STUDIES ON *LECANICILLIUM MUSCARIUM* AS A MYCOPARASITE OF THE SOYBEAN RUST FUNGUS, *PHAKOPSORA PACHYRHIZI*

By

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DISSERTATION SUMMARY

Soybean rust (SBR) caused by *Phakopsora pachyrhizi* Syd. & P. Syd is the most important foliar disease of soybean. The disease starts on the lower canopy and from there it colonizes the whole plant. When conditions are favorable for disease development, SBR can cause serious damage and is able to cause up to 100% of yield loss. Spraying with fungicides is currently considered to be the only effective method for SBR control. However, there are undesirable consequences such as fungicide resistant strains of *P. pachyrhizi* and increased costs of production. Therefore, alternative control strategies, aiming for integrated disease management practices, are needed to control rust on soybean.

An isolate of *Lecanicillium spp*, strain Nesta-08, was observed colonizing and feeding on *Hemileia vastatrix* Berk. and Broome, the rust of coffee at the Assagay Coffee Farm, Cato Ridge, KwaZulu-Natal, South Africa. The fungus was isolated on Sabouraud dextrose yeast extract agar (SDYA) and the pure culture obtained was subcultured onto potato dextrose agar (PDA). Morphological studies were done using light and scanning electronic microscopy (SEM). Polymerase chain reaction (PCR) was performed to amplify the internal transcribed spacer (ITS), and specifically the portion of the mitochondrial encoded NADH dehydrogenase subunits 1 (MT-ND1) and 3 (MT-ND3) genes. Based on morphology and molecular studies, the isolate Nesta-08 was identified as a strain of *Lecanicillium muscarium* Zare et Gams and it was deposited into the National Collection of Fungi (accession number PPRI 13715).

Optimization of growing conditions is an essential aspect that must be taken into consideration to produce an economically viable biocontrol agent. Laboratory experiments were conducted to assess the effects of different growing conditions (temperature, artificial growing media, natural substrates and UV radiation) on colony growth and conidia production. Strain N-08 of *L. muscarium* was cultured on plates and incubated in the dark at five different temperatures: 18, 21, 24, 25 and 28°C for 30 days. The best growth was observed at 24°C. It was also grown on four different media, namely potato dextrose agar (PDA), malt extract agar (MEA), V-8 juice agar (V8A), and Sabouraud dextrose agar (SDA). The best growth was seen on V8A. Six different agro-industrial solid substrates were tested for suitability for sporulation by *L. muscarium* strain Nesta-08. These were wheat bran (*Triticum aestivum* L.), rice (*Oryza sativa* L.), rolled oats (*Avena sativa* L.), pearl

millet [*Pennisetum glaucum* (L.) R.Br.], pearled barley (*Hordeum vulgare* L.), and sorghum (*Sorghum bicolor* L.). The best production of conidia occurred on pearl millet followed by wheat bran and pearled barley. The lowest conidial production was observed on rolled oats. Under UV light mycelial growth was unaffected.

The effect of *L. muscarium* strain Nesta-08 on SBR was investigated using a bioassay on detached leaves, and in a greenhouse experiment. In a bioassay study conducted on soybean leaves infected with *P. pachyrhizi*, 1ml of a conidial suspension (10^6 conidia ml^{-1}) was sprayed on the abaxial surface of detached infected leaves using a hand sprayer. The isolate was observed growing and colonizing the SBR fungus. Under ESEM (Environmental Scanning Electron Microscope), the mycelium of *L. muscarium* was observed coiling around *P. pachyrhizi* urediospores. Penetration holes were also observed, where the infection hyphae had pulled away during sample preparation. *L. muscarium* Nesta-08 was further tested against *P. pachyrhizi* under greenhouse conditions. Potted soybean plants were artificially infected with *P. pachyrhizi* at Stage R_1. Once SBR pustules emerged, the plants were sprayed by conidia of *L. muscarium* at various conidial doses (10^8, 10^6 and 10^4 conidia ml^{-1}). All treatments significantly reduced SBR severity (P=0.0001) by 83.92%, 79.86% and 70.15%, respectively.

L. muscarium strain Nesta-08 was used in a field experiment at three doses (10^8, 10^6 and 10^4 conidia ml^{-1}), using the fungicide Score® as a comparison. All tested treatments significantly reduced SBR severity (P=0.0001). There were no significance differences in the disease ratings of plots treated with three doses of *L. muscarium* and Score® (0.0001). However, none of the treatments caused a significant increase in either plant dry weight or seed weight. Larger field trials are needed to test the effect of *L. muscarium* strain Nesta-08 on *P. pachyrhizi* for yield increases in soybean.

Keywords: Biocontrol, *Lecanicillium muscarium*, soybean rust, *Phakopsora pachyrhizi*.

DECLARATION

I, Jean Pierre HAVUGIMANA, declare that:

i. The research reported in this dissertation, except where otherwise indicated, is my original work.

ii. This dissertation has not been submitted for any degree examination at any other university.

iii. This dissertation does not contain other persons' data, pictures, graphs or other information, unless acknowledged as being sourced from other persons.

iv. This dissertation does not contain other's person writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted. Then:

   a) Their words have been re-written but the general information attributed to them has been referenced.

   b) Where their exact words have been used, then their writing has been placed inside quotation marks and referenced.

v. This dissertation does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the references sections.

Signed: …….

Mr. JP. HAVUGIMANA

Signed: …….

Prof. M.D. LAING (Supervisor)

Signed: …….

Dr. K.S. YOBO (Co-supervisor)

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DEDICATION

To the Havugimana Family:

Thank you for your love, encouragement and support throughout my study.

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DISSERTATION INTRODUCTION

The Soybean, or Greater Bean (*Glycine max (*L.) Merrill), is native to Manchuria, China. The crop is considered one of the five oldest cultivated crops and was utilized by the Chinese as a source of food before 2500 BC (Ward, 2011). However, it was only utilised in the western world as a source of oil and protein in the 19th century (DAFF, 2010). Soybean seed is a major of source of vegetable seed oil and protein, and contains about 40% basic protein and 20% oil. It is also a source of calcium, iron, carotene and ascorbic acid. Soybean oilcake meal has become the principal protein source for poultry and livestock in many countries (Ghaly & Sutherland, 1982). Recently, Sensoz and Kaynar (2005) investigated the production of bio-oil from soybean oil cake.

Soybeans are grown in temperate to tropical regions of the world, with production being highest in Argentina, Brazil, China, India and the USA (Karr-Lilienthal *et al*., 2004). Emphasis in research has been on breeding of soybeans appropriate for tropical environments (Hartman *et al*., 1999, Hartman *et al*., 2005; Bonde *et al*., 2006). In the past 30 years, world production of soybeans increased to more than 100 million metric tons. Of this, 51% is produced in the USA, 20% in Brazil, 10% in Argentina and 10% in China. The first report of soybeans in South Africa is believed to be in the Cedara Memoirs of 1903. Due to an increase in soybean production throughout the world, diseases that affect this crop have also increased in number and severity (Nunkumar, 2006; Hartman *et al.*, 2015).

Soybean is attacked by a variety of fungi, bacteria, nematodes and viruses (Wrather and Koenning, 2006; Kevin *et al*., 2009). The United States Department of Agriculture reported soybean disease losses of over 15% in the USA and over 10% worldwide (Wrather, 1997; Wrather and Koenning, 2006). Asian soybean rust (SBR) is one of the most important diseases for soybean. It is caused by *Phakopsora pachyrhizi* Syd. & P. Syd, a fungus that was first reported in Japan, hence the name, Asian Soybean Rust (Hennings, 1903; Kendrick *et al*., 2011).

Since then the disease has caused major crop losses in many Asian countries including Japan, China, Taiwan, Thailand and India (Sinclair and Hartman, 1999). In Africa, the pathogen was first reported in East African countries: Uganda, Kenya and Rwanda, arriving via monsoon winds from India in 1996 (Oloka *et al*., 2008). After a short time SBR appeared in Zimbabwe and Zambia, and it then reached Nigeria in 1999, Mozambique in 2000, and South Africa (SA) in 2001 (Pretorius *et*

al., 2001, Caldwell *et al.*, 2002, du Preez *et al.*, 2005; Nunkumar *et al*., 2008). Finally, in 2004, *P. pachyrhizi* was found in the USA, infecting soybean and kudzu bean in many southern states (Schneider *et al*., 2005).

Soybean rust (SBR) is the most destructive foliar infection of soybean, especially in regions with moderate winters, such as Central-East Africa, Asia and southern America, where crop losses caused by *P. pachyrhizi* are estimated to range from 10 to 80% (Miles *et al.*, 2011). The disease can have a significant financial impact in affected areas. According to Fanglin (2013) and Yorinori *et al.* (2005), in the 2001-2002 growing seasons, the Brazilian government estimated losses of 41.9 million tons, equivalent to US$125.5 million. This was the first report of SBR in Brazil. After only 5 years (2007), the yield losses caused by the same pathogen increased up to US$10 billion. The same year, Japan faced crop losses of up to 40%, while in Taiwan the losses were up to 80%. In Eastern-Central African countries, where this pathogen first appeared on the continent, SBR continues to cause large yield losses. Studies done at Makerere University, Uganda, estimated crop loss due to SBR at 15-80% (Oloka et al., 2005). In South Africa, yield losses caused by *P. pachyrhizi* are reported to be 10-80% (Nankumar, 2006). Worldwide yield losses due to *P. pachyrhizi* have been estimated at 10-90%. The highest yield losses have been reported in Taiwan and Africa, where the disease can cause crop losses of up to 100% (Pivonia and Yang, 2004; Glen *et al.*, 2005).

Since its discovery, many studies have been done on *P. pachyrhizi* and its management to reduce yield loss due to the pathogen. Many practices to decrease soybean crop loss have been suggested and adopted, such as cultural practices and fungicide applications (Hassan *et al.*, 2014). Fungicide applications have shown a positive effect in reducing crop damage caused by SBR; however, the pathogen tends to develop resistance to fungicides such as triazoles and strobilurins (Ward, 2011), and the cost of fungicides means an increase in production costs (Hassan *et al.*, 2014). Few studies have been done on biological control as an option to control the SBR pathogen. Recently, studies were conducted on *Simplicillium lanosoniveum* Zare & W. Gams to control SBR (Ward *et al.*, 2012). However, there are no registered or commercial biocontrol products against SBR, currently.

In February 2013, the fungus *Lecanicillium muscarium* Zare et Gams was observed attacking and feeding on *Hemileia vastatrix* Berkeley and Broome, the cause of coffee rust, at the Assagay Coffee Farm at Cato Ridge, KwaZulu-Natal, South Africa. The mycoparasitic fungus has been isolated

and tested on various plant rusts, e.g., oxalis rust (*Puccinia oxalidis* Dietel and Ellis) (Hlatshwayo, 2014; Nxumalo, 2015) and, with this current study, on SBR. This local strain of *L. muscarium* could be a successful biological control agent against SBR. If this mycoparasite is used to control SBR, this can reduce the frequent and costly use of fungicides on soybeans. This would reduce input cost for farmers, and increase yield by reducing SBR severity to low levels.

Study aim and objectives

The primary aim of the present study was to evaluate the potential of *L. muscarium* to control *P. pachyrhizi*. The specific objectives included:

1. Review the available literature on SBR to properly understand the pathogen, *P. pachyrhizi*, and the use of fungi as biocontrol agents against plant rusts.

2. Isolate a local strain of *L. muscarium* from coffee leaves infected by *H. vastatrix*, where the hyperparasite was seen colonizing the coffee rust pustules.

3. Identify the fungus using morphological and DNA based criteria.

4. Obtain pure culture of *L. muscarium;* store them under short- and long-term conditions; determine the optimum conditions for its growth and conidial production in the laboratory.

5. Undertake colonization and mycoparasitic studies using *L. muscarium* on *P. pachyrhizi*. This will involve *L. muscarium-P. pachyrhizi* interaction studies using environmental scanning electron microscopy (ESEM), as well as a leaf disc bioassay using *L. muscarium* and *P. pachyrhizi*.

6. Evaluate the effects of *L. muscarium* on *P. pachyrhizi* under greenhouse conditions using potted soybean plants as subjects for the study. The greenhouse study will also identify the optimal dose level for disease control and assess disease development over time.

7. Evaluate the development of *L. muscarium* on SBR at the Ukulinga Research Farm of UKZN. This will involve rating the impact of *L. muscarium* on SBR in the field, using disease incidence and severity studies. This will allow for the assessment of yield increases due to *L. muscarium* and fungicide sprays.

This dissertation is structured in the form of five chapters. Each chapter covers specific objectives of the research that was conducted. Except for Chapter One, the literature review, the other four chapters were independent studies and were written in the form of discrete research chapters, each as a stand-alone research paper. This format is the standard dissertation model that has been adopted by the University of KwaZulu-Natal because it facilitates the publishing of research out of the dissertation far more easily than the older monograph form of dissertation. As such, there is some unavoidable repetition of references, methods and some introductory information between chapters.

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CHAPTER 1: LITERATURE REVIEW

1.1.Introduction

Soybean [*Glycine max* (L.) Merrill)] is one of most valuable crops in the world, not only because of its oil for human and livestock consumption, but also as a source of protein for animal and human nutrition, and as a biofuel feedstock (Hartman *et al.*, 2011). Masuda and Goldsmith (2009) reported an increase in global soybean production from 24.7 million ha in 1961-1965 to 94.1 million ha in 2005-2007.

Worldwide the production of soybean is negatively affected by various pathogens. At the top of the list is the soybean rust (SBR) fungus, *Phakopsora pachyrhizi* Syd. & P. Syd (Murithi *et al*., 2016). Fungicides are usually used for SBR control (Levy, 2005; Miles *et al*., 2007). This review covers the biology and current control of *P. pachyrhizi*.

1.2. Soybean

Soybean is an important crop worth US$ 215 billion/year grown on 93.9 million ha of cultivable area (USDA, 2009). Around the world, soybean is a major source of vegetable oil for human consumption and industrial use, and protein-rich meal for livestock feed (Sign *et al*., 2004; Pham *et al.*, 2010). Soybean has been a major source of food for animals and humans, as well as being used in medicine to treat human diseases (Hartman *et al*., 1999; Barret, 2006). Studies have suggested that increasing soy consumption could be related to the decreased risk of occurrence of breast cancer (He and Chen, 2013; Batra and Sharma, 2013) and could reduce the risk for cardiovascular disease (Xiao, 2008). Soybean has various industrial uses from adhesives to biofuels (Young, 2012).

Almost 96% of soybean is produced by Brazil, Argentina, USA, Paraguay, Canada and China. In Africa, soybean production was estimated to be only about 0.5% of the World soybean production. In 2002, Africa produced 179.9 million tonnes (Sign *et al*., 2004). In South Africa (SA), soybean is an important crop not only because of its oil products, but as a source of protein for human and animal food supplements. Utilization of soybean products in SA far exceeds production. The Protein Research Foundation (PRF) report in 2015 indicated that 728,150 tonnes of soy oilcake were imported into SA in 2013/2014 (PRF, 2015).

1.3.Soybean Rusts

Soybean is affected by a variety of fungal, bacterial, viral and nematode diseases. The most devastating soybean disease has been reported as SBR (Ward *et al*., 2012). The disease was first reported in Africa in 1996, South America in 2001 and the United States of America (USA) in 2004 (Miles *et al*. 2003; Schneider *et al*. 2005). The yield losses caused by this pathogen is estimated to be 10-90% worldwide, and is highest in Taiwan and Africa where the disease can cause damage of up to 100% (Pivonia and Yang, 2004; Glen *et al*., 2005; Pandey *et al*., 2011; Jorge *et al*., 2015).

Phakopsora pachyrhizi and *Phakopsora meibomiae* (Arthur), are the causative agents of SBR. The current study focuses more on *P. pachyrhizi* as it is the most aggressive of the SBR pathogens (Goellner *et al.,* 2010; Li *et al*., 2012). The fungus is an obligate plant parasite. It depends on the plant host for its growth and reproduction. Asian Soybean Rust originated in Asia, but now is found around the world, affecting soybean and other leguminous plants (Goellner *et al.,* 2010).

1.3.1. *Phakopsora pachyrhizi*

Phakopsora pachyrhizi has spread all over the world, wherever soybean is produced. The pathogen, first reported in Japan in 1902 (Young, 2012), spread to other Asian countries within a few years. The pathogenic fungus came to Africa through East African countries in 1996 from Kenya, Uganda and Rwanda (Miles *et al*., 2003; Nunkumar, 2006) (Fig 1.1). *P. pachyrhizi* spread to southern Africa, where reports indicate its presence in Zambia and Zimbabwe in 1998, Mozambique in 2000 and South Africa in 2001 (Pretorius *et al.*, 2001). In 2001, SBR was reported in Paraguay, in 2002 in Argentina and in 2003 in Brazil and Bolivia. Schneider *et al.* (2005) confirmed the existence of SBR in the USA from 2004. After its report in Louisiana in 2004, the disease was found in 15 states by 2006 (Park *et al.*, 2008).

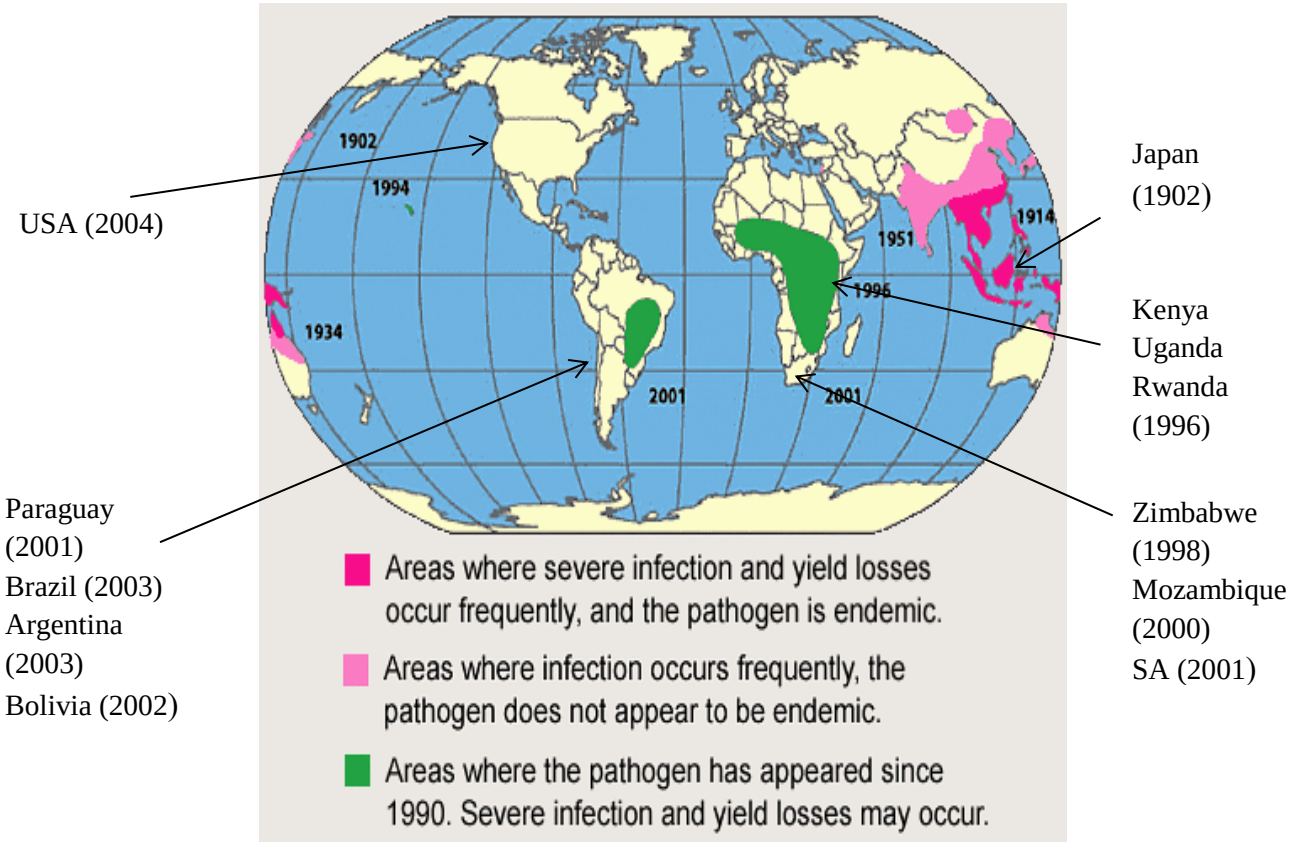

Figure 1.1. Distribution of soybean rust caused by *Phakopsora pachyrhizi* (adapted from Miles *et al.*, 2003)

1.3.2. Economic importance

Worldwide, SBR is the most destructive foliar disease of soybean (Miles *et al.*, 2003; Christiano *et al.*, 2007). In many Asian countries where the pathogen is endemic, yield reduction caused by *P. pachyrhizi* has been reported up to 80% in the absence of control methods (Yang *et al.*, 1991). According to studies conducted by Chakraborty (2009), SBR is the most serious and important menace to soybean.

SBR affects soybean production via premature defoliation, and a reduction of pod numbers and seed weight. In general, the pathogen reduces the soybean plant's biomass as it is able to infect all aerial parts of the plant. The severity of yield loss depends on the time of the infection and the growth stage of the infected soybean plants (Bromfield, 1980; Nunkumar, 2006).

Yield losses caused by SBR have been reported all over the world, especially in regions with moderate winters such as Central-East Africa, Asia and southern America (Miles *et al.*, 2011). The

disease can have a significant economic impact in affected areas. According to Schem (2008), in the 2001-2002 growth seasons, the Brazilian government estimated rust associated losses of 41.9 million tons equivalent to US $125.5 million. In Brazil, after only 5 years (2007), the yield losses caused by the same pathogen increased up to US $10 billion. Also in 2007, Japan faced crop losses up to 40%, while in Taiwan the losses were up to 80% (Jarvie, 2009).

In East-Central African countries where this pathogen first appeared on the continent, SBR continues to cause substantial yield losses. Studies done at Makerere University, Uganda, estimated yield loss due to SBR at 15-80% (Oloka *et al*., 2008). In South Africa, yield losses caused by *P. pachyrhizi* are reported to be 10-80% (Levy, 2005).

1.3.3. Taxonomy and morphology

Phakopsora pachyrhizi Syd. & P. Syd belongs to the Kingdom Fungi; Phylum Basidiomycota; Order Uredinales; Class Urediniomycetes; Family Phakopsoraceae; Genus *Phakopsora* (Goellner et al., 2010). A related rust fungus, *Phakopsora meibomiae*, is less infectious than *P. pachyrhizi*, but can also infect soybean (Bonde *et al.,* 2006). The most accurate way to differentiate these two species is the use of polymerase chain reaction (PCR) with specific primers (Frederick *et al.,* 2002).

P. pachyrhizi forms circular uredial pustules. The urediospores are obovoid to approximately elliptical, with thick cell walls. The size of urediospores is highly variable, depending on different conditions of host and environment. They vary from 16.23 -21.37µm in diameter (Figure 1.2). The inner wall of uredia is surrounded by paraphyses, united at the base and forming a cover over the sporophores. Paraphyses form a volcano-like structure with urediospores on sporophores inside the structure. They are cylindrical to clavate, hyaline to yellowish-brown, and sized from 25-50 x 6-14µm. There is an opening (ostiole) at the top of the volcano-like structure where urediospores are released. *P. pachyrhizi* is among the few rust fungi that can infect the host plant directly without appressorium formation. Telia have been observed on the abaxial leaf surface, mixed with uredia and at the ends of lesions. From orange-brown or light brown at young age, they become dark-brown to black as they mature. They have an irregular shape, mostly round and approximately 150-250µm in diameter (Sinclair and Backman, 1989).

1.3.4. Symptoms

Phakopsora pachyrhizi is capable of infecting host plants at any growth stage (Dias, 2008). The infection usually begins in the lower leaves of the host plant, mostly during or after the flowering

stage, but is only visible when the pods are established (Caldwell *et al*., 2002; Goellner *et al.,* 2010). The common visible SBR symptom on soybean is small dark brown or reddish brown, rounded or many-sided lesions (0.5-5mm^2) on the leaves.

The early visual symptoms give the impression of small water- soaked lesions, which grow and change colour from grey to bronze or brown. The release of observable rust spores is one of characteristic symptoms of the SBR (Caldwell *et al*., 2002). Early symptoms of SBR might be confounded with bacteria pustules (*Xanthomonas campestris* pv. *glycinea*) and bacterial blight (*Pseudomonas savastanoi* pv. *glycinea*), which are also capable of causing defoliation and discoloration of infected soybean leaves.

SBR lesions are commonly seen on the abaxial leaf surface (Bromfield *et al.,* 1980 (Fig 1.2). However, in several situations, they can be observed on petioles, pods and stems of soybean plants (Singh *et al.,* 1977; Vikili, 1981; Caldwell *et al.,* 2002). Each lesion contains multiple urediospores and can be used for pathogen diagnosis. As soon as lesions develop, infected leaves turn yellow and prematurely defoliate, which results in a reduced number of leaves and consequently, a reduced yield of pods and seeds (Akinsanmi *et al*., 2001; Bonde *et al*., 2006).

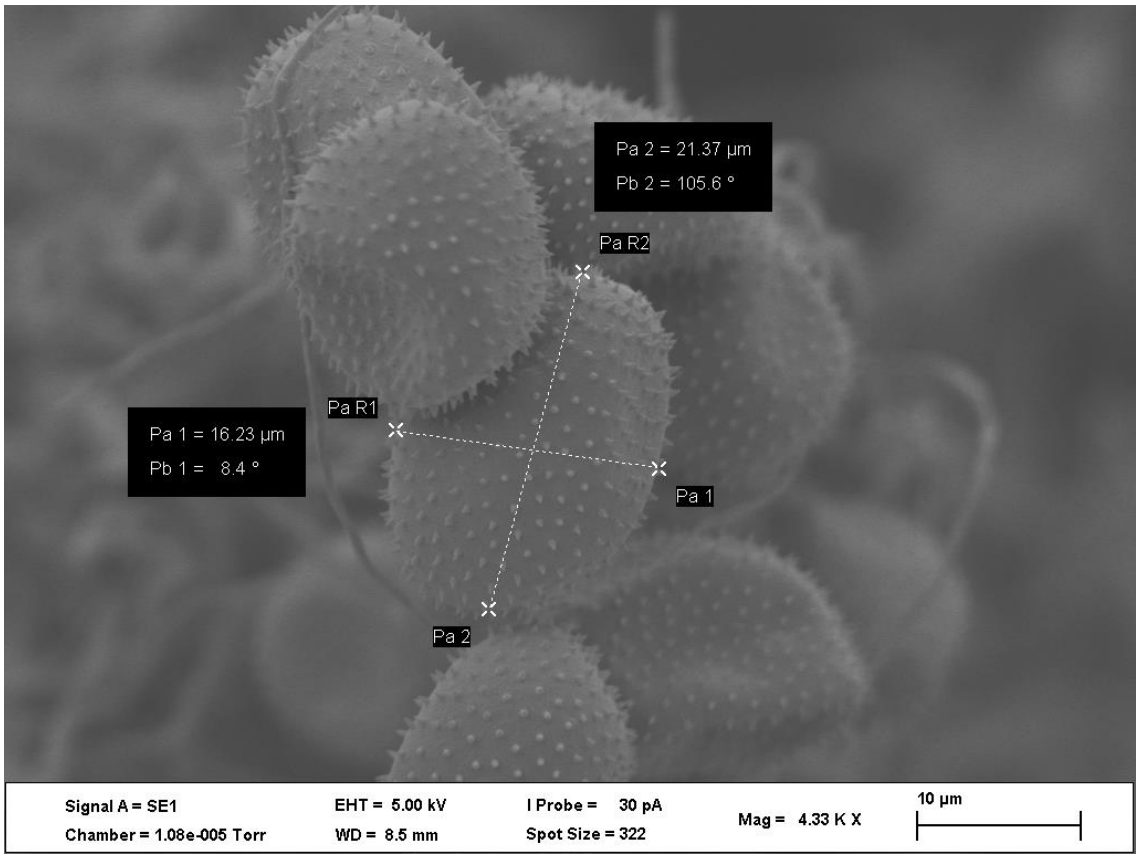

Figure 1.2**.** SEM (Scanning electronic microscope) view of *Phakopsora pachyrhizi* urediospores (photograph by J.P. Havugimana, 2015)

1.3.5. Host range

A plant is called a host of a pathogen when the pathogen can infect, grow and finalise its life cycle on it (Thordal, 2003; Schulze and Panstruga, 2011; Bettergenhaeuser *et al.,* 2014). *P. pachyrhizi* has a wide host range and can infect leaf tissues of many leguminous plants. In the field, more than 31 legumes species in 17 genera have been found to be infected. Under laboratory conditions, it has been shown that the pathogen can infect 60 plant species in 26 genera (Goellner *et al.,* 2010). *Glycine max, Glycine sojae* Siebold & Zuccarini, *Pachyrhizus erosus (*L.*)* Urban, *Pueraria lobate* Siebold & Zuccarini and *Vigna unguiculata* (L) Walp. are the major hosts (USDA, 2009). As with other obligate parasites, *P. pachyrhizi* uses alternative hosts to survive unfavourable conditions (Miles *et al.*, 2003).

1.3.6. Infection Process and Epidemiology

Rust fungi represent a group of plant pathogens that infect many plant species. They have a complex life cycle, depending on the rust species. For rust fungi to complete their life cycle, five spore phases are involved. According to Perez-Hernandez (2007), the following phases are seen:

Phase 0: spermogonia carrying spermatia (n) and receptive hyphae (n)

Phase I: aecia carrying aeciospores (n + n)

Phase II: urediospores (n + n)

Phase III: telia carrying teliospores (n + n: 2n)

Phase IV: basidia carrying basidiospores (n)

According to the above reproductive stages, rust fungi have been divided into three distinct groups: macrocyclic, demicyclic and microcyclic. *P. pachyrhizi* is a microcyclic fungus because it only produces urediospores, teliospores and basidiospores (Alexopoulos *et al.*, 1996; Perez- Hernandez, 2007) (Fig 1.3).

As mentioned earlier, *P. pachyrhizi* is one of the few rust fungi that are capable of penetrating the tissues of the host plant directly (Bonde *et al.*, 1976; Kosh *et al.*, 1983; Du Preez, 2005). Kosh *et al.* (1983) showed that the infection of *P. pachyrhizi* usually starts with the direct penetration by the pathogen of the cuticle of soybean leaves (Fig 1.4). Sometimes appressoria occur over stomata, in which case, the rust fungus enters through the guard cell rather than through the stomatal opening, as occurs with most rusts.

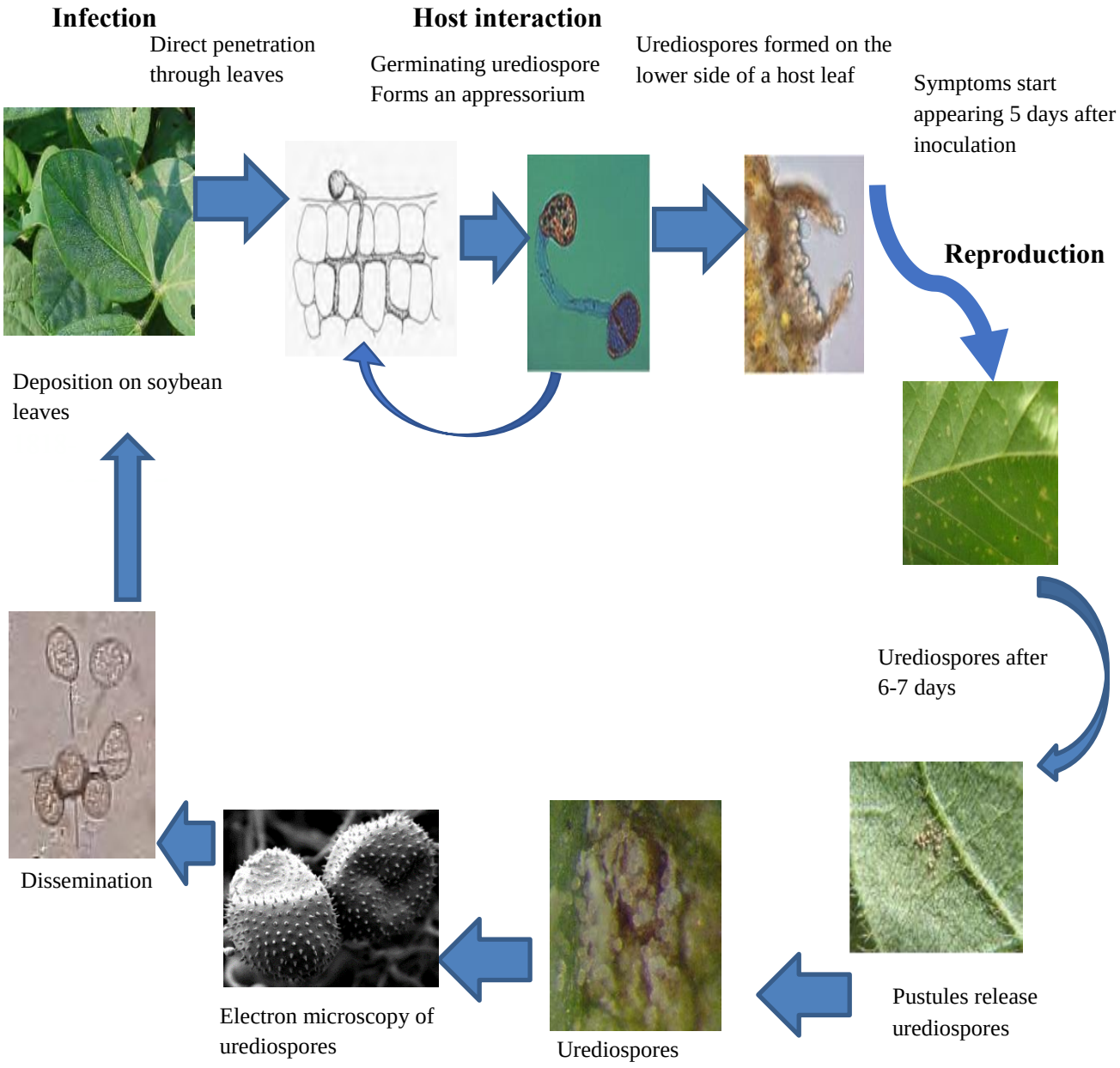

Figure 1.3. Disease cycle of *Phakopsora pachyrhizi* (adapted from Gollner et al., 2010; Young, 2012).

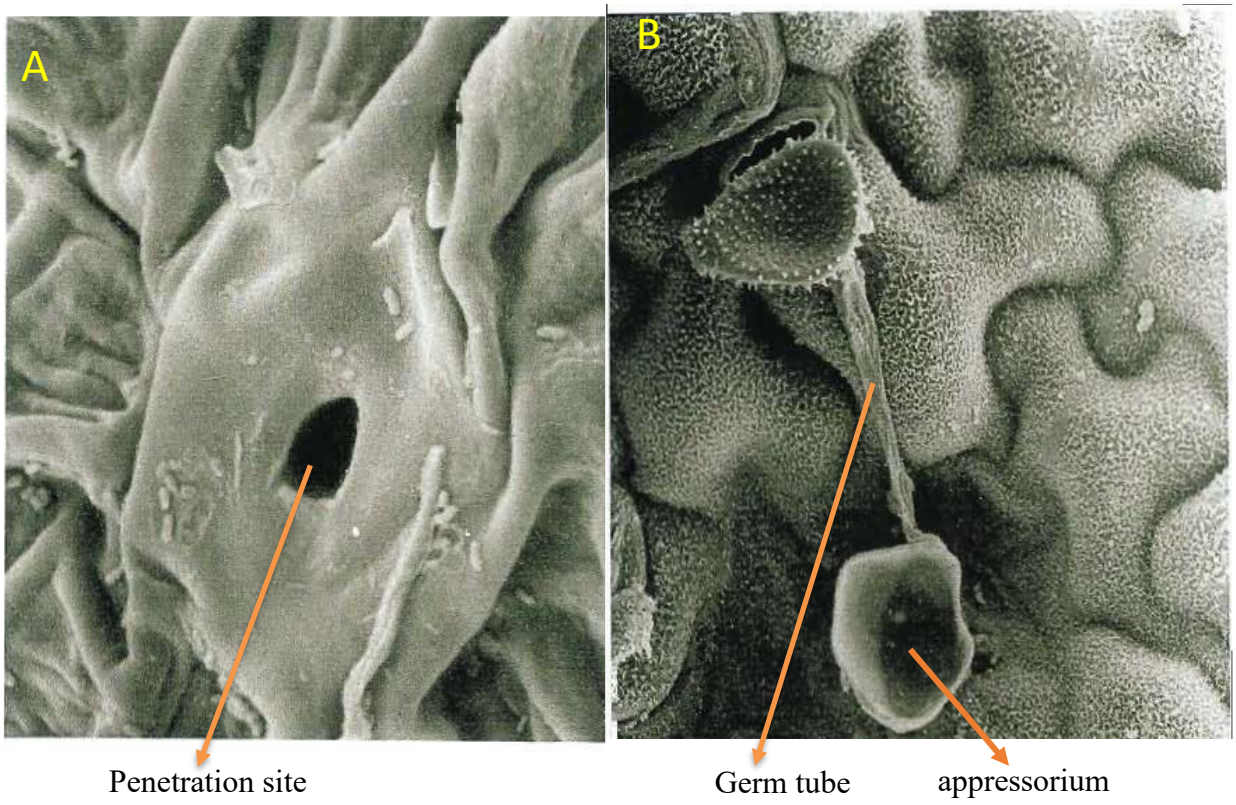

Figure 1.4. Scanning electron micrograph showing a penetration site at which *P. pachyrhizi* directly enters the epidermis of soybean leaf (1.4.A) and germ tube (1.4.B) (Du Preez, 2005).

The disease cycle starts with the presence of urediospores on the soybean leaf. Urediospores are predominately wind dispersed and serve as the only known infective type. The infection process begins with the germination of urediospores on the host tissues. This occurs within 2 hours after spore deposition, under favourable environmental conditions (Young, 2012) (Table 1.1).

Table 1.1. The sequence of events over time in development of SBR caused by *P. pachyrhizi* (Melching *et al.*, 1975; Bonde *et al.*, 1976; McLean, 1981; Koch *et al.*, 1983; Miles *et al.*, 2003; Du Preez, 2005).

Sequence of events	**Time**
1. A urediospore lands on soybean leaf surface over epidermal cell	0 hpi
2. Germ tube development (5-400µm)	12 hpi
3. An appressorium cone formed	16 hpi
4. Penetration hyphae formed	16 hpi
5. First hyphal septum formed	18-20 hpi
6. Primary hyphae produced	18-20 hpi
7. Collapse of epidermal cell	24 hpi
8. Haustorium formed	24-48 hpi
9. Branching into secondary hyphae	48-72 hpi
10. Mycelial development inside spongy mesophyll and intercellular space	3 dpi
11. Collapse of appressorium and penetration hyphae	4 dpi
12. Necrotic lesions appear on leaf	6 dpi
13. Runner hyphae passing through mesophylls	7 dpi
14. Hyphae aggregate, uredial primordia formed	9 dpi
15. Urediospore mature	11-12 dpi

hpi: hours post infection; dpi: days post infection

Many studies have been done on the epidemiology of *P. pachyrhizi* (Bromfield, 1980; Tschanz and Wang, 1980; Casey, 1981; Yang *et al.*, 1991; Del Ponte *et al*., 2006). The presence of receptive host plants, infectious rust spores, and favourable conditions are crucial for *P. pachyrhizi* infection to take place and for its development. Both the host plant and the pathogen need favourable environmental conditions to develop. The optimum temperature for *P. pachyrhizi* urediospore to germinate ranges between 13-27^{o}C (Du Preez, 2005; Bonde *et al.*, 2012). It has been shown that urediospores germinate better in the dark and that the presence of light inhibits or delays germination. In the presence of favourable conditions and host plant leaf tissue, *P. pachyrhizi* urediospores takes about 6 hours to germinate (Jarvie, 2009; Bonde *et al.*, 2012).

1.3.7. Disease management

Successful SBR management currently involves the use of suitable fungicides and cultural practices (Abawi and Widmer, 2000; Twizeyimana and Hartman, 2017).

1.3.7.1.Cultural control

The use of cultural practice is an approach to reduce SBR incidence, or to delay disease development. The cultural control measures can be practised alone or in combination with fungicides to manage SBR (Du Preez, 2005; Baley *et al*., 2009).

SBR infection is intense at different times of the year. In KwaZulu-Natal, it was observed that the disease appeared late in the growing season. Thus, early planting can help to avoid severe losses (Caldwell and McLaren, 2004; Du Preez, 2005). According to DAFF (2010), planting early to mid-November is the most appropriate for optimum yield production in South Africa.

Spacing between rows might reduce disease levels. The closer the infected plants are, the easier it is to contaminate neighbouring plants. Caldwell and McLaren (2004) showed that a wider row-spacing of about 90 cm allowed the symptoms to be seen early, and to facilitate disease management, while narrow rows promoted disease dispersion and prevented the fungicides from penetrating the canopy.

Cultural practices also involve the control of alternate host plant because *P. pachyrhizi* can infect many legume species on which the fungus can overwinter to infect the next soybean crop. Crop rotation may reduce this disease (Sinclair and Hartman, 1996).

1.3.7.2.Chemical control

The use of fungicides appears to be the only effective approach for SBR management currently available. It includes preventive and curative fungicide applications (Hartman *et al.,* 1999). However, to be effective, fungicides need frequent applications (3-4 applications per season), which makes this approach expensive for soybean farmers.

Since the appearance of SBR, much research has been done on fungicide applications to control the rust fungus. Glogoza (1998) listed propiconazole, chlorothalonil and maneb as the major fungicides used at that time for rust control in Minnesota and North Dakota, USA. According to Patil and Anahosur (1998), triazole fungicides were used for SBR control just prior to 1998.

In southern Africa, fungicide trials were conducted in Zimbabwe after the appearance of SBR in 1998 (Levy, 2003). Later, USDA conducted a study on fungicide efficacy for SBR control in 3 different countries: South Africa, Zimbabwe and Paraguay (Levy, 2004; Miles *et al*, 2004; Monte *et al*., 2004; Mueller *et al*., 2009).

Triazoles have been shown to be the most effective group of fungicides for SBR control. However, continuous use of triazoles without alternating with other fungicide groups, SBR will develop resistance to the fungicides. The rotational use of fungicides is recommended together with combinations, for example, a strobilurin-triazole mixture. Abacus (epoxiconazole), Bayfidan® 250DC (triadimenol), Capitan® 250EW (flusilazole), D-Zole 250EC (difenoconazole), Folicur 250®EW (tebuconazole), Impact (flutriafol), Lyric® 250EW (flusilazole), Lyric® C (carbendazim/ flusilazole), Pronto 250EC (difenoconazole), Punch® C (carbendazim/ flusilazole), Punch®-xtra (carbendazim/ flusilazole), Scope 250 (difenoconazole), Score® 250EC (difenoconazole), Shavit 250EC (triadimenol), Tebucure (Tebuconazole), and Tristar EC (triadimenol) are fungicides registered in South Africa for SBR control (Laing and Caldwell, 2014).

1.3.7.3.Resistance breeding

Breeding for SBR resistance seems to be the best control option for farmers. Partial resistance to SBR has been reported, from both classical breeding and molecular approaches (Goellner *et al.*, 2010). However, at present, there is no available commercial cultivar that is resistant to all *P. pachyrhizi* strains (Yorinori *et al.*, 2005; Goellner *et al.*, 2010).

Some soybean varieties show tolerance to SBR. In studies conducted in Taiwan, the variety Shih-shih had good yields even while heavily rusted (Bromfield *et al.*, 1980). In South Africa, 23 soybean cultivars were evaluated for SBR resistance during the 2003/04 and 2004/05 at Cedara (McLaren, 2008)

Four SBR vertical resistance genes have been identified (Rpp1, Rpp2, Rpp3 and Rpp4) (Garcia *et al*., 2008; Monteros *et al*., 2007; Goellner *et al*., 2010). However, these genes are rapidly matched by virulent races of SBR (Pham *et al*., 2009).

1.3.7.4.Biological control

Biocontrol is the use of natural enemies to reduce the impact of a damaging organism. Such natural enemies include pathogenic and competitor microorganisms. This disease control method has been

practised for around two millennia (Joop and Lenteren, 2011). Many fungi species have been shown to have the capacity to supress growth of rust fungi. In an effort to find an alternative approach to controlling SBR, several mycoparasitic fungi have been tested against *P. pachyrhizi*, such as *Verticillium psalliotae* Treschow (Saksirirat and Hoppe, 1991; Goellner *et al*., 2010), *Trichothecium roseum* (Pers.) (Kumar and JHA, 2002), *Gliomastrix* and *Trichothecium* species (Ward, 2011), *Tuberculina* species, *Verticillium lecanii* (Zimm.) (Saksirirat and Hoppe, 1991) and *Simplicillium lanosoniveum* Zare & W. Gams (Ward *et al.*, 2011). Many other fungi have been seen colonising other rusts, such as *Verticillium, Cladosporium, Lecanicillium* and *Simplicillium spp* (Saksirirat and Hope, 1990; Assante *et al.*, 2004; Ward, 2011).

Ward (2011) at Louisiana State University highlighted the potential of *S. lanosoniveum* to control SBR *in vitro*. In a microscopy study several hyphae were observed binding urediospores and colonizing *P. pachyrhizi* sori (Fig 1.5). Scanning Electron Microscope examinations showed that the fungus appeared only on soybean leaves if rust spores were present. The nature of interaction between both fungi, *P. pachyrhizi* and *S. lanosoniveum,* is not yet fully explored. Studies are needed on its mode of action and to determine if it is either mycoparasitism, cohabitation or necrotrophic growth.

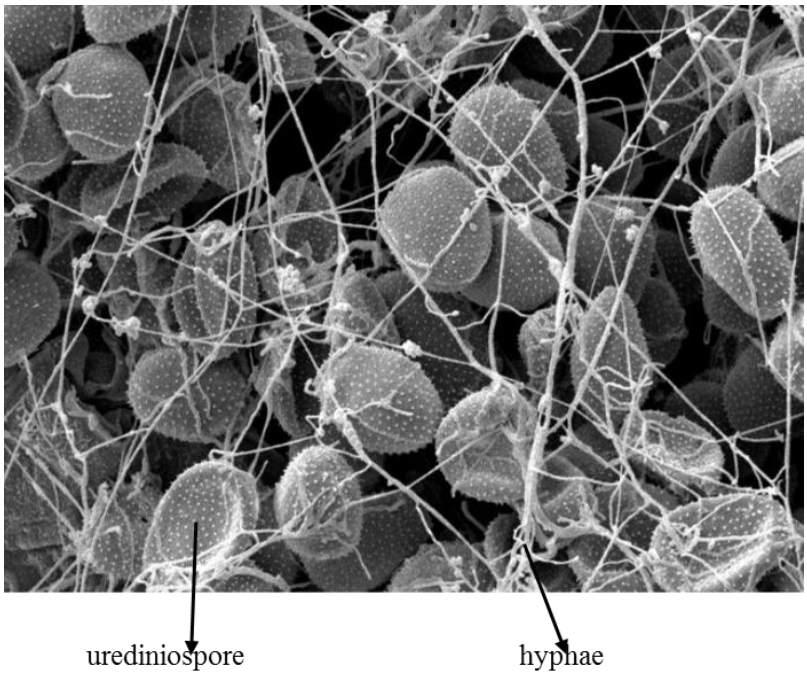

Figure 1.5. Colonisation of *P. pachyrhizi* by *S. lanosoniveum* a under scanning electron microscope (Ward *et al*., 2011).

In February 2013, the author noted a fungus, later identified as *Lecanicillium muscarium (*Petch) Zare et Gams, parasitizing coffee rust (*Hemileia vastatrix* Berkeley and Broome) at the Assagay Coffee Farm at Cato Ridge, near Pietermaritzburg, South Africa. It is hypothesised that this mycoparasite fungus could be useful for controlling SBR. The successful application of *L. muscarium* as a biocontrol agent against SBR could reduce the frequent and costly use of fungicides on soybean. This would reduce input costs for farmers and increase yield by reducing SBR severity to a low level.

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CHAPTER 2: IDENTIFICATION OF A STRAIN OF *LECANICILLIUM* SPECIES AS A POTENTIAL BIOCONTROL AGENT FOR SOYBEAN RUST

Abstract

Soybean rust (SBR) is the most damaging foliar disease in many soybean growing areas of the World. Fungicide sprays seems to be the only method available for SBR control. An alternative to fungicides is needed, considering that some *Phakopsora pachyrhizi* strains have shown resistance to triazole fungicides. The main objective of this chapter was to isolate and identify a *Lecanicillium* spp. that could be used for SBR control. An isolate of *Lecanicillium spp* strain Nesta-08 was isolated from the Assagay Coffee Farm, Cato Ridge, KwaZulu-Natal, South Africa, where it was observed parasitizing *Hemileia vastatrix*, the causal agent of the coffee rust. The fungus was isolated on Sabouraud dextrose yeast extract agar (SDYA) and the pure culture obtained was subcultured onto potato dextrose agar (PDA). Under light microscopy, conidia were found to be ovoid to ellipsoidal. Under scanning electronic microscopy, the size of conidia ranged between 1.9-3.9µm. Based on nBLAST using ITS and ITS4 sequences, the isolate Nesta-08 presented 99.8% similarity to strain CBS 318.70B of *L. muscarium*. Based on the DN_3 gene, isolate Nesta-08 shows a common ancestor with *L. muscarium* at a low bootstrap value (58-59). Based on DN_1, isolate Nesta-08 shows a common ancestor with *L. muscarium* and is supported with a high bootstrap value of 94. Based on morphological and molecular studies the isolate was identified as *Lecanicillium muscarium*. It was deposited into the National Collection of Fungi with an accession number PPRI 13715.

2.1. Introduction

In South Africa, and worldwide, plant rusts are recognised among the most destructive plant diseases (Du Preez, 2004; McLaren, 2008). Rust fungi are obligate parasites that feed directly on the host plant tissues, mostly by feeding on the leaf-tissue, which affect the photosynthetic process and lead to early senescence and defoliation. In recent years, the development of effective biological control for plant pest and disease has prompted considerable interest among agricultural researchers and commercial industries (Cuthbertson et *al.*, 2005; Ravensberg, 2011).

Lecanicillium muscarium (previously known as *Verticillium lecanii*) has been studied and used as biocontrol agent against many arthropods (Askary *et al.*, 2007; Cuthbertson *et al.*, 2008). Research

reports have highlighted the hyperparasitic potential of *Lecanicillium spp.* against nematodes, insects, and fungi (Goettel *et al.*, 2008; Shinya *et al.*, 2008). *Lecanicillium muscarium* belongs to the phylum Ascomycota, order Hypocreales (Zare *et al*., 2000; Grams and Zare, 2001; Kouvelis *et al*., 2008). Researchers and commercial biocontrol companies are interested in *L. muscarium* due to its wide host range, e.g., aphids, scales, whitefly, phytopathogenic fungi and plant parasitic nematodes (Hall, 1984; Yang *et al*., 2005). Mycotal® (Koppert BV, Netherlands) is a commercial biocontrol product of *Lecanicillium* spp. A related commercial product is Vertalec® (Koppert BV, Netherlands), based on the entomopathogenic mitosporic fungus *Lecanicillium longisporum*. Studies have shown its suppressive effect against powdery mildews and aphids (Askary *et al.*, 1998; Miller *et al*., 2004; Kim *et al*., 2008). However, no commercial product has been developed to control rust fungi.

The aim of this study was to isolate and identify a locally adapted strain of *Lecanicillium* spp for SBR control. The efficacy of a biocontrol agent against rust fungi could reduce the costs associated with fungicide use for SBR control.

2.2. Materials and Methods

2.2.1. Sample collection

Coffee leaves were sampled from the Assagay Coffee Farm located in Cato Ridge, KwaZulu-Natal, South Africa (Fig 2.1), where *Lecanicillium* spp. was identified hyperparasiting coffee rust (*Hemileia vastatrix*) (Fig 2.2).

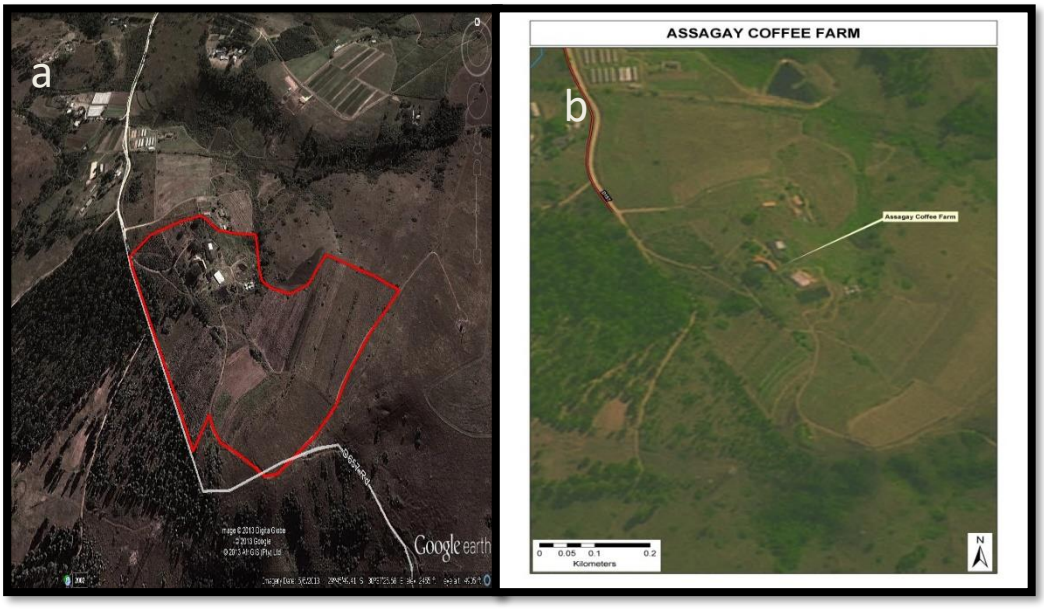

Figure 2.1. Aerial photograph (a) and locality map (b) of the Assagay Coffee Farm.

A local strain of *Lecanicillium* spp was observed feeding on pustules of *H. vastatrix*. It was hypothesized that this adapted local strain of *Lecanicillium* spp. could be developed and used as a biocontrol agent for controlling rust fungi.

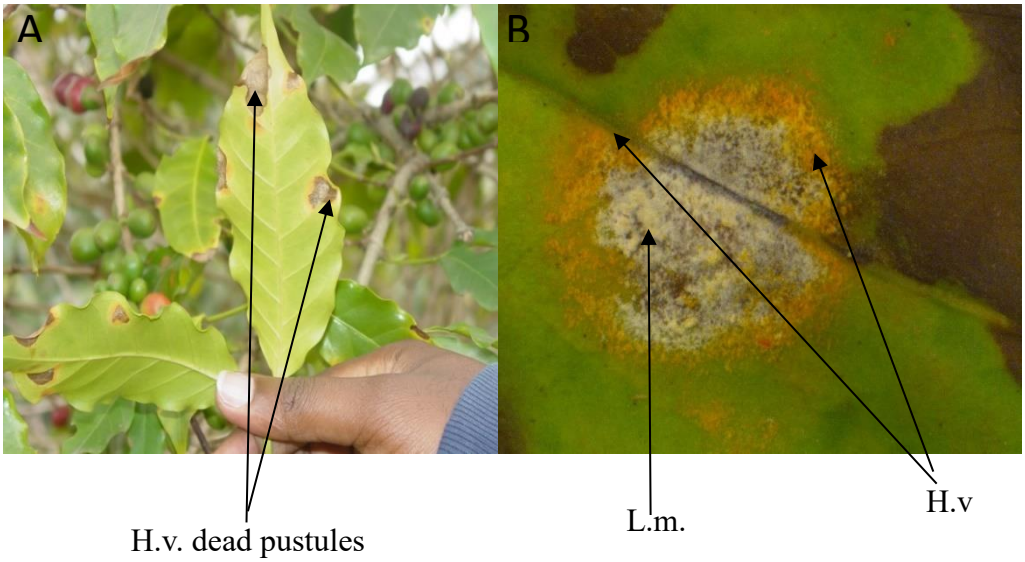

Figure 2.2. *Lecanicillium* spp (L.m.) hyperparasiting the rust fungus of coffee, *H. vastatrix* (H.v.), at the Assagay Coffee Farm, Cato Ridge, South Africa.

Coffee leaves with both *Hemileia vastatrix* and *Lecanicillium spp* were collected from the Assagay Coffee Farm, transferred to a University of KwaZulu-Natal (UKZN) Plant Pathology laboratory and stored overnight at room temperature (25-26^{o} C) until further use.

2.2.2. Isolation of *Lecanicillium* spp

The *Lecanicillium* spp was isolated on Sabouraud dextrose yeast extract agar (SDYA). The medium consisted of (10g mycological peptone, 40g dextrose (D-glucose), 2g yeast extract, 15g agar) (Gerritsen and Cornelissen, 2006). A whitish powder of *L. muscarium* conidia was washed off the coffee leaves and transferred onto SDYA. The plates were incubated at 25^{o} C in the dark. Fungal growth was monitored every 3 days for a period of 12 days. After 12 days, pure colonies were selected and subcultured onto potato dextrose agar (PDA). The *Lecanicillium* isolate was maintained through periodic re-inoculation of pure culture on PDA. For short-term preservation, double sterilized distilled water was used (Richter and Bruhn, 1989). This method can keep the culture alive for 3 to 6 months (Johnson and Martin, 1999). Agar slants were used for medium term storage.

2.2.3. Morphology of the *Lecanicillium* isolate

The shape, colour, mycelia type and length of conidia were recorded. The pure culture of the isolate grown on PDA was subjected to different microscopy studies to confirm the morphology of the isolate. Light microscopy and Scanning Electron Microscopy (SEM) were used to examine spores size and structure.

Conidia were harvested from PDA plates by washing the fungal mycelia with sterile distilled water using a sterile L-shape glass rod and the suspension filtered through cheese cloth. Spore suspension was examined under optical microscopy. Images were taken with Zeiss Axiocam ERC5S camera (Carl Zeiss Microscopy, USA).

Agar disks ($4mm^2$) covered with mycelia of *Lecanicillium* were placed on SEM specimen stubs and fixed in 3% buffered glutaraldehyde for 1 hour. Samples were washed twice in sodium cacodylate buffer for 5 min. After the buffer wash, specimens were dehydrated (5 min in each concentration) in 10% ethanol, 30% ethanol, 50% ethanol, 70% ethanol, 90% ethanol and finally twice in 100% ethanol, each for 15 min. Dehydrated sample stubs were transferred into the sputter coat and examined under SEM using a ZEISS EVO LS 15 (Carl Zeiss Microscopy, USA).

2.2.4. Genomics

2.2.4.1.DNA extraction, PCR, cloning and sequencing

The DNeasy Plant Mini Kit was used to extract total DNA from mycelium growing on PDA plate according to the manufacturer's instructions (Qiagen, Germany) (Fredrick *et al*., 2005). A polymerase chain reaction (PCR) was performed to amplify the internal transcribed spacer (ITS), and specifically the portion of the mitochondrial encoded NADH dehydrogenase subunits 1 (MT-ND1) and 3 (MT-ND3) genes (Prochazka et al., 2010; Schoch *et al*., 2012). PCR was run according to the settings described by Kouvelis *et al.* (2008), using the KAPA2G HotStart ReadyMix and the primers listed in Table 2.1.

Table 2.1. Primers used and PCR settings

Name	Sequence (5'-3')	PCR settings*	Target amplicon size	Reference
VLITS1	GTCCCTGCCCTTTGTA	95°C-15s 95°C-15s 72°C-15s	ITS 758 bp	Kouvelis *et al.*, 2008
VLITS2	CCTGGTGGTTTCTTTTCC			
*nad*1A	ATGGCIAGTATGCAAAGAAGA	95°C-15s 95°C-15s 72°C-15s	MT-ND1 493 bp	Kouvelis *et al.*, 2008
*nad*1B	GCATGTTCTGTCATAAAICCACTAAC			
*nad*3A	ATTTGAATGTGGTTTTCAT	95°C-15s 95°C-15s 72°C-15s	MT-ND3 225 bp	Kouvelis *et al.*, 2008
*nad*3B	AATGCAITTTTACCTAATTCA			

*: 35 cycles of PCR were performed.

The sizes of the amplicons generated were assessed on a 2% agarose gel in TAE buffer pre-stained with SYBR Safe (ThermoScientific, USA) (Fukushima *et al*., 2003; Nam *et al*., 2004). Amplicons of the expected sizes were gel-purified using the QIAquick® gel extraction kit (Qiagen, Germany) before being cloned to the pCR2.1 vector of the TOPO® TA cloning kit (ThermoScientific, USA), according to the manufacturer's instructions. Two recombinant clones of each amplicon were sequenced in forward and reverse directions, using the M13 primers (Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa).

2.2.4.2.Phylogenetic analyses

The data generated from sequencing were aligned in order to determine the consensus sequences. The NCBI BLAST tool was used to identify the organism under study. *Lecanicillium* spp. from the BLAST results were selected for phylogenetic analyses (Ramanujam *et al.*, 2011; Kumar *et al.,*

2015). All the alignments were done using the Muscle program implemented in MEGA 6 software (Tamura *et al.*, 2013). Tamura 3 parameters (Tamura, 1992) with gamma distribution were identified as the best substitution model for this study and were used to infer the maximum likelihood trees with 500 bootstrap replicates. The initial trees for the heuristic search were obtained automatically by applying the maximum parsimony method (Sourdis and Nei, 1988; Kumar *et al.,* 2008). Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013).

2.3. Results

2.3.1. Morphology of *Lecanicillium* isolate

The *Lecanicillium* isolate grown on PDA produced white mycelia (Fig 2.3). Under the light microscope, conidia were found to have ovoid to ellipsoidal shapes. Under SEM, conidia ranged between 1.9-3.9µm (Fig 2.4).

Figure 2.3. Light microscopy image of a *L. muscarium* colony on a PDA plate.

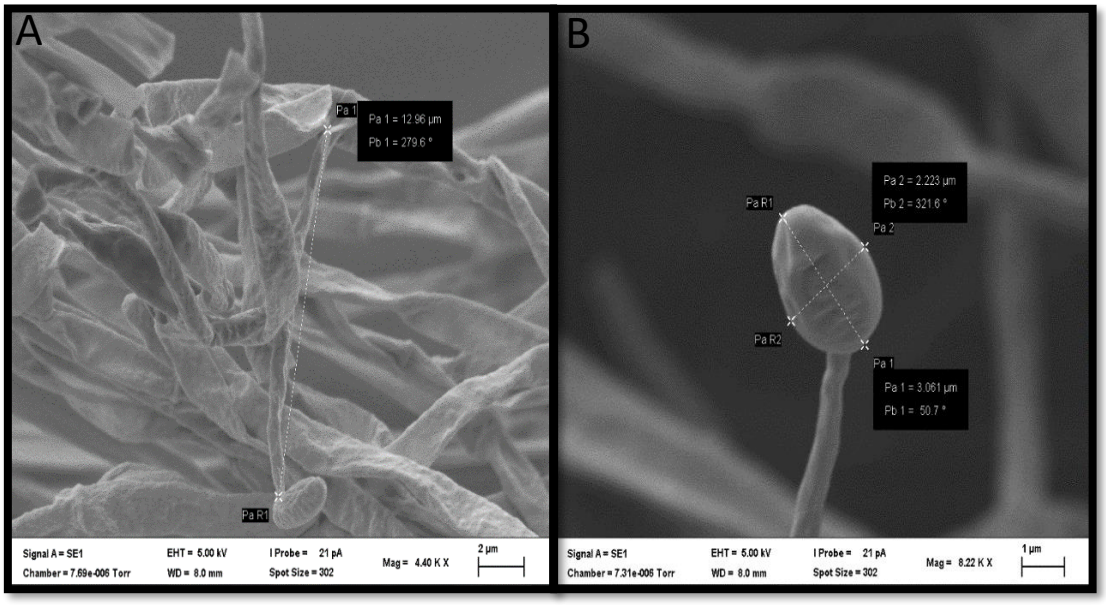

Figure 2.4. An SEM view and measurement of a *L. muscarium* conidium.

2.3.2. Genomics

2.3.2.1.Phylogenetic analyses

The BLAST hits of the consensus sequences matched various species of *Lecanicillium*. This pattern was also noticeable in the phylogenetic tree generated. The phylogenetic trees of MT-ND1 and MT-ND3 indicated a distant relationship between the South African isolate (Nesta-08) and *L. muscarium* (Figs 2.5 & 2.6). Isolate Nesta-08 formed a unique cluster when using the phylogenetic results of the ITS regions (Fig 2.7). The DN_3 gene in isolate Nesta-08 showed a common ancestor with *L. muscarium* at a low bootstrap value (58-59). The DN_1 gene in Isolate Nesta-08 shows a common ancestor with *L. longisporum* and *L. muscarium*, supported with a high bootstrap value of 94. The ITS region showed a common ancestor with *L. lecanii* with a bootstrap value of 96. nBLAST analysis of the ITS and ITS4 sequences of isolate Nesta-08 determined a 99.8% similarity to strain CBS 318.70B of *L. muscarium.*

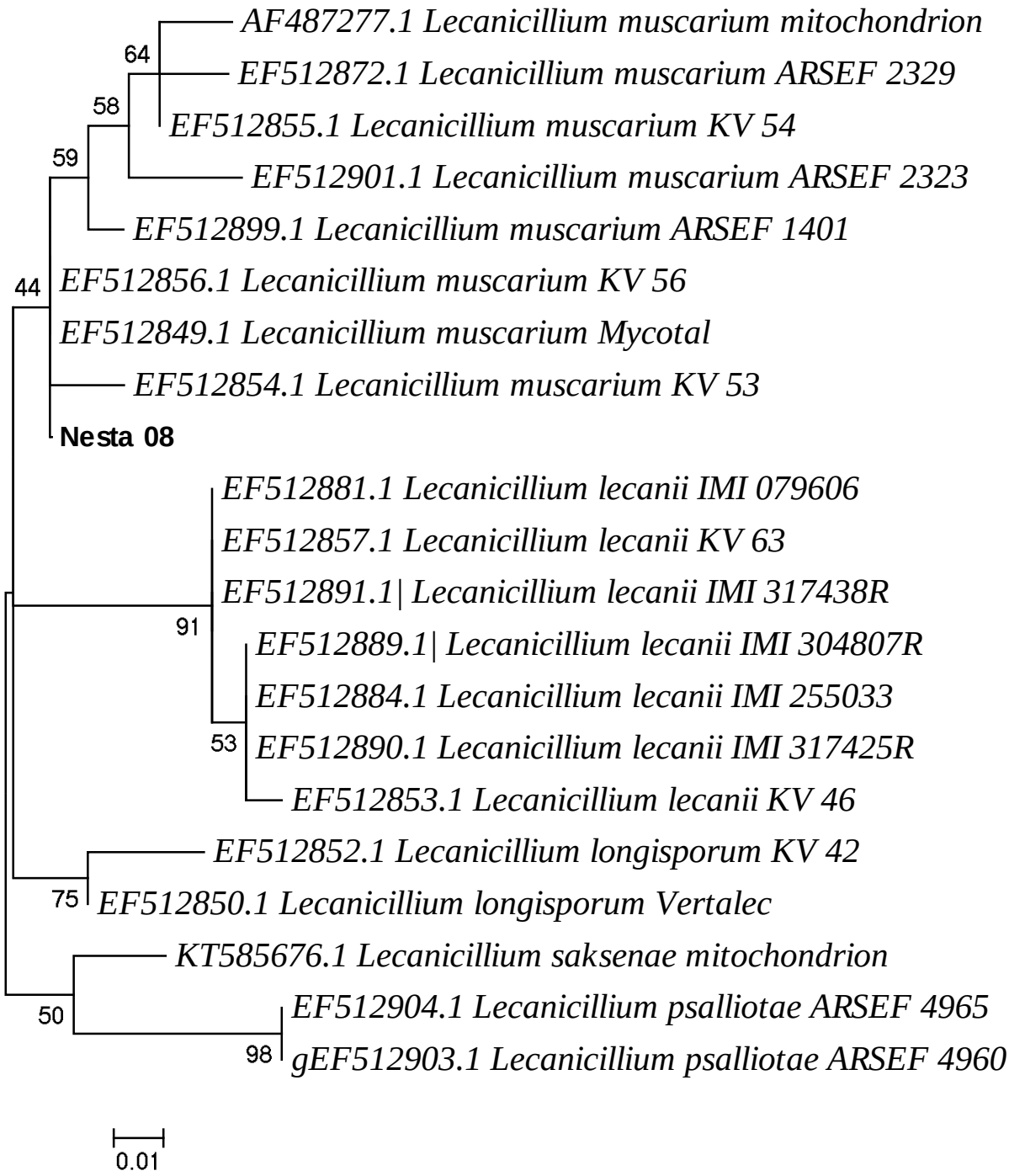

Figure 2.5. A phylogenetic tree from the maximum likelihood analysis of the MT-ND3 gene. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

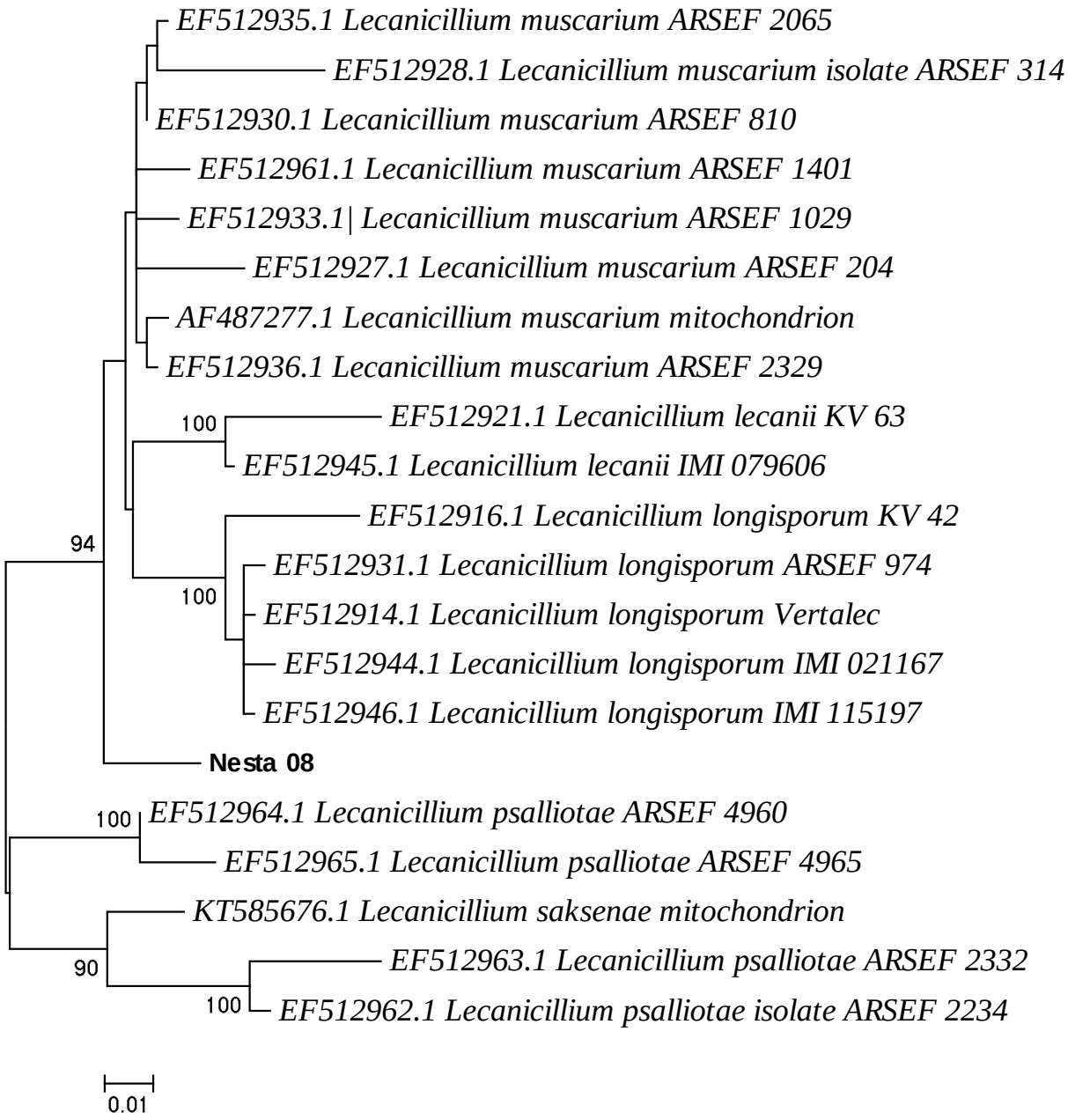

Figure 2.6. A phylogenetic tree from the maximum likelihood analysis of the MT-ND1 gene. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

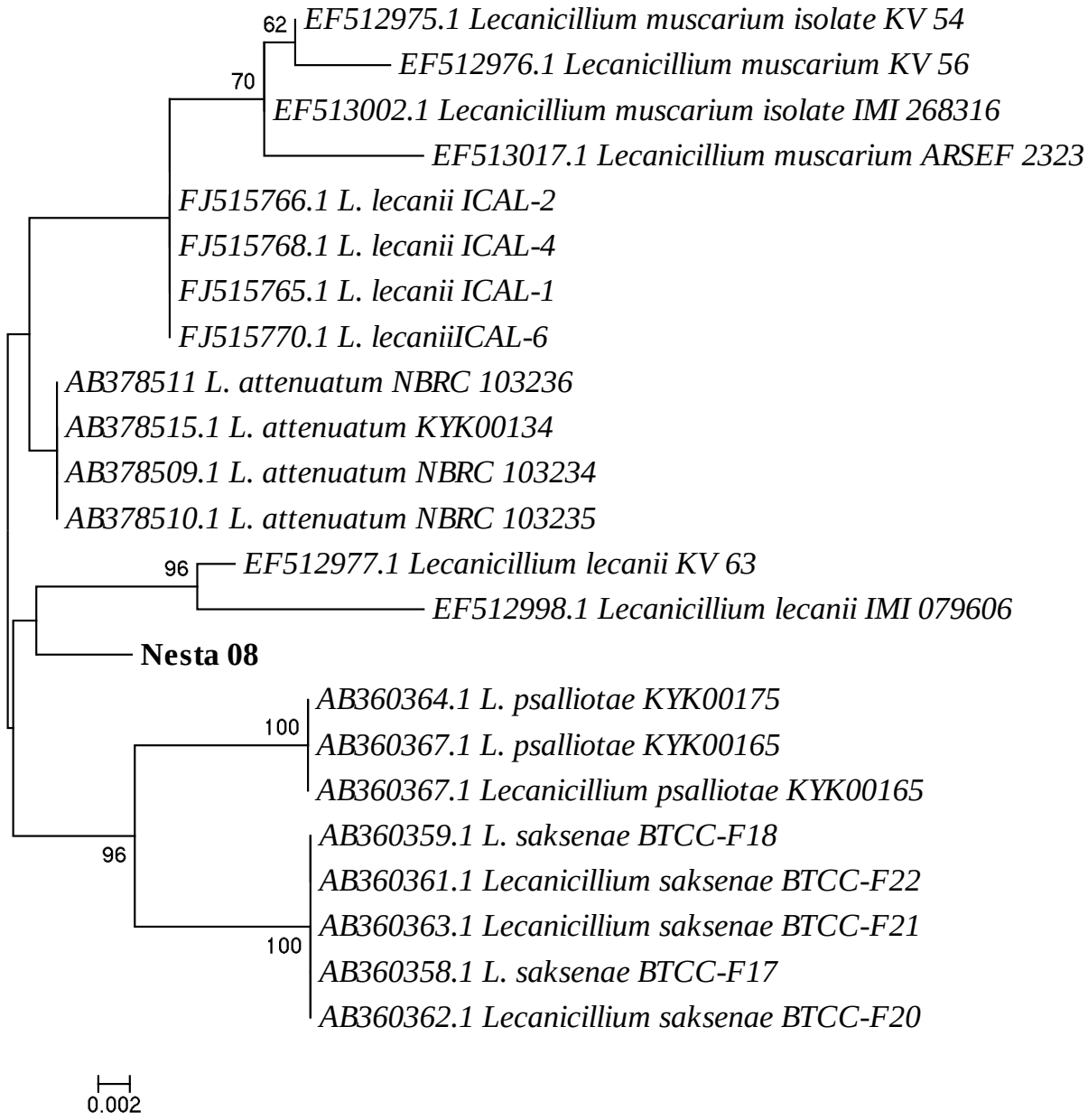

Figure 2.7. A phylogenetic tree from the maximum likelihood analysis of the ITS region. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

2.4. Discussion

According to Cortez-Madrigal *et al.* (2003), the selection of virulent isolates adapted to local environmental conditions is one of the essential aspects for the development of efficient biological control agents. The main aim of this chapter was to isolate and identify a strain of *Lecanicillium* spp. which could be used as a biological control agent for SBR control

An isolate of *Lecanicillium spp* strain Nesta-08 was isolated from Assagay Coffee Farm, Cato Ridge, KwaZulu-Natal, South Africa, where it was observed parasitizing *Hemileia vastatrix*, the causal agent of the coffee rust. The fungus was isolated on Sabouraud dextrose yeast extract agar (SDYA) and the pure culture obtained was subcultured onto potato dextrose agar (PDA). On PDA plates, isolate Nesta-08 produced a white yellowish mycelium and under light microscopy, conidia were found to be ovoid to ellipsoidal. Under scanning electronic microscopy, the size of conidia ranged between 1.9-3.9µm. These results confirm the research done previously where *Lecanicillium* spp. were found to be white in colour and 2.27 µm in length (Diaz *et al*., 2009).

Fungi are morphologically complex organisms. The structure of conidia and spores vary, depending on the life cycle stage and environmental conditions (Papagianni, 2004). To identify isolate Nesta-08, molecular studies were done in addition to morphology studies. The internal transcribed spacer (ITS) region is one of the wider sequenced fungal DNA region and has been used widely as a phylogenetic marker (Hillis and Dixon 1991; Salazar *et al.,* 1999; Diaz *et al.,* 2009). The kind of ambiguities noticed in this study have already been reported by some authors such as Kouvelis *et al*. (2008) and Diaz *et al.* (2009). Taxonomic ambiguity observed with phylogenetic analyses is not uncommon in the classification of *Lecanicillium* species. Kouvelis *et al.* (2008) reported a similar finding from their studies using the same regions of the genome used in this study. For very close-related species is not easy to identify and differentiate them based only on the ITS region alone, analysis of the ITS region was combined with analysis of the DN_3 and DN_1 genes.

Isolate Nesta-08 was considered to be a strain of *L. muscarium* because of its distant relationship with other *L. lecanii* isolates. The phylogenetic trees of MT-ND1 and MT-ND3 indicated a distant relationship between the isolate Nesta-08 and it is supported with a very high bootstrap value.

Based on morphological and molecular studies, the entomopathogenic fungus was identified as *L. muscarium*. The Isolate N-08 was deposited into the National Collection of Fungi with accession number PPRI 13715.

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CHAPTER 3: STUDIES ON THE GROWTH AND PRODUCTION OF *LECANICILLIUM MUSCARIUM* STRAIN NESTA-08

Abstract

Optimization of growing conditions is an essential aspect that must be taken into consideration to produce an effective biocontrol agent. Laboratory experiments were conducted to assess the effects of different growing conditions (temperatures, artificial growing media, natural substrates and UV radiation) on colony growth and conidia production on *L. muscarium* strain Nesta-08. *L. muscarium* strain Nesta-08 grew best at temperatures ranging between 21 to 25^{o}C, and the best radial growth was observed at 24^{o}C after 30 days. V8 tomato juice agar was the best medium for colony growth followed by Sabouraud dextrose agar (SDA). *L. muscarium* strain Nesta-08 was exposed to 370 nm UV-A radiation for 30 days. UV light did not affect mycelial growth. When *L. muscarium* strain Nesta-08 was grown on various industrial food sources, the most conidial production occurred on pearl millet grain, followed by wheat bran and pearled barley.

3.1. Introduction

Lecanicillium muscarium has been shown to parasitize and kill or seriously disable a range of insects (Marshall *et al*., 2003; Goettel *et al*., 2008; Kim *et al*., 2008; Lazreg *et al*., 2009; Cuthbertson *et al*., 2010). The fungus infects whitefly, aphids and thrips (Hall 1976; Schreiter *et al*., 1994; Andrew *et al*., 2005; Shi *et al*., 2007). Due to its entomopathogenic potential, *L. muscarium* has been developed into several commercial biopesticides (Goettel *et al.*, 2005; De Maria and Wraight, 2007; Goettel *et al*., 2008). Recent research has demonstrated the activity of *Lecanicillium* spp. against nematodes, powdery mildew and other fungal plant pathogens (Miller *et al*., 2004; Goettel *et al*., 2008; Yu *et al*., 2015). However, there are no studies done on *Lecanicillium* spp. infecting plant rusts.

One of the factors that needs to be taken into consideration for the success of pathogen control using hyperparasitic fungi is the virulence of the isolate (Aiuchi *et al.*, 2007; Khan *et al.*, 2012). The virulence of isolates is determined by their exertion of mechanical forces and production of degradative enzymes. For *Lecanicillium* spp., three specific enzymes have been observed:

chitinase, protease and lipase. Research done by Xie *et al*. (2010) and Reyes *et al*. (2012) on *Lecanicillium* spp. enzyme production, showed that chitinase was the enzyme most secreted, whereas the other two are produced in small quantities. Other important factors to consider are the rate of mycelial growth and the number of conidia produced (Vu et al., 2007).

Choosing an entomopathogenic fungal isolate for use as a biocontrol agent demands a good understanding of its growth and production requirements including the optimum temperature for mycelial growth and sporulation (Vidal *et al*., 2003; Kope *et al*., 2008). The purpose of this study was to evaluate the effect of different growing media, temperatures and UV-light on growth and conidial production of *L. muscarium* strain Nesta-08.

3.2. Materials and methods

3.2.1 Lecanicillium muscarium

In the current study, a strain of *Lecanicillium muscarium* (Nesta-08) was used, which was isolated from the Assagay Coffee Farm in Cato Ridge, KwaZulu-Natal, South Africa (29˚45´49.41´´S and 30˚37´25.68´´E). This strain had been observed colonising pustules of coffee rust (*Hemileia vastatrix*). To optimize growing and sporulation conditions, it was cultured on different growing media, incubated on different temperatures and exposed to UV light.

3.2.2 Effects of growing media and temperatures on *L. muscarium* colony growth

Strain N-08 of *L. muscarium* was cultured on plates and incubated in the dark at 5 different temperatures: 18, 21, 24, 25 and 28˚C for 30 days. During this experiment four different media were used, namely potato dextrose agar (PDA), malt extract agar (MEA), V-8 juice agar (V8A), and Sabouraud dextrose agar (SDA). The media had the following ingredients: MEA (malt extract: 30g, peptone: 5g and agar: 15g), V-8 (V8 juice:200ml, $CaCO_3$:3g, agar:15g), SDA (peptone:10g; dextrose:40g, agar:15g) and 39g of commercial potato dextrose agar powder (dextrose: 20g, agar: 15g, and potato starch: 4g) per litre of distilled water (Dutton and Penn, 1989; Mulyati *et al*., 2015). Three replicates were prepared for each medium and temperature.

Colony inocula constituted of 5 mm^2 plugs cut from the edge of 12 days old culture grown on PDA plates. Inverted plugs were placed in the centre of each plate. To facilitate measurement,

perpendicular lines were drawn on the bottom of 90 mm petri dishes. Radial growth was assessed every 2 days for a period of 30 days.

3.2.3. Effect of UV radiation on *L. muscarium* growth

The irradiation experiment was conducted in a box with two UV light (Philips, F40 T8 40w BLB) (Figure 3.1). Each light produces long wave UV-A light at approximately 370 nm. 5mm plugs were cut off from the edge of 12 days old culture grown on PDA as described above and were transferred onto different media. Three replicate plates were prepared for each media and incubated at 25°C in the dark for a period of 7 days. After 7 days, plates with growing fungal mycelia were transferred to the black box and exposed to UV radiation for 30 days. Radial growth was measured every 2 days.

Figure 3.1. Inside a UV light box with two UV light of 40 w each. L (length) = 1.43 m; W (width) = 0.39 m; and H (height) = 0.41 m. UV light from 2 x Philips, F40 T8 40w BLB fluorescent tubes.

3.2.4. Evaluation of various nutrient substrates for conidial production

Six different agro-industrial solid substrates were tested as food sources to support the growth and sporulation of *L. muscarium* strain Nesta-08. These were wheat bran (*Triticum aestivum* L.), rice (*Oryza sativa* L.), rolled oats (*Avena sativa* L.), pearl millet [*Pennisetum glaucum* (L.) R.Br.], pearled barley (*Hordeum vulgare* L.), and sorghum (*Sorghum bicolor* L.). Before inoculation, substrates were prepared as follows:

►500g of each substrate was soaked in distilled water, for a period of 24 hours, to achieve the desired moisture content of approximately 50%.

►Free water was removed using cheesecloth and the media were air dried.

►After drying the grains, 30g were placed in 500ml Erlenmeyer and autoclaved for 20 minutes at 121°C.

►As soon as flasks were removed from the autoclave they were shaken vigorously to avoid lumping of grains.

►Substrates were allowed to sit over-night.

► Substrates were autoclaved for the second time for 20 min at 121°C.

► The media were cooled to ambient temperature and inoculated the following day.

► Eight blocks of 5mm x 5mm were cut from PDA plates and served as inocula for each solid media. The inoculated media was then incubated at 25° C.

► The media were shaken manually every 48 hours. After 21 days, CONIDIA were harvested by rinsing the culture with sterilized water and filtered through a double layer of cheesecloth. Conidia were viewed under a light microscope and counted using a standard haemocytometer (Wyss *et al*., 2001; Vu *et al*., 2008).

► The experiment was replicated twice.

3.2.5. Data analysis

Mycelial growth was measured at different times and results were subjected to analysis of variance (ANOVA) using Statistical Analysis System (SAS) software version 9.3 (SAS Institute Inc, 2011). Treatment means were separated using Duncan multiple range test.

3.3. Results

3.3.1. *L. muscarium* colony growth at different temperatures and on different media

Lecanicillium muscarium isolate Nesta-08 grew at temperatures of 18°C to 28°C. The mean growth rate increased as temperature increased up to 24°C. Temperature above 24°C resulted in reduced radial growth of the isolate (Fig 3.2). The growth was assessed over time and the following results represent the growth at Days 6, 12, 18, 24, and 30.

Day 6: From the first day of inoculation, the best growth at the temperature of 24°C (Fig 3.2) occurred on SDA.

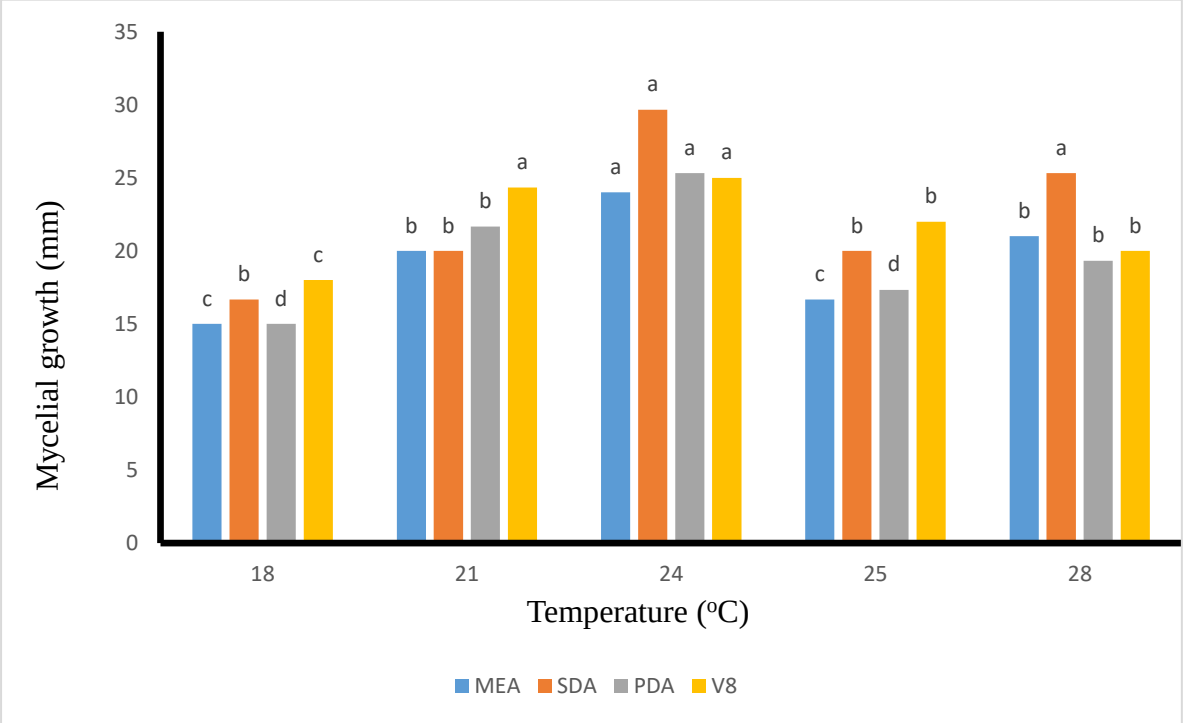

Figure 3.2. Mycelial growth (mm) of *Lecanicillium muscarium* at Day 6. Treatment values followed by the same letters for the same temperature were not significantly different per Duncan's

multiple range test. MEA (F =14.31; P = 0.0004; CV% = 8.44); SDA (F = 13.68; P = 0.0005; CV% = 10.78); PDA (F = 48.9; P = 0.0001; CV% = 4.61); V8 (F = 17.57; P = 0.0002; CV% = 5.53).

L. muscarium Nesta-08 grew at all tested temperatures, with the best growth at 24ºC, and the lowest growth at 18ºC. On Day 6, there were a temperature and media response where the best mycelia growth was 29.667 mm on SDA at 24ºC (Fig 3.2).

Day 12: At day 12, SDA was the best media for *L. muscarium* growth with a mean value of 44 followed by V8 with a mean value of 43.66 both at 24°C. Higher growth was observed again on V8 at 21°C with a mean value of 41.66 (Fig 3.3).

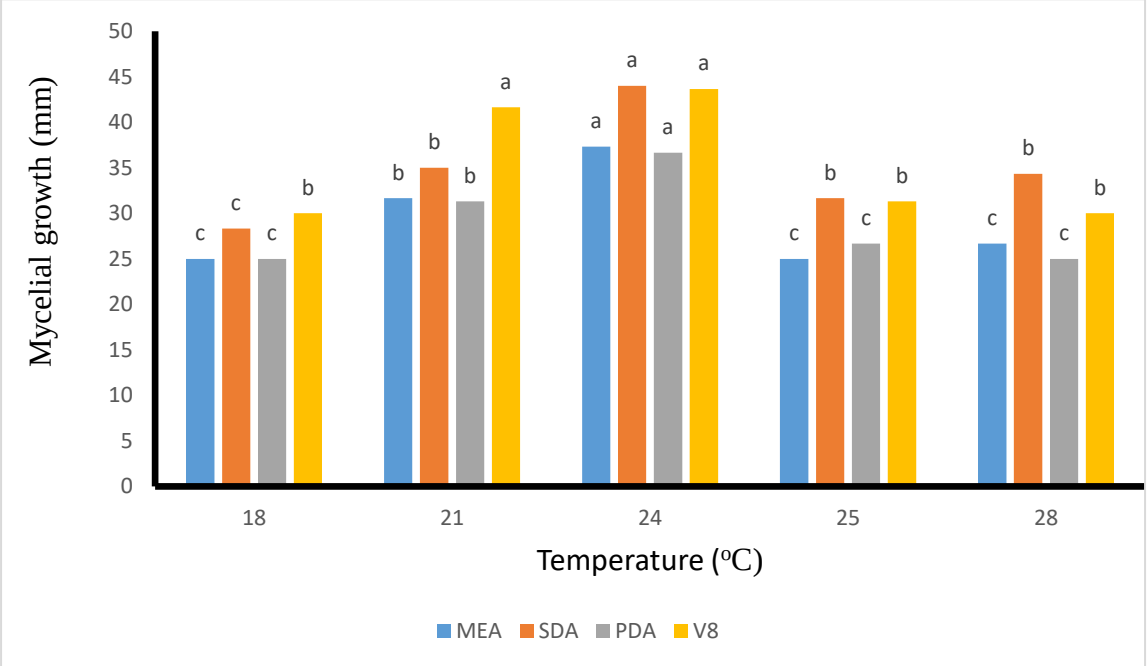

Figure 3.3. Mycelial growth (mm) of *Lecanicillium muscarium* at Day 12. Treatment values followed by the same letters for the same temperature are not significantly different per Duncan's multiple range test. MEA (F = 14.73; P = 0.0003; CV% = 8.26); SDA (F = 12.79; P = 0.0006; CV% = 8.15); PDA (F = 17.33; P = 0.0002; CV% = 7.24); V8 (F = 45.61; P = 0.0001; CV% = 4.90).

There was increase of *L. muscarium* Nesta-08 mycelia growth from Day 6 to Day 12, SDA and V8 had the best mycelia growth of 43.667 mm at 24°C. During this period, mycelia growth increased considerably by 29.667 mm to 43.667 mm for SDA and at 24°C. MEA had the lowest growth value of 25 mm at 18°C.

Day 18: At day 18, V8 was the best growing media at both 21°C and 24°C with mean values of 59.33 and 60, respectively (Figure 3.4).

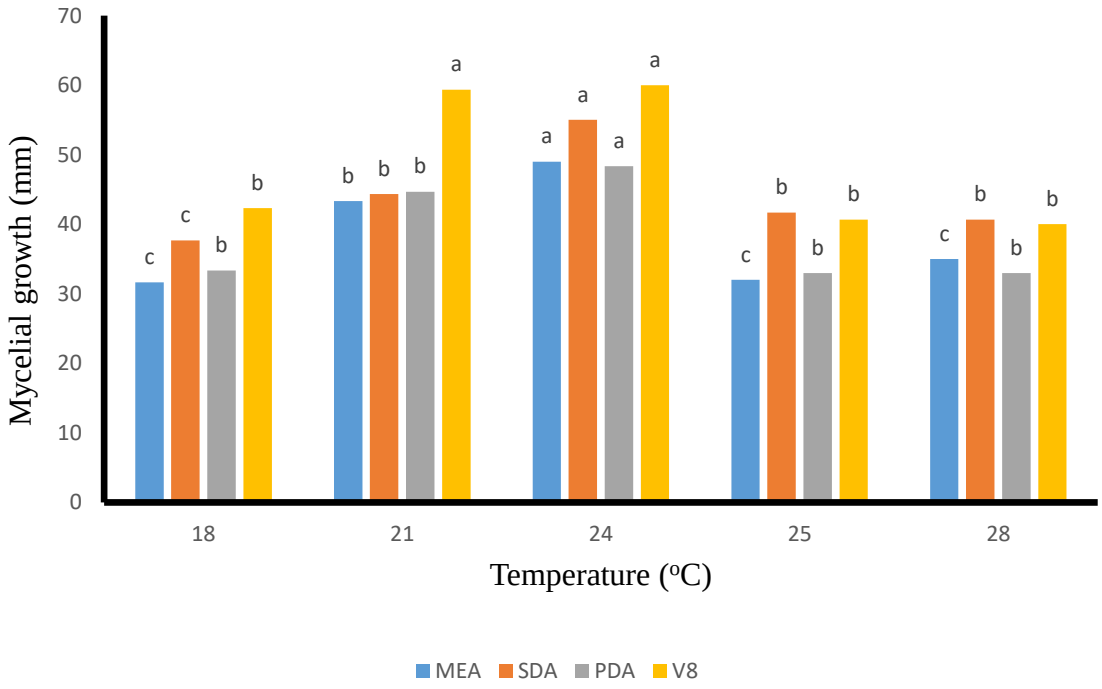

Figure 3.4. Mycelial growth (mm) of *Lecanicillium muscarium* at Day 18. Treatment values followed by the same letters for the same temperature were not significantly different per Duncan's multiple range test. MEA (F =22.73; P =0.0001; CV% =7.27); SDA (F =32.77; P =0.0001; CV% =4.59); PDA (F =16.00; P = 0.0002; CV% =8.38); V8 (F = 175.52; P =0.0001; CV% =2.76).

From Day 12 to Day 18, mycelia growth increased considerably by 43.667 mm to 60 mm on V8 at 24$^{\circ C}$. There was no significant difference between 21 and 24$^{\circ C}$ where the best mycelia growth was 59.33 and 60 mm, respectively. 18°C had the lowest mycelia growth of 33.3 mm on MEA.

Day 24: At day 24, V8 was the best media for *L. muscarium* mycelial growth at all tested temperatures (Fig 3.5).

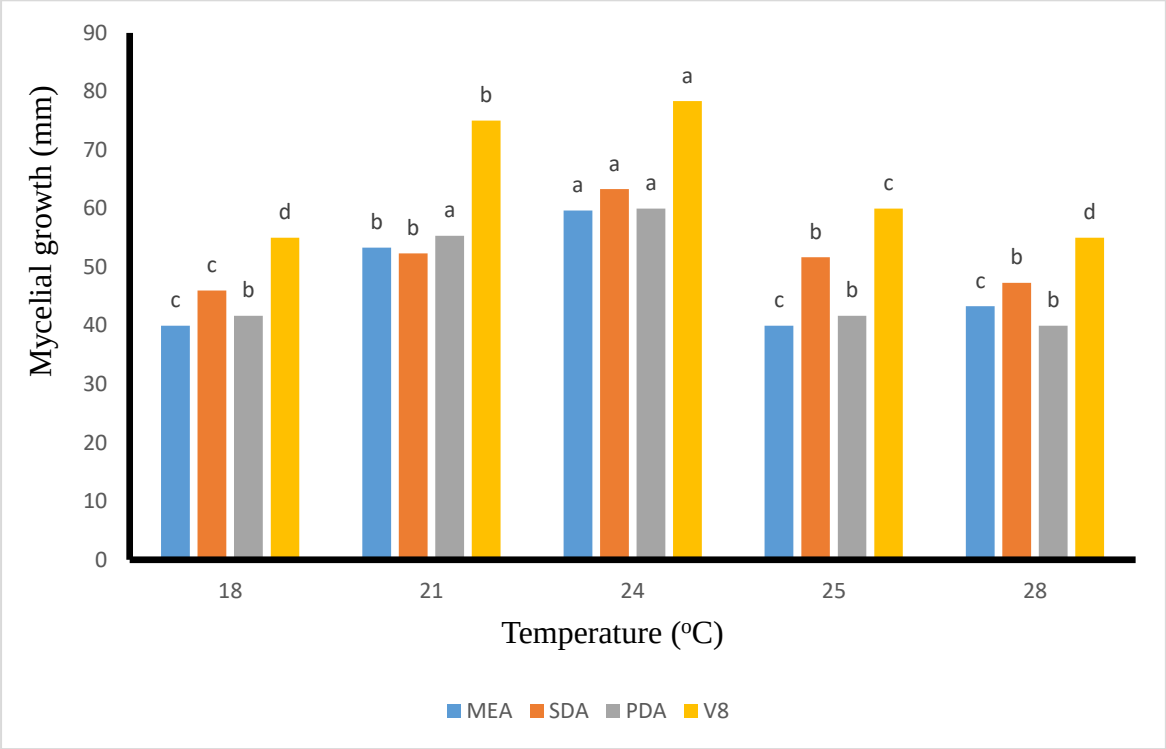

Figure 3.5. Mycelial growth (mm) of *Lecanicillium muscarium* at Day 24. Treatment values followed by the same letters for the same temperature are not significantly different per Duncan's multiple range test. MEA (F =35.41; P =0.0001; CV% =5.43); SDA (F =13.61; P =0.0005; CV% =6.14); PDA (F =24.64; P =0.0001; CV% =6.75); V8 (F =226; P =0.0001; CV% =1.99).

From Day 18 to Day 24, V8 was still the best media for mycelia growth at 24°C. The mycelial growth increased considerably by 60 mm to 78.333 mm. The lowest mycelia growth was recorded at 28°C on PDA (40mm).

Day 30: At the last day of rating, the highest mycelial growth was on V8. The graph shows the best growth was at 21℃ and 24°C (Fig 3.6).

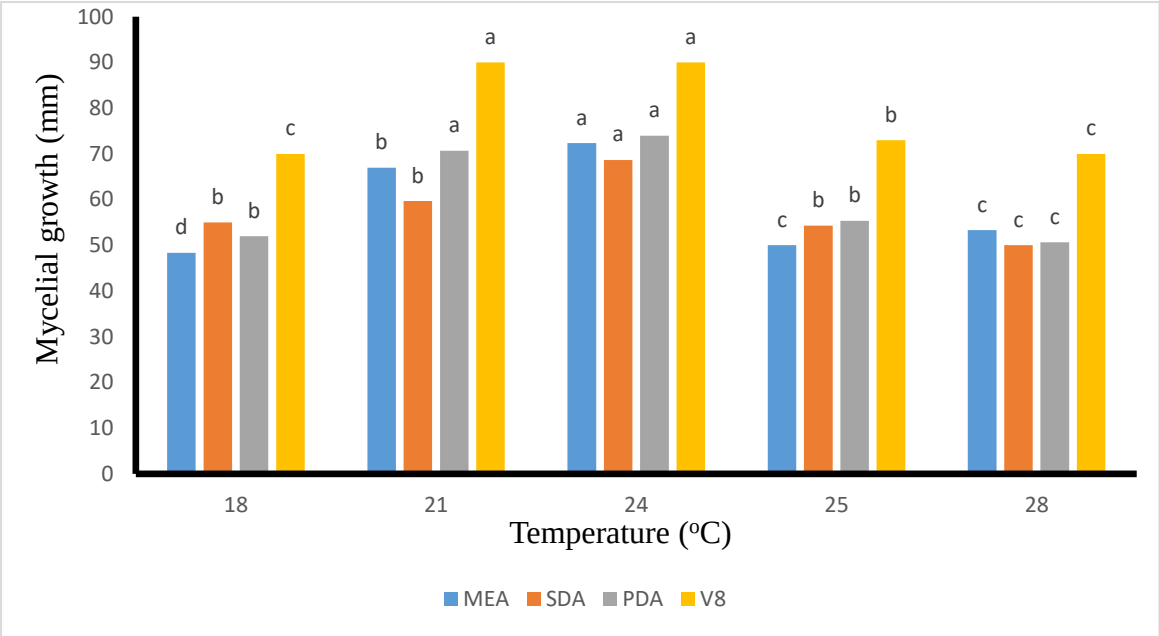

Figure 3.6. Mycelial growth (mm) of *Lecanicillium muscarium* at Day 30. Treatment values followed by the same letters for the same temperature are not significantly different per Duncan's multiple range test. 18°C (F = 130.24; P = 0.0001; CV = 2.56%); 21°C (F = 29.47; P = 0.0001; CV = 5.54%); 24°C (F= 60.45; P = 0.0001; CV = 2.75%); 25°C (F = 265.24; P = 0.0001; CV = 1.85%); and 28°C (F = 110.71; P = 0.0001; CV = 2.77%).

At the last day of rating, the best mycelia growth for all temperatures tested was on V8 agar. 24°C was the best temperature for mycelia growth of *L. muscarium* strain Nesta-08 with the following radial growth: 90 mm, 74 mm, 72.33 mm, and 68.66 mm on V8, PDA, MEA, and SDA, respectively. MEA had the lowest growth (67 mm) at 18°C. There was time response on mycelial growth: as *L. muscarium* Nesta-08 mycelia growth increased by time, the highest mycelia growth was recorded at the last day.

3.3.2. Effect of UV radiation on *L. muscarium* growth

L. muscarium strain Nesta-08 grew at different rates on four media when exposed to UV radiation. The isolate grew best on V-8 agar. Radial growth on all four media are presented in Fig 3.7.

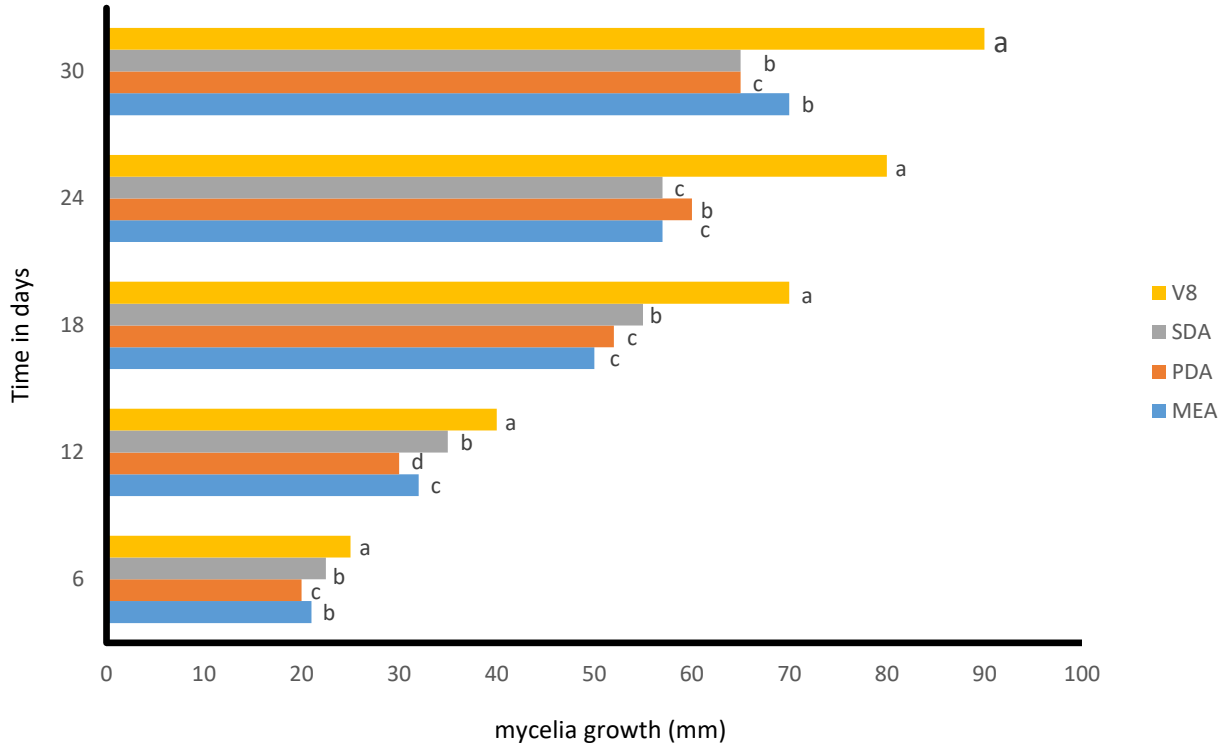

Figure 3.7. Effect of UV-light on *L. muscarium* Nesta-08 mycelium growth. Means with the same letter were not significantly different per Duncan's multiple range test. Day 6 (F = 15.13; P = 0.0120; CV = 3.57%); Day 12 (F = infinity; P = 0.0001; CV = 0%); Day 18 (F = infinity; P = 0.0001; CV = 0%); Day 24 (F = infinity; P = 0.0001; CV = 0%); and Day 30 (F = infinity; P = 0.0001; CV = 0%).

V8 was the best growth medium from Day 6 to Day 30. There was time response: *L. muscarium* grew by 25-40 mm from Day 6-12; 40-70 mm from Day 12-18; 70-80 mm from Day 18-24 and by 80-90 mm from Day 24 to Day 30.

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3.3.3. Evaluation of natural substrates for conidial production

Lecanicillium muscarium strain Nesta-08 sporulated on all substrates used. The greatest production of conidia occurred on pearl millet grain followed by wheat bran and pearled barley. The lowest conidial production was observed on rolled oats (Fig 3.4).

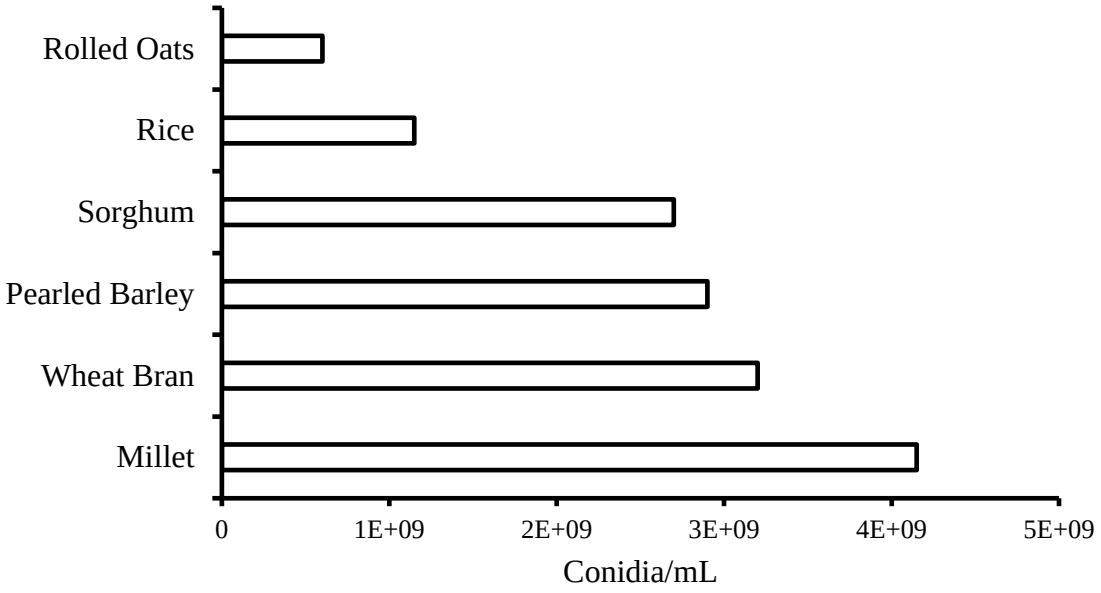

Figure 3.8. Sporulation of *L. muscarium* strain Nesta-08 on different source of substrates (25^{o}C) for 21 days.

Pearl millet was the best substrate for sporulation of *L. muscarium* Nesta-08 with 4.15 x 10^{9} conidia ml^{-1} being produced.

3.4. Discussion

Development of an effective entomopathogenic fungus for biocontrol use requires a good understanding of its growth conditions. In this study, the effects of temperature, artificial media, UV radiation and natural substrates were investigated in terms of *L. muscarium* Nesta-08 mycelial growth and conidial production.

The optimal colony growth for *L. muscarium* strain Nesta-08 was observed at 24°C. Our results confirm findings from previous studies which reported the genus *Lecanicillium* to be mesophilic, with optimum growth at 25°C, and no growth has been recorded at temperatures over 34°C (Li *et al*., 1991; Lopez-Lorca and Carbonell, 1998; Vu *et al*., 2007; Rivas *et al*., 2014). The use of *L. muscarium* strain Nesta-08 for soybean rust control should be advantageous because the ideal temperatures ranged from 21°C to 25°C, which are the same optimum temperatures for *P. pachyrhizi* sporulation (Bonde *et al*., 2012).

The isolate grew on all artificial media tested but the best growth was observed on V8 juice agar. When exposed to UV radiation, there were significant difference on radial growth for all media tested. Galvao and Bettiol (2014) evaluated the effect of UV radiation on *Lecanicillium* spp. and found that the level of sensitivity to UV light was different for the ten *Lecanicillium* strains tested. Biocontrol agents react to UV radiation depending of their geographic origin, with strains from regions with higher incidences of UV radiation being less affected (Piazena, 1996). A positive relationship has been shown for some entomopathogenic fungi between the origin and their tolerance on UV radiation (Fargues *et al.*, 1996; Fargues *et al.*, 1997).

The best substrate for spore production was pearl millet. *L. muscarium* strain Nesta-08 produced conidia on all substrates used. Our results confirm studies done previously by Shinde *et al*. (2010) who found pearl millet to be a suitable substrate for *Lecanicillium* spp. conidial production (10.17×10^{10} conidia/100g). *L. muscarium* strain Nesta-08 grew and produced conidia on all natural substrates used. This is beneficial for biomass and conidial production, especially where high concentrations of conidia are needed.

In conclusion, *L. muscarium* strain Nesta-08 grew on all used media and the best growth was observed on V8, at 24°C. It produced conidia on all substrates used, especially pearl millet. This strain was tolerant of UV light.

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CHAPTER 4: BIOCONTROL OF SOYBEAN RUST, *PHAKOPSORA PACHYRHIZI*, USING *LECANICILLIUM MUSCARIUM*

Abstract

The use of *Lecanicillium muscarium* Nesta-08 against *Phakopsora pachyrhizi* was investigated in the laboratory and greenhouse. In bioassay studies on soybean leaves infected by *P. pachyrhizi*, the *Lecanicillium muscarium* Nesta-08 was observed growing and colonizing the soybean rust (SBR) fungus. Under ESEM (Environmental Scanning Electron Microscope), long *L. muscarium* mycelia were observed wrapping tightly around and penetrating *P. pachyrhizi* urediospores. Greenhouse experiments were conducted to evaluate the efficacy of *L. muscarium* strain Nesta-08 on *P. pachyrhizi,* the causal agent of SBR. Under greenhouse conditions, three conidial doses (10^4, 10^6 and 10^8 *L. muscarium* Nesta-08 conidia/ml) significantly reduced rust pustules on soybean leaves ($P = 0.0001$). Compared to the control, the lowest disease severities were recorded at conidial doses of 10^8 (12.6%) and 10^6 (15.0%).

4.1. Introduction

The Asian soybean rust (SBR), *Phakopsora pachyrhizi* Syd. & P. Syd., is one of the most devastating diseases of soybean. It can cause losses of more than 80% (Miles *et al.,* 2003; Caldwell and McLaren, 2004). In the absence of a control measure, the rust fungus is able to cause complete yield loss (Stewart *et al.,* 2005; Twizeyimana *et al.,* 2009). Due to its wide distribution and its capacity to cause severe yield losses, SBR has been reported to be the most serious foliar disease of soybean crops worldwide (Hartman *et al*., 2011). The pathogen causes premature defoliation which reduces the areas of photosynthetic tissues and negatively affects the yield (Hartman et al., 1991; Ivancovich, 2005). During the last decade, the Asian soybean rust fungus has spread from Asia to Africa, America and recently to the USA (Kwawuki *et al.,2003;* Yorinori *et al.,* 2005; Twizeyimana *et al.,* 2007).

Present control measures against SBR are based on the use of fungicides. Due to the costs of application and sourcing, the use of foliar fungicide sprays for SBR control is not a viable option for soybean production in most developing countries (Kawuki et al., 2003; Twizeyimana *et al.*, 2007). Planting resistant cultivars would be an economical way to manage SBR. Six vertical

resistance genes have been identified for SBR resistance: Rpp_1-Rpp_6 (Hartman et al., 2005; Vuong *et al*., 2016). These 6 single dominant genes impact resistance against some of rust isolates but not all races. There are some cultivars with partial resistance. However, there is no available commercial cultivar that can resist all *P. pachyrhizi* isolates. Hence, most commercial cultivars are highly susceptible to SBR (Hartman et al., 2005; Vuong *et al*., 2016).

An alternative to fungicides is the use of biocontrol agents. A number of hyperparasitic fungus have been found associated with *P. pachyrhizi*. Sangit (2002) observed *Gliomastrix* spp. and *Trichothecium* spp. growing in association with *P. pachyrhizi* and reported that they were able to decrease the number of rust pustules. In Thailand, *Tuberculina sp*., *Verticillium psalliotae* Treschow and *Verticillium lecanii* Zimm. were reported to be hyperparasitic on *P. pachyrhizi* (Saksirirat and Hope, 1990; Saksirirat and Hope, 1991). Recently, *Simplicillium lanosoniveum* Zare & W. Gams was isolated on rust pustules, and empirical studies were run on its use as a biocontrol agent for *P. pachyrhizi* (Ward *et al*., 2012).

The primary control method for SBR is to use fungicides of triazole and strobilurin families. However, resistance will develop if this remains the sole control method (Laing and Caldwell, 2014). However, there are no registered commercial biological agents for SBR control yet.

The primary aim of the present study was to evaluate the potential of *Lecanicillium muscarium* Zare et Gams to control *P. pachyrhizi.* The specific objectives were:

a. to undertake colonization and mycoparasitic studies of *L. muscarium* on *P. pachyrhizi*. This involved *L. muscarium*-*P. pachyrhizi* interaction studies using environmental scanning electron microscopy (ESEM), as well as a leaf disc bioassay using *L. muscarium* and *P. pachyrhizi*.

b. to evaluate the effects of *L. muscarium* on *P. pachyrhizi* under greenhouse conditions using potted soybean plants as subjects for the study. The greenhouse study was also used to identify the optimal dose level for disease control and assess disease development over time.

4.2. Material and Methods

4.2.1. *In vitro* dual culture bioassay

P. pachyrhizi, the causal agent of the SBR, is an obligate parasite. The only way of manipulating this pathogen is to keep it on the living host tissue. In the detached leaf assay, therefore, the primary challenge is to maintain the green colour of the soybean leaves (Xie *et al*., 1998; Foolad *et al*.,

2000). An *in vitro* dual culture bioassay was done to identify the biocontrol mechanism of the isolate Nesta-08 of *L. muscarium* on *P. pachyrhizi*.

4.2.1.1. Preparation of plant tissue for bioassay study

Soybean leaves infected with the SBR pathogen, *P. pachyrhizi*, were randomly sampled from the disease garden of University of KwaZulu-Natal, Pietermaritzburg, South Africa. As an obligate parasite, *P. pachyrhizi* must be kept on the living host tissue. Immediately after sampling, leaves were packed in a dry paper bag and taken to the laboratory. Samples were aseptically manipulated under the lamina flow cabinet. Each leaflet was cut into 5 cm x 5 cm pieces and individually placed with abaxial side upwards in a sterile petri dish (9cm) containing sterile filter paper saturated with distilled water to maintain high humidity (Twizeyimana and Hartman, 2010; Wiebke-Strohm *et al.*, 2015).

4.2.1.2. *L. muscarium* inoculum preparation and inoculation

Conidia of *L. muscarium* were harvested from PDA plates, previously grown as indicated in Chapter 3, Section 3.2.2, by washing fungal mycelia with sterilized distilled water using a sterilized hockey stick and filtered with sterilized cheese cloth. Conidial suspensions were examined under a light microscope, counted using a haemocytometer and adjusted to 10^6 conidia ml^{-1}. Then 1 ml of a *L. muscarium* Nesta-08 conidial suspension was sprayed on the abaxial surface using a hand sprayer. Leaves were allowed to dry up for 1 hr before incubation. During this experiment, the entire leaf surface was sprayed with the *L. muscarium* conidial suspension. The petri dishes were incubated at 24°C with a 12/12 light/dark cycle for 14 days. On the 14^{th} day of incubation, rotting leaves or other leaves showing other disease symptoms were selected and eliminated. The remaining samples were subjected to an Environmental Scanning Electron Microscope (SEM) study to observe colonization of *P. pachyrhizi* urediospores by *L. muscarium* Nesta-08.

4.2.1.3. SEM sample preparation

Specimen preparation for conventional ESEM involved four operational phases: (a) primary fixation: leaf disks covered with mycelia of both fungi were placed on ESEM specimen stubs and fixed in 3% buffered glutaraldehyde for 1 hour; (b) buffer wash: samples were washed twice in sodium cacodylate buffer for 5 minutes; (c) dehydration: specimens were dried in 10% ethanol for 5 minutes, 30% ethanol for 5minutes, 50% ethanol for 5 minutes, 70% ethanol for 5 minutes, 90% ethanol for 5 minutes and finally twice in 100% ethanol; (d) coating: sample stubs were transferred

into the sputter coat (Pathan *et al.,* 2008). Dried, coated samples were viewed with ESEM, using a ZEISS EVO LS 15 (Carl Zeiss Microscopy, USA).

4.2.2. Effect of *L. muscarium* strain Nesta-08 on *P. pachyrhizi* in the glasshouse

Soybean seeds were germinated in germination trays, 120 health seedlings were selected and planted in the middle of 12 cm diameter pots filled with composted pine bark (Potting Mix, National Plant Foods (Pty) Ltd, Camperdown, South Africa) and maintained in a glasshouse with uniform irrigation and fertilization with Easygro starter (NPK) 2:1:2 (43) Ag-Chem Africa (Pty) Ltd, Silverton, South Africa. The pots were arranged in a randomized complete block design, except the untreated control, to avoid *L. muscarium* cross contamination, and all treatments were replicated six times. Treatments consisted of (1) Untreated control, (2) 10^4conidia ml^{-1}, (3)10^6 conidia ml^{-1} and (4) 10^8 conidia ml^{-1}. The *L. muscarium* inoculum was prepared using pearled barley as the growing substrate as described in Section 3.2.5. and 0.01% Breakthru® (Vonik Degussa Africa (Pty) Ltd, South Africa) were added to the suspension.

At stage R_1 (open flower at any node on the main stem), all plants were inoculated with *P. pachyrhizi* urediospores. Soybean leaves infected with *P. pachyrhizi* were harvested from the UKZN disease garden. All plants were inoculated by attaching the infected leaves to the healthy leaves using a stapler. Each plant was stapled with 2 infected leaves and covered by a plastic bag for 24 hrs to augment humidity. At the appearance of the first visible rust pustule (approximately 7 days after rust inoculation), soybean plants were sprayed by *L. muscarium* until run off, using a 1liter hand sprayer. All plants were sprayed on the same day according to the treatments. The controls were sprayed with tap water. Disease severity and *L. muscarium* growth were assessed 4 times from the first day after the sighting of white mycelia typical of *L. muscarium*. The assessment was done every 3 days. The visual scale of SBR severity that was used was as follows: 0.6%; 2.0%; 7.0%; 18.0%; 42.0% and 78.5% (Godoy *et al*., 2006).

4.2.3. Statistical analysis

The AUDPC values and the final disease severity values were subjected to analysis of variance (ANOVA) using Statistical Analysis System (SAS) software, Version 9.3 (SAS Institute Inc., 2011). Treatment means were separated using Duncan multiple range test.

4.3. Results

4.3.1. Effect of *Lecanicillium muscarium* on *Phakopsora pachyrhizi* using detached leaves

The bioassay studies using detached soybean leaves inoculated with *P. pachyrhizi* under controlled laboratory conditions showed *L. muscarium* hyphae developing on *P. pachyrhizi* sori (Figure 4.1).

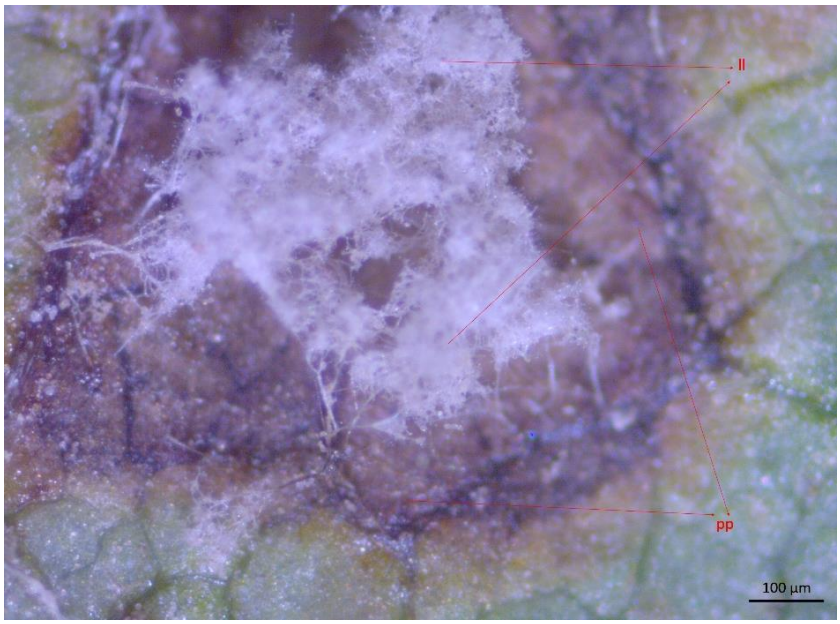

Figure 4.1. Development of *L muscarium* (ll) on *P. pachyrhizi* (pp) sori on detached soybean leaf.

The Environmental Scanning Electron Microscope (ESEM) observation of the interactions between the two fungi showed a mycophilic attraction of *L. muscarium* to *P. pachyrhizi* urediospores. Hyphae of *L. muscarium* were observed coiling tightly around urediospores of *P. pachyrhizi* (Figs 4.2 A & B). Some infection holes were also observed.

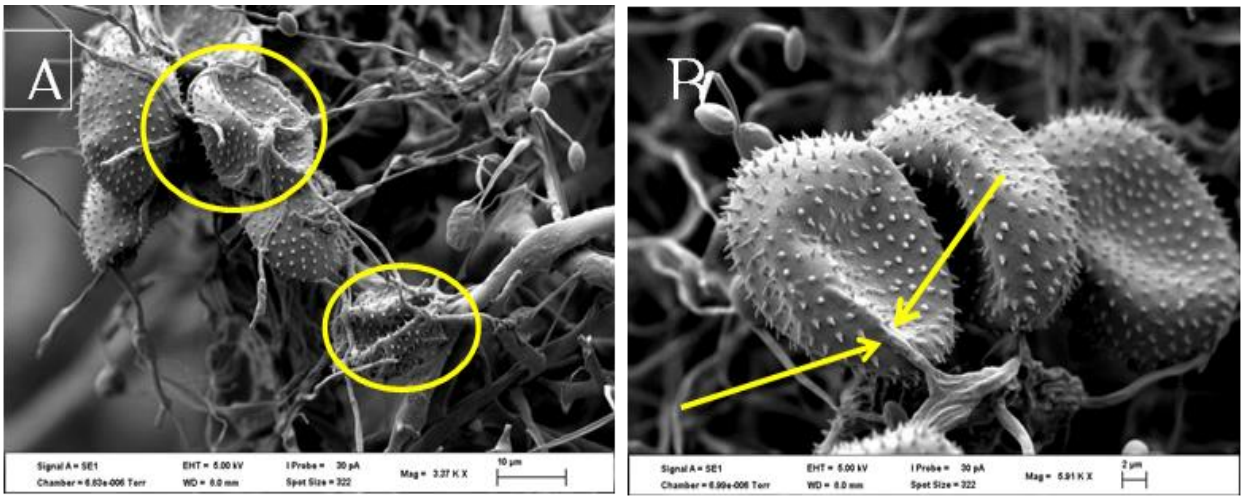

Figure 4.2 (A): *L. muscarium* mycelia surrounding and squeezing tightly around *P. pachyrhizi* urediospores (encircled) and (B): penetration of *L. muscarium* inside urediospore (arrowed).

4.3.2. Effect of *L. muscarium* on *P. pachyrhizi* in the glasshouse

Greenhouse trials were conducted to determine the most effective dose for field application. Four *L. muscarium* dosages were tested (0, 10^4, 10^6 and 10^8 conidia ml^{-1}). It was found that 10^6 and 10^8 were more effective and 10^6 was chosen as the optimum dose for field application. Compared to the untreated control, all three treatments significantly reduced the number of active rust pustules on soybean leaves (Table 4.1). On Day Four, the lowest disease severities were recorded for Treatment 10^6 and 10^8 conidia ml^{-1}, with mean values of 15.32% and 12.24%, respectively. The highest disease severity (76.12%) was recorded on the untreated control (Table 4.1). Table 4.1 and 4.2 show correlation between *L. muscarium* doses and rust pustules reduction. Compared to the control, the hyperparasite grew on rust pustules on all treated plants, covering 17.2%, 18.1%, and 14.3% for doses of 10^8, 10^6, 10^4, respectively (Fig 4.3.). As was mentioned above, the untreated control (no *L. muscarium* inoculation) was separated from the rest of plants to avoid cross infection, therefore there was no *L. muscarium* growth on the control (Table 4.2.).

Table 4.1. Effect of different treatments (different dosage of *L. muscarium*) on SBR severity.

Treatments	**Soybean rust severity**	**% of disease reduction**	**AUDPC**
Control	76.117 a	0	368.84 a
$1.e^4$ *L muscarium*	22.717 b	70.15	171.37 b
$1.e^6$ *L muscarium*	15.323 b	79.86	136.91 b
$1.e^8$ *L muscarium*	12.240 b	83.92	129.28 b
	F value = 37.87		F value = 10.98
	P = 0.0001		P = 0.0002
	CV = 37.79010		CV = 41.43035

1. Visual ratings of foliar disease severity (0 – 100).
2. Within each column, values followed by the same letter indicate that there was no significant difference between the means, based on Duncan multiple range test (DMRT).
3. AUDPC = Area Under the Disease Progress Curve, based on disease severity on assessment dates.

SBR infection was severe on the control treatment, with an AUDPC value of 368.84. All *L. muscarium* doses reduced SBR severity considerably by 70.17-83.92%. There was not clear a dose response.

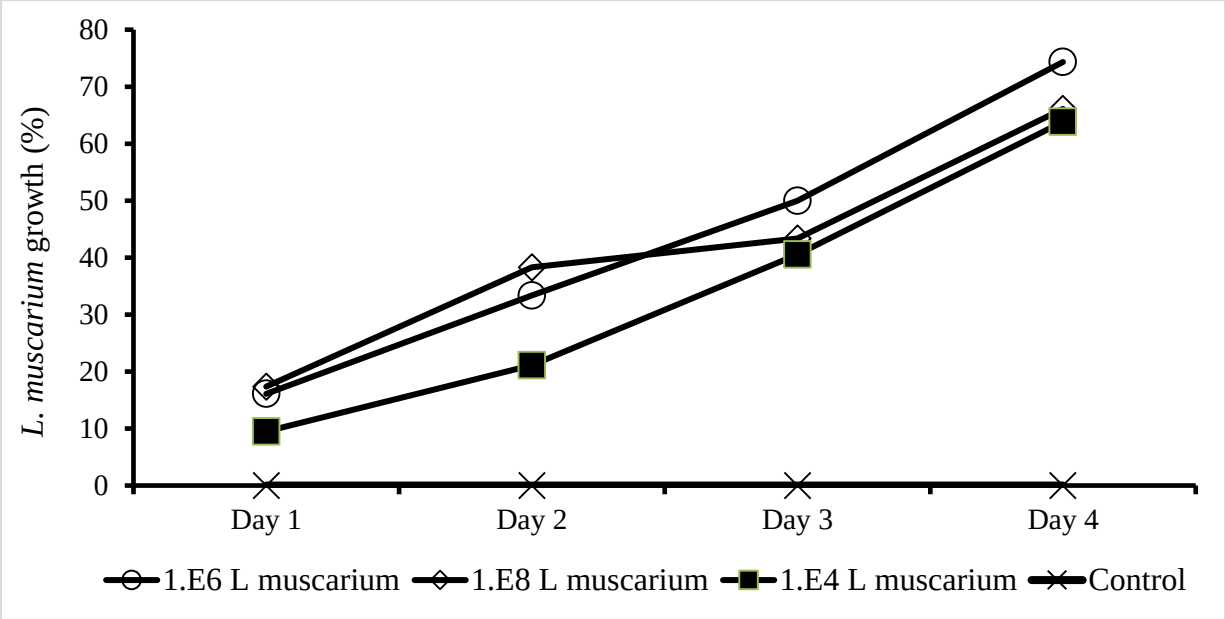

Figure 4.3. The colonization by *Lecanicillium muscarium* on *Phakopsora pachyrhizi* on different dates and according to the following concentration (untreated control, 10^4 conidia ml^{-1}, 10^6 conidia ml^{-1}, and 10^8 conidia ml^{-1}).

L. muscarium growth increased over time. There was a dose response on the last day (Day 4) but not much.

Table 4.2. *Lecanicillium muscarium* growth on *Phakopsora pachyrhizi* over time. Treatments applied are control, 10^4 conidia ml^{-1}, 10^6 conidia ml^{-1} and 10^8 conidia ml^{-1}.

Treatments	*L. muscarium* growth	AUDPC
Control	0 b	0 b
1.E4 L muscarium	63.833 a	317.10 a
1.E6 L muscarium	74.333 a	385.65 a
1.E8 L muscarium	66.100 a	382.75 a
	F value = 32.84	F value = 24.55
	P = 0.0001	P = 0.0001
	CV = 28.74600	CV = 33.46114

1. Visual ratings of *Lecanicillium muscarium* growth (0 – 100).
2. Within each column, values followed by the same letter indicate no significant difference, based on Duncan Multiple range test (DMRT).
3. AUDPC = Area Under the Disease Progress Curve based on disease severity on assessment dates.

All *L. muscarium* treatments resulted in an increase in the colonization of rust pustules, increasing according to the three concentrations.

4.4. Discussion

Nowadays, the use of fungi as biocontrol agents against plant pathogens is considered to be a viable alternative to fungicides use, for example, for SBR control. The use of natural enemies for plant disease control will positively impact in yield quantity maintenance and food quality, and it may reduce the use of toxic chemicals (Jyoti and Singh, 2016). The objective of the present study was to investigate the biocontrol effect of *L. muscarium* strain Nesta-08 on *P. pachyrhizi*, the causal of SBR.

To achieve the above objective, co-inoculum of *L. muscarium* strain Nesta-08 on *P. pachyrhizi* was done in the laboratories using detached soybean leaves infected by *P. pachyrhizi*. During this experiment, *L. muscarium* Nesta-08 developed on *P. pachyrhizi* and the hyperparasite produced many long hyphae that ramified through the pustule and sporulated. A mycophilic attraction of *L. muscarium* to *P. pachyrhizi* urediospores was observed. Under ESEM, long *L. muscarium* Nesta-08 mycelia were observed wrapping tightly around *P. pachyrhizi* urediospores. Askary and Yarmand (2008) reported the secretion of a mucilaginous matrix by *L. muscarium* wherever mycelia made contact with the host. At the point of contact, extracellular enzymes were active in hydrolysing the host tissue (Yan *et al.*, 1996). Recently, *L. muscarium* isolate CCFEE 5003 was reported to produce cold-tolerant chitinolytic enzymes. The enzymes caused mycelial damages, cell lysis, inhibition of conidia germination, formation and bursting of protoplast (Fenice, 2016).

In addition to the bioassay experiment using detached leaves, the effect of *L. muscarium* Nesta-08 on *P. pachyrhizi* was investigated under greenhouse conditions. Approximately 5.0 x 10^3 to 10^7 conidia ml^{-1} of *L. lecanii* were used in the greenhouse experiment against powdery mildew on strawberry (Miller *et al.*, 2004). In the greenhouse, the biocontrol efficacy of *L. muscarium* Nesta-08 reduced the level of SBR compared to the control ($P = 0.0001$). Compared to the control, lower disease severities were recorded at 10^8 (12.24 %) and 10^6 (15.32 %). There was no significant difference between the two different doses, therefore 10^6 conidia/ml were adopted and used as a standard dose for field experiments).

To the best of our knowledge, there is no registered biocontrol agent for SBR control and this was the first trial of *L. muscarium* against *P. pachyrhizi*. However, there have been other studies done on biocontrol of SBR where different mycoparasitic fungi were observed colonizing *P. pachyrhizi in vivo*. *Simplicillium lanosoniveum* was observed growing on *P. pachyrhizi* but replicated

biocontrol trials were not undertaken (Ward *et al*., 2012). Another similar study was done under greenhouse conditions using an inoculation of mixed spores of *Trichothecium roseum* and *P. pachyrhizi*. During this glasshouse trial, *T. roseum* was observed colonizing *P. pachyrhizi* (More and Kamble, 2009).

In the presence of SBR, *L. muscarium* Nesta-08 colonized *P. pachyrhizi* uredinia and wrapped around urediospores within 3 days. *L. muscarium* Nesta-08 was not seen growing on leaves that had not been infected by *P. pachyrhizi*. When *L. muscarium* Nesta-08 was sprayed onto to *P. pachyrhizi* uredinia, structures were observed that resembled penetration sites on the surface of urediospores. This confirms the study done previously where *L. muscarium* was found to produce high levels of enzymes and to penetrate host tissues after coiling around these (Hussain *et al*., 2016). Based on these observations, this similarity in mode of action suggests that *L. muscarium* is a mycoparasite of *P. pachyrhizi*, which requires further study.

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CHAPTER 5: FIELD EVALUATION OF *LECANICILLIUM MUSCARIUM* NESTA-08 AS A BIOCONTROL AGENT OF *PHAKOPSORA PACHYRHIZI,* THE SOYBEAN RUST FUNGUS

Abstract

Worldwide, soybean rust (SBR) caused by *Phakopsora pachyrhizi* is classified as the most destructive foliar disease of soybean. SBR leads to premature leaf loss and yield reduction. To evaluate the impact of *Lecanicillium muscarium* strain Nesta-08 on SBR, two field experiments were conducted in the 2014-2015 and 2015-2016 growing seasons at Ukulinga Research Farm, Pietermaritzburg, South Africa. Treatments with *L. muscarium* or the fungicide Score caused a significant disease decrease compared to the untreated control, but there was no significant difference in yield. The lowest area under disease progress curve (AUDPC) value was observed for the treatment with Score (172.2), and the highest was on untreated control plots (1716.8). Based on Duncan's multiple range test (P = 0.0001), there were no significant difference between Score and *L. muscarium* treated plots. *L. muscarium* reduced the SBR severity at a level of 89.1%.

5.1. Introduction

Soybean is an important crop worldwide for human and animal feed (Sharma et al., 2012). In South Africa, the growing of soybean, *Glycine max* L., was first reported in Cedara in 1903. According to the report, soybean seed was imported from China to South Africa. Out of all provinces, Mpumalanga is reported to be the most important producer of soybean, representing 40% of South African soybean production (DAFF, 2010). Soybean oil cake is the most important protein component for South African animal feed industry (Nyinawamwiza *et al.*, 2007; Njobeh *et al*., 2012).

The increased demand for soybean products has resulted in increases in soybean farming worldwide. Soybean is attacked by many pests and disease, which decrease soybean production. One of the most important diseases is Asian soybean rust. Soybean rust (SBR) was first reported in Japan in 1903 (Hennings, 1903; Hartman *et al*., 2005). It occurs all over the world where soybean is grown, including in many African countries (Goellner et al., 2009). Depending on climate and

time of infection, this disease has caused yield losses of up to 100% (Kumudini *et al*., 2008; Murithi *et al*., 2014). The only effective method for SBR control currently is spraying with fungicides. There are no resistant cultivar available on the market, nor are there any registered biocontrol agents.

Lecanicillium muscarium Zare and Gams (previously known as *Verticillium lecanii*) is well known as entomopathogen, it has been developed into commercial pesticides (Gindin *et al*., 1994; Faria and Wraight, 2007; Goettel *et al*., 2008; Ren *et al*., 2010). The effect of *L. muscarium* on arthropods has been investigated under laboratory conditions where it was found to produce cuticle-degrading enzymes (Ali *et al*., 2013). However, *Lecanicillium spp.* also have activity against fungal plant pathogens (Dik *et al*., 1998; Meyer *et al*., 1996; Benhamou and Bordeur, 2000). The purpose of this chapter was to evaluate the effect of *L. muscarium* Nesta-08 on SBR in field trials. This involved rating the impact of *L. muscarium* on SBR incidence and severity, and to compare its effect with the triazole fungicide Score® on crop losses caused by SBR.

5.2. Material and methods

Field experiments were conducted for two consecutive seasons (2014-2015 and 2015-2016) at Ukulinga Research Farm, KwaZulu-Natal, South Africa (29°24´E; 30°24´S) to evaluate the effects of *L. muscarium* isolate Nesta-08 and the fungicide Score® on SBR severity and incidence. The soil was classified as a Hutton, with 50% clay.

5.2.1. Soybean rust inoculum, biocontrol agent, fungicide and seed source

Soybean leaves naturally infected with the SBR, *P. pachyrhizi*, were randomly sampled from the disease garden of University of KwaZulu-Natal, Pietermaritzburg, South Africa. As an obligate parasite, the pathogen was maintained on living soybean plant leaves as a source of field inoculum. During this study, soybean plants at stage R1 were inoculated with *P. pachyrhizi*, as described in Chapter 4, Section 4.2.2. All middle row plants were inoculated by attaching infected leaves to healthy ones using a stapler. Inoculated plants had two infected leaves attached to each plant. During the greenhouse trial, infected plants were covered with plastic bags for 24 hours to optimize humidity. For the field trials, plants were not covered with plastic bags, but were irrigated after sunset to optimize formation of dew overnight.

Inoculum of *L. muscarium* Nesta-08 used in the field experiments was isolated from infected soybean leaves in greenhouse experiments as described in Chapter 2, Section 2.2.3. As described in Chapter 3, Section 3.3.2, pearled barley was found to be one of the best media for the production of conidia of *L. muscarium*. The inoculum was prepared using barley as described in Chapter 3, Section 3.2.3. An adjuvant, Breakthru®, was added at 0.01% to enhance the spread of the biocontrol agent. Soybean plants were hyper-inoculated with *L. muscarium,* as described in Chapter 4, Section 4.2.2.

Score fungicide (Score 250EC a.i. difenoconazole) was sourced from the local agent of Syngenta AG. The fungicide was applied twice at the same time as biocontrol sprays at the recommended dose.

The soybean cultivar, DM5953RSF, used in this study was obtained from Pannar Seeds (Pty) Ltd, Greytown, South Africa.

5.2.2. *L. muscarium* assessment

Ten days after treatment sprays, soybean leaves were harvested from the *L. muscarium* Nesta-08 treated plots (Ward *et al*., 2011). Samples were subjected to ESEM study. The specimens for ESEM were prepared as described in Chapter 4, Section 4.2.1.3.

5.2.3. Disease severity assessments

Assessments for disease severity were done from the first day of inoculation with *L. muscarium* Nesta-08 and spraying with Score®. According to Kawuki *et al.* (2003), Hartman *et al*. (1991) and Yang *et al*. (1990), SBR severity measurements should be done on a weekly basis. Therefore, during this study, SBR severity was assessed after a week and 7 times after that, until defoliation in the control plots interfered with assessments.

In the 2014-2015 field trial, on each date of evaluation, severity was rated as a percentage of leaf area covered with uredinia and associated chlorosis within the canopy. The diagrammatic scale used for the first trial was adopted from Claudia *et al*. (2005). Values of percentage of diseased leaf area above 80% are difficult to observe in the field because rust infections accelerate leaf senescence and defoliation. The diagrammatic scale used in this field trial had six severities levels: 0.6, 2.0, 7.0, 18.0, 42.0, and 78.5% of leaf area infected with SBR lesions (Claudia *et al.* 2005).

In the 2015-2016 field trial, a few modifications were made to the diagrammatic scale, considering the following points: SBR starts at the bottom of plants and develops up to the top leaves. Therefore, there can be green leaves on top of a plant, and yellow or brown leaves below. As the soybean plants age, especially after they get to the R8 stage, they start to yellow and defoliate naturally. It is difficult to separate this from early maturity as a result of SBR. It is therefore important to do the ratings at stages well before the R7 and R8 stages. To measure the healthy green and photosynthesizing leaves, the following diagrammatic scale with 7 levels was adopted:

Levels	Percentages
1	zero spots, entirely green leaves
2	1% of leaves speckled; still green
3	3% of leaves speckled, first tinge of yellow
4	6% of the plants have leaves brown; noticeable yellow leaves low down.
5	12% of the plants have leaves brown or yellow; some very yellow lower leaves.
6	25% of the plants have leaves brown, lots of yellow leaves low down. Start of defoliation.
7	50% of the plants have leaves brown, yellow or defoliated
8	complete defoliation

5.2.4. Yield assessments

Yield was assessed by weighing dry plants and seed after harvest. Plants from middle rows were harvested, packed into paper bags and allowed to dry naturally for 12 days. After weighing the dried plants, soybean pods were shelled to obtain seed weight. The crop yield was recorded and evaluated in g per plot for each treatment. For the 2014-2015 trial, soybean pods were shelled manually, whereas for 2015-2016, a shelling machine was used.

5.2.5. Trial design, layout and data analysis

During these field experiments, treatments were laid out in a randomized complete block design. The field site was pre-treated with Kleenup® (glyphosate + adjuvants)

(http://www.henchem.co.za), and Karate® (2.5g l^{-1} lambda-cyhalothrin Syngenta AG) (3 ℓ/ha) to remove weeds and cutworms, respectively. The site had a size of 144 m^2, which was divided into five blocks of 28.8 m^2 each. Each block represented one replicate and was divided into six plots (3m x 1.5m). Five plots represent 5 different treatments (0, 10^4, 10^6, 10^8 *L. muscarium* conidia ml^{-1} and Score®. The 6th extra plot had a protective role against bird and other pest damage. Each plot had four rows in which 20 plants were planted. Plant spacing was 40cm x 15cm, intra-row and extra-row, respectively. No borders were created between blocks or plots.

5.2.6. Statistical analysis

The AUDPC values and the final disease severity values were subjected to analysis of variance (ANOVA) using Statistical Analysis System (SAS) software, Version 9.3 (SAS Institute Inc., 2011). Treatment means were separated using Duncan's multiple range test.

5.3. Results

5.3.1. *L. muscarium* assessments

L. muscarium was observed colonizing *P. pachyrhizi* under field conditions. The ESEM examination of leaves from the experimental site showed *L. muscarium* mycelia growing around *P. pachyrhizi* urediospores (Figure 5.1). The biocontrol agent was even seen colonizing *P. pachyrhizi* in the control plots as well as in the fungicide treated plots, i.e., it spread from treated plots to neighboring plots.

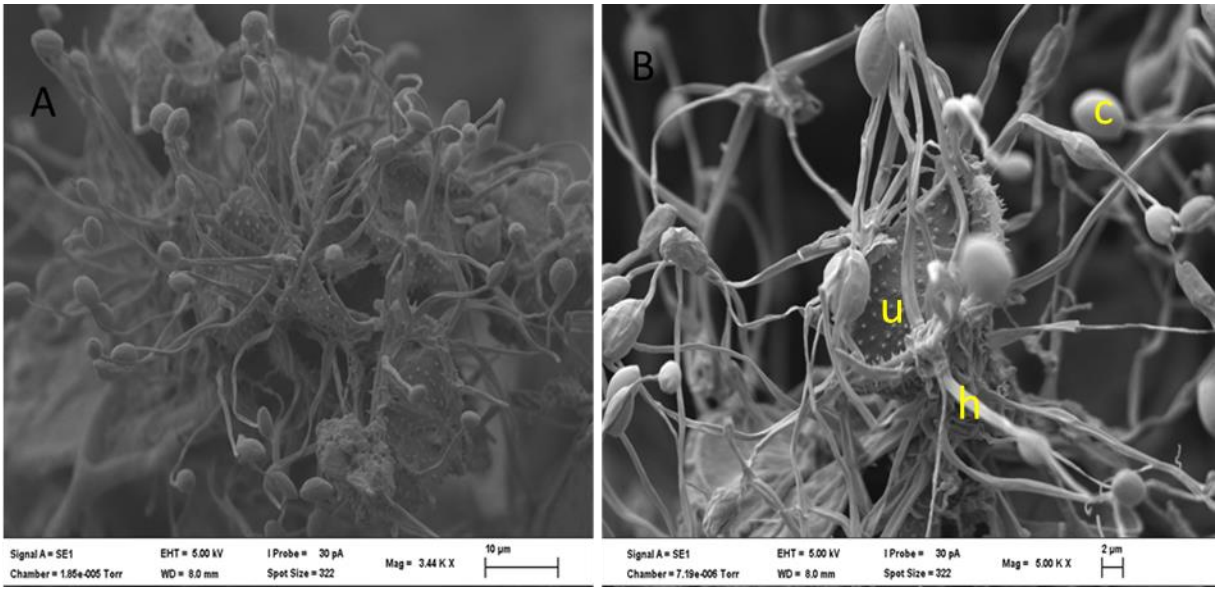

Figure 5.1. Micrographs of *L. muscarium* Nesta-08 colonizing *P. pachyrhizi* sori in the field, observed under ESEM. Figure 5.1.A shows *L. muscarium* N-08 sporulating actively around *P. pachyrhizi;* Figure 5.1.B shows a single urediospore of *P. pachyrhizi* surrounded by *L. muscarium* hyphae. (c) conidia, (h) hyphae, (u) urediospore.

5.3.2. Disease severity

For both field trials, *L. muscarium* Nesta-08 caused a significant decrease of SBR severity when compared to the control.

5.3.2.1. 2014-2015 Trial

During this trial, all *L. muscarium* doses were able to decrease disease severity compared to the control plot (Table 5.1). The dose of the biocontrol agent did not have a significant effect as there were no significant differences between treatments at 10^6 conidia ml^{-1} (202.16 AUDPC units) and 10^8 conidia ml^{-1} (186.2 AUDPC units). The lowest AUDPC value was observed after treatment with Score (172.2), while the highest was observed in control plots (1716.6) (Table 5.1).

Table 5.1. Effect of *L. muscarium* strain Nesta-08 on SBR severity

Treatments	Soybean rust severity	% of disease reduction	AUDPC
Control	78.50 a	0	1561.35 a
1.E4 *L. muscarium*	12.60 b	83.949	457.80 b
1.E6 *L. muscarium*	1.16 c	98.5223	202.16 c
1.E8 *L. muscarium*	0.60 c	99.2357	186.20 c
Score®	0.60 c	99.2357	172.20 c
	F value = 489.84		F value = 171.10
	P = 0.0001		P = 0.0001
	CV = 18.28189		CV = 19.75258

1. Visual ratings of foliar disease severity (0 – 100).
2. Values followed by the same letter indicate no significant difference, based on Duncan multiple range test.
3. AUDPC = Area Under the Disease Progress Curve, based on disease severity on seven assessment dates.

The control plots developed high levels of SBR, with a mean AUDPC value of 1716.8. Application of *L. muscarium* Nesta-08 reduced AUDPC values considerably, by 70-88%. There was a dosage response as the concentration of *L. muscarium* conidia increased. Treatment with the fungicide Score resulted in the lowest SBR levels, with a mean AUDPC value of 172.2. However, there was no significant difference between the mean AUDPC values of the *L. muscarium* and Score® plots.

5.3.2.2. 2015-2016 Trial

Again, there were no significant differences between the mean AUDPC values of the *L. muscarium* treated plots (10^6 and 10^8 conidia ml^{-1}plots had AUDPC values of 319.9 and 284.9, respectively), and the Score treated plots (259.7).

The study on disease severity over time showed no difference between *L. muscarium* treated plots compared to the control for the first two weeks after inoculation. Disease severity was 1% the first week after inoculation, and 3% after the second week (Table 5.2). After the second *L. muscarium* spray, significant differences developed in SBR severity in the *L. muscarium* treated plots and the control plots. On the last day of rating, on the 20th March 2016, disease severities were 70% for the control, 30% for 10^4 conidia ml^{-1}, 17.2% for 10^6 conidia ml^{-1}, and 9.6% for 10^8 conidia ml^{-1} (Table 5.2).

Table 5.2. Soybean rust severity, AUDPC Units, percentage of disease reduction as a result of five treatments (0, 10^4, 10^6, 10^8 *L. muscarium* conidia/ml and score fungicide).

Treatments	Soybean rust severity	% of disease reduction	AUDPC
Control	70 a	0	1053.50 a
1.e^4 *L. muscarium*	30 b	57.14	462.70 b
1.e^6 *L. muscarium*	17.20 bc	75.43	319.90 c
1.e^8 *L. muscarium*	12 bc	82.85	284.90 c
Score®	7.20 c	89.71	259.70 c
	F value = 17.22		F value = 85.83
	P = 0.0001		P = 0.0001
	CV = 50.07122		CV = 16.83794

1. Visual ratings of foliar disease severity (0 – 100).
2. Within each column, values followed by the same letter indicate no significant difference, based on Duncan's multiple range test (DMRT).
3. AUDPC = Area Under the Disease Progress Curve based on disease severity on seven assessment dates.

The control plots developed high levels of SBR, with a mean AUDPC value of 1053.50. Application of *L. muscarium* Nesta-08 reduced AUDPC values by 462.70-284.90 (about 50-75%). There was a dosage response as *L. muscarium* conidia concentration increased but the increase was not substantially. All *L. muscarium* doses decreased SBR severity considerably by 57.14 to 82.85%. There were significant differences between the AUDPC values of the plots treated with *L.* muscarium or Score®. and Score® reduced the disease severity the most, by 89.71%.

5.3.3. Yield assessments

5.3.3.1. Field trial 2014-2015

Plots treated by *L. muscarium* Nesta-08 had increased yields when compared to the control. However, they were no yield difference between plots treated with *L. muscarium* or Score (Table 5.3).

Table 5.3. Plant dry weight and seed weight

	Season 2014-2015	
	Seed weight	plant dry weight
Control	375.80 b	691.81 b
10^4 *L. muscarium*	452.64 a	768.16 ab
10^6 *L. muscarium*	482.53 a	802.16 a
10^8 *L. muscarium*	482.55 a	813.57 a
Score®	500.85 a	855.14 a
	F value = 7.80	F value = 3.27
	P =0.0006	P = 0.0322
	CV% = 8.6	CV% = 9.6

L. muscarium Nesta-08 increased yield on both plant and seed weight, but not by much. There was a dose response as *L. muscarium* concentrations increased plant dry weight by 452 to 482 g and plant weight by 768 to 813g. Score treatments increased plant and seed weight more than the treatments with *L. muscarium* but not by much.

5.3.3.1. 2015-2016 season

Plots treated with *L. muscarium* Nesta-08 had increased yields when compared to the control. However, they were no significant difference between in yields in the *L. muscarium* treated plots treatments and those treated with Score® (Table 5.4).

Table 5.4. Plant dry weight and seed weight

	Season 2015-2016	
	Seed weight	plant dry weight
Control	16.22 b	901.6 b
10^4 *L. muscarium*	386.10 a	1280 ab
10^6 *L. muscarium*	405.07 a	1370 a
10^8 *L. muscarium*	414.45 a	1382 a
Score	466.91 a	1503 a
	F value = 4.77	F value = 2.90
	P =0.0073	P = 0.0481
	CV% = 32.87984	CV% = 23.42813

The fungicide Score® and an experimental biocontrol agent, *L. muscarium* strain Nesta-08 both increased plant and seed weight relative to the untreated control. There was a significant difference between the control yields compared with the *L. muscarium* and Score® yields.

5.4. Discussion

L. muscarium strain Nesta-08 showed the potential to control *Phakopsora pachyrhizi in vitro* using detached leaves, and under greenhouse conditions (Chapter 4). Under field conditions, however, fungal biocontrol agents are known to be affected by weather conditions (Wraight & Ramos, 2002; Gatarayiha, 2009). The growing season of 2015-2016 appeared to favor rust development more than the 2014-2015 season.

ESEM examinations of soybean leaves collected from the field show the high levels of colonization and sporulation of *L. muscarium* Nesta-08 on *P. pachyrhizi* urediospores.

L. muscarium and Score® treatments both caused reductions of SBR severity, increased plant dry weight and yield in the field. During both field trials, three different doses of *L. muscarium* used reduced SBR severity. However, the greatest SBR severity reduction was caused by 10^8 conidia ml^{-1}. These results confirmed previous results (Chapter 4) from *in vitro* and greenhouse studies. ESEM examination showed *L. muscarium* colonizing *P. pachyrhizi in vitro* in detached leaf bioassays. In the greenhouse studies a reduction of SBR severity was caused by *L. muscarium* sprays. The present field trials also confirmed another previous study using the same biocontrol isolate (*L. muscarium* Nesta-08) where it had been observed colonizing the rust of *Oxalis* spp *in vitro* and under greenhouse conditions (Nxumalo, 2015).

The majority of studies for SBR control have been focusing on the use of fungicides. There is no commercial biocontrol agent for SBR available on the market. The cost of fungicide applications reduces the profit margins for soybean production. This study showed that there was no significant difference in yield increases after applications of *L. muscarium* and Score®. However, this study needs to be continued to confirm the efficacy and the biocontrol use of this isolate, using larger trials to reduce the effects of inter-plot interference.

In conclusion, *L. muscarium* strain Nesta-08 reduced SBR severity under field conditions. Compared to Score®, they were no significance differences (P = 0.0001.). This shows that *L. muscarium* strain Nesta-08 could be developed into a useful biological control against SBR. In the 2013-2014 season, the cost of SBR management globally was estimated to be US$ 2.2 billion (Godoy et al., 2016). This underscores the urgent need to develop a biocontrol agent to control SBR which will substantially reduce the costs of managing the disease.

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DISSERTATION OVERVIEW

Introduction

Soybean, *Glycine max* L. Merrill., is an important and strategic crop in South Africa (SA). The demand of soybean in SA far exceeds the local production (De Jager, 2016). To meet local demand 842 107 tons of oilcake were imported in the 2005/2006 soybean production season (Visser, 2007). A South African trade map (1999-2015) shows an increase in soybean imports from US$ 0.96 million in 2010 up to US$ 51 million in 2014 (Ncube *et al*., 2016). The oilcake import was reported to be the highest in 2013, when it was estimated to reach the value of US$ 70.5 million (Nahman and de Lange, 2013). The increase in soybean product imports results from increases in human and animal consumption, and yield losses in locally grown crops due to due to soybean diseases. The most threatening disease of the soybean plant was reported to be the soybean rust (SBR) (Hartman *et al.*, 2005). SBR caused by *Phakopsora pachyrhizi* was first reported in South Africa in February 2001 in the Province of KwaZulu-Natal (Pretorius et al., 2001). Since its discovery, the rust fungus has been causing a lot of yield losses (Levy, 2005).

Control of SBR is based on the use of foliar fungicides. Alternatives to fungicides are needed because of the costs of fungicide applications, and because *P. pachyrhizi* develops resistance to systemic fungicides. Many fungal biocontrol agents against plant diseases have been successful registered and commercialised all over the world as alternatives to fungicide use. However, there is no biocontrol product on the market for SBR control. Therefore, the study of a biocontrol agent for SBR control was undertaken to find an alternative to fungicide use. Consequently, the study focused on:

1. Isolation and identification a strain of *L. muscarium* from coffee leaves infected by *H. vastatrix*;

2. Obtaining a pure culture of *L. muscarium*, store under short- and long-term conditions;

3. Determining the optimum conditions for its growth and spore production in the laboratory;

4. Studying the colonization by *L. muscarium* on *P. pachyrhizi*. This involved *L. muscarium*-*P. pachyrhizi* interaction studies using environmental scanning electron microscopy (ESEM), as well as a leaf disc bioassay using *L. muscarium* and the SBR pathogen;

5. Evaluating of the effects of *L. muscarium* on *P. pachyrhizi* under greenhouse conditions using potted soybean plants. The greenhouse experiment also determined the optimal dose level for disease control;

6. Evaluating the efficacy of *L. muscarium* against *P. pachyrhizi* under field conditions. This involved rating the impact of *L. muscarium* on SBR in the field, on disease incidence and severity studies

7. Comparing the costs of biocontrol agent and fungicide against crop losses caused by SBR.

Chapter 2: IDENTIFICATION OF A STRAIN OF *LECANICILLIUM* SPECIES AS A POTENTIAL BIOCONTROL AGENT OF SOYBEAN RUST

Major findings:

- ✓ Based on nBLAST using ITS and ITS4 sequences, the isolate N-08 showed 99.8% similarity to strain CBS 318.70B of *L. muscarium*. ITS region has been used widely as a phylogenetic marker (Hillis and Dixon 1991; Schoch et al., 2012).
- ✓ The ITS region had a common ancestor with *L. lecanii* with a very high bootstrap value of 96.
- ✓ DN_1, of isolate N-08, showed a common ancestor with *L. longisporium* and *L. muscarium* and this was supported by a very high bootstrap value of 94.
- ✓ DN_3 gene of isolate Nesta-08 showed a common ancestor with *L. muscarium* but with a low bootstrap value (58-59).
- ✓ Based on morphological and molecular studies, the entomopathogenic fungus was identified as *L. muscarium.*
- ✓ The isolate Nesta-08 was deposited into the National collection of fungi with accession number PPRI 13715

Implications of findings:

An isolate of a *Lecanicillium* sp., Nesta-08, was isolated and identified as a strain of *Lecanicillium muscarium* using morphological and DNA identification tools. The mycoparasitic fungus was

observed colonising coffee rust and there is a possibility that it could also feed on the rust of soybean. Hence it might be suitable as a biocontrol agent of SBR and to other important rusts.

Chapter 3: STUDIES ON GROWTH AND PRODUCTION OF *LECANICILLIUM MUSCARIUM* STRAIN NESTA-08

Major findings:

- ✓ The fungus was tested in a series of experiments to determine the most suitable temperatures for culturing of the fungus, the best artificial media, and natural substrates. Colonies were exposed to UV light to determine their tolerance of UV, and whether it enhanced sporulation.
- ✓ Temperature had a significant effect on the colony radial growth and spore production. The optimal colony growth for *L. muscarium* strain N-08 was observed from 21 to 24°C.
- ✓ The mean growth rate increased as temperature increased up to 24°C. Temperature above 24°C resulted in reduced radial growth of the isolate.
- ✓ The isolate grew on all artificial media tested, the best growth was observed on V8 juice agar (42.75mm).
- ✓ The best bulk substrate for spore production was pearl millet grain with a mean of 4.15×10^9spores ml^{-1}, followed by pearled barley (2.9×10^9spores ml^{-1}).
- ✓ When exposed to UV radiation, radial growth of the mycelium did not change on any of the tested media.

Implications of findings:

Optimisation of growth media is an important issue to resolve in order to produce an effective biocontrol agent. *L. muscarium* Nesta-08 grew on all tested artificial media. This was useful for the small amounts of conidia needed for laboratory experiments. For large amounts of conidia, pearl millet or pearled barley could be used.

Tolerance of UV radiation and ambient temperatures confirmed that *L. muscarium* Nesta-08 has the capacity to be used in the field, where UV radiation and temperature fluctuations might be intense.

Chapter4: BIOCONTROL OF SOYBEAN RUST, *PHAKOPSORA PACHYRHIZI*, USING *LECANICILLIUM MUSCARIUM*

In vitro studies were conducted on the effect of *L. muscarium* isolate Nesta-08 on pustules of *P. pachyrhizi*.

Major findings:

- ✓ Co-inoculation studies of *L. muscarium* and *P. pachyrhizi* using a bioassay (detached soybean leaves inoculated with *P. pachyrhizi*) showed that *L. muscarium* Nesta-08 grew on, and colonized, *P. pachyrhizi* pustules.
- ✓ Samples from the colonized zones of the leaves (colonized by both fungi sequentially) were observed under the light microscopy and SEM.
- ✓ Microscope studies show the interaction between both fungi, *L. muscarium* hyphae were observed wrapping tightly around *P. pachyrhizi* urediospores. Points of penetration were also observed.
- ✓ On Day Four, the lowest disease severities were recorded for the biocontrol treatments with 10^8 and 10^6 conidia ml^{-1}, with mean values of 15.32% and 12.24%, respectively.
- ✓ The highest level of disease severity (76.12%) was recorded on the untreated control.

Implications of findings:

The mycoparasitism by *L. muscarium* of *P. pachyrhizi* was evaluated in the greenhouse. Under greenhouse conditions, the strain Nesta-08 reduced SBR severity. The optimum dose for *L. muscarium* was the dose of 10^6 spores ml^{-1}. This dose of inoculum was then used in the field experiments.

Chapter 5: FIELD EVALUATION OF *LECANICILLIUM MUSCARIUM* NESTA-08 EFFICACY FOR THE CONTROL OF *PHAKOPSORA PACHYRHIZI*, THE SOYBEAN RUST FUNGUS

Major findings:

- ✓ In the field experiments, plots treated by *L. muscarium* Nesta-08 showed a reduction in disease severity of up to 89%.

- ✓ Comparing to the control, all *L. muscarium* doses that were tested were able to reduce disease severity.
- ✓ The fungicide, Score®, reduced disease severity by 90%.
- ✓ There were no significant differences between SBR severity after treatments with the fungicide and the *Lecanicillium* treatments.
- ✓ Both *Lecanicillium* and fungicide sprays increased soybean yields compared to the control, but were not significantly different from each other.

Implications of findings:

In both field experiments *L. muscarium* strain Nesta-08 proved to be capable of reducing SBR severity. Therefore, it can be developed into a useful biocontrol agent for SBR management as an alternative to fungicides. Studies need to be continued on various aspects such as optimizing the efficacy of *L. muscarium*, correlating the number of sprays, and timing of sprays with weather conditions, and costing of its use.

In the long term, an industry partner will need to undertake full registration trials, and develop large scale production protocols, formulations and storage conditions in order to create a commercial product that farmers will be able to use.

Future Research

The research outcomes of this MSc dissertation suggested the following research topics could be undertaken productively:

- ✓ Conduct light and electron microscopy studies, with biochemical assays, to clarify the mycoparasitic mechanisms of *L. muscarium* infecting *P. pachyrhizi.*
- ✓ Conduct large scale field trials at multiple sites to compare the impact of *L. muscarium* and commonly used fungicides for SBR control, and to quantify soybean yield increases.

- ✓ The cost effectiveness of *L. muscarium* Nesta-08 needs to be compared with commonly used fungicides for SBR control, relative to their efficacy in control of SBR, and hence the relative incomes of crops protected by a biocontrol agent or a fungicide.
- ✓ Compare the effectiveness of *L. muscarium* with other mycoparasitic fungi for SBR control. This will require a fresh project to discover new biocontrol fungi and bacteria that colonize rust pustules.

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