Evaluation of Mycoparasitic Fungi as Potential Biological Control Agents of Wattle Rust (*Uromycladium acaciae*)

By

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DISSERTATION SUMMARY

Uromycladium acaciae (Cooke) P. Syd. & Syd., the causative agent of wattle rust, is an economically significant pathogen affecting black wattle (*Acacia mearnsii* De Wild) in the Republic of South Africa (RSA). Initial symptoms are brown powdery pustules on petioles, stems, and leaves which further develop into matting of leaves and pinnules, and rachis malformation. In severe cases, infection results in stunting of seedlings and mortality of young trees. Yield losses of up to 40% have been reported. The current control measures for wattle rust consist of azoxystrobin and triazole-based fungicides and the deployment of resistant or tolerant lines of *A. mearnsii.* However, due to undesirable environmental effects posed by fungicides, the substantial costs involved with both fungicide usage and the deployment of tolerant clones of *A. mearnsii* and increasing regulations around the use of fungicides in commercial forestry, there is a need for alternative control methods for wattle rust.

The use of biological control presents a cost-effective and environmentally friendly alternative method for the management of wattle rust. Several mycoparasites of various rust diseases have been evaluated on different rust diseases as potential biological control agents. In this study, the mycoparasites *Sphaerellopsis filum* (Biv.-Bern. ex Fr.) Sutton [=*Darluca filum* (Biv.-Bern. ex Fr.) Berk.] and *Akanthomyces uredinophilus* Manfrino et al. were evaluated for biocontrol efficacy on wattle rust using an *in vitro* detached leaf assay. Leaves of black wattle infected with *U. acaciae* were collected from a local commercial plantation in Richmond, KwaZulu-Natal, South Africa. Rust-infected leaves were sprayed with conidial suspensions of 1 $g.L^{-1}$ of *S. filum* ($5.8x10^{8}$ $conidia.ml^{-1}$) and *A. uredinophilus* ($1x10^{6}$ $conidia.ml^{-1}$) and incubated in plastic containers containing moist paper towels to create conditions of high humidity. Colonisation of rust pustules by *A. uredinophilus* and *S. filum* was assessed under a light microscope and a percentage colonisation for each treatment was estimated at four weeks post-inoculation. Colonised leaves were also viewed under an environmental scanning electron microscope (ESEM) to examine the interaction between the mycoparasitic fungi and *U. acaciae*. At four weeks post inoculation, *A. uredinophilus* and *S. filum* were able to colonise 41% and 44% of rust pustules,

respectively. Under the ESEM, *S. filum* and *A. uredinophilus* demonstrated mycoparasitic activity on the teliospores of *U. acaciae*. Hyphae of *S. filum* and *A. uredinophilus* coiled around and punctured rust teliospores, causing them to rupture and deflate. Overall, conidia of both *S. filum* and *A. uredinophilus* were able to colonise and mycoparasitize *U. acaciae* teliospores.

Optimal Integrated Pest Management (IPM) for disease suppression requires the seamless and coordinated integration of multiple compatible control strategies. Therefore, any biological agents that may be considered for use to suppress a disease should be compatible with the range of agrochemicals and adjuvants that are being used on the crop of interest, in order for the management strategy to be effective. To this end, the compatibility of two isolates of mycoparasitic fungi (*Sphaerellopsis filum* and *Akanthomyces uredinophilus*) and a commercial insect-pathogenic fungus (*Beauveria bassiana,* Eco-Bb®) with an insecticide (deltamethrin) and an adjuvant (Break-thru®) was evaluated. The experiment utilized an amended bioassay method with *S. filum*, *A. uredinophilus*, and *B. bassiana* being cultured on potato dextrose agar (PDA) amended with 250 $\mu l.L^{-1}$, 125 $\mu l.L^{-1}$, and 500 $\mu l.L^{-1}$ of Break-thru® , or amended with 1 $ml.L^{-1}$, 0.5 $ml.L^{-1}$ and 2 $ml.L^{-1}$ of deltamethrin. Unamended media served as a negative control, whilst media amended with the commercial rust fungicide Amistar Top® served as a positive control. After three weeks, radial growth was measured from the centre of the fungal colony, and a percentage inhibition efficiency (IE) of Break-thru®/ deltamethrin on the growth of the biological control agent was calculated. There was no significant inhibition in any of the biological agents by either Break-thru® or deltamethrin at all dosages tested. Therefore, *S. filum, A. uredinophilus* and *B. bassiana* are compatible with the insecticide (deltamethrin) and the adjuvant (Break-thru®), and they could be combined in an integrated control program.

Further evaluation of the two mycoparasitic fungi as potential biological control agents of wattle rust were undertaken in nursery pot trials. Pot Trial One investigated the biological control efficacy of mycoparasitic strains of *S. filum* and *A. uredinophilus* for control of wattle rust on three wattle varieties. Rust-infected cuttings of varieties PSO14, FW54 and SP644 were treated with *S. filum* and *A. uredinophilus* every two

weeks for 12 weeks. They were also evaluated for rust levels every two weeks, as percentage leaf area infected. Experimental controls included a fungicide control (positive control) and untreated rust control (negative control). Bi-weekly applications of *S. filum* significantly reduced ($p < 0.001$) rust disease progression. Although applications with *A. uredinophilus* did reduce disease progression, it did not produce a significant reduction compared to the untreated rust control. After six and nine weeks of bi-weekly treatment applications, characteristic white mycelial growth of *A. uredinophilus* and *S. filum,* respectively, were observed on rust pustules. Characteristic spore morphology that resembled that of S. *filum* and *A. uredinophilus* was confirmed after re-isolation. There was no significant difference ($p = 0.09605$) between the wattle varieties in their response to the treatments. Overall, both *S. filum* and *A. uredinophilus* have the potential to control wattle rust.

In Pot Trial Two, the optimal dosage and application frequency of *S. filum* and *A. uredinophilus* was evaluated on the FW54 (susceptible, frost tolerant) commercial wattle variety. Conidial suspensions of the mycoparasitic strains were prepared at dosages of $\frac{1}{2}$x, 1x and 2x at a quantity of $5x10^5$ conidia.ml^{-1}, $1x10^6$ conidia.ml^{-1}, and $2x10^6$ conidia.ml^{-1} of *A. uredinophilus*, and 0.5 gram, 1 gram and 2 gram.L^{-1} of *S. filum* (5.8×10^8 conidia.ml^{-1}). The conidial suspensions were sprayed at three frequencies which were once-off, bi-weekly (every two weeks), and monthly. Prior to every bi-weekly spray treatment, percentage rust severity (0 to 100%) was scored for all treatment frequencies on the fully expanded leaves directly below the new flush. The percentage rust severity was used to calculate the area under disease progress curve (AUDPC). For *S. filum* and *A. uredinophilus*, dosage and frequency of application had no significant interaction. All treatments reduced wattle rust relative to the untreated control treatment. Notably, the bi-weekly treatment with a 2x dosage of *A. uredinophilus* resulted in the least amount of rust disease, which was not significantly different to that of the fungicide treated control.

In Pot Trial Three, the use of silicon fertilization in conjunction with *A. uredinophilus* (the best performing biocontrol treatment as identified in Pot Trial Two) for optimal control of wattle rust was evaluated. A spray application of $2x10^6$ conidia.ml^{-1} of *A.*

uredinophilus was combined with silicon fertilization at concentrations of 100, 200, and 400 mg.L^{-1} for evaluation of the control of wattle rust on the commercial wattle variety FW54. The *A. uredinophilus* spore suspension was sprayed bi-weekly (every two weeks), and treated seedlings were drenched every week with 10L of water containing silicon at 100, 200, and 400 mg L^{-1}. Prior to every bi-weekly *A. uredinophilus* spray treatment, percentage rust severity (0 to 100%) was scored on the fully expanded leaves directly below the new flush. Experimental control treatments included a fungicide control (positive control, Amistar® Top, Syngenta) and an untreated rust control (negative control). At 16 weeks, leaf samples were taken from seedlings to evaluate the uptake and distribution of silicon using Field Emission Gun-Environmental Scanning Electron Microscopy (FEG-ESEM) and Energy Dispersive X-ray Spectroscopy (EDX). All treatments reduced wattle rust relative to the untreated control treatment. Treatment with *A. uredinophilus* + 400mg.L^{-1}, silicon resulted in the least amount of rust disease. However, the *A. uredinophilus* + 0mg.L^{-1} silicon treatment also produced a significant reduction in rust. The EDX spectra and mapping showed that silicon was not taken up by treated plants. The combined application of *A. uredinophilus* and silicon fertilization did not result in the enhanced control of wattle rust because wattle appears not to take up silicon as a micronutrient.

The overall results of this study suggest that both mycoparasites can be used as biological control agents for wattle rust. Bi-weekly applications of *A. uredinophilus* at 2×10^6 conidia.ml^{-1} was the best performing biocontrol treatment in these pot trials. However, it is unknown whether the nursery application of *A. uredinophilus* would yield sufficient fungal inoculum to control wattle rust under field conditions. Therefore, field trials should be implemented to establish the biocontrol efficacy of *S. filum* and *A. uredinophilus* under field conditions.

DECLARATION

I, Nonkululeko Poswa, 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:
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I dedicate this dissertation to the following people:

My mother Zibuyile Poswa,

My father Sandile Poswa,

My sister Mbalenhle Poswa, and two brothers Sbongakonke and Kwanele Poswa

Thank you, family, for believing in me.

INTRODUCTION

Plantation forestry contributes 1% to the gross domestic product (GDP) of South Africa (SA). There are three main genera of forest trees that are planted in South Africa, namely, *Pinus* (48.6%), *Eucalyptus* (44.2%), and *Acacia* (6.8%) (DFFE, 2020). The two main *Acacia* species are *Acacia decurrens* J.C. Wendl. Willd (green wattle) and *Acacia mearnsii* De Wild (black wattle). Both species are planted on over 100,000 ha, predominantly in the KwaZulu-Natal, Mpumalanga, and Limpopo Provinces (McTaggart *et al*., 2015). Black wattle is a species of commercial significance and is harvested for its bark and timber products (Beck-Pay *et al*., 2023). It is currently planted in 110 000 ha of which 86 000 ha (78%), 600 ha (4%), and 2000 ha (18%) are owned by commercial farmers, corporate growers and small-scale growers, respectively (Chan *et al*., 2015, Little and Payn, 2016). Black wattle is susceptible to several economically significant pests and diseases, including wattle mirid (*Lygidolon laevigatum* Reut.), wattle bagworm (*Kotochalia junodi* Hyalaerts) and wattle rust (*Uromycladium acaciae* (Cooke) P. Syd. & Syd.) (Payn and Little, 2017, Chan and Isik, 2019). Wattle rust was initially observed in 2013 in the KwaZulu-Natal Midlands region and is now considered a major epidemic of wattle plantations causing long-term economic losses (Chan and Isik, 2019, Oumar *et al*., 2019).

The use of fungicides and rust tolerant/resistant clones are the two primary control methods used to control wattle rust (Fraser *et al*., 2019). However, fungicides are not desirable due to their impact on the environment, and the potential for fungicide resistance due the need for repeated applications. Moreover, the use of fungicides has been restricted by international and local certification schemes. Therefore, there is a critical need for other environmentally sustainable and cost-effective control methods for the management of wattle rust.

Biological control agents have frequently been evaluated for the control of various diseases as alternative methods to chemical pesticides (Havugimana, 2017, Nxumalo, 2018). *Sphaerellopsis* spp. and *Akanthomyces* spp. are known mycoparasites of rust fungi (Gordon and Pfender, 2012, Trakunyingcharoen *et al*., 2014, Nxumalo, 2018).

Sphaerellopsis filum (Biv.-Bern. ex Fr.) Sutton [=*Darluca filum* (Biv.-Bern. ex Fr.) Berk.] was confirmed as a hyperparasite of *Puccinia recondita* Rob. ex. Desm. f. sp. *tritici* Eriks. (wheat leaf rust) and *Puccinia graminis* Persoon (stem rust of cereals) (Plachecka, 2005). Fraser *et al*. (2021) also identified *Sphaerellopsis macroconidialis* as a hyperparasite of *U. acaciae*. An *Akanthomyces muscarium* Zare et Gams strain was found to reduce soybean rust (*Phakopsora pachyrhizi* Syd. & P. Syd.) uredinospores by 89% (Havugimana, 2017). Nxumalo (2018) also found that 10^6 conidia.ml^{-1} of *Akanthomyces attenuatum* Zare and Gams strain colonised 77.3% of wheat rust (*Puccinia triticina* Eriks.). Therefore, this study aimed to evaluate mycoparasitic fungi *Sphaerellopsis filum* (Biv.-Bern. ex Fr.) Sutton [=*Darluca filum* (Biv.-Bern. ex Fr.) Berk.] and *Akanthomyces uredinophilus* Manfrino et al., pers. comm. Mwevelasi as potential biological control agents of wattle rust (*U. acaciae*).

The study objectives were to:

1. Review the available literature on *U. acaciae*, to understand its epidemiology as a disease of black wattle, and to review the use of biological control agents as a management strategy for rusts in general and wattle rust in particular.
2. Evaluate the ability of the mycoparasites to colonise and mycoparasitise *U. acaciae* using a detached leaf assay and electron microscopy.
3. Evaluate the compatibility of the mycoparasitic fungi (*Sphaerellopsis filum* and *Akanthomyces uredinophilus*) and a commercial insect-pathogenic fungus (*Beauveria bassiana*, Eco-Bb®) with an insecticide (deltamethrin) and an adjuvant (Break-thru®).
4. Evaluate the biocontrol efficacy of mycoparasitic fungi (*Sphaerellopsis filum* and *Akanthomyces uredinophilus*) on three wattle varieties with varying levels of susceptibility to wattle rust *in vivo* (Pot Trial One).
5. Determine the optimal dosage and application frequency of the mycoparasitic fungi (*Sphaerellopsis filum* and *Akanthomyces uredinophilus*) for control of wattle rust *in vivo* (Pot Trial Two).
6. Evaluate the best performing treatment from Pot Trial Two (*Akanthomyces uredinophilus*, applied bi-weekly at $2x10^6$ conidia.ml^{-1}) in combination with silicon fertilization for optimal control of wattle rust *in vivo* (Pot Trial three).

This dissertation consists of seven chapters with Chapters 1 to 6 addressing a specific objective. Chapters 4, 5 and 6 are presented as series chapters. Chapter 7 provides a summary of the main findings, their implications, and recommendations for future studies.

This dissertation is structured in a standard format adopted by the University of KwaZulu-Natal, because it facilitates the publication of the chapters of the dissertation in scientific journals more easily than the older, monograph format. Thus, repetition of references, and some information in the Materials and Methods sections will be encountered between the chapters.

This research was undertaken at the Institute for Commercial Forestry Research (ICFR) in collaboration with the Discipline of Plant Pathology, at the University of KwaZulu-Natal, Pietermaritzburg Campus under the supervision of Prof. Mark Laing (UKZN) and Dr Benice Sivparsad (ICFR).

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CHAPTER 1: LITERATURE REVIEW

1.1 Abstract

South Africa's forestry plantation sector includes *Acacia decurrens* (J.C. Wendl.) Willd. (green wattle) and *Acacia mearnsii* De Wild (black wattle), which are planted on over 100,000 ha, predominantly in the KwaZulu-Natal, Mpumalanga, and Limpopo Provinces. Black wattle is a plantation species of economic importance and is used to produce hardwood, pulpwood, charcoal, tannin extract, and fluorescent. In the Republic of South Africa, wattle rust (*Uromycladium acaciae (*Cooke) P. Syd. & Syd.) is considered to be the most important disease threatening wattle production. Wattle rust was discovered in black wattle plantations during the 2013 growing season in KwaZulu-Natal Midlands region. Initial symptoms consist of brown powdery pustules on petioles, stems, and leaves. Symptoms progress into matting of leaves, pinnule and rachis malformation, and can potentially reduce production by 20–40% in one growing season. Fungicides (azoxystrobin and difenoconazole) and the use of tolerant and resistant *A. mearnsii* cultivars are the current options used for the management of wattle rust. However, a change of approach to pesticide usage by international forestry certification schemes such as the Forest Stewardship Council ® (FSC®) has restricted the use of fungicides. Therefore, there is an urgent need for the identification and development of alternative control products to be used as part of an integrated pest management strategy for wattle rust. The use of biological control agents would be advantageous because they are environmentally safe and long-lasting. This review aims to: (1) provide an overview of *A. mearnsii* (black wattle) production in RSA; (2) highlight the significance of *Uromycladium acaciae (*Cooke) P. Syd. & Syd. (wattle rust) as a disease of black wattle; and (3) provide a synopsis of literature where the mycoparasitic fungi *Sphaerellopsis filum* (Biv.-Bern. ex Fr.) Sutton [=*Darluca filum* (Biv.-Bern. ex Fr.) Berk.] and *Akanthomyces uredinophilus* Manfrino et al. have been used in the control of rust diseases.

1.2 Introduction

The Republic of South Africa (RSA) has approximately 1,265,811 ha of planted commercial forestry species in the Mpumalanga, Limpopo, KwaZulu-Natal, Western Cape and Eastern Cape Provinces (FSA, 2015). The forestry industry in RSA contributes 1% of the national gross domestic product and 1.4% of the total formal employment, where it provides employment to 111 600 people: 67,500 are in formal employment, 30,000 are contract workers and 39,500 are small growers (DAFF, 2017).

Three genera are grown commercially in SA: *Pinus* species, *Eucalyptus* species and *Acacia* species (FSA, 2015). *Acacia* species, commonly referred to wattle, are native to Australia (Welgama *et al*., 2019). Wattle are excellent colonisers or pioneer plants because they grow easily from seed (de Neergaard *et al*., 2005). *Acacia mearnsii* De Wild (black wattle), *Acacia dealbata* (L.) Willd (silver wattle), *Acacia longifolia* (Andrews) Willd (Sydney golden wattle) and *Acacia decurrens* (J.C. Wendl.) Willd (green wattle) are amongst the *Acacia* species introduced in South Africa (de Neergaard *et al*., 2005; McTaggart *et al*., 2015). Wattle species were brought across from Australia for several reasons, including the supply of fuel, timber, and for stabilizing the soil (Aitken *et al*., 2009).

Black wattle is a commercially valuable tree species for plantations, as well as a socially important tree species in RSA rural communities because of its various beneficial uses such as heating, cooking and building materials (De Beer, 1986). The black wattle tree has a wide range of applications, including specialized hardwood, pulpwood, charcoal, tannin extract, and fluorescent dyes (De Beer, 1986). Tannin extracted from black wattle bark is used all over the world for tanning leather. The extensive planting of black wattle in commercial plantations in KwaZulu-Natal led to the development of a significant and successful export industry (De Beer, 1986).

There are a variety of fungal pathogens that cause diseases on black wattle. These include: *Uromycladium acaciae* (Cooke) P. Syd. & Syd., *Ceratocystis albifundus* M.J. Wingf. & Crous, *Botryosphaeriaceae canker* J. de Wet & M.J. Wingf, *Phytophthora* rot and *Phytophthora cinnamomi* Rands. *U. acaciae* (wattle rust) was identified in 2013 and is the most recent pathogen affecting black wattle in RSA (Fraser *et al*., 2017).

When wattle rust spread throughout the wattle industry by 2014, it reduced potential production by 20–40% during one growing season (Peerbhay *et al*., 2022). Fungicides and the production of rust-tolerant clones are two current control options used to manage wattle rust (Little and Payn, 2016; Fraser *et al*., 2019). However, forest certification bodies such the Forest Stewardship Council (FSC) have restricted the use of pesticides and mandate the need for alternative environmentally sustainable approaches to pest and disease management (Forest Stewardship Council, 2018). Several pesticides, including propizole, which contains propiconazole, the active ingredient against wattle rust, as well as cyproconazole have been banned from usage since June 1, 2024 (Meyer, 2023).

Mycoparasitic fungi such *Sphaerellopsis filum* (Biv.-Bern. ex Fr.) Sutton [=*Darluca filum* (Biv.-Bern. ex Fr.) Berk.], *Trichoderma* species and *Akanthomyces* spp. could be used as biological control alternatives that can form part of a broader integrated management strategy for wattle rust (Trakunyingcharoon *et al*., 2014). The purpose of this review is to highlight the significance of *U. acaciae* (wattle rust) as a disease of *A. mearnsii* (black wattle) and to evaluate the potential of mycoparasitic fungi, such as *S. filum and Akanthomyces* species as potential biological control agents for wattle rust.

1.3 Wattle plantations in the Republic of South Africa

The Republic of South African mining sector expanded in the second part of the 19th century, which led to an increase in demand for timber. Experimental plantations of wattle and eucalypts were established due to the scarcity of local timber (Aitken *et al*., 2009). Eucalypt species were imported from Australia for use as timber, paper, and poles (Hirsch *et al*., 2020). In 1858, black wattle trees were planted as shade trees, and used as a source of firewood until the high tannin of its bark was discovered. Black wattle was then planted extensively in commercial plantations in KwaZulu-Natal and its production quickly developed into the core of a significant and successful export industry (De Beer, 1986). In 1978, black wattle planting declined to cover about 16 000 ha, or about 15% of the total plantation area of all tree species in the Republic of South Africa (De Beer, 1986).

Today, timber plantations cover 1.2 million hectares across five of the country's nine provinces. Pine (49%) and eucalypts (43%) are the two most common commercial tree genera, followed by a smaller quantity of wattle (7%) (Forestry South Africa, 2020). The forestry value chain provides over 158 000 jobs and adds R69 billion to our country's economy annually (Forestry South Africa, 2020).

Acacia species grown by the plantation forestry sector include *A. decurrens* (green wattle) and *A. mearnsii* (black wattle), which are planted on over 100,000 ha, predominantly in the KwaZulu-Natal, Mpumalanga, and Limpopo Provinces (McTaggart *et al*., 2015). Plantation-grown *A. mearnsii* wood is utilised commercially in the RSA largely for the manufacturing of kraft and soda anthraquinone paper pulp (McTaggart et al., 2015).

Black wattle extraction, harvesting, and export of its bark and bark extract constitute activities of major economic importance, where the wood produced is used in paper pulp, fuel or mining timber (Stephens and Goldschmidt, 1939; Brown and Ko, 1997). The bark extracts of black wattle are used to produce tanbark liquor for the leather industry (Aitken et al., 2009). Due to the limited demand by the local pulp and paper industry in the 1970s, RSA began exporting black wattle woodchips (Chan *et al*., 2015). The RSA exports *A. mearnsii* woodchips to Japan, where they are mostly used to make kraft pulp (Fraser *et al.,* 2017) and recently to China for bleached hardwood kraft pulp (Chan *et al*., 2015).

Acacia mearnsii (black wattle) is also used for charcoal manufacturing for soil reclamation (Oumar, 2020). Furthermore, there are many unmanaged wattle stands and woodlots that are used by rural communities as a source of wood for heating and cooking, as well as for building materials. Therefore, *A. mearnsii* is a commercially valuable tree species for plantations, as well as a socially important tree species in RSA rural communities (Oumar *et al*., 2019).

The seed of green wattle is used to make a green dye, while the blossoms are used to make yellow dye (Health Benefit Times, 2021). It also has been grown as a windbreak, a firewood source, and a shelter tree (Doran *et al*., 1997). The prevailing assumption is that green wattle is simpler to maintain than black wattle, particularly in challenging environments (Philp and Sherry, 1946). Green wattle is bagworm tolerant, grows faster on poor sites, and is quicker to recover from hail, drought, and insect damage (Philp and Sherry, 1946). It is also tolerant to frost, and is rust resistant (Chan *et al.*, 2015). Green wattle has kraft pulp yields comparable to commercial pulpwoods, along with low bulk, strong bursting strength, and breaking length, making it appropriate for fine paper end product such as writing and printing papers (Clark *et al*., 1992). However, the tannins extracted from green wattle have a low tanning quality (Moffett, 1956) because it is reddish, which is considered to decrease the value of leather (Luyt and Gwaze, 1987).

From these *Acacia* species, an income of 15% is generated from the bark and 85% from the timber (Little and Payn, 2016). Bark extract and wood chip exports are projected to be valued more than 150 million USD per year, with the business employing 36,000 people. Each year RSA generates approximately 45,000 tons of bark extract (Fraser *et al.,* 2017). The proportional contribution of wattle to the overall RSA hardwood chip exports increased from 36% in 2008 to 66% in 2013 as a result of a consistent decline in the exports of eucalypt woodchips (Chan *et al*., 2015).

Pest and pathogen outbreaks have gradually developed in the forestry sector as natural destructive agents in plantation forestry (Peerbhay *et al*., 2022). In RSA, the brown wattle mirid (*Lygidolon laevigatum* Reut.), wattle bagworm (*Kotochalia junodi* Hyalaerts), and different lappet moths of the Lasiocampidae family are the most frequent pests of black wattle (Payn and Little, 2017). Black wattle is frequently affected by *Ceratocystis albofundus* M.J. Wingfield, De Beer & M.J. Morris, as well as several *Phytophthora* and *Botryosphaeriaceae* species (Payn and Little, 2017).

The rust disease *Uromycladium acaciae (*Cooke) P. Syd. & Syd. was discovered in a black wattle plantation during the 2013 growing season in the KwaZulu-Natal Midlands region (Oumar *et al*., 2019). It is now considered to be an epidemic disease on black wattle in the RSA (Fraser *et al*., 2021a, Fraser *et al*., 2021b) and can reduce potential production by 20-40% during one growing season (Peerbhay *et al*., 2022). The disease affects wattle of all age classes, stunts growth, and in severe cases results in mortality. The damage is greatest in trees at ages 0-2 years due to defoliation, which inhibits initial growth and has an impact on stand management (Chan and Isik, 2019). Dual infection of wattle rust and the wattle mirid causes defoliation and deformation in freshly produced shoots or leaves during the first two years after establishment, causing extreme damage in some areas (Chan and Isik, 2019).

Furthermore, the *Melanterius* weevil (also known as stem borer) has recently been identified in the KwaZulu-Natal Midlands as a novel pest affecting black wattle plantation (South African Forestry, 2024). Its symptoms involve small perforations on the shoots and twigs causing swelling around the entry holes due to larval growth and feeding. These symptoms develop to shoot cracking, yellowing and eventually dieback of branches (South African Forestry, 2024). Another new pest in Melmoth, KwaZulu-Natal, has been found attacking black wattle (Slippers and Hurley, 2024). The cause of the damage was identified as larvae of a wood-boring beetle from the Buprestidae family, also known as flatheaded borers or jewel beetles (Slippers and Hurley, 2024). These beetles typically target stressed or dead trees, but some can infest trees that also seem to be healthy. They lay eggs on the outer tissue of plants, such as in the cracks in bark, and the larvae tunnel into the inner bark and outer sapwood (Slippers and Hurley, 2024). This infestation can result in tree mortality. However, trees with strong immunity fight back by producing resin (Slippers and Hurley, 2024). Further investigation to confirm the identification and to determine the distribution and impact of both these new insect pests of black wattle is ongoing (Slippers and Hurley, 2024).

In addition to pests and diseases, the sustainable production of black wattle is also threatened by frost and snow (Gujrathi and Babu, 2007). The sensitivity of black wattle to frost damage and insect attack, as well as poor soil quality and low and irregular rainfall, are the primary causes for poor black wattle cultivation (Gujrathi and Babu, 2007). To manage these risks, the RSA black wattle breeding program was established to develop and release frost and rust-tolerant cultivars. The black wattle breeding program has been running for more than 70 years (Chan and Isik, 2019). Incorporating germplasm from cultivars of Australian origin that are cold hardy into the frost-susceptible populations has been shown to improve the genetic variation for frost tolerance in wattle plantations (Chan and Isik, 2021).

1.4 *Uromycladium acaciae* (wattle rust)

1.4.1 History and taxonomy

Uromycladium acaciae was first discovered in New Zealand on an Australian *Acacia* species and named as *Uredo acaciae* by Cooke in 1890 (Cooke, 1890, McTaggart *et* *al*., 2015, Doungsa-Ard *et al.*, 2018). In 1905, McAlpine established a genus *Uromycladium* for rust fungi on *Acacia* based on the arrangement and number of teliospores and vesicles on the pedicels (McAlpine, 1905, McTaggart et al., 2015). In 1915, Sydow and Sydow transferred *Uredo acaciae* Cooke to *Uromycladium acaciae* (McTaggart *et al*., 2015, Doungsa-Ard *et al*., 2018). *Uromycladium acaciae* (Cooke) P. Syd. & Syd. first appeared in RSA in the mid-1980s in its uredinial form, but no significant damage was observed on *A. mearnsii* (Morris *et al*., 1988, Fraser *et al*., 2017). In 1988, when the uredinial stage of *U. acaciae* on *A. mearnsii* in RSA caused mild symptoms on the leaflet, it was initially identified as *U. alpinum*. However, the morphology of the teliospores and host range matched *U. acaciae* rather than *U. alpinum* (McTaggart *et al*., 2015). Infections induced by telia and spermogonia of *U. acaciae*, which resulted in serious infection in *A. mearnsii* were not detected in the RSA until 2013 (Fraser *et al*., 2017).

The taxonomy of *Uromycladium acaciae* is described as:

Domain: Eukaryota
Kingdom: Fungi
Phylum: Basidiomycota
Subphylum: Pucciniomycotina
Class: Pucciniomycetes
Order: Pucciniales
Family: Pileolariaceae
Genus: *Uromycladium*
Species: *Uromycladium acaciae*
Common Name: Wattle rust
Scientific Name: *Uromycladium acaciae* (Cooke) P. Syd. & Syd.
Synonyms: *Uredo acaciae* Cooke, *Uromycladium bisporum* McAlpine

1.4.2 Symptoms

Initial symptoms consist of brown powdery pustules on petioles, stems, and leaves (Fig. 1.1A, McTaggart *et al*., 2015). Symptoms further develop into matting of leaves (Fig. 1.1B), pinnule and rachis malformation (Fig. 1.1C) and can consequentially induce severe stunting of seedlings and young trees (McTaggart et *al*., 2015). During periods of prolonged leaf wetness, a black slime appears on pustules on leaves and stems (Fig. 1.1D). Recently, a new symptom of well-developed galls was found on rust-infected *A. mearnsii* trees in Paulpietersburg. Phylogenetic analyses of DNA sequences from brown/ grey powdery rust teliospores found on the surface of these galls showed that the rust was *U. acaciae*. This is the first time that gall symptoms have been associated with this rust and further studies need to be undertaken to determine the basis for this unusual symptom development in a relatively well-known rust (Slippers, 2022).

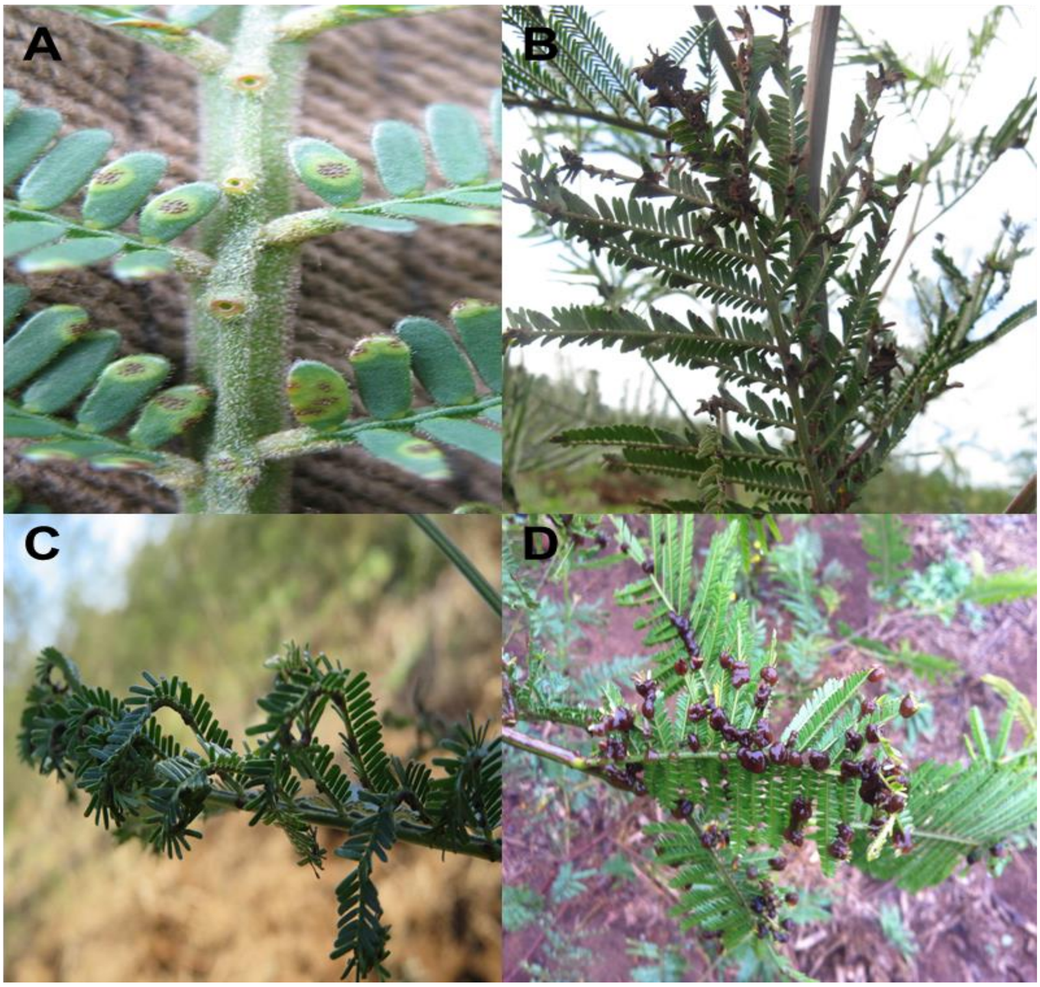

Figure 1.1: Symptoms of wattle rust (*Uromycladium acaciae*) on black wattle (*Acacia mearnsii*) include (A) brown pustules, (B-C) matting and malformation of pinnules, and (D) slime formation.

1.4.3 Economic impact

There is limited information available on the impact of the wattle rust on growth and productivity (Nxumalo *et al*., 2017). There have been reports of mortality rates of over 30%, which is because of loss of growth, forking, and branching in infected trees (South African Forestry, 2014). Two studies have indicated that wattle rust can result in yield losses of 20% to 40% (Little and Payn, 2016, Nxumalo *et al*., 2017).

1.4.4 Life cycle

The rust fungus *Uromycladium acaciae* is an autoecious rust fungus, which means it can complete its entire life cycle on *A. mearnsii* and it is also considered as a microcyclic fungus because it consists only of spermogonia and a telial stage (Fraser *et al*., 2017, FABI, 2023). Rust fungi produce spermogonia, which are haploid structures that grow on the surface of the infected tissues of the host plant (*Acacia mearnsii*) (Aime *et al*., 2014). The cross-fertilization of compatible nuclei (n+n) occurs within the spermogonia. Subsequently, plasmogamy occurs leading to dikaryotization of mycelia (n+n) (Aime *et al*., 2014, Alan Wood, ARC, personal communication, 2023). The dikaryotic phase progresses into the telial stage whereby within the teliospores, two haploid nuclei fuse to produce a diploid nucleus (2n) by the karyogamy process (Newcombe, 2004, Alan Wood, ARC, personal communication, 2023). Teliospores (2n) which are asexual spores are normally generated dry, but when exposed to moisture, they produce dark, slimy masses (Fraser *et al*., 2017). Rain or water splashes are the primary means of teliospore dissemination (Fraser *et al*., 2021b). The teliospores then germinate, and the diploid (2n) nucleus goes through meiosis producing four haploid (n) basidiospores (sexual spores) which infect newly formed host tissue (Fraser *et al*., 2017). Rust infection occurs under wet conditions (Fraser *et al*., 2021b).

1.4.5 Role of mirids as vectors of wattle rust

The wattle mirid (*Lygidolon laevigatum* Reuter) is a commonly encountered Hemiptera species in KwaZulu-Natal that feeds on black wattle (Connell, 1970). Adult wattle mirids are black or chestnut in colour with a yellow spot on the back (Fig. 1.2A). The nymphs still have developing wings, are green in colour and are highly energetic, jumping from tree to tree. Adults and nymphs suck sap from the vulnerable, developing edges of immature pinnules of black wattle plants (Ingham, 1995), and cause significant damage in young wattle trees in KwaZulu-Natal. While feeding, insects drain sap and inject toxic saliva into the leaves (Govender, 2005). Punctures form in the pinnules as a result of necrotic tissues (Fig. 1.2B). Damaged leading shoots develop several branches, resulting in poor tree shape and the formation of witches broom (Fig. 1.2C, Govender, 2005).

Dual infections of mirid and wattle rust are commonly encountered in commercial wattle plantations. As such, there has been speculation over the possible role of mirid as an insect vector of wattle rust spores. Young telia are powdery, but under wet conditions they release slime from hygroscopic cysts located the base of the teliospores. Numerous fungal spores that cause foliar diseases are sticky or have appendages that adhere to the legs and other body parts of insects, thus enabling long distance dispersal (Agrios, 2008). Rust fungi develop receptive hyphae and spermatia in the same spermogonia. Spermatia may be carried on the outside of insects that are visiting leaves with adhesive spermogonia (Agrios, 2008). The insect picks up the reproductive cells, particularly, the spermatia, which are the male reproductive cells and transfer them to the next compatible structure of a female referred to as a receptive hyphae, as they move around and feed on the infected plants. This results in fertilization, allowing the continuity of the sexual reproductive cycle of *U. acaciae* (Agrios, 2008). The adult wattle mirids are approximately 3 mm in size (FABI, 2024). *Uromycladium acaciae* teliospores are 15–20 µm in size (McTaggart *et al*., 2015). Therefore, it is high likely that wattle rust can be vectored by mirids. However, to date, no studies have confirmed the role of the mirid as a vector of wattle rust.

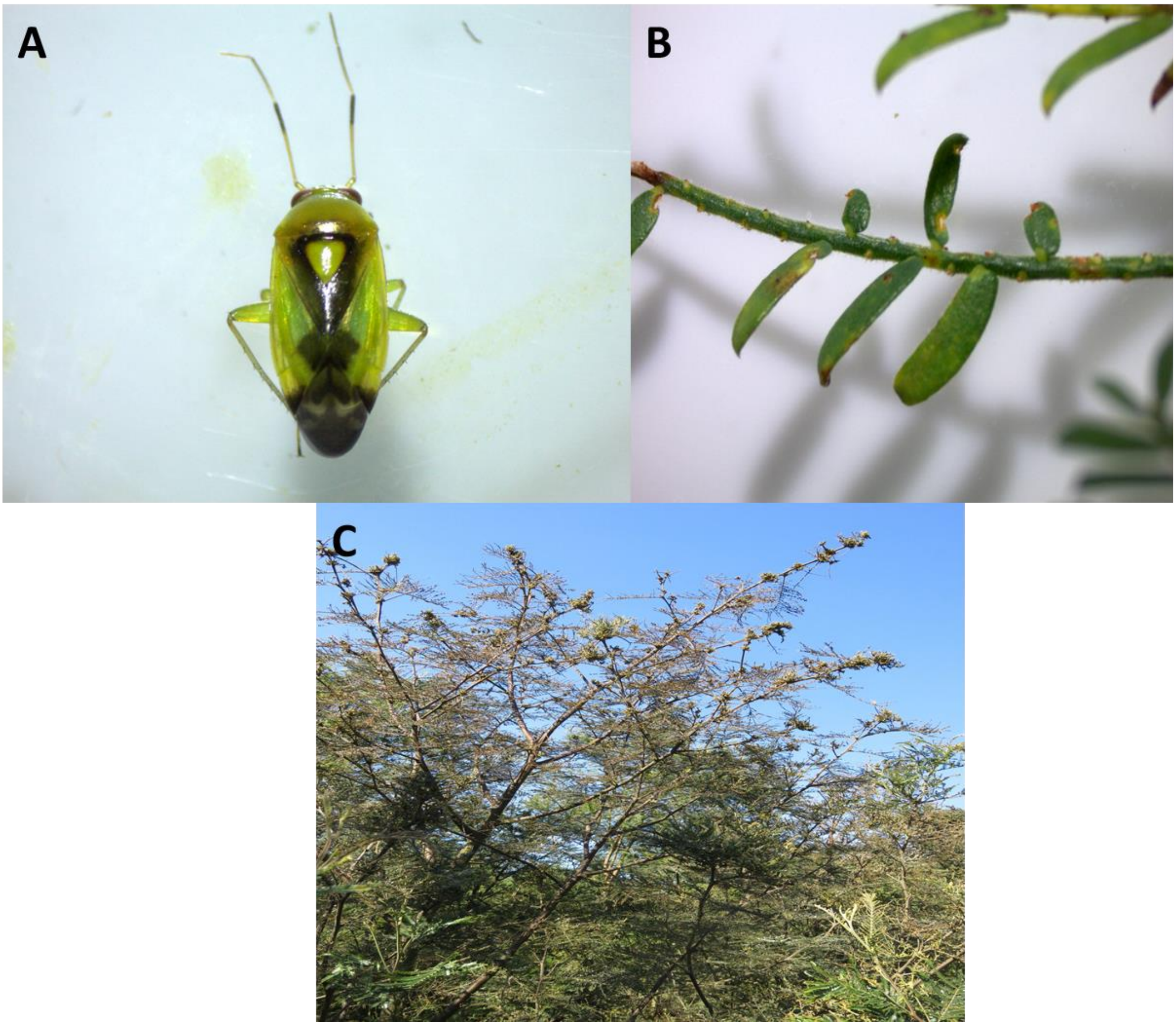

Figure 1.2: The wattle mirid (*Lygidolon laevigatum* Reuter): (A) Mirid adult, (B) initial necrotic lesions on pinnules resulting in (C) poor tree shape and the formation of witches broom.

Based on observations made of insects linked with rust pustules, two species of parasitic Hymenoptera are involved in coffee leaf rust dissemination. *Lestodiplosis* spp. and *Mycodiplosis* spp. have been identified as the disseminators (Crowe, 1963). These parasitic species are similar in size and form, and are easily carried by the wind due to their small size (average body length: 1 mm). Consequently, they appear to function as vectors of coffee rust, and aid in long distance dispersal of the rust (Crowe, 1963). Another example of an insect vectored rust is *Puccinia punctiformis* (Strauss) Röhling, which can be transmitted by a weevil, *Ceratapion onopordi* Kirby (Wandeler and Bacher, 2006). The female weevils deposit their eggs on new root growth of

Cirsium arvense (L.) Scop. The larvae feed inside the above-and below-ground shoots before pupating occurs in the root collar (Wandeler and Bacher, 2006). The female weevil transports urediniospores and injects them into the stem base via ovipositional punctures (Cripps *et al*., 2009).

1.4.6 Disease management

Current management of wattle rust is undertaken using registered fungicides (Little and Payn, 2016) and the production of rust-tolerant clones (Fraser *et al*., 2019).

1.4.6.1 Fungicides

Azoxystrobin, difenoconazole, and cyproconazole are fungicides that are currently registered for the treatment of wattle rust. However, cyproconazole is no longer accessible (Payn and Little, 2017). Amistar Top® is a registered fungicide containing both azoxystrobin and difenoconazole (McTaggart *et al*., 2015). Azoxystrobin and difenoconazole inhibit mitochondrial respiration and ergosterol production in the higher fungi, respectively (Payn and Little, 2017).

The early growth of wattle is negatively impacted by a number of pests and diseases, including brown wattle mirid and wattle rust. Combining an insecticide and a fungicide in a single tank, and applying the mixture is effective for the control of mirid and rust (Little, 2021). However, due to the high cost and the need for frequent applications throughout the growing season, the use of fungicides to control wattle rust is problematic. The potential for the disease to acquire chemical resistance, as well as potential environmental harm are also drawbacks of frequent fungicide applications (Chan and Isik 2019). In addition, a change of approach to pesticide usage by international forestry certification schemes such as the Forest Stewardship Council (FSC) has limited the number of applications of fungicides that can be made per season (Forest Stewardship Council, 2018). Moreover, the RSA government has gazetted the ban of several pesticides including propizole containing propiconazole, the active ingredient against wattle rust, as well as cyproconazole from 1 June 2024 (Meyer, 2023; Meyer, 2024). Therefore, investigating alternative biological or chemical control agents is essential to mitigate resistance development and adherence to pesticide regulations (Little, 2021).

1.4.6.2 Wattle clones for rust tolerance

Wattle rust can be controlled by producing and promoting *A. mearnsii* cultivars that are tolerant or resistant (Chan and Isik, 2019). The RSA wattle landraces are not resistant to wattle rust. However, it is believed that germplasm infusion of rust-resistant Australian black wattle into the RSA landraces of black wattle should increase wattle rust resistance in the RSA wattle population (Chan and Isik, 2021). Since October 2014, the Institute for Commercial Forestry Research (ICFR) and the NCT Forestry Co-operative have collaborated in clonal breeding programs to produce rust-and frost-tolerant clones (Thompson, 2018). The first commercial wattle clone from the program is called SP 644, and was selected for its tolerance to rust and its rapid growth. Other wattle clones have already showed increased early growth and rust tolerance; however, the more recent wattle clones are not going to be released until they have undergone extensive testing (Thompson, 2018). Ezigro Seedlings produced another clone AF01, that is tolerant to wattle rust (Candy, 2022). One frost-tolerant wattle clone, FW54, is available for commercial production. It is, however, highly susceptible to wattle rust (South African Forestry, 2022).

1.5 Mycoparasitic fungi as biological control agents of wattle rust

Biological control using mycoparasitic fungi can be a cost-effective and environmentally safe option that could constitute a part of the broader integrated pest management strategy for wattle rust. Fungi that have been shown to mycoparasitize rust fungi include *Sphaerellopsis filum*, *Trichoderma* species and *Akanthomyces lecanii (*synonym *Lecanicillium lecanii)*. *Trichoderma* species including *T. aggressivum*, *T. andinense*, *T. parareesei*, *T. petersenii* and *T. pseudopyramidale,* and *Akanthomyces* sp. were found to be mycoparasites of coffee rust (*Hemileia vastatrix*

Berkeley & Broome*)* in Africa and America respectively (Rodríguez *et al*., 2021). These fungi were found to not only colonise the coffee leaf and parasitize the surface rust pustules, but also to attack the rust's invasive intercellular mycelium (Bae *et al*., 2011; Poveda *et al*., 2019).

1.5.1 Sphaerellopsis filum

Sphaerellopsis filum, also known as *Eudarluca caricis* (teleomorph) or *Darluca filum*, is a mycoparasite of rust fungi (Uredinales). *Eudarluca caricis* is less frequently seen than *S. filum* (Trakunyingcharoen *et al*., 2014*)*. *Sphaeria filum* was originally isolated from a rust species. It was assigned to the genus *Phoma* before the genus *Darluca* was established, and *Sphaeria filum* was classified as a synonym (Trakunyingcharoen *et al*., 2014). The term *Eudarluca* was given to an ascomycete linked to uredinia of a rust on *Conna* species on the hypothesis that it was the sexual morph of *Darluca*, which was later confirmed to be correct (Keener, 1951). *Darluca filum* was then assigned to the genus *Sphaerellopsis* (Trakunyingcharoen *et al*., 2014).

Sphaerellopsis filum can be found on a variety of rusts (Trakunyingcharoen *et al*., 2014). This fungus colonises rust pustules and further produces a significant number of conidia in cirri (Hunter *et al*., 2003). Due to its broad host range as a rust mycoparasite, its potential as a biocontrol agent has attracted considerable interest (Trakunyingcharoen *et al*., 2014). In Australia, *S. filum* infection on a gall-forming *Uromycladium* sp. on *Acacia stricta* has been observed to lower gall spore output by 50%. (Burges, 1934). In 2016, Fraser *et al*. (2021a) found conidiomata of a species of *Sphaerellopsis* parasitizing telia of *U. acaciae* on foliar samples of *A. mearnsii* taken from three wattle plantations in Mpumalanga and suggested that *Sphaerellopsis* species be investigated as potential biocontrol agents for wattle rust.

Sphaerellopsis filum (as *Eudarluca caricis)* was reported to infect the urediniospores of *Puccinia* (Wang *et al*., 2020). *Sphaerellopsis filum* can limit the production of Willow rust (*Melampsora larici-epitea* Kleb.) spores by up to 98% in experimental studies (Hunter e*t al*., 2003). It is also a mycoparasite of southern fusiform rust of pine (*Cronartium fusiforme* Hedgc. & Hunt ex Cumm.) and rust in southern live oaks (*Cronartium strobilinum* (Arthur) Hedgc. & Hahn). It infected 93% of the *C. strobilinum* sori; and as a result, telia were found in only 0.8% of the sori (Kuhlman *et al.*, 1978). Willow rust became infected after being exposed to *S. filum* isolates from *Melampsora* spp. and crown rust *(Puccinia coronata* Corda.) (Hunter *et al*., 2003). *Sphaerellopsis filum* naturally develops on the uredosori of various rusts (Nicolas and Villanueva, 1965).

1.5.2 Akanthomyces spp.

Akanthomyces spp. are fungal and insect pathogens and are efficient biological control agents for many plant diseases and plant-parasitic nematodes (Zhou *et al*., 2022). The entomopathogenic fungus species formerly known as *Verticillium lecanii* (Zimmerman) Viegas was given the official name *Lecanicillium lecanii* (Li, 2018). However, some *Lecanicillium* spp. have been renamed *Akanthomyces* spp. (Nishi *et al*., 2022*).* The genus *Lecanicillium* was rejected in a new classification system, and some of its species were moved to the genus *Akanthomyces* (Zhou *et al*., 2022). Currently, *Lecanicillium* has 35 officially identified species, and five of these species have been moved to the *Akanthomyces* genus. However, it is still unclear to which genus the majority of the original *Lecanicillium* species should be assigned (Zhou *et al*., 2022).

Species of the fungal genus *Akanthomyces* are naturally present on rust-infected leaves and are identifiable by a white mycelium that appears on the rust pustules (Nxumalo, 2018). Studies have shown that *Akanthomyces* spp. are potential biocontrol agents of rust diseases. *Akanthomyces lecanii* is one of the fungi that was reported to infect the urediniospores of *Puccinia* (Wang *et al*., 2020). The pustules of *Puccinia allii* (the causative agent of leek rust) were colonised by *A. lecanii*, which completely coated the pustules in thick, white masses of mycelium and spores (Uma and Taylor, 1987). In addition, *A. lecanii* naturally hyperparasites *Hemileia vastatrix* (coffee leaf rust) decreasing its severity and lowering disease incidence (Galvão and Bettiol, 2014, Havugimana, 2017, Nxumalo, 2018). In greenhouse and field tests, foliar sprays of *A. lecanii* conidia at 100, 1000, or 10 000/ml infected *Coleosporium domingense* (H. & P. Sydow) Dietel & Holw (Frangipani rust) and stopped the defoliation of Frangipani trees (*Plumeria rubra*) (McMillan, 1985; Weeraratne and Adikaram, 2006).

1.6 Conclusion

Wattle rust is a severe disease of black wattle that can result in considerable yield and quality losses. Increasing regulations around the use of fungicides in commercial forestry in RSA has created a major challenge for the forestry industry. Therefore, the management of wattle rust needs be dealt with in a sustainable manner. The use of biological control agents would be advantageous, as they are environmentally safe and could be used on a sustainable basis. Mycoparasites such as *S. filum* and *A. lecanii* on rust diseases have been observed and evaluated for biocontrol efficacy. *S. filum* has been shown to reduce up to 50% or more of spore output of different rust fungi including *Uromycladium* species (Burges, 1934, Hunter *et al*., 2003). It is likely that *S. filum* can reduce overwintering telia and the initial inoculum for the following epidemic season, thus reducing wattle rust infections (Fraser *et al*., 2021a). In addition, *Akanthomyces* isolates have been demonstrated to colonise and reduce coffee and soybean rust in RSA. It is unknown whether *S. filum* and *A. uredinophilus* can reduce the incidence of wattle rust specifically. Therefore, studies to evaluate the biocontrol efficacy of *S. filum* and *Akanthomyces uredinophilus* Manfrino et al. on wattle rust are warranted.

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CHAPTER 2: *In vitro* detached leaf bioassay and microscopy of interactions between mycoparasitic fungi and *Uromycladium acaciae*

2.1 Abstract

The aim of this study was to assess the ability of *Akanthomyces uredinophilus* Manfrino et al., pers. comm. Mwelasi and *Sphaerellopsis filum* (Biv.-Bern. ex Fr.) Sutton [=*Darluca filum* (Biv.-Bern. ex Fr.) Berk.] to colonise and mycoparasitize *Uromycladium acaciae* (Cooke) P. Syd. & Syd.. using a detached leaf bioassay. Leaves of black wattle (*Acacia mearnsii* De Wild) infected with *U. acaciae* were collected from a local commercial plantation in Richmond, KwaZulu-Natal, South Africa. Rust-infected leaves were sprayed with conidial suspensions of 1 $g.L^{-1}$ of *S. filum* ($5.8x10^8$ $conidia.ml^{-1}$) and *A. uredinophilus* ($1x10^6$ $conidia.ml^{-1}$) and incubated in plastic containers containing moist paper towels to create conditions of high humidity. Colonisation of rust pustules with *A. uredinophilus* and *S. filum* was assessed under a light microscope and a percentage colonisation for each treatment was estimated at four weeks post-inoculation. Colonised leaves were also viewed under an environmental scanning electron microscope (ESEM) to examine the interaction between the mycoparasitic fungi and *U. acaciae*. At four weeks post-inoculation, *A. uredinophilus* and *S. filum* were able to colonise 41% and 44% of rust pustules, respectively. Under the ESEM, *S. filum* and *A. uredinophilus* demonstrated mycoparasitic activity on the teliospores of *U. acaciae*. Hyphae of *S. filum* and *A. uredinophilus* coiled around and punctured rust teliospores, causing them to rupture and deflate. Overall, conidia of both *S. filum* and *A. uredinophilus* were able to colonise and mycoparasitize *U. acaciae* teliospores.

2.2 Introduction

Black wattle (*Acacia mearnsii* De Wild) is a valuable plantation species that is used for the production of hardwood, pulpwood, charcoal, tannin extracts, and fluorescent dyes (De Beer, 1986). However, numerous pests and diseases have an adverse effect on its production in South Africa. Wattle rust, caused by *Uromycladium acaciae* (Cooke) P. Syd. & Syd., is the most economically important disease currently affecting black wattle plantations in South Africa (Chan and Isik, 2019).

Identifying management tactics for the sustainable control of wattle rust is crucial to minimize its detrimental effects on black wattle productivity. Triazole and azoxystrobin-based fungicides, and genetic resistance or tolerance to the disease have been used in the management of wattle rust (Oumar *et al*., 2019). However, recent legislation changes and environmental concerns have mandated the restricted use of registered fungicides to control wattle rust. In 2023, the Timber Industry Pesticide Working Group (TIPWG) highlighted the immediate need for biological alternatives for the management of wattle rust following a government gazetted ban of currently registered rust fungicides from June 2024 (Meyer, 2023).

Potential biocontrol agents for a variety of rust species have been studied, including *Sphaerellopsis filum* (Teleomorph: *Eudarluca caricis*) and *Akanthomyces lecanii* (formerly known as *Verticillium lecanii*) (Trakunyingcharoen *et al*., 2014, Wang *et al*., 2020). *Akanthomyces* species are active against a variety of phytopathogenic fungi, such as *Pythium*, rusts, powdery mildews, and green moulds. Antibiosis and mycoparasitism are modes of actions that will inhibit a plant pathogenic fungus (Goettel *et al*., 2008). Various *Akanthomyces* species have distinct modes of action that vary depending on the host pathogen. When hyphae of *Akanthomyces* spp. come into contact with the mycelia and conidia of powdery mildew, they produce enzymes such chitinase which degrade cell walls, allowing hyphae to penetrate and kill the pathogen (Goettel *et al*., 2008). *Akanthomyces* species have also been observed entering and colonising the uredial sori of *Puccinia coronata* (Goettel *et al.,* 2008). A second mycoparasite, *Sphaerellopsis filum,* has at least 369 rust hosts, including

Cronartium strobilinum on dwarf live oaks (*Quercus minima)*, *Melampsora larici-epitea* on willows (*Salix* spp.), *Phragmidium violaceum* on raspberries (*Rubus* spp.), and *Puccinia graminis* on wheat (*Triticum* spp.) (Sendhilvel and Raguchander, 2020). The fungus is also able to produce antimicrobial isocyanide metabolites such as Darlucin A (1) and B (2) (Sendhilvel and Raguchander, 2020).

The ability of these mycoparasites to colonise and mycoparasitize *U. acaciae* has not been investigated. In this study, an initial screening was conducting using a detached leaf bioassay to determine whether *Akanthomyces uredinophilus* and *Sphaerellopsis filum* have the potential to colonise and mycoparasitize *U. acaciae.*

2.3 Methods and Materials

2.3.1 Wattle rust-infected leaves

Leaves of black wattle (*Acacia mearnsii*) infected with *Uromycladium acaciae* were collected from a local wattle plantation (MSD Timbers, Richmond, KwaZulu-Natal, Republic of South Africa). Fully expanded leaves displaying a moderate level of wattle rust infection were selected and immediately placed in a brown paper bag for use in the detached leaf assay experiment. Once in the laboratory, the detached leaves were kept alive by putting moist sterile cotton wool around the cut ends of detached leaves and then incubating the leaves at 25^{o}C in transparent containers containing sterile moist paper towels to maintain humidity.

2.3.2 Mycoparasitic fungal isolates

Samples of a formulated product containing *S. filum* were obtained from Andermatt-PHP (Nottingham Road, KZN). A total of 1 gram of *S. filum* (5.8 x 10^8 conidia.ml^{-1}) product was added to 1000 ml of sterile distilled water amended with 250 µl of Break-Thru (Evonik Operations GmbH, Germany). The suspension was shaken and allowed to stand for 30 minutes before spraying, to allow for the release of conidia into suspension. For *A. uredinophilus*, sporulating cultures were obtained from the University of KwaZulu-Natal (PhD student – Phumelela Peace Mwelasi, Department of Plant Pathology, PMB). Conidia of *A. uredinophilus* were harvested from a culture on a PDA plate by washing fungal mycelia with sterilized distilled water using a sterilized hockey stick and filtering it through with sterilized cheese cloth. The conidial suspension was examined under a light microscope, counted using a haemocytometer and the concentration of conidia adjusted to $1x10^6$ conidia.ml^{-1}. A conidial concentration of $1x10^6$ conidia.ml^{-1} of *A. uredinophilus* is the recommended dosage, as previous studies have found it to be more efficient in controlling various rust diseases (Havugimana, 2017). The conidial suspension was made up to 1000 ml and amended with 250 µl of Break-Thru. The conidial suspensions of *S. filum* (1 g.L^{-1} of *S. filum* product (5.8 x 10^8 conidia.ml^{-1})) and *A. uredinophilus* ($1x10^6$ conidia.ml^{-1}) were sprayed onto the leaves until run-off (~8ml).

2.3.3 Detached leaf bioassay and ultrastructure studies under ESEM

After inoculation, the detached leaves were placed back in the transparent containers. Control leaves were sprayed to run-off with sterile distilled water only. Treated containers were arranged in a randomized complete block design (Fig 2.1). The design incorporated three treatments (*A. uredinophilus*, *S. filum* and the water-treated control), with each treatment replicated four times. Two containers represented an experimental unit and the experiment was repeated twice. At four weeks post-inoculation, colonisation of rust pustules with *A. uredinophilus* and *S. filum* was assessed under a light microscope (Zeiss Axio-Imager M2 Microscope, Carl Zeiss AG) and a percentage colonisation of rust pustules was estimated for each treatment.

Figure 2.1: Layout of a detached leaf assay to determine whether *Akanthomyces uredinophilus* and *Sphaerellopsis filum* have the potential to colonise and mycoparasitize *Uromycladium acaciae* pustules*.*

Leaves with rust pustules displaying high levels of colonisation by *A. uredinophilus* and *S. filum* were taken to the Microscopy and Microanalysis Unit (MMU, UKZN, PMB campus) for viewing under an environmental scanning electron microscope (ESEM) (ZEISS EVO® LS 15, Carl Zeiss AG). Interactions between the biological control agents and *U. acaciae* were examined on *A. uredinophilus*-and *S. filum*-infected leaves and compared to that of the rust-only control leaves.

A standard SEM procedure for biological samples was followed: (i) primary fixation: colonised leaf samples were cut into small disks and placed onto ESEM specimen stubs and fixed for 1 hour in 3% buffered glutaraldehyde; (ii) buffer wash: leaf disk samples were soaked in sodium cacodylate buffer for 5 minutes twice; (iii) dehydration: the specimens were immersed in a series of 10%, 30%, 50%, 70% and 90% ethanol each for 5 minutes, and twice then for 5 minutes in 100% ethanol; (iv) coating: the stubs with samples were placed into a Quorum Q150R ES Gold Sputter Coater for coating; (v) critical point drying: A Quorum Q150 RES Critical Point Dryer was used to dry the samples to keep them intact. After drying and coating, the samples were viewed under a ZEISS EVO® LS 15 ESEM.

2.4 Results

2.4.1 Detached leaf bioassay

At four weeks post-treatment, characteristic black pycnidia of *Sphaerellopsis filum* were observed producing cirri where the mycoparasite was colonising pustules of *U. acaciae* (Fig. 2.2A and B). At one week post-treatment, characteristic white mycelial growth of *Akanthomyces uredinophilus* was observed on colonised pustules of *U. acaciae* (Fig. 2.2C and D). In Trial one, *S. filum* colonised 49% of rust pustules and *A. uredinophilus* colonised 21%. In Trial two*, S. filum* colonised 39% of wattle rust and *A. uredinophilus* colonised 60%. Taken together, results showed that *A. uredinophilus* and *S. filum* were able to colonise 41% and 44% of rust pustules respectively.

2.4.2 Interaction studies using ESEM

Mycoparasitic activity of *S. filum* and *A. uredinophilus* on *U. acaciae* was observed using ESEM of infected leaf samples from the detached leaf assay (Fig 2.3). In untreated rust-infected leaves, teliospores appeared in good condition with no visible puncture holes or distortion (Fig 2.3A). In rust-infected leaves inoculated with *S. filum* and *A. uredinophilus*, hyphae of the mycoparasites were seen attaching to and puncturing rust teliospores, causing the teliospores to rupture and deflate (Fig 2.3 B-D).

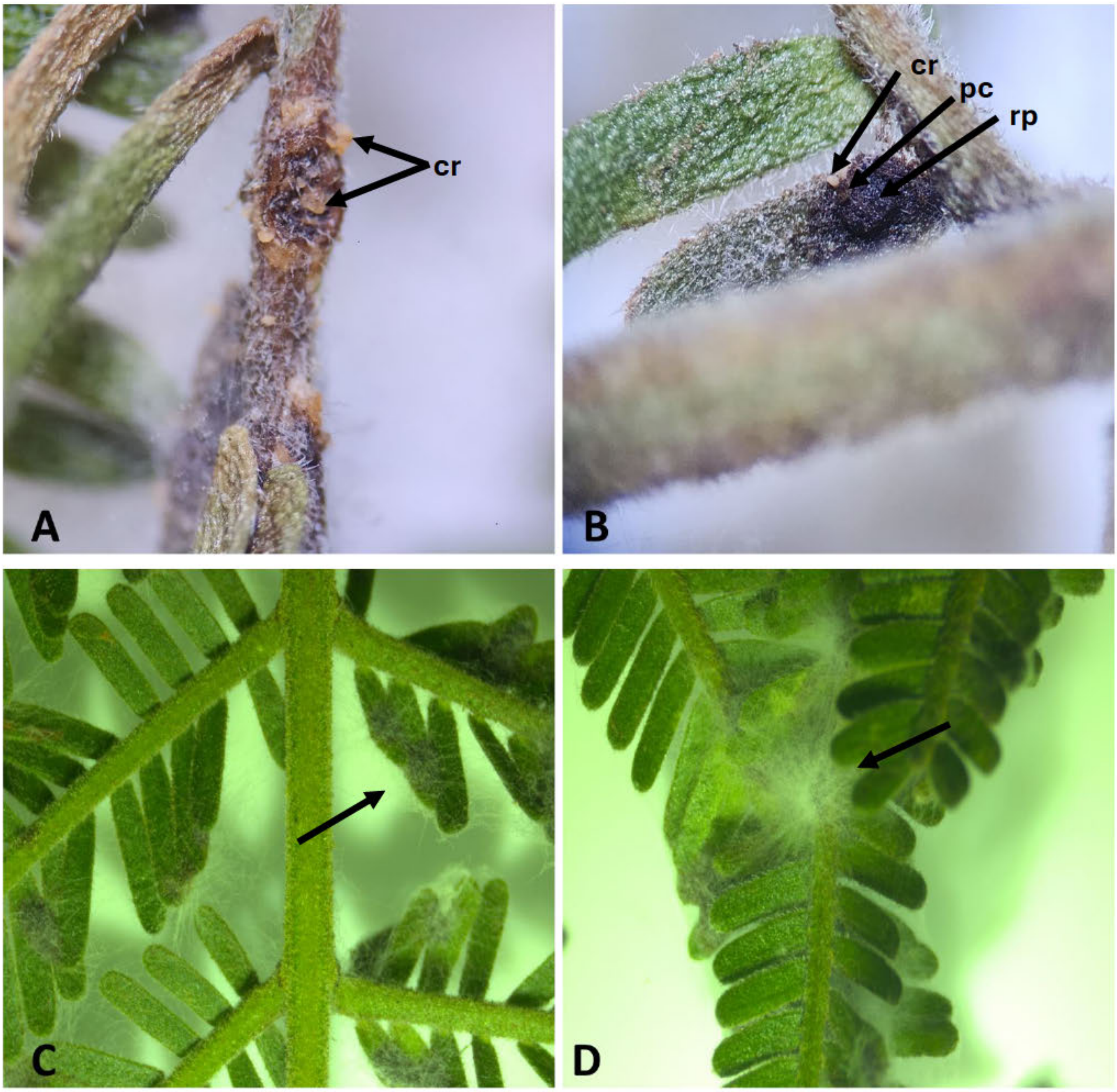

Figure 2.2: Colonisation of pustules of *Uromycladium acaciae* by (A-B) *Sphaerellopsis filum* and (C-D) *Akanthomyces uredinophilus* viewed at 1.65x under a dissecting microscope. Cirri (cr) were being secreted from pycnidia (pc) of *Sphaerellopsis filum* colonising wattle rust pustule (rp). The arrows in C and D are pointing at wattle rust pustules colonised by *Akanthomyces uredinophilus*, which is visible as white strands in both C-D.

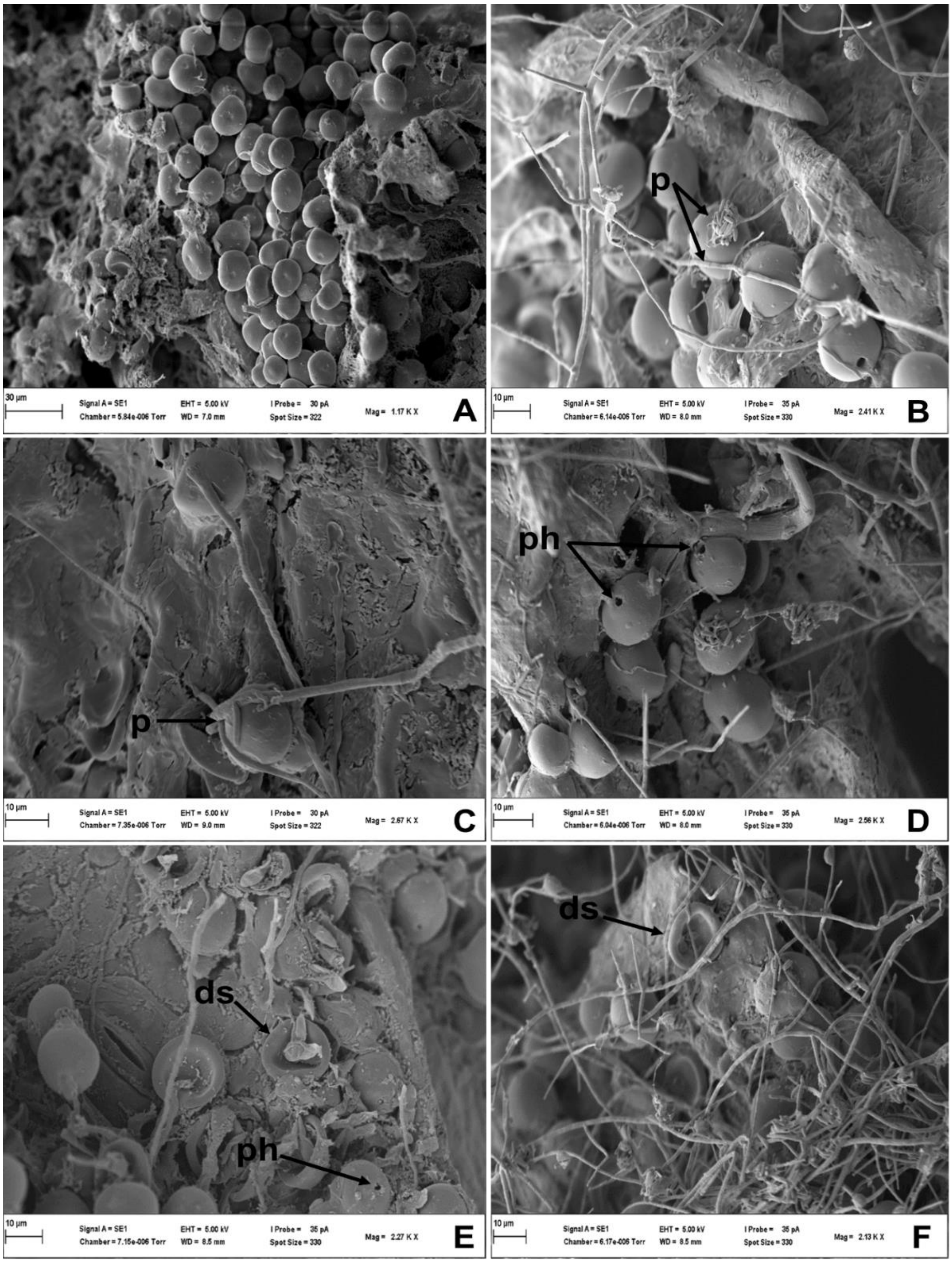

Figure 2.3: Environmental Scanning Electron microscopic observations of interactions between wattle rust *Uromycladium acaciae* (Cooke) P. Syd. & Syd. and two mycoparasitic fungi, *Akanthomyces uredinophilus* Manfrino et al. and *Sphaerellopsis filum* (Biv.-Bern. ex Fr.) Sutton [=*Darluca filum* (Biv.-Bern. ex Fr.) Berk.]. (A) Untreated, healthy wattle rust teliospores; (B) Cluster of *Akanthomyces* conidia and hyphae attaching onto rust teliospores (p); (C): Cluster of *Sphaerellopsis filum* conidia and hyphae attaching onto rust teliospores (p); In D-F: puncture holes (ph), resulting in deflated rust teliospores (ds).

2.5 Discussion

Conidia of both *S. filum* and *A. uredinophilus* have the ability to colonise and mycoparasitize teliospores of *U. acaciae.* Around 40% of wattle rust pustules were colonised by *S. filum* and *A. uredinophilus* by four weeks post-inoculation, which indicated that both *S. filum* and *A. uredinophilus* could be used as biocontrol agents against wattle rust.

Under the SEM, mycoparasitic activity of both *S. filum* and *A. uredinophilus* was observed on the teliospores of *U. acaciae*. Teliospores were punctured and deflated, rendering them inactive. This mechanism would reduce teliospore viability and a subsequent reduction of overwintering inoculum for the next season (Gordon and Pfender, 2012). Biological control mechanisms that cause the destruction of rust spores have been reported in previous studies (Plachecka, 2005, Raguchander and Durgadevi, 2021). Hyphae of *S. filum* were shown to hyperparasitize the uredinospores of *Puccinia recondita* Rob. ex Desm. f. sp. *tritici* (Erikss.) in wheat, causing a reduction in their production and spread, thus considerably reducing the severity of the disease (Plachecka, 2005). *Sphaerellopsis filum* was also reported to decrease the spore germination of Willow rust (*Melampsora epitea)* by up to 98% (Raguchander and Durgadevi, 2021). *Sphaerellopsis filum* was shown to colonise and extract nutrients from the sori of various rust diseases such as willow rust and wheat rust (Moricca and Ragazzi, 2008). Furthermore, Gordon and Pfender (2012) also showed that pycnidia of *S. filum* on dead rust pustules of *Puccinia graminis* subsp. *graminicola* on dead leaves of rye grass (*Lolium perrene* L.) served as a source of overwintering inoculum for the biocontrol fungus. Therefore, *S. filum* is most likely to be effective as a biocontrol agent after the overwintering period (Gordon and Pfender, 2012).

The mode of action of *S. filum* includes enzymatic processes and physical means to disintegrate the cell walls of the host rust (Moricca and Ragazzi, 2008). Furthermore, *S. filum* produces antibiotics such Darlucin A and B that inhibit pathogenic fungi (Durgadevi *et al*., 2023). The ability of hyperparasitic fungi to apply physical pressure and produce degradative enzymes is a key indicator of their virulence (Aiuchi *et al*., 2007; Khan *et al*., 2012). However, in this study, an investigation into the possible biocontrol activity caused by antifungal metabolites and chitinolytic enzymes was not carried out.

Akanthomyces lecanii Zimmermann (1901) is a species that generates hydrolytic enzymes such as proteases and chitinases (Nana *et al*., 2023). Specifically, chitinases have been linked to the degradation of the cuticle of host fungi. Urediniospore walls can be hydrolyzed by these enzymes (Nana *et al*., 2023). Numerous publications have demonstrated that hydrolytic enzymes are key to the mycoparasitic capabilities of mycoparasites (Xie *et al*., 2010, Reyes *et al*., 2012). Three distinct enzymes have been identified from *Akanthomyces* spp., which are a lipase, a protease, and a chitinase (Xie *et al*., 2010, Reyes *et al*., 2012). *Akanthomyces lecanii* synthesizes hydrolytic enzymes that break down the host cell wall. Subsequently, this fungus destroys the plasma membrane of the host cell, and subsequently breaks down the cytoplasm (Moricca and Ragazzi, 2008). This mechanism could account for the distortion of the teliospores of wattle rust in the presence of both the hyperparasites. The repeated observation of penetration holes showed that the fungi were able to penetrate the teliospores of *U. acaciae*.

Overall, results from the detached leaf assay and the microscopic observations revealed that both *S. filum* and *A. uredinophilus* can colonise and parasitize teliospores of *U. acaciae.* The biocontrol efficacy of mycoparasitic fungi on wattle rust in practice will be determined through a series of potted nursery trials.

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CHAPTER 3: Compatibility of two mycoparasitic fungi (*Sphaerellopsis filum* and *Akanthomyces uredinophilus*) and *Beauveria bassiana* with an insecticide (deltamethrin) and an adjuvant (Breakthru®)

3.1 Abstract

Optimal Integrated Pest Management (IPM) for disease suppression requires the seamless and coordinated integration of multiple compatible control strategies. Therefore, any biological agents that may be considered for use to suppress a disease should be compatible with the range of agrochemicals and adjuvants that are being used on the crop of interest, in order for the management strategy to be effective. This study evaluated the compatibility of two isolates of mycoparasitic fungi (*Sphaerellopsis filum* and *Akanthomyces uredinophilus*) and a commercial insect-pathogenic fungus (*Beauveria bassiana,* Eco-Bb®) with an insecticide (deltamethrin) and an adjuvant (Break-thru®). The experiment utilized an amended bioassay method with *S. filum*, *A. uredinophilus*, and *B. bassiana* being cultured on potato dextrose agar (PDA) amended with 250 $\mu l.L^{-1}$, 125 $\mu l.L^{-1}$, and 500 $\mu l.L^{-1}$ of Break-thru® , or amended with 1 $ml.L^{-1}$, 0.5 $ml.L^{-1}$ and 2 $ml.L^{-1}$ of deltamethrin. Unamended media served as a negative control, whilst media amended with the commercial rust fungicide Amistar Top® served as a positive control. After three weeks, radial growth was measured from the centre of the fungal colony, and a percentage inhibition efficiency (IE) of Break-thru®/ deltamethrin on the growth of the biological control agent was calculated. There was no significant inhibition of any of the biological agents by either Break-thru® or deltamethrin at any of the dosages tested. Therefore, *S. filum, A. uredinophilus* and *B. bassiana* are compatible with the insecticide (deltamethrin) and the adjuvant (Break-thru®), and they could be combined in an integrated control program.

3.2 Introduction

Optimal Integrated Pest Management (IPM) for pest suppression entails the seamless and coordinated integration of multiple compatible control strategies (Malinga and Laing, 2024). Applying biological plant protection products, particularly microbiological preparations, poses challenges due to the sensitivity of the microorganisms to factors such as temperature, humidity, and sunlight (Holka and Kowalska, 2023). In addition, these biologicals need to be compatible with the agrochemicals currently used on the target crop. To optimize the physico-chemical properties of spray solutions and maximize the efficacy of agrochemicals applied to foliage, surfactants, commonly referred to as adjuvants, are often utilized in agricultural pesticide applications (Slabbert and den Berg, 2009). Currently approved fungicides for wattle rust treatment include difenoconazole and azoxystrobin (Payn and Little, 2017). Amistar Top® (Syngenta Inc.) is the registered fungicide containing both these ingredients (McTaggart *et al*., 2015). However, black wattle is attacked by both the wattle mirid and wattle rust in commercial wattle plantations. Spray treatments with deltamethrin are being used for the control of the wattle mirid by the wattle industry in South Africa. To this end, combining the insecticide (deltamethrin) and a fungicide in a single tank and applying the mixture has become common practice for the control of wattle mirid and wattle rust (Little, 2021).

A shift towards biological control presents a promising avenue, reducing the necessity for chemical insecticides. This transition not only benefits farmers but also offers environmental advantages (Oliveira *et al*., 2003). Control of the wattle rust may be possible using the mycoparasites *Sphaerellopsis filum* (Biv.-Bern. ex Fr.) Sutton [=*Darluca filum* (Biv.-Bern. ex Fr.) Berk.] and *Akanthomyces uredinophilus* Manfrino et al., pers. comm. Mwelasi, which are being developed in South Africa for the control of rusts of commercial crops. In parallel, control of the wattle mirid may be possible using the entomopathogenic fungus *Beauveria bassiana* (Bals.-Criv.) Vuill. (Forlani *et al*., 2014), which has already been commercialized in South Africa (andermatt-php.co.za; www.agro.basf.co.za). Understanding the compatibility of mycoparasitic and entomopathogenic fungi with other crop protection methods is crucial for preserving naturally occurring mycoparasites and entomopathogens, or those introduced for insect and disease control (Sahayaraj *et al*., 2011). A synergistic approach utilizing both entomopathogenic fungi and plant-based pesticides could offer a cost-effective, sustainable solution for long-term pest and disease management (Sahayaraj *et al*., 2011).

Within integrated pest management systems, biological plant protection serves as a pivotal component, providing a safer alternative to chemical interventions, thereby mitigating environmental and public health risks (Shah *et al*., 2009, Thoudam and Dutta, 2014). The efficacy of fungi in the field is significantly influenced by their compatibility with other chemical protection measures (Challa and Sanivada, 2014). Certain biological control agents may exhibit synergistic interactions with chemical pesticides, thereby augmenting their efficacy when utilized concurrently (Malinga and Laing, 2024). In this study, two mycoparasites, *S. filum* and *A. uredinophilus*, and the entomopathogenic fungus, *B. bassiana,* were evaluated in an *in vitro* experiment for their compatibility with an insecticide (deltamethrin – [100g.L^{-1}], Decis Forte®, Bayer CropScience Limited) and an adjuvant (Break-thru® S 240 – organo-modified siloxane, Evonik Industries, Germany).

3.3 Materials and Methods

3.3.1 Inoculum preparation

A sporulating culture of an *A. uredinophilus* used in this study was obtained from a PhD student at the University of KwaZulu-Natal (Phumelela Peace Mwelasi, Department of Plant Pathology, UKZN, Pietermaritzburg). A culture of *S. filum* was obtained from Andermatt-PHP (Nottingham Road, KZN) as a formulated product and a sporulating culture of *B. bassiana* was also obtained from Andermatt-PHP (Nottingham Road, KZN). Each fungus was cultured on potato dextrose agar (PDA) at 21-25˚C for three weeks. Fungal discs (1 x 1cm) were cut from each culture and were used in the bioassay.

3.3.2 Amended media bioassay

A compatibility test was conducted *in vitro* using actively growing cultures of *A. uredinophilus*, *S. filum* and *B. bassiana*. The 1 x 1cm disc of each culture was placed aseptically in the centre of each plate containing potato dextrose agar (PDA) amended with 250 $\mu l.L^{-1}$, 125 $\mu l.L^{-1}$ and 500 $\mu l.L^{-1}$ dosages of Break-thru®, or amended with 1 $ml.L^{-1}$, 0.5 $ml.L^{-1}$ and 2 $ml.L^{-1}$ dosages of deltamethrin ([100$g.L^{-1}$]. Unamended media served as a negative control, whilst media amended with a commercial rust fungicide containing difenoconazole and azoxystrobin (125g^{-1} + 200$g.L^{-1}$, respectively; sold as Amistar Top® (Syngenta), used at one dosage of 1$ml.L^{-1}$) served as a positive control. Plates were incubated at 21˚C-25˚C for three weeks. Each treatment was replicated three times and arranged in a randomized complete block design. The experiment was repeated twice.

3.3.3 Data capture and analysis

At three weeks post-inoculation, the radial growth from the centre of each fungal disc of *S. filum, A. uredinophilus* and *B. bassiana* was measured. The inhibition efficiency (IE) of Break-thru®, deltamethrin and the control fungicide (Amistar Top®) on the growth of the three biological control agents was calculated using the formula:

$$IE(\%) = \left[\frac{diameter\ of\ untreated\ control - diameter\ of\ treatment}{diameter\ of\ untreated\ control}\right] X\ 100$$

Data were analysed using the R statistical software package (R Development Core Team, 2012). The Kruskal-Wallis non-parametric test was utilized to determine whether there were any significant differences between the treatments. Dunn's post hoc test with Bonferroni adjustments was utilized to identify specific pairwise differences between treatment groups.

3.4 Results

For *S. filum*, a low level of inhibition of approximately 20% was observed for the 0.5 ml.L^{-1} and 1 ml.L^{-1} treatments of deltamethrin and 125 µl.L^{-1} treatment of Break-Thru® but this level of inhibition was not significantly greater than the unamended control. Amended media with the fungicide control (Amistar Top®) significantly inhibited *S. filum* growth (Fig 3.1, *p*-value= 0.0229, x^2= 14.826, df= 7).

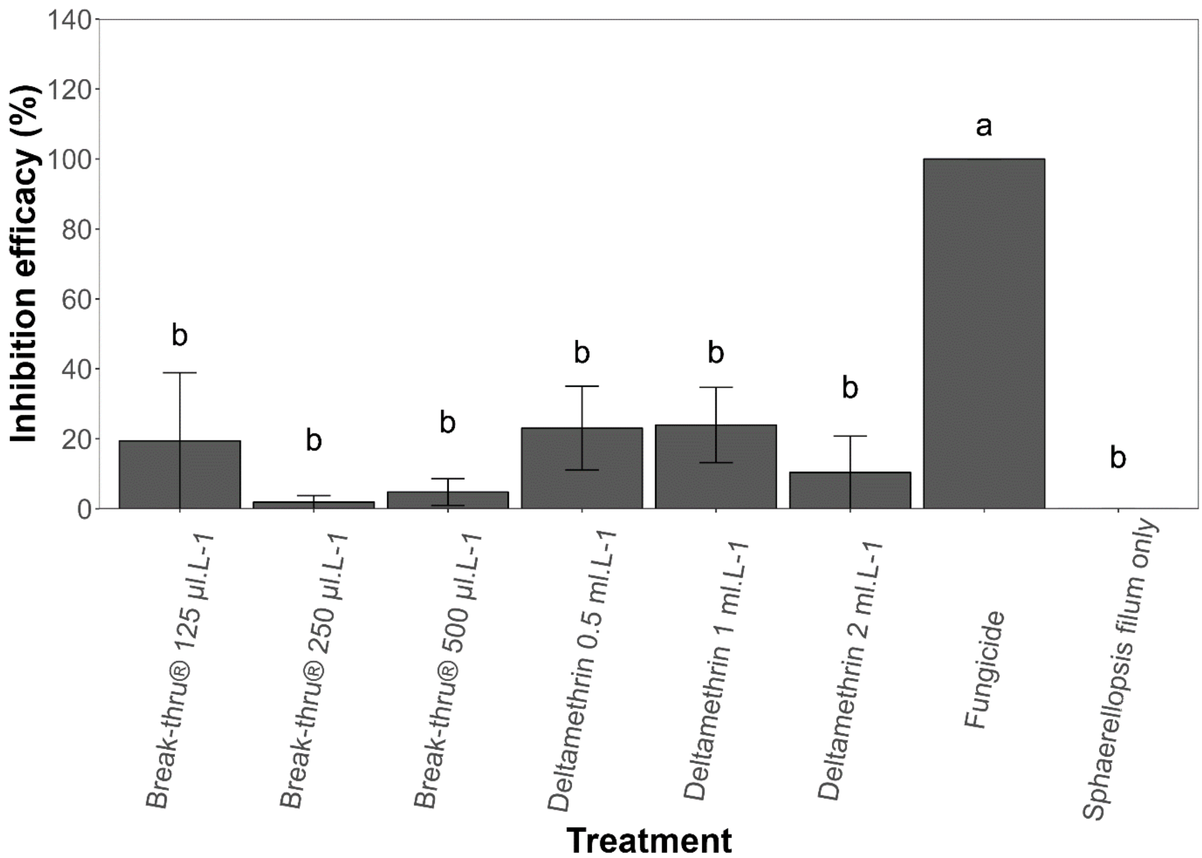

Figure 3.1: Effect of different dosages of an adjuvant (Break-Thru®) and an insecticide (deltamethrin) on radial growth of *Sphaerellopsis filum* in an amended bioassay experiment. The fungicide control used was Amistar Top®. Means with the same letter are not significantly different at *p*<0.05.

For *A. uredinophilus*, a low percentage inhibition of ~5% was observed for the 250 µl.L^{-1} treatment with Break-Thru®, which was not significantly greater than the unamended control. Amended media with the fungicide control significantly inhibited *A. uredinophilus* growth (Fig 3.2, *p*-value= 0.0117, x^2= 19.5, df= 7).

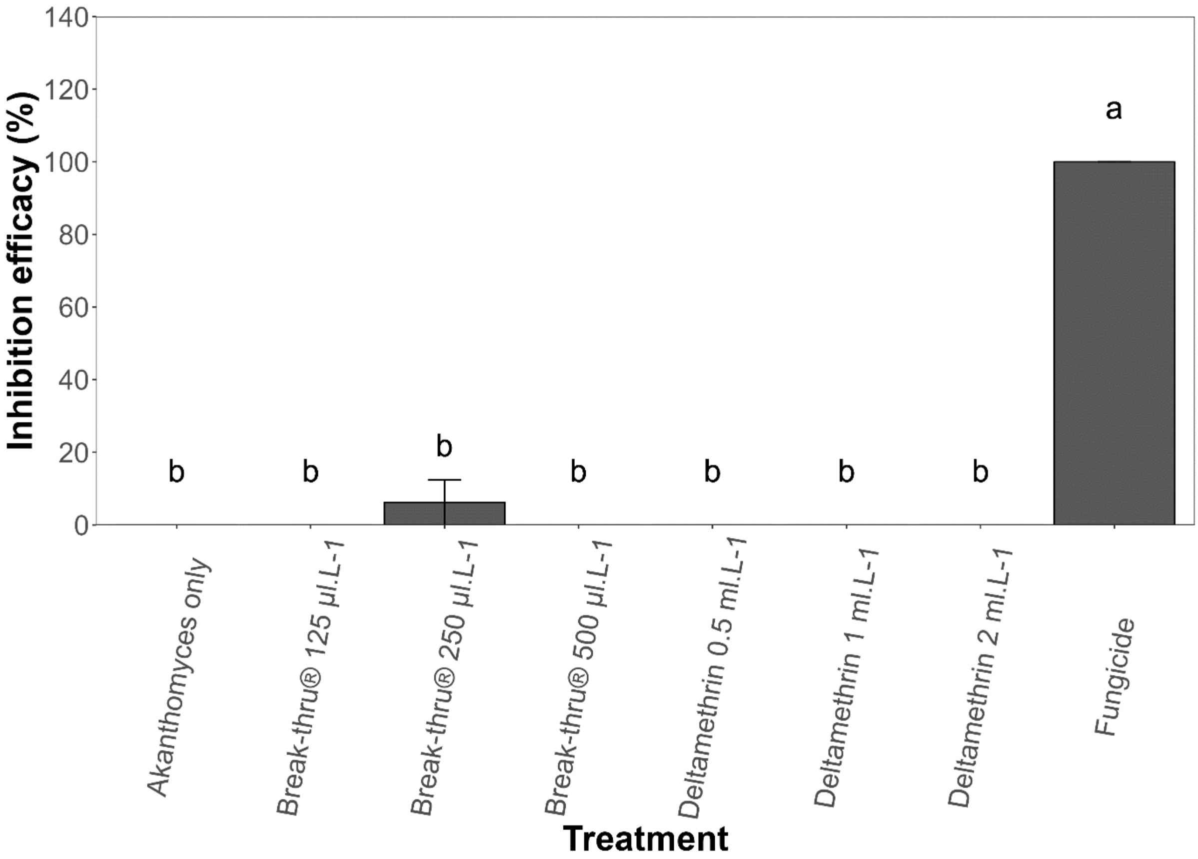

Figure 3.2: Effect of different dosages of an adjuvant (Break-Thru®) and an insecticide (deltamethrin) on radial growth of *A. uredinophilus* in an amended media bioassay experiment. The fungicide control used was Amistar Top®. Means with the same letter are not significantly different at $p<0.05$.

For *B. bassiana*, a low percentage inhibition of ~5-30% was observed for the 125 µl.L^{-1} and 250 µl.L^{-1} treatments of Break-Thru® and 0.5 ml.L^{-1} treatment of deltamethrin, which was not significantly greater than the unamended control. Amended media with the fungicide control significantly inhibited *B. bassiana* growth (Fig 3.3, *p*-value= 0.0381, x^2= 17.22, df=7).

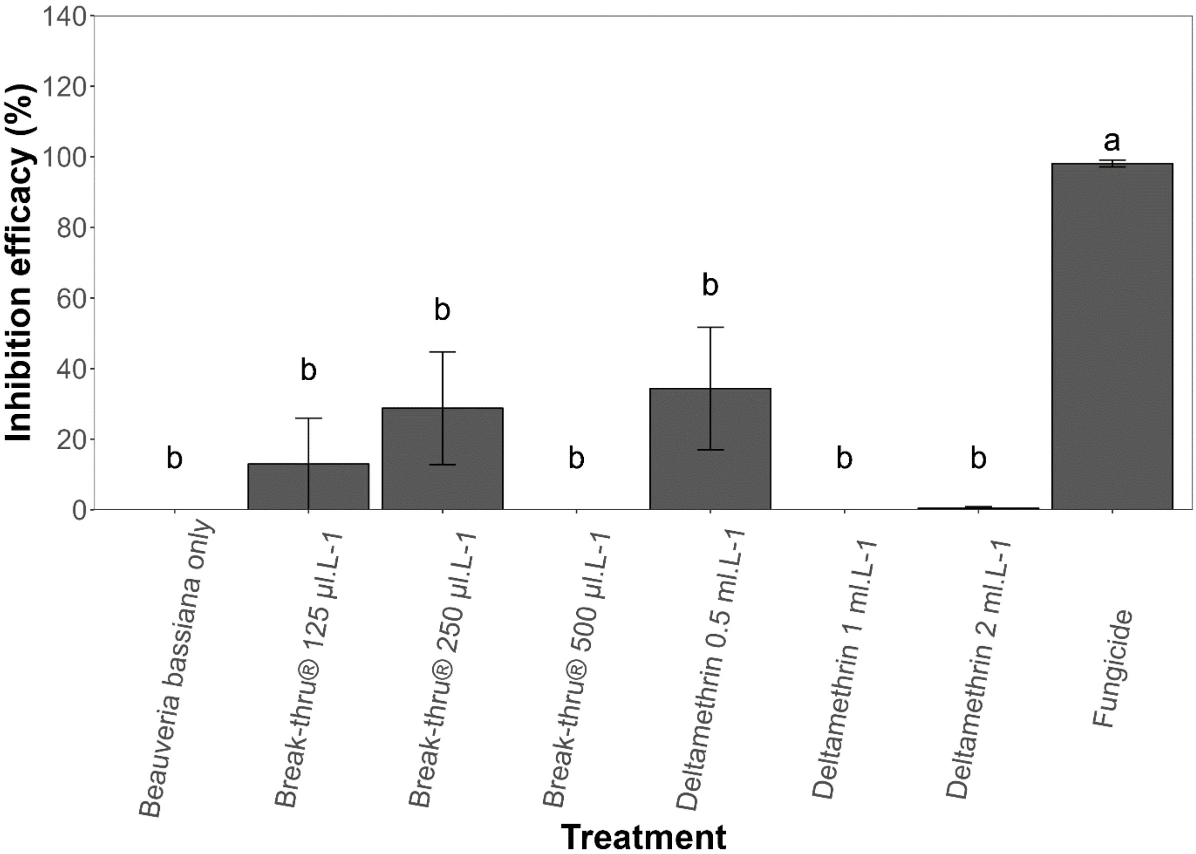

Figure 3.3: Effect of different dosages of an adjuvant (Break-Thru®) and an insecticide (deltamethrin) on radial growth of *Beauveria bassiana* in an amended media bioassay experiment. The fungicide control used was Amistar Top®. Means with the same letter are not significantly different at $p<0.05$.

3.5 Discussion

Synthetic agrochemicals and biological control agents are the principal strategies used to control agricultural pests and diseases (Zibaee, 2019). To maximise efficiency and reduce costs associated with repetitive applications, biologicals are often used in conjunction with agrochemicals. In this study, deltamethrin, an insecticide registered for the control of wattle mirid, and Break-Thru® were found to be compatible with three biocontrol fungi, *A. uredinophilus*, *S. filum* and *B. bassiana*.

Previous studies on compatibility testing have shown similar results. The insecticide deltamethrin, which is frequently employed in combating sunn pests (*Eurygaste*r spp.), which are major pests of wheat and barley in the Middle East and Africa, was also found to be compatible with *Akanthomyces muscarium* (Petch) Spatafora, Kepler & B. Shrestha (Gül *et al*., 2022). Kim and Kim (2007) also found that deltamethrin did not inhibit *Akanthomyces attenuatum* Zare and Gams. However, Saindane (2007) found that deltamethrin was incompatible with *Verticillium lecanii* Zimmerman due to changes in pH of media, which became either acidic or alkaline. These findings suggest that the compatibility of deltamethrin with *Akanthomyces* spp. may vary. Break-Thru® and *B. bassiana* were found to work well together, exhibiting synergistic insecticidal efficacy against whitefly (*Bemisia tabaci* Gennadius*)* (Mascarin *et al*., 2014). Havugimana (2017) showed that 0.01% of Break-Thru® was compatible with *A. muscarium* and the combination significantly reduced soybean rust (SBR) *Phakopsora pachyrhizi* Syd. & P. Syd.

In conclusion, Break-Thru® and deltamethrin had little or no effect on the growth of *A. uredinophilus*, *S. filum* and *B*. *bassiana*. Based on these results, biocontrol agents *A. uredinophilus* and *S. filum* could be used with deltamethrin and Breakthru®, or *A. uredinophilus* and *S. filum* could be used with *B. bassiana* and Breakthru® in an integrated biological control programme for the control of both wattle mirid and wattle rust concurrently.

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CHAPTER 4: Pot trial evaluation of the biocontrol efficacy of selected strains of *Sphaerellopsis filum* and *Akanthomyces uredinophilus* on wattle rust

4.1 Abstract

Wattle rust, caused by *Uromycladium acaciae* (Cooke) P. Syd. & Syd., is the most economically important disease currently affecting black wattle (*Acacia mearnsii* De Wild) plantations in South Africa. A severe infection of this disease will result in reduced growth, and even mortality, of young wattle trees. Selected isolates of *Sphaerellopsis filum* (Biv.-Bern. ex Fr.) Sutton [=*Darluca filum* (Biv.-Bern. ex Fr.) Berk.] and *Akanthomyces uredinophilus* Manfrino et al., pers. comm. Mwelasi are potential biological control agents of wattle rust. In this study, the biological control efficacy of mycoparasitic strains of *S. filum* and *A. uredinophilus* were evaluated for control of wattle rust. Rust-infected cuttings of three wattle varieties (PSO14, FW54, SP644) were treated with *S. filum* and *A. uredinophilus* every two weeks for 12 weeks. They were evaluated for rust levels every two weeks, as percentage leaf area infected. Experimental controls included a fungicide control (positive control) and untreated rust control (negative control). Bi-weekly applications of *S. filum* significantly reduced ($p < 0.001$) rust disease progression. Although applications with *A. uredinophilus* did reduce disease progression, it did not produce a significant reduction compared to the untreated rust control. After six and nine weeks of bi-weekly treatment applications, characteristic white mycelial growth of *A. uredinophilus* and *S. filum,* respectively, were observed on rust pustules. Characteristic spore morphology that resembled that of S. *filum* and *A. uredinophilus* was confirmed after re-isolation. Overall, both strains of *S. filum* and *A. uredinophilus* have the potential to control wattle rust.

4.2 Introduction

Wattle rust, caused by *Uromycladium acaciae* (Cooke) P. Syd. & Syd., is the most economically important disease currently affecting black wattle plantations in South Africa. Since its initial outbreak in the KwaZulu-Natal Midlands during the 2013/2014 summer, the disease has spread rapidly throughout the main growing areas and is now considered to be a major threat to the long-term economic sustainability of the South African black wattle industry (Chan and Isik, 2019). The disease causes matting of leaves, pinnule and rachis malformation and severe stunting of cuttings and young trees (McTaggart *et al*., 2015). Little and Payn (2016) reported yield losses of up to 40% in untreated trees.

Azoxystrobin-based fungicides and the development and deployment of resistant or tolerant lines of *A. mearnsii* are the two currently available control measures for wattle rust (Little and Payn 2016, Chan and Isik, 2019). However, the development of wattle rust-tolerant clones is expensive and time consuming. Furthermore, registered fungicides with active ingredients of cyproconazole and propiconazole have been prohibited for use by the South African registrar (Act 36 of 1947) due to their toxicity according to the criteria Carcinogenicity, Mutagenicity and Reproductive (CMR) Toxicity and will be phased out from June 2024 (Meyer, 2023). In addition, excessive fungicide utilization may promote the generation of fungicide resistance in rust strains, which would result in the reduced effectiveness of chemical control methods. Therefore, there is an urgent need to develop alternative control measures for wattle rust that will comply with Forest Stewardship Council (FSC) regulations and also meet the criteria of CMR toxicity.

Biological control is a cost effective and environmentally safe option that could constitute a broader part of the integrated pest management strategy for wattle rust. *Sphaerellopsis filum* (Biv.-Bern. ex Fr.) Sutton [=*Darluca filum* (Biv.-Bern. ex Fr.) Berk.] and *Akanthomyces uredinophilus* Manfrino et al. are amongst the potential biological control agents of wattle rust. They parasitize a variety of rusts and are well-known mycoparasites globally (Trakunyingcharoen *et al.,* 2014). *Sphaerellopsis filum* can be found on a variety of rusts (Trakunyingcharoen *et al*., 2014). In Australia, *Eudarluca filum*, (the teleomorph of *Sphaerellopsis filum)*, has been shown to reduce gall spore output by 50% on gall forming *Uromycladium* sp. on *Acacia stricta* ((Andrews) Willd.)

(Burges, 1934). In experimental studies, it can also infect *Puccinia* urediniospores and reduce rust spore generation by up to 98% (Hunter *et al*., 2003). In 2016, Fraser *et al*. (2021) found conidiomata of a species of *Sphaerellopsis* parasitizing telia of *U. acaciae* on foliar samples of taken from three wattle plantations in Mpumalanga and suggested that *Sphaerellopsis* species be investigated as a potential biocontrol agent for wattle rust. *Akanthomyces* spp. also parasitize many rust fungi, including coffee rust (*Hemileia vastatrix* Berkeley & Broome), Frangipani rust (*Coleosporium plumeriae* Pat.) and leek rust (*Puccinia allii* Rudolphi) (Uma and Taylor, 1987; Weeraratne and Adikaram, 2006; Havugimana, 2017). This study aimed to investigate the efficacy of mycoparasitic strains of *S. filum* and *A. uredinophilus* as biological control agents for wattle rust.

4.3 Methods and Materials

4.3.1 Commercial wattle varieties

The efficacy of bi-weekly applications of mycoparasitic strains of *S. filum* and *A. uredinophilus* were evaluated on three commercial wattle varieties with varying levels of susceptibility to wattle rust. Seedling trays with cuttings of PSO14 (susceptible), SP644 (moderately resistant) and FW54 (susceptible, frost tolerant) were obtained from Sunshine Seedlings Services (Pietermaritzburg, KZN). At least two weeks before inoculations, cuttings were pruned back to 10–20 cm to allow for the development of new growth. To ensure uniform rust infection, cuttings were artificially inoculated with a teliospore suspension of *U. acaciae.* Teliospores were harvested from rust-infected leaf material collected from a wattle plantation in Hodgsons (Sappi, KZN Midlands) by placing the infected leaf material in sterile distilled water containing 0.05% Tween 20 and stirring with a magnetic stirrer. The teliospore suspension (10^6 spores ml^{-1}) was sprayed onto the wattle cuttings in seedling trays to the point of runoff, and seedling trays were immediately placed in transparent plastic bags to ensure maximum humidity. The inoculated cuttings were incubated in a dark room at 18ºC for 48 hours. The cuttings were then returned back to the nursery for symptom development. Infected cuttings displaying ~5% rust infection was selected and transplanted into

20cm pots containing seedling mix (Gromor, Cato Ridge, KZN). Adequate overhead irrigation was utilized to ensure ideal conditions for further rust infections, and hyperparasitism by the two putative biocontrol agents. The cuttings were regularly treated with Deltamethrin (1ml.L^{-1}) to ensure the control of infestations of the brown wattle mirid (*Lygidolon laevigatum* Reuter).

4.3.2 S. filum and A. uredinophilus preparation, treatment and disease assessments

Samples of a formulated product containing *S. filum* were obtained from Andermatt-PHP (Nottingham Road, KZN). A total of 1 gram of *S. filum* (5.8 x 10^8 conidia.ml^{-1}) product was added to 1000 ml of sterile distilled water, amended with 250 µl of Break-Thru (Evonik Industries, Germany) to provide 0.025% of the silicone adjuvant in the suspension. The suspension was shaken and allowed to stand for 30 minutes before spraying to allow adequate release of conidia from pycnidia. For inoculum of *A. uredinophilus*, sporulating cultures were obtained from University of KwaZulu-Natal (PhD student-Peace Mwelasi, Department of Plant Pathology, Pietermaritzburg). Conidia of *A. uredinophilus* were harvested from PDA plates by washing fungal mycelia with sterilized distilled water using a sterilized hockey stick and filtering it through sterilized cheese cloth. The conidial suspension was examined under a light microscope, counted using a haemocytometer and adjusted to 10^6 conidia ml^{-1}. The conidial suspension was made up to 1000 ml and amended with 250 µl of Break-Thru (Evonik Industries, Germany). Every two weeks, cuttings were sprayed with the *S. filum* and *A. uredinophilus* suspensions till the point of runoff. Prior to every spray treatment, percentage rust severity (0 to 100%) was scored on the fully expanded leaves directly below the new flush. A total of six spray treatments and disease ratings were done over a period of 12 weeks.

4.3.3 Experimental design and layout

A two-factor factorial arranged in a randomized complete block design was utilized. The factorial design consisted of four levels of chemical treatments (*A. uredinophilus*, *S. filum*, fungicide-treated control and untreated control) and three levels of wattle varieties (PSO14, SP644, FW54). Two pots formed an experimental unit that was used to compare treatments, with six replicates per treatment. The trial was maintained under overhead irrigation in a shaded greenhouse at the Institute for Commercial Forestry Research (ICFR), Pietermaritzburg, South Africa (29.6265° S, 30.4050° E) for 12 weeks.

4.3.4 Data analysis

Data were analysed using the R statistical software package (R Development Core Team, 2012). The percentage (%) rust severity over 12 weeks period was used to calculate the area under disease progress curve (AUDPC) (Shaner and Finney, 1977). An analysis of variance (ANOVA) was used to determine treatment effects. Significant difference between treatments at a 5% probability level was determined using Fisher's unprotected least significant difference (LSD) test. The effect of treatments of *A. uredinophilus* and *S. filum* on rust disease progression was determined by comparing the AUDPC values to that of the fungicide-treated control and the untreated control.

4.3.5 Confirmation of A. uredinophilus and S. filum colonisation of rust pustules

Rust pustules on pinnules from treated plants were routinely inspected for characteristic white mycelial growth of *S. filum/ A. uredinophilus*. Leaves with rust pustules with suspected *S. filum/ A. uredinophilus* colonisation was selected, surface sterilized, dried on a laminar flow bench, and plated onto selective PDA media amended with chloramphenicol (50mg.ml^{-1}). The resulting culture was inspected under light microscopy for confirmation of fungal spore morphology.

4.4 Results

Results of the ANOVA (Table 4.1) showed that the treatments yielded a significant response (p<0.001). The choice of wattle variety was not significant and there was no significant interaction between treatment and wattle varieties.

Table 4.1: Summary of analysis of variance (ANOVA) showing significance level (p) of variance ratios for AUDPC values in the pot trial evaluation of biocontrol efficacy of *Sphaerellopsis filum* and *A. uredinophilus* on wattle rust

Source of variation		**d.f.**	**AUDPC**	
treatment		3	<0.001***	
Wattle variety		2	0.09605	
rep		5	0.69624	
treatment.wattle variety		6	0.97852	
Residuals		55		
Grand mean			893	
Treatment means				
		Fungicide treated control	405	a
		S. filum	714	ab
		A. uredinophilus	1081	bc
		Untreated rust control	1370	c
Sed	10.51			
%cv	37			
Bartlett test for homogeneity of variances	0.08^{ns}			
Shapiro-Wilk test for normality	0.34^{ns}			

*** denotes significance at p< 0.001 and ns = not significant.

In comparison to the untreated rust control and fungicide-treated plants, applications with *S. filum* significantly reduced rust disease progression. Although applications with *A. uredinophilus* did reduce rust disease progression, it did not produce a significant reduction when compared to the untreated rust control.

For wattle variety response to treatment (Fig 4.1), it was observed that FW54 and PSO14 plants treated with the fungicide yielded the least amount of rust. Untreated FW54, PSO14 and SP644 plants yielded the highest levels of wattle rust. Treatment with *S. filum* to PSO14 produced the most significant reduction in rust disease progression. Applications with *A. uredinophilus* did not produce a significant reduction when compared to the untreated rust control of all wattle varieties.

The ICFR had previously ranked the wattle cultivars PSO14 as susceptible, SP644 as moderately resistant and FW54 as susceptible. However, in this trial SP644 developed the most disease, and PSO14 and FW54 developed an equal level of disease. Statistically, the three cultivars were equal in their susceptibility to wattle rust.

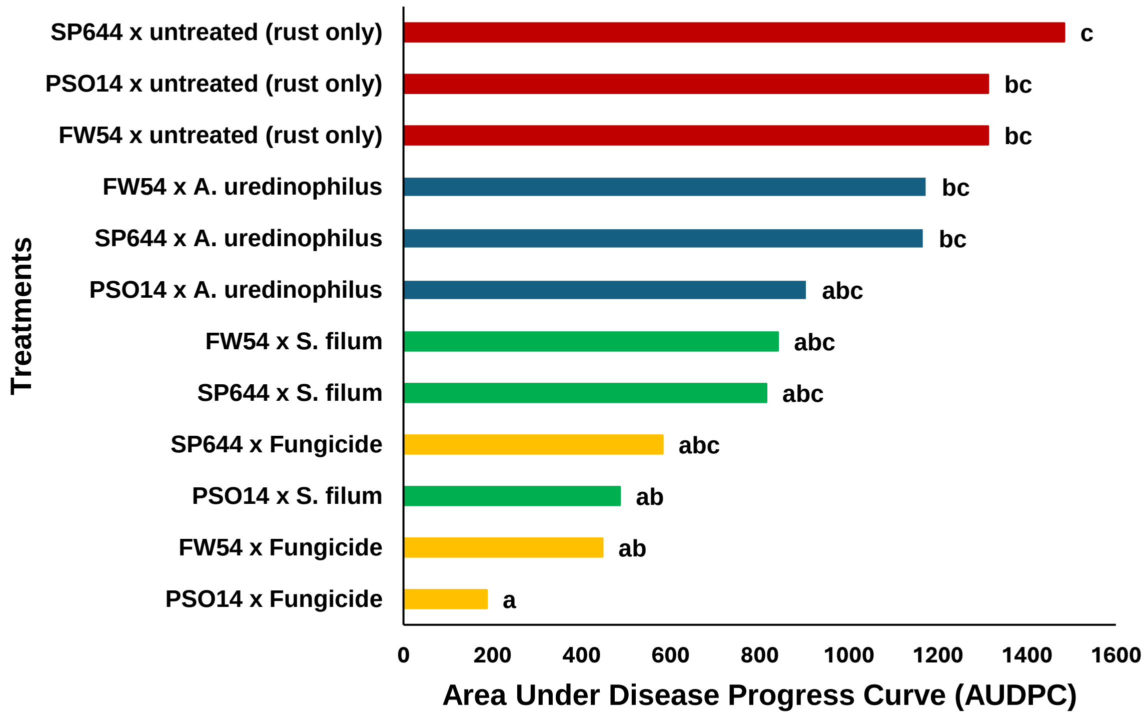

Figure 4.1: Effect of treatment with *Akanthomyces uredinophilus* and *Sphaerellopsis filum* on different wattle varieties.

Microscopy of re-isolated *A. uredinophilus* and *S. filum* from treated plants confirmed their colonisation of rust pustules (Fig 4.2). White mycelial growth was observed on rust pustules from Week Six for *A. uredinophilus* and from Week Nine for *S. filum*. Characteristic spore morphology that resembles that of *S. filum* and *L. lecanii* was confirmed after re-isolation.

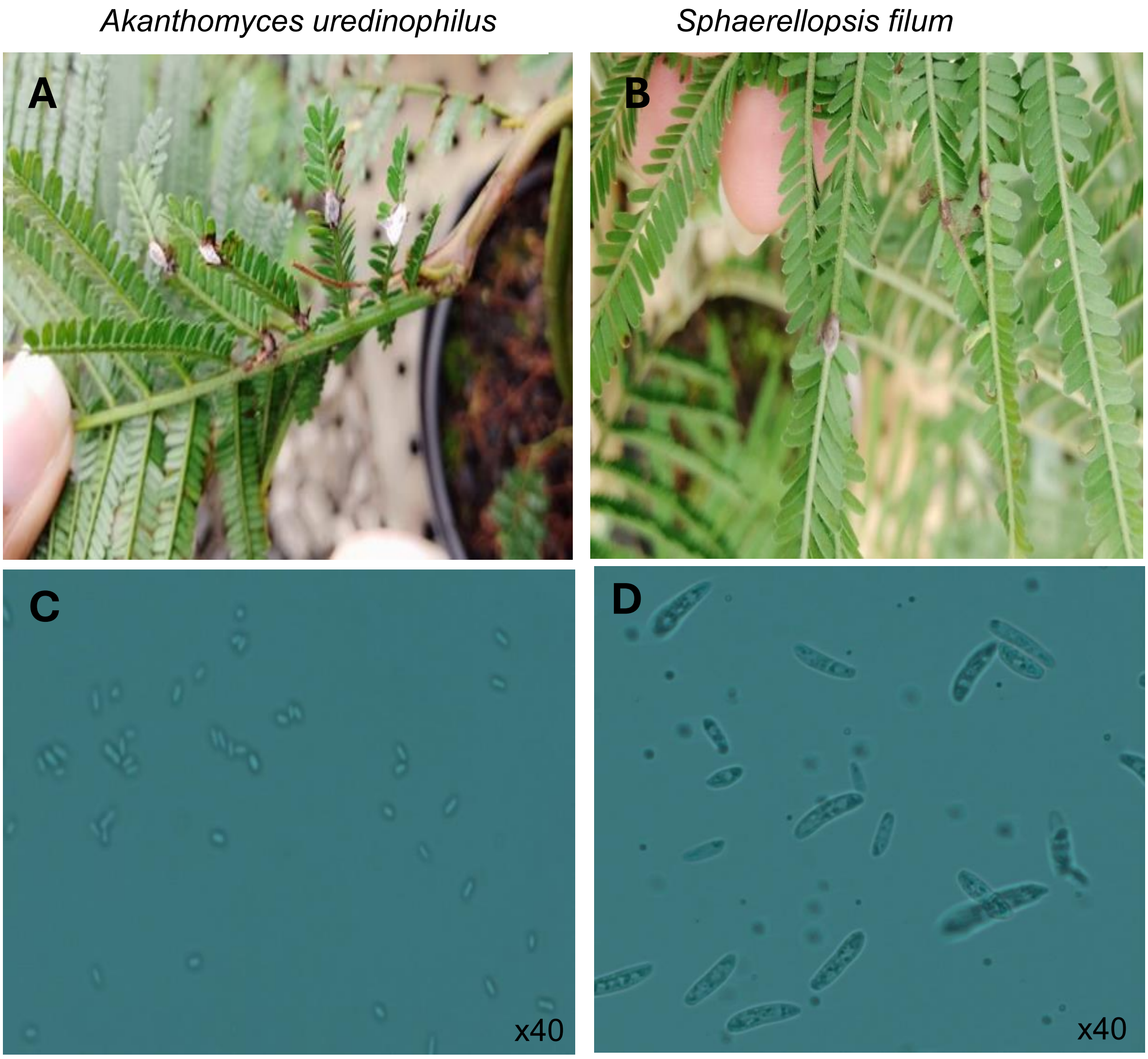

Figure 4.2: Confirmation of *Akanthomyces uredinophilus* and *Sphaerellopsis filum* colonisation of pustules of *Uromycladium acaciae*. (A) *Akanthomyces uredinophilus* colonising rust pustules at Week Six, and (C) conidia morphology examined under light microscopy. (B) *Sphaerellopsis filum* colonising rust pustules at Week Nine, and (D) conidia morphology examined under light microscopy.

4.5 Discussion

In this study, the three wattle varieties did not differ significantly (*p*= 0.09605) in their resistance to rust (untreated controls), nor in their response to treatments with the fungicide, *S. filum* and *A. uredinophilus* In addition, there were no significant interactions (*p*= 0.97852) between the treatments and the wattle varieties. PSO14 plants treated with *S. filum* produced the least reduction in rust progression relative to the untreated control treatment. However, at a plantation level, this wattle variety is genetically improved for desired characteristic (Chan, 2019). Plantations containing genetically modified trees may be more vulnerable to pests and diseases since there is less genetic variation amongst them (Häggman *et al*., 2013). The SP644 wattle clone is recognized for its rust tolerance and high capacity for growth. It is, however, more vulnerable to frost damage (Thompson, 2018), and in this trial, it was not more rust resistant than the other two cultivars. Choosing frost-tolerant clones might help growers to achieve more consistent yields as frost is a major impediment to wattle production in RSA. Hence, the frost tolerant FW54 wattle clone was included in this study and based on the results of this pot trial, this clone reacted favourably to treatments with both *A. uredinophilus* and *S. filum* and was selected for further pot trial experiments.

Treatment with *S. filum* produced a statistically significant reduction in wattle rust. However, although treatment with *A. uredinophilus* did reduce wattle rust progression, the reduction was not significant when compared to the untreated rust control. Nevertheless, both *A. uredinophilus* and *S. filum* can be seen as effective mycoparasites, reducing wattle rust disease on all tested wattle varieties. *Lecanicillum* spp. have been shown to effectively lower the occurrence of several rust diseases in greenhouses at 10^6 conidia ml^{-1} (Havugimana, 2017; Nxumalo, 2018). Based on the results of this pot trial, both *A. uredinophilus* and *S. filum* produced satisfactory reductions in wattle rust disease and were carried forward into further pot trials.

In this study, the observation of characteristic white mycelial growth and spore morphology was used as an indication of successful colonisation of rust pustules by *S. filum* and *A. uredinophilus* Colonisation of rust pustules by *A. uredinophilus* and *S. filum* was visible at six weeks and nine weeks, respectively. This occurrence might be seen as delayed colonisation of rust pustules. However, the observation of white mycelial growth indicates colonisation; and a delay of colonisation does not imply reduced biological control efficiency. Optimum prolonged conditions of temperature and humidity are needed by both *S. filum* and *A. uredinophilus* to sporulate and manifest as characteristic growth, even if infection and colonisation of rust pustules took place much earlier. Therefore, a plausible explanation for the delayed occurrence of mycelial growth could be that nursery conditions up to six to nine weeks of the experiment were not conducive to sporulation of *S. filum* and *A. uredinophilus*

Results of this first pot trial showed that bi-weekly treatments of *A. uredinophilus* and *S. filum* reduced disease progression and that they exhibit potential for wattle rust management. Further experiments are required to determine the ideal dosage and application frequency of *S. filum* and *A. uredinophilus* for optimized control of wattle rust.

4.6 References

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CHAPTER 5: Pot trial evaluation of optimal dosage and application frequency of *Sphaerellopsis filum* and *Akanthomyces uredinophilus* for control of wattle rust

5.1 Abstract

Initial *in vitro* and pot trial screening of *Sphaerellopsis filum* and *A. uredinophilus* showed that both mycoparasites were able to colonise rust teliospores and reduce disease progression of wattle rust (*Uromycladium acaciae* (Cooke) P. Syd. & Syd.). The study aimed to evaluate the optimal dosage and application frequency of *Sphaerellopsis filum* and *Akanthomyces uredinophilus* for control of wattle rust. Mycoparasitic strains of *S. filum* and *A. uredinophilus* were evaluated on the FW54 (susceptible, frost tolerant) commercial wattle variety in a potted nursery trial. Conidial suspensions of the mycoparasitic strains were prepared at dosages of $\frac{1}{2}$x, 1x and 2x at a quantity of $5x10^5$ conidia.ml^{-1}, $1x10^6$ conidia.ml^{-1}, and $2x10^6$ conidia.ml^{-1} of *A. uredinophilus*, and 0.5 gram, 1 gram and 2 gram.L^{-1} of *S. filum* (5.8 x 10^8 conidia.ml^{-1}). The conidial suspensions were sprayed at three frequencies which were once-off, bi-weekly (every two weeks), and monthly. Prior to every bi-weekly spray treatment, percentage rust severity (0 to 100%) was scored for all treatment frequencies on the fully expanded leaves directly below the new flush. The percentage rust severity was used to calculate the area under disease progress curve (AUDPC). For *S. filum* and *A. uredinophilus*, dosage and frequency of application had no significant interaction. All treatments reduced wattle rust relative to the untreated control treatment. Notably, the bi-weekly treatment with a 2x dosage of *A. uredinophilus* resulted in the least amount of rust disease, which was not significantly different to that of the fungicide treated control.

5.2 Introduction

The most commercially significant disease currently affecting black wattle plantations in South Africa is wattle rust (*Uromycladium acaciae* (Cooke) P. Syd. & Syd.) (Fraser *et al*., 2017). *Uromycladium acaciae* is currently controlled primarily through application of azoxystrobin and difenoconazole fungicides (Payn and Little, 2017). However, a change of approach to pesticide usage by international forestry certification schemes such as the Forest Stewardship Council (FSC) has limited the number of applications of fungicides that can be made per season (Forest Stewardship Council, 2018). In addition, registered rust fungicides with the active ingredients of cyproconazole and propiconazole have been prohibited for use by the South African Registrar (Act 36 of 1947) due to their toxicity according to the criteria Carcinogenicity, Mutagenicity and Reproductive (CMR) Toxicity and will be phased out from June 2024 (Meyer, 2023, Meyer, 2024). Hence, Forestry South Africa (FSA) together with the Timber Industry Pesticide Working Group (TIPWG) have mandated the need for evaluating biological control agents as a new and safer alternative control method to traditional synthetic pesticides (Meyer, 2023).

Biological control exploit living agents, customarily those referred to as antagonists or competitors of the causative agents to control plant disease (Tripathi *et al*., 2010). Fungal biocontrol agents exert no negative environmental impact, and their mode of action promotes their high effectiveness against a broad spectrum of insects, pests, weeds, and diseases, without inducing resistance (Savita and Sharma, 2019).

In this study two mycoparasitic fungi, *Sphaerellopsis filum* (Biv.-Bern. ex Fr.) Sutton [=*Darluca filum* (Biv.-Bern. ex Fr.) Berk.] and *Akanthomyces uredinophilus* Manfrino et al., pers. comm. Mwelasi, were shown to colonise (Chapter 2) and reduce the disease progression (Chapter 4) of wattle rust. *Sphaerellopsis filum* can mycoparasitize 369 species in 30 genera of rust fungi worldwide and they are differentially effective against different hosts (Black, 2012, Gordon and Pfender, 2012). Switchgrass rust (*Puccinia emaculata* Schw.), leaf rust (*P. triticina*), stem rust (*P. graminis f. sp. tritici*), oak rust (*C. fusiforme*), willow rust (*M. epitea*) and wattle rust (*Uromycladium acaciae*) are among the spectrum of susceptible hosts (Black, 2012, Gordon and Pfender, 2012, Fraser *et al*., 2021). In addition, *Akanthomyces* spp. are also known mycoparasites of various rust diseases (Trakunyingcharoen *et al.,* 2014). *Akanthomyces lecanii* (Zimmermann) Zare & Gams was reported to infect the fungal pathogen *Puccinia striiformis* f. sp. *tritici* (*Pst.* Eriks) causing wheat stripe rust (Zhan *et al*., 2014). *Akanthomyces* spp. can be entomopathogenic fungi and a mycoparasite capable of combating both the green coffee scale (*Coccus viridis* Green) and coffee leaf rust *(Hemileia vastatrix* Berkeley & Broome) (Jackson *et al*., 2012).

Based on favourable results of a potted nursery trial (Chapter 4), a second trial was initiated to evaluate the optimal dosage and application frequency of *Sphaerellopsis filum* and *Akanthomyces uredinophilus* for control of wattle rust.

5.3 Materials and Methods

5.3.1 Wattle seedlings

The optimal dosage and application frequency of mycoparasitic strains of *S. filum* and *A. uredinophilus* were evaluated on the FW54 (susceptible, frost tolerant) commercial wattle variety. Trays of seedlings of FW54 were obtained from Sunshine Seedlings Services (Pietermaritzburg, KZN). Seedlings were pruned back to 10–20 cm at two weeks before inoculation to promote the production of new growth that will be susceptible to rust infection. To achieve uniform rust infection across the seedlings, artificial inoculation using a teliospore suspension of *U. acaciae* was employed. Teliospores were harvested from rust-infected leaf material collected at MSD Timbers in Richmond, KZN. The rust-infected leaf material was immersed in sterile distilled water containing 0.05% Tween 20 and the suspension was stirred gently for at least one hour to ensure the release of adequate teliospores. The wattle seedlings were uniformly sprayed with the teliospore suspension (10^6 spores ml^{-1}) to the point of runoff. Immediately after inoculation, seedling trays were enclosed in transparent plastic bags to maintain optimal humidity levels. Subsequently, the inoculated seedlings were placed in a dark room and incubated at a temperature of 18°C for a period of 48 hours to induce ideal conditions for the establishment of rust infection. Trays were then maintained in the nursery to allow for adequate levels of rust infection. Infected seedlings displaying ~5% rust infection was selected and transplanted into 20cm pots containing seedling mix (Gromor, Cato Ridge, KZN). Adequate overhead irrigation was utilized to ensure ideal conditions for further rust infections. To mitigate infestations of the brown wattle mirid (*Lygidolon laevigatum* Reuter), seedlings were regularly treated with Deltamethrin (1ml. L^{-1}).

5.3.2 S. filum and A. uredinophilus inoculum preparation, treatment and disease assessments

For *A. uredinophilus*, white rice (Nalma brand) was double autoclaved at 121˚C for 15 minutes to remove impurities. After cooling, 1 cm discs of *A. uredinophilus* culture from potato dextrose agar (PDA) plates were used to inoculate the rice for mass sporulation. At three weeks, a conidial suspension of *A. uredinophilus* was harvested from the rice culture by washing the colonised rice grains with sterilized distilled water and filtering it through sterilized cheese cloth. The conidial suspension was examined under a light microscope, counted using a haemocytometer and adjusted to dosage $\frac{1}{2}$x, 1x and 2x at a quantity of $5x10^5$ conidia.ml^{-1}, $1x10^6$ conidia.ml^{-1}, and $2x10^6$ conidia.ml^{-1}, respectively. A total of 250 µl of Break-thru® (Evonik Industries, Germany) was added to 1000ml of spore suspension. For *S. filum*, dosage $\frac{1}{2}$x, 1x and 2x was prepared by adding 0.5 grams, 1 gram, and 2 grams to 1000ml of sterile distilled water respectively and amended with 250 µl of Break-thru® (Evonik Industries, Germany). The resulting spore suspensions were sprayed at three frequencies which were once-off, bi-weekly (every two weeks), and monthly. Bi-weekly treated plants received a total of five spray treatments, while monthly treated plants received a total of three spray treatments. The *S. filum* and *A. uredinophilus* suspensions were sprayed till the point of runoff. Prior to every bi-weekly spray treatment, percentage rust severity (0 to 100%) was scored for all treatment frequencies on the fully expanded leaves directly below the new flush. The disease ratings were done over a period of 13 weeks. Experimental control treatments included a fungicide control (positive control Amistar® Top, Syngenta) and untreated rust control (negative control).

5.3.3 Experimental design

A three-factor factorial arranged in a split plot design was utilized. The factorial design consisted of four levels of chemical treatments (*A. uredinophilus*, *S. filum*, fungicide-treated control and untreated control), three levels of biological control dosages ($\frac{1}{2}$x, 1x, 2x) and three levels of application frequencies (Once-off, Bi-weekly and Monthly). Two pots formed an experimental unit that was used to compare treatments, with three replicates per treatment. The trial was maintained under overhead irrigation in a shaded greenhouse at the Institute for Commercial Forestry Research (ICFR), Pietermaritzburg, South Africa (29.6265° S, 30.4050° E) for 13 weeks.

5.3.5 Data analysis

The data were analysed using the R statistical software package (R Development Core Team, 2012). To assess the progression of rust severity over a 13-week period, the percentage (%) rust severity was utilized to compute the area under the disease progress curve (AUDPC) (Shaner and Finney, 1977). Treatment effects were investigated through an analysis of variance (ANOVA). The square root transformation was used when the data did not meet the assumptions of ANOVA. The significant differences between treatments at a 5% probability level were determined utilizing Fisher's unprotected least significant difference (LSD) test. The effect of treatments of *A. uredinophilus* and *S. filum* on rust disease progression was determined by comparing the AUDPC values to that of the fungicide treated control and the untreated control.

5.4 Results

For *Akanthomyces uredinophilus*, all the treatments reduced wattle rust relative to the untreated control treatment. Notably, the bi-weekly 2x dosage yielded the least amount of rust disease which was not significantly different to that of the fungicide treated control. Half dosage and 1x dosage applied monthly yielded the least reduction of wattle rust compared to the untreated control. In addition, there was no significant (p= 0.16683) interaction between the dosage and frequency (Table 5.1, Fig 5.1).

Table 5.1: Summary of analysis of variance (ANOVA) showing significance level (p) of variance ratios for AUDPC values in the pot trial evaluation of optimal dosage and application frequency of *Sphaerellopsis filum* and *A. uredinophilus* on wattle rust

	Akanthomyces uredinophilus		*Sphaerellopsis filum*	
Source of variation	Df		Df	
Dosage	2	0.02608*	2	0.07691^{ns}
Frequency	2	0.05935^{ns}	2	0.34531^{ns}
Replicates	2	0.43693^{ns}	2	0.31632^{ns}
Dosage: frequency	4	0.16683^{ns}	4	0.57408^{ns}
Residuals	14		11	
Sd		360.2214		5.0477
Coefficient of variation (%)		26.48256		12.9673
Homogeneity of variance (Bartlett's test)		0.4302^{ns}		0.02046
Normality (Shapiro Wilk test)		0.8376^{ns}		0.009045

For *Sphaerellopsis filum*, all the treatments reduced wattle rust relative to the untreated control treatment. However, there was no significant difference between all the treatments relative to each other and all treatments were significantly different from both untreated (negative control) and fungicide-treated (positive control). In addition, there was no significant (p= 0.57408) interaction between the dosage and frequency (Table 5.1, Fig 5.2).

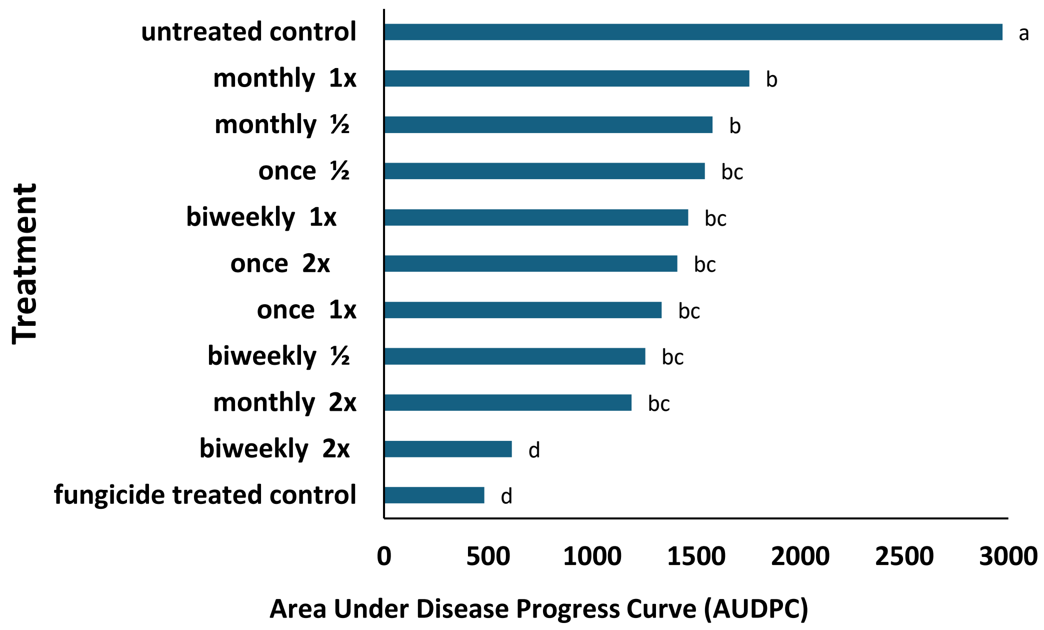

Figure 5.1: Effect of *Akanthomyces uredinophilus* applied at 1x, $\frac{1}{2}$x and 2x dosages and at once-off, bi-weekly and monthly frequencies on the disease progression of wattle rust (*Uromycladium acaciae*).

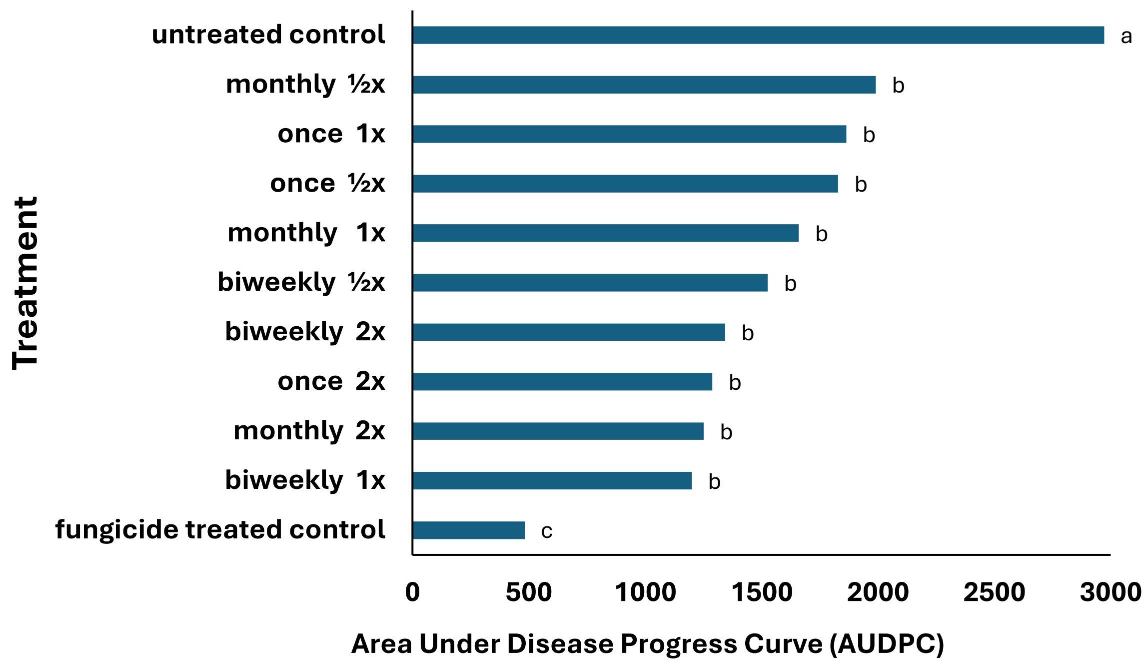

Figure 5.2: Effect of *Sphaerellopsis filum* applied at 1x, $\frac{1}{2}$x and 2x dosages and at once-off, bi-weekly and monthly frequencies on the disease progression of wattle rust (*Uromycladium acaciae*).

5.5 Discussion

In this study, an application of *Akanthomyces uredinophilus* at $2x10^6$ conidia ml^{-1} (2x dosage) every two weeks yielded the highest reduction of wattle rust. This reduction of disease was not significantly different to that of the fungicide control. Nxumalo (2018) showed that maximum pustule colonisation was observed at the dose of $1x10^6$ conidia ml^{-1} of *Akanthomyces attenuatum* (Zimm.) Zare and Gams in a dose trial conducted to control *Puccinia oxalidis* Dietel & Ellis (oxalis rust) pustules on *Oxalis latifolia* Kunth leaves (Nxumalo, 2018). This concentration is equivalent to the standard dosage in this study. Results indicate that a higher dosage applied more frequently can suppress disease progression to levels similar to that obtained using a fungicide. All tested dosages sprayed once-off were not significantly different from each other, but they did significantly reduce wattle rust compared to the untreated control. Applying treatments at intervals has been shown to effectively keep pathogen populations below the threshold level (Heydari and Pessarakli, 2010). In addition, low dosages of the biocontrol agents may result in poor efficacy (Nxumalo, 2018). Application of treatments at high dosages account for the high levels of control (Miller *et al*., 2004). In this study, bi-weekly treatments achieved a high reduction of wattle rust. This would imply that repeated application would be required to replenish the biocontrol fungal inoculum to a level that could establish effective colonisation of the rust pathogen (Folorunso *et al*., 2022: Miller *et al*., 2004).

For *Sphaerellopsis filum*, all treatments did reduce the amount of wattle rust relative to the untreated rust control. However, this reduction was not comparable to that of the fungicide control. Gordon (2011) demonstrated that both single application and monthly applications of *S. filum* resulted in a notable rise in the proportion of *Puccinia graminis* subsp. *graminicola* (Pgg) Eriksson & Henning pustules infected with *S. filum*, in comparison to the untreated control treatment. *Sphaerellopsis filum* has the ability to decrease sporulation and limit the lifespan of rust pustules once it has colonised (Gordon, 2011). In this study, *S. filum* did demonstrate an acceptable level of control over the three-month duration of the trial. Over an extended period of time, the rate of colonisation may increase which could result in greater disease reduction.

Overall, *A. uredinophilus* applied at 2x10^6 conidia ml^{-1} (2x dosage) every two weeks was the superior treatment. This application regime showed significant promise for advancement as a biological control method for wattle rust due to its quick reduction in disease levels. Further evaluations should adopt this application regime.

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CHAPTER 6: Combining silicon fertilization with a biocontrol agent (*Akanthomyces uredinophilus*) for the management of wattle rust

6.1 Abstract

Fertilization with potassium silicate (silicon) can be used in agriculture to mitigate abiotic and biotic stress in some crops, for example due to its ability to enhance plant resistance to insect pests. This study evaluated the use of silicon fertilization in conjunction with *Akanthomyces uredinophilus* Manfrino et al., pers. comm. Mwelasi (the best performing biocontrol treatment as identified in Chapter 5) for optimal control of wattle rust (*Uromycladium acaciae* (Cooke) P. Syd. & Syd.) in a nursery pot trial. A spray application of $2x10^6$ conidia.ml^{-1} of *A. uredinophilus* was combined with silicon fertilization at concentrations of 100, 200, and 400 mg.L^{-1} for evaluation of the control of wattle rust on the commercial wattle variety FW54. The *A. uredinophilus* spore suspension was sprayed bi-weekly (every two weeks), and treated seedlings were drenched every week with 10L of water containing silicon at 100, 200, and 400 mg.L^{-1}. Prior to every bi-weekly *A. uredinophilus* spray treatment, percentage rust severity (0 to 100%) was scored on the fully expanded leaves directly below the new flush. Experimental control treatments included a fungicide control (positive control, Amistar® Top, Syngenta) and an untreated rust control (negative control). At 16 weeks, leaf samples were taken from seedlings to evaluate the uptake and distribution of silicon using Field Emission Gun-Environmental Scanning Electron Microscopy (FEG-ESEM) and Energy Dispersive X-ray Spectroscopy (EDX). All treatments reduced wattle rust relative to the untreated control. Treatment with *A. uredinophilus* + 400mg.L^{-1}, silicon resulted in the least amount of rust. However, the *A. uredinophilus* + 0mg.L^{-1} silicon treatment also produced a significant reduction in rust levels. The EDX spectra and mapping showed that silicon was not taken up by the treated plants. The combined application of *A. uredinophilus* and silicon fertilization did not result in the enhanced control of wattle rust because wattle appears not to take up silicon as a micronutrient.

6.2 Introduction

Silicon fertilization has been adopted by the forestry industry due to its role in increasing plant resistance to insect pests (de Queiroz *et al.*, 2016). Silicon is absorbed by some plant species by their roots in silicon's soluble form, monosilicic acid (H_4SiO_4) (Meena *et al*., 2014). Its uptake is directed to the cell wall of the epidermis where it supports and strengthens cell tissue, thus providing a structural defence from insects and invading pathogens (Soomro, 2000, de Queiroz *et al*., 2016).

Accumulated content of monosilicic acid in plant sap may polymerize into amorphous silica (SiO_2-nH_2O) if the concentration reaches a critical level (Meena *et al*., 2014, Kubheka, 2015). The deposited silica, particularly in the cell wall, enhances cell wall strength and rigidity upon interacting with polyphenols and pectin (Kubheka, 2015). Silicon enhances plant resistance of some plants, such as monocotyledons, to insect attack by forming phytoliths in plant leaves, which increase tissue rigidity, weakening herbivore mouthparts, and reduce leaf digestibility (Verma *et al*., 2021). To inhibit pathogen colonisation, silicon primes plants for enhanced levels of systemic acquired resistance (SAR), which results in the synthesis of antimicrobial compounds by promoting plant tolerance capacity associated with signalling pathway activation and gene expression (Verma *et al*., 2021).

Kang *et al*. (2023) reported that the combination of silicon and biological control agents may increase biocontrol efficacy. The synergistic effect of silicon (applied as potassium silicate) and *Beauveria bassian*a (Bals.-Criv.) Vuill resulted in the control of red spider mites (*Tetranychus urticae*, Koch) on tomato plants (Laing *et al*., 2006). Similarly, a 92% mortality of spider mites was induced by the combination of potassium silicate and the fungus *B. bassiana* (Verma *et al*., 2021). *Metarhizium robertsii* (Strain 2017) formulated as blastospores and metabolites, combined with silicon dioxide nanoparticles with an amide group, caused enhanced mortality of Colorado potato beetle (*Leptinotarsa decemlineata* Say.) larvae and crucifer flea beetles (*Phyllotreta* sp. L.) (Shatalova *et al*., 2022).

Currently, cost effective and environmentally friendly approaches are prioritised in the control of wattle rust. Silicon fertilization plays a vital role in integrated pest management of pests and diseases because it is environmentally friendly and is compatible with other management strategies such as biological control (Laing *et al*., 2006). Hence, this study evaluated the use of silicon fertilization in conjunction with *Akanthomyces uredinophilus* Manfrino et al., pers. comm. Mwelasi, a biocontrol treatment evaluated previously (Chapter 5). The combination was evaluated in the hope of finding additive or synergistic control of wattle rust (*Uromycladium acaciae* (Cooke) P. Syd. & Syd.).

6.3 Materials and Methods

6.3.1 Wattle seedlings

Trays of FW54 wattle seedlings were obtained from Sunshine Seedlings Services (Pietermaritzburg, KZN) and artificially inoculated with a teliospore suspension of *U. acaciae* according to the method outlined in Section 4.3.1 (Chapter 4). Infected seedlings displaying ~5% rust infection were selected and transplanted into 20cm pots containing artificial growing media (Organics for Africa, Greytown, KZN). Overhead irrigation was utilized to ensure ideal conditions for further rust infections. To mitigate infestations of the brown wattle mirid (*Lygidolon laevigatum* Reuter), the seedlings were regularly treated with deltamethrin (1ml.L^{-1}).

6.3.2 Akanthomyces uredinophilus preparation and treatment

White rice (Nalma brand) was double autoclaved at 121˚C for 15 minutes to remove contaminants. After cooling, 1 cm discs of *A. uredinophilus* culture from potato dextrose agar (PDA) plates were used to inoculate the rice for mass sporulation. After three weeks, a conidial suspension of *A. uredinophilus* was harvested from the rice culture by washing the colonised rice grains with sterilized distilled water and filtering it through sterilized cheese cloth. The conidial suspension was examined under a light microscope, counted using a haemocytometer and adjusted to dosage $2x10^6$ conidia.ml^{-1}. A total of 250 µl of Break-thru® (Evonik Industries, Germany), a silicon adjuvant, was added to 1000ml of the spore suspension. The spore suspension was sprayed bi-weekly (every two weeks) to the point of runoff. Experimental control treatments included a fungicide control (positive control, Amistar® Top, Syngenta) and an untreated rust control (negative control).

6.3.3 Application of soluble silicon

Silicon fertilization was employed where silicon was applied to the seedlings in the form of potassium silicate (K_2SiO_3) (21% a.i.) using a drenching method. Treatments commenced four weeks prior to artificial inoculation to allow for sufficient uptake and were continued every week for the duration of the trial. Drenches consisted of 10L of water containing silicon at concentrations of 0, 100, 200, or 400 $mg.L^{-1}$.

6.3.4 Disease assessments

Prior to every bi-weekly *A. uredinophilus* treatment, rust severity as a percentage of leaf area infected (0 to 100%) was scored on the fully expanded leaves directly below the new flush. The disease ratings were done over a period of 11 weeks.

6.3.5 Experimental design

A two-factor factorial arranged in a randomised complete block design was utilized. The factorial design consisted of three levels of control treatment (*A. uredinophilus*, fungicide-treated control and untreated control), and four levels of silicon dosage (0 $mg.L^{-1}$, 100 $mg.L^{-1}$, 200$mg.L^{-1}$, 400$mg.L^{-1}$,). Three pots formed an experimental unit, with six replicates per treatment. The trial was maintained under overhead irrigation in a shaded greenhouse at the Institute for Commercial Forestry Research (ICFR), Pietermaritzburg, South Africa (29.6265° S, 30.4050° E) for 11 weeks.

6.3.6 Data analysis

The data were analysed using the R statistical software package (R Development Core Team, 2012). To assess the progression of rust severity over an 11-week period, the percentage (%) rust severity was utilized to compute the area under the disease progress curve (AUDPC) (Shaner and Finney, 1977). Treatment effects were investigated through an analysis of variance (ANOVA). Where data did not meet the assumptions of ANOVA, a cube-root transformation was applied. Significant differences between treatments at a 5% probability level were determined utilizing Fisher's unprotected least significant difference (LSD) test. The effect of treatments of *A. uredinophilus* combined with 0, 100, 200 and 400 $mg.L^{-1}$, silicon on wattle rust disease progression was determined by comparing the AUDPC values to that of the fungicide treated control and the untreated control.

6.3.7 Uptake and distribution of silicon using EDX-ESEM

At 16 weeks, leaf samples were taken from seedlings of each treatment to evaluate the uptake and distribution of silicon. A standard SEM procedure for biological samples was followed: (i) primary fixation: the leaf samples were placed onto ESEM specimen stubs and fixed for 1 hour in 3% buffered glutaraldehyde; (ii) buffer wash: leaf disk samples were soaked in sodium cacodylate buffer for 5 minutes twice; (iii) dehydration: the specimens were immersed in a series of 10%, 30%, 50%, 70% and 90% ethanol each for 5 minutes, and twice then for 5 minutes in 100% ethanol; (iv) coating: the stubs with samples were placed into a Quorum Q150R ES Gold Sputter Coater for coating; (v) critical point drying: A Quorum Q150 RES Critical Point Dryer was used to dry the samples to keep them intact. After drying and coating, the samples were viewed under a ZEISS EVO LS 15 (Carl Zeiss AG) ESEM. FEG-ESEM and EDX were used for visualization of the surface of the samples and identification of the area of interest (lamina containing veins) for subsequent elemental analysis.

6.4 Results

6.4.1 Akanthomyces uredinophilus and silicon for control of wattle rust

The wattle seedlings of the untreated control developed the most wattle rust, but the rust level was not significantly different from the treatment of *A. uredinophilus* combined with 100 $mg.L^{-1}$ silicon. There was no significant difference between the *A. uredinophilus* + 100 $mg.L^{-1}$ silicon and *A. uredinophilus* + 200 $mg.L^{-1}$ silicon treatments. *A. uredinophilus* with 0 $mg.L^{-1}$ silicon and *A. uredinophilus* with 200 $mg.L^{-1}$ silicon significantly reduced wattle rust in comparison with the untreated control, but not in comparison with the fungicide control. *A. uredinophilus* with 400 $mg.L^{-1}$ silicon significantly reduced wattle rust disease relative to the fungicide control treatment. The fungicide control produced the most significant reduction in wattle rust severity (Fig 6.1).

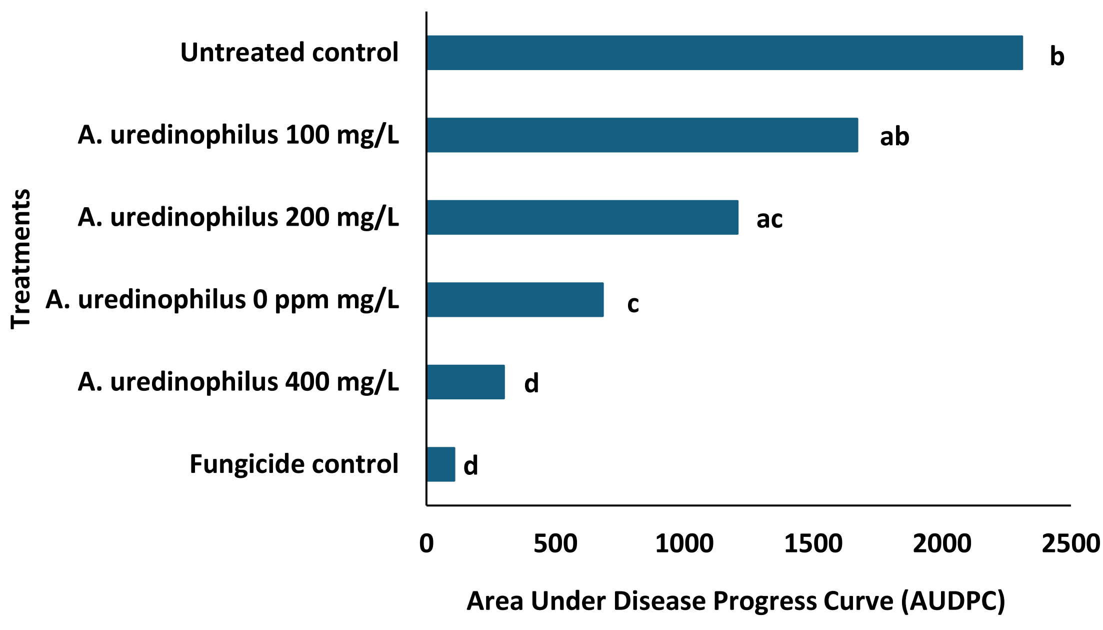

Figure 6.1: Effect of *A. uredinophilus* applied at dosage $2x10^6$ $conidia.ml^{-1}$ alone, or combined with silicon fertilization at three concentrations of 100, 200, and 400 $mg.L^{-1}$ on the disease progress of wattle rust (*Uromycladium acaciae*, *p*= <0.001***, sd= 3.31, cv= 36%, LSD= 1.56).

6.4.2 *EDX-ESEM analysis*

The Energy Dispersive X-ray (EDX) spectra (Fig. 6.2) and EDX mapping (Fig. 6.3) showed that the silicon was present in both treated and untreated plants in very small quantities. The weight percentage of silicon in 0 mg L^{-1} (no silicon) treated plants was 0.37% while in 100 mg L^{-1}, 200 mg L^{-1} and 400 mg L^{-1} treated plants showed a weight percentage of 0.28, 0.34 and 0.07%, respectively. These results suggest that the applied silicon fertilizer was not taken up by the plants, and the amount of silicon detected was naturally present.

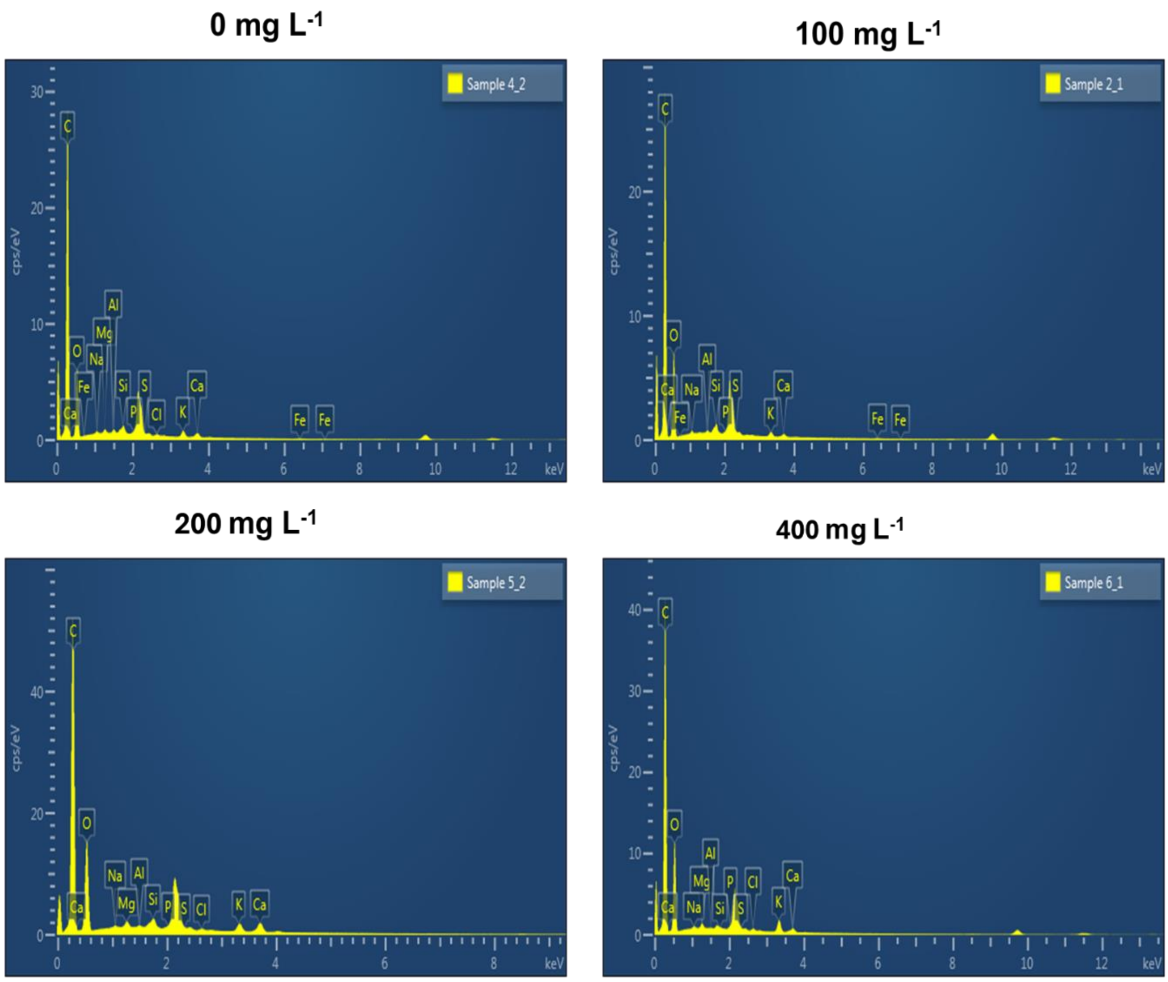

Figure 6.2: Energy dispersive X-ray (EDX) spectra showing elemental composition in black wattle leaves after treatment with 0 mg.L^{-1}, 100 mg.L^{-1}, 200 mg.L^{-1}, and 400 mg.L^{-1} of silicon.

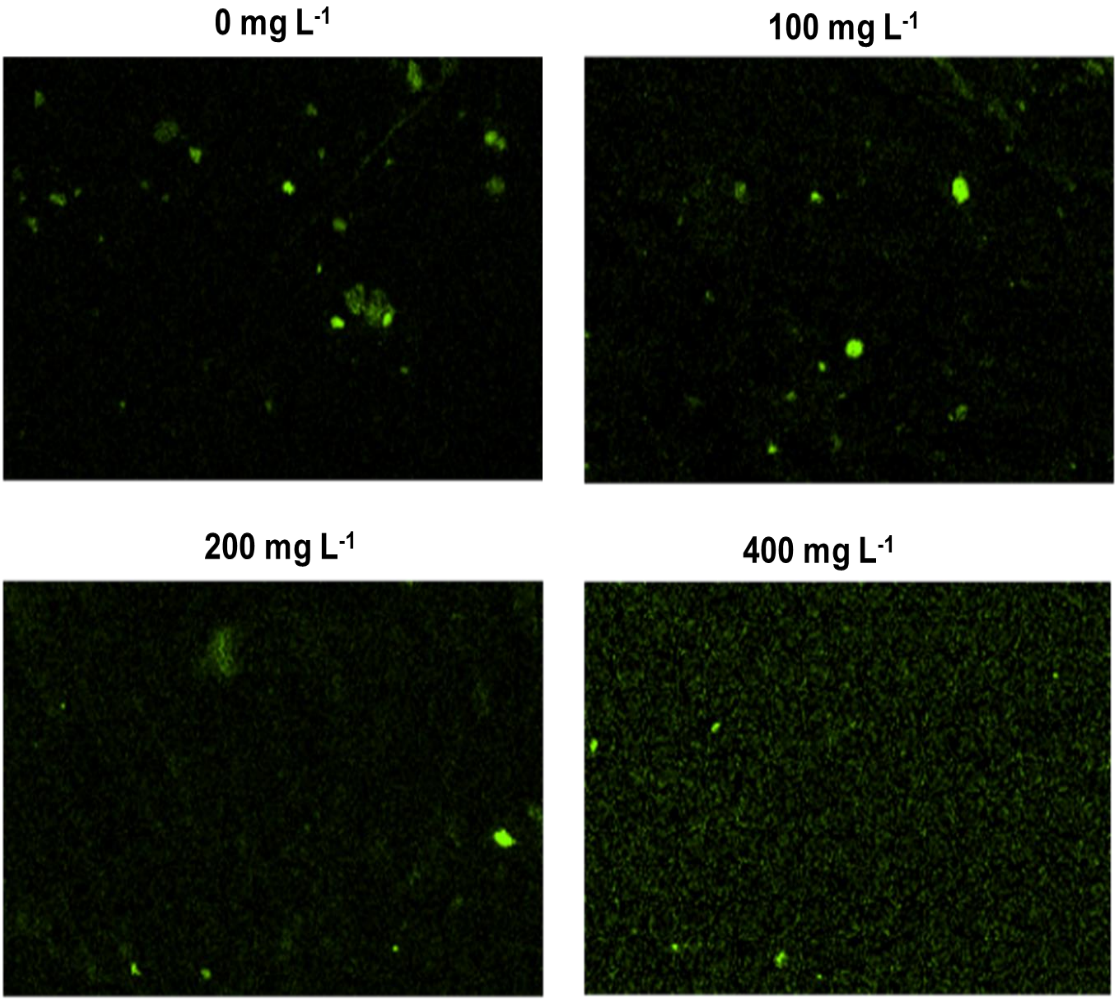

Figure 6.3: EDX mapping of silicon in black wattle leaves after treatment application with 0 mg.L^{-1}, 100 mg.L^{-1}, 200 m. L^{-1}, and 400.mg L^{-1} of silicon.

6.5 Discussion

In this study, fertilization with silicon (Si) in a form of potassium silicate (K_2SiO_3) was combined with an effective biological control treatment (the best performing biocontrol treatment from Pot Trial 2) to determine whether the combination of the two treatments would enhance wattle rust control. Treatment with *A. uredinophilus* combined with 400 $mg.L^{-1}$ of silicon resulted in the least amount of rust. However, the combination of *A. uredinophilus* with 0 $mg.L^{-1}$ silicon treatment also produced a significant reduction in wattle rust. Furthermore, the weight percentage of silicon in untreated plants (0 $mg.L^{-1}$) was 0.37% while the 400 $mg.L^{-1}$ treated plants contained 0.07% weight percentage of silicon. Thus, the EDX analysis of leaf material showed that silicon was not taken up the plant by active supplementation to growing media. This would account for the lack of enhanced control in plants treated with both silicon and *A. uredinophilus*. Additionally, the potassium in the KSilicate could have made a difference in treatments. This is the first study to evaluate the use of silicon to enhance disease resistance in black wattle.

Dik *et al*. (1998) found that silicon did not enhance the performance of the biological control agents *Ampelomyces quisqualis* Cesati, *Verticillium lecanii* Zimmerman and *Sporothrix flocculosa* Traquair, Shaw & Jarvis in controlling cucumber powdery mildew (*Sphaerotheca fuliginea* (Schlecht.:Fr.) Pollacci). However, individual treatments of silicon or biological control agents significantly reduced cucumber powdery mildew. Combinations of isolates of *Bacillus* spp., and *Trichoderma harzianum* Rifai B77 (T.77) with potassium silicate (KSil 255®) had no significant effect on the reduction of grey leaf spot (GLS) caused by *Pyricularia grisea* (Cooke) Sacc. (Dammie, 2017). Moreover, the combination effect of silicon fertilization with various biological control agents may differ.

In this study, the growing medium contained a low level of silicon, as evident in the low levels of silicon in the treatment where silicon fertilization was not applied (0 $mg.L^{-1}$, 0.37% of silicon present). Kanto *et al*. (2006) and Liang *et al*. (2005) noted that silicon fertilizers are more effective when used preventively than curatively. Once symptoms appear on a susceptible plant, applying silicon as a curative measure has little impact because the disease can affect the whole plant within few days. In this study, silicon was applied four weeks prior to rust infection. It could be that this time period was too short to allow for sufficient levels of silicon to be taken up by the plants. Additionally, wattle may be one of several genera of plants that do not take up and use silicon as a physiologically active element that enhances disease resistance expression (Epstein, 1999).

This is the first study to evaluate the combination of silicon fertilization with *A. uredinophilus* to control wattle rust. However, other studies have shown the combination of biocontrol agents with silicon fertilization may be effective in controlling various diseases of other crops (Tesfagiorgis *et al*., 2014, de Queiroz *et al*, 2016; Kang *et al*., 2023). Adding potassium silicate to a combination of *Trichothecium roseum* (Pers.) Link and *Serratia marcescens* Bizio increased silicon content in the leaves of zucchini resulting in the suppression of powdery mildew incidence (Tesfagiorgis *et al*., 2014). Potassium and calcium silicate enhanced *Eucalyptus camaldulensis* Dehnhardt plant resistance against *Glycaspis brimblecombei* Moore*,* in the field and in the nursery (de Queiroz *et al*, 2016). Combining *Cytobacillus firmus* (Bredemann and Werner) Patel and Gupta JBRS159 (JBRS159) with K_2SiO_3 or SiO_2 nanoparticles significantly reduced the disease incidence of bacterial seedling rot (BSR) of rice plants more than K_2SiO_3 or SiO_2 nanoparticles individually (Kang *et al*., 2023). Further evaluations are needed to investigate the effect of silicon fertilization alone as a prolonged preventative measure for the control of wattle rust. Such studies could introduce silicon fertilization early in the nursery, at least six to twelve weeks prior to planting with weekly additional doses after planting, or the use of a slow release silicon fertilizer in the field.

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Chapter 7: Dissertation overview

Introduction

Wattle rust, caused by *Uromycladium acaciae* (Cooke) P. Syd. & Syd., is a severe disease that has caused extensive damage to black wattle plantations in South Africa for over a decade (Chan and Isik, 2019). The use of fungicides is the primary method in the management of wattle rust in South Africa (Oumar *et al*., 2019). However, changes to the list of acceptable fungicides mandated by international and national forestry certification schemes such as the Forest Stewardship Council® (FSC®) [FSC-POL-30-001 V3-0 EN] and the South African Registrar (Act 36 of 1947) have resulted in the banning of all registered rust fungicides (Meyer, 2023). This study was initiated to address the immediate need of the forestry industry to develop alternative control measures for wattle rust that will align with the regulations of the international and national forestry certification schemes (Forest Stewardship Council, 2018; Meyer, 2023). Biological control is an effective and environmentally safe option for the management of wattle rust (Trakunyingcharoen *et al*. 2014). There is currently no commercial biological control agent available for the management of wattle rust. This study evaluated two mycoparasitic fungi, *Sphaerellopsis filum* and *A. uredinophilus*, as potential biological control agents for use in an integrated pest management (IPM) program to manage wattle rust.

The study objectives focused on:

1. Evaluating the ability of the mycoparasites to colonise and mycoparasitise *U. acaciae* using a detached leaf assay and electron microscopy.
2. Evaluating the compatibility of the mycoparasitic fungi (*Sphaerellopsis filum* and *Akanthomyces uredinophilus*) and a commercial insect-pathogenic fungus (*Beauveria bassiana,* Eco-Bb®) with an insecticide (deltamethrin) and an adjuvant (Break-thru®).
3. Evaluating the biocontrol efficacy of two mycoparasitic fungi (*Sphaerellopsis filum* and *Akanthomyces uredinophilus*) on three wattle varieties with varying levels of susceptibility to wattle rust *in vivo* (Pot Trial 1).

4. Determining the optimal dosage and application frequency of the two mycoparasitic fungi (*Sphaerellopsis filum* and *Akanthomyces uredinophilus*) for control of wattle rust *in vivo* (Pot Trial 2).

6. Evaluating the best performing treatment from Pot Trial 2 (*Akanthomyces uredinophilus*, applied bi-weekly at $2x10^6$ conidia.ml^{-1}) in combination with silicon fertilization for enhanced control of wattle rust *in vivo* (Pot Trial 3).

Chapter 2: *In vitro* detached leaf bioassay and microscopy of interactions between mycoparasitic fungi and *Uromycladium acaciae*

Major findings

- Pycnidia of *Sphaerellopsis filum* producing cirri were observed inside wattle rust pustules.
- White mycelial strands of *A. uredinophilus* were observed inside wattle rust pustules.
- *Sphaerellopsis filum* and *A. uredinophilus* colonised ~40% of wattle rust pustules at four weeks post-inoculation.
- Both *S. filum* and *A. uredinophilus* demonstrated mycoparasitic activity on teliospores of *U. acaciae*.

Implications

- Both mycoparasites were able to colonise rust pustules by four weeks post-inoculation.
- Mycoparasitic activity on the over-wintering teliospores of *U. acaciae* would result in a reduction of teliospore viability and a subsequent reduction of overwintering inoculum for the next season. Hence, initial applications may not necessarily result in an immediate decrease in disease severity but rather a decline in the next generation of disease.

Chapter 3: Compatibility of mycoparasitic fungi (*Sphaerellopsis filum* and *A. uredinophilus*) and *Beauveria bassiana* with an insecticide (deltamethrin) and an adjuvant (Breakthru®)

Major findings

- *Sphaerellopsis filum, A. uredinophilus* and *B. bassiana* are compatible with the insecticide (deltamethrin) and the adjuvant (Break-thru®).

Implications

- With dual infections of black wattle mirid and wattle rust commonly encountered in black wattle plantations, the results of this trial indicated that all three tested biological control agents could be incorporated into an IPM program for both wattle mirid and wattle rust.
- The biocontrol efficacy of *B. bassiana* on wattle mirid is not known and requires further investigation if a fully biological approach to pest management is required by the forestry industry.

Chapter 4: Pot trial evaluation of the biocontrol efficacy of selected strains of *Sphaerellopsis filum* and *A. uredinophilus* on wattle rust

Major findings

- Bi-weekly (every two weeks) application of $1x10^6$ conidia.ml^{-1} dosage of *A. uredinophilus* and 1 g.L^{-1} of *S. filum* ($5.8x10^8$ conidia.ml^{-1}) resulted in a significant reduction of wattle rust of all tested wattle varieties.
- Although the wattle variety SP644 developed the most disease, statistically the three tested black wattle cultivars (FW54, PSO14 and SP644) were equal in their susceptibility to wattle rust.
- *Sphaerellopsis filum* and *A. uredinophilus* colonised wattle rust pustules at nine and six weeks post treatment, respectively.

Implications

- Both *S. filum* and *A. uredinophilus* colonised and reduced wattle rust, and could be developed as biological control agents for wattle rust management.
- Different colonisation times and disease development in plants treated with *S. filum* and *A. uredinophilus* might indicate that, although *A. uredinophilus* colonised rust pustules faster, *S. filum* might be a more effective biological control agent in the long term.
- More wattle varieties need to be included in subsequent studies to evaluate their response to treatment with biological control agents to control wattle rust.

CHAPTER 5: Pot trial evaluation of optimal dosage and application frequency of *Sphaerellopsis filum* and *Akanthomyces uredinophilus* for control of wattle rust

Major findings

- All the dosages and frequencies of *S. filum* and *A. uredinophilus* reduced wattle rust disease relative to the untreated control.
- The bi-weekly treatment with $2x10^6$ conidia.ml^{-1} of *A. uredinophilus* resulted in the least amount of rust disease, which was not significantly different to that of the fungicide-treated control.

Implications

- In this study, bi-weekly treatments at the higher tested dose achieved a greater reduction of wattle rust. This would imply that repeated application would be required to replenish the biocontrol fungal inoculum to a level that would rapidly colonise new rust pustules that develop on new leaves.
- In order to meet the practical requirements of spraying in a commercial forestry setting, nursery treatments would need to be followed by field applications of the chosen biocontrol agent. The optimal frequency and dosage would have to be established in subsequent field trials.

CHAPTER 6: Combining silicon fertilization with a biocontrol agent (*Akanthomyces uredinophilus*) for the management of wattle rust

Major findings

- Silicon fertilization at four weeks post-inoculation, combined with bi-weekly applications of *A. uredinophilus* at $2x10^6$ conidia.ml^{-1}, did not enhance control of wattle rust.
- The EDX spectra and mapping showed that silicon was not taken up by treated plants. Therefore, the lack of enhanced control was due to the insufficient uptake of silicon.

Implication

- Either wattle plants take up silicon slowly or not at all. If they take it up slowly, then early silicon fertilization of seedlings or cuttings would be required to allow for the sufficient uptake of silicon by the plants by the time the plants become vulnerable to infection by wattle rust.

Future studies

The overall results of this study suggest that both mycoparasites can be used as biological control agents for wattle rust. Bi-weekly applications of *A. uredinophilus* at $2x10^6$ conidia.ml^{-1} was the best performing biocontrol treatment in these pot trials. However, it is unknown whether the nursery application of *A. uredinophilus* would yield sufficient fungal inoculum to control wattle rust under field conditions. Therefore, field trials should be implemented to establish the biocontrol efficacy of *S. filum* and *A. uredinophilus* under field conditions. These trials would need to test dosage and frequency of application of the biocontrol agents. It would also be valuable to test the potential application methods for trees, such as a mistblower versus drone application. Such a regime would need to be effective, practical to apply, and economically affordable for it to be adopted by the forestry industry.

Dual infection involving wattle mirid (*Lygidolon laevigatum* Reuter) and wattle rust are frequently observed, leading to speculation that mirids are possibly important vectors of the rust pathogen. Further studies should evaluate the possible relationship between wattle mirid and wattle rust, using quantitative Polymerase Chain Reaction (qPCR) and electron microscopy to determine if wattle mirids are responsible for the local and long-distance spread of wattle rust. If found to be true, the proposed IPM treatment regime should also include an insecticide and insecticidal biocontrol agents (such as *Beauveria bassiana*) for the control of wattle mirid and wattle rust, concurrently.

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