

Inoculation techniques and evaluation methodologies for *Sclerotinia sclerotiorum* head and stem rot in sunflower and soybean

by

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Declaration

I, Marlese Christine Bester, declare that the dissertation hereby submitted by me for the degree of Magister Scientiae Agriculture at the University of the Free State, is my own independent work and has not previously been submitted by me at another University/Faculty. I cede copyright of this dissertation to the University of the Free State.

Marlese Christine Bester

A handwritten signature in dark ink, appearing to read 'M. Bester', is centered on the page. The signature is stylized and cursive.

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30 January 2019

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Preface

The research reported in this dissertation was carried out in the Division of Plant Pathology, at the University of the Free State, Bloemfontein, under the supervision of Professor N.W. McLaren and Miss L.A. Rothmann.

This dissertation includes four chapters. The overall aim of this study was to optimise *Sclerotinia* inoculum production in the laboratory and to evaluate the response of sunflower and soybean cultivars to inoculation techniques under greenhouse and field environmental conditions.

Chapter 1 is a literature review on sunflower and soybean production, importance, survival, germination and infection of *Sclerotinia sclerotiorum*, *Sclerotinia* disease management and resistance.

In Chapter 2 *S. sclerotiorum* inoculum production, in the form of sclerotia and ascospores, was optimised in the laboratory and their survival in different forms of *S. sclerotiorum* mycelium infected sunflower stubble was evaluated under controlled conditions for 12 months.

In Chapter 3 the viability and efficacy of *S. sclerotiorum* inoculum source, application method and timing on disease incidence were evaluated under both controlled and natural field conditions.

Chapter 4 covers an evaluation of the responses of soybean and sunflower cultivars to *S. sclerotiorum* inoculum under field conditions at different planting dates.

Chapter 1

Sclerotinia stem and head rot of soybeans and sunflower

1.1. General introduction

Plant disease epidemics arise when a susceptible plant, virulent pathogen and favourable environment for the infection process and spread of disease, change the intensity, including both severity and incidence, of disease in the host population over space and time (Campbell and Madden, 1990).

Sclerotinia sclerotiorum is the causal organism of the monocyclic disease, Sclerotinia stem and head rot (white mould disease or cottony rot) in soybeans and sunflowers. The mode of penetration used by this fungus to invade host tissue depends on the type of inoculum, availability of exogenous nutrients, defence properties of the host and prevailing environmental conditions. Two types of inoculum are generated by *S. sclerotiorum* to initiate infection namely, airborne ascospores and hyphae produced from sclerotia. Consequently sclerotia can either germinate myceliogenically to produce hyphae or carpogenically to produce apothecia (Purdy 1979). Typical signs of this pathogen are the mass of cotton-like white mycelium and black sclerotia, on and within plant tissue. Sclerotinia diseases are omnipresent in South Africa and were detected in eight of the nine provinces during 2006-2015. Sclerotinia stem rot of soybean was most prevalent during the 2010 season while Sclerotinia head rot of sunflower was most prevalent during 2006 (LA Rothmann personal communication).

In this review the effect of the pathogen's life cycle and epidemiology in relation to soybean and sunflower are reviewed.

1.2. Crop production

1.2.1. Soybeans

Soybeans (*Glycine max* L.) Merril.) are produced globally on more than 121 million ha of land with a total production of 334 894 085 t in 2016 (FAO, 2016). The three major producers of soybeans in the world are the United States of America with 117 208 380 t, Brazil with 96 296 714 t and Argentina with 58 799 258 t. Soybeans were first cultivated in South Africa in 1903 (Shurtleff and Aoyagi, 2009). The area planted to soybean in South Africa in the 2017/2018 season was more than 780 000 ha with a total production of 1 395 000 t (Table 1.1).

Table 1.1: Soybean production in South Africa in 2017/2018 (The South African Grain Laboratory, 2018).

Province	Area planted '000 (ha)	Production '000 (ton)	Yield (t/ha)
Eastern Cape	2.40	2.40	1.00
Free State	345.00	517.50	1.50
Gauteng	30.00	60.00	2.00
Kwazulu-Natal	40.00	124.00	3.10
Limpopo	20.00	55.00	2.75
Mpumalanga	310.00	373.50	1.85
North West	36.00	52.20	1.45
Northern Cape	3.00	9.00	3.00
Western Cape	0.80	1.20	1.50
Total in South Africa	787.20	1395	1.77

Soybeans are produced in nine provinces in South Africa with the Free State and Mpumalanga provinces as the largest producers, with a harvest of 517 000 t and 373 000 t respectively. In Kwazulu-Natal only 40 000 ha soybeans were planted, but 124 000 t were harvested, which resulted in the highest yield per area unit of 3.1 t/ha.

Yields in the Free State and Mpumalanga provinces, despite the highest area under production, averaged 1.5 t/ha and 1.9 t/ha respectively.

1.2.2. Sunflowers

The most important oilseed crop in South Africa is sunflower (*Helianthus annuus* L.). Sunflowers are produced globally on 47 345 036 ha of land with a total production of 26 205 337 t in 2016 (FAO, 2016). Ukraine, Russian Federation and Argentina are the biggest sunflower producers in the world with 13 626 890 t, 11 010 197 t and 3 000 367 t respectively, produced in 2016. The sunflower planting area in South Africa in the 2017/2018 season exceeded 600 000 ha with almost 750 000 t produced. Sunflowers are produced in seven of the nine South African provinces (Table 1.2). The Free State and North West Provinces were the biggest producers in the 2017/2018 season and delivered the highest yields of sunflower seeds per area unit of 1.3 t/ha and 1.2 t/ha, respectively.

Table 1.2: Sunflower production in South Africa in 2017/2018 (The South African Grain Laboratory, 2018).

Province	Area planted '000 (ha)	Production '000 (ton)	Yield (t/ha)
Eastern Cape	0.00	0.00	0.00
Free State	314.00	423.90	1.35
Gauteng	5.50	5.50	1.00
Kwazulu-Natal	0.00	0.00	0.00
Limpopo	45.00	36.00	0.80
Mpumalanga	2.30	2.18	0.95
North West	233.00	279.60	1.20
Northern Cape	1.60	1.92	1.20
Western Cape	0.10	0.10	1.00
Total in South Africa	601.50	749.21	1.25

1.3. Pathogen: *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum (Lib.) de Bary, first described in 1837 as *Peziza sclerotiorum*, is a soil-borne, hemibiotroph and omnivorous fungal plant pathogen that attacks more than 500 plant species, which include sunflowers and soybeans (Purdy, 1979; Saharan and Mehta, 2008; Kabbage *et al.*, 2015). *S. sclerotiorum* belongs to the Kingdom Eumycota, Sub-kingdom Dikarya, Phylum Ascomycota, Sub-phylum Pezizomycotina, Class Leotiomyces, Sub-class Leotiomycetidae, Order Helotiales, Family Sclerotiniaceae, Genus *Sclerotinia* and epithet *sclerotiorum* (Mycobank, 2018). *S. sclerotiorum* has no conidial anamorph and is assumed to be homothallic, having the ability to reproduce sexually (Kohn, 1979). This fungus is mainly a threat to dicotyledonous crops such as sunflower, soybean, dry bean, oilseed rape (known as canola in South Africa), chickpea, peanut, lentils and numerous vegetable crops, but may also pose a threat to several monocotyledonous species i.e. wheat, barley and maize (Purdy, 1979; Saharan and Mehta, 2008). After infecting host plant tissue or colonising plant debris, this fungus produces a survival structure, enabling it to stay viable for up to five years, which makes this pathogen highly successful (Adams and Ayers, 1979).

The primary survival structure or resting body, known as a sclerotium, is an asexual black, large and irregularly shaped structure comprising masses of hyphae. The size and shape of this structure is host and substrate specific. Sclerotia have a protective exterior rind consisting of melanin pigments that make them highly tolerant to degradation, by either adverse environmental conditions or microbes. The interior of a sclerotium, the medulla, is light cream/pink in colour consisting of β -1, 3 glucans and protein rich fungal cells (Le Tourneau, 1979). Sclerotia stay dormant in soil or plant stubble until favourable environmental conditions initiate germination. As inferred above, the sclerotium can undergo two types of germination, namely myceliogenic germination (asexual stage) and carpogenic germination (sexual stage) (Le Tourneau, 1979). The type of germination is dependent on prevailing environmental conditions (Le Tourneau, 1979). Figure 1.1 illustrates sclerotia incubated under the same environmental conditions with one sclerotium producing hyphae while the other produces stipes which will mature into apothecia suggesting that sclerotium source may also play a role.

1.3.1. Germination and infection

1.3.1.1. Myceliogenic germination

Sclerotia produced by *S. sclerotiorum* consist of specialised hyphal cells which are hyaline, septate, branched, multinucleated and start off as white but turn black as they mature (Kohn, 1979). The production of hyphae from a sclerotium is a process referred



Figure 1.1: Sclerotia under the same environmental conditions. One sclerotium produces hyphae and the other, producing stipes (Picture M.C. Bester).

to as myceliogenic germination. The hyphae have the ability to directly penetrate shoot system tissue, as well as to infect sub-terranean plant tissue (Huang and Dueck, 1980; Perveen *et al.*, 2010). The factors under which myceliogenic germination can be observed are temperatures between 20 - 25°C, 80% Relative humidity (RH), pH of 5 and high levels of exogenous nutrients. As nutrients become depleted, the mycelium aggregates and transforms into sclerotia which are retained in plant debris. No asexual conidia are produced by *S. sclerotiorum*; however, microconidia, also known as phialospores, are produced from phialides in sporodochia (Kohn, 1979), but these conidia do not germinate, and their biological function has not yet been established.

1.3.1.2. Carpogenic germination

The influential factors for carpogenic germination are different to those for myceliogenic germination. Literature suggests that most *Sclerotinia* epidemics are the result of infection caused by ascospores, which are the end product of carpogenic germination, making this a crucial event in the life cycle of this pathogen (Schwartz and Steadman, 1978; Abawi and Grogan, 1979; Steadman, 1979). This type of germination is essential since it allows for sexual recombination. Dormant sclerotia buried in soil are stimulated to germinate carpogenically by temperatures between 4 - 20°C, water potentials of -0.01kPa to -0.1kPa, light intensity of 160 -190 mol⁻¹ms⁻¹ and low levels of nutrients (Sun and Yang, 2000; Clarkson *et al.*, 2003; Hao *et al.*, 2003; Wu and Subbarao, 2008). Most published studies suggest that apothecial stipes will only be produced from preconditioned sclerotia (Kohn, 1979; Dillard *et al.*, 1995; Hao *et al.*, 2003; Ghasolia and Shivpuri, 2005; Pethybridge *et al.*, 2015). Preconditioned sclerotia are those that have been exposed to long periods (2 - 8 weeks) of low temperature (4 - 10°C), which are typically associated with winter.

The sexual ascospores are produced in asci within a fruiting structure known as an apothecium (Figure 1.2) on a matured apothecial stipe (Abawi and Grogan, 1979). Ascospores are released from asci in the mature apothecial disk into the air. Changes in RH and air pressure are triggers that force asci to discharge ascospores. Each apothecium can produce up to a million ascospores over a period of two weeks (Clarkson *et al.*, 2003; Sharma *et al.*, 2015). Ascospores need to be in contact with susceptible plant tissue under suitable environmental conditions to germinate and cause infection. These conditions include RH higher than 25%, continuous leaf wetness of 42 - 72 hours and a temperature less than 28°C but higher than 5°C with an optimum temperature of 18°C (Abawi and Grogan, 1979; Young *et al.*, 2004; Harikrishnan and Del Rio, 2006). Senescing blossoms and tissue are assumed to be the most important source of nutrients for ascospore germination and infection (Abawi and Grogan, 1979; Sedun and Brown, 1987; Turkington and Morral 1993). The birds nest fungus (Figure 1.3) can easily be mistaken for *S. sclerotiorum* apothecia.

1.3.2. Infection

Disease development can be divided into five phases namely (1) production of inoculum (2) inoculum dispersal (3) infection i.e. formation of a pathogenic or parasitic relationship (4) incubation period i.e. development of symptoms and (5) latent period i.e. becoming infectious or completion of a generation (van der Plank, 1963; Campbell and Madden, 1990). There are two infection processes by which *S. sclerotiorum* invades host tissue, firstly, infection by germinated ascospores or secondly, by hyphae produced directly by sclerotia. The epidemiology of these two infection types differ.



Figure 1.2: Field produced apothecia (Pictures M.C. Bester).



Figure 1.3: The birds nest fungus, *Cyathus sp.*, that is easily confused with the apothecia of *S. sclerotiorum* (Picture M.C. Bester).

1.3.2.1. Ascosporic hypha

The first type of infection is by means of germinated ascospores. A number of studies suggested that most *Sclerotinia* infections on annual crops occur by means of ascospores, e.g. lettuce drop (Clarkson *et al.*, 2014); stem rot on tomato (Purdy and Bardin, 1953); stem rot of potatoes (Johnson and Atallah, 2014); leaf blight of sunflowers (Sedun and Brown, 1987); pod rot of peas (Huang and Kokko, 1992); stem rot in soybeans (Boland and Hall *et al.*, 1988) and rot of carrots (Kora *et al.*, 2005a; Foster *et al.*, 2011). Infection of healthy plant tissue through ascosporic hyphae is mostly directly through the cuticle and not the stomata (Lumsden and Dow, 1973; Abawi *et al.*, 1975). Studies have also concluded that senescing or dead tissue, external nutrients and/or wounded plant tissue are needed for ascospores to initiate infection (Sedun and Brown, 1987; Kora *et al.*, 2005a; Bolton *et al.*, 2006; McQuilken, 2011). In the presence of these factors, a single appressorium is produced from a germinating ascospore which is capable of invading and colonising the host (Abawi *et al.*, 1975; Sutton and Deverall, 1983).

1.3.2.2. Sclerotial hypha

The second means of infection is by hyphae. It is thought that infection caused from hyphae directly arising from sclerotia are not as effective as the primary infection initiated by ascospores. This is assumed to be due to the reduced ability of the hyphae to compete saprophytically, meaning that the mycelium first infects senescent material and then proceeds to living host tissues (Saharan and Mehta, 2008). However, active colonisation involving several processes has been described by which hyphae of *S. sclerotiorum* hyphae branch over the host surface, form an appressorium after physical contact with the host surface, penetrate the cuticle mechanically and form an inflated vesicle that gives rise to an infection hypha which invades and colonises inter- and intracellularly in both dead and living plant tissues (Lumsden and Dow, 1973; Saharan and Mehta, 2008).

Studies have indicated that the direct penetration of hyphae through the cuticle can be observed 12h after inoculation and after 24h, hyphae have colonised both inter- and intracellularly (Lumsden and Dow, 1973; Davar *et al.*, 2012). A greenhouse study

conducted by Botha *et al.* (2011) to evaluate different forms of *S. sclerotiorum* mycelium inoculum, found that the optimum conditions for disease development were 20.9°C at a RH of >90%. An association has been found between penetration and lesion expansion and cell degrading enzymes, e.g. cellulases, hemicellulases, and pectinolytic enzyme; proteases and oxalic acid (Maxwell and Lumsden, 1970; Riou *et al.*, 1991; Cessna *et al.*, 2000).

1.3.2.3. Role of oxalic acid

Oxalic acid is a toxic substance if produced by pathogenic micro-organisms, while that produced by animals, plants and non-pathogenic micro-organisms is a chemically inactive end-product of their metabolisms (Donaldson *et al.*, 2001; Kim *et al.*, 2008). Oxalic acid changes the pH of infected plant tissue to close to the optimum pH for the activity of cell wall degrading enzymes which favours the process of plant cell death (Cessna *et al.*, 2000; Kabbage *et al.*, 2013, Williams *et al.*, 2014).

S. sclerotiorum produces oxalic acid and this is used as a determinant of this fungus's pathogenicity (Zhou and Boland, 1999; Hegedus and Rimmer, 2005). This involvement has been proved by the recovery of oxalic acid from infected plant tissues. After injection of oxalate into plants, disease symptoms can be observed (Cessna *et al.*, 2000; Dai *et al.*, 2006). The outcomes of a study conducted by El-Argawy (2012) confirmed the association with *S. sclerotiorum*'s pathogenicity by measuring the production of oxalic acid in *S. sclerotiorum* isolates that differ in their level of virulence. A highly virulent *S. sclerotiorum* isolate produced high levels of oxalic acid while an isolate that was weakly virulent produced small amounts of oxalic acid. Livingstone *et al.* (2005) developed a transgenic peanut plant, containing the oxalate degenerating oxalate oxidase gene, which made the plant more resistant to *Sclerotinia minor*. The importance of oxalic acid as a pathogenicity factor was shown in relation to the suppressing effect of oxalate oxidase on the expression of oxalic acid.

1.3.3. Survival

S. sclerotiorum stays dormant as sclerotia produced within and on plant debris (Kohn,

1979). Available data on the longevity of sclerotia under field conditions is variable with a range from a few weeks to several years (Adams and Ayers, 1979; Grogan, 1979; Weiss *et al.*, 1980). Some studies have reported that this resting propagule can remain viable in soil and host crop debris from three to eight years under dry conditions and when non-host crops are planted (Adams and Ayers, 1979). The structure and texture of sclerotia maintain the fungus in a dormant state until favourable conditions for germination prevail. The ability of sclerotia to survive in soil is dependent on several factors including the source of sclerotia (naturally produced or cultured), sclerotial size, type of micro-organisms present, moisture level of soil, soil temperature, burial depth within soil, UV light, and RH (Adams and Ayers, 1979; Caesar and Pearson, 1983; Cosic *et al.*, 2012).

Caesar and Pearson (1983) found that exposure of ascospores to 25 - 30°C and RH of >60% resulted in survival that was limited to two to eight days, with a mortality of 50.0% which corresponds with a study conducted by Clarkson *et al.* (2003). In a second experiment, Caesar and Pearson (1983) studied the impact of lower temperatures and results indicated that ascospores exposed to temperatures between 5 - 10°C at a RH of 80.0% did not survive for more than 16 days, while results from the Clarkson *et al.* (2003) study indicated that ascospore under similar conditions survived more than 16 weeks. These conflicting results could be due to different isolates used to conduct these experiments and is also an indication that more research on factors affecting survival need to be conducted. Steyn (2015) cultured 18 South African *S. sclerotiorum* isolates on potato dextrose agar at 15°C, 20°C, 25°C and 30°C, to determine the optimum growth temperature. Results indicated that the optimum growth temperature is between 20 - 25°C and a significant isolate x temperature interaction was observed. This indicated variation between different *S. sclerotiorum* isolates and how adaption to different temperature regimes have evolved in the local pathogen population.

1.4. Symptoms and signs

1.4.1. Soybeans

Studies have shown that the incidence of *Sclerotinia* stem rot in soybeans is related to flowering onset, canopy closure, tillage and irrigation, which influence disease progression (Blad *et al.*, 1978; Pennypacker and Risius, 1991, Kim *et al.*, 2000; Kurle *et al.*, 2001). Symptoms of stem rot generally appear during the early stages of pod development, (R3 and R4). The first symptom is foliar related, observed above canopy level (Abawi and Grogan, 1979; Purdy, 1979; Petlier *et al.*, 2012). Foliar symptoms include chlorosis and wilting. As the disease develops and spreads, leaves become entirely necrotic, curled and shredded. Stem lesions initially appear at the nodes, having a grey water-soaked appearance. The pathogen rapidly advances into tissues above and below the nodes which lead to an increase in lesion size. White mycelium covers the lesion, and black sclerotia differentiate (Figure 1.4a and 1.4b) from the hyphae that can also be observed within the stem. When infected crops reach maturity, they have a shredded appearance with poorly developed pods and a large number of sclerotia present in the pith as shown in Figure 1.5.



Figure 1.4: (a) *S. sclerotiorum* mycelium developing on soybean stems and (b) sclerotia forming within the stems (Picture M.C. Bester).



Figure 1.5: A sclerotium present in the developed soybean pith (Picture M.C. Bester).

1.4.2. Sunflowers

S. sclerotiorum infection on sunflowers causes both stalk/stem and head rot (Purdy, 1979). Brown water-soaked lesions can appear at any growth stage on the roots usually the result of myceliogenic infection but are more evident at flower- and seed development stages. The lesions spread to stems (Figure 1.6) causing stem rot, and leaves begin to wilt. With the onset of wilting the fibrous roots in the upper soil layer are entirely damaged. It is common for diseased stems to break at the point of infection.

Head rot infection, generally the result of ascosporic infection, usually starts at the back of the sunflower head (Figure 1.7) and spreads over the developing head and down the stem (Saharan and Metha, 2008; Ramusi and Flett, 2015). As the infection spreads, the front of sunflower heads becomes covered with mycelium (Figure 1.8). Subsequently, the mycelium transforms into a black net of sclerotia, covering the entire front of the head (Figure 1.9). As the disease becomes more aggressive, the entire plant develops a shredded appearance (Figure 1.10) with sclerotia in between the plant fibres (Saharan and Metha, 2008; Ramusi and Flett, 2015).



Figure 1.6: (a) *S. sclerotiorum* mycelium developing on the stems of sunflowers (b) which will later transform into black sclerotia (Picture M.C. Bester).



Figure 1.7: Brown water soaked lesions covered with white cotton-like mycelium, develop at the back of sunflower heads (Picture M.C. Bester).



Figure 1.8: *S. sclerotiorum* mycelium developing on the face of sunflowers (Picture L.A. Rothmann).



Figure 1.9: *S. sclerotiorum* mycelium that developed on the sunflower face transformed into a net of black sclerotia (Picture L.A. Rothmann).



Figure 1.10: Shredded appearance of sunflowers caused by *S. sclerotiorum* (Picture M.C. Bester).

1.5. Economic importance

Sunflowers and soybeans are an important source of income around the world, and thus the effect of diseases can disturb the economic welfare of many countries (South African Grain Laboratory, 2016). Changes in environmental conditions, management practices and germplasm susceptibility have led to an increase in the importance of Sclerotinia diseases worldwide. The higher disease severities are associated with lower yields (Grau and Radke, 1984).

For many years *S. sclerotiorum* has been recognised as an important pathogen of various vegetable crops, but it was only in 2001 that Sclerotinia was recognised as becoming a major yield-limiting disease on soybeans (Kurle *et al.*, 2001). *Sclerotinia* species can affect seedlings, mature plants as well as harvested grains. The annual economic loss due to *S. sclerotiorum* on the five crops studied by the United States Agricultural Research Service National Sclerotinia Research Initiative which include soybeans, sunflowers, dry edible beans, canola, peas and lentils was \$US 482 million. It has been reported that the loss to sunflowers was \$US 100 million and \$US 300

million to soybeans (National Sclerotinia Initiative, 2016). There is a continuing annual increase in yield loss due to Sclerotinia diseases, illustrating the importance of the need to develop appropriate management strategies to fight this devastating pathogen. The management of Sclerotinia diseases remains difficult, unreliable and inefficient especially due to the extensive host range and enduring survival structure, the sclerotium (Grogan, 1979; Gulya *et al.*, 1997).

1.6. Disease Management

1.6.1. Agronomic practices

1.6.1.1. Crop rotation

Crop rotation studies have shown inconsistent and conflicting results. Crop rotations of 2 - 4 years were shown to reduce the severity of Sclerotinia stem rot of soybean by reducing the number of sclerotia present in soil (Gracia-Garza *et al.*, 2002; Rousseau *et al.*, 2007) contrary to an earlier study that indicated that a three year crop rotation did not reduce the population of sclerotia significantly (Schwartz and Steadman, 1978).

Workneh and Yang (2000) suggested that planting maize as a preceding crop can result in greater numbers of apothecia within a field than soybean as the preceding crop. Even though maize is not recognised as a host crop, it serves as a growth medium for the pathogen.

Due to the long-term survival of sclerotia, a field's cropping history can be of great importance in the determination of the level of inoculum present in the soil and thus, the effect of crop rotation systems. Crop rotation may not be able to decrease inoculum levels over the short term, but could prevent inoculum build-up if no weed-hosts are present in the rotation system (Schwartz and Steadman, 1978; Mueller *et al.*, 2002a). In South Africa several common weeds serve as alternative hosts which include *Amaranthus deflexus* (pigweed), *Bidens formosum* (cosmos), *Bidens pilosa* (common blackjack) and *Tagetes minuta* (khaki weed) (Purdy 1979; Phillips, 1992). Since *S. sclerotiorum* has a broad host range and a high survival ability, crop rotation hosts are limited and rotation therefore is not an adequate control measure on its own.

1.6.1.2. Soil solarisation

Due to the persistence of sclerotia in soil, Sclerotinia disease management through conventional methods has yielded unsatisfactory results (Phillips, 1990). Solarisation of soil has great potential in managing the population number of soil-borne pathogens particularly in intensive, high value cropping systems. This is a common agricultural practice in New Zealand for reducing the viability of sclerotia of *S. sclerotiorum* in horticultural soil (Swaminathan *et al.*, 1999). This method entails the use of clear plastic laid on the surface of the soil for a few weeks during hot periods, magnifying sun penetration and heating of the ground. Temperatures as high as 40°C can be reached. They suggested that the use of integrated soil solarisation with biocontrol agents, especially as the cool wet summers in New Zealand favour *S. sclerotiorum* (Swaminathan *et al.*, 1999).

The number of the recovered sclerotia after 15 days of solarisation at depths of 5, 15 and 30 cm were 53.3%, 76.6% and 80.0% respectively with respective viabilities of 29.0%, 45.0% and 52.0% (Cartia *et al.*, 1994). Gullino *et al.* (1998) applied metham sodium and methyl bromide, chemicals used to control several soilborne pathogens in many crops in the past, to soil which was subsequently covered with a low-density polyethylene film. This improved the fumigants' efficacy and decreased the period of solarisation required to reduce sclerotial viability. A secondary benefit from soil solarisation is the decrease in weed populations (Phillips, 1990). However, it must be noted that methyl bromide was phased out and brought to a complete ban in South Africa in 2015 (Agricultural remedies that are banned or restricted for use in the Republic of South Africa, 2017).

1.6.1.3. Tillage

Sclerotia can stay viable in the soil for up to eight years (Adams and Ayers, 1979). Tillage practices can positively and negatively contribute to a reduction in sclerotia in soil. Shallow harrowing maintains infested stubble on or near the soil surface, increasing favourable conditions for carpogenic germination (Mueller *et al.* 2004). This is in contrast to an earlier study that indicated that the number of apothecia, i.e. primary inoculum, was reduced in no-tilled soybean fields (Gracia-Garza *et al.*, 2002).

Workneh and Yang (2000) evaluated the prevalence of Sclerotinia stem rot in no-till, minimum-till and conventional-till soybean fields, previously infested with sclerotia and those in which sclerotia were absent. This study found that Sclerotinia stem rot incidence was not affected by tillage, of any sort, in soybean fields previously infested with *S. sclerotiorum*.

In a simulation of no-till, Brustolin *et al.* (2016) indicated that sclerotia left on the soil surface of a soybean field, lost their viability after 12 months. Where *S. sclerotiorum* infested soil is deeply ploughed, sclerotia are buried deeper into the soil providing a survival potential of up to eight years. However, increased organic matter content in the soil profile associated with this practice favours micro-organisms and could lead to an increase in microbial activity which may speed up sclerotial degradation. As with crop rotation, tillage or zero tillage showed conflicting results, which could be attributed to stubble type, soil characteristics and other environmental variables.

1.6.1.4. Planting date

Most of the studies carried out to evaluate the effect of planting date on Sclerotinia disease incidences, have suggested that earlier planted crops are at a lower risk of infection than crops in later plantings. A study conducted in Ohio, United States of America, indicated that alfalfa sown earlier, i.e. beginning of August, had 4.0% Sclerotinia disease severity compared with alfalfa sown later, i.e. late August, which had 41.0% Sclerotinia disease severity (Sulc and Rhodes, 1997). This is due to disease escape in that the earlier planted crops are exposed to less favourable environmental conditions during respective critical growth stages compared with conditions later in the season.

In contrast, Gupta *et al.* (2004) in India, studied the effect of planting dates which included, late October, early November, early December, late December and early January on Sclerotinia incidence in rapeseed. Their results indicated that planting in late October yielded the highest levels of Sclerotinia blight (10.5%), while disease decreased with each delay in planting, with early January yielding no Sclerotinia disease. These results indicate that the effect of planting date on Sclerotinia diseases

is locality/regionally dependent and associated with variation in environmental cycles over years.

1.6.1.5. Crop density and modification of microclimate

Crop density and subsequent canopy structure is the primary factor when considering microclimate relationships with *Sclerotinia* disease incidence and severity. This relationship applies to most crops affected by *S. sclerotiorum*. Blad *et al.* (1978) indicated that a dry bean cultivar that produces a dense canopy and which is liberally irrigated, creates a cool and wet microclimate which results in high disease severity while a less viny dry bean cultivar, which is less dense, together with less frequent irrigation formed a dry canopy with little disease. Crop populations that form a dense canopy can thus create a micro-environment that may favour infection by *S. sclerotiorum* (Gracia-Garza *et al.*, 2002). *Sclerotinia* requires moisture to germinate, either myceliogenically or carpogenically and regulating the level of free water present in the field by means of canopy density, may assist in managing the development of the disease as well as apothecia formation (Rotem and Palti, 1969). Clipping a carrot plants' canopy for example, can reduce the number of apothecia that develop due to the creation of a less favourable microclimate without affecting the carrot root weight at harvest (Kora *et al.*, 2005b).

Planting fewer plants per row as well as a wider row spacing may increase the inter-row rate of evaporation while the period of favourable environmental conditions for disease development decreases due to delayed canopy closure (Steadman *et al.*, 1973). Wide row spacing (more than 76 cm) and cultivars with a low canopy density and an upright posture, have resulted in reduced levels of *Sclerotinia* infection (Saindon *et al.*, 1995). Lee *et al.* (2005) indicated that disease severity in a soybean field was lower when the population of soybean plants was decreased from 560 000 seeds/ha to 430 000 seeds/ha using 19 cm rows. Decreasing the soybean population was more effective than increasing inter-row spacing in managing *Sclerotinia* disease in irrigated soybean fields.

Hoes and Huang (1976) in a similar study on sunflower, indicated that at crop densities of 27 500 to 55 000 seeds/ha, yield loss due to *S. sclerotiorum* induced wilting, was

lower than at a density of 82 000 seeds/ha. Wider inter- and intra-row spacing is crucial when trying to reduce disease incidence, because free air circulates more effectively through widely spaced plants, leading to a drier microclimate and consequently, fewer apothecia. In a field where plants are widely spaced, the contact between healthy and infected roots is also limited leading to less spread and subsequent wilt incidence (Young and Morris, 1927; Huang and Hoes, 1980). Inter-row spacing of 36 cm or higher, together with a plant population of 26 000 to 49 000 plants/ha has the potential of reducing yield loss (Hoes and Huang, 1985).

1.6.1.6. Sanitation

Reducing the amount of inoculum may not always lead to less disease over the short-term due to inoculum thresholds often being exceeded. However, activities that prevent or minimise the re-introduction of inoculum into the field may be an option for managing disease (Steadman, 1979). Sanitation activities may either reduce or eradicate the *Sclerotinia* inoculum present in planting material, field or storeroom and it will also avoid spreading the disease to uninfected plants and uninfested fields (Agrios, 2005).

Sclerotia can accidentally be harvested with soybeans, sunflowers, rapeseed or other seed crops. Planting of certified seed will minimise the risk of introduction of *S. sclerotiorum* inoculum into uninfested fields. The re-introduction of inoculum into fields, through the use of infected plant residues, contaminated planting and harvesting equipment must be avoided (Steadman, 1979). Burning infected alfalfa (*Medicago sativa*) stubble was shown to be a highly effective method for controlling *Sclerotinia* inoculum, by destroying 95.0% of sclerotia (Gilbert, 1991). According to Hind-Lanoiselet *et al.* (2005) destroying infected wheat stubble with fire was not as effective as exposing sclerotia to temperatures higher than 121°C in an oven. *S. sclerotiorum* has a wide host range and sanitation as a disease management strategy needs to take account of the various weeds that may serve as alternative hosts for the pathogen. Their presence can also increase the density of the canopy, favouring disease development (Phillips, 1992). Thus, theoretically performing complete field sanitation, can reduce inoculum levels and delay distribution.

An overflow of water from an infected field to an uninfected field may lead to the introduction of *Sclerotinia* inoculum and emphasises the need to include the course of drainage as a factor in sanitation and disease control (Schwartz and Steadman, 1978).

1.6.2. Chemical control

Breeding for resistant germplasm is a continuing process. However, due to limited success, the application of fungicides to suppress or control *Sclerotinia* diseases remains one of the primary control measures available to producers although no fungicide provides complete control (Oplinger *et al.*, 2007). The inhibition of *S. sclerotiorum* infection through fungicide application occurs differentially and is dependent on the type of fungicide applied (Peltier *et al.*, 2012). Similarly, the timing of application, by implication the correct growth stage, application method, active ingredient, the degree of coverage and penetration into the canopy are primary factors that determine the efficacy and success of the chemical. Applying fungicides to soybeans at the R1 growth stage will maximise the efficacy of the fungicide due to the epidemiological relationship with flowering compared to a later application at R3 stage (Mueller *et al.*, 2002b, Mueller *et al.*, 2004). Sumida *et al.*, (2015) indicated that fungicides, Fluazinam and Procymidone, reduced the incidence of *Sclerotinia* stem rot by more than 70.0%. Agriculture and Horticulture Development Board conducted a study in 2015 on the effect of fungicides on *Sclerotinia* stem rot in canola, and the results showed that Proline (275 g/l prothioconazole), Filan (500 g/kg boscalid), Pictor (200g/l dimoxystrobin and 200 g/l boscalid) and Amistar (200 g/l azoxystrobin and 200 g/l difenoconazole) reduced the levels of *Sclerotinia* disease significantly with up to 90% control (Agriculture and Horticulture Development Board, 2016).

In some host crops, fungicide application is a less practical option due to the difficulty of penetrating the site of infection due to canopy density, and host growth habit. Furthermore, applying fungicides to lesser value crops increases the cost of production, to the point that returns do not warrant chemical inputs. Chemicals may also be harmful to the environment. In these circumstances, a biological alternative may be more eco-friendly, sustainable and practical (Fernando *et al.*, 2004; Saharan and Mehta, 2008; Davidson *et al.*, 2016).

1.6.3. Biological control

The attention given to biological control agents (BCA) over recent years has increased significantly, and may be considered the appropriate alternative to control *Sclerotinia* disease intensity by inhibiting the carpogenic germination of sclerotia and attacking the hyphae of myceliogenic germination using micro-organisms (McLaren *et al.*, 1996; Pérez-García *et al.*, 2011; Kamal *et al.*, 2015). Understanding the behaviour of a BCA population under different production and ecological systems is of great importance in disease management strategies (Utkhede, 1996).

Antagonistic bacteria and fungi can be used to manage *S. sclerotiorum* infections (Huang and Erickson, 2008; Kamal *et al.*, 2016). Sumida *et al.* (2015) evaluated the use of a commercial product based on *Trichoderma harzianum* at 5×10^{10} conidia.ha⁻¹ as an agent to inhibit carpogenic germination and ascospore germination of *S. sclerotiorum* in soybeans. Their results revealed that *T. harzianum* did not reduce the incidence of *S. sclerotiorum* in soybean fields, which was considered an indication of the difficulty in effectively establishing BCA under field conditions. Zhang *et al.* (2016) demonstrated in dual culture tests that the *T. harzianum* isolate, T-aloe, inhibited *S. sclerotiorum* growth with up to 56.3%. *T. harzianum* hyphae grew parallel with *S. sclerotiorum* and also produced contact hooked branches which is a sign of mycoparasitism towards *S. sclerotiorum*. The study also illustrated the increase in the activity of catalase and peroxidase as well as an increase in chlorophyll and total phenol content in plants, indicating that *Trichoderma* spp. does not just act as a pathogenic fungus but also enhances plant health. Although these two studies were carried out under different environmental conditions, this was the first report indicating the potential of *Trichoderma* spp. in the control of soybean stem rot.

Coniothyrium minitans is reported to be an effective antagonistic organism to control *S. sclerotiorum* in fields infested with *S. sclerotiorum* sclerotia (Huang and Hoes, 1976). The survival of *C. minitans* requires the presence of sclerotia in the soil to parasitise (Elsheshtawi *et al.*, 2017). The application of Contans® (*Coniothyrium minitans*) together with reduced doses of the fungicide Sumiscler, suppressed the growth of *S. sclerotiorum* which led to a 90.0% plant survival in beans in Egypt. In the United States of America, Zeng *et al.* (2012) treated soil of soybean field trials three times, 2007,

2008 and 2009, with three BCS, which were *C. minitans*, *Streptomyces lydicus* and *Trichoderma harzianum*. Comparison of BCA effects on Sclerotinia disease index (DSI) and the number of sclerotia present in soil indicated that *C. minitans* was the most effective BCA and reduced the DSI by almost 69.0%, while *Streptomyces lydicus* and *Trichoderma harzianum* reduced the DSI by 43.1% and 38.5% respectively. *C. minitans* also reduced the number of sclerotia in the soil by as much as 95.3%. They suggested that the effectiveness of these three BCA may be due to the fact that the population of all three BCA stayed stable throughout the season.

Bacillus amyoliquefaciens and *Pseudomonas chlororaphis* can be used as bacterial biocontrol agents to manage *S. sclerotiorum* (Fernando *et al.*, 2004). These bacteria suppress both carpogenic and myceliogenic germination through the production of antimicrobial substances. These two bacteria inhibited *S. sclerotiorum* infection in a canola field. Yuen *et al.* (1994) found that dry edible bean plants, under controlled environmental conditions, pre-treated with *Erwinia herbicola* had less Sclerotinia stem rot than the control plants. However, *E. herbicola* was not successful in reducing Sclerotinia under field conditions.

1.6.4. Resistance

The resistance of plants can be expressed when exposed to both abiotic and biotic factors (Bi *et al.*, 1994; Wang *et al.*, 2003). Plants have several mechanisms that recognise infections caused by pathogenic micro-organisms and have the ability to respond effectively to these attacks by activating antifungal defence responses such as proteins and peptides (Stintzi *et al.*, 1993). Plants have primary barriers such as waxes, cell walls containing lignin, antifungal peptides and proteins which may also be initial obstacles for invading pathogens. Elicitors in pathogens that have the ability to overcome these barriers by degradation, induce another set of defence responses within plant cells which include the production of phytoalexins, phenolics and pathogenesis-related (PR) proteins (Hematy *et al.*, 2009). PR proteins were first reported after a hypersensitive response in the tobacco leaf to tobacco mosaic virus, was recorded (van Loon and Kammen, 1970). Chitinase and β -1, 3-glucanase are PR proteins that are intensively studied for their role in plant defence against pathogens

(Van Loon *et al.*, 2006; Balasubramanian *et al.*, 2012). However the effect of chitinase and β -1, 3-glucanases on *S. sclerotiorum* has not been widely studied.

1.6.4.1. Chitinase

Chitinases are PR proteins that belong to the PR-3 family. These proteins catalyse the hydrolysis of chitin and chitosan (Jitonnom *et al.*, 2011). Chitin and chitosan are present in the exoskeleton of invertebrates, bacteria and in fungal cell walls (Li and Roseman, 2004). Dai *et al.* (2006) recorded an induction of chitinase produced by the plant, after *Arabidopsis thaliana* L. became infected with *S. sclerotiorum*, indicating that this enzyme plays a role in plant defence. The *in planta* effects of chitinase on Sclerotinia diseases have not been established. It has also been found that chitinase in combination with β -1, 3-glucanases can inhibit fungal growth by lysing hyphal tips (Mauch *et al.*, 1988b).

1.6.4.2. β -1, 3-glucanases

β -1, 3-glucanases are PR proteins that belong to the PR-2 family. These enzymes play important roles in flower formation through to the maturation of seeds, cell division, transportation of materials through the plasmodesmata, endurance of abiotic stresses including heavy metals, drought and low temperatures (Romero *et al.*, 2008; Pirsellova *et al.*, 2011; Gregorová *et al.*, 2015). The β -1, 3-glucanase substrate is a major component of fungal cell walls (Balasubramanian *et al.*, 2012). This enzyme has the ability to defend plants against pathogenic micro-organisms either on its own or in combination with chitinase (Mauch *et al.*, 1988). β -1, 3-glucanases have been extensively studied for their abilities to inhibit the growth of, and infection by fungal pathogens by acting as a barrier controlling the spread of pathogens and lesion size (Castresana *et al.*, 1990; Balasubramanian *et al.*, 2012). Chatterton and Punja (2009) concluded in their study that this enzyme inhibited conidial and hyphal germination and the growth of *Fusarium oxysporum* as well as *Pythium aphanidermatum*. It was also found that there is an increase in the expression of β -1, 3-glucanases in the fungus, *Coniothyrium minitans* during a mycoparasitic interaction with *S. sclerotiorum* (Giczey *et al.*, 2001). The *in planta* effects of β -1, 3-glucanases on Sclerotinia diseases have not been established.

1.6.4.3. Lignin

As plants mature, they become more fibrous and rigid. This is due to lignin binding the fibres within plants by deposition in cell walls of leaves and stems. These depositions serve as a structured protective barrier (Jung and Allen, 1995). It has been suggested that the increase in the lignin content of cell walls decreases plant cell permeability and thus water loss and also acts as mechanical barrier to microbial degradation.

In contrast to the above, Peltier *et al.* (2009) indicated that *S. sclerotiorum* resistance is associated with low levels of lignin within soybean stems and suggested that lignin concentrations within the stems of plants can be used as a biological marker when selecting genotypes for resistance. Limited research has been done on the effect of lignin concentrations on *S. sclerotiorum* and it is suggested that more research needs to be done on the effect of lignin on pathogenic micro-organisms.

1.6.4.4. Techniques of screening for resistance

Identifying resistant germplasm would aid in breeding for Sclerotinia resistance. Resistant genotypes are considered the safest, easiest, least expensive and most effective means for managing plant diseases (Agrios, 1997). Although resistance to *S. sclerotiorum* is limited, studies have found that some soybean genotypes may have partial resistance to *S. sclerotiorum* (Auclair *et al.*, 2004; Bastein *et al.*, 2012)). Various escape mechanisms and physiological characteristics have also been associated with resistance of soybeans to stem rot under field and greenhouse conditions (Nelson *et al.*, 1991; Kim and Diers, 2000).

Several inoculation techniques have been developed and evaluated in screening for resistance to Sclerotinia diseases under both field and greenhouse conditions. Some of these inoculation techniques are labour intensive and showcase inconsistent results (Nelson *et al.*, 1991; Hoffman *et al.*, 2002). These techniques include (a) inoculation of cotyledons of 14-day old seedlings with mycelium disks cut from a *S. sclerotiorum* colony (Kim *et al.*, 2000; Kull *et al.*, 2003); (b) detached leaf assay where leaves are cut from the plant and inoculated with mycelium disks cut from *S. sclerotiorum* colony (Chun *et al.*, 1987); (c) spray mycelium method where a mycelium suspension grown

in potato dextrose broth (PDB) is homogenised and sprayed onto plants (Chen and Wang, 2005); (d) cut petiole inoculation where plants are mechanically wounded and subsequently inoculated with *S. sclerotiorum* (Del Rio *et al.*, 2001); (e) milled grain mycelium where sorghum grains are inoculated with an agar plug of *S. sclerotiorum*, incubated for three weeks, air-dried once the mycelium has colonised the grains and milled; the milled mycelium is then distributed over the plants at critical growth stage (Nelson *et al.*, 1988); (f) ascospore suspension where ascospores collected from apothecia are suspended into sterile distilled water and sprayed to the plant at flowering (Van Becelaere and Miller, 2004).

Several cultivar evaluation studies have been conducted in both the greenhouse and field. Results from these two environments are variable and low correlations between genotype responses to inoculation with *S. sclerotiorum* in greenhouse and field trials have been recorded (Vuong *et al.*, 2004; Huzar-Novakowski *et al.*, 2017). It is assumed that the response of cultivars to Sclerotinia disease under controlled environments are due to physiological resistance while the reaction of cultivars to Sclerotinia under field conditions is due to both physiological and escape resistance (Boland and Hall, 1987; Nelson *et al.*, 1991; Kim *et al.*, 2000). Disease escape mechanisms are mostly associated with early flowering which leads to earlier maturity and avoiding disease favourable conditions and an upright and more open canopy that provides a less favourable microclimate (Miklas *et al.*, 2013). McLaren and Craven (2008) emphasised the strong interactions between the genotype and environment and questioned the value of resistance screening methodologies under controlled environmental conditions. They developed a multi-environment, regression based screening methodology which quantifies the genotype x environment responses of germplasm to changing disease potentials.

Botha *et al.* (2011) evaluated the reaction of four soybean cultivars to six mycelium based inoculation techniques in a greenhouse. One of the techniques used, the spray mycelium (Chen and Wang, 2005), yielded the highest level of Sclerotinia stem rot. They also recorded a technique x cultivar interaction in addition to cultivar and technique main effects. A field study carried out by Ebrahimi *et al.* (2013) on the reaction of sunflower cultivars to various inoculation techniques, also found significant

differences between cultivars, indicating that cultivars react differently to different forms of inoculum.

1.7. Conclusion

Despite the extensive literature related to *Sclerotinia* diseases, this disease remains a problem in many economically important crops. The spread of the disease into important crop production areas of South Africa in recent years and the associated crop losses, highlights the need for effective control measures and the urgency for their deployment. Losses due to *Sclerotinia* disease can be reduced when an integration of different management practices such as cultural practices, chemical or biological control with varieties tolerant to increasing disease potentials is used.

The following study emphasises the development of effective inoculation techniques which replicate natural infection and provide a non-invasive protocol which can be applied to screening for resistance or tolerance to increasing disease potential and thus risk reduction. These techniques also serve as the basis for fungicide and BCA evaluations, crop modelling experiments and pathogen survival studies currently in progress at the University of the Free State, South Africa.

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Chapter 2

Laboratory generation and optimising *Sclerotinia sclerotiorum* inoculum

2.1. Abstract

Sclerotia of *Sclerotinia sclerotiorum* can germinate to produce either hyphae, the result of myceliogenic germination, or apothecia, which are the result of carpogenic germination. Both these germination types can lead to infection and disease development in susceptible crops, although environmental conditions required to initiate disease, differ. The production of apothecia is more complex than the production of mycelium. According to literature, the production of ascospores requires sclerotia to be pre-conditioned for several weeks at 4°C. The aim of this study was to produce laboratory grown sclerotia and the subsequent production of myceliogenic and ascosporic inoculum. Sclerotia were produced on several substrates to determine the substrate that yields the most and largest sclerotia. The tested substrates included; sorghum grain, maize grain, sunflower seed, wheat grain, soybeans, soybean stubble, wheat grain meal, soybean meal, maize meal, sorghum meal, sunflower meal and sunflower stubble. Maize meal and soybean stubble yielded the largest sclerotia, with an average mass of 0.0192 mg per sclerotium. Sunflower meal yielded the smallest sclerotia with an average mass of 0.0099 mg per sclerotium. Sclerotia were incubated on 1.5% water agar at 16°C, 18°C, 20°C, 22°C, 24°C, 26°C, 28°C and 30°C respectively, to determine the optimal temperature for apothecial development. Rapid stipe development occurred at 16°C while no apothecial stipes were produced at 22°C and higher temperatures. After determining that 16°C was the optimal temperature for apothecia development, the required sclerotial pre-conditioning period was studied. Freshly harvested sclerotia from an infected sunflower field, sorghum grain substrate and 1.5% water agar were either maintained at 16°C with no pre-conditioning or pre-conditioned at 4°C at 2-week intervals for 8 weeks and subsequently incubated at 16°C until apothecia developed. This procedure was conducted on both 1.5% water agar and sandy soil as substrates. The sclerotia harvested from the sunflower field produced the most apothecial stipes, with a mean of 16 apothecial stipes per sclerotium, while the sclerotia produced on 1.5% water agar and sorghum grain produced a mean of two apothecial stipes. Sclerotia pre-conditioned on sandy soil produced a mean of 10 apothecial stipes while sclerotia pre-conditioned between two layers of 1.5% water agar produced a mean of three apothecial stipes. The sclerotia collected from infected sunflower fields pre-conditioned at 4°C on sandy soil for 8 weeks, produced a mean of 48 apothecial stipes after 83 days, while a pre-conditioning for 4 weeks produced only three apothecial stipes after 73 days. Sclerotia with a length of 27 mm produced a mean of 118 apothecial stipes while those with a length of 5 mm produced a mean of 14 apothecial stipes. The viability of the pathogen infected sunflower stems milled into a fine powder, shredded into pieces of approximately 2.5 cm by 1.0 cm, shredded into pieces of 8 cm by 3 mm and stubble left whole, were evaluated at monthly intervals under laboratory conditions for 12 months. All the stubble forms were able to produce *S. sclerotiorum* mycelium and sclerotia after 12 months on Potato Dextrose Agar.

2.2. Introduction

Sclerotinia sclerotiorum is a soil-inhabiting fungal pathogen. It is considered one of the most successful plant pathogens, particularly since this fungus has more than 500 plant hosts and two germination pathways. The means of germination are dependent on the prevailing environmental conditions (Willets and Wong, 1980). Myceliogenic germination occurs when hyphae are produced from sclerotia under temperatures ranging from 20 - 25°C, 80% Relative humidity (RH), pH of 5 and exogenous nutrients (Huang and Erickson, 2008). As nutrients become depleted, the mycelium aggregates and transforms into a sclerotium. The *in vitro* production of sclerotia is mostly based on mycelium infested grains and are the preferred inoculum source rather than the production of ascospores which are the result of carpogenic germination (Kull *et al.*, 2003; Rousseau *et al.*, 2004).

Carpogenic germination is a result of apothecial stipes being produced from pre-conditioned sclerotia. Pre-conditioning has been reported to be an essential factor in the initiation of carpogenic germination (Kohn, 1979; Dillard *et al.*, 1995; Hao *et al.*, 2003; Ghasolia and Shivpuri, 2015; Pethybridge *et al.*, 2015). Pre-conditioned sclerotia are those that have been exposed to long periods (2 - 8 weeks) of low temperature (4 - 10°C), which are normally associated with winter. The apothecial stipes or initials transform into mature apothecia, mushroom-like structures, which release the asci-formed ascospores into the air. Ascospore release is triggered by a change in air pressure and RH, favouring long-distance dispersal as well as infection. Numerous *in vitro* techniques have been evaluated for apothecia production, although the protocols are punctuated with inconsistencies and contradictions.

Phillips (1987) reviewed available information on carpogenic germination at that time. He suggested that in some *S. sclerotiorum* isolates, the germination process is controlled by a constitutive dormancy element. It is a type of dormancy that is delayed due to the presence of an innate property in the dormant structure. This type of dormancy can be bypassed by the exposure of sclerotia to sandy soil at temperatures of 10 - 20°C. Dillard *et al.* (1995) tested several conditioning methods which included, sclerotia placed on wet filter paper in glass Petri dishes, sclerotia planted in 10 cm pots containing masonry sand and watered three times per week for four weeks and

subsequently twice a week, sclerotia placed in flasks filled with 200 ml water and constantly aerated and sclerotia placed in flasks filled with 200 ml water without aeration. All the treatments were conditioned at 8°C. Sclerotia were removed 1, 2, 4 and 8 weeks after the onset of each conditioning treatment. The results indicated that all these methods were able to stimulate carpogenic germination and as the weeks of pre-conditioning increased so did apothecia production. However, 8 weeks of pre-conditioning produced the highest number of apothecial stipes. These authors studied the optimum temperature for pre-conditioning using 24 isolates of *S. sclerotiorum*. This ranged from 8 – 16°C with significant differences between isolates. They also found that as the sclerotial size increased, the number of stipes produced also increased.

Letham (1976), evaluated 23 *S. sclerotiorum* isolates for their ability to produce apothecia as well as the duration of apothecia development without any form of sclerotial pre-conditioning. Ten sclerotia from each isolate were placed in a glass tube containing 1.5% tap-water agar, plugged with cotton wool and incubated at 18 - 20°C in indirect sunlight. All 23 *S. sclerotiorum* isolates produced apothecial initials. Forty-four percent of the isolates produced up to 19 apothecial initials, 17.0% produced up to 39 apothecial initials, 22.0% produced up to 59 apothecial initials and 17.0% produced up to 79 apothecial initials. However, the time taken to produce these initials differed between isolates and ranged from 3 – 10 weeks.

Most studies related to the survival of *S. sclerotiorum* are based on sclerotia and ascospores. Little information is available on the survival of mycelium especially within host plant stubble. In Canada, Huang and Kozub (1993) evaluated the overwintering ability of *S. sclerotiorum* mycelium in the stems of dry bean, canola, and sunflower under both field and laboratory conditions. The mycelium had a higher survival rate when stored at -10°C in the laboratory compared with 20°C. Mycelium infected stems buried to a depth of 7 cm in sandy soil did not survive the winter, which is from November to March, while mycelium within the infected stems placed on the surface of the sandy soil had a survival rate of 69%. Although they concluded that the burial of infected stems may hasten the death process of mycelium, this may not be a practical and effective cultural practice since sclerotia are also produced in infected stems and can survive in the sandy soil for several years. It was suggested that the

removal of infected stems may be the best practice to managing *S. sclerotiorum* populations in the field.

The current study was aimed at optimising the production of *S. sclerotiorum* sclerotia and ascospores in the laboratory to provide inoculum for field and greenhouse trials. The survival of *S. sclerotiorum* mycelium on sunflower stubble of various textures under laboratory conditions, was also quantified.

2.3. Materials and Methods

2.3.1. Sclerotia production

2.3.1.1. Isolate

Sclerotia were collected from infected sunflower fields in Candy, Free State in 2016. Sclerotia were removed from sunflower stubble, washed in tap water and surface sterilised in 3% sodium hydrochloride for 4 min. The sodium hypochlorite was discarded and 76% ethanol was added for further sterilisation for 2 min. The 76% ethanol was discarded and followed by two washes in sterilised distilled H₂O. Sclerotia were placed on 90 mm Potato Dextrose Agar (PDA) plates, containing 1.5 ml Streptomycin per litre PDA. The Petri dishes were incubated for five days at 25°C under natural 12-hour light and dark cycles. The cultures were sub-cultured and left for seven days before use. Accepting the fact that the South African *S. sclerotiorum* population is clonal (Steyn, 2015), only one isolate was used in the trial.

2.3.1.2. Substrate preparation

Eleven different plant based substrates were used to produce sclerotia under laboratory conditions. These substrates include maize grain, maize meal, sorghum grain, sorghum meal, soybeans, soybean meal, soybean stubble, sunflower seed, sunflower seed meal, sunflower stubble, wheat grain and wheat meal. CONSOL jars (1 l) were filled with 150 g of each substrate. Water (500 ml) was added to each jar and left overnight. The water was drained from the jars and 85 ml distilled water was added. The jars were autoclaved for 20 min at 121°C and upon cooling, re-autoclaved.

The autoclaved jars were cooled and inoculated with ten, 5 mm diameter *S. sclerotiorum* mycelium agar plugs from seven day old *S. sclerotiorum* colonies on PDA. The jars were incubated at 25°C under natural 12-hour light and dark cycles until the substrates were fully colonised.

2.3.1.3. Harvesting sclerotia

As soon as the sclerotia had matured on the respective substrates they were harvested. The jars were shaken to loosen all the sclerotia from the substrate. The jars were emptied on to a flat surface and air dried. The sclerotia were collected by soaking the mixture of substrate and sclerotia in water. The sclerotia floated on the water surface while the substrates sank to the bottom. Sclerotia were scooped up using a tea-strainer and left to air dry in a laminar flow bench. The lengths (mm) of 50 random sclerotia and their respective masses (mg) were recorded.

2.3.2. Apothecia production

2.3.2.1. Temperature requirement

Sclerotia collected from sunflower fields at Candy, were surface sterilised as described above. Five sclerotia were plated onto 1.5% water agar (Sigma) in 90 mm Petri dishes and covered by another layer of 1.5% water agar, thus keeping the sclerotia moist. Sclerotia were conditioned at 16°C, 18°C, 20°C, 22°C, 24°C, 26°C, 28°C and 30°C. The trial was repeated five times for each temperature. The Petri dishes were left in the incubators until the first set of stipes developed.

2.3.2.2. Pre-conditioning

Sclerotia were harvested from (1) an infected sunflower field at Candy/Greytown; (2) sorghum grain substrate; and (3) 1.5% water agar culture. Sclerotia were surface sterilised as described above. All three sources of sclerotia were maintained in a Petri dish either filled with saturated sandy soil or sandwiched between 1.5% water agar as described above. The sclerotia planted in the sandy soil filled Petri dishes were watered with sterile dH₂O twice a week. Both these media were either kept at 16°C

without pre-conditioning or pre-conditioned at 4°C at 2-week intervals for 8 weeks and subsequently incubated at 16°C until stipes developed.

2.3.2.3. Stipe formation associated with sclerotium mass

Ten sclerotia within eight size classes (Table 2.1) from infected sunflower fields from Greytown, were weighed, measured and surface sterilised as described above. The sclerotia were plated onto wet sandy soil according to size class. The process was repeated five times. Every seven days the stipe formation of each sclerotium was counted and recorded.

2.3.3. Survival of *S. sclerotiorum* in sunflower stubble

Sclerotinia infected sunflower stubble was collected from an infected sunflower field in Greytown, KwaZulu-Natal. The sunflower stubble was used in four forms, which included infected sunflower stems milled into a fine powder using a coffee grinder, shredded into pieces of approximately 2.5 cm by 1.0 cm, shredded into pieces of approximately 8.0 cm by 3.0 mm and pieces left whole. All four types were stored at room temperature from April 2017 until April 2018. Every 30 days three stubble pieces and 0.5 g of the milled sample were plated onto PDA in two replicates and incubated at 25°C for seven days. The stubble segments with *S. sclerotiorum* colonies were determined.

2.3.4. Statistical analysis

Data were analysed using NCSS (Hintze, 2007). Data related to treatment effects on sclerotial production, sclerotial mass and number of apothecial stipes were analysed using Analysis of variance (ANOVA). Relationships between variables and temporal analyses were determined using linear and non-linear regression analysis. Pair-wise comparisons were conducted using the t-test.

2.4. Results

2.4.1. Sclerotia production

Analysis of variance (ANOVA) of the effect of substrates on sclerotia production (Table 2.2) indicated a significant difference ($P < 0.05$) in the mass (mg) of sclerotia produced on the different substrates (Figure 2.1). Maize meal and soybean stubble yielded the largest sclerotia, having an average mass of 0.0192 mg per sclerotium although these masses did not differ significantly from wheat meal. No significant differences in sclerotium size on sunflower meal, soybean meal, sunflower seed, sorghum meal, maize kernels and wheat grain were recorded. Sunflower meal yielded the smallest sclerotia, with an average mass of 0.0099 mg per sclerotium. Sunflower stubble and soybeans produced no sclerotia.

2.4.2. Apothecia production

Preliminary temperature tests revealed rapid stipe development at 16°C while no apothecial stipes were produced at 22°C and higher temperatures. Thus, 16°C was accepted as the appropriate temperature for apothecial stipe development.

ANOVA of the maximum number of apothecial stipes produced per sclerotium indicated the significant effects of medium, source of sclerotia and weeks pre-conditioned as well as a medium x sclerotia source x weeks pre-conditioned interaction (Table 2.3). Sclerotia produced in the field produced the most apothecial stipes with an average of 16 per sclerotium, while sclerotia produced on sorghum grain and 1.5% water agar in the laboratory produced the lowest number of apothecial stipes, with averages of less than two apothecial stipes per sclerotium (Figure 2.2).

Sclerotia conditioned on the sandy soil medium (Figure 2.3) produced an average of 10 apothecial stipes per sclerotium while sclerotia conditioned between two layers of 1.5% water agar (Figure 2.4) produced an average of three apothecial stipes per sclerotium (data presented in Figure 2.5).

The field produced sclerotia pre-conditioned on saturated sandy soil for 8 weeks reached a maximum of 48 apothecial stipes after 83 days, while the field produced sclerotia pre-conditioned between two layers of 1.5% water agar for 4 weeks, produced a maximum of 3 apothecial stipes after 73 days (Figure 2.6). The field produced sclerotia pre-conditioned in two layers of 1.5% water agar for 4 weeks reached its maximum stipe production of 28 apothecial stipes after 77 days, while pre-conditioning for 8 weeks produced no apothecial stipes (Figure 2.7). A linear relationship was observed between weeks pre-conditioned and days until maximum number of apothecial stipes had developed on both substrates, with a $R^2=0.91$ ($P<0.05$) and $R^2=0.99$ ($P<0.05$) on sandy soil and water agar substrates, respectively (Figure 2.8). Regression parameters indicated that for each week of pre-conditioning, it took approximately five days longer for apothecial stipe development. Intercepts recorded from regression analyses ranged from 34 – 42 days depending on the source of sclerotia and the substrate, indicating that the *S. sclerotiorum* isolates used in this study did not require pre-conditioning and were able to produce apothecial stipes within approximately one month after incubation at 16°C.

Sclerotial mass was highly correlated with sclerotial length class ($r = 0.93$), thus sclerotial mass was used in subsequent analyses. ANOVA of the maximum number of apothecial stipes produced indicated significant differences ($P<0.05$) between sclerotial mass classes (Table 2.4; Figure 2.9). After 60 days of incubation at 16°C, class 8 which was 27 mm in length with an average mass of 0.9341 mg per sclerotium, produced 118 apothecial stipes while class 1 which was 5 mm in length with an average mass of 0.0217 mg, produced 14 apothecial stipes. A significant linear relationship between sclerotium mass and number of stipes produced was recorded. Figure 2.10 illustrates that the onset and subsequent rates of stipe development are also affected by sclerotial size. In class 8, onset occurred after approximately 22 days while in class 1 this occurred after approximately 28 days of incubation. An unexplained deviation from this tendency was observed with size class 7.

2.4.4. Survival of *S. sclerotiorum* in sunflower stubble

All four stubble types produced mycelium and sclerotia after 12 months (Figure 2.11).

2.5. Discussion

The ability of *S. sclerotiorum* to germinate both myceliogenically and carpogenically makes it a dynamic plant pathogen. Both the end products of these two germination processes have the ability to successfully infect living and senescing plant material. These two end products are hyphae and ascospores, which are both produced from sclerotia although the respective processes are initiated by different sets of environmental conditions. The aim of the current study was to optimise the production of inoculum for use in greenhouse and field trials. However, results of the current study raise numerous questions related to the local epidemiology of Sclerotinia diseases compared with northern hemisphere cropping systems influences on Sclerotinia diseases.

The size of sclerotia is dependent on the type of host (Taylor *et al.*, 2018). The sclerotia produced on sunflower heads, for example are much larger and flatter than those produced in the stems of soybean, which are long and narrow. In the current study, significant differences were recorded between the number and size of sclerotia on the different substrates used for sclerotia production. Notable was the greater response to non-host crop stubble, in particular maize meal. Maize and wheat are non-host crops of *S. sclerotiorum* and often the suggested rotation crops for Sclerotinia disease management. The prolific growth on non-host material indicates the saprophytic ability of the pathogen and supports the notion that non-host stubble management could be an important consideration in Sclerotinia disease management. The observation could explain in part, why a reduction in sclerotial numbers were not recorded with crop rotation systems (Schwartz and Steadman, 1976) and the increase in apothecial numbers recorded following maize as preceding crop (Workneh and Yang, 2000).

Several studies on the effect of temperature on the formation of apothecia have indicated that 16°C is the appropriate temperature for stipe production (Dillard *et al.*, 1995; Mila and Yang, 2008). Mila and Yang (2008) evaluated sandy soil temperatures of 4°C, 8°C, 12°C and 16°C, and found that the 8°C resulted in the greatest stimulation of apothecia production. In the current study the upper limit of their temperature range was found to be optimal which could suggest local adaptation to higher temperatures within the local *S. sclerotiorum* population. This was proposed by Steyn (2015) who

reported that 20 - 25°C was the optimum growth temperature for the pathogen. She also reported a significant isolate x temperature interaction with some isolates able to grow optimally at 30°C.

Literature has indicated that pre-conditioning of sclerotia at 4°C for several months is essential for the formation of apothecial stipes (Dillard *et al.*, 1995; Cobb and Dillard, 1996). In contrast, the current study has indicated that apothecia can germinate from sclerotia that have not been exposed to 4°C for extended periods and in fact from relatively freshly harvested sclerotia. Exposure of sclerotia to 16°C, whether planted in sandy soil or sandwiched between two layers of 1.5% water agar was sufficient for the production of apothecia. Comparisons with northern hemisphere isolates would have to be conducted to verify any extent of local adaptation. It should be noted that the first report of *S. sclerotiorum* on soybean in South Africa was from Lydenburg (Thompson and Van der Westhuizen, 1979), an area characterised by mild winter temperatures. Extended pre-conditioning of sclerotia did, however, increase the number of apothecia produced while also delaying carpogenic germination. The relationship between the length of the pre-conditioning period and the number of apothecia produced is in accordance with the report by Dillard *et al.* (1995) and suggests a mechanism of ensuring a constant source of inoculum during crop-favourable conditions but with a later growth stage peak in ascospore levels to coincide with critical crop growth stages i.e. later-flowering infection (Sedun and Brown, 1987; Kora *et al.*, 2005; Bolton *et al.*, 2006; McQuilken, 2011).

The size of the sclerotium also play a critical role in the number of apothecia produced. The results of this study indicated that the largest sclerotia produced the most apothecial stipes, which agrees with a study by Taylor *et al.* (2018). They also found that the rate of carpogenic production increases as the size of the sclerotium increases which is accordance with the results of the current study.

In the current study *S. sclerotiorum* mycelium was able to survive for 12 months at room temperature in different forms of sunflower stubble. However, further assessments are required to determine survival period, as well as survival of mycelium on the various forms of stubble available under natural, field conditions.

2.6. References

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Table 2.1: Sclerotia size classes used in the determination of stipe formation and the concomitant sclerotial masses.

Sclerotia	Length x1 (mm)	Mass x1 (mg)
1	5.00	0.02
2	8.00	0.06
3	11.00	0.08
4	14.00	0.12
5	16.00	0.21
6	20.00	0.36
7	24.00	0.53
8	27.00	0.93

Table 2.2: Analysis of variance to compare the effect of substrates on the mass of sclerotia produced.

Analysis of Variance Table					
Source Term	Df	Sum of Squares	Mean Square	F-Ratio	Probability Level
Type of substrate	9	0.0005	0.0001	2.480	0.0274*
Reps	4	0.0001	0.00002	1.010	0.4142
Error	33	0.0008	0.00002		
Total (Adjusted)	46	0.0013			
Total	47				

Table 2.3: Analysis of variance to compare the maximum number of apothecial stipes produced from different sclerotia sources, media and weeks pre-conditioned.

Analysis of Variance Table					
Source Term	Df	Sum of Squares	Mean Square	F-Ratio	Probability Level
Medium	1	46.182	46.182	89.200	0.0000*
Sclerotia	2	93.834	46.917	90.600	0.0000*
Medium x Sclerotia source	2	0.944	0.472	0.910	0.4047
Weeks pre-conditioned	4	7.007	1.752	3.380	0.0117*
Medium x Weeks pre-conditioned	4	1.332	0.333	0.640	0.6326
Sclerotia source x Weeks pre-conditioned	8	6.966	0.871	1.680	0.1100
Medium x Sclerotia source x Weeks pre-conditioned	8	16.204	2.026	3.910	0.0004*
Reps	4	4.137	1.034	2.000	0.0993
Error	116	60.049	0.518		
Total (Adjusted)	149	236.655			
Total	150				

Table 2.4: Analysis of variance of eight mass classes and the maximum number of apothecial stipes produced per sclerotium.

Analysis of Variance Table					
Source Term	Df	Sum of Squares	Mean Square	F-Ratio	Probability Level
Sclerotial mass	7	39616.980	5659.568	4.87	0.0011*
Rep	4	15360.100	3840.025	3.30	0.0245*
Error	28	32549.900	1162.496		
Total (Adjusted)	39	87526.980			
Total	40				

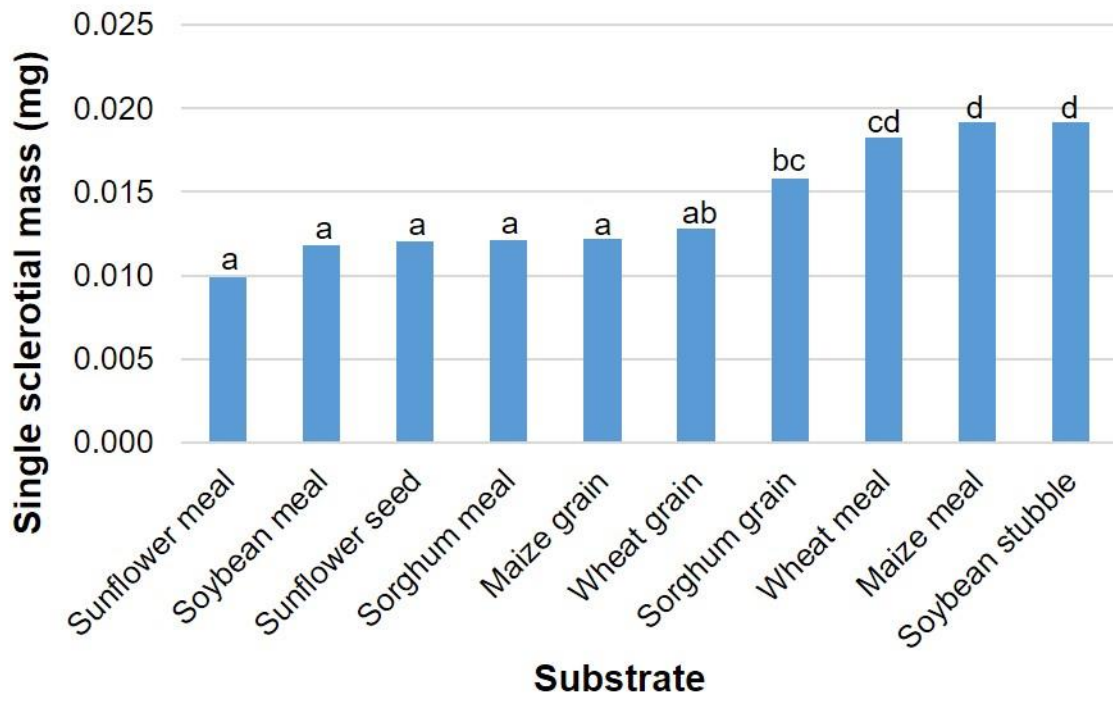


Figure 2.1: Mean mass (mg) of sclerotia produced on different substrates in laboratory cultures.

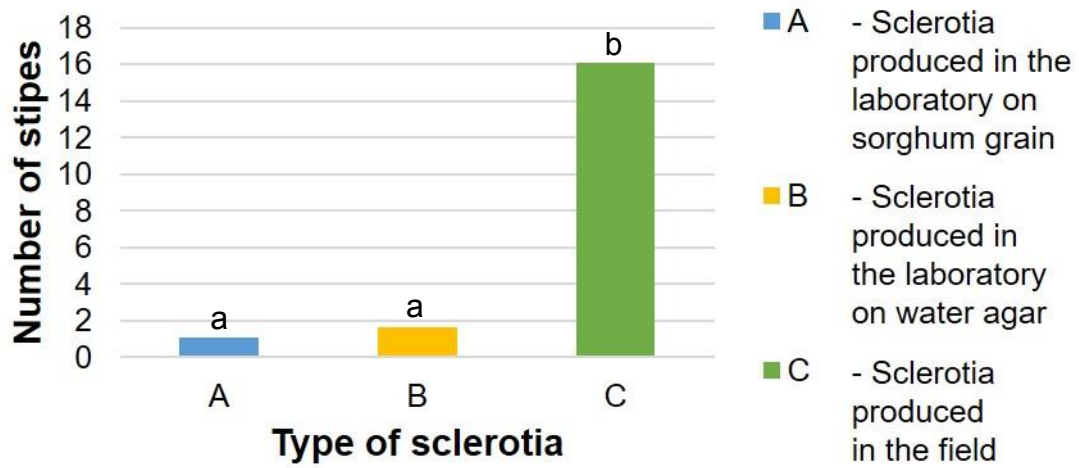


Figure 2.2: Number of apothecial stipes produced from sclerotia from two laboratory sources and those harvested in the field.



Marlese Bester

Figure 2.3: Apothecial stipe production from sclerotia conditioned in sandy soil (Picture M.C. Bester).



Figure 2.4: Apothecial stipe production from sclerotia conditioned between two layers of 1.5% water agar (Picture M.C. Bester).

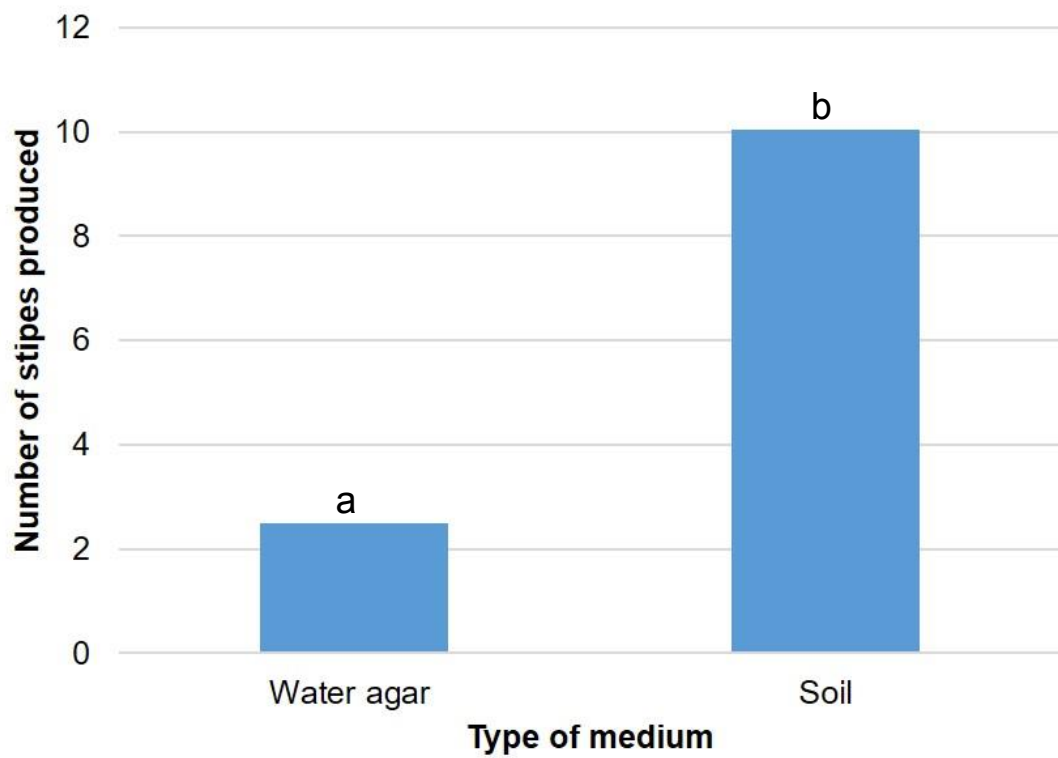


Figure 2.5: Number of apothecial stipes produced from sclerotia placed on 1.5% water agar and saturated sandy soil.

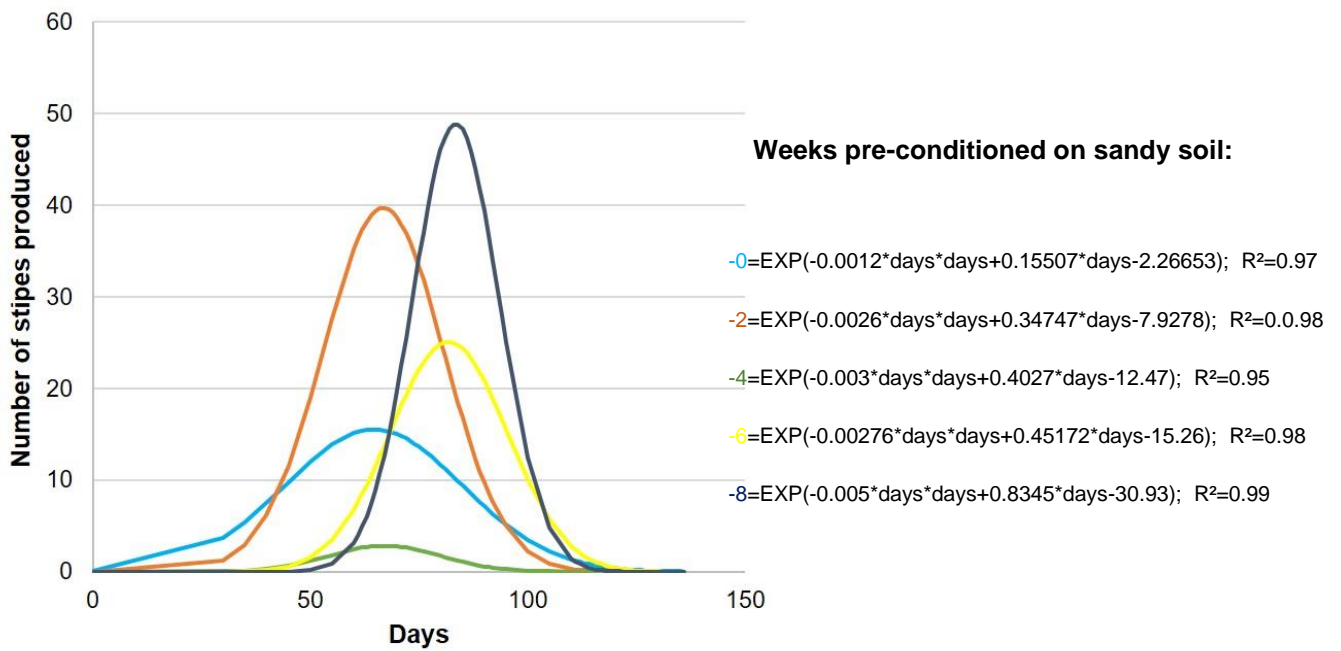


Figure 2.6: Temporal production of apothecial stipes from sclerotia without pre-conditioning and pre-conditioned for 2-8 weeks on saturated sandy soil.

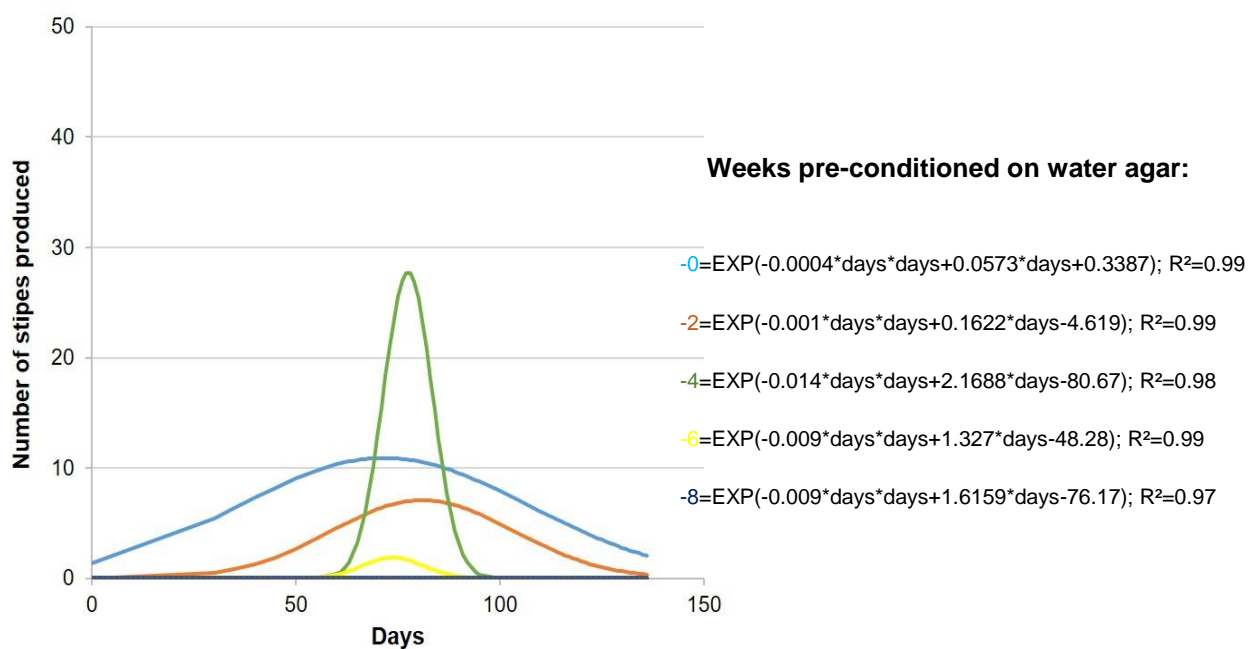


Figure 2.7: Temporal production of apothecial stipes from sclerotia without pre-conditioning and pre-conditioned for 2-8 weeks between two layers of 1.5% water agar.

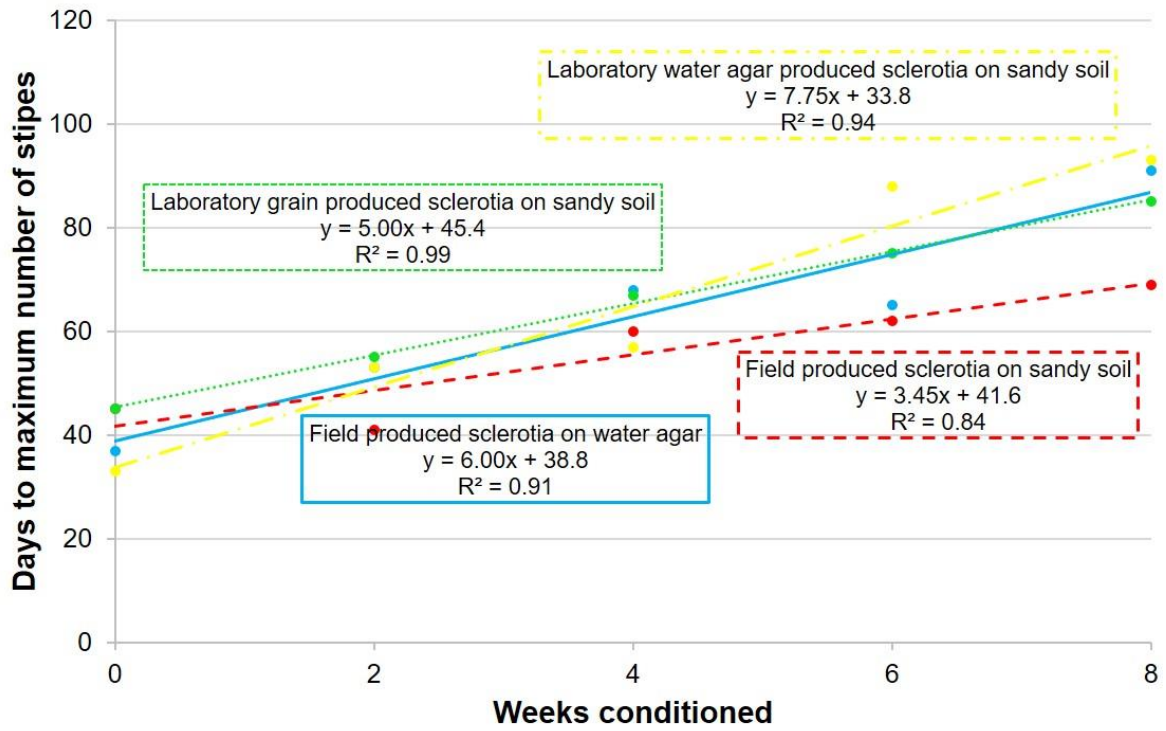


Figure 2.8: Relationship between weeks of sclerotial pre-conditioning at 4°C and days to maximum number of apothecial stipes from laboratory and field produced sclerotia.

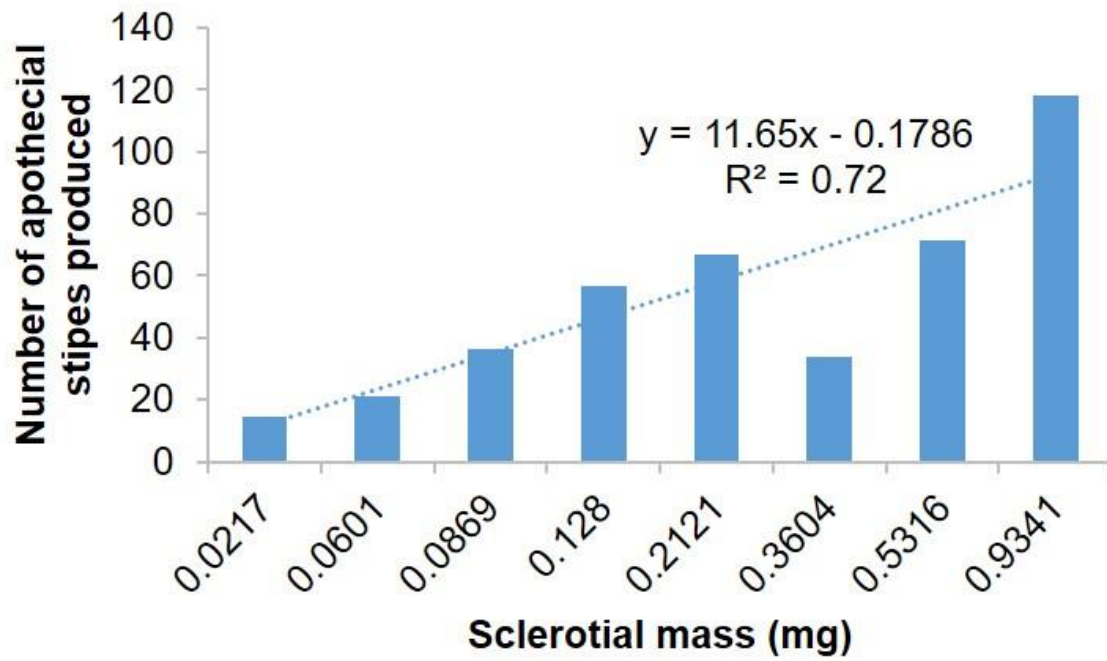


Figure 2.9: Number of apothecial stipes produced from a range of sclerotium mass classes.

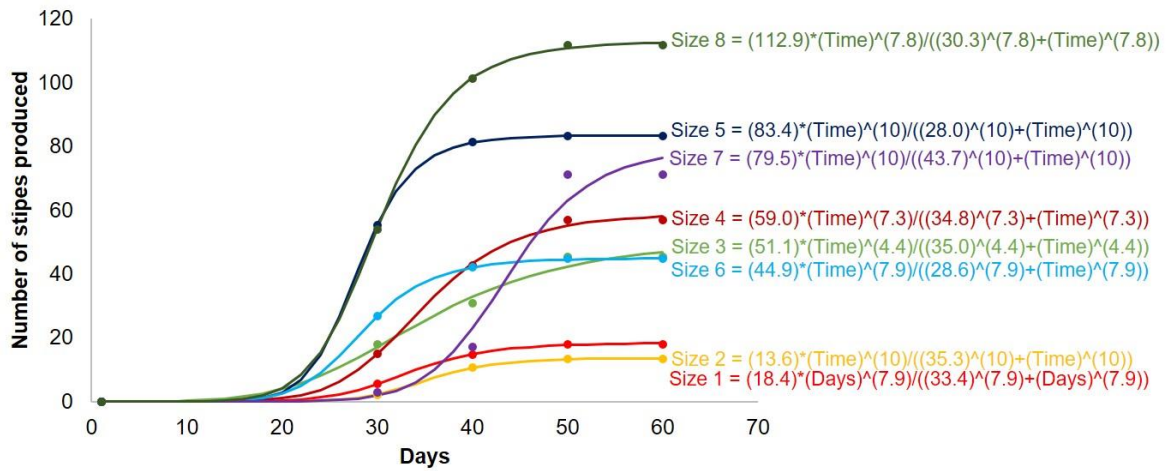


Figure 2.10: Temporal relationship between stipe development and size classes of *S. sclerotiorum* sclerotia. Size 1 = 0.0220 mg; Size 2 = 0.0600 mg; Size 3 = 0.0870 mg; Size 4 = 0.1280 mg; Size 5 = 0.2120 mg; Size 6 = 0.3600 mg; Size 7 = 0.5320 mg and Size 8 = 0.9320 mg.

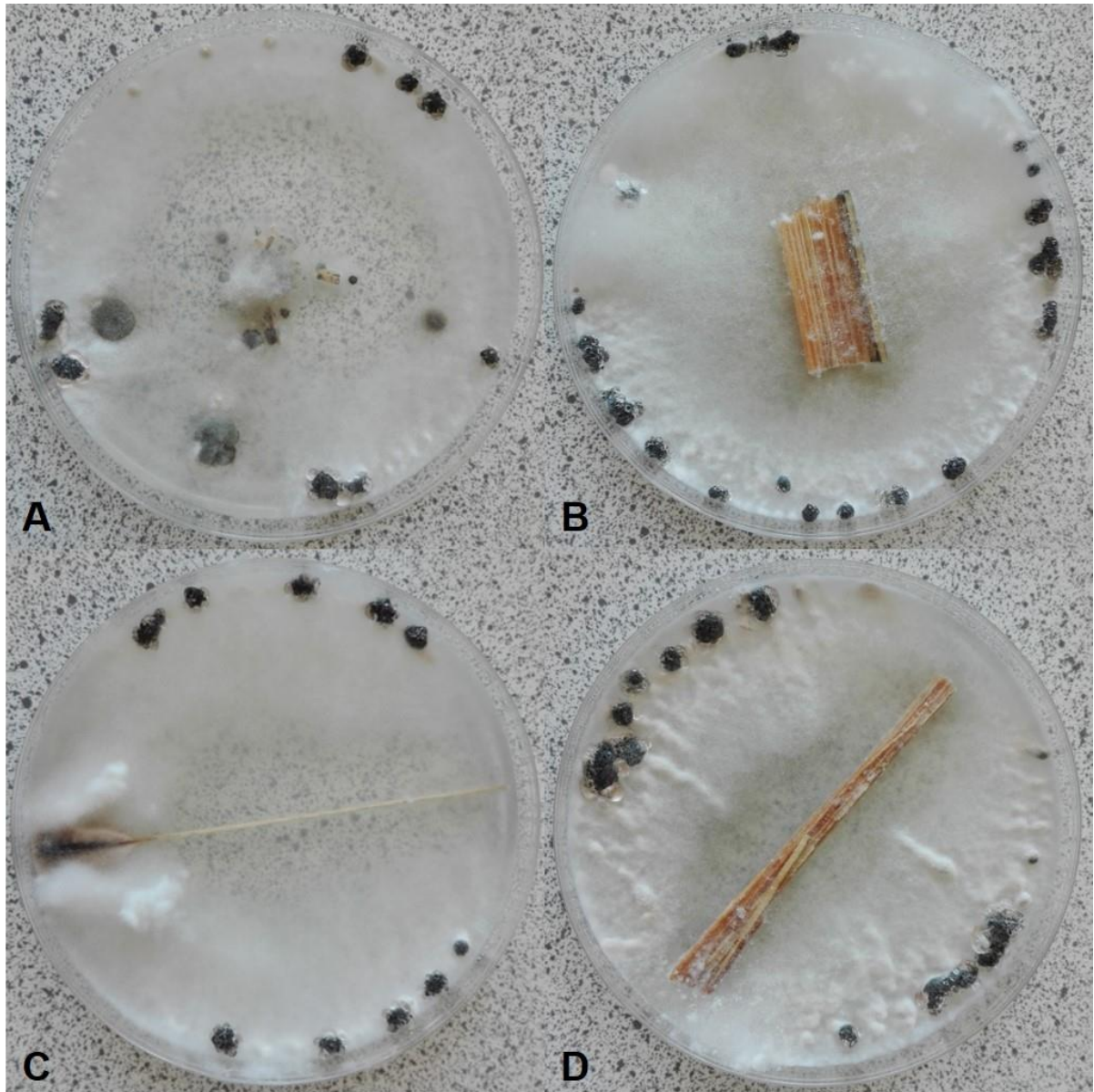


Figure 2.11: Growth of *S. sclerotiorum* from different textures of infected sunflower stubble after 12 months on PDA. A = milled into a fine powder using a coffee grinder; B = shredded into pieces of approximately 2.50 cm by 1 cm; C = shredded into pieces of approximately 8 cm by 3 mm and D = pieces left whole.

Chapter 3

Evaluation of inoculation techniques for *Sclerotinia* stem and head rot in soybean and sunflower under greenhouse and field conditions in Mpumalanga and Kwazulu-Natal

3.1. Abstract

Stem and head rot of soybean and sunflower, caused by *Sclerotinia sclerotiorum*, are responsible for economic losses of up to 100%. Resistance screening requires inoculation techniques (IT) that consistently mimic natural field reactions of the host to pathogen. The aim of this study was to optimise the viability and efficacy of inoculum sources, application methods and timing on disease incidence in both greenhouse and field trials. Split-split plot experimental designs were used with IT as the whole plot factor and number of applications and growth stage as sub-plot factors in soybean and sunflower trials respectively. The soybean field trial was further split into an early inoculation i.e. 50% flowering over all plots with a second inoculation 14 days later on the second plot, thereby providing a double inoculation. Sunflower field trials were further split to include three commercial cultivars. Techniques used were sclerotia planted adjacent to seedlings, liquid spray mycelium, milled grain mycelium and an ascospore suspension. A head punch technique was also added, whereby a hole is punched into a flowering head and a *S. sclerotiorum* colonised grain is inserted, was also applied to sunflowers. Treatments were applied to sunflower at bud, flower and head-ripening growth stage while on soybean, the liquid spray mycelium, milled grain mycelium and ascospore suspension were applied at flowering stage. IT and cultivar main effects were observed in sunflower field trials but no IT x cultivar interactions was recorded. IT x growth stage interactions were observed in sunflowers in both greenhouse and field trials. An ascospore suspension sprayed at flowering resulted in the highest level of *Sclerotinia* head rot in the greenhouse, while the liquid spray mycelium inoculated at bud stage yielded the highest *Sclerotinia* head rot in the field. A significant difference in IT was observed in both the soybean greenhouse and field trials. The liquid spray mycelium and sclerotia planted adjacent to seedlings yielded the highest incidence of *Sclerotinia* stem rot in the greenhouse while the milled grain mycelium produced the highest level of *Sclerotinia* stem rot in field trials. The additional, later application of inoculum yielded twice the amount of *Sclerotinia* stem rot compared to the earlier single application. A negative correlation was observed between soybean greenhouse and field trials, while a poor correlation was observed between sunflower greenhouse and field trials. Results highlighted the need for multiple considerations when screening for *S. sclerotiorum* resistance.

3.2. Introduction

S. sclerotiorum (Lib.) de Bary is a hemibiotroph, omnivorous and soil-borne fungal plant pathogen with a host range of more than 500 plant species that include soybeans (*Glycine max* L.) Merrill.) and sunflowers (*Helianthus annuus* L.) (Purdy, 1979; Saharan and Mehta, 2008). Sclerotinia stem and head rot in soybeans and sunflowers can cause up to 100% crop loss depending on the disease potential (i.e. inoculum and environmental conditions) and concomitant disease incidence. *S. sclerotiorum* is a devastating pathogen, especially since it has two types of germination, namely myceliogenic and carpogenic germination (Le Tourneau, 1979). The method of germination is dependent on the prevailing environmental conditions.

Myceliogenic germination occurs when sclerotia produce hyphae under temperatures ranging from 20 - 25°C in the presence of 80% Relative humidity (RH), pH of 5 and exogenous nutrients (Huang and Erickson, 2008). As nutrients become depleted, mycelium of *S. sclerotiorum* aggregates and reverts to sclerotia. Carpogenic germination results in apothecial stipes produced from pre-conditioned sclerotia, as suggested in literature (Kohn, 1979; Dillard *et al.*, 1995; Hao *et al.*, 2003; Ghasolia and Shivpuri, 2015; Pethybridge *et al.*, 2015). Conditioned sclerotia are those that have been exposed to long periods (2 - 8 weeks) of low temperatures (4 - 10°C), which typically occurs during the winter season. The apothecial stipes transform into mature apothecia, mushroom-like structures, in which asci are produced and which subsequently release ascospores into the air when there is a change in air pressure and RH, favouring field to field dispersal and infection.

The fungus can remain dormant under environmental conditions that are not favourable for germination and infection, in the form of mycelium in infected plant residues and sclerotia in both plant residues and soil. Only sclerotia found in the first 5 cm of soil are considered epidemiologically important, since the apothecial stipes produced from the sclerotia are triggered by light. A change in environmental conditions, management practices and the susceptibility of germplasm have led to an increase in the importance of Sclerotinia diseases worldwide. The greater the incidence of the disease, the lower the yield (Grau and Radke, 1984).

Several inoculation techniques have been developed and evaluated for the screening of soybean and sunflower for resistance to *Sclerotinia* rot (Kim *et al.*, 2000). Most of these techniques are mycelium-based as opposed to using ascospores (Kull *et al.*, 2003). While the primary inoculum of *S. sclerotiorum* is ascospores, the challenge of ascospore production, collection and preservation *in vitro* is the primary reason for mycelium-based techniques being favoured in screening for *Sclerotinia* resistance (Kull *et al.*, 2003; Rousseau *et al.*, 2004). The first and most commonly used technique used to screen for resistance is where cotyledons of 14-day old seedlings are inoculated with *S. sclerotiorum* mycelium (Kim *et al.*, 2000; Kull *et al.*, 2003). Some of the other techniques include (1) detached leaf assay where leaves are removed from a healthy plant and inoculated with mycelium disks cut from a *S. sclerotiorum* colony (Chun *et al.*, 1987); (2) spray mycelium method where Potato Dextrose Broth (PDB) is inoculated with *S. sclerotiorum* mycelium and incubated for a week; the mycelium suspension is homogenised and sprayed onto healthy plants at critical growth stages (Chen and Wang, 2005); (3) cut petiole inoculation where plants are mechanically wounded by the removal of petioles and then inoculated with a *S. sclerotiorum* mycelium agar plug contained in a plastic straw (Del Rio *et al.*, 2001); (4) milled grain mycelium where sorghum grains are inoculated with agar plugs of *S. sclerotiorum* and incubated for three weeks, air-dried once the mycelium has colonised the grains and milled mycelium is then distributed over the plants at critical growth stages (Nelson *et al.*, 1988) and (5) ascospore suspension where ascospores are collected from apothecia and suspended into sterile distilled water and sprayed onto the plant at the critical growth stages (Van Becelaere and Miller, 2004).

Various field and greenhouse resistance screening trials have been performed to evaluate germplasm using these inoculation techniques but results have been contradictory. Low to negative correlations have been found between resistance to *S. sclerotiorum* recorded in controlled/greenhouse and field assays. The response of plants to *S. sclerotiorum* under field conditions is assumed due to physiological resistance combined with escape mechanisms whereas under controlled conditions responses are assumed due only to physiological resistance (Boland and Hall, 1987; Nelson *et al.*, 1991; Kim *et al.*, 2000). Disease escape mechanisms are related to early flowering and early maturity and thus, avoidance of disease-favourable conditions, as well as growth habit and a canopy that's more open and upright (Miklas *et al.*, 2013).

Due to the high genotype x environment interactions, the value of Sclerotinia resistance screening methodologies under controlled environmental conditions are questioned (McLaren and Craven, 2008) and the use of only one set of environmental conditions may not be sufficient to correctly identify Sclerotinia resistance in crops. Since Sclerotinia diseases are highly dependent on environmental conditions, both for their effect on the pathogen as well as host predisposition, several environments/localities must be considered when evaluating crops against *S. sclerotiorum*.

Potential sources of variation as well as reproducibility of results, remains a problem when screening for resistance to Sclerotinia diseases (Steyn, 2015). It is important to identify an effective screening method that can simulate natural interactions, is reliable, accurate, repeatable and not labour intensive. The aim of this study was to compare and evaluate the viability and efficacy of inoculum sources, application methods and timing on disease incidence. These techniques were optimised in a greenhouse as well as a series of multiple-environment field trials.

3.3. Materials and Methods

3.3.1. Plant production

3.3.1.1 Soybeans

3.3.1.1.1 Greenhouse trial

One popular commercial soybean cultivar, PAN 1454, was planted into 5 l pots filled with steam sterilised soil consisting of 1:1 soil:peatmoss and placed in the greenhouse at the University of the Free State. Ten seeds were planted per pot and after emergence, plants were thinned to five plants per pot. The plants were maintained at a 25/20°C day/night regime with a 14/10-hours light/dark cycle. Plants were watered daily with tap water and fertilised once a week with a mixture of 5 ml Seagro B2051 Fish Emulsion (Efekto) in 1 l water. A randomised block experimental design was used with five replicates for each of four inoculation techniques. Plants were grown to flowering growth stage.

3.3.1.1.2. Field trial

Field plots at DMS Genetics in Delmas, Mpumalanga, were fertilised with a 200 kg/ha mixture of nitrogen, phosphorus and potassium at a 0:1:2 ratio. One commercially treated soybean cultivar, RA 626, was planted on 29 January 2017 at a plant density of 360 000 plants per hectare. The late planting was used in anticipation of late season cooler and wetter conditions which would favour disease incidence. Subsequent to planting trials were sprayed with 1 l/ha Palladium 960EC (chloro-acetamide, Villa Crop Protection), 25 g/ha Zeona (triazolopyrimidine sulfonanilide, Villa Crop Protection), 50 ml/ha Judo Secure (pyrethroid, Villa Crop Protection). After emergence, plants were sprayed with 2 l/ha Touchdown Total (glyphosate, Syngenta) and 50 ml/ha Judo Secure (pyrethroid, Villa Crop Protection). A 3-replicate randomised split-split plot experimental design was used. The whole plot factor consisted of four inoculation techniques (Figure 3.2). The sub-plot consisted of two inoculation times i.e. early inoculation (February) and late inoculation (March). Sub-sub plots consisted of two inoculations where each inoculation time was divided into a single application of inoculum, followed by a second application 14 days later (Table 3.1).

3.3.1.2. Sunflowers

3.3.1.2.1 Greenhouse trial

One popular commercially treated sunflower cultivar, PAN 7049, was planted into 5 l pots filled with steam sterilised soil consisting of a 1:1 of soil:peatmoss and placed in the greenhouse at the University of the Free State. Ten seeds per pot were planted and after emergence, plants were thinned to 5 plants per pot. The plants were kept at 25/20°C day/night regime with a 14/10-hour light/dark cycle. Pots were watered daily with tap water and fertilised once a week with a mixture of 5 ml Seagro B2051 Fish Emulsion (Efekto) in 1 l water. The greenhouse trial layout was randomised block experimental design with five replicates. Treatments included three inoculation techniques, as described below, applied at three growth stages i.e. bud, flowering and head-ripening stage. Each inoculation technique x growth application was regarded as a single treatment. This was due to the head punch and sclerotia techniques (see below) not including growth stages and therefore resulting in an unbalanced

experimental design. Plants were maintained until the respective growth stages were reached.

3.3.1.2.2. Field trial

The field trial was conducted at PANNAR Seed Co. in Greytown, KwaZulu-Natal. The plots were fertilised with a 250 kg/ha mixture of nitrogen, phosphorus and potassium in 2:3:4 (28%) ratio and 0.50% zinc as well as 300 kg of urea (46%). Three sunflower cultivars, PAN 7089 HO, PAN 7057 and PAN 7049, were planted in two row plots on two planting dates, 13 December 2016 and 5 January 2017 at a plant density of 30 000 plants per hectare. At planting, 70 ml/ha of Decis Forte (pyrethroid, Bayer) was sprayed over the soil. This was followed by 1,3 l/ha of Dual Gold (chloro-acetanilide, Syngenta). The cultivars were arranged in a 2-replicate split-plot experimental design with 10 inoculation techniques (as applied in the greenhouse trial described above) as the whole-plot factors and the three cultivars as sub-plot factors. Plants were maintained until the respective growth stages had been reached.

3.3.2. Inoculum production and application

3.3.2.1. Sclerotia

Sclerotia collected from infected sunflower fields in Candy, Free State in 2016, were used in both the soybean and sunflower greenhouse trials as well as sunflower field trials in Greytown. Sclerotia collected from soybean fields in Delmas, Mpumalanga in 2017 were used in soybean field trials. In both greenhouse and field trials, sclerotia were planted adjacent to seedlings at a distance of approximately 2 cm. Accepting the fact that the South African *S. sclerotiorum* population is clonal (Steyn, 2015), only one isolate was used per trial.

3.3.2.2. Milled grain mycelium (Nelson *et al.*, 1988)

CONSOL jars (1 l) were filled with 150 g sorghum grains and soaked in 500 ml water overnight. The water was drained from the jars and 85 ml distilled water was added. The jars were autoclaved for 20 min at 121°C and upon cooling, re-autoclaved. The

autoclaved jars were cooled and inoculated with ten, 5 mm diameter *S. sclerotiorum* mycelium plugs from seven day old *S. sclerotiorum* colonies on Potato Dextrose Agar (PDA). The jars were incubated at 25°C until the grains were fully colonised. The colonised grains were air dried for five days and milled using a coffee grinder (Mellerware). The plants were sprayed with water to run-off to aid adhesion. The milled grains (2 g per m) were distributed over the wet soybean plants at the onset of flowering and at bud, flowering and head-ripening stage for sunflower. After inoculation, plants in the greenhouse were covered with a clear plastic bag for seven days to increase RH levels favourable for disease development while inoculum and infection in the field were exposed to prevailing weather conditions.

3.3.2.3. Liquid spray mycelium (modified from Chen and Wang, 2005)

Potato Dextrose Broth (PDB) (Merck) was prepared in 4 l Erlenmeyer flasks and autoclaved for 20 min at 121°C. The broth was cooled and inoculated with ten, 5 mm diameter mycelium plugs from seven day old *S. sclerotiorum* colonies on PDA. The Erlenmeyer flasks containing the inoculated broth were incubated at 25°C for seven days. Once the fungus had colonised the medium and formed a mycelium mat on the surface of the liquid, the inoculum was homogenised using a Heidolph SilentCrusher M and stored at 4°C until required. The mycelium suspension was placed in a hand atomiser. The suspension was sprayed onto soybeans at the onset of flowering and at bud, flowering and ripening head growth stages for sunflower. After inoculation, plants in the greenhouse were covered with a clear plastic bag for seven days to increase RH levels favourable for disease development, while field trials remained exposed to prevailing weather conditions.

3.3.2.4. Ascospore suspension (Van Becelaere and Miller, 2004)

Sclerotia collected from sunflower fields in Candy, Free State were surface sterilised with 3% sodium hypochlorite for 4 min. The sodium hypochlorite was discarded and 76% ethanol was added to the beaker containing sclerotia and sterilisation proceeded for a further 2 min. The 76% ethanol was discarded and followed by two washes in sterilised distilled H₂O. Sclerotia were placed on 1.5% water agar in Petri dishes and maintained at 16°C until stipes and subsequently apothecia had developed.

Ascospores were collected by slightly lifting the Petri dish lid, but still having the lid in close proximity to the apothecia, thereby providing a pressure change. This pressure change stimulates the asci within apothecia to actively discharge ascospores onto the inside of the Petri dish lid which contained condensation droplets and promoted ascospores adhering to the inner lid. The ascospores were washed from the lid using sterilised distilled H₂O. The ascospores were counted with a haemocytometer and adjusted to 1x10⁴ spores per ml. The ascospore suspension was placed in a hand atomiser and sprayed over soybean plants at 50% flowering and on sunflowers at bud, flowering and head-ripening growth stages. After inoculation, plants in the greenhouse were covered with a clear plastic bag for seven days to increase RH levels favourable for disease development, while field trials remained exposed to prevailing weather conditions.

3.3.2.5. Head punch – whole grain mycelium

This method of inoculation included the punching of a small hole at the back of the sunflower head at full flowering. Use was made of a 3 mm diameter corkborer and the hole depth was approximately 1 cm. A *S. sclerotiorum* colonised grain, as prepared for the milled grain mycelium method above (3.3.2.3.) was inserted into the hole. After inoculation, plants in the greenhouse were covered with a clear plastic bag for seven days to increase RH levels favourable for disease development, while those in field trials were exposed to prevailing weather conditions.

3.3.3. Disease assessment and statistical analysis

One soybean cultivar and sunflower cultivar was analysed for their response to Sclerotinia inoculation technique in the greenhouse while in the field trial one soybean cultivar was rated and three sunflower cultivars. Disease severity ratings were conducted on both soybeans and sunflower greenhouse trials, 10 days after inoculation. Field trial evaluations were conducted 30 days after inoculation in soybean field trials and 14 days after inoculation in sunflowers. The disease was quantified in soybean trials as the percentage of wilted plants per row showing the typical *S. sclerotiorum* mycelial growth while Sclerotinia head rot in sunflower was quantified based on the presence of *S. sclerotiorum* mycelium on the heads as well as shredded,

diseased tissue. Data were statistically analysed using Analysis of Variance (ANOVA) in NCSS (Hintze, 2007) after being assessed using Shapiro-Wilk test for normality. Natural log transformation (Ln-transformation) was applied where a deviation from normality was indicated. Sunflower and soybean reactions to inoculation techniques in greenhouse and field trials were ranked using Spearman rank correlation analysis.

3.4. Results

3.4.1. Soybeans

ANOVA of greenhouse soybean data (Table 3.2) indicated a significant difference ($P < 0.05$) between inoculation techniques (Figure 3.1). Sclerotia planted adjacent to soybean seedlings as well as liquid spray mycelium, produced high levels of Sclerotinia stem rot, with incidences of 48.1% and 46.0%, respectively. The ascospore suspension resulted in an incidence of 15.3%.

ANOVA, subsequent to Ln-transformation to account for a deviation from normality according to the Shapiro-Wilk test for normality, indicated significant differences ($P < 0.05$) between inoculation techniques in the soybean field trial (Table 3.3) and the time of application in the field trial (Figure 3.2). No inoculation technique x time of inoculation interaction was recorded. No significant differences were recorded between the milled grain mycelium, ascospore suspension and liquid spray mycelium inoculation methods which yielded mean disease incidences of 9.0%, 10.5% and 10.2%, respectively. Sclerotia planted adjacent to seedlings yielded the lowest level (1%) of Sclerotinia stem rot. A later application of *S. sclerotiorum* inoculum yielded almost twice as much Sclerotinia stem rot as a single earlier application (Figure 3.2). A negative, albeit non-significant correlation ($r = -0.20$), was recorded between greenhouse and field trials.

3.4.2. Sunflower

ANOVA of greenhouse sunflower Sclerotinia head rot incidences (Table 3.4) indicated significant differences ($P < 0.05$) between inoculation techniques (Figure 3.3). Ascospore suspension sprayed at flowering growth stage as well as punching the back

of ripening sunflower heads and inserting a single colonised grain, resulted in high incidences of *Sclerotinia* head rot of 95.8% and 98.8%, respectively. Ascospore suspension sprayed at bud and head-ripening growth stage and inoculation with milled grain mycelium at ripening stage produced 0% and 5.0% disease incidence, respectively.

In the field trial, ANOVA indicated inoculation technique main effects on *Sclerotinia* head rot incidence, while no inoculation technique x cultivar interaction was recorded. As a result cultivar data were pooled (Table 3.5). Inoculation techniques that yielded the highest *Sclerotinia* head rot incidence were milled grain mycelium and liquid spray mycelium at bud (94.7% and 98.3%, respectively) and flower (82.5% and 92.3%, respectively) growth stages. The ascospore suspension at all the growth stages yielded lower levels of disease (44.2%, 53.3% and 40.4% at bud, flower and head-ripening stages respectively). Similarly, sclerotia planted adjacent to seeds produced lower disease incidences (40.8%) (Figure 3.4). ANOVA of field trial data indicated that when data from liquid spray mycelium, milled mycelium and ascospore suspension was included in a factorial analysis (i.e. fully balanced data sets), no interaction between inoculation technique and sunflower growth stage was recorded (data not shown). No significant correlation ($r = 0.14$) was recorded between greenhouse and field trials.

3.5. Discussion

It is essential when selecting for disease resistance in crops, to develop inoculation techniques that are cost and time effective, repeatable, require minimal labour and reflects the natural interaction between the host, the pathogen and the environment. Several artificial inoculation techniques have been developed and applied to evaluate the reaction of host crops to *S. sclerotiorum* in an attempt to select genotypes with physiological resistance to the pathogen. Most of the inoculation methods used such as petiole inoculation method (Del Rio *et al.*, 2001), straw inoculation method (Auclair *et al.*, 2004) and detached leaf assay (Chun *et al.*, 1987) require plant injury before inoculation. This might lead to the by-pass of some escape mechanisms that may form part of a process of reduced disease incidence (Danielson *et al.*, 2004). It is thus preferable in the current study that all the soybean and sunflower structural barriers

remain included in Sclerotinia disease assessment and, with the exception of head punch method, which served as an extreme, invasive method, emphasis was on non-invasive inoculation methods.

Despite most inoculation techniques evaluated in this study not requiring any form of plant damage prior to inoculation, the results of the greenhouse trials indicated that the sunflowers inoculated with ascospores at flowering stage produced very similar incidences of Sclerotinia head rot to the head punch method, which requires plant injury before inoculation. Botha *et al.* (2009), used four South African soybean cultivars and six mycelium-based inoculation techniques under controlled environmental conditions, reported similar reactions to the pathogen. The methods used were spray mycelium, drop mycelium, cut stem, cotyledon inoculation, straw inoculation and petiole inoculation. The results indicated that some of the inoculation techniques which require wounding such as the cotyledon, petiole and straw inoculation methods produced almost similar levels of Sclerotinia wilt as those recorded with the non-wounding, drop mycelium method. Natti (1971) indicated that direct infection of stems, branches, leaves and pods of bean plants occurs with subsequent enzymatic degradation of cells, while the respective tissues are apparently being equally susceptible. Cellular disruption and permeability due to oxalic acid production also predisposes cells to colonisation. Results, therefore indicate that *S. sclerotiorum* does not necessarily require host wounding prior to infection and that Sclerotinia head and stem rot in sunflowers and soybeans can be accurately measured using inoculation techniques that do not damage plant tissue before the application of Sclerotinia inoculum. Non-invasive methods, are less time-consuming, laborious and costly and enable larger populations of germplasm to be evaluated.

Inoculation of sunflower heads at flowering with ascospores, produced the highest incidence of Sclerotinia head rot in the greenhouse. However, in the sunflower field trials, flowers inoculated with ascospores were less severely infected. This may be due to the former being covered with plastic bags to maintain high RH, while sunflowers in field trials were exposed to alternating weather conditions. The current observation suggests that ascospores may be sensitive to weather fluctuations. This is in contrast to soybeans where similar responses to the ascospore suspension and liquid spray mycelium method were recorded in the greenhouse. Similarly, in the field

responses to these techniques, in addition to the milled grain mycelium, as single and double application produced similar levels of Sclerotinia stem rot. Harikrishnan and Del Rio (2006) reported that in growth chamber experiments, both ascospores and mycelium applied to dry bean flowers were equally effective in producing 100% Sclerotinia wilt incidence under high RH (90%) conditions, five days after inoculation. However under low RH (25%) the mycelium were more efficient than the ascospores, producing 20.0% and 10.0% Sclerotinia incidence five days after inoculation, respectively. The contrast between observations on sunflower and soybean in the current field trials may be due to the germination and infection by ascospores requiring free water which may be maintained within the closed soybean canopy for extended periods subsequent to night time dew, while the exposed sunflower head dries more rapidly, thus making infection more dependent on mycelium that has the ability to tolerate lower RH than ascospores. Thus, despite ascospores being successfully used to evaluate soybean and sunflower reaction to Sclerotinia, the liquid spray mycelium and milled grain mycelium inoculation methods are the preferred techniques as inoculum production is considerably less time-consuming and labour intensive compared to the production of ascospores and remain viable for longer periods under variable weather conditions.

The poor correlations and contradictory reports between field and greenhouse responses of sunflower and soybean remains a concern. Vuong *et al.* (2004) conducted greenhouse and field trials to evaluate the resistance of sunflower, soybean and dry bean cultivars to *S. sclerotiorum* using the cut stem inoculation method. This method requires a *S. sclerotiorum* mycelium agar plug being placed on artificially wounded sites on stems, these usually being severance between the second and third node. A positive correlation was recorded between greenhouse and field trials for soybeans, but a negative correlation was observed between the sunflower greenhouse and field trials. Kim *et al.* (1999), Yang *et al.* (1999) and Kim and Diers (2000) have also reported the high genotype x environment interactions resulting in poor correlations between evaluation trials. The importance of developing techniques that consider changes in the reaction of the host to changing environmental conditions is highlighted by the poor correlations between the responses of germplasm to *S. sclerotiorum* in greenhouse and field trials. This too, is an indication of the importance of using multiple environments when screening for Sclerotinia resistance especially

under natural field conditions, which take into account the high genotype x environment interactions. For this reason, performing resistance screening in the greenhouse only, will limit the value of the screening methodologies used (McLaren and Craven, 2008).

3.6. References

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Table 3.1: Summary of application time and dosage applied to soybeans in field trials in Delmas, Mpumalanga.

Early or Late	Date	Number of Application
Early inoculation	19 February 2018	Single
Early inoculation	27 February 2018	Double
Late inoculation	15 March 2018	Single
Late inoculation	3 April 2018	Double

Table 3.2: Analysis of Variance indicating the effect of *S. sclerotiorum* inoculation techniques on soybean in greenhouse trials.

Analysis of Variance Table					
Source of variation Term	Df	Sum of Squares	Mean Square	F-Ratio	Probability Level
Inoculation techniques	3	3423.750	1141.250	3.740	0.0400*
Rep	4	1112.500	278.130	0.910	0.4900
Error	12	3657.500	304.790		
Total (Adjusted)	19	8193.750			
Total	20				

Table 3.3: Analysis of variance indicating the effect of *S. sclerotiorum* inoculum techniques on soybean in a field trial at Delmas.

Analysis of variance					
Source of variation Term	Df	Sum of Squares	Mean Square	F- Ratio	Probability level
Rep stratum	2	9.271	4.636	5.230	
Inoculation techniques	7	28.966	4.138	4.670	0.0070*
Residual	14	12.397	0.886	3.780	
Time of application	1	2.969	2.969	12.690	0.0030*
Inoculation technique x Time of application	7	1.060	0.152	0.650	0.7120
Residual	16	3.744	0.234		
Total	47	58.407			

Table 3.4: Analysis of variance indicating the effect of inoculation techniques and growth stage on Sclerotinia head rot of sunflower in the greenhouse.

Analysis of Variance Table					
Source Term	Df	Sum of squares	Mean Square	F-Ratio	Probability level
Inoculation techniques	10	71730.580	7173.058	44.270	0.0000*
Rep	4	4043.527	1010.882	6.240	0.0005*
Error	40	6480.873	162.022		
Total (Adjusted)	54	82254.980			
Total	55				

Table 3.5: Analysis of variance evaluating the inoculation techniques and growth stage in Greytown sunflower field trials.

Analysis of variance					
Source of variation	Df	Sum of squares	Mean square	F- Ratio	Probability level
Rep stratum	1	5.519	5.519	7.400	
Inoculation technique	10	101.203	10.120	13.560	0.001*
Residual	10	7.461	0.746	0.180	
Cultivar	2	25.339	12.669	3.000	0.070*
Inoculation technique x Cultivar	20	49.925	2.496	0.590	0.878
Residual	22	92.760	4.216	7.300	
Total	131	320.305			

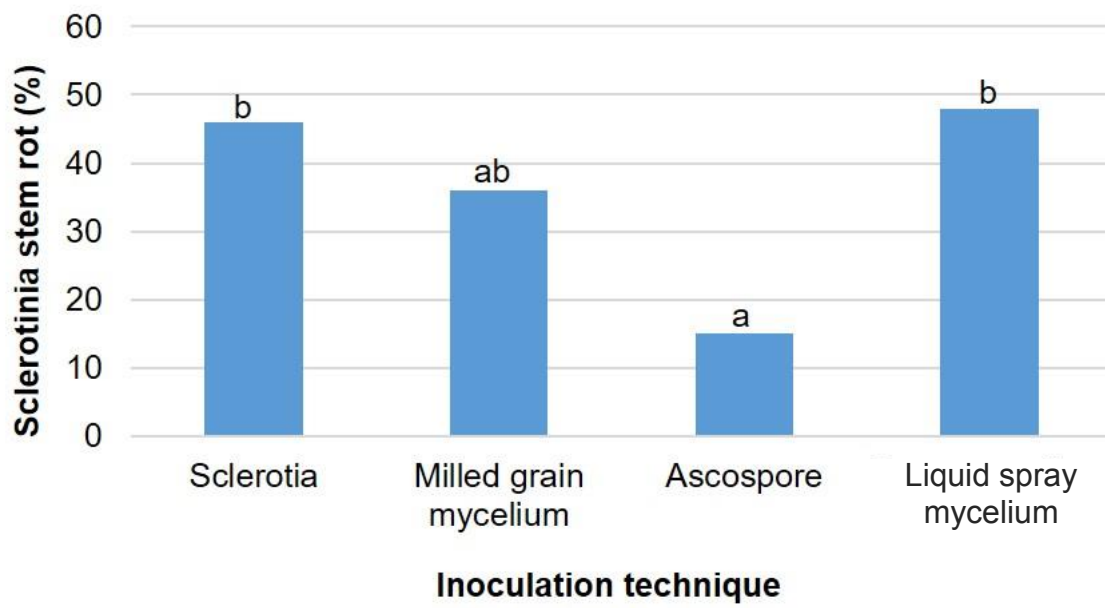


Figure 3.1: Sclerotinia stem rot incidence induced by different *S. sclerotiorum* inoculation techniques on soybean in greenhouse trials.

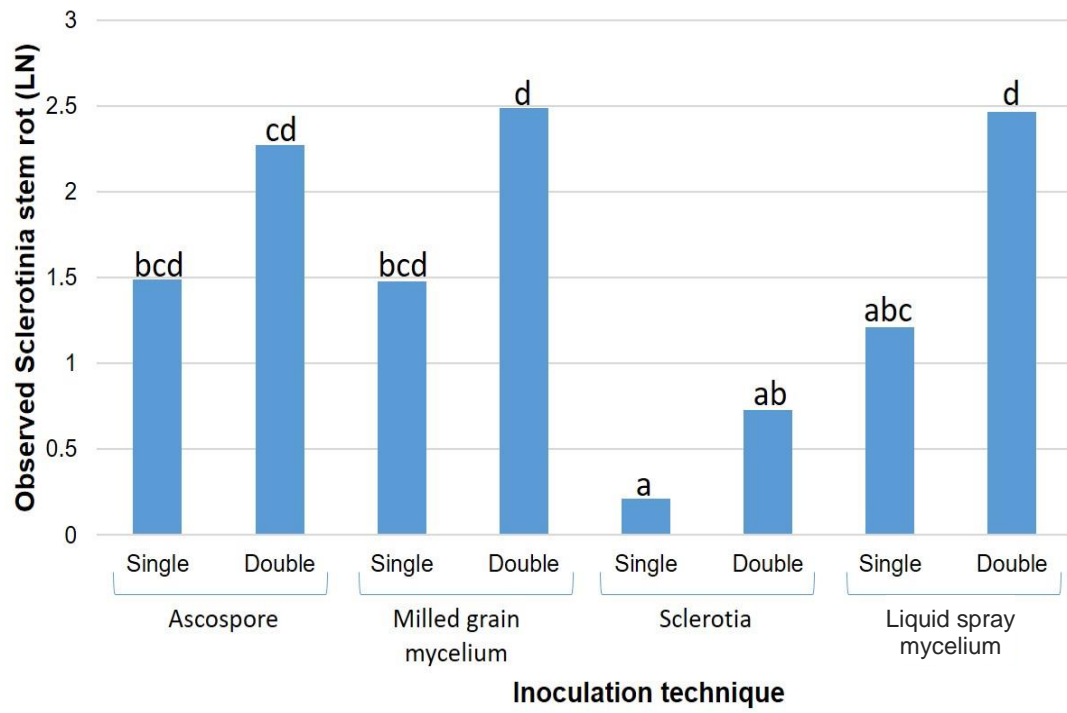


Figure 3.2: Incidence of Sclerotinia stem rot (Ln-transformed) induced by *S. sclerotiorum* inoculation techniques on soybean in a field trial at Delmas.

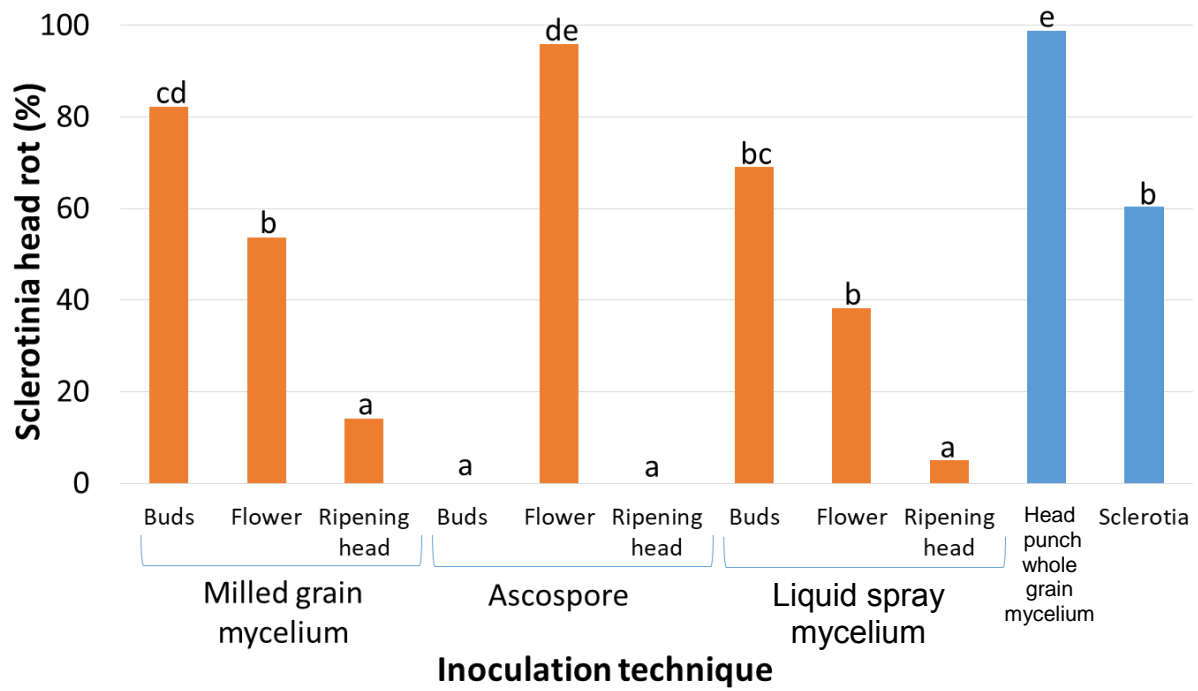


Figure 3.3: Sclerotinia head rot incidence induced by *S. sclerotiorum* inoculation techniques at different sunflower growth stages in the greenhouse.

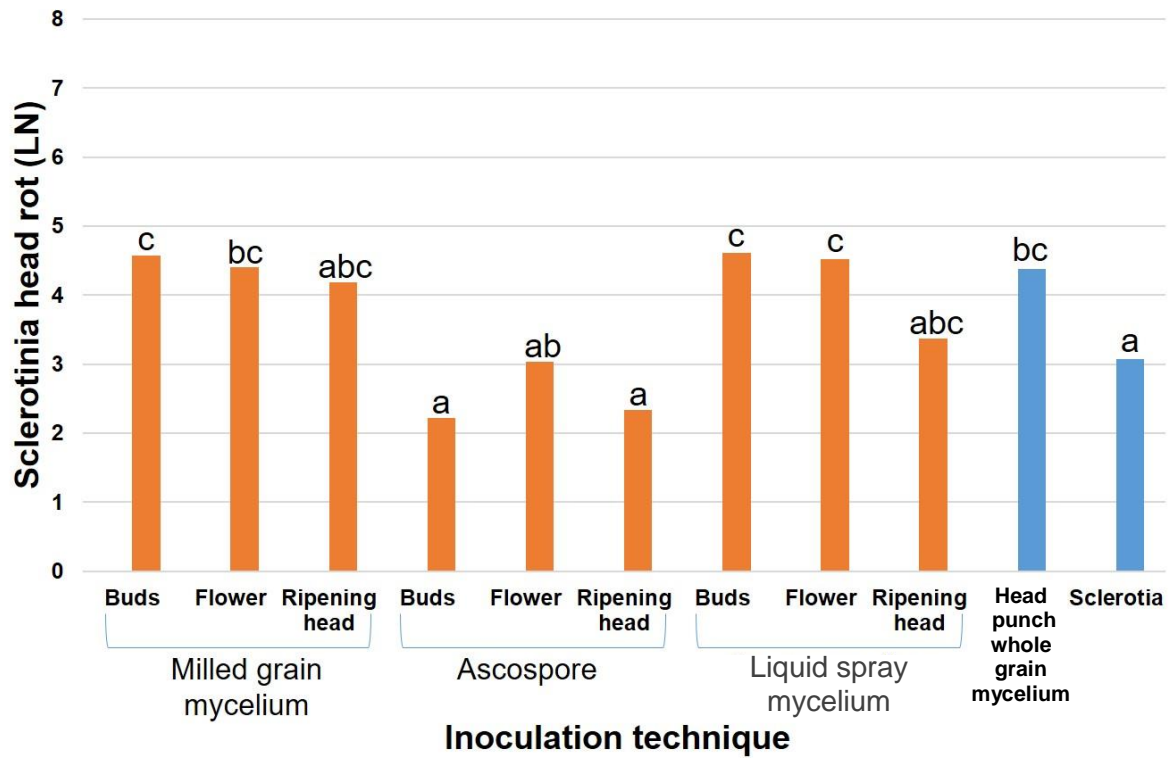


Figure 3.4: Sclerotinia head rot incidence induced by *S. sclerotiorum* inoculation techniques at different sunflower growth stages in Greytown field trials.

Chapter 4

Response of soybean and sunflower cultivars to *Sclerotinia sclerotiorum* under field conditions in Mpumalanga and Kwazulu-Natal

4.1. Abstract

The incidence and severity of *Sclerotinia* diseases, caused by *Sclerotinia sclerotiorum*, are highly dependent on environmental conditions. The high genotype x environment interaction reported in many studies emphasises the importance of screening for tolerance to *Sclerotinia* under diverse natural field conditions, if stable resistance is to be identified. Seven sunflower cultivars were evaluated in field trials in Greytown, KwaZulu-Natal during 2016/17 and 19 soybean cultivars in Delmas, Mpumalanga, during 2017/18. Plantings were conducted sequentially from November to January to create a range of weather conditions during each season of evaluation. Sunflowers were inoculated with both milled grain and liquid spray mycelium at bud growth stage and the percentage head rot incidence was assessed. Soybean blocks were split and inoculated with milled mycelium colonised grain and a liquid spray mycelium at onset of flowering. Soybean cultivar responses to inoculum type were quantified as percentage stem rot incidence. Milled grain mycelium yielded a mean of 56.0% disease incidence in soybean while the liquid spray mycelium yielded a mean of 10.0% *Sclerotinia* stem rot. Regression analysis were used to determine response type and the relationship between observed stem or head rot incidence within a cultivar and disease potential. The latter was quantified as the mean disease severity within a planting over all cultivars. The regression model $Y=aX^b$ was used where Y is the observed incidence within a cultivar and X is the disease potential. Three cultivar response types were identified based on the model b parameter i.e. cultivars tolerant to increasing disease potential, cultivar intolerant to increasing disease potential and cultivars having a linear relationship with changing disease potential. The *Sclerotinia* potential required to initiate disease onset and the rate of disease increase subsequent to onset were calculated by re-arrangement of model parameters. The soybean cultivar, PAN 1454, responded less rapidly to changing stem rot potentials, with an onset potential of 19.3% and a subsequent response rate of 0.4 per potential unit (p.p.u.) increase, making it more “escape resistant” to *S. sclerotiorum* while P 64 T 39 was less tolerant of increasing *S. sclerotiorum* potential and reacted more quickly to changing stem rot potential, with an onset potential of 1.0% and a response rate of 3.4 p.p.u. The sunflower cultivar, P 65 LC 54, responded less rapidly to changing stem rot potentials, with an onset potential of 18.9% and a response rate of 0.6 p.p.u. respectively while P 65 LL 02 had an onset potential of 2.1% and a response rate of 1.9 p.p.u. Leaves of soybean and sunflower cultivars were collected at different growth stages. Lignin, total protein, chitinase and β -1, 3-glucanase levels were quantified to determine a correlation with *Sclerotinia* disease incidence. No correlation was recorded. Results highlight the need for multiple considerations when screening for *S. sclerotiorum* resistance.

4.2. Introduction

Soybean and sunflower are a vital source of income globally. As a result, diseases can disrupt the economic welfare of local communities and of many countries (The South African Grain Laboratory, 2018). In several countries, the production of soybean and sunflower has increased tremendously, which indirectly suggests that the impact of diseases affecting these crops has also increased (Barber, 2017). Managing plant diseases will not only improve yield but also plant health and quality.

S. sclerotiorum is an important fungal plant pathogen of soybean and sunflower, as well as numerous other crop plants. The pathogen is responsible for causing Sclerotinia white rot (Purdy, 1979; Saharan and Mehta, 2008). The ability of the pathogen to produce significant yield losses makes Sclerotinia diseases some of the most important diseases in economically important crops (Wrather *et al.*, 1997). Reports from the United States Agricultural Research Services (ARS) National Sclerotinia Research Initiative have indicated that the annual economic losses in sunflowers and soybeans due to *S. sclerotiorum* are in excess of \$US 100 million and \$US 300 million, respectively (National Sclerotinia Initiative, 2016).

Due to an annual increase in losses caused by Sclerotinia diseases, appropriate management approaches to control the pathogen have grown in importance. Managing Sclerotinia diseases remains a challenging task especially due to its wide host range and its durable survival structure, the sclerotium, which has the ability to remain viable in the soil for up to eight years (Grogan, 1979; Gulya *et al.*, 1997). The identification of soybean and sunflower cultivars with resistance to *S. sclerotiorum* would provide a cost-effective management strategy and reduce the dependence on fungicides to control Sclerotinia diseases. Resistant genotypes are considered the easiest, safest, most effective and least expensive means of managing plant diseases in economically important crops (Agrios, 2005). No soybean and sunflower cultivars have yet been identified with resistance to *S. sclerotiorum* which is sufficient to be of economic value.

Several inoculation methodologies have been evaluated in both greenhouse and field tests to assess cultivar reactions to *S. sclerotiorum*. Some of these techniques are (a)

inoculation of cotyledons of 14-day old seedlings with mycelium disks cut from a *S. sclerotiorum* colony (Kim *et al.*, 2000; Kull *et al.*, 2003); (b) detached leaf assay where leaves are removed from a healthy plant and inoculated with mycelium disks cut from *S. sclerotiorum* colony (Chun *et al.*, 1987); (c) spray mycelium method where Potato Dextrose Broth (PDB) is inoculated with *S. sclerotiorum* mycelium, incubated for a week, homogenised and sprayed onto healthy plants (Chen and Wang, 2005); (d) cut petiole inoculation where plants are mechanically wounded by removal of petioles and inoculated with a *S. sclerotiorum* mycelium agar plug, contained in a plastic straw (Del Rio *et al.*, 2001); (e) milled grain mycelium where sorghum grains are inoculated with agar plugs of *S. sclerotiorum*, incubated for three weeks, dried and milled and distributed over the plants at critical growth stage (Nelson *et al.*, 1988) and (f) ascospore suspension where ascospores are collected from apothecia, suspended in sterile distilled water and sprayed on the plant at the critical growth stage (Van Becelaere and Miller, 2004). Results from most *Sclerotinia* resistance screening studies have provided variable responses in genotypes to the pathogen.

Despite the numerous inoculation techniques developed and used to screen germplasm for *Sclerotinia* stem rot resistance under controlled conditions, no method has been identified that reproduces reactions that constantly correlate with results recorded in the field (Kim and Diers, 2000; Kim *et al.*, 2000; Hoffman *et al.*, 2002; Kull *et al.*, 2003; Vuong *et al.*, 2004). This may be due to the high disease x environment interactions and different responses of host cultivars to the *S. sclerotiorum* under fluctuating conditions (Kim *et al.*, 1999; McLaren and Craven, 2008).

Botha *et al.* (2008) evaluated the reaction of four soybean cultivars to six mycelium-based inoculation methodologies in a greenhouse. The methods used were spray mycelium, drop mycelium, cut stem, cotyledon inoculation, straw inoculation and petiole inoculation. The spray mycelium yielded the highest level of *Sclerotinia* stem rot although a significant technique x cultivar interaction was found in addition to the cultivar and technique main effects. It was suggested that these interactions may be due to the difference in defence mechanisms in cultivars and the effect of invasive vs. non-invasive inoculation techniques. The spray mycelium methods require no wounding of plant tissue, which means that the structural barriers of the plants remain intact. A field study in Iran into the reaction of sunflower cultivars to inoculation, with a

S. sclerotiorum mycelium plug, an oxalic acid solution and *S. sclerotiorum* infested wheat seeds found no correlation between inoculation techniques and the mycelium plug method produced significantly more disease than the other two methods tested (Ebrahimi *et al.*, 2013). The authors also found significant difference between cultivars, but more importantly that cultivars reacted differently to inoculum sources.

Vuong *et al.* (2004) evaluated the resistance of sunflower, soybean and dry bean cultivars to *S. sclerotiorum* using the cut stem inoculation method. In this method, a *S. sclerotiorum* mycelium agar plug is placed on artificially wounded sites on cut stems, usually between the second and third node. Significant differences were found among soybean and dry bean cultivars in both greenhouse and field trials as well as a positive correlation between the field and greenhouse trials. A significant difference was found among sunflower hybrids, however, a negative correlation was observed between the field and greenhouse incidences. The negative correlations between field and controlled environment response have been attributed to physiological factors being the primary driving variables under controlled environmental conditions, whereas under natural field conditions, physiological resistance as well as escape resistance are being measured (Nelson *et al.*, 1991; Kim *et al.*, 2000). These disease escape mechanisms are associated with early flowering, meaning earlier maturity and avoidance of disease favourable conditions and a canopy that's more open and upright (Miklas *et al.*, 2013). Due to the high genotype x environment interactions, the value of resistance screening methodologies under controlled environmental conditions is questioned (McLaren and Craven, 2008) and the use of only one environment may not be sufficient to correctly identify Sclerotinia resistance in cultivars. They suggested that since Sclerotinia diseases are highly dependent on environmental conditions, several environments/localities must be considered when evaluating cultivar response to *S. sclerotiorum*.

Although significant differences were found between soybean and sunflower cultivars using the cut stem method, this technique has a number of negative features. This method as well as several other methods, such as petiole inoculation method (Del Rio *et al.*, 2001), straw inoculation method (Auclair *et al.*, 2004) and detached leaf assay (Chun *et al.*, 1987) requires plant damage prior to inoculation, leading to a by-pass of potential escape mechanisms that could reduce disease incidence. These methods

are also labour intensive in field trials where a large number of cultivars need to be screened (Danielson *et al.*, 2004).

The present study was aimed at evaluating and identifying soybean and sunflower cultivars with tolerance or partial resistance to *S. sclerotiorum* artificially inoculated at different disease potentials under field conditions. The concentration of total protein, chitinase and β -1, 3-glucanase at various growth stages in both soybean and sunflower cultivars were determined as well as the levels of lignin in the stems of soybean cultivars. These were aimed at determining possible physiological markers associated with host response to the pathogen.

4.3. Materials and Methods

4.3.1. Plant production

4.3.1.1. Soybean

The plots at DMS Genetics in Delmas, Mpumalanga, were fertilised with a 200 kg/ha mixture of nitrogen, phosphorus and potassium in a 0:1:2 ratio. Twenty-two soybean cultivars (Table 4.1) were planted over four planting dates from late October to late December. Plantings were replicated on 13 October 2017, 7 November 2017, 1 December 2017 and 18 December 2017. Cultivar plots consisted of two rows, 10 m in length with a row spacing of 76 cm and a plant density equivalent to 360 000 plants per hectare. Subsequent to planting trials were sprayed with 1 l/ha Palladium 960EC (chloro-acetamide, Villa Crop Protection), 25 g/ha Zeona (triazolopyrimidine sulfonanilide, Villa Crop Protection), 50 ml/ha Judo Secure (pyrethroid, Villa Crop Protection). After plant emergence, plants were sprayed with 2 l/ha Touchdown Total (glyphosate, Syngenta) and 50 ml/ha Judo Secure (pyrethroid, Villa Crop Protection).

4.3.1.2. Sunflower

The plots at PANNAR in Greytown, KwaZulu-Natal, were fertilised with a 250 kg/ha mixture of nitrogen, phosphorus and potassium in 2:3:4 ratio and 0.50% zinc as well as 300 kg Urea (46%). Seven sunflower cultivars (Table 4.2) were planted over three

dates; 13 December 2016, 28 December 2016 and 5 January 2017. Cultivar plots consisted of single rows, 10 m in length with a row spacing of 90 cm and a plant density equivalent to 30 000 plants per hectare. At planting, 70 ml/ha Decis Forte (pyrethroid, Bayer) was sprayed over the soil. Post-planting 1,3 l/ha Dual Gold (chloro-acetanilide, Syngenta) was sprayed over the soil.

4.3.2. Inoculum production and application

4.3.2.1. Isolation from sclerotia

Sclerotia were collected from infected sunflower fields at Candy, Free State in 2016. Sclerotia were removed from sunflower stubble, washed in water and surface sterilised in 3% sodium hydrochloride for 4 min. The sodium hypochlorite was discarded and 76% ethanol was added into the beaker containing sclerotia and which were further sterilised for 2 min. The 76% ethanol was discarded and followed by two washes in sterilised distilled H₂O. Sclerotia were placed on 90 mm Potato Dextrose Agar (PDA) plates, containing 1.5 ml Streptomycin per 1 l PDA. These *Sclerotinia* cultures were incubated for five days at 25°C under natural 12-hour light and dark cycles. Accepting the fact that the South African *S. sclerotiorum* population is clonal (Steyn, 2015), only one isolate was used per trial.

4.3.2.2. Milled grain mycelium (Nelson *et al.*, 1988)

CONSOL jars (1 l) were filled with 150 g sorghum grains and soaked in 500 ml water overnight. The water was drained from the jars and 85 ml distilled water was added. The jars were autoclaved twice for 20 min at 121°C. The autoclaved jars were cooled and inoculated with ten, 5 mm diameter *S. sclerotiorum* mycelium plugs from seven-day old *S. sclerotiorum* colonies on PDA that had been incubated for five days at 25°C under natural 12-hour light and dark cycles. The jars were incubated at 25°C until the grains were fully colonised with mycelium. The colonised grains were air dried for five days and milled using a coffee grinder (Mellerware).

At the onset of soybean flowering, half of a single row was inoculated with milled grain mycelium (2 g per m) while the remaining half row served as an un-inoculated control.

The plants were sprayed with water to run-off to aid adhesion of inoculum to plant surfaces. The milled grains were distributed over the heads of the seven sunflower cultivars at bud growth stage.

4.3.2.3. Liquid spray mycelium (modified from Chen and Wang, 2005)

PDB (Merck) was prepared in 4 l Erlenmeyer flasks and autoclaved for 20 min at 121°C. The broth was cooled and inoculated with ten, 5 mm diameter *S. sclerotiorum* mycelium plugs from seven-day old *S. sclerotiorum* colonies on PDA. The Erlenmeyer flasks containing the inoculated broth were incubated at 25°C for seven days, homogenised using a Heidolph SilentCrusher M and stored at 4°C until required for inoculations. The mycelium suspension was placed in a hand atomiser and sprayed over the heads of the seven sunflower cultivars at bud growth stage. The spray rate was approximately 100 ml/l. At the onset of soybean flowering, half of a single row was inoculated with liquid spray mycelium at the above rate, while the remaining half row served as an un-inoculated control.

4.3.3. Disease assessment and statistical analysis

Nineteen of the 22 soybean cultivars and the seven sunflower cultivars were analysed for their response to *Sclerotinia* inoculation. Disease severity ratings were done on soybeans, 30 days after inoculation and 14 days after inoculation on sunflowers. *Sclerotinia* stem rot was quantified as the percentage of wilted plants per row showing the typical *S. sclerotiorum* mycelial growth on soybeans while on sunflowers *Sclerotinia* head rot was quantified based on the presence of *S. sclerotiorum* mycelium on the heads as well as shredded diseased tissue. Data were statistically analysed using NCSS (Hintze, 2007). Regression analysis was carried out on the respective crop data using the model $Y=aX^b$ to determine the relationship between observed incidence within a cultivar (Y) and disease potential (X) where a and b are regression parameters (Table 4.3) (*sensu* McLaren and Craven, 2008). Disease potential was determined as the mean disease severity over all soybean or sunflower cultivars within a planting date. Soybean cultivar analysis was done using six disease potentials based on inoculation method and planting date i.e. natural infection/control; milled grain mycelium; liquid spray mycelium over plantings 3 and 4. The disease potentials used

were (1) soybean cultivars planted on 1 December 2017 inoculated with milled grain mycelium; (2) soybean cultivars planted on 1 December 2017 inoculated with liquid spray mycelium; (3) soybean cultivars planted on 18 December 2017 inoculated with milled grain mycelium; (4) soybean cultivars planted on 18 December 2017 inoculated with liquid spray mycelium and (5) soybean cultivars not inoculated with laboratory produced inoculum used as control. The disease potentials used for sunflower cultivar analysis were (1) sunflower cultivars inoculated with both milled grain mycelium and liquid spray mycelium on 13 December 2016; (2) sunflower cultivars inoculated with both milled grain mycelium and liquid spray mycelium on 28 December 2016 and (3) sunflower cultivars inoculated with both milled grain mycelium and liquid spray mycelium on 5 January 2017. The Sclerotinia stem and head rot potential (SOP) needed to initiate disease, arbitrarily taken at 5.0 % incidence, was determined by rearranging the model i.e. $SOP = \text{Exp} (\text{Ln}(5\%) - \text{Ln}(A))/B$. The rate of response of a cultivar to changing disease potential was quantified as $r=a*b*(X)^{(b-1)}$.

Soybean and sunflower cultivars within the sequential plantings at Delmas and Greytown were ranked using Spearman rank correlation analysis (NCSS, Hintze, 2007).

4.3.4. Cell wall-degrading enzymes

The leaves of 22 soybean and seven sunflower cultivars at four growth stages i.e. pre-flowering, flowering, post-flowering and mid-vegetative growth stages, were collected in Delmas and placed on ice for transportation. Tissues were powdered in liquid nitrogen using a pestle and mortar. The leaf powder (1 g) was placed into a 15 ml Falcon tube with 4 ml of a sodium acetate buffer with a pH of 5.9. This extraction buffer also contained 5% polyvinyl pyrrolidone, 2 mM ethylenediaminetetraacetic acid, B-mercaptoethanol, 10% sodium dodecyl sulphate. The buffer served to protect the proteins during the extraction process and remove most phenolics. The samples were centrifuged in Optima BHG 700 at 14 000 g for 10 min. The extracted enzymes were removed and stored at -80°C until needed for the determination of proteins, chitinase and β -1, 3-glucanase activity (Mohase, personal communication, Department of Plant Sciences, University of the Free State).

4.3.4.1. Total protein concentration in soybeans and sunflower leaves

Protein concentrations were determined using a modification of the dye-binding assay method of Bradford (1976). Each reaction contained 40 μl Bio-Rad protein assay, 150 μl distilled sterile water and 10 μl enzyme extract or 10 μl γ -globulin in the standard curve reaction. The samples were analysed in triplicate and assayed in a Biotek ELx808 microplate reader at a wavelength of 590 nm.

4.3.4.2. Chitinase activity in soybeans and sunflower leaves

A modified method of Wirth and Wolf (1990) as described by Steyn (2015) was used for quantification of chitinase activity. This method is based on the precipitability of carboxymethyl-chitin-remazol brilliant violet 5R (CM-chitin-RBV; Loewe Biochemica GmbH) in a 2 N hydrochloric buffer. Each reaction contained 600 μl 0.1 M sodium acetate buffer (pH 5.1), 200 μl (2 mg/ml) CM-chitin-RBV, 20 μl enzyme extract. This reaction mixture was incubated at 37°C for 30 min. This reaction was stopped with 200 μl hydrochloric acid. The mixtures were left on ice for 10 min after which they were centrifuged with a Hermle ZZ33 M-2 at 14 000 g for 5 min. The absorbance of the supernatant was measured at 550 nm using a T60 UV-VIS spectrophotometer and expressed as mg^{-1} protein h^{-1} .

4.3.4.3. β -1, 3-glucanase activity in soybeans and sunflower leaves

A modified method of Fink *et al.* (1988) was used to quantify β -1, 3-glucanase activity. Each reaction contained 240 μl 50 mM sodium acetate buffer (pH 4.5), 250 μl 2 mg/ml Laminarin and 20 μl enzyme extract which was incubated at 37°C for 10 min, after which 500 μl Somogyi's reagent (1952) was added. After the samples were incubated at 100°C for 10 min, 500 μl Nelson's (1944) reagent was added. The absorbance was measured at 540 nm using a T60 UV-VIS spectrophotometer and produced glucose concentration was determined and expressed as mg glucose mg^{-1} protein h^{-1} .

4.3.5. Lignin (Kneebone, 1962)

Soybean stems were transversely cut into 2 mm pieces and placed onto a microscope slide. A stain solution was prepared by dissolving 2 g of phloroglucinol in 80 ml of 20% ethanol following the addition of 20 ml of hydrochloric acid (12 N). A drop of

phloroglucinol-HCl was added onto the piece of stem on the microscope slide. The samples were examined using an Olympus SZX10 stereo microscope and visually evaluated based on the presence of red-violet colouration.

4.4. Results

4.4.1. Soybeans

The third and fourth planting in Delmas (1 December 2017 and 18 December 2017) were the only plantings that produced consistent levels of Sclerotinia stem rot. T-test ($t=10.67$; $p<0.05$) indicated significant differences between the mean levels of disease in planting three and four (Figure 4.1). Planting three had a mean of 6.7% while planting four yielded a mean of 33.4% Sclerotinia stem rot. T-test also indicated significant differences in Sclerotinia stem rot response to milled grain mycelium and liquid spray mycelium within plantings (Figure 4.1). The milled grain mycelium inoculation yielded a mean of 56.0% disease incidence while the liquid spray mycelium yielded a mean of 10.0% stem rot. Rank correlations ranging from $r = 0.7$ to $r = 0.2$ were recorded between cultivars from different disease potentials (Table 4.3) with only four of the 10 relationships being statistically significant ($p<0.05$).

Three response types were recorded between observed Sclerotinia stem rot incidence in cultivars and Sclerotinia stem rot potential as illustrated in Figure 4.2. Where $b > 1$, e.g. PAN 1454, greater tolerance to increasing disease potential is indicated with a higher potential required to initiate disease onset (termed the onset potential). Where $b \approx 1$, e.g. PAN 1521 R, a linear relationship is indicated, while $b < 1$ e.g. P 64 T 39, indicates a low tolerance to increasing disease potential in the tested genotypes. Cultivar P 64 T 39 was the most susceptible to *S. sclerotiorum* with a b-parameter of 0.7 while PAN 1454 had a more stable response towards increasing *S. sclerotiorum* potential with a b-parameter = 1.7 (Table 4.4).

Sclerotinia stem rot onset potential (SOP) was calculated as the disease potential required to initiate 5% (arbitrary value) disease incidence in a test cultivar. Cultivar P 64 T 39 had an SOP of 1.0% indicating high susceptibility while PAN 1454 was more stable with a 19.314% SOP (Table 4.4). The rate of response (ROR) at SOP was calculated using $Y=AbX^{(b-1)}$. The value indicates the rate at which disease incidence

responds to increasing disease potential at onset. Cultivar P 64 T 39 yielded a rate of 3.1% per potential unit compared with PAN 1454 that yielded a rate of 0.4%. This is an indication that PAN 1454 responds less rapidly to increasing Sclerotinia stem rot potential, while P 61 T 38 will respond more quickly to changes in Sclerotinia stem rot potentials.

Mean chitinase concentration differed significantly (Table 4.5; $P < 0.05$) in soybean cultivars (Figure 4.3) as well as at different growth stages (Figure 4.4). P 64 T 39 had the highest level of chitinase at $1.7 \text{ h}^{-1} \text{ mg}^{-1} \text{ protein h}^{-1}$ while RA 660 produced the lowest at $0.497 \text{ h}^{-1} \text{ mg}^{-1} \text{ protein h}^{-1}$. Analysis of Variance of the β -1, 3-glucanase concentration in soybean cultivars (Table 4.6) indicated significant differences ($P < 0.05$) between cultivars (Figure 4.5) and at the different growth stages (Figure 4.6). PAN 1454 produced the highest level of β -1, 3-glucanase, i.e. $3.3 \text{ mg glucose mg}^{-1} \text{ protein min}^{-1}$, while RA 660 only produced $1.3 \text{ mg glucose mg}^{-1} \text{ protein min}^{-1}$. Figure 6 illustrates that the level of β -1, 3-glucanase at the third growth stage was $2.5 \text{ mg glucose mg}^{-1} \text{ protein min}^{-1}$, while the second growth stage yielded the lowest levels of β -1, 3-glucanase, which was $1.9 \text{ mg glucose mg}^{-1} \text{ protein min}^{-1}$. Enzyme levels at the different growth stages did not correlate with Sclerotinia stem rot severity.

Figure 4.7 illustrates the total lignin in cross sections of the stems in each cultivar. Soybean cultivars differed in the amount of lignin present in the stems. Cultivar 1 (PAN 1454) and 5 (DM 5953) had the highest level of red saturation while Cultivar 13 (RA 568) and 14 (RA 660) had the lowest level of red saturation. The level of red saturation present in the soybean stems did not correlate with Sclerotinia stem rot incidence.

4.4.2. Sunflowers

Sclerotinia head rot quantified as percentage head rot incidence was recorded in all three planting dates at Greytown. A significant decline in Sclerotinia head rot potential (mean head rot incidence) was observed over the three planting dates as indicated by t-test (Figure 4.8). The first planting yielded the most disease while the third planting had less disease. This was associated with early season high rainfall and moderate temperatures compared with warmer and dryer conditions later in the season. A poor rank correlation of $r = 0.5^{n/s}$ was observed between cultivars in the first and third

planting dates, while inverse, albeit non-significant correlations, were recorded between the first and second planting dates and second and third planting dates (Table 4.7).

Regression analysis was used to determine the relationship between observed head rot incidence within a cultivar and disease potential (*sensu* McLaren and Craven, 2008). The three response types recorded between observed *Sclerotinia* head rot in a test cultivar and *Sclerotinia* head rot potential are illustrated in Figure 4.9 and were similar to those described above for soybean. Cultivar P 65 LL 02 was the most susceptible to *S. sclerotiorum* with a b-parameter of 0.8 while P 65 LC 54 had a more stable response towards *S. sclerotiorum* indicated by a 2.2 b-parameter (Table 4.8). *Sclerotinia* head rot onset potential (SOP) required to initiate 5 % disease incidence was calculated as indicated above. Cultivar P 65 LL 02 had an SOP of 2.1 % indicating high susceptibility while P 65 LC 54 was more stable with 18.9 % SOP (Table 4.8). The rate of response (ROR) to SOP was calculated. Cultivar P 65 LL 02 had a rate of 1.9 compared with P 65 LC 54 which had a rate of 0.5. This is an indication that P 65 LC 54 responds less rapidly to increasing *Sclerotinia* head rot potential.

Analysis of variance indicated a significant difference in levels of chitinase production (Table 4.9; $P < 0.05$) within the three different sunflower growth stages (Figure 4.10) but no significant difference was observed between cultivars. The flowering stage produced $0.3 \text{ h}^{-1} \text{ mg}^{-1} \text{ protein h}^{-1}$ of chitinase, being the lowest production while the bud stage had the highest level of chitinase, $0.6 \text{ h}^{-1} \text{ mg}^{-1} \text{ protein h}^{-1}$ which was almost double the amount produced by the flowers. Analysis of variance of the β -1, 3-glucanase concentration (Table 4.10) indicated no significant differences in sunflower cultivars ($P < 0.05$) but a significant difference was found at different growth stages (Figure 4.11). Figure 4.11 illustrates that levels of β -1, 3-glucanase production declined from the bud growth stage through to the ripening heads from $2.7 \text{ mg glucose mg}^{-1} \text{ protein min}^{-1}$ to $1.1 \text{ mg glucose mg}^{-1} \text{ protein min}^{-1}$. Enzyme levels and growth stages did not correlate with *Sclerotinia* stem rot severity.

4.5. Discussion

Managing plant diseases using host tolerance and resistance may contribute to an increase in food production. Host tolerance to plant diseases describes the plants response to infection and its ability to endure the disease without dying or suffering serious yield loss (Agrios, 2005). However, host resistance is a plants ability to overcome the damaging effects or factors of a pathogen, either completely or partially (Politowski and Browning, 1978). The level of yield produced by an infected host plant will determine whether the plant is tolerant or resistant to the plant pathogen, i.e. if a high yield of good quality is attained in the presence of disease, it is said that the plant is tolerant to that disease.

Sclerotinia resistant cultivars have previously been used to reduce the risk of Sclerotinia diseases, although Mueller *et al.* (2002) indicated that germplasm responses changed as inoculum potential changes. McLaren and Craven, 2008 illustrated that soybean cultivars differed significantly in their reaction to Sclerotinia diseases at different localities and seasons, and suggested that these different rankings of cultivars to Sclerotinia diseases may be due to differential interactions of host cultivars with environmental variables and inoculum potential.

The observed vs. potential approach to the evaluation of cultivars to diseases characterised by a higher genotype x environment interaction provides multiple criteria which can be applied to aid the understanding of genotype behaviour (McLaren, 1992; McLaren and Flett, 1994). The different reactions of cultivars to disease potential were based on the b-parameter ($b < 1$; $b \approx 1$; $b > 1$), 5% Sclerotinia onset potential and the rate of change in disease incidence at increasing disease potential subsequent to onset (McLaren and Craven, 2008). Thus, a high value for b and SOP and low r-parameter could indicate a lower risk cultivar. This regression approach was successfully used to determine differences in maize hybrids to changing *Stenocarpella maydis* ear rot potentials (Flett and McLaren, 1994) as well as the different reactions of sorghum genotypes to ergot potentials caused by *Claviceps africana* (McLaren, 1992). These parameters also provided selection criteria in breeding programs which resulted in escape resistance in sorghum sufficient to be of commercial value (Reed *et al.*, 2002).

Most of the 19 soybean and seven sunflower cultivars evaluated in the current study showed limited tolerance to *Sclerotinia* disease potentials. In the context of sorghum ergot, McLaren (1992) found that b and SOP values exceeding 5.0 and 20.0% for a 1.0% SOP were needed to provide a sufficient reduction in the risk of disease. In the current studies b -values < 1.5 were recorded in all but three of the 19 soybean cultivars while SOP were also generally $< 10.0\%$ disease potential. Similar values were recorded in sunflower with the exception of P 65 LC 54 with a b -parameter of 2.2 and SOP of 18.9%.

The low rank correlations observed between the different planting dates and thus, disease potentials in the soybean and sunflower trials in the current study, is an indication that a single environment is not sufficient to evaluate *Sclerotinia* disease escape in cultivars. Different levels of tolerance to *Sclerotinia* stem and head rot potentials were recorded in both soybean and sunflower cultivars. No cultivar, however had complete resistance to *S. sclerotiorum* or potential tolerance levels to be of significant economic impact.

The importance of the rate of breakdown as evaluation criterion is indicated by soybean cultivars RA 437 R and SSS 5449. The cultivars had similar SOP between 17.0 - 18.0% but respective rates of 0.2 and 0.3 p.p.u. implying that the latter cultivar will react more rapidly to changes in disease potential subsequent to disease onset and therefore, making the former the preferred cultivar to reduce disease risk. It can therefore be assumed that the impact of increasing disease potential will be less on the cultivars displaying a lower rate of response to changing disease potential than those cultivars with higher rate parameters. Boland and Hall (1987) reported variation in cultivars reaction to *Sclerotinia* in different cultivar evaluation trials as well as cultivars which were relatively resistant in the first year of evaluation being susceptible in the subsequent season and *vice versa*. The interaction between onset and the subsequent rate is a factor of final disease incidences, indicates the need for multiple criteria when screening for *Sclerotinia* resistance.

Steyn (2015) indicated that the regression methodology applied in this study (McLaren, 1992) could be used to accurately model the cultivar response to disease potential, which was supported by high R^2 values. This was confirmed in the current

study despite the relatively low number of disease potential applied, particularly in the sunflower trials. It is recommended that evaluations be conducted over a wider range of potentials by increasing planting dates to create a diversity of potentials. These could be created by shifting the first planting date to a later time and increasing the number of planting dates to six as opposed to four since at least five different disease potentials are required to optimise this regression analysis. In addition, the number of localities could be increased.

Planting of host cultivars having levels of tolerance to Sclerotinia disease potentials indicated by the current study, will only provide a limited amount of control and cannot be used solely to reduce the risk of disease. A large volume of literature on *S. sclerotiorum*, suggests that the adaption of crop production systems may decrease disease potential and enhance the limited Sclerotinia resistance in crop varieties. Several cultural strategies have been suggested for the control of Sclerotinia diseases including crop rotation (Mueller *et al.*, 2002a), tillage (Brustolin *et al.*, 2016), crop microclimate modification (Hoes and Huang, 1976), sanitation (Steadman, 1979), planting date (Kim and Diers, 2000) and soil solarisation (Swaminathan *et al.*, 1999). The integration of these strategies with lower risk genotypes is essential if the risk of Sclerotinia diseases is to be minimised.

Previous studies (Van Loon *et al.*, 2006; Zhao *et al.*, 2007; Balasubramanian *et al.*, 2012) on the effect of chitinase and β -1, 3-glucanases on soybean and canola have suggested that these can act as barriers and reduce Sclerotinia infection. However, in this study, no correlation was recorded between chitinase or β -1, 3-glucanases and Sclerotinia stem and head rot severity, despite significant differences recorded between cultivars. Petlier *et al.* (2009) found that low soybean stem lignin concentrations are related to resistance, while Liu *et al.* (2018) suggested that high lignin concentration provided disease resistance. No correlation was found between the degree of lignification (phloroglucinol:HCl) in the soybean stems and the levels of Sclerotinia stem rot observed in the current study, PAN 1454 which had a 32.0% Sclerotinia incidence, had high levels of red saturation, while SSS 5052 with a similar Sclerotinia incidence (30.0%) had lower levels of red saturation. The same contradictory results were indicated by RA 565 with a 90.0% Sclerotinia incidence and low levels of red saturation while P 61 T 38 with a Sclerotinia incidence of 80.0% had

high levels of red saturation. It would appear that high levels of lignin do not necessarily mean low *Sclerotinia* incidence in soybean cultivars.

This study indicates that the reaction of soybean and sunflower cultivars screened for resistance to *S. sclerotiorum* changed according to disease potential. It is recommended that multiple criteria are considered when evaluating crops for resistance to *Sclerotinia* at different disease potentials, since a single criterion cannot define the actual behaviour of crops to pathogens. The regression methodology (McLaren, 1992; Flett and McLaren, 1998; McLaren and Craven, 2008) used in this study proved to be an effective and accurate technique to quantify cultivar response to disease potential. The milled grain mycelium (Nelson *et al.*, 1988) evaluated in this study, proved to be an effective inoculation technique to evaluate cultivar response to *S. sclerotiorum*, especially since plants were not injured before inoculation, meaning that no mechanical barrier of the plants to the pathogen were compromised. This study illustrated that the level of lignin, β -1, 3-glucanases and chitinase within crops do not necessarily play a role in resistance to *S. sclerotiorum*.

4.6. References

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Table 4.1: Soybean cultivars planted at Delmas during 2017/18 for the evaluation of resistance to Sclerotinia stem rot caused by *S. sclerotiorum*.

#	Cultivar	Seed company
1	PAN 1454	Pannar
2	PHB 94 Y 80	Pioneer
3	RA 437 R	DMS Genetics
4	SSS 5449	Sensako
5	DM 5953	Agricol
6	RA 560 R	DMS Genetics
7	DM 5302	Agricol
8	DM 5609	Agricol
9	PAN 1521 R	Pannar
10	PAN 1523 R	Pannar
11	RA 565	DMS Genetics
12	SSS 5052	Sensako
13	RA 568	DMS Genetics
14	RA 660	DMS Genetics
15	LS 6164 R	Link Seed
16	LS 6261 R	Link Seed
17	P 61 T 38	Pioneer
18	P 64 T 39	Pioneer
19	PAN 1614 R	Pannar
20	PAN 1623 R	Pannar
21	RA 626	DMS Genetics
22	P 96 T 06	Pioneer

Table 4.2: Sunflower cultivars planted at Greytown during 2016/17 for the evaluation of resistance to Sclerotinia head rot caused by *S. sclerotiorum*.

#	Cultivar	Seed company
1	SYN 390	Syngenta
2	P 65 LC 54	Pioneer
3	PAN 7095 CL	Pannar
4	PAN 7049	Pannar
5	P 65 LL 02	Pioneer
6	PAN 7031 LC	Pannar
7	PAN 7117 CLP	Pannar

Table 4.3: Rank correlations of 19 soybean cultivars at five disease potentials. Disease potential 1: soybean cultivars planted on 1 December 2017 and inoculated with milled grain mycelium; Disease potential 2: soybean cultivars planted on 1 December 2017 and inoculated with liquid spray mycelium; Disease potential 3: soybean cultivars planted on 18 December 2017 and inoculated with milled grain mycelium; Disease potential 4: soybean cultivars planted on 18 December 2017 and inoculated with liquid spray mycelium; Disease potential 5: soybean cultivars not inoculated with laboratory produced inoculum (control).

	Disease potential 1	Disease potential 2	Disease potential 3	Disease potential 4	Disease potential 5
Disease potential 1	1.000	0.700***	0.470*	0.120 ^{n/s}	0.400 ^{n/s}
Disease potential 2		1.000	0.220 ^{n/s}	-0.040 ^{n/s}	-0.110 ^{n/s}
Disease potential 3			1.000	0.280 ^{n/s}	0.570**
Disease potential 4				1.000	0.580**
Disease potential 5					1.000

* Significant (P<0.05); ** significant (P<0.01); *** significant (P<0.01); n/s non-significant

Table 4.4: Calculated parameters for the relationship between observed Sclerotinia stem rot and Sclerotinia stem rot potential in soybean cultivars, 5% Sclerotinia stem rot onset potential (SOP) and rate at breakdown.

Cultivar	R²	a- parameter	b- parameter	Sclerotinia onset potential (SOP) (5%)	Rate of response (ROR)
PAN 1454	0.990	0.030	1.732	19.314	0.448
PHI 94 Y 80	0.591	2.758	0.699	2.344	1.491
RA 437 R	0.760	0.348	0.936	17.252	0.271
SSS 5449	0.968	0.176	1.175	17.309	0.339
DM 5953	0.893	0.805	0.896	7.680	0.583
DM 5302	0.976	0.085	1.627	12.246	0.664
DM 5609	0.988	0.435	1.260	6.951	0.906
PAN 1521 R	0.947	1.491	0.935	3.650	1.280
PAN 1523 R	0.948	2.263	0.850	2.542	1.672
RA 565	0.923	1.558	0.942	3.448	1.366
SSS 5052	0.999	0.347	1.379	6.932	0.994
RA 568	0.991	0.831	1.100	5.112	1.076
RA 660	0.936	1.043	0.834	6.552	0.636
LS 6164 R	0.999	0.128	1.580	10.159	0.777
LS 6261 R	0.981	2.925	0.770	2.007	1.917
P 61 T 38	0.968	1.069	1.054	4.321	1.219
P 64 T 39	0.834	4.958	0.691	1.012	3.414
PAN 1614 R	0.968	0.607	1.159	6.163	0.940
PAN 1623 R	0.713	2.353	0.732	2.799	1.308

Table 4.5: Analysis of variance indicating for chitinase concentration in soybean cultivars at different growth stages.

Analysis of Variance					
Source Term	Df	Sum of squares	Mean Square	F-Ratio	Probability level
Cultivar	21	16.173	0.770	3.760	0.000*
Growth stage	2	24.751	12.375	60.490	0.000*
Cultivar x Growth stage	42	31.035	0.738	3.610	0.000*
Rep	2	0.244	0.122	0.600	0.551
Error	130	26.597	0.204		
Total (Adjusted)	197	98.802			
Total	198				

Table 4.6: Analysis of variance indicating β -1, 3-glucanase concentration in soybean cultivars at different growth stages.

Analysis of Variance					
Source Term	Df	Sum of squares	Mean Square	F-Ratio	Probability Level
Cultivar	21	32.563	1.550	2.600	0.0005*
Growth stage	2	13.509	6.754	11.330	0.0000*
Cultivar x Growth stage	42	56.750	1.351	2.270	0.0002*
Rep	2	2.385	1.192	2.000	0.139
Error	130	77.468	0.595		
Total (Adjusted)	197	182.677			
Total	198				

Table 4.7: Rank correlations of seven sunflower cultivars at three disease potentials at Greytown. The disease potentials used for sunflower cultivar analysis were Disease potential 1: sunflower cultivars inoculated with both milled grain mycelium and liquid spray mycelium on 13 December 2016; Disease potential 2: sunflower cultivars inoculated with both milled grain mycelium and liquid spray mycelium on 28 December 2016 and Disease potential 3: sunflower cultivars inoculated with both milled grain mycelium and liquid spray mycelium on 5 January 2017.

	Disease potential 1	Disease potential 2	Disease potential 3
Disease potential 1	1.000 ^{n/s}	-0.460 ^{n/s}	0.460 ^{n/s}
Disease potential 2		1.000	-0.640 ^{n/s}
Disease potential 3			1.000

n/s non-significant

Table 4.8: Calculated parameters for the relationship between observed Sclerotinia head rot and Sclerotinia head rot potential in sunflower cultivars, 5% Sclerotinia head rot onset potential (SOP) and rate at breakdown (RAB).

Cultivar name	a - Parameter	b - Parameter	R²	Sclerotinia onset potential (SOP) (5%)	Rate at breakdown (RAB)
SYN 3970	0.243	1.423	0.700	8.385	0.848
P 65 LC 54	0.007	2.231	0.970	18.906	0.590
PAN 7095 CL	0.366	1.323	0.950	7.226	0.915
PAN 7049	1.798	0.941	0.930	2.965	1.587
P 65 LL 02	2.581	0.849	0.970	2.180	1.947
PAN 7031 LC	0.072	1.698	0.810	12.133	0.700
PAN 7117 CLP	0.188	1.471	0.680	9.316	0.789

Table 4.9: Analysis of variance indicating chitinase concentration in different sunflower cultivars at different growth stages.

Analysis of Variance Table					
Source Term	Df	Sum of squares	Mean Square	F-Ratio	Probability level
Cultivar	6	0.540	0.090	0.720	0.6350
Growth stage	2	1.012	0.506	4.050	0.0251*
Cultivar x Growth stage	12	3.255	0.271	2.170	0.0334*
Rep	2	0.243	0.122	0.970	0.3860
Error	40	5.004	0.125		
Total (Adjusted)	62	10.056			
Total	63				

Table 4.10: Analysis of variance indicating β -1, 3-glucanase concentration in different sunflower cultivars at different growth stages.

Analysis of Variance Table					
Source Term	Df	Sum of squares	Mean Square	F-Ratio	Probability level
Cultivar	6	9.505	1.584	1.770	0.1310
Growth stage	2	39.644	19.822	22.080	0.0000*
Cultivar x Growth stage	12	29.087	2.423	2.700	0.0092*
Rep	2	0.596	0.298	0.330	0.7190
Error	40	35.905	0.897		
Total (Adjusted)	62	114.739			
Total	63				

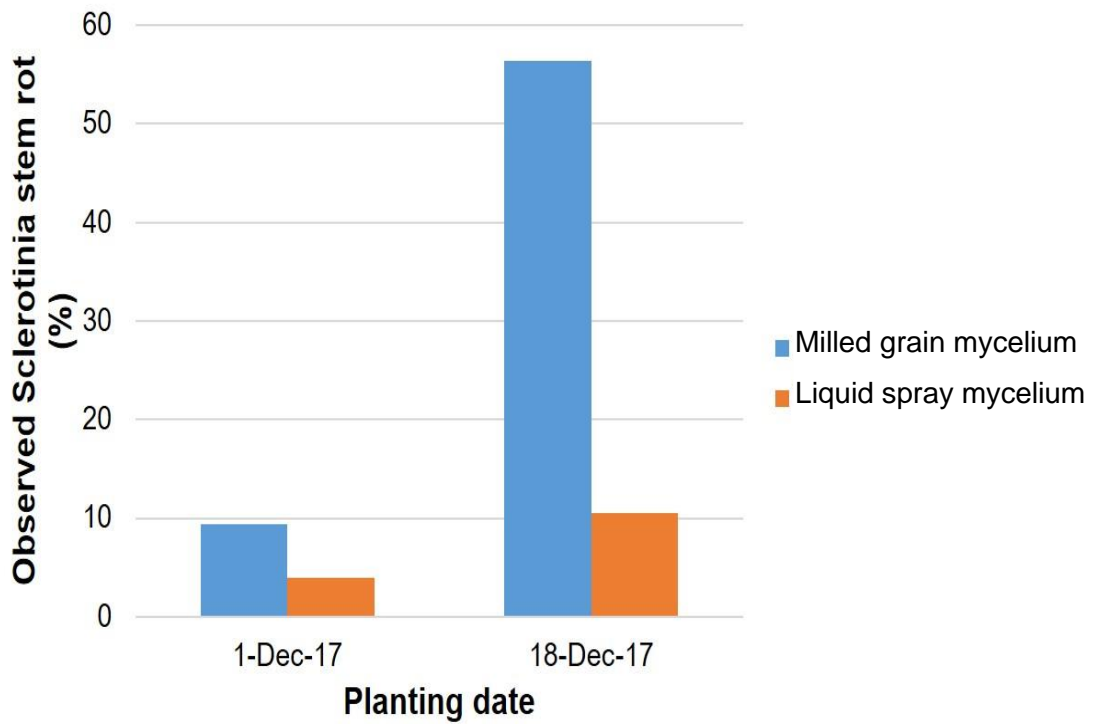


Figure 4.1: Mean Sclerotinia stem rot incidence in 19 soybean cultivars associated with planting date and inoculation technique at Delmas during 2017/18. T-value between 1 Dec 17 and 18 Dec 17 = 10.670.

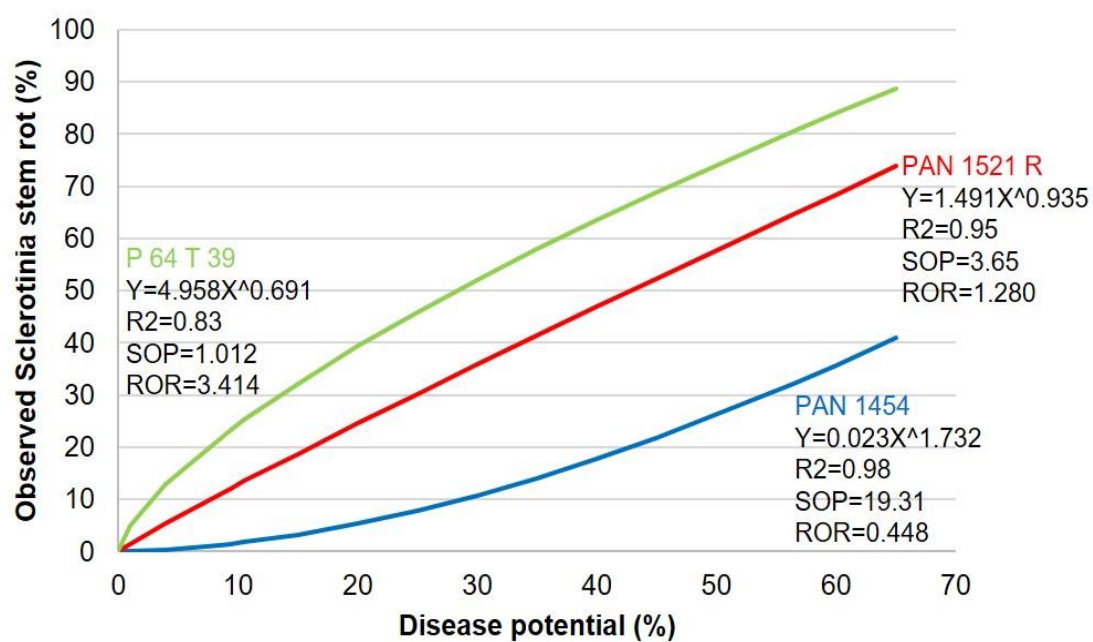


Figure 4.2: Response-types in soybean cultivars subjected to Sclerotinia stem rot potentials at Delmas during 2017/18.

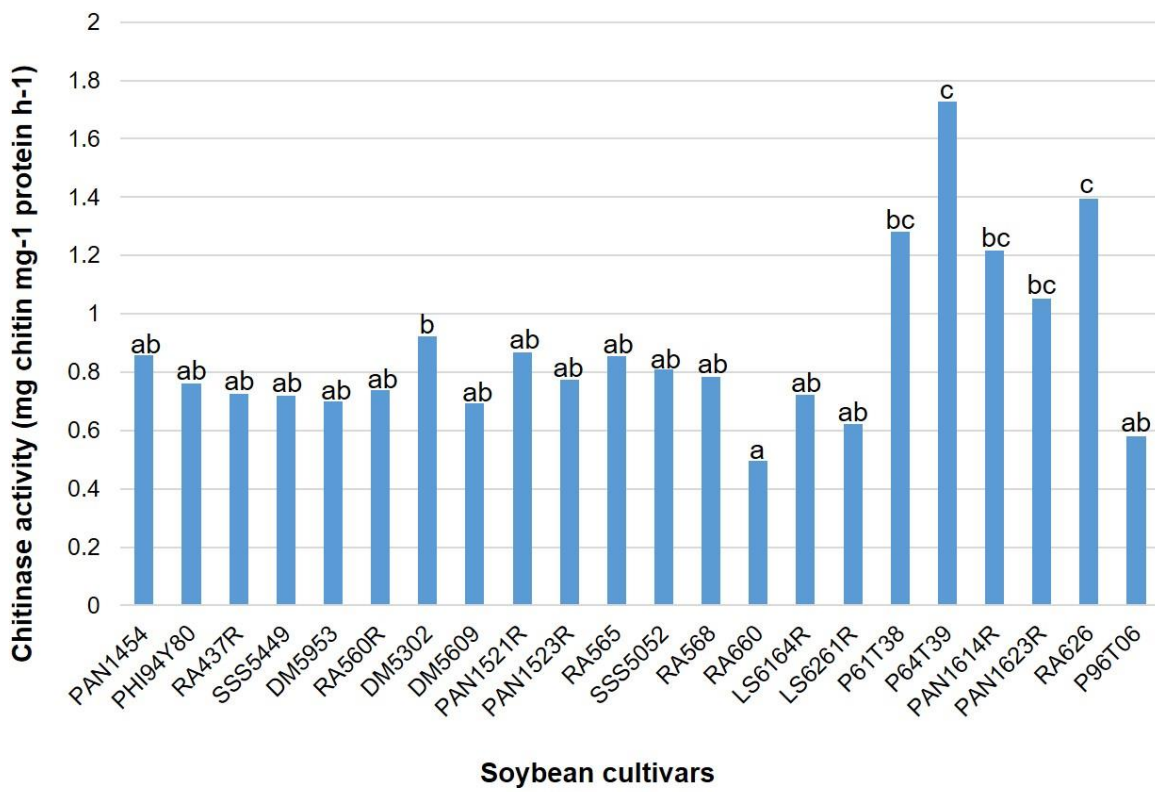


Figure 4.3: Mean chitinase concentrations in soybean cultivars planted at Delmas during 2017/18.

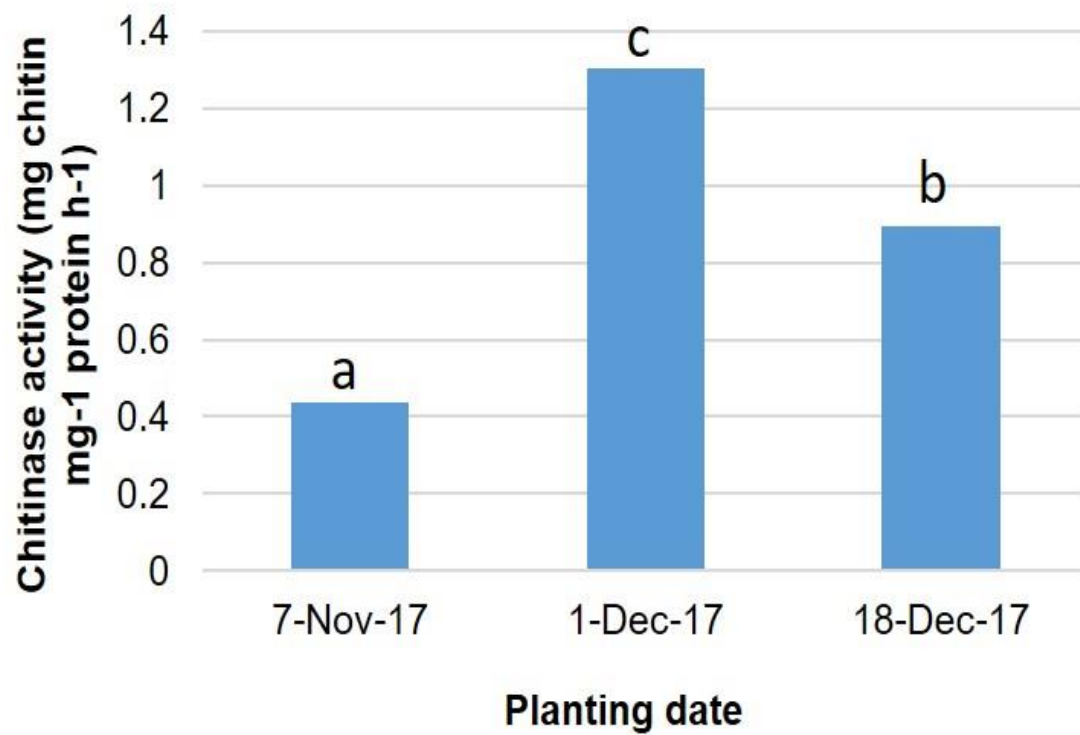


Figure 4.4: Total chitinase concentrations in soybean cultivars planted at Delmas during 2017/18, collected at three different growth stages.

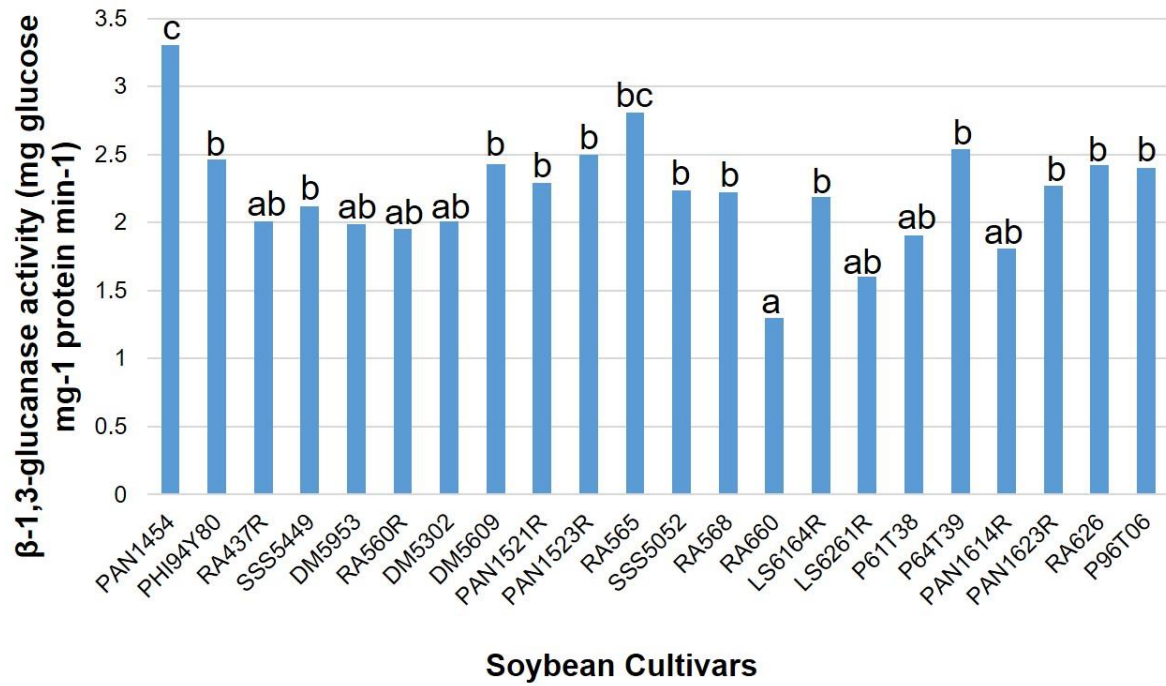


Figure 4.5: Mean β -1, 3-glucanase concentrations in soybean cultivars planted at Delmas during 2017/18.

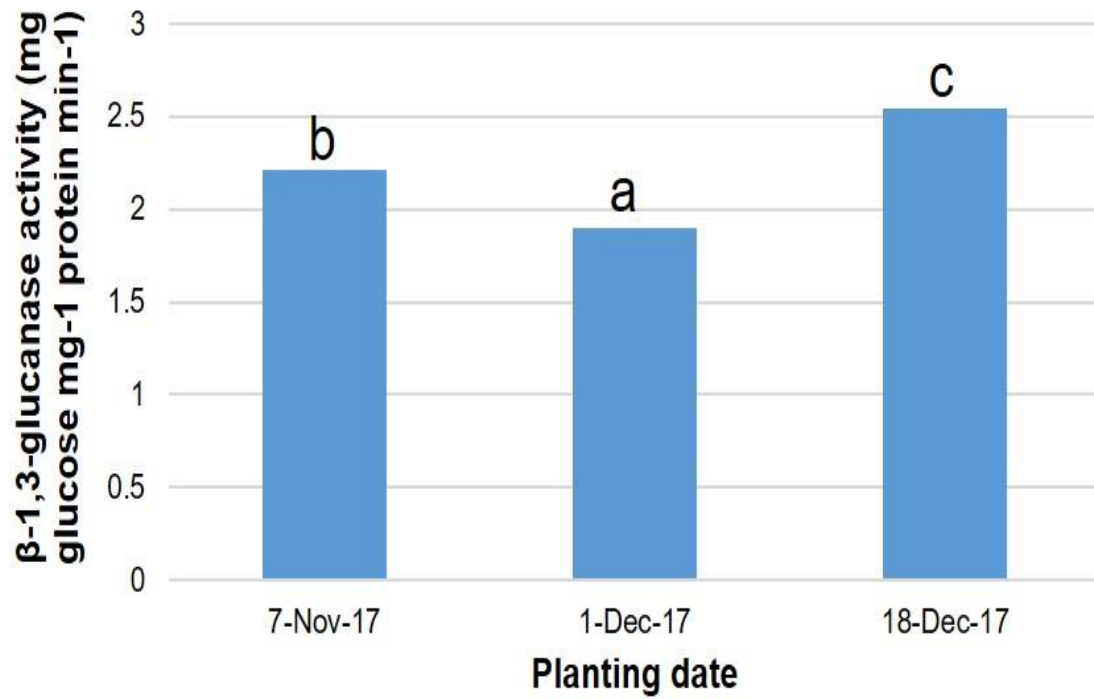


Figure 4.6: Total β -1, 3-glucanase concentrations in soybeans planted at Delmas during 2017/18, collected at three different growth stages.

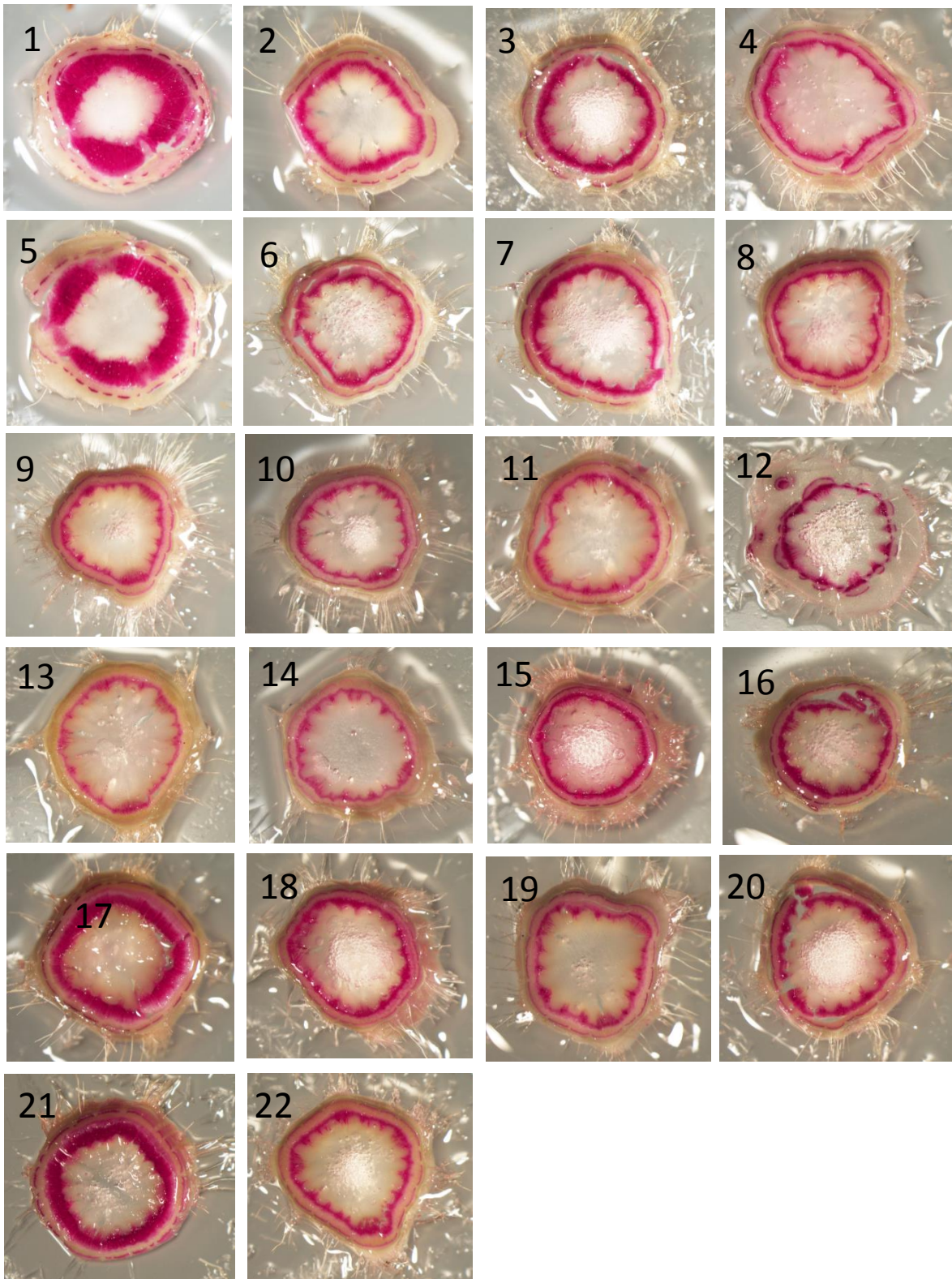


Figure 4.7: Stem cross sections of 22 soybean cultivars stained with phloroglucinol to indicate the presence of lignin (Picture M.C. Bester).

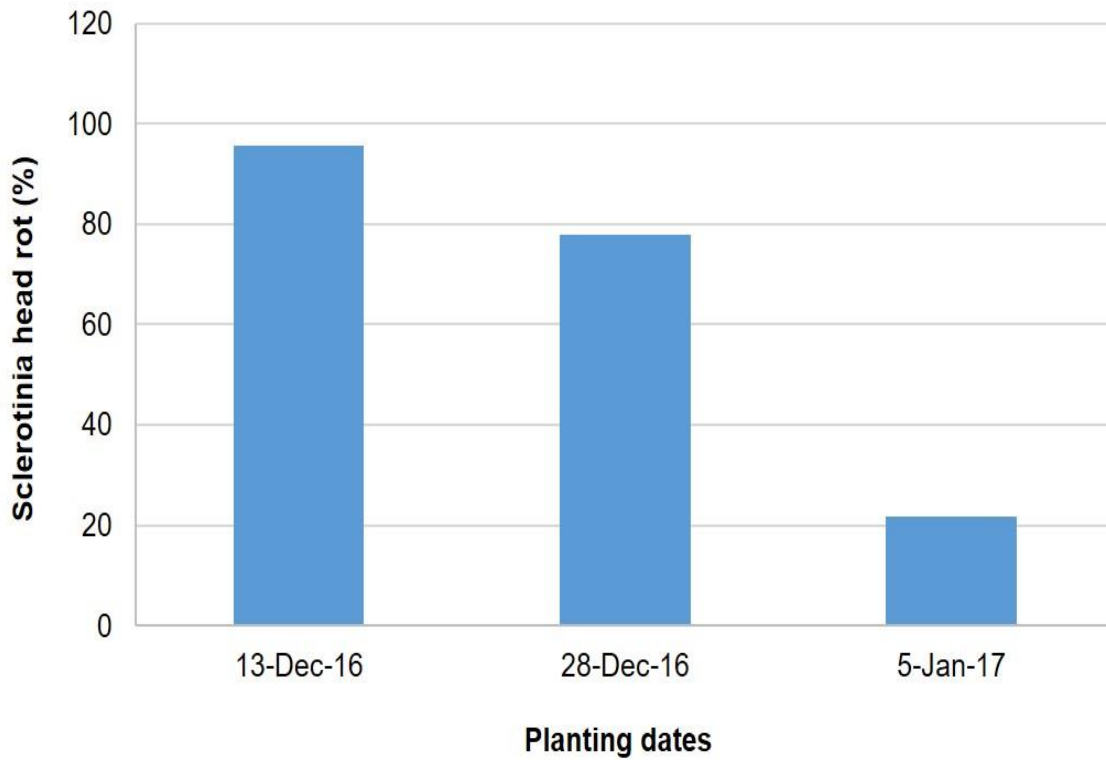


Figure 4.8: Mean Sclerotinia head rot incidence in seven sunflower cultivars associated with different planting dates at Greytown during 2016/17. T-value between 13 Dec 16 and 28 Dec 16 = 2.365; T-value between 13 Dec 16 and 5 Jan 17 = 9.867 and T-value between 28 Dec 16 and 5 Jan 17 = 4.385.

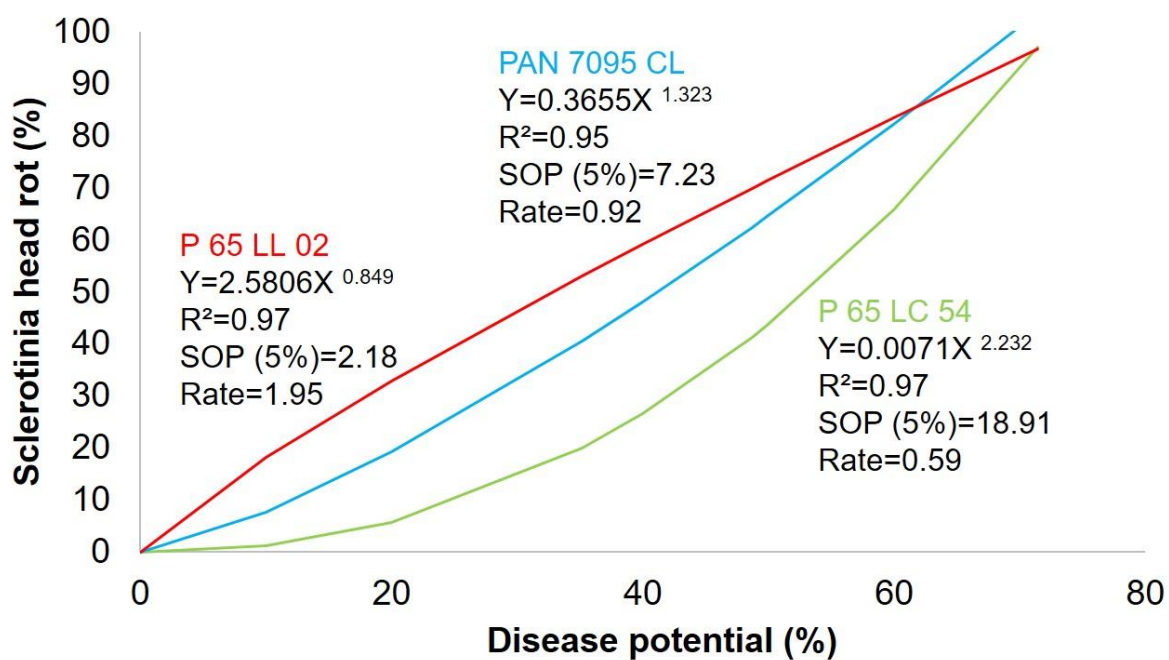


Figure 4.9: Response types recorded in sunflower cultivars to Sclerotinia head rot potential at Greytown during 2016/17.

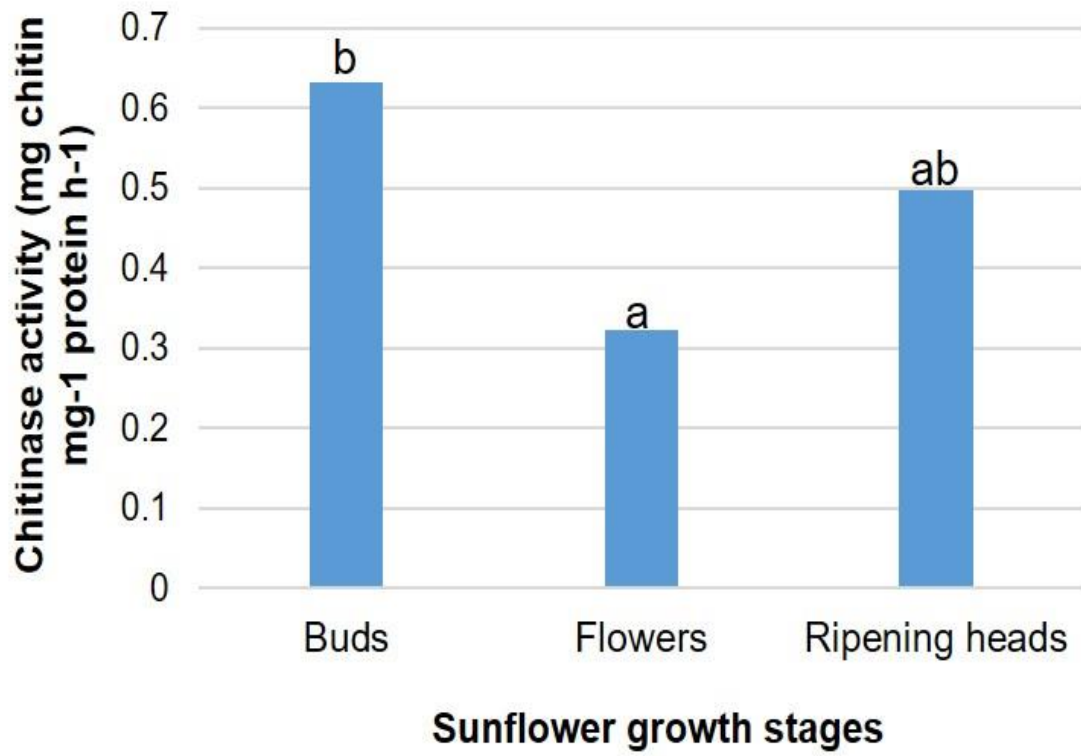


Figure 4.10: Total chitinase concentrations in sunflower cultivars planted at Greytown during 2016/17 and collected at different growth stages.

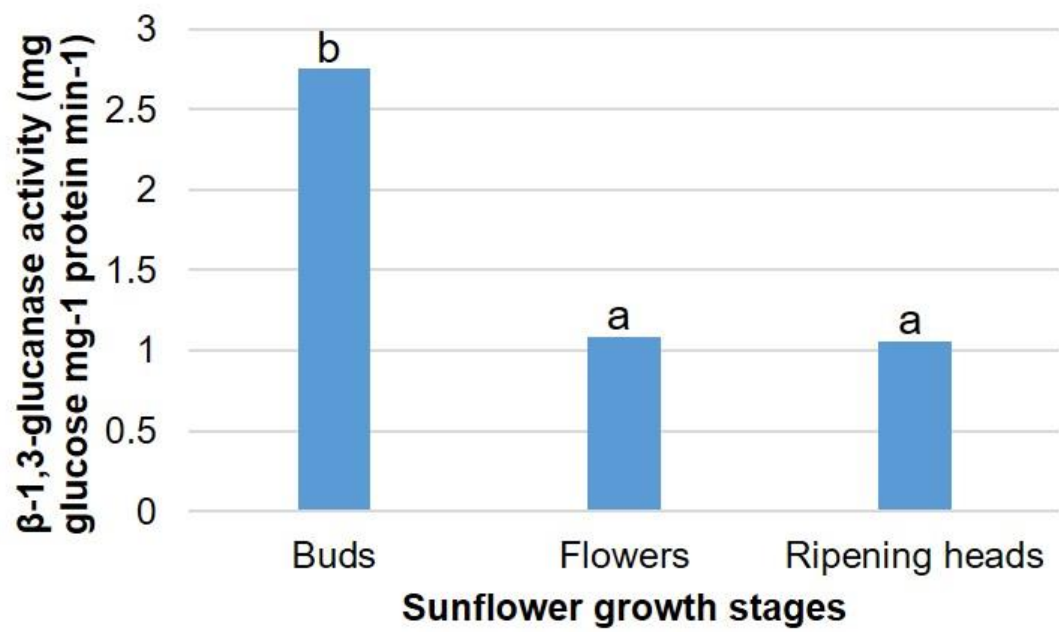


Figure 4.11: Total β -1, 3-glucanase concentrations in sunflower cultivars planted at Greytown and collected at different growth stages.

Summary

Sclerotinia stem and head rot on soybean and sunflower are caused by the fungal plant pathogen *Sclerotinia sclerotiorum*. This pathogen can be responsible for up to 75.0% yield loss. A major constraint in the effective control of Sclerotinia in sunflowers and soybeans is the absence of stable resistance. Cultivar selection as a management strategy remains the ultimate goal in reducing Sclerotinia outbreaks among South African producers. Quantification of cultivar responses to pathogen and environmental stimuli could assist the selection and breeding of cultivars with tolerant to high disease potentials.

The production of *S. sclerotiorum* ascospores and sclerotia were optimised in the laboratory. Maize meal and soybean stubble as substrates for sclerotia production, produced the largest sclerotia with mean masses 0.0292 mg per sclerotium, while sunflower meal produced the lightest sclerotia with a mean mass of only 0.0099 mg per sclerotium. Several literature sources have stated that a pre-conditioning step of 4 - 10°C is needed in order for sclerotia to germinate carpogenically. Results of this study indicate that no pre-conditioning is needed for South African isolates of *S. sclerotiorum* to form apothecial stipes and that the optimum temperature for apothecia development is 16°C. However, results indicated that extending pre-conditioning of sclerotia at 4°C by 4 - 8 weeks, will produce a higher number of apothecia. Regression analysis indicated that apothecial stipes take approximately five days longer to develop for each week of pre-conditioning. Size of sclerotia also affected the number of apothecial stipes and sclerotia 27 mm in length produced up to 118 apothecial stipes, while the smallest sclerotia, 5 mm in length only produced 14 apothecial stipes.

The viability of the pathogen in infected sunflower stems whether milled into a fine powder or shredded into pieces of various sizes, was evaluated every 30 days for 12 months under laboratory conditions. All the infected stubble sizes were able to produce both *S. sclerotiorum* mycelium and sclerotia on Potato Dextrose Agar after 12 months.

After the optimisation of *Sclerotinia* inoculum, efficacy of inoculum source, application technique and timing on disease severity under greenhouse and field conditions were evaluated on both soybean and sunflower. The techniques used were liquid spray mycelium, milled grain mycelium, sclerotia planted adjacent to seedlings and an ascospore suspension. A head punch method, whereby a hole is punched into a flowering head and a *S. sclerotiorum* colonised sorghum grain is inserted, was also applied to sunflowers. Treatments were applied to sunflower at bud, flower and head-ripening growth stage while on soybean, the liquid spray mycelium, milled grain mycelium and ascospore suspension were applied at flowering stage. A significant difference was observed between both sunflower and soybean greenhouse and field trials. Overall the technique that yielded the highest levels of *Sclerotinia* disease was the milled grain mycelium. A negative correlation was observed between soybean greenhouse and field trials, while a poor correlation was observed between sunflower greenhouse and field trials. This illustrated the importance of using multiple environments when screening for *Sclerotinia* resistance, especially under field conditions, as well as the need to consider the high genotype x environment interactions that occur. Performing resistance evaluations in the greenhouse alone, will limit the value of screening techniques.

Field evaluations of resistance in soybean and sunflower cultivars were carried out in Delmas and Greytown respectively, over several planting dates. The cultivars were inoculated with milled grain mycelium and liquid spray mycelium. Regression analyses were used to determine the response type as well as the relationship between observed stem or head rot incidence within a cultivar and disease potential. The disease potential was defined as the mean disease severity within a planting date over all the cultivars within a trial. The regression model $Y=aX^b$ was used where Y is the observed incidence within a cultivar, X is the disease potential and a and b are regression parameters. Three cultivar response types were identified based on the b parameter i.e. cultivars tolerant of increasing disease potential ($b>1$), cultivar intolerant to increasing disease potential ($b<1$) and cultivars showing a linear relationship with changing disease potential ($b\approx 1$). The *Sclerotinia* potential required to initiate disease onset and the rate of disease increase subsequent to onset were calculated by rearrangement of model parameters. The soybean cultivar, PAN 1454, responded less rapidly to changing stem rot potentials, having an onset potential of 19.3% and a

subsequent response rate of 0.5 per potential unit increase, making it more tolerant to increasing disease potentials, while P 64 T 39 had an onset potential of 1.0% and a response rate of 3.4 per potential unit increase, making it less tolerant of increasing *Sclerotinia* potential. The sunflower cultivar, P 65 LC 54, responded less rapidly to changing head rot potentials, having an onset potential of 18.9% and a response rate of 0.6 per potential unit, while P 65 LL 02 had an onset potential of 2.2% and a response rate of 1.9 per potential unit increase. The level of lignin, total proteins, chitinase and β -1, 3-glucanase in the leaves of soybean and sunflower cultivars collected at different growth stages, were evaluated to determine the correlation between these resistance components and *Sclerotinia* disease development. No correlation was recorded.

It was concluded that the reaction of soybean and sunflower cultivars to changing disease potentials is an essential component of any resistance screening study and genotype x environment interactions must be quantified. The use of multiple criteria is essential if the behaviour of the pathosystem is to be understood and if stable resistance of commercial value is to be obtained.

Key words: *Sclerotinia sclerotiorum*, soybean, sunflower, inoculation techniques, resistance screening.