Biological control of head rot and damping-off of sunflower using yeasts, *Bacillus* spp. and *Trichoderma* spp.

by

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DECLARATION

The experimental work presented in this thesis was carried out in the School of Agriculture, Earth and Environmental Sciences, University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Professor Mark D. Laing and Dr Kwasi Sackey Yobo.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others it is duly acknowledged in the text.

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DISSERTATION ABSTRACT

The soil-borne fungi *Sclerotinia sclerotiorum*, *Sclerotium rolfsii* and *Rhizoctonia solani* are ubiquitous plant pathogens with a wide host range. They are among the most widespread pathogens, and cause destructive diseases of many crops, including sunflower (*Helianthus annus* L.), an economically important oilseed crop. Chemical fungicides are available for control of seedling damping-off caused by these pathogens, but there are environmental concerns and the possibility of developing tolerance in pathogen populations, which have led to a drastic reduction in their usage and increased the pressure to find alternative means of disease control. Additionally, there are no registered fungicides that effectively control *Sclerotinia* head rot of sunflower caused by *S. sclerotiorum* in South Africa.

Successes in biological control (biocontrol) and plant growth promotion research have led to the development of various *Trichoderma* and *Bacillus* products, which are available commercially. This study was conducted to evaluate the effect of three strains of *Bacillus* spp., one yeast and one commercial strain of *Trichoderma* spp., and their respective combinations, on *Sclerotinia* head rot on sunflower. An additional commercial strain of *Trichoderma* spp. was also evaluated for the control of damping-off. *In vitro* biological control and growth promotion studies were carried out under greenhouse conditions with the use of foliar spray treatment as the method of application for head rot, and seed and soil drench treatments for damping-off.

In vitro screening was undertaken to select the best *Bacillus* and yeast isolates from 136 *Bacillus* spp. and 100 yeasts isolated from local wild sunflower heads. Dual-culture bioassays were undertaken and isolates were assessed for antagonism by examining the radial growth of *S. sclerotiorum* mycelium. A scale was used to group the isolates, based on their inhibition ability in order to select the best isolates to screen *in vivo*. Seventeen *Bacillus* isolates achieved a Class 3 rating (≥70% inhibition of pathogen mycelial growth), while only 4 yeast isolates achieved a Class 2 rating (41-69% inhibition). The isolates, along with *T. atroviride* strain 77 (T77), were further screened *in vivo* under greenhouse conditions for antagonistic activity against *Sclerotinia* head rot of sunflower cv. PAN7080 plants, when plants were at the R6 reproductive stage.

Disease incidence was recorded 14 days after inoculation with BCAs and *S. sclerotiorum*, and grain was harvested, dried and weighed 85-115 days after planting. A total of 20 yeast and *Bacillus* isolates were screened against *S. sclerotiorum* and 4 *Bacillus* isolates and 1 yeast isolate reduced disease incidence by ≥50%, compared to the disease control. *Bacillus* B16 resulted in complete disease suppression, followed by B24, B26 and T77, which reduced disease incidence to 12.5%. Seven of the 20 yeast and *Bacillus* isolates, along with T77, significantly improved grain yield. B16 resulted in the highest grain yield, followed by T77.

The effect of inoculum concentration was evaluated for the best performing yeast and *Bacillus* spp. isolates. A concentration of 1 x 10^8 cells mL^{-1} for yeast Y79, and 1 x 10^9 cfu mL^{-1} for B16, B24 and B26 caused the greatest disease suppression and improvement in grain yield. In comparison to the *Bacillus* isolates, Y79 was the poorest performing biocontrol agent (BCA), reducing the incidence of head rot the least. In addition, it was not as effective at improving grain yield and failed to perform consistently between the first, second and third greenhouse screening.

Sunflower heads treated with single and combined inoculations of T77, Y79 and B16, B24 and B26 exhibited improved grain yield. Combined inoculations of B16 + B26 and B26 + B24 provided over 10.0% increase in grain yield (12.8% and 15.5%, respectively) over the disease-free control. Y79, when inoculated in combination with B16 and B24, scored reduced disease incidences of 62.5% and 37.5% as well as improved grain yields of 15.8 g and 36.0 g, respectively.

In vitro dual-culture assays carried out with *T. asperellum* strain kd (Tkd) showed effective antibiosis activity and marked mycoparasitism of *S. sclerotiorum*, *R. solani* and *S. rolfsii*, despite the BCA performing poorly according to the Bell rating scale in dual culture plates. Greenhouse trials were carried out in Speedling 24® trays, and Tkd was applied as a seed treatment alone and/or a monthly-bimonthly soil drench. Various other greenhouse trials were set up to evaluate the potential of Tkd to suppress damping-off of sunflower caused by the three pathogens, and several growth parameters were measured.

Seed treatment in combination with a monthly or bimonthly soil drench significantly increased seedling, shoot, root and head dry weight, along with root area, when tested against all three pathogens- effectively reducing disease incidence. Reduced disease

incidence and enhanced seedling and plant growth were also achieved when Tkd was applied as a seed treatment alone, drench at planting alone, and drench at planting + bimonthly drench, but at lower levels.

A number of methods were adapted from studies carried out in other parts of the world with the objective of finding a fast and reliable method of inducing sclerotia of *S. sclerotiorum* to germinate carpogenically and produce ascospores. However, none of the published techniques worked under the conditions tested. Only one method, adapted from a study conducted by a fellow South African researcher, resulted in stipe formation, but not in ascospore production. The failure of these published techniques to work under the local conditions may be attributed to the fungus having stringent requirements for environmental conditioning before it will sporulate carpogenically. It appears that these requirements vary with the geographic source of the sclerotia, and that effective conditioning parameters in one place may not work in other geographic locations.

The results presented in this dissertation confirm the concept of biological control by *Trichoderma* spp. and *Bacillus* spp. as a viable disease control strategy to manage *S. sclerotiorum* of sunflower. Furthermore, this dissertation forms a basis for further *Trichoderma*-*Bacillus*-Yeast interaction studies to determine whether strains of these three organisms could be combined to enhance biocontrol and plant growth promotion.

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Finally, I would like to thank God and my ancestors for seeing me through this study.

DEDICATION

To my dearest Mother, for your never-ending love and support.

And to myself, for not giving up.

INTRODUCTION

Sclerotinia head rot is a global disease of sunflower (*Helianthus annuus* L.) caused by the fungus *Sclerotinia sclerotiorum* (Lib.) de Bary that leads to major yield losses. In addition to yield losses, head rot can also affect the quality of seed by reducing the oil content in the seed by 10 to 15%, and increasing the content of free fatty acids, leading to rancidity of the oil (Gulya *et al*., 2019). Currently, there are three fungicide groups registered on sunflower for use against these diseases, but the efficacy data are not available, or are not consistent across years and/or locations (Seiler *et al*., 2017).

To a lesser extent, damping-off of sunflower seed and seedlings caused by *S. sclerotiorum,* and the fungi *Rhizoctonia solani* Kuhn and *Sclerotium rolfsii* Sacc. also lead to significant economic losses to local and international growers, and are difficult to control. Although fungicides are available, widespread use of fungicides has failed to eliminate damping-off caused by these pathogens. Furthermore, these fungicides are subject of public concern due to the harmful effect they have on non-target organisms and consumers. The current research was motivated by the need to find non-chemical approaches to control the diseases caused by these pathogens on sunflower under greenhouse conditions using yeasts, *Bacillus* spp. and two *Trichoderma* isolates (Eco-T® and Eco-77®).

The overall aim of this research was to isolate and investigate the efficacy of newly isolated strains of yeasts and *Bacillus* spp, as well as commercial strains of *Trichoderma* spp., and their combinations, to manage infection of sunflower heads and seedlings by *S. sclerotiorum*, *S. rolfsii* and *R. solani*.

To achieve these aims, the following approaches were taken:

1. Review available literature on the importance, survival, germination, infection and management mainly of *Sclerotinia sclerotiorum*, briefly on damping-off caused by *S. sclerotiorum*, *S. rolfsii* and *R. solani*, and on the potential of using yeasts, *Bacillus* spp, and *Trichoderma* spp. for biological control (Chapter 1).
2. Isolate and screen yeasts and *Bacillus* spp. for biological control activity against *S. sclerotiorum* in *vitro* (Chapter 2).

3. Document the failure of published techniques to induce carpogenic germination of the sclerotia of *S. sclerotiorum* to produce ascospores for use in *in vivo* screening of isolated yeasts and *Bacillus* spp. (Chapter 3).
4. Screen isolated strains of yeast and *Bacillus* spp. and a commercial strain of *Trichoderma atroviride in vivo,* and evaluate the effects of single and dual inoculations of these for biological control of *S. sclerotiorum* head rot of sunflower (Chapter 4).
5. Evaluate the potential of a commercial strain of *Trichoderma asperellum* to control damping-off of sunflower caused by *S. sclerotiorum*, *S. rolfsii* and *R. solani in vitro* and *in vivo*, and to investigate the effect of application method and frequency on the level of biological control (Chapter 5).

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CHAPTER 1

Literature Review

1.1 Introduction

Sunflower (*Helianthus annuus* L.) is widely used in the production of cooking oil and can be eaten as a seed, in addition to being grown as an ornamental plant. Ukraine is presently the largest sunflower producer globally. Sunflower is the fourth largest grain crop produced in South Africa (SA) after maize, wheat and soybeans and it's production is well suited for South African climatic conditions (SAGL, 2014). Sunflower is a valuable source of vegetable oil in SA, with production being most prevalent in the summer rainfall areas. Production for sunflower seed in SA ranges between 500 000 to 700 000 tons (DAFF, 2010). However, the crop is susceptible to several diseases, the most serious being those caused by fungi. Stalk and head rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is one of the most devastating diseases around the world. Over 408 species and 278 genera of plants have been reported to be hosts of *S. sclerotiorum* (Boland and Hall, 1994), which persists in infested soils for several years in resting structures called sclerotia. Those structures can infect plants through myceliogenic and carpogenic processes (Bolton *et al*., 2006).

More than sixty different names have been used to refer to diseases caused by *S. sclerotiorum* (Purdy, 1979). These include cottony rot, watery soft rot, stem rot, drop, crown rot, blossom blight and most commonly, white mold. Widespread crop damage, lack of high levels of host resistance and the general difficulty of managing diseases caused by *S. sclerotiorum* have been the impetus for sustained research on this pathogen (Bolton *et al*., 2006). Although it also has a broad ecological distribution, it is prevalent mostly in temperate regions. The first report of *S. sclerotiorum* infecting sunflower was made in 1861, causing root, steam and head rot (Gulya *et al*., 1997).

Biological control refers to the use of microorganisms to control (or antagonize) other microorganisms or plant pathogens. Biocontrol provides an alternative to chemicals in crop protection programmes. Many studies have shown that filamentous fungi and yeasts that inhabit plant surfaces have the potential to suppress plant diseases (Zhou and Reeleder, 1989; Madrigal *et al*., 1994; Petersson and Schnurer, 1998; Helbig, 2002; Calvo *et al*., 2003; Lima *et al*., 2003). The fungus *Trichoderma* is amongst the

most commonly studied biocontrol microbes (Altomare *et al*., 1999). It is an important biocontrol agent (BCA) of several soil borne phytopathogens. Their high reproductive capacity, rhizospheric modification ability, aggressiveness towards phytopathogenic fungi, and efficiency in promoting plant growth and disease defence mechanisms are some of the attributes that make *Trichoderma* strains successful BCAs (Benitez *et al*., 2004).

Among the antagonistic microorganisms, natural yeasts have been widely used as BCAs (Zhimo *et al*., 2014). Yeasts are particularly attractive as biological control agents as they are widely used for various food and industrial purposes and thus considerable information is available with regards to genetic manipulation techniques, production and yeast cell storage (Hofstein *et al*., 1994; Hamilton *et al*., 2003). Possessing numerous important properties, yeasts are ideal candidates to be used for biocontrol purposes: they do not produce allergenic spores or mycotoxin, unlike many mycelial fungi, or antibiotics, often produced by bacterial antagonists (El-Tarabily and Sivasithamparam, 2006).

Bacillus species' ability to colonize plant rhizosphere, suppress competing phytopathogenic bacteria and fungi and stimulate plant growth make them ideal for use in biocontrol (Qiao *et al*., 2014). Antagonistically important *Bacillus* species are increasing very rapidly in terms of use and number. *Bacillus* species are unique in their rapid replicative ability, tolerance and resistance to adverse environmental conditions and boast a broad spectrum of biocontrol ability. The genus has become attractive biological control agents due to their ability to produce antibiotics that control a broad range of plant pathogens, and to survive as tough endospores (Cavaglieri *et al*., 2005). Some *Bacillus* species also produce volatile compounds which play an integral role in plant growth promotion and plant defence mechanism activation by triggering induced systemic resistance (ISR) in plants (Compant *et al*., 2005).

The aim of this review is to describe the economically important pathogen *S. sclerotiorum*, in addition to the soil-borne pathogens *Sclerotium rolfsii* Sacc. and *Rhizoctonia solani* Kühn which cause damping-off of sunflower. The potential of *Trichoderma*, yeast and *Bacillus s*pp. to prevent/reduce yield losses caused by these pathogens on sunflower is also discussed.

1.2 The sunflower crop

1.2.1 Taxonomy and botany

The genus *Helianthus* is in the tribe Heliantheae of the Compositae family and is made up of both annual and perennial species. Commonly known as sunflower, the cultivated species *H. annuus* has close wild species relatives. The genus *Helianthus* contains approximately fifty species, mostly native to North America; many are indigenous to the Rocky Mountains, others to tropical America, while a few species are found in Peru and Chile.

Sunflower is an annual herb with a rough, hairy stem that grows 1-4 m high. The leaves are broad, coarsely toothed, rough and 80-300 mm in length. They have circular heads of flowers, 80-150 mm wide in wild specimens and often 300 mm or more in cultivated specimens. The flower heads are made up of numerous small tubular flowers, organized compactly on a flattish disk. The outer row flowers have long strap-shaped corollas which form the rays of the composite flower.

1.2.2 Agronomy

In SA, sunflower derives most of its economic value from the extracted oil, with the remaining value from the meal. The achenes of oilseed sunflower are usually black. Of the total value of the oilseed sunflower crop, the oil extracted from the achenes accounts for about 80% (Fick and Miller, 1997).

When selecting a cultivar, factors such as yield, maturity, oil percentage, seed size (for non-oilseed markets), bird resistance and lodging should be considered. Growers are inclined to choose cultivars which best are suited their area of production A summary of the agronomic requirements of sunflower are shown in Table 1.1.

Table 1.1 Agronomic requirements of sunflower (DAFF, 2010)

Agronomic Feature	Requirement
Temperature	Tolerant to low and high temperatures but more so to low temperatures; Seed germination: 14 to 21°C; Plant growth: 23 to 34°C (optimum is 23 to 28°C)
Rainfall	Rainfall requirement ranges from 500-1000 mm
Soil requirements	Grows in a wide range of fertile soil types; Optimum growth in sandy loam to clays with pH 6.0-7.5; Good soil drainage is required
Planting density	25 000-35 000 plants per hectare (depends on the yield potential of the area); Row width ranges from 90-100 cm; In-row spacing is approximately 30 cm
Planting depth	In predominantly clay soils, seeds are planted 25 mm below the soil surface; In predomantly sandy soils, seeds can be planted up to 50 mm deep
Fertilisation	50 to 100 kg/ha of a 3:2:1 (nitrogen: phosphorus: potassium) (25) fertiliser mixture applied at planting is sufficient for a 1000-1 500 kg/ha yield potential
Planting date	In South Africa, planting in the eastern areas takes place from early November to the end of December and from early November to mid-January in the western areas
Herbicides	The following are some of the commonly used herbicides: AIM (carfentrazone-ethyl)- Targets small weeds ; Assert (imazamethabenz)- Tagets wild mustard; Assure II and Targa (Quizalofop)- Targets annual grasses and quackgrass; BroadAxe (sulfentrazone)- Targets kochia, other broadleaves and grasses like foxtail; Dual MAGNUM- Targets grasses and some broad leaf weeds; Eptam 7E (S-ethyl dipropylthiocarbamate)- Targets annual grasses and annual broadleaf weeds; Glyphosate- Targets emerged grass and broadleaf; Paraquat- Targets emerged annual grasses and broadleaf weeds

1.2.3 Economic importance

Sunflower is the most economically important oilseed crop in South Africa. Sunflower seed production measured nearly 681 000 tonnes in 2018/2019 in comparison to 859 000 tonnes produced during 2017/18. Average yield was 1.32 t/ha in 2018/19. Production is concentrated in the Free State (FS) and North West (NW) provinces, together accounting for roughly 80% of the farmland planted to sunflower (South Africa Online, 2021). The FS and NW provinces produced the highest grain yields of 1.3 t/ha and 1.2 t/ha, respectively, during the 2017/2018 season. (Table 1.2).

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

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

Historically, sunflower in SA has been grown as an alternative to maize in cases where biotic and abiotic constraints e.g. drought, have hampered maize crop production or as part of a crop rotation system (Schoeman, 2003). According to The Bureau for Food and Agricultural Policy (BFAP) Baseline, Agricultural Outlook 2014 – 2023, sunflower yields can be expected to increase gradually over time to reach a national average of almost 1.6 tons per hectare over a ten-year period (SAGL, 2014).

1.3 The pathogens

1.3.1 *Sclerotinia* head rot

a) Taxonomy and morphology

Sclerotinia head 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).

Two 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). Sclerotial germination may be myceliogenic or carpogenic, where sporocarps in the form of apothecia producing asci and ascospores develop (Willets and Wong, 1980). Hence, the various structures produced by *S. sclerotiorum* are sclerotia, mycelia and ascospores (Grau and Hartman, 1999).

Sclerotia can be defined as multi-cellular, asexual (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 (Fig 1.1), are formed by the aggregation of mycelia (Grau, 1988) and appear as hard, black and irregularly shaped structures (McGee, 1992).

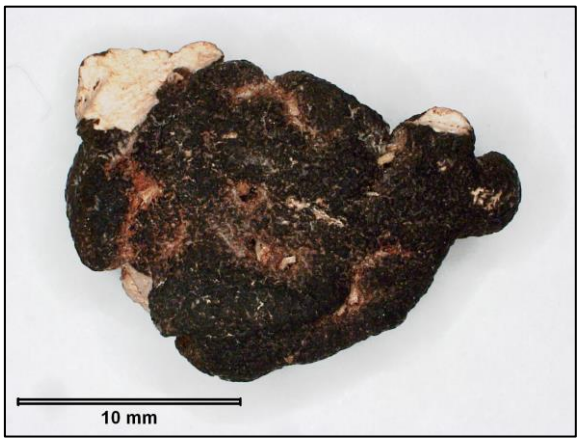

Fig 1.1 Vegetative sclerotium of *Sclerotinia sclerotiorum* (Visser, 2007).

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

1988). Mycelia (Fig 1.2) from sclerotia placed on potato dextrose agar (PDA) are typically white or pale grey (Mordue and Holliday, 1976).

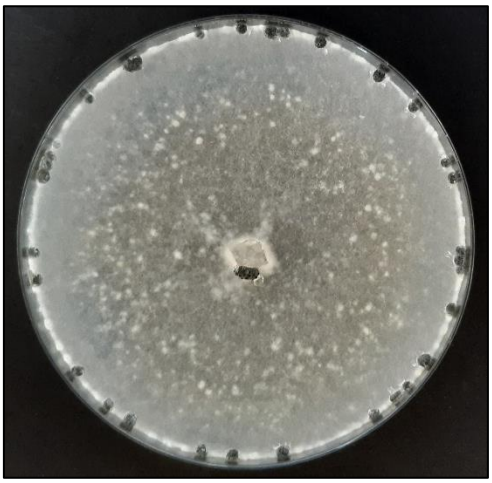

Fig 1.2 Mycelium of *Sclerotinia sclerotiorum* on Potato dextrose agar. Black sclerotia can also been seen on the edges of the plate after 7 days of incubation at 25°C (Picture A.J. Moody, 2019).

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

Fig 1.3 Fully developed apothecia from germinated sclerotia of *Sclerotinia sclerotiorum*, from which ascospore dispersal may now occur (Visser, 2007).

b) Epidemiology

The vast reproductive potential in combination with capability for long-term survival makes sclerotia chief components in the epidemiology of *S. sclerotiorum* diseases (Bolton *et al*., 2006). Sclerotia of *S. sclerotiorum* remain viable in the soil for many years. They imbibe moisture from moist soil and this leads to germination of the sclerotia. Sclerotia may germinate directly and produce mycelium (myceliogenic germination) (Fernando *et al*., 2004). Hyphal germination occurs when soil is cool and wet. Near-saturated wetness with soil temperatures of 12-24°C are optimal for hyphal germination. Plant infection following this type of germination is favoured by similar soil conditions, and is restricted to roots, crowns and other plant parts located within 1-2 cm of the sclerotia (APS, 2012). These conditions are created by prolonged rainy periods, or by irrigation events in combination with soil shading due to closure of the crop canopy (Agrios, 1988).

Sclerotia can also germinate to produce apothecia (carpogenic germination) (Fernando *et al*., 2004). Rapid apothecial development occurs when soils are saturated and temperatures are in the range of 10-20°C (Abawi and Grogan, 1975). These subsequently produce ascospores which infect aerial tissues of plants. Most of the diseases caused by this pathogen are initiated by ascospores (Schwartz and Steadman, 1978; Abawi and Grogan, 1979; Steadman, 1979).

c) Life cycle and symptoms

Sclerotia that germinate carpogenically produce apothecia which in turn produce ascospores, which infect aerial portions of the plant (Figs 1.4-1.5). Myceliogenic germination produces hyphae which are septate, hyaline, branched and multinucleate. The mycelium is white to tan in colour. The fungus does not produce asexual conidia. Once established on the host, the fungus secretes oxalic acid and acidic lytic enzymes which are released by the advancing mycelium (Bolland and Hall, 1998).

Most ascospores remain within the field where they are produced (Wegulo *et al*., 2000) although it is common for some to be carried to neighboring fields in air currents (Li *et al*., 1994). The sticky mucilage with which ascospores are coated enables adhesion to the substrate on which they land. They are capable of surviving on plant tissue for roughly 2 weeks, but this depends on environmental conditions (Clarkson *et al*., 2003).

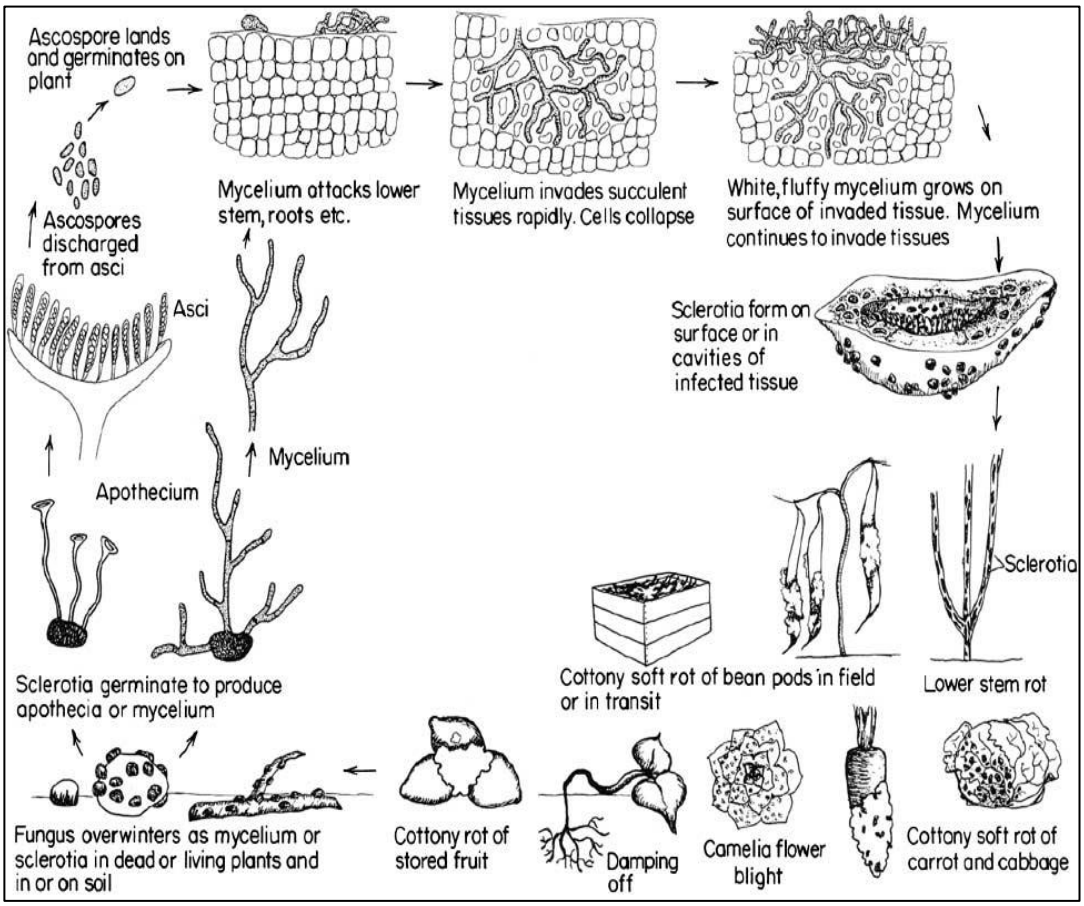

Fig 1.4 Development and symptoms of diseases of vegetables and flowers caused by *Sclerotinia sclerotiorum* (Agrios, 1988).

Ascospores may germinate on the surface of healthy plant tissue but infection will not occur without an exogenous nutrient source and moisture. Thus, senescing or dead plant tissue provide the nutrient source required to initiate ascosporic germination, subsequently leading to mycelial infection of the host plant (Abawi and Grogan, 1979; Lumsden, 1979; McLean, 1958). Flowering is a crucial host factor associated with most ascospores-initiated diseases. Senescing floral parts serve as the primary source of nutrients (Inglis and Boland, 1990; Turkington and Morrall, 1993). The time of canopy closure coincides with the flowering stage. As a result, sources of nutrients are available when environmental conditions are conducive for disease development (Bolton *et al*., 2006).

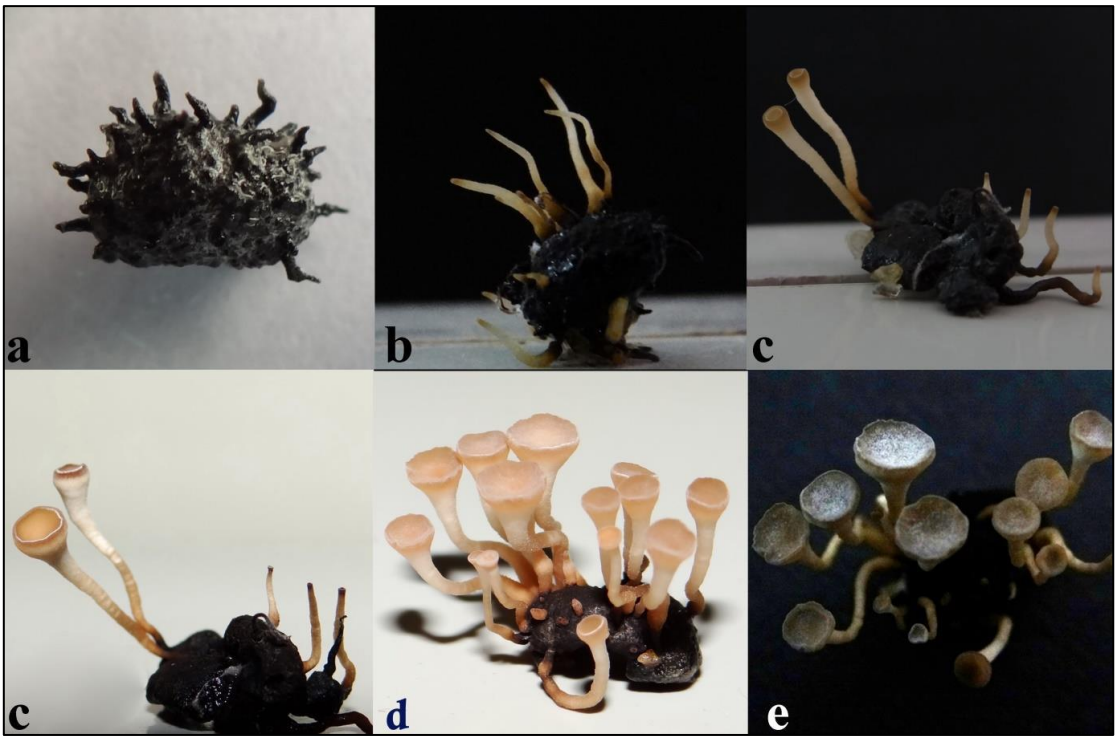

Fig 1.5 (a) Stipe initials arising from a sclerotium, (b) Melanization and elongation of stipe initials, (c) Disc differentiation initiation, (d) Growing disc, (e) Mature apothecia, (f) Apothecial disc containing ascospores (Vinodkumar *et al*., 2015).

Infection caused by myceliogenic germination seldomly takes place in most crops. Mycelium may directly attack susceptible root tissues of certain crops. When sclerotia germinate myceliogenically, mycelia are produced which can directly penetrate plant tissue (Le Tourneau, 1979). In sunflower, infection is often initiated in the roots and progresses upwards into the stem thereafter. Sclerotia are the primary long-term inoculum in the development of *Sclerotinia* head rot of sunflower. Thus, the amount of sclerotia in the soil is directly proportional to disease incidence (Holley and Nelson, 1986). Development of mycelia often continues even after certain vegetables have been harvested, resulting in storage rot (Lumsden, 1979).

While host species express symptoms differently, there are a number of similarities. Common symptoms include water-soaked, irregularly-shaped spots on fruits, stems, leaves, or petioles (Agrios, 1988). The spots expand and the affected area becomes covered with a cottony mycelium. As the fungus spreads, plant tissue becomes a soft, slimy, water-soaked mass (Ferreira and Boley, 1992). In sunflower, diseases occur in

different phases as root rot, stem rot, and head rot. The taproot and fibrous roots also show the water-soaked lesions.

The back of the head becomes infected, causing it to become soft, light brown and spongy. The infection penetrates the developing head (Fig 1.6- Photo 1) and develops within the stalk until eventually only the fibrous strands at the back of the head and upper stalk remain (Fig 1.6- Photo 2). Infected seed becomes too heavy and eventually falls out of the head (Fig 1.7- Photo 3). Large, black sclerotia develop below the seed layer and around the seeds (Fig 1.7- Photo 4).

Photo 1: Sclerotinia infection extending into developing sunflower head. Photo: André Nel, ARC-GCI

Photo 2: Fibrous strands at upper end of stalk and head as a result of Sclerotinia head rot damage.

Fig 1.6 Sunflower heads infected with *Sclerotinia sclerotiorum* (GRAINSA, 2012).

Head and stem rot outbreaks occur after periods of rainy weather (Agrios, 1988). Head rot reduces the number, weight and oil content of seed. Seed grade and market value of the crop is reduced due to the presence of sclerotia in seed.

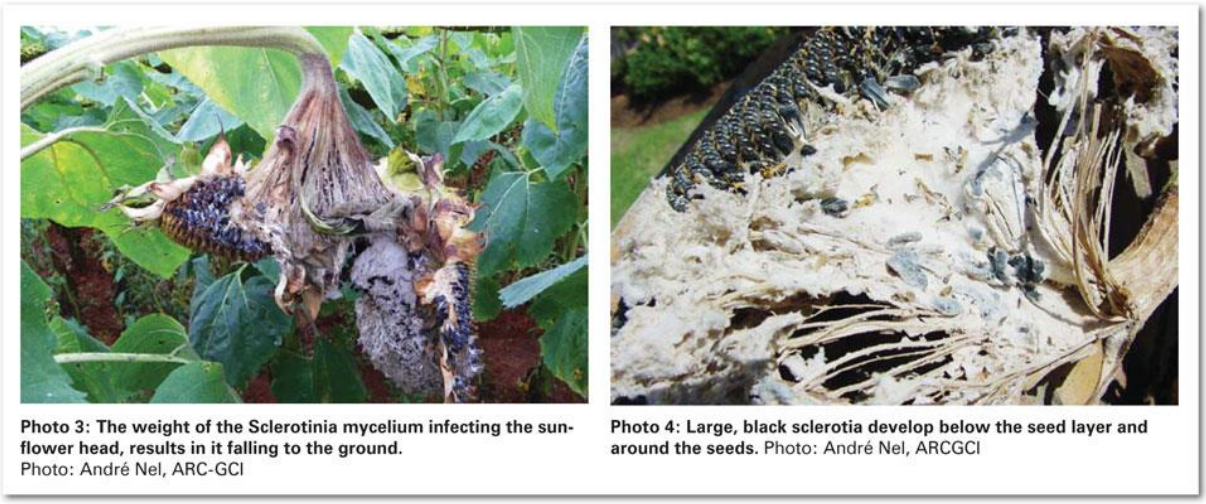

Photo 3: The weight of the Sclerotinia mycelium infecting the sunflower head, results in it falling to the ground. Photo: André Nel, ARC-GCI

Photo 4: Large, black sclerotia develop below the seed layer and around the seeds. Photo: André Nel, ARCGCI

Fig 1.7 Disintegrated sunflower head (left) and development of sclerotia below and around seeds (right) caused by *Sclerotinia sclerotiorum* (GRAINSA, 2012).

In the field, infected heads often disintegrate and this results in seed fall (Berlin and Arthur, 2000) (Fig 1.8). Despite the absence of toxins in contaminated seed, high levels of sclerotia in seed is deemed unacceptable for human or animal consumption (Berlin and Arthur, 2000). Additionally, sunflower seed containing sclerotia is likely to be rejected at foreign ports.

Fig 1.8 Left: A sunflower field infested with *Sclerotinia sclerotiorum* (The Western Producer, 2010); Right: Sunflower receptacle displaying shredded vascular tissues and many sclerotia (Bolton *et al*., 2006).

Sudden leaf wilting is also characteristic of the disease (Mukhtar, 2009). Grey to brown basal and stem lesions appear initially but as the disease progresses, the entire plant wilts and dries up. Stems shred into vascular strands and become straw coloured on

drying. In the case of head rot, white mycelial growth is observed. Under moist conditions, white, fluffy mycelium can also be found within or outside infected tissues. Mycelia aggregate into sclerotia that form within and outside the plant stem as nutrients are exhausted. Thereafter, the sclerotia fall to the ground where they may overwinter for 3-5 years (Schwartz and Steadman, 1978). Movement of sclerotia occurs between fields by natural or human-assisted movement of soil (Smith, 1988).

d) Disease management

Managing diseases caused by *Sclerotinia sclerotiorum* has been difficult. Lack of adequate levels of host resistance in major host crops has led to much damage (Bolton *et al*., 2006). As a result, the use of synthetic fungicides has been the main component in controlling diseases caused by this pathogen (Steadman, 1979; Bardin and Huang, 2001). Commercially, successful control has been achieved using synthetic fungicides on a small number of crops such as dry bean, soybean, oilseed rape and some vegetables (Twengstrom *et al*., 1998; Bailey *et al*., 2000; Budge and Whipps, 2001; Del Rio *et al*., 2004). However, the build-up of fungicide resistance is a concern (Gossen *et al*., 2001). The only fungicides commercially available to prevent infection by *S. sclerotiorum* on sunflower are those containing benomyl and procymidone (Rothmann and McLaren, 2018), none of which suppress sclerotial germination. To be effective, chemical fungicides are required to be applied multiple times at critical stages of growth, making chemical control costly and tedious (GRAINSA, 2016). Adequate spray coverage is also necessary and applications should be synchronized with the discharge of ascospores, which is difficult. This results in control being inconsistent. Additionally, heavy reliance on and intensive use of chemicals to control crop diseases has shown to have harmful effects on the environment. Therefore, cultural control methods have largely used to manage *S. sclerotiorum* diseases.

Zero-tillage, together with crop rotation, can reduce the risk of attack by the necrotrophic pathogen *S. sclerotiorum.* Sclerotia are found within the top 2-3 cm of soil (Davis, 1925; Tu, 1986). Carpogenic germination of sclerotia takes place in the upper 5 cm of the soil (Kurle *et al*., 2001; Duncan, 2003). Once the soil is ploughed, the sclerotia are buried deeper in the soil and can survive for several years. At minimum, a five-year rotation of two non-host crops of *S. sclerotiorum* is necessary to reduce infection levels. This is not, however, economically viable for growers.

S. sclerotiorum persists in infected seeds as dormant mycelia, and is capable of surviving in testae and cotyledons for longer than three years (Tu, 1998). Disease reduction has been shown with seed treatments using Captan and thiophanate-methyl (Tu, 1989).

Globally, *S. sclerotiorum* has been one of the most difficult fungal plant pathogens to manage. Since *S. sclerotiorum* has such a wide host range the current management techniques do not work well. Aside from sclerotia being able to persist in the soil for many years, carpogenic germination of sclerotia releases millions of ascospores. Senescing floral tissue provide nutrient sources, enabling ascospores to proliferate and establish pathogenicity. Hence, only managing sclerotia persisting in the soil is inadequate to control *S. sclerotiorum*. It is essential, therefore, to protect the infection court i.e. petals and leaves from ascosporic infection (Fernando *et al*., 2004).

1.3.2 Damping-off

Damping-off is a disease of seeds (pre-emergence damping-off) and seedlings (post-emergence damping-off or seedling blight) that can be caused by many fungi, primarily *Pythium s*pp. and *Fusarium* spp., but also *Penicillium* spp., *Rhizoctonia* spp., *Sclerotium* spp., *Aspergillus* spp., *Alternaria s*pp., and others. These fungi can be found in soils worldwide and are capable of infecting a wide range of crops. They survive between growing seasons as dormant resting structures (oospores, sclerotia), as saphrophytes in crop debris, and pathogenically on weeds and other hosts (Agrios, 1988).

Sclerotinia sclerotiorum, *Sclerotium rolfsii* and *Rhizoctonia solani* all cause this disease in sunflower and often result in significant yield loss. The soil-borne fungus *R. solani* causes damping-off both pre- and post-emergence, seed rot, stem canker, root rot, fruit decay and foliage diseases (Agrios, 1997). It is distributed globally and infects most plant families (Anderson, 1982; Ogoshi, 1987). Although growth and virulence are highly variable between individual isolates of *R. solani*, isolates can be grouped based on anastomosis- the process of hyphal fusion (Sneh *et al*., 1991). Like *R. solani, S. rolfsii* is soil-borne, distributed worldwide and has a wide host range. Crown and root rot, stem canker, damping-off and resulting diseases called southern wilt, blight or stem rot are some of the diseases caused by *S. rolfsii* (Punja, 1985).

In sunflowers, these pathogens invade and kill seed prior to or directly after germination. The decaying seed often serves as food for the fungi, which subsequently spread through the soil to infect adjacent seeds (Davis *et al*., 1997). In other cases, the seed may germinate, but the fungi infect and kill the seedling prior to emergence (Smith, 1988). The fungi also attack juvenile seedlings upon or post-emergence. Initially, roots may show elongated, water-soaked lesions 1-3 weeks after planting (Adams, 1988; Agrios, 1997). As most of the main root system is destroyed, the overall growth of the plant is diminished. The water-soaked lesions may progress above ground level to the stem and eventually dry out, appearing tan to brown in colour and somewhat sunken (Agrios, 1988; Smith, 1988).

Polygalacturonase enzymes produced by the fungi during post-emergence infection of young seedlings leads to tissue breakdown and weakening of stems which eventually collapse (Smith, 1988). Low quality and damaged seed (caused by other biotic and abiotic diseases) increases susceptibility to damping-off fungi (Smith, 1988).

1.4 Biological control agents (BCAs)

Since the 1990's, biological control of fungal diseases of crops has been shown to be a promising alternative to chemical control (Wisniewski and Wilson, 1992). Fungi, yeasts and bacteria have been studied and used to control plant pathogenic fungi, both biotrophic and neurotrophic. Biotrophic fungi e.g. powdery mildew fungi, only grow and reproduce on the living host plant (obligate parasites), whereas necrotrophs e.g. *Botrytis cinerea*, which causes gray mold disease, are opportunistic fungi that grow and reproduce on plant debris or organic matter but can rapidly invade wounded or senescing plant tissues (Punja and Utkhede, 2003). A thorough understanding of the disease cycle (beginning from the onset of infection to colonization and reproduction of *S. sclerotiorum*) and life cycle of *S. sclerotiorum* is crucial for any disease management strategy to be effective. The majority of plant pathogens affecting economically important crop species, such as the ones discussed in this review, have been studied in-depth and information on their biology is readily available (Agrios, 1997).

Successful use of fungi, yeasts and bacteria to manage crop diseases requires a disruption of one or more stages of the disease- or life cycle of the pathogen. Some of the mechanisms through which this has been achieved are: prevention of infection,

reduction in colonization of host tissues and reduction of sporulation and survival of the pathogen. Each of these may provide a level of disease control using biological control agents (Punja and Utkhede, 2003). Many of these exist naturally as epiphytes or saprophytes on or near plant tissues, utilizing nutrients available in various niches.

1.4.1 Yeasts

Natural yeasts have been widely used as biocontrol agents (Irtwange, 2006). Some yeasts are capable of colonizing plant surfaces or wounds for extended periods under dry conditions and can secrete extracellular polysaccharides which enhance their survival and also impede pathogen colonization sites (Wisniewski *et al*., 1991; Chand-Goyal and Spotts, 1997). Therefore, the plant/fruit surface is a good source of naturally occurring antagonistic yeasts.

Biocontrol activity of antagonistic yeasts has been attributed to a number of mechanisms. These include competition for nutrients and space, production of cell wall-degrading enzymes, production of antifungal metabolites, induction of host resistance, and mycoparasitism (El-Tarabily and Sivasithamparam, 2006). Although there are many yeasts with ideal biocontrol characteristics, the only yeasts that have been registered for application as biocontrol products are *Candida oleophila* strains I-182 and O (under the trade names Aspire® and Nexy®), *Cryptococcus albidus* (YieldPlus®), *Aureobasidium pullulan* strains DSM 14940 (CF 10) and DSM 14941 (CF 40) in mixture (Blossom-Protect® and Boni-Protect®), *Metschnikowia fructicola* isolate NRRL Y-30752 and *Saccharomyces cerevisiae* (Romeo®) (Freimoser *et al*., 2019).

1.4.2 *Trichoderma* spp.

The genus *Trichoderma* is among the most prominent and commonly used organisms for biological control of plant pathogens (Tronsmo and Hjeljord, 1998). These filamentous Deuteromycetes are commonly found in all soils and occur naturally on plant surfaces (Samuels, 1996). Most species of the genus are photosensitive and sporulate easily on a range of natural and artificial media (Papavizas, 1985). Numerous studies have reported the successful use of *Trichoderma* spp. to suppress pathogenic fungi on many economically important crops (Lewis *et al*., 1996; Ahmed *et al*., 1999; Mathre *et al*., 1999; Harman, 2000). *T. harzianum* is well known for its parasitic ability against several important soil pathogens, including those belonging to the genera *Rhizoctonia* and *Sclerotinia* (Bell *et al*., 1982; Hadar *et al*., 1984; Coping 2009). The

fungus demonstrates hyperparasitism against pathogens by coiling around the hyphae and absorbing nutrients from the pathogen (Elad 2000; Coping 2009). The success of *T. harzianum* ss as BCAs is attributed to their high reproductive capability, ability to survive under unfavourable conditions, adeptness in the utilization of nutrients, capacity to alter the rhizosphere, strong aggressiveness against phytopathogenic fungi, and efficiency at inducing plant growth defence mechanisms (Benítez *et al*., 2004). Many *Trichoderma* spp. have been registered for use as biocontrol products including *T. atroviride* strain 77 (Eco-77®), *T. harzianum* strain 22 (Trianum-G®) and *T. gamsii* strain ICC080 (Remedier®) (Woo *et al*., 2014).

1.4.3 *Bacillus* spp.

The genus *Bacillus* belongs to the family Bacillaceae. Species belonging to this genus are rod-shaped bacteria and are generally motile. One important advantage of this genus is their motility since it allows the bacteria to scavenge more efficiently for limited nutrients from root exudates (Brock and Madigan, 1991). Present in a large palette of environments, Bacilli can be found in sea water to soil and have even been found in extreme environments like hot springs (Hoch e*t al*., 1993). This genus has several valuable traits that render it a major source of potential microbial biopesticides (Ongena and Jacques, 2008). *Bacillus* spp. have been extensively used for many years in research aimed at increasing plant growth and suppressing the activities of soil-borne plant pathogens (Turner and Backman, 1991; Holl and Chanway, 1992; Kim *et al*., 1997a; Paulitz and Bélanger, 2001). However, studies attempting to use *Bacillus* spp. against foliar pathogens are not as extensive.

Bacilli, such as *B. subtilis*, are well-studied organisms, which facilitates their rational use. The US Food and Drug Administration (USFDA) given the "generally regarded as safe" (GRAS) status to *B. subtilis,* and is thus recognized as non-pathogenic (Harwood and Wipat, 1996). This is essential regarding its commercialization as a biopesticide. Bacilli produce endospores (Piggot and Hilbert, 2004), which are dormant forms capable of withstanding high temperatures, unfavourable pH, lack of nutrients or water, etc. The bacteria produce endospores when environmental conditions are unfavourable, enabling these microorganisms to survive in the phytosphere. The phenomenon can also be exploited in industrial production as sporulation can be induced at the end of cultures (Monteiro *et al*., 2005). There are numerous *Bacillus* spp. registered for use as biocontrol products including *B. pumilus* strain GB34

(YieldShield®), *B. subtilis* strain FZB24 (RhizoPlus®) and *B. amyloliquefaciens* strain FZB42 (RhizoVital®42).

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CHAPTER 2

Isolation and screening of yeast and *Bacillus* species for biological control of *Sclerotinia sclerotiorum*

Abstract

In South Africa, root wilt and stem and head rot caused by *Sclerotinia sclerotiorum* lead to the biggest yield losses in sunflower. Heavy relience on chemical fungicides poses a threat to human health and the environment, and the development of tolerance in pathogen populations is a growing concern. The use of Biological Control Agents (BCAs) to control the disease provides an alternative to instensive chemical usage, or to be included in Integrated Pest Management (IPM) systems. Sunflower heads growing wildly on local roadsides were collected and the petals harvested. A total of 100 and 136 native yeast and *Bacillus* strains, respectively, were isolated from the petals, which were free from fungicide or other substances of synthetic origin and cultured on selective media (Sabouraud dextrose agar for yeasts and Tryptone soy agar for *Bacillus*). The isolates were sub-cultured according to their macroscopic features visible to the naked eye and thereafter, light microscopy was used to identify the microstructures unique to each. Dual-culture bioassays were undertaken and assessed for evidence of antagonism and rated according to an inhibition scale. More than 53% of the yeast strains showed inhibition against *S. sclerotiorum*, the highest being achieved by Yeast Y86, which inhibited mycelial growth by 60.7%. Over 96% of the *Bacillus* strains inhibited *S. sclerotiorum*, the highest inhibition being achieved by B26 at 81.2%. None of the yeast isolates achieved a Class 3 rating (≥ 70% inhibition) while 17 *Bacillus* isolates achieved Class 3 ratings. In addition, the highest mean yeast inhibition percentage (60.7%) was significantly lower than the lowest mean *Bacillus* inhibition percentage (68.8%). Clear zones of inhibition could be seen between pathogen mycelia and *Bacillus* streaks in the dual-culture bioassays even after 21 days of incubation. Aerial mycelium of *S. sclerotiorum* was shorter and less dense than the control mycelia, its hyphae were shorter and more compact in the area closer to the antagonists, when viewed under stereomicroscope. Overall, *Bacillus* spp. isolated and screened in this study showed to be more highly antagonistic against *S. sclerotiorum* compared to the yeast isolates *in vitro*.

2.1 Introduction

Sclerotinia sclerotiorum (Lib.) de Bary, the causal agent of *Sclerotinia* head rot, is listed in the top five most economically important sunflower pathogens worldwide (DAFF 2010). In South Africa (SA), root wilt, stem and head rot caused by *S. sclerotiorum* lead to the biggest yield losses in sunflower (Davar *et al*., 2012). Epidemics resulting in 60% disease severity and losses in grain of up to 65% were reported during the 2013/14 growing season (Crave *et al*., 2016). The use of chemical fungicides is currently the main strategy to control diseases caused by *S. sclerotiorum*. Widespread, intensive use of chemical fungicides has become a serious concern due to the disastrous effects they have on non-target organisms, carcinogenetic effects and the development of tolerance in pathogen populations. This has increased the need to find alternative means of disease control. One approach might be the use of biocides or biological control agents (BCAs). In phytopathology, the term biological control refers to the use of introduced or resident living organisms to contain or suppress pathogen populations.

Bacillus spp. have widely been used for many years in extensive research in an attempt to increase plant growth and suppress plant pathogens (Turner and Backman, 1991; Holl and Chanway, 1992; Gutierrez Mañero *et al*., 1996; Kim *et al*., 1997; Paulitz and Bélanger, 2001). Several studies have shown that epiphytic yeasts inhabiting the surface of plant tissue have the potential to suppress plant diseases (Zhou and Reeleder, 1989; Madrigal *et al*., 1994; Helbig, 2002; Calvo *et al*., 2003; Lima *et al*., 2003). The majority of these antagonistic *Bacillus* and yeasts occur naturally on fruit and vegetable surfaces (Suzzi *et al*., 1995) but can be also isolated from other sources in the phyllosphere (Kalogiannis *et al*., 2006), the rhizosphere (Long *et al*., 2005) or the soil (Zhao *et al*., 2012). An advantage of using naturally occurring yeast and *Bacillus* strains is that they are already adapted to the biophysical and biochemical specificities of the contaminated niches.

The first step in developing biocontrol agents is isolation of the potential BCA and thereafter, screening to identify inhibitory effects of the BCA towards the pathogen *in vitro*. The best sources of antagonistic microorganisms are their natural environments where they compete with plant pathogens (Janisiewicz and Korsten, 2002; Droby *et al*., 2009). This is followed by *in vitro* screening of the isolates for biological control activity (Chanway *et al*., 1988; Aziz *et al*., 1997).

The aims of the research presented in this chapter were to isolate a selection of naturally occurring yeast and *Bacillus* cultures from healthy sunflower heads and thereafter subject them to *in vitro* screening to assess for biological control activity against *S. sclerotiorum*.

2.2 Materials and Methods

2.2.1 Isolation of *Sclerotinia* sp.

The *Sclerotinia sclerotiorum* strain used in this study was provided by Miss L.A. Rothmann[1] in the form of sclerotia. The sclerotia were surface sterilized by rinsing with 70% ethanol for 30 seconds. The 70% ethanol was discarded, and 1% sodium hypochlorite solution was added for further sterilization for 30 seconds. The 1% sodium hypochlorite solution was discarded and followed by two washes in sterilised distilled water (dH_2O). Surface sterilized sclerotia were placed on sterilised paper towel on a laminar flow bench and allowed to dry for 1 minute. Sclerotia were placed on 85 mm Petri dishes containing potato dextrose agar (PDA) (Lasec[2]) and incubated for 5 days at 25°C. The cultures were sub-cultured and left for 7 days before use. Since the South African *S. sclerotiorum* population is clonal (Steyn, 2015), only one isolate was used in this study.

2.2.2 Isolation of yeast and *Bacillus* species

Random samples of sunflowers were collected from roadsides in and around Pietermaritzburg, KwaZulu-Natal, South Africa. Flower heads were processed either immediately, after being stored for one to 2 days at room temperature, or after storage in a cold room at 8±1°C for five to 7 days.

Yeast and *Bacillus* isolates were recovered from the petals of the sunflower heads. The petals were removed from the heads using forceps dipped in 70% ethanol and flame sterilized. Petal samples, weighing 10g, were placed in separate 250 mL Erlenmeyer flasks containing 100 mL sterilised dH_2O and shaken at 250 rotations per minute (rpm) (MRC Orbital Shaker Incubator, Germany) for 30 minutes at 25°C. Flower petals were removed and the liquid suspension was used to make a serial dilution of 10^{-1}, 10^{-2} and 10^{-3}. Aliquots of 0.2 mL from each dilution were plated in triplicate on Sabouraud dextrose agar (SDA) (Appendix 1) using the spread plate method, for recovery of yeast isolates. Plates were incubated at 25°C for 3 days. Discrete colonies were then selected and streaked separately onto freshly prepared SDA plates. For isolation of *Bacillus*, the same serial dilution prepared for the yeast isolates was used, after heat shock treatment at 80°C for 10 minutes. Aliquots of 0.2 mL from each dilution

[1] Miss Lisa Rothmann, Department of Plant Sciences, University of the Free State, Bloemfontein, 9300, South Africa.
[2] Lasec SA (Pty) Ltd., 52 Old Mill Road, Ndabeni, Cape Town, 7405, South Africa.

were plated in triplicate on tryptone soy agar (TSA) (Lasec[3]) using the spread plate method, for recovery of *Bacillus* isolates. Plates were incubated at 28°C for 3 days. Representative colonies were selected and streaked onto freshly prepared TSA plates to obtain single colonies. Both the yeast and *Bacillus* cultures were incubated at 28°C for 3 days.

Yeast and *Bacillus* colonies were isolated from streaked SDA and TSA plates, respectively, and sub-cultured according to their macroscopic features (texture, surface, margin, elevation and colour). Their morphology was confirmed by light microscopic (Zeiss, Germany) observation. Yeast isolates were identified by observing microstructures unique to yeasts such as budding (Fig 2.1), which they use to reproduce asexually. *Bacillus* isolates were identified using a gram-staining technique to identify endospores, resistant asexual spores that develop inside gram-positive bacterial cells such as those of *Bacillus s*pecies. Gram-positive bacteria such as *Bacillus* retain crystal violet dye during gram staining and thus remain a blue-grey colour when washed with alcohol. Additionally, endospores appear transparent within the blue-grey stained *Bacillus* cells (Fig 2.2).

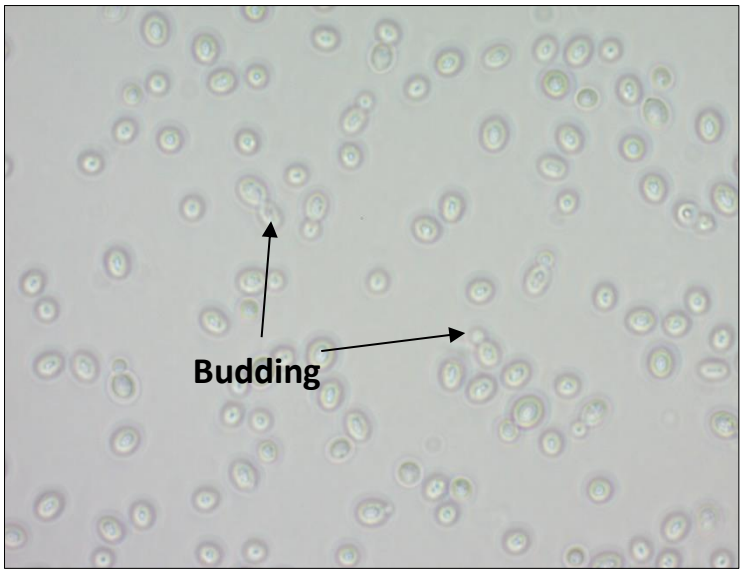

Fig 2.1 Globose yeast cells under a light microscope undergoing budding (40X magnification).

[3] Lasec SA (Pty) Ltd., 52 Old Mill Road, Ndabeni, Cape Town, 7405, South Africa.

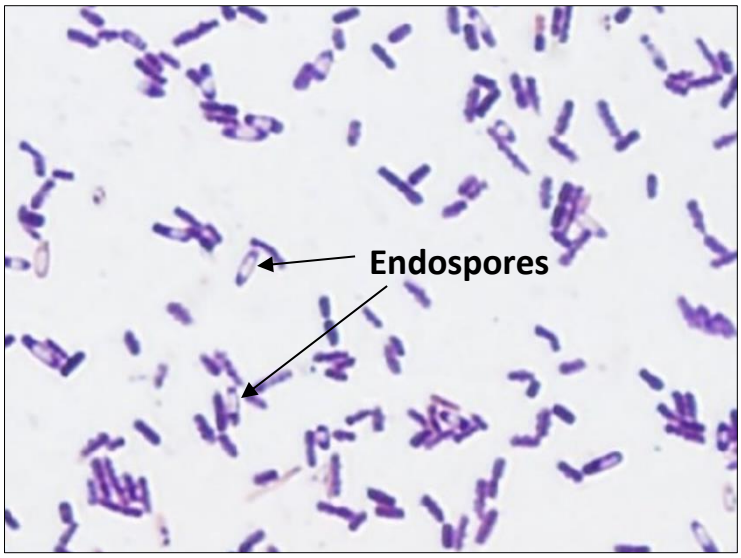

Fig 2.2 Gram-positive *Bacillus* under light microscope containing endospores (100X magnification).

Each yeast and *Bacillus* isolate was assigned an isolate code and were stored at –80°C (SANYO Ultra Low VIP series Ultra Low temperature freezer, Model MDF-U71V, SANYO Electrical Co., Ltd Japan) in 50% glycerol. Yeast and *Bacillus* isolates were plated on SDA and TSA respectively, when needed.

2.2.3 *In vitro* interaction between *S. sclerotiorum* and yeast and *Bacillus* isolates

The radial growth inhibition of *S. sclerotiorum* strains by antagonistic yeasts and *Bacillus* was tested according to the modified eclipse screening method described by Perez *et al*., 2016. Two loops of 3-4 day old yeast cells grown on PDA plates were streaked parallel to each other 15 mm from the edge of the plate on PDA. A single mycelial cube (4x4 mm, cut from the actively growing edge of a 4-day old mycelial mat on PDA) colonized with *S. sclerotiorum* was placed at the centre of a PDA plate equidistant from the yeast streaks. The same method was adopted for *Bacillus* isolates. Plates inoculated solely with *S. sclerotiorum* served as a control. Each bioassay was replicated three times and incubated in the dark at 25°C until control plates were fully colonized. Thereafter, antagonism was determined by calculating the percent of relative growth inhibition of *S. sclerotiorum*. The growth diameter of *S.*

sclerotiorum exposed to the yeast and *Bacillus* isolates was measured and growth inhibition percentage calculated in relation to the controls by the following formula:

L= $\frac{(C-T)}{C}$ x 100 where,

L= inhibition of radial mycelial growth; C= average radial growth of pathogen in control; T= average radial growth of pathogen in the presence of antagonist

The bioassay was repeated once.

Based on the results obtained from the primary screening above, ten yeasts and ten *Bacillus* isolates that reduced growth of *S. sclerotiorum* the most were selected for secondary screening which was a repetition of the above protocol. The bioassay was repeated once. A scale was developed which grouped the isolates based on their inhibition range in which Class 1 contained isolates that achieved ≤ 40% inhibition, Class 2 contained isolates that achieved between 41-69% inhibition and Class 3 contained isolates that achieved ≥ 70% inhibition. Based on the results obtained from the secondary screening, the seven best performing yeasts and the thirteen best performing *Bacillus* isolates were selected for greenhouse trials (Chapter 3).

Furthermore, observation, through a stereomicroscope, of pathogen mycelia that showed growth inhibition was conducted 10 days after incubation. They were compared with the corresponding controls to detect macroscopic differences.

2.2.4 Statistical analysis

Data was subjected to an analysis of variance (ANOVA) using Genstat® (18th edition) statistical analysis software (GenStat, 2016) to determine differences between treatment means. If the values were significant at $P < 0.05$, the means were separated using the Duncan's Multiple Range Test.

2.3 Results

2.3.1 Isolation

A total of 100 and 136 native yeast and *Bacillus* strains, respectively, were isolated from the heads of wild sunflowers.

2.3.2 *In vitro* interaction between *S. sclerotiorum* and yeast and *Bacillus* isolates

In the control plates, *S. sclerotiorum* grew to cover the entire surface of the plate after only 4 days of incubation (Fig 2.3 A) but mycelial growth was stunted in the presence of selected yeast and *Bacillus* isolates (Fig 2.3 B-D). The dual-culture plate tests revealed that over 53% of the yeast strains inhibited growth of *S. sclerotiorum* (Appendix 2). The highest inhibition was achieved by Yeast Y86, which inhibited mycelial growth of *S. sclerotiorum* by 60.7% after 4 days (Table 2.1) and also after 30 days of screening. Over 96% of the *Bacillus* strains inhibited *S. sclerotiorum* after 4 days (Appendix 3), with the highest inhibition being achieved by B26 at 81.2% (Table 2.1) and also after 30 days of screening.

None of the yeast isolates achieved a Class 3 rating (Table 2.1) and Y56, Y71, Y79 and Y86 were the only yeasts that achieved Class 2 ratings. This was in stark contrast to the performance of the *Bacillus* isolates, of which 17 isolates achieved Class 3 ratings. It is also important to note that the highest mean yeast inhibition percentage (60.7%) was significantly lower than the lowest mean *Bacillus* inhibition percentage (68.8%).

Table 2. 1 Mean percentage inhibition of *S. sclerotiorum* by selected potential yeast antagonists and their class ratings

Isolate code	Average inhibition (%)	Class
Y58	26.0 a	1
Y54	30.0 bc	1
Y81	30.0 cd	1
Y76	28.3 b	1
Y90	32.0 e	1
Y92	29.5 cd	1
Y41	30.6 b	1
Y60	30.7 d	1
Y91	30.7 d	1
Y29	34.4 f	1
Y68	34.5 f	1
Y93	35.4 fg	1
Y89	35.6 g	1
Y75	35.9 g	1
Y78	37.2 h	1
Y85	40.5 i	1
Y56	45.5 j	2
Y71	45.8 j	2
Y79	52.7 k	2
Y86	60.7 l	2
F-ratio	**536.8**	
P-level	**< 0.001**	
LSD	**1.1**	
CV%	**1.8**	

Means followed by different letters are significantly different (Duncan's Multiple Range Test, $P < 0.05$).

Table 2. 2 Mean percentage inhibition of *S. sclerotiorum* by selected potential *Bacillus* antagonists and their class ratings

Isolate code	Average inhibition (%)	Class
B76	68.8 a	2
B91	69.2 ab	2
B17	69.9 abc	2
B75	70.0 abcd	3
B136	70.4 abcde	3
B54	70.9 bcdef	3
B77	71.7 cdef	3
B61	71.8 def	3
B36	71.9 ef	3
B53	72.1 ef	3
B80	72.4 f	3
B57	72.5 f	3
B62	75.7 g	3
B63	76.7 g	3
B84	76.7 g	3
B122	76.9 g	3
B89	77.0 g	3
B16	77.1 g	3
B24	80.7 h	3
B26	81.2 h	3
F-ratio	**39.6**	
P-level	**< 0.001**	
LSD	**1.7**	
CV%	**1.4**	

Means followed by different letters are significantly different (Duncan's Multiple Range Test, $P < 0.05$).

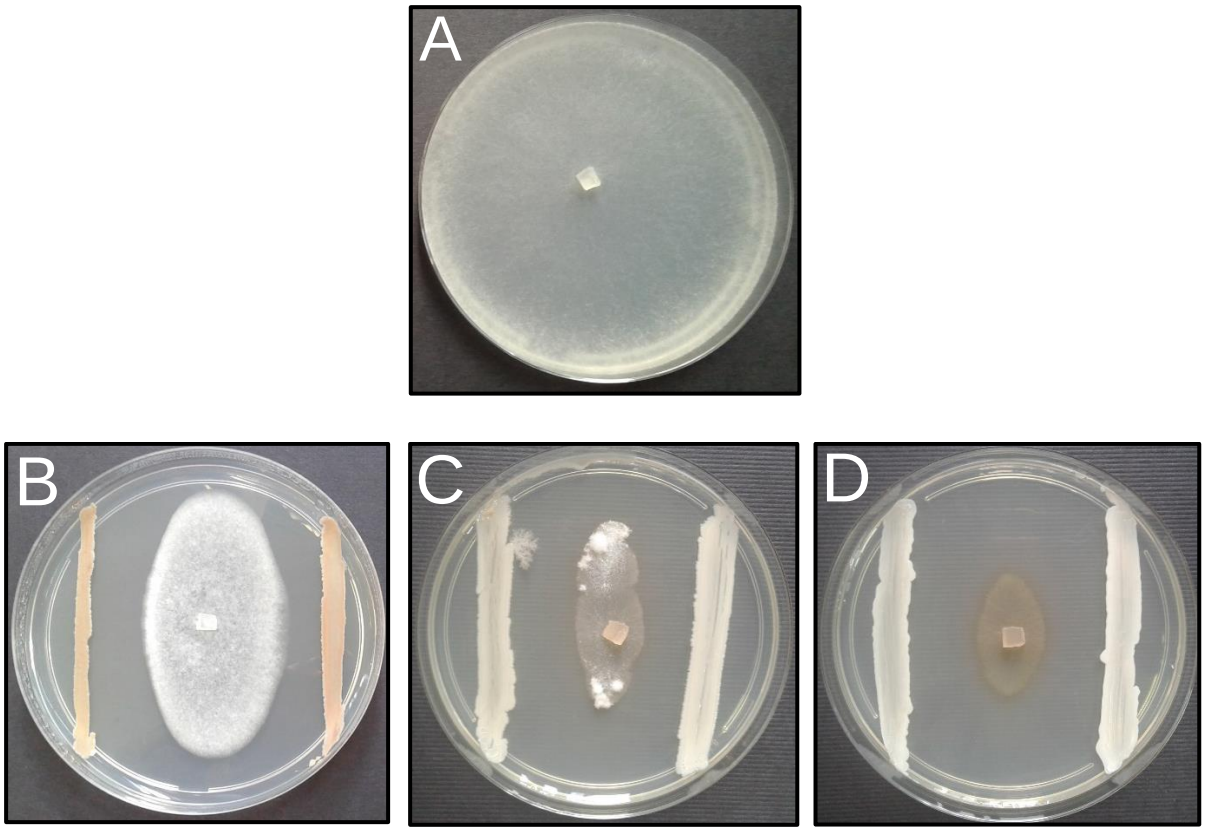

Fig 2.3 *In vitro* inhibitory activity of yeast and *Bacillus* isolates against *S. sclerotiorum* on Potato dextrose agar medium after 4 days incubation at 25°C. (A) Control plate inoculated only with *S. sclerotiorum*; (B) Y86; (C) B24 and (D) B26.

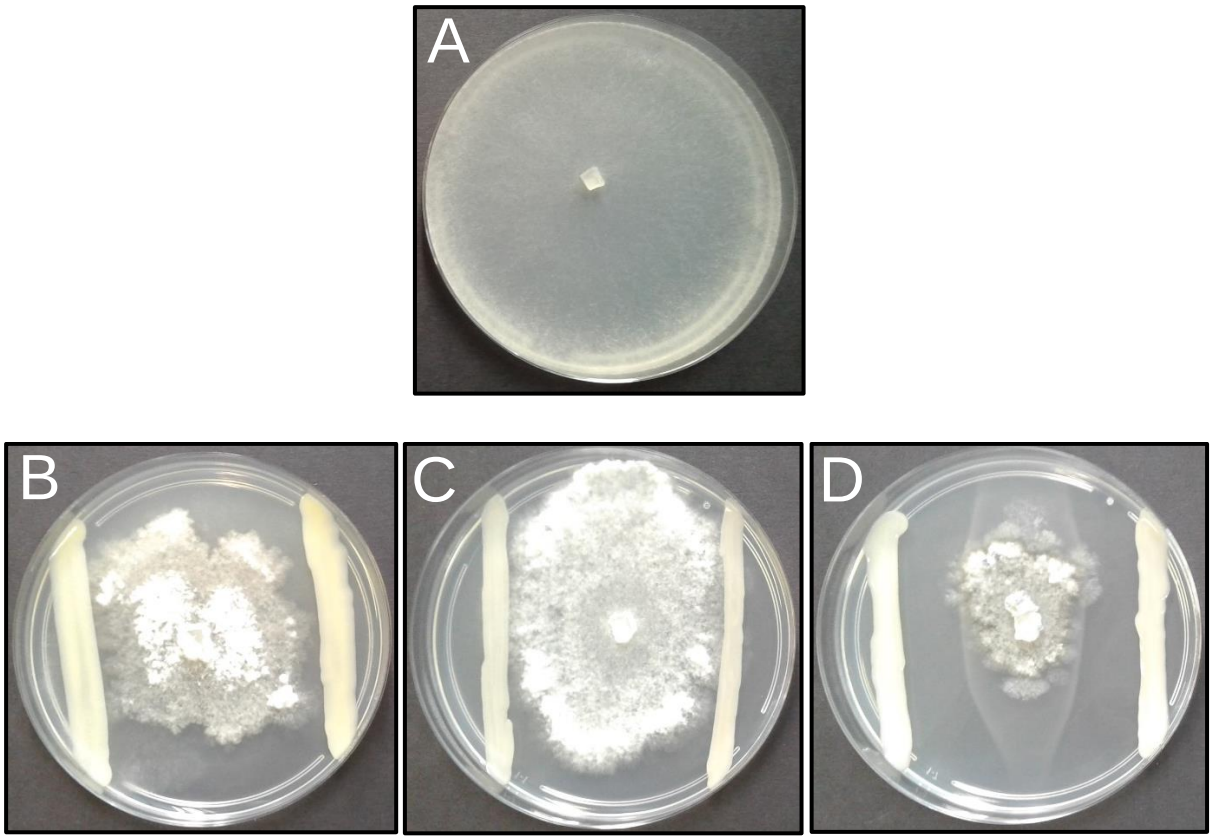

Fig 2.4 *In vitro* inhibitory activity of yeast isolates on Potato dextrose agar medium after 4 days incubation at 25°C. (A) Control plate inoculated only with *S. sclerotiorum*; (B) Y85, (C) Y86 and (D) Y75.

Stunted and deformed mycelial growth of *S. sclerotiorum* was caused by many of the yeast isolates that failed to significantly inhibit growth of the pathogen (Fig 2.4). Additionally, many of the *Bacillus* isolates that inhibited *S. sclerotiorum* less than B26 slowed down the growth of *S. sclerotiorum* by day 4 and also prevented pathogen mycelia from making contact even after 21 days, producing clear zones of inhibition (Fig 2.5). Sclerotial formation could also be seen where mycelial growth reached the edge of the petri plate (Fig 2.5 F, G, K and L).

On the tenth day of incubation an observation through a stereomicroscope of aerial mycelia that experienced growth inhibition was conducted. They were compared with the corresponding controls to detect macroscopic differences. In general, growth of the aerial mycelia of *S. sclerotiorum* was more dense than the control mycelia. Additionally, radial growth of pathogen hyphae was stunted along the edges of the colony parallel to the antagonists (Fig 2.6).

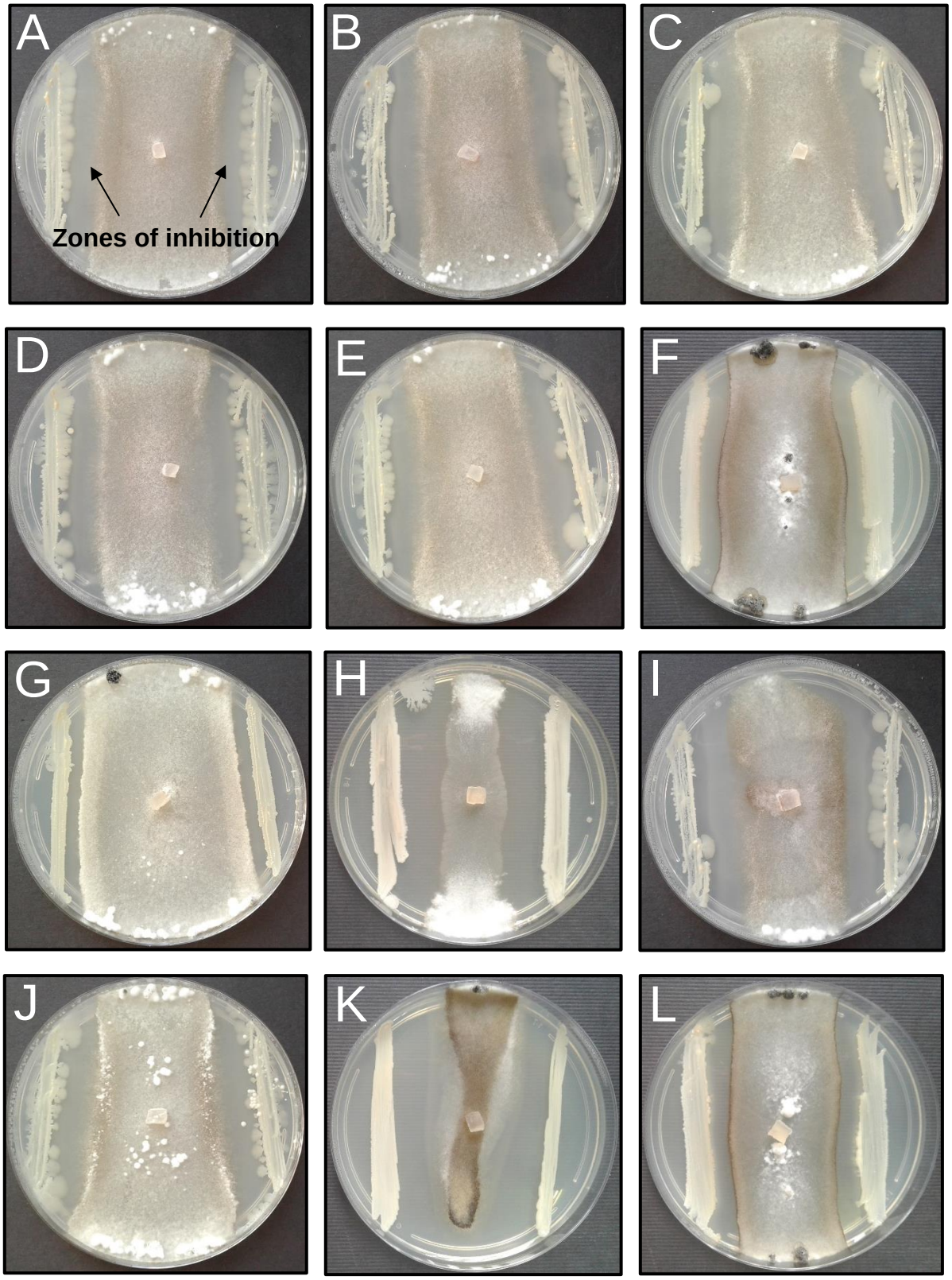

Fig 2.5 Numerous *Bacillus* isolates inhibiting growth of, and preventing contact with, *S. sclerotiorum* mycelium after 21 days of incubation at 25°C. (A) B1; (B) B6; (C) B10; (D) B11; (E) B12; (F) B13; (G) B15; (H) B16; (I) B20; (J) B91; (K) B122 and (L) B133.

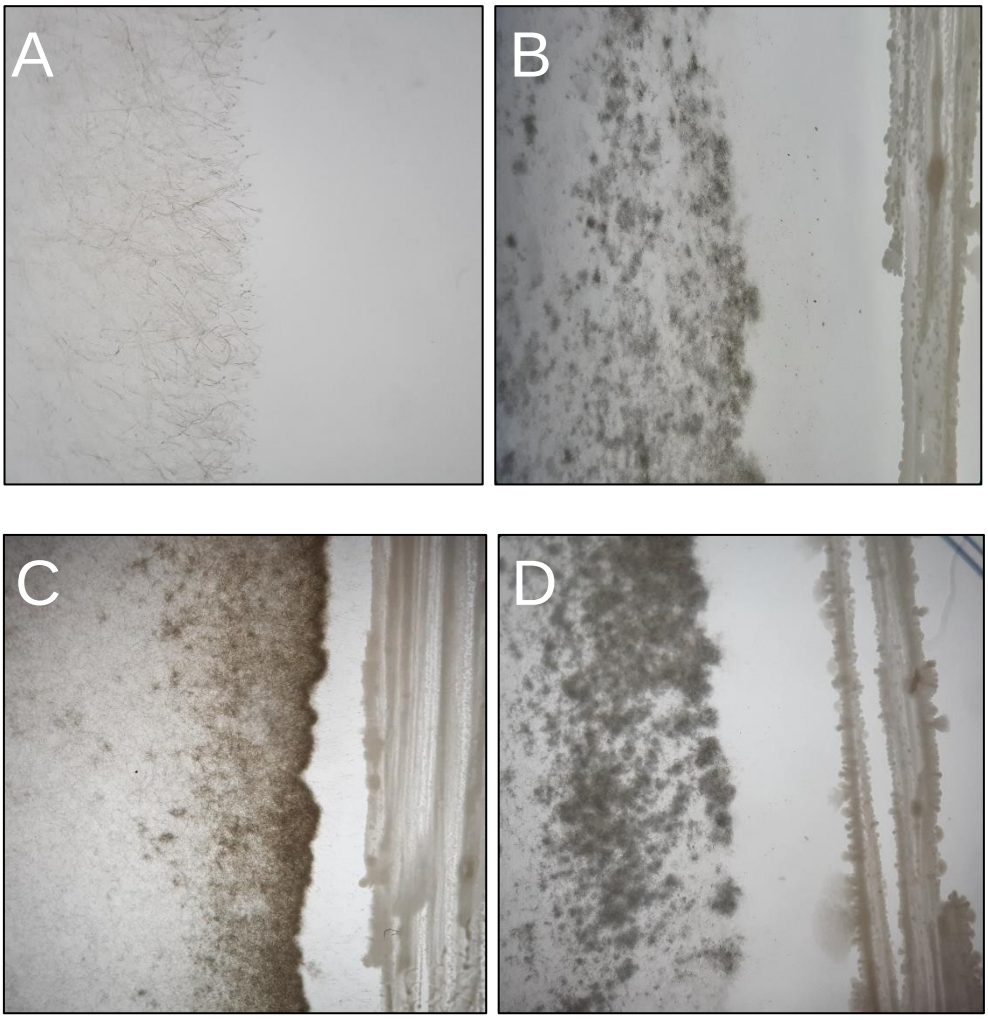

Fig 2.6 *In vitro* inhibition of *S. sclerotiorum* by selected yeast and *Bacillus* isolates against *S. sclerotiorum* on Potato dextrose agar medium after 10 days incubation at 25°C under stereomicroscope (6.5X). (A) *S. sclerotiorum* in the absence of antagonist; (B-D) *S. sclerotiorum* in the presence of *Bacillus* isolates.

2.4 Discussion

Sclerotinia sclerotiorum is an aggressive pathogenic fungus which causes significant yield losses in major sunflower growing regions. Limited success in disease control has been achieved with the application of chemical fungicides. In addition, phytotoxic effects, the development of resistance by pathogenic strains, and the hazards chemical fungicides pose to human health and the environment is a growing concern. Alternative control measures thus need to be investigated. Biological control uses natural enemies of the pathogen to suppress disease caused by the pathogen. Isolation and *in vitro*

screening is the first step towards identifying these enemies. This sets the foundation for the development of non-hazardous biological products which may be used as stand-alone treatments or concurrently with existing control measures i.e. chemical fungicides, cultural practices, planting tolerant sunflower varieties etc.

The major objective of this study was to isolate strains of yeasts and *Bacillus* spp. antagonistic to *S. sclerotiorum*. This approach has been reported by other researchers (Reeleder, 2004; Cavalcanti *et al*., 2020; Rahman *et al*., 2016; Wu *et al*., 2014). However, it is the first report in South Africa whereby native yeasts and *Bacillus* have been isolated and screened for potential antagonistic ability against *S. sclerotiorum*.The best sources of antagonistic microorganisms are their natural environments in which they compete with naturally colonized microflora among which are also plant pathogens or spoilage microorganisms. Isolation of antagonists from a chosen geographical region has shown to be more effective with enhanced specificity against the strains of a pathogen found in that region (Vero *et al*., 2002; Bouzerda *et al*., 2003). For that reason, sources of antagonistic microorganisms that inhabit the surface of sunflower heads and compete with plant pathogens were systematically screened.

Dense bacterial and yeast populations were found, with little growth of filamentous fungi. This is an indication that microorganisms present on healthy sunflower heads growing wild on roadsides may be used as a rich source of yeast and *Bacillus* isolates. The presence of these microorganisms on mature sunflower heads indicate that they are tolerant to the harsh conditions present on the surface of the heads such as low levels of readily available nutrients, exposure to UV radiation and highly variable climatic conditions (Leibinger *et al*., 1997). It also reflects their capability to inhabit and persist on the sunflower heads.

Of the 100 yeast isolates recovered from the surface of the sunflower heads, only two, Y79 and Y86, inhibited growth of *S. sclerotiorum* mycelia by >50%. These results differed greatly from the inhibition obtained from the *Bacillus* isolates, most of which inhibited growth of *S. sclerotiorum* by >50%. *Bacillus* isolates also achieved much higher inhibition percentages than the yeast isolates overall, the highest being 81.2% by B26 compared to 60.7% by Y86. This is an indication that *Bacillus* strains have

greater potential to antagonize and subsequently reduce disease caused by *S. sclerotiorum*.

Antibiotics produced by biological control agents (BCAs) *in vitro* in most instances have been regarded as the principle compounds responsible for biological control *in vivo* (Leifert *et al*., 1995). Inhibition zones produced by many of the *Bacillus* isolates remained constant after 21 days of incubation, suggesting that the antifungal compound produced was fungicidal in nature. *Bacillus* spp. have been reported to produce an array of antibiotics *in vitro* against several plant pathogens (Leifert *et al*., 1995; Asaka and Shoda, 1996). Although antibiotic production *in vitro* alone cannot be regarded as sufficient proof of the involvement of antibiotics in biological control *in vivo*, it is regarded as a useful tool for pre-screening potential BCAs *in vitro*.

Selection of BCAs based on *in vitro* production of extracellular enzymes, siderophores, antibiotics and other metabolites can be regarded as a useful screening procedure to reduce the large number of isolates at the initial stage for further testing *in vivo* (Kloepper *et al*., 1992). However, the presence of inhibition zones produced by many of the *Bacillus* isolates does not guarantee that they will perform well as BCAs *in vivo*, and neither does its absence guarantee that they are not BCAs. This can also be said for the yeast isolates that did not produce these inhibition zones. Ultimately, *in vivo* and field testing is required to ratify the choice and selection of BCAs. Future studies on this work could be to identify the modes of action used by the yeast isolates that successfully inhibited the growth of *S. sclerotiorum*. Furthermore, the antibiotics produced by the best performing *Bacillus* isolates could be identified.

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Appendix 1.

Composition of Sabouraud dextrose agar (SDA)

Peptone	10g
Agar (Lasec[1])	12g
D-Glucose	40g
Distilled water	1L

Mix and autoclave for 15min at 121°C.

Before pouring, antibiotics (ampicillin and streptomycin) and dodine were dissolved in 10 mL sterilised distilled water, then filter sterilized and added to the cooled medium at 0.1g/L, which was agitated to mix evenly.

Appendix 2. Mean percentage inhibition of isolated yeasts

Isolate	Inhibition %	Isolate	Inhibition %
Y1	0.58	Y51	3.72
Y2	14.52	Y52	3.92
Y3	0.00	Y53	0.00
Y4	3.33	Y54	31.97
Y5	5.28	Y55	0.00
Y6	13.15	Y56	41.20
Y7	15.68	Y57	6.67
Y8	0.00	Y58	24.10
Y9	3.92	Y59	0.00
Y10	14.90	Y60	37.85
Y11	15.10	Y61	14.90
Y12	2.95	Y62	0.00
Y13	6.12	Y63	0.00
Y14	14.30	Y64	0.00
Y15	0.00	Y65	3.15
Y16	0.00	Y66	0.00
Y17	0.58	Y67	1.57
Y18	0.00	Y68	34.33
Y19	0.00	Y69	4.52
Y20	0.00	Y70	3.70
Y21	6.65	Y71	35.95
Y22	0.00	Y72	8.82
Y23	1.58	Y73	0.00
Y24	0.00	Y74	9.62
Y25	9.15	Y75	33.35
Y26	2.93	Y76	27.45
Y27	0.00	Y77	10.97
Y28	1.18	Y78	36.07
Y29	28.82	Y79	39.42
Y30	5.30	Y80	13.92
Y31	0.98	Y81	33.92
Y32	0.00	Y82	0.00
Y33	0.00	Y83	0.00
Y34	0.00	Y84	13.93
Y35	12.93	Y85	31.38
Y36	0.00	Y86	61.38
Y37	11.38	Y87	0.58
Y38	21.77	Y88	30.80
Y39	0.00	Y89	28.05
Y40	0.00	Y90	35.10
Y41	31.57	Y91	31.57
Y42	0.00	Y92	29.60
Y43	0.00	Y93	35.10
Y44	3.52	Y94	0.00
Y45	11.77	Y95	0.00
Y46	0.00	Y96	0.00
Y47	0.00	Y97	0.00
Y48	10.78	Y98	0.00
Y49	0.00	Y99	0.00
Y50	0.00	Y100	0.00
F-ratio 35.47 **P-level < 0.001** **LSD 6.44** **CV% 38.90**			

Appendix 3. Mean percentage inhibition of isolated *Bacillus* spp.

Isolate	Inhibition %	Isolate	Inhibition %
B1	59.00	B69	52.15
B2	50.97	B70	55.10
B3	50.00	B71	52.73
B4	58.03	B72	56.47
B5	49.22	B73	54.30
B6	60.58	B74	66.48
B7	61.75	B75	69.43
B8	49.42	B76	70.20
B9	57.07	B77	70.58
B10	59.40	B78	49.23
B11	47.27	B79	63.32
B12	57.03	B80	72.73
B13	59.98	B81	66.10
B14	59.18	B82	66.87
B15	61.73	B83	63.12
B16	76.07	B84	73.53
B17	70.00	B85	55.48
B18	45.10	B86	78.63
B19	0.00	B87	61.17
B20	60.55	B88	64.48
B21	64.32	B89	75.30
B22	54.30	B90	63.33
B23	0.00	B91	69.42
B24	80.75	B92	52.35
B25	56.83	B93	0.00
B26	79.78	B94	58.42
B27	64.32	B95	59.20
B28	40.40	B96	43.72
B29	43.30	B97	45.48
B30	42.15	B98	50.60
B31	44.10	B99	49.20
B32	45.90	B100	50.18
B33	45.12	B101	0.00
B34	48.42	B102	57.25
B35	54.90	B103	0.00
B36	71.78	B104	53.70
B37	43.50	B105	59.02
B38	32.15	B106	51.57
B39	41.17	B107	50.02
B40	61.55	B108	46.90
B41	48.25	B109	61.37
B42	22.72	B110	49.62
B43	48.02	B111	54.72
B44	47.65	B112	46.67
B45	51.95	B113	34.32
B46	48.07	B114	51.55
B47	41.35	B115	53.90
B48	53.32	B116	53.90
B49	58.22	B117	52.55
B50	56.47	B118	53.32
B51	47.07	B119	43.52
B52	44.50	B120	52.52
B53	71.75	B121	54.10
B54	71.57	B122	75.50

Isolate	Inhibition %	Isolate	Inhibition %
B55	62.95	B123	58.02
B56	48.43	B124	53.12
B57	70.98	B125	52.92
B58	67.55	B126	49.62
B59	49.40	B127	53.30
B60	52.13	B128	52.95
B61	71.00	B129	42.35
B62	73.73	B130	54.28
B63	76.25	B131	48.43
B64	65.90	B132	50.38
B65	52.33	B133	52.93
B66	68.45	B134	51.57
B67	59.98	B135	57.23
B68	58.60	B136	70.18
F-ratio 198.28 **P-level < 0.001** **LSD 2.91** **CV% 3.30**			

CHAPTER 3

Carpogenic germination of sclerotia of *Sclerotinia sclerotiorum*

Abstract

Ascospores are the preferred source of inoculum in resistance screening, and chemical and biological fungicide testing programs involving crops that are naturally infected by ascospores of *Sclerotinia sclerotiorum*. However, the many factors affecting the ability of sclerotia of *S. sclerotiorum* to carpogenically germinate, and the lack of a consistent, reliable method of production has limited ascospore use. The aim of this study was to find a reliable method to routinely produce ascospores for use in greenhouse trials. Various protocols in which carpogenic germination of sclerotia and subsequent production of ascospores were successful, mostly adapted from research conducted with Northern Hemispheric strains of *S. sclerotiorum*, were tested. Sclerotia were produced on barley, carrot disks, maize and wheat meal and harvested. After air drying, sclerotia were subjected to various conditioning processes in which length of conditioning, temperature, humidity, substrate, light exposure and other environmental conditions differed. All these methods failed to produce ascospores. One method, adapted from a study conducted by a fellow South African researcher, led to stipe formation, but also failed to produce ascospores. Results presented in this study bring into question the local epidemiology of Sclerotinia diseases compared with northern hemispheric farming systems and their influences on Sclerotinia diseases. An adapted mycelial inoculation technique was used for greenhouse trials in Chapter 4.

3.1 Introduction

Sclerotia are the primary survival structures of the fungus, *Sclerotinia sclerotiorum*. Sclerotia play a major role in disease cycles as they produce inoculum (Willetts and Wong, 1980) and are able to persist in the soil for up to 8 years (Adams and Ayers, 1979). A sclerotium is a mass of hyphae surrounded by a black coating which contains melanin. Melanin has been found to protect against adverse conditions and microbial degradation in numerous fungi (Bell and Wheeler, 1986; Henson *et al*., 1999). In some instances, a higher melanin content in the rind of sclerotia may lead to the fungus being more virulent. The size of sclerotia of *S. sclerotiorum* differ greatly between hosts. On sunflower, a sclerotium casing the seed layer may be 1cm thick and more than 35cm in diameter, while on dry bean, sclerotia are globose and 2-10mm in diameter. Generally, the formation of sclerotia occurs when mycelia encounter a nutrient-limited environment (Christias and Lockwood, 1973).

Sclerotia develop in three stages (Townsend and Willetts, 1954): (i) initiation (aggregation of hyphae to form a white mass called sclerotial initials), (ii) development (hyphal growth and further aggregation to increase size), and (iii) maturation (surface delimitation, melanin deposition in peripheral rind cells, and internal consolidation). Much has been studied and reported on the factors influencing sclerotial development (Chet and Henis, 1975; Le Tourneau, 1979; Willetts and Wong, 1980; Willetts and Bullock, 1992). Sclerotia germinate to produce mycelia (myceliogenic germination) or apothecia (carpogenic germination), and infection occurs by mycelia arising from the sclerotia, or by ascospores produced in the apothecia (Abawi and Grogan, 1979; Willets and Wong, 1980). Mycelial infections predominantly occur at the soil line and cause basal stem rots, or mycelia from sclerotia make contact with the roots to cause root rot (Huang and Deuck, 1980; Holley and Nelson, 1986). Above-ground infections, particularly in inflorescences, occur via ascospores (Abawi and Grogan, 1979; Boland and Hall, 1987; Gulya *et al*., 1989). Ascosporic inocula are the chief drivers of epidemics in many crops (Purdy, 1956; Newton and Sequera, 1972; Blad *et al*., 1978; Schwartz and Steadman, 1978; Purdy, 1979; Phillips, 1986; Boland and Hall, 1987).

Studies have shown that the main environmental factors that govern carpogenic germination and development of apothecia are soil temperature, soil moisture and light (Phillips 1987, Bardin and Huang 2001). Ascospores are the ideal source of inoculum when screening for resistance and testing fungicides in crops that are infected by

ascospores in nature. However, the many factors affecting the ability of sclerotia of *S. sclerotiorum* to germinate carpogenically, and the lack of a consistent, reliable method of production has limited ascospore use. Sclerotia of most isolates must be conditioned for a prescribed length of time to overcome dormancy and to trigger carpogenic germination (Coley-Smith and Cooke, 1971; Willets and Wong, 1980). The initial objective of this study was to develop a fast and reliable method of inducing sclerotia of *S. sclerotiorum* to germinate carpogenically and produce ascospores for use in greenhouse trials on sunflower. However, since numerous methods were tested and ascospores were not produced, the aim of this Chapter became to review those methods.

3.2 Materials and Methods

3.2.1 Production of Sclerotia

a) Substrate preparation

Several different substrates were used to produce sclerotia. Barley was allowed to soak overnight in tap water and then dried under a laminar flow hood for 5-8 hours. One hundred and fifty grams and 250 g of barley was then transferred to 500 mL and 1 L conical flasks, respectively, and autoclaved for 20 min at 121°C. Once cool, the barley was re-autoclaved. The barley was allowed to cool and then inoculated with 4-day old colonized agar squares (4 x 4 mm) of *S. sclerotiorum*. The flasks were incubated at 25°C under natural 12-hour light and dark cycles until the substrates were fully colonised.

Similarly, carrot disks, maize meal and wheat meal (150 g) were placed in 1 L CONSOL glass jars. Water (500 mL) was added to each jar and left overnight. The water was drained from the jars and 85 mL distilled water (dH_2O) was added. The jars were autoclaved for 20 min at 121°C and upon cooling, re-autoclaved. The autoclaved jars were cooled and inoculated with *S. sclerotiorum* and incubated as before, until the substrates were fully colonised.

b) Harvesting sclerotia

Once the sclerotia had matured on the respective substrates they were harvested. The flasks and jars were shaken at 250 rotations per minute (rpm) (MRC Orbital Shaker Incubator, Germany) for 30 minutes at 25°C to remove the sclerotia from the substrate. Sclerotia were then placed on to a flat surface and air dried. The substrate/sclerotia mixture was soaked in water until sclerotia rose to the surface while the substrate sank to the bottom. After being strained, sclerotia were placed under a laminar flow hood and stored at room temperature for a maximum period of 4 weeks.

3.2.2 Conditioning of Sclerotia to Induce Carpogenic Germination

Various methods were used in an attempt to induce carpogenic germination of *S. sclerotiorum*:

a) Method 1

Sclerotia were placed in sterilised glass vials and refrigerated at 4°C for 4, 6 or 8 weeks. After the refrigeration period, the sclerotia were transferred to 85 mm Petri dishes containing filled with Umgeni sand, steam pasteurised potting mix or steam

pasteurised composted pine bark[4]. The sclerotia were pushed down slightly in order to be covered by a layer of sand/soil and the plates were incubated at 25°C or 30°C for 4, 6 or 8 weeks. Thereafter, plates were placed under near-UV light at 22°C for four to 15 days and inspected daily under stereomicroscope for apothecia. The trial was run thrice.

b) Method 2- Cosic *et al*., 2012

This method was adapted from Cosic *et al*., 2012. In the lab, sclerotia were washed under tap water (2 h), surface sterilized (97% alcohol for 2 minutes), rinsed twice in distilled water and air-dried. In Petri dishes, 10 sclerotia were placed on threefold filter paper saturated with distilled water and kept on a lab desk at 22±3°C with a natural light/dark regime. In every Petri dish there were five small (3-4 mm) and five larger (≥4 mm) sclerotia. Filter paper moisture was maintained daily with distilled water. Inspections under stereomicroscope were done every 3 days for 27 days. The trial was run twice.

c) Method 3- Ekins, 1999

This method was adapted from Ekins, 1999. Sclerotia were transferred to deep Petri dishes (96 mm x 25 mm) with 30 mL sterilised dH_2O and incubated for 6-8 weeks in the dark at 15°C for 8 hours and 10°C for 16 hours. When stipes formed between 4-8 weeks, Petri dishes were transferred to another incubator and illuminated for 8 hours/day at 15-18°C for fourteen to 21 days under day light fluorescent tubes until apothecial discs were formed. The trial was run twice.

d) Method 4- Pethybridge *et al*. 2015

This method was adapted from Pethybridge *et al*. 2015. Sclerotia were placed in cheesecloth bags and tie closed. Bags were placed in a bucket filled with tap water in an incubator (7°C). Lights were kept on continuously and air was bubbled through the water using an aquarium pump (Fig 3.1). The water was discarded on a weekly basis and fresh water added to avoid algal build-up.

[4] Gromor (Pty) Ltd., P.O. Box 89, Cato Ridge, KwaZulu-Natal, 3680, South Africa

Fig 3.1 Conditioning sclerotia in aerated water in a cold room (Picture A.J. Moody, 2018).

Sclerotia were kept in the cold water bath for four to eight weeks. After 8 weeks, bags were checked weekly for sclerotia with initials that had protruded through the cheesecloth. The trial was run twice.

e) Method 5- Clarkson *et al*. 2003

This method was adapted from Clarkson *et al*. 2003. Pre-conditioned sclerotia were evenly placed on 100 g steam pasteurised compost in clear plastic boxes (600 mL) and covered with a further 30 g pasteurised compost (0.5 cm depth). The boxes were sealed and placed in a cooled glasshouse at 15-22°C or in a controlled environment cabinet at 15°C (12 h light/dark). The compost moisture content was maintained at 30% (w/w) by adding an appropriate amount of water initially and maintaining the weight of each box by further additions each week. Apothecia were expected to appear after approximately 3–5 weeks. The trial was run twice.

f) Method 6- O'Malley *et al*., 2015

This method was adapted from O'Malley *et al*., 2015. Briefly, sclerotia were placed on V8 juice agar, incubated in darkness at room temperature and sub-cultured at 7-day intervals. After three transfers on V8 juice agar, isolates were transferred to wholemeal agar plates and incubated at 20°C in darkness for a further 4 weeks. Sclerotia were scraped onto sterilised filter paper and dried for 2 days. Sclerotia were then placed in 10 mL sterilised dH_2O and incubated at 15°C for 8 h, followed by 10°C for 16 h for 12 weeks. Isolates were placed at 10°C for a further 14 days in darkness, before exposure

to 15W fluorescent daylight tubes illuminated with an 8 h photoperiod at 10°C for 12 weeks. The trial was run twice.

g) Method 7- Bester, 2018

The following methods were adapted from Bester, 2018.

i) Sclerotia were surface sterilised in 3% sodium hydrochloride for 4 min and the sodium hydrochloride was discarded thereafter. Sclerotia were then sterilised further with 76% ethanol for 2 min. The 76% ethanol was discarded and sclerotia were rinsed twice in sterilised dH_2O. Five sclerotia were placed onto 1.5% water agar (WA) in 90 mm Petri dishes and covered by another layer of 1.5% water agar, in order to keep sclerotia moist. Sclerotia were conditioned at 16°C until the first set of stipes developed.

ii) Sclerotia were surface sterilised as before (Method 7- i) and five sclerotia were placed onto a Petri dish filled with saturated sandy soil. Petri dishes were watered with sterilised dH_2O twice a week and kept at 16°C until the first set of stipes developed.

Both trials were run thrice.

3.3 Results

Methods 2-5 failed to induce stipe formation and subsequently, apothecial and ascospore production.

Method 1 and Method 7 (i) were successful at initiating carpogenic germination and induced stipe formation. However, apothecia and ascospores were not produced. Additionally, stipes showed wilting and discolouration. Fig 3.2 shows poorly formed stipes induced by Method 7 (i). Method 7 (ii) failed to induce stipe formation and instead induced myceliogenic germination within the sandy soil.

Fig 3.2 Apothecial stipe production from sclerotia conditioned between two layers of 1.5% water agar (Picture A.J. Moody, 2019).

3.4 Discussion

Attempts at mass production of ascospores inoculum have been hampered by unreliable carpogenic germination of *S. sclerotiorum*. Sclerotia of most isolates of *S. sclerotiorum* exhibit constitutive and exogenous dormancy (Coley-Smith and Cooke, 1971; Willets and Wong, 1980; Phillips, 1987). For some isolates, incubation of sclerotia at low temperatures breaks dormancy (Coley-Smith and Cooke, 1971; Willets and Wong, 1980; Huang and Kozub, 1981; Smith and Boland, 1989; Saito, 1997) or in sandy soil (Keay, 1939; Newton and Sequeira, 1972; Bester, 2018) for specific durations. Occasionally, sclerotia of some isolates are also found to germinate carpogenically without conditioning when taken directly from pure cultures on agar media and incubated in water (Huang and Kozub, 1981; Letham, 1976).

A great deal of work has been undertaken to determine the environmental factors such as temperature, light, moisture, and burial depth required to trigger sclerotia to germinate carpogenically (Willets and Wong, 1980). However, results are inconsistent and contradictory (Phillips, 1987; Wu and Subbarao, 2008). For example, although it is known that apothecia develop rapidly at 10 to 20°C (Willets and Wong, 1980), there have been reports of this occuring at 7°C to 25°C (Phillips, 1987). Various researchers recommend a range of temperatures: 0°C (Kohn, 1979), 3°C (Saito, 1977), 4°C (Clarkson *et al*., 2003), 5°C (Phillips, 1986), 7°C (Cobb and Dillard, 1996), 8°C (Dillard *et al*., 1995), and 10°C (Huang and Kozub, 1991). In contrast, isolates that germinate without pre-conditioning have been reported (Bedi, 1956; Ramsey, 1925). The length of the preconditioning period suggested to induce carpogenic germination also differs frequently, ranging from seven to 10 days to 8 weeks (Abawi and Grogan, 1975; Phillips, 1987; Mila and Yang, 2008).

Moisture is also considered an important factor in carpogenic germination, and even mild osmotic stress may prevent apothecia from forming (Williams and Western, 1965; Grogan and Abawi, 1975; Abawi and Grogan, 1979; Stun and Yang, 2000; Wu and Subbarao, 2008). Light has also been described to play a key role in the development of mature apothecia but not essential for stipe formation (Steadman and Nickerson, 1975; Thaning and Nilsson, 2000). The source and burial depth of sclerotia (Willets and Wong, 1980; Smith and Boland, 1989; Wu and Subbarao, 2008) also has an impact on apothecial production of *S. sclerotiorum.* Since numerous different variables impact carpogenic germination, it is unsuprising that reproducing different individual

methods in diverse geographical locations has been largely unsuccessful. This makes it hard to link published findings on carpogenic germination to geographical locations. (Grogan, 1979; Mylchreest and Wheeler, 1987; Smith and Bolland, 1989; Dillard and Ludwig, 1995).

Such differences in germination behaviour of the sclerotia of the *S. sclerotiorum* isolates could partially be due to the geographical origin (Huang and Kozub, 1991) or the temperature conditions under which sclerotia were formed (Huang and Kozub, 1989). This is the most likely explanation as to why the method adapted from Bester (2018), which was the only method shown to successfully induce carpogenic germination of *S. sclerotiorum* in South Africa, was one of the two methods that led to stipe formation. The rest of the methods adapted from studies performed in other parts of the world were unsuccessful at inducing stipe formation. Results presented in this study raise questions regarding the local epidemiology of Sclerotinia diseases compared with northern hemispheric farming systems and their influences on Sclerotinia diseases.

Due to the failure of any of the tested protocols to produce ascospores, the inoculation technique of Bester (2018) was adopted, which uses a suspension of mycelial fragments in water to inoculate sunflower heads. This was shown to be effective at causing head rot of sunflower (and stem rot of soybean) and due to the time constraints placed on a Masters project, it was decided that this method would be used to carry out subsequent greenhouse screening of the yeast and *Bacillus* spp. isolated and screened *in vitro* in Chapter 1.

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CHAPTER 4

In vivo screening of Yeast, *Bacillus spp.* and *Trichoderma atroviride* against head rot of sunflower caused by *Sclerotinia sclerotiorum*

Abstract

Ten yeasts, 10 *Bacillus* spp. isolates and one commercial *Trichoderma* strain, *T. atroviride* (Eco-77®) were tested *in vivo* for biological control of *Sclerotinia sclerotiorum* head rot of sunflower. Three *Bacillus* isolates, B16, B24 and B26, and one yeast isolate, Y79, significantly reduced disease incidence and improved grain yield. The effect of three inoculum concentrations (1×10^7, 1×10^8 and 1×10^9 cells mL^{-1}/cfu mL^{-1}) of the yeast isolate and the *Bacillus* isolates on disease control was evaluated. Inoculum concentration of 1×10^8 cells mL^{-1} for Y79 reduced disease incidence by 50.0% and significantly improved grain yield. All three *Bacillus* isolates prevented infection at 1×10^9 cfu mL^{-1}. The highest grain yields were measured when the *Bacillus* isolates were applied at 1×10^9 cfu mL^{-1}. A further study of inoculum concentration on disease control was done with *Bacillus* isolates, B16 and B26. Application of B16 at a concentration of 7.5×10^8 cfu mL^{-1} to sunflower heads completely suppressed head rot, while the optimal concentration for B26 was 2.5×10^9 cfu mL^{-1} and reduced disease incidence to 10.0%. Grain yield was also significantly improved at these concentrations. A final study was conducted in which Y79, B16, B24, B26 and T77 were tested *in vivo*, singly and each in combination for plant growth promotion and biological control. Combined inoculations of B26 and B24 gave the highest mean grain yield of sunflower, which was significantly greater than eight of the seventeen single/combined inoculation treatments. This study showed that there is potential in using mixtures of *Trichoderma* spp., *Bacillus* spp. and yeasts for improving sunflower growth and biological control of head rot caused by *S. sclerotiorum*.

4.1 Introduction

Plant disease control has been achieved following foliar treatments with selected strains of yeasts, *Bacillus* spp. or *Trichoderma* spp. (Elad *et al*., 1994; Inbar *et al*., 1994; Podile and Prakash, 1996; Elad, 2000; Harman, 2000; Fernando *et al*., 2007; Ziedan and Farrag, 2011). However, most of the research into using these microorganisms as biological control agents (BCAs) has focused on seed, soil and post-harvest applications (Raspor *et al*., 2010; Yobo *et al*., 2011; Singh *et al*., 2013; Zhimo *et al*., 2014)

Yeasts are particularly attractive as biological control candidates because they are widely used for various food and industrial purposes, and thus there is a substantial amount of information available with with regards to techniques for genetic manipulation, production, and storage of yeast cells (Hofstein *et al*., 1994; Hamilton *et al*., 2003). Yeast antagonists are potential biological control agents because they proliferate rapidly by using available nutrients; they can synthesize extracellular polysaccharides that enhance their survival; they colonize wounds in plants for long periods; and they are tolerant of most agrochemicals (Janisiewicz, 1988; Richard and Prusky, 2002). Given the wide genetic diversity amongst yeast-like fungi, different mechanisms of biocontrol are likely to operate in different taxa (Reeleder, 2004).

Bacillus spp. are also attractive biological control agents due to their ability to reproduce as highly resistant endospores, and the production of antibiotics that control a wide range of plant pathogens (Cavaglieri *et al*., 2005). Various modes of action have been demonstrated by *Trichoderma* spp. which can suppress or control fungal plant pathogens. These include the synthesis of extracellular antibiotics (Ghisalberti and Sivasithamparam, 1991; Calistru *et al*., 1997), enzymes such as chitinase (Lima *et al*., 1997) and β-1,3-glucanase (Menendez and Godeas, 1998), siderophore production (Scher and Baker, 1982), mycoparasitism (Benhamou and Chet, 1993; Kumar *et al*., 1998; Gupta *et al*., 1999) and competition for key nutrients or elements (Elad, 1996). Elad (1996) reported that *T*. *harzianum* T39 successfully competes with *Botrytis cinerea* for nutrients or elements responsible for activating germination of *S. sclerotiorum* propagules for infection.

The rhizosphere is buffered in the biological, chemical and physical soil environment whereas the phyllosphere and the fructosphere are exposed to the harsh gaseous

atmosphere and sharp fluctuations in temperature, surface wetness humidity, as well as air pollutants, wind, and UV radiation. These environments differ drastically and the microbial populations found in each of them differ significantly, too. The rhizosphere is best suited for proliferation and activities of diverse microfloral species comprised of filamentous fungi, bacteria, actinomycetes, protozoa and algae. In contrast, the phyllosphere is colonized mostly by specialized bacteria and yeasts, while the filamentous fungal species occur mainly as spores (Lindow and Brandl, 2003). As a result of the fluctuations in the physical, chemical and nutritional environment of the phyllosphere, the microbial populations inhabiting this environment change frequently.

Thus, the phyllosphere and fructosphere are considered difficult sites for biological control because the applied biocontrol agent is required to establish, proliferate and retain its antagonistic potential in the fluctuating and harsh foliar environment. *Sclerotinia* head rot of sunflower is difficult to control once it starts, and renders the head unmarketable, in addition to the creation of inoculum and a rapid spread of *S. sclerotiorum* to other plants. It is therefore imperative to prevent the initial infection of sunflower heads. Studies on the biological control of *Sclerotinia sclerotiorum* in the fructosphere of sunflower with potential biocontrol agents, including yeasts, *Trichoderma* spp. and bacterial antagonists, have not been reported previously.

The aim of this study was to investigate the biocontrol of *Sclerotinia* head rot of sunflower caused by *Sclerotinia sclerotiorum* by selected strains of yeasts and *Bacillus* spp. and a commercial biocontrol agent, *T. atroviride* strain 77, with the goal of preventing or reducing infection of sunflower heads.

4.2 Materials and Methods

4.2.1 Inoculum preparation of *S. sclerotiorum*

As in Chapter 2, the *S. sclerotiorum* isolate used was initially isolated from sclerotia provided by Miss L.A. Rothmann[5]. The sclerotia were surface sterilized by rinsing with 70% ethanol for 30 seconds. The 70% ethanol was discarded and 1% sodium hypochlorite solution was added for further sterilization for 30 seconds. The 70% ethanol was discarded and followed by two washes in sterilized distilled water (dH_2O)

[5] Miss Lisa Rothmann, Department of Plant Sciences, University of the Free State, Bloemfontein, 9300, South Africa.

and placed on sterile paper towel on a laminar flow bench and allowed to dry for 1 minute. Sclerotia were placed on 85 mm Petri dishes containing potato dextrose agar (PDA) (Lasec[6]) and incubated for 5 days at 25°C. The cultures were sub-cultured and left for 7 days before use. The South African *S. sclerotiorum* population is considered to be clonal (Steyn, 2015). Thus, only one isolate was used in the trial.

The spray mycelium method (Botha *et al*., 2009) was used. Potato dextrose broth (PDB) (Lasec[1]) was prepared and autoclaved in 1 L conical flasks. Three agar plugs were cut from a 4-day old *S. sclerotiorum* colony and placed into each flask containing PBD. Flasks were placed in a shaker for 3 days at 25°C, at 10 rotations per minute (rpm). A kitchen blender (Braun MQ100 Soup Hand Blender) was then used to homogenize the mycelium for 15-20 seconds. The mycelium suspension was placed in a 1 L hand atomizer (MTS Manual Handheld Pressure Sprayer) before being sprayed evenly onto plants to the point of run-off.

The average hyphal diameter of the *S. sclerotiorum* strain used in this study was found to be 0.61 µm. Fig 4.1 shows the hyphae taken from a 4-day old culture under a light microscope.

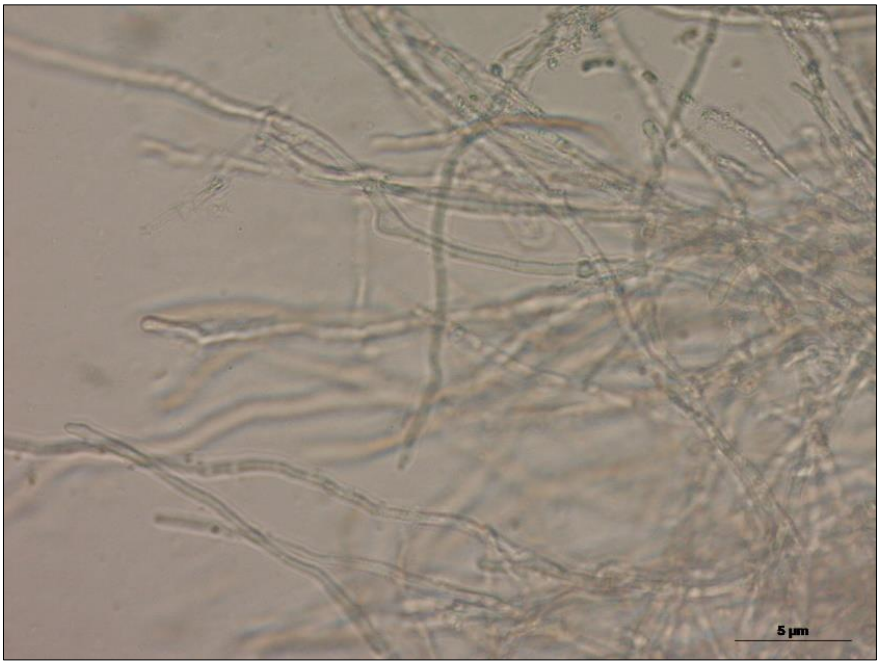


Fig 4.1 Hyphae of *Sclerotinia sclerotiorum* under a light microscope.

[6] Lasec SA (Pty) Ltd., 52 Old Mill Road, Ndabeni, Cape Town, 7405, South Africa.

4.2.2 Inoculum preparation of biocontrol agents

For yeast isolates, stock solutions were made by transferring three loops of 4-day old cultures into 100 mL bottles containing sterilized dH_2O. A dilution series was used to make suspensions of each isolate at three different concentrations: 1 x 10^7 cells mL^{-1}, 1 x 10^8 cells mL^{-1} and 1 x 10^9 cells mL^{-1}, adjusting the concentration using a haemocytometer (Marienfeld Neubauer Improved Counting Chamber).

For *Bacillus* isolates, a single colony of each strain was inoculated and grown in tryptone soy broth (TSB) (Lasec[1]) in a shaker incubator (MRC Orbital Shaker Incubator, Germany) with constant shaking at 150 rpm for 48 hours at room temperature. The cultures were centrifuged at 6000 rpm for 10 min and the bacterial cells were resuspended in a phosphate buffer (100 mM, pH 7.0). The cell concentration was adjusted spectrophotometrically to approximately 1 x 10^7 cfu mL^{-1}, 2.5 x 10^7 cfu mL^{-1}, 1 x 10^8 cfu mL^{-1} and 1 x 10^9 cfu mL^{-1}.

A commercial formulation of *T. atroviride* strain 77 (T77)[7] was applied to sunflower heads as a foliar spray at a rate of 1 g per 2 L sterile dH_2O. Concentration of the biofungicide is 2 x 10^9 conidia mL^{-1}.

All suspensions were transferred into separate hand atomizers before being sprayed evenly onto sunflower heads to the point of runoff.

4.2.3 Greenhouse trials

Sunflower seeds (PAN 7080[8]) were washed eight times with sterile distilled water and air-dried under a laminar flow before being planted in cylindrical plastic pots (300 mm diameter) filled with steam pasteurized composted pine bark. The pots were drip irrigated daily and arranged in a randomised complete block design in a polycarbonate greenhouse tunnel, which was maintained at temperatures of between 26 to 28°C with a relative humidity (RH) of 75 to 85%. For each treatment two seeds were planted into each pot. To avoid possible competition between plants, seedlings were thinned to one plant per pot 1 week after germination. Sunflowers were allowed to flower and were inoculated with the biocontrol agents and *S. sclerotiorum* inoculum at the R5.8-R5.9 reproductive stage whereby 80%-100% of the head area (disk flowers) had completed or were in flowering.

[7] Eco-77®, Plant Health Products, Nottingham Road, South Africa
[8] PANNAR (Pty) Ltd., PO Box 19, Greytown, Kwa-Zulu Natal, 3250, South Africa.

a) Preventative action of T77 and selected yeast and *Bacillus* isolates antagonistic to *S. sclerotiorum* on sunflower heads

Ten yeasts, 10 *Bacillus* isolates and T77 were tested for their preventative action against *S. sclerotiorum*. Sunflower heads were sprayed until runoff with suspensions of the 10 yeasts (1 x 10^8 cells mL^{-1}) and 10 *Bacillus* spp. isolates (1 x 10^8 cfu mL^{-1}) that best performed *in vitro* (Chapter 2), in addition to T77 as a foliar spray (2 x 10^9 spores mL^{-1}). There were four plants per treatment. The biocontrol agents were given 48 hours to colonize the sunflower heads before they were inoculated with the mycelial suspension of *S. sclerotiorum*. Disease controls were sprayed with distilled water and then with the mycelial suspension of *S. sclerotiorum* after 48 hours. Plants were visually examined for infection after 14 days and disease incidence was calculated using the following formula:

DI = $\frac{IP}{T}$ x 100 where,

DI= % disease incidence; T= total number of plants; IP= infected plants

Sunflower heads were left to dry and turn brown while still on the stalk before being harvested (85-115 days after planting). Seeds were hand harvested by briskly rubbing the heads to dislodge them from the head, and were then air-dried for 48 hours under a laminar flow bench before being weighed. The trial was run twice and the data was pooled.

b) (i) Dose effect of the best performing yeast and *Bacillus* isolates applied preventatively on sunflower heads for the control of *S. sclerotiorum*

The effect of various concentrations of the most promising yeast (Y79) and *Bacillus* isolates (B24, B26 and B16) were subsequently studied for their preventative action on sunflower heads against *S. sclerotiorum*. Sunflower heads were sprayed until runoff with suspensions of the yeast (1 x 10^7 cells mL^{-1}, 1 x 10^8 cells mL^{-1} and 1 x 10^9 cells mL^{-1}) and *Bacillus* isolates (1 x 10^7 cfu mL^{-1}, 1 x 10^8 cfu mL^{-1} and 1 x 10^9 cfu mL^{-1}). There were four plants per treatment. After 48 hours, the sunflower heads were inoculated with a mycelial suspension of *S. sclerotiorum*. Disease controls were sprayed with distilled water and then with the mycelial suspension after 48 hours. Plants were visually examined for infection after 14 days and disease incidence was

calculated as in 4.2.3 (a). Seeds were harvested, dried and weighed. The trial was run twice and the data was pooled.

(ii) Dose effect of two *Bacillus* isolates, B16 and B26, applied preventatively on sunflower heads for the control of *S. sclerotiorum*

The effect of various concentrations of two *Bacillus* isolates, namely B16 and B26, were studied for their preventative action on sunflower heads against *S. sclerotiorum*. Sunflower heads were sprayed until runoff with different concentrations of the suspensions of both the *Bacillus* isolates: 2.5 x 10^8 cfu mL^{-1}, 5 x 10^8 cfu mL^{-1}, 7.5 x 10^8 cfu mL^{-1} and 2.5 x 10^9 cfu mL^{-1}. There were five plants per treatment. The biocontrol agents were given 48 hours to colonize the sunflower heads before being sprayed until runoff with the mycelial suspension of *S. sclerotiorum*. Disease controls were sprayed with distilled water and then inoculated with the mycelial suspension after 48 hours. A disease-free control was introduced to determine possible growth promotion effects of the *Bacillus* spp. Disease-free control plants were sprayed with distilled water initially and then again with distilled water after 48 hours. Plants were visually examined for infection after 14 days and disease incidence was calculated was calculated as in 4.2.3 (a). Seeds were harvested and weighed. The trial was run twice and the data was pooled.

c) Comparison of single and dual inoculations of selected yeasts, *Bacillus* isolates and T77 applied preventatively on sunflower heads for the control of *S. sclerotiorum*

Combined inoculations were done by mixing formulations of the biocontrol agents before inoculation onto sunflower heads. Y79 and *Bacillus* isolates B16, B24 and B26 were applied at a concentration of 1 x 10^8 cells mL^{-1}/cfu mL^{-1}. T77 was applied at the recommended dosage as before (2 x 10^9 spores mL^{-1}). Treatments were made up of combinations of selected isolates. Sunflower heads were sprayed until runoff with the suspensions. There were four plants per treatment. The biocontrol agents were given 48 hours to colonize the sunflower heads before the heads were inoculated with a mycelial suspension of *S. sclerotiorum*. The mycelial suspension was also sprayed evenly until runoff. Disease controls were sprayed with distilled water until runoff and then inoculated with the mycelial suspension after 48 hours. A disease-free control

was introduced to determine possible growth promotion effects of the isolates. Disease-free control plants were sprayed with distilled water initially and then again with distilled water after 48 hours. Plants were monitored for infection for 14 days and disease incidence was calculated as before. Seeds were harvested and weighed was calculated as in 4.2.3 (a). The trial was run twice and the data pooled.

4.2.4 Statistical analysis

Data was subjected to an analysis of variance (ANOVA) using Genstat® (Version 18) statistical analysis software (GenStat, 2016) to determine differences between treatment means. If the values were significant at $P \leq 0.05$, the means were separated using the Duncan's Multiple Range Test.

4.2.5 Isolate Identification

The best performing yeast and *Bacillus* isolates were sent to Inqaba Biotechnical Industries (Pty) Ltd (Hatfield, South Africa) to be sequenced using the ITS region, and 16S rDNA sequencing, respectively, and identified to the species level. The primers used can be shown in the table below:

16S Primers sequences

Name of Primer	Target	Sequence (5' to 3')
16S-27F	16S rDNA sequence	AGAGTTTGATCMTGGCTCAG
16S-1492R	16S rDNA sequence	CGGTTACCTTGTTACGACTT

4.3 Results

4.3.1 Greenhouse screening of T77, and selected yeast and *Bacillus* isolates against *S. sclerotiorum* on sunflower heads

Table 4.1 (Appendix 4) shows fourteen of the twenty yeast and *Bacillus* isolates screened *in vivo* reduced disease incidence by ≥ 50.0% compared to the disease control. Only one of the isolates, *Bacillus* isolate B16, resulted in 0% disease incidence. B24 and B26, as well as T77, reduced disease incidence to 12.5% compared to the disease control.

Only seven of the twenty isolates significantly improved grain yield compared to the disease control at $P \leq 0.05$. Application of B24, B26, B16 and T77 resulted in grain

yields that were not significantly different to each other ($P \leq 0.05$). Application of B16 resulted in the best grain yield of 58.5 g, followed by T77 (53.6 g).

Fig 4.2 illustrates the reduction in disease incidence and increase in grain yield caused by the fourteen yeast and *Bacillus* isolates, and T77 that reduced head rot in the first *in vivo* evaluation. Disease incidence was closely related to reduced grain yield. Only one yeast isolate (Y79) significantly reduced disease incidence and improved grain yield. In contrast, three *Bacillus* isolates (B24, B26 and B16) produced significant results, controlling the disease and increasing grain yield ($P \leq 0.05$).

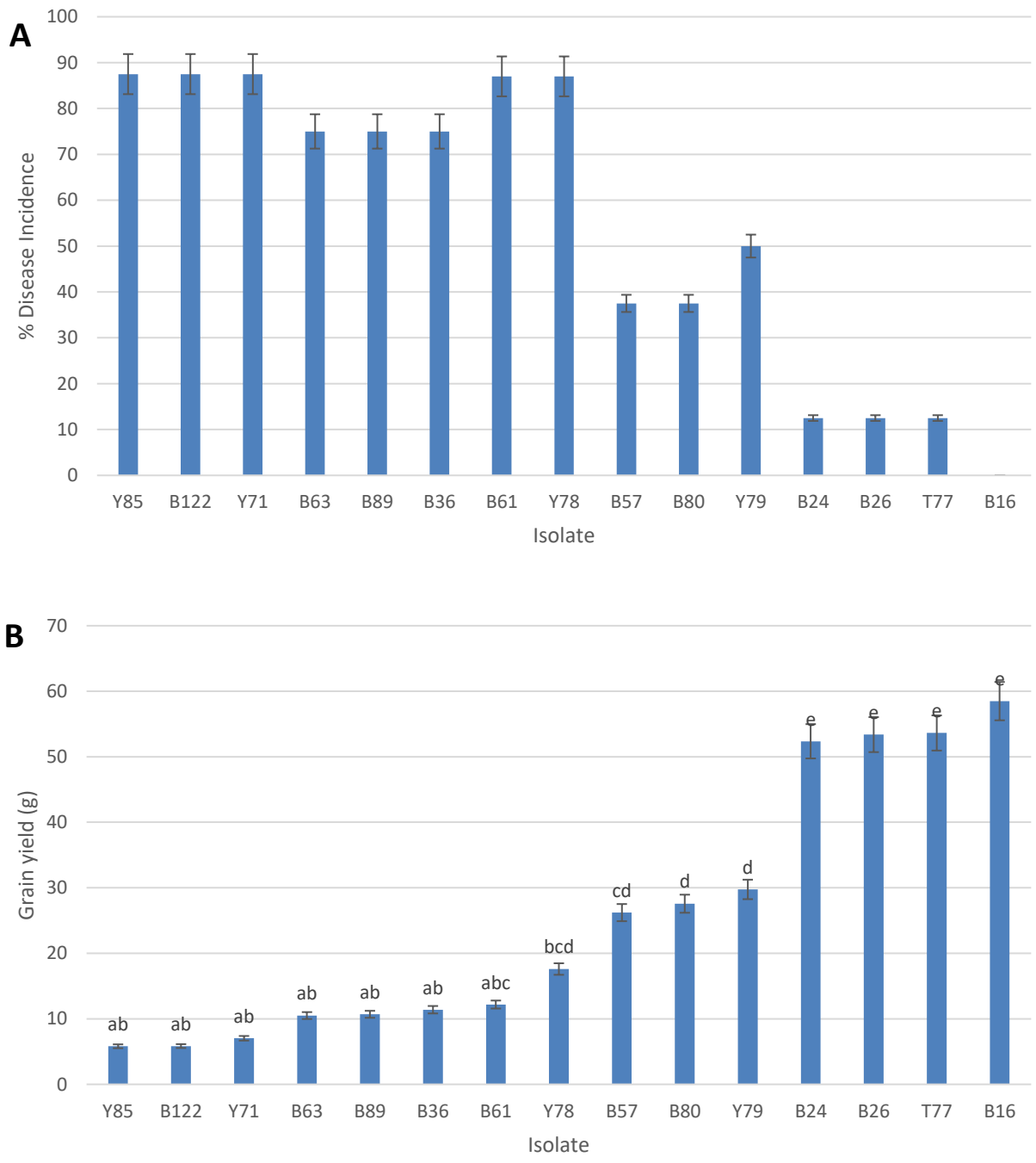
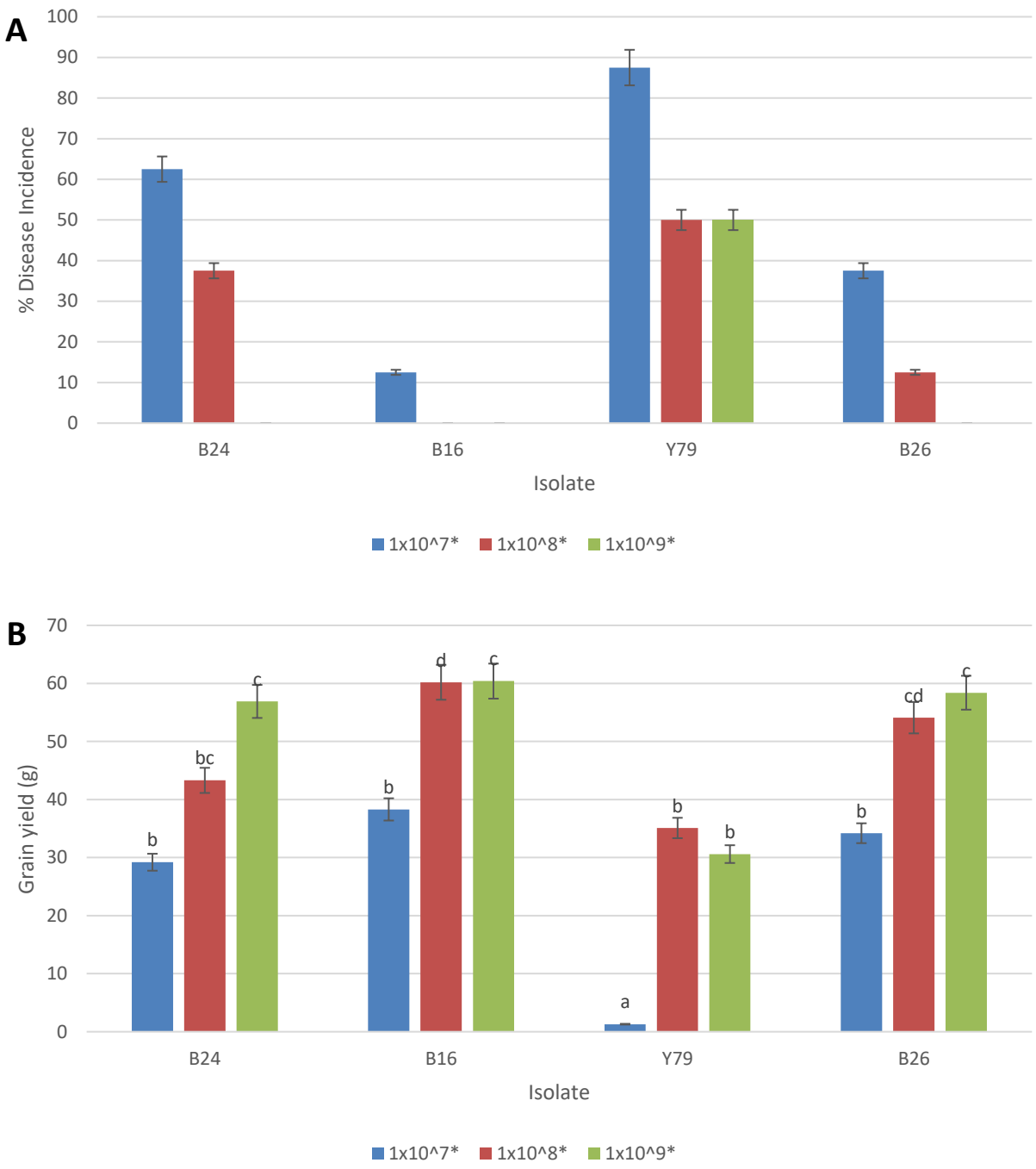


Fig 4.2 Yeast and *Bacillus* isolates which reduced disease incidence (A) and increased grain yield (B) of sunflower heads inoculated with *S. sclerotiorum* in the greenhouse. Different letters above bars indicate significant difference between means (Duncan's Multiple Range Test, $P < 0.05$).

4.3.2 (i) Dose effect studies of selected yeast and *Bacillus* antagonistic to *S. sclerotiorum*

Inoculum levels of the biocontrol agents was important. A concentration of 1 x 10^7 cells mL^{-1} or cfu mL^{-1} was found to be the least effective at reducing head rot incidence and improving grain yield for all four isolates tested (Table 4.2 - Appendix 4). Applying Yeast Y79 to sunflower heads at 1 x 10^8 cells mL^{-1} and 1 x 10^9 cells mL^{-1} both resulted in 50.0% reduction in disease incidence. However, grain yield was 4.5 g higher when Yeast Y79 was applied at 1 x 10^8 cells mL^{-1} compared to 1 x 10^9 cells mL^{-1} (Fig 4.3). *Bacillus* B16 was the only isolate to successfully prevent infection of sunflower heads when applied at 1 x 10^8 cfu mL^{-1}, resulting in 0% disease incidence. All three *Bacillus* isolates prevented infection at 1 x 10^9 cfu mL^{-1}. The highest grain yields were measured when *Bacillus* isolates were applied at 1 x 10^9 cfu mL^{-1}. However, grain yield did not differ significantly between them ($P \leq 0.05$).

Fig 4.3 shows that even at the lowest concentration of 1 x 10^7 cfu mL^{-1}, *Bacillus* B24 was still able to improve grain yield, even in the presence of *S. sclerotiorum* head rot. Of the four isolates, Yeast Y79 reduced disease incidence and improved grain yield the least (Fig 4.3). Table 4.2 (Appendix 4) shows, however, that Yeast Y79 applied at 1 x 10^8 and 10^9 cells mL^{-1} improved grain yield significantly, compared to the disease control at $P \leq 0.05$.



* cells mL^{-1} and cfu mL^{-1} for yeast and *Bacillus* isolates, respectively.

Fig 4.3 Effect of concentration of yeast and *Bacillus* suspensions on head rot incidence (A) and grain yield (B) of sunflower heads, inoculated with *S. sclerotiorum* in the greenhouse. Different letters above bars of the same colour indicate significant difference between means (Duncan's Multiple Range Test, *P* < 0.05).

(ii) Dose effect of B16 and B26 applied preventatively on sunflower heads for the control of *S. sclerotiorum*

B16 completely prevented *S. sclerotiorum* infection at 7.5 x 10^8 cfu mL^{-1} (Table 4.3 - Appendix 4). Applied at 2.5 x 10^9 cfu mL^{-1}, B16 did not prevent infection, resulting in disease incidence of 100.0%. The highest grain yield was obtained when B16 was applied at 7.5 x 10^8 cfu mL^{-1} ($P \leq 0.05$). B26 also significantly improved grain yield compared to the disease-free control ($P \leq 0.05$) when applied at 2.5 x 10^9 cfu mL^{-1}.

Fig 4.5 illustrates that applying B16 and B26 at a concentration of 2.5 x 10^9 cfu mL^{-1} and 7.5 x 10^8 cfu mL^{-1}, respectively resulted in poor grain yield that was not significantly different ($P \leq 0.05$) to the disease control (Table 4.3 - Appendix 4). B16 and B26 provided different levels of protection to *S. sclerotiorum* at different concentrations (Fig 4.4).

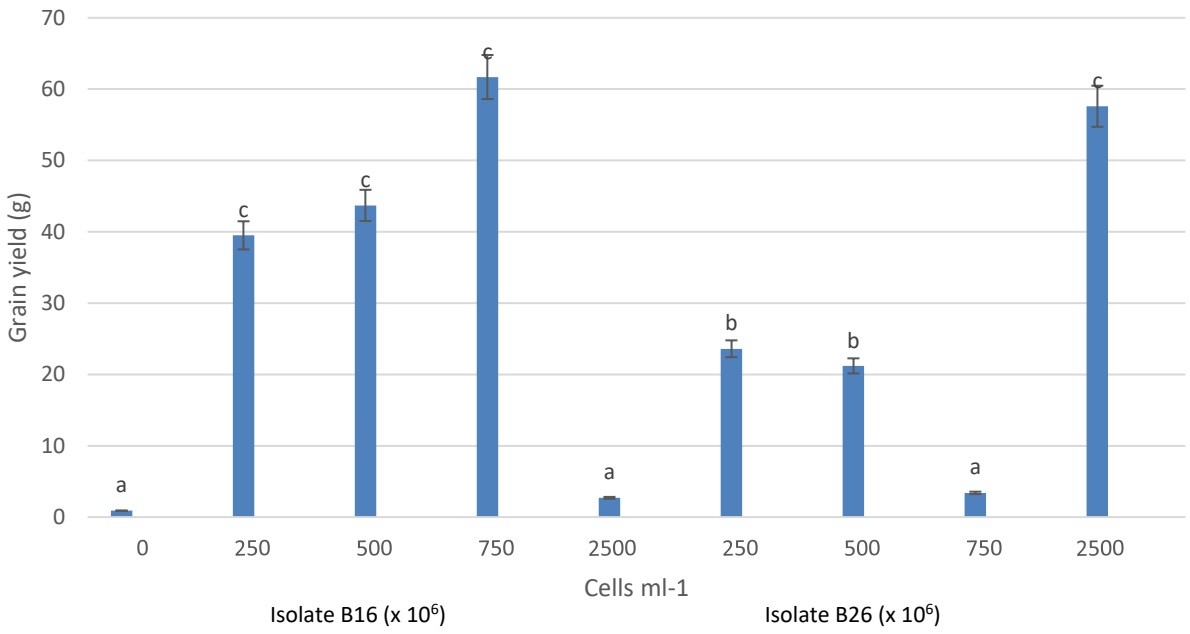


Fig 4.4 Effect of concentration of B16 and B26 on grain yield of sunflower heads inoculated with *S. sclerotiorum* in the greenhouse. Different letters above bars indicate significant difference between means (Duncan's Multiple Range Test, *P* < 0.05).

4.3.3 Single and dual inoculations of selected yeasts, *Bacillus* isolates and T77 to control head rot on sunflower caused by *S. sclerotiorum*

Table 4.4 (Appendix 4) shows that a combined inoculation of *Bacillus* B26 and B24 resulted in the highest mean grain yield of sunflower (65.8 g) which was significantly greater ($P \leq 0.05$) than 8 of the 17 treatments. This was closely followed by single inoculations of B16, T77, B26 and B24, and combined inoculations of B16 + B26, T77 + B26 and B16 + B24 (Fig 4.5 B). Although 6 of the treatments achieved a higher grain yield than the disease-free control (50.2 g), they did not differ significantly ($P \leq 0.05$). B24, B26, T77 + B26, B16, B16 + B26 and B26 + B24 all effectively prevented infection, resulting in 0% disease incidence (Fig 4.5 A).

Only two treatments, B16 + B26 and B26 + B24, provided over 10.0% increase in grain yield (12.8% and 15.5%, respectively) over the disease-free control (Table 4.4 - Appendix 4). The remainder of the single treatments and/or combinations recorded yield increases between 3.0-9.6% over the disease-free control, with the rest of the treatments recording grain yields lower than the uninoculated control (Table 4.4).

Yeast Y79 performed poorly; disease incidence was 87.5% and grain yield was 11.8 g. These findings do not correlate with those obtained in prior trials in Sections 4.3.1 and 4.3.2 (i). However, when co-inoculated with B16 or B24, disease incidence was lower (62.8% and 37.5%, respectively) (Fig 4.5 A). Sunflower treated with Y79 + B16 and Y79 + B24 also had higher grain yields (15.8 and 36.0 g, respectively) than a single inoculation of Y79 (11.8 g) (Table 4.4 - Appendix 4). As can be seen in Fig 4.6 B, B24, B16, B26, T77 and their respective combinations resulted in grain yields that did not differ significantly to each other ($P \leq 0.05$).

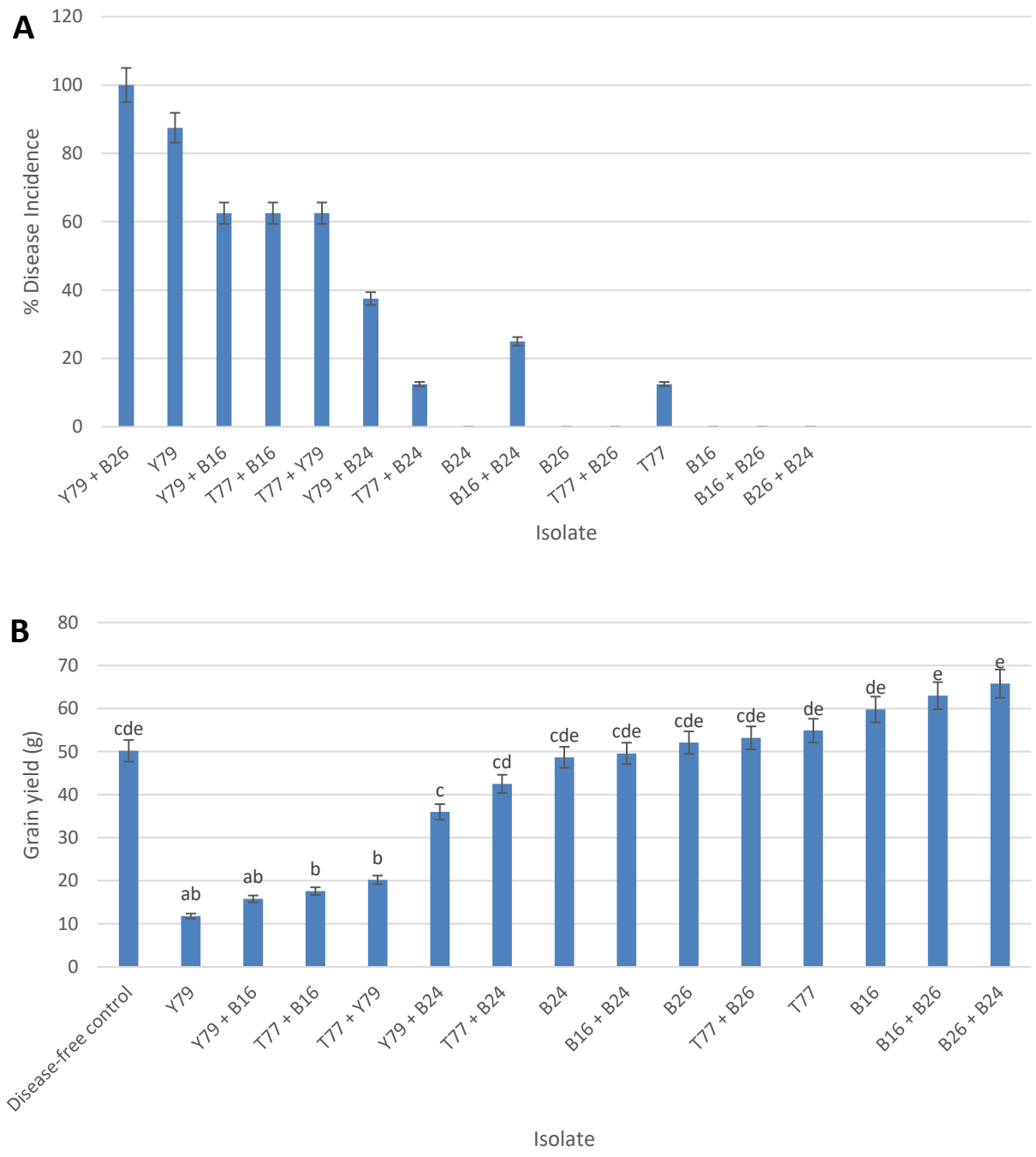


Fig 4.5 Effects of single, dual and triple BCA combinations of Yeast Y79, *Bacillus* isolates and *Trichoderma* T77 inoculated with *S. sclerotiorum* in the greenhouse. Different letters above bars indicate significant difference between means (Duncan's Multiple Range Test, $P < 0.05$).

4.3.4 Isolate identification

The isolates were identified as follows:

Table 4.1 BLAST identification for selected yeast and *Bacillus* isolates

Name of sample **Identified organism**	B16 *Bacillus subtilis*
Name of sample **Identified organism**	B24 *Bacillus subtilis* or *Bacillus tequilensis*
Name of sample **Identified organism**	B26 *Bacillus siamensis or Bacillus amyloliquefaciens (B. velezensis)*
Name of sample **Identified organism**	Y79 *Filobasidium oeirense,* synonym *Cryptococcus oeirensis*

4.4 Discussion

A lack of effective fungicides and concern over the impact of chemical pesticides on the environment has resulted in an increased interest in biocontrol strategies for the management of *Sclerotinia sclerotiorum*. The use of biological control to reduce losses due to sunflower head rot caused by *S. sclerotiorum* has not been widely investigated. There are currently no registered biofungicides for use against head rot in sunflower. Greenhouse screening of natural enemies of *S. sclerotiorum* with the aim of preventing or reducing disease incidence and yield loss is necessary for future development of biofungicides.

The major objective of this study was to investigate the potential biological control ability of a selected yeast and several *Bacillus* strains previously isolated (Chapter 2) using *in vivo* trials, compared with a commercial strain of the fungus *Trichoderma atroviride* (strain 77). Dosage effects were tested on the four best performing isolates. The potential to enhance biocontrol using combined applications of these isolates and T77 was investigated thereafter. *Bacillus* isolates showed significant control of head rot in greenhouse studies, while the yeast isolates did not provide as effective control of head rot.

The most widely studied biocontrol agents for the management of *S. sclerotiorum* include mycoparasitic fungi and hypervirulent strains of the target fungus (Fernando *et al*., 2007).The parasitic fungus *Coniothyrium minitans*, under the trade name Contans®, has been widely studied for it's antagonistic potential against *S.*

sclerotiorum. Drenching soil with *C. minitans* suppresses carpogenic germination of sclerotia (Vrije e*t al*., 2001). However, few attempts have been made to explore the possibility of bacterial and fungal biocontrol agents for the management of *Sclerotinia* head rot of sunflower.

In this greenhouse screening of 20 yeast and *Bacillus* isolates, several *Bacillus* isolates demonstrated antagonism against *S. sclerotiorum* head rot. These findings confirmed *in vitro* screenings conducted in Chapter 2, in which more *Bacillus* isolates successfully inhibited mycelial growth of *S. sclerotiorum in vivo* than yeast isolates. Additionally, the isolates that demonstrated effective inhibition of *S. sclerotiorum* mycelial growth *in vitro* also significantly reduced disease incidence in the greenhouse. This was contrary to the findings and recommendations of Williams and Asher (1996) who found no correlation between biological control *in vivo* and antifungal activity *in vitro* when bacterial isolates that showed strong antifungal activity against *Pythium ultimum in vitro*, were tested *in vivo*. They further concluded that the bacterial isolates that showed strong antifungal action *in vitro* were not necessarily good biological control agents *in vivo*. However, Askew and Laing (1994) reported that out of 118 *Trichoderma* isolates, 92% identified as antagonistic to *Rhizoctonia solani in vitro* significantly reduced damping-off in the nursery.

The best performing yeast isolate, Y79, later identified as *Filobasidium oeirense* (synonym *Cryptococcus oeirensis*), reduced disease incidence by 50.0% in the first and second evaluations but did not provide consistent results in the third evaluation when high disease incidence occurred (87.5%). This may indicate that the isolate would not provide reliable protection against *Sclerotinia* head rot as a commercial BCA. Previous studies have reported antifungal activity of a closely related yeast, *C. laurentii,* against postharvest gray mold, blue mold and *Rhizopus* decay of peach caused by *Botrytis cinerea*, *Penicillium expansum* and *Rhizopus stolonifera,* respectively, and against anthracnose of mango caused by *Colletotrichum gloeosporioides* (Zhang *et al*., 2007; Bautista-Rosales *et al*., 2014). However, this is the first reported study in which *F. oeirense* has been isolated in South Africa and tested for its antagonistic activity against *S. sclerotiorum*.

A greenhouse study of the biocontrol activities of different concentrations of the best performing yeast and three *Bacillus* isolates against *S. sclerotiorum* head rot

demonstrated that greater biocontrol efficacy was achieved when Yeast Y79 was applied at a concentration of 1 x 10^8 cells mL^{-1}. This is in agreement with findings of Tian *et al*. (2002) in which *Candida guilliermondii* and *Penicillium membranefaciens* completely controlled infection by *R. stolonifer* on peaches and nectarines at a concentration of 10^8 cells mL^{-1}. Isolate B16 also completely prevented infection at 10^8 cfu mL^{-1}, whereas B26 and B24 only completely prevented infection when applied at the higher concentration of 10^9 cfu mL^{-1}. However, application of all three *Bacillus* isolates at a concentration of 10^9 cfu mL^{-1} to sunflower resulted in the highest grain yields. Yobo *et al*. (2011) reported similar findings when *Bacillus* sp. cell suspensions were applied to dry bean and cucumber at 10^9 cfu mL^{-1} and suppressed damping-off caused by *R. solani*.

A further look at the effect of concentration of application of B24 and B26, the two best performing isolates, revealed that optimal concentration of two isolates of *Bacillus*, isolated from the same source, in this case, sunflower heads, can differ. With B16, a concentration of 7.5 x 10^8 cfu mL^{-1} resulted in complete disease control and the highest grain yield, whereas at that dose no disease control was provided by B26. Conversely, B26 applied at a concentration of 2.5 x 10^9 cfu mL^{-1} greatly reduced disease incidence, whereas B16 applied at the same concentration was ineffective at controlling the disease.

Both *Bacillus* isolates appeared to cause significantly higher grain yields than the disease-free control, indicating that B16 and B26 may be plant growth promoting bacteria (PGPB). The majority of PGPB have been studied in the soil environment, especially in the rhizosphere and rhizoplane. However, some of them move onto the aerial parts of plants (Compant e*t al*., 2010). The scientific data regarding the positive interaction of certain bacteria strains and plants are increasing. Researchers are now trying to optimize Soil-Plant-PGPB systems (Ruzzi and Aroca, 2015), in order to maximize the positive effects of PGPB. The mode of action utilized by PGPB to enhance plant growth and yield is not clearly defined. However, many researchers have reported that certain species and strains of PGPB have the ability to produce growth regulators, are responsible for N_2 fixation, create an antagonistic environment for phytopathogens and they can solubilize mineral phosphates (Nieto and Frankenberger, 1989; Kumar *et* al., 2001; Fernando *et al*., 2007; Ahmed *et al*., 2008; Corréa *et al*., 2014; Ahmadi-Rad *et al*., 2016; Romero-Perdomo, 2017). The results

obtained in this study, however, are by no means conclusive as greenhouse conditions provide a favourable environment for plant growth and this may to mask the effect of the different concentrations of the isolates. It is more likely that the effects on plant growth will be expressed more clearly under periods of stress, as found in the field (Rabeendran *et al*., 2000). Schroth and Bevker (1990) found erratic and inconsistent performances of PGPB under field conditions and Kloepper *et al*. (1989) reported increases in yield in response to inoculation of PGPB to a range of crops with decreases in yield also being common in trials. Field trials are thus needed in this regard.

Single and dual inoculation trials in the greenhouse demonstrated all isolates and their various combinations reduced the incidence of sunflower head rot caused by *S. sclerotiorum*, with the exception of a combined inoculation of Yeast Y79 and *Bacillus* B26, which failed to prevent or reduce infection. A general trend was that those isolates that reduced disease when inoculated alone, also worked well in combinations. In the cases of combined inoculations of T77 + B26 and B16 + B26, they completely prevented head rot when inoculated in combination with other isolates. Furthermore, it was found that single inoculations of T77 and B16 and combined inoculations of T77 and B26 as well as B16 and B26 increased grain yield. These combinations appear to have a synergistic effect. The synergistic effect between different biocontrol agents in combination in general is due to different components expressing different protection mechanisms (Jetiyanon and Kloepper, 2002; Yobo *et al*., 2011; Boer e*t al*., 2003). The association of different biocontrol agents have been shown to intensify disease control, for example, with a combination of *Pichia guilliermondii* and *B. mycoides* on strawberry leaves affected by *B. cinerea*, where the mechanism of protection, parasitism and production of toxic compounds acted together (Guetsky *et al*., 2001). Combination may also increase the spectrum of diseases controlled, as seen in the combination of *B. pumilus*, *Curtobacterium flaccumfaciens* and *B. subtilis* inducing resistance and producing antibiotics to control *Pseudomonas syringae* pv. *lachrymans*, *Erwinia tracheiphila* and *C. orbiculare* in cucurbits (Raupach and Kloepper, 1998).

The results presented in this study suggest that using mixtures of yeasts, *Trichoderma* and *Bacillus* spp. show potential to enhance biological control of *S. sclerotiorum* and/or increase seedling growth and establishment. Several reports have shown that individual yeasts, *Trichoderma* and *Bacillus spp*. can suppress plant pathogen

activities and enhance plant growth. As shown in this study, a combination of these organisms could lead to an increase in disease suppression and plant growth. Modes of action and compatibility of the intended isolates to be combined will determine whether or not this combination performs maximally. Modes of action that complement each other between organisms can be exploited, leading to increased synergism and activity, particularly under variable environmental conditions (Raupach and Kloepper, 1998) and where more than one plant pathogen is present. Mixtures of those organisms may greatly benefit organic farming (Raupach and Kloepper, 1998) or may be used in concurrence with a reduced rate of chemical fungicides. The *Bacillus* isolates were the main contributors to disease suppression as well as growth promotion, and are unlikely to be killed by fungicides.

Selected *Bacillus* isolates and T77 provided consistent control of *S. sclerotiorum* head rot on sunflower heads compared with the best yeast isolate. In practice, due to production of spores by *Trichoderma* and *Bacillus* spp., these BCAs are easier to formulate than other organisms such as fluorescent pseudomonads. However, combining the two organisms as a mixture and as a commercial product may be impractical considering the higher production and registration costs than would be incurred by registering a single strain (Schiller *et al*., 1997).

16S rDNA sequence analysis revealed that the best performing isolate, B16, was *B. subtilis*, while B24 and B26 were predicted to be *B. subtilis* or *B. tequilensis*, and *B. siamensis* or *B. amyloliquefaciens*, respectively. *B. tequilensis* has been reported as an endophyte (Eldeen e*t al*., 2015; Li *et al*., 2018; Bhattacharya e*t al*., 2019) with biocontrol potential against *Magnaporthe oryzae* (rice blast), *Fusarium oxysporum* (tomato wilt) and pathogenic bacterial strains infecting mangrove plants. As there are no reports of this *Bacillus* species also existing as an epiphyte and since all the isolates in this study were isolated from the surface of sunflower heads, it is likely that B24 is in fact a *B. subtilis*. Similarly, *B. siamensis* has more widely been reported as a rhizobacterium (Hussain and Khan, 2020) in which it demonstrated biocontrol against *Macrophomina phaseolina* on bean plants and *Alternaria alternata* brown spot on tobacco, respectively. It is thus more likely that B26 is *B. amyloliquefaciens*.

B. subtilis and *B. amyloliquefaciens* have been reported to control tomato wilt disease and powdery mildew in cucumber and *Sclerotinia s*tem rot in cucumber, respectively

(Zhang and Xue, 2010; Chen e*t al*., 2013). However, this is the first reported study in which these *Bacillus* species have been isolated in South Africa and their potential antagonistic ability assessed against *S. sclerotiorum* of sunflower.

Further research would be to conduct field trials in various sunflower production areas across South Africa where *Sclerotinia* head rot is prevalent, using popular sunflower cultivars. Other research would test the ability of the biocontrol agents to control *S. sclerotiorum* curatively wherein the BCAs are applied to already infected sunflower heads, under controlled conditions and then in the field, if successful.

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Appendix 4.

Table 4.2 Biocontrol activity of selected strains of yeasts and *Bacillus* spp, and *Trichoderma* T77 against head rot caused by *S. sclerotiorum* on sunflower heads

Isolate/Treatment	Disease Incidence (%)	Grain yield (g)
Disease control	100.0	0.8 a
Y86	100.0	0.0 a
Y56	100.0	0.0 a
Y68	100.0	0.7 a
B62	100.0	1.0 a
B53	100.0	1.3 a
B84	100.0	1.5 a
Y85	87.5	5.8 ab
B122	87.5	5.8 ab
Y71	87.5	7.0 ab
B63	75.0	10.7 ab
B89	75.0	10.7 ab
B36	75.0	11.4 ab
B61	87.5	12.2 abc
Y78	87.5	17.6 bcd
B57	37.5	26.2 cd
B80	37.5	27.6 d
Y79	50.0	29.7 d
B24	12.5	52.4 e
B26	12.5	53.4 e
T77	12.5	53.6 e
B16	0.0	58.5 e
F-ratio		**27.3**
P-level		**< 0.001**
LSD		**13.3**
CV%		**62.1**

Means followed by different letters are significantly different (Duncan's Multiple Range Test, $P < 0.05$).

Table 4.3 Mean disease incidence and grain yield of sunflower inoculated with various concentrations of a selected yeast, *Bacillus* isolates and *Trichoderma* T77, and subsequently inoculated with *S. sclerotiorum*

Isolate/Treatment	Disease Incidence (%)			Grain yield (g)		
	1 x 10^7*	**1 x 10^8***	**1 x 10^9***	**1 x 10^7***	**1 x 10^8***	**1 x 10^9***
Disease control	100.0	100.0	100.0	0.8 a	0.8 a	0.8 a
Y79	87.5	50.0	50.0	1.3 a	35.1 b	30.6 b
B24	62.5	37.5	0.0	29.2 b	43.3 bc	56.9 c
B26	37.5	12.5	0.0	34.2 b	54.1 cd	58.4 c
B16	12.50	0.0	0.0	38.3 b	60.2 d	60.4 c
F-ratio				**14.9**	**19.9**	**47.9**
P-level				**< 0.001**	**< 0.001**	**< 0.001**
LSD				**13.6**	**15.0**	**10.7**
CV%				**64.5**	**38.1**	**25.4**

Means followed by different letters are significantly different (Duncan's Multiple Range Test, $P < 0.05$).

* cells mL^{-1} and cfu mL^{-1} for yeast and *Bacillus* isolates, respectively.

Table 4. 4 Mean disease incidence and grain yield of sunflower treated with different concentrations of *Bacillus* isolates B16 and B26, and subsequently inoculated with *S. sclerotiorum*

Isolate/Treatment	Disease Incidence (%)				Grain yield (g)			
	2.5 x 10^{8}*	5 x 10^{8}*	7.5 x 10^{8}*	2.5 x 10^{9}*	2.5 x 10^{8}*	5 x 10^{8}*	7.5 x 10^{8}*	2.5 x 10^{9}*
Disease control	100.0	100.0	100.0	100.0	0.9 a	0.9 a	0.9 a	0.9 a
Disease-free control	0.0	0.0	0.0	0.0	50.6 c	50.6 c	50.6 b	50.6 b
B26	40.0	50.0	100.0	10.0	23.6 b	21.2 b	3.4 a	57.6 c
B16	50.0	20.0	0.0	100.0	39.5 c	43.7 c	61.7 c	2.7 a
F-ratio					**22.3**	**39.9**	**1025.2**	**188.4**
P-level					**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
LSD					**13.1**	**10.3**	**2.8**	**6.3**
CV%					**50.3**	**38.8**	**10.7**	**25.0**

Means followed by different letters are significantly different (Duncan's Multiple Range Test, $P < 0.05$).

* cfu mL^{-1} per *Bacillus* isolate.

Table 4.5 Mean disease incidence and grain yield of sunflower after single and dual inoculations of Yeast Y79, *Bacillus* isolates and *Trichoderma* T77, and subsequently inoculated with *S. sclerotiorum*

Isolate/Treatment	Disease Incidence (%)	Grain yield (g)	% Grain yield (% of disease-free control)
Disease control	100.0	1.0 a	n/a
Disease-free control	0.0	50.2 cde	100.0 [0]
Y79 + B26	100.0	0.0 a	n/a
Y79	87.5	11.8 ab	61.6 [-38.4]
Y79 + B16	62.5	15.8 ab	65.6 [-34.4]
T77 + B16	62.5	17.6 b	67.4 [-32.6]
T77 + Y79	62.5	20.2 b	70.0 [-30.0]
Y79 + B24	37.5	36.0 c	85.8 [-14.2]
T77 + B24	12.5	42.5 cd	92.3 [-7.7]
B24	0.0	48.7 cde	98.4 [-1.56]
B16 + B24	25.0	49.6 cde	99.4 [-0.61]
B26	0.0	52.1 cde	98.1 [-1.9]
T77 + B26	0.0	53.2 cde	103.0 [3.0]
T77	12.5	54.9 de	104.7 [4.7]
B16	0.0	59.8 de	109.6 [9.6]
B16 + B26	0.0	63.0 e	112.8 [12.8]
B26 + B24	0.0	65.8 e	115.5 [15.5]
F-ratio		**16.8**	
P-level		**< 0.001**	
LSD		**15.0**	
CV%		**40.2**	

Means followed by different letters are significantly different (Duncan's Multiple Range Test, $P < 0.05$).

CHAPTER 5

The use of *Trichoderma* spp. to control damping-off of sunflower caused by *Sclerotinia sclerotiorum*, *Sclerotium rolfsii* and *Rhizoctonia solani*

Abstract

The aim of this study was to evaluate the potential of a commercial *Trichoderma* strain, *T. asperellum* strain kd (Tkd) to control damping-off of sunflower caused by *Sclerotinia sclerotiorum*, *Rhizoctonia solani* and *Sclerotium rolfsii*. *In vitro* antifungal activity of Tkd on all three pathogens was assayed. Each dual-culture bioassay was assessed for antibiosis, antagonism and/or invasion ability. Tkd effectively inhibited the growth of pathogen mycelium and was able to rapidly invade and overgrow pathogen mycelium within 7 days. Ultrastructural studies of each of the Tkd-pathogen interactions demonstrated evidence of mycoparasitism by Tkd against all three pathogens. In the greenhouse, the effect of application method of the bio-fungicide on disease suppression and growth promotion was investigated. Seed treatment with Tkd alone yielded the highest increase in germination for seeds inoculated with *R. solani*, while seed treatment in combination with a bi-monthly drench of Tkd lead to maximum germination of seeds inoculated with *S. sclerotiorum* and *S. rolfsii*. Applying Tkd as a seed treatment at planting and as a monthly to bi-monthly soil drench was the most effective at controlling damping-off caused by the three pathogens. Rhizotron studies revealed that the application of Tkd either as a seed treatment and/or soil drench significantly improved dry shoot and root weight and root area of seedlings, even in the presence of each pathogen, which was inoculated into the growth media at planting. Similarly, plant height and dry head weight of mature sunflower plants improved significantly, compared to the disease and disease-free controls, despite the presence of the pathogens in the growth media.

5.1 Introduction

Root diseases are one of the many factors that preclude the full expression of the inherited potential of a crop and are some of the most widespread, destructive diseases of several economically important crops, including sunflowers. Soil-borne pathogens belonging to the *Fusarium*, *Phytophthora*, *Pythium*, *Rhizoctonia* and *Sclerotinia* genera are endemic in most soils and often cause severe damage to seedlings. Diseases such as seed rot, pre-emergence and post-emergence damping-off may subsequently lead to significant stand losses upon plant establishment (Altier and Thies, 1995). Damping-off is a disease that causes decay of germinating seeds and death of young seedlings, and is a major yield cont both in nurseries and fields (Lamichhane e*t al*., 2017). Economic losses caused by damping-off are represented by a direct cost as a result of damage to seed or seedlings, and indirectly, by necessitating replanting and reduced yield due to later planting dates (Babadoost and Islam, 2003; Bacharis *et al*., 2010; Horst, 2013).

Also concerning is the environmental impact due to widespread use of chemical fungicides to manage this frequently occurring disease. Intensive use of chemical fungicides has resulted in the build-up of resistance in isolates which has added to the management challenges for farmers (Moorman *et al*., 2002; Taylor *et al*., 2002; Lamichhane *et al*., 2016). In light of the significant economic impact of damping-off and the undesirable environmental impact caused by conventional fungicide-intensive control strategies, sustainable alternatives to manage the disease need to be developed. Integrated pest management (IPM) provides a sustainable approach to this aim by combining measures (e.g., enhancement of seed health, which represents the core of resilient agroecosystems) in addition to effective cultural and agronomic practices first and pesticide-based control as the last option (Lamichhane *et al*., 2017).

Using biological control in an integrated management system offers a cost-effective, practical and non-toxic method to prevent damping-off of sunflower by *Sclerotinia sclerotiorum*, *Rhizoctonia solani* and *Sclerotium rolfsii*, soil-borne phytopathogens which cause damping-off. *Trichoderma* species are widely known for their ability to parasitize a number of important soil-borne pathogens, including *Rhizoctonia* and *Sclerotinia* (Bell *et al*., 1982; Hadar *et al*., 1984; Coping 2009). Several species belonging to these genera are hyperparasitic to pathogenic fungi and have shown to coil around pathogen hyphae and subsequently leach nutrients from the pathogen

(Elad 2000; Coping 2009). Mycoparasitism involves the degradation of a fungal cell wall by an antagonist via the production of lytic enzymes (Lorito *et al*., 1993; Lima *et al*., 1997). Mycoparasitism of plant pathogenic fungi by *Trichoderma* species has often been reported in *in vitro* studies. Enzymes that hydrolyse fungal cell wall components such as chitinase and glucanase have been shown to play a crucial role in cell wall lysis (Lorito *et al*., 1993; Lorito *et al*., 1994; Lima *et al*., 1997; Menendez and Godeas, 1998). The mycoparasitic action of *Trichoderma* on phytopathogens has been suggested to be a synergistic action of hydrolytic enzymes such as chitinases, lipases, proteases and glucanases (Benhamou and Chet, 1996).

T. asperellum strain kd (Tkd) and *T. atroviride* strain 77 (T77) are registered, formulated biocontrol products (Eco-T® and Eco-77®, respectively) known for their ability to colonise damaged or senescing plant tissues, thus preventing entry of harmful pathogens. The present research aimed to investigate the biocontrol potential of these specific strains of *Trichoderma* species against damping-off of sunflower caused by *Sclerotinia sclerotiorum, Rhizoctonia solani* and *Sclerotium rolfsii*. The primary objectives of this research were (1) to investigate and demonstrate the antagonistic ability of these *Trichoderma* strains in supressing growth of the pathogens in *in vitro* laboratory tests and (2) to evaluate the potential of *Trichoderma* to reduce and possibly prevent damping-off under greenhouse conditions.

5.2 Materials and methods

5.2.1 Source of inoculum

The *Sclerotium rolfsii* strain used in this study was isolated from an indigenous lily, *Clivia miniata* (Lindley) Regel, obtained from the display garden of Prof. M.D. Laing[9] in Wembley, Pietermaritzburg, KwaZulu-Natal, South Africa. The plant exhibited typical disease symptoms characteristic to this pathogen; white mycelial mats and sclerotia could be seen at and near the soil surface on infected plant tissues. Sections (1 cm long) of diseased lily leaves were surface sterilized by rinsing with 70% ethanol for 30 seconds. The 70% ethanol was discarded and 1% sodium hypochlorite solution was added for further sterilization for 30 seconds. The 70% ethanol was discarded and followed by two washes in sterilized distilled water (dH_2O) and placed on sterilized

[9] Prof. M.D. Laing, Discipline of Plant Pathology, University of KwaZulu-Natal, Private Bag X01, Scottsville, 3209, South Africa.

paper towel on a laminar flow bench and allowed to dry for 1 minute. The leaf sections were placed on 85 mm Petri dishes containing Potato Dextrose Agar (PDA) (Lasec[10]) and incubated for 5 days at 25°C. The cultures were sub-cultured and examined daily for evidence of mycelial growth. Wet mount slides of the culture were prepared and viewed using a light microscope (Axiolab 5, ZEISS, Germany . Samples were confirmed to be *Sclerotium* sp. based on characteristic morphological structures as described in Bolton *et al*. (2006).

Three isolates of *Rhizoctonia solani* were provided by Dr M.J. Morris[11] in the form of mycelia grown on PDA. All three isolates were sub-cultured onto fresh PDA and incubated at 25°C for five to 7 days. A mini seedling trial using susceptible cucumber cultivar, Ashley (Starke Ayres[12]) was carried out to identify the *R. solani* isolate which was the most virulent. Speedling 24® trays (24 cells per tray) were filled with seedling mix (Gromor[13]) and one cucumber seed was planted into each cell of the Speedling 24® trays, and inoculated with a fungal colonized agar block placed 4 cm from the seed at planting. The seedlings were allowed to germinate and the isolate which resulted in the highest damping-off incidence and severity was selected for use in this study.

The *Sclerotinia sclerotiorum* strain used in this study was provided by Miss L.A. Rothmann[14] in the form of sclerotia.

The *Trichoderma* spp. used in this study were those found in the commercial strains Eco-T® and Eco-77®[15] (active ingredients *Trichoderma asperellum* strain kd and *T. atroviride* strain 77, respectively). Both powders were inoculated onto PDA and incubated at 25°C for 5 days. The cultures were then sub-cultured and incubated at the same temperature for three to 5 days before use.

[10] Lasec SA (Pty) Ltd., 52 Old Mill Road, Ndabeni, Cape Town, 7405, South Africa.
[11] Dr. M.J. Morris, Managing Director, Plant Health Products (Pty) Ltd., Nottingham Road, KwaZulu-Natal, 3280, South Africa.
[12] Starke Ayres (Pty) Ltd., Willowton Road, Willowton, Pietermaritzburg, KwaZulu-Natal, 3201, South
[13] Gromor (Pty) Ltd., P.O. Box 89, Cato Ridge, KwaZulu-Natal, 3680, South Africa.
[14] Miss Lisa Rothmann, Department of Plant Sciences, University of the Free State, Bloemfontein, 9300, South Africa.
[15] Plant Health Products (Pty) Ltd., P.O. Box 207, Nottingham Road, KwaZulu-Natal, 3280, South Africa.

5.2.2 *In vitro* antagonism of *Trichoderma* spp. against *S. sclerotiorum*, *S. rolfsii* and *R. solani*

a) Dual-culture bioassay

In vitro dual-culture bioassays were performed by placing colonized agar squares (4 x 4 mm) of a selected *Trichoderma* isolate and the test pathogen on opposite sides of a Petri dish (85mm diameter) containing PDA medium. The bioassay was replicated three times for each *Trichoderma* isolate and plates were incubated in the dark for 5 days at 25°C. Controls for each *Trichoderma* isolate as well as the test pathogens were also established on PDA medium and incubated with the test plates. The experiment was repeated once.

After the control plates were fully colonized by the test pathogens, each dual-culture bioassay plate was assessed for antibiosis, antagonism and/or invasion ability. Antimicrobial activity was interpreted as zones of inhibition on the dual-culture test plates. A distinct zone of inhibition between a *Trichoderma* isolate and test pathogens was attributed to the production of antimicrobial compounds. The degree of antagonism of each isolate towards the test pathogens was rated according to the rating system of Bell *et al*. (1982), based on a scale of 1-5:

Class 1 = *Trichoderma* completely overgrew the test pathogen and covered the entire medium surface;

Class 2 = *Trichoderma* overgrew at least two thirds of the medium surface;

Class 3 = *Trichoderma* and the test pathogen each colonized approximately one-half of the medium surface and neither organism appear to dominate the other;

Class 4 = The test pathogen colonized at least two-thirds of the medium surface and appear to withstand encroachment by *Trichoderma*;

Class 5 = The test pathogen completely overgrew the *Trichoderma* and occupied the entire medium surface.

According to Bell *et al*. (1982), a *Trichoderma* isolate is considered to be antagonistic towards a fungal pathogen if the mean score is ≤2, but is not highly antagonistic if the number is ≥3. Plates were subsequently incubated for a further 2 days and then rated again for invasion ability using a rating system developed by Yobo (2005), which was adapted to apply to all three damping-off pathogens used in this study.

Class 1 = *Trichoderma* completely overgrew the test pathogen and invaded the entire plate. Sporulation of *Trichoderma* was apparent on all sections of the plate after 7 days. The test pathogen mycelium turned brown;

Class 2 = *Trichoderma* completely overgrew the test pathogen and invaded the entire plate. Sporulation of *Trichoderma* was evident on all sections of the plate after 7 days. No discolouration of the test pathogen mycelium occurred;

Class 3 = *Trichoderma* colonized 50% of the plate from the point of contact with the test pathogen and patches of sporulation of *Trichoderma* were evident on sections of plate where *Trichoderma* invaded the test pathogen; and

Class 4 = *Trichoderma* colonized less than 50% of the test pathogen from the point of contact with the test pathogen, and little or no sporulation of *Trichoderma* was evident on invaded sections.

Trichoderma judged as either Classes 1 or 2 were considered to be strongly antagonistic against the pathogen.

b) Ultrastructure studies of *Trichoderma* spp. interactions with *S. sclerotiorum*, *S. rolfsii* and *R*. *solani* under ESEM

Colonized agar squares (4 x 4 mm), cut from the actively growing edge of a 4-day old mycelial mat on PDA of a single antagonist and the pathogen and were placed opposite each other on a 85mm diameter petri dish containing PDA medium. Each bioassay was replicated four times and was incubated for 5 days at 25°C in the dark. Mycelial squares (4 x 4 mm) from regions of mycoparasitic interaction were collected 7 days post-inoculation, fixed in 3% (v/v) buffered glutaraldehyde overnight, and washed twice in a sodium cacodylate buffer (0.1M; pH 7.0). The specimens were dehydrated in a graded ethanol series [10, 30, 50, 70, 90% (v/v)] for 10 minutes each and three times in 100% (v/v). Samples underwent critical point drying and then mounted on copper stubs with double-sided carbon tape and sputter coated with gold-palladium and were kept in a desiccator until examination with Zeiss EVO LS 15 Environmental Scanning Electron Microscope (ESEM) on high vacuum at 5kV. Three samples per *Trichoderma*-pathogen interaction were examined.

5.2.3 *In vivo* screening of *Trichoderma* spp. against damping-off caused by *S. sclerotiorum*, *S. rolfsii* and *R. solani*

a) Growth and preparation of pathogen inoculum

Fungal inoculum for *in vivo* trials was prepared by soaking barley seeds in 500 mL Erlenmeyer flasks overnight with 40 mL tap water per 100 g of seed. Soaked seeds were drained and autoclaved at 121°C for 15 minutes on 2 consecutive days. Autoclaved barley seeds were then incubated for 4 days at room temperature to ensure that no contamination had taken place. After incubation at room temperature, flasks were inoculated with 4-day old colonized agar squares (4 x 4 mm) of either *S. sclerotiorum, S. rolfsii* or *R. solani* and incubated for a further 3 weeks to allow the fungus to completely colonize the barley seeds. The colonized barley seeds were air dried and stored in paper bags at 4°C until needed.

b) Seed treatment and soil drench preparation

Sunflower seeds (PAN 7080[16]) were washed eight times with sterilized distilled water and air-dried under a laminar flow bench. Seeds were treated by soaking them in a conidial suspension (10 mL) of Eco-T® (2 x 10^8 spores L^{-1}) (Mao et al., 1998) using a 2% solution of carboxymethylcellulose (CMC) (Sigma[17]) as a sticking agent. Approximately 80-90 seeds were added to each batch of spore-sticker suspension and allowed to soak for 1 hour with intermittent wrist swirling. The treated seeds were removed from the suspension, placed in a sterilized Petri dish and air dried on a laminar flow bench for 24 hours. For the soil drench, Eco-T® was mixed with water at a rate of 1g per 4L water and applied to steam pasteurised growth media.

c) Speedling 24® trial

Speedling 24® trays were filled with steam pasteurised composted pine bark[18] and one sunflower seed was planted into each cell of the Speedling 24® tray, and inoculated with two fungal colonized barley grains, placed equidistant from the seed during planting. Treatments were as follows: (T_1) uninoculated control; (T_2) diseased control; (T_3), application of *T. asperellum* strain kd (Eco-T®) as a seed treatment; (T_4), application of *T. asperellum* strain kd as a soil drench at planting only; (T_5), application

[16] PANNAR (Pty) Ltd., PO Box 19, Greytown, Kwa-Zulu Natal, 3250, South Africa.
[17] Sigma Capital Enterprises, PO Box 62, New Germany, KwaZulu-Natal, 3620, South Africa.
[18] Gromor (Pty) Ltd., P.O. Box 89, Cato Ridge, KwaZulu-Natal, 3680, South Africa

of *T. asperellum* strain kd as a soil drench at planting and bimonthly; and (T_6), application of *T. asperellum* strain kd as a seed treatment and as a soil drench bimonthly. Disease-free controls using seeds coated solely with CMC sticker and kaolin powder[19] (anhydrous aluminium silicate- $Al_2Si_2O_5(OH)_4$ were established. This was to ensure any effects of *T. asperellum* on the seedlings was due solely to the activity of the biocontrol agent and not the carrier powder. Disease-free control trays received uninoculated barley with no pathogen, while diseased control trays received inoculated barley with the pathogen but no *Trichoderma*. There were three replicate trays for each treatment. The trays were watered and arranged in a randomised complete block design in a polycarbonate greenhouse tunnel maintained between 26 to 28°C with a relative humidity (RH) of 75 to 85%. The trays were irrigated three times a day by microjet overhead irrigation containing NPK soluble fertilizer [2:1:2 (43)] at a rate of 1.8 g/l. The trial was terminated 30 days after planting (dap) to determine the effects of treatments on germination (%) and dry biomass of the seedlings post inoculation with the test pathogens using the following equation:

$$\text{Germination (\%)} = \frac{\text{no. of healthy seedlings / tray x 100}}{\text{no. of sown seeds / tray}}$$

The plant material was harvested at soil level and subsequently dried at 70°C for 48 hours to determine the total dry biomass of seedlings per plot (tray). Only above-ground stems and leaves were weighed. The experiment was repeated once and results pooled for statistical analysis.

d) Rhizotron studies

Trichoderma strain kd was used in rhizotron studies to assess its ability to protect root and shoot growth of sunflower seedlings from damping-off. Treatments were the same as in the Speedling24® trial. Rhizotrons were made by placing two Plexiglas (100 x 150 mm) plates together, secured with butterfly screws and separated by a silicone tube spacer (15 mm diameter). The nature and design of the rhizotrons was similar to that described by James et al. (1985). The rhizotrons were filled with potting mix[20] that had

[19] Plant Health Products (Pty) Ltd., P.O. Box 207, Nottingham Road, KwaZulu-Natal, 3280, South Africa.

[20] Gromor (Pty) Ltd., P.O. Box 89, Cato Ridge, KwaZulu-Natal, 3680, South Africa

been previously sifted (2 mm pore size sieve) and sterilized. There were four rhizotrons per treatment and one seed was planted per rhizotron. Each rhizotron was wrapped in two layers of aluminium foil to prevent light from reaching the roots, watered with tap water and placed in a germination room at 20-24°C for 2 days. The rhizotrons were then moved into a polycarbonate greenhouse tunnel maintained between 26 to 28°C with a relative humidity (RH) of 75 to 85%. Once the seedlings had germinated, each was watered daily (25 mL) with NPK soluble fertilizer [2:1:2 (43)] at a rate of 1.8 g l^{-1}. After 2 weeks, the volume of water was increased to 50 mL per rhizotron and subsequently, to two watering a day (mornings and evenings) from the third week until the end of the experiment. Seedling growth was monitored for 30 days. The experiment was repeated once and results pooled for statistical analysis.

i) Root area measurements (image analysis)

Seedlings from each rhizotron were harvested at the base after 5 weeks of growth. The roots were gently washed five times in plastic buckets containing tap water. Root samples from replicate treatments were finely spread on a dark background and images were captured, calibrated, manipulated and then root area measurements taken using ImageJ 1.52a image analysis software. Four measurements were made per replicate root sample and the mean area measurement determined.

ii) Shoot and root dry biomass measurements

Roots (after image analysis) and shoots of seedlings from each rhizotron were both dried at 70°C for 48 hours in an oven and their respective dry biomass was determined.

c) Pot trial

Sunflower seeds were planted in cylindrical plastic pots (300 mm diameter) filled with steam pasteurized composted pine bark. For each treatment two seeds were planted into each of four pots (four replicates per treatment). Treatments were as follows: (T_1) uninoculated control; (T_2) diseased control; (T_3), application of *T. asperellum* strain kd (Eco-T®) as a seed treatment; (T_4), application of *T. asperellum* strain kd as a soil drench at planting only; (T_5), application of *T. asperellum* strain kd as a soil drench at planting and monthly; (T_6), application of *T. asperellum* strain kd as a soil drench at planting and bimonthly; (T_7), application of *T. asperellum* strain kd as a seed treatment and as a soil drench monthly; and (T_8), application of *T. asperellum* strain kd as a seed treatment and as a soil drench bimonthly. The pots were watered and arranged in a

randomised block design in a polycarbonate greenhouse tunnel maintained between 26 to 28°C with a relative humidity (RH) of 75 to 85%. To avoid possible competition between plants, seedlings were thinned to one plant per pot 1 week after germination. The pots were drip irrigated twice a day. After 80 days (80 dap), plant height (cm) and the head dry biomass (g) were evaluated to determine the effect of treatments on sunflowers. The experiment was repeated once and results pooled for statistical analysis.

5.2.5 Statistical analysis

Data was subjected to an analysis of variance (ANOVA) using Genstat® (Version 18) statistical analysis software (GenStat, 2016) to determine differences between treatment means. If the values were significant at $P < 0.05$, the means were separated using the Duncan's Multiple Range Test.

5.3 Results

5.3.1 *In vitro* dual-culture bioassay and ultrastructure studies of *Trichoderma* spp. pathogen interactions under ESEM

The results for dual-culture test are shown in Table 5.1. The tests revealed that contact between the pathogens and each of the *Trichoderma* isolates occurred 3 days after inoculation. *T. asperellum* produced zones of inhibition against *S. sclerotiorum* and *S. rolfsii*, but failed to produce a zone of inhibition when screened against *R. solani.* Conversely, based on the invasion ability rating scale, *T. asperellum* achieved a Class 1 rating against *R. solani* and a Class 3 and 4 rating against *S. sclerotiorum* and *S. rolfsii,* respectively. *T. atroviride* also successfully produced a zone of inhibition against *S. sclerotiorum* and *S. rolfsii* but failed to do so against *R*. *solani*. *T. atroviride* achieved only a Class 4 rating against all three pathogens, indicating poor invasion ability. Based on the Bell rating scale, neither of the *Trichoderma* screened scored better than a Class 3 rating after 5 days with the exception of *T. atroviride* which scored a Class 2 rating when screened against *S. sclerotiorum* (Table 5.1). Zones of inhibition were produced by both *Trichoderma* spp. against *S. sclerotiorum* and *S. rolfsii*, but not against *R. solani*.

A brownish discolouration was produced when *T. atroviride* came into contact with *S. rolfsii*. This discolouration was also seen in the *T. asperellum* and *R. solani* dual-

culture plates. However, the discolouration spread over the *R. solani* culture as the *T. asperellum* subsequently invaded the entire plate. Complete invasion of dual-culture plates by *T. asperellum* against *R. solani* occurred within 5 days of inoculation (Fig 5.1). Sporulation on all sections of the plate was also apparent. Partial invasion by *T. asperellum* occurred against *S. sclerotiorum* after 5 days but spread over the entire *S. sclerotiorum* culture within 7 days. No overgrowth of *S. rolfsii* by *T. asperellum* occurred after 5 days (Fig 5.1). However, overgrowth was observed eight to 10 days post inoculation. Based on these results, it was decided that the *in vivo* trials in this chapter be carried out using *T. asperellum* alone. However, since *T. atroviride* achieved a Class 2 Bell rating against *S. sclerotiorum*, it was screened for antagonistic ability against the head rot-causing pathogen in the fructosphere (Chapter 4) alongside the yeast and *Bacillus* strains which were isolated and screened against the pathogen *in vitro* (Chapter 2).

Coiling by *T. asperellum* hyphae occurred when they came into contact with hyphae of *S. sclerotiorum*, *R. solani* and *S. rolfsii* was observed with ESEM (Fig 5.2). In addition, an appressorium produced by *T. asperellum* was observed (Fig 5.2 c) as well as cell destruction (Fig 5.2 d) as a result of dense coiling by *T. asperellum* hyphae of pathogen hyphae.

5.3.2 *In vivo* screening of *T. asperellum* against damping-off caused by *S. sclerotiorum*, *S. rolfsii* and *R. solani*

a) Speedling 24® trial

Percentage seedling survival for the controls in the *S. sclerotiorum* test trays ranged from 54.9% for the diseased control to 90.0% for the disease-free control ($P \leq 0.05$) (Table 5.2 - Appendix 5). The mean dry seedling biomass for the diseased control was 64.7% of that obtained for the disease-free control. Compared to the diseased control, *T. asperellum* significantly increased seedling survival from 54.9% to 87.3% when applied as a seed treatment and bimonthly soil drench, and dry seedling biomass from 64.7% to 107.9% of the biomass of the disease-free control (Table 5.2 - Appendix 5) for the same treatment. Seed treatment in combination with a bimonthly drench showed an increase of 32.4% in germination (Fig 5.7 A) and an increase of 6.0 g in dry seedling mass (Fig 5.7 B) compared to the disease control.

In the *R. solani* test trays, percentage seedling survival for the controls ranged from 29.6% for the diseased control to 89.6% for the disease-free control ($P \leq 0.05$) (Table 5.3 - Appendix 5). The mean dry seedling biomass for the diseased control was 65.9% of that obtained for the disease-free control. Compared to the diseased control, seedling survival increased from 29.6% to 84.3% when *T. asperellum* was applied as a seed treatment. However, treating seeds with *T. asperellum a*s well as applying it as a bimonthly soil drench yielded the highest dry seedling biomass, which increased from 65.9% to 112.3% of the biomass of the disease-free control (Table 5.3 - Appendix 5). Compared to the disease control, an increase in germination of 54.7% (Fig 5.7 A) was achieved by seed treatment alone. However, seed treatment in combination with a bimonthly drench achieved the highest increase in dry seedling mass of 6.4 g (Fig 5.7 B).

Similarly, the bimonthly drench had the highest dry seedling biomass in the *S. rolfsii* test trays, which increased from 54.7% to 90.6% of the biomass of the disease-free control ($P \leq 0.05$) (Table 5.4 - Appendix 5). This treatment was also the best for percent germination (75.0%) in comparison to the diseased control (31.7%). The mean dry seedling biomass for the diseased control was 54.7% of that obtained for the disease-free control (Table 5.4 - Appendix 5). Seed treatment in combination with a bimonthly drench showed an increase of 43.3% in germination (Fig 5.7 A) and increased dry seedling mass by 5.0 g (Fig 5.7 B) compared to the disease control.

b) Rhizotron studies

The data in Table 5.5 (Appendix 5) shows an increase in the shoot and root dry biomass, and root area of sunflower seedlings arising from inoculations with *T. asperellum* in the presence of *S. sclerotiorum*. Maximum shoot dry biomass of 108.3% of the disease-free control was obtained by treating seeds in combination with a bimonthly soil drench. This treatment showed significant ($P \leq 0.05$) increase in the shoot dry biomass over the disease control in addition to the disease-free control, indicating possible growth promotion effects, even in the presence of *S. sclerotiorum*. Similarly, dry root biomass obtained by this treatment, which was 106.7% of the disease-free control, was greater than that of both the disease control and the disease-free control. However, these results did not differ significantly from those obtained by the disease-free control ($P \leq 0.05$). Lastly, seed treatment in combination with a bimonthly drench also resulted in the greatest root area (100.4% of the disease-free

control), which was higher than that of the disease control and the disease-free control, although not significantly different from the latter ($P \leq 0.05$). None of the applications of *T. asperellum* produced a root area significantly different to the root area of the disease-free control (Fig 5.3). Seed treatment in combination with a bimonthly drench increased dry shoot biomass (2.1 g) (Fig 5.8 A); dry root biomass (1.0 g) (Fig 5.8 B); and root area (15570 mm^2) (Fig 5.8 C) compared to the disease control.

The highest increase in dry shoot biomass in plants infected with *R. solani* was obtained by treating seeds in combination with a bimonthly drench and found to be 62.5% of the disease-free control (Table 5.6 - Appendix 5). The results were significantly higher than those obtained by the disease control but not significantly different ($P \leq 0.05$) to the three other *T. asperellum* application treatments. Dry root biomass obtained by the same treatment was identical to that of the disease-free control, despite the presence of the pathogen. The increase in this parameter was also significantly different ($P \leq 0.05$) from those obtained by the three other applications. This treatment also obtained the highest root area of sunflower seedlings which was 97.1% of the disease-free control but was not significantly different from the root area obtained by other application treatments as well as the disease-free control (Fig 5.8). Both seed treatment and seed treatment in combination with a bimonthly drench application increased dry shoot biomass (1.1 g) (Fig 5.8 A). The latter application also increased dry shoot biomass (1.2 g) and root area (18352 mm^2) (Fig 5.8 B and C) compared to the disease control.

Similarly, the greatest increase in dry shoot and root biomass was obtained by seed treatment in combination with a bimonthly soil drench (Table 5.7 - Appendix 5). Dry shoot biomass obtained by this treatment was significantly higher ($P \leq 0.05$) than all other treatments including the disease-free control (108.3% of the disease-free control). However, dry root biomass obtained by this treatment was not higher than that of the disease-free control (86.7% of the disease-free control) and was not significantly different from the root biomass obtained by the other applications. Root area was also greatest (97.4% of the disease-free control) with seed treatment in combination with a bimonthly soil drench (Fig 5.3). However, these results were not significantly different from those obtained by drenching soil at planting as well as bimonthly and a single seed treatment. Seed treatment in combination with a bimonthly drench had increased dry shoot biomass (2.1 g) (Fig 5.8 A); dry root biomass (1.2 g)

(Fig 5.8 B); and root area (18909 mm^2) (Fig 5.8 C) compared to the disease control. None of the rhizotrons in which seeds were treated or were drenched with *T. asperellum* showed any formation of sclerotia.

c) Pot trial

Treating seeds with *T. asperellum* along with applying it as a soil drench at planting significantly increased both plant height and dry head weight of plants infected with *S. sclerotiorum* by 99.7% and 99.4%, respectively, of the plant height and dry head weight of the disease-free control (Table 5.8 - Appendix 5). As shown in Table 5.8 (Appendix 5), there were no significant differences in plant height between a single seed treatment, soil drench at planting as well as monthly, seed treatment and monthly drench, drench at planting as well as bimonthly, and seed treatment as well as a bimonthly drench ($P \leq 0.05$). Plants were taller with seed treatment in combination with a monthly drench and an increase in plant height (168.7 cm) (Fig 5.9 A) and dry head biomass (12.3 g) (Fig 5.9 B) compared to the disease control. The effect of this treatment on plant height is shown in Fig 5.6.

In contrast, plants infected with *R. solani* performed best when seeds were treated with *T. asperellum* in addition to drenching the soil bimonthly. Plant height and head dry weight were 98.4% and 95.0%, respectively, of the disease-free control (Table 5.9 - Appendix 5). However, this treatment did not differ significantly in comparison to a single seed treatment, and seed treatment in combination with a monthly drench with regards to plant height ($P \leq 0.05$). Seed treatment in combination with a bimonthly soil drench increased plant height (69.9 cm) (Fig 5.9 A) and dry head biomass (14.3 g) (Fig 5.9 B) compared to the disease control. The effect of this treatment on plant height is shown in Fig 5.7.

The same treatment also led to the tallest plants and heaviest dry head weight that was 90.7% and 90.4%, respectively, of the disease-free control in plants infected with *S. rolfsii* (Table 5.10 - Appendix 5). However, plant height obtained by this treatment did not differ significantly with plant height obtained by the soil drench at planting and bimonthly treatment. Similarly, seed treatment in combination with a monthly soil drench and seed treatment in combination with a bimonthly drench did not differ significantly with regards to dry head weight ($P \leq 0.05$). An increase in plant height (186.4 cm) (Fig 5.9 A) and in dry head biomass (14.4 g) (Fig 5.9 B) compared to the

disease control was achieved by seed treatment in combination with a bimonthly drench. The effect of this treatment on plant height is shown in Fig 5.8.

Table 5.1 *In vitro* screening of *Trichoderma* spp. against *S. sclerotiorum*, *S. rolfsii* and *R. solani* using dual-culture bioassays

Treatment		Bell rating[a]	Invasion Ability[b]	Antibiosis[c]
Biocontrol	**Pathogen**			
T. asperellum	*S. sclerotiorum*	3	3	+
	S. rolfsii	3	4	+
	R. solani	3	1	+
T. atroviride	*S. sclerotiorum*	2	4	+
	S. rolfsii	3	4	+
	R. solani	3	4	-

[a]*Trichoderma* isolate is considered to be antagonistic towards a fungal pathogen if the mean score is ≤2, but is not highly antagonistic if the mean score is ≥3.

[b]*Trichoderma* isolate is considered to be highly invasive towards a fungal pathogen if the mean score is ≤2, but is not highly invasive if the mean score is ≥3.

[c] – +, negative or positive for antibiosis, inhibition zones observed before *Trichoderma* hyphae made physical contact with the pathogen hyphae.

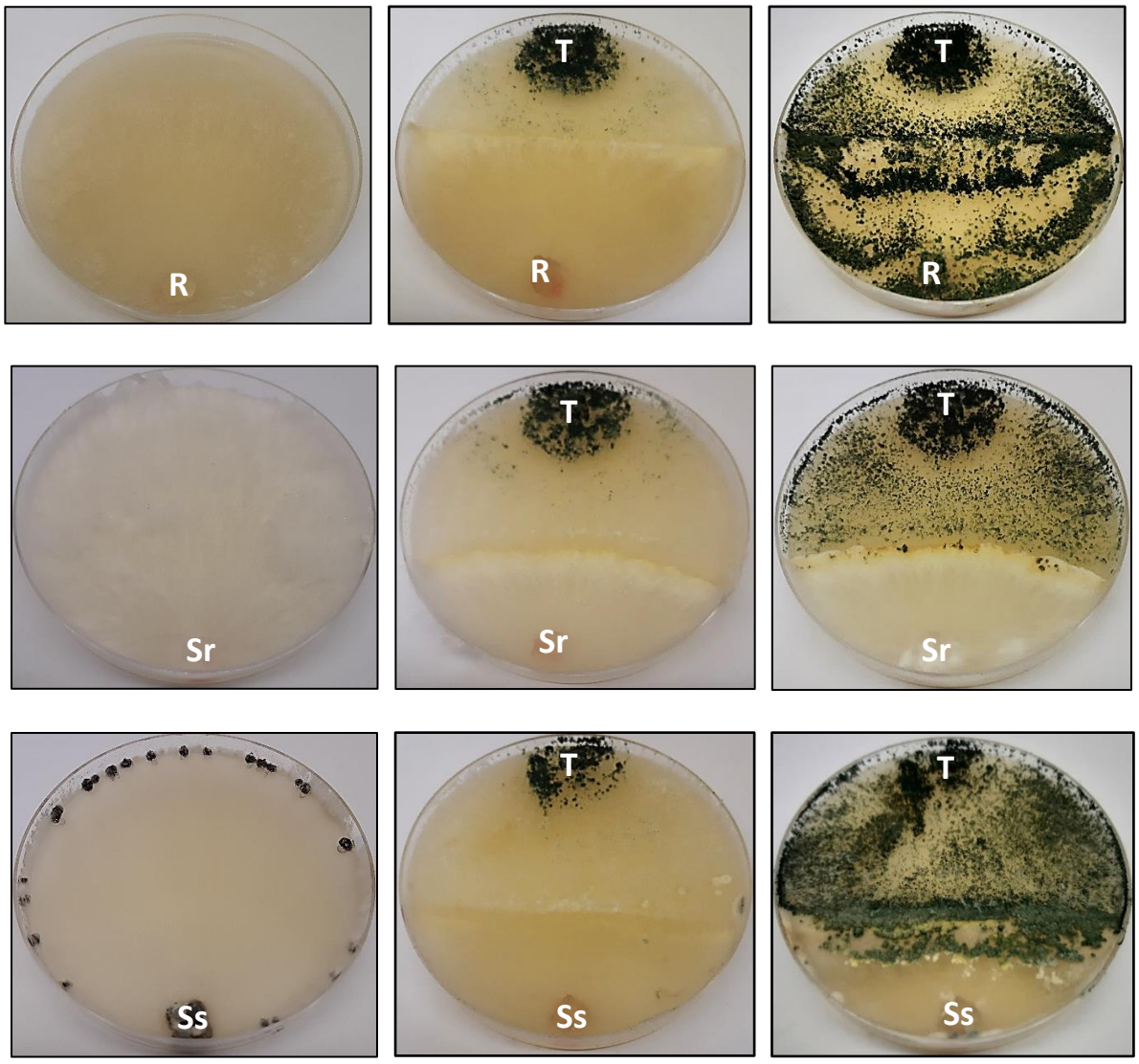

Fig 5.1 *In vitro* interactions between *T. asperellum* (T) and *R. solani* (R), *S. rolfsii* (Sr) and *S. sclerotiorum* (Ss) on Potato dextrose agar. Controls are shown on the left; inhibitory response by *T. asperellum* against the pathogens 3 days post inoculation is shown in the middle and; varying levels of overgrowth by *T. asperellum* 5 days post inoculation is shown on the right.

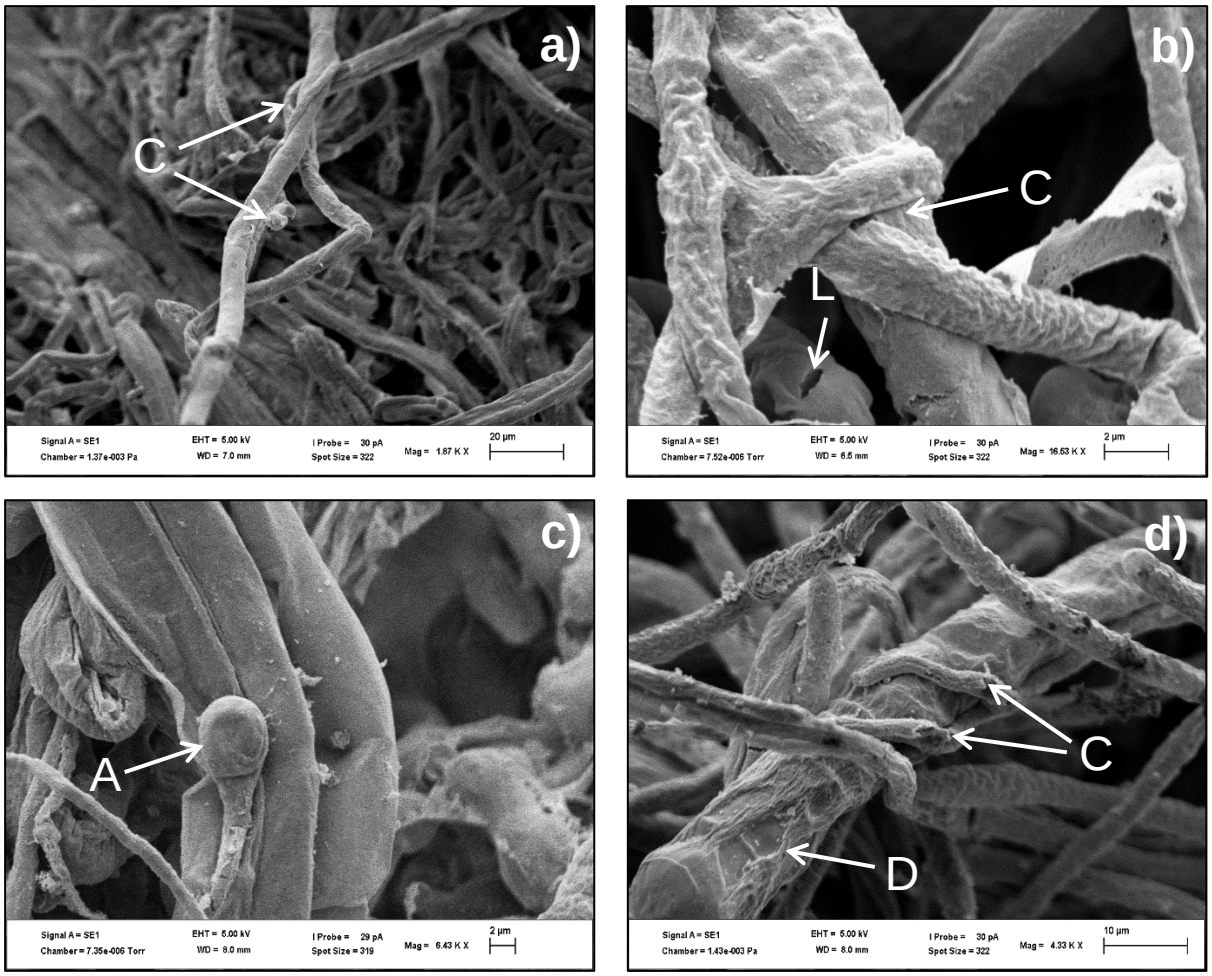

Fig 5.2 Environmental scanning electron micrographs of hyphae of *T. asperellum* mycoparasitising pathogen hyphae at 7 days post inoculation. a) Hypha of *T. asperellum* attached to the hypha of *S. sclerotiorum* showing initial signs of coiling (C); b) Hypha of *T. asperellum* attached to the hypha of *R. solani* showing advanced signs of coiling (C); c) Formation of an appressorium (A) by *T. asperellum* was evident on the surface of a partially degraded *R. solani* hypha; d) Dense coiling by *T. asperellum* hyphae of *S. rolfsii* hypha (C) and subsequent cell destruction (D) of the hypha.

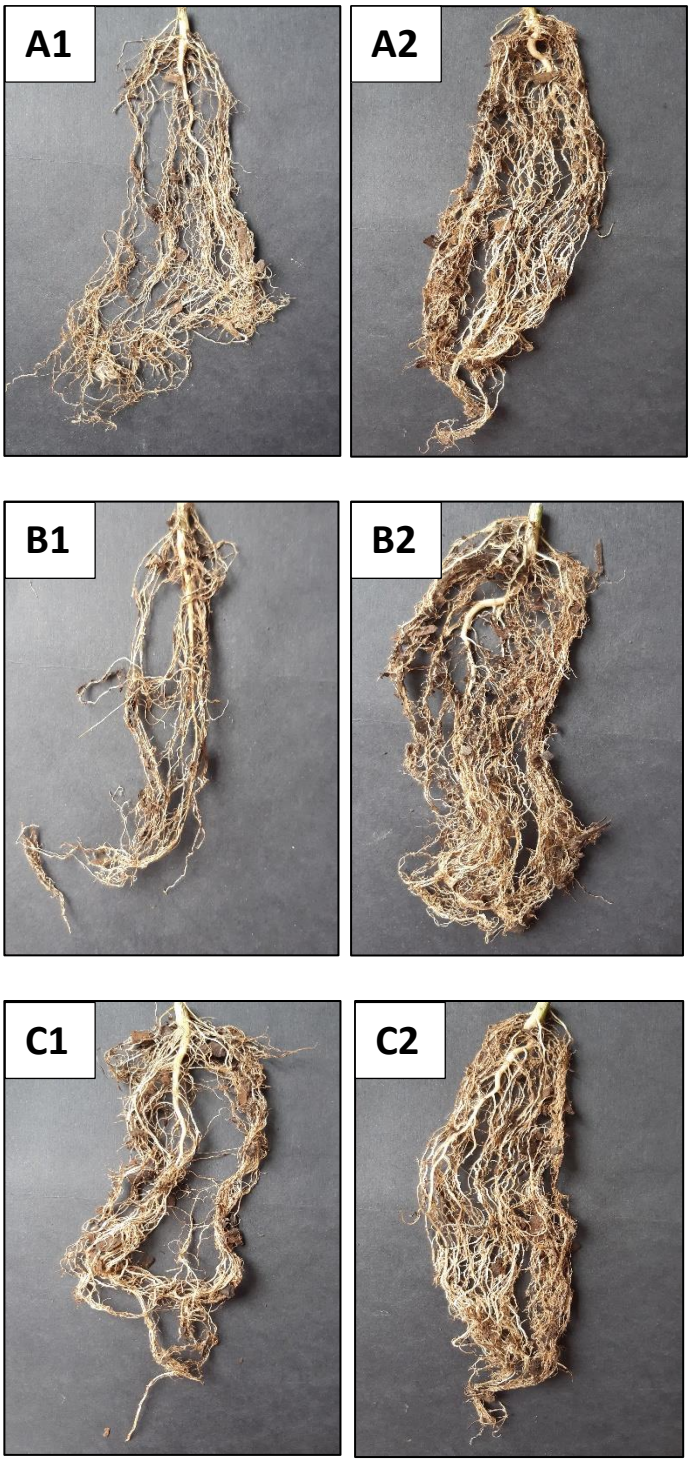

Fig 5.3 Improvement of root development by application of *T. asperellum* despite the presence of *S. rolfsii* (A2), *R. solani* (B2) and *S. sclerotiorum* (C2) compared to their respective disease controls (A1, B1 and C1).

Fig 5.4 Effect of seed treatment in combination with bimonthly soil drench on the height of 80-day old sunflower plants infected with *S. rolfsii* at planting (A) compared to the disease control (B).

Fig 5.5 Effect of seed treatment in combination with bimonthly soil drench on the height of 80-day old sunflower plants infected with *R. solani* at planting (A) compared to the disease control (B).

Fig 5.6 Effect of seed treatment in combination with a monthly soil drench on the height of 80-day old sunflower plants infected with *S. sclerotiorum* at planting (A) compared to the disease control (B).

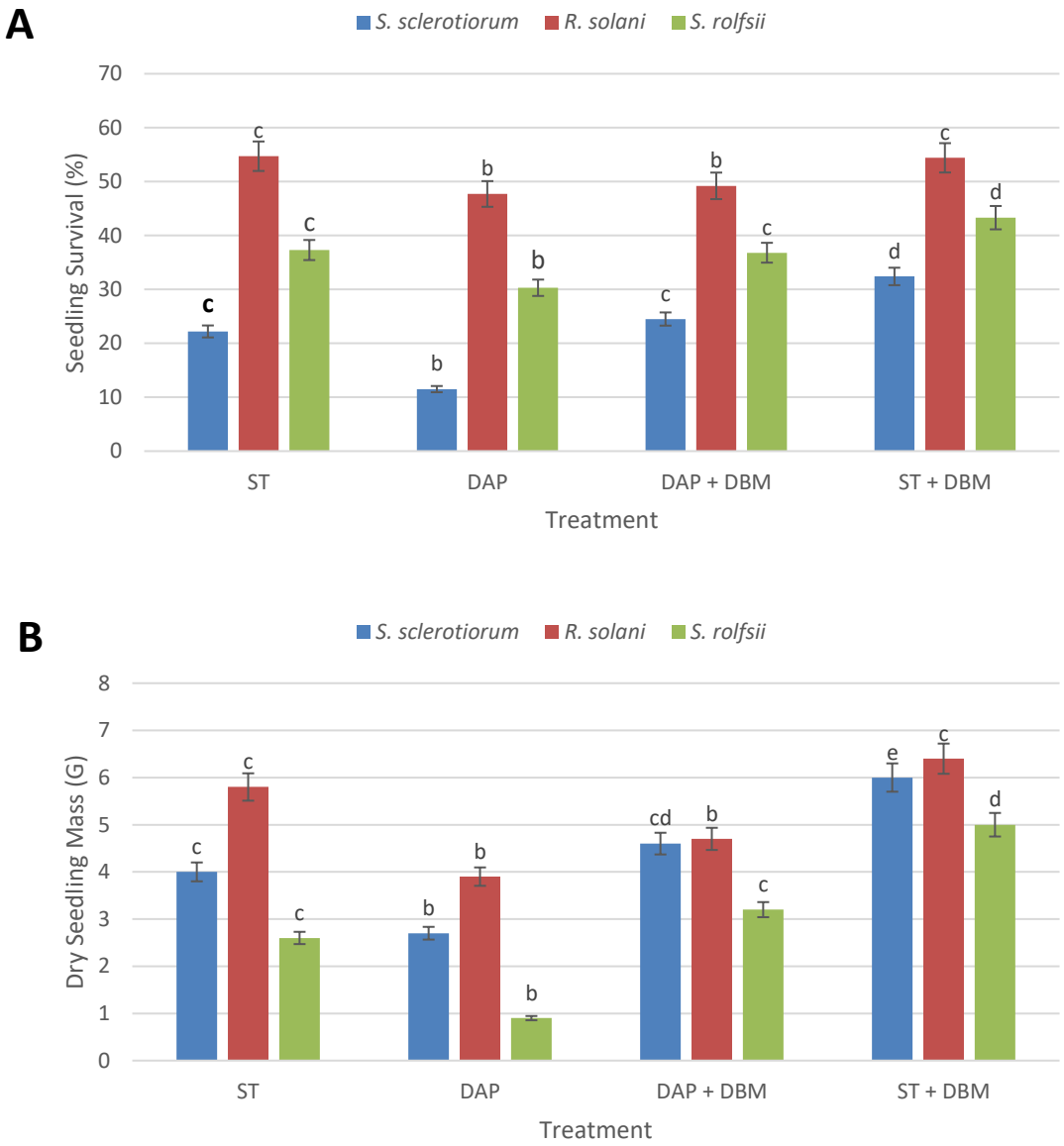

Fig 5.7 Increases in germination (A) and dry seedling biomass (B) in response to treatment with *T. asperellum* as a seed coat (ST), soil drench at planting (DAP), soil drench at planting and bimonthly (DAP + DBM) and seed coat and soil drench bi-monthly (ST + DBM) post inoculation with the pathogens. Different letters above bars of the same colour indicate significant difference between means (Duncan's Multiple Range Test, $P < 0.05$).

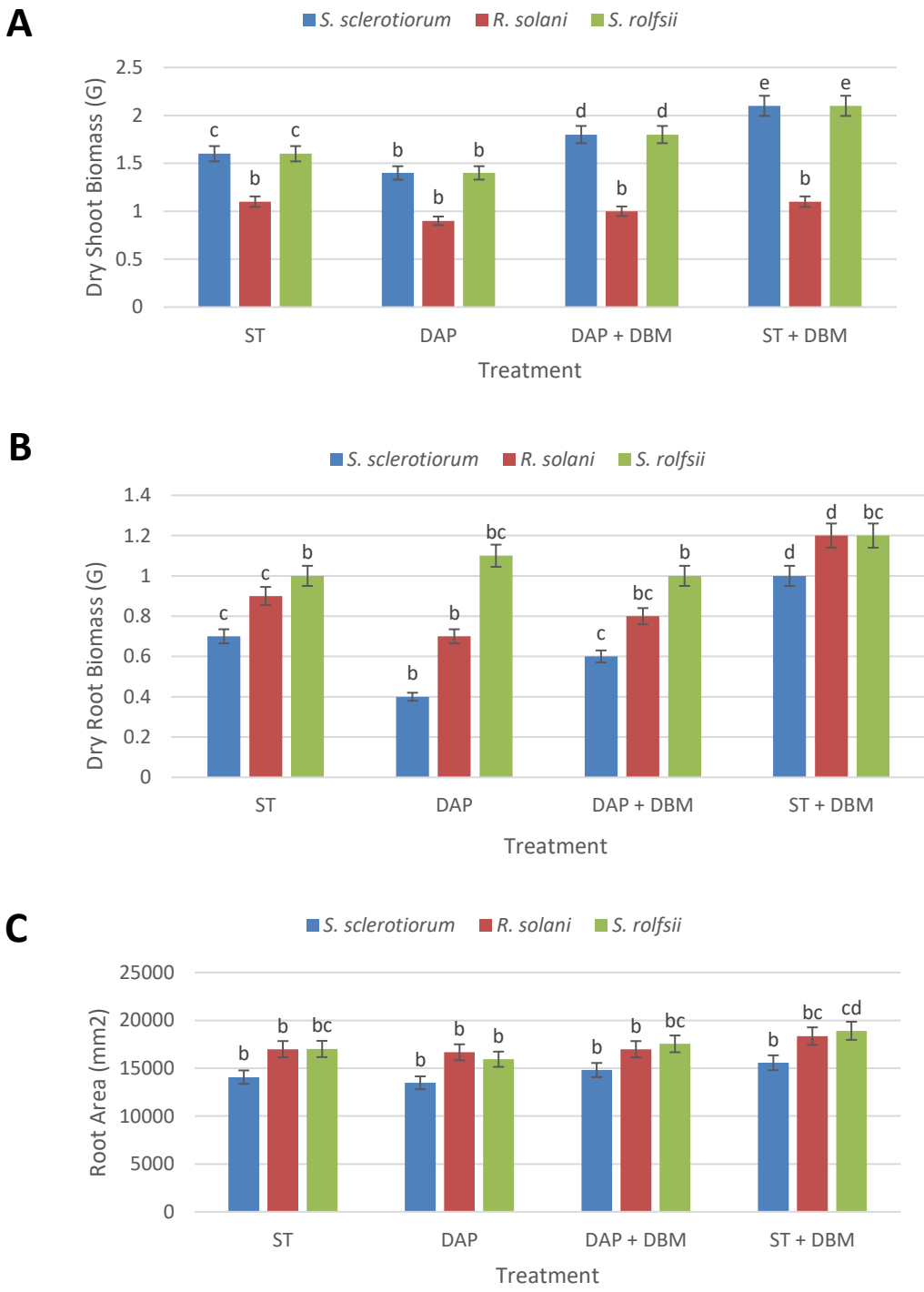

Fig 5.8 Increases in dry shoot biomass (A), root biomass (B) and root area (C) in response to treatment with *T. asperellum* as a seed coat (ST), soil drench at planting (DAP), soil drench at planting and bimonthly (DAP + DBM) and seed coat and soil drench bimonthly (ST + DBM) post inoculation with the pathogens. Different letters above bars of the same colour indicate significant difference between means (Duncan's Multiple Range Test, $P < 0.05$).

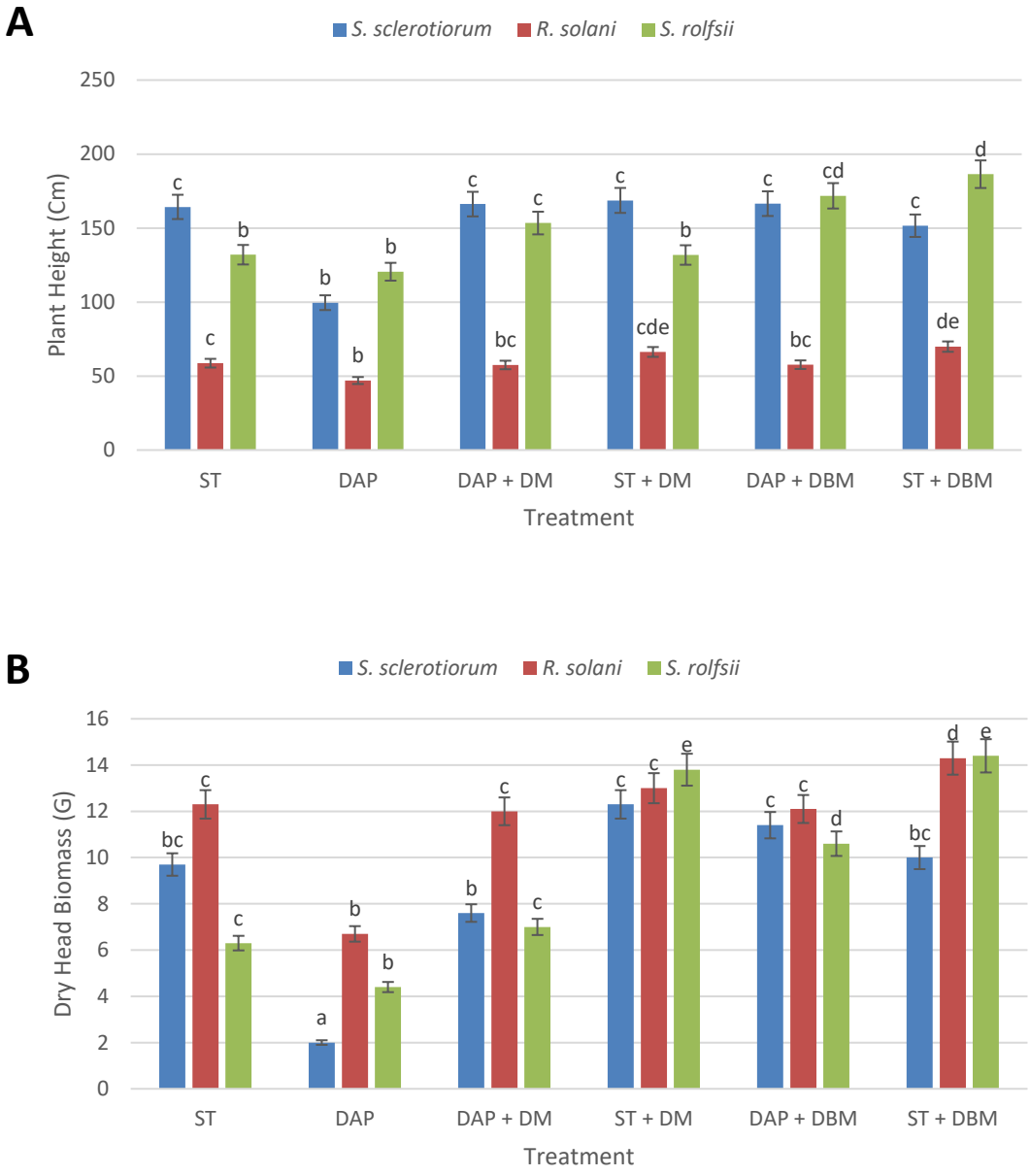

Fig 5.9 Increases in plant height (A) and dry head biomass (B) in response to treatment with *T. asperellum* as a seed coat (ST), soil drench at planting (DAP), soil drench at planting and monthly (DAP + DM), seed coat and soil drench monthly (ST + DM), soil drench at planting and bimonthly (DAP + DBM) and seed coat and soil drench bimonthly (ST + DBM) post inoculation with the pathogens. Different letters above bars of the same colour indicate significant difference between means (Duncan's Multiple Range Test, $P < 0.05$).

5.4 Discussion

Seedling germination and emergence contributes directly to the economic success of all commercial crops (Finch-Savage and Bassel, 2016). Since roots function to anchor the plant to the soil/growth medium and to provide a large surface area (which is increased by the presence of root hairs) to facilitate the uptake and absorption of nutrients and water, their health or lack thereof has a significant effect on the size and vigour of plants, adaption to certain soils and response to cultural practices (Syngenta, 2011). Finding ways to reduce losses due to damping-off at seed and seedling level are vital at ensuring healthy, vigiorous plant stands that have better chances of resisting/recovering from attack by pest and diseases or abiotic stressors.

In this study, *in vitro* screening of two commercial strains of the *Trichoderma* fungus was carried out, followed by *in vivo* screenings of one of the strains, *Trichoderma asperellum* strain kd, to control damping-off of sunflower caused by *Sclerotinia sclerotiorum*, *Sclerotium rolfsii* and *Rhizoctonia solani*. In the greenhouse screening, various application methods were investigated and it was found that control of damping-off by *T. asperellum* differed between the three pathogens but overall, seed treatment in combination with drenching of the soil was most effective at reducing losses caused by all three pathogens. Suppression of damping-off pathogens using biological control has been widely reported (Schroth and Hancock, 1981). Damping-off caused by *Pythium, Rhizoctonia* spp., *Sclerotinia* and other soilborne plant pathogens has been suppressed by seed and soil applications of strains of the fungus *Trichoderma* (Sivan *et al*., 1984; Papavizas, 1985).

In vitro results from the present study revealed that Eco-77® was not effective at inhibiting the growth of *S. sclerotiorum*, *S. rolfsii* and *R. solani*. A Class 3 Bell rating was scored against all three pathogens. However, Eco-T® was successful at invading *R. solani* and *S. sclerotiorum* and scored a Class 1 and Class 3 rating, respectively, producing spores on the entire plate and on sections of the plate where *T. asperellum* invaded the pathogen. A Class 4 invasion ability rating was recorded for *T. asperellum* against *S. rolfsii*, with little sporulation at the point of contact. Antibiosis against all three pathogens was demonstrated by the production of a zone of inhibition and brown discolouration at the point of contact between *T. asperellum* and pathogen mycelium. The pigmentation has been attributed to production of toxic metabolites and/or enzymes and is thought to give a good

indication of mycoparasitism (Lorito *et al*., 1993; Calistru *et al*., 1997; Menendez and Godeas, 1998). This was later confirmed in the Environmental Scanning Electron Microscopy (ESEM) study. Suppression of damping-off pathogens using biological control has been widely reported (Schroth and Hancock, 1981). Damping-off caused by *Pythium, Rhizoctonia* spp., *Sclerotinia* and other soilborne plant pathogens has been suppressed by seed and soil applications of strains of the fungus *Trichoderma* (Sivan *et al*., 1984; Papavizas, 1985).

Findings from the Bell rating system did not correlate well with the *in vivo* plant screening. The findings were similar to a study by Williams and Asher (1996) who found no correlation between biological control *in vivo* and antifungal activity *in vitro* when bacterial isolates showed strong antifungal activity against *Pythium ultimum* Trow *in vitro*, were tested *in vivo*. This was contrary to the findings and recommendations of Askew and Laing (1994), who reported that out of 118 *Trichoderma* isolates, 92% identified as antagonistic to *R. solani in vitro* significantly reduced damping-off in the nursery. The Bell rating system makes the assumption that *Trichoderma* isolates rated ≥ 3 are not highly antagonistic (Bell *et al*., 1982). However, in this study, *T. asperellum* significantly reduced damping-off *in vivo* compared to the diseased control. Thus, the Bell rating system alone is not the only scale that should be used to identify antagonistic *Trichoderma* species, as it is inadequate. Additional rating scales such as the Invasion Ability scale used in this study, as well as the antibiosis test, should also be used to assess biocontrol ability.

T. asperellum successfully invaded colonies of *S. sclerotiorum* and *R. solani* but not *S. rolfsii*. The rapid invasion *by T. asperellum* observed during *in vitro* bioassays may be a possible mechanism of biological control. Zones of inhibition were produced by *T. asperellum* against all three pathogens before making physical contact with them, suggesting the secretion of antimicrobial compounds (Askew and Laing, 1994; Calistru *et al*., 1997). These results indicate that the selection criteria for choosing isolates can be very subjective and do not always correlate well with *in vivo* studies. Inhibition zones produced by *T. asperellum* were observed before making physical contact with the pathogens. This suggests that antimicrobial substances were produced (Askew and Laing, 1994; Calistru *et al*., 1997). Antibiotic substances, volatile and non-volatile compounds have been implicated in biological control of plant pathogens and other fungal species (Fravel, 1988; Calistru *et al*., 1997; Wheatley *et al*., 1997).

Trichoderma produces volatile metabolites that have either a fungistatic effect e.g. acetaldehyde and/or a fungicidal effect e.g. alkyl pyrones (Claydon *et al*., 1987) and non-volatile metabolite. Cellulase or chitinase enzymatic exudates produced by the fungus degrade the cell walls of pathogens, suppressing their growth (Graeme-Cook and Faull, 1991). However, poor correlations have been found between *in vitro* and *in vivo* studies (Williams and Asher, 1996). For example, a *T. harzianum* isolate that effectively controlled damping-off on eggplant (*Solanum melongena* L.), bean (*Phaseolus vulgaris* L.) and tomato (*Lycopersicon esculentum* L.) but which failed to produce antibiotics *in vitro*, was found to hyperparasitize the cell walls of *R. solani* (Hader *et al*., 1979).

Mycoparasitism of fungal plant pathogens is one of the mechanisms employed in the control of plant diseases (Zhang *et al*., 1999). The *in vitro* bioassay coupled with ESEM ultrastructure studies indicated that *T. asperellum* actively parasitized the pathogen mycelium. Cell wall disruption and lysis of pathogen mycelium points towards lytic enzymes being involved in the mycoparasitic process. The coiling action of *T. asperellum* hyphae of pathogen hyphae allows both entry into the lumen of the parasitised pathogen hyphae and the subsequent assimilation of the cell contents (Chiuraise *et al*., 2015). The resulting death of the pathogen was evident in degradation and cell wall lysis of pathogen mycelium. Previous studies have also shown that *Trichoderma* spp. utilize mycoparasitism against other pathogens such as *Pythium*, *Botrytis* and *Alternaria* (Bell *et al*., 1982; Bellows and Hassel, 1999). Although *in vitro* screening is necessary, ultimately, *in vivo* and field testing is required to ratify the choice and selection of biological control agents (BCAs).

The present study showed that *T. asperellum* suppressed pathogenicity of *R. solani*, *S. sclerotiorum* and *S. rolfsii* as measured by % germination, dry weight, plant height and root area of sunflowers. This suppressive effect varied with application method and frequency. The reason for this study was to investigate which application method would achieve optimal results with regards to the above mentioned parameters, if at all. The results showed seed treatment alone was sufficient for *T. asperellum* to colonize the seed surface and surrounding rhizosphere and prevent *R. solani* from attacking the seed before it could germinate and the hypocotyl after emergence. Soil containing *S. sclerotiorum* and *S. rolfsii*, however, required a bi-monthly drench with *T. asperellum* to achieve maximum germination. This suggests that these pathogens not

only attack the seed but are capable of entering the host plant through roots, stem and other tissues exposed to their mycelium and spores, making it difficult to control these pathogens with seed treatment alone.

The highest dry seedling biomass in the Speedling 24® trials was obtained when seeds were treated and soil drenched bi-monthly, for all three pathogens. These results correlate with the seedling biomass obtained in the rhizotron trials for the same treatment in rhizotrons inoculated with *S. sclerotiorum* and *S. rolfsii*. For those inoculated with *R. solani*, however, seedling biomass was the same with seed treatment alone and seed treatment in combination with a bi-monthly drench. Optimal root area was obtained when seeds were treated in combination with a bi-monthly drench for all three pathogens. This is likely due to the soil drench applications enhancing the establishment of *T. asperellum* populations in the soil, thereby reducing the amount of sclerotia in the soil produced by the pathogens, subsequently reducing sources of infection or preventing sclerotial germination. In addition, treating seeds in combination with a bi-monthly drench also achieved the highest dry root mass. These results suggest a correlation between root mass and root area. Competition for nutrients and space is one of the likely mechanisms involved in the biological control of plant pathogens (Tronsmo and Hjeljord, 1998). Usually the biological control agent grows and out competes the pathogen for nutrients and space. The pathogen is suppressed in the process leading to a population reduction, which no longer becomes a problem (Anonymous, 2001). Sivan and Chet (1989) suggested competitive displacement and free nutrient competition as the mechanism involved in the biological control of *F. oxysporum* on cotton by *T. harzianum T35*.

It was interesting to note that plants inoculated with *R. solani* did not die but were stunted, compared to the disease-free control and also those inoculated with *S. sclerotiorum* or *S. rolfsii*. This indicated that sub-lethal infection of sunflower plants by *R. solani* in the soil may stunt sunflower growth. Plant height and dry head weight of sunflower plants inoculated with *S. sclerotiorum* showed the most growth with seed treatment in combination with a monthly drench, whereas those inoculated with *R. solani* and *S. rolfsii* showed the most growth with seed treatment in combination with a bi-monthly drench.

The significant improvement in sunflower biomass agreed with earlier reports where fresh weight, shoot length, dry weight and leaf area of cucumber seedlings as well as seedling weight of cabbages were increased significantly by the application of *T. harzianum* and *T. viride* (Raviv *et al*., 1998; Yedidia *et al*., 2001). Increases in root area of dry bean and plant height of sunflower by application of *Trichoderma* spp. as a seed treatment have also been reported (Yobo, 2005; Elungi, 2009). Results presented in this chapter effectively demonstrate the potential of *T. asperellum* to control damping-off caused by *S. sclerotiorum*, *S. rolfsii* and *R. solani*.

An improvement on this study would be to evaluate the ability of *T. asperellum* strain kd to colonize and kill the sclerotia of *S. sclerotiorum*, *R. solani* and *S. rolfsii* and to replicate this study under field conditions.

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Appendix 5.

Table 5.2 Effect of *T. asperellum* on the germination and dry seedling biomass of sunflower seedlings grown in Speedling 24® trays and inoculated with *S. sclerotiorum in vivo*

Treatment	Mean seedling survival 30 dap (%)	% Seedling survival*	Mean dry seedling biomass 30 dap (g)	% Dry seedling biomass*
DFC	90.0 d	100.0 [0]	13.9 d	100.0 [0]
DC	54.9 a	61.0 [-39.0]	9.0 a	64.7 [-35.2]
ST	77.1 c	85.7 [-14.3]	13.0 c	93.5 [-6.5]
DAP	66.4 b	73.8 [-26.2]	11.7 b	84.2 [-15.8]
DAP + DBM	79.4 c	88.2 [-11.8]	13.6 cd	97.8 [-2.2]
ST + DBM	87.3 d	97.0 [-3.0]	15.0 e	107.9 [7.9]
F-ratio	**69.1**		**73.0**	
P-level	**< 0.001**		**< 0.001**	
LSD	**4.6**		**0.7**	
CV%	**5.1**		**4.8**	

dap= days after planting; DFC= disease-free control; DC= disease control; ST= seed treatment; DAP= drench at planting; DBM= drench bimonthly; *% of uninoculated, untreated control; Means followed by different letters are significantly different (Duncan's Multiple Range Test, $P < 0.05$); Values in parentheses indicate percentage increase/decrease in seedling survival or dry seedling biomass over the uninoculated + untreated control.

Table 5.3 Effect of *T. asperellum* on the germination and dry seedling biomass of sunflower seedlings grown in Speedling 24® trays and inoculated with *R. solani in vivo*

Treatment	Mean seedling survival 30 dap (%)	% Seedling survival*	Mean dry seedling biomass 30 dap (g)	% Dry seedling biomass*
DFC	89.6 d	100.0 [0]	13.8 b	100.0 [0]
DC	29.6 a	33.0 [-67.0]	9.1 a	65.9 [-34.1]
ST	84.3 c	94.1 [-5.9]	14.9 c	108.0 [8.0]
DAP	77.3 b	86.3 [-13.7]	13.0 b	94.2 [-5.8]
DAP + DBM	78.8 b	87.9 [-12.0]	13.8 b	100.0 [0]
ST + DBM	84.0 c	93.7 [-6.25]	15.5 c	112.3 [12.3]
F-ratio	**221.0**		**59.0**	
P-level	**< 0.001**		**< 0.001**	
LSD	**4.3**		**0.8**	
CV%	**4.9**		**5.4**	

dap= days after planting; DFC= disease-free control; DC= disease control; ST= seed treatment; DAP= drench at planting; DBM= drench bimonthly; *% of uninoculated, untreated control; Means followed by different letters are significantly different (Duncan's Multiple Range Test, $P < 0.05$); Values in parentheses indicate percentage increase/decrease in seedling survival or dry seedling biomass over the uninoculated + untreated control.

Table 5.4 Effect of *T. asperellum* on the germination and dry seedling biomass of sunflower seedlings grown in Speedling 24® trays and inoculated with *S. rolfsii in vivo*

Treatment	Mean seedling survival 30 dap (%)	% Seedling survival*	Mean dry seedling biomass 30 dap (g)	% Dry seedling biomass*
DFC	91.4 e	100.0 [0]	13.9 e	100.0 [0]
DC	31.7 a	34.7 [-65.3]	7.6 a	54.7 [-45.3]
ST	69.0 c	75.5 [-24.5]	10.2 c	73.4 [-26.6]
DAP	62.0 b	67.8 [-32.2]	8.5 b	61.1 [-38.8]
DAP + DBM	68.5 c	74.9 [-25.0]	10.8 c	77.7 [-22.3]
ST + DBM	75.0 d	82.1 [-17.9]	12.6 d	90.6 [-9.3]
F-ratio	**217.9**		**87.1**	
P-level	**< 0.001**		**< 0.001**	
LSD	**3.8**		**0.7**	
CV%	**4.9**		**5.9**	

dap= days after planting; DFC= disease-free control; DC= disease control; ST= seed treatment; DAP= drench at planting; DBM= drench bimonthly; *% of uninoculated, untreated control; Means followed by different letters are significantly different (Duncan's Multiple Range Test, $P < 0.05$); Values in parentheses indicate percentage increase/decrease in seedling survival or dry seedling biomass over the uninoculated + untreated control.

Table 5.5 The effect of *T. asperellum* on the dry shoot and root biomass and root area of sunflower grown in rhizotrons and inoculated with *S. sclerotiorum in vivo*

Treatment	Mean dry shoot biomass 30 dap (g)	% Dry shoot biomass*	Mean dry root biomass 30 dap (g)	% Dry root biomass*	Mean root area 30 dap (mm^2)	% Root area*
DFC	2.4 d	100.0 [0]	1.5 d	100.0 [0]	22801 b	100.0 [0]
DC	0.5 a	20.8 [-79.2]	0.6 a	40.0 [-60.0]	7332 a	32.2 [-67.8]
ST	2.1 c	87.5 [-12.5]	1.3 c	86.7 [-13.3]	21401 b	93.9 [-6.1]
DAP	1.9 b	79.2 [-20.8]	1.0 b	66.7 [-33.3]	20812 b	91.3 [-8.7]
DAP + DBM	2.3 d	95.8 [-4.2]	1.2 c	80.0 [-20.0]	22143 b	97.1 [-2.9]
ST + DBM	2.6 e	108.3 [8.3]	1.6 d	106. 7 [6.7]	22902 b	100.4 [0.4]
F-ratio	**163.8**		**35.7**		**83.0**	
P-level	**< 0.001**		**< 0.001**		**< 0.001**	
LSD	**0.2**		**0.2**		**1882.1**	
CV%	**9.2**		**16.0**		**10.7**	

dap= days after planting; DFC= disease-free control; DC= disease control; ST= seed treatment; DAP= drench at planting; DBM= drench bimonthly; *% of uninoculated, untreated control; Means followed by different letters are significantly different (Duncan's Multiple Range Test, $P < 0.05$); Values in parentheses indicate percentage increase/decrease in seedling survival or dry seedling biomass over the uninoculated + untreated control.

Table 5.6 The effect of *T. asperellum* on the dry shoot and root biomass and root area of sunflower grown in rhizotrons and inoculated with *R. solani in vivo*

Treatment	Mean dry shoot biomass 30 dap (g)	% Dry shoot biomass*	Mean dry root biomass 30 dap (g)	% Dry root biomass*	Mean root area 30 dap (mm^2)	% Root area*
DFC	2.4 c	100.0 [0]	1.5 d	100.0 [0]	22724 c	100.0 [0]
DC	0.4 a	16.7 [-83.3]	0.3 a	20.0 [-80.0]	3715 a	16.3 [-83.6]
ST	1.5 b	62.5 [-37.5]	1.2 c	80.0 [-20.0]	20704 b	91.1 [-8.9]
DAP	1.3 b	54.2 [-45.8]	1.0 b	66.7 [-33.3]	20384 b	89.7 [-10.3]
DAP + DBM	1.4 b	58.3 [-41.7]	1.1 bc	73.3 [-26.7]	20703 b	91.1 [-8.9]
ST + DBM	1.5 b	62.5 [-37.5]	1.5 d	100.0 [0]	22067 bc	97.1 [-2.9]
F-ratio	**73.0**		**55.8**		**126.9**	
P-level	**< 0.001**		**< 0.001**		**< 0.001**	
LSD	**0.2**		**0.2**		**1882.8**	
CV%	**16.4**		**17.3**		**11.1**	

dap= days after planting; DFC= disease-free control; DC= disease control; ST= seed treatment; DAP= drench at planting; DBM= drench bimonthly; *% of uninoculated, untreated control; Means followed by different letters are significantly different (Duncan's Multiple Range Test, $P < 0.05$); Values in parentheses indicate percentage increase/decrease in seedling survival or dry seedling biomass over the uninoculated + untreated control.

Table 5.7 The effect of *T. asperellum* on the dry shoot and root biomass and root area of sunflower grown in rhizotrons and inoculated with *S. rolfsii in vivo*

Treatment	Mean dry shoot biomass 30 dap (g)	% Dry shoot biomass*	Mean dry root biomass 30 dap (g)	% Dry root biomass*	Mean root area 30 dap (mm^2)	% Root area*
DFC	2.4 d	100.0 [0]	1.5 c	100.0 [0]	22649 d	100.0 [0]
DC	0.5 a	20.8 [-79.2]	0.1 a	6.7 [-93.3]	3158 a	13.9 [-86.1]
ST	2.1 c	87.5 [-12.5]	1.1 b	73.3 [-26.7]	20169 bc	89.0 [-10.9]
DAP	1.9 b	79.2 [-20.8]	1.2 bc	80.0 [-20.0]	19099 b	84.3 [-15.7]
DAP + DBM	2.3 d	95.8 [-4.2]	1.1 b	73.3 [-26.7]	20703 bc	91.4 [-8.6]
ST + DBM	2.6 e	108.3 [8.3]	1.3 bc	86.7 [-13.3]	22067 cd	97.4 [-2.6]
F-ratio	**23.4**		**23.4**		**133.5**	
P-level	**< 0.001**		**< 0.001**		**< 0.001**	
LSD	**0.3**		**0.3**		**1808.2**	
CV%	**29.5**		**29.5**		**11.2**	

dap= days after planting; DFC= disease-free control; DC= disease control; ST= seed treatment; DAP= drench at planting; DBM= drench bimonthly; *% of uninoculated, untreated control; Means followed by different letters are significantly different (Duncan's Multiple Range Test, $P < 0.05$); Values in parentheses indicate percentage increase/decrease in seedling survival or dry seedling biomass over the uninoculated + untreated control.

Table 5.8 The effect of *T. asperellum* on the plant height and dry head biomass of sunflower grown in pots and inoculated with *S. sclerotiorum in vivo*

Treatment	Mean plant height 80 dap (cm)	% Plant height*	Mean dry head biomass 80 dap (g)	% Dry head biomass*
DFC	238.6 c	100.0 [0]	17.7 c	100.0 [0]
DC	69.3 a	29.0 [-71.0]	5.3 a	29.9 [-70.1]
ST	233.6 c	97.9 [-2.1]	15.0 bc	84.7 [-15.2]
DAP	168.9 b	70.8 [-29.2]	7.3 a	41.2 [-58.8]
DAP + DM	235.5 c	98.7 [-1.3]	12.9 b	72.9 [-27.1]
ST + DM	238.0 c	99.7 [-0.2]	17.6 c	99.4 [-0.6]
DAP + DBM	235.8 c	98.8 [-1.2]	16.7 c	94.3 [-5.6]
ST + DBM	220.9 c	92.6 [-7.4]	15.3 bc	86.4 [-13.6]
F-ratio	**13.9**		**24.2**	
P-level	**< 0.001**		**< 0.001**	
LSD	**45.3**		**2.7**	
CV%	**22.1**		**20.1**	

dap= days after planting; DFC= disease-free control; DC= disease control; ST= seed treatment; DAP= drench at planting; DBM= drench bimonthly; *% of uninoculated, untreated control; Means followed by different letters are significantly different (Duncan's Multiple Range Test, $P < 0.05$); Values in parentheses indicate percentage increase/decrease in seedling survival or dry seedling biomass over the uninoculated + untreated control.

Table 5.9 The effect of *T. asperellum* on the plant height and dry head biomass of sunflower grown in pots and inoculated with *R. solani in vivo*

Treatment	Mean plant height 80 dap (cm)	% Plant height*	Mean dry head biomass 80 dap (g)	% Dry head biomass*
DFC	238.3 e	100.0 [0]	17.9 d	100.0 [0]
DC	67.9 a	28.5 [-71.5]	2.7 a	15.1 [-84.9]
ST	207.8 cd	87.2 [-12.8]	15.0 c	83.8 [-16.2]
DAP	180.0 b	75.5 [-24.5]	9.4 b	52.5 [-47.5]
DAP + DM	204.9 bc	86.0 [-14.0]	14.7 c	82.1 [-17.9]
ST + DM	226.0 cde	94.8 [-5.2]	15.7 c	87.7 [-12.3]
DAP + DBM	205.5 bc	86.2 [-13.8]	14.8 c	82.7 [-17.3]
ST + DBM	234.4 de	98.4 [-1.6]	17.0 d	95.0 [-5.0]
F-ratio	**35.9**		**128.7**	
P-level	**< 0.001**		**< 0.001**	
LSD	**26.0**		**1.2**	
CV%	**13.3**		**9.3**	

dap= days after planting; DFC= disease-free control; DC= disease control; ST= seed treatment; DAP= drench at planting; DBM= drench bimonthly; *% of uninoculated, untreated control; Means followed by different letters are significantly different (Duncan's Multiple Range Test, $P < 0.05$); Values in parentheses indicate percentage increase/decrease in seedling survival or dry seedling biomass over the uninoculated + untreated control.

Table 5.10 The effect of *T. asperellum* on the plant height and dry head biomass of sunflower grown in pots and inoculated with *S. rolfsii in vivo*

Treatment	Mean plant height 80 dap (cm)	% Plant height*	Mean dry head biomass 80 dap (g)	% Dry head biomass*
DFC	237.4 e	100.0 [0]	17.8 f	100.0 [0]
DC	29.0 a	12.2 [-87.8]	1.7 a	9.5 [-90.4]
ST	161.0 b	67.8 [-32.2]	8.0 c	44.9 [-55.1]
DAP	149.5 b	63.0 [-37.0]	6.1 b	34.3 [-65.7]
DAP + DM	182.4 c	76.8 [-23.2]	8.7 c	48.9 [-51.1]
ST + DM	160.8 b	67.7 [-32.3]	15.5 e	87.1 [-12.9]
DAP + DBM	200.8 cd	84.6 [-15.4]	12.3 d	69.1 [-30.9]
ST + DBM	215.4 d	90.7 [-9.3]	16.1 e	90.4 [-9.5]
F-ratio	**86.0**		**126.1**	
P-level	**< 0.001**		**< 0.001**	
LSD	**19.3**		**1.4**	
CV%	**11.6**		**13.1**	

dap= days after planting; DFC= disease-free control; DC= disease control; ST= seed treatment; DAP= drench at planting; DBM= drench bimonthly; *% of uninoculated, untreated control; Means followed by different letters are significantly different (Duncan's Multiple Range Test, $P < 0.05$); Values in parentheses indicate percentage increase/decrease in seedling survival or dry seedling biomass over the uninoculated + untreated control.

DISSERTATION OVERVIEW

Sunflower is grown worldwide as a food crop for domestic use and bird feed, but more so for oilseed production. Sunflower oil is of great commercial importance in Australia, China, India, South Africa (SA) and Turkey (Semelczi-Kovacs, 1975). Sunflower is susceptible to damage caused by numerous pests and pathogens, particularly fungi, which lead to significant losses in grain yield. *Sclerotinia sclerotiorum*, the causal agent of sunflower head and stem rot, is an aggressive fungus which attacks and infects almost 400 plant species (Steadman *et al*., 1994). This pathogen can be responsible for losses up to 75.0% in sunflower as a result of head and stem rot.

S. sclerotiorum also causes damping-off of sunflower seedlings pre- and post-emergence, along with fungal pathogens *Rhizoctonia solani* and *Sclerotium rolfsii*. This problem often leads to poor or erratic plant stands. The causal agents are commonly found in soils worldwide and have broad host ranges. They are capable of survival between crops and planting seasons as hard, dormant resting structures (sclerotia), in crop debris (saprophytic) and on weeds and other hosts (pathogenic).

Inappropriate, continuous and non-discriminative use of chemicals causes undesirable effects such as residual toxicity, environmental pollution, development of pathogen resistance, health hazards to humans and animals, and increased farm expenditure for plant protection. Instead, some plant pathologists have decided to shift their attention to developing environmentally safe, long-lasting and effective biocontrol methods for the management of plant diseases.

The contributions of beneficial fungal and bacterial species to the development of sustainable agriculture has been emphasised in literature (Lewis and Papavizas, 1991; Schippers *et al*., 1995). Such contributions range from control of plant diseases, increased plant growth as well as enhanced mineral uptake by plants. *Trichoderma* and *Bacillus* spp. are among the beneficial fungal and bacterial species that have been widely studied for their role in biological control of plant diseases and in plant growth promotion (Yobo, 2005). Moreover, species belonging to these two genera have been commercialised and are currently available commercially (Woo *et al*., 2014; Borriss, 2015). However, isolation of new antagonistic strains is necessary to improve biological control methods and to enhance control of plant diseases.

The findings presented in this dissertation resulted from the evaluation of three *Bacillus* isolates and one yeast isolate that were recovered from wild sunflower, and a commercial strain of *Trichoderma* spp., for biological control of *S. sclerotiorum* head rot of sunflower and possible growth promotion. A second commercial strain of *Trichoderma* spp. was screened for biocontrol of damping-off caused by *S. sclerotiorum*, *S. rolfsii* and *R. solani* on sunflower. Experimental trials were all conducted under greenhouse conditions with the use of foliar spray application for screening yeasts, *Bacillus* spp. and *Trichoderma* strain 77 against head rot, and seed treatment and soil drench as the method of application when screening *Trichoderma* strain kd against damping-off. The data showed:

- *Bacillus* spp. isolates were more widespread on wild sunflower heads than yeasts.
- Of the yeasts and *Bacillus* spp. isolated, a greater proportion of *Bacillus* isolates were effective at inhibiting growth of *S. sclerotiorum* mycelia during *in vitro* dual-culture bioassays.
- Carpogenic induction of ascospores of *S. sclerotiorum* was highly dependent on temperature, moisture, light and length of sclerotial conditioning. Without the right combination of these environmental factors, dormancy of sclerotia was not broken, and no carpogenic germination occured.
- None of the methods to stimulate carpogenic germination, published by researchers in the Northern Hemisphere, worked with sclerotia in this study, which were sourced locally.
- In the greenhouse, the *Bacillus* isolates tested against *Sclerotinia* head rot were the most effective agents of biocontrol in this study, and were also the most effective agents of plant growth promotion.
- *T. atroviride* strain 77 (Eco-77®), or T77, was effective at controlling *Sclerotinia* head rot in the greenhouse, despite not being registered for use against *S. sclerotiorum* head rot of sunflower on the product label.
- Dual inoculations of *Bacillus* isolates and T77 to control *Sclerotinia* head rot were generally better than single inoculations but not significantly different to single inoculations of *Bacillus* spp.
- The optimal inoculum concentration/density of yeast and *Bacillus* isolates varied greatly, impacting the level of control of *Sclerotinia* head rot.

- During *in vitro* dual-culture bioassays, *T. asperellum* strain kd (Eco-T®), or Tkd, effectively inhibited mycelial growth and overgrew cultures of *S. sclerotiorum*, *R. solani* and *S. rolfsii* and mycoparasitism could be seen via scanning electron microscopy ultrastructure studies.
- In the greenhouse, Tkd was found to be effective at controlling damping-off of sunflower seedlings caused by *R. solani*, *S. sclerotiorum* and *S. rolfsii*, despite not being recommended for use against the latter two pathogens on the product label.
- A combination of seed treatment and soil drenching was found to be most effective at controlling damping-off caused by *S. sclerotiorum*, *S. rolfsii* and *R. solani* in the greenhouse.

Many isolates of the genus *Bacillus* are antagonistic towards a broad range of phytopathogenic microorganisms in agricultural crops, such as rice, maize, fruit trees, and others (Wang *et al*., 2014; Li *et al*., 2015). Several species of the *Bacillus* genus (*B. subtilis*, *B. pumilus*, *B. amyloliquefaciens* and *B. licheniformis*) have been studied as potential biocontrol agents to mitigate the incidence of diseases of importance to agriculture (Raaijmakers and Mazzola, 2012). These strains reduce the establishment and development of phytopathogenic organisms through various mechanisms, which include (a) the production of antibiotics, (b) siderophores, (d) lytic enzymes, (d) toxins and (e) inducing the systemic resistance of the plant (ISR) (Layton *et al*., 2011; Tejera-Hernández *et al*., 2011). They are fast-growing, grow effectively in low cost media and sporulate under undesirable conditions, making *Bacillus* isolates attractive candidates for application as biocontrol agents. The demand for such agents is growing rapidly since it is expected that global market for biopesticides will significantly expand over the next 3-5 years (www.bccresearch.com/market-research/chemicals/biopesticides-chm029e.html).

In this study, it was found that combined inoculations of B16 + B24 and B24 + B26 were most effective at suppressing *Sclerotinia* head rot and enhancing growth. This may be a result of additive effects of the different modes of action of each *Bacillus* antagonist. However, there have been no reports in literature of dual inoculations of two or more different antagonistic *Bacillus* species. Combined inoculations of T77 + B26 also completely suppressed disease. The enhancement of biological control using

combinations of bacteria and fungi has been previously reported (Stevez de Jensen *et al*., 2002; Sundaramoorthy *et al*., 2011; Alizadeh *et al*., 2013; Marimuthu *et al*., 2013; Jain *et al*., 2015; Izquierdo-García *et al*., 2020). Yobo (2005) evaluated different isolates of *Trichoderma* spp. and *Bacillus* spp. to control *R. solani* and promote growth of cucumber and beans. However, in his study, none of the combinations were able to improve the efficacy shown by the individual activity of three isolates of *Trichoderma* spp. and only plant growth promotion by the combined inoculation was observed. The results presented in this study suggest that *Trichoderma* and *Bacillus* could be used together. Unravelling the necessary conditions under which these two organisms could be used together efficiently and effectively could help reduce inconsistencies reported with the use of single organisms. Ideally, a combination of biological control and growth promoting traits would be advantageous and would result in a better biological control and plant growth promotion effects when compared to any of the organisms used alone (Yobo, 2005).

A question that needs to be addressed is to determine what the optimum combination of biological traits required from each organism are, in order to achieve a synergistic effect. Jisha and Alagawadi (1996) combined phosphate solubilizing *B*. *polymyxa* and cellulolytic *T*. *harzianum* to increase yield and nitrogen uptake in sorghum (*Sorghum bicolor* L. Moench) compared to single inoculations of each organism. The work presented in this dissertation considered foliar treatment as the only method of BCA application; the question of whether different inoculation systems would improve biological control and growth promotion efficiencies in a dual inoculation system needs to be looked at.

Mycoparasitism is one of the mechanisms used by *Trichoderma* spp. for biological control of plant pathogens (Tronsmo and Hjeljord, 1998). Ultrastructure studies showed extensive mycoparasitism by Tkd against *S. rolfsii*, *S. sclerotiorum* and *R. solani.* The rhizosphere is rich in nutrients and pathogen and the introduced biocontrol agent (antagonist) compete for the availability of space and nutrients. *Trichoderma* spp. mycelia grow rapidly and can inhibit or slow down development of the pathogen population in the rhizosphere, subsequently reducing disease. These fungi colonize the root epidermis and outer cortical layers, releasing bioactive molecules that induce pathways for resistance in plants, increase plant growth and nutrient uptake (Benitez

et al., 2004; Harman, 2006). Seed treatment and soil drench with Tkd effectively enhanced the growth of sunflower seedlings and plants when tested against *S. rolfsii* and *S. sclerotiorum*. However, seed treatment alone provided sufficient disease suppression and growth promotion when tested against *R. solani*. This agrees with the findings of Yobo (2005) in which seed treatment of Tkd alone was effective at suppressing disease and enhancing growth of dry bean plants when tested against *R. solani*.

This dissertation forms the basis of *Trichoderma-Bacillus* interaction studies and proposes that with the right combinations, these two organisms can be used together to enhance plant growth and biological control of plant diseases. What remains to be resolved is how these two organisms can be formulated together as a single commercial product for use on agricultural crops. Factors that would have to be considered at length and studied are formulation and storage conditions, cost and shelf life. Larkin and Fravel (1998) suggested that combinations of fungi and bacteria might provide protection against plant diseases at different times and/or under different conditions. Commercial strains of *Trichoderma* spp., under the trade names Eco-77® and Eco-T®, clearly show potential at suppressing foliar and seedling diseases of sunflower caused by the pathogens used in this study. However, field testing under natural conditions is necessary. This study is also the first to demonstrate that the heads of wild sunflower plants are good sources from which to isolate native bacterial antagonists that effectively control *Sclerotinia* head rot in the greenhouse.

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