Development of a Biocontrol Agent, *Lecanicillium attenuatum* (Zare & Gams), of Wheat Leaf Rust (*Puccinia triticina* Eriks.)

By

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Dissertation Summary

Wheat leaf rust is a common fungal disease caused by *Puccinia triticina* Eriks. The disease is favoured by high relative humidity, cool nights and the presence of dew. Under these favourable conditions, the polycyclic disease develops rapidly, causing severe yield losses. *P. triticina* affects wheat production globally. Understanding the epidemiology of *P. triticina* is important for the management of the disease. Management of wheat leaf rust has been based on the use of resistant wheat cultivars, and chemical and cultural control strategies. However, under conducive conditions, rapid disease development often overwhelms these strategies. The development of new pathogen races that can infect previously resistant cultivars has resulted in pressure on fungicide application. The development of resistance to some fungicides by rusts has limited their effectiveness. Biological control agents provide an alternative approach to disease control. Fungal agents, including *Lecanicillium* spp., have been considered as promising alternatives to synthetic fungicides.

Species of the fungal genus *Lecanicillium* can be found naturally on rust infected leaves, and is characterized by a white mycelium occurring on the rust pustules. Isolation of *Lecanicillium* strains was done by extracting samples of the white mycelium from oxalis rust (*Puccinia oxalidis*) using a needle and culturing these samples on potato dextrose agar (PDA) plates. Clean colonies of putative *Lecanicillium* spp. were transferred onto fresh PDA plates to obtain a pure culture. One pure culture of *Lecanicillium* spp. was used for identification and was sent to Inqaba Biotech for DNA identification of the fungus. The sample was found to be *Lecanicillium attenuatum,* with the GenBank Accession number AB378513.1.

A trial to evaluate three doses of conidia of *L. attenuatum* (at 10^2, 10^4, and 10^6 conidia ml^{-1}) was done on rust pustules on *Oxalis latifolia* Kunth plants. This host plant was used as a proxy for commercial crops, as it is highly susceptible to *P. oxalidis*. Each of the prepared doses was applied to 12 *Oxalis* plants, while the remaining 12 plants served as the control. Colonisation of *P. oxalidis* pustules was evident on the inoculated pustules but not the pustules of the untreated control. *L. attenuatum* controlled the oxalis rust

caused by *P. oxalidis* on *O. lateralis* plants. The three doses of *L. attenuatum* conidia provided various colonization percentages: 10^2 conidia ml^{-1} providing 25% colonisation, 10^4 conidia ml^{-1} providing 26.49% colonisation and 10^6 conidia ml^{-1} providing 70% colonisation, while the control pustules had a mean of 4.63% colonization.

The three doses of *L. attenuatum* (10^2, 10^4, and 10^6 conidia ml^{-1}) were tested under greenhouse conditions on 48 wheat rust infected plants that were planted in 18 cm pots, each containing three plants. *L attenuatum* greatly reduced the development of wheat leaf rust caused by *P. triticina*. At all three doses of *L. attenuatum* conidia rust pustules were significantly (P = 0.001) colonised, relative to the control plants. The dose of 10^2 conidia ml^{-1} provided a colonization of 52.83% of the initial rust pustules, which was 65.22%, while 10^4 conidia ml^{-1} colonised 50.42% of the 71.81% of the rust pustules on leaves. The most infection of rust pustules was achieved by the dose of 10^6 conidia ml^{-1}, which colonised of 81.64% of the initial 76.79% of rust pustules.

The aggressiveness of *L. attenuatum* on rust was further tested in the field at the Ukulinga research farm owned by the University of KwaZulu-Natal. The control treatment developed 81.7% rust infection, of which 2.73% of the pustules were colonized by *L. attenuatum.* At a dose of 10^2 conidia ml^{-1} colonisation of the rust pustules reached 51.60% (at a rust level of 52.10%), while at 10^4 conidia ml^{-1} 60.52% of the rust pustules were colonised (at a rust level of 60.70%). At a dose of 10^6 conidia ml^{-1}, 77.29% of the rust pustules were colonised (at a rust level of 73.20%).

Hyperparasitism studies between *L. attenuatum* and *P. triticina* were done under scanning electron microscope, which showed the infection hyphae emerging from *L. attenuatum* conidia and penetrating the urediospores of *P. triticina.* After penetration, the urediospores became deformed.

DECLARATION

I, Thembani Nxumalo, declare that:

1. The research reported in this dissertation, except where otherwise indicated, is my original research
2. This dissertation has not been submitted for any degree examination at any other university
3. This dissertation does not contain other person's data, pictures, graphs or other information, 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;
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4. 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

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Mr. T. Nxumalo

Signed: …….

Prof. M.D. Laing (Supervisor)

Signed: ……..

Dr K.S. Yobo (Co-Supervisor)

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DEDICATION

To my families, Nxumalo and Ntanzi,

My Grandmother, Nyokana Ntanzi,

My brothers, Mfungeleni Nxumalo and Velani Nxumalo

My sisters, Zethu Mantazi Khoza, Nosipho MaNtanzi Biyela, Xolile Ntanzi

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Dissertation Introduction

Wheat (*Triticum aestivum* L. Em Thell.) is a staple food for approximately two billion people world-wide (Bhardwaj et al., 2016). However, it is attacked by many fungal pathogens, including the rust fungus *Puccinia triticina* Eriks (Bolton et al., 2008). *P. triticina* is considered to be the most destructive pathogen on wheat, due to its global distribution, and its potential to form new pathotypes that cause epidemics in previously resistant cultivars (Hamdy et al., 2001; French, 2010). Under conducive conditions, *P. triticina* may cause high levels of yield losses, which historically has resulted in famines (Atiq et al., 2017).

Managing the disease is currently based on chemical and cultural control strategies, and by planting resistant cultivars. However, under conducive environmental conditions, these strategies have not been consistently effective. Therefore, the development of resistance to some fungicides by rusts has limited their effectiveness. The use of biological control measures has gained extensive attention as an alternative means to chemical control (Sheroza et al., 2003).

Rust pustules may be attacked by a number of fungal hyperparasites, including species of *Lecanicillium*, therefore the overall aims of this study were to isolate strains of *Lecanicillium attenuatum* from rust pustules of *Oxalis latifolia*; to investigate the ability of the strains to parasitize wheat leaf rust; and to estimate the level of biocontrol that the best of the strains, at an optimum dose of conidia, could provide against wheat leaf rust.

The objectives of this study were to:

1. Conduct a review of *P. triticina*, its causal agent, host range, dissemination, symptoms, infection process, epidemiology and management strategies, including biological control options and potential options;
2. Isolate and evaluate the potential of *L. attenuatum* as a biocontrol agent of wheat leaf rust using oxalis plants as a proxy for commercial crops;
3. Evaluate *L. attenuatum* as a biological control agent of wheat leaf rust under greenhouse conditions;

4. Test the pathogenicity of *L. attenuatum* on wheat leaf rust in the field.
5. Conduct histological studies on the hyperinfection of rust pustules by *L. attenuatum* using scanning electron microscopy.

This thesis is presented in the form of four chapters. Individual chapter covers specific objectives of the study conducted.

This dissertation follows a standard format that has been adopted by the University of KwaZulu-Natal because it facilitates the publishing of research out of the dissertation far more readily than the older monograph form of dissertation. As such, there is some unavoidable repetition of references, methods and some introductory information

This research was undertaken in the Discipline of Plant Pathology, at the University of KwaZulu-Natal, Pietermaritzburg Campus, under the supervision of Prof. M.D. Laing and Dr. K.S. Yobo.

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CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

Wheat (*Triticum aestivum* L. Em Thell) has been the staple cereal of humans for the past several decades, and is the world's primary crop plant, followed by maize (*Zea mays* L.) and rice (*Oryza sativa* L.) (Nevo et al., 2000). The first archeological record of the use of wheat originates in Israel. Evidence of the use of wild tetraploid wheat, *Triticum dicoccoides*, was found and estimated to be 19,000 years old (Abara, 2007). Wheat grasses belong to the botanical tribe known as *Triticeae* in the grass family *Poaceae*, which also comprises the related crop species rye (*Secale cereale* L.) and barley (*Hordeum vulgare* L.) (Huerta-Espino et al., 2011; Nevo et al., 2000; Snape and Pankova, 2006).

Wheat leaf rust is caused by *Puccinia triticina* Eriks. This is a destructive pathogen in South Africa, and infects wheat under high relative humidity conditions with cool nights and the presence of dew (Kloppers and Tweer, 2010; Hollaway, 2014). Under these conducive conditions, the disease develops rapidly, causing yield losses due to decreased numbers of kernels per head and lower kernel weights on infected plants (Melvin et al., 2008). *P. triticina* is now known to be the most significant pathogen in wheat production globally, causing important yield losses across large geographical areas (Melvin et al., 2008).

The rust pathogens are amongst the most destructive in important crops, such as maize (*Zea mays* L.), coffee (*Coffea arabica L.*) and wheat (Robert, 1991). Wheat is highly susceptible to three different rusts, namely leaf (*P. triticina*), stem (*Puccinia graminis* Pers.) and stripe (*Puccinia striiformis* var. *striiformis* W. (Barreto and Evans, 1988; Robert, 1991), each of which can have a devastating effect and reduce the yield (Markell et al., 2001)

Wheat leaf rust is commonly controlled by resistant cultivars, but it causes problems where susceptible varieties are grown (Hollaway, 2014), or if novel virulent races evolve. Under such circumstances, chemical control measures have contributed significantly towards controlling rust diseases for a number of decades. However, in some instances this practice has resulted in the development of fungicide resistance in pathogens and increased input costs. Safer and cheaper alternative control strategies of wheat rust diseases are therefore needed (Sheroza et al., 2003).

A genus of fungi, *Lecanicillium* spp., is both entomopathogenic and antagonistic to some fungal diseases due to its ability to hyperparasitise the pathogen spores (Zare and Gams, 2001). It has the potential to be used as a biocontrol agent for rust pathogens. However, to test for the effectiveness of a formulated biocontrol agent, a plant is required that is easily grown and which is highly susceptible to rust (Roy et al., 2001).

1.2 Commercial use and economic importance of wheat

Wheat is the most important cereal crop universally, and is the main food for nearly two billion people.The global economic role of wheat production is significance in terms of food supply, employment and commerce. South Africa as a Developing Country is a net importer of wheat, importing about 300 000 tons yearly (Agriculture Forestry and Fisheries, 2016).

1.3 The pathogen and the disease

1.3.1 Taxonomy and morphology of wheat leaf rust

All three wheat rust fungi (*P. graminis* f. sp. *tritici*, *Puccinia striiformis* f. sp. *tritici,* and *P. triticina*) belong to the Order Uredinales within the Basidiomycetes. The taxonomic classification of the pathogen that causes wheat leaf rust has been changed due to several revisions (Melvin et al., 2008), originally was known as a species different from the other rusts and in 1815 de Candolle termed it *Uredo rubigo-vera* (Roelfs and Bushnell, 1985). In 1894 Eriksson described *Puccinia dispersa*. which comprised leaf rusts of wheat and rye. In 1899, Eriksson described wheat leaf rust as *P. triticina*. In 1932, Mains

sectioned *P. rubigo-vera* into 56 *formae speciales*, and of which one, *f. sp. tritici*, matched to Eriksson's *P. triticina* (Bowers, 1982;; Anon 2003). Later In 1956, Cummins and Caldwell suggested that *Puccinia recondita* was the useable name for the leaf rusts of grasses. The fungus currently known as *P. triticina* has telial hosts in many species of grasses, rye and wild wheat (Melvin et al., 2008). Smith et al (2005) and Bowers (1982) noted that wheat leaf rust has *Thalictrum speciosissimum* L. (Family *Ranunculaceae*) as the alternate host.

1.3.2 Host range of *Puccinia triticina*

The common hosts of *P. triticina* includes hexaploid wild wheat (Melvin et al., 2008; Kloppers and Twee, 2010), tetraploid durum wheat (*Triticum turgidum ssp. durum* D.), domesticated and wild emmer wheat (*Triticum dicoccum* S.), and triticale (*Triticum triticosecale* W.) (Chai et al., 2016). *P. triticina* also occurs on common goat grass (*Aegilops triuncialis* L.) in some countries, including the USA (Melvin et al., 2008). Not all races of *P. triticina* are virulent on non-hexaploid wheat hosts, which indicate a very high degree of telial host specificity (Roelfs, 1992; Chai et al., 2016). According to Melvin et al (2008), *P. triticina* infections do not occur on wild wheat species such as *Triticum urartu* T. on which infections only occur with artificial inoculations (Aime, 2006). *P. triticina* does not occur on *Thalictrum spp* on a regular basis and is found more frequently on *Triticum turgidum var. speciosissimum* L. (Roelfs, 1992).

1.3.3 Dissemination of *Puccinia triticina*

Splash from rainfall is the main means of rust local spread from infected leaf material (Melvin et al., 2008). The spores of the most destructive rust pathogens, including *P. triticina,* may be scattered by heavy water droplets through sprinkler irrigation or rainfall to other plants in the field (Avise, 2004; Bolton et al., 2008). Verhaar et al (1996) suggested that long distance dispersal is significant for the survival and reproduction strategy to most of rust pathogens, as is the case with *P. triticina,* which can travel thousands of kilometers in the air (Arthur, 1934; Bolton et al., 2008). Wherever the urediospores land, the host must be susceptible for the epidemic or disease progress to

occur successfully (Bolton et al., 2008). In greenhouse trials, many plants become infected after the occurrence of a single infection in one plant (Melvin et al., 2008). The accumulation of rust spores on the infected leaf makes its dissemination process easier under the influence of wind, as they are not firmly held on the leaf surface, which results in the spores sticking to workers' clothes in the field and being introduced to new areas (Bowers, 1982; Melvin et al., 2008).

1.3.4 Symptoms of the disease

Leaf rust is known for its aggressive attacks on wheat leaves. It can be distinguished by the dusty, reddish-orange to reddish-brown urediospores occurring in oval pustules on the upper surface (Figure 1.1) (Chai et al., 2016; Marsalis and Goldberg, 2016). Pustules are small, approximately 1.5 mm long, and unlike stripe rust, do not form in stripes (Figure 1.2) (Marsalis and Goldberg, 2016). Leaf rust can be found from autumn through to crop maturity, but is most common in spring (Marsalis and Goldberg, 2016). Black pustules (telia) may be produced in leaf rust lesions late in spring, but their teliospores do not cause disease on wheat (Robert, 1991; Melvin et al., 2008). Leaf rust normally presents uniformly across the field, but in overwintering locations is more severe on the lower leaves. When it blows in from distant fields during the same season, it is most severe on the upper leaves (Figure 1.1) (Robert, 1991; Bolton et al., 2008).

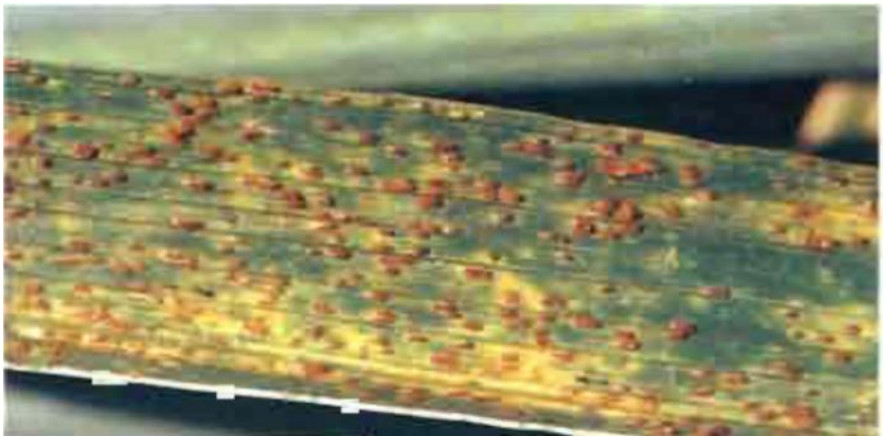

Figure 1.1 Dusty, reddish-orange to reddish-brown urediospores occurring in oval pustules on the upper surface of leaves (Robert, 1991).

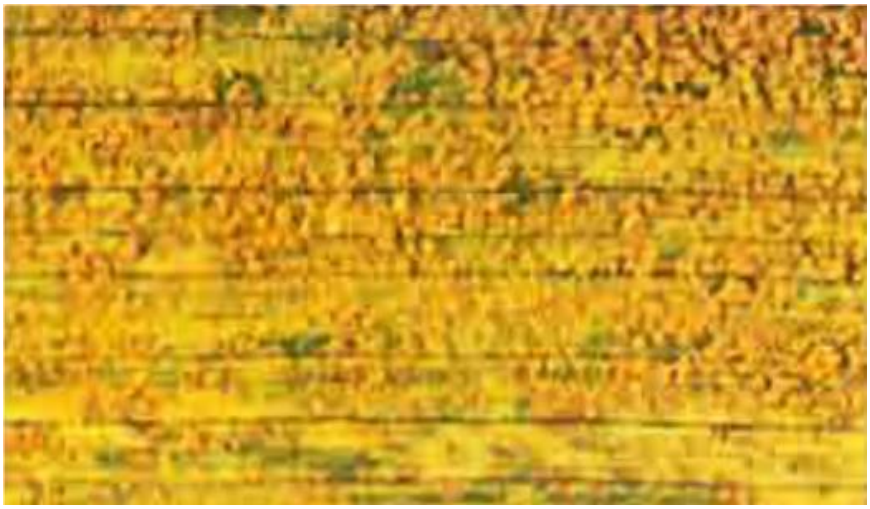

Figure 1. 2 Severe leaf rust infection occurring on the lower leaves of wheat plants (Mark and Natalie, 2006).

The pustules are characterized by masses of orange powdery spores, which can spill off from pustules and form a grainy orange dust on the leaf surface around the pustule (Chai et al., 2016). When the degree of pathogenicity by rust is very high, field scouts may observe the orange dust on hands and clothing (Marsalis and Goldberg, 2016). As leaves age, pustules begin to produce dark black instead of orange spores, which look like tar spots, and are most easily seen on the lower leaf surface and sheaths. Although leaf rust may initiate tiny orange spots on culms and heads, it does not form large, open pustules on these organs, which helps to distinguish leaf rust from stem rust. Stem rust is uncommon, and usually only occurs late in the growing season, as it requires warm temperatures. Leaf rust pustules occur randomly across the leaf, this distribution distinguishing it from stripe rust, which has narrow yellow stripes of pustules. (Robert, 1991). Chlorosis, or yellowing of leaves, can be evident with both leaf and stripe rust, and fields with plants displaying severe symptoms may be easily detectable from a distance (Figure 1.3) (Bolton et al., 2008; Melvin et al., 2008; Marsalis and Goldberg, 2016).

Figure 1.3. Chlorosis on leaves caused by wheat leaf rust infection (Robert, 1991).

1.3.5 Life cycle, infection process, and epidemiology of *Puccinia triticina*

Rust fungi have compound life cycles that may involve two unambiguously host plants, and have up to five spore stages, both sexual and asexual (Marsalis and Goldberg, 2016). Rust diseases that comprise two host plants to complete their life cycle normally have what is identified as an economic crop and an alternate host, usually a weed (Chai et al., 2016). The economic host in this study is wheat, while the alternate host is typically a weed (Figure 1.4C) or native plant (Gillin and Kasnakoglu, 2003). *Thalictrum* spp. are the primary alternate host for *P. triticina.* Its infection result in circular, yellow-to-red coloured pustules on the leaves (Melvin et al., 2008; Marsalis and Goldberg, 2016).

P. triticina is known as a heteroecious and macrocylic fungus and produces urediospores, basidiospores and teliospores (Melvin et al., 2008). Urediospores that are produced (Figure 1.4 A), infect the wheat host when the temperature ranges between 10-25^{o}C and there is free water on the leaf, such as rain or dew. Teliospores are produced in mature pustules (Figure 1.4 B). These spores allow the fungus to withstand unfavorably high summer temperatures and then infect the alternate host in autumn (Melvin et al., 2008).

Studies by Hiratsuka and Sato (1982) showed that the basidiospores penetrate and infect epidermal cells on the primary host, and subsequently develop pycnia, which are characterized by yellow pustules on leaf material (Hiratsuka and Sato, 1982). Fertilization takes place when the pycniospores and receptive hyphae fuse in compatible pairs of opposite mating types (Figure 1.4D). The dikaryotic mycelium multiplies through the leaf, and on the lower leaf surface it develops an aecium, typically formed directly below the pycnium (Figure 1.4E). *P. triticina* completes its life cycle when the aeciospores are dispersed by air, land on the primary host, germinate grow and penetrate the stomata of leaves of the primary host. Subsequently, the mycelium starts to develop in the leaf, and forms uredial pustules. These shed asexual urediospores, which continue the asexual reproductive cycle (Melvin et al., 2008)

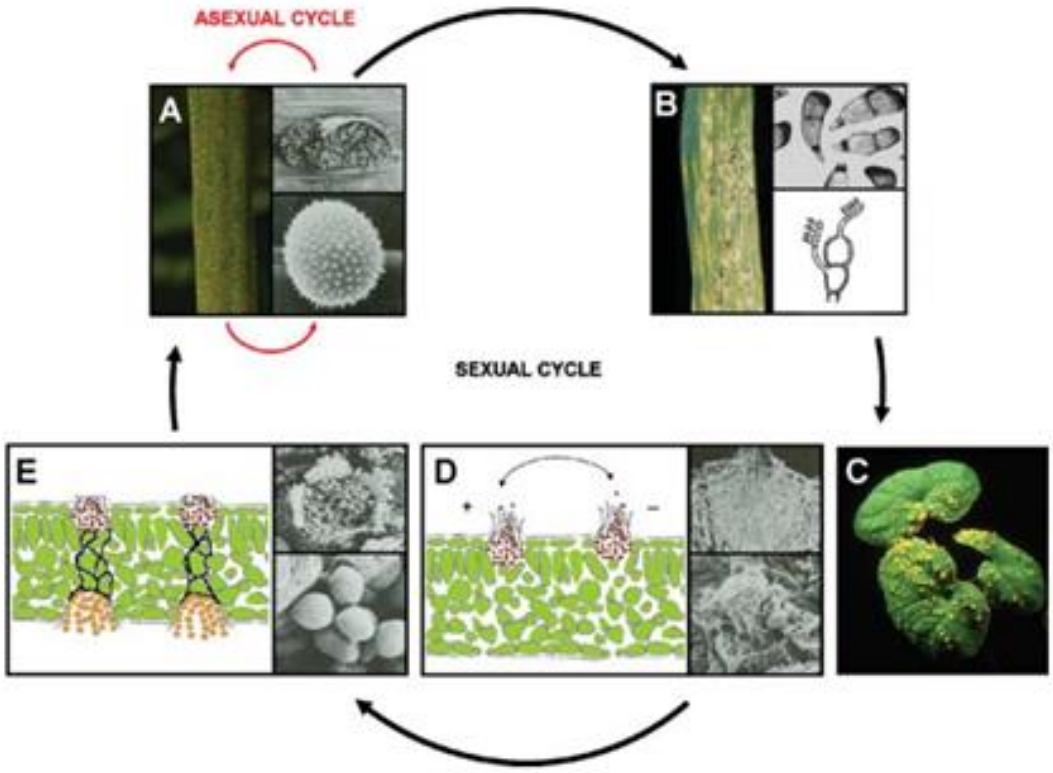

Figure 1.4 The five stages of the life cycle of wheat leaf rust, showing the primary and alternate hosts (Marsalis and Goldberg, 2016; Roelfs, 1992).

The fungus can infect with dew periods of 3 hours or less, with more infections occurring with longer dew periods. Temperatures of approximately 20°C are required. At cooler

temperatures, longer dew periods are needed, for example, at 10°C, a 12-hour dew period is necessary. Few, if any infections occur where temperatures are above 32°C or below 2°C (Table 1.1) (Robert, 1991).

Table1 .1 Environmental conditions required by wheat leaf rust (Robert, 1991)

Stage of leaf rust	Temperature			Light	Free water
	Minimum	Optimum	Maximum		
Germination	2	20	30	Low	Essential
Germling	5	15-20	30	Low	Essential
Appressorium		15-20		None	Essential
Penetration	10	20	30	No effect	Essential
Growth	2	25	35	High	None
Sporulation	10	25	35	High	None

Leaf rust reduces the crop yields because diseased leaves die prematurely. The earlier the leaves wilt, the more severe the yield loss becomes. Losses may vary depending on the variety's ability to fill from the stem, glumes, and awns (Robert, 1991). Each 1% increase in leaf rust severity decreases yield weight by 40.07 kg ha^{-1} and 1000 kernel weight by 0.13 g (Walid et al., 2015)

1.4 Managing *Puccinia triticina*

1.4.1 Chemical control

Chemical methods have been used for many years in various countries to control *P. triticina* (Martinez-Espinoza et al., 2014). In Brazil, Paraguay and South Africa, chemicals are used on infected wheat to manage an array of diseases (Hamdy et al., 2001, Turra et al., 2017). Susceptible wheat plants can be protected from rust with foliar fungicides that include Tilt (propiconazole), Quadris (azoxystrobin), and mancozeb (dithiocarbamate), these being the main fungicides for controlling leaf rust (Wise, 2016). Chemical control

strategies are most effective when rust diseases are identified at the early stage of the growing season when the plants are young. Acanto (picoxystrobin), Insignia (pyraclostrobin), Capitan (flusilazole), Folicur (tebuconazole), Flint (trifloxystrobin) and Soprano (epoxiconazole) are the fungicides currently used in South Africa to control wheat rust, and have contributed to maximizing the yield (Agriculture, Forestry and Fisheries, 2016. According to Kloppers and Tweer (2010), some systemic fungicides provide adequate control of wheat leaf rust, such as the strobilurins and triazoles (Kloppers and Tweer, 2010; Wise, 2016). Keeping the flag leaf disease free is essential, as it contributes 70% of the content of the head weight (Kloppers and Tweer, 2010; Wise 2016).

1.4.2 Resistant varieties

While wheat leaf rust can be controlled by planting resistant cultivars (Wegulo and Byamukama, 2012), the development of new races by the fungus can overcome this resistance (Oxly and Bayles, 2012 ;Wegulo and Byamukama, 2012). A study by Hei (2016) on Ethiopian cultivars, which included Pavon 76, Bonny, Africa Moyo, Qulqulu, Galili, Senqegna, and Hawi, documented low disease severity in the tested wheat varieties (Hei, 2016). In many countries, including South Africa and Australia, the resistance genes active against wheat leaf rust are known, and include the Lr37 gene (McIntosh et al., 1995, Huerta-Espino et al., 2011, Terefe, 2016).

Pitic 62, Penjamo 62, Sanora 64, Rojo 64, Siete Cerros Lerma, Xindong33, Xinjiang 15, Xindong 17, Xinjiang 25, Xindong18, Xinjiang 30, Xindong 20, Xinjiang 5, Xindong 22, Africa Moyo, Qulqulu, Galili, Senqegna, Hawi and Thatcher are some of the leaf rust resistant cultivars used in South Africa, China, India and other countries, and contribute significantly to the yields of bread wheat (Khan et al., 2013, Terefe et al., 2014, Ren et al., 2017). The majority of these cultivars have sustained their resistance for five and more years, which is the agronomic life expectancy of resistant cultivars where appropriate breeding platforms exist (Adam et al., 2000; Mark and Natalie, 2006). However, Roelfs (1992) noted that some cultivars lost their resistance before they were grown on more

than a fraction of the desired cultivated acreage, which highlights the need for new methods of controlling the wheat leaf rust, such as biological control (Kolmer, 1996).

1.4.3 Cultural practices

Cultural practices can serve as a strategy for partial control of wheat leaf rust epidemics. They include crop rotation and removing debris, which significantly enhances the present resistance on cultivated wheats (Singh et al., 1999). Removing volunteer susceptible wheat will reduce the quantity of inoculum for the following crop (Hollaway, 2014, Martinez-Espinoza et al., 2014, Chai et al., 2016). A good understanding of the epidemiology of the pathogen is essential before embarking on a control strategy, especially one including cultural means (Robert, 1991). Field scouting is essential for monitoring of wheat leaf rust (Melvin et al., 2008; Marsalis et al., 2016).

1.4.4 Biological control

To maintain healthy crops, biotic disease causing organisms have been mostly been controlled through the application of agrochemicals (Brent and Hollomon, 2007), which have been effective and quick to apply (Hart and Trevors 2005). However, their excessive application has resulted in numerous complications (Emmert and Handelsman, 1999), such as soil and water pollution, the accumulation of unwanted residues and development of resistance by plant pathogens, nematodes and insects (Dias, 2012). Therefore there is substantial pressure from consumers for agriculture to develop safer methods to control these pests, such as biological control (Tjamos et al., 2010).

Biological control of plant pathogens includes harnessing disease-suppressing microorganism to booster plant health, hence improving the yield of commercial crops (Handelsman and Stabb, 1996). Biological control can provide sustainable and affordable options to manage plant pathogens (Bale et al., 2008; Grzywacz et al., 2014). Disease suppression by biological control agents requires sustained interaction between the pathogen and biocontrol agents (Suarez-Estrella et al., 2013; Naher et al., 2014; Singh., 2014). In a study by Sheroze et al (2003) in Russia, where five microorganisms were tested against wheat leaf rust, applications of conidia of *Verticillium lecanii* N. *(*now

Lecanicillium lecanii R.*)* reduced rust development compared to the combinations of *Paecilomyces fumosoroseus* Br. and *Beauveria bassiana* V.*,* and to the untreated control.

1.5 *Lecanicillium* spp. as biological control agents

Lecanicillium spp. are known as entomopathogenic fungi (Goettel et al., 2008), but they can control both insects and plant pathogens (Owley et al., 2010). *Lecanicillium* spp. have an extremely wide host range, which includes aphids, whitefly, phytopathogenic fungi, and plant parasitic nematodes (Aiuchi et al., 2008). In addition, some strains can trigger induced systemic resistance. This has been reported on both monocotyledons and dicotyledonous plant species, including soybean plants infected by *Phakopsora pachyrhizi* S. (soybean rust) (Owley et al., 2010).

1.5.1 *Lecanicillium* spp. as a pathogen of plant pathogens and parasites

Coffee leaf rust is caused by *Hemileia vastatrix* P. (Berk & Broome (1869). It is a destructive disease globally. This rust fungus is naturally hyperparasitized by *Lecanicillium* spp., indicating its potential as a biocontrol agent (Galvao and Bettiol, 2014). In a study conducted by Galvao and Bettiol (2014) in Brazil, two strains of *Lecanicillium* reduced the levels of coffee rust. *Lecanicillium* spp. have been used as biological control agents for or against pea aphids (*Acyrthosiphon pisum* H.), as reported by Ramezani et al. (2013). Kumar et al (2015) reported the infectivity of *Lecanicillium* spp. on cardamom thrips (*Sciothrips cardamom* R.). In a similar study conducted in Chile, cypress aphids (*Cinara cupressi* B.), which are listed amongst the hundred most destructive invasive pests in the world, were controlled by *L. attenuatum* under laboratory experiments (Montalva et al., 2017).

1.5.2 *Lecanicillium* spp. as a pathogen of plant parasitic nematodes and powdery mildew

Some of the *Lecanicillium* spp., including *L. lecanii* and *L. attenuatum,* have been used as parasites or biopesticides for insects and a few rust pathogens (Table 1.2.) (Shinde et al., 2010).

Lecanicillium spp. are potentially beneficial biocontrol agent of soybean nematode (*Heterodera glycines* I.*)* (Smith et al., 2005; Shinya et al., 2008). Three different strains studied by Shinya et al (2008) were found to reduce the numbers of nematode eggs in the soil by 20%. Some strains of *Lecanicillium* spp. attack powdery mildew conidia and mycelia (Goettel et al., 2008). Miller et al (2004) controlled strawberry powdery mildew caused by *Sphaerotheca macularis* (Wallr. ex Frier) Cooke f.*sp. fragariae* using *L. lecanii* collected from fields in California.

Table 1.2 *Lecanicillium* spp that have been used as biopesticides against insect pests and fungal pathogens

Antagonist	Host	Comment	Reference
Lecanicillium psalliotae	thrips (*Sciothrips cardamomi*)	At the dose of $1x10^{7}$conidia ml^{-1}, 62.9 % mortality was recorded in the thrips population ten days after inoculation.	Kumar et al., 2015
Lecanicillium attenuatum,	Cypress aphid (*Cinara cupressi*)	At the dose of $1x10^{8}$conidia ml^{-1} aphid population decreased	Montalva et al., 2017
Lecanicillium lecanii	Coffee rust (*Hemileia vastatrix*), green coffee scale (*Coccus viridis*), and soybean nematode (*Heterodera glycines*)	Simultaneous suppression of *Hemileia vastatrix* and *Coccus viridis* was observed.	Jackson et al., 2012, Shinya et al., 2008
Lecanicillium longisporum	Pea aphid (*Acyrthosiphon pisum*)	*L. longisporum* was suggested as a compatible biocontrol agent of pea aphids.	Ramezani et al., 2013
Lecanicillium muscarium	Planthopper (*Ricania simulans*)	Mortality of *R. simulans* nymphs was in the range of 51 to 75%.	Guclu et al., 2010

1.6 Mode of action to suppress plant pathogens?

The modes of action employed by antagonistic fungi and other biocontrol agents on plant parasitic organisms has been the topic for several studies (Goettel et al., 2008, Sandhu et al., 2012). *Lecanicillium spp*. use both hydrolytic enzymes and mechanical forces to directly penetrate the integument of target insects, or the cell wall of fungal plant pathogens (Goettel et al., 2008). When *Lecanicillium* spp. conidia interacts with an insect integument, they stick to the epicuticle and then germinate. Germinated conidia produce germ tubes that penetrate the cuticle directly (Shinde et al., 2010). The germ tubes penetrate through the epicuticle and the procuticle. The fungus produces a diverse range of extra cellular enzymes that are essential during cuticle penetration (Shinde et al., 2010), such as Phosphorylase-rupturing (Pr1), Proteases, Chitinases, Esterase, Endoprotease, N-Acetylglucosaminase, Aminopeptidase, Lipase, and Carboxypeptidase (Shinde et al., 2010; Hasan et al., 2013). The enzyme Pr1 acts as a cuticle degrading enzyme, with its highest dose being at the point of the penetration peg (Shinde et al., 2010). The insect dies as a result of mechanical pressure due to the fungal growth inside (Shinde et al., 2013).

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CHAPTER TWO

Evaluation of strains of *Lecanicillium* spp. as biocontrol agents of rust diseases, using *Puccinia oxalidis* on *Oxalis lateralis* as the test system

Abstract

The pathogenicity of isolates of *Lecanicillium* spp. were tested on Oxalis rust (*Puccinia oxalidis* D.) growing on plants of a common weed, *Oxalis lateralis.* The biological control agent, *Lecanicillium* spp., occurred naturally on infected Oxalis leaves, characterized by white mycelium on rust pustules. Isolation of *Lecanicillium* spp. was done by picking the white mycelium using a needle and culturing it onto Potato Dextrose Agar (PDA) plates. Clear colonies of suspected *Lecanicillium* sp. from non-contaminated PDA plates were transferred onto fresh PDA plates to obtain a pure culture, with one pure culture being used for genomic identification by Inqaba Biotech (http://www.inqababiotec.co.za/). The sample was found to be *Lecanicillium attenuatum* W., with the GenBank Accession number AB378513.1. Three doses of conidia of *Lecanicillium* spp. (10^2, 10^4 and 10^6 conidia $ml^{-1)}$ were prepared for supra-inoculation on Oxalis rust pustules. The dose of 10^6 conidia ml^{-1} provided more control (70% pustule colonisation) than 10^4 conidia ml^{-1} (26.49% pustule colonisation) and 10^2 conidia ml^{-1} (25% pustule colonisation), while on the control 4.63% pustule colonisation occurred. Spore penetration by *Lecanicillium* spp. on urediospores was viewed by scanning electron microscope, observing pustules that had been inoculated with *L. attenuatum* at 10^6 conidia ml^{-1}. At lower conidial doses (both 10^2 and 10^4 conidia ml^{-1}), only hyperparasitism was observed without urediospore penetration.

Key words: *Lecanicillium attenuatum*, *Puccinia oxalidis*, hyperparasitism

2.1 Introduction

Rust pathogens are destructive diseases affecting important crops such as wheat (*Triticum aestivum* L.), coffee (*Coffea arabica* L) and maize (*Zea mays* L) (Brian, 2013), causing losses ranging from 20-30% a year (Food Agriculture Organization, 2017). Wheat is a staple food worldwide (Anand et al., 2009), but wheat plants are vulnerable to many diseases (Avelino et al., 2004), including rusts (World Summit of Food Security, 2009; Ahmad et al, 2010). Three types of rusts are found on wheat: (i) leaf rust (brown rust), caused by *Puccinia triticina* Eriks (Groth et al., 2015) ii), stripe rust (yellow rust), caused by *Puccinia striiformis var tritici* W. (Murray et al., 2005) iii), and stem rust (black rust), caused by *Puccinia graminis* Pers (Beddow et al., 2015). Leaf and stripe rusts appear periodically on wheat crop and cause yield losses of 20 to 25% (Ahmad et al, 2010). Numerous epidemics of leaf rust on wheat crops have been reported, and in the absence of widely available effective and affordable control mechanisms, this pathogen will continue to be a major threat to wheat production (Wegulo and Byamukama, 2012).

Fungicides and cultural practices have remained the most important components of wheat rust management strategies (Walia et al., 2014). However, under rapid disease development, cultural control strategies do not provide sufficient management of wheat rusts diseases (Terefe and Delport, 2014). The occurrence of resistance to fungicides by wheat rust pathogens limits the effectiveness of chemical control strategies. While picoxystrobin, fluoxastrobin, propiconazole and tebuconazole have been extensively used as systemic fungicides to control wheat rust pathogens (Melvin, 2010), their extensive application has resulted in the development of resistant fungal strains (Bradley, 2002).

Biocontrol control agents are a promising alternative to fungicides to manage plant diseases (Askary and Yarmand, 2007). These methods involve the use of microorganisms that are antagonistic to plant pathogens to reduce plant disease development to a tolerable level (Pal and McSpadden, 2006). The entomopathogenic fungus *Lecanicillium attenuatum* has been shown to be antagonistic to some fungal pathogens due to its ability to hyperparasitise many pathogen spores (Cuthbertson and Walters, 2005; Doug et al., 2012; Junaid et al., 2013; Rachel et al., 2009; Zare and Gams, 2001). Kim et al. (2006) noted a reduction in severity of powdery mildew

caused by *Sphaerotheca fuliginea* P. in their study where *L. attenuatum* suppressed the disease development. Therefore this study aimed to isolate *L. attenuatum* and to evaluate its potential as a biocontrol agent of wheat leaf rust, using the *Oxalis latifolia* / *Puccinia oxalidis* pathosystem as a proxy for wheat / wheat leaf rust pathosystem. The concept of using the weed pathosystem was driven by the ongoing rust infections of *O. latifolia* plants in Spring, Summer and Autumn (9 months of the year), whereas the wheat rust pathosystem is limited to Autumn and Spring in South Africa (about 3-4 months of the year).

2.2 Materials and Methods

2.2.1 Oxalis plants for rust infections

Oxalis seedlings (200) were planted in 18 cm pots filled with composted bark (potting mix) and placed in 2 L ice cream tubs containing water with a hydroponic fertilizer (2.3.2 (38). The pots were kept in a greenhouse at 26^{o}C and relative humidity of approximately 80%, and the plants were allowed to grow for 14 days, with a maximum of three leaves each. Infected *O. latifolia* plant materials were collected from a property in Woodhouse Road, Scottsville, Pietermaritzburg, and used to inoculate the potted plants. The healthy *O. latifolia* plants were dusted with the urediospores from the leaves of rust infected plants, after which they were covered with freezer bags for 48 hours to provide high relative humidity to enhance the *P. oxalidis* infections on the leaves, after which the bags were removed. Rust symptoms appeared 4 to 5 days after inoculation. Ten days post-inoculation the rust infected leaves were taken to the Microscopy Microanalysis Unit (MMU) to view the rust pustules under a Scanning Electron Microscope (SEM) (Zeiss EVO LS15, Germany), operated at high vacuum, chamber vacuum = 1.26e – 005 Torr, gun vacuum = 5.08e – 007 Torr.

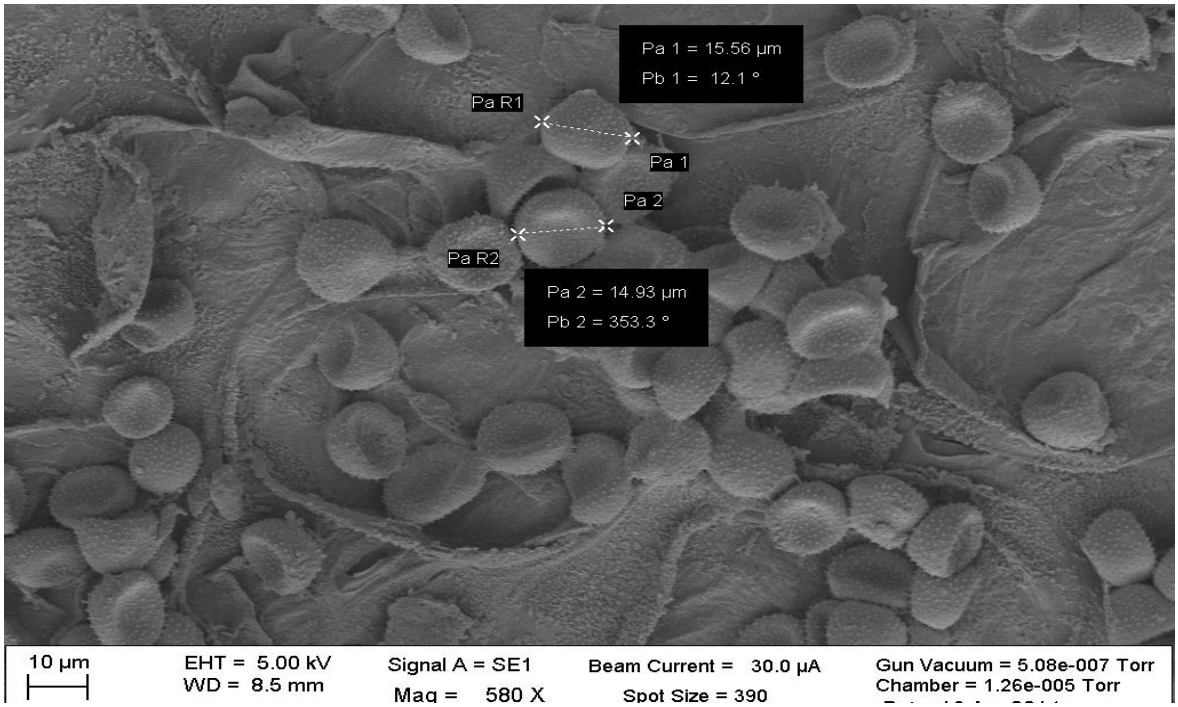

Figure 2.1 Urediospores of the rust of *Oxalis latifolia* viewed under SEM and found to be *P. oxalidis*

2.2.2 Isolation of *Lecanicillium sp.*

Lecanicillium spp*.* occurred naturally, where the rust pustules were clustered (Figure 2.2). White mycelium of a *Lecanicillium* spp*.* was picked of infected pustules using a needle, and cultured on Potato Dextrose Agar (PDA) plates. Inoculated PDA plates were incubated at 28^{o}C for 21 days, the growth being observed daily after three days. Clear colonies of suspected *Lecanicillium* spp. from non-contaminated PDA plates were transferred onto fresh PDA plates to obtain pure cultures (Figure 2.3).

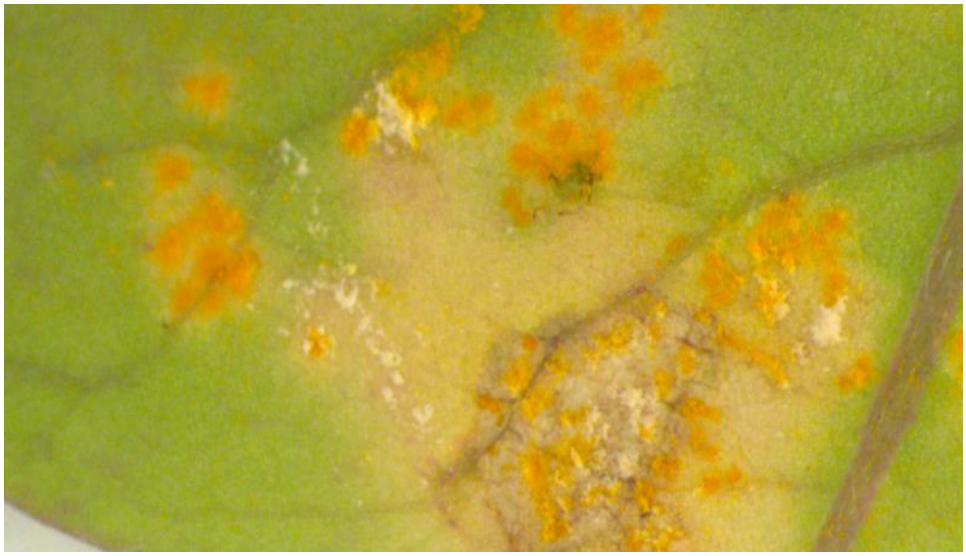

Figure 2.2 Natural infections by *Lecanicillium* spp. on the *P. oxalidis* pustules (from the inoculated *O. latifolia* leaves in the greenhouse).

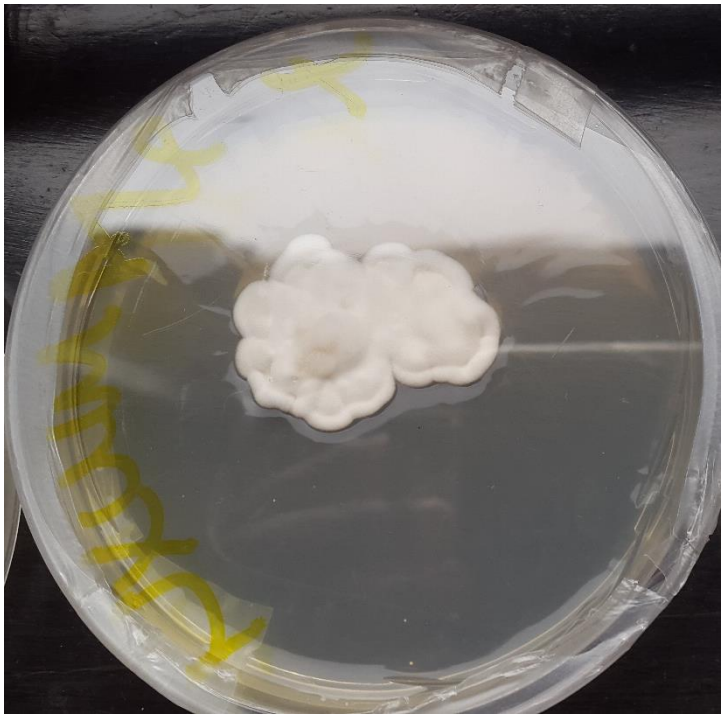

Figure 2.3 A pure colony of isolated *Lecanicillium* spp. culture on PDA.

2.2.3 Second inoculation with *Puccinia oxalidis* urediospores

The study was conducted in the green house at the University of KwaZulu-Natal, Pietermaritzburg. A randomized complete block design was used for the experiment. A total of 16 pots were used as blocks. Four pots constituted one block and accommodated 12 plants. The four treatments were randomized in each block. A total

of 48 *O. latifolia* plants, each with three leaves (total of 144 leaves), were inoculated with urediospores from rust pustules of *P. oxalis*, each host leaf being inoculated independently. The pots containing the plants were covered with freezer bags for 48 hours to provide a high relative humidity to enhance *P. oxalidis* infections on the plant leaves. Symptoms of *P. oxalidis* were observed within 4-5 days.

2.2.4 Supra-inoculation with *Lecanicillium* spp. on rust pustules

Twenty PDA plates containing colonies of *Lecanicillium* spp. were placed under UV light to stimulate sporulation. The sporulating cultures of *Lecanicillium* spp. on the PDA plates were flooded with 200 ml of sterile distilled water. The conidia were then dislodged using an L-bent glass rod to harvest them. The suspension was filtered through four layers of sterile cheesecloth to remove the mycelium, and the number of conidia ml^{-1} of the filtrate was determined using a haemocytometer. From the prepared suspension, three doses of conidia of *Lecanicillium* spp were prepared at concentrations of 10^2, 10^4, and 10^6 conidia ml^{-1}. Each of the prepared doses was applied to 12 *O. latifolia* plants. The remaining 12 plants served as the control and were not inoculated. The rust pustules infected by *Lecanicillium* spp. were viewed under the SEM to determine the interaction between the *Lecanicillium* spp. and urediospores of *P. oxalidis*.

2.2.5 Interaction studies of *Lecanicillium* spp on rust spores under SEM

Primary fixation was performed by dipping the *O. latifolia* infected leaf samples in a 3% glutaraldehyde buffer for 1-3 hours, after which they were put in a sodium cacodylate buffer twice for 5 minutes each time. Samples were dehydrated in a graduated ethanol series (10, 30, 50, 70, 90% and twice in 100% ethanol), and then the specimens were transferred into a critical point drying (CPD) (Quorum Q150R K850, UK) unit under 100% ethanol. This resulted in dry and intact specimens that were mounted on copper stubs with a double sided sticky tape, and sputter coated with gold palladium, with three coated specimens being viewed under the SEM at a time.

2.2.6 Molecular identification of *Lecanicillium* spp.

Genomic deoxyribonucleic acid (DNA) was extracted from the cultures of suspected *Lecanicillium* sp. using a ZR Fungal DNA Kit™ (Zymo Research, Catalogue No. D6005). The ITS target region was amplified using EconoTaq® PLUS GREEN 2X Master Mix (Lucigen) with the ITS1 and ITS4 primers, and the polymerase chain reaction (PCR) products were run on a gel. The gel products were extracted using a Zymo Research, Zymoclean™ Gel DNA Recovery Kit, Catalogue No. D4001. The extracted fragments were sequenced in the forward and reverse directions (Applied Biosystems, ThermoFisher Scientific, Big Dye terminator kit v3.1) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were run on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific) for each reaction for the sample. CLC Bio Main Workbench v7.6 was used to analyse the ab1 files generated by the ABI 3500XL Genetic Analyzer. The results were analysed using a BLAST search (NCBI). The sample was matched to be *Lecanicillium attenuatum* W., with the GenBank Accession number AB378513.1.

2.2.7 Disease rating, data collection and analysis

A visual rating scale (percentage) was used to rate for levels of rust disease and biocontrol hyper-infection. These were for: (i) % leaf area covered with rust infections; and (ii) the % of rust pustules infected with *Lecanicillium* spp. The data was collected for each of the treatments: control, 10^2, 10^4, and 10^6 conidia ml^{-1} doses. The data was collected after 10 days from 12 *O. latifolia* plants per treatment, with three leaves being rated per plant, resulting in 144 leaves being rated for the four treatments. The data sets for the treatments were subjected to analysis of variance (ANOVA) using GenStat (14^{th} edition).

2.3 Results

2.3.1 Dose response of *Lecanicillium attenuatum* on *Puccinia oxalidis*

The mycelium of *Lecanicillium* spp. colonised the urediospores of *P. oxalidis*. Figure 2.4A shows how the *Lecanicillium* spp. mycelium penetrated the *P. oxalidis* spores. In Figure 2.4B, the germ tube produced by *Lecanicillium* spp. coiled around the urediospores of *P. oxalidis*. Application of various doses of *Lecanicillium* spp. conidia

resulted in a range of colonisation levels. The dose of 10^2 conidia ml^{-1} that was supra-inoculated on rust pustules provided 25.70% colonisation, 10^4 conidia ml^{-1} provided 26.50%, 10^6 conidia ml^{-1} provided 70.10%, and the control developed a level of rust of 4.63% (Table 2.1).

Table 2.1 Colonisation of rust pustules by *Lecanicillium* spp three doses, plus an uninoculated control

Treatment (conidia ml^{-1})	*Lecanicillium* sp. level (%)	Rust Level (%)
10^2	25.70 a	56.44
10^4	26.50 a	51.30
10^6	71.10 b	72.30
Control	4.63 c	65.80
F values	18.97	1.06
P- Value	0.001	0.284
CV %	57.3	
S.E.D	9.61	

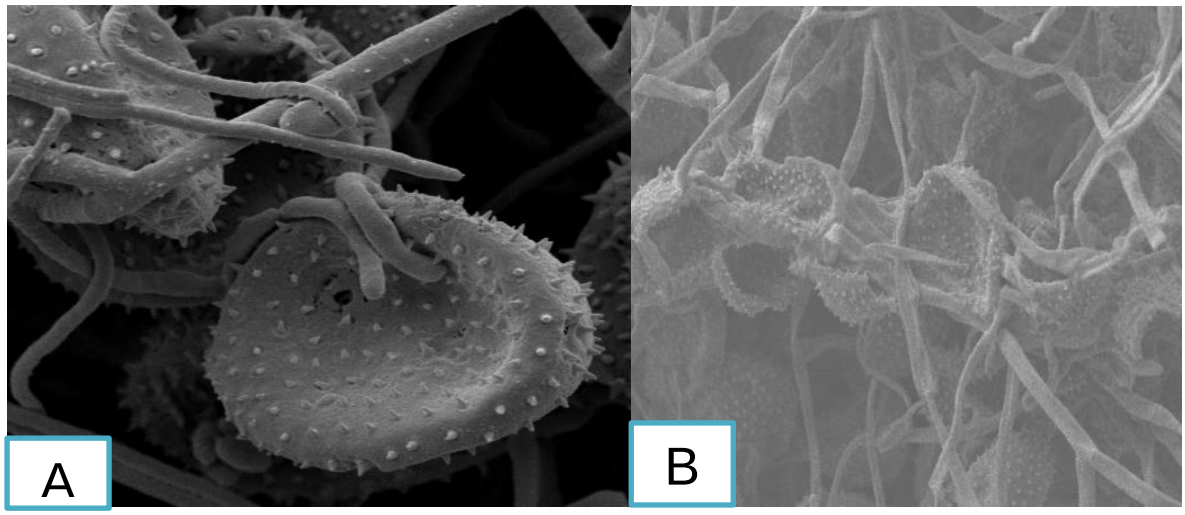

Figure 2.4 *Lecanicillium* spp. colonizing (B), and penetrating (A) the urediospores of *P. oxalidis*.

2.3.2 Confirmation of the pathogen

Due to the susceptibility of *O. latifolia* plants to two different rust pathogens, *Puccinia sorghi* S. and *P. oxalidis*, leaves with infected abaxial surfaces were viewed under SEM. The spores in the pustules were observed to be the urediospores of *P. oxalidis,* which are characterized by a round urediospores with sharp spines, with an average spore diameter of 15µm (Figure 2.1) (Vandermeer et al., 2009; Versluys, 2012)

2.3.3 Interaction between *Lecanicillium* spp. and *P. oxalidis* under SEM

The SEM results showed that *Lecanicillium* spp. coils on the urediospores of *P. oxalidis* (Figure 2.4). Spore penetration and colonization of rust spores by *Lecanicillium* spp. were found after supra-inoculation with *Lecanicillium* spp. on the rust pustules. The urediospores of *P. oxalidis* were deformed in shape, as a result of the *Lecanicillium* spp. infections. Figure 2.4A shows the *Lecanicillium* spp. penetrating the spore of *P. oxalidis* In Figure 2.4B hyperparasitism by *Lecanicillium* spp. on the urediospores of *P. oxalidis* was observed.

2.4 Discussion

Lecanicillium spp. was successfully isolated from rust pustules (*P. oxalidis*) of leaves of *O. latifolia*. Molecular identification found the pathogen to be matched by the profile of *L. attenuatum.*

A dose trial was run on the control of rust pustules on *O. latifolia* leaves. *L. attenuatum* should be applied in relatively high doses in order to attack the urediospores of *P. oxalidis* (Xie et al., 2015). The highest level of pustules colonization occurred at the dose of 10^6 conidia ml^{-1}. This compares well with other studies investigating the potential of *L. attenuatum* to control pathogens, such as coffee rust (*Hemileia vastatrix* Berk & Broome*)*, and powdery mildew on strawberries (*Podosphaera aphanis* W.) (Hall, 1980). Studies conducted by Kurze et al (2001) showed that *L. attenuatum* is also a mycoparasite of strawberry powdery mildew.

The rust pustules of *P. oxalidis* did not appear to succumb to infection when *L. attenuatum* conidia were inoculated at a dose of 10^2 conidia ml^{-1}. This reflects the poor efficacy of biocontrol agent when applied at low doses. According to Goettel et al (2008), the biocontrol agent (*L. attenuatum*) has limitations; for example, the rust

pustules have to be present in relative high levels for the spores to infect the urediospores

Lecanicillium attenuatum was found to directly attack urediospores. Similarly, Yan (2016) found that *L. attenuatum* suppressed pathogens through direct hyperparasitic action. Coiling around urediospores was followed by spore penetration, where the mycelia of *L. attenuatum* created holes into urediospores, apparently killing them. This is supported by excessive colonization, whereby the biocontrol agent coils over the spore of *P. oxalidis* after it creates the hole (Faria and Wraight, 2007).

Oxalis latifolia leaves remained green when infected with the rust alone, which appeared to allow the host to continue with the photosynthetic processes (Eken and Demirci, 1997; Naher, 2014). However, once hyperparasitism occurred, and the rust pustules die, the infected leaf tissues also died.

The highest level of hyperparasitism occurred after inoculation with a dose of 10^6 conidia ml^{-1}dose, which was as expected.

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CHAPTER THREE

Evaluation of *Lecanicillium attenuatum* as a biocontrol agent of wheat leaf rust under a greenhouse environment

Abstract

Puccinia triticina is a major rust disease of wheat, causing huge losses worldwide. This study aimed to evaluate a strain of *Lecanicillium attenuatum* for its potential to control wheat leaf rust under greenhouse conditions. Three different inoculum levels were evaluated (10^2, 10^4, and 10^6 conidia ml^{-1}). Two week old wheat plants previously inoculated with *P. triticina* were sprayed with the three levels of conidia of *L. attenuatum* after the appearance of rust symptoms. The dose of 10^6 conidia ml^{-1} resulted in the colonization of more rust pustules than the other treatments. All three treatments (doses) significantly (*P*= 0.001) controlled rust development compared to the control. The potential of *L. attenuatum* as a biocontrol agent of *P. triticina* was confirmed under greenhouse conditions.

Key words: *Lecanicillium attenuatum, Puccinia triticina*

3.1 Introduction

Wheat leaf rust, caused by *Puccinia triticina* Eriks, is the most damaging rust disease on wheat (*Triticum aestivum* L) (Kloppers and Tweer, 2010; Afzal et al., 2007) due to its wide distribution, ability to form new pathotypes that infect previously resistant cultivars, its potential to travel long distances, and its capacity to develop promptly under favorable conditions (Huerta-Espino et al., 2011; Wegulo and Byamukama, 2012). Under endemic environments, *P. triticina* has caused famines that have severely affected economies and communities (Dubin and Brennan, 2009). The most severe losses occur when the flag leaves become severely rusted prior to the flowering stage (Wegulo and Byamukama, 2012).

Managing wheat leaf rust is mainly dependent on chemical and cultural control strategies (Roelfs et al., 1992; Adam et al., 2000; Audsley et al., 2007), which can be

ineffective under conditions that favour rapid disease development (Hamdy et al., 2001). Injudicious application of synthetic chemicals to manage the disease can have negative consequences for animal and human health, as well as agroecosystem (Shabana et al., 2017). The development of resistance to fungicides by plant pathogens restricts the effectiveness of chemicals in managing the disease (Shabana et al., 2017). The development of new pathogen strains that infect resistant cultivars has resulted to inadequate control of the disease (Lowe et al., 2011).

Eco-friendly or biological control measures that directly or partially inhibit disease development have received considerable attention as alternative means to chemicals (Shabana et al., 2017). Fungal organisms including *Lecanicillium* spp. (Montalva et al., 2017), *Trichoderma* spp. (Naher et al., 2014) and *Bacillus* spp. (Ashwini and Srividya, 2014) have been considered as promising alternatives to synthetic fungicides. In a study by Sheroza et al (2003), wheat leaf rust was partially controlled with the combination of several bio-control agents: *Verticillium* sp., *Paecilomyces* sp., *Beauveria bassiana* V., *Cladosporium cladosporioides* V. and *Metarrhizium anisopliae* S., with the combination *Verticillium* sp and *Paecilomyces* sp substantially reducing the development of wheat leaf rust. This study aimed to evaluate *L. attenuatum* as a biological control agent of wheat leaf rust under greenhouse conditions.

3.2 Materials and methods

3.2.1 Planting wheat seeds and inoculating with *Puccinia triticina*

The study was conducted in the green house at the University of KwaZulu-Natal, Pietermaritzburg. A randomized complete block design was used for the experiment. A total of 16 pots were used as blocks. Four pots constituted one block and accommodated 12 plants. The four treatments were randomized in each block Wheat seed were grown in 18 cm pots. A total of 48 plants were planted in 16 pots, each pot had three wheat plants (Duzi cultivar). The pots were kept in the greenhouse at a temperature of approximately 26^{o}C and relative humidity of about 80%. Wheat leaf rust (*P. triticina*) inoculum was collected from the Pannar Seed research farm in Greytown. The rust infected leaves of wheat were placed in brown paper bags and refrigerated in -8^{o}C. All wheat plants were inoculated with *P. triticina* two weeks before

flowering stage. Inoculation was done by suspending infected leaf material in 2 liters of distilled water for 30 minutes. While the leaf infected material was suspended in water, lesions or pustules were scraped with a blade in order to harvest *P. triticina* spores. All 48 wheat plants were sprayed with the prepared wheat rust inoculum. The pots containing the plants were then covered with freezer bags for two days to ensure high relative humidity to enhance *P. triticina* infections on plant leaves. Leaf rust symptoms were observed to develop 7 - 10 days post-inoculation.

3.2.2 Supra-inoculation with *Lecanicillium attenuatum* on rust pustules of wheat leaf rust

Thirty potato dextrose agar (PDA) plates containing *L. attenuatum* colonies were placed under UV light to stimulate sporulation. The *L. attenuatum* cultures were mixed with 200 ml of non-sterile distilled water, and conidia were dislodged using an “L” shaped glass rod in order to harvest the conidia. The suspension was filtered through four layers of sterile cheesecloth to remove the mycelium. The number of conidia per ml of the filtrate was determined using a haemocytometer. From the prepared suspension three different doses of conidia of *L. attenuatum* were made. These were of 10^2, 10^4, and 10^6 conidia ml^{-1}. Each of the prepared doses of the biocontrol agent was applied to 12 wheat plants and other 12 plants were used as a control. The rust pustules infected by *L. attenuatum* were also viewed with scanning electron microscopy (SEM) in order to check the interaction between *L. attenuatum* and urediospores of *P. triticina*.

3.2.3 Sample preparation for viewing with SEM

Samples of *L. attenuatum* treated wheat leaves with *P. triticina* were taken to the Microscope and Micro-analysis Unit of UKZN for hyper-parasitism studies. Primary fixation was performed by sinking infected wheat leaf samples in a 3% buffered glutaraldehyde for one to three hours. Samples were then immersed in a sodium cacodylate buffer twice for five minutes. Samples were then dehydrated in a classified ethanol series (10%, 30%, 50%, 70%, 90%), and twice in 100% ethanol for 10 minutes. Specimens were transferred to a critical point drying unit (CPD) (Quorum Q150R K850, UK) under 100% ethanol, whereby the ethanol was substituted with a liquid CO_2 that was heated and pressurized to its critical point at which point the liquid changed to gas without the damaging effects of surface tension on the sample. This resulted in

dry and intact specimens that were mounted on copper stubs with double sided sticky tape. The specimen stubs were sputter coated with gold palladium. Three coated specimens were viewed at a time.

3.3 Disease rating, data collection and analysis

To evaluate *L. attenuatum* as a biological control agent of wheat leaf rust under greenhouse conditions, two data sets were collected 10 days after inoculation. The first was the level of rust infections measured as the percentage leaf area infected (L.A.I.). The second was the level of infection of the rust lesions colonized by *L. attenuatum*. The initial rust infection percentage relative to the leaf area was taken as 100% when rating the level of pustule colonization by *L. attenuatum,* with the infections being rated relative to the area of rust pustules invaded by *L. attenuatum*, and converted to percentage. The data was collected for each of the treatments (control, 10^2, 10^4, and 10^6 conidia ml^{-1}) and this was subjected to analysis of variance (ANOVA) using GENSTAT (18^{th} edition).

3.4 Results

3.4.1 Effects of different doses of *Lecanicillium attenuatum* conidia applied on diseased wheat plants

All three treatments significantly (P = 0.001) colonized rust pustules compared to the control (Table 3.1). The dose of 10^2 conidia ml^{-1} provided a colonization percentage of 52.8% on the rust pustules (65.2% L.A.I.), while 10^4 conidia ml^{-1} infected 50.4% of the rust pustules (with an L.A.I. level of 71.8%). The best control was achieved by the dose of 10^6 conidia ml^{-1}, which infected 81.6% of the rust pustules (with an L.A.I of 72.8%).

Table 3.1 Levels of rust pustule infection three *Lecanicillium attenuatum* conidial doses and a control applied to wheat plants infected with wheat leaf rust

Treatment (conidia ml^{-1})	Pustule infection by *L. attenuatum* (%)	LAI(%)
10^2	52.8 a	65.2
10^4	50.4 a	71.8
10^6	81.6 b	72.8
Control	5.5 c	76.5
F Test Values	23.27	1.04
P-Values	0.001	0.383
LSD values	18.57	13.07
CV %	47.47	22.2

3.4.2 Hyperparasitism between *Lecanicillium attenuatum* and *Puccinia triticina*

In the interaction studies under SEM germ tubes could be seen emerging from conidia of *L. attenuatum*, before penetrating urediospores of *P. triticina* (Figure 3.1). Infection holes can be seen on urediospores, where the infection peg has pulled away.

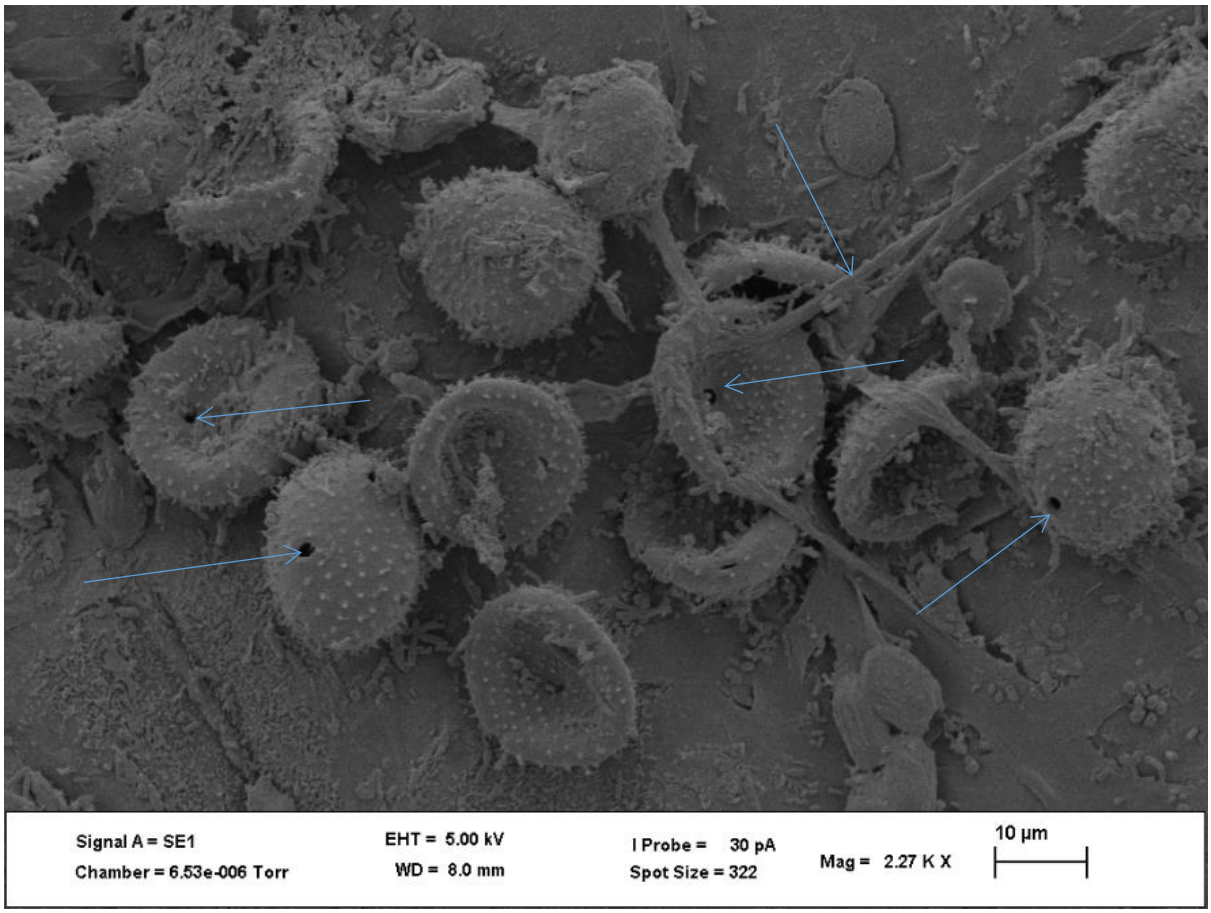

Figure 3.1 Invasion of *P. triticina* urediospores by germ tubes emerging from *L. attenuatum* conidia

3.5 Discussion

In this current study, conidia of *L. attenuatum* that had been supra-inoculated onto rust pustules directly infected the urediospores of *P. triticina*. This correlates well with the mechanism displayed by this fungus in killing the cypress aphid (*Cinara cupressi* B.) by penetrating the integuments of the aphid (Montalva et al 2017). When applied at a high dose of conidia (10^6 conidia ml^{-1}), *L attenuatum* colonized 81.6% of *P. triticina* pustules. In a similar study carried out in Brazil with coffee leaf rust, 40% of the rust pustule area was colonized by *Lecanicillium* spp. (Galvao and Bettiol, 2014). The level of conidia of *Lecanicillium* spp used as a biocontrol agent impacts upon its ability to control various diseases (Kim, 2007; Goettel et al., 2008; Machado et al., 2010; Ramezani et al., 2013 Sumalatha, 2014).

Infection of urediospores by *L. attenuatum* has been shown to result from the production of enzymes that degrade the cell walls of urediospores of *P. triticina* (Pal and Gardener, 2006; Lu et al., 2015). Upon adherence to the urediospores, the germinated *L. attenuatum* conidia formed germ tubes that penetrated the rust spores (Shinde et al., 2010). The production of extracellular enzymes, which included proteases, esterase, chitinase, N-acetylglucosamine, aminopeptidase, endoprotease, carboxypeptidase, Lipase and prl-chymoelastase serine protease, are required to allow the hyperparasite to penetrate the urediospores (Shinde et al., 2010).

Lecanicillium attenuatum infected the majority of rust pustules when supra-inoculated in high doses of conidia. Kim et al (2007) found that a high concentration of *L. attenuatum* conidia (10^9 ml^{-1}) controlled the development of cucumber powdery mildew. In a dose similar that used in this study, Romero et al (2007) found *Lecanicillium* spp. applied at 5 x 10^5 conidia ml^{-1} to control cucurbit powdery mildew on greenhouse-grown melon.

At a concentration of 10^6 conidia ml^{-1} the strain of *L. attenuatum* used in this study infected 81.6% of the rust pustules present, effectively reduced the development of *P. triticina*. At the two lower doses (10^2 and 10^4 conidia ml^{-1}) a lower level of colonization occurred (52.8 and 50.4%), and reduced the development of wheat leaf rust. This study confirmed the concept that *L. attenuatum* could be used as an applied biological control agent to manage wheat rust.

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CHAPTER FOUR

Field evaluation of *Lecanicillium attenuatum* as a biocontrol agent of wheat leaf rust

Abstract

This study tested the pathogenicity of *L. attenuatum* on wheat leaf rust in the field. Wheat plants in four seed beds were inoculated with *Puccinia triticina* prior to flowering, and the symptoms were observed after 10-14 days post-inoculation. Subsequently, *Lecanicillium attenuatum* was applied at 0, 10^2, 10^4 10^6 conidia ml^{-1} to each of 48 plants infected with wheat leaf rust as a supra-inoculation, totaling 192 applications. With all three biocontrol treatments the rust pustules were significantly colonized compared to the control. The concentration of 10^2 conidia ml^{-1} provided a colonization level of 51.6% of rust pustules (which covered 52.1% of the leaf area), while at 10^4 conidia ml^{-1} the hyperparasite colonized 60.5% of the pustules (at 60.7% leaf infected). The highest level of colonization of 77.3% was achieved by inoculation with 10^6 conidia ml^{-1} (73.2% leaves are infected with leaf rust).

Key words: *Lecanicillium attenuatum*, *Puccinia triticina*, Wheat

4.1 Introduction

Lecanicillium spp. have been mainly used as an entomopathogens of insects, diseases and nematodes. The biocontrol potential of this fungus has been identified for the control of fungal pathogens (Gurulingappa et al., 2011; Koike et al., 2011; Xie et al., 2015). Some *Lecanicillium* spp. have been developed as commercial biopesticides. These species use both hydrolytic enzymes and mechanical forces to infect kill pests (Pal and Gardener, 2006). They directly penetrate the integument of insects and the cell wall of fungi (Goettel et al., 2008; Vu et al., 2008; Mondal et al., 2016). A few *Lecanicillium* spp. that occur routinely in nature (include *L. attenuatum* and *L. longisporum* (R)) have been reported to suppress the growth of *Sphaerotheca fuliginea* S. and *Podosphaera fusca* (Fr) B. spore production, which is the causal organism of cucumber and cucurbit powdery mildew (Romero et al., 2007; Goettel et al., 2008; Kim and Kim 2008; Kim et al., 2008). Several studies have evaluated *Lecanicillium* spp., including that of Kim et al (2010), but not on wheat leaf rust.

The efficacy of *L. attenuatum* under field conditions when supra-inoculated on rust diseases is unknown. Studies on *Lecanicillium spp* have largely been conducted under controlled environments, such as in the study of Fadayivata et al (2014), where it was found that *L. longisporum* significantly reduced the population of aphids compared to the control.

Successful control of wheat leaf rust using an eco-friendly, and cheap products would be a breakthrough for wheat farmers in South Africa and other countries. This study therefore aimed to evaluate the effectiveness of *L. attenuatum* for the control of wheat leaf rust under field conditions.

4.2 Materials and Methods

4.2.1 Field layout

The study was conducted at the Ukulinga Research Farm, University of KwaZulu-Natal, Pietermaritzburg. A randomized complete blocks deign was used for the experiment. Four seedbeds were prepared, as blocks, each being 2m long and 1.4 meters wide. Each block accommodated four rows with 12 plants per row. The spacing between the planted seeds was 15cm in the row, and 40cm between the rows. Each row constituted a plot. The four treatments (three biocontrol and one control) were randomized in each block. To mitigate the effect of interplot interference when spraying each treatment row, all non-target plants in each plot were first covered with plastic before spraying was undertaken.

4.2.2. Inoculation with *Puccinia triticina*

All 192 wheat plants (Duzi cultivar) were inoculated with *P. triticina*. The inoculum was harvested from Pannar seed research farm in Greytown. Rust infected leaves of wheat were put in brown paper bags and kept at -8^{o}C until used. All wheat plants in the field were inoculated with *P. triticina* two weeks before flowering stage. Inoculation was performed by suspending infected leaf material in 4 liters of distilled water for one hour. The rust pustules were scraped with a blade in order to harvest *P. triticina* spores into the water. All 192 wheat plants in each seedbed were sprayed with the

urediospores in suspension. The inoculated plants were covered with freezer bags for 24 hours to provide high relative humidity to enhance *P. triticina* infections on the plant leaves. Rust symptoms were observed 10-14 days post-inoculation.

4.2.3 Supra-inoculation with *Lecanicillium attenuatum* on rust pustules on wheat leaf rust

Fifty cultures of *L. attenuatum* grown on PDA Petri plates were placed under UV light to enhance sporulation. The *L. attenuatum* cultures were mixed with 2.5 liters of non-sterile distilled water and conidia were dislodged using a bent glass rod to harvest the conidia. The suspension was filtered through four layers of sterile cheesecloth to remove the mycelium. The number of conidia ml^{-1} of the filtrate was determined using a haemocytometer. From the prepared suspension, three doses of conidia of *L. attenuatum* were made, these being 10^2, 10^4, and 10^6 conidia ml^{-1}. Each of the prepared doses were applied to 12 wheat plants per row, and three rows of wheat plants per seedbed were sprayed with each treatment while the fourth row was used as a control.

4.2.4 Disease rating, data collection and analysis

Disease severity was rated as a percentage of leaf area infected (L.A.I). The level of biocontrol by *L attenuatum* was rated as a percentage of infection of all the rust pustules present on each leaf. The initial rust infection relative to the leaf area was taken as 100% when rating the colonization percentage. The data was collected 10 days after inoculation from 48 plants for each treatment. The data for the treatments were subjected to analysis of variance (ANOVA) using GenStat 18^{th} Edition. Differences within treatment means were determined using Fisher's unprotected Least Significant Difference test at a 5% significance level ($P = 0.05$).

4.3 Results

4.3.1 Effects of different doses of conidia of *Lecanicillium attenuatum* applied on wheat plants infected with leaf rust

The three doses of *L. attenuatum* (10^2, 10^4, 10^6 conidia ml^{-1}) significantly ($P = 0.001$) colonized rust pustules or lesions development compared to the control (Table 4.1). The dose of 10^2 conidia ml^{-1} colonized 51.6% of the rust pustules (at 52.1% L.A.I.). At 10^4 conidia ml^{-1} 60.5% of the rust pustules were infected (60.7% L.A.I.). The highest

level of hyperparasitism was achieved by the dose of 10^6 conidia ml^{-1}, which resulted in 77.3% pustules being infected (73.2% L.A.I).

Table 4.1 Levels of infection of rust pustules by four doses of conidia of *Lecanicillium attenuatum* wheat plants infected with leaf rust

Treatment (conidia ml^{-1})	Infection of rust pustules by *L. attenuatum* (%)	Rust level (L.A.I.)(%)
10^2	51.6 a	52.1
10^4	60.5 a	60.7
10^6	77.3 b	73.2
Control	2.7 c	81.7
F test	79.80	5.83
P-Value	0.001	0.001
CV %	51.7.	40.2
LSD	10.00	10.03

L.A.I Leaf Area Infected

4.5 Discussion

Various *Lecanicillium* spp. have been used as biopesticides, suppressors of nematodes and biofungicides of powdery mildew fungi (Kim, 2007; Kim et al., 2007; Chirivi-Salomon et al., 2015). This study showed that selected strains of *L. attenuatum* can be used as a biological control agent of wheat leaf rust caused by *P. triticina*.

Montalva et al (2017), Ownley et al (2010) demonstrated that *L. attenuatum* could control a cypress aphid (*Cinara cupressi* B) population. Their study microscopically displayed *L. attenuatum* employing mechanical forces to penetrate cypress aphid's integument causing death of the infected population. The mechanism employed is similar when this entomopathogenic fungus antagonistically controls the development of *P. triticina* under field and green house conditions.

The conidial dose plays an important role in the level of colonization of pustules of urediospores, with the highest colonization of rust pustules resulting from the dose of

10^6 conidia ml^{-1}. These results compares well with the study by Kim and Roberts (2012), which investigated the effect of conidial dose, moulting and insect development stages on cotton aphid susceptibility, and found that the insects' population was significantly reduced after the application of a high conidial dose.

There is wide range of organisms that *L. attenuatum* can infect, which includes insects and some powdery mildew pathogens, in addition this species (*L. attenuatum*) can also be used to control wheat leaf rust (Brodeur, 2012). The results of this study serve as advancement of controlling rust pathogens through the utilization *L. attenuatum*, this is supported by the work of Galvao and Bettiol (2014) whereby *Lecanicillium* spp suppressed the development of coffee leaf rust. The results of this study also confirm that the pathogenicity of *L. attenuatum* is viable to control wheat leaf rust under field conditions. *L attenuatum* could be used to supplement or replace triazole and strobilurin fungicides, without impacting on non-target organisms (Bye and Charnley, 2008), in that it only attacks targeted organisms.

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Chapter Five

Thesis overview of the major findings and their implications

5.1 Introduction

Wheat leaf rust caused by *Puccinia triticina* is of the most devastating disease on wheat. This is because of its wide distribution, ability to form new races that infect previously resistant cultivars, its capacity to travel long distances, and its capacity to develop quickly under favorable conditions. Management of *P. triticina* is currently on chemical and cultural control strategies. Under rapid disease development, these strategies provide inadequate control of the disease. The development of resistance to fungicides by plant pathogens restricts the effectiveness of fungicides in managing the disease in the long term. Therefore finding an alternative control strategy is essential for the future management of rust diseases of wheat.

The aims of this study were to isolate *Lecanicillium attenuatum* strains; evaluate their capacity to parasitize pustules of wheat leaf rust; and estimate the levels of biocontrol of severe rust infection that *L. attenuatum* could provide.

The specific objectives were as follows:

1. To conduct a literature review on *P. triticina:* the causal organism, its host range, dissemination, symptoms, infection process, epidemiology, and management strategies;
2. To isolate and evaluate the potential of *L. attenuatum* as biocontrol agent of rust using *Oxalis lateralis* plants as proxy for commercial crops;
3. To evaluate *L. attenuatum* as a biological control agent of wheat leaf rust under greenhouse conditions;
4. To evaluate *L. attenuatum* as a biological control agent of wheat leaf rust under field conditions.

Chapter 2: To isolate and evaluate the potential of *Lecanicillium* spp. as a biocontrol agent of rust diseases using the *Oxalis lateralis* / *Puccinia oxalidis* pathosystem as a proxy for the wheat / leaf rust pathosystem

Major findings:

- *Lecanicillium* sp. was successfully isolated from *O. lateralis* plants. The fungus involved was identified as *L. attenuatum* with the gene bank accession number AB378513.1.
- *L. attenuatum* controlled the oxalis rust caused by *P. oxalidis* on *O. lateralis* plants.
- Under the SEM the mycelia of *L. attenuatum* was observed to directly penetrate the urediospores of *P. oxalidis.*
- The dose of 10^6 conidia ml^{-1} was the most effective for the colonization of rust pustules of *P. oxalidis.*

Implications:

L. attenuatum could be used as a bio-pesticide of rust fungi. This suggests that this fungus should be tested against the rust diseases of commercial crops. *L. attenuatum* is the natural parasite of *P. oxalidis,* as it occurred without being inoculated, prior to isolation.

Chapter 3: Evaluation of *Lecanicillium attenuatum* as a biocontrol agent of wheat leaf rust under greenhouse conditions

Major findings:

- *L. attenuatum* greatly reduced the development of wheat leaf rust caused by *P. triticina*.
- *L. attenuatum* directly infected rust pustules
- There was a considerable difference in the levels of rust colonization by *L. attenuatum* with doses of conidia ranging between 10^2 and 10^6 conidia ml^{-1}.
- The dose of 10^6 conidia ml^{-1} caused the highest level of pustule colonization.

Implications:

This study creates a breakthrough in the combating of wheat rust. It showed that *L attenuatum* can be used as an alternative to synthetic chemicals for the management of wheat leaf rust. However, further studies are required to test the biocontrol agent in the field, and against the three wheat rust diseases.

Chapter 4: To evaluate *L. attenuatum* as a biocontrol agent of wheat leaf rust in the field

Major findings:

- Three doses of conidia of *L. attenuatum* were applied on wheat leaf rust, and were assessed against an untreated control. All three doses resulted in high levels of colonization of rust pustules compared to the control.
- The highest level of rust infection due to the biocontrol agent was achieved by the dose of 10^6 conidia ml^{-1}, which was 77.3% infection of the rust infection (73.2% L.A.I.).

Implications:

The highest dose of conidia (10^6 conidia ml^{-1}) provided the best control of wheat rust; therefore further studies with the application of higher doses are required.