

Analysis of efficacy of different Penicillium fungal species as bio- control agents against Fusarium oxysporum

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the degree *Master of Science in Crop Science* at the North-
West University

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DECLARATION

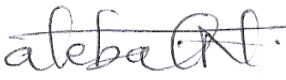
I, Galaletsang Patronella Segone declare that this dissertation with the research title “Analysis of biosafety of different *Penicillium* species for potential use as biocontrol agents against *Fusarium oxysporum*” submitted to North-West University is my original work. I have not previously in any form or part submitted to any other university. All sources used in this work have been acknowledged through references.

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DEDICATION

To my parents; Tidimalo and Solomon Segone

The sacrificial care and immense love have carried me this far and made it possible for me to
complete this work

My grandmother; Gothatamang Keebine

And my family

The support, prayers and encouragement are much appreciated.

“Don’t stop believing” – Journey

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LIST OF ABBREVIATIONS AND ACRONYMS

ABI:	Applied Biosystems
AMF:	Arbuscular Mycorrhizal fungi
ANOVA:	Analysis of variance
Aw:	Water activity
BLAST:	Basic Local Alignment Search Tool
CCI:	Chlorophyll Concentration Index
CRD:	Completely Randomized Design
DNA:	Deoxyribose Nucleic Acid
ECM:	Ectomycorrhizae
<i>F. sp.:</i>	<i>Formae specialis</i>
FOL:	<i>F. oxysporum f.sp. lycopersici</i>
FORL:	<i>F. oxysporum f.sp. radicis lycopersici</i>
G.A:	Gibberellic Acid
ITS:	Internal Transcribed Spacer
K:	Potassium
KOH:	Potassium Hydroxide
Mg:	Magnesium
N:	Nitrogen
P:	Phosphorus
PCR:	Polymerase Chain Reaction
PDA:	Potato Dextrose Agar

pH:	Power of Hydrogen
spp:	Species
VAM:	Vesicular-arbuscular mycorrhizae
VI:	Vigor Index
Zn:	Zinc

LIST OF PAPERS

- i. Segone. G.P. and Ramachela. K. (2020). ‘*In-vitro* analysis of suppressive effect of fungal isolates from compost amended soils on commonly occurring soil borne pathogens’, Presented at the *DSI-Howard University Women in STEM Conference*. Eastern Cape province, South Africa.

ABSTRACT

The fungal genus *Penicillium* and many other soil-borne fungi have widely been reported to create soil myco-ecological conditions that influence plant growth. These fungal species have been noted to have the ability to produce and release a diverse range of phytochemicals and exogenous compounds with variable physiological influence on plants, plant growth and other microbes. To positively utilize these unique characteristics of *Penicillium* species, the study was conceived and designed to investigate the suppressive effects of *Penicillium* and *Aspergillus* species against *F. oxysporum f.sp. radicis lycopersici* (FORL). Different experiments were carried out, i.e. (i) Isolation and identification of soil fungal isolates in the semi-arid agro-ecological region, (ii) Dual anti-fungal assessment of the isolates against *F. oxysporum f.sp. radicis lycopersici* (FORL), (iii) Analysis of isolates' phytopathological safety on selected vegetables and (iv) An *in-vivo* evaluation of the effect of *P. concavorugulosum-2* and various rhizosphere conditions against *F. oxysporum f.sp. radicis lycopersici* (FORL) on *S. lycopersicum*. This was carried out to determine the myco-suppressiveness of the local and well-adapted *Penicillium* species and *Aspergillus sp.* against *F. oxysporum f.sp. radicis lycopersici* (FORL). Five fungal species were isolated from cultivated soils by use of the serial dilution method. The isolates were morphologically identified to genus level using colony characteristics, i.e. conidia, hyphal form, and conidiophores. Molecular identification was carried out to identify the isolates to species level by use of the PCR method. To investigate the dual anti-fungal association, an *in-vitro* analysis was carried out by use of mycelial disc method. Safety of these isolates on the commonly grown vegetables viz. *Spinacia oleracea*, *Beta vulgaris* and *Solanum lycopersicum* was assessed by inoculating them with respective fungal isolates. Furthermore, *in -vivo* evaluation was carried out by assessing the effect of the promising isolate; *P. concavorugulosum-2* under five (5) rhizosphere amended conditions.

The identification of the fungal soil isolates resulted in the confirmation of the isolate identities to be as follows: *Penicillium commune-1* (MK660351.1), *Penicillium commune-2* (MK660335.1), *Aspergillus fumigatus* (MN178806.1), *Penicillium concavorugulosum-1* (MK841454.1) and *Penicillium concavorugulosum-2* (MK841454.1). Analysis of their dual interaction showed the occurrence of various relationships such as antibiosis, competition and mycoparasitism. High inhibition effect of *P. concavorugulosum-2* (67.9%) and *A. fumigatus* (65.36%) on *F. oxysporum f.sp. radicis lycopersici* (FORL) was noted. A strong positive linear relationship ($r = 0.785$) was exhibited between *P. commune-1* and *F. oxysporum f.sp. radicis lycopersici* (FORL). Their effect on the bio-safety study on selected vegetables showed that

the treatments had statistically significant differences in their effect on the germination, radicle and plumule growth of *S. oleracea* and *B. vulgaris* ($P < 0.05$). There was, however, a relatively non-significant positive response on *S. lycopersicum*. Of interest, is the germination of *S. lycopersicum* seeds that were treated with *P. concavorugulosum*-2, *P. commune*-2 and control which all resulted in 100% germination.

In an *in-vivo* study where the effect of *P. concavorugulosum*-2 under different rhizosphere conditions was analyzed, there were statistically significant differences in the seedling biomass for the different treatments ($P < 0.05$).

The development of *P. concavorugulosum*-2 as a potential bio-control agent is recommended as it exhibited plant growth stimulatory and protective properties against *F. oxysporum*. It is recommended that *P. concavorugulosum*-2 be studied under a situation where it is combined with other promising isolates to explore possible synergistic effects.

Keywords: *Fusarium oxysporum f.sp. radicis lycopersici* (FORL), *Penicillium species*, Biosafety, *Solanum lycopersicum*, Mycoparasitism, Antibiosis, Mycorrhizae and Biochar.

CHAPTER ONE

1.1. GENERAL INTRODUCTION

Crop pests and diseases have persistently caused crop yield losses and are widely considered a major global constraint to crop production (Savary *et al.*, 2019). Oerke and Dehne, (2004) reported that in South Africa, between 1996 and 1998 plant diseases caused crop yield losses of up to 9.9%. Additionally, about 10-16% loss in harvest is still being claimed by pathogens between 2009 and 2011 (Chakraborty and Newton, 2011) . The study also highlighted that yield losses increased to 14.9% when chemical control measures were not applied. Lindenthal *et al.*, (2005) further highlighted that fungi and bacteria resulted in an average of 7% to 15% crop losses in major world crops.

Extensive use of chemicals such as insecticides and fungicides in the control of plant pests and diseases has contributed to the high cost of crop and horticultural production (De Bon *et al.*, 2014, Panth *et al.*, 2020). Furthermore, these chemicals have, of late, been widely viewed negatively because of their adverse effects on the environment. A certain number of these agro-chemicals have strongly been associated with carcinogenic effects on humans (Wightwick & Allison, 2007).

Although these agro-chemicals have been noted to harm the environment, the high crop yield losses due to pests and diseases has resulted in the extensive global continual use of these chemicals. *Fusarium* species, among many fungal pathogens, have been reported to have a widespread destructive impact on both field and vegetable crops (Srinivas *et al.*, 2019). *Fusarium oxysporum* has particularly been noted to be one of the most destructive soil-borne fungal species. This pathogen has a wide range of hosts and has specialized forms (*formae specialis* (f.sp.)) that cause a variety of diseases (Fravel *et al.*, 2003; Michielse, 2009). There are two forms that result in extensive economic losses in tomatoes i.e. *F. oxysporum f.sp. radices-lycopersici* (FORL) and *F. oxysporum f. sp. lycopersici* (FOL) [(Baysal *et al.*, 2009; Debbi *et al.*, 2018; Srinivas *et al.*, 2019)]. FORL attacks the root crown whereas FOL attacks the fine roots and goes up the xylem, causing wilting – Fusarium wilt. These *formae specialis* forms also include the non-pathogenic *F. oxysporum* species that have endophytic properties that generate plant defensive mechanism against most soil-borne pathogens (de Lamo & Takken, 2020). The endophytic strain can therefore mediate or reduce the effect of vascular diseases caused by pathogenic strains of *F. oxysporum* (de Lamo & Takken, 2020). The *Fusarium* fungal species are generally known to be facultative parasites i.e. in the absence of a

host they can live as saprophytes. In the presence of a susceptible host and under suitable environmental conditions they become parasitic (Srivastava *et al.*, 2018). The pathogenic strains can also co-exist with the host without injury but exert harm once the host defence mechanism is compromised, resulting in vascular wilts of seedlings (de Lamo & Takken, 2020). These fungal species can result in high seedling mortality of vegetables such as tomatoes (*Solanum lycopersicum*), eggplant (*Solanum melongena*), and peppers (*Capsicum annum*). These pathogens cause diseases such as vascular wilts, stalk and cob rots, collar rots of seedlings and tuber rots (Pietro *et al.*, 2003; Gordon, 2017; Srivastava *et al.*, 2018). Most susceptible host plants are vegetable crops from the *Solanaceae* family i.e. *S. lycopersicum*, *S. tuberosum*, *S. melongena*, etc. and the *Cucurbitaceae* family which include the following: *Cucumis sativus*, *Cucumis melo* and *Cucurbita*. Another susceptible plant family is the *Fabaceae* family that includes crops such as *Pisum sativum*, *Cicer arietinum* and *Glycine max*. The fungus *F. oxysporum* attacks its host by gaining entry through the plant roots and grows in the plants' xylem vessels, eventually blocking the vascular system, preventing transport of water and nutrients to various parts of the plant (Michielse, 2009, Rana *et al.*, 2017, Joshi, 2018).

The wilts and mortality of most vegetable seedlings are a result of the destruction of vascular systems. For instance, Naseri and Tabande (2017), highlighted that 108 epidemics of chickpea (*Cicer arietinum*) affected by *Fusarium* wilt have been reported worldwide. *Fusarium* wilt is one of the most prevalent and problematic diseases caused by *Fusarium oxysporum* and other soil-borne pathogens and is known to drastically impact plant growth and development (Fravel *et al.*, 2003; Srivastava *et al.*, 2018). Effects of *Fusarium* wilt on *S. lycopersicum* have been reported to have a direct impact on the economic wellbeing of *S. lycopersicum* growers because of the high cost of chemicals, fumigants and various control measures that are used to control the diseases (Amini and Sidovich, 2010; Koehler and Shew, 2019).

Various soil amendments and management practices have been investigated on their suppressive effect on *F. oxysporum*. These management practices include the use of fungicides, soil sterilization and fumigation. Their effectiveness has however, been found to be variable between different studies (Oerke and Dehne, 2004; Koehler and Shew, 2019). The use of methyl bromide has for a long time been effective and widely used by farmers. Its use has, however, been discontinued because of the concern on its contribution to methane emission that is destructive to the ozone layer. The other control method that has been commonly employed is the use of resistant cultivars. The use of resistant cultivars has been compromised

by the development of new strains of *F. oxysporum* that can infect and cause seedling mortality in cultivars that are traditionally known to have been resistant to *F. oxysporum* (Tanyolaç and Akkale, 2010). To develop effective and environmentally safe methods of controlling this pathogen, the use of biological agents has been investigated by various researchers (da Silva *et al.*, 2017, Diblasi *et al.*, 2015, Qualhato *et al.*, 2013). Results of these studies have generated interest among many plant pathologists. One of the interesting results is the study by da Silva *et al.* (2017) who reported that the colonization of host root rhizosphere by non-pathogenic *F. oxysporum* as well as *P. oxalicum* resulted in the reduction of *Fusarium* wilt diseases.

Additionally, *Trichoderma*, *Aspergillus* and *Penicillium* species are some of the most reported species to show promising results as biological control agents against soil-borne pathogens viz. *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, *F. solani*, etc. For instance, Bastakoti *et al.*, (2017) reported that *Trichoderma* species are more effective as biological control agents against *Sclerotium rolfsii* than other soil-borne pathogens. Similarly, a study by Urooj *et al.*, (2018) highlighted the interesting suppressive effects of *Penicillium* species against destructive soil-borne pathogens viz. *F. solani*, *F. oxysporum*, *R. solani*. Toghueo and Boyom, (2020) reported on some species of the *Penicillium* genus as endophytic and these were *P. restrictum* and *P. asperum*. These endophytic *Penicillium* species were noted to exhibit protection to host plants against various soil-borne pathogens. Toghueo and Boyom (2020) further highlighted the anti-microbial, anti-parasitic, insecticidal and biocontrol properties that these species contain. This, therefore, shows the potential effectiveness of *Penicillium* species as biocontrol agents against the commonly occurring soil-borne fungal pathogens. This study was therefore undertaken to investigate the following: (i) Isolation, morphological and molecular identification of soil-borne *Penicillium* species and other soil-borne fungi from various cultivated sites in a semi-arid region. (ii) *In-vitro* analysis of the effect of identified *Penicillium* spp. and other fungal isolates against *F. oxysporum*. (iii) Screening of *Penicillium* species and other fungal isolates on its phytopathological biosafety on selected vegetable species. (iv) To assess the effect of biochar and mycorrhizae as rhizosphere amendments for enhancement of *Penicillium* spp suppressiveness on *F. oxysporum* in tomato seedlings.

1.1.1. Problem statement

The excessive use of fungicides, soil fumigants and other agro-chemicals results in adverse effects on the environment. This occurs because of their residual persistence in the soil which alters soil biochemical and physical properties resulting in polluted terrestrial ecosystems and

waterways (Larena *et al.*, 2003; Wightwick & Allinson, 2007). For example, Eijsackers *et al.* (2005) and Wightwick *et al.* (2008) highlighted the concerns over the excessive use of copper-based fungicides which resulted in the accumulation of copper in the soil. These high levels of copper have been reported to negatively affect beneficial soil microbial populations such as mycorrhizae and saprophytes fungi that play an important ecological role in the soil.

In an effort to find an ecologically safe technology that can be employed to control soil-borne pathogens, the use of bio-control agents and various soil sterilization techniques have been investigated. For example, biological agents which include among others; fungal microorganisms such as *Trichoderma* species, and bacterium species like *Pseudomonas* and *Bacillus subtilis* have been explored (Larena *et al.*, 2003). However, *Trichoderma* species are more effective in moist and slightly acidic soils. They have been noted to be less effective when used in soils that are not amended with compost or farmyard manure (Kumar *et al.*, 2014; Hamed *et al.*, 2015; Konappa *et al.*, 2018). The species are also not highly persistent in the soil. The poor persistence in the soil results in the need for repeated application and therefore causing excessive usage. This makes its use rather expensive and not easily accessible to smallholder farmers who are generally not financially well-resourced. However, there is ongoing research on *Trichoderma* that is looking at the development of *Trichoderma* formulations with longer field persistence and suitability for dry weather conditions (Kumar *et al.*, 2014). In addition to the studies on *Trichoderma*, other efforts are exploring other soil-borne fungi isolates that can be used as soil bio-control agents for the management and control of the commonly occurring soil-borne plant pathogens.

1.1.2. Justification

Various studies on the use of various fungal species such as *Penicillium* and certain bacterial species as a biological control of soil-borne pathogens agent have shown interesting results. This study, therefore, seeks to identify *Penicillium* species and any other fungal isolates with myco-suppressive effects that are endemic to the semi-arid region and therefore well adapted to the conditions. These well-adapted fungal biotypes can be used as a bio-control agent in vegetable seedling nurseries against *F. oxysporum* and other problematic soil-borne fungal pathogens. It is the goal of this study to develop an ecologically stable low-cost bio-control formulation that is affordable for smallholder farmers in the semi-arid regions.

1.1.3. Research Objectives

Main objective:

To investigate the suppressive effect of *Penicillium* species and other soil-borne fungi on *Fusarium oxysporum* f.sp. *radicis lycopersici* (FORL) in tomato seedlings.

Specific objectives:

- (i) Isolation, morphological and molecular identification of soil-borne *Penicillium* species and other soil-borne fungi from various cultivated sites in a semi-arid region.
- (ii) To carry out *In- vitro* analysis of the effect of identified *Penicillium* spp. and other fungal isolates against *F. oxysporum*.
- (iii) To carry out screening of *Penicillium* species and other fungal isolates on its phytopathological biosafety on selected vegetable species.
- (iv) To assess the effect of biochar and mycorrhizae as rhizosphere amendments for enhancement of *Penicillium* spp suppressiveness on *F. oxysporum* in tomato seedlings.

1.1.4. Research hypotheses

- (i) *Penicillium* species and other soil-borne fungal isolates' effectiveness against *F. oxysporum* will vary. The variation will depend on the presence of anti-fungal (exogenous compounds) properties by the species.
- (ii) Different *Penicillium* species and other soil-borne fungal isolates will enhance seed germination of the three selected vegetables by providing phytochemicals/growth-promoting hormones that improve seed germination.
- (iii) Different rhizosphere amendments will enhance *Penicillium* suppressiveness against *F. oxysporum* by improving soil properties; soil fertility, soil structure and soil water holding capacity.

1.2. LITERATURE REVIEW

The extensive and excessive use of agrochemical compounds such as fungicides to control soil-borne pathogens has become a global concern. These agrochemicals, among many other concerns are toxic, unstable and have been noted to cause environmental pollution (Jayaraj *et al.*, 2016).

The use of copper-based fungicides and other compounds as soil applications for control of soil-borne pathogens has been reported to decrease growth, survival, as well as behavioural aspects of a wide range of beneficial soil microorganisms (Eijsackers *et al.*, 2005). Repetitive uses of fungicides result in residual persistence and fungicidal drift into the air and waterways, which pose a health hazard to both human health as well as aquatic organisms' survival (Wightwick *et al.*, 2010). This has led agricultural scientists to explore initiatives that can lead to the development of environmentally safe methods that can be used to manage and control the problematic soil-borne pathogens.

The ecologically safe control methods that have been investigated include the use of organic amendments such as green manure, animal manure, compost, and peats. These organic amendments have been noted to decrease the occurrence and severity of various soil-borne disease, *albeit* to different levels (Magid *et al.*, 2001). Although some reports indicated the effectiveness of organic amendments as suppressants of various soil-borne pathogens, Bonanomi *et al.* (2010) highlighted that these organic amendments may increase the occurrence and severity of certain species of pathogens. Extensive research on various organic amendments has been carried out, however, no soil amendment management technology for the control of soil-borne pathogens has been successfully developed. It has however, been established that organic amendments aid in the reduction of chemical damage and toxicity. They have also been noted to play an important ecological role in soil health. They serve as a food source for antagonistic microorganisms that are found in the soil and these aid in the reduction of seedling attack by certain soil-borne pathogens.

Fungal pathogens such as *Fusarium oxysporum* are known to cause detrimental effects on plant growth and development of different crops and vegetables. A number of management strategies have then been initiated and used to mitigate the impact and pathogenicity of *F. oxysporum*. These include the use of different microorganisms that have shown effectiveness against pathogenic fungal species, for example, the use of *Trichoderma* species in plant production to suppress growth of pathogenic microbes while improving plant growth and productivity (Bastakoti *et al.*, 2017).

1.2.1. Current initiatives on management of *F. oxysporum*

Fusarium oxysporum f.sp. radicis lycopersici is commonly known as Fusarium crown rot and has been reported to cause severe economic losses in both field and greenhouse productions (Manzo *et al.*, 2016). The pathogen has been ranked 5th among the top 10 fungal pathogens

(Husaini *et al.*, 2018) is reported to be a major challenge in agricultural production world-wide (McGovern, 2015). Studies conducted to understand this pathogen resulted in a variety of integrated strategies such as chemical, host-resistance, biological and physical control (Szczeczura *et al.*, 2013, McGovern, 2015, Manzo *et al.*, 2016, Zhao *et al.*, 2017, Srinivas *et al.*, 2019). A study by (Yuce *et al.*, 2010) analysed the effectiveness of the integration of solarization combined with hydrogen peroxide and benzoic acid on the pathogen and concluded that the combination could be an effective environmentally friendly chemical that can suppress the impact of FORL and FOL. Furthermore, a study on biological control analysis of antifungal effects of medicinal plants viz. *Zygophyllum album*, *Euphorbia guyoniana*, *Atriplex halimus* etc. on FORL was conducted by (Wassima, 2017). The study found a 75,25% inhibition rate and effect of *Z. album* on FORL after 8 days and concluded that an aqueous extract of the plant showed promising results against the pathogen. A number of biological control agents by use of fungal microorganisms such as *Glomus intraradices* and *Trichoderma harzianum* have shown suppressiveness against FORL and are considered natural enemies or antagonists of the pathogen (Akköprü and Demir, 2005, Martínez-Medina *et al.*, 2010, Castillo *et al.*, 2019).

1.2.2. Ecology of *Penicillium* species

Penicillium is an omnipresent fungal genus that is prevalent in soil, food, feed, and can be classified as an opportunistic fungus (Perrone and Susca, 2017; Park *et al.*, 2019). These fungal species are generally considered facultative i.e. can survive as both saprophytes and parasites. Most species exhibit optimal growth at moderate to low temperatures and can grow at water activities (a_w) below 0.9 (Pitt, 2002). Water activity may be defined as the measurement of the energy status of water in a food, soil and plant systems (Alkorta *et al.*, 2017; Lenovich, 2017). *Penicillium* species vary in their ecological effects as most may result in food and feed deterioration whereas certain species can produce metabolites while a few are endophytes, parasitic or saprophytes (Pitt *et al.*, 2000; Pitt, 2002; Pitt and Hocking, 2009).

Growth of *Penicillium* species or any other fungal growth and production of metabolites are influenced by various factors such as host characteristics and climatic factors. Pointing out a single factor would therefore be scientific inappropriate as they generally function collectively (Hyde *et al.*, 2019). These factors can be categorized as climatic factors, competitive actions, plant properties, and other environmental factors such as pH, water activity (a_w), and oxygen content (Popovski and Celar, 2013). Temperature and water activity (a_w) are the most important set of conditions influencing the growth and metabolite production

of fungi such as *Penicillium* and *Fusarium* species (Abellana *et al.*, 2001). The influence and interactions between this set of conditions on the growth of three *Penicillium* species – *P. aurantiogriseum*, *P. chrysogenum*, and *P. corylophilum*, and metabolites/mycotoxin production were studied by Abellana *et al.*, (2001) and Sanchis *et al.*, (2004). The researchers concluded that growth rates showed a strong dependence on water activity and temperature. The minimum water activity values for growth of these species were 0.85 – 0.9. The conclusion was, however drawn and established that an increase in water activity results in an increase in temperature since a water activity of 0.85 - 0.90 showed the optimum growth of *Penicillium* species between the temperature of 20 °C and 25 °C.

Studies by Frisvad *et al.*, (2006, 2007 and Frisvad *et al.*, (2007) showed that various *Penicillium* species produce metabolites and the importance of these compounds depends on the ecology and biology of the species. Frisvad *et al.*, (2006) highlighted that for the influence on the production of the metabolites to be evident, the species should be grown in a suitable host plant or food and the temperature should favour germination and water activities should be optimal. Metabolites production by *Penicillium* species is however complex as the formation of the compounds differs from species to species. Most species produce a single toxin whereas some produce more than one toxin (Pitt *et al.*, 2006). For example, *Penicillium expansum* and *Penicillium crustosum* produce two toxins whereas other species such as *Penicillium islandicum* and *Penicillium simplicissimum* produce four toxins (Pitt *et al.*, 2000, 2006; Pitt, 2002).

1.2.3. Identification of *Penicillium* species

Various laboratory techniques are used in the identification of fungal micro-organism. The traditional approach of morphological evaluations, as well as different methods of molecular identification, are some of the approaches that are often used (Bandh *et al.*, 2011; Bechem *et al.*, 2017). Similar approaches are also applied in *Penicillium* fungal species identification whereby conventional traditional and microscopic morphological identification and molecular analysis are used. These methods include evaluation of the fungal colony for characteristic features such as colour, shape/form, size, spores/conidia, and fruiting bodies/conidiophores (Kim *et al.*, 2013; Alsohaili and Bani-Hasan, 2018). Molecular analysis methods, on the other hand, include subjecting *Penicillium* mycelium to DNA extraction procedures, PCR, gel electrophoresis, ITS and phylogenetic analysis for species determination (Houbraken *et al.*, 2011; Peterson, 2012; Demirel *et al.*, 2013; Visagie *et al.*, 2014; Bechem *et al.*, 2017; Koffi *et al.*, 2019; Sawant *et al.*, 2019). Among the conventional microbial identification methods,

microscopy identification is still mostly used as it is less expensive. However, various studies have proven that the use of molecular analysis is more accurate and recommended for *Penicillium* species. This is because the *Penicillium* fungal species belong to a large genus that shows little to no variation in media cultures and in morphology which often leads to inaccuracies in morphological identification procedure (Visagie *et al.*, 2014).

1.2.4. Secondary metabolites production by *Penicillium*

Metabolite production is the synthesis of secondary metabolic substances by some fungal microorganisms which they use to defend themselves when exposed to unfavourable conditions (Ismail and Papenbrock, 2015; Cinar and Onbaşı, 2019). Various fungal species such as *Penicillium*, *Aspergillus* and *Fusarium* genera are amongst the fungal microorganisms that produce harmful toxic biochemicals (Desjardins and Proctor, 2007). Various studies have also highlighted the effects of fungal metabolites on agricultural production, food production and human health (Zain, 2011; Mokogwu *et al.*, 2017; Cinar and Onbaşı, 2019; Nguyen *et al.*, 2019).

The *Penicillium* genus has over 300 recorded species and they are recognised as multifunctional due to their production of diverse secondary metabolites (Perrone and Susca, 2017). Biochemical compounds such as ochratoxin A & B, aflatoxin B1, citrinin, acanin, pestalotin, 7-hydroxypestalotin and questiomycin A were regarded and reported as mycotoxins by Bräse *et al.*, (2009). They were further reported to be *Penicillium* produced (Olsen *et al.*, 2019). Metabolite effects are reported on seed quality reduction, negative effect on germination, root growth and seedling vigour (Ismail and Papenbrock, 2015; Perrone and Susca, 2017). Despite the negative effects exerted on plants and plant growth by certain species in this genus, they can, however, be investigated for their potential use as biocontrol agents i.e. their use in the reduction of soil-borne pathogens.

1.2.5. Rhizosphere eco-zones and Plant root microbial interactions

The rhizosphere is a closely compacted area of the soil that surrounds plant roots and is influenced by plant root exudates and soil factors surrounding the roots. The micro-ecology of the region is such that plant roots compete with the invading soil-borne microorganisms such as fungi, bacteria and various insects for space, nutrients, water, and minerals (Giri *et al.*, 2003). Similarly, the root rhizosphere is influenced by secretion of a diverse mixture of compounds or chemicals by the roots. Sugiyama and Yazaki, (2014) highlighted that the compounds

secreted by plant roots serve an important role as chemical attractants or repellents to various soil microorganisms in the rhizosphere. These exudates assist in the classification of different zones and components that play a significant role in determining characteristics of a specific rhizosphere component (Bazin *et al.*, 1990; York *et al.*, 2016), [Table 1].

Table 1.1: Rhizosphere components and their eco-zones*

Component	Size	Definition
Rhizosphere	~cm	Soil influenced by roots
Rhizoplane	1 mm	Root epidermis, mucigel, and adhering soil
Rhizosheath	1 mm	Soil adhered by root hairs and mucilage
P depletion zone	3 mm	Concentration gradient of P in soil solution due to uptake
N depletion zone	2 cm	Concentration gradient of N in soil solution due to uptake
Accumulation zone	1 mm	Calcium from mass flow but not adsorbed
Soil structure modification	1 cm	Changes in soil porosity, soil architecture modification
Oxygen depletion	3 mm	Oxygen uptake due to root and microbial respiration
CO₂ Accumulation	3 mm	Respired carbon dioxide from roots and microbes
Exudation zone	2 mm	Sugars, mucilage, acids, allelochemicals released by roots
Microbe	µm–m	Fungal mycelia transcend six orders of magnitude in scale

*Source: York *et al.*, (2016)

There are various interactions between microorganisms, plant roots, and the soil, that alter the soil chemical and physical properties. These interactions, in turn determine the microbial population in the rhizosphere (Huang *et al.*, 2014). These interactions can be beneficial to the soil environment as well as the plants (Nadeem *et al.*, 2014). They may be classified as positive as well as negative interactions and are mediated by plant roots. Plants are influenced positively through mechanisms such as nitrogen fixation, increased biotic and abiotic stress tolerance by the presence of endophytic microorganisms, and mycorrhizal association (Bais *et al.*, 2006; Weir *et al.*, 2010). Various studies on rhizosphere influences have highlighted that microorganisms are attracted to compounds produced through root exudation (Bais *et al.*, 2006; Nadeem *et al.*, 2014), thus signalling and initiating colonization by various beneficial as well as non-beneficial microorganisms.

Microorganisms present in the rhizosphere include bacteria, fungi, actinomycetes, and protozoa. However, fungi and bacteria are generally the most abundant (Nadeem *et al.*, 2014).

There is a symbiotic relationship between mycorrhizal fungi and plant roots which aids in the increase of the plant root surface area, enabling the plant to absorb water and nutrients such as phosphorus and nitrogen more efficiently (Sugiyama *et al.*, 2014). There are two types of mycorrhizae involved in the symbiotic relationship, the ectomycorrhizae (ECM) and the arbuscular mycorrhizal fungi - AMF (Nadeem *et al.*, 2014). Arbuscular mycorrhizae are the most abundant in agricultural soils and they assist the ecosystem through their role in nutrient cycling (Christie *et al.*, 2004).

A study by Brito *et al.* (2013) established enhanced growth and productivity of various vegetable crops that were associated with root colonization by mycorrhizal fungi. Arbuscular mycorrhizal fungi can increase the availability and supply of slowly diffusing ions and can provide macro- and micro-nutrients such as N, K, Mg, and Zn to the plants particularly in soils where the nutrients are available in less soluble forms (Smith *et al.*, 2008; Nadeem *et al.*, 2014).

1.2.5 Biochar as a soil amendment

Biochar may be defined as carbonized biomass produced under low oxygen (Yao *et al.*, 2017) and it may be used as a soil amendment to enhance agricultural and environmental value. The carbonized biomass can also assist in retaining water and nutrients in the soil thus improving water uptake by plants.

The use of biochar as a soil amendment is on the increase among farmers because of its ability to improve soil quality and crop productivity (Zheng *et al.*, 2010). Biochar can adsorb and immobilize heavy metal ions found in contaminated soils (Hossain *et al.*, 2010; Hayyat *et al.*, 2016; Zhou *et al.*, 2017; Wang *et al.*, 2019). Efficiency and effectiveness of biochar as a soil amendment depend on the plant type used, soil type, biochar production temperature and the rate of application (Rizwan *et al.*, 2016). Application rate also influences the availability of microbes in the soil as biochar modifies the soil physical and biochemical properties and facilitates soil fungi reproduction and spore germination including fungi such as arbuscular mycorrhizae (Zhang *et al.*, 2013; Hammer *et al.*, 2015).

1.2.6 Mycorrhizae as a soil amendment

Mycorrhizal fungi have been termed and considered to be natural bio-fertilizers that aid in the enhancement of plant water and nutrients uptake as well as protection against pathogenic soil microorganisms (Berruti *et al.*, 2016). Numerous studies with various aims and objectives have

been undertaken by several researchers with the aim of analysing effects that mycorrhizal fungi have on plant growth, nutrient uptake and mechanisms involved in plant root colonisation. For example, Watts-Williams *et al.* (2017) analysed benefits of AMF under both zinc (Zn) deficiency and toxic conditions to determine the type of transporter gene involved in root colonisation under various zinc stress conditions. Other studies by Amballa and Bhumi (2016) and Igiehon *et al.* (2017) explored different influences and benefits of AMF interactions with other microorganisms and abiotic soil factors on plant growth, health and nutrition. These studies generally concluded that mycorrhizal fungi interactions with rhizosphere organisms and influence plant health, soil fertility and crop productivity positively.

1.2.7 Influence of biochar and mycorrhizae as soil amendments

Symbiotic influence of mycorrhizal fungi on plants and plant roots has been commended for various improvements in plant growth and development (Paymaneh *et al.*, 2018). Biochar on the other hand has been advocated for its numerous positive effects on soil physical and biochemical properties as well as plant development. These positive influences in plant growth and soil improvement have resulted in several researchers investigating the effect of a combination of mycorrhizae and biochar as a soil amendment. A study by Hammer *et al.*, (2015) showed that combined application of biochar and arbuscular mycorrhizae had a synergistic effect on nutrient cycling. This blended soil amendment also influenced the sequestration of heavy metals and thereby reducing their negative effect on microbial activity. Zhang *et al.*, (2013) also highlighted that there were studies that reported and justified the use of biochar together with arbuscular mycorrhizal fungi. It has however remained unclear whether the combination can synergistically increase availability of beneficial microbes and decrease heavy metal uptake by plants. Therefore, mechanical and physiological protection induced by mycorrhizae and the ability to sequester trace elements in combination with biochar as a source of carbon for the mycorrhizae are investigated as soil amendments in this study through an in-vivo experiment.

CHAPTER TWO

Isolation and identification of soil-borne *Penicillium* spp and other fungal species in a semi-arid agro-ecological region.

2.1 INTRODUCTION

Penicillium species are a widespread group of facultative fungi which are found in various habitats such as soil, air and decaying plant matter (Pitt and Hocking, 2009; Visagie *et al.*, 2014). Various studies have reported the genus to contain several species which possess different roles in agriculture and human health. For example, species such as *Penicillium cyclopium* and *Penicillium citrinum* have been reported to be contaminants in corn, soybean, and dried beans (Munkvold *et al.*, 2019). Whereas *Penicillium expansum* and many other *Penicillium* species have been reported to cause post-harvest losses in pome fruits and apples (Pitt and Hocking, 2009; Wu *et al.*, 2019).

Many *Penicillium* species produce extracellular enzymes such as cellulolytic enzymes, polysaccharases and pectic enzymes that aid in the breakdown of organic materials and use in antibiotic production, cell degeneration, as well as food production (Yoon *et al.*, 2007; Rabha and Jha, 2018). Production of these enzymes and phytohormones by *Penicillium* fungal species that influence plant physiological processes was also reported by Sanders, (2011) as well as Chanclud and Morel, (2016). Tiwari *et al.* (2011) also highlighted *Penicillium* species as some of many fungal microorganisms that possess multifunctional properties that enable them for use in various agro-industries, biological, medicinal, and commercial uses.

Therefore, commercial and industrial use of the different fungal species including *Penicillium* species require accurate identification. Identification of fungal microorganisms is based on analyzing morphological features of the mycelial colony and their respective spores. Features such as hyphal type and the presence or absence of special characteristics such as conidia and conidiophores etc. are used in determining the genera of most fungal microorganisms (Bandh *et al.*, 2011). The use of morphological features is however not very accurate and has on many occasions lead to inaccurate identification. This has led to the development and utilization of molecular identification techniques. This technology uses polymerase chain reaction (PCR). Fungal microorganisms are mostly analyzed using the PCR method to determine the ITS region so that there could be accurate differentiation of species amongst the genus (Johnston, 2011).

The objective of this study was therefore to carry out identification and differentiation of fungal isolates from cultivated soils using morphological characteristics and molecular analysis using PCR techniques.

2.1.1 Specific objective(s):

- i. To isolate and morphologically identify *Penicillium* species using the serial dilution technique and microscopic examination
- ii. To molecularly identify the isolated fungal species using PCR methodology.

2.2 MATERIALS AND METHODS

2.2.1 Isolation of the fungal species

Four soil samples were collected from different sites at the North-West University Mafikeng agricultural farm. These sites had previously been subjected to different agronomic practices such as minimum soil tillage, weed control, fertilizer management and controlled irrigation. Soil sub-sample suspensions were prepared by mixing 100g of each of the four sampled soils with 700ml of distilled water, respectively.

Single spore isolation of fungal species growing in the respective soil samples was made by doing serial dilutions (Sarker *et al.*, 2006). One (1) ml of the sixth test tube of the serial dilution was pipetted onto the Petri dishes containing potato dextrose agar (PDA) growth media and incubated at $25\pm 1^{\circ}\text{C}$ for 48 hours. Re-isolation from colonies was carried out after 48 hours of incubation. Agar plug were cut by use of dissecting pins and cultured into PDA media.

2.2.2 Morphological identification of the isolated fungal species

Morphological identification, i.e. macroscopic and microscopic was carried out by analysing colony colour and texture as well as microscopic characteristics such as type of mycelial structure (septa) and fungal spores (shape, colour, and fruiting body). ZEISS ZEN 2016 software compound microscope at 400x magnification.

2.2.3 Molecular identification of the isolated fungal species

Genomic DNA was extracted by aseptically scraping mycelia of the different isolates from the respective PDA Petri-plates. Mycelial DNA was extracted using the Quick-DNA™ fungal/bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005) (White *et al.*, 1990).

2.2.4 Polymerase chain reaction (PCR) amplification

Amplification of the target genes was carried out by use of OneTaq® Quick-load® 2×Master Mix (NEB, Catalogue No. M0486) with primers presented in Table 1. Each Eppendorf tube comprised of 10µl of NEB OneTaq 2X MasterMix with Standard Buffer (Catalogue No. M0482S), 1µl of genomic DNA (10-30ng/µl), 1µl forward primer (10µM), 1µl reverse primer (10µM), and 7µl nuclease-free water (Catalogue No. E476). Amplification cycles had an initial denaturation at 94 °C for 10 mins, 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 50 °C, elongation at 68 °C for 1 minute and final elongation at 72 °C for 10 mins. The PCR amplicons were stored at 4 °C until electrophoresis.

Table 2. 1: Internal Transcribed Spacer Primers sequences that were used for amplification of the ITS regions.

Name of Target primer	Sequence (5' to 3')	Cycling conditions
ITS 1 Small Sub-unit	TCCGTAGGTGAACCTTGCGG	$\left. \begin{array}{l} 94^{\circ}\text{C for } 30 \text{ sec} \\ 50^{\circ}\text{C for } 30 \text{ sec} \end{array} \right\} 35 \text{ cycles}$
ITS 4 Large Sub-unit	TCCTCCGCTTATTGATATGC	$\left. \begin{array}{l} 68^{\circ}\text{C for } 1 \text{ min} \\ 68^{\circ}\text{C for } 10 \text{ min} \end{array} \right\} 35 \text{ cycles}$

2.2.5 Agarose Gel Analysis

The integrity of the PCR amplicons was visualized on a 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd) stained with EZ-vision® Bluelight DNA Dye. The gel ladder used for analysis is shown in Figure 2.1.

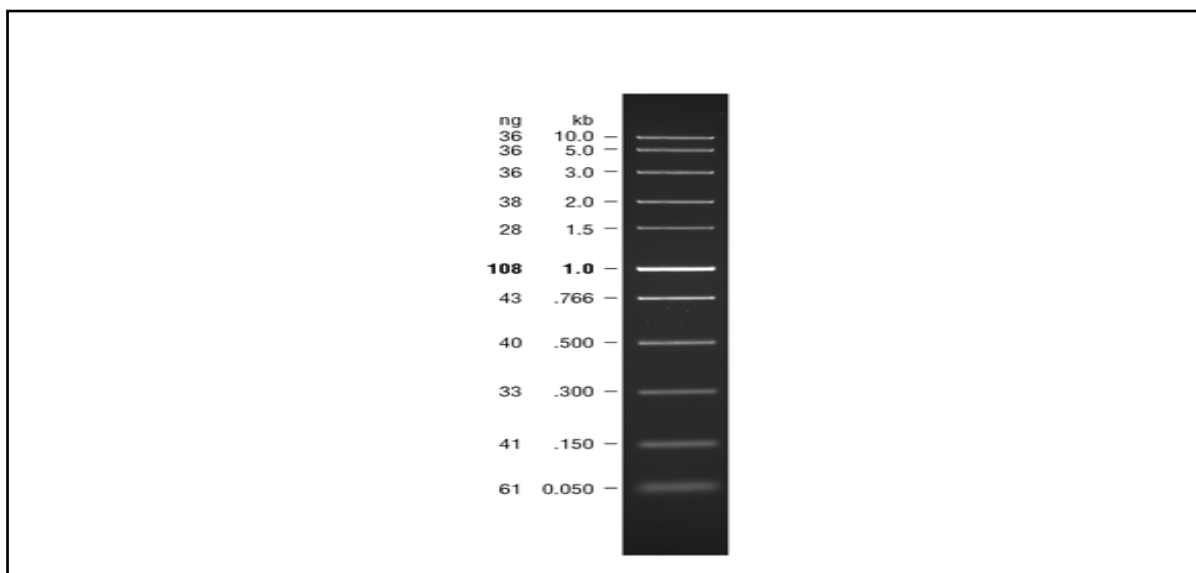


Figure 2. 1: Ladder details that were used in electrophoresis analysis.

2.2.6 ITS sequencing of the extracted fragments

The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit v3.1, BRD3-100/1000) and were purified using (Zymo Research, ZR-96 DNA Sequencing clean-up Kit™, Catalogue No. D4050).

2.2.7 Phylogenetic analysis

The purified fragments were analysed on the ABI 3500xl Genetic analyser (Applied Biosystems, ThermoFisher Scientific) for each reaction and every sample. CLC Bio Main Workbench v7.6 was used to analyse the ab1 files generated by the ABI 3500xl Genetic analyser and the results were obtained by conducting a BLAST search (NCBI) (Stephen *et al.*, 1997).

2.3 RESULTS

2.3.1 Morphological identification of isolated species

Morphological characteristics may be defined as a variation in shape, size, and structure of an organism (Wang *et al.*, 2015). This variation assists in the classification of organisms to their respective genera. Morphological identification of the fungal isolated species was carried out based on such characteristics. Figure 2.2 shows different macroscopic features of different isolates i.e. colony colour, form and shapes. Figures 2.2 and 2.3 show macroscopic and microscopic features of the isolated fungal isolates.

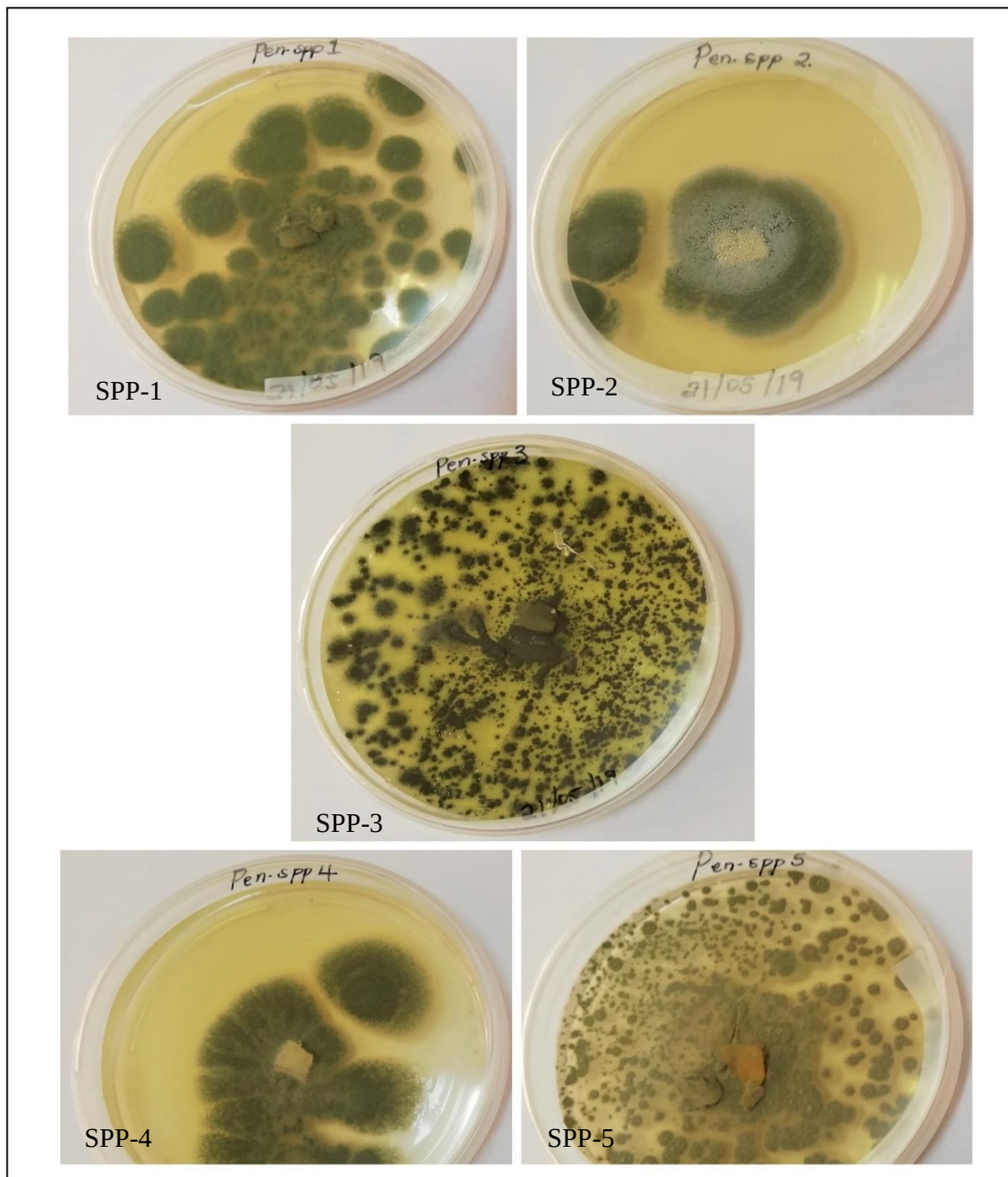


Figure 2. 2: (SPP-1 – SPP-5): Varying shapes and colours of different *Penicillium* cultures.

Microscopic morphological characteristics of spores and fruiting bodies of different isolated fungal species are shown in Fig. 2.3. Special structures such as conidiophores, hyphae and conidia were noted from the various species.

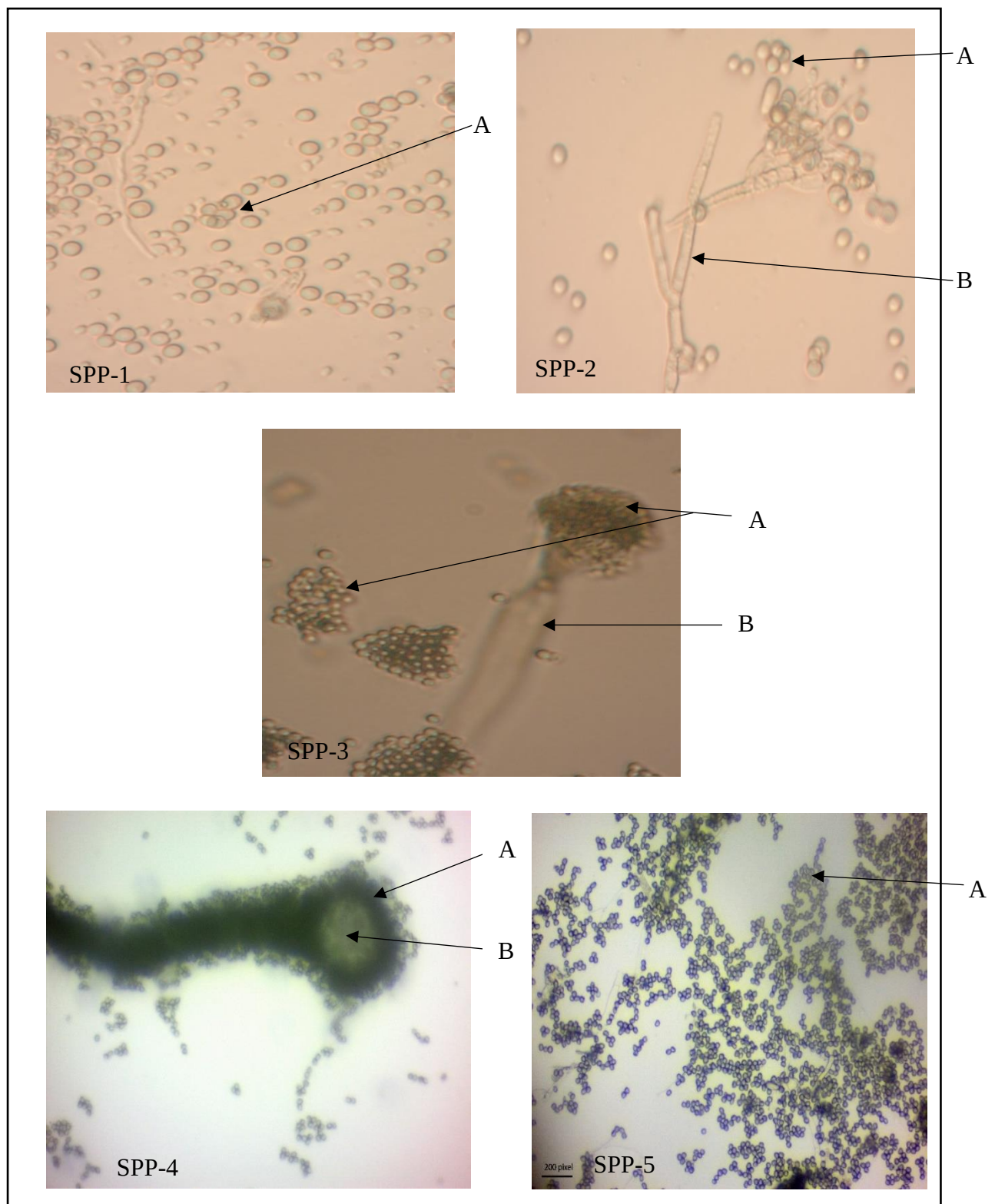


Figure 2. 3 (SPP1 – SPP5): Microscopic images of isolated fungal species. SPP1: A- Conidia/spores. SPP2: A- Conidia/spores, B- Hyphae. SPP3: A- Conidia, B- Stipe. SPP4: A- Conidia/spores, B- Fruiting body. SPP5: A- Conidia.

Fungal features of isolate SPP-1 showed conidia that had a light green colour at the centre and had a round shape. Isolate SPP-2 showed septate hyphae that were surrounded by round conidia, and isolate SPP-3 produced brush-like conidiophore, round clustered conidia with a light green colour at the centre. Isolate SPP-4 produced green coloured conidia surrounding a conidiophore and the conidia were spheroid in shape. Isolate SPP-5 produced oval-shaped conidia with a green tinge. The conidia appeared in abundance and were clustered (Fig 2.3). Different macroscopic and microscopic features are presented in Table 2.2.

Table 2. 2: Macroscopic and microscopic characteristics of different isolated *Penicillium* species.

Sample number	Probable ID	Growth rate	Macroscopic characteristics		Microscopic characteristics	
					Nature of hyphae	Presence of special structure
			Colony colour			
			Top	Bottom		
SPP-1	Penicillium	Moderate	Dark green	White	Smooth	Conidia
SPP-2	Penicillium	Slow	Fern green	Cream with yellow spots	Septate and smooth	Hyphae Conidia
SPP-3	Penicillium	Rapid	Forest green	Cream white	Apical and septate	Conidia Conidiophore
SPP-4	Penicillium	Moderate	Olive green	Cream white	Smooth and septate	Circular conidia
SPP-5	Penicillium	Rapid	Moss green	Cream with orange spots	Septate	Spheroidal conidia

Mycelial growth assessment was carried out daily for seven (7) days by use of vanier calipers. The fungal isolate SPP-1 colony formed dark green separate concentric rings and with white colour at the back and base. Based on these characteristics the isolate was identified to be *Penicillium* spp-1. Isolate SPP-2 grew slowly with numerous concentric rings that were dark

green with white mycelial edges and a cream back view with yellow spots and was identified as *Penicillium* spp-2.

Isolate SPP-3 on the other hand was a rapid grower with forest green powdery mycelia that covered the whole media and had cream back view and it was identified as *Penicillium* spp-3. Isolate SPP-4 was olive green with a moderate growth rate and cottony mycelial form and a cream white back view, based on these characteristics it was identified as *Penicillium* spp-4. Isolate SPP-5 had a pale green front view and a cream back view that had orange spots and formed powdery mycelia that were shaped like small granular rings that filled the entire PDA media, it was therefore identified as *Penicillium* spp-5.

2.3.2 Molecular identification of isolated *Penicillium* species

On completion of morphological identification, these samples were further subjected to molecular analysis to determine the accuracy of the morphological identification and to also identify the respective isolates to species level. The respective fungal isolates were processed and subjected to PCR analysis to generate DNA fragments. Fig.2.4 shows a photographic image of an agarose gel indicating DNA fragments generated by the ITS1/ITS4 PCR.

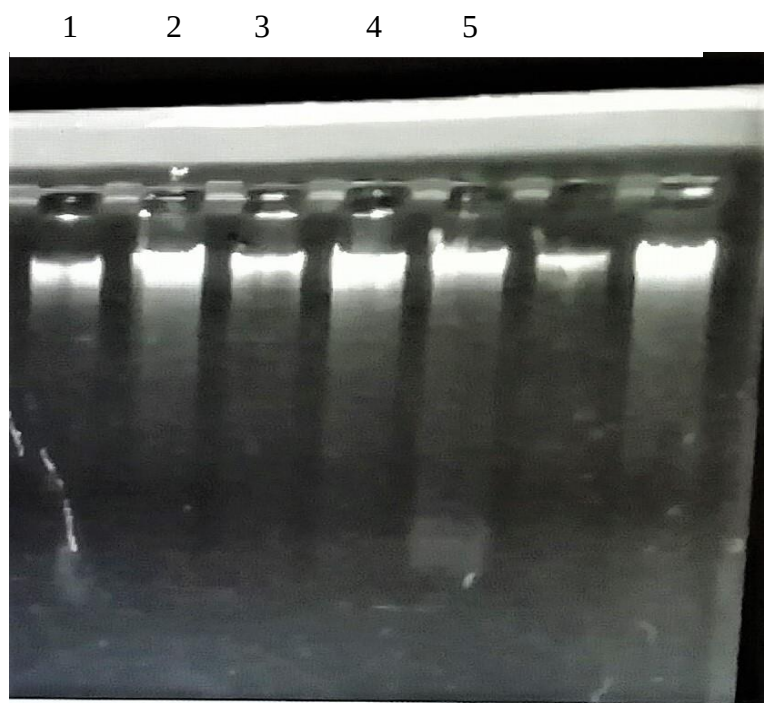


Figure 2. 4: ITS1/ITS4 generated DNA bands of the five isolated fungal species. Molecular weight markers (base pairs ladder) are indicated on the left side of the figure.

Key: 1 to 5 indicates the PCR reactions of the isolated fungal species.

2.3.3 BLAST outputs

The generated DNA sequences of the respective fungal isolates were queried by assessing their similarity against the biological sequence within the NCBI database, and the following outputs were obtained.

Table 2. 3: BLAST generated outputs.

	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5
Name of Isolate SPP-1 sample	Isolate SPP-2	Isolate SPP-3	Isolate SPP-4	Isolate SPP-5	
Request ID	STGXS6RS0 15	STGZW2FT0 15	SW337SHV0 14	T38PRS7401 5	T38R4RF801 5
Predicted Organism	<i>Penicillium commune</i>	<i>Penicillium commune</i>	<i>Aspergillus fumigatus</i>	<i>Penicillium concavorugulosum</i>	<i>Penicillium concavorugulosum</i>
GenBank Accession number	MK660351.1	MK660335.1	MN178806.1	MK841454.1	MK841454.1

The BLAST nucleotide sequencing results and the use of the GENBANK (www.ncbi.nlm.nih.gov) gave various *Penicillium* species and one *Aspergillus* species and these are shown in Table 2.3. Isolate SPP-1 and SPP-2 were identified as *Penicillium commune*, Isolates SPP-4 and SPP-5 were both identified as *Penicillium concavorugulosum*, while isolate SPP-3 was identified as *Aspergillus fumigatus*, which was initially morphologically identified as a *Penicillium* species.

2.4 DISCUSSION

The results indicate that although the identification of *Penicillium* fungal isolates by analyzing morphological characteristics had limited accuracy, it is relatively useful. Differences in the mycelial colour were noted on all *Penicillium* species (Figure 2.2). These could probably be

explained by the inherent genetic differences and/or environmental influences such as temperature and pH of the soil where they were isolated from. Influence of environmental factors such as temperature and pH on mycelial pigmentation of *Penicillium* species were previously noted and reported by various researchers (Mendez *et al.*, 2011; Afshari *et al.*, 2015). For example, Mendez *et al.*, (2011) reported that *T. purpurogenum* (formerly known as *P. purpurogenum*) produces colourants in both solid and liquid growth media. They further highlighted that the colour differences occurred because of the effect of varying growth temperatures.

All five species were morphologically identified to the genus level as *Penicillium* fungi, but one isolate was later identified by using molecular analysis technique to belong to genus *Aspergillus*. It should be noted that morphological differences of fungal species can be influenced by environmental factors as reported by Mendez *et al.* (2011) along with Afshari *et al.* (2015). Macroscopic characteristics i.e. colony colour/pigmentation and mycelial form of these species were noted to be different (Figure 2.3). This could have been interpreted to mean that the species were of different strains or that variations in environmental conditions such as temperature, moisture, pH and light from the different soils could have caused the difference in their morphological features. A study by Shrestha *et al.*, (2006) reported an effect of varying light conditions, temperature, pH and moisture have on pigmentation, texture, and mycelial density of fungal cultures. It can therefore be deduced that the differences in morphological features (colour) of the fungal isolates SPP-1, SPP-2, SPP-3, SPP-4 and SPP5 could have resulted from both genetic and environmental factor influences. Confirmation through molecular analysis was therefore needed for the morphologically identified *Penicillium* species.

Penicillium species are relatively difficult to differentiate to species level by use of morphological characteristics. This is because of the close similarities in microscopic features among species of this genus (Cardoso *et al.*, 2007). This, therefore, necessitates the need to use molecular identification techniques for identification at the species level. In this study, the use of molecular identification addressed the morphological misidentification of *Aspergillus* species as a *Penicillium* species. Molecular identification of these fungal species relied on analysis of DNA band sizes and DNA nucleotide sequences of the test samples. DNA fragments generated on a 1% agarose gel were of similar size at 1.0kb for all the isolates. According to various researchers, agarose electrophoresis is the most practical and effective method used for separation of DNA molecules based on their sizes (Voytas, 2001; Raja *et al.*,

2017; Green and Sambrook, 2019). Isolate SPP-1 generated the faintest and low DNA band while isolate SPP-4 and SPP-5 generated high and thicker matching DNA bands at the same size.

The SPP-3 fungal isolate, although initially morphologically identified as *Penicillium* was however identified through molecular analysis as *A. fumigatus*. This highlighted the limitation of the morphological identification procedure. Furthermore, the limitation of this procedure to identify these isolates to the species level highlights a grey area that limits a detailed understanding of the individual *Penicillium* species' fungal biology and ecological interactions. The determination of the inaccuracy of morphological identification of fungal isolates is an important finding. This is particularly critical when there is a need to separate *Penicillium* species that have the potential to be developed as ecologically stable biocontrol agents from those that harm both human beings and the environment.

As earlier indicated in the above paragraph, isolate SPP-3 which had been morphologically identified as *Penicillium* species was later molecularly identified as *A. fumigatus*. Similar morphologically microscopic misidentification of *Aspergillus* and *Penicillium* species were also reported by Tsang *et al.*, (2018). He reported close similarities of *Penicillium* and *Aspergillus* respective spore shapes, textures, colours, and sizes (Fig 2.3).

Although morphologically identified to be different, *Penicillium* SPP-1 and SPP-2 were later established through molecular analysis to contain the same DNA nucleotide sequences and were both identified as *P. commune*. On the other hand, *Penicillium* SPP-4 and SPP-5 were both identified as *P. concavorugulosum*. This, therefore, means SPP-1 and SPP-2 are genetically the same *Penicillium* species. Similarly, SPP-4 and SPP-5 were also identified to be genotypically the same species. The morphological differences probably occurred because of environmental effects. This deduction is based on the reports that the growth of certain fungal microorganisms in different growth media conditions have been found to affect filamentous fungi mycelial development and growth rate (Agudelo-Escobar *et al.*, 2017). It is, therefore, possible that the source of the respective *Penicillium* species could have influenced the morphological colony characteristics when grown in PDA. There is also a possibility of the existence of sub-biotype or strains within these *Penicillium* fungal species that were identified as SPP-1 and SPP-2. These fungal biotypes could be genetically similar but possessing varying *P. commune* strain properties. This is particularly so as they were assigned with different GenBank Accession numbers (MK660351.1 and MK660335.1) respectively. For example,

genetic diversity of *F. oxysporum* strains that are pathogenic and non-pathogenic have been reported on various studies. The *F. oxysporum* pathogenic strain and some non-pathogenic i.e. endophytic strain have been established to be genetically similar but differ in their eco-physiological impact on the host (Nirmaladevi *et al.*, 2016). The possibility of the existence of different strains of *Penicillium* species is strengthened by Alapont *et al.* (2014) who established the existence of 24 strains of *P. commune*. The occurrence of various *P. commune* strains in the soil rhizosphere in semi-arid eco-region of sub-Saharan Africa should therefore be further investigated.

The four *Penicillium* species and one *A. fumigatus* species that were identified in the study should be investigated for their suppressive properties/effect against *F. oxysporum*. *Penicillium* and *Aspergillus* species are known for their ability to produce secondary metabolites that contain the potential to influence growth and survival of other fungal microorganisms (Pitt *et al.*, 2000; Pitt, 2002; Svahn, 2015). Different soil microorganisms have been noted to vary in the suppressive effect on various soil-borne pathogens. These different suppressive properties include the following: Antagonism, myco-parasitism and ecological competitiveness (Frisvad *et al.*, 2007). These characteristics have been noted to vary between fungal species including *Penicillium* species within the same genera (Frisvad *et al.*, 2006). In addition to the study on the suppressive effect, they should also be evaluated for phytopathological biosafety i.e. negative effect on commonly grown vegetables. This investigation should be considered on a wide range of soil and climatic factors including the semi-arid regions. Costa and Nahas (2012) reported on the importance of soil and environmental factors on the adaptations, growth and survival mechanisms of fungal microorganisms.

Based on the differences in morphological features and similarities in molecular identification in this study, the test fungal isolates have therefore been named as follows:

Table 2. 4: Names of the *Penicillium* fungal isolates used in this dissertation

Isolate	Predicted organism	Name in dissertation
Isolate 1 (SPP-1)	<i>P. commune</i>	<i>P. commune-1</i>
Isolate 2 (SPP-2)	<i>P. commune</i>	<i>P. commune-2</i>
Isolate 4 (SPP-4)	<i>P. concavorugulosum</i>	<i>P. concavorugulosum-1</i>
Isolate 5 (SPP-5)	<i>P. concavorugulosum</i>	<i>P. concavorugulosum-2</i>

CHAPTER THREE

***In-vitro* analysis of the efficacy of four *Penicillium* spp. and *Aspergillus fumigatus* on *Fusarium oxysporum* f.sp. *radicis lycopersici* (FORL).**

3.1. INTRODUCTION

Penicillium and *Aspergillus* species are part of many filamentous fungi that produce a range of secondary metabolites and mycotoxins that result in various influences on plants, soil and other various microbes (Frisvad *et al.*, 2006; Frisvad *et al.*, 2007). These secondary metabolites include Fumagillin/Pseurotin, Aflatoxins etc. and cause a variety of interactions/relationships such as antibiosis, competition and mycoparasitism between microorganisms (Whipps, 2001, Perotto *et al.*, 2013, Verma *et al.*, 2020). Antibiosis may be defined as an antagonistic response induced by microbial secondary metabolites which neutralizes metabolic activities of other microorganisms (Verma *et al.*, 2020). Mycotoxins have been defined as secondary metabolites that are produced by fungal microorganisms that cause diseases in humans, animals, and plants (Kamei & Watanabe, 2005; Alshannaq *et al.*, 2017). Aflatoxins, gliotoxins, ochratoxins etc. are many of various and recognizable toxins produced by *Penicillium* and *Aspergillus* species and they have been reported to also have antimicrobial effects as well as immune-suppressive properties on other microorganisms (Kamei & Watanabe, 2005).

These species are of economic importance in agriculture because of their multifunctional properties that may be beneficial as well as harmful to plants and other soil micro fauna (Bhatnagar *et al.*, 2002). For example, some pathogenic effect on plants may be exerted by various *Penicillium* species due to metabolite influence on the physiological growth as reported by Shuping and Eloff (2017). This may be classified as an economic effect as economic losses are experienced when yields decrease. Two of the several pathogenic *Penicillium* species that cause citrus green rot and onion rot/decay are *P. digitatum*, *P. italicum* and *P. glabrum*, resulting in economic losses (Agrios, 2005).

Studies on *Penicillium* and *Aspergillus* species have exhibited their abilities to positively influence plant growth and development by production of certain phytohormones and secondary metabolites (Kumar *et al.*, 2018). These fungal species inherently possess the potential to be developed as both plant growth enhancers and root protection against soil-borne pathogens.

3.1.1. Specific objective

- i. To carry out *in-vitro* analysis of the effect of four *Penicillium* spp isolates and *Aspergillus fumigatus* against *Fusarium oxysporum* f.sp. *radicis lycopersici* (FORL).

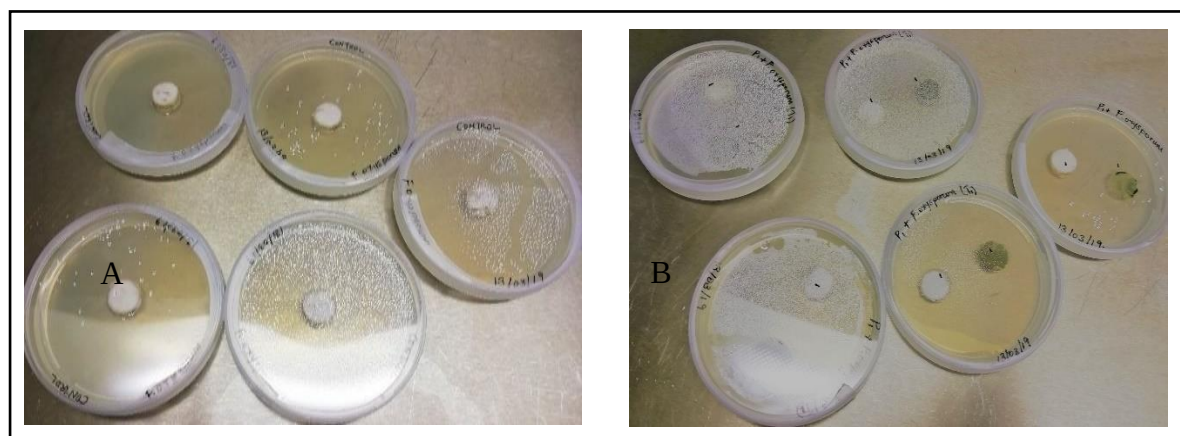
3.2. MATERIALS AND METHODS

The five fungal isolates viz. *P. commune*-1 (MK660351.1), *P. commune*-2 (MK660335.1), *A. fumigatus* (MN178806.1), *P. concavorugulosum*-1 (MK841454.1) and *P. concavorugulosum*-2 (MK841454.1) were used in an *in-vitro* study to evaluate their efficacy as biocontrol agents against *F. oxysporum* f.sp. *radicis lycopersici* (FORL).

3.2.1 Experimental description

a. Experimental Design and layout

An *in-vitro* experiment was carried out using each of the respective *Penicillium* species isolated from different soil types, i.e. *P. commune*-1, *P. commune*-2, *A. fumigatus*, *P. concavorugulosum*-1 and *P. concavorugulosum*-2. Each of the respective fungal isolates was cultured in PDA and their mycelia cut into 2-cm diameter discs and analysed for their suppressive effect against *F. oxysporum*. This was carried out by placing the respective test fungal isolates alongside two (2) centimetre diameter discs of *F. oxysporum*. The discs were each placed 3cm apart in each petri dish that made up an experimental unit. The experimental treatment combinations were computed as follows: FT0, FT1, FT2, FT3, FT4, and FT5; where F= *F. oxysporum*, T1= *P. commune*-1; T2= *P. commune*-2; T3= *A. fumigatus*; T4= *P. concavorugulosum*-1 and T5= *P. concavorugulosum*-2. Making a total of six treatment combinations. These treatment combinations were each replicated five times making a total of thirty (30) experimental units and these were laid out in a Completely Randomized Design (CRD) in an incubator set at 25°C - 27°C for ten (10) days.



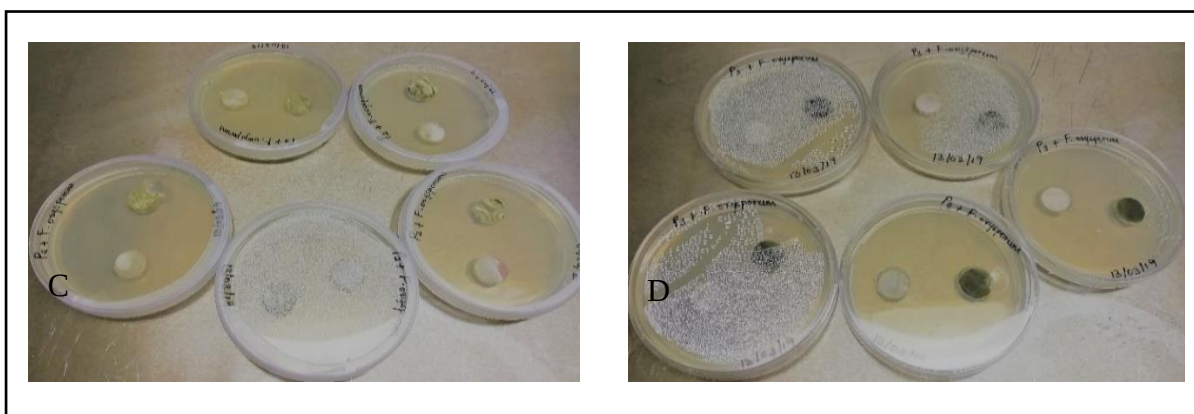


Figure 3. 1: Experimental setup of the dual anti-fungal analysis: Prior incubation. (A) *F. oxysporum* discs in PDA growth media (control). (B) *P. commune-1* and *F. oxysporum* discs in PDA growth media. (C) *P. commune-2* and *F. oxysporum* discs in PDA growth media. (D) *A. fumigatus* and *F. oxysporum* in PDA growth media.

b. Data collection and analysis

- i. Mycelial growth assessment was carried out on a two-day interval for ten days whereby the diameter of *F. oxysporum* and respective *Penicillium* species and *A. fumigatus* were measured using a Vernier calliper.
- ii. The correlation coefficient between the growth of pathogen and respective test antagonists was determined using Pearson's correlation coefficient.
- iii. Inhibition percentages were calculated using the controls- pathogen growing alone and pathogen growing with isolate respectively using the formula:

$$IP = \frac{\bar{X} T1(A0) - \bar{X} T1(A1)}{\bar{X} T1(A0)} \times 100 \quad (3.1)$$

Where:

- IP = Inhibition percentage
 - $\bar{X}$ = average/mean
 - T1(A0) = Pathogen growing alone
 - T1(A1) = Pathogen growing with respective isolate
- iv. Analysis of variance (ANOVA) was carried out to test the significance of the relationship/effect at 5% probability level using GENSTAT software seventeenth edition (Copyright 2014, VSN International Ltd.).

3.3 RESULTS

3.3.1 Dual antifungal relationships of selected test isolates against *F. oxysporum*

Mycelial growth diameter, mycelial form and various growth relationships such as antibiosis, mycoparasitism and competition of the respective species were assessed at a two-day interval for a week. Fig.3.2(a) and Fig.3.2 (b) show mycelial discs growth of isolated *Penicillium* and *Aspergillus spp* against *F. oxysporum* in PDA growth media after 10 days of incubation.

a. Dual antifungal relationship assessments.

Figure 3.2(a) and (b) show various interactions between the five isolates with *F. oxysporum* f. *sp. radicis lycopersici* (FORL).

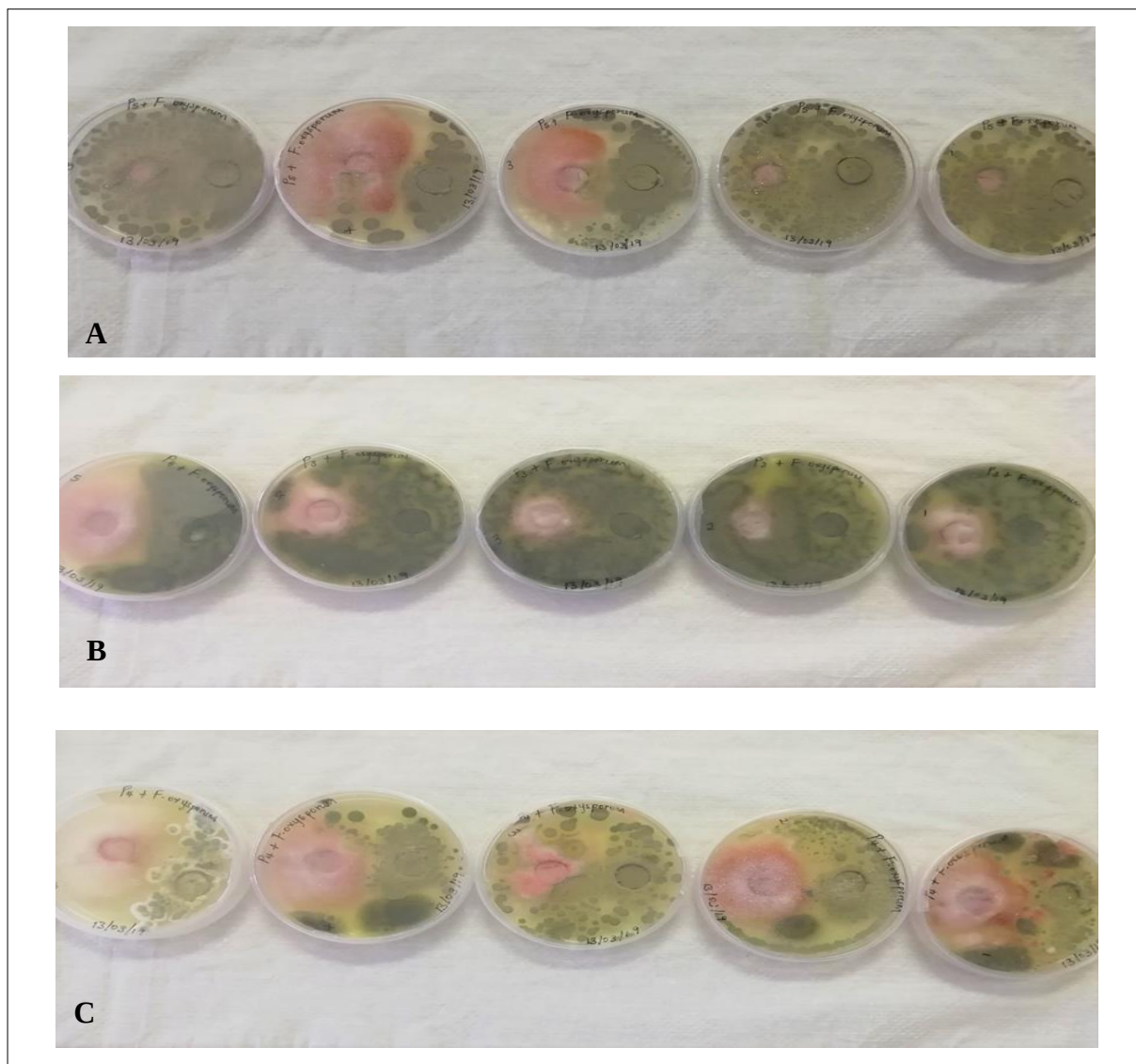


Figure 3.2(a): Fungal growth and interactions between selected fungal species after 7 days: (A): *P. concavorugulosum*-2 and *F. oxysporum*, (B): *A. fumigatus* and *F. oxysporum*, (C): *P. concavorugulosum*-1 and *F. oxysporum*.

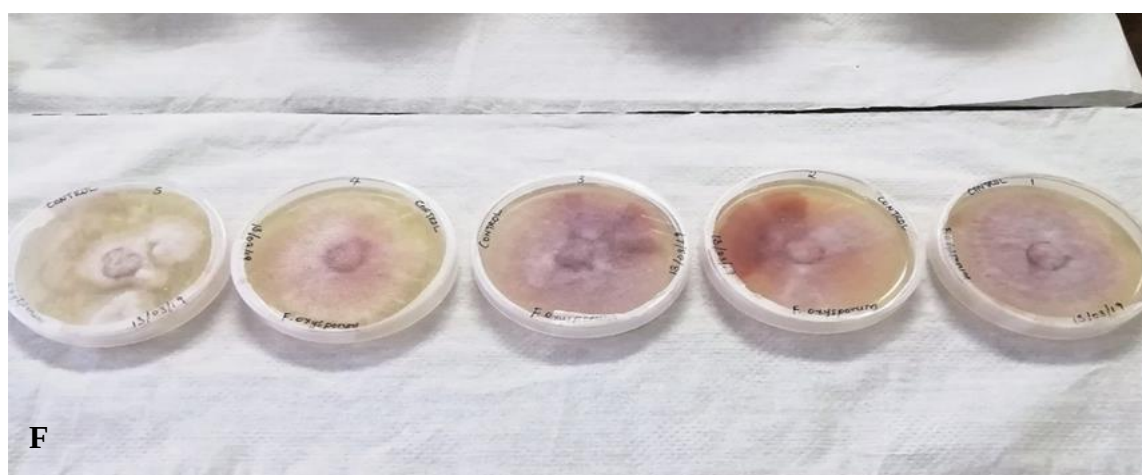
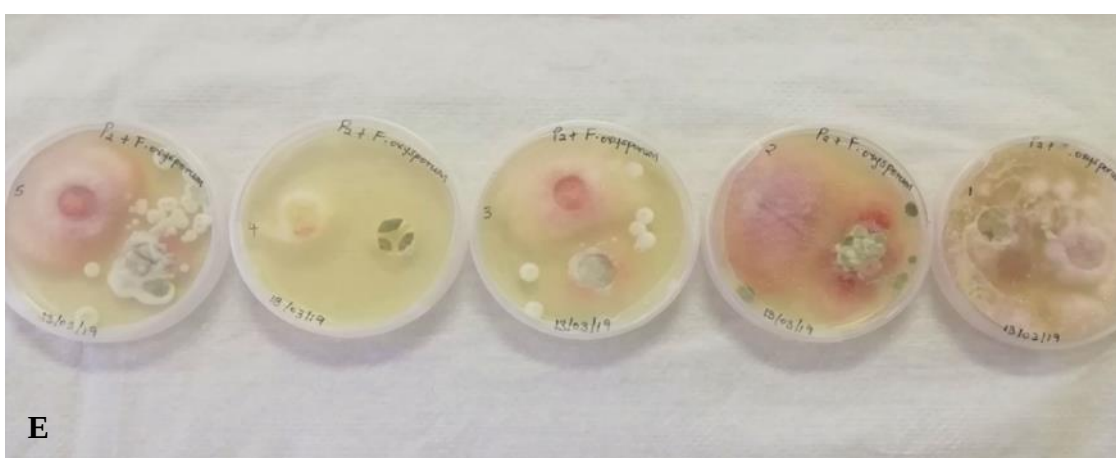
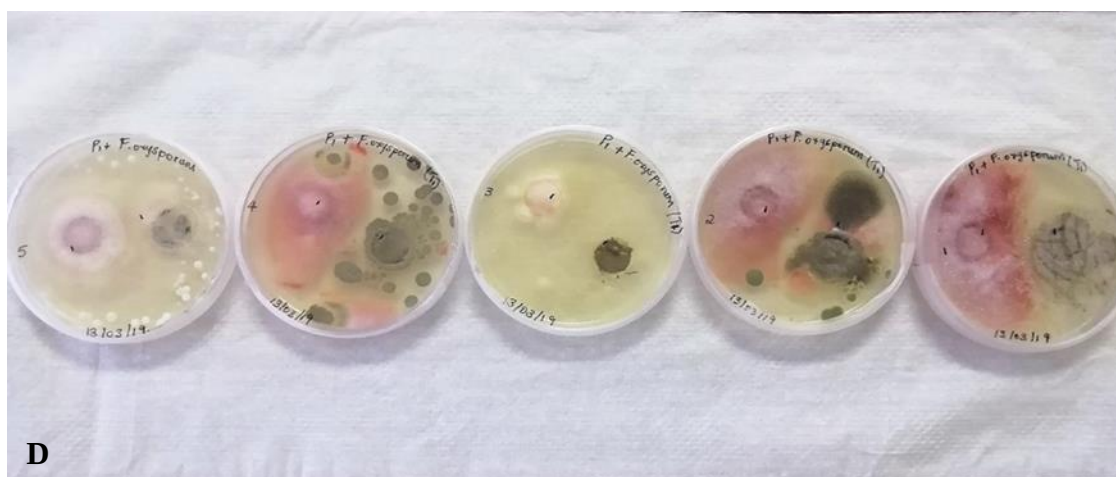


Figure 3.2(b): Fungal growth and interactions between selected fungal species after 7 days: (D): *P. commune-1* and *F. oxysporum*, (E): *P. commune-2* and *F. oxysporum*, (F): *F. oxysporum* growing alone.

Rapid mycelial growth and multiple suppressive characteristics i.e. competitiveness, antibiosis and mycoparasitism were noted for *P. concavorugulosum-2* against *F. oxysporum*. *P.*

concavorugulosum-2 rapidly grew around and over *F. oxysporum* while slowly reducing its growth and size (Fig 3.2b (A)). Multiple suppressive effects were also exhibited by *A. fumigatus* against *F. oxysporum* where strong competitive, antibiosis and mycoparasitic effect were noted. *A. fumigatus* rapidly grew around *F. oxysporum* while slowly covering the isolate (Fig 3.2b (B)). Slow growth was noted on *P. concavorugulosum*-1 as it grew away from *F. oxysporum*, exhibiting signs of antibiosis between fungal isolate and *F. oxysporum f.sp. radicis lycopersici* (FORL) [Fig 3.2b (C)] Notably, antibiosis effect of *F. oxysporum* against *P. commune*-1 was evident. The decline of *P. commune*-1 mycelial growth when compared to *F. oxysporum* was indicative of its restricted growth (Fig 3.2a (D)). Less growth rate was further noted on *P. commune*-2 when compared to *F. oxysporum*. There was an indication of antibiosis and competitiveness exhibited by *F. oxysporum* (Fig 3.2a (E)).

b. Mycelial growth rate assessments

The mycelial growth rate of the respective isolates and *F. oxysporum f.sp. radicis lycopersici* (FORL), and their ecological relationships are shown in Figures (3.3 – 3.8).

An inversely proportional relationship between *P. concavorugulosum*-2 and *F. oxysporum* was noted. With a rapid growth rate for *P. concavorugulosum*-2, there was a corresponding decline of *F. oxysporum* (Fig 3.3).

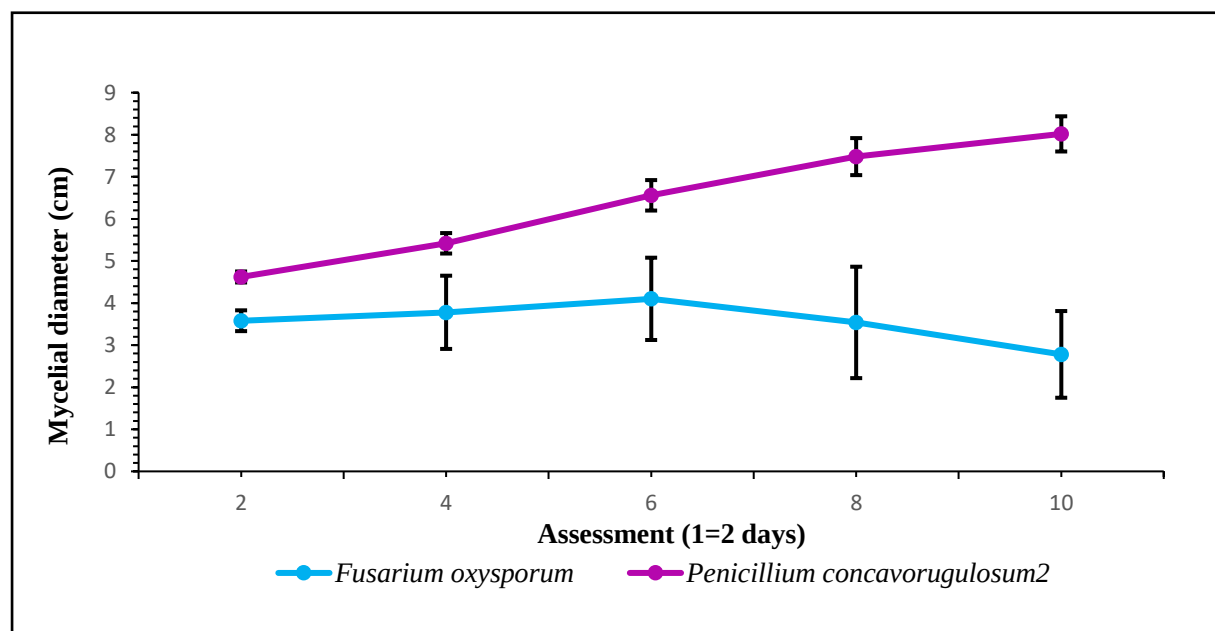


Figure 3.3: Mycelial growth comparison of *P. concavorugulosum* against *F. oxysporum f.sp. radicis lycopersici* (FORL): Growth rate and interaction collected at a two-day interval for 10 days.

A. fumigatus exhibited a continual increase on all the assessments whereas *F. oxysporum* followed a parabolic path with a peak of 3,92cm at assessment 3. A gradual decline was however noted at assessment 4 and 5 for *F. oxysporum* (Fig 3.4).

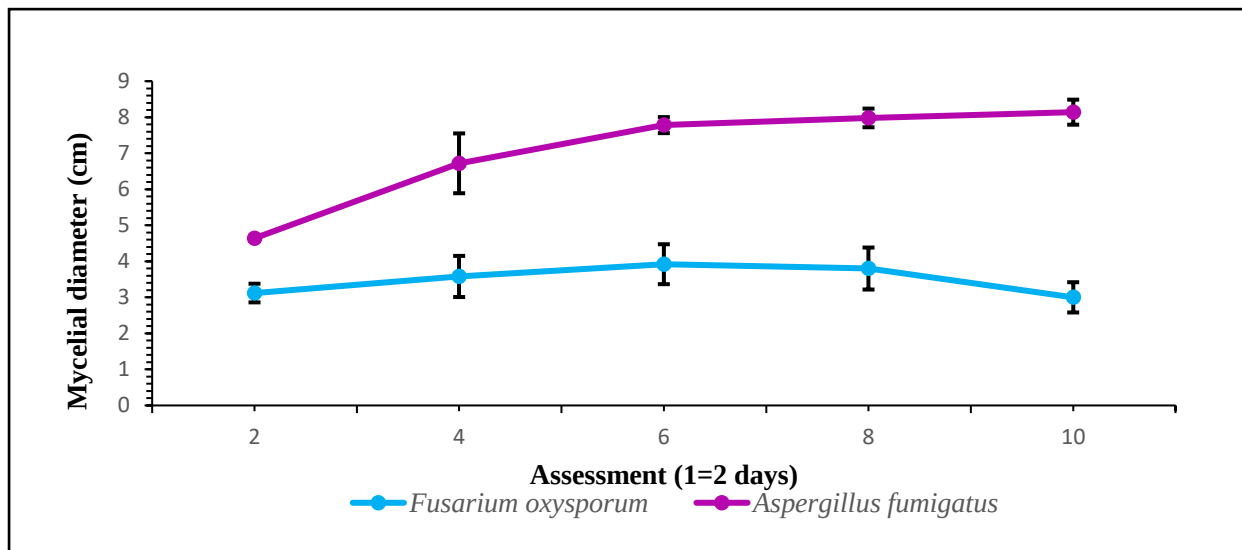


Figure 3.4: Mycelial growth comparison of *A. fumigatus* against *F. oxysporum f.sp. radicle lycopersici* (FORL): Growth rate and interaction collected at two-day interval for 10 days.

Although *A. fumigatus* exhibited multiple suppressive characteristics against *F. oxysporum*, *P. concavorugulosum-1* was not as strongly expressive. This is evident by the higher *F. oxysporum* growth rate (Fig 3.5).

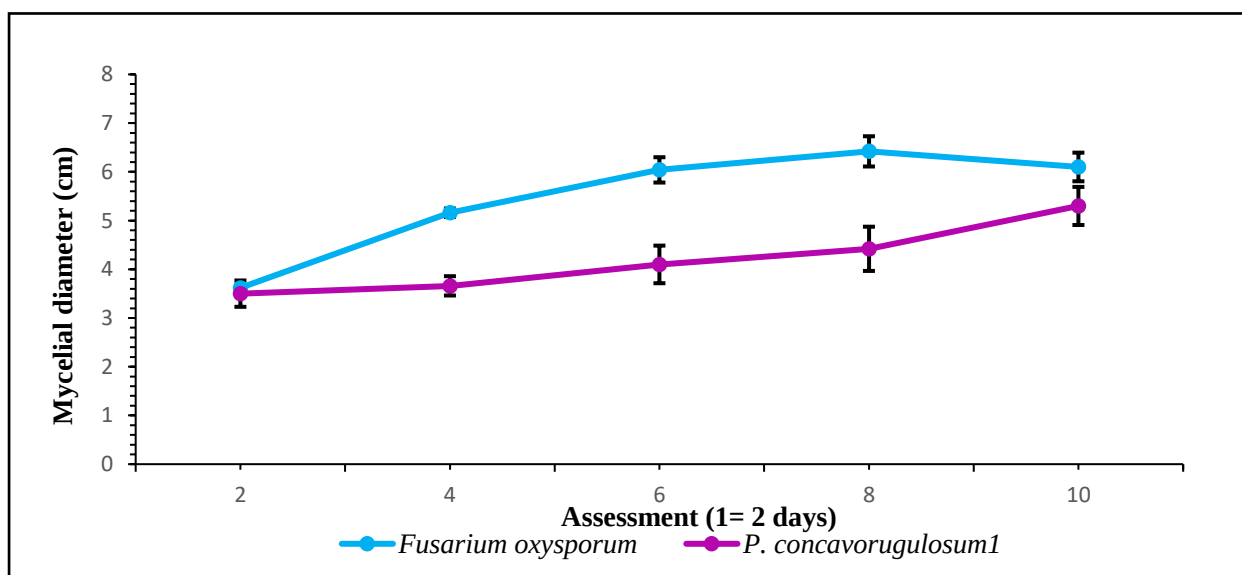


Figure 3.5: Mycelial growth comparison of *P. concavorugulosum-1*: against *F. oxysporum f.sp. radicle lycopersici* (FORL). Growth rate and interaction collected at a two-day interval for 10 days.

P. commune-1 exhibited a reduced growth rate against *F. oxysporum*, this indicates the rapid growth of the pathogen. Competition in favour of *F. oxysporum* was noted between the species. This resulted in subdued mycelial growth of *P. commune-1* (Fig 3.6).

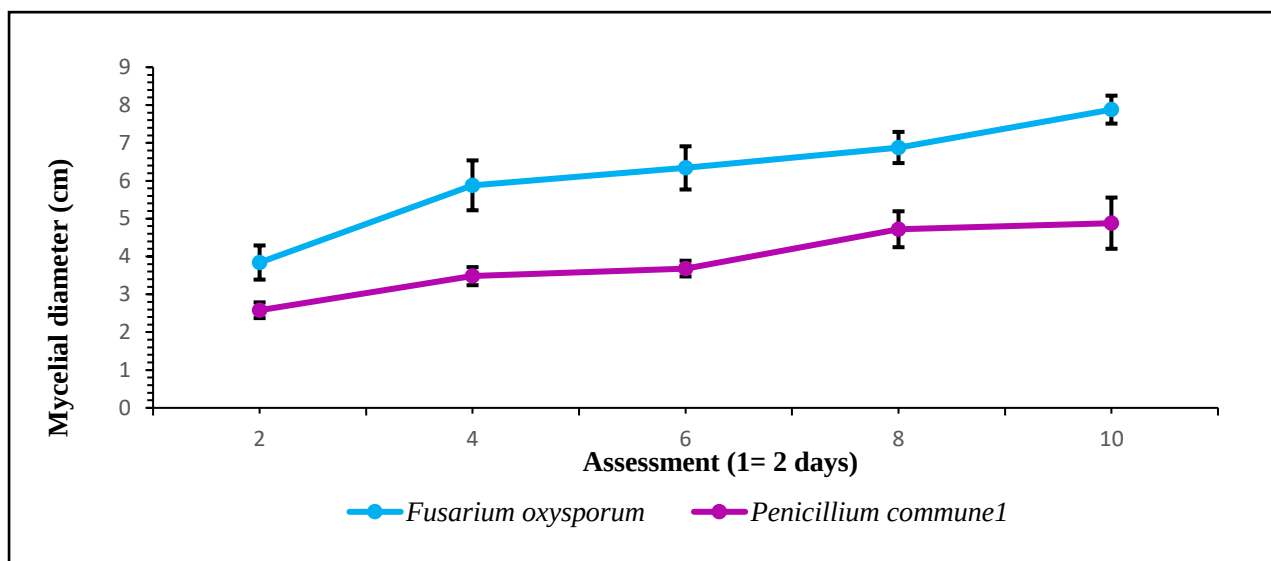


Figure 3.6: Mycelial growth comparison of *P. commune-1* against *F. oxysporum f.sp. radicis lycopersici* (FORL). Growth rate and interaction of *P. commune1* against *F. oxysporum* collected at two-day interval for 10 days.

A gradual decline in mycelial growth was noted on *P. commune-2* whereas a steep elevation was noted on *F. oxysporum*, expressing less suppressiveness of *P. commune-2* on *F. oxysporum*. (Fig 3.7).

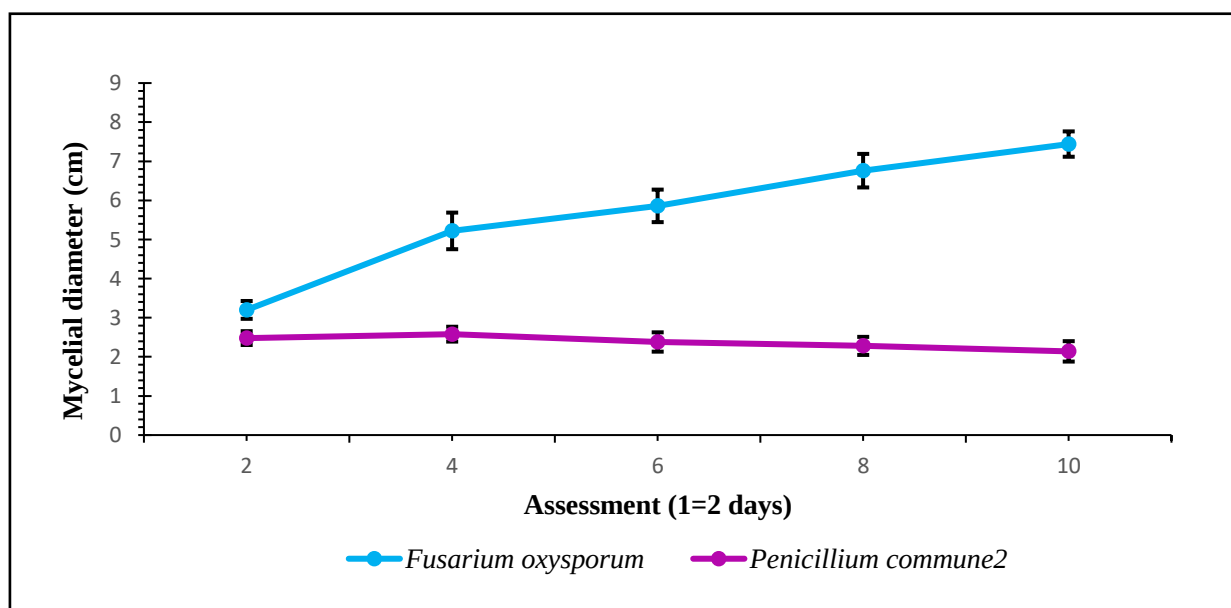


Figure 3.7: Mycelial growth comparison of *P. commune-2* against *F. oxysporum f.sp. radicis lycopersici* (FORL): Growth rate and interaction collected at a two-day interval for 10 days.

Varying mycelial growth rates of fungal isolates against *F. oxysporum f.sp. radicis lycopersici* (FORL) shown in (Figures 3.3 – 3.7) depict and highlight the range of suppressiveness of the respective isolates against *F. oxysporum*.

3.3.2 Correlation coefficient

Correlation analysis determines the statistical relationship or association and strength between two variables. Statistical relationships of the test isolate against *F. oxysporum f.sp. radicis lycopersici* (FORL) were therefore determined and are exhibited in Figures 3.8 to Fig 3.12.

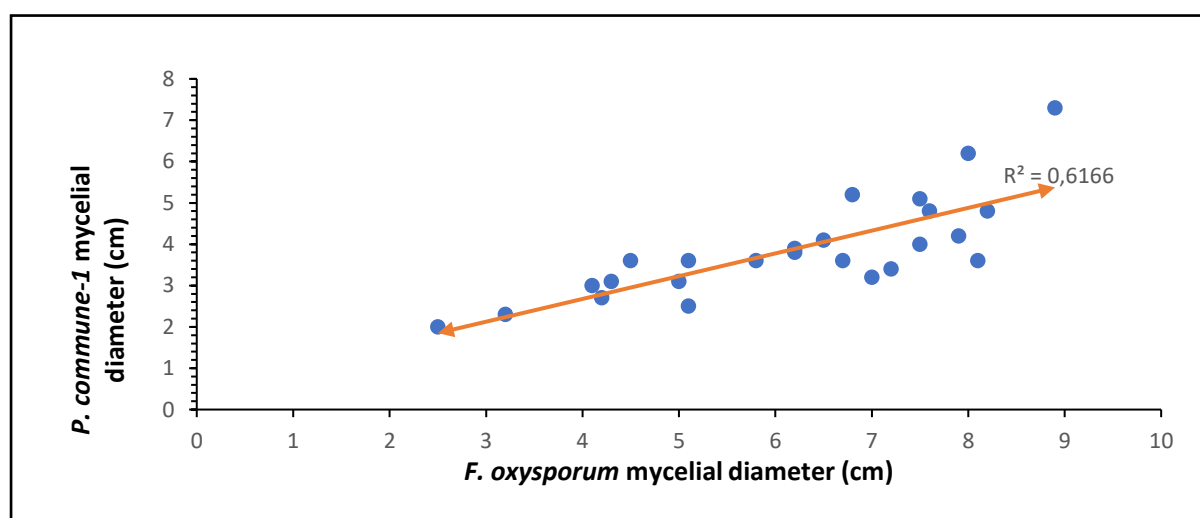


Figure 3.8: Moderate positive relationship between *P. commune-1* and *F. oxysporum f.sp. radicis lycopersici* (FORL). $r = 0.785$

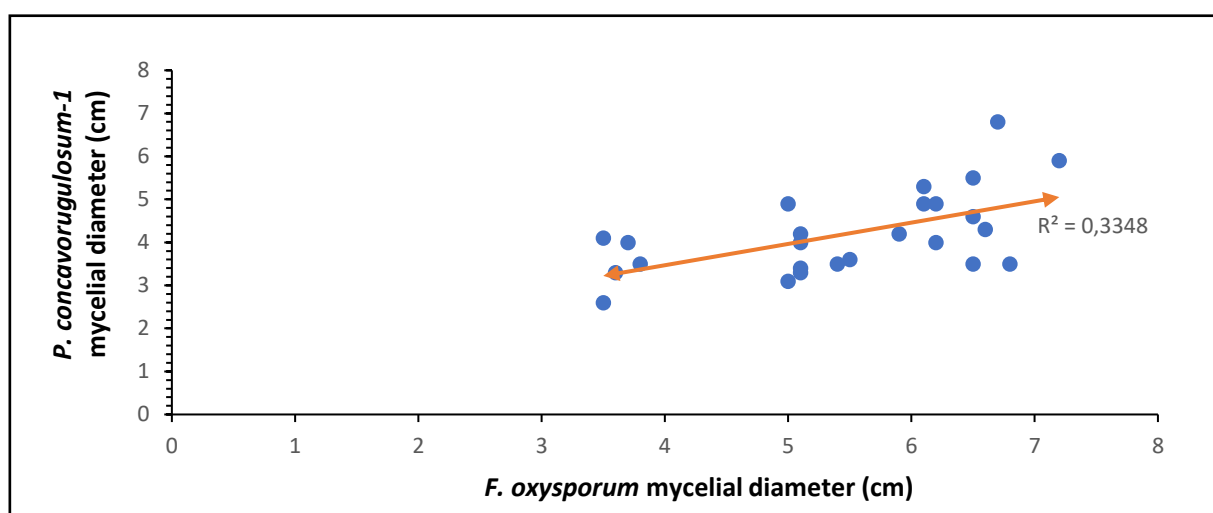


Figure 3.9: Weak positive association between *P. concavorugulosum-1* and *F. oxysporum f.sp. radicis lycopersici* (FORL). $r = 0.579$

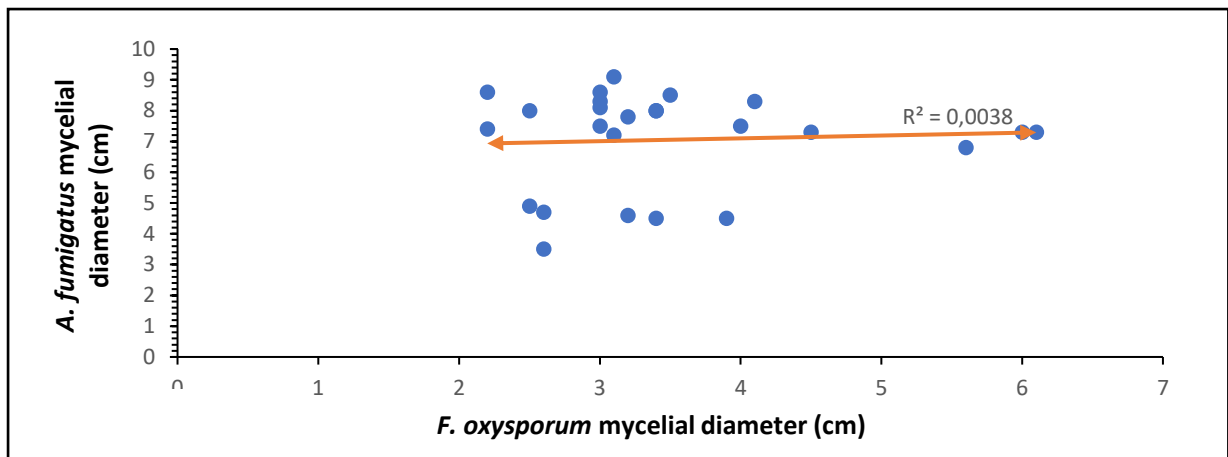


Figure 3.10: Very weak positive relationship/association between *A. fumigatus* and *F. oxysporum f.sp. radicis lycopersici* (FORL). $r = 0.062$

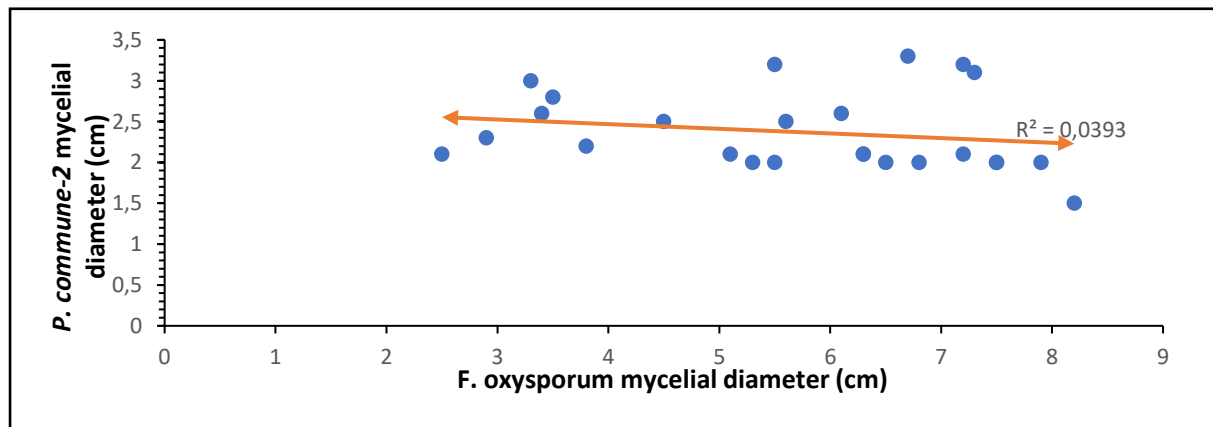


Figure 3.11: Very weak negative relationship/association between *P. commune-2* and *F. oxysporum f.sp. radicis lycopersici* (FORL). $r = -0.198$

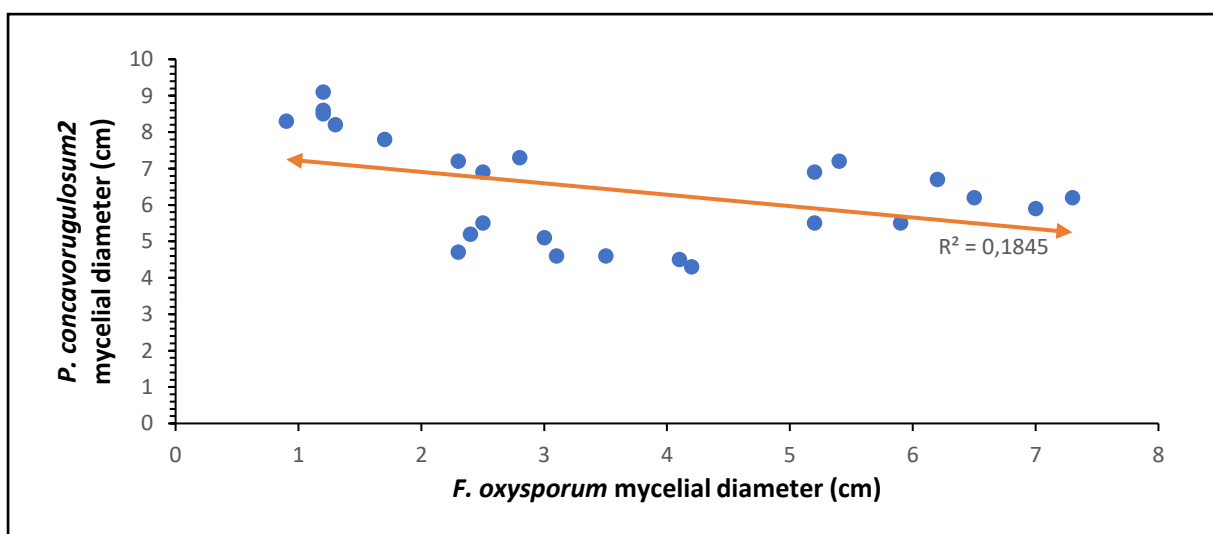


Figure 3.12: Weak negative association between *P. concavorugulosum2* and *F. oxysporum f.sp. radicis lycopersici* (FORL). $r = -0.429$

Although there was an evident correlation between the respective test isolates and *F. oxysporum f.sp. radicis lycopersici* (FORL) albeit relatively weak, there was notable inhibition effects due the test fungal isolates.

3.3.3 Inhibition percentage

Percentage inhibitions of the test isolates against *F. oxysporum* are graphically presented in Fig 3.13 where *P. concavorugulosum*2 and *A. fumigatus* exhibited the highest inhibition percentage against *F. oxysporum*. The lowest inhibition was noted on *P. commune*1 against *F. oxysporum f.sp. radicis lycopersici* (FORL). Inhibition percentage may be explained as the measure of inhibitory influence by subtracting inhibited growth from normal growth of the same object and dividing by the normal growth of the same object (Himratul-Aznita *et al.*, 2011, Harun *et al.*, 2013).

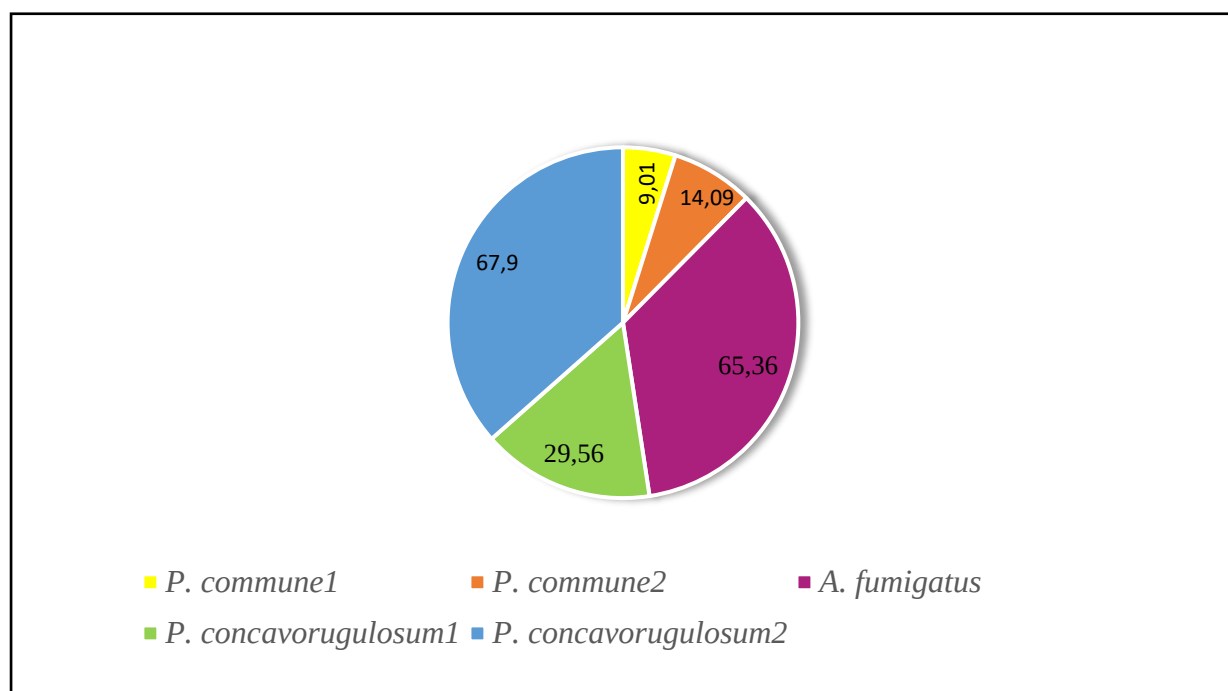


Figure 3.13: Inhibition percentages of the four *Penicillium* isolates and *A. fumigatus* against *F. oxysporum f.sp. radicis lycopersici* (FORL).

3.4 DISCUSSION

The *in-vitro* results exhibited various relationships such as competition, antibiosis, and mycoparasitism of the respective test isolates against *F. oxysporum f.sp. radicis lycopersici* (FORL) [Fig 3.2(a) and 3.2(b)]. This fungus-on-fungus interaction may probably be explained by the production of labile substances that impact the development and survival of the

microorganisms that they come into contact with (Zeilinger-Migsich & Mukherjee, 2014; Schmidt *et al.*, 2015). *A. fumigatus* and *P. concavorugulosum*-2 were noted to overgrow *F. oxysporum*, which could possibly be indicative of mycoparasitism/mycoparasitic behavior (Fig 3.2(a), insert A and B). This is explained as a relationship that occurs when one fungal species parasitizes on another (Moore *et al.*, 2011; Qualhato *et al.*, 2013). This was probably because of the production of gliotoxin by *A. fumigatus*. Gliotoxin is a pleiotropic mycotoxin that is produced by *A. fumigatus* and has been reported to cause cytotoxicity in mammals and various other microorganisms (Nieminen *et al.*, 2002; Bulgari *et al.*, 2020). This could have caused the rapid degradation and reduced growth of *F. oxysporum* observed in [Fig 3.2(a) insert B]. The overgrowth of *P. concavorugulosum*-2 against *F. oxysporum* [Fig 3.2(a), insert A] could also be because *F. oxysporum* released extrolites that acted as a nutritional source for *P. concavorugulosum*-2. *A. fumigatus* and *P. concavorugulosum*-2 demonstrated rapid growth and a feeding mechanism on *F. oxysporum* [Fig 3.2(a), insert A and B] as their mycelia expressed a degradation effect. This was noted with mycelia of these respective isolates overlaying *F. oxysporum* mycelia. The faster growth rate exhibited by *A. fumigatus* and *P. concavorugulosum*-2 when grown against *F. oxysporum* could be explained by the differences in inherent biological properties that could have influenced their parasitism, adaptation and competitiveness.

Additionally, the two test isolates could have possibly catabolized metabolites produced by *F. oxysporum* to decrease its growth and survival rate. Studies by Chatterjee *et al.* (2016) reported that species-specific metabolite catabolism by the producer or an organism in the same growth environment is possible. The good performance of *P. concavorugulosum*-2 against *F. oxysporum* is indicative of its potential use as a biocontrol agent against the pathogen. The *Penicillium* species could be used as a single agent or be used in combination with other *Penicillium* isolates (e.g. *P. digitatum*) that also showed a suppressive effect against *Fusarium* pathogens. It is expected that a synergistic effect of collective activity against *F. oxysporum* of the combined fungal species could be generated. Further studies should therefore be undertaken to determine whether such synergistic effect could be achieved. Furthermore, *in-vivo* analysis of the antifungal efficiency of *P. concavorugulosum*-2 and *A. fumigatus* in the plant rhizosphere should also be carried out. Besides, their effect on germination and the establishment of various other vegetables of economic importance should also be investigated.

Mycelial disc growth comparison of *A. fumigatus* against *F. oxysporum* (Fig 3.4) showed that *A. fumigatus* had a faster growth rate throughout all five (5) assessments. *P.*

*concolorugulosum*2's mycelial growth also exhibited higher and rapid growth than *F. oxysporum* (Fig 3.3). This is indicative that *A. fumigatus* and *P. concolorugulosum*-2 contain stronger competitive and anti-microbial characteristics against the growth of *F. oxysporum*. Similar ecological relationships have been established in *Trichoderma* studies against certain pathogenic fungi. The competitive advantage of a biocontrol agent against a pathogen has been established in the studies on *Trichoderma* species. Go *et al.* (2019) established that some *Trichoderma* species have expeditious growth mechanisms and can utilize available resources faster while decreasing the growth of another pathogenic fungal species. *P. concolorugulosum*-2 and *A. fumigatus* could therefore possess similar characteristics.

Although a positive outcome was noted on *P. concolorugulosum*-2 and *A. fumigatus*, a weak development rate of *P. commune*-1 was noted against *F. oxysporum* (Fig 3.6). This was probably because a competitive interaction was triggered on both fungal species which triggered their chemical defenses, therefore reducing the growth of *P. commune*-1 while the growth of *F. oxysporum* increased. Künzler, (2018) reported on various fungi defense mechanisms when sensing microbial competition. The dominant action being chemical defense. Competition occurs when one organism outgrows and survives more aggressively than the other by utilizing vital resources such as carbon, trace elements and space faster than the other (Hassani *et al.*, 2018). This could therefore mean that occupation of space and utilization of nutrients by *F. oxysporum* could occur faster than the microbial bio-control agent (*P. commune*-1) thus increasing the chances of disease occurrence under field conditions. This competitive aspect however depends on growth conditions that the microorganisms are subjected to, which can either enhance or depress their growth as well as disease occurrence (Alkorta *et al.*, 2017; Köhl *et al.*, 2019).

The antibiosis interaction (growth inhibition zone – colonies growing away from each other) of *P. commune*-2 against *F. oxysporum* that was established (Fig 3.7) was possibly because of the production of metabolites that can weaken and reduce competitiveness and growth of other microorganisms. For example, Perincherry *et al.* (2019) reported on the production of toxic secondary metabolites by *Fusarium* species during various interactions with plants or other microbes. These metabolites can therefore influence/weaken the growth of other microbial species. *Fusarium* species have been reported to produce mycotoxins such as zearalenone, deoxynivalenol, *fumonisin*s, trichothecenes, etc. that can invade cell membranes, alter microbial growth patterns and increase pathogen multiplication (Mendoza *et al.*, 2015; Perincherry *et al.*, 2019). Therefore, this might have given *F. oxysporum* growth and

multiplication advantage over *P. commune*-2. Furthermore, the growth inhibition zone observed in (Figure 3.2 insert E) may be explained by the production of inhibitory metabolites by *F. oxysporum* that led to the growth-free zone, allowing both species to grow but giving *F. oxysporum* excess growth (Mendoza *et al.*, 2015; Perrone and Susca, 2017).

There is a variation in the strength of the relationship between test isolates and *F. oxysporum* ranging from weakly negative to moderate positive associations. Correlation between *A. fumigatus* and *F. oxysporum* showed a very weak positive relationship where $r = 0.062$ (Fig 3.11) compared to moderate correlation value between *P. concavorugulosum*-2 and *F. oxysporum* that showed a moderate negative relationship of $r = -0.43$ (Figure 3.13). This means *P. concavorugulosum*2 expressed a range of fungal suppressive properties i.e. competitive, antibiotic and mycoparasitic properties. A similar expression was established between *A. fumigatus* and *F. oxysporum* albeit at a weaker level (Figure 3.11). The moderate positive association between *P. commune*-1 and *F. oxysporum* shows no indication of pathogen suppressiveness (Figure 3.9). There was no evident relationship between *P. commune*-1 and *F. oxysporum* on all the five assessments. There is, however, a relatively weak negative association of *P. commune*-2 and *F. oxysporum* indicating a decline of *P. commune*-2 and an increase of *F. oxysporum*

The negative correlation established between *P. concavorugulosum*-2 and *F. oxysporum* is indicative of the inhibition ability of the test fungus against the pathogen. The inhibition characteristics of a test isolate exerted on *F. oxysporum* that was calculated and expressed in percentages showed *P. concavorugulosum*-2 had the highest inhibitory effect against *F. oxysporum* (67.9%) followed by *A. fumigatus* (65.36%) [Figure 3.14]. The other test isolates' inhibition was not very significant as they ranged from 9% to 29%.

A conclusion can be drawn that *P. concavorugulosum*-2 and *A. fumigatus* contain potential antagonistic properties that enable suppression of growth of *F. oxysporum* (Figure 3.2b insert A and B). These fungal isolates should be considered as good candidates for development as commercial biocontrol inoculum as individuals or in combination. However, these species' suppressive properties should be evaluated under natural soil conditions. *In-vivo* studies will determine their influence in the presence of various soil microorganisms and under different soil factors. They should also be investigated for their phytopathological and ecological biosafety status. *A. fumigatus* was however reported to produce mycotoxins that are harmful to

human health when exposure to high amounts occurs. It is therefore recommended to exclude it from further studies.

CHAPTER FOUR

Screening of pathogenicity of four *Penicillium* isolates and one *Aspergillus fumigatus* species on selected vegetables.

4.1 INTRODUCTION

Seed germination is one of the most important stages of plant establishment and growth. It is a complex physiological process that is easily influenced as well as affected by various factors such as moisture availability, oxygen, light, and the presence or absence of plant pathogens (Ali and Elozeiri, 2017, Bareke, 2018). Soil-borne pathogens are amongst the most devastating pathogens for both field and vegetable crops and result in significant losses when infection occurs during the germination process.

Soil-borne pathogens such as *F. oxysporum* are amongst the most destructive phytopathogenic fungi that are abundant in the soil. The species have a wide range of plant hosts due to its several specialized forms (*formae specialis (f.sp.)*) (Pietro *et al.*, 2003; Rana *et al.*, 2017) that enables it to attack a wide range of vegetables, cereal crops as well as ornamentals.

The utilization of fungal microorganisms with different ecological properties i.e. secondary metabolite production, mycoparasitism, competition or antibiosis as biological control agents has been initiated in order to mitigate the extensive use of chemical control methods (Larena *et al.*, 2003; Heydari and Pessarakli, 2010). However, their behaviour and effectiveness have been reported to vary under different ecological interactions. A study by Knudsen and Dandurand (2014), reported the detailed influence of fungal biocontrol on soil-borne pathogens. The study highlighted that the complexity of the ecosystem i.e. varying environmental conditions such as temperature, pH and a variety of microorganisms may negatively or positively influence the behaviour of these biocontrol agents thus impacting on its effectiveness. For example, Brunner *et al.* (2005) indicated the short-fall of various *Trichoderma* species on glucose oxidase products, thus limiting utilization of its disease control and plant growth-promoting properties. The study, therefore, looked at enhancing the disease control effect of *Trichoderma harzianum* to its directiveness on fungal pathogen inhibition and to incite resistance in plants.

Furthermore, the impact of the complexity of microbial interaction and rhizosphere interaction was explained by Whipps (2001), highlighting that fungal biocontrol agent-fungal pathogen association governed by competition, antibiosis, induced resistance and multiple microbe interactions have different modes of action. Therefore, these different modes of action influence the response of the host plant to the biocontrol agent.

In search of alternative bio-control agents that are not harmful to plant growth and development, the test fungi that were investigated in chapter three must be screened for their bio-phytopathological and ecological safety. This study was therefore undertaken to examine the pathogenic effect of all the test fungi i.e. *Penicillium* species: *P. commune*-1, *P. commune*-2, *P. concavorugulosum*-1, *P. concavorugulosum*-2 and *A. fumigatus* had on germination and seedling growth of three vegetable seeds: *Spinacia oleracea*, *Beta vulgaris* and *Solanum lycopersicum*.

4.1.1 Specific objectives:

- (i) To investigate pathogenic effect of four (4) *Penicillium* species and *A. fumigatus* on the germination of *S. oleracea*, *B. vulgaris* and *S. lycopersicum*.
- (ii) To investigate pathogenic effect of four (4) *Penicillium* isolates and *A. fumigatus* on the post-emergence seedling growth.

4.2 MATERIALS AND METHODS

4.2.1 Inoculum preparation and seed inoculation

The respective four (4) *Penicillium* species that were identified in chapter 2 of this dissertation viz. *P. commune*-1 (MK660351.1), *P. commune*-2 (MK660335.1), *P. concavorugulosum*-1 (MK841454.1), *P. concavorugulosum*-2 (MK841454.1) and *A. fumigatus* (MN178806.1), were prepared by scraping mycelia of the respective actively growing cultures into 50 ml individual beakers. A sub-sample of 10g fungal mycelia of the respective species were mixed with 5g of talc powder. The talc powder was added as seed binding material to respective mycelial samples. 20 ml of sterile water was poured into each respective mixture of mycelia and talc powder. The respective suspensions were stirred until homogenous slurries were achieved.

Seed surface was sterilized using 70% ethanol for 3 minutes (Oyebanji *et al.*, 2009, Jan *et al.*, 2013). A batch of 30 sterilized seeds each for the selected vegetable types i.e. *Spinacia oleracea* (STAR 1801), *Beta vulgaris* (Detroit dark red) and *Solanum lycopersicum* (Moneymaker) were individually added into 50ml beakers containing respective homogenous mixtures of the slurry treatments i.e. slurry with mycelial samples of the following fungal isolates; *P. commune*-1, *P. commune*-2, *A. fumigatus*, *P. concavorugulosum*-1 and *P. concavorugulosum*-2. The slurry coated seeds were placed on a sterilized laminar flow cabinet to dry. These were kept in vials for later use to carrying out germination tests.

4.2.2 Experimental description and design

Three sub-experiments were laid out whereby each of the three vegetable seeds i.e. *S. oleracea*, *B. vulgaris* and *S. lycopersicum* were subjected to the following slurry treatments: C, P1, P2, P3, P4 and P5, where C= control, P1= *P. commune*-1, P2= *P. commune*-2, P3= *A. fumigatus*, P4= *P. concavorugulosum*-1 and P5= *P. concavorugulosum*-2. Five (5) respective test fungi slurry treated seeds and the control treatment were placed in between paper towels that were rolled around 8ml test tubes. Each of the respective sub-experimental units was replicated three (3) times making a total of eighteen (18) experimental units. These experimental units were placed in beakers containing 4mm level of distilled water that moistened the paper towels through capillary action. The moistened paper towel provided suitable conditions for seed germination. This seed germination technique is described in detail by Bicksler (2011). The respective experimental units for each of the sub-experiment were laid in a completely randomized design (CRD) in an incubator set at a temperature of 25°C.



Figure 4. 1: Seed germination experimental setup.

Germination percentage of the respective test fungi treated vegetable seeds was assessed for all the three sub-experiments to screen for phytopathological safety of different vegetable seeds when exposed to all the test fungal isolates. The effect of the test fungal isolates on respective vegetable seeds was assessed by counting the number of germinated seeds at a two-day interval for a period of two weeks. The radicle and plumule lengths for all the selected vegetables were assessed using vernier calipers. Disease occurrence or pathogenicity of the test isolates was assessed by checking for necrosis and discoloration on the radicles and plumules of each respective vegetable.

4.2.3 Data collection

- i. Germination rate was carried out at a two-day interval (Number of germinated seeds per assessment). The germination percentage was calculated.
- ii. The growth rate of respective radicles and plumules of the germinated seeds.
- iii. Vigour indices were calculated using the following mathematical formula as described and explained by (Khokhar *et al.*, 2013, Rao *et al.*, 2014) and suggested by (Abdul-Baki and Anderson, 1973):

Vigour index (VI) determines the potential for rapid and uniform germination/emergence under various conditions (Chen *et al.*, 2015, Huang *et al.*, 2017). It was calculated using the addition of the mean length of plumule and mean length of radicles multiplied by germination percentage (Abdul-Baki and Anderson, 1973).

$$VI = (lp + lr) * 100 \quad (4.2)$$

Where

VI= vigour index

lp= length of plumule (cm)

lr= Length of radicle (cm)

4.2.4 Statistical Analysis

Analysis of variance (ANOVA) of the data from all the experiments was carried out using GEN STAT software seventeenth edition (VSN International Ltd.) to test the significant difference of the effect of all treatments on germination and growth of the seed of the three vegetable

types: germination rate, plumule and radicle length. The mean comparison was interpreted by the use of Bonferroni test at 5% probability level.

4.3 RESULTS

Among the three vegetables selected for the biosafety study, treatments had a statistically significant effect on the germination, radicle and plumule growth of *S. oleracea* and *B. vulgaris*. There was, however, a relatively non-significant weak positive effect on *S. lycopersicum*.

4.3.1 Effect of different treatments on seed germination of the selected vegetables

a. ANOVA for germination of selected vegetables: *S. oleracea* and *B. vulgaris*

Analysis of variance of the effect of four *Penicillium* isolates: *P. commune*-1, *P. commune*-2, *P. concavorugulosum*-1, *P. concavorugulosum*-2 and *A. fumigatus* on the germination of *S. oleracea* and *B. vulgaris* showed statistically significant effects on seed germination ($P < .001$) [Tables 4.1 and 4.2]. Treatment means comparison for *S. oleracea* and *B. vulgaris* are presented in Table 4.3. Means with the same superscripts are not statistically significantly different.

Table 4. 1: ANOVA on the effect of all treatments on *S. oleracea* germination

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	1.000	0.500	0.41	
Rep. *Units* stratum					
Treatment	5	30.7619	6.1524	5.10	<.001
Residual	118	142.238	1.205		
Total	125	174.0000			

Table 4. 2: ANOVA on the effect of all treatments on *Beta vulgaris* seed germination

Source of variation	d. f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	6.778	3.389	3.23	
Rep. *Units* stratum					
Treatment	5	93.111	18.622	17.74	<.001

Residual	118	123.889	1.050
Total	125	223.778	

Table 4. 3: Mean comparison and grouping of *S. oleracea* and *B. vulgaris* seed germination

Treatment	<i>S. oleracea</i> germination means*	Treatment	<i>B. vulgaris</i> germination means*
<i>P. concavorugulosum2</i>	4.048 ^a	<i>P. commune2</i>	2.381 ^a
<i>P. concavorugulosum1</i>	4.238 ^a	<i>P. concavorugulosum1</i>	3.429 ^b
<i>A. fumigatus</i>	4.333 ^{ab}	<i>P. commune1</i>	3.667 ^b
Control	4.810 ^{ab}	<i>A. fumigatus</i>	3.714 ^b
<i>P. commune1</i>	5.286 ^b	<i>P. concavorugulosum2</i>	4.238 ^b
<i>P. commune2</i>	5.286 ^b	Control	5.238 ^c

Comparison-wise error rate = 0.0033

*The mean comparison treatments with the same superscripts are not significantly different from one another.

b. Assessment of germination rate of *S. oleracea* and *B. vulgaris*

The trend of the germination rate of *S. oleracea* is graphically presented in figure 4.2. *P. commune-1* and *P. commune-2* exhibited a higher stimulatory effect on germination of *S. oleracea* seeds than *P. concavorugulosum-1*, *P. concavorugulosum-2* coated seeds and control. Among the various *Penicillium* isolates and *A. fumigatus* used as slurry seed treatments, control, *P. commune-1*, and *P. commune-2* recorded the highest germination percentages (100%) on *S. oleracea* (Fig 4.1).

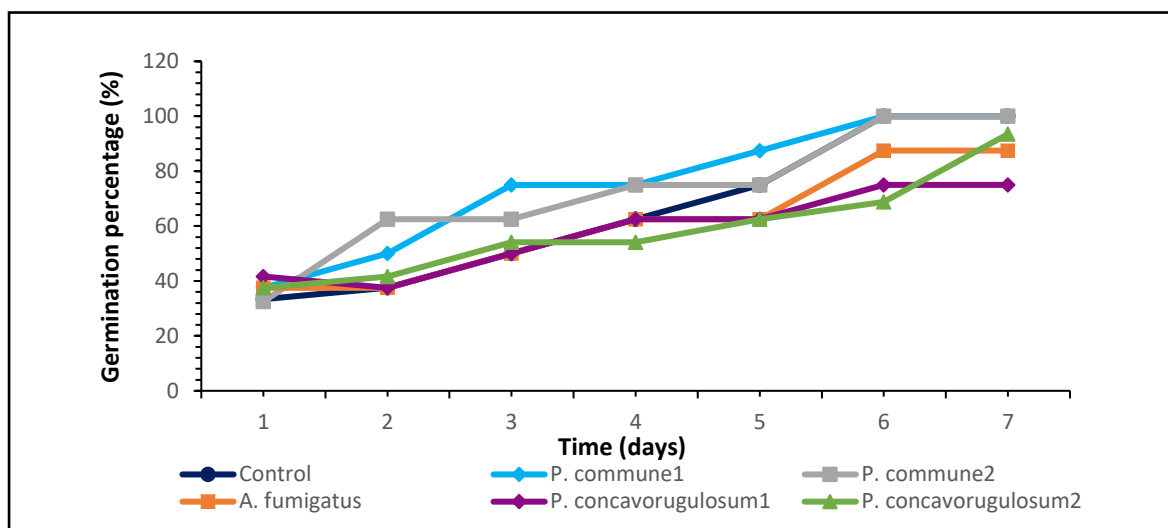


Figure 4.2: *S. oleracea* seed germination rate over a period of 14 days.

On the other hand, the control treatment showed full seed germination (100%), whereas *P. commune-2* inoculated seeds exhibited low germination percentage compared to all the treatments (Figure 4.2).

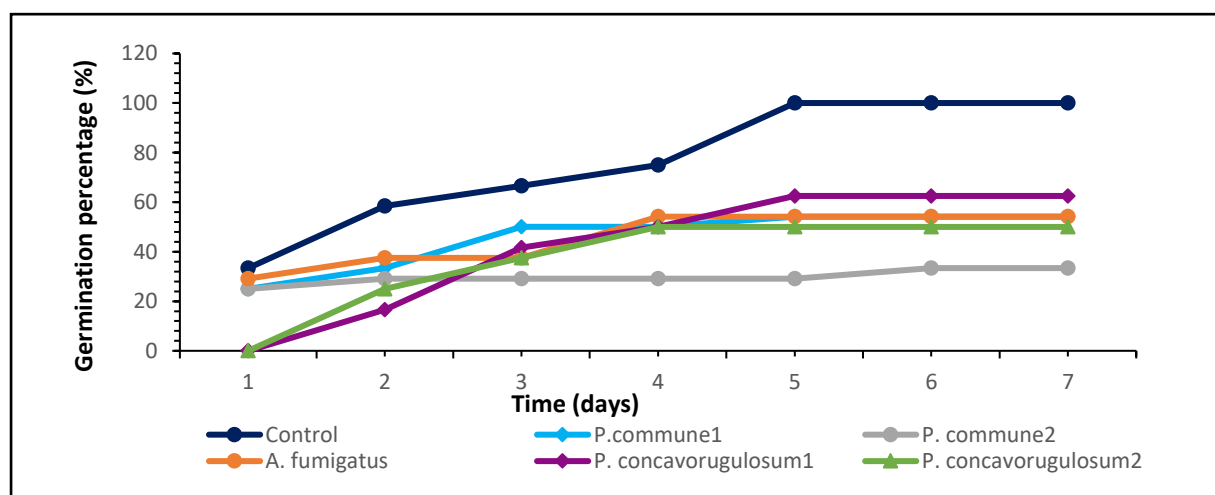


Figure 4.3: *B. vulgaris* seed germination rate. Germination was collected at the two-days intervals for two weeks.

Positive germination was noted for all treatments on *S. lycopersicum*; however, a not statistically significant difference output was observed on seed germination.

4.3.2 Effect of the treatments on the radicle and plumule response

a. ANOVA for *S. oleracea* and *B. vulgaris* radicle and plumule growth assessments

In addition to the stimulatory effect of different *Penicillium* and *Aspergillus* fungal species on germination of *S. oleracea* and *B. vulgaris* seeds, their effect was also noted on the plumule growth rate. A non-statistically significant effect was however noted on the radicle for all three vegetables. Analysis of variance on the effect of the treatments on plumule response shows a

statistically highly significant effect on *S. oleracea* and *B. vulgaris* ($p < .001$) (Table 4.4) and (Table 4.5).

Table 4. 4: ANOVA on the effect of different treatments on *S. oleracea* plumule response.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Assessment stratum	6	228.7838	38.1306	52.56	
Assessment*Units*stratum					
Treatment	5	75.8088	15.1618	20.90	<.001
Residual	114	82.7072	0.7255		
Total	125	387.2998			

Table 4. 5: ANOVA showing the effect of all treatments on plumule growth.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	0.5303	0.2652	0.94	
Rep. *Units* stratum					
Treatment	5	219.2228	43.8446	155.31	<.001
Residual	118	33.3109	0.2823		
Total	125	253.0639			

b. Radicle and Plumule growth assessment: *S. oleracea* and *B. vulgaris*

***S. oleracea* radicle and plumule growth**

A graphical presentation of *S. oleracea* radicle and plumule response is shown in figure 4.4(a) and figure 4.4(b). For instance, Control, *P. commune1* and *A. fumigatus* treatments showed higher radicle and plumule growth response than *P. commune2*, and *P. concavorugulosum1* and *P. concavorugulosum2* treatments.

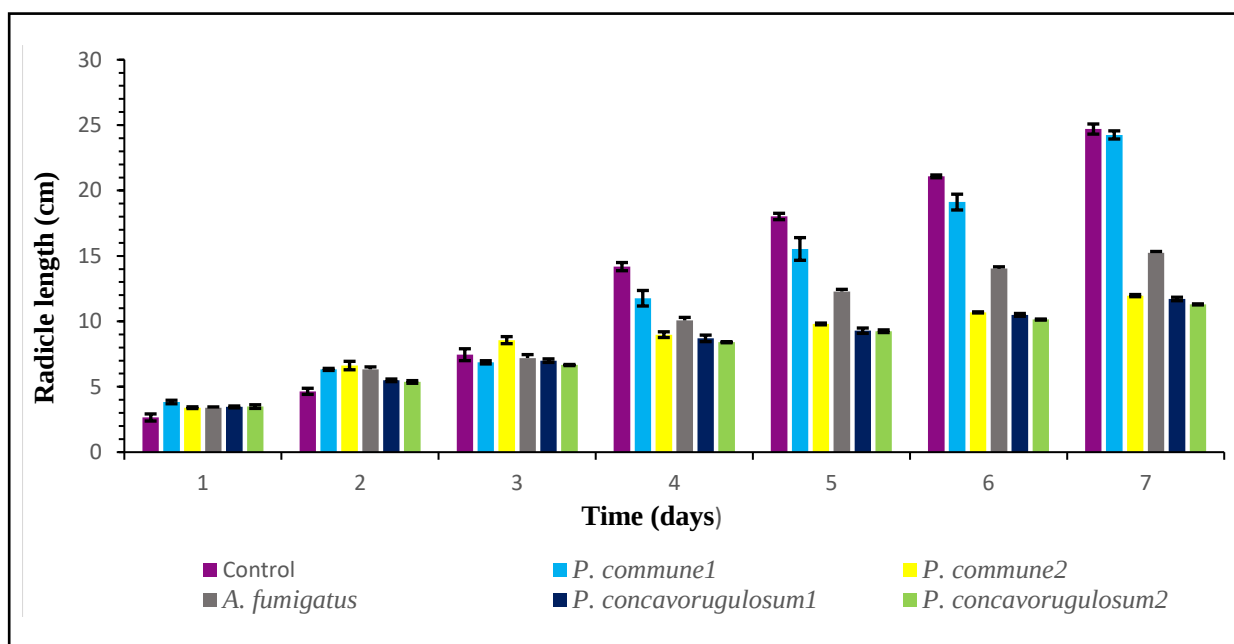


Figure 4.4(a): Two weeks assessment of *S. oleracea* radicle growth.

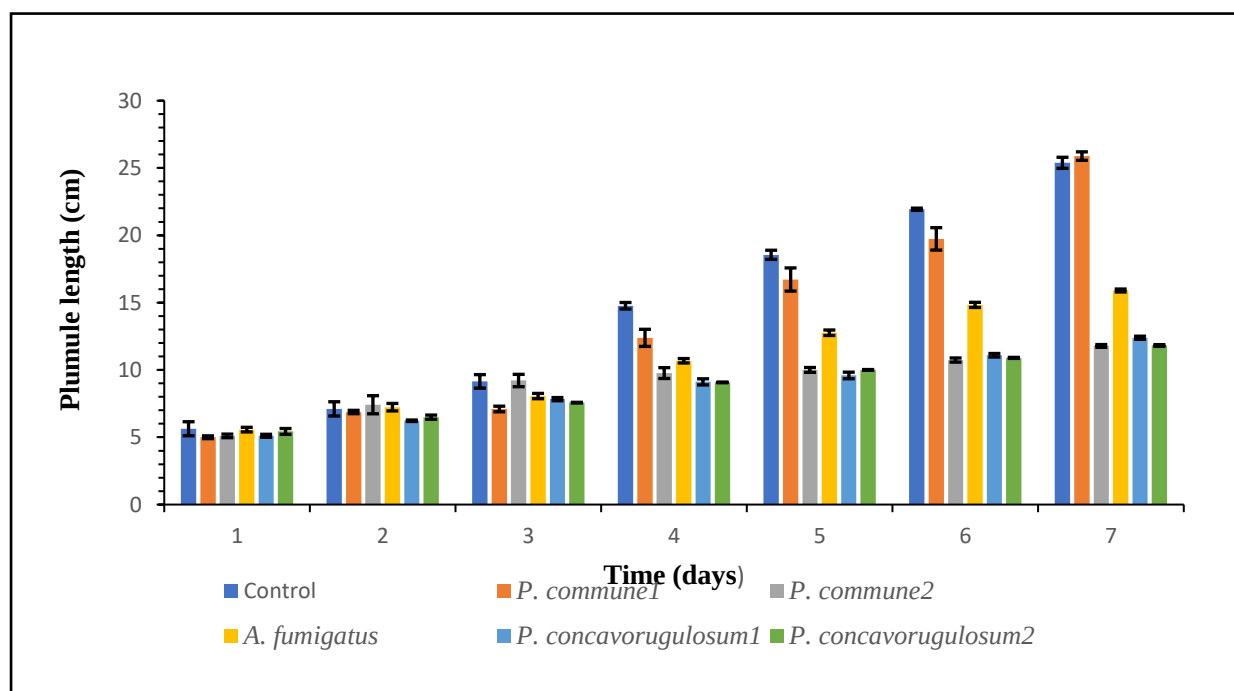


Figure 4.4 (b): Two weeks assessment of *S. oleracea* plumule growth.

***B. vulgaris* radicle and plumule growth assessment**

On the contrary, *P. concavorugulosum-2* and *A. fumigatus* coated seeds exhibited high radicle growth whereas a high plumule growth was noted on *P. concavorugulosum-1* and *P.*

concolorugulosum-2 treated *B. vulgaris* seeds (Fig 4.6) and (Fig 4.7). Control treatment exhibited the highest growth on both the radicle and plumule for *B. vulgaris*.

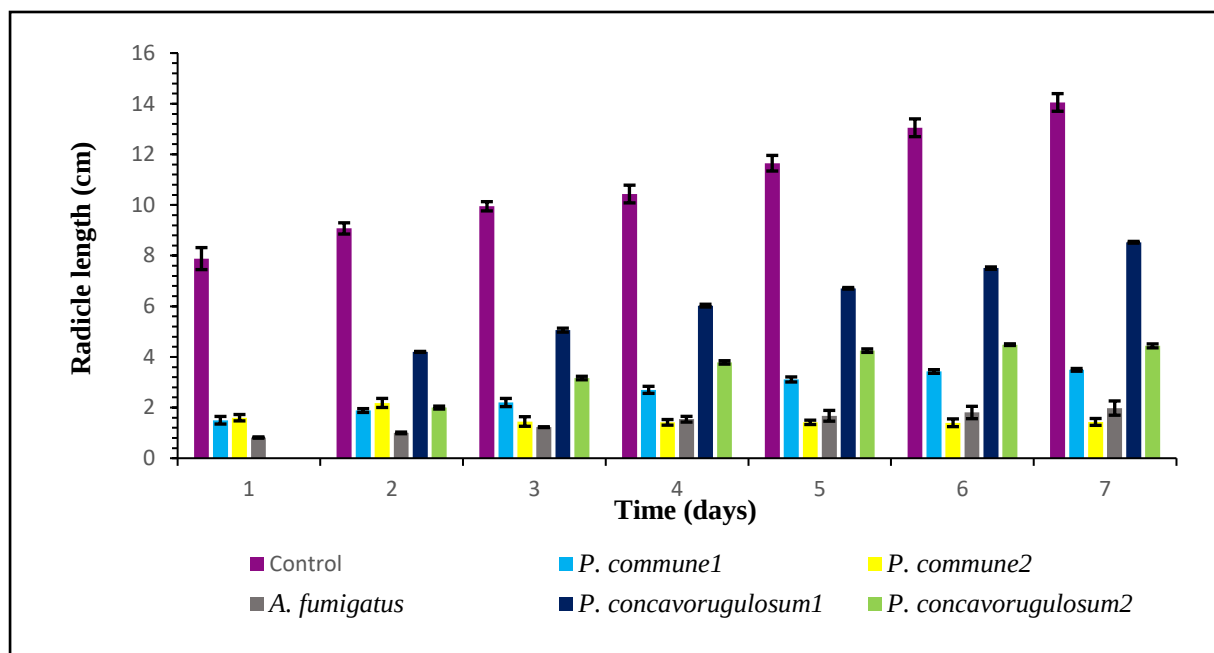


Figure 4.5(a): *B. vulgaris* radicle growth assessed at two-day intervals for two weeks.

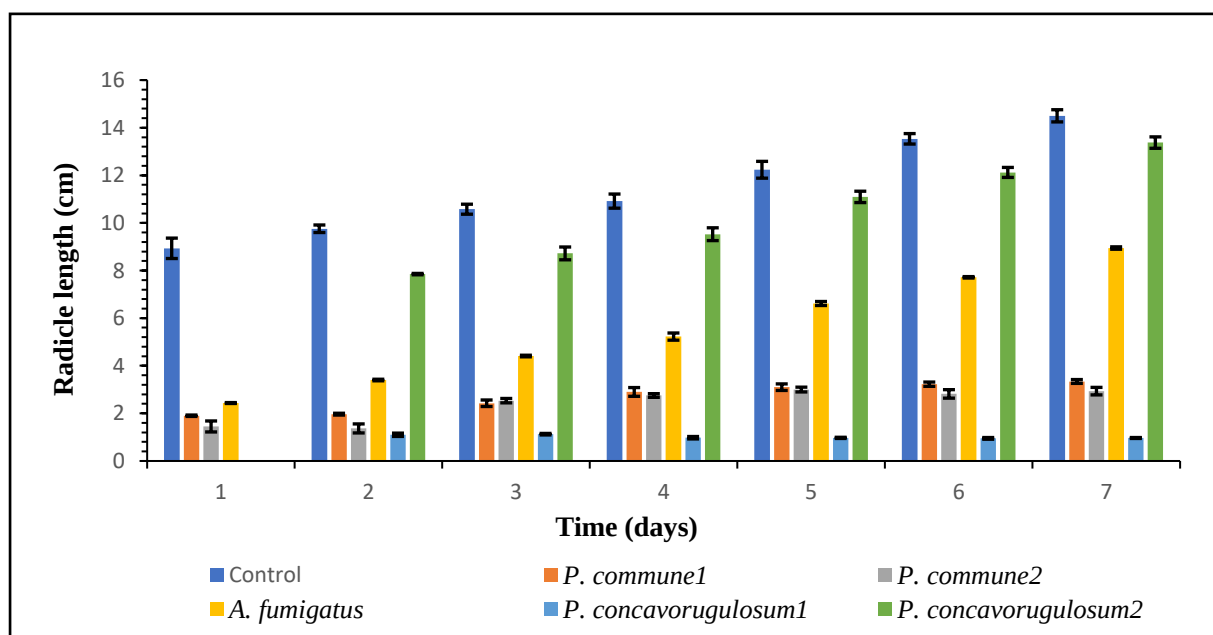


Figure 4.5(b): *B. vulgaris* plumule growth assessed at two-day intervals for two weeks.

4.3.3 Effect of the treatments on seedling size and health: Seedling Vigour

a. Seeding inspection and diagnosis

There was no necrosis nor discoloration noted on *S. oleracea* radicle and plumule for all the treatments (Fig 4.6).



Figure 4. 6: *Spinacia oleracea* seedlings showing no signs and symptoms of disease occurrence

Evidence of growth suppression and the presence of parasitism were noted on *B. vulgaris* radicle and plumule for all treatments. The control treatment also showed some evidence of necrosis on the plumule and radicle [Figure 4.7(a) & (b)]. The seeds and seedlings exhibited reduced and slower germination, discoloration and necrosis on the radicles and plumules.

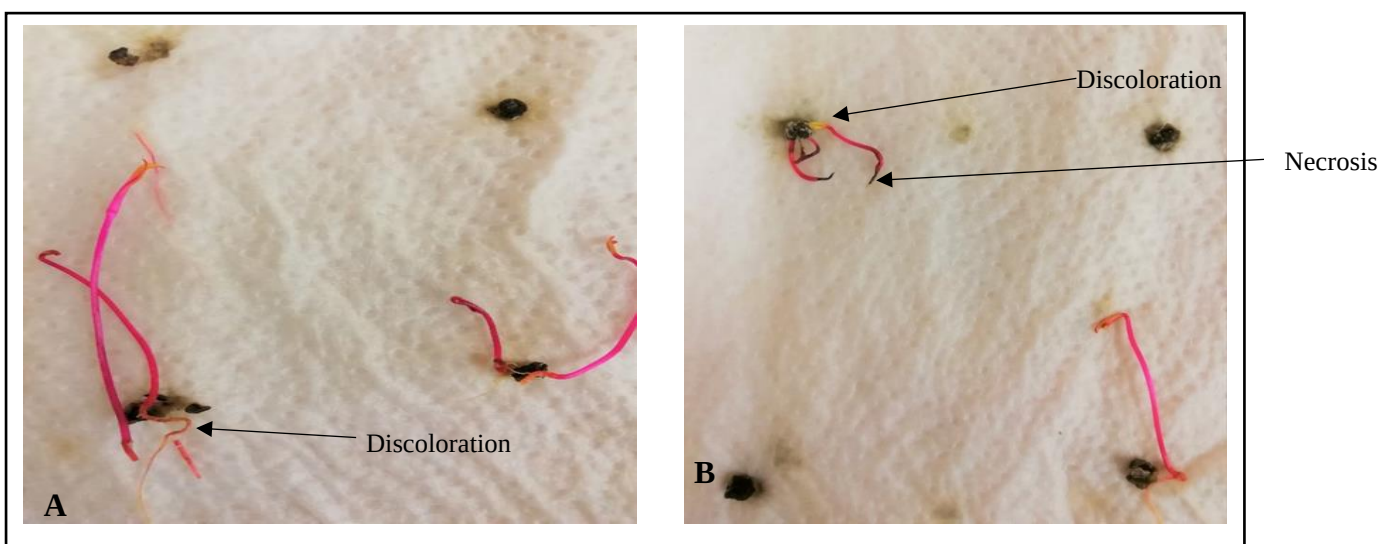


Figure 4. 7(a): (A) Discoloration symptoms on control treatment. (B) Radicle and plumule necrotic signs on *P. commune1* treatment.

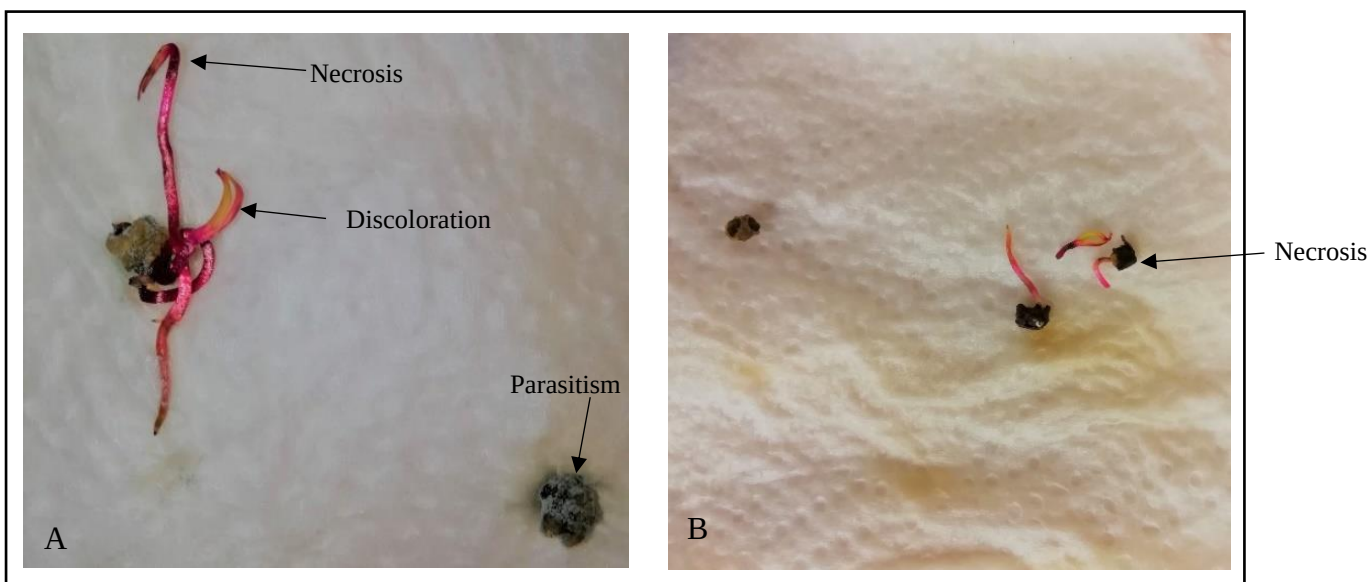


Figure 4.7(b): (A) Parasitism, discoloration and necrosis symptoms on *A. fumigatus* treated seeds. (B) Necrosis signs noted on seeds treated with *P. concavorugulosum*1.

The biosafety inspection on *S. lycopersicum* showed no evidence of infection for all treatments (Fig 4.8).

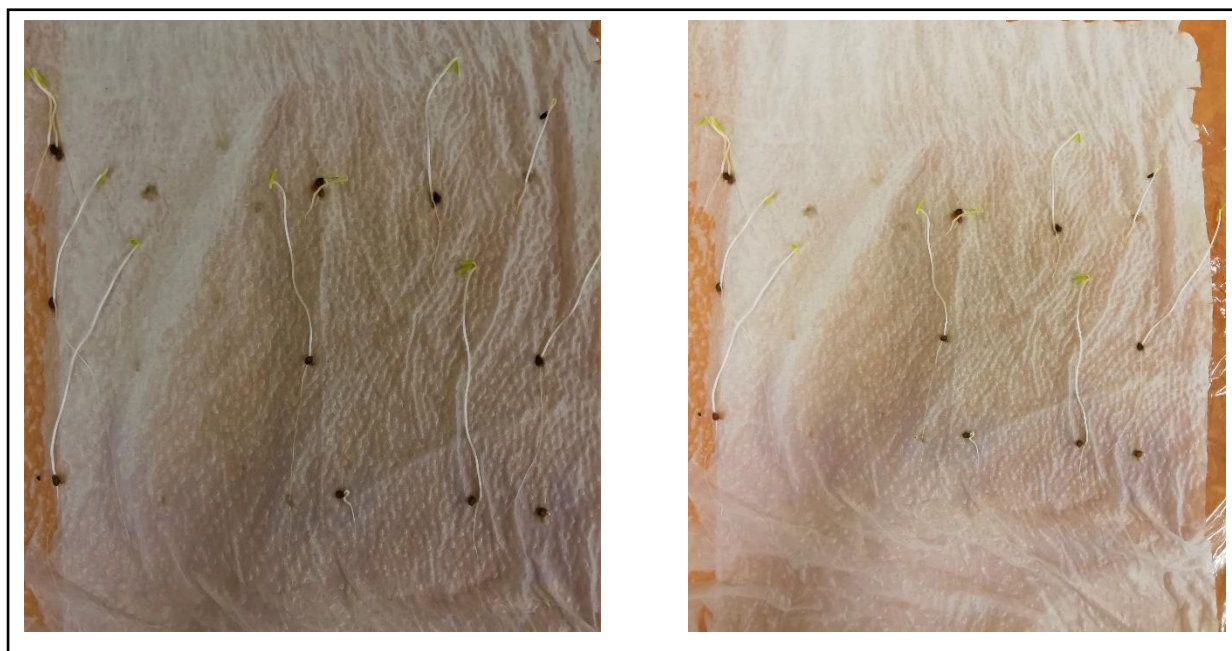


Figure 4. 8: *S. lycopersicum* germinated seedlings.

b. Vigour index assessment for *S. oleracea*, *B. vulgaris* and *S. lycopersicum*

To further analyse the effect of different *Penicillium* and *Aspergillus* isolates on the germination, plumule, and radicle growth rate, the seedling vigour (vigour index) was

calculated. A high vigour index was recorded for *S. oleracea* treated with *P. commune*-1 and the control in comparison to other treatments where a low vigour index was recorded on *P. concavorugulosum*-2 treated seeds (Table 4.6). The control treatment was observed to be the highest in vigour index for *B. vulgaris* whereas a low vigour index was noted on *P. commune*-2 (Table 4.7). Also, a positive increase in the growth of both the radicle and plumule on *S. lycopersicum* resulted in high vigour indices (Table 4.8).

Table 4. 6:Table showing germination percentages and vigour index of *S. oleracea*.

Treatments	Seed germination %	Plumule length	Radicle length	Vigour Index
Control	100	25.38	24.70	5008
<i>P. commune</i> 1	100	25.88	24.50	5038
<i>P. commune</i> 2	100	11.78	11.96	2374
<i>A. fumigatus</i>	83	15.89	15.89	2583.79
<i>P. concavorugulosum</i> 1	78	12.39	12.39	1879.8
<i>P. concavorugulosum</i> 2	72	11.82	11.82	1661.76

Table 4. 7: Table showing germination percentages and vigour index of *B. vulgaris*.

Treatments	Seed germination %	Plumule length	Radicle length	Vigour Index
Control	100	14.5	14.05	2855
<i>P. commune</i> 1	72	3.34	3.49	491.76
<i>P. commune</i> 2	44	2.93	1.43	191.84
<i>A. fumigatus</i>	72	8.95	1.98	786.96
<i>P. concavorugulosum</i> 1	83	0.95	8.53	786.84
<i>P. concavorugulosum</i> 2	67	13.38	4.44	1193.94

Table 4. 8: Table showing germination percentages and vigour index of *S. lycopersicum*.

Treatments	Seed germination %	Plumule length	Radicle length	Vigour Index
Control	100	25.73	28.53	5426
<i>P. commune</i> 1	100	29.83	31.58	6141
<i>P. commune</i> 2	100	32.1	34.48	6658
<i>A. fumigatus</i>	89	27.78	28.87	5041.85
<i>P. concavorugulosum</i> 1	95	26.15	26.72	5022.65
<i>P. concavorugulosum</i> 2	100	29.45	30.11	5956

4.4 DISCUSSION

The stimulatory effect of the *Penicillium* species and *A. fumigatus* on the germination of *S. oleracea* (Fig 4.2) and *S. lycopersicum* (Table 4.8) seeds could have been caused by the exogenous production of plant growth hormones such as auxins, gibberellic acids, abscisic acid, ethylene and jasmonic acid. Many fungal microorganisms have been reported to produce plant hormones that either promote plant growth and development (Leitao and Enguita, 2016; Altaf *et al.*, 2018) or are harmful. *Penicillium* and *Aspergillus* species have been reported in various studies to produce gibberellic acid which is needed in the mitotic division of seed germination (Leitao and Enguita, 2016; Ali *et al.*, 2017; Altaf *et al.*, 2018).

Plant growth hormones have been reported to control and trigger plant growth and development by promoting cell division, seed germination, stem elongation, etc. (Gupta and Chakrabarty, 2013; Chanclud and Morel, 2016; Wolny *et al.*, 2018). Germination is achieved by positively influencing the production of plant growth hormones that influence the development of radicle and plumule physiological processes (Sanders, 2011; Chanclud and Morel, 2016). This is also evident on *S. oleracea* seeds for all the treatments, where a statistically significant effect was noted on *S. oleracea* seed germination (Table 4.1). The control, *P. commune*-1 and *P. commune*-2 treatments showed total seed germination (100%) for *S. oleracea* (Fig 4.2) whereas on the other hand, control, *P. commune*-1, *P. commune*-2 and *P. concavorugulosum*-2 had the highest germination (100%) for *S. lycopersicum*. This varying germination percentage/rate of *S. oleracea* and *S. lycopersicum* seeds could have occurred because of inherent differences in

the stimulatory effect of phytohormones produced by the *Penicillium* and *Aspergillus* fungi on seed germination. Ali *et al.* (2017) and Altaf *et al.* (2018) highlighted the influence of *Penicillium* species in plant growth promotion and that the effectiveness of the production of secondary metabolites and plant growth-promoting regulators vary under different ecological conditions. This in turn results in different responses by plants growing under different ecological conditions. Since seed germination is a process that is triggered when the embryo undergoes mitotic division which is influenced by the presence or absence of plant growth hormones i.e. abscisic acid (ABA) and gibberellic acids (GA). Absciscic acid is known to inhibit germination whereas gibberellic acids trigger mitotic division in the embryonic cells (Leitao and Enguita, 2016). Control had 100% germination whereas *P. concavorugulosum-2*, *P. concavorugulosum-1* and *A. fumigatus* showed suppressive effects on *S. oleracea* (Table 4.6). A similar trend was noted on *S. lycopersicum* where *A. fumigatus* and *P. concavorugulosum-2* exhibited suppressive effects on seed germination (Table 4.8). Therefore, it can be deduced that the isolates either suppressed production of gibberellic acids or stimulated production of abscisic acid which has a negative effect on seed germination.

Similarly, the effect of the isolates on *B. vulgaris* seemed to be more severe as suppressive effects were noted for all isolates. The seed germination was more severely suppressed in seeds treated with *P. commune-2* (44% germination) whereas the control treatment exhibited a total germination rate (100%) compared to the other treatments, *albeit* at a slower rate (Table 4.7). This could be because there were no antagonistic factors that could have affected or reduced response to water uptake to initiate the germination process in the *B. vulgaris* control treatment. The slow germination rate could, however, be because of inherent factors in beet seeds that play a role in reduced seed germination rate. For example, a study by Taylor *et al.* (2003) explained that factors such as mucilaginous layer in beet seeds can lower and suppress germination when layers are too thick and when antagonists such as fungal microorganisms are present. Possibly, this could have resulted in the slow germination rate for other treatments that had introduced fungal isolates and not the control treatment.

Furthermore, the high nitrate content of *B. vulgaris* seeds have been reported to suppress germination by the effect of ammonia that is generated during the nitrification process (Habib, 2010). A few fungal species such as *Penicillium* and *Aspergillus wentii* species have been reported to be involved in the nitrification process (Hayatsu *et al.*, 2008). Therefore, this could have contributed to the suppression of seed germination on *P. commune-2* and *P. concavorugulosum-2* treated seeds. It is also possible that some of the *Penicillium* species used

in the study may be ammonia sensitive fungi, i.e. could be suppressed by high amounts of ammonia or other nitrogenous materials. These ammonia-sensitive fungal species are generally described as a chemo-ecological group of fungi (Barua *et al.*, 2012; Suzuki, 2017). This could have resulted in a decreased germination because of the limited production of plant growth hormones due to antagonistic interaction between the *Penicillium* species and ammonia bacteria on *B. vulgaris* seeds (Habib, 2010).

On the other hand, the poor seed germination in *B. vulgaris* that is inoculated with different fungal isolates could be because of poor nutrient uptake by the germinating seed occurring because of competition for nutrients between microbes that had been introduced around the rough seed coat. A study by Mancini and Romanazzi, (2014) on microbial inoculation of seeds for improved crop growth showed that rough seed coats can impair nutrient uptake as well as extrolites penetration while promoting fungal or microbial penetration in germinating seeds.

As germination is a physiological process that happens in the cotyledon and embryo, followed by the next stage i.e. elongation of both the radicle and the plumule, the factors that influenced germination would be expected to also influence the post-germination growth processes. A positive and statistically significant effect of *P. commune-1*, *A. fumigatus*, and control treatments on radicle and plumule growth was noted for *S. oleracea* on [Fig 4.4(a) and Fig 4.4(b)]. This could have been because of gibberellins and metabolites producing *Penicillium* species. *Penicillium* species have been reported and noted to produce plant hormones and growth-promoting compounds (Altaf *et al.*, 2018). A study conducted by Waqas *et al.* (2015) reported on gibberellins producing *Penicillium* species that aid in the promotion as well as an acceleration of growth on plants. These plant growth regulators are known for their role in plant physiological processes such as cell division and elongation, shoot growth stimulation, as well as stem elongation (Gray, 2004; Gupta and Chakrabarty, 2013).

A positive response in root and shoot length for *S. lycopersicum* was noted on seeds treated with *P. commune-1*, *P. commune-2*, *P. concavorugulosum-2* and the control (Table 4.8). It is noted that this fungal-seed interaction positively influenced seed germination also influenced radicle and plumule growth by accelerating length and improving vigour of the radicle and plumule growth of *S. oleracea* and *S. lycopersicum*. Similarly, *B. vulgaris* radicle growth rate was higher on the control and *P. concavorugulosum-2* inoculated seeds compared to *P. commune-1*, *P. commune-2*, *A. fumigatus* and *P. concavorugulosum-1* [Fig 4.5 (a) & 4.5(b)].

As elongation of the radicle and plumule determine growth and establishment of seedlings, seed vigour was therefore computed to determine the influence that various *Penicillium* and *Aspergillus* treatments had on the vigour of seedlings during the establishment. The high seed vigour index established in *S. oleracea* and *S. lycopersicum* seeds for all the treatments was therefore expected. This is indicative of the high potential for good seedling establishment (Table 4.6 and Table 4.8). A higher seed vigour index indicates a vigorous seed lot whereas a lower seed vigour index indicates a weak seed lot that would lead to a poor seedling establishment (Chen *et al.*, 2015). *B. vulgaris* had the lowest seed vigour index (Table 4.7) on all *Penicillium*, and *Aspergillus* treated seeds when compared to *S. oleracea* and *S. lycopersicum* (Tables 4.6 and 4.8). On the other hand, control (non-treated) *B. vulgaris* seeds showed high seed vigour index. The low/reduced vigour index for the other treatments on *B. vulgaris* might have resulted from the slow response to the *Penicillium* treatments or the pathogenic influence that the species had on germination as well as plumule and radicle growth [Fig 4.7 (a) and 4.7(b)]. *B. vulgaris* high seed vigour index on control could have been because there were no antagonistic influences on the seeds during germination as well as radicle and plumule elongation. This could also mean that the inherent beet seed factors that reduce germination had no extreme effect on the control treatment than other treatments as there was no competition for oxygen and nutrients by the naturally occurring microbial load on the untreated seed coats and the germinating beet seeds, which probably occurred on seeds with introduced fungal isolates.

The antagonistic effect on *B. vulgaris* seeds treated with different test isolates could have occurred because of the presence of seed-borne or seed contaminants which acting together with the introduced fungi used in seed coating could have drained the seeds of nutrients. This could have affected its source of nutrients to support the development and growth of radicle and plumule [Fig 4.4(a) and 4.4(b)]. Hamim *et al.*, (2014) reported similar studies which established that suppressive effect on seed germination occurred because the interaction between different microorganism on the seed coat depleted nutrients before germination. The presence of the seed-borne or seed contaminants that were isolated from *B. vulgaris* could explain the poor seed vigour of *B. vulgaris*. Hypocotyl lesions were also noted on the control treatment seedlings. This could mean either the pathogenic species were seed-borne or seed contaminants [Fig 4.7(a)]. This infection did not, however, seem to affect the seedling vigour for the control. The severity of the infection was high on seeds treated with *A. fumigatus* than the other treatments. This could mean that *A. fumigatus* has pathogenic properties on *B.*

vulgaris. It has been reported that *A. fumigatus* does produce gliotoxin. This is a mycotoxin that is reportedly known to have different potential suppressive effects on plant growth and to possibly cause a variety of immunosuppressive effects on human health (Smith *et al.*, 2016). Phyto- pathological effect of these fungal isolates should be investigated.

Findings of this study that exhibited slight positive responses on germination and seedling growth on *S. oleracea* and *S. lycopersicum* demonstrated the potential use of *P. concavorugulosum*-2 and *A. fumigatus* as seed inoculants for stimulatory growth of *S. oleracea* and *S. lycopersicum* seedlings. There could also be a possibility of endophytic properties by these test isolates, particularly *P. commune*-1, *P. concavorugulosum*-2, and *A. fumigatus*. Fungal endophytes may be explained as microorganisms that can inhabit plant tissues, aiding in plant development and disease defensiveness without exerting negative effects on the plant (Ali *et al.*, 2017; Khiralla *et al.*, 2017; Rana *et al.*, 2019). For example, Waqas *et al.* (2015) reported on *Penicillium* and *Aspergillus* species viz. *P. citrinum* and *A. terreus*, which aid and promote plant growth characteristics. It can therefore be deduced that *P. commune*-1, *P. concavorugulosum*-2 and *A. fumigatus* had some endophytic elements. The use of these plant growth enhancers could help promote the establishment of good seedling stands. A lower response was however noted on *B. vulgaris*. It is therefore recommended that further investigations assessing the effect of the two fungal species is investigated for their endophytic properties. Considering that *A. fumigatus* has been reported for its immunosuppressive diseases such as Aspergillosis on human health (Smith *et al.*, 2016), this fungal isolate should be excluded for further research work. *P. concavorugulosum*-2 was therefore selected as a potential test bio-control agent for further research. This test fungal isolate exhibited minimum negative effects on germination and showed positive effects on germination, radicle and plumule growth of the selected vegetables. *P. concavorugulosum*-2 also showed antibiosis and mycoparasitic properties against *F. oxysporum* (Chapter 3 in this dissertation), indicating suppressive effects against *F. oxysporum*. The fungi could therefore be used in combination with *Penicillium* species such as *P. commune*-1 and *P. concavorugulosum*-1 that have a stimulatory effect on plant growth but no suppressive effects on *F. oxysporum*.

CHAPTER FIVE

***In- vivo* analysis of the suppressive effect of *P. concavorugulosum*2 on *F. oxysporum f.sp. radidis lycopersici* (FORL) in tomato (*Solanum lycopersicum*) seedlings under different soil amendments**

5.1 INTRODUCTION

The success of *Fusarium* species is influenced by its inherent biological properties and the way they interact with the root rhizosphere and the surrounding soil environment (Abdel-Azeem *et al.*, 2019). These environmental factors include divergent biotic and abiotic factors in different agro-ecological regions. For instance, *Fusarium* species have inherent properties of long persistence in the soil and they are adapted to attack a wide range of hosts (Leslie and Summerell, 2006, Abdel-Azeem *et al.*, 2019). These adaptive properties make it difficult to manage them. The occurrence of different strains under varying environmental conditions directly or indirectly affect plant root rhizosphere and thereby influencing pathogen infection processes (Validov *et al.*, 2011). This, therefore, influences disease development and exacerbates the disease situation (Elmer, 2015). Furthermore, the use of resistant host or cultivars is slowly being compromised as *Fusarium* pathogens develop new strains that can overcome host resistance (Andersen *et al.*, 2018; Hermida-Montero *et al.*, 2019). This is reported to be achieved by the *F. oxysporum* bio-pathotypes that break plant defense mechanisms by use of extrolites to disarm host plants, or by developing multiple infection levels whereby two or more pathogens release competitive exudates that affect the normal functioning of a plant, reducing its ability to defend itself (Abdullah *et al.*, 2017).

To mediate the impact of soil-borne pathogens on plants, use of organic amendments and biological control agents were initiated. For example, studies by Matsubara *et al.*, (2002) and Elmer and Pignatello, (2011) analysed the influence and interaction of biochar, mycorrhizae, *F. oxysporum* and *F. proliferatum* on asparagus. The studies showed that plants grown on biochar and mycorrhizae amended soils had more pathogen resistance than plants grown on unamended soils (Wiednera *et al.*, 2013). This study was therefore carried out to assess the effects of biochar and mycorrhizae amended soil rhizosphere on the suppressive effect of *P. concavorugulosum*-2 on the infection process of *F. oxysporum f.sp. radidis lycopersici* (FORL).

5.1.1 Specific objectives

- (i) To evaluate suppressive effects of *P. concavorugulosum*-2 on *F. oxysporum f.sp. radicis lycopersici* (FORL), and the establishment and growth of *S. lycopersicum* seedlings.
- (ii) To evaluate effects of mycorrhizae as a rhizosphere amendment on the suppressive effects of *P. concavorugulosum*-2 on *F. oxysporum f.sp. radicis lycopersici* (FORL), and the establishment and growth of *S. lycopersicum* seedlings.
- (iii) To evaluate effects of biochar as a rhizosphere amendment on the suppressive effects of *P. concavorugulosum*-2 (MK841454.1) on *F. oxysporum f.sp. radicis lycopersici* (FORL), and the establishment of *S. lycopersicum* seedlings.

5.2 MATERIALS AND METHODS

5.2.1 Soil collection and experimental site description

The soil was sampled at North-West University Mafikeng Campus Agricultural Research fields (25° 48' S, 45° 38' E; 1012 m.a.s.l). The sampled soil was of the Hutton soil type as classified by the soil classification handbook. The study was carried out at North-West University Agricultural farm under a temperature-controlled glasshouse with temperatures ranging between 26°C-32°C.

5.2.2 Laboratory analysis description

a. Soil sterilization and preparation

Batches of thirty (30) five-kilogram plastic bags of soil were autoclaved for thirty minutes at 121°C under the pressure of 140 kPa to eliminate microbial population and spores that might alter the objectives of the study. The autoclaved soil was cooled and stored in the laboratory in hessian bags for later use.

b. Soil analysis

A 100g of soil was sub-sampled and used to carry out pH and texture analysis. Soil pH was determined by use of a standard pH method of analysis. A ratio of 1:5 (soil: water) suspension as described by Rayment and Higginson (1992) was used. Soil texture was analysed using the Bouyoucos hydrometer test as described by Van Reeuwijk (2002). The availability or presence

of pathogenic microorganisms was analyzed using the serial dilution method (Sarker *et al.*, 2006).

Soil Texture	Soil pH
• Silt (%) 11.3 • Clay (%) 25.6 • Sandy (%) 63	6.6

c. Mycorrhizae and biochar inoculum preparation

Vesicular–arbuscular mycorrhizae (VAM) and hardwood biochar were used as soil amendments in the study. VAM inoculum containing a combination of the following species: *Rhizophagus clarus*, *Gigaspora gigante*, *Claroideoglossum etunicatum* and *Paraglossum occulum* was used.

A portion of the autoclaved soil was apportioned into two equal quantities (thirty kilograms per quantity) and amended with 200g of hardwood biochar for the respective portions. Soil amendment with biochar was carried out by mixing with the soil before sowing. Mycorrhizae inoculation was undertaken at planting. Three 20g portions of the mycorrhizae were placed at 5mm below seeds during planting to enable early mycelial contact with seed radicle.

5.3 Experimental description and design

5.3.1 Experimental description

In-vivo analysis of the effectiveness of *P. concavorugulosum*-2 as a bio-control agent against *F. oxysporum f.sp. radicis lycopersici* (FORL) on *S. lycopersicum* was evaluated under different root rhizosphere amendments.

a. Seed treatment/rhizosphere amendment

Seed inoculation with *P. concavorugulosum*-2 was applied in a form of a slurry. The slurry was prepared by mixing a 10 g sub-sample of *P. concavorugulosum*-2 mycelia with 5 g of talc powder. A total of 20 ml sterile water was poured into the mixture and stirred until a homogenous mixture was achieved.

The experiment was laid out in a temperature-controlled glasshouse. Experimental treatments were as follows: (i) Un-amended rhizosphere + untreated seeds (T0), (ii) Un-amended rhizosphere + untreated seeds + *F. oxysporum f.sp. radicis lycopersici* (FORL) inoculation (T1), (iii) Un-amended rhizosphere + *P. concavorugulosum2* treated seeds (T2), (iv) *P. concavorugulosum2* treated seeds + mycorrhizae amended soil (T3), (v) *P. concavorugulosum2* treated seeds + biochar amended soil (T4), (vi) *P. concavorugulosum2* treated seeds + Mycorrhizae + Biochar amended soil (T5).

Seeds that had been subjected to three respective treatments were directly sown at a depth of about 20 mm in 140 mm polythene pots. Three 20 g portions of the mycorrhizae were placed at 5 mm below seeds during planting to enable early mycelial contact with seed radicle. The treatments were replicated ten times (10) making a total of sixty (60) experimental units. The treatments were laid out in a completely randomized design (CRD) in a temperature-controlled glasshouse with temperatures ranging between 20°C to 24°C. Seedlings grown in the five respective treatments i.e. T1, T2, T3, T4 and T5 were challenged with *F. oxysporum*.

Inoculations with the pathogenic *F. oxysporum f.sp. radicis lycopersici* (FORL) inocula were carried out by use of a syringe, 7.5ml of the spore suspension was administered per pot. Inoculation was carried out a week after germination of seedlings for all the treatments i.e., T1, T2, T3, T4 and T5. Experimental units were irrigated with 500 ml of water when necessary to meet their moisture requirements. Weeding was carried out by hand whenever necessary.

5.3.2 Data collection

Seedling emergence assessment was carried out at a two-day interval, starting fifteen (15) days after planting until no new seedling emergence was recorded in any of the experimental units. Data collection on plant height, number of leaves and chlorophyll content (CCI) was carried out at one (1) week interval for seven (7) weeks, starting three weeks after full emergence i.e. one week after inoculation with *F. oxysporum*. Chlorophyll content readings were taken on an individually tagged single leaf by use of a CCM – 200 plus chlorophyll meter. Daily temperatures were recorded during the period of the study.

Plant height was measured by use of a measuring tape; the number of leaves was manually counted per seedling. Seedling mortality was recorded at one-week intervals. Occurrence and severity of disease development were assessed at the end of the experiment scoring for root rots and lesions.

Total fresh shoot and root biomass of the individual experimental units was determined using the Symmetry PR Precision scale at the end of the experiment. Total root and shoot dry biomass were assessed for the respective experimental units after 72 hours of oven drying at 60°C.

5.4 Mycorrhizae colonization analysis

Evaluation for the success of mycorrhizal fungi root colonization was carried out by microscopic examination of the Trypan blue stained roots. Staining was carried out to detect the presence of special structures such as arbuscules and vesicles (Dodd *et al.*, 2000; Vierheilig *et al.*, 2005).

5.5 Statistical analysis

Data of the assessed growth parameters *i.e.*, seedling emergence, seedling height, number of leaves, chlorophyll content and total shoots and roots biomass was subjected to analysis of variances using GENSTAT software seventeenth edition (Copy right 2014, VSN International Ltd.).

5.6 RESULTS

Among all the parameters investigated *i.e.* seedling emergence, the number of leaves, seedling height, chlorophyll content and biomass, the effect of different treatments was only significant on seedling dry biomass.

5.6.1 Effect of treatments on seedling biomass.

Analysis of variance (ANOVA) showed that the effect of treatments was statistically significant on seedling dry biomass ($p < .005$) [Table 5.1].

Table 5. 1: ANOVA showing the effect of treatments on seedling dry biomass.

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr
Treatment	5	1.37831	0.27566	4.24	0.003
Residual	54	3.51271	0.06505		
Total	59	4.89102			

Table 5. 2: Treatment means comparison of seedling dry biomass

Treatment	Means
T0	0.1660 ^a
T5	0.3610 ^{ab}
T1	0.3810 ^{ab}
T3	0.4210 ^{ab}
T4	0.4660 ^{ab}
T2	0.6780 ^b

T2 - Seeds treated with *P. concavorugulosum*-2, challenged with *F. oxysporum f.sp. radicis lycopersici* (FORL) produced a statistically higher dry biomass than T0 - 0control. No statistically significant effect was noted on the following treatments; T5 – Treated seeds + mycorrhizae and biochar amended rhizosphere, T1 – Untreated seeds +unamended rhizosphere, T3 - Treated seeds + mycorrhizae amended rhizosphere, and T4 - Treated seed + biochar amended rhizosphere (Table 5.2).

The effect of treatments on seedling biomass is graphically presented in Fig 5.1.

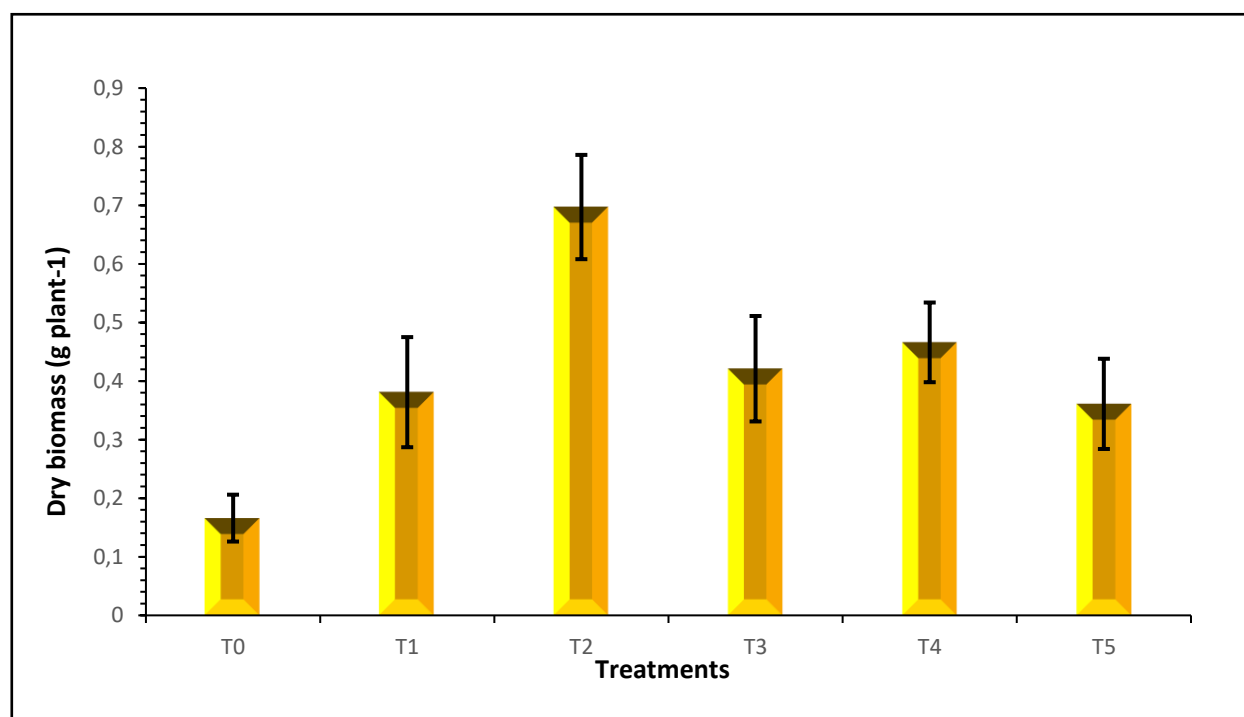


Figure 5.1: Effect of different soil rhizosphere conditions on *S. lycopersicum* seedlings dry biomass. T0 = Un-amended rhizosphere + untreated seeds, T1 = Un-amended rhizosphere + untreated seeds + *Fusarium* inoculation, T2 = Un-amended rhizosphere + *P. concavorugulosum*2 treated seeds, T3 = *P. concavorugulosum*2 treated seeds + mycorrhizae amended soil, T4 = *P. concavorugulosum*2 treated seeds + biochar amended soil, T5 = *P. concavorugulosum*2 treated seeds +Mycorrhizae + Biochar amended soil (T5).

5.6.2 *F. oxysporum f.sp. radicis lycopersici* (FORL) infection assessment

Evidence of crown/stem rot, lower leaf necrosis and root lesions/necrosis were noted on six (6) seedlings that were grown on T4 - Treated seeds + biochar amended rhizosphere seedlings that were inoculated with *F. oxysporum f.sp. radicis lycopersici* (FORL).

Infection percentage was calculated using the number of seedlings showing crown rots, lower leaf necrosis and symptoms of damping off.

$$\begin{aligned}\text{Infection percentage (\%)} &= \frac{\text{Number of infected seedlings}}{\text{Total number of seedlings}} \times 100 \\ &= \frac{6}{10} \times 100 \\ &= 60\%\end{aligned}$$

A total of 60% of the seedlings grown on T4 showed *F. oxysporum* infection symptoms where crown rots and lower leaf necrosis and discoloration were noted (Fig 5.2).





Figure 5. 2: Stem/crown rots, root rots, lesions and leaf necrosis *S. lycopersicum* seedlings.

Seedlings subjected to T0 = Un-amended rhizosphere + untreated seeds are shown in Fig 5.3 to highlight and compare symptom occurrence related to *F. oxysporum f.sp. radicis lycopersici* (FORL).



Figure 5. 3: Seedlings grown in the control treatment.

5.6.3 Mycorrhizal root colonization

Root samples from T3 and T5 which had mycorrhizal inoculation were evaluated for mycorrhizal colonization. Special features such as arbuscules and vesicles were noted on the root samples from both treatments. Figure 5.3 shows mycorrhizal colonization in *S. lycopersicum* seedling roots.

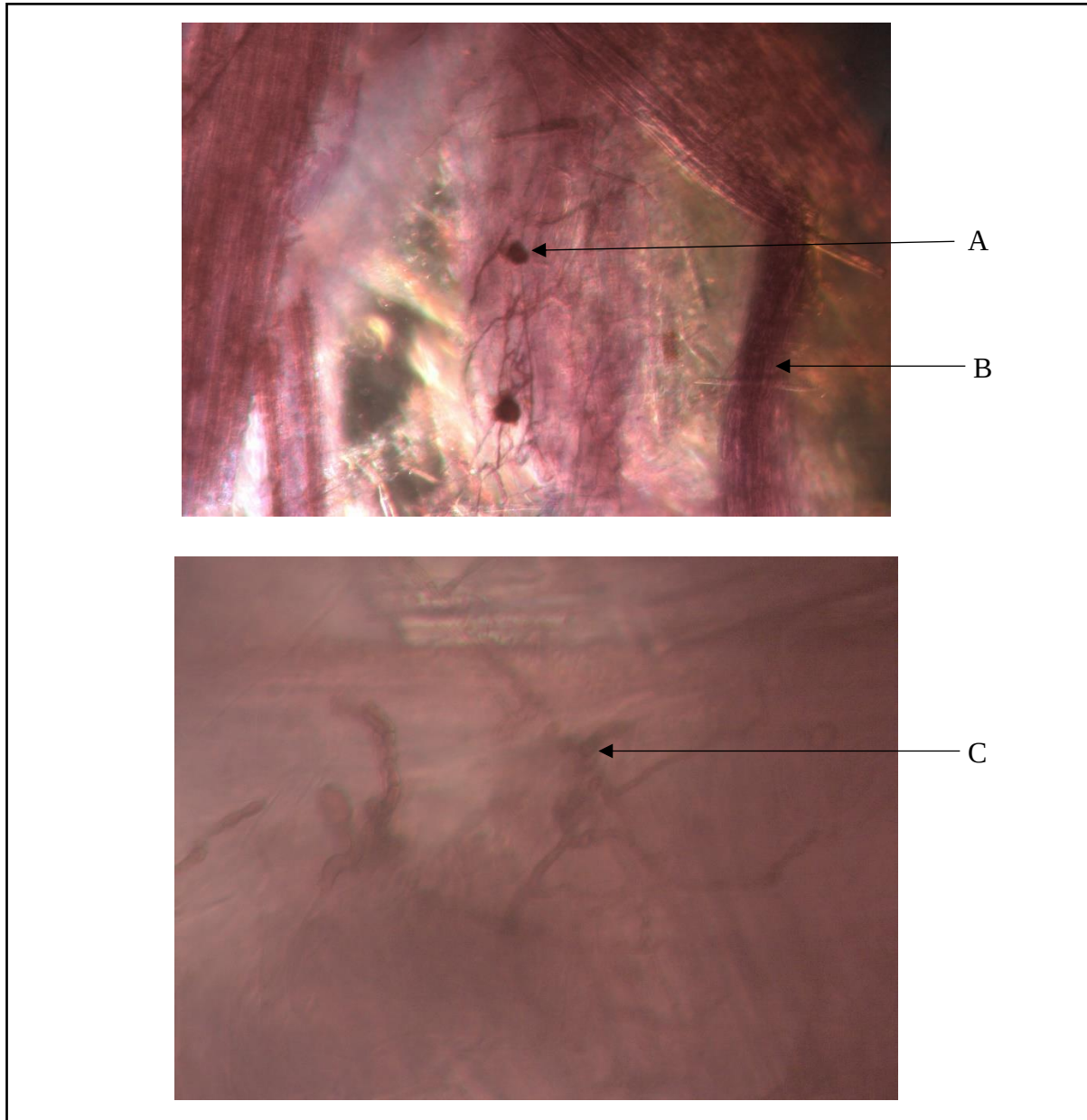


Figure 5. 4: Mycorrhizal colonization on T3 and T5 *S. lycopersicum* seedling roots. (A) Vesicles, (B) Root, (C) Arbuscules.

5.7 DISCUSSION

Seedlings that were inoculated with *P. concavorugulosum*-2 and challenged with *F. oxysporum* (T2) had significantly higher biomass than the control (T0). There was however no significant difference with other treatments (T1, T3, T4 and T5). This could be explained by the possible effect of biochar on fungal growth. Biochar as a carbon source can stimulate fungal growth and multiplication (Wiedner *et al.*, 2013). *F. oxysporum f.sp. radicis lycopersici* (FORL) could have therefore accessed and utilized nutrients faster than *P. concavorugulosum*-2, increasing its establishment which is evident in T4 (Fig 5.2) where *Fusarium* infection signs were noted. Treatments T4 and T5's lower biomass than T2 could have occurred due to the suppressive effect of carbon on the properties of *P. concavorugulosum*-2. High levels of carbon and excessive addition of carbon in the soil have been reported to, at times, have an inhibitory effect on the growth and colonization of *Penicillium* species (Costa and Nahas, 2012).

Other than biochar being contributory to the suppression of *P. concavorugulosum*-2, the *Penicillium* could also have experienced a suppression by the highly competitive growth of mycorrhizae. Mycorrhizae have been noted to have some competitive effect on certain fungal species when grown together at their earliest stages of seedling growth. Mycorrhizal fungi are reported to normally compete with the host or microorganisms around the host during the early stages of their establishment (Dar and Reshi, 2017). In this aspect, the only microorganism which was present was *P. concavorugulosum*-2 as a seed treatment. Therefore, mycorrhizae could have competed with *P. concavorugulosum*-2, thus the expected positive effect of *P. concavorugulosum*-2 could have been compromised. Ingham and Molina (1991); Wehner *et al.* (2010) and Dar and Reshi (2017) reported on the excessive competitiveness of arbuscular mycorrhizae during its establishment and colonization in an environment with competing microorganisms. Vesicular-Arbuscular mycorrhizal (VAM) fungal colonization on host plant roots is known to trigger and signal plant defense mechanisms. However, these defense responses have been reported to improve mycorrhizal colonization while straining plant growth and development (Gadkar *et al.*, 2001; Evelin *et al.*, 2009). This could also have possibly compromised early seedling growth parameters such as leaf number and shoot height.

Considering the suppressive effects on the early seedling establishment of both amendments i.e. high levels of carbon and mycorrhizal competitiveness, it is also therefore possible that the combination of biochar and mycorrhizae (T5) would equally have a cumulative effect on the suppression of *P. concavorugulosum*-2.

The unexpected results i.e. non-significant effect of treatments on the seedling growth parameters, could be because the effect of these treatments had not been realized in the early growth stages of seedlings growth. This would probably be the stage when the mycorrhizae effect would have exerted a negative effect on its host. Although there was successful mycorrhizal infection/colonization (Fig 5.3), unexpected mycorrhizal effects with depressed growth of early vegetative parameters were noted. This is reported in several mycorrhizae research studies. Growth is reportedly known to be eventually compensated at a later growth stage after colonization and establishment (Machineski *et al.*, 2018; Pinheiro *et al.*, 2019; de Sousa Cruz *et al.*, 2020). The significant positive effect on the biomass could be explained by skewed photosynthetic partition in favour of stem and branch thickness. This, however, needs to be investigated

Although there was evidence of a significant difference of the independently applied *P. concavorugulosum*-2 over the control, it could be indicative that *P. concavorugulosum*-2 is better acting on its own than in combination with other soil amendments. This, however, is inconclusive as there was no significant difference in other growth parameters, viz. the number of leaves, plant height and chlorophyll content. This could be due to the aspect of the delayed expression of mycorrhizal benefit as mycorrhizal symbiosis is influenced by both soil and environmental factors that either aid in the improvement or delay infection and plant growth (Jamiołkowska *et al.*, 2019). Since successful colonization was established (Fig 5.3), the aspect of lack of significant difference on chlorophyll content, plant height and number of leaves could have been due to early harvesting and early termination of the study. That is before mycorrhizae could physiologically express their effects.

P. concavorugulosum-2 showed stronger effects when inoculated on its own than in combination with biochar or mycorrhizae. The study however cannot conclusively recommend it as an appropriate biocontrol for *F. oxysporum f.sp. lycopersici* (FOL) or other soil-borne pathogens. There is therefore a need for further studies to investigate various levels of biochar and mycorrhizae inoculations. This proposed study should particularly investigate the long-term growth responses of various vegetables under different biochar and mycorrhizae treatments. Furthermore, *P. concavorugulosum*-2 should be subjected to *in-vivo* tests in unsterilized conditions to evaluate its response when in the presence of other microorganisms that would presumably minimize the suppressive effect of high carbon levels on the artificially inoculated agent i.e. *P. concavorugulosum*-2.

The remarkable performance by *P. concavorugulosum*-2 (T2) inoculated independently could be explained in two ways : (i) a possibility that *P. concavorugulosum*-2 eliminated *F. oxysporum* while promoting seedling growth, (ii) *P. concavorugulosum*-2 produced plant growth-promoting hormones as evidenced in Chapter 4 paragraph 1. This difference could have influenced the seedling stem growth as there was no significant difference on the other growth parameters other than seedling biomass. It seems that the effect of *P. concavorugulosum*-2 was more focused on the enhancement of stem thickness and branching, which could have influenced increasing the seedling biomass. This possibility could be explained from the results of studies by Chanclud and Morel (2016) who established that *Penicillium* species produced phytohormones such as cytokinin, auxins and gibberellic acids. These plant growth hormones would have been responsible for the promotion of seedling branches, stem growth and elongation (cell division). These parameters which were found to be non-significant would therefore need to be re-evaluated or assessed in *in-vivo* studies where long-term effects of the treatments should be investigated.

CHAPTER SIX

6.1 General discussion and conclusion

In the search for a bio-control agent for the management of *F. oxysporum f.sp. radicis lycopersici* (FORL) and other soil-borne pathogens that are well adapted to dry hot semi-arid regions, several experiments were carried out. This included isolation of fungal species from different soils in semi-arid regions and evaluated them for their suppressive effect against *F. oxysporum f.sp. radicis lycopersici* (FORL). These isolated fungal isolates were also evaluated for their phytopathological biosafety for commonly grown vegetables. These fungal species were initially identified by the use of visual and microscopical examination of morphological characteristics. Morphological identification resulted in the identification of all fungal species to the genus level. These were later identified by DNA molecular technology. The molecular identification procedure enabled the achievement of more accurate identification of isolate *A. fumigatus* which had initially been morphologically misidentified as *Penicillium* species.

Through molecular identification technology, the test fungi were therefore conclusively identified to be the following species: *P. commune-1*, *P. commune-2*, *A. fumigatus*, *P. concavorugulosum-1* and *P. concavorugulosum-2*.

The identified fungal isolates were evaluated for suppressive properties against *F. oxysporum f.sp. radicis lycopersici* (FORL). The study revealed a diversity of inherent biological properties between the different isolates. *P. concavorugulosum-2* exhibited a multiplicity of superior suppressive properties that included antibiosis, growth competitiveness and mycoparasitism. The second most suppressive isolate was *A. fumigatus* with similar properties. The other fungal isolates such as *P. commune-1*, *P. commune-2*, *P. concavorugulosum-1* did not exhibit worthwhile antagonism against *F. oxysporum f.sp. radicis lycopersici* (FORL). *F. oxysporum* appeared to outgrow them.

In addition to evaluating their efficacy against *F. oxysporum f.sp. radicis lycopersici* (FORL), these test fungal isolates were examined for their phytopathological biosafety on selected commonly grown vegetables namely: tomatoes (*S. lycopersicum*), spinach (*S. oleracea*) and beetroot (*B. vulgaris*). These fungal isolates exhibited a diversity of effects that included various degrees of a stimulatory effect on the germination, growth of radicle and plumule of all test vegetables. Out of all the test fungal isolates, four species i.e. *A. fumigatus*, *P. commune-1*, *P. commune-2* and *P. concavorugulosum-2* showed interesting growth response to all the test vegetables except for *B vulgaris*.

The overall conclusion of the study is that although *P. commune-1* and *P. commune-2* showed less suppressiveness against *F. oxysporum*, they had a stimulatory effect on *S. oleracea* and *S. lycopersicum* germination, radicle and plumule growth. Therefore, they should be further investigated for their possible contributory function to improve seedling vigour. They could particularly be investigated for their possible synergistic effect on seedling growth enhancement when used in combination with *P. concavorugulosum-2*. The negative effect of these test fungal isolates on the *B. vulgaris* needs to be investigated.

As *P. concavorugulosum-2* exhibited the most biocontrol properties against *F. oxysporum f.sp. radicis lycopersici* (FORL), it was further subjected to *in-vivo* evaluation under varying tomato (*S. lycopersicum*) seedling rhizospheres conditions. The varying rhizosphere conditions included those generated by inoculation with VAM mycorrhizae, and biochar amended conditions. This study generated interesting results as evidenced by the statistically significant response in biomass accumulation for seedlings growing in un-amended conditions ($P < 0.05$). The study, however, revealed the limitation of using sterilized soil conditions. Sterilized soil conditions did not reflect a realistic situation where the biocontrol inocula would compete and interact with a diverse soil microbial population. Furthermore, the short period of the study did not give an indication of the persistence of the introduced inocula and this short period did not also allow assessment for the long term benefits of mycorrhizae particularly for the species that are known to be late colonizers. There is, therefore, need for a long-term study particularly investigating whether there is variation in the effect of these biocontrol measures between the different forms i.e. FORL and FOL. It is also recommended that the effect of these measures on the endophytic *F. oxysporum* should be established.

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APPENDICES

APPENDIX A: BLAST CONSENSUS RESULTS FOR ALL ISOLATED SPECIES (CHAPTER TWO)

BLAST consensus for *Penicillium spp1* results showed that the following species produces alignments that are significant to the species.

RID: STGXS6RS015

Database: nt_v5

Query= *Penicillium spp1* Consensus

		Score E	
Sequences producing significant alignments:		(Bits)	Value
gi 1595603195 gb MK660351.1	<i>Penicillium commune</i> strain QLM2 ...	1026	0E00
gi 1595603185 gb MK660341.1	<i>Penicillium commune</i> strain P6 sm...	1026	0E00
gi 1595603179 gb MK660335.1	<i>Penicillium commune</i> strain M170 ...	1026	0E00
gi 1595603178 gb MK660334.1	<i>Penicillium commune</i> strain M145 ...	1026	0E00
gi 1678639092 ref NR_163685.1	<i>Penicillium caseifulvum</i> CBS 10...	1024	0E00
gi 1678639076 ref NR_163669.1	<i>Penicillium fuscoglaucum</i> CBS 2...	1024	0E00
gi 1531397796 gb MK268135.1	<i>Penicillium commune</i> isolate R972...	1024	0E00
gi 1531397779 gb MK268118.1	<i>Penicillium commune</i> isolate R971...	1024	0E00
gi 1531397170 gb MK267509.1	<i>Penicillium commune</i> isolate E204...	1024	0E00
gi 1531397169 gb MK267508.1	<i>Penicillium commune</i> isolate E204...	1024	0E00
gi 1531397121 gb MK267460.1	<i>Penicillium commune</i> isolate E204...	1024	0E00
gi 1531397089 gb MK267428.1	<i>Penicillium commune</i> isolate E203...	1024	0E00
gi 1531397088 gb MK267427.1	<i>Penicillium commune</i> isolate E203...	1024	0E00
gi 1531397087 gb MK267426.1	<i>Penicillium commune</i> isolate E203...	1024	0E00
gi 1531397086 gb MK267425.1	<i>Penicillium commune</i> isolate E203...	1024	0E00
gi 1531397083 gb MK267422.1	<i>Penicillium commune</i> isolate E203...	1024	0E00
gi 1523715712 gb MK226538.1	<i>Penicillium palitans</i> isolate CBS...	1024	0E00
gi 1473249778 gb MH862722.1	<i>Penicillium caseifulvum</i> strain C...	1024	0E00
gi 1473245902 gb MH858846.1	<i>Penicillium verrucosum</i> strain CB...	1024	0E00
gi 1473243408 gb MH856352.1	<i>Penicillium commune</i> strain CBS 2...	1024	0E00
gi 1473242118 gb MH855062.1	<i>Penicillium fuscoglaucum</i> strain ...	1024	0E00
gi 1465463445 gb MG733678.1	<i>Penicillium oxalicum</i> strain APBS...	1024	0E00
gi 1450319269 gb MF803944.1	<i>Penicillium palitans</i> isolate DTO...	1024	0E00
gi 1149033129 gb KY643771.1	<i>Penicillium commune</i> strain LCZ5 ...	1024	0E00
gi 1040718938 dbj LC145288.1	<i>Penicillium</i> sp. T7508-1-2 genes...	1024	0E00
gi 996130059 gb KR233455.1	<i>Penicillium oxalicum</i> strain PO15 ...	1024	0E00
gi 731445980 gb KP132490.1	<i>Penicillium commune</i> strain IHEM 1...	1024	0E00
gi 443429719 gb KC009817.1	<i>Penicillium commune</i> strain H09-10...	1024	0E00
gi 443429716 gb KC009814.1	<i>Penicillium commune</i> strain H09-09...	1024	0E00
gi 443429704 gb KC009802.1	<i>Penicillium commune</i> strain H09-08...	1024	0E00
gi 401878799 gb JX171184.1	<i>Penicillium commune</i> isolate LKF10...	1024	0E00
gi 259018338 gb GQ458026.1	<i>Penicillium commune</i> strain MA09-A...	1024	0E00
gi 187884378 gb EU664481.1	<i>Penicillium camemberti</i> strain 095...	1024	0E00
gi 187884358 gb EU664463.1	<i>Penicillium camemberti</i> strain 082...	1024	0E00
gi 187884357 gb EU664462.1	<i>Penicillium camemberti</i> strain 095...	1024	0E00
gi 3925707 emb AJ004813.1	<i>Penicillium commune</i> DNA for 5.8S r...	1024	0E00
gi 1584734763 gb MK580820.1	<i>Penicillium commune</i> isolate gel ...	1022	0E00
gi 1561008385 gb MK424124.1	<i>Penicillium</i> sp. isolate Cr_FuPnl...	1022	0E00
gi 1046760650 gb KU561925.1	<i>Penicillium commune</i> strain aT3 1...	1022	0E00
gi 34809385 gb AY373905.1	<i>Penicillium commune</i> strain ATCC 10...	1022	0E00
gi 597900478 ref NR_111143.1	<i>Penicillium commune</i> CBS 311.48 ...	1022	0E00
gi 1735093903 gb MN413150.1	<i>Penicillium cavernicola</i> strain C...	1019	0E00
gi 1678639091 ref NR_163684.1	<i>Penicillium cavernicola</i> CBS 10...	1019	0E00
gi 1473249765 gb MH862709.1	<i>Penicillium cavernicola</i> strain C...	1019	0E00

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gi|1473245998|gb|MH858942.1| Penicillium camemberti strain CB... 1019 0E00
gi|1473243411|gb|MH856355.1| Penicillium camemberti strain CB... 1019 0E00
gi|1473242121|gb|MH855065.1| Penicillium commune strain CBS 2... 1019 0E00
gi|1473241639|gb|MH854583.1| Penicillium camemberti strain CB... 1019 0E00
gi|1450319270|gb|MF803945.1| Penicillium palitans isolate DTO... 1019 0E00
gi|1386784040|gb|MG490880.1| Penicillium cf. discolor strain ... 1019 0E00

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ALIGNMENTS

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>gb|MK660351.1| Penicillium commune strain QLM2 small subunit ribosomal RNA
gene, partial
sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and
internal transcribed spacer
2, complete sequence; and large subunit ribosomal RNA gene, partial sequence
Length=589
Score = 1025.6 bits (1136), Expect = 0E00
Identities = 568/568 (100%), Gaps = 0/568 (0%)
Strand = Plus/Minus

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Query 1      TTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCAACCTGGATAAAAAATTTGGGTTGA 60
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Sbjct 568    TTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCAACCTGGATAAAAAATTTGGGTTGA 509

Query 61     TCGGCAAGCGCCGGCCGGGCCTACAGAGCGGGTGACAAAGCCCCATACGCTCGAGGACCG 120
             |||
Sbjct 508    TCGGCAAGCGCCGGCCGGGCCTACAGAGCGGGTGACAAAGCCCCATACGCTCGAGGACCG 449

Query 121    GACGCGGTGCCGCCGCTGCCTTTTCGGGCCCCGTCCCCCGGAGATCGGAGGACGGGGCCCAA 180
             |||
Sbjct 448    GACGCGGTGCCGCCGCTGCCTTTTCGGGCCCCGTCCCCCGGAGATCGGAGGACGGGGCCCAA 389

Query 181    CACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCCCGGAATACC 240
             |||
Sbjct 388    CACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCCCGGAATACC 329

Query 241    AGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTTGCAATTCACATTACG 300
             |||
Sbjct 328    AGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTTGCAATTCACATTACG 269

Query 301    TATCGCATTTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCGTTGTTGAAAGTT 360
             |||
Sbjct 268    TATCGCATTTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCGTTGTTGAAAGTT 209

Query 361    TTAAATAATTTATATTTTCACTCAGACTTCAATCTTCAGACAGAGTTCGAGGGTGTCTTC 420
             |||
Sbjct 208    TTAAATAATTTATATTTTCACTCAGACTTCAATCTTCAGACAGAGTTCGAGGGTGTCTTC 149

Query 421    GGCGGGCGCGGGCCCGGGGGCGTGAGCCCCCGGCGGCCAGTTAAGGCGGGCCCGCCGAA 480
             |||
Sbjct 148    GGCGGGCGCGGGCCCGGGGGCGTGAGCCCCCGGCGGCCAGTTAAGGCGGGCCCGCCGAA 89

Query 481    GCAACAAGGTAAAATAAACACGGGTGGGAGGTTGGACCCAGAGGGCCCTCACTCGGTAAT 540
             |||
Sbjct 88     GCAACAAGGTAAAATAAACACGGGTGGGAGGTTGGACCCAGAGGGCCCTCACTCGGTAAT 29

Query 541    GATCCTTCCGCAGGTTACCTACGGAAG 568
             |||
Sbjct 28     GATCCTTCCGCAGGTTACCTACGGAAG 1

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BLAST consensus for *Penicillium spp2* results showed that the following species produces alignments that are significant to the species.

RID: STGZW2FT015

Database: nt_v5

Query= *Penicillium spp2* Consensus

			Score	E
Sequences producing significant alignments:			(Bits)	Value
gi 1595603179 gb MK660335.1	Penicillium commune strain M170 ...	1059	0E00	
gi 1595603185 gb MK660341.1	Penicillium commune strain P6 sm...	1054	0E00	
gi 1595603178 gb MK660334.1	Penicillium commune strain M145 ...	1054	0E00	
gi 1678639076 ref NR_163669.1	Penicillium fuscoglaucum CBS 2...	1054	0E00	
gi 1531397796 gb MK268135.1	Penicillium commune isolate R972...	1054	0E00	
gi 1531397779 gb MK268118.1	Penicillium commune isolate R971...	1054	0E00	
gi 1531397170 gb MK267509.1	Penicillium commune isolate E204...	1054	0E00	
gi 1531397169 gb MK267508.1	Penicillium commune isolate E204...	1054	0E00	
gi 1531397121 gb MK267460.1	Penicillium commune isolate E204...	1054	0E00	
gi 1531397089 gb MK267428.1	Penicillium commune isolate E203...	1054	0E00	
gi 1531397088 gb MK267427.1	Penicillium commune isolate E203...	1054	0E00	
gi 1531397087 gb MK267426.1	Penicillium commune isolate E203...	1054	0E00	
gi 1531397086 gb MK267425.1	Penicillium commune isolate E203...	1054	0E00	
gi 1531397083 gb MK267422.1	Penicillium commune isolate E203...	1054	0E00	
gi 1523715712 gb MK226538.1	Penicillium palitans isolate CBS...	1054	0E00	
gi 1473242118 gb MH855062.1	Penicillium fuscoglaucum strain ...	1054	0E00	
gi 1465463445 gb MG733678.1	Penicillium oxalicum strain APBS...	1054	0E00	
gi 1450319269 gb MF803944.1	Penicillium palitans isolate DTO...	1054	0E00	
gi 996130059 gb KR233455.1	Penicillium oxalicum strain PO15 ...	1054	0E00	
gi 443429719 gb KC009817.1	Penicillium commune strain H09-10...	1054	0E00	
gi 443429716 gb KC009814.1	Penicillium commune strain H09-09...	1054	0E00	
gi 259018338 gb GQ458026.1	Penicillium commune strain MA09-A...	1054	0E00	
gi 187884378 gb EU664481.1	Penicillium camemberti strain 095...	1054	0E00	
gi 3925707 emb AJ004813.1	Penicillium commune DNA for 5.8S r...	1054	0E00	
gi 1584734763 gb MK580820.1	Penicillium commune isolate gel ...	1052	0E00	
gi 1561008385 gb MK424124.1	Penicillium sp. isolate Cr_FuPnl...	1052	0E00	
gi 34809385 gb AY373905.1	Penicillium commune strain ATCC 10...	1052	0E00	
gi 597900478 ref NR_111143.1	Penicillium commune CBS 311.48 ...	1052	0E00	
gi 1735093903 gb MN413150.1	Penicillium cavernicola strain C...	1049	0E00	
gi 1450319270 gb MF803945.1	Penicillium palitans isolate DTO...	1049	0E00	
gi 1386784040 gb MG490880.1	Penicillium cf. discolor strain ...	1049	0E00	
gi 1386784037 gb MG490877.1	Penicillium cf. discolor strain ...	1049	0E00	
gi 1386784029 gb MG490869.1	Penicillium cf. discolor strain ...	1049	0E00	
gi 1386784028 gb MG490868.1	Penicillium cf. discolor strain ...	1049	0E00	
gi 1386784027 gb MG490867.1	Penicillium cf. discolor strain ...	1049	0E00	
gi 1386784026 gb MG490866.1	Penicillium cf. discolor strain ...	1049	0E00	
gi 1334684241 gb KY469068.1	Penicillium camemberti strain KA...	1049	0E00	
gi 1334684240 gb KY469067.1	Penicillium camemberti strain KA...	1049	0E00	
gi 1334684239 gb KY469066.1	Penicillium camemberti strain KA...	1049	0E00	
gi 1334684238 gb KY469065.1	Penicillium camemberti strain KA...	1049	0E00	
gi 1334684237 gb KY469064.1	Penicillium camemberti strain KA...	1049	0E00	
gi 1334684236 gb KY469063.1	Penicillium camemberti strain KA...	1049	0E00	
gi 1334684235 gb KY469062.1	Penicillium camemberti strain KA...	1049	0E00	
gi 699340823 gb KM232463.1	Penicillium sp. 5H1-P0-P5-3 strai...	1049	0E00	
gi 530692585 gb KF212277.1	Fungal sp. APA-2013 clone LJ133Ms...	1049	0E00	
gi 443429721 gb KC009819.1	Penicillium commune strain H09-10...	1049	0E00	
gi 373938716 gb JN642222.1	Penicillium solitum strain 20-01 ...	1049	0E00	
gi 262118032 dbj AB479314.1	Penicillium camemberti genes for...	1049	0E00	
gi 259018351 gb GQ458039.1	Penicillium camemberti strain ATC...	1049	0E00	
gi 259018332 gb GQ458020.1	Penicillium camemberti strain MA0...	1049	0E00	

ALIGNMENTS

>gb|MK660335.1| Penicillium commune strain M170 small subunit ribosomal RNA gene, partial
sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer
2, complete sequence; and large subunit ribosomal RNA gene, partial sequence
Length=589
Score = 1059.0 bits (1173), Expect = 0E00
Identities = 588/589 (99%), Gaps = 0/589 (0%)
Strand = Plus/Minus

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Query 1   TCCTCCCGCTTATTGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCAAC 60
          |||||
Sbjct 589 TCCTCCCGCTTATTGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCAAC 530

Query 61   CTGGATAAAAATTTGGGTTGATCGGCAAGCGCCGGCCGGGCTACAGAGCGGGTGACAAA 120
          |||||
Sbjct 529 CTGGATAAAAATTTGGGTTGATCGGCAAGCGCCGGCCGGGCTACAGAGCGGGTGACAAA 470

Query 121  GCCCCATACGCTCGAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGGGGCCCGTCCCCCGG 180
          |||||
Sbjct 469 GCCCCATACGCTCGAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGGGGCCCGTCCCCCGG 410

Query 181  AGATCGGAGGACGGGGCCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGAC 240
          |||||
Sbjct 409 AGATCGGAGGACGGGGCCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGAC 350

Query 241  AGGCATGCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACT 300
          |||||
Sbjct 349 AGGCATGCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACT 290

Query 301  GAATTTGCAATTCACATTACGTATCGCATTTTCGCTGCGTTCTTCATCGATGCCGGAACCA 360
          |||||
Sbjct 289 GAATTTGCAATTCACATTACGTATCGCATTTTCGCTGCGTTCTTCATCGATGCCGGAACCA 230

Query 361  AGAGATCCGTTGTTGAAAGTTTTAAATAATTTATATTTTCACTCAGACTTCAATCTTCAG 420
          |||||
Sbjct 229 AGAGATCCGTTGTTGAAAGTTTTAAATAATTTATATTTTCACTCAGACTTCAATCTTCAG 170

Query 421  ACAGAGTTCGAGGGTGTCTTCGGCGGGCGCGGGCCCGGGGCGTGAGCCCCCGGCGGCC 480
          |||||
Sbjct 169 ACAGAGTTCGAGGGTGTCTTCGGCGGGCGCGGGCCCGGGGCGTGAGCCCCCGGCGGCC 110

Query 481  AGTTAAGGCGGGCCCGCCGAAGCAACAAGGTAAAATAAACACGGGTGGGAGGTTGGACCC 540
          |||||
Sbjct 109 AGTTAAGGCGGGCCCGCCGAAGCAACAAGGTAAAATAAACACGGGTGGGAGGTTGGACCC 50

Query 541  AGAGGGCCCTCACTCGGTAATGATCCTTCCGCAGGTTACCTACGGAAG 589
          |||||
Sbjct 49   AGAGGGCCCTCACTCGGTAATGATCCTTCCGCAGGTTACCTACGGAAG 1

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BLAST consensus for *Penicillium spp3* results showed that the following species produces alignments that are significant to the species.

RID: SW337SHV014

Database: nt_v5

Query= *Penicillium spp3* Consensus

					Score	E
Sequences producing significant alignments:					(Bits)	Value
gi 1704721198 gb MN178806.1	Aspergillus	fumigatus	isolate TB...	807	0E00	
gi 1704721198 gb MN178806.1	Aspergillus	fumigatus	isolate TB...	64	6E-06	
gi 1677556527 gb MN006422.1	Aspergillus	fumigatus	isolate AB...	807	0E00	
gi 1677556527 gb MN006422.1	Aspergillus	fumigatus	isolate AB...	139	3E-28	
gi 1655483383 gb MK952424.1	Aspergillus	fumigatus	strain AfB...	807	0E00	
gi 1655483383 gb MK952424.1	Aspergillus	fumigatus	strain AfB...	84	6E-12	
gi 1653966441 gb MK054270.1	Aspergillus	sp. isolate Z2 inter...	807	0E00		
gi 1653966441 gb MK054270.1	Aspergillus	sp. isolate Z2 inter...	85	6E-12		
gi 1624800035 gb MK841450.1	Aspergillus	fumigatus	voucher NW...	807	0E00	
gi 1624800035 gb MK841450.1	Aspergillus	fumigatus	voucher NW...	75	3E-09	
gi 1624800021 gb MK841436.1	Aspergillus	fumigatus	voucher NW...	807	0E00	
gi 1624800021 gb MK841436.1	Aspergillus	fumigatus	voucher NW...	71	1E-07	
gi 1624800018 gb MK841433.1	Aspergillus	fumigatus	voucher NW...	807	0E00	
gi 1624800018 gb MK841433.1	Aspergillus	fumigatus	voucher NW...	66	2E-06	
gi 1624800016 gb MK841431.1	Aspergillus	fumigatus	voucher NW...	807	0E00	
gi 1624800016 gb MK841431.1	Aspergillus	fumigatus	voucher NW...	75	3E-09	
gi 1622715532 gb MK817529.1	Aspergillus	fumigatus	strain 355...	807	0E00	
gi 1622715532 gb MK817529.1	Aspergillus	fumigatus	strain 355...	77	9E-10	
gi 1607212468 gb MK751709.1	Aspergillus	fumigatus	strain M4 ...	807	0E00	
gi 1605372086 gb MH892837.1	Aspergillus	fumigatus	isolate S1...	807	0E00	
gi 1605372086 gb MH892837.1	Aspergillus	fumigatus	isolate S1...	164	8E-36	
gi 1591436826 gb MK630344.1	Aspergillus	fumigatus	strain ZC-...	807	0E00	
gi 1591436826 gb MK630344.1	Aspergillus	fumigatus	strain ZC-...	84	6E-12	
gi 1590834702 gb MK623263.1	Aspergillus	fumigatus	isolate C1...	807	0E00	
gi 1590834702 gb MK623263.1	Aspergillus	fumigatus	isolate C1...	157	1E-33	
gi 1531381882 gb MK267099.1	Aspergillus	fumigatus	isolate RS...	807	0E00	
gi 1531381882 gb MK267099.1	Aspergillus	fumigatus	isolate RS...	162	3E-35	
gi 1524758986 gb MK243451.1	Aspergillus	fumigatus	isolate GL...	807	0E00	
gi 1524758986 gb MK243451.1	Aspergillus	fumigatus	isolate GL...	71	1E-07	
gi 1524758966 gb MK243397.1	Aspergillus	fumigatus	isolate GL...	807	0E00	
gi 1524758966 gb MK243397.1	Aspergillus	fumigatus	isolate GL...	81	7E-11	
gi 1476494148 gb MH911420.1	Aspergillus	fumigatus	strain MF2...	807	0E00	
gi 1476494148 gb MH911420.1	Aspergillus	fumigatus	strain MF2...	158	3E-34	
gi 1476494144 gb MH911416.1	Aspergillus	fumigatus	strain MF2...	807	0E00	
gi 1476494144 gb MH911416.1	Aspergillus	fumigatus	strain MF2...	162	3E-35	
gi 1476494125 gb MH911397.1	Aspergillus	fumigatus	strain MF2...	807	0E00	
gi 1476494125 gb MH911397.1	Aspergillus	fumigatus	strain MF2...	164	8E-36	
gi 1476494112 gb MH911384.1	Aspergillus	fumigatus	strain MF2...	807	0E00	
gi 1476494112 gb MH911384.1	Aspergillus	fumigatus	strain MF2...	146	2E-30	
gi 1573068568 gb MH484011.1	Aspergillus	fumigatus	isolate BN...	807	0E00	
gi 1573068568 gb MH484011.1	Aspergillus	fumigatus	isolate BN...	69	5E-07	
gi 1564308349 gb MK461083.1	Aspergillus	fumigatus	strain IR3...	807	0E00	
gi 1564308349 gb MK461083.1	Aspergillus	fumigatus	strain IR3...	124	7E-24	
gi 1564307561 gb MK460927.1	Aspergillus	fumigatus	strain CR3...	807	0E00	
gi 1564307528 gb MK460917.1	Aspergillus	fumigatus	strain CR2...	807	0E00	
gi 1564307528 gb MK460917.1	Aspergillus	fumigatus	strain CR2...	132	5E-26	
gi 1562063245 gb MK439477.1	Aspergillus	sp. isolate CK392 sm...	807	0E00		
gi 1562063245 gb MK439477.1	Aspergillus	sp. isolate CK392 sm...	162	3E-35		
gi 1546451089 gb MK351862.1	Aspergillus	fumigatus	small subu...	807	0E00	
gi 1546451089 gb MK351862.1	Aspergillus	fumigatus	small subu...	156	1E-33	
gi 1444363069 gb MH725579.1	Aspergillus	fumigatus	isolate HN...	807	0E00	
gi 1444363069 gb MH725579.1	Aspergillus	fumigatus	isolate HN...	82	2E-11	

gi 1444356936 gb MH725577.1	Aspergillus fumigatus isolate HN...	807	0E00
gi 1444356936 gb MH725577.1	Aspergillus fumigatus isolate HN...	79	3E-10
gi 1444345339 gb MH725575.1	Aspergillus fumigatus isolate HN...	807	0E00
gi 1444345339 gb MH725575.1	Aspergillus fumigatus isolate HN...	82	7E-11
gi 1442330471 gb MG462851.1	Uncultured Aspergillus clone GM5...	807	0E00
gi 1442330471 gb MG462851.1	Uncultured Aspergillus clone GM5...	84	6E-12
gi 1406194617 gb MH497165.1	Fungal sp. isolate 14A2 internal...	807	0E00
gi 1406194617 gb MH497165.1	Fungal sp. isolate 14A2 internal...	69	5E-07
gi 1389448002 gb MH345956.1	Aspergillus fumigatus isolate 11...	807	0E00
gi 1389448002 gb MH345956.1	Aspergillus fumigatus isolate 11...	72	4E-08
gi 1389447917 gb MH345871.1	Aspergillus fumigatus isolate 31...	807	0E00
gi 1389447917 gb MH345871.1	Aspergillus fumigatus isolate 31...	67	2E-06
gi 1389447910 gb MH345864.1	Aspergillus fumigatus isolate 24...	807	0E00
gi 1389447910 gb MH345864.1	Aspergillus fumigatus isolate 24...	71	1E-07
gi 1389447906 gb MH345860.1	Aspergillus fumigatus isolate 20...	807	0E00
gi 1389447906 gb MH345860.1	Aspergillus fumigatus isolate 20...	66	2E-06
gi 1389447903 gb MH345857.1	Aspergillus fumigatus isolate 17...	807	0E00
gi 1389447903 gb MH345857.1	Aspergillus fumigatus isolate 17...	79	3E-10
gi 1389447902 gb MH345856.1	Aspergillus fumigatus isolate 16...	807	0E00
gi 1389447902 gb MH345856.1	Aspergillus fumigatus isolate 16...	71	1E-07
gi 1389447901 gb MH345855.1	Aspergillus fumigatus isolate 15...	807	0E00
gi 1389447901 gb MH345855.1	Aspergillus fumigatus isolate 15...	77	9E-10
gi 1389447896 gb MH345850.1	Aspergillus fumigatus isolate 10...	807	0E00
gi 1389447896 gb MH345850.1	Aspergillus fumigatus isolate 10...	75	3E-09
gi 1389447894 gb MH345848.1	Aspergillus fumigatus isolate 8 ...	807	0E00
gi 1389447894 gb MH345848.1	Aspergillus fumigatus isolate 8 ...	71	1E-07
gi 1389387579 gb MF374838.1	Aspergillus fumigatus isolate AS...	807	0E00
gi 1384787902 gb MH270614.1	Aspergillus fumigatus strain ND1...	807	0E00
gi 1384787902 gb MH270614.1	Aspergillus fumigatus strain ND1...	79	3E-10
gi 1384787896 gb MH270608.1	Aspergillus fumigatus strain ND1...	807	0E00
gi 1384787896 gb MH270608.1	Aspergillus fumigatus strain ND1...	77	9E-10
gi 1384787890 gb MH270602.1	Aspergillus fumigatus strain NR7...	807	0E00
gi 1384787890 gb MH270602.1	Aspergillus fumigatus strain NR7...	67	2E-06
gi 1384787878 gb MH270590.1	Aspergillus fumigatus strain NR6...	807	0E00
gi 1384787878 gb MH270590.1	Aspergillus fumigatus strain NR6...	85	6E-12
gi 1384787860 gb MH270572.1	Aspergillus fumigatus strain NR4...	807	0E00
gi 1384787857 gb MH270569.1	Aspergillus fumigatus strain NR4...	807	0E00
gi 1384787857 gb MH270569.1	Aspergillus fumigatus strain NR4...	78	9E-10
gi 1384787843 gb MH270555.1	Aspergillus fumigatus strain NR2...	807	0E00
gi 1384787843 gb MH270555.1	Aspergillus fumigatus strain NR2...	60	2E-04
gi 1354562356 gb KY827337.1	Aspergillus fumigatus strain SCA...	807	0E00
gi 1354562356 gb KY827337.1	Aspergillus fumigatus strain SCA...	77	9E-10
gi 1349758592 gb MG991675.1	Aspergillus fumigatus strain CMX...	807	0E00
gi 1349758592 gb MG991675.1	Aspergillus fumigatus strain CMX...	164	8E-36

ALIGNMENTS

```
>gb|MN178806.1| Aspergillus fumigatus isolate TB1 internal transcribed
spacer 1, partial sequence;
5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence;
and large subunit
ribosomal RNA gene, partial sequence
Length=568
Score = 807.4 bits (894), Expect = 0E00
Identities = 447/447 (100%), Gaps = 0/447 (0%)
Strand = Plus/Minus
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Query 1   TTCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCAAC 60
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Sbjct 567 TTCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCAAC 508
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Query 61  CTTAGAAAAATAAAGTTGGGTGTCGGCTGGCGCCGGCCGGGCCTACAGAGCAGGTGACAA 120
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 507  CTTAGAAAAATAAAGTTGGGTGTCGGCTGGCGCCGGCCGGGCCTACAGAGCAGGTGACAA 448

Query 121 AGCCCCATACGCTCGAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCCGTCCCCCG 180
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 447  AGCCCCATACGCTCGAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCCGTCCCCCG 388

Query 181 GGAGAGGGGGACGGGGGCCCCAACACACAAGCCGTGCTTGAGGGCAGCAATGACGCTCGGA 240
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 387  GGAGAGGGGGACGGGGGCCCCAACACACAAGCCGTGCTTGAGGGCAGCAATGACGCTCGGA 328

Query 241 CAGGCATGCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCAC 300
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 327  CAGGCATGCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCAC 268

Query 301 TGAATTCTGCAATTCACATTACTTATCGCATTTTCGCTGCGTTCTTCATCGATGCCGGAAC 360
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 267  TGAATTCTGCAATTCACATTACTTATCGCATTTTCGCTGCGTTCTTCATCGATGCCGGAAC 208

Query 361 CAAGAGATCCGTTGTTGAAAGTTTTAACTGATTACGATAATCAACTCAGACTGCATACTT 420
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 207  CAAGAGATCCGTTGTTGAAAGTTTTAACTGATTACGATAATCAACTCAGACTGCATACTT 148

Query 421 TCAGAACAGCGTTCATGTTGGGGTCTT 447
          ||||||||||||||||||||||||||||
Sbjct 147  TCAGAACAGCGTTCATGTTGGGGTCTT 121

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BLAST consensus for *Penicillium spp4* results showed that the following species produces alignments that are significant to the species.

RID: T38PRS74015

Database: nt_v5

Query= *Penicillium spp4* Consensus

Sequences producing significant alignments:	Score	E
	(Bits)	Value
gi 1624800039 gb MK841454.1 <i>Penicillium concavorugulosum</i> vou...	992	0E00
gi 1547055358 gb MK359013.1 <i>Talaromyces</i> sp. isolate OUCMDZ-5...	992	0E00
gi 1547054995 gb MK358958.1 Fungal sp. isolate OUCMDZ-5185 s...	992	0E00
gi 1042744258 gb KU839281.1 Fungal sp. strain S212T internal...	992	0E00
gi 1042744257 gb KU839280.1 Fungal sp. strain S212S internal...	992	0E00
gi 1042744256 gb KU839279.1 Fungal sp. strain S212R internal...	992	0E00
gi 1042742701 gb KU837725.1 Fungal sp. strain P279A internal...	992	0E00
gi 1004525520 gb KU216713.1 <i>Talaromyces variabilis</i> strain XQ...	992	0E00
gi 725095821 gb KF984800.1 <i>Talaromyces sublevissporus</i> strain ...	992	0E00
gi 299767731 gb GQ999323.1 Uncultured fungus clone LX042768-...	992	0E00
gi 673921011 emb LN482471.1 <i>Talaromyces radicus</i> genomic DNA ...	992	0E00
gi 326579935 gb HM469413.1 <i>Talaromyces radicus</i> strain KUC167...	992	0E00
gi 171464364 gb EU262660.1 <i>Talaromyces radicus</i> strain MET090...	992	0E00
gi 725095818 gb KF984797.1 <i>Talaromyces variabilis</i> strain NRR...	988	0E00
gi 725095816 gb KF984795.1 <i>Talaromyces variabilis</i> strain DTO...	988	0E00
gi 299767729 gb GQ999321.1 Uncultured fungus clone LX042768-...	988	0E00
gi 299767727 gb GQ999319.1 Uncultured fungus clone LX042768-...	988	0E00
gi 511783057 gb KC466524.1 <i>Talaromyces variabilis</i> 18S riboso...	988	0E00
gi 311235642 gb HQ288049.1 <i>Penicillium variabile</i> strain NRRL...	986	0E00
gi 299767730 gb GQ999322.1 Uncultured fungus clone LX042768-...	983	0E00
gi 1594472747 gb MK646059.1 <i>Talaromyces radicus</i> isolate JJGG...	981	0E00
gi 321172822 gb HQ698594.1 <i>Penicillium variabile</i> strain NRRL...	980	0E00
gi 361052000 gb JN851019.1 <i>Talaromyces radicus</i> strain SCSGAF...	979	0E00
gi 1584169938 gb MK577438.1 <i>Penicillium</i> sp. isolate QM-M sma...	978	0E00

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gi|1628757350|gb|MH935987.1| Talaromyces sp. strain 6HVCE1 sm... 972 0E00
gi|1511001243|gb|MG957181.1| Talaromyces variabilis isolate 4... 971 0E00
gi|1005417929|gb|KT958549.1| Talaromyces radicus strain DspF... 971 0E00
gi|961554357|gb|KT264430.1| Talaromyces sp. isolate AM081-P10... 971 0E00
gi|498922946|gb|KC920846.1| Talaromyces radicus isolate CrP20... 971 0E00
gi|1526299447|gb|MH006605.1| Talaromyces variabilis isolate 2... 967 0E00
gi|1347308147|gb|MG976292.1| Talaromyces wortmannii isolate 4... 965 0E00
gi|1473252768|gb|MH865712.1| Talaromyces wortmannii strain CB... 965 0E00
gi|1473245354|gb|MH858298.1| Talaromyces wortmannii strain CB... 965 0E00
gi|1473243468|gb|MH856412.1| Talaromyces wortmannii strain CB... 965 0E00
gi|1042744486|gb|KU839509.1| Fungal sp. strain S252D internal... 965 0E00
gi|1042744460|gb|KU839483.1| Fungal sp. strain S248J internal... 965 0E00
gi|1042744442|gb|KU839465.1| Fungal sp. strain S246M internal... 965 0E00
gi|1042744441|gb|KU839464.1| Fungal sp. strain S246I internal... 965 0E00
gi|1042744440|gb|KU839463.1| Fungal sp. strain S246H internal... 965 0E00
gi|1042744439|gb|KU839462.1| Fungal sp. strain S246G internal... 965 0E00
gi|1042744250|gb|KU839273.1| Fungal sp. strain S212K internal... 965 0E00
gi|1042744118|gb|KU839141.1| Fungal sp. strain S195T internal... 965 0E00
gi|1042744112|gb|KU839135.1| Fungal sp. strain S194T internal... 965 0E00
gi|1042744111|gb|KU839134.1| Fungal sp. strain S194S internal... 965 0E00
gi|1042744109|gb|KU839132.1| Fungal sp. strain S194N internal... 965 0E00
gi|1042744104|gb|KU839127.1| Fungal sp. strain S194C internal... 965 0E00
gi|1042744103|gb|KU839126.1| Fungal sp. strain S194B internal... 965 0E00
gi|1042744102|gb|KU839125.1| Fungal sp. strain S194A internal... 965 0E00
gi|1042744052|gb|KU839075.1| Fungal sp. strain S179B internal... 965 0E00
gi|1127908509|gb|KY425712.1| Talaromyces variabilis strain El... 965 0E00

```

ALIGNMENTS

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>gb|MK841454.1| Penicillium concavorugulosum voucher NWUSeq42 internal
transcribed spacer 1,
partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer
2, complete sequence;
and large subunit ribosomal RNA gene, partial sequence
Length=570
Score = 992.2 bits (1099), Expect = 0E00
Identities = 550/551 (99%), Gaps = 0/551 (0%)
Strand = Plus/Minus

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Query 1 TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTAGCCCTACCTGATCCGAGGTCAACC 60
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Sbjct 569 TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTAGCCCTACCTGATCCGAGGTCAACC 510

Query 61 GGAAGAGGCGCACCCCGAAGGGCGGCCTAAAGGGAAGACCAGCGCCGACCGAGTCCCTCC 120
|
Sbjct 509 GGAAGAGGCGCACCCCGAAGGGCGGCCTAAAGGGAAGACCAGCGCCGACCGAGTCCCTCC 450

Query 121 CGAGCGGGTGACAAAGCCCCATACGCTCGAGGACCCGACGCGGCGCCGCGCCACTGCTTTTG 180
|
Sbjct 449 CGAGCGGGTGACAAAGCCCCATACGCTCGAGGACCCGACGCGGCGCCGCGCCACTGCTTTTG 390

Query 181 GGGCGTGTCCCCGGGGGGACAGCGCCCAACACCCAGCCGTGCTGGAGGGCAGAAATGACG 240
|
Sbjct 389 GGGCGTGTCCCCGGGGGGACAGCGCCCAACACCCAGCCGTGCTGGAGGGCAGAAATGACG 330

Query 241 CTCGGACAGGCATGCCCCCGGAATGCCAGGGGGCGCAATGTGCGTTCAAAGATTCGATG 300
|
Sbjct 329 CTCGGACAGGCATGCCCCCGGAATGCCAGGGGGCGCAATGTGCGTTCAAAGATTCGATG 270

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Query 301 ATTCACGGAATTCTGCAATTCACATTACTTATCGCATTTTCGCTGCGTTCTTCATCGATGC 360
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Sbjct 269 ATTCACGGAATTCTGCAATTCACATTACTTATCGCATTTTCGCTGCGTTCTTCATCGATGC 210
Query 361 CGGAACCAAGAGATCCGTTGTTGAAAGTTTTTAATGATTTCAAATCTCACTCAGACTCACT 420
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 209 CGGAACCAAGAGATCCGTTGTTGAAAGTTTTTAATGATTTCAAATCTCACTCAGACTCACT 150

Query 421 ATTCAGGCAGGGTTCTAGGGTTCGTCGGCGGGCGCGGGCCCGGGGGCAGAAGCCCCCGG 480
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 149 ATTCAGGCAGGGTTCTAGGGTTCGTCGGCGGGCGCGGGCCCGGGGGCAGAAGCCCCCGG 90

Query 481 CGACCGGGGCCAGGCSCCAGTGGGCCCCGCCGAGGCAACGCGGTAACGGTAAACACGGGTG 540
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 89 CGACCGGGGCCAGGCCCCAGTGGGCCCCGCCGAGGCAACGCGGTAACGGTAAACACGGGTG 30

Query 541 GGAGGTTGGGC 551
          |||||||||
Sbjct 29 GGAGGTTGGGC 19

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BLAST consensus for *Penicillium spp5* results showed that the following species produces alignments that are significant to the species.

RID: T38R4RF8015

Database: nt_v5

Query= *Penicillium spp5* Consensus

		Score	E
Sequences producing significant alignments:	(Bits)	Value	
gi 1624800039 gb MK841454.1 <i>Penicillium concavorugulosum</i> vou...	797	0E00	
gi 1624800039 gb MK841454.1 <i>Penicillium concavorugulosum</i> vou...	91	1E-13	
gi 1547055358 gb MK359013.1 <i>Talaromyces</i> sp. isolate OUCMDZ-5...	797	0E00	
gi 1547055358 gb MK359013.1 <i>Talaromyces</i> sp. isolate OUCMDZ-5...	159	3E-34	
gi 1547055358 gb MK359013.1 <i>Talaromyces</i> sp. isolate OUCMDZ-5...	797	0E00	
gi 1547055358 gb MK359013.1 <i>Talaromyces</i> sp. isolate OUCMDZ-5...	159	3E-34	
gi 1547054995 gb MK358958.1 Fungal sp. isolate OUCMDZ-5185 s...	797	0E00	
gi 1547054995 gb MK358958.1 Fungal sp. isolate OUCMDZ-5185 s...	149	2E-31	
gi 1547054995 gb MK358958.1 Fungal sp. isolate OUCMDZ-5185 s...	797	0E00	
gi 1547054995 gb MK358958.1 Fungal sp. isolate OUCMDZ-5185 s...	149	2E-31	
gi 1042744258 gb KU839281.1 Fungal sp. strain S212T internal...	797	0E00	
gi 1042744258 gb KU839281.1 Fungal sp. strain S212T internal...	115	4E-21	
gi 1042744257 gb KU839280.1 Fungal sp. strain S212S internal...	797	0E00	
gi 1042744257 gb KU839280.1 Fungal sp. strain S212S internal...	144	7E-30	
gi 1042744256 gb KU839279.1 Fungal sp. strain S212R internal...	797	0E00	
gi 1042744256 gb KU839279.1 Fungal sp. strain S212R internal...	119	3E-22	
gi 1042742701 gb KU837725.1 Fungal sp. strain P279A internal...	797	0E00	
gi 1042742701 gb KU837725.1 Fungal sp. strain P279A internal...	115	4E-21	
gi 1004525520 gb KU216713.1 <i>Talaromyces variabilis</i> strain XQ...	797	0E00	
gi 1004525520 gb KU216713.1 <i>Talaromyces variabilis</i> strain XQ...	159	3E-34	
gi 1004525520 gb KU216713.1 <i>Talaromyces variabilis</i> strain XQ...	77	9E-10	
gi 725095821 gb KF984800.1 <i>Talaromyces sublevissporus</i> strain ...	797	0E00	
gi 725095821 gb KF984800.1 <i>Talaromyces sublevissporus</i> strain ...	129	2E-25	
gi 725095818 gb KF984797.1 <i>Talaromyces variabilis</i> strain NRR...	797	0E00	
gi 725095818 gb KF984797.1 <i>Talaromyces variabilis</i> strain NRR...	129	2E-25	
gi 299767731 gb GQ999323.1 Uncultured fungus clone LX042768-...	797	0E00	
gi 299767731 gb GQ999323.1 Uncultured fungus clone LX042768-...	159	3E-34	
gi 299767729 gb GQ999321.1 Uncultured fungus clone LX042768-...	797	0E00	
gi 299767729 gb GQ999321.1 Uncultured fungus clone LX042768-...	155	4E-33	
gi 673921011 emb LN482471.1 <i>Talaromyces radicus</i> genomic DNA ...	797	0E00	
gi 673921011 emb LN482471.1 <i>Talaromyces radicus</i> genomic DNA ...	157	1E-33	
gi 361052000 gb JN851019.1 <i>Talaromyces radicus</i> strain SCSGAF...	797	0E00	

gi 361052000 gb JN851019.1	Talaromyces radicus strain SCSGAF...	69	5E-07
gi 326579935 gb HM469413.1	Talaromyces radicus strain KUC167...	797	0E00
gi 326579935 gb HM469413.1	Talaromyces radicus strain KUC167...	157	1E-33
gi 311235642 gb HQ288049.1	Penicillium variabile strain NRRL...	797	0E00
gi 311235642 gb HQ288049.1	Penicillium variabile strain NRRL...	92	4E-14
gi 171464364 gb EU262660.1	Talaromyces radicus strain MET090...	797	0E00
gi 171464364 gb EU262660.1	Talaromyces radicus strain MET090...	159	3E-34
gi 1594472747 gb MK646059.1	Talaromyces radicus isolate JJGG...	793	0E00
gi 1594472747 gb MK646059.1	Talaromyces radicus isolate JJGG...	144	7E-30
gi 725095816 gb KF984795.1	Talaromyces variabilis strain DTO...	792	0E00
gi 725095816 gb KF984795.1	Talaromyces variabilis strain DTO...	129	2E-25
gi 299767730 gb GQ999322.1	Uncultured fungus clone LX042768-...	792	0E00
gi 299767730 gb GQ999322.1	Uncultured fungus clone LX042768-...	159	3E-34
gi 299767727 gb GQ999319.1	Uncultured fungus clone LX042768-...	792	0E00
gi 299767727 gb GQ999319.1	Uncultured fungus clone LX042768-...	159	3E-34
gi 511783057 gb KC466524.1	Talaromyces variabilis 18S riboso...	792	0E00
gi 511783057 gb KC466524.1	Talaromyces variabilis 18S riboso...	153	1E-32
gi 321172822 gb HQ698594.1	Penicillium variabile strain NRRL...	790	0E00
gi 321172822 gb HQ698594.1	Penicillium variabile strain NRRL...	79	3E-10
gi 1628769687 gb MK240332.1	Talaromyces variabilis strain Fe...	789	0E00
gi 1628769687 gb MK240332.1	Talaromyces variabilis strain Fe...	59	2E-04
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gi 1584169938 gb MK577438.1	Penicillium sp. isolate QM-M sma...	147	6E-31
gi 1270026405 gb KY379802.1	Talaromyces sp. strain NT5-17 in...	789	0E00
gi 748806741 gb KM279975.1	Talaromyces radicus isolate UASWS...	789	0E00
gi 748806741 gb KM279975.1	Talaromyces radicus isolate UASWS...	57	9E-04
gi 1666020307 gb MK183804.1	Talaromyces variabilis strain NQ...	784	0E00
gi 1666020307 gb MK183804.1	Talaromyces variabilis strain NQ...	91	4E-14
gi 1628757350 gb MH935987.1	Talaromyces sp. strain 6HVCE1 sm...	784	0E00
gi 1628757350 gb MH935987.1	Talaromyces sp. strain 6HVCE1 sm...	159	3E-34
gi 1511001243 gb MG957181.1	Talaromyces variabilis isolate 4...	782	0E00
gi 1511001243 gb MG957181.1	Talaromyces variabilis isolate 4...	140	9E-29
gi 1511001243 gb MG957181.1	Talaromyces variabilis isolate 4...	782	0E00
gi 1511001243 gb MG957181.1	Talaromyces variabilis isolate 4...	140	9E-29
gi 1005417929 gb KT958549.1	Talaromyces radicus strain DsppF...	782	0E00
gi 1005417929 gb KT958549.1	Talaromyces radicus strain DsppF...	138	3E-28
gi 961554357 gb KT264430.1	Talaromyces sp. isolate AM081-P10...	782	0E00
gi 961554357 gb KT264430.1	Talaromyces sp. isolate AM081-P10...	159	3E-34
gi 536708330 gb KF053672.1	Talaromyces radicus strain H9 int...	782	0E00
gi 498922946 gb KC920846.1	Talaromyces radicus isolate CrP20...	782	0E00
gi 498922946 gb KC920846.1	Talaromyces radicus isolate CrP20...	94	1E-14
gi 498922944 gb KC920844.1	Talaromyces radicus isolate CrP18...	782	0E00
gi 498922944 gb KC920844.1	Talaromyces radicus isolate CrP18...	65	6E-06
gi 498922943 gb KC920843.1	Talaromyces radicus isolate CrP17...	782	0E00
gi 498922943 gb KC920843.1	Talaromyces radicus isolate CrP17...	65	6E-06
gi 1347308147 gb MG976292.1	Talaromyces wortmannii isolate 4...	779	0E00
gi 1347308147 gb MG976292.1	Talaromyces wortmannii isolate 4...	76	3E-09
gi 1473252768 gb MH865712.1	Talaromyces wortmannii strain CB...	779	0E00
gi 1473252768 gb MH865712.1	Talaromyces wortmannii strain CB...	117	1E-21
gi 1473245354 gb MH858298.1	Talaromyces wortmannii strain CB...	779	0E00
gi 1473245354 gb MH858298.1	Talaromyces wortmannii strain CB...	146	2E-30
gi 1042744486 gb KU839509.1	Fungal sp. strain S252D internal...	779	0E00
gi 1042744486 gb KU839509.1	Fungal sp. strain S252D internal...	105	7E-18
gi 1042744118 gb KU839141.1	Fungal sp. strain S195T internal...	779	0E00
gi 1042744118 gb KU839141.1	Fungal sp. strain S195T internal...	127	2E-24
gi 1042744112 gb KU839135.1	Fungal sp. strain S194T internal...	779	0E00
gi 1042744112 gb KU839135.1	Fungal sp. strain S194T internal...	131	5E-26
gi 1042744111 gb KU839134.1	Fungal sp. strain S194S internal...	779	0E00
gi 1042744111 gb KU839134.1	Fungal sp. strain S194S internal...	130	2E-25
gi 1042744109 gb KU839132.1	Fungal sp. strain S194N internal...	779	0E00
gi 1042744109 gb KU839132.1	Fungal sp. strain S194N internal...	131	5E-26

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gi|1042744104|gb|KU839127.1| Fungal sp. strain S194C internal... 779 0E00
gi|1042744104|gb|KU839127.1| Fungal sp. strain S194C internal... 101 8E-17
gi|1042744103|gb|KU839126.1| Fungal sp. strain S194B internal... 779 0E00
gi|1042744103|gb|KU839126.1| Fungal sp. strain S194B internal... 101 8E-17
gi|1042744102|gb|KU839125.1| Fungal sp. strain S194A internal... 779 0E00
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ALIGNMENTS

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>gb|MK841454.1| Penicillium concavorugulosum voucher NWUSeq42 internal
transcribed spacer 1,
partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2,
complete sequence;
and large subunit ribosomal RNA gene, partial sequence

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Length=570

Score = 796.6 bits (882), Expect = 0E00

Identities = 441/441 (100%), Gaps = 0/441 (0%)

Strand = Plus/Minus

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Query 1   CCGCTTATTGATATGCTTAAGTTCAGCGGGTAGCCCTACCTGATCCGAGGTCAACCGGAA 60
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Sbjct 565 CCGCTTATTGATATGCTTAAGTTCAGCGGGTAGCCCTACCTGATCCGAGGTCAACCGGAA 506

Query 61   GAGGCGCACCCCGAAGGGCGGCCTAAAGGGAAGACCAGCGCCGACCGAGTCCCTCCCGAG 120
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Sbjct 505 GAGGCGCACCCCGAAGGGCGGCCTAAAGGGAAGACCAGCGCCGACCGAGTCCCTCCCGAG 446

Query 121  CGGGTGACAAAGCCCCATACGCTCGAGGACCCGACGCGGCGCCGCGCCACTGCTTTTGGGGC 180
          |||
Sbjct 445 CGGGTGACAAAGCCCCATACGCTCGAGGACCCGACGCGGCGCCGCGCCACTGCTTTTGGGGC 386

Query 181  GTGTCCCCGGGGGGACAGCGCCCAACACCCAGCCGTGCTGGAGGGCAGAAATGACGCTCG 240
          |||
Sbjct 385 GTGTCCCCGGGGGGACAGCGCCCAACACCCAGCCGTGCTGGAGGGCAGAAATGACGCTCG 326

Query 241  GACAGGCATGCCCCCGGAATGCCAGGGGGCGCAATGTGCGTTCAAAGATTCGATGATTC 300
          |||
Sbjct 325 GACAGGCATGCCCCCGGAATGCCAGGGGGCGCAATGTGCGTTCAAAGATTCGATGATTC 266

Query 301  ACGGAATTCTGCAATTCACATTACTTATCGCATTTTCGCTGCGTTCTTCATCGATGCCGGA 360
          |||
Sbjct 265 ACGGAATTCTGCAATTCACATTACTTATCGCATTTTCGCTGCGTTCTTCATCGATGCCGGA 206

Query 361  ACCAAGAGATCCGTTGTTGAAAGTTTTAATGATTTCAAATCTCACTCAGACTCACTATTC 420
          |||
Sbjct 205 ACCAAGAGATCCGTTGTTGAAAGTTTTAATGATTTCAAATCTCACTCAGACTCACTATTC 146

Query 421  AGGCAGGGTTCTAGGGTGCGT 441
          |||
Sbjct 145 AGGCAGGGTTCTAGGGTGCGT 125

```

APPENDIX B: ANOVA TABLES FOR CHAPTER THREE

ANOVA tables for *Penicillium commune*1 and *F. oxysporum* cultured together.

Variate: *P. commune*1

<i>Source of variation</i>	<i>d.f.</i>	<i>s.s.</i>	<i>m.s.</i>	<i>v.r.</i>	<i>F.pr</i>
<i>Day</i>	4	17.9744	4.4936	5.41	0.004
<i>Residual</i>	20	16.6000	0.8300		
<i>Total</i>	24	34.5744			

Variate: *F. oxysporum*1

<i>Source of variation</i>	<i>d.f.</i>	<i>s.s.</i>	<i>m.s.</i>	<i>v.r.</i>	<i>F. pr</i>
<i>Day</i>	4	44.250	11.212	8.81	<.001
<i>Residual</i>	20	25.448	1.272		
<i>Total</i>	24	70.298			

ANOVA tables for *Penicillium commune*2 and *F. oxysporum* cultured together.

Variate: *P. commune*2

<i>Source of variation</i>	<i>d.f.</i>	<i>s.s.</i>	<i>m.s.</i>	<i>v.r.</i>	<i>F. pr</i>
<i>Day</i>	4	0.5864	0.1466	0.58	0.678
<i>Residual</i>	20	5.0240	0.2512		
<i>Total</i>	24	5.6104			

Variate: *F. oxysporum*2

Source of variation	d.f.	s.s.	m.s.	v.r.	F. pr
Day	4	53.286	13.321	18.12	<.001
Residual	20	14.704	0.735		
Total	24	67.989			

ANOVA tables for *Aspergillus fumigatus* and *F. oxysporum* cultured together.

Variate: *A. fumigatus*

Source of variation	d.f.	s.s.	m.s.	v.r.	F. pr
Day	4	42.5144	10.6286	11.40	<0.001
Residual	20	18.6480	0.9324		
Total	24	61.1624			

Variate: *F. oxysporum*3

Source of variation	d.f.	s.s.	m.s.	v.r.	F. pr
Day	4	3.330	0.832	0.68	0.612
Residual	20	24.384	1.219		
Total	24	27.714			

ANOVA tables for *Penicillium concavorugulosum*1 and *F. oxysporum* cultured together.

Variate: *P. concavorugulosum*1

Source of variation	d.f.	s.s.	m.s.	v.r.	F. pr
Day	4	10.2496	2.5324	4.13	0.013
Residual	20	12.4200	0.6210		
Total	24	22.6696			

Variate: *F. oxysporum*4

Source of variation	d.f.	s.s.	m.s.	v.r.	F. pr
Day	4	25.714	6.428	24.54	<.001
Residual	20	5.240	0.262		
Total	24	30.954			

ANOVA tables for *Penicillium concavorugulosum*2 and *F. oxysporum* cultured together.

Variate: *P. concavorugulosum*2

Source of variation	d.f.	s.s.	m.s.	v.r.	F. pr
Day	4	39.7160	9.9290	17.14	<0.001
Residual	20	11.5840	0.5792		
Total	24	51.3000			

Variate: *F. oxysporum*5

Source of variation	d.f.	s.s.	m.s.	v.r.	F. pr
Day	4	4.746	1.186	0.26	0.901
Residual	20	91.856	4.593		
Total	24	96.602			

APPENDIX C: CHAPTER FIVE EXPERIMENT PICTURES



Figure C.1: 25cm diameter pots containing sown *S. lycopersicum* seeds in different treatments.



Figure C.2: *Solanum lycopersicum* seedlings



Figure C.3: Control seedling and T5 seedlings



Figure C.4: T2 seedlings before and after termination