Isolation and characterisation of entomopathogenic fungi

By

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Declaration

I, Fhatani Kwinda (student number: 1831425), declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Masters of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

Signature: ~~*F. Kwinda*~~

Date: 10/06/2024

Abstract

The purpose of the study was to isolate and identify fungal isolates in soil samples, followed by virulence characterisation to study their effectiveness in controlling insect pests using *Tenebrio molitor* as our model organism. Lastly, a combination study was conducted to evaluate the effectiveness of the joint use of two entomopathogenic microorganisms. For isolation, *T. molitor* was used as bait then the isolated fungal isolates were identified using molecular and morphological characterisation. Morphological characterisation included macroscopic (fungal cultures) and microscopic (conidia shape and size) analysis while molecular characterisations included extraction of DNA, amplification of the internal transcribed spacer region and sequence alignment. Once identification was done, virulence was assessed through in-vitro virulence parameter like vegetative growth and in-vivo assessment where bioassays were done against *T. molitor*. Lastly, entomopathogenic fungi were combined with *Cruznema* sp. NTM-2021 in a soil assay. From the study, two of the five isolates were identified as entomopathogenic fungi, *Metarhizium anisopliae* ARSEF 7487. *M. anisopliae* had the slowest vegetative growth but was the highest in virulence. When used for a single application in a soil environment it reaches 97.8% mortality and its combination with *Cruznema* sp. NTM-2021 resulted in a 57.8% mortality and an additive interaction. In conclusion, *M. anisopliae* used alone was effective in its control of *T. molitor*.

Dedication

I dedicate this work to my loving parents, Azwifarwi Aaron Kwinda and Lufuno Kone, for their continual support and encouragement

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Most of all, I thank God for his grace that carried me throughout the whole journey

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List of abbreviations

ANOVA – Analysis of Variance

BLAST – Basic Local Alignment Tool

DNA – Deoxyribonucleic acid

EPFs – Entomopathogenic Fungi

EPNs – Entomopathogenic nematodes

IJs – Infective juveniles

IPM – Integrated Pest Management

ITS – Internal transcribed spacer region

LC – Lethal concentration

MEGA – Molecular Evolutionary Genetics Analysis

MUSCLE – Multiple Sequence Comparison by Log-Expectation

NCBI – National Center for Biotechnology Information

PCR – Polymerase chain reaction

PDA – Potato Dextrose Agar

rDNA – Ribosomal deoxyribonucleic acid

Chapter 1:

Literature review

1.1. Chemical insecticides

Arthropod pests cause serious damage to crops therefore causing a decline in the crop yield and specific pests cause damage to specific types of crops (Harith-Fadzilah *et al*., 2021; Manosathiyadevan, Bhuvaneshwari and Latha, 2017; Thomas, 1999). It was estimated that there is about 34% of annual crop loss globally and these major crops include potatoes, fruits, vegetables and fibre crops (Sharma, Kooner and Arora, 2017). Most of these losses occur in the field with greater losses occurring in developing countries such as India and African countries like South Africa (Sharma, Kooner and Arora, 2017). To reduce such losses, chemical insecticides were introduced and were essential for the control of problematic pests to meet the demands of the increasing human population (Ansari, Moraiet and Ahmad, 2014). Their ease of application and high usefulness made them favourable but their effectiveness over the years has decreased due to the development of resistance of pests to these chemical insecticides (Sharma and Sharma, 2021; Bertomeu-Sánchez, 2019). Chemical insecticides were found to be problematic due to being, a) toxic to the environment, b) harmful to the health of humans due to the toxic residues, c) farm workers being exposed to the toxins, and d) harmful to non-target organisms (Bertomeu-Sánchez, 2019; Abdelghany, 2018; Ansari, Moraiet and Ahmad, 2014; Hennessy, 2012). For effective pest control, the use of biological control, namely, entomopathogenic microorganisms, has been considered a safe alternative. Biocontrol is the introduction of living (pathogenic) organisms in an area where the pests are present to reduce the presence of the pest in that particular area (Hennessy, 2012). The taxa of these entomopathogenic microorganisms belong to, 1) Fungi, 2) Bacteria, 3) Nematodes, 4) Protozoa and 5) Viruses (Youssef, 2014).

1.2. Fungi

Fungi are the second largest eukaryotic group that has an estimated number of 1.5 to 5.1 million species (Raja *et al.*, 2017; Blackwell, 2011; O'Brien *et al.*, 2005; Hawksworth, 1991). They are also heterotrophic because they obtain their food source and nourishment from the external environment and suck in products through the cell wall (McConnaughey, 2014). They can be either unicellular (yeast) or multi-cellular (Bava *et al.*, 2022). Fungal species are very diverse

in their morphology, metabolism, ecology and phylogeny (Raja *et al.*, 2017). They can be divided into two groups, namely, macroscopic and microscopic fungi. Macroscopic fungi that are well-known include mushrooms and moulds whereas microscopic fungi are yeasts and spores (Bava *et al.*, 2022; Cole, 1996).

The structure of the fungi consists of the hyphae which is the body of the fungi. The hyphae have a cell wall which is rigid and the cell wall contains chitin and glucans (Bava *et al.*, 2022; Garcia-Rubio *et al.*, 2020; Cole, 1996). The hyphae can either be septate or non-septate. A network of hyphae is known as the mycelium which is the main body of the fungi (Cole, 1996). Mycelium gives rise to fruiting bodies and the main function of these fruiting bodies is to produce spores (Figure 1.1.2). Spores are the reproductive structures of fungi (Bava *et al.*, 2022). Reproduction in fungi can either occur asexually, sexually or both. Asexual reproduction takes place with the formation of spores which are reproductive cells that are a result of mitosis of the parent cell (budding, binary fission and fragmentation) (Bava *et al.*, 2022; Cole, 1996). Sexual reproduction involves the union of sex organs, nuclei, and cells formed by sexed spores (Bava *et al.*, 2022; Cole, 1996).

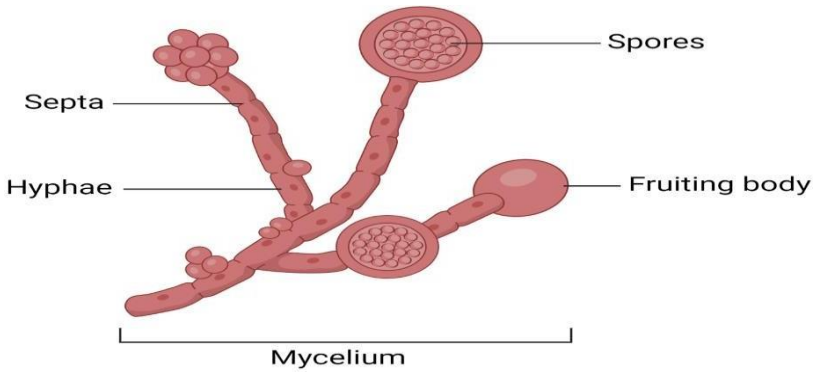

Figure 1.1.2. Structure of fungi.

Fungi have a heterotrophic metabolism therefore they are dependent on the host for survival. They may have a mutualistic, symbiont or parasitic relationship with the host (Bava *et al.*, 2022). This study focussed on parasitic fungi (entomopathogenic fungi) that are harmful to insects.

1.3. History of entomopathogenic fungi

Agostino Bassi in 1834 was the first to refer to the entomopathogens due to infection of silkworms by a white muscardine disease which was later identified as *Beauveria bassiana* (Mantzoukas *et al.*, 2022; Sharma and Sharma, 2021; Gilbert and Gill, 2010; Roberts and

Hajek, 1992). Years after this discovery, Elias Metschnikoff (1888) discovered a green muscardine which was initially identified as *Entomopthora anisopliae* against the wheat cockchafer, Anisoplia austriaca but was later identified as *Metarhizium anisopliae*, which is a fungal disease that kills insects (Mantzoukas *et al.*, 2022; Sharma and Sharma, 2021; Dauda *et al.*, 2018). The mass production of *Metarhizium anisopliae* on a large scale was done by Krassilstschik in the year 1888 against *Bothynoderes punctiventris* (sugar beet weevil) in Russia (Sharma and Sharma, 2021). In the 19th century, more assays were done to test whether these fungi can be used as a potential pest control agent (Mantzoukas *et al.*, 2022). The advancement of these experiments was due to the previous discoveries that were made. The rise of chemical insecticides put a pause on the establishment of entomopathogenic fungi on pest management (Mantzoukas *et al.*, 2022; Gilbert and Gill, 2010). This was also followed by the introduction of the bacteria, *Bacillus thurengiensis*, which played a role as a biological agent against insects therefore further delaying the advancement of entomopathogenic fungi in pest management (Mantzoukas *et al.*, 2022).

1.4. Entomopathogenic fungi

Entomopathogens refer to organisms that infect and kill insects and terrestrial arthropods such as spiders, mites and ticks (Bava *et al.*, 2022; Mora, Castilho and Fraga, 2018; Motta Delgado and murcia ordoñez, 2011). Entomopathogenic fungi (EPFs) are known to kill insects and are used as biological pest control agents (Wei *et al.*, 2021). They are soil organisms that act either as a biocontrol for pests or endophytes which play a role in improving crop performance and crop health (Uzman *et al.*, 2019). There are more than 100 genera of EPFs and most are found in the order Hypocreales of the Ascomycota (Bava *et al.*, 2022; Sharma and Sharma, 2021). EPFs are considered natural enemies to arthropod pests and are found in many countries (Meyling and Eilenberg, 2007).

EPFs have many attributes, namely, a) they are endophytes, b) they colonise internal plant tissue and rhizosphere, and c) they promote plant growth and plant fitness (Mantzoukas *et al.*, 2022). They help with enhancing tolerance to environmental conditions such as drought and they also act as an antagonist against plant pathogens by taking up vital nutrient spaces which will cause the plant's systemic resistance to be activated (Mantzoukas *et al.*, 2022).

What has led to the success of EPFs affecting destructive arthropods in agriculture, forestry and livestock is that during infection they produce different extracellular enzymes and release secondary metabolites which are toxic to the pests (Shahriari *et al.*, 2021). The benefits of using

EPFs are: a) safe to the environment, b) little to no effect on non-target organisms, c) reduces the need for chemical insecticides, and d) pest resistance caused by them is less likely to occur due to their mode of action (Shahriari *et al.*, 2021; Dauda *et al.*, 2018). Their limitations are, a) they are sensitive to environmental conditions such as temperature changes and UV radiation b) require specific environmental conditions for infection to occur, and c) slow acting (Dauda *et al.*, 2018; Starnes, Liu and Marrone, 1993). Therefore there is a need to isolate strains that can easily adapt to certain environmental conditions for formulations and field applications (Shahriari *et al.*, 2021).

1.5. EPFs as endophytes

The function of EPFs as endophytes is to protect the host plant against pathogens and plant-eating animals (Uzman *et al.*, 2019). It helps with increasing plant growth and productivity and provides the plant with nitrogen that is derived from insects (Uzman *et al.*, 2019). Endophytic fungi have been isolated from a variety of plants such as soybeans, tomatoes, wheat and bananas (Sharma and Sharma, 2021).

Endophyte EPFs develop inside the plant tissues that are above ground and do not produce any infection symptoms on the plant (Sharma and Sharma, 2021). The most commonly known entomopathogenic fungi that are endophytes include *Beauveria bassiana* and *Metarhizium anisopliae* (Sharma and Sharma, 2021; Akutse *et al.*, 2013). They are beneficial to the host plant as they provide protection against many pests and they enhance the plant hosts responses through inducing resistance in the host plants (Sharma and Sharma, 2021). An example of endophytes being beneficial to the host plant is when conidia of *M. anisopliae* were applied to corn seeds before being planted and this helped increase the fresh weight and density of the corn (Sharma and Sharma, 2021; Kabaluk and Ericsson, 2007).

1.6. Life cycle of EPFs

The infection process starts when a suitable host comes into contact with EPFs spores. The spores adhere to the epicuticle of the host (Bava *et al.*, 2022; Barra-Bucarei, France Iglesias and Pino Torres, 2019). Adherence of the spores to the epicuticle, germination of the spores and appression are critical steps in the infection process. The sites where the fungi penetrate the host appear as dark and melanotic areas (Bava *et al.*, 2022). For entry to the host, the combined action of two mechanisms is needed, namely, enzymatic degradation and mechanic pressure mechanisms (Starnes, Liu and Marrone, 1993). To attach to the waxy epicuticle of the host, EPFs produce hydrophobic conidia. Not only attaching to the epicuticle by producing

hydrophobic conidia but the fungus synthesizes molecules called adhesins that help with the binding process (Bava *et al.*, 2022). After binding, mucus that has strong adhesion properties is secreted and this mucus also contains degrading enzymes. The well-known degrading enzymes that have been found in EPFs species are proteases, lipases and chitinases (Bava *et al.*, 2022; Barra-Bucarei, France Iglesias and Pino Torres, 2019; Vega *et al.*, 2012).

A specialized structure called appressorium is a narrow peg that concentrates physical and chemical energy on a small area of the cuticle (Bava *et al.*, 2022; Barra-Bucarei, France Iglesias and Pino Torres, 2019). When the fungus is successfully attached to the cuticle, the growth of the hyphae then follows. The hyphae grow through the cuticle interstices and move into the haemocoel of the host. The filamentous hyphae growing within the hemocoel then grow small thin-walled hyphal bodies which circulate the haemolymph and proliferate (Bava *et al.*, 2022; Starnes, Liu and Marrone, 1993). The cellular form of the hyphae helps with increasing the rate of nutrient acquisition which is used for growth and reproduction for the fungi (Barra-Bucarei, France Iglesias and Pino Torres, 2019; Cole, 2012). This is then followed by the production of toxins that help in their pathogenicity, for example, *M. anisopliae* produces a toxin called dextruxins that causes paralysis and death of the host (Wang and Wang, 2017; Cole, 2012; Starnes, Liu and Marrone, 1993). The fungus returns to mycelium growth when the nutrients are depleted and then starts invading the host's internal tissues and organs (Bava *et al.*, 2022; Mora, Castilho and Fraga, 2018; Vega *et al.*, 2012).

Due to the progressive infection, the body of the host hardens because of the uptake/absorption of liquids by the fungus (Bava *et al.*, 2022). After the death of the host, the hyphae emerge through the holes of the body and intersegmental membrane of the host (Vega *et al.*, 2012). Spores are therefore produced which partially or fully cover the cadaver body and allow the conidia to spread and affect other hosts (Bava *et al.*, 2022; Barra-Bucarei, France Iglesias and Pino Torres, 2019; Meyling and Eilenberg, 2007).

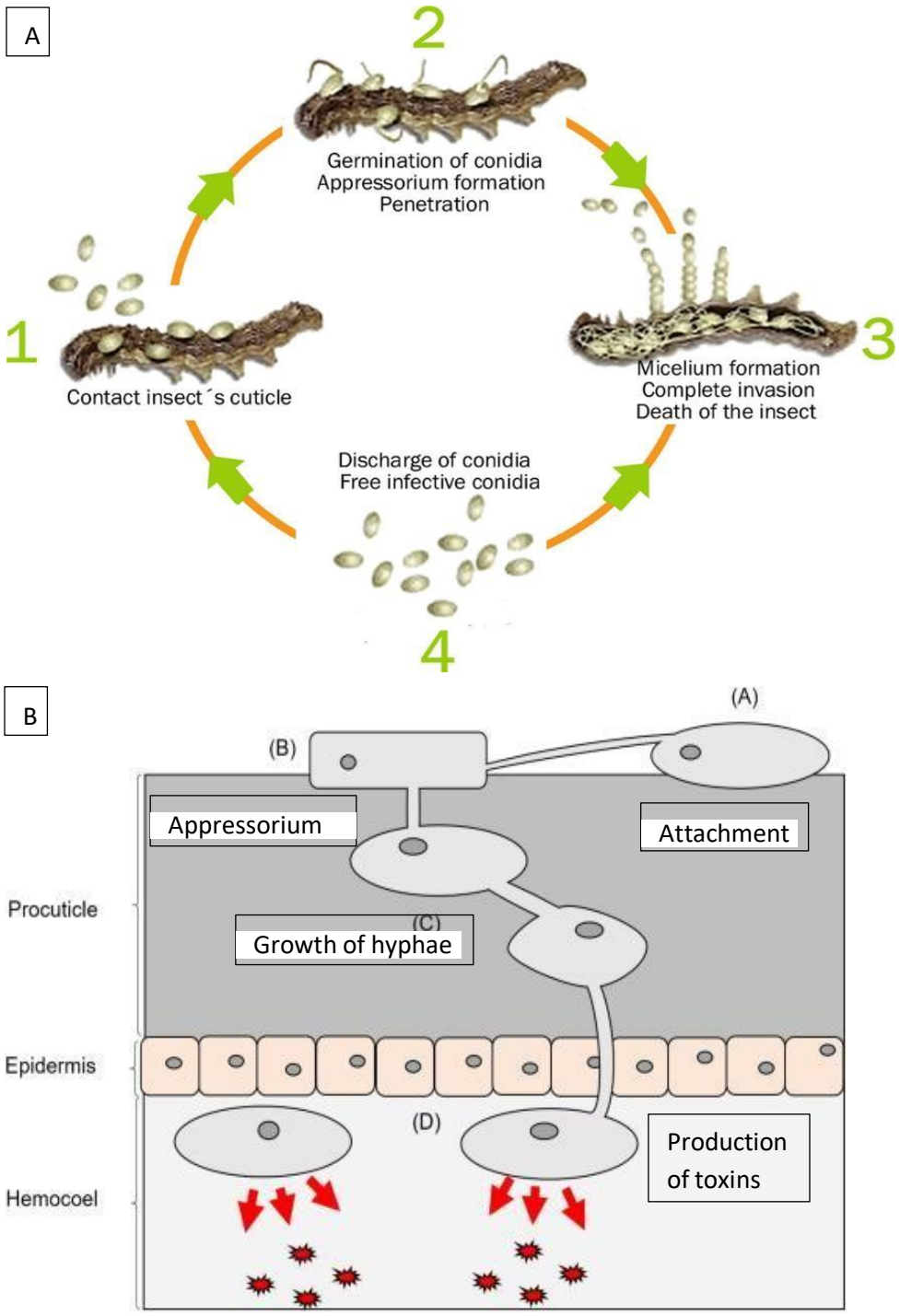

Figure 1.1.6. Infection process of entomopathogenic fungi. A) Overview of the life cycle of EPFs and B) Overview of the mechanism of infection (Bava *et al.*, 2022; Harith-Fadzilah *et al.*, 2021).

1.7. EPFs as biocontrol agents

EPFs are currently used commercially against agricultural pests and mosquitoes which transmit diseases to humans (Wei *et al.*, 2021). What makes EPFs good biocontrol agents is their

adherence properties, their ability to degrade the host's cuticle and their ability to avoid the host's defence mechanisms (Bava *et al.*, 2022). EPFs in the market are sold as powders and emulsified oil which contains additives that help with protecting or improving certain factors of the EPFs (Bava *et al.*, 2022). Oils are often added in liquid and powdered formulations to improve shelf-life and increase their adhesion to the waxy cuticle of the host (Bava *et al.*, 2022).

Formulated products of EPFs are commercially available for a variety of crops (Uzman *et al.*, 2019). Isolates of EPFs that are already commercialized in different countries as biocontrol agents might be ineffective in controlling some strains due to strain differences to the host and environmental suitability (Shahriari *et al.*, 2021). Therefore there is a need to find local isolates that are more environmentally and ecologically suited to local pests and have lower hazards to non-target organisms as compared to exotic strains (Shahriari *et al.*, 2021; Barra-Bucarei, France Iglesias and Pino Torres, 2019; García-Gutiérrez and González-Maldonado, 2010).

The use of EPFs as microbial insecticides has gained much attention due to their high virulence, and broad host range not forgetting that it is found in a wide range of soil habitats mainly cultivated and forest soils (Gebremariam *et al*., 2021). The genus of EPFs used worldwide for the control of insect pests are namely, *Metarhzium*, *Beauveria* and *Paecilomyces* (Gebremariam *et al*., 2021). *Metarhizium anisopliae* (green muscardine fungi) and *Beauveria bassiana* (white muscardine fungi) play a major role in integrated pest management strategies and are mostly used for the control of sucking and chewing agricultural insect pests (Gebremariam *et al*., 2021). The commercial products of these two species have been used for the control of a wide range of insect hosts. The challenge that comes with using these commercial products is that isolates are non-adaptable to certain agro-ecological conditions and the application of these isolates for a long time tends to have reduced efficacy in that particular ecosystem (Gebremariam *et al*., 2021). How this can be overcome through the isolation and identification of local EPFs that are already adapted to the local environmental conditions (García-Gutiérrez and González-Maldonado, 2010). This will allow for effective pest management and it has been reported that locally isolated EPFs are effective in the control of a wide range of agricultural pests under local conditions (Gebremariam *et al*., 2021).

The use of local EPFs is much more effective and better suited for the control of insect pests compared to the use of exotic EPFs isolates (Gebremariam *et al*., 2021). What makes local EPFs suitable is their environmental suitability with pest species (Gebremariam *et al*., 2021). This now emphasizes the importance of isolating, identifying and screening local EPFs

pathogenicity for their use in pest management strategies which can in future be used under greenhouse and field conditions (García-Gutiérrez and González-Maldonado, 2010).

The following factors are important to look at when selecting EPFs for their use as biological agents, i) their rate of development, ii) their ability to infect and kill the target insect host and iii) their suitability to be used in artificial production (Santos *et al.,* 2018).

1.8. Entomopathogenic nematodes

Entomopathogenic nematodes (EPNs) are soil-dwelling micro-organisms that are found in diverse habitats across the world (Tarasco *et al.,* 2008). They have a mutualistic relationship with pathogenic bacteria that are able to infect and kill insects within 24 to 48 hours. The entomopathogenic nematodes discovered are *Heterorhabditis, Steinernema* and *Oscheius* genus with their symbiotic bacteria being *Photorhabdus*, *Xenorhabdus* and *Serratia*, respectively (Lephoto and Gray, 2015). EPNs carry their symbiotic bacteria in their midgut, with *Xenorhabdus* covered with a special vesicle called a receptacle in anterior region of the *Steinernema* gut (Sajnaga and Kazimierczak, 2020).

EPNs have a free-living third stage of development, non-feeding infective juveniles (IJs), which can persist in the soil for a long period of time even under adverse conditions (Lephoto and Gray, 2015). These IJs are the only infectious stage of the EPNs while the other stages of the EPNs are found and developed in the host (Dillman and Sternberg, 2012). IJs are developmentally arrested and have the ability to locate their host through various strategies such as cruiser and ambusher strategy. *Heterorhabditis* use the cruiser strategy involves the IJs actively searching for their target host. *Steinernema* use either cruiser or ambusher strategy or both, the ambusher strategy involves the IJs waiting in one place for their target host ((Didiza *et al.,* 2021). When the IJs have located their target host, the infection process begins.

They enter the insect host through natural openings such as the mouth or anus while some *Heterorhabdus spp.* can enter their hosts through penetrating the cuticle (Garcia-del-Pino *et al*, 2018). After infiltrating their host, they shed their outer cuticle and start taking in the haemolymph which allows for the release of their symbiotic bacteria either by defecation (*Steinernema spp.*) or regurgitation (*Heterorhabditis spp*) (Garcia-del-Pino *et al.*, 2018). When bacteria is released in the haemocoel of the insect host it releases compounds that aid with killing the insect host. The compounds consist of toxins, antimicrobials, insecticidal as well as proteases which help breakdown the insect tissue and suppresses the hosts' immune system (Didiza *et al.*, 2021). They enter the haemolymph of the insect and produce toxins that weaken

the immune system of the insect therefore killing the host in 24 to 48 hours through toxaemia or septicaemia (Garcia-del-Pino *et al.*, 2018). The nematode feeds on the cadaver and reproduces up to 2 to 3 generations depending on the availability of nutrients. The bacteria also produces some antimicrobials that keep opportunistic organisms away. When the nutrients are depleted, the non-feeding IJs carries their symbiotic bacteria and moves from the cadaver to the soil and await its next insect target (Lephoto et al., 2015; Lephoto and Gray, 2019).

EPNs are good biological controls because of many factors such as their ability to search for hosts and how safe they are to the environment, beneficial organisms and humans. Another thing that is gaining them attention is their wide host range and the antimicrobial and insecticidal compounds that the symbiotic bacteria produces (Flores *et al.*, 2021). EPNs can be used as an alternative to chemical insecticides that are harmful to the environment and people.

1.9. Research Motivation

Crops are prone to attacks by insect pests causing serious damage to crops therefore causing a decline in crop production and yield (Harith-Fadzilah *et al.*, 2021). Food security is also threatened due to the loss of crops of which some are considered to be important staple crops for the country. To slow down the damage caused by insect pests, chemical insecticides were introduced as a primary control measure of insect pests (Sharma and Sharma, 2021). The use of chemical insecticides causes environmental pollution and has adverse effects on the health of humans. It also causes an increase in the resistance of pests to these insecticides, has detrimental effects on beneficial organisms and results in residual toxicity (Narmen A Youssef, 2014). The increasing disadvantages of using chemical insecticides led to the search for an alternative safe insecticide such as entomopathogenic microorganisms in this case EPFs (Hennessy, 2012).

Currently used commercialized EPFs biological agents do have their disadvantages such as their environmental limitation (Shahriari *et al.*, 2021). One of the ways to combat this is by accurately identifying and characterizing EPFs isolates that have traits that are more useful than the ones that are currently being used as biological agents against insect pests

1.10. Aims and Objectives

Aim 1

To Isolate and identify the fungi found in the collected soil samples.

Objectives

- To bait the soil samples with *T. molitor*.
- To do molecular and morphological identification of fungi isolates.
- To identify the fungal species using the National Center for Biotechnology Information database.

Aim 2

To determine the virulence of fungal isolates for the control of *T. molitor*.

Objectives

- To immerse *T.molitor* in the fungal suspension of the isolates.
- To perform a dose-dependent assay.

Aim 3

To evaluate the efficacy of using *Cruznema* sp. NTM-2021 and EPFs in combination for the control of *T. molitor*.

Objectives

- To apply the microbial agents onto the soil surface.
- To do a single and combined application of *Cruznema* sp. NTM-2021 and EPFs.
- To determine the relationship of the combined effect of *Cruznema* sp. NTM-2021 and EPFs on *T. molitor*.

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Chapter 2

Isolation, Identification and characterisation of fungi.

2.1 Introduction

In the past 70 years, chemical insecticides have been the preferred method for control of problematic pests (Pérez Álvarez *et al.*, 2022). During these past years, there has been an increasing number of cases that speak on the risks that chemical insecticides pose to the health of the environment and animals. This has led to the need of wanting to reduce the use of chemical insecticides and lean more toward the use of integrated pest management (IPM) systems (Bueno *et al.*, 2017). Therefore, strategies have been put in place to find an alternative to the use of chemical insecticides this being the use of biological controls (Pérez Álvarez *et al.*, 2022). Biological control in recent years has been gaining momentum as it is an environmentally safe alternative compared to chemical insecticides and has been proposed to be a component in IPM (Świergiel *et al.*, 2016). Biological control includes entomopathogenic microorganisms such as fungi, bacteria, nematodes and viruses that can potentially be used for the control of insect pests (Bich *et al.*, 2021). Entomopathogenic fungi (EPFs) are one of the biological controls that have increasingly been studied and are known to be a sustainable biological control agent and this has led to them being manufactured (Pérez Álvarez *et al.*, 2022; Bich *et al.*, 2021). The most studied genus of EPFs is *Beauveria* and *Metarhizium* (Barra-Bucarei, France Iglesias and Pino Torres, 2019).

The soil environment contains a diverse population of EPFs, therefore, EPFs can be isolated from soil samples. A particular habitat contains different EPFs species that may differ in their genetic composition within the species (González-Jartín *et al.*, 2019). For isolation of EPFs, the insect bait method is a highly sensitive detection method that makes use of a susceptible insect host and is considered a direct method of isolation (Shin *et al.*, 2013; Vega and Kaya, 2012). The susceptible insect hosts often used in insect baiting are *Galleria mellonella* (greater wax moth; Lepidoptera) and *Tenebrio molitor* (yellow mealworm; coleopteran) (Keller, Kessler and Schweizer, 2003). This is a commonly used method that has been used in the isolation of many indigenous species of EPFs. A few studies have considered this method to be a very selective method because EPFs species are selected based on the type of insect species used as bait (Shin *et al.*, 2013; Klingen, Eilenberg and Meadow, 2002). Dryness, stiffness and colour change are what characterise infection by EPFs. This is followed by the growth of

hyphae on the exterior of the cadaver. The hyphae growth increases with time resulting in the cadaver being fully covered by the fungi (Ayala-Zermeño *et al.*, 2015).

To produce biological insecticides there is a need to identify and select the most suitable EPFs isolates (Ayala-Zermeño *et al.,* 2015). For the production of biological insecticides, research needs to be done which may take time as proper identification of EPFs isolates is required (Bich *et al.,* 2021; Ayala-Zermeño *et al.,* 2015). The use of both morphological and molecular characterisation of fungi is recommended. The identification of fungi at the species level is a basic important step that most researchers do and the use of scientific names allows other researchers to find other strains that are closely related to the isolate (Raja *et al.,* 2017). Taxonomic identification also allows for the fungal strains to be used in many of their other functions such as their use in the industrial and pharmaceutical industry (Raja *et al.,* 2017). For the selection of a suitable EPFs isolate, one needs to ensure that the isolate will not threaten the health of humans and animals then this is followed by evaluating the virulence of the fungal strains (Ayala-Zermeño *et al.,* 2015).

In the past, only morphology was the sole means of identifying fungi species (Raja *et al.*, 2017). This involved macroscopic and microscopic characterisation of fungi (Bich *et al.*, 2021). For microscopic identification, conidia size and shape would be considered when identifying *Beauveria* and *Metarhizium* species. The advantage of using morphological traits was seeing the evolution of the morphological characters. The traditional way of identifying fungi has its challenges such as the genetic variability in the traits of the fungal strains and the phenotypic plasticity (Bich *et al.*, 2021). Another challenge is the limited number of phenotypic traits that can be used for identification. Some fungi species do not sporulate therefore identification would not be possible (Raja *et al.*, 2017). These challenges have led to the emergence of molecular identification of fungi species. Sequence-based identification of fungi involved the use of DNA barcoding using the internal transcribed spacer (ITS) region (Raja *et al.*, 2017). DNA barcoding involves comparing the unknown species against a sequence database such as the Genbank of the National Centre of Biotechnology Information; Genbank NCBI which identifies species based on species similarity (Raja *et al.,* 2017).

Molecular techniques used for the identification of fungi include the use of DNA sequences and DNA markers which help with identifying fungi at the inter-or-intra-species level (Ahmed, Khalil and Sahab, 2022). ITS is the official barcode marker for fungi due to its large barcode gap, its ease of amplification and its widespread use (Raja *et al.*, 2017). ITS is used for species-

level identification and is one of the most commonly used and trusted markers in providing proper identification of fungi (Schoch *et al.*, 2012). Challenges in using the ITS region as a barcode marker include its failure in being able to properly identify high-specious genus such as *Fusarium* and *Aspergillus* due to its narrow or non-existent barcode gap (Samson *et al.*, 2014; Schoch *et al.*, 2012). To verify the identity of the fungi, the ITS sequence is submitted to the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990). ITS as well as ribosomal DNA (rDNA) can be used when looking at the phylogenetic relationship of different EPFs groups (Bich *et al.*, 2021).

A study has shown that about 31% of fungi are identified based on morphology only, while about 27% are identified mainly by using molecular techniques such as ITS. Approximately 14% make use of both morphological and molecular identification (Raja *et al.*, 2017).

To understand the natural biodiversity of fungi found in a certain area or region, characterisation of the fungal species is essential and this adds to the pool of potential EPFs species that can be used as biocontrol agents (Ahmed, Khalil and Sahab, 2022). It has been found that the use of fungal strains isolated from natural habitats is preferred to the introduction of exotic species (Ahmed, Khalil and Sahab, 2022). Native EPFs are more adapted to the environmental conditions and are better suited to killing the local problematic pests which are their natural enemy. This reduces the risk of non-target organisms from being harmed whereas the exotic species might potentially kill some of the non-target organisms (Kushiyev *et al.*, 2022).

When selecting a fungal strain to be used as a biological agent, the essential prerequisite is to identify the fungi morphologically and molecularly (Bich *et al.*, 2021). This study aimed to isolate and identify, morphologically and molecularly, the fungi found in the soil collected.

2.2. Methods and materials

2.2.1. Rearing of insect larvae

Tenebrio molitor (*T.molitor*) also known as yellow wheat mealworm was bought at an Amazon pet shop in Lenasia. They were reared in the laboratory and fed an artificial diet. They were placed in 2L plastic containers that had a diet consisting of oat bran and slices of carrots. The lid of the containers had holes to allow for air circulation then the containers were stored at room temperature.

2.2.2. Soil collection

The soil was collected at different areas at the University of Witwatersrand (Table 2.2.2) and it was collected at a depth of 15cm using a hand shovel then it was sieved to get fine soil free from rocks and debris. The soil was placed in 2L containers. The bait method was used to isolate fungi from the soil. *T. molitor* was used as bait where six *T. molitor* larvae each were placed in six containers containing fine sieved sandy loam soil (Figure 2.2.2). The containers were closed, inverted then placed at room temperature. Inverting the containers allows for the larvae to move around the soil and increases the probability of making contact with the fungi. Mortality was checked daily and dead larvae were then placed on white traps.

Table 2.2.2. Co-ordinates of the collected soil samples at the University of Witwatersrand.

Areas	Co-ordinates
Witwatersrand west campus area 1	26˚11'23.706" S, 28˚1'34.68" E
Witwatersrand west campus area 2	26˚11'23.135" S, 28˚1'32.988" E
Witwatersrand west campus area 3	26˚11'17.296" S, 28˚1'33.217" E
Witwatersrand west campus area 4	26˚11'16.537" S, 28˚1'33.285" E

Figure 2.2.2. Soil baiting with *Tenebrio molitor*.

2.2.3. Modified white traps

A white trap consisted of a 90mm petri dish, a 38mm glass watch and a Whatman No.1 filter paper. The glass watch was placed in the middle of the large petri dish. The filter paper was

placed on top of the glass watch then distilled water was added to create a moist environment. The dead cadaver was first cleaned with 1% sodium hypochlorite and rinsed in distilled water twice. The sterilized cadaver was then placed in the white trap to allow for external sporulation. If the external sporulation occurred, this was an indication that the larvae were infected by the fungi.

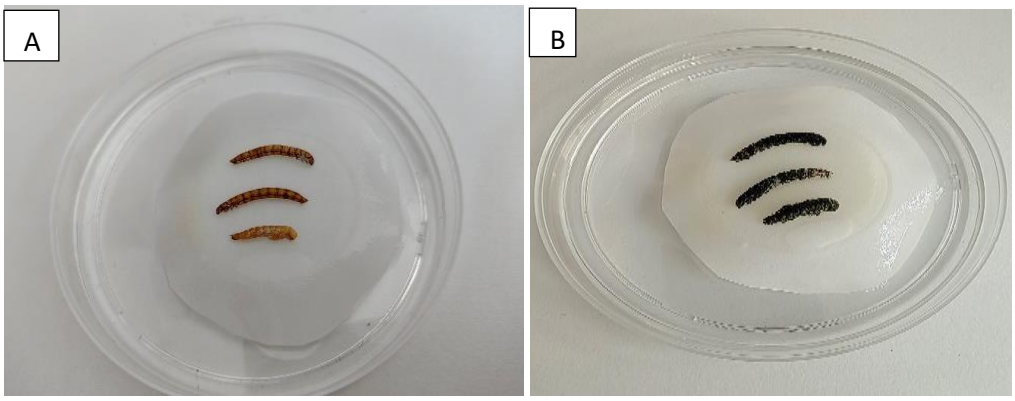

Figure 2.2.3. White trap set up with insect cadaver. A) Insect cadaver that has been cleaned and placed on a white trap and B) insect cadaver with external sporulation.

2.2.4. Genomic DNA extraction and Polymerase Chain Reaction amplification

The fungus from the surface of the dead larvae was scraped off using sterile forceps and put into a sterile Eppendorf tube. The samples were sent to Inqaba Biotechnical Industries (Pty) Ltd, South Africa for sequencing. The following protocol was used for the genomic DNA extraction of fungi:

The fungal genomic DNA was extracted using the Quick-DNA™ Fungal Miniprep Kit #D6005. Fungal isolates were suspended in 200 µl of isotonic buffer (Phosphate-buffered saline) in a ZR BashingBead™ Lysis Tube then 750 µl of BashingBead™ Buffer was added to the tube. This was placed in a bead beater and vortexed at a maximum speed for ≥ 5 minutes. The ZR BashingBead™ Lysis Tube was centrifuged at 10,000 xg for 1 minute. Up to 400 µl of the supernatant was transferred to a Zymo-Spin™ III-F Filter in a Collection Tube and centrifuged at 8,000 xg for 1 minute. 1,200 µl of Genomic Lysis Buffer was added to the filtrate in the Collection Tube and 800 µl of the mixture was added to a Zymo-Spin™ IICR Column 3 in a Collection Tube and centrifuged at 10,000 xg for 1 minute. The flow was discarded from the Collection Tube and the previous step of adding 800 µl of the mixture was added to a Zymo-Spin™ IICR Column 3 in a Collection Tube and centrifuged at 10,000 xg for 1 minute. 200 µl of DNA Pre-Wash Buffer was then added to the Zymo-Spin™ IICR Column in a new

Collection Tube and centrifuged at 10,000 xg for 1 minute. 500 µl of g-DNA Wash Buffer was added to the Zymo-Spin™ IICR Column and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin™ IICR Column was transferred to a clean 1.5 ml microcentrifuge tube and 100 µl of DNA Elution Buffer was added directly to the column matrix. This was centrifuged at 10,000 xg for 30 seconds to release the DNA from the matrix. DNA was stored at 4˚C until further use.

The PCR contents and the quantity used are listed in Table 2.2.4 and the following universal primers were used: Forward ITS1 (5'TCC GTA GGT GAA CCT GCG G-3') and Reverse ITS4 (5'TCC TCC GCT TAT TGA TAT GC-3').

Table 2.2.4. PCR contents with their quantity for the amplification of the ITS region.

PCR contents	**Quantity (µl)**
NEB One Taq 2X MasterMix	10
Genomic DNA (ng)	1
Forward primer (10µM)	1
Reverse primer (10µM)	1
Nuclease free water	7
Total reaction volume	20

The following PCR conditions were followed for the amplification of the ITS region:

Initial denaturation before cycling at 94˚C for 5 minutes

35-cycle amplification series:

Denaturation at 94˚C for 30 seconds

Annealing at 50˚C for 30 seconds

Extension at 68˚C for 1 minute

Final extension at 68˚C for 10 minutes

The PCR products were then sequenced

2.2.5. Sequence alignment

The sequences from Inqaba Biotech were edited and corrected using Bioedit version 7.2.5.0 (biological sequence alignment editor). The edited sequences were uploaded onto the basic alignment search tool (Blastn) on the National Center of Biotechnology Information (NCBI) database. The sequences are aligned against the sequences in the database to find a match with the highest similarity.

2.2.6. Fungi cultures

This method was adapted from Jaber *et al* (2016). The insect cadavers with external sporulation were taken and suspended in a 0.05% Tween solution. The solution was shaken vigorously for the suspension of the spores into the solution. 100 µL of the suspension was plated on Potato Dextrose Agar (PDA). The cultures were grown in complete darkness for 15 days at 25˚C ± 2˚C. The fungal growth was monitored every 2nd day.

2.2.7. Morphological Characterisation

The five fungal cultures grown for 15 days on PDA were preliminarily identified by their morphological characteristics. This included macroscopic characterisation based on the shape, size, elevation, margin and colour on the front and reverse sides of the plate. Microscopic traits observed were the size and shape of the conidia. The slide culture technique was used and the Olympus BX63 microscope from the Microscope and Microanalysis Unit (MMU) was used for viewing and taking pictures.

2.2.8. Phylogenetic tree

DNA sequences were aligned using the MUSCLE program found on MEGA 11 version 11.0.6 software. The phylogenetic tree was generated using the maximum likelihood method using the ITS sequence based on the Tamura-Nei method with a bootstrap of 1000 replicates.

2.3. Results

The larvae of *T. molitor* used as bait in the soil to recover fungi were found to be covered with fungal growth. The five isolates were subjected to molecular identification therefore they were sequenced and uploaded onto BLAST and the results are seen in Table 2.3.1.

Table 2.3.1. Identification of the isolated fungi.

No.	Isolate name	Strain	Genbank accession no.	Identification %
1	*Metarhizium anisopliae*	ARSEF 7487	NR_132017.1	99.72
2	*Fusarium foetens*	CBS 110286	NR_159865.1	100
3	*Metarhizium anisopliae*	ARSEF 7487	NR_132017.1	99.74
4	*Neocosmospora rubicola*	CBS 101018	NR_154277.1	100
5	*Aspergilus insuetus*	NRRL 279	NR_131292.1	99.71

From the results obtained in Table 2.3.1, only *Metarhizium anisopliae* (isolates 1 and 3) was identified as entomopathogenic fungi.

Each isolate was subjected to morphological characterisation. The macroscopic colony traits of the isolated fungi grown for 15 days on PDA medium were recorded (Table 2.3.2). Most of the fungi were Filamentous with a flat surface.

Table 2.3.2. Macroscopic traits of the isolated fungi.

No.	Shape	Size	Elevation	Margin	Front view	Rear view
1	Circular Filamentous	Medium	Flat to slightly raised	Filamentous		

2	Circular Filamentous	Large	Flat	Filamentous		
3	Circular Filamentous	Large	Flat to slightly raised	Filamentous		
4	Circular Filamentous	Large	Flat	Filamentous		
5	Circular Filamentous	Medium	Raised	Filamentous		

The microscopic traits of each isolate are recorded in Table 2.3.3 and Figure 2.3.4.

Table 2.3.3. Microscopic traits and morphometrics of the isolated fungi.

No.	Conidia shape	Conidia length (n=10) Mean±SD	Conidia width (n=10) Mean±SD
1	Oblong oval	7.218±0.658	2.752±0.365
2	Ovoid	7.272±0.496	3.874±0.436
3	Oblong oval	7.527±0.648	3.346±0.288
4	Fusoidal to ellipisoidal	13.727±3.358	5.593±0.803
5	Oval	4.922±0.605	4.922±0.605

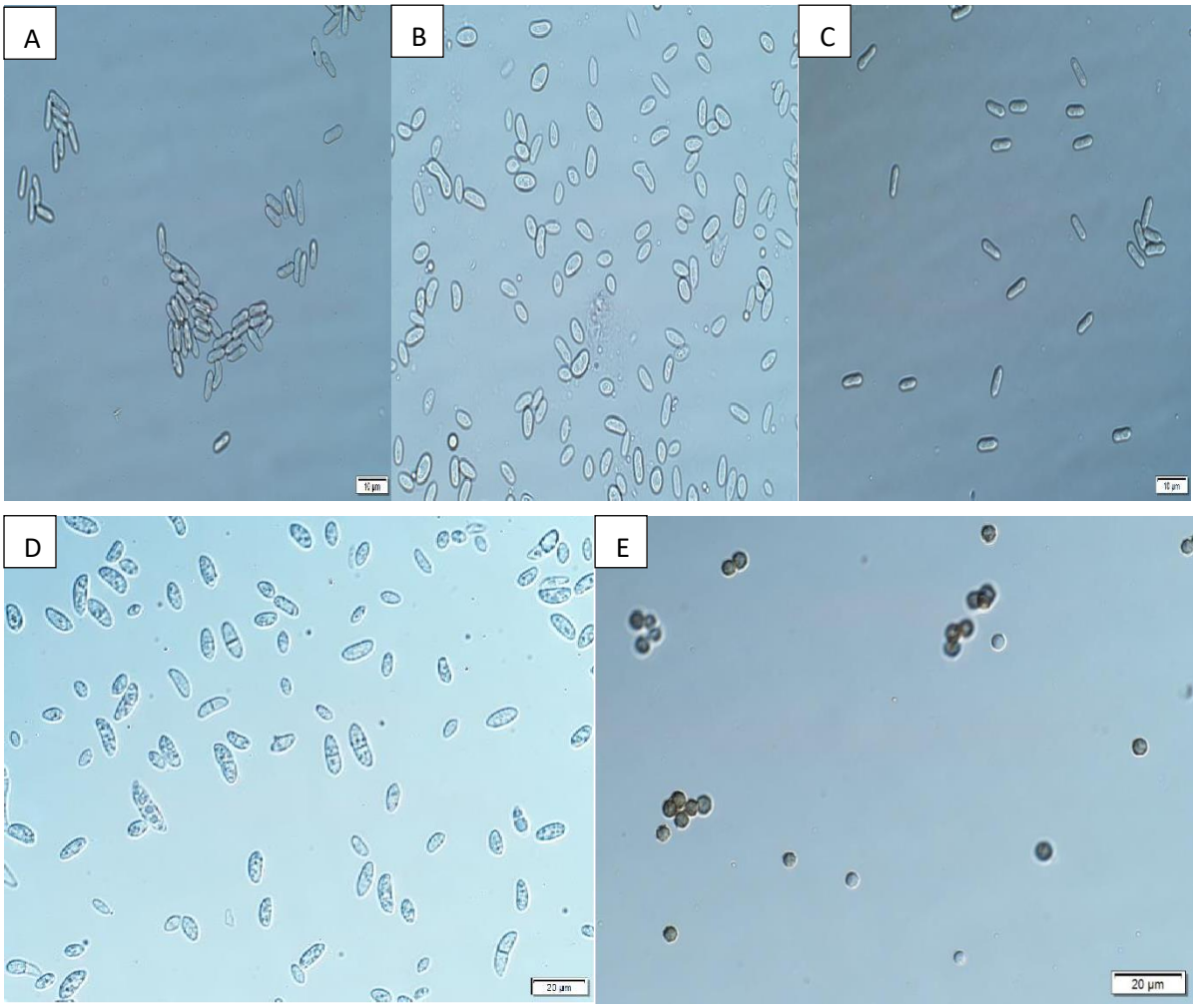

Figure 2.3.4. Microconidia of the five isolates. A) *Metarhizium anisopliae* (Green) B) *Fusarium foetens*, C) *Metarhizium anisopliae* (Yellow), D) *Neocosmospora rubicula*, E) *Aspergillus insuetus*. Scale bar (A-C) 10 µm and (D-E) 20 µm.

The two *M. anisopliae* isolates (1 and 3) were named *M. anisopliae* (Green) and *M. anisopliae* (Yellow) to differentiate them based on their fungal culture colour.

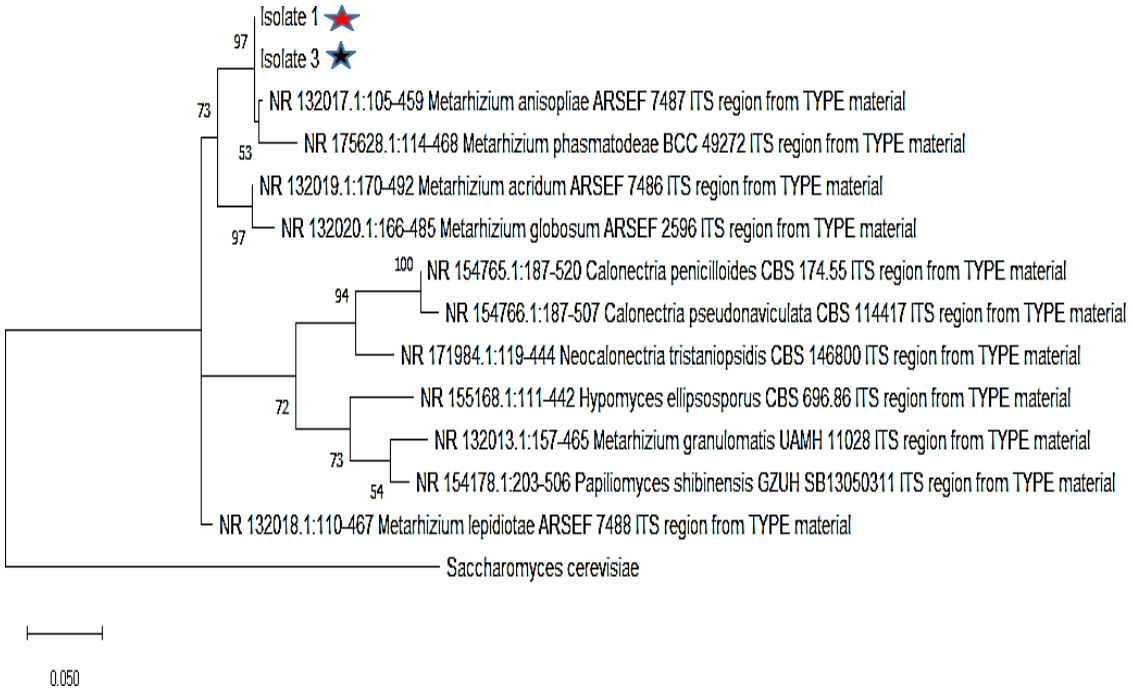

Figure 2.3.5. Phylogenetic relationships between the initially unknown isolate 1 and 3 and 12 other species based on the ITS rDNA region. The outgroup *Saccharomyces cerevisiae* was used to root the tree. Isolate 1 is indicated by a red star while isolate 3 is indicated by a black star. The phylogenetic tree was generated with the maximum likelihood method based on the Tamura-Nei model with a bootstrap of 1000 replicates. The tree was drawn to scale.

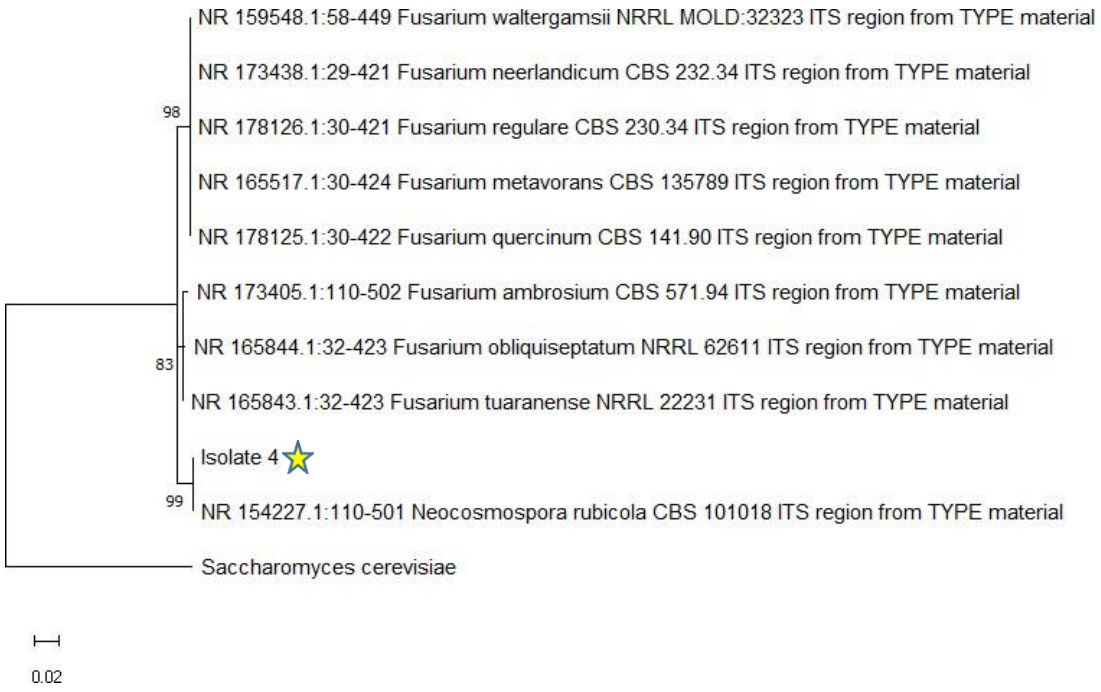

Figure 2.3.6. Phylogenetic relationships between the initially unknown isolate 4 and 10 other species based on the ITS rDNA region. The outgroup *Saccharomyces cerevisiae* was used to root the tree. Isolate 1 is indicated by a yellow star. The phylogenetic tree was generated with the maximum likelihood method based on the Tamura-Nei model with a bootstrap of 1000 replicates. The tree was drawn to scale.

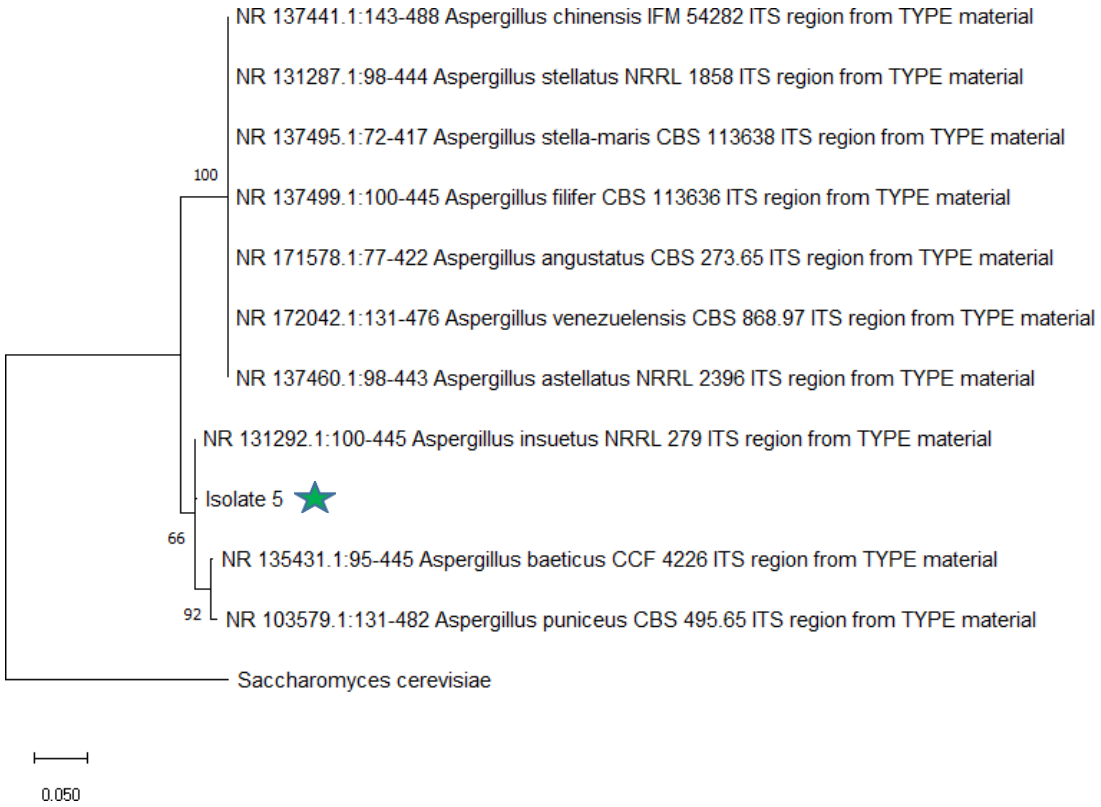

Figure 2.3.7. Phylogenetic relationships between the initially unknown isolate 5 and 11 other species based on the ITS rDNA region. The outgroup *Saccharomyces cerevisiae* was used to root the tree. Isolate 1 is indicated by a green star. The phylogenetic tree was generated with the maximum likelihood method based on the Tamura-Nei model with a bootstrap of 1000 replicates. The tree was drawn to scale.

2.4. Discussion

Fungal isolates were isolated from soil samples which were studied using molecular and morphological identification.

Metarhizium anisopliae

Two of our fungal isolates (isolates 1 and 3) were identified as *Metarhizium anisopliae* as shown in Table 2.3.1. *Metarhizium* is the most common genera of entomopathogenic fungi that are used as a biological control agent throughout the world (Gebremariam, Chekol and Assefa, 2021). They are found in soil, in the rhizosphere of plants, cadavers of arthropods as saprophytes and are known to be parasites to insects and ticks (Schrank and Vainstein, 2010).

They are used in controlling chewing and sucking agricultural insect pests and play a vital role in the IPM (Gebremariam, Chekol and Assefa, 2021). They are also known as green muscardine fungus that infects about 200 insect pests (Gebremariam, Chekol and Assefa, 2021).

In this study, *M. anisopliae* (isolate 1) was mostly white with a dark green centre and a dark green circle towards the end. The reverse side was mostly white with a faded olive-green colour while the centre was mostly olive green as shown in Table 2.3.2. Whereas, *M. anisopliae* (isolate 3) was mostly yellow with some white and olive green in the centre while the reverse side was yellow with the outskirts being white as shown in Table 2.3.2. Similarly, when looking at the morphology of *Metarhizium* at a macroscopic level, their colonies have been reported to be greenish on the front whereas the rear side of the colonies are brownish, white, and yellowish-white (Gebremariam, Chekol and Assefa, 2021). This was also in agreement with Bridge *et al* (1993) who described *M. anisopliae* as being yellowish green, olive green, and dark-herbage green in colour. This showed that as much as *Metarhizium* species may vary due to phenotypic plasticity and genetic variability as they are found in different locations, some consistent morphological features help with their identification.

Both *M. anisopliae* had a flat to slightly raised elevation and a circular colony shape (Table 2.3.2) which were in agreement with the results by Gebremariam *et al* (2021), where *M. anisopliae* was described to have a round colony shape and flat to slightly raised elevation. The microconidia of *M. anisopliae* have been said to be ellipsoid, cylindrical or oval in shape (Gebremariam, Chekol and Assefa, 2021; Erler *et al.*, 2015) which is contrary to the results obtained in our study as ours was found to be oblong oval for both isolates (Table 2.3.3). *M. anisopliae* (isolate 1) had conidia which was 7.218 ± 0.685 while *M. anisoplae* (isolate 3) was 7.527 ± 0.365 as seen in Table 2.3.3. Conidia of *M. anisopliae* has been reported to be 7–9 µm long (Erler *et al.*, 2015) whereas it has also been reported to be 5-8 and 10-14 µm long (Zimmermann, 2007). The conidia in this study were within the size range that was reported by these two studies. This further classifies the isolates to be *Metarhizium* species.

The phylogenetic analysis in Figure 2.3.5 showed that the initially unknown isolates 1 and 3 are isolates of the *Metarhizium* species. Both isolates formed a clade with *M. anisopliae* ARSEF 7487 and *Metarhizium phasmatosae* BCC 49272. They are closely related to *M. anisopliae* ARSEF 7487 and the lack of branching between the two isolates (1 and 3) shows that the isolates are identical.

Fusarium foetens

The second isolate was identified to be 100% *Fusarium foetens* CBS 110286 as shown in Table 2.3.1. The *Fusarium* genus consists of filamentous fungi that vary in their morphological and ecological traits (Mirghasempour *et al.*, 2022). They are known to cause damage to agricultural products and the genus also consists of opportunistic human pathogens (Mirghasempour *et al.*, 2022). These species have been found in Europe, Canada and the USA (González-Jartín *et al.*, 2019). In this study, *F. foetens* was mostly purple-red with brownish-white outskirts, while the reverse side had the same colour as the front but much darker and the colony covered the entire petri dish evenly (Table 2.3.2). The colony morphology of *F. foetens* CBS 110, 286 reported by Liu *et al* (2023) had some similarities in terms of the colony covering the entire petri dish and the reverse colony having a purplish-red colour in the centre. What was contrary was that the front colony formed thick white tufts whereas in this study it had thin white aerial mycelium but the main colour was purple-red that spread from the centre till towards the ends.

The microconidia was mostly ovoid with a few conidia irregularly shaped. *F. foetens* CBS 110,286 caused root rot of Lavender in China, has microconidia that are oval to elliptical and were 4.2–12.0 μm × 2.0-3.8 μm in size (Wei *et al.*, 2023). The results in Table 2.3.3 were similar to the report by Wei *et al* (2023) with the size of the conidia being within the given range of the conidia reported in their study.

Neocosmospora rubicola

The fourth isolate was identified as *Neocosmospora rubicola* CBS 101018 as shown in Table 2.3.1. *Neocosmospora* genus (Hypocreales, Nectriaceae) are found widely distributed in soil, plant debris, and water and air (Sandoval-Denis, Lombard and Crous, 2019). This genus consists of a plant (Riaz *et al.*, 2022) pathogenic group that is considered to be important and has almost 500 different plant hosts that have been recorded with the main one being potatoes which causes potatoes to dry up and causes their roots to rot (Sandoval-Denis, Lombard and Crous, 2019). Species of this genus survive both in living and non-living organisms (Riaz *et al.*, 2022). The results showed that the colony of *N. rubicola* covered the entire petri dish evenly and had a pale yellow colour on both sides with a thin white aerial mycelium in the front view (Table 2.3.2). These morphological features were identical to the findings of Kim *et al* (2017) and Riaz *et al* (2022) for *N. rubicola* (FCBP 1565). *N. rubicola* strain CBS 320.73 had a cream to pale yellow colour on both sides for their colony grown on PDA which was similar to our results. The conidia of our *N. rubicola* were fusoidal to ellipsoidal with some curving at both

ends (Table 2.3.4) and had a size of 13.727±3.358 and 5.593±0.803 (Table 2.3.3). On the contrary, it has been reported to be ellipsoidal to cylindrical with their size being 2-4 x 1-2 µm (Riaz *et al.*, 2022) while Lombard *et al* (2015) reported the shape to be fusiform to ellipsoidal which was in agreement with this study. Micro conidia reported by Riaz *et al* (2022) were 8-12 x 1-2 µm in size which is slightly smaller than the size obtained in this study.

The phylogenetic analysis in Figure 2.5.6 showed that the unknown isolate 4 is an isolate of the *Neocosmospora* species. Isolate 4 formed a clade with *N. rubicola* CBS 101018 and the lack of branching within the clade shows that isolate is *N. rubicola* CBS 101018.

Aspergillus insuetus

The fifth and last isolate was identified as *Aspergillus insuetus* NRRL 279 as shown in Table 2.3.1. The results show *A. insuetus* to be black/ greyish in the centre and white at the margin which is floccose (Table 2.3.2). The morphological features are closely similar to *A. insuetus* CBS 107.25T, which was isolated in South Africa (Houbraken *et al.*, 2007). *A. insuetus* CBS 107.25T = NRRL 279 which is the same as the strain obtained in this study (Samson, 2014). There was not much information provided on this species concerning its isolation, identification and characterisation.

The phylogenetic analysis in Figure 2.5.7 showed that the unknown isolate 5 is an isolate of the *Aspergillus* species. Isolate 5 formed a clade with *A. insuetus* NRRL 279, *Aspergillus bacticus* CCF 4226 and *Aspergillus puniceus* CBS 495.65. The isolate was closely related to *A. insuetus* NRRL 279 and the lack of branching within the clade shows that isolate is *A. insuetus* NRRL 279.

In conclusion, the use of molecular and morphological techniques is useful in the identification of fungal isolates. Morphological identification for certain fungal strains may be inconsistent therefore the use of molecular identification helped with the accurate identification of fungal isolates.

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Chapter 3:

Virulence characterisation of five fungal isolates against *Tenebrio molitor* larvae.

3.1. Introduction

Insects can easily adapt to a variety of terrestrial habitats (Vigneron *et al.*, 2019). They are considered to be beneficial when they play a major role in activities that are advantageous to humans, or the environment such as being involved in pest control or being used as a viable food source (Vigneron *et al.*, 2019). They are considered to be pests when they cause damage to crops therefore decreasing crop production or when they pose a threat to the health of humans (Pestano *et al.*, 2019).

Tenebrio molitor (Coleoptera: Tenebrionidae), a yellow mealworm, is indigenous to Europe and widely distributed worldwide (Ramos-Elorduy *et al.*, 2002). They have a short life cycle and are reared on wheat bran and fruits and vegetables are also included in their diet (Makkar *et al.*, 2014). They are classified both as a pest and a beneficial organism (Vigneron *et al.*, 2019). They are considered to be beneficial insects due to their ability to break down polystyrene and plastic waste. They are also an essential food source due to their high nutrient content and can potentially be used in animal feed providing protein (Vigneron *et al.*, 2019). They are considered pests because they infest stored food products and cause damage to plant products by affecting their total mass and nutritive value (Siemianowska *et al.*, 2013). They not only consume but also contaminate stored products by their shedding and waste matters (Siemianowska *et al.*, 2013). Adult beetles cause high losses of grain products and seeds (Houbraken *et al.*, 2016). They are a host to a range of pathogens and parasites which includes protozoa and entomopathogenic microorganisms (Vigneron *et al.*, 2019).

Virulence characterisation of fungal isolates is important for the identification of virulent isolates that can potentially be used as biological control agents for insect pests (Mascarin *et al.*, 2013). The virulence of fungi has many facets and depends on abiotic factors (Mantzoukas *et al.*, 2022). A single factor does not determine the pathogenicity of a fungal isolate but is dependent on many pathogenicity determinants (Shah, Wang and Butt, 2005).

Four steps are involved in the virulence of entomopathogenic fungi (EPFs) which are adhesion, germination, differentiation and penetration. Virulence is first seen when the fungus attaches to the host's cuticle (Shahid *et al.*, 2012). Adhesion of the spores to the host's cuticle allows

for successful infection. Following adhesion, the next virulent factor is the enzymes produced which function to hydrolyse the cuticle of the host. These enzymes are lipases, proteases and chitinases that are produced sequentially (Smith, Pekrul and Grula, 1981).

Virulence characterisation also includes in-vitro assessment where primary parameters such as spore germination, vegetative growth and spore production are observed as well as in-vivo assessment (bioassay evaluation against an insect host) (Gebremariam, Chekol and Assefa, 2021). Fast growth rate and rapid sporulation or high spore number are some of the characteristics that are considered to be important for fungal virulence (Dotaona *et al.*, 2015). Several other factors known to influence fungal virulence to a certain extent include initial host infection time, how long the disease incubation period lasts, the rate at which the fungus spreads and environmental factors (Mantzoukas *et al.*, 2022). Interestingly, conidia also have specific traits that lead to virulence; these include their germination rate, spore size and adhesion (Shah, Wang and Butt, 2005).

EPFs vary based on their mode of action and their virulence (Shahid *et al.*, 2012). The ability of EPFs to adhere to the cuticle and penetrate the host integument determines whether the infection will be successful (Shahid *et al.*, 2012). After the death of the insect pests, there is external sporulation (Mantzoukas *et al.*, 2022). The spores are infectious agents and when other insect pests come across it, they become infected (Mantzoukas *et al.*, 2022).

This study, mainly looked at vegetative growth rate and the ability of the fungal isolates to cause infection resulting in mortality. The ability of a fungal isolate to cause death implies that it successfully went through the four steps that are involved in virulence (adhesion, germination, penetration and differentiation).

3.2. Methods and Materials

3.2.1. Fungi source

Five fungal isolates were used for this study, namely, two *Metarhizium anisopliae* ARSEF 7487, *Fusarium foetens* CBS 110286, *Neocosmospora rubicola* CBS 101018 and *Aspergillus insueius* NRRL 279. We had previously isolated and characterized these isolates.

3.2.2. Vegetative growth rate

The method was adapted from (Bugeme *et al.*, 2008)

Each of the fungal isolates (1ml suspension of 10^8 spores/ml) was spread on fresh Potato Dextrose agar (PDA) plates using a spreader. Parafilm was used to seal the plates and incubated at 25℃ for 72 hours under complete darkness. A 5mm mycelium plug was cut from the fungi cultures and then placed on the centre of fresh PDA plates; three replicates were done for each isolate. The plates were sealed and then incubated at 25℃ for 15 days under complete darkness. The mycelium growth was recorded every 2^{nd} day for 15 days.

3.2.3. Counting

Well-sporulated plates were used and 6-8ml of sterile aqueous solution containing 0.05% Tween 80 was pipetted onto the plates. Tween 80 acts as an emulsifier and allows for the spores on the plate to easily de-attach from the plates. A sterile loop was used to scrape off the fungal growth therefore releasing the spores. The solution was collected and transferred into a sterile 50ml falcon tube using a pipette. The solution was vigorously mixed using a vortex mixer for 1-2 minutes at 2850 rpm. This was filtered into a sterile falcon tube using a double-layered cheesecloth, which removes the mycelium from the solution. This was transferred into 2ml Eppendorf tubes then centrifuged for 10 minutes at 1800xg and the supernatant was poured off leaving about 0.5ml or the last few drops. The conidia was re-suspended with 1ml of Tween 80 solution and was vortexed at 2850 rpm. This was placed in a 4˚C incubator until further use.

A 1:100 dilution series was done and a Neubauer haemocytometer was used to count the fungal spores.

Calculation:

Area of large squares= 1mm x 1mm = $1mm^2$

Depth of haemocytometer view side: 0.1mm

Volume= 0.1mm x $1mm^2$ = $0.1mm^3$

$0.1mm^2$ = 0.1µl = 100 nl

Formula:

Average cell count per square x Dilution factor x 10^4

3.2.4. Virulence test

Ten fresh larvae were surface sterilized with 70% ethanol and then immersed into a spore suspension (10^8) of each isolate (five isolates in total) for 10-20 seconds in a sterile petri dish

whereas the control was immersed in a sterile 0.05% Tween 80 solution. The larvae were placed on sterile dry filter paper to remove the excess suspension liquid and dried under a laminar flow. After drying, the larvae were then transferred into 30ml plastic cups lined with a double layer of wet sterile filter paper to create a moist environment then closed and incubated at 25 °C. Each treatment was replicated three times and the number of dead larvae was recorded every 2nd day for 8 days as infection takes longer using fungi. The entire bioassay was repeated three times to ensure biases and reliability.

Figure 3.2.3. Set up for the virulence test. A 30ml cup layered with two moist filter papers with 10 larvae.

3.2.5. Dose-dependent virulence test

The highest virulent fungal isolate was selected and subjected to a dose-dependent test. Ten *T. molitor* larvae were immersed in two different concentrations of the spore suspension (10^6, 10^7) that were prepared using a Neubauer haemocytometer. The control was immersed in a sterile Tween 80 solution and each treatment was replicated three times. The rest of the methods done were the same as the above experiment for 8 days.

3.2.6. Data analysis

An Analysis of variance (ANOVA) test was done to calculate the statistical difference. The test was considered significantly different when the p-value < 0.05. If the ANOVA is significantly different, a Tukey post hoc test was done to find out which groups were significantly different from one another.

3.3. Results

Five fungal isolates were subjected to a virulence test even though only two fungal isolates were identified as EPFs (*M. anisopliae* ARSEF 7487). The other isolates were tested to see whether they were potentially parasitic to insects. The vegetative growth was a preliminary test before testing for virulence was done. Figure 3.3.1, served to show as an example of how the measurements were taken as seen by the markings on the plates and showed how growth progressed between experimental days. Figure 3.3.2 illustrates the growth of all isolates.

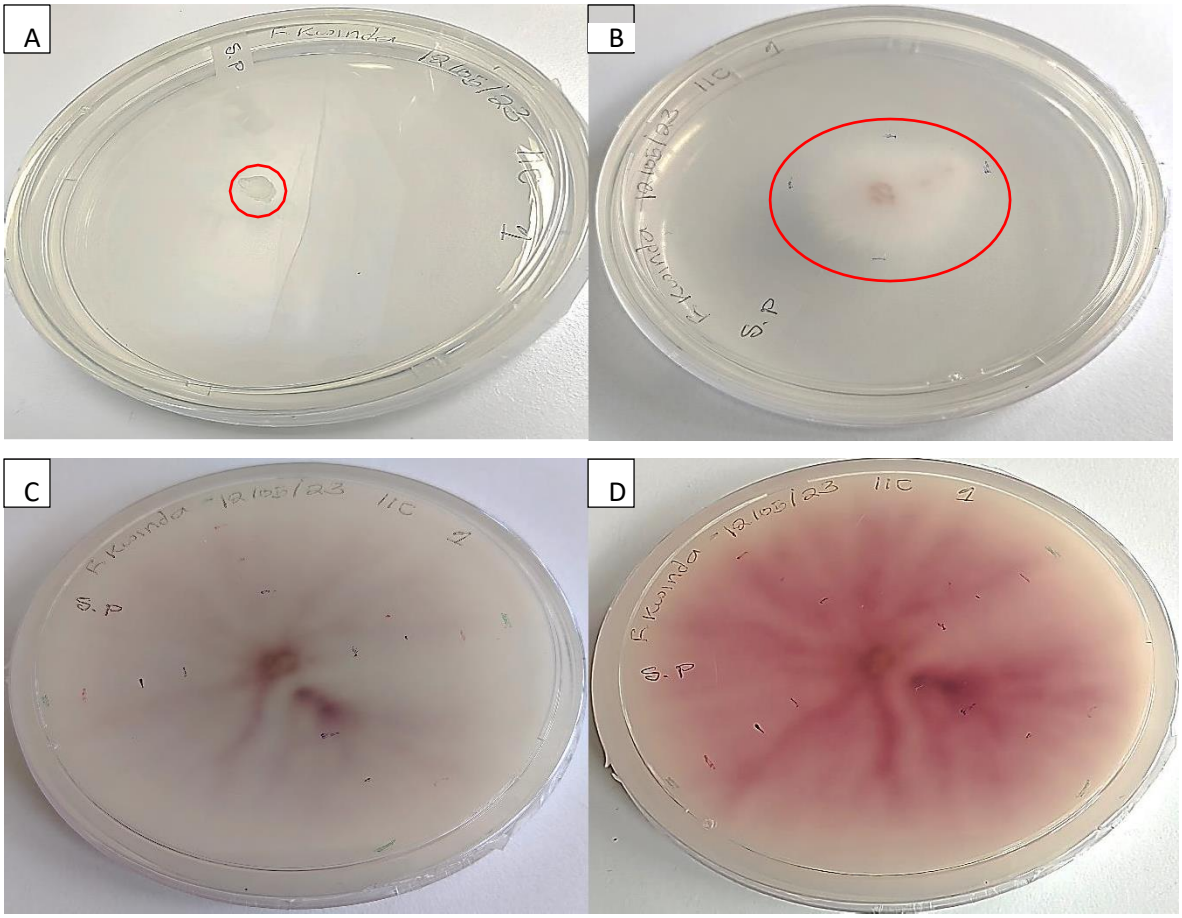

Figure 3.3.1. Vegetative growth rate progress of *F. foetens* CBS 110286. A) 5mm mycelium plug on the first day, B) Growth on the 3rd day, C) Growth on the 9th day and D) Growth on the 15th day.

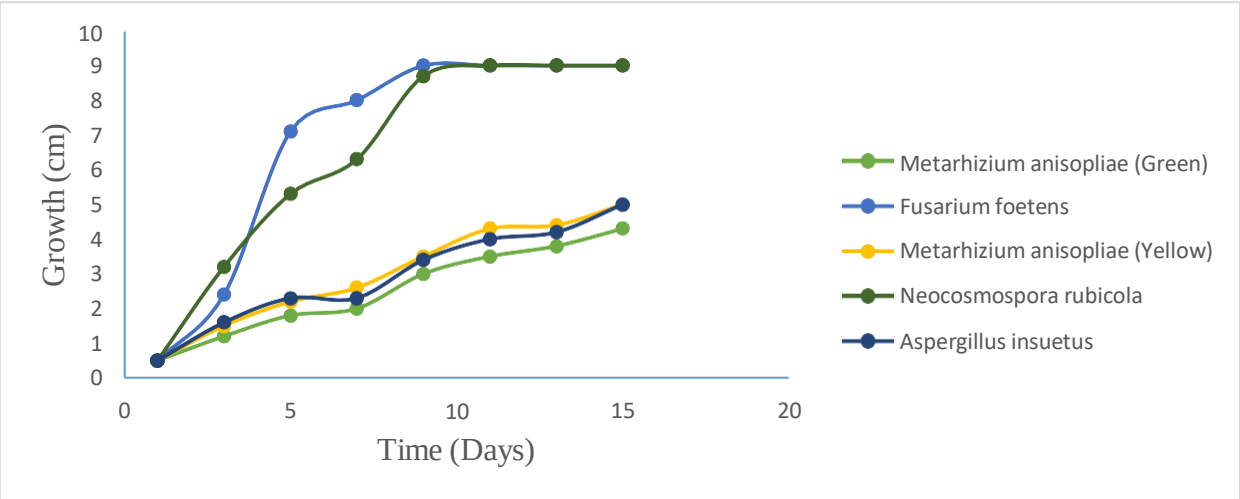

Figure 3.3.2. Vegetative growth (cm) of each fungal isolate taken every second day for 15 days.

From the results obtained for the vegetative growth rate, *F. foetens* and *N. rubicola* reached their final growth of 9 cm on the ninth day while *M. anisopliae* had the lowest growth rate. The statistical analysis indicated significant differences (p-value < 0.05) among the isolates (Table 3.3.2, supplementary information). The means of the following pairs are significantly different: *M. anisopliae* (Green) vs *F. foetens*, *M. anisopliae* (Green) vs *N. rubicola*, *F. foetens* vs *M. anisopliae* (Yellow), *F. foetens* vs *A. insueius*, *M. anisopliae* (Yellow) vs *N. rubicola*, *N. rubicola* vs *A. insueius*. The two *M. anisopliae* isolates were named *M. anisopliae* (Green) and *M. anisopliae* (Yellow) to differentiate them based on their fungal culture colour.

The larvae, *T. molitor*, were immersed in the fungal solution of each fungal isolate and the mortality was recorded as seen in Figure 3.3.3.

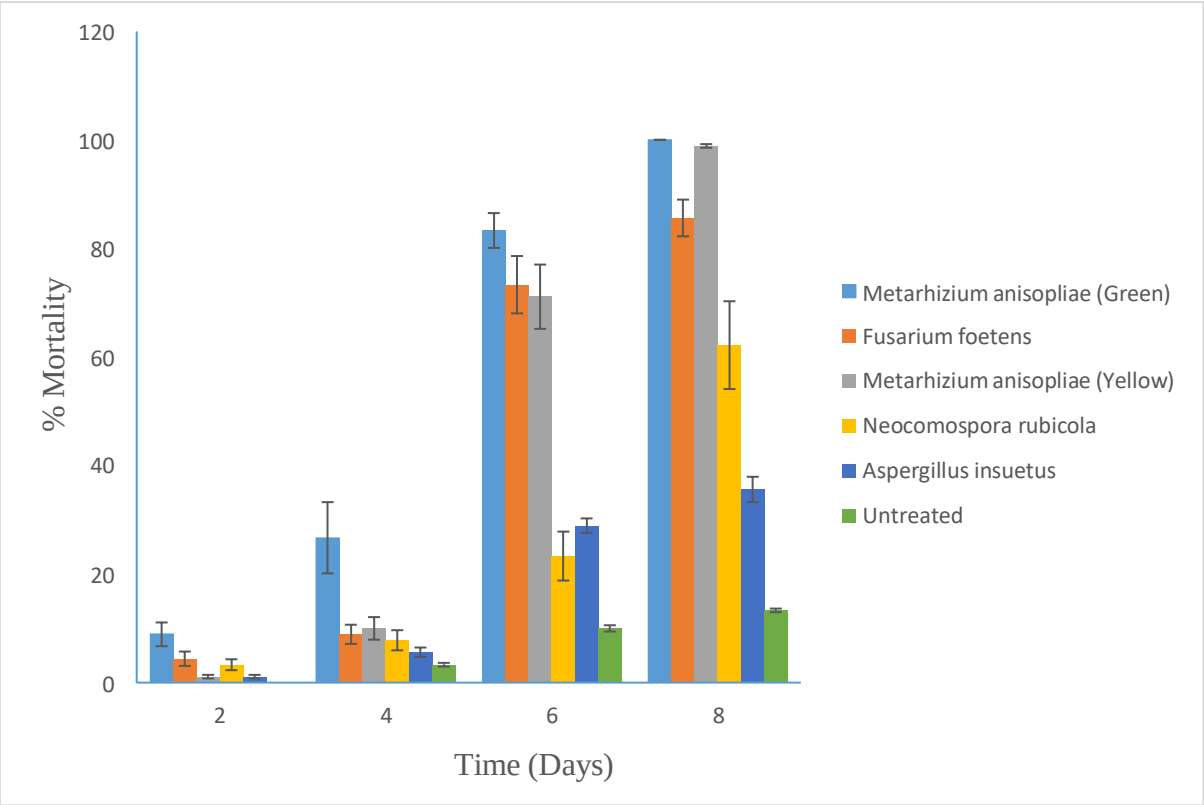

Figure 3.3.3. Mean mortality (%) of *T. molitor* when treated with five different fungal isolates. The error bars represent the standard error of three replicates.

In the figure above, the mortality percentage for each isolate increased as the days went by with only *M. anisopliae* (Green) achieving 100% mortality on the last day (8^{th} day). *M. anisopliae* strains are shown to be highly virulent. The statistical analysis indicated a significant difference (p-value < 0.05) therefore the null hypothesis (there is no difference between group means) was rejected (Table 3.3.3a, supplementary information). The mean mortality (%) of the following days were shown to be significantly different: 2 vs 6, 2 vs 8, 4 vs 6 and 4 vs 8. While day 2 vs 4 and day 6 vs 8 were not significantly different (Table 3.3.3b, supplementary information).

In Figure 3.3.3, the results recorded were the total mortality meaning that whether or not mortality was caused by fungal isolates it was recorded. Larvae that died due to fungal infection showed symptoms such as hardening of the body and external sporulation when placed on a white trap (moist environment) for a few days as seen in Figure 3.3.4.

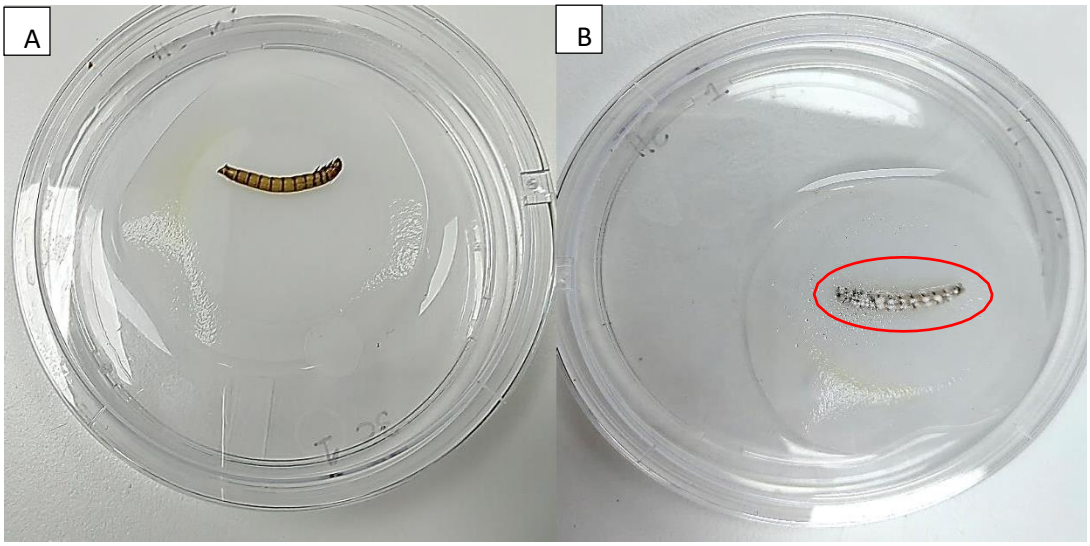

Figure 3.3.4. The development of symptoms on an infected insect in a white trap. A) Dead hardened larvae placed on a white trap, B) larvae developing external sporulation.

The mortality caused by fungal infection was considered the final result and is recorded in Figure 3.3.5.

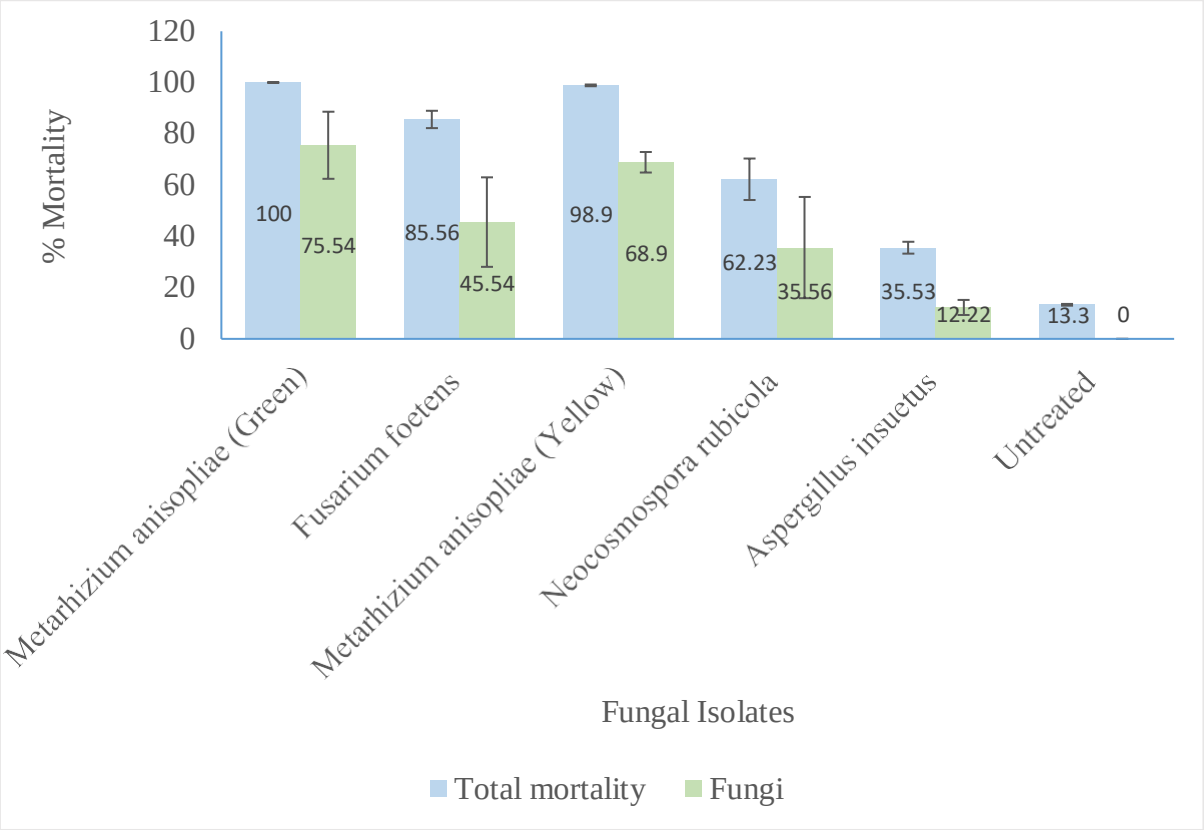

Figure 3.3.5. The total mortality vs actual mortality that was caused by the fungal isolate. The blue bars represented the total mortality that did not consider whether or not the death of larvae was caused solely by the fungal isolate. While the green bars were the mortality caused solely by the fungal isolate. The error bars represent the standard error.

M. anisopliae (Green) had the highest mortality (75.54%), followed by *M. anisopliae* (Yellow) with 68.8% mortality, while *A. insuetus* has the lowest mortality (12.22%). Since the p-value < 0.05 it means that there was a significant difference among the isolates and the control. The mean mortality of the following pairs was statistically different: *M. anisopliae* (Green) vs *A. insuetus*, *M. anisopliae* (Green) vs control, and lastly, *M. anisopliae* (Yellow) vs control.

The highest virulent isolate which was *M. anisopliae* (Green) in this case was subjected to a dose-dependent test. This was to evaluate whether mortality would remain the same if a lower concentration was used. The 10^8 spores/ml result was taken from the results in Figure 3.3.5.

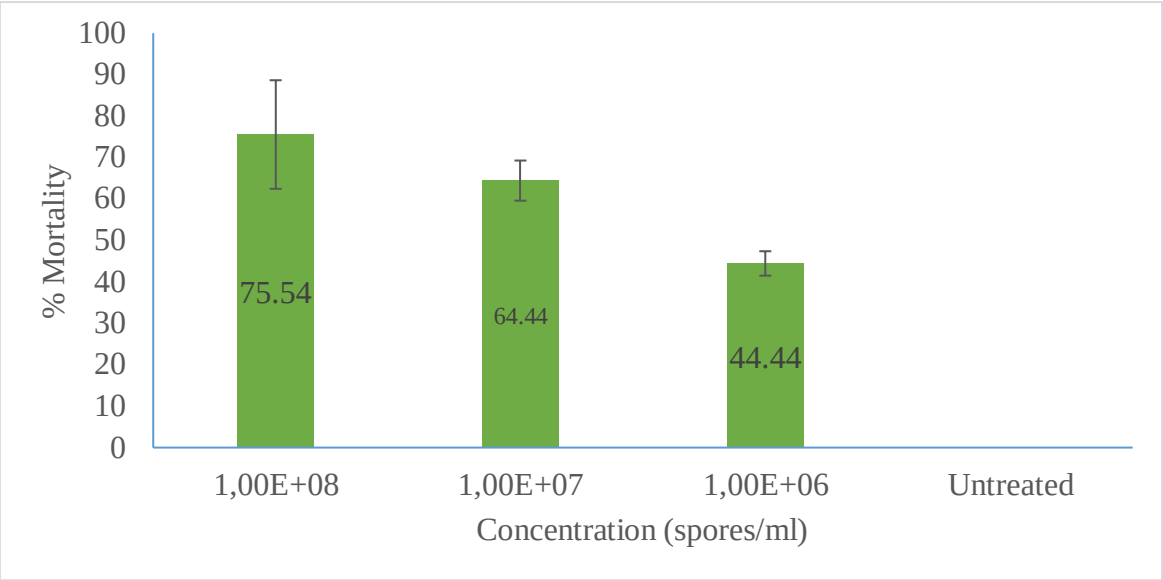

Figure 3.3.6. Dose-dependent test of *M. anisopliae* (Green) against *T. molitor*. The error bars represented the standard error.

Mortality percentage decreased with decreasing concentration. The error bars were a representation of the standard error for the three replicates which accounts for why there is an error bar for the untreated. There was a significant difference (p-value < 0.05) (Table 3.3.6a, supplementary information), however, the significant difference was between each concentration with the control. There was no significant difference between the concentrations meaning there is no significant difference in mortality caused by the different concentrations.

The LC_{50} was calculated and found to be 1.9×10^6 spores/ml meaning that half of the population can be killed using this specified concentration (Table 3.3.6b, supplementary information).

3.4. Discussion

When it comes to selecting the highest virulent strain for the control of insect pests, it is important that we also look at other virulence factors such as vegetative growth (Rodrigues, Luz and Humber, 2017). Common to the life cycle of EPFs, vegetative growth occurs in the insect haemocoel. This vegetative growth allows the fungi to disperse and colonize the host's circulatory system (Shahid *et al.*, 2012). Fast growth is a pathogenicity determinant that positively correlates with fungal virulence therefore, screening of this parameter could be important in determining highly virulent isolates (Gebremariam, Chekol and Assefa, 2021).

M. anisopliae ARSEF 7487 had a colony diameter of 20 mm (Green) and 26 mm (Yellow) for its isolate on the 7th day (Figure 3.3.2). Contrary to this study, a study by Gebremariam, Chekol and Assefa (2021), a radial growth ranging from 5.17 to 9.83 mm/day was recorded which would be 36.19 to 68.8 mm on the 7^{th} day which was higher than the observed growth in this present study. The colony diameter of *F. foetens* CBS 110286 was 90mm after 7 days (as seen in Figure 3.3.2). In a study by Schroers *et al* (2004) and Liu *et al* (2023) colonies of *F. foetens* on PDA ranged from 51 to 68 mm in diameter after 7 days which was much higher as compared to the colony diameter recorded in the present study. The colony diameter of *N. rubicula* CBS 101018 on the 7^{th} day was 63 mm (Figure 3.3.2). Whereas, In a study by Kim *et al* (2017) and Lombard *et al* (2015), the colony on PDA reached 35 to 40 mm in diameter after 7 days which was much higher than the reported in the present study. For the four fungal isolates what can be observed is that there was a difference in the colony diameter that was recorded in the present study to those that are recorded by other studies. The variability of the colony growth might be influenced by the fungal species and the geographical origin of these isolates (Gebremariam, Chekol and Assefa, 2021). For *A. insuetus* NRRL 279 the colony diameter on the 7^{th} day was 23 mm which is similar to the one recorded by (Houbraken *et al.*, 2007), where the colony diameter on the 7^{th} day ranged from 23 mm to 41 mm.

Therefore in the results presented, *F. foetens* and *N. rubicola* were the fastest-growing fungi while *M. ansiopliae* (Green) had the slowest growth. Fast growth was said to be correlated with high virulence but this was later found to not be true in this study. What was found is that the growth rate does not relate to the virulence.

Members of the *Metarhizium* genus are entomopathogenic fungi (Inglis *et al.*, 2008). Both of the *M. anisopliae* ARSEF 7487 isolates in this study were highly virulent with a mortality greater than 50%. Only *M. Anisopliae* (Green) managed to reach 75% mortality against *T. molitor* (as seen in Figure 3.3.5). Similarly, in another study, *M. ansiopliae* caused 75% mortality of whiteflies after 7 days (Sanchez-Pena, Lara and Medina, 2011). This was also in agreement with a study by Habtegebriel *et al* (2016), where 75% mortality of *Galleria mellonella* was recorded when a concentration of 10^8 spores/ml was used. Fungal isolates vary greatly among each other when it comes to viability and pathogenicity (Shahid *et al.*, 2012), therefore explaining the differences in virulence of the two *M. anisopliae* isolates (Figure 3.3.5).

Fusarium is a soil-borne pathogen that causes huge losses in yields of potatoes which are the fourth largest crop with China and India using it as a staple crop (Liu *et al.*, 2023). The first report made of *F. foetens* was when it caused wilt in hybrid begonia (Liu *et al.*, 2023). *F. foetens* has also been reported as a pathogen of rooibos seedlings in South Africa and Solanaceae crops, such as tomatoes, bell peppers, and cayenne peppers (Liu *et al.*, 2023). Rooibos is an indigenous legume crop in South Africa and rooibos seedlings in nurseries are susceptible to damping off with the disease caused by *fusarium* (Lamprecht and Tewoldemedhin, 2017). This was the first report made in South Africa on *F. foetens* (Lamprecht and Tewoldemedhin, 2017). *F. foetens* CBS 110286 was the third most virulent isolate that caused almost 50% of mortality on *T. molitor* as shown in Figure 3.3.5. In a study done by Pestano *et al* (2019), the *Fusarium* genus was categorized to be entomopathogens; parasitic to insects. Since it was also classified as a plant pathogen, this may suggest that it has multiple lifestyles. The mortality was below average therefore it might be a weak entomopathogen.

N. rubicola is a filamentous fungus which causes rots in crops such as fruits, vegetables and oilseeds (Riaz *et al.*, 2022; Mukherjee and Vilcinskas, 2018). Since *N. rubicola* is commonly identified as a plant pathogen, therefore mortality of *T. molitor* caused by this fungi (35.56%) as shown in Figure 3.3.5 might be caused by something else such as the mycotoxins they produce. They produce harmful toxins not only to the health of the plants but also to animals and humans (Riaz *et al.*, 2022; Mukherjee and Vilcinskas, 2018). *N. rubicola* is a new threat therefore not much information has been given on the mycotoxins that they produce (Riaz *et al.*, 2022). However, various *Neocosmospora* species are associated with certain mycotoxins that play various activities and are associated not only with plants but with animals and humans (Riaz *et al.*, 2022). Two of the known toxic metabolites produced by *Neocosmospora* species include furanoterpenoids (promote necrotic activity) and ipomeanols (interfere with normal cell growth) which might have been the cause of *T. molitor* mortality (Riaz *et al.*, 2022).

A.insuetus are found in abundance in terrestrial habitats but have also been found in marine ecosystems (Chi *et al.*, 2020). In Figure 3.3.5, the *A. insuetus* NRRL 279 caused the lowest mortality compared to the rest of the fungal isolate. In a study by Pestano *et al* (2019), some *Aspergillus* species such as *Aspergillus niger, Aspergillus flavus* and *Aspergillus versicolor* were categorized to be non-pathogenic colonizers meaning that they are unable to parasitize an insect. Therefore, this might also be the case for *A. insuetus* used in this study.

In conclusion, *M. ansiopliae* ARSEF 7487 had the slowest growth but had the highest virulence therefore the vegetative growth of fungal isolates is not related to its virulence. Other factors that contribute to virulence such as germination rate should be looked at. Larval mortality was significantly affected by the type of fungal isolate used, parasitic fungal isolates had the highest mortality while plant or non-parasitic fungal isolates had the lowest mortality.

3.5. References

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Chapter 4:

Effect of combined application of putative entomopathogenic nematode and entomopathogenic fungi against *Tenebrio molitor* larvae.

4.1 Introduction

Microbial control agents are seen as important crop protection tools in the European Union (EU) (Erler *et al.*, 2015). These microbial control agents include fungi and nematodes that are known to kill problematic insect pests.

Nematodes have different modes of life which are; free-living, parasitic to plants and animals, insect associates, entomopathogenic, terrestrial and marines (Heonil Kang, 2013; Sudhaus, 2011). The free-living living is known for being beneficial to the terrestrial and marine ecosystems (Heonil Kang, 2013). *Cruznema* is a nematode genus that belongs to the family of Rhabditidae and it is distributed all around the world except for Antarctica (Dongzhen *et al.*, 2020; Tahseen *et al.*, 2012). It can either be free-living nematodes that feed on bacteria, bacterivores, or it can be a parasite to crickets and molluscs (Dongzhen *et al.*, 2020). As usual with some other rhabditids, some species of *Cruznema* are associated with insects and their association is often with Coleopterous and Orthopterous species (Tahseen *et al.*, 2012). Some examples are *Cruznema magdalensis* which has been found in crickets in the United States and Brazil (Reboredo and Camino, 1998), *Cruznema campestris* target host is *Coremia signaticollis*, which belongs to a family of beetles (Reboredo and Camino, 2000), *Cruznema brevicaudutum* found in the intestines of mole cricket in India (Latheef and Seshadri, 2011). Some other *Cruznema* species are found in soil or plant residues (Tahseen *et al.*, 2012).

For fungi, the most common entomopathogenic fungi (EPFs) species for control of problematic insect pests are from the *Metarhizium* genus. *Metarhizium* is one of the most studied genus that are effective in controlling 200 insects, especially the Coleopteran insect order (Pilz *et al.*, 2011). *Metarhizium* resides in the rhizosphere and rhizoplane, making them a promising candidate for controlling soil-dwelling insect pests. With this ability, the use of *Metarhizium* species is being exploited for the control of insect pests (Shah, Ash and Wilson, 2023). *Metarhizium anisopliae* strains (Hypocreales: Cordycipitaceace) are present in soils throughout the world (Shahid *et al.*, 2012). Their discovery as a biological control agent was found in the 1880s and they are presently used to control four types of insect pests; beetles, termites, locusts

and spittlebugs (Shahid *et al.*, 2012). They are a member of Hyphomycetes which can infect a wide range of insect order and kill them using the toxins that they produce which can overcome the host defence responses (Mantzoukas *et al.*, 2022; Shahid *et al.*, 2012). Hyphomycetes are also considered to be hemibiotrophic meaning that they have a well-defined parasitic phase inside the host and a saprophytic phase on the death of their hosts (Shahid *et al.*, 2012).

The use of EPNs is increasing as many people are taking an interest in them but they also face certain restrictions (Acevedo *et al.*, 2007). One of them is that when they are used in a laboratory setting, they can cause death to the insect host within 24-48 hours but when the setting is the field their infectivity is unpredictable (Georgis and Gaugler, 1991). As for the use of EPFs, environmental factors such as moisture, pH and temperature affect the growth of *M. ansiopliae* (Journal *et al.*, 2017). Therefore, there are some limiting factors in making use of *M. anisopliae* alone since the environmental factors may affect their infectivity as well as EPNs.

Therefore, researchers have been looking at the combination of control agents to increase pathogen efficiency in the field. A promising application for increased pest control is the joint use of nematodes which mainly attack soil-dwelling insect pests with fungi (Acevedo *et al.*, 2007). In the establishment of the combined use of microbial agents is that we expect that it will result in more efficient pest control and the kill time will be greatly reduced (Acevedo *et al.*, 2007). This approach of combined application promises to provide a superior efficacy for controlling insect pests than the single application of the microbial agents (Wakil, Yasin and Shapiro-Ilan, 2017).

There is also growing evidence that the combination of microbial agents may greatly improve the control of pests and this may be mainly due to the synergistic and additive effects of the microbial agents (Ansari, Shah and Butt, 2008). The use of both fungi and nematodes has shown to be a synergistic and additive interaction and they are both safe for the environment and safe for non-target organisms (Wakil, Yasin and Shapiro-Ilan, 2017). Therefore, exploring more of their use together may help with controlling problematic insect pests effectively (Wakil, Yasin and Shapiro-Ilan, 2017). Koppenhofer and Grewal (2005) stated that an additive effect is when there is no interaction between the two agents (act independently), whilst synergistic or antagonistic effects are when the interaction between agents either results in more or less control of pests. The synergy between two biocontrol agents allows for the acceleration of killing the insect pest therefore it is favoured to use such agents for reduction in killing time

(Journal *et al*., 2017). The mechanism of how the combined agents result in synergism remains unclear but it is postulated that one agent alters the behaviour of the host therefore making it more susceptible to the infection of the other agent (Ansari, Shah and Butt, 2008). For example, *M. anisopliae* infected insects tend to be less mobile therefore allowing EPNs to have more time to penetrate the host (Ansari, Shah and Butt, 2008; Ansari, Tirry and Moens, 2005; Ansari et al., 2004). On the contrary, antagonistic interactions are also a possible outcome when two agents are combined, with one example being the one where the combination of *M. anisopliae* with *Steinernema carpocapsae* resulted in an antagonistic interaction (Wakil, Yasin and Shapiro-Ilan, 2017).

This study aimed to do a single application of *M. anisopliae* ARSEF 7487 and *Cruznema* sp. NTM-2021. Subsequently, a combination study of both *M. anisopliae* and *Cruznema* sp. NTM-2021 to see whether the combined application of both microbial agents would result in better control of *Tenebrio molitor* larvae as compared to the single application.

4.2. Methods and materials

4.2.1. A single application of putative EPNs

4.2.1.1. Nematode source

Cruznema nematodes were previously isolated and obtained in the Nematology lab, University of Witwatersrand.

4.2.1.2. Pathogenicity test

Silica sand was used as the media for the pathogenicity test. *Cruznema* sp. NTM-2021 was inoculated in the soil and sterile distilled water was added to achieve 10% soil moisture. *T. molitor* larvae were added and this was closed with parafilm and placed at room temperature. The test was done to confirm that *Cruznema* sp. NTM-2021 causes mortality and to increase the number of infective juveniles (IJs) for the downstream experiments.

Figure 4.2.1.2. Pathogenicity test set up with *T. molitor* larvae as bait. A view of the plate with silica sand inoculated with *Cruznema* sp. NTM-2021 and baited with *T. molitor* larvae.

4.2.1.3. White trap

A white trap consisted of a 90mm petri dish, a 38mm glass watch and a Whatman No.1 filter paper. The glass watch was placed in the middle of the large petri dish. The filter paper was placed on top of the glass watch then distilled water was added. Cadavers from the pathogenicity test were placed on top of the filter paper. IJs emerge from the cadaver and go into the water then collected and counted.

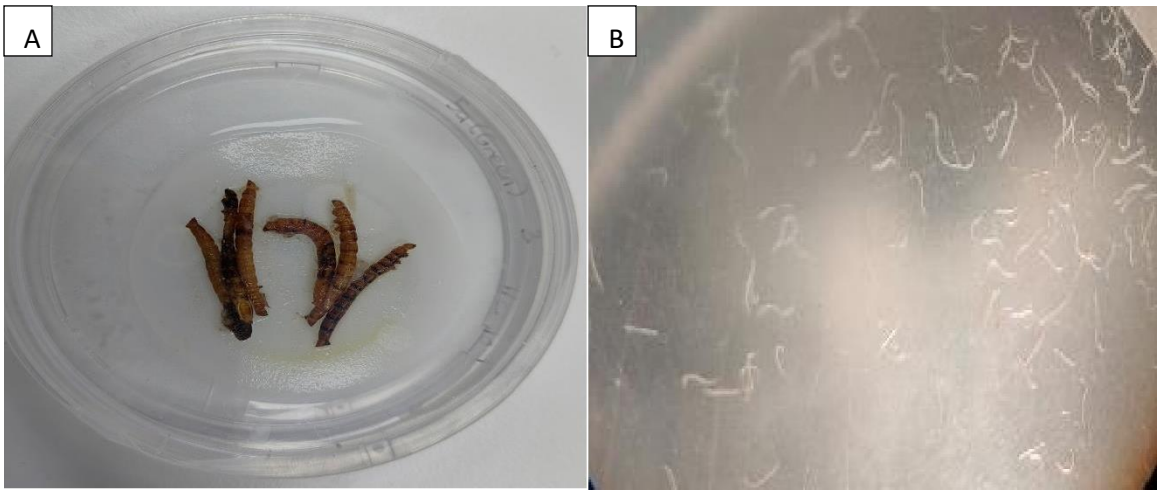

Figure 4.2.1.3. White trap set-up with larvae cadavers. A) Larvae cadavers that were placed in a white trap and B) the nematodes that were in the water.

4.2.1.4. Single application bioassays for EPNs

Sandy loamy soil was autoclaved and dried to ensure that it had 0% soil moisture. 25g of the sterile soil was added in 310 ml of plastic cups and 14% soil moisture of soil field capacity was achieved. The 14% soil moisture included 1ml of the nematode solution which consisted of 1500 IJs. An insect diet (slices of carrot) was added to each container as a food source. There were three replicates with 10 larvae per replicate. The control groups used distilled water and had no nematode solution. Mortality was checked every 2nd day.

Figure 4.2.1.4. An experimental set up of the single application of *Cruznema* sp. NTM-2021. A) Top-view of the set-up before the food source was added and B) Complete set-up with three replicates and one control.

4.2.2. Single application of EPFs

4.2.2.1. Fungi source

Metarhizium anisopliae ARSEF 7487 was used in this experiment. We had previously isolated and identified the fungi which had been proven to be virulent.

4.2.2.2. Single application bioassay for EPFs

Sandy loamy soil was autoclaved and dried to ensure that it had 0% soil moisture. 25g of the sterile soil was added in 310 ml of plastic cups and 14% soil moisture of soil field capacity was achieved. The 14% soil moisture included 1 ml of the spore solution of 10^8 spores/ml. An insect diet (slices of carrot) was added to each container as a food source. The containers were covered with plastic for increased moisture and humidity. There were three replicates with 10 larvae per replicate. The control groups used distilled water and had no fungal solution. Mortality was checked every 2nd day.

Figure 4.2.2.2. An experimental set up of the single application of *M. anisopliae* ARSEF 7487. A view of the set-up with three replicates and one control.

4.2.3. Combined application

Sandy loamy soil was autoclaved and dried to ensure that it had 0% soil moisture. 25g of the sterile soil was added in 310 ml of plastic cups and 14% soil moisture of soil field capacity was achieved. The 14% soil moisture included 1ml of the spore solution of 10^8 spores/ml and 1ml of the nematode solution which consisted of 1500 IJs. An insect diet (slices of carrot) was added to each container as a food source. There were three replicates with 10 larvae per replicate. The control groups used distilled water with no microbial agent. Mortality was checked every 2nd day.

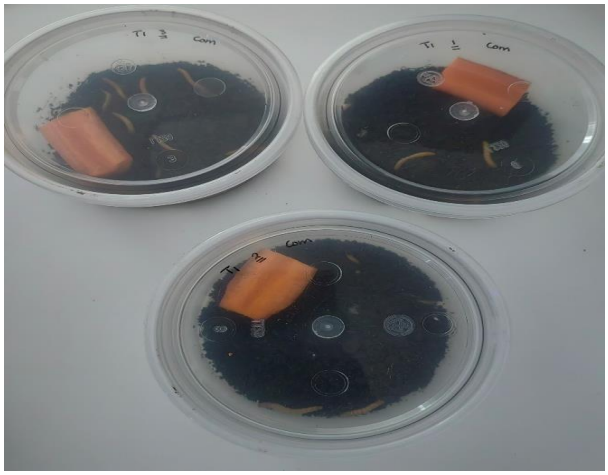

Figure 4.2.3. An experimental set up of the combined application of *Cruznema* sp. NTM-2021 and *M. anisopliae* ARSEF 7487. A view of the set-up with three replicates.

4.2.4. Data analysis

An ANOVA test was done to calculate the statistical difference. The test was considered significantly different when the p-value < 0.05. If the ANOVA is significantly different, a Tukey post hoc test was done to find out which groups were significantly different from one another.

4.2.4.1. Nematode-fungus interaction

A chi-square (X^2) test was used to test the interaction of EPNs and EPFs. A modified procedure by McVay *et al* (1977) was used to determine the type of interaction

For the expected additive proportional mortality, the following formula was used:

$M_E = M_N + M_F (1- M_N)$

M_N and M_F are the observed mortalities caused by the application of EPNs and EPFs alone

$X^2 = (M_{NF} – M_E)\ x2 / M_E$

M_{NF} is the observed mortality rate for the combined application of EPNs and EPFs

The x^2 value was compared to the table value.

- If x^2 values > table value = non-additive effect.
- If the differences $M_{NF} – M_E = D$
    - If D is a positive value then it is synergistic.
    - If D is a negative value it is antagonistic.

4.3. Results

A single application test of *Cruznema* sp. NTM-2021 and *M. anisopliae* ARSEF 7487 were performed so that we would know how the isolates perform when used individually. A more natural environment was created by making use of soil and a food source (slices of carrots).

For the single application of *Cruznema* sp. NTM-2021, 1500 IJs were used per treatment and the mortality is as recorded in the figure below.

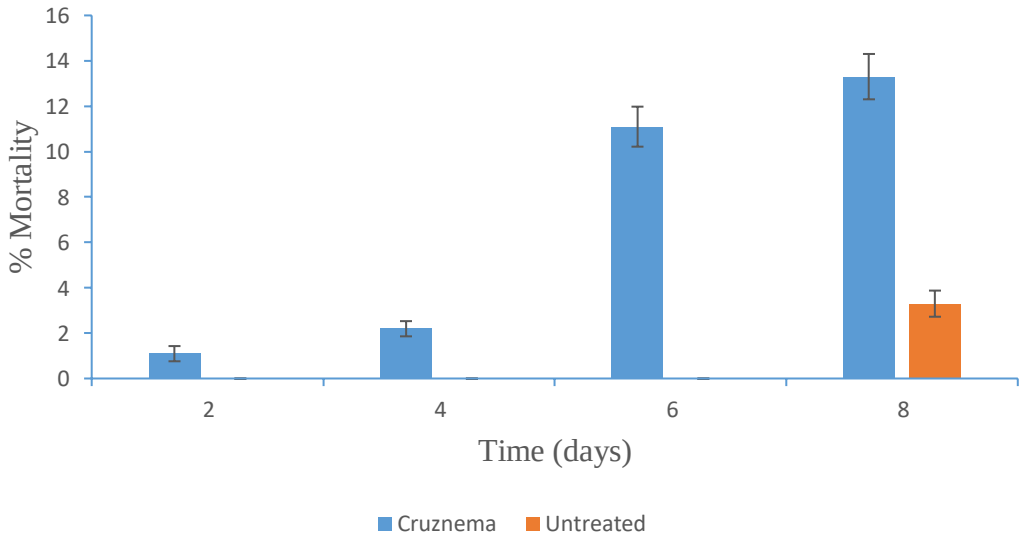

Figure 4.3.1. Mean mortality (%) of *T. molitor* when treated with *Cruznema* sp. NTM-2021. *Cruznema* sp. NTM-2021 was applied at 1500 IJs. ml^{-1}.

The total mortality on the last day (8^{th} day) was recorded to be 13.33% and all dead larvae were shown to be infected by the *Cruznema* sp. NTM-2021 due to the emergence of IJs when placed in a white trap.

ANOVA was done and it showed the test to be significantly different, $p<0.05$, therefore the null hypothesis (there is no difference among group means) was rejected (Table 4.3.1a, supplementary information). Due to the significance difference, a Tukey post-hoc test was done (at least one group differs significantly from the overall mean of the dependent variable). For the untreated, there was no significant difference on any of the days. For the *Cruznema* sp. NTM-2021 treatment, day 2 vs 6, day 2 vs 8, day 4 vs 6 and day 4 vs 8 were significantly different (Table 4.3.1b, supplementary information).

Next, a single application of the highly virulent *M. anisopliae* strain was done. The mortality observed in 8 days was recorded as shown in the figure below.

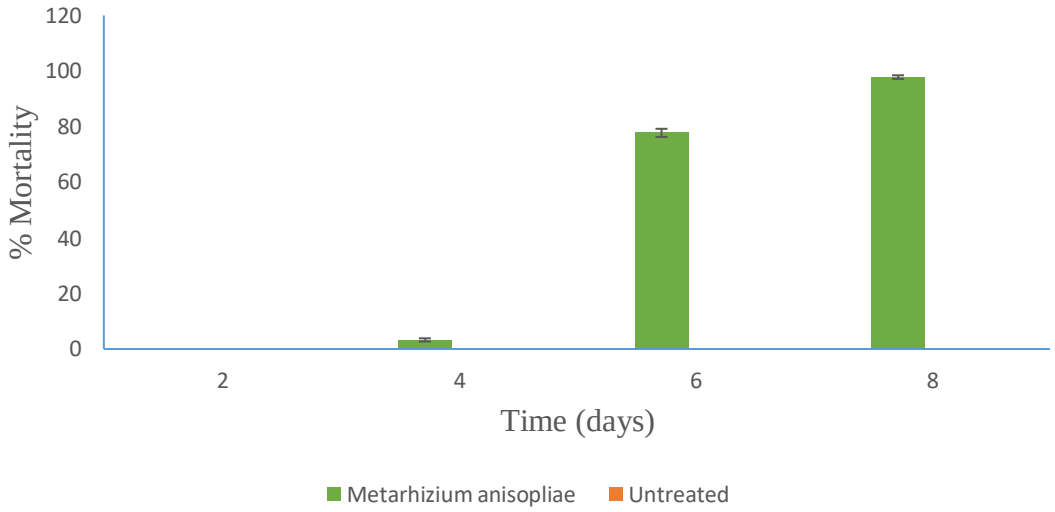

Figure 4.3.2. Mean mortality (%) of *T. molitor* treated with *M. anisopliae* ARSEF 7487. *M. anisopliae* was used at 10^8 spores/ml.

The application of *M. anisopliae* alone resulted in a very high mortality of 97.8%. We assumed that the mortality would have resulted in 100% if the two larvae that had died due to their fitness and injury, had been alive. The larvae had even shown signs that their cause of death was due to the fungi due to their hardening and external sporulation. There was no need to place them in a moist environment to promote external sporulation as they had already been fully covered by spores when checked on the last day as seen in Figure 4.3.3.

The test was significantly different, p-value < 0.05 (Table 4.3.2a, supplementary information). For the untreated, there was no significant difference since there was no mortality in any of the days. For the treatments, the following days were significantly different from one another; 2 vs 6, 2 vs 8, 4 vs 6, 4 vs 8 and 6 vs 8 (Table 4.3.2b, supplementary information).

Figure 4.3.3. Dead *T. molitor* larvae that were treated with *M.anisopliae* ARSEF 7487. The larvae were fully covered with green granular spores on the 8th day of the experiment.

Although, the *Cruznema* sp. NTM-2021 was not highly effective in the control of *T. molitor*, a combined study was still done. The expectation was that if we use the joint use of highly virulent EPFs and a poorly virulent nematode, we might obtain an additive interaction. Therefore, *Cruznema* sp. NTM-2021 and *M. anisopliae* ARSEF 7487 were applied simultaneously and the results are seen in Figure 4.3.4.

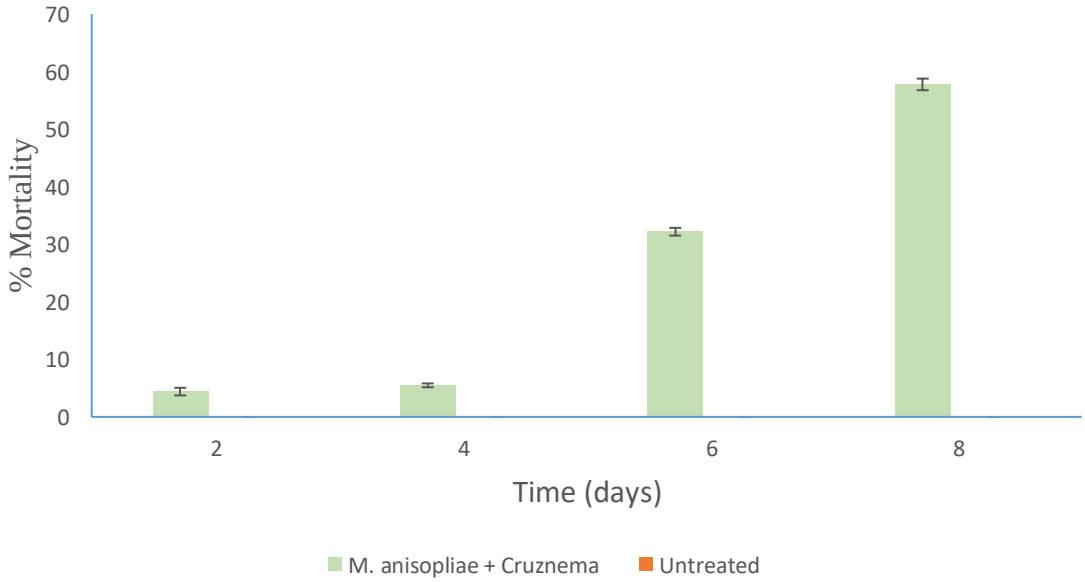

Figure 4.3.4. Mean mortality (%) of *T.molitor* when treated simultaneously with both *M. anisopliae* ARSEF 7487 and *Cruznema* sp. NTM-2021. *M. anisopliae* was used at 10^8 spore/ml and *Cruznema* sp. NTM-2021 was applied at 1500 IJs.ml^{-1}.

The total mortality of the combined applied on the 8th day was 57.8% which was average. The test was significantly different, p-value <0.05 (Table 4.3.4a, supplementary information). For the untreated, there was no significant difference since there was no mortality in any of the days. For the treatments, the following days were significantly different from one another; 2 vs 6, 2 vs 8, 4 vs 6, 4 vs 8 and 6 vs 8 (Table 4.3.4b, supplementary information).

The dead larvae were placed in a white trap to see whether the death was caused by a pathogen or if both pathogens worked together to cause infection therefore resulting in death. The larvae that had died due to fungi (*M. anisopliae*) was 33.3% and this was recorded based on the symptoms being that the larvae were hard and had external sporulation. Death caused by nematodes (*Cruznema* sp. NTM-2021*)* was 7.8% and was confirmed with the symptoms being that the cadavers were soft and IJs emerged. There were a few that showed symptoms of both pathogens and resulted in 5.6% of mortality. The larvae would generally have the upper region consisting of the head and legs infected with fungi while the middle region was infected by the nematodes. Two larvae showing such symptoms are seen in Figure 4.3.5a and Figure 4.3.5b.

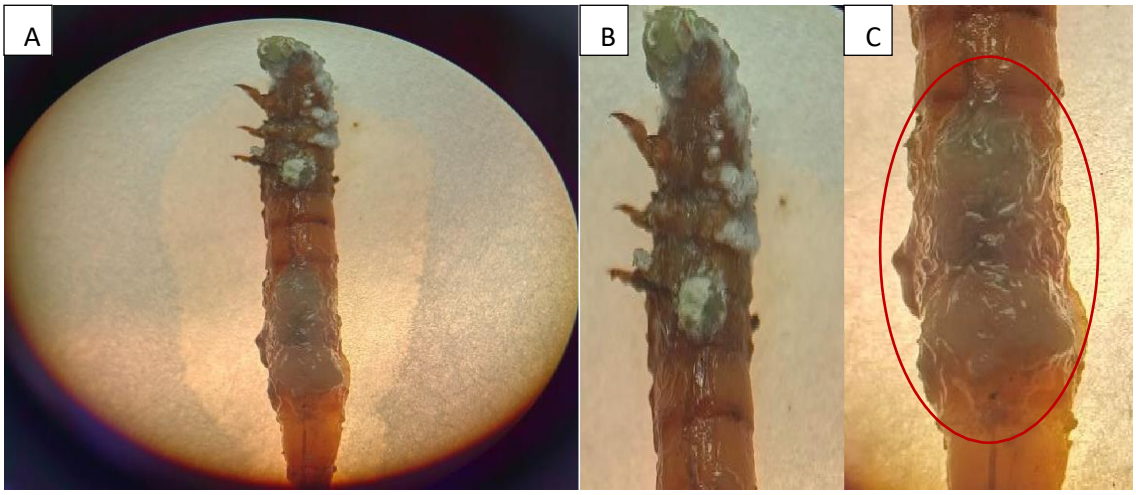

Figure 4.3.5a. *T. molitor* larvae infected by both *M. anisopliae* ARSEF 7487 and *Cruznema* sp. NTM-2021. A) A full body picture of the larvae showing both symptoms of the two pathogens, B) the upper region covered with fungi that is white and green and C) a clump of nematodes in the middle section of the larvae. This was viewed under a dissecting microscope.

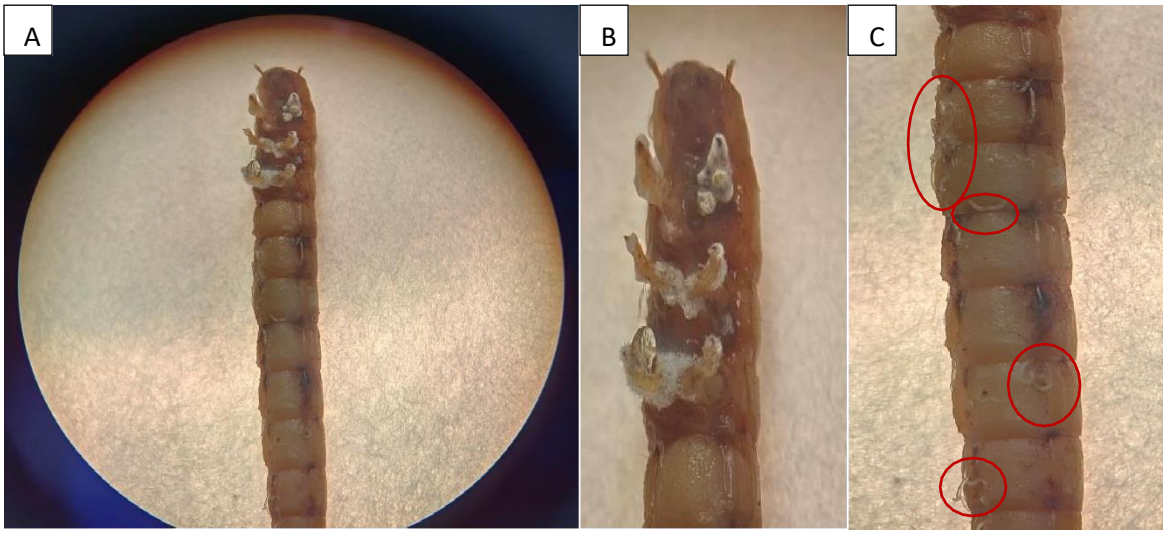

Figure 4.3.5b. *T. molitor* larvae infected by both *M. anisopliae* ARSEF 7487 and *Cruznema* sp. NTM-2021. A) A full body picture of the larvae showing both symptoms of the two pathogens, B) the upper region covered with fungi that are white and green, especially on the legs and C) a few nematodes in the middle section of the larvae circled in red. This was viewed under a dissecting microscope.

The summary of the results obtained in this study is shown in Figure 4.5.6.

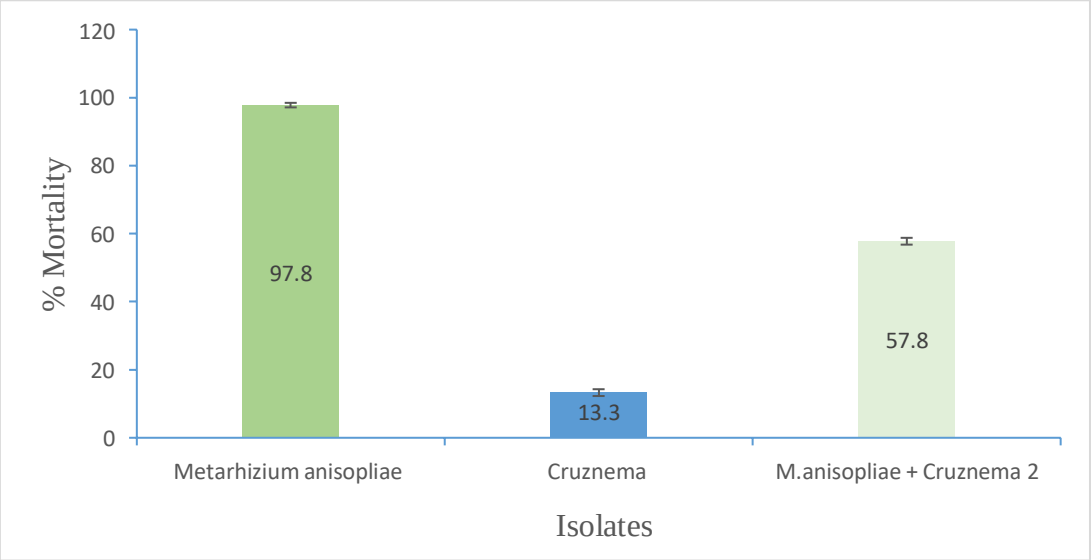

Figure 4.3.6. Summary of the mean mortality (%) of *T. molitor* in the single and combined application.

4.3.7. Nematode-Fungi interaction

$M_E = M_N + M_F (1- M_N)$

$= 4.7 + 29.3 (1-4.7)$

$= -103.71$

$X^2 = (M_{NF} – M_E) x2 / M_E$

$= (17.3 – (-103.71))^2 – 103.71$

$= -141.2$

$X^2 = -141.2$

X^2 value < table value therefore it is an additive effect

4.4. Discussion

Single and combined applications of *Cruznema* sp. NTM-2021 and *M. anisopliae* ARSEF 7487 were done to see if the combined application of both pathogens is more efficient in controlling *T. molitor* larvae as well as their interaction.

In the single application of *Cruznema* sp. NTM-2021, what was found is that the application of this microbial agent resulted in low mortality as shown in Figure 4.3.1. The low mortality shows that it is not effective in the control of *T. molitor* larvae in the given concentration. The limitation of the study was that only one concentration (1500 IJs) was used whereas if higher concentrations had been used, maybe there would have been higher mortality. Studies on *Cruznema* species report mainly based on characterisation and where they were found such as on agricultural pests where *Gryllodes laplatae Sauss* (Orthoptera: Gryllidae) was found to be parasitized by *Cruznema lincolnensis* (Reboredo and Camino, 1998). In another study, *Cruznema tripartitum* was recovered from soil in the Eastern Free State potato production area in South Africa (Shokoohi *et al.*, 2022). It was recovered from mealworms (Coleoptera: Tenebrionidae) suggesting that it is an entomophathogen. This was the first report made on an entomophathogenic nematode, *C. tripartitum*, in South Africa (Shokoohi *et al.*, 2022). In the single application of *M. anisopliae, M. anisopliae* proved to be highly virulent as shown in Figure 4.3.2. In agreement with the results, in a study by (Gebremariam *et al.*, 2021), where *M. anisopliae* was used in a concentration of 10^8 spores/ml, and had a mortality percentage ranging

from 86.67% to 100%. This pathogen proved that it can be used alone in the controlling of *T. molitor* larvae. Another observation made is that the dead larvae also had external sporulation that covered their entire body on the last day of the experiment as shown in Figure 4.3.3. *M. anisopliae* is a parasitic fungi therefore it was expected that it would perform fairly well due to it being a natural enemy of insect pests (Gebremariam, Chekol and Assefa, 2021).

In the combined application, both pathogens were applied simultaneously and resulted in a mortality of 57.8% (Figure 4.3.4) which is lower than the single application of *M. anisopliae*. The interaction was also found to be additive, meaning that the two pathogens act independently. According to Wakil, Yasin and Shapiro-Ilan (2017), when *M. anisopliae* is used simultaneously with nematodes, there is more of an additive interaction whereas when there is a delayed application of the nematodes, the interaction moves towards synergy. Similarly, Ansari, Shah and Butt (2008) and Koppenhöfer and Grewal (2005) suggest that when the nematodes and fungi are simultaneously applied, their interaction results in an additive effect on host mortality. In most of the studies above, the expectation is that the simultaneous application of fungi and nematodes would at least result in an additive interaction but if application of the nematodes is delayed then synergistic interaction is possible. For this study, the results are in agreement with the mentioned studies, as the combination resulted in an additive effect. To achieve a synergistic interaction, application time would need to be taken into account as it is postulated that the synergistic effects between fungi and nematodes are due to the longer exposure of the host to the fungi which weakens it and makes it possible for the EPNs to infect it (Ansari, Shah and Butt, 2008). When the fungi weaken the host, the host will respire more therefore the EPNs follow the Carbon dioxide gradient leading them to the host (Ansari, Shah and Butt, 2008). This study could have potentially resulted in synergistic interaction if only there was a delay in the application of nematodes because *M. anisopliae* is highly virulent (as shown in Figure 4.3.2) and would have made the *T. molitor* larvae more susceptible to the less effective *Cruznema* sp. NTM-2021 (as shown in Figure 4.3.1).

In conclusion, the single application of *M. anisopliae* ARSEF 7487 was more effective in the control of *T. molitor* compared to the joint use of *M. anisopliae* and *Cruznema* sp. NTM-2021. Most commonly an additive interaction occurs when fungi and nematodes are applied simultaneously but if synergy is desired, one needs to consider the application time and make careful selection of pathogens that will be used.

4.5. Reference

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5. Summary

Local EPFs are preferred because of their ecological and environmental suitability therefore making them more effective in the control of insect pests. When selecting a fungal strain to be used as a biological agent, the essential prerequisite is to identify the fungi morphologically and molecularly. Fungi were isolated from soil samples collected at the University of Witwatersrand using *Tenebrio molitor* as bait. This was followed by the identification of the fungal isolates using morphological (macroscopic and microscopic) and molecular (DNA, amplification of the internal transcribed spacer region, sequence alignment and phylogenetic tree analysis) techniques. From the five isolates, only two were identified as entomopathogenic fungi, *Metahizium anisopliae* ARSEF 7487.

For virulence characterisation of the five fungal isolates, namely, two *Metarhizium anisopliae* ARSEF 7487, *Fusarium foetens* CBS 110286, *Neocosmospora rubicola* CBS 101018 and *Aspergillus insueius* NRRL 279, factors such as vegetative growth (in-vitro parameter) and a bioassay against *T. molitor* larvae (in-vivo assessment) were done. Fast vegetative growth is often associated with high virulence but in this study, *M. anisopliae* (Green) which had the slowest vegetative growth had the highest virulence. Therefore, vegetative growth is not always related to virulence. *M. anisopliae* is also a parasitic fungi so it was expected that it would be highly virulent. In the future, more tests can be done such as viability and germination tests in order to further characterize the isolates. A whole genome test should also be done in order to know which genes are responsible for virulence of a particular isolate.

It is believed that the combined application of two microbial control agents may result in the superior control of insect pests. The combined application of *M. anisopliae* ARSEF 7487 and *Cruznema* sp. NTM-2021 in this study did not result in the superior control of *T. molitor* larvae. The use of *M. anisopliae* ARSEF 7487 alone had proven to be more effective in the control of *T. molitor* larvae with a high mortality of 97.8%. To achieve a superior control of *T. molitor* using both microbial agents, application time would have to be taken into account. Future work should take into account the application time.

6. Appendix (Supplementary information)

select all *10 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Metarhizium anisopliae ARSEF 7487 ITS region; from TYPE material	Metarhizium anisopliae	651	651	100%	0.0	99.72%	525	NR_132017.1
Metarhizium pinghaense CBS 257.90 ITS region; from TYPE material	Metarhizium pinghaense	641	641	100%	0.0	99.16%	526	NR_077205.1
Metarhizium robertsii ARSEF 2575 ITS region; from TYPE material	Metarhizium robertsii	640	640	100%	0.0	99.16%	644	NR_132011.1
Metarhizium gryllidicola BCC 82988 ITS region; from TYPE material	Metarhizium gryllidicola	636	636	100%	0.0	98.88%	521	NR_175627.1
Metarhizium guizhouense CBS 258.90 ITS region; from TYPE material	Metarhizium guizhouense	627	627	100%	5e-180	98.60%	500	NR_163550.1
Metarhizium brunneum ARSEF 2107 ITS region; from TYPE material	Metarhizium brunneum	625	625	100%	2e-179	98.32%	502	NR_132023.1
Metarhizium majus ARSEF 1914 ITS region; from TYPE material	Metarhizium majus	625	625	100%	2e-179	98.32%	527	NR_152952.1
Metarhizium phasmatodeae BCC 49272 ITS region; from TYPE material	Metarhizium phasmatodeae	601	601	100%	3e-172	97.19%	557	NR_175628.1
Metarhizium lepidiotae ARSEF 7488 ITS region; from TYPE material	Metarhizium lepidiotae	566	566	100%	1e-161	95.29%	536	NR_132018.1
Metarhizium baoshanense CCTCC M2016589 ITS region; from TYPE material	Metarhizium baoshanense	520	520	99%	8e-148	92.88%	536	NR_166220.1

Figure 2.3.1a. Blast results for isolate 1. Isolate 1 has a percentage identity of 99.72% with *Metarhizium anisopliae* ARSEF 7487.

Metarhizium anisopliae ARSEF 7487 ITS region; from TYPE material

Sequence ID: NR_132017.1 Length: 525 Number of Matches: 1

Range 1: 105 to 459 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
651 bits(352)	0.0	355/356(99%)	1/356(0%)	Plus/Plus

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Query  1    GGGGACCCAAACCTTCTGAATTTTTTAATAAGTATCTTCTGAGTGGTTaaaaaaaaTGAA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  105  GGGGACCCAAACCTTCTGAATTTTTTAATAAGTATCTTCTGAGTGGTTAAAAAAAATGAA  164

Query  61   TCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  165  TCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC  224

Query  121  GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  225  GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGC  284

Query  181  CCGTCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACGCCCCTCAAGTCCCCTGT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  285  CCGTCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACGCCCCTCAAGTCCCCTGT  344

Query  241  GGGACTTGGTGTTGGGGATCGGCGAGGCTGGTTTTCCAGCACAGCCGTCCCTTAAATTAA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  345  -GGACTTGGTGTTGGGGATCGGCGAGGCTGGTTTTCCAGCACAGCCGTCCCTTAAATTAA  403

Query  301  TTGGCGGTCTCGCCGTGGCCCTCCTCTGCGCAGTAGTAAAGCACTCGCAACAGGAG  356
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  404  TTGGCGGTCTCGCCGTGGCCCTCCTCTGCGCAGTAGTAAAGCACTCGCAACAGGAG  459
```

Figure 2.3.1b. Pairwise alignment of isolate 1 with *Metarhizium anisopliae* ARSEF 7487 using blast.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Fusarium foetens CBS 110286 ITS region; from TYPE material	Fusarium foetens	717	717	100%	0.0	100.00%	511	NR_159865.1
Fusarium inflexum NRRL 20433 ITS region; from TYPE material	Fusarium inflexum	701	701	100%	0.0	99.23%	521	NR_152941.1
Fusarium ficicrescens CBS 125178 ITS region; from TYPE material	Fusarium ficicrescens	691	691	100%	0.0	98.72%	421	NR_152915.1
Fusarium succisae NRRL 13613 ITS region; from TYPE material	Fusarium succisae	691	691	100%	0.0	98.72%	522	NR_174876.1
Fusarium circinatum CBS 405.97 ITS region; from TYPE material	Fusarium circinatum	691	691	100%	0.0	98.72%	560	NR_120263.1
Fusarium denticulatum CBS 407.97 ITS region; from TYPE material	Fusarium denticulatum	688	688	100%	0.0	98.71%	444	NR_138359.1
Fusarium bactridioides CBS 100057 ITS region; from TYPE material	Fusarium bactridioides	686	686	100%	0.0	98.46%	557	NR_120262.1
Fusarium pseudocircinatum CBS 449.97 ITS region; from TYPE material	Fusarium pseudocircinatum	686	686	100%	0.0	98.46%	502	NR_163683.1
Fusarium pseudonygamai NRRL 13592 ITS region; from TYPE material	Fusarium pseudonygamai	686	686	100%	0.0	98.46%	522	NR_137162.1
Fusarium pseudoanthophilum CBS 414.97 ITS region; from TYPE material	Fusarium pseudoanthophilum	680	680	100%	0.0	98.21%	557	NR_163682.1

Figure 2.3.1c. Blast results of isolate 2. Isolate 2 is identical to *Fusarium foetens* CBS 110286 with a percentage identification of 100%.

Fusarium foetens CBS 110286 ITS region; from TYPE material

Sequence ID: NR_159865.1 **Length:** 511 **Number of Matches:** 1

Range 1: 59 to 446 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
717 bits(388)	0.0	388/388(100%)	0/388(0%)	Plus/Plus

```
Query  1    GCGGATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  59   GCGGATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTT  118

Query  61   TCTATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  119  TCTATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCT  178

Query  121  TGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  179  TGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATT  238

Query  181  CAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  239  CAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCC  298

Query  241  TGTTCGAGCGTCATTTCAACCCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTCGC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  299  TGTTCGAGCGTCATTTCAACCCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTCGC  358

Query  301  GTTCCTCAAATTGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  359  GTTCCTCAAATTGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGT  418

Query  361  TACTGGTAATCGTCGCGGCCACGCCGTT  388
            ||||||||||||||||||||||||||||
Sbjct  419  TACTGGTAATCGTCGCGGCCACGCCGTT  446
```

Figure 2.3.1d. Pairwise alignment of isolate 2 with *Fusarium foetens* CBS 110286 using blast.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Metarhizium anisopliae ARSEF 7487 ITS region; from TYPE material	Metarhizium anisopliae	713	713	100%	0.0	99.74%	525	NR_132017.1
Metarhizium pinghaense CBS 257.90 ITS region; from TYPE material	Metarhizium pinghaense	704	704	100%	0.0	99.23%	526	NR_077205.1
Metarhizium robertsii ARSEF 2575 ITS region; from TYPE material	Metarhizium robertsii	702	702	100%	0.0	99.23%	644	NR_132011.1
Metarhizium gryllidicola BCC 82988 ITS region; from TYPE material	Metarhizium gryllidicola	693	693	100%	0.0	98.72%	521	NR_175627.1
Metarhizium guizhouense CBS 258.90 ITS region; from TYPE material	Metarhizium guizhouense	689	689	100%	0.0	98.72%	500	NR_163550.1
Metarhizium brunneum ARSEF 2107 ITS region; from TYPE material	Metarhizium brunneum	688	688	100%	0.0	98.47%	502	NR_132023.1
Metarhizium majus ARSEF 1914 ITS region; from TYPE material	Metarhizium majus	682	682	100%	0.0	98.21%	527	NR_152952.1
Metarhizium phasmatodeae BCC 49272 ITS region; from TYPE material	Metarhizium phasmatodeae	664	664	100%	0.0	97.44%	557	NR_175628.1
Metarhizium lepidiotae ARSEF 7488 ITS region; from TYPE material	Metarhizium lepidiotae	612	612	100%	1e-175	94.95%	536	NR_132018.1
Metarhizium baoshanense CCTCC M2016589 ITS region; from TYPE material	Metarhizium baoshanense	579	579	100%	1e-165	93.25%	536	NR_166220.1

Figure 2.3.1e. Blast results of isolate 3. Isolate 3 has a percentage identity of 99.74% with *Metarhizium anisopliae* ARSEF 7487.

Metarhizium anisopliae ARSEF 7487 ITS region; from TYPE material

Sequence ID: NR_132017.1 **Length:** 525 **Number of Matches:** 1

Range 1: 88 to 476 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
713 bits(386)	0.0	389/390(99%)	1/390(0%)	Plus/Plus

```
Query  1    GGGACTTCGCGCCCGCCGGGGACCCAAACCTTCTGAATTTTTTAATAAGTATCTTCTGAG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  88   GGGACTTCGCGCCCGCCGGGGACCCAAACCTTCTGAATTTTTTAATAAGTATCTTCTGAG  147

Query  61   TGGTTaaaaaaaaTGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  148  TGGTTAAAAAAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGA  207

Query  121  AGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  208  AGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCT  267

Query  181  TTGAACGCACATTGCGCCCGTCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  268  TTGAACGCACATTGCGCCCGTCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACG  327

Query  241  CCCCTCAAGTCCCCTGTGGGACTTGGTGTTGGGGATCGGCGAGGCTGGTTTTCCAGCACA  300
            |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  328  CCCCTCAAGTCCCCTGT-GGACTTGGTGTTGGGGATCGGCGAGGCTGGTTTTCCAGCACA  386

Query  301  GCCGTCCCTTAAATTAATTGGCGGTCTCGCCGTGGCCCTCCTCTGCGCAGTAGTAAAGCA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  387  GCCGTCCCTTAAATTAATTGGCGGTCTCGCCGTGGCCCTCCTCTGCGCAGTAGTAAAGCA  446

Query  361  CTCGCAACAGGAGCCCGGCGCGGTCCACTG  390
            ||||||||||||||||||||||||||||||
Sbjct  447  CTCGCAACAGGAGCCCGGCGCGGTCCACTG  476
```

Figure 2.3.1f. Pairwise alignment of isolate 3 with *Metarhizium anisopliae* using blast.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Neocosmospora rubicola CBS 101018 ITS region; from TYPE material	[Neocosmospora] rubicola	725	725	100%	0.0	100.00%	627	NR_154227.1
Fusarium solani CBS 140079 ITS region; from TYPE material	Fusarium solani	719	719	100%	0.0	99.74%	632	NR_163531.1
Fusarium ornamentatum CBS 562.70 ITS region; from TYPE material	Fusarium ornamentatum	684	684	100%	0.0	98.21%	490	NR_160126.1
Fusarium falciforme CBS 475.67 ITS region; from TYPE material	Fusarium falciforme	682	682	100%	0.0	97.97%	586	NR_164424.1
Fusarium tonkinense CBS 115.40 ITS region; from TYPE material	Fusarium tonkinense	682	682	100%	0.0	97.97%	534	NR_170733.1
Fusarium suttonianum NRRL 32858 ITS region; from TYPE material	Fusarium suttonianum	676	676	100%	0.0	97.72%	479	NR_172216.1
Fusarium crassum CBS 144386 ITS region; from TYPE material	Fusarium crassum	675	675	100%	0.0	97.72%	449	NR_178123.1
Fusarium obliquiseptatum NRRL 62611 ITS region; from TYPE material	Fusarium obliquiseptatum	669	669	100%	0.0	97.46%	549	NR_165844.1
Fusarium tuaranense NRRL 22231 ITS region; from TYPE material	Fusarium tuaranense	669	669	100%	0.0	97.46%	549	NR_165843.1
Fusarium variasi CMW 53734 ITS region; from TYPE material	Fusarium variasi	669	669	100%	0.0	97.47%	594	NR_177139.1

Figure 2.3.1g. Blast result of isolate 4. Isolate 4 is matched identically with *Necosmospora rubicola* CBS 101018 with a percentage identity of 100%.

Neocosmospora rubicola CBS 101018 ITS region; from TYPE material

Sequence ID: NR_154227.1 **Length:** 627 **Number of Matches:** 1

Range 1: 110 to 501 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
725 bits(392)	0.0	392/392(100%)	0/392(0%)	Plus/Plus

```
Query  1    CAGACGGCCCTGTAACAACGGGCCGCCCCCGCCAGAGGACCCCTAACTCTGTTTTTATAA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  110  CAGACGGCCCTGTAACAACGGGCCGCCCCCGCCAGAGGACCCCTAACTCTGTTTTTATAA  169

Query  61   TGTTTTTCTGAGTAAACAAGCAAATAAATTAAAACTTTCAACAACGGATCTCTTGGCTCT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  170  TGTTTTTCTGAGTAAACAAGCAAATAAATTAAAACTTTCAACAACGGATCTCTTGGCTCT  229

Query  121  GGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  230  GGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA  289

Query  181  TCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  290  TCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGA  349

Query  241  GCGTCATTACAACCCTCAGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAAGCCCCCTGT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  350  GCGTCATTACAACCCTCAGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAAGCCCCCTGT  409

Query  301  GGGCACACGCCGTCCCTCAAATACAGTGGCGGTCCCGCCGCAGCTTCCATTGCGTAGTAG  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  410  GGGCACACGCCGTCCCTCAAATACAGTGGCGGTCCCGCCGCAGCTTCCATTGCGTAGTAG  469

Query  361  CTAACACCTCGCAACTGGAGAGCGGCGCGGCC  392
            ||||||||||||||||||||||||||||||||
Sbjct  470  CTAACACCTCGCAACTGGAGAGCGGCGCGGCC  501
```

Figure 2.3.1h. Pairwise alignment of isolate 4 with *Necosmospora rubicola* CBS 101018 using blast.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Aspergillus insuetus NRRL 279 ITS region; from TYPE material	Aspergillus insuetus	634	634	100%	0.0	99.71%	591	NR_131292.1
Aspergillus thesauricus CCF 4166 ITS region; from TYPE material	Aspergillus thesauricus	628	628	100%	1e-180	99.42%	590	NR_135432.1
Aspergillus keveii CBS 209.92 ITS region; from TYPE material	Aspergillus keveii	628	628	100%	1e-180	99.42%	570	NR_175108.1
Aspergillus keveioides HMAS 242394 ITS region; from TYPE material	Aspergillus keveioides	625	625	100%	2e-179	99.14%	533	NR_173250.1
Aspergillus asper NRRL 35910 ITS region; from TYPE material	Aspergillus asper	619	619	99%	8e-178	99.13%	522	NR_151788.1
Aspergillus pseudodeflectus NRRL 6135 ITS region; from TYPE material	Aspergillus pseudodeflectus	617	617	100%	3e-177	98.84%	591	NR_135372.1
Aspergillus contaminans CBS 142451 ITS region; from TYPE material	Aspergillus contaminans	612	612	99%	1e-175	98.83%	495	NR_156339.1
Aspergillus sigurros PPRI 15889 ITS region; from TYPE material	Aspergillus sigurros	612	612	99%	1e-175	98.83%	726	NR_171611.1
Aspergillus monodii CBS 435.93 ITS region; from TYPE material	Aspergillus monodii	595	595	98%	1e-170	97.97%	469	NR_135427.1
Aspergillus granulosus NRRL 1932 ITS region; from TYPE material	Aspergillus granulosus	590	590	99%	6e-169	97.41%	593	NR_135348.1

Figure 2.3.1i. Blast result of isolate 5. Isolate 5 has a percentage identity of 99.71% with *Aspergillus insuetus* NRRL 279.

Aspergillus insuetus NRRL 279 ITS region; from TYPE material

Sequence ID: NR_131292.1 **Length:** 591 **Number of Matches:** 1

Range 1: 100 to 445 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
634 bits(343)	0.0	345/346(99%)	0/346(0%)	Plus/Plus

```
Query  1    TAGCCGCCGGAGACCACATTGAACCTCTTGTCTTTAGTGTTGTCTGAGCTTGATAGCAAA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  100  TAGCCGCCGGAGACCACATTGAACCTCTTGTCTTTAGTGTTGTCTGAGCTTGATAGCAAA  159

Query  61   CCTATTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  160  CCTATTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAA  219

Query  121  CTGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  220  CTGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATT  279

Query  181  GCGCCCCCTGGCATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTTCAAGCCC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  280  GCGCCCCCTGGCATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTTCAAGCCC  339

Query  241  GGCTTGTGTGTTGGGTCGTCGTCCCCCCTGGGGGACGGGCCCGAAAGGCAGCGGCGGCAC  300
            |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  340  GGCTTGTGTGTTGGGTCGTCGTCCCCCCCGGGGGACGGGCCCGAAAGGCAGCGGCGGCAC  399

Query  301  CGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCCGCTCGATTA  346
            |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  400  CGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCCGCTCGATTA  445
```

Figure 2.3.1j. Pairwise alignment of isolate 6 with *Aspergillus insuetus* NRRL 279 using blast.

Table 3.3.2. Two-way ANOVA results for vegetative growth rate.

Anova: Two-Factor With Replication							
SUMMARY	Metarhizium anisopliae (Green)	Fusarium foetens	Metarhizium anisopliae (Yellow)	Neocosmospora rubicola	Aspergillus insuetus	Total	
1							
Count	3	3	3	3	3	15	
Sum	1,5	1,5	1,5	1,5	1,5	7,5	
Average	0,5	0,5	0,5	0,5	0,5	0,5	
Variance	0	0	0	0	0	0	
3							
Count	3	3	3	3	3	15	
Sum	3,7	7,7	4,4	12,6	4,9	33,3	
Average	1,233333	2,566667	1,466667	4,2	1,633333	2,22	
Variance	0,023333	0,253333	0,373333	0,03	0,003333	1,367429	
5							
Count	3	3	3	3	3	15	
Sum	5,4	21,4	6,6	15,8	6,8	56	
Average	1,8	7,133333	2,2	5,266667	2,266667	3,733333	

Variance	0,07	2,613333	0,01	0,093333	0,013333	5,146667	
7							
Count	3	3	3	3	3	15	
Sum	5,9	24,1	7,8	19	6,9	63,7	
Average	1,966667	8,033333	2,6	6,333333	2,3	4,246667	
Variance	0,043333	0,703333	0,01	0,003333	0,03	6,625524	
9							
Count	3	3	3	3	3	15	
Sum	8,9	27	10,4	26,1	10,1	82,5	
Average	2,966667	9	3,466667	8,7	3,366667	5,5	
Variance	0,043333	0	0,243333	0,27	0,023333	8,138571	
11							
Count	3	3	3	3	3	15	
Sum	10,6	27	12,9	27	12	89,5	
Average	3,533333	9	4,3	9	4	5,966667	

Variance	0,043333	0	0,07	0	0,01	6,65381	
13							
Count	3	3	3	3	3	15	
Sum	11,3	27	13,3	27	12,5	91,1	
Average	3,766667	9	4,433333	9	4,166667	6,073333	
Variance	0,023333	0	0,043333	0	0,013333	6,17781	
15							
Count	3	3	3	3	3	15	
Sum	12,9	27	14,8	27	15	96,7	
Average	4,3	9	4,933333	9	5	6,446667	
Variance	0,01	0	0,083333	0	0,28	4,774095	
Total							
Count	24	24	24	24	24		
Sum	60,2	162,7	71,7	156	69,7		
Average	2,508333	6,779167	2,9875	6,5	2,904167		

Variance	1,653841	10,56259	2,30288	8,69913	2,063025		
ANOVA							
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*	
Sample	465,7613	7	66,53732	490,146	6,48E-63	2,126324	
Columns	428,6622	4	107,1655	789,4331	2,07E-63	2,485885	
Interaction	104,8525	28	3,744732	27,5855	1,91E-30	1,617112	
Within	10,86	80	0,13575				
Total	1010,136	119					

Table 3.3.3a. Two-way ANOVA results for virulence of each isolate.

Anova: Two-Factor With Replication							
SUMMARY	Metarhizium anisopliae (Green)	Fusarium foetens	Metarhizium anisopliae (Yellow)	Neocomospora rubicola	Aspergillus insueius	Untreated	Total
2							
Count	3	3	3	3	3	3	18
Sum	8	4	1	3	1	0	17
Average	2,666667	1,333333	0,333333	1	0,333333	0	0,944444
Variance	14,33333	5,333333	0,333333	3	0,333333	0	3,584967
4							
Count	3	3	3	3	3	3	18
Sum	24	8	9	7	5	1	54
Average	8	2,666667	3	2,333333	1,666667	0,333333	3
Variance	129	9,333333	13	10,33333	2,333333	0,333333	25,41176
6							
Count	3	3	3	3	3	3	18

Sum	75	66	64	21	26	3	255
Average	25	22	21,33333	7	8,666667	1	14,16667
Variance	31	84	105,3333	61	5,333333	1	119,4412
8							
Count	3	3	3	3	3	3	18
Sum	90	77	89	56	32	4	348
Average	30	25,66667	29,66667	18,66667	10,66667	1,333333	19,33333
Variance	0	34,33333	0,333333	196,3333	16,33333	0,333333	145,6471
Total							
Count	12	12	12	12	12	12	
Sum	197	155	163	87	64	8	
Average	16,41667	12,91667	13,58333	7,25	5,333333	0,666667	
Variance	172,9924	156,2652	186,9924	102,0227	25,69697	0,606061	

ANOVA							
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*	
Sample	4209,167	3	1403,056	46,57446	2,97E-14	2,798061	
Columns	2118,278	5	423,6556	14,06325	1,8E-08	2,400851	
Interaction	1435,167	15	95,67778	3,176026	0,001184	1,880175	
Within	1446	48	30,125				
Total	9208,611	71					

Table 3.3.3b. Tukey post hoc test.

Turkey test for total averages					
k	4				
n.obv	12				
dff	48				
ms	30,125				
Combination	6				
Q	4,39				

Comparison	Absolute value	Critical value	Results		
2 to 4	2,055555556	6,955645264	means are not significantly different		
2 to 6	13,22222222	6,955645264	means are significantly different		
2 to 8	18,38888889	6,955645264	means are significantly different		
4 to 6	11,16666667	6,955645264	means are significantly different		
4 to 8	16,33333333	6,955645264	means are significantly different		
6 to 8	5,166666667	6,955645264	means are not significantly different		

Table 3.3.6a. One-way ANOVA for the dose-dependent test for *M. anisopliae*

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Dose 1	3	226,63	75,54333	514,1966		
Dose 2	3	193,33	64,44333	70,31853		
Dose 3	3	133,33	44,44333	25,92963		
Untreated	3	0	0	0		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*

Between Groups	9994,026	3	3331,342	21,82895	0,00033	4,066181
Within Groups	1220,89	8	152,6112			
Total	11214,92	11				

Table 3.3.6b. Calculations for lethal concentration 50

Concentration	log10	Mean mortality	Probit of kill
1,00E+08	8	76	5,71
1,00E+07	7	64	5,36
1,00E+06	6	44	4,85
Intercept	2,296667		
X Variable 1	0,43		
y=ax+b			
y=0,43x+2,30			
5-2,3=0,43x			
x=(5-2,3)/0,43			
x=6,27907			
LC50= antilog x			
LC50= antilog 6,28		1905461	
LC50= 1905461			

Table 4.3.1a. ANOVA test for a single application of *Cruznema* sp. NTM-2021.

Anova: Two-Factor With Replication							
SUMMARY	Cruznema 2	Control	Total				
2							
Count	3	3	6				
Sum	1	0	1				
Average	0,333333	0	0,166667				
Variance	0,333333	0	0,166667				
4							
Count	3	3	6				
Sum	2	0	2				
Average	0,666667	0	0,333333				
Variance	0,333333	0	0,266667				
6							
Count	3	3	6				
Sum	10	0	10				
Average	3,333333	0	1,666667				
Variance	2,333333	0	4,266667				
8							
Count	3	3	6				
Sum	12	3	15				

Average	4	1	2,5				
Variance	3	1	4,3				
Total							
Count	12	12					
Sum	25	3					
Average	2,083333	0,25					
Variance	3,901515	0,386364					
ANOVA							
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*	
Sample	22,33333	3	7,444444	8,507937	0,001315	3,238872	Significant
Columns	20,16667	1	20,16667	23,04762	0,000196	4,493998	Significant
Interaction	10,83333	3	3,611111	4,126984	0,024024	3,238872	Significant
Within	14	16	0,875				
Total	67,33333	23					

Table 4.3.1b. Tukey post-hoc test for a single application of *Cruznema* sp. NTM-2021.

Tukey test (for cruznema)									Tukey test (for control)							
k	4		Q	4,05												
n.obv	3															
df (error)	16															
ms (error)	0,875															
combinati	6															
comparisc	Absolute	critical val	result						comparisc	absolute v	critical val	result				
2 vs 4	0,333333	2,18725	means are not significantly different						2 vs 4	0	2,18725	means are not significantly different				
2 vs 6	3	2,18725	means are significantly different						2 vs 6	0	2,18725	means are not significantly different				
2 vs 8	3,666667	2,18725	means are significantly different						2 vs 8	1	2,18725	means are not significantly different				
4 vs 6	2,666667	2,18725	means are significantly different						4 vs 6	0	2,18725	means are not significantly different				
4 vs 8	3,333333	2,18725	means are significantly different						4 vs 8	1	2,18725	means are not significantly different				
6 vs 8	0,666667	2,18725	means are not significantly different						6 vs 8	1	2,18725	means are not significantly different				

Table 4.3.2a. ANOVA test results for a single application of *M. anisopliae.*

Anova: Two-Factor With Replication							
SUMMARY	Metarhizium anisopliae (Green)	Control	Total				
2							
Count	3	3	6				
Sum	0	0	0				
Average	0	0	0				
Variance	0	0	0				
4							
Count	3	3	6				
Sum	3	0	3				
Average	1	0	0,5				

Variance	1	0	0,7				
6							
Count	3	3	6				
Sum	70	0	70				
Average	23,33333	0	11,66667				
Variance	6,333333	0	165,8667				
8							
Count	3	3	6				
Sum	88	0	88				
Average	29,33333	0	14,66667				
Variance	1,333333	0	258,6667				
Total							
Count	12	12					
Sum	161	0					
Average	13,41667	0					
Variance	188,6288	0					
ANOVA							
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*	
Sample	1028,792	3	342,9306	316,5513	1,88E-14	3,238872	significant

Columns	1080,042	1	1080,042	996,9615	7,66E-16	4,493998	significant
Interaction	1028,792	3	342,9306	316,5513	1,88E-14	3,238872	significant
Within	17,33333	16	1,083333				
Total	3154,958	23					

Table 4.3.2b. Tukey post-hoc test for a single application of *M.anisopliae.*

Tukey test (for fungi)							Tukey test (for control)						
k	4												
n.obv	3												
df (error)	16												
ms (error)	1,083333												
combinati	6												
Q	4,05												
comparisc	absolute c	critical val	results				comparisc	absolute c	critical val	results			
2 vs 4	1	2,433747	means are not significantly different				2 vs 4	0	2,433747	means are not significantly different			
2 vs 6	23,33333	2,433747	means are significantly different				2 vs 6	0	2,433747	means are not significantly different			
2 vs 8	29,33333	2,433747	means are significantly different				2 vs 8	0	2,433747	means are not significantly different			
4 vs 6	22,33333	2,433747	means are significantly different				4 vs 6	0	2,433747	means are not significantly different			
4 vs 8	28,33333	2,433747	means are significantly different				4 vs 8	0	2,433747	means are not significantly different			
6 vs 8	6	2,433747	means are significantly different				6 vs 8	0	2,433747	means are not significantly different			

Table 4.3.4a. ANOVA test results for the combined application of *M. anisopliae* and *Cruznema* sp. NTM-2021.

Anova: Two-Factor With Replication							
SUMMARY	M.anisopliae + Cruznema 2	Untreated	Total				
2							
Count	3	3	6				
Sum	4	0	4				

Average	1,333333	0	0,666667				
Variance	1,333333	0	1,066667				
4							
Count	3	3	6				
Sum	5	0	5				
Average	1,666667	0	0,833333				
Variance	0,333333	0	0,966667				
6							
Count	3	3	6				
Sum	29	0	29				
Average	9,666667	0	4,833333				
Variance	1,333333	0	28,56667				
8							
Count	3	3	6				
Sum	52	0	52				
Average	17,33333	0	8,666667				
Variance	1,333333	0	90,66667				
Total							
Count	12	12					
Sum	90	0					
Average	7,5	0					

Variance	48,09091	0					
ANOVA							
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*	
Sample	260,1667	3	86,72222	160,1026	3,84E-12	3,238872	significant
Columns	337,5	1	337,5	623,0769	3,07E-14	4,493998	significant
Interaction	260,1667	3	86,72222	160,1026	3,84E-12	3,238872	significant
Within	8,666667	16	0,541667				
Total	866,5	23					

Table 4.3.4b. Tukey post-hoc test for the combined application of *M.anisopliae* and *Cruznema* sp. NTM-2021.

Tukey test (for combination)				Tukey test (for control)			
k	4						
n.obs	3						
df	16						
ms	0,541667						
combinati	6						
Q	4,05						
comparisc	Absolute v	critical val	result	comparisc	Absolute v	critical val	result
2 vs 4	0,333333	1,72092	means are not significantly different	2 vs 4	0	1,72092	means are not significantly different
2 vs 6	8,333333	1,72092	means are significantly different	2 vs 6	0	1,72092	means are not significantly different
2 vs 8	16	1,72092	means are significantly different	2 vs 8	0	1,72092	means are not significantly different
4 vs 6	8	1,72092	means are significantly different	4 vs 6	0	1,72092	means are not significantly different
4 vs 8	15,66667	1,72092	means are significantly different	4 vs 8	0	1,72092	means are not significantly different
6 vs 8	7,666667	1,72092	means are significantly different	6 vs 8	0	1,72092	means are not significantly different