Lecanicillium uredinophilum, a potential biological control mycoparasite of *Phakopsora pachyrhizi*, the soybean rust pathogen

By

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Thesis Summary

Soybean [*Glycine max* (L.) Merr.] is the largest and most significant contributor to global oilseed production, which is driven by its high oil and protein composition. Although the crop is native to China, Brazil, the USA and Argentina are the three largest producers in the world. South Africa is the top soybean producer in Africa. Soybean production globally is affected by plant pathogens and insect pests. Plant pathogens pose the greatest and most significant threat to soybean production and yield, and the Asian soybean rust (ASR) disease caused by the biotrophic fungus *Phakopsora pachyrhizi* Syd. & P. Syd., is the most significant among the foliar pathogens of soybeans. ASR disease became a pandemic over two decades ago, and to date, soybean producing areas have experienced epidemics despite the widespread application of fungicides. The costs associated with fungicides for managing the ASR pathogen are substantial, with Brazil alone spending approximately US$3 billion annually. Efforts on breeding for durable ASR genetic resistance, despite significant efforts, have not been successful as the ASR pathogen quickly overcomes single gene resistance. The reliance on chemical fungicides has led to the development of pathogen resistance to these fungicides. Therefore, this study sought to isolate indigenous fungal hyperparasites of rusts, such as *Lecanicillium* spp., which could serve as biological control agents, as alternatives to chemical control. The dearth of current and scientifically updated information on the mechanism of action of soybean rust hyperparasites is a gap addressed in this thesis. Understanding the mechanisms of action of hyperparasites may assist in the development of effective formulations of biocontrol agents.

Hyperparasitic fungi were isolated from ASR pustules on soybean. This was followed by identification of the fungi using morphological characterisation, followed by genomic DNA extraction and Sanger sequencing of the internal transcribed spacers (ITS). These identification steps classified the isolated hyperparasites as two isolates of *Akanthomyces muscarium*, a *Lecanicillium* spp. (*Aphanocladium araneurum*) and a *Simplicillium lanosoniveum* isolate. Further phylogenetic analyses could not conclusively resolve the taxonomy of the suggested species, leading to additional marker genes being added to enhance the species level identification of the isolates. Two isolates, PP2018-001 and PP2018-003, were selected on the basis of their superior pathogenicity on ASR. The Isolate PP2018-001, and another isolate of *Lecanicillium* (PP2018-005) obtained from a local biological control company (Andermatt-PHP) were further characterized for their identity. The multi-locus phylogenetic analysis with

the additional DNA-polymerase II second subunit (RPB2), the transcription elongation factor-1α (TEF) identified the two isolates as being *Lecanicillium uredinophilum.* The PP2018-001 isolate, originally isolated from wild strawberry rust pustules, showed potential as a biocontrol agent of ASR.

Ten locally marketed commercial adjuvants (Break-Thru®, Bond®, Aquawet®, Designer®, Ballista®, Tronic®, Summit Super®, Wetcit®, Nufilm P® and Sprayfilm 10®) as well as seven vegetable oils (canola, macadamia, olive, peanut, organic sesame, and sunflower) were sourced from the local market, and were subjected to bioefficacy evaluations for their compatibility with the target fungi. This was measured using mycelial radial growth and colony forming units (CFU) counts of *Lecanicillium* isolates PP2018-001 and PP-2018-003. The compatibility of the fungi varied significantly with different adjuvants and vegetable oils, compared with the control treatments, for both fungal isolates. The vegetable oils showed consistency in enhancing both radial growth and CFU counts with an increase in concentration compared to the control treatment. All the vegetable oils can be evaluated in future glasshouse and field trials. However, amongst the commercial adjuvants there were significant differences in their effects on the radial mycelial growth and CFU counts, with a consistent decrease in fungal growth with an increase in concentration for all adjuvants, compared to the control treatment. The best commercial adjuvant was Break-Thru® at 0.01% and 0.05%. Ballista® and Bond® showed promising compatibility at 0.01%, and further studies on reduced concentrations might reveal better compatibility. Wetcit® drastically reduced radial growth and CFU counts, and inhibited growth at the 1% concentration or higher. The results of the study would be crucial in the selection of adjuvants for bio-formulations and practical applications in either controlled or field environments for these two biocontrol agents. They may also be applicable to other fungal propagules.

The mechanism of action of the *L. uredinophilum* Isolate (PP2018-001) was investigated. Ultrastructural examinations of fungus-to-fungus interactions (*P. pachyrhizi* to *L. uredinophilum*) were done through high throughput, high-end microscopy. Investigations used confocal laser-scanning microscopy (CLSM) employing a green fluorescent protein (GFP) transformant of PP2018-001. Tracking the hyperparasite's infection process was successful through the CLSM, which revealed the ability *of L. uredinophilum* to penetrate and to colonise *P. pachyrhizi* urediniospores. In Scanning Electron Microscopy (SEM) studies, *L. uredinophilum* first attached and germinated on *P. pachyrhizi* urediniospores, forming an

intense mycelial network and coiling around the urediniospores. Furthermore, *L. uredinophilum* both directly penetrated urediniospores, and entered through germ pores, after which there was a loss of cellular integrity, as evidenced by the breakdown and multiple perforations of the infected urediniospores. In Transmission Electron Microscopy (TEM) studies at the cellular level, the mode of entry of *L. uredinophilum* into the urediniospores was confirmed and showed the growth of *L. uredinophilum* hyphae inside the urediniospores and the loss of cellular integrity inside infected urediniospores, compared with uninfected urediniospores. This study confirmed that *L. uredinophilum* employed direct mycoparasitism as its mode of action against *P. pachyrhizi* urediniospores.

The *L. uredinophilum* Isolate PP2018-001 was evaluated for colonization of the *P. pachyrhizi* urediniospores using three conidial concentrations of *L. uredinophilum* in greenhouse studies. Three concentrations (1.5×10^2, 1.5×10^4 and 1.5×10^6 conidia.ml^{-1}) of *L. uredinophilum* were employed for this study, which investigated the level of colonization of the urediniospores by *L. uredinophilum,* as well as its effect on the ASR disease severity. Colonization of urediniospores by *L. uredinophilum* mycoparasite was visible 3-10 days post inoculation (dpi). All treatments achieved some level of colonization of the *P. pachyrhizi* urediniospores, with the highest concentration achieving almost 100% colonization at 10 dpi, whilst the other two concentrations achieved approximately 45% and 34%, in order of their concentration levels. All the treatments significantly impacted ASR severity as assessed at 0 dpi and 10 dpi. The result of this study provided evidence that *L. uredinophilum* conidial suspension at the highest concentration could be used to stop any further development of *P. pachyrhizi* and could be used as a curative strategy to control ASR.

A framework for evaluating biocontrol deployment in soybean fields was conducted through the physical installation of automatic weather stations (AWS), which facilitated the measurement of various climatic parameters such as relative humidity, wind speed, wind direction, air temperature, dew temperature, and wet bulb temperature in two soybean fields. This study evaluated six machine learning (ML) algorithms to predict leaf wetness (LW) and leaf wetness duration (LWD). The algorithms also estimated the degree of importance of each environmental parameter on the prediction of LW and LWD. While LW and LWD prediction models are mainly developed for disease early warning systems, they could also be used to assess the suitability of field conditions for the deployment of biological control agents.

The results presented in this thesis reinforce the importance of studies on biological control in the development of an alternative control strategy for the Asian soybean rust pathogen, *P. pachyrhizi*. *Lecanicillium uredinophilum* provides a curative approach for control of ASR.

Declaration

I, Phumelela Peace Mwelasi, declare that:

(i). The research contained in this thesis, except where otherwise indicated, is my original work.

(ii). This thesis has not been submitted for any degree or examination at any other University.

(iii). This thesis does not contain other person's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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

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Date: 04 February 2025 Signature: [redacted]

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Date: 18th July 2025 Signature: [redacted]

Professor Kwasi Sackey Yobo (Supervisor)

Date: 19 July 2025 Signature: [redacted]

Professor Mark Delmege Laing (co-supervisor)

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Dedication

To the MOLOI/NDLOVU, MWELASE/MANYATHELA and BHEBHE clans,
who together provided the moral support, and unceasing prayers in the face of all adversity.
To God be the Glory, great things He has Done!

Publications

- P. P. Mwelasi, M.D. Laing, J.D. Ibaba, R. Rogers, M.Z. Németh & K.S. Yobo (2025) Ultrastructural examination of the fungus-to-fungus interactions of *Lecanicillium uredinophilum* and *Phakopsora pachyrhizi*. *Plant-Environment Interactions* (https://onlinelibrary.wiley.com/journal/25756265) **(Under review).**

Table of Contents

Chapter 1: General Introduction

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1.1 Introduction

Soybean [*Glycine max* (L.) Merr.] is an edible legume with native origins in Asia (China), which has become a crop of global importance due to its high protein (35-40%), oil (20%) and dietary fibre (9%) content (Qin et al., 2022). Soybean is cultivated in six out of the nine provinces in South Africa. Since its introduction, there has been an increase in both average yields per hectare and the area under cultivation (https://sagrainmag.co.za/2024/05/06/investment-in-soybean-industry-creates-new-opportunities/). South Africa is the largest producer of soybean in Africa (FAO, 2024), and in 2023, the value of soybean production at the farm level was estimated at R20 billion (https://www.grainsa.co.za/soybean-farming-to-measure-is-to-know). In Africa, the gross soybean production value stood at US$2 billion in 2021, but this decreased to US$ 1.8 billion in 2022, a 15.14% decrease (FAO, 2024). South Africa contributed 41.5% to the African total soybean production in 2021, 35.8% in 2022, and 0.7% globally (FAO, 2024). Soybean production in South Africa is a success story, considering the history and milestones achieved. South Africa exported over 200 MT of soybeans in 2022 (Valerie Cilliers, 2023). However, Africa's contribution to global soybean production remains below 1%, despite the climatic suitability of soybean production in many parts of Africa (Moosa et al., 2023; Engelbrecht et al., 2020). On a global scale, soybean contributes an average of 60% of the world's vegetable oilseed production (Soystats, 2024; Engelbrecht et al., 2020). The global soybean gross production value was US$172.6 billion (2021) and US$175.3 billion (2022) (FAO, 2024). The top three producing countries in 2022/2023 were Brazil at 162 million MT (43% of the world total), the United States at 116 million MT (31% of the world total) and Argentina at 25 million MT (7% of the world total) (Soystats, 2024).

Soybean is a crop that is susceptible to many insect pests and diseases worldwide, with combined losses to pests of 26-29% globally (Wang, 2024; Da Silva et al., 2022; Gaur and Mogalapu, 2018; Heinrichs and Muniappan, 2018). The potential yield of soybeans is

drastically reduced by Asian soybean rust (ASR) caused by the pathogen *Phakopsora pachyrhizi* Syd. & P. Syd. In Africa, losses of 10-90% have been recorded (Sileshi and Gebeyehu, 2021), whereas losses of 10-100% have been recorded in in Asia (Hossain et al., 2024). In South Africa, handbooks detailing soybean rust trends were published in 1995, 2000, and 2010 (Van Wyk and Smit, 2010), and soybean rust epidemics have been experienced every season since the initial outbreak in 2001 (A. Jarvie, personal communication, 25 April 2023). ASR remains the most significant threat to soybean yields globally (Hao et al., 2024; Sileshi and Gebeyehu, 2021).

Currently, the management of ASR predominantly relies on the use of fungicides (Bandara et al., 2020). Breeding efforts have been made in the past; however, the ASR pathogen rapidly overcame the resistance genes that were deployed to date, no cultivar with durable resistance is available for cultivation (Chicowski et al., 2024). Despite the failure to breed for durable resistance, a novel gene (Rpp6907-07) has been discovered by Hao et al. (2024), and experimental results indicate that it provides for resistance, albeit for an unknown period. Other efforts have focused on developing transgenic soybean varieties, an approach that has shown positive results towards resistance against the ASR pathogen (Chicowski et al., 2024; Soto et al., 2020). The chemical control of ASR, despite achieving significant success resulting in increased yields (Felipe-Victoriano et al., 2024; Delaney et al., 2018), is expensive. The cost of fungicide applications against ASR is approximately US$2.8 billion in a single season, for Brazil alone (Hao et al., 2024). In addition, the number of fungicides used to manage soybean diseases has more than doubled in less than a decade (US$0.95 billion in 2009 to over US$2.36 billion in 2017) (Winter and Fehr, 2021). Another compounding factor threatening the continued dependency on fungicides is the development of resistance to the primary fungicides by the ASR pathogen (Machado et al., 2022; Dorighello et al., 2020; Dalla Lana et al., 2018).

The challenges outlined above have created a demand for biological control products as alternative control measure (Muniz et al., 2023). The genus *Lecanicillium* Zare & Gams is a well-studied source of mycoparasites and entomopathogens that have been isolated from rust sori (Vandermeer et al., 2009), from insects such as beetles (Tanyeli et al., 2010), from arachnids such as spiders (Chen et al., 2017; Mitina et al., 2017), ticks (Kalsbeek et al., 1995), Lepidopteran larvae (Chen et al., 2020), and also acts as an endophyte (Yuningsih et al., 2022; Nicoletti and Becchimanzi, 2020). It is also a genus undergoing substantial reclassification (Zhou et al., 2022). Of particular interest is the species *Lecanicillium lecanii*, formerly *Verticillium lecanii* (Zimm) Zare & Gams, which is an entomopathogenic fungus (EPF), that

can be used as a biological control agent against a variety of insect pests in agriculture: thrips (Subramaniam et al., 2021), whitefly (Abdulle et al., 2020; Xie et al., 2019), aphids (Hanan et al., 2020), psyllids (Lu et al., 2015), scales (Liu et al., 2009), and grasshoppers (Dakhel et al., 2020; Khachatourians, 1992). It has also been investigated for its potential to control anopheles mosquitoes, which are vectors of malaria, dengue, Zika, Japanese encephalitis and chikungunya, which threatens human health (Sogan et al., 2023). However, the use of *Lecanicillium* spp. for the control of the ASR pathogen is a relatively novel concept, with limited scientific research to date (Havugimana, 2017).

In 2013, the University of KwaZulu-Natal (UKZN) began exploring potential hyperparasites to control rusts, starting with a survey of parasites of coffee rust pustules (*Hemileia vastatrix* Berkeley & Broome) at the Assagay Coffee Farm (29°45'45.5"S; 30°37'13.6"E), which is near the UKZN, Pietermaritzburg campus (29°37'18.48"S; 30°23'50.28"E). The study led to the isolation, identification and characterization of a hyperparasite fungus, *Lecanicillium muscarium* Petch, (Havugimana, 2017). Elsewhere, studies have evaluated *Lecanicillium* spp. as a potential biocontrol agent for the coffee rust pathogen (Das et al., 2024; Vandermeer et al., 2009), and for ASR (*P. pachyrhizi)* (Saksirirat and Hoppe, 1990). However, there are no other publications on the evaluation of *Lecanicillium* spp. as a biocontrol agent of ASR. This study, through the specific objectives below, documents further evidence of rust hyperparasites found in nature and their application as potential biocontrol agents of *P. pachyrhizi*. The findings in this study will contribute to the body of knowledge on rust hyperparasites, with particular attention to *Lecanicillium* spp. and their mechanism of action.

1.2 Specific Objectives

The thesis is organized into eight chapters with their respective specific objectives as follows:

1) Chapter 1: General Introduction
    i) Gives an introduction and overview of the thesis.
2) Chapter 2: Literature Review–
    i) Reviews the potential of hyperparasites to control ASR, with special reference to *Lecanicillium uredinophilum.*
3) Chapter 3: Morphological and molecular characterization of mycoparasitic fungi targeting *Phakopsora pachyrhizi* in South African soybeans;
    i) Is a preliminary study that deals with isolation and identification of fungal hyperparasites on rust sori with special emphasis on finding *Lecanicillium* spp.;

ii) Characterization of the hyperparasites using morphological and molecular tools;
iii) Phylogenetic analysis of the selected isolates and confirm their identities.

4) Chapter 4: Assessing the bio-efficacy of ten commercial adjuvants and seven edible oils as potential performance enhancer for *Lecanicillium uredinophilum*, a mycoparasite of soybean rust pathogen (*Phakopsora pachyrhizi*)
    i) Evaluates seventeen commercial adjuvants for their compatibility with isolates of *Lecanicillium uredinophilum, in vitro;*
    ii) Evaluates seven edible oils for their compatibility with isolates of *Lecanicillium uredinophilum, in vitro;*
5) Chapter 5: Ultrastructural examination of the fungus-to-fungus interactions of *Lecanicillium uredinophilum* and *Phakopsora pachyrhizi*
    i) Documents the colonization of soybean leaves by *Phakopsora pachyrhizi*
    ii) Visualizes both surface and internal colonization of the rust urediniospores, using a combination of three microscopy tools: scanning electron microscopy (SEM); transmission electron microscopy (TEM), and confocal laser-scanning microscopy (CLSM);
    iii) Ascertains whether *Lecanicillium uredinophilum* only colonizes rust urediniospores, or whether it has any visible interactions with the host plant.
6) Chapter 6: Evaluation of colonization of soybean rust urediniospores by three conidial concentrations of the mycoparasite *Lecanicillium uredinophilum*.
    i) Assess the colonization dynamics of *Lecanicillium uredinophilum* visually and microscopically on ASR sori.
7) Chapter 7: Machine learning-based prediction of leaf wetness duration: A framework for evaluating biocontrol deployment feasibility in soybean fields
    i) Uses a Machine Learning model to predict the duration of soybean leaf wetness before field deployment of *Lecanicillium uredinophilum;*
    ii) Identifies the key agrometeorological factors governing leaf wetness and leaf wetness duration, and their relative levels of importance.
8) Chapter 8: General Conclusions and Recommendations
    i) Provides the general conclusions and recommendations, based on the research outcomes.

1.3 Thesis Layout

The exposition of the research study is outlined in eight chapters. The thesis employed the Harvard referencing style, with specific use of the format used by "*Molecular Plant Pathology*" (with descending date order format, and then alphabetic order of authors as well as visible digital object identifier). Each chapter is presented as a research paper, apart from the first and last chapters. The first chapter gives a general introduction to the thesis while the last chapter covers the thesis' conclusions and recommendations. The thesis format is the standard format adopted by the University of KwaZulu-Natal (UKZN), which creates some repetition of information, notably introductions, references, and some portions of research methods.

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Chapter 2: Literature Review: The potential of fungal entomopathogens to control the soybean rust pathogen with specific reference to *Lecanicillium* species.

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2.1 Introduction

Soybean [*Glycine max* (L.) Merr.] is an important crop worldwide due to its versatility in its nutritive value and products apart from health benefits (Soystats, 2024, Miransari, 2016). The yields of soybeans globally are seriously undermined by *Phakopsora pachyrhizi*, Syd. & P. Syd., the causal pathogen of the Asian soybean rust (ASR) (Godoy et al., 2016). In Brazil alone, since the outbreak of ASR, the country has lost more than US$18 billion in soybean crop losses (Yorinori & Hartman, 2021, Langenbach et al., 2016). Furthermore, it is reported that in one season, Brazil spent more than US$2 billion on three fungicide applications countrywide for the control of ASR (Godoy et al., 2016). Asia has reported losses of 10-100% due to ASR. (Hossain, 2024) Chemical control of ASR through fungicides is undoubtedly common worldwide. The highly effective fungicides, the succinate dehydrogenase inhibitors (SDHIs) introduced in 2013, face severe pressure due to their increased usage, thus imposing a vulnerability in selection for resistance to *P. pachyrhizi* isolates (Oliver, 2014). Fungal resistance is, therefore, an impeccable reality (Shcherbakova, 2019, Hahn, 2014, Van den Bosch et al., 2011). To augment protection efforts in the control of ASR, further research on soybean breeding programmes worldwide and in South Africa are yet to release cultivars which are resistant to a multiple of *P. pachyrhizi* isolates (Chicowski et al., 2024, Langenbach et al., 2016).

Successful control of plant pathogens hinges on understanding the environment promoting both the crop and the pathogen and the host-pathogen interaction, apart from the pathogen interactions with other environmental elements. The rise in the literature on biological control of plant pathogens offers a promising sustainable solution in that they are regarded as relatively safer for human populations and the environment (Jiang & Wang, 2023). Furthermore, biological control agents are not expected to be rapidly eroded in their efficacy since they are already present within the ecosystem (Nchu, 2024). Fungal entomopathogens such as from the *Lecanicillium* genus (Zare & Gams) have been commercialised for their effectiveness under

controlled environmental conditions in controlling various insects and mites (Chen et al., 2017, Cuthbertson et al., 2011, Goettel et al., 2008, Kim et al., 2007, Hayden et al., 1992). Therefore, further research is needed to optimize using *Lecanicillium* formulations in field studies. Investigations into the mechanism of action of *Lecanicillium* species highlighted a multifaceted approach, as endophytes, hyperparasites, use of enzymes for degradation of insect structures, mycoparasitism as well as competition (Liu et al., 2023, Nicoletti & Becchimanzi, 2020, Mannino et al., 2019, Altinok, 2019, Hasan et al., 2013, Chandler et al., 1993). They also have been revealed to use various substrates as food sources, including rust urediniospores (García-Nevárez & Hidalgo-Jaminson, 2019, Jackson et al., 2012). It is, therefore, hypothesized that the *Lecanicillium* species and their various mechanisms of action could act as potential biological control agents of *P. pachyrhizi*. The attractiveness of biological control agents as an alternative to chemical control is their deemed safety on the environment and non-target organisms (Jiang & Wang, 2023).

Successful application and commercialisation of biological control agents (BCAs) requires proper development of formulations, and as such, apart from benefits, limitations also exist as far as successful application in field conditions. By an interdisciplinary approach, this review seeks to advance the understanding of biological control strategies, with specific reference to *Lecanicillium* spp. to contribute to the management of the soybean rust disease, a globally significant disease of soybean production.

2.2 Economic importance of soybeans in South Africa and globally

The introduction of soybeans into South Africa in 1903 has seen a marked increase since then due to the South African government's promotion at both production and policy levels (Van der Linde, 2023, Miransari, 2016, Dlamini et al., 2014). The Fig 2.1A shows production trends in the ten years of 2014 to 2024. In the 2014/15 season, soybean production stood at 1.07 million tonnes on 687 000 ha in South Africa (DAFF, 2021) with a market value of approximately R5 billion. In contrast, the market value was around R100 million in 1990/91 at 135,200 tonnes (NAMC, 2017). The soybean industry in South Africa is currently estimated at R20 billion at farm level (Mcpherson, 2023), and the top five African-producing countries are led by South Africa, as shown in Fig 2.1B.

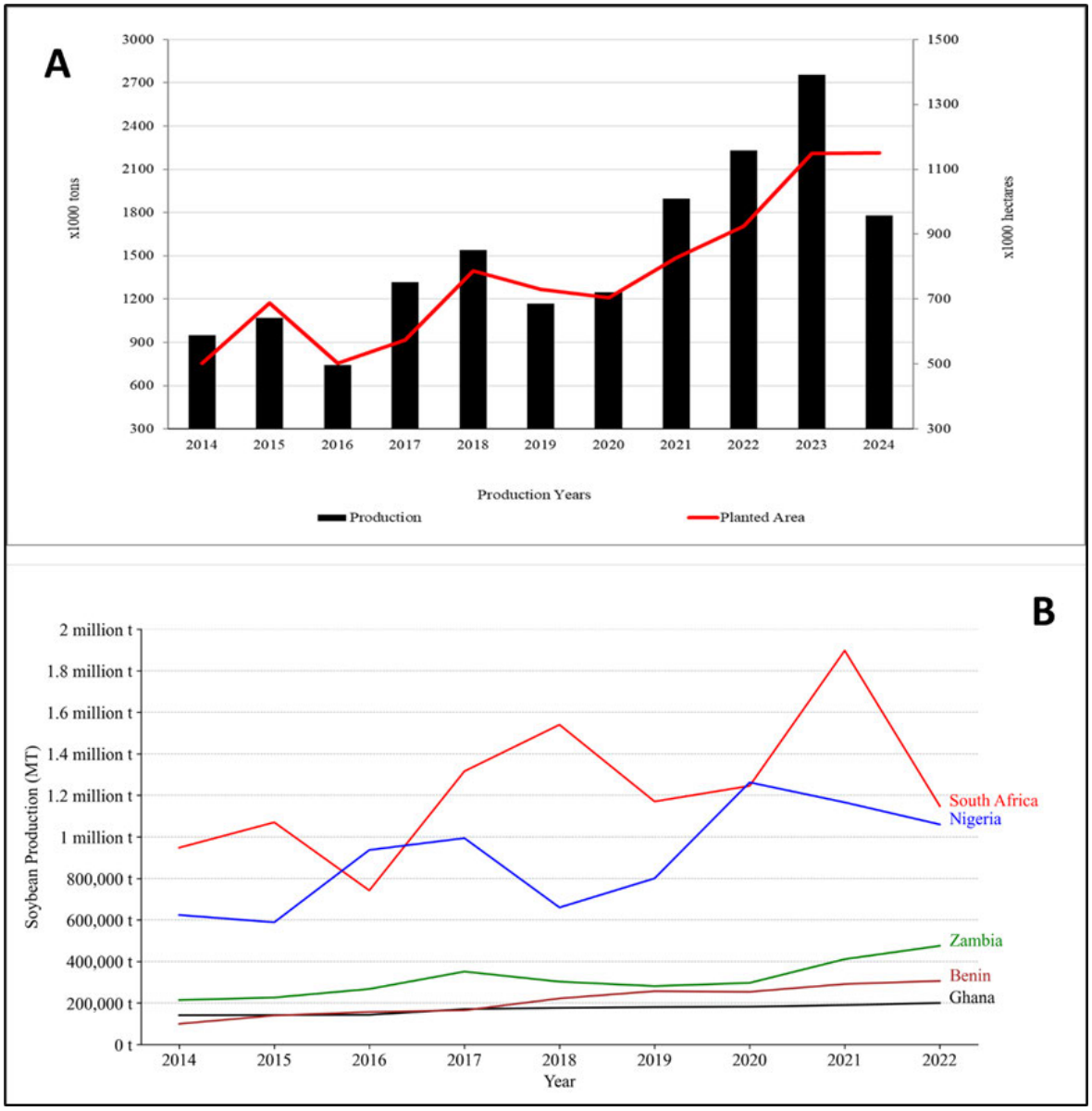

Fig 2.1 A: Soybean production and area planted from 2014 to 2024 in South Africa (Data source: GrainSA (2024); B: Soybean production in Africa's top five producing countries (Data source: FAO, 2024).

The soybean plant and its wild ancestor (*Glycine soja* Siebold & Zuccarini) are believed to have originated in China (Da Silva et al., 2017, Miransari, 2016). Botanically, soybean belongs to the subfamily *Papilionoideae* under *Leguminosae/Fabaceae* (Da Silva et al., 2017) and bears a C_3 photosynthetic pathway (Shibles et al., 1987). Agronomically, soybean can grow in a wide temperature range (10-40 ℃) (Singh, 2010) and optimally, 18/12℃ and 26/20℃ day/night minimum and maximum, respectively (Miransari, 2016). Despite the noted temperature range, yield parameters and global distribution of the soybean crop are temperature-dependent (Liu et

al., 2008). Short-day photoperiods are required with noted cultivar differences on dark period maxima to induce flowering (Singh, 2010).

In the 2022 season, South Africa produced 1.15 million MT (25.3% of Africa's total), Nigeria 1.06 million MT (23.4% of Africa's total) and Zambia 475,353 MT (10.5% of Africa's total) of the total Africa production of 4.5 million MT (FAO, 2024, FAO, 2023). Despite leading Africa in production, South Africa imported 97.1% high-protein soybean meal from Argentina https://oec.world/en/profile/bilateral-product/soybean-meal/reporter/zaf. Most of the processed soybeans in South Africa find their use in the industry: oilcake and oil (68%), animal feed (15%), human consumption (<3%)(DAFF, 2021). At a global scale in 2022, the leading soybean-producing countries (top three) were: Brazil, producing 120.7 million MT (34.6% of the world total); United States of America (USA), producing 116.4 million MT (33.4% of the world total), and Argentina 43.9 million MT (12.6% of the world total) (FAO, 2023). Africa contributed just 1.3% to world soybean production (4.5 million MT) in 2022 (FAO, 2023).

Nutritionally, the soybean pods on a dry matter basis constitute high protein quality and quantity (35-40%)(Soystats, 2024), soybean oil (18-22%) (Parle et al., 2014), carbohydrate with high fibre quantity (35%) (Kanchana et al., 2016) amongst other important constituents (Singh et al., 2008). Soybean is a versatile crop whose protein contains all the essential amino acids required in animal diets (Kanchana et al., 2016). In 2016 alone, soybeans accounted for 61% of the world's total oilseed production (Soystats, 2017). Soybean is also used for a plethora of products, high protein stock feeds, soybean oil, biodiesel, and other value-added products such as defatted soy flour, soy protein isolates, soymilk, soy biscuits, soy bread, soybean lecithin, miso, natto, tofu, just to mentioned but a few (Kanchana et al., 2016, Parle et al., 2014, Day, 2013, Die, 2011, Dourado et al., 2011, Singh, 2010, Gandhi, 2009, Singh et al., 2008). The website www.soyinfocenter.com has pioneered extensive information on soybeans (soy products or uses, history, bibliographies) that spans centuries ago (1100 B.C.) to date.

2.3 Economic importance of the soybean rust pathogen, *Phakopsora pachyrhizi*

Soybean rust is caused by the fungus *Phakopsora pachyrhizi*, and is one of the most economically significant of soybean diseases worldwide and the most severe disease of the crop (Godoy et al., 2016, Dean et al., 2012, Hartman et al., 2011b, Garcia et al., 2008, Isard et al., 2005, Hartman et al., 1999). Losses due to the soybean rust pathogen have been reported to be in the range of 10-90% globally (Murithi et al., 2016, Ribeiro et al., 2008) and escalate to 100% under favourable conditions (Hossain et al., 2024, Hartman et al., 2011b, OSU, 2007,

Sweets et al., 2004, Hartman et al., 1991, Kuchler et al., 1984, Bonde et al., 1976). A 10-80% yield loss range has been reported in South Africa, Brazil, Zimbabwe, Paraguay, Argentina, and Asia (NDPN, 2017). In the global map, countries in the Eastern Hemisphere record the highest soybean yield losses due to soybean rust disease. Some examples are India (10-90%), Japan (40%), Southern China (10-50%), Taiwan (23-90%) and Thailand (10-40%) (Hartman et al., 2017). As part of the Eastern Hemisphere, African soybean-producing countries have suffered soybean rust epidemics with a high impact on yield since the respective countries reported the disease. South Africa's first ASR report was in 2001, and epidemics were recorded in 2008 (Jarvie, 2009). South Africa has experienced soybean rust outbreaks since 2001 to date (A. Jarvie, personal communication, 22 July 2024).Yield losses due to ASR in sub-Saharan Africa have remained in the range of 10-90% and an aggregate of approximately 6% annually (Sileshi & Gebeyehu, 2021).

The potential soybean yield is undermined by abiotic, edaphic, and biotic factors such as weeds, insect pests, and plant pathogens, which are mainly a threat to global food security (Sierotzki & Scalliet, 2013, Dean et al., 2012, Chakraborty & Newton, 2011, Strange & Scott, 2005). The ASR pathogen is characteristically an obligate biotrophic fungus capable of completing its life cycle only in one suitable living host (Godoy et al., 2016). Urediniospores of *P. pachyrhizi* (infective stage) are imperative in epidemics of the ASR disease (Godoy et al., 2016). Urediniospores germinate by forming an appressorial cone within the appressorium, and thus directly penetrating the host epidermal cells, leading to their collapse (Campe et al., 2014, Goellner et al., 2010, Keogh et al., 1980). Therefore, the rust fungus causes leaf lesions, leading to premature defoliation. As a result, highly compromised photosynthetic output due to the soybean rust attack culminates in significant yield losses in all soybean genotypes (Goellner et al., 2010). The mechanism of soybean yield loss is attributed to premature defoliation, which leads to compromised photosynthetic output. The ASR lesions reduce the canopy green leaf area, net dry matter accumulation due to a compromise in photoabsorption by non-affected leaf area. A net effect is the reduction in partitioning of accumulated dry matter to grain yield (harvest index) (Picanço et al., 2023). It has been established experimentally that the extent of yield loss is dependent upon the soybean growth stage infected; for example, if infection occurred after flowering (R2 growth stage), yield losses would be higher (66-68%) as compared to seed filling (R5 growth stage) which showed lower yield losses (35-39%) (Kumudini et al., 2008). Earlier research recorded losses ranging from 50-80% with onset of

infection at R1 growth stage and up to 50% with infection at R5 growth stage (Yang et al., 1992, Hartman et al., 1991).

2.4 Strategies for control of soybean rust disease caused by *Phakopsora pachyrhizi*

The importance of developing sustainable crop protection measures against plant invaders such as insect pests, weeds and plant pathogens dates innumerable years in retrospect. Despite the in-vogue synthetic agrochemicals introduced over many years ago plant diseases are still on the rise and undermine crop quality and quantity (Narayanasamy, 2013, Regnault-Roger, 2012, Walters, 2012, Deacon, 2006). Soybean is an important crop worldwide due to its versatility in its nutritive value and products, apart from its health benefits (Miransari, 2016, El-Shemy, 2011). The soybean yields are seriously undermined by *P. pachyrhizi*. In Brazil alone, between 2001 and 2009/10, the country lost more than US$10 billion in yield losses, US$7.1 billion cost of control (2-3 fungicide sprays) and US$1.3 billion revenue lost on grain lost. Thus, the period recorded over US$18 billion in economic losses (Yorinori & Hartman, 2021). Figure 2.2 shows the strategies used in ASR management, which include chemical control, exploitation of different genetic resources such as non-host and host resistance, and using biological control agents (Langenbach et al., 2016).

2.4.1 Chemical control of the soybean rust pathogen

Hartman et al. (2011a) submit that strategies to combat ASR disease began more than half a century ago, and as such, there is well-documented literature on chemical control through fungicides (Machado et al., 2022, Zambolim et al., 2021, Leadbeater, 2015, De et al., 2014). Chemical control through fungicides offers a first line of defence. The agrochemical industry has existed over the past seventy years (Siegwart et al., 2015), and chemical control against ASR has had a fair success as the most effective control means (Langenbach et al., 2016). South Africa is one of the leading nations in Africa in importing pesticides for agricultural use (Quinn et al., 2012), and in 2022 imported US$720 million worth of pesticides (https://oec.world/en/profile/bilateral-product/pesticides/reporter/zaf). Fungicide control is achieved by 1-2 sprays (Laing & Caldwell, 2014), and more could be used in severe cases. Studies on chemical control established important parameters (effective dosage rates, timing in application and frequency (Du Preez, 2005). South Africa has primarily depended on demethylation inhibitors (DMIs) fungicides (e.g. triazoles) and quinone outside inhibitors (QoIs) fungicides (e.g. strobilurins). The Fungicide Resistance Action Committee (FRAC) recommendations are followed on fungicide rotation for 2-4 sprays to help combat the build-

up of fungicide resistance (Laing & Caldwell, 2014). Challenges faced by chemical control are ineffective control when sprays are done in the presence of 20-30% canopy infection (OSU, 2007), the presence of resistant isolates in a population and lack of durable resistance in cultivars of soybean (Chicowski et al., 2024).

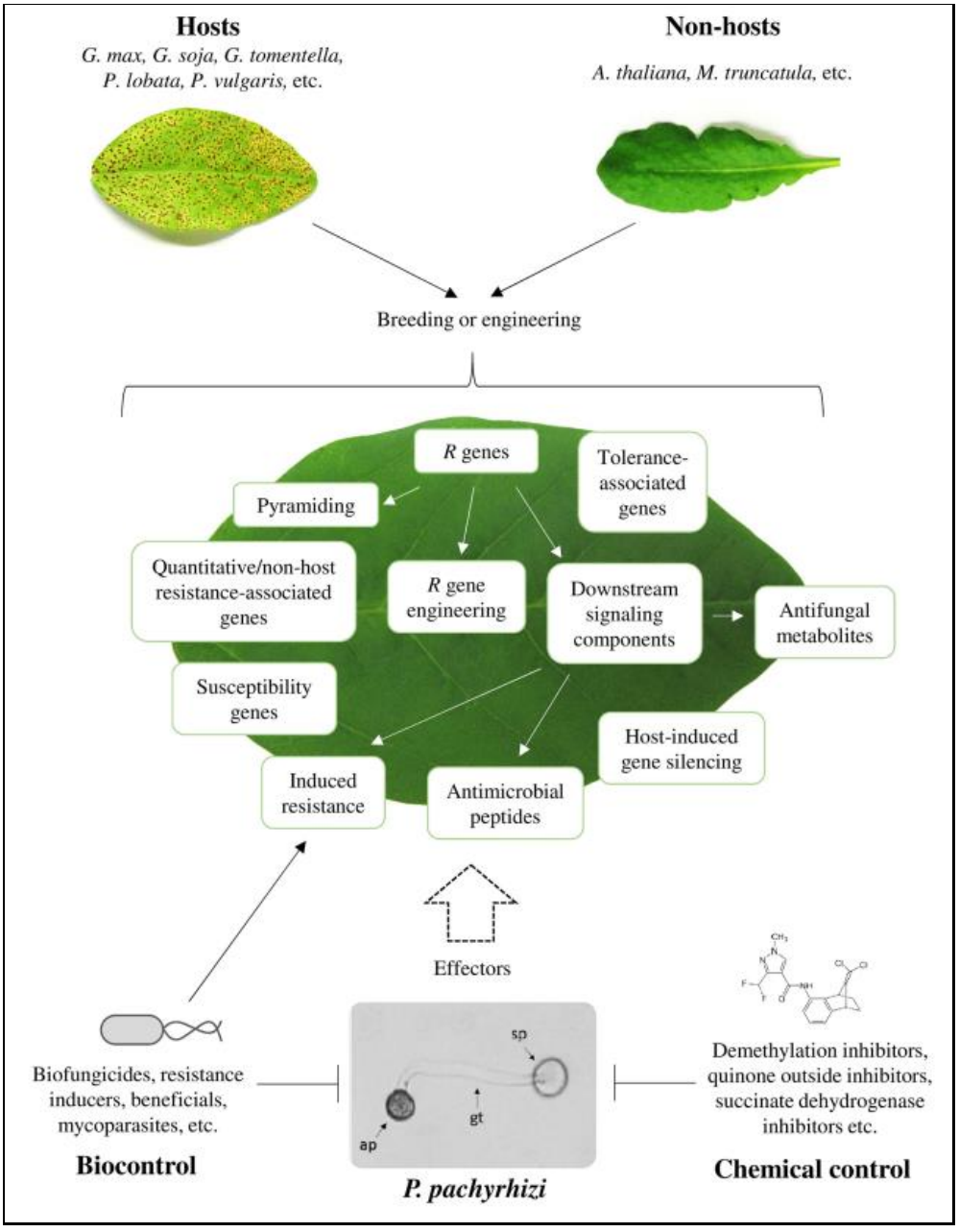

Fig 2.2 Strategies in ASR control (exploitation of genetic resources, chemical fungicides, biological control agents) to combat *Phakopsora pachyrhizi*. appressorium (ap); germ tube (gt) and urediniospore (sp). Adapted from Langenbach et al. (2016).

2.4.2 Cultural control of the soybean rust pathogen

Critical to cultural control of ASR is varietal maturity and timing in planting dates to allow maturity before conditions favour disease proliferation (Young et al., 2011, Du Preez, 2005). In South Africa, optimal planting dates range from end-November to end-December in very hot areas, mid-November to mid-December for warm regions (e.g. northern KwaZulu-Natal) and end-October to end-November in cool areas (e.g. southern KwaZulu-Natal) (Pannar, 2006). Soybean rust infection was observed to appear late in the growing season in northern KwaZulu-Natal; hence, early planting could improve yields; however, it is not sufficient to achieve effective control (Du Preez, 2005, Caldwell & McLaren, 2004). The survival and viability of ASR urediniospores whilst airborne are highly influenced by climatic conditions (solar radiation, relative humidity and temperature, ultraviolet radiation) (Del Ponte & Esker, 2008). Solar radiation (≥27.3 MJ/m^2) and ultraviolet radiation (≥1.2 MJ/m^2) exposure proved fatal to the germination of ASR urediniospores (Isard et al., 2006). Other cultural strategies to control ASR development could target breaking the cycle of leaf wetness and duration of irrigating at a time that would allow leaf drying before dew (Caldwell et al., 2002). Leaf wetness and duration are essential factors in ASR development (Beruski et al., 2019, Nunkumar et al., 2009, Wang & Hartman, 1992, Marchetti et al., 1976).

2.4.3 Soybean resistance breeding and biotechnology as methods of control

The second line of defence against the ASR pathogen is soybean resistance breeding through conventional means (Garcia et al., 2008, Kiryowa et al., 2008, Ribeiro et al., 2008, Ribeiro et al., 2007) and genetic engineering or transgenic soybean plants (Soto et al., 2020, Chander et al., 2019, Da Silva et al., 2017, Langenbach et al., 2016). Genetic control through developing soybean cultivars with resistance to ASR can reduce the agrochemical environmental burden and offer an economically viable option (Yamanaka et al., 2016). The worldwide genetic collection of soybean accessions is approximately 170,000 maintained in 70 countries, led by China (26,000 accessions) and the USA (19,000 accessions) (Da Silva et al., 2017, Pratap & Kumar, 2011, Stacey, 2008). Soybean breeding efforts have focused on genetic analysis and mapping of soybean-resistant (*R*) genes. The whole genome sequencing of soybeans was completed in 2008 (Kalaitzandonakes et al., 2019, Wilson, 2012), therefore providing more insights into breeding strategies such as mapping race-specific *R* genes (Langenbach et al., 2016, Yamanaka et al., 2016, Li et al., 2012, Ray et al., 2009, Chakraborty et al., 2009, Garcia et al., 2008) as well as *R* gene pyramiding to circumvent race-specific resistance inadequacies

(Pathania et al., 2017, Hartman et al., 2016, Lemos et al., 2011, Tukamuhabwa & Maphosa, 2011, Yamanaka et al., 2010, Garcia et al., 2008, McLean, 1979). Breeding against *P. pachyrhizi* considers three types of characterized responses to infection: complete resistance (immunity) in soybean is asymptomatic, whereas incomplete/partial resistance exhibits red-brown (RB) type lesions with 1-2 uredinia with sparse sporulation, and susceptible soybean varieties produce characteristically tan-coloured (TAN) type lesions with at least 2-5 uredinia profusely sporulating (Kato, 2017, Vuong et al., 2016, Twizeyimana et al., 2014, Lu, 2013, Pandey et al., 2011, Goellner et al., 2010, Pham et al., 2009, Calvo et al., 2008). Some challenges faced in breeding include the inability of *R* genes to confer resistance to all *P. pachyrhizi* isolates, the limited genetic base of soybean germplasm, molecular breeding challenges such as R-gene slow rate and complex R-gene clusters in soybean (Gupta, 2016, Board, 2013, Wilson, 2012, Tukamuhabwa & Maphosa, 2011). Other approaches that have been extensively researched and show some promise are non-host resistance (NHR) by *Arabidopsis* and *Medicago truncatula* Gaertn. RNA interference and host-induced gene silencing studies are also on the rise. Despite all these efforts, soybean cultivars with durable resistance to ASR remain elusive (Chicowski et al., 2024, Langenbach et al., 2016).

2.4.4 Biological Control Agents against *Phakopsora pachyrhizi*

In February 2013, a University of KwaZulu-Natal (UKZN) team visited the Assagay Coffee Farm (29°45'45.5"S 30°37'13.6" E) at Cato Ridge, near Pietermaritzburg and found the coffee rust fungus, *Hemileia vastatrix* Berkeley & Broome having been colonized by a mass of white mycelium. Upon isolation and molecular characterisation, the fungicolous fungus was identified as *Lecanicillium muscarium* (Petch) Spatafora et al. (Havugimana, 2017). The sample was given an accession number PPRI 13715 after being deposited into the National Collection of Fungi under the Agricultural Research Council of South Africa (ARC). Initial *in vitro* and greenhouse studies indicated that it could be used under field conditions against the rust pathogen (Havugimana, 2017). There is a dearth of detailed studies of *P. pachyrhizi* control through biological agents despite the interest in the biological control of soybean rust shown in the past four decades (Langenbach et al., 2016, Goellner et al., 2010). Four decades ago, more than 30 genera of fungi were shown to inhabit various rust pustules from different crop species (Littlefield, 1981). Some close relatives of rusts (*Tuberculina* spp.) have been reported to be mycoparasites of rusts (Blakeman & Fokkema, 1982, Casey, 1979). **Table 2.1** profiles

some of the specific biological control studies on *P. pachyrhizi* and their proposed mode of action (M.O.A), type of experiment (s) and results over the years.

Table 2.1 Some Biological Control Agents (BCAs) with the potential to control *Phakopsora pachyrhizi*, the soybean rust pathogen.

Organism	Proposed M.O.A[†]	Type of experiment	Results	Reference(s)
Metarhizium anisopliae (MABR-01) *Metarhizium humberi* (MHBR-03)	not investigated	*In vitro* bioassays, greenhouse experiments	The *M. humberi* and *M. anisopliae* cell-free culture filtrates *in vitro* significantly inhibited the *P. pachyrhizi* urediniospores germination by 96% and 85%, respectively. The conidial suspensions did not affect ASR urediniospores in vitro; however, they showed activity in planta experiments (51% efficacy for *M. anisopliae*). Soil drench application reduced soybean rust severity by 40% (*M. anisopliae*), and *M. humberi* treatment did not affect soybean rust. Foliar applications reduced ASR severity by 86% for *M. humberi* and M. *anisopliae* by 50% or 75% for cell-free culture treatments.	Holz et al. (2023)
Bacillus subtilis (QST-713); *Trichoderma harzianum* (T-22)	not investigated	*In vitro* bioassays, greenhouse experiments	*B. subtilis* (QTS-713) significantly reduced ASR sporulation in detached leaf bioassays and greenhouse experiments across all (4) application times. In contrast, the *T. harzianum* (T-22) effect on SBR was variable depending on cultivar and time of application, with some treatments showing no significant reduction.	Twizeyimana and Hartman (2019)
Lecanicillium muscarium	mycoparasitism	*In vitro* bioassays were done with visualization on SEM	SEM[†] was able to elucidate coiling around uredinia as well as abnormal spore morphology due to *L. muscarium* infection.	Havugimana (2017)
Bacillus subtilis (QST-713), AP-3, and AP-51 and *Bacillus pumilus* (QST-2808	not investigated	*In vitro* bioassays, greenhouse and field trials	On germination tests, *B. subtilis* QST-713, *B. pumilus* QST-2808 and fungicide exhibited 100% urediniospore germination inhibition. Other species: *B. subtilis* AP-3 (11.5%); *B. subtilis* AP-51, B. subtilis (BS), *B. licheniformis* (BL) and a mixture (BS + BL) had <6% germination inhibition. *In vitro* bioassays on detached leaves percentage reduction in disease severity (as shown in parenthesis): *B. subtilis* (QST-713) (98.6%), AP-3 (75.3%), and AP-51 (61.2%) and *B. pumilus* (QST-2808) (97.7%); the other species (BS, BL, BS + BL) showed 22.3%, 33.9% and 42.0% respectively. Greenhouse trials showed a reduction in the AUDPC for all *Bacillus* isolates except the AP-51 isolate. Limited effectiveness of *Bacillus* species in reducing disease severity was observed under field conditions.	Dorighello et al. (2015)

Organism	Proposed M.O.A†	Type of experiment	Results	Reference(s)
Simplicillium lanosoniveum	mycoparasitism	*In vitro* bioassays were done with visualization on SEM, TEM† and CLSM† microscopy. *Simplicillium lanosoniveum* transformed with green fluorescent protein (GFP) gene.	The fungus *S. lanosoniveum* wrapped around *P. pachyrhizi* urediniospores before colonization and penetrated through germ pores 24 hours after inoculation. The *S. lanosoniveum* could colonize 90% of urediniospores within 5 days of inoculation. The *S. lanosoniveum* hyphae ramified through the urediniospores and erupted after 7 days post-inoculation. Confocal microscopy clearly showed the GFP *S. lanosoniveum* transformant as it ramified throughout the urediniospores 3 days after co-inoculation.	Gauthier et al. (2014)
Simplicillium lanosoniveum	mycoparasitism	*In vitro* bioassays were done with visualization on SEM. Field studies to determine colonization of uredinia by *S. lanosoniveum* were done.	The fungus *S. lanosoniveum* was revealed to be mycophilic as it grew only when *P. pachyrhizi* was present.	Ward et al. (2012)
Simplicillium lanosoniveum	mycoparasitism	*In vitro* bioassays were done with visualization on SEM	Microscopic observations showed 90% colonization of sori 3-4 days after sorus eruption. Light and SEM micrographs revealed heavy colonization and wrapping of urediniospores by *S. lanosoniveum* hyphae.	Ward et al. (2011)
Trichothecium rosae	mycoparasitism and enzymatic action	*In vitro* bioassays were done with visualization on SEM	*T. rosae* reduced urediniospore germination by 90%. SEM showed heavy mycelial colonization of the uredinia, resulting in shrinkage and hypertrophy. Crater-like structure on the spore wall in SEM was suggestive of enzymatic action.	Kumar and Jha (2002)
Verticillium psalliotae Treschow and *Verticillium lecanii* (Zimm.) Viegas	mycoparasitism and enzymatic action	*In vitro* studies	Isolates of *V. psalliotae* readily grew on autoclaved urediniospores in liquid culture secreting enzymes (β-1,3-glucanase, chitinase, protease). *V. lecanii* isolate could not secret quantifiable chitinase and produced lower levels of the other enzymes than *V. psalliotae*. Isolates from both species were comparable in their lipolytic activity. *V. lecanii* showed amylolytic activity, and *V. psalliotae* could not. The experiment documented differences in protein and protease patterns for both species.	Saksirirat and Hoppe (1991b)
Verticillium psalliotae Treschow	mycoparasitism and enzymatic action	*In vitro* studies	*V. psalliotae* isolates cell-free cultures were able to produce enzymes (β-1,3-glucanase, chitinase, protease) responsible for the degradation of urediniospores breaking them down to various products (carbohydrates, amino acids, N-acetyl hexosamine and other	(Saksirirat & Hoppe, 1991a)

Organism	Proposed M.O.A†	Type of experiment	Results	Reference(s)
			unknown products). The rapid growth of *V. psalliotae* was attributed to enzyme activities which make nutrients available for the mycoparasite.	
Verticillium psalliotae	mycoparasitism and enzymatic action	*In vitro* and growth chamber	Four Asian *V. psalliotae* isolates and five mycoparasitic isolates (from different origins) were compared on urediniospores of soybean rust and common bean rust (*Uromyces appendiculatus*). In the growth chamber experiments (75-85 RH%), *V. psalliotae* had greater than 80% colonization of urediniospores; however, penetration was less than 10%. *V. psalliotae* were intensely damaged; however, there was no visible interior growth of mycelium. Extracellular enzymes were allegedly implicated in urediniospore damage other than damage by penetration. Light and electron microscopy studies confirmed penetration of urediniospores by *V. psalliotae;* however, the most heavily damaged urediniospores had no mycelium inside. Lytic enzyme secretion by *V. psalliotae* was suggested.	Saksirirat and Hoppe (1990b); Saksirirat and Hoppe (1990a)

†**M.O.A**–Mechanism of action; **SEM**–Scanning electron microscopy; **TEM**–Transmission electron microscopy; **CLSM**–Confocal laser-scanning microscopy;

2.4.5 *Lecanicillium* and other *Akanthomyces* spp. as biological control agents

The fungus *Akanthomyces muscarius* (Petch) Spatafora, Kepler & B. Shrestha, 2017 (previously designated *Lecanicillium muscarium*) belongs to the phylum (*Ascomycota*), class (*Sordariomycetes*), order (*Hypocreales*) and family of *Cordycipitaceae* (Kepler et al., 2017, Maharachchikumbura et al., 2016, Maharachchikumbura et al., 2015, Ainsworth, 2008, Sung et al., 2007). The multi-gene phylogenetically-based nomenclature studies on *Cordycipitaceae* found *Akanthomyces* distinguishable dichotomies from that of *Beauveria* and *Cordyceps* (Kepler et al., 2017) and reclassified *Lecanicillium* spp. (*L. lecanii*, *L. attenuatum*, *L. muscarium*, *L. sabanense*) into the already existing genus *Akanthomyces* Lebert (which has taken precedence over *Lecanicillium*) (Zare & Gams, 2001), leading to the rejection of the *Lecanicillium* as a genus name (Kepler et al., 2017, Zare & Gams, 2001). Despite the taxonomic reclassification, there remained a prominent unresolved challenge of attribution of the original species in the *Lecanicillium* genus (Zhou et al., 2022). More recent studies on the taxonomy of *Lecanicillium* have made attempts to ascribe some five original *Lecanicillium* members to, for example, a new genus, *Gamszarea*, based on ITS and multilocus sequence data (Zhang et al., 2021). Another study transferred three *Lecanicillium* members to another new genus, *Flavocillium*, based on ITS sequence data (Wang et al., 2020). A total of 35 species of *Lecanicillium* are recognised as having been formerly described and listed in the Index Fungorum. The works of Kepler et al. (2017) only transferred five of these species to *Akanthomyces*. Zhou et al. (2022) have provided more data on further *Lecanicillium* attribution through divergence age estimates. Zhou et al. (2022) further propose the transfer of four species into *Akanthomyces* and suggest merging the genus *Parengyodontium* with *Gamszarea*. In their phylogenetic analysis, *Lecanicillium huhutii* Zou & Zhou, a spider-pathogenic fungus formed an independent branch in clade with *Lecanicillium tenuipes* (Petch) Zare & Gams (Zhou et al., 2022).

Lecanicillium spp. was also formerly synonymized as a single species of *Verticillium lecanii* (Aiuchi et al., 2008, Goettel et al., 2008) as well as several revisions of nomenclature (Zhou et al., 2022, Goettel et al., 2008, Zare & Gams, 2001, Zare et al., 2000) with some share of notable confusions emanating from non-verification of rDNA sequences (Koike et al., 2007, Sugimoto et al., 2003). *Akanthomyces* spp. are morphologically characterized by conidiophores that arise from aerial hyphae, which may bear whorls of phialides (1-2); conidiophores can be prostrate, exhibiting solitary to multiple phialides; conidia clump together in heads which may be slimy. The conidia take an ellipsoidal to sub-cylindrical architecture with different sizes and shapes

depending on specific species with an upward attenuation (Vinit et al., 2018, Chen et al., 2017, Havugimana, 2017, Chiriví-Salomón et al., 2015, Lu et al., 2015, Park et al., 2015, Zare & Gams, 2003, Sung et al., 2001, Zare & Gams, 2001, Hsieh et al., 1997, Mains, 1950).

There are extensive worldwide studies on entomopathogenic fungi evaluated as biocontrol agents of both plant pathogens and insect pests by the Hypocreales fungi (Canassa et al., 2019, Jaber & Alananbeh, 2018, Jaber & Araj, 2018, Jaber & Ownley, 2018, Yun et al., 2017, Ownley et al., 2010, Askary et al., 1998). *Akanthomyces longisporum* (Petch) Spatafora et al. conferring dual suppression of cotton aphids (*Aphis gossypii* Glover) and powdery mildew [*Sphaerotheca fuliginea* (Schlechtendahl) Braun & Takamatsu] in cucumbers (Kim et al., 2010, Kim et al., 2008, Kim et al., 2007) is a classic example. *Akanthomyces muscarius* (Petch) Spatafora et al. has been isolated in a wide range of hosts and substrates: soil (Xie et al., 2015, Kaifuchi et al., 2013, Sukarno et al., 2009); insects and some arthropods (Wei et al., 2018, Mitina et al., 2017, Luz et al., 2010, Sukarno et al., 2009, Askary & Yarmand, 2007); plant tissue (Vinit et al., 2018), powdery mildew (Miller et al., 2004, Rao & Pavgi, 1977), coffee rust (Havugimana, 2017); uredinia of *Phragmidium* spp. on *Salix* and *Robus* spp.(Mitina et al., 2017); carnation rust, *Uromyces dianthi* (Persoon) Niessl (Spencer, 1980); uredinia of frangipani rust, *Coleosporium domingense* Patouillard (McMillan Jr, 1985) and other *Akanthomyces* spp. have been isolated from rusts and various other sources (Wei et al., 2018, Mitina et al., 2017, Sukarno et al., 2009). *Akanthomyces* spp. potential in invading phytopathogens, arthropods, and nematodes have been reported (Goettel et al., 2008), and an extensive review on biocontrol of *Heterodera glycines* Ichinohe, the soybean cyst nematode using hybrid isolates of *Akanthomyces* spp.(Koike et al., 2011). *Akanthomyces muscarius* application (10^7 spores ml^{-1}) on powdery mildew (*S. fuliginea*) of cucumber leaves was characterized by mycoparasitism of *S. fuliginea* cells with appressorial formation at the end of some germ tubes, secretion of mucilaginous matrix, profuse sporulation on mycelia and conidia as well as mycelia deformation observed under Scanning Electron Microscopy (SEM) (Askary & Yarmand, 2007). Field trial and repeated applications of *A. muscarius* (10^{13} spores ha^{-1}) on *Podosphaera aphanis* Wallr. (strawberry powdery mildew) suppressed the disease (Miller et al., 2004). *Akanthomyces muscarius* lytic enzymatic production and successful suppression of phytopathogenic fungi (*Botrytis cinerea* Persoon and *Mucor mucedo* Fresenius) and oomycetes [*Pythium aphanidermatum* (Edson) Fitzpatrick and *Phytophthora palmivora* (E.J. Butler) E.J. Butler] has been observed (Fenice & Gooday, 2006). Further analysis of the type of lytic enzymes revealed that *A. muscarius* released more than five (5) chitinolytic enzymes with cell

wall degrading enzymes such as chitinases as well as glucanolytic, which were implicated in the biocontrol agent's mycoparasitic role (Fenice & Gooday, 2006). The outer layer of insects (exoskeleton), some arthropods as well as fungal cell walls are chiefly composed of chitin amongst other components, and chitinases degrade this barrier during the mycoparasitism process by entomopathogenic fungi (Berini et al., 2018, Berini et al., 2016, Mondal et al., 2016, Hajek et al., 2013, Hartl et al., 2012).This process has been demonstrated by chitinases produced by *Trichoderma harzianum* Rifai and *T. viride* Persoon against the silkworm [*Bombyx mori* (Linnaeus)] larvae (Berini et al., 2016). Other enzymes profiled in *Akanthomyces* spp. include proteases, lipases as well as catalases (Mondal et al., 2016), and catalase production in *A. muscarius* has been shown to influence infection processes (conidia germination, stress tolerance and virulence) (Huang et al., 2012). Multifarious activities on plant growth promotion by *Akanthomyces* spp. [*A. psalliotae* (Treschew) Zare & Gams] have been observed. This include solubilization of inorganic minerals (Zinc, and phosphates), production of phytohormones (Indole-3-Acetic Acid), enzymes (α-amylases, cellulases and proteases), which degrade fungal cells walls (Kumar et al., 2018). Subsequently, *A. psalliotae* has been reported to be infectious to thrips of cardamom, *Sciothrips cardamomi* (Ramakrishna Ayyar) (Kumar et al., 2018).

Akanthomyces muscarius application (10^2,10^3,10^4 conidia ml^{-1}) effectively reduce frangipani (*Plumeria rubra* Linnaeus) leaf area covered with *Coleosporium domingense* Patouillard pustules as well as the reduction in defoliation in both glasshouse studies and field trials (McMillan Jr, 1985). Earlier studies had shown the bean rust *Uromyces appendiculatus* (Persoon) Unger was suppressed by foliar applications of *A. muscarius* with apparent hyperparasitism, however, with no lysis of the urediniospores (Allen, 1982). Later in the years, some studies revealed the secretion of enzymes by *Verticillium* spp. (*V. psalliotae* and *V. lecanii*) during studies on biocontrol agents of soybean rust (Saksirirat & Hoppe, 1991b, Saksirirat & Hoppe, 1991a). Subsequently, *Vertillicium* (= *Lecanicillium*) spp. (*V. hemileiae*) have also been known to parasitize *H. vastatrix* (coffee rust) (Yirga, 2020), later infection of coffee rust was associated with infection of green coffee scale insect *Coccus viridis* (Green) in a mutualistic relationship with an ant (*Azteca instabilis* Antwiki) forming a complex web of interactions (Vandermeer et al., 2009). Parasitism by *V. lecanii* was observed on *U. dianthi* (carnation rust) both in detached leaf assays as well as glasshouse trials (Spencer, 1980). Microscopy studies confirmed mycoparasitism by *V. lecanii* on carnation rust as well as wheat leaf rust (*Puccinia recondita* Desmazières) during glasshouse studies (Spencer & Atkey, 1981).

Studies investigating *Akanthomyces* spp. as potential biocontrol fungi on various rusts species and insect control have been reported (Wei et al., 2018, Park et al., 2015, Vandermeer et al., 2009, Sharma et al., 2002, Whipps, 1993, Leinhos & Buchenauer, 1992, Allen, 1982) as well as extensive reviews of rust diseases biocontrol by various fungicolous fungi such as *Tuberculina* spp., *Verticillium* spp., *Cladosporium* spp., *Sphaerellopsis filum* (teleomorph: *Eudarluca caricis*), *Scytalidium uredinicola*, *Aphanocladium album* and many more others (Moricca & Ragazzi, 2008, Gowdu & Balasubramanian, 1988, Kranz, 1981).

Ascomycota, as a phylum and mainly in the order *Hypocreales*, infects 13 insect orders (Wu et al., 2018, Leland & Gore, 2017, Shin et al., 2017, Lovett & St.Leger, 2016). Studies that evaluate BCAs on insect control makes frequently make mention of *A. muscarius* amongst other *Akanthomyces* spp., *Beauveria bassiana* (Balsamo) Vuillemin, *Metarhizium anisopliae* (Mechnikov) Sorokin, *Isaria fumosorosea* (Wize) Kepler et al. as some of the key entomopathogenic genera (Kumar et al., 2019, Karimi et al., 2019, Wu et al., 2018, Lovett & St.Leger, 2016, Lu et al., 2015, Lacey et al., 2015, Gurulingappa et al., 2011) with some species commercially formulated as biopesticides of insect pests (Humber, 2016, Aiuchi et al., 2008, De Faria & Wraight, 2007). Notable are Koppert Biological Systems, Netherlands commercialized Vertalec® (active bioagent, formerly *L. longisporum* now *Akanthomyces longisporum*) for the control of aphids, and Mycotal® (active bioagent, formerly *L. muscarium*, now *Akanthomyces muscarius*) for the control whiteflies and trips in protected environments (Aiuchi et al., 2008, De Faria & Wraight, 2007). These registered commercial formulations have been shown to have the potential to control other pests. Vertalec® (a.i. *L. longisporum*) isolates have been evaluated against dual control of aphids and powdery mildew (Kim et al., 2010, Kim et al., 2008) amongst other various studies (Ghaffari et al., 2017, Goettel et al., 2008); Mycotal® (a.i. *L. muscarium*) have been evaluated against the control of *Myzus persicae* Sulzer (green peach aphid) Mohammed and Hatcher (2017) amongst other studies (Ghaffari et al., 2017, Hamdi et al., 2011, Khachatourians, 2009, Bardin et al., 2008, Goettel et al., 2008, Askary & Yarmand, 2007). *Lecanicillium lecanii* as the active bioagent has had many formulations by various companies worldwide for control of mostly insect pests: Biocatch® (whitefly, India); Microgermin® (thrips, Denmark); Euvert® (USA) for the control of insect pests (thrips, whitefly, aphids), mites (red spider mite), plant pathogens (rust, powdery mildew) and nematodes; Ag Biocontrol *Verticillium* 50 (insect orders Homoptera and Diptera, Colombia); Vertisol® (insect orders Thysanoptera, Hemiptera and Homoptera, Mexico) and

Nutrio-Life Myco-Force® (Australia), *L. lecanii* in combination with *B. bassiana* (scale, thrips, whitefly, aphids) (De Faria & Wraight, 2007).

In Thailand, *A. muscarius* has been reported as an endophyte of *Nypa fruticans* (nipa plum) Vinit et al. (2018). Earlier in the years, *Verticillium* (= *Lecanicillium*) *lecanii,* which has been believed to be synonymous with *A. muscarius* endophytic associations, were recorded in *Arctostaphylos uva-ursi* (Linnaeus) Sprengel (bearberry) as well as in ironwood (Vinit et al., 2018). Endophytic behaviour of *Akanthomyces* spp. has also been noted in *Cucurbita maxima* Duchesne (pumpkin) Gurulingappa et al. (2010), *Gossypium hirsutum* Linnaeus (cotton) Gurulingappa et al. (2010) and Gurulingappa et al. (2011), as well as in the *Araceae* family (Bamisile et al., 2018). In some studies, *V. lecanii* induced host defence against *Pythium ultimum* Trow (root rot pathogen) infection in cucumber roots by evoking structural and biochemical barriers (Bamisile et al., 2018). There is growing literature on endophytic entomopathogens (Dara, 2019, Barra-Bucarei et al., 2019) which may be explained by plant ecology and/or evolution interests in fungal symbionts, saprobes as well as the observed phenomenon of plant pathogens entering a latent phase (Doilom et al., 2017, Sung et al., 2008).

2.5 Mechanisms of biological control

Principally, literature on the mechanism/mode of action (MOA) of biological control agents (BCAs) on plant diseases shows a preponderance on elaborating about four to five MOAs namely: (i) antibiosis (mixed path antagonism), a direct interaction with pathogens, (ii) parasitism (direct antagonism with pathogens), (iii) infection pressure by competition (direct interaction with pathogens), (iv) induced systemic resistance (ISR), an indirect interaction with pathogens and (v) reduction of pathogen virulence (Mycovirus-mediated cross-protection, MMCP), the last one being the least discussed (Köhl et al., 2019, Ghorbanpour et al., 2018). Despite the five MOAs listed above, Köhl et al. (2019) argue that more MOAs have not yet been detected and that BCAs in nature may employ rather more complex MOAs, which may actually be combinations or mixed, rather than single-based MOA (Stenberg et al., 2021). Moreover, the growing technologies such as metagenomics and metatranscriptomic, part of the New Generation Sequencing (NSG), have added new light on the microbiomes (composition and function). Three different hosts: *S. fuliginea* (causal agent of powdery mildew on cucurbits), *Macrosiphum euphorbiae* Thomas (an aphid of the potato plant) and *Aphidius nigripes* Ashmead (a parasitoid wasp that feeds on aphids) were treated with a conidial suspension of *L. muscarium* (DAOM 19849), initially isolated from *Cydia pomonella* (Linnaeus), codling moths, Canada) to investigate fungal-host interactions (Askary &

Yarmand, 2007). The experiment yielded interactions of *L. muscarium* with its hosts: fungal-fungal interactions and fungal-insect interactions, which showed that successful interaction (infection process), regardless of host, follows a 5-stepwise approach or process (Askary & Yarmand, 2007). The BCA (*L. muscarium*) from the studies showed these five steps: (i) BCA attachment to hosts, (ii) BCA conidia germination leading to germ tube formation, (iii) BCA proliferation through mycelial growth on the hosts, (iv) BCA proliferation through penetration of the hosts and growth either externally or internally and (v) BCA spore production on hosts surfaces (Askary & Yarmand, 2007). Various studies and detailed complimentary microscopy work, some with green fluorescent protein (GFP) transformation, have further elucidated the observed infection process in different BCA-host interactions, either part of the steps or fully (Zhu et al., 2022, Németh et al., 2019, Gauthier et al., 2014, Ward et al., 2012, Ward et al., 2011, Charnley, 2003, Kumar & Jha, 2002, Clarkson & Charnley, 1996, Saksirirat & Hoppe, 1991b, Saksirirat & Hoppe, 1991a). **Table 2.2** shows examples of biological control agents and their possible mechanism of action and Fig 2.3 shows a simplistic mechanism of action employed by fungal biological control agents.

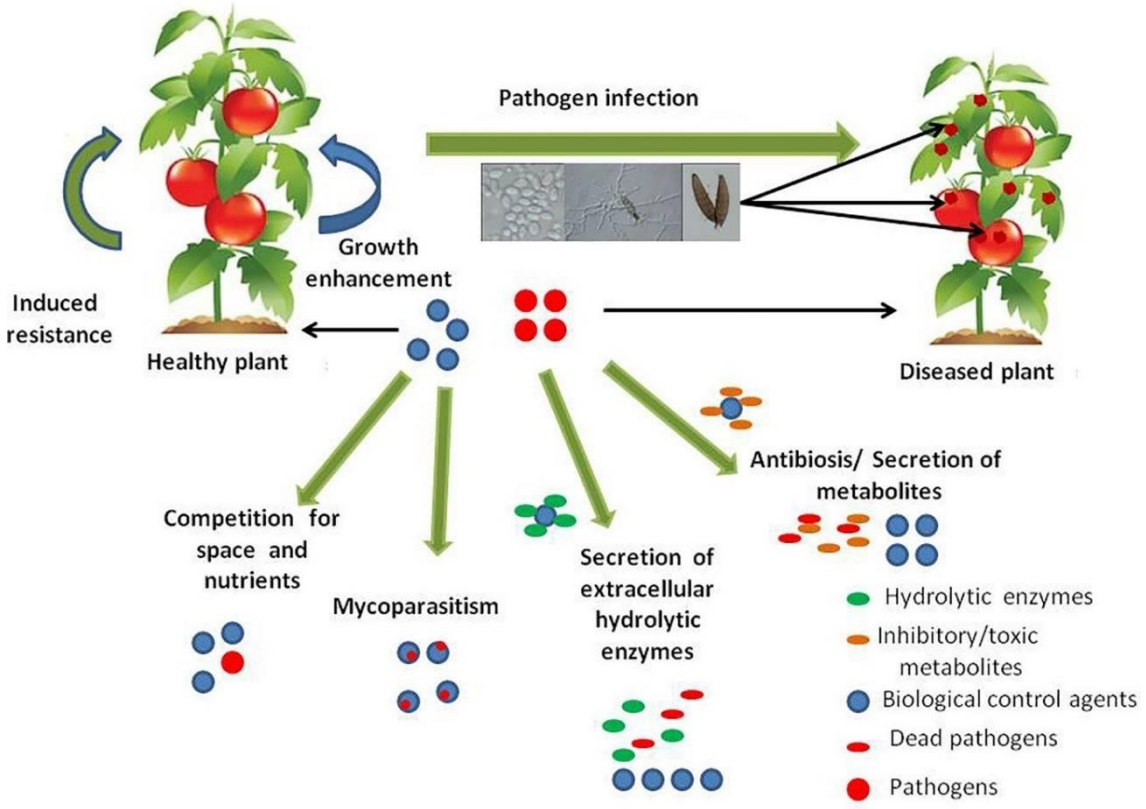

Fig 2.3 Fungi vs. fungi in biocontrol: Illustrated mechanism of action of fungi biocontrol agent on disease suppression (Thambugala et al., 2020)*(Reproduced with permission*–see Appendix 1*)*.

Table 2.2 Some biological control agents of plant pathogens and their possible mechanism of action

Type of BCA	Host Plant	Target Pathogen	Proposed MOA	Summary MOA/Effect on target pathogen	Reference
Bacillus amyloliquefaciens (SF14), *Alcaligenes faecalis* (ACBC1)	nectarines	*Monilinia fructigena* (brown rot disease)	Competition (infection pressure)	ACBC1 showed more significant inhibition due to rapid growth on wounds compared to SF14. Both biological control agents improved inhibition when in combination with SA at 0.5% and 3.5% Salicylic Acid (SA).	Lyousfi et al. (2021)
Bacillus amyloliquefaciens B10W10 and *Pseudomonas* sp. B11W11	apple	*Monilinia fructigena* (brown rot disease)	Antibiosis and competition (infection pressure)	A total of 13 bacterial isolates plus a commercial fungicide were tested with B10W10 and B11W11, which provided better control (*in vitro* and *in planta*). The bacteria were tested against the production of lytic enzymes, hydrocyanic acid and lipopeptides.	Lahlali et al. (2020)
Trichoderma harzianum ITEM 3636	peanut	*Fusarium solani* (peanut brown rot)	Mycoparasitism (enzymatic production) and antibiosis	Direct mycoparasitism was observed with perforations and physical alterations of *Fusarium solani* hyphae. High amounts of hydrolytic enzymes are observed and suggest aid in mycoparasitism. Secondary metabolites had significant inhibitory effects on the pathogen.	Erazo et al. (2021)
Ampelomyces isolates (*Ampelomyces quisqualis* M10)	tomato, barley, melon, red clover, strawberry	*Pseudoidium neolycopersici* (tomato powdery mildew); *Blumeria graminis* f. sp. *hordei* (barley powdery mildew); *Erysiphe trifoliorum* (red clover powdery mildew); *Podosphaera aphanis* (strawberry powdery mildew); *Podosphaera xanthii* (melon powdery mildew);	Mycoparasitism	*Ampelomyces quisqualis* (biocontrol agent) spores germinated on tomato foliage and directly penetrated powdery mildew fungi on host plants, leading to atrophy of pathogen conidiophores. The BCA produced new intracellular pycnidia in *Pseudoidium neolycopersici* conidiophores.	Németh et al. (2021)
Clonostachys rosea (formerly *Gliocladium catenulatum*, commercial – Gliomix® Prestop)	various crops	Soil-borne diseases of various crop species, e.g., *Pythium*, *Rhizoctonia* spp. causing dumping-off diseases	Competition in rhizosphere, mycoparasitism, CWDEs, antibiosis, induced resistance	Microscopy (SEM, light) revealed the ability of *Clonostachys rosea* to coil around pathogen and produce penetrating appressorium against pathogen hyphae. The BCA can produce CWDEs that help in its MOA. Induced resistance and antibiosis have been reported to suppress clubroot disease in canola.	Mcquilken et al. (2001), Abdellatif et al. (2022), Chatterton & Punja (2009), Lahlali & Peng. (2014)
Clonostachys rosea	numerous plant fungal diseases	*Botrytis cinerea*	Mycoparasitism	*Clonostachys rosea* efficient isolate tagged with green fluorescence gene (GFP) successfully produced infection structures leading to mycoparasitism, which was elucidated using confocal microscopy and SEM.	Hasan et al. (2022)

2.5.1 Antibiosis (mixed path antagonism)

Antibiosis is a confrontation of phytopathogens by chemical compounds secreted by the BCAs. Principally, the BCAs secrete a battery of chemicals such as allelochemicals such as cell-wall-degrading enzymes, antibiotics, lipopeptides, biosurfactants, bacteriocins, microbial volatile organic compounds (MVOCs) which drastically alter or provide for aberrations in phytopathogen anatomy and physiology (Zhao et al., 2023, Köhl et al., 2019, Borriss et al., 2019, Quintana-Rodriguez et al., 2018). The broad spectrum of secondary metabolites or chemical compounds produced by BCAs and their action through antibiosis or as elicitors of ISR in host plants have been extensively reviewed in the literature (Zhao et al., 2023, Suresh et al., 2022, Suresh et al., 2021, Borriss et al., 2019, Andreolli et al., 2019, Gu et al., 2017, Quecine Maria et al., 2016). Antibiotics produced by BCAs are preferred due to their environmental friendliness, biodegradability, and low toxicity (Kang et al., 2021). Some antibiotics produced by various microorganisms for the control of some soil-borne pathogens include: 2,4-diacetylphloroglucinol (*Pseudomonas fluorescens* Migula), gliotoxins [*Gliocladium/Trichoderma virens* (Miller et al.) Arx], Iturin A (*Bacillus amyloliquefaciens* Priest et al.), phenazine-1-carboxamide (*Pseudomonas fluorescens*), phenazine-1-carboxylic acid (*Pseudomonas chlororaphis* Bergey et al.) and Pyoluteorin (*Pseudomonas protegens* Migula) (Arseneault & Filion, 2017). Fungal biocontrol agents are also known to produce various antimicrobials (ketones, alcohols, ethylene, hydrogen cyanide, aldehydes, harzianolide, anthraquinone, viridin, viridiol, gliovirin, gliotoxin and heptelidic acid) which are essential in antibiosis and other mechanisms of biocontrol (Ayaz et al., 2023, Quintana-Rodriguez et al., 2018).

2.5.2 Parasitism (direct antagonism)

Microbe-microbe interactions have been investigated to some extent to unravel key signal transduction pathways required for such relationships (Sun et al., 2019, Syed Ab Rahman et al., 2018). Fungal-fungal interactions that result in the invasion of the fungi hosts leading to nutrient extraction by the invading fungi are characteristic of parasitism, referred to as mycoparasitism (Lahlali et al., 2022, Mukherjee et al., 2022, Palmieri et al., 2022, Köhl et al., 2019). Mycoparasites can either exhibit necrotrophic or biotrophic parasitism. In necrotrophic parasitism, the invading pathogen ultimately kills its host and extracts nutrients, whereas in biotrophic parasitism, living host structures benefit the invading parasite to continue extracting nutrients (Lahlali et al., 2022). Other microbes like bacteria and viruses can have parasitic

relationships with other microbes, viruses parasitising bacteria (bacteriophages) and bacteria parasitising fungi (mycophagy) and distinguishable from mycoparasitism, which is fungi on fungi (Raymaekers et al., 2020, Köhl et al., 2019, Syed Ab Rahman et al., 2018). Mycoparasites apart from direct penetration of their host, have been shown to breakdown host cells through secretion of various cell-wall degrading enzymes (CWDEs) such as amylase, β-1,3-glucanase, pectinase, chitinase, proteinase and cellulases which are under gene regulation (Cesa-Luna et al., 2020, Andreolli et al., 2019, Quecine Maria et al., 2016, Daguerre et al., 2014). Some specific hydrolases (Ras-GTPases) have been reported in the regulation of various crucial fungi cues such as fungal-fungal interactions, production of conidia, growth and development, and even essential for virulence of the mycoparasite (Dautt-Castro et al., 2020).

2.5.3 Infection pressure by competition

Infection pressure by competition or competition for nutrients and space is a common mode of action in nature and through which interacting microbial populations, one population group outcompetes other population groups and thrive due to an overlap of the demand of the same resource (Lahlali et al., 2022, Janisiewicz et al., 2000). The classical competition involves the antagonist (BCA) outcompeting the pathogen in both space and the colonisation rate (Zeilinger-Migsich & Mukherjee, 2014, Garrett, 1975). Studies in postharvest disease control have demonstrated the competitive advantage yeasts have over pathogens, leading to successful fruit protection from the pathogens (Janisiewicz et al., 2000). Some yeast isolates were able to reduce by 86% and 97% the disease severity of tomato and apple fruits caused by *Penicillium expansum* Link and *B. cinerea* (Fernandez-San Millan et al., 2021). In the same study, the yeasts also reduced disease severities of the soil pathogens *Fusarium oxysporum* (Padwick) Matuo & Sato (50%) and *Verticillium dahliae* Klebahn (40%). In other ecological relationships, mycoparasites such as *Lecanicillium* species isolates can compete for the common host (used as the substrate) and the competitive isolate establishes its significance. In their study, Chandler et al. (1993) reported two *V. lecanii* species competing for a host and one more competitive isolate being recovered from the host after co-inoculations. **Table 2.2** provides more examples of the discussed MOA.

2.5.4 Induced systemic resistance (ISR)

Induced systemic resistance (ISR) is defined as a complex defence mechanism by plants brought on by the presence of either a pathogenic microbe to the host plant or an opportunistic microbe which does not cause harm to the plant, leading to successfully warding off other phytopathogens (Yu et al., 2022, Kamle et al., 2020). The host immune response mechanism triggers systemic acquired resistance (SAR) distal to the host infection site. Phytopathogens or endophytic microbes induce ISR and SAR (Datta et al., 2022, Nicoletti & Becchimanzi, 2020, Choudhary et al., 2007). The mechanism of ISR is complex and usually involves complex signal transduction pathways that employ the jasmonic acid (JA) and ethylene (ET) pathways, amongst other mechanisms (Mandal & Ray, 2011). Whilst it does not directly kill pathogens, the induction of ISR confers resistance to future attacks (Kamle et al., 2020).

On the other hand, SAR induced through salicylic acid (SA) signal transduction pathways leads to putative defence gene expression in the host plant, leading to durable resistance against a broad spectrum of plant pathogens (Durrant & Dong, 2004). *Trichoderma* species are well documented for their ability to colonize different parts of plant organs, leading to changes in the plant's metabolism and transduction pathways (SA, JA, ET) that induce ISR and SAR (Gupta & Bar, 2020). For example, *Trichoderma atroviride* Bissett uses chitinases as elicitors to trigger ISR in apples to ward off *Venturia inaequalis* (Cooke) Winter, which causes apple scab disease (Faize et al., 2003). Further work by Gupta et al. (2022) showed that *T. harzianum* T39, *Metarhizium brunneum* Petch and *B. bassiana* are capable of inducing plant immunity (ISR) in tomatoes, leading to conferring resistance against various pests and plant pathogens. Non-pathogenic isolates of *Fusarium* species have been profiled for their ability to confer resistance to *Fusarium* wilt disease through ISR induction and competition (Patil & Sriram, 2020). *Lecanicillium* and *Akanthomyces* isolates are also profiled to induce ISR against pathogens (Nicoletti & Becchimanzi, 2020).

2.6 Development of fungal commercial BCAs and their formulations

Fungi are among the most widely studied BCAs, and their potential for commercialization is increasingly recognized and a topic of interest in the agricultural industry (Omidvari et al., 2023, Priyashantha et al., 2023a, 2023b). The market for BCAs is continuously rising, valued at US$7.53 billion (2023), expected to rise to US$8.73 billion (2024), and projected to reach US$28.61 billion by 2032 (https://www.fortunebusinessinsights.com/industry-reports/biopesticides-market-100073). Fungi such as *Trichoderma* spp., *Lecanicillium* spp. and

B. bassiana have been studied and many commercial formulations have been developed from their isolates (Guzmán-Guzmán et al., 2023, Rodrigues et al., 2023, Saldaña-Mendoza et al., 2023,Yao et al., 2023, Ghorbanpour et al., 2018, Chandler et al., 2011). Other fungal BCAs, such as *Metarhizium* spp. and *Paecilomyces* spp., have also been commercially developed to control plant pathogens (Savita & Sharma, 2019). The initial stages of commercialisation are isolation and screening, identification, bioassays, and pilot studies for a potential BCA (Martinez et al., 2023, Collinge et al., 2022). A potential BCA must have desirable characteristics such as high virulence, ease in production, host specificity, some displayed mechanism of action, and environmental adaptability, amongst many more traits (Ghorbanpour et al., 2018). After selecting a promising BCA isolate, patenting is often done, followed by a multiple-step process towards mass production. A multidisciplinary approach is usually employed in mass production techniques such as solid-state fermentation, submerged fermentation, and other bioprocessing techniques leading into formulations such as liquid formulations, encapsulated formulations, oil-based formulations, wettable powders, granules as notable examples (Loera-Corral et al., 2016, de Faria & Wraight, 2007, Wraight, 2001). The patented BCA undergoes rigorous processes such as toxicological tests, environmental compatibility and impact evaluations, analytical methods and many more (Teixidó et al., 2022). These processes overlap and are interlinked. The efficacy of BCAs is affected by many aspects of the three groups (abiotic, biotic, formulation) as significant factors (Boro et al., 2022, Ghorbanpour et al., 2018). Formulation and storage follow as the BCA product undergoes registration processes. Formulation development is a complex and multidisciplinary approach which seeks to ensure, among other things: (i) a stable BCA formulation from bioprocessing company to end user, (ii) handling and safety, (iii) viability and bioefficacy as stated on the BCA product, and (iv) protection from harmful abiotic and biotic factors (Martinez et al., 2023, Teixidó et al., 2022). Formulation, therefore, is a bridge between product development and application, and the various requirements of the user, technological requirements, regulation laws, and costs determine the success of commercialisation (Jiang & Wang, 2023, Teixidó et al., 2022). Pilot or field trials evaluate the efficacy of the developed BCA, assessing various factors, including the impact on the targeted pests and beneficial organisms paving the way towards commercialization (Collinge et al., 2022). Less industrialized countries face astronomic costs towards producing viable and dependable BCAs (Grzywacz et al., 2023). In South Africa, developing BCAs takes at least nine years, of which five are committed to early research two years to field trials, and the last two years on registration and overall costs of not less than R7 million (M.D. Laing, personal communication, April 2024). Overall, developing

commercial isolates of BCAs is a promising avenue for controlling plant pathogens, and further research is needed to optimize their use in agricultural practices.

2.7 Delivery systems of BCAs through adjuvants

Adjuvants are substances (inert or may have varied synergistic effects) added to pesticides, biocontrol agents' formulations, or nutrient solutions to improve the mixing, application, or effectiveness of the active ingredient (Encerrado-Manriquez et al., 2024, Nairn & Forster, 2024, Holka & Kowalska, 2023, Wu et al., 2023, Beestman, 2018, Hagedorn et al., 2017, Mesnage & Antoniou, 2017, Mulqueen, 2003). The performance of biological control agents can be enhanced by adjuvants like surfactants, carriers, emulsifiers, protective agents, and nutritional adjuvants (Stevenson et al., 2017, Castro et al., 2013). Adjuvants' physicochemical properties determine their function and impact on biological activity, and thus, understanding the critical parameters of adjuvants is crucial for solving specific delivery problems (Ferreira et al., 2020, Courtney et al., 2019, Prado et al., 2016, Castro et al., 2013, Holloway, 1998). Adjuvants modify the properties of spraying solutions to enhance the effectiveness of active substances applied to soybean crops (Tavares & Cunha, 2023, Gimenes et al., 2013). These adjuvants work by reducing the surface tension of the liquid improving the spread and adhesion on the leaf surface (Wang et al., 2022, Gaskin et al., 2005). Adjuvants are, therefore, critical in fungicide and biocontrol applications as they help in emulsifying, dispersing, and spreading the active agents on the leaf surfaces and are manufactured to have varied practical application properties desirable to the farmer for effective pest control (Lin et al., 2023, Castro et al., 2013, Czarnota & Thomas, 2013). For example, surfactants facilitate the fungicide action against soybean rust by ensuring that the fungicide covers and adheres to the leaf surface more effectively (Garcia et al., 2016, Cunha & Peres, 2010). Vegetable oil additives, adjuvants, or carriers play a crucial role in enhancing the effectiveness of BCAs (Senthilkumar, 2019a, 2019b, Batta et al., 2011). Atplus PFA® (an alkoxylated alcohol) is a commercially registered adjuvant that is flowable and noted explicitly for its efficacy in enhancing the action of fungicides against soybean rust by improving the spread and retention of fungicides on the leaf surfaces (https://www.crodacropcare.com/en-gb/product-finder/product/312-atplus_1_pfa).

Soybean leaves possess properties such as a waxy outer layer and hairy abaxial surface, and the canopies have leaves situated at an angle which provides for water repelling or roll-off, uneven spread and runoff, respectively (Badawy et al., 2023, Gimenes et al., 2013, Taylor, 2011). Water sprays on waxy leaves result in minimal wetted areas, high contact angle (>90º),

and the failure of droplets to spread (Xu et al., 2010). Leaf surface permeability or leaf physiochemical properties are crucial to agrochemical applications (Nairn & Forster, 2024, Fernández et al., 2017). Contact angle provides for quantification of the wettability of a leaf surface with lower contact angle (<60º) and high contact (>100º) regarded as "easier" and "very difficult" to wet, respectively (Damato et al., 2017, Gaskin et al., 2005); and vary with growth stages in soybean (Puente & Baur, 2011). The soybean leaf properties compounded by high water surface tension reduce the coverage and effectiveness of water-based application solutions to properly adhere the active ingredients of the spray treatment to the target surfaces and thus increase off-target losses (Xu et al., 2010).

2.8 Benefits and limitations of fungal biological agents of plant pathogens

The entomopathogens *Trichoderma* spp., *B. bassiana*, *M. anisopliae*, and *L. lecanii* are some of the top five fungi studied as biological control agents with *Trichoderma* spp. as the most widely studied (Alfiky & Weisskopf, 2021, Thambugala et al., 2020). Interestingly, some *Trichoderma* spp. such as *Trichoderma afroharzianum* Chaverri et al. is now known to cause the "*Trichoderma* ear rot on maize" disease (Pfordt et al., 2020). The fungal BCAs and their multimodal mechanisms of action discussed in earlier sections make them attractive and are also considered eco-friendly with no expected deleterious effects on the natural ecosystems (https://ibma-global.org/biocontrol-benefits; Hulot and Hiller (2021) and possible postponement of resistance development (Zaki et al., 2020). There are various reviews on biological control agents and their use in either controlled environments or field studies enumerating their potential, success and challenges (Ayaz et al., 2023, Jiang & Wang, 2023, Mesguida et al., 2023, Priyashantha et al., 2023a, Palmieri et al., 2022, Collinge et al., 2022, Islam et al., 2021, Zaki et al., 2020, Gerbore et al., 2014, Schmutz et al., 2010, Butt et al., 2001). A Google Scholar (https://scholar.google.com/) search of the keywords "control of plant fungal diseases by fungal biological control agents" from 1980 to 2024 generated 19,400 articles.

Despite the increasing research in this area, literature records quite a small number of categorized fungi (approximately 300) with biocontrol potential against plant pathogens. In addition, these belong to 13 classes comprising 113 genera (Thambugala et al., 2020). Zaki et al. (2020) showed that mycopesticides are inundated with various challenges ranging from politics of development, the mode of action of the fungi, the physiological state of the active ingredient, and legislation, among other discussed factors (Grzywacz et al., 2023). In South Africa, developing and commercialising a BCA takes at least nine years, of which five are

committed to early research, two years to field trials, and the last two years on registration and overall costs of not less than R7 million (M.D. Laing, personal communication, April 2024). Overall, developing commercial isolates of BCAs is a promising avenue for controlling plant pathogens, and further research is needed to optimize their use in agricultural practices. Moreover, the registration of BCAs is done through the same daunting and expensive route for chemical synthetic pesticides (Humber, 2016). Farmers who have been used to applying chemical pesticides are more keen to see pest eradication rather than management, for example, the visible accumulation of dead insect pests as observed when insecticides are applied (Humber, 2016). Humber (2016) also discusses other challenges, such as the disruptiveness of changing terminology, errors in genomic databases, fidelity in culture collections, storage, and experimental procedures which often prove challenging to reproduce and incapacitated institutions fail to properly preserve specimens. Other numerous factors that curtail the use of fungi as BCAs include both biotic (host plant, pathogen, BCA's nature, virulence, biodiversity), and abiotic (temperature, relative humidity, leaf wetness, UV-B) factors, and BCAs are known to show high variability and sensitivity to these (Fenta & Mekonnen, 2024, Ayaz et al., 2023, Lahlali et al., 2022).

2.9 Conclusion

There is a growing concern about the environmental impact of synthetic agricultural pesticides, which affect human health, kill pollinators such as bees and destroy natural ecosystem balance. Soybean is one of the top five most valuable crops in the world. There is heavy usage of fungicides for soybean diseases, including ASR pathogen *P. pachyrhizi*. On one hand, the reliance on fungicides for the ASR pathogen has met pathogen resistance, requiring fungicides rotation scheme and the use of fungicides with multiple modes of action, which has raised costs for the production of the soybean crop worldwide. Fungicide use is also coupled with challenges of timing in application as ASR, even in low severity levels, renders the protectant fungicides ineffective for the polycyclic and proficient spore-producing ASR pathogen. On the other hand, breeding efforts, despite some successes, have had to contend with the short durability of developed soybean cultivars as the ASR pathogen in no time overcomes the resistance owing to its intricate pathosystem, genetic structure and diversity. The search for biological control options for phytopathogens, such as *P. pachyrhizi*, the soybean rust pathogen, is increasing. Fungal entomopathogens such as *Lecanicillium* species have been commercially developed as biological control options primarily for insect pests under controlled environmental facilities. However, even though the fungal species have shown

potential, there is still a need for more research on their development as biological control options for plant pathogens both under controlled environments and in the field. Several biological control options have been developed for other plant diseases; however, there is limited work for soybean rust disease, mainly due to the problematic nature of biotrophic plant pathogens. Whilst acknowledging the challenges faced in developing viable biological control of soybean rust, this review supports the evaluation of *Lecanicillium* species, particularly *L. uredinophilum,* as a promising hyperparasite that needs further development. The development of fungal biological control agents also requires the ability to understand the environmental requirements that make them proliferate, as well as their delivery systems. This is a crucial area of development that optimizes their performance.

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Chapter 3: Morphological and molecular characterization of mycoparasitic fungi targeting *Phakopsora pachyrhizi* biocontrol in South African soybeans

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Abstract

Soybean is a crop of global importance and South Africa is a leading producer in Africa. Soybean rust disease caused by *Phakopsora pachyrhizi* is of global economic significance because it causes up to 100% losses. It is currently managed through the use of fungicides. The study aimed to find mycoparasites of this rust as eco-friendly biocontrol alternatives. Four isolates were successfully isolated from pustules of rusts from two plants (*Oxalis* spp. and *Fragaria vesca* L.). Isolates were initially identified through genomic DNA extraction and Sanger sequencing of the internal transcribed spacers (ITS) regions as targets, after which inconclusive phylogenetic analysis necessitated additional marker genes. Therefore, the DNA-directed RNA polymerase II second largest subunit (RPB2) and the transcription elongation factor-1α (TEF) were used to resolve the taxonomic ambiguity. Isolates were also visually evaluated through morphological characterization using microscopy. The isolates were screened using a modified Koch's Postulate protocol, which also served as a pathogenicity test. Visual and microscopic morphological characterization showed Isolate PP2018-001, and PP2018-003 were similar in colony morphology, although PP2018-001 was white-coloured, whilst PP2018-003 was creamy-yellow. The spores of both isolates were ovoid, oblong, and ellipsoidal, with a few cylindrical spores produced in whorls or singular phialides or 3-4 nodes of phialides. Scanning electron microscopy revealed a range of phialide lengths: 15-40 µm, a spore length of 1.1-3.5 µm for Isolate PP2018-001, and a phialide length of 20-50 µm and a spore length of 1.6-6.7 µm for Isolate PP2018-003. The results from the pathogenicity test were used to select isolates PP2018-001 and PP2018-003 as potential mycoparasites through the extent of colonization of rust pustules of soybean rust and *Plumeria* spp. rust. Initially, ITS analysis identified isolates PP2018-001 and PP2018-003 as *Akanthomyces muscarium*, isolate

PP2018-003 as *Lecanicillium* spp. or *Aphanocladium araneurum* and isolate PP2018-004 as *Simplicillium lanosoniveum*. Further phylogenetic analyses using the additional gene markers on the screened isolate PP2018-001, and another isolate obtained from Andermatt-PHP (PP2018-005) successfully resolved the taxonomic uncertainty, identifying both isolates as *Lecanicillium uredinophilum* isolates. This is the first study demonstrating that *Lecanicillium uredinophilum* isolated from wild strawberry rust pustules is a potential biocontrol agent for soybean rust in South Africa.

Keywords: Morphological characterization, Molecular characterization, Mycoparasitic fungi, *Phakopsora pachyrhizi*, South African soybeans, Biocontrol

3.1 Introduction

Soybean [*Glycine max* (L.) Merr.] is one of the world's important crops (Dilawari et al., 2022), grown on 6% total arable land (Hamza et al., 2024). Soybean seeds, on a dry matter basis, contain high protein quality (balanced amino acids (Kanchana et al., 2016) and protein quantity (35-40%) (Hartman et al., 2011), a high quality cooking oil (18-22%) (Parle et al., 2014), and carbohydrates combined with a high content of fibre (35%) (Kanchana et al., 2016), amongst other important constituents such as vitamins, antioxidant polyphenols and mineral elements (Singh et al., 2008). Production of soybeans on a global scale is led by Brazil (36%), the USA (34%), and Argentina (12%), which accounted for 82% of the global total (348.9 million MT). The global value of soybean was US$175.3 billion in the 2022 season (FAO, 2024; Soystats, 2023; Soystats, 2022). South Africa, in 2022, led soybean production in Africa, accounting for 35.8% of Africa's total production. However, Africa contributes less than 1% of the global output (FAO, 2024). Soybean in South Africa has a monetary potential of over R20 billion at the farm level (Mcpherson, 2023). Consumption estimates are 67.8%, 15.29%, and 2.67% for protein, oilcake and human consumption, respectively, whilst the gross value of agricultural production increased from over R2 billion in 2011 to R8 billion in 2020 (van de Linde, 2023; DAFF, 2021).

Soybean production is particularly susceptible to the Asian soybean rust (ASR), a fungal disease caused by *Phakopsora pachyrhizi* Syd. & P. Syd., which is considered to be its most destructive foliar disease (Garcia et al., 2008; Miles et al., 2003), contributing to global losses of 10-90% (Murithi et al., 2016). In Asia, losses of 10-100% have been recorded (Hossain et al., 2024). The disease reduces seed size, weight, oil content, and yield (Hoffmann et al., 2022).

Phakopsora pachyrhizi has many hosts, infecting at least 41 species in 17 genera, and has a wide variation in virulence (Zambolim et al., 2022; Zambolim et al., 2021). According to Savary et al. (2019), the aggregate annual production losses due to ASR are approximately 5.9% across sub-Saharan Africa. Moreover, soybean varieties lack durable resistance to the pathogen (Chicowski et al., 2024; Murithi et al., 2016).

Efforts to control *P. pachyrhizi* have focused on chemical control using fungicides as the first-line of defence (Langenbach et al., 2016). The second line strategy has involved breeding soybean cultivars resistant or tolerant to ASR (Da Silva et al., 2017; Tukamuhabwa et al., 2012; Sneller et al., 2005). The third line of defence, a relatively new approach, has been biological control, which in the past 30 years has developed as a viable large scale option for the control of pests and diseases (Nega, 2014). Chemical control of soybean rust is not always feasible because of specific application requirements. For instance, preventative applications of protectant fungicides must be accurately timed because they may not be effective if the disease is already present at low levels; 20-30% of canopy infections render fungicides ineffective (Mueller et al., 2009; Dorrance et al., 2007). In addition, the *P. pachyrhizi* has evolved and has developed resistance, particularly to triazole fungicides (Hahn, 2014; Oliver, 2014; Reddy, 2012). In South Africa, and historically, ASR control has primarily relied on the rotation of different chemical fungicides with different modes of action, such as quinone outside inhibitors (QoI)/strobilurins, demethylation inhibitors (DMI)/triazoles and succinate dehydrogenase inhibitors (SDHIs)/carboxamide fungicides available on the market (Laing and Caldwell, 2014). More recently, combination fungicides are used for the management of ASR, for example, Custodia™ 320 SC (a.i. full dose azoxystrobin and tebuconazole) provides protection for up to 28 days (https://www.adama.com/south-africa/en/soybean-farming/soybean-farming-south-africa). Pesticides have proven to have adverse effects on the health of human populations (farm workers, consumers, farmers and rural people) and the environment (water quality, soil health, pollinators and wildlife habitats) (Raine and Rundlöf, 2024; Khan et al., 2023; Almeida et al., 2021; Jepson et al., 2020; Anderson and Harmon-Threatt, 2019; Carvalho, 2017; Damalas, 2009). Despite being used to control or manage pests, pesticides can also create conditions that ultimately favour plant disease outbreaks through their disruptive nature to natural ecosystems, selection pressure, pesticide resistance, and impeding plant natural defence mechanisms (Yin et al., 2023).

The quest to find an alternative to chemical control strategies and eco-friendly solutions to various phytopathogens has promoted research on the development of biological control agents (Ajuna et al., 2024; Bean et al., 2024; De Brida et al., 2024; Dowd and Johnson, 2024; Dubey et al., 2024; Khulbe and Batra, 2024; Konthoujam Ambedkar et al., 2024; Nchu, 2024; Swathy et al., 2024; Teles et al., 2024; Lahlali et al., 2022; Lyousfi et al., 2021; Lahlali et al., 2020; Lahlali and Peng, 2014; Lahlali et al., 2011). In South Africa, various biological control agents (BCAs) have been registered for insect pests and fungal plant pathogens, but none so far for the soybean rust pathogen. The beneficial nematode (*Steinernema feltiae*) formulation (Nemaplus® Depot P) controls both fungal gnats and Western flower thrips (https://biobee.co.za/solutions/nemaplus-depot-p/). The product Eco-77® (*Trichoderma atroviride*) can control *Botrytis*, *Sclerotinia sclerotiorum* and *Eutypa lata* whilst Eco-BB® (*Beauveria bassiana*) is employed against whitefly, spider mites, *Tuta absoluta* and other insects pests (https://andermatt-php.co.za/t-77-eco-77/). The control of ASR using BCAs has not yet been fully explored; however, there have been reports of antagonistic microorganisms to *P. pachyrhizi* (Gauthier et al., 2014; Ward et al., 2012a; Ward et al., 2012b; Ward et al., 2011; Saksirirat and Hoppe, 1991a; Saksirirat and Hoppe, 1991b; Saksirirat and Hoppe, 1990a). After extensive studies on the biocontrol agent, the fungus *Simplicillium lanosoniveum* was reported to colonize soybean leaves infected with *P. pachyrhizi* (Gauthier et al., 2014; Ward et al., 2012a). Ward et al. (2012a) were able to show a significant (fourfold less) and successful reduction of urediniospores by two isolates of *S. lanosoniveum* co-inoculated with *P. pachyrhizi* in detached leaf assays, compared with the control treatments. Their scanning electron microscope (SEM) results showed *S. lanosoniveum* coiling around ASR uredinia, although no visible penetration marks were observed. In field experiments the authors evaluated levels of *P. pachyrhizi* in control plots and plots of rust-infected soybeans treated with *S. lanosoniveum* through DNA extraction and quantitation using qPCR assays. The results revealed statistically significant differences in the levels of ASR between untreated controls and biocontrol-treated soybean leaves. Gauthier et al. (2014), through their extensive confocal laser-scanning microscopy (CLSM), SEM, and transmission electron microscopy (TEM), as well as using a green fluorescent protein (GFP)-labelled *S. lanosoniveum* isolate, were able to provide evidence that S. *lanosoniveum* was indeed a mycoparasite that can coil around and penetrate urediniospores and degrade cell contents.

In Taiwan and Thailand, several fungal hyperparasites growing on *P. pachyrhizi* urediniospores were isolated and identified as species of *Trichothecium*, *Tuberculina* and *Verticillium*

(Saksirirat and Hoppe, 1990b; Saksirirat and Hoppe, 1990a). Kumar and Jha (2002) isolated *Trichothecium roseum* (Persoon) Gray from ASR-infected leaves. Scanning electron microscopy (SEM) revealed that the fungus attacked *P. pachyrhizi*, causing shrinkage and hypertrophy of urediniospores as well as crater-like depressions on urediniospore walls, which were attributed to enzyme activity by *T. roseum.* The same study revealed the ability of *T. roseum* cell-free culture filtrate to inhibit urediniospores germination. Saksirirat and Hoppe (1991b) used light and SEM microscopy to show that the mycoparasite *V. psalliotae* was able to colonize urediniospores of *P. pachyrhizi*; most urediniospores appeared heavily damaged without any visible mycelium inside, as a result of the production of lytic enzymes by *V. psalliotae* (Saksirirat and Hoppe, 1991b).

From these examples, it is hypothesized that rust sori provide a substrate for various mycoparasitic fungi, which may offer an alternative to chemical control of ASR. Therefore, this study sought to isolate and identify mycoparasites from a range of rust sori as potential biological control agents against *P. pachyrhizi*.

3.2 Materials and Methods

3.2.1 Sample collection

The isolation of mycoparasites is dependent on field observations of diseased plant species. In the case of rust mycoparasites, periodical observations are made on plant species (cultivated and non-cultivated) that are affected by rust. In this study, close attention was given to the following plant species: wood sorrel (*Oxalis* spp.), wild strawberry (*Fragaria vesca* L.), coffee (*Coffea* spp.) and frangipani (*Plumeria* spp.). These plants are all infected by different rusts, namely: *Puccinia oxalidis* Dietel & Ellis (1895), *Phragmidium mexicanum* (Mains) H.Y.Yun, Minnis & Aime, *Hemileia vastatrix* Berk. & Broome (1869) and *Coleosporium plumeriae* Patouillard (1902), respectively. Professor Mark Laing (Discipline of Plant Pathology, University of KwaZulu-Natal, Pietermaritzburg, South Africa) brought in oxalis and wild strawberry plants that were infected with rusts and mycoparasites from his home garden. These were placed in the disease tunnel (high humidity tunnel~90-95% RH) for 1-5 days before isolation could be made. Other *Oxalis* plants were found at the University of KwaZulu-Natal (UKZN), College of Agriculture, Engineering and Science (CAES), around the controlled environment facilities near experimental plots (29° 37' 18.48" S; 30° 23' 50.28" E). Figure 3.1

and 3.2 are examples of rust-infected *Oxalis* plants and wild strawberry plant samples, with visible colonization of rust sori.

Fig 3.1 Non-symptomatic *Oxalis* spp. in a garden (A); Potted rust-infected *Oxalis* spp. in a tunnel (B); Mycoparasitic colonization developing on rust-infected *Oxalis* spp. leaves; Advanced mycoparasitic colonization of rust pustules on *Oxalis* spp. leaves (D).

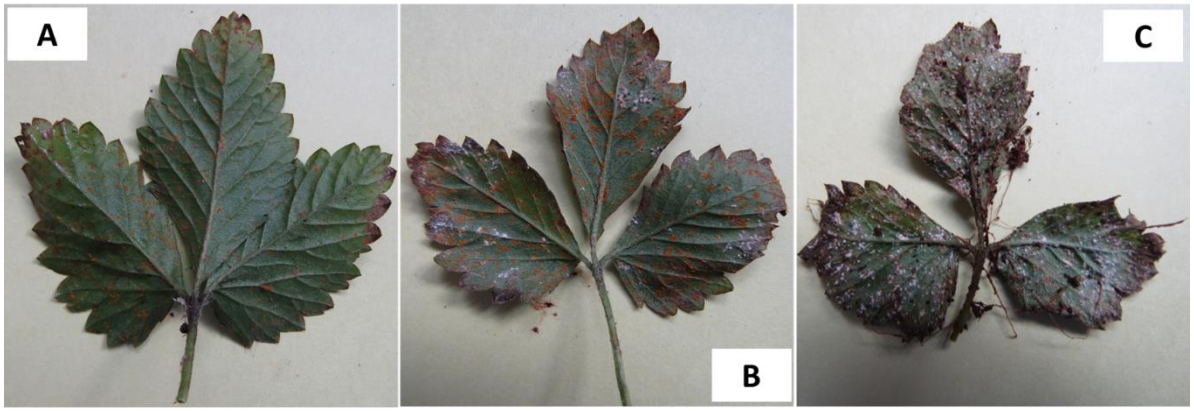

Fig 3.2 Wild Strawberry leaves infected with rust in a garden (A); Rust-infected wild strawberry leaves showing early mycoparasitic colonization in a tunnel (B); Advancing mycoparasitic colonization on the rust-infected wild strawberry leaves (C).

3.2.2 Isolation of potential rust sori mycoparasites as biocontrol agents

The isolation of potential mycoparasite candidates followed the methods outlined by Senanayake (2020) with modifications. Fig 3.3 below outlines a brief chronogram required to achieve a pure isolate. Briefly, suspected and visually apparent signs of fungi-fungi

interactions, as shown in Fig 3.1 and Fig 3.2, were considered potential candidates of mycoparasitic associations and incubated for 1-5 days at 21-28℃ and 90-95% RH. Aseptically, a sterile inoculating needle was used to pick out visible mycelia/fruiting body (with and without a stereomicroscope) from the samples and to point inoculate fungi onto potato dextrose agar (PDA) plates prepared according to manufacturer's instructions (Merck Pty Ltd., Johannesburg, South Africa). One hundred (100) PDA plates for each plant species were inoculated through needle-point inoculation from various rust pustules of wood sorrel and wild strawberry plants. The inoculated PDA plates were inverted and incubated at 25±1℃ in a biological oxygen demand (BOD) incubator with daily observations for the development of fungal growth. Fungal colonies obtained from direct isolations were subcultured by point-inoculation onto freshly prepared PDA plates until pure colonies were achieved. After 3, 7 and 14 days, wet mounts of pure fungal cultures were prepared and viewed under a light microscope (X400) to observe fruiting bodies, spores, and mycelial growth. To help classify an isolate as a potentially useful mycoparasite, it had to produce spores within 14 days post-incubation at 25±1℃, failure of which such an isolate would be treated with ultra-violet (UV) light for a further 7 days to induce sporulation. Failure to sporulate after UV light treatment rendered such isolates unsuitable for further studies. Isolates were organized into nine (9) groups, each consisting of 20 replicates, except for Groups 1, 6, and 9, which had 21 replicates each. Based on the ability to sporulate within 14 days post-culture, Groups 1, 4, 5, and 8 were give identities as PP2018-001 (1), PP2018-002 (4), PP2018-003 (8), and PP2018-004 (5) as they were suitable for further studies as potential mycoparasites. Groups 2, 3, 6 and 7 did not produce any spores under 14 days post-culture and the further 7 days under UV light treatments and these were not given any identities. The pure cultures for the sporulating isolates were subsequently stored on PDA slants.

3.2.3 Koch's postulate as pathogenicity tests for selected fungal isolates

The spore suspensions of the four isolates (PP2018-001, PP2018-002, PP2018-003, and PP2018-004) were prepared by washing 14-day-old cultures on full-strength PDA with aqueous Break-Thru® (250 ml) 0.01% (v/v) (a non-ionic surfactant) and filtered through a double layer of cheesecloth. The spore suspensions were adjusted with a haemocytometer to achieve 1 x 10^6 spores/ml. The determination that no fungal mycoparasite was present on the soybean-rust-infected leaves was done by first spraying a mist of sterile distilled water on five selected soybean-rust-infected plants growing in a greenhouse and covered with transparent plastic bags for three days. A similar treatment was done on the leaves of *Plumeria* spp.

(frangipani) infected with rust on five branches of the frangipani tree, which were covered with transparent plastic bags for three days. After the 3-day period, a magnifying glass was used to inspect for any fungal growth on the rust pustules. All the leaves showed no signs of development of any fungi on the rust pustules for either plant species. A hydraulic spray bottle (100 mL) was used to spray the spore suspensions of the respective isolates until run-off on four soybean-rust-infected plants; the fifth plant was only treated with sterile 0.01% aqueous Break-Thru® suspension as a control treatment. The plants were covered with new transparent plastic bags and left in the greenhouse (90-95% RH, temperatures of 26-28 °C for 10 days. For the frangipani plant, the rust-infected leaves covered with clear plastic bags were sprayed with spore suspensions from the respective isolates (PP2018-001, PP2018-002, PP2018-003, and PP2018-004) until run-off and covered again with new plastic bags. The leaves on the fifth branch were treated with 0.01% aqueous Break-Thru® as a control. After 10 days of monitoring, rust spore colonisation was visually inspected and re-isolated, and light microscope evaluations were done on spores to confirm Koch's postulate (Bhunjun et al., 2021). Koch's postulate served as a pathogenicity test, and isolates showing complete or near complete colonization of the rust pustules were selected for further studies.

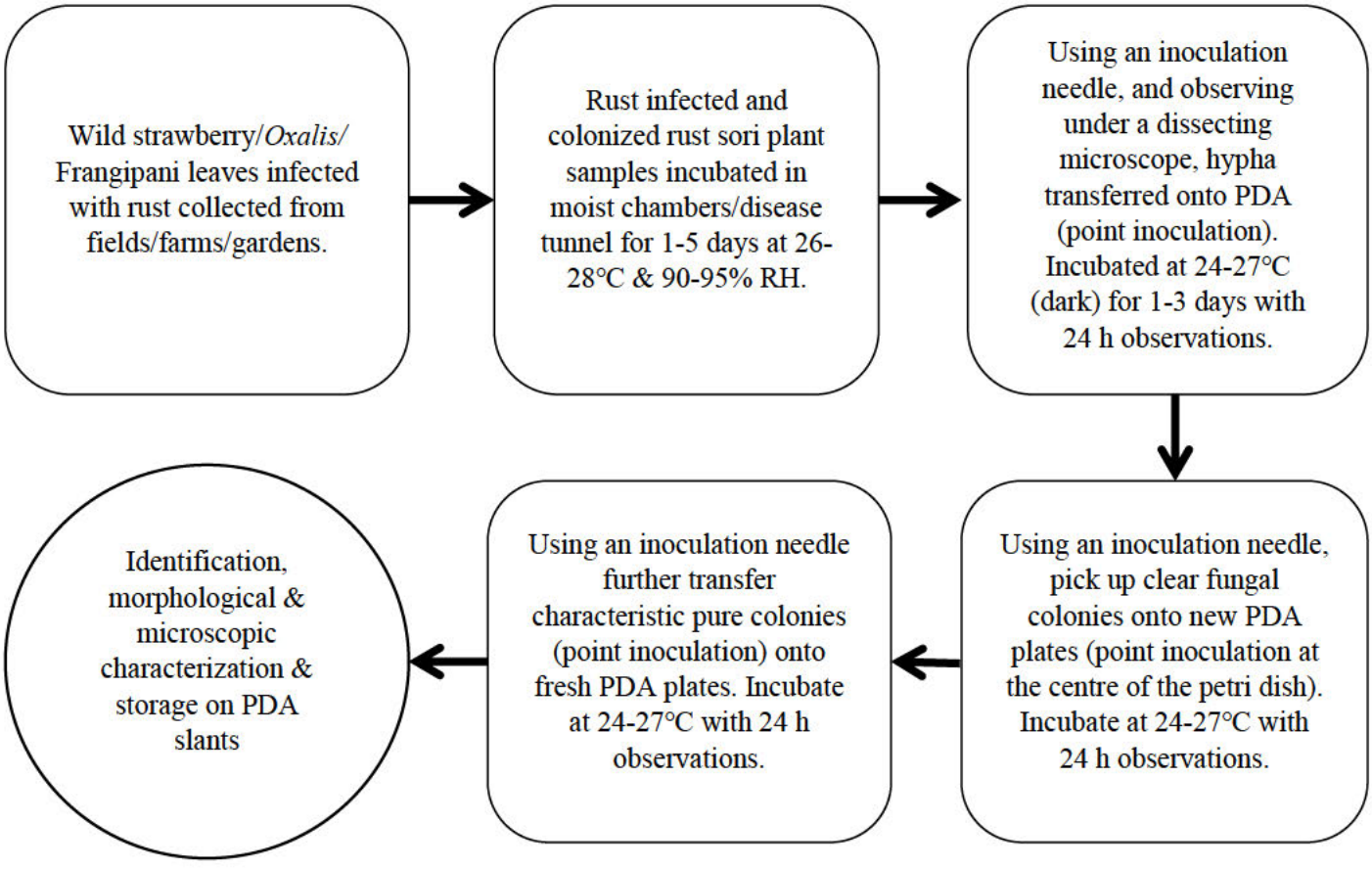

Fig 3.3 Flow diagram of the process followed for the isolation of potential mycoparasites

3.2.4 Morphological characteristics of the suspected fungal mycoparasites

The pure fungal isolates obtained in Section 3.2.2 above were subjected to observations. Firstly, visual characteristics were noted on colony morphological traits such as observed from previous studies (typical mycelium structure, size, shape, elevation, margin, colouration, opacity) before visualization of wet mount structures under the microscopes (light microscope and SEM for successfully selected isolates). The structures under the microscope, such as hyphae and spores, were done on Days 3, 7, and 14 of growth of the pure cultures on PDA plates. Fungal cultures that had not produced spores within the first 14-day period were regarded as non-rust invaders and possibly phylloplane fungi. These were further subject to UV treatment for an extended 7 days, after which, upon their failure to produce spores, they were discarded as unsuitable for further studies.

3.3 Phylogenetic analyses of suspected rust sori mycoparasites

3.3.1 Fungal ID using the internal transcribed spacers (ITS) regions markers, DNA Extraction, PCR Amplification and Sequencing

To extract the genomic DNA from the four fungal isolates (PP2018-001, PP2018-002, PP2018-003, and PP2018-004), spore and mycelia were scraped from the surfaces of 14-day-old cultures grown on full-strength PDA. The Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005) was employed for genomic DNA extraction from the four isolates according to the method of Lee and Taylor, (1990). Polymerase chain reaction (PCR) targeting the internal transcribed spacers (ITS) regions were run in 20 µl reactions containing 10µl OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486), 1µl of 10 µM forward and reverse primer each, 1 µl genomic DNA (10-30 ng/µl) and 7 µl nuclease-free water. The sequences of the primers used are shown in **Table 3.1**. The PCR was performed using the following cycling parameters: initial denaturation at 94 °C for 5 min, and 35 cycles at 94°C for 30 s, 50°C for 30 s, 68°C for 1 min, followed by a final extension at 68°C for 10 min. The PCR products were run on a 1% agarose gel and purified using Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Catalogue No. D4001). Direct Sanger sequencing of the PCR product were performed at Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa.

Table 3.1 Description of primers used for PCR and Sanger sequencing for identification of four samples

Target regions	Primer names	Sequence	References
Small Sub-Unit	ITS1	5'-TCCGTAGGTGAACCTGCGG-3'	(White et al., 1990)
Large Sub-Unit	ITS4	5'-TCCTCCGCTTATTGATATGC-3'	(White et al., 1990)

3.3.2 Fungal ID by ITS, RPB2 and TEF markers, DNA extraction, polymerase chain reaction, sequencing, and phylogenetic analysis

Isolate PP2018-001, initially named as University of KwaZulu-Natal Wild Strawberry Isolate (UKZN WSI), as well as an isolate that had shown a potential to be developed as a commercial product, PP2018-005, initially designated as PHP 2205, was obtained from Andermatt-PHP (Pty) Ltd, Nottingham Road, South Africa were grown on full-strength PDA. After 14 days, two PDA plates of each isolate were sent to Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa, for genomic DNA extraction, PCR and Sanger sequencing. PCR and sequencing targeted the internal transcribed spacers (ITS) regions, the DNA-directed RNA polymerase II second largest subunit (RPB2) and the transcription elongation factor-1α (TEF) using the primers listed in **Table 3.2**.

Table 3.2 Description of primers used for PCR and Sanger sequencing.

Target regions	Primer names	Sequence	References
ITS	ITS1	5'-TCCGTAGGTGAACCTGCGG-3'	(White et al., 1990)
	ITS4	5'-TCCTCCGCTTATTGATATGC-3'	
RPB2	RPB2F	5'-GACGACCGTGATCACTTTGG-3'	(Van den Brink et al., 2012)
	RPB2R	5'-CCCATGGCCTGTTTGCCCAT-3'	
TEF	EF1AF	5'-GCCCCCGGCCATCGTGACTTCAT-3'	

	EF1AR	5'-ATGACACCGACAGCGACGGTCTG-3'	(Van den Brink et al., 2012)

For phylogenetic analysis, the consensus sequences of the isolates of interest were aligned with selected taxa based on the studies of (Zhou et al., 2022) on *Lecanicillium,* downloaded from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). The trees were generated according to the evolutionary model, which best fitted our data for each locus and the combined ITS, RPB2 and TEF sequences with bootstraps based on 500 replicates. Multiple sequence alignment, selecting the best evolutionary models and tree drawing were all performed using MEGA 11, software version 11.0.13 (Tamura et al., 2021). *Simplicillium lanosoniveum* IMI 317442 (Accession number AJ292395.1), isolate CBS 704.86 (Accession number AJ292396.1) and *Simplicillium lamellicola* CBS 116.25 (Accession number AJ292393.1) were used to root the trees. Branches with bootstrap values below 75% were collapsed.

3.4 Results

3.4.1 Isolation of potential fungal mycoparasites from rust-infected plants

In this study, a total of nine (9) distinct groups exhibiting diverse morphological characteristics, as shown in Fig 3.4, were obtained with Groups 2, 3, 4, 5, 7 and 8 (each with 20 replicates) and Groups 1, 6 and 9 (each with 21 replicates). Screening of the 9 groups was achieved by creating wet mounts on Days 3, 5, 7 and 14. The groups (1, 4, 5, and 8 seen in Fig 3.4) produced distinct mycelial growth and spores. These four groups were preserved in PDA slants and stored for further studies. Groups 2, 3, 6, 7 and 9 failed to produce any spores under normal incubation and extended incubation under UV treatment and thus were discarded as unsuitable for further studies. Groups 1, 2, 6 and 8 were isolated from the wild strawberry rust pustules, whilst Groups 3, 4, 5 and 9 were isolated from *Oxalis* spp. rust pustules. Isolates 1, 4, 5, and 8 were designated identities as PP2018-001 (1), PP2018-002 (4), PP2018-003 (8), PP2018-004 (5).

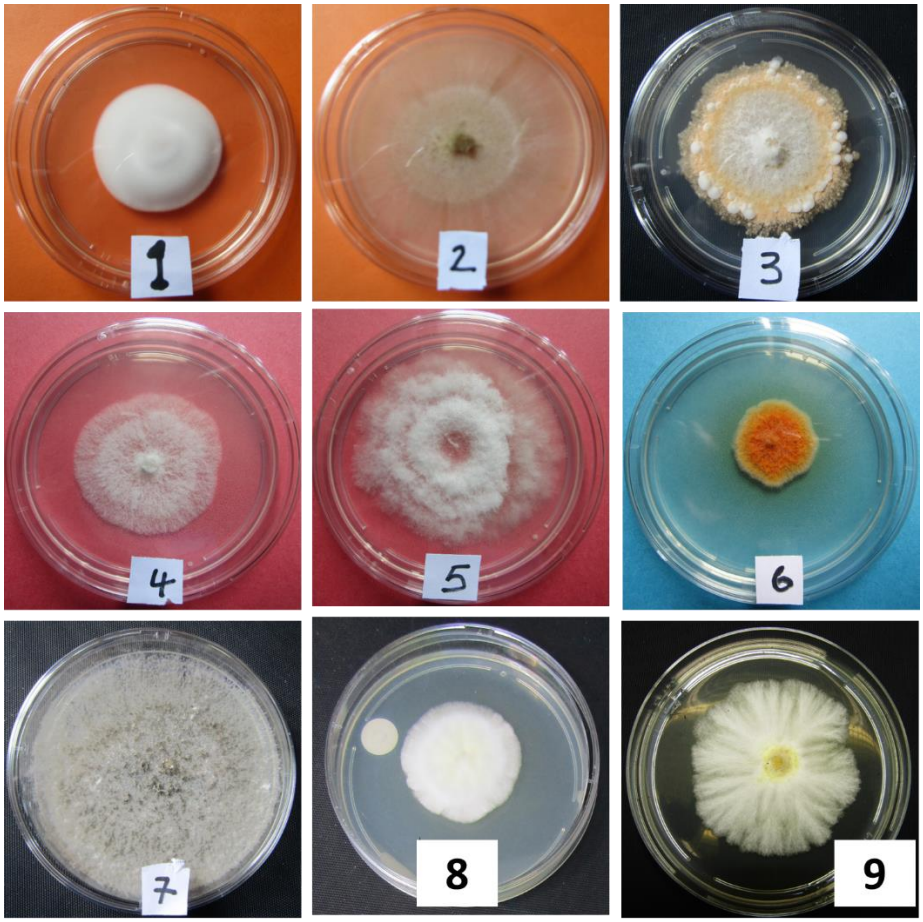

Fig 3.4 Representative groups of fungal isolates from rust sori of infected plants.

3.4.2 Koch's Postulate as pathogenicity tests for selected fungal isolates

The pathogenicity test results for Isolates PP2018-001 and PP2018-003 showed successful colonization of the soybean and frangipani rust pustules, as shown in Fig 3.5 (only isolate PP2018-001 images are provided). Re-isolation of the suspected mycoparasites through needle-point inoculations successfully produced pure cultures of the suspected mycoparasites. Isolates PP2018-001 and PP2018-003 were tagged for further studies, whilst isolates PP2018-002 and PP2018-004 were not selected for further studies, due to their poor colonization of the rust pustules of soybean and frangipani (images not shown).

Fig 3.5 Koch's Postulate/pathogenicity test showing: Soybean rust uredinia colonized by isolate PP2018-001 (A) and the control (B); and colonization of frangipani rust pustules (C) and the corresponding control (D).

3.4.3 Morphological characteristics of suspected rust sori mycoparasites

The four suspected mycoparasites that did produce spores have their identities as isolate PP2018-001, PP2018-002, PP2018-003, and PP2018-004. Isolate PP2018-001 (Fig 3.6a, 3.6b, 3.6c) and Isolate PP2018-003 (Fig 3.6e, 3.6f, 3.6g) showed typical morphological characteristics of *Lecanicillium* spp. The colony size of isolate PP2018-001 after 14 days of incubation at 25±1℃ ranged from 48-53 mm in diameter, with an entire margin, flat elevation, velvet to cottony texture, opaque and both the obverse and reverse side being white in colour (Fig 3.6a, 3.6b). Isolate PP2018-003 of the abovementioned characteristics differed in size (40-44 mm diameter) and colouration of creamy-yellowish on the reverse side (Fig 3.6e, 3.6f). For Isolate PP2018-001, under light microscopy (X400 magnification) (Fig 3.6c) spores were ovoid, ellipsoidal, oblong, or cylindrical shapes and of different sizes. Similar observations were made for Isolate PP2018-003 (Fig.6g). The spores of Isolates PP2018-001 and PP2018-003 ranged in size under SEM observation (Fig 3.7 and 3.8), with phialides occurring singly or as whorls, in prostrate branches of 3-4, ranging in size from 15-40 µm for isolate PP2018-

001 and 20-50 μm for isolate PPP2018-003. The length of the spores varied: spores of isolate PP2018-001 were 1.1 - 3.5 μm long, and isolate PP2018-003 were 1.6 - 6.7 μm long. Scanning electron microscopy (SEM) observations were not done for isolates PP2018-002, and PP2018-004; however, they were sent together with PP2018-003 and PP2018-001 for genomic identification through ITS.

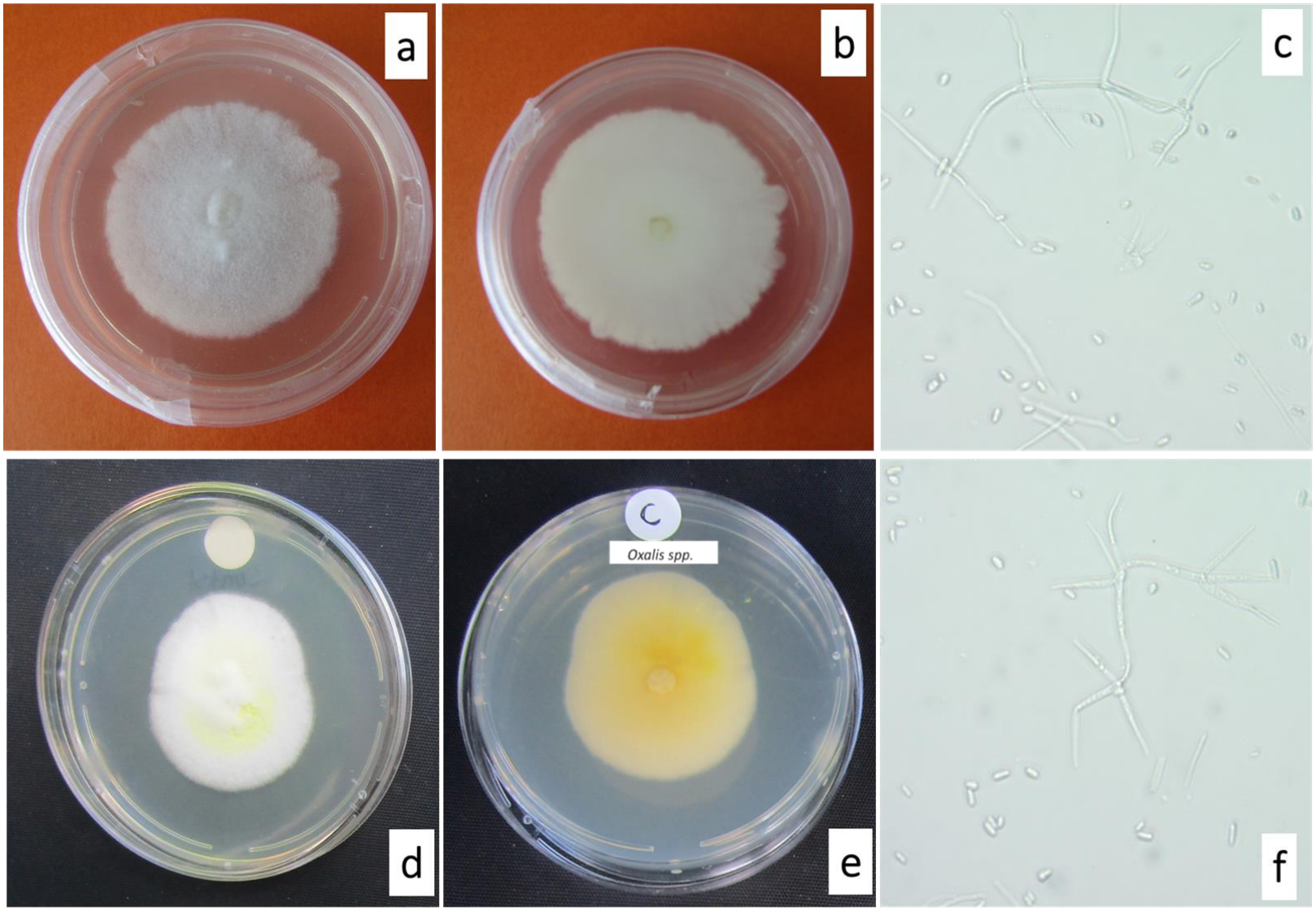

Fig 3.6 Growth characteristics (a, b and c) on PDA for fungal isolate PP2018-001 and (d, e, f) for isolate PP2018-003.

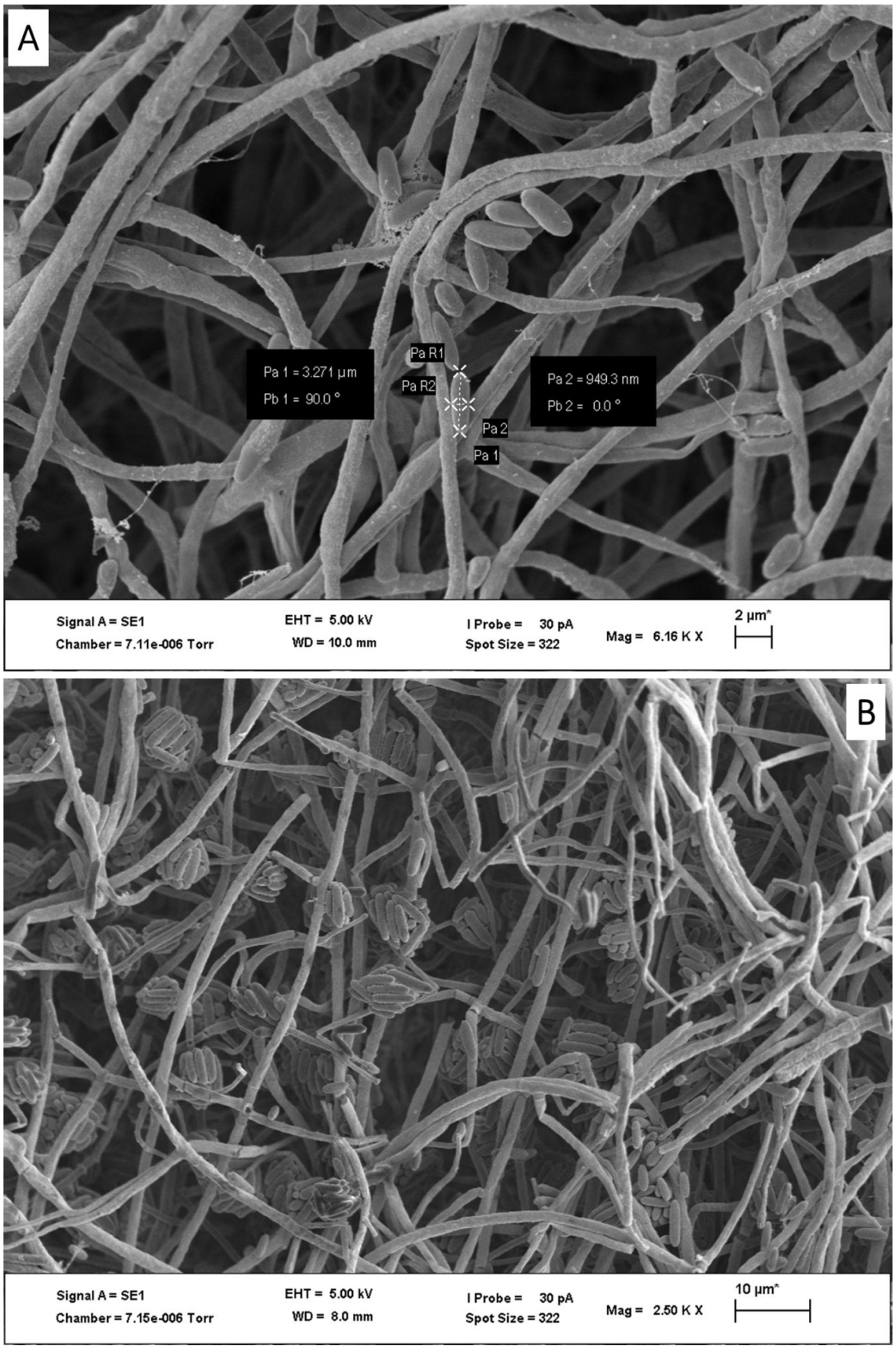

Fig 3.7 Scanning electron microscopy (SEM) view of isolate PP2018-001 showing spore dimensions (A) and phialides with clustered spores (B).

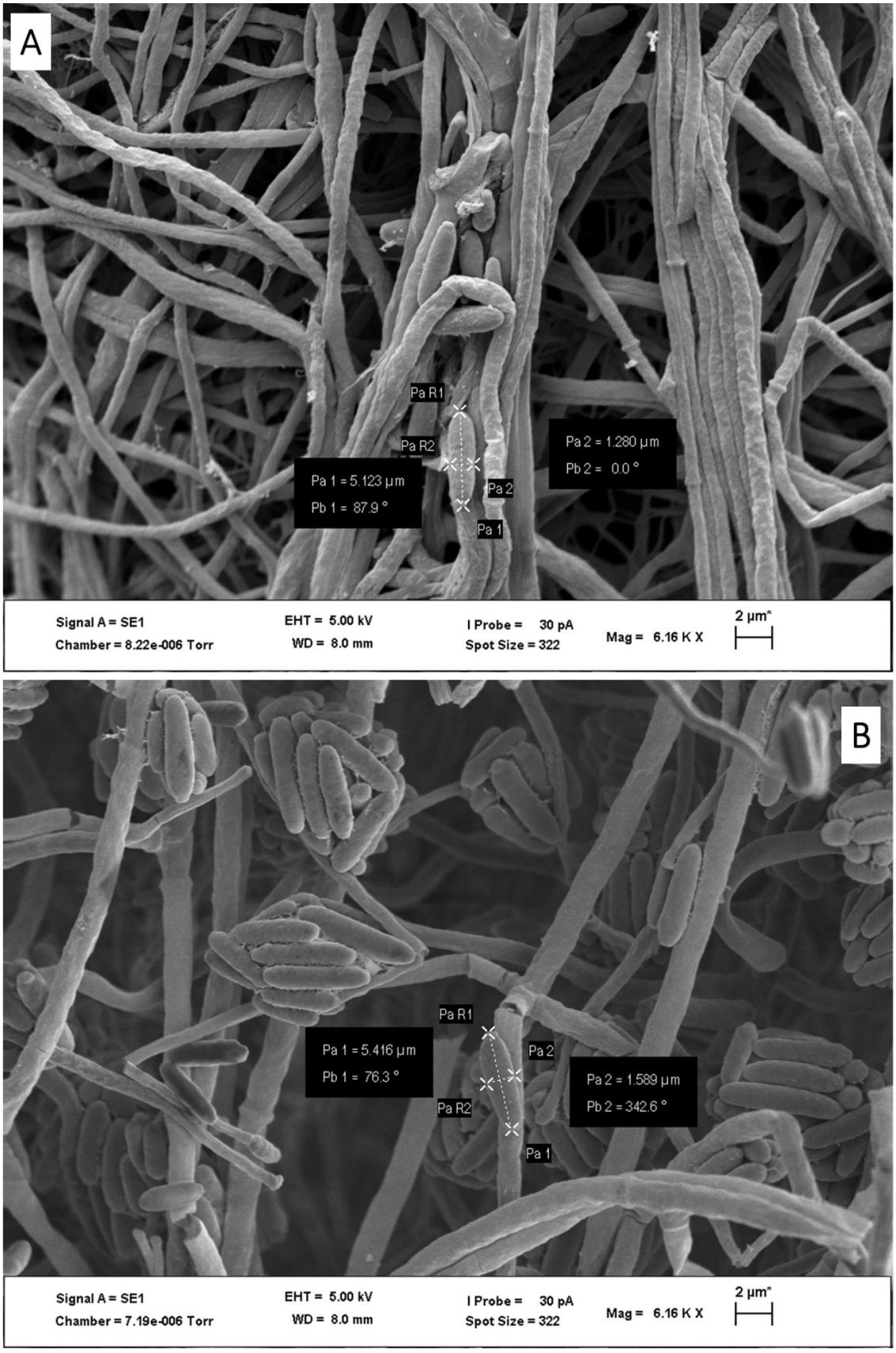

Fig 3.8 Scanning electron microscopy view of isolate PP2018-003 showing spore dimensions (A) and phialides with clustered spores (B).

3.4.4 Fungal ID by ITS1 and ITS4 markers only, DNA Extraction, PCR Amplification and Sequencing

The BLAST of processed sequences generated through ITS identified isolates PP2018-001 and PP2018-003 as *Akanthomyces muscarium* isolates. Isolate PP2018-002 was identified as a isolate of either *Lecanicillium* spp. or *Aphanocladium aranearum* and PP2018-004 was identified as *Simplicillium lanosoniveum*. However, there was a taxonomic problem on the construction of phylogenetic trees, which was inconclusive, rendering identification by the ITS primers used as inadequate.

3.4.5 Fungal ID by ITS, RPB2 and TEF markers

The consensus sequences for each locus and the ITS of two isolates (PP2018-001 initially designated as UKZN WSI, and an isolate obtained from Andermatt-PHP (PP2018-005) initially designated as PHP 2205 were submitted to the National Centre for Biotechnology Information (NCBI) and were given accession numbers **(Table 3.3)**.

Table 3.3 Accession numbers of the sequences of selected isolates

Isolates	ITS	RPB2	TEF
UKZN WSI (PP2018-001)	PP259072.1	PP273269.1	PP273271.1
PHP 2205 (PP2018-005)	PP259071.1	PP273270.1	PP273272.1

The phylogenetic tree of the ITS regions was generated using the maximum likelihood method and the general time reversible model with a discrete gamma distribution and evolutionarily invariable sites. The ITS data involved 34 nucleotide sequences, including the outgroup sequences. It was observed that the isolate PP2018-001 formed a lineage of its own, while Isolate PP2018-005 was grouped with *Lecanicillium uredinophilum* isolates (Fig 3.9). The RPB2 data consisted of 38 nucleotide sequences, including those of the outgroup. The tree was generated using the maximum likelihood method and Kimura 2-parameters model (Kimura, 1980) with a discrete gamma distribution and evolutionarily invariable sites. Both isolates under study clustered with *L. uredinophilum* only, as shown in Fig 3.10. The same topology was observed from the TEF tree, which was inferred using the maximum likelihood method

and Tamura-Nei model (Tamura and Nei, 1993) with a discrete gamma distribution and evolutionarily invariable sites, as shown below (Fig 3.11). The TEF data had 37 nucleotide sequences. The phylogenetic analysis of the combined loci and ITS regions also placed PP2018-001 and PP2018-005 within the *L. uredinophilum* lineage with a high bootstrap value of 100, as shown in Fig 3.12 The maximum likelihood method and Tamura-Nei model (Tamura and Nei, 1993) with a discrete gamma distribution and evolutionarily invariable sites were identified as fitting best the dataset that consisted of 33 nucleotide sequences.

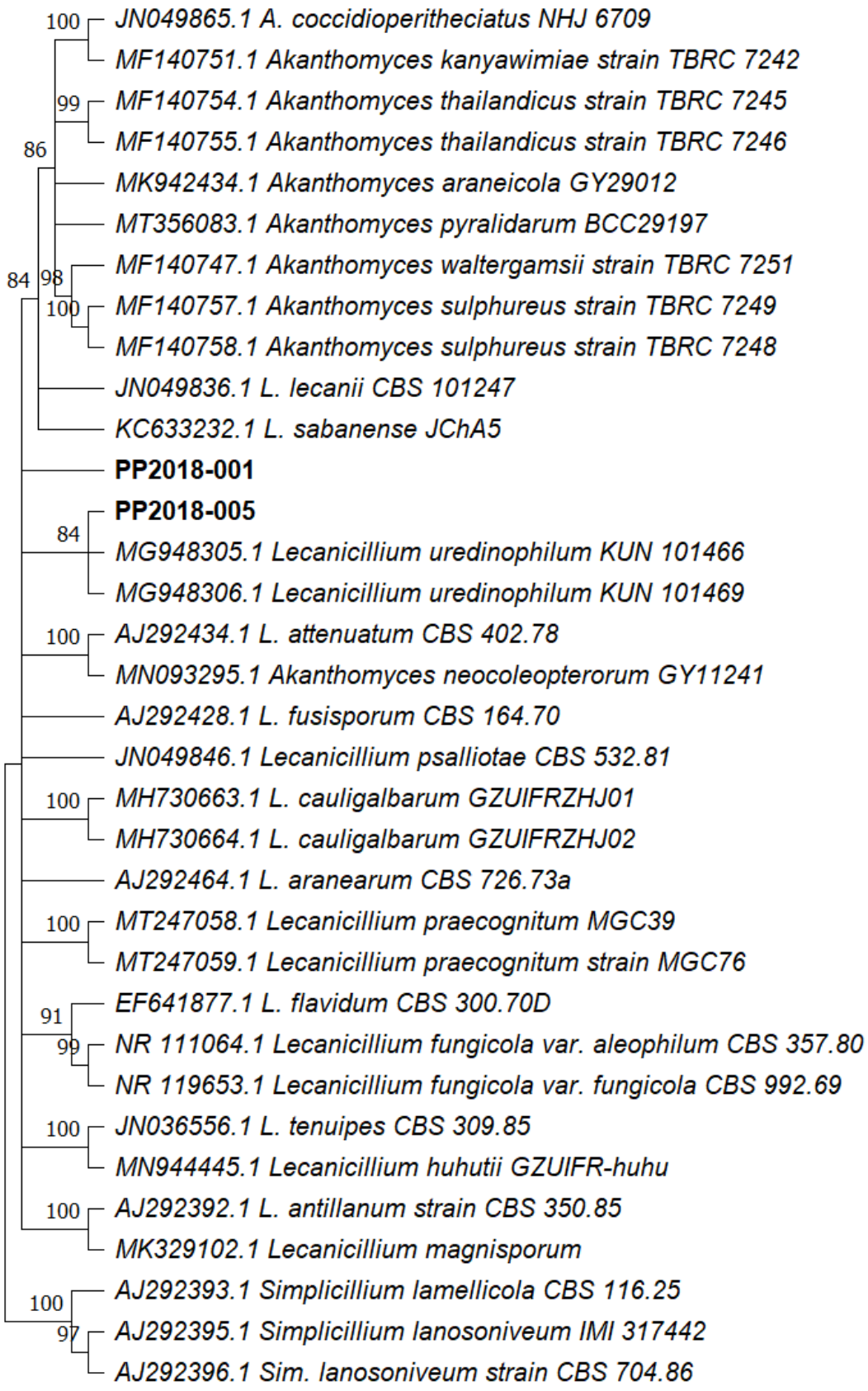

Fig 3.9 Phylogenetic tree based on internal transcribed spacers (ITS) sequences illustrating the genetic relationships.

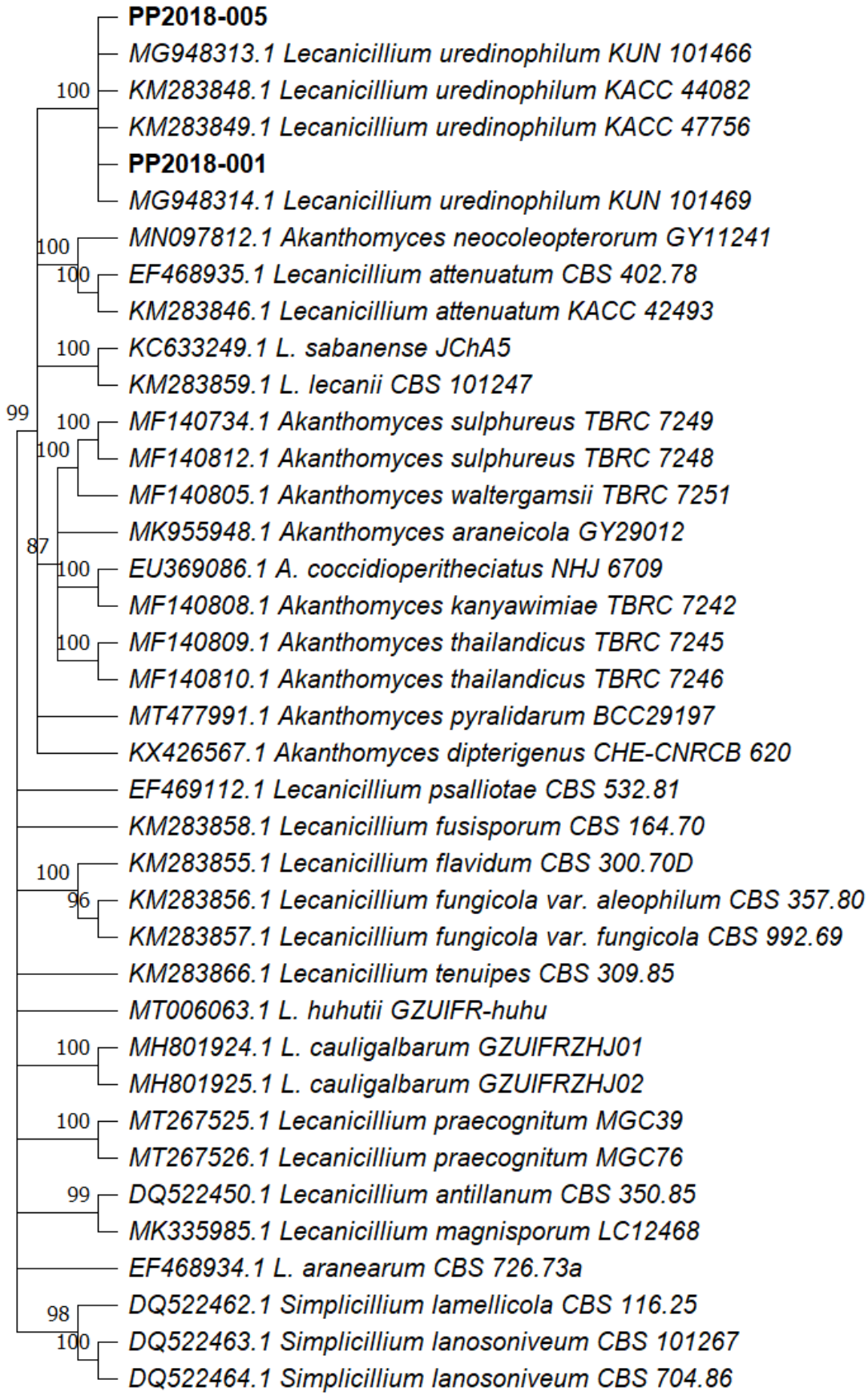

Fig 3.10 Phylogenetic analysis of the DNA-directed RNA polymerase II second largest subunit (RPB2).

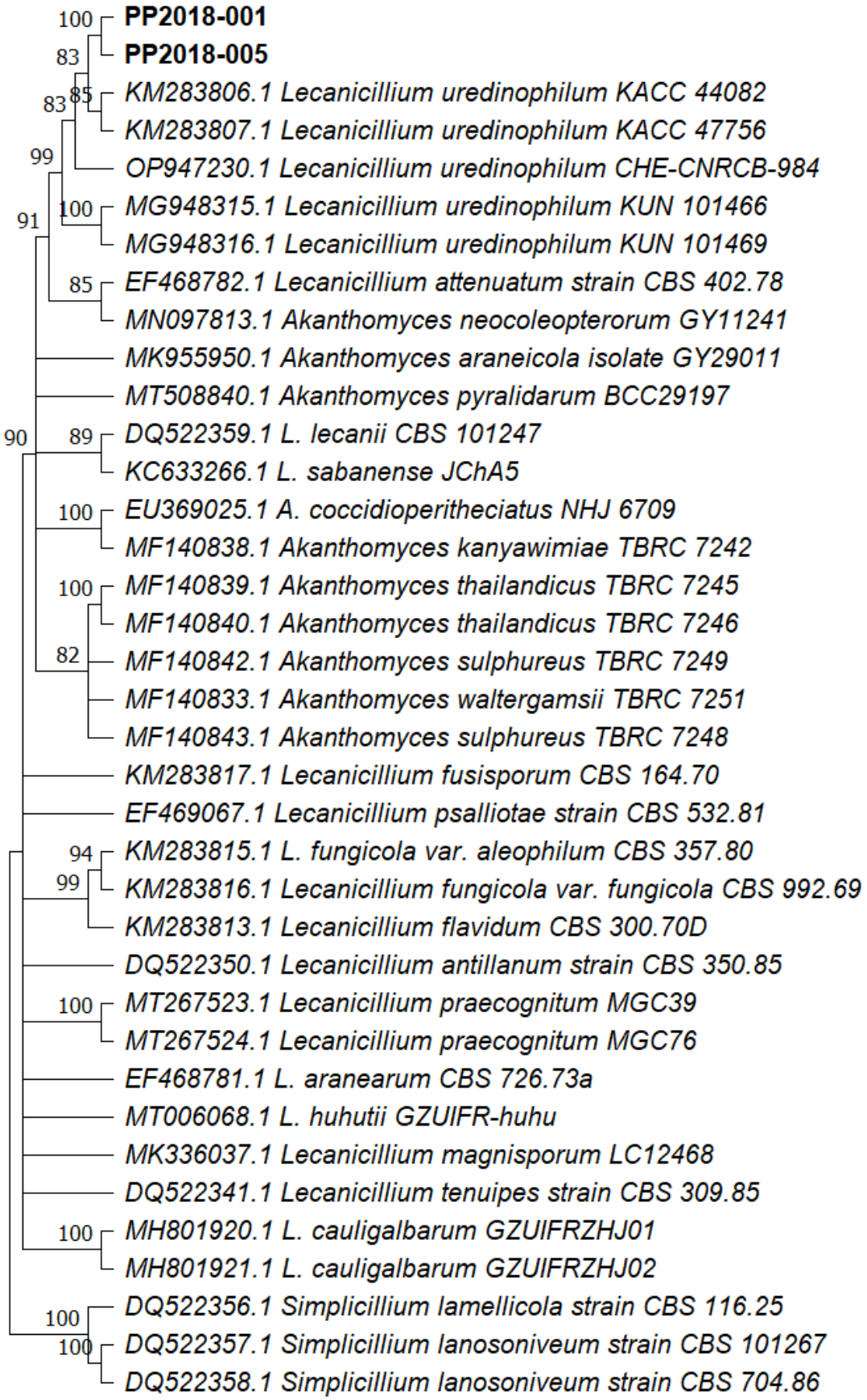

Fig 3.11 Phylogenetic analysis of the translation elongation factor (TEF) gene sequences

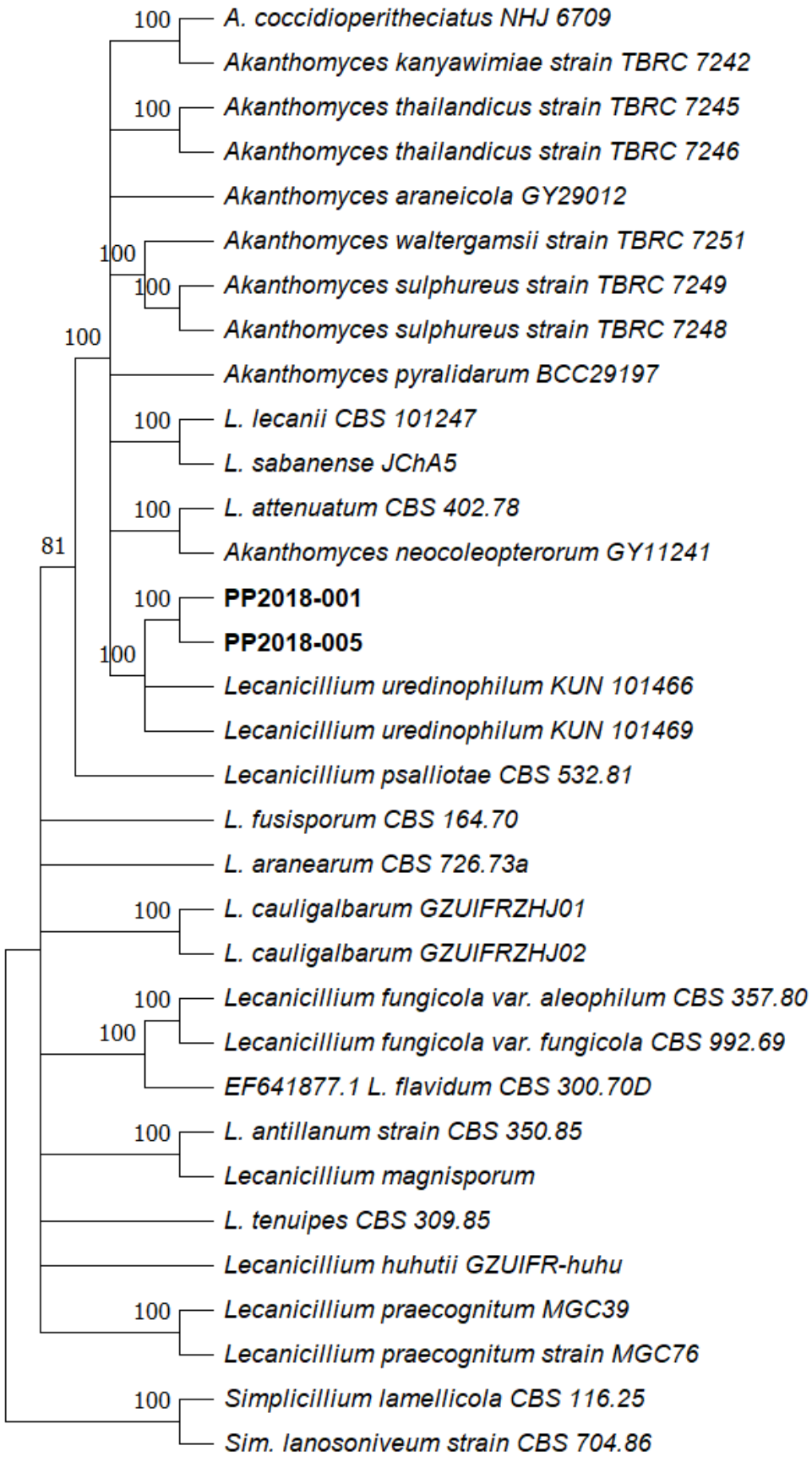

Fig 3.12 The phylogenetic analysis of the combined loci and ITS regions.

3.5 Discussion

The main objective of this study was to isolate potential mycoparasites naturally infecting rust pustules for the biocontrol of the soybean rust pathogen *P. pachyrhizi*. Microbial screening and characterization are an initial laboratory-scale stage towards the development of biological control agents (Palmieri et al., 2022), with subsequent steps being industrial-scale production, formulation, packaging etc.. Whilst this study was not directly aimed at commercializing the isolated successful mycoparasite, it is noteworthy that the preliminary step was initiated.

When resources are not limited, the best a way to find potential mycoparasites in a local environment is scouting and surveying many habitats, particularly during rust infections of wild and cultivated plant species (Den Breeyen et al., 2022). This study, albeit limited to a few plant species, discovered novel potential mycoparasites by examining rust pustules on wild species of plants and checking these for suspected colonization of rust sori. This study initially isolated nine (9) distinct groups of mycoparasites from two wild plant species (wild strawberry and *Oxalis* spp.), which were infected by their pathogenic rusts. From the four groups that sporulated, four isolates were identified using their internal transcribed spacers (ITS) as *Akanthomyces muscarium* (PP2018-001), *Lecanicillium* spp. or *Aphanocladium aranearum* (PP2018-002), *Akanthomyces muscarium* (PP2018-003) and *Simplicillium lanosoniveum* (PP2018-004). The two *Akanthomyces muscarium* isolates (isolates PP2018-001 and PP2018-003) were isolated from wild strawberry rust pustules, whilst *Lecanicillium* spp. or *Aphanocladium aranearum* and *Simplicillium lanosoniveum* were isolated from *Oxalis* spp. rust pustules.

In a similar study, *Lecanicillium uredinophilum* isolated from fungal rust host *Pucciniastrum agrimoniae* on an *Agrimonia pilosa* plant (Park et al., 2016) in Korea, as a first report. This study also included the taxonomic classification of isolates PP2018-001 and PP2018-005. Similarly, this study is the first record of *L. uredinophilum* being found on wild strawberry rust pustules in South Africa. Other studies have isolated *L. uredinophilum* from insects (Wei et al., 2018) in China. The *L. uredinophilum* morphological characterization in this study, visually and microscopically, showed characteristic morphologies such as ovoid, oblong, ellipsoidal, and cylindrical in spore shapes, which agrees with observations by Park et al. (2016), as well as Wei et al. (2018), who recorded similar characteristics. The study also revealed that isolates of *L. uredinophilum* exhibited different sizes in phialide length ranging from 15-50 µm whilst spores ranged from 1.1-6.7 µm. Park et al, (2016) and Wei et al., (2018) generally measured

much smaller sizes; however, such variability is expected in fungal isolates of the same species (Hong et al., 2024).

The ability of *L. uredinophilum* to parasitize insects, arachnids and plant pathogenic fungi shows a wide genetic variability and an interesting phenomenon, particularly in the mechanism of action. Moreover, whether an isolated isolate can successfully exhibit multiple biological control abilities on cultivated plants is yet to be fully explored. The *Cordycipitaceae* family houses various popular biocontrol genera such as *Lecanicillium*, *Beauveria*, *Simplicillium*, *Cordyceps*, amongst others, and are characteristically parasitic fungi (Lu et al., 2023; Wang et al., 2020). Various *Lecanicillium* spp. have been developed as insect biocontrol agents (Armand et al., 2024; Perumal et al., 2024; Bajsa et al., 2023; Hajji-Hedfi et al., 2023; Liu et al., 2023; Mantzoukas et al., 2023; Manfrino et al., 2022; Meng et al., 2022; Reddy, 2020; Reddy and Sahotra, 2020; Zhou et al., 2020; Liu et al., 2011).

In nature, rusts can be simultaneously hyperparasitized by various fungal species, which can coexist whilst using the rust urediniospores as their substrate for nutrition, growth and development (Den Breeyen et al., 2022). In their investigation of hyperparasites on cereal crops, (Wang et al., 2022; Wilson et al., 2020) isolated *Clonostachys rosea f. rosea* (Link) Schroers (1999) and *Lecanicillium psalliotae* (Treschew) Zare & Gams (2001) from wheat rust *Puccinia triticina* Eriksson (1899) pustules.

The ITS regions, especially ITS1, ITS2, and ITS4 regions, have been the preferred marker for studies of fungi of little or no taxonomic ambiguity (Vu et al., 2016; Irinyi et al., 2015; Pryce et al., 2003) despite some potential biases or deficiencies in ITS data having been identified (Raja et al., 2017; Bellemain et al., 2010). The identification of the taxonomic affiliation of the isolates under study started with an approach focused only on the ITS. This approach did not produce definitive taxonomic identities, requiring a more comprehensive method to be used that considered other marker genes or multi-locus gene sequencing. Based on related literature, the RPB2 and TEF sequences were added to the ITS sequences for the multiple marker genes analysis. In a related study, (Zhou et al., 2020) used six genes (ITS, SSU, LSU, TEF, RPB1, RPB2) to reconstruct the *Lecanicillium* spp. phylogeny, and conclusions made were that the ITS combined with TEF, RPBI and RPB2 could be used to resolve the phylogenetic analysis of the genus. Further, they revealed that the SSU and LSU alone were insufficient to resolve the taxonomy of the samples for their study. In this study, the first tree (Fig 3.9) constructed

using ITS sequences only agrees with other studies (Blaalid et al., 2013; Manter and Vivanco, 2007; Gams and Zare, 2001).

Fungal identification using multiple marker genes has gained momentum over the years and has become a standard procedure (Zhou et al., 2022; Zhou et al., 2020; Wei et al., 2018; Kepler et al., 2017; Sung et al., 2007). The inclusion of RPB2 and TEF in the analysis was useful, especially in resolving the taxonomy of isolate PP2018-001. All the trees generated in this study showed that the two isolates under study (PP2018-001 and PP2018-005) are *L. uredinophilum*. Both the RPB2 and TEF are now widely used for phylogenetic studies in fungi, providing a higher resolution and reliable locus (Wang et al., 2020; Rehner and Buckley, 2005). The tree thus obtained from the RPB2 region (Fig 3.10) and its apparent grouping of PP2018-001 and PP2018-005 isolates with *L. uredinophilum* showcase an alignment with studies in the past, thus making the RPB2 gene marker complementary to the ITS sequences in the taxonomy of *Lecanicillium* (Zhou et al., 2022; Zhou et al., 2020). Furthermore, the TEF region encoding translation elongation factor, 1-alpha, is also useful due to its high sequence variability in all eukaryotes. The TEF tree (Fig 3.11) corroborates well with previous studies (Sung et al., 2007) and highlights its potential to resolve phylogenetic ambiguity for the *Lecanicillium* genus and closely related genera. This study also used combined loci for phylogenetic analysis and thus sought to provide a comprehensive taxonomic understanding. The combined loci phylogenetic tree (Fig 3.12) provides a more detailed picture, as would be expected when multiple points of genetic variability are integrated. A strong bootstrap support for the clade, including *L. uredinophilum*, underscored the value of using multiple loci and its robustness. Resolution of complex taxonomic groups requires the high resolution power provided by multiple locus phylogenetic studies, which has been corroborated by the strong bootstrap values they provide (Chen et al., 2020; Qiu et al., 2020; Wang et al., 2020; Vinit et al., 2018; Mazard et al., 2012; Udayanga et al., 2012).

Keeping up with changes in the taxonomy of isolates of interest was another lesson learned through this study of taxonomic identification. Doing this study, the previously identified isolates changing their names from *L. muscarium* to *Akanthomyces muscarium* and to its current taxonomic rank. The evolutionary history of the genus *Lecanicillium* in the family *Cordycipitaceae* has been investigated several times in an attempt to resolve taxonomic issues (Johnson et al., 2009; Sung et al., 2007). The attribution problem of most species in the original genus *Lecanicillium* remains unsolved. A proposal was made that includes *L. uredinophilum*

to be moved to the genus *Akanthomyces* (Zhou et al., 2022). Earlier taxonomic studies had transferred some species (*L. lecanii*, *L. attenuatum*, *L. muscarium*, and *L. sabanense*) into the *Akanthomyces* genus (Kepler et al., 2017). The proposed name for *Lecanicillium uredinophilum* is *Akanthomyces uredinophilus* (Manfrino et al., 2022).

3.6 Conclusion

The study successfully isolated and identified mycoparasites that are associated with rusts in nature. Wild strawberry and *Oxalis* spp. plants are ubiquitous in most regions in South Africa as wild, non-cultivated plants. They are hosts to biotrophic pathogenic rusts that attack them. In turn, these rusts serve as substrates for mycoparasites. Particular attention was paid to isolate PP2018-001, which was initially identified as *Akanthomyces muscarium*, but subsequently, using multi-loci gene phylogenetics, identified as *Lecanicillium uredinophilum.* The study also revealed that although low-cost ITS primers are used to identify *Lecanicillium* species, ambiguities in taxonomic classifications often occur, requiring more powerful genomic tools to be used such as multi-loci phylogenetic analyses. Two additional primers (RPB2 and TEF) were able to offer a conclusive resolution to the identity of PP2018-001. During the study, *Plumeria* spp. (frangipani) was found to be an ideal model plant because it is attacked by a biotrophic rust that persists on the leaves for a long time. Furthermore, the two isolates of *L. uredinophilum* were able to colonize soybean rust pustules as well as *Plumeria* spp. rust pustules, although it was isolated from wild strawberry rust pustules. Therefore, further studies could focus on the compatibility of *L. uredinophilum* spores with commercial adjuvants or oil formulations so that formulations of the spores can be used in glasshouse and field trials on the biocontrol of ASR.

3.7 References

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Chapter 4: Assessing the effects of ten commercial adjuvants and seven edible oils on the artificial growth of *Lecanicillium uredinophilum*, a mycoparasite of soybean rust pathogen (*Phakopsora pachyrhizi*)

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Abstract

Biocontrol of the Asian soybean rust pathogen (*Phakopsora pachyrhizi* Syd. & P. Syd.) could provide an eco-friendly and cost-effective alternative to synthetic chemical fungicides. This study evaluated the effects of ten commercial adjuvants and seven edible oils on the growth of *Lecanicillium uredinophilum* isolates (PP20180-001 and PP2018-003), a mycoparasite of *P. pachyrhizi*. Seventeen treatments were divided into three groups (adjuvants made with edible vegetable oils, commercial adjuvants and a control). Six concentration levels of the treatments (0.01, 0.05, 0.1, 0.5, 1, and 2%) were included in potato dextrose agar (PDA), and 5x5 mm^2 plugs of a 14-day old culture of *L. uredinophilum* was placed at the centre of the PDA plates and incubated at 25±2ºC. Radial growth of the colony was measured 18 days post-inoculation. Counts of colony-forming units (CFUs) were determined by spreading 0.5 ml of a conidial suspension (5.0 x 10^2 conidia.ml^{-1}) on PDA plates, and colony counts were made after 48 hours of incubation at 25±2ºC. Non-parametric data analysis shows that the three groups were significantly different ($\rho < 0.05$). The vegetable oil adjuvants, and the concentration levels achieved the most radial growth ($40\ \text{mm} \leq \tilde{x} < 65\ \text{mm}$) and highest CFU counts ($200 \leq \tilde{x} < 250$) for PP2018-001 as the oil concentration increased, but it varied for PP2018-003. The commercial adjuvants drastically reduced radial growth and CFU counts as their concentrations increased and provided the lowest radial growth ($0\ \text{mm} \leq \tilde{x} < 20\ \text{mm}$) and lowest CFU counts ($0 \leq \tilde{x} < 100$). For BCA PP2018-001, only Break-Thru® and Ballista® supported high mean radial growth, with Break-Thru® achieving 52.6 mm at 0.01%, and Ballista® achieving 44.7 mm and 42.6 mm at 0.01% and 0.05%, respectively. All other adjuvants (Spayfilm 10®, Wetcit®, Tronic®, Summit Super®, Nufilm P®, Designer®, Bond®, and Aquawet®) resulted in low to moderate mean radial growth for this BCA dependent on concentration and BCA type. In the case of BCA PP2018-003, high mean radial growth (40.2 mm) was observed only with Bond at 0.01%, while the remaining adjuvants yielded moderate

growth at the same concentration. For CFU counts, Sprayfilm 10®, Aquawet®, and Ballista® achieved high mean CFU levels at 0.01% and 0.05% for BCA PP2018-001. Notably, Ballista® supported high mean CFUs across all concentrations tested (0.01%–2%), while Break-Thru® supported high CFUs at 0.01%, 0.05%, and 0.1%. The remaining adjuvants exhibited CFU counts ranging from low to moderate depending on concentration and type. For BCA PP2018-003, only Break-Thru® enabled high mean CFUs at 0.01%, whereas all other adjuvants resulted in low to moderate CFU levels, again dependent on concentration and adjuvant type. All the edible vegetable oil adjuvants could be used for *L. uredinophilum* formulations, at any of the six concentrations tested. These results are useful when selecting the type, category and concentrations of adjuvants for *L. uredinophilum* formulations, and probably other fungal biocontrol agents.

Keywords: *Lecanicillium uredinophilum*, bio-efficacy, compatibility, adjuvant, radial growth, colony-forming units

4.1 Introduction

Soybean [*Glycine max* (L.) Merr.] is an important global and local crop contributing to an average of 60% of the world's oilseed production (Soystats, 2023; Soystats, 2022; Engelbrecht et al., 2020). However, potential yields may be drastically reduced by the Asian Soybean Rust (ASR), which can cause losses of 10-100% (Hossain et al., 2024). The ASR pathogen (*P. pachyrhizi*) has a complex life cycle. Its urediniospores spread rapidly over long distances and infect a wide spectrum of hosts, which serves as an inoculum for rust epidemics of soybean crops under favourable conditions (Goellner et al., 2010). The control of ASR typically involves the use of fungicides (Hossain et al., 2024; Netto et al., 2020). However, the ASR pathogen has progressively developed resistance to the limited spectrum of fungicides being used globally (Oliver and Beckerman, 2022; Zambolim et al., 2021; Simões et al., 2018; Hahn, 2014; Schmitz et al., 2014).

The biological control of the ASR pathogen through the use of *Lecanicillium uredinophilum*, (Park, Hong & Shin), is a promising control option as an alternative to chemical fungicides, through its ability to infect and kill rust urediniospores (Ayaz et al., 2023; Nega, 2014). *Lecanicillium* is a well-studied genus of entomopathogens and mycoparasites that have been isolated from various sources, including rust sori (Vandermeer et al., 2009). Notably, *Lecanicillium lecanii* ((Zimmerman) Zare & Gams) (formerly *Verticillium lecanii*), an

entomopathogenic fungus (EPF), is utilised as a biological control agent against various agricultural pests. These include thrips (Subramaniam et al., 2021), whitefly (Abdulle et al., 2020; Xie et al., 2019), aphids (Hanan et al., 2020), psyllids (Lu et al., 2015) and scales (Liu et al., 2009). All these applications require enhanced formulation or adjuvants for effectiveness.

The hairy and hydrophobic nature of soybean leaves is considered "difficult" to wet (Yao et al., 2014). This characteristic necessitates using commercially available adjuvants to overcome the surface tension to enhance the spread and wetting of soybean leaves by water-based fungicide formulations. By improving coverage, adhesion, and penetration, adjuvants make fungicide treatments more reliable and effective (Holka and Kowalska, 2023; Garcia et al., 2016; Gaskin et al., 2005). Similarly, the successful use of biological control agents (BCAs) requires efficient spray application technologies, while maintaining the spore viability and virulence of the formulations (Holka and Kowalska, 2023). The viability and virulence of BCAs are affected by both abiotic and biotic factors, and at the phyllosphere level, these are constantly fluctuating (Elad, 2003). Ultra-violet radiation (UV-B) and natural light (290-400nm) affect foliar applications of BCAs (Fătu et al., 2020). Adverse temperatures (below 10℃ and above 35℃) and low relative humidity impede the growth and development of entomopathogenic fungi (Ignoffo, 1992). To circumvent these challenges, adjuvants such as surfactants, humectants, and emulsifiers may be used to enhance the formulation of biocontrol agents by improving leaf adhesion, reducing evaporation, and enhancing the penetration of the fungal appressoria into the targeted host (Murillo-Alonso et al., 2023; Mascarin et al., 2014; Jarrold, 2001). For example, glycerol as an adjuvant helps retain water within the spore structure, reducing desiccation and extending the shelf life of the biocontrol product (Stevenson et al., 2017).

Despite *L. uredinophilum* having shown potential as a mycoparasite of the ASR, there is little information available on its interaction with commercially available adjuvants and natural adjuvants, such as vegetable oils, on its *in vitro* growth and viability. Understanding these interactions would assist in the development of formulations that maximise the biocontrol potential of *L. uredinophilum*. Consequently, this study aimed to evaluate the compatibility of 10 commercial adjuvants and seven adjuvants made up with vegetable oils on the radial growth, colony-forming units (CFU), and qualitative growth characteristics of *L. uredinophilum*, isolates PP2018-001 and PP2018-003, under *in vitro* conditions.

4.2 Materials and methods

4.2.1 Preparation of biological control agents

Two BCAs, labelled as PP2018-001 and PP2018-003, previously identified as *Akanthomyces muscarium* (Petch) isolates (syn. *Lecanicillium uredinophilum*) (Chapter 3), were grown on full-strength potato dextrose agar (PDA). This was done by inoculating at the centre of each petri dish with a 5x5 mm^2 disc carrying mycelia from pure culture plates. The plates were incubated at 25±2°C for 14 days in preparation for the radial growth and CFU count experiments. For the preparation of the CFU count experiments, Petri dishes with 14-day-old cultures of isolates PP2018-001 and PP2018-003 were washed with sterile de-ionized water by gently scraping the mycelia off the agar surface with a sterile glass rod. Three layers of sterilized cheesecloth were used to filter off mycelial fragments to make a stock conidial suspension solution of 500 ml for each isolate. The initial concentration of the stock conidia suspension was determined using a haemocytometer (model 3048–13, Hausser Scientific, Horsham, PA, USA). Serial dilutions were made to a conidia suspension of 500 conidia per ml for each BCA and labelled PP2018-001 for PP2018-001 and PP2018-003 for PP2018-003. The adjusted conidia suspension was stored at 4°C until use.

4.3 Preparation of vegetable oil formulations

A 10% stock volume of 40 ml of each vegetable oil formulation was mixed with Triton X-100 (a non-ionic surfactant, **Table 4.1**), LUDOX® HS-30 colloidal silica (a stabilizer, **Table 4.1**) and sodium carbonate (Na_2CO_3)(an anti-foaming agent, **Table 4.1**). Anhydrous sodium carbonate (5g) was weighed and quantitatively transferred into a 50 ml volumetric flask. Deionized water was gradually added to the flask while stirring with a glass rod to dissolve the sodium carbonate completely. The solution was brought to the 50 ml calibration mark by adding more deionized water and labelled as 10% Na_2CO_3. The Triton X-100 glass bottle was placed in a 37ºC water bath for 30 minutes to make the contents less viscous, and 5 ml pipetted into a 50 ml volumetric flask. Deionized water was added to bring the volume to 50 ml whilst gentle stirring to avoid foaming and labelled as 10% Triton X-100. A 10% colloidal suspension of LUDOX® HS-30 colloidal silica was prepared by measuring 16.7 ml into a 50 ml volumetric flask, and deionized water added to bring to the 50 ml calibration mark. These components were mixed with the following vegetable oils: avocado, sunflower, organic sesame, olive, canola, macadamia, and peanut oil. The formulation of each vegetable oil formulation consisted of 10% surfactant (v/v), 10% stabilizer (v/v), 10% anti-foaming agent (v/v), 10% vegetable oil

(v/v), and 60% water, totalling 100% v/v. A magnetic stirrer was used to homogenize this mixture for 1 h, and then it was sterilized in an autoclave for 15 minutes at 121 ºC.

4.4 Preparation of commercial adjuvants

In preparing a 10% stock volume of 40 ml of the commercial adjuvants, 4 ml of each adjuvant was pipetted into a 100 ml sterile Schott bottle, and 36 ml of sterile de-ionized water was added to bring the volume to 40 ml. The mixture was stirred with a sterile glass rod to ensure uniform dispersion.

4.5 Experimental Procedure

Full-strength PDA media was prepared by weighing 4g of PDA powder (Merck Pty Ltd., Johannesburg, South Africa) into 250 ml-Schott bottles, 100 ml of de-ionized water was added, and the mixture was homogenized through hand stirring. The mixture was sterilized at 121ºC for 15 minutes. After cooling to about 50 ºC, each volume of the media was standardized by removing 10, 50, 100, 500, 1000 and 2000 µl respectively, from each subset of six 250 ml-Schott bottles, labelled with each adjuvant name and concentrations of 0.01, 0.05, 0.1, 1 and 2%, respectively. To make up the six concentrations, the amounts of media removed were compensated by adding the corresponding volumes of the stock oil emulsions or commercial adjuvants into the labelled concentration and adjuvant type. The media-adjuvant mixture was aseptically stirred with a sterile glass rod and poured into labelled Petri dishes. The plates were allowed to set overnight. Under aseptic conditions, 5×5 mm² mycelial discs from 14-day-old pure cultures of each BCA were excised using a sterile cork borer, placed at the centre of individual agar plates, and incubated at 25±1°C for 18 days. For the CFU count experiment, 0.5 ml of the previously prepared conidia suspension was dispensed onto the surface of the PDA-adjuvant media and spread evenly using an L-shaped sterile glass rod. The agar plates were inverted and incubated at 25±1ºC for 48 h. The experiment employed a complete randomized design with a factorial arrangement of two factors: adjuvant type with 17 levels, six concentration levels and a control group, resulting in 540 experimental units. Each treatment combination was replicated five times, and the experiments were repeated in two independent trials. The commercial adjuvants and vegetable oils used are presented in **Table 4.1**.

Table 4.1 List of commercial adjuvants and oil formulations evaluated for the artificial growth of *Lecanicillium uredinophilum* isolates.

Name of Product	Type of Product	Source	Manufacturer*
Designer®	Commercial Adjuvant	Nulandis	UAP Crop Care
Break-Thru®	Commercial Adjuvant	Nulandis	Evonik Degussa
Tronic®	Commercial Adjuvant	Nulandis	Gouws and Scheepers (Pty) Ltd/(Edms) Bpk
Wetcit®	Commercial Adjuvant	Nulandis	Citrus Oil Products
Nufilm P®	Commercial Adjuvant	Farmers Agricare	Miller chemical and fertilizer
Bond®	Commercial Adjuvant	Nulandis	Nulandis (AECI Limited)
Ballista®	Commercial Adjuvant	Nulandis	Bayer (Pty) Ltd/ (Edms) Bpk
Summit super®	Commercial Adjuvant	Farmers Agricare	Villa Crop Protection (Pty) Ltd.
Sprayfilm 10®	Commercial Adjuvant	Farmers Agricare	UPL South Africa (Pty) Ltd
Aquawet®	Commercial Adjuvant	Farmers Agricare	Rolfes Agri (Pty) Ltd.
Sunflower	Edible Vegetable Oil	Woolworth	Southern Oil
Avocado	Edible Vegetable Oil	Woolworth	Wilson Foods
Organic sesame	Edible Vegetable Oil	Woolworth	Wilson Foods
Olive	Edible Vegetable Oil	Woolworth	Wilson Foods
Canola	Edible Vegetable Oil	Woolworth	Southern Oil
Macadamia	Edible Vegetable Oil	Woolworth	Wilson Foods
Peanut oil	Edible Vegetable Oil	Woolworth	Wilson Foods
Triton X-100	Surfactant/emulsifier	Merck (Sigma-Aldrich)	Merck (Sigma-Aldrich)
Sodium Carbonate	Anti-foaming agent	Merck (Sigma-Aldrich)	Merck (Sigma-Aldrich)
LUDOX® HS-30 colloidal silica	Stabiliser	Merck (Sigma-Aldrich)	Merck (Sigma-Aldrich)

*All manufacturers were from South Africa

4.6 Data Collection and Statistical Analysis

4.6.1 Radial Growth Data

Data collection for the radial growth of the two BCAs, PP2018-001 and PP2018-003, was done by measuring the diameter of the radial growth (in mm) using a 150-mm ruler. All data analyses were performed using R Statistical Software (v4.3.2, R Core Team, 2021). The data for the two independent trials for each BCA was subjected to the Levene test to check for homogeneity of variances ($\rho > 0.05$) and subsequently pooled for each BCA. Due to the non-conformity of the data to transformations (logarithmic, square root, cube root and Box-Cox), the data was subjected to non-parametric analysis using the Kruskal-Wallis Test together with the Gaussian mixture model (GMM). Post-hoc tests after the Kruskal-Wallis test were done through Dunn's test with ρ-adjustment done through the Benjamini-Hochberg method to provide for the statistical significance within adjuvants (17 adjuvants and controls) at each level of the six concentrations (within adjuvants) and between adjuvants across all the six levels of concentrations.

4.6.2 Colony Forming Units Data

Colony forming units (CFUs) were determined through physical counts using a laboratory counter for each experimental unit for the two independent trials. All data analyses were performed using the R Statistical Software (v4.3.2, R Core Team, 2021). The CFU count data were subjected to normality tests using the Shapiro-Wilk normality test for each independent trial and were found to be statistically not normally distributed (all $\rho < 0.05$). The Levene test for homogeneity of variances was conducted for each trial for the BCA types, and data for the respective trials for each BCA type were pooled accordingly. A non-parametric test (Kruskal-Wallis) was employed to assess differences between adjuvants. In addition, the CFU data was also subjected to five statistical models developed for count data, namely: Poisson, quasi-Poisson, negative binomial, generalized additive model (GAM) and generalized additive model for location, scale, and shape (GAMLSS). Post-hoc pairwise comparisons were conducted using an estimated marginal means (EMMs) package to evaluate the effects of different adjuvants and concentration levels on CFU counts. Negative binomial models were fitted separately for each BCA type, and EMMs were computed for each combination of adjuvant and concentration. Pairwise comparisons of these EMMs were performed with adjusted ρ-values using the Benjamini-Hochberg method to control for multiple comparisons.

4.7 Results

4.7.1 Radial growth

4.7.1.1 Morphological growth characteristics of BCAs grown on adjuvant amended growth media

The adjuvant amended growth media affected the radial growth of both BCAs similarly, with increased concentrations of commercial adjuvants drastically reducing radial growth, leading to total inhibition of growth in some cases. The mycelial growth, colony characteristics, and CFUs per ml differed considerably for different adjuvants at different concentrations, compared to the control treatments (Fig 4.2). The *L. uredinophilum* isolates (PP2018-001 and PP2018-003) showed some differences in growth characteristics, with PP2018-001 achieving greater mean radial growth than that of PP2018-003. Colouration for both BCA isolates on the obverse was characteristically white, mostly circular in form, with entire margins and were flat in appearance. The reverse side for PP2018-001 was creamy white, while for PP2018-003, it exhibited a yellowish colouration. The radial growth of PP2018-001 and PP2018-003 isolates on PDA amended with adjuvants at the six concentrations is illustrated, showing samples for

the 0.01% and 2% concentration for each adjuvant (Fig 4.2) at the end of the 18-day growth period (data for PP2018-003 plates are not shown).

4.7.1.2 Effect of adjuvant type and concentration on radial growth of *Lecanicillium uredinophilum* isolates

The adjuvant types (commercial or vegetable oil) were evaluated for their effect on compatibility with two *L. uredinophilum* isolates (PP2018-001 and PP2018-003) by assessment of their radial growth. The mean radial growth for PP2018-001 was generally higher than that of PP2018-003, with the mean radial growth for the control groups being 54.2 mm (PP2018-001) and 35.3 mm (PP2018-003). Heatmaps to visualize radial growth data [Fig 4.3(a), 4.3(b)] categorized the radial growth ranges in colour maps, blue ($0\ \text{mm} \leq \tilde{x} < 20\ \text{mm}$) indicative of lowest radial growth, pale blue shade ($20\ \text{mm} \leq \tilde{x} < 40\ \text{mm}$), for intermediate radial growth and yellow shade ($40\ \text{mm} \leq \tilde{x} < 65\ \text{mm}$), for the highest radial growth. The greatest radial growth of the BCAs exposed to commercial adjuvants was on PDA with 0.01% Break-Thru® and Ballista®, 0.05% Ballista® (PP2018-001), and 0.01% Bond® (PP2018-003). Most commercial adjuvants (Wetcit®, Tronic®, Summit Super®, Nufilm P®, Designer®, Break-Thru®, Bond®, Balista®, Aquawet®) generally caused a reduction in mean radial growth for *L. uredinophilum* isolates as the adjuvant concentrations were increased. All radial growth measurements were in the blue colour range for concentrations greater than 0.5%. Wetcit® at 1% and 2% concentrations completely inhibited the growth of both *L. uredinophilum* isolates. The addition of vegetable oil adjuvants exhibited resulted in the greatest radial growth, in the yellow shade for cultures of *L. uredinophilum* isolate PP2018-001 [Fig 4.2(a), 4.2(b)].

The pooled data of radial growth was subjected to non-parametric tests (Kruskal-Wallis and Dunn's tests for post-hoc analysis) with the Benjamini-Hochberg ρ-value adjustments and results presented in **Table 4.2**. The type of adjuvant (commercial or vegetable oil), concentration, and interaction effects had a significant impact on radial growth. Post-hoc analysis for between adjuvant and within adjuvants across all concentrations showed varied effects on radial growth depending on the adjuvant type, concentration and *L. uredinophilum* isolate. For example, at all concentration levels, use of vegetable oil adjuvants resulted in the most significant differences between adjuvants pairwise comparisons (triangular heatmaps for ρ-values as addendums in Appendix II and III) for both *L. uredinophilum* isolates. Vegetable oil adjuvants generally promoted radial growth for both *L. uredinophilum* isolates with increased concentrations. Greater and increasing mean radial growth was typically shown to

occur with increasing concentrations of vegetable oil adjuvants for both *L. uredinophilum* isolates, with PP2018-003 showing the greatest mean growth (64 mm) whereas PP2018-001 achieved a maximum diameter of 55.6 mm. Commercial adjuvants showed the least statistical differences between adjuvants at all concentration levels for both *L. uredinophilum* isolates. For example, at 0.01%, the control and Aquawet® consistently showed significant differences with other treatments, while Ballista® and Designer® showed fewer significant pairwise differences. Within adjuvant differences across all concentrations showed similar trends (albeit a nonsignificant difference for most comparisons), regardless of adjuvant type and *L. uredinophilum* isolate (triangular heatmaps for ρ-values as addendums in Appendix IV and V). Generally, significant differences were mostly observed in comparisons at the lowest concentrations (0.01 and 0.05%), whereas non-significant differences were observed as concentrations increased. For example, with isolate PP2018-001 growing on Aquawet® media, its radial growth was significantly different for its growth on the control medium at all concentrations of Aquawet® ($\rho < 0.05$). For isolate PP2018-001 growing on avocado oil medium, all radial growth comparisons between concentrations were not statistically different ($\rho > 0.05$) but were all greater than growth on the control medium.

The Gaussian mixture model (GMM) was used to analyse the three categories (commercial adjuvant, vegetable oil adjuvant and control group) (Fig 4.1), which were all significantly different ($\rho < 0.05$). Differences in means were significant for all three categories ($\rho < 0.05$), and the greatest differences were between the control group and commercial adjuvant, followed by the vegetable oil adjuvant and commercial adjuvant [Fig 4.1(b), PP2018-001] and [Fig 4.1(d), PP2018-003]. A negative difference was observed between the vegetable oil adjuvant and the control groups (meaning that the vegetable oil adjuvants media provided for greater radial growth than the control group) [Fig 4.1(b)] for PP2018-001. Differences amongst the three categories were also visualized by boxplots [Fig 4.1(a), PP2018-001; Fig 4.1(c), PP2018-003].

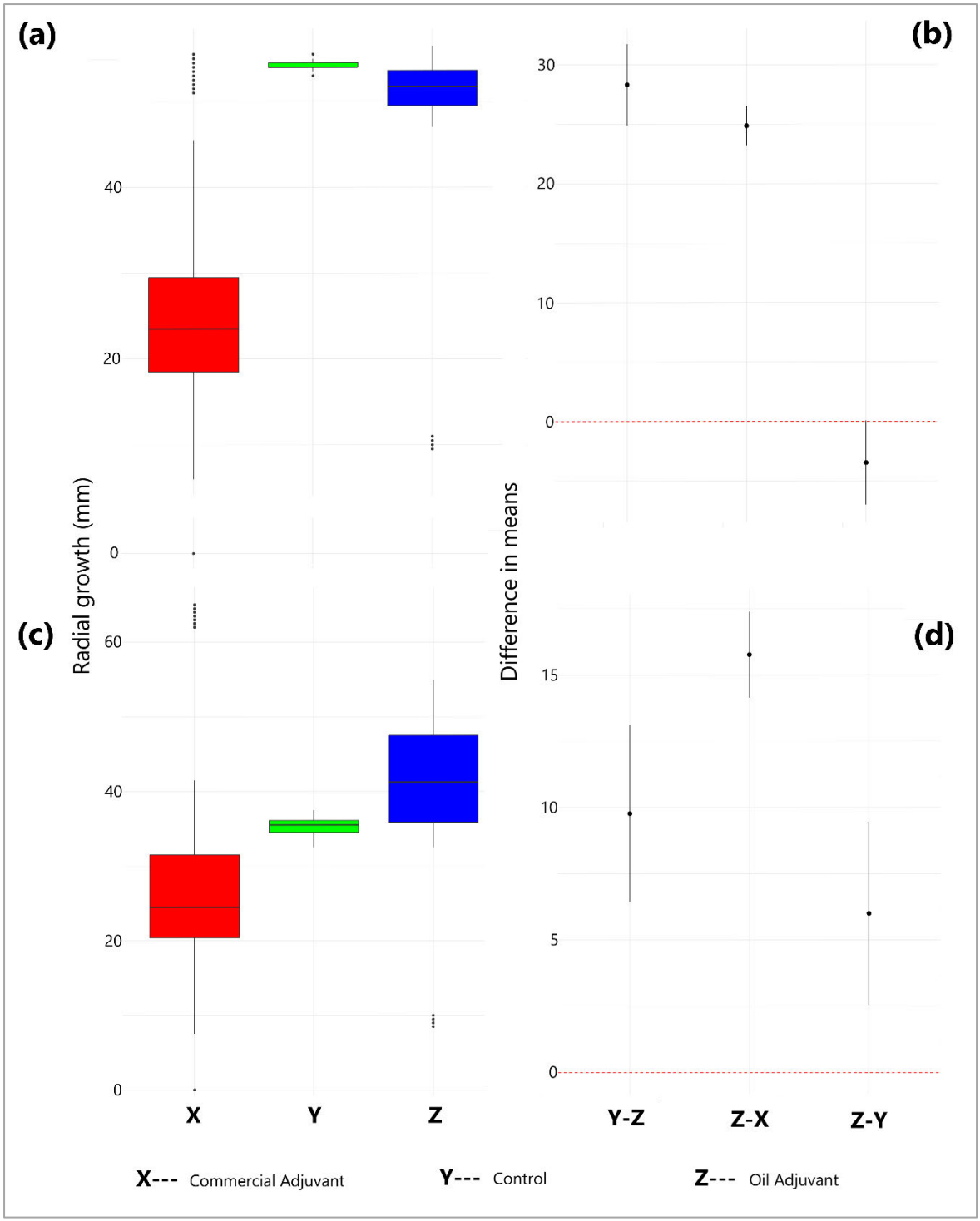

Fig 4.1 Comparisons between the effects of the commercial adjuvants, the vegetable oil adjuvants and the control group on the radial growth of two biocontrol agents.

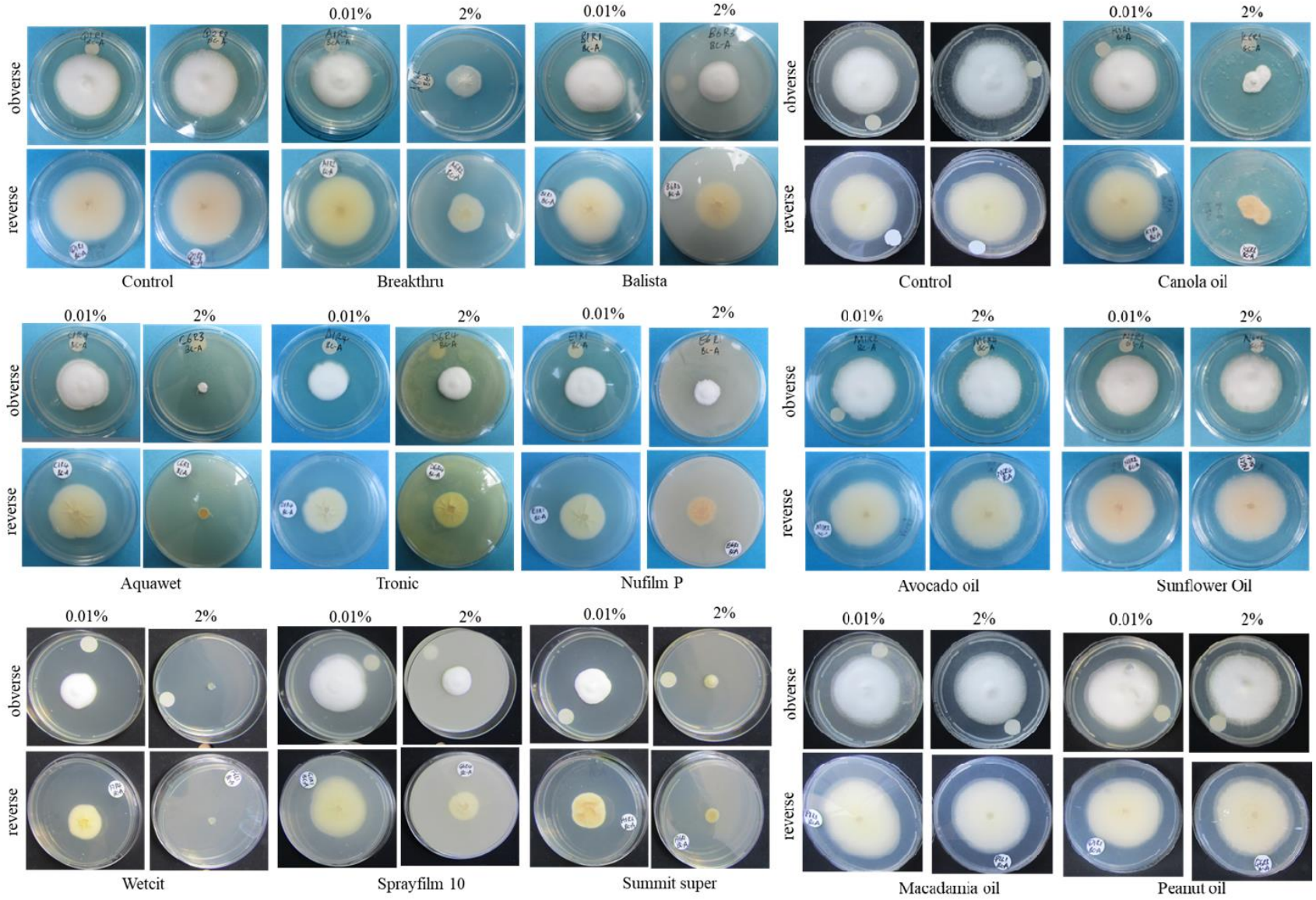

Fig 4.2 Comparison of selected means of radial growth for isolate PP2018-001 on lowest and highest adjuvant concentrations and the control group.

Table 4.2 Effect of adjuvants and oil formulations on radial growth of *Lecanicillium uredinophilum* isolates PP2018-001 and PP2018-003.

Adjuvant	BCA isolate: *Lecanicillium uredinophilum* (PP2018-001) Radial growth (mm) [mean ± SD (n)]							BCA isolate: *Lecanicillium uredinophilum* (PP2018-003) Radial growth (mm) [mean ± SD (n)]						
	Concentration (%)						Rank	Concentration (%)						Rank
	0.01	0.05	0.1	0.5	1	2	[0.01%]	0.01	0.05	0.1	0.5	1	2	[0.01%]
Aquawet®	37.6 ± 0.5	27.9 ± 0.5	23.5 ± 0.3	21.4 ± 0.3	13.8 ± 0.6	6.9 ± 0.5	12	24.0 ± 0.4	21.5 ± 0.4	19.5 ± 0.4	23.5 ± 0.4	13.1 ± 0.4	8.1 ± 0.4	16
Avocado oil	49.9 ± 0.5	52.2 ± 1.6	49.9 ± 0.7	52.3 ± 1.1	50.1 ± 0.8	50.9 ± 0.3	7	42.8 ± 0.6	36.7 ± 0.6	35.9 ± 0.6	34.7 ± 0.6	35.7 ± 0.6	34.3 ± 0.6	3
Ballista®	44.7 ± 0.5	42.7 ± 0.8	34.3 ± 0.3	28.2 ± 0.3	29.2 ± 2.0	26.4 ± 1.2	9	36.0 ± 0.3	31.1 ± 0.4	27.3 ± 0.3	25.0 ± 0.4	26.3 ± 0.3	27.1 ± 0.4	8
Bond®	28.6 ± 0.8	26.2 ± 0.3	24.2 ± 0.4	22.8 ± 0.6	21.5 ± 3.4	20.0 ± 4.8	16	40.3 ± 1.0	33.0 ± 0.4	34.1 ± 0.4	35.1 ± 0.4	30.6 ± 0.4	25.1 ± 0.4	4
Break-Thru®	52.7 ± 1.5	32.7 ± 0.8	24.7 ± 0.8	23.0 ± 0.4	21.7 ± 0.9	21.1 ± 0.4	3	37.7 ± 0.6	34.3 ± 0.6	28.2 ± 0.6	24.1 ± 0.6	26.3 ± 0.6	21.2 ± 0.6	6
Canola oil	55.6 ± 0.5	53.7 ± 0.3	53.9 ± 0.3	53.8 ± 0.3	51.7 ± 0.3	10.1 ± 0.5	1	45.7 ± 0.6	39.8 ± 0.6	48.7 ± 0.6	47.9 ± 0.6	44.3 ± 0.6	9.3 ± 0.6	2
Designer®	39.0 ± 0.8	23.9 ± 0.3	16.9 ± 0.7	16.1 ± 0.4	12.1 ± 0.9	9.8 ± 0.6	11	36.8 ± 0.6	24.2 ± 0.6	23.8 ± 0.6	16.7 ± 0.6	12.7 ± 0.6	14.8 ± 0.6	7
Macadamia oil	52.1 ± 0.4	53.6 ± 0.3	54.3 ± 0.8	53.7 ± 0.3	53.5 ± 1.3	54.8 ± 0.5	5	30.6 ± 0.8	36.1 ± 0.8	38.6 ± 0.8	63.1 ± 0.8	63.0 ± 0.8	64.0 ± 0.8	13
Nufilm P®	28.9 ± 0.5	24.2 ± 0.3	24.3 ± 0.8	22.0 ± 0.4	21.3 ± 0.3	18.0 ± 0.7	14	24.8 ± 0.6	24.2 ± 0.6	23.2 ± 0.6	23.2 ± 0.6	20.8 ± 0.6	13.2 ± 0.6	15
Olive oil	52.2 ± 0.6	50.1 ± 0.5	54.2 ± 0.4	51.3 ± 2.0	51.7 ± 2.1	49.8 ± 0.4	4	54.1 ± 0.8	53.5 ± 0.8	52.6 ± 0.8	50.1 ± 0.8	50.0 ± 0.8	33.5 ± 0.8	1
Org. Sesame oil	53.6 ± 1.8	52.7 ± 1.7	52.7 ± 0.8	51.7 ± 0.8	51.5 ± 2.3	50.5 ± 3.4	2	35.7 ± 0.6	36.9 ± 0.6	33.8 ± 0.6	42.7 ± 0.6	46.3 ± 0.6	44.2 ± 0.6	9
Peanut oil	50.3 ± 1.6	53.3 ± 0.3	54.2 ± 0.6	54.0 ± 0.4	54.3 ± 0.3	54.5 ± 0.4	6	34.2 ± 0.6	37.9 ± 0.6	35.8 ± 0.6	34.8 ± 0.6	38.3 ± 0.6	42.7 ± 0.6	10
Sprayfilm 10®	39.2 ± 0.6	29.8 ± 0.6	24.2 ± 1.0	23.0 ± 0.8	22.6 ± 1.4	20.3 ± 1.1	10	32.7 ± 0.6	31.7 ± 0.6	31.2 ± 0.6	25.3 ± 0.6	22.2 ± 0.6	21.2 ± 0.6	12
Summit Super®	29.5 ± 0.3	24.0 ± 0.4	22.5 ± 0.2	18.8 ± 0.6	15.3 ± 1.1	10.9 ± 0.7	13	33.5 ± 0.4	28.1 ± 0.4	25.0 ± 0.4	19.5 ± 0.4	21.0 ± 0.4	19.1 ± 0.4	11
Sunflower oil	49.0 ± 0.4	53.8 ± 0.3	50.6 ± 0.5	49.9 ± 0.7	48.6 ± 0.5	48.9 ± 0.4	8	39.6 ± 0.8	38.5 ± 0.8	51.5 ± 0.8	52.1 ± 0.8	47.1 ± 0.8	44.6 ± 0.8	5
Tronic®	28.9 ± 0.6	24.3 ± 0.5	23.0 ± 0.8	23.0 ± 0.8	18.4 ± 0.5	16.4 ± 0.8	15	26.1 ± 0.4	22.5 ± 0.4	21.5 ± 0.4	21.1 ± 0.4	14.1 ± 0.4	9.1 ± 0.4	14
Wetcit®	23.4 ± 0.3	16.2 ± 0.3	12.3 ± 0.3	8.6 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	17	20.5 ± 0.4	15.9 ± 0.6	9.0 ± 0.8	8.0 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	17
Control	54.2 ± 0.6							35.3 ± 1.3						
P-Value Adjuv.	<0.00001							<0.00001						
Conc_n	<0.00001							<0.00001						
Adjuv.*Conc_n	<0.00001							<0.00001						

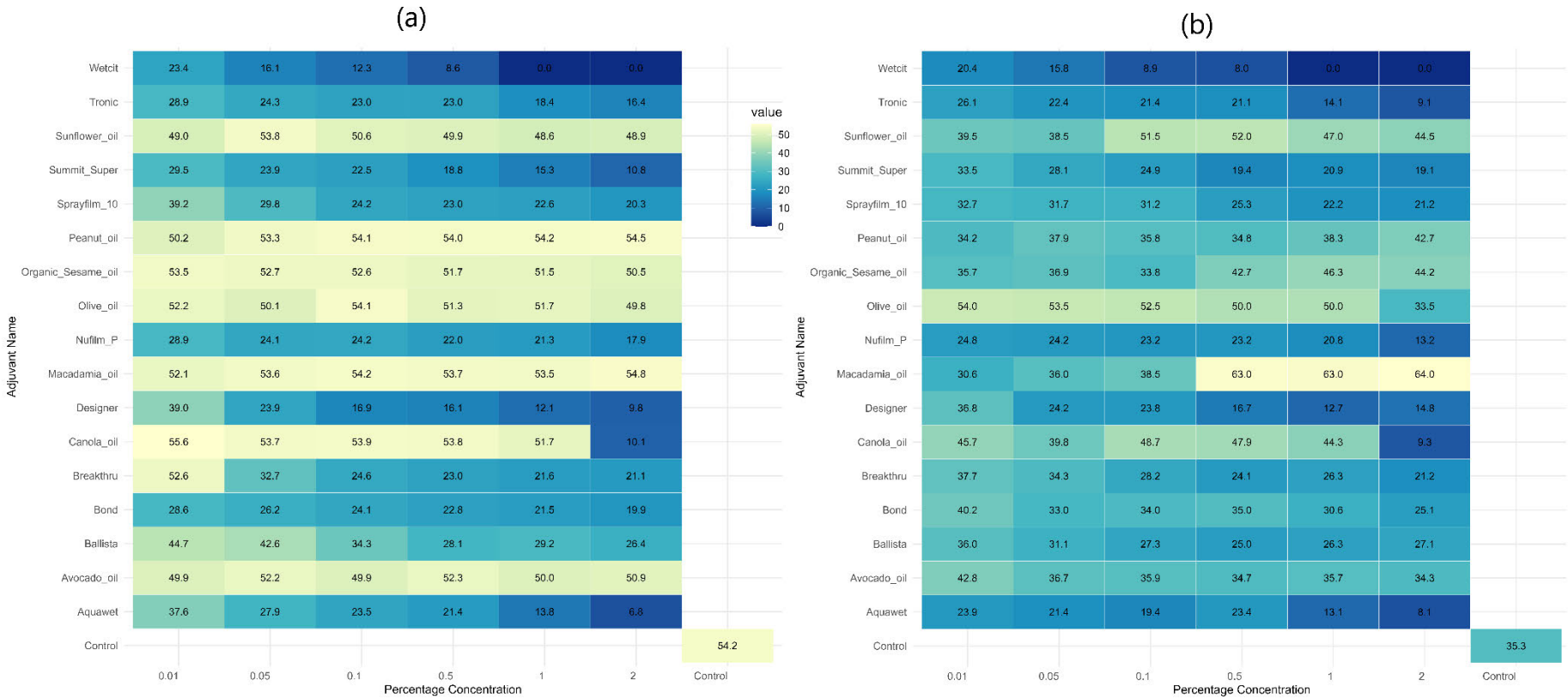

Fig 4.3 Heatmaps showing effect of adjuvant type and concentration on mean radial growth for *L. uredinophilum* isolates PP2018-001 (a) and PP2018-003 (b) showing the effect of adjuvant type and concentration on mean radial growth.

4.7.2 Colony forming units count

4.7.2.1 Effect of adjuvant type and concentration on colony-forming units across adjuvant concentration

The Kruskal-Wallis test showed that the effects due to the adjuvants, concentrations and their interaction were statistically significant in contributing to the differences in colony-forming unit (CFU) counts for the two *L. uredinophilum* isolates (**Table 4.3**). The effect of the adjuvant type across all concentrations for each BCA isolate was investigated, and the mean CFU counts per mL are represented by heatmaps for *L. uredinophilum* isolates PP2018-001 [Fig 4.4(a)] and PP2018-003 [Fig 4.4(b)]. The colour gradients range from blue to green to yellow, indicating CFU count values from low (blue) to high (yellow). The maximum CFU achieved was 245 (PP2018-001) and 206 (PP2018-003) CFU ml^{-1}, respectively, as indicated by the scale in Fig 4.4. Vegetable oil adjuvants (peanut oil, macadamia oil, organic sesame oil, sunflower oil, avocado oil, olive oil and canola oil) consistently enhanced CFU counts ($200 \leq \tilde{x} < 250$) for isolate PP2018-001 across all concentration levels (0.01% to 2%), which implies they promoted higher CFU counts than the Control group. Despite the increasing trend of CFU counts with increasing concentration for the vegetable oil adjuvants, a sharp drop in CFU count at the 2% concentration was a notable outlier, as the CFU count decreased from 186 (1%) to 10.6 (2%). Some media with commercial adjuvants allowed for high CFU counts ($200 \leq \tilde{x} < 250$) only at lower concentrations for isolate PP2018-001: Bond®, Nufilm P®, Summit super® and Tronic® only at 0.01%, Aquawet® at 0.01% and 0.05%, and Break-Thru® at 0.01% to 0.1%. Only Ballista®, media resulted in high CFU counts ($200 \leq \tilde{x} < 250$) at all concentration levels (0.01% to 2%). Moderate CFU counts ($100 \leq \tilde{x} < 200$) occurred on media with commercial adjuvants at specific concentrations: Designer® (0.01%), Tronic® and Summit super® (0.05% to 0.5%), Bond® (0.05% to 2%), Sprayfilm 10® (0.1% to 2%), Nufilm P® (0.05% to 1%) and Aquawet® (0.1% and 0.5%). Low CFU counts ($0 \leq \tilde{x} < 100$) occurred on media amended with commercial adjuvants, which showed a general trend of decreasing CFU counts with increased adjuvant concentrations. Designer® and Wetcit® were notable for causing inhibitory effects on the CFU counts of BCAs at all concentration levels, with the highest concentration of Wetcit® completely inhibited both BCA isolates.

The trends for PP2018-003 were similar to those of PP2018-001. Only on a few media amended with vegetable oil adjuvants and one commercial adjuvant had high CFU counts ($200 \leq \tilde{x} < 250$). For example, peanut oil (0.05% to 2%), macadamia oil (0.5% to 2%), and the commercial

adjuvant Break-Thru® (0.01%) allowed for high CFU counts. The general trend for PP2018-001 was that increased concentration levels of the commercial adjuvants resulted in decreasing CFU counts. On the other hand, the vegetable oil adjuvants promoted CFU counts, higher than the Control treatments.

The negative binomial models fitted with estimated marginal means (EMMs) provided insights into the main effects of adjuvant and concentration, and their interaction effects, which were statistically significant in contributing to differences in CFU counts for both *L. uredinophilum* isolates. Specifically, the interaction between adjuvant type and concentration levels significantly influenced CFU counts, indicating that the effect of concentration on CFU counts varied, depending on the adjuvant used (triangular matrixes not shown). Vegetable oil adjuvants such as avocado oil (est. 0.064, $\rho = 0.027$) slightly increased CFU counts compared to the control treatment. Designer®, a commercial adjuvant (est. -0.591, $\rho < 0.00001$), caused a significant decrease in CFU counts compared to the control group. Wetcit®, a commercial adjuvant (est. -1.259, $\rho < 0.00001$), had a strong negative effect on CFU counts. The commercial adjuvant, Break-Thru® (est. 0.098, $\rho = 0.00053$), and a vegetable oil adjuvant, canola (est.0.098, $\rho = 0.00053$), showed significant positive effects on CFU counts compared to the control treatment group. Interaction effects showed that most vegetable oil adjuvants exhibiting positive interaction effects, pointing to potential synergistic effects in CFU counts.

On the other hand, the commercial adjuvants showed adverse effects on CFU counts, with strong negative effects produced by Wetcit® (est. -1.017, $\rho < 0.00001$ at 0.05% concentration), in particular, pointing towards detrimental effects on CFU counts with both *L. uredinophilum* isolates. Within adjuvants, pairwise comparisons across concentration levels differed significantly according to adjuvant categories (vegetable oil adjuvants, commercial adjuvants and control group). Other adjuvant types exhibited statistically significant differences ($\rho < 0.05$) at all concentration levels. Other adjuvants (Ballista®, Bond®, avocado oil, olive oil) within concentration effects were non-significant on CFU counts across all concentration levels. The type of adjuvant and the concentration level had a profound effect on CFU unit counts, with vegetable oil adjuvants positively affecting CFU counts while the commercial adjuvants negatively affected CFU counts increasingly with increased concentration.

Table 4.3 Effect of adjuvant on colony forming units (CFUs) of *Lecanicillium uredinophilum* isolates PP2018-001 and PP2018-003.

Adjuvant	BCA isolate: *Lecanicillium uredinophilum* (PP2018-001)							BCA isolate: *Lecanicillium uredinophilum* (PP2018-003)						
	Colony Forming Units (CFU.ml^{-1}) [mean ± SD (n)]							Colony Forming Units (CFU.ml^{-1}) [mean ± SD (n)]						
	Concentration (%)						Rank	Concentration (%)						Rank
	0.01	0.05	0.1	0.5	1	2	[0.01%]	0.01	0.05	0.1	0.5	1	2	[0.01%]
Aquawet®	219 ± 1	214 ± 1	141 ± 2	162 ± 25	45 ± 1	24 ± 1	10	184 ± 1	176 ± 1	112 ± 1	105 ± 1	26 ± 1	20 ± 1	8
Avocado oil	234 ± 1	235 ± 1	230 ± 2	226 ± 1	229 ± 1	231 ± 2	5	196 ± 1	196 ± 1	192 ± 1	187 ± 1	191 ± 1	191 ± 2	4
Ballista®	226 ± 1	225 ± 1	217 ± 1	214 ± 1	212 ± 1	210 ± 1	7	194 ± 1	191 ± 1	190 ± 1	186 ± 1	188 ± 1	189 ± 1	6
Bond®	200 ± 1	180 ± 2	174 ± 1	175 ± 1	175 ± 1	175 ± 1	15	161 ± 1	141 ± 1	136 ± 1	134 ± 1	136 ± 1	134 ± 1	14
Break-Thru®	245 ± 1	215 ± 1	200 ± 2	197 ± 2	170 ± 2	169 ± 2	1	200 ± 1	194 ± 1	183 ± 1	179 ± 1	182 ± 1	146 ± 1	2
Canola oil	243 ± 1	237 ± 2	246 ± 1	246 ± 1	238 ± 3	10 ± 1	2	202 ± 1	197 ± 1	208 ± 1	186 ± 1	179 ± 1	11 ± 1	1
Designer®	131 ± 1	88 ± 1	21 ± 1	17 ± 1	16 ± 1	13 ± 1	16	92 ± 1	48 ± 1	15 ± 1	11 ± 1	9 ± 2	5 ± 1	16
Macadamia oil	224 ± 1	227 ± 1	234 ± 1	243 ± 2	238 ± 1	239 ± 2	9	184 ± 1	187 ± 1	195 ± 1	206 ± 1	200 ± 1	200 ± 1	9
Nufilm P®	210 ± 1	195 ± 1	188 ± 1	187 ± 1	185 ± 1	53 ± 1	13	148 ± 1	148 ± 1	146 ± 1	145 ± 1	35 ± 1	16 ± 1	15
Olive oil	235 ± 1	234 ± 1	245 ± 1	234 ± 1	241 ± 2	237 ± 1	3	196 ± 1	195 ± 1	207 ± 1	195 ± 1	202 ± 1	198 ± 1	5
Org. Sesame oil	225 ± 1	223 ± 1	225 ± 1	226 ± 1	224 ± 1	224 ± 1	8	183 ± 1	183 ± 2	184 ± 1	186 ± 1	184 ± 1	184 ± 1	10
Peanut oil	235 ± 1	243 ± 1	245 ± 1	246 ± 1	244 ± 1	245 ± 1	4	197 ± 1	205 ± 1	206 ± 1	205 ± 1	204 ± 1	206 ± 1	3
Sprayfilm 10®	214 ± 1	209 ± 1	185 ± 2	182 ± 1	183 ± 1	176 ± 1	11	176 ± 1	169 ± 1	166 ± 1	150 ± 1	145 ± 1	145 ± 1	11
Summit Super®	212 ± 1	190 ± 2	185 ± 1	134 ± 1	98 ± 1	13 ± 1	12	170 ± 1	149 ± 1	145 ± 1	95 ± 1	58 ± 1	5 ± 1	12
Sunflower oil	227 ± 1	241 ± 2	227 ± 1	225 ± 1	224 ± 1	223 ± 1	6	188 ± 1	203 ± 1	189 ± 1	185 ± 1	184 ± 1	184 ± 1	7
Tronic®	206 ± 1	189 ± 2	186 ± 1	187 ± 1	58 ± 1	55 ± 1	14	168 ± 1	152 ± 1	148 ± 1	146 ± 1	38 ± 1	15 ± 1	13
Wetcit®	67 ± 1	22 ± 1	16 ± 1	9 ± 1	0 ± 0	0 ± 0	17	48 ± 1	18 ± 1	12 ± 1	6 ± 1	0 ± 0	0 ± 0	17
Control	246 ± 1							194 ± 1						
P Value Adjuv.	<0.00001							<0.00001						
Conc_n	<0.00001							<0.00001						
Adjuv. * Conc_n	<0.00001							<0.00001						

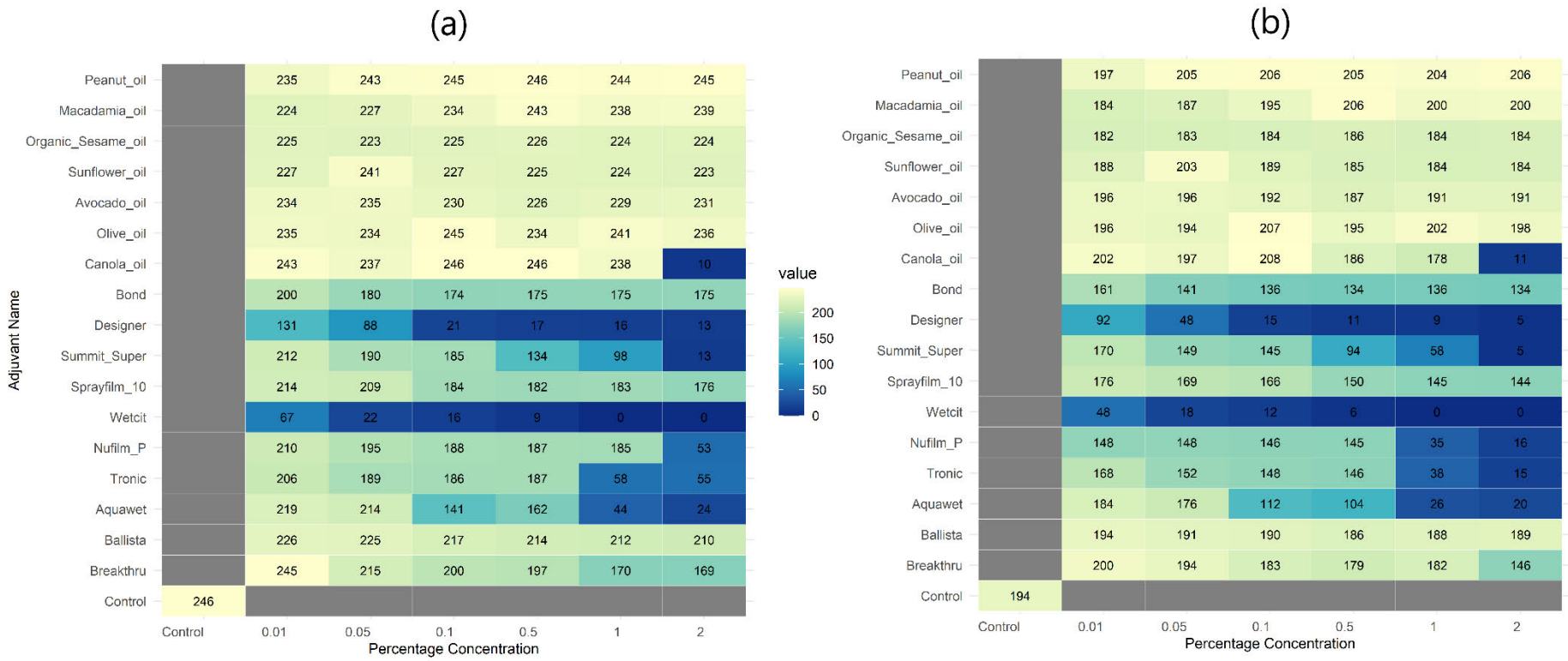

Fig 4.4 Heatmaps showing effect of adjuvant type and concentration on mean colony forming units (cfu.ml^{-1}) for *L. uredinophilum* isolates PP2018-001 (a) and PP2018-003 (b) showing the effect of adjuvant type and concentration on mean colony forming units (cfu.ml^{-1})

4.8 Discussion

The successful use of *L. uredinophilum* as a BCA hinge on maintaining the virulence and viability of the spores before application and during their infection process on the target host. This study sought to investigate the compatibility of ten commercial adjuvants and seven edible vegetable oil adjuvants on the radial growth, and CFU counts of two isolates of *L. uredinophilum*. Ideally, adjuvant application should enhance bio-efficacy (Holka and Kowalska, 2023). However, adjuvants exhibit various physiological and physiochemical properties, which may render them either synergistic or antagonistic, hence the need to evaluate their compatibility before further assays such as glasshouse or field studies (Castro et al., 2014; Castro et al., 2013; Van Zyl et al., 2010). The commercial adjuvants were chiefly selected for their expected effect on the soybean leaf surface, considering their physicochemical characteristics (spreader, penetrant, wetting agent, sticker, depositor, and surfactant), amongst other properties. The vegetable oils were selected based on their chemical or physicochemical properties, such as their fatty acids (saturated, mono-saturated, poly-saturated), unsaturated versus saturated ratio, and their viscosity at a given temperature. All ten commercial adjuvants and the seven (7) vegetable oil adjuvants appear to have not been assessed before on their compatibility with *L. uredinophilum* for controlling foliar plant diseases. The results of this study should assist in the development of formulation of *L. uredinophilum* and other fungi as biocontrol agents.

The current study revealed that adjuvants and their concentration levels affected both the radial mycelial growth and CFU counts for both of the tested *L. uredinophilum* isolates. Generally, the vegetable oil adjuvants provided synergistic effects (radial and CFU counts), with the canola oil adjuvant emerging as the best vegetable oil adjuvant. On the other hand, both radial mycelial growth and CFU counts were negatively affected by the inclusion of commercial adjuvants. However, Break-Thru® at 0.01% concentration showed positive effects on radial growth of isolate PP2018-001, ranking third, and being the only commercial adjuvant with comparable effects to the control group and the vegetable oil adjuvants. Break-Thru® at 0.01% ranked first for CFU counts of isolate PP2018-001, and second for CFU counts of isolate PP2018-003. Notably, all other commercial adjuvants had strong negative effects on both radial mycelial growth and CFU counts for both *L. uredinophilum* isolates, and these increased as the concentrations increased. Wetcit® completely inhibited mycelial growth and CFU counts at 1% and 2% concentrations for both *L. uredinophilum* isolates. While inhibitory effects were profound for commercial adjuvants, the level at which such inhibitory effects start was not

determined. Further studies may be necessary to determine the lowest limits of inhibitory effects. However, at these lower levels, the adjuvants may fail in their primary role, which is to enhance the spread and distribution of the biocontrol agent on soybean leaves.

In this study, all seven of the vegetable oil adjuvants (canola, olive, avocado, sunflower, organic sesame, macadamia and peanut) promoted the growth of the *L. uredinophilum* isolate, PP2018-001. They contributed to strong radial growth ($40\ \text{mm} - 65\ \text{mm}$) and high CFU counts ($200 - 250$) at all concentrations, except for canola oil at 2%. For *L. uredinophilum* isolate PP2018-003, strong radial growth and high CFU counts resulted from the use of some vegetable oil adjuvants, at various concentrations: sunflower oil (0.1% to 2%); peanut oil (2%); organic sesame oil and macadamia oil (0.1% to 2%); olive oil and canola oil (0.01% to 1%) and avocado oil (0.01%). These differences could be attributed to isolate variations of *L. uredinophilum* and their ability to utilize the oils as food sources. Various studies have shown that vegetable oil adjuvants work synergistically with entomopathogenic fungi used as BCAs in promoting germination, growth, sporulation (Shahid et al., 2024; Yong Lee et al., 2023; Castruita-Esparza et al., 2020; Kaiser et al., 2020; Luz and Batagin, 2005) and virulence (Shahid et al., 2024; Mohamed, 2016; Luz and Batagin, 2005; Prior et al., 1988). However, some studies have shown a negative effect of some vegetable oils on both spore germination and mycelial growth (Visalakshy et al., 2006; Luz and Batagin, 2005). *Lecanicillium lecanii* formulations in various vegetable oil adjuvants (peanut oil, sesame oil, sunflower oil, coconut oil, mustard oil) caused high levels of mortality in cabbage aphids, with the use of peanut oil and sesame oil adjuvants resulting in a 97% mortality, and more than 93% mortality with the use of sunflower, coconut, and mustard seed oil adjuvants (Varun et al., 2022). In the current study, the peanut oil adjuvant was amongst the top five best performing adjuvants in enhancing radial growth ($40\ \text{mm} - 65\ \text{mm}$) and CFU counts ($200 - 250$). The fatty acids in vegetable oils are principally oleic and linoleic acid (Hamilton, 1993). Vegetable oil adjuvants are known to enhance the solubility of active ingredients due to reduced surface tension and increased solubility (Holka and Kowalska, 2023). Oil formulations of biocontrol agents allow for their enhanced adhesion to plant surface layers, or the waxy cuticle layers of pests, providing more contact time for the BCA to interact with the targeted host. Surfactants are incorporated into vegetable oil adjuvants to emulsify the fats, and to enable the even dispersion of BCA propagules in the adjuvant emulsion (Holka and Kowalska, 2023). The compatibility of microorganisms with surfactants is known to be correlated to their physicochemical properties, such as their lipophilic nature and their alkyl chain length, which play a crucial role (Leal et

al., 2009). Therefore, further research is necessary to understand the biochemistry of vegetable oils that promote BCA germination, vegetative growth, sporulation and virulence.

Commercial adjuvants varied and adverse effects on *L. uredinophilum* radial mycelial growth and CFU counts could be attributed to their physicochemical properties. In a study by Dos Santos et al. (2021), the effects of six different adjuvants, both wettable powder and suspension concentrate products, were tested with *Bacillus thuringiensis* (*Bt*) for their effects on vegetative growth, sporulation, and efficacy on the target pest, bollworm (*Spodoptera frugiperda*, J.E. Smith). Only one adjuvant (LI-700) showed compatibility, increased *Bt* growth and sporulation, and promoted mortality of *S. frugiperda*. The other five adjuvants were found to be highly toxic to *Bt* growth and development. The study revealed that different active ingredients and concentrations of adjuvants, which share common properties with some agricultural chemicals, may be toxic to BCAs (Lin et al., 2023; Iglesias et al., 2020; Janků et al., 2018; Pakdaman Sardrood and Mohammadi Goltapeh, 2018; Chen et al., 1974). In this current study, the non-ionic surfactant adjuvants were Break-Thru®, Wetcit®, Summit Super®, Designer® and Bond®. However, their effects varied significantly on the mycelial radial growth and CFU counts of *L. uredinophilum*. The physicochemical properties of adjuvants affect different microorganisms differently (Lin et al., 2023). Break-Thru®, a silicone wetting agent, was tested for its compatibility and adjuvant properties with *Beauveria bassiana* (Balsamo) Vuillemin control of *Tetranychus urticae* (Koch) (red spider mite). It was fully compatible at 0.01% to 0.04% Break-Thru® and enhanced the mortality of the target pest under glasshouse conditions (Gatarayiha et al., 2010). In the current study, the 0.01% and 0.05% of Break-Thru® showed the best radial growth and CFU counts. Another study showed that Break-Thru® synergistically increased mortality by 2% in the control of biotype B whiteflies (*Bemisia tabaci* Gennadius) (Mascarin et al., 2014). On the other hand, Wetcit®'s adverse and inhibitory effects on *L. uredinophilum* could be due to the properties of its primary ingredients, alcohol ethoxylate and orange oil (primarily limonene) (10% of each in an emulsion) (Jian et al., 2023). When combined with borax, it creates a product with insecticidal, miticidal and fungicidal activity (Rovensanext, 2022). Wetcit® is used alone for the control of thrips in blueberries in South Africa (Dlamini et al., 2020). Wetcit® has also been shown to inhibit the growth of *Fusarium graminearum* (Schwabe) with increasing concentration (0.0025% completely inhibited growth) (Jian et al., 2023). Essential oils have been shown to be toxic to fungi via multiple target sites, damaging cellular integrity by negatively affecting the functions of various cell organelles and cellular components (Jian et al., 2023; Zhang et al., 2022;

Dassanayake et al., 2021; Naveen Kumar et al., 2016). Sprayfilm 10® contains terpenes extracted from pine resins. Terpenoids are known to inhibit mycelial and spore germination of fungi (An et al., 2019), which could account for the reduced radial growth and CFU counts observed with Sprayfilm 10® in this study.

This study was limited to evaluating *in vitro* compatibility between the 17 adjuvants and two isolates of *L. uredinophilum*. In the future, the performance of these isolates, combined with compatible adjuvants, will need to be tested under glasshouse or field conditions to evaluate the efficacy of the selected adjuvants in enhancing the field performance of the BCAs.

4.9 Conclusion

This study evaluated the *in vitro* compatibility of two isolates of *L. uredinophilum* with ten commercial adjuvants available in the South African market, together with seven laboratory-developed vegetable oil adjuvants. There were significant differences in the compatibility of the fungi with the commercial adjuvants and the vegetable oil adjuvants. There were also interaction effects between the adjuvants and their concentrations.

At a low concentration (0.01%), the commercial adjuvants Break-Thru® (a non-ionic surfactant) and Ballista® (a penetrant/spreader) supported mean radial colony growth of BCA isolate PP2018-001 that fell within the highest classification range ($40\ mm \leq \tilde{x} < 65\ mm$). In contrast, for BCA isolate PP2018-003, Bond®, a non-ionic surfactant with wetting, sticking, spreading, and depositing properties was the only commercial adjuvant to support mean radial growth within this highest range. The vegetable oil adjuvants were compatible with both fungal isolates at all concentrations, and higher concentration enhanced growth and CFU counts. *L. uredinophilum* isolate PP2018-001 performed better in radial growth and CFU counts for the commercial adjuvants and control group, whilst *L. uredinophilum* isolate PP2018-003 performed better with the vegetable oil adjuvants treatments. Future studies should focus on evaluating the selected adjuvants on soybean rust-infected plants to assess their spreading ability, persistence, and overall impact on disease control in greenhouse and field trials. This study provides information on the compatibility of ten commercial adjuvants and seven vegetable oil adjuvants, and their concentrations, that could be used in deciding on which adjuvant formulations to use for *in planta* greenhouse and field trials of *L. uredinophilum* against *P. pachyrhizi*.

4.10 References

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Chapter 5: Ultrastructural examination of the fungus-to-fungus interactions of *Lecanicillium uredinophilum* and *Phakopsora pachyrhizi*

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Abstract

Asian soybean rust (ASR) is caused by the biotrophic fungus *Phakopsora pachyrhizi* Syd. & P. Syd. and is one of the most important diseases of soybean [*Glycine max* (L.) Merr.], with recorded yield losses of up to 100%. The fungus *Lecanicillium uredinophilum* isolate PP2018-001, originally isolated from wild strawberry rust pustules, revealed hyperparasitic abilities on the rust urediniospores of soybean and frangipani (*Plumeria* spp.). This study examined the fungus-to-fungus interactions of the hyperparasite (*L. uredinophilum*) and *P. pachyrhizi* using confocal laser-scanning microscopy (CLSM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM) to reveal whether *L. uredinophilum* isolate PP2018-001 employed mycoparasitism as its mechanism of action. The CLSM used an *Agrobacterium tumefaciens*-mediated transformation (AGTM) of *L. uredinophilum* isolate PP2018-001 with the green fluorescent protein (GFP) gene to clearly track the hyperparasite infection process. The SEM and TEM investigations used both *in vivo* and *in vitro* co-inoculations to examine the extent and type of damage caused by *L. uredinophilum* on the urediniospores of *P. pachyrhizi.* Confocal microscopy revealed the ability of *L. uredinophilum* to penetrate and intensely colonise urediniospores within 36 hours. In SEM studies, *L. uredinophilum* hyphae extensively coiled around urediniospores after both the *in vivo* and *in vitro* co-inoculations, with clear penetration and damage of outer urediniospore walls, which with time, produced visible perforations and loss of cell integrity. TEM revealed the infection of urediniospores by *L. uredinophilum,* penetrating hyphae, germ tube pores and the collapse of urediniospores. This

study captured the parasitic nature of *L. uredinophilum* on *P. pachyrhizi* fungus-fungus interactions. It showed that mycoparasitism definitely occurred, and possibly enzymatic activity occurred, resulting in the degradation of urediniospore outer walls and germ tubes. The results are important for the for potential registration of isolates of *L. uredinophilum* for the biological control of soybean rust.

Keywords: biotrophic, *Glycine max*, *Phakopsora pachyrhizi*, *Lecanicillium uredinophilum*, hyperparasite, mycoparasitism

5.1 Introduction

Soybean [*Glycine max* (L.) Merr.] is a global crop that contributes substantially to food security, provides essential nutrients and is a significant source of protein and oil (Hamza et al., 2024; Dourado et al., 2011). Its cultivation is critical for food security and economic stability in many countries (Nair et al., 2023). However, the profitability of soybean production is negatively impacted by the biotrophic fungus *Phakopsora pachyrhizi* Syd. & P. Syd., the causative agent of Asian soybean rust (ASR) (Hossain et al., 2024). This soybean pathogen is of great economic significance, resulting in yield losses and significant financial repercussions for farmers and the agricultural industry (Hossain et al., 2024; Meira et al., 2020). Fungicides in the classes of succinate dehydrogenase inhibitors (SDHIs) and quinone outside inhibitors (QoIs) effectively control ASR. However, in Brazil alone, the amount of fungicide required to control the ASR pathogen has increased since the first outbreak and, thus, raised the costs of soybean production (Meira et al., 2020). Furthermore, there is evidence of resistance development against both classes of fungicides (QoIs and SDHIs) (Klosowski et al., 2018).

Developing disease-resistant crops and applying chemical pesticides are the primary approaches for controlling plant diseases such as ASR (Hao et al., 2024; Langenbach et al., 2016). However, the sole use of a few classes of fungicides has led to pathogens developing resistance, restricting their application in the agricultural sector (Tudi et al., 2021; Sharma et al., 2019). In the quest for sustainable farming practices, biological control agents against the ASR pathogen have emerged as a promising alternative to chemical fungicides (Twizeyimana et al., 2023; Si et al., 2022; Dorighello et al., 2020). These agents can potentially manage plant diseases with reduced environmental impact and little risk of the pathogen developing resistance. Among the potential biocontrol agents, isolates of *Lecanicillium* spp. Zare & W. Gams. (syn. to *Verticillium* spp., *Akanthomyces* spp.)[(Kepler et al., 2017)] are effective biological control agents for numerous insect pests (Manfrino et al., 2022; Zhou et al., 2020)

and have been evaluated on *Hemileia vastatrix* (Berk & Broome), the rust pathogen of coffee (*Coffea arabica* L.) (Das et al., 2024; Luiz et al., 2024; Romero and Castillo-Arévalo, 2023; Belachew Bekele, 2022; García-Nevárez and Hidalgo-Jaminson, 2019) as well as *Puccinia arachidis* Speg., the peanut (*Arachis hypogaea* L.) rust pathogen (Nana et al., 2024; Nana et al., 2023).

The present study's central question is whether the fungus *L. uredinophilum* functions as a true mycoparasite. Specifically, the study aims to determine if the fungus directly penetrates the host tissue of *P. pachyrhizi* to extract nutrients from the biotrophic fungus as substrate. Although studies of *L. lecanii* on the peanut rust pathogen revealed a confrontation of urediniospores, including internal invasion, as well as some presence of mucilaginous matrix suggestive of enzyme activity (Nana et al., 2023), *L. uredinophilum* is yet to be investigated on *P. pachyrhizi*, the soybean rust pathogen. Understanding the mode of action of *L. uredinophilum* isolate PP2018-001 is crucial for several reasons. If *L. uredinophilum* isolate PP2018-001 is a true mycoparasite of the ASR pathogen, it may directly attack and kill the rust fungus, providing a robust biocontrol strategy. Alternatively, if it primarily uses enzymatic action, then this knowledge could inform the development of formulations that enhance its enzymatic activity, thereby improving its effectiveness as a biocontrol agent. Previous studies found that *Lecanicillium* spp. employed mechanical force and enzymes to penetrate their insect hosts (Hajji-Hedfi et al., 2023).

This study sought to elucidate the parasitic nature of *L. uredinophilum* isolate PP2018-001 through a series of detailed microscopic investigations. Confocal microscopy using a green fluorescent protein (GFP) transformant to enhance the visualisation of the hyperparasitic nature of the *L. uredinophilum* interactions with *P. pachyrhizi* urediniospores. Scanning electron microscopy (SEM) further sought to elucidate the interactions of *L. uredinophilum* and *P. pachyrhizi* in *in vivo* and *in vitro* assays. The SEM examinations could reveal direct penetration or infection of *P. pachyrhizi* by *L. uredinophilum* through the germination pores. In addition, transmission electron microscopy (TEM) was employed to visualise internal interactions of the hyperparasite with the ASR pathogen at the cellular level, in *in vivo* and *in vitro* assays. These insights advance the understanding of fungal biocontrol mechanisms and contribute to developing more effective and sustainable integrated pest management strategies for soybean cultivation.

5.2 Materials and methods

5.2.1 Soybean host plants preparation

Seed of an unnamed soybean cultivar susceptible to the Asian soybean rust (ASR) was provided by Pannar Seed Company (Corteva), Greytown, KwaZulu-Natal (GPS Coordinates: -29,058956, 30,592695). To plant the soybean seed, composted pine bark growth media (3 kg) was prepared by mixing with 15g of controlled release fertiliser (Osmocote Exact Mini 5-6M 15-3.9-9.1+1.2Mg+TE, Greenhouse Products (Pty) Ltd, South Africa) in 30cm-containers and subsequently moved to a passive glasshouse facility (University KwaZulu-Natal controlled environmental facilities). Osmocote is a slow-release water-soluble fertiliser that can provide balanced fertilization for 3-6 months, depending on the application rate and the crop. Four to five seeds were planted in each pot and irrigated every 1-3 days using a manually controlled drip irrigation system (1 h each time). Three to four planting dates with an interval of 7-10 days were scheduled to allow for different ages of the soybean host plants, whilst accessing inoculum of *P. pachyrhizi*. The initial *P. pachyrhizi* inoculum was obtained from the Ukulinga Research Farm, University of KwaZulu-Natal (GPS:29° 40′S; 30° 24′E; 809 m a.s.l). Diseased leaves were pinned on trifoliates of soybean plants in pots to allow for natural infection by ASR. Fourteen days post-inoculation, most potted plants (>80%) had ASR symptoms. The infections were allowed to further proliferate for at least 4-6 weeks before urediniospore collection. A total of five pots each with four (4) soybean plants were kept in a separate glasshouse establishment to prevent them from being infected by the soybean rust pathogen.

5.2.2 Use of frangipani rust (*Coleosporium plumeriae*) as a host to maintain the pathogenicity of isolate PP2018-001 of *L. uredinophilum*

In the grounds of the greenhouse facility of the University of KwaZulu-Natal, Pietermaritzburg campus, is a large frangipani tree (*Plumeria* spp.), approximately 40 years old. Annually, the leaves are infected by the frangipani rust (*Coleosporium plumeriae* Patouillard) from late October to late June in the subsequent year, summer to autumn. Over the years of this study, 15-20 potted trees were grown from cuttings, to be used in glasshouses. A conidial suspension of isolate PP2018-001 of *L. uredinophilum* was inoculated onto the *C. plumeriae* sori twice annually and re-isolated as a process to retain the pathogenicity of the biocontrol agent on rusts. This was done to curtail the loss of virulence, probably through epigenetics, which can occur when cultures are continuously sub-cultured on artificial growth media.

5.2.3 Soybean rust spore collection and germination tests

Soybean plants previously infected with ASR provided the required urediniospores. These were collected 48 h before each planned experiment. They were collected with a soft paintbrush, and dusted into weighing boat, before being transferred into 20 ml scintillation vials and stored at 4±2°C till required, particularly for confocal laser-scanning microscopy (CLSM) and transmission electron microscopy (TEM). For extended storage periods, the vials containing the urediniospores were left with lids open in a desiccator for 24 h to dry and then stored at -80°C till required. (Bonde et al., 2006) recommended that using urediniospores previously stored at -80°C requires careful rehydration, which is achieved by transferring a required quantity of urediniospores onto a 150 mm glass petri dish lid placed inside a glass desiccator with a film of water underneath the petri dish, and left *in situ* for 24 h. Germination tests were run to confirm their viability and visualisation in the CLSM. Urediniospores were dusted onto 1.25% water agar. After 24 h, the germinated urediniospores were viewed under a light microscope (Carl Zeiss light microscope, Model GmbH 37,081, Gottingen, Germany) at x400 resolution. Samples with 80-100% germination rate were used in subsequent experiments (De Paula et al., 2022; Juliatti et al., 2017; Koïta et al., 2017; Nunkumar et al., 2009).

5.2.4 *Lecanicillium uredinophilum* preparation

The biological control agent for this study was a isolate previously identified as isolate PP2018-001 of *L. uredinophilum* with accessions as PP259072.1 (ITS), PP273269.1 (RPB2) and PP273271.1 (TEF) (Chapter 3). Isolate PP2018-001 was isolated from colonised pustules of the wild strawberry rust, *Phragmidium mucronatum* (Pers.) Schltdl. Previous test experiments (data not shown here) with isolate PP2018-001 had achieved at least 80% colonisation of soybean rust pustules *in vivo* (greenhouse test experiment), and in detached leaf experiments on its colonization of pustules of *C. plumeriae*. These experiments confirmed that it is an antagonist capable of infecting multiple rust species. Koch's postulate postulates were verified by conducting experiments to ascertain that no other opportunistic antagonist could have been present as latent infections of the rust sori of the two rust species, and the results were positive. *C. plumeriae* is present from as early as October each year. It persists on the leaves until June/July (winter) on most plants, thus providing an excellent experimental model on which to test potential soybean rust mycoparasites. Working cultures of *L. uredinophilum* were maintained on Petri dishes of full-strength PDA. The plates were stored at 4°C under continuous darkness. In order to track the biocontrol agent *in vivo*, isolate PP2018-001 was

transformed with the green fluorescent protein (GFP) and used in subsequent experiments. The transformed isolate PP2018-001 of *L. uredinophilum* shall be referred to as isolate PP2018-001-GFP.

5.2.5 GFP transformation of *Lecanicillium uredinophilum*

Agrobacterium tumefaciens (syn. *Rhizobium radiobacter*) isolate AGL1 (Lazo et al., 1991) was used to transform *L. uredinophilum* (isolate PP2018-001) with the binary vector pCBCT (Gorfer et al., 2007) that codes for the reporter gene GFP, as described by (Németh et al., 2019). Briefly, 15 to 20 small fungal colony fragments, approx. 2-4 mm x 2-4 mm, were transferred onto sterile cellophane sheets placed on malt extract agar (MEA; Merck) plates (d = 9 cm). Colonies of *L. uredinophilum* (isolate PP2018-001) were grown for four days in the dark at 25°C on the MEA. The cellophane sheets bearing the growing fungal colonies were then transferred to plates containing Moser induction medium (MoserIND) (Gorfer et al., 2007). *Agrobacterium tumefaciens* isolate AGL1 carrying the binary vector pCBCT was grown overnight at 28°C in 4 ml Lysogeny Broth (L.B. broth) containing 0.1% glucose supplemented with 50 $\mu g ml^{-1}$ kanamycin under continuous shaking (180 rpm). Bacteria were then pelleted by centrifugation for nine min at 3,800 *g* and resuspended in *Agrobacterium* Induction Medium (AtIND) (Gorfer et al., 2007). Induction of bacteria was achieved after incubation for 6 h under continuous agitation at 180 rpm at 28°C. Aliquots (50-100 µl) of the induced bacterial culture were pipetted directly onto *L. uredinophilum* colonies growing on cellophane sheets placed on MoserIND and wetted by pipetting the suspension up and down. Co-culture plates were incubated for four days at room temperature. The cellophane sheets from these plates were then transferred to new plates containing selective medium (MEA with 50 $mg l^{-1}$ hygromycin B and 100 $mg l^{-1}$ cefotaxime, Duchefa Biochemie) and incubated in the dark at 22°C for 2 to 4 weeks, until the emergence of visible fungal colonies. Hyphae of actively growing putative transformants were illuminated with blue light, and colonies exhibiting green fluorescence were transferred to a new 6 cm diameter Petri dish containing the same selective medium. Colonies exhibiting fluorescence when excited with blue light were considered *L. uredinophilum*-GFP transformants. GFP expression of the subcultured colonies was verified with a fluorescence microscope (Zeiss Axioskop 2 Plus microscope (Carl Zeiss Microscopy GmbH) (Fig 5.1). The transformants were subcultured every 4-6 weeks on MEA plates. GFP expression of hyphae was verified with fluorescent microscopy before every subculturing. Hygromycin B was not added to MEA used to maintain the transformants in culture after the

third transfer, as these remained stable without selection pressure during the duration of this work.

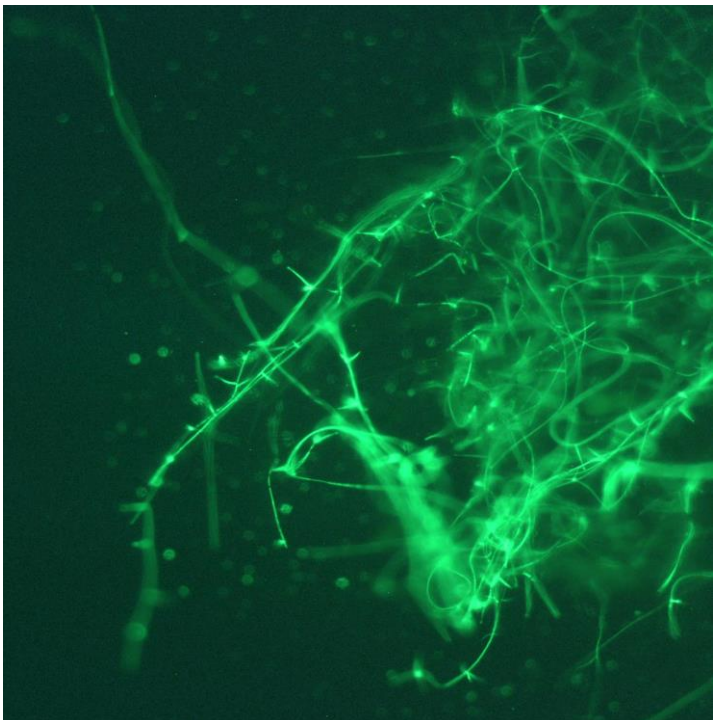

Fig 5.1 Fluorescence microscopy of a sample of *Lecanicillium uredinophilum* (PP2018-001-GFP), confirming its successful GFP transformation

5.2.6 Scanning electron microscopy (SEM)

5.2.6.1 Soybean plants preparation and inoculation with soybean rust

Colonised rust sori appear to be covered with the mycelium of the mycoparasite; however, visual examination does not reveal the nature of the microbe-microbe interactions. Scanning electron microscopy (SEM) was employed: (i) to examine the topographical characteristics of the hyperparasitized sori of *P. pachyrhizi* and *C. plumeriae*; and (ii) to assess the integrity of the urediniospores after colonisation, aiming to determine the extent of damage, if any, to the urediniospores. The SEM studies were conducted according to (Gauthier et al., 2014) and (Adendorff, 1998), with some modifications. At the time of the successful transformation of *L. uredinophilum* with the green fluorescent protein (GFP), there were a range of ASR-infected soybean plants and non-infected plants (growth stages V1 to R6) to examine. The R6 growth stage was defoliating due to extensive infection by *P. pachyrhizi.* Some infected plants were used to infect naturally V3 and R1 ASR-free soybean plants to prepare the *in vivo* assays for the SEM studies.

5.2.6.2 Hyperparasite preparation and inoculation

Ten (10) successful transformants, labelled PP2018-001-GFP_01 through PP2018-001-GFP_10 (Fig 5.2), were subcultured after transformation. Inoculum for transformants was prepared from subculturing on full-strength PDA for 14 days and harvesting conidia. A 0.05% Break-Thru® solution (an adjuvant) was prepared and used to wash off conidia from plates of the ten PP2018-001-GFP transformants. The suspension was filtered through a double layer of cheesecloth to create conidial suspensions for the ten PP2018-001-GFP transformants. The concentrations of the conidial suspensions were adjusted using an improved Neubauer haemocytometer (model 3048–13, Hausser Scientific, Horsham, PA, USA) to achieve 1 x 10^6 conidia ml^{-1} in the *in vivo* assays. After 28 days of soybean rust growth on previously uninfected V3 and V1 soybean plants, three plants with leaves varying in rust disease symptom severity were sprayed to runoff using a pneumatic spray bottle. The plants were allowed to dry in the shade, then covered with plastic bags and transferred to the glasshouse to allow colonisation of the rust pustules. After conidia of *L. uredinophilum* were sprayed onto detached rust-infected leaves of frangipani plants, the leaves were placed in transparent polyethylene lunch boxes (25 x 15 x 10 cm) to act as humidity chambers. The boxes were incubated at 25 ±2℃.

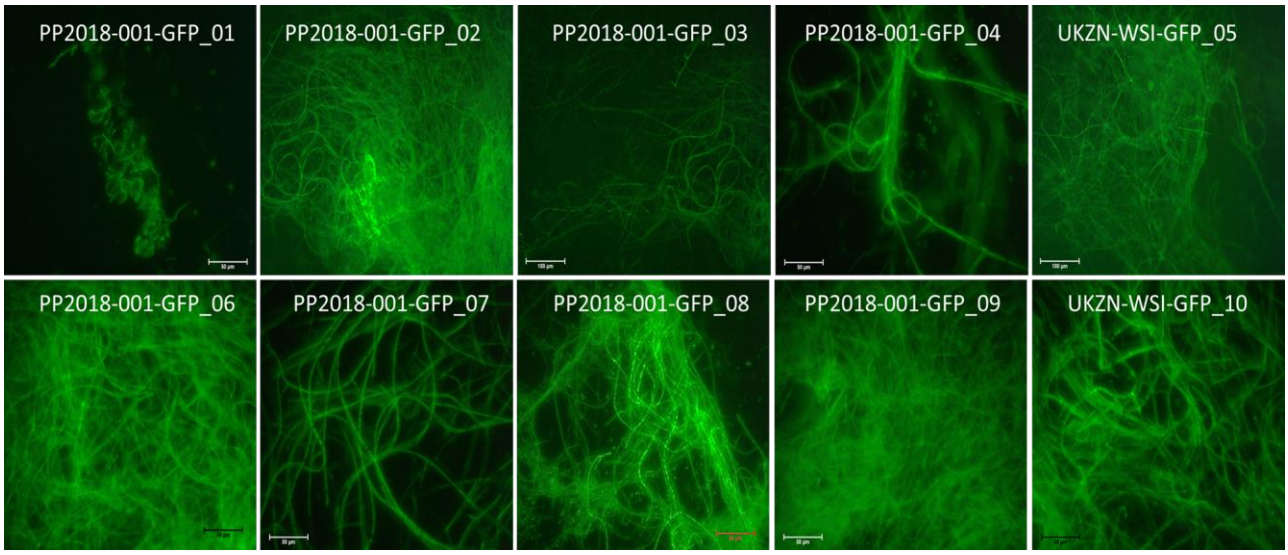

Fig 5.2 Sample images of ten transformed isolates of *Lecanicillium uredinophilum* Isolate PP2018, demonstrating the intensity of the fluorescence of the GFP.

5.2.6.3 Leaf sample preparation for scanning electron microscopy

Leaf samples for SEM and TEM were chosen based on the extent of hyperparasitic colonisation of sori (assessed visually) on days 5, 10, and 15. Three samples were taken for each sampling

day and each host, totalling 18 SEM-leaf samples. Before primary fixation for SEM imaging, soybean leaf cuttings and frangipani leaf cuttings with visible colonisation by *L. uredinophilum* were viewed under a stereomicroscope fitted with a Leica 450FC camera, equipped with Leica LAS imaging software (Leica MZ16 Stereo Light Microscope, Germany). From the colonised portions of the leaf samples, about 8-10 samples of 4 mm^2 were cut and fixed (primary fixation) in 3% buffered glutaraldehyde in 0.05 M sodium cacodylate buffer, allowed to stand for 1 h, before storage at 4℃ for 4 weeks

5.2.6.4 Co-inoculations on cornmeal agar filter paper culture

The PP2018-001-GFP transformants of *L. uredinophilum* were grown on 1% cornmeal agar (CMA) topped with Whatman #10 filter paper before the agar had set (CMA-filter-paper-culture). After the media had set, 1000 µl of PP2018-001-GFP conidial suspension (1x 10^6 spores ml^{-1}) was pipetted onto the plate surface and spread with a sterile L-shaped glass rod. The plates were incubated at 25±2℃ for 14 days. On day 15, urediniospores (1 mg) previously stored for 48 h (germination of 80-100%) were dusted onto the 14-day CMA-filter-paper-culture with the aid of a paintbrush and incubated at 25±2℃ for 5 days. Four segments of 4mm^2 of the CMA-filter-paper-culture, post inoculation with urediniospores, were cut every 24-36 hrs using a sharp razor blade and submerged in 3% glutaraldehyde in 0.05 M sodium cacodylate buffer, held at room temperature for 1 h before refrigeration at 4℃. After the primary fixation of both leaf (Section 5.2.5.3 above) and CMA-filter-paper samples, 2 x 5 minutes of buffer washes were made in 0.05 M sodium cacodylate buffer. The samples were rinsed in 0.05 M sodium cacodylate buffer again for the same time and rinsed twice in the sodium cacodylate buffer. This was followed by a series of dehydration steps in ethanol started at 10% and then incrementally increased to 30%, 50%, 70%, and 90%, each at 10 minutes and a single wash. Dehydration at 100% ethanol was done three times at 10-minute intervals. After this series of dehydration, the samples were critical point dried (Quorum Q150 RES Critical Point Dryer, Quorum Technologies Ltd, United Kingdom) using carbon dioxide as the transition fluid followed by the affixation of 2-3 samples of 4 mm^2 to stubs, which were subsequently sputter-coated with 60:40 gold: palladium (Quorum Q150R ES Gold Sputter Coater, Quorum Technologies Ltd, United Kingdom). The SEM samples were viewed using a Zeiss EVO LS 15 Variable Pressure Scanning Electron Microscope, VP SEM (Carl Zeiss, Oberkohen, Germany).

5.2.7 Confocal laser-scanning microscopy (CLSM)

Scanning electron microscopy (SEM) offers extensive imagery to resolve some of the critical aspects of anatomical structures of fungi; however, it cannot visualise internal structures. The CLSM studies were done using the techniques of Gauthier et al. (2014). Briefly, optical sectioning was possible through the CLSM instrument (Zeiss LSM 710 Confocal-Laser Scanning Microscope, Zeiss, Oberkochen, Germany). Conidia of the ten PP2018-001-GFP transformants were actively grown on full-strength PDA and incubated at 25±2℃ for 14 days. After 14 days, wet mounts from each of the isolates were viewed under a compound microscope (Olympus AX70, Olympus USA, Melville, NY) to confirm green fluorescence (the microscope was equipped with a GFP filter with 450-490 nm excitation, 500 nm emission) and with a Nikon DS Fil camera for imaging. Previously, the ten PP2018-001-GFP transformants successfully colonised urediniospores when viewed under SEM in *in vivo* preparation experiments. The transformant PP2018-001-GFP_08 had a slightly higher GFP signal than other transformants. However, all the ten transformants could have been used, based on *in vivo* SEM preparation experiments. Sample images are presented in Fig 5.1. Growth media (1% CMA) was prepared and topped with Whatman #10 filter paper before the CMA set. 1000 µ*l* of a conidial suspension (1 x 10^6 sporesmL^{-1}) of *L. uredinophilum* transformant PP2018-001-GFP_08 was pipetted onto the surface of the growth media and gently spread across the plate using a sterile glass rod and incubated at 25±2℃ for 14 days. Approximately 1 mg of urediniospores of freshly stored (48 hrs at 4℃) urediniospores of ASR with a germination percentage of 80-100% were dusted onto the 14-day-CMA-filter-paper cultures of PP2018-001-GFP_08 with the aid of a small paint brush and incubated at 25±2℃ for 5 days. Each day of the 5 days, urediniospores were picked onto a glass slide, covered with a cover slip and edges fixed onto the glass slides with nail wax polish. The slides were immediately observed using a confocal microscope (Zeiss LSM 710 Confocal Laser Scanning Microscope, Jena, Germany) equipped with a Zeiss camera and Zeiss Zen imaging software for image acquisition and processing, respectively. Confocal images were generated with a 63 x 1.4 NA Apo lens and in conjunction with a 488 nm krypton/argon laser. The laser was able to excite the GFP in *L. uredinophilum* (hyphae and conidia), as well as auto fluorescent compounds in *Phakopsora pachyrhizi* urediniospores. The PP2018-001-GFP_08 image acquisition spectra were 510-535 nm, whereas urediniospores autofluorescence spectra were 550-650 nm.

5.2.8 Transmission electron microscopy (TEM)

The TEM experiment followed the protocol of Gauthier et al. (2014) with some modifications. Briefly, TEM samples were highly dependent on SEM output to limit the number of samples to less than ten out of the 23 SEM samples (to maximise the time of the quite elaborate TEM processes and limit unnecessary duplications). Stored samples (both leaf and the CMA-filter-paper cultures) in the primary fixation phase (3% glutaraldehyde in 0.05 M in sodium cacodylate buffer) were retrieved and washed in 0.05 M sodium cacodylate buffer four times at 30-minute intervals and rinsed in the same, but fresh, buffer for 24 h in vials, with the buffer completely covering the submerged samples. After removal of the overnight buffer, osmium tetroxide (2%) in 0.05 M sodium cacodylate buffer was used to post-fix the samples for a duration of 3 h (or until the samples turned black), followed by rinsing the samples in 0.05 M cacodylate buffer twice at 30-minute intervals. A graded series of ethanol dehydration of samples from 10-100% at 10-minute washing periods was maintained up to the 90% wash and culminated in two 10-minute washes in 100% ethanol. After dehydration, samples were placed in 100% propylene oxide for two 15-minute intervals. An infiltration process using LR White resin (polyhydroxylated acromatic acrylic resin) was followed, using a graded series at 1 h intervals (25%, 50%, 75%, 100% resin mixed with 75%, 50%, 25% and 0% of propylene oxide). Samples were left overnight in 100% resin. Moulds for samples were filled up to 50% with 100% resin and each sample was placed in the mould and topped up with 100% resin to create a convex shape. Embedded samples in the moulds were polymerised in the oven at 70°C overnight (16-24 h), removed from the oven to cool, the blocks removed from the moulds and were subsequently labelled accordingly. Glass knives for sectioning were cut using an LKB 780 1A Knifemaker (Stockholm-Bromma, Sweden). After blocks were acquired, they were rough trimmed and thin sectioned (at 500 nm) with a Reichert-Jung Ultracut E Ultramicrotome (DiATOME, USA) and ultra-thin sectioned (at 120 nm) with a Leica UC7 Ultramicrotome (Wetzlar, Germany). Thin sections were picked up and dried onto glass slides using a hot plate and stained with toluidine blue. Ultrathin sections were picked up onto copper grids and post-stained with 2% uranyl acetate (for 5 minutes) and lead citrate (for 5 minutes) with a 30 second wash in deionized water in between. Images were acquired with Gatan's Digital Micrograph (DMG) software using a Gatan Orius SC-600 CCD camera (AMETEK, Inc. USA) in the TEM (JEM-1400, JEOL, TEM, Tokyo, Japan).

5.3 Results

5.3.1 Scanning electron microscopy (SEM)

Soybean leaf surfaces were not penetrated by *L. uredinophilum;* however, hyphae could be seen extending towards where there were urediniospores (Fig 5.3B) and *in vivo* inoculations did result in the colonisation of rust pustules (Fig 5.3A for *P. pachyrhizi* and Fig 5.3C for C. *plumeriae*). At 7 days post inoculation (dpi) of pustules of *P. pachyrhizi* and *C. plumeriae* showed clear perforations on urediniospores of both *P. pachyrhizi* (Fig 5.4B) and *C. plumeriae* (Fig 5.4D) as compared to control (Fig 5.4A, *P. pachyrhizi*) and (Fig 5.4C, *C plumeriae*). Moreover, the integrity of the urediniospores for both rust species appeared compromised (compared to the control), and some mycelia could be seen growing inside the urediniospore of *C. plumeriae* (Fig 5.4D). The PP2018-001-GFP filter paper cultures showed urediniospores extensively coiled in a mat of *L. uredinophilum* hyphae (Fig 5.5A). It showed microbe-microbe interactions and a concentration of growth around the uredinia after 3 days on the filter-paper-culture. Individual urediniospores could be seen under attack by *L. uredinophilum* (Fig 5.5B), suggesting some signalling that draws the mycoparasite to the uredinia. An extensive hyphae network and conidia on the surface of the uredinia could also be seen (Fig 5.5C). The *L. uredinophilum* hyphae could be seen protruding out, and some ramifying through the *P. pachyrhizi* urediniospore (Fig 5.5D). The CMA-filter-paper culture co-inoculations with *C. plumeriae* at 5 dpi were able to show *L. uredinophilum* hyphae growing through perforations of urediniospores. Mycelia growing inside the urediniospores after their gritty outer layers were compromised or peeled off (Fig 5.6A and Fig 5.6B) were clearly visible. Moreover, the *C. plumeriae* urediniospore could be seen disintegrating with *L. uredinophilum* mycelia ramifying through the disintegrating urediniospore (Fig 5.6C) and more perforations and disintegration was observed at 10 dpi (Fig 5.6D). Soybean rust urediniospores also did show clear penetration by *L. uredinophilum* (Fig 5.7A). Mycelia of *L. uredinophilum* growing inside the urediniospore viewed through a pore on the urediniospore (Fig 5.7B). A series of visibly damaged urediniospores and loss of urediniospore membrane wall could be seen (Fig 5.7C), as well as after 10 dpi, the outer membrane and urediniospores could be seen with clear disintegration of the urediniospores (Fig 5.7D).

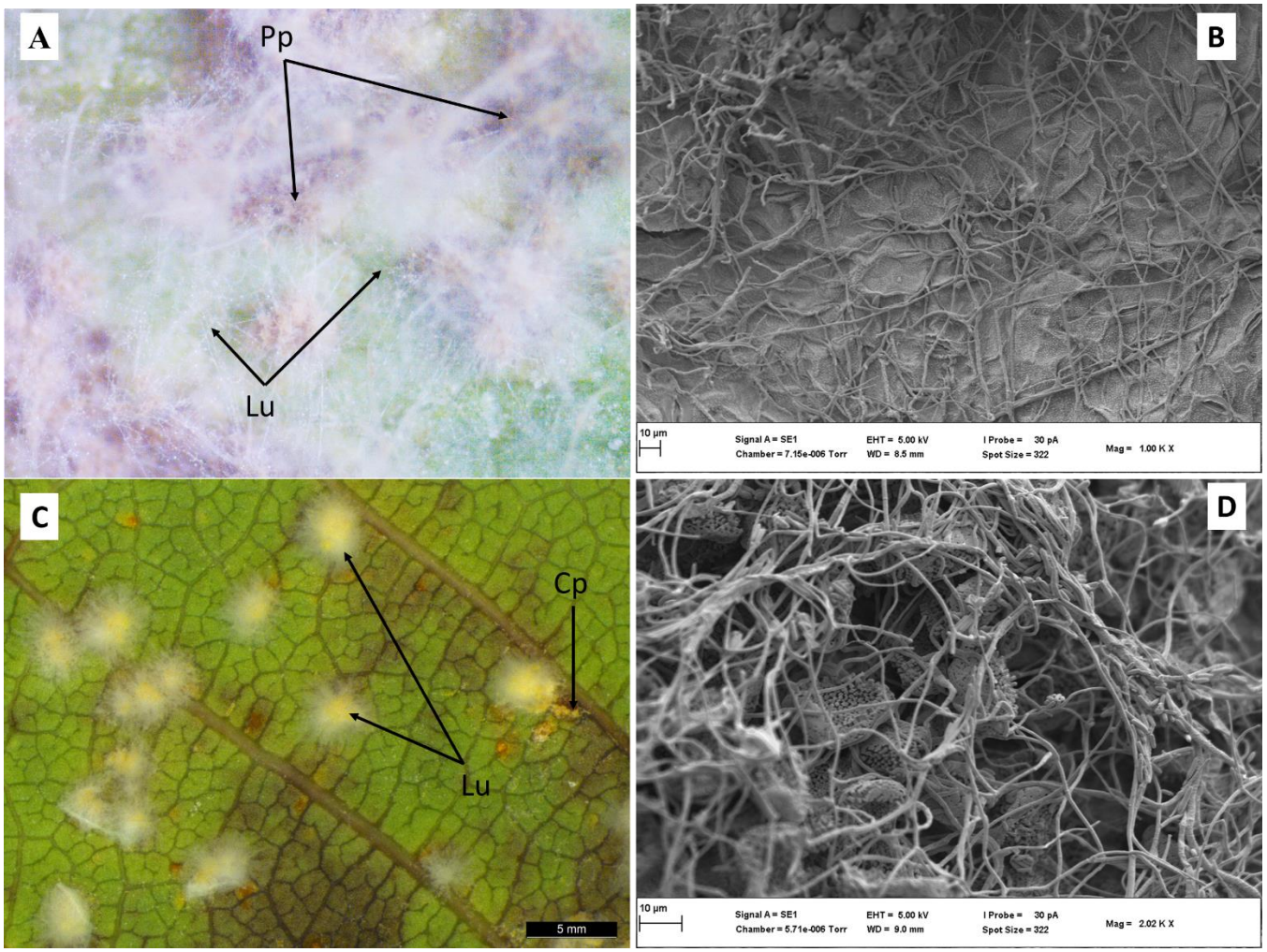

Fig 5.3 Light and SEM views of hyperparasitism by *L. uredinophilum*: Stereomicroscope views of a soybean leaf cutting infect by *P. pachyrhizi* (Pp) rust pustules (brown in colour) colonised by *L. uredinophilum* (Lu) white mycelia (A); Scanning electron micrograph showing a network of *L. uredinophilum* on a soybean leaf surface (B); A frangipani (*Plumeria* spp.) leaf cutting infected by *C. plumeriae* (Cp) forming pustules, which are colonised by *L. uredinophilum* (Lu), 5 dpi (C); Scanning micrograph showing *L. uredinophilum* on colonised pustules of *C. plumeriae* (D).

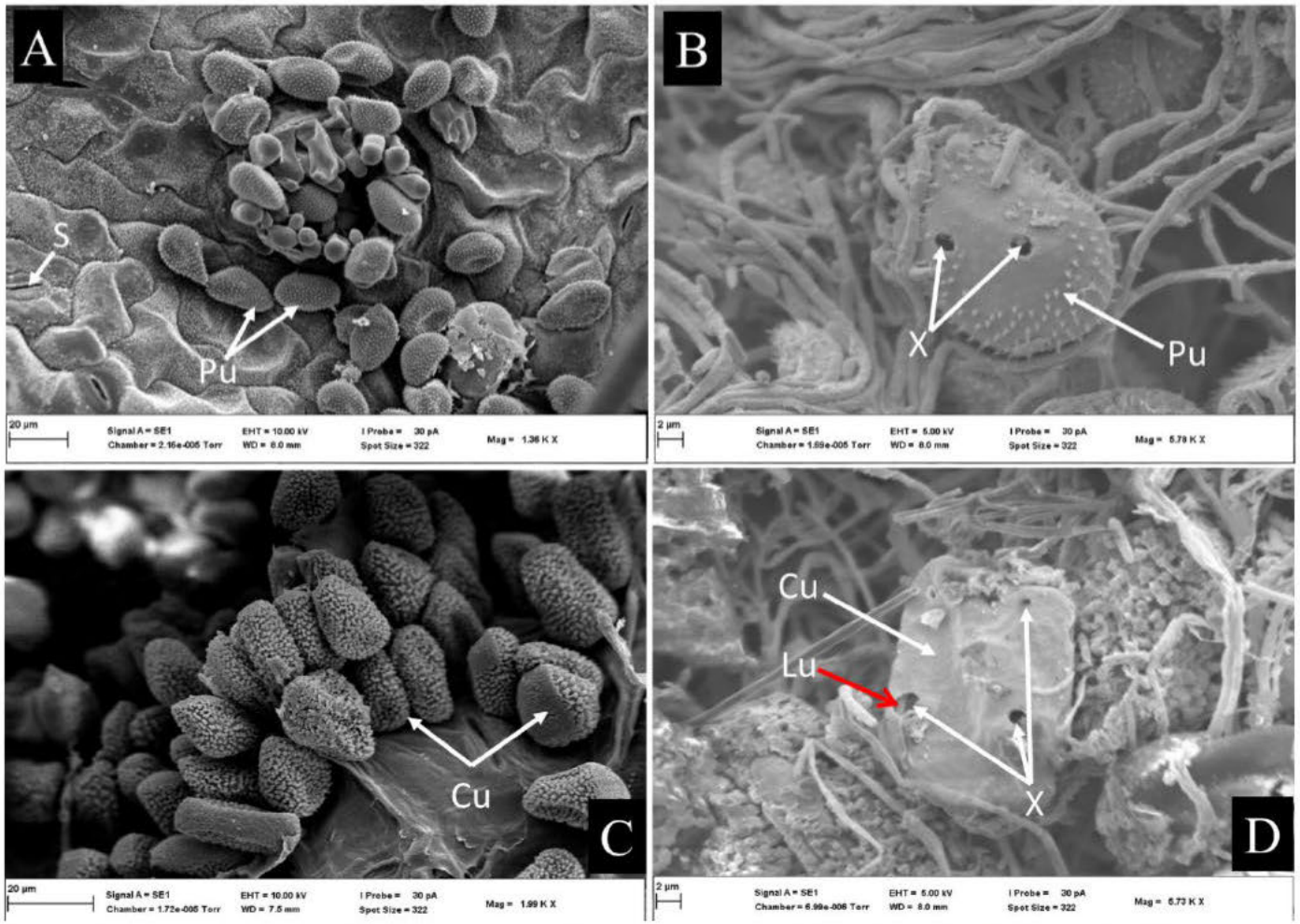

Fig 5.4 Scanning electron microscope view *Phakopsora pachyrhizi* urediniospores (Pu) not treated with *L. uredinophilum* spore suspension (A); *Phakopsora pachyrhizi* urediniospores (Pu) with perforations (X) on them (B); Urediniospores of *C. plumeriae* (Cu) not treated with *L. uredinophilum* suspension spores, (C); A perforated urediniospore of *C. plumeriae* (Cu) and the red arrow shows a hypha of *L uredinophilum* (Lu) growing inside the urediniospore of *C. plumeriae* (D).

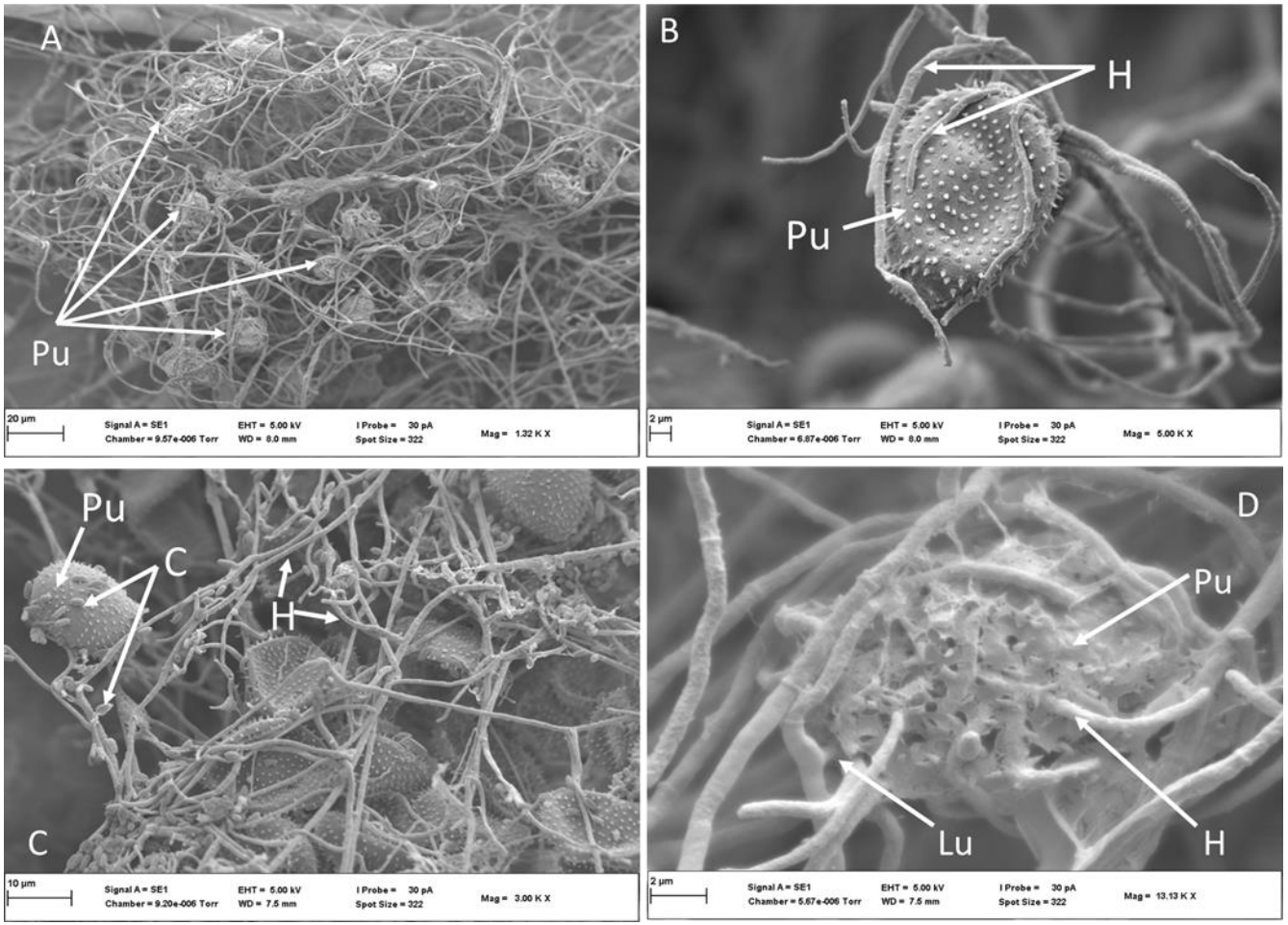

Fig 5.5 Scanning electron microscope view of the interactions of *L. uredinophilum* and *P. pachyrhizi*: An extensive hyphal network of *L. uredinophilum*, with some hyphae tightly coiled around urediniospores of *P. pachyrhizi* (Pu) 3 dpi (A); An individual urediniospore of *P. pachyrhizi* (Pu) being colonized by *L. uredinophilum* hyphae, 3dpi (B); Conidia (C) on the surface of urediniospores (Pu), as well as a network of hyphae (H), (C); A urediniospore (Pu), with a disintegrating cell wall, with penetration by hyphae (H) of *L. uredinophilum* (Lu), 5 dpi (D).

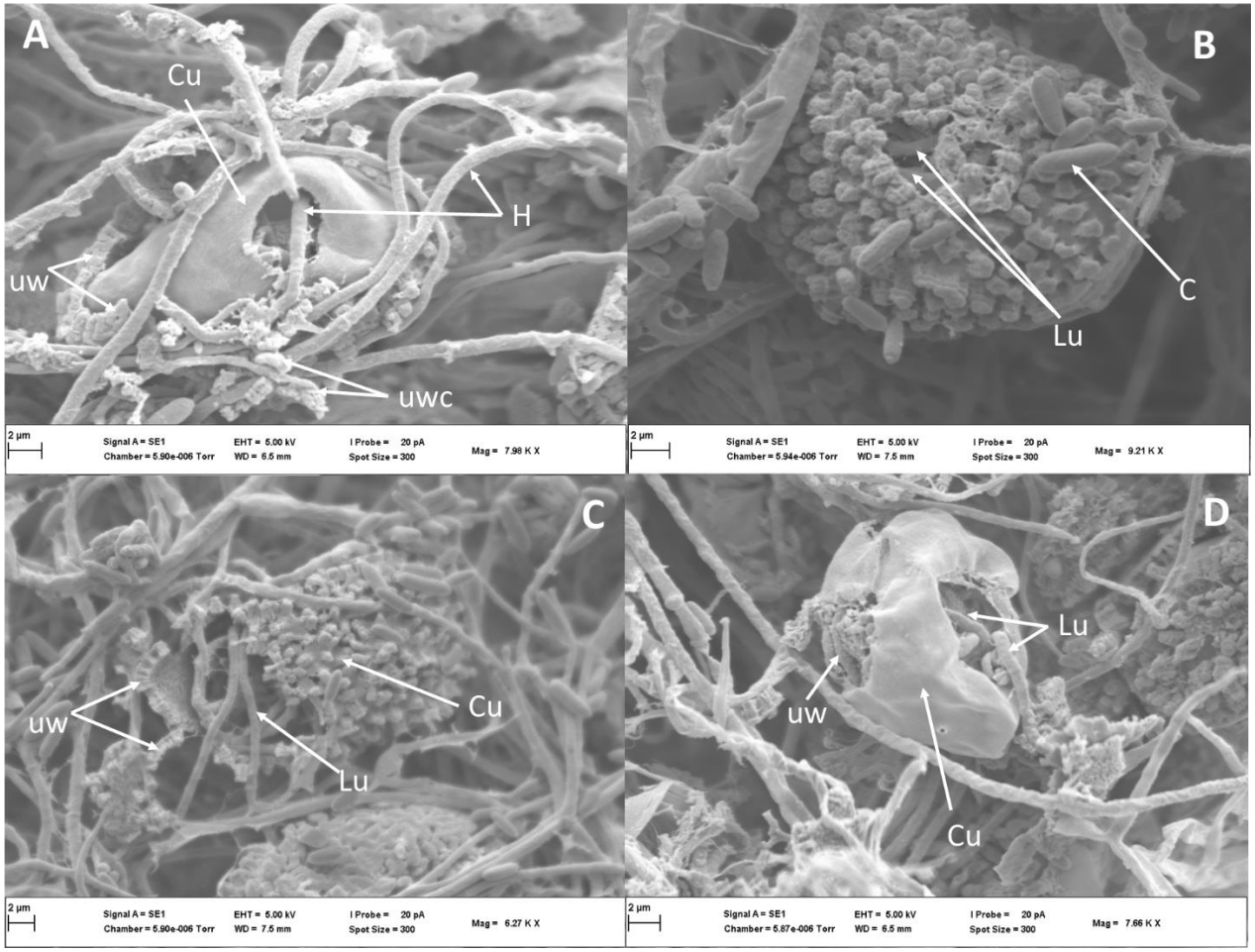

Fig 5.6 Scanning electron microscope views of interactions of *L. uredinophilum* and *C. plumeriae*: A urediniospore of *C. plumeriae* (Cu) showing a substantial hole, with hyphae of *L. uredinophilum* penetrating through the perforation, as well as breakdown of the outer membrane of the urediniospore, and cell wall damage (urediniospore wall component - uwc) (A); Conidia and mycelia growing inside and outside a urediniospore, after the outer membrane of the urediniospore had been compromised (B); Breakdown of the urediniospore cell wall (uw), 5dpi (C); Extensive damage to a *C. plumeriae* urediniospore (Cu), showing a damaged cell wall (uw), and hyphae of *L. uredinophilum* (Lu)., 10 dpi (D).

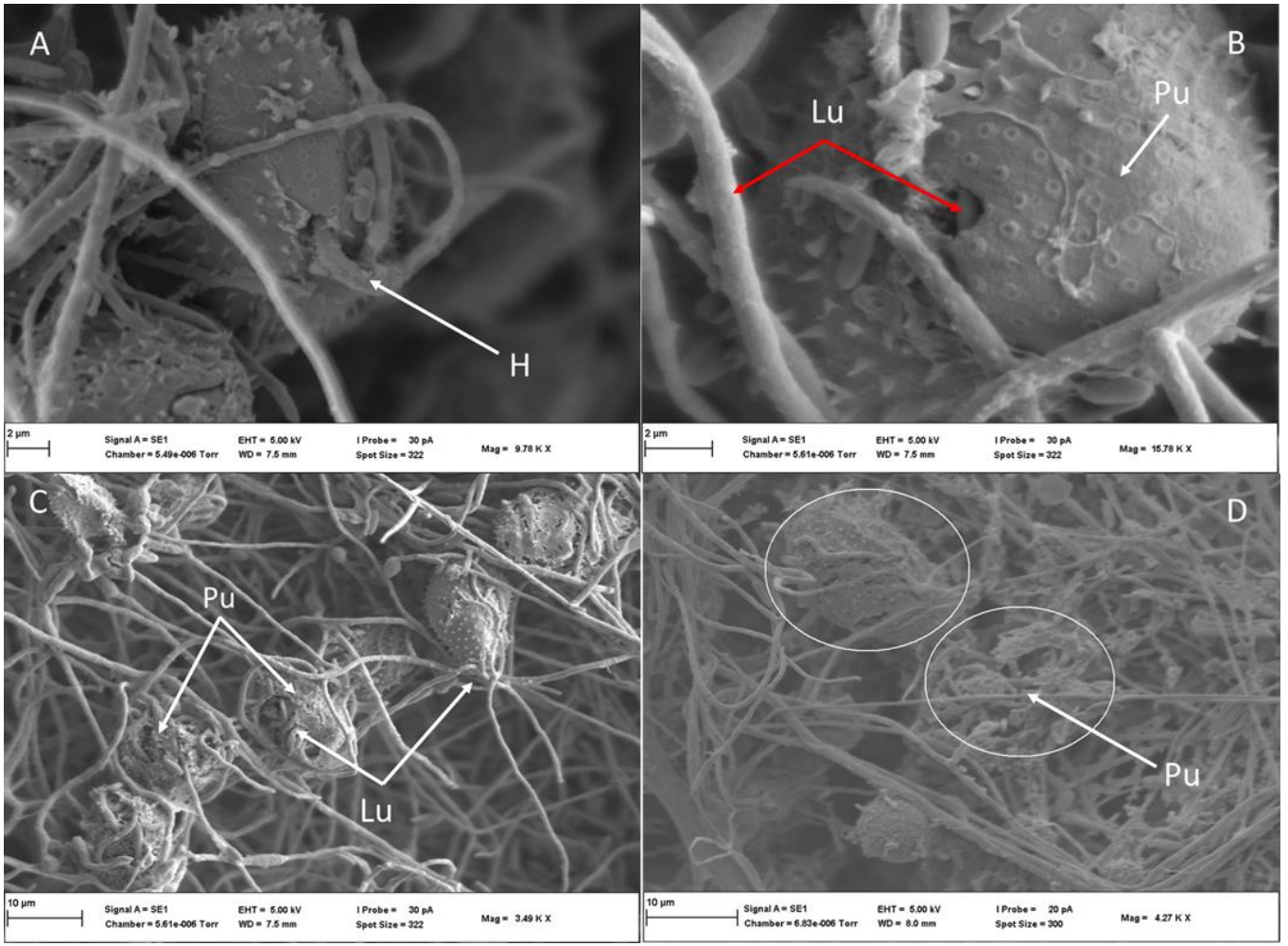

Fig 5.7 Scanning electron microscope view hyperparasitism of urediniospores of *P. pachyrhizi*: A urediniospore being penetrated by a hypha (H) of *L. uredinophilum* (A); A hypha of *L. uredinophilum* growing inside a urediniospores, viewed through a perforation (B); Several urediniospores (Pu) being parasitised by hyphae of *L. uredinophilum* (Lu), resulting in damaged cell walls (C); Interactions in a filter-paper-culture, 10 dpi, several urediniospores (Pu) can be seen to have started disintegrating (D).

5.3.2 Confocal laser-scanning microscopy (CLSM)

The PP2018-001-GFP_08 (*L. uredinophilum* transformant with GFP) colonised *P. pachyrhizi* urediniospores in both leaf cultures and the CMA-filter-paper cultures. After 76 h post-co-culture, at least 60-80% of urediniospores were colonised and showed fluorescence due to the GFP. They showed extensive growth inside urediniospores (Fig 5.8A, Fig 5.8C). The soybean rust urediniospores auto fluoresced red (514 nm wavelength), whilst the PP2018-001-GFP_08 showed fluorescence at a wavelength of 488 nm. The CLSM revealed possible penetration sites through either germ tube pores of urediniospores and possible direct penetration (Fig 5.9B), and the *L. uredinophilum* hyphae extensively and tightly coiled inside the urediniospores.

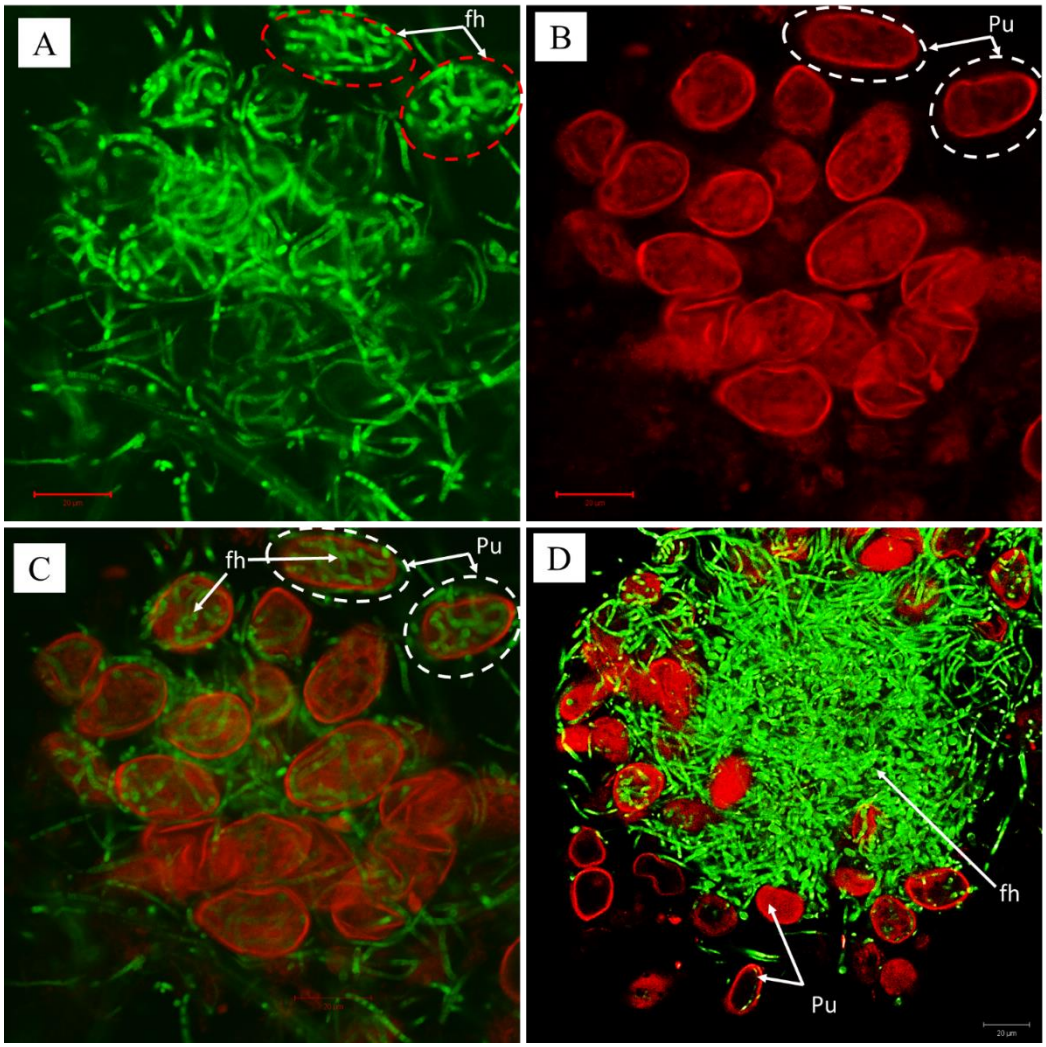

Fig 5.8 Confocal laser-scanning microscope views: Green fluorescence shown by a GFP *L. uredinophilum* transformant at the 510-535 nm emission spectra; circled in red visible hyphae (A); *Phakopsora pachyrhizi* urediniospores (Pu), non-circled and circled in white, obtained at 550-650 nm emission spectra (B); Circled in white are urediniospores with the green fluorescent GFP-*L. uredinophilum* hyphae inside the urediniospores (C); A large mass of fluorescing GFP-*L. uredinophilum* hyphae (fh) with several of these hyphae appearing to be inside or on the surface of *P. pachyrhizi* urediniospores (D).

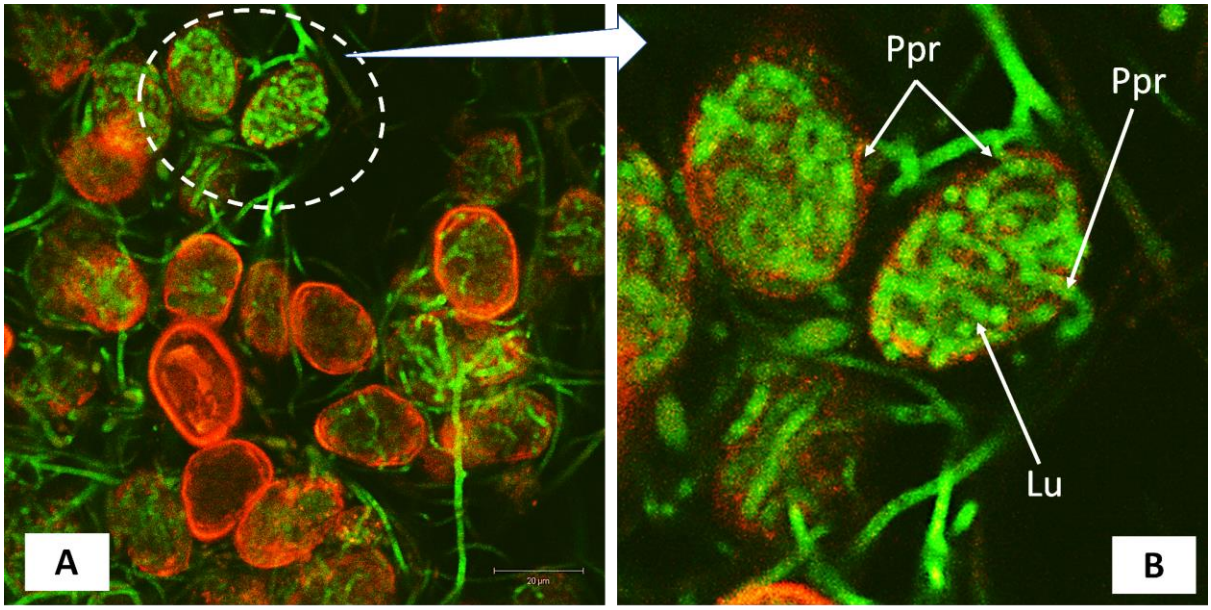

Fig 5.9 Confocal laser-scanning microscope views: An extensively colonised urediniospores of *P. pachyrhizi* by *L. uredinophilum* (Lu), 72 h after co-inoculations on a CMA-filter-paper-culture (A); An enlarged image of the same site, showing possible points of penetration (Ppr), or exit, of hyphae from the urediniospore by *L. uredinophilum* (B).

5.3.3 Transmission electron microscopy (TEM)

The ultra-thin TEM section for the co-inoculations in the CMA-filter-paper-culture *in vitro* studies (24-36 h) revealed the soybean urediniospores' cellular integrity (although organelles were not distinctive) as shown in Fig 5.10A, germ pore sites (Fig 5.10A, Fig 5.10B). Urediniospores were internally colonised by growing hyphae of the hyperparasite (Fig 5.10B). Some urediniospores were visibly distorted in shape possibly due to infection by the hyperparasite (Fig 5.10C). while the control did not show any visible distortions. The hyperparasite *L. uredinophilum* hyphae and spores (Fig 5.10D) were visible around the urediniospores. No urediniospore germination were observed in the *in vitro* studies. Further observation on the infection sites on urediniospores indicated possibly three strategies prior to infection: (i) the hyperparasite spores or hyphae attached itself to the urediniospores through somewhat mucilaginous matrix (Fig 5.11A); (ii) infection through penetrating urediniospores via germ tube degradation as observed in Fig 5.11B; and (iii) direct penetration through mechanical force (Fig 5.11C). After these infection strategies, the hyperparasite showed putative hyphal growth inside urediniospores leaving clear germ pore entry sites (Fig 5.11D). The *in vivo* studies (5-10 days) revealed some burst soybean rust pustules with some urediniospores colonised by the hyperparasite *L. uredinophilum* hyphae (Fig 5.12A, Fig 5.12B, Fig 5.12D). Urediniospores of the soybean rust lost cellular integrity and shape and some

visible germ pore sites as well as direct penetrating hyperparasite *L. uredinophilum* hypha and hyphal growth into the cell or out of the urediniospores (Fig 5.12C, Fig 5.12D). The cytoplasm of the colonised urediniospore varied in appearance, some not clearly showing contents, or some became vacuolated with clear distortions of cellular contents or emptying of contents following successful infection by the hyperparasite. Further *in vivo* results revealed putative hyphal growth of *L. uredinophilum* (Fig 5.13A, Fig 5.13B, Fig 5.13C), a break in a cell wall of a urediniospore (Fig 5.13A). A collapse and distortion in shape of urediniospores confronted by *L. uredinophilum* was also observed (Fig 5.13C) whilst in a burst pustule, a urediniospore colonised by the hyperparasite was observed (Fig 5.13D).

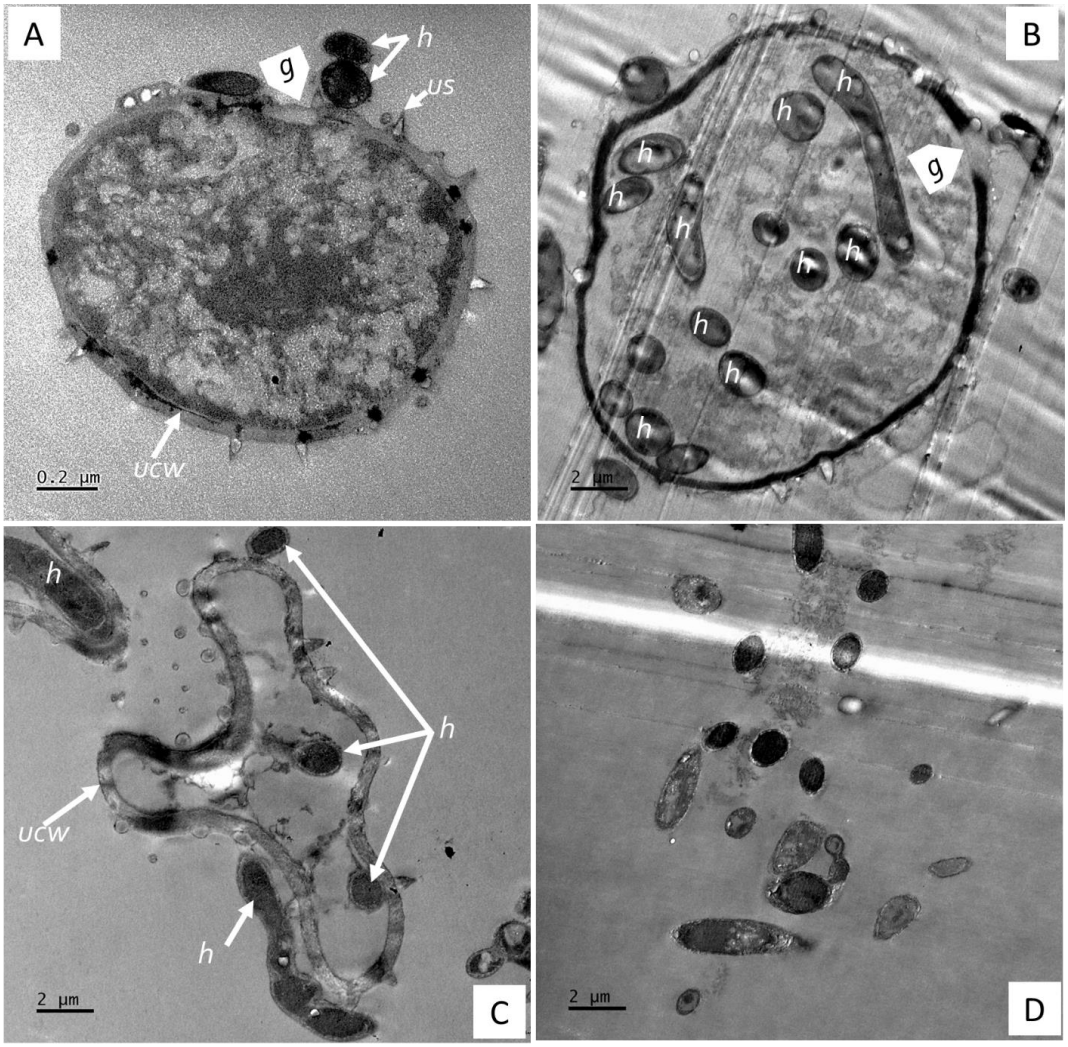

Fig 5.10 Transmission electron microscope view of the soybean rust urediniospores (*Phakopsora pachyrhizi*) interacting with the hyperparasite (*L. uredinophilum*) on CMA-filter-paper-culture 24-36 h post-co-inoculations: A urediniospore with a visible germ pore point (arrowhead) (g) before colonisation by the hyperparasite (A). Note the urediniospores spine (us), urediniospores cell wall (ucw) and hyphae (h) of the hyperparasite growing outside the urediniospore of soybean rust; A germ tube point of infection (g) and numerous hyphae (h) inside the urediniospores (B); A distorted urediniospore cell wall (cw) shape, with hyphae (h) inside the urediniospores (C); A cross-section of conidia of *L. uredinophilum* (D).

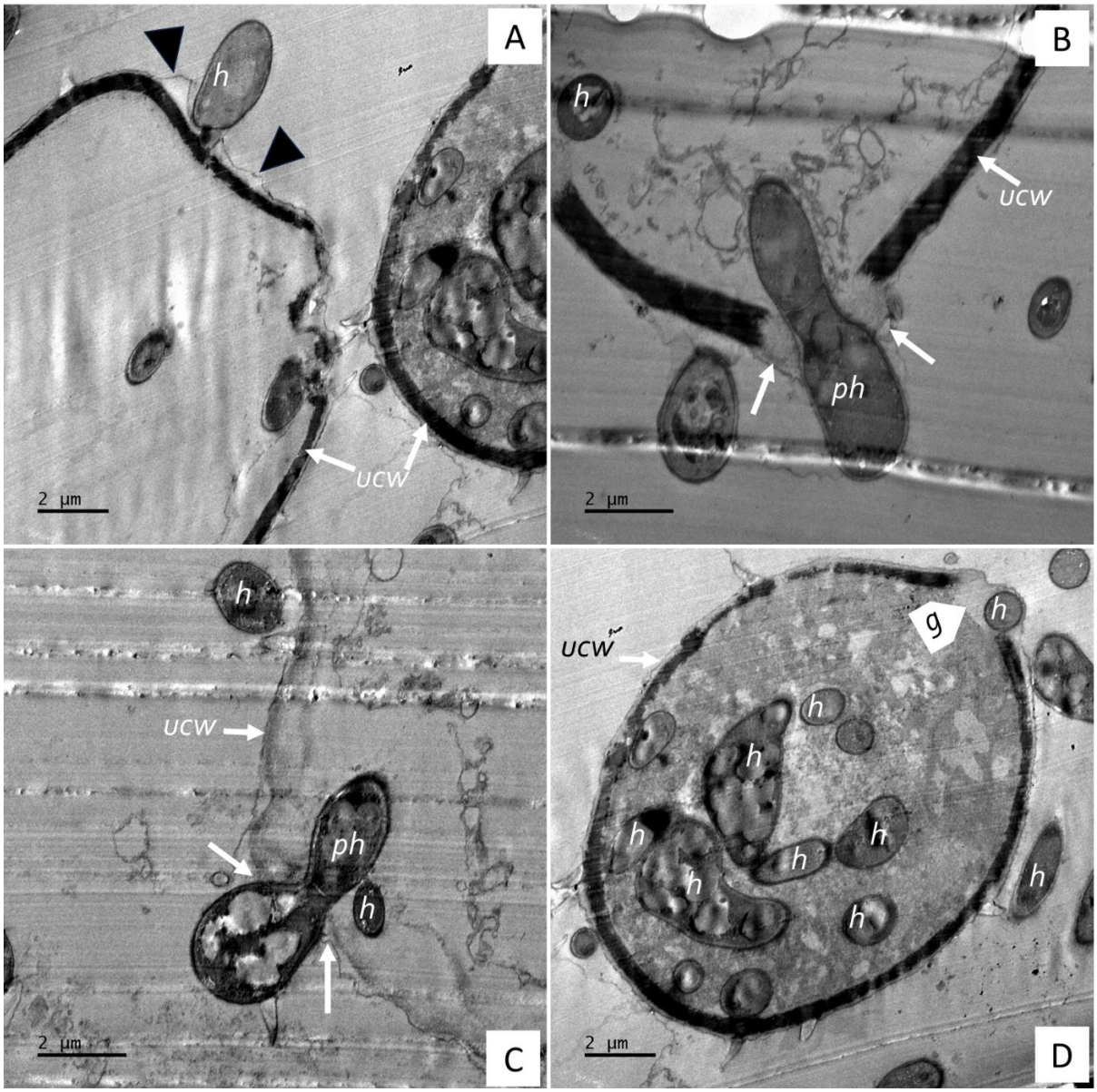

Fig 5.11 Transmission electron microscope view of the soybean rust urediniospores (*P. pachyrhizi*) interacting with the hyperparasite (*L. uredinophilum*) on CMA-filter-paper-culture 24-36 h post-co-inoculations: A conidium of *L. uredinophilum* in direct contact with a urediniospore, with mucilaginous matrix (black arrowheads), probably gluing the conidium to the urediniospore (A); Penetration by a hypha-penetrating hypha (*ph*) of *L. uredinophilum* into a urediniospore, showing degradation of germ pores (*g*) indicated by arrows, or with a matrix (arrows) on the outside of the hyphae at the point of penetration (B); Another view of a hypha of *L. uredinophilum* penetrating a urediniospore, with a matrix (arrows) on the outside of the hyphae at the point of penetration (C); Hyphae (h) growing inside a urediniospore (D).

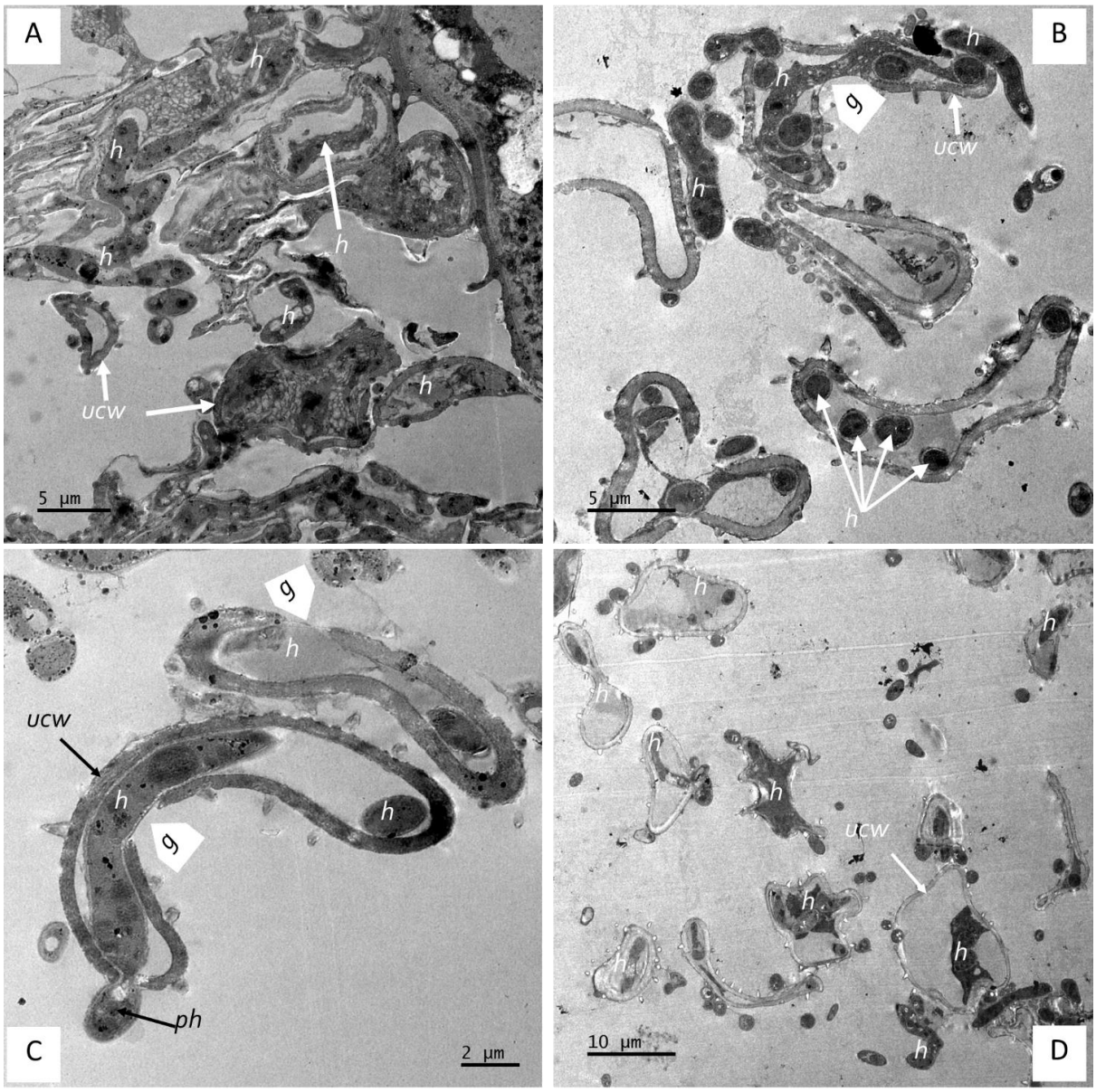

Fig 5.12 Transmission electron microscope view of the soybean rust urediniospores (*P. pachyrhizi*) interacting with the hyperparasite (*L. uredinophilum*) *in vivo*, 5 days post spraying with hyperparasite spores: A burst soybean rust pustule showing infected urediniospores and the hyphae (*h)* of *L. uredinophilum* (A); Urediniospores colonized by hyphae (*h*) of *L. uredinophilum*, the cell walls of the urediniospores remain intact but distorted (B); A hypha (*ph*) of *L. uredinophilum* penetrating a urediniospore and expanding inside. The cell wall (*uwc)* remains intact, and a germ pore can be seen *(g*) (C); Hyphal growth in several soybean rust urediniospores collapsed urediniospores due to hyperparasitism (C). Urediniospores germ pores (*g*); urediniospores cell wall (*ucw*) hyphae (*h*) of the hyperparasite growing inside the urediniospore of soybean rust, penetrating hypha (*ph*) (D).

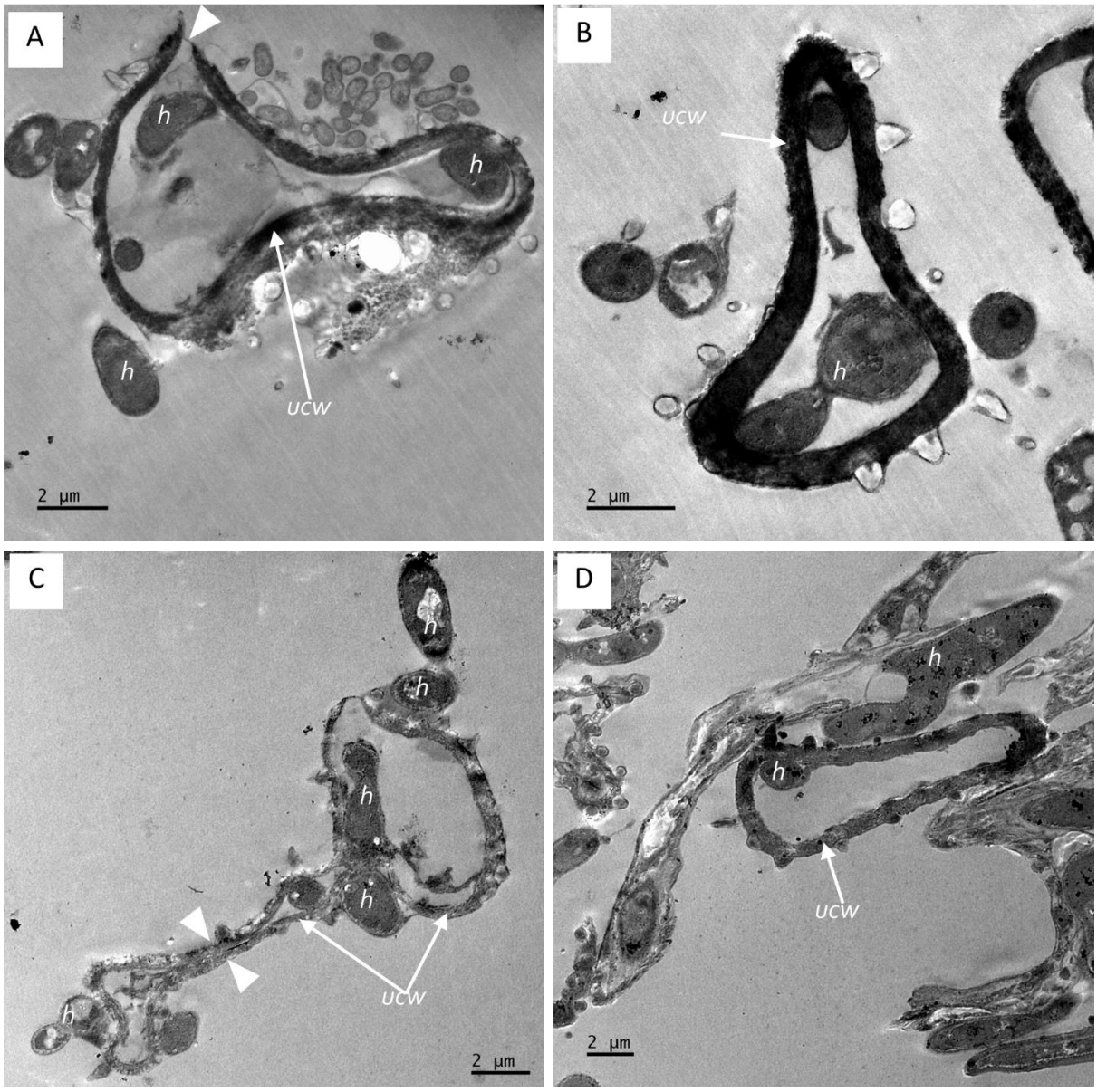

Fig 5.13 Transmission electron microscope view of the soybean rust urediniospores (*P. pachyrhizi*) interacting with the hyperparasite (*L. uredinophilum*) *in vivo*, 10 days post spraying with hyperparasite spores: A urediniospore with a broken cell wall (*uwc*) (arrowhead) and hyperparasite colonisation, with hyphae inside (*h*) (A); Advanced hyperparasite hyphal growth (*h*) in a urediniospore. The cell wall (*uwc*) remains intact (B); Two collapsed urediniospore, with their cell walls still intact (*uwc*) (white arrows), and hyphae (*h*) of growing inside an adjacent urediniospore (C); A urediniospore in a burst pustule, colonised by *L. uredinophilum* hyphae (*h)* (D).

5.4 Discussion

Fungal parasitism has been evaluated and targeted as a potential biological control strategy for phytopathogens, for example, effective control of mildew on grapes and other crops by *Ampelomyces quisqualis* (Németh et al., 2023; Saha et al., 2021; Menge and Makobe, 2017; Sztejnberg, 1993). The current study was able to document the fungus-fungus parasitism of *L. uredinophilum* isolate PP2018-001 on *P. pachyrhizi* both *in vivo* and *in vitro,* and such antagonist interactions as have been observed are well supported by definition as mycoparasitism (Wang et al., 2023; Mukherjee et al., 2022; Karlsson et al., 2017; Zeilinger-Migsich and Mukherjee, 2014; Van Den Boogert, 1996; Barnett, 1964). The fungi genera *Lecanicillium* spp. (formerly *Verticillium* for some species) are well known as hyperparasites forming fungus-fungus antagonist interactions with lesions of various fungal diseases of plants, such as, rusts (Setiawati et al., 2021; Wei et al., 2018; Park et al., 2016), powdery mildews (Kim et al., 2010; Kim et al., 2007; Miller et al., 2004; Askary et al., 1997), leaf spots (Nana et al., 2022) and others reviewed by (Goettel et al., 2008). The hyperparasite *L. uredinophilum* could infect *P. pachyrhizi* urediniospores within 24 h, making their contents a nutritive substrate and rendering the parasitised urediniospores non-viable. Through mycoparasitic interactions, the *L. uredinophilum* hyphae tightly coiled around urediniospores, directly penetrated urediniospore cell walls and degraded germ pore sites to gain access into and exit from the *P. pachyrhizi* urediniospores. Similar observations were recorded for parasitised rust urediniospores of *C. plumeriae*. There are documented studies of rust pathogens parasitised by other fungi. For example, the coffee rust fungus (*Hemileia vastatrix* Berk. & Broome) has been reported to be hyperparasitized by the entomopathogenic fungus *Cordyceps cateniannulata* Z.Q. Liang, through mycoparasitism. This fungus also acted as an endophyte and entomopathogenic to *Tenebrio molitor* (mealworms), *Hypothenemus hwamei* (coffee berry borer beetle) and a leaf miner (*Leucoptera coffeella*) (Pereira et al., 2024). Other documented examples include *Akanthomyces lecanii* on peanut rust (*Puccinia arachidis* Speg.) (Nana et al., 2024; Nana et al., 2023), *Hemileia vastatrix* coffee rust hyperparasitized by *Lecanicillium lecanii* (Das et al., 2024; Belachew Bekele, 2022; Jackson et al., 2012; Vandermeer et al., 2009), *Verticillium psalliotae* Treschow on soybean rust (*P. pachyrhizi*) (Saksirirat and Hoppe, 1991; Saksirirat and Hoppe, 1990) and *Puccinia* spp. hyperparasitized by *Cladosporium uredinicola* (Barros et al., 1999; Traquair et al., 1984).

The CLSM investigations allowed the visualisation of the *L. uredinophilum* GFP transformant putative and near-complete internal colonisation of *P. pachyrhizi* urediniospores and entry or exit sites within 36-96 h from PDA-filter-paper-culture co-inoculations similar to Gauthier et al. (2014) in their study using *Simplicillium lanosoniveum* GFP transformant as their hyperparasite on *P. pachyrhizi* urediniospores. The same authors also observed extensive branching of hyperparasite hyphae upon entry into *P. pachyrhizi* urediniospores and over 90% colonisation on day 5, similar to the current study. The rapid hyperparasitic infection of *P. pachyrhizi* urediniospores is crucial in that it has the potential to drastically reduce the secondary inoculum of the highly polycyclic and sporulating ASR pathogen and therefore, curtail the rate at which ASR epidemics can occur. Gauthier et al. (2014) successfully revealed this strategy in their field studies with *S. lanosoniveum* against ASR.

Lecanicillium uredinophilum, through mycoparasitism, formed appressorium-like structures in confrontation and infection of the urediniospores. The infection led to the formation of penetration structures similar to those observed by (Nana et al., 2023) in their studies of *Akanthomyces lecanii* against *Puccinia arachidis* (peanut rust) and similarly observed attachment to urediniospores through the mucilaginous matrix which has been suggested to create a conducive environment for enzymatic activity and insect cuticle penetration by *V. lecanii* (Schreiter et al., 1994). Other previous studies, such as mycoparasitism of *Uromyces appendiculatus* (bean rust) by *Cladosporium tenuissimum*, revealed possible attack of urediniospores by mechanical force and suggested enzymatic activity (Assante et al., 2004). Gauthier et al. (2014) recorded similar results when *S. lanosoniveum* parasitised urediniospores of *P. pachyrhizi*, and some of their observations suggested possible enzymatic activity. This study also suggests enzymatic activities in the infection process due to the presence of the mucilaginous matrix, which aid in the attachment of the hyperparasite to the host and in the process, releasing lytic enzymes.

Furthermore, the current study reveals, through the SEM, the disintegration or peeling of the outer urediniospore walls and detachment of spines from the urediniospores. This act is phenomenal to enzymatic activity. Early studies, for example, by Saksirirat and Hoppe (1991), were able to reveal that *V. psalliotae* and *V. lecanii* secreted a battery of enzymes (glucanases, chitinases, proteases) upon culture with autoclaved *P. pachyrhizi* urediniospores. *Verticillium lecanii* was reported to produce amylases, lipases, and proteases in another study by Hasan (2013), whereas Askary et al. (1997) through their cytochemical investigation on *V. lecanii* against *Sphaerotheca fuliginea* (Schlechtendahl) U. Braun & S. Takamatsu (cucumber

powdery mildew) implicated release of cell-wall degrading enzymes (CWDEs) after attachment to their fungal host. Investigations on the nematode *Meloidogyne incognita* (Kofold & White) Chitwood eggshell degradation identified chitinase as the responsible key enzyme in *Lecanicillium antillanum* (Nguyen et al., 2007). *Lecanicillium attenuatum* applied to rice fields to control the brown planthopper [*Nilaparvata lugens* (Stål)] upon transcriptome sequencing analysis showed high gene expression in the production of four principal enzymes (proteases, phospholipases, cutinases, chitinases) which are responsible for digestion of the insect tissue (Zhang et al., 2024); virulence to insects in *L. lecanii* and other entomopathogenic fungi are attributed to chitinase (Huang et al., 2024). In addition, the groundnut rust (*Puccinia arachidis*) hyperparasite (*Fusarium chlamydosporum* Wollenweber & Reinking) uses chitinases to degrade urediniospore germ tubes, urediniospore walls and also inhibits their germination (Mathivanan et al., 1998). Similar studies revealed chitinases from *L. lecanii* prohibiting conidial germination and mycelial growth of pathogenic fungi (Nguyen et al., 2015). The current study did not carry out an enzymatic analysis, which may suggest future studies to help further elucidate the mycoparasitic nature of *L. uredinophilum* as a combination of strategies.

Transmission scanning microscopy is endowed with the ability to resolve microbe-microbe interactions such as those of *L. uredinophilum* versus *P. pachyrhizi*, as well as microbe-plant interaction, *P. pachyrhizi* versus the soybean plant at the cellular level. Microbe-microbe interactions in biological control are essential in elucidating the mechanism of the infection process, which the TEM study corroborated well with the other microscopy work undertaken (SEM and CLSM). The TEM experiment investigated whether *L. uredinophilum* gained access into the urediniospores via direct urediniospores wall penetration, germ tube pores and evidence or lack thereof of degraded urediniospore outer membranes before colonisation and infection. The TEM also sought to examine subcellular membranes post-*L. uredinophilum* urediniospores hyperparasitism.

Several studies investigating mycoparasitism (including the current study) have shown quite striking similarities such as degradation of urediniospore germ pores, extensive hyphae growth inside the host fungi urediniospores, disintegration of urediniospore contents and loss of cellular integrity (Nana et al., 2023; Gauthier et al., 2014; Askary et al., 1997; Spencer and Atkey, 1981; Spencer, 1980). The SEM investigations revealed that the outer walls of urediniospores peeled off and spines were removed, which could be due to enzymatic activity or mechanical penetration by the *L. uredinophilum* hyperparasitism; the TEM did not reveal degradation or lack of urediniospore cell walls for most observed cells although some images

suggested partial digestion. Urediniospore exhibited cell wall breaks and multiple germ tube sites. In contrast, another TEM study Mendgen (1981) observed partial and dissolved urediniospore walls upon confrontation of stripe rust (*Puccinia striiformis*) with *V. lecanii*. Despite these deviations, the current research extensively elucidates the attack of *P. pachyrhizi* urediniospores by *L. uredinophilum*, which gains nutrition through this characteristic interaction and a convincing act of mycoparasitism (Nana et al., 2023; Gohel et al., 2022; Gauthier et al., 2014; Assante et al., 2004).

5.5 Conclusion

This current study demonstrated that *L. uredinophilum* employs mycoparasitism as one of its major strategies for effective infection and killing *P. pachyrhizi* urediniospores. Through high-end microscopy (CLSM, SEM and TEM) for treatments done for the CMA-filter-culture co-inoculations and on soybean rust-infected living host, a summary of at least four major events can be deduced through which *L. uredinophilum* acts as a hyperparasite and mycoparasite of *P. pachyrhizi*. The four events observed in the current study are (i) attachment of the hyperparasite *L. uredinophilum* to host fungi urediniospores (coiling of hyphae around urediniospores and spore attachment to urediniospores); (ii) mechanical force through direct penetration of urediniospore walls by *L. uredinophilum* or clear entry through degradation of germs pores (suggestive of enzymatic activity); (iii) Penetration and active growth of the hyperparasite *L. uredinophilum* hyphae inside urediniospores; and (iv) Digestion of urediniospore contents by *L. uredinophilum* leading to collapse and contortion of urediniospores and release of hyperparasite from dead urediniospores. Therefore, the isolation of *L. uredinophilum* from wild strawberry rust was not a coincidence. It strongly suggests, and has been investigated, that it can use rusts as a substrate for its nutrition through mycoparasitism, which gives rise to its potential use in the biological control of the ASR pathogen. Molecular fungus-fungus interaction could be studied to elucidate the understanding of the mechanism of action, including how genes influence the establishment of successful interactions. Application studies of *L. uredinophilum* could focus on the controlled environmental conditions required for effective control of the ASR pathogen and focus on producing formulations that can be used for field trial applications.

5.6 References

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Chapter 6: Evaluation of colonization of soybean rust urediniospores by three conidial concentrations of the mycoparasite *Lecanicillium uredinophilum*

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Abstract

Soybean leaves, already infected with Asian soybean rust (ASR), were inoculated with *Lecanicillium uredinophilum* and then evaluated for its mycoparasitic colonization of rust uredinia and urediniospores, in a greenhouse experiment under elevated relative humidity (RH) (80-90%). Four treatments were applied: the control treatment (untreated control) and three different concentrations of *Lecanicillium uredinophilum* (1.5×10^2, 1.5×10^4 and 1.5×10^6 conidia ml^{-1}). These caused various levels of mycoparasite colonization of ASR urediniospores, which were rated visually. Statistically significant differences were recorded between the treatments ($\rho < 0.05$) after 3-10 days post-inoculation (dpi). Inoculation with conidia of *L. uredinophilum* at 1.5×10^6 conidia ml^{-1} resulted in an extensive mat of mycelial growth covering almost 100% (98.01%) of the urediniospores that were exposed to the treatment. Application of *L. uredinophilum* conidia at 10^4 conidia ml^{-1} resulted in 44.91% urediniospores colonization, whilst at 10^2 conidia ml^{-1} treatment, 33.85% of the ASR urediniospores being colonized. All biocontrol treatments impacted significantly on ASR levels, assessed at 0 dpi and 10 dpi ($\rho < 0.05$), except for *L. uredinophilum* applied at 1.5×10^6 conidia ml^{-1}, which resulted in a non-significant difference ($\rho = 0.568$) with the Control. The rank-biserial showed a strong correlation between assessment time (0 dpi vs. 10 dpi) and ASR severity between the Control ($r = 0.97$), *L. uredinophilum* at 1.5×10^4 conidia ml^{-1} ($r = 0.94$) and *L. uredinophilum* at 1.5×10^2 conidia ml^{-1} ($r = 0.83$). Treatment of *L. uredinophilum* at 1.5×10^6 conidia ml^{-1} ($r = 0.05$) did not suppress rust severity significantly, however, mycoparasite colonization effect was strong ($r = 1.00$). Further post hoc analysis using Tukey's HSD test contrasts revealed statistically significant differences among pairwise comparisons ($\rho < 0.0001$). The severity of ASR (comparing pre- and post-inoculation assessments on Day 0 and Day 10) were significantly different for all treatments ($\rho < 0.0001$), except for *L. uredinophilum* applied at 1.5×10^6 conidia ml^{-1} comparing rust levels at 0 dpi and 10 dpi, which was not significantly different ($\rho = 0.652$). Pairwise comparisons of all mycoparasite colonization of urediniospores

post-inoculation were statistically different ($\rho < 0.0001$). These results indicate that *L. uredinophilum* at 1.5 x 10^6 conidia ml^{-1} can achieve a 98% colonization of ASR urediniospores. The colonization of ASR urediniospores by *L. uredinophilum* as a mycoparasite is a promising curative strategy for ASR disease management.

Keywords: *Lecanicillium uredinophilum*; mycoparasite; colonization, urediniospores; elevated humidity, soybean rust

6.1 Introduction

Soybean (*Glycine max* (L.) Merr.) is an important crop worldwide that contributes significantly to protein consumption (69%), oilseed production (59%) and vegetable oil consumption (28%), coming second after palm seed oil (35%) http://soystats.com/ (Soystats, 2024). The Asian soybean rust (ASR), caused by *Phakopsora pachyrhizi* Syd. & P. Syd., is a major fungal disease that causes considerable yield losses globally. In Asia, losses of 10-100% have been recorded (Hossain et al., 2024), whilst in Africa, losses of 10-90% have also been reported (Sileshi and Gebeyehu, 2021). Losses to ASR in sub-Saharan Africa (SSA) are estimated at US$0.06 billion per annum (Sileshi and Gebeyehu, 2021). Since its first report in 2001, ASR has consistently occurred across all major soybean-producing areas in South Africa each season, though the severity of outbreaks has varied (A. Jarvie, personal communication, May 23, 2023).

The life cycle of *P. pachyrhizi* has implications for its management strategy (Murithi et al., 2021). Infections of soybean leaves by *P. pachyrhizi* occur after rust urediniospores land on soybean leaf surfaces. The urediniospores germinate and directly penetrate the soybean leaves when favourable conditions exist, such as a high relative humidity (RH) (75-100%), appropriate temperatures (21-24ºC), and the presence of moisture on the leaves for more than 6 hours, leading to maximum infection if free moisture persists for 10-12 hours (Beruski et al., 2019). Uredinia of ASR develop 5-8 days post-infection, and the first urediniospores can be released after 10 days post-infection, with sporulation continuing for up to 21 days (Goellner et al., 2010). A single inoculation can produce uredinia for up to 28 days, and the formation of secondary uredinia from the edges of the initial infection can extend the sporulation period to beyond 56 days. Moreover, a single infectious lesion at 20-29ºC can potentially maintain urediniospore production for up to 15 weeks, producing 2000 – 6600 urediniospores in its lifespan (Kassie et al., 2023). The life cycle of *P. pachyrhizi* exhibits a polycyclic disease function (sigmoid curve) such that if no control is applied, it quickly moves from a lag phase to an exponential phase (Kassie et al., 2023). Thus, the exponential production of

urediniospores often leads to exodemic infections and ultimately, regional ASR epidemics (Santiago-Pérez et al., 2022).

Globally control of ASR largely revolves around the use of systemic fungicides, such as tetraconazole, applying 3-5 sprays to control ASR and other soybean diseases. Fungicide applications need to be timed accurately relative to ASR incidence if they are to be effective (Beruski et al., 2020), as well as relative to the crop growth stages, which are represented by different phenological stages (Vx–V1, for example, is the first trifoliate, R1– onset of flowering, R8 – full maturity) (https://crops.extension.iastate.edu/soybean/production_growthstages.html). Even low levels of disease severity (0.05%) can impact on soybean yields (Scherm et al., 2009), and the first application of fungicides should be done when the ASR severity level is less than 1% (Kassie et al., 2023).

Mycoparasitism, as a potential biological control strategy, offers an alternative to chemical fungicides for the management of plant diseases. It provides a curative rather than a protectant approach because the plant pathogen must first be present on the host plant before the hyperparasite can establish itself on the pathogen, and then start reducing the reproductive success of the pathogen (Colmán et al., 2021). Mycoparasites are widespread in nature, and use other fungi as their food substrate for their nutrition, growth and development (Khonsanit et al., 2024; Prahl et al., 2023). One promising biocontrol agent is *Lecanicillium uredinophilum* Park et al., a versatile hyperparasitic fungus known for colonizing and parasitizing urediniospores of rust fungi (Luiz et al., 2024), and infecting insect pests (Manfrino et al., 2022). Isolates of *Lecanicillium* spp., as entomopathogenic fungi, have gained considerable attention in their use in the biological control of insect pests under controlled environments (Reddy, 2020). Some isolates such as *Lecanicillium muscarium* (Petch) Zare and Gams [syn. *Akanthomyces muscarius* (Petch), (Kepler et al., 2017)] have been developed into commercial formulations (Mycotal®) that are currently in use (Bohatá et al., 2024). Previous studies on related mycoparasites, such as *Simplicillium lanosoniveum* (Beyma) Zare and Gams, demonstrated the potential of targeting ASR uredinia under controlled conditions, and in field studies, leading to reduced urediniospore production, reduced germination of colonized urediniospores, and a reduction in disease severity in the crop (Gauthier et al., 2014; Ward et al., 2012b; Ward et al., 2011). *Lecanicillium* spp. is often found as a mycoparasite on the coffee rust pathogen, (*Hemileia vastatrix* Berk. & Broome) (Das et al., 2024; Romero and Castillo-Arévalo, 2023; Vandermeer et al., 2009).

This study aimed to evaluate the efficacy of *L. uredinophilum* in colonizing urediniospores of *P. pachyrhizi* under elevated relative humidity conditions (80-90%), a factor known to promote rust development, as well as the growth and development of mycoparasites. It was further hypothesized that increased humidity would enhance mycoparasite colonization of soybean urediniospores at various concentrations of *L. uredinophilum* conidial suspension concentrations, which would offer insights into optimizing the application of this biological control agent under field conditions. This study investigated the interaction between *L. uredinophilum* and *P. pachyrhizi* under controlled conditions to evaluate the potential of the mycoparasite as a primary biological control agent for suppressing ASR and preventing its progression to severe or epidemic levels. It was predicted that the mycoparasitism of ASR urediniospores by *L. uredinophilum* should interfere with the polycyclic disease progress of ASR by reducing the reproductive rate of ASR, directly reducing the inoculum available for exodemic infections. In particular, it was expected that ASR uredinia would be colonized, with the potential to reduce urediniospore release and viability resulting in a slowdown of disease progression. The combined effect would reduce the rate of progress of ASR epidemics ("r") and the duration of ASR epidemics ("t"), in the equation of $X_1 = X_0 e^{rt}$, which is the basic equation to predict plant disease progress (Van der Plank, 1963).

6.2 Materials and methods

6.2.1 Host plant preparation and ASR inoculum

Soybean seed (an unnamed variety that is known to be susceptible to ASR) was provided by Pannar Seed Company Pty (Ltd) (GPS Coordinates: 29º 03' 45" S, 30º 35' 33" E; Greytown, KwaZulu-Natal). A total of 68 pots (mouth diameter-19.5 cm, height-17.0 cm and base-13.5 cm) were each filled with 1.5 kg of a composted pine bark growth medium mixed with 7.5 g slow-release fertilizer (Osmocote Exact Mini 5-6M 15-3.9-9.1+1.2Mg+TE, Greenhouse Products (Pty) Ltd, South Africa) and watered in preparation for planting. Forty (40) pots were placed in the disease tunnel. Another set of 28 pots were placed in a separate glasshouse facility. Six (6) soybean seeds were planted in each pot, followed by thinning to two soybean seedlings 7 days after germination. Watering of the plants in the disease tunnel was done manually every 3 days, whilst the plants in the glasshouse facility were drip irrigated every 3 days for an hour. Composted pine bark has both poor nutrient and water-holding capacity, and the watering interval was sufficient to maintain healthy growth, and the slow-release fertilizer lasts up to 4 months. The glasshouse facility soybean plants were maintained disease-free. After reaching

the vegetative (V2-V3) stage, the disease tunnel pots were placed side-by-side with ASR-infected plants prepared from previous experiments to naturally infect them for the growth stages between vegetative growth to flowering.

6.2.2 Soybean rust inoculum preparation and urediniospores germination test

The ASR-infected plants in the tunnels were used to provide urediniospores inoculum. Using a sterile brush and weighing boat, urediniospores from the ASR-infected plants were lightly dusted into a small plastic dish. Germination of urediniospores was checked following the method by (JIRCAS, 2016) with slight modifications. The collected urediniospores were suspended in sterile water combined with 0.01% Break-Thru® (100 ml) [as a surfactant], and the conidial concentration was adjusted to 1.5 x 10^4 urediniospores ml^{-1} using an improved Neubauer-haemocytometer (Neubauer-improved, Superior Marienfeld, Germany) and the prepared suspension was stored at 4ºC until required. For the germination test, 5 microscope glass slides were prepared by spreading sterilized 1 ml of 0.5% water agar media on approximately 1 cm^2 area on the glass slides, and the media was allowed to set. After setting, 20 µl of the prepared urediniospores suspension was dispensed onto the 1cm^2 water agar on each glass slide. A sterile paper towel was soaked in sterile distilled water and spread in 5 sterilized 150 mm diameter glass petri dishes, followed by placing a 60 mm petri dishes at the centre of each 150 mm glass petri dish. The inoculated slides were pivoted (one per glass petri dish) on top of the 60 mm petri dishes, covered and incubated at 25±2ºC for 12 hours in the dark. Germinated urediniospores were counted using a stereomicroscope and counter. Germination was considered to have occurred if a germination tube emerged that was as long as the longest dimension of the urediniospores. Ungerminated urediniospores remained without any visible germ tube emergence and elongation. A total of 200 spores were counted for each glass slide, and the percentage germination was calculated. A one-sample t-test performed against a hypothesized mean germination of 80% was used to establish if germination was successful (Gauthier et al., 2014).

6.2.3 Detached leaf assay

Soybean plants were allowed to grow, and trifoliate leaves (V3 to V5) were detached using a method developed by Mo et al. (2007), with modifications. Whatman® 10 sterile filter paper was placed in twelve (12) 150 mm sterile glass petri dishes, and the trifoliate leaves were placed horizontally on each filter paper (one trifoliate per glass petri dish). Each leaf petiole was

pushed through a rectangular block of sterilized sponge The sponge was supported by cotton wool soaked in sterile, de-ionized water. The sponge was wetted using a laboratory wash bottle filled with sterile de-ionized water. Care was taken not to flood the petri dishes with water. The glass petri dishes were covered and placed in two germination chambers (9 in each germination chamber) with a 14-h light /10-h darkness cycle at a temperature of 25±2ºC and relative humidity (85-90%). The periodic addition of de-ionized sterile water to the sponge and cotton wool was done using a laboratory wash bottle. In about 18-21 days, roots had developed on all the trifoliate petioles. To inoculate the adaxial soybean trifoliate surfaces, the sponge with the developed trifoliate root network was turned upside down to orient the adaxial side of the soybean trifoliate leaves upward. The previously prepared urediniospores suspension was sprayed onto the soybean trifoliate adaxial surfaces (as well as abaxial leaf surfaces) using a 100 ml fine spray bottle and allowed to air dry. Control treatments (trifoliate leaves in 3 glass petri dishes) were finely sprayed with 0.01% Break-Thru® in water, without the addition of urediniospores. The soybean trifoliate leaves were lightly sprayed with sterile distilled water to achieve wetness without runoff and turned to orient the adaxial surfaces back to their previous position. The glass petri dishes were then covered with their lids and put in darkness for 24 h at 25±2ºC. They were then transferred back to the germination chambers, and the previously described environmental conditions were maintained. Rust development was checked with a hand magnifying lens (20X) from Day 7 post-inoculation.

6.2.4 Pathogenicity of *Lecanicillium uredinophilum* on frangipani rust and its re-isolation

Colonies of previously sub-cultured (up to 6 times) on potato dextrose agar (PDA) *L. uredinophilum* isolate PP2018-001 and cultures stored in McCartney bottles PDA slants were retrieved from storage. Batches of PDA were prepared by measuring 23.4g of PDA powder and quantitatively transferring it into 600 ml distilled water in 1 L Schott bottles, stirred, and sterilized in an autoclave at 121ºC for 15 minutes. The PDA media was allowed to cool (48-50ºC), before being poured into 90 mm Petri dishes and allowed to set overnight. An inoculation loop was used to aseptically transfer chunks of *L. uredinophilum* mycelium from the McCartney bottle-PDA slants onto the centre of the PDA petri dishes and incubated at 25±2ºC for 14 days. After 14 days, *L. uredinophilum* plates (80 plates) were washed with 500 ml 0.01% Break-thru® solution by scraping the colonies using a glass rod. The freshly washed *L. uredinophilum* conidia suspension was filtered through 3 layers of cheesecloth to remove PDA solid residues, as well as mycelia. The filtrate stock solution concentration of conidia was

adjusted to 1.5 x 10^6 conidia ml^{-1} using an improved Neubauer haemocytometer (Neubauer improved, Superior Marienfeld, Germany). The adjusted conidia suspension was stored at 4ºC till further use. Plastic lunch boxes (length-23 cm, width-16 cm, height-7.5 cm) were lined with wet filter paper to serve as humidity chambers. Using a 100 ml fine spray bottle, the *L. uredinophilum* conidia suspension (1.5 x 10^6 conidia ml^{-1}) was sprayed onto ten fresh leaves of frangipani (*Plumeria* sp. L.) infected with frangipani rust (*Coleosporium plumeriae* Pat.) till runoff and allowed to air-dry under airflow laminar bench in the laboratory. After drying, two sprayed frangipani leaves per chamber were placed inside five humidity chambers. The petioles were covered by wet cotton wool and lightly misted with 0.01% Break-Thru® solution to leaf wetness without runoff. The control leaves (rust-infected frangipani leaves) were sprayed with a sterile aqueous 0.01% Break-Thru® solution, and two leaves per chamber were also placed in two humidity chambers. The humidity chambers were initially incubated at 25±2ºC for 24 h in the dark and then with a 14-h light, 10-h dark cycle, relative humidity (90-95%) for 10 days. After 6-8 days, *L. uredinophilum* was re-isolated and plated onto fresh PDA plates. Microscopic observations were made to confirm the presence of characteristic *L. uredinophilum* mycelium and conidia. Fresh *L. uredinophilum* conidia suspensions were prepared, and the concentrations were adjusted as previously described, to three different concentration levels (1.5 x 10^2, 1.5 x 10^4 and 1.5 x 10^6 conidia ml^{-1}) and stored at 4ºC until required.

6.2.5 Training in disease severity rating of ASR

Training to better estimate ASR severity was done through an online training program (TraineR2) https://delponte.shinyapps.io/traineR2/ designed to train users in the assessment of disease, which is expressed as the per cent area infected, in realistic online images (leaves or fruit lesions). Differences in rating the same leaves by different people are the chief source of inaccuracy in estimating ASR disease severity (Bock et al., 2021). A standard visual severity scale for ASR (Franceschi et al., 2020) was adopted, and used in conjunction with the TraineR2 Shiny app to train the first author to rate accurately and consistently. Franceschi et al. (2020) developed an image-based visual ASR assessment scale that consists of 10 images estimating various levels of ASR severity (0.2%, 1.0%, 3.0%, 5.0%, 10.0%, 25.0%, 40.0%, 55.0%, 70.0%, 84.0%). The training was done using 10 independent training exercises on the TrainerR2 Shiny app for the 39 available ASR images. The program reveals the actual levels of ASR severity of each test leaf at the end of a training exercise. The 39 ASR infected leaves appear in random order in each training exercise. The TraineR2 Shiny app provided data analysis at the end of

each training exercise and an option to download the training data as an Excel file. The training data was downloaded and stored for reference.

6.2.6 Treatment of ASR infected leaves with *L. uredinophilum*

Twenty-four (24) pots out of the 40 pots previously placed in the disease tunnel to develop rust infections naturally were selected, based on the healthy status of the plants and the level of ASR development. The 24 selected pots were labelled and laid in a randomized complete block design (RCBD) of four blocks, with four treatment groups: *L. uredinophilum* at 1.5 x 10^2 (Lu_conc_n01), 1.5 x 10^4 (Lu_conc_n02), 1.5 x 10^6 (Lu_conc_n03) conidia ml^{-1} concentration levels, and a control. The control treatment consisted of 0.01% Break-Thru® aqueous solution. Six soybean trifoliate leaves per pot (three trifoliate leaves per plant) were marked using white, red, blue and black plastic ring labels for the treatment groups. Marked trifoliate soybean leaves per pot were visually rated on their adaxial surfaces for ASR severity using the (Franceschi et al., 2020) image-based scale for ASR severity assessment. An initial assessment for all the marked leaves in the 24 pots (432 leaves) was done and recorded as the pre-inoculation baseline of infection, i.e., Day 0. Inoculation with the *L. uredinophilum* conidial suspensions was done as a single spray using a 100 ml hand spray bottle that created a fine spray. All leaves were sprayed till runoff. The control treatment single spray was applied using the 0.01% Break-Thru® aqueous solution in a similar hand sprayer. A second ASR severity rating was done 10 days post-inoculation (10 dpi) with *L. uredinophilum*. Assessment of ASR severity for all treatments at 10 dpi was done on the adaxial leaf surfaces. For assessment of the levels of colonization of urediniospores and uredinia by the mycoparasite, 432 leaves with rust colonized by mycoparasite were detached for image processing to accurately measure the levels of colonisation of ASR. The RH and temperature measurements were acquired hourly through data loggers, which the University of KwaZulu-Natal (UKZN) Department of Agrometeorology set up in the tunnel. The measurements could be viewed from an online dashboard but were also stored online.

6.2.7 Effect of *L. uredinophilum* on ASR severity

Ten days after inoculation with *L. uredinophilum* conidial suspensions, the control leaves and the mycoparasite treatments were assessed for ASR severity, as previously described. To measure the levels of colonization of ASR by the mycoparasite, the 432 leaves with rust colonized by mycoparasite were detached from the plants and photographed (abaxial surfaces) with a contrasting background, using a still camera. The level of mycoparasite colonization

was evaluated using a plant image analysis package (pliman), which is a software routine in the R statistical package that was developed for disease severity analyses (leaf and seed analyses) (Olivoto et al., 2022; Olivoto, 2022). The pliman package uses palettes that distinguish between the image background, symptoms (leaf lesions or diseased portions) and healthy portions (unaffected by the disease). For the analysis of mycoparasite level of colonization, a modification was done with the palettes: the background remained unchanged, as the image background; the ASR symptoms and healthy leaf portions were developed as palettes that were labelled as "healthy portions"; and the "symptoms" palette was used to label areas of visible mycoparasite mycelia colonizing the ASR pustules and urediniospores. At 10 dpi, ASR severity was assessed on the adaxial surfaces, while the mycoparasite colonization was assessed on the abaxial leaf surfaces.

6.2.8 Statistical analyses

Data analyses were conducted in R programming (v4.4.1, R Core Team, 2021). Data for training to rate ASR severity was visualized through correlations (Lin's concordance correlation coefficient (Lin's CCC), which gives a clear relationship of accuracy in rating versus the actual ratings for the ASR sample leaves in TraineR2 Shiny app. Data for percentage germination was analysed through one-sample t-test against a hypothesized 80% germination percentage. Data on ASR severity and mycoparasite colonization of ASR urediniospores by *L. uredinophilum* under elevated relative humidity was first tested for normality using the Shapiro-Wilk test. Data were not normally distributed, and the Wilcoxon signed-rank test (non-parametric test) was used to analyse the repeated measures. Post-hoc analyses were done by the Wilcoxon signed-rank test. A generalized additive model (GAM) was employed for fitting both disease severity and mycoparasite colonization variables, using smoothing terms for the block and pot ID, and including factors for treatment and assessment time. Further post-hoc analyses were done using the Tukey's HSD test through pairwise comparisons, combined with the Benjamini-Hochberg correction to reveal significant differences between treatment groups regarding the interaction between treatment and assessment days. Further analysis included calculating the effect size using the rank-biserial correlation coefficient (r) to quantify the magnitude of change in assessments, taken at 0 dpi and 10 dpi. Additionally, Spearman's rank correlation coefficient was used to examine the relationship between disease severity and mycoparasite colonization.

6.3 Results

6.3.1 Urediniospores germination test

All the five glass slides had germination percentages falling into the acceptable range (79.0%, 83.0%, 81.0%, 85.0% and 84.0%), which were not statistically different ($t = 2.23$, $\rho = 0.09$) against a hypothesized value of 80%. Germination rates considered acceptable for inoculation are at least 75%, and therefore, the urediniospores suspensions were suitable for inoculations in detached leaf assays.

6.3.2 Detached leaf assay

The detached leaf assay inoculation with the urediniospore suspension three weeks post-inoculation showed that the soybean trifoliate leaves did not produce any rust symptoms. However, the trifoliate soybean leaves looked relatively healthy (Fig 6.1), and the roots continued developing. After a second inoculation, this experiment was discarded due to a faulty germination chamber with unstable light and temperature cycles, and intermittent load shedding without a power backup, a problem affecting South Africa at that time.

Fig 6.1 Soybean detached trifoliate setup (A) and rooted petiole of soybean trifoliate leaves (B).

6.3.3 Pathogenicity of *Lecanicillium uredinophilum* on frangipani rust and its re-isolation

The rust uredinia (pustules) on frangipani leaves are characteristically powdery and hypophyllous, yellow-orange or bright yellow, and very distinct on the leaves where they are formed (Fig 6.2A). The treatment with *L. uredinophilum* conidial suspension resulted in successful infection of the frangipani rust urediniospores through mycoparasitism (previously

demonstrated in Chapter 5). The colonized frangipani rust pustules were progressively covered by the distinct white mycelia of *L. uredinophilum* from about 3-5 dpi, and most pustules (80-90%) were entirely colonized by 10 dpi (Fig 6.2B). The colonized frangipani rust pustules were cleared off the leaf and the treated leaves only showed the presence of the *L. uredinophilum,* indicating that the rust pustules had been engulfed and consumed by the hyperparasite (Fig 6.2B). A sterile needle was used to pick off samples of the white mycelia and were point inoculated onto fresh PDA plates. Morphological characteristics of *L. uredinophilum* were confirmed in pure cultures of the fungus on PDA and in microscopic observations (Fig 6.2C and Fig 6.2D).

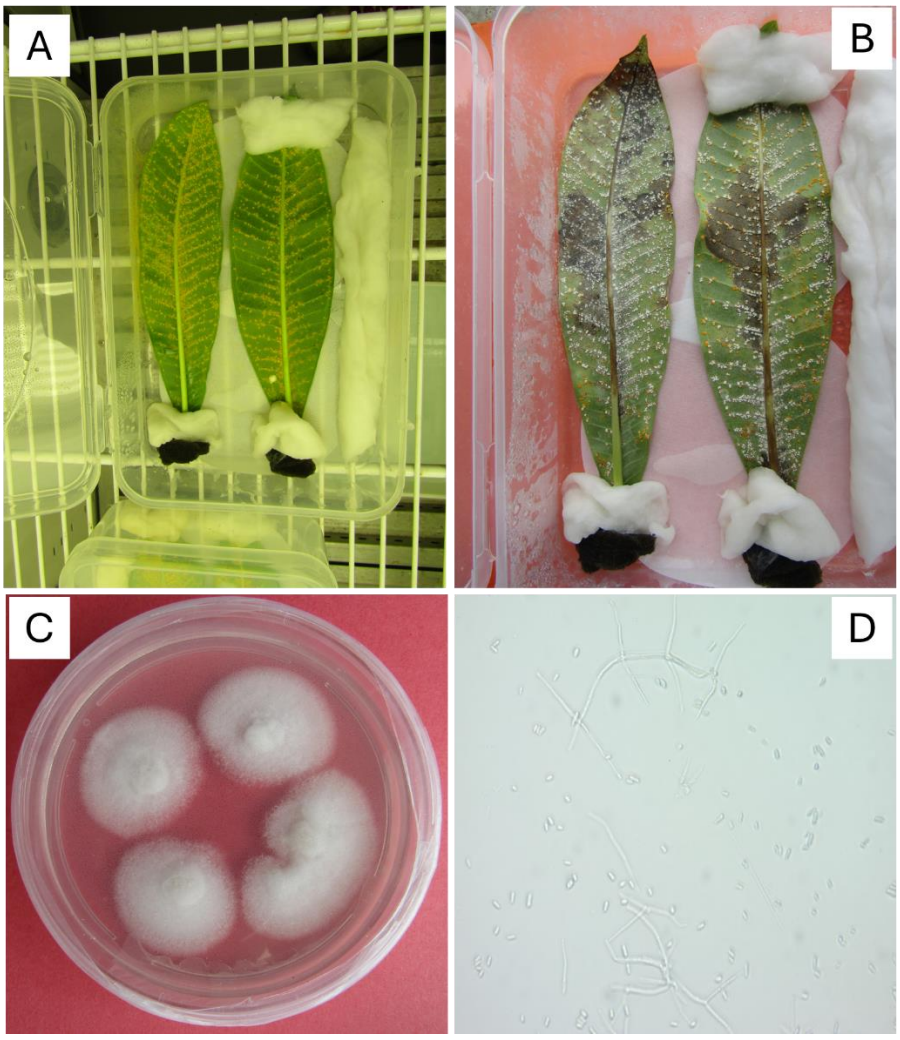

Fig 6.2 Leaves of Frangipani, infected with rust, and inoculated with *L. uredinophilum* (A); 6 dpi with *L. uredinophilum* (B); Re-isolated pure colonies of *L. uredinophilum* (C); light microscopy (x400) of *L. uredinophilum* conidia and mycelia from a re-isolated pure culture (D).

6.3.4 Training in disease severity rating of ASR

The training using the TrainerR2 Shiny app for visually estimating ASR severity consistently improved in all ten (10) trials. Trial 1 achieved a Lin's concordance correlation coefficient (CCC) of 0.80 and precision of 0.72, whilst, from Trials 5-10, the Lin's CCC and precision averaged 0.99 to 1.00, indicating a considerably higher level of accuracy. Higher concordance (Lins's CCC close to 1.00) denotes higher accuracy or agreement of the ratings to the known actual ratings. A precision of 1.00 is the highest value possible. Before estimating ASR levels in trials, refresher training was done using the TrainerR2 Shiny app, set for estimating ASR levels.

6.3.5 Treatment of ASR leaves with *L. uredinophilum*

Physical observations of the ASR control treatment leaves by unaided eye and a 20X hand lens did not reveal any *L. uredinophilum* infection of the ASR urediniospores. The ASR lesions appeared normal on the adaxial leaf surface, and the abaxial leaf surface showed typical pale-brown ASR pustules (Fig 6.3A, Fig 6.4L1, 6.4L2). Three days after inoculation (3 dpi) with *L. uredinophilum*, a rapidly developing white mycelium was visible on rust pustules through the 20X hand lens and with the unaided eye. At 10 dpi, the level of colonization of the ASR urediniospores by *L. uredinophilum* differed according to treatment concentrations, ranging from limited colonization (Fig 6.3B, *L. uredinophilum* at 1.5×10^2 conidia ml^{-1}), to moderate colonization (Fig 6.3C, *L. uredinophilum* at 1.5×10^4 conidia ml^{-1}) and to a high level of colonization (Fig 6.3D, *L. uredinophilum* at 1.5×10^6 conidia ml^{-1}). In contrast, at 10 dpi the soybean leaflets of the control treatment (Fig 6.4L adaxial leaf surface) showed ASR uredinia without any visible colonization by *L. uredinophilum*. The abaxial soybean leaf surface (Fig 6.4L2) revealed the lesions caused by the ASR pathogen. Where the mycoparasite was present, there was extensive colonization of urediniospores by *L. uredinophilum* (10 dpi) (Fig 6.4R1). On a soybean leaflet at stage R3 (an enlarged image of R1/R2) shows the abaxial leaf surface that were analyzed using the pliman program in R. The arrows labelled with an "X" show some areas of rust pustules on the leaf that *L. uredinophilum* had not colonized yet. The RH data were acquired hourly and averaged 84.1% for the 10-day period; however, episodes of low RH (34.1%) and highest RH (93.3%) were recorded (Fig 6.6). The hourly internal tunnel temperature (T) showed a similar trend to RH, averaging (22.8 ºC) and with lowest T (16.6 ºC) and highest T (33.2 ºC) for the duration of the 10 days (Fig 6.6).

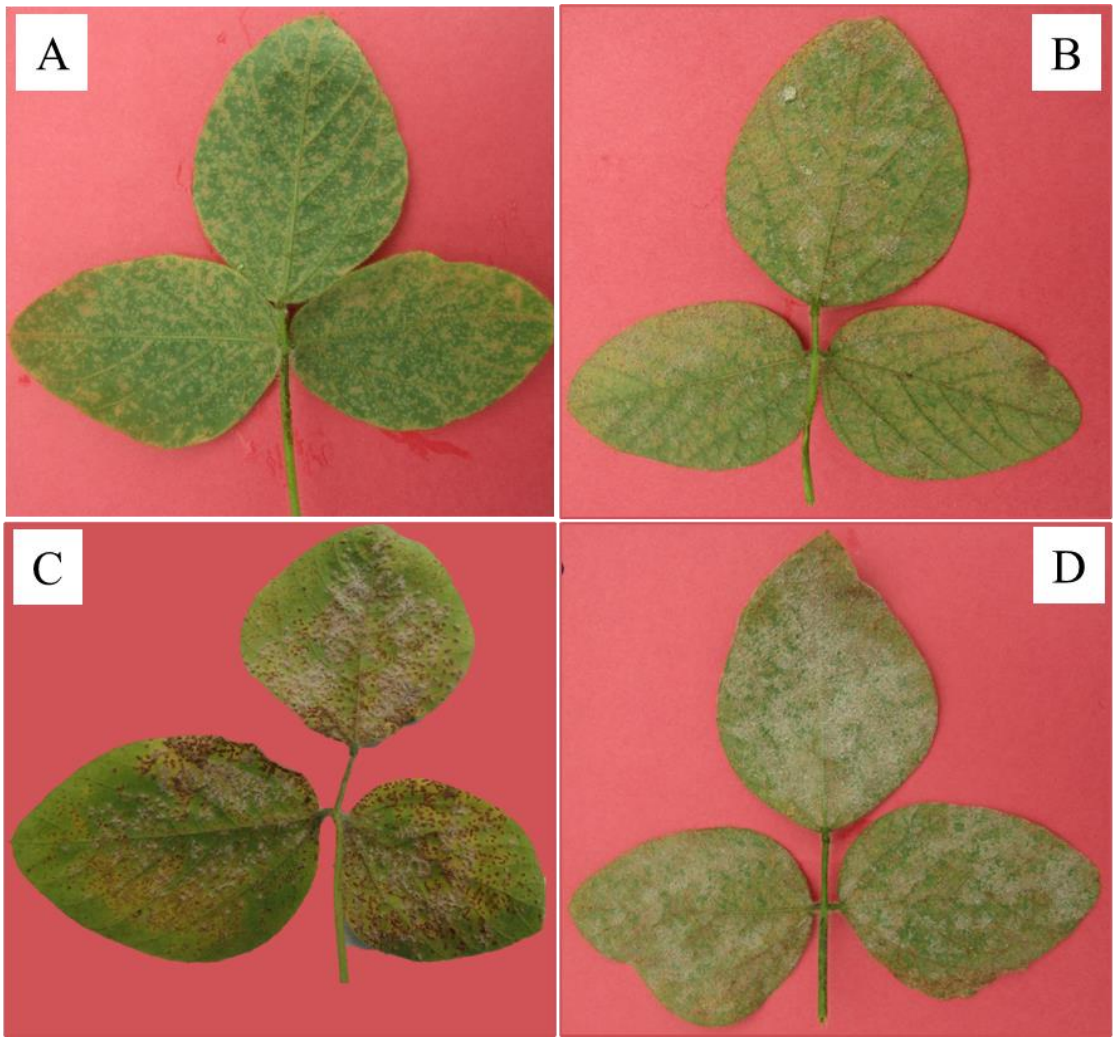

Fig 6.3 Representative soybean trifoliate leaves 10 dpi: control treatment, soybean leaves infected with *P. phakopsora* (A); *L. uredinophilum* treatments at 1.5 x 10^{2} conidia ml^{-1} (B), 1.5 x 10^{4} conidia ml^{-1} (C), and 1.5 x 10^{6} conidia ml^{-1} (D), reflecting various levels of mycoparasitism (see the white mycelium on the rust pustules).

6.3.6 Effect of *L. uredinophilum* on ASR severity

The Wilcoxon signed-rank test on disease severity and mycoparasite colonization data showed statistically significant differences in both the levels of disease severity, and the levels of mycoparasite colonization of the ASR urediniospores, comparing levels at 0 dpi with levels at 10 dpi. The post-hoc pairwise Wilcoxon test on ASR severity and mycoparasite colonization showed statistical differences within treatment comparisons (all $\rho < 0.05$). Meaningful comparisons (Wilcoxon rank sum test) were drawn from the interaction of treatment and assessment date, comparing the severity of ASR on the two assessment dates, 0 and 10 dpi. The mean disease severity ratings and the levels of mycoparasite colonization of ASR urediniospores are provided in **Table 6.1**. Soybean disease severity levels (assessed at 0 and

10 dpi) were all significantly different ($\rho < 0.05$), except for the treatment of *L. uredinophilum* at 1.5 x 10^6 conidia ml^{-1} treatment, which recorded a non-significant difference ($\rho = 0.568$). The mycoparasite colonization of the ASR urediniospores was also significantly different ($\rho < 0.05$) for all concentration levels of *L. uredinophilum* used in the study. The mycoparasite colonization of urediniospores can be visualized graphically as presented in Fig 6.5A (treatment effects with assessment date), whilst Fig 6.5B shows the mean ASR progression in the 10 days between 0 dpi to 10 dpi. The rank-biserial correlation measured the link between the severity of ASR and mycoparasite colonization of ASR urediniospores, at 0 dpi and 10 dpi. There was a strong correlation between assessment date (0 dpi versus 10 dpi) and ASR severity for the control (r = 0.97), *L. uredinophilum* at 1.5 x 10^4 conidia ml^{-1} (r = 0.94) and *L. uredinophilum* at 1.5 x 10^2 conidia ml^{-1} (r = 0.83). The treatment of *L. uredinophilum* at 1.5 x 10^6 conidia ml^{-1} (r = 0.05) suppressed an increase in disease severity as the disease levels at 0 and 10 dpi were almost equal. The Tukey's HSD test results revealed significant differences in the levels of ASR severity among various treatment groups and dates. After applying the Benjamini-Hochberg adjustment method to control for false discovery rate, several contrasts emerged as statistically significant ($p < 0.0001$). Notably, the severity of soybean disease was significantly lower in *L. uredinophilum* at 1.5 x 10^6 conidia ml^{-1} (at 10 dpi) compared to the control (at 10 dpi) (estimate = 8.2, SE = 1.5). Additionally, significant reductions in the severity of soybean disease were observed in all treatment groups at the 10 dpi assessment compared to the control on the initial assessment at 0 dpi (estimates ranging from 14.6 to 38.6). Furthermore, pairwise comparisons among treatment groups at 0 dpi showed significant differences, with *L. uredinophilum* at 1.5 x 10^6 conidia ml^{-1} (0 dpi) causing no statistical difference in the severity of ASR compared to other groups at the 10 dpi assessment. Fig 6.5B shows the progression of ASR severity and how each treatment affected the severity of ASR, and most of all *L. uredinophilum* applied at 1.5 x 10^6 conidia ml^{-1}, as seen in the 0 dpi and 10 dpi assessments. All treatment groups showed significant differences in the ASR severity at the 10 dpi compared to the control at 10 dpi ($p < 0.0001$). Application of *L. uredinophilum* at 1.5 x 10^6 conidia ml^{-1} resulted in the most extensive mycoparasite colonization of ASR urediniospores (98.01%) compared to the control at 10 dpi (estimate = - 47.6, SE = 0.8). Pairwise comparisons among treatment groups at 10 dpi revealed significant differences, with *L. uredinophilum* at 1.5 x 10^6 conidia ml^{-1} being more effective than *L. uredinophilum* at 1.5 x 10^4 conidia ml^{-1} (44.91%) and *L. uredinophilum* at 1.5 x 10^2 conidia ml^{-1} (33.85%) in the levels of ASR urediniospores that were colonized over the 10 days between the two assessment dates. Fig 6.5C shows the treatment mean severity of ASR for all treatments at 0 and 10 dpi, whilst

Fig 6.5D shows the proportion per treatment of soybean urediniospores colonized by *L. uredinophilum* and the proportions of non-colonized urediniospores at 10 dpi. The *L. uredinophilum* at 1.5 x 10^6 conidia ml^{-1} treatment was the most effective (98.01%) in colonizing ASR urediniospores.

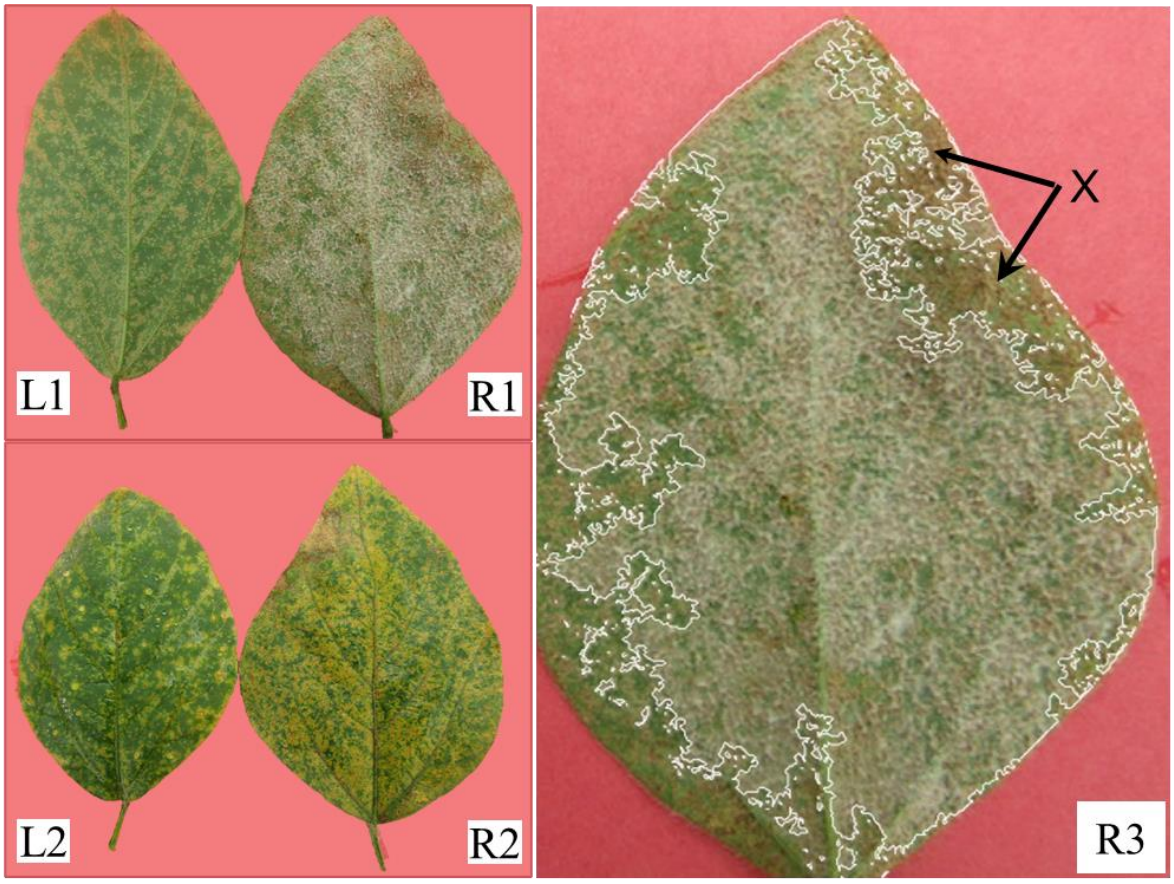

Fig 6.4 Representative soybean leaves surfaces 10 dpi: control treatment abaxial surface (L1), and adaxial surface (L2 at approx. 35% ASR severity); *L. uredinophilum* treatment at 1.5 x 10^6 conidia ml^{-1}, abaxial leaf surface (R1 showing *L. uredinophilum* mycoparasitism on soybean rust urediniospores adaxial leaf surface (R2 at approx. 55% ASR severity); Mycoparasite colonization percentage (60%) estimated through pliman package image analysis in R programming (R3), X shows the regions on the leaf where the urediniospores are not colonized.

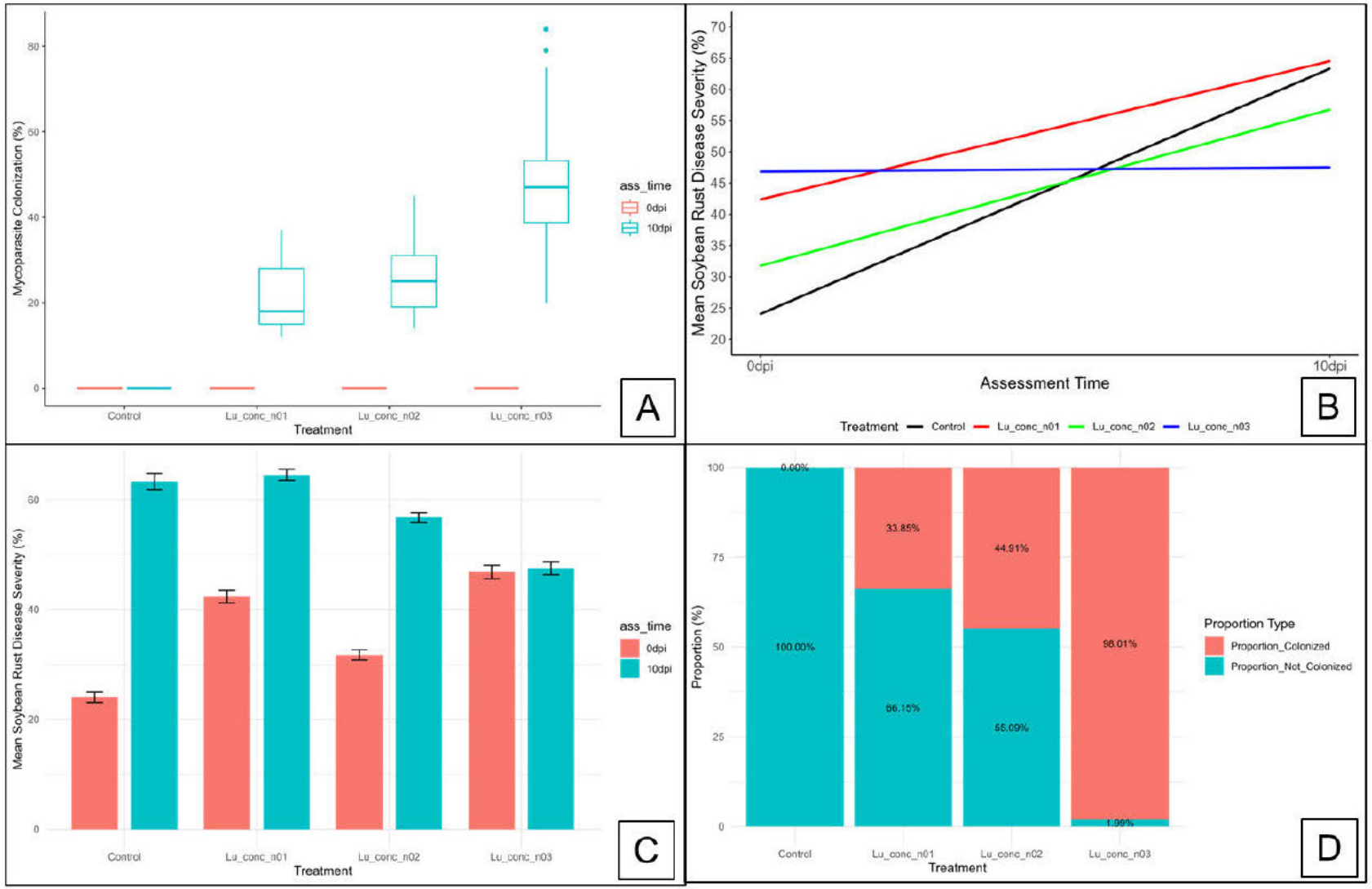

Fig 6.5 Assessments of levels of colonization of ASR pustules by the mycoparasite at 0 and 10 dpi with the mycoparasite (*L. uredinophilum*) (A); Mean ASR severity progression between assessment dates (B); Mean ASR severity on Day 0 and Day 10 of the mycoparasite treatment (C); Proportion of colonized urediniospores by *L. uredinophilum* and non-colonized urediniospores 10 dpi (D).

Table 6.1 Interaction of three inoculum levels of *L. uredinophilum* with soybean rust urediniospores and assessment times on disease severity and mycoparasite colonization proportions over a period of 10 days.

Treatment	Concentration Conidia.ml^{-1}	Assessment Time	Disease severity (%) mean ± SD	*P-adjusted (BH)	Treatment	Concentration Conidia.ml^{-1}	Assessment Time	Mycoparasite colonization proportion (%) mean ± SD	*P-adjusted (BH)
Control	-	Day 10	63.3 ± 15.3	0.000	Control	-	Day 10	0.0 ± 0.0	-
		Day 0	24.0 ± 9.8				Day 0	0.0 ± 0.0	
L. uredinophilum	1.5 x 10^2	Day 10	64.5 ± 10.5	0.000	*L. uredinophilum*	1.5 x 10^2	Day 10	21.8 ± 7.2	0.000
		Day 0	42.3 ± 11.8				Day 0	0.0 ± 0.0	
	1.5 x 10^4	Day 10	56.7 ± 9.1	0.000		1.5 x 10^4	Day 10	25.5 ± 7.7	0.000
		Day 0	31.7 ± 9.7				Day 0	0.0 ± 0.0	
	1.5 x 10^6	Day 10	47.5 ± 12.4	0.568		1.5 x 10^6	Day 10	46.6 ± 12.6	0.000
		Day 0	46.8 ± 12.3				Day 0	0.0 ± 0.0	

*P-value adjusted through the Benjamini-Hochberg procedure.

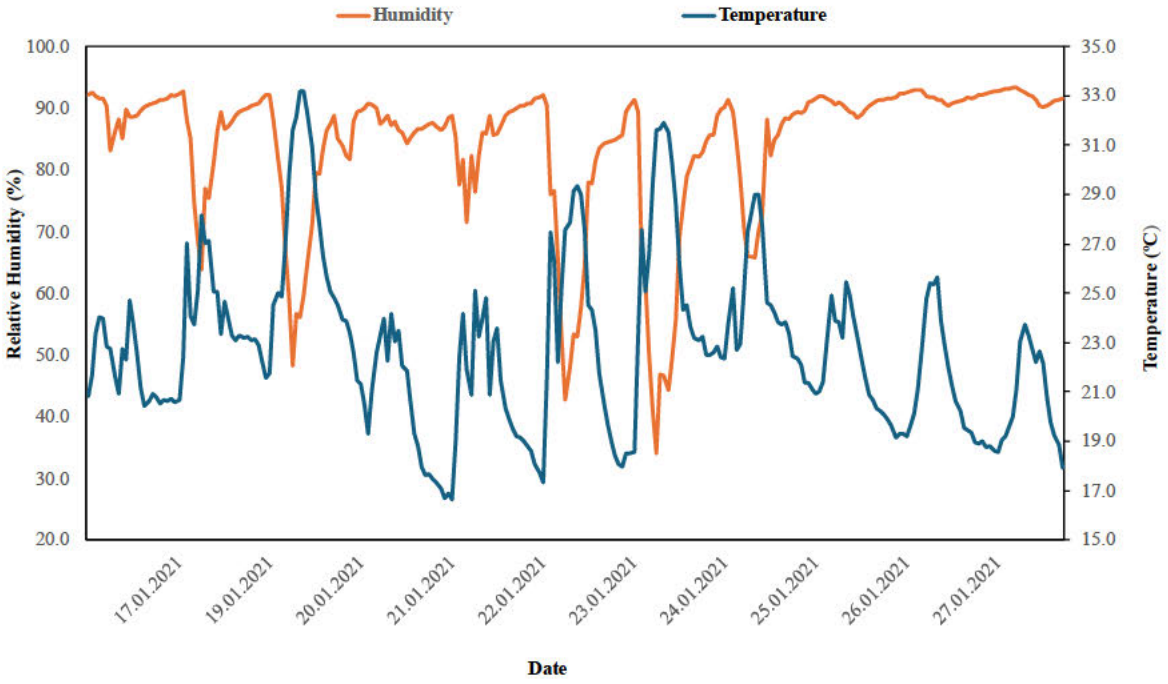

Fig 6.6 Relative humidity and temperature over the 10-day period of treatment of soybean rust with *L. uredinophilum* conidial suspensions. Climate data source: Savage, MJ. Agrometeorology Discipline, School of Agricultural, Earth, and Environmental Sciences, University of KwaZulu-Natal, Pietermaritzburg, South Africa

6.4 Discussion

Soybean rust commences with successful infection of soybean leaves by urediniospores, leading to visible symptoms characterized by continual production of more urediniospores and, consequently, more infections. This study revealed that the mycoparasite, *L. uredinophilum*, successfully colonized ASR urediniospores under elevated RH conditions. The study also showed the potential of *L. uredinophilum* as a biological control agent, particularly in reducing inoculum production. Colonized urediniospores cannot be dispersed, and colonized pustules lose the ability to release new urediniospores. The management of ASR epidemics requires a drastic reduction of urediniospore production by curative measure, such as the use of a mycoparasite. Fungicides currently used for ASR management are a combination of protectant and curative fungicides, and work effectively before the establishment of ASR infection, but also kill existing ASR pustules. The current study was able to show that a single spray of *L. uredinophilum* at the highest concentration in the study (1.5×10^6 conidia ml^{-1}) could result in the colonization of an estimated 98.01% of the observed ASR urediniospores. *L. uredinophilum* uses ASR urediniospores as a substrate for its growth and development, eliminating uredinia and killing the urediniospores in uredinia (Chapter 5). However, the disease severity assessed from the abaxial leaf surface was still recorded to be approximately the same on Day 0 and Day 10 post-inoculation (no statistical difference). This can be explained by the fact that the estimation of soybean disease severity is based on lesions on the adaxial leaf surface due to the biotrophic nature of *P. pachyrhizi*, even at the lowest detectable ASR severity. The lesions would subsist and worsen over time if no treatment is provided, eventually leading to accelerated leaf abscission due to infection load (Whitham et al., 2016). The soybean crop's loss of photosynthetic tissue, coupled with pod abscission, leads to widespread yield loss (Yorinori, 2021). Leaf loss, though not quantified in this study, was observed for a few ASR control treatments, whereas plants treated with *L. uredinophilum* conidia suspensions did not lose their leaves despite the similarity in some ASR severity ratings. Future work could focus on yield components, measurement of the photosynthetic capacity of infected leaves, pre- and post-inoculation with *L. uredinophilum*, and the rate of defoliation of mycoparasite-treated leaves versus control leaves. These studies did not take place because of the limitations of the passive tunnel that was used for these studies, and the frequent occurrence of loading shedding of power by the national grid, which created quite capricious climatic conditions, as shown in the RH and temperature data. Therefore, more work has to be done under fully controlled environmental conditions to properly investigate the hypotheses that treatment of ASR with a

mycoparasite should delay leaf abscission compared to non-treated leaves. This has the potential to reduce the impact of ASR on soybean crop losses. This study was not able to directly quantify urediniospore reduction, mainly due to the mycelial mat covering the urediniospores and leaving only the non-parasitized urediniospores uncovered. A study by Zhang et al. (2022) was able to quantify the reduction of *Puccinia striiformis* f. sp. *Tritici* (*Pst*) Eriksson urediniospore production (uredinia per leaf area) as well as DNA biomass decrease when treated by the mycoparasite *Cladosporium cladosporioides* (Fresenius) de Vries. The same study showed a decline (up to 80% reduction) of urediniospore germination with the increase in days post-treatment of *P. striiformis* f.sp. *tritici* with *C. cladosporioides*. As with the current study, Zhang et al. (2022) provided evidence of mycoparasite mycelia covering the urediniospores, and they noted the change of colour of colonized urediniospores. In a similar study on the biological control of coffee rust (*Hemileia vastatrix*) by *Lecanicillium lecanii* Zare & Gams there was evidence of a significant reduction in the germination of rust urediniospores *in vitro* after all *L. lecanii* treatments, and treatments with culture filtrates of *L. lecanii*, and the application of conidial suspensions of *L. lecanii* (Das et al., 2024). The same study showed that *L. lecanii* reached a level of 63% of mycoparasitic colonization of urediniospores on one of the 11 coffee cultivars used in the experiment, and 51% mycoparasitic colonization of urediniospores on another coffee cultivar. Measurement of airborne *P. pachyrhizi* urediniospores could be helpful in quantifying urediniospores before treatment and post-treatment. The quantity of airborne urediniospores and their distribution have been shown to correlate with ASR incidence, the severity of ASR and levels of crop loss (Santiago-Pérez et al., 2022).

This study revealed that the mycoparasite *L. uredinophilum* requires the presence of ASR for the hyperparasite-host interactions to be established because on leaves where there was no ASR, no colonies of *L. uredinophilum* developed. This observation is similar to Ward et al. (2012a; 2011), who established that the host fungus *P. pachyrhizi* has to be present previously for successful hyperparasite-host interactions with *S. lanosoniveum*. Furthermore, their study also found a decrease in disease severity of ASR (decrease in *P. pachyrhizi* DNA amounts) when the ASR on soybean leaves was colonized by the mycoparasite. In the current study, application of conidia of *L. uredinophilum* suppressed disease progress of ASR by parasitizing uredinia and urediniospores of *P. pachyrhizi*. Si et al. (2022) evaluated the use of *S. lanosoniveum* against *Puccinia graminis* f. sp. *tritici* (*Pgt*), and found that the mycoparasite reducing the *Pgt* urediniospore germination, as well as suppressed of urediniospore production.

The lack of standardized methods for estimating mycoparasite colonization of rust urediniospores is a limitation in this field. The current study quantified the level of mycoparasitic colonization of ASR urediniospores using the pliman package in the R programming package, a well-developed package using image analysis to estimate disease severity (Olivoto et al., 2022). Despite the advancement in image analysis, the pliman R package estimation, just like the estimation of disease severity through visual scales, depends on distinctive palettes developed for the "background", "symptoms", and "healthy" leaf tissues (https://r4pde.net/). The current study needed an additional layer, which is the mycoparasite infecting the ASR uredinia and urediniospores. Therefore, additional image palettes, or alternative non-DNA estimation methods are needed. In their study, Das et al. (2024) employed a quantification of mycoparasite colonization of coffee rust urediniospores by using a proportional formula (total coffee rust infected leaves showing colonization of urediniospores by *L. lecanii* divided by total number of coffee rust infected leaves observed). However, this formula is very limited because it factors in the initial level of severity of coffee rust prior to biocontrol treatments. It only captures disease incidence, making it a challenge to quantify the effect of *L. lecanii* in reducing disease severity.

In this study, the estimated levels of mycoparasite colonization of uredinia and urediniospores differed statistically with the different concentrations of conidia applied, with the dose of *L. uredinophilum* at 1.5 x 10^6 conidia ml^{-1} provided an average of 98.01% colonization, whereas the dose of *L. uredinophilum* at 1.5 x 10^4 conidia ml^{-1} provided 44.91% colonization, and *L. uredinophilum* applied at 1.5 x 10^2 conidia ml^{-1} achieved 33.85% colonization. Misting nozzles provided the moisture required in the greenhouse providing an elevated average of 84.1% RH and a range of 34.1-93.3% over the study period. The lowest level of RH recorded in the study was influenced by electricity power cuts however, there was no net effect observed on the experiment. Some isolates of *Lecanicillium* spp. have demonstrated ability to grow under a wide range of RH. For example, a commercial formulation of *L. muscarium* operated across a wide range of humidity levels (55-90% RH) against the green peach aphid (Mohammed and Hatcher, 2016). Other fungi, such as *Aschersonia aleyrodis* (Webber), showed efficacy in the control of *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae), a sweet potato whitefly under suboptimal RH (≤45%) (Bohatá et al., 2024). Such elasticity and an ability to provide efficacy at a wide range of RH is a desirable trait for biocontrol agents under field conditions, which show a wide variability of daily climatic parameters. Reddy (2020) reviewed *Lecanicillium* spp. as a potential biocontrol agent of different insect pests, and temperature and RH were

identified as major factors affecting their success. Weather conditions are critical to development of ASR epidemics (Hossain et al., 2024). In the field, the infection processes and the reproduction of *P. pachyrhizi* are affected by a number of weather variables (temperature, RH, leaf wetness, leaf wetness duration, solar radiance, wind) (Beruski et al., 2019). Soybean plants on commercial farms in South Africa are exposed to moisture through rainfall, dew, mist and irrigation. Soybean rust infections occur in the presence of moisture for a period of greater than 6 h, combined with RH levels of 75-100% (Nunkumar et al., 2009). Biocontrol fungi, in general, and entomopathogenic fungi in particular, have a requirement for a high RH (93-97%) for successful host infection, and subsequent growth and development (Quesada-Moraga et al., 2024).

The requirement of the presence of ASR by mycoparasites such as *L. uredinophilum* to suppress *P. pachyrhizi* urediniospore production, therefore, presents a significant challenge as far as ASR epidemics are concerned and potential yield losses. A 1% production loss of soybean in Africa is estimated to be equivalent to an income loss of US$0.01 billion (0.02 MT) (Sileshi and Gebeyehu, 2021), yet aggregated annual losses in sub-Saharan Africa (SSA) are estimated to be around 6% (Savary et al., 2019). ASR typically causes crop losses of 1-5% in soybean crops in South Africa. However, if commercial farmers optimize their fungicide applications, they can reduce these losses substantially (A. Jarvie, personal communication, May 23, 2023). Biocontrol agents such as *L. uredinophilum* need to perform as well as or better than these registered fungicides that commercial farmers used for control. Large-scale field trials are required to determine whether mycoparasites such as *L. uredinophilum* can reduce soybean crop losses to ASR as efficiently as the currently used fungicides, and therefore, to replace these fungicides in the long term.

6.5 Conclusion

This study demonstrated that the mycoparasite *L. uredinophilum* applied at a dose of 1.5×10^6 conidia ml^{-1} can be used as a biological control agent to control ASR. This potential was demonstrated by the ability of *L. uredinophilum* to colonize 98% of ASR urediniospores, and to stop any increase in leaf area infected by ASR. Although the direct reduction of urediniospore production was not measured, stopping any increase in disease severity indicated that no new uredinia grew and no new urediniospores were produced. that the dose of *L. uredinophilum* conidial suspension applied was an important factor. The lower concentrations of *L. uredinophilum* at 1.5×10^2 and 1.5×10^4 conidia ml^{-1} were not as effective in initiating the colonization of ASR uredinia and urediniospore. Despite the demonstrated potential of

L. uredinophilum as a mycoparasite of ASR, more studies need to be done. For example, whilst it was valuable to have achieved such high levels of colonization, there remains the requirement to establish the lower limits that *L. uredinophilum* can achieve the highest possible ASR urediniospore colonization because field conditions would often be more variable than partially controlled or actively controlled environments. In contrast to fungicides used for ASR management, which take both a protective and curative approach, *L. uredinophilum* takes only a curative approach, and there remains a question on how the least present disease severity prior to full colonization of the urediniospores would affect the photosynthetic potential of the soybean plant and yield components. The study also employed freshly harvested *L. uredinophilum* spores, which is certainly possible for limited application. Extensive applications in the field would mainly require the development of reliable formulations. In addition, this study did not employ any use of fungicides; however, practically, the soybean crop is normally protected by a host of pesticides for insect pests and plant pathogens, which may synergistically or negatively interfere with the effectiveness of *L. uredinophilum.*

6.6 References

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Chapter 7: Machine learning-based prediction of leaf wetness duration: A framework for evaluating biocontrol deployment feasibility in soybean fields

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Abstract

Leaf wetness (LW) is critical for the growth, development, and spread of fungal phytopathogen and their hyperparasites. The effectiveness of fungal biocontrol agents in the field may be compromised due to climatic and microclimatic variables, especially LW. Leaf wetness is essential for both the growth and development of the Asian soybean rust (ASR) pathogen, *Phakopsora pachyrhizi,* and its biocontrol antagonist, *Lecanicillium uredinophilum*. To monitor the periods of LW and leaf wetness duration (LWD), this study used automatic field weather stations in two soybean-growing locations (Greytown and Cedara) in KwaZulu-Natal Province, South Africa. Machine learning (ML) algorithms were employed to predict the LWD of the soybean canopy. This would be useful for the future deployment of a mycoparasite such as *L. uredinophilum* against *P. pachyrhizi*. Six ML algorithms – Random Forest Model (RFM), CatBoost Model (CBM), Gradient Boosting Models (GBM), Extra Trees Models (ETM),

AdaBoost Model (ADM) and Decision Tree Models (DTM) were evaluated for their efficacy in predicting LWD. The ML algorithms employed eight explanatory variables (relative humidity, air temperature, dew temperature, wet bulb temperature, day_night, day_part, wind speed and wind direction). The response variable used was LW measurement in kilo-Ohms (kOhms), with LW identified by resistance values below 501 kOhms. Confusion matrices and F1 scores were used to assess the performance of the models, and the Shapley additive exPlanations (SHAP) values elucidated the importance of each of the variables. The six ML algorithms were employed to classify LWD data from three LW sensors (LWS1, LWS2, LWS3) deployed at three soybean canopy levels: 0.45m (lower), 0.60m (middle) and 0.90m (upper), to capture spatial variability at each of the two study sites. All six ML algorithms performed comparatively well on F1 score ratings (harmonic mean of precision and recall) while further precision and recall were explained by confusion matrices for the best-performing ML algorithm for each study site and LW sensor spatial position. At the Greytown site, the best-performing ML algorithm for LW sensors F1 scores was ADM: LWS1 (0.94), LWS2 (0.90) and LWS3 (0.89) on the test sets. At the Cedara site, the best-performing ML algorithms for LW sensors F1 scores were shared by multiple algorithms: LWS1 (0.94, equal for GBM, ETM, ADM), LWS2 (0.95, equal for RFM, CBM, GBM, ETM, ADM) and LWS3 (0.96, for CBM, ADM) for the test sets. The range of F1 scores for all the ML algorithms was 0.82 to 0.95 for the test sets, indicating that all six ML algorithms could be employed to predict LW, although the ADM showed the highest F1 scores with some LW sensors. The SHAP values identified relative humidity (RH) as the most influential explanatory variable, regardless of the ML algorithm, spatial position of LW sensors, and study site. Other explanatory variables changed ranks, depending on the ML algorithm and the spatial position of LW sensors. The six ML algorithms may be used to predict predicting LW and LWD. Despite the limited data collection period, the 15-minute data resolution provided sufficient data points to assess the performance metrics of the models for their portability. While LW predictions are mostly made for disease early warning systems, the same approach could provide useful information required for the deployment of biological control agents.

Keywords: Leaf wetness duration, biological control, mycoparasite, machine learning, algorithms, *Lecanicillium uredinophilum*

7.1 Introduction

Soybean [*Glycine max* (L.) Merr.] is a crop of global importance due to its high protein content (36-38%) (Soystats, 2024) and contribution to stock feed, amongst other various value-added products (Oviedo-Rondón et al., 2024). However, soybean is susceptible to a range of plant diseases (Henning et al., 2024). Leaf wetness (LW) and leaf wetness duration (LWD) are critical microclimate variables in plant pathology, as they are linked to plant disease development in the classical plant disease triangle (Agrios, 2024). For example, the Asian soybean rust (ASR) disease caused by the fungal pathogen *Phakopsora pachyrhizi* Syd. & P. Syd. requires at least 6-8 h of continuous LW for successful infection of soybean leaves, and longer LWD periods provide for even more favourable conditions (Kassie et al., 2023). Biological control agents (BCAs), such as mycoparasites, that are employed to combat plant diseases in both controlled environments and field conditions require LW conditions or high relative humidity at the plant leaf level, often performing best under elevated RH conditions (Quesada-Moraga et al., 2024). Conditions that promote plant disease development also tend to encourage the growth and development of mycoparasites. Leaf wetness in plant foliage is facilitated through various means, such as irrigation, precipitation, and dew formation, which provide free water to the leaf surfaces, crop canopy, or plant surfaces (Rowlandson et al., 2015). Rowlandson et al. (2015) assert that the acquisition of accurate LWD data is not only a priority but a necessity for the effective management of plant diseases, both chemically and through biological control strategies.

While the development of plant disease models began in the mid-1900s, there has been a growing technological advancement, including increasingly sophisticated sensor technology, for the collection of microclimatic variables, which has provided useful insights into disease forecasts (González-Domínguez et al., 2023). Numerous models based on LWD have been developed, and some are operational for enhanced disease management. The modelling of LWD estimates the risk of disease incidence, which is then categorized to provide a threshold that invokes the commencement of fungicide application. For example, the Strawberry Advisory System (SAS) is an LWD early warning system (EWS) model that is in operation in the USA, used by farmers for the effective management of the highly destructive fruit rot strawberry fungal diseases caused by *Colletotrichum acutatum* Simmonds and *Botrytis cinerea* Pers. (http://www.agroclimate.org/tools/sas/). Additionally, the progress of ASR epidemics has been modelled using a simple LWD model, which utilizes RH as its only explanatory variable, with critical limits on the duration of RH equal to or above being the 90% threshold (NHH90)

to trigger the application of fungicides (Beruski et al., 2019). de Oliveira Engers et al. (2024) successfully developed an LWD and temperature-based model, which, through critical timing of fungicide control, reduced the number of sprays required per season. While these LWD models were developed to enhance chemical control of ASR, this study proposes that such modelling could also be used to optimize the application of biocontrol agents of plant pathogens and pests. For example, as with chemical control, LWD modelling can optimize the timing of the deployment of biocontrol antagonists (Rowlandson et al., 2015). Other LWD-based models and the evaluation of their application (ability to be used as early warning systems), precision, and accuracy have been explored, including their combinations. For example, dew point depression (DPD), and classification and regression tree (CART) models provided more accurate LWD predictions than the NHH90 and Penman-Monteith (PM) models (Gama et al., 2022). Models to estimate LW and LWD can be divided into physical and empirical methods. The physical methods use meteorological variables such as energy balance, mass balance or other variables such as water evaporation, the interception of water vapour, cloud cover, and albedo to estimate LW and LWD (Solís and Rojas-Herrera, 2021). Empirical methods use data-driven strategies by estimating LWD using relationships established between commonly measured meteorological variables and actual LW measurement using sensors (Asadi and Tian, 2021). Many weather stations do not comprehensively measure all the variables required for the physical methods, rendering them expensive and inaccessible. Hence, empirical models are more useful in practice.

Machine learning (ML) in crop protection has gained traction and has found considerable functionality and utility in pest and disease prediction models (De Oliveira Aparecido et al., 2020; Ip et al., 2018). The emergence of advanced ML algorithms has provided a reliable approach for estimating LWD for disease management (Park et al., 2019). The advancement of ML algorithms allows for the handling of the highly dimensional and complex microclimate and meteorological variables with ease, while providing high levels of accuracy (Park et al., 2019). For example, Park et al. (2019) in their application of ML algorithms, used multiple explanatory variables (air temperature, wind speed, short wave radiation, time, latitude. longitude, RH) to predict LW and LWD. The ML algorithms can automatically learn the complex patterns and relationships among multitudes of microclimate and meteorological variables, and their impact on LW and LWD (Solís and Rojas-Herrera, 2021; Wang et al., 2019). The ML algorithms and their higher computational power in handling complex data, provide for faster, data-driven decision-making, informed by highly accurate predictions based

on LWD that far exceed the capabilities of traditional empirical models (Gama et al., 2022; Asadi and Tian, 2021).

Biological control studies that have attempted to use mycoparasites for the control of ASR typically conduct controlled environment studies before deploying the BCA in field studies (Ward et al., 2012). However, rarely do such studies establish the suitability of field conditions before deploying the mycoparasites for field studies. This study hypothesized that ML algorithms can learn from physically collected microclimate variables to predict LWD. In addition, it evaluated the variables or meteorological variables that promote LW and evaluated whether such conditions can support the deployment of a mycoparasite for use in the field. Moreover, predicting the LWD and associated variables helps reduce confounding variables often experienced in field studies and thus gives a better insight into the variables to focus on, should a deployed mycoparasite fail. Various field studies generally implicate environmental conditions such as RH and LWD as limiting the successful use of fungal-based BCAs in field pest management (Ayaz et al., 2023). This study further hypothesized that ML algorithms can offer robust predictive power for LW and LWD, and either affirmed or ruled out LWD as a limiting factor for mycoparasite establishment in field studies.

To test these hypotheses, six ML algorithms were employed to predict LW and LWD based on data generated by four automatic weather stations (AWS), at two sites, to capture various climatic variables associated with LW, as well as directly measuring LW through the deployment of LW sensors. The study sought to develop and validate ML algorithms for estimating LW and LWD in soybean growing sites. Furthermore, the study sought to identify the most important climatic variables influencing LW amongst the selected variables recorded by the AWS. Additionally, the study also aimed to evaluate the accuracy of the different ML algorithms employed for predicting LW and LWD. The selected ML algorithms are briefly reviewed below.

7.2 Machine learning classification background

Machine learning (ML) classification is a fundamental artificial intelligence (AI) technique that categorises data into classes (https://www.ibm.com/topics/machine-learning). The technical mathematical processes and documentation can be accessed through Scikit-learn (https://scikit-learn.org/stable/supervised_learning.html). It consists of supervised and unsupervised learning, which are distinct in their algorithm approach. On the one hand,

supervised learning builds predictive models from labelled data, while on the other hand, unsupervised learning identifies patterns in unlabelled data (Priya and Ramesh, 2020). The ML algorithms have diverse applications in plant pathology and precision agriculture (Jafar et al., 2024). Traditional models such as regression and random forests have been used extensively to predict LWD (Shin et al., 2020). Deep learning (https://www.ibm.com/topics/deep-learning) has been applied successfully in plant disease detection models (Shi et al., 2023). The classification models used in this study were selected as some of the commonly used high-performance algorithms known to solve complex problems, and for their ability to handle complex data such as agrometeorological data (Escobar and Morales-Menendez, 2024).

7.2.1 Random Forest Model (RFM)

The random forest classification model (RFM) is a versatile ML algorithm that combines multiple decision trees to create a robust and accurate classification model (Shin et al., 2021). The algorithm operates on the principle of generating multiple decision cutting points within the provided data (decision trees), each trained on a random set of the data, which are then used in parallel to make a prediction by majority vote or average (Solís and Rojas-Herrera, 2021). As a robust and popular model, the RFM has performed well in predicting LWD, a critical parameter for managing plant diseases in precision agriculture (Solís and Rojas-Herrera, 2021). Agrometeorological data is often highly heterogeneous, requiring applications such as the RFM that can handle diverse data types (Yao et al., 2023).

7.2.2 CatBoost (CBM)

CatBoost (CBM) is a robust gradient-boosting ML algorithm capable of high performance in diverse classification and regression applications (Krishnan et al., 2023). It is capable of handling complex data types (Castro-Valdecantos et al., 2024) and serves as a highly effective tool for predicting LWD (Arostegi et al., 2022) and other precision agriculture applications (Albattah et al., 2022). Additionally, CatBoost provides variable importance information, which can offer valuable insights into the key environmental drivers of LWD, informing the development of more targeted disease management strategies (Mazumder et al., 2023). As precision agriculture continues to evolve, the integration of advanced machine learning algorithms like CatBoost will be crucial in unlocking the full potential of data-driven decision-making (Escobar and Morales-Menendez, 2024).

7.2.3 Gradient Boosting Model (GBM)

The gradient boosting model (GBM) is an ML algorithm that iteratively improves a sequence of constructed weak learners (decision trees) to build a strong predictive model (Shoaib et al., 2023). Gradient boosting models excel at handling complex, non-linear relationships in diverse datasets, reducing overfitting and providing variable-importance insights (Jafar et al., 2024). The gradient boosting models can manage intricate, non-linear relationships in diverse dataset, which aid in reducing undesirable overfitting and enhancing variable insights (Jafar et al., 2024). In the context of plant disease management, gradient boosting algorithms have demonstrated promising performance in predicting parameters like LWD (Shin et al., 2020).

7.2.4 Extra Trees Models (ETM)

The extra trees classifier models (ETM) are ML algorithms that represent a variant of the Random Forest Model (RFM), employing higher randomization in the tree construction, which leads to improved performance in complex and non-linear data (Shahhosseini and Hu, 2021). The ETMs at application level have shown potential in predicting LWD for plant disease monitoring in plant protection for precision agriculture (Wang et al., 2019). These algorithms can capture intricate patterns by exploring a wider range of split points and thus ultimately provide for more robust and accurate predictions while handling heterogeneous agricultural data (Mesías-Ruiz et al., 2023). Variable importance insights from the ETMs can guide targeted disease management strategies focused on influential environmental factors (Shoaib et al., 2023).

7.2.5 Adaboost Model (ADM)

The Adaboost model (ADM) is a widely recognised machine learning (ML) algorithm that has been successfully applied in plant pathology and precision agriculture (Jafar et al., 2024). The ADM algorithm employs a strategy of iteratively training weak learners (such as decision trees), and then combines them to formulate a robust predictive model (Duarte-Carvajalino et al., 2021). Adaboost has demonstrated its effectiveness in predicting variables for leaf disease application (Yao et al., 2023). Its ability to focus on challenging instances and provide insights into variable importance make it a valuable tool for developing targeted disease management strategies (Hu et al., 2020).

7.2.6 Decision Tree Model (DTM)

Decision tree models (DTMs) are well-known ML algorithms that are widely used with a broad range of applications in plant disease detection models (Shoaib et al., 2023). The DTM algorithms operate on a principle of recursively partitioning the variable space into a tree-like structure, where internal nodes represent decisions based on variables, and the leaf nodes denote the final predictions (Costa and Pedreira, 2023). One key advantage of DTMs is their inherent interpretability, which allows for a better understanding of relationships between inputs and the target (Costa and Pedreira, 2023). In plant disease management, decision trees have predicted variables such as LWD which is crucial for monitoring and controlling disease spread (Wang et al., 2019). Furthermore, the DTMs provide valuable insights into variable importance, informing targeted disease management strategies focused on influential environmental factors (Jafar et al., 2024).

7.3 Materials and Methods

7.3.1 Study Site

The study involved two soybean growing sites for the collection of macroclimate and microclimate data. The first site was located in Greytown Pannar/Corteva Soybean Research station (GPS Coordinates: 29º 03' 45" S, 30º 35' 33" E, 1046 masl), 74 km from Pietermaritzburg central. The second site was a field near Cedara Agricultural Research Station (GPS Coordinates: 29º 32' 54" S, 30º 14' 46" E, 1113 masl), about 15 km from Pietermaritzburg central. Both sites were located in the KwaZulu-Natal Province, South Africa (Fig 7.1). The Cedara climate is generally warm summers (24-26ºC) and cool winters (2-5ºC), with annual precipitation of 840 – 1140mm (https://www.meteoblue.com/en/weather/historyclimate/climatemodelled/cedara_south-africa_1013840). The climate in Greytown is similar to that of Cedara, with slightly higher summer temperatures (25-27ºC), winter temperatures (4-8ºC) and annual precipitation ranging from 750 – 1080mm https://www.meteoblue.com/en/weather/historyclimate/climatemodelled/greytown_south-africa_1000006. The soybean season generally starts in late October to early March for the early crop, depending on the cultivar. Optimum planting time is between 20 October to 20 November in the KwaZulu-Natal province.

Fig 7.1 Site locations, Greytown (A) and Cedara (B), KwaZulu-Natal Province, South Africa.

7.3.2 Meteorological and microclimate data acquisition

Two automatic weather stations (AWS) were set up (Fig 7.2A) at each site, Greytown (from 9 March 2023 to 23 May 2023) and Cedara (18 March 2023 to 12 April 2023). The installation of the AWS was planned for the beginning of the soybean season; however, delays in instrument purchase curtailed the starting time, resulting in data collection for only a limited period. An AWS system **(Table 7.1)** box was attached to a 2.5 m pole to house all data loggers, batteries and any electronic gadgets required for system functionality. The resolution measurement for the AWS was set for 15 minutes, as well as hourly and daily data. The atmospheric RH, windspeed/wind direction, and air temperature (^{o}C) (average dew temperature, wet-bulb temperature, average air temperature) were acquired 2m above ground, measured every minute and logged, based on the AWS set resolution. A tipping bucket rain gauge with a sensitivity of 0.2 mm was installed 1.2 m above the ground, ensuring it was clear of any interference, and connected to the data logger system. Three LW sensors (237-L Campbell Scientific, USA) were positioned at 45^{o} and mounted at varying heights above ground near the soybean leaf canopy (0.45 m, 0.60 m, and 0.90 m) to capture spatial variability (Fig 7.2B). Leaf wetness data were logged every 15 minutes, with acquisitions made at 1-minute intervals. For accurate LW measurements, the 237-L LW sensors were calibrated (Gama et al., 2022), using soybean field conditions and wetness experienced in the soybean leaf canopy after 5 days of installation. Dielectric unit measurements in Ohms of less than 500 kOhms were considered as wetness on the leaf, while above this threshold, values were considered leaf dryness (Fig 7.2C1, 7.2C2). Each AWS recorded additional meteorological variables (not shown in Table 1), which were concurrently logged by dataloggers. The AWS systems were powered by a 12V 7AHr battery, charged by a 20W solar panel and 10A regulator

placed 1.5 m above ground, facing north. To view the acquisition of data in near real-time, a dashboard was created showing various graphics for the recorded data using an internet-based data information system for each study site. At the end of the study period, data was downloaded as DAT files, processed with Notepad, and saved as Microsoft Excel spreadsheets or comma-separated-values (CSV) files for further analysis. The AWS installed at the Cedara site acquired a total of 2304 data records for all the variables (19 March 2023 to 12 April 2024) while the Greytown site acquired 6693 data records (14 March 2023 to 23 May 2023) after calibration.

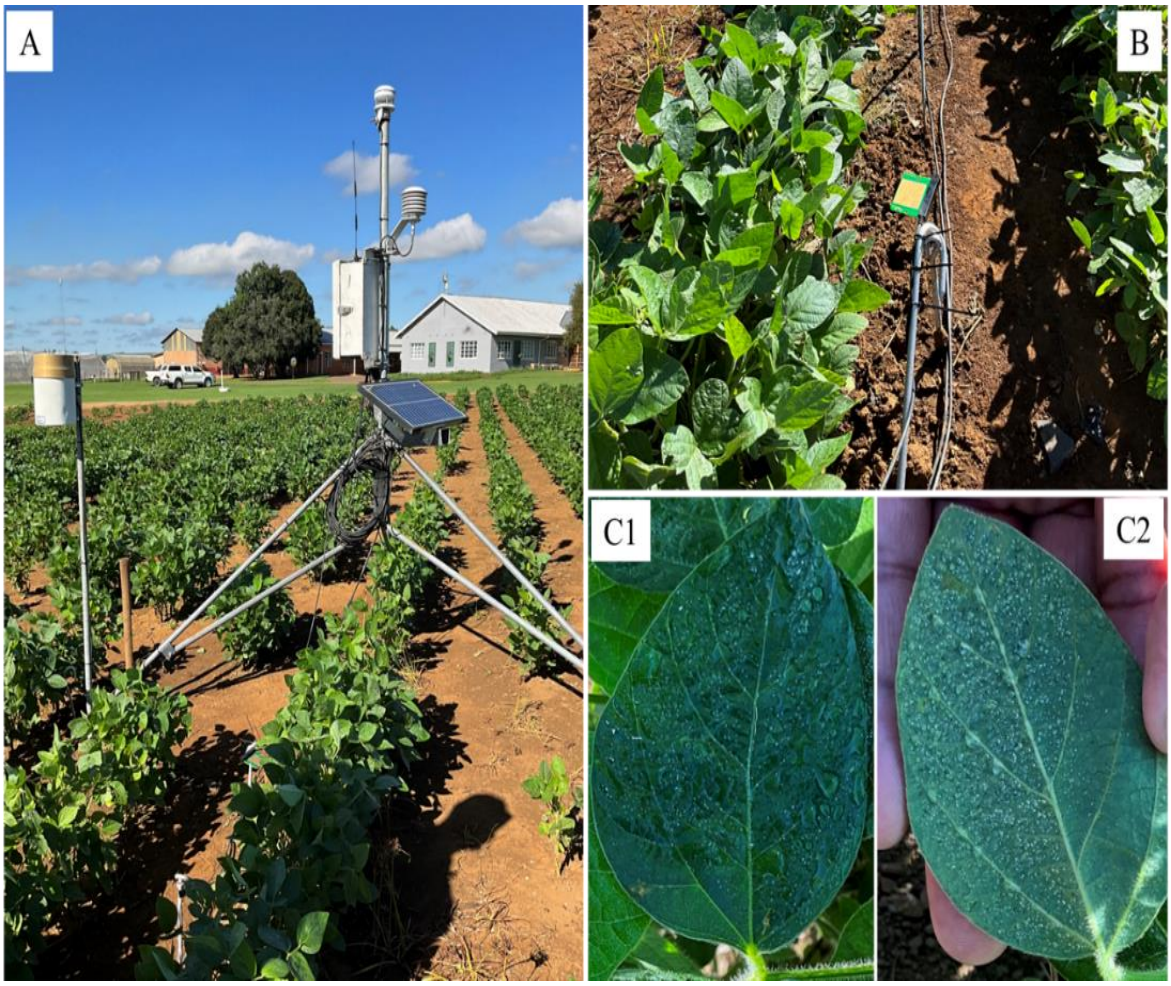

Fig 7.2 Automatic weather station (AWS) setup in Greytown site (A); Leaf wetness sensor setup showing a single sensor (B); Wetness due to dew on adaxial soybean leaf surface (C1) and wetness due to dew on abaxial leaf surface (C2), both taken during sensor field calibrations.

Table 7.1 Automatic field weather station measurements and relevant station system details

Station details/ variables	**Automatic Field Station details**
Sensor	Leaf Wetness Sensor (LWS) [(237-L[1]), LWS1, LWS2, LWS3]; RH and air temperature (CS215[2]) at 2m; Rain gauge[3] – rim at 1.2 m; Wind Speed and Direction ATMOS 22[4] at 2m;
Field dataloggers	CR1000 datalogger[5]
Field-to-base station	Datalogger-attached RF416[5] in turn connected to a panel antenna[5] in line-of-sight with the base station
Grounding	The datalogger was earthed using a lighting and grounding rods
Power	12V 7AHr battery charged by a 20W solar panel and 10A regulator
SW12 Timed Control Modem	The Datalogger was connect to a GSM modem and controlled by the datalogger to switch on and off at an hourly interval during the day from 6am until 6pm for data downloads.
Software	Base station software included Loggernet2 for data downloads. RTMC Pro version 3.0[5]was used to create a web-based display of data, graphics and alerts of daily, weekly and monthly LWD totals
timestamp	time data logger recorded data on the datalogger system, including resolution
air_tc_avg	average air temperature in degrees Celsius for the time resolution
td_c_avg	average dew temperature in degrees Celsius
tw_c_avg	wet-bulb temperature, Celsius, average
rh	atmospheric relative humidity as a percentage, measured at 2m above the ground
rain_mm_tot	rainfall in mm data for the resolution time
ws_ms_s_wvt	wind speed in metres per second
wind_dir_d1_wvt	wind direction in degrees
kOhms_1_avg	Leaf Wetness Sensor 237-L measurement (LWS1-placed at 0.45 m above ground), electrical resistance on the surface of the sensor (a wet surface is less resistant), threshold is 500 Ohms for wetness; measurement >501 Ohms mean dryness
kOhms_2_avg	Leaf Wetness Sensor 237-L measurement (LWS3-placed at 0.60 m above ground), electrical resistance on the surface of the sensor (a wet surface is less resistant), threshold is 500 Ohms for wetness; measurement >501 Ohms mean dryness
kOhms_3_avg	Leaf Wetness Sensor 237-L measurement (LWS2-placed at 0.9 m above ground), electrical resistance on the surface of the sensor (a wet surface is less resistant), threshold is 500 Ohms for wetness; measurement >501 Ohms mean dryness
lws_wet_tot_1	Leaf Wetness Sensor duration of wetness in the data logger resolution time set, that is, 15mins (LWS1)
lws_wet_tot_2	Leaf Wetness Sensor duration of wetness in the data logger resolution time set, that is, 15mins (LWS2)
lws_wet_tot_3	Leaf Wetness Sensor duration of wetness in the data logger resolution time set, that is, 15mins (LWS3)

[1] Campbell Scientific, Inc., Logan, Utah, USA – 237-L leaf Wetness Sensor
[2] Campbell Scientific, Inc., Logan, Utah, USA – Temperature & Relative Humidity Sensor
[3] Campbell Scientific, Inc., Logan, Utah, USA – TE525-L Raingauge (0.2 mm resolution)
[4] Campbell Scientific, Inc., Logan, Utah, USA – ATMOS 22 Ultrasonic Anemometer (0.01m/s resolution; 1º resolution)
[5] Campbell Scientific, Inc., Logan, Utah, USA – datalogger

7.3.3 Data analysis and prediction models through machine learning algorithms

7.3.3.1 Data handling and cleaning

Data cleaning is an essential step prior to the application of any ML algorithms or statistical analysis (Côté et al., 2024). Data cleaning in this study involved removing data recordings before field calibration of LW sensors. Data name variables such as the timestamp were converted into a date / time format and variable names for readability were adjusted for both data sets. The datasets used in this study included various climatic and environmental variables recorded at the 15-minute resolution, as described in **Table 7.1**. Variable engineering was used to create new variables such as day_night and day_part (define in Table 1) to capture the time of the day and its impact on LW (response variable). The data was checked for missing values. The Cedara site data had no missing values and was used with only data transformation to achieve the desired data format. On the other hand, the Greytown data had missing values for some LW resistance sensors (0.90 m aboveground sensor). However, less than 20% of data was missing. Imputation of missing values was therefore considered. All missing LW sensor data, by inspection of the other nearest sensor (0.60 m aboveground), had records above the wetness threshold of 500 kOhms. Hence data imputation for the missing values was assigned as the highest possible (10, 000 Ohms). The rainfall variable was dropped (rainfall was less than the 0.25 mm threshold) for the Cedara site, and the Greytown dataset's rain gauge did not record any precipitation due to a mechanical malfunction.

7.3.3.2 Exploratory data analyses and prediction models

Initial exploratory data analysis (EDA) was done through R programming Statistical Software (v.4.4.1 R Core Team, 2021). Further, EDA provided data visualizations and correlation analysis. Leaf wetness duration provided by total LW for every 15 minutes of data logged was statistically tested for normality using the Anderson-Darling test ($\rho < 0.05$). Thus, non-parametric tests were considered. Statistical analyses of LWD's effect on spatial position from the ground was conducted using Friedman's test and post hoc analysis done using the Wilcoxon signed-rank test with the Bonferroni correction. Traditional statistical model fitting (linear regression) was compared with four machine learning models (random forest, support vector machine, gradient boosting, and AdaBoost), which were evaluated through their root mean squared error (RMSE), mean absolute error (MAE) and R-squared (R^2) metrics. The RMSE measures the average magnitude of errors between predicted and actual values. Low RMSE values indicate better predictive accuracy. The MAE indicates the average absolute errors,

providing a straightforward measure of prediction error magnitude, and similarly, lower MAE values show more precise predictions. The R-squared represents the proportion of variance explained by the model, with values closer to 1 indicating better explanatory power. The EDA, therefore, together with the literature, provided a guide on the ML models to explore further. From the EDA, the random forest model (RFM), AdaBoost model (ADM) and gradient boosting model (GBM) were selected for further ML exploration. Three more ML algorithms were added (CatBoost, Extra decision trees and decision trees), while the support vector machine algorithm was excluded.

7.3.3.3 Model development through machine learning algorithms

This study employed six machine learning algorithms, namely, Random Forest (RFM), CatBoost (CBM), Gradient Boosting (GBM), Extra Trees (ETCM), AdaBoost (ADM), and Decision Tree (DTM) for the prediction of LW and LWD in soybean fields at two study sites. The explanatory variables included atmospheric relative humidity (rh), wind speed (ws_ms_s_wvt), wind direction (wind_dir_d1_wvt), day/night (day_night), day part (day_part), rainfall (Cedara site only), and temperature measurements: air (air_tc_avg), dew (td_c_avg), wet bulb (tw_c_avg). The RFM, CBM, GBM, ETCM, ADM and DTM models were created using Python's scikit-learn package (https://scikit-learn.org/stable/) (Gupta et al., 2024; Pedregosa et al., 2011). Dataset pre-processing involves additional checks for missing values, encoding categorical variables and normalizing variables. Both, Greytown and Cedara data were split into a training set (70%), testing set (15%) and validation set (15%). For the Greytown site data, each model underwent training on the prepared data, with hyperparameters tuning via grid and random searches to optimize performance. Each classifier's training involved specific hyperparameters tailored to its algorithm, such as maximum depth, number of estimators, learning rate, and base estimator adjustments. The ML models were evaluated through performance metrics (accuracy, precision, recall, F1-score, confusion matrix), which were assessed on the testing subset as a standard procedure (Jafar et al., 2024). The models were subsequently deployed for LW predictions on unseen data, a standard procedure in ML processes (Arostegi et al., 2022). The SHapley Additive exPlanations (SHAP) were also employed to elucidate on variable importance, and to analyse and interpret each model's predictive contributions (Antwarg et al., 2021). Determining the importance of each variableusing SHAP provided insights into the key variables influencing LW prediction.

7.4 Results

7.4.1 Exploratory data analyses and prediction models

Data visualization was part of the exploratory data analysis (EDA) and revealed the general trends in the multi-dimensional dataset for the study sites. The three LW sensors (LWS) revealed a dichotomy between day and night data and amongst them due to their spatial positions. The spatial LW positions aboveground were LWS1 (0.45 m), LWS2 (0.60 m) and LWS3 (0.90 m). The highest readings of LW sensor resistance (> 501 kOhms) indicative of leaf dryness were recorded much during the day (06:00 – 17:59 h), as shown in Fig.4A (7-day period sample data for the Greytown site). Leaf wetness sensor readings (< 501 kOhms) were primarily recorded during the night-time period (18:00 – 05:59 h), as shown in Fig 7.1B. Variability of LW sensors was affected by the time of the day as well as position of the sensor from the ground level (Cedara site data not shown). **Table 7.3** presents sample data (Greytown site) for a 28-day period for selected LW predictors and total LWD (LWD) per hour integrated into daily readings. Statistically, Friedman's test showed significant differences ($\rho < 0.05$) in the LWD for the entire study period due to the LW sensor position from the ground for both study sites. The LWD for the Greytown site were statistically different ($\rho < 0.05$) in pairwise comparisons by the Wilcoxon signed-rank test (Bonferroni adjustment). The Cedara site pairwise comparisons provided statistical differences ($\rho < 0.05$) for LWS1 vs. LWS3, LWS1 vs. LWS3, and LWS2 vs. LWS3 LWD but non-significant difference for LWS1 vs. LWS2 ($\rho > 0.05$). The mean wind speed remained stable for both day and night sample Greytown site readings (7-day period), while the mean air temperature was higher during the day than at night, as expected (Fig 7.4C, Fig 7.4D). The 7-day sample period (Greytown site) atmospheric RH varied considerably during the day with recorded values dropping below 50% (Fig 7.4C) while night readings remained relatively higher (75-100%) over the same period (Fig 7.4D).

The EDA for the LW predictive models: linear regression (LM), decision trees (DTM), support vector machine (SVM), random forest (RFM) and gradient boosting (GBM) were done for each sensor using explanatory variables discussed earlier. Among all the sensors, the RFM exhibited the highest predictive accuracy and explanatory power in both training and testing metrics (LWS1: RMSE = 2.24, MAE = 1.18, R^2 = 0.81; LWS2: RMSE = 2.38, MAE = 1.32, R^2 = 0.85; LWS3: RMSE = 2.58, MAE = 1.38, R^2 = 0.88), followed by the GBM (LWS1: RMSE = 2.77, MAE = 1.36, R^2 = 0.68; LWS2: RMSE = 2.72, MAE = 1.48, R^2 = 0.78; LWS3: RMSE = 2.89, MAE = 1.58, R^2 = 0.85) for the training test sets metrics. Figures 1, 2 and 3 shows the visualization of all model performances for LW sensors (LWS1, LWS2 and LWS3, Greytown site). The SVM, LM and DTM exhibited the lowest predictive accuracy

and explanatory power, as depicted in Fig 7.2. The Cedara site predictive LW models' performance metric is shown in **Table 7.2** for all the LW sensors (LWS1, LWS2 and LWS3), which showed similar trends as the Greytown study site. The top three performing models in EDA (RFM, GBM and DTM) were further analysed to enhance machine learning (ML) predictive performance. The LM and SVM were dropped, and in their place, the Adaboost model (ADM), Extra trees classifier model (ETM) and CatBoost model (CBM) were added and evaluated together with RFM, GBM and DTM in ML model development.

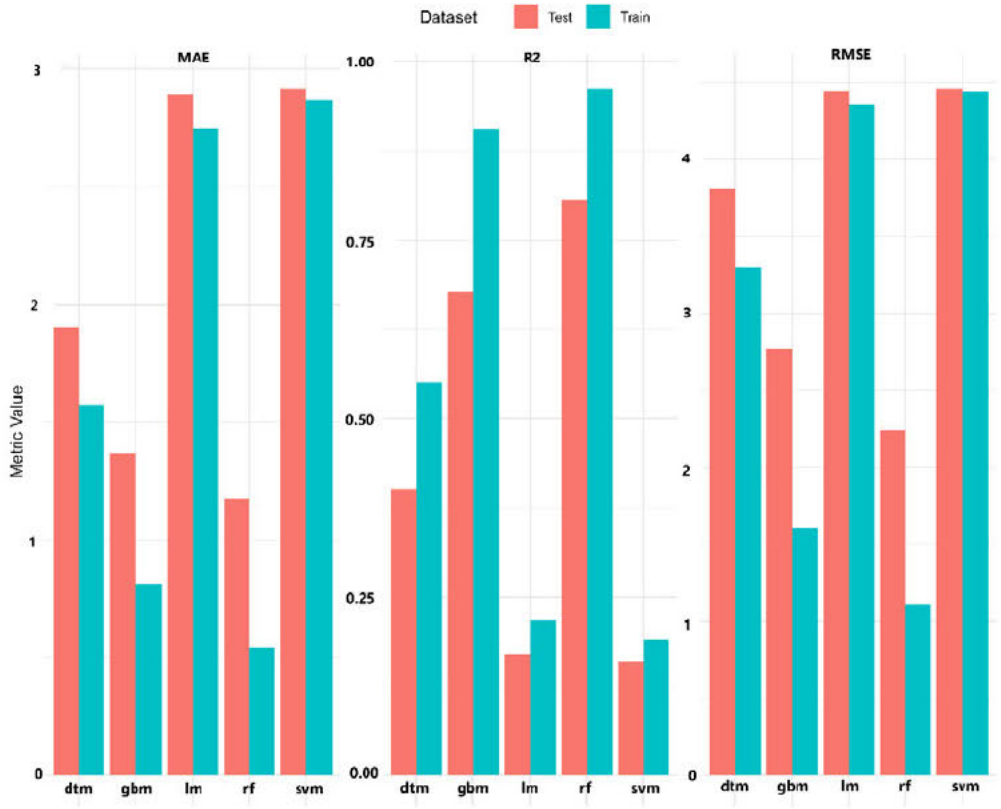

Fig 7.1 Performance metrics predictive models on leaf wetness sensor (LWS1), Greytown

Table 7.2 Leaf wetness predictive model performance metrics (Cedara study site)

	Sensor 1 (LWS1)						Sensor 2 (LWS2)						Sensor 3 (LWS3)					
	RMSE		MAE		R2		RMSE		MAE		R2		RMSE		MAE		R2	
model	Train	Test	Train	Test	Train	Test	Train	Test	Train	Test	Train	Test	Train	Test	Train	Test	Train	Test
dtm	1.81	2.68	0.49	0.74	0.73	0.26	1.93	2.63	0.55	0.72	0.82	0.53	2.40	2.97	0.88	1.08	0.68	0.40
gbm	0.34	2.12	0.16	0.58	0.99	0.48	0.39	1.56	0.17	0.52	0.99	0.84	0.56	2.81	0.25	1.04	0.99	0.49
lm	3.11	2.79	1.67	1.56	0.20	0.10	4.02	3.75	2.45	2.33	0.22	0.12	3.62	3.77	2.12	2.18	0.28	0.12
rf	0.82	1.84	0.27	0.54	0.96	0.57	0.81	1.60	0.29	0.57	0.97	0.83	1.11	2.22	0.45	0.89	0.95	0.64
svm	3.33	2.87	1.58	1.41	0.09	0.03	4.23	3.62	2.57	2.27	0.14	0.14	3.91	3.60	2.27	2.15	0.18	0.07

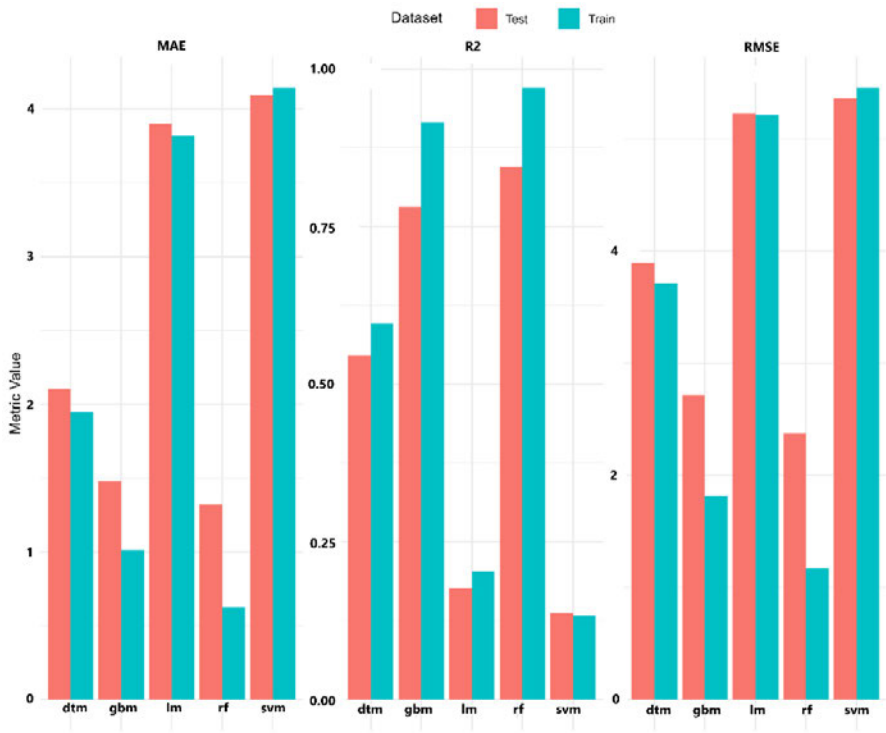

Fig 7.2 Performance metrics predictive models on leaf wetness sensor (LWS2), Greytown

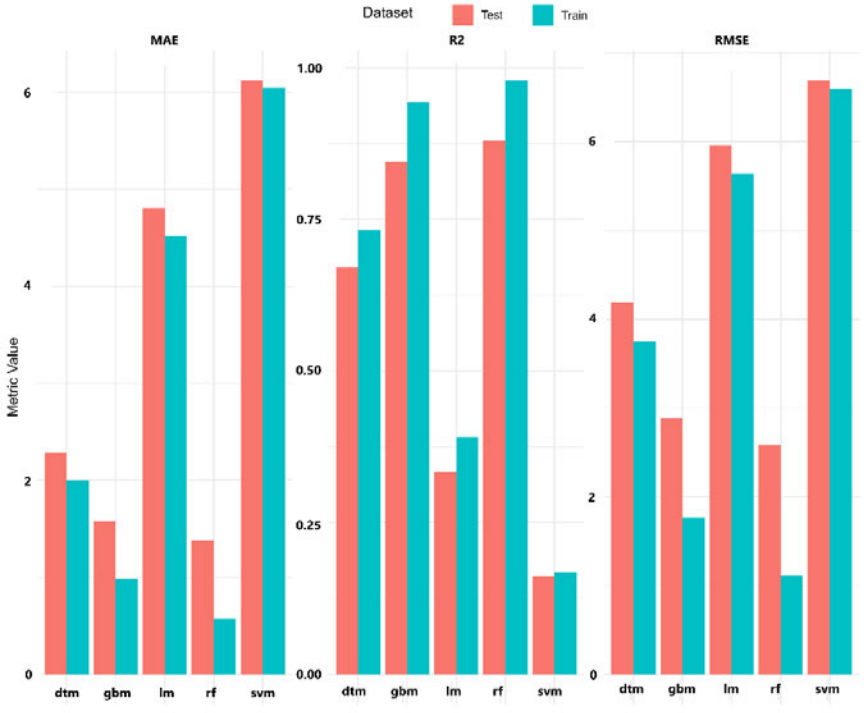

Fig 7.3 Performance metrics predictive models on leaf wetness sensor (LWS3), Greytown

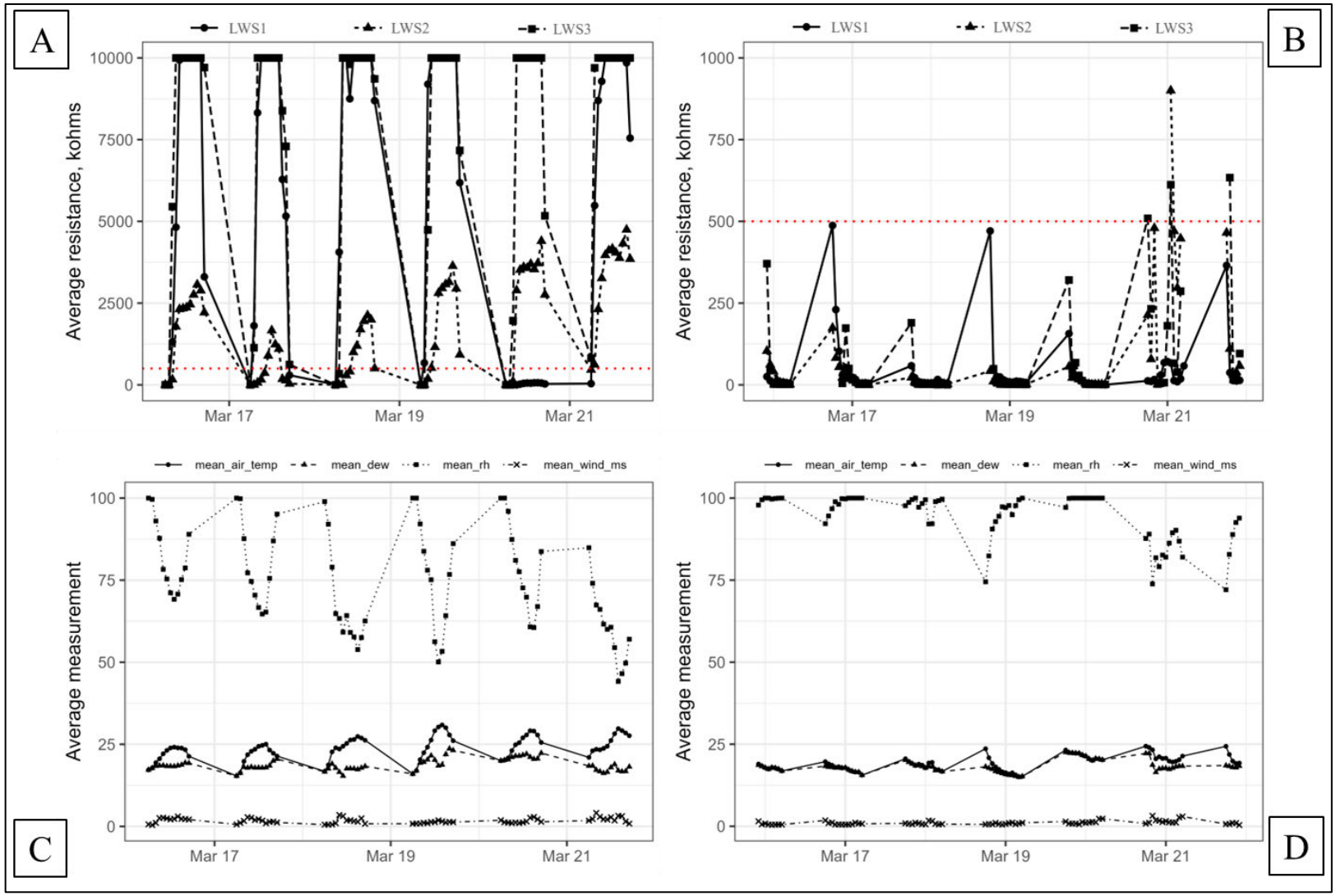

Fig 7.4 Sample Greytown site 7-day period (16-22 March 2023) climatic measurements: Leaf wetness sensor day readings showing much dryness during the day (A); Leaf wetness sensor night readings showing much wetness during the night (B). Red dotted line at 500 kOhms demarcates dryness (above the line) and wetness (below) the line. Mean air, dew temperature, mean atmospheric RH and mean wind speed during the day (C) and at night (D).

Table 7.3 Sample Greytown site leaf wetness duration (LWD, h) for a 28-day period (20 March –16 April 2023), for three leaf wetness sensor (LWS) units (showing LWD in 24 h), air temperature (T_{air}), atmospheric relative humidity (RH), wet bulb temperature (T_{wb}), dew temperature (T_{dew}), windspeed (W_s), wind direction (W_d), and daily total rainfall (mm). Asterisks (*) indicate days with rain.

Date/Day	LWD (h)											Rainfall† (mm)
	LWS1	LWS2	LWS3	Mean	SD	T_{air} (ºC)	RH (%)	T_{wb} (ºC)	T_{dew} (ºC)	W_s (m/s)	W_d (º)	
2023-03-20	15.0	7.7	7.8	10.1	4.2	23.2	85.4	21.2	20.4	1.6	187.5	0.2*
2023-03-21	7.7	4.1	5.1	5.6	1.8	23.1	73.8	19.4	17.8	1.8	197.0	0.0
2023-03-22	11.3	6.6	6.7	8.2	2.7	21.1	90.9	19.8	19.2	1.4	131.5	1.8*
2023-03-23	7.8	2.5	1.8	4.0	3.3	21.4	87.4	19.7	18.9	1.4	143.5	0.0
2023-03-24	7.3	10.6	4.1	7.3	3.3	20.3	86.6	18.5	17.7	1.4	130.0	0.0
2023-03-25	4.7	10.8	5.6	7.0	3.3	22.4	76.3	19.2	17.8	2.2	103.7	0.0
2023-03-26	7.5	13.5	7.5	9.5	3.5	18.8	86.9	17.2	16.4	1.6	212.6	7.6*
2023-03-27	4.8	1.2	4.3	3.4	1.9	14.2	84.9	12.6	11.6	2.1	164.0	0.2*
2023-03-28	1.4	1.0	1.9	1.4	0.4	18.1	78.3	15.4	14.0	1.7	147.8	0.0
2023-03-29	5.4	6.0	5.1	5.5	0.5	19.8	74.2	16.4	14.7	1.9	171.6	0.0
2023-03-30	10.8	10.6	7.2	9.5	2.1	14.4	95.4	13.9	13.7	1.1	174.7	2.4*
2023-03-31	9.5	5.6	7.9	7.7	2.0	16.2	93.6	15.4	15.1	1.2	114.6	0.0
2023-04-01	6.1	4.9	7.2	6.1	1.2	19.0	91.0	17.8	17.3	1.1	161.5	1.0*
2023-04-02	11.7	5.2	7.0	8.0	3.3	20.6	84.2	18.5	17.5	1.4	166.3	0.2*
2023-04-03	8.5	0.4	1.1	3.3	4.5	21.0	73.3	17.4	15.6	1.9	224.7	0.0
2023-04-04	6.9	5.7	5.1	5.9	0.9	16.7	84.5	14.8	13.7	1.9	165.3	2.0*
2023-04-05	5.9	5.7	6.8	6.2	0.6	16.8	84.8	14.9	13.8	1.1	180.4	0.0
2023-04-06	7.7	11.8	8.6	9.4	2.1	15.6	86.4	13.8	12.9	1.1	150.7	0.0
2023-04-07	7.2	9.8	8.4	8.4	1.3	18.6	80.1	15.6	14.2	1.0	180.8	0.0
2023-04-08	11.3	10.4	7.1	9.6	2.2	20.1	89.1	18.6	18.0	1.4	149.4	0.0
2023-04-09	8.5	7.4	7.4	7.8	0.6	21.3	81.8	18.9	17.8	1.7	183.4	3.6*
2023-04-10	6.4	4.2	9.7	6.8	2.8	17.4	90.2	16.2	15.6	1.5	164.0	0.0
2023-04-11	8.6	8.3	8.0	8.3	0.3	15.9	89.6	14.6	13.9	1.5	150.2	0.8*
2023-04-12	10.5	11.6	9.5	10.5	1.1	16.7	82.2	14.4	13.0	1.3	152.9	0.0
2023-04-13	8.5	9.2	8.1	8.6	0.6	18.8	77.9	15.5	13.7	1.1	214.4	0.0
2023-04-14	8.2	8.1	7.5	7.9	0.4	17.0	87.5	15.5	14.7	1.8	185.6	0.0
2023-04-15	9.4	8.1	3.0	6.8	3.4	14.3	73.5	11.2	9.2	1.1	108.6	0.0
2023-04-16	10.1	10.8	9.4	10.1	0.7	14.1	81.2	11.5	10.0	0.9	130.2	0.0
Total	228.8	202.0	178.7	203.1		516.9	2351.0	457.8	428.4	41.3	4546.8	19.8
Total*	142.4	132.1	114.8	129.8		345.8	1561.5	301.2	278.4	28.0	3013.8	

*Excludes days with rain

†**NB:** The daily rainfall data was obtained from an independent Pannar Seed Research weather station, some 50 m from this study AWS).

7.4.2 Model development through machine learning algorithms

7.4.2.1 ML algorithms F1-scores performance metrics

The LW and LWD were predicted through eight explanatory variables: RH, day_night, day_part, air temperature, dew temperature, wet bulb temperature, wind speed, and wind direction. The six ML algorithms employed in the study to predict LW for each LW sensor at its spatial position were all quite robust in their accuracy, obtaining high F1-scores (harmonic mean of precision and recall) values **(Table 7.4)**. The Adaboost (ADM) ML algorithm had the highest F1-score values: LWS1 (0.94), LWS2 (0.90) and LWS3 (0.89), which was also equivalent to the Gradient Boosting (GBM) ML algorithm for the Greytown site LW predictions. The F1-scores for the Cedara site were similar for the ML algorithms, with LWS1 (GBM, ETM, ADM, F1-score = 0.94 for all), LWS2 (RFM, CBM, GBM, ETM, ADM, F1-score = 0.95 for all), and LWS3 (CBM, ETM, F1-score = 0.96 for all). Table 4 shows both the training and test F1-score values, and from all the ML algorithm performances, there were neither over-fitting nor under-fitting models.

Table 7.4 F1-score performance metrics for the ML leaf wetness predictive models

Leaf Wetness Sensors, Greytown site						
	Sensor 1 (LWS1) F1-score		Sensor 2 (LWS2) F1-score		Sensor 1 (LWS3) F1-score	
model	Train	Test	Train	Test	Train	Test
Random Forest (RFM)	0.94	0.92	0.90	0.88	0.89	0.87
CatBoost (CBM)	0.93	0.92	0.88	0.88	0.85	0.85
Gradient Boosting (GBM)	0.94	0.93	0.90	0.89	0.89	0.89
Extra Trees (ETM)	0.93	0.92	0.89	0.88	0.87	0.87
AdaBoost (ADM)	1.00	0.94	1.00	0.90	1.00	0.89
Decision Trees (DTM)	0.93	0.91	0.87	0.84	0.86	0.82
Leaf Wetness Sensors, Cedara site						
	Sensor 1 (LWS1) F1-score		Sensor 2 (LWS2) F1-score		Sensor 1 (LWS3) F1-score	
model	Train	Test	Train	Test	Train	Test
Random Forest (RFM)	0.97	0.93	0.97	0.95	0.98	0.95
CatBoost (CBM)	0.96	0.93	0.95	0.95	0.96	0.96
Gradient Boosting (GBM)	0.96	0.94	0.97	0.95	0.96	0.95
Extra Trees (ETM)	0.96	0.94	0.95	0.95	0.96	0.96
AdaBoost (ADM)	1.00	0.94	1.00	0.95	1.00	0.95
Decision Trees (DTM)	0.97	0.93	0.96	0.93	0.97	0.94

7.4.2.2 ML algorithms confusion matrixes

The precision and recall of the six ML algorithms in predicting LW in the three LW sensors were well explained by the confusion matrices for the best performing model. The confusion

matrices are presented for LWS1, LWS2 and LWS3 at the Greytown site [Fig 7.5(a) – Fig 7.5(c)] and [Fig 7.5(d) – Fig 7.5(f)] for the Cedara site. The confusion matrices for the Greytown site demonstrated that the AdaBoost model for the three LW sensors had true positives of 0.91 (LWS1), 0.93 (LWS2) and 0.90 (LWS3). These true positive rates, expressed as percentages, indicate that 91% (LWS1), 93% (LWS2) and 90% (LWS3) of the time the AdaBoost model correctly predicted LW, when it was indeed present on the sensors. The levels of false negatives of 0.09 (LWS1), 0.065 (LWS2), and 0.096 (LWS3) show that 9.0% (LWS1), 6.5% (LWS2) and 9.6% (LWS3) times the model mistakenly predicted the absence of LW when there was LW. The false negatives adversely affect recall, which measures how many of the actual positives LW cases were correctly identified. More false negatives result in a lower recall, as they represent missed positive cases. The true negatives (0.90) mean that the ADM predicted no LW 90% of the time when there was no LW. The false positive (0.099), indicates that 9.9% of the time the ADM incorrectly predicted LW when there was no LW. False positives negatively impact precision, which measures how many of the predicted positives LW cases were correct. More false positives reduce precision, as they inflate the number of incorrect positive predictions. In summary, an F1-score of 0.94 **(Table 7.4)** indicates a good balance between precision and recall, implying the ADM model performed well overall in predicting both classes, with only a few false positives and false negatives. The Cedara site metrics can be explained, as was done for the Greytown site, following the selected best-performing models described in Section 7.4.2.1 above and **Table 7.4**.

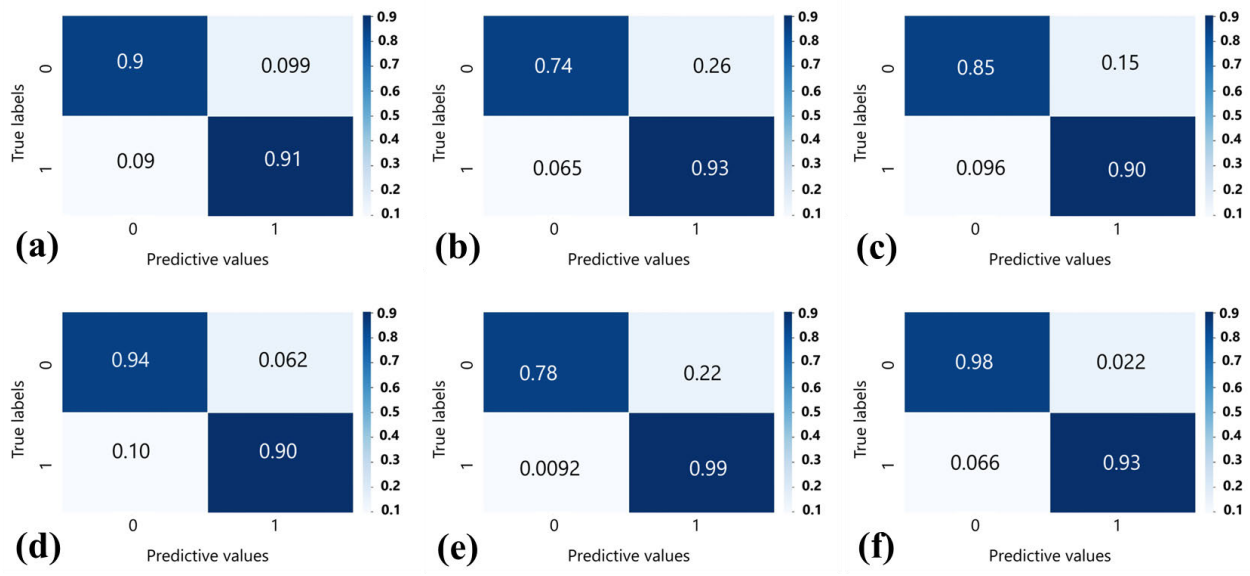

Fig 7.5 Best performing model confusion matrixes from which precision and recall performance metrics can be deduced; LWS1 (a), LWS2 (b) and LWS3 (c), Greytown site; and LWS1 (d), LWS2 (e) and LWS3 (f) for Cedara site.

7.4.2.3 ML algorithms variable importance

The six ML algorithms' Shapley additive exPlanations (SHAP) values elucidated the importance of explanatory variables for the best-performing models predicting LW on the three LW sensors across the two study sites. The impact of SHAP values on model impact showed some similarities and differences in variable importance (Fig 7.6)

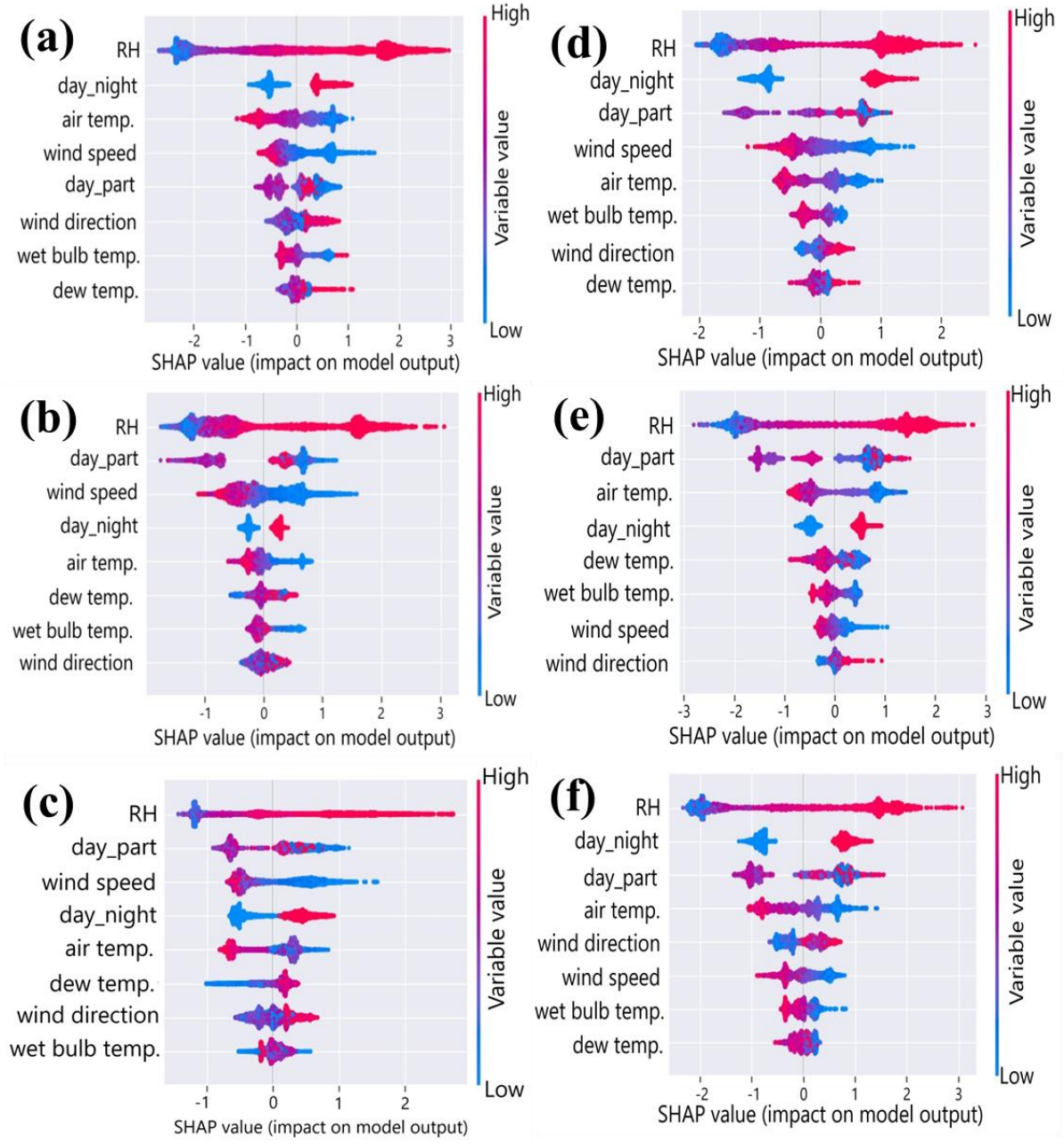

Fig 7.6 Best performing models showing the SHAP value impact on model output; LWS1 (a), LWS2 (b) and LWS3 (c), Greytown site; and LWS1 (d), LWS2 (e) and LWS3 (f) for Cedara site.

The most important variable in both study sites, and regardless of sensor spatial position, was the atmospheric relative humidity (RH) in each of the best performing ML model for each of the LW sensors. Higher values of RH (represented in red) tend to influence the prediction towards LW, while lower values (represented in blue) push the target value towards no LW (Fig 7.6). The importance of the other variables varied according to the best-performing model and the spatial position of the LW sensor. The general interpretation, regardless of the hierarchy of importance, corresponds to the SHAP value, reflecting the impact that each value had on the model. The day_night variable frequently ranked second, showing that it had a significant impact on the LW. Leaf wetness predictions varied considerably, depending on the whether it was day (blue) or night (red). Night generally pushed the prediction towards LW and day pushed the outcome towards no wetness. The air temperature variable showed a high to moderate importance, with higher temperatures (red) tending to push LW predictions towards no wetness, while lower temperatures (blue) were associated with LW. The day-part variable had high to moderate importance, with certain parts of the day (red) tending to increase the likelihood of LW, while the other parts of the day (blue), would tend to reduce the likelihood of predicting LW. Wind speed and direction displayed high variability, ranging from moderate to low importance; higher wind speed, depending on its direction (red), reduced the likelihood of LW, pushing prediction towards no LW. Lower wind speed (blue), depending on direction, and pushing predictions toward no LW. The dew temperature and wet bulb temperature had more moderate effects, and lower values (blue) tended to increase the likelihood of LW, while higher values (red), tended to push predictions towards no LW.

7.5 Discussion

The study, involving two AWS installed at two soybean growing sites, successfully recorded climatic and microclimate data at 15-minute resolution, which was used by ML algorithms to predict LW and LWD. The ML algorithms input variables included the average air temperature, average atmospheric RH, dew temperature, wet bulb temperature, wind speed and direction, day_part and day_night explanatory variables. The input variables were included due to previous studies that have successfully used them, and because ML algorithms have shown robustness in handling multi-dimensional data that include complex interactions between micrometeorological variables (Alsafadi et al., 2024; Gillespie et al., 2021). Exploratory data analysis (EDA) provided correlograms that showed interactions between the variables used (figures not shown). Moreover, ML algorithms are capable of handling patterns that do not

assume linear relationships. The EDA showed that the linear model tested had the high MAE, RMSE and lowest R^2 (Fig 7.1 – Fig 7.3), whereas whilst some of the ML models tested provided better performances (RFM, ADM, and GBM). Moreover, dew formation is an interplay of conditions prevailing at night, clear skies, with radiative cooling of plant surfaces dissipating the heat to the sky, lowering of air surface temperature to the dew point, which leads to condensation and dew formation (Kuwagata et al., 2024). Technically, dew formation is highly dependent upon and directly influenced by RH, air temperature, effective radiation, and exchange velocity (how quickly water vapour is transported between the leaf surface and the surrounding air) (Kuwagata et al., 2024). Previous studies by Sentelhas et al. (2008) demonstrated the suitability of RH as an important meteorological variable for estimating LW and LWD. The variable engineering in Python (https://scikit-learn.org/stable/) created the new variables (day_night, day_part) in the datasets to enable ML algorithms to read the patterns in the datasets. LW is a binary variable, and night and day conditions differ in their influence on LW and LWD, with sunlight reducing the duration of LW whereas night conditions allow leaves to remain wet for a longer period (Kuwagata et al., 2024; Merle et al., 2022; Asadi and Tian, 2021). The wind speed and direction affect LW and LWD on plant canopies. Llorens et al.(2014) observed rapid loss of LW when wind speed exceeded 1.5 ms^{-1} above the plant canopy. Machine learning models have extensively used various meteorological variables in estimating LW and LWD (Asadi and Tian, 2021). Atmospheric RH and air temperature have been frequently used as the primary meteorological variables in ML models (Park et al., 2019), whilst wind speed and solar radiation have been used in more complex models (Perondi et al., 2020). Leaf wetness and its duration are critical in the development of plant diseases due to the requirement of wet plant surfaces by most pathogens for successful infection of the leaves. While most LW and LWD models have been developed for disease warning systems (Gillespie et al., 2021; Dalla Marta et al., 2005), the same models could be used to enhance biological control strategies (Alsafadi et al., 2024; Rowlandson et al., 2015). Most biological control fungal antagonists require the same environmental conditions that promote plant pathogen growth and development (Patel, 2024).

The study successfully explored and evaluated six ML algorithms for predicting LW (LW) and LWD (LWD), with all algorithms yielding highly accurate predictions. Despite the limited dataset, the ML algorithms managed to predict LW and LWD. Whilst predictive models are typically developed through the use of extensive datasets (Gillespie et al., 2021), the current

study datasets can still be used to assess the performance metrics of the models for their portability (Park et al., 2019).

A study conducted by Asadi and Tian (2021) employed RFM, SVM, kernelized SVM, feedforward neural network (FNN) and classification and regression tree (CART) ML models. The best performing model, according to the performance metrics used (accuracy, MAE, RMSE), was RFM in estimating LW. In this study, RH was the most important variable in the all the ML models used, regardless of site and spatial LW sensor position (SHAP summary plots in Fig 7.6 only show figures for the best performing models). The top five explanatory variables used in the current study were RH, air temperature, windspeed, day_part and day_night. The rank of importance was dependent on LW position, the best model selected, as well as the LW sensor spatial position. The interpretation of SHAP values illustrated the contributions of the explanatory variables, irrespective of their ranking of importance. In their study, Asadi and Tian (2021) used sixteen (16) explanatory variables, and the RFM model emerged supreme, and the top four most important variables according to rank were potential evaporation, RH, dew point depression and net solar radiation. Air temperature was in the sixth position. The current study did not measure potential evaporation or net solar radiation, which were used in their study. Dew point depression could have been included as it is defined as the difference between air temperature and dew point temperature. Saturated vapour pressure (SVP) was measured in this current study but was not used. In their study, Asadi and Tain (2021), SVP was the 5th most important variable of the 16 variables used. Use of all the variables provided a better estimation of LW and LWD than using four or eight variables. Saturated internal vapor pressure can lead to condensation, affecting LW (Li et al., 2023). In another study, Gillespie et al. (2021) employed seven ML algorithms (SVM, Gaussian Naïve Bayes, DTM, RFM, K nearest neighbours, logistic regression, and multi-layer perceptron) to predict LW and LWD using datasets from 31 weather stations that were further sub-divided to four regional subsets. The explanatory variables were rainfall, RH, solar radiation and wind speed, and like the current study, RH emerged as the most important variable across all the ML algorithms. The multi-layer perceptron (MLP) model had the best performance metrics on the uncategorized dataset, whilst the RFM had better performance using regional subset data. In the current study rainfall data for the Cedara site was dropped because it did not reach the threshold for a rainy day (≤0.25mm). At the Greytown site, the rain gauge did not record any data. However, confirmation from another weather station at the same research centre indicated that it rained on several days. The resolution of the rainfall data (daily average) was too low to

determine the specific times it rained and the duration. A prediction of daily LWD employed three ML algorithms (RFM, support vector regression and MLP) and seventeen explanatory variables from a 5-year period, managed to provide high performance metrics for the RF model (Alsafadi et al., 2024). The weather variables RH, dew point depression (DPD), vapour pressure deficit (VPD), evapotranspiration (ETos) and latent heat flux (LE) were identified as the most influential variables for predicting LW. The relative importance of variables identified by most of the ML algorithms (Patel et al., 2022; Park et al., 2019) provides useful information for the timing of disease management activities. Biological control strategies require such critical information for the design of field experiments (Patel, 2024).

7.6 Conclusion

This study was able to show that several microclimate variables that do not normally fall under the World Meteorological Organisation (WMO) as standard weather station variables, are essential for the prediction of LW and LWD. The emergence of ML algorithms and their extensive applications allow for their use in such experiments as LW and LWD predictions, which may provide high levels of precision and accuracy. This study employed six ML algorithms, which all provided for high levels of accuracy and precision for the test sets, regardless of the ML algorithm and the study sites. The SHAP value impact on the model output provided an estimate of the relative importance of variables to LW and LWD predictions. The SHAP summary plots helped to visualize the importance of each variable, providing not only rank but the impact or influence of each of the explanatory variables. Whilst RH was the most influential explanatory variable, there is correlation of the other explanatory variables used, such that predicting LW and LWD with all the explanatory variables provided better precision and accuracy than using just one or a few. Leaf wetness and LWD are critical for both disease development as well as disease management. While most LW and LWD models are primarily developed as early warning systems for disease forecasting or optimizing fungicide application timing, their integration may also improve the effectiveness of biological control strategies, including the application of fungal BCAs. Soybean is prone to many pathogens as a crop, and facilitation in infection processes are aided by LW and LWD amongst other variables Future work could improve on the ML algorithm used by collecting far more weather data over a longer period, employing other ML algorithms, and the inclusion of more explanatory variables such as radiation, dew point depression and vapour pressure deficit.

7.7 References

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Chapter 8: General Conclusions and Recommendations

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8.1 General Overview

Soybean is an important crop in sub-Saharan Africa, with South Africa being the leading producer in Africa. By August 2024, South Africa had exported R191 million worth of soybean meal versus R86 million worth of imports (https://oec.world/en/profile/bilateral-product/soybean-meal/reporter/zaf). In the 2022/2023 season, South Africa harvested 2.75 million MT over an area of 1.148 million ha. Production is expected to grow by at least 50% in the next five years with the introduction of new germplasm and production technologies (https://sagrainmag.co.za/2024/05/06/investment-in-soybean-industry-creates-new-opportunities/). Despite these projections, soybean yield potential is threatened by pests and diseases, with Asian soybean rust (ASR) being the most important (Hossain et al., 2024; Hossain, 2024). Currently, the primary control strategy for ASR is chemical fungicides , their cost and their environmental impact are factors that have promoted a search for biological control measures (Hossain et al., 2024). The use of rust hyperparasites could provide a curative approach to ASR, reducing the current reliance on the three to four sprays of chemical fungicides that are typically required for ASR control (Twizeyimana et al., 2023). The broad objectives of this study were to seek fungal rust hyperparasites that could control the ASR pathogen, possibly leading to the commercialization of a new biocontrol product for the control of rusts. The study also sought to understand the mechanism of action of potential rust hyperparasites, with particular emphasis on the genus *Lecanicillium*. The research information gained from this study will help contribute to the body of knowledge on recent developments in the use of fungal rust hyperparasites as potential ASR biocontrol agents in South Africa. The major findings, their implications, and recommended research for future studies are summarized in this chapter.

8.2 General Conclusions

8.2.1 Morphological and molecular characterization of mycoparasitic fungi targeting *Phakopsora pachyrhizi* on South African soybeans

Many wild species of plants develop rust symptoms. In some cases, these rusts are attacked by hyperparasitic fungi. The initial steps towards finding biological control agents are by closely observing hyperparasitic fungal-fungal associations. Hyperparasitic associations were observed in wild sorrel (*Oxalis* spp.) and wild strawberry (*Fragaria vesca*) plants infected with rust. The hyperparasites were subsequently isolated and identified. Morphological characterisation suggested that a number of similar fungal species were present. Molecular characterization was conducted, using the internal transcribed spacer (ITS) regions, including ITS1 and ITS2, which are widely used for taxonomic and phylogenetic studies of fungi due to their variability and ease of amplification. However, in this study, the phylogenetic analysis using only the ITS regions resulted in taxonomic ambiguities, which were successfully resolved upon the application of multi-locus gene markers. Earlier studies by Zhou et al. (2020) successfully employed multi-locus gene marker analysis to resolve the identity of a number of *Lecanicillium* spp. The screened rust hyperparasites isolated from wild strawberry rust pustules were identified as *Lecanicillium uredinophilum*. This study provides the first report confirming that *L. uredinophilum* isolated from wild strawberry rust could be utilised as a potential biocontrol agent for the ASR pathogen.

8.2.2 Assessing the effects of ten commercial adjuvants and seven edible oils on the artificial growth of *Lecanicillium uredinophilum*, a mycoparasite of soybean rust pathogen (*Phakopsora pachyrhizi*)

Greenhouse or field applications of potential biological control agents (BCAs) require appropriate delivery systems to enhance their bio-efficacy. Formulations of biocontrol agents often employ adjuvants to enhance their efficacy. Two previously identified *Lecanicillium uredinophilum* isolates were subjected to bio-efficacy tests on ten South African commercial adjuvants and seven edible vegetable oils. Growth and development of *L. uredinophilum* was accessed through radial mycelial growth and counts of colony-forming units (CFU). The study revealed that all the commercial adjuvants had an adverse effect on both growth parameters at all of the six concentrations evaluated in the study on the two isolates (Appendix II–Appendix V). On the other hand, all the edible vegetable oils provided enhanced growth in both parameters. Of the 10 commercial adjuvants evaluated, Break-Thru® at 0.01% emerged as the

best adjuvant whilst at 0.05% shared similarities with some. The study indicated that, despite literature suggesting certain adjuvants positively support specific BCAs, generalizations regarding applications can lead to undesirable and unexpected outcomes. Different fungal BCAs may respond differently to commercial adjuvants' physico-chemical properties. Therefore, bio-efficacy studies on compatibility are imperative. Moreover, different adjuvants may show similar physico-chemical properties but produce different effects on BCAs. Similarly, different isolates of the same fungus used as a BCA may not be affected by adjuvants in the same way. Therefore, researchers are advised to carry out comprehensive bio-efficacy studies to preclude any deleterious effects arising from the use of available adjuvants.

8.2.3 Ultrastructural examination of the fungus-to-fungus interactions of *Lecanicillium uredinophilum* and *Phakopsora pachyrhizi*

The journey towards the registration of a potential BCA requires comprehensive details on the mechanism of action of the BCA (Adejumo and Voegele, 2021; Sparado and Droby, 2016). To elucidate the mechanism of action of *Lecanicillium uredinophilum* on *Phakopsora pachyrhizi*, three microscopy studies were conducted, including confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The CLSM, using a green fluorescent protein (GFP) transformant of *L. uredinophilum*, produced visualizations that showed the penetration and mycelial growth of *L. uredinophilum* inside *P. pachyrhizi* urediniospores. The SEM studies provided evidence of adhesion of *L. uredinophilum* conidia to the *P. pachyrhizi* urediniospores, with subsequent coiling of mycelia around urediniospores. Furthermore, damage to the outer membranes of urediniospores, the detachment of spikes, the loss of urediniospore integrity, and presence of perforations of urediniospores were documented. Using TEM, cellular level fungus-to-fungus interactions were visualized, which corroborated the CLSM and SEM studies through evidence of direct penetration of the urediniospores, degradation of germ pores and the development of a hyphal network inside the urediniospores. These studies confirmed that, the mechanism of action of *L. uredinophilum* on *P. pachyrhizi* is as a direct mycoparasite.

8.2.4 Evaluation of colonization of soybean rust urediniospores by three conidial concentrations of the mycoparasite *Lecanicillium uredinophilum*

Under conditions of high RH, conidial suspensions of *L. uredinophilum* were sprayed on soybean plant leaves exhibiting various levels of ASR severity. ASR severity and *L. uredinophilum* levels of mycelial colonization of the *P. pachyrhizi* urediniospores were

assessed on Day 0 and Day 10. All concentrations of *L. uredinophilum* varied in their level of colonization of soybean rust urediniospores, with the highest concentration achieving 98% colonization. Therefore, biocontrol agents using *L. uredinophilum* will require the highest possible concentration of conidia to achieve effective control. Commercial products often use high concentrations to ensure efficacy, for example, Mycotal® (a.i. *Lecanicillium muscarium*) contains 10^{10} viable conidia per gram; and for the control of peach aphids, a concentration of 1x 10^{8} conidia.ml^{-1} is recommended (Mohammed and Hatcher, 2017).

Controlled environmental facilities (CEF) studies can be used to track the impact of environmental parameters, such as relative humidity, and their effect on BCA's performance. Future studies could undertake infection studies under controlled environmental conditions to confirm the role of the environmental parameters on the efficacy of the BCA.

8.2.5 Machine learning-based prediction of leaf wetness duration: A framework for evaluating biocontrol deployment in soybean fields

Field trials of BCAs can be used to conduct pre-feasibility studies to collect data to optimize their successful deployment. Various models have been developed using leaf wetness (LW) and leaf wetness duration (LWD) to forecast the risk of disease development, or as early warning systems (EWS) for the development of diseases and pests (de Oliveira Engers et al., 2024). Fungal BCAs thrive under a fairly narrow range of environmental conditions, with a few exceptions, where some fungi have been shown to exhibit a wider range of environmental elasticity (Bohatá et al., 2024). This study collected meteorological data (using automatic weather stations) and microclimate data (LW and LWD through leaf wetness sensors) in two soybean fields. The highly complex meteorological data was subjected to machine learning (ML) algorithms to help predict LW and LWD, and to calculate the level of importance of each predictor or explanatory variable. Relative humidity emerged as the most crucial predictor of LW and LWD, and all the models predicted LW and LWD with high accuracy. Therefore, this study gives valuable insights into the future deployment of *Lecanicillium uredinophilum* in large-scale field trials.

8.3 Recommendations

- In the isolation and screening of rust hyperparasites from the wild strawberry rust pustules, four fungal hyperparasites were obtained concurrently. Preliminary characterization identified the species isolated as *Simplicillium lanosoniveum* and *Akanthomyces muscarium*. This suggests that more than one hyperparasite can infect

rust pustules concurrently. A study by Mitina and Sokornova (2013) evaluated a combination of *Lecanicillium* isolates for the control of *Trialeurodes vaporariorum* Westwood (whitefly), and discovered a synergistic effect by multiple isolates. A synergistic effect between *S. lanosoniveum* and *A. muscarium* could exist. Moreover, this study screened for two *Lecanicillium uredinophilum* isolates that exhibited differences in their growth rate. A further study could combine the isolates and evaluate their combined effect.

- The transmission electron microscopy (TEM) studies that were undertaken did not detect any mycoparasitic interactions occurring inside soybean leaves infected with *P. pachyrhizi.* The confocal laser scanning microscopy (CLSM) observations were made using *in vitro* infection studies. However, CLSM could be used to track *L. uredinophilum* infection of *P. pachyrhizi* mycelia inside soybean leaves, as the plant infection progresses.
- Despite confirmation of the mechanism of action by *L. uredinophilum* as mycoparasitism, research is recommended to determine the type of enzymes that are produced by *L. uredinophilum*, which are active in the infection process of urediniospores, leading to their destruction.
- The molecular and genetic basis for the recognition of host fungi by *L. uredinophilum* could be studied to understand the process.
- While this study investigates the effect of *Lecanicillium uredinophilum* under elevated relative humidity conditions, the environmental elasticity of *L. uredinophilum* under suboptimal conditions was not established. A study on the effect of a range of relative humidity levels, and temperatures could determine their effect on the minimum environmental requirements of *L. uredinophilum* to infect *P. pachyrhizi* urediniospores.
- Weather stations do not normally record leaf wetness and leaf wetness duration as standard meteorological variables espoused under the World Meteorological Organization (WMO), and this presents challenges when such data is needed for plant pathological modelling. To establish more robust models, there is a need to record LW and LWD data or variables that can be used to predict LW. Such data measurements must be recorded over multiple years and at high resolution. Moreover, such studies require a collaborative interdisciplinary effort in funding and execution.

8.4 References

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Appendices

Appendix I: Permission to use illustration from a published scientific journal

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>
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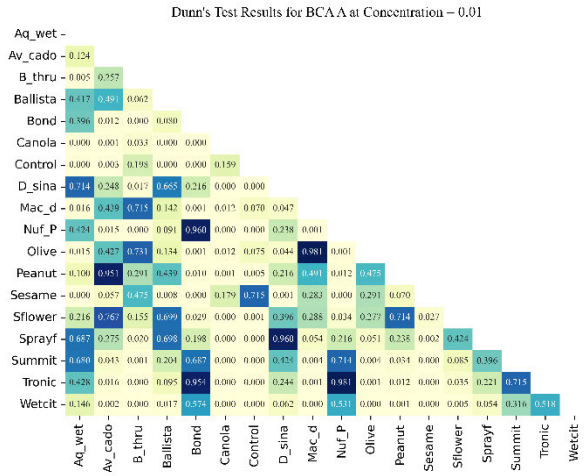
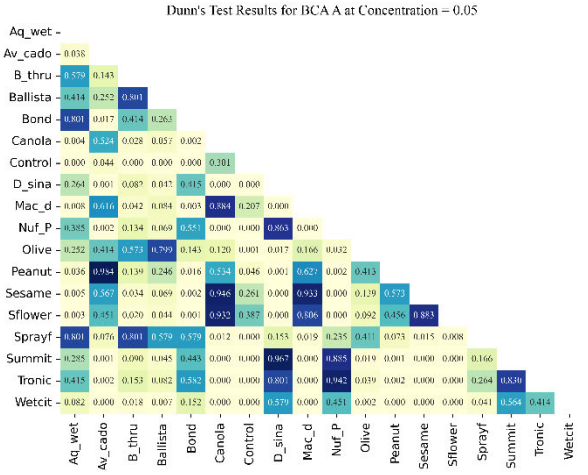
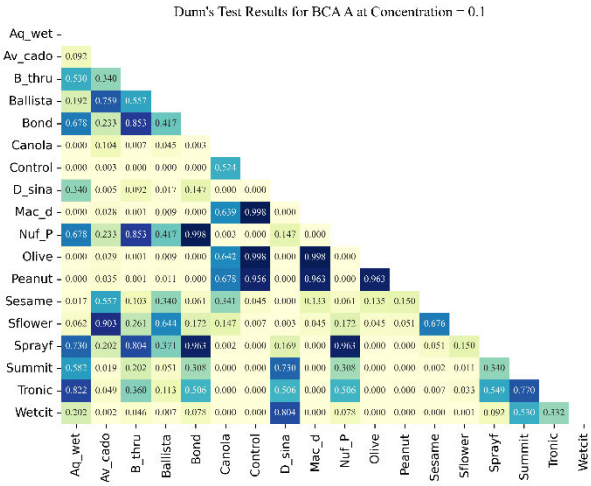
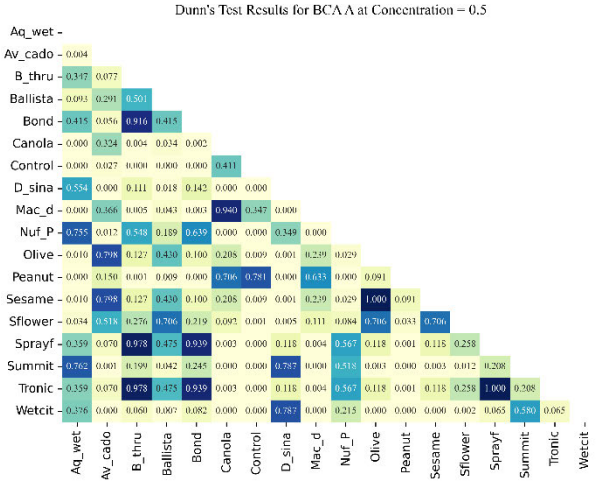
Faculty of Applied Sciences,
University of Sri Jayewardenepura,
Nugegoda,
Sri Lanka.
Phone: +94 715687752

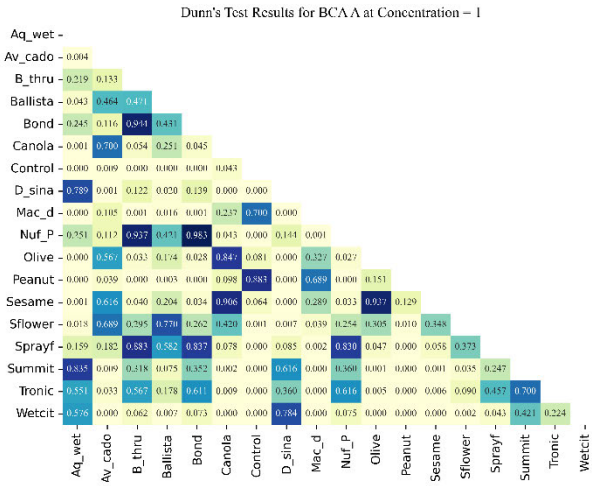
Appendix II: Triangular Heatmaps (ρ-values) for *Lecanicillium uredinophilum* (PP2018-001) radial growth between adjuvant types across all concentrations

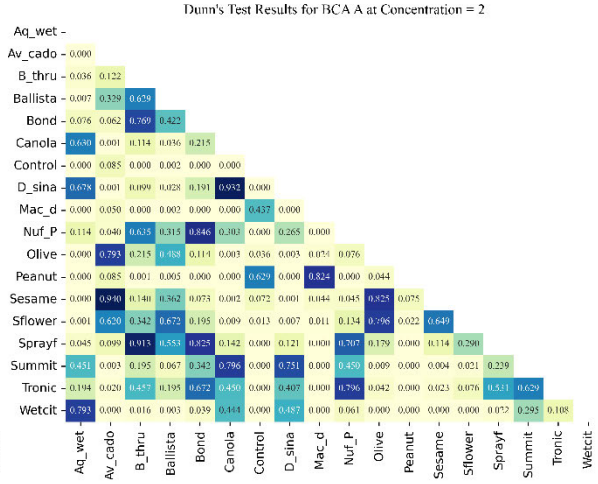




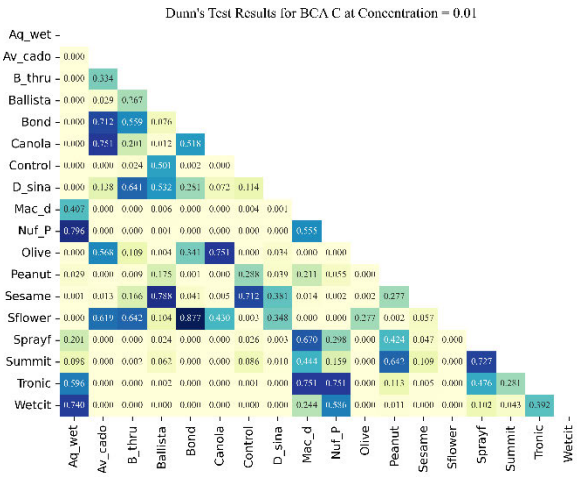


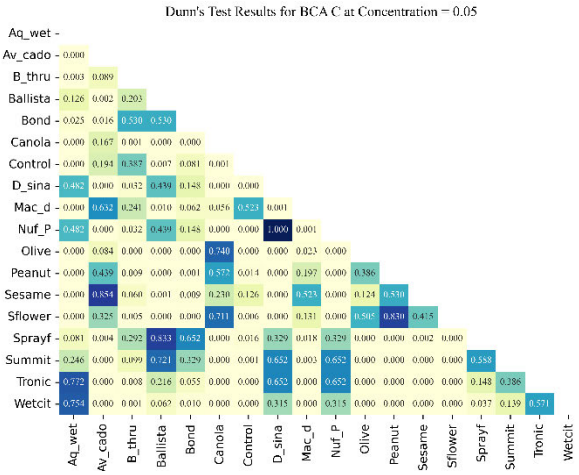


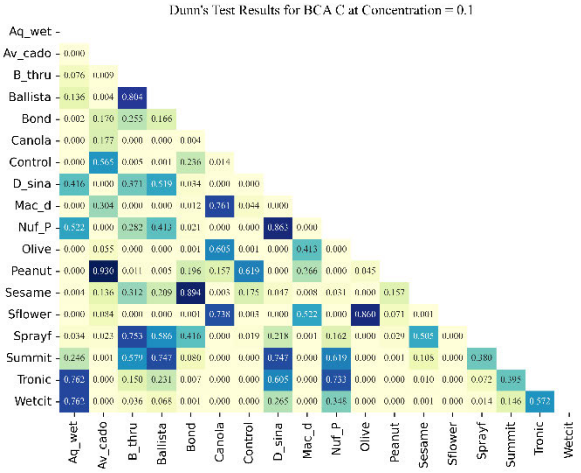


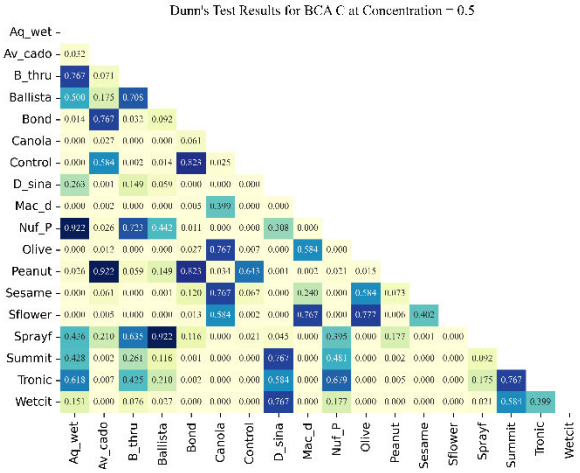


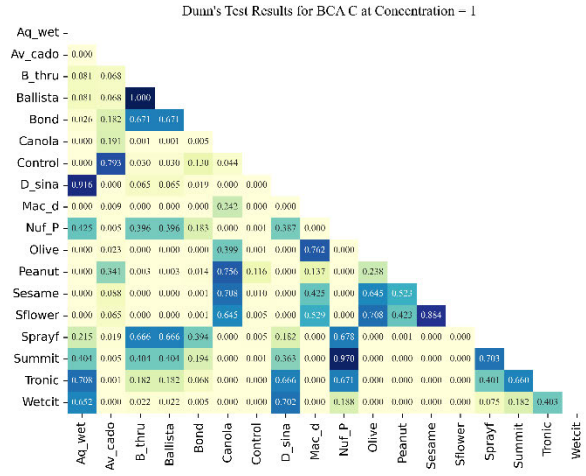
Appendix III: Triangular Heatmaps (ρ-values) for *Lecanicillium uredinophilum* (PP2018-003) radial growth between adjuvant types across all concentrations

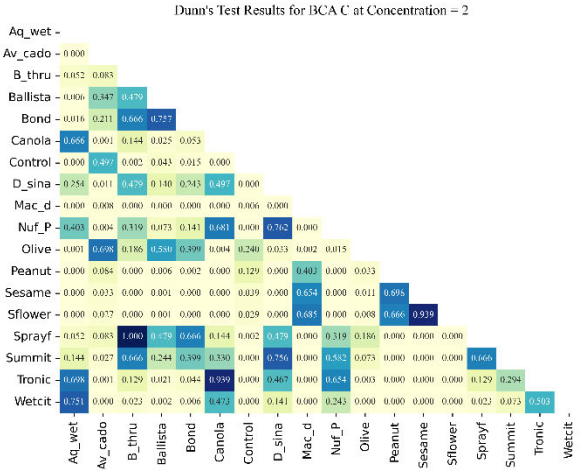




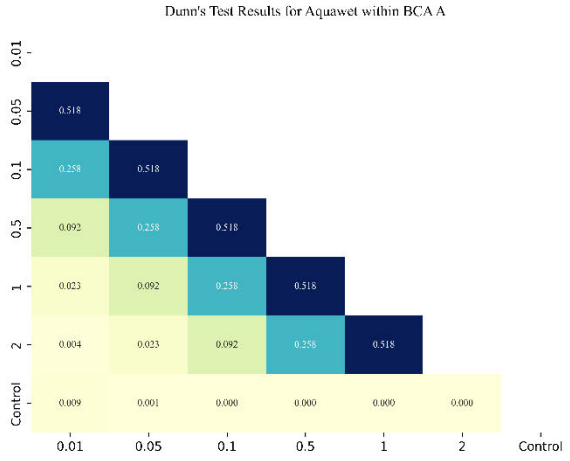


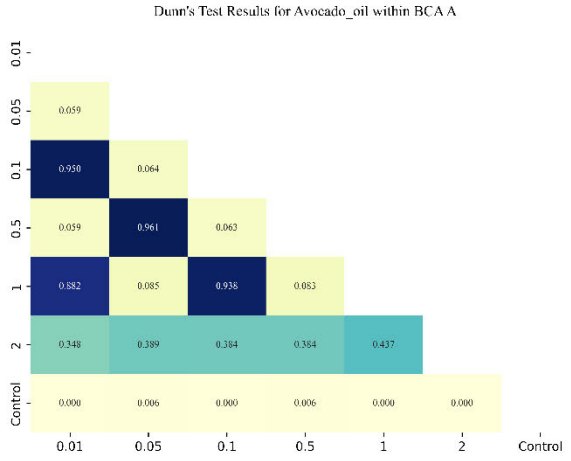


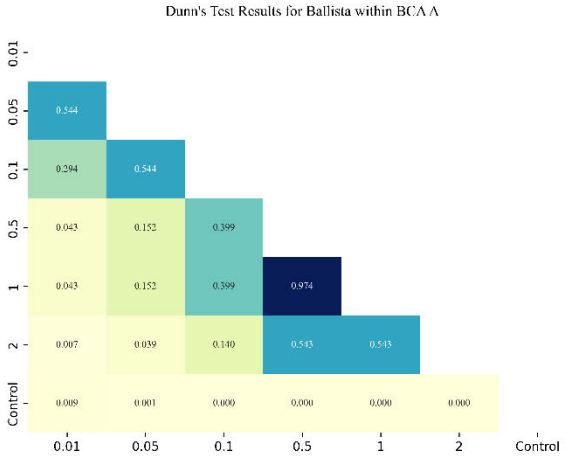


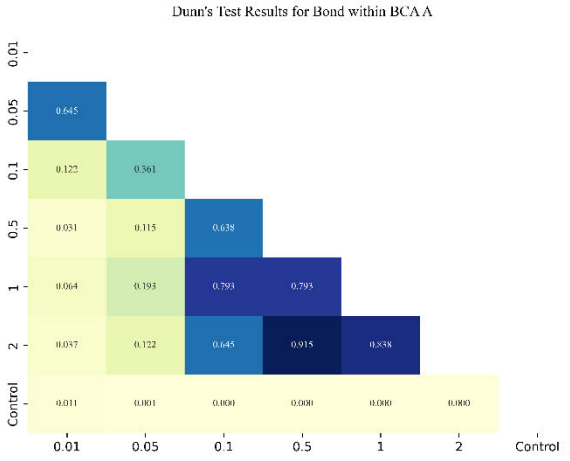


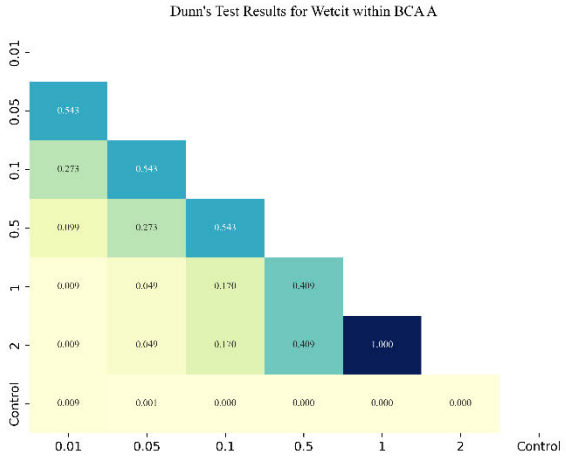
Appendix IV: Triangular Heatmaps (ρ-values) for *Lecanicillium uredinophilum* (PP2018-003) radial growth within adjuvant types across all concentrations

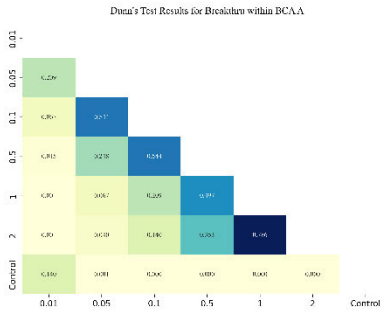








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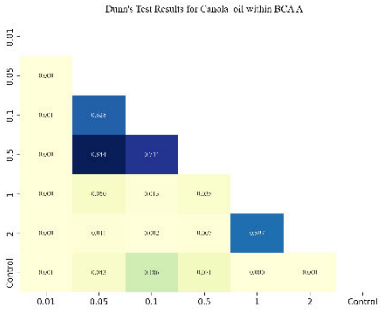
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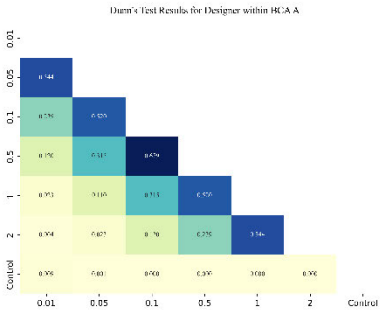
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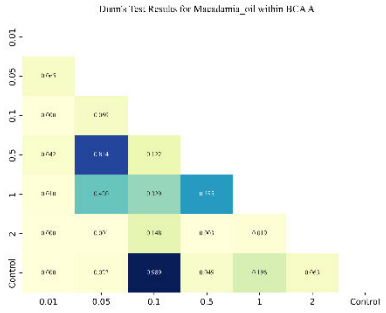
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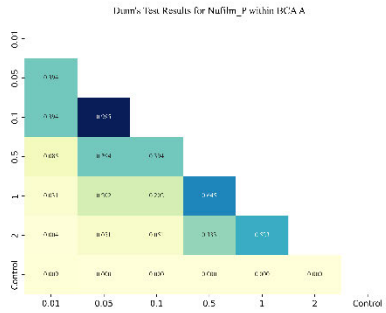
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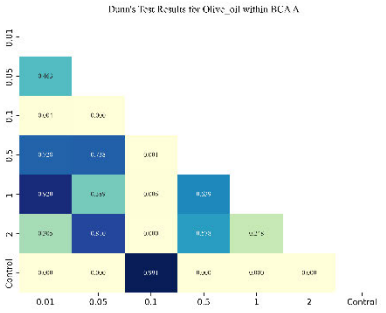
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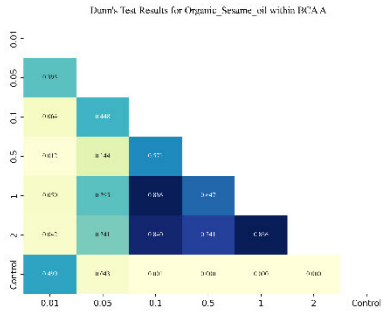
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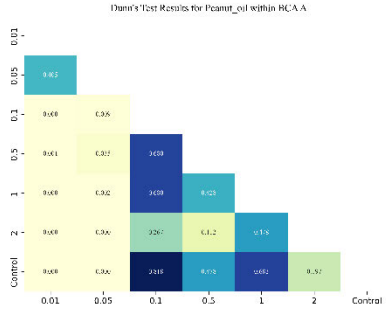
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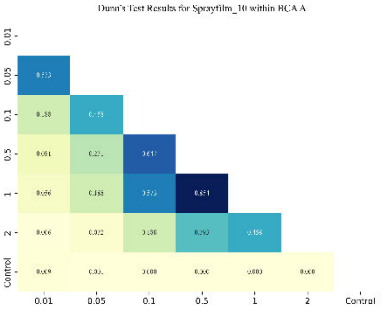
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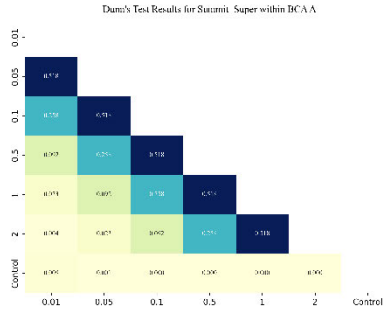
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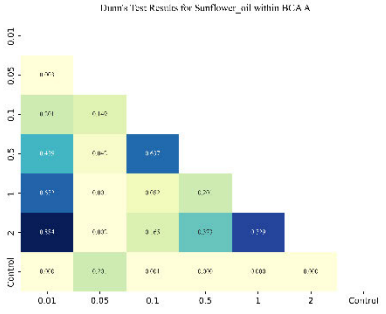
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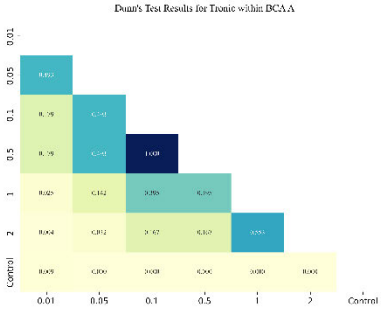
Dunn's Test Results for Tronic within BCAA
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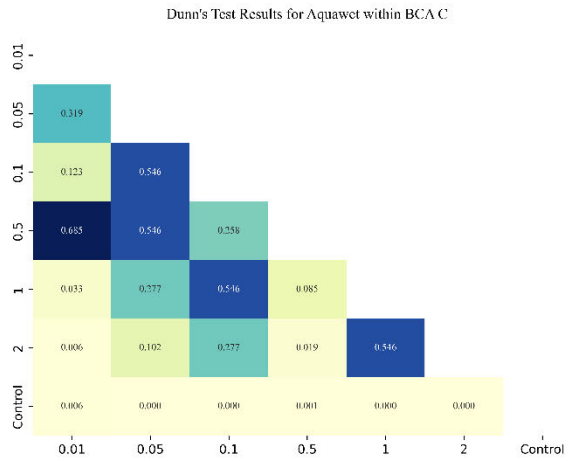
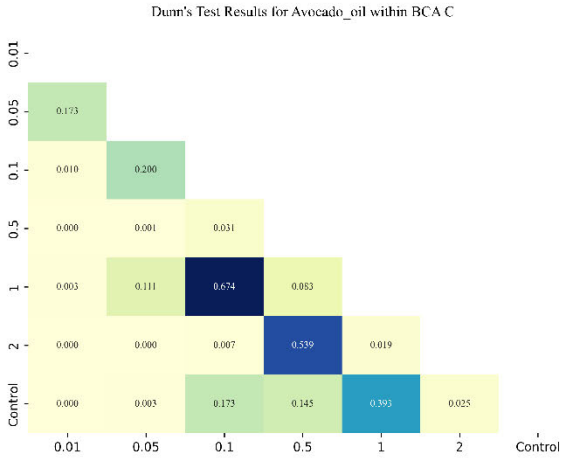
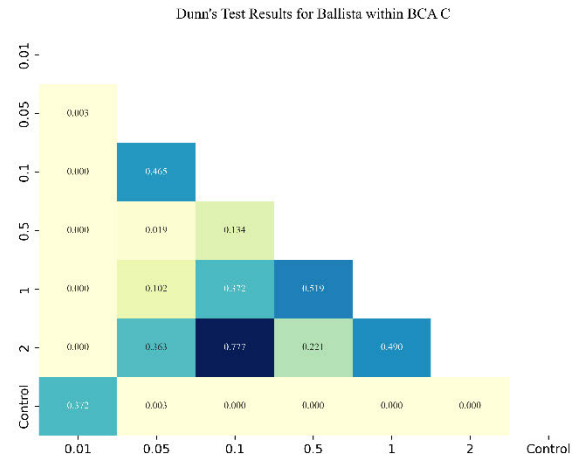
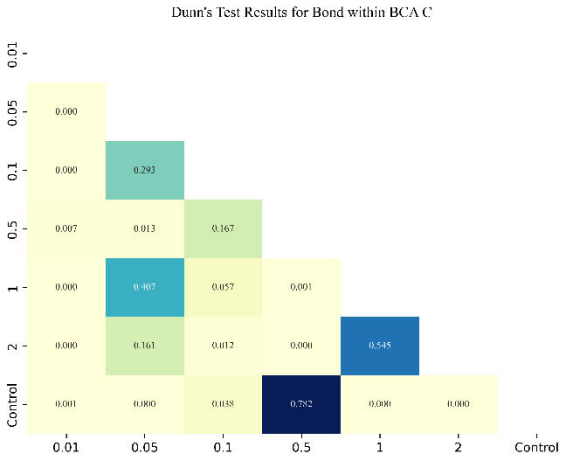
Control

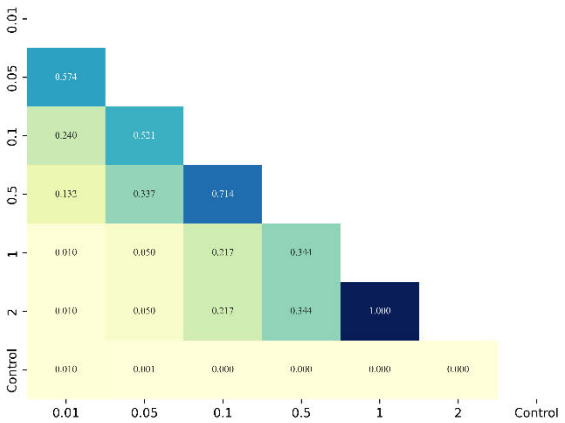
Appendix V: Triangular Heatmaps (ρ-values) for *Lecanicillium uredinophilum* (PP2018-003) radial growth within adjuvant types across all concentrations

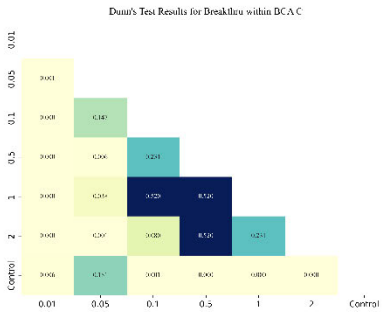


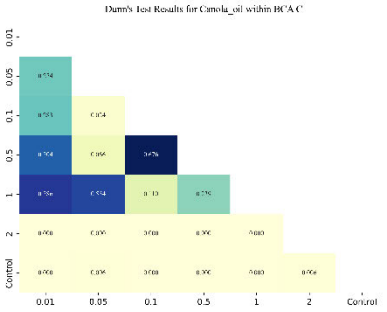


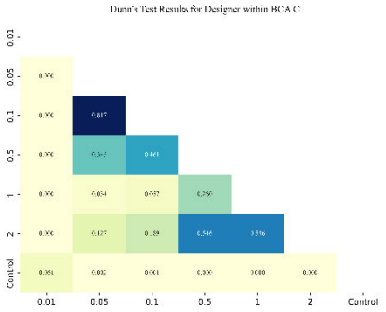


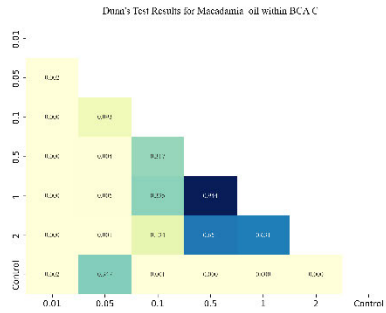


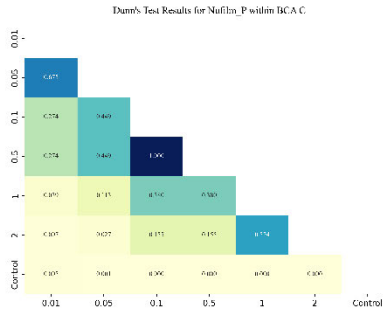


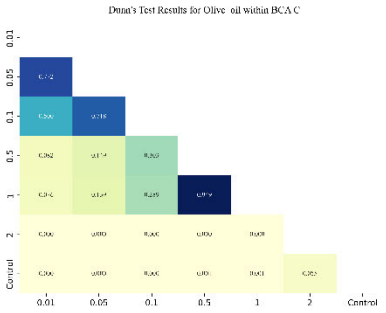

Dunn's Test Results for Breakthru within BCA C
0.01
0.05
0.1
0.5
1
2
Control

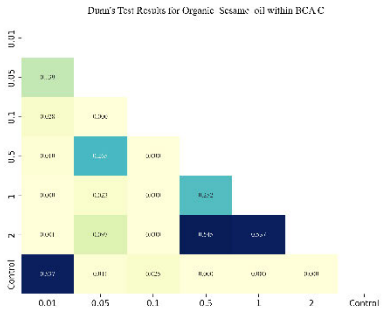

Dunn's Test Results for Canola_oil within BCA C
0.01
0.05
0.1
0.5
1
2
Control

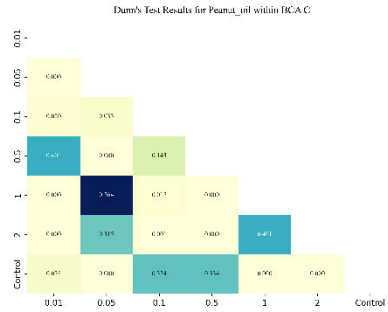

Dunn's Test Results for Designer within BCA C
0.01
0.05
0.1
0.5
1
2
Control

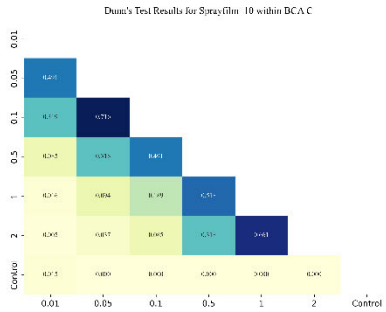

Dunn's Test Results for Macadamia oil within BCA C
0.01
0.05
0.1
0.5
1
2
Control

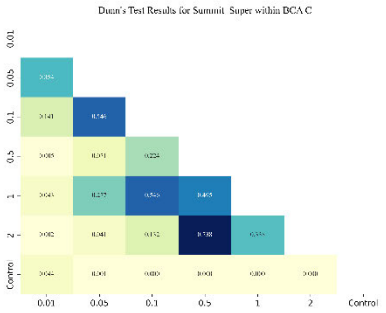

Dunn's Test Results for Nufilm_P within BCA C
0.01
0.05
0.1
0.5
1
2
Control

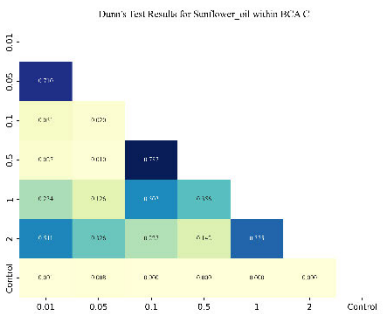

Dunn's Test Results for Olive oil within BCA C
0.01
0.05
0.1
0.5
1
2
Control

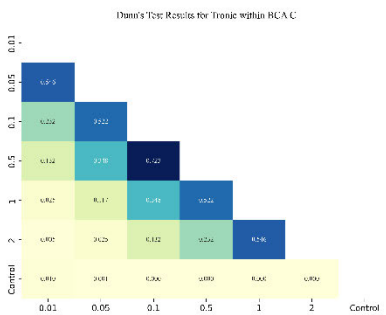

Dunn's Test Results for Organic Sesame oil within BCA C
0.01
0.05
0.1
0.5
1
2
Control


Dunn's Test Results for Peanut_oil within BCA C
0.01
0.05
0.1
0.5
1
2
Control


Dunn's Test Results for Sprayfilm 10 within BCA C
0.01
0.05
0.1
0.5
1
2
Control


Dunn's Test Results for Summit Super within BCA C
0.01
0.05
0.1
0.5
1
2
Control


Dunn's Test Results for Sunflower_oil within BCA C
0.01
0.05
0.1
0.5
1
2
Control


Dunn's Test Results for Tronic within BCA C
0.01
0.05
0.1
0.5
1
2
Control