Alternative products in the inhibition of the plant pathogen *Sclerotinia sclerotiorum* on potato production

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Alternative products in the inhibition of the plant pathogen *Sclerotinia sclerotiorum* on potato production

By

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Submitted in fulfilment of the requirements for the degree of Masters of Technology in Agriculture at the Nelson Mandela Metropolitan University

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Supervisor: Mr P.R. Celliers
DECLARATION

In accordance with Rule G4.6.3, I hereby declare that the above-mentioned thesis is my own work and that it has not previously been submitted for assessment to another University or for another qualification.

SIGNATURE: ____________________________________________________

DATE: ______________________________________________________
DEDICATION

This thesis is dedicated to my late father, Herbert Hlebeni Dhliwayo. Rest in peace.
**ABSTRACT**

White mold caused by *Sclerotinia sclerotiorum* attacks a wide host range of broad-leafed plants which includes potatoes. Current control is limited to the use of chemicals, but biological control has emerged as an eco-friendly alternative. For the study, 19 bacterial strains and 18 fungal strains obtained from soil samples taken from a potato field naturally infested with the pathogen *S. sclerotiorum* were tested for the effects on *S. sclerotiorum* mycelium growth and sclerotia viability *in vitro*.

A total of eight bacterial strains and six fungal strains proved to be effective in the inhibition of *S. sclerotiorum* mycelium growth on PDA plate using a dual culture technique. These antagonistic microbes were six *Bacillus subtilis, Bacillus pumilis, Bacillus marisflavi, Fusarium solani, Fusarium equiseti, Fusarium chlamydosporum, Aspergillus niger, Aspergillus insuetus* and *Penicillium citrinum*. There was a significant difference (p<0.05) in the interaction between *S. sclerotiorum* mycelium growth and the time after inoculation with a *S. sclerotiorum* mycelium plug for both bacterial and fungal soil isolates. An additional sclerotia viability test was carried out using four of the eight antagonistic bacteria, and six of the antagonistic fungi. The results showed that two *Bacillus* species, namely *B. pumilis* and *B. marisflavi* can effectively reduce sclerotia viability. The other two bacteria (both *B. subtilis*), recorded low percentage reduction in sclerotia viability. As for the six antagonistic fungi, the viability test proved to be less effective in determining sclerotia viability. However, the result of this study supports the use of bio-control agents, not only because they are environmentally friendly, but because they are also an effective way of controlling the plant pathogen, *S. sclerotiorum*. 

iii
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TABLE OF CONTENTS

DECLARATION i
DEDICATION ii
ABSTRACT iii
ACKNOWLEDGEMENTS iv
TABLE OF CONTENTS v
LIST OF TABLES viii
LIST OF FIGURES ix
LIST OF APPENDICES xi

CHAPTER ONE

INTRODUCTION 1

1.1 OBJECTIVES 4
1.2 SUMMARY 4

CHAPTER TWO

LITERATURE REVIEW 5

2.1 INTRODUCTION 5
2.2 LIFE CYCLE OF SCLEROTINIA SCLEROTIORUM 7
2.3 SCLEROTINIA SCLEROTIORUM MODE OF INFECTION 9
2.4 CONTROL 11

2.4.1 Cultural Control 11
2.4.1.1 Site selection 11
2.4.1.2 Reduced tillage 12
2.4.1.3 Crop rotation 13
2.4.1.4 Soil fertility and organic matter 13
2.4.1.5 Choice of cultivar 14

v
CHAPTER THREE

MATERIALS AND METHODS

3.1 INTRODUCTION

3.1.1 Origin of *S. sclerotiorum* test bacterial and fungal cultures

3.1.2 Microbial population and isolation of soil microbes

3.2 EXPERIMENT 1: COLONY INTERACTION AND COMPETITION IN DUAL CULTURE

3.3 EXPERIMENT 2: SCLEROTIA VIABILITY TEST

3.4 STATISTICAL ANALYSIS

3.5 SUMMARY

CHAPTER FOUR

RESULTS

4.1 INTRODUCTION

4.2 EXPERIMENT 1: EVALUATION OF TEST MICROBIAL ANTAGONISM USING THE DUAL CULTURE TECHNIQUE

4.2.1 Test microbial isolates and identification.
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 4.1</td>
<td>Antagonistic bacterial identification</td>
<td>30</td>
</tr>
<tr>
<td>Table 4.2</td>
<td>Antagonistic fungal identification</td>
<td>30</td>
</tr>
<tr>
<td>Table 4.3</td>
<td>Percentage reduction of <em>S. sclerotiorum</em> mycelial growth in dual culture with bacterial strains</td>
<td>33</td>
</tr>
<tr>
<td>Table 4.4</td>
<td>Percentage reduction in sclerotia viability</td>
<td>49</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Figure 2.1</td>
<td>Life cycle of <em>S. sclerotiorum</em></td>
<td>9</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Dual culture of bacteria and <em>S. sclerotiorum</em></td>
<td>24</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Dual culture of test fungi and <em>S. sclerotiorum</em></td>
<td>25</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>Growth of <em>S. sclerotiorum</em> in dual culture with isolated bacterial strains obtained from the soil</td>
<td>32</td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>Number of sclerotia formed three weeks after incubation</td>
<td>34</td>
</tr>
<tr>
<td>Figure 4.3</td>
<td><em>S. sclerotiorum</em> growth inhibition by inhibitory volatiles produced by <em>Bacillus pumilis</em> (B4)</td>
<td>35</td>
</tr>
<tr>
<td>Figure 4.4</td>
<td>Inhibition of mycelium of <em>S. sclerotiorum</em> by strain <em>Bacillus subtilis</em> (B6) on PDA</td>
<td>36</td>
</tr>
<tr>
<td>Figure 4.5</td>
<td><em>S. sclerotiorum</em> growth inhibition by strain <em>Bacillus subtilis</em> (B7)</td>
<td>36</td>
</tr>
<tr>
<td>Figure 4.6</td>
<td>Inhibition of <em>S. sclerotiorum</em> mycelium by strain <em>Bacillus subtilis</em> (B2)</td>
<td>37</td>
</tr>
<tr>
<td>Figure 4.7</td>
<td><em>S. sclerotiorum</em> growth inhibition by inhibitory volatiles produced by <em>Bacillus subtilis</em> (B8)</td>
<td>37</td>
</tr>
<tr>
<td>Figure 4.8</td>
<td><em>S. sclerotiorum</em> growth inhibition by strain <em>Bacillus subtilis</em> (B9)</td>
<td>38</td>
</tr>
<tr>
<td>Figure 4.9</td>
<td><em>S. sclerotiorum</em> inhibition by strain <em>Bacillus subtilis</em> (B1) on PDA</td>
<td>39</td>
</tr>
<tr>
<td>Figure 4.10</td>
<td><em>S. sclerotiorum</em> growth inhibition by inhibitory volatiles produced by <em>Bacillus marisflavi</em> (B3) on PDA</td>
<td>39</td>
</tr>
<tr>
<td>Figure 4.11</td>
<td><em>S. sclerotiorum</em> mycelium growth without bacteria on the plates, control plate</td>
<td>40</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>------------</td>
<td>-------------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Figure 4.12</td>
<td>Growth of <em>S. sclerotiorum</em> in dual culture with test fungi I, F, H, E, D and C</td>
<td>41</td>
</tr>
<tr>
<td>Figure 4.13</td>
<td>Percentage reductions in <em>S. sclerotiorum</em> mycelium growth seven days after incubation</td>
<td>42</td>
</tr>
<tr>
<td>Figure 4.14</td>
<td>Number of sclerotia formed 21 days after incubation</td>
<td>43</td>
</tr>
<tr>
<td>Figure 4.15</td>
<td><em>S. sclerotiorum</em> growth inhibition by strain <em>Aspergillus insuetus</em> (F) on PDA</td>
<td>44</td>
</tr>
<tr>
<td>Figure 4.16</td>
<td><em>S. sclerotiorum</em> growth inhibition by strain <em>Penicillium citrinum</em> (D)</td>
<td>45</td>
</tr>
<tr>
<td>Figure 4.17</td>
<td>Inhibition of <em>S. sclerotiorum</em> mycelium by strain <em>Fusarium equiseti</em> (E) on PDA</td>
<td>46</td>
</tr>
<tr>
<td>Figure 4.18</td>
<td><em>S. sclerotiorum</em> inhibition by inhibitory volatiles produced by <em>Fusarium chlamydosporum</em> (C)</td>
<td>46</td>
</tr>
<tr>
<td>Figure 4.19</td>
<td><em>S. sclerotiorum</em> inhibition by inhibitory volatiles produced by <em>Fusarium solani</em> (I)</td>
<td>47</td>
</tr>
<tr>
<td>Figure 4.20</td>
<td>Inhibition of <em>S. sclerotiorum</em> mycelium by strain <em>Aspergillus niger</em> (H) on PDA</td>
<td>48</td>
</tr>
</tbody>
</table>
# LIST OF APPENDICES

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appendix A</td>
<td><em>Sclerotinia sclerotiorum</em> microscopic identification</td>
<td>70</td>
</tr>
<tr>
<td>Appendix B</td>
<td><em>Fusarium chlamydosporum</em> (C) microscopic identification</td>
<td>71</td>
</tr>
<tr>
<td>Appendix C</td>
<td><em>Penicillium citrinum</em> (D) microscopic identification</td>
<td>72</td>
</tr>
<tr>
<td>Appendix D</td>
<td><em>Fusarium equiseti</em> (E) microscopic identification</td>
<td>73</td>
</tr>
<tr>
<td>Appendix E</td>
<td><em>Aspergillus insuetus</em> (F) microscopic identification</td>
<td>74</td>
</tr>
<tr>
<td>Appendix F</td>
<td><em>Aspergillum niger</em> (H) microscopic identification</td>
<td>75</td>
</tr>
<tr>
<td>Appendix G</td>
<td><em>Fusarium solani</em> (G) microscopic identification</td>
<td>76</td>
</tr>
<tr>
<td>Appendix H</td>
<td>ANOVA table of the interaction between <em>S. sclerotiorum</em> mycelium growth and bacterial treatment</td>
<td>77</td>
</tr>
<tr>
<td>Appendix I</td>
<td>ANOVA table of the interaction between <em>S. sclerotiorum</em> mycelium growth and fungal treatment</td>
<td>77</td>
</tr>
<tr>
<td>Appendix J</td>
<td>ANOVA table of the number of sclerotia formed three weeks after inoculation in dual plate with <em>S. sclerotiorum</em> and test bacteria</td>
<td>78</td>
</tr>
<tr>
<td>Appendix K</td>
<td>ANOVA table of the number of sclerotia formed three weeks after inoculation in dual plate with <em>S. sclerotiorum</em> and test fungi</td>
<td>78</td>
</tr>
<tr>
<td>Appendix L</td>
<td>Bacterial identification phylogenetic tree done at Stellenbosch University by Dr Karin Jacobs</td>
<td>79</td>
</tr>
<tr>
<td>Appendix M</td>
<td>Fungal identification phylogenetic tree done at Stellenbosch University by Dr Karin Jacobs</td>
<td>80</td>
</tr>
</tbody>
</table>
The potato belongs to the genus *Solanum* in the family *Solanaceae*. The most widely cultivated species is *Solanum tuberosum*. In South Africa, it is the third most important food crop grown after maize and wheat. South Africa has 52 000 hectares under potatoes and produces 1, 7 million tonnes of potatoes per year (Potatoes South Africa, 2007). Although potatoes are grown almost exclusively for human consumption, today they also have a wide variety of table, processed, livestock feed, and industrial uses. Potatoes are rich in carbohydrates, making them a good source of energy. They also have the highest protein content (approximately 2.1 % on a fresh weight basis) in the family of root and tuber crops. The protein is of a fairly high quality with an amino-acid pattern that is well matched to human requirements. They are also rich in vitamin C with a single medium-sized potato containing about half the recommended daily intake. In addition, no other food, including soybean can match the potato for production of food energy and food value per unit of land area. However, potato production is facing many challenges especially pests and diseases, which have led to low productivity.

White mold, caused by the fungi *Sclerotinia sclerotiorum* (*S. sclerotiorum*) is a problem in most broad leafed plants. In recent years, losses from this disease have averaged as high as 20%, with a few individual field losses exceeding 65% (Schwartz & Steadman,
2004). In South Africa, white mold is now becoming a serious problem in the Gamtoos River Valley (Eastern Cape, South Africa, co-ordinates: 33°58′8.75″S 25°1′58.08″E) where many vegetables are grown. This fungus attacks a wide range of hosts and has a worldwide distribution on numerous field crops and vegetables. White mold has gained significance as a disease with the increased use of sprinkler irrigation and maintenance of high fertility levels. Its ability to form sclerotia (persistent resting structure) in combination with its wide host range makes it difficult to control (Phillips & Botha, 1990).

Control of S. sclerotiorum is currently limited to the use of cultural and chemical control methods. In search of alternatives, biological control has emerged as a way of managing this pathogen. Biological control is the reduction in the amount of inoculum or disease producing activity of a pathogen accomplished through one or more organisms other than man (Tu, 1997). It can be targeted towards reducing the number of sclerotia in the soil with microbial parasites or inhibiting infection of host tissues by propagules of the pathogen (Rabeendran, Jones & Stewart, 1998).

Numerous antagonistic and mycoparasitic fungi or bacteria have proved to be effective against S. sclerotiorum. Isolates of Coniothyrium minitans (Budge, McQuilken, Fenlon & Whipps, 1995) and Trichoderma spp. (Dos Santos & Dhingra, 1980) have shown sclerotial parasitic characteristics in glasshouse and field trials. Two
fungi, *Sporidesmium sclerotivorum* and *Gliocladium* spp., have also been identified as having promising antagonistic effects on the pathogen (Adams & Ayers, 1979). Other mycoparasites that belong to the genera, *Acrostalagmus, Fusarium, Hormodendrum, Mucor, Penicillium, Aspergillus, Stachybotrys* and *Verticillium*, have also been described as parasites of sclerotia (Bedi, 1961).

The fungus, *C. minitans*, is the most investigated microbe, and is a commercial bio-control product (Whipps & Gerlagh, 1992). Other micro-organisms that have been reported to suppress mycelial growth or the germination of the ascospores may also have potential as bio-control agents, however, little data is available. Therefore, the need for research on potential mycoparasites to *S. sclerotiorum* is evident.

Organic matter has also proved to be an alternative in the control of *S. sclerotiorum*. Cattle, poultry and pig waste have all exhibited an inhibitory effect on many fungal pathogens. The addition of compost to soil also significantly reduces carpogenic germination (Couper, Litterick, & Leifert, 2001).

In this study, soil samples were taken from a field naturally infested with *S. sclerotiorum* and selected microbes present were tested against *S. sclerotiorum* using the dual culture technique and the sclerotia viability test. This was done so as to evaluate the selected microbe’s potential pathogenicity in the absence of microbial competitors under optimum conditions of constant temperature and high humidity.
1.1 OBJECTIVES

The objectives of this study are to:

- Isolate soil microorganisms from soil naturally infested with *S. sclerotiorum*
- Test for antagonistic action of the isolated soil microorganisms to mycelium and sclerotia of *S. sclerotiorum* *in vitro*
- To identify microorganisms that are possible bio-control agents of *S. sclerotiorum*

1.2 SUMMARY

Chapter 1 was the introduction of this study. It defined the problem as the plant pathogen *S. sclerotiorum* on potatoes and briefly explained how this study wishes to address the problem of *S. sclerotiorum*. The objectives of this study were clearly stated. The next chapter will briefly review the potato crop, and then broadly analyse the plant pathogen, *S. sclerotiorum*. The possible control measures of the disease will also be discussed.
CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION

Potatoes (\textit{Solanum tuberosum} L.) are the third most important food crop grown in South Africa. About 40% of South Africa's potato crop is grown in the high-lying areas of the Free State and Mpumalanga. Limpopo, the Eastern, Western and Northern Cape, and the high-lying areas of KwaZulu-Natal are also important production areas. Of the total crop, 50% is delivered to fresh produce markets and a further 18% is processed (South African farming sector, 2007). South Africa has 52 000 hectares under potatoes and produces 1, 7 million tonnes of potatoes per year (Potatoes South Africa, 2007). There is no certainty about how and when this commodity reached South Africa. It can, however, be accepted with reasonable accuracy that it came to South Africa with the Dutch East India Company from the Netherlands (Harris, 1992).

The potato is a major world food crop and by far the most important vegetable crop in terms of quantities produced and consumed worldwide (FAO, 2005). The potato is exceeded only by wheat (\textit{Triticum aestivum} L.), rice (\textit{Oryza sativa} L.) and maize (\textit{Zea mays} L.) in world production for human consumption (Bowen, 2003). Potato tubers give an exceptionally high yield per land area unit and have a wide variety of table, processing
livestock feed and industrial uses (Talburt, 1987). As potatoes provide nutritious food in a diversity of environments, they can be an important food source for the increasing world population, especially with its potential for increased vitamin C and protein content.

Culled potatoes and, in some years, surplus production of potatoes are also a source of high-energy feed for livestock. In addition, some alternative therapeutic uses of potato by-products, although unproven, include its potential as an antacid, antispasmodic, antiscorbutic or poultice to reduce inflammation, to promote healing (cicatrizant), act as a diuretic and preventative treatment for heart attacks, to reduce certain eye irritations (an old Creole remedy), to treat neuralgia as well as act as a remineraliser (MSCOMM, 2003). Of the total South African potato production of 21, 1 million bags, 7% (10, 3 million bags), is for export, while the rest, 80 % (126, 4 million bags), is for consumption (Potato profile, 2003).

Potato production is facing a number of constraints which include the fungal disease white mold caused by \textit{S. sclerotiorum}. \textit{S. sclerotiorum} is a necrotrophic, phytopathogenic, filamentous ascomycete (Harel, Bercovich & Yarden, 2006). It belongs to the genus \textit{Sclerotinia} in the family of the \textit{Sclerotiniaceae} (Laemmlen, 2007). There are two other species of \textit{Sclerotinia}, namely, \textit{S. trifoliorum} and \textit{S. minor}. \textit{S. trifoliorum} is known only on alfalfa and forage legumes, while \textit{S. minor} is primarily a lettuce and peanut pathogen. \textit{S. trifoliorum} is similar in biology and morphology to \textit{S. sclerotiorum},
but *S. minor* produces much smaller sclerotia and generally does not produce apothecia in nature. In general, *S. minor* sclerotia are more numerous, smaller, and more angular than *S. sclerotiorum* (Laemmlen, 2007).

*S. sclerotiorum* infects more than 400 botanical species and causes major economic losses of crops worldwide (Bedi, 1961). In South Africa, it has been reported to cause diseases on vegetables including cabbage, cauliflower, lettuce, carrots, tomatoes and potatoes. Yield losses due to *Sclerotinia* diseases in susceptible crops vary and may be as high as 100% (Purdy, 1979). Losses in some crops from diseases caused by *S. sclerotiorum* and other species of *Sclerotinia* amount to millions of dollars annually. The losses are directly from loss of yield and indirectly from reduced quality (loss in grade) (Willetts & Wong, 1980). Indirect loss caused by this pathogen, resulting from production lost due to abandonment of fields for growing preferred crops for less lucrative non-host crops, or to non crop plants, weeds or fallow (Purdy, 1979).

### 2.2 LIFE CYCLE OF *SCLEROTINIA SCLerotIORUM*

*S. sclerotiorum* survives in the soil as irregular shaped, hard, black survival structures called sclerotia. These structures survive in the soil during the winter and other periods of adverse environmental conditions. Depending on the environmental conditions, sclerotia can germinate myceliogenically (sexual stage) or carpogenically (asexual stage), the latter being the most infectious (Willetts & Wong 1980). Carpogernic germination is
when sclerotia germinate to produce small trumpt-shaped, mushroom-like structures called apothecia (Abawi & Hunter, 1979). There are numerous environmental conditions for the formation of apothecia of *S. sclerotiorum*. For example, sclerotia should be on the surface or in the top 2 to 3 centimetres (cm) of the soil surface, the crop canopy must shade the ground and soil moisture must remain high for several days (Wharton & Kirk, 2007). Apothecia are the sexual reproductive structures which bear millions of ascospores that are forcibly ejected from the ascus and wind or air currents will carry them until some land on plant tissue. Ascospores are sticky, therefore, they can stick easily on the nearest plant tissue.

The surface of mature and senescent blossoms has nutrients essential for ascospore germination on plants, thus most infections begin on flowers and occur within one to two weeks of peak blooming (Abawi & Hunter, 1979). When flowers or other plant parts, such as petioles, covered with ascospores fall to the ground, the ascospores can colonise the decaying plant matter (Olsen, Miller, Nolte & Miller, 2003). Hyphae then grow out of senescing tissues onto healthy stems and leaves in the lower plant parts, and sclerotia are produced internally or on the surface of plant stems within 10 to 14 days (Wharton & Kirk, 2007). When the infected stems dry out and the host dies, sclerotia return to the soil, thus completing the disease cycle (Figure 2.1).

Sclerotia can also germinate myceliogenically. When conditions are too dry for apothecium formation, sclerotia can germinate directly to form mycelium. The mycelium

8
can colonise senescent and dead plant material in contact with them and eventually colonise healthy plants also in contact (Abawi & Hunter, 1979). Sclerotia will be formed and the disease cycle repeats. Figure 2.1 illustrates the life cycle of *S. sclerotiorum*.

![Figure 2.1 Life cycle of *S. sclerotiorum* (Agrios, 1997)](image)

### 2.3 SCLEROTINIA SCLEROTIORUM MODE OF INFECTION

The mode of infection by *S. sclerotiorum* is not completely understood, but initial colonisation of the host plant occurs mainly by enzyme tissue dissolution (Willetts & Wong, 1980). It was reported that *S. sclerotiorum* produces pectin methylesterase and polygalacturonase on wheat bran (Echandi & Walker 1957).
Oxalic acid (OA) production has been found to be correlated with sclerotia development and has been shown to be an important factor in the pathogenicity of *S. sclerotiorum* (Donaldson *et al.*, 2001). OA is a virulence factor of several phytopathogenic fungi, including *S. sclerotiorum* (Ferrar & Walker, 1993). OA has also been shown to act synergistically with pectic and cellulolytic enzymes for destruction of host tissues (Maxwell & Lumsden, 1970).

Metabolic disorders and infectious diseases are linked to oxalate formation in plant tissues (Holmes & Assimos, 1998). It was also established that mutant *S. sclerotiorum* isolates, deficient in oxalate biosynthesis, were less pathogenic than the wild-type fungus (Godoy, Steadmen, Dickman & Dan 1990).

Enzymes that catabolise oxalate protect plants from *S. sclerotiorum* infection. Expression of wheat oxalate oxidase, an enzyme that converts oxalate into H2O2 and C02, enhances resistance of soybean to *S. sclerotiorum*. Similarly, over-expression of oxalate decarboxylase from the fungus *Collybia velutipes* protects tobacco and tomatoes against *S. sclerotiorum* (Donaldson *et al*, 2001).

OA’s mechanism of action during infection is not fully understood, however, oxalate has been proposed to remove calcium ions bound to pectins, which expose host cell walls to catabolic enzymes of fungal origin (Bateman & Beer, 1965).
A number of techniques and strategies for managing white mold have been identified, but there are no simple ways of achieving total control of this disease. Producers are encouraged to address the problem on several fronts, rather than settling on single control measures. White mold can basically be controlled using cultural, chemical and biological methods.

2.4.1 Cultural control

Although some cultural practices tend to avoid or reduce *Sclerotinia* diseases, none effectively control *S. sclerotiorum* on their own. Cultural practices are often used to prevent the spread of the pathogen from one host to another by killing the pathogen or by providing unfavorable conditions for the pathogen to develop. Such practices include site selection, reduced tillage, crop rotation, soil fertility, planting date, choice of cultivar, row width, plant density, control of weeds, rouging and irrigation management (Tu, 1997).

2.4.1.1 Site selection

*S. sclerotiorum* infestation varies between fields. Such variation contributes in part to the difference in disease incidence in the fields ranging from 0 to 85% (Tu, 1986). Therefore,
not planting a susceptible host in a field with a previous history of severe white mold is encouraged. Rather a resistant crop like corn or small *graminaceous* crops should be planted.

2.4.1.2 Reduced tillage

The effect of tillage on sclerotia survival is poorly studied and no generalisations can be made to aid managing the pathogen. There is evidence that most of the sclerotia in the upper 2 to 3 cm of soil will deteriorate within a year (Tu, 1986) and sclerotia buried deeper in the soil will have a higher rate of survival. Thus deep ploughing can bury the sclerotia, and repeated ploughing and other convectional tillage operations eventually distribute long-surviving sclerotia throughout the plough zone, while leaving plenty on the soil surface to germinate each season (Kuepper, 2001).

Fields in which no-till is a practiced result in the disease being confined to patches rather than spreading it throughout the fields. Therefore, if it becomes necessary to plant a susceptible host in a field with a history of white mold, no-till, combined with crop rotation can reduce disease risk (Tu, 1997).
2.4.1.3 Crop rotation

Since white mold sclerotia can survive for many years in the soil, crop rotation generally
does not prevent infection (Schwartz & Steadman, 2004). However, this practice does help reduce the number of sclerotia within the field, and hence the potential for disease loss. With crop rotation, the sclerotia will germinate, but the fungus will not have a suitable host to infect and will be unable to continue its life cycle (Wharton & Kirk, 2007). If a field has a history of white mold it is encouraged to avoid rotating with susceptible hosts including beans, alfalfa, potato, sunflower, onion, carrots, tomato, garlic, peppermint, pea, canola and many other broad-leafed plants. While rotation to non-host crops may not be feasible, research has demonstrated that shorter two to three year rotations with non-host crops is still highly effective in reducing sclerotia in the soil and should be seriously considered as part of any overall disease management strategy (Kuepper, 2001).

2.4.1.4 Soil fertility and organic matter

Good fertility management that reduces excessive vine growth will suppress white mold by improving aeration of the crop canopy and hence moisture. High levels of organic matter have also been shown to be effective in lowering disease incidence (Asirifi, Morgan & Parbery, 1994). Neem oil and organic compost have shown the ability to inhibit in vitro growth of *S. sclerotiorum* (Mello, Lourencio & Amorium, 2005). This is
attributed to the fact that both neem oil and organic compost produce a high amount of CO₂ and, therefore, stimulate soil microbial activities.

The amount of sclerotia formed in the soil and the number of diseased lettuce plants can also be reduced by applying cattle, equine and poultry waste (Asirifi, *et al*., 1994). Therefore, organic compost could be part of soil management practices to control *S. sclerotiorum* for its effective growth inhibition of the pathogen while not affecting soil microbial activities. However, there is evidence to indicate that the application of excessive quantities of manure is associated with increased white mold severity (Lumsden, Lewis & Millner, 1983). The reason for this is that manure increases canopy density and this creates a favourable environment for the disease development. In a Minnesota experiment, application of very high rates of nitrogen in swine manure resulted in a slight increase in white mold severity (MN Soybean Production, 2007).

2.4.1.5 Choice of cultivar

Cultivars that produce a thick, dense canopy have a higher risk of white mold than cultivars that produce a sparser canopy (Olsen, *et al*, 2003). Early maturing varieties also have a higher risk of the disease as they flower at the same time as *S. sclerotiorum* liberation and dispersal of spores start to shower. It was formerly believed that resistance to *S. sclerotiorum* did not exist as breeding programs aimed at developing increased physiological resistance had limited success, possibly because resistance to *S.*
sclerotiorum is governed primarily by an additive gene action. More recently, however, progress has been made in the development of cultivars resistant to Sclerotinia diseases in crops such as bean, safflower, sunflower and soybean (Steadman, 1979).

To date, potato cultivar resistant to S. sclerotiorum has not yet been discovered and there is a need for more research on the control of this disease on potatoes.

2.4.1.6 Row-width and plant density

Narrow rows and high-plant density reduce air circulation and trap moisture in the canopy (Tu, 1997). These contribute to a higher incidence and more severe white mold than convectional row-width and reduced plant density. Narrow rows and high plant density also increase early senescence and contact of plant parts, the latter facilitating intra-row spread of the disease. Air circulation between rows can be improved by planting the rows parallel to the prevailing winds, reducing seeding rates and practicing stringent weed control.

2.4.1.7 Control of weeds and rouging

There are a number of weeds that are hosts of S. sclerotiorum, and it is critical to remove all weeds even when the field is in fallow for a year or more. Weeds should be controlled as they provide additional sites for sporulation and a favorable microclimate for infection (Dillard & Cobb 2005). Susceptible weeds include pigweed, nightshade, vetch, crabgrass
and many other broad-leaf weeds. Another control measure that should be considered is rouging infected plants. Roughed plants should be placed in a wagon or sack immediately, including the surrounding organic debris. The diseased material should then be burnt and not used as compost because of the risk of spreading the disease via long surviving sclerotia (Joy & Hudelson, 2003).

An alternative to rouging is the use of a propane torch or similar device in the field. The flame should be aimed at the soil surface and the lower 25.4 cm of stem. The use of rouging or torching may be more suitable for small plantings or when first signs of the disease appear (Kucharek, 2000).

The use of the herbicide Lactofen for white mold control has received considerable attention. Cobra application on soybean at the beginning of flowering has been shown to reduce white mold severity and increase yields when white mold occurs (MN soybean production, 2007).

2.4.1.8 Irrigation management

Proper irrigation management is a critical factor in dealing with potential white mold problems. Canopy wetness should be reduced and excessive irrigation avoided whenever possible. This can be achieved by scheduling irrigation to allow the foliage to dry before night fall (Tu, 1997). Reduction in the frequency of irrigation during periods of ascospore
production can reduce the duration of leaf wetness and so reduce disease. However water deprivation during this period could increase the number of malformed tubers and reduce yield, therefore, reducing irrigation frequency may be impractical in production areas with a high demand for water. Research has shown that use of drip irrigation can dramatically reduce both factors near the soil surface and, thereby, reduce the disease (Laemmlen, 2007).

2.4.2 Chemical control

Currently registered chemicals are protectants and, therefore, need to be applied prophylactically before infection. Although protectant fungicides have no curative properties, they can reduce the spread of the pathogen to new tissues. Proper timing of spray application and methods of application have a great impact on the disease reduction. Fungicides should be applied at row closure before the environment becomes conducive for sclerotia germination and infestation, for chemical control to be effective. Spores are usually discharged over a period of two to eight weeks after row closure (Schwartz, McMillan & Lienert, 1994). Effective fungicides include iprodione, dichloran, fluazinam), azoxystrobin, thiophanate-methyl and boscalid (Hammerschmidt, Dann & Diers, 1998).

In South Africa, there is no chemical registered for the control of S. sclerotiorum on potatoes but in the Gamtoos River Valley, procymidone 500 SC is used to control the pathogen.
2.4.3 Biological control

Control of *S. sclerotiorum* relies mostly on the use of fungicides but these have become less effective owing to increased disease pressure, development of pathogen resistance and problems with enhanced microbial degradation of fungicides in soil (Slade, Fullerton, Hoyte & Stewart, 1995). Any biological measure should target the dormant sclerotia in the field when the pathogen has little mobility, or at the germination stage, when the pathogen is most vulnerable to attack (Tu, 1997).

When applying biological control, no-tillage should be practiced; since the pathogen should remain on the soil surface where bio-control agents can work effectively (Tu, 1986). No-till also means that there will be maximum weathering and biodegradation, which leads to sclerotia developing cracks in the rinds. Cracked sclerotia leak nutrients and are easily colonised by other micro-organisms and decomposing. Biological activity is also at its highest near the soil surface.

There are many fungi, bacteria and other soil organisms that parasitise or utilise sclerotia as carbon sources. One reason that crop rotation is recommended for *S. sclerotiorum* is to allow the natural microbial population to degrade sclerotia. Two important fungal parasites are *Coniothyrium minitans* and *Sporidesmium sclerotivorum* (McLaren, Huang, Kozub and Rimmer, 1994). Both these fungi have been touted as possible bio-control
agents for sclerotia, but their use to control *S. sclerotiorum* on potatoes has not been adequately studied.

A number of antagonistic and/or mycoparasitic fungi and bacteria are also known to be effective bio-control agents of *S. sclerotiorum*. These are *Trichoderma* species and *Gliocladium* species (Adams & Ayers, 1979). Other mycoparasites that belong to the genera *Acrostalagmus, Fusarium, Hormodendrum, Mucor, Penicillium, Aspergillus, Stachybotrys*, and *Verticillium* (Bedi, 1961) have been described as parasites on sclerotia. Although *G. virens* shows the potential to infect sclerotia of *Sclerotinia* spp., it has not been evaluated on a large scale against *S. sclerotiorum*. In contrast, *C. minitans* applied to soil as a solid-substrate inoculum can infect sclerotia of *S. sclerotiorum* year-round and effectively reduce their number and viability in the soil (Budge *et al.*, 1995).

Little information is available about the effect of bacteria on the survival of sclerotia. However, damaged sclerotia appear more susceptible to bacterial invasion. Bacteria are consistently found in decomposed sclerotia as are many other saprophytic fungi. Thus, many bacteria, such as *P. fluorescence, P. putida*, and *B. subtilis*, may have a direct or indirect impact on the survival of the sclerotia, particularly those which produce antibiotics (Tu, 1997).

Plant associated bacteria can act as agents for the stimulation of plant growth and managing of soil and plant health, thus inducing systemic resistance (ISR). These bacteria are known as plant growth promoting bacteria (PGRB). ISR to *S. sclerotiorum* has been
observed in crops like beans, cucumber, radish and tomato (Van Loon, Baker & Pieterse, 1998).

Rhizobacteria and endophytic bacteria use some of the same mechanisms to promote plant growth and control phytopathogens like *S. sclerotiorum*. These include competition for an ecological niche or a substrate, production of inhibitory allelochemicals, and induction of systemic resistance in host plants to a broad spectrum of pathogens (Glick, 1995). IRS involves such elements as synthesis of phenolics (Chen, *et al*., 2000) and accumulation of PR protein (Viswanathan & Samiyappan, 1999). An example of a PR protein is chitinase (PR-3) which hydrolyse chitin which is a component of fungal pathogen cell wall.

### 2.4.4 Integrated control

An integrated approach to the control of sclerotial pathogens is likely to be most effective. More rational use of chemicals through disease forecasting has been combined with biological control to make decisions, whether to spray biological control agents or fungicide (Shtienberg & Elad, 1997). Biological control agents can also be integrated with reduced fungicide applications, which often improve the consistency of disease control. Budge & Whipps, (2001) showed that *S. sclerotiorum* was effectively controlled with a combination of soil application of *C. minitans* and reduced foliar iprodione...
application and this integrated control strategy did not require a fungicide tolerant isolate of *C. minitans*.

Biological control agents can also be integrated with soil solarisation or application of crop residues. Although the biological control agent *Trichoderma virens* cannot survive solarisation, treatment of pre-solarised soil could provide additional management of *Sclerotium rolfsii* on tomato (Ristaino, Perry & Lumsden, 1996). Solarisation also enhanced the effect of some cruciferous amendments on reducing the viability of *Sclerotium rolfsii* sclerotia in soil (Stapleton & Duncan, 1998).

2.5 SUMMARY

The plant pathogen, *S. sclerotiorum*, has become a significant problem in the Gamtoos River Valley. Potato crop production in this region is being affected mainly owing to the extensive use of overhead irrigation and planting of *S. sclerotiorum* susceptible hosts in the same field for a long period of time. Control of *S. sclerotiorum* on the potato crop in the Gamtoos River Valley has mainly been through the use of fungicides, which has adverse effects on non-target organisms and allows the pathogen to develop resistance. There are also increasing risks of fungicides residues being harmful to the soil and water environment. As a result of the public concerns over fungicide use, alternative methods of disease control, such as biological control, should be considered. The next chapter explains the materials and methods used in this study.
CHAPTER THREE

MATERIALS AND METHODS

3.1 INTRODUCTION

For this study, *in vitro* antagonistic action of soil isolated microbes on *S. sclerotiorum* was carried out in two experiments. The first experiment was a dual culture technique and the second test experiment was a sclerotia viability test. All soil isolated microbes were tested for their effects on *S. sclerotiorum* mycelium in the first experiment, but only those soil microbes that proved to be antagonistic to *S. sclerotiorum* mycelium in the first experiment, were tested on sclerotia of *S. sclerotiorum* in the second test experiment.

3.1.1 Origin of *S. sclerotiorum*, test bacterial and fungal cultures

*S. sclerotiorum* used in the study was obtained from an infested potato field in Gamtoos River Valley. Test microbes were obtained from a soil sample taken from a potato field naturally infected with the pathogen *S. sclerotiorum* in the Gamtoos River Valley. The soil sample was taken from the upper 2-3cm of the soil. Test microbes were also obtained from the surface of sclerotia found on the potato crop.
3.1.2 Microbial population and isolation of microbes

The microbial population was evaluated using plate count serial dilutions on appropriate nutrient media in petri dishes. Using a spatula, one gram of a soil sample was placed in nine millilitres of saline in a test tube and shaken well. This made up a dilution of 1:10. Then 1ml of the first dilution (1:10) was taken and placed into another 9ml test tube of saline and this mix was shaken well to make up 1:100 dilution and, lastly, 1ml of the second dilution (1:100) was added to another 9ml of saline to make up 1:1000 dilution. Then from each dilution, 0.1 ml was placed on PDA (for fungi isolation) and nutrient agar (NA) (for bacterial isolation) petri dishes and incubated at 25°C for between one and five days. Three replicates were made for each dilution. The test fungi and bacteria were isolated from the PDA and NA petri dishes and sub-cultured to give pure cultures of the test microbes (Microbiology procedure, 2007).

The pure cultures of both bacterial and fungal isolates were incubated at 25°C. The test bacteria were sub-cultured every two weeks, and the test fungi were sub-cultured every four weeks. The sclerotia of *S. sclerotiorum* obtained from the infested potato crop was plated on PDA and incubated at 25°C. The fungal pathogen was then sub-cultured every four weeks.
3.2 EXPERIMENT 1: COLONY INTERACTION AND COMPETITION IN DUAL CULTURE

The growth medium used in the dual plating was PDA. Bacterial cells from a 48 hour old NA culture of each test bacteria were scraped using a sterile loop and diluted in saline to the same cloudiness of a 1.0 McFarland standard suspension. Two streaks were made on either side of the central point on the PDA plate and incubated at 25°C for 24 hours. After 24 hours, a 1cm x 1cm mycelium plug of a seven day PDA culture of *S. sclerotiorum* was inoculated centrally (Figure 3.1). An additional plate with just the *S. sclerotiorum* plug placed centrally was made as a control plate. All treatments were replicated three times and incubated for three to five days at 25°C. (Whiteman & Stewart, 1998).

![Diagram](image)

*Figure 3.1 Dual culture of bacteria and *S. sclerotiorum*

A 1cm x 1cm plug of the respective test fungi was inoculated at a position diametrically opposite a 1cm x 1cm plug of the pathogen *S. sclerotiorum* which was inoculated 48 hours after inoculation of the test fungi (Figure 3.2). A control plate with only a 1cm x 1cm plug of the pathogen *S. sclerotiorum* was positioned one centimetre from the edge of
the petri dish. All treatments were replicated three times and then incubated for eight days at 25°C (Sitepu & Wallace, 1984).

**Figure 3.2 Dual culture of test fungi and S. sclerotiorum**

The growth of *S. sclerotiorum* was recorded everyday and measurements were taken on the day before contact, or after 28 days if no contact between the colonies occurred. The growth of *S. sclerotiorum* mycelium (average of width of mycelium) on control plates was taken as reference for computing the antagonistic activity of bacteria or fungi with the following equation:

\[
\text{Reduction in mycelium growth \%} = \frac{\text{Control} - \text{Treatment}}{\text{Control}} \times 100
\]

(Treatment = Mycelium growth of *S. sclerotiorum* in plate with staked test bacteria or fungus)

(Control = Mycelium growth of *S. sclerotiorum* in plate without test bacteria or fungus)

The number of sclerotia present in the plates was also counted in each dual-plate (Sivakumar *et al.*, 2000).
3.3 EXPERIMENT 2: SCLEROTIA VIABILITY TEST

From the previous study, some antagonistic bacteria were identified as being in the same subgroup, therefore, only one from the similar bacteria was selected and used in this experiment. Therefore 4 antagonistic bacteria were used for this experiment. All six antagonistic fungi from Experiment 1 were used in this experiment.

Spore suspensions of test fungi were prepared by adding 10ml of 0.01% Tween 80 in sterile distilled water (SDW) to each PDA petri dish and rubbing the surface with a sterile glass spreader. The resulting spore suspension was adjusted to $1 \times 10^6$ spore/ml using a haemocytometer (McLean, Madsen & Stewart, 2004). Bacterial suspensions for the test bacteria were prepared by using the McFarlands standard to adjust to give $1 \times 10^6$ spore/ml.

The substrate used for placing the sclerotia, was sterile sand which was obtained from a potato field in the Gamtoos River Valley. The sand was sterilised by rinsing five times in tap water and drying in a $160^\circ$C oven for 24 hours. Twenty grams of dry sand was transferred to each petri dish (McLean, et al., 2004). Prior to treating the sclerotia in the spore suspension, sclerotia were surface sterilised by agitating in 3% NaOCl for three minutes, rinsing twice in SDW. Twelve sclerotia were immersed in 10ml of spore suspensions of the test bacteria and fungi for ten minutes (Jones & Stewart, 2000) and blotted on sterile filter paper. Sclerotia were placed in sterile sand in petri dishes, sealed
with cling film and incubated at 28°C for 30 days. Four sclerotia were placed in each petri dish. Control sclerotia was washed in SDW and treated similarly. The experiment was arranged in a Randomised Complete Block Design (RCBD) with four bacteria and six fungi treatments, replicated three times. After 30 days, the sclerotia were recovered from the substrate by washing in tap water. All sclerotia from each replicate plate were soaked in ethanol and 17% NaOCl for three minutes. The sclerotia were rinsed twice for 15 minutes in SDW and two sclerotia from each plate were bisected with both halves being placed on a PDA. The remaining two sclerotia were also bisected but both halves were placed on PDA containing 50 microliters of ampicillin and streptomycin sulfate salt. The addition of ampicillin and streptomycin sulfate salt was to reduce contaminants. All plates were incubated at 20°C and mycelium growth was measured every second day for ten days (Van Toor, 2002).

3.4 STATISTICAL ANALYSIS

All measurements were analysed using ANOVA generated from STATISTICA version statistical package. Repeated measure ANOVA was used to analyse S. sclerotiorum mycelium growth.
3.5 SUMMARY

This chapter explained the methodology of the study. Basically there were two experimental tests, namely dual culture test, and sclerotia viability test. The next chapter gives the results of the two experimental tests.
CHAPTER FOUR

RESULTS

4.1 INTRODUCTION

In experiment 1, 19 bacteria and 18 fungi were isolated from the soil. There was a significant difference (p<0.05) in the interaction between \textit{S. sclerotiorum} mycelium growth and bacterial treatment (Appendix H). There was also a significant difference (p<0.05) in the interaction between \textit{S. sclerotiorum} mycelium growth and fungal treatment (see Appendix M). A total of eight bacteria, and six fungi inhibited the growth of \textit{S. sclerotiorum}. In Experiment 2, only two antagonistic bacteria from Experiment 1, managed to reduce sclerotia viability.

4.2 EXPERIMENT 1: EVALUATION OF TEST MICROBIAL ANTAGONISM USING THE DUAL-CULTURE TECHNIQUE

4.2.1 Test microbial isolates and identification

Nineteen bacterial strains and 18 fungal strains were obtained from the soil sample. Of the 19 isolate bacteria, eight inhibited \textit{S. sclerotiorum} growth. The eight antagonistic bacteria were sent for identification at Stellenbosch University and all eight bacterial strains, were rod shaped and gram-positive. For this reason, the identification was based
on the 16s ribosomal gene region. The eight antagonistic bacteria were all identified as
*Bacillus* species (see Table 4.1).

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>B 1</td>
<td><em>Bacillus subtilis</em></td>
</tr>
<tr>
<td>B 2</td>
<td><em>Bacillus subtilis</em></td>
</tr>
<tr>
<td>B 3</td>
<td><em>Bacillus marisflavi</em></td>
</tr>
<tr>
<td>B 4</td>
<td><em>Bacillus pumilis</em></td>
</tr>
<tr>
<td>B 6</td>
<td><em>Bacillus subtilis</em></td>
</tr>
<tr>
<td>B 7</td>
<td><em>Bacillus subtilis</em></td>
</tr>
<tr>
<td>B 8</td>
<td><em>Bacillus subtilis</em></td>
</tr>
<tr>
<td>B 9</td>
<td><em>Bacillus subtilis</em></td>
</tr>
</tbody>
</table>

Six fungal strains inhibited *S. sclerotiorum* mycelium growth. These were identified as
three *Fusarium* spp., two *Aspergillus* spp. and one *Penicillium* sp. (Table 4.2). The
identification of these antagonistic bacteria and fungi was done at Stellenbosch
University by Dr Karin Jacobs (Appendices A-G).

<table>
<thead>
<tr>
<th>Fungal strain</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td><em>Fusarium solani</em></td>
</tr>
<tr>
<td>F</td>
<td><em>Aspergillus insuetus</em></td>
</tr>
<tr>
<td>H</td>
<td><em>Aspergillus niger</em></td>
</tr>
<tr>
<td>E</td>
<td><em>Fusarium equiseti</em></td>
</tr>
<tr>
<td>D</td>
<td><em>Penicillium citrinum</em></td>
</tr>
<tr>
<td>C</td>
<td><em>Fusarium chlamydosporum</em></td>
</tr>
</tbody>
</table>
4.2.2 Bacterial isolates

There was a significant difference (p<0.05) in the interaction between \textit{S. sclerotiorum} mycelium growth and bacterial treatment (Appendix H). Antagonism was observed in eight \textit{bacillus} species, namely, \textit{B. marisflavi} (B3), \textit{B. pumilis} (B4) and six \textit{B. subtilis} (B1, B2, B6, B7, B8, B9) (Figure 4.1). Two \textit{B. subtilis} strains (B1 and B9) completely restricted any vertical or horizontal mycelium growth of \textit{S. sclerotiorum}, making these two \textit{B. subtilis} the most effective inhibitors of \textit{S. sclerotiorum} mycelium growth. \textit{B. sutilis} (B8) managed to completely inhibit \textit{S. sclerotiorum} growth horizontally, but slight growth was recorded vertically.

Figure 4.1 shows the growth of \textit{S. sclerotiorum} in dual culture with all test bacteria. The maximum growth that \textit{S. sclerotiorum} can grow in the PDA petri dish is 9 cm. The control plate and plates with non-inhibitory bacteria reached nine centimetres, 96 hours after inoculation. At 24 hours after inoculation of \textit{S. sclerotiorum} mycelium plug, the mycelium growth of the pathogen in dual culture with 11 of the non-inhibitory bacteria had already grown more than two centimetres and continued growing rapidly until it had grown all over the petri dish. Mycelium growth in five antagonistic bacteria, B2, B3, B4, B6 and B7 was slightly slow and stopped at 72 hours after inoculation of \textit{S. sclerotiorum} mycelium plug.
The growth of *S. sclerotiorum* in dual culture with antagonistic bacteria B3 started faster than the other antagonistic bacteria. At 24 hours after inoculation of *S. sclerotiorum* plug, the growth of *S. sclerotiorum* mycelium in dual culture with B3 was similar to the control plate (Figure 4.1). However, the growth of *S. sclerotiorum* was then reduced by B3 after the 24 hours and completely stopped at 72 hours after inoculation.

![Figure 4.1](image)

Figure 4.1 Growth of *S. sclerotiorum* in dual culture with isolated bacterial strains obtained from the soil

The percentage reduction in *S. sclerotiorum* mycelium growth was relatively high in all eight antagonistic bacteria (Table 4.3). Three *B. subtilis* species, namely, B1, B8 and B9,
had the highest percentage reduction, whereas all non-inhibiting bacteria had no percentage reduction in mycelium growth.

Table 4.3 Percentage reduction of *S. sclerotiorum* mycelial growth in dual culture with bacterial strains

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th><em>S. sclerotiorum</em> growth eight days after incubation (cm)</th>
<th>% reduction in <em>S. sclerotiorum</em> growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1*</td>
<td>1</td>
<td>88.9</td>
</tr>
<tr>
<td>B2*</td>
<td>1.13</td>
<td>85.2</td>
</tr>
<tr>
<td>B3*</td>
<td>2.47</td>
<td>72.6</td>
</tr>
<tr>
<td>B4*</td>
<td>1.37</td>
<td>84.8</td>
</tr>
<tr>
<td>B5</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>B6*</td>
<td>1.6</td>
<td>82.2</td>
</tr>
<tr>
<td>B7*</td>
<td>1.13</td>
<td>87.4</td>
</tr>
<tr>
<td>B8*</td>
<td>1</td>
<td>88.9</td>
</tr>
<tr>
<td>B9*</td>
<td>1</td>
<td>88.9</td>
</tr>
<tr>
<td>B10</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>BA</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>BB</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>BC</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>BD</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>BE</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>BF</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>BI</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>BJ</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>BM</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>0</td>
</tr>
</tbody>
</table>

NB: Maximum *S. sclerotiorum* growth in petri dish is 9 cm, hence B5, B10, BA, BB, BC, BD, BE, BF, BI, BJ and BM did no inhibit *S. sclerotiorum* mycelium growth.

*Antagonistic bacteria

Sclerotia formation was recorded in all dual plates with non-inhibitory bacteria and also in the control plate, the latter having the highest sclerotia formation (Figure 4.2).

However, only one antagonistic bacterium, *B. pumilis* (B3), recorded sclerotia formation in its dual plate with *S. sclerotiorum*, whilst all the other antagonistic bacteria managed to restrict any sclerotia formation.
Figures 4.3 to 4.11 illustrate the inhibition of *S. sclerotiorum* by eight *B. subtilis* species. Bacteria were streaked twice on opposite ends on PDA and incubated at 25°C for 24 hours before the inoculation of *S. sclerotiorum* mycelia plug. Inhibition was observed eight days after *S. sclerotiorum* mycelia inoculation.

A clear zone of inhibition was observed between *S. sclerotiorum* mycelium and six antagonistic bacteria (Figures 4.3 to 4.7).
S. sclerotiorum mycelium growth was initiated, but the bacteria, *B. pumilis* (B4) completely inhibited the growth of *S. sclerotiorum* two days after inoculation of *S. sclerotiorum* plug (Figure 4.3). A distinct line of precipitation was formed between *B. pumilis* (B4) and *S. sclerotiorum*, six days after inoculation of *S. sclerotiorum* plug.

There was also some *S. sclerotiorum* growth the first two days after inoculation of *S. sclerotiorum* mycelium plug but the bacterial strain, *B. subtilis* (B6) successfully inhibited *S. sclerotiorum* growth three days after inoculation of mycelium plug. A slight line of precipitation was also observed (Figure 4.4).
Figure 4.4 Inhibition of mycelium of *S. sclerotiorum* by strain *Bacillus subtilis* (B6) on PDA.

Figure 4.5 *S. sclerotiorum* growth inhibition by strain *Bacillus subtilis* (B7)
B. subtilis (B7) and B. subtilis (B2) both inhibited S. sclerotiorum growth and a distinct zone of inhibition were observed with the formation of line of precipitation (Figure 4.5 and 4.6).
*B. subtilis* (B8) inhibited any horizontal growth of *S. sclerotiorum*. There was a slight vertical growth of *S. sclerotiorum* mycelium (Figure 4.7). *B. subtilis* (B8) is a fast growing bacteria as seen by the large surface area which it took in the PDA plate. After seven days, a line of precipitation was also observed between the bacteria *B. subtilis* (B8) and *S. sclerotiorum*.

Two bacteria, namely *B. subtilis* (B9) and *B. subtilis* (B1) completely inhibited *S. sclerotiorum* mycelium growth on PDA (Figure 4.8 and 4.9). These two bacteria were the most effective in the inhibition of *S. sclerotiorum* mycelium as neither vertical nor horizontal mycelium growth was recorded.

![Figure 4.8 S. sclerotiorum growth inhibition by strain Bacillus subtilis (B9)](image)
Figure 4.9 *S. sclerotiorum* inhibition by strain *Bacillus subtilis* (B1) on PDA.

Figure 4.10 illustrates inhibition of *S. sclerotiorum* by *B. marisflavi* (B3). *S. sclerotiorum* growth was only evident vertically, and owing to this vertical growth, the dual plate between *S. sclerotiorum* and *B. marisflavi* (B3) recorded a few sclerotia formation.

Figure 4.10 *S. sclerotiorum* growth inhibition by inhibitory volatiles produced by *Bacillus marisflavi* (B3) on PDA.
*S. sclerotiorum* mycelium grow very fast in the control plate. Sclerotia formation started five days after the inoculation of the *S. sclerotiorum* mycelium plug. Three weeks after inoculation, sclerotia were fully grown (Figure 4.11).

![Figure 4.11 S. sclerotiorum mycelium growth without bacteria on the plates, control plate](image)

4.2.3 Fungal isolates

Of the 18 isolated fungal strains, six fungi, namely, *Aspergillus insuetus* (F), *Penicillum citrinum* (D), *Fusarium equiseti* (E), *Fusarium chlamydosporum* (C), *Fusarium solani* (I) and *Aspergillus niger* (H), inhibited the growth of *S. sclerotiorum* with the latter being the most effective. There was a significant difference (p<0.05) in the interaction between *S. sclerotiorum* mycelium growth, the time after inoculation of *S. sclerotiorum* mycelium plug and test fungal treatment (Figure 4.12).
The growth of *S. sclerotiorum* mycelium in dual plate with inhibitory fungi was similar to the control plate at 24 and 48 hours after inoculation of *S. sclerotiorum* mycelium. But after 72 hours of incubation, the *S. sclerotiorum* growth in dual plates with the inhibitory fungi was constant, whereas the *S. sclerotiorum* mycelium growth in the control plate increased rapidly until it had grown all over the plate at 120 hours of incubation (Figure 4.12).

Figure 4.12 Growth of *S. sclerotiorum* in dual culture with test fungi I, F, H, E, D and C

All antagonistic fungi reduced *S. sclerotiorum* growth by more than 50%, with the highest percentage reduction in *S. sclerotiorum* growth being recorded in dual plates with
test fungi H (*Aspergillus niger*) (Figure 4.13). The lowest percentage reduction in *S. sclerotiorum* growth was recorded with test fungi F (*Aspergillus insuetus*) and E (*Fusarium equeseti*), with both of them recording a 50% reduction in *S. sclerotiorum* growth.

Sclerotia formation was recorded in all dual plates, except dual plates with test fungi H (*Aspergillus niger*) which recorded no sclerotia formation. The control plate had the highest number of sclerotia formed (Figure 4.14).
Figure 4.14 Number of sclerotia formed 21 days after incubation

There was a distinct zone of inhibition for all six inhibitory fungi 20 days after inoculation of *S. sclerotiorum* mycelium. Figures 4.15 to 4.20 illustrate the inhibition of *S. sclerotiorum* by the six antagonistic fungal strains, namely, *Aspergillus insuetus* (F), *Penicillium citrinum* (D), *Fusarium equiseti* (E), *Fusarium chlamydosporum* (C), *Fusarium solani* (I) and *Aspergillus niger* (H).

The growth of *S. sclerotiorum* mycelium was inhibited by *A. insuetus* (F), with a clear zone of inhibition being formed between *S. sclerotiorum* and *A. insuetus* (F) (Figure
Sclerotia formation was evident in the dual plate between *S. sclerotiorum* and *A. insuetus* (F).

Figure 4.15 *S. sclerotiorum* growth inhibition by strain *Aspergillus insuetus* (F) on PDA. Strain F was inoculated on the right side of the plate, and incubated at 25°C for 48 hours before the inoculation of *S. sclerotiorum* mycelium plug on the left side of the plate.

The fungal strain, *P. citrinum* (D) showed antagonistic action to *S. sclerotiorum* mycelium (Figure 4.16). The growth of *S. sclerotiorum* was clearly inhibited. *P. citrinum* (D) is a relatively fast growing fungi and it grow faster than *S. sclerotiorum*. A clear zone of inhibition was observed between *S. sclerotiorum* and *P. citrinum* (D). Sclerotia of *S. sclerotiorum* were formed in the dual plate.
Figure 4.16 *S. sclerotiorum* growth inhibition by strain *Penicillium citrinum* (D). Strain D was also inoculated on right side of the plate, but incubated at 25°C for 24 hours before the inoculation of *S. sclerotiorum* mycelium plug on the left side of the plate.

Two fungi, namely, *F. equiseti* (E) and *F. chlamydosporum* (C), managed to inhibit the growth of *S. sclerotiorum* (see Figure 4.17 and 4.18). Although *S. sclerotiorum* mycelium growth was faster than both *F. equiseti* (E) and *F. chlamydosporum* (C), the two antagonistic fungi, *F. equiseti* (E) and *F. chlamydosporum* (C), managed to inhibit *S. sclerotiorum* growth with a clear zone of inhibition being formed between *S. sclerotiorum* and the two antagonistic fungi. The zone of inhibition between *S. sclerotiorum* and *F. equiseti* (see Figure 4.17) was bigger than the zone of inhibition between *S. sclerotiorum* and *F. chlamydosporum* (see Figure 4.18). Sclerotia of *S. sclerotiorum* were formed in dual plates with *S. sclerotiorum* and both *F. equiseti* (E) and *F. chlamydosporum* (C).

45
Figure 4.17 Inhibition of *S. sclerotiorum* mycelium by strain *Fusarium equiseti* (E) on PDA. Strain E was inoculated on right side of the plate, and incubated at 25°C for 48 hours before the inoculation of *S. sclerotiorum* mycelium plug on the left side of the plate.

Figure 4.18 *S. sclerotiorum* inhibition by inhibitory volatiles produced by *Fusarium chlamydosporum* (C). Strain (C) was inoculated on the right side of the plate, and incubated at 25°C for 48 hours before the inoculation of *S. sclerotiorum* mycelium plug on the left side of the plate.
*F. solani* (I) inhibited mycelial growth of *S. sclerotiorum* (Figure 4.19). A clear zone of inhibition was observed between *S. sclerotiorum* mycelium and *F. solani* (I) mycelium. There was also formation of sclerotia of *S. sclerotiorum*.

![Image of inhibition and sclerotia formation](image)

Figure 4.19 *S. sclerotiorum* inhibition by inhibitory volatiles produced by *Fusarium solani* (I). Strain (I) was inoculated on the right side of the plate, and incubated at 25°C for 24 hours before the inoculation of *S. sclerotiorum* mycelium plug on the left side of the plate.

The highest percentage inhibition of *S. sclerotiorum* was recorded by *A. niger* (I) (Figure 4.13). The growth of *S. sclerotiorum* mycelium was slower than the growth of *A. niger* mycelium and a clear zone of inhibition was observed between the two fungi (Figure 4.20).
4.3 EXPERIMENT 2: SCLEROTIA VIABILITY TEST

In Experiment 2, all six antagonistic fungi from Experiment 1 were used, but only four antagonistic bacteria were used. This was because, among the eight Bacillus species, 6 were identified as being Bacillus subtilis, namely, B1, B2, B6, B7, B8 and B9, with the other two being Bacillus pumilis (B4) and Bacillus marisflavi (B3). Of the 6 B. subtilis, there were two sub-groups as is evident in the phylogenetic tree (Appendix L). Therefore since four of antagonistic B. subtilis, namely B6, B7, B8 and B9 were identified in a similar sub-grouped, only one was select to be used in Experiment 2, which was B9. In addition, two of the other antagonistic B. subtilis, namely, B1 and B2, were also grouped
in another similar subgroup, and only B1 was used in the second Experiment. Therefore, the six antagonistic bacteria used in experiment 2, were *B. subtilis* (B1), *B. marisflavi* (B3), *B. pumilis* (B4) and *B. subtilis* (B9).

Two of the four selected test bacteria, resulted in a high percentage sclerotia viability reduction. These two were *B. marisflavi* (B3) and *B. pumilis* (B4) (Table 4.4).

The other two test bacteria, which were both *B. subtilis*, did not manage to reduce sclerotia viability with one recording a 33% reduction in sclerotia viability and the other recording no reduction in sclerotia at all.

Table 4.4 Percentage reduction in sclerotia viability

<table>
<thead>
<tr>
<th>Test bacteria</th>
<th>PDA</th>
<th>% Reduction of sclerotia viability</th>
<th>% Contaminants</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>+ antibiotics</td>
<td>33</td>
<td>17</td>
</tr>
<tr>
<td>B1</td>
<td>- antibiotics</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>B3</td>
<td>+ antibiotics</td>
<td>83</td>
<td>0</td>
</tr>
<tr>
<td>B3</td>
<td>- antibiotics</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>B4</td>
<td>+ antibiotics</td>
<td>83</td>
<td>0</td>
</tr>
<tr>
<td>B4</td>
<td>- antibiotics</td>
<td>0</td>
<td>83</td>
</tr>
<tr>
<td>B9</td>
<td>+ antibiotics</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B9</td>
<td>- antibiotics</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Control</td>
<td>+ antibiotics</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Control</td>
<td>- antibiotics</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

+antibiotics = PDA with two antibiotics (ampicillin and streptomycin sulfate salt)  
-antibiotics = PDA without any additives.

A high percentage of contaminants were recorded on all bacterial PDA plates without the two antibiotics, streptomycin sulfate salt and ampicillin. Whereas PDA plates with the two antibiotics had relatively lower contaminants and in some cases there was no contaminants recorded, as in B4, B9 and B3 (Table 4.4).
As for the fungi, all the plates recorded the growth of each test fungi treatment. All the sclerotia in both PDA plates with and without the antibiotics did not grow.

4.4 SUMMARY

The results of this study were highlighted in this chapter. A total of 14 micro-organisms inhibited mycelium growth of *S. sclerotiorum in vitro*. Among these 14 microbes, two bacteria, namely, *B. marisflavi* and *B. pumilis*, reduced viability of sclerotia of *S. sclerotiorum*. The next chapter will discuss the results obtained from this study.
Nineteen bacteria strains were isolated from the soil sample, and eight of these bacterial isolates inhibited the growth of *S. sclerotiorum* mycelium. These eight bacterial isolates were *Bacillus* spp. The soil sample was taken from the upper rhizosphere of the soil and according to Mahafee & Kloepper, (1997), *Bacillus* spp. are one of the most dominant bacteria in the rhizosphere. In addition, Weller (1988) found that *Bacillus* spp. are more prevalent in the rhizosphere and are also good bio-control agents mainly because they reduce endospores that are tolerant to heat and desiccation. For sclerotia of *S. sclerotiorum* to germinate, it needs to be on the surface of the soil, and since *Bacillus* species are more dominant around the soil surface, there is a high probability that these antagonistic *Bacillus* species will be in contact with the sclerotia and will attack them before they germinate and spread.

In this study, two *B. subtilis* strains, namely B1 and B9 completely inhibited both horizontal and vertical growth of *S. sclerotiorum* mycelium, making these two *B. subtilis* the most effective bacterial isolates in the inhibition of *S. sclerotiorum* mycelium *in vitro* (Figure 4.8 and 4.9). However, another *B. subtilis* strain, B8, managed to completely inhibit horizontal growth of *S. sclerotiorum* mycelium, but slight vertical growth of mycelium was recorded (Figure 4.10). This slight vertical mycelium growth shows that
the distance between *S. sclerotiorum* and its antagonist is an important factor in the inhibition of the plant pathogen. This emphasizes the fact that when applying bio-control agents like *B. subtilis*, no-tillage should be practiced, since the pathogen would remain on the soil surface where these bio-control agents can work effectively (Tu, 1986).

Although all antagonistic bacteria were identified as *Bacillus* spp., the percentage reduction in mycelium growth was similar for only three of the antagonists, namely B1, B8 and B9. These three bacterial antagonists recorded the highest percentage reduction in *S. sclerotiorum* mycelia growth. The difference in percentage reduction of *S. sclerotiorum* mycelia growth might be due to the fact that *Bacillus* spp. protection mechanism differs between strains. *Bacillus* species have different mode of action that include antibiosis, parasitism, and induced systemic resistance (Jacobsen, Zidack & Larson, 2004).

All eight bacteria that managed to inhibit *S. sclerotiorum* mycelial growth, all inhibited sclerotia formation totally in their dual plates except for *B. marisflavi* (B3) (Figure 4.2). This was because, *S. sclerotiorum* mycelium managed to grow vertically until it reached the edge of the plate (Figure 4.10). However *B. marisflavi* (B3) inhibited the horizontal growth of *S. sclerotiorum*, thus antagonising the plant pathogen. All non-antagonistic bacteria, together with the control plate yielded very high numbers of sclerotia formation.
Bacillus species have shown to have broad suppressive properties due to their ability to produce a great abundance of antibiotics (Stein, 2005). In this study, S. sclerotiorum and six of the antagonistic Bacillus species, produced lines of precipitation between each other in dual plates. This could have been due to volatile or non-volatile inhibitory substances produced by the Bacillus species, causing the inhibition of S. sclerotiorum growth. Similar results were recorded by Zhang (2004) who found that three Bacillus species, namely, B. subtilis, B. cereus and B. amyloliquefaciens, all had an inhibitory effect on S. sclerotiorum on PDA and LBA plates.

Of the four selected bacterial isolates that inhibited the growth of S. sclerotiorum mycelium on PDA, only two managed to reduce sclerotia viability. These two were B. marisflavi (B3) and B. pumilis (B4) (Figure 4.4). This therefore suggests that, B. marisflavi (B3) and B. pumilis (B4) are effective bio-control agents for controlling mycelium and sclerotia growth in S. sclerotiorum. Further studies in this regard are needed to confirm the use of B. marisflavi and B. pumilis as biological control agents of S. sclerotiorum. Unfortunately, the other two, which were both B. subtilis (B1 & B9), did not reduce sclerotia viability. This suggests that both of these B. subtilis were effective in inhibiting S. sclerotiorum mycelium growth on PDA, but could not antagonise the sclerotia of S. sclerotiorum.

Of the eighteen fungal isolates, six inhibited the growth of S. sclerotiorum mycelium. Of these six antagonistic fungi, three were identified as being of the Fusarium spp. These
were *F. solani* (I), *F. equiseti* (E) and *F. chlamydosporum* (C). *Fusarium* species have been shown to be effective antagonists of *S. sclerotiorum*. *F. equiseti* and six strains of *F. solani* inhibited the growth of *S. sclerotiorum* mycelium on PDA, in dual plates (Zazzerini & Tosi, 1985). Sitepu & Wallace (1984), also observed that *F. lateritium* effectively inhibits the germination of ascospores and the growth of mycelia of *S. sclerotiorum in vitro*. Although these *Fusarium* spp. inhibit *S. sclerotiorum*, their mode of action is not known. Some *Fusarium* species have pathogenitic characteristics. For example, *F. solani* has been shown to cause damage to the potato crop as it causes potato dry rot. There are other *Fusarium* spP. which are considered to cause this disease as well, and these include, *F. coeruleum, F. sambucinum, F. oxysporum, F. culmorum* and *F. solani* (Dorozhkin & Belskaya, 1979). The composition of dry rot causes may vary in areas, depending on the *Fusarium* flora of the soils (Loiveke, 2006). Hence the pathogenicity of these *Fusarium* spp. should be tested further, before their use as bio-control agents.

Two of the six antagonistic fungal strains were identified as *Aspergillus* spp. These were, *A. niger* (H) and *A. equiseti* (F). Similar results were obtained by Rai and Saxena (1975). In this study, *A. niger* (H) was the most effective in the inhibition of *S. sclerotiorum* mycelium (Figure 4.12). This suggests that *A. niger* (H) produces very effective inhibitory volatiles or non-volatiles. The fungi *A. niger* also produces a large number of spores, making it more competitive, and thus increasing its inhibitory characteristics.
The highest percentage inhibition was also recorded in dual plates with *A. Niger* (Table 4.13). Overall, *A. niger* was the most effective test fungi inhibiting *S. sclerotiorum* mycelium growth, however, some reports have linked *A. niger* as a health hazard fungus. It can be dangerous to humans in sufficient quantities if inhaled causing a chronic lung disease called Aspergillosis (Springs, 2008). Therefore, care is needed when handing or using *A. niger* as a bio-control agent against *S. sclerotiorum*.

Another relatively fast growing fungus, *P. citrinum* (D), also recorded a high percentage in *S. sclerotiorum* growth. *P. Citrinum* has been reported to produce mycotoxin(s) that inhibit mycelial growth of *Sclerotinia* species. Mycelial growth of *S. minor* and *S. rolfsii* was completely inhibited on media amended with 20% filtrate of *P. citrinum* (Melouk & Akem, 1987). This supports the results obtained in this study, since *P. citrinum* managed to inhibit *S. sclerotiorum* mycelium growth, and also reduce the formation of sclerotia.

Five of the antagonistic fungi, namely, *F. equiseti* (E) *F. chlamydosporum* (C), *F. solani* (I), *A. insuetus* (E) and *P. citrinum* (D) recorded sclerotia formation in their dual plates with *S. sclerotiorum*. However, *A. niger* (H) was the most effective in inhibiting sclerotia formation, as no sclerotia was formed in its dual plate with *S. sclerotiorum* (see Figure 4.14).
A clear zone of inhibition was observed between *S. sclerotiorum* and the six antagonistic fungal strains. This might be caused by antibiotics produced by the antagonistic fungal strains, and these antibiotics cause the inhibition of *S. sclerotiorum* mycelia growth.

Three fungal strains, namely, *A. insuetus* (E), *A. niger* (H) and *P. citrinum* (D), which effectively inhibited *S. sclerotiorum* mycelium growth in this study, have no detrimental effect on the growth and development of the potato crop. This suggests that the use of *A. insuetus* (E), *A. niger* (H) and *P. citrinum* (D) as biological control agents for the control of *S. sclerotiorum* on potatoes is possible.

Results from the viability test showed that the addition of streptomycin sulphate salt and ampicillin to PDA will reduce contamination on sclerotia with bacteria treatments. However, the addition of streptomycin and ampicillin did not have an effect on all sclerotia treated with fungal treatment since only test fungal growth was recorded. This suggests that either the 17% NaOCl was not effective in the removal of the test fungi on the surface of the sclerotia, and/or the addition of streptomycin sulphate salt and ampicillin was not effective in reducing test fungal contaminants on the sclerotia.
CONCLUSIONS AND RECOMMENDATIONS

Mycelium growth of *S. sclerotiorum* can be effectively inhibited by soil isolated microbes. The objectives of this study were to isolate, test and identify soil microorganisms that can be used as possible alternative biological control agents of the plant pathogen *S. sclerotiorum*. Biological control of plants by microorganisms is a very promising alternative to the extended use of pesticides, which are often expensive and accumulate in plants, having adverse effects on humans.

In this study, eight *Bacillus* species, namely, *B. pumilis, B. marisflavi* and six strains of *B. subtilis*, proved to be effective in the inhibition of mycelia growth of *S. sclerotiorum in vitro*. These results indicate that *Bacillus* spp., which are prominent in most soils, have a devastating effect on the mycelium of *S. sclerotiorum* if in contact. Hence, there is need of a management system that ensures that these antagonistic bacteria are in close proximity to the mycelia growth of *S. sclerotiorum*, so as to ensure inhibition of the plant pathogen. These findings suggest that if biological control is to be effective, cultural practices such as reduced tillage should be incorporated together with the biological control agent.

However, further tests of these antagonistic *Bacillus* species, on sclerotia of *S. sclerotiorum*, showed that only two of the eight *Bacillus* species which showed antagonistic action on *S. sclerotiorum* mycelium, can inhibit sclerotia viability. These two
were *B. marisflavi* (B3) and *B. pumilis* (B4). Sclerotia are the resting structure of *S. sclerotiorum*, and any mechanism that suppresses the sclerotia of *S. sclerotiorum* is an effective way of controlling the disease. From these findings, it can be suggested that *B. pumilis* (B4) and *B. marisflavi* (B3) are effective bio-control agents of the plant pathogen *S. sclerotiorum*.

As for the fungal soil isolated microbes, six fungi, namely, *Aspergillus insuetus* (F), *Penicillium citrinum* (D), *Fusarium equiseti* (E), *Fusarium chlamydosporum* (C), *Fusarium solani* (I) and *Aspergillus niger* (H), effectively inhibited mycelium growth of *S. sclerotiorum in vitro*. However the effects of these six antagonistic fungi on sclerotia of *S. sclerotiorum* need further to be researched on, since the sclerotia viability test did not give enough evidence on the effects of the six fungi on sclerotia of *S. sclerotiorum*.

The results of this study have shown that biological control of *S. sclerotiorum* with soil isolated microorganisms is possible. Two bacterial strains, namely, *B. marisflavi* (B3) and *B. pumilis* (B4), have proven to be effective in inhibiting both mycelia and sclerotia of *S. sclerotiorum*. Therefore *B. marisflavi* (B3) and *B. pumilis* (B4) are promising alternative products in the inhibition of the plant pathogen *S. sclerotiorum*. 
RECOMMENDATIONS

- It is of paramount importance to test the effects of the fungal strains (antagonistic to *S. sclerotiorum* mycelium), on sclerotia of *S. sclerotiorum*.

- It would be of great value if the observed microorganisms, that showed antagonistic action to *S. sclerotiorum in vitro*, would be tested in actual field environment (*in vivo*).

- From an environmental point of view, understanding the different mechanisms such as direct antagonism or induced systemic resistance employed by these potential bio-control bacteria would give more options to consider and integrate them into a disease management program for the plant pathogen *S. sclerotiorum*. 
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APPENDICES

The ITS sequencing shown in Appendix A to G was done at the Stellenbosch University by Dr Karin Jacobs.

APPENDIX A

Sclerotinia sclerotiorum microscopic identification

![Image of microscopic identification]

ITS sequence:

5’-GAAGGGTGAGACCTCACCCTTTGTTGACTTTTCCTTTGCTTTGGCGAGCTGCTCTTCGGGGCCTTGGATGCTCGCCAGAGAATATCAAAACTCTTTTATTAAATGTCGTCTGAGTACTATAATAATAGTTAAAAACTTTCAACAA CGGATCTCTTGTTCTGTGCATCGAAGAACGCACAGGAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCTCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAG CTCACTCTGGTATTGGGACTCATGAGTAGTATTGGACCCAGGGTCCATTTGATTGCAGGTTTGGGCCGCTGGGTCCTGAACGTAGTAAATCTCTCGTTACAGGTTCCTTCGGGTGGCTTCTGCCAAAACCCAAATTTTCTATGGTTGACCTCGGATCAGGTAGGGATACCGCCTGAACCTTAAGCATATCAATAAGCGGAGA-3’
**Fusarium chlamydosporum (C) microscopic identification**

5'-CTCCCAACCCCTGTGAACATACCTATACGTTGCTCGCGATCAGCCCGGCCTCCGCAAAAAGGGAC
GGCCGGCCGGAGGACCCCTAAACTCTGTTTTTAGTGGAACCTCTGAGTAAAACAAATCAAAACTTTCAAC
AAGGGATCTCTTGTGTCTGTCGATGAAAGACGTACCTTATCTTACGAGAACAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGA
TCATCAGATCTTTGAAAGCAGACATTTGCGGCCGCCATCTCTGTCGAGCAGTCAATTTCTCAACCTCTCA
AGCTCAGCTGTTGTTGGGACTCGCGGTACCCGGTCTCCAAAATGATTGGCGGTCACGTCGACGTCTAGCGT
AGTAATCTGTTTAATGGGCTGCAGCCCGCAACGGCCTGAAAACCAACTTCTGAAATGTGGACCTCGGATC
AGGTAGGAATTACCGCGCTGAATTAAACCATATCAATAAGCGGAGGA-3'
APPENDIX C

Penicillium citrinum (D) microscopic identification

ITS sequence

5'-TCGGGGCCCAACCTCACCCGTGTTGCGGAACCTATGTTGCTCGGCGGGCCGGCCGGCCGGCCGGACGGCCCCCCTGAACGCTGTCTGAAGTTGCAGTCTGAGACCTATAACGAAATTAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCCCTCTCTTGATTTCCGGAGGGCATGCCTGTCGAGCGTCATTGCTGCCCTCAAGCCCGGTGTGTTGGGCCCCGTCCCCCCCGCCGGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCGGTCCTCGAGCGTGTGGGGCTTCGTCACCCGCTCTAGTAGGCCCGGCCGGCGCCAGCCGACCCCCAACCTTTAATTATCTCAGGTTGACCTCGGATAGGATAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAA-3'}
APPENDIX D

_Fusarium equiseti_ (E) microscopic identification

**ITS sequence**

5'-CTCCCAACCCCTGTGACATACCTATACGTTGCGCTCGCGGATCAGCCCGCGCCCCGTAAAAGGGACGGGCCCGCCCGAGGACCCCTAAACTCTGTTTTAGTGGAACTTCTGAGTAAAACAAACAAATAAATCAAATTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAACCTTGTTGGGACTCGCGGTAACCCGCGTTCCCCAATCGATTGGCGGTCACGTCGAGCTTCCATAGCGTGTAATCATACACCCCTGTACTGTGTAATCCTGCGGCCACGCCGTAAAACCCCAACTTCTGAATGTTGACCTCGGATCGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA-3'
APPENDIX E

Aspergillus insuetus (F) microscopic identification

ITS sequence

5’-TCTGCCCGCCGGCAGCGCTAACCCTCCACCCCGTGAATACTGACCAACGTGCTTCGGCGGTGCGCCC
CCCGGCGGTTGACGCCCGGGAGACCAACCGGAACCTCCTGCTTTAAGTTGTTGCTAGCTTTGATAGCAAAACCTATTTAA
ACTTTCAACAGGATCTTCTTTGGCTGCCGCACTGATGAAAGCAGACACGAACCTCCTGAGTAAAGTAATGTGAATTGCAAAAG
TTCAGTGAAATCTCAGTCTTTTTGAACGCAGACCTCGCATTCTCCTGCCAAGGGCCGCTGTGCAGCGTACTGTCCTCCGGC
CTGGCCCTTACGCCAGCTTGTGTCGTCGTCGTCGTCCCGGGGACGGGGACCGGCGCCGACCCGC
GTCCGGTCTCTGAGCGTATGGGCTTTTGTATTACCAGGTCTCAGATACCAGGGATACCGCAGTGAAGTAAC-3’
APPENDIX F

Aspergillus niger (H) microscopic identification

ITS sequence

5'-CTTTGGGCCACCTCCCATCCGTTATTTGATACCCCTGTGCTTTTGCGGCGCCCGCCGGGTGGCCGGCCGGGGGGCGCCTCTGCCGGGGGGCGCCCGGGGGCCCGTGCCCGCCGGAGACCCCAACACGAACACTGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAATCAGTTAAAACTTTCAACAAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAA TGCGATAACTAAATGTGAATTTACATGAAATTCATCGATCGTTTTAAGACATGCGGCCCCCCTGGTATTCCGGGGGGCATGCCTGAGCGTCATTGCTGCCCTCAAGCCGGCTTGTGTGTTGGGTCGCCGTCCCCCTCTCCGGGGGCACGGGCCACCCCGCAGCCCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACATGCTCTGTAGGATTGGCCGGCGCCTGCCGACKTTTTCAACCATTTTTCWGGTTGACCCTGAGTACGGGATACCACCTGGCTGAACTT-3'
APPENDIX G

Fusarium solani (I) microscopic identification

ITS sequence

5'-CACTCATCAACCTGTGAACATACTAACCCTGATATGCTGTGCCGCGGGAAGACGCGGCCCGTAACACCGGGCCGCCCCCGCCAGAGGACCCCCTAACTCTGTTTCTTTAATATTTTTCTCTGAGTAAACAAGAAATTAATTTAAAACATTTCAACACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACAACCCTAGGCCCCGCCTCCCTGCGGGAATGCACCCCGCGTCCCGCCCCGCAGCTTCCATTGCGTAGTAGCTAACACCTCGCAACTGGAGAGCGGCGCGGCCACGCCGTAAAACACCCAACCTCTGAATGTTGACCTCGAATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA-3'
**APPENDIX H**

ANOVA table of the interaction between *S. sclerotiorum* mycelium growth and bacterial treatment

<table>
<thead>
<tr>
<th>Effect</th>
<th>SS</th>
<th>Degr. of Freedom</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>4046.709</td>
<td>1</td>
<td>4046.709</td>
<td>43031.03</td>
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<tr>
<td>Test bacteria</td>
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<td>19</td>
<td>67.957</td>
<td>722.63</td>
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<td>Error</td>
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<td>40</td>
<td>0.094</td>
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<tr>
<td>GROWTH</td>
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<td>3</td>
<td>196.511</td>
<td>2603.28</td>
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<td>GROWTH*Test bacteria</td>
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**APPENDIX I**

ANOVA table of the interaction between *S. sclerotiorum* mycelium growth and fungal treatment

<table>
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<tr>
<th>Effect</th>
<th>SS</th>
<th>Degr. of Freedom</th>
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<th>F</th>
<th>p</th>
</tr>
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<tr>
<td>Label</td>
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<td>20.875</td>
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<td>GROWTH</td>
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<td>29.192</td>
<td>313.406</td>
<td>0.000000</td>
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<td>GROWTH*Label</td>
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<td>2.949</td>
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<td>Error</td>
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<td>0.093</td>
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</table>
### APPENDIX J

ANOVA table of the number of sclerotia formed three weeks after inoculation in dual plate with *S. sclerotiorum* and test bacteria

<table>
<thead>
<tr>
<th>Effect</th>
<th>SS</th>
<th>Degr. of Freedom</th>
<th>MS</th>
<th>F</th>
<th>p</th>
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</thead>
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<tr>
<td>Intercept</td>
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### APPENDIX K

ANOVA table of the number of sclerotia formed three weeks after inoculation in dual plate with *S. sclerotiorum* and test fungi

<table>
<thead>
<tr>
<th>Variable</th>
<th>Analysis of Variance (N scler 3 weeks.sta)</th>
<th>Marked effects are significant at p &lt; .05000</th>
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<td>SS Effect</td>
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<tr>
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</table>
APPENDIX L

Bacterial identification phylogenetic tree done at Stellenbosch University by Dr Karin Jacobs
APPENDIX M

Fungal identification phylogenetic tree done at Stellenbosch University by Dr Karin Jacobs

NMMU2 (C) Fusarium chlamydosporum
NMMU4 (E) Fusarium equiseti
AB425996 Fusarium equiseti
EU326202 Fusarium equiseti

EF611087 Fusarium equiseti
AB369435 Fusarium chlamydosporum

EU314961 Fusarium incarnatum
EU214561 Fusarium chlamydosporum
EU714391 Gibberella moniliforme

NMMU7 (I) Fusarium solani
EU126189 Fusarium solani
EU625405 Fusarium solani
EU442586 Fusarium oxysporum
EF556218 Nectria haematococca

NMMU1 (Se) Sclerotinia sclerotiorum
EU082466 Sclerotinia sclerotiorum
EF091809 Sclerotinia sclerotiorum

Z99672 Sclerotinia tetraspore
EF153017 Monilinia laxa
AF455503 Myriosclerotinia cibo
AF067644 Sclerotinia borealis

NMMU3 (D) Penicillium citrum
EF634428 P. citrinum
EF634427 P. citrinum
AF033423 P. westlingii
AF033421 P. sartory

EF652481 A. minutes; AY373876 A. ustus
EF652432 A. insuetus
EF652507 A. pseudodeflectus, NMMU5 Aspergillus insuetus

EF652500 E. herterothallica
EF652498 A. puniceus, EF652469 A. puniceus
EF652430 A. granulosus

NMMU6 (H) Aspergillus niger, FJ195350 Aspergillus niger, EF661189 A. niger, AY373852 A. niger

EF567948 A. niger