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Microsclerotia production in *Sclerotinia sclerotiorum*: A potential bioherbicide for Californian Thistle and Giant Buttercup

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A thesis
submitted in partial fulfilment
of the requirements for the Degree of
Master of Science
at
Lincoln University
by
Jonty Mills

Lincoln University
2023

Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Master of Science

Microsclerotia production in *Sclerotinia sclerotiorum*: A potential bioherbicide for Californian Thistle and Giant Buttercup

by

Jonty Mills

The plant pathogen, *Sclerotinia sclerotiorum,* was further investigated as a potential bioherbicide targeting the problematic weeds *Cirsium arvense* (Californian thistle) and *Ranunculus acris* (Giant buttercup). Previous research has found that *S. sclerotiorum* can be effective at controlling both weeds with bioherbicide work primarily focused on solid substrate production of fungal mycelium, which has proven cost-prohibitive to scale up.

The primary objective of this research was to investigate whether *S. sclerotiorum* could produce microsclerotia (MS) in liquid fermentation. Liquid fermentation is more cost-effective and scalable than solid substrate production. MS are small and resilient fungal propagules with characteristics such as desiccant tolerance and shelf stability, that make them attractive as propagules to be used as an active ingredient for a bioherbicide. Although MS production has been demonstrated for other species of fungi, it has not previously been reported for *S. sclerotiorum*. The suitability of MS incorporated into a *S. sclerotiorum* bioherbicide formulation was additionally assessed.

Six *S. sclerotiorum* isolates (S36, S37, GGB1, GGB2, G57 and G64) were screened for their suitability for future bioherbicide development. The growth of the isolates was compared under a range of illumination (12 hr photoperiod or 24 hr darkness) and temperature (15 °C, 20 °C and 25 °C) conditions. Their pathogenicity on excised leaves of *C. arvense* (provenances G20 and G27) and *R. acris* (haplotypes A, A2, G and J) was compared. GGB2 was found to be the fastest growing isolate and was one of the most pathogenic isolates on both host species. This isolate is a potential candidate for future research.

Seven liquid media with varying compositions were screened for their ability to induce MS formation with *S. sclerotiorum* isolate S36. A single media was identified that resulted in MS production, yielding 4.5 x 10^3 MS per mL after 14 days of fermentation at 300 rpm. Bioassays using formulations of these MS as inoculum on excised leaves of *C. arvense* and *R. acris* initially showed promising

results. MS inoculum caused lesions on both species covering a large proportion of the leaf area and resulted in lesions significantly larger than those from a sclerotia inoculum ($P < 0.001$). Replications of the bioassay resulted in no lesions with the MS formulations, which led to further investigation of the formulation and drying process.

The survival of the MS at each step of the formulation process was examined. The survival of the structures decreased dramatically when dried to below 30% moisture. Adjusting the fermentation time and carrier used did not improve MS viability after the drying step. Microscopy of the MS ultrastructure indicates that the drying process may be causing damage to the hyphal cell membranes, resulting in lysis of the cell and potentially causing low viability.

Production of *S. sclerotiorum* MS has been demonstrated for the first time. Despite the inconsistency of MS of *S. sclerotiorum* to survive drying and cause infection, MS remain a promising propagule for a future bioherbicide. Optimization of the fermentation and formulation should be the focus of future work. If reliable production of desiccant tolerant MS of *S. sclerotiorum* can be achieved, this would present an opportunity to enable the sustainable management of *C. arvense* and *R. acris.*

Keywords: *S. sclerotiorum*, microsclerotia, *R. acris*, *C. arvense*, biological control, bioherbicide

Acknowledgements

Thank you to my supervisors Seona Casonato and Laura Villamizar for their help and expertise that have guided me throughout the course of my studies. A special thank you to Graeme Bourdot and Mike Cripps for the mentoring and discussions that helped shaped the work of this thesis. Additional thanks to Lincoln University staff Eirian Jones and Sandy Hammond for all their work.

I also extend my thanks to Gloria Barrera (Agrosavia, Colombia) for her support with the electron microscopy analysis. The images she provided gave me useful insights that would not have been possible without her assistance.

I am grateful for all the friends I have met while at Lincoln University, many of whom have helped me out with laboratory work that is presented here.

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Chapter 1
Introduction

1.1 Introduction

Cirsium arvense (L.) Scop. (Californian thistle) and *Ranunculus acris* (L.) Sp. Pl. (Giant Buttercup) are two of the costliest weeds to pastoral production in New Zealand, causing an estimated annual productivity loss of ~$700 million and ~$210 million respectively (Saunders et al., 2017). Productivity losses are caused by the establishment of the weed species in pasture, where weed infestations outcompete the sward and overtake significant percentages of total grazing area (Bourdôt, Hurrell, & Saville, 2014). Current control tools are inadequate for *C. arvense* and *R. acris*. Of particular concern is the identification of multiple herbicide resistances in *R. acris* populations that are attributed to historical herbicide exposure (Jackman, Bourdôt, Noble, Lamoureaux, & Ghanizadeh, 2020). New control tools are needed to enable long-term control of these weeds.

Sclerotinia sclerotiorum (Lib.) de Bary is a hemibiotrophic pathogenic fungus with the potential to be used as a biological control agent for *C. arvense* and *R. acris* (Bourdôt, Harvey, Hurrell, & Alexander, 1993; Hurrell & Bourdôt, 2011; Kabbage, Yarden, & Dickman, 2015). *S. sclerotiorum* belongs to Sclerotiniaceae and has a broad host range. Natural populations of *S. sclerotiorum* can be found on both weed species in New Zealand, although the inoculum load is not high enough to provide adequate control. Using augmentative biological control, the amount of inoculum in a localized area of a weed population can be artificially increased (Van Lenteren, 2012). Augmentative biological control using formulations of *S. sclerotiorum* has been demonstrated to cause high rates of mortality in *C. arvense* and *R. acris.* Long-term population decline has been observed in *C. arvense* after application of *S. sclerotiorum* as a bioherbicide (Bourdôt, Hurrell, & Saville, 2011; Bourdôt, Hurrell, Saville, & Leathwick, 2006). In field experiments, mortality of *R. acris* has been achieved with no negative effects on pasture grasses and legumes (such as clovers) (Cornwallis et al., 1999). These findings suggest that *S. sclerotiorum* has potential as a bioherbicide, if a cost-effective formulation to enable commercial production is developed (Hurrell & Bourdôt, 2011).

Previous bioherbicide formulations of *S. sclerotiorum* have been based on solid substrates such as mycelium-on-grain and brewers waste, which are cost-prohibitive to commercialize at scale (Bourdôt et al., 1993; Smith, Casonato, Noble, & Bourdôt, 2016). Liquid culture production is

generally regarded as the gold standard for efficient production of fungal propagules (Jackson, 1997). Liquid culture production of a novel structure called microsclerotia (MS) may be a cost-effective way to produce *S. sclerotiorum* bioherbicide inoculum. MS are resilient, compact (100-1000 µm), melanized hyphal aggregates that germinate when rehydrated and can initiate infection (Jackson & Jaronski, 2012). MS are well suited to be used as bioherbicide propagules as they are desiccant tolerant propagules that can be produced cheaply in high concentrations (>10^3 per mL) via liquid fermentation (Jackson & Schisler, 1995). MS production in liquid culture has been reported for several entomopathogenic and phytopathogenic fungi, but not in Sclerotiniaceae (Jackson & Schisler, 1995; Song, Yin, Jiang, Liu, & Wang, 2014; Villamizar et al., 2018).

1.2 *Cirsium arvense*

1.2.1 Background & Distribution

Cirsium arvense is a perennial herb native to Eurasia that is now found naturalized throughout most of the world's temperate latitudes (Tiley, 2010). *C. arvense* is considered among the world's worst weeds due to its successful establishment in pasture and crop systems, resulting in significant losses (Holm, Plucknett, Pancho, & Herberger, 1977). *C. arvense* is New Zealand's most costly pastoral weed responsible for an estimated cost to productivity of nearly $700 million annually (Bourdôt, Hurrell, Trolove, & Saville, 2016). *C. arvense* reduces the productivity of pasture by outcompeting pasture sward and livestock avoiding feeding around the thistles due to their spines. As a result, there is a reduction to livestock weight gain and total livestock production from infested pastures (Cripps et al., 2011).

1.2.2 Life History

Cirsium arvense primarily spreads through its creeping rhizomatous root system (Leathwick & Bourdôt, 2012). The creeping root system survives through the winter, providing a source of energy for the plant even after the shoots die off in autumn (McAllister & Haderlie, 1985). The root system is replaced annually (Bourdôt, Leathwick, & Hurrell, 2000) and contains many dormant adventitious root buds, each capable of developing into clonal shoots that break dormancy overwinter and emerge above ground in spring (Bourdôt et al., 2006; Moore, 1975). The dormant root buds act as an important reservoir to generate new shoots if the initial shoots fail (Leathwick & Bourdôt, 2012). The amount of root biomass that overwinters is linearly correlated to the above-ground vegetation that accumulates during the growing season, reflecting the plant's photosynthetic opportunity (Bourdôt, Leathwick, Hurrell, & Saville, 1998). The plant spreads rapidly through this clonal mechanism and if left uncontrolled, can form dense expanding clusters of individual plants

(Leathwick & Bourdôt, 2012). The movement of root fragments by farm machinery and livestock may also contribute to short-range spread of the weed (Donald, 1994). *C. arvense* is dioecious, reproducing sexually via seed, which contributes to the establishment of new populations in crops and pasture (Donald, 1994). In contrast, vegetative spread is an important mode of recruitment in established infestations.

1.2.3 Impacts

1.2.4 Control

Defoliation is the most effective strategy to control *C. arvense*. Defoliation programmes aim to reduce the above-ground biomass of *C. arvense* which reduces the plants photosynthetic capacity, thereby reducing the production of new root biomass (Bourdôt et al., 1998). Energy reserves in the root system must be utilized to replace the defoliated shoots and as a result less root biomass is available to overwinter to produce new shoots in the spring. At least two defoliations a year are required for population reduction (Bourdôt, Basse, & Cripps, 2016; Mitchell & Abernethy, 1995). Defoliation can be achieved by mowing, herbicides, and biological control agents. Mowing has shown to be an effective control technique to reduce *C. arvense* populations, which is enhanced when mowing is conducted during rainfall (Bourdôt, Hurrell, Skipp, Monk, & Saville, 2011). Defoliation of *C. arvense* can also be achieved with various herbicides available in New Zealand including 2,4-D, MCPA/MCPB, glyphosate, clopyralid, aminopyralid, dicamba, and triclopyr + picloram (AgPest, n.d.). Most of the available herbicides will damage clover, thus negatively impacting productivity. Steep terrain can limit access by farm machinery and make control through mowing or herbicide spraying difficult or infeasible (Cripps, Jackman, & van Koten, 2019). For this reason, numerous classical biological control agents for *C. arvense* have been investigated, but success has been limited. Several fungal pathogens already present in New Zealand have been investigated as potential augmentative biological control agents. These fungi include *S. sclerotiorum*, *Verticillium dahliae*, *Puccinia punctiformis* and *Plectosphaerella cucumerinam* (Bailey et al., 2017; Skipp et al., 2013). No commercial products based on these fungi are currently available for control of *C. arvense*. There is a demonstrated need for novel control strategies and bioherbicides based on these pathogenic fungi present a promising opportunity.

1.3 *Ranunculus acris*

1.3.1 Background & Distribution

Ranunculus acris (Giant buttercup) is one of 14 *Ranunculus* species present in New Zealand (Bourdôt, Lamoureaux, Jackman, Noble, & Chapman, 2019). The plant originates from a native range

in Central and Northern Europe where *R. acris* is highly variable, leading to the plants being classified into a number of subspecies (Coles, 1971). In New Zealand only one subspecies, *Ranunculus acris* subsp. *acris,* is present (referred to as *R. acris* from this point). This subspecies has naturalized and become an economically important weed of dairy pastures in addition to inhabiting other moist environments such as roadsides, wastelands and river flats (Webb & Sykes, 1988).

R. acris has been dispersed from its native range and is now considered to have circumpolar distribution (present throughout many longitudes at high latitudes) (Lamoureaux & Bourdôt, 2007). The naturalized range extends from artic northern Europe through to northern Africa, most of the northern United States and southern Canada and parts of South Africa, Australia and New Zealand (Lamoureaux & Bourdôt, 2007; Webb & Sykes, 1988). This reflects the high climatic suitability of this plant to a broad range of environments.

The weed arrived in New Zealand with Early European settlers and *R. acris* has naturalized (Lamoureaux and Bourdôt, 2007). New Zealand is the country where the species has become the most problematic as a pasture weed. In six of the 17 dairy farming regions (South Auckland, Hawkes Bay, Taranaki, Wairarapa and Horowhenua) in New Zealand, *R. acris* is considered a serious weed (Bourdôt & Saville, 2010). Given the weeds high climatic adaptability there is likely no climatic constraint on the plant growing in all dairy farming regions within New Zealand. This is reflected by observations of sporadic populations along roadsides in regions where the weed is not yet considered a problem to pasture (Lamoureaux & Bourdôt, 2007).

1.3.2 Life History

Ranunculus acris is a short lived rhizomatous perennial herb that flowers and produces seeds for several years before senescing (Tuckett, 1961). Its stems are hairy and erect, growing up to 1 m tall and are typically highly branched. The species overwinters as a rosette with flowering stems dying off by May which helps to increase the plants frost tolerance (Popay, Edmonds, Lytile, & Phung, 1989). The rosette rapidly produces 40-50 larger leaves in August and September (Southern Hemisphere spring) which are produced from the apex of the rhizome (Popay et al., 1989). The flowers are vibrant yellow with five petals (Harper, 1957). The defining feature of the subspecies that is naturalized in New Zealand is the three main divisions of leaves that are stalkless (Tuckett, 1961). The leaf margins are jagged compared to other *Ranunculus* species and the three divisions of leaves may be less obvious. Due to the high degree of variability within the species, other leaf traits can be variable.

The plant can reproduce both asexually, through the production of clonal rhizome shoots, and sexually, via flowering. In New Zealand both modes of reproduction are known to occur but is unknown if one plays a more significant role than the other (Lusk, 2012). Vegetative reproduction occurs during autumn when dormant axillary buds formed at the base of the terminal aerial shoot are stimulated to develop, producing new daughter shoots that develop a few millimeters from the parent plant (Lamoureaux & Bourdôt, 2007). Over time the rhizomatous connection between the clonal plant and the parent is reduced. Subsequently, the clonal plant becomes a ramet independent from the parent, producing its own rhizome, which continues this cycle. This type of reproduction facilitates the spread of the weed over short distances forming dense clonal colonies of the weed (Sarukhan & Harper, 1973). In New Zealand, clonal colonies are commonly observed in dairy pastures and can extend more than 1 m in diameter (Lamoureaux & Bourdôt, 2007). Rhizomes often rot away by the time of flowering in well aerated soils, but rhizomes can persist for one to two years, with increased persistence in peaty soils (Harper, 1957; Lamoureaux & Bourdôt, 2007).

Sexual reproduction begins when a mature *R. acris* plant begins to flower. In New Zealand, flowering begins in early October and continues through to April, with peak floral density observed in December (Popay et al., 1989). In some locations, flowers can be observed all year. *R. acris* produces bright yellow hermaphroditic flowers that are self-incompatible and are insect pollinated. The flowers are protogynous, meaning the female floral organs are mature before pollen is released from the anthers (Lusk, 2012). Each plant can produce up to 5000 seeds per year and seed production is reportedly size independent (Hemborg & Karlsson, 1998; Tuckett, 1961). The seeds are 1.5 mm in diameter and are usually not dispersed far from the parent plant (Kiviniemi & Eriksson, 1999). A small hook on the seed aids in the long-range dispersal of the plant by latching onto fur of livestock, clothing and farm machinery (Tuckett, 1961). The seed bank of *R. acris* can reach 2600 seeds per m^2 in severely infested New Zealand pastures (Tuckett, 1961). Seeds of *R. acris* typically do not last for more than one year when buried in the top 20 mm of soil, but the long-term viability of seeds increases with burial depth. A small percentage of seeds remain viable for an estimated 51 years when buried at 200 mm in some soil types, although these are the exception with only a minority of seeds surviving for more than three years in soil (James & Rahman, 1999; James, Rahman, & Trivedi, 2010; Roberts & Boddrell, 1985). Seeds from *R. acris* establish in autumn and spring, with recruitment at its highest rate in patches of pasture with reduced competition such as bare ground. Bare ground patches can be caused by factors related to poor pasture management such as overgrazing or trampling, as well as environmental factors that result in disturbance to pasture sward such as flooding and drought (Lusk & Lamoureaux, 2009; Tozer, Bourdot, & Edwards, 2011). Farmers have reported that in poorly drained soils the weed is particularly problematic to

control, and this is thought to be a result of increased pugging, resulting in more suitable bare ground for *R. acris* seedling recruitment (DairyNZ, 2018a). Production of hay from infested paddocks can spread seeds of the plant over longer distances if used as feed in paddocks where the weed was not already present. The movement of infected hay on trucks is the likely source of spontaneous populations of *R. acris* observed on roadsides and responsible for the spread of the weed to new regions and pastures (Bourdôt & Saville, 2010).

Ranunculus acris, much like other related members of the *Ranunculus* family, produces the glycoside ranunculin that upon digestion is converted into vesicant protoanemonin (Lamoureaux & Bourdôt, 2007). Protoanemonin causes blistering of livestock lips and tongue and can cause problems in the digestive tract. In extreme cases, poisoning of the livestock can be fatal and result in cardiac or respiratory failure; no cases of severe livestock poisoning have been reported in New Zealand (Connor, 1977; Harper, 1957). Due to its toxicity, livestock avoid eating the giant buttercup and potentially palatable plants in the immediate vicinity of the weed, resulting in productivity losses (Bourdôt & Lamoureaux, 2002).

1.3.3 Control

Cultural Control of *R. acris*

Mowing is the most effective non-chemical control method against *R. acris.* This removes the above ground part of the plant and can reduce the amount of flowering and seeding that occurs (Lusk, 2012).The best time to mow in New Zealand is before the plant flowers in November and prior to grazing (AgPest, 2017). Mown plants lose their toxicity and can be eaten by cows (DairyNZ, 2018a). Mowing after flowering can spread seeds throughout the pasture and is not advisable. Bourdôt (2019) found that the effects of mowing on *R. acris* density within infested Golden Bay farms was not significant until 7 months post treatment. Bourdôt et al. (2019) hypothesize the effect of mowing became more apparent with time due to the cumulative effect of repeated defoliation that reduces the rhizomatous energy stores of the *R. acris* plants, reducing their ability to regenerate. Long term, the effect of mowing is a decrease in *R. acris* cover within a pasture and a near 1:1 ratio increase in cover by palatable pasture sward species such as ryegrasses and clovers. Mowing as a control method is effective, independent of other control methods, such as herbicide application and thus is an important component of integrated management of *R. acris* (Bourdôt et al., 2019).

Due to the increased recruitment of *R. acris* in wet, poorly drained soils and bare ground, proper grazing management to reduce pugging and overgrazing can contribute to the control of the weed. Keeping pasture dense and competitive throughout the year can help reduce recruitment to some

extent (Bourdôt & Lamoureaux, 2002). Improved control of *R. acris* through the application of plant growth promoters, such as gibberellin and nitrogen (N) fertilizer that increase pasture competitiveness, have been reported by some New Zealand farmers. Bourdôt (2019) examined these claims but found no significant effect of either N fertilization or gibberellin on the control of *R. acris* across nine different Golden Bay farms over a three-year period. Plant growth promoters have been reported to have little effect on *R. acris* growth and abundance in Austria (Buchgraber & Sobotik, 1995), Slovenia (Leskosek, 1996) and Russia (Babenko, 1991). The effect may be highly variable depending on the soil type and condition, with studies from Russia and Greece on low yielding natural grassland finding that fertilizer applications reduced the relative abundance of *R. acris* and increased the proportion of palatable grasses (Melnikov, Pilishvili, & Kuznetsov, 1975; Tzialla, Papakosta, & Veresoglou, 2002). Overall, in the case of New Zealand, plant promotors are unlikely to effect the control of *R. acris* (Brown, 1993; Lamoureaux & Bourdôt, 2007).

Farmers have reported improvements in *R. acris* control by cropping an infested pasture but this is yet to be tested experimentally (Lusk, 2012; Tuckett, 1961). It is likely the improvement in control will only be temporary as some plants will remain in the cropped field and seeds, that can last in the soil for several seasons, will likely repopulate the new pasture (Tuckett, 1961). Another issue with *R. acris* control is hay produced from pasture infested with *R. acris.* In such a situation, the hay is likely to contain many seeds of the weed. Thus, it is not advisable to use feed hay from an infested pasture in a paddock where *R. acris* is not present (AgPest, 2017). Avoiding this may not directly contribute to control of an existing infestation but may prevent accelerating the spread of the weed throughout a farm. The regional management of hay from infested pastures is a potential point of interest for internal biosecurity, as this is a likely vector for the weed throughout the dairy regions of New Zealand (Bourdôt & Lamoureaux, 2002).

Chemical Control of *R. acris*

Five chemical herbicides are available in New Zealand under various brand names that have label claims to be effective against *R. acris.* Additionally, the broader spectrum herbicide combinations Aminopyralid and Aminopyralid + triclopyr are also known to be effective against the weed (DairyNZ, 2018b; Lusk, 2012). The utilised chemicals belong to four mode of action groups and resistance has been recorded to two of these modes of action groups, with the others considered high risk. Table 1 shows a summary of the available herbicides in New Zealand that are known to control *R. acris* in pastures.

Table 1.1 Summary of available herbicides known to have an effect on the pasture weed *R. acris*. Table adapted from a DairyNZ (2018a) figure produced by Graeme Bourdôt available at www.dairynz.co.nz.

Herbicide	Mode of Action	Clover Damage	Damage to Grass	Known Resistance
Aminopyralid	O3[1]	Yes	No	No
Aminopyralid + triclopyr	O3	Yes	No	No
Bentazone + MCPB	C3[2]/O1[3]	No	No	No
MCPA	O1	Yes	No	Yes
MCPB	O1	No	No	Yes
Thifensulfuron-methyl	B[4]	Yes	Yes	No
Flumetsulam	B	No	No	Yes

[1]O3 mode of action group- Auxin mimics. Pyridine carboxylates
[2]C3 mode of action group - Inhibition of photosynthesis at photosystem II
[3]O1 mode of action group - Auxin mimics. Phenoxy-carboxyllic-acids
[4]B mode of action group – Inhibition of acetolactate synthase ALS (acetohydroxyacid synthase AHAS)

The first herbicides to be available in New Zealand with label claims of effectiveness against *R. acris* were MCPA and MCPB, belonging to the O1 mode of action group (Tuckett, 1961). Both of these herbicides are synthetic auxins that mimic the natural plant hormone responsible for growth regulation and cell differentiation. Application of the herbicides on susceptible plants causes a hormone overdose resulting in deformed stems, leaves and flowers. Within a few weeks' chlorosis, wilting and necrosis occurs, leading to plant death (Grossmann, 2010). MCPA has been widely used as a broadleaf selective herbicide in pastures since the 1950s largely due to its relatively low cost (Lamoureaux & Bourdôt, 2007). Unfortunately, the herbicide also targets beneficial legumes like clover that are important for pasture vigour (particularly in New Zealand where feed supplementation is less utilized than overseas). The reduction in clover caused by application can lead to reduced pasture performance for several months after application (Popay et al., 1989). MCPB is not as effective as MCPA at reducing *R. acris* biomass but this is somewhat balanced by the

fact that it does not damage clovers (Lusk, 2012). It is suggested that farmers should alternate annually between application of MCPA and MCPB. These herbicides provided effective control up until the 1970s but after 30 years of use they have become almost totally ineffective in pastures where resistant populations have developed (Bourdôt, Hurrell, & Saville, 1990). The mechanism of resistance is unknown but it is likely a single dominant nuclear gene (Leathwick & Bourdôt, 1991). Resistance in populations is correlated with historical exposure of that pasture to MCPA/MCPB herbicide treatment, indicating that the use of these herbicides is providing the selective pressure that has driven the development of resistance (Bourdôt et al., 1990).

The second group of herbicides used in New Zealand to control *R. acris* are the acetolactate synthase enzyme inhibitors (ALS inhibitors) that belong to the O3 mode of action group. Two herbicides thifensulfuron-methyl and flumetsulam are available from this group (Bourdôt & Lamoureaux, 2002). These herbicides prevent the production of essential branched chain amino acids (BCAAs) needed for plant growth and survival and result in the gradual death of the plant (Babczinski & Zelinski, 1991). Compared to the auxin mimics, control from these herbicides takes longer to be observed due to reserves of these BCAAs within the plant tissues. Results are usually evident after several months. Thifensulfuron-methyl is known to be damaging to clovers and thus potentially reducing pasture competitiveness and productivity and potential increases to *R. acris* seedling recruitment (Lamoureaux & Bourdôt, 2007; Sanders & Rahman, 1994). In a survey reported by Lusk (2012) most Golden Bay farmers interviewed who had used thifensulfuron-methyl said they had only used it once or twice and then did not use it again as they found it to be far too damaging to their pastures. Limited scientific research has been done to prove these claims but observations reported in Harris and Husband (1997) support the observations of farmers. Flumetsulam is the preferred ALS inhibitor that is more commonly used by dairy farmers to control *R. acris* as it does not cause damage to clovers when applied at the label rate (Jackman et al., 2020). It has been known since the first use of ALS inhibitors that there was a high risk of weed populations developing resistance, which had been observed in other weed species where ALS inhibitors had been relied upon (Bourdôt et al., 2019). Resistant populations in *R. acris* have since been identified (Jackman et al., 2020; Lusk, Hurrell, Harrington, Bourdôt, & Saville, 2015). Application above the recommended label rate can cause clover damage, and this may have implications on pastures where *R. acris* populations develop resistance to flumetsulam therefore requiring higher doses of the herbicide to be effective (Lusk, Harrington, & Hurrell, 2011). Bentazone is another herbicide that is effective against *R. acris* from a third mode of action group C3. It must be used in conjunction with MCPA (NOVACHEM, 2020). Bentazone works by inhibiting photosystem II and thus preventing photosynthesis. It is not taken up by plant roots and is most effective when applied in late August to mid-September while *R. acris*

plants are in the overwintering rosette stage (NOVACHEM, 2020). As Bentzone must be used in conjugation with MCPA there is limited scope for rotation of mode of action groups in the integrated control of *R. acris* and associated pasture weeds.

Due to the limited variety of different mode of action herbicides available for the control of *R. acris* and the necessity to use rotate mode of actions to avoid developing multiple resistant populations, some broad-spectrum herbicides can be used every few years. An effective application tool that can enable selective control using non-selective herbicides are weed wipers, also known as rope wick applicators (Thompson, 1983). This tool works particularly well on *R. acris* since it is avoided by grazing cows in pasture and has long stems that rise above the grass and clovers. Aminopyralid and the combination aminopyralid + triclopyr are general broadleaf selective herbicides that are highly effective against *R. acris* in pasture. Bourdôt et al. (2019) found that of all herbicides with label claims against *R. acris*, the aminopyralid based formulations were the most consistent and effective at reducing weed coverage even 31 months after treatment. Coverage of *R. acris* treated with these herbicides showed 60% reductions 31 months after treatment. While herbicides like Aminopyralid are damaging to clovers if sprayed over entire pastures, the use of a weed wiper can help mitigate this risk and target just the weeds that extend above grass level.

1.3.3.3 Biological Control

Two potential biological control agents have been investigated in New Zealand for use against *R. acris*; both are plant pathogenic fungi found on naturalized populations of the weed (Hardwick, Close, & Field, 1993; Verkaaik, Hurrell, & Saville, 2004). Ideally, these fungal bioherbicides should provide selective control on *R. acris* without damage to clover or pasture species. The first species examined was a *Gnomonia* species that was observed causing anthracnose-type lesions on the stems and petioles of *R. acris*. When tested experimentally the fungus was able to infect 30 – 40% of the plants but no mortality was recorded and thus it did not have potential to be an effective biocontrol (Hardwick et al., 1993). The second species investigated was *S. sclerotiorum* that causes a soft rot of the buttercup foliage.

1.4 Sclerotinia sclerotiorum

Sclerotinia sclerotiorum is a hemibiotrophic, homothallic pathogen belonging to the phylum Ascomycota (order Helotiales) (Kabbage et al., 2015). The fungus is known to cause disease on a broad range of host plants including economically significant crops (Pennycook, 1989).
S. sclerotiorum has a wide ecological distribution favouring cool, humid conditions but also occurs in semi-arid environments, particularly when irrigation is used (Heffer Link & Johnson, 2007). Infection

by the pathogen is characterized by the presence of water-soaked lesions that often have an apparent margin on stems and foliage (Smolińska & Kowalska, 2018). Fluffy white mycelium may be visible on necrotic lesions. These lesions develop as a result of invading hyphae which release enzymes and oxalic acid to facilitate the infection (Dutton & Evans, 1996). Lesions spread down petioles and onto stems (Bolton, Thomma, & Nelson, 2006). Secondary symptoms include the shredding, wilting and bleaching of the aerial foliage (Abawi & Grogan, 1979). As lesions expand the pathogen can girdle stems of the plant, causing tissue supplied by the constricted vasculature to wilt and become necrotic (Figure 1.1). As the disease progresses, fluffy white mycelium develops into spherical hyphal aggregates (Heffer Link & Johnson, 2007). The aggregates mature into melanized, irregularly shaped sclerotia that vary in size from a few millimetres to a few centimetres (Hao, Subbarao, & Duniway, 2003). Sclerotia are the overwintering structures of the fungus and enable the pathogen to remain viable in soil independent of host plants. Sclerotia can survive in soil for as long as 5 - 10 years, although viability declines significantly after the first year (Brustolin, Reis, & Pedron, 2016; Harvey, Foley, & Saville, 1995).

Figure 1.1 A *Ranunculis acris* plant (left) exhibiting characteristic symptoms following infection with *Sclerotinia sclerotiorum*, compared to a healthy *R. acris* plant (Right). Image provided by Grame Bourdôt.

Sclerotia can germinate carpogenically or myceliogenically (Figure 1.2). In *S. sclerotiorum,* carpogenic germination is the more commonly observed mechanism that involves the production of apothecia

which contain asci from which sexual ascospores are produced (Smolińska & Kowalska, 2018). Sclerotial size is an important factor determining the production of apothecia – larger sclerotia germinate more frequently and produce more apothecia per sclerotium (Dillard, Ludwig, & Hunter, 1995). A single apothecium produces approximately 2 x 10^6 ascospores over several days (Schwartz & Steadman, 1978). Ascospores are carried by the wind to aerial tissues of host plants where they land and can germinate. Infection is favoured under cool conditions and extended periods of leaf wetness over 16 to 48 hrs (Heffer Link & Johnson, 2007). The presence of external nutrition, which may come from senescing plant material, or from sugars secreted from the host plant that accumulate in areas such as leaf axils, is required for the pathogen to develop appressoria, enabling the invasion of healthier plant tissues (Sedun & Brown, 1987; Turkington & Morrall, 1993).

The second germination mechanism is an asexual process known as myceliogenic germination whereby sclerotia germinate via the direct emergence of hyphae that directly infect host tissues (Smolińska & Kowalska, 2018). These hyphae induce infection in areas of the plant within 1 - 2 cm of the sclerotia, such as the roots and crown (Heffer Link & Johnson, 2007). Myceliogenic germination occurs at temperatures between 12 - 24°C when the soil is near saturation following irrigation or periods of rain. Myceliogenic germination is less commonly observed in *S. sclerotiorum* than in related species such as *Sclerotinia minor* (Schwartz & Steadman, 1978) however, sclerotia of some isolates of *S. sclerotiorum* have been observed to preferentially germinate via this mechanism (Personal observation, J. Mills, March 2021).

S. sclerotinia is a monocyclic disease meaning that it has only a primary disease cycle and lacks a secondary infection cycle. Infected host plants do not produce asexual spores that are capable of initiating new infections within the same growing season (Heffer Link & Johnson, 2007). New infections are induced by ascospores or hyphae produced by germinating sclerotia in the soil. Windborne ascospores are blown from apothecia of germinating sclerotia, often resulting in distinct foci of infection within an infected crop, where the spores have spread out from the source of infection (Heffer Link & Johnson, 2007). Ascospores can be dispersed by the wind over long distances to nearby fields and dispersal distances of 25 m to several hundred metres have been reported (Abawi & Grogan, 1979). de Jong et al. (2002) developed a mathematical model to predict the spread of ascospores from sites where the pathogen has been used as a biological control agent. The presence of ascospores from an area where the fungus has been applied as a bioherbicide is undetectable compared to background levels at 2.5 – 7.9m away (Bourdôt, Hurrell, Saville, & DeJong, 2001).

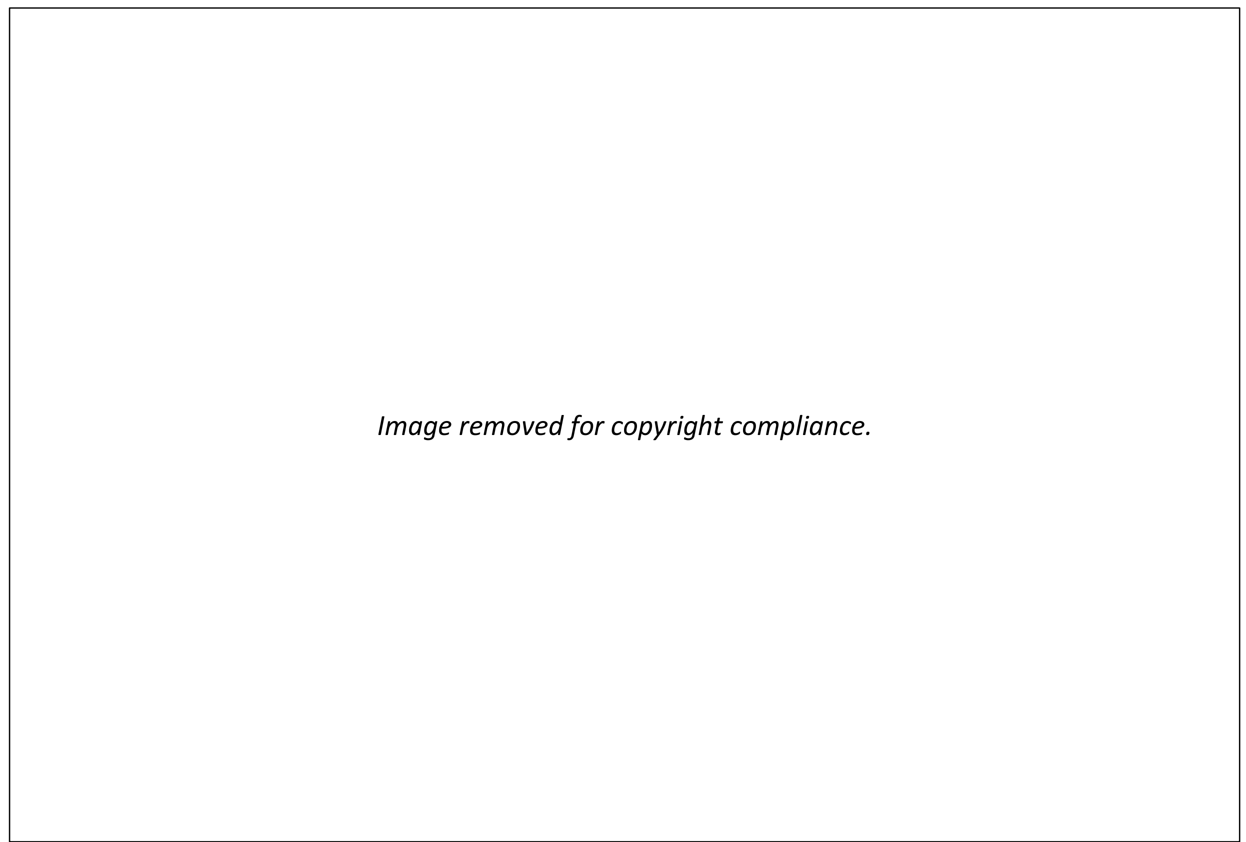

Figure 1.2 Lifecycle and symptoms of *Sclerotinia sclerotiorum* (Agrios, 2005).

1.4.1 Use as an inundative biological control agent

Unlike classical biological control programs that require the introduction of new species, *S. sclerotiorum* is already found naturalized in New Zealand and has been found on both *C. arvense* and *R. acris* in the field. Background levels of *S. sclerotiorum* are not high enough to enable control of weed species but the amount of inoculum in a localized area can be increased. This is known as inundative or augmentative biological control.

Brosten & Sands (1986) were the first to investigate the use of *S. sclerotiorum* as a biological control agent of *C. arvense*. They found that *S. sclerotiorum* caused significant shoot mortality and reductions in shoot density in the subsequent growing season. This result suggested that *S. sclerotiorum* had caused significant damage to the root system, which was supported by the re-isolation of the pathogen up to 25 cm deep into the *C. arvense* root system. Further experiments have since shown that *S. sclerotiorum* has potential as a bioherbicide. Bourdôt *et al.* (1993) tested a mycelium on kibbled wheat preparation of *S. sclerotiorum* which was tested for its ability to control *C. arvense* in grazed pastures and found it caused high shoot mortality and reduced the coverage of the weed, as a result of damage to the root system. Bourdot (2006) investigated the long-term effects of *S. sclerotiorum* bioherbicide applications on *C. arvense* populations and reported long-

term reductions in root biomass, again suggesting its potential as an inundative biological control agent.

In 1995, 19 isolates of *S. sclerotiorum* were screened and tested for their virulence against another highly problematic weed in New Zealand, *R. acris* (Green, Bourdot, & Harvey, 1995). Subsequent research focused on screening a wide range of isolates on *R. acris* and found that the best isolates provided a similar level of control as the herbicide MCPA was achieving at the time (Cornwallis et al., 1999). The bioherbicide also caused no damage to clover or grasses, unlike MCPA. Since this time, MCPA has become almost completely ineffective against many populations of *R. acris* due to resistance to the herbicide (Lusk et al., 2015). In 2004, a formulation of the biocontrol was tested on *R. acris* infested pastures and was found to kill >60% of the individual *R. acris* plants at an application rate of 530 kg/ha (Bourdôt, 2011). Surviving plants had stunted growth. Lower doses of granular forms of the biocontrol at 100 kg/ha also showed potential to reduce *R. acris* biomass by up to 50% with more success in Takaka than Taranaki. Liquid forms of the biocontrol were tested in 2006 on 10 farms at various application rates and found that it did not affect *R. acris* control, while a follow-up in 2007 found on average a 12% reduction in Giant Buttercup coverage the year after application, although on some farms *R. acris* coverage had increased (Bourdôt, 2011). In both weed species, the efficacy of *S. sclerotiorum* has varied between populations perhaps due to genetic variation. *C. arvense* plants from 32 provinces around New Zealand varied in their susceptibility to *S. sclerotiorum* (Smith et al., 2016).

Previous research on *S. sclerotiorum* has primarily utilized solid substrate production to produce bioherbicide inoculum such as mycelium on-grain (Harvey, Alexander, Waipara, & Saville, 1994) and barley brewer's waste (Smith et al., 2016). Solid substrate production systems suffer from challenges in scale-up, with sterilization, adequate gas exchange and contamination becoming issues at scale (Jaronski, 2014). The use of solid substrates colonized by *S. sclerotiorum* as inoculum also requires high application rates (> 300kg/ha) creating further logistical difficulties and costs (Hurrell & Bourdôt, 2011). Compared to solid substrate fermentation, liquid culture fermentation offers improved scalability, fermentation times and control of conditions (Jackson, 1997). This has led to the investigation of various other fermentation and formulations processes that are more cost-effective to produce in bulk products such as mycelial slurries and gels (Bourdôt, Hurrell, & Saville, 2011). More recently, sclerotia have been investigated as a potential fungal propagule to be used as bioherbicide. Bulk production of sclerotia faces similar issues to mycelium production in solid fermentation as the sclerotia must be produced and harvested from a solid substrate or from agar. A

new cost-effective production method of fungal propagules has been demonstrated in several species of entomopathogenic fungi, by producing MS in a suspended liquid culture (Jackson & Schisler, 1995; Villamizar et al., 2018). MS formulations of *S. sclerotiorum* may provide a cost-effective way to formulate a new bioherbicide.

1.5 Microsclerotia (MS)

MS are small melanized, hyphal aggregates that are structurally similar to sclerotia but have smaller diameters (100 -1000µm)(Song, 2018). MS are multicellular aggregations of filamentous hyphae, that are typically pigmented and can tolerate some degree of desiccation (Jackson & Payne, 2016). Like sclerotia, MS are thought to be survival structures that help preserve the fungus through adverse conditions and enable it to continue growing when favourable conditions return, utilising endogenous nutritional reserves within structure to facilitate new growth (Coley-Smith & Cooke, 1971; Jackson & Payne, 2016).

MS are produced by some phytopathogenic fungi in their natural disease cycles such as *Verticillium dahliae* (Buchwaldt, Morrall, Chongo, & Bernier, 1996). More recently, production of MS has been induced using specific fermentation conditions in submerged liquid cultures – including for fungi that have not been observed producing MS in nature. Induced MS production was first demonstrated in *Colletotrichum truncatum* and was explored as a bioherbicide propagule targeting *Sesbania exalta*, a weed in cotton, soybean and rice (Jackson & Schisler, 1995). Since then, MS research has predominantly investigated production of MS in entomopathogenic fungi, to base biopesticides, with good results achieved. Fungi such as *Metarhizium* spp. and *Beauvaria* spp. have been shown to produce high yields of MS per mL with propagules capable of surviving drying to < 5% and retaining viability when rehydrated (Song et al., 2014; Villamizar et al., 2018). Villamizar et al. (2018) showed that in a New Zealand isolate of *Beauveria pseudobassiana* AgR-704 MS were produced at a concentration of 8.46×10^3 per mL and could survive drying as a storable formulation with 100% germination after 6 months storage at 4 °C and 68% germination after 6 months at 20°C.

To create a cost-effective biopesticide product, fungal propagules must be efficient to produce, have adequate shelf stability and be capable of consistently infecting hosts (Jackson, 1997). MS produced in submerged liquid culture are well suited as a biopesticide propagules for several reasons. Firstly, liquid fermentation, which is what is used to produce MS, is regarded as the most efficient method for production of fungal based biopesticide products (Jackson & Schisler, 1995; Jaronski, 2014). Liquid fermentations use conventional fermentation equipment where variables such as

temperature, pH and nutrition can be easily adjusted to optimize conditions for production (Jackson, 1997). Liquid fermentations can be upscaled into larger fermenters easily, unlike solid substrate fermentations where difficulties with sterilization and control of conditions limit the viability of upscaling (Jackson & Jaronski, 2012). MS produced in some species have been shown to be shelf stable when dried with a carrier and when rehydrated retain high viability, germinating to produce mycelium or infective conidia (Jackson & Jaronski, 2009). The shelf stability of MS is thought to be due to the pigmented aggregation of the structure which provides protection against desiccation and UV (Song, Shen, Zhong, Yin, & Wang, 2016). Particles of media trapped within aggregates may also provide endogenous nutrition that facilitates germination/infection when the structures are rehydrated (Coley-Smith & Cooke, 1971). MS can be produced at high concentrations per mL of liquid culture, with some entomopathogenic fungi species have achieved yields as high as 2.9×10^5 MS per mL (Jackson & Jaronski, 2009). A high number of infective propagules per unit volume means that a final formulation will have many infective points when applied onto the target pest and/or weed.

Published literature on MS-producing fermentations share similarities in culture conditions and media composition. The culture conditions that result in MS production in other fungi such as *Metarhizium spp.* and *Beauveria spp.*, as well as *Trichoderma sp.* have been investigated (de Lira, Mascarin, & Júnior, 2020; Jackson & Jaronski, 2009; Jackson & Schisler, 1995; Kobori, Mascarin, Jackson, & Schisler, 2015; Mascarin, Kobori, de Jesus Vital, Jackson, & Quintela, 2014; Song et al., 2016; Song et al., 2014; Villamizar et al., 2018). All published media that result in MS formation across different fungal genera share some similarities in terms of nutrition and aeration. They all contain a carbon source, a nitrogen source, and basal salts, and are grown in cultures under high rotation (300 rpm). The carbon-to-nitrogen ratio and level of oxidative stress, caused by shaking and oxidizing agents in the media, are known to influence MS formation. Alkylating and oxidizing materials can promote sclerotia formation and this is hypothesized to apply to MS formation as well (Georgiou, Patsoukis, Papapostolou, & Zervoudakis, 2006). Increasing the concentration of ferrous sulphate was shown to increase the yield of MS produced by *Metarhizium rileyi* (Song et al., 2014).

MS production has not been demonstrated in the family Sclerotiniaceae previously. If achieved, a *S. sclerotiorum* bioherbicide formulation, based on MS produced in submerged liquid culture, may be a cost-effective way to produce inoculum to use as an inundative biological control of some of New Zealand's costliest weeds. Production and survival of *S. sclerotiorum* MS must be examined.

1.6 Aims and Objectives

This research aims to produce MS of *S. sclerotiorum* in suspended liquid culture. MS production has not previously been reported in the family Sclerotinaceae. Initial media compositions to be tested will be based on formulations used by previous research to produce MS in other fungi. Produced MS will be used to create a novel bioherbicide formulation of *S. sclerotiorum.* The effectiveness of MS formulations of *S. sclerotiorum* as a bioherbicide will be examined using excised leaf bioassays. Six potential bioherbicide isolates of *S. sclerotiorum* (S36, S37, GGB1, GGB2, G57, and G64) will be screened for attributes relevant to formulation as a bioherbicide. The most suitable isolate will be recommended for future bioherbicide development.

Objective 1: Compare the pathogenicity, growth, and apothecia production of various isolates of *S. sclerotiorum* excised from the target hosts *C. arvense* and *R. acris* to evaluate the potential for their use in bioherbicide production.

a) Examine whether the isolates can produce apothecia.

b) Examine the effect of photoperiod on the germination and growth of sclerotia of each isolate.

c) Compare the growth of each isolate under different temperature conditions.

d) Compare the pathogenicity of the isolates on excised leaves of *C. arvense* and *R. acris*.

Objective 2: Produce MS of *S. sclerotiorum* in submerged liquid culture and evaluate the infectivity of the produced structures.

a) Screen different culture media to determine if MS formation can be induced in *S. sclerotiorum*.

b) Examine the infectivity of MS of *S. sclerotiorum* on the target weed *R. acris* and *C. arvense*.

Objective 3: Evaluate aspects of *Sclerotinia sclerotiorum* MS production that may influence viability of the produced structures.

a) Compare the survival of MS structures during the formulation process.

b) Compare the infectivity of MS at different fermentation times.

c) Examine the effects of different formulation agents on MS survival.

Chapter 2

Investigation of Growth and Germination of *S. sclerotiorum* Isolates

2.1 Chapter Introduction

Sclerotinia sclerotiorum is a ubiquitous plant pathogen that can infect more than 400 species of plants, across 278 genera and 75 families, including economically important crop and weed species (Boland & Hall, 1994). *S. sclerotiorum* reproduces asexually via myceliogenic germination of sclerotia as well as sexually via the production of ascospores. As a homothallic fungus, *S. sclerotiorum* ascospores are capable of self-fertilisation, through haploid selfing, and therefore dispersal via sexual ascospores can also reinforce a clonal population structure (Attanayake, Xu, & Chen, 2019; Billiard, López-Villavicencio, Hood, & Giraud, 2012). Despite both modes of reproduction, *S. sclerotiorum* populations are primarily clonal, although genetic diversity within local and distant populations exists. The diversity amongst populations of *S. sclerotiorum* was historically assessed by methods of mycelial compatibility to define Mycelial Compatibility Groupings (MCGs) and the use of DNA fingerprinting (Sleight, 2001). More recently these techniques have been replaced with modern molecular techniques using specific PCR primers targeting a region called the Mating Type Locus (*MAT*) and through next generation sequencing that enables cost-effective analysis of larger genome regions (Attanayake, Xu, & Chen, 2019; Chitrampalam, Inderbitzin, Maruthachalam, Wu, & Subbarao, 2013). On pastoral farms in New Zealand, several clonal lineages of *S. sclerotiorum* can be found within single fields with some overlapping MCGs. With increasing spatial separation of populations, such as across regions, Carpenter, Frampton, and Stewart (1999) found little mycelial compatibility between isolates of *S. sclerotiorum* in New Zealand with differences also reflected in the genetic fingerprints of the isolates (Carpenter et al., 1999). Results from Canada and the USA similarly report the detection of many distinct clones within local populations of *S. sclerotiorum* (Cubeta, Cody, Kohli, & Kohn, 1997; Kohn, 1995).

For this research, six isolates of *S. sclerotiorum* (S36, S37, GGB1, GGB2, G57 and G64) have been selected to examine their potential as bioherbicide agents of *Ranunculus acris* and *Cirsium arvense*. The objective of this chapter was to investigate various aspects of each of the isolates relevant to use as a bioherbicide and identify the most suitable isolates to continue with bioherbicide development. The six isolates included in this research originate from infections of the two target weeds within New Zealand pastures as outlined in Table 2.1. This is advantageous for bioherbicide development to target these weeds, as the isolates are known to infect populations of one of the

two target weeds, so are expected to be more pathogenic than isolates from other host plants. *S. sclerotiorum* has an extensive host-range and there is little evidence of host specialization (Liang & Rollins, 2018), so isolates from one of the two target weed species may be pathogenic on the other target weed. For the specific isolates chosen, with the exception of S36, this has not been previously investigated.

Table 2.1 Isolates of *Sclerotinia sclerotiorum* investigated in this study for suitability to create a bioherbicide formulation targeting the pasture weeds *Cirsium arvense* and *Ranunculus acris*. Host origin of the initial isolate, location and year of collection is indicated.

Isolate	Host plant originally excised from	Location collected	Year collected	Tested as a bioherbicide? (Y/N) Fermentation type (if applicable)
S36	*Cirsium arvense*	Tai Tapu, Canterbury, NZ	1998	Yes. Solid substrate (Smith et al., 2016), and as a gel produced after liquid fermentation (Bourdôt, Hurrell, & Saville, 2011).
S37	*Cirsium arvense*	Limestone Valley, Canterbury, NZ	2005	Yes. Solid substrate (Smith, 2018).
GGB1	*Ranunculus acris*	Golden Bay, Tasman, NZ	2020	No
GGB2	*Ranunculus acris*	Golden Bay, Tasman, NZ	2020	No
G57	*Ranunculus acris*	Takaka, Tasman, NZ	1995	Yes. Solid substrate (Cornwallis et al., 1999).
G64	*Ranunculus acris*	Takaka, Tasman, NZ	1995	Yes. Solid substrate (Cornwallis et al., 1999).
CS14	*Cirsium arvense*	Leeston, Canterbury	1999	No

S. sclerotiorum isolate S36 is the most well documented of the six isolates, originally isolated from an infected *C. arvense* plant in Canterbury. S36 has been extensively investigated as a bioherbicide, applied as both liquid and solid formulations and has shown to be pathogenic towards *C. arvense* and *R. acris* (Smith et al., 2016; Verkaaik et al., 2004). Limitations in the development of a cost-effective formulation have prevented further commercialization. S37 is closely related to S36 with few detectable genetic differences but has been shown to significantly differ from S36 in pathogenicity to specific genotypes of *C. arvense* (Smith, 2018). G57 and G64 are isolates that were collected by AgResearch from infected *R. acris* plants and were two of five isolates that caused mortality of *R. acris* plants on field populations of the weed in a screening of 35 isolates (Cornwallis et al., 1999). Some growth rate data have been published for S36, S37, G57 and G64 to varying extents (Cornwallis et al., 1999; Sleight, 2001; Smith, 2018). This may provide useful comparison to see if these isolates have changed, as some have been in lab cultures for almost 20 years. GGB1 and GGB2 were isolated in 2020 from infected *R. acris* plants. A literature search did not reveal any publications for these isolates.

S. sclerotiorum sclerotia can germinate carpogenically to form apothecia. Apothecia release ascospores which initiate new infections and are known to be important in the disease cycle of the pathogen on some crops (Abawi & Grogan, 1979). When *S. sclerotiorum* is applied as an inundative biological control agent onto pasture weeds, apothecial production is undesirable due to concerns about increasing the pathogen inoculum to nearby cropping or horticultural areas (Sleight, 2001). Despite these concerns, modelling has shown that apothecia load from inundative application would be undetectable from background levels within short distances (de Jong et al., 2002). For the purpose of this research, isolates will be screened for their ability to produce apothecia *in vitro*. There is some anecdotal evidence that the six selected isolates do not produce apothecia *in vitro* (Casonato, Senior Lecturer Lincoln University, pers. comm., 2021). This will be tested by storing sclerotia of all of the isolates under optimal conditions for apothecial production and excluding apothecia producing isolates from further tests (Phillips, 1987).

The growth rates and sclerotia production of each isolate in response to different light and temperature conditions will also be investigated in this chapter. Sclerotia of each of the isolates will be cultured on agar under a range of temperature and light conditions. The growth rates and sclerotia production will be compared between the *S. sclerotiorum* isolates. The experiments in this chapter were designed to be preliminary tests, to give some insight into the initial set of conditions to be used in the fermentation to maximize produce bioherbicide propagules. Future work will be needed to optimize the fermentation.

Finally, the pathogenicity of the isolates towards *R. acris* and *C. arvense* will be examined. This will be done through excised leaf bioassays in which leaves of *R. acris* and *C. arvense* will be inoculated with mycelium of each of the isolates and the development of lesions will be compared. While not comparable to the results of a whole plant trial or field trial, excised leaves provide an accessible method for such a preliminary stage of testing.

The hypothesis to be tested in this chapter are:

1. Biological control isolates of *S. sclerotiorum* will not produce apothecia under *in vitro* conditions that favour carpogenic germination in other isolates*.*

2. Growth rates and sclerotia production of *S. sclerotiorum* isolates vary in response to abiotic conditions.

3. The pathogenicity of *S. sclerotiorum* towards *C. arvense* and *R. acris* varies among isolates.

4. Isolates of *S. sclerotiorum* excised from one target weed species are more pathogenic towards that weed than the alternative host species.

2.2 Materials and Methods

2.2.1 Apotheica production in candidate bioherbicide isolates of *S. sclerotiorum*

2.3 Petri plates (referred to as plates henceforth) containing potato dextrose agar (PDA) were inoculated in a laminar flow with stored *S. sclerotiorum* sclerotia of each isolate: S36, S37, GGB1, GGB2, G57, G64 and CS14 (Table 2.1). One sclerotia was placed into the centre of each plate. Plates were incubated at 20 °C in 24 hr dark for two weeks to grow the cultures which produced sclerotia. After two weeks, two sclerotia originating from a single *S. sclerotiorum* isolate, were removed from the PDA plates and placed aseptically into a McCartney vial containing water agar prepared as in Appendix A.1. Ten replicate vials, each containing two sclerotia, were prepared for each of the seven *S. sclerotiorum* isolates. Only whole sclerotia of a size >2mm were selected, and any sclerotia lacking a complete rind or appearing to be multiple sclerotia that had coalesced, were excluded. These were placed into ten randomized blocks and incubated at 18 °C under a 14 hr light, 10 hr dark photoperiod in a top-lit incubator with LED lights (Memmert, Peltier-cooled incubator IPP410eco, Cold White 6500K). The tubes were checked once every week for 8 weeks, then once every 8 weeks thereafter (had been planned more frequently but adjusted due to disruption from COVID-19). The sclerotia in each tube were assessed for stipe development upon which apothecia are borne. Any development of a stipe was noted, defined as a sclerotia whereby the rind had ruptured, and an

accompanying length of compacted mycelium, a stipe had begun growing. A positive control was included, isolate CS14, which was previously observed to produce apothecia after 16 weeks (Madhavi Dassanayaka, PhD student Lincoln University, pers. comm., 2021).

2.3.1 *In vitro* Growth Rates and Sclerotia Production of *S. sclerotiorum* isolates

Sclerotia of each of the six *S. sclerotiorum* isolates (S36, S37, GGB1, GGB2, G57 and G64) were aseptically harvested from 10 day old cultures growing on PDA plates at 20 °C. Each isolate culture had been recovered from stored stock culture on agar fragments kept at -80 °C in glycerol and subcultured three times to ensure pure culture. The only exception was S37, where the culture was 28 days old and had been subcultured twice from -80 °C stored stock cultures. An individual sclerotia was transferred into the centre of a fresh 1/5 strength PDA plate (1/5 PDA). Only whole sclerotia of a size >2mm were selected, and any sclerotia lacking a complete rind or appearing to be multiple sclerotia that had coalesced were excluded. It is important to note, the sclerotia used within this study were not cold pre-conditioned. For each isolate, 60 plates were prepared. On the underside of these plates, a mark where the sclerotium was placed and two perpendicular lines intersecting the centre point, were made (Figure 2.1). The 60 plates of each isolate were randomly assigned, and 20 plates were placed into individual incubators at 15 °C, 20 °C and 25 °C with a 12 hr photoperiod (12 hr light: 12 hr dark) (Contherm, BioSyn 6000CP, Cold white 6500K bulbs). For each of the 20 plates containing an individual isolate, 10 were wrapped in tinfoil to simulate 24 hr darkness. There were 10 replicates of each isolate and illumination per temperature regime: 12 hr photoperiod or 24 hr dark and isolate S36, S37, GGB1, GGB2, G57 or G64. Plates were set up into 10 blocks in a completely randomised block design. The experimental design is as follows:

6 isolates * 3 temperatures (3 incubators each at 15 °C, 20 °C and 25 °C) * 2 illumination treatments (12 hr photoperiod, 24 hr dark).

After 96 hr, the growth of mycelium from the initial sclerotium was recorded along the lines previously marked onto the plate (Figure 2.1). Fourteen days after inoculation, the number of sclerotia on each plate were counted and weighed. Only mature sclerotia were counted, defined by being completely melanised as in Figure 2.2. The mature sclerotia from each culture were then removed using tweezers and placed into 1.5mL Eppendorf tubes, ensuring no media was transferred. The weight of the sclerotia was measured by weighing the Eppendorf tube on scales zeroed with an empty tube. The average sclerotia weight was calculated for each plate by dividing the total sclerotia weight by the number of completely melanised sclerotia present.

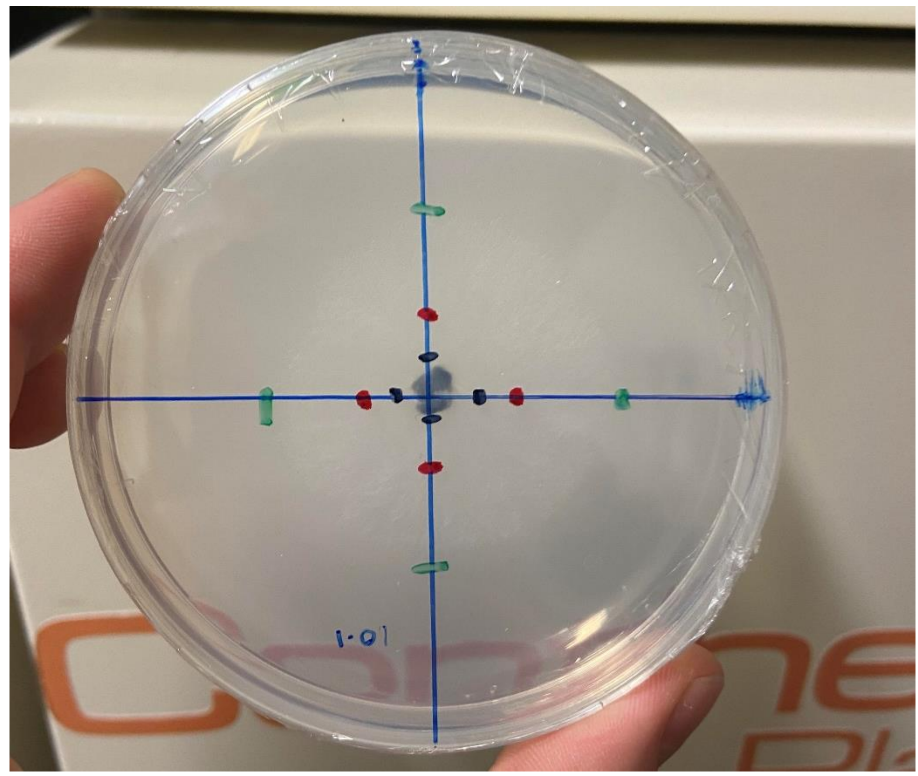

Figure 2.1 Example plate showing centre point, perpendicular lines and growth assessments after different durations shown by coloured dashed lines.

The data were checked for normality and outliers were removed. S37 grew abnormally, potentially due to contamination, and was therefore excluded from statistical analysis. The growth data were analysed using a TOBIT (syn. censored regression model) linear mixed model, with right censoring at 85 mm, as this was the maximum possible growth measurement due to the mycelium in some plates reaching the edge of the Petri plate (Ø 90 mm) by the day 4 measurement. The other response data collected were analysed using linear mixed models.

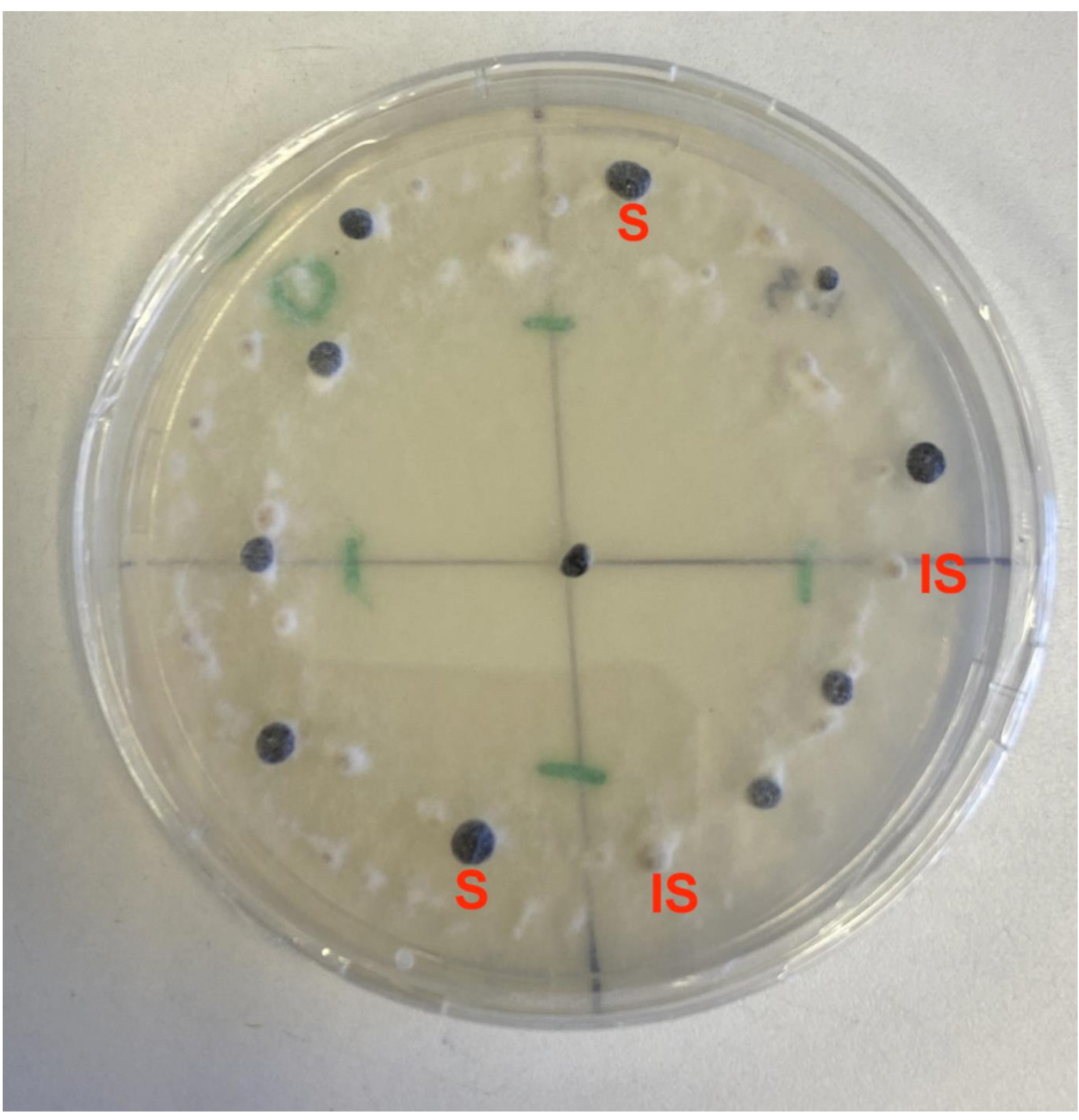

Figure 2.2 Example plate of *S. sclerotiorum*. Green lines mark the colony growth after 96 hr. Mature sclerotia (S) were counted compared. Immature sclerotia (IS) were not counted.

2.3.2 Pathogenicity Assay on Detatched Leaves

Sixty leaves were collected from *C. arvense* (G20 provenance) and *R. acris* (J haplotype) plants grown in the Lincoln University shadehouse that received natural daylight hours and was at ambient temperature. The plants were grown in potting mix in 3 L pots (Appendix A.2) From each of 10 individual plants for each species, six leaves were collected. The leaves were then pooled together. Both species of plants had aphids at the time of harvesting and the leaves were surface sterilised.

Surface sterilisation was undertaken in a laminar flow hood by washing in 1% bleach (MaxKleen™ Pure; active sodium hypochlorite ~10%) solution for 1 min, then double rinsed in sterile water for an additional 1 min each. Each leaf was placed into a clear lidded 750 mL container onto a paper towel (Tork™; Green Singlefold Hand Towel) that was folded to fit into the container and then moistened with 20 mL of sterile water.

Six isolates (S36, S37, GGB1, GGB2, G57 and G64) of *S. sclerotiorum* had been previously cultured on PDA plates. Agar plugs (15 mm Ø) from 7 day old cultures of these isolates were excised using a sterile 10 mL Falcon tube as a guide, and transferred to the central lobe of each *R. acris* leaf and the centre of each *C. arvense* leaf. Ten replicates of each combination of plant species and isolate of *S. sclerotiorum* was prepared. Leaves within each container were arranged into 10 replicate blocks in an incubator set at 20 °C under a 12 hr photoperiod. Images of leaf lesions were taken after 6 days with a camera and the lesion area measured using ImageJ (Schneider, Rasband, & Eliceiri, 2012). The lesions size was recorded, along with the leaf size, using a known object to calibrate the scale of each image. Differences were compared using ANOVA in GenStat (VSN International, 2022) with leaf size as a covariate and multiple comparisons undertaken using a post-hoc Bonferroni test (95%).

2.4 Results

2.4.1 Apothecia Production

After 10 weeks of incubation, stipes were observed on two sclerotia of the positive control isolate CS14 (10% of the CS14 sclerotia included in trial) across the vials. After 18 weeks, eight of the CS14 sclerotia had developed stipes (40% of the CS14 sclerotia included in trial). At 24 weeks, 13 sclerotia of CS14 (positive control) had developed stipes (65% of the CS14 sclerotia included in trial). No signs of stipe development was observed in any replicate vials of the other isolates (S36, S37, GGB1, GGB2, G57 and G64). At this point, the trial was concluded. Most of the stipes produced by CS14 did not develop into complete apothecia with caps, but only the presence or absence of any initiation of apothecia was measured with no differentiation between apothecia at different stages of development. Figure 2.3 shows two sclerotia of CS14, both of which have produced stipes, where one stipe has differentiated into an apothecium by producing a cap.

Figure 2.3 Apothecia of CS14 isolate of *S. sclerotiorum* after 24 weeks incubation. Both sclerotia would have been assessed as having initiated stipe development. C indicates the apothecia cap, while S labels the stipe.

2.4.2 Growth Rates and Sclerotia Production

2.3.2.1 Growth after 96 hr

The isolate S37 performed abnormally and had inconsistent sclerotia germination and was excluded from the analyses in this section. All other isolates germinated and grew on agar under all temperature and illumination treatments. Table 2.2 shows the growth rates of all the isolates expressed in mm per day. The total growth split by all isolates and treatments is shown in Figure 2.4

Table 2.2 Growth rates (mm per day) of different isolates of *S. sclerotiorum* under different temperature and illumination conditions, grown on 1/5 PDA for 96 hr. Letters represent significantly different groups from the results of a Bonferroni test at P =0.05

Temperature (°C)	Illumination	Isolate				
		G57	G64	GGB1	GGB2	S36
15	Dark	15.3^{cde}	10.8^{a}	13.8^{bc}	16.7^{defg}	12.3^{ab}
	Light	19.1^{ghijk}	13.9^{bcd}	17.6^{efghi}	19.7^{hijkl}	15.7^{cdef}
20	Dark	18.9^{ghijk}	18.2^{fghij}	20.1^{ijkl}	19.6^{hijkl}	16.1^{cdef}
	Light	17.8^{efghi}	17.3^{efgh}	20.3^{jkl}	19.9^{hijkl}	16.8^{efg}
25	Dark	20.0^{jkl}	19.2^{hijkl}	20.6^{kl}	21.3^{m}	19.0^{ghijk}
	Light	20.5^{kl}	20.5^{jkl}	21.0^{l}	21.3^{m}	20.5^{jkl}

There was a significant interaction between isolate and temperature (P < 0.001), although the F-statistic was small in comparison to the factors independently (16.35). There were significant differences (P < 0.001) in the growth after 96 hr between different temperature treatments. Based on the F-statistics, temperature is the factor that accounts for the most variance seen in the growth response (F-statistic = 368.85). Averaged across all isolates and illumination, growth was greatest at 25 °C at 88 mm per 96 hr, decreasing to 74 mm per 96 hr at 20 °C and 62 mm per 96 hr at 15 °C (Figure 2.4). There was no significant (P = 0.618) three-way interaction between isolate, temperature and illumination.

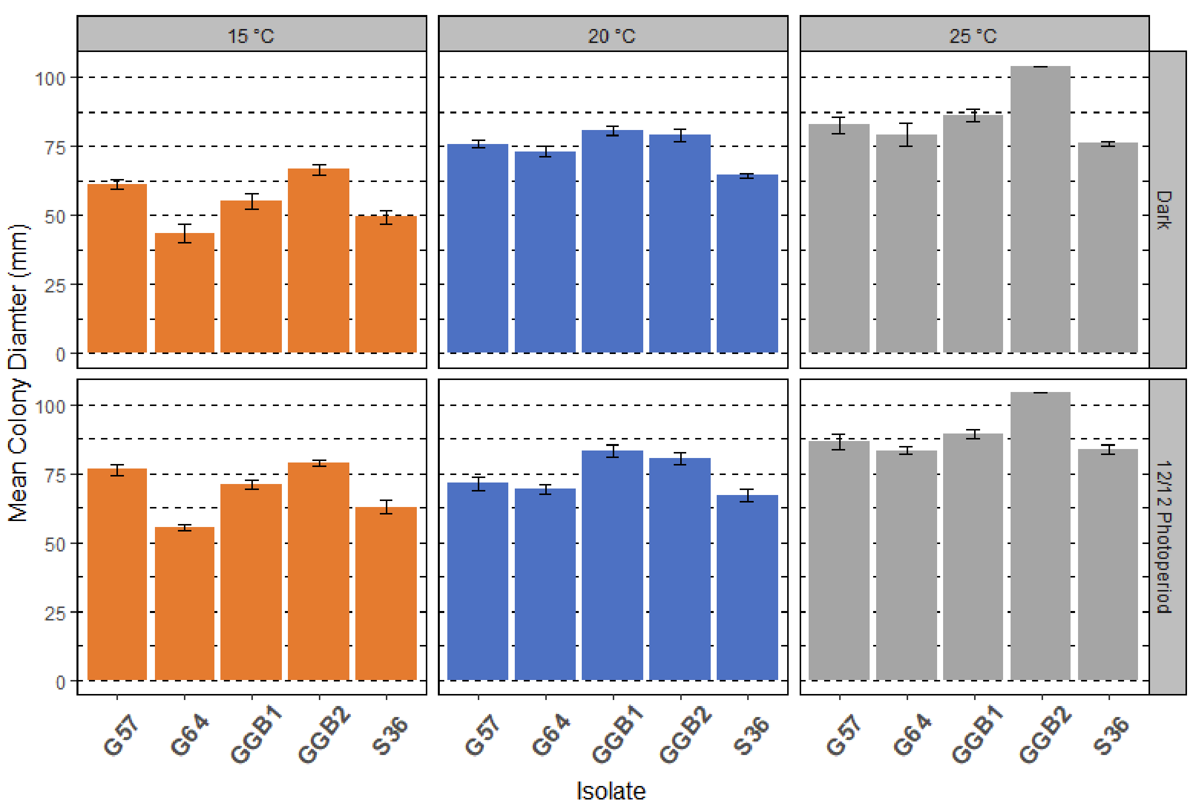

Figure 2.4 Mean growth of five *S. sclerotiorum* isolates (G57, G64, GGB1, GGB2, S36) after 4 days across three incubators set at different temperatures (15 °C, 20 °C, 25 °C) with two light treatments (dark or 12 hr photoperiod). Error bars indicate standard error.

The mean growth after 4 days averaged across all temperature and light treatments was significantly (P < 0.001) different among the isolates of *S. sclerotiorum* (Figure 2.5). Isolate was the factor with the second largest F-statistic (156.31). Averaged across all temperature and light treatments, *S. sclerotiorum* isolate GGB2 was the fastest growing reaching a mean diameter of 86 mm after 96 hr which differed significantly (P <= 0.001) from all other isolates (Figure 2.5). *S. sclerotiorum* isolates G57 and GGB1, which grew to a mean diameter of 75 and 77 mm respectively after 96 hrs (Figure 2.5), did not significantly differ (P > 0.05) from each other but did differ significantly (P < 0.05) from

isolates G64 and S36. *S. sclerotiorum* isolates G64 and S36 did not differ significantly (P > 0.05) after 96 hrs with both reaching a mean diameter of 67 mm (Figure 2.5).

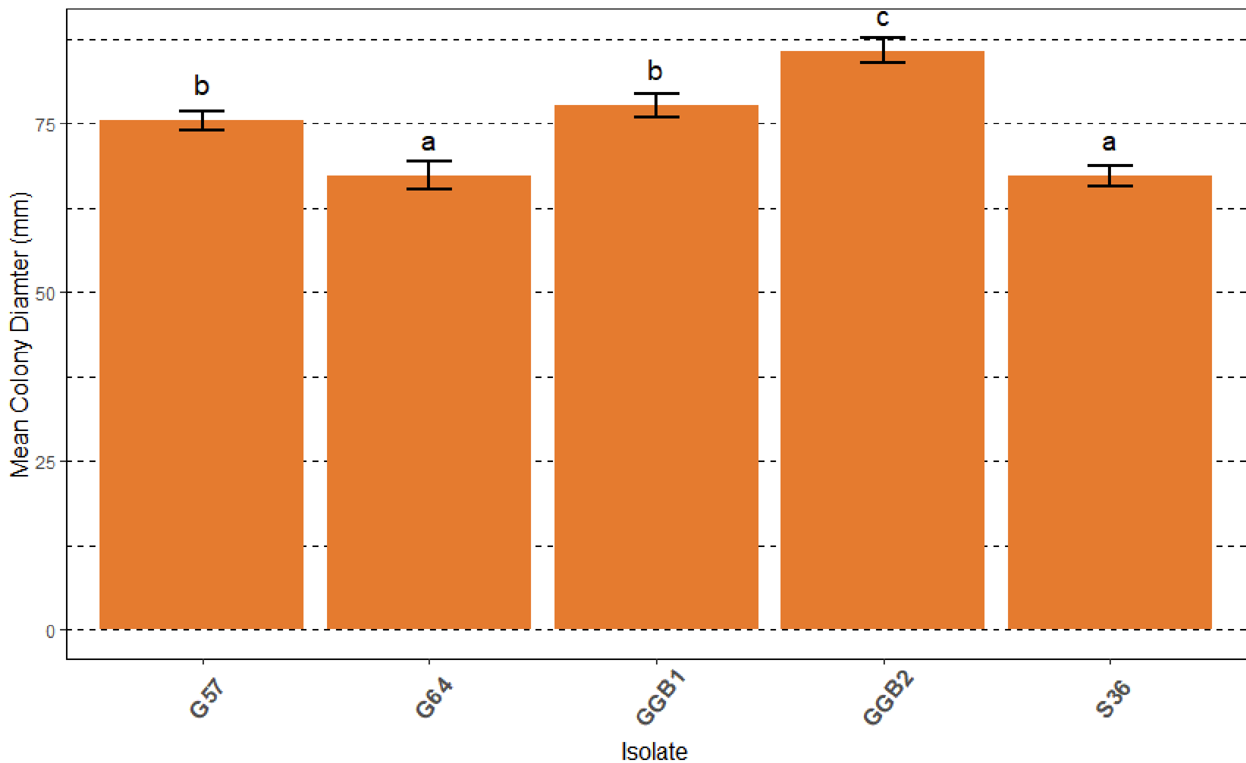

Figure 2.5 Mean growth of five isolates of *S. sclerotiorum* averaged across all illumination (dark or 12 hr photoperiod) and temperature (15 °C, 20 °C, 25 °C) treatments. Error bars indicate standard error. Letters indicate significantly different groups from the results of a Bonferroni test at P=0.05.

Illumination (12 hr photoperiod or 24 hr dark) had a significant (P < 0.001) effect on the mean colony diameter. The F-statistic for illumination was much smaller than the other factors, indicating that this factor is contributing to a lesser amount of variation in the growth response (F-statistic = 28.11). Mean growth under the 12 hr photoperiod was significantly higher for isolates GGB1 and S36 (P <= 0.05) compared to growth in darkness (Figure 2.6). For the other isolates, the mean growth under the 12 hr photoperiod was greater than in darkness, however the differences were not significant at P=0.05. Averaged across all isolates and temperatures the mean predicted growth after 96 hr was 71.9 ± 1.3mm in the dark and 77.4 ± 1.1mm under a 12 hr photoperiod. Overall there was no significant interaction between isolate and illumination (12 hr photoperiod or 24 hr darkness) (P = 0.455).

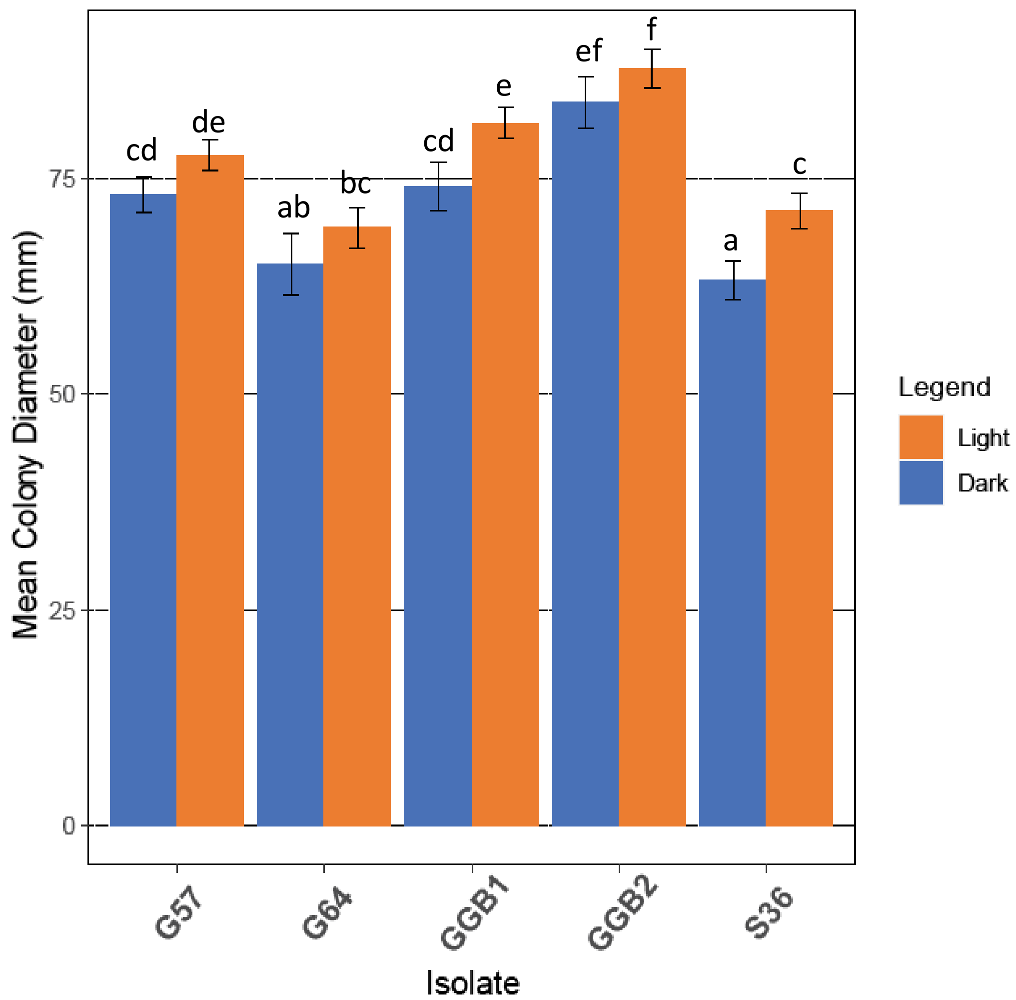

Figure 2.6 Mean diameter of five isolates of *S. sclerotiorum* under either a 12 hr photoperiod (orange bars) or darkness (blue bars) averaged across three temperature (15 °C, 20 °C, 25 °C) treatments. Error bars indicate standard error of the mean (SEM). Letters indicate significantly different groups from the results of a Bonferroni test at P =0.05.

2.3.2.2 Day 14 Sclerotia

At day 14 there was a significant interaction between isolate and temperature (P < 0.001) and the number of sclerotia produced. For *S. sclerotiorum* isolates S36 and GGB2, the number of sclerotia produced increased significantly at 25 °C compared to at 15 °C (Figure 2.7). For G57 there was no significant difference in sclerotia production across any of the temperature treatments (P > 0.05) (Figure 2.7). For isolate G64, 15.4 ± 0.9 per sclerotia per plate were produced at 20 °C, which was significantly greater than the number of sclerotia produced at 25 °C which was 10.0 ± 0.9, but was not significantly different from the number produced at 15°C which was 11.2 ± 0.4 (Figure 2.7). Sclerotia produced by S36 at 25 °C, resulted in the highest average number of sclerotia per plate at 22.4 ± 0.9, and this treatment was significantly different from all other treatment combinations (P = 0.05). Temperature also had a significant (P < 0.001) effect on the number of sclerotia produced

after 14 days. Across all isolates a mean of 10.1 ± 0.3, 12.2 ± 0.5 and 13.1 ± 0.5 sclerotia were produced at 15 °C, 20 °C and 25 °C, respectively.

At day 14, there were significant (P < 0.001) differences in the number of sclerotia among the five isolates (G57, G65, GGB1, GGB2 and S36) of *S. sclerotiorum* when averaged across temperature and illumination treatments. The most sclerotia per plate were produced by isolate S36 with an average of 16.1 ± 0.7 per plate, followed by GGB2 with 12.3 ± 0.6, G57 with 13.3 ± 0.7, G64 with 12.2 ± 0.6, and GGB1 with 10.4 ± 0.5 per plate. Illumination (12 hr photoperiod and 24 hr dark) did not have a significant effect on the number of sclerotia produced (P = 0.580). There were no significant interactions between illumination and temperature (P = 0.788) or illumination and isolate (P = 0.200), and the three-way interaction of illumination, temperature and isolate was not significant (P = 0.897).

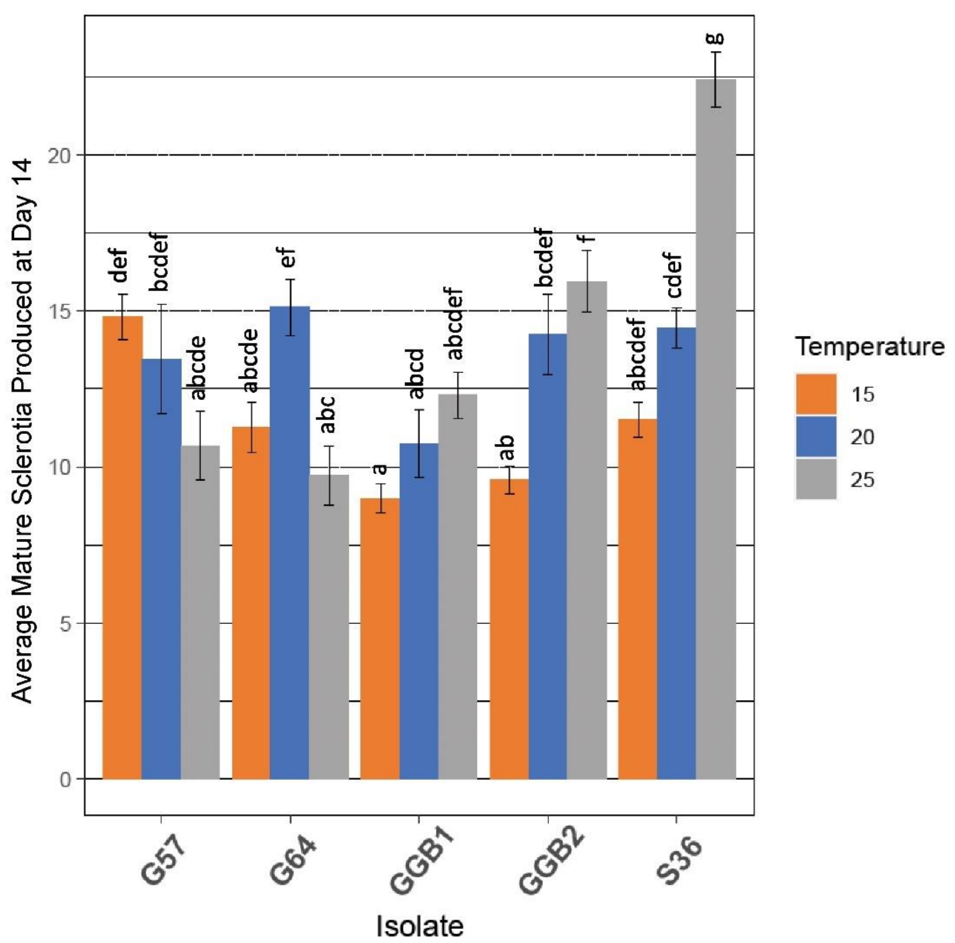

Figure 2.7 Average number of sclerotia produced by six isolates of *S. sclerotiorum* after 14 days at different temperature (15 °C in orange, 20 °C in blue, 25 °C in grey) treatments. Average across both illumination treatments (dark or 12 hr photoperiod). Error bars indicate standard error of the mean (SEM). Letters indicate significantly different groups from the results of a Bonferroni test at P=0.05.

2.3.2.3 Total Sclerotia Weight

The interaction between temperature and isolate was significant (P < 0.001). Total sclerotia biomass was significantly higher at 15 °C than at 20 °C and 25 °C, for all isolates other than S36 at P =0.05. Isolates G57 and G64 produced significantly more biomass of sclerotia at 20 °C than at 25 °C (P=0.05). The other isolates also produced more biomass on average at 20 °C than at 25 °C however the difference was not significant (P > 0.05). At 15 °C, *S. sclerotiorum* isolates G57, G64, GGB1 and GGB2 produced respective total sclerotia weights per plate of 0.128 ± 0.004 g, 0.148 ± 0.005 g, 0.149 ± 0.004 g and 0.136 ± 0.003 g, which was almost double the biomass the same isolates produced at 25 °C at 0.071 ±0.003 g, 0.073 ± 0.004 g, 0.069 ± 0.002 g and 0.085 ± 0.003 g respectively (Figure 2.8). The differences in mean total sclerotia weight per plate across each of the temperature treatments was not as pronounced for S36, although the trend was the same with mean weights averaging 0.134 ± 0.004 g at 15 °C, decreasing to an average of 0.108 ± 0.003 g at 25 °C. For S36 the difference between total sclerotia weight at 20 °C was not significantly different to the weights at 15 °C or 25 °C at a 95% confidence interval.

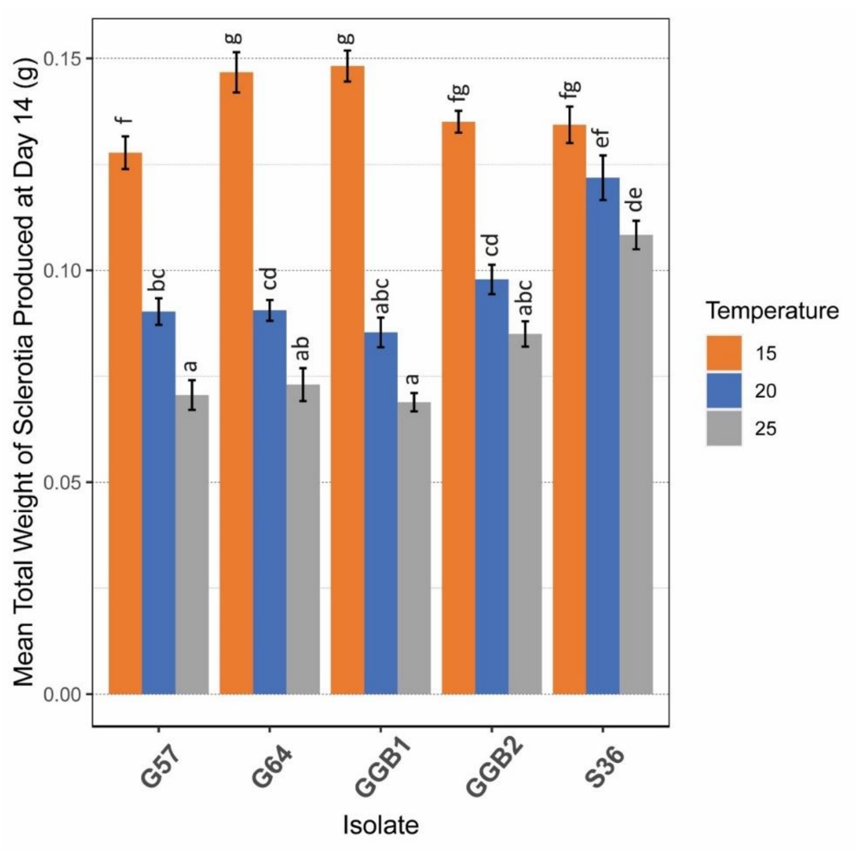

Figure 2.8 Mean total weight of sclerotia produced after 14 days of incubation of five isolates of *S. sclerotiorum* under different temperatures (15 °C in orange, 20 °C in blue, 25 °C in grey), averaged across illumination treatments (dark or 12 hr photoperiod). Error bars indicate standard error of the mean (SEM). Letters indicate significantly different groups from the results of a Bonferroni test at P=0.05.

There were significant (P < 0.001) differences in total sclerotia weight per plate between isolates of *S. sclerotiorum*. Averaged across all temperature and light treatments, S36 produced significantly more sclerotia biomass than the other isolates with an biomass of 0.122 ± 0.02g (Figure 2.9). This was driven by the increased weight of S36 at lower temperatures (15 °C and 20 °C) compared to the other isolates (Figure 2.8). G64, GGB1 and GGB2 did not significantly differ in biomass from each other producing 0.105 ± 0.014g, 0.101 ± 0.013g and 0.106 ±0.014g respectively (P = 0.05). G57 differed significantly from all isolates other than GGB1 (P = 0.05), producing an average sclerotial biomass of 0.097 ± 0.12g.

Temperature also had a significant effect (P < 0.001) with the total weight of sclerotia produced decreasing with increasing temperature from 0.139 ± 0.002g at 15 °C, to 0.0977±0.002g at 20 °C, to 0.81 ±0.002g at 25 °C. Illumination also had a significant (P = 0.016) effect on the mean sclerotia biomass per plate. On average the biomass of sclerotia per plate was increased in the dark at 0.109 ±0.003g compared to 0.104±0.002 in the light, although the difference is small.

The interactions between isolate and illumination, and temperature and illumination were not significant (P = 0.053 and P = 0.659 respectively). The three-way interaction between isolate, temperature and illumination was also not significant (P = 0.261).

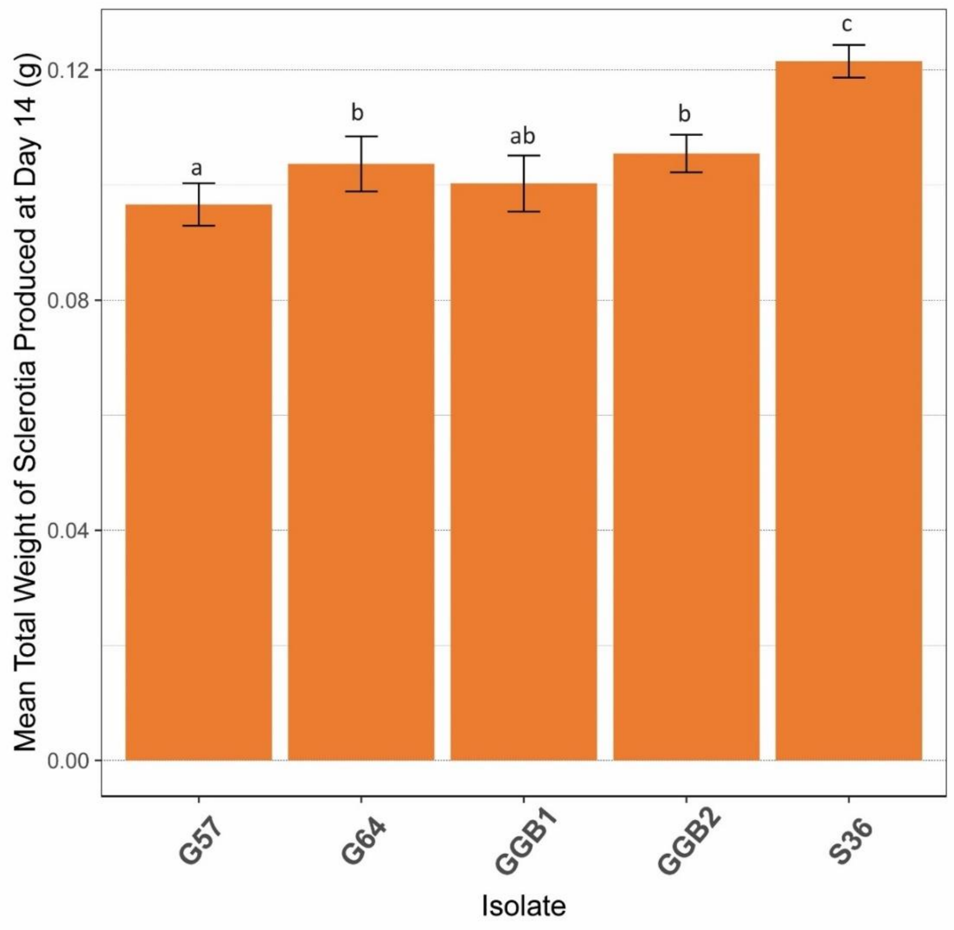

Figure 2.9 Mean sclerotia biomass per plate produced by six isolates of *S. sclerotiorum* averaged across temperature (15 °C, 20 °C, 25 °C) and illumination treatments (dark or 12 hr photoperiod). Error bars indicate standard error of the mean (SEM). Letters indicate significantly different groups from the results of a Bonferroni test at P=0.05.

2.3.2.4 Average sclerotia weight

There was a significant interaction between isolate and temperature that resulted in differences in average sclerotia weight (P < 0.001). There were no significant differences in the average weight of sclerotia at 20 °C compared to 25 °C for any of the isolates at P = 0.05. For G57, there was no significant difference (P = 0.05) in average sclerotia weight across the temperature treatments of 15, 20 and 25 °C. In contrast, isolates G64, GGB1, GGB2 produced significantly (P < 0.05) heavier sclerotia at 15 °C compared to 20 °C and 25 °C (Figure 2.10). The difference was most pronounced in GGB1 with an average sclerotia weight of 17.4 ± 1.2mg at 15 °C, decreasing significantly to 6.2 ± 0.7mg at 25 °C (P = 0.05). Isolate S36 produced significantly heavier sclerotia on average at 15 °C (12.1 ± 0.5mg) compared to 25 °C (5.0 ± 0.3mg), while the difference in biomass between 15 °C and 20 °C (8.5 ± 0.25mg), and 20 °C and 25 °C was not statistically significant (P = 0.05). The interactions between isolate and illumination, and illumination and temperature were not significant (P = 0.141 and P = 0.058 respectively).

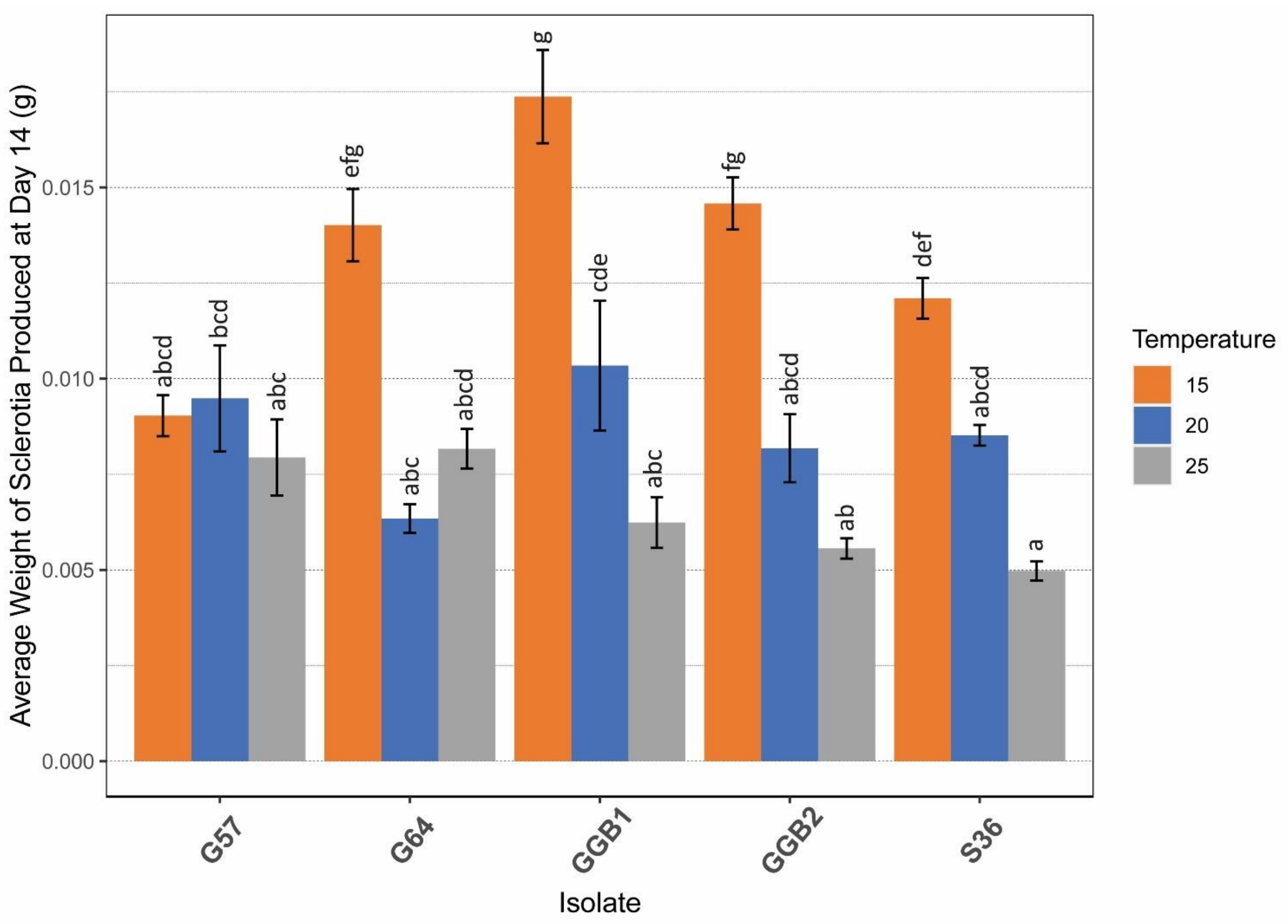

Figure 2.10 Average sclerotia weight (g) after 14 days growth of five isolates of *S. sclerotiorum* grown at three different temperatures (15 °C in orange, 20 °C in blue, 25 °C in grey). Data averaged across illumination treatments (dark or 12 hr photoperiod). Error bars indicate standard error of the mean (SEM). Letters indicate significantly different groups from the results of a Bonferroni test at P=0.05.

There were significant (P = 0.032) differences between *S. sclerotiorum* isolates in average sclerotia weight averaged across all temperature and illumination treatments (Figure 2.11). Isolates G64, GGB1 and GGB2 produced the heaviest sclerotia on average (9.5 ± 0.6 mg, 11.2 ± 0.9 mg, 9.4 ± 0.6 mg, respectively) and did not differ significantly (P = 0.05). GGB1 produced the heaviest sclerotia on average (11.2 ± 0.9 mg) and differed significantly (P < 0.05) compared with isolates G57 and S36, which produced sclerotia with the lowest average weight (8.8 ± 0.6 mg, 8.5 ± 0.4 mg respectively) (Figure 2.11). The average sclerotia weight of isolates G57 (8.8 ± 0.6 mg), G64 (9.5 ± 0.5mg), GGB2 (9.4 ± 0.9mg) and S36 (8.5 ± 0.4 mg) did not significantly differ averaged across all temperature and illumination treatments. Significant differences existed between temperatures (P < 0.001) averaged across all isolates and illuminations, with the greatest average sclerotia weight at 15 °C at 13.4 ± 0.4 mg, decreasing to 8.6 ± 0.5 mg at 20 °C, and decreasing again to 5.0 ± 0.3 mg at 25 °C. There was also a significant difference in average sclerotia weight between illumination treatments (P = 0.036).

The average weight of sclerotia produced in the dark was marginally significantly (P = 0.05) heavier at 10.1 ± 0.5 g compared to 8.9 ± 0.3 g when produced under a 12 hr photoperiod when averaged across isolate and temperature treatments.

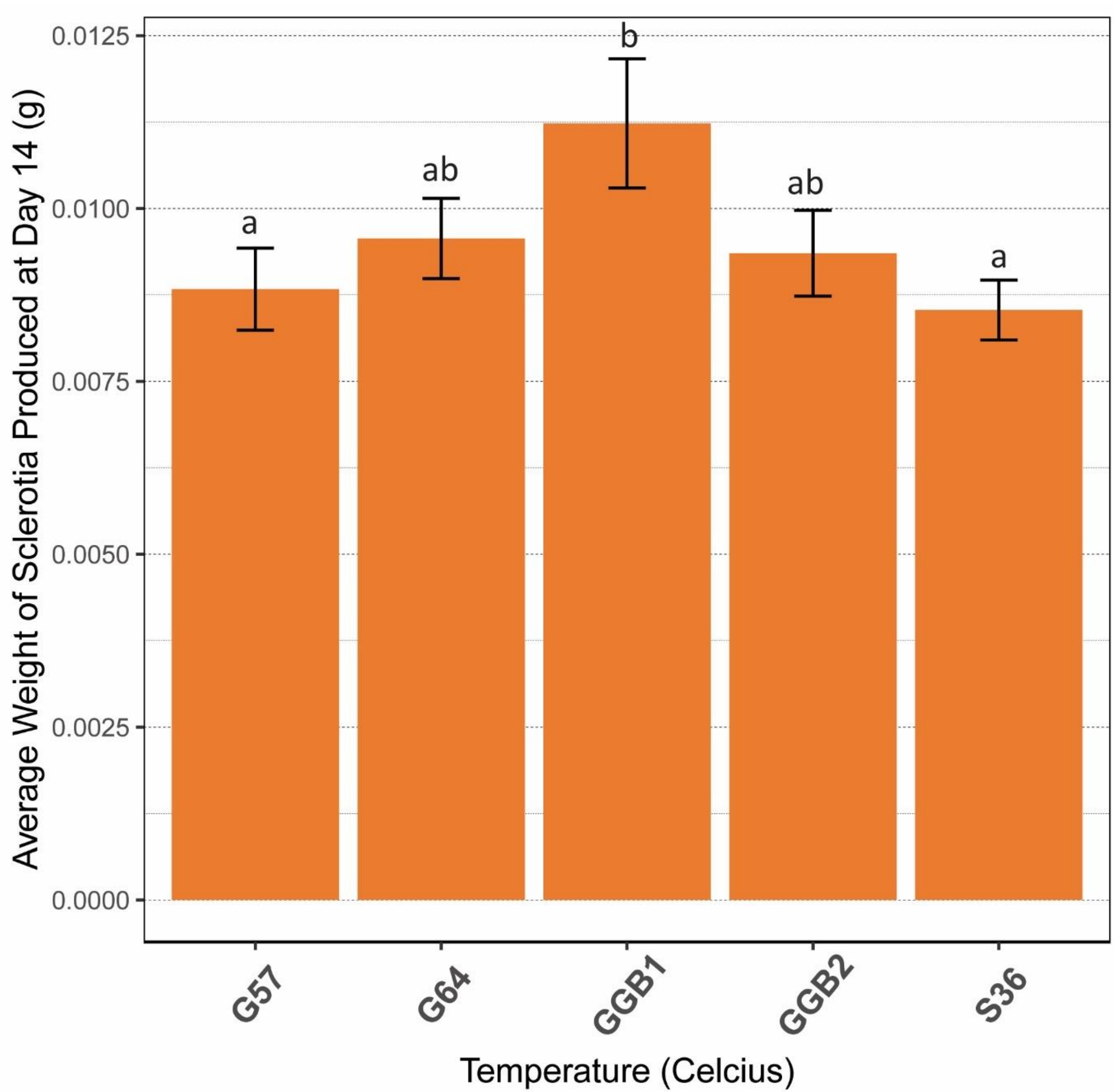

Figure 2.11 Differences in average sclerotia weight among six isolates of *S. sclerotiorum* averaged across three temperature (15 °C, 20 °C, 25 °C) and two light treatments (dark or 12 hr photoperiod). Error bars indicate standard error of the mean (SEM). Letters indicate significantly different groups from the results of a Bonferroni test at P=0.05.

2.4.3 Pathogenicty Comparison

There were significant differences in lesion size between target species (P = 0.001), with the mean lesions resulting from the six *S. sclerotiorum* isolates being significantly smaller on *R. acris* than on *C. arvense* (48.4% compared to 94.9%). The effect of the isolate of *S. sclerotiorum* on the average

lesion size was significant (P = 0.001). Analysing each target species independently, GGB1 caused the smallest lesions on *C. arvense* with an average necrotic area of 41.9% after 6 days, compared to the other isolates that all caused lesions covering between 86 to 99.3% of the leaf area (Figure 2.12, left trellis) On *R. acris* the mean necrotic area percentage varied among isolates with lesions caused by both S36 and G57 being significantly smaller than those caused by GGB2 and S37 (P = 0.037)(Figure 2.12, right trellis). G57 and G64 both produced smaller lesions on *R. acris* than on *C. arvense* which resulted in the significant interaction between isolate and target species (P = 0.001). The host that the isolates of *S. sclerotiorum* were originally excised from did not have a significant effect on the necrotic area observed on either target weed (P = 0.365). Isolates originally excised from *R. acris* caused lesions that covered an average of 71.4 ± 5.4% of the leaf area after 6 days, compared to isolates from *C. arvense* that caused lesions that covered an average of 75.8 ± 3.7% of the leaf area.

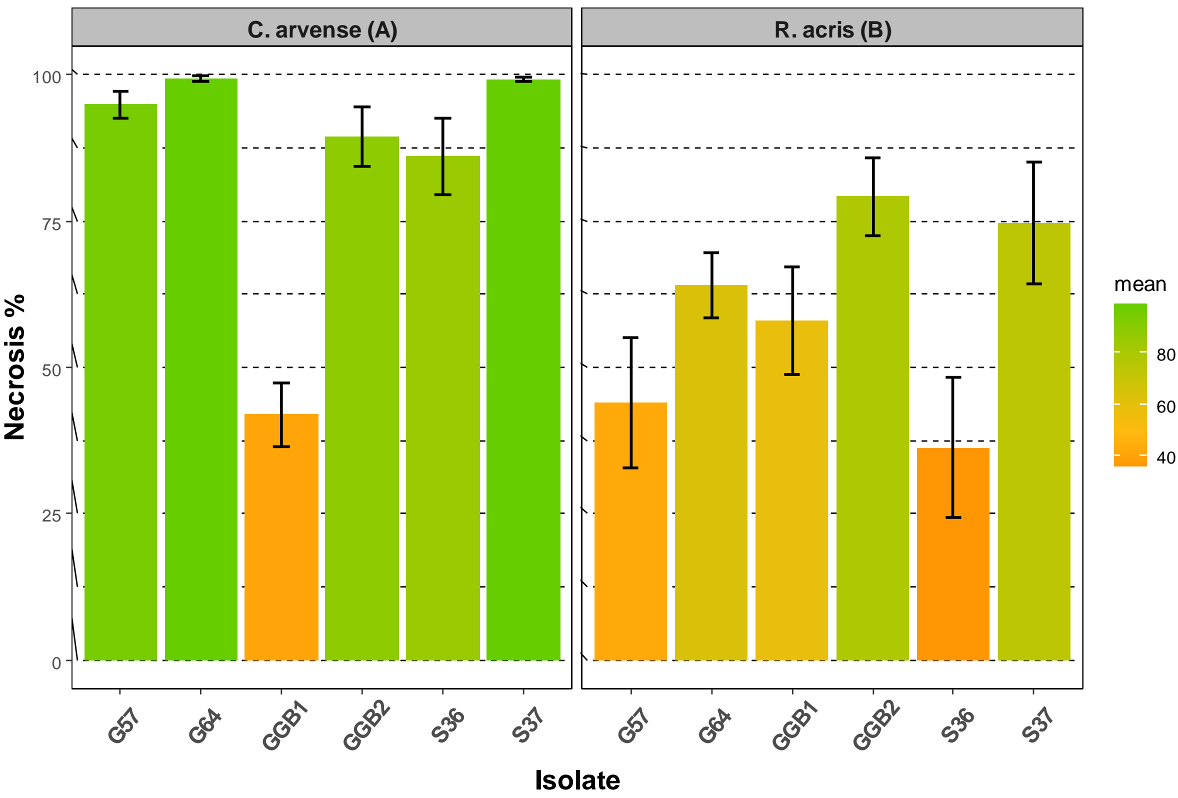

Figure 2.12 Necrotic leaf area of detached leaves of *C. arvense* (A) and *R. acris* (B) six days after inoculation with different isolates of *S. sclerotiorum*. Error bars indicate standard error of the mean (SEM.

2.4.4 Isolate Combined Performance Score

Using the results collected from the previous experiments it was possible to create a performance index score that collates information from across the experiments to predict which isolate will be

the best to use for a bioherbicide. A growth score was calculated from the results of the growth on agar trial, along with a pathogenicity score from the detached leaf assays.

$$1.\ Growth\ Score\ =\ Mean\ predicted\ growth\ after\ 4\ days\ \times (Average\ sclerotia\ weight * 0.5)$$
$$(scaled\ so\ highest\ performing\ isolate\ recives\ a\ value\ of\ 1)$$

$$2.\ Pathogenicity\ Score = \ Average\ lesion\ size\ on\ host\ leaf\ after\ 6\ days$$
$$(expressed\ as\ proportion\ of\ total\ leaf\ area)$$

$$3.\ Performance\ Score = Growth\ score + \ Pathogenicity\ Score\ on\ C.arvense + Pathogenicity\ Score\ on\ R.acris$$

The overall rankings of the isolates in terms of performance score from highest to lowest was GGB2 at 2.83, G64 at 2.54, G57 at 2.32, GGB1 at 2.15 and S36 at 1.98 (Figure 2.13). The highest possible score in any one index was 1, making the maximum possible score in the combined performance index 3. GGB2 had the fastest growth averaged across all temperatures and illuminations (Figure 2.5), was the most pathogenic isolate on *R. acris,* and also performed well on *C. arvense* (Figure 2.13). From the performance score rankings, S36 was the least favourable isolate to use for bioherbicide development.

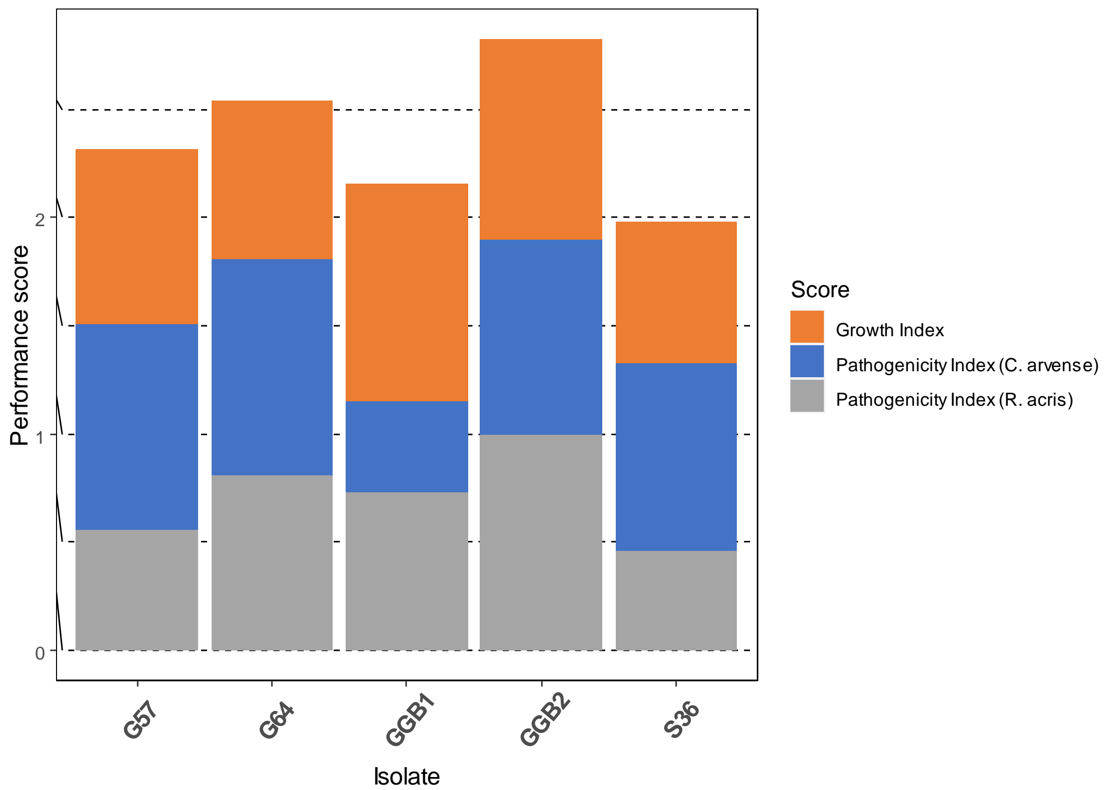

Figure 2.13 Performance scores for each of the tested isolates of *S. sclerotiorum*. The height of the bars indicates the performance score which factors in the relative growth of the

isolates (orange), as well as relative performance on both target hosts *C. arvense* (blue) and *R. acris* (grey).

2.5 Discussion

2.5.1 Apothecia production.

None of the *S. sclerotiorum* isolates chosen as potential candidates for inundative biocontrol developed apothecia in this current study, and as such they appear to only myceliogenically germinate under the given experimental conditions. Only one *S. sclerotiorum* isolate tested, CS14, produced apothecia under the conditions. This isolate was included as a positive control for apothecial production. The majority of the germinating sclerotia of CS14 only produced one to two apothecia initials that never matured into an ascocarp, though some, as indicated in Figure 2.3, did develop completely into an apothecium. There are several reasons why the other isolates may not have produced apothecia. Cold conditioning of sclerotia was not used in this study, and this may have contributed to the isolates not producing apothecia. The positive control, isolate CS14 produced relatively few apothecia, indicating cold conditioning was required as this isolate had been observed to readily produce apothecia in culture (Madhavi Dassanayaka, PhD student Lincoln University, pers. comm., 2021). Cold conditioning has been shown to be important to induce carpogenic germination in some studies (Kohn, 1979), but others have found that cold conditioning is not required (Bedi, 1956). For a New Zealand isolate of *S. sclerotiorum,* Kalvelage (1996) found no significant effect of cold conditioning at 10 °C for eight weeks on the rates of carpogenic germination on water agar, but when incubated on sand, cold conditioning significantly increased carpogenic germination. The hypothesis that the biological control isolates do not carpogenically germinate under conditions favourable for carpogenic germination in other isolates can be accepted. However, further experiments should be undertaken to confirm that these isolates do not carpogenically reproduce after cold conditioning.

Another reason the *S. sclerotioroum* isolates S36, S37, GGB1, GGB2, G57 and G64 may not have carpogenically reproduced is that the light and temperature conditions supplied were not optimal. Temperature and light are known to be important factors influencing the carpogenic germination of sclerotia of *S. sclerotiorum* (Abawi & Grogan, 1979; Huang & Kozub, 1991). Reported optimal temperatures for carpogenic germination of sclerotia vary widely (Sun & Yang, 2000). This may be a result of different populations of *S. sclerotiorum* having varying requirements due to local geographic adaptations. With New Zealand isolates of *S. sclerotiorum*, Kalvelage (1996) reported rates of carpogenic germination on sand to be better at 5 °C and 20 °C compared to 15 °C. The temperature used in the current research was 18 °C, which was also used by B. Wu, Peng, Qin, and

Subbarao (2007) to induce carpogenic germination of isolates of *S. sclerotiorum* from lettuce. The temperature during sclerotia formation also influences carpogenic germination. The temperature during formation of sclerotia for the current research was constant (20 °C) and within the range reported to maximize apothecia production (Kalvelage, 1996; Purdy, 1956). The light cycle used in the current research was 14 hr light, 10 hr dark and was based on the results of Kalvelage (1996). Kalvelage (1996) found higher carpogenic germination of New Zealand *S. sclerotiorum* isolates in the light, with a 14 hr photoperiod, than in the dark. Letham (1975) found that apothecia germinated faster and at a higher rate under ambient natural lighting than in total darkness. Light intensity significantly affects the carpogenic germination of sclerotia with more apothecia produced more quickly and in higher quantities under higher light intensities (Sun & Yang, 2000). Light intensity was not measured in the present study but should be included in future experiments to determine if this factor has an effect on apothecial formation.

The results of this experiment suggest that under conditions which are favourable for apothecia production for other *S. sclerotiorum* isolates, isolates S36, S37, GGB1, GGB2, G57 and G64 do not produce apothecia. Apothecial production of the *S. sclerotiorum* isolates, if applied as an innovative biological control agent onto pasture weeds, is incorrectly seen as undesirable due to concerns about increasing inoculum of the pathogen to nearby cropping systems (Sleight, 2001). Investigations have shown that this concern is unfounded for several reasons. Firstly, the isolates of *S. sclerotiorum* used to date in bioherbicide development in New Zealand have all originally been isolated from natural populations of the weed species that are present on farms. No new species are being introduced and the cropping systems already exist in the presence of the pathogen. It could be argued that despite the pathogen already being present, inundative biocontrol is increasing the inoculum load within a local area. Further investigation has shown that this concern is also unfounded. Ascospore load from an inundative application of a *S. sclerotiorum* bioherbicide was undetectable compared to background levels at a maximum distance of 7 m away (Bourdôt et al., 2001). A biological control application of an *S. sclerotiorum* based bioherbicide would limit the use of certain susceptible species such as forage brassicas and fodder beet in the same area of localised application. This could be limited by proposing a withholding period on a future bioherbicide product, with Bourdôt, Saville, Hurrell, Harvey, and De Jong (2000) finding that the sclerotia population declined to background levels after four years in the soils of a Canterbury sheep pastures in which experimental *S. sclerotiorum* bioherbicides had been applied. The isolates that did not produce apothecia in this trial were included in further growth and pathogenicity trials. These will further inform which isolates are the best candidates for bioherbicide development.

2.5.2 Growth Trial

The S37 isolate had abnormal performance in the growth trial and was excluded from analysis. The growth and sclerotia production of this isolate was highly inconsistent and varied between subcultures. Initial cultures of this isolate were maintained in stock at -80 °C and had been subcultured twice, with the third subculture not exhibiting normal growth. The sclerotia used of this isolate were therefore 28 days old, compared to 10 days old for all the other isolates which also may have caused the abnormal performance. A more likely explanation is that the culture of this isolate had become contaminated with a fungal mycoparasite or virus which can significantly reduce the growth of the fungus (Whipps & Budge, 1990; Zeng, Wang, Kirk, & Hao, 2012).

There were significant differences among the six isolates of *S. sclerotiorum* in all the collected response variables (day 4 growth, day 14 sclerotia, total sclerotia weight, and average sclerotia weight). Across all isolates there was increased growth with increasing temperature. This result was consistent with findings for other isolates of *S. sclerotiorum* which exhibited increasing growth at warmer temperatures up to 25 °C (Humpherson-Jones & Cooke, 1977). The fastest growing isolate in the present study was GGB2, which grew the fastest at 25 °C regardless of light conditions to an average predicted diameter of 103.97mm after 96 hr. This rate of growth equates to 25.9 mmd^{-1} is consistent with the findings reported from of an isolate from infected eggplants (*Solanum melongena*) from Kuwait, which grew at 25.6 mmd^{-1} when grown at 25 °C on full strength PDA (Abdullah, Ali, & Suleman, 2008). This study also reported differences in growth rate between 20 and 25 °C on PDA which matched the findings of the current research on 1/5 PDA. The growth of isolate S36 in the current study at 25 °C was 75.95 mm after 96 hr, which was faster than previously reported by Smith (2016) under the same conditions at 68.4mm after 96 hr. The mean growth for all the isolates was significantly greater under a 12 h photoperiod than in complete darkness. This result suggests that light may be an important factor when considering fermentation conditions for bioherbicide production. Commercial fermentation typically uses liquid media and the current results were obtained on solid agar, and it is not known if the same results would be observed with liquid fermentation.

Sclerotia production was significantly affected by temperature and the nature of the interaction between temperature treatments was dependant on the isolate. For *S. sclerotiorum* isolates GGB2 and S36, the number of sclerotia produced was significantly greater at 25 °C than at 15 °C (Figure 2.7). For isolates G57 and GGB1 this trend was not observed with no significant difference across the three temperatures. Isolate G64 produced less sclerotia at 25 °C than at 20 °C, while sclerotia production at 15 °C was not different from at 20 °C or 25 °C. The greatest number of sclerotia were

produced by the *S. sclerotiorum* isolate S36 at 25 °C, with an average of 22.4 sclerotia per plate. In the experiment, temperature treatments were split across three different incubators with only one time replication. A data-logger should be included in future to ensure that the temperature is accurate and therefore the temperature factor is not confounding with the incubator used.

Averaged across all isolates and illuminations, mean sclerotia biomass produced per plate was greatest at 15 °C and for most isolates tended to decrease with increasing temperature, with an almost two-fold reduction at 25 °C compared to 15 °C. A reason for this may be that the fungus is able to accumulate more nutrition from the agar at lower temperatures, however as growth increased with increasing temperature this seems unlikely. Another possible explanation for the decreasing weight is that sclerotia produced at the higher temperature of 25 °C may have less moisture content due to drying out at the time of measurement than those produced at cooler temperatures. It was noted that there was more water condensation (water droplets on the lids) in Petri plates incubated at a higher temperature, suggesting that more water evaporated from the agar and the biomass under these conditions. Future experiments could check if this was the case by drying the sclerotia for a further period before weighing the sclerotia, to enable a comparison of the dry weights. The total yield of sclerotia collected per plate was low compared to other studies that have used PDA, which is not surprising since the media used in this work was 1/5 PDA so provides less nutrition for the culture (Abdullah et al., 2008). Darkness also increased mean sclerotia biomass produced per plate when averaged across all treatments, which differed to the results of Humpherson-Jones and Cooke (1977) who found no significant differences in total sclerotia biomass in dark grown colonies compared to those grown under four different spectra of light (blue, green, red, far red).

The average weight of sclerotia only varied significantly between isolate GGB1, which produced heavier sclerotia than G57 and S36 averaged across all temperature and light treatments (Figure 2.11). The results observed in this study are similar to those from Akram, Iqbal, Ahmed, Iqbal, and Ghafoor (2008) who identified 16 isolates of *S. sclerotiorum* from chickpea in Pakistan and found the growth rates, sclerotia production and average weight of sclerotia to significantly vary between isolates. The range of weights they measured was from 0.06 to 0.014 g which overlapped similarly with the results of the present study finding the smallest mean sclerotia weight being 0.0048 g (S36 isolate, 25 °C, 12 h photoperiod) and the heaviest being 0.0165 g (GGB1 isolate, 15 °C, dark).

Isolates of *S. sclerotiorum* can vary significantly in their growth traits and pathogenicity, as observed in this current study. There is sufficient evidence to accept the hypotheses that growth rates and sclerotia production of the *S. sclerotiorum* isolates vary in response to abiotic conditions. Genetic

diversity between *S. sclerotiorum* isolates collected from various locations and hosts may contribute to these differences. All isolates tested in this trial were collected from the South Island of New Zealand and only one (S36) was collected outside of Golden Bay. Differences in isolates therefore do not seem to be due to adaptation due to geographic separation. Growth differences may reflect adaptation to different ecological conditions and microclimate in the locations where the isolates were originally isolated from host plants. Phenotypic traits of *S. sclerotiorum* have been shown to be highly variable among isolates and even between colonies of the same isolate (Lehner et al., 2014). It is disputed whether the values of these variables are informative in comparing isolates due to the innately high variability. Significant differences were observed between isolates in these experiments with ten replicates of each treatment. There was variability in the results within an isolate. Another potential source of variation is that the isolates have been in culture for different periods of time. Some fungal isolates have been subcultured many times since they were originally isolated from the field, although they had been repassaged. Attenuation of fungal cultures is associated with successive subculturing and it possible after long periods of storage that the isolates may have reduced in virulence and vigour (Ansari & Butt, 2011; Chang et al., 2020). To minimize this, the inoculum for the isolates used in the experiments came from stored stock sclerotia and had been subcultured as little as possible. Repeating another time replication of these experiments would reduce these sources of uncertainty and increase the confidence in the findings of this chapter.

2.5.3 Isolate Pathogenicity Trial on Detatched Leaves of *C. arvense* and *R. acris*

There were substantial differences between the *S. sclerotiorum* isolates pathogenicity and the resulting lesions on the target species *R. acris* and *C. arvense* after 6 days. *C. arvense* leaves were more susceptible to *S. sclerotiorum* with a greater percentage of necrotic area after 6 days than for *R. acris*. The host plant the *S. sclerotiorum* isolate was originally excised from had no effect on pathogenicity, observed as lesion size, to the target weeds. This agrees with the findings of Ekins (1999) who found that isolates of *S. sclerotiorum* were no more aggressive to the host from which they were originally isolated than on other hosts. Comparatively, Durman, Menéndez, and Godeas (2003) found that isolates derived from sunflowers were more aggressive on non-sunflowers host. In the current research, some results concur with this as observed with isolates G57 and G64 which were significantly more virulent on *C. arvense* than *R. acris* despite being originally isolated from infected *R. acris* plants. Genetic studies on New Zealand population of *S. sclerotiorum* found evidence of greater similarities between isolates infecting the same host species compared with isolates collected from other hosts (Carpenter et al., 1999). This suggests that the isolates are not physiologically specialized to better infect certain hosts, and have a broad host range, which is

characteristic of *S. sclerotiorum* (Liang & Rollins, 2018). From these findings, there is evidence to reject the hypothesis that bioherbicide isolates of *S. sclerotiorum* excised from one target weed species are more pathogenic towards that weed than the alternative host species.

The *S. sclerotiorum* isolate S36, that had been studied previously the most, caused lesions on *C. arvense* that were not statistically different from G57, G64, GGB2 or S37. The lesions, however, caused by this isolate on *R. acris* were on average the smallest, being significantly smaller than those caused by isolates GGB2 and S37. Previous studies on isolates S36 and S37, also reported that on some genotypes of *C. arvense*, S37 was significantly more virulent than S36, but on other genotypes the virulence was not significantly different (Smith, 2018). The isolate that performed the best across both target weed species was GGB2, which was originally isolated from *R. acris*. GGB2 resulted in lesions that after 6 days covered 89.4% of the total leaf area on *C. arvense* and 79.1% of the leaf area on *R. acris*. Despite abnormal performance in the growth trials in Section 2.4.2, S37 was among the most virulent isolates on both *C. arvense* and *R. acris*. There is sufficient evidence to accept the hypothesis that the pathogenicity of *S. sclerotiorum* towards *C. arvense* and *R. acris* varies among isolates.

In this present research, the pathogenicity on detached leaves was found to statistically differ between isolates, and on the target host plant. On *C. arvense,* isolate GGB1 was less virulent than the other isolates which all fell within one significant group. On *R. acris* the virulence of the isolates was more varied, with isolates GBB2 and S37 grouping into a significantly different group than the other isolates. Differences in virulence between isolates of *S. sclerotiorum* has also been reported on soybean (Koga, Bowen, Godoy, Oliveira, & Hartman, 2014; Kull, Pedersen, Palmquist, & Hartman, 2004), canola (Kohli et al., 1995), sunflower (Ekins, Hayden, Aitken, & Goulter, 2011) and *Brassica* sp. (Garg, Kohn, et al., 2010; Ge, You, & Barbetti, 2015). The differences between isolates may be a result of genetic differences between isolates that influence virulence. Genetic studies on isolates of *S. sclerotiorum* in New Zealand from cauliflower and *R. acris* have identified high levels of genetic variation (Carpenter et al., 1999). Genetic studies, investigating the *MAT* loci of the isolates used in this study would provide insight into the genetic differences between the isolates (Chitrampalam et al., 2013). A potential explanation of the differing virulence of *S. sclerotiorum* isolate may be to do with oxalic acid production which is known to be an important pathogenicity factor in the fungus and facilitates its' broad host range (Liang & Rollins, 2018). Attanayake, Porter, Johnson, and Chen (2012) developed a semiquantitative method, using a pH sensitive agar, to measure differences in oxalic acid production between *S. sclerotiorum* isolates but found no correlation between oxalic acid production and virulence of the isolates tested. It is possible that the isolates tested in the present

work produce variable levels of oxalic acid that result in the observed variable virulence on *C. arvense* and *R. acris*.

The current work investigated the pathogenicity of six isolates of *S. sclerotiorum* on a single genotype of *C. arvense* and one of *R. acris*, therefore the interaction between pathogen isolates and host genotype was not investigated. In some pathogen-host systems of *S. sclerotiorum*, significant interactions between pathogen isolate and host genotype have been found such as on *Brassica* sp. in which some genotypes show consistent virulence responses while others where highly variable depending on the isolate (Garg, Kohn, et al., 2010; Ge et al., 2015). In other cases, such as a screening of six *S. sclerotiorum* isolates on six *Glycine max* (soybean) genotypes found no significant interaction between host cultivar and pathogen isolate (Kull et al., 2004). In terms of use of *S. sclerotiorum* as a bioherbicide, investigations of the S36 isolate have shown that significant variability exists in the susceptibility of different populations of *C. arvense* to the pathogen, at a local population level (Smith et al., 2016). Since the host-pathogen interaction can significantly influence virulence in some cases it is not clear whether the pathogenicity results from this trial would be reflective of the pathogens performance across genetically diverse populations of the two target weeds *C. arvense* and *R. acris*.

2.5.4 Isolate Performance Scores and Concluding Recommendations

The information collected from each of the sections of this chapter were combined into a final score to suggest which isolates would be the best to move forward with in a bioherbicide development program. Combined performance scores from the growth trial and pathogenicity on *C. arvense* and *R. acris* suggest that GGB2 is the top performing isolate across the range of tests. This isolate did not produce apothecia at 18 °C under 14 h light and 10 h dark photoperiod but has yet to be tested after cold conditioning.

There are several significant limitations of this performance score. Firstly, the model makes assumptions that the growth of the isolates is important for a bioherbicide. This assumption may be invalid as in other isolates of *S. sclerotiorum*, it has been shown that the colony diameter in culture has no correlation to the aggressiveness and pathogenicity of the isolates on young *Brassica napus* plants (Garg, Kohn, et al., 2010). Another assumption is that the pathogenicity of the isolates on detached leaf-assays is a reliable indicator of performance on whole plants in the field or greenhouse. Performance of *S. sclerotiorum* pathogenicity screenings under laboratory conditions have been shown to vary from actual observed field responses in several cases (Kim, Sneller, & Diers, 1999; Nelson, Helms, & Olson, 1991; Wegulo, Yang, & Martinson, 1998). Kull et al. (2003) found that

cut-stem assays were a more precise method of detecting the main effects and interactions of different *S. sclerotiorum* isolates on soybean than detached leaf assay methods and cotyledon assays. Detached leaf-assays were found to be the least repeatable. Despite this, in the current work, the part of the plant that would be targeted by any future bioherbicide would be the foliage, so it was logical to test the performance of the inoculum on the leaf surface or centre of whole plants. Due to time and resource constraints, the use of detached leaves was considered the best option for this work but should be expanded to whole plant assays in further experiments.

Despite sub-optimal performance in the experiments in this chapter, the S36 isolate was selected to continue forward with for the rest of the work of this thesis. The justification for this was that this isolate has been the most researched as a bioherbicide previously, including being shown to cause population reductions to both *C. arvense* and *R. acris* when applied as an inundative bioherbicide (Smith et al., 2016; Verkaaik et al., 2004). Despite better growth and pathogenicity performance in detached leaf assays, other isolates such as GGB2 do not have proven efficacy on field populations of the weeds, with no publications on its potential as bioherbicide *in vitro* or in the field.

Chapter 3
Microsclerotia Production

3.1 Chapter Introduction

Sclerotinia sclerotiorum has been previously demonstrated to reduce populations of the pasture weeds *Ranunculus acris* and *Cirsium arvense* in small-scale experiments (Bourdôt, Hurrell, & Saville, 2011; Cornwallis et al., 1999; Verkaaik et al., 2004; Waipara, Harvey, & Bourdôt, 1993). The earliest experiments used solid-state fermentation (SSF) to produce *S. sclerotiorum* inoculum by growing mycelium on grains that were then dried and milled into granules for further use (Waipara et al., 1993). More recently, sclerotia have been investigated as an alternative fungal propagule to develop a bioherbicide product (Smith, 2018), for which production is also carried out by SSF. SSF is commonly used to produce fungal propagules for biopesticides. SFF has several limitations with scaling-up and downstream processing due to the cost and variability of solid substrates, high energy requirements for sterilization, high labour demand and challenges with adequate gas exchange and contamination (Jaronski, 2014; Villamizar et al., 2018). Solid substrates colonized with *S. sclerotiorum* have been successfully assessed as a potential bioherbicide in pastures under field conditions but the high application rates (300 to 500 kg per ha) required reduced the technical and economic feasibility of this product (Hurrell & Bourdôt, 2011). These factors mean that solid substrate fermentation is not likely to be a cost-effective process to commercially produce a *S. sclerotiorum* bioherbicide.

In this context, liquid-state fermentation (LSF) is an alternative method of production that has several advantages. LSF offers ease of scalability, sterilization, reduced fermentation times and labour and greater control of conditions than SSF (Jackson, 1997). This type of fermentation was used by Bourdôt, Hurrell, and Saville (2011) to produce *S. sclerotiorum* mycelium, which was then formulated as a gel and tested for pathogenicity against five common pasture weeds including both *R. acris* and *C. arvense.* Results showed that application rates of 200 µL gel per plant caused a 90% area reduction to the green leaf area of the plants. Even though this bioherbicide prototype showed some potential to control the weeds, the short shelf-life of this product prevented it from continuing the development process.

In order to overcome the mentioned limitations for fungal production and stabilization, an alternative method involving production of microsclerotia (MS) in LSF has been developed for several biocontrol fungi such as *Trichoderma harzianum* (Kobori et al., 2015), *Metarhizium anisopliae*

(Jackson & Jaronski, 2009), *Metarhizium brunneum* (Jackson & Jaronski, 2012), *Metarhizium rileyi* (Song, Lin, Du, Yin, & Wang, 2017) and *Paeilomyces lilacinum* (Song et al., 2016). MS are compact, melanized aggregates that can be mass produced in liquid fermentation, are shelf stable, tolerant to adverse conditions and able to germinate producing infective mycelium and conidia when rehydrated. Structurally they are similar to sclerotia, but are much smaller, typically between 100 – 1000 µm in diameter (Jackson & Schisler, 1995). MS are tolerant structures that can use their own endogenous reserves to sustain germination and growth when rehydrated without the immediate need for exogenous nutrition (Rodrigues et al., 2021). For these reasons, it is of interest to investigate if MS formation can be induced in *S. sclerotiorum* using LSF and determine the potential of these propagules to be used as the active ingredient for a novel bioherbicide formulation.

The media and culture conditions that result in MS production across a range of filamentous fungi including *Metarhizium* spp., *Beauveria* spp., *Trichoderma* sp. and *Colletotrichum truncatum* have been well documented (de Lira et al., 2020; Jackson & Jaronski, 2009; Jackson & Schisler, 1995; Kobori et al., 2015; Mascarin et al., 2014; Song et al., 2016; Song et al., 2014; Villamizar et al., 2018). All published media that result in MS formation across different fungal genera share similarities in terms of nutrition and aeration. They all contain a carbon source, a nitrogen source, and basal salts and are grown in shaking cultures under rapid rotation (300 rpm) which induces oxidative stress. One hypothesis for MS formation is that they are produced in response to oxidative stress, as the cells of the fungi attempt to isolate themselves from molecular oxygen, resulting in spherical aggregates (Georgiou et al., 2006). The globose to ovate shapes of the MS minimize the surface area to volume ratio of the structures, which protects the inner medulla cells from external oxidative stress, aligning with this hypothesis. This hypothesis also links to nutritional depletion as under nutritional stress, cells have less resources to producing reducing enzymes, resulting in the accumulation of molecular oxygen from respiration, resulting in increased intracellular oxygen (Georgiou et al., 2006). Melanisation of MS as they reach maturation may also be in response to oxidative stress as the nutrition in the media becomes depleted. Melanin can scavenge free radicals and reactive oxygen species (ROS). The melanisation of MS has been linked to increased thermotolerance and resistance to UV, similar to the rind of sclerotia in *S. sclerotiorum* that protects against desiccation and adverse conditions (Butler & Day, 1998).

From the best of our knowledge, the method to produce MS structures in *S. sclerotiorum* by using LSF has not been previously investigated. In this context, this chapter addresses objective two of this thesis and aims to develop the conditions to produce MS from *S. sclerotiorum* by using LSF and to test whether any produced MS are infective towards the target weeds *R. acris* and *C. arvense*. Fungal

propagules must be infective to be useful as a bioherbicide. The infectivity of MS will be tested using detached leaf bioassays on the two target weed species, *R. acris* and *C. arvense*, under humid conditions. The high humidity favours fungal growth and provides maximum challenge to the detached weed leaves. Detached leaves bioassays are a quick and effective way of determining the virulence of a pathogens. In this case these assays were used to screen the pathogenicity of MS of *S. sclerotiorum*. Detached leaf assays are often used to rapidly assess plant varieties for resistance to diseases and pests, and the results of these assays have been shown to correlate with the results of whole plant assays (Foolad, Sullenberger, & Ashrafi, 2015; Miller-Butler, Smith, Babiker, Kreiser, & Blythe, 2018).

The hypothesis to be tested in this chapter are:

1. MS formation in *S. sclerotiorum* can be induced in liquid state fermentation under specific nutritional and culture conditions.

    Seven media have been selected for an initial pilot screening. The seven media covered a range of carbon and nitrogen sources at various carbon to nitrogen ratios (C:N) which are known to be important factors in MS formation.

2. MS formulations of *S. sclerotiorum* are infective on the target weeds *R. acris* and *C. arvense*.

    Detached leaf bioassays will be undertaken to test the pathogenicity of *S. sclerotiorum* MS (isolate S36) across four haplotypes of *R. acris,* including two which have known herbicide resistance, and two *C. arvense* lineages originating from different provenances.

3. MS should be more pathogenic on *C. arvense* as this is the host from which the isolate S36 was originally excised from.

3.2 Materials and Methods

3.2.1 Screening of liquid media to induce MS formation in *S. sclerotiorum*

Seven media with different compositions were trialled to induce formation of MS in liquid fermentation. Six media had previously been used to produce MS in other species of fungi (de Lira et al., 2020; Jackson & Schisler, 1995; Kobori et al., 2015; Mascarin et al., 2014; Song et al., 2014; Villamizar et al., 2018) with slight modifications (specific details unavailable due to intellectual property restrictions). The seventh medium was a new composition based on a literature review of

conditions favouring sclerotia production in *S. sclerotiorum*. The media contained different carbon and nitrogen sources at various concentrations plus differing concentrations of minerals and trace elements. Table 3.1 shows details of the compositions of the seven media, which were selected to cover a broad range of carbon and nitrogen sources at various C:N ratios.

For each treatment, the dry ingredients (excluding the trace elements) were added to 500 mL Schott bottles. In a separate Schott bottle 100 mL of a 100x trace element solution was prepared for each treatment by combining 10x the desired final amount per litre in 100 mL of distilled water. Before autoclaving, 297 mL of distilled water was added to each of the Schott bottles containing the dry ingredients along with 3 mL of the 100x trace element solution to reach a final volume of 300 mL. This volume was divided into 100 mL within three 250 mL conical flasks (Schott Duran®) per media (replicates), to which cotton bungs and tinfoil were applied prior to autoclaving at 121 °C at 15 psi for 15 min. After the medium in the flasks had cooled, each flask was inoculated with five sclerotia (with intact rinds) of *S. sclerotiorum* S36 (similar size) aseptically collected from cultures grown in petri plates on potato dextrose agar (PDA) in a biosafety cabinet. The flasks were then placed into a shaking incubator running at 300 rpm and 20 °C in darkness.

The flasks were checked on day 4, 7, 11 and 14 post-inoculation. On days 4 and 7 during observation of the flasks, mycelium rings developing on the glass of the flasks were removed using a sterile inoculation loop in a laminar flow hood. At each observation time, 100 µL samples of each treatment medium were transferred onto a glass slide, using a wide-mouth pipette tip, and observed under an Olympus BX50 light microscope with visible structures photographed using an Olympus DP72 digital camera. If MS were visible, a one-tenth dilution of that treatment media was prepared by adding 1 mL of the media to 9 mL of 0.02% Tween 80 (Sigma). For each of these dilutions, 20 µL samples were transferred onto microscope slides, with 10 slides prepared for each dilution. All MS present on each slide were counted and averaged for all 10 slides. The original concentration of MS in the media was then calculated by:

$$\boldsymbol{Initial\ MS\ concentration = A * 50 * 10}$$

Where A is equal to the average number of MS counted per 20 µL sample, "50" is the factor to give the concentration per 1 mL of diluted media and "10" is the dilution factor of the counted dilution.

The first 50 MS to be counted were also photographed and measured using cellSans Olympus software to obtain information on the average size of the MS.

Table 3.1 Seven liquid media compositions evaluated to induce MS formation in *S. sclerotiorum* isolate S36.

Conditions	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6	Treatment 7
Reference	Wang et al (2019) & Song et al (2016)	de Lira et al (2020)	Mascarin et al (2013)	Jackson & Schisler (1995)	Song et al (2014)	Villamizar et al (2018)	Original Sclerotinia Media
Species	*Metarhizium rileyi* and *Purpureocillium lilacinum*	*Metarhizium sp.*	*Metarhizium sp.*	*Colletotrichum truncatum*	*Metarhizium rileyi*	*Beauveria sp.*	*Sclerotinia scleroti orum*
Carbon source	Glucose	Glucose	Glucose	Glucose	Glucose	Corn steep liquor	Glucose
Nitrogen Source	Peptone + yeast extract	Yeast extract	Yeast extract	Casamino acids	Ammonium citrate	Casamino acids	Casamino acids
C:N	20:1	4:1	54:1	~30:1	50:1	5:1	50:1
Mineral and Trace Element Solution Conc.	100%	100%	100%	50%	100%	25%	100%

3.2.2 Electron Microscopy

MS produced by any of the media were formulated with a carrier, diatomaceous earth (DE; Celite 81), which was added to each flask (rate of 5 g per 100 mL of media). After being mixed thoroughly, the suspension was filtered through a #1 Whatman filter in a Buchner funnel to remove the spent media. The filter was wetted with deionized water and a vacuum applied to dry out the filter cake. The filter cakes were left to dry for 24 h in a laminar flow hood at 20 ^{o}C on a plastic tray. To obtain samples for electron microscopy, 250 mg of the formulation was resuspended in 1 mL of 0.05% Tween® 80 (Sigma) which was poured into a dish from which individual MS were isolated and prepared for transmission and scanning electron microscopy (TEM and SEM, respectively). To protect the structure during processing for TEM, samples were embedded in an agarose matrix (0.5% w/v), which was immersed in glutaraldehyde 2.5% prepared in buffer phosphate (pH 7.4) for 12 h at 4°C. Samples were then post-fixed in osmium tetroxide (1%) for 2 h and dehydrated with ethanol in ascendant concentrations. Finally, the samples were embedded in acrylic resin (LR white medium grade) and polymerised at 60°C. The blocks were thin sectioned (40 nm) and contrasted with uranyl acetate and lead citrate as described previously (Reynolds, 1963). The samples were observed in TEM (JEOL 1400 plus). For SEM, MS were directly fixed with glutaraldehyde 2.5% (pH 7.4) and dehydrated with ethanol in ascendant concentrations. The samples were sputtered with colloidal gold for 30 s (300 V, 10 mA, ~3 nm) and observed under an SEM (JEOL JSM 7000 F).

3.2.3 Maximum Challenge Leaf Bioassay

3.2.3.1 MS harvesting/formulation and treatments for Bioassay

MS were produced via fermentation in four replicate conical flasks using the methodology described in Section 3.2.1, using only media six (Table 3.1). Two flasks were used to created the "Formulated MS" treatment and two were used to create the "Unformulated MS" treatment. Three inoculum treatments were tested for pathogenicity against the two target weeds, *R. acris* and *C. arvense*. The "Formulated MS", the "Unformulated MS" and a final inoculum denominated "Sclerotia", consisted of sclerotia removed from cultures on PDA. The processing of each of the inoculum treatments is described below.

3.2.3.2 Formulated MS

Two flasks from the fermentation above were randomly selected and used for this formulation treatment. This formulation process is commonly used for MS harvesting and is based on a protocol by Jackson and Payne (2016). The carrier consisted of DE (Celite 81), which was added to each flask (rate of 5 g per 100 mL of media). After being mixed thoroughly, the suspension was filtered through a #1 Whatman filter in a Buchner funnel to remove the spent media. The filter was wetted with

deionized water and a vacuum applied to dry out the filter cake. The filter cakes were left to dry for 24 hr in a laminar flow hood at 20 °C. The next day the moisture content of the samples was measured by using an infrared moisture analyser (AND, MX-50 Moisture Analyzer. End point 100 °C). Once an average moisture content of less than <6.5% was achieved, the preparations were collected and sealed in a 50 mL centrifuge tube which was stored in a refrigerator at 4 °C until use. The colour of the final formulation is a light brown, similar in colour to the spent liquid media (Figure 3.1).

Figure 3.1 Granules of *S. sclerotiorum* MS formulated with DE.

3.2.3.3 Unformulated MS

Two replicate flasks were combined and then added to 1 L of distilled water. The MS were left to settle to the bottom for around 10 min and then the liquid fraction from the top was removed using a peristaltic pump. The remaining bottom fraction containing the MS was made up to 1 L by adding fresh distilled water (Figure 3.2A). This fractional dilution process was repeated several times until the liquid was clear. Subsequently the MS were left to settle for a final time before as much liquid was removed as possible. The bottom fraction containing the MS was then poured over plastic trays

and left to dry for 24 hr in a laminar flow hood (Figure 3.2B). When dried, the dry MS were collected and stored in a plastic tube and kept refrigerated, in the dark, at 4 °C (Figure 3.2C).

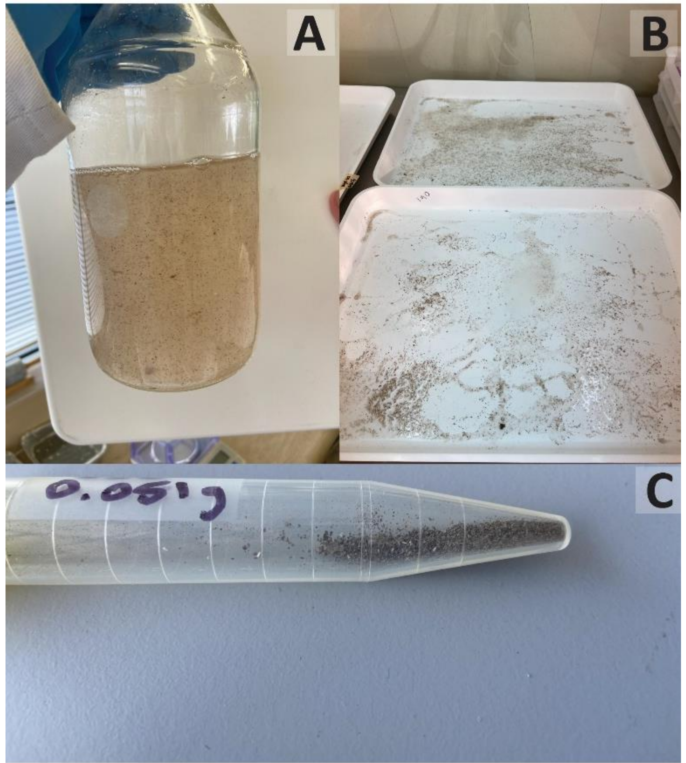

Figure 3.2 MS suspended in water during the washing process (A). Trays with MS suspension used for the drying process (B). Dry MS within a centrifuge tube (C).

3.2.3.4 Sclerotia

The final treatment to be included in the bioassay was a sclerotia treatment. This is a more traditional approach to use as the inoculum for *S. sclerotiorum* bioherbicide and so provided a point of comparison for the MS treatments. Sclerotia were collected from two-week-old cultures of isolate S36 grown on PDA plates (Ø 90 mm).

3.2.3.5 Bioassay

Plants from four *R. acris* cytotypes (A, A2, J and G) and two *C. arvense* provenances (G20 and G27) were grown in the shade house at Lincoln University, in standard potting mix (Appendix A.2) in 3 L pots. An original individual for each plant type had been previously separated into many clones via ramet separation for *R. acris* and root fragments of *C. arvense*. Ten replicate leaves of each plant type were collected. Leaves were selected to be approximately the same age, with newer healthier leaves chosen with old or leaves visibly infected with other pathogens excluded.

In a laminar flow hood, these leaves were surface sterilized in 1% bleach solution (MaxKleen™ Pure; active sodium hypochlorite ~10%) for 1 min whilst being agitated. The leaves were subsequently rinsed twice, for 1 min each, in separate baths of sterile distilled water. Each leaf was placed upon

sterile paper towel, moistened with 20 mL of sterile water, contained within a 500 mL plastic container. Three circular Vaseline rings were applied onto each leaf, on separate lobes on the *R. acris* leaves and spaced as far away as possible along the axis of *C. arvense*. The rings were applied using the end of a 1 mL pipette tip as a circular guide. Then, each dry treatment was randomly placed onto one of the Vaseline rings of each leaf as in Figure 3.3. Sclerotia were transferred using sterile tweezers, with one sclerotium (complete rind) per leaf. The two MS treatments were applied to each leaf using a sterile 1/32 tsp (1 Smidgen) spoon, to equate to approximately the same volume of inoculum as a single sclerotium (Figure 3.3). The treatments were set-up and applied to leaves contained within single block. Once a replicate block was completed the leaves were misted with sterile water before being sealed in the container. The containers were placed into an incubator with a cycle of 20 °C with lights on for 12 hr and 16 °C with lights off for 12 hr (Contherm, BioSyn 6000CP, Cold white 6500K bulbs). The experiment were set up in a randomized block design. The leaves in the containers were photographed at day 4, 6 and 7 post-inoculation. The size of the lesion caused by each of the treatments was measured using the software ImageJ (Schneider et al., 2012). The images were processed to calculate the percentage of necrotic leaf area, by dividing the total leaf area by the size of the lesion at each time of photographing (day 4, 6 and 7 post-inoculation). The lesion size data were also collected as a percentage of the lobe for *R. acris,* although only one inoculum treatment resulted in lesions. The data were transformed using an arcsine transformation, checked for normality and then analyzed using ANOVA with the treatment structure Inoculum Treatment * Haplotype/Species, using a Bonferroni test for multiple comparisons (95%). At the end of the trial a subsample of ten leaves were randomly selected. From the necrotic area of each leaf a fragment was excised using a scalpel and placed into the centre of a petri dish containing 1/5th PDA. These plates were examined after 14 days and the presence of *S. sclerotiorum* was determined based on the morphology of the culture and the presence of sclerotia.

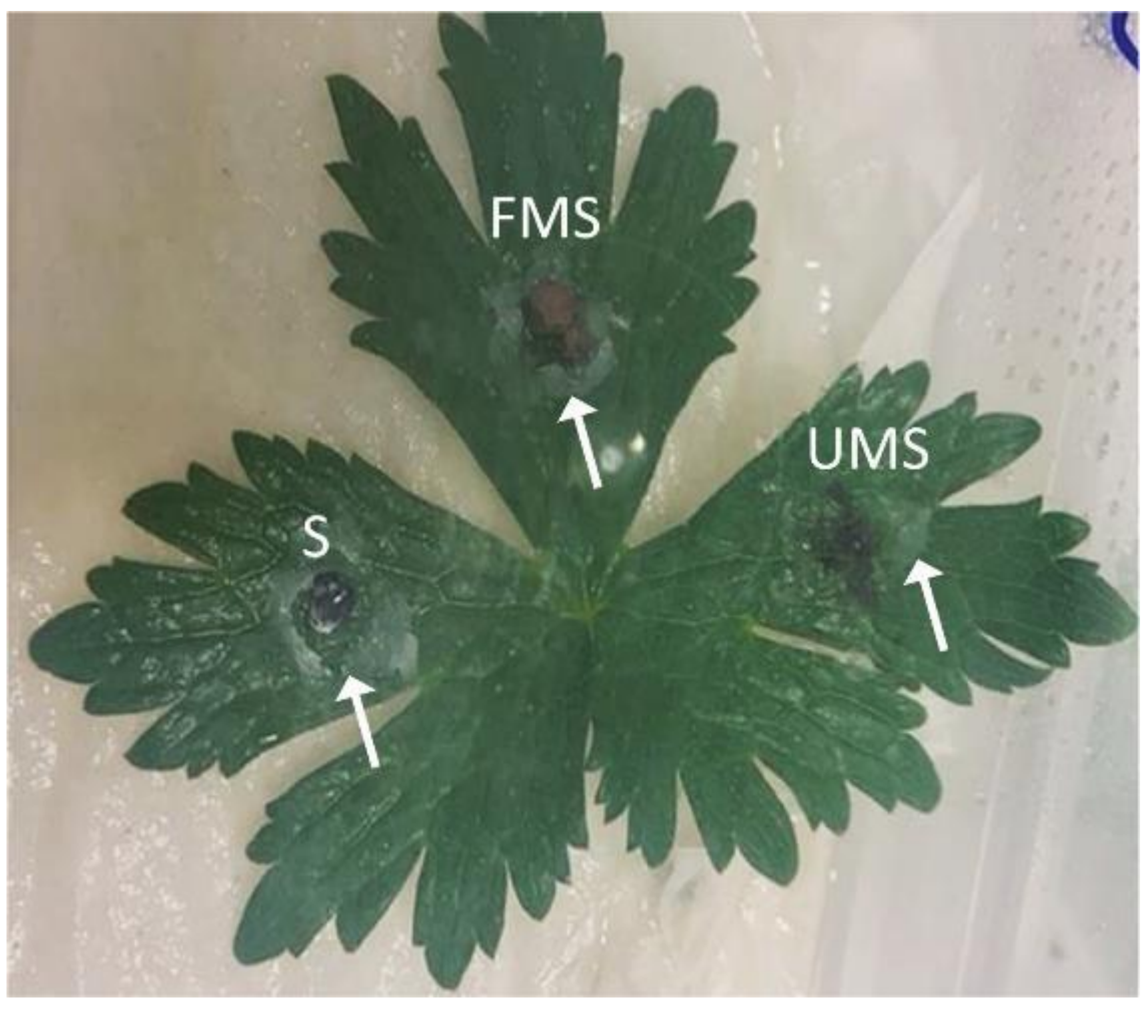

Figure 3.3 Example of the three inoculum treatments on an *R. acris* leaf for the maximum challenge leaf assay. Sclerotia (S), formulated MS (FMS) and unformulated MS (UMS) within Vaseline® rings (arrows).

3.3 Results

3.3.1 Screening of liquid media to induce MS formation in *S. sclerotiorum*

Sclerotinia sclerotiorum was able to grow in all the seven tested liquid media, presenting different characteristics (colour, viscosity, biomass) after 2 weeks of fermentation (Figure 3.4).

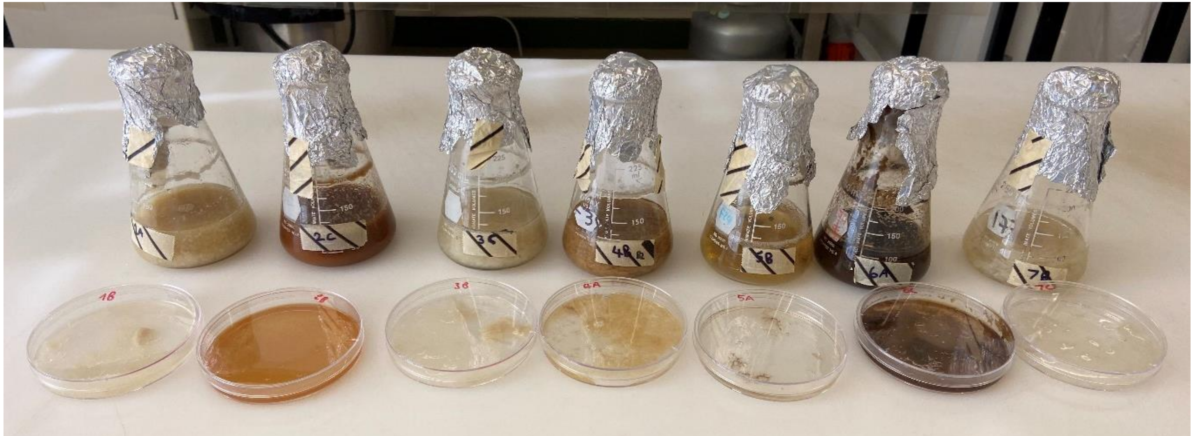

Figure 3.4 Final appearance (2 weeks fermentation) of the seven different media tested to induce MS formation in *S. sclerotiorum*. Colour differences reflect compositional differences in the media.

In media 1, 3, 4, and 7 the culture became very viscous with thick mycelial growth forming large, macroscopic conglomerates of hyphae. Structures from these flasks sampled at day 14 and observed under the microscope appeared as large, wispy, mycelial aggregates (Figure 3.5a-f). Media 5, which used an inorganic nitrogen source, had minimal growth apart from small hyphal developments growing from around the sclerotia used as inoculum. Media 2 produced some small aggregates, resembling MS, in the early stages of formation but after 14 days of fermentation there was little to no compaction of these structures and no melanisation was observed (Figure 3.5b).

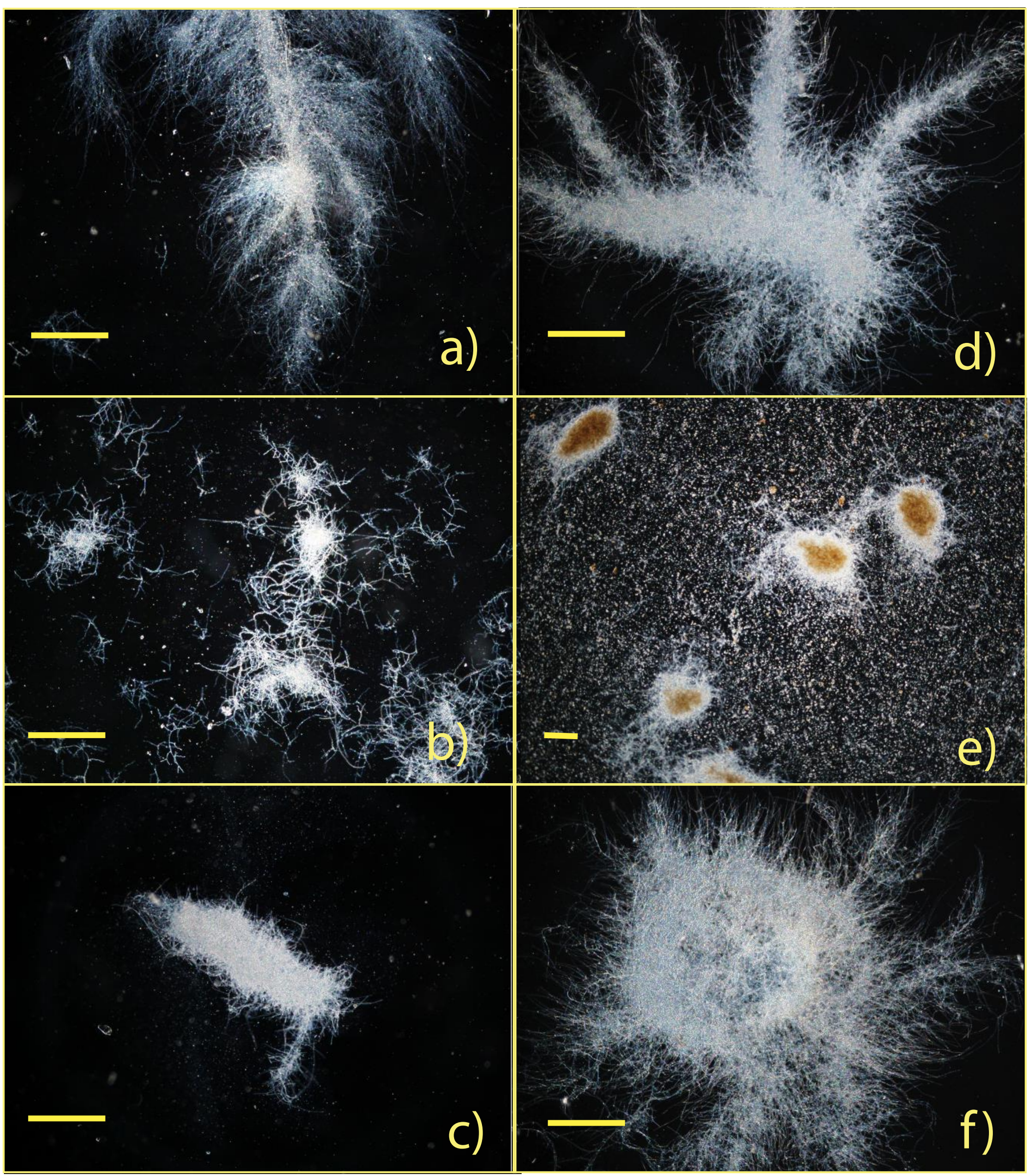

Figure 3.5 (a – f): Figures a, b, c, d, e, and f show the structures from the media 1, 2, 3, 4, 6 and 7. The compositions of these media can be found in detail in Table 3.1. MS were only produced in medium 6 (e). Medium 5 not shown due to minimal growth. Photos taken under phase contrast microscopy at 100x magnification except (e), which was taken at 40x. Scale bars indicate 200 µm.

MS-like aggregates were only formed in medium 6, which was based on a composition published by Villamizar et al. (2018) that was developed to induce MS formation in *Beauveria* sp. Medium 6 uses corn steep liquor and casamino acids as the carbon and nitrogen sources, with a carbon to nitrogen ratio of 5:1. This medium also has reduced concentrations of micronutrients compared to the other tested compositions. Initially the growth in this medium was slow, with minimal hyphal growth at the day 4 post-inoculation (Figure 3.6), in comparison to the rapid appearance of viscous mycelial growth that was seen in other media. Mycelial aggregates started to be observed at day 6 and at day 10

these aggregates had increased in size and density but still had floccose margins (Figure 3.6). By day 14, mycelial aggregates had become visibly more compact, appeared brown under the light and phase contrast microscopy with less floccose mycelium around the margins of the structures observed (Figure 3.5e and Figure 3.6).

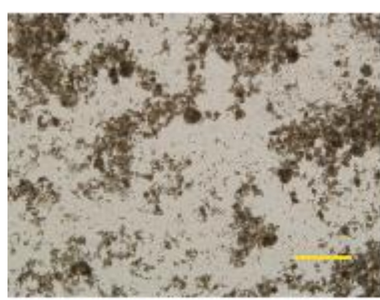

Figure 3.6 Development of MS of *S. sclerotiorum* in medium 6. Left panel shows observation at day 4, middle panel at day 10, and right panel day 14. Scale bar indicates 200 µm.

After day 10 of the fermentation, a colour change occurred in the medium 6, with the culture becoming darker brown (Figure 3.7). The colour change occurred across all three replicate flasks between days 10 and 14, but did not occur at the same time in each flask. This colour change coincided with differences between structures observed under microscopy at day 14 compared to day 10, with MS becoming notably denser, melanized and developing more defined margins (Figure 3.8). The initial pH of the media was 4.34 and only slightly increased to 4.57 after 14 days of fermentation.

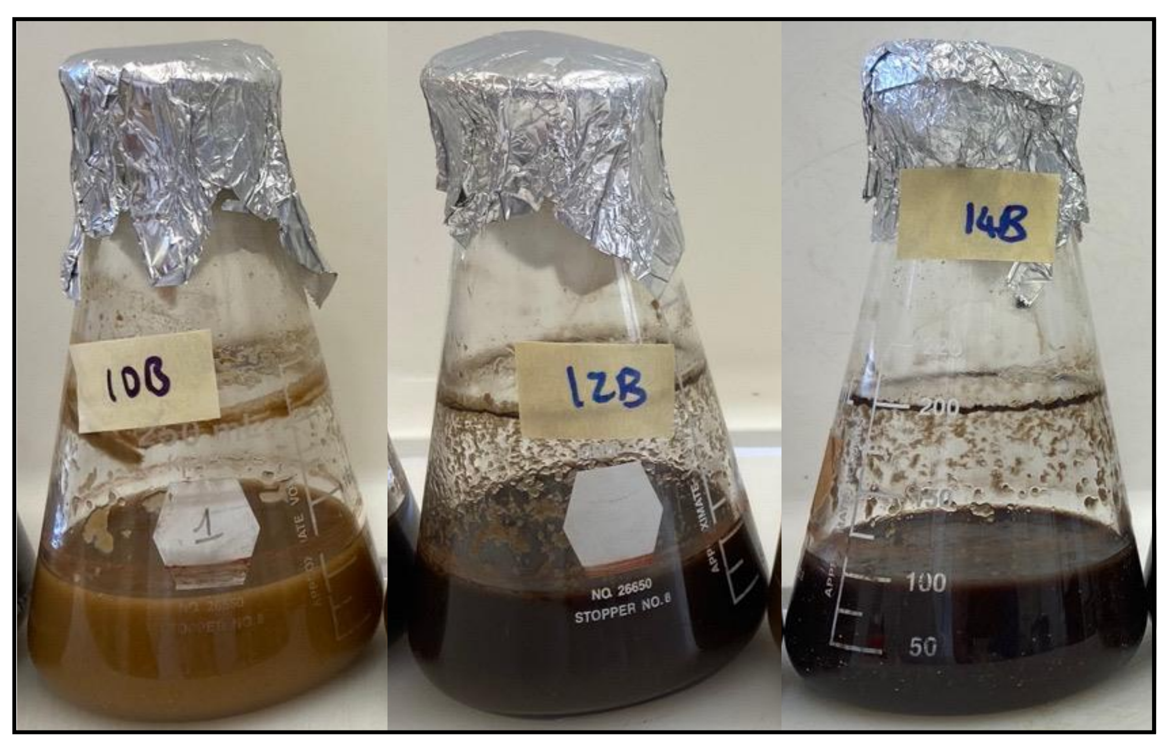

Figure 3.7 Colour change of medium 6 that produced MS of *S. sclerotiorum.* The colour change between day 10 (left), day 12 (middle) and day 14 (right), coincided with the appearance of melanized MS aggregates which can be seen on the wall of the flask in day 12 and 14 images.

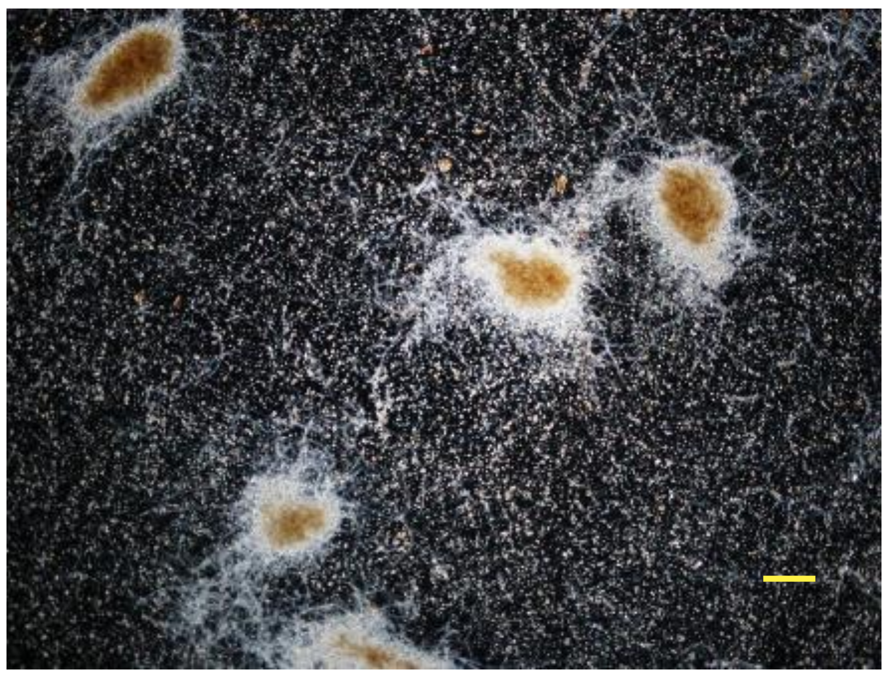

Figure 3.8 MS of *S. sclerotiorum* at day 14 of the fermentation. 40X magnification. Scale bar shows 200 µm.

In media 6, MS were present at day 14 at an average concentration of $4.8 \times 10^3 \pm 449$ MS per mL. There was no significant difference in the concentration of MS produced across the three replicate flasks of media 6 ($P = 0.47$) (Figure 3.9). MS were mostly spherical to ovoid in shape, with the average MS having a diameter of 269 ± 8 μm. There was no significant difference in the size of MS among the three replicate flasks ($P = 0.35$).

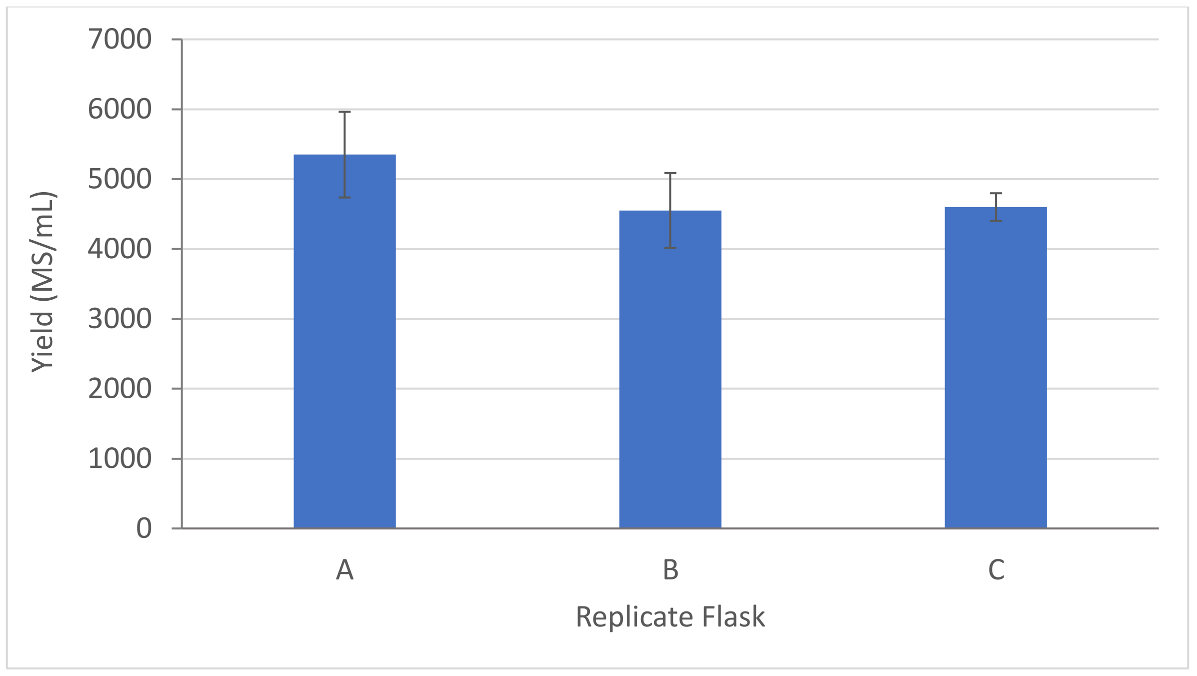

Figure 3.9 Yield (MS/mL) of *S. sclerotiorum* MS across the three replicate flasks after 14 days of fermentation. Error bars correspond to standard error of the mean MS/ml from 10 subsamples from each replicate flask.

3.3.2 Electron Microscopy of MS

From the scanning electron micrographs of the surface of the MS (Figure 3.10), significant compaction and intertwining of the hyphae was observed. Some hyphae appeared turgid (Figure 3.10A-B), while others appeared to be shrivelled/deflated (Figure 3.10C). A number of hyphae had visible holes, suggesting that these cells may be damaged (Figure 3.10A-B). A substance with an irregular granular appearance can be observed surrounding the hyphae, which is likely extracellular matrix. In Figure 3.10C, there appears to be a large amount of extracellular matrix surrounding the shrivelled hyphae, perhaps due to lysing cells. Particles with regular, patterned structures were also visible on the surface of the MS and this was identified as the DE that was used to formulate the MS.

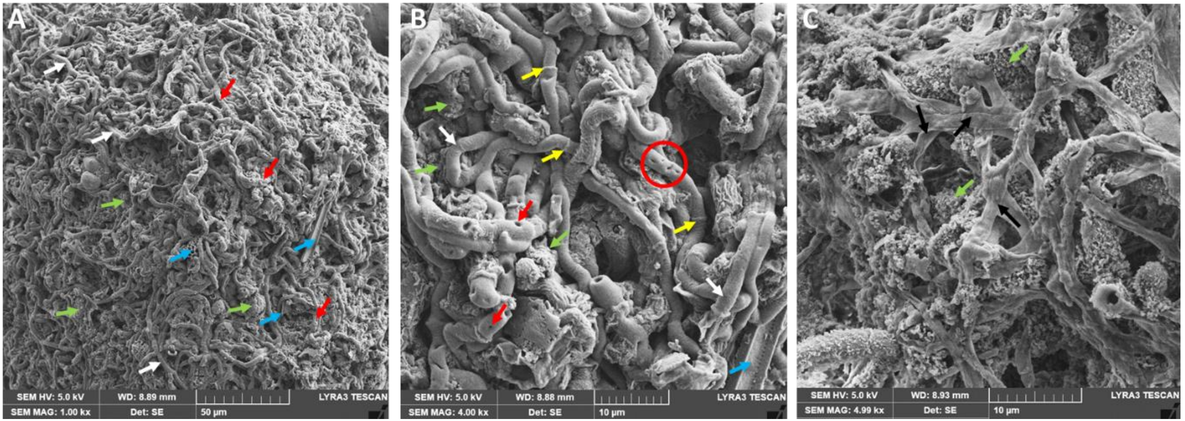

Figure 3.10 Scanning electron micrographs of MS formed by *S. sclerotiorum* (14 days liquid fermentation) after harvesting and drying for 16 hr with diatomaceous earth. (A) Intricated mycelium covering the MS surface. (B) Detailed surface showing hyphae with holes. (C) Detailed surface showing collapsed hyphae. White arrows show turgent hyphae, red arrows/circle show holes, blue arrows show diatomaceous earth particles, yellow arrows show septum, green arrows show extracellular matrix, black arrows show shriveled hyphae.

TEM of ultrathin sections of the interior of the MS show a small number of electrodense cells, corresponding to alive cells. Regular oval shaped grey areas were interspersed within the structures which are hypothesized to be lipid/metabolite droplets (Figure 3.11A-B). Extracellular matrix was also observed within the structures, visible as irregular, granular electrodense material filling the spaces between cells (Figure 3.11A-B). Some swollen cells were observed that had an electrodense material surrounding the exterior of the cells (Figure 3.11C).

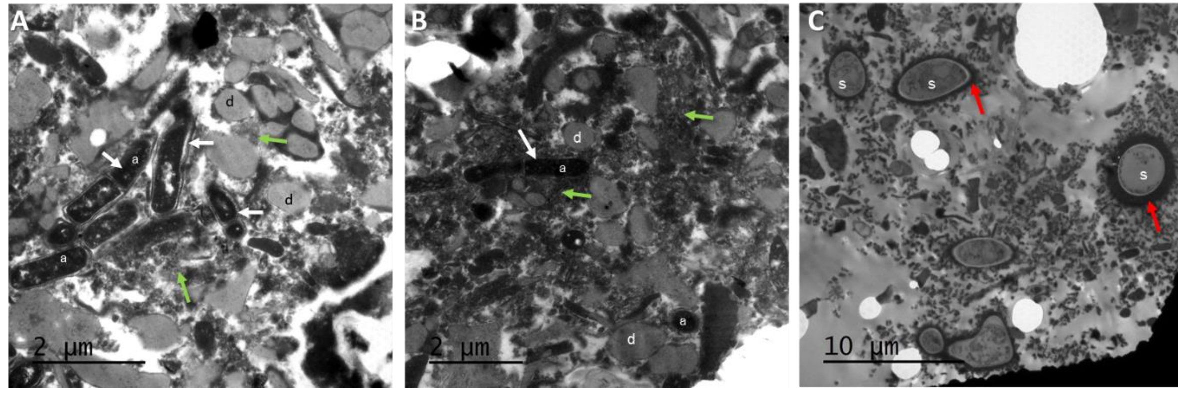

Figure 3.11 Ultrathin sections of MS formed by *S. sclerotiorum* (14 days liquid fermentation) after harvesting and drying for 16 hr with diatomaceous earth. (A and B) Internal ultrathin sections showing loose structure with few electrodense alive cells (a) and several lipid drops (d). (C) Internal ultrathin section showing swollen cells (s) surrounded by an electrodense material. White arrows show hyphae, green arrows show the extracellular matrix and red arrows show electrodense material.

3.3.3 Maximum Challenge Leaf Bioassay

The MS yield in the fermentation carried out for this experiment was 5458 ± 1100 MS/mL which was not significantly different from the average yield obtained in the first media trail (P = 0.76) (Section 3.3.1). The final moisture content of the dried formulated MS was 3.92% after 24 hr of drying.

There was a significant (P < 0.001) interaction between the inoculum treatment and the haplotype of the plant (species-specific) on the size of the lesions observed. Treatment (formulated MS, unformulated MS or sclerotia) had the largest significant (P < 0.001) effect on the size of the lesions with the formulated MS inoculum resulting in lesions on both *R. acris and C. arvense* across all haplotypes (Figure 3.12 and Figure 3.13). For the other inoculum treatments (sclerotia and unformulated MS), lesion size did not vary significantly across the haplotypes (P = 0.05), with sclerotia only causing small lesions on *C. arvense* and none on *R. acris*, and the unformulated MS not resulting in lesions on either host (Figure 3.12 and Table 3.2). For the formulated MS treatment, the inoculum resulted in lesions on both *R. acris and C. arvense* across all haplotypes and with significant differences (P < 0.05) in lesion size across the haplotypes, which drove the interaction between inoculum and treatment. The largest lesions developed on the *R. acris* A2 haplotype, with lesions covering an average of 56.3 ± 9.9% of the leaf green area after 6 days (Table 3.2) but this did not significantly differ (P > 0.05) to *R. acris* haplotype J and *C. arvense* provenance G27 for which were lesions covered 33.0 ± 4.8% and 39.3 ± 6.8% of the green leaf area, respectively. *R. acris* haplotype A, G and *C. arvense* provenance G20 did not differ significantly (P > 0.05) from each other, with smaller lesions at 21.8 ± 4.6%, 18.3 ± 6.4% and 18 ± 9.5%, respectively. The lesions caused by the formulated MS were significantly (P = 0.05) larger than those caused by sclerotia across the host species and haplotypes, with the single exception of *C. arvense* provenance G20 that did not differ significantly from the sclerotia inoculum treatment (Table 3.2). Averaged across all haplotypes, the average lesion size 6 days after inoculation as a percentage of the total leaf area was 30.5 ± 3.2% for the formulated MS, 1.07 ± 0.53% for the sclerotia treatment and 0% for the unformulated MS. The haplotypes of the plant species also differed significantly (P < 0.001) in their susceptibility, but this was only apparent for the formulated MS. The host species did not have a significant effect on the necrotic leaf area observed (P = 0.900). *S. sclerotiorum* was able to be reisolated from a subsample of 10 infected leaves, fulfilling Koch’s postulates.

Table 3.2 Mean Lesion Percentage (as % of green leaf area) caused by different *S. sclerotiorum* inoculums on *C. arvense* 6 days after leaf inoculation in a maximum challenge leaf assay. Letters indicate significantly different values from the results of a Bonferoni test at P = 0.05 and analysed across all treatments and haplotypes.

Species	Haplotype/ Provenance	Inoculum Treatment		
		Formulated MS	Sclerotia	Unformulated MS
C. arvense	G20	18 ±9.5^{bc}	3.7 ±1.9^{ab}	0 ±0^{a}
C. arvense	G27	39.3 ±6.8^{ef}	2.8 ±2.6^{ab}	0 ±0^{a}
R. acris	A	21.8 ±4.6^{cde}	0.0 ±0^{a}	0.0 ±0^{a}
R. acris	A2	56.3 ±9.9^{f}	0.0 ±0^{a}	0.0 ±0^{a}
R. acris	G	18.3 ±6.4^{bcd}	0.0 ±0^{a}	0.0 ±0^{a}
R. acris	J	33.0 ±4.8^{def}	0.0 ±0^{a}	0.0 ±0^{a}

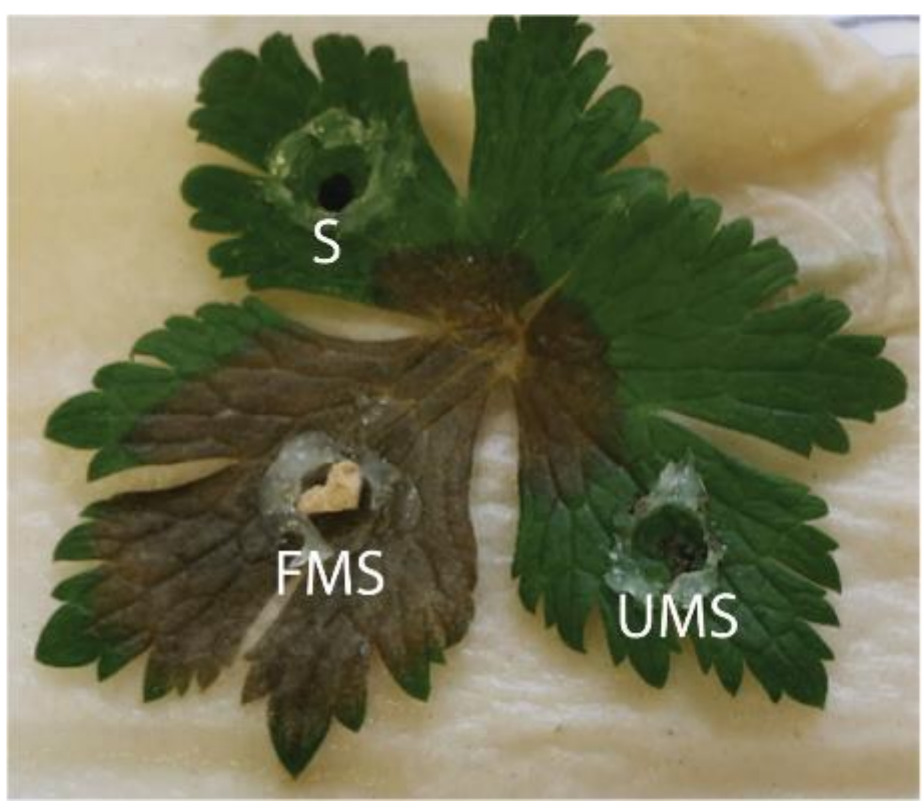

Figure 3.12 An infected *R. acris* leaf, 6 days after inoculation with *S. sclerotiorum* (S36 isolate). Sclerotia (S), Formulated MS (FMS) and unformulated MS (UMS).

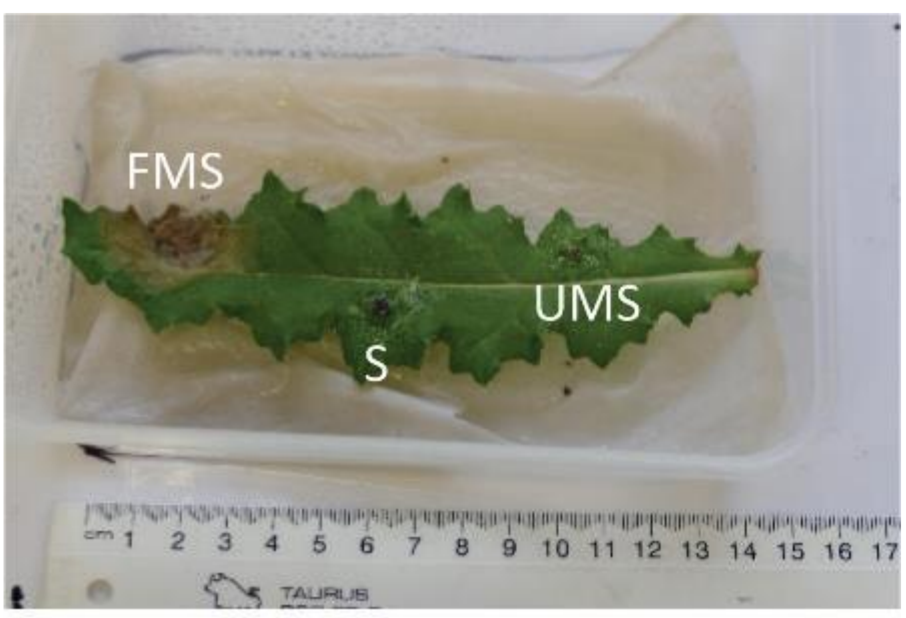

Figure 3.13 An excised leaf of *C. arvense* four days after inoculation with *S. sclerotiorum* (S36 isolate). Sclerotia (S), Formulated MS (FMS) and unformulated MS (UMS)

Across all *R. acris* haplotypes the formulated MS caused infection on >90% of all replicate leaves except for the G haplotype on which only 50% of inoculations resulted in infection (Figure 3.14). On *C. arvense* both the formulated MS and sclerotia inoculum caused infection. No lesions were observed in the unformulated MS. Across all *C. arvense* leaves the formulated MS caused lesions on 80% of replicate leaves while the sclerotia inoculum resulted in lesions on 25% of replicate leaves (Figure 3.15).

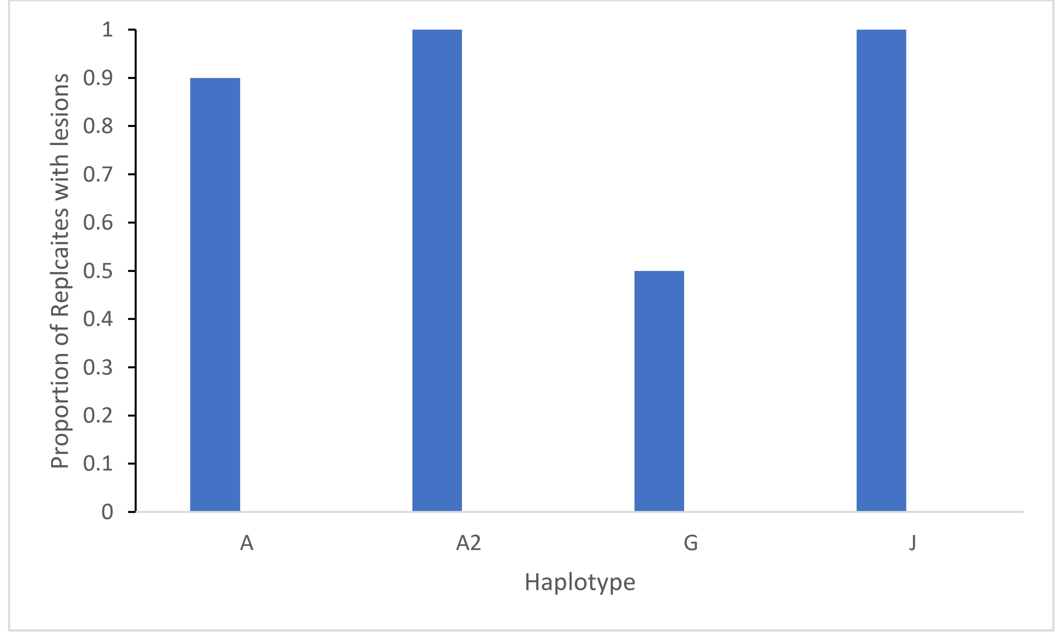

Figure 3.14 Proportion of replicates of formulated MS of *S. sclerotiorum* causing a lesion of any size on four haplotypes of *R. acris.* Sclerotia did not cause any lesions on *R. acris*. N=10 (with the exception of A2 where n=9)

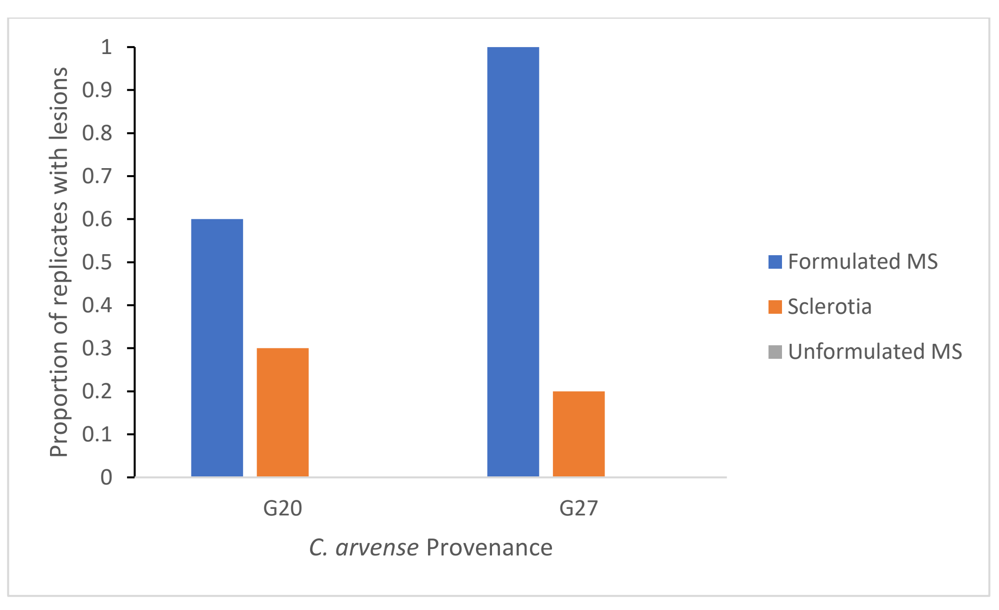

Figure 3.15 Proportion of replicate leaves where inoculum treatments (Sclerotia in orange. Formulated MS in blue.) caused lesions of any size across *C. arvense* haplotypes. G20 (n=10). G27 (n=9).

For the formulated MS treatment there were significant differences (P < 0.001) in the size of the lesions depending on the time since inoculation. Averaged across all haplotypes/species the average necrotic area increased with time post inoculation from 7.9 ± 1.3% at day 4, to 30.6 ± 3.3% at day 6 to 50.9 ± 4.5% at day 7. For all *R. acris* haplotypes lesions did not vary significantly in size between days 4 and 6 or between days 6 and 7 (P > 0.05), but the difference in lesion size between days 4 and 7 was significant (P < 0.05) (Figure 3.16). For *C. arvense* provenance G20, there was no significant difference in lesion size between days post inoculation (Figure 3.17). For *C. arvense* provenance G27, lesions did not vary significantly in size between days 4 and 6 or between days 6 and 7 (P > 0.05), but the difference in size between days 4 and 7 was significant (P < 0.05) (Figure 3.17).

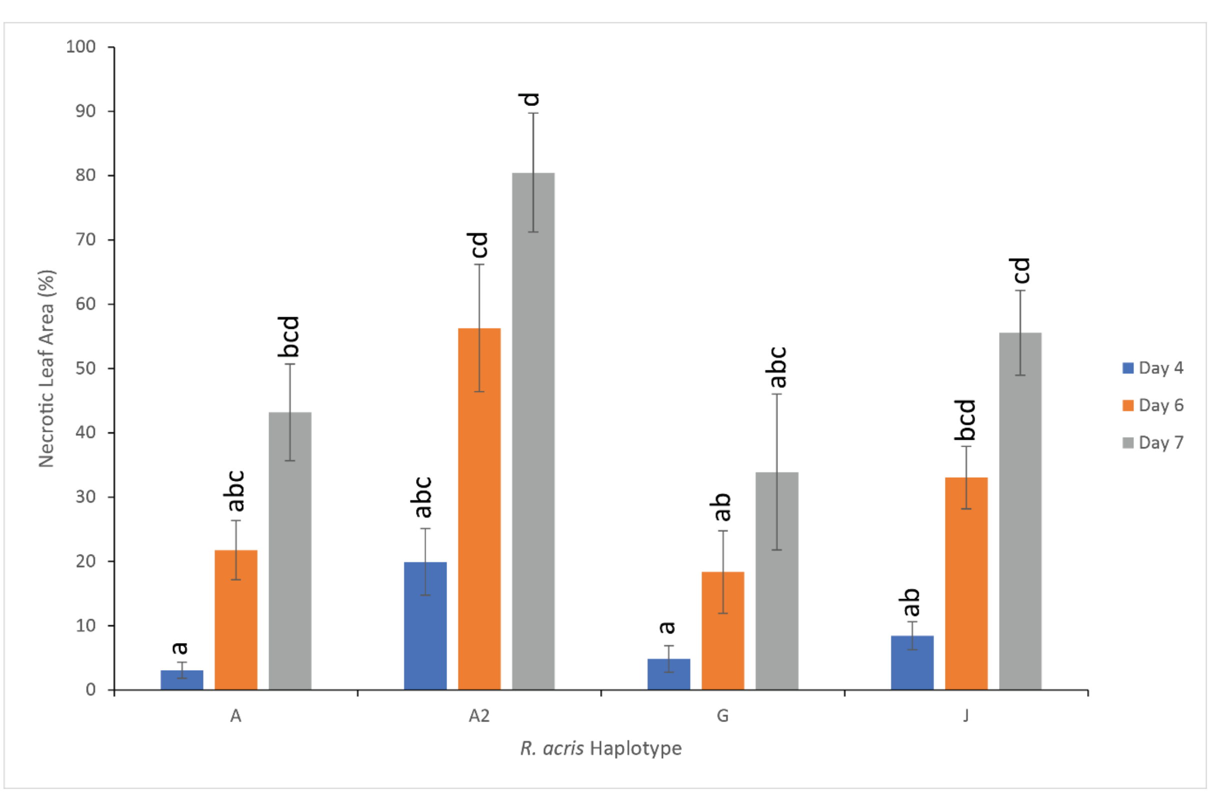

Figure 3.16 Average % necrosis from MS of *S. sclerotiorum* isolate S36 after inoculation with formulated MS on four *R. acris* haplotypes (A, A2, G and J). Day 4 (blue), day 6 (orange) and day 7 (grey). Error bars indicate standard error of the mean (SEM). Letters indicate significantly different groups from the results of a Bonferroni test at P=0.05.

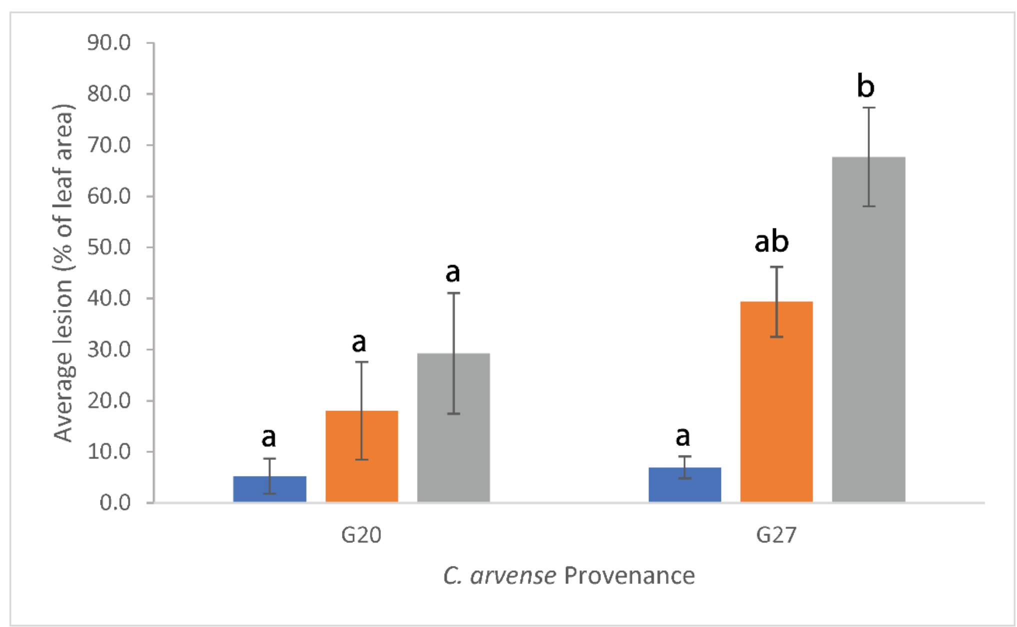

Figure 3.17 Average % necrosis of MS from *S. sclerotiorum* isolate S36 after inoculation with formulated MS on two *C. arvense* provenances (G20 and G27). Day 4 (blue), day 6 (orange) and day 7 (grey). Error bars indicate standard error of the mean (SEM). Letters indicate significantly different groups from the results of a Bonferroni test at P=0.05.

After these initial results, a replicate experiment was carried out using treatments prepared in an identical manner to the first attempt. A second replicate experiment was conducted without the use of surface sterilisation or Vaseline rings. However, in these two replicate trials the MS treatments did not cause any visible infection on either host species.

3.4 Discussion

Only a single media tested resulted in the production of MS of *S. sclerotiorum.* This medium was based on the composition reported by Villamizar et al. (2018) to produce MS in *Beauveria* species. At day 14, structures in the media were observed as compact aggregates, with dark brown pigmentation with short halos of mycelium, with an average diameter of 269 μm, conferring with the definition of MS. MS were produced at a concentration of 4.8 x 10^3 ± 449 MS per mL. In LSF, formation of MS is thought to be induced in response to nutritional depletion and oxidative stress caused by the composition and the shaking of the culture (Jaronski & Mascarin, 2017).

MS yields achieved in this study were in the order of 5 x 10^3 MS/mL, similar to the yield achieved by Villamizar et al. (2018) for MS of *Beuaveria bassiana* (6.00 x 10^3 MS/mL)*, B. pseudobassiana* (8.46 x 10^3 MS/mL) *and B brogniartii* (6.18 x 10^3 MS/mL) *, Metarhizium humberi* (3.6 x 10^3 MS/mL) by de Lira et al. (2020) and *Mycoleptodiscus terrestris* (1.5 x 10^3 MS/mL) by Shearer and Jackson (2006). Higher yields have been reported in *Trichoderma harzianum* (1.5 x 10^5 MS/mL) and *M. rileyi* (2.2 x 10^4 MS/mL), but often these have been achieved after optimisation of the fermentation process (Kobori et al., 2015; Song et al., 2017). For a pilot screening of media with potential for MS formation, the yields achieved in the present work are appreciable, and will provide a good starting point for future MS media development for *S. sclerotiorum*. It is important to note that for the end goal of a bioherbicide, the quality and quantity of MS are equally important. As such, viability of the structures, as well as yield, need to be measured when developing the fermentation further.

S. sclerotiorum used in this current study had very limited growth during the first 4 days, with mycelium aggregation beginning around day 6. Mycelial aggregation increased with structures becoming more compact by day 10 and melanised between day 10 and day 14. In contrast, in LSF of other species, MS formation has occurred between 4 to 10 days after inoculation (Kobori et al., 2015; Mascarin et al., 2014; Song et al., 2014; Villamizar et al., 2018). The slower onset of MS formation and growth of *S. sclerotiorum* in the liquid medium could be related with the type of propagule used as inoculum (Jackson & Schisler, 1995; Song et al., 2014; Villamizar et al., 2018). The use of sclerotia as the inoculum may be contributing to an initial lag phase in the growth of *S. sclerotiorum*, as it takes some time for the sclerotia to myceliogenically germinate, due to the protective rind (Huang, 1985). Sclerotia were selected as the inoculum to use in the present work, as they required the least amount of handling and were relatively easy to manipulate. Aseptically transferring sclerotia from

colonized agar plates into the flasks was a simple process, with no additional processing of the inoculum required, reducing contamination potential. In LSF of other fungi, conidial suspensions are commonly used as the inoculum source, typically using 10^4 to 10^8 conidia per mL (Jackson & Schisler, 1995; Song et al., 2014; Villamizar et al., 2018). The high concentration of conidia provides many inoculation points that enables the fungi to homogenously colonise the media and begin to form aggregates. At the beginning of this research, it was hypothesized that ground sclerotia may act similarly to conidia but this inoculum source did not significantly change the rate of colonization of the media compared to using unground sclerotia or agar plugs in preliminary experiments (data not shown). *S. sclerotiorum* does not produce conidia but does rapidly produce abundant sclerotia when cultured on agar plates. This is the reason why sclerotia were selected as inoculum to develop the production experiment. Another disadvantage of sclerotia as inoculum is that sclerotia are irregular in shape and size which may contribute to variability between replicate liquid cultures, especially as only five sclerotia are being used to inoculate each flask. An alternative inoculation technique, is to use a preculture containing many short hyphal fragments as the inoculum for the MS media. (Shearer & Jackson, 2006). This should be undertaken in future work with *S. sclerotiorum* as it may facilitate faster colonisation of the liquid broth and subsequent MS production. From a bioherbicide production standpoint this would make fermentation more commercially viable.

The only media that induced formation of MS in *S. sclerotiorum* had a C:N ratio of 5:1. Corn steep liquor (CSL) and casamino acids (CA) were the primary nutritional sources, supplemented with basal salts and trace elements. MS production in other species of filamentous fungi has been induced in liquid culture using media compositions primarily with high carbon to nitrogen ratios (between 30 and 50:1) and utilizing simple sugars such as glucose as the carbon source and yeast extract or casein as the nitrogen source (de Lira et al., 2020; Jackson & Schisler, 1995; Mascarin et al., 2014; Song et al., 2016). With *S. sclerotiorum* in the present study, media tested with high C:N ratios (20-50:1) resulted in viscous hyphal cultures, with structures that were weakly aggregated with large halos of ropey mycelium and no melanisation (Figure 3.8a, b, c, d, and f). These findings suggest that a rich media with a low C:N ratio is necessary to produce MS in *S. sclerotiorum*. Production of MS is known to be affected by different factors such as carbon and nitrogen sources, C:N ratio, pH, oxidative stress and iron concentrations (Song, 2018). No MS were produced in media 2, which had a similar C:N of 4:1 but used glucose and yeast extract as the primary nutritional sources. Both media had similar total concentrations of carbon and nitrogen (Media 2: 18g/L carbon, 4.95 g/L nitrogen; Media 6: 16.5g/L carbon, 3.6g/L nitrogen) and yet MS were only produced in media 6. This result suggests the carbon and nitrogen sources have a significant effect on MS production in *S. sclerotiorum* and further investigation will be needed to determine in detail, how the C:N ratio is influencing MS production in this species. Future work could be undertaken by comparing MS production in media with varying

C:N ratios whilst keeping the nutritional sources of CSL and CA constant. Kobori et al. (2015) used this method to compare MS production by *T. harzianum* at a range of C:N ratios and found that the biomass of MS was significantly higher at a 10:1 C:N ratio than at 30:1 or 50:1, although the difference in number of MS was not significant. *T. harzianum* MS produced at a C:N of 10:1 also produced greater numbers of conidia upon rehydration. In *Trichoderma asperellum* MS yield was also increased in richer media with a C:N ratio of 10:1 compared to 30:1 (Locatelli et al., 2022).

SEM of the produced MS revealed an ultrastructure consisting of intricate layers of hyphae, surrounded by depositions of extracellular matrix. The ultrastructure of the MS are similar to the surface of macroscopic sclerotia of *S. sclerotiorum,* as reported by Ordóñez-Valencia et al. (2015) and Brustolin et al. (2016). Ultrathin sections observed under TEM showed few electrodense cells surrounded by extracellular matrix. This result is very similar to the findings of Garcia Riaño (2022) who found that within mature MS of *Metarhizium robertsii*, only a small number of cells were still alive but were embedded within an extracellular matrix containing carbohydrates. This may facilitate the survival of the remaining cells, thus enabling the germination and growth of the MS after drying. It was observed under light microscopy in the current study that solid precipitate particles in the media would become trapped by growing mycelial aggregates and eventually become part of the MS structures. It has been hypothesized in other species of fungi that these media particles contribute to endogenous nutritional reserves within MS, along with nutritional material that accumulates in the extracellular matrix through the autolysis of cells within the structure (Villamizar et al. 2020). Autolysis has been shown to be an important process in biofilms, where during starvation, autolysing cells provide nutrition for the surviving cells (Allocati, Masulli, Di Ilio, & De Laurenzi, 2015). In *Mycoleptodiscus terrestris,* accumulations of carbohydrates, specifically mannitol and trehalose, were detected within the extracellular matrix of MS. As these compounds have also been found in the sclerotia of *S. sclerotiorum* (Tourneau, 1966), further research into carbohydrates accumulation within MS of *S. sclerotiorum* is warranted.

The main nutritional source in the media that produced MS in *S. sclerotiorum* is CSL. Compared to monosaccharides such as glucose, CSL is a more complex nutritional source and contains approximately 20.2% carbon and 5.2% nitrogen by weight along as well as a rich array of micronutrients, trace elements and vitamins (including B vitamins), and essential amino acids (Corn Refiners Association, 2006; Villamizar et al., 2018). CSL is an agro-industrial by-product, so its exact composition is undefined and depends on the source of raw materials and the processing methods employed. It is possible that some unidentified component or growth factor was present in this nutrition source that contributed to the formation of MS that was not present in the other media based on more purified constituents. The use of agro-industrial by-products for fermentation of a bioherbicide products is desirable as they can be obtained at a low cost compared to pure reagents,

allowing fermentations to be commercially feasible. The use of by-products to create something of value, such as a bioherbicide, would divert this waste product from being disposed and thus contribute to the sustainability of any final product.

The colour change from light brown to dark brown between day 10 and day 14, which was associated with the melanisation and maturation of MS, could be related with the production of pigmented metabolites (Villamizar, Barrera, Hurst, & Glare, 2021). In the plant pathogenic fungus *Colletotrichum truncatum*, the initiation of formation of hyphal aggregates into MS was associated with the depletion of nitrogen in the culture, and the MS became melanised with the depletion of carbon (Jackson & Schisler, 1995). The carbon and nitrogen levels in the media were not measured in this work but may provide an insight into the nutritional conditions that are associated with MS formation in *S. sclerotiorum*. It is well known that sclerotial development in *S. sclerotiorum* is initiated when mycelium of the fungus is grown in a nutrient depleted environment (Christias & Lockwood, 1973). Measuring the carbon and nitrogen levels in the broths during this time period using the methodology by Jackson and Schisler (1995), could generate useful insights into the nutritional conditions associated with MS formation in *S. sclerotiorum*.

Oxidative stress is another important factor associated with MS formation. All MS production protocols use shaking cultures that result in high concentrations of dissolved oxygen. Fe^{2+} ions contribute to oxidative stress by catalysing reactions that generate reactive oxygen species (ROS) such as hydroxide radicals (Song et al., 2013). The concentration of pure ferrous sulphate in the medium composition that produced MS in *S. sclerotiorum* was low at a concentration of just 25 mg/L compared to other published MS media that typically contain 100- 200mg/L (de Lira et al., 2020; Song et al., 2014). Fe^{2+} ions have been demonstrated to be a critical factor in inducing MS differentiation in *M. rileyi* in which MS production was maximized with the addition of ferrous sulphate at a rate of 0.15 g/L (Song et al., 2014). Although the amount of iron sulphate added to media 6 was measured, iron was also present in the CSL used in this media. The exact amount in the CSL used in this present work is unknown. Measurements from the Corn Refiners Association (CRA) indicate that iron is present in generic CSL at an approximate rate of 110 ppm which would equate to 110 mg/L (Corn Refiners Association, 2006). Adjusting the ferrous sulphate concentration could be a key factor to increase the yield and optimize the fermentation process developed in this research.

The initial pH of the media was 4.34 and only slightly increased to 4.57 after 14 days of fermentation. The pH of the media may also be important in the development of MS. Oxalic acid is an important metabolite produced by *S. sclerotiorum* that plays a role in pathogenicity as well as in sclerotial morphogenesis. Accumulation of oxalic acid as the fermentation progresses may influence the onset of MS formation. *S. sclerotiorum* has an increased growth rate at lower pH, and pH is known to be an

important regulator of oxalic acid production, pathogenicity and sclerotial metamorphosis (Rollins & Dickman, 2001; T. Xu et al., 2018). As well as the production of oxalic acid, numerous other pathogenicity factors are produced by *S. sclerotiorum* such as cell wall-degrading enzymes (CDWEs) that can be expected to accumulate in the fermentation broth (Lumsden, 1979). It is possible that these metabolites could be included into a formulation of a *S. sclerotiorum* bioherbicide to increase its efficacy. Liquid chromatography to investigate the array of secondary metabolites present in the broth may provide interesting insights.

The maximum challenge leaf bioassay carried out in the present work showed significant differences between the inoculum treatments, with the formulated MS causing larger lesions than the sclerotial inoculum at all observation times (days 4, 6 and 7). In the initial bioassay, the formulated MS germinated and formed lesions on both *R. acris* and *C. arvense*. The high proportion of infection and large necrotic area caused by formulated MS on both target species suggest that this formulation of MS has potential to be developed as a novel bioherbicide.

There were no significant differences in the lesion area caused by the formulated MS between the two species *R. acris* and *C. arvense*. This is not a surprising result as *S. sclerotiorum* is a pathogen that is renowned for its ability to infect and colonise host tissues in a non-discriminant manner across a broad range of hosts (Xu et al., 2018). As a plant pathogen, this makes *S. sclerotiorum* problematic as it can infect many different species, but from a bioherbicide perspective the ability to target multiple problematic weeds with one product is advantageous. In pasture, *S. sclerotiorum* has been shown to have no negative effects towards pasture grasses or legumes but can potentially target several economically significant weeds (Bourdôt, Hurrell, & Saville, 2011; Cornwallis et al., 1999). The results of this detached leaf assay are in agreement with findings from Bourdôt, Hurrell, and Saville (2011) who found approximately 75 - 80% reductions in green leaf area on both *C. arvense* and *R. acris* after inoculation with *S. sclerotiorum* formulated as a gel onto whole plants. The gel formulation of Bourdot (2011) also resulted in similar reductions in green leaf area on other economically important weeds such as the annual thistle *Carduus tenuiflorus* and *Carduus nutans*. The isolate used by Bourdôt, Hurrell, and Saville (2011) was S36, which was the same isolate used to produce MS in the current research. It can be expected that the infective MS formulations produced with the S36 isolate would also be able to infect these other target weeds.

The sclerotial inoculum treatment did not cause any infection on *R. acris* leaves but did infect a small proportion of *C. arvense* leaves. Previous research investigating the S36 isolate as a bioherbicide has also found low rates of germination of whole sclerotia (<1%) on *C. arvense* leaves (Smith, 2018). When they ground sclerotia with a mortar and pestle exposing the medulla, the rate of infection increased to 75% (Smith, 2018). In the present work, the rate of sclerotia germination on *C. arvense*

was slightly higher (>25%), but subsequent lesion development was significantly smaller than those caused by the formulated MS. The poor performance of sclerotia may be explained by the presence on an intact rind (Huang, Chang, & Kozub, 1998). Since the S36 isolate was originally excised from an infected *C. arvense* plant, there may be some degree of host-specificity influencing the myceliogenic germination of the sclerotia, resulting in germination only on *C. arvense* and not on *R. acris*. This result is unexpected as *S. sclerotiorum* is a pathogen with a wide host range and there is little evidence that host-specialisation exists within isolates of the species (Liang & Rollins, 2018).

Mycelial forms of *S. sclerotiorum* are apparently less selective and are more readily able to infect a range of hosts. This was evident in formulated MS of S36 in this research and by the mycelial gel formulations reported by Bourdôt, Hurrell, and Saville (2011) which consistently infected a range of target weeds including non-thistles. The ability of the MS formulation to cause appreciable lesions across both host species may also have been improved by metabolites and virulence factors that accumulated during the fermentation and were then captured by the DE carrier during the formulation process. These metabolites were not investigated but likely include oxalic acid which is known to be important in the pathogenicity of *S. sclerotiorum* (Liang & Rollins, 2018). In contrast, the sclerotia were not within a formulation so the infection process was led by the mycelium using endogenous reserves within the medulla. Any metabolites and pathogenicity factors would have to be actively produced to enable the fungus to infect the host tissues.

All of the plant haplotypes and provenances of both *C. arvense* and *R. acris* were susceptible to infection from the formulated MS of *S. sclerotiorum*, with no resistance to infection observed. Despite this, significant differences in lesion size were found between haplotypes of *R. acris* with two significantly different groups. The G haplotype of *R. acris* had significantly smaller mean lesion area after 6 days indicating it may be less susceptible than the other three haplotypes, resulting in a lower mean lesion area. Similarly, on *C. arvense*, the G27 lineage was significantly more affected by the formulated MS than the G20 lineage. On G20, the formulated MS did not cause significantly larger lesions than the sclerotial formulation. These differences in susceptibility are most likely due to genetic differences between the haplotypes and provenances of the *R. acris* and *C. arvense,* respectively, that result in changes to the pathogen-host interaction. The data here is in agreement with findings presented by Smith et al. (2016) who found that different *C. arvense* provenances varied in susceptibility to application of *S. sclerotiorum* used as mycelium-colonized grains. The differences in susceptibility are most probably due to genetic differences between the plant lineages that result in differences in the host-pathogen interaction. Differences in susceptibility exist between cultivars of crops that *S. sclerotiorum* infects such as *Brassica napus*. The difference between susceptible and resistant plants is reported to occur at the cellular level after the pathogen has penetrated into the plant tissues (Garg, Li, Sivasithamparam, Kuo, & Barbetti, 2010). It is possible

that the differences in susceptibility observed in this study result from past exposure to *S. sclerotiorum* in the field that may have created a selective pressure favouring partial resistance to the pathogen in the weeds. Another possibility is that large variability exists in susceptibility even between individual plants and the environment in which the plant has grown in, and that the 10 replicates used in this work are not sufficient to represent these differences. Further experiment with more replicates and whole plants to draw any conclusions about differences in susceptibility between haplotypes of *R. acris* and provenances of *C. arvense* to formulations of *S. sclerotiorum* need to be undertaken.

Unformulated MS did not cause lesions on either host species. Formulated MS resulted in lesions that covered 50.9% of the total leaf area after 7 days, averaged across all leaf haplotypes/provenances. The difference in these inoculum treatments suggests that the presence of a carrier is critical for the survival of the structures during the drying process. A carrier-only treatment, where DE alone was placed onto the leaf surface, was not included in this experiment. It seems unlikely that this treatment would result in damage as DE is commonly used in foliar insecticide formulations without damage to leaves (El-Wakeil & Saleh, 2009). Despite this, it would be worthwhile to include a carrier only treatment as a control if the research was repeated, to observe whether the carrier alone impacts the excised leaves. Carriers are used as a filter aid to assist in separating the fungal biomass from the spent liquid media (Hynes & Boyetchko, 2006). Typically, carriers are inert compounds such as DE or kaolin clay. The primary purpose of the carrier is to support the structures during filtration and drying and preventing the structures from adhering to each other. Adherence of MS to each not only other reduces the total number of infective propagules in the formulation but may also result in increased mortality due to rupturing of cell membranes as the fungal agents are dried and granulated (Jackson & Jaronski, 2012). In most reports of microsclerotia production in other species of fungi, DE is used as the carrier as was used in this research (Jaronski & Mascarin, 2017). Jackson and Jaronski (2012) found for *M. brunneum* F52 that the microsclerotia could survive storage even when dried without the use of a carrier but found that even with the addition of 60 g/L of DE, samples produced comparable numbers of conidia after 12 months storage to dried MS formulations without any DE.

Successful biological control propagules must have high virulence resulting in consistent infection on the target host (Song et al., 2014). The failure of the formulated MS to cause any infection in subsequent replications of this experiment on excised leaves highlights the need to further investigate attributes of *S. sclerotiorum* MS to work towards more consistent and repeatable production and formulation. Different batches of MS were used in subsequent attempted replications of the experiment but is as yet unclear why the MS from these batches did not have the same activity as those from the first batch. Additionally, the *S. sclerotiorum* isolate was repassaged

through the *R. acris* host, and attempting the excised leaf assay again, the MS formulations still failed to cause infection. All conditions during the fermentation, processing and bioassay were kept as similar as possible. The excised leaves used in the maximum challenge assay were exposed to a number of treatments that may have introduced sources variability. Namely, surface sterilisation and the use of tweezers and pipette tips near the leaf surface, both could have introduced cuticular damage. The inclusion of a blank control leaf would be a useful inclusion in future experiments to control for these effects. The assay was repeated without any surface sterilisation or Vaseline rings and still no infection from the MS was observed. Therefore, these possible sources of variance do not seem to be the factor driving the failure of the MS to infect the leaves.

The electron microscopy images reveal that some of the hyphae in the MS are damaged, with holes visible in the cell membrane as in Figure 3.10A-B. The damage observed appears similar to the ultrastructure damage caused to *S. sclerotiorum* cell membranes after exposure to antifungal extracts of the endophyte *Phomopsis sp.* reported by (Huang et al., 2019). The damaged hyphae resulted in leakage of the cellular contents, detected as an increase in carbohydrates and products of lipid peroxidation. Wu et al. (2015) found antifungal volatile organic compounds to cause cell wall damage to *S. sclerotiorum* hyphae that resulted in inhibition of germination of sclerotia, and microscopy of these hyphae showed damage that was similar to that observed in the present study. This suggests that damage to the cellular membranes during the drying and formulation process in the current work, may be resulting in lysis of the cells within the MS, resulting in their poor performance.

The next steps are to scrutinize the formulation and drying process to determine the points at which the MS are becoming inactive, leading to the inconsistent infection on excised leaves. Once a stable formulation is achieved other important factors such as long-term storage stability and production of MS by other isolates of *S. sclerotiorum* can be investigated. Modifications to the fermentation medium were outside the scope of this research due to time limitations but will be important to investigate in the future optimization of the MS fermentation. Minor changes to the fermentation media/formulation have resulted in major differences in the production, survival, and infectivity of MS in other species of filamentous fungi (Huarte-Bonnet et al., 2019; Shearer & Jackson, 2006; Song et al., 2016; Song et al., 2014).

Previous work has demonstrated that *S. sclerotiorum* isolates excised from *R. acris* and *C. arvense* can cause significant damage when applied onto these species as an augmentative biological control agent (Bourdôt, Hurrell, & Saville, 2011; Cornwallis et al., 1999; Verkaaik et al., 2004; Waipara et al., 1993). The development of a *S. sclerotiorum* bioherbicide based on solid substrates was impeded by the high costs of sterilization and production, while attempts at liquid fermentations produced

mycelial inoculum that while infective had issues with stability and storage. MS combine the stability and storage benefits of a solid substrate formulation, while being produced efficiently in liquid culture. The results of this chapter indicate that MS of *S. sclerotiorum*:

a) can be produced in submerged liquid culture.

b) are capable of surviving drying to <4% with a DE carrier.

c) Formulated MS were infective on excised leaves of both *R. acris* and *C. arvense*.

Future work needs to focus on improving the consistency of infection and testing on whole plants in the greenhouse and the field. Literature on *S. sclerotiorum* sclerotia formation is often drawn upon to hypothesize and understand findings of MS formation due to the similarities of the structures. This work will enable future work to compare the production of MS and sclerotia within one species to better understand the similarities and differences between the structures and their production.

Chapter 4

Effect of formulation process on survival of *S. sclerotiorum* microsclerotia

4.1 Introduction

When formulating biopesticides, a carrier can be used to support the microbial propagules (mycelium, conidia, microsclerotia, blastospores etc.) produced by a fermentation during the downstream processing (filtration and drying) (Ravensberg, 2011). Carriers are principally inert inorganic or organic compounds such as diatomaceous earth (DE), peat, vermiculite, clays and polysaccharides (Sahu & Brahmaprakash, 2016; Vassilev et al., 2020). Carriers protect microbial propagules by preventing them from adhering to each other, helping to maintain structure and micrometric size, and protection from extreme desiccation (Behle & Birthisel, 2023). DE is the most used carrier in MS formulation and has been used to create active granular MS formulations for a number of species (Jackson & Payne, 2016; Jackson & Schisler, 1995; Mascarin et al., 2014; Villamizar et al., 2018).

Microsclerotia (MS) production of *Sclerotinia sclerotiorum* has been demonstrated consistently in fermentations with the MS media described in Chapter 3. When MS were harvested and prepared as a dried formulation, as in Section 3.2.1, the efficacy of the MS formulation was not consistent over time replications of the experiment. The first experiment on excised leaves of *Ranunculus acris* and *Cirsium arvense* resulted in large necrotic lesions, characteristic of *S. sclerotiorum* infection, and *S. sclerotiorum* was able to be isolated from all infected leaves that were sampled, fulfilling Koch's postulates (Section 3.3.2). However, in later replications of the experiment MS formulations did not result in any infection on excised leaves.

The objective of research presented in this chapter, was to investigate the fermentation, formulation and processing of the MS to better understand what caused inconsistent performance when MS were applied to excised leaves. The first experiment measured the germination of MS throughout each step of the formulation and drying processes to determine the effect on MS viability on agar and pathogenicity on excised leaves of *R. acris*. The hypothesis was that MS would survive up until the drying step. The second experiment tested the germination of MS produced after different lengths of fermentation. The hypothesis was that as the MS mature, the structures grow and become dense, with an increasing amount of extracellular matrix overtime with reducing viability of the mycelium inside the medulla of the structures. This phenomenon was observed by Garcia Riaño

(2022) with maturing MS of *Metarhizium robertsii*. The third experiment examined the effect of the carrier used in the formulation on the survival and germination of the MS. The alternative carriers, corn starch and kaolin were compared to DE, which was the only carrier tested in previous chapters. These carriers have been used in formulations of MS with other species of fungi and are commonly used in formulations to create water-dispersible granules (Jackson & Payne, 2016).

The hypothesis to be tested in this chapter are:

1. MS of *S. sclerotiorum* survive the formulation process up until the air-drying step.
2. MS of *S. sclerotiorum* have reduced viability with increasing fermentation time.
3. Formulating MS of *S. sclerotiorum* with different carriers does not affect the viability of the structures.

4.2 Methods

4.2.1 Effect of formulation process on MS viability and virulence

4.2.1.1 MS production

MS medium (2 L) was prepared as in Section 3.2 and subsequently aliquoted into 20 x 250 mL conical flasks (Schott Duran®). Due to supply issues, there was a modification to the original media composition. The original media used Casamino Acids, Technical (Bacto™) which contains 8.3% total nitrogen, 5.9% amino nitrogen, 36% ash, 30% salt and is not supplemented with inorganic salts or growth factors (Appendix A.3) This product could not be sourced for this experiment so was replaced with Casamino Acids (AG Scientific) which contains a similar profile of 8.7% total nitrogen, 5.4% amino nitrogen, 32% Ash and 37% salt (Appendix A.4). Flasks were prepared, inoculated with six sclerotia each of the *S. sclerotiorum* S36 isolate from ten-day old cultures grown in darkness at 20°C, then incubated for 14 days as described in Section 3.2.1.

At days 6, 8, and 14, growth rings of mycelium that accumulated on the inside walls of the flask were removed from all of the flasks using a sterile inoculation loop within a laminar flow hood. Early in the fermentation procedure, growth rings are not easily removed as they mainly consist of granular precipitate that is not held together. By day 8, the rings are easier to remove as they are held together by mycelium as the fungus establishes in the media (Observation, J. Mills, April 2021). On day 8, 100 µL samples were taken from all 20 flasks and spread onto PDA plates using a sterile plastic spreader in a laminar flow hood and were incubated at 25°C. Plates were assessed 6 days later for the growth of *S. sclerotiorum* and any potential contaminants. If contaminants were observed, the

flask contents from which they came, were discarded. On the same day (day 14), *S. sclerotiorum* flasks were harvested.

4.2.1.2 MS Harvesting and Formulation Process

Harvesting followed the same protocol as for MS formulation in Section 3.2.1. During the harvesting stage, aseptic technique was used where practicable. Samples were collected at each step in the processing of the fresh fermentation broth, through to the final dried formulations as shown in the flow chart in Figure 4.1. The process is described in detail here. From each of the 20 flasks, an 11 mL sample was taken from the 14-day-old fermentation broth and transferred into a 15 mL centrifuge tube. From each of the 11 mL samples, 1 mL was transferred into separate 15 mL centrifuge tubes (Nest®), to obtain the “Fresh” treatment for bioassays and plating (Figure 4.1, “Fresh” treatment). After this, the contents of all flasks were combined to create the “Bulked” treatment for the experiment (Figure 4.1). Flasks with any signs of contamination were not mixed. After mixing thoroughly by hand for 1 min, a 15 mL sample was taken for analysis. Then, DE (Celite 281) was added at a rate of 5 g per 100 mL to the bulked broths and mixed by swirling until the mixture was homogenous. Of this sample, 15 mL was used to create the “DE” treatment for plating and bioassays (Figure 4.1, “DE” treatment). The mixture was then vacuum filtered for approximately 90 sec through Whatman Number #1 filter paper until it reached a dough-like consistency, to form a filter cake. For optimal liquid extraction, this process was done in several small batches, before each of the filter cakes were combined together into one homogenous mass. At this point, the dough still contained a substantial amount of liquid, so the entire combined dough was vacuum filtered for a final time. After this final vacuuming process, 15 g of the dough were taken to create the “Dough” treatment for plating and bioassays (Figure 4.1, “Dough” treatment). The remaining dough was weighed and split equally into two 141 g samples. These two samples were spread into thin layers on aluminium trays, with grooves added with a spatula to increase the surface area to improve the drying. One of the samples was air dried for 24 hr in a laminar flow cabinet and had its moisture content measured (AND, MX-50 Moisture Analyzer. 100 °C) and was then packaged and sealed in a plastic zip-lock bag (Esselte, 100 mm x 155 mm) to create the “Air Dried” treatment for plating and bioassays, which was stored at 4 °C until use (Figure 4.1, “Air Dried” treatment). The other dough sample was freeze-dried for 48 hr to reach a final moisture content of 2.99% and was then sealed in a plastic zip-lock bag to create the “Freeze Dried” treatment for plating and bioassays (Figure 4.1, “Freeze Dried” treatment).

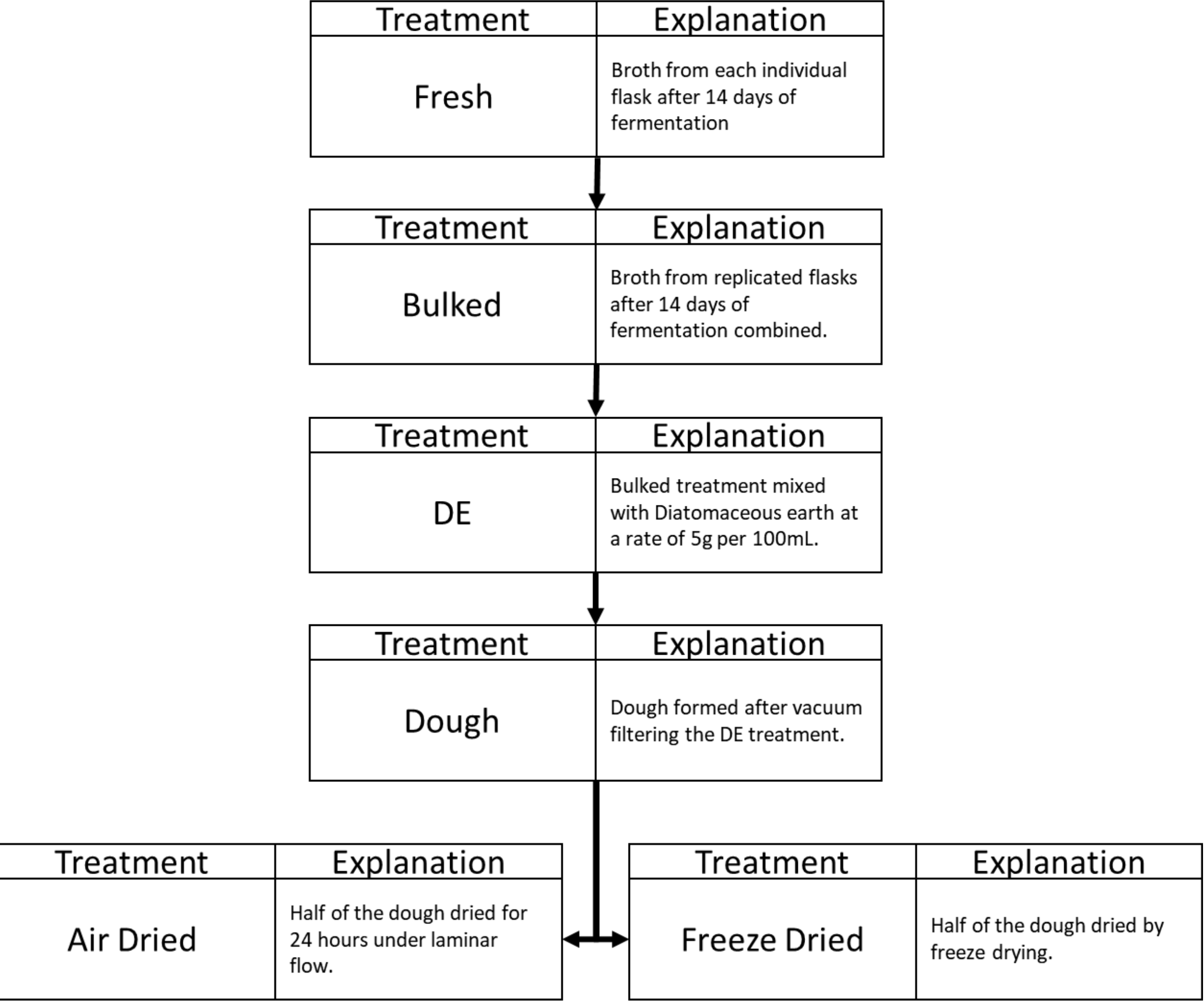

Figure 4.1 Flow chart describing the processing steps to create a formulation from a fresh fermentation broth containing microsclerotia of *S. sclerotiorum*. Samples were taken at each processing step and tested for germination on agar and pathogenicity on excised *R. acris* leaves.

4.2.1.3 *R. acris* leaf collection

R. acris leaves of the cytotype “J” were collected from potted plants located in the Lincoln University shadehouse. Leaves in the plant were categorised into large and medium/small with the diameter across the leaf >70 mm or 30 – 70 mm, respectively. The large leaves were not used for the bioassay as they were visibly infected with *Alternaria* spp. and other foliar pathogens. Small to medium leaves that had less visible signs of fungal infection and appeared healthier were used in the bioassays. All of the collected leaves were cut into three lobes, with any lobes showing signs of *Alternaria* or damage discarded. In total, 200 healthy lobes were collected. These lobes were randomly mixed and split into two lots of 100. One lot was used in for the bioassay using only “Fresh” and the other was used in a concurrent experiment comparing all of the bulked treatments from the drying process (“Bulked”; “DE”; “Dough”; “Air Dried”; “Freeze Dried”).

4.2.1.4 Leaf Assay – Fresh Treatment

Five lobes of *R. acris* leaves were placed into each clear plastic container (175 x 120 x 55 mm) (n=20) on a paper towel dampened with 20 mL of sterile water (Figure 4.2). These containers were arranged to create five replicate blocks. Each block consisted of four containers, with five lobes of *R. acris* per container. The samples of broth from each of the 20 replicate flasks were randomly allocated to one of the leaves within a block. For each individual leaf lobe, 50 μL of fresh MS broth from a flask was pipetted onto the leaf surface. The clear lids were put on the containers and then incubated at 20 °C with a 12 hr photoperiod. Leaves were photographed after 24, 48 and 72 hr. By 72 hr, all leaves had become infected.

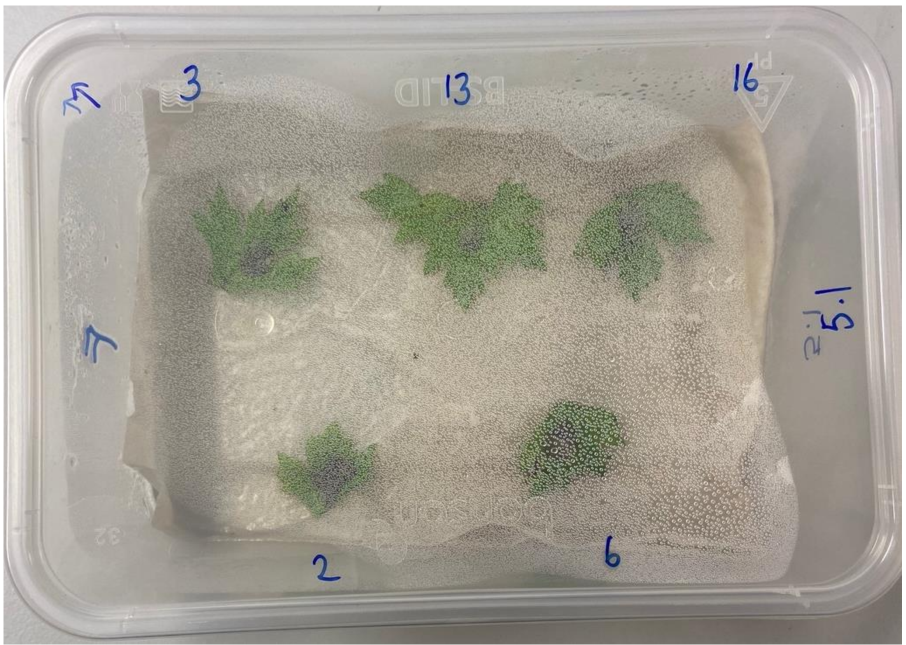

Figure 4.2 Container with *R. acris* lobes inoculated with 50 μL of fresh broth from a MS fermentation of *S. sclerotiorum.* The numbers indicate the replicate flasks from which each leaf was inoculated. Four containers formed one replicate block.

4.2.1.5 Leaf Assay – Drying Treatments (Bulked)

Four lobes of *R. acris* leaves were placed into each of the 20 plastic containers on a paper towel dampened with 20 mL of sterile water, as in Figure 4.3. Within each container there were four treatments, with each lobe randomly reciveing one treatment: “Bulked”, “DE”, “Dough” and “Air Dried”. For the treatments in a liquid state, 50 μL of each treatment was pipetted onto the leaf lobes. For the dry treatment 1/32th of a teaspoon (1 smidgen) was transferred onto the leaf lobes.

Containers were arranged in randomized blocks and incubated at 20 °C with a 12 hr photoperiod. Leaves were photographed after 24, 48 and 72 hr.

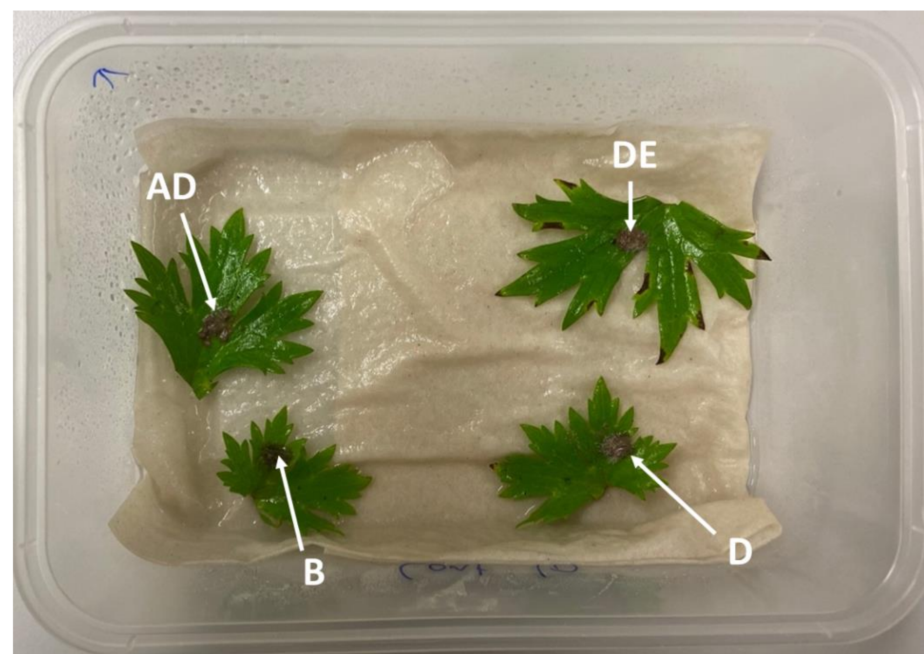

Figure 4.3 ***R. acris*** **leaves inoculated with MS across 4 stages of the formulation process. "B" indicates the Bulked treatment, that was created by mixing all 20 replicate flasks. "DE" indicates the treatment where the bulked sample was mixed with diatomaceous earth. "D" indicates the Dough treatment, which was sampled after vacuum filtration of the microsclerotia and diatomaceous earth mixture. "AD" indicates the Air Dried treatment which was the sample after the dough was left to air dry for 24 hours under laminar flow.**

4.2.1.6 Viability Assay - Fresh

On 22 June 2022, 15 mL samples of fresh broth from individual replicate flasks were harvested and stored in a 1.5 mL centrifuge tube which was placed in a refrigerator overnight at 4 °C prior to use in the bioassay on 23 June 2022. Samples for the germination test could not be processed immediately and had to be stored in the fridge for an additional 24 hr, which facilitated their germination and aggregation, thus making the evaluation impossible.

4.2.1.7 Viability Assay - Drying Treatment Samples

The "Bulked" and "DE" samples were each stored as 15 mL overnight in a refrigerator at 4 °C, with individual MS clearly visible in the sample. For the "Dough", "Air Dried" and "Freeze Dried samples", 1 g of the samples was re-suspended in 9 mL of water to create a liquid suspension. For each of the samples, 100 µL was pipetted onto five replicate plates containing 1/5 strength potato dextrose agar (1/5 PDA) and spread with sterile plastic spreaders. After 24 hr, the first 20 observed MS on each

plate were assessed for germination and again after 60 hr a further 20 MS were observed. Structures were considered germinated if there was mycelial growth where the length was greater than half the diameter of the MS.

4.2.2 Effect of age (fermentation time) on MS viability

MS broth (1200 mL) was prepared as in Section 3.2.1 and divided into 12 flasks (each containing 100 mL). The fermentation was set up as Section 3.2.1 with three replicate flasks harvested at the following time intervals: 10 days, 12 days and 14 days post-inoculation of the broth.

At each of the harvesting times, flasks were divided so that 50 mL of broth from each flask were formulated and air dried following the method in section 3.2.1, while the remaining 50 mL was retained as fresh broth. From each of the fresh broth samples, 1 mL was taken to estimate MS production under light microscopy using the method in section 3.2.1.

A 1/10 dilution was made from the fresh and dried samples of each flask, by suspending 1 mL or 1 g (dependent on whether the sample was liquid or solid) in 9 mL of 1% Tween®80 (Sigma). To determine MS viability, 100 µL of each of the dilutions was pipetted onto 5 replicate plates of 1/5 PDA and spread using a sterile plastic spreader. After 40 hr, the first 100 MS observed across the 5 plates of each of the samples was assessed for germination.

To prepare samples for microscopy, 1 mL of fresh broth from one of the replicate flasks was spread across a sterile plastic petri plate. Using a compound microscope and an inoculation loop, 30 individual MS were transferred into 1 mL of 2.5% glutaraldehyde solution to be fixed. MS from the tube were used for observation under an Olympus BX50 light microscope and structures were photographed using an Olympus DP72 digital camera.

4.2.3 Effect of drying time and formulation with different carriers on MS viability

MS medium was prepared and 100 mL placed into each of the 250 mL conical flasks. Three treatments were randomly assigned to five replicate flasks. The flasks were randomly placed within a shaking incubator and incubated for 14 days at 20 °C and 300 rpm as in Section 3.2.1 to produce MS. At days 8, 10 and 12, rings were removed from each of the flasks using aseptic technique. After 14 days, 100 µL samples of a 1/10 dilution made from each of the flasks was plated on 1/5 PDA and spread with a sterile plastic spreader, then incubated for 48 hr at 20 °C. To assess the germination (%) as a measurement of the viability of fresh MS directly recovered from the culture medium, the germination of 100 MS from each plate were assessed under the microscope. The flasks (five per carrier) were then mixed with the carriers: DE (Celite 281), kaolin (Pure Nature, NZ) and corn flour (Edmonds), at a rate of 5 g per 100 mL of broth.

After the vacuum filtration step, as in Section 3.2.1, the MS containing dough was placed in a laminar flow hood to dry on a plastic tray. Beginning at time 0, 0.1 g samples of the dough were taken, and the moisture content measured in a moisture analyser (AND, MX-50 Moisture Analyzer. 100 °C). Another 0.5 g sample was mixed with 4.5 mL of distilled water to create a suspension, of which 100 µL was pipetted onto four 1/5 PDA plates. These plates were incubated for 48 hr at 20 °C under darkness before the germination of the first 100 MS observed across the four plates from each replicate flask were assessed. The moisture analysis and germination plating were repeated every 8 hr for 24 hr.

4.3 Results

4.3.1 MS Production

Plated samples from all 20 replicate flasks showed growth of only *S. sclerotiorum* and no contaminant species were detected. On the cotton bungs of three replicate flasks, contamination was observed and so these flasks were excluded from being combined into the bulked treatments.

4.3.2 Effect of formulation process on MS viability and virulence

4.3.2.1 Virulence

The fresh broth from individual replicate flasks (“Fresh”) resulted in the highest proportion of infected lobes, with 97% of lobes showing necrotic lesions after 24 hr (n=100) (Figure 4.4). The “Bulked” treatment resulted in no lesions observed after 24 hr and 55% of the inoculated lobes with observable lesions after 48 hr (n=20). The proportion of lobes with lesions after 72 hr was 100% for the “Fresh” treatment (n=100), 65% for the “Bulked” and “DE” treatments (n=20), 85% for the “Dough” treatment (n=20) and 0% for the “Air Dried” treatment (n=20). The effect of these treatments on the proportion causing infection was significant ($P < 0.001$), with the “Air Dried” treatment not causing any lesions after 72 hr. The proportion of leaves with lesions between the “DE”, “Dough” and “Bulked” treatments was not significantly ($P = 0.279$) different after 72 hr. The null hypothesis that the proportion of infected leaves will be the same at each stage of MS processing can be rejected at a 95% confidence interval. Koch’s postulates were not tested to see if *S. sclerotiorum* could be reisolated from the infected leaves, but the mycelium, lesions and sclerotia observed on the infected leaves after ~7 days, were characteristic of *S. sclerotiorum* infection.

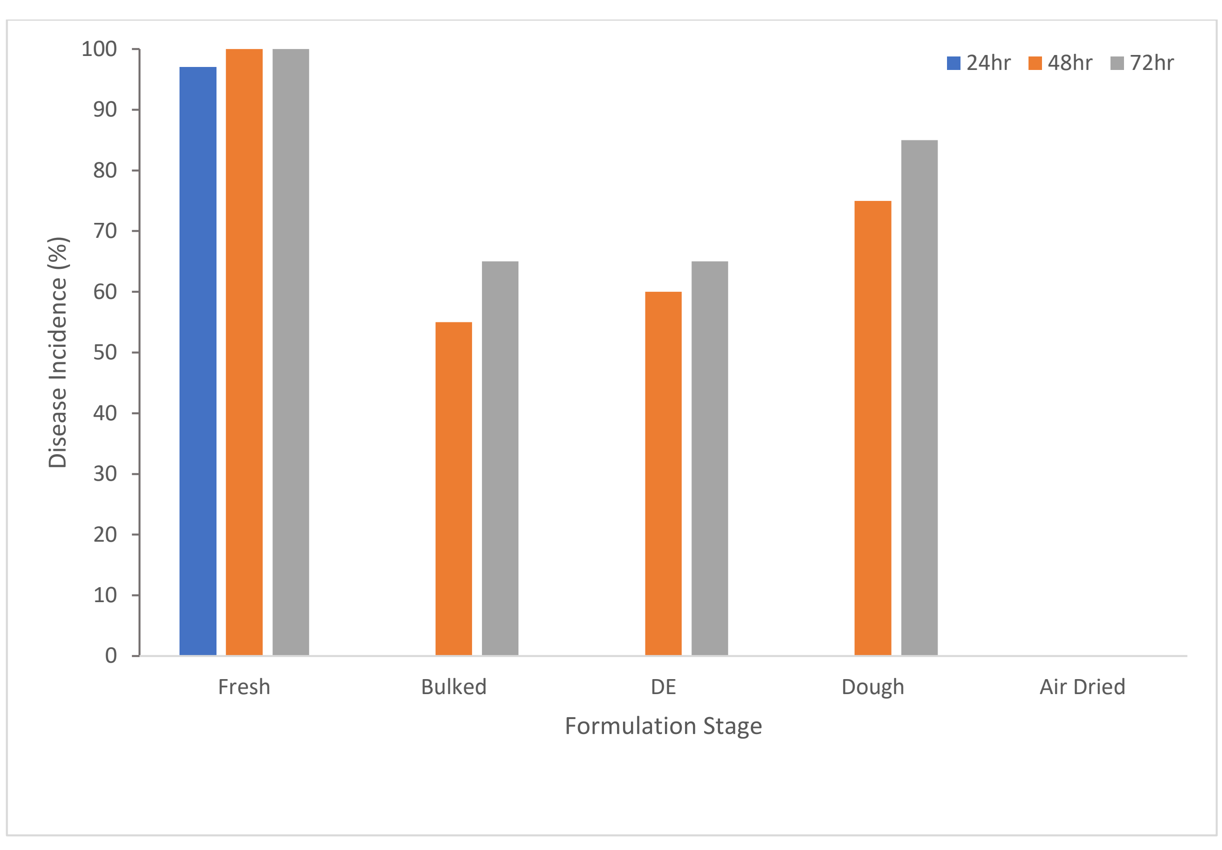

Figure 4.4 Disease incidence on *R. acris* lobes assessed after 24, 48 and 72 hr after inoculation with MS of *S. sclerotiorum*. Each formulation stage represents a further step of processing as outlined in Figure 4.1. "Fresh" samples were taken directly from individual replicate flasks. The "Bulked" samples were taken after mixing all 20 replicate flasks. "DE" samples were taken after the bulked broth was mixed with a diatomaceous earth carrier. "Dough" samples were taken after the formulation with diatomaceous earth was vacuum filtered. "Air Dried" samples were taken following 24 hours of air drying of the dough.

4.3.2.2 Viability

In both dried treatments, "Air Dried" and "Freeze Dried", no MS were observed to be germinated at either observation time. At 24 hr, the average germination of MS on agar was not significantly ($P > 0.05$) different between the "Bulked" and "DE" treatments, with 84% and 89% of MS germinated respectively, which increased to 99% and 100% respectively after 60 hr of incubation (Figure 4.5). The germination percentage of the "Dough" treatment was in a significantly ($P < 0.05$) different group at 24 hr, with lower germination at 27%, but after 60 hr 100% of the MS observed had germinated and did not differ significantly ($P > 0.05$) from the "Fresh", "Bulked" or "DE" treatments (Figure 4.5). After 60 hr, no MS had germinated from either dried treatment. These differences drove the interaction effect between treatment and time, which had a significant ($P < 0.001$) effect on germination. The effects on the individual factors of treatment were also significant ($P < 0.001$), as was the effect of time ($P < 0.001$).

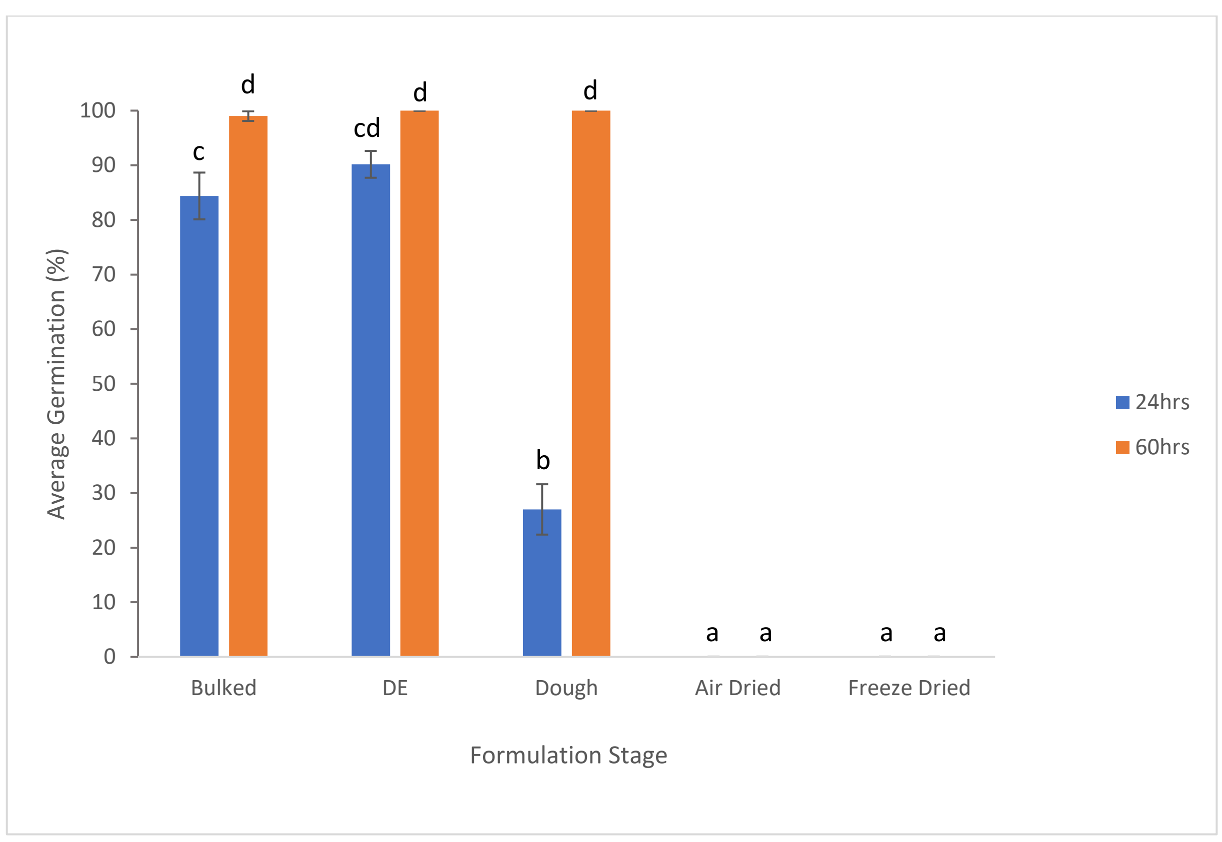

Figure 4.5 Germination of *S. sclerotiorum* MS on agar at each step of the formulation process as outlined in Figure 4.1. The "Bulked" samples were taken after mixing all 20 replicate flasks. "DE" samples were taken after the bulked broth was mixed with a diatomaceous earth carrier. "Dough" samples were taken after the formulation with diatomaceous earth was vacuum filtered. "Air Dried" samples were taken following 24 hours of air drying of a subsample of the dough. "Freeze Dried" samples were taken following the freeze drying of a subsample of the dough. Error bars indicate standard error of the mean (SEM). Letters indicate significantly different groups from the results of a Bonferroni test at P=0.05.

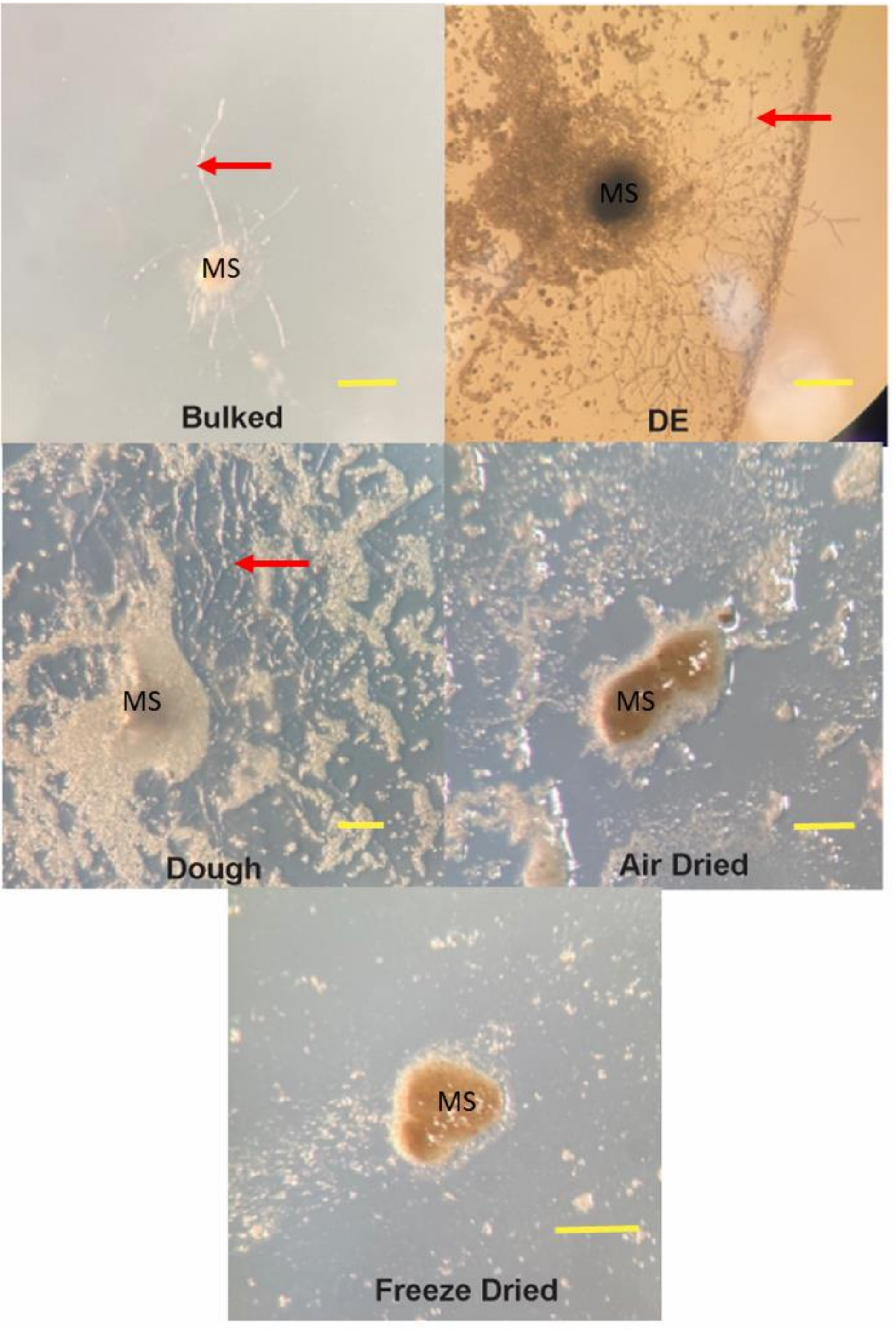

Figure 4.6 MS of *S. sclerotiorum* from each formulation processing step after 60 hr incubation on 1/5 strength PDA at 20 °C. MS indicates microsclerotia and red arrows indicate germinating mycelium. Scale bars indicate ~200µm. The "Bulked" samples were taken after mixing all 20 replicate flasks. "DE" samples were taken after the bulked broth was mixed with a diatomaceous earth carrier. "Dough" samples were taken after the formulation with diatomaceous earth was vacuum filtered. "Air Dried" samples were taken following 24 hours of air drying of a subsample of the dough. "Freeze Dried" samples were taken following the freeze drying of a subsample of the dough.

4.3.3 Effect of age (fermentation time) on MS viability

The total number of structures per mL (including mature MS and immature aggregates) were 3900, 3420 and 3860 at day 10, 12 and 14 post-inoculation, respectively and did not vary significantly (P = 0.834) between samples from the three fermentation times. The number of mature MS (compact and melanised, without highly floccose margins) observed did not significantly differ (P = 0.053) between the three fermentation times, when all replicate flasks were included (Figure 4.8). The mean MS per mL after 14 days was 1760 compared to 420 at day 10. It was clear from microscopic observations that the MS structures became increasingly melanized and compact over time, with less wispy mycelium around the margins (as in Figure 4.6, Chapter 3). Two of the replicate flasks at day 14, had floccose loosely aggregated structures as in Figure 4.7, unlike what had been observed in previous fermentations, that were not counted as mature MS so had very low MS counts at day 14 (300 and 700). When flasks that were considered to have these floccose aggregates were excluded from analysis, the differences in MS yields between fermentation times were significantly different (P = 0.01). The average MS per mL at day 10 was 525 ± 149, increasing significantly (P = 0.05) to 2600 ± 950 by day 14. Having only two categories of assessment (immature or mature) for counting MS limited the scope of the data set as it did not account for the maturation of the MS after they became "mature" aggregates or the unexpected floccose aggregates in some of the flasks (Figure 4.7).

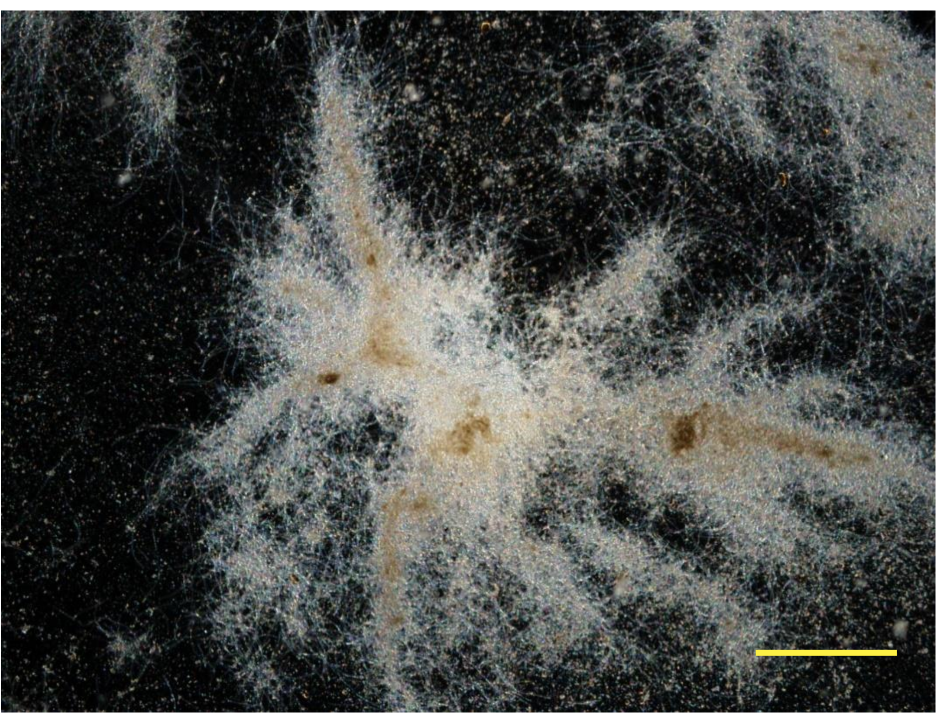

Figure 4.7 Mycelial aggregate on day 14 of fermentation in the MS broth. Two replicate flasks exhibited structures like this that were floccose and lacked the characteristic features of MS. These aggregates had high rates of germination after drying compared to the replicates that contained MS-like structures. Scale bar indicates 200 µm.

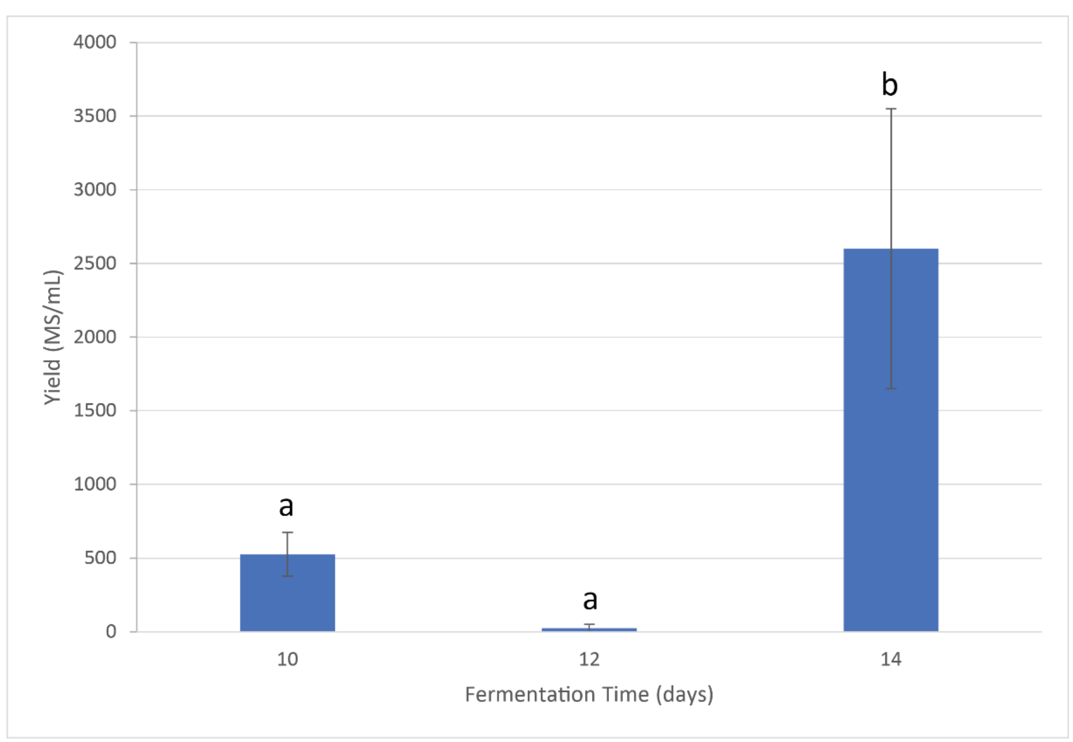

Figure 4.8 Yield (MS/mL) of mature MS from *S. sclerotiorum* in subsamples of broth after different fermentation times excluding replicate flasks that only produced floccose aggregates. There were significant differences in yield between fermentation lengths (P = 0.01). Error bars indicate standard error (n=5). Letters indicate significantly different groups from the results of a Bonferroni test at P=0.05.

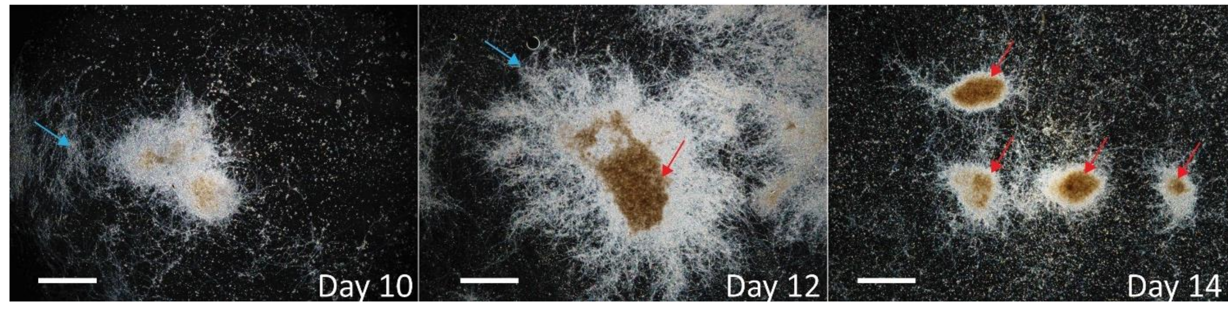

Figure 4.9 Development of MS from *S. sclerotiorum* over time under 40x magnification phase contrast microscopy. White scale bar in each panel represents a length of 200 µm. Red arrows indicate mature MS, noting the melanisation of the aggregates. Blue arrows indicate the mycelium halos.

Fermentation time had a significant (P = 0.025) effect on the viability of MS when averaged across both fresh and dried treatments. Fresh from the broth, structures had 97.3 ± 1.7% viability, that was not significantly (P = 0.068) different between the fermentation times, although the means were numerically higher with increasing fermentation time (Day 10 = 92.8 ± 4.5%, Day 12 = 99.0 ± 1.0%, Day 14 = 100 ± 0%) (Figure 4.10). The effect of fermentation time on MS viability was more noticeable with the dried and formulated MS, with viability increasing from 5.5 ± 1.9% at day 10, to 19.5 ± 5.9% at day 12 to 47.4 ± 19.1% at day 14. The drying treatment (fresh or formulated and dried) had a significant effect on the viability of the MS (P < 0.001) with drying of MS having substantially

less germination than fresh. At all fermentation times, the effect of drying had a significant (P < 0.05) reduction in the viability of MS (Figure 4.9). There was variability within a single treatment during experimentation with two of the replicate flasks at day 14 exhibiting very low germination after being dried, 0% and 3%, while the other three replicates had 85%, 84% and 65% germination (Figure 4.11). It was noted that the two replicate flasks that had the highest germination after being dried, both had the lowest counts of mature MS structures and with most structures floccose, loosely compact mycelial aggregates as shown in Figure 4.7. The results indicate that these mycelial aggregates survive the drying process better than the MS structures observed, although the sample size is too small to make definitive conclusions (two of five replicate flasks). The interaction between fermentation time and drying treatment did not have a significant effect on the viability of MS (P=0.117).

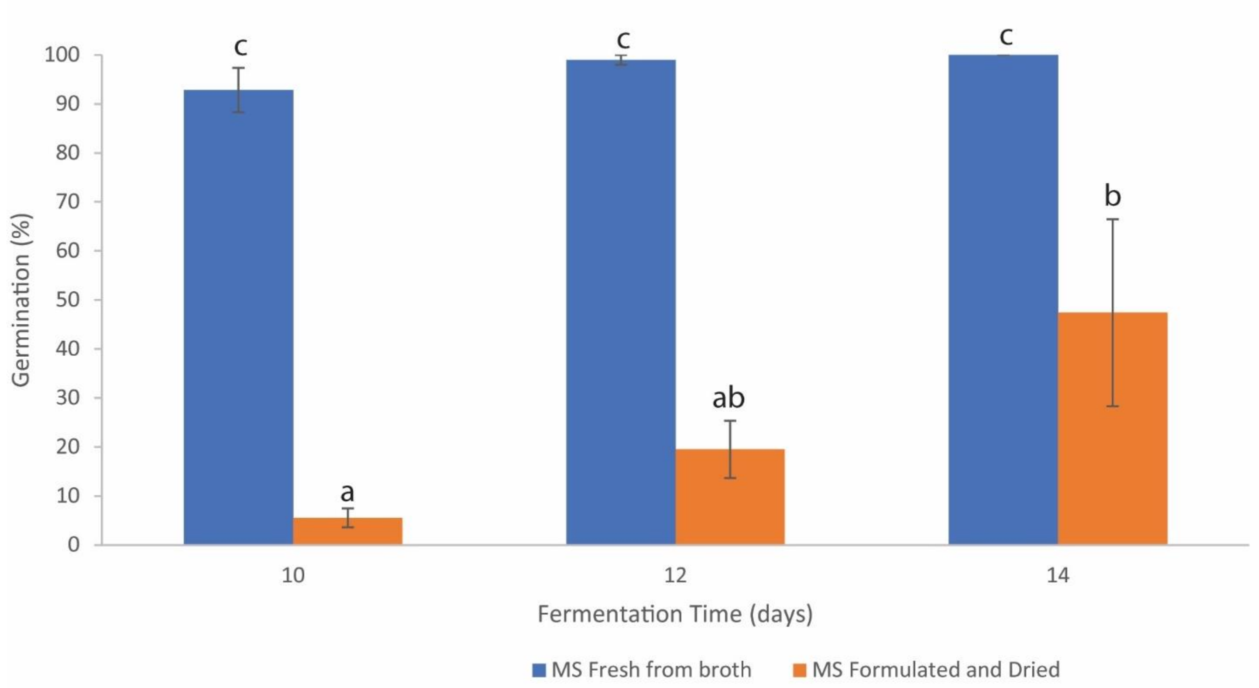

Figure 4.10 Viability of MS from *S. sclerotiorum* harvested at different fermentation times and assessed before and after formulation into a dry powder. Blue bars show MS fresh from broth. Orange Bars show MS after being formulated and dried. Error bars indicate standard error of the mean (SEM). Letters indicate significantly different groups from the results of a Bonferroni test at P=0.05.

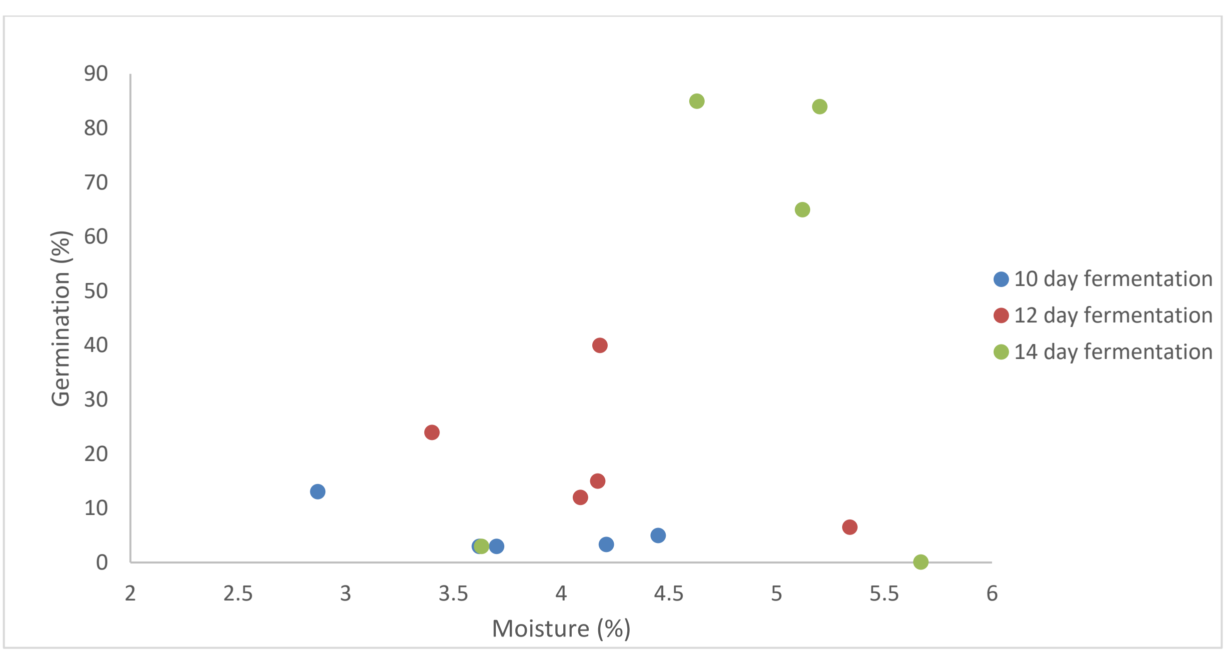

Figure 4.11 Survival and moisture content of dry formulations based on MS from *S. sclerotiorum* harvested at different fermentation times. X-axis shows the final moisture content of the dried samples. 10 day of fermentation (blue). 12 day fermentation (red). 14 day fermentation (green).

The mean moisture content after MS were formulated and dried for 24 hr increased with fermentation time, from 3.7 ± 0.5% at day 10, to 4.2 ± 0.6% at day 12, to 4.9 ± 0.7% at day 14 (Figure 4.12), but the difference between these was not significant (P=0.087).

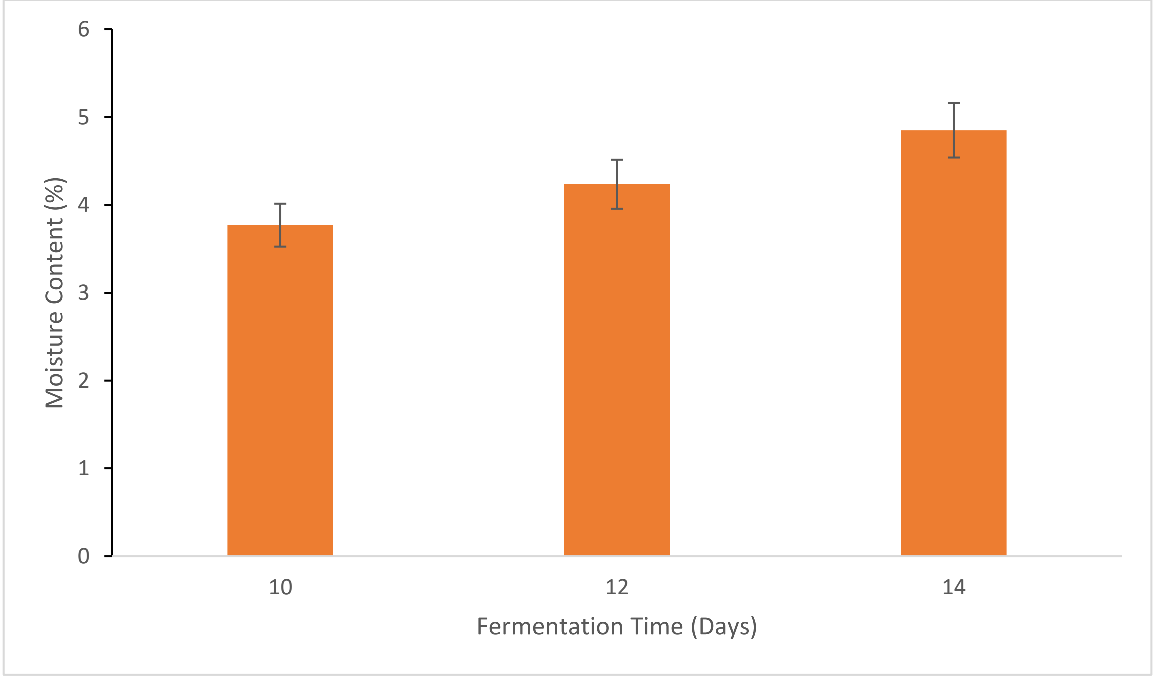

Figure 4.12 Mean moisture content of 24 hr dried formulation containing MS harvested after different fermentation times (10 days, 12 days, 14 days). Error bars indicate standard error of the mean (SEM).

4.3.4 Effect of drying time and formulation with different carriers on MS viability

The interaction between drying time and carrier treatment had a significant effect on the moisture content of the MS formulations ($P<0.001$). The interaction was largely driven by drying time, which had a significant effect on the formulation moisture content ($P<0.001$), with moisture content decreasing across all treatments with increasing drying time (Figure 4.13). The DE formulation contained the highest initial moisture content after vacuum filtration at time 0, at an average of 68.25%, but dried at a faster rate than the other carriers (kaolin and cornflour), at a rate of 2.6% moisture loss per hr (Figure 4.13). The moisture content of the DE treatment remained higher than the other two carrier treatments after 8 hr, but by 16 hr it was not significantly different from the corn-starch treatment. After 24 hr of drying there was no significant ($P < 0.05$) difference in the moisture content across the three treatments, with the average moisture after 24 hr drying being 7.06 ± 0.54%. There was no significant ($P=0.783$) difference in moisture content between the kaolin and corn starch treatments at any sampling time. Kaolin and corn starch formulations both had a similar initial moisture content after vacuum filtration of 46.8% and 42.5% and decreased at a rate of 1.9% and 1.6% per hr respectively.

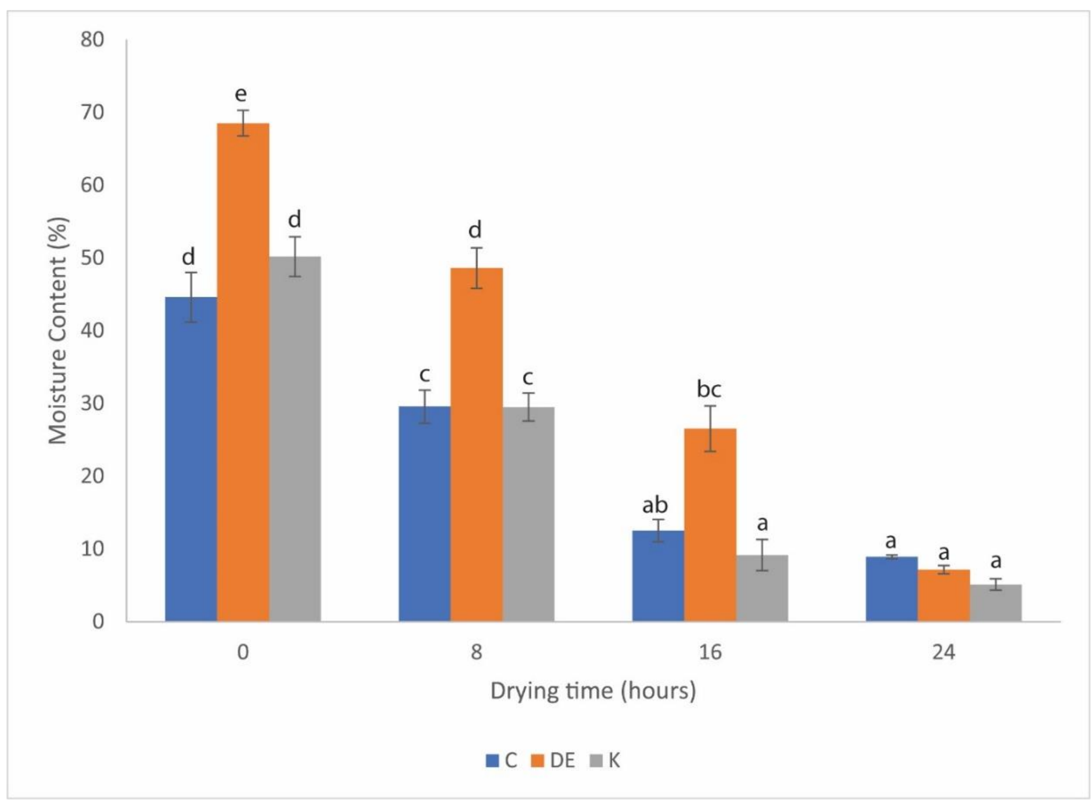

Figure 4.13 Moisture content of *S. sclerotiorum* MS formulated with different carriers during drying process under laminar flow at 22°C. C = corn starch, DE = diatomaceous earth, K = kaolin. Error bars show standard error of the mean (n=5). Letters indicate significantly different groups from the results of a Bonferroni test at P=0.05.

The germination of MS was significantly affected by the drying time (P = 0.03) and the carrier treatment (P < 0.001). The interaction between drying time and carrier was also significant (P < 0.001). The moisture content of the formulations was included as a co-variate in the ANOVA analysis but was not significant (P = 0.066).

MS formulated with corn starch had the most rapid decrease in viability, with a reduction from 97% germination to 9% after 8 hr of drying (Figure 4.14). After 16 hours of drying MS formulated with DE maintained significantly higher germination (86 ± 6%) than MS formulated with kaolin (29 ± 16%) and corn starch (9 ± 4%) (P < 0.05). After 24 hr of drying, the average germination of MS with DE decreased to 21%, that did not significantly differ (P = 0.175) from the germination of MS in formulations with the other carriers.

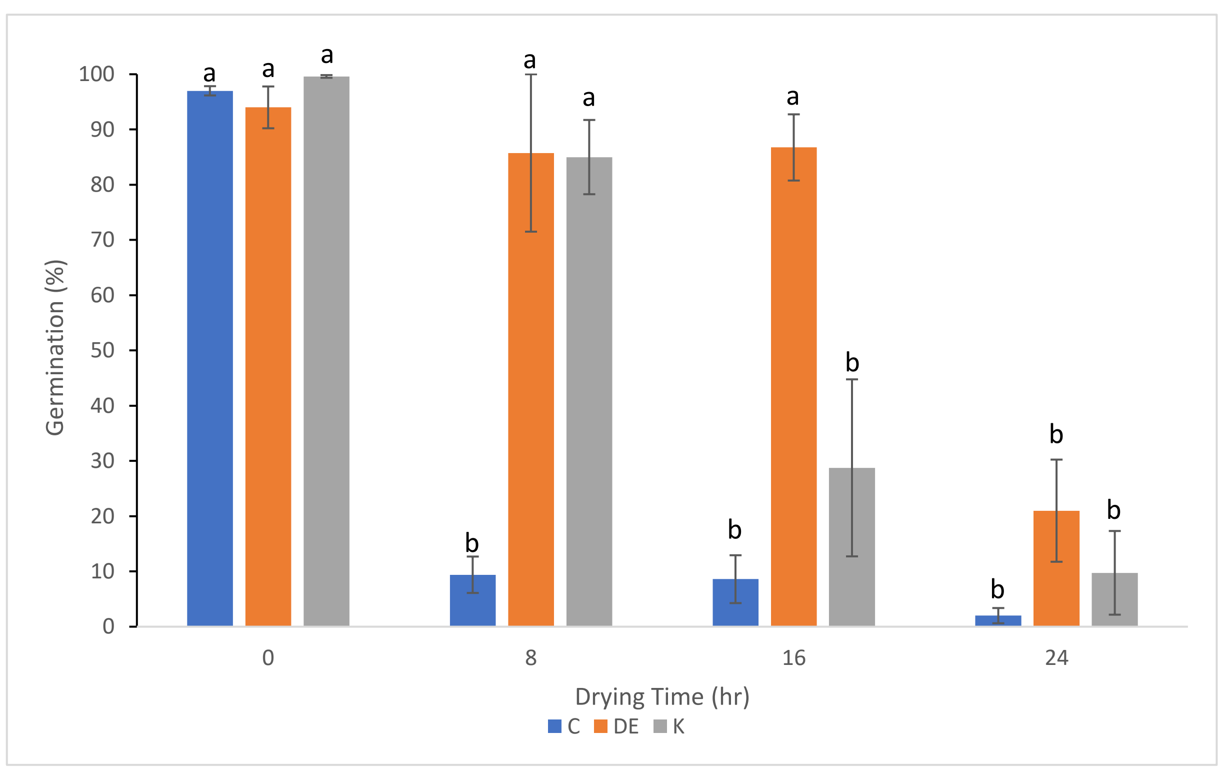

Figure 4.14 Germination of *S. sclerotiorum* MS formulated with different carriers at drying times. (C = corn starch, DE = diatomaceous earth, K = Kaolin). Error bars show standard error of the mean (n=5). Letters indicate significantly different groups from the results of a Bonferroni test at P=0.05.

4.4 Discussion

The purpose of this chapter was to investigate the formulation and drying process to identify at which stage the MS of *S. sclerotiorum* were becoming inactive. The results of this chapter have identified that it is the drying step that is significantly reducing the survival of the MS. A critical moisture threshold of around 30% was identified, below which, the survival of the MS was very low.

Adjusting the fermentation time from 14 days to 10 or 12 days did not improve the survival of MS after drying. Similarly, changing the carrier used in the formulation did not improve the survival of MS after drying.

4.4.1 Effect of formulation process on MS viability and virulence

The purpose of this investigation was to understand at what point the MS were becoming inactive, and subsequently lose the ability to germinate on 1/5 PDA plates or plant host tissue. Air drying the formulation containing MS caused a reduction in viability of the structures. The air-dried formulation did not cause infection on any of the excised *R. acris* lobes after 72 hr incubation. This result is possibly related with the drop in viability because MS from dry treatments had 0% germination on agar after 60 hr incubation. Evidently it is at this stage of production, the air-drying step, at which the *S. sclerotiorum* MS lose viability and are predominantly dead suggesting that the produced MS are susceptible to desiccation. During drying of the formulation, the cells undergo dehydration that causes damage and is most likely what is resulting in the MS dying at this stage. During desiccation, cell membranes can be irreparably damaged due to a loss of structural integrity which results in membranes becoming leaky and a resultant loss of the cell contents (Crowe, Oliver, & Tablin, 2002). The scanning electron microscopy images show that some of the hyphae within the produced MS have damaged cell membranes (Figure 3.10A-B), and some cells have completely lysed and shrivelled (Figure 3.10C), providing evidence towards the hypothesis that cell membrane damage during the drying process could be the cause of the low viability of the MS. On the other hand, Garcia Riaño (2022) found that as MS of *Metarhizium robertsii* matured, the number of viable cells within these structures decreased, with an increasing amount of extracellular matrix and space between the viable cells. In the current work, transmission electron microscopy of ultrathin sections of MS showed the interior containing low density of electrodense (alive) cells surrounded by extracellular matrix (Figure 3.11) similar to the findings of Garcia Riaño (2022). However, in the current study microscopy was only undertaken on 14-day old MS, so it is not known how the densities of cells might change as MS of *S. sclerotiorum* mature. It is hypothesized that the few live cells within the MS of *S. sclerotiorum* is also related to the loss of viability during drying. The inability of the MS of *S. sclerotiorum* to survive drying contrasts with the findings of MS research using other species of fungi, in which MS dried with the same process as used in the current research have initial germination rates of 100% after drying to below 4% moisture (Jackson & Jaronski, 2012; Jackson & Schisler, 1995; Kobori et al., 2015; Villamizar et al., 2018). It is not yet clear why MS of other species of fungi are desiccant tolerant while those of *S. sclerotiorum*, which produced desiccant tolerant macro sclerotia (Wu, Subbarao, & Liu, 2008), produce MS that do not survive drying. Freeze drying *S. sclerotiorum* MS with DE was also unsuccessful with no germination on agar or infection observed on excised leaves. Storage trials on *S. sclerotiorum* in the form of sclerotia have also found that

freeze drying is not suitable for the long-term storage of sclerotia of the fungus, with low survival and germination rates (Pottinger, Ridgway, Bourdôt, & Stewart, 2008).

The performance of fresh MS from individual fermentation flasks ("Fresh" treatment) significantly differed in virulence compared with fresh MS from the "Bulked" treatment prepared by mixing the broth from all the flasks. Of the lobes of *R. acris* leaves, 97% were infected after 24 hr when treated with the MS from individual flasks before mixing. Comparatively with the "Bulked" broth, there was no visible infection on *R. acris* leaves after 24 hr. This result suggests that a combination of MS-containing broth from several replicates (flasks) negatively affected the virulence of these fungal structures, even though the viability on agar was not affected (>80% germination after 24 hr). The virulence *in planta* was reduced, with reduced lesion development on *R. acris*. The cause of this reduction in pathogenicity is unknown but there are several possibilities that are proposed.

One possibility is that mycoviruses may have been present in one of the samples that were mixed into the bulked sample. Mycoviruses are viruses that cause infection to fungi and can be RNA or DNA based and have been associated with hypovirulence of fungi (Xie & Jiang, 2014). Boland (1992) identified a hypovirulent isolate of *S. sclerotiorum* that was associated with high levels of dsRNA, characteristic of infection with a mycovirus. This isolate had reduced virulence compared to isolates that were free of dsRNA, resulting in smaller lesions to celery leaves in bioassays. A range of mycoviruses have been identified from *S. sclerotiorum*. Mycoviruses of *S. sclerotiorum* have been associated with downregulation in gene expression of 150 genes, across a range of biological functions (Li et al., 2008). An important gene that was found to be supressed by the mycovirus SsDVR is the *S. sclerotiorum* integrin-like gene (*SSITL*). When the SSITL was silenced, significant reductions in virulence and growth rates resulted (Zhu et al., 2013). It is possible that a mycovirus had established in one of the flasks used during this study and resulted in the reduced virulence observed in the current research once the flasks were bulked. The high rates of germination of fresh MS on agar indicates that the structures were still viable, but the lack of infection on *R. acris* suggests a reduction in vigour of the propagules. Viability on agar can overestimate the quality of fungi based biopesticides, as propagules can be viable on agar after extended periods of incubation but would not cause infections on hosts (Faria, Lopes, Souza, & Wraight, 2015). For *Beauveria bassiana* the vigorous conidia that germinate rapidly resulted in the majority of lethal infections (Faria et al., 2015). It is possible mycoviruses in the current work reduced the vigour of the structures, and the effects of this were only apparent as reduced infection of the host. It would be possible to test for mycoviruses within cultures in future work by using molecular techniques to purify dsRNA (Son, Yu, & Kim, 2015).

Another possible explanation is that one or more of the flasks may have been contaminated with another organism that had an inhibitory effect on the *S. sclerotiorum,* resulting in reduced rates of infection on the excised leaf lobes. Some contaminant fungi such as isolates of *Trichoderma* have been found to have an inhibitory effect on the growth of *S. sclerotiorum* and may have resulted in inhibition of germination in the experiment (Matroudi & Zamani, 2009). This explanation seems unlikely as samples from all twenty individual flasks caused infection when tested on their own and quality control samples only resulted in pure cultures of *S. sclerotiorum*. Contamination was observed on the cotton bungs of some of the replicate flasks at day 14 and these were excluded from the bulked samples. It is possible that contamination was introduced to the broth from the bung during the removal of mycelial rings from the walls of the flasks. All work to remove the mycelial rings was undertaken in a laminar flow to limit contamination. In future experiments, growth rings should be left until day 8 before attempting to remove them, as before this time they are not easily removed as they are mostly precipitate from the media, that is not well held together by mycelium yet. This will reduce both the number of times the cotton bung is removed during the fermentation and the duration that it is necessary to remove it for, thereby reducing the risk of contamination entering the flasks.

The addition of the DE carrier and vacuum filtration did not result in any significant changes to the virulence on *R. acris* or viability on agar of the formulation containing MS of *S. sclerotiorum* when compared to the bulked treatment. This result indicates that the DE carrier did not negatively impact the survival of *S. sclerotiorum* MS within the formulation. DE is an inert carrier that is commonly used to formulate MS produced in liquid fermentation (Goble, Gardescu, Jackson, & Hajek, 2016; Jackson & Payne, 2016). After vacuum filtration to create the dough treatment, there was a significant reduction in the germination of the MS structures after 24 hr of being inoculated on agar. However, after 60 hr incubation, 100% of the MS had germinated. This result suggests that the MS at this stage of processing are still viable but require a longer period of time to germinate at the same rates as prior to vacuum filtration. The vacuum filtration process removes a large amount of spent liquid media from the microsclerotia/diatomaceous earth mixture, reducing the total moisture content to around 60% (as at T0 in Figure 4.13). The delayed germination at this stage suggests that the microsclerotia are still viable but require a longer period of time to germinate *in vitro* – i.e. the vigour of the MS was reduced. In the entomopathogen *Beauveria bassiana* it has been found that the vigorous conidia (those germinating within the first 16 hrs of incubation) contribute to nearly all lethal infections of the target host, while conidia that germinate after 48 hr or more, are mostly avirulent (Faria et al., 2015). These findings suggest that for viability studies, data collected after a short period of incubation will provide a more reliable indication of true virulence, while a longer incubation is likely to overestimate the quality of fungal preparations. Less vigorous conidia do

eventually germinate although they do not contribute significantly to infection of the host. In relation to this research, it may indicate that after vacuum filtration of the MS formulation into a dough the structures showed slower germination on agar due to a reduction in vigour. An alternative possibility is that germinating mycelium from this treatment was difficult to see due to it being surrounded with DE, as seen in Figure 4.5. It was noted during data collection that due to the DE surrounding the microsclerotia, which by the "Dough" step in processing was quite concentrated, that mycelium emerging from the structures was difficult to identify due to being the same colour as the DE under light microscopy. This may have resulted in an under-estimation of the true viability at the 24 hr observation time-point. The results of the leaf bioassay with *R. acris* found no lesions caused by the "Bulked", "DE", or "Dough" treatments during the first 24 hr, with lesions only becoming visible after 48 hr. This indicates that there was a reduction in vigour of the propagules when compared to the MS from individual flasks which caused lesions on 97% of lobes within 24 hr (Figure 4.3).

A prolonged period of high humidity is a requirement of many pathogenic fungi to initiate germination and infection of a host and is often a limiting factor to the field success of biopesticide applications (Jones & Burges, 1998). This moisture requirement is often a limiting factor in the success of biopesticides in the field, and additives to the formulation such as glycerine or PEG-400 which act as humectants can be added to optimize a biopesticides formulation (Catão et al., 2021). Future work could look into the addition of humectants or protective agents into the formulation to improve the survival of the MS of *S. sclerotiorum* after drying.

There are some differences between the fermentation in the current research compared to those used to produce MS in other species of fungi, and these may give insight into why MS differed. One difference is the duration of the fermentation. In other published studies MS appear at day 3 – 6 and are typically harvested between days 5 – 11 (Jackson & Schisler, 1995; Kobori et al., 2015; Villamizar et al., 2018). The current work differs in that the onset of MS formation takes longer, with the structures developing at around day 10 and become pigmented (maturing) by day 14. In similar work by Garcia Riaño (2022), electron microscopy was used to examine the development of MS of *Metarhizium robertsii* over the course of fermentation. Developing MS at day 4 and 8 contained a growing mesh of hyphae that became more compact by day 16, with increased cell lysis of hyphae embedded with the development of an encompassing extracellular matrix. After 16 days the MS had become highly compact, with the external layer totally covered by extracellular matrix, and an inner medulla that contained mostly extracellular matrix and few cells. A possible explanation for the low survival rates of the *S. sclerotiorum* MS is that due to the comparatively long length of the fermentation, most of the cells within the MS at the time of harvesting have lysed, leaving the few remaining cells susceptible to the drying process. This hypothesis led to the second experiment in

this chapter examining the effect of fermentation length on the survival of fresh and dried microsclerotia.

The carbon: nitrogen ratio used in the MS media in this research was 5:1 and MS production media typically use more carbon rich media with C:N ratios of 30:1 or 50:1 (Jackson & Schisler, 1995; Kobori et al., 2015; Shearer & Jackson, 2006). Culture conditions can have a significant impact on cell viability and resistance to desiccation during harvesting and formulation (Fu & Chen, 2011). In particular, the addition of compatible solutes to the culture medium such as the non-reducing disaccharides sucrase and trehalose, have been shown to protect the cell membranes from osmotic stress during dehydration, due to their accumulation within cells (Welsh, 2000). The carbohydrates trehalose and mannitol accumulate in sclerotia of *S. sclerotiorum* (Tourneau, 1966) and have also been shown to be important in desiccation tolerance of other microorganisms (Fu & Chen, 2011). Future experiments testing the fermentation of *S. sclerotiorum* with modified versions of the MS media, such as with modified C:N ratios and the inclusion of compatible solutes, may be able to produce MS of *S. sclerotiorum* that are better able to survive desiccation.

The MS produced in this research did not have a defined melanised rind like sclerotia but it is possible that the melanin produced in the MS may contribute to the dormancy of the structures. It has been demonstrated in previous work, that immature sclerotia of *S. sclerotiorum* lacking a complete rind can germinate more readily than mature sclerotia that are fully melanized (Huang & Kozub, 1994). Coley-Smith & Cooke (1971) and Huang (1985) have concluded that formation of the melanin rind induces sclerotial dormancy as well as acting as a physical barrier to myceliogenic germination. MS formed by *S. sclerotiorum* in the present work were dark, suggesting melanin presence, with the concentration appearing to increase inside of the structures during drying although this was not measured. The melanin concentration may have an inhibitory effect on myceliogenic germination.

The reason MS of *S. sclerotiorum* are not surviving is not yet clear and results have been inconsistent. In Chapter 3, the first bioassay with a dried formulation of *S. sclerotiorum* MS caused infection on *R. acris* and *C. arvense* leaves, but this result was not reproducible in future experiments. For MS to be considered a feasible propagule for a *S. sclerotiorum* bioherbicide, these reproducibility issues need to be understood.

4.4.2 Microsclerotia Age

At day 10 of the fermentation, developing MS were characterized by large mesh like aggregations of hyphae, with particles of the media trapped inside the structures. As the aggregates matured, the centre of the structures became more compact and darker as melanin was produced. By day 14,

there was a notable reduction in the amount of floccose mycelium surrounding the compact MS. The mean number of mature MS was increased at day 14 compared to day 10 and day 12, however the difference was not significant. In *Metarhizium brunneum*, extending the length of fermentation from 4 days to 7 days, resulted in no significant differences in the yield of MS (Jackson & Jaronski, 2012), similar to the results presented in the current research for *S. sclerotiorum.*

Two of the replicate flasks from the 14-day fermentation had very few MS per mL (<1000) which resulted in a large deviation in the dataset, causing the differences in means to not be significant. Conditions of the fermentation were tightly controlled across replicates and batches to reduce sources of variation such as the media composition in addition to the brand and size of flasks, which were kept constant. The positions in the incubator were randomised. The difference between replicates observed in a number of experiments in this research most likely came as a result of using sclerotia as inoculum. In liquid fermentation of other fungi, conidial suspensions are commonly used as the inoculum source, typically using 10^4 to 10^8 conidia per mL (Jackson & Schisler, 1995; Song et al., 2014; Villamizar et al., 2018). The high concentration of conidia provides many inoculation points that enables the fungi to homogenously colonise the media and begin to form aggregates. Another disadvantage of sclerotia as inoculum is they are irregular in shape and size which may contribute to variability between replicate liquid cultures, especially as only five sclerotia are being used to inoculate each flask. An alternative inoculation technique that has been successfully utilized to produced MS in *Mycoleptodiscus terrestris* is to use a preculture, containing many short hyphal fragments, as the inoculum for the MS media (Shearer & Jackson, 2006). This may have the additional benefit of shortening the fermentation time, reducing any delay caused by the time it takes for the myceliogenic germination of sclerotia, as the complete rind induces a dormancy effect on the sclerotia (Huang, 1985).

Germination of MS was near 100% when in broth, but subsequently reduced after drying to 5.5% and 19.5% after fermentation times of 10 days and 12 days, respectively. This is again evidence to suggest that the issue is that the MS of *S. sclerotiorum* are not surviving the drying process. In contrast to the findings of this research, in *Metarhizium brunneum*, the maturation and melanisation of MS was found to have no significant impact on the survival of MS with structures produced after 4, 5, 6 and 7 days all having 100% hyphal germination after drying (Jackson & Jaronski, 2012). These researchers noted that melanised fungal cells are more resistant to microbial attack in soil, so melanised MS may be more effective in the field. The mean germination increased with increasing fermentation time, but the difference was not significant as a result of high variation within the data. At day 14 the average germination of the structures after drying was 47%, however the variation between replicates was large. The hypothesis is that as MS age, the number of live cells within the structure decreases, with an increase in extracellular matrix and melanisation. The number of live cells is

critical to the survival of the MS, but the propagules need to have a balance between the number of living cells, extracellular matrix, the level of compaction, and melanin content. The low survival of mature MS in this work suggests that these structures have low numbers of viable cells and are not able to survive drying. Garcia Riaño (2022) reported similar findings with MS of *M. robertsii,* finding MS produced with a 20 day fermentation had poor storage survival compared to MS harvested after 8 and 16 days, as the number of viable cells within the structures decreased. In the present research, the two replicate flasks at day 14 that produced structures with the highest survival rates after drying, 85% and 84%, were the two with the lowest counts of mature MS. Microscopy had revealed that the structures in these flasks were morphologically different, consisting of floccose, loose mycelium compared to the other flasks that contained compact, melanized microsclerotia-like structures. The high survival of these structures after drying, suggest that there was a high number of live cells within these structures and provides evidence that cells of the fungus can survive the drying process. This finding suggests that formulations based on mycelial aggregates of *S. sclerotiorum* may be of interest to investigate in further bioherbicide development research.

The variability in the dataset can be partially explained by these morphological differences in the structures produced between replicate flasks, however the reason that these differences are occurring is not understood. It is possible that the differences in growth and MS morphogenesis between replicate flasks arise from differences in the sclerotial inoculum used to inoculate the flasks as other factors such as the media and culture conditions were kept consistent across all the replicates. As previously suggested, future work could use a preculture *S. sclerotiorum* liquid culture as the inoculum source to reduce the variation between flasks. A larger number of replicates would also enable more confidence to determine if any of the trends observed were significant.

4.4.3 Carrier Comparison

This experiment was the first to test the survival of dried MS of *S. sclerotiorum* with a range of different carriers. Three carriers were tested: DE, kaolin clay and corn flour. There were no significant differences in the germination of MS after 24 hr of drying with different carriers, and overall survival rates were low with an average germination of 21% with DE, 9.75% with Kaolin and 2% with corn flour. Using alternative carriers resulted in no improvement to the survival of the MS of *S. sclerotiorum,* suggesting that the poor survival rate of the MS after drying is not due to the carrier, but rather related to the detrimental effect of desiccation. When working with MS produced by liquid fermentation, the primary purpose of using a carrier is to support the structures during the downstream processing (filtration and drying), preventing them from adhering to each other and maintaining their shape and micrometric size, as well as protecting from extreme desiccation. Adherence of several MS that touch during filtration or drying leads to their aggregation into larger

structures, which may result in increased mortality due to rupturing of cell membranes as the fungal agents are granulated and dried (Jackson & Jaronski, 2012). Therefore, carriers play an important role during the formulation process of microbial-based products, being principally inert inorganic or organic compounds such as DE, peat, vermiculite, clays and polysaccharides (Sahu & Brahmaprakash, 2016; Vassilev et al., 2020) . DE is the most commonly used carrier in MS harvesting/formulation and has been used to create active granular MS formulations for a number of species (Jackson & Payne, 2016; Jackson & Schisler, 1995; Mascarin et al., 2014; Villamizar et al., 2018). Goble et al. (2016) evaluated the performance of different carriers of *Metarhizium brunneum* F52 for activity against Asian Longhorn Beetle and found few biologically relevant differences in conidia production and efficacy between the carriers' microcrystalline cellulose, DE and kaolin clay. The difference in this study from the present work is that all carriers tested resulted in high rates of germination and conidia production, whereas no carriers resulted in high germination in this test when using *S. sclerotiorum* MS harvested after 14 days of fermentation. These findings provide further evidence that death of the MS is most likely related to the detrimental effect of desiccation, than other factors in the formulation process such as the choice of carrier.

The rate of drying was approximately linear for all carriers tested, although the initial moisture after vacuum filtration and rates of drying between carriers differed. After 24 hr drying, the granular formulations had a final average moisture content of 7% with no significant difference between the different carriers used. Jackson and Payne (2016) suggest that MS be dried to moisture contents below 4% to increase the storage stability of formulations. In this carrier comparison the final moisture contents were greater than 4% at an average of ~7% moisture. Jackson and Jaronski (2012) found that MS of *M. brunneum* in DE granules with moisture contents of 2.5% to 6% had higher conidial production during the first four months of storage, but granules with moisture contents <2.5% produced more conidia when germinating after 12 months of storage. These results suggest that lower moisture contents aid in the prolonged shelf life of MS granules, but that higher moisture contents such as those used in the current research should not have negatively impacted the initial germination.

In the current work, the drying profiles differed between carriers, reflected in both the moisture content and germination as the samples dried. DE retained the highest initial moisture content after vacuum filtration but had the highest rate of drying. The drying profiles of cornflour and kaolin did not significantly differ, with a lower initial moisture after vacuum filtration than DE and a reduced rate of drying. Despite retaining the same moisture content as the kaolin, the corn flour carrier had a major effect on the germination of the MS with a reduction from 97% at time zero to 9.4% 8 hr post-drying. The kaolin maintained 85% germination of MS after the same duration of drying, with the same duration of drying and moisture contents. MS in DE maintained high germination, >85%, after

16 hr of drying but decreased after to 24 hr to 21%. This result is probably since DE retained a higher moisture content than the other carriers after 16 hr, but by 24 hr had dried to the same moisture content as the other carriers at ~7%. These results suggests that there is some interaction between the choice of carrier and the survival of the MS during the drying process, but the survival seems to be linked to the moisture content more that the carrier as after 24 hr drying, germination across all three carriers was low. All carriers in the present work were incorporated at a rate of 5 g/100mL of culture broth based on the protocol of Jackson and Payne (2016). Jackson and Jaronski (2012) found that with MS of *M. brunneum,* granules made with increasing rates of DE resulted in reduced conidial production of the granules after storage, up until 12 months of storage, after which there was no difference between any rates of DE included. Considering the reduction in germination obtained for *S. sclerotiorum* MS when dried with the three evaluated carriers to <7% moisture, further work will be needed to understand the damage caused during the dehydration process. Future experiments could evaluate alternatives to overcome this problem, such as by testing different drying rates, altering the final moisture content or by adding specific compounds that act as membrane stabilizers/drying protectants.

4.4.4 Concluding Comments

The results of this chapter have identified that the MS of *S. sclerotiorum* produced in liquid fermentation by using the conditions developed in Chapter 3 are not consistently able to survive desiccation. The structures survive the initial vacuum filtration but after air-drying for 24 hr, the viability drops, resulting in no germination on agar or infection of excised leaves. This result indicates that the MS structures produced in this research are behaving differently to those produced in other species of fungi, which are consistently reported as being desiccant tolerant and can survive drying to moisture contents below 4% (Jackson & Payne, 2016; Jackson & Schisler, 1995; Villamizar et al., 2018). It is unclear whether the difference in survival of MS is due to structural differences or differences in physiology between fungal species.

Investigation of different fermentation length and different carriers did not improve the survival of the MS. These results suggest that conditions, including medium composition and C:N ratio, inoculum type, incubation temperature, pH, and fermentation time, developed in the present work for inducing formation of MS from *S. sclerotiorum,* need to be optimized. This will be necessary to improve yields as well as propagule robustness. The use of sclerotia as inoculum was consistently found to be a source of significant variation among replicates. Future investigations into the fermentation should use a preculture of mycelium as inoculum to reduce this variation, and may potentially reduce the length of the fermentation, producing more consistent microsclerotia in less time.

Chapter 5
General Discussion

This thesis investigated the potential of *Sclerotinia sclerotiorum* as a bioherbicide of the pasture weeds *Ranunculus acris* and *Cirsium arvense*. Previous research on the potential use of this fungus as a bioherbicide has primarily focused on solid substrate production (Smith et al., 2016) which has been found to be cost-prohibitive to scale up (Bourdôt, 2011). Liquid culture production of fungal propagules is regarded as the most efficient and scalable way to produce fungal propagules (Jaronski, 2014). Microsclerotia (MS) are fungal propagules that have been produced in submerged liquid culture for other species of fungi and have characteristics such as desiccant tolerance and shelf stability that make them attractive as biopesticide propagules (Jackson & Payne, 2016). The main objective of this research was to ascertain if *S. sclerotiorum* could be induced to produce MS in submerged liquid culture and test the activity of these propagules against the hosts, *R. acris* and *C. arvense*. A comparison in growth and virulence was also undertaken between six isolates of *S. sclerotiorum* from isolates that were originally excised from the two target hosts, *R. acris* and *C. arvense*.

The six *S. sclerotiorum* isolates included two isolates excised from *C. arvense* (S36 and S37) and four isolates (GGB1, GGB2, G57 and G64) excised from *R. acris*. Under the trial conditions using a 16 hr light, 8 hr dark photoperiod at 18 °C, none of these isolates could be induced to form apothecia. Further trials should investigate the potential of cold conditioning to induce apothecial development of the sclerotia (Huang & Kozub, 1989, Huang & Kozub, 1993). The temperature at which sclerotia were formed is essential for carpogenic germination and Huang and Kozub (1991) further observed that the geographic origin of isolates was important, with isolates from cool climatic areas producing apothecia readily at cool temperatures (10 °C), while isolates from warmer climates required cold conditioning below 10 °C to produce apothecia. The isolates used in this research came from temperate climates within the South Island of New Zealand, and therefore it is possible that cold conditioning is a requirement to induce apothecia formation. In growth trials on 1/5 PDA, the growth rates of all isolates increased with increasing temperature up to 25 °C and under a 12 hr photoperiod compared to darkness, suggesting optimal conditions for future fermentations. This was in line with previous research that has found the optimum temperature for growth to be slightly above 20 °C, with temperature minima (0 - 7 °C) and maxima (26 - 32 °C) for growth, which were outside the range tested in the current work (Uloth, You, Cawthray, & Barbetti, 2015). Significant differences in growth rates were found between isolates of *S. sclerotiorum* tested with the fastest growing isolate found to be GGB2 with an average rate of 25.9 mmd^{-1}. Isolate GGB2 was originally isolated from

R. acris and had the best pathogenic performance across both host species (*R. acris and C. arvense*) in excised leaf bioassays. The resultant lesions caused by GGB2 covered an average of 89.4% of the total leaf area on *C. arvense* and 79.1% of the leaf area on *R. acris* after 6 days. GGB2 had not previously been included in bioherbicide research and is a promising isolate for future bioherbicide development. Only one time repetition of this experiment was conducted. Another exact repetition of the growth trial experiment and pathogenicity assays under the same conditions would be useful to increase the confidence of the findings presented here. Expanding the pathogenicity assays to include tests on whole potted plants prior to in field testing is more analogous to the final use case of any potential bioherbicide and would be valuable to include in future research.

To investigate the potential of *S. sclerotiorum* to produce MS, a fermentation testing seven different liquid media was undertaken, with the media compositions based on those used to produce MS in other species of fungi (de Lira et al., 2020; Jackson & Jaronski, 2009; Jackson & Schisler, 1995; Kobori et al., 2015; Mascarin et al., 2014; Song et al., 2016; Song et al., 2014; Villamizar et al., 2018). The seven media encompassed a range of carbon and nitrogen sources at various carbon to nitrogen ratios. MS production in other species of filamentous fungi has been induced in liquid culture using media compositions primarily with high carbon to nitrogen ratios (between 30 and 50:1) and utilizing simple sugars such as glucose as the carbon source and yeast extract or casein as the nitrogen source (de Lira et al., 2020; Jackson & Schisler, 1995; Mascarin et al., 2014; Song et al., 2016). In the current work media with high C:N ratios (20-50:1) resulted in viscous hyphal cultures, with structures that were weakly aggregated with large halos of ropey mycelium and no melanisation. Only one medium tested, based on the composition published by Villamizar et al. (2018), resulted in the production of MS and had a C:N ratio of 5:1. This medium, based on cornsteep liquor and casamino acids, resulted in the production of MS after 14 days of fermentation at a yield of 4.5×10^3 MS per mL. This yield is comparable to MS yields achieved by Villamizar et al. (2018) for MS of *Beuaveria bassiana, B. pseudobassiana and B. brogniartii,* de Lira et al. (2020) for *Metarhizium humberi* and by Shearer and Jackson (2006) for *Mycoleptodiscus terrestris*. At day 14, structures in the media were observed as compact aggregates, with dark brown pigmentation with short halos of mycelium, with an average diameter of 269 μm – consistent with the definition of MS (Song, 2018). These findings suggest that a rich media with a low C:N ratio is necessary to produce MS in *S. sclerotiorum*. No MS were produced in media 2, which had a C:N of 4:1 but used glucose and yeast extract as the primary nutritional sources. Both media had similar total concentrations of carbon and nitrogen and yet MS were only produced in media 6. This result suggests that the carbon and nitrogen sources have a significant effect on MS production in *S. sclerotiorum* and further investigation will be needed to determine in detail how the C:N ratio is influencing MS production in this species. This experiment could be done

by comparing MS production in media with varying C:N ratios keeping the nutritional sources of cornsteep liquor and casamino acids constant.

MS produced by this fermentation were dried to <4% moisture with a diatomaceous earth (DE) carrier to create a simple granular formulation according to the process outlined by Jackson and Payne (2016). This granular formulation produced large lesions on both *R. acris* and *C. arvense* leaves in an initial excised leaf bioassay when inoculated with 1/32 tsp per leaf. The lesions caused by the MS formulation were significantly larger than those caused by sclerotial inoculum of the same *S. sclerotiorum* isolate (S36). MS formulations caused infection to both target species with lesions covering large proportions of the total leaf area after 6 days, while sclerotia only caused infection on *C. arvense* with smaller lesions. Sclerotia did not result in any infection or lesion development on *R. acris*. This finding adds to previous results which have found the whole sclerotia of *S. sclerotiorum* cause low rates of infection on *C. arvense* (Smith, 2018), while mycelial inoculum has been shown to be capable of readily infecting a range of hosts (Bourdôt, Hurrell, & Saville, 2011). Metabolites that accumulate during the fermentation may become captured in the final formulation during the process of vacuum filtration with the DE carrier. These metabolites were not investigated but likely include oxalic acid which is known to be important in the pathogenicity of *S. sclerotiorum* (Liang & Rollins, 2018), and cell wall degrading enzymes (Liang & Rollins, 2018; Lumsden, 1979). Oxalic acid production in *Sclerotinia minor* was doubled by adjusting the culture medium to have an initial pH of 6.0 as opposed to 4.3 when not adjusted (Briere, Watson, & Hallett, 2000). Incorporating small quantities of the culture media containing oxalic acid was shown to increase the virulence of a granular bioherbicide formulation of *S. minor* towards the turf weed *Taraxacum officinale* (Dandelion) (Briere et al., 2000). *S. sclerotiorum* has an increased growth rate at lower pH, and pH is known to be an important regulator of oxalic acid production, pathogenicity and sclerotial metamorphosis (Rollins & Dickman, 2001; T. Xu et al., 2018). In the current work pH of the media was not adjusted and changed only slightly from an initial 4.34 to 4.57 after 14 days of fermentation. Future work testing the production and virulence of MS of *S. sclerotiorum* produced with media with amended pH may provide interesting insights.

All of the populations of *R. acris* (4 haplotypes: A, A2, J and G) and *C. arvense* (two provenances: G20 and G27) tested were susceptible to the MS formulation. Significant differences in susceptibility between different haplotypes of *R. acris* were identified, with the G haplotype found to be the least susceptible to infection compared to the other three haplotypes tested (Lange, Goeke, Hickman, Podolyan, & Houliston, 2018). Similarly, with *C. arvense* the G27 population was found to be more susceptible to infection by the MS formulation of *S. sclerotiorum* than G20 (Cripps et al., 2020). It is important to note that these results were obtained from excised leaf assays. Leaves were chosen as this is the plant structure that would be targeted by future formulations of *S. sclerotiorum* applied as

a foliar spray or water dispersible granule. Additionally, results of excised leaf assays from other plant-pathogen systems have reported good correlation between results observed in detached leaf assays and whole plants in glasshouse or field trials (Foolad et al., 2015; Goth & Keane, 1997; Miller-Butler et al., 2018; Vleeshouwers et al., 1999). Previously, infection of *C. arvense* in excised leaf bioassays inoculated with solid substrate formulations of *S. sclerotiorum* did not result in the same performance when whole plants in a greenhouse were inoculated (Smith, 2018). This highlights the importance of testing MS formulations in future experiments on whole plants on the same *R. acris* haplotypes and *C. arvense* provenances in a greenhouse setting. The results from this will provide greater understanding of the accuracy of excised leaf bioassays for evaluating the performance of *S. sclerotiorum* based bioherbicide formulations.

The results of Chapter 3 indicated that MS of *S. sclerotiorum* have potential as an infective propagule for the development of a bioherbicide targeting both *C. arvense* and *R. acris*. Unfortunately, further attempts to repeat the initial bioassay were unsuccessful with no germination or infection observed from the MS formulations. Successful biological control propagules must have a high level of pathogenicity and result in consistent infection on the target host (Song et al., 2014). The failure of the MS formulations to show any activity in subsequent replications of this treatment on excised leaves highlighted the need to further investigate attributes of *S. sclerotiorum* MS to enable consistent and repeatable performance.

Germination assays at each step of the formulation process revealed that the MS had 100% germination when taken fresh from the broth and maintained high germination up until the final air-drying step, after which no germination was observed on agar or on excised leaves. This result showed that the MS produced have limited tolerance to desiccation and are likely dying due to cellular damage occurring during the drying. During desiccation, cell membranes can be irreparably damaged due to a loss of structural integrity which results in membranes becoming leaky and a resultant loss of the cell contents (Crowe et al., 2002). Scanning electron microscopy (SEM) images of the surface of the produced MS showed signs of damage to the hyphal cell membranes. The finding that the structures produced do not survive drying contrasts with MS research using other species of fungi, in which MS dried with the same process have an initial germination of 100% after drying to below 4% moisture (Jackson & Jaronski, 2012; Jackson & Schisler, 1995; Kobori et al., 2015; Villamizar et al., 2018). This research was the first time that MS had been produced by a fungus within the family Sclerotiniaceae, and by a fungus that produces macroscopic sclerotia as a normal part of its lifecycle. A possibility is that *S. sclerotiorum* behaves differently to previously studied fungi due to the regulation of genes that are involved with sclerotia formation. The regulatory pathways involved in sclerotia formation in *S. sclerotiorum* have been characterised and investigating the transcription levels of these genes during MS production would be beneficial in future work (Xu et al., 2022).

A difference between the current work and other MS production research is the length of fermentation. MS of *S. sclerotiorum* did not begin to form until day 10 and matured by day 14. In contrast, in liquid fermentation of other species MS formation has occurred between 4 to 10 days after inoculation (Kobori et al., 2015; Mascarin et al., 2014; Song et al., 2014; Villamizar et al., 2018). We hypothesised that the longer length of fermentation in the present research could be contributing to the inactivity of the MS after drying, due to lysis of the cells within the MS and replacement with extracellular matrix, as was found in *M. robertsii* with increasing fermentation time (Garcia Riaño, 2022). Sampling of *S. sclerotiorum* MS at different fermentation lengths of 10, 12 and 14 days did not find any fermentation time that increased the survival of the MS after drying. This result showed that fermentation time was not the factor resulting in the inactivity of the MS, and that it likely relates to the inability of the structures themselves to tolerate desiccation. An interesting finding was that in two replicates that did not produce MS, but had floccose, loose aggregates of mycelium, survival after drying was high. This finding suggests that formulations based off mycelial aggregates of *S. sclerotiorum* may be of interest to investigate in further bioherbicide development research.

Formulating the MS with two alternative carriers, kaolin and corn flour did not result in an increase in survival of the MS with DE retaining the highest germination after 24 hr drying. Differences in viability were found after varying drying periods with each of the carriers, with DE found to retain moisture for a longer period and maintained the survival of the structures for a longer period, however after 24 hr of drying there were no significant differences in the viability of the MS across any of the carriers.

Despite the inconsistency in effectiveness of MS of *S. sclerotiorum* to germinate on agar and cause infection after drying, MS remain a promising propagule for a future bioherbicide. Future work should focus on optimizing fermentation to produce MS that remain viable after drying. In other species of fungi small changes to the fermentation media have resulted in large differences in MS yield and survival (Shearer & Jackson, 2006). Substituting the nitrogen sources has been shown to have significant effects on the yield and viability of MS and should be tested with *S. sclerotiorum*, building on the findings of Chapter 3 in this research. For example, in *Mycoleptodiscus terrestris*, using corn steep liquor powder (CSLP) as a nitrogen source yielded 7.5×10^2 MS per mL that had low viability after drying at 20% (Shearer & Jackson, 2006), similar to the low viability of *S. sclerotiorum* MS results after 24 hr of drying reported in Chapter 4. Substituting the CSLP for cottonseed meal, resulted in an increase in MS yield to 1.5×10^3 per mL that had 100% viability after drying. Using yeast extract as a nitrogen source produced MS that had 0% viability after drying (Shearer & Jackson, 2006). These results show that minor changes to the fermentation media can have substantial

impacts on both the quantity and quality of MS that are produced. Thus, future work should focus on the use of various nitrogen sources for MS production and MS survival after drying.

This research has identified a media composition that results in MS production in *S. sclerotiorum* and will provide a good basis for future research focused on developing this medium to improve the viability of MS after drying. Testing MS production in modified versions of this media composition with a range of carbon to nitrogen ratios may provide some insight into the optimal conditions for MS production. Since both the quantity and quality of MS are equally important, these studies should include both yield and viability measurements. Finally, Fe^{2+} ions have been demonstrated to be a critical factor in inducing MS differentiation. An experiment examining the effect of adjusted Fe^{2+} concentrations on MS in *S. sclerotiorum*, from a range of concentrations from 0.05 g/L up to 0.3 g/L, as was done by Song et al. (2014) for *Metarhizium rileyi,* would be useful as iron ions have been identified as an important factor to induce the formation of MS.

This research will also be useful for comparing the production of MS to the production of sclerotia. *S. sclerotiorum* is a well-studied model species for sclerotia morphogenesis (Chet & Henis, 1975; Erental, Dickman, & Yarden, 2008). Literature on MS formation often draws upon literature on sclerotia formation in *S. sclerotiorum* due to the similarities of the structures. As MS production has been demonstrated in *S. sclerotiorum*, there is scope for future research to compare the development of sclerotia and MS within a single species. Investigating the gene expression associated with MS formation in *S. sclerotiorum* and comparing this to the genes involved in sclerotia formation may provide some interesting insights.

A consistent issue that was found in several experiments in this research was high variability between replicate flasks during the same fermentation. This was most likely caused by using sclerotia as the inoculum for the liquid cultures, which are irregular in shape and size and must myceliogenically germinate before the mycelium can colonize the broth. In liquid culture production of other fungi, conidial suspensions are commonly used as the inoculum source, typically using 10^4 – 10^8 conidia per mL (Jackson & Schisler, 1995; Song et al., 2014; Villamizar et al., 2018). Since *S. sclerotiorum* does not produce conidia in culture, preliminary trials were conducted that tested ground sclerotia as an inoculum. It was hypothesized that ground sclerotia may colonise the broth faster than sclerotia due to having many starting points of inoculation, more analogous to a conidia inoculum. Anecdotally the ground sclerotia did not appear to improve the colonisation of the liquid media so sclerotia were used in further experiments. This is an area for improvement and warrants further investigation. An alternative approach of inoculating with a preculture, which contains many short hyphal fragments would be beneficial to test for future experiments. This approach has been used successfully to produced MS in *Mycoleptodiscus terrestris* (Shearer & Jackson, 2006). A

preculture inoculum would provide many more points of inoculation of the media than sclerotia and may have the added effect of reducing the fermentation time required to produce MS, as well as reducing variability between replicates.

This research marks the first time that MS have been produced by *S. sclerotiorum*. Initial MS formulations showed promising infection on the target weed species *C. arvense* and *R. acris*, although inconsistencies in the survival of MS after drying from different production batches were observed. MS of *S. sclerotiorum* are promising propagules to be incorporated into a future bioherbicide and warrant more research. Future work should focus on the optimization of the fermentation media to improve the viability of the MS after drying. Once storage stable MS can be produced, it will be important for future assays to test the effectiveness of MS formulations on whole potted plants and plants in the field, as opposed to only excised leaves as was used in this research.

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Appendix A

Supplementary Material

A.1 Preparation of Mc Cartney Vials

400 mL water agar was prepared by dissolving 6g of agar into 400mL of RO water using a stir plate at 60 °C with a magnetic stir bar. 5mL samples of the molten water agar were transferred via pipette to 28mL McCartney vials, while being mixed by the stir plate to ensure the media was homogenous. The vials were autoclaved for 15 min at 121°C at 15psi. After autoclaving, while the agar was still molten, the vials were placed in tube racks tilted on a 30° angle to produce slants. Once cooled and set, these vials were kept at room temperature for two weeks prior to use and any vials with contamination were discarded.

A.2 Standard Potting Mix Recipe

Standard potting mix per 500 litres

Media
400L composted bark, grade 2
100L pumice, grade 1 1-4mm

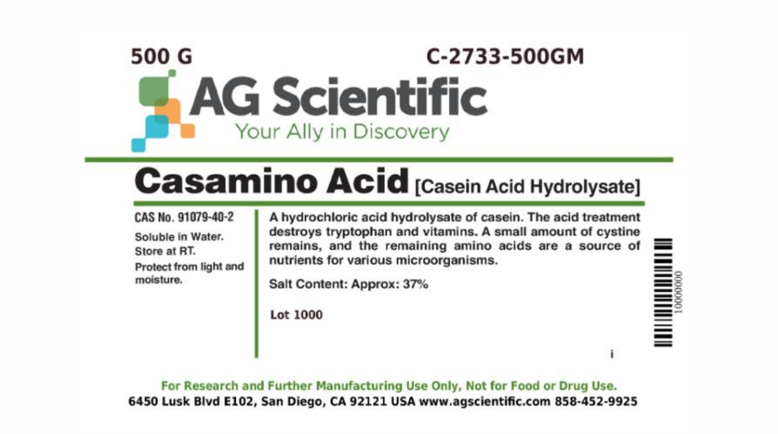
Fertilisers
Osmocote exact 16 – 3.9 -10 (3-4 months) 1500g.
Horticultural Lime 500g
Hydraflo 500g

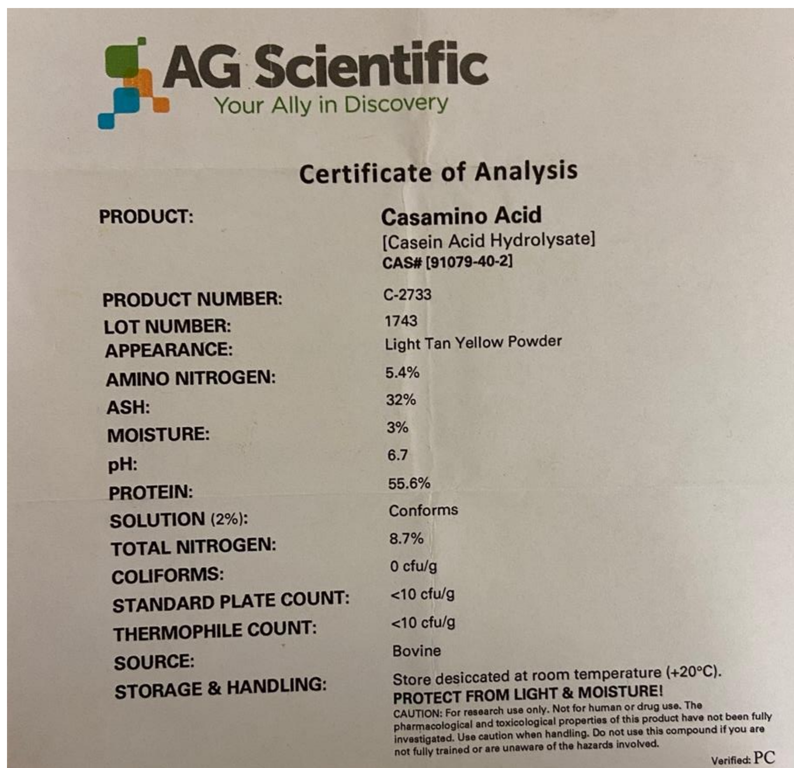
A.3 BD Bacto™ Casamino Acids, Technical Specifications

BD Animal Origin Peptides Technical Specifications retrieved from BDbiosciences.com/advbio

Product Name	Total Nitrogen (%)	Amino Nitrogen (%)	AN/TN	Total Carbohydrate (mg/g)	Ash (%)	Loss on Drying (%)	NaCl (%)	pH (1% Solution)	Calcium (µg/g)	Iron (µg/g)	Magnesium (µg/g)	Potassium (µg/g)	Sodium (µg/g)	Chloride (%)	Sulfate (%)	Phosphate (%)	Alanine (% Free)	Alanine (% Total)	Arginine (% Free)	Arginine (% Total)	Asparagine (% Free)	Aspartic Acid (% Free)
BD BBL™ Acidicase™ Peptone	8.5	6.2	0.73	0.29	36.8	5.3	32.3	6.8	229	4.9	36	383	140900	16.99	0.25	1.42	1.6	2.1	1.3	1.9	0.0	3.4
BD BBL™ Biosate™ Peptone	13.4	6.0	0.45	32.98	7.7	6.6	0.3	7.1	258	56.2	398	21320	17100	0.07	0.43	3.19	2.4	4.2	2.1	2.9	0.9	0.9
BD Bacto™ Casamino Acids	10.8	9.4	0.87	0.00	18.3	4.8	12.1	6.4	59	1.3	143	4098	88090	6.74	0.55	2.56	3.0	3.0	2.4	2.5	0.0	0.7
BD Bacto™ Casamino Acids, Technical	8.3	5.9	0.71	0.15	36.0	1.2	30.1	6.9	110	6.2	48	1361	145667	18.25	0.26	1.53	2.1	4.4	1.1	1.7	0.0	3.1

A.4 AG Scientific Casamino Acid, Technical Specifications





AG Scientific
Your Ally in Discovery

Certificate of Analysis

PRODUCT:	Casamino Acid [Casein Acid Hydrolysate] CAS# [91079-40-2]
PRODUCT NUMBER:	C-2733
LOT NUMBER:	1743
APPEARANCE:	Light Tan Yellow Powder
AMINO NITROGEN:	5.4%
ASH:	32%
MOISTURE:	3%
pH:	6.7
PROTEIN:	55.6%
SOLUTION (2%):	Conforms
TOTAL NITROGEN:	8.7%
COLIFORMS:	0 cfu/g
STANDARD PLATE COUNT:	<10 cfu/g
THERMOPHILE COUNT:	<10 cfu/g
SOURCE:	Bovine
STORAGE & HANDLING:	Store desiccated at room temperature (+20°C). PROTECT FROM LIGHT & MOISTURE! CAUTION: For research use only. Not for human or drug use. The pharmacological and toxicological properties of this product have not been fully investigated. Use caution when handling. Do not use this compound if you are not fully trained or are unaware of the hazards involved.

Verified: PC