UNIVERSITY OF
KWAZULU-NATAL

STUDIES ON *SCLEROTINIA SCLEROTIORUM* (SCLEROTINIA STEM ROT) ON SOYBEANS

by

Dael Desiree Visser

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School of Biochemistry, Genetics, Microbiology and Plant Pathology
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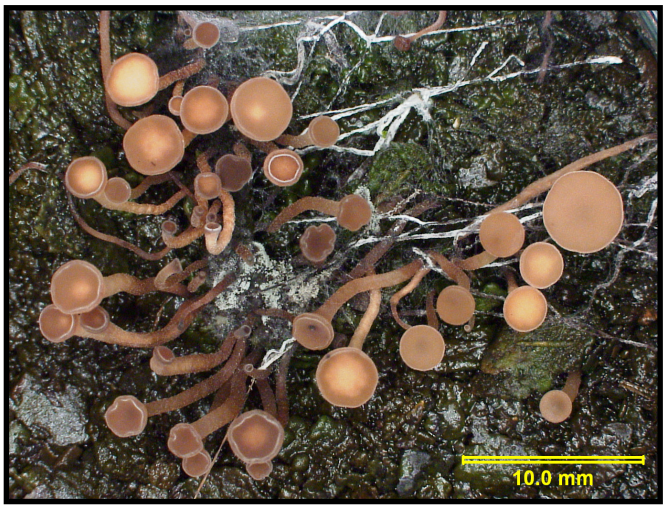
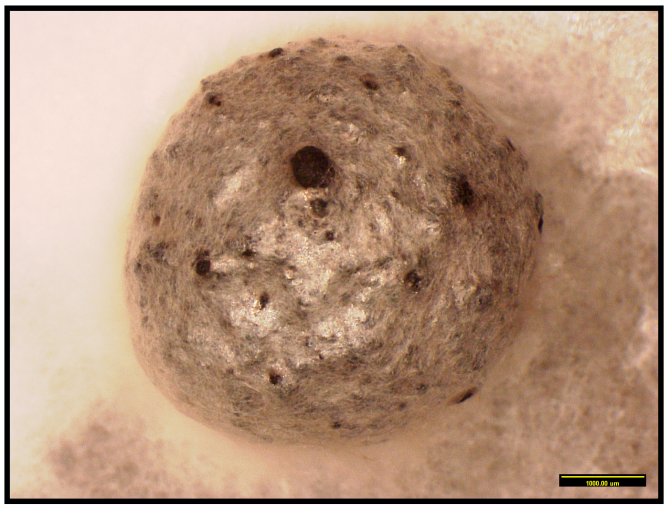
FRONTISPIECE: SCLEROTINIA STEM ROT CAUSED BY *SCLEROTINIA SCLEROTIORUM*

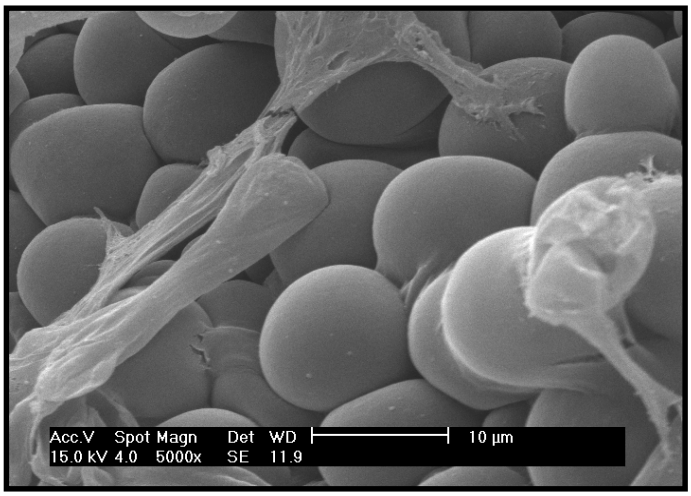
Cryogenic section of an ascus showing ascospores.



Carpogenic germination of sclerotia to form apothecia.



A young sclerotium, showing pigmented rind cells.



Surface of a sclerotium, showing bulbous rind cells.

ABSTRACT

Soybeans, *Glycine max*, are an economically and strategically important crop in South Africa (SA). In order to meet local demands, large imports of soybeans are required, e.g., in the 2005/2006 soybean production period, 842 107 tonnes of oilcake were imported. Due to an increase in soybean production throughout the world, diseases that affect this crop have also increased in incidence and severity.

Sclerotinia sclerotiorum, the causal organism of sclerotinia stem rot (SSR), is an important yield limiting disease of soybeans, as well as numerous other crops. The pathogen was first reported in SA in 1979. However, it was only in 2002 that this fungus was considered a major pathogen of soybeans in SA.

The research reported in this thesis was conducted to investigate the epidemiology of *S. sclerotiorum* and examine numerous potential control methods for this pathogen, i.e., resistant cultivars, biocontrol, chemical control and seed treatments. A *S. sclerotiorum* isolate was obtained from sunflowers in Delmas, Mpumulanga, SA, in the form of sclerotia. This isolate was cultured and sent for identification and deposition in the Plant Protection Research Institute collection. This isolate, in the form of mycelia, was used for the duration of the study.

For epidemiology studies, the effect of temperature, leaf wetness duration (LWD) and relative humidity (RH) were examined for their effect on rate of pathogen development. Twenty four combinations of temperature (19°C, 22°C, 25°C and 28°C), LWD (24, 48 and 72 hr) and RH (85 and 95%) were investigated. No interaction between temperature, LWD and RH was found. Temperature alone was the only factor that affected disease development. At 22°C, the rate of pathogen development (0.45 per unit per day) was significantly higher than all other temperatures, indicating that this temperature is optimum for disease development.

Thirteen different soybean cultivars, i.e., LS6626RR, LS6710RR, LS666RR, LS555RR, LS6514RR, LS678RR, Prima 2000, Pan 626, AG5601RR, AG5409RR, 95B33, 95B53 and 96B01B, commercially grown in SA were investigated for their reaction to *S. sclerotiorum*. Prima 2000, 96B01B, 95B33 and AG5409RR were considered to be the least susceptible as they showed a significantly low rate of pathogen development (0.28, 0.28, 0.24, 0.23 per unit per day, respectively) and produced a significantly low number of sclerotia (3.03, 3.42, 3.21, 2.38, respectively). LS6626R and LS666RR may be considered most susceptible because of their significantly high rate of pathogen development (0.45 and 0.42 per unit per day, respectively) and high sclerotia production (8.16 and 7.50, respectively). Regression analysis showed a positive correlation coefficient (R^2=0.71) between rate of growth of the pathogen and number of sclerotia produced, indicating that a higher rate is associated with a higher number of sclerotia.

In vitro dual culture bioassays were performed to identify the biocontrol mechanisms of the biocontrol agents, EcoT® (a seed treatment) and Eco77® (a foliar treatment), against hyphae and sclerotia of *S. sclerotiorum*. Ultrastructural studies revealed that mycoparasitism is the probable mode of action as initial signs of hyphae of EcoT® and Eco77® coiling around hyphae of *S. sclerotiorum* were observed. Surface colonization of sclerotia by hyphae of EcoT® and Eco77® was also observed.

In vitro antagonism of EcoT® against *S. sclerotiorum* on soybean seed was performed to determine pre-emergence and post-emergence disease. There was no significant difference in percentage germination between seeds treated with EcoT® and plated with the pathogen, untreated seeds and no *S. sclerotiorum*, and the control (i.e. no EcoT® and no pathogen). However, percentage non infected seedlings from seeds not treated with EcoT® was significantly lower, suggesting that EcoT® may be successfully used as a seed treatment for the control of SSR. *In vivo* trials were performed to investigate the effect of silicon (Si) alone, and in combination with Eco77®, on the effect of the rate of disease development. Plants treated with Eco77® had a significantly lower rate of disease development (0.19 per unit per day for plants treated with Eco77® and *S. sclerotiorum* and 0.20 per unit per day for plants treated with Eco77®, *S. sclerotiorum*

and Si), compared to plants not treated with Eco77® (0.29 per unit per day for plants treated with *S. sclerotiorum* and 0.30 per unit per day for plants treated with *S. sclerotiorum* and Si), regardless of the application of Si. Similarly, plants treated with Eco77® had a significantly lower number of sclerotia (0.46 for plants treated with Eco77® and *S. sclerotiorum* and 0.91 for plants treated with Eco77®, *S. sclerotiorum* and Si), compared to plants not treated with Eco77® (3.31 for plants treated with *S. sclerotiorum* and 3.64 for plants treated with *S. sclerotiorum* and Si). The significantly lower rate of disease development coupled with a significant reduction in sclerotia showed that Eco77®, and not Si, was responsible for reducing the severity of SSR. A strong positive correlation between rate of disease development and the number of sclerotia produced (R^2=0.79) was observed.

For the investigation of various fungicides for the control of *S. sclerotiorum*, *in vitro* trials to determine the potential of three different fungicides at different rates, i.e., BAS 516 04F (133 g a.i. ha^{-1}), BAS 516 04F (266 g a.i. ha^{-1}), BAS 512 06F (380 g a.i. ha^{-1}) and Sumisclex (760 g a.i. ha^{-1}) were initially conducted. The control (non-amended PDA) had a significantly higher area under mycelial growth curve (243.0) than all fungicides tested. BAS 516 04F (at both concentrations) and BAS 512 06F completely inhibited the mycelial growth of *S. sclerotiorum*. Sumisclex inhibited the fungus by 89.07%. For *in vivo* trials, preventative treatments, i.e., BAS 516 04F (133 g a.i. ha^{-1}), BAS 516 04F (266 g a.i. ha^{-1}), BAS 512 06F (380 g a.i. ha^{-1}), curative treatment, i.e. Sumisclex (760 g a.i. ha^{-1}) and a combination preventative/curative treatment, i.e., BAS 512 06F (380 g a.i. ha^{-1})/Sumisclex (570 g a.i. ha^{-1}) were investigated. No significant difference in disease severity index (DSI) was found between fungicide treatments and the inoculated control. BAS 512 06F and BAS 512 06F/Sumisclex had significantly lower grain yields (6.09 g and 5.96 g, respectively) compared to all other treatments. There was a positive correlation coefficient (R^2=0.76), between DSI and grain yield, indicating that a high DSI is correlated with low grain yield.

Trials to evaluate the effect of commercially available and currently unregistered seed treatments for the control of *S. sclerotiorum* on soybean seeds *in vivo* and *in vitro* were performed. Seed germination tests were performed to determine if seed treatments had any negative effects on seed germination *in vitro*. All seed treatments tested, i.e., BAS 516 03F (8, 16 and 32 ml a.i. 100 kg^{-1} seed), BAS 512 00F (7.5, 15 and 32 ml a.i. 100 kg^{-1} seed), Celest XL (100, 125, 200 and 250 ml a.i. 100 kg^{-1} seed), Sumisclex (5 and 10 ml a.i. 100 kg^{-1} seed), Benomyl (150 g a.i. 100 kg^{-1} seed), Captan (240 ml a.i. 100 kg^{-1} seed), Thiulin (180 g a.i. 100 kg^{-1} seed) and Anchor Red (300 ml a.i. 100 kg^{-1} seed), showed no negative effect on seed germination. For *in vivo* trials, BAS 516 03F (16 and 32 ml a.i. 100 kg^{-1} seed), BAS 512 00F (7.5, 15 and 32 ml a.i. 100 kg^{-1} seed), Celest XL (100, 125, 200 and 250 ml a.i. 100 kg^{-1} seed), Sumisclex (5 and 10 ml a.i. 100 kg^{-1} seed), Benomyl and Anchor Red had significantly similar percent germination and percent seedling survival as the untreated/uninoculated control. These seed treatments should be recommended for the control of *S. sclerotiorum*, as they protected seed during germination and subsequent seedling development. BAS 516 03F (8 ml a.i. 100 kg^{-1} seed) should not be recommended for the control of SSR, as it gave the lowest percent germination and percent seedling survival.

The results presented in this thesis have helped to identify optimal environmental conditions for the development of *S. sclerotiorum*, which is important for the development of forecasting models for disease control. The least and most susceptible cultivars of those tested have been identified. Biocontrol using Eco77® as a foliar application showed great potential.

The effect of Si needs to be further investigated, including the testing of more frequent applications and higher concentrations. The fungicides tested in this research did not show any potential for the control of SSR. However, the spray programme tested is for the control of soybean rust (*Phakopsora pachyrhizi*), and was investigated for its potential for the control of SSR. The spray programme, fungicide application and rating scale needs to be modified, to determine the true potential of these fungicides for the control of SSR. Numerous seed treatments have shown potential for the control of seed

infection by SSR. Due to difficulties in producing ascospores, which are the primary source of inoculum for this pathogen in the field, all studies in this research were conducted with mycelia and not ascospores. The production, collection and storage of ascospores needs to be thoroughly investigated, and research conducted with ascospores.

DECLARATION

I, Dael Desiree Visser, declare that the research reported in this thesis, except where otherwise indicated, is my own original research. This thesis has not been submitted for any degree or examination at any other university.

_____

Dael Desiree Visser
CANDIDATE

_____

Dr P. M. Caldwell
SUPERVISOR

FOREWORD

The research reported, was undertaken in the Discipline of Plant Pathology, at the University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Dr P. M. Caldwell.

Sclerotinia sclerotiorum, (Lib.) de Bary, the causal organism of sclerotinia stem rot (SSR), has become a major pathogen of soybeans in South Africa (SA), particularly in the wetter growing areas. Soybean is an economically and strategically important crop in SA. Not only is soybean oil economically important, but soybean protein is critical for animal feeds and human nutritional supplements. Consumption in SA for soybean derived commodities far exceeds production, resulting in an annual import of 600 000-800 000 tonnes of oilcake in order to meet local demands.

The main objectives of this research were to develop a balanced and objective approach to an integrated disease management programme for stem rot caused by *S. sclerotiorum*. The scope of this research is broad, encompassing many aspects of the biology of *S. sclerotiorum*, and traversing seven chapters.

Chapter One is a review of the literature on pathogen taxonomy and morphology, host range, symptoms, dissemination, history, economic importance, geographic distribution, infection process, epidemiology and disease management.

Chapter Two reports on the effect of temperature, leaf wetness duration and relative humidity on infection of soybeans by *S. sclerotiorum*.

Chapter Three covers the evaluation of different soybean cultivars for resistance to *S. sclerotiorum* using the cut stem method.

Chapter Four covers the biological control of *S. sclerotiorum* by two commercially available biocontrol agents i.e., EcoT® and Eco77® (*Trichoderma harzianum*), and

includes the biological control of mycelia and sclerotia (overwintering structures) of the pathogen *in vitro*, and *in vivo* trials using Eco77® and silicon.

Chapter Five reports on the efficacy of BAS 516 04F, BAS 512 06F and Sumisclex at various rates for the control of *S. sclerotiorum* of soybeans.

Chapter Six reports on the efficacy of seed treatments against *S. sclerotiorum* on soybeans and crop tolerance, including various new and commercially available seed treatment products.

Chapter Seven reviews the experimental results, conclusions and allows for the recommendation of the development of a balanced and objective approach to an integrated disease management programme for *S. sclerotiorum*.

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My sister Lythann Emslie, for her encouragement and love.

My parents, John and Elizabeth Visser, for always believing in my ability and helping me realise my dream, for their support, both emotionally and financially, and without whom this thesis would not be possible.

DEDICATION

To my parents, John and Elizabeth Visser,
for their love and selfless investment in my education

Whatever you can do, or dream you can do, begin it.

Boldness has genius, power, and magic in it.

Johann Wolfgang van Goethe

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LIST OF ACRONYMS

ANOVA	=	Analysis of variance
AUMGC	=	Area under mycelial growth curve
Ca	=	Calcium
CMC	=	Carboxymethyl cellulose
cv%	=	Coefficient of variance
Dpi	=	Days post inoculation
DSI	=	Disease severity index
ESEM	=	Environmental Scanning Electron Microscope
K	=	Potassium
KZN	=	KwaZulu-Natal
PCNB	=	Pentachloronitrobenzene
l.s.d.	=	Least significant difference
LWD	=	Leaf wetness duration
N	=	Nitrogen
P	=	Phosphorus
PDA	=	Potato dextrose agar
PDB	=	Potato dextrose broth
PPRI	=	Plant Protection Research Institute
R^2	=	Correlation coefficient
RCBD	=	Randomized complete block design
RH	=	Relative humidty
SA	=	South Africa
SBR	=	Soybean rust
s.e.d.	=	Standard error of deviation
SEM	=	Scanning Electron Microscopy
Si	=	Silicon
SSR	=	Sclerotinia stem rot
TEM	=	Transmission Electron Microscopy
USA	=	United States of America
USDA	=	United States Department of Agriculture

LIST OF APPENDICES

CHAPTER ONE

LITERATURE REVIEW

1.1 INTRODUCTION

Soybeans, *Glycine max* (L.) Merrill., are a major source of vegetable oil and protein in the world (Singh *et al*., 2004). This crop has been used for thousands of years for human and animal food as well as in medicine to treat many human diseases (Hartman *et al*., 1999). Soybeans are considered the most important legume, as well as one of the five sacred grains cultivated in China (Hinson and Hartwig, 1977). About 96% of the total soybean production in the world is produced by Argentina, Brazil, Canada, China, India, Paraguay and the United States of America (USA). The total soybean production in 2002 in Africa, however, only accounted for 0.5% of the total global soybean production of 179 917 000 tonnes (Singh *et al*., 2004). Soybeans were introduced into Africa by missionaries and explorers at the beginning of the 20th century (Naik *et al*., 1987; Root *et al*., 1987). However, it was only in the late 1960s that soybean cultivation increased in popularity in a few countries in Africa. In Africa soybean cultivation has increased from 72 000 tonnes of soybeans that were produced on an area of 191 000 ha in 1961 to 989 000 tonnes of soybean produced on an area of 1 090 000 ha in 2002 (Singh *et al*., 2004).

Due to an increase in soybean production throughout the world, diseases that affect this crop have also increased in incidence and severity (Grau and Hartman, 1999). During the 1997-1998 soybean growing season, an estimated 28.5 x 10^6 tonnes of soybeans were lost due to various diseases, with *Sclerotinia sclerotiorum* (Lib.) de Bary being the third most important yield limiting disease in the top ten soybean producing countries (Argentina, Bolivia, Brazil, Canada, China, India, Indonesia, Italy, Paraguay, USA) (Wrather *et al*., 2001). Soybeans are affected by more than 100 pathogens, of which

approximately 35 are of economic importance (Earthington *et al*., 1993). All parts of the soybean plant are susceptible to pathogens, resulting in a reduction in quality and quantity of grain yields (Sinclair and Backman, 1989).

Soybean is an economically and strategically important crop in South Africa (SA). Not only is soybean oil economically important, but soybean protein is critical to animal feeds and human nutritional supplements. Consumption of soybean in SA far exceeds production, resulting in the import of 842 107 tonnes of oilcake in the 2005/2006 soybean production period, in order to meet local demands (Joubert, 2006).

Recently, sclerotiorum-forming fungi have received much attention, due to an increase in crop losses caused by these pathogens (Willets, 1978). Fungi which form sclerotia are considered more destructive plant pathogens than non-sclerotial forming fungi, due to their ability to form sclerotia, survive adverse environmental conditions and remain viable for extended periods (Entwisle, 1987).

Sclerotinia stem rot (SSR) of soybeans, caused by the fungus *S. sclerotiorum* is an important disease of soybeans as well as numerous other plant species (Purdy, 1979; Boland and Hall, 1994). *Sclerotinia sclerotiorum* is an important pathogen as it causes substantial losses in crop production throughout the world (Boland and Hall, 1994). However, it was only recently that SSR was considered a major pathogen of soybeans (Grau and Hartman, 1999). In SA, SSR was first reported in the Badfontein area of Lydenburg (Thomson and Van Der Westhuizen, 1979). Sporadic outbreaks have recently been reported in the Winterton-Underberg areas and more commonly in the Piet Retief areas of KwaZulu-Natal (KZN), particularly in wet years. Sporadic outbreaks have also been observed on the Highveld in the Ermelo area. Outbreaks of SSR are becoming increasingly common and more severe, rapidly spreading through fields and in some instances totally destroying the crop. In 2003, SSR was so severe on the Swaziland border of KZN, farmers harvested their crop early in the season for use as silage, as it was predicted that there would be no grain yield. Sclerotinia stem rot, together with the arrival of soybean rust caused by *Phakopsora pachyrhizi* Sydow in

2001 threatens the viability of soybean production, which plays a crucial role in agriculture and the downstream industry (Caldwell, pers. comm.[1]).

1.2 BACKGROUND INFORMATION

1.2.1 History

Much controversy occurred over the naming of this fungus before it came to be known as *S. sclerotiorum*. The fungus was first described in 1837 as *Peziza sclerotiorum* (Libert) (Purdy, 1979) by Madame M. A. Libert (Wakefield, 1924). Fuckel then transferred the species *Peziza* to the genus *Sclerotinia*, which he created and described in 1870 (Purdy, 1979). The name, however, was changed to *S. libertiana* (Fuckel), as it is presumed that Fuckel disliked the combination of *S. sclerotiorum* (Wakefield, 1924). Fuckel chose to honour M. A. Libert by retaining the species as *libertiana* (Purdy, 1979). Synonyms of *S. libertiana*, such as *P. sclerotiorum* (Libert) and *P. sclerotii* (Fuckel), have also been cited by Fuckel (Willets and Wong, 1980).

This name change by Fuckel was accepted and used until 1940, when it was shown to be inconsistent with the International Rules of Botanical Nomenclature (Purdy, 1979) and, therefore, there was also no justification for the continuation of Fuckel's name (Wakefield, 1924). Massee was then cited as the correct authority of *S. sclerotiorum* (Lib.) Massee, as he used this combination in 1895 in his British Fungal Flora (Wakefield, 1924; Purdy, 1979). However, it was then identified that de Bary used this combination in his contribution in 1884, and the name was again changed to *S. sclerotiorum* (Lib) by de Bary (Purdy, 1979).

[1] Dr P. M. Caldwell, Discipline of Plant Pathology, School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of KwaZulu-Natal, Private Bag X01, Scottsville, 3209, Pietermaritzburg, South Africa

Korf and Dumont (1972) also proposed a name change, and the fungus was transferred to the genus, *Whetzelinia*, which was created by Korf and Dumont (Pratt, 1992), in honor of Professor Whetzel (Korf and Dumont, 1972). This proposal was accepted by some taxonomists, but the new genus was considered unnecessary. Subsequent proposals for name changes were put forward by Korf and Dumont (1972). However, it was recommended by the General Committee of the International Association of Plant Taxonomists (Willets and Wong, 1980) that the name *S. sclerotiorum* is used to describe this fungus (Kohn, 1979). The correct name and authority has been concluded to be *S. sclerotiorum* (Lib.) de Bary (Purdy, 1979).

1.2.2 Economic importance

Sclerotinia sclerotiorum has been recognized for many years as a serious pathogen of numerous vegetables both in the field and during their transit (Willets and Wong, 1980). With the expansion of soybean production into regions where other susceptible crops are grown, concern about *S. sclerotiorum* has increased (McGee, 1992). Sclerotinia stem rot, previously considered a minor pathogen of soybeans (Grau and Hartman, 1999), has recently become an increasingly important yield limiting disease of soybeans (Kurle *et al*., 2001).

An increase in SSR incidence may be due to numerous factors, e.g., the cultivation of soybeans following sclerotinia-susceptible crops, the expansion of soybean production into areas with favourable environmental conditions for disease development and the introduction of cultural practices such as irrigation and narrower row spacing (Boland and Hall, 1987). Crop losses due to SSR range from 0-100% (Purdy, 1979) and are dependent on susceptible varieties and environmental conditions conducive for pathogen development (Bell *et al*., 1990). Disease severity is positively correlated with the number of sclerotia and ascospores within infested soil (Boland and Hall, 1988a) and prolonged periods of foliar wetness, during and after crop flowering (Boland and Hall, 1988a).

Serious crop losses have been reported in China, France and SA (McGee, 1992). In Argentina, SSR on soybeans was considered the most important disease until the mid 1990s, where yield losses of up to 55% were reported. The impact of this disease has decreased due to the implementation of improved management practices (Plopper, 2004).

In 1994, the total losses due to SSR in the top ten soybean producing countries was 1 061 200 tonnes. Yield losses were greatest in China and Argentina, with losses of 77 300 tonnes of the 16 x 10^6 tonnes produced and 183 300 tonnes of the 12.40 x 10^6 tonnes produced, respectively (Wrather *et al*., 1997). In 1998, the total number of tonnes of soybeans lost in the top ten soybean producing countries increased to 14 742 000 tonnes. Yield losses were greatest in India and Argentina, with losses of 438 560 tonnes of the 5.7 x 10^6 tonnes produced and 423 200 tonnes of the 17 x 10^6 tonnes produced, respectively (Wrather *et al*., 2001).

In 1994, SSR was also ranked as the leading cause of soybean yield loss in the North Central soybean producing area in the USA, after soybean cyst nematode (Yang *et al*., 1999). Sclerotinia stem rot was still ranked as the second most yield-reducing soybean disease in the USA in 2004, causing a yield loss of 1.63 million tonnes (Chen and Wang, 2005).

1.2.3 Geographic distribution

Sclerotinia stem rot is known to occur in all cool and moist areas of the world where soybeans are grown (Mordue and Holliday, 1976). The pathogen was first reported on soybeans in 1924 in Hungary (Grau and Hartman, 1999). In the USA, the pathogen was first observed in 1946 in Illinois (Hartman *et al*., 1999) and Ontario, but was not considered a threat to crop production (Boland and Hall, 1988b). Later, severe localized outbreaks were reported in Arizona, Minnesota, Virginia and Wisconsin in the USA. The pathogen has since spread to neighbouring states. However, SSR is limited to northern soybean producing areas of the USA (Hartman *et al*., 1999). It has since been reported

in Argentina, Brazil, Canada, China, India, Italy, Nepal (Grau and Hartman, 1999) and South Africa (Thompson and van der Westhuizen, 1979).

1.3 THE PATHOGEN

1.3.1 Taxonomy and morphology

Sclerotinia stem rot is caused by the fungus *Sclerotinia sclerotiorum*, which belongs to the Kingdom Fungi, Division Eumycota, Subdivision Ascomycotina, Class Discomycetes, Order Heliotales, Family Sclerotiniaceae, Genus *Sclerotinia* and species *sclerotiorum* (Agrios, 1997).

Three types of germination from sclerotia for the genus *Sclerotinia* can be distinguished, these being determined by the final structure produced (Coley-Smith and Cooke, 1971). Germination of sclerotia may be myceliogenic, sporogenic, where asexual spores (conidia) are produced and carpogenic, where sporocarps in the form of apothecia producing asci and ascospores develop (Willets and Wong, 1980).

However, in *S. sclerotiorum* only carpogenic and myceliogenic germination occurs (Le Tourneau, 1979). Hence, the various structures produced by *S. sclerotiorum* are sclerotia, mycelia and ascospores (Grau and Hartman, 1999).

Sclerotia may be defined as asexual, multicellular (Chet and Henis, 1975), vegetative resting bodies which are composed of a compact mass of thick-walled, interwoven, special sized hyphal cells (Shurtleff and Averre, 1997). Sclerotia (Figure 1.1), are formed by the aggregation of mycelia (Grau, 1988) and appear as hard, black and irregularly shaped structures (McGee, 1992).

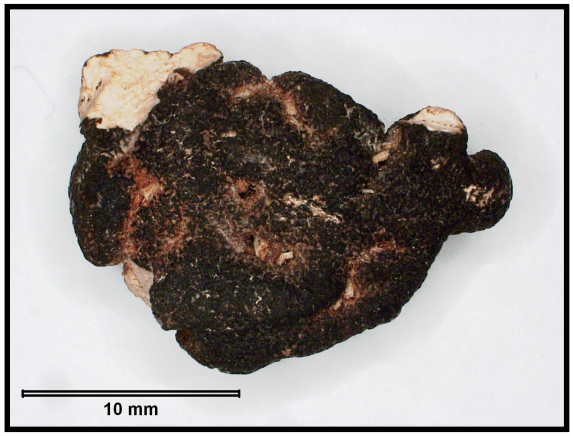

Figure 1.1 Vegetative sclerotia, of *Sclerotinia sclerotiorum* (Photograph by Dael Visser).

Sclerotia are 2-20 mm in diameter (Grau and Hartman, 1999). They may form on, or within, diseased soybean tissue and function as resting structures (Grau, 1988).

Mycelia (Figure 1.2) from sclerotia placed on potato dextrose agar (PDA) are typically white or pale grey (Mordue and Holliday, 1976).

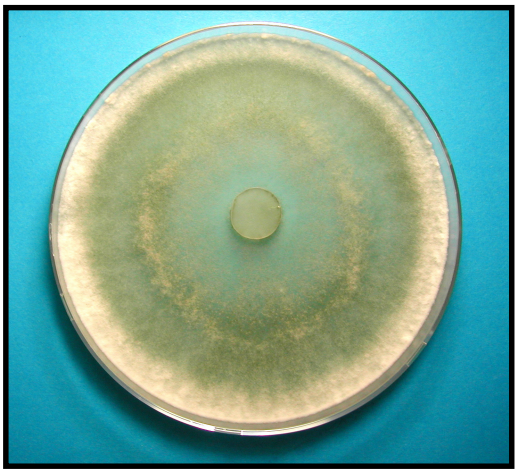

Figure 1.2 White and pale grey mycelium on potato dextrose agar from a single sclerotium of *Sclerotinia sclerotiorum* (Photograph by Dael Visser).

Stipes arise from sclerotia to form cup-shaped apothecia (Figure 1.3), which are 0.5-2 mm in diameter and light to tan-brown in colour (Anonymous, 2005a).

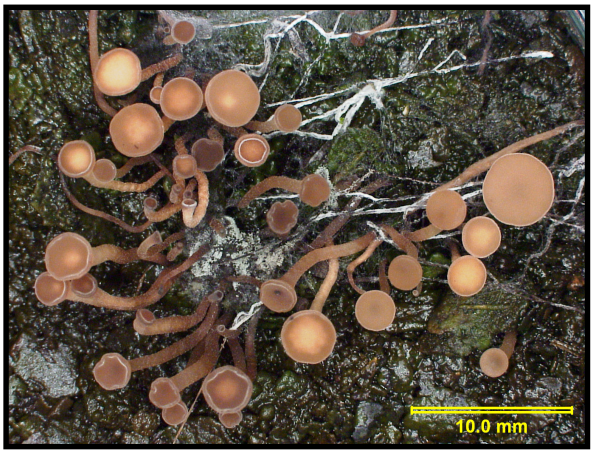

Figure 1.3 Cup-shaped apothecia, which result from the carpogenic germination of sclerotia of *Sclerotinia sclerotiorum* (Photograph by Dael Visser).

The hymenium consists of closely stacked, narrow and cylindrical asci (Figure 1.4), which are 4-22 x 81-252 µm in size, and are interspersed with slender hyaline paraphyses (Grau and Hartman, 1999). Asci produce eight ascospores (Figure 1.5), which are 9-15 x 4-7 µm in size (Domsch *et al*., 1980).

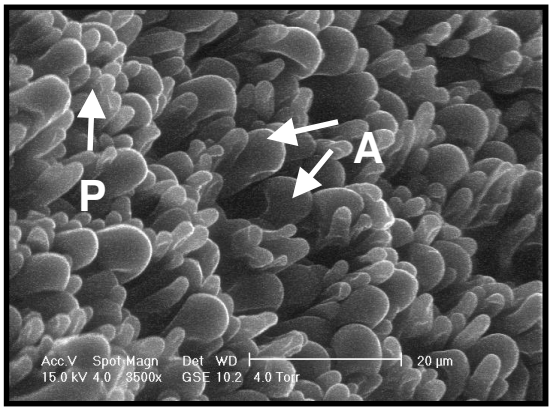

Figure 1.4 Scanning electron micrograph of a mature apothecium of *Sclerotinia sclerotiorum* showing asci (A) and paraphyses (P) (Photograph by Dael Visser).

Figure 1.5 Cryogenic longitudinal section of a mature apothecium of *Sclerotinia sclerotiorum* showing eight ascospores in an ascus (Photograph by Dael Visser).

1.3.2 Symptoms

Symptoms may be observed on stems, leaves, pods and seed (McGee, 1992). Initial symptoms of SSR are visible during pod development (Steadman *et al*., 1996), where the sudden wilting of plants or small groups of infected plants in irregular patches in a field occurs(Martens *et al*., 1994). This can be seen in Figure1.6.

Figure 1.6 Wilting of soybean plants infected with *Sclerotinia sclerotiorum*, in irregular patches within a field (Anonymous, 2004).

In years following initial infection, the size and number of these patches of diseased plants increases. In extreme cases, large areas of a field may be killed prematurely (Scott *et al*., 2005). Blossoms are the primary site of infection (Steadman *et al*., 1996). Leaves become greyish green and eventually become necrotic, tattered and curled, remaining attached to plants (Grau and Hartman, 1999). Foliar symptoms of SSR may easily be confused with late season phytopthora root rot, brown stem rot and stem canker (Grau, 1988).

Stem lesions develop at nodes approximately 10-50 cm above the soil line (Grau and Hartman, 1999). These appear grey and water-soaked (Grau, 1988). Lesions may change from tan to white (McGee, 1992) as the disease progresses in stem tissues. This results in the development of a fluffy white mould (Figure 1.7) on the surface of infected stems (Scott *et al*., 2005).

Figure 1.7 Appearance of fluffy white mould on the surface of infected soybean stems infected with *Sclerotinia sclerotiorum* (Steadman *et al*., 1996).

Stems become girdled with lesions (Scott *et al*., 2005), which disrupts the transport of water, mineral nutrients and photosynthates to developing pods. This results in a reduction in pod development and pod fill above stem lesions (Grau and Hartman, 1999). At crop maturity, stems appear white and shredded (McGee, 1992). A reddish discolouration is frequently interspersed within diseased stem tissues and at the borders of lesions (Grau, 1988).

Sclerotia may develop on stems in the white mould growth (Figure 1.8) and within the pith (Martens *et al*., 1994) and occasionally in pods (Scott *et al*., 2005).

Figure 1.8 Formation of sclerotia of *Sclerotinia sclerotiorum* on infected soybean stem (Anonymous, 2004).

Diseased pods are outwardly white in appearance, with mycelia and sclerotia are readily observed within (Grau, 1988). Seeds appear flattened and shrivelled and may be replaced by sclerotia (Grau and Hartman, 1999).

1.3.3 Host range

Sclerotinia sclerotiorum can infect a wide range of plants including crop, vegetable, ornamental, fruit and weed species (Table 1.1) (Scott *et al*., 2005). The index of plant hosts of *S. sclerotiorum* contains 408 species, 42 subspecies, 75 families and 278 genera. All hosts occur in the Gymnospermae and Angiospermae classes, except for one species in the Pteridophyta, of the division Spermatophyta. Although rare, monocotyledonous plants, such as plants from the Arum, Grass, Iris, Lily and Banana Families, in the subclass Monocotyledonae, may also be considered hosts of *S. sclerotiorum* (Boland and Hall, 1994).

Table 1.1 Partial list of agronomic and vegetable crops on which *Sclerotinia sclerotiorum* has been reported (Grau, 1988).

Crop	Scientific name
Table beet	*Beta vulgaris*
Rapeseed (canola)	*Brassica napus*
Cole crops (cauliflower etc)	*Brassica oleracea*
Watermelon	*Citrullus vulgaris*
Muskmelon	*C. melo*
Cucumber	*C. sativus*
Winter squash	*C. maxima*
Pumpkin	*C. pepo*
Summer squash	*C. pepo* var. *melopepo*
Peppermint	*Mentha piperita*
Crownvetch	*Coronilla varia*
Lentil	*Lens culinaris*
Birdsfoot Trefoil	*Lotus corniculatus*
Alfalfa	*Medicago sativa*
Sweetclovers	*Melilotus* spp.
Sainfoin	*Onobrychis viciifolia*
Scarlet runner bean	*Phaseolus coccineus*
Lima bean	*P. limensis*
Green and dry bean	*P. vulgaris*
Pea	*Pisum sativum*
Field pea	*P. sativum*
Clovers (red, white etc.)	*Trifolium* spp.
Cowpea	*Vigna sinensis*
Onion	*Allium cepa*
Flax	*Linum flavum*
Cotton	*Gossypium hirsutum*
Peanut	*Arachis hypogea*
Buckwheat	*Fagopyrum esculenthum*
Tomato	*Lycopersicum esculentum*
Tobacco	*Nicotiana tabacum*
Potato	*Solanum tuberosum*
Carrot	*Daucus carota* var. *sativa*
Parsnip	*Pastinaca sativa*
Sunflower	*Helianthus annuus*
Lettuce	*Lactuca sativa*

Alternative hosts include weeds such as bleedingheart, buckhorn, black medic, cocklebur, common chickweed, chicory, chokeberry, crabgrass, curled dock, dandelion, dog fennel, evening primrose, garlic goldenrod, horse nettle, Jerusalem artichoke, lambsquaters, milkweed, mullein, mustard (black), oxeye daisy, parsnip, penneycross, peppergrass, pigweed, purslane, prickely lettuce, ragweed, sheperdspurse, thistles, wild sweet potato, velvetleaf and yellow rocket (Scott *et al*., 2005), which may contribute towards inoculum build-up (Boland and Hall, 1994).

1.3.4 Dissemination

1.3.4.1 Short distance dissemination

The major means of dissemination from field to field of SSR is by windblown ascospores (Adams and Ayers, 1979). SSR may also be disseminated in the form of sclerotia or mycelia in infected host tissue by soil adhering to seedlings, contaminated equipment, animals or humans (Agrios, 1997). Dispersal distances of ascospores have been found to range between 25 m to several hundred meters (Wegulo *et al*., 2000).

Yang *et al.* (1997) found that ascospores cause infection of soybeans in a field 50 m away from the source of inoculum. In other studies by Yang *et al*. (1992), results showed that 12% of infected seeds produced sclerotia. Therefore, it was concluded that internally infected soybean seed may serve as a means of dissemination of *S. sclerotiorum* (Yang *et al*., 1992).

Wegulo *et al*. (2000), found that disease incidence decreased with distance from point sources of apothecial inoculum. Disease was observed at a maximum of 10-12 m from the edges of inoculum areas. The results from Wegulo *et al*. (2000) are in agreement with studies by Hartill (1980) and Suzui and Kobayashi (1972), indicating that most ascospores are deposited within a few metres of the inoculum source, thereby implying that short distance dissemination is mainly due to the production of ascospores within the field (Wegulo *et al*., 2000).

1.3.4.2 Long distance dispersal

The greatest potential for long distance dispersal of SSR is via mycelia or sclerotia mixed with seed (Adams and Ayers, 1979). Long distance dispersal (several tens to hundreds of metres) may be from many inoculum point sources making up an area source (Wegulo *et al*., 2000).

1.4 INFECTION PROCESS AND EPIDEMIOLOGY

1.4.1 Life cycle and infection process

Three principal modes of infection by *S. sclerotiorum* have been identified. Infection may result from infection at the stem base from mycelia that develops from germinating sclerotia (Blodgett, 1946; Purdy, 1958), germination and penetration of ascospores at wound sites (Mclean, 1958; Abawi and Grogan, 1975) and the germination of ascospores on senescent flowers or leaves and organic matter in contact with the host (Purdy and Bardin, 1953; Mclean, 1958; Newton and Sequeira, 1972; Abawi and Grogan, 1975; Abawi *et al*., 1975). However, it is the germination of ascospores on senescent flowers which is epidemiologically important as the majority of epidemics occur after flowering (Sutton and Deverall, 1983).

The fungus remains dormant during unfavourable environmental conditions in the form of sclerotia or as mycelia in infected plant residues. Sclerotia may survive on or in the soil (Scott *et al*., 2005) or in debris (Steadman *et al*., 1996) for approximately seven years (Scott *et al*., 2005), thereby assuring inoculum when the host crop is planted or conditions are favourable for germination (Steadman, 1983).

The life cycle of *S. sclerotiorum* is shown in Figure 1.9. According to environmental conditions, sclerotia either undergo myceliogenic or carpogenic germination. Myceliogenic germination results in the production of mycelial strands from sclerotia that infect young stems directly (Agrios, 1997).

Carpogenic germination of sclerotia occurs after prolonged periods of wet and cool weather. Sclerotia on, or just below, the soil surface germinate by means of a stipe (Scott *et al*., 2005). Stipes develop from small centra or nests of hyphae within sclerotia (Kosasih and Willets, 1975) and grow towards the soil surface (Anonymous, 2005a). Stipe primordia form mainly in the cortex of sclerotia, but may also develop within the medulla. Hyphae in the primordia grow rapidly, resulting in the appearance of raised areas on the surface of sclerotia.

Continued growth of these initials causes the rind to rupture, resulting in the appearance of stipes (Figure 1.10). Many primordia develop, but not all form stipes (Phillips, 1987).

Figure 1.10 The initial stages of carpogenic germination of sclerotia in *Sclerotinia sclerotiorum*, where the elongation of raised areas on sclerotia leads to stipe formation (Photograph by Dael Visser).

As the tips of the stipes are exposed to sunlight, they differentiate into cup-shaped apothecia (Figure 1.11). Apothecia are usually produced after the crop canopy has partially closed, thereby shading the soil surface (Grau and Hartman, 1999), where the microclimate is suitable for production and discharge of ascospores (Willets and Wong, 1980).

Figure 1.11 Fully developed apothecia from germinated sclerotia of *Sclerotinia sclerotiorum*, where ascospore dispersal may now occur (Photograph by Dael Visser).

Asci are produced in fully developed apothecia (Anonymous, 2005a) and ascospores are forcibly ejected from asci (Grau and Hartman, 1999) and dispersed by air currents over short distances (Willets and Wong, 1980). Air-borne ascospores land on flowers, stems, branches or pods of soybean plants and cause infection (Grau and Hartman, 1999) during the reproductive phase of soybean growth (Kurle *et al*., 2001).

Usually, ascospores germinate by means of a germ tube, which subsequently swells to form an appressorium (Lumsden, 1979). This formation of appressoria has been shown during infection of beans, lettuce, oilseed rape and chrysanthemums (Tariq and Jeffries, 1984). However not many detailed studies on the infection process in soybeans exists (Sutton and Deverall, 1983).

In infection studies reported by Sutton and Deverall (1983), infection of soybeans by *S. sclerotiorum* commences with short germ tubes which were subpolar to ascospores, and occasionally central to ascospores. Scanning electron microscopy (SEM) studies indicated the presence of a sheath (Figure 1.12), which arises from the fungus, and may

aid in the attachment of the ascospore to the host. Neither SEM nor optical microscopy revealed a morphologically distinct appresorium (Sutton and Deverall, 1983).

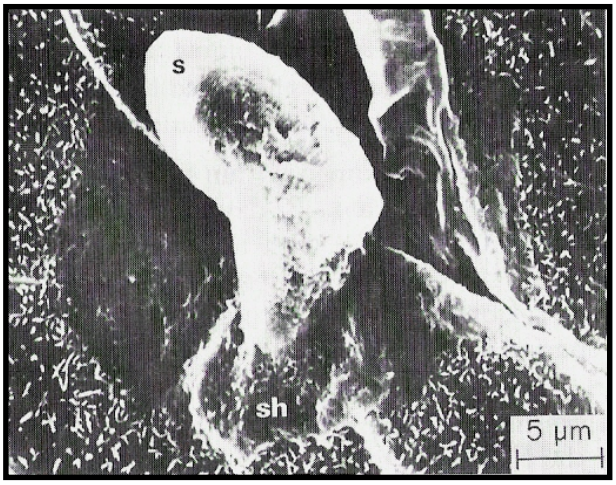

Figure 1.12 Scanning electron micrograph of a trifoliate soybean leaf, 24hrs post inoculation, showing a germinated ascospore (s) of *Sclerotinia sclerotiorum* with surrounding sheath (sh) (Sutton and Deverall, 1983).

As ascospores on leaf surfaces begin to germinate, the cytoplasm of epidermal cells begins to turn brown. Slight granulation also occurs. Sinking of the leaf surface at the infection sites to below the level of the surrounding unaffected epidermal cells occurs due to the collapse of groups of necrotic cells. Large necrotic areas may be apparent, due to the germination of several ascospores in close proximity. The necrotic epidermal cells are then penetrated by fine hyphae that arise from the distal end of the germ tube, which is in contact with the leaf surface (Figure 1.13). Penetrating hyphae form swollen hyphae within the necrotic epidermal cells (Figure 1.14) (Sutton and Deverall, 1983).

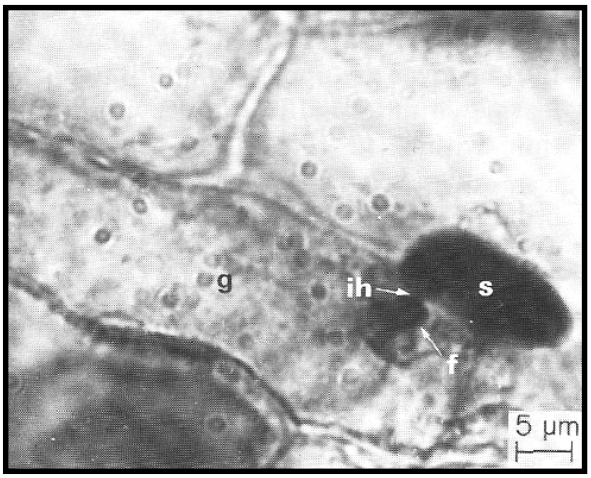

Figure 1.13 Penetration of a soybean leaf epidermal cell by a fine infection hypha (ih), arising from a germinated ascospore (s) of *Sclerotinia sclerotiorum*. An intercellular hypha (f) develops within the cell and granulation of the penetrated cell (g) occurrs (Sutton and Deverall, 1983).

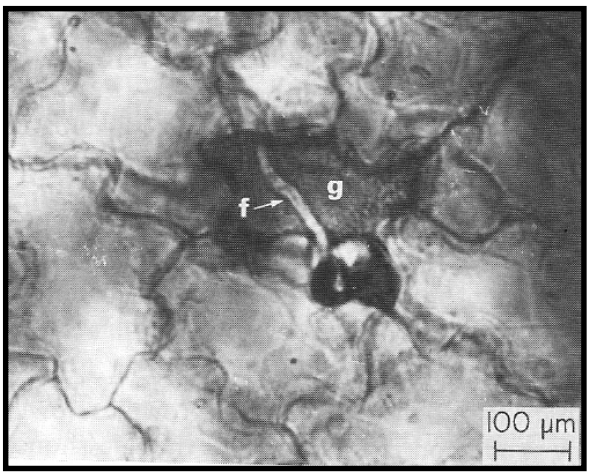

Figure 1.14 Intercellular hyphae (f) of *Sclerotinia sclerotiorum* are limited to penetrated granular epidermal cell (g), 21 days post inoculation (Sutton and Deverall, 1983).

Ascospores that are deposited on flower petals, germinate when free water is present on plant surfaces. Petals are used as an exogenous nutrient base (Abawi and Grogan, 1979; Boland and Hall, 1988b). Mycelia colonize flowers and thereafter spread throughout the host (Sutton and Deverall, 1983). Plant tissue becomes macerated by oxalic acid which is released by advancing mycelia (Abawi and Grogan, 1979; Boland and Hall, 1988b).

Once infection of flowers, pods or leaves has occurred, mycelia grow into plant structures (Anonymous, 2005a) and progress towards the main stem (Scott *et al*., 2005). Plant tissues become damaged, particularly in the vascular system. Portions of the stem, above the area of infection die (Anonymous, 2005a). Ramifying hyphae emerge from host tissue (Lumsden, 1979) appearing as a white fungal mycelial mat that is commonly observed on stems (Anonymous, 2005a). As nutrients become exhausted, fungal mycelia aggregate into sclerotia, which may form within or on the surface of the stem (Kurle *et al*., 2001).

Three developmental phases are distinguished during sclerotial formation, i.e., initiation, development and maturation (Le Toureneau, 1979; Willets and Wong, 1980). During initiation (Phase 1), a white mass of interwoven mycelial strands form, as seen in Figure 1.15 (Colotelo, 1974; Chet and Henis, 1975; Le Toureneau, 1979, Willets and Wong, 1980).

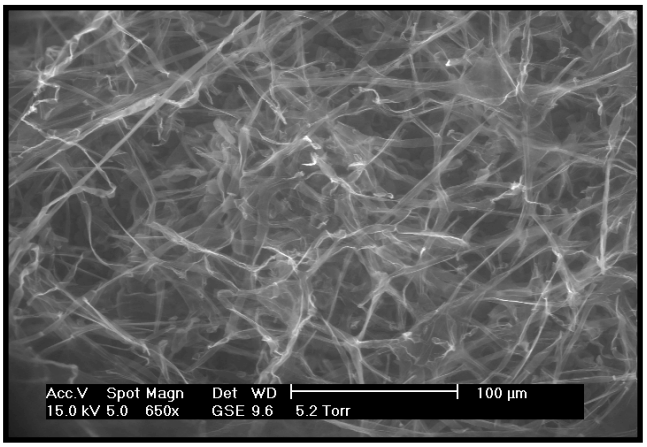

Figure 1.15 Interwoven white mass of mycelia of *Sclerotinia sclerotium*, at the initial stages of development of a sclerotium (Photograph by Dael Visser).

It is believed that the initiation of sclerotial formation is due to contact of growing mycelia with a mechanical barrier (Chet and Henis, 1975; Willets and Wong, 1980). *In vitro* formation of sclerotia occurs when the colony reaches the edge of the container in which it is growing (Le Tourneau, 1979). In nature, sclerotia form in host debris when there is a depletion of nutrients or other factors such as light, temperature, pH (Chet and Henis, 1975; Willets and Wong, 1980), oxygen and carbon dioxide, staling products, microbial excretions and antibiotics, internal morphogenetic factors, hyphal branching, interweaving and fusion of hyphae, translocation, sclerotial exudation, carbon dioxide metabolism, protein synthesis, enzyme action and changes in RNA (Chet and Henis, 1975).

The growth/development phase (Phase 2), results in the formation of a full-sized sclerotium (Chet and Henis, 1975; Le Tourneau, 1979). These interwoven mycelial strands are covered with droplets of liquid, each of which is surrounded by membranous material. Sub-surface mycelial cells swell and become pigmented, resulting in the formation of bulbous rind cells (Figure 1.16) which darken with age (Colotelo, 1974).

As the size and number of swellings of peripheral sub-surface cells increase, a complete rind is formed. Rind cells are observed to be prominent beneath the surface mycelial network with a membranous material overlying the rind cells (Colotelo, 1974).

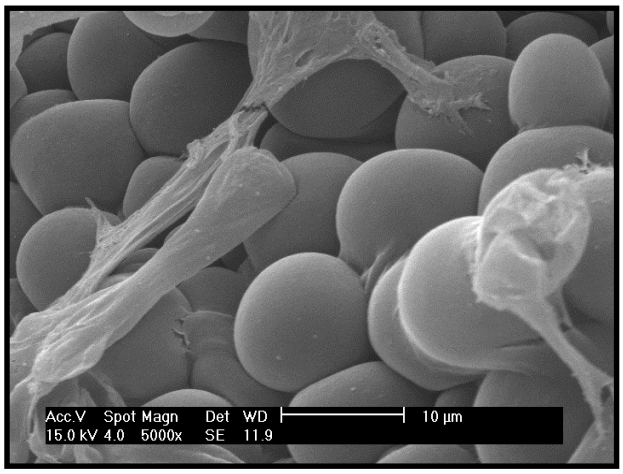

Figure 1.16 Surface of a sclerotium, showing dark bulbous rind cells of *Sclerotinia sclerotiorum* (Photograph by Dael Visser).

Although the exterior of young sclerotia are loosely woven, the interior is compact (Colotelo, 1974). As sclerotia mature, structural and storage polysaccharides are deposited, a pigment is formed and tissues dehydrate (Willets and Wong, 1980). The exudates of sclerotia contain cations, lipids, ammonia, amino acids, proteins and various enzymes (Colotelo *et al*., 1971).

When mature (Phase 3), sclerotia consist of a black rind (Colotelo, 1974), 2-3 cells wide, a cortex of 2-4 cells wide and a medullary region with numerous darkly stained, loosely interwoven hyphae imbedded in an amorphous matrix (Huang, 1983). Once mature, sclerotia are capable of resisting adverse conditions, and serve as resting structures (Willets and Wong, 1980).

Sclerotia may be dislodged by wind or during harvesting and be deposited on the soil surface (Schwartz and Steadman, 1978) or they may remain with seed during harvesting (Anonymous, 2005a). Sclerotia may be distributed throughout the vertical soil profile by tillage practices and between fields via humans, animals, irrigation runoff water and contaminated equipment (Schwartz and Steadman, 1978).

Sclerotia may then undergo carpogenic or myceliogenic germination and continue the life cycle, or remain in the soil or on crop debris until favourable conditions for germination occur (Anonymous, 2005a).

1.4.2 Epidemiology

The disease cycle of any pathogen, beginning at spore germination and ending in colonization and sporulation, is influenced by biotic factors of the pathogen and host, as well as abiotic factors of the environment (Bromfield, 1984).

As infection of soybeans by SSR may either be via sclerotia or ascospores, the epidemiology of these two types of infection is different and the effects of weather on their incidence and development differs considerably (Abawi and Grogan, 1979). In order to identify and implement possible control measures, it is imperative that the epidemiology of *S. sclerotiorum* is understood. However, information on optimum environmental conditions for disease development of soybeans by SSR is limited (Boland and Hall, 1988a; Grau and Hartman, 1999).

Sclerotia are known to be highly resistant to dry heat of up to 70˚C and prolonged periods of freezing and thawing (Grau and Hartman, 1999). For apothecial development, low soil temperatures of 5-15˚C and soil matrix potentials of less than - 5 bars for 10-14 days are optimum. Sun and Yang (2000) found that at a light intensity of 160-190 $mol^{-1}ms^{-1}$ and 20˚C, the highest number of apothecial initials were produced. Greater than 80% of these initials developed into apothecia at 25˚C. The greatest number of apothecia were produced at 20˚C with a high light intensity. Apothecia at a high light intensity were larger in size than those produced at a low light intensity.

Results suggest that apothecia may be produced at a wide moisture range at a low light intensity, but at a high light intensity, more apothecia are produced at a broader temperature-moisture range (Sun and Yang, 2000).

A method for inducing apothecia from sclerotia has been developed by Mylchreest and Wheeler (1987). Mature sclerotia must be preconditioned at 4˚C for 4 weeks, kept in moist compost at 10˚C for approximately 6 weeks and then be exposed to near-UV light at 22˚C. Although this method was developed for sclerotia from oilseed rape, 35 other isolates were also tested using this procedure. For sclerotia from soybeans, 8 weeks at 10˚C was required for stipe production but only 34 apothecia from the 40 sclerotia were produced. Although fewer apothecia were formed, compared to the number on the original isolate from oilseed rape for which the method was developed, this method gives an indication of optimal environmental conditions required for apothecial production (Mylchreest and Wheeler, 1987).

Generally, prolonged cool and wet conditions are the primary factors influencing SSR development via ascospores (Blad *et al*., 1978; Boland and Hall, 1988b). Of the few studies done on ascospores of *S. sclerotiorum* on soybeans, canopy temperatures of <28℃, coupled with a leaf wetness durations (LWD) of 12-16 hr reoccurring on a daily basis or a continuous LWD of 42-72 hr are optimal (Grau and Hartman, 1999). Harikrishnan and del Rio (2006) found that at low RH's (25% RH), ascospores were less efficient than mycelia in causing disease, postulating that ascospores need free standing water to germinate, while mycelia are more tolerant to desiccation. Both 18℃ and 22℃ were conducive for infection via ascospores. However, at 18℃ disease developed faster (Harikrishnan and del Rio, 2006).

Boland and Hall (1988a) concluded that the development of the crop, reproduction of the pathogen and initiation of disease and optimum environmental factors are all interrelated. It is the development of a closed crop canopy that provides extended periods of high soil moisture, and is a prerequisite for carpogenic germination and apothecial development (Boland and Hall, 1988a).

1.5 DISEASE MANAGEMENT

No single disease management practice effectively prevents infection of soybeans by *S. sclerotiorum* (Steadman *et al*., 1996). However, the integration of various measures may reduce disease severity and minimize yield loss (Steadman *et al*., 1996). Management practices (Figure 1.17) may be implemented at every stage in the pathogen's life cycle, ranging from prevention of sclerotial development to preventing apothecial formation and ascospore germination (Anonymous, 2005b).

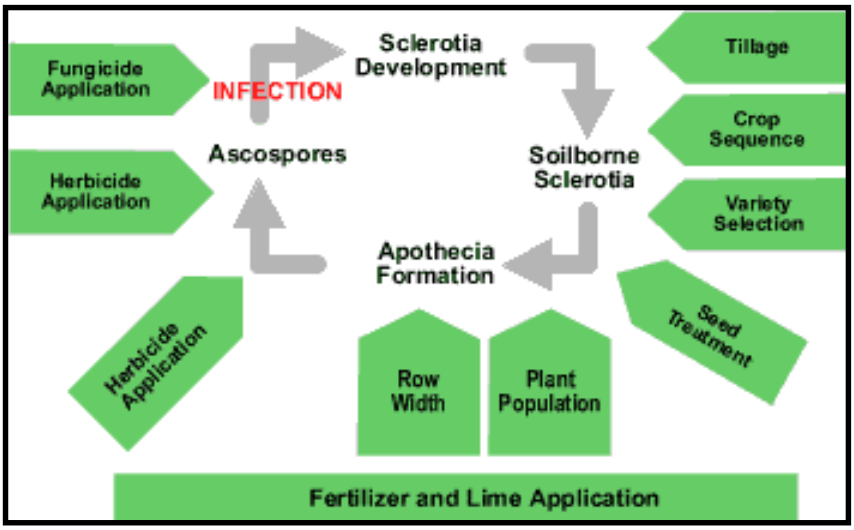

Figure 1.17 Soybean crop management interactions with *Sclerotinia sclerotiorum* of soybeans (Anonymous, 2005b).

1.5.1 Chemical control

Foliar fungicides

In order for fungicides to work effectively to control SSR, it is imperative to ensure complete coverage of blossoms (Mueller *et al*., 2002). In Brazil, foliar fungicides such as procimidone, iprodione and vinclozolin have been used successfully to reduce infection. However, problems occur with ensuring correct coverage (McGee, 1992). In SA, procymidone (300 ml 100 ℓ^{-1}) is currently registered for SSR control, at the onset of

flowering (Nel *et al*., 2003). In the USA, benomyl and thiophanate methyl applied at flowering are registered for control of SSR on soybeans (Grau, 1988). Trials by Wegulo *et al*. (1996) showed that benomyl was effective in decreasing disease incidence by 59%. Soybean yields increased by 37% when plants were sprayed with benomyl, compared to the untreated control (Wegulo *et al*., 1996). Benomyl and thiophanate methyl are effective for control of SSR in dry beans and canola. They are both effective on soybeans, but are not economically viable (Anonymous, 2005b).

Trials to determine the effects of iprodione on mycelia of *S. sclerotiorum* showed that the mycelial growth of the fungus in both solid and liquid media was inhibited by 3 µM of the fungicide (Reilly and Lamoureux, 1981). In trials by Mueller *et al*. (1999), the effects of six fungicides, i.e., captan, fludioxonil, metalaxyl, pentachloronitrobenzene (PCNB), thiabendazate and thiram on mycelial on fungicide amended agar were tested. Fludioxonil was the most effective fungicide for decreasing the radial growth of *S. sclerotiorum*. However, the radial growth of *S. sclerotiorum* was significantly reduced on all agar plates amended with the test fungicides compared to the non-amended control (Mueller *et al*., 1999).

In vitro studies to determine the effect of four fungicides i.e., benomyl, tebuconazole, thiophanate methyl and vinclozolin on the radial growth of *S. sclerotiorum* on fungicide amended agar showed that vinclozolin inhibited mycelial growth at 1 µg a.i. $m\ell^{-1}$ of agar, benomyl and tebuconazole inhibited mycelial growth at 10 µg a.i. $m\ell^{-1}$ of agar and thiophanate methyl inhibited growth at 50 µg a.i. $m\ell^{-1}$ of agar (Mueller *et al*., 2002).

In greenhouse studies, soybean seedlings infected with *S. sclerotiorum* and treated at the V2 stage with benomyl, thiophanate methyl and vinclozolin at recommended rates, did not express symptoms of SSR. Infected soybeans treated with tebuconazole developed lesions on the leaves, but no symptoms were observed on stems. The untreated control showed foliar symptoms which resulted in defoliation, fungal colonization of stems and some dead plants. Although this inoculation technique, i.e., using mycelia to cause natural infection resulting from direct contact within plants,

thereby initiating secondary infection does not involve the primary source of infection, i.e., ascospores, these results have identified a potential method of controlling secondary spread of SSR (Mueller *et al*., 2002). Stipe development may be inhibited by the application of benomyl, dichlozine, quitozene, thiophanate methyl and PCNB (Partyka and Mai, 1962).

Foliar herbicides

The application of lactofen (Cobra) at the beginning of flowering reduces SSR severity and increases yield. However, soybean cultivar and SSR severity determines the effectiveness of Cobra (Anonymous, 2005b).

Soil fumigants

The application of PCNB to soil decreases sclerotial germination (Scott *et al*., 2005). Dazomet (Jones, 1974), metam-sodium, chloropicrin and methyl bromide (Partyka and Mai, 1958) prevent carpogenic germination and are fungicidal (Radke and Grau, 1986). Herbicides such as linuron and DNBP applied to soil, were found to inhibit germination of sclerotia and apothecial development (Radke and Grau, 1986).

1.5.2 Biological control

Soilborne fungi are commonly known to invade sclerotia (McGee, 1992). Fungi that are effective against *S. sclerotiorum* include *Trichoderma harzianum* Rifai, *T. viride* Pers., *T. koningii* Rifai, *T. pseudokoningii* Rifai (Jones and Watson, 1969; dos Santos and Dhingra, 1982, Artigues and Davet, 1984; Zazzerini and Tosi, 1985; Davet, 1986), *Gliocladium virens* Miller & Foster (Tu, 1980; Phillips, 1986; Artigues and Davet, 1984), *G. roseum* (Link) Bain. (Ervio *et al*., 1964; McCredie and Sivasithamparam, 1985), *Coniothyrium minitans* Campbell (Huang and Hoes, 1976; Turner and Tribe, 1976; Tu, 1984; Huang and Kokko, 1987, Campbell, 1947; Whipps and Gerlagh, 1992; Huang, 1980; Budge and Whipps, 1991), *Paecilomyces lilacinus* (Thom) Samson (Karhuvaara 1960; Ervio *et al*., 1964) and *Sporidesmium sclerotivorum* Uecker, Ayers and Adams (Uecker *et al*., 1978; Adams, 1989). Recently, a nematode-trapping Hyphomycete,

Monacrosporium janus sp. Nov., has been identified as a potential biocontrol agent of sclerotia and hyphae of *S. sclerotiorum* on soybeans (Li *et al*., 2003).

1.5.3 Resistance

No soybean varieties with resistance are currently available (Grau and Bissonnette, 1974; Steadman *et al*., 1996). However, tolerance to SSR has been identified (Grau *et al*., 1982; Boland and Hall, 1987; Nelson *et al*., 1991). As commercial cultivars are currently limited in their resistance to SSR, yield losses may not be completely prevented (Hoffman *et al*., 2002). The inheritance of resistance to SSR in soybeans is not fully understood and hence the development of resistance breeding is currently limited (Kim and Diers, 2000).

Problems exist in the use of inoculation techniques to identify resistance as well. Most inoculation techniques and controlled environment screening methods have not consistently predicted field reactions to SSR on soybeans (Nelson *et al*., 1991; Wegulo *et al*., 1998; Kim *et al*., 2000) and dry beans (Vuong *et al*., 2004). Under field conditions, reactions of cultivars to SSR are the result of physiological resistance and escape mechanisms (Boland and Hall, 1987), whereas under glasshouse or laboratory conditions, cultivar reactions are due to physiological resistance only, as there is little chance for escape (Grau and Bissonnette, 1974; Nelson *et al*., 1991), thus resulting in inconsistent disease ratings in the field (Kim *et al*., 2000).

Of all the methods tested, the cut stem inoculation technique has proven to be the most reliable (Kull *et al*., 2003). Kull *et al*. (2003) showed that of three different inoculation techniques tested (i.e., mycelial plug inoculations of cotyledons, cut stem and detached leaves), the cut stem method was statistically proven to be better than the other two methods for determining cultivar resistance under controlled environmental conditions (Kull *et al*., 2003). Greenhouse inoculation techniques, however, do provide preliminary information on the resistance of cultivars (Kim *et al*., 2000).

The use of ascospores to identify resistance also showed no correlation between glasshouse and field studies. Soybean flowers inoculated with ascospores aborted, thereby preventing subsequent spread of *S. sclerotiorum* towards the stem (Cline and Jacobsen, 1983). Considering this, and the difficulty in producing and storing ascospores, such trials with ascospores are not practical. In trials with ascospores, Rousseau *et al*. (2004) also found no correlation between flower inoculation and stem inoculation, suggesting that there are two distinct resistant components to *S. sclerotiorum* expressed in the flower as well as the stem of soybeans (Rousseau *et al*., 2004).

1.5.4 Cultural control

Sanitation

Sanitation involves all activities which may eliminate or decrease the amount of inoculum present in a plant, field or warehouse and prevent the spread of the fungus to uninfected plants (Agrios, 1997). Hence, all equipment must be cleaned of soil which may contain sclerotia and plant debris when moving between fields (Scott *et al*., 2005). Certified seed should also be used, thereby reducing the risk of introducing the pathogen into previously uninfested fields, as sclerotia may often be mixed with soybean seed, as seen in Figure 1.18 (Steadman, 1979).

Figure 1.18 Contaminated seed, showing soybean seed mixed with sclerotia (Steadman *et al*., 1996).

Rotation

Crop rotation is not considered effective for SSR due to the longevity of sclerotia (Steadman *et al*., 1996). Sclerotinia stem rot also has a wide host range therefore limiting the diversity of crops which may be used in rotation (Willets and Wong, 1980). Planting of soybeans after SSR infected crops should be avoided (Scott *et al*., 2005).

Monocotyledons, such as maize and sorghum, which are not hosts of SSR, may be used as rotation crops, if crop rotation is to be implemented. Continuous cropping of soybeans in fields with a history of SSR should also be avoided (Steadman *et al*., 1996).

Rotation should be used as a supplement to other control measures (Grau, 1988), such as wider row spacing or lower plant populations (Anonymous, 2005b), as it is not effective as a control measure by itself (Grau, 1988). Rotational crops may, however, stimulate sclerotial germination and thereby deplete soilborne sclerotia (Anonymous, 2005b).

Irrigation

Excessive irrigation should be avoided until after flowering (Grau and Hartman, 1999) as this promotes ascospore germination (Steadman *et al*., 1996). Grau and Radke (1984) found that the severity of SSR was affected by the timing of overhead irrigation in relation to the time of flowering. In two of the three years that the trial was run, disease severity was greatest with biweekly irrigation throughout the growth season. Where irrigation was at the preflower and postflower growth stages, disease severity was less than when plants were irrigated throughout the growing season (Grau and Radke, 1984).

Tillage

Tillage practices have the potential to influence the development of SSR both positively and negatively (Hart, 1998). Tillage is known to affect the placement and density of sclerotia, apothecial development and soybean stand (Anonymous, 2005b). According to McGee (1992), development of SSR is more severe under no-till, as opposed to

conventional tillage. In studies by Wegulo *et al*. (1996), disease incidence was significantly greater in no-till, when compared to mouldboard ploughing. In contrast, Workneh and Yang (2000) found that in no-till fields, less SSR was observed.

Deep tillage is recommended, as this prevents bringing sclerotia to the surface (Scott *et al*., 2005), and also buries sclerotia beyond the emerging capability of apothecia (Workneh and Yang, 2000). However, according to Ferreira and Boley (1992), all tillage assures the presence of sclerotia at or near the soil surface as in subsequent tillage operations, deeply buried sclerotia would be returned to the surface.

In studies on the effect of tillage systems on SSR, Mueller *et al*. (2002) found that tillage did not affect the total number of apothecia observed. However, mouldboard ploughing did delay the emergence of apothecia as compared to no-till (Mueller *et al*., 2002).

Kurle *et al*. (2001) found that sclerotial density was highest in the 0-2 cm of soil profile for no-till and chisel plough. Lowest sclerotial densities for these tillage practices were observed at a depth of 10-20 cm. The average sclerotial viability was highest in no-till, and was the same at depths of 0-2 cm and 2-10 cm. However, viability decreased at the 10-20 cm soil depth, for mouldboard and chisel ploughing possibly due to parasitism (Kurle *et al*., 2001).

Although much research on the effects of tillage on SSR exists, controversy over the actual advantages and disadvantages of this practice remain. The relationship between SSR incidence and tillage is not clear (Anonymous, 2005b) and more research needs to be conducted to clarify these unanswered questions.

Weed control

Due to the wide host range of SSR, many weeds may act as alternative hosts, resulting in a build-up of inoculum or the carry-over of inoculum to the following season. Weeds should therefore be eliminated (Scott *et al*., 2005).

Row width

In order to decrease SSR incidence, wide row widths and/or low plant densities are vital (McGee, 1992) as this decreases the build-up of humidity beneath and between plants. Excessive plant densities may result in heavy, early canopy closure and lodging (Ferreira and Boley, 1992).

In studies by Grau and Radke (1984) and Wegulo *et al*. (1996), row widths of 38 cm had a higher disease severity incidence (DSI) and a lower yield than those with row widths of 76 cm. Buzzell *et al*. (1993) found that disease incidence was not affected by row widths of 23, 45 and 69 cm. However, a trend towards more SSR in row widths of 23 cm was apparent (Buzzell *et al*.,1993). Generally, row widths less than 76 cm should be avoided (Grau, 1988; Grau and Hartman, 1999).

Cultivar age

Buzzell *et al*. (1993) found that cultivar differences in disease incidence were partially related to maturity. Early-maturing cultivars had less disease possibly because of disease escape mechanisms (Buzzell *et al*., 1993) and are therefore preferred (Gracia-Garza *et al*., 2002).

1.5.5 Seed treatment

Seed treatments may prevent the spread of SSR inoculum in infected soybean seed. Seedborne mycelia may be present in a significant percentage of seeds harvested from infected fields (Anonymous, 2005b). Captan, benomyl, thiobendazole and thiram at rates of 1.5; 1.0; 0.2 and 2.1 g kg^{-1}, respectively gave complete control of infected seed in Brazil (McGee, 1992). In Romania, carbendazim and thiram, carboxin and thiram, benomyl and captan, thiopanate-methyl and captan gave satisfactory control (McGee, 1992). Generally, most commonly available seed treatments provide effective control (Anonymous, 2005b).

1.6 DISCUSSION AND CONCLUSION

Sclerotinia stem rot is not only a disease of soybeans, but of numerous other crops. A combination of cultural practices and the use of soybean cultivars with higher levels of resistance need to be implemented in order to avoid serious yield losses (Hart, 1998).

However, although resistant cultivars may provide sufficient means of control, little is known about the inheritance of resistance of soybeans to SSR. Consequently, this has resulted in limited development of optimal breeding strategies. It is also unknown what proportion of resistance in the field is a result of physiological resistance or escape mechanisms (Kim and Diers, 2000). The United States Department of Agriculture (USDA) Soybean Germplasm Collection has recently been evaluated for new sources of resistance to *Phialophora gregata* (Allington and Chamberlain) W. Gams and *Phytophthora sojae* Kaufmann and Gerdemann and Kűhn. However, the collection has not yet been evaluated for resistance to SSR (Hoffman *et al*., 2002). In recent intensive studies by Hoffman *et al*. (2002), including 16 researchers in eight institutions, various sources of resistance that may aid in increasing the levels of resistance in existing germplasm have been identified. Research into the use of biocontrol agents against *S. sclerotiorum* is gaining in popularity as an alternative to possible chemical control options due to the lack of resistant varieties and concern over fungicide residues in the environment (Pan, 1998; Gossen *et al*., 2001).

In KZN, SA, serious soybean yield losses are already occurring due to the arrival of soybean rust in 2001. With this new pathogen which is now endemic in SA, together with an increase in SSR, the future of soybean production in SA is under threat. Sufficient knowledge about *S. sclerotiorum* on various hosts such as beans and sunflowers is available. However, for *S. sclerotiorum* on soybeans, research is lacking especially in SA, in particular the epidemiology and infection process of this pathogen. It is imperative that the epidemiology of this pathogen is investigated and understood, as this provides a tool for developing control measures. The information available about *S. sclerotiorum* on other hosts only provides guidelines for what occurs on soybeans.

As more research is conducted, the better our understanding of this pathogen will become.

The paucity of research indicates that it is clearly imperative that an effective, economically viable and integrated disease management programme should be developed for SA soybean farmers to implement to ensure the success and increase of soybean production in SA in the years ahead and the concomitant reduction in expensive imports of this important crop.

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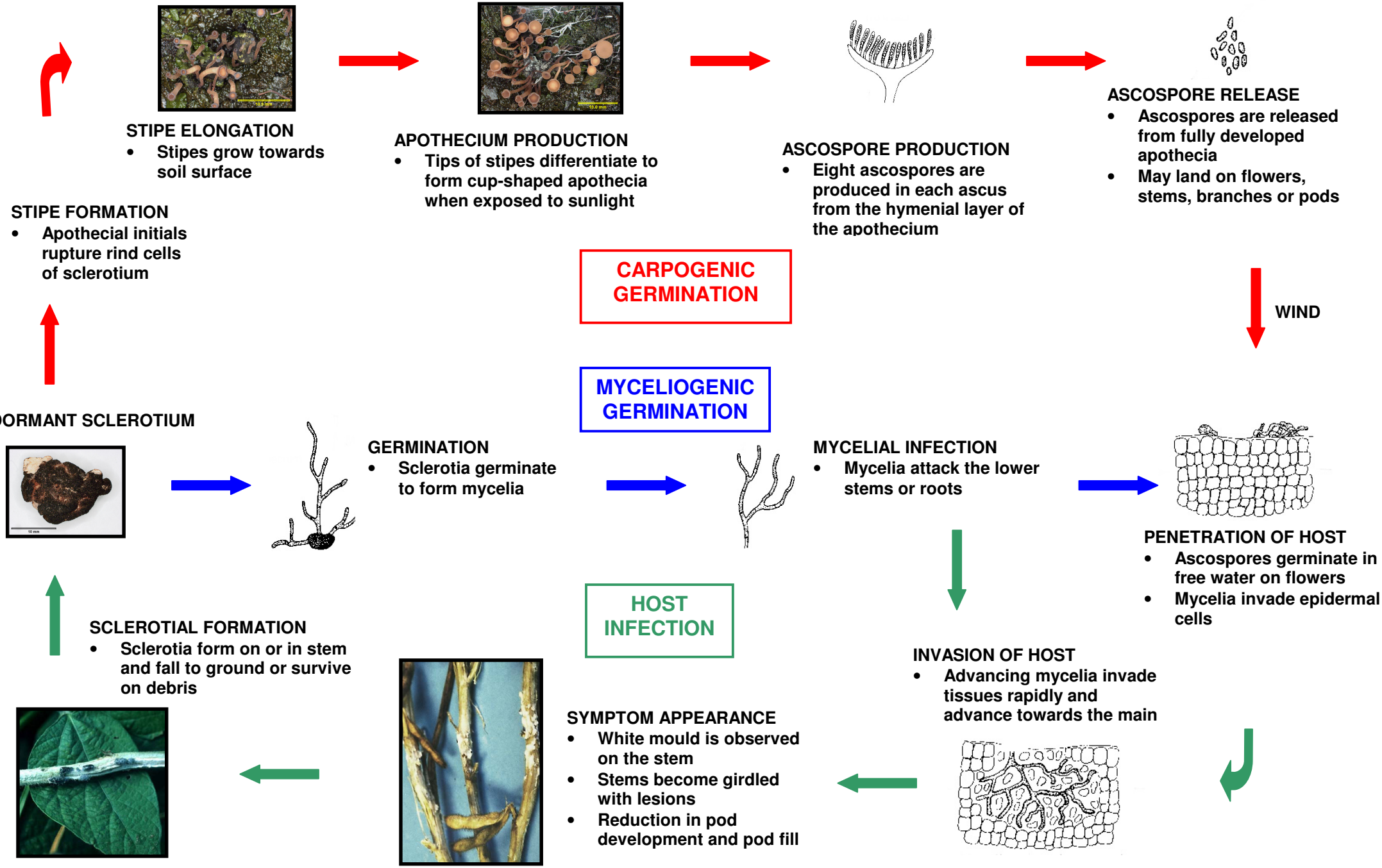

Figure 1.9 Life cycle of *Sclerotinia sclerotiorum* on soybeans (By Dael Visser).

CHAPTER TWO

DEVELOPMENT OF *SCLEROTINIA SCLEROTIORUM* AT DIFFERENT TEMPERATURES, RELATIVE HUMIDITIES AND LEAF WETNESS DURATIONS

D.D. Visser[1], P.M. Caldwell[1], N. W. McLaren[2]

[1]Discipline of Plant Pathology, University of KwaZulu-Natal, Private Bag X01, Scottsville, 3209, South Africa

[2]Department of Plant Sciences, University of the Free State, Bloemfontein, 9300, South Africa

ABSTRACT

Understanding the epidemiology of *Sclerotinia sclerotiorum* of soybeans (*Glycine max*), is important for the development of disease prediction models, but has not been well researched. *In vivo* studies were conducted to determine the optimum conditions for infection and the effects of temperature, relative humidity (RH) and leaf wetness duration (LWD), under controlled environmental conditions. The main stems of soybean plants (Prima 2000) at the V4 growth stage were horizontally severed approximately 0.5-1 cm above the third node and inoculated by dipping the cut stem surface into a homogeneous inoculum source. Disease was quantified at twenty four combinations of temperature (19°C, 22°C, 25°C and 28°C), RH (85 and 95%) and LWD (24, 48 and 72 hr). Inoculated plants were placed in a dew chamber at the relative temperature and RH, and removed after the required LWD before being placed in a conviron™ at 22-24°C for 21 days. Lesion lengths were measured 7, 10, 13, 17 and 21 days post inoculation. Rate of growth of the pathogen was then calculated using linear regression analysis. A significant interaction was found between temperature and RH, but not between these factors and LWD. At 22°C at 85% and 95% RH, the rate of growth of the

pathogen was significantly higher (0.46 and 0.43 per unit per day, respectively) than all other temperatures, indicating that this temperature is optimum for infection of *S. sclerotiorum* by mycelia. This information is an indication of the optimum conditions required for disease development of *S. sclerotiorum* under controlled environmental conditions. Further studies, including field trials, shorter LWD and inoculation with ascospores, are required in order to use this information for developing disease prediction models.

2.1 INTRODUCTION

Epidemiology is defined as the study of factors that lead to an increase in disease in a population (Bowen, 2004). Detailed and quantitative epidemiological data are critical for the development of effective control programs (Abawi and Grogan, 1979).

Sclerotinia stem rot (SSR) of soybeans (*Glycine max* (L.) Merrill.), caused by the fungus *Sclerotinia sclerotiorum* (Lib.) de Bary is an important disease of soybeans as well as numerous other plant species (Purdy, 1979; Boland and Hall, 1994). In 2004 it was considered to be the second most important yield-limiting soybean disease in the United States of America (USA) (Chen and Wang, 2005). In South Africa (SA), sporadic outbreaks have recently been reported in the Winterton-Underberg areas and more commonly in the Piet Retief areas of KwaZulu-Natal (KZN), as well as on the Highveld in the Ermelo area, particularly in wet years. In 2003, SSR was so severe on the KZN side of the Swaziland border, farmers harvested their crop early in the season for use as silage, as it was predicted that there would be no grain yield (Caldwell, pers. comm.[1]).

[1] Dr P. M. Caldwell, Discipline of Plant Pathology, School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of KwaZulu-Natal, Private Bag X01, Scottsville, 3209, Pietermaritzburg, South Africa.

Temperature and leaf wetness are considered the most influential of the various abiotic factors influencing disease caused by *S. sclerotiorum* (Abawi and Grogan, 1979). Information regarding optimal environmental conditions that favour disease development of SSR on soybeans is limited (Abawi and Grogan, 1979; Boland and Hall, 1988; Grau and Hartman, 1999). It has been suggested that the development of SSR coincides with crop flowering, extended below average air temperatures and moist weather. Cool conditions, e.g., canopy temperatures of <28°C and a leaf wetness periods of 12-16 hr recurring on a daily basis or continuously for 42-72 hr are essential for disease development (Abawi and Grogan, 1979; Grau and Hartman, 1999).

Ascospores are the primary source of infection, which germinate in adequate moisture when they land on senescent flowers (Abawi and Grogan, 1975; Tu, 1989). As the fungus grows, it girdles stems which disrupts the transport of water, mineral nutrients and photosynthates to developing pods (Grau and Hartman, 1999). Foliar symptoms include necrotic leaves, bleached stems and pods, white fluffy mycelia on stems, and the presence of sclerotia on or in stem surfaces and pods (Boland and Hall, 1987; Kim *et al*., 2000).

The aim of this study was to determine optimum conditions for infection of soybeans by mycelia of *S. sclerotiorum*, by investigating the effects of temperature, relative humidity (RH) and leaf wetness duration (LWD), on the rate of stem lesion development.

2.2 MATERIALS AND METHODS

2.2.1 Plant production

Soybean seeds of the cultivar Prima 2000 (Pannar[2]) were planted in composted pine bark (Growmor[3]) in seedling containers (3 x 3 x 5 cm). Seedling containers were placed in plastic containers (8 x 15 x 21 cm) (Food Packaging Distributors[4]) and watered every second day (Figure 2.1).

Figure 2.1 Soybean plants grown in seedling containers and placed in plastic containers (8 x 15 x 21 cm).

Plants were fertilized weekly with a mixture of Hortichem[5] 3:1:3 (N:P:K) and $CaNO_3$ (19.5% Ca, 15.5% N) at a rate of 1 g ℓ^{-1}. Plants were grown to the V4 growth stage (Fehr *et al*., 1971) in a growth room at 25ºC, 60% RH, a photoperiod of 14 hr and a light intensity of 347.17 $\mu Em^{-2}s^{-1}$.

[2] Pannar Seed, PO Box 19, Greytown, 3250, KwaZulu-Natal, South Africa
[3] Growmor, PO Box 89, Cato Ridge, 3680, KwaZulu-Natal, South Africa
[4] Food Packaging Distributors, 157 Victoria Rd, Pietermaritzburg, 3209, KwaZulu-Natal, South Africa
[5] Ocean Agriculture Pty Ltd., PO Box 741, Muldersdrift, 1747, Gauteng, South Africa

2.2.2 Isolate and inoculum production

A *S. sclerotiorum* isolate was obtained from sunflowers (*Helianthus annuus*) in Delmas, Mpumalanga, SA in February, 2005 (McLaren[6]) in the form of sclerotia. The *Sclerotinia* isolate used in this study was sent to Dr E. J. van der Linde[7] for identification and deposition in the Plant Protection Research Institute (PPRI) collection, and was confirmed to be *S. sclerotiorum* (PPRI Accession number 8374). Initially, sclerotia were surface sterilized for 3 min in 70% ethanol, washed twice in sterilized distilled water, and plated onto potato dextrose agar (PDA) (Merck[8]) in 9 cm diameter Petri dishes.

Petri dishes were sealed with Parafilm® (Industricord[9]) and incubated in the dark at 20°C for 4 weeks. The resulting sclerotia were harvested, surface sterilized for 3 min in 70% ethanol, washed twice in sterilized distilled water, placed on filter paper in a Petri dish and left to air dry overnight on a laminar flow bench. Sclerotia were placed into a sterile Petri dish and sealed and stored at 12°C in the dark until needed. This stock culture was also maintained by subculturing mycelia onto PDA slants and kept in the dark at 20°C.

To produce inoculum for greenhouse trials, a sclerotium was surface sterilized, washed twice in sterile distilled water, placed on PDA and allowed to germinate myceliogenically. After 7 days, when the mycelia reached the edge of the Petri dish, a single mycelial plug was cut from the margin of the growing colony with an 11 mm diameter cork borer and aseptically transferred to the centre of a new PDA plate. Plates were incubated for 4 days in the dark at 21 °C. Mycelia were sub-cultured by cutting mycelial plugs from the margin of the growing colony and aseptically transferred to new PDA plates. After 4 days, mycelial plugs were cut from the margin of the growing colony and used to inoculate potato dextrose broth (PDB) (Anatech Instruments[10]). Three plugs were

[6] Prof N. W.l McLaren, Department of Plant Sciences, University of the Free State, Bloemfontein, 9300, South Africa
[7] Dr E. J. van der Linde , Biosystematics Division, Agricultural Research Council (ARC), Plant Protection Research Institute (PPRI), Queenswood, 0121, Pretoria, South Africa
[8] Merck, Biolab Diagnostics (Pty) Ltd, 259 Davidson Rd, Wadeville, 1428, Gauteng, South Africa
[9] Industricord, PO Box 243, Pavilion, 3611, KwaZulu-Natal, South Africa
[10] Anatech Instruments, PO Box 98485, Sloane Park, 2152, South Africa

aseptically transferred to 100 ml PDB in 250 mℓ Erlenmeyer flasks. Inoculated flasks were placed on an SMS platform shaker at a speed of 100 rpm at room temperature (21°C -22°C).

After 4-5 days on the shaker, when mycelial masses were observed in the flasks (Figure 2.2), mycelia were harvested by filtering the liquid through cheesecloth for 10 min, and then weighed. Mycelia were blended for 20 sec using a Waring Commercial Blender at a medium speed and made up to a concentration of 2 g $m\ell^{-1}$ with sterile distilled water. Two grams of D(+) Glucose ($C_6H_{12}O_6$) (Merck[9]) per 100 mℓ of mycelial suspension were added to serve as an energy base for the fungus until infection occurred. The absorbance of the mycelial suspension was also measured at 600 nm using a Milton Roy Spectronic 301 spectrophotometer. The optical density of the mycelial suspension was adjusted to a value of 1.5-2.0 to ensure uniform concentration of inoculum prepared at different times during the course of the experimental work.

Figure 2.2 Mycelial masses of *Sclerotinia sclerotiorum* grown in potato dextrose broth after 4-5 days on a platform shaker.

2.2.3 Inoculation procedure

The main stems of soybean plants at the V4 growth stage (Fehr *et al*., 1971) were horizontally severed approximately 0.5-1 cm above the third node using a sterile razor blade (Figure 2.3).

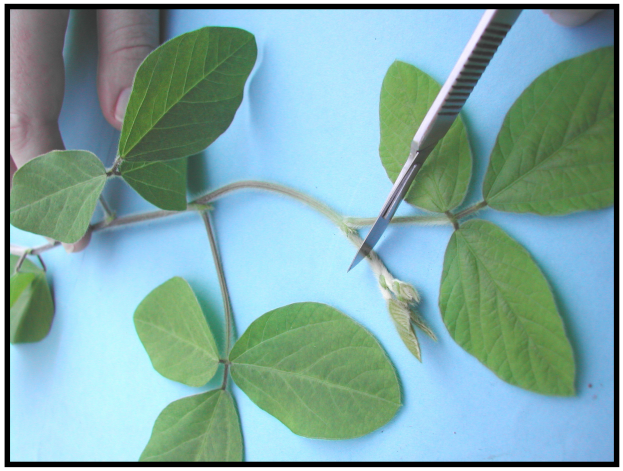

Figure 2.3 Horizontal severing of a soybean plant stem above the 3rd node.

The tip of the severed surface of the stem was dipped in the mycelial suspension (Figure 2.4) for 5 sec. Inoculated plants were left to dry for 15 min before spraying plants with distilled water to ensure the start of the leaf wetness period. Inoculated plants were placed in a dew chamber at the required temperature, RH and LWD with a photoperiod of 12 hr and a light intensity of 120 $\mu Em^{-2}s^{-1}$.

The capacity of the dew chamber is 6 m^3. Light is provided by fluorescent lamps at the top of the chamber, radiating through plexiglass. Light intensity was measured with a light meter and controlled by adding or removing fluorescent lamps. The light period was controlled by a timer. Humidity is achieved with an ultrasonic humidifier and controlled by the measurement of RH. Temperature is controlled with a reversed cycle refrigeration unit and heating elements.

After the required LWD, plants were transferred to a conviron™ (22°C-24°C, 80% RH, 14 hr photoperiod and a light intensity of 109 $\mu Em^{-2}s^{-1}$) for 21days.

Figure 2.4 Dipping of cut surface of soybean plant in mycelial inoculum of *Sclerotinia sclerotiorum*.

2.2.4 Treatments

Twenty four combinations of temperature (19℃, 22℃, 25℃ and 28℃), LWD (24, 48, 72 hr) and RH (85 and 95%) were investigated.

2.2.5 Experimental design

Five plants with three replicates were used in each trial. Plants were arranged in a randomized complete block design. The trial was repeated.

2.2.6 Disease assessment

The progression of lesion length (mm), on each stem was measured 7, 10, 13, 17 and 21 days post inoculation (dpi) from the severed tip of the stem.

2.2.7 Statistical analysis

Rate of growth of the pathogen was calculated as the linear regression of Vanderplank's logistic model (Vanderplank, 1963). Proportions were initially calculated for each day for each set of environmental conditions by dividing lesion length by the maximum lesion length obtained in the experiment. The proportion of disease, y, was then substituted into Vanderplank's equation for the logistic model, r = (ln (y/(1-y)). These values were used to calculate rate of growth of SSR using linear regression analysis, by the mean sum of squares. Rates were calculated using data from 1-13 dpi, by which time lesions had extended to the base of the main stem in many cases (McLaren, pers. comm.[6]).

All data were subjected to an analysis of variance (ANOVA) using Genstat® Executable Release 9.1 Statistical Analysis Software (Anonymous, 2006) to determine differences between treatment means and interactions between temperature, LWD and RH. Least significant differences were determined at $P<0.05$.

2.3 RESULTS

Trial 2 confirmed results obtained in Trial 1 as similar trends and patterns were observed in both trials. According to the ANOVA, trials did not differ, and data were therefore pooled. Lesion lengths (mm) for Trial 1 and 2 at various temperatures, LWD and 85% RH are shown in Appendix 2a and at 95%RH in Appendix 2b. Rates of growth of the pathogen at various temperatures, LWD and RH are shown in Appendices 2c and d, for Trial 1 and 2, respectively. Rates of growth of the pathogen at various temperatures and RH are shown in Appendices 2e and f, for Trial 1 and 2, respectively.

Infection was observed initially as water soaked lesions on the main stem, which later turned light brown in colour. When the margin of the lesion reached the lower nodes of the stem, leaves wilted and died but remained attached to the stem (Figure 2.5). Symptoms were observed on plants inoculated at all combinations of temperature, LWD and RH.

Figure 2.5 Macroscopic symptoms of *Sclerotinia sclerotiorum* on soybeans, seen as a light brown lesion on the main stem.

No significant 3 way interaction was found between temperature, LWD and RH (Appendix 2g). However, a 2 way interaction was found between temperature and RH (Appendix 2h). At 85% RH, the rate of growth (0.46 per unit per day) at 22℃ was significantly higher than all other temperatures. Although the rate of growth was significantly lower at all other temperatures, they were not significantly similar at 19℃, 25℃ and 28℃ (0.36, 0.31, 0.28 per unit per day, respectively) (Appendix 2h and Figure 2.6). At 28℃ (0.28 per unit per day), the rate of growth was significantly lower than at 19℃ (0.36 per unit per day), whereas at 95% RH, the rate of growth at 19℃, 25℃ and 28℃ (0.33, 0.37, 0.34 per unit per day, respectively), were significantly similar, but significantly lower than at 22℃ (0.43 per unit per day) (Appendix 2h and Figure 2.7).

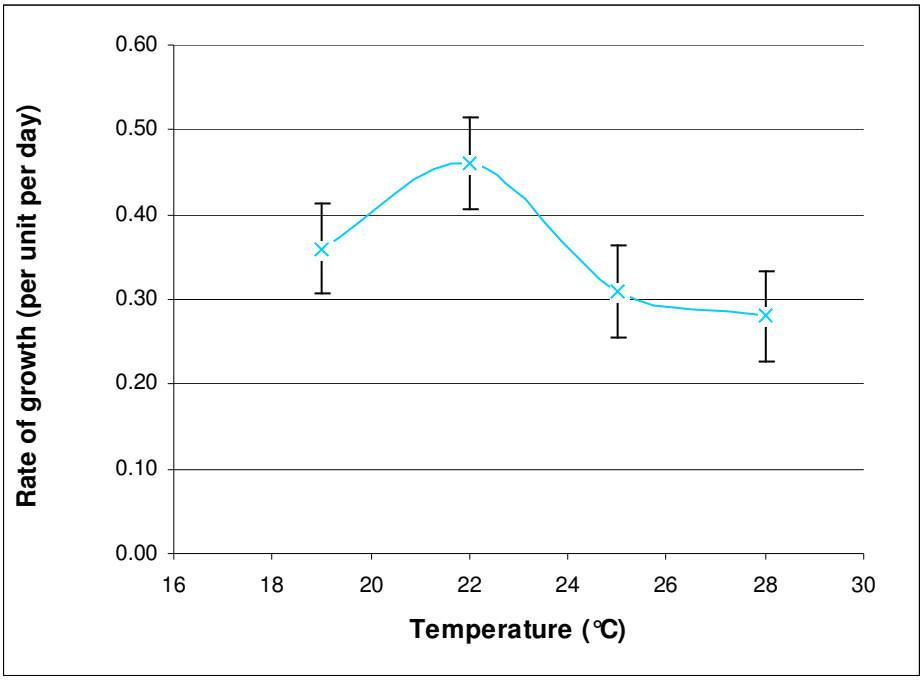

Figure 2.6 Rate of growth of the *Sclerotinia sclerotiorum* over time at various temperatures at 85% relative humidity on Prima 2000.

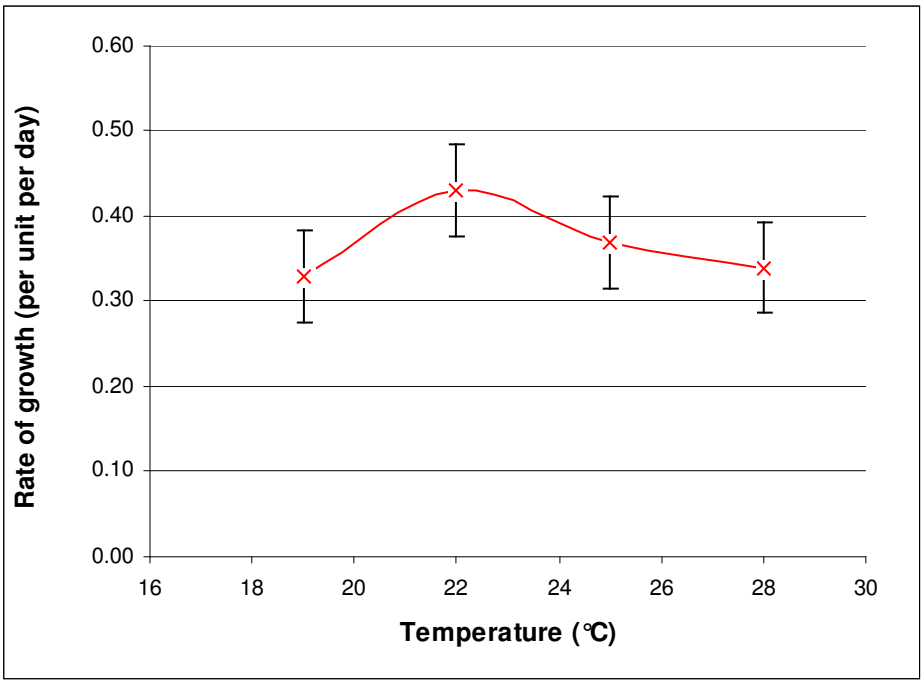

Figure 2.7 Rate of growth of the *Sclerotinia sclerotiorum* over time at various temperatures at 95% relative humidity on Prima 2000.

2.4 DISCUSSION

Infection of soybeans by mycelia of *S. sclerotiorum* occurred at all four temperatures investigated in this trial. Weiss *et al*. (1980) also found that infection of beans (*Phaseolus vulgaris* L.) by mycelia of *S. sclerotiorum* occurred over a temperature range of 10°C-30°C. It is generally known that prolonged, cool moist conditions, with temperatures less than 28°C are optimal for *S. sclerotiorum* (Grau and Hartman, 1999). In this research, infection of plants by *S. sclerotiorum* occurred at sub optimal temperatures (19°C and 28°C) outside the normal range for growth of the pathogen. Although the rate of growth was significantly slower at sub optimal temperatures, infection still occurred indicating that low and high temperatures in the field would not stop disease development. The optimum temperature in this study for infection by mycelia was 22°C at 85% and 95% RH, which falls in the range of 20-25°C determined in other studies (Abawi and Grogan, 1975; Weiss *et al*., 1980; Boland and Hall, 1987).

It is important to note that LWD showed no significant effect on the rate of growth of *S. sclerotiorum* in this study. However, ascospores were not used as inoculum due to their difficulty in producing, collecting and storing. It was concluded that due to the small area (stem diameter), which was inoculated with mycelium, as opposed to leaf area and flower area (as with the inoculation of ascospores), LWD possibly did not play a significant role in the rate of infection. The effect of LWD should, however, be investigated with ascospore inoculations. It has been observed that longer LWD periods are required in controlled environmental studies to initiate infection, than in the field (Abawi and Grogan, 1975; Boland and Hall, 1987), which may also have attributed to the absence of an interaction with LWD. Shorter LWD periods, i.e., less than 24 hr, should be included in future studies with mycelia, to determine whether shorter periods would have a significant effect on the rate of growth.

Harikrishnan and del Rio (2006) found that ascospores and mycelia were equally effective in causing disease at high RH levels irrespective of temperature. However, at low RH levels, mycelia were more effective. They postulated that mycelia are more tolerant to desiccation than ascospores, which require free standing water to germinate

(Harikrishnan and del Rio, 2006). Hence, infection observed at all temperatures and both RH levels tested, may be due to the tolerance of mycelia to withstand desiccation. The fact that LWD also had no significant effect on disease development may also be due to the tolerance of mycelia to desiccation, indicating mycelia may be able to survive long periods of adverse conditions. Studies on the influence of interrupted periods of LWD on the infection of beans by ascospores showed that continuous leaf wetness was not necessary for infection (Phillips, 1994). Phillips (1994) also postulated that germ tubes are susceptible to desiccation during dry periods which follow wet periods, thereby delaying infection. However, it was suggested that in the field, apothecia continually produce ascospores, thereby renewing inoculum sources (Phillips, 1994).

No studies on fluctuating temperatures and the influence of interrupted periods of LWD on infection by ascospores or mycelia have been carried out on soybeans. Information on the effect of fluctuating temperatures and the influence of interrupted periods of LWD, as well as fluctuating RH levels is needed as these factors are not constant in the field (Harikrishnan and del Rio, 2006). This information is essential for developing a system to predict when infection may be expected. Phillips (1994) suggests that fungicides should be applied before the tissues are invaded by *S. sclerotiorum* for successful control. In order to achieve this, it is imperative that research be conducted to develop disease prediction models, allowing for the prediction of the phase before infection approaches, so that fungicides may be timeously applied (Phillips, 1994).

Minimal literature on the epidemiology of *S. sclerotiorum* of soybeans using ascospores (the primary source of inoculum under field conditions) is currently available. Due to the difficulty of ascospore production, as well as collecting and preserving ascospores *in vitro* (Kull *et al*., 2003; Rousseau *et al*., 2004), they are less widely used, hence the use of mycelia to inoculate trials. Harikrishnan and del Rio (2006) suggest that information on infection by mycelia may potentially be useful, due to its tolerance to desiccation under different environmental conditions, which may be further exploited for resistance screening.

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CHAPTER THREE

EVALUATION OF SOYBEAN CULTIVARS FOR PHYSIOLOGICAL RESISTANCE TO *SCLEROTINIA SCLEROTIORUM* USING THE CUT STEM METHOD

D.D. Visser[1], P.M. Caldwell[1], N. W. McLaren[2]

[1]Discipline of Plant Pathology, University of KwaZulu-Natal, Private Bag X01, Scottsville, 3209, South Africa

[2]Department of Plant Sciences, University of the Free State, Bloemfontein, 9300, South Africa

ABSTRACT

Sclerotinia sclerotiorum, the causal agent of sclerotinia stem rot (SSR) has only recently been considered a major pathogen of soybeans (*Glycine max*) in South Africa (SA). This study was aimed at identifying the cultivar reaction of 13 soybean cultivars, i.e., LS6626RR, LS6710RR, LS666RR, LS555RR, LS6514RR, LS678RR, Prima 2000, Pan 626, AG5601RR, AG5409RR, 95B33, 95B53 and 96B01B, to *S. sclerotiorum*. The cut stem method was used which involved inoculating 6-7 week old soybeans (V6), grown in a glasshouse (25˚C), by horizontally severing the main stem 1 cm above the fifth node, before placing a mycelial disk on the cut surface. After inoculation, plants were placed in a dew chamber at 22˚C and 90% relative humidity (RH) for 48 hr and thereafter returned to the glasshouse. Lesion length was measured 2, 3, 4, 7, 8, 9 and 10 days post inoculation (dpi) and the number of sclerotia produced in the main stem counted 10 dpi. The rate of growth of the pathogen was calculated using linear regression analysis to determine susceptible and tolerant cultivars. All plants showed symptoms of SSR. Prima 2000, 96BO1B, 95B33 and AG5409RR, may be considered least susceptible and LS6626RR and LS666RR may be considered as the most susceptible of the cultivars

tested. Regression analysis showed that there was a positive correlation between rate of growth of the pathogen and the number of sclerotia produced (R^2 = 0.71), indicating that fewer sclerotia were always associated with a lower rate of growth of the pathogen. These results have aided in identifying the tolerance of various cultivars of soybeans commonly grown in SA.

3.1 INTRODUCTION

Soybeans, *Glycine max* (L.) Merrill., are a major source of vegetable oil and protein in the world (Singh *et al*., 2004) and have been used for thousands of years for human and animal food as well as in medicine to treat many human diseases (Hartman *et al*., 1999). Soybeans were introduced into Africa by missionaries and explorers at the beginning of the 20th century (Naik *et al*., 1987; Root *et al*., 1987). However, it is only since the 1970s that soybean cultivation has increased in popularity.

Soybean is an economically and strategically important crop in South Africa (SA). Not only is soybean oil economically important, but soybean protein is critical to animal feeds and human nutritional supplements. Consumption of soybean derived commodities in SA far exceeds production, resulting in the import of 842 107 tonnes of oilcake in the 2005/2006 soybean production period, in order to meet local demands (Joubert, 2006).

Due to an increase in soybean production throughout the world, diseases that affect the crop have increased in number and severity (Grau and Hartman, 1999). Combating plant disease increases not only increases yield, but also improves the quality of plant product available for use. The use of resistant varieties is considered to be the least expensive, easiest, safest and one of the most effective means of controlling plant diseases in crops (Agrios, 1997).

Sclerotinia stem rot (SSR) of soybeans, caused by the fungus *Sclerotinia sclerotiorum* (Lib.) de Bary, is an important disease of soybeans as well as numerous other plant species (Purdy, 1979; Boland and Hall, 1994) and causes substantial losses in crop production throughout the world (Boland and Hall, 1994). However, it was only recently that SSR was considered a major pathogen of soybeans (Grau and Hartman, 1999).

Due to an increase in the prevalence of SSR, breeding for resistance has become imperative in many soybean cultivar development programs (Kim *et al*., 2000). Although high levels of resistance to SSR in soybeans and dry beans are difficult to find (Kull *et al*., 2003), extensive field evaluations to assess soybean resistance to SSR have been reported (Kim *et al*., 1999; Kim *et al*., 2000; Hoffman *et al*., 2002). However, most inoculation techniques and controlled environment screening methods have not consistently predicted field reactions to SSR on soybeans (Nelson *et al*., 1991; Wegulo *et al*., 1998; Kim *et al*., 2000) and dry beans (Vuong *et al*., 2004).

Varietal differences in resistance to SSR in soybean have been reported from field, glasshouse and laboratory evaluations (Grau *et al*., 1982; Cline and Jacobsen, 1983; Boland and Hall, 1986; Boland and Hall, 1987; Chun *et al*., 1987; Nelson *et al*., 1991; Kim *et al*., 2000). Under field conditions, reactions of cultivars to SSR are the result of physiological resistance and escape mechanisms (Boland and Hall, 1987), whereas under glasshouse or laboratory conditions, cultivar reactions are due to physiological resistance only, as there is little chance for escape (Grau and Bissonnette, 1974; Nelson *et al*., 1991), thus resulting in inconsistent disease ratings in the field (Kim *et al*., 2000).

Numerous inoculation techniques have been developed to evaluate soybeans for resistance to SSR (Kim *et al*., 2000). The majority of inoculation techniques use a mycelial-infested substrate, as opposed to ascospores (Kull *et al*., 2003). Although ascospores are the primary source of inoculum, they are less widely used due to difficulties in producing and conditioning sclerotia, and collecting and preserving ascospores *in vitro* (Kull *et al*., 2003; Rousseau *et al*., 2004). The first technique used to evaluate soybeans for resistance to SSR was by Grau and Bissonnette (1974) which

involved inoculating the cotyledons. This method has been commonly used (Hartman *et al*., 2000; Kim *et al*., 2000; Kull *et al*., 2003). One of the more common inoculation techniques is the inoculation of excised stems or detached leaves of soybeans and dry beans with *S. sclerotiorum* mycelia (Chun *et al*., 1987; Leone and Tonneijck, 1990; Nelson *et al*., 1991; Miklas *et al*., 1992; Steadman *et al*., 1997; Wegulo *et al*., 1998; Kull *et al*., 2003). The cut stem method is a more recently introduced inoculation technique (Vuong and Hartman, 2002; Kull *et al*., 2003). The use of oxalic acid, which is associated with pathogenesis by *S. sclerotiorum*, has also been used to evaluate cultivars for resistance (Noyes and Hancock, 1981; Tu, 1985; Kolkman and Kelly, 2000).

The aim of this study was to evaluate various soybean cultivars for resistance to SSR in the greenhouse using the cut stem method to identify possible resistant cultivars.

3.2 MATERIALS AND METHODS

3.2.1 Soybean cultivars

Thirteen commonly grown commercial cultivars in SA, i.e., LS6626RR[1], LS6710RR[1], LS666RR[1], LS555RR[1], LS6514RR[1], LS678RR[1], Prima 2000[2], Pan 626[2], AG5601RR[3], AG5409RR[3], 95B33[4], 95B53[4] and 96B01B[4] were used.

3.2.2 Plant production

Soybean seed of the various cultivars were planted in composted pine bark (Growmor[5]) in 12 cm diameter plastic pots (675 cm^3) (Highfeld Packaging[6]). Pots were placed in

[1] Link Seed (Pty) Ltd, PO Box 755, Greytown, 3250, KwaZulu-Natal, South Africa
[2] Pannar Seed, PO Box 19, Greytown, 3250, KwaZulu-Natal, South Africa
[3] Monsanto Company, Monsanto House Building 4, Fourways Office Park, Randburg, 2055, Gauteng, South Africa
[4] Pioneer Hi-Bred RSA (Pty) Ltd, PO Box 8010, Centurion, 0046, Gauteng, South Africa
[5] Growmor, PO Box 89, Cato Ridge, 3680, KwaZulu-Natal, South Africa
[6] Highfield Packaging, 3 Chesterfield Rd, Willowton, 3201, KwaZulu-Natal, South Africa

Plastic containers (8 x 15 x 21 cm) (Food Packaging Distributors[7]) and watered every second day to maintain a depth of approximately 1 cm of water (Figure 3.1).

Figure 3.1 Soybean cultivars grown in 12 cm diameter pots and placed in plastic containers (8 x 15 x 21 cm).

Plants were fertilized weekly with a mixture of Hortichem[8] 3:1:3 (N:P:K) at a rate of 1 g ℓ^{-1} and $CaNO_3$ (19.5% Ca, 15.5% N) at a rate of 1 g ℓ^{-1}. Plants were grown till the V6 growth stage (Fehr *et al.*, 1971) in a glasshouse at 25ºC.

3.2.3 Isolate and inoculum preparation

A *S. sclerotiorum* isolate was obtained from sunflowers (*Helianthus annuus)* in Delmas, Mpumalanga, SA in February, 2005 (McLaren[9]) in the form of sclerotia. The *Sclerotinia*

[7] Food Packaging Distributors, 157 Victoria Rd, Pietermaritzburg, 3209, KwaZulu-Natal, South Africa

[8] Ocean Agriculture Pty Ltd., PO Box 741, Muldersdrift, 1747, Gauteng, South Africa

[9] Prof N. W. McLaren, Department of Plant Sciences, University of the Free State, Bloemfontein, 9300, South Africa

isolate used in this study was sent to Dr E. J. van der Linde[10] for identification and deposition in the Plant Protection Research Institute (PPRI) collection, and was confirmed to be *S. sclerotiorum* (PPRI Accession number 8374). Initially, sclerotia were surface sterilized for 3 min in 70% ethanol, washed twice in sterilized distilled water, and plated onto potato dextrose agar (PDA) (Merck[11]) in 9 cm Petri dishes.

Petri dishes were sealed with Parafilm® (Industricord[12]) and incubated in the dark at 20°C for 4 weeks. The resulting sclerotia were harvested, surface sterilized for 3 min in 70% ethanol, washed twice in sterilized distilled water and placed on filter paper in a Petri dish and left to air dry overnight on a laminar flow bench. Sclerotia were placed into a sterile Petri dish and sealed and stored at 12°C in the dark until needed. This stock culture was also maintained by subculturing mycelia onto PDA slants and kept in the dark at 20°C.

To produce inoculum for greenhouse trials, a sclerotium was surface sterilized, washed twice in sterile distilled water and placed on PDA and allowed to germinate myceliogenically. After 7 days, when the mycelia reached the edge of the Petri dish, a single mycelial plug was cut from the margin of the growing colony with an 11 mm diameter cork borer and aseptically transferred to the center of a new PDA plate. Plates were incubated for 4 days in the dark at 21°C. Mycelia were sub-cultured by cutting mycelial plugs from the margin of the growing colony and aseptically transferred to new PDA plates. After 4 days, mycelial plugs were cut with a 5 mm diameter cork borer at the edge of the growing colony and used to inoculate plants.

[10] Dr E. J. van der Linde , Biosystematics Division, Agricultural Research Council (ARC), Plant Protection Research Institute (PPRI), Queenswood, 0121, Pretoria, South Africa
[11] Merck, Biolab Diagnostics (Pty) Ltd, 259 Davidson Rd, Wadeville, 1428, Gauteng, South Africa

[12] Industricord, PO Box 243, Pavilion, 3611, KwaZulu-Natal, South Africa

3.2.4 Inoculation procedure

The main stems of soybean plants at the V6 growth stage (Fehr *et al*., 1971) were horizontally severed approximately 0.5-1 cm above the fifth node using a sterile razor blade (Figure 3.2), and a single mycelial plug (5 mm in diameter) was placed, mycelial side down, on the cut stem (Figure 3.3).

Figure 3.2 Horizontal severing of soybean plant above the 5th node.

Figure 3.3 A 5 mm diameter mycelial plug of *Sclerotinia sclerotiorum*, was placed mycelial side down on horizontally severed stem of soybean plant at the V6 growth stage.

A sterilized 100/200 µℓ micropipette tip (Polychem Supplies CC[13]) was placed over the cut stem and mycelial disk, to prevent inoculum from falling off or drying out (Figure 3.4). Pipette tips were removed 3 days post inoculation (dpi) to facilitate rating.

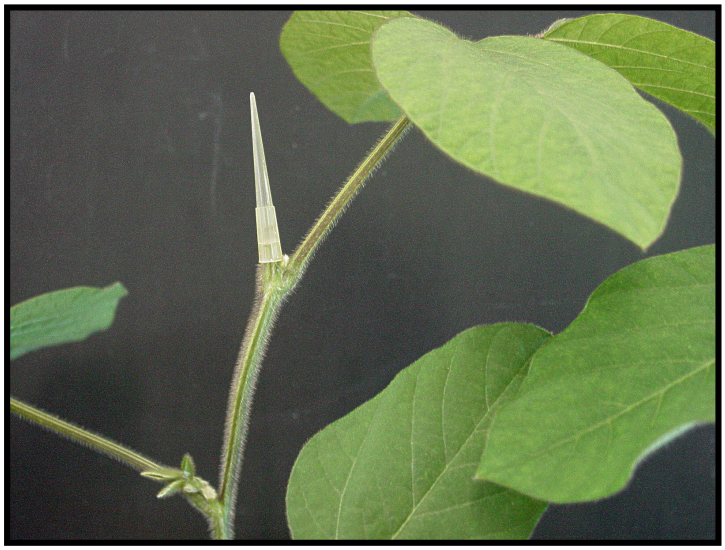

Figure 3.4 Micropipette tip placed over cut stem of soybean plant and mycelial disk of *Sclerotinia sclerotiorum* to prevent dessication.

Inoculated plants were placed in a dew chamber at 22℃ and 90% relative humidity (RH) for 48 hr.

The capacity of the dew chamber is 6 m^3. Light is provided by fluorescent lamps at the top of the chamber, radiating through plexiglass. Light intensity was measured with a light meter and controlled by adding or removing fluorescent lamps. The light period was controlled by a timer. Humidity is achieved with an ultrasonic humidifier and controlled by the measurement of RH. Temperature is controlled with a reversed cycle refrigeration unit and heating elements.

[13] Polychem Supplies CC, PO Box 17254, Congella, 4013, KwaZulu-Natal, South Africa

Thereafter, plants were transferred back to the glasshouse where they were previously grown.

3.2.5 Experimental design

Five plants with three replicates were used in each trial. Plants were placed in a randomized complete block design (RCBD) in the glasshouse and dew chamber. The trial was repeated.

3.2.6 Disease assessment

Lesion length (mm) on each stem was measured 2, 3, 4, 7, 8, 9 and 10 dpi from the point of inoculation downwards. After 10 dpi, the stems were harvested by horizontally severing them at their base. Using a sharp razor blade stems were split vertically and the number of sclerotia formed inside the stem was counted.

3.2.7 Statistical analysis

Rate of growth of the pathogen was calculated as the linear regression of Vanderplank's logistic model (Vanderplank, 1963). Proportions were initially calculated for each day for each cultivar by dividing lesion length by the maximum lesion length obtained in the experiment. The proportion of disease, y, was then substituted into Vanderplank's equation for the logistic model, $r = (\ln (y/(1-y))$. These values were used to calculate rate of growth of SSR using linear regression analysis, by the mean sum of squares (McLaren, pers. comm.[9])

All data were subjected to an analysis of variance (ANOVA) using Genstat® Executable Release 9.1 Statistical Analysis Software (Anonymous, 2006) to determine differences between treatment means. Least significant differences were determined at $P<0.05$.

3.3 RESULTS

Trial 2 confirmed results obtained in Trial 1 as similar trends and patterns were observed in both Trials. According to the ANOVA, experiments did not differ, and data were therefore pooled. Lesion lengths (mm) are shown in Appendices 3a and b, for Trial 1 and 2, respectively. Rates of growth of the pathogen and number of sclerotia produced are shown in Appendices 3c and d, for Trial 1 and 2, respectively. Regression analysis for Trial 1 and Trial 2 are shown in Appendix 3e.

All inoculated cultivars showed typical symptoms of SSR. Symptoms initially appeared as water soaked lesions on the main stem. These later turned light brown in colour (Figure 3.5), and developed cottony white mycelia.

When the margin of the lesion reached the lower node of the cut stem section, leaves wilted and died but remained attached to the stem (Figure 3.6), as commonly observed in the field. Sclerotia were evident in the main stem of all cultivars (Figure 3.7).

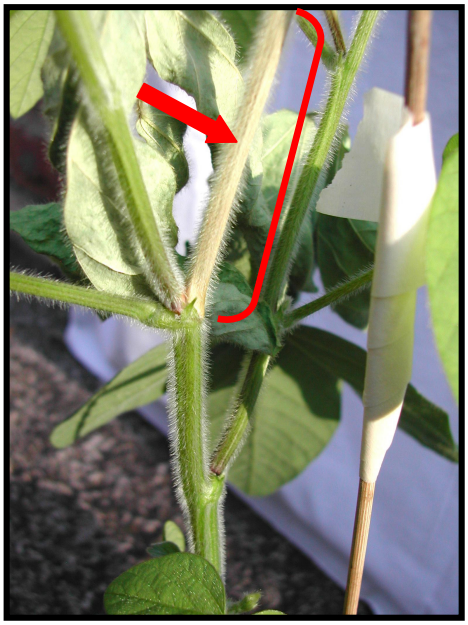

Figure 3.5 Macroscopic symptoms of sclerotinia stem rot on soybeans, seen as a light brown lesion on the main stem.

Figure 3.6 Appearance of soybean plants with wilted leaves still attached to the stem due to infection by sclerotinia stem rot.

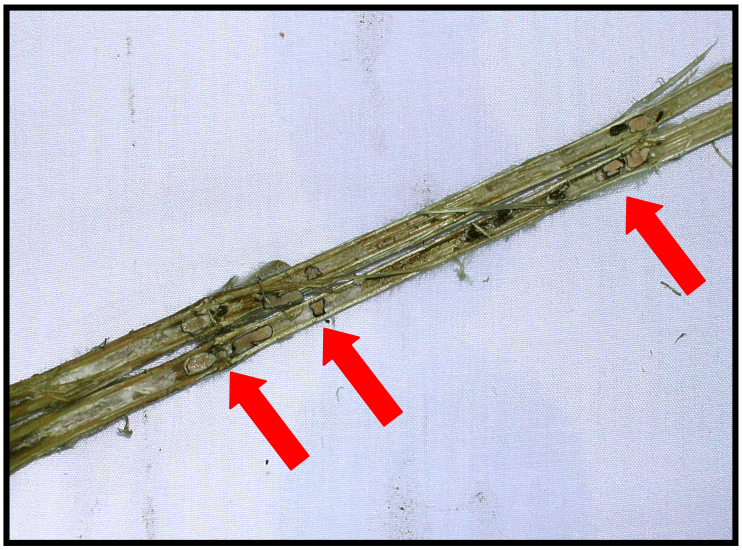

Figure 3.7 Appearance of sclerotia in stems of soybean plants 10 days post inoculation with *Sclerotinia sclerotiorum*.

Cultivars LS6626RR, LS666RR, LS555RR, LS6514RR, LS678RR and LS6710RR had a significantly higher rate of growth of the pathogen than AG5601RR, Pan 626, 95B33, Prima 2000, AG5409RR, 95B33 and 96BO1B (Appendix 3f and Figure 3.8).

However, of the cultivars with the significantly highest rate of growth of the pathogen, only LS6626RR and LS666RR produced the significantly highest number of sclerotia (Appendix 3f and Figure 3.9). Of the cultivars with the significantly lowest rate of growth of the pathogen, Prima 2000, 96BO1B, 95B33 and AG5409RR produced the significantly lowest number of sclerotia (Appendix 3f and Figure 3.9).

Regression analysis showed a positive correlation coefficient between rate of growth of pathogen and number of sclerotia, i.e. $R^2 = 0.71$ (Figure 3.10).

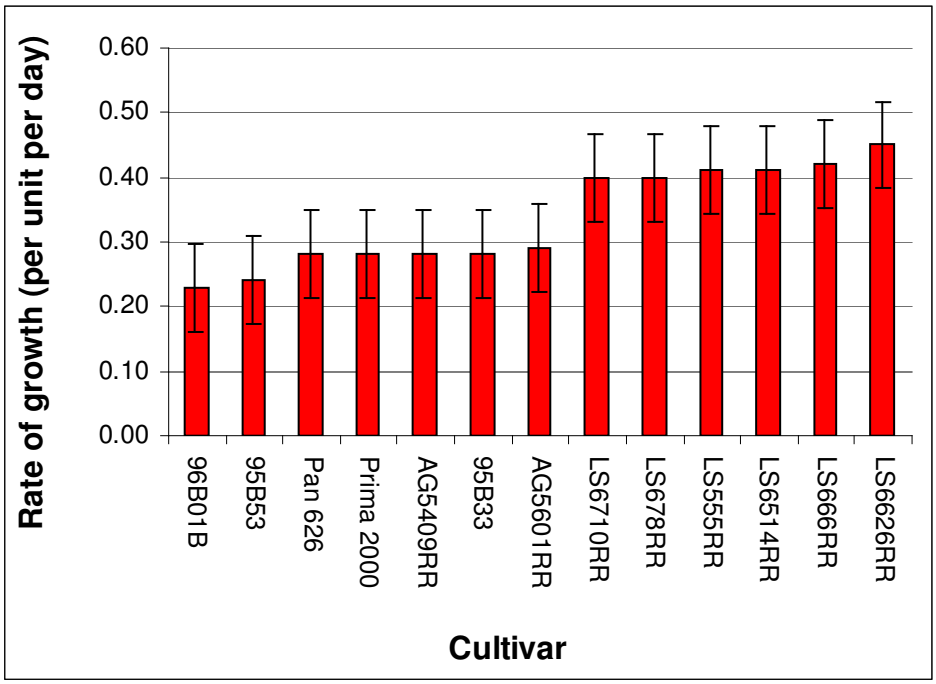

Figure 3.8 Rate of growth of pathogen (per unit per day) of different soybean cultivars inoculated with *Sclerotinia sclerotiorum*.

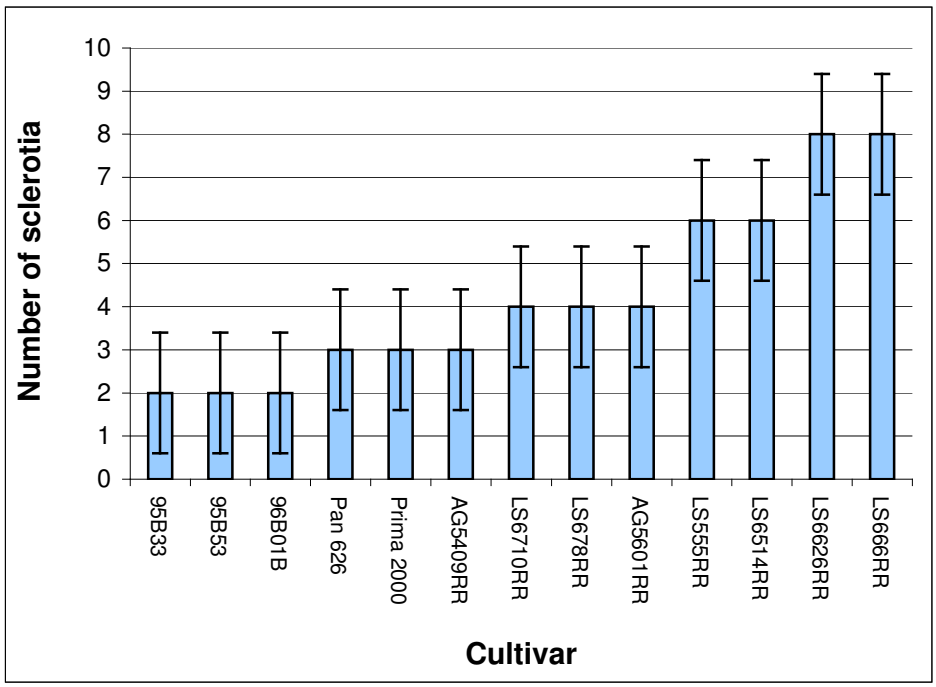

Figure 3.9 Number of sclerotia produced at 14 days post inoculation for different soybean cultivars inoculated with *Sclerotinia sclerotiorum*.

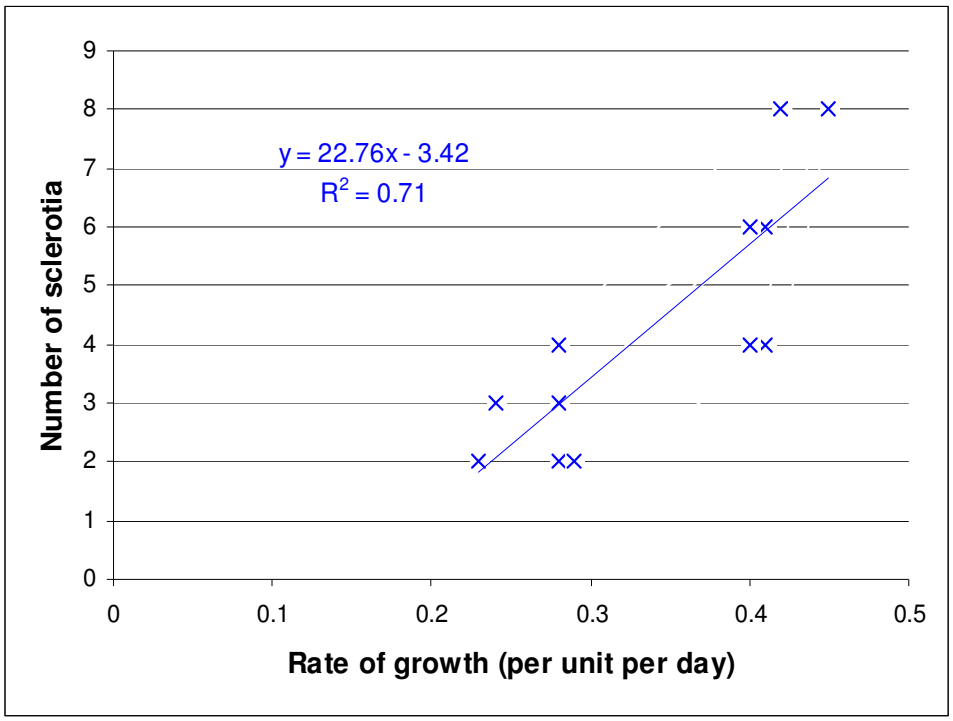

Figure 3.10 Regression analysis of rate of growth of pathogen (per unit per day) versus number of sclerotia of different soybean cultivars inoculated with *Sclerotinia sclerotiorum*.

3.4 DISCUSSION

Although many techniques have been developed for evaluating the resistance of soybeans and many other crops to *S. sclerotiorum*, the cut stem inoculation technique has proven to be the most reliable (Kull *et al*., 2003). It has been argued that the cut stem method allows the pathogen to bypass early defence mechanisms of the plant, but many trials have been performed to identify the reliability of the cut stem method (Kull *et al*., 2003; Vuong *et al*., 2004). Chen and Wang (2005) also argue that the cut stem method does not allow for the detection of resistance due to preformed structural barriers, and have recently developed two new methods, i.e., the spray-mycelium and drop-mycelium methods, where no pre-wounding of plants occur. Positive correlation coefficients of 0.64 and 0.63 ($P<0.01$) were found between the drop-mycelium and the cut stem method, respectively for lesion length and plant mortality (Chen and Wang, 2005). In trials by Kull *et al*. (2003) three different inoculation techniques were used, i.e., mycelial plug inoculations of

cotyledons, cut stem and detached leaves to compare the efficacy of resistance screening methods. The cut stem method was statistically proven to be better than the other two methods for determining cultivar resistance under controlled environmental conditions (Kull *et al*., 2003).

Many researchers have found no correlation or no consistent correlation between field and artificial inoculations (Boland and Hall, 1986; Chun *et al*., 1987; Nelson *et al*. 1991; Wegulo *et al*., 1998; Kim *et al*., 2000).

Kim *et al*. (2000) found moderate correlation with field resistance when greenhouse plants were inoculated using infested oat seed, mycelial plugs and excised leaves. They also observed that none of their inoculation techniques could accurately predict resistance in the field. It was suggested that greenhouse inoculation techniques may provide preliminary information on the resistance of cultivars, but field trials are required to confirm these initial results (Kim *et al*., 2000).

When soybean flowers were inoculated with ascospores, Cline and Jacobson (1983) found that inoculated flowers aborted, thereby preventing subsequent spread of *S. sclerotiorum* towards the stem. Considering this, and the difficulty in producing and storing ascospores, such trials with ascospores are not practical. Rousseau *et al*. (2004) suggests that there are two distinct resistant components to *S. sclerotiorum*, the first, which is expressed in the flower and the second, which is expressed in the stem of soybeans (Rousseau *et al*., 2004).

Results obtained from these trials indicate that preliminary information on the resistance of the SA cultivars tested may be determined. Cultivars with a significantly lower rate of growth and significantly lower number of sclerotia, such as Prima 2000, 96BO1B, 95B33 and AG5409RR, may be considered least susceptible to this isolate of *S. sclerotiorum*, whereas LS6626RR and LS666RR may be considered as the most susceptible. However, field trials need to be conducted to confirm the above, and also determine whether the cut stem method can be correlated to field work.

More importantly is the positive correlation coefficient (R^2=0.71) that was observed for rate of growth of pathogen versus number of sclerotia. This indicates that the higher the rate of pathogen development, the more sclerotia produced. Number of sclerotia found in the stems is an important observation as the fewer sclerotia produced in the main stem of the plant, the lower the propagation or over-wintering potential of the pathogen, and spread of the pathogen thereafter.

Reliable and accurate screening methods are imperative to enable the correct identification of levels of resistance to *S. sclerotiorum* in SA. Results of the present study have identified susceptible and tolerant cultivars which are commonly planted in SA with this isolate of *S. sclerotiorum*. As no resistant cultivars were identified, screening of a wider range of cultivars should be performed. Field trials should also be conducted, to confirm these controlled environmental condition trials (Kull *et al*., 2003), as well as the use of various inoculation methods to detect different resistant components. The results of this study are also specific for this particular isolate of *S. sclerotiorum*. Kull *et al*. (2003) showed that the range in aggressiveness of *S. sclerotiorum* isolates may impact on cultivar performance.

Evaluation of different isolates of *S. sclerotiorum* in greenhouse trials coupled with field trials will aid in identifying the best soybean cultivars with resistance to *S. sclerotiorum* in SA (Rousseau *et al*., 2004) and also the implementation of effective programmes to identify resistant germplasm in soybeans to *S. sclerotiorum* (Kull *et al*., 2003).

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CHAPTER FOUR

BIOLOGICAL CONTROL OF *SCLEROTINIA SCLEROTIORUM* USING *TRICHODERMA HARZIANUM* AND SILICON

D.D. Visser[1], P.M. Caldwell[1], N. W. McLaren[2]

[1]Discipline of Plant Pathology, University of KwaZulu-Natal, Private Bag X01, Scottsville, 3209, South Africa

[2]Department of Plant Sciences, University of the Free State, Bloemfontein, 9300, South Africa

ABSTRACT

Sclerotinia sclerotiorum, the causal organism of sclerotinia stem rot (SSR) has become a major pathogen of soybeans (*Glycine max*) in South Africa (SA), particularly in the wetter growing areas. *In vitro* dual culture bioassays were performed to identify possible bio-control mechanisms of EcoT® and Eco77® against hyphae and sclerotia of *S. sclerotiorum*. Ultrastructural studies using environmental scanning electron microscopy (ESEM) revealed that mycoparasitism is the probable mode of action as initial signs of hyphae of EcoT® and Eco77® coiling around hyphae of *S. sclerotiorum* were observed. Surface colonization of sclerotia by hyphae of EcoT® and Eco77® was also observed. *In vitro* antagonism of EcoT® against *S. sclerotiorum* on soybean seed was performed to determine pre-emergence and post-emergence disease. Seeds were placed in a Petri dish where one half was covered with sterile vermiculite and the other with potato dextrose agar (PDA). Two treatments were examined; seeds treated with a conidial suspension of EcoT® (2 x 10^8 spores $m\ell^{-1}$) and plated with a mycelial disk of *S. sclerotiorum*; untreated seeds plated with a mycelial disk of and *S. sclerotiorum* and a control of untreated seeds with no *S. sclerotiorum*. Percentage germination and percentage healthy seeds was determined. Seeds treated with EcoT® were not infected

by *S. sclerotiorum*. There was no significant difference in percentage germination between both treatments (seeds treated with EcoT® and plated with no *S. sclerotiorum*, and seeds not treated with EcoT® and plated with no *S. sclerotiorum*) and the control. However, percentage healthy seedlings of seeds not treated with EcoT® was significantly lower, suggesting that EcoT® may be successfully used as a seed treatment for the control of SSR. The effect of Eco77® together with silicon (Si), was tested *in vivo* against *S. sclerotiorum*. Soybean plants were inoculated using the cut stem method. Silicon (100 ppm), in the form of potassium silicate, was applied to the soil weekly from the V1 stage in the irrigation water. Eco77® was sprayed onto plants 2 days before inoculation. Plants treated with Eco77® had a significantly lower rate of disease development (0.19 per unit per day for plants treated with Eco77® and *S. sclerotiorum* and 0.20 per unit per day for plants treated with Eco77®, *S. sclerotiorum* and Si), compared to plants not treated with Eco77® (0.29 per unit per day for for plants treated with *S. sclerotiorum* and 0.30 per unit per day for plants treated with *S. sclerotiorum* and Si), regardless of the application of Si. Similarly, plants treated with Eco77® had a significantly lower number of sclerotia (0.46 for plants treated with Eco77® and *S. sclerotiorum* and 0.91 for plants treated with Eco77®, *S. sclerotiorum* and Si), compared to plants not treated with Eco77® (3.31 for plants treated with *S. sclerotiorum* and 3.64 for plants treated with *S. sclerotiorum* and Si). The significantly lower rate of disease development coupled with a significant reduction in sclerotia showed that Eco77®, and not Si, was responsible for reducing the severity of SSR. A strong positive correlation between rate of disease development and the number of sclerotia produced ($R^2 = 0.79$) was observed.

4.1 INTRODUCTION

Biological control of plant pathogens is increasing in popularity due to growing public concerns over the potentially harmful effects that chemical pesticides pose to human health and the environment and for specific diseases where no, or partial control methods are currently available (Cook, 1993). Due to the absence of resistant cultivars,

as well as a decrease in fungicide use, there is a requirement for the use of alternative methods of control (Clarkson and Whipps, 2005). Biological control measures involve the use of natural or modified organisms, genes or gene products to decrease the effects of undesirable organisms such as plant pathogens (Ownley and Windham, 2004).

Although the biological control of soil-borne plant pathogens by introduced microorganisms has been studied for more than 65 years, this method of control was originally not considered to be feasible (Cook and Baker, 1983; Baker, 1987). However, since 1965, interest and research into this method of control has increased along with a shift in the opinion of the impact biocontrol could have in agriculture (Weller, 1988). Sclerotia-forming fungi have received much attention in biocontrol due to an increase in crop losses caused by these pathogens (Willets, 1978).

Sclerotinia sclerotiorum (Lib.) de Bary, a soil-borne plant pathogen is considered one of the two most important and economically damaging soil-borne sclerotial plant pathogens (Clarkson and Whipps, 2005). It is a widespread soil-borne pathogen affecting 408 species, 42 subspecies, 75 families and 278 genera (Boland and Hall, 1994).

Elimination of sclerotia, the overwintering structures of *S. sclerotiorum* (Bruehl, 1987), is of paramount importance (Whipps and Budge, 1990) as they not only survive for up to seven years in the soil (Scott *et al*., 2005), but also provide the primary source of inoculum, i.e., ascospores, when they germinate carpogenically (Purdy, 1979). Large numbers of ascospores may be produced which have the potential to infect plants over a wide area (Budge and Whipps, 1991). Antagonists that either inhibit sclerotial production, induce sclerotial degeneration or suppress infection by ascospores may be used to control *Sclerotinia* diseases.

Since 1979, more than 30 species of fungi, bacteria, insects and other organisms have been reported as antagonists of *Sclerotinia* species (Steadman, 1979). The genus *Trichoderma* contains numerous mycoparasitic species (Wells *et al*., 1972; Grosclaude

et al., 1973; Gindrat *et al*., 1977; Tronsmo and Dennis, 1977; Chet and Baker, 1980; Huang, 1980; Dos Santos and Dhingra, 1982) such as *T. harzianum* Rifai, *T. viride* Pers., *T. koningii* Rifai, *T. pseudokoningii* Rifai (Jones and Watson, 1969; dos Santos and Dhingra, 1982, Artigues and Davet, 1984; Zazzerini and Tosi, 1985; Davet, 1986) and *Trichoderma roseum* (pers.:Fr.) Link (Huang and Kokko, 1993). Other mycoparasites of *S. sclerotiorum* include *Gliocladium virens* Miller & Foster (Artigues and Davet, 1984; Tu, 1980; Phillips, 1986), *G. roseum* (Link) Bain. (Ervio *et al*., 1964; McCredie and Sivasithamparam, 1985), *Coniothyrium minitans* Campbell (Campbell, 1947; Huang and Hoes, 1976; Turner and Tribe, 1976; Huang, 1980; Trutmann *et al*., 1980; Tu, 1984; Huang and Kokko, 1987; Budge and Whipps, 1991; Whipps and Gerlagh, 1992; Bennett *et al*., 2006), *Talaromyces flavus* (Klöcker) A.C. Stolk and R.A. Samson (McLaren *et al*., 1986; Mclaren *et al*., 1994) *Paecilomyces lilacinus* (Thom) Samson (Karhuvaara 1960; Ervio *et al*., 1964) *Ulocladium atrum* Preuss (Li *et al*., 2003) and *Sporidesmium sclerotivorum* Uecker, Ayers and Adams (Uecker *et al*., 1978; Adams, 1989).

Trichoderma harzianum is well known for its ability to parasitize sclerotia forming plant pathogenic fungi, such as *Rhizoctonia* and *Sclerotinia* (Hadar *et al*., 1979; dos Santos and Dhingra, 1982; Elad *et al*., 1983; Agrios, 1997). *Trichoderma koningii* has been successfully shown as an antagonist of *S. sclerotiorum* (Trutman and Keane, 1990) but few reports on the biocontrol potential of *T. harzianum* on *S. sclerotiorum* exist.

Silicon (Si), the second most abundant element in soil is well known for its important role in plant growth, mineral nutrition and mechanical strength (Epstein, 1994). Since the 1930s it has also been shown to effectively protect plants, specifically rice, against fungal diseases (Suzuki, 1935; Wagner, 1940) such as *Magnaporthe grisea* (Herbet) Barr (rice blast), *Cochliobolus miyabeanus* Drechs ex Dastur (brown spot), *M. salvinii Catt.* (stem rot), *Monographella albescens* Theum (scald), grain discolouration and *Rhizoctonia solani* Kűhn (sheath blight) (Winslow, 1992). Silicon has been associated with inducing resistance to powdery mildew, *Sphaerotheca fuliginea* (Schlechtend.: Fr.) Pollacci (Menzies *et al*., 1991; Menzies *et al*., 1992) and *Pythium* spp. (Cherif *et al*.,

1992a; Cherif *et al*., 1992b; Cherif *et al*., 1994) in cucumber which has lead to research into the potential of Si on other diseases in different crops (Bélanger *et al*., 1995) i.e., *Pyricularia grisea* (Cooke) Sacc. in rice (Yoshida *et al*., 1962; Datnoff *et al*., 1997); *Erysiphe graminis* DC. f. sp. *hordei* Ém. Marchal in barley (Carver *et al*., 1987) and *Erysiphe graminis* DC. f. sp *tritici* Ém. Marchal in wheat (Bélanger *et al*., 2003; Yang *et al*., 2003).

The objective of this study was to: (i) test the ability of two commercially available biocontrol agents, EcoT® and Eco77®, both strains of *T. harzianum*, to attack hyphae and sclerotia of *S. sclerotiorum in vitro*, (ii) evaluate the pre-emergence and post-emergence effect of EcoT® and *S. sclerotiorum* on soybean seeds *in vitro*, and (iii) evaluate the effect of Si, alone and in combination with the biocontrol agent Eco77®, for the control of *S. sclerotiorum* .

4.2 MATERIALS AND METHODS

4.2.1 *In vitro* dual culture bioassay and ultrastructural studies of Eco77® and EcoT® and *Sclerotinia sclerotiorum*

4.2.1.1 Sources of pathogen and biocontrol organisms

Two commercially available biocontrol agents Eco77® and EcoT® (Plant Health Products[1]) were tested. Biocontrol agents were initially plated onto half strength V8 agar in a Petri dish (Appendix 4a). After 4 days, when the mycelia reached the edge of the plate, a single mycelial plug was cut from the margin of the growing colony with a 5 mm diameter cork borer and aseptically transferred to the center of a new potato dextrose agar (PDA) (Merck[2]) plate. Plates were incubated for 4 days in the dark at 25°C. Mycelia were sub-cultured by cutting mycelial plugs from the margin of the new growing colony and aseptically transferred to fresh PDA plates. After 4 days, mycelial plugs of

[1] Plant Health Products (pty) Ltd, PO Box 207, Nottingham Road, 3280, KwaZulu-Natal, South Africa
[2] Merck, Biolab Diagnostics (Pty) Ltd, 259 Davidson Rd, Wadeville, 1428, Gauteng, South Africa

the biocontrol agents were cut with a 5 mm diameter cork borer at the edge of the growing colony and used to inoculate new Petri dishes.

A *S. sclerotiorum* isolate was obtained from sunflowers (*Helianthus annuus)* in Delmas, Mpumalanga, SA in February, 2005 (McLaren[3]) in the form of sclerotia. The *Sclerotinia* isolate used in this study was sent to Dr E. J. van der Linde[4] for identification and deposition in the Plant Protection Research Institute (PPRI) collection, and was confirmed to be *S. sclerotiorum* (PPRI Accession number 8374). Initially, sclerotia were surface sterilized for 3 min in 70% ethanol, washed twice in sterilized distilled water, and plated onto PDA in 9 cm Petri dishes.

Petri dishes were sealed with Parafilm® (Industricord[5]) and incubated in the dark at 20°C for 4 weeks. The resulting sclerotia were harvested, surface sterilized for 3 min in 70% ethanol, washed twice in sterilized distilled water and placed on filter paper in a Petri dish and left to air dry overnight on a laminar flow bench. Sclerotia were then placed in a sterile Petri dish and sealed and stored at 12°C in the dark until needed. This stock culture was also maintained by subculturing mycelia onto PDA slants and kept in the dark at 20°C.

To produce inoculum for dual culture bioassays, a sclerotium was surface sterilized, washed twice in sterile distilled water and placed on PDA and allowed to germinate myceliogenically. After 7 days, when the mycelia of *S. sclerotiorum* reached the edge of the Petri dish, a single mycelial plug was cut from the margin of the growing colony with an 11 mm diameter cork borer and aseptically transferred to the center of a new PDA plate. Plates were incubated for 4 days in the dark at 21°C. Mycelia were sub-cultured by cutting mycelial plugs from the margin of the new growing colony and then aseptically transferred to fresh PDA plates. After 4 days, mycelial plugs were cut with a 5 mm

[3] Prof. N. W. McLaren, Department of Plant Sciences, University of the Free State, Bloemfontein, 9300, South Africa
[4] Dr E. J. van der Linde , Biosystematics Division, Agricultural Research Council (ARC), Plant Protection Research Institute (PPRI), Queenswood, 0121, Pretoria, South Africa
[5] Industricord, PO Box 243, Pavilion, 3611, KwaZulu-Natal, South Africa

diameter cork borer at the edge of the growing colony and used to inoculate Petri dishes.

4.2.1.2 *In vitro* dual culture bioassays of Eco77® and EcoT® and *Sclerotinia sclerotiorum*

For *in vitro* antagonism, testing hyphal-hyphal interactions (hyphae of Eco77® and EcoT® and hyphae of *S. sclerotiorum*), as well as hyphal-sclerotial interactions (hyphae of Eco77® and EcoT® and sclerotia of *S. sclerotiorum*) were investigated.

Hyphal-hyphal interactions

Mycelial plugs of the pathogen and biocontrol agents were placed at opposite sides of 90 cm PDA plates, 1 cm away from the wall of each Petri dish (Figure 4.1).

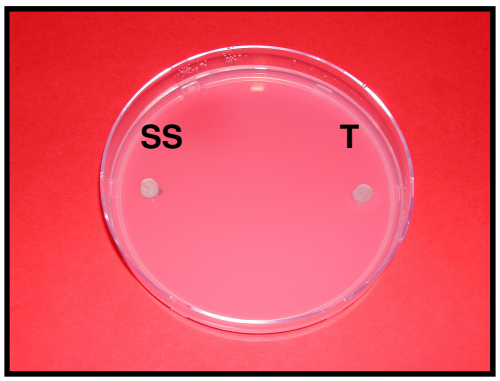


Figure 4.1 Hyphal-hyphal interaction between mycelial plugs of Eco77® or EcoT® (T) and *Sclerotinia sclerotiorum* (SS).

Hyphal-sclerotial interactions

A sclerotium of the pathogen and either Eco77® or EcoT® was placed at opposite ends of PDA plates, 1 cm away from the wall of each Petri dish (Figure 4.2).

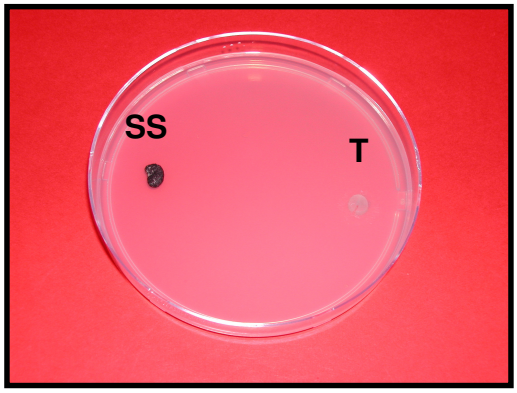


Figure 4.2 Hyphal-sclerotial interaction between mycelial plugs of Eco77® or EcoT® (T) and a sclerotium of *Sclerotinia sclerotiorum* (SS).

Controls

Petri dishes of PDA were inoculated with mycelial plugs of *S. sclerotiorum*, Eco77® and EcoT® alone as controls.

All inoculated plates were incubated in the dark at 25°C, and evaluated at 3, 5 and 7 days post inoculation (dpi).

4.2.1.3 Experimental design

Five Petri dishes with three replicates were used for each trial and the experiment was repeated. Plates were arranged in a randomized complete block design (RCBD).

4.1.2.4 Assessment for antagonism, invasion ability and antibiosis

Hyphal-hyphal interactions

After 3 and 5 days, each dual culture bioassay plate was assessed for the degree of antagonism, invasion ability and antibiosis. The degree of antagonism of Eco77® and EcoT® towards *S. sclerotiorum* was rated on a Bell rating scale of 1-5 according to the rating system of Bell *et al*. (1982), where 1 = Eco77® or EcoT® completely overgrew *S. sclerotiorum* and covered the entire medium surface; 2 = Eco77® or EcoT® overgrew

at least two thirds of the medium surface; 3 = Eco77® or EcoT® and *S. sclerotiorum* each colonized approximately one half of the medium surface and neither organism appeared to dominate the other; 4 = *S. sclerotiorum* colonized at least two thirds of the medium surface and appeared to withstand encroachment by Eco77® or EcoT®; 5 = *S. sclerotiorum* completely overgrew Eco77® or EcoT® and occupied the entire medium surface. Bell *et al*. (1982) considered a *Trichoderma* isolate to be antagonistic towards the fungal pathogen if the mean score was ≤2, but not highly antagonistic if the mean score is ≥3.

After 7 days, plates were rated for invasion ability on a scale of 1-5, adopted from Yobo (2005), where 1 = Eco77® or EcoT® completely overgrew *S. sclerotiorum*, invaded the entire plate, sporulation was apparent on all sections of the plate after 7 days and the pathogen turned brown; 2 = Eco77® or EcoT® completely overgrew *S. sclerotiorum*, invaded the entire plate, sporulation was apparent on all sections of the plate after 7 days, but no discolouration of the *S. sclerotiorum* mycelium occurred; 3 = Eco77® or EcoT® colonized 50% of the plate from the point of contact with *S. sclerotiorum*, patches of sporulation were evident on sections of the plate where Eco77® or EcoT® invaded *S. sclerotiorum*; 4 = Eco77® or EcoT® colonized less than 50% of the pathogen from the point of contact with *S. sclerotiorum* and little or no sporulation was evident on invaded sections. A *Trichoderma* isolate rated as 1 or 2 for invasion ability was considered to be highly invasive against *S. sclerotiorum.*

Antibiosis was identified as zones of inhibition on dual culture bioassay plates, where a distinct zone of inhibition between Eco77® or EcoT® and *S. sclerotiorum* was attributed to the production of antimicrobial compounds.

Hyphal-sclerotial interactions

Sclerotia were macroscopically observed 4, 6 and 8 dpi for colonization by hyphae of Eco77® or EcoT® and sporulation of Eco77® or EcoT® using a Leica Dissecting Microscope. Sclerotia were removed from the Petri dish and cut in half to determine whether sclerotia remained intact or disintegration had occurred.

All plates (hyphal-hyphal and hyphal-sclerotial interaction) were further incubated for 3 weeks and examined for the formation of sclerotia.

4.2.1.5 Environmental Scanning Electron Microscopic (ESEM) observation on the interaction between Eco77® and EcoT® and *Sclerotinia sclerotiorum*

Mycelial plugs (approximately 3-4 mm in diameter) were cut from the interaction zone between Eco77® or EcoT® and *S. sclerotiorum* for the hyphal-hyphal interaction. For the hyphal-sclerotial interaction, sclerotia were removed and fixed overnight in 3% gluteraldehyde in 0.05 M sodium cacodylate buffer (pH 6.99). Samples were washed twice in a 0.05 M cacodylate buffer (pH 7.16) for 30 min, followed by dehydration in a graded ethanol series, from 10% to 100%. Samples were then critical-point dried with carbon dioxide as a transitional fluid in a Hitachi HCP-2. Critical-point dried samples were mounted onto aluminum stubs previously coated with double sided tape and thereafter sputter coated with gold/palladium in an E5100 Polaron Equipment Ltd SEM coating unit. Samples were viewed with a Phillips Environmental Scanning Electron Microscope (ESEM) on high vacuum at 15 Kv.

4.2.2 *In vitro* antagonism of EcoT® against *Sclerotinia sclerotiorum* on soybean seed

4.2.2.1 Seed sterilization

Soybean seeds of the cultivar Prima 2000 (Pannar[6]) were surface sterilized for 3 min in NaOCl (4%), washed three times in sterile distilled water and air dried overnight on a laminar flow bench.

[6] Pannar Seed, PO Box 19, Greytown, 3250, KwaZulu-Natal, South Africa

4.2.2.2 Seed treatment

Sterilized seeds were coated by soaking seeds in a conidial suspension of EcoT® (2 x 10^8 spores $m\ell^{-1}$) using 1% (w v^{-1}) carboxymethyl cellulose (CMC) (Sigma[7]) as a spore-sticker for 5 min. Coated seeds were air dried for 1 hr on a laminar flow bench.

4.2.2.3 Petri dish set-up

Petri dishes were set-up containing sterile vermiculite (Kompel[8]) in one half and PDA in the other half (Figure 4.3). Two soybean seeds were placed in each Petri dish on the moist vermiculite. A 5 mm agar disk of *S. sclerotiorum* was placed on the PDA. The following treatments were used: *Trichoderma* treated seeds and a plug of agar containing *S. sclerotiorum*; untreated seeds and a plug of agar containing *S. sclerotiorum*, untreated seeds and no plug of agar containing *S. sclerotiorum* (control). Petri dishes were placed in a conviron™ (22-23°C, 60% relative humidity (RH), 14 hr photoperiod and a light intensity of 261 $\mu Em^{-2}s^{-1}$) for 14 days.

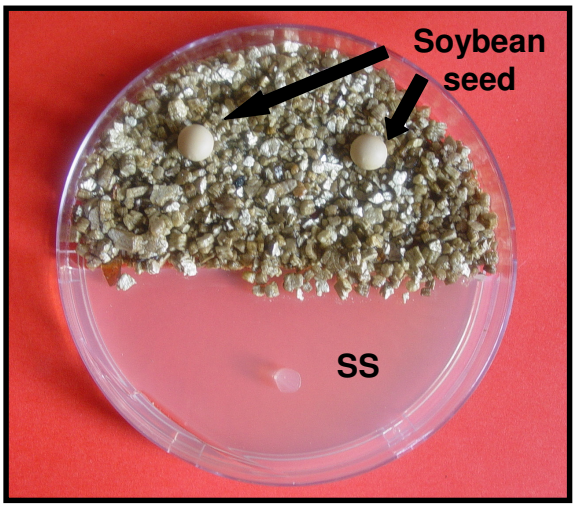


Figure 4.3 Petri dish setup for *in vitro* antagonism of EcoT® against *Sclerotinia sclerotiorum* (SS) on soybean seed, showing sterile vermiculite in one half and PDA in the other half.

[7] Sigma, Capital Enterprises, PO Box 62, New Germany, 3620, KwaZulu-Natal, South Africa
[8] Kompel, Starke Ayres, PO Box 304, Eppindust, 7475, Western Cape, South Africa

4.2.2.4 Experimental design

Two seeds per Petri dish with twenty replicates were used in each trial and the experiment was repeated. Petri dishes were arranged in a RCBD.

4.2.2.5 Statistical analysis

The number of germinated seeds and uninfected plants were recorded to determine the pre-emergence and post-emergence effect of *S. sclerotiorum* on treated and untreated soybean seeds.

All data were subjected to an analysis of variance (ANOVA) using Genstat® Executable Release 9.1 Statistical Analysis Software (Anonymous, 2006) to determine differences between treatment means. Least significant differences were determined at $P<0.05$.

4.2.3 *In vivo* effect of potassium silicate and Eco77® on *Sclerotinia sclerotiorum*

4.2.3.1 Plant production

Soybean seeds of the cultivar Prima 2000 (Pannar[6]) were planted in composted pine bark (Growmor[9]) in 12 cm diameter plastic pots (675 cm^3) (Highfeld Packaging[10]). Pots were placed in plastic containers (8 x 15 x 21 cm) (Food Packaging Distributors[11]) and watered every second day to maintain a depth of approximately 1 cm of water (Figure 3.1).

[9] Growmor, PO Box 89, Cato Ridge, 3680, KwaZulu-Natal, South Africa
[10] Highfield Packaging, 3 Chesterfield Rd, Willowton, 3201, KwaZulu-Natal, South Africa
[11] Food Packaging Distributors, 157 Victoria Rd, Pietermaritzburg, 3209, KwaZulu-Natal, South Africa

Plants were fertilized once a week with a mixture of Hortichem[12] 3:1:3 (N:P:K) at a rate of 1 g ℓ^{-1} and $CaNO_3$ (19.5% Ca, 15.5% N) at a rate of 1 g ℓ^{-1}. Plants were grown to the V6 growth stage (Fehr *et al*., 1971) in a glasshouse at 25ºC.

4.2.3.2 Potassium silicate application

Silicon, in the form of potassium silicate[13] was added in irrigation water at a rate of 100 ppm, once every 7 days from the V1 stage (Fehr *et al*., 1971) until harvest. No Si was added to the control plants.

4.2.3.3 Eco77® application and inoculation procedure

The main stems of soybean plants at the V6 growth stage (Fehr *et al*., 1971) were horizontally severed approximately 0.5-1 cm above the fifth node using a sterile razor blade (Figure 3.2). A single mycelial plug was placed, mycelial side down, on the cut stem. For controls, an agar plug of PDA (with no *S. sclerotiorum*) was placed on the cut stem (Figure 3.3). A sterilized 100/200 µℓ micropipette tip (Polychem Supplies CC[14]) was placed over the cut stem and mycelial disk, to prevent inoculum from falling off or drying out (Figure 3.4). Pipette tips were removed 3 dpi to facilitate rating.

Eco77® was applied at the recommended rate (1g 100 ml^{-1}) onto cut soybean stems two days before inoculation with *S. sclerotiorum*, to allow *T. harzianum* to colonize the cut surface. No Eco77® was added to the control plants.

Inoculated plants were placed in a dew chamber at 21ºC and 80% RH for 48 hr. The capacity of the dew chamber is 6 m^3. Light is provided by fluorescent lamps at the top of the chamber, radiating through plexiglass. Light intensity was measured with a light meter and controlled by adding or removing fluorescent lamps. The light period was controlled by a timer. Humidity is achieved with an ultrasonic humidifier and controlled by the measurement of RH. Temperature is controlled with a reversed cycle refrigeration unit and heating elements.

[12] Ocean Agriculture Pty Ltd., PO Box 741, Muldersdrift, 1747, Gauteng, South Africa
[13] Ineos Silicas S.A. (Pty.) Ltd, Private Bag 12062, Jacobs, 4026, KwaZulu-Natal, South Africa
[14] Polychem Supplies CC, PO Box 17254, Congella, 4013, KwaZulu-Natal, South Africa

Thereafter, plants were transferred back to the glasshouse where they were previously grown.

4.2.3.4 Experimental design

Four plants with four replicates were used in each trial. Plants were placed in a RCBD in the glasshouse and dew chamber. The trial was repeated.

4.2.3.5 Disease assessment

Lesion length (mm), on each stem was measured 2, 3, 4, 6, 7, 8, 9, 10, 11, 13 and 14 dpi, from the point of inoculation downwards. After 14 dpi, the stems were harvested by severing them horizontally at their base. Using a sharp razor blade, stems were split vertically and the number of sclerotia formed inside the stem was counted.

4.2.3.6 Statistical analysis

Rate of growth of the pathogen was calculated as the linear regression of Vanderplank's logistic model (Vanderplank, 1963). Proportions were initially calculated for each day for each cultivar by dividing lesion length by the maximum lesion length obtained in the experiment. The proportion of disease, y, was then substituted into Vanderplank's equation for the logistic model, $r = (\ln (y/(1-y))$. These values were used to calculate rate of growth of SSR using linear regression analysis, by the mean sum of squares (McLaren, pers. comm.[3]).

All data were subjected to an ANOVA using Genstat® Executable Release 9.1 Statistical Analysis Software (Anonymous, 2006) to determine differences between treatment means. Least significant differences were determined at $P<0.05$.

4.3 RESULTS

Trial 2 confirmed results obtained in Trial 1. Similar trends and patterns were observed in both Trials. According to the ANOVA, experiments did not differ, and data were therefore pooled.

4.3.1 *In vitro* dual culture bioassay and ultrastructural studies of Eco77® and EcoT® and *Sclerotinia sclerotiorum*

4.3.1.1 *In vitro* dual culture bioassays of Eco77® and EcoT® and *Sclerotinia sclerotiorum*

Hyphal-hyphal interactions

The results of antagonism, invasion ability and antibiosis are shown in Table 4.1. Dual culture bioassays for hyphal-hyphal interactions revealed that contact between *S. sclerotiorum* and Eco77® and EcoT® occurred 3 dpi. Eco77® and EcoT® showed no inhibitory response towards *S. sclerotiorum*. Hyphae of Eco77® and EcoT® overgrew hyphae of *S. sclerotiorum* and 7 dpi, Petri dishes showed heavy sporulation of Eco77® and EcoT® over the entire Petri dish (Table 4.1, Figure 4.4i to vi). Further incubation of plates revealed no formation of sclerotia on any plates.

Table 4.1 *In vitro* screening of Eco77® and EcoT® against *Sclerotinia sclerotiorum* using dual culture bioassays

	Bell rating		Antibiosis*		Invasion ability
	3 dpi	5 dpi	3 dpi	5 dpi	7 dpi
Eco77®	3	2	-	-	2
EcoT®	3	2	-	-	2

*- (negative) or + (positive) for antibiosis

Figure 4.4 Petri dishes showing the *in vitro* interactions between hyphae of *Sclerotinia sclerotiorum* and Eco77® and EcoT® 3 and 5 days post inoculation.

Plate i: Initial contact between hyphae of *Sclerotinia sclerotiorum* and Eco77® 3 days post inoculation.

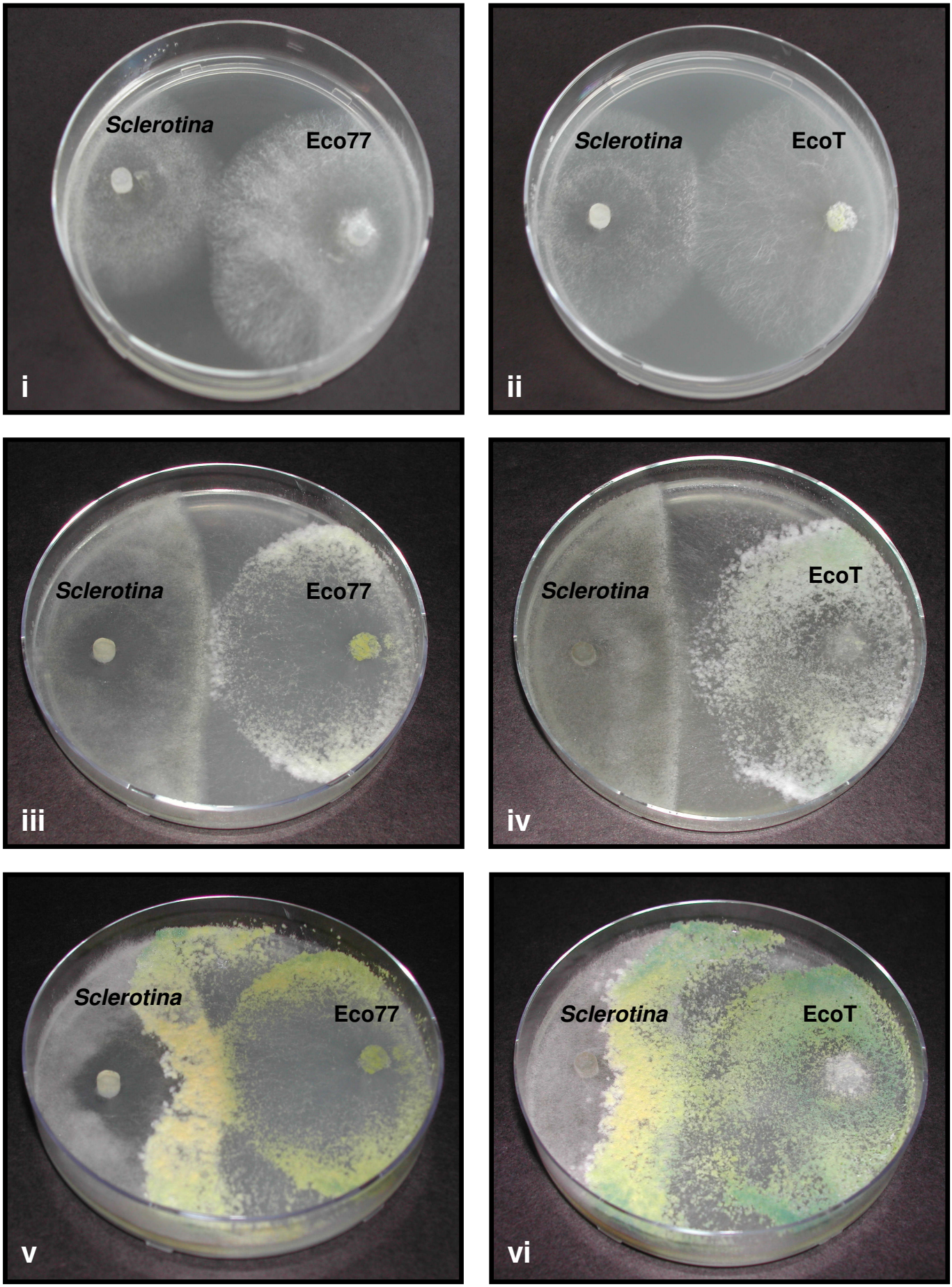
Plate ii: Initial contact between hyphae of *Sclerotinia sclerotiorum* and EcoT® 3 days post inoculation.

Plate iii: Advanced stages of mycoparasitism showing hyphae of Eco77® completely overgrowing hyphae of *Sclerotinia sclerotiorum* 5 days post inoculation.

Plate iv: Advanced stages of mycoparasitism showing hyphae of EcoT® completely overgrowing hyphae of *Sclerotinia sclerotiorum* 5 days post inoculation.

Plate v: Advanced stages of mycoparasitism hyphae of *Sclerotinia sclerotiorum* completely overgrown by a sporulating mycelial mat of Eco77® 7 days post inoculation.

Plate vi: Advanced stages of mycoparasitism hyphae of *Sclerotinia sclerotiorum* completely overgrown by a sporulating mycelial mat of EcoT® 7 days post inoculation.


Sclerotina
Eco77
i
Sclerotina
EcoT
ii
Sclerotina
Eco77
iii
Sclerotina
EcoT
iv
Sclerotina
Eco77
v
Sclerotina
EcoT
vi

Hyphal-sclerotial interactions

Sclerotia appeared to be colonized more rapidly by hyphae of Eco77® (Figure 4.5i, Figure 4.6ii), compared to sclerotia colonized by hyphae of EcoT® (Figure 4.5ii, Figure 4.6ii). Sparse to dense conidiophores and conidia were observed on the surface of all sclerotia thereafter (Figure 4.5iii and iv, Figure 4.6iii and iv), coinciding with the initial stages of disintegration, when sclerotia became soft. Further incubation of plates did not result in the formation of sclerotia for either biocontrol agents.

Figure 4.5 Petri dishes showing the *in vitro* interactions between sclerotia of *Sclerotinia sclerotiorum* and hyphae of Eco77® and EcoT® 4 and 6 days post inoculation.

Plate i: Initial contact between hyphae of Eco77® and sclerotia of *Sclerotinia sclerotiorum* 4 days post inoculation.

Plate ii: Initial contact between hyphae of EcoT® and sclerotia of *Sclerotinia sclerotiorum* 4 days post inoculation.

Plate iii: Later stages of colonization of sclerotia, showing the beginning of sporulation of Eco77® on sclerotia, 6 days post inoculation.

Plate iv: Later stages of colonization of sclerotia, showing the beginning of sporulation of EcoT® on sclerotia, 6 days post inoculation.

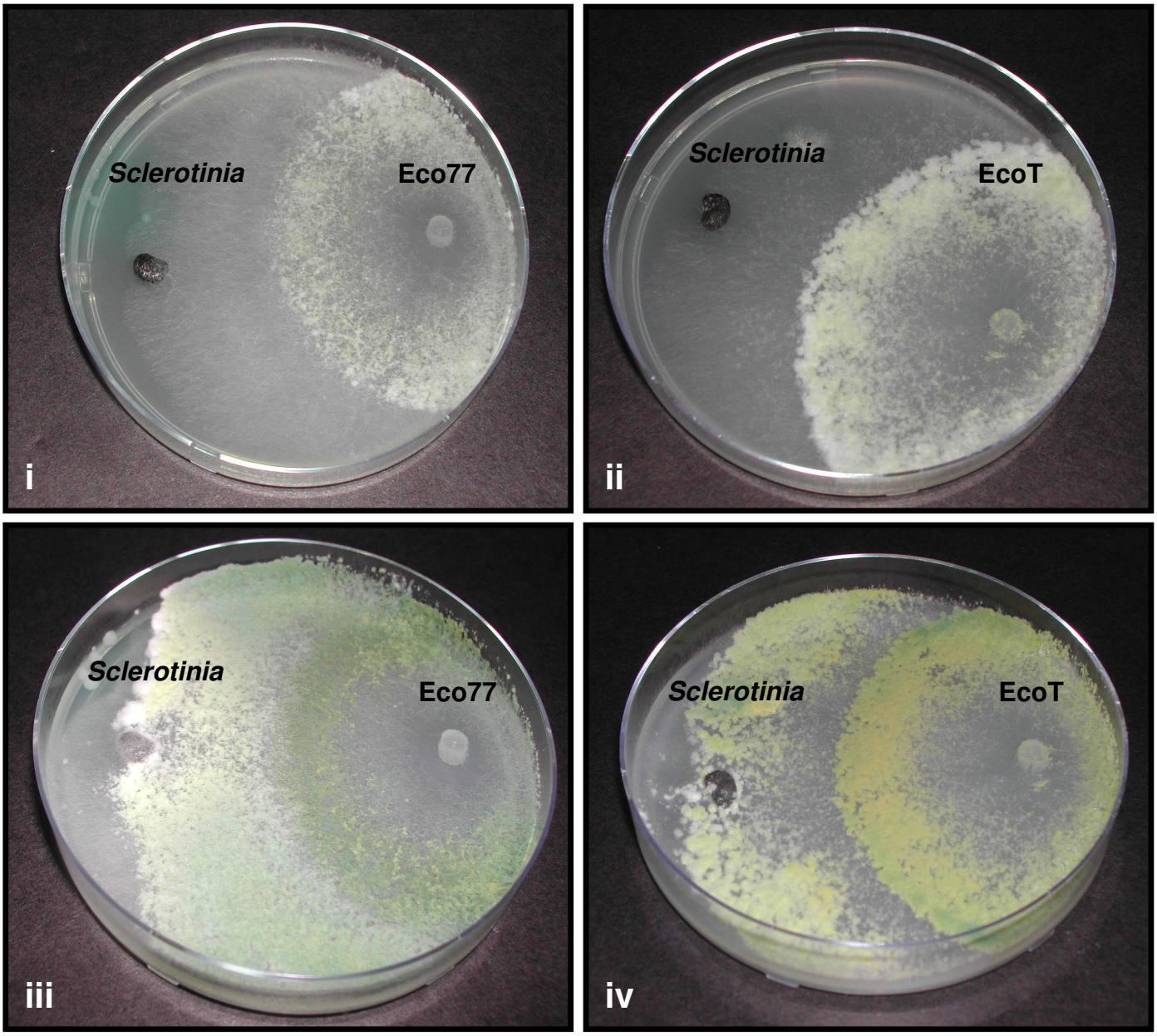

Sclerotinia
Eco77
i
Sclerotinia
EcoT
ii
Sclerotinia
Eco77
iii
Sclerotinia
EcoT
iv

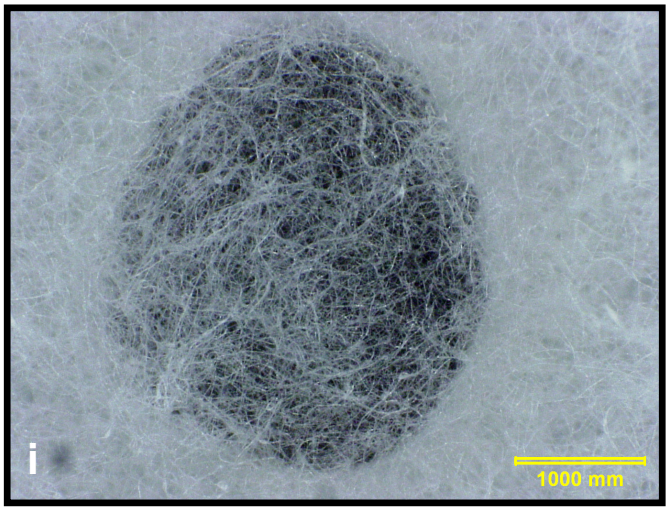
Figure 4.6 *In vitro* interactions between sclerotia of *Sclerotinia sclerotiorum* and hyphae of Eco77® and EcoT® at 4 and 8 days post inoculation.

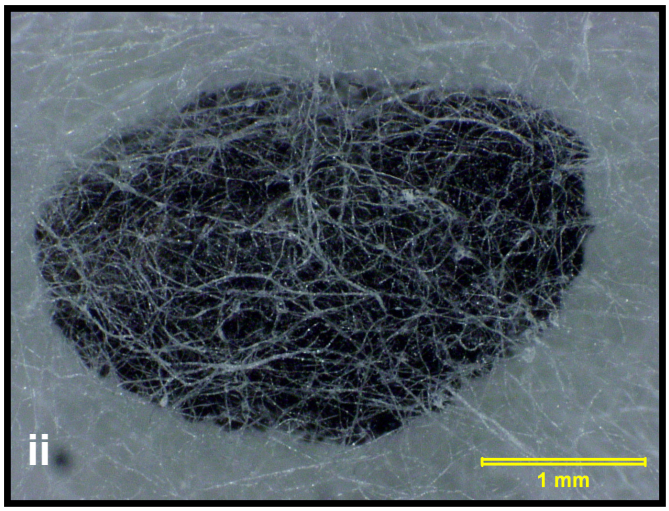
Plate i: Colonization of sclerotia by hyphae of Eco77® and sclerotia of *Sclerotinia sclerotiorum* 4 days post inoculation.

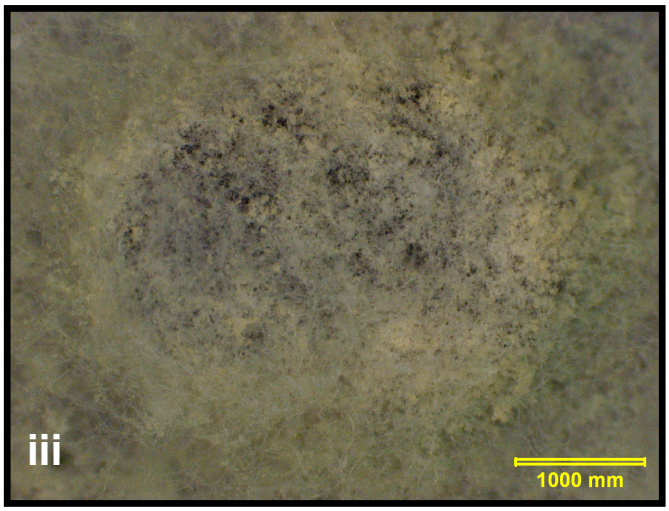
Plate ii: Colonization of sclerotia by hyphae of EcoT® and sclerotia of *Sclerotinia sclerotiorum* 4 days post inoculation.

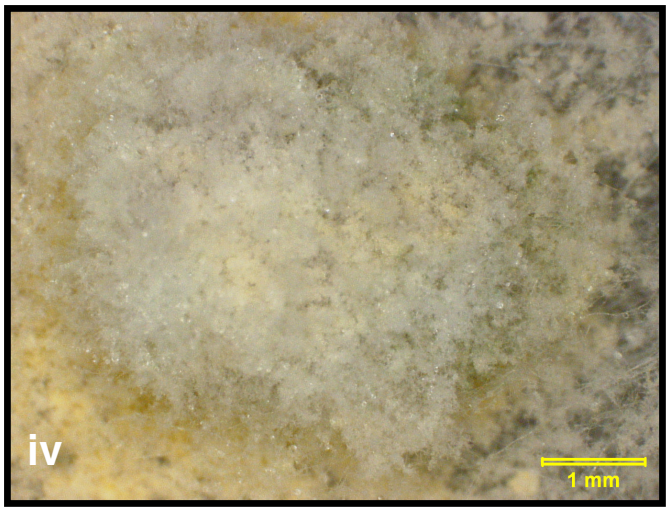
Plate iii: Profuse sporulation of Eco77® on sclerotia, 8 days post inoculation.

Plate iv: Profuse sporulation of EcoT® on sclerotia, 8 days post inoculation.


i
1000 mm


ii
1 mm


iii
1000 mm


iv
1 mm

4.3.1.2 Environmental Scanning Electron Microscopic (ESEM) observations on the interaction between Eco77® and EcoT® and *Sclerotinia sclerotiorum*

Hyphal-hyphal interaction

Environmental scanning electron microscopy revealed that both Eco77® and EcoT® hyphae showed initial signs of mycoparasitism, i.e., coiling of hyphae of the antagonist (Eco77® and EcoT®) around hyphae of *S. sclerotiorum* (Figure 4.7i to iv and Figure 4.8i and ii), causing cell wall lysis (Figure 4.7ii) and degradation (Figure 4.7iii). No advanced stages of coiling were observed.

Hyphal-sclerotial interaction

Environmental scanning electron microscopy revealed that sclerotia were more colonized by hyphae of Eco77® (Figure 4.8i), than sclerotia colonized by hyphae of EcoT® (Figure 4.8ii).

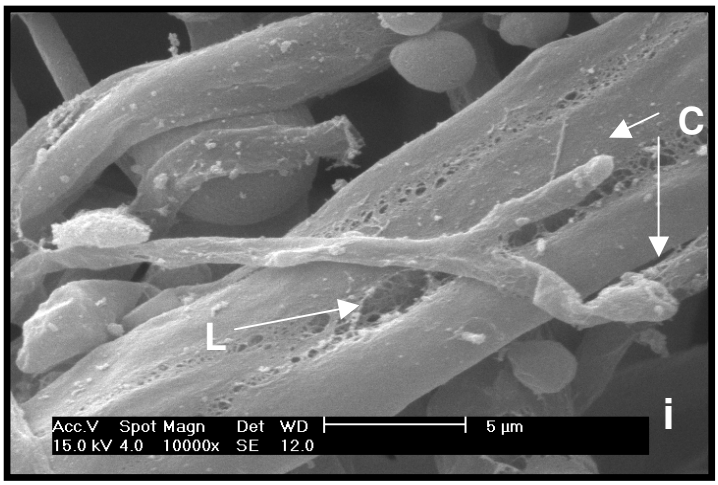
Figure 4.7 Environmental scanning electron micrographs of hyphae of Eco77® and EcoT® mycoparasiting hyphae of *Sclerotinia sclerotiorum* at 3 days post inoculation.

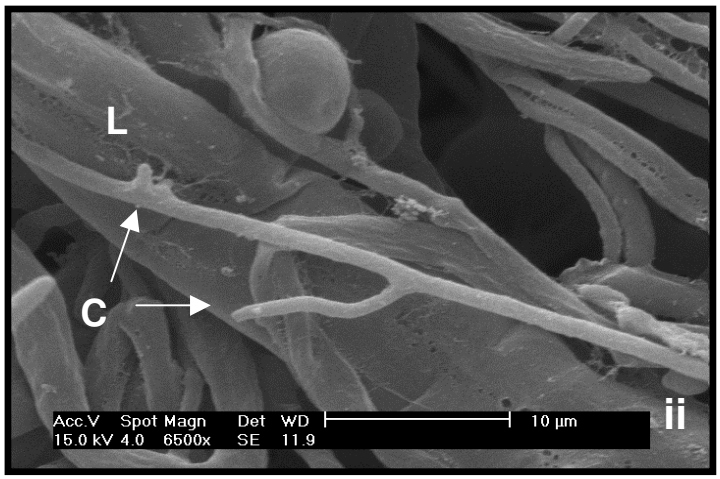
Plate i: Hypha of Eco77® attached to the hypha of *Sclerotinia sclerotiorum*, showing initial signs of coiling (C) and lysis (L) of the cell wall of *Sclerotinia sclerotiorum*, 3 days post inoculation.

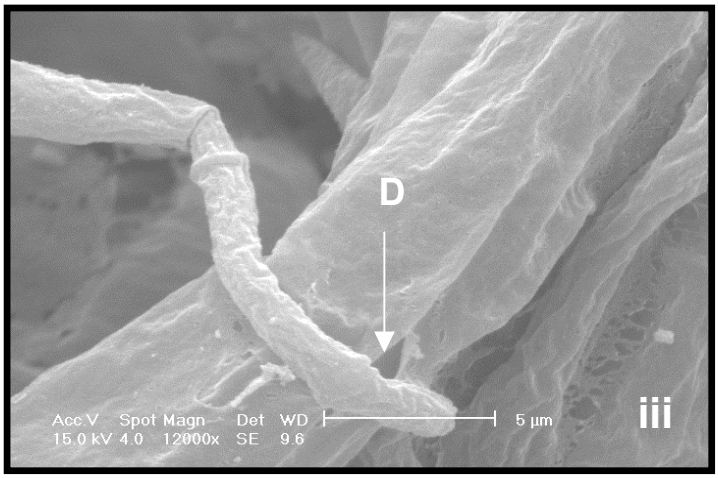
Plate ii: Hypha of Eco77® attached to the hypha of *Sclerotinia sclerotiorum*, showing initial signs of coiling and lysis of the cell wall of *Sclerotinia sclerotiorum*, 3 days post inoculation.

Plate iii: Hypha of EcoT® attached to the hypha of *Sclerotinia sclerotiorum*, showing initial signs of coiling, and degradation (D) of the cell wall of *Sclerotinia sclerotiorum*, 3 days post inoculation.

Plate iv: Coiling of hyphae of EcoT® around hypha of *Sclerotinia sclerotiorum,* 3 days post inoculation.


C
L
Acc.V Spot Magn Det WD 5 µm
15.0 kV 4.0 10000x SE 12.0
i


L
C
Acc.V Spot Magn Det WD 10 µm
15.0 kV 4.0 6500x SE 11.9
ii


D
Acc.V Spot Magn Det WD 5 µm
15.0 kV 4.0 12000x SE 9.6
iii

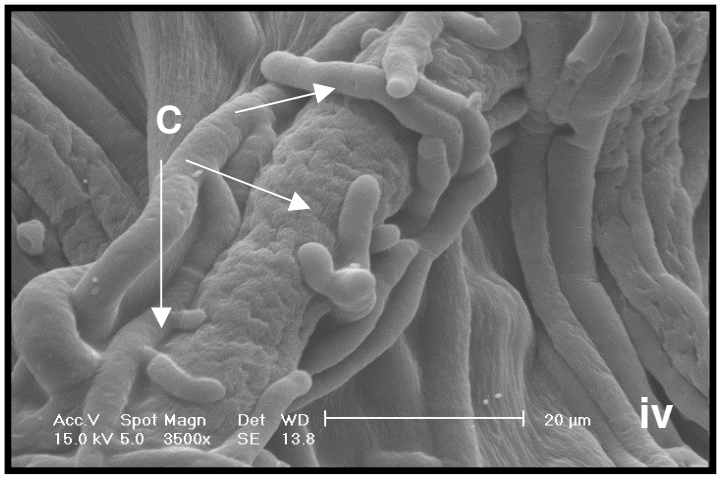
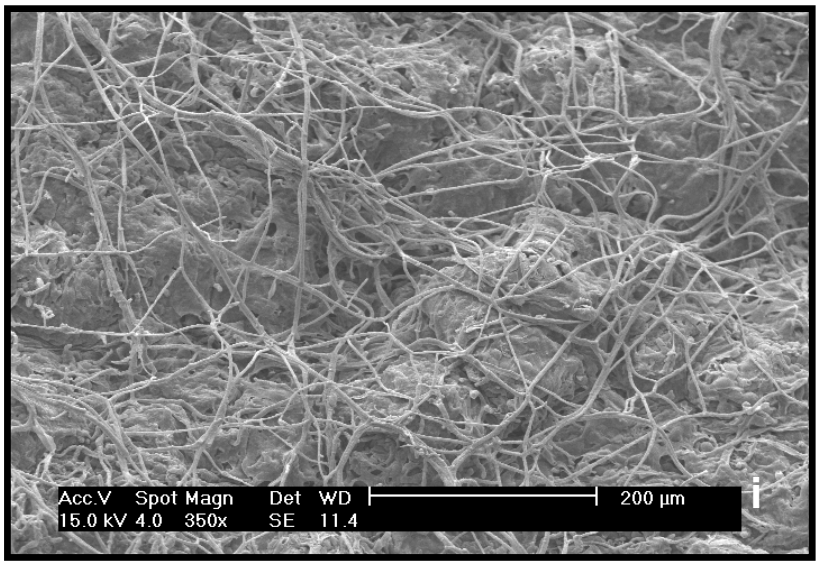
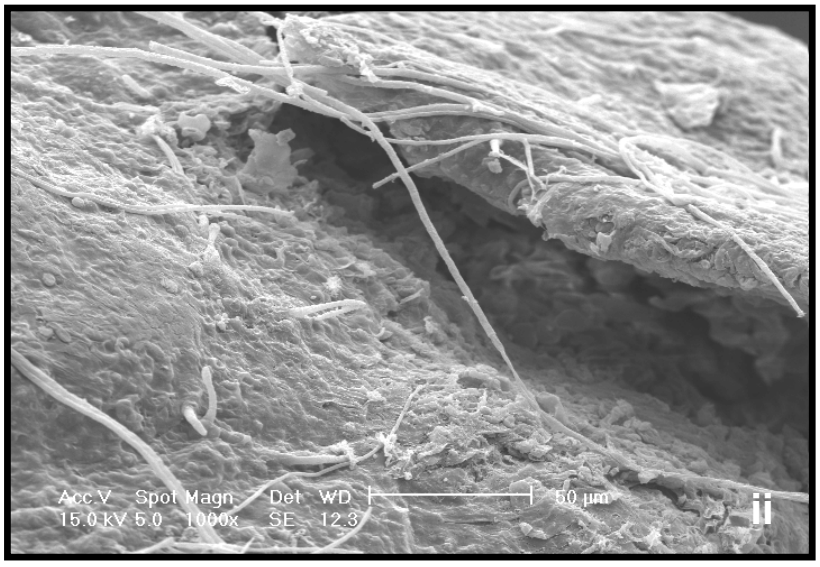

C
Acc.V Spot Magn Det WD 20 µm
15.0 kV 5.0 3500x SE 13.8
iv

Figure 4.8 Environmental scanning electron micrographs of hyphae of Eco77® and EcoT® colonizing sclerotia of *Sclerotinia sclerotiorum* at 4 and 6 days post inoculation.

Plate i: Hyphae of Eco77® colonizing the surface of a sclerotium, 4 days post inoculation.

Plate ii: Hyphae of EcoT® colonizing the surface of a sclerotium, 6 days post inoculation.


Acc.V Spot Magn Det WD 200 µm
15.0 kV 4.0 350x SE 11.4
i


Acc.V Spot Magn Det WD 50 µm
15.0 kV 5.0 1000x SE 12.3
ii

4.2.3 *In vitro* antagonism of EcoT® against *Sclerotinia sclerotiorum* on soybean seed

Results of percentage germination and percentage non infected seedlings 14 dpi for *Trichoderma* treated seeds and *S. sclerotiorum*; untreated seeds and *S. sclerotiorum* and the control are shown in Appendices 4b and c for Trial 1 and Trial 2, respectively.

No significant difference in percentage germination was found between the two treatments, i.e., EcoT® treated seeds plated with *S. sclerotiorum*; and untreated seeds plated with *S. sclerotiorum*; and the control (Appendix 4d and Figure 4.9). Conidia of EcoT® were observed growing across the vermiculite and colonizing the PDA area where the pathogen was placed for the EcoT® treated seeds (Figure 4.10ii). The EcoT® treated seeds and the control were not infected at 14 dpi (Figure 4.10i and iii).

In untreated seeds, a significantly lower percentage of non infected seedlings (31.2%) was recorded (Figure 4.9, Appendix 4d). Lesions due to infection by the pathogen were observed on those seedlings which were not treated with EcoT®.

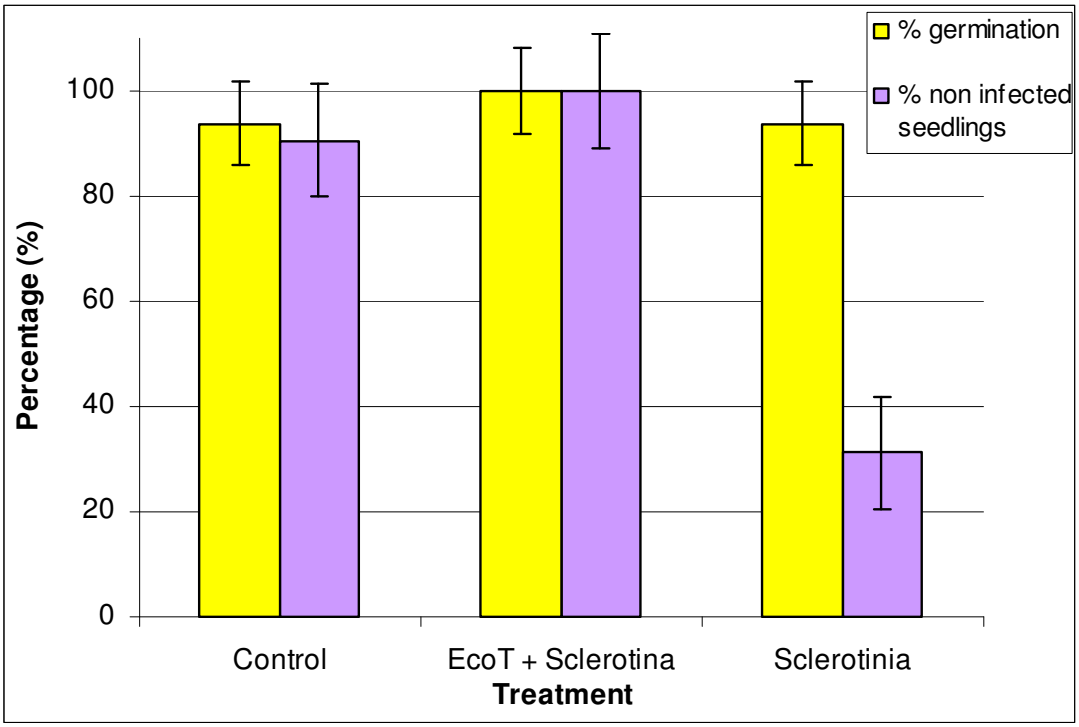


Figure 4.9 Percentage germination and percentage non infected seedlings 14 days post inoculation for *Trichoderma* treated seeds and *Sclerotinia sclerotiorum*; untreated seeds and *Sclerotinia sclerotiorum* and the control.

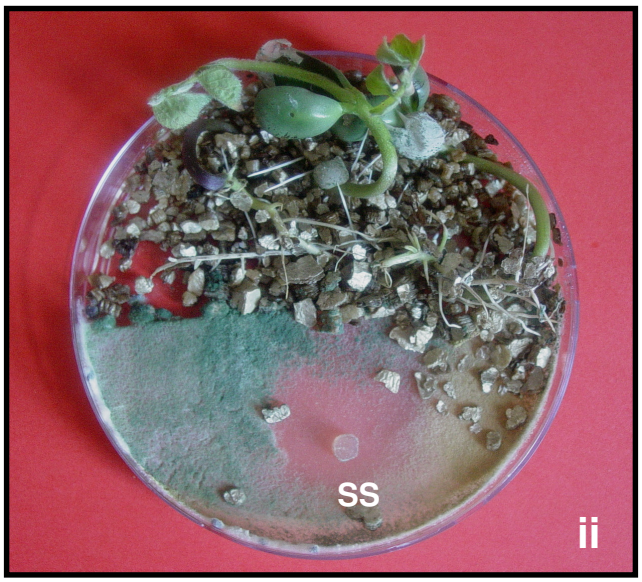
Figure 4.10 Petri dishes showing the *in vitro* antagonism of EcoT® against *Sclerotinia sclerotiorum* on soybean seed 14 days post inoculation.

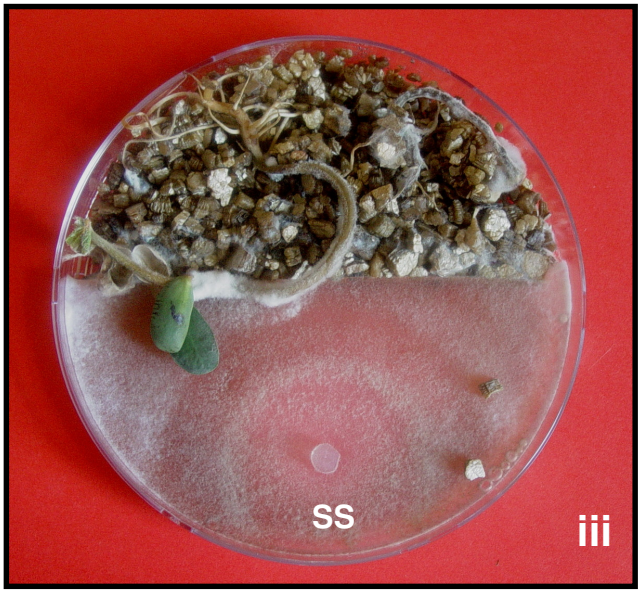
Plate i: Non infected seeds of the control.

Plate ii: *Trichoderma* treated seeds plated with *Sclerotinia sclerotiorum* (SS), showing non infected seedlings.

Plate iii: Untreated seeds plated with *Sclerotinia sclerotiorum* (SS), showing infected seedlings.


i


SS
ii


SS
iii

4.3.3 *In vivo* effect of potassium silicate and Eco77® on *Sclerotinia sclerotiorum*

All plants inoculated with mycelial plugs of *S. sclerotiorum* showed typical symptoms of SSR. Sclerotia were present in the main stems of all plants treated with the pathogen. Initially, symptoms appeared as water soaked lesions on the main stem, which later turned a light brown colour. When the margin of the lesion reached the lower node of the cut stem section, leaves wilted and died but remained attached to the stem.

Results of lesion length (mm) are shown in Appendices 4e and f for Trial 1 and 2, respectively. Rates of growth of the pathogen and number of sclerotia produced are shown in Appendices 4g and h, for Trial 1 and 2, respectively. Regression analysis for Trial 1 and 2 are shown in Appendix 4i.

Plants treated with Eco77® (Appendix 4j, Figure 4.11 i and ii and Figure 4.12) had a significantly slower rate of growth of the pathogen compared to plants not treated with Eco77® (Appendix 4j, Figure 4.11 iii and iv and Figure 4.12), regardless of the application of Si. There was also a significant difference in the number of sclerotia between plants treated with Eco77®, regardless of Si application, and plants not treated with Eco77® (Appendix 4j and Figure 4.13).

Regression analysis showed a strong positive correlation coefficient between rate of growth of pathogen and number of sclerotia (14 dpi), i.e., $R^2 = 0.79$ (Figure 4.14).

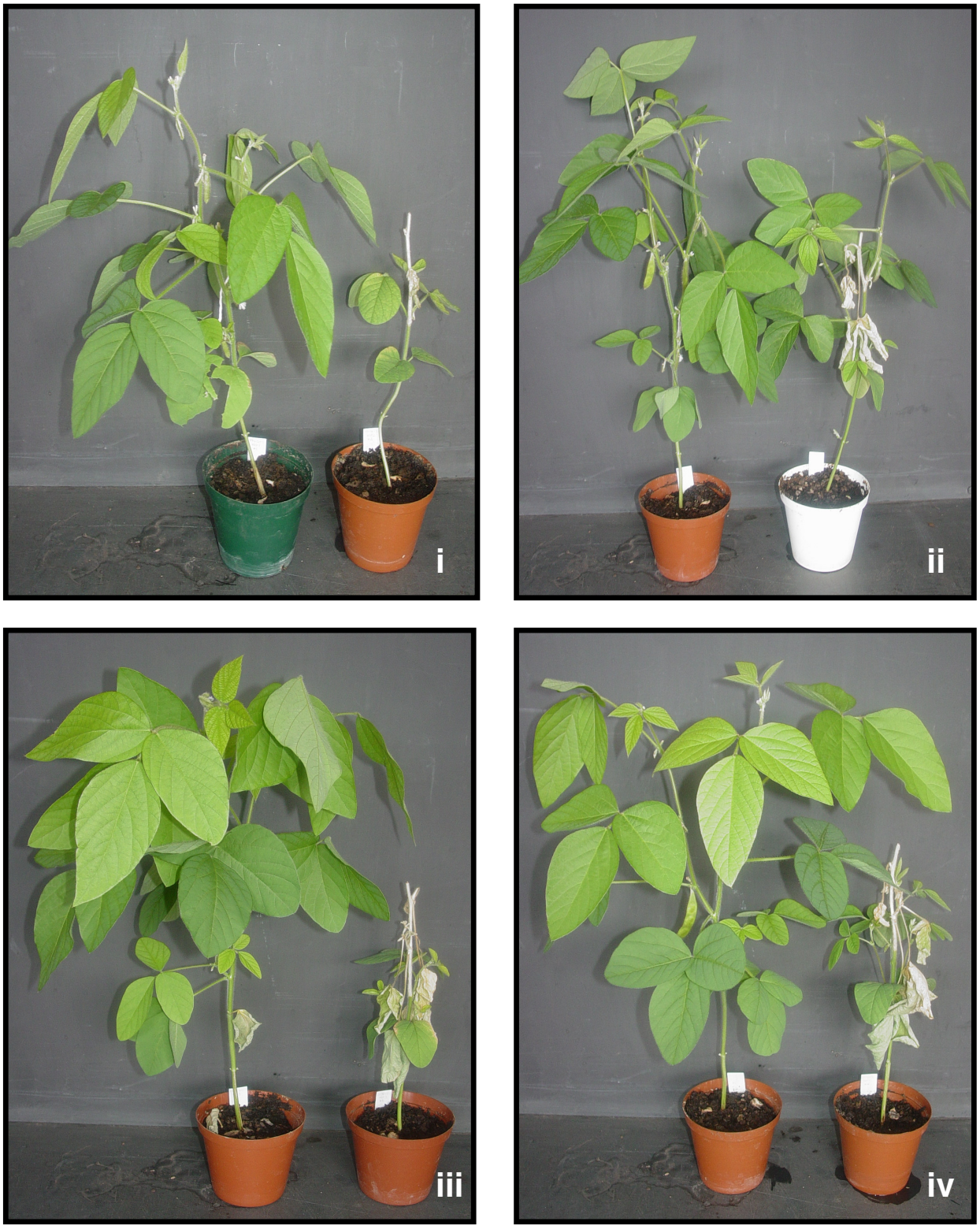
Figure 4.11 *In vivo* effect of potassium silicate (Si) and Eco77® on *Sclerotinia sclerotiorum* on soybean plants 14 days post inoculation.

Plate i: Soybean plants treated with Eco77® only (left) and Eco77® and *Sclerotinia sclerotiorum* (right).

Plate ii: Soybean plants treated with Eco77® and Si (left) and Eco77®, *Sclerotinia sclerotiorum* and Si (right).

Plate iii: Soybean plants not treated (left) and treated with *Sclerotinia sclerotiorum* only (right).

Plate iv: Soybean plants treated with Si only (left) and *Sclerotinia sclerotiorum* and Si (right).


i
ii
iii
iv

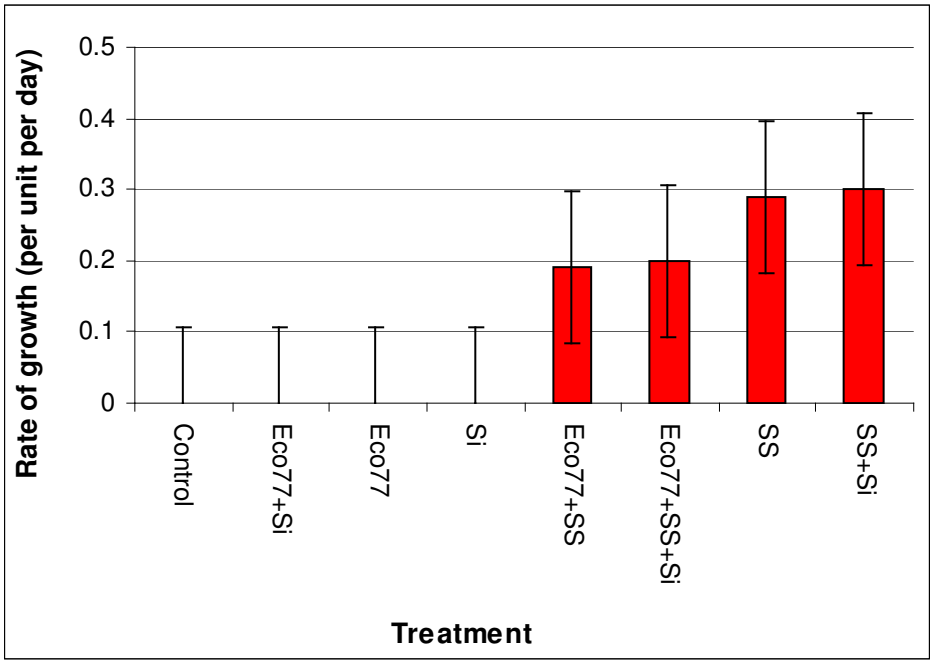


Figure 4.12 Rate of growth of the pathogen in Prima 2000 plants treated with combinations of potassium silicate (Si), Eco77® (Eco77) and *Sclerotinia sclerotiorum* (SS).

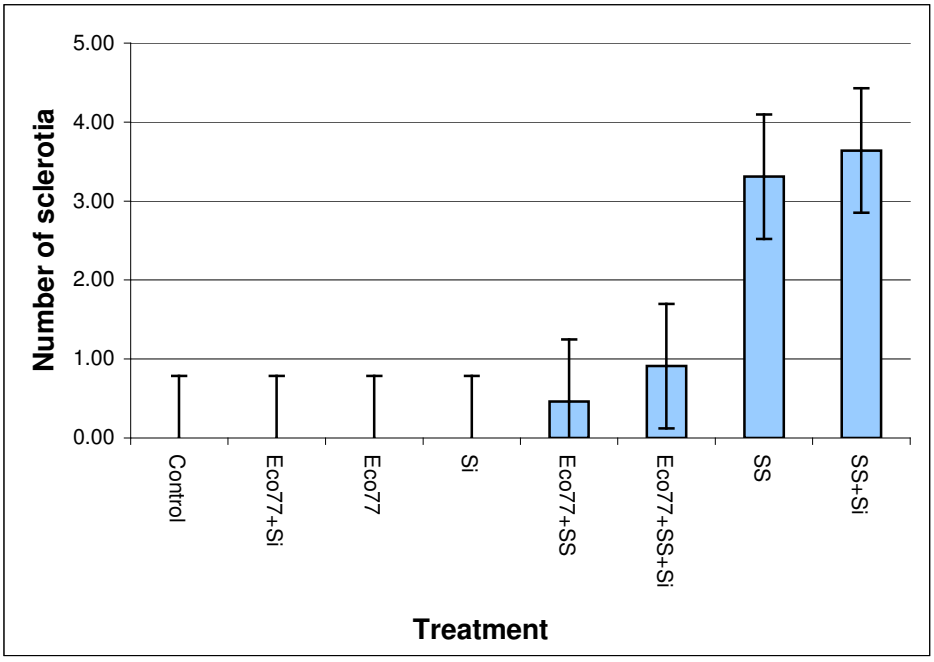


Figure 4.13 Number of sclerotia produced at 14 days post inoculation in Prima 2000 plants treated with combinations of potassium silicate (Si), Eco77® (Eco77) and *Sclerotinia sclerotiorum* (SS).

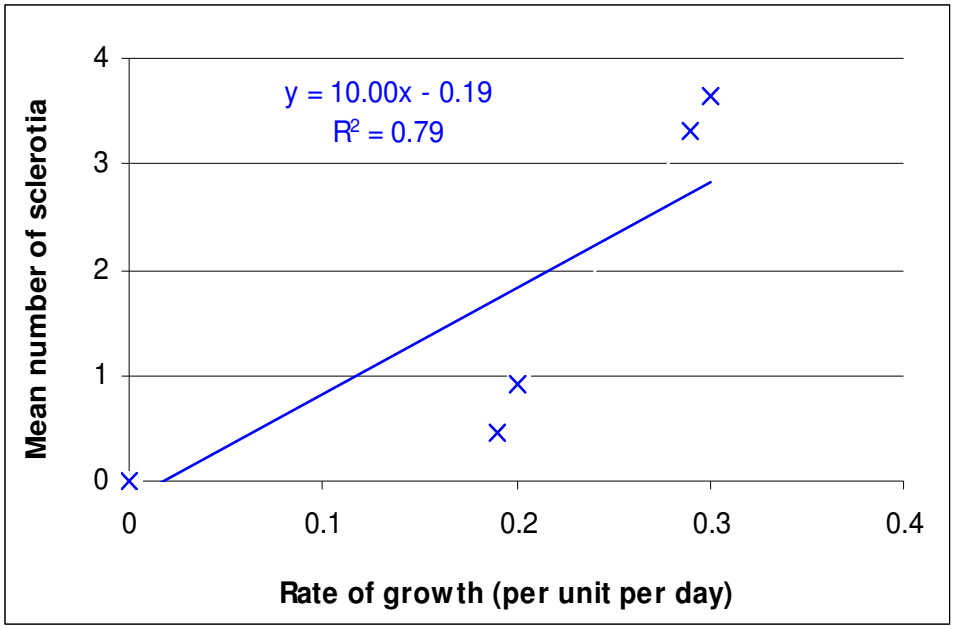


Figure 4.14 Regression analysis of rate of growth of pathogen (per unit per day) versus number of sclerotia produced in Prima 2000 plants treated with combinations of potassium silicate, Eco77® and *Sclerotinia sclerotiorum*.

4.4 DISCUSSION

Trichoderma spp. are well known for their antagonistic activity against many plant pathogens, including soil-borne pathogens such as *S. sclerotiorum* (Merriman, 1976). In this study, initial signs of coiling of both Eco77® and EcoT® hyphae around hyphae of *S. sclerotiorum* was observed, suggesting mycoparasitism as the probable mode of action. Whipps (1987) observed that occasionally, and only with one isolate of *S. sclerotiorum*, hyphae of *Trichoderma* spp. coiled around hyphae of *S. sclerotiorum.* However, mycoparasitism, i.e., coiling, as the mode of action of *Trichoderma* spp. and other antagonists on various pathogens has been successfully determined (Jones and Watson, 1969; Hadar *et al*., 1979; Tu, 1980; Bell *et al*., 1982; Dos Santos and Dhingra, 1982; Elad *et al*., 1983; Huang and Kokko, 1983; McLaren *et al*., 1986; Whipps, 1987; Singh, 1991; Inbar *et al*., 1996; Menendez and Godeas, 1998; Omarjee, 2002; Li *et al*.,

2003; Yobo *et al*., 2004; Kidane, 2004). Slight coiling of *C. minitans* around hyphae of *S. sclerotiorum* has also been observed (McLaren *et al*., 1986). Further investigations into the hyphal-hyphal interaction and mode of action of Eco77® and EcoT® on *S. sclerotiorum* are required. Eco77® and EcoT®, however, did inhibit the formation of subsequent sclerotia by the pathogen.

Environmental scanning electron microscopy revealed Eco77® and EcoT® on the surface of sclerotia. From these studies it cannot be confirmed whether hyphae of Eco77® and EcoT® penetrated the medulla of sclerotia. It is thus important that Transmission Electron Microscopy (TEM) studies, which may reveal whether hyphae of Eco77® and EcoT® penetrate the medulla and determine whether penetration is superficial, inter-cellular or intra-cellular, be conducted. *Coniothyrium minitans* has been shown to invade sclerotia of *S. sclerotiorum*, inter-cellularly and intra-cellularly using TEM (Huang and Kokko, 1987).

Using TEM to determine the degree of destruction of sclerotia is important as sclerotia germinate to produce mycelia or ascopores, the primary source of inoculum of *S. sclerotiorum*. Baker and Cook (1974) suggested that hyperparasites should be most effective against the survival structures of pathogens, in this case sclerotia. *Coniothyrium minitans* is effective in controlling the production of apothecia, which in turn produce ascospores, thereby decreasing inoculum potential of *S. sclerotiorum* under canopies of host and non-host crops (McLaren *et al*., 1996). *Talaromyces flavus* is also able to successfully destroy sclerotia of *S. sclerotiorum* (McLaren *et al*., 1983).

Not only is it important that either Eco77®, EcoT® or both antagonists to degrade preformed sclerotia and inhibit subsequent sclerotial production, they must also be able to degrade large numbers of sclerotia in order to successfully control *S. sclerotiorum*, as only a few surviving sclerotia are capable of initiating an epidemic (Trutmann and Keane, 1990). It is also important to ensure that these antagonists are able to penetrate dry stems of soybeans which are left in the field after harvest, as it is here, in the pith of

soybean stover, where sclerotia remain as an important source of inoculum for the subsequent season (Merriman *et al*., 1979).

Although the *in vitro* antagonism of EcoT® against *S. sclerotiorum* on soybean seed may not indicate what happens in the soil, it does however give an indication of the potential of EcoT® in controlling *S. sclerotiorum* on soybean seed. The observation of sporulation of EcoT®, i.e., conidia on the vermiculite and colonization of the PDA area where the pathogen was placed, shows that coating soybean seeds with EcoT® protects the seed from infection by *S. sclerotiorum*. Coupled with the fact that uncoated seeds were attacked by *S. sclerotiorum*, resulting in a significantly lower percentage of healthy seeds, coating soybean seeds with EcoT® holds great potential. Hadar *et al*. (1984) showed that conidia of *T. harzianum* inoculated in the soil with seeds effectively controlled damping-off of bean, tomato and eggplant by *R. solani*. *Trichoderma harzianum* and *T. atroviride* P. Karsten were both successfully shown to increase percentage germination when cucumber seedlings were coated with conidia against *R. solani* (Yobo *et al*., 2004).

Silicon applications to plants have been associated with resistance to numerous diseases (Yoshida *et al*., 1962; Carver *et al*., 1987; Menzies *et al*., 1991; Cherif *et al*., 1992a; Cherif *et al*., 1992b; Menzies *et al*., 1992; Cherif *et al*., 1994; Datnoff *et al*., 1997; Bélanger *et al*., 2003; Yang *et al*., 2003). However, Si did not show any effect on resistance as no significant differences in lesion length of SSR were found with root applications of Si. However, the significant difference in lesion length between plants treated with Eco77® and those not treated with Eco77® as well as the significant difference in number of sclerotia between plants treated with Eco77® and those not treated with Eco77®, regardless of Si application indicates that Eco77®, and not Si, is responsible for decreasing lesion length and sclerotial production. By significantly reducing the production of sclerotia, which leads to carpogenic or myceliogenic germination, resulting in the production of ascospores or mycelia, inoculum for secondary cycles may be reduced.

There may be numerous reasons why Si may not have been as effective in controlling SSR as observed with other diseases, i.e., the Si concentration tested or the application method of Si (Yoshida *et al*., 1962; Carver *et al*., 1987; Menzies *et al*., 1991; Cherif *et al*., 1992a; Cherif *et al*., 1992b; Menzies *et al*., 1992; Cherif *et al*., 1994; Datnoff *et al*., 1997; Bélanger *et al*., 2003; Yang *et al*., 2003).

It is suggested that the optimal concentration of Si is 100 ppm for sufficient control of powdery mildew (*P. xanthii*) and root rot diseases (*Phythium* spp.) in cucumber (Menzies *et al*., 1991; Cherif and Bélanger, 1992). Higher concentrations of Si, or an increase in the number of Si applications per week, could be examined for their effect on the control of SSR in soybeans.

In most studies on the effectiveness of Si to control diseases, root application of Si has been studied, with little focus on foliar application (Liang *et al*., 2005). Foliar application and root application of Si was studied by Liang *et al*. (2005) for its control of powdery mildew (*P. xanthii*) on cucumber. It was found that foliar application effectively controls infection by *P. xanthii* via the physical barrier of Si deposited on leaf surfaces but Si cannot enhance systemic accquired resistance induced by inoculation. However, root application of Si can enhance systemic required resistance induced by inoculation (Liang *et al*., 2005). Bowen *et al*. (1992) found that root application of Si had no effect on disease severity of *Erysiphe necator* (Schwein.) on grape leaves. However, when Si was sprayed onto the leaf surface 1 dpi, the development of disease substantially decreased. In studies on soybean rust (*Phakopsora pachyrhizi* Sydow), percentage area leaf infected with soybean rust decreased with an increase in Si application. Leaves with foliar application of Si at 200 ppm had 25% leaf area infected, whereas leaves treated with 50 ppm, 45% of the leaf area was infected (Laing, pers. comm.[15]).

It may be useful to study the foliar application of Si, including application onto flowers, and inoculating leaves and flowers with ascospores, the primary source of inoculum of SSR, to give an indication of the potential of Si to control ascospores of the pathogen.

[15] Prof M. Laing, Discipline of Plant Pathology, School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of KwaZulu-Natal, Private Bag X01, Scottsville, 3209, Pietermaritzburg, South Africa

In vitro studies showed that at Si doses of 5-80 ml ℓ^{-1} agar, growth of *S. sclerotiorum* was completely inhibited. Silicon has an inhibitory effect on the growth of *S. sclerotiorum in vitro* and exhibits direct fungitoxic action, suggesting the potential of Si in resistance to *S. sclerotiorum* (Labuschange, pers. comm.[16]).

From these studies, it can be concluded that Eco77® and EcoT® may be considered potential biocontrol agents of *S. sclerotiorum*. *In vitro* antagonism of Eco T® against *S. sclerotiorum* on soybean seed suggests that EcoT® has potential as a seed treatment for protection against *S. sclerotiorum.* Although further research is needed, e.g., foliar application, higher concentrations and more frequent applications of Si as a root application, to determine whether Si increases growth and/or yield of plants, this element does show potential.

[16] Dr N. Labuschagne, Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, 0002, South Africa

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CHAPTER FIVE

EFFICACY OF FUNGICIDES ON *SCLEROTINIA SCLEROTIORUM* AND THEIR POTENTIAL FOR CONTROL OF SCLEROTINIA STEM ROT ON SOYBEAN

D.D. Visser[1], P.M. Caldwell[1], N. W. McLaren[2]

[1]Discipline of Plant Pathology, University of KwaZulu-Natal, Private Bag X01, Scottsville, 3209, South Africa

[2]Department of Plant Sciences, University of the Free State, Bloemfontein, 9300, South Africa

ABSTRACT

Currently only one fungicide, i.e. procymidone (250 g a.i. ℓ^{-1}), is registered for the control of *Sclerotinia sclerotiorum* on soybeans (*Glycine max*) in South Africa (SA). With the increase in the prevalence of sclerotinia stem rot (SSR), effective control measures need to be identified. The current spray programme for the control of soybean rust (*Phakopsora pachyrhizi*) in SA was investigated for its potential for the control of SSR. *In vitro* trials were conducted to determine the potential of three fungicides at different rates, i.e., BAS 516 04F (133 g a.i. ha^{-1}), BAS 516 04F (266 g a.i. ha^{-1}), BAS 512 06F (380 g a.i. ha^{-1}) and Sumisclex (760 g a.i. ha^{-1}). Fungicides were added to tempered potato dextrose agar (PDA). A mycelial plug of *S. sclerotiorum* was added to the centre of each Petri dish, and the fungal diameter measured (mm) daily for four days post inoculation. Thereafter, the area under mycelial growth curve (AUMGC) and percent inhibition was calculated. The control (uninoculated PDA) had a significantly higher AUMGC (243.0) than all fungicides tested. Complete inhibition of *S. sclerotiorum* by BAS 516 04F (at both concentrations) and BAS 512 06F occurred. Sumisclex inhibited the fungus by 89.07%. *In vivo* trials were conducted on soybean plants (Prima 2000) which were grown to the R1 growth stage before inoculum and fungicides were applied.

Inoculum was applied in the form of *S. sclerotiorum* inoculated barley grain, which was dusted by hand onto plant foliage. Preventative treatments, i.e., BAS 516 04F (133 g a.i. ha^{-1}), BAS 516 04F (266 g a.i. ha^{-1}), BAS 512 06F (380 g a.i. ha^{-1}), curative treatment, i.e. Sumisclex (760 g a.i. ha^{-1}) and a combination preventative/curative treatment, i.e., BAS 512 06F (380 g a.i.h a^{-1})/Sumisclex (570 g a.i. ha^{-1}) were applied at different periods after flowering. Plants were rated on a scale of 1-6, which was then converted to a disease severity index (DSI). Grain yield was also determined. No significant difference in DSI was found between fungicide treatments and the inoculated control. BAS 512 06F and BAS 512 06F/Sumisclex had significantly lower grain yields (6.09 g and 5.96 g, respectively) compared to all other treatments. There was a positive correlation coefficient (R^2=0.76), between DSI and grain yield, indicating that a high DSI is correlated with low grain yield. The use of ascospores, number of sprays per treatment and curative versus preventative fungicides need to be further investigated in order to determine the true potential of these fungicides. Nozzle arrangement to ensure complete coverage of lower foliage also needs to be investigated.

5.1 INTRODUCTION

Sclerotinia stem rot (SSR) of soybeans (*Glycine max* (L.) Merrill.), caused by the fungus *Sclerotinia sclerotiorum* (Lib.) de Bary is an important disease of soybeans as well as numerous other plant species (Purdy, 1979; Boland and Hall, 1994). It causes substantial losses in crop production throughout the world (Purdy 1979). In 2004 it was considered the second most important yield-limiting soybean disease in the United States of America (USA) (Chen and Wang, 2005). In South Africa (SA), SSR was first reported in 1979 (Thompson and van der Westhuizen, 1979). In recent years, particularly in years with high rainfall, there have been sporadic outbreaks of SSR in the Winterton-Underberg and Piet Retief areas of KwaZulu-Natal (KZN), as well as on the Highveld in the Ermelo area, causing significant yield reductions in soybeans. In 2003, SSR was so severe in eastern KZN near the Swaziland border, that farmers harvested

their crop early in the season for use as silage, as it was predicted that there would be no grain yield (Caldwell, pers. comm[1].).

Sclerotinia sclerotiorum can infect a wide range of plants including agriculturally important crops such as cereals, vegetables, fruit, ornamentals and weed species (Scott *et al*., 2005). The index of plant hosts of *S. sclerotiorum* contains 408 species, 42 subspecies, 75 families and 278 genera (Boland and Hall, 1994). This broad host range is important as it restricts the number of non-host crops that could be included in crop rotations designed to reduce sclerotia in infested soils (Abawi and Grogan, 1975).

The recent widespread occurrence of SSR in soybeans may be attributed to changes in management practices, susceptible germplasm and the occurrence of favourable environmental conditions (Hartman *et al*., 1998; Hoffman *et al*., 1998; Mueller *et al*., 1999). The incidence of SSR of soybeans may be reduced with the use of partially resistant cultivars and the alteration of cultural practices that favour disease development. However, with conducive environmental conditions, outbreaks of SSR may still occur (Kim *et al*., 2000; Kurle *et al*., 2001; Gracia-Garza *et al*., 2002; Mueller *et al*., 2002).

No single disease management practice effectively prevents infection of soybeans by *S. sclerotiorum*. However, the integration of various control measures may reduce disease severity and minimize yield loss (Steadman *et al*., 1996). Management practices may be implemented at every stage in the pathogen's life cycle, ranging from prevention of sclerotial development to preventing apothecial formation and ascospore germination (Anonymous, 2005).

[1] Dr P. M. Caldwell, Discipline of Plant Pathology, School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of KwaZulu-Natal, Private Bag X01, Scottsville, 3209, Pietermaritzburg, South Africa.

Fungicides therefore, present an option for control as part of an integrated pest management approach to reduce disease severity and minimize yield loss (Steadman *et al*., 1996). Fungicides constitute crop protection chemicals which have shown the potential to control SSR (Dann *et al*., 1999; Nelson *et al*., 2002). Most of the research on the use of fungicides to control SSR has been carried out on common bean (*Phaseolus vulgaris* L.). Although this may be used as a guideline for the control of SSR on soybeans, there are sufficient fundamental differences in growth habits between the two crops to warrant additional research (Hunter *et al*., 1978; Steadman, 1983; Morton and Hall, 1989). Only one fungicide, i.e. procymidone (250 g a.i. ℓ^{-1}), is registered for the control of *S. sclerotiorum* on soybeans in SA. No disease loss estimates for SSR on soybeans in SA are available.

The objectives of this study were to investigate the effect of different registered and unregistered fungicides on the mycelial growth of *S. sclerotiorum* on fungicide amended agar and to evaluate the efficacy of these different fungicides for the control of *S. sclerotiorum* on soybean in the greenhouse.

5.2 MATERIALS AND METHODS

5.2.1 *In vitro* growth of *Sclerotinia sclerotiorum* on fungicide amended agar

5.2.1.1 Media preparation and treatments

Potato dextrose agar (PDA) (Merck[2]) was autoclaved at 121°C for 15 min and cooled to 45°C-50°C. Fungicides were aseptically added to the cooled agar. Three fungicides were evaluated, at various concentrations: BAS 516 04F[3] (133 g a.i. ha^{-1}) (350 mℓ in 300 ℓ water), BAS 516 04F[3] (266 g a.i. ha^{-1}) (700 mℓ in 300 ℓ water), BAS 512 06F[3] (380

[2] Merck, Biolab Diagnostics (Pty) Ltd, 259 Davidson Rd, Wadeville, 1428, Gauteng, South Africa
[3] BASF SA, PO Box 2801, Halfway House, 1685, South Africa

g a.i. ha^{-1}) (1000 mℓ in 300 ℓ water) and Sumisclex[4] (760 g a.i. ha^{-1}) (2000 mℓ in 300 ℓ water). For the control, non-amended PDA was used. Approximately 20 mℓ of agar was aseptically poured into 14 cm diameter Petri dishes and left overnight on a laminar flow bench.

5.2.1.2 Isolate, inoculum preparation and media inoculation

A *S. sclerotiorum* isolate was obtained from sunflowers (*Helianthus annuus*) in Delmas, Mpumalanga, SA in February, 2005 (McLaren[5]) in the form of sclerotia. The *Sclerotinia* isolate used in this study was sent to Dr E. J. van der Linde[6] for identification and deposition in the Plant Protection Research Institute (PPRI) collection, and was confirmed to be *S. sclerotiorum* (PPRI Accession number 8374). Initially, sclerotia were surface sterilized for 3 min in 70% ethanol, washed twice in sterilized distilled water, and plated on PDA in 9 cm diameter Petri dishes.

Petri dishes were sealed with Parafilm® (Industricord[7]) and incubated in the dark at 20°C for 4 weeks. The resulting sclerotia were harvested, surface sterilized for 3 min in 70% ethanol, washed twice in sterilized distilled water, placed on filter paper in a Petri dish and left to air dry overnight on a laminar flow bench. Sclerotia were placed in a sterile Petri dish and sealed and stored at 12°C in the dark until needed. This stock culture was also maintained by subculturing mycelia onto PDA slants and kept in the dark at 20°C.

To produce inoculum for *in vitro* trials, a sclerotium was surface sterilized, washed twice in sterile distilled water, placed on PDA and allowed to germinate myceliogenically. After 7 days, when the mycelia reached the edge of the Petri dish, a single mycelial plug was cut from the margin of the growing colony with an 11 mm diameter cork borer and aseptically transferred to the centre of a new PDA plate. Plates were incubated for

[4] Philagro SA (Pty) Ltd., PO Box 36213, Menlo Park, 0102, South Africa
[5] Neal McLaren, Department of Plant Sciences, University of the Free State, Bloemfontein, 9300, South Africa
[6] Dr E. J. van der Linde , Biosystematics Division, Agricultural Research Council (ARC), Plant Protection Research Institute (PPRI), Queenswood, 0121, Pretoria, South Africa
[7] Industricord, PO Box 243, Pavilion, 3611, KwaZulu-Natal, South Africa

4 days in the dark at 21°C. Mycelia were sub-cultured by cutting mycelial plugs from the margin of the growing colony and aseptically transferred to new PDA plates. After 4 days, mycelial plugs were cut with a 5 mm diameter cork borer at the edge of the growing colony and used to inoculate amended and non-amended plates. Plates were incubated at 21°C in the dark.

5.2.1.3 Experimental design

Four Petri dishes with six replicates were used in each trial. Petri dishes were placed in a randomized complete block design (RCBD) in the incubator. The trial was repeated.

5.2.1.4 Mycelial assessment

The length and width of the radial growth of the mycelial mat was measured daily for 4 days post inoculation (dpi). The average of the length and width of the colony was used to calculate the diameter of radial growth.

5.2.1.5 Mycelial analysis and percentage inhibition

The area under mycelia growth curve (AUMGC) was then calculated using colony diameter as the dependant variable and the 4 dates as independent variables, using the following formula:

$$AUMGC = \Sigma^{n-1}_{i=1}[(X_{i+1} + X_i)/2]t_{i+1} - t_i]$$

where X_i = colony diameter expressed in mm at the i^{th} observation, t_i = time (dpi) at the i^{th} observation, and n = total number of observations (Mueller *et al*., 1999; Mueller *et al*., 2002).

Percentage inhibition was calculated using the following formula:

$$\text{Percentage inhibition} = \frac{(C - T)}{T} \times 100$$

where C = colony diameter (mm) of control and T = colony diameter (mm) of the test plate (Labuschange, pers. comm.[8]).

5.1.2.6 Statistical analysis

All data were subjected to an analysis of variance (ANOVA) using Genstat® Executable Release 9.1 Statistical Analysis Software (Anonymous, 2006) to determine differences between treatment means. All least significant differences were determined at $P<0.05$.

5.2.2 *In vivo* effect of fungicides on the control of *Sclerotinia sclerotiorum* on soybean plants

5.2.2.1 Plant production

Five soybean seeds (Prima 2000[9]) were planted in composted pine bark (Growmor[10]) in 25 cm diameter pots (6225 cm^3) (Highfield Packaging[11]). Plants were grown in a tunnel at 25°C and 60% relative humidity (RH), where plants were irrigated, supplemented with NPK fertilizer [3:1:3], four times a day for 5 min. Plants were grown to the R1 growth stage (Fehr *et al*., 1971) in a RCBD.

5.2.2.2 Inoculum preparation

To produce inoculum for greenhouse trials, mycelia were sub-cultured by cutting mycelial plugs from the margin of a growing colony and aseptically transferred to new PDA plates. After 4 days, mycelial plugs from the edge of the growing colony were cut with an 11 mm diameter cork borer. Initially 50 g of barley were placed in 250 mℓ Erlenmeyer flasks with 100 mℓ water and allowed to soak overnight. Flasks were autoclaved at 121°C for 15 min on two consecutive days. Three mycelial plugs were used to inoculate the autoclaved barley.

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[8] Dr N. Labuschagne, Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, 0002, South Africa
[9] Pannar Seed, PO Box 19, Greytown, 3250, KwaZulu-Natal, South Africa
[10] Growmor, PO Box 89, Cato Ridge, 3680, KwaZulu-Natal, South Africa
[11] Highfield Packaging, 3 Chesterfield Rd, Willowton, 3201, KwaZulu-Natal, South Africa

Inoculated flasks (Figure 5.1) were left at room temperature for 5-6 days, and shaken by hand every second day to ensure even distribution and growth of the fungus. Thereafter colonized barley grain was aseptically removed from flasks and placed on trays and allowed to dry in a conviron™ at 25°C for 3-4 days in the dark. The inoculated grain was then ground in a commercial coffee grinder and stored at 4°C until needed.

Figure 5.1 Autoclaved barley inoculated with mycelial disks of *Sclerotinia sclerotiorum*.

5.2.2.3 Fungicide application

Four fungicides with different fungicidal actions and dose rates were evaluated (Table 5.1). This spray programme has been tested for the control of *Phakopsora pachyrhizi* Sydow, and was tested for its potential for the control of SSR (Hackland, pers. comm[12].). Plants to be treated with preventative fungicides were first sprayed with fungicides before inoculation, whereas plants to be treated with curative fungicides were first inoculated with the pathogen before applying fungicides. A herbicide sprayer with a 8002 flat fan nozzle at 2 bar pressure (Figure 5.2) was used to apply fungicides immediately after plants flowered.

[12] Nigel Hackland, BASF SA, PO Box 2801, Halfway House, 1685, South Africa

Table 5.1 Fungicides and dose rates (g a.i. ha^{-1}) applied for the control of *Sclerotinia sclerotiorum* on soybeans

Trade name*	Fungicide action	Fungicide formulation**	Active ingredient*	Dose rate (g a.i. ha^{-1})
Untreated control	-	-	-	-
BAS 516 04F	preventative	SC	unknown*	133
BAS 516 04F	preventative	SC	unknown*	266
BAS 512 06F	preventative	SC	unknown*	380
Sumisclex	curative	SC	procymidone	760
BAS 512 06F/Sumisclex	preventative/ curative	SC	unknown*/ procymidone	380/570

* Fungicide treatments are not commercially registered and active ingredients and trade names currently unavailable for release

** SC = suspension concentrate

After the initial fungicide application, plants were left in a glasshouse at 25°C for 2 days, to prevent irrigation water washing off the chemicals. Plants were hand watered during this time. Fungicides were applied on certain days after flowering (Table 5.2).

Figure 5.2 Herbicide sprayer used to apply fungicides to soybean plants.

Table 5.2 Application times of fungicides for the control of *Sclerotinia sclerotiorum* on soybeans

Treatment	Dose rate (g a.i.ha^{-1})	At 1st flowering	Flowering + 10 days	Flowering + 20 days	Flowering + 30 days
Untreated control	-	-	-	-	-
BAS 516 04F	133	X		X	
BAS 516 04F	266	X		X	
BAS 512 06F	380	X			X
Sumisclex	760	X	X	X	
BAS 512 06F/Sumisclex	380/570	X		X	X

5.2.2.4 Inoculation procedure

Plants were lightly sprayed with distilled water. Approximately 2.5 mℓ of inoculum was placed in a McCarthy bottle covered with cheesecloth and lightly dusted by hand evenly onto leaves of each plant (Figure 5.3).

Figure 5.3 Inoculation of soybean plants at the R1 growth stage with barley inoculated with *Sclerotinia sclerotiorum*.

Leaves were lightly sprayed with water to ensure the start of the leaf wetness duration (LWD) period before plants were placed in a dew chamber at 22°C, 95% RH with a 12 hr photoperiod and a light intensity of 110 $\mu Em^{-2}s^{-1}$, for 4 days. The capacity of the dew chamber is 6 m^3. Light is provided by fluorescent lamps at the top of the chamber, radiating through plexiglass. Light intensity was measured with a light meter and controlled by adding or removing fluorescent lamps. The light period was controlled by a timer. Humidity is achieved with an ultrasonic humidifier and controlled by the measurement of RH. Temperature is controlled with a reversed cycle refrigeration unit and heating elements. Plants were then returned to the tunnel where they remained for the duration of the trial.

5.2.2.5 Disease assessment

Plants were assessed at the R7 growth stage. Plants were individually rated based on visual assessment of lesions on leaves of inoculated plants on a scale of 1-6 (adjusted from Kolkman and Kelly, 2000), where:

1 = no symptoms/healthy plants
2 = <10% of the plant with lesions
3 = 10 to 25% of the plant with lesions
4 = 25 to 50% of the plant with lesions
5 = 50 to 90% of the plant with lesions
6 = >90% of the plant with lesions or plants dead

5.2.2.6 Grain yield

At the R7 growth stage, plants were moved to a shade house and left to dry for 2 weeks. At physiological maturity, pods were hand-harvested and dried to <12% moisture in a drying oven at 50°C for 2 days. Thereafter soybean grain was removed from pods and weighed to determine yield.

5.2.2.7 Statstistical analysis

Visual ratings of lesions on leaves was converted to a 0-100 disease severity index (DSI), using the following formula from Kolkman and Kelly (2000):

$$DSI = [\sum (\text{rating of each plant}) / 6 (\text{number of plants rated})] \times 100$$

All data were subjected to an analysis of variance (ANOVA) using Genstat® Executable Release 9.1 Statistical Analysis Software (Anonymous, 2006) to determine differences between treatment means and interactions between temperature, LWD and RH. Least significant differences were determined at $P<0.05$.

5.3 RESULTS

Trial 2 confirmed the results that were obtained in Trial 1 as similar trends and patterns were observed in both trials. According to the ANOVA, experiments did not differ, and data were therefore pooled.

5.3.1 *In vitro* growth of *Sclerotinia sclerotiorum* on fungicide amended agar

Mycelial diameters (mm) on the Petri dish for Trial 1 and 2 are shown in Appendix 5a.

5.3.1.1. Area under mycelial growth curve

Area under mycelial growth curve is shown in Appendices 5b and c, for Trial 1 and 2, respectively.

Non-amended agar (control) had a significantly higher AUMGC (243.0), compared to that of Sumisclex (23.31). No radial growth was observed on agar amended with all BAS treatments, hence an AUMGC of 0 (Appendix 5d and Figure 5.4).

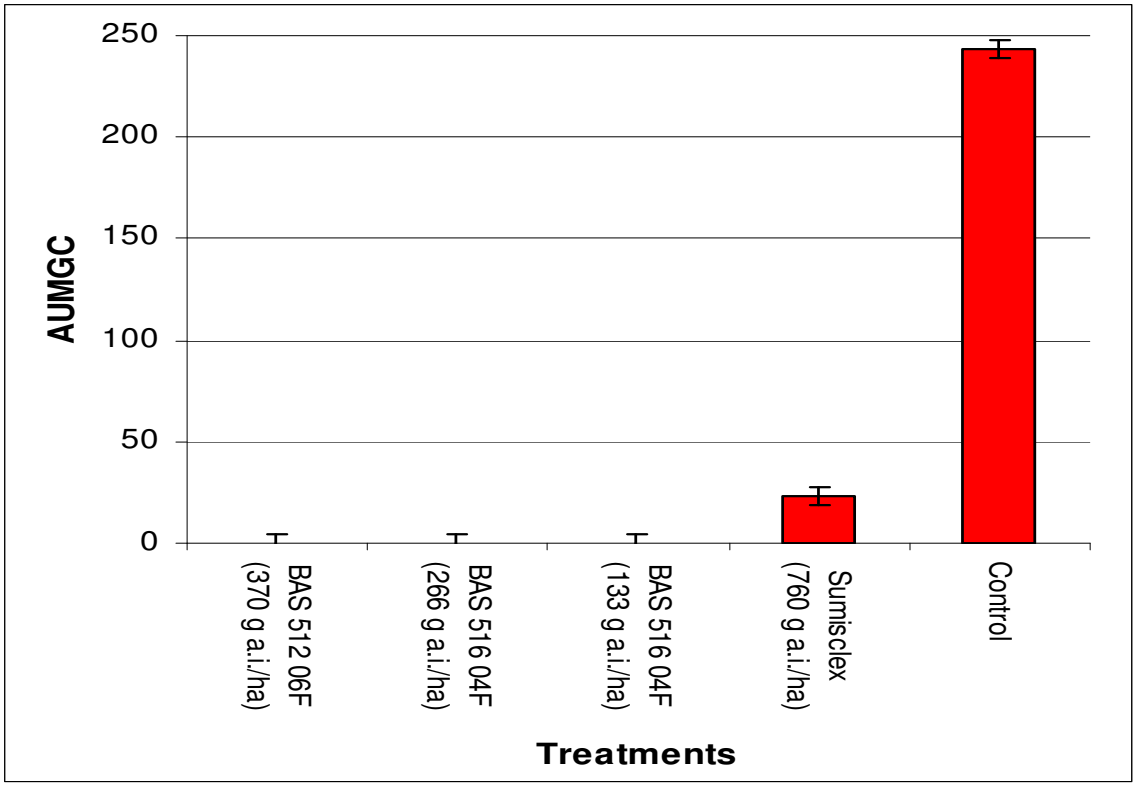

Figure 5.4 Area under mycelial growth curve (AUMGC) of fungicide amended agar inoculated with *Sclerotinia sclerotiorum.*

5.3.1.2. Percentage inhibition

Percentage inhibition is shown in Appendices 5b and c, for Trial 1 and 2, respectively.

Agar amended with BAS 516 04F at 133 and 266 g a.i. ha^{-1}, and BAS 512 06F completely inhibited mycelial growth of *S. sclerotiorum* (100%) (Appendix 5d and Figure 5.5). Sumisclex inhibited the growth of *S. sclerotiorum* by 89.07% (Appendix 5d and Figure 5.5).

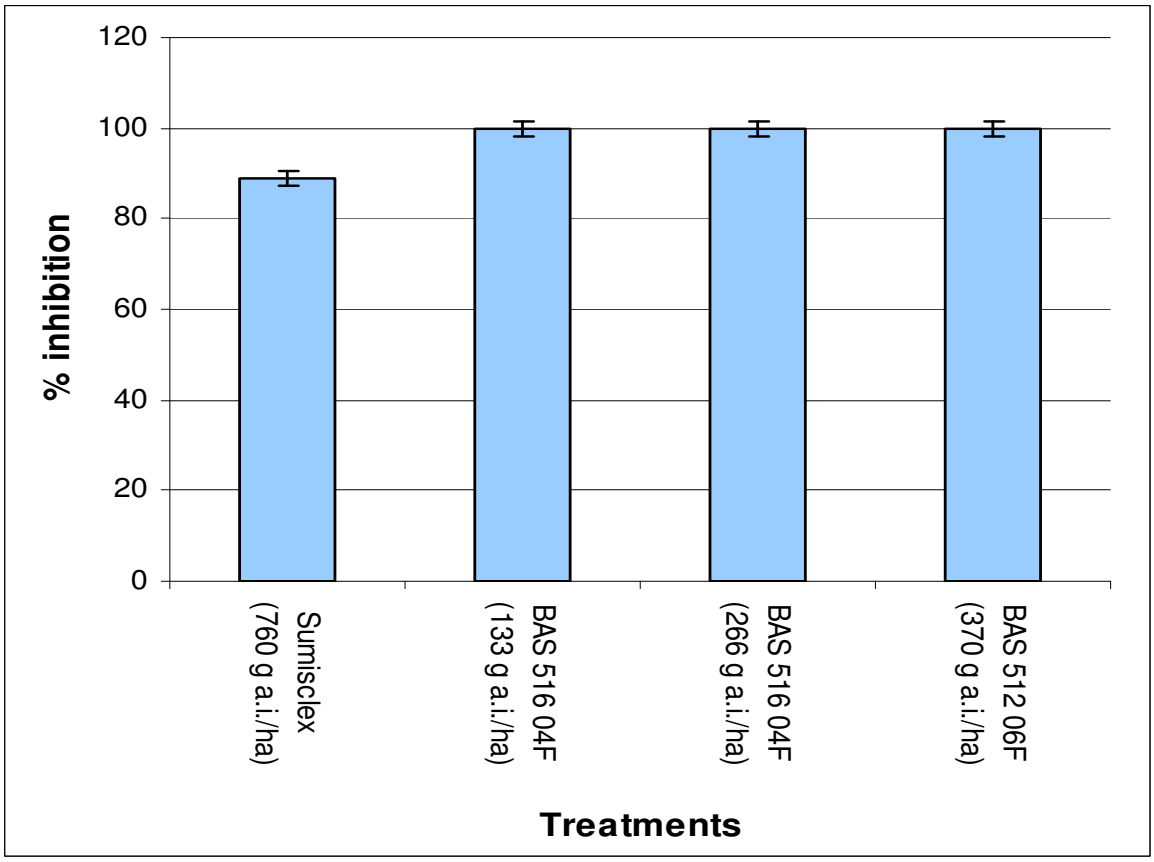

Figure 5.5 Percentage inhibition of *Sclerotinia sclerotiorum* on fungicide amended agar.

5.3.2 *In vivo* effect of fungicides on the control of *Sclerotinia sclerotiorum* on soybean plants

In both trials, light brown lesions on leaves were observed on all plants inoculated with the fungus (Figure 5.6). In pods of inoculated controls, small sclerotia were observed. However, in all fungicide treated plants, no sclerotia were observed in pods.

Figure 5.6 Symptoms of mycelial infection of soybean leaves by *Sclerotinia sclerotiorum*.

5.3.2.1. Disease severity index

Disease severity index ratings are shown in Appendices 5e and f, for Trial 1 and 2, respectively.

Disease severity index was not significantly different between the inoculated control and all fungicides tested (Appendix 5g and Figure 5.7).

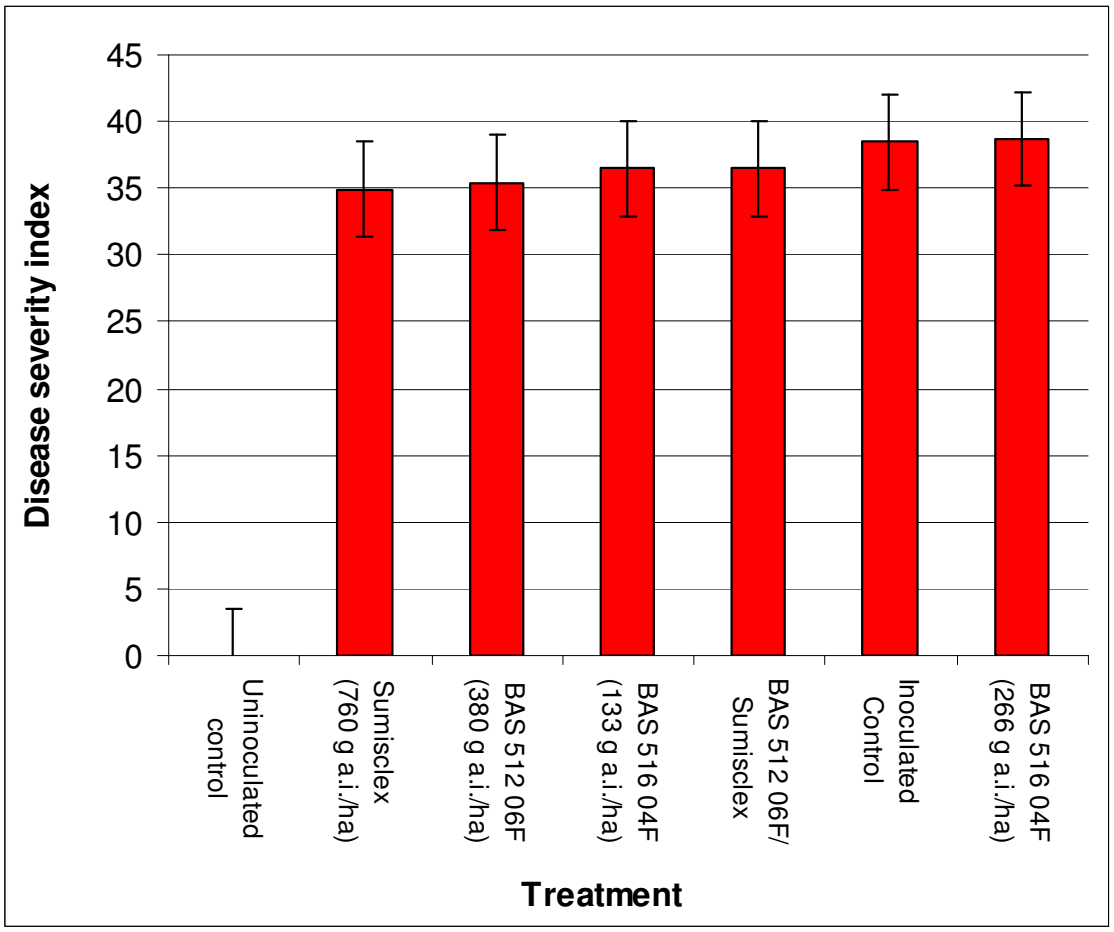

Figure 5.7 Disease severity index of Prima 2000 inoculated with *Sclerotinia sclerotiorum* and sprayed with various fungicides, at the R7 growth stage.

5.3.2.2. Grain yield

Grain yields are shown in Appendices 5e and f, for Trial 1 and 2, respectively.

The uninoculated control showed a significantly higher grain yield (26.07 g) than all other treatments (Appendix 5g). BAS 512 06F (380 g a.i. ha^{-1}) and BAS 512 06F/ Sumisclex had the significantly lowest yield (6.09 and 5.96 g, respectively) (Appendix 5g and Figure 5.8). BAS 516 04F (both concentrations) and Sumisclex were not significantly different to the inoculated control (Appendix 5g and Figure 5.8).

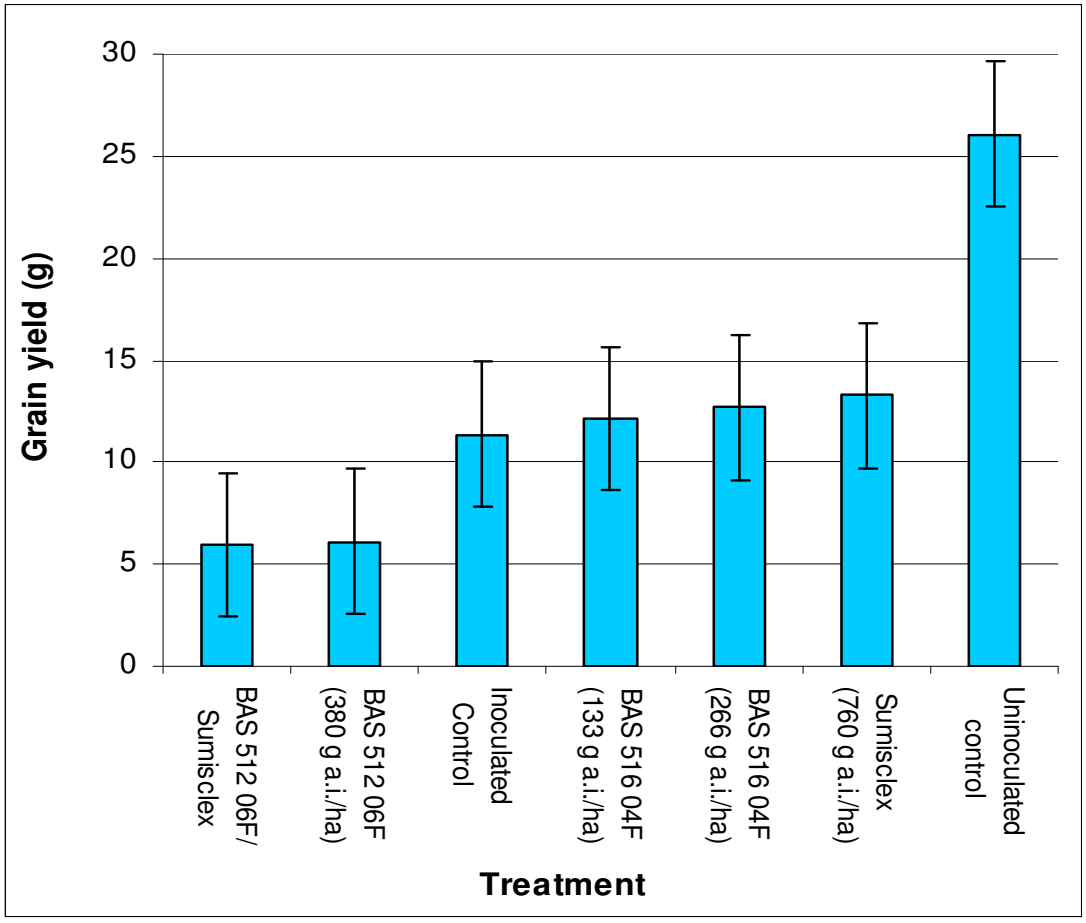

Figure 5.8 Grain yield of Prima 2000 inoculated with *Sclerotinia sclerotiorum* and sprayed with various fungicides, at the R7 growth stage.

5.3.2.3. Regression analysis

Regression analysis is shown in Appendix 5h, for Trial 1 and 2, respectively.

Regression analysis showed a strong positive correlation coefficient between disease severity index and grain yield of SSR at the R7 growth stage, i.e., $R^2 = 0.76$ (Figure 5.9).

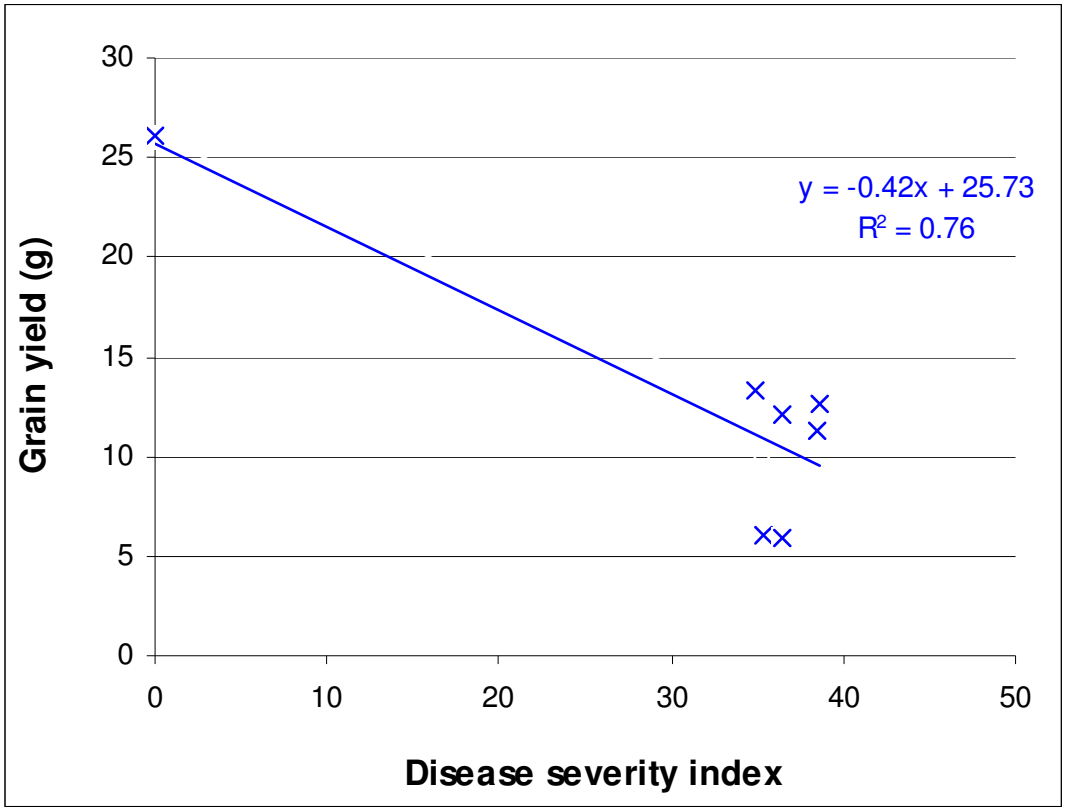

Figure 5.9 Regression analysis between disease severity index and grain yield of *Sclerotinia sclerotiorum* at the R7 growth stage.

5.4 DISCUSSION

From the *in vitro* studies it was observed that complete inhibition of mycelial growth of *S. sclerotiorum* was achieved by all BAS treatments i.e., BAS 516 04F (133 g a.i. ha^{-1}), BAS 516 04F (266 g a.i. ha^{-1}) and BAS 512 06F (380 g a.i. ha^{-1}), whereas Sumisclex inhibited mycelial growth by 89.07%, indicating that these fungicides have potential for disease control in the field. From Hawthorne and Jarvis's (1973) extensive study on the differential activity of fungicides on various stages of the life cycle of *S. sclerotiorum*, they concluded that *in vitro* studies may be used to identify specific fungicides and their rates for fungicidal activity against *S. sclerotiorum*.

However, in greenhouse trials, plants sprayed with those fungicides which produced the significantly lowest grain yields (BAS 512 06F (380 g a.i. ha^{-1}) and BAS 512 06F (570 g a.i. ha^{-1})/Sumisclex (380 g a.i. ha^{-1})), did not have significantly higher DSI's than the inoculated control. However, the regression analysis showed a strong correlation coefficient (R^2 = 0.76) indicating that a low grain yield is associated with a high DSI. Although these two treatments, i.e., BAS 512 06F and BAS 512 06F/Sumisclex had significantly lower grain yields, but a significantly similar DSI to the inoculated control, this could be attributed to phytotoxicity. Severe defoliation was observed on all plants treated with BAS 512 06F, both on its own and in combination with Sumisclex, thereby reducing the number of leaves available for carbohydrate production and therefore grain production, hence a lower grain yield. In this trial, only leaves present on the plant were rated for the presence of lesions, therefore if there was severe defoliation, as in the case with BAS 512 06F and BAS 512 06F/Sumisclex, only the few leaves present on the plant were rated for the presence of lesions as the whole plant could not be taken into consideration.

As there was no significant difference in the DSI or grain yield between the curative treatment (Sumisclex) and preventative treatments, it may be concluded that preventative and curative fungicide treatments did not control disease development.

The current spray programme that was tested is commonly used for the control of soybean rust (*P. pachyrhizi*) (Bromfield, 1984) in SA. This spray programme (Table 5.2) was adapted for the control of SSR on soybeans however, these trials have shown that the rates, timing and frequency of fungicide application are not applicable for the control of SSR on soybeans. Currently, procymidone (250 g a.i. ℓ^{-1}) is the only fungicide currently registered for SSR control in SA, at the onset of flowering (Nel *et al*., 2003).

In this study, only one isolate of the fungus was tested. However, to gain a more conclusive indication of the potential of these fungicides against *S. sclerotiorum*, further isolates should be tested. Mueller *et al*. (2002) found that benomyl did not control any of the 100 isolates of *S. sclerotiorum* tested. This was attributed to fungicide resistant

isolates, which has also been reported in other studies (Detweiler *et al*., 1983; Brenneman *et al,* 1987; Hubbard *et al*., 1997). In addition, small sclerotia (those of *S. minor*) were found to be more sensitive to fungicides compared to the bigger sclerotia produced by *S. sclerotiorum* (Hawthorne and Jarvis, 1973).

Another reason why the fungicides did not work *in vivo*, could be due to nozzle arrangement of the spray apparatus. In this trial, one flat fan nozzle was used in the herbicide sprayer for fungicide application. Due to the plant architecture of Prima 2000, a determinate variety, plants were bushy and it was observed that fungicides did not penetrate between the lower leaves completely. Morton and Hall (1989) found nozzle arrangement important for the control of SSR in beans. The level of disease was directly related to the number of blossoms within the canopy which were covered by the fungicide. The best control using benomyl (1.1 kg a.i. ha^{-1} in 550 ℓ water) applied at full bloom, was achieved when it was applied using 3 flat fan nozzles per row, where one nozzle was above the row to direct spray downwards, and one on either side of the row to direct spray at the base of plants (Morton and Hall, 1989). Complete coverage of foliage has been found to control SSR (Hunter *et al*., 1978; Morton and Hall, 1989; Bowerman and Gladders, 1993). Plant architecture of dry beans has also been found to affect SSR development (Steadman, 1983; Kim *et al*., 1999; Hoffman *et al*., 2002). In Brazil, foliar fungicides such as procimidone (1000 and 750 g a.i. ha^{-1}), iprodione (1000 and 200 g a.i. ha^{-1}) and vinelazolin (500 g a.i. ha^{-1}) reduced infection. However, problems occurred with ensuring correct coverage (McGee, 1992).

The potential of these fungicides may also be determined by evaluating their effect on all four stages of the pathogen's life cycle, i.e., germination of ascospores, germination of sclerotia to form apothecia, germination of sclerotia to form mycelia and the growth of mycelia, as each stage has the potential for inoculum build up and/or inoculum spread. Fungicides with the most potential for control would be those which inhibit the germination of sclerotia, which leads to either myceliogenic or carpogenic germination (Hawthorne and Jarvis, 1973). Because of the difficulty in producing apothecia and

ascospores, the effect of the fungicides on the germination of sclerotia to produce apothecia and the germination of ascopores could not be tested.

There are numerous factors that need to be improved or reinvestigated. Glasshouse trials need to be performed using ascospores to identify which fungicides show potential. As there was poor coverage of lower foliage using the herbicide sprayer, lower blossoms would most likely also receive minimal fungicide. Hence, the method of application needs to be changed. In addition, the method of rating plants needs to be improved, possibly including wilting and defoliation as an indication of disease severity. This could help eliminate results obtained in this trial with BAS 512 06F and BAS 512 06F/Sumisclex where DSI ratings were not significantly different whereas grain yields were significantly different between treatments.

The number of sprays per treatment were not examined in this trial. Morton and Hall (1989) found that a single spray at the correct time was most effective. More extensive research into the effect of curative versus preventative fungicides needs to be conducted. A more suitable rating scale needs to be developed, as well as the possible effects of phytotoxicty, must be included. Hence it is imperative to determine timing of spraying, nozzle arrangement and the number of applications in order to achieve maximum control. Field trials also need to be coupled with greenhouse trials.

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CHAPTER SIX

EFFICACY AND CROP TOLERANCE OF SEED TREATMENTS AGAINST *SCLEROTINIA SCLEROTIORUM* OF SOYBEANS

D.D. Visser[1], P.M. Caldwell[1], N. W. McLaren[2]

[1]Discipline of Plant Pathology, University of KwaZulu-Natal, Private Bag X01, Scottsville, 3209, South Africa

[2]Department of Plant Sciences, University of the Free State, Bloemfontein, 9300, South Africa

ABSTRACT

Research on the use and effect of seed treatments to control *Sclerotinia sclerotiorum* on soybean seeds is limited. Trials to evaluate the effect of commercially available and currently unregistered seed treatments for the control of *S. sclerotiorum* on soybean seeds *in vivo* and *in vitro* were carried out. Seed germination tests were performed to determine if seed treatments had any negative effects on seed germination *in vitro*. "Paper dolls" were used to evaluate seed germination according to the International Seed Testing Association (ISTA). Seeds were surface sterilized and coated with the various seed treatments, i.e., BAS 516 03F (8, 16 and 32 mℓ a.i. 100 kg^{-1} seed), BAS 512 00F (7.5, 15 and 30 mℓ a.i. 100 kg^{-1} seed), Celest XL (100, 125, 200 and 250 mℓ a.i. 100 kg^{-1} seed), Sumisclex (5 and 10 mℓ a.i. 100 kg^{-1} seed), Benomyl (150 g a.i. 100 kg^{-1} seed), Captan (240 mℓ a.i. 100 kg^{-1} seed), Thiulin (180 g a.i. 100 kg^{-1} seed) and Anchor Red (300 mℓ a.i. 100 kg^{-1} seed). The control consisted of untreated seeds. Seeds were evaluated 5 and 8 days after treatments for percent normal seedling germination, abnormal seedling germination, dead seed, hard seeds and fresh ungerminated seeds. All seed treatments showed tolerances below the maximum range allowable, indicating that these seed treatments had no negative effect on seed germination. For *in vivo* trials, coated seeds were placed with an agar plug of *S. sclerotiorum* in composted pine

bark in Speedling® 24 trays. Seeds were evaluated 5 days post inoculation (dpi) for percent germination and 14 dpi for percent seedling survival. Seeds treated with BAS 516 03F (16 and 32 mℓ a.i. 100 kg^{-1} seed), BAS 512 00F (7.5, 15 and 30 mℓ a.i. 100 kg^{-1} seed), Celest XL (100, 125, 200 and 250 mℓ a.i. 100 kg^{-1} seed), Sumisclex (5 and 10 mℓ a.i. 100 kg^{-1} seed), Benomyl and Anchor Red had significantly similar percent germination and percent seedling survival as the untreated/uninoculated control. These seed treatments should be recommended for the control of *S. sclerotiorum*, as they suppressed seed colonization by *S. sclerotiorum* and protected seedlings during subsequent seedling development. BAS 516 03F (8 ml a.i. 100 kg^{-1} seed) should not be recommended for the control of SSR, as it resulted in a very low percent germination.

6.1 INTRODUCTION

Seeds are important in the reproduction of most food crops. Approximately 90% of the worlds food crops are propagated via seed. However, seeds are passive carriers of pathogens which are transmitted when seeds are planted and emerge under favourable conditions for pathogen development (Maude, 1996). Fungi form the major group of pathogens that may be transmitted via seeds (Agarwal and Sinclair, 1987).

The use of seed treatments for the protection of seeds from microorganisms dates back to 60AD. The introduction and use of copper sulphate in 1761 began the use of seed treatments (Jeffs, 1986). The famine of Japan in the 1930s and 1940s, due to the rice blast fungus, *Pyricularia oryzae* (Cooke) (Teleomorph *Magnaporthe grisea* (T. T. Herbert)) (Webster and Gunnell, 1992) and the loss of $5.6 million in the Pacific North-West from the downgrading of wheat because of the bunt fungus, *Tilletia tritici* (Bjerk) Wint. (Wiese, 1987) resulted in an increase in research and a developing appreciation for seed-borne organisms (Maude, 1996).

Sclerotinia stem rot (SSR) of soybeans (*Glycine max* (L.) Merr.), caused by the fungus *Sclerotinia sclerotiorum* (Lib.) de Bary was considered the second most important yield-limiting soybean disease in the United States of America (USA) in 2004 (Chen and Wang, 2005). Sporadic outbreaks of SSR on soybeans have recently been reported in South Africa (SA), particularly in the Winterton-Underberg and Piet Retief areas of KwaZulu-Natal (KZN), as well as on the Highveld in the Ermelo area. In 2003, SSR was so severe on the KZN side of the Swaziland border, farmers harvested their crop early in the season for use as silage, as it was predicted that there would be no grain yield (Caldwell, pers. comm.[1]).

Sclerotinia sclerotiorum spreads from field to field via windblown ascospores during the growing season (Adams and Ayers, 1979; Muckel and Steadman, 1981), which are the primary source of inoculum (Abawi and Grogan, 1975; Tu, 1988). Long distance dispersal of SSR is via sclerotia in contaminated soil, equipment or irrigation water, or by seeds infected with mycelium (Adams and Ayers, 1979; Mueller *et al*., 1999). Soil-borne sclerotia are considered a major source of inoculum (McGee, 1992). Sclerotia may undergo myceliogenic germination resulting in the production of mycelial strands that may then infect stems directly (Agrios, 1997).

No single disease management practice effectively controls SSR. Management practices need to be implemented at every stage in the pathogen's life cycle. The planting of certified seed where sclerotia and poor quality seeds have been removed should be practiced (Steadman *et al*., 1996). The use of seed treatments to prevent infection of seed in fields with a history of SSR may also be introduced (Anonymous, 2005).

[1] Dr P. M. Caldwell, Discipline of Plant Pathology, School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of KwaZulu-Natal, Private Bag X01, Scottsville, 3209, Pietermaritzburg, South Africa.

Seed treatments such as captan, benomyl, thiobendazole and thiram at rates of 1.5; 1.0; 0.2 and 2.1 g a.i. kg^{-1} seed, respectively gave complete control of infected seed in Brazil (Yorinori and Homechin, 1985). In Romania, carbendazim and thiram, carboxin and thiram, benomyl and captan, thiopanate methyl and captan gave satisfactory control (Drăgoescu, 1981). Generally, most commonly available seed treatments provide effective control (Anonymous, 2005).

The aim of this study was to determine the effect of various commercially available and unregistered seed treatments on germination of soybean seeds *in vitro* and their effect against mycelial infection of seeds by *S. sclerotiorum in vivo*.

6.2 MATERIALS AND METHODS

6.2.1 *In vitro* germination of soybean seeds coated with seed treatments

Germination tests were performed and evaluated according to the International Seed Testing Association (ISTA) rules (Anonymous, 1999).

6.2.1.1 Seed sterilization

Soybean seeds of the cultivar Prima 2000 (Pannar[2]) were surface sterilized for 3 min in NaOCl (4%), washed three times in sterile distilled water and allowed to air dry on a laminar flow bench.

[2] Pannar Seed, PO Box 19, Greytown, 3250, KwaZulu-Natal, South Africa

6.2.1.2 Seed treatments

Sterilized seeds were coated with various commercially available and unregistered seed treatments (Table 6.1). No adjuvants were used for any of the treatments. Treated seeds were placed in sterile 90 mm diameter Petri dishes and air dried on a laminar flow bench overnight.

Table 6.1 Seed treatments and dose rates (ml or g 100 kg^{-1} seed) applied for the control of *Sclerotinia sclerotiorum* on soybeans

Trade name*	Active ingredient*	Fungicide formulation**	Dose rate (mℓ or g a.i. 100 kg^{-1} seed)
Untreated/uninoculated control	-	-	-
Untreated/inoculated control	-	-	-
BAS 516 03F* [3]	unknown*	FS	8
			16
			32
BAS 512 00F* [3]	unknown*	FS	7.5
			15
			30
Celest XL[4]	fludioxonil/mefenoxam	FS	100
			125
			200
			250
Sumisclex [5]	procymidone	SC	5
			10
Benomyl[6]	benomyl	WP	150
Captan[7]	captan	FS	240
Thiulin[8]	thiram	DS/WS	180
Anchor Red[8]	carboxin/thiram	FS	300

* Fungicide treatments are not commercially registered and active ingredients and trade names currently unavailable for release

** FS = flowable concentrate; SC = suspension concentrate; WP = wettable powder; DS = powder for dry seed treatment; WS = water dispersable powder

[3] BASF South Africa (Pty) Ltd., PO Box 2801, Halfway House, 1685, South Africa
[4] Syngenta SA (Pty) Ltd., PO Box X60, Halfway House, 1685, South Africa
[5] Philagro SA (Pty) Ltd., PO Box 36213, Menlo Park, 0102, South Africa
[6] Dow Agrosciences Southern Africa (Pty) Ltd., PO Box 912-055, Silverton, 0127, South Africa
[7] Kombat (Pty) Ltd., PO Box 514, Greytown, 3500, South Africa
[8] Bayer (Pty) Ltd., PO Box 143, Isando, 1600, South Africa

6.2.1.3 "Paper dolls"

Moistened seed germination paper (Agricol[9]) was laid flat on surface sterilized laboratory bench tops and 10 treated seeds were evenly spaced on the paper (Figure 6.1). Another moistened piece of seed germination paper was then placed on top of the seeds (Figure 6.2) and rolled up (Figure 6.2) to form a "paper doll" (Figure 6.3).

Figure 6.1 Moist germination paper with treated soybean seeds spaced evenly on the surface.

Figure 6.2 Rolling of germination paper to form a "paper doll".

[9] Agricol, Eagle Street, Brackenfell, 7560, South Africa

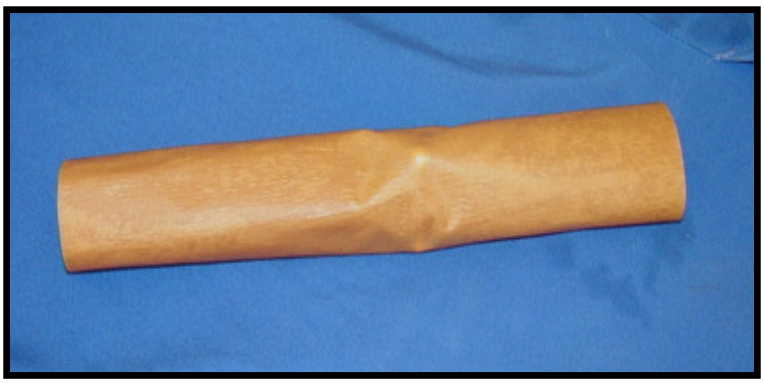

Figure 6.3 Formation of a “paper doll”.

Five “paper dolls” were placed in a plastic packet and heat-sealed. Plastic packets were placed vertically in plastic containers (Figure 6.4), allowing shoots to grow straight up and roots to grow straight down. These were then placed in a conviron™ (24°C, 80% RH, 14 hr photoperiod and a light intensity of 109 $\mu Em^{-2}s^{-1}$) to allow germination to take place.

Figure 6.4 Vertical placement of “paper dolls” in plastic containers before placement in a conviron™.

6.2.1.4 Experimental design

One hundred seeds with four replicates were used in each trial. “Paper dolls” were arranged in a randomized complete block design (RCBD). The trial was repeated.

6.2.1.5 Seedling assessment

“Paper dolls” were assessed 5 and 8 days after treatments for normal seedling germination, abnormal seedling germination, hard seeds, fresh ungerminated seeds and dead seed. Normal seedlings were classified as those which possessed all essential structures that are indicative of their ability to produce plants under favourable conditions. Abnormal seedlings were classified as those which do not show the capacity for continued development into normal plants when grown in good quality soil, under favourable conditions of water supply, temperature and light. Hard seeds were classified as those seeds which remain hard at the end of the prescribed test period because they have not absorbed water due to an impermeable seedcoat. Fresh ungerminated seeds were classified as those seeds, other than hard seeds, which remain firm and viable after the appropriate time period required for breaking dormancy. Dead seeds were classified as those which at the end of the test period are neither hard nor fresh and have not produced seedlings

Seedlings classified as normal at the initial count (5 days after treatment) were removed from the trial in order to allow the remaining seedlings to develop. Fungal infected seeds were also removed in the initial count to prevent possible contamination.

6.2.1.6 Data analysis

The number of normal, abnormal, dead, hard and fresh ungerminated seeds are reported as a percentage for each replicate. The difference between the maximum and minimum percent germination of the four replicates for each treatment was calculated. The average percent germination in all replicates was also determined.

6.2.1.7 Tolerance testing

To determine if the variation between replicates was due to random variation or improper test conditions, a tolerance table was used (Table 6.2) (Anonymous, 1999). The tolerance, or allowable difference, in germination results between replicates reported in the tolerance table indicates random variation.

The average percent germination for each treatment is located in the tolerance table (Table 6.2) and then the maximum range correlated with the percent germination is shown. If the difference between the maximum and minimum percent germination of each treatment is less than the maximum range from the tolerance table (Table 6.2), the difference is considered to be due to random variation only. If the difference between the maximum and minimum percent germination of each treatment is greater than the maximum range from the tolerance table (Table 6.2), the test needs to be repeated. If the second test is within the maximum range allowable, then the average is reported. If the difference between the maximum and minimum percent germination is again greater than the maximum range allowable, then the test needs to be repeated a third time. If the average percent germination falls between the first and second test, and the difference is within the maximum range allowable, the average of all three tests is reported (Anonymous, 1999). If on the third test the difference is not within the maximum range allowable, it was concluded that the seed treatments applied were toxic and showed a negative effect on germination.

Retesting may also be performed if dormancy is expected, when the result is considered unreliable due to phytotoxicity or spread of fungi or bacteria, when it is difficult to evaluate a number of seedlings or when there is evidence of errors in test conditions (Anonymous, 1999).

Table 6.2 Maximum tolerated range between four replicates of 100 seeds in one germination test (two-way test at 2.5% significance level) (adopted from Anonymous, 1999)

Average percentage germination	Maximum range
99	5
98	6
97	7
96	8
95	9
93 to 94	10
91 to 92	11
89 to 90	12
87 to 88	13
84 to 86	14
81 to 83	15
78 to 80	16
73 to 77	17
67 to 72	18
56 to 66	19
51 to 50	20

6.2.2 *In vivo* effect of seed treatments on the infection of soybean seeds by *Sclerotinia sclerotiorum*

6.2.2.1 Isolate and inoculum preparation

A *S. sclerotiorum* isolate was obtained from sunflowers (*Helianthus annuus*) in Delmas, Mpumalanga, SA in February, 2005 (McLaren[10]) in the form of sclerotia. The *Sclerotinia* isolate used in this study was sent to Dr E. J. van der Linde[11] for identification and deposition in the Plant Protection Research Institute (PPRI) collection, and was confirmed to be *S. sclerotiorum* (PPRI Accession number 8374). Initially, sclerotia were surface sterilized for 3 min in 70% ethanol, washed twice in sterilized distilled water, and plated on PDA in 9 cm diameter Petri dishes.

Petri dishes were sealed with Parafilm® (Industricord[12]) and incubated in the dark at 20°C for 4 weeks. The resulting sclerotia were harvested, surface sterilized for 3 min in 70% ethanol, washed twice in sterilized distilled water, placed on filter paper in a Petri dish and left to air dry overnight on a laminar flow bench. Sclerotia were placed in a sterile Petri dish and sealed and stored at 12°C in the dark until needed. This stock culture was also maintained by subculturing mycelia onto PDA slants and kept in the dark at 20°C.

To produce inoculum for *in vitro* trials, a sclerotium was surface sterilized, washed twice in sterile distilled water, placed on PDA and allowed to germinate myceliogenically. After 7 days, when the mycelia reached the edge of the Petri dish, a single mycelial plug was cut from the margin of the growing colony with an 11 mm diameter cork borer and aseptically transferred to the centre of a new PDA plate. Plates were incubated for 4 days in the dark at 21°C. Mycelia were sub-cultured by cutting mycelial plugs from the margin of the growing colony and aseptically transferred to new PDA plates. After

[10] Neal McLaren, Department of Plant Sciences, University of the Free State, Bloemfontein, 9300, South Africa
[11] Dr E. J. van der Linde , Biosystematics Division, Agricultural Research Council (ARC), Plant Protection Research Institute (PPRI), Queenswood, 0121, Pretoria, South Africa
[12] Industricord, PO Box 243, Pavilion, 3611, KwaZulu-Natal, South Africa

4 days, mycelial plugs were cut with an 11 mm diameter cork borer at the edge of the growing colony and used to inoculate trials.

6.2.2.2 Seed sterilization

Soybean seeds of the cultivar Prima 2000 (Pannar[2]) were surface sterilized for 3 min in NaOCl (4%), washed three times in sterile distilled water and allowed to air dry on a laminar flow bench.

6.2.2.3 Seed treatments

Sterilized seeds were coated with various commercially available and unregistered seed treatments (Table 6.1). No adjuvants were used for any of the treatments. Treated seeds were placed in sterile 90 mm diameter Petri dishes and air dried on a laminar flow bench overnight.

6.2.2.4 Tray preparation

Speedling® 24 trays were filled with composted pine bark (Growmor[13]) and watered with tap water. Previcur N (Bayer[8]) was then applied to kill any possible damping off pathogens, e.g., *Phythium, Rhizoctonia* and *Fusarium* in the planting medium. Trays were placed in a tunnel at 25°C and 60% RH where trays were irrigated, supplemented with NPK fertilizer [3:1:3], four times a day for 5 min for the duration of the trial.

6.2.2.5 Inoculation procedure

An 11 mm agar disc containing the pathogen was placed in each cell of the Speedling® 24 trays and the treated seeds placed directly on top of the agar disc (Figure 6.5). Inoculated trays were initially hand watered in the tunnel for 3 days and then moved under the overhead irrigation for the remainder of the trial.

[13] Growmor, PO Box 89, Cato Ridge, 3680, KwaZulu-Natal, South Africa

Figure 6.5 Inoculation of Speedling® 24 trays with the *Sclerotinia sclerotiorum* agar disc and treated seeds.

6.2.2.6 Experimental design

Five seeds with three replicates were used in each trial. Trays were arranged in a RCBD. The trial was repeated.

6.2.2.7 Disease assessment

Trays were assessed 5 days post inoculation (dpi) for percent germination and 14 dpi for percent seedling survival. From the seedlings that germinated, the percent seedling survival was calculated as the percent of seedlings that were not attacked by *S. sclerotiorum* of the seedlings that germinated.

6.2.2.8 Statistical analysis

All data were subjected to an analysis of variance (ANOVA) using Genstat® Executable Release 9.1 Statistical Analysis Software (Anonymous, 2006) to determine differences between treatment means. Least significant differences were determined at $P<0.05$.

6.3 RESULTS

6.3.1 *In vitro* germination trials of soybean seeds coated with seed treatments

Percent normal seedlings, abnormal seedlings and dead seeds in germination trials of soybean seeds treated with various seed treatments are shown in Appendix 6a. No hard or fresh ungerminated seeds were observed in all trials. Average percent germination, differences between replicates and allowed tolerances for germination trials of soybean seeds treated with various seed treatments are shown in Appendix 6b.

All seed treatments, except two, i.e., Celest XL (100 mℓ a.i. 100 kg^{-1} seed) and Sumisclex (5 mℓ a.i. 100 kg^{-1} seed), showed tolerances below the maximum range allowable (Appendix 6b), indicating that these seed treatments had no negative effect on seed germination.

The two treatments, i.e., Celest XL (100 mℓ a.i. 100 kg^{-1} seed) and Sumisclex (5 mℓ a.i. 100 kg^{-1} seed) were repeated, and the difference between maximum and minimum percent germination was found to be below the maximum range allowable (Appendices 6c and d), and therefore the trial was not repeated a third time.

Results of the repeat of the two treatments together with the other treatments are shown in Table 6.3.

Table 6.3 Average percentage germination, difference between maximum and minimum averages and allowed tolerances for soybean seeds treated with various seed treatments

Treatment	Dose rate (mℓ or g a.i. 100 kg^{-1} seed)	Average % germination	Difference between maximum and minimum averages	Allowed tolerated range according to ISTA*
Untreated control	-	94	3	10
BAS 516 03F	8	96	5	8
	16	90	5	12
	32	87	10	13
BAS 512 00F	7.5	94	4	10
	15	91	5	11
	30	92	5	11
Celest XL	100	88	7	7
	125	95	9	9
	200	92	5	11
	250	95	4	9
Sumisclex	5	88	13	13
	10	88	6	13
Benomyl	150	86	7	14
Captan	240	85	7	14
Thiram	180	95	4	9
Anchor Red	300	89	4	12

* International Seed Testing Association

6.3.2 *In vivo* effect of seed treatments on the infection of soybean seeds by *Sclerotinia sclerotiorum*

Trial 2 confirmed results obtained in Trial 1 as similar trends and patterns were observed in both Trials. According to the ANOVA, experiments did not differ, and data were therefore pooled.

6.3.2.1 Percent germination

Percent germination is shown in Appendices 6e and f, for Trial 1 and 2, respectively.

Where seeds did not germinate, white cottony mycelia of *S. sclerotiorum* were observed covering the disintegrating seeds. The untreated/uninoculated control was significantly similar to BAS 516 03F (16 and 32 mℓ a.i. 100 kg^{-1} seed), BAS 512 00F (7.5, 15 and 30 mℓ a.i. 100 kg^{-1} seed), Celest XL (100, 125, 200 and 250 mℓ a.i. 100 kg^{-1} seed), Sumisclex (5 and 10 mℓ a.i. 100 kg^{-1} seed), Benomyl and Anchor Red (Figure 6.6, Figure 6.7, Appendix 6g), indicating that these treatments prevented the fungus from killing the seed. The untreated/inoculated control showed a significantly lower percent germination compared to all other treatments (Figure 6.6, Appendix 6g). BAS 516 03F (8 mℓ a.i. 100 kg^{-1} seed) gave the least control, with an average percent germination of 47%, which was significantly lower than all other treatments (Figure 6.7, Appendix 6g). Captan and Thiulin showed a significantly lower percentage germination than the untreated/uninoculated control (Figure 6.7, Appendix 6g).

Figure 6.6 Speedling® 24 trays of treated soybean seeds inoculated with *Sclerotinia sclerotiorum*, showing the untreated/inoculated control (left), where no germination occurred, compared to the BAS 516 03F (8 mℓ a.i. 100 kg^{-1} seed) treatment (middle) and the untreated/uninoculated control (right), where percentage germination was significantly higher, at 8 days post inoculation.

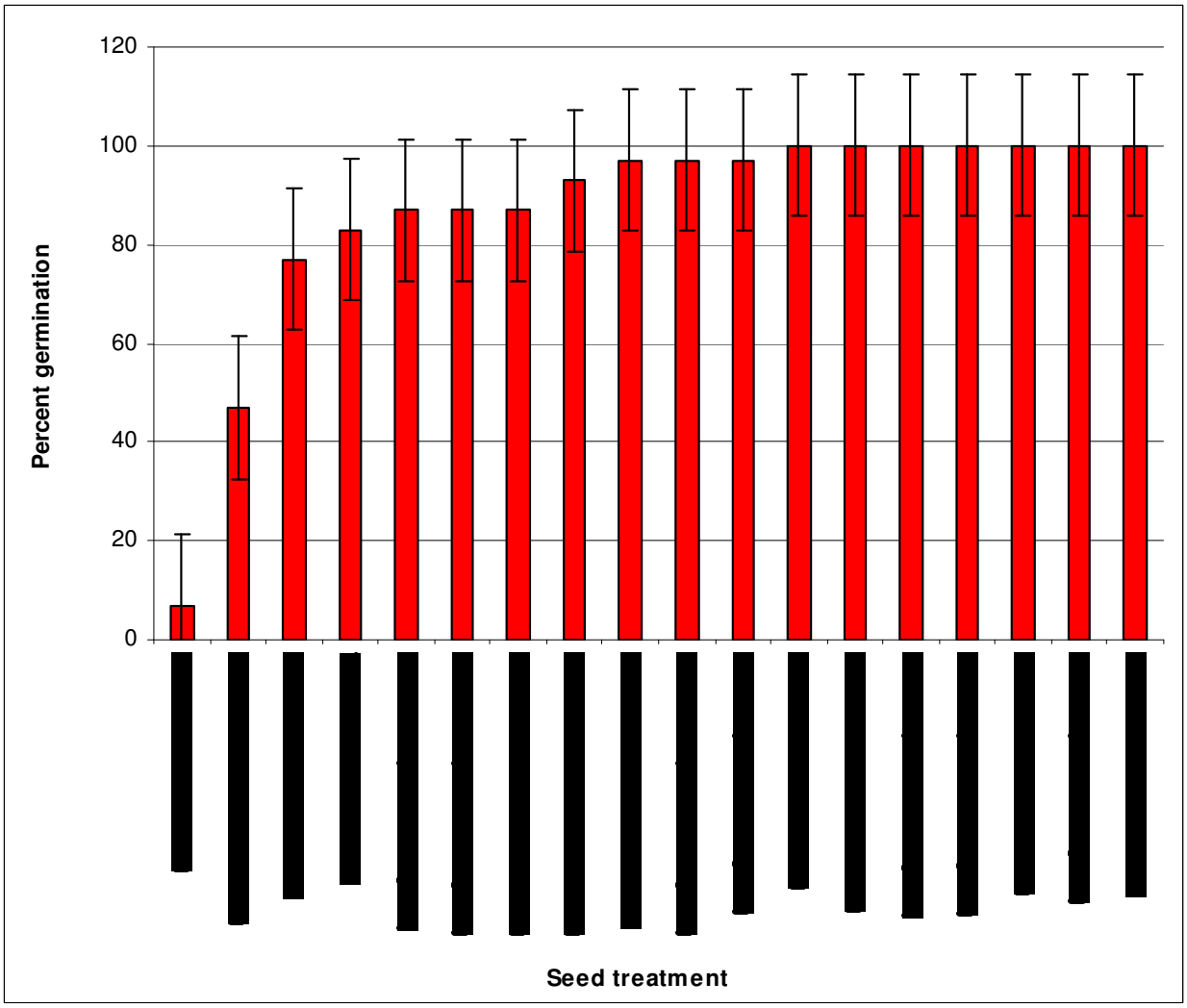

Figure 6.7 Percentage germination for soybean seeds treated with various seed treatments and inoculated with *Sclerotinia sclerotiorum*.

6.3.2.2 Percent seedling survival

Percent seedling survival is shown in Appendices 6e and f, for Trial 1 and 2, respectively.

A few seedlings which germinated and appeared healthy, became infected after the initial percentage germination rating. A white cottony mycelium was observed on seedling stems. Seedlings later fell over and died (Figure 6.8). BAS 516 03F (16 and 32 mℓ a.i. 100 kg^{-1} seed), BAS 512 00F (7.5, 15 and 30 mℓ a.i. 100 kg^{-1} seed), Celest XL (100, 125, 200 and 250 mℓ a.i. 100 kg^{-1} seed), Sumisclex (5 and 10 mℓ a.i. 100 kg^{-1} seed), Benomyl and Anchor Red, were significantly similar to the untreated/uninoculated control. Celest XL (100, 125, 200 and 250 mℓ a.i. 100 kg^{-1} seed), Sumisclex (5 and 10 ml a.i. 100 kg^{-1} seed) and Benomyl showed a significantly higher percent seedling survival than BAS 516 03F (8 mℓ a.i. 100 kg^{-1} seed), Captan, and Thiulin (Figure 6.9, Appendix 6g).

Figure 6.8 Speedling® 24 tray showing seedlings treated with BAS 512 00F (7.5 mℓ a.i. 100 kg^{-1} seed) which germinated, but was infected by *Sclerotinia sclerotiorum* (arrows) (14 days post inoculation).

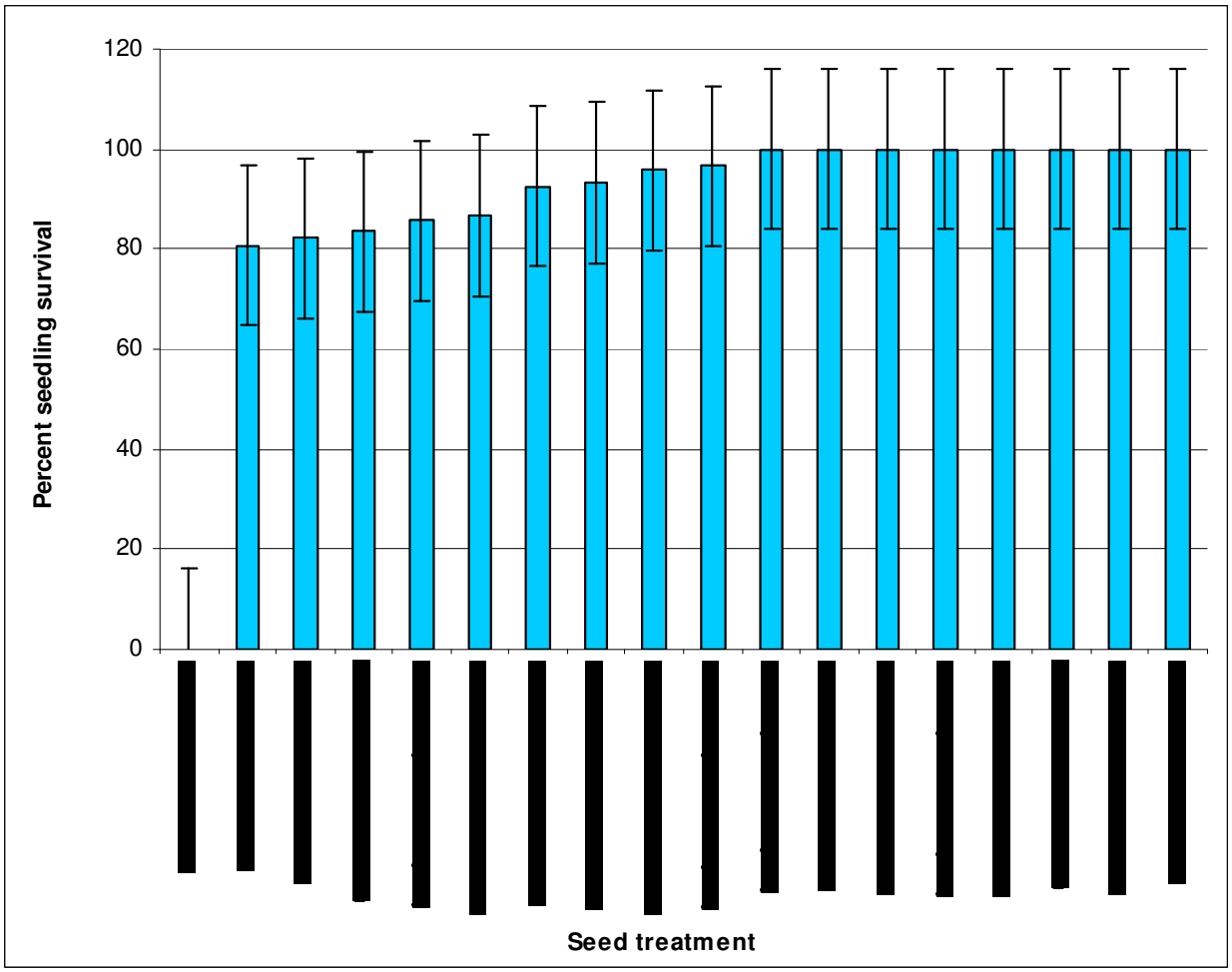

Figure 6.9 Percentage seedling survival for soybean seeds treated with various seed treatments and inoculated with *Sclerotinia sclerotiorum*.

6.4 DISCUSSION

In vitro germination tests are valuable for determining the effect, if any, on the germination of seeds. As no negative effect on germination occurred due to seed treatments, i.e., repetition of germination tests for a third time resulting in tolerances outside the allowed range, it may be concluded that these unregistered seed treatments, i.e., BAS 516 03F (8, 16 and 32 mℓ a.i. 100 kg^{-1} seed) and BAS 512 00F (7.5, 15 and 32 mℓ a.i. 100 kg^{-1} seed), are safe for use on soybean seed.

All seed treatments that were not significantly different in percent germination to the untreated/uninoculated control, i.e., BAS 516 03F (16 and 32 mℓ a.i. 100 kg^{-1} seed), BAS 512 00F (7.5, 15 and 30 mℓ a.i. 100 kg^{-1} seed), Celest XL (100, 125, 200 and 250 mℓ a.i. 100 kg^{-1} seed), Sumisclex (5 and 10 mℓ a.i. 100 kg^{-1} seed), Benomyl and Anchor Red, were also not significantly different to the untreated/uninoculated control in percent seedling survival, indicating that these treatments protect seeds from *S. sclerotiorum* whilst germinating and during subsequent seed development. BAS 516 03F (8 mℓ a.i. 100 kg^{-1} seed) should not be recommended for the control of SSR, as this treatment had the significantly lowest percent germination of all treatments. Although the percent seedling survival for BAS 516 03F (8 mℓ a.i. 100 kg^{-1} seed) was significantly similar to Captan and Thiulin, these treatments gave the significantly lowest percent seedling survival. Captan and Thiulin should also not be recommended for the control of SSR, as these treatments were significantly lower in percent germination from the untreated/uninoculated control, indicating they did not protect seeds sufficiently during germination, nor did they protect seedlings during development, as they had the significantly lowest percent seedling survival.

Not much research on the use of seed treatments for the control of *S. sclerotiorum* on soybeans is available. Yorinori and Homechin (1985) found that benomyl (150 g a.i. kg^{-1} seed) gave complete control of seed infection. The same concentration of benomyl was examined in this trial, where all seeds were protected whilst germinating, and seedlings were not attacked after germination, indicating that this seed treatment gives complete

control of SSR of soybean seeds. Thiram and captan were also tested by Yorinori and Homechin (1985), and gave complete control. However, in this study, captan and thiram (Thiulin) did not give complete control of *S. sclerotiorum*.

A few reports on internally seed-borne mycelia of *S. sclerotiorum* are available (Nicholson *et al*., 1972; Steadman, 1975; Thompson and van der Westhuizen, 1979; Tu, 1988; Yang *et al*., 1998). Research on the effect of fungicidal seed treatments on the control of seed-borne *S. sclerotiorum* has been investigated. Herd and Phillips (1988) found when infected seeds were treated with benomyl (100 and 200 g kg^{-1} seed) and procymidone (100 and 200 g kg^{-1} seed), the growth of S. *sclerotiorum* was inhibited.

Mueller *et al*. (1999) found thiram (0.55 mℓ a.i. kg^{-1} seed) reduced mycelial growth of SSR from seed by 90% *in vitro*. Mueller *et al*. (1999) also found that combinations of active ingredients, i.e., captan + pentachloronitrobenzene + thiabendazole, carboxin + thiram, and thiabendazole + thiram, completely inhibited mycelial growth from infected seed. No such combinations were investigated in this study, but should possibly be investigated due to the potential shown by Mueller *et al*. (1999). Seeds treated with seed treatments produced significantly lower numbers of sclerotia compared to seeds not treated with seed treatments, indicating that seed treatments provide an efficient means of reducing the production of sclerotia from infected seed and therefore spread of the pathogen (Mueller *et al*., 1999). Tu (1988) found that dormant mycelia in infected seed plays an important role in reducing the dissemination of this pathogen.

BAS 512 00F (7.5, 15 and 30 mℓ a.i. 100 kg^{-1} seed), Celest XL (100, 125, 200 and 250 mℓ a.i. 100 kg^{-1} seed) and Sumisclex (5 and 10 mℓ a.i. 100 kg^{-1} seed) showed the same control at all concentrations tested. Possibly lower concentrations may be examined, especially for BAS 512 00F, (which is currently not registered for the control of *S. sclerotiorum* on soybean seed), to make these seed treatments more economic to use commercially.

No economic studies have been conducted to determine whether any of the seed treatments which provided complete control of *S. sclerotiorum* are, in fact, economically viable for the control of *S. sclerotiorum* on soybeans.

6.5 REFERENCES

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CHAPTER SEVEN

THESIS OVERVIEW

Soybean, *Glycine max* L. Merrill., is an economically and strategically important crop in South Africa (SA). Not only is soybean oil economically important, but soybean protein is critical to animal feeds and human nutritional supplements. Consumption of soybean in SA far exceeds production, resulting in the import of 842 107 tonnes of oilcake in the 2005/2006 soybean production period, in order to meet local demands (Joubert, 2006).

In recent years, sporadic outbreaks of *Sclerotinia sclerotiorum* (Lib.) de Bary, the causal organism of sclerotinia stem rot (SSR), on soybeans in the Winterton-Underberg areas and more commonly in the Piet Retief areas of KwaZulu-Natal (KZN) have been reported, as well as on the Highveld in the Ermelo area. Outbreaks have become increasingly common and more severe in recent years, with the pathogen spreading rapidly through fields and sometimes completely destroying the crop. In 2003, SSR was so severe on the KZN side of the Swaziland border that farmers harvested their crop early in the season for use as silage, as it was predicted that there would be no grain yield. The distribution of this pathogen in SA is currently unknown. Sclerotinia stem rot, together with the outbreak of soybean rust (caused by *Phakopsora pachyrhizi* Sydow) in 2001 threatens the viability of the soybean crop, which plays a crucial role in agriculture and the downstream food industry (Caldwell, pers. comm.[1]).

The research in this thesis aimed to develop a balanced and objective approach to an integrated pest management (IPM) programme for SRR, focusing particularly on the epidemiology of the pathogen, cultivar trials, biocontrol using commercially available

[1] Dr P. M. Caldwell, Discipline of Plant Pathology, School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of KwaZulu-Natal, Private Bag X01, Scottsville, 3209, Pietermaritzburg, South Africa

biocontrol agents and silicon (Si), chemical and seed treatments for the control of the pathogen.

It was established that:

- a 3 way interaction of temperature, leaf wetness duration (LWD) and relative humidity (RH) had no significant effect on the rate of disease development. However, temperature and RH (2 way interaction) had a significant effect on the rate of disease development, with the significantly highest rate occurring at 22ºC

- of the 13 commercially available South African cultivars tested, none were resistant to *S. sclerotiorum*. Some cultivars, i.e., Prima 2000, B01B96, 95B33 and AG5409RR were considered less susceptible, whereas LS6626RR and LS666RR were considered the most susceptible of the cultivars tested

- *in vitro* dual culture bioassays identified the probable mode of action of EcoT® and Eco77® as mycoparasitism

- seeds treated with EcoT® were not infected by *S. sclerotiorum*, whereas seeds not treated with EcoT® were infected by *S. sclerotiorum*, i.e., suggesting EcoT® may be used to protect soybean seed from infection by *S. sclerotiorum*

- Eco77®, regardless of Si application, reduced the rate of disease development in soybean plants

- all fungicides tested, BAS 516 04F (133 g a.i. ha^{-1}), BAS 516 04F (266 g a.i. ha^{-1}), BAS 512 06F (380 g a.i. ha^{-1}), Sumisclex (760 g a.i. ha^{-1}) and BAS 512 06F (380 g a.i.h a^{-1})/Sumisclex (570 g a.i. ha^{-1}) showed a significantly similar disease severity index (DSI) to the inoculated control. Plants treated with BAS 512 06F (380 g a.i. ha^{-1}) and BAS 512 06F (380 g a.i. ha^{-1})/Sumisclex (570 g a.i. ha^{-1}) resulted in a significantly lower grain yield, which was attributed to phytotoxicity

- BAS 516 03F (16 and 32 ml a.i. 100 kg^{-1} seed), BAS 512 00F (7.5, 15 and 30 ml a.i. 100 kg^{-1} seed), Celest XL (100, 125, 200 and 250 ml a.i. 100 kg^{-1} seed), Sumisclex (5 and 10 ml a.i. 100 kg^{-1} seed), Benomyl and Anchor Red may be recommended for the control of SSR

7.1 Production of ascospores

Ascospores are the primary source of inoculum for *S. sclerotiorum* (Abawi and Grogan, 1975). However, throughout the duration of this study mycelia were used as an inoculum source. Initially, time was spent trying to produce ascospores, but with little success. A few apothecia were produced, which in turn produced ascospores, but numbers were insufficient to conduct trials. These apothecia were photographed macroscopically, microscopically and cryogenic sections cut. Ascospores were also collected using a vacuum pump and stored on filter paper. However, it proved difficult to collect and recover ascospores.

Mylchreest and Wheeler (1987) extensively evaluated the literature to develop a convenient method to induce apothecial production consistently from sclerotia. They were specifically interested in developing a method for apothecial production for a *S. sclerotiorum* isolate from oilseed rape, but also investigated 35 other isolates from other crops, including soybean. They found that the optimum method developed for the oilseed rape isolate was not optimum for other isolates, with the production of fewer apothecia from these isolates. They suggested this method needs to be modified to induce apothecial formation for specific isolates (Mylchreest and Wheeler, 1987). Huang and Kozub (1991) found that isolates of different geographic origin have different temperature optima for carpogenic germination. The effect of environmental factors on ascospore release and survival are important for pathogen life cycle and development, yet only one study by Caesar and Pearson (1983) on this exists. Ascospore release and survival form key stages in the life cycle and it is essential these are understood in order to add to disease control strategies.

No studies on South African isolates have been conducted to identify optimum temperatures for carpogenic germination. It is essential such studies are conducted to identify periods of high inoculum potential in the field which in turn would allow for disease forecasting and a more rational approach to the chemical control of SSR.

7.2 Inoculum application

Inoculum application proved difficult as mycelial inoculations had to be used. For the epidemiology of *S. sclerotiorum*, plants were initially inoculated with homogenized liquid mycelium which was applied via a herbicide sprayer with a large nozzle, as used for fungicide application. Difficulties were encountered with congestion of the nozzle due to mycelial fragments. The spray distributed onto plants was not even as the inoculum formed big droplets on the leaf surfaces. Large volumes of inoculum were also required for this inoculation technique. Inoculated plants developed lesions but lesions did not spread over the 21 day period investigated, suggesting that mycelial fragments penetrated epidermal tissues but did not ramify through it. Hence, for the epidemiology studies, the cut stem method (as used for the cultivar reaction trials) was adjusted by dipping the cut stems into liquid inoculum.

For the fungicide trials, inoculum was applied using barley grain inoculated with the pathogen. A great deal of literature on this method exists (Chun *et al*., 1987; Kim *et al*., 1999; Hoffman *et al*., 2000; Kim and Diers, 2000; Kim *et al*., 2000; Vuong *et al*., 2004). In the research conducted for this thesis, each trifoliate leaf was dusted with inoculum and this was found to be very time consuming. Due to the difficulty of ascospore production, it is essential that a practical method to apply mycelial inoculum is developed.

7.3 Fungicide application

In this research, the current spray programme used for soybean rust was tested for it's efficacy against SSR. However, this did not provide sufficient control of SSR as no fungicide completely inhibited infection by SSR. This spray programme needs to be adjusted for SSR, including changing frequency and rates of application, especially for BAS 512 06F (alone and in combination with Sumisclex) as phytotoxicity was observed on plants sprayed with this fungicide. It is felt that the effect of preventative versus curative fungicide application has not been correctly identified and this aspect needs to be further investigated. The apparatus used to apply fungicides, i.e., the herbicide sprayer, also needs to be reassessed, as sufficient coverage of plants was not obtained.

This spray programme may, however, work well for inoculation using ascospores, where flowers are infected and spraying commences at the first appearance of flowering. However, if such trials are to be conducted at the University of KwaZulu-Natal, the apparatus would need to be adjusted to ensure complete coverage of flowers.

7.4 Disease assessment

As mycelium was used for all inoculations and not many different rating scales have been developed for this, it was difficult to measure disease development. A few rating scales are available, with a 0 -3 scale commonly used for field trials, and thereafter converted to disease severity index (DSI) (Chun *et al*., 1987; Kim *et al*., 1999; Hoffman *et al*., 2000; Kim and Diers, 2000; Kim *et al*., 2000; Vuong *et al*., 2004). Lesion lengths were measured for the epidemiology, cultivar reactions and biocontrol trials to assess disease severity. Rate of disease development was then calculated from lesion lengths using the linear regression of Vanderplank's logistic model (Vanderplank, 1963).

The rating scale used in the fungicide trial was adjusted from an existing rating scale (Kolkman and Kelly, 2000) to eliminate the rating of lateral branches with lesions as in the 0-3 scale, as none were observed. Rating for the fungicide trial proved difficult. It was felt that the adjusted rating scale facilitated better rating for plants where all leaves were inoculated.

7.5 Field trials

All trials in this thesis were conducted *in vitro* and *in vivo*. It is important that field trials are conducted to identify if there is a correlation between results found between those glasshouse trials and field trials. Field trials also need to be conducted at different locations.

7.6 Isolates

Only one isolate was investigated in this research. The genetic diversity of *S. sclerotiorum* isolates in SA has not been documented. As mentioned, isolates from different geographic origins optimally germinate to produce apothecia at different temperatures (Huang and Kozub, 1991). Kull *et al*. (2003) found that isolates ranging in aggressiveness impacts on cultivar reactions.

A range in aggressive isolates should be included, to determine the true potential of all aspects investigated in this research. However, this may not be practical due to time consuming inoculation techniques.

7.7 Silicon

Foliar application of silicon (Si) against powdery mildew on cucumber (*Sphaerotheca fuliginea* (Schlechtend.:Fr) Pollacci) (Liang *et al*., 2005) and soybean rust (*Phakopsora pachyrhizi* Sydow)[2] has shown much promise in the control of these diseases. This method of application, i.e., foliar application, needs to be tested with the primary source of inoculum, i.e., ascospores for the control of SSR.

[2] Prof M. Laing, Discipline of Plant Pathology, School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of KwaZulu-Natal, Private Bag X01, Scottsville, 3209, Pietermaritzburg, South Africa

7.8 Overall conclusion

Although numerous problems were encountered during this research due to mycelial inoculum application, it is believed that the difficulty in using mycelia as a form of inoculum was overcome to the best of our ability.

The results presented in this body of research provide a good starting point for developing a balanced and objective approach to an integrated pest management (IPM) programme for SRR. However it is imperative that the problem of producing ascospores is overcome as it is essential trials are conducted with this form of inoculum.

7.9 Proposed future research priorities

- find a reliable method to produce, collect and store ascospores to conduct trials
- investigate the epidemiology of ascospores to better understand *S. sclerotiorum*
- investigate different isolates from SA in order to determine the effect of their aggressiveness on all trials
- improve and refine mycelial inoculations for all trials to be conducted with this pathogen if ascospores are not available
- improve disease assessment of *S. sclerotiorum*, i.e., developing new rating scales

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APPENDIX 2

Appendix 2a

Lesion length (mm) at 7-13 days post inoculation produced on Prima 2000 inoculated with *Sclerotinia sclerotiorum* at different temperatures and leaf wetness durations at 85% RH (Trial 1 and Trial 2)

Temperature (°C)	Leaf wetness duration (hr)	Trial 1			Trial 2		
		7	10	13	7	10	13
19	24	75.33	130.67	182.67	116.13	171.20	227.67
	48	67.40	116.47	172.53	130.12	196.52	264.05
	72	87.44	129.33	177.67	111.42	165.27	223.71
22	24	87.25	139.00	206.75	62.53	128.81	198.78
	48	94.78	154.08	201.78	80.85	145.68	218.58
	72	95.42	147.97	198.42	97.22	161.97	218.58
25	24	142.67	217.08	298.50	87.83	133.83	187.33
	48	118.86	193.72	271.47	115.33	170.89	218.56
	72	124.50	190.44	260.00	115.89	162.06	304.89
28	24	141.55	200.77	259.98	93.17	123.44	187.61
	48	128.67	177.67	221.83	121.17	169.83	222.17
	72	87.61	128.33	176.00	101.17	150.62	211.43

Appendix 2b

Lesion length (mm) at 7-13 days post inoculation produced on Prima 2000 inoculated with *Sclerotinia sclerotiorum* at different temperatures and leaf wetness durations at 95% RH (Trial 1 and Trial 2)

Temperature (°C)	Leaf wetness duration (hr)	Trial 1			Trial 2		
		7	10	13	7	10	13
19	24	26.27	50.42	101.09	41.31	78.67	120.83
	48	50.33	91.67	153.67	34.67	56.67	102.50
	72	22.61	38.83	91.78	45.92	76.03	113.75
22	24	36.94	84.33	111.56	73.61	117.38	159.99
	48	50.56	106.28	136.92	75.96	118.24	168.82
	72	36.50	87.06	119.83	78.00	119.42	162.42
25	24	40.58	78.08	109.00	35.56	69.33	111.00
	48	36.33	72.50	128.67	32.50	56.25	98.00
	72	32.50	68.50	109.75	32.22	62.61	104.72
28	24	34.83	54.42	88.00	68.58	124.92	180.00
	48	37.39	64.75	110.50	53.67	102.50	147.67
	72	34.89	47.17	90.03	49.92	106.17	154.92

Appendix 2c

Rate of growth of *Sclerotinia sclerotiorum* over time (1-13 days) on Prima 2000 inoculated with *Sclerotinia sclerotiorum* at different temperatures, leaf wetness durations and relative humidities (Trial 1)

Temperature (°C)	Leaf wetness duration (hr)	Rate of growth (per unit per day)	
		85%RH	95%RH
19	24	0.38	0.31
	48	0.39	0.34
	72	0.36	0.30
22	24	0.44	0.39
	48	0.47	0.57
	72	0.43	0.44
25	24	0.33	0.32
	48	0.33	0.42
	72	0.39	0.38
28	24	0.37	0.30
	48	0.27	0.42
	72	0.22	0.31
F probability		0.654	
s.e.d.		0.059	
l.s.d.		0.118	
cv%		19.4	

Appendix 2d

Rate of growth of *Sclerotinia sclerotiorum* over time (1-13 days) on Prima 2000 inoculated with *Sclerotinia sclerotiorum* at different temperatures, leaf wetness durations and relative humidities (Trial 2)

Temperature (°C)	Leaf wetness duration (hr)	Rate of growth (per unit per day)	
		85%RH	95%RH
19	24	0.31	0.35
	48	0.40	0.34
	72	0.30	0.32
22	24	0.47	0.41
	48	0.50	0.40
	72	0.45	0.40
25	24	0.25	0.39
	48	0.21	0.32
	72	0.32	0.36
28	24	0.24	0.35
	48	0.24	0.31
	72	0.30	0.35
F probability		0.910	
s.e.d.		0.062	
l.s.d.		0.124	
cv%		21.9	

Appendix 2e

Rate of growth of *Sclerotinia sclerotiorum* over time (1-13 days) on Prima 2000 inoculated with *Sclerotinia sclerotiorum* at different temperatures and relative humidities (Trial 1)

Temperature (°C)	Rate of growth (per unit per day)	
	85%RH	95%RH
19	0.38 [b]	0.32 [ab]
22	0.45 [c]	0.46 [c]
25	0.35 [b]	0.37 [b]
28	0.29 [a]	0.34 [ab]
F probability	0.125	
s.e.d.	0.034	
l.s.d.	0.068	
cv%	19.4	

Means with the same letter are not significantly different at P<0.005

Appendix 2f

Rate of growth of *Sclerotinia sclerotiorum* over time (1-13 days) on Prima 2000 inoculated with *Sclerotinia sclerotiorum* at different temperatures and relative humidities (Trial 2)

Temperature (°C)	Rate of growth (per unit per day)	
	85%RH	95%RH
19	0.34 [b]	0.34 [b]
22	0.47 [c]	0.40 [bc]
25	0.26 [a]	0.36 [b]
28	0.26 [a]	0.34 [b]
F probability	0.007	
s.e.d.	0.036	
l.s.d.	0.072	
cv%	21.9	

Means with the same letter are not significantly different at P<0.005

Appendix 2g

Rate of growth of *Sclerotinia sclerotiorum* over time (1-13 days) on Prima 2000 inoculated with *Sclerotinia sclerotiorum* at different temperatures, leaf wetness durations and relative humidities (Combination of Trial 1 and Trial 2)

Temperature (℃)	Leaf wetness duration (hr)	Rate of growth (per unit per day)	
		85%RH	95%RH
19	24	0.35	0.33
	48	0.40	0.34
	72	0.33	0.31
22	24	0.45	0.40
	48	0.48	0.48
	72	0.44	0.42
25	24	0.29	0.35
	48	0.27	0.37
	72	0.36	0.37
28	24	0.31	0.32
	48	0.26	0.36
	72	0.26	0.33
F probability		0.707	
s.e.d.		0.019	
l.s.d.		0.039	
cv%		16.0	

Appendix 2h

Rate of growth of *Sclerotinia sclerotiorum* over time (1-13 days) on Prima 2000 inoculated with *Sclerotinia sclerotiorum* at different temperatures and relative humidities (Combination of Trial 1 and Trial 2)

Temperature (°C)	Rate of growth (per unit per day)	
	85%RH	95%RH
19	0.36 ^{bc}	0.33 ^{abc}
22	0.46 ^{d}	0.43 ^{d}
25	0.31 ^{ab}	0.37 ^{c}
28	0.28 ^{a}	0.34 ^{bc}
F probability	0.018	
s.e.d.	0.027	
l.s.d.	0.054	
cv%	15.8	

Means with the same letter are not significantly different at P<0.005

APPENDIX 3

Appendix 3a

Lesion length (mm) at 1-10 days post inoculation on different soybean cultivars inoculated with *Sclerotinia sclerotiorum* (Trial 1)

Cultivar	Days post inoculation						
	2	3	4	7	8	9	10
LS6626RR	15.13	30.07	50.40	96.00	116.00	138.33	154.27
LS666RR	13.93	26.53	42.27	76.33	96.67	119.13	139.80
LS6514RR	14.87	38.83	54.17	94.50	107.90	130.33	144.47
LS6710RR	17.00	33.33	58.00	96.00	107.00	131.00	145.67
LS555RR	16.00	27.73	47.00	86.27	102.73	124.80	144.33
LS678RR	14.47	29.07	48.67	87.47	101.53	125.13	143.60
AG5601RR	14.46	26.44	38.22	72.56	85.11	93.67	99.11
PAN626	20.22	36.04	48.87	86.71	98.27	108.56	118.56
AG5409RR	16.50	29.50	37.33	72.00	81.17	92.67	105.17
Prima 2000	19.73	33.07	42.13	78.60	88.93	101.53	112.13
95B33	15.06	25.11	32.39	65.67	78.94	87.50	93.56
96B01B	21.17	34.50	45.50	70.17	81.50	86.33	89.93
95B53	16.73	27.67	36.00	60.67	68.47	74.87	81.27

Appendix 3b

Lesion length (mm) at 1-10 days post inoculation on different soybean cultivars inoculated with *Sclerotinia sclerotiorum* (Trial 2)

Cultivar	Days post inoculation						
	2	3	4	7	8	9	10
LS6626RR	19.88	42.03	60.90	115.62	131.98	149.67	169.03
LS666RR	18.92	38.90	55.30	105.10	116.72	129.75	142.77
LS6514RR	15.31	34.08	50.86	99.56	109.33	126.17	135.86
LS6710RR	14.55	39.56	58.56	110.67	129.11	144.89	162.33
LS555RR	14.09	33.40	46.53	95.47	110.98	125.78	134.50
LS678RR	17.78	36.98	57.95	99.83	112.73	125.83	136.98
AG5601RR	15.36	25.19	42.33	55.72	66.14	80.61	88.11
PAN626	16.58	25.33	50.44	60.94	74.94	86.25	94.78
AG5409RR	15.83	24.17	45.42	57.33	68.25	79.08	84.83
Prima 2000	13.08	13.33	36.69	47.89	57.75	63.78	70.64
95B33	14.86	20.39	41.28	52.89	66.28	77.53	83.67
96B01B	16.75	22.42	39.83	47.67	57.42	67.25	74.42
95B53	16.44	24.11	42.44	51.94	62.22	74.50	83.11

Appendix 3c

Rate of growth of *Sclerotinia sclerotiorum* over time (1-10 days) and mean number of sclerotia produced on different soybean cultivars inoculated with *Sclerotinia sclerotiorum* (Trial 1)

Cultivar	Rate of growth (per unit per day)	Mean number of sclerotia
LS6626RR	0.49 ^{c}	7.87 ^{ef}
LS666RR	0.46 ^{c}	8.07 ^{f}
LS6514RR	0.45 ^{c}	6.73 ^{def}
LS6710RR	0.43 ^{bc}	6.33 ^{cdef}
LS555RR	0.43 ^{bc}	7.87 ^{ef}
LS678RR	0.43 ^{bc}	6.87 ^{ef}
AG5601RR	0.32 ^{ab}	1.78 ^{a}
PAN626	0.32 ^{a}	5.96 ^{cde}
AG5409RR	0.32 ^{a}	4.67 ^{bc}
Prima 2000	0.31 ^{a}	4.77 ^{bcd}
95B33	0.31 ^{a}	2.17 ^{a}
95B53	0.25 ^{a}	3.80 ^{ab}
96B01B	0.25 ^{a}	2.50 ^{a}
F probability	<0.001	<0.001
s.e.d.	0.055	0.995
l.s.d.	0.113	2.053
cv%	18	23

Means with the same letter are not significantly different at P<0.005

Appendix 3d

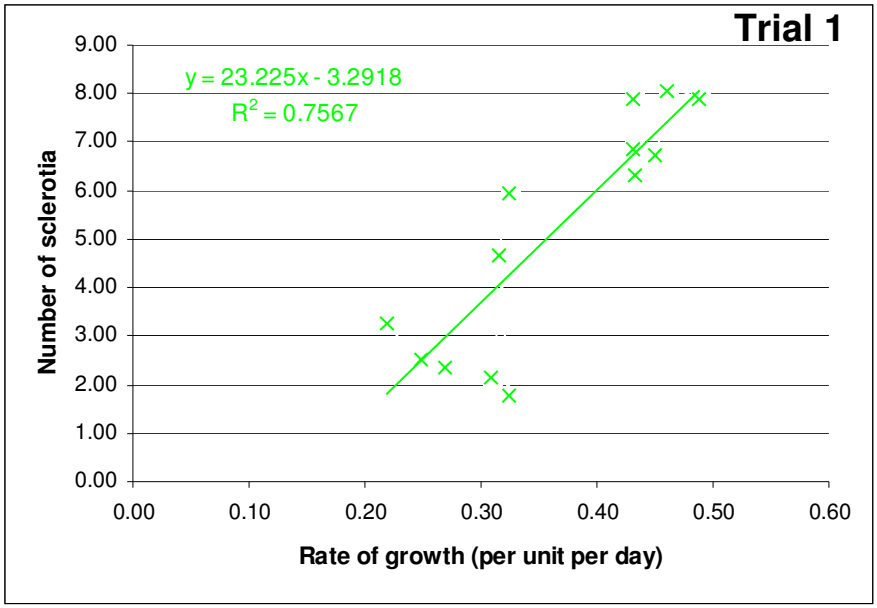
Rate of growth of *Sclerotinia sclerotiorum* over time (1-10 days) and mean number of sclerotia produced on different soybean cultivars inoculated with *Sclerotinia sclerotiorum* (Trial 2)

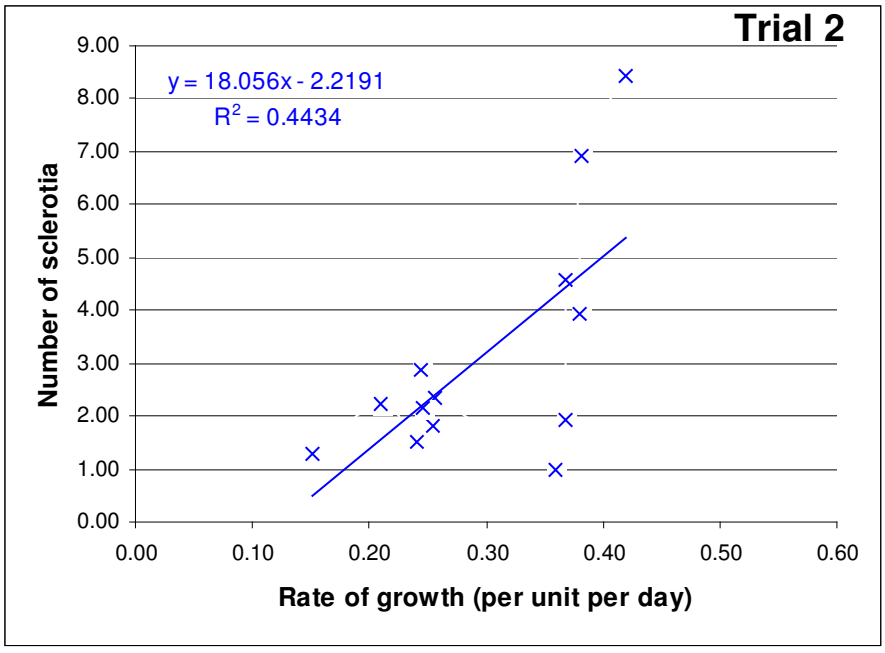
Cultivar	Rate of growth (per unit per day)	Mean number of sclerotia
LS6626RR	0.42 [b]	8.45 [d]
LS666RR	0.38 [b]	6.93 [d]
LS555RR	0.38 [b]	3.93 [bc]
LS678RR	0.37 [b]	4.58 [c]
LS6514RR	0.37 [b]	1.94 [a]
LS6710RR	0.36 [b]	1.00 [a]
95B33	0.26 [a]	2.36 [ab]
AG5601RR	0.25 [a]	1.83 [a]
AG5409RR	0.25 [a]	2.17 [ab]
PAN626	0.24 [a]	2.89 [abc]
Prima 2000	0.24 [a]	1.33 [a]
95B53	0.23 [a]	2.61 [ab]
96B01B	0.21 [a]	2.25 [ab]
F probability	<0.001	<0.001
s.e.d.	0.035	0.940
l.s.d.	0.072	1.940
cv%	14	35

Means with the same letter are not significantly different at P<0.005

Appendix 3e

Regression analysis of rate of growth of *Sclerotinia sclerotiorum* versus number of sclerotia produced on different soybean cultivars inoculated with *Sclerotinia sclerotiorum* (Trial 1 and Trial 2)





Appendix 3f

Rate of growth of *Sclerotinia sclerotiorum* over time (1-10 days) and number of sclerotia produced on different soybean cultivars inoculated with *Sclerotinia sclerotiorum* (Combination of Trial 1 and Trial 2)

Cultivar	Rate of growth (per unit per day)	Mean number of sclerotia
LS6626RR	0.45 [b]	8.16 [h]
LS666RR	0.42 [b]	7.50 [h]
LS555RR	0.41 [b]	5.90 [g]
LS6514RR	0.41 [b]	4.34 [de]
LS678RR	0.40 [b]	5.73 [fg]
LS6710RR	0.40 [b]	3.67 [cd]
AG5601RR	0.29 [a]	1.81 [a]
PAN626	0.28 [a]	4.42 [def]
95B33	0.28 [a]	2.26 [ab]
Prima 2000	0.28 [a]	3.03 [abcd]
AG5409RR	0.28 [a]	3.42 [bcd]
95B53	0.24 [a]	3.21 [bcd]
96B01B	0.23 [a]	2.38 [abc]
F probability	<0.001	<0.001
s.e.d.	0.033	0.673
l.s.d.	0.068	1.390
cv%	12	19

Means with the same letter are not significantly different at P<0.005

APPENDIX 4

Appendix 4a

Half strength V8 agar medium (composition)

V8 tomato juice	100 ml
$CaCO_3$	3.0 g
Agar (Merck)	20.0 g
Distilled water	800 ml

Mix and autoclave for 15 min at 121℃

Appendix 4b

Percentage germination and percentage non infected seedlings 14 days post inoculation for *Trichoderma* treated seeds and *Sclerotinia sclerotiorum*; untreated seeds and *Sclerotinia sclerotiorum* and the control (Trial 1)

	% germination	% non infected seedlings
Control	93.8 [a]	81.2 [a]
***Trichoderma* treated seeds**	100.0 [a]	100.0 [a]
Untreated seeds	87.5 [a]	31.2 [b]
F probability	0.224	0.029
s.e.d.	6.59	19.32
l.s.d.	16.12	47.27
cv%	9.9	38.6

Means with the same letter are not significantly different at P<0.005

Appendix 4c

Percentage germination and percentage non infected seedlings 14 days post inoculation for *Trichoderma* treated seeds and *Sclerotinia sclerotiorum*; untreated seeds and *Sclerotinia sclerotiorum* and the control (Trial 2)

	% germination	% non infected seedlings
Control	93.8 [a]	100.0 [a]
***Trichoderma* treated seeds**	100.0 [a]	100.0 [a]
Untreated seeds	100.0 [a]	31.2 [b]
F probability	0.422	0.018
s.e.d.	5.10	19.32
l.s.d.	12.49	47.27
cv%	7.4	35.4

Means with the same letter are not significantly different at P<0.005

Appendix 4d

Percentage germination and percentage non infected seedlings 14 days post inoculation for *Trichoderma* treated seeds and *Sclerotinia sclerotiorum*; untreated seeds and *Sclerotinia sclerotiorum* and the control (Combination of Trial 1 and Trial 2)

	% germination	% non infected seedlings
Control	96.9 [a]	90.6 [a]
***Trichoderma* treated seeds**	100.0 [a]	100.0 [a]
Untreated seeds	93.8 [a]	31.2 [b]
F probability	0.244	0.001
s.e.d.	3.29	10.72
l.s.d.	8.06	26.24
cv%	4.8	20.5

Means with the same letter are not significantly different at P<0.005

Appendix 4e

Lesion length (mm) at 1-14 days post inoculation and mean number of sclerotia produced on Prima 2000 treated with combinations of potassium silicate (Si), Eco77® (Eco77) and *Sclerotinia sclerotiorum* (SS) (Trial 1)

Treatment	Days post inoculation										
	2	**3**	**4**	**6**	**7**	**8**	**9**	**10**	**11**	**13**	**14**
Eco77+SS	6.33	10.38	15.23	21.65	25.06	27.40	30.69	34.67	37.81	41.88	43.21
Eco77+SS+Si	5.48	9.08	12.00	18.96	22.77	25.88	28.23	30.54	32.21	37.25	40.35
SS+Si	16.75	28.81	39.25	55.88	63.56	69.31	74.81	79.38	83.56	95.00	99.50
SS	16.39	24.89	34.89	50.83	61.50	67.61	73.22	79.06	82.33	94.56	99.22
Eco77	0	0	0	0	0	0	0	0	0	0	0
Eco77+Si	0	0	0	0	0	0	0	0	0	0	0
Si	0	0	0	0	0	0	0	0	0	0	0
Control	0	0	0	0	0	0	0	0	0	0	0

Appendix 4f

Lesion length (mm) at 1-14 days post inoculation and mean number of sclerotia produced on Prima 2000 treated with combinations of potassium silicate (Si), Eco77® (Eco77) and *Sclerotinia sclerotiorum* (SS) (Trial 2)

Treatment	Days post inoculation										
	2	**3**	**4**	**6**	**7**	**8**	**9**	**10**	**11**	**13**	**14**
Eco77+SS+	4.63	12.50	17.25	21.75	24.00	32.50	34.50	41.00	42.63	46.13	51.88
Eco77+SS+Si	4.17	14.25	17.25	21.25	24.25	36.75	38.88	43.25	45.25	48.13	56.38
SS+Si	12.15	24.69	33.65	45.13	52.31	73.14	76.40	85.52	88.48	99.33	106.19
SS	6.25	21.75	29.50	36.00	47.25	68.75	71.88	77.88	81.50	91.50	96.00
Eco77	0	0	0	0	0	0	0	0	0	0	0
Eco77+Si	0	0	0	0	0	0	0	0	0	0	0
Si	0	0	0	0	0	0	0	0	0	0	0
Control	0	0	0	0	0	0	0	0	0	0	0

Appendix 4g

Rate of growth of *Sclerotinia sclerotiorum* over time (1-14 days) on Prima 2000 treated with combinations of potassium silicate (Si), Eco77® (Eco77) and *Sclerotinia sclerotiorum* (SS) (Trial 1)

Treatment	Rate of growth (per unit per day)	Mean number of sclerotia
Eco77	0 [a]	0 [a]
Eco77 + Si	0 [a]	0 [a]
Si	0 [a]	0 [a]
Control	0 [a]	0 [a]
Eco77 + SS	0.17 [b]	0.92 [b]
Eco77 + SS + Si	0.19 [b]	1.06 [b]
SS + Si	0.28 [c]	3.25 [c]
SS	0.28 [c]	2.31 [c]
F probability	<0.001	<0.001
s.e.d.	0.020	0.489
l.s.d.	0.041	1.021
cv%	24.4	73.5

Means with the same letter are not significantly different at P<0.005

Appendix 4h

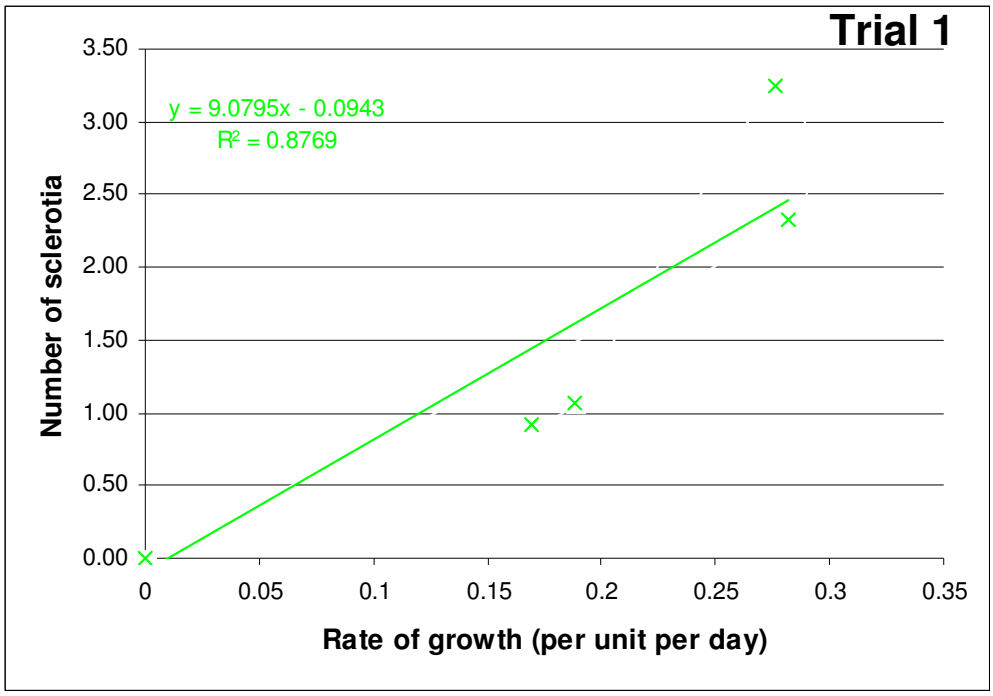
Rate of growth of *Sclerotinia sclerotiorum* over time (1-14 days) on Prima 2000 treated with combinations of potassium silicate (Si), Eco77® (Eco77) and *Sclerotinia sclerotiorum* (SS) (Trial 2)

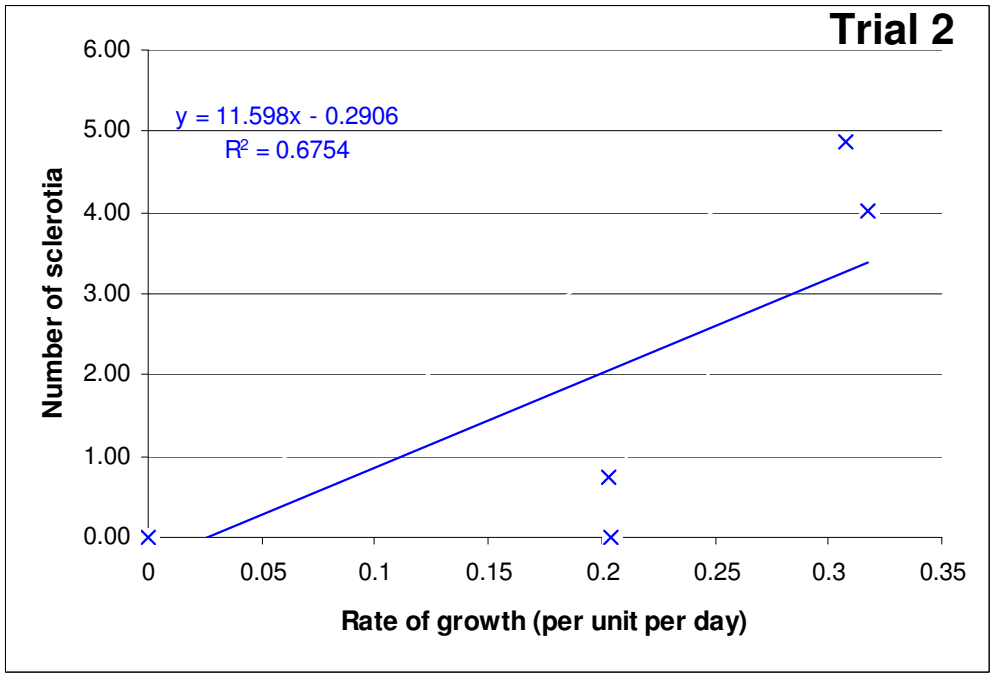
Treatment	Rate of growth (per unit per day)	Number of sclerotia
Eco77	0 [a]	0 [a]
Eco77 + Si	0 [a]	0 [a]
Si	0 [a]	0 [a]
Control	0 [a]	0 [a]
Eco77 + SS	0.20 [b]	0 [b]
Eco77 + SS + Si	0.20 [b]	0.75 [b]
SS + Si	0.32 [c]	4.02 [c]
SS	0.31 [c]	4.88 [c]
F probability	<0.001	<0.001
s.e.d.	0.030	0.447
l.s.d.	0.063	0.929
cv%	33.2	52.4

Means with the same letter are not significantly different at P<0.005

Appendix 4i

Regression analysis of rate of growth of *Sclerotinia sclerotiorum* versus number of sclerotia produced in Prima 2000 treated with combinations of potassium silicate (Si), Eco77® (Eco77) and *Sclerotinia sclerotiorum* (SS) (Trial 1 and Trial 2)





Appendix 4j

Rate of growth of *Sclerotinia sclerotiorum* over time (1-14 days) on Prima 2000 treated with combinations of potassium silicate (Si), Eco77® (Eco77) and *Sclerotinia sclerotiorum* (SS) (Combination of Trial 1 and Trial 2)

Treatment	Rate of growth (per unit per day)	Number of sclerotia
Eco77	0 [a]	0 [a]
Eco77 + Si	0 [a]	0 [a]
Si	0 [a]	0 [a]
Control	0 [a]	0 [a]
Eco77 + SS	0.19 [b]	0.46 [b]
Eco77 + SS + Si	0.20 [b]	0.91 [b]
SS + Si	0.30 [c]	3.64 [c]
SS	0.29 [c]	3.31 [c]
F probability	<0.001	<0.001
s.e.d.	0.022	0.379
l.s.d.	0.045	0.788
cv%	25.1	51.5

Means with the same letter are not significantly different at P<0.005

APPENDIX 5

Appendix 5a

Mycelial diameter (mm) at 1 to 4 days post inoculation on fungicide amended potato dextrose agar inoculated with *Sclerotinia sclerotiorum* (Trial 1 and Trial 2)

Fungicide	Rate (g a.i.ha^{-1})	Mycelial diameter (mm)							
		Trial 1				Trial 2			
		1	2	3	4	1	2	3	4
Control	-	26.75	64.03	101.56	129.94	27.91	62.78	101.69	127.28
BAS 516 04F	133	0	0	0	0	0	0	0	0
	266	0	0	0	0	0	0	0	0
BAS 512 06F	380	0	0	0	0	0	0	0	0
Sumisclex	760	0	7.94	9.31	9.97	0	9.38	11.13	12.47

Appendix 5b

Area under mycelial growth curve (AUMGC) on fungicide amended potato dextrose agar inoculated with *Sclerotinia sclerotiorum* (Trial 1)

Fungicide	Rate (g a.i.ha^{-1})	AUMGC	% inhibition
Control	-	243.9 ^{c}	-
Sumisclex	760	22.2 ^{b}	90.87 ^{a}
BAS 516 04F	133	0 ^{a}	100 ^{b}
	266	0 ^{a}	100 ^{b}
BAS 512 06F	380	0 ^{a}	100 ^{b}
F probability		<0.001	<0.001
s.e.d.		3.73	0.205
l.s.d.		8.12	0.464
cv%		9.9	0.3

Means with the same letter are not significantly different at P<0.005

Appendix 5c

Area under mycelial growth curve (AUMGC) on fungicide amended potato dextrose agar inoculated with *Sclerotinia sclerotiorum* (Trial 2)

Fungicide	Rate ($g\ a.i.ha^{-1}$)	AUMGC	% inhibition
Control	-	242.07 [c]	-
Sumisclex	760	26.74 [b]	86.22 [a]
BAS 516 04F	133	0 [a]	100 [b]
	266	0 [a]	100 [b]
BAS 512 06F	380	0 [a]	100 [b]
F probability		<0.001	<0.001
s.e.d.		1.139	2.244
l.s.d.		2.482	5.077
cv%		3.0	3.3

Means with the same letter are not significantly different at P<0.005

Appendix 5d

Area under mycelial growth curve (AUMGC) on fungicide amended potato dextrose agar inoculated with *Sclerotinia sclerotiorum* (Combination of Trial 1 and Trial 2)

Fungicide	Rate (g a.i.ha^{-1})	AUMGC	% inhibition
Control	-	243.0 ^{c}	-
Sumisclex	760	23.31 ^{b}	89.07 ^{a}
BAS 516 04F	133	0 ^{a}	100 ^{b}
	266	0 ^{a}	100 ^{b}
BAS 512 06F	380	0 ^{a}	100 ^{b}
F probability		<0.001	<0.001
s.e.d.		1.962	0.763
l.s.d.		4.276	1.725
cv%		5.2	1.1

Means with the same letter are not significantly different at P<0.005

Appendix 5e

Disease severity indices (DSI) and grain yield of soybean plants infected with *Sclerotinia sclerotiorum* and periodically sprayed with fungicides (Trial 1)

Fungicide	Rate ($g\ a.i.ha^{-1}$)	DSI (at R7 growth stage)	Yield (g)
Uninoculated control	-	0.00 [a]	24.83 [b]
Inoculated Control	-	25.00 [c]	8.94 [a]
BAS 516 04F	133	22.60 [bc]	12.56 [a]
BAS 516 04F	266	19.00 [bc]	9.96 [a]
BAS 512 06F	380	25.00 [c]	8.28 [a]
Sumisclex	760	18.00 [b]	10.51 [a]
Sumisclex/BAS 512 06F	380/570	25.00 [c]	6.02 [a]
F probability		<0.001	<0.001
l.s.d.		6.388	3.193
s.e.d.		3.095	6.604
cv%		25.4	43.6

Means with the same letter are not significantly different at P<0.005

Appendix 5f

Disease severity indices (DSI) and grain yield of soybean plants infected with *Sclerotinia sclerotiorum* and periodically sprayed with fungicides (Trial 2)

Fungicide	Rate (g a.i.ha^{-1})	DSI (at R7 growth stage)	Yield (g)
Uninoculated control	-	0.00 ^{a}	27.25 ^{c}
Inoculated Control	-	50.40 ^{bc}	13.77 ^{b}
BAS 516 04F	133	44.80 ^{b}	11.74 ^{b}
BAS 516 04F	266	42.40 ^{b}	15.45 ^{b}
BAS 512 06F	380	60.00 ^{c}	5.64 ^{a}
Sumisclex	760	46.40 ^{b}	16.01 ^{b}
Sumisclex/BAS 512 06F	380/570	60.00 ^{c}	5.90 ^{a}
F probability		<0.001	<0.001
l.s.d.		7.17	2.743
s.e.d.		11.37	5.675
cv%		25.9	31.7

Means with the same letter are not significantly different at P<0.005

Appendix 5g

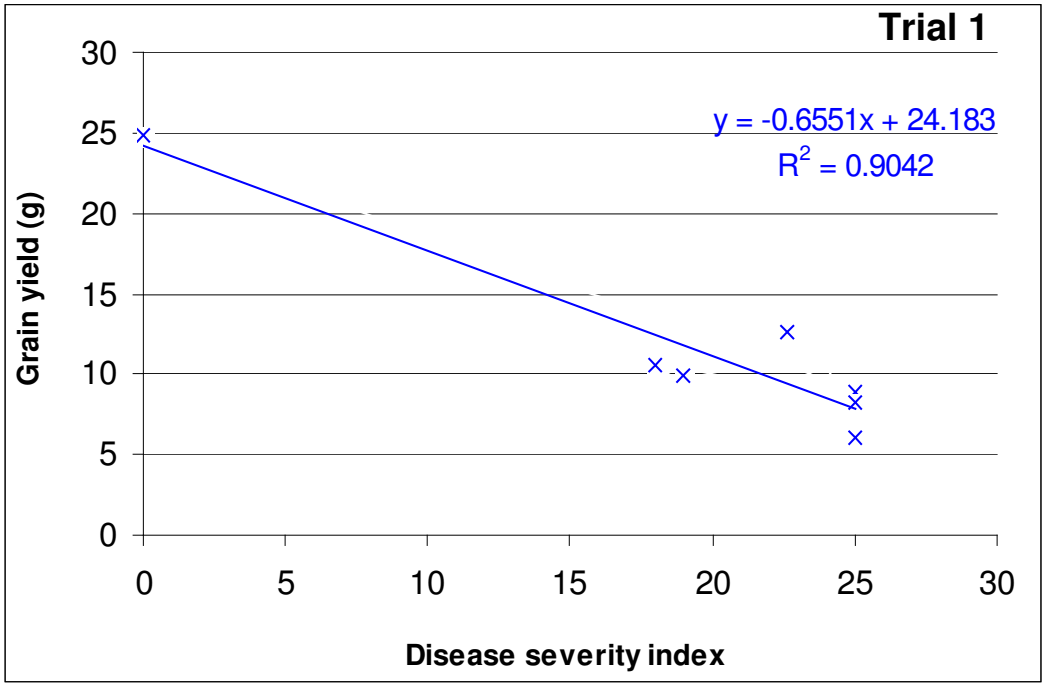
Disease severity indices (DSI) and grain yield of soybean plants infected with *Sclerotinia sclerotiorum* and periodically sprayed with fungicides (Combination of Trial 1 and Trial 2)

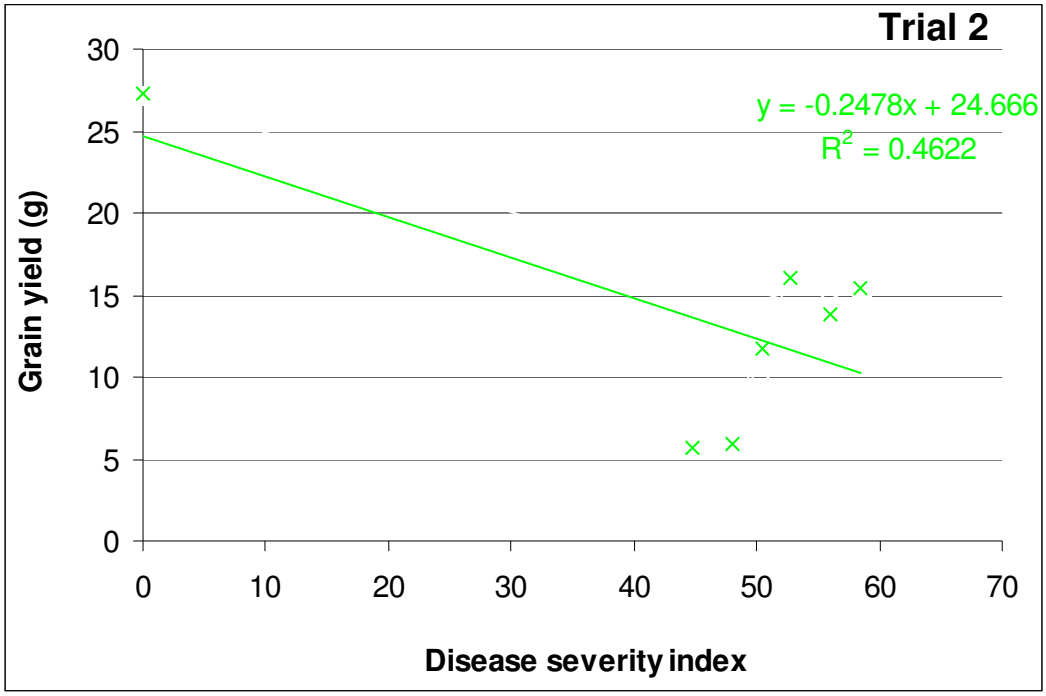
Fungicide	Rate (g a.i.ha^{-1})	DSI (at R7 growth stage)	Yield (g)
Uninoculated control	-	0.00 [a]	26.07 [c]
Inoculated Control	-	38.50 [b]	11.36 [b]
BAS 516 04F	133	36.50 [b]	12.15 [b]
BAS 516 04F	266	38.70 [b]	12.71 [b]
BAS 512 06F	380	35.40 [b]	6.09 [a]
Sumisclex	760	34.90 [b]	13.26 [b]
Sumisclex/BAS 512 06F	380/570	36.50 [b]	5.96 [a]
F probability		<0.001	<0.001
l.s.d.		3.549	2.090
s.e.d.		7.324	4.325
cv%		17.8	26.4

Means with the same letter are not significantly different at P<0.005

Appendix 5h

Regression analysis of disease severity index versus grain yield (g) on Prima 2000 inoculated with *Sclerotinia sclerotiorum* (Trial 1 and Trial 2)





APPENDIX 6

Appendix 6a

Percent normal seedlings, percent abnormal seedlings and dead seeds in germination trials of soybean seeds treated with various seed treatments

Treatment	Dose rate (ml or g a.i. 100kg^{-1})	Replicate	% normal seedlings	% abnormal seedlings	% dead seedlings
Untreated control	-	1	95	3	2
		2	93	3	4
		3	95	4	1
		4	92	6	2
BAS 516 03F	8	1	94	4	0
		2	99	0	1
		3	98	2	0
		4	94	3	3
	16	1	89	5	6
		2	92	4	4
		3	90	7	3
		4	87	9	4
	32	1	83	13	4
		2	93	5	2
		3	88	9	3
		4	84	11	5
BAS 512 00F	7.5	1	92	5	3
		2	96	3	1
		3	96	3	1
		4	93	5	2
	15	1	93	6	1
		2	87	6	7
		3	91	8	1
		4	88	9	3
	30	1	91	4	5
		2	93	7	0
		3	94	5	1
		4	89	9	2
Celest XL	100	1	97	2	1
		2	95	1	4
		3	86	8	6
		4	97	2	1
	125	1	96	2	2
		2	95	3	2
		3	98	1	1
		4	89	4	7
	200	1	94	1	5
		2	89	4	7
		3	91	5	3
		4	93	3	4
	250	1	96	1	3
		2	94	2	4
		3	97	0	3
		4	93	3	4
Sumisclex	5	1	77	18	5
		2	90	8	2
		3	86	12	2
		4	96	3	1
	10	1	87	11	2
		2	85	11	4
		3	91	7	2
		4	89	8	3
Benomyl	150	1	89	9	2
		2	89	7	4
		3	82	12	6
		4	83	14	3
Captan	240	1	83	12	5
		2	82	14	4
		3	86	13	1
		4	89	11	0
Thiram	180	1	93	6	3
		2	95	3	2
		3	94	4	2
		4	97	3	0
Anchor Red	300	1	88	8	4
		2	92	7	1
		3	88	10	2
		4	89	8	3

Appendix 6b

Average percent germination, differences between replicates and allowed tolerances for germination trials of soybean seeds treated with various seed treatments

Treatment	Dose rate (ml or g a.i. 100 kg^{-1} seed)	Average % seed germination	Difference between maximum and minimum averages	Allowed tolerated range according to ISTA*
Untreated control	-	94	3	10
BAS 516 03F	8	96	5	8
	16	90	5	12
	32	87	10	13
BAS 512 00F	7.5	94	4	10
	15	91	5	11
	30	92	5	11
Celest XL	100	94	11	10
	125	95	9	9
	200	92	5	11
	250	95	4	9
Sumisclex	5	87	19	13
	10	88	6	13
Benomyl	150	86	7	14
Captan	240	85	7	14
Thiulin	180	95	4	9
Anchor Red	300	89	4	12

* International Seed Testing Association

Appendix 6c

Percent normal seedlings, percent abnormal seedlings and dead seeds in repeated germination trials of soybean seeds treated with various seed treatments

Treatment	Dose rate (ml or g a.i. 100 kg^{-1} seed)	Replicate	% normal seedlings	% abnormal seedlings	% dead seedlings
Celest XL	100	1	87	10	3
		2	85	10	5
		3	86	7	7
		4	92	7	1
Sumisclex	5	1	92	3	5
		2	80	18	2
		3	85	9	6
		4	93	4	3

Appendix 6d

Average percent germination, differences between replicates and allowed tolerances for repeated germination trials of soybean seeds treated with various seed treatments

Treatment	Dose rate (ml or g a.i. 100 kg^{-1} seed)	Average% seed germination	Difference between maximum and minimum averages	Allowed tolerated range according to ISTA*
Celest XL	100	88	7	13
Sumisclex	5	88	13	13

* International Seed Testing Association

Appendix 6e

Percent germination and survival for soybean seeds (Prima 2000) treated with various seed treatments and inoculated with *Sclerotinia sclerotiorum* (Trial 1)

Treatment	Dose rate (ml or g a.i. 100 kg^{-1} seed)	% seed germination	% seedling survival
Untreated/uninoculated control	-	100 ^{e}	100 ^{d}
Untreated/inoculated control	-	0 ^{a}	0 ^{a}
BAS 516 03F	8	60 ^{b}	89.0 ^{bcd}
	16	80 ^{bcde}	91.7 ^{cd}
	32	73 ^{bcd}	93.3 ^{d}
BAS 512 00F	7.5	100 ^{e}	73.3 ^{b}
	15	93 ^{de}	91.7 ^{cd}
	30	100 ^{e}	100 ^{d}
Celest XL	100	93 ^{de}	100 ^{d}
	125	100 ^{e}	100 ^{d}
	200	100 ^{e}	100 ^{d}
	250	100 ^{e}	100 ^{d}
Sumisclex	5	100 ^{e}	100 ^{d}
	10	100 ^{e}	100 ^{d}
Benomyl	150	100 ^{e}	100 ^{d}
Captan	240	80 ^{bcde}	100 ^{d}
Thiulin	180	67 ^{bc}	75.0 ^{bc}
Anchor Red	300	87 ^{cde}	91.7 ^{cd}
F probability		<0.001	<0.001
s.e.d.		11.57	8.45
l.s.d.		23.52	17.17
cv%		16.6	11.6

Means with the same letter are not significantly different at P<0.005

Appendix 6f

Percent germination and survival for soybean seeds (Prima 2000) treated with various seed treatments and inoculated with *Sclerotinia sclerotiorum* (Trial 2)

Treatment	Dose rate (ml or g a.i. 100 kg^{-1} seed)	% seed germination	% seedling survival
Untreated/uninoculated control	-	100 ^{d}	100 ^{c}
Untreated/inoculated control	-	13 ^{a}	0 ^{a}
BAS 516 03F	8	33 ^{b}	82.6 ^{bc}
	16	93 ^{cd}	80.0 ^{bc}
	32	100 ^{d}	93.3 ^{c}
BAS 512 00F	7.5	87 ^{cd}	100 ^{c}
	15	100	93.3 ^{c}
	30	93 ^{cd}	93.3 ^{c}
Celest XL	100	100 ^{d}	100 ^{c}
	125	100 ^{d}	100 ^{c}
	200	100 ^{d}	100 ^{c}
	250	100 ^{d}	100 ^{c}
Sumisclex	5	100 ^{d}	100 ^{c}
	10	100 ^{d}	100 ^{c}
Benomyl	150	100 ^{d}	100 ^{c}
Captan	240	73 ^{c}	64.3 ^{b}
Thiulin	180	100 ^{d}	86.7 ^{bc}
Anchor Red	300	87 ^{cd}	100 ^{c}
F probability		<0.001	<0.001
s.e.d.		10.44	11.89
l.s.d.		21.1	24.19
cv%		14.6	16.4

Means with the same letter are not significantly different at P<0.005

Appendix 6g

Percent germination and survival for soybean seeds (Prima 2000) treated with various seed treatments and inoculated with *Sclerotinia sclerotiorum* (Combination of Trial 1 and 2)

Treatment	Dose rate (ml or g a.i. 100 kg^{-1} seed)	% seed germination	% seedling survival
Untreated/uninoculated control	-	100 ^{e}	100 ^{c}
Untreated/inoculated control	-	7 ^{a}	-
BAS 516 03F	8	47 ^{b}	83.5 ^{b}
	16	87 ^{cde}	85.8 ^{bc}
	32	87 ^{cde}	93.3 ^{bc}
BAS 512 00F	7.5	93 ^{de}	86.7 ^{bc}
	15	97 ^{de}	92.5 ^{bc}
	30	97 ^{de}	96.7 ^{bc}
Celest XL	100	97 ^{de}	100 ^{c}
	125	100 ^{e}	100 ^{c}
	200	100 ^{e}	100 ^{c}
	250	100 ^{e}	100 ^{c}
Sumisclex	5	100 ^{e}	100 ^{c}
	10	100 ^{e}	100 ^{c}
Benomyl	150	100 ^{e}	100 ^{c}
Captan	240	77 ^{c}	82.2 ^{b}
Thiulin	180	83 ^{cd}	80.8 ^{b}
Anchor Red	300	87 ^{cde}	95.8 ^{bc}
F probability		<0.001	<0.001
s.e.d.		7.06	7.88
l.s.d.		14.34	16.02
cv%		10.0	10.9

Means with the same letter are not significantly different at P<0.005