRESSED GENE 5	17,813.8	6,51	Plays a role in mRNA binding.
NLTP_MAIZE	Non-specific lipid-transfer protein	11,705.0	8,72	Plant non-specific lipid-transfer proteins transfer phospholipids as well as galactolipids across membranes.
PIP26_MAIZE	Aquaporin PIP2-6	30,191.5	8,25	Aquaporins facilitate the transport of water and small neutral solutes across cell membranes.

RLA3_MAIZE	60S acidic ribosomal protein P3	12,219.0	4,56	Plays an important role in the elongation step of protein synthesis.
SODC5_MAIZE	Superoxide dismutase [Cu-Zn] 4AP	15,070.2	6,1	Destroys radicals which are normally produced within the cells, and which are toxic to biological systems.
TBA3_MAIZE	Tubulin α-3 chain (α-3-tubulin)	50 000	5.24	Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the βchain and one at a non-exchangeable site on the αchain.
TIP21_MAIZE	Aquaporin TIP2-1 (Tonoplast intrinsic protein 2-1) (ZmTIP2-1) (ZmTIP2;1)	25 000.0	5.64	Aquaporins facilitate the transport of water and small neutral solutes across cell membranes.

6.2.3 Effect of biopriming with *P. simplicissimum* A4 on the functional characteristics of maize roots proteins under *Fusarium* infection

The identified proteins from the different treatment were characterized based on their involvement in biological processes, molecular functions and cellular localization. The functional characterisation of the identified unique proteins in the control roots showed that 38 % were involved in biological processes, 27 % were involved in molecular functions, and 31 % were involved in cellular localizations. Additionally, the functional characterisation of the identified unique proteins in roots infected with *F. proliferatum* showed that 43 % were involved in biological processes, 41 % were involved in molecular functions, and 38 % were involved in cellular localizations. Lastly, the functional characterisation of the identified unique proteins in roots bio-primed with *P. simplicissimum* A4 showed that 19 % were involved in biological processes, 32 % were involved in molecular functions, and 32 % were involved in cellular localizations.

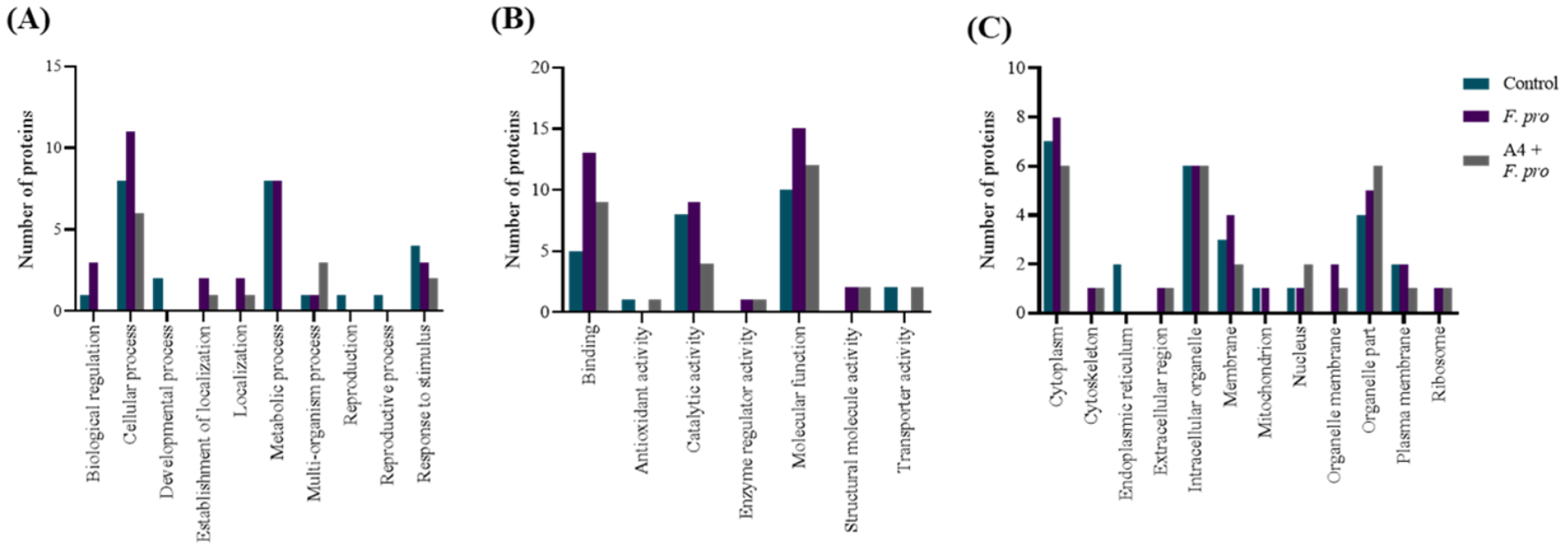


Figure 6.3 Effect of bio-priming with *P. simplicissimum* A4 on the functional characteristics of maize roots proteins under *Fusarium* infection. (A) The biological processes of unique maize root proteins across all treatments. (B) The molecular functions of unique maize root proteins across all treatments. (C) The cellular localizations of unique maize root proteins across all treatments.

6.3 Discussion

Plants possess sophisticated defence mechanisms against pathogen invasion. Biochemical modulations play a significant role in defence against pathogens, particularly microbial infections. The pathogens dismantles cellular barriers and infiltrates the cuticle and cell wall for colonization and disease progression (Akbar et al., 2023). A good example of this response is the changes in the pattern of protein synthesis wherein various enzymes produced has the ability to inhibit or attack the pathogens (Liu et al., 2019b; Verburg et al., 1992). From this study, different proteins associated with plant defence and protection against pathogens were identified. Majority of the identified proteins are involved in biosynthetic processes, storage protein, signalling responses, and anti-pathogenic proteins (Table 6.1). For example, glutamine synthetase (GLNA2_MAIZE) and sucrose-phosphatase 1 (SPP1_MAIZE) were identified in the root of maize infected with *F. proliferatum* (Table 6.1). It has been observed that plants express glutamine synthetase during disease progression, particularly in the first stage of infection. Additionally, transcripts of the fungal glutamine synthetase were increased by nitrogen deprivation in axenic cells. Numerous investigations into plant–fungus interactions have indicated that nutrient scarcity in axenic culture may replicate the growth circumstances present during the initial phases of the infection process (Pageau et al., 2006). The pathogen genes induced by nitrogen deprivation may significantly contribute to pathogenicity. Therefore, the presence of this protein is an indication that the plant is under severe abiotic stress. Furthermore, sucrose-phosphatase catalyses the final step of sucrose biosynthesis where sucrose-6-phosphate is irreversibly hydrolysed to sucrose. The activity of sucrose-phosphatase has been found in different species of vascular and non-vascular plants. The presence of this enzyme suggested that there is an increase in the demand for energy in the early stages of root growth. Proteins that were present in response to priming with *P. simplicissimum* A4 prior to infection included proteins that were involved in anti-pathogen defence as well as antioxidant activity (Table 6.1).

Other important proteins identified from all the treatment cohort were found to be associated with different biological process responsible for the growth, development and protection of the maize plant against pathogens. For example, lysophosphatidic acid acyltransferase (LPAAT) is an important enzyme responsible for the synthesis of cell lipids such as phosphatidylinositol. Phosphatidylinositol is a major constituent of membranes and functions in important metabolic processes within plants (dos Santos Maraschin et al., 2019; Ridgway, 2021). Additionally, LPAATs possess various protein-to-protein interactions. For instance, the interaction between

glycerol-3-phosphate acyltransferase and gycerol-3-phosphate dehydrogenase which plays a critical role in lipid metabolism and energy balance (Table S6.5; Figure S6.1). The presence of this protein is an indication that the plant required energy necessary to withstand the stress imposed by the pathogen. Furthermore, during the early stage of plant development, huge amount of energy is required for various metabolic process such as glycolysis (Figure 6.3 A).

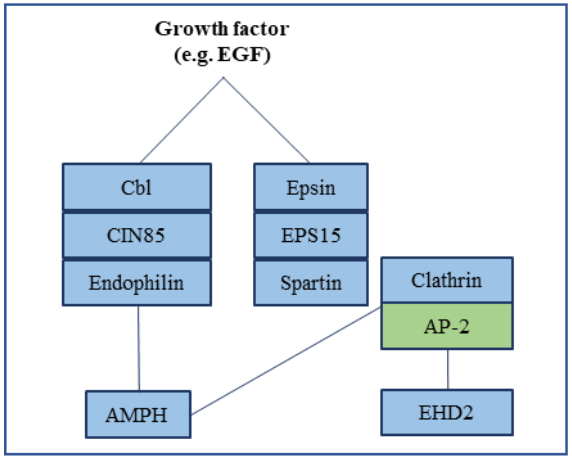
Translational elongation factors (EFT's) are proteins that play significant roles in the elongation cycle of protein biosynthesis localized in the ribosome (Maloy & Hughes, 2013). In this study, EFTS_MAIZE protein was identified in the root of maize infected with *F. proliferatum.* This is another protein involved in protein-to-protein interactions such as elongation factors and ribosome recycling factors (Table S6.6; Figure S6.2). This interaction is essential for coordinating the activities of various translational components such as the binding of aminoacyl-tRNA to the ribosome's A-site during elongation (Figure 6.3 A). From this study, EFTS_MAIZE protein participated in cellular and metabolic processes and was involved in binding (Figure 6.3 B), within the cytoplasm, the intracellular organelle, mitochondrion, and the organelle part (Figure 6.3 C).

Ribosome is made up of two subunits which are both required for translation. The small subunit (40S) decodes genetic messages and the large subunit (60S) is responsible for catalysing the peptide bond formation (Gregory et al., 2019). From this study, 60S acidic ribosomal protein RLA2A_MAIZE and RLA3_MAIZE were identified in the roots of maize bio-primed with *P. simplicissimum* A4 and roots of maize under *F. proliferatum* respectively (Table S6.7; Figure S6.3). These 60S acidic ribosomal proteins are involved in cellular and metabolic processes, and in response to stimulus (Figure 6.3 A), it has binding activity and structural molecule activity (Figure 6.3 B), and was localized in the cytoplasm, intracellular organelle, organelle part, and the ribosome (Figure 6.3 C) in bio-primed roots.

6.3.1 Maize root proteins in response to *F. proliferatum* infection

The heterotetrameric adaptor protein 2 (AP-2) [AP2S1_MAIZE] was identified in the roots of maize infected with *F. proliferatum.* This protein play an important role in the clathrin-mediated endocytosis (CME)-adaptor complex which interrelates with the membrane, clathrin and CME accessory proteins (Figure 6.4) (Brodsky, 2012; Di Rubbo et al., 2013; Kitakura et al., 2011; McMahon & Boucrot, 2011; Wang et al., 2013; Yamaoka et al., 2013). The protein is associated with a set of proteins that link cargo molecules to the clatherin-coated vesicles (CCV), enhancing their internalization into the cell. During infection of host plants, fungal

pathogens secrete effectors such hydrolytic enzymes that possess the ability to kill plant tissues (Souibgui et al., 2021). These hydrolytic proteins are then transferred from the golgi apparatus (GA) and the endoplasmic reticulum (ER) via intracellular vesicles to the extracellular space (Souibgui et al., 2021). Pathogens exploit this intake of membrane and extracellular compounds to gain access to host plant cells (Latomanski & Newton, 2019; Robinson et al., 2018; Souibgui et al., 2021; Yang & Shen, 2020).



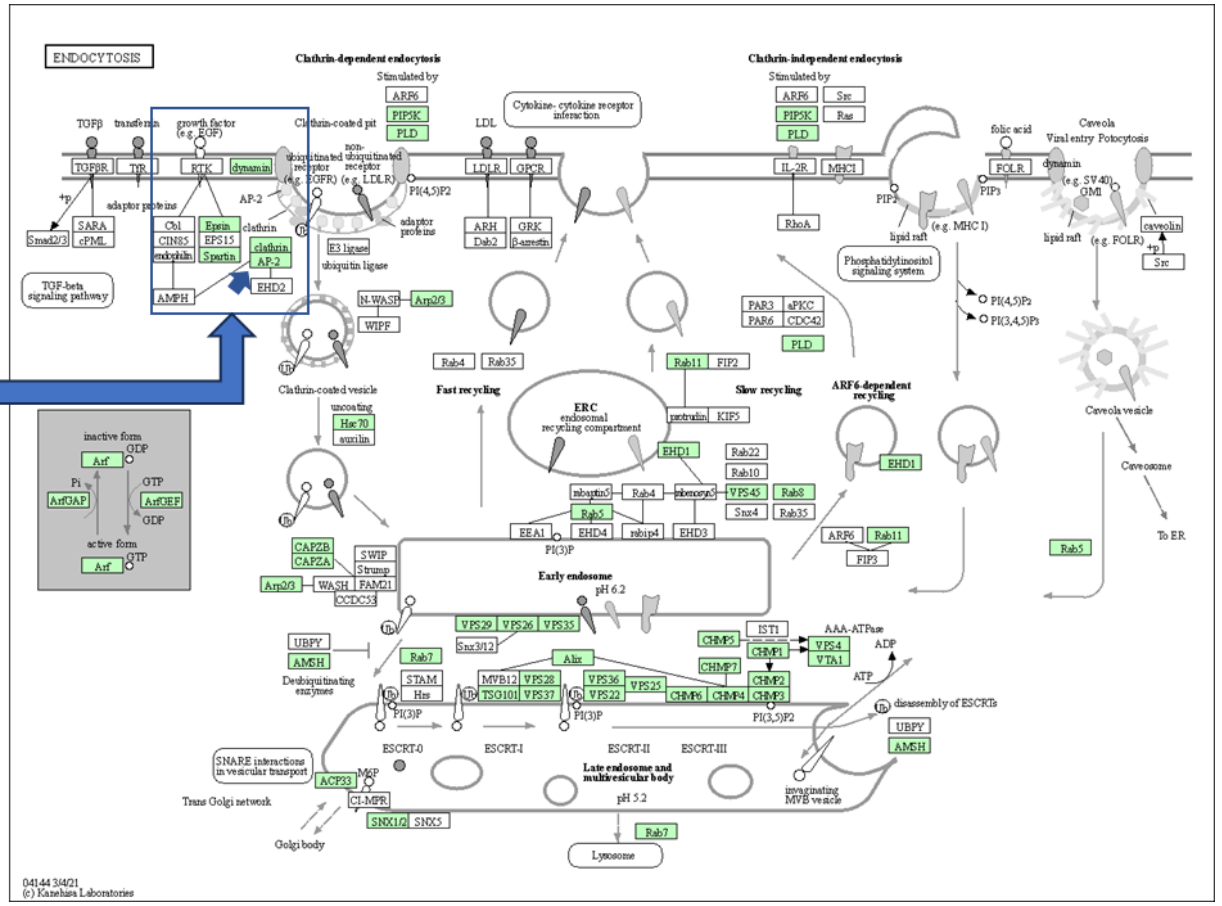


Figure 6.4 KEGG pathway analysis revealing the role of AP-2 complex subunit sigma in clathrin-dependent endocytosis. Endocytosis involves the removal of nutrients, ligands, lipids and plasma membrane (PM) proteins from the surface of the cell, thus moving them into the interior of the cell. Transmembrane proteins that enter the cell via clathrin-dependent endocytosis (CDE) possess sequences within their cytoplasmic domains which binds to adaptor-related protein complexes (APs) which enables their removal from the PM.

Despite the importance clathrin in vesicular trafficking during fungal growth, not much is known about it in filamentous fungi (Shoji et al., 2014), however, Souibgui et al. (2021) showed that clathrin is an integral part of the infectious process in the phytopathogenic fungus *B. cinerea.* The study also showed that the clathrin proteins play roles in the release of cell death-inducing proteins (CDIPs), associated to the degradation of cells walls (CWs) and ROS production. Furthermore, the authors revealed that clathrin is essential in pathogenic fungi's infectious cushion development which are structures utilized for the penetration and early destruction of host tissue. Interestingly, Bairwa et al. (2019) observed that when encapsulated yeast *Cryptococcus neoformans* lacked the clathrin heavy chain-encoding gene, it was defective in haemoglobin uptake which is the main iron source for fungal pathogens, and the biosynthesis of two important virulence factors such as the capsule and melanin. Likewise in our study, AP-2 complex subunit sigma present in the roots of the infected maize was shown to be involved in establishment of localization, clathrin-dependent endocytosis, response to pathogen infection (Figure 6.3 A), it possesses binding activity (Figure 6.3 B), within the cytoplasm, intracellular organelle, organelle membrane, organelle part, and the plasma membrane (Figure 6.3 C).

Chitinases possess the ability to degrade CW components, such as chitin, which renders the CW unstable, and they are involved in the protein-to-protein interaction with squamosa promoter-binding-like protein 15 (Table S6.8; Figure S6.4). From this study, CHIA_MAIZE a protein associated with chitinase activity in the roots of infected maize. Maize possesses a variety of chitinase genes, however, there is limited evidence that shows maize chitinases are directly active against maize fungal pathogens (Dowd et al., 2018). Alterations in the expression of chitinases is relative to pathogen resistance under unchallenged conditions, and in some cases chitinase expression is decreased when climatic conditions are favourable for infection by some pathogens of maize plants (Dowd & Johnson, 2015; Luo et al., 2010). To our knowledge, there have been no studies focusing on the chitinase protein changes in maize roots in the presence of the pathogen *F. proliferatum*, however, similarly, to our study, Cordero et al. (1994) reported the presence of endochitinase A in maize seed roots infected with *F. proliferatum* (Table 6.1). According to their report, the overexpression of this protein enhances the germination rate of maize seed under *Fusarium moliforme* infection. Lastly, Pechanova et al. (2013) reported the presence of chitinase in developing maize kernels of two inbred lines 48 hours post-infection with *Fusarium* sp. which appeared to be a major contributor to the plants' resistance to infection. From this study, functional characterization of endochitinase A

showed that the protein is involved in cellular and metabolic processes, multi-organism processes and was in response to stimulus (Figure 6.3 A), it was also involved in binding and catalytic activity (Figure 6.3 B), within the extracellular region (Figure 6.3 C).

Dehydrins (DHN) present as DHN1_MAIZE in the roots of infected maize is part of the group II LEA proteins which are known as stress-related proteins as they possess important roles in the plants' adaptation and responses to abiotic stress (Hanin et al., 2011; Liu et al., 2017b). According to N'guyen et al. (2019), dehydrin proteins were significant in *Alternaria brassicicola* to accomplish key mechanisms within its pathogenic life cycle. The study showed that the single deletion of the mutants that were deficient of the fungal hydrophilin-like proteins portrayed that dehydrin-like proteins impacts the conidial survival when exposed to high and freezing temperatures and oxidative stress tolerance. Additionally, double-dehydrin mutants displayed an increase in compromised pathogenicity and a decline in aggressiveness on the leaves of *Brassica oleracea* as well as a decrease in its ability to be transmitted via siliques in *Arabidopsis* seeds. There are limited studies on the role of dehydrin protein in plants under biotic stress, Manzo et al. (2016) reported acellular damage in *Momor* plants under *F. oxysporum* infection resulted in loss of water and high accumulation of dehydrin in the plants could restore osmotic stress in resistant plants.. Functional characterization of this protein showed its involvement in plant's response to stimulus (Figure 6.3 A) and a binding activity (Figure 6.3 B), within the membrane, organelle membrane, and the organelle part (Figure 6.3 C).

The protein glutamine synthetase root isozyme 2 was identified in the roots of maize infected with *F. proliferatum*, while glutamine synthetase root isozyme 4 was identified in bio-primed maize roots. The proteins were involved in cellular and metabolic processes (Figure 6.3 A), as well as binding and catalytic activity (Figure 6.3 B). Glutamine synthetase root isozyme 2 also possessed protein-to-protein interactions with ferredoxin-dependent glutamate synthase, chloroplastic and GMP synthase (Table S6.9; Figure S6.5). Nitrogen (N) is an important mineral required for the development of plants (Lam et al., 1996; Prinsi & Espen, 2015). Glutamine synthetase (GS) isozymes play important roles in N assimilation pathway (Bernard & Habash, 2009; Harper et al., 2010; Manzo et al., 2016; Martin et al., 2006; Thomsen et al., 2014; Wei et al., 2018; Wei et al., 2020). The changes in host glutamate metabolism (GM) in response to various pathogen infections can be carried out in two ways. It either supports the ongoing defence strategy to manipulate a successful resistance response or it is exploited by

pathogens to facilitate and promote their infection (Seifi et al., 2013). Throughout the plant-pathogen interaction, the hosts' glutamate metabolism is changed, and this leads to either a metabolic state known as "evasion" where cell death occurs or "endurance" where cell viability is maintained (Seifi et al., 2013; Swartzberg et al., 2008).

Heat shock proteins (HSPs) plays important roles as a molecular chaperone in quality control of pattern recognition receptor (PRR's) as well as intracellular proteins against pathogenic invaders (Abou-Deif et al., 2019; Baniwal et al., 2004; Li et al., 2021; Park & Seo, 2015; Piterková et al., 2013; Swindell et al., 2007). From this study, HSP82_MAIZE was identified in maize roots infected with *F. proliferatum*. According to Campo et al. (2004), HSP were up-regulated in maize embryos infected with *Fusarium verticillioides.* Furthermore, Pegoraro et al. (2011) reported that HSP were differentially expressed in maize plants infected with *U. maydis* 48, 96, 108, and 192 hours post infection. Their results showed that HSPs were associated with plant defence mechanisms against pathogen attack (Figure 6.5). Finally, Chen et al. (2007) reported an upregulation of HSPs in maize plants infected by *Aspergillus flavus*. Functional characterization of HSPs in this study showed that they are involved in biological regulation, cellular processes and in enhance plant's response to stimulus (Figure 6.3 A). The protein also possessed binding and catalytic activity (Figure 6.3 B), and was localized in the cytoplasm, plasma and cell membrane (Figure 6.3 C). Lastly, they were involved in protein-to-protein interactions with heat shock factor protein HSF30 and cysteine and histidine-rich domain-containing protein RAR1 (Table S6.10; Figure S6.6).

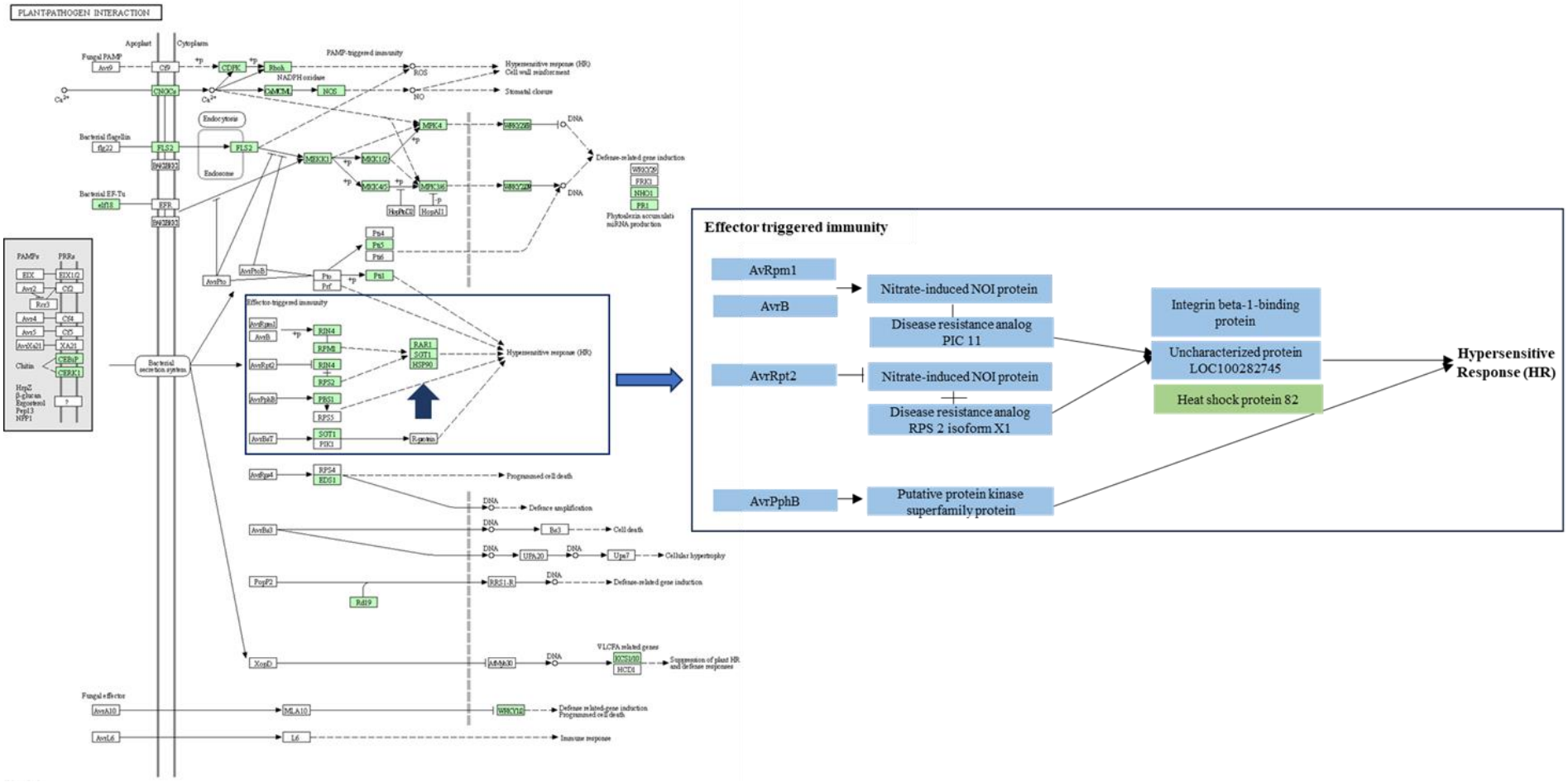


Figure 6.5 KEGG pathway analysis revealing the role of heat shock protein 82 in the effector-triggered immunity which elicits a hypersensitive response during plant-pathogen interactions.

6.3.2 *P. simplicissimum* A4 assists with antioxidant activity in maize roots

Antioxidants play a critical role during pathogen infection by mitigating oxidative stress and supporting immune responses. Pathogen infections often trigger an imbalance in reactive oxygen species (ROS) levels, which can damage host cells and affect pathogen survival (Lee & Song, 2021). Superoxide dismutase (SOD) is an enzyme responsible for the conversion of superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2) and oxygen molecules (O_2) (Thirach et al., 2007), and possessed interactions with several proteins such as superoxide dismutase [Cu-Zn] 4A and copper chaperone for SOD (Table S6.11; Figure S6.7). The observed antioxidant proteins identified from this study (Table 6.1; Figure 6.3 B) is similar to the reported proteins in a study carried out by Geddes et al. (2008); Yang et al. (2010); and Zhou et al. (2006). They reported the presence of proteins associated with different antioxidant activity in barley and wheat infected with *Fusarium graminearum* during grain filling. Conversely, Yang et al. (2010) reported a decrease in SOD activity in *F. graminearum* infected roots of barley seeds. According to his report, pathogen infection causes a breakdown in the plant ROS-defence system hence making them vulnerable to attack by the pathogen. Based on the results from our study, we hypothesize that the presence of *P. simplicissimum* A4 enabled the infected maize roots to regain the production of antioxidants enzymes to protect itself from the increase in ROS when exposed to *F. proliferatum* (Figure 5.2 A-B).

6.3.3 Proteins associated with plant defence against *Fusarium proliferatum* infection

Proteins play critical roles in plant defence against pathogens by recognizing invaders, activating immune responses, and executing defence mechanisms. These proteins operate at various levels of the plant immune system to protect plants from a wide range of pathogens, including bacteria, fungi, viruses, and nematodes (Boutrot & Zipfel, 2017; Pandey et al., 2016). Aquaporins (AQP's) are channel proteins responsible for the transportation of water and neutral metabolites across various biological membranes (Chaumont et al., 2001; Zhang et al., 2019a). In plants, the movement of water is essential for the homeostasis of numerous physiological processes such as stomata guard cell opening and cell elongation as well as the transport of nutrients and hormones (Lopez et al., 2003). From this study we identified the presence of PIP26_MAIZE and TIP21_MAIZE, aquaporin channel proteins in the roots of maize bio-primed with *P. simplicissimum* A4. Similar study by Chaumont et al. (2000); Dixit et al. (2001); Marin-Olivier et al. (2000); and Moshelion et al. (2002), showed the presence of PIP2 proteins in *Xenopus oocytes.* However, Zhang et al. (2019a) showed that PIPs may also possess a function in plant's immunity against pathogenic infection their by protecting plant

from pathogen attack. In this study, aquaporin PIP2-6 possessed transporter activity (Figure 6.3 B) and was localized in the membrane and the plasma membrane (Figure 6.3 C).

Although plants do not have specialized cells that are equivalent to the immune system in animals, they still possess the ability to respond to pathogenic infection or physical injury. One of these systems is wound response, where wounding causes a change in gene expression of several proteins which includes proteinase inhibitors, glycine-rich proteins, or hydroxyproline-rich glycoproteins (Rohrmeier & Lehle, 1993). The Maize Wip1 gene encodes for the wound-induced bowman-birk inhibitor (BBI) protein which is a serine protease inhibitor expressed during wounding or infection, and thus it confers resistance against pathogens and pests (QI et al., 2005). From this study IBBWP_MAIZE, a protein associated with plant immune boosting, was identified in the roots of maize seed bio-primed with *P. simplicissimum* A4 (Table 6.1). The protein possessed enzyme regulatory activity (Figure 6.3 B) and was localized in the extracellular region of the plant (Figure 6.3 C). Qu et al. (2003) reported seven BBI genes in rice and the overexpression of the rice BBI2-2 confers resistance to fungal pathogens in transgenic rice plants.

The intracellular movement of phospholipids requires phospholipid-transfer proteins (PLTP) mainly located in the cytosol of organs such as the ovaries, germinating and maturing seeds, stems, anthers, roots, leaves, and pollen (Han et al., 2001; Tchang et al., 1988; Wei & Zhong, 2014). These PLTPs play significant roles in membranes biosynthesis and renewal as well as the transport of hydrophobic compounds (Arondel et al., 1991; Tchang et al., 1988). Plant non-specific phospholipid-transfer proteins (nsLTP's) transport cuticular components needed for the inhibition of bacterial and fungal pathogens (Molina et al., 1993; Terras et al., 1992). Studies on plant innate immunity showed that endophytic fungi are able to protect plants against various pathogens via the production of toxic- secondary metabolites (SMs), elicitors and enzymes, which induces systemic resistance in plants (Choudhary et al., 2007; Gozzo, 2003; Li et al., 2008; Wei & Zhong, 2014). These SMs are used as BCAs as an alternative to chemical methods of disease management (Odintsova et al., 2019). From this study, NLTP_MAIZE was identified the roots of maize bio-primed with *P. simplicissimum* A4. Several studies have observed that the overexpression of lipid transfer protein genes in transgenic plants enhances pathogen resistance. Sun et al. (2008) showed that overexpressing wheat nsLTP genes in transgenic plants enhanced disease resistance where *in vitro* antifungal assays were done with eight wheat nsLTPs against three non-wheat and eight wheat pathogens.

The authors also reported the differential inhibition of spore germination and mycelial growth. Additionally, all the wheat nsLTPS showed activity against *F. graminearum*. Zhu et al. (2012) reported that overexpression of wheat lipid transfer protein gene (TaLTP5) enhanced their resistance to *F. graminearum.* Finally, Safi et al. (2015) reported that the expression of TdLTP4 gene in *Arabidopsis thaliana* enhanced their resistance to *Alternaria solani* and *B. cinerea*. From this study, the identified non-specific lipid-transfer protein was involved in localization as well as the establishment of localization (Figure 6.3 A). The protein has binding activity (Figure 6.3 B) and was shown to interact with the protein putative homeobox DNA-binding domain superfamily protein (Table S6.12; Figure S6.8).

The result of this study confirmed that *P. simplicissimum* A4 elicited proteomic changes in maize roots which was responsible for ROS-detoxification and defence against fungal pathogens in the presence of the fungal pathogen *F. proliferatum*.

CHAPTER 7

CONCLUSION AND FUTURE WORK

This study comprehensively evaluated the biocontrol potential of *Penicillium simplicissimum* A4 against *Fusarium proliferatum*, a major phytopathogen threatening maize production. Through the combination of whole-genome sequencing, untargeted metabolomic profiling, *in vitro* assays, biochemical assays, *in planta* assays, and proteomic analyses, this research elucidated the multifaceted mechanisms employed by *P. simplicissimum* A4 in mitigating the pathogenic effects of *F. proliferatum*. To the best of our knowledge, this is the first study investigating the response of germinating maize seeds prior to infection with the pathogenic fungus *F. proliferatum* which can cause the decline in germination, as well as the biocontrol capabilities of *P. simplicissimum* A4 against *F. proliferatum* which assists the plants' recuperation during infection.

The *in silico* genomic insights revealed a repertoire of genes encoding proteins which were critical for mycoparasitism and detoxification. Additionally, the *in silico* metabolomic profiling identified metabolites with strong antifungal activities, which further emphasized the biocontrol potential of *P. simplicissimum* A4. Enzymatic and biochemical assays demonstrated the antagonistic impact of *P. simplicissimum* A4 on *F. proliferatum*, including the disruption of cell wall (CW) integrity, enzymatic inhibition which effectively impaired the pathogen's virulence, and oxidative stress induction.

Moreover, bio-priming maize seeds with *P. simplicissimum* A4 enhanced root growth and resilience under *F. proliferatum* infection via the modulation of reactive oxygen species (ROS) levels and thus the reduction of oxidative damage, and the improvement of antioxidant enzyme activities. Proteomic analysis provided further evidence of the defensive role of specific proteins activated in maize roots by *P. simplicissimum* A4, which highlighted its role in boosting host plant defense mechanisms.

The findings emphasised the potential of *P. simplicissimum* A4 as a sustainable and eco-friendly biocontrol agent (BCA), offering a promising alternative to chemical fungicides. This study lays the foundation for further exploration of fungal endophytes for the promotion of global food security and environmental sustainability, including their application in enhancing

crop resilience, reducing dependence on chemical inputs, and addressing global food security challenges.

Future work will include antifungal assays using liquid media and using heat-treated controls to better understand the growth patterns of *P. simplicissimum* A4 under various conditions. Additionally, future work will include chemically synthesizing the list of antifungal metabolites to determine its capabilities to control *F. proliferatum in vitro* as well as the characterization of unknown SMs produced by *P. simplicissimum* A4. These SMs can be used to produce biofertilizer that can be utilized as a seed-priming agent that small-scale and large-scale farmers can use to decrease the growth of *F. proliferatum* thereby increasing growth and overall yield Moreover, gene expression studies can be done on genes responsible for the production of the candidate metabolites to maximise the production and use as biological control agents. Furthermore, field-scale validation should be done to maximize the efficacy and commercial viability of *P. simplicissimum* A4 as a BCA. Furthermore, studies can be done to determine the effect that *P. simplicissimum* A4 has on the production of mycotoxins in *F. proliferatum in vitro* and *in planta*. Lastly, future studies will test the role of NAGase and glucanase activities against other *Fusarium* pathogens.

CHAPTER 8

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