STUDY OF INOCULATION AND DISEASE EVALUATION TECHNIQUES FOR SCLEROTINIA STALK ROT (*SCLEROTINIA SCLEROTIORUM*) OF SOYBEAN

By

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5. Evaluation of isolate variation and pathogenicity of *Sclerotinia sclerotiorum* isolates on Soybeans in South Africa

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I wish to dedicate this work to my family who gave me the opportunity,

love, trust and encouragement to fulfil my dreams

Declaration

I declare that the dissertation hereby submitted by me for the degree Magister Scientiae Agriculturae at the University of the Free State is my own independent work and has not previously been submitted by me at another university/faculty. I furthermore cede copyrights of the dissertation in favour of the University of the Free State.

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Chrisna Botha

General Introduction

Soybeans have been grown in South Africa for many years, but have only become a major cash crop over the last 30 years (Pschorn-Strauss & Baijnath-Pillay, 2004). The crop is mostly cultivated in areas such as Bergville, Bethal, Ermelo, Newcastle, Warden, Winterton, Vryheid and Vrede (Anonymous, 2007). Around the world, soybean is a significant source of income and factors that reduce soybean production such as diseases, insects, weeds and environmental influences can affect the economic welfare of many countries (Wrather *et al.*, 2001).

Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is increasing in importance on soybeans. Severe epidemics of sclerotinia stem rot occurred in the Gauteng production areas during the late 1970's and early 1980's (Phillips & Botha, 1990) and the disease has subsequently spread to all production areas. This is mainly due to changes in management practices, germplasm susceptibility and favourable weather conditions (Mueller *et al.*, 2004). Diseases caused by *S. sclerotiorum* are common in many areas of South-Africa (Gorter, 1977) especially due to the wide host range of this pathogen which contains 408 plant species. It however causes severe losses in dicotyledonous crops such as sunflower, soybean, edible dry bean, chickpea, peanut and dry bean (Bolton *et al.*, 2006).

Disease incidence is dependent on weather (Phillips & Botha, 1990), specifically microclimate conditions within the crop canopy. Temperature has been shown to have a significant effect on apothecial formation, ascospore germination, mycelial growth, initiation of infection and expansion of lesions (van den Berg & Lentz, 1968) together with moisture. Not only is moisture necessary for ascospore production but also for initiation and development of infection (Steadman, 1983; Tu, 1989). Control of this pathogen is best achieved when several practices are combined taking into consideration that it is applied at the correct

time and proper procedures are followed. Sufficient knowledge of the biology of this pathogen is important, however in order to put such systems in place and to properly control this pathogen.

On soybean, *S. sclerotiorum* reduces yield of susceptible soybean cultivars and disease severity and yield are statistically correlated (Grau & Radke, 1984). Losses are generally directly from loss of yield and indirectly from reduced grain quality (loss in grade). Development of resistant soybean cultivars appears to be a feasible, effective and economic strategy for the control of Sclerotinia stem rot (Boland & Hall, 1987) and are important in the management of Sclerotinia stem rot (Yang, Lundeen & Uphoff, 1999). For this to succeed, a controlled-environment screening method that reflects differences in disease susceptibility and also has the ability to accurately predict the reaction of soybean germplasm that could be expected in field environments is needed (Kim *et al.*, 2000, Kull *et al.*, 2003; Vuong *et al.*, 2004). Several methods for inoculation to screen for germplasm for resistance under artificial conditions have been developed but not one method has been identified that produces reactions that consistently correlate with field results (Hoffman *et al.*, 2002; Kim & Diers, 2000; Kim *et al.*, 2000; Kull *et al.*, 2003; Vuong *et al.*, 2004). Evaluation of varietal resistance in the field only permits one cycle of evaluations during the growing season. This is a lengthy process and often subject to failure due to unfavourable weather (Chun, Kao & Lockwood*,* 1987). Therefore such an inoculation technique would reduce the reliance on natural environmental conditions for the evaluation of soybean varieties and avoid possible disease escapes that occur in field trials (Auclair *et al.*, 2004).

Screening for Sclerotinia stem rot resistance is affected by numerous potential sources of variation and reproducibility of results remains a problem. Moreover, a non-destructive approach would be useful in genetic studies and breeding programs where progeny tests to measure seed productivity are often required (Vuong *et al.*, 2004) and this would greatly aid in the recurrent selection program and breeding effort (Neslon *et al.,* 1991). It is especially important to know whether

pathogen genotypes have evolved that are better adapted and more pathogenic to soybean (Hambleton, Walker & Kohn, 2002). This information of the genetic basis of *S. sclerotiorum* isolates will assist in the breeding of resistant and improved cultivars with durable resistance (Zhao & Meng, 2003).

The aim of the current study was to:

1. Review the literature on Sclerotinia stem rot of soybean with emphasis on control strategies.
2. Evaluate inoculation techniques for *S. sclerotiorum* and evaluate soybean germplasm under greenhouse conditions.
3. Evaluate and determine the conditions optimal for disease development on soybeans in the greenhouse and field.
4. Compare chemical and biological control of Sclerotinia stem rot in the greenhouse.
5. Evaluate isolate variation and pathogenicity of *S. sclerotiorum* isolates collected from local fields using *in vitro, in vivo* and AFLP analyses.

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CHAPTER 1

An overview of Sclerotinia stem rot of soybean

1.1. Introduction

The first record of soybeans (*Glycine max*) being planted in South Africa was in 1903 (Anonymous, 2007). However, it is only relatively recently that more attention has been given to soybean as a major cash crop (Pschorn-Strauss & Baijnocth-Pillay, 2004). Approximately 10 000 t were produced during the 1970's and this grew to 190 000 t in 2001. The 2001 harvest made up only 0.7% of total agricultural economy. The province of Mpumalanga is the main soybean growing region followed by Kwazulu-Natal and the Free State. The crop is mostly cultivated in areas such as Bergville, Bethal, Ermelo, Newcastle, Warden, Winterton, Vryheid and Vrede (Anonymous, 2007)

Around the world, soybean is a significant source of income and factors that reduce soybean production such as diseases, insects, weeds and environmental fluctuations affect the economic welfare of many countries (Wrather *et al.*, 2001). This crop is an important source of high quality protein and oil with a content of approximately 40% and 20% respectively (Anonymous, 2006).

Species of the genus *Sclerotinia* cause destructive diseases on numerous plants worldwide affecting seedlings, mature plants and their harvested products (Agrios, 1997). Sclerotinia stem rot is becoming increasingly important on soybeans due to changes in management practices, germplasm susceptibility and favourable weather conditions (Mueller *et al.*, 2004). Diseases caused by *Sclerotinia sclerotiorum* (Lib.) de Bary are common in many areas of South-Africa on soybean, sunflower and dry beans (Gorter, 1977).

S. sclerotiorum is among the world's most omnivorous and successful plant pathogens, together with the closely related species, *S. minor* and *S. trifoliorum*.

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S. sclerotiorum does the most damage to vegetables and oilseed species, *S. minor* to peanut and lettuce and *S. trifloliorum* to forage legumes (Steadman, 1983). *S. minor* and other *Sclerotinia* spp. are known to cause root and stem rots, fruit and vegetable rots while *S. cepivorum* causes white rot of onions and other *Allium* spp. (Alexander & Stewart, 1994).

Disease control measures commonly used include the applications of fungicides, seed treatments, crop rotations, sanitation, moisture control planting adaptations and microclimate regulations (Steadman, 1983). This chapter will review the importance of *S. sclerotiorum* diseases and their control. Included is a brief overview of the causal organism, symptoms, host range, geographic distribution and its epidemiology.

1.2. Causal Organism

S. sclerotiorum is a necrotrophic soil-inhabiting fungus (Bolton, Thomma & Nelson, 2006). The fungus belongs to the phylum Ascomycota, class Discomycetes and order Helotiales, family Sclerotiniaceae and genus *Sclerotinia.* The hyphae are hyaline, septate, branched and multinucleate and appear to be white to tan in culture and on plants. The sclerotium is the main survival structure of the fungus and 90% of the life-cycle occurs in the soil (Alexander & Stewart, 1994).

S. sclerotiorum produces large, smooth, rounded sclerotia, 2 to 10mm in diameter compared to *S. minor* which produces small, rough, angular sclerotia, 0.5mm to 2mm in diameter (Laemmlen, 2006). Sclerotia have a light coloured interior portion called the medulla, consisting of fungal cells rich in β-glucans and proteins. The rind, a black outer protective covering contains melanin pigments which are highly resistant to degradation. The size and shape of the sclerotia depend on the host and where they are produced in the infected plant (Anonymous, 2005). The formation of secondary sclerotia increases inoculum density (Adams & Ayers, 1979).

Sclerotia can survive and remain viable for up to ten years under dry conditions (Laemmlen, 2006). According to Cook *et al.* (1975) sclerotia can survive in soil for at least three years. When the sclerotia fail to completely melanize during maturation

or if the rind layer becomes naturally or artificially injured, the ability of the sclerotium to function as a dormant propagule is altered (Huang, 1985). Soil temperatures, pH and moisture appear to have little direct effect on their survival, but a combination of high temperatures and high moisture appears to encourage the degradation of the sclerotia near the soil surface (Ferreira & Boley, 1992).

1.3. Symptoms

Symptoms caused by *S. sclerotiorum* vary according to host and environment. The disease is known by a variety of descriptive names, such as cottony rot, white mould, stem rot and blossom blight, to name a few (Agrios, 1997) and literature has revealed more than sixty names used to refer to disease caused by this omnivorous pathogen (Purdy, 1979). On most plants early symptoms are associated with the appearance of a white, fluffy mycelial growth on the infected tissue with the sclerotia developing later. They are white at first and later become black with a hard exterior (Agrios, 1997).

Symptoms on most host plants usually result indirectly from mycelium that colonizes flower petals and moves into branch axils, after which colonization progresses into the main stem (Boland & Hunter, 1988; Grau, Radke; & Gillespie, 1982). Branches become infected, leaves become yellow and wilted and soon abscise. Sclerotia and mycelia are sometimes present on the stem surface(Natti, 1971) (Figure 1 a & b).

a

b

Figure 1 a & b: Symptoms of *S. sclerotiorum* showing white bleached stems, white mycelial growth and sclerotia (Nelson, 2004).

Beans with white mould display a characteristic bleached stem and the formation of sclerotia on the stem surface as well as internally (Figure 1 a & b). First symptoms can usually be seen as scattered wilted leaves in a field. Close investigation of the vines shows soft watery spots on leaves, pods and stems (Steadman, 1983). On soybeans, darker coloured areas with watersoaked

margins that gradually enlarge are visible (Thompson & van der Westhuizen, 1979). Lesions later become rotten and covered by white mycelium. After infection, stems and branches become wilted and eventually die and take on a bleached appearance. The epidermal layers become dried and cracked (Steadman, 1983) and the softer intervascular tissue in the stems disintegrates while the leaves become brittle exposing the vascular strands (Thompson & van der Westhuizen, 1979).

Direct infection of host tissues may also occur with the stems, branches, leaves and pods of beans plants apparently being equally susceptible (Natti, 1971). Penetration of the host cuticle is achieved by mechanical pressure since there is no evidence of pre-penetration dissolution of the cuticle by enzymes. Penetration is followed by rapidly disorganized tissues as the result of enzymatic processes that affect the middle lamella between cells. As fungal activity continues, it results in the total destruction of parenchymatous tissues and the remaining vascular and structural elements of the stalks, stems, branches and twigs take on a shredded appearance (Purdy, 1979). Death of the whole plant can occur as fungal activity continues. Seedpods remained undeveloped and flattened and in some cases dark brown sclerotia form within the pods (Thompson & van der Westhuizen, 1979). The white cottony mycelium growth is visible over the diseased areas. Later black elongated sclerotia develop on the mycelia and within the stem tissue (Ryley, 2006).

Secondary spread is associated with abundant mycelial growth from infected tissues. Contact between diseased and healthy plants results in disease spread and distribution of disintegrating tissues by environmental factors can also contribute to secondary infections (Natti, 1971). Factors such as rain duration and frequency, dew, wind, aeration, row width and plant morphology affect duration of free moisture which will have an effect on disease incidence and severity (Tu, 1989), which also means that the epidemiology of white mould is dependent on a wide range of factors (Tu, 1987).

Figure 2: Sclerotia present in soybean stems
(Botha, 2006; Personal obserservation).

1.4. Host Range

According to Boland & Hall (1994) the host index of *S. sclerotiorum* contains 42 sub-species or varieties, 408 species, 278 genera and 75 plant families. Except for *Rumohra adiantiformis* in the Pteridophyta, all hosts of *S. sclerotiorum* fall within the plant division Spermatophyta. No hosts have been found among the Thallophyta or Bryophyta. Within the Spermatophyta, hosts have been identified among the classes Gymnospermae and Angiospermae.

The pathogen can cause severe losses in dicotyledonous crops such as sunflower, soybean, oilseed rape, edible dry bean, chickpea, peanut, dry bean and lentils and in some monocotyledonous species such as onion and tulips (Bolton *et al.*, 2006).

The majority of reported hosts are herbaceous plants from the sub-class Dicotyledonae of the Angiospermae. Families that contain the largest number of hosts included Asteraceae, Fabaceae, Brassicaceae, Solanaceae, Apiaceae

and Ranunculaceae, in decreasing order of host numbers. Several perennial, woody hosts such as apple, horse-chestnut and oak have also reported from this sub-class. Within the monocotyledonae, there are at least 25 hosts classified within the families Dipsacaceae, Iridaceae, Liliaceae, Musaceae and Poaceae (Boland & Hall, 1994). However, according to Mclaren, Huang & Rimmer (1996), wheat and barley are non-host crops together with maize and sorghum (Phillips & Botha, 1990).

In South Africa, *S. sclerotiorum* has been reported on vegetables including cabbage, cauliflower, lettuce, Brussels sprouts, dry beans, green beans, soybeans, carrots and tomatoes. Sunflower, cotton and lupins are also highly susceptible crops (Phillips & Botha, 1990). Weeds can play a significant role in the disease cycle, e.g. marsh elder, lambsquarters, pigweed and wild mustard that act as alternative hosts (Anonymous, 2005).

1.5. Geographic distribution

S. sclerotiorum is geographically cosmopolitan and has a broad ecological distribution. It is most common in temperate regions and was originally believed to occur only in cool, moist areas but is now known to occur in hot dry areas as well (Ferreira & Boley, 1992). This pathogen is much less active at temperatures approaching freezing point (0°C) or temperatures greater than 32°C (Purdy, 1979).

1.6. Economic importance

In 2005, 95.2 million ha was under soybean cultivation in the world with a total production of 212.6 million tonnes. In South Africa, approximately 150 000 ha are cultivated with soybean (Anonymous, 2006a). *S. sclerotiorum* reduces yields of susceptible soybean cultivars and disease severity and yield are statistically correlated (Grau & Radke, 1984). Losses result directly from loss of yield and indirectly from reduced grain quality (loss in grade). According to Purdy (1979), losses of up to 5-10% occur annually in dry bean and a 40% loss in the grade of potatoes has been reported, resulting in losses of \$48-50,000 and \$4,000,000 respectively in the United States. Estimated soybean yield losses in the U.S. were

estimated at \$3.1 x 10^9 in 1998 compared to \$0.9 x 10^9 in 1994 which indicates a significant increase over a relatively short period. Losses due to Sclerotinia stem rot were estimated at 509 000 t in 1998 (Wrather *et al*., 2001).

According to the United States national *Sclerotinia* initiative, annual losses due to this disease reached \$26 million in dry beans, \$13 million in snap beans and \$94 million in canola in North Dakota and Minnesota from 1991-2002. In the Midwest areas, about 2% is lost every year and annual losses of up to \$300 million often occur. In sunflower production, \$15 million in losses is recorded every year and in the years 1999 and 2004, losses reached \$100 million each year (Anonymous, 2006).

Recently, annual losses in the United States exceeded \$200 million (Bolton *et al*., 2006). According to Wrather *et al*. (2001), the reduction in soybean yields (in thousand metric tons) for the top ten soybean producing countries in the world was greatest for the United States (509.0) followed by India (438.5) and Argentina (423.2). No losses were recorded in Bolivia and Indonesia.

In Argentina, soybean is an important crop due to entry into the international market. In the early 1990's diseases began to threaten the soybean production with some such as *S. sclerotiorum* causing major losses in 1998. *S. sclerotiorum* was one such disease. In 1997-1998, favourable disease conditions included mild temperatures and abundant rainfall. Monocropping and no-till production practices promoted disease spread (Wrather *et al*., 2001).

In Canada production has increased from 83,6000 ha in 1994 to 96,6000 ha in 1998, due to the development of short-season cultivars adapted to cool conditions. Sclerotinia stem rot has become a serious production contraint (Wrather *et al*., 2001) having increased from affecting 60 000 ha in 1994 to 100 000 ha in 1998.

In India, soybean is the number two oilseed crop. During 1998, extensive yield losses occurred because of diseases such as Fusarium root rot and stem blight, Rhizoctonia foliar blight and Sclerotinia stem rot (Wrather *et al*., 2001). Sclerotinia caused reduction in soybean yields of approximately 438 500 metric tons in 1998.

1.7. Epidemiology

Plants can become infected by *S. sclerotiorum* in three ways, i.e. by mycelium from infected branches or other plant parts, mycelium present on the ground from infected, dead leaves and from mycelium that originates from germinated ascospores (Purdy, 1979).

Sclerotia in the soil can germinate in one of two ways to initiate infection namely carpogenically or myceliogenically. In myceliogenic germination, the sclerotium produces mycelia that infect root tissues directly (Bardin & Huang, 2001) while with carpogenic germination large numbers of ascospores are released for aerial dispersal.   The latter is the main method of infection and has an advantage in that spores have the ability to be dispersed over large areas (Steadman, 1983).

1.7.1 Carpogenic germination

Sclerotia that result from infected plants will not germinate until they have been preconditioned, also known as physiological maturation. This usually occurs during winter or the non-cropping season. In most cases freezing is not necessary, but adequate moisture and temperatures of 4 – 20°C trigger the conversion of a dormant sclerotium unto an active one. After preconditioning, sclerotia germinate to form apothecia (Figure 3). Stipes are seldom longer than 5cm in length and therefore only sclerotia within 5cm of the soil surface will complete ascospore production (Steadman, 1983). Light, temperature and moisture are considered the most important factors for carpogenic germination (Sun & Yang, 2000; Hao, Subbarao & Duniway, 2003). When moisture is at an optimum, germination of sclerotia decreases significantly at temperatures >30°C.   Similarly, when temperatures are ≤ 5°C the rate of carpogenic germination is low and the incubation time required is longer (Hao *et al.*, 2003).

Sclerotia must be within 2-10cm of the soil surface for apothecial production to occur (Steadman, 1983; Cook *et al.*, 1975). Cook *et al.* (1975) found that sclerotia

at greater depths remained under exogenous dormancy due to moisture fluctuations which serve as a germination stimulus. After germination, stipes or apothecial stalks form and although they may be formed above ground, light is necessary to stimulate formation of the inoperculate asci from brownish, stiptate ascospore-containing disks of about 2-8mm in diameter at the end of the stipes (Steadman, 1983).

Figure 3: Apothecia of S. sclerotiorum (Lamley & Bradley, 2003).

Ascospores are formed in the apothecial disks and when ripened, large numbers of 10 000 to 30 000 spores mature simultaneously. Relative humidity is a trigger for discharge and a sudden change will trigger forcible discharge, releasing many ascospores and causing a "puffing" phenomenon (Figure 4) (Steadman, 1983). When ascospores are released, each apothecium can release 2–30 million spores over a period of several days (Venette, 1998). Ascospores escape to above the canopy and have been detected on leaves 50-100 m from the source (Steadman, 1983). In most cases this disease is initiated by ascospores (Bolton *et al.*, 2006) which infect the aerial tissues and result in stem blight, stalk rot, head rot, pod rot and blossom blight of plants (Bardin & Huang, 2001). Disease incidence increases with increasing ascospore concentrations.

Figure 4. Production of apothecia *in vitro* and ascospore discharge (Cobb & Dillard, 2004).

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White mould becomes a problem in bean fields usually 10-14 days after full bloom. This supports the view that senescent or dead flowers are involved in disease development (Natti, 1971). Necrotic tissues resulting from injury or other pathogens are ideal for infection (Tu, 1989). Dillard & Cobb (1995) investigated the effect of wounds on cabbage infection and found that injuries penetrating several leaf layers of cabbage plants resulted in cell damage and plant exudates. Exudates serve as a source of nutrients for the germination of ascospores and infection of the cabbage head by *S. sclerotiorum*.

Although moisture is an important factor in infection and disease development, ascospores can infect flowers and initiate disease even at relative humidities as low as 25%. Ascospores of *S. sclerotiorum* can remain viable on colonized flowers for up to 144h of drying and still cause disease if favourable conditions subsequently appear (Harikrishnan & Del Rio, 2006). Young *et al.* (2004) also found that infection may occur when inoculated plants were maintained for 144h at a RH of 50%.

Harikrishnan & Del Rio (2006) made inoculum from re-hydrated dry bean flowers that had been dipped in an ascospore suspension or mycelium suspension and found that a RH as low as 25% and temperatures of 18°C to 22°C are sufficient for ascospores to infect seedlings. Mycelium was however more efficient in causing disease than ascospores under these conditions.

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Even with ascospore concentrations as low as two ascospores per flower, 20 to 40% white mould incidence was recorded. When RH was 90%, ascospores and mycelia were equally effective in causing disease irrespective of the temperature. Differences could be due and absence of free water available on the leaf surface at 25% RH and as a result ascospores germinate at a lower rate than ascospores incubated at 90% RH (Harikrishnan & Del Rio, 2006). This is in contrast to mycelium which is less affected by low RH.

According to Abawi & Grogan (1975), moisture is the most important climatic factor affecting white mould development. It is essential for the production of ascospores as well as the initiation and development of infection and needs to be accompanied by low to moderate temperatures (Kurle *et al*., 2001).

Leaf infections by ascospores require 2 to 3 days of continuous leaf wetness (Couper, 2003). When plants were inoculated with ascospore suspensions and exposed to a range of wetness durations or different RH and temperature combinations, all plants developed disease and no relationship was observed between leaf wetness duration or humidity and disease incidence (Young *et al*., 2004). Moisture plays an important role in infection and disease development and a free moisture period of 48-72h is required for establishment of infection and lesion expansion.   In the absence of free moisture lesion development quickly stops and the fungus remains quiescent in the lesion until it can be reactivated upon the return of free moisture (Tu, 1989).

Couper (2003) stated that the release of ascospores requires rain or high humidity. Senescing flowers need to be wet for approximately 48h for spores to germinate and mycelium to colonize host tissues. Temperatures between 16°C and 25°C are best for germination (Harikrishnan & Del Rio, 2006). When conditions are favourable, the fungus proceeds from senescent blossoms to healthy tissue in about 16 to 24h (Venette, 1998). Under continuous leaf wetness, ascospores inoculated on lettuce leaves germinated within 2-4h at 15-25°C, 10h at 10°C with little to no germination at 30°C (Young *et al*., 2004).

1.7.2. *Myceliogenic Germination*

Myceliogenic germination plays a major role in the disease cycle of Sclerotinia wilt of sunflower (Anonymous, 2005). It is a particular feature of small-sclerotia types that require the invasion of organic matter as an energy source for the formation of mycelium and subsequent infection of the host plant (Purdy, 1979). Sclerotia germinate in the presence of exogenous nutrients and produce hyphae that invade non-living organic matter. The mycelium subsequently penetrates the host cuticle by mechanical pressure (Ferreira & Boley, 1992). Mycelial infection, however, is unlikely to occur in plants located more than 2cm from a sclerotium (Ferreira & Boley, 1992). *S. sclerotiorum* and *S. minor,* have the ability *to* survive between crops as mycelium in infected plant debris (Laemmlen, 2006).

The melanized rind of the sclerotium serves as a protective layer and as a dormancy-controlling site preventing myceliogenic germination (Huang, 1985). Huang, Chang and Kozub (1998) found that myceliogenic germination of sclerotia occurred easily at high temperatures (20 – 25°C) and at high humidity and when they are dry prior to incubation. This is assumed to be due to injury to the rind, cortex and outer medullary tissues of the sclerotia by extreme dehydration thus resulting in the release of nutrients that support the growth of the germinating hyphae.

Any time after seedling emergence bean plants are susceptible to *S. sclerotiorum.* Sclerotia in the soil can germinate to form mycelium which can attack seedlings and result in a damping-off symptoms (Purdy, 1979). Lettuce seedlings placed directly on *S. sclerotiorum* cornmeal inoculum, were colonized and the fungus spread from plant to plant across a 12cm gap in 5 days at 16°C, 3 days at 20°C and 4 days at 24°C. Inoculations however did not succeed if the initial inoculum was farther than 2cm away from the plants (Newton & Sequeira, 1972).

Studies conducted by Natti, (1971) showed that mycelium could serve as inoculum on beans, but its importance *in vivo* could not be established. Grau *et al*. (1982) stated that ascospores remain the primary inoculum and not mycelia. Cook *et al*. (1975) found that *S. sclerotiorum* does not survive as mycelium in soil or residue. Mycelia from germinated sclerotia initiated less than 10% of the initial infections of beans in the field

and greenhouse and most of the initial infections in the field and greenhouse started in the plant canopy due to mycelium from colonized senescent flowers adhering to various plant organs (Cook *et al.,* 1975).

In Nebraska, mycelium did not overwinter in plant debris, but survived in bean seed (Cook *et al.*, 1975). Huang & Kozub (1993) found that mycelia of *S. sclerotiorum* do not survive in stems of dry beans, sunflower and canola, but could overwinter in infected seeds. Poor survival of mycelium could be due to humidity effects and microorganisms in the soil. In Nebraska, mycelium lost viablilty rapidly after four to eight months in the field. When seedlings come into direct contact with surviving mycelia these can serve as primary inoculum for seedling infection (Huang & Kozub, 1993).

Moisture and temperature seem to be the most important factors affecting the mycelial survival. Mycelia in air-dried stems survived better at 10°C than at 20°C. Therefore, when sub-freezing temperatures occur in fields, mycelial survival in diseased stems may be prolonged. At temperatures > 20°C viability is quickly lost (Huang & Kozub, 1993). Mycelium are more tolerant to desiccation than ascospores and therefore may be able to cause disease under lower RH conditions (Harikrishnan & Del Rio, 2006).

1.8. Control

The ability of cultural control to minimize plant diseases makes this practice an essential component of any disease strategy (Steadman, 1979). Sclerotia survive in the soil for at least three years (Steadman,1983; Cook *et al.*, 1975), and factors that affect survival include soil type, previous crop, initial population of sclerotia and environmental conditions (Anonymous, 2005). A complex of abiotic (soil moisture content, texture and pH) together with biotic (fungal, bacteria and nematodes) in addition to sclerotia immobility influences survival (Alexander & Stewart, 1994). Cultural control, although sometimes advised for the control of *Sclerotinia* diseases, is not effective for the control of *S. sclerotiorum*, that form large sclerotia (Steadman, 1979).

1.8.1. Tillage

In addition to reduced tillage having positive effects on the soil structure, soil erosion and water conservation, Gracia-Garza *et al.* (2002), showed that a reduction in the numbers of apothecia and apothecial clumps are associated with no-tillage and no-tillage with chopped residue. However, according to Mueller *et al.* (2004), reduced tillage or no-till systems allowed soil borne inoculum to accumulate on or near the soil surface, increasing apothecia formation and the release of airborne ascospores (Gracia-Garza *et al.*, 2002).

Kurle *et al.* (2001) compared three tillage practices for their effect on sclerotia distribution and viability. Sclerotia were buried and redistributed throughout the soil profile by mouldboard and to a lesser extent by chisel plow. Viability of sclerotia in the upper 2cm of soil was reduced by mouldbord and chisel plough compared to no-till.

No-till resulted in no reduction in sclerotial density and little reduction in viability in the upper soil profile. The greatest density of viable sclerotia was obtained in the upper 2cm of the soil with no-till. Loss in viability of sclerotia from the mouldboard and the chisel plough could be the result of parasitism of the buried sclerotia. Sclerotia on the soil surface, on the other hand, showed reduced

parasitism activity probably reduced by periodic drying (Kurle *et al.*, 2001). Despite this, however, the greatest number of apothecia and the highest disease incidence were found in plots where mouldboard ploughing was applied. These authors suggested that tillage systems affect disease incidence indirectly by their effect on soybean emergence, plant population and crop canopy structure. Higher soybean plant populations were observed with mouldboard and chisel plough than with no-till. Thus, microclimate conditions within the crop canopy in no-till were not sufficiently favourable for apothecia formation and disease development compared with mouldboard and chisel ploughing (Kurle *et al.*, 2001).

Duncan *et al*. (2006) investigated the time and burial depths that influenced the viability and colonization of sclerotia of *S. sclerotiorum* by bacteria. They found that viability was lowest at a depth of 10cm, followed by 5cm. Sclerotia placed on the soil surface showed high viability. Bacterial populations associated with sclerotia at a 10cm depth were greatest and thought to play a role in viability. Sclerotia at the soil surface had the lowest bacterial colonization levels.

In contrast to the above, Tu (1986) and Alexander & Stewart (1994) reported that most sclerotia in the upper 2-3cm of soil deteriorate within a year compared deeper buried sclerotia which showed a higher rate of survival. Anonymous (2005) suggested that more dramatic changes in temperature and moisture occur on the soil surface and these factors are deleterious to sclerotia. However, temperature studies showed that normal soil temperatures (10-30°C) did not affect survival. It was only when a temperature of 35°C was kept constant for three weeks or more that the survival of sclerotia was negatively affected (Adams & Ayers, 1979).

According to Merriman *et al.* (1979), deep ploughing is recommended to bury crop residue because survival of sclerotia is greater at soil surface than when buried. However, various factors need to be taken into account that could affect the survival of sclerotia in the soil. Soil temperature, the effect of soil wetting and drying fluctuations and soil microflora directly or indirectly influence sclerotia (Alexander & Stewart, 1994). During a cropping cycle, wetting and drying of soil is a very effective method in reducing the number of active sclerotia in the soil

(Laemmlen, 2006). The type of soil and depth to which sclerotia were buried influenced their survival. Experiments showed that sclerotia buried 1 and 2cm deep produced most apothecia, but less sclerotia were recovered as it appears that after sclerotia produced apothecia, they becomes more liable to decay (Mitchell & Wheeler, 1990). Alexander & Stewart (1994) also found that *S. sclerotium* and *S. rolfsii* survived for longer due to the fact that they produce larger sclerotia and therefore, have more food reserves to persist than *S. minor* and *S. cepivorum* that have smaller sclerotia. Survival of *S. sclerotiorum* in pastures in Canterbury showed that sclerotia smaller than 4mm degenerate over a seven month period while larger sclerotia persist for considerably longer.

1.8.2. Crop rotation

Crop rotation is a common practice in the control of white mould of dry beans (Steadman, 1983). However, in the North Platte Valley in Western Nesbraska, USA, rotation of dry bean crops with corn and sugar beets every third year is not effective (Cook *et al.,* 1975). In plots planted continuously with soybean greater apothecia production was observed compared with rotations using crops such as corn and wheat. According to Mclaren *et al.* (1996) wheat and barley are not hosts of *S. sclerotiorum*. These are often used in rotation with susceptible crops such as bean and canola to reduce disease potential.

Wheat appears to be a better choice for use in rotation as higher germination frequencies of sclerotia were recorded in trials when precipitation coincided with the peak of plant canopy development. In corn, lower germination frequencies were associated with a less dense canopy (Gracia-Garza *et al*., 2002). Kurle *et al.* (2001) however found that the reduction in disease incidence following corn or a small grain does not appear to be caused by a reduction in soilborne inoculum as no significant difference in sclerotial density or apothecial numbers were recorded. However, no explanation of why a reduction in disease incidence was observed was provided.

Crop sequences consisting of soybean and other susceptible crops result in the accumulation of soilborne inoculum (Mueller *et al.*, 2004). Rotation with non-

host crops together with the use of no-till were both effective in reducing SSR incidence. When these practices are used in combination with a moderately resistant cultivar, their effectiveness in reducing SSR was mutually complementary.

Kurle *et al.* (2001) reported that although disease is reduced after planting a non-host crops, the reduction recorded was less than is obtained when a resistant cultivar is planted. This could be attributed to alternate hosts and it was emphasized that, if an infested area is planted with a non-host crop, it is important that proper weed control practices are employed in order to reduce inoculum (Mclaren *et al.*, 1996).

1.8.3. Crop Density

Row spacing and plant density of have been shown to affect stem rot incidence and severity. Buzzel, Welacky & Anderson (1993) found that there was no significant increase in stem rot incidence with decreasing row width and with increasing plant populations per hectare. However, it is generally accepted that cultural practices such as narrower row spacing, higher plant populations and optimum fertilizer application create dense plant canopies, that result in high humidity and cooler temperatures within the canopy and favour infection and disease development (Mueller *et al.*, 2004). Narrow rows and high plant densities reduce air circulation and trap moisture within the canopy. These conditions increase early senescence and hence susceptibility to the pathogen. A denser canopy also increases contact between plant parts which promotes the plant to plant spread of inoculum (Tu, 1997).

When soybean plants were planted in 90cm row widths, canopy closure was slower resulting in similar canopy and air temperatures. This reduced canopy density results in soil and canopy temperatures being more greatly influenced by air temperatures in 90cm row widths and results in less disease development (Grau & Radke,1984). In beans, control can be enhanced by improving air circulation between rows by planting the rows parallel to prevailing winds (Tu, 1997), the use of wire trellises to raise foliage from the ground, the pruning of

branches (Ferreira and Boley, 1992) and reducing the seeding rate and practicing stringent weed control (Tu, 1997).

Grau and Radke (1984) found that Sclerotinia stem rot was more severe in soybeans planted at row widths of 25cm compared to 75cm rows. This may be due to fluctuations in soil moisture in the more shaded rows being less therefore resulting in lower mortalities of sclerotia during the growing season. Reducing planting density however can cause reductions in yields. Yield trials in Wisconson, USA showed a general yield increase of 21% when soybeans were grown in 25cm rows compared to 75cm row widths in the absence of the disease. When the same cultivars were grown at narrow row spacing in the presence of *S. sclerotiorum,* yields were reduced by 42%.

1.8.4. Planting date

A study conducted in China showed that earlier sown plots had a greater incidence of Sclerotinia stem rot and that more serious disease was present in varieties that mature early than with the ones maturing later. Although results indicated that disease could be avoided or minimized in later sowing, it also showed that yield in later sown crops declined sharply (Hu *et al*., 1999). The consistency of this method of disease escape will however, depend on local weather cycles and the consistency of seasonal wet and dry cycles.

1.8.5. Irrigation

Due to the relationship between moisture and disease development, a reduction in the number of irrigation cycles applied to the crop could reduce disease incidence especially towards the end of the season when crop development coincides with the susceptible period. A disadvantage of this however is that a reduction in irrigation often results in a reduction in yield (Steadman, 1983). In Nebraska both apothecial production and disease severity were reduced when the irrigation frequency was reduced and this was correlated with a yield increase. It was proposed that plants be watered thoroughly until a continuous

canopy forms, after which irrigation frequencies should be reduced to ensure less sclerotinia infection and yield stability (Steadman, 1983).

1.8.6. Sanitation

Sanitation is important in preventing the introduction of inoculum into uninfested fields but will not significantly reduce the disease potential in fields that are already infested (Tu, 1989). Sanitation practices such as not using irrigation runoff water will reduce the spread of sclerotia, mycelia or ascospores from one field to another. If contaminated water is re-used, application to non-host crops will minimize pathogen dissemination in irrigation water (Steadman, 1983).

Sanitation practices that could contribute to disease control include clean seed programmes that keep sclerotia out of seed lots (Figure 5) (Anonymous, 2005). It is therefore important to plant only cleaned seed if *S. sclerotiorum* has been present (Steadman *et al.*, 1996). Infected seed is especially important in spreading white mould into new white bean (*Phaseolus vulgaris*) crops and low quality seed from infested fields should therefore not be used for planting. Growth chamber studies suggest that seed infested with *S. sclerotiorum*, upon germination, can provide a nutrient source that stimulates sclerotia germination (Lundeen, 1998).

Thompson & van der Westhuizen (1979) showed that the fungus is internally seedborne in a considerable proportion of undersized seed. Sanitation is a necessary practice when it comes to harvesting of crops such as sunflower, pea and bean. Seed beans contaminated with sclerotia, infected seeds or both may be an important source of inoculum, especially to fields that are not already infested with *S. sclerotiorum* (Hoffman *et al.,* 1998). Certified seed will prevent the introduction of *Sclerotinia* into disease-free areas and care should be taken not to redistribute inoculum or infested residues back into fields leading to inoculum build-up (Steadman, 1979).

Figure 5: Sclerotia of *S. sclerotiorum* in soybean seed lots (Anonymous, 2007a).

1.8.7. Weed Control

Weeds often serve as alternate hosts for plant pathogens and proper field sanitation plays a vital role in reducing inoculum survival and dissemination. In South Africa, weeds that serve as alternate hosts include *Amaranthus deflexus* (Moq) (pigweed), *Bidens formosum* L. (cosmos), *Bidens pilosa* L (common blackjack) and *Tagetes minuta* L (tall Khaki weed) (Purdy 1979; Phillips, 1992). *Datura* sp. and *Xanthium strumarium* were also reported as hosts of *S, sclerotiorum* but are less susceptible (Phillips, 1992).

1.8.9. Fertilization

Soil fertility can influence a number of plant diseases especially those that produce soilborne resting structures like *sclerotinia* diseases (Gilbert, Mclaren & Grant, 2000).   Sun & Huang, (1985) formulated a S-H mixture for the control of soilborne diseases, containing 4.4% bagasse, 8.4% rice husks, 4.25% oyster shell powder, 8.25% urea, 1.04% potassium nitrate, 13.16% calcium superphosphate and 60.5% mineral ash. This mixture proved to be effective in controlling Fusarium wilt of radish and Pythium rot of cucumber (Gilbert *et al*., 2000). These authors also found that viability of sclerotia of *S. sclerotiorum* was 94% in the untreated plots compared to 62% and 48% in soil amended with 1% and 2% S-H mixture, respectively. It is understood that these products release inhibitory substances that prevent the growth of stipes and the development of apothecia. The effective ingredients in the S-H mixture

seem to accelerate the weakening of sclerotia, thereby enhancing their colonization by soil microorganisms (Gilbert *et al*., 2000).

Huang *et al*. (2006) reported that, when calcium cyanamide (Perlka®) was added to soil, sclerotial survival and sclerotial carpogenic germination were reduced. No viable sclerotia were found after two weeks and carpogenic germination did not occur. However, breakdown products of Perlka® can negatively affect seed germination and plant growth in a number of crops and it is recommended for that if a soil treatment is to be used, application should be done before sowing or planting. This timing varies with crops and the amounts applied (Huang *et al*., 2006).

When the levels of nitrogen were increased in soils, the number of apothecia was reduced and a delay in apothecia production were also noticed (Mitchell & Wheeler, 1990). However, excessive N fertilization may also promote disease development due to an increase in leaf area, succulence of the crop and earlier canopy closure (Gilbert *et al.,* 2000). Gilbert *et al*. (2000) found that the application of a urease inhibitor applied with urea-ammonium nitrate (UAN) or urea resulted in less disease than with the application of UAN or urea alone. Urease enzymes convert urea-N to ammonium-N and due to slower availability to the plant, will have the same effect as delayed application of N.

S. sclerotiorum has a wide growth range. Growth is limited at a pH of 10 by alkaline conditions induced with sodium hydroxide and pH 8 with sodium phosphate. *S. sclerotiorum* however has a tendency to change the hydrogen ion concentration of its nutrient solution that rapidly changes the pH to 3.2. The wide pH adaptation of *S. sclerotiorum* implies that liming of soils to the required inhibitory levels may not be practical and could also explain the wide host range and soil conditions under which the pathogen is found (Zeliff, 1928).

1.9. Chemical Control

Infection due to ascospores occurs on senescent flowers and fungicides, therefore need to be applied prophylactically to prevent colonization (Steadman, 1979). First flowers appear towards the middle of the plant and as growth continuous most flowers are produced on the new nodes towards the top of the plants. However, flowers continue to be produced near the bottom of the plant and thus, flowering occurs over an extended period. Some flowers may only become infected later during the growing season. The increased control with two fungicide applications of thiophanate-methyl suggests that protection of new flowers that form on lower nodes after the first application is essential and could lead to higher yields and lower disease incidences provided that the first application occurs prior to infection (Mueller *et al.*, 2004).

Fungicides should be directed at flower petals especially those in the lower canopy (Mueller *et al.*, 2002). Although several fungicides are effective against *S. sclerotiorum*, timing to protect blossoms from becoming infected is critical. Chemicals should be applied during early bloom and a second application may be necessary if favourable disease conditions occur. In order to provide thorough coverage of the blossoms, stems and leaves, chemicals should be applied in sufficient water to ensure deep penetration of the canopy (Wrather *et al.*, 2001). If the disease has already developed with visible symptoms, fungicide sprays can still provide effective control by preventing further spread. Proper timing of sprays and method of application, impacts on results (Tu, 1997) especially in the case of aerial application where coverage may be affected by the growth habit of the plants and the canopy density (Steadman, 1979). It is understood that aerial application is relatively ineffective when compared to ground application because the latter gives better penetration of the spray into the canopy (Tu, 1997).

1.9.1 Effective chemicals

Benomyl has been reported to control *Sclerotinia* diseases of sunflower, cabbage and beans (Ferreira & Boley, 1992). The fungicide thiram, when applied with Benomyl, delayed biological degradation of the latter and improved

absorption from soil. Thiram appears to prolong the persistance of Benomyl in soil which reduces apothecial emergence under field conditions (Ferreira & Boley, 1992). When plots sprayed with Benomyl and Topsin M were compared with untreated plots, white mould incidence was 61% and 72% in the two trails respectively compared to 48% and 34% respectively in the treated plots. This resulted in a yield difference of 30% and 21% (Lamley, 1998).

Complete plant coverage rather than dependence on systemic movement of Benomyl is important for good control. Efficacy of Benomyl is often diminished because of microbial degradation of the compound in soil (Ferreira & Boley, 1992) emphasizing the need for effective plant coverage. Natti (1971) reported that the efficacy of Benomyl was due to the absorption of the fungicide by the foliage of the crop and translocation of the chemical to the developing blossoms and buds. However, according to Hunter *et al*. (1978), Benomyl is not translocated into developing blossoms in beans at levels that are considered effective, despite being a systemic fungicide that moves acropetally in plants. A possible explanation may be that the distribution of the chemical is dependant on transpiration rate and since bean petals have no or limited stomata, translocation to these critical organs is limited.

Benomyl, Thiophanate-methyl, Tebuconazol and Vinclozolin were tested in a greenhouse trail against Sclerotinia stem rot on soybeans. No symptoms were observed with Benomyl, Thiophanate-methyl and Tinclozolin. Tebuconazole treated plants had both restricted and expanded lesions on leaves, but no stem symptoms were observed (Mueller *et al*., 2002). The dicarboximide fungicides rovral, ronilan and procymidine provided effective *Sclerotinia* control on lettuce and peanuts and restricted the development of established lesions (Ferreira & Boley, 1992). The herbicide Lactofen (Cobra®) (Dann, Diers & Hammerschmidt, 1999) was effective in suppressing disease development especially in locations and years in which disease incidence was high. This was attributed to alterations in the canopy environment reduction of sites on the plant that are natural infection sites or delayed flowering, senescence and maturity in Lactofen treated plots compared to controls (Dann *et al.,* 1999).

In a seedling trial in Ontario, 88-100% of seeds infected with *S. sclerotiorum* failed to germinate. Seedlings from infected seeds subsequently died from white mould at an early stage (Tu,1988). Sclerotia that formed on these plants could become a source of inoculum in subsequent plantings and therefore chemical seed treatment is an important factor in the control of *S. sclerotiorum.* Mueller, Hartman & Pedersen (1999) tested several seed treatment fungicide-amended agar, including Thiram, Captan, Fludioxonil, Metalaxyl, Pentachloronitrobenzene, Thiabendazole and and found that radial growth of *S. sclerotiorum* was significantly reduced on agar amended with these fungicides. Captan and Thiophanatemethyl were used as seed treatments and were 100% effective in eradicating the pathogen from infected seeds (Tu, 1989). These results showed that the application of fungicide seed treatments is an effective method of reducing sclerotium germination in infected seed.

1.9.2. *Timing of chemical sprays*

Since flower petals are the primary infection court, timing of fungicides must be coordinated with crop growth and development to achieve optimum control of SSR in soybean (Mueller *et al.,* 2004). Application must be coordinated with flowering and timing and direction of fungicides must be such that complete coverage of flower petals is obtained, especially those at the lower nodes of the plant. Coverage of plant parts with a chemical such as Benomyl, especially the blossoms, determines the efficacy. Unsatisfactory coverage of blossoms can be due to the flowering pattern of different varieties especially in the case of beans with an intermediate flowering habit (Steadman, 1979).

Studies in Illinois and Wisconsin to determine the optimum number of sprays and timing of thiophanate-methyl applications necessary to control Sclerotinia stem rot show that high yields and low disease severities are achieved when the chemical is applied at full rate before the desposition of inoculum on flower petals. Two applications during flowering, but before infection occurred, gave the best results (Mueller *et al.*, 2004).

1.9.3. *Application methods*

Poor results are often achieved due to improper timing and inefficient application procedures. According to Steadman (1983), studies in Nebraska showed that aerial application of Benomyl on beans for the control of white mould can be just as effective as ground application in covering the first set of blossoms. However, efficacy is determined by applications that thoroughly cover blossoms and sprays that are applied 3-5 days before full bloom. Failures of control by Benomyl has been reported when aerial sprays are applied to dense plantings that limit penetratrion of the compound into the canopy (Hunter *et al.*, 1978). In Idaho, sprinkler application of Benomyl gave effective control and in Florida, aerial application combined with an earlier ground application and an in-furrow treatment gave excellent control of white mould on the upright open canopy of pole beans (Steadman, 1983).

Foliar sprays of Benomyl during the active growing period of the blossoms and buds provides maximum control. However when Benomyl was applied after full bloom to mature and dead blossoms, control was not achieved. Benomyl when applied at flower can remain effective even after the blossoms have senesced (Natti, 1971).

Application of fungicides such as Vinclozolin, Procymidone and Fliazinam through irrigation water (chemigation) on dry beans is a common control method in Minas Gerias, Brazil. Conventional fungicide application requires 30-1000 ℓ ha^{-1} of water. Chemigation requires a minimum water volume of 2.5mm (25 000 ℓ ha^{-1} of water) using a centre pivot. Therefore, chemigation exceeds the maximum volume of water used by conventional ground sprayers by at least 25 times (Vieira *et al.*, 2003) but ensures canopy penetration by the chemical.

Hunter *et al.* (1978) found that Benomyl on blossoms was resistant to removal by rain even when rain occurred shortly after application. When oil was added to Benomyl, efficacy in the control *Cercospora* leaf spot of peanuts, scab and powdery mildew of apples was improved. However, this did not improve the control of white mould on snap beans (Hunter *et al.*, 1978).

1.10. Biological Control

Although biological control is a promising alternative for chemical control, not all chemical control will be replaced in the near future (Adams & Wong, 1991). The increasing environmental concern over the use of chemical pesticides has promoted a strong interest in biocontrol of *Sclerotinia* diseases (Tu, 1997). A number of research programs have focused on the application of biocontrol agents as a spray for control of diseases such as white mould of bean, stem rot of canola or rapeseed and lettuce drop, and as soil treatment for the control of diseases such as sunflower wilt (Bardin & Huang, 2000).

At least 30 species of parasites and antagonists have been identified for biological control of *S. sclerotiorum* (Steadman, 1979). Filamentous fungi are able to suppress white mould of beans. Control however is variable within and between individual taxa. Even though some are able so suppress disease, not all taxa have the ability to do so and the potential for biological control appears to be an isolate-specific character (Boland, 1997).

1.10.1. Biological agents

Alternaria alternata (Fr) Keissler, *Bacillus subtilis, Cladosporium cladosporioides* (Fr) de Vries, *Coniothyrium minitans* Campbell, *Drechlera* sp., *Epicoccum nigrum (*Link), *E. purpurascens* Ehrenb. Ex Schlect, *Fusarium acuminatum, F. graminearum, Myrothecium verrucaria, Penicillium* spp. and *Trichoderma viride* Pers. Ex Fr. were recorded as suppressive organisms by Boland (1997) and Inglis & Boland, (1992). All except *Penicillium* spp. significantly reduced lesion diameters and the incidence of disease by > 95% in a growth room, co-application trial (Inglis & Boland, 1992).

Adams (1989), evaluated reported antagonists of *Sclerotinia* spp. for their ability to destroy sclerotia and found that *Penicillium citrinum, Talaromyces flavus*, *Trichoderma* sp. and *Gliocladium virens* failed to reduce the survival of sclerotia of *Sclerotinia minor. T. flavus* was also found as ineffective in reducing apothecial production by Mclaren *et al.* (1996).

A biological agent that has achieved world wide success in the control of *S. sclerotiorum* is the coelomycete *C. minitans* Campbell. Studies on the efficiency of this mycoparasite have shown that, although the number of sclerotia were reduced, disease levels were not reduced. These results indicate that *C. minitans* may be effective as a bio-control agent of *S. sclerotiorum* but only at a low disease pressure. *S. sclerotiorum* has the ability to infect large areas with only a few apothecia and therefore *C. minitans* may be more successful against other sclerotia-forming pathogens such as *S. minor*, which generally germinate to form hyphae (Budge & Whipps, 1991).

In order to apply a biological agent, it is essential to understand population dynamics of the organism in use. A decrease in the number of sclerotia does not necessarily mean a decrease in disease and in yield losses. Research for the biocontrol of *Sclerotinia* applying the antagonists to the site of entrance i.e. the petals, needs to be investigated (Savchuk & Fernando, 2004).

1.10.2. Mode of Action

It appears that the most important factor that affects sclerotia survival is the biological activity in the soil (Adams & Ayers, 1979). In the soil, deleterious environmental factors such as soil type, soil moisture and temperature play a role in the declining populations of sclerotia together with microbial degradation. Numerous fungi, bacteria and other soil organisms parasitize and utilize sclerotia as a carbon source (Anonymous, 2005).

Important fungal parasites involved in the natural degradation of sclerotia are *Coniothyrium minitans, Sporidesmium sclerotivorum* (Anonymous, 2005) as well as *Alternaria* spp., *Epicoccum* spp., *Fusarium* spp., *Gliocladium roseum*, *Mucor* spp., *Penicillium* spp. *Trichoderma* spp. and *Trichothecium roseum* (Ferreira & Boley, 1992). Although these fungi have been shown to be effective against *Sclerotinia* spp. in culture their efficacy under natural conditions still needs to be established. Isolates of certain *Trichoderma* spp. have been show to be parasitic on sclerotia. Under natural conditions *C. minitans* is a well established destructive mycoparasite of *Sclerotinia sclerotiorum* (Adams & Ayers, 1979).

Sclerotia of *S. minor* were soaked in spore suspensions of *C. minitas, Dictyosporium elegans* and *Teratosperma oligocladum* before being incorporated into soil. The numbers of viable sclerotia declined. When spores of antagonists *T. oligocladum* and *Spirodesmium sclerotivorum* were incorporated into soil infected with *S. minor* only *S. sclerotivorum* markedly reduced the survival of *S. minor* (Adams, 1989). This was attributed to the macroconidia of these two fungi being able to germinate in soil when they were adjacent to sclerotia of *Sclerotinia* spp. The mycoparasites could grow out into the soil mass because infected sclerotia served as a food base, thus allowing them to compete with other microorganisms. In the long term, an organism which can invade sclerotia and destroy them will prove to be a more effective biological agent (Whipps & Budge, 1990)

Trichoderma spp. were found to have no effect on sclerotial viability (Budge & Whipps, 1991) and failed to reduce the survival of sclerotia (Adams, 1989). However, Gracia-Garza, Reeleder & Paulitz (1997), found that an isolate of *Trichoderma hamatum* tested under *in vitro* conditions in combination with *Bradysia coprophila* (fungus gnats) showed that sclerotial survival was highly affected by the activity of these organisms. Fungus gnat larvae were very effective in reducing the survival of sclerotia due to the removal of the melanized rind in the early phase of consumption of the sclerotia. Damage due to larval feeding increased the ability of *T. hamatum* to degrade sclerotia (Gracia-Garza *et al.*, 1997).

Inglis & Boland (1992) tested 126 isolates of *Alternaria alternata, C. cladosporioides* and *E. purparascens* and recorded from 100% to no suppression of *Sclerotinia* isolates. According to Boland & Hunter (1988) *A. alternata* or *C. cladosporioides* lack strong inhibitory effects on ascospore germination and mycelium growth of *S. sclerotiorum*. Although they restricted disease incidence in inoculation tests, this mainly have been due to the fact that these agents are more successful competitors for nutrients within the blossom tissues and not necessarily by parasitism or antibiosis.

The efficacy of *C. minitans* was tested by applying the agent either as a conidial suspension or as maize meal-perlite inoculum to soil (Jones, Mead & Whipps, 2003). When these mixtures were applied at full rate (10^7 cfu cm^{-3}) they were effective in reducing both the number of apothecia and the viability of the sclerotia. However, when high disease pressure occurred, the time between *C. minitans* inoculum application and a recorded reduction in sclerotia viability was increased significantly. Due to temperature and soil water potential affecting the efficacy of *C. minitans,* there is a need for optimization of the interval between treatment and planting of the crop (timing) and the rate of application in order for this treatment to be cost-effective for disease control (Jones, Mead & Whipps, 2003). This mycoparasite is, however, effective in controlling the production of apothecia under the canopy of host and non-host crops, thereby reducing the inoculum potential of *S. sclerotiorum* and making this a valuable soil treatment (Mclaren *et al.,* 1996).

C. minitans applied to soil at the seedling stage appears to invade bean tissues indicating that this hyperparasite has the ability to move from soil to aerial tissue. This ability to spread from areas removed from the site of application to sites infected makes *C. minitans* very attractive for the use as a biological agent (Mueller *et al.*, 1996). *C. minitans* has also been found to improve bean quality due to the reduction of sclerotia in harvested seed. It is the only mycoparasite shown to have the ability to attack sclerotia produced on diseased plants. It has the ability to survive for a period of at least 2 weeks in contact with leaf and soil micro flora (Gerlagh *et al.*, 1994).

1.10.3. Factors affecting efficacy

The efficacy of biological control depends on the extent of the interactions between the environment and the biological agent (Boland, 1997), the timing of application in relation to crop development, the type and concentration of propagules, the ability of the biological agent to survive and colonize plant tissues and the concentration of naturally occurring inoculum of the pathogen (Inglis & Boland, 1992). Adams (1989) suggested that a mycoparasite must be able to infect and destroy a plant pathogen, as well as be able to grow through

natural soil and infect healthy propagules of the pathogen. Therefore, it should be an aggressive mycoparasite as opposed to a passive parasite.

The effect of air temperature and relative humidity on biological control of white mould of bean was investigated by Hannusch & Boland (1996). Relatively small changes in air temperature or RH often produced dramatic changes in disease suppression. The suppression of disease was most effective under environmental conditions that were least conducive for disease (Boland, 1997; Hannusch & Boland, 1996).

Savchuk & Fernando (2004) studied the timing of applications of biological control agents by applying *Pseudomonas* spp. to Canola, relative to the application of the pathogen. They found that where the bacterium was applied up to 24h after the pathogen, disease was still significantly reduced compared with control treatments. The bacterium was able to compete with disease already established and therefore had a curative potential. However, under normal crop growth conditions and due to the extended flower period the bacterium needs to remain viable for at least two weeks after application to achieve best control. Results showed however that populations decreased after 7d of inoculation.

1.10.4. Combined Control

Boland (1997) found that biological agents were not able to control disease when highly favourable disease conditions reigned compared to fungicides that provided effective suppression of the disease under these conditions. It appears, however that biological control combined with fungicides can control the disease and that this combination provided improved control while reducing the amount of chemical applied.

Zhou & Reeleder (1989) investigated the combined effect of *Epicoccum pupurascens* and the fungicide Iprodione. *E. pururescens* has the potential to reduce the incidence and disease index of white mould (Inglis & Boland 1992; Huang *et al.,* 2000) while also being tolerant of the chemical. When the two

however were combined, the level of control was not greater than those where the agent was applied alone indicating that there were no additive or synergistic effects. However, three to four applications of the biocontrol agent were needed and further work is required before it can be offered as a sole alternative to chemical control (Zhou & Reeleder, 1989).

When *C. minitans* was applied to plots of beans and compared with Benomyl as a foliar spray, it did not compare with the efficacy Benomyl application for disease control in the season of application but did so in relation to longer term reduction of inoculum. Thus, the combination of the short-term effectiveness of Benomyl with the longer-term effectiveness of *C. minitans* appears feasible. The fungicide can be applied as a protectant to the growing crop and the hyerparasite could serve as a post-harvest treatment of sclerotia associated with crop residues before they are buried (Trutmann *et al.,* 1982).

Adams and Wong (1991) tested fungicides by applying them to the surface of soil columns and showed that when applied in the usually manner, these may not adversely affect *Sporidesmium sclerotivorum* in the soil.   Fungicides Benomyl, Iprodione and Vinclozoline are highly toxic to *S. sclerotivorum in vitro* but when applied to soil surface columns, only Benomyl at a concentration of 100 µg in the upper 2cm of the soil reduced the activity of the mycoparasite significantly, by preventing sclerotia from becoming infected by *S. sclerotivorum*. The other fungicides had little adverse effect on mycoparatism when applied at label rates in the field. Studies indicate that applying both chemical and biological control agents is both practical and prudent. Chemical treatment may be useful to control the disease until activity of the biological control agent is sufficient to suppress the pathogen (Adams & Wong, 1991).

1.11. Breeding for resistance

Chemical and biological control are often expensive or ineffective and their efficacy is affected by environmental conditions and agricultural practices. Genetic control remains the most economic method of disease control as well as being more applicable to sustainable agriculture and minimizing harm to the environment. Identification of resistance, however is hampered by factors such as easy and reliable screening procedures and a minimum of genetic variability for resistance to Sclerotinia stem rot (Grafton, 1998). The goal of breeding for resistance should be to select cultivars with high resistance to all forms of *S. sclerotiorum* attack found in the region of cultivation (Castano, Vear & Tourvieille de labrouhe, 1993).

Soybean is highly susceptible by *S. sclerotiorum* and the impact of infection on the crop is high. Current knowledge of sources of resistance in soybean to *S. sclerotiorum* and their stability across localities is limited because the relatively low number of lines evaluated to date. The nature of inheritance of resistance and the relative importance of physiological resistance and disease escape mechanisms under field conditions still requires investigation. Development of soybean cultivars with resistance to this pathogen appears feasible and may represent an effective and economic strategy for disease control in areas where this disease is prevalent (Boland & Hall, 1987). Physiological resistance and plant architecture have been identified as mechanisms to reduce white mould (Grafton, 1998).

Resistance is controlled by several genes and therefore the use of avoidance mechanisms such as upright and open plant structure, less dense canopies and branching patterns, elevated pod set and reduced lodging have been suggested to reduce the damage by *S. sclerotiorum*. Changing or incorporating these factors into a single host plant could result in better penetration of the canopy by sunlight and air circulation, making the microclimate within the plant canopy less favourable for disease development (Grafton, 1998).

Resistance to white mould was discovered in white bean (Tu & Beversdorf, 1982). In 1980, ExRico 23 was compared with Fleetwood, a cultivar of similar yield and maturity, in a field with a history of severe white mould. Results showed that ExRico 23 had a consistently lower disease incidence than Fleetwood (Tu 1989a). Benomyl spray slightly improved yield in Fleetwood, it did not prevent yield losses of up to 50% due to white mould (Tu & Beversdorf, 1982).

Initial disease incidence in ExRico 23 and Fleetwood were similar but progression of disease was considerably slower in ExRico 23. ExRico 23 showed a high cellular tolerance to oxalic acid than susceptible cultivars such as Fleetwood (Tu, 1989). The low disease incidence however, suggests that it may be more difficult for the fungus to initiate infection in ExRico 23 than in susceptible cultivars. The lower severity may be attributable to a slower disease progress within the infected plant (Tu, 1985). However, according to Tu & Beversdorf, 1982), further study is needed to determine if resistance results from genetic or structural characteristics.

Tu (1985) evaluated three white beans cultivars, the tolerant cultivar, ExRico 23 and two susceptible cultivars, Seafarer and Kentwood. Seedlings susceptible to *S. sclerotiorum*, showed water-soaked lesions much earlier and within six days the entire hypocotyl or petiole was affected and total collapse occurred by the seventh or eighth day. The development of brown spots was rapid and the leaf tissue disintegrated in 2-3 d. The earliest sign of infection was noticed on the fifth day and the lesions on the hypocotyl and petiole was remarkably smaller than those on the susceptible cultivars. The water-soaked zone around the brown-rot like lesions was wider in susceptible cultivars and spread rapidly from veins to interveinal tissue compared to leaves of ExRico 23 where initial symptoms were largely confined to the veins near the leaf base and the spread of brown rot-like symptoms from the veins to interveinal tissue was somewhat restricted (Tu, 1985).

1.11.1. Production of oxalic acid

Pathogenicity of *S. sclerotiorum* is closely related to the production of oxalic acid. Mutant isolates, not able to produce oxalic acid are non-pathogenic (Godoy *et al.*, 1990). Oxalic acid (ethanedioic acid) occurs in nature as a free acid and commonly as soluble potassium, sodium oxalate or insoluble calcium oxalate (Guimaraes & Stotz, 2004) and is known as one of the strongest organic acids with a pKa value of 1.3 and 4.3 (Caliskan, 1998).

Durman *et al*. (2005) showed that isolates collected from different hosts differed in their ability to release acids oxalic acid in particular. This suggests that the host may have some selective pressure on *S. sclerotiorum* isolates. Studies showed that chloroplasts, other cellular organelles and the plasma membrane are disrupted by oxalic acid. However for the oxalic acid to affect the chloroplast, it must alter the semi-permeable nature of the chloroplast membrane and also change the semi-permeability of the plasma membrane (Tu, 1989a). Guimaraes & Stotz (2004) found that stomatal guard cell are disrupted due to the inhibition of cellular responses.

Studies have indicated that tolerance of ExRico 23 to white mould is associated with the tolerance of the plasma membrane of cells to oxalic acid. This is associated with membrane stability of the cells (Tu, 1989a). Studies also showed that the response of excised leaves to uptake of oxalic acid was less in ExRico 23 than in the susceptible cultivars and smaller areas of brown rot like symptoms appeared in the excised leaves compared to cultivars Kentwood and Seafarer. This might have been caused by either greater cellular tolerance or impeded permeability to oxalic acid by ExRico 23 (Tu, 1985). Chipps *et al.* (2005) also found that oxalate contributes to lesion expansion.

A transgenic peanut line with resistance to *S. minor* was developed by Livingstone *et al.* (2005) based on an oxalate degenerating oxalate oxidase gene. They found that lesion type varied between transgenic lines and controls and that resistance was stable in the transgenic peanut line when oxalic acid was applied even at concentrations up to 20 x patho-physical levels.

Cessna *et al.* (2000) demonstrated a site of action of fungal oxalate in the facilitation of fungal infections and a suppression of the oxidative burst of the host plant. Many plant species can generate an oxidative burst to resist pathogens. Results indicated that oxalate is effective in suppressing the burst in soybean and tobacco as stimulated by a variety of elicitors. If the pathogen manages to discharge sufficient quantities of oxalate before the plant can detect the intruder, a primary site of disease resistance will be compromised.

1.12. Conclusion

Despite the vast volume of published research on topics such as epidemiology, symptoms, control and biology of this pathogen, *S. sclerotiorum*, remains a problem in many countries. Biocontrol of *S. sclerotiorum* is an aspect that still requires considerable research and progress is slow due to insufficient knowledge in the areas of mass production of inoculum, an effective delivery system for the agents, the shelf-life of biocontrol agents and their efficacy under various environmental conditions (Bardin & Huang, 2001).

Breeding for resistance and a more open plant canopy requires additional research. When management practices like adequate crop rotation, cultural practice modifications, correct irrigation practices and application of fungicides are combined with these new varieties, losses due to white mould can be efficiently reduced (Schwarts *et al.*, 2004).

Chemical sprays are not economical although the disease can be controlled to a significant degree by a combination of seed treatments and the use of less susceptible cultivars (Tu, 1989). It is clear however that control of this pathogen is best achieved when several practices are integrated. Proper timing and correct procedures are also important as well as knowledge of the biology of the pathogen.

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Chapter 2

Evaluation of greenhouse inoculation techniques for screening for Sclerotinia stem rot resistance in soybeans

2.1. Abstract

Numerous inoculation methods for screening of soybean germplasm for resistance to Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum* have been developed. This present study was done to compare and evaluate inoculation and disease evaluation techniques. Four soybean cultivars, SNK 500, PAN 520 RR, Wenner and CRN 5550 were planted in the greenhouse and grown to V3 (third trifoliate) growth stage. Six inoculation methods were evaluated namely a spray mycelium, drop mycelium, cut stem, cotyledon inoculation, straw inoculation and a petiole inoculation method. Plants were inoculated using the respective methods and were covered for nine days with transparent plastic bags. The number of infected plants were counted and lesion development and degree of wilting were scored using 0 to 5 rating scale. The spray mycelium method yielded the highest incidence of wilting although a significant cultivar x inoculation technique ($P<0.05$) was recorded. Results suggest that cultivar responses are affected by the degree and area of tissue damage. The spray mycelium method was used to evaluate 27 cultivars in three separate greenhouse experiments. Results of cultivars from the different experiments were poorly correlated and were dependent on the conditions during inoculation and subsequent disease development. Regression analysis was used to quantify the cultivar environment interaction. LS 580 proved more resistant and was least affected by changes in the environment while LS 555 was most susceptible. Results indicate the importance of quantifying the host x pathogen x environment interaction when evaluating cultivar response to Sclerotinia stem rot.

2.2. Introduction

Sclerotinia sclerotiorum is one of the most destructive plant pathogens in temperate regions of the world (Steadman, 1983). The host range includes 361 plant species in 64 families and new hosts are constantly being added to the list (Phillips & Botha, 1990). Sclerotinia stem rot caused by S. *sclerotiorum* is a major disease on soybeans (Hoffman *et al.,* 1998) and crop losses range from 0 to 100% depending on time and degree of infection (Purdy, 1979). Bean production in some highly desirable locations was discontinued because of repeated, heavy losses due to this pathogen (Natti, 1971). In South Africa, the disease was first reported on soybean in 1979 (Thompson & van der Westhuizen, 1979) although *S. sclerotiorum* had been previously reported on *Brassica* spp., *Citrus* spp., *Lupinus* spp., and *Phaseolus vulgaris* (Gorter, 1977) long before then. Severe epidemics of Sclerotinia stem rot occurred in the Gauteng production areas during the late 1970's and early 1980's (Phillips & Botha, 1990) and the disease has subsequently spread to all local production areas.

Cultivars with field resistance are an integral component of managing Sclerotinia stem rot. The disease requires cool, wet weather for development (Kim *et al.*, 2000) which may not occur in field conditions at the appropriate time of crop development and field trials, hence, cannot always be depended upon to produce reliable results (Nelson, Helms & Olson*.,* 1991). The evaluation of varietal resistance in the field only permits one cycle of evaluations during the growing season, making this a lengthy process particularly where failures occur due to unfavourable weather (Chun, Kao & Lockwood*.,* 1987). As a result, there is a need for a controlled-environment screening method that reflects differences in disease susceptibility and accurately predicts the reaction of soybean germplasm that may be expected in field environments (Kim *et al.*, 2000, Kull *et al.*, 2003; Vuong *et al.*, 2004). Such an inoculation technique would reduce the reliance on natural environmental conditions for the evaluation of soybean varieties and avoid possible disease escapes that occur in field trials (Auclair *et al.*, 2004a).

Several methods for inoculation to screen germplasm for resistance to Sclerotinia stem rot under artificial conditions have been developed but no single method has been identified that reproduces reactions that consistently correlate with field results (Hoffman *et al.*, 2002; Kim & Diers., 2000; Kim *et al.*, 2000; Kull *et al.*, 2003; Vuong *et al.*, 2004). This has been attributed to high disease x environment interactions and differential responses of host cultivars to the pathogen under varying conditions (Kim, Sneller & Diers (1999); McLaren & Craven, 2007). Chun *et al.*, (1987) used an excised stem method to evaluate germplasm for resistance to *S. sclerotiorum* but found reproducibility of results a limitation under varying environmental conditions. Nelson *et al.*, (1991) using the same methodology found no correlation between laboratory testing and field testing. Wegulo, Yang & Martinson, (1998) found that detached leaf assays had a better correlation with field performance compared with other methods, but that it was also the least repeatable. In addition, Kim *et al.* (1999) and Auclair *et al.* (2004b) cited the specific type of inoculation method used as a source of variation in response of the soybean cultivars to *S. sclerotiorum.* This may be due to the techniques requiring plant damage or injury [e.g. the cut stem method (Kull *et al.*, 2003), Punched stem method (Auclair *et al.*, 2004a]. Although these techniques may be helpful in identifying physiological resistance, they by-pass escape mechanisms that could mask weak physiological resistance but which form and integral mechanism of reduced disease incidence.

Screening for Sclerotinia stem rot is affected by numerous potential sources of variation and reproducibility of results remains a problem. An effective screening method that can detect interactions and is reliable and accurate needs to be identified. Moreover, a non-destructive approach would be useful in genetic studies and breeding programmes where progeny tests to measure seed productivity are often required (Vuong *et al.*, 2004). This would greatly aid in the recurrent selection program and breeding effort (Nelson *et al.,* 1991). As a result this study was done to: (i) compare and evaluate greenhouse inoculation techniques for Sclerotinia stem rot on soybeans, (ii) quantify the extent of variation among repetitive screening and (iii) develop a method that

accommodates and quantifies host x environmental interactions under greenhouse conditions.

2.3. Methods and Materials

2.3.1. Evaluation of inoculation techniques

Four cultivars, PAN 520 RR, SNK 500, CRN 5550 and Wenner, from the National Cultivar Trails were planted into 1 ℓ pots filled with steam sterilized soil (composition of soil:peatmoss 1:1) in the greenhouse. Pots were over-seeded and after emergence plants were thinned out to 45 plants per pot. A randomized block design with 8 replicates for each of six inoculation techniques was used. Pots were watered daily and maintained at a 25/20°C day/night regime with a 14/10 hours light/dark cycle. Plants were grown to the V3 growth stage.

2.3.1.1. Inoculum production and inoculation

Sclerotia obtained from infected fields in Greytown during the 2005/06 season were washed in running tap water and surface sterilized in a 3% sodium hypochloride solution for 3 minutes, rinsed twice in distilled water and placed onto potato dextrose agar (PDA) (Biolab) in 90cm petri dishes. Petri dishes were incubated at 20°C for 3 days. These cultures served as stock cultures for all the techniques evaluated to minimize variation due to culture age.

Six inoculation techniques were used as follows:

(a*) Spray mycelium method* (Chen & Wang, 2005): Potato dextrose broth (PDB) was prepared by cutting 200g of unpeeled, washed potatoes in cubes and boil in 1 ℓ of water until soft approximately 25min. The potatoes are drained and the remaining water filled up to 1 ℓ. Dextrose (20 g) was added and mixed well. The suspension was autoclaved for 20min at 121kpa pressure and left to cool. Three agar plugs were cut from the three day old colony of *S. sclerotiorum* using a 5mm cork borer and placed into each or five 0.5 ℓ Erlen-Meyer flask

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containing 200ml of the broth. Flasks were placed in a shaker for 3 days and homogenized for 15–20 seconds using a Heidolph Silentcrusher M to make sure the mycelium is evenly suspended.

The mycelium suspension was placed in a hand atomizer and 250ml of the suspension was atomized evenly on the plants in 32 pots (four varieties and eight replicates).

(b) *Drop mycelium method* (Chen & Wang, 2005): Inoculum was prepared as above. However, plants were inoculated by dropping 1 ml/plant of the PDB mycelial suspension onto the apical meristem of each test plant using a pipette.

(c) Cut stem method (Vuong *et al.*, 2004): Agar plugs were cut from the three day old cultures of *S. sclerotiorum* colony using a 5mm cork borer. The stems of each of the 32 tests plants were horizontally severed between the second and the third node, and the agar plug placed mycelium side down on the severed stem.

(d) *Cotyledon method* (Kull *et al.*, 2003): Since cotyledons had abscised by the V3 growth stage, this method was modified whereby a 5mm agar plug was placed on the unifoliate leaves adjacent to and touching the stem.

(e) *Petiole inoculation method* (Del Rio, Kurtzweil & Grau, 2001): The petiole of the second trifoliate was cut approximately 2.5cm from the main stem. Plastic drinking straws were inserted into the PDA dish containing the pathogen. The unstapled straw containing a mycelium plug was trimmed and placed over the petiole that the severed end of the petiole was in direct contact with the mycelium.

(f) *Straw inoculation method* (Auclair *et al.*, 2004b): This procedure was similar to the petiole inoculation method except that the apical meristem of the plants was severed and a straw containing an agar plug with the pathogen was placed over the cut. The straws were stapled at the non-mycelial end to ensure the plug remained inside and to prevent desiccation.

After inoculation all plants were covered with transparent plastic bags with four 5mm holes punched at the top to ensure a high relative humidity while allowing respiration and gas exchange.

2.3.1.2. Disease assessment and analysis

Assessments commenced after 9 days. Bags were removed and readings were taken daily for two weeks.

The disease rating scale was as follows;

    0 = no visible symptoms on leaves,

    1 = leaves showing small symptoms (spots, browning of leaves and curling up);

    2 = Some leaves yellow, large brown lesion visible or lesion visible underneath the straw, moving downward on the stem;

    3 = infected leaves and petioles are brown, mycelium growth visible in some cases, lesions moved further down stems, leaves start to die;

    4 = 90% of leaves are dead, curled up or have been shed. Leaves and apical meristem wilted and lodging occurs. Mycelial growth visible;

    5 = all leaves infected, dead or shed, mycelial growth and sclerotia visible whole plant dead in some cases.

Wilting was assessed as follows;

    0 = no wilting visible;

    1 = only infected leaves show signs of wilting;

    2 = top part of plants wilted (apical meristem and upper leaves);

    3 = infected leaves and petioles wilted, wilting of trifoliate leaves underneath straw visible. Lesion moved downwards towards the middle of plant stem;

    4 = main stem and primary leaves wilted;

    5 = whole plant and main stem wilted, leaves dead and shed;

Data were analyzed using NCSS (Hintze, 2001). A General Linear Model (GLM) ANOVA was conducted to each treatment, cultivar and treatment x

cultivar interaction effects. Means were compared using Fishers Least Significance Difference (LSD) at P < 0.05.

2.3.2. Cultivar evaluation

Soybean cultivars (Table 2.2) selected from the National Cultivar Trials (ARC-Grain Crops Institute, Potchefstroom) were evaluated for Sclerotinia stem rot resistance in three separate greenhouse experiments. Greenhouse ports were filled with steam sterilized field soil and four pots per replicate were over-seeded with each cultivar. Pots were maintained at 25/22°C day night regime with a 14/12 hour light/dark cycle and were watered daily. Subsequent to emergence, seedlings were thinned out to four plants per pot.

After 5 weeks, plants were inoculated with *S. sclerotiorum* using the spray mycelium method referred to above. Plants were covered for 7 days with transparent plastic bags to ensure high humidity to promote development of the disease. Plants were assessed using the above criteria.

Trails were analysed using GLM-ANOVA and the analytical software NCSS (Hintze, 2001). Rank correlation analysis was used to determine the relationship between cultivar reactions to the pathogen in the different trails.

2.4. Results

2.4.1. Evaluation of inoculation techniques

Responses to the inoculation techniques are illustrated in Figure 2.1. A significant (P<0.05) inoculation technique, cultivar and inoculation technique x cultivar interaction was recorded for lesions, wilting and degree of wilting. Despite the significant interactions, the spray mycelium method consistently induced the highest disease incidence and severities. Mean leaf lesion severities and degree of wilting ratings of 4.3 and 3.8 were recorded, respectively with a mean wilting incidence of 83.59% (Table 2.1). The cut stem, drop mycelium method and straw inoculation methods did not differ significantly

and resulted in significantly less disease than the spray mycelium method and wilting severities of 53.13, 39.84 and 49.22% respectively. The petiole and cotyledon inoculation method yielded the lowest mean disease intensities.

SNK 500 was the most susceptible cultivar although it was slightly less susceptible to wilting induced by the spray mycelium method than CRN5550 (96.88% and 100% respectively). In contrast wilting indicated by the cut stem methods was 46.88% in CRN5550 compared with 81.25% in SNK 500 suggesting that cultivars react differently to tissue damage induced by the inoculation method.

2.4.2. Cultivar evaluation

Analysis of variance indicated that wilting incidence in cultivars differed significantly (P<0.05). Trial means did not differ significantly (Table 2.2) and no trial x cultivar interaction was recorded. However, trial variation was high and analysis of variance indicated that replicates and error accounted for 63% of the total variance. Cultivars only accounted for 23% of the total variance indicating that a large proportion of the variance is due to outside influences. This is supported by poor rank correlations of r=0.15, r=0.01 and r=0.31 between trials 1 and 2, 1 and 3 and 2 and 3 respectively and re-enforced by the poor correlation between individual blocks of cultivars across trials (Table 2.3) where only 8 out of 25 correlations proved significant.

The method of McLaren & Craven (2007) was used to determine the relationship between disease potential in the individual cultivar blocks and the observed disease incidence in the test cultivars. Disease potential of a trial block was defined as the mean disease incidence over all cultivars within the specific block. The model $Y=AX^b$ was used in a regression analysis where Y was the observed disease incidence, X the disease potential and A and b are regression parameters.

Three relationships could be identified between potential and observed disease incidence (Figure 2.2) and these are defined by the b-parameter. A linear relationship between disease potential and observed disease incidence within a cultivar was indicated by b=1 (Figure 2.2B); b>1 indicates initial resistance to the disease, despite increasing disease potential (Figure 2.2A) while susceptibility, despite a low disease potential, is indicated by b<1 (Figure 2.2C).

Applying calculated A and b parameters to the model, Sclerotinia stem rot potential required to induce an arbitrary disease (Y) level could be calculated. In the present study, this was calculated for Y= 10% and was the disease potential required to induce 10% disease incidence in the test cultivar (termed the "sclerotinia onset potential" of the cultivar). This value ranged from 0.9% in the susceptible SNK 500 to 49.4% in the more resistant LS580 (Table 2.4). Egret, LS580 and PAN522 RR were the only cultivars that required an onset potential of >20%. A second criterion determined from the regression analysis, namely the rate of response to increasing Sclerotinia stem rot potential at the point of disease onset could be determined by the model $dy/dx = AbX^{(b-1)}$. This ranged from a 1.8% increase in stem rot incidence per potential unit in the susceptible PAN 421 to 0.00% in LS580 and 0.62% in Egret which exhibited slower responses to increasing disease potential. Area under the disease progress curve was also calculated and served as an indication of the total host response to changing disease potentials and ranged form 2325.6 to 265.7 in LS555 and LS580 respectively.

2.5. Discussion

2.5.1. Inoculation techniques

Artificial inoculation techniques and screening methodologies need to be effective in order to determine differences in disease susceptibility among host cultivars that compare with field resistance. This is the essential element that determines the effectiveness of resistance screening techniques in greenhouse studies. The selection of an effective screening method needs to detect

interactions and allows identification of resistance or susceptibility of cultivars different to isolates of the pathogen (Kull *et al.*, 2003).

Significant differences were recorded in the cultivar reactions to *S. sclerotiorum* using different inoculation techniques. Results indicate that the spray mycelium method was the most effective, causing the highest amount of leaf damage and wilting. High method x cultivar interaction in both leaf damage and wilting, indicates however that care needs to be taken when interpreting the relative resistance of germplasm to *S. sclerotiorum*.

The current data indicate that the spray mycelium technique provides a reliable result. An advantage of this method is that no wounding of plants occurs before inoculation and in the evaluation of resistance of plants due to performed structural barriers to infection by the fungus (Chen & Wang, 2005). Pierson *et al.* (1994) suggested that the use of mycelia in artificial inoculations to screen for resistance provides an increase in expression of resistance in the field.

The cut stem method was the second most effective technique evaluated. Kull *et al.* (2003) found that this method had the smallest coefficient of variation and was able to detect interaction. They concluded that this method was statistically better than the detached leaf assay and cotyledon inoculation method. In the present study the cotyledon inoculation method was also the least effective inoculation method. According to Vuong *et a*l. (2004) the cut stem method may require more time and space than would be practical for application in breeding programs where large amounts of cultivars are screened for resistance. However since the technique enabled scientists to evaluate response of genotypes to disease infection on the basis of quantitative measurements it may be useful for evaluation resistance to small plant populations in genetic studies. Wounding on plants may however affect results due to the fact that necrotic tissue that occur due to the injury of the host plant which provides an ideal location for infection and biased plant reactions (Tu, 1989).

When two cultivars, Triall and MN0301 were evaluated, no differences between genotypes were recorded although Triall was known to be susceptible to

Sclerotinia stem rot. According to Danielson and Nelson & Helms (2004) this may be due because of this form of artificial inoculation bypassing resistance traits or escape mechanisms that are normally expressed under natural infection. Dillard & Cobb (2000) investigated the effect of wounds on infection of cabbage by *S. sclerotiorum* and found that plants with cuts or bruise wounds showed a lower disease incidence than plants that were injured by bruising. This suggested that injury resulting from bruising penetrates several cell layers and cause severe cell damage.

Cultivars tested respond differently to the various inoculation techniques used. Differential responses to environmental conditions have also been reported (Wegulo *et al.*, 1998). Fluctuations in cultivar rankings for resistance when different inoculation methods are used may be due to the differences in defense mechanisms among cultivars (Wegulo *et al.*, 1998).

2.5.2. Cultivar evaluation

Low rank correlations were recorded between the different greenhouse experiments. This emphasizes the problem of the reactions of cultivars in one environment to another and the dangers that emerge due to the high genotype x environment interactions that occur (Kim *et al.*, 1999; Yang *et al.*, 1999; Kim & Diers, 2000). The need to consider changes in the host reaction with changing environments is emphasized by the poor correlations between individual blocks planted in the greenhouse and that they differ by virtue of their positive reactions relative to light and ventilation. Regression parameters show that cultivars respond to changing SSR potentials even under greenhouse conditions. Egret and LS 580 were resistant with a low disease incidence as indicated by relative high onset potentials. SNK 500 and LS 555 were the more susceptible cultivars with relative low onset potentials. The latter cultivar had a potential high disease incidence (Table 2.2). The high potentials required to induce disease onset in LS 580 and a relatively low rate response to changing conditions is an indication that this has a more stable response to the pathogen compared with LS 555 which reacted rapidly to changing disease potentials. This stability is reflected in the AUDPC.

The advantage of using multiple criteria in the analysis of disease resistance lies in the inabililty of a single criterion to define the behaviour of soybeans to the pathogen under different evaluation conditions. AUDPC, for example, cannot distinguish between early and late onset and slower and faster rates of development (Campbell & Madden, 1990) and this criterion therefore needs to be used in associations with the other criteria listed (McLaren & Craven, 2007). This study emphasizes the need to take fluctuations in weather and inoculum into account when assessing germplasm for resistance to Sclerotinia stem rot. The regression methodology applied here takes three fluctuations into account.

Shortcomings of many studies currently is that greenhouse and field results are not comparable. When the current data was compared with greenhouse and field area under the disease progress curves (AUDPC's) a significant relationship was observed (Figure 2.3). Although this methodology improves the relationship between greenhouse and field results, the relative low R^2 value of 0.53 suggests that there is still a need for improving the accuracy of the applied methodologies.

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Table 2.1: Sclerotinia stem rot incidence and severity induced by six inoculation techniques on four soybean cultivars in the greenhouse.

Disease Assessment	Cultivar	Inoculation method						Cultivar mean
		Spray mycelium	Cut stem	Straw	Drop mycelium	Petiole	Cotyledon	
Leaf Lesions (0-5)	Wenner	4.25[n]	2.25[gh]	2.00[f]	1.25[bc]	1.13[b]	0.38[a]	1.88[a]
	SNK 500	3.50[l]	2.13[fg]	2.63[j]	2.25[gh]	1.50[d]	1.75[e]	2.29[b]
	CRN 5550	2.88[k]	2.38[hi]	1.38[cd]	2.25[gh]	1.12[b]	1.25[bc]	1.87[a]
	PAN 520 RR	3.88[m]	3.63[l]	2.00[f]	2.50[ij]	2.13[fg]	2.13[fg]	2.71[c]
	Treatment mean	3.63[d]	2.59[c]	2.00[b]	2.06[b]	1.47[b]	1.38[b]	2.19
Wilting incidence (%)	Wenner	78.13[k]	31.25[cd]	65.63[j]	43.75[f]	37.50[e]	43.75[f]	50.00[a]
	SNK 500	96.88[l]	81.25[k]	50.00[gh]	53.13[h]	53.13[h]	53.13[h]	64.58[b]
	CRN 5550	100.00[l]	46.88[fg]	46.88[fg]	18.75[b]	28.13[c]	9.38[a]	41.67[a]
	PAN 520 RR	59.38[i]	53.13[h]	34.38[de]	43.75[f]	28.13[c]	28.13[c]	41.15[a]
	Treatment mean	83.59[d]	53.13[c]	49.22[bc]	39.84[ab]	36.72[a]	33.59[a]	49.35
Degree of wilting (0-5)	Wenner	3.13[i]	1.25[c]	2.63[h]	1.75[e]	1.50[d]	1.75[e]	2.00[a]
	SNK 500	3.88[j]	3.25[i]	2.00[f]	2.13[f]	2.13[f]	2.13[f]	2.58[b]
	CRN 5550	4.00[j]	1.88[e]	1.88[e]	0.75[b]	1.13[c]	0.38[a]	1.67[a]
	PAN 520 RR	2.38[g]	2.13[f]	1.38[d]	1.75[e]	1.13[c]	1.13[c]	1.65[a]
	Treatment mean	3.34[d]	2.13[c]	1.97[bc]	1.59[ab]	1.47[a]	1.34[a]	1.97

Table 2.2: Wilting incidence (%) in soybean cultivars evaluated for resistance to Sclerotinia stem rot in the greenhouse.

Cultivar	Trial 1	Trial 2	Trial 3	Mean
		Wilting Incidence (%)		
A5409RG	42.92	44.80	32.17	**30.00** [hj]
AG5601	38.33	22.73	12.65	**22.62** [bcde]
AG6101	34.02	31.27	31.03	**30.15** [defgh]
CRN5550	37.62	17.10	20.72	**23.19** [bcdef]
Dumela	39.17	34.50	32.33	**33.38** [gh]
Egret	22.62	12.40	7.93	**12.36** [a]
Highveld Top	35.00	31.13	43.02	**34.43** [gh]
Knap	36.04	41.10	36.67	**35.98** [hi]
LS444	33.21	23.33	24.61	**25.10** [bcdefg]
LS555	48.98	45.53	48.25	**45.63** [i]
LS580	6.6	18.37	0.00	**6.39** [a]
LS666	32.50	22.77	18.93	**22.77** [bcde]
LS677	29.36	40.33	22.56	**28.79** [cdefgh]
Marula	52.70	40.20	23.00	**36.67** [hi]
PAN421RR	45.2	34.10	26.67	**33.38** [gh]
PAN520RR	21.67	22.50	16.57	**18.28** [b]
PAN522RR	31.67	22.10	15.08	**20.99** [bcd]
PAN626	42.50	38.40	20.26	**31.76** [efgh]
PAN660	31.97	40.00	20.36	**28.82** [cdefgh]
PAN809	21.67	20.53	0.00	**12.11** [a]
Prima2000	21.67	17.13	25.43	**19.45** [bc]
SNK500	60.85	37.20	44.44	**45.54** [bc]
Sonop	35.95	32.57	36.00	**32.88** [fgh]
Stork	31.67	47.63	33.33	**35.59** [h]
Wenner	36.67	42.93	22.52	**32.08** [efgh]
Mean	**28.96**	**31.23**	**24.58**	**28.25**

Table 2.3: Rank correlation coefficients of the individual replicates in greenhouse evaluation of 25 soybean cultivars for resistance to Sclerotinia stem rot.

	Trial 1		Trial 2			Trial 3		
	R1	R2	R1	R2	R3	R1	R2	R3
R1	1.00	0.35	0.54*	0.73*	0.51*	0.45	0.23	0.40
R2		1.00	0.46	0.21	0.21	0.26	0.42	0.09
R1			1.00	0.57*	0.66*	0.52*	0.11	0.20
R2				1.00	0.59*	0.46	0.40	0.38
R3					1.00	0.14	0.05	0.05
R1						1.00	0.44	0.59*
R2							1.00	0.32
R3								1.00

Table 2.4:   Regression parameters, onset potential and response rate   of cultivars evaluated for Sclerotinia stem rot potential at Bethlehem and Greytown.

Cultivar	A Parameter	B parameter	R^2	Sclerotinia onset potential (%)	Response rate (potential unit-1)	AUDPC
A5409RG	6.06	0.54	0.92	2.53	0.38	1771.508
AG5601	7.44	1.65	0.68	1.20	5.73	1062.34
AG6101	1.82	0.85	0.71	7.42	0.69	1337.54
CRN5550	0.69	1.04	0.73	13.08	1.09	1119.25
Dumela	7.66	0.45	0.82	1.81	0.37	1647.56
Egret	0.01	2.01	0.88	31.08	0.62	609.98
Highveld top	1.81	0.89	0.79	6.82	0.77	1830.62
Knap	1.39	0.98	0.88	7.49	0.95	1953.99
LS444	2.73	0.68	0.81	6.75	0.45	1336.07
LS555	4.8	0.68	0.74	2.94	0.48	2325.59
LS580	1.00E-21	12.99	0.97	49.39	0.00	265.73
LS666	3.23	0.6	0.82	6.58	0.36	1106.18
LS677	0.93	1.03	0.88	10.03	1.07	1428.29
Marula	3.06	0.74	0.75	4.95	0.53	1572.03
Pan421RR	7.76	1.78	0.78	1.15	8.66	1996.12
Pan520RR	3.15	2.47	0.91	1.60	40.58	966.09
Pan522RR	5.03	2.39	0.87	1.33	47.28	1186.47
Pan626	0.46	1.25	0.9	11.74	1.61	1563.25
Pan660	1.47	0.9	0.82	8.42	0.79	1375.47
Pan809	2.63	0.48	0.16	16.16	0.21	377.48
Prima2000	0.4	1.17	0.78	15.66	1.40	1134.01
SNK500	10.61	0.44	0.81	0.87	0.45	2142.04
Sonop	5.11	0.57	0.83	3.25	0.38	1613.98
Stork	0.75	1.16	0.81	9.33	1.40	1921.06
Wenner	1.38	0.94	0.8	8.22	0.87	1411.53
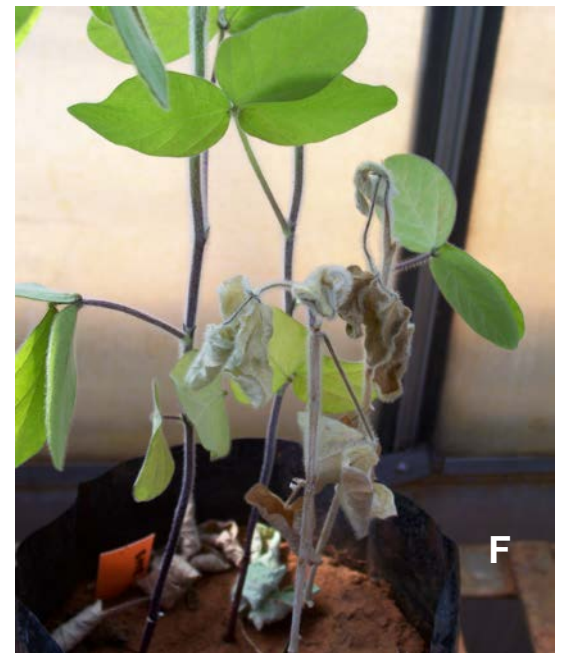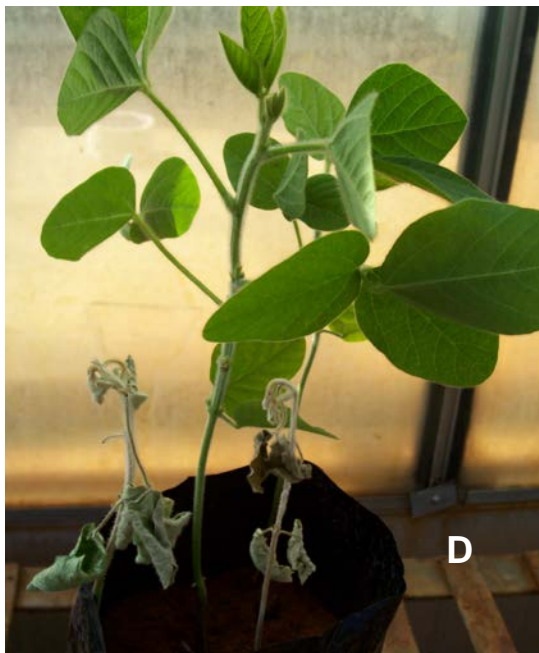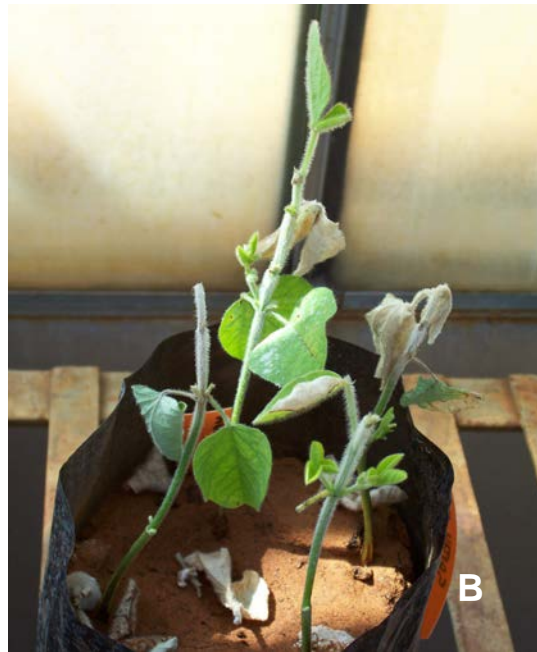
Figure 2.1. Various inoculation techniques evaluated:

(a) Cut stem method on the cultivar SNK 500.
(b) Spray mycelium method on the cultivar Wenner
(c) Drop mycelium method on the cultivar PAN 520 RR
(d) Straw inoculation method on the cultivar SNK 500
(e) Petiole inoculation method on the cultivar Wenner
(f) Cotyledon inoculation method on the cultivar SNK 500
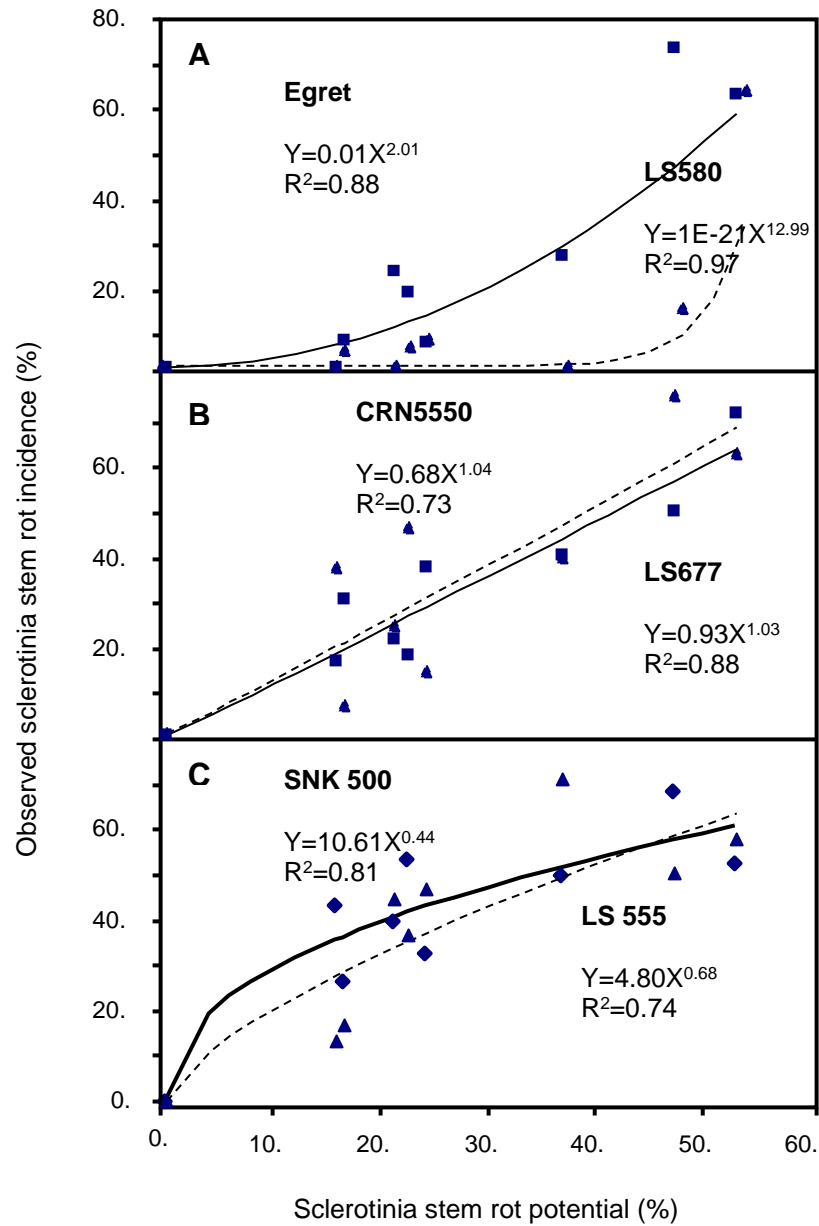
Figure 2.2: Regression parameters illustrating the response of cultivars to changing disease potential under field conditions.
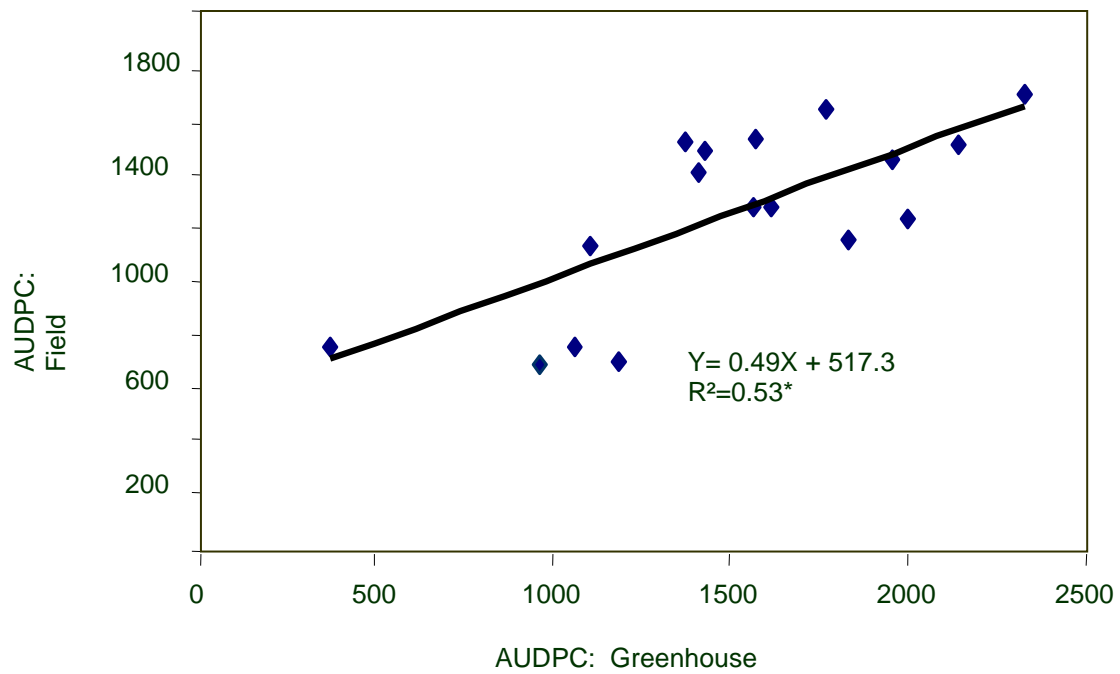
Figure 2.3: Relationship between field AUDPC's (McLaren & Craven, 2007) and greenhouse AUDPC's using the regression methodology.

Chapter 3

The effect of leaf wetness duration and temperature on the development of Sclerotinia stem rot on soybeans

3.1. Abstract

The severity of *Sclerotinia sclerotiorum*, the causal organism of Sclerotinia stem rot of soybeans is dependant on weather, specifically microclimate conditions within the crop canopy. Temperature and moisture are the main variables that affect disease development. This study was done to evaluate the effect of temperature and humidity on disease development initiated by mycelium of *S. sclerotiorum* in the greenhouse and the field. Soybean cultivar SNK 500 was planted in the greenhouse, grown to V3 growth stage and inoculated using a spray mycelium inoculum method. Pots were covered with transparent plastic bags and randomly placed in each of six incubation cabinets set at 18°C, 20°C, 22°C, 24°C, 26°C and 28°C. Control pots were placed directly on the greenhouse bench, without being bagged. Plants were scored for wilting incidence and leaf damage using percentage and 0-5 rating scales respectively. Field trails consisting of 30 commercial cultivars were inoculated at flowering with grain-mycelium inoculum at Bethlehem in the Free State Province from 2004/05 to 2005/06 and Greytown in Kwazulu Natal province during 2005/06. Trials consisted of sequential plantings spaced approximately 14 days apart to create a range of conditions during the post-flowering period. Disease potential in each planting was quantified as the mean Sclerotinia stem rot incidence (wilting) measured over all cultivars. Weather data were analysed using analysis of variance to determine main effects and interaction effects while correlation and regression analyses were used to quantify and establish correlation patterns between disease potential. Results from greenhouse trials indicated that temperature and leaf wetness periods significantly ($P<0.05$) affected both wilting severity and leaf damage. Greatest disease wilting severity (47%) and leaf damage rating (2.7) were recorded at a temperature of 22°C and leaf wetness of nine days. Maximum temperature and humidity were the only variables that were significantly correlated with disease potential in field trials. An $R^2=0.81$ using multiple regression analysis indicated that these variables

accounted for most of the variation potential while using the 3-dimensional Guassian equation this value increased to R²=0.92. This study confirms the temperature and humidity limits of Sclerotinia stem rot and lays a foundation for further studies to develop a disease forecasting model.

3.2. Introduction

Sclerotinia sclerotiorum, the causal organism of Sclerotinia stem rot of soybeans is one of the most destructive diseases in the world's temperate regions where cool and wet seasons prevail (Purdy, 1979). Disease incidence is largely dependant on weather (Phillips & Botha, 1990), specifically microclimate conditions within the crop canopy. According to Huang & Kozub (1993) temperature and moisture are the most important variables that affect mycelial survival.

Temperature has been shown to have a significant effect on apothecial formation, ascospore germination, mycelial growth, initiation of infection and expansion of lesions. Growth rate of *S. sclerotiorum* increases with temperature to a maximum of 20°C. At 35°C mycelium dies within a few days while at temperatures below 0°C the fungus still manages to grow (Van den Berg & Lentz, 1968). Vuong *et al.* (2004) found that *in vitro* growth was slow at 15°C, very fast at 20°C and 25°C and suppressed at 30°C. Inoculated plants showed similar results as lesion development occurred at 25°C and was suppressed at 30°C. Hannush and Boland (1996) similarly reported that disease development was optimal at 15 to 25°C with no disease at 30°C. Lesions did not develop when inoculated plants are incubated at 5 or 30°C (Abawi & Grogan, 1979).

Pennypacker & Risius, (1999) evaluated the exposure of soybeans to temperatures of less than 19°C, 19-22°C and greater than 22°C to determine genotype x environment interactions and to predict the proportion of infection by *S. sclerotiorum* under different conditions. Despite regression coefficients not differing within the temperature ranges evaluated, a slight positive slope in cultivar response to temperatures less than 19°C was detected and disease ratings increased with increased time of exposure to the lower temperatures.

This response corresponds with similar studies by Nelson (1998), Abawi & Grogan (1979) and Boland & Hall (1987).

Moisture is one of the most important weather variables in the development of Sclerotinia epidemics and is necessary for ascospore production and the initiation and development of infection. Optimum disease development requires daily plant surface moisture duration of 12-16 h or continuous surface moisture for 42-72h (Abawi & Grogan, 1975; Steadman, 1983; Tu, 1989; Nelson, 1998). Abawi & Grogan (1979) suggested that infection of moist bean blossoms by actively growing mycelium requires 16-24h while dry bean blossoms require 72 h for infection to occur. Tu (1989) and Abawi & Grogan (1975), found that expansion of lesions requires free moisture and that lesion development becomes inhibitied when the infected surface dries. *S. sclerotiorum* survived for a shorter period at 90-95%, than at 100% relative humidity (Van den Berg & Lentz, 1968). In contrast, Harikrishnan & Del Rio (2006) found that mycelium can serve as an inoculum source under varying environmental conditions due to its tolerance to desiccation.

The temperature x humidity interaction plays a critical role in disease development (Harikrishnan & Del Rio, 2006). A leaf wetness period of at least 48 hours is necessary with an air temperature of 20°C to 25°C for germination of ascospores (Phillips & Botha, 1990). Plant surface wetness periods of at least 54h at 20°C are needed for symptoms to become apparent and longer periods are required at 15°C and 28°C. These conditions apply to both controlled environment and field conditions (Boland & Hall, 1987). Tu (1989) found no relationship between precipitation and the prevalence of Sclerotinia stem rot suggesting that the amount of precipitation *per se* may not be important as long as moisture is available. Under these conditions, temperature was more dominant in affecting disease development.

This study was done to evaluate the effect of temperature and humidity on disease development initiated by mycelium of *S. sclerotiorum* in the greenhouse and under field conditions.

3.3. Materials and methods

3.3.1. Greenhouse evaluation

3.3.1.1. Planting

Soybean cultivar SNK 500 was planted into 155, 1ℓ pots filled with steam sterilized soil (composition of soil:peatmoss 1:1) in the greenhouse. Pots were over seeded and after emergence, were thinned to 4 plants per pot. Pots were watered daily and maintained at a 20/22°C day night regime with a 14/10h light/dark cycle. Plants were grown to V3 growth stage for inoculation.

3.3.1.2. Inoculum production and inoculation

A spray mycelium inoculum was used to inoculate plants (Chen & Wang, 2005). Potato dextrose broth was prepared by cutting 200g of unpeeled, washed potatoes into cubes and boiling until soft in 1ℓ of water (approximately 25 min). The liquid was drained into a flask and made up to 1ℓ with distilled water. Dextrose (20g) was added and dissolved by shaking. The mixture placed in five Erlenmeyer flasks. Three agar plugs were cut from a three day old colony of *S. sclerotiorum* with a 5mm cork borer and placed into the flasks. The broth was placed in a rotary shaker for 3d. After incubation, the broth was homogenized for approximately 15-20 seconds using a Heidolph Silentcrusher M homogenizer to ensure that the mycelium was evenly suspended.

The mycelial suspension was placed in a hand spray bottle and 15ml was sprayed over each pot. After inoculation each of 150 pots was covered with a transparent plastic bag with fours punched holes at the top to allow gas exchange while ensuring high humidity. Thirty five pots were randomly placed in each of six incubation cabinets set at 18°C, 20°C, 22°C, 24°C, 26°C and 28°C. The remaining five pots served as control pots and were placed directly on the greenhouse bench, without being bagged. This was regarded as 0 days leaf wetness duration.

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Three days after inoculation, five pots (representing five replicates) from each incubation cabinet were randomly selected. The bags were removed and the pots were placed on the greenhouse bench. The remaining plants were similarly treated six, nine and 12 days after inoculation.

3.2.1.3. Evaluation and data analysis

Plants were scored daily for two weeks for wilting incidence and leaf damage using percentage and 0-5 disease rating scales respectively. Leaves were scored as follows:

0 = no visible symptoms on leaves;

1 = leaves showing small symptoms (spots, browning of leaves and curled up);

2 = some leaves are yellow, large brown lesions visible;

3 = infected leaves and petioles are brown, mycelial growth visible in some cases;

4 = 90 % of plant leaves are dead and curled up or have been shed, mycelial growth visible;

5 = all leaves infected, dead or shed, myclial growth and sclerotia visible in most cases.

Wilting was scored as follows:

0% = no wilting visible on leaves;

10% = only infected leaves show signs of wilitng;

30% = upper parts of plants wilted (Apical meristem and top leaves);

50% = infected leaves and petioles wilted; 80% = leaves and apical meristem wilted and lodged (apical meristem turned brown and dead);

100% = whole plant and main stem wilted, leaves dead and shed.

Data were analysed using analysis of variance (Hintze, 2001) to determine main and interaction effects on disease incidence and severity. Means were calculated for each treatment. The relationship between temperature, wetness treatment and disease severity were analyzed using the 3-dimentional Gaussian model of SigmaPlot 9.0.

3.3.2. Field evaluation

3.3.2.1. Planting

National Soybean Cultivar field trails (ARC-Grain Crops Institute, Potchefstroom) were used to quantify the effect of weather conditions on Sclerotinia stem rot severity. Experiments were conducted at Bethlehem in the Free State Province from 2004/05 to 2005/06 and Greytown in Kwazulu Natal province during 2005/06. Blocks of the cultivars were planted sequentially at Bethlehem during 2003 on 19 November and 3 and 15 December, 17 November, 1 and 15 December during 2004 and on 2 and 17 November and 1 and 22 December 2005. In Greytown, cultivars were planted on 4 and 18 November and 2 and 23 December 2005.

Plots consisted of four rows, 8m in length with a 45cm inter-row spacing. The plant density was equivalent to 360 000 plants ha^{-1}. Plots were treated with 300kg ha^{-1} super phosphate and 50kg ha^{-1}, KCl. Commercial rhizobium (*Bradyrhizobium japonicum*) was mixed with water (100g 151^{-1} water) and added to furrows at planting.

3.3.2.2. Inoculum production and inoculation

Inoculum of *S. sclerotiorum* was prepared from sclerotia collected from the preceding season at Bethlehem. Sclerotia were surface sterilized and grown on potato dextrose agar at 22°C for three days and maintained at 4°C until used. Glass fruit flasks (500ml) were filled with 400ml sorghum grain and tap water was added to fill jars. Flasks were drained after 24 hours and 30ml of modified Fries Bosal medium (Chambers, 1987) was added after which jars were autoclaved for 30min at 120°C on two consecutive days. Each jar was inoculated with a 5mm agar plug cut from an actively growing mycelium colony and incubated at 22°C until the sorghum grains were fully colonized. Flasks were shaken every two days to ensure even colonization of grains. After the

incubation period, grains were removed from the jars, air dried for five days at room temperature and milled. Inoculum was stored at 4°C until use.

At flowering (R1 growth stage) 25g of inoculum was hand-distributed over each test plot. During 2004/05 and 2005/06 a second application was done after 14 days. During 2005/06 at Bethlehem and Greytown the second application consisted of a mycelium spray prepared by blending 30, 10 day old PDA cultures grown at 22°C in 10 ℓ of distilled water and spraying plants to run-off using a knapsack sprayer.

3.3.2.3. Data collection and analysis

The disease potential in each planting was quantified as the mean Sclerotinia stem rot incidence (wilting) measured over all cultivars (McLaren and Craven, 2007). Wilting incidence was recorded at R7 growth stage.

Weather data for Bethlehem during each season was provided by the ARC-Institute for Soil, Climate and Water. These included maximum and minimum daily temperatures, maximum and minimum relative humidity and rainfall. Weather data for Greytown was supplied by the South African Weather Service. Data were analysed using the NCSS program (Hintze, 2001). Analysis of variance was used to determine main effects and interaction effects. A correlation matrix was used to establish correlation patterns between disease potential and sequential pentads for each weather variable for the period subsequent to inoculation.   Identified pentad values were subsequently analysed using the 3-dimensional Gaussian equation (SigmaPlot 9.0)

3.4. Results

3.4.1. Greenhouse evaluation

Analysis of variance revealed that temperature and leaf wetness periods significantly ($P<0.05$) affected both wilting severity and leaf damage caused by

S. sclerotiorum. Significant (P<0.05) interaction effects between these variables were recorded.

Greatest mean wilting severity (47%) and leaf damage rating (2.7) were recorded with a temperature of 22°C (Figure 3.1) and leaf wetness of nine days (Table 3.1; Figure 3.2). Regression analysis using the Gaussian equation provided similar optimum values for wilting severity (temperature = 20.95°C; wetness = 8.96 d), (Figure 3.3) while for leaf damage variation was such that an optimum value for wetness duration of 16.36 days (Figure 3.4) was projected. The former value is probably the more reliable since an $R^2=0.91^{**}$ was recorded with wilting severity (Figure 3.3) compared with $R^2=0.79^*$ with leaf damage (Figure 3.4).

3.4.2. Field evaluation

Disease potentials varied from 0 to 47.9 % and yielded a sufficiently large range of data for multiple regression analyses (Table 3.2). During each season a distinct increase in disease severity was recorded with each subsequent planting. The correlation matrix yielded a clustering of significant correlations in the early post-inoculation period and the pentad period of days 1-5 post-inoculation was selected for further analysis. Initial stepwise multiple regression analysis, after transformation of data to ensure linearity, selected pentad values for maximum temperature and humidity as the only variables having significant relationships with disease potential and these could account for 81% of the variation in disease potential ($R^2=0.81^{**}$). No significant relationships were recorded with minimum temperature, rainfall or rain days. Re-analysis with these selected variable using the non-linear, 3-dimentional Gaussian equation yielded a highly significant relationship ($R^2=0.92^{***}$, Figure 3.5). Optimum temperature and humidity were calculated at 22.75°C and 95.37% respectively. Application of the regression model indicates that less than 10% disease incidence can be expected at temperatures greater than 25°C and no disease above 28°C. The model also indicates that very small fluctuations in relative humidity below the optimum of 95% RH can reduce disease incidences significantl

3.5. Discussion

All studies to date have shown that Sclerotinia stem rot severity increases under cool, moist conditions. This was confirmed in both greenhouse and field conditions in the current study. Disease develops best at temperatures between 20°C and 25°C (Nelson, 1998; Philips & Botha, 1990) and these figures correspond with *in vitro* studies that showed that 20-25°C was the best for mycelial growth with poor growth at 5°C and no growth 30°C (Abawi & Grogan,1975). Abawi & Hunter (1979) showed that the period required for infection was only 24h at temperatures between 15°C and 20°C. In the current study optimum temperatures determined from the field and greenhouse models show the optimum temperatures to lie within a narrow range of 20.9°C to 22.8°C. Rearrangement of model parameters indicate that under field conditions at optimum humidity (95%), no disease can be expected at temperatures below 7.6°C and above 27.6°C. Under greenhouse conditions limits were calculated at 7.90°C and 34.2°C, suggesting that under conditions with limited fluctuations in conditions, disease may occur at higher temperatures (Van den Berg & Lentz, 1968).

Moisture is a critical component in the infection cycle of *S. sclerotiorum*. Abawi & Grogan (1975) evaluated different time periods for leaf wetness and concluded that free water is required for disease initiation and the development of lesions. At incubation temperatures of 18°C and 22°C, disease development was best at RH of 90% compared to a low RH of 25% (Harikrishnan & Del Rio, 2006). Abawi & Hunter (1979) and Philips & Botha (1990) suggested that more than 72h of continuous leaf wetness are required for optimal infection of bean plants. However 48h appears sufficient for disease initiation and in the case of secondary spread on already colonized plant parts, only 16-24h of available moisture is required.

The above references indicate that tissue surface wetness is a critical factor in the initiation of disease. In the greenhouse, no disease occurred in plants that were not covered while disease was recorded at all the test temperatures after 3 days (36h) of high humidity facilitated by the plastic bag covers. Infection

increased significantly the longer the high humidity was ensured. Similarly in field trials, disease occurred at 95% RH but rapidly declined as humidity declined. The corresponds to reports by Hannush and Boland (1996) who showed that a small change in temperature or RH could produce large changes in disease incidence. Care therefore needs to be taken when relating disease potential to temperature, but particularly to RH. Although RH may fluctuate considerably beyond the crop canopy, dense canopies tend to trap moisture and may alter the relationships predicted by the current models (Turkington *et al*, 1991).

Although the current study shows distinct relationships between weather and moisture, the models developed, particularly under field conditions, are based on a limited number of data points. Further data are needed to ensure the accuracy of regression parameters and subsequent testing to determine the forecasting potential of the models is required. An accurate forecasting model is particularly important in relation to chemical control of the disease. Currently only procymidone is registered in South Africa for the control of Sclerotinia stem rot of soybean ("A guide for the control of plant diseases by the National Department of Agriculture.) but systematic spraying is not profitable due to the high cost. A model that can accurately predict the need to spray is needed. Furthermore, the current models do not take into account inoculum potential and are based on the assumption that inoculum is present. Further models should also take into account both the number of sclerotia present in a field and the conditions required to induce germination of sclerotia (Twengstrom *et al*, 1998).

This study confirms the temperature and humidity limits of Sclerotinia stem rot and lays a foundation for further studies to develop a disease forecasting model.

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Table 3.1: Effect of temperature and leaf wetness duration on leaf damage and wilting severity of soybeans caused by *S. sclerotiorum* in the greenhouse.

	Temperature (°C)						
Wetness (Days)	**18**	**20**	**22**	**24**	**26**	**28**	**Mean**
	Wilting (%)						
0	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**
3	24.28	21.43	30.00	32.86	10.00	30.0	**24.76**
6	38.57	50.00	67.14	35.71	27.14	32.86	**41.90**
9	50.00	50.00	80.00	58.57	12.86	30.00	**46.91**
12	58.57	50.00	58.57	35.71	10.00	30.00	**40.48**
Mean	**34.28**	**34.29**	**47.14**	**32.57**	**12.00**	**24.57**	**30.81**
Wetness (Days)	**18**	**20**	**22**	**24**	**26**	**28**	**Mean**
	Leaf damage (0-5)						
0	0.00	0.00	0.00	0.00	0.00	0.00	**0.00**
3	2.29	2.14	2.42	2.86	2.00	2.14	**2.31**
6	3.00	3.29	3.86	3.00	2.29	2.86	**3.05**
9	3.29	3.57	4.00	3.29	2.00	2.57	**3.12**
12	3.57	3.00	3.43	3.00	2.00	2.14	**2.86**
Mean	**2.43**	**2.4.0**	**2.74**	**2.43**	**1.66**	**1.94**	**2.27**

LSD (P>0.05)	Wilting	Leaf damage
Temperature (Main effect)	**6.67**	**0.32**
Wetness (Main effect)	**5.33**	**0.26**
Temperature x Wetness (Interaction)	**5.96**	**0.04**

Table 3.2: Disease potentials recorded at Bethlehem and Greytown in sequential plantings from 2003/04 to 2005/06.

Locality	Inoculation date	Wilting (%)
Bethlehem	16/01/04	7.8
Bethlehem	6/02/04	16.3
Bethlehem	20/02/04	47.9
Bethlehem	19/01/05	9.9
Bethlehem	27/01/05	24.4
Bethlehem	17/02/05	28.9
Bethlehem	17/01/06	0.0
Bethlehem	31/01/06	3.8
Bethlehem	14/02/06	4.6
Bethlehem	21/02/06	5.4
Greytown	18/01/06	0.0
Greytown	1/02/06	0.0
Greytown	15/02/06	17.5
Greytown	15/02/06	25.8
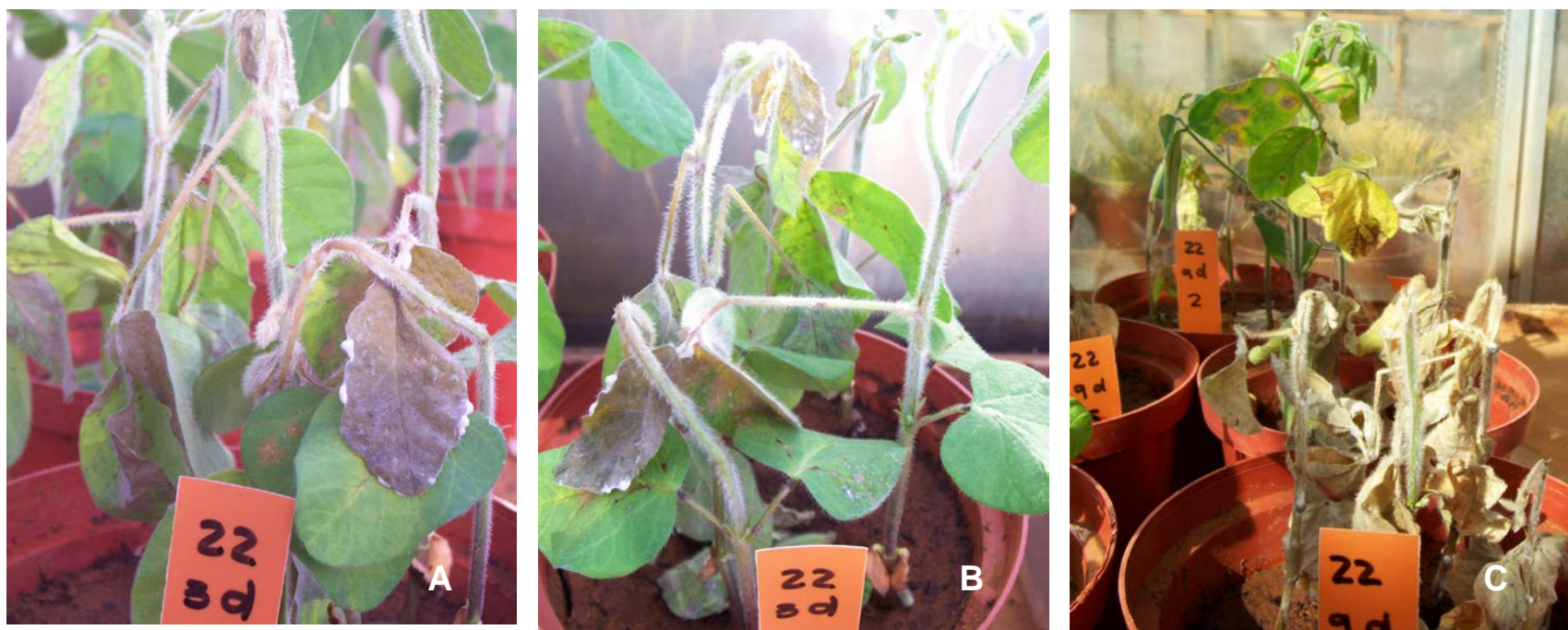
Figure 3.1: Leaf damage and wilting incidence of plants incubated at 22°C with 3 days leaf wetness (a) and 9 days leaf wetness (b & c).

Figure 3.2: Cultivar SNK 500 inoculated with spray mycelium method and incubated at at 22°C with 0, 3, 6, 9 and 12 days leaf-day wetness.

$$Y=66.95*\exp(-0.5*(((Temp-20.95)/4.65)^2 + ((Wet-8.96)/4.38)^2$$

$$R^2=0.91$$
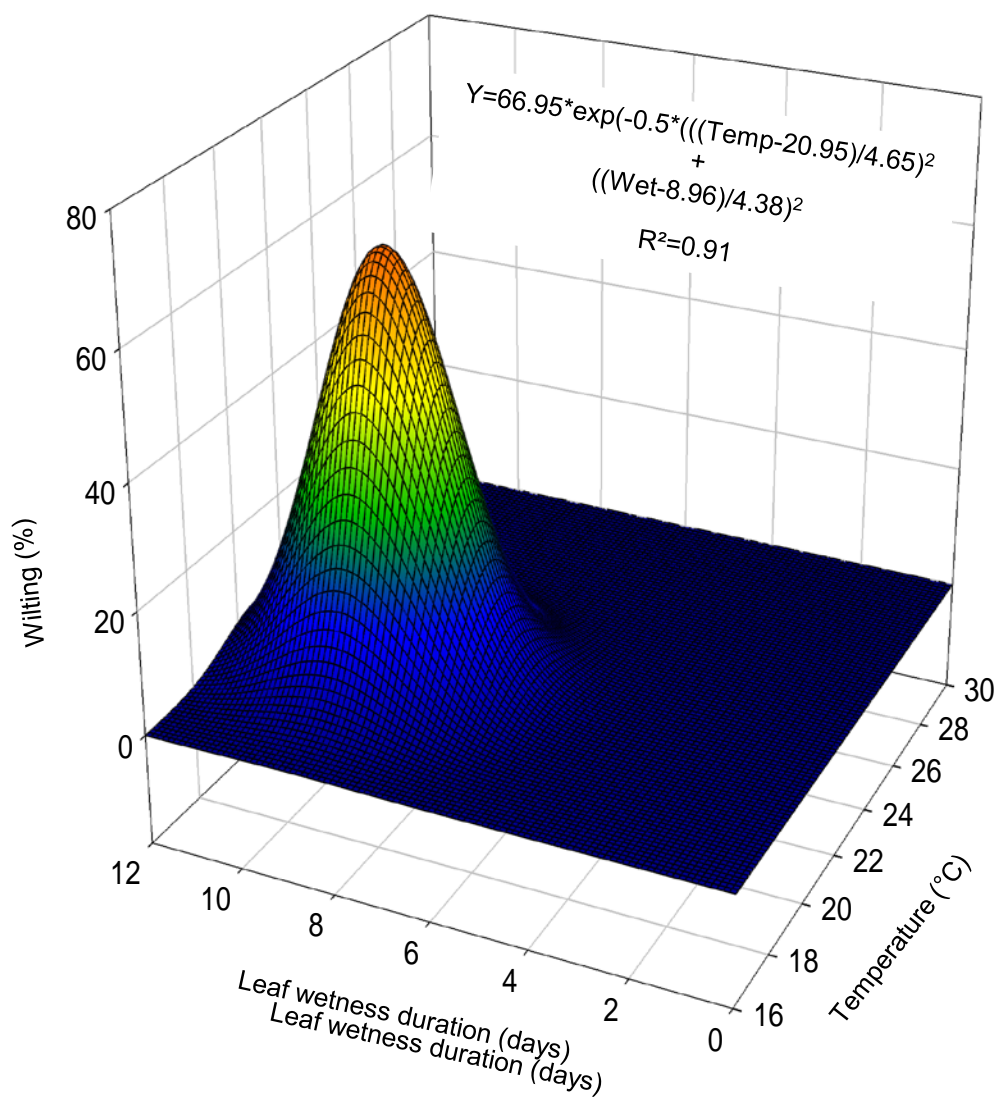
Figure 3.3: Relationship between temperature and leaf wetness duration on wilting of soybean caused by *S. sclerotiorum* in the greenhouse.

$$Y=3.80*\exp(-0.5*(((Temp-20.90)/8.21)^2 + ((Wet-16.36)/11.72)^2)$$

$$R^2=0.79$$
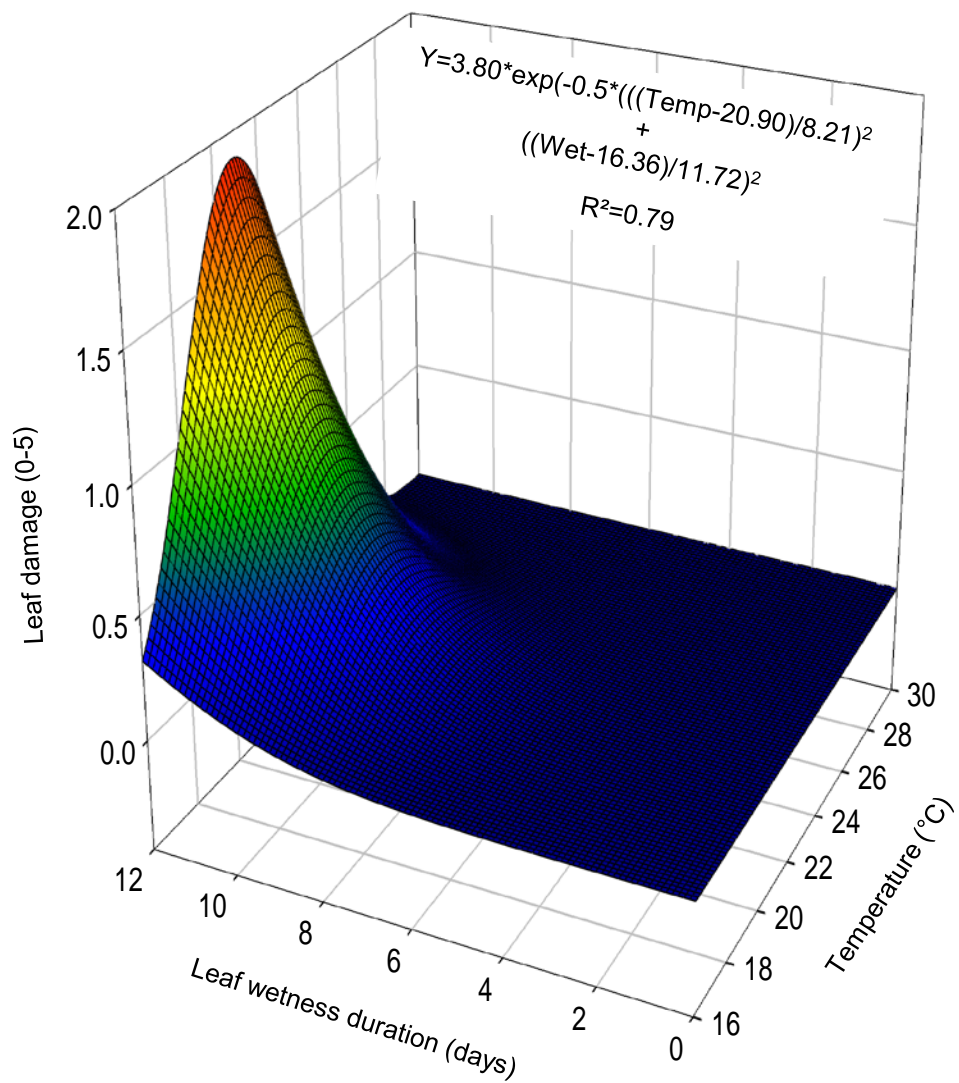
Figure 3.4: Relationship between temperature and leaf wetness duration on leaf damage to soybean caused by *S. sclerotiorum* in the greenhouse.
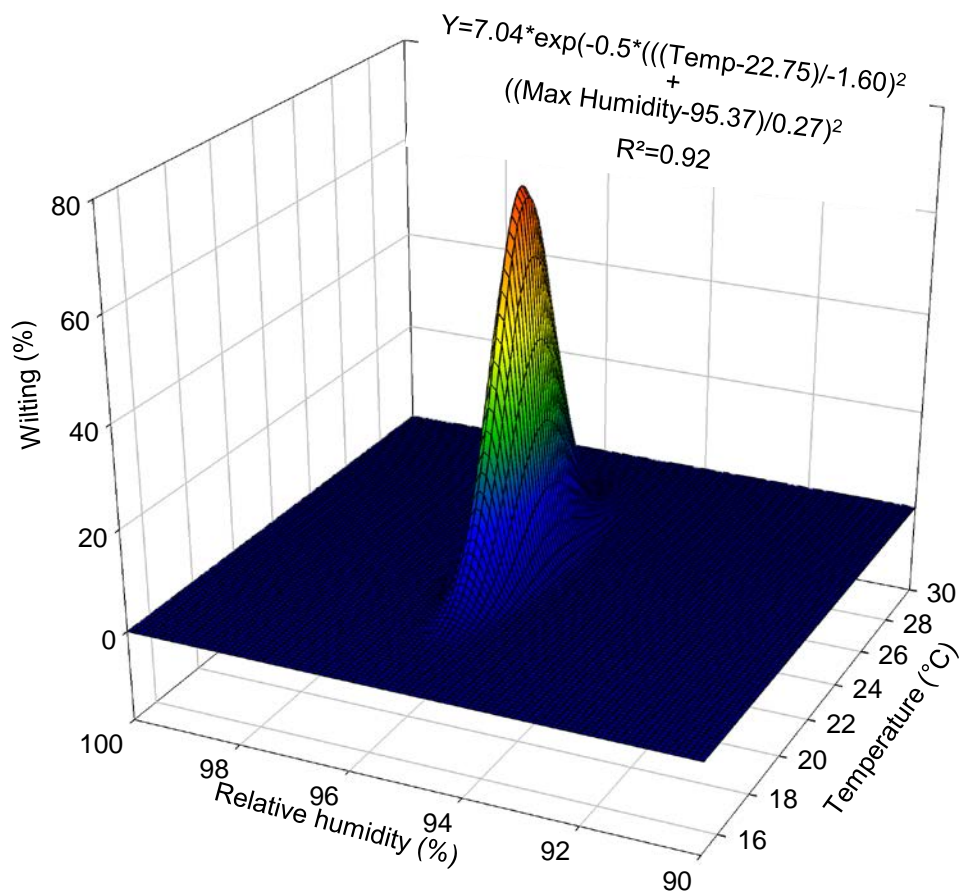
Figure 3.5: Relationship between temperature and humidity on leaf damage to soybean caused by *S. sclerotiorum* in the field subsequent to mycelial inoculation.

Chapter 4

Comparison of selected chemical and biological control strategies for Sclerotinia stem rot caused by *Sclerotinia sclerotiorum*.

4.1. Abstract

Sclerotinia sclerotiorum, the causal agent of Sclerotinia stem rot, causes major disease losses either from direct yield reductions or due to losses in grain quality. Reliable and economic control methods are essential to reduce the financial repercussions of the disease. Two fungicides, Benomyl and procymidone, a plant growth promoter, Extrasol, two *Trichoderma* species, Eco T and Eco 77 and an experimental plant extract (designated S007) were evaluated for efficacy against *S. sclerotiorum*. Treatments were applied according to regulatory rates and at half and double these rates except for Eco T and Eco 77. Isolates 2, 3, 11, 12, 16 and 18 were used for evaluation. Treatments were evaluated *in vitro* on amended PDA using colony growth rate as the evaluation criterion. The susceptible cultivar, SNK 500 was grown to V4 growth stage in the greenhouse and inoculated with a mycelium suspension of *S. sclerotiorum.* Treatments were applied three days prior to inoculation, at inoculation and three days after inoculation. A detached leaf assay was used to determine treatment efficacy. Treatments were applied to cultivar SNK500 at V4 growth stage at the regulatory rates and nine trifoliate leaves were randomly picked from the top, middle and bottom part of the plants on the day of application and at three day intervals until 12 days after application. Detached leaves were inoculated with *S. sclerotiorum* and lesions were measured daily for three days. Results from all trials revealed that Benomyl and Procymidone were the most effective in the suppression *S. sclerotiorum* growth and disease development subsequent to inoculation with the pathogen. Bio-agents EcoT and Eco77 were effective in inhibiting growth of *S. sclerotiorum* on amended PDA but was less effective when applied to plants in the greenhouse. Time of application to plants relative to inoculation with the pathogen proved important with biological agents whereas chemical fungicides provided a wider window of

control. In all trials a significant (P<0.05) isolate x treatment interaction was recorded indicating that some isolates are more sensitive to treatments than others.

4.2. Introduction

Sclerotinia sclerotiorum (Lib.) de Bary, an ubiquitous phytopathogenic fungus, causes Sclerotinia stem rot on a wide range of plant species in temperate regions of the world (Li *et al.*, 2005). Losses due to this disease occur either from direct yield reductions or indirectly, due to losses in grain quality (Purdy, 1979). Reliable and economic control methods are essential to reduce the financial repercussions of the disease. The impact of chemicals on the environment has become an increasing concern due to residues in soil and water and this, in turn has resulted in increased interest in biological control and other alternative control methods (Budge & Whipps, 1991, Huang *et al.*, 2000).

Numerous fungal parasites have been associated with the natural degradation of sclerotia including *Coniothyrium minitans, Sporidesmium sclerotivorum* (Anonymous, 2005) as well as *Trichoderma* spp. (Ferreira & Boley, 1992). Fungi that proved to be effective against *S. sclerotiorum in vitro* are *Gliocladium roseum*, *Trichothecium roseum*, *Fusarium* spp., *Mucor* spp., *Alternaria* spp., *Epicoccum* spp. and *Penicillium* spp. Menendez and Godeas (1998) found that greenhouse soybeans treated with *T. harzianum* survived more readily than untreated plants while the application of *T. harzianum* in the field resulted in reduced infection by *S. sclerotiorum* and 40% or more of the plants survived.

Bacteria, including *Erwinia*, *Pseudomonas* and *Bacillus* spp. have been widely used to control Sclerotinia stem rot. *Bacillus polymyxa* inhibited mycelial growth of *S. sclerotiorum in vitro,* reduced germination of ascospores and inhibited germ tube growth. However, when applied to bean foliage this bacterium did not have any effect on white mould severity despite surviving on bean leaves, (Yuen *et al.*, 1991). Savchuck and Fernando (2004) found that the timing of the antagonist application is critical and that disease incidence is highest when ascospores, the primary infection agents, were present prior to application of

the bacterial antagonists. When bacteria were applied at the time of inoculation with ascospores, complete inhibition of the disease was achieved. This was attributed to reduced pathogenicity of the pathogen through nutrient competition.

To be effective, a biological agent must be able to persist on the plant. Many antagonists are able to inhibit *in vitro* growth of the pathogen but can rapidly loose their efficacy when applied to healthy leaves, as a result of conditions that favour the pathogen and enable it to penetrate the leaf and escape competition (Fokkema, 1993). As a result, the general tendency is that biological agents are most efficacious under conditions that are less than optimal for disease development. Biological agents failed to control disease under environmental conditions highly favourable for disease development in contrast with fungicides which continued to provide effective suppression of disease under these conditions (Boland, 1997). As a result fungicides still remain the most viable option. Benomyl is a widely used fungicide in the control of Sclerotinia stem rot, effectively suppressing disease development (Mueller *et al.*, 2002, Hunter, Abawi & Crosier, 1978) and reducing sclerotial germination and apothecial formation (Yarden *et al.*, 1986). The use of fungicides, and in particular Benomyl, has been limited by resistance in *S. sclerotiorum*. Gossen and Rimmer (2001) reported that two isolates from Canadian prairies were very resistant to Benomyl (EC50>200mg/liter) which in turn has lead to an increase in the application rate of fungicides applied (Paula *et al.,* 2006).

As with the application of biological control agents, the timing of fungicide sprays is critical for the control of *S. sclerotiorum* and a difference in application time of a few days affects the control. One spray applied at full blossom of primary flowers is more effective than two to three applications at row closure which does not give satisfactory control (Johnson and Atallah, 2006). Hunter *et al.*, (1978) suggested that sprays should be applied three to five days before full bloom. Morton and Hall (1989) found that Sclerotinia stem rot incidence was reduced significantly by three to six sprays at full bloom compared with three to six sprays applied at late bloom.

The above illustrates that a reliable and economic control method for the control of Sclerotinia stem rot has yet to be developed. The present study was, thus undertaken to compare the efficacy of selected chemical and biological agents against Sclerotinia stem rot of soybeans in the laboratory and the greenhouse.

4.3. Materials and Methods

4.3.1. Laboratory evaluation

Two fungicides, Benomyl [Dow Agrosciences Southern Africa (PTY) LTD] and procymidone (Orica Limited), a plant growth promoter, Extrasol (Stimuplant), two *Trichoderma* species, EcoT and Eco77 and an experimental plant extract (designated S007, Prof. S. Pretorius, University of the Free State) (Table 4.1) were evaluated for efficacy against *S. sclerotiorum*. Four isolates of the pathogen were selected based on variation in prior growth studies and designated isolate 2, 3, 11, 12, 16 and 18 (Chapter 5).

Concentrations of each treatment were determined from the regulatory pamphlet. Each treatment with the exception of EcoT and Eco77 were applied at the registered rate and half and double registered. These are indicated in Table 4.1.

Each isolate of *S. sclerotiorum* was cultured on potato dextrose agar (PDA) (Biolab) for three days at 20°C before evaluations commenced. Benomyl, procymidone and Extrasol were evaluated by placing a sterile, 5mm assay disk approximately 1 cm from the side of a 90 cm Petri dish containing PDA and pipetting 50µl of the compound onto the disk. A 5mm agar disk of the respective three day old *S. sclerotiorum* test colony was placed mycelium side down at the opposite side of the Petri dish. Each isolate x treatment combination was replicated five times. After three days incubation at 20°C the inhibition zone between the assay disk and *S. sclerotiorum* culture was measured.

Eco T and Eco 77, (0.5g of the powder formulation) were placed on PDA and grown for 5d at 20°C, transferred to a new PDA petri dish and grown for a further three days. A 5mm agar plug from a three day old colony of *S. sclerotiorum* was placed on the one side of a PDA dish and a plug of a three day old Eco T or Eco 77 was placed on the opposite side. Each treatment x isolate combination was replicated five times. The cultures were incubated as above and the inhibition zone between the antagonist and *S. sclerotiorum* was measured.

S007 was evaluated as directed by Prof. S. Pretorius. A sample of 0.075g of the plant extract was placed in a sterile, glass Petri dish in a laminar flow cabinet. The extract was covered with 96 % ethanol and left overnight until the ethanol had evaporated. Malt extract agar (MEA) (Biolab) was prepared and autoclaved for 20 min at 121 kpa. The medium was cooled to 50°C and 0.3 ml streptomycin and the S007 extract was added. The medium was poured into 90 mm Petri dishes. A 5mm plug from a 3 day old colony of the respective *S. sclerotiorum* culture was placed in the center of the Petri dish and grown for three days at 20°C after which the colony diameter was measured. MEA was prepared as above but without the plant extract and served as control plates. Each treatment x isolate combination was replicated five times.

4.3.2. Greenhouse evaluation

4.3.2.1. Plant production

Ninety-five pots were filled with 1kg of steam sterilized soil (composition of soil:peatmoss 1:1) and over seeded with Sclerotinia stem rot susceptible cultivar, SNK 500. These were maintained in the greenhouse at a 23/17°C day/night regime. Pots were watered daily and after emergence, were thinned to four plants per pot. Plants were grown to V4 growth stage before inoculation with *S. sclerotiorum*. Pots were arranged in a randomized block design to accommodate six treatments, three application times relative to inoculation, a control treatment and five replications.

4.3.2.2. Inoculation

A spray mycelium inoculation method was used (Chen & Wang, 2005). Potato dextrose broth (PDB) was prepared by cutting 200g of unpeeled, washed potatoes into cubes and boiling these in 1ℓ of water until soft (approximately 25 min). The potatoes are drained and the remaining water was made up to 1ℓ. Dextrose (20g) was added and mixed well by shaking. The suspension was autoclaved for 20 min at 121 kpa pressure and left to cool. The suspension was decanted into five Erlenmeyer flasks and three agar plugs, cut from a three day old, PDA colony of *S. sclerotiorum* using a 5mm cork borer, were placed into each flask. Flasks were placed on a shaker for three days after which the colonies were homogenized for 15–20 seconds using a Heidolph Silentcrusher M to ensure that the mycelium was evenly suspended.

The mycelium suspension (15ml) was evenly sprayed over the plants in each pot with a hand spray bottle. After inoculation, plants were covered with transparent plastic bags with four holes at the top to ensure high humidity while allowing respiration and gaseous exchange.

4.3.2.3. Treatments

Benomyl, procymidone, Extrasol, EcoT, Eco77 and S007 were applied as a foliar spray to the plants in five pots each using the rates recommended in the regulatory pamphlet (Table 4.3). Treatments were applied at different times relative to inoculation i.e. 3 days prior to inoculation, at inoculation and three days after inoculation. Nine days after inoculation, bags were removed and the disease incidence and severity were recorded. Leaf and stem symptoms were assessed as follows:

0 = no visible symptoms on leaves;

1 = leaves showing small symptoms (spots, browning of leaves and curling up);

2 = Some leaves yellow, large brown lesion visible or lesion visible underneath the straw, moving downward on the stem;

3 = infected leaves and petioles are brown, mycelium growth visible in some cases, lesions moved further down stems, leaves start to die;

4 = 90% of leaves are dead, curled up or have been shed. Leaves and apical meristem wilted and lodging occurs. Mycelial growth visible;

5 = all leaves infected, dead or shed, mycelial growth and sclerotia visible whole plant dead in some cases.

The degree of wilting was assessed as follows:

0% = no wilting symptoms visible;

5% = infected leaves wilted and dead, new small leaves on apical
 meristem dead;

30% = infected leaves and stems wilted and dead;

50% = top part of plant wilted and dead;

80% = most of plant wilted and dead, only unifoliate leaves visible;

100% = entire plant wilted and dead;

4.3.3. Detached leaf assay

The susceptible soybean cultivar SNK500 was planted into 42 pots in the greenhouse. After emergence, plants were thinned to four plants per pot and maintained to V4 growth stage using the above procedure.   Six pots each were treated with Benomyl, Procymidone, Extrasol, EcoT, Eco77 and S007 as a foliar spray using the recommended rates in the regulatory pamphlet.   Nine trifoliate leaves were randomly picked from the top, middle and bottom parts of the plants on the day of application and at three day intervals until 12 days after application. The procedure was replicated three times.

Glass Petri dishes (90 mm) lined with a double layer of Whatman No. 3 filter paper were autoclaved. Weigh boats were placed upside down inside each dish and leaves were placed on top of each weigh boat. A three day old colony

of *S. sclerotiorum* (isolate 1) was cut with a 5 mm cork borer and plugs were placed mycelium side down on each leaf. The filter paper was moistened with sterilized water. Leaf lesions (mm) were measured daily for 3 days using a digital caliper.

4.3.4. Data Analysis

Data were analyzed using the NCSS program (Hintze, 2001). Analysis of Variance was used to distinguish between treatments, dosage rate, application times and interaction effects. Fishers Least Significant Difference (LSD) at $P = 0.05$ was used to compare means.

4.4. Results

4.4.1. Laboratory evaluation

Growth of *S. sclerotiorum* was suppressed *in vitro* in all treatments (Table 4.2; Figures 4.1-4.4) with the exception of the plant extract S007 at half and the recommended rate. The latter rates, in fact significantly ($P<0.05$) stimulated growth by 44% and 34% respectively. Benomyl was the only treatment that showed a significant ($p>0.01$) application rate response, with an increase in inhibition zone from 5.77 mm to 14.64 mm from half to double the regulatory rates respectively. A slight increase in the inhibition zone was recorded with procymidone applied at double rates compared with half and regulatory rates. *Trichoderma* spp., EcoT and Eco77 were the most effective in inhibiting *S. sclerotiorum in vitro* with mean inhibition zones of 21.99mm and 26.00mm respectively (Figure 4.3).   Extrasol yielded a consistent inhibition zone of approximately 7mm at all concentrations.

A significant ($P<0.05$) treatment x isolate interaction was recorded indicating that isolates differed in their sensitivity to treatments. Isolate 11 was particularly insensitive to Benomyl at half and regulatory rates, while isolate 3 was less sensitive to double rates of Benomyl. In contrast, isolate 11 was more sensitive to procymidone than the remaining isolates with an inhibition zone of 11.13mm

compared to a treatment mean of 9.62mm. Isolates 12, 16 and 18 were less affected by the plant extract S007 with mean colony sizes of 60.48 to 69.40mm compared with 54.76, 51.41 and 57.57mm in the remaining isolates.

4.4.2. Greenhouse evaluation

Analysis of Variance showed treatment, treatment x application time interaction effects on leaf rating and treatment, application time and treatment x application time effects on degree of wilting although levels of infection were very low. Benomyl and procymidone resulted in the lowest mean leaf ratings (0.72 and 0.94) respectively compared with control treatments and the plant extract S007 with mean ratings of 1.89 and 1.67 respectively (Table 4.3).

Benomyl applied at the time of inoculation with the pathogen yielded the lowest mean leaf rating (0.33) followed by procymidone applied three days prior to inoculation, Extrasol on the day of inoculation and Benomyl three days after inoculation, all with a mean leaf rating of 0.83. The latter indicates that Benomyl can restrict infection subsequent to infection and has some curative efficacy. EcoT and S007 had a limited effect on disease severity.

Benomyl and procymidone also yielded the lowest degree of wilting at all application times. The highest wilting incidence was observed with Eco77 (Table 4.3). S007 did not reduce disease severity relative to the control treatment.

4.4.3. Detached leaf assay

The effect of fungicides and biological agents on lesion development using a detached leaf assay is illustrated in Figures 4.5 and 4.6. A significant ($P<0.05$) treatment, time after application and treatment X time after application interaction was detected. Procymidone totally inhibited lesion development followed by Benomyl (mean lesion size = 0.28 mm) indicating the high efficacy of chemical control on soybeans in a controlled environment (Table 4.4). In

contrast with *in vitro* laboratory evaluations, EcoT had a limited effect on lesion development with a mean lesion size of 6.81mm followed by Eco77 and S007 with 5.71mm and 5.70mm, respectively. These treatments proved to be the least effective in controlling or suppressing disease (Table 4.4).

Data suggest that the greatest restriction in lesion size occurred six days after treatment. Extrasol and Eco77 were effective on the day of application (0.00 mm and 2.64 mm respectively) but had a limited residual period. EcoT required 6 days to become established in the host but had the largest lesion size after 12 days.

4.5. Discussion

In vitro laboratory evaluations, greenhouse inoculation studies and detached leaf assays all indicated that Benomyl and procymidone effectively inhibited the growth of *S. sclerotiorum.* Procymidone is currently the only fungicide registered for use on soybean in South Africa (National Department of Agriculture, personal communication) while Benomyl has been shown to be effective in controlling this disease in the greenhouse (Hunter *et al.* 1978; Mueller *et al.,* 2002); and in the field (Hunter *et al.,* 1978; Thompson, Thomas & Evans, 1884; Morton & Hall. 1989). However, the significant interaction (P<0.05) recorded between Benomyl's efficacy and *S. sclerotiorum* isolate in the current study suggests that some components of the pathogen population may display levels of resistance to the fungicide. This result is similar to the report by Gossen and Rimmer (2001) who found a similar tendency in Canadian populations of *S. sclerotiorum* that necessitated increased application rates to counter the reduced efficacy of this fungicide. Numerous other reports of resistance to Benomyl by *S. sclerotiorum* have been made (Brenneman *et al.*, 1987; Mueller *et al.*, 2002).

Suppression of *S. sclerotiorum* by *Trichoderma* spp. has been documented (Merriman, 1976). In the current study EcoT and Eco77 proved very effective in restricting the growth of *S. sclerotiorum* in i*n vitro* evaluations. Wells, Bell & Jaworski, (1972) observed that some isolates of *T. harzianum* only grew until

they met the leading edge of the pathogen colony at which time the growth of the latter stopped and eventually became overgrown with *T. harzianum*. *T. harzianum* parasitizes the hyphae of *S. sclerotiorum* and dense coils of the *Trichoderma* sp. form around the host mycelium. Extensive digestion of *S. sclerotiorum* mycelium can be observed. Inbar, Menendez and Chet (1996) on the other hand, showed that a clear zone was formed between mycelia of *T. harzianum* and *S. sclerotiorum* indicating the excretion of an inhibitory substance by the biocontrol agent. The latter phenomenon was observed in the current study.

The efficacious inhibition of the pathogen with EcoT and Eco77 was not reflected in plant tissues. Application of EcoT to soybean plants three days prior to inoculation resulted in higher mean disease ratings than applications at inoculation or subsequent to inoculation. The efficacy of biological control depends on the extent of the interactions between the environment and the biological agent (Boland, 1997), the timing of application in relation to crop development, the type and concentration of propagules, the ability of the biological agent to survive and colonize plant tissues and the concentration of naturally occurring inoculum of the pathogen (Inglis & Boland, 1990). Adams (1989) suggested that a mycoparasite must be able to infect and destroy a plant pathogen, as well as be able to grow and infect healthy propagules of the pathogen. Although these interactions were not quantified using the biological control agents in the current study, the extent of these interactions reduces the dependability of biological control compared with chemical control. This was also reflected in the detached leaf assays where biocontrol agents either had a limited residual period or required an establishment period before becoming efficacious.

Yuen *et al*. (1994) found that bacterial populations decrease in the first 24h after application. Savchuck and Fernando (2004) found that Sclerotinia stem rot disease was more severe when the pathogen was applied to plants before the application of *Pseudomonas* spp. to plants. When bacteria were applied before or at inoculation, *S. sclerotiorum* was completely inhibited. This was attributed to bacteria being able to effectively compete with the pathogen for nutrients and

could account for the early inhibition of lesion development in the detached leaf assays with Extrasol.

Data from the current study would suggest that biological control agents can inhibit *S. sclerotiorum in vitro* but are less efficacious than chemical control agents when applied to soybean plants in the greenhouse. However, low infection levels in the greenhouse require repetition of the trials before any definite deductions are made.

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Table 4.1: Chemical and bio-control agents evaluated for *Sclerotina sclerotiorum* control *in vitro*.

Treatment	Description	Recommended Rate	Rate in vitro	Half rate	Double rate
Benomyl	A wettable powder, systemic fungicide with the active ingredient of Benzimidazole at 500g/kg	300g/100 l water	1.5g / 500ml water	0.75g/500 ml water	3g/ 500 ml water
Procymidone	Sumisclex 500 is a fungicide with a particular activity against diseases caused by *Botrytis* and *Sclerotinia*. The suspension concentrate contains the active ingredient procymidone at 500g/ℓ	500ml/100 l water.	2.5ml/500ml water	1.25ml/500ml water	5ml/500ml water
Extrasol	Extrasol is a commercially available plant growth promoter that contains three *bacillus* spp., *Azomonas* sp. and a pseudomonas sp at 1 x 10^9 cfu/ml.	2l/ha in 120-150l water	6.67ml/500ml water	13.33ml/500 ml water	3.33 ml/500 ml water
Eco T	Eco T. This is used mostly for the control of many crops especially seedlings. It is based on a strain of *Trichoderma harzianum* isolated form soils and have shown to be effective against *S. sclerotiorum*.	200g of powder with 400 l of water	0.25g/500 ml water	0.50g/500 ml water	0.13g/500 ml water
Eco 77	Eco 77 is a strain of *T. harzianum* selected for above ground activity. It is registered for commercial use.	200g of powder with 400 l of water	0.25g/500 ml water	0.50g/500 ml water	0.125g/500 ml water
S 007	Natural organic plant extract	15 mg/75 ml water	0.105g/500ml water	0.210g/500ml water	0.525g/500ml water

Table 4.2: Inhibition of *S.sclerotiorum* on potato dextrose agar by chemical and biological control agents.

Treatment	Application rate	Isolate						Mean
		2	**3**	**11**	**12**	**16**	**18**	**Mean**
		Inhibition zone (mm)						
Benomyl	Half	5.82	5.27	4.54	6.19	6.87	5.96	**5.77**
	Recommended	9.42	10.90	0.00	10.43	13.79	10.90	**9.24**
	Double	12.31	9.75	15.03	16.65	16.95	17.14	**14.64**
Sumisclex	Half	9.30	8.85	11.13	9.56	8.80	10.08	**9.62**
	Recommended	10.86	12.09	12.13	12.91	12.58	13.13	**12.28**
	Double	9.59	12.12	14.00	13.14	13.01	12.81	**12.45**
Extrasol	Half	10.95	9.57	6.50	5.49	4.88	4.42	**6.97**
	Recommended	11.00	8.96	8.82	5.70	5.11	3.99	**7.26**
	Double	10.72	9.59	6.95	4.47	6.30	5.85	**7.31**
Eco T	Recommended	12.60	21.57	22.46	25.31	23.11	26.87	**21.99**
Eco 77	Recommended	19.24	23.49	28.29	26.89	31.40	26.69	**26.00**
Mean		**11.07**	**12.02**	**11.81**	**12.43**	**12.98**	**12.53**	**12.14**

LDS (P=0.05) Treatment means = 4.16
            Interaction table = 3.42

`	Application rate	Colony growth (mm)						
S 007	Recommended	75.74	76.78	90	90	78.44	90	**83.49**
	Half	57.61	62.65	85.73	90	79.86	90	**77.64**
	Double	26.43	23.08	34.93	18.12	18.54	14.66	**22.63**
MEA	Control	59.27	43.14	19.62	76.88	65.07	82.96	**57.83**
Mean		**54.88**	**51.41**	**57.57**	**68.75**	**60.48**	**69.40**	**60.40**

LSD (P=0.05) Treatment means =11.86
            Isolate mean   = 6.93
            Interaction table = 11.24

Table 4.3: Interactions of treatments applied at different inoculation times indicating leaf rating and wilting (%) on soybeans inoculated with *S. sclerotiorum.*

Treatment	Concentration	Leaf rating				Wilting (%)			
		Treatment relative to inoculation							
		3 days before	day of inoculation	3 days after	Mean	3 days before	day of inoculation	3 days after	Mean
Benomyl	0.25g/80 ml water	1.00	0.33	0.83	**0.72**	0.0	0.0	0.0	**0.0**
Sumisclex	0.42ml/80 ml water	0.83	1.00	1.00	**0.94**	0.0	0.0	0.0	**0.0**
Extrasol	1.11ml/ 80 ml water	1.50	0.83	1.17	**1.17**	5.0	0.0	0.0	**1.7**
Eco T	0.042g/80 ml water	2.00	1.33	1.67	**1.67**	5.0	5.0	0.0	**3.3**
Eco 77	0.042g/80 ml water	1.67	1.50	1.67	**1.61**	5.0	5.0	5.0	**5.0**
S 007	0.0175g/80 ml water	2.00	2.00	1.00	**1.67**	5.0	5.0	0.0	**3.3**
Control	80 ml distilled water	2.16	2.00	1.50	**1.89**	5.0	5.0	0.0	**3.3**
Mean		**1.59**	**1.28**	**1.26**	**1.38**	**3.57**	**2.86**	**0.71**	**2.38**
LSD (P=0.05)	Treatment means Time of inoculation Interaction table =	0.92 N/S 0.47				2.33 1.87 3.13			

Table 4.4: Mean lesion size on detached soybean leaves treated with chemical and biological control organisms at different times relative to inoculation with *S. sclerotiorum*.

| Treatment | Time after treatment and inoculation | | | | | Mean |
	Day of treatment	3d after	6d after	9d after	12d after	
	Lesion size (mm)					
Benlate	0.71	0.71	0.00	0.00	0.00	0.28
Sumisclex	0.00	0.00	0.00	0.00	0.00	0.00
Extrasol	0.00	7.23	3.21	6.27	6.64	4.67
Eco T	8.85	7.55	0.57	5.63	11.42	6.81
Eco 77	2.64	7.20	6.41	8.97	3.34	5.71
S 007	3.28	8.03	3.88	7.38	5.89	5.70
Control	2.40	5.47	2.22	3.66	6.64	3.85
Mean	**2.55**	**5.17**	**2.33**	**4.56**	**4.85**	**4.10**
LSD (P=0.05)	Treatment means Time after treatment Interaction table	3.46 2.11 4.84				
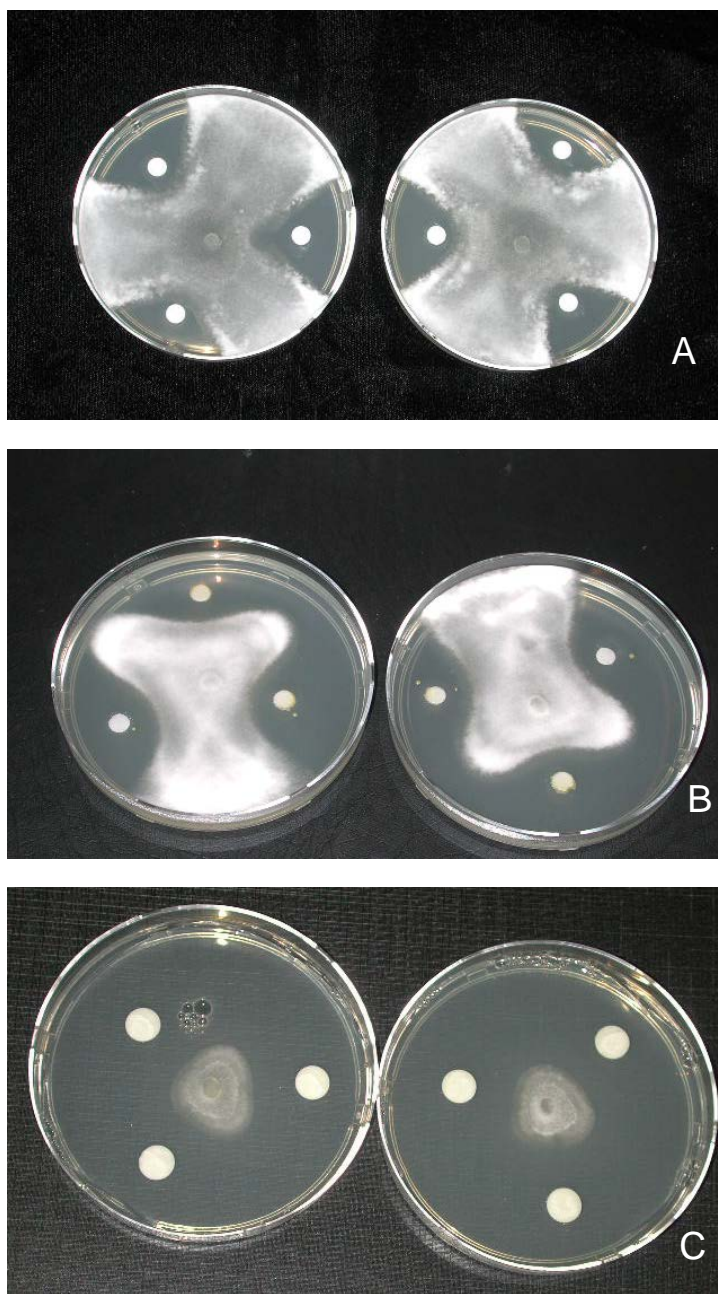
Figure 4.1. *In vitro* effect of benomyl applied at regulatory rate (a), half rate (b) and double rate (c) on mycelial growth of *Sclerotinia sclerotiorum* on potato dextrose agar.

Figure 4.2. *In vitro* effect of procymidone applied at regulatory rate (a), half rate (b) and double rate (c) on mycelial growth of *Sclerotinia sclerotiorum* on potato dextrose agar.

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Figure 4.3. *In vitro* effect of biological control agents Eco77 (a) and EcoT (b) on *S. sclerotiorum* on mycelial growth of *Sclerotinia sclerotiorum* on potato dextrose agar.
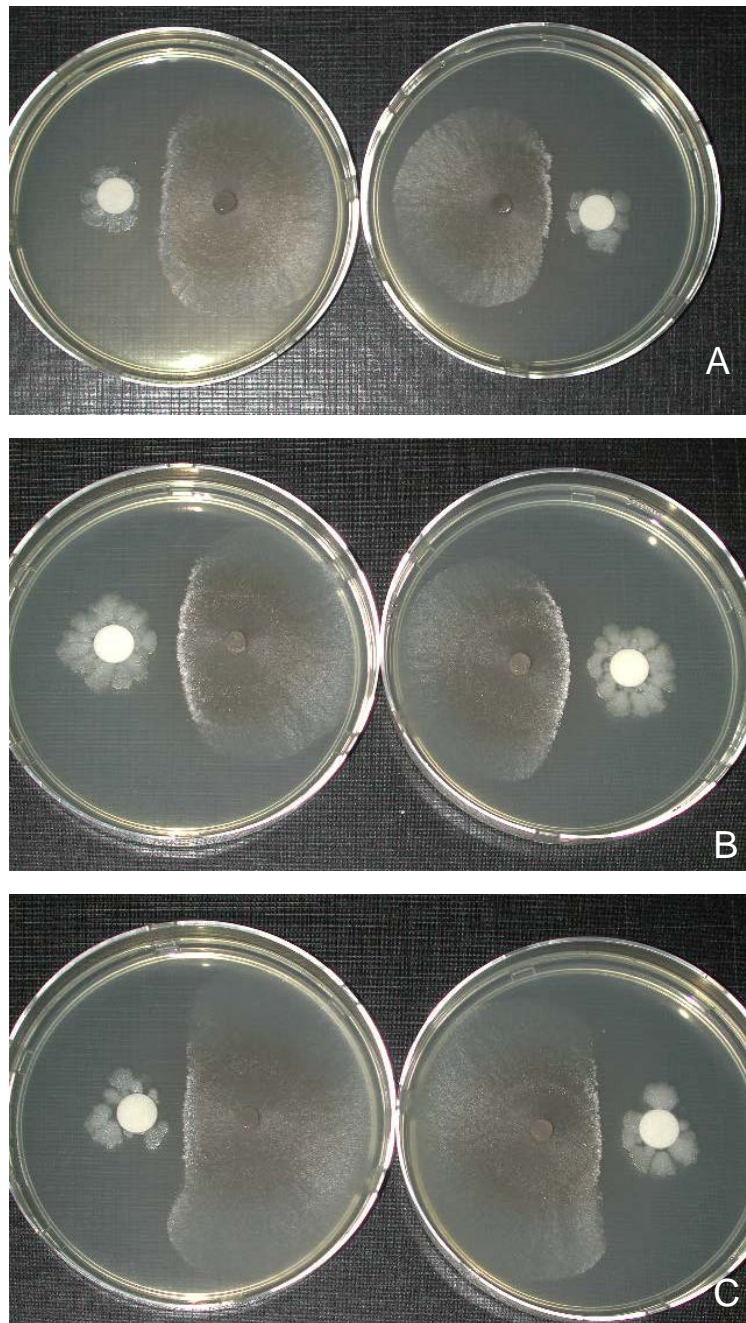
Figure 4.4. In vitro effect of Extrasol applied at regulatory rate (a), half rate (b) and double rate (c) on mycelial growth of *Sclerotinia sclerotiorum* on potato dextrose agar.
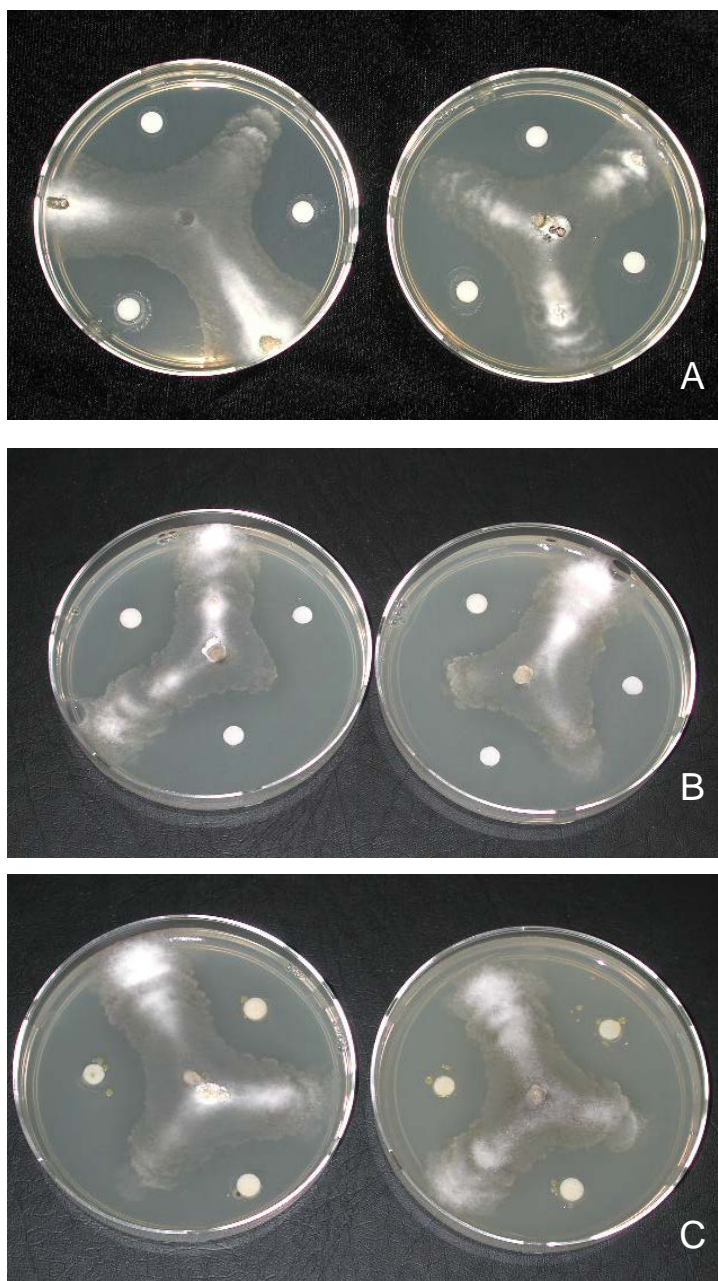
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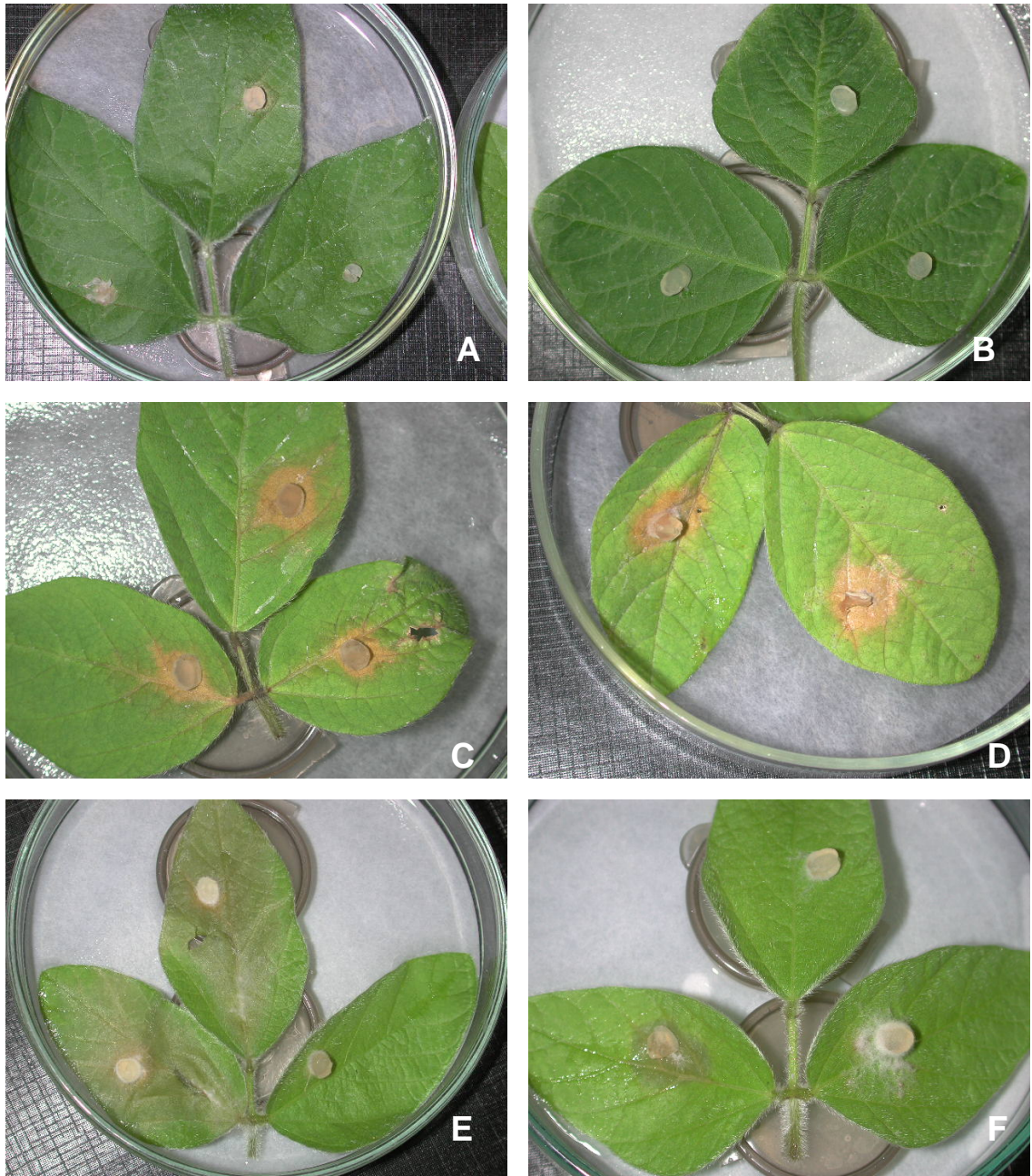
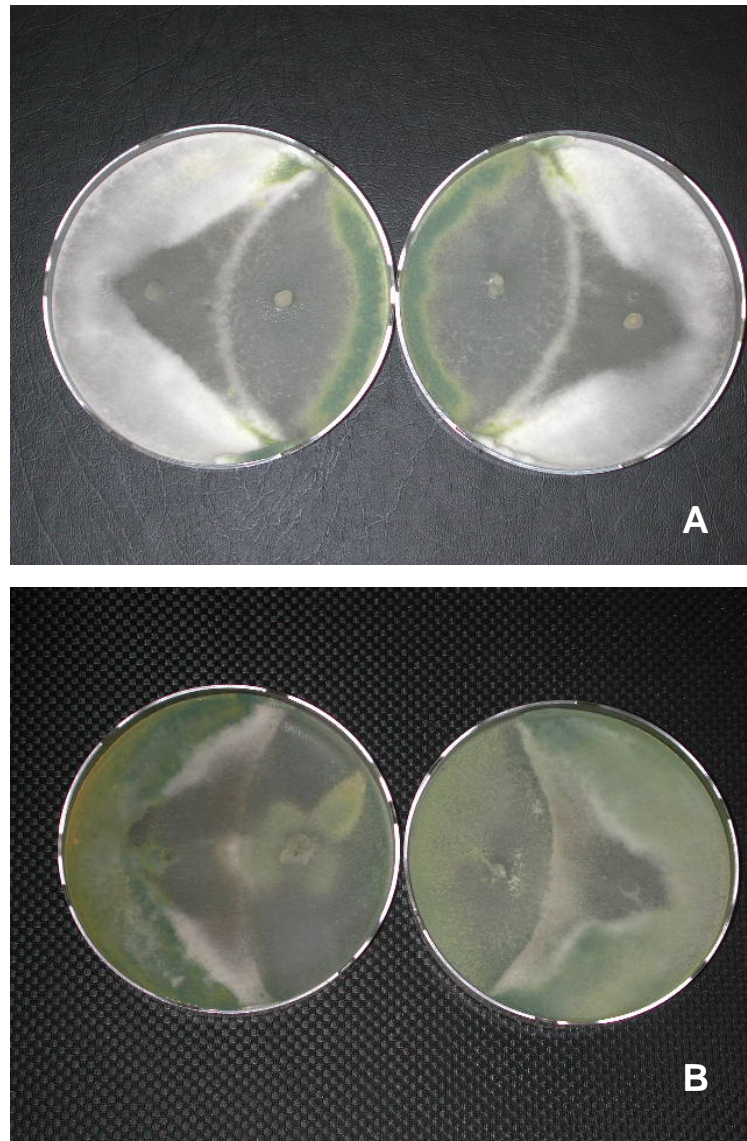Figure 5.5: Detached leaf assay used in the evaluation of control agents against *S. sclerotiorum* on soybeans inoculated on the day of treatment application – (a) benomyl (b) procymidone (c) Extrasol (d) S 007 (e) Eco 77 and (f) Eco 77.

Figure 5.6: Detached leaf assay used in the evaluation of control agents against *S. sclerotiorum* on soybeans inoculated 6 days after application – (a) benomyl (b) procymidone (c) Extrasol (d) S 007 (e) Eco 77 and (f) Eco 77.

Chapter 5

Evaluation of isolate variation and pathogenicity of *Sclerotinia sclerotiorum* isolates on soybeans in South Africa

5.1 Abstract

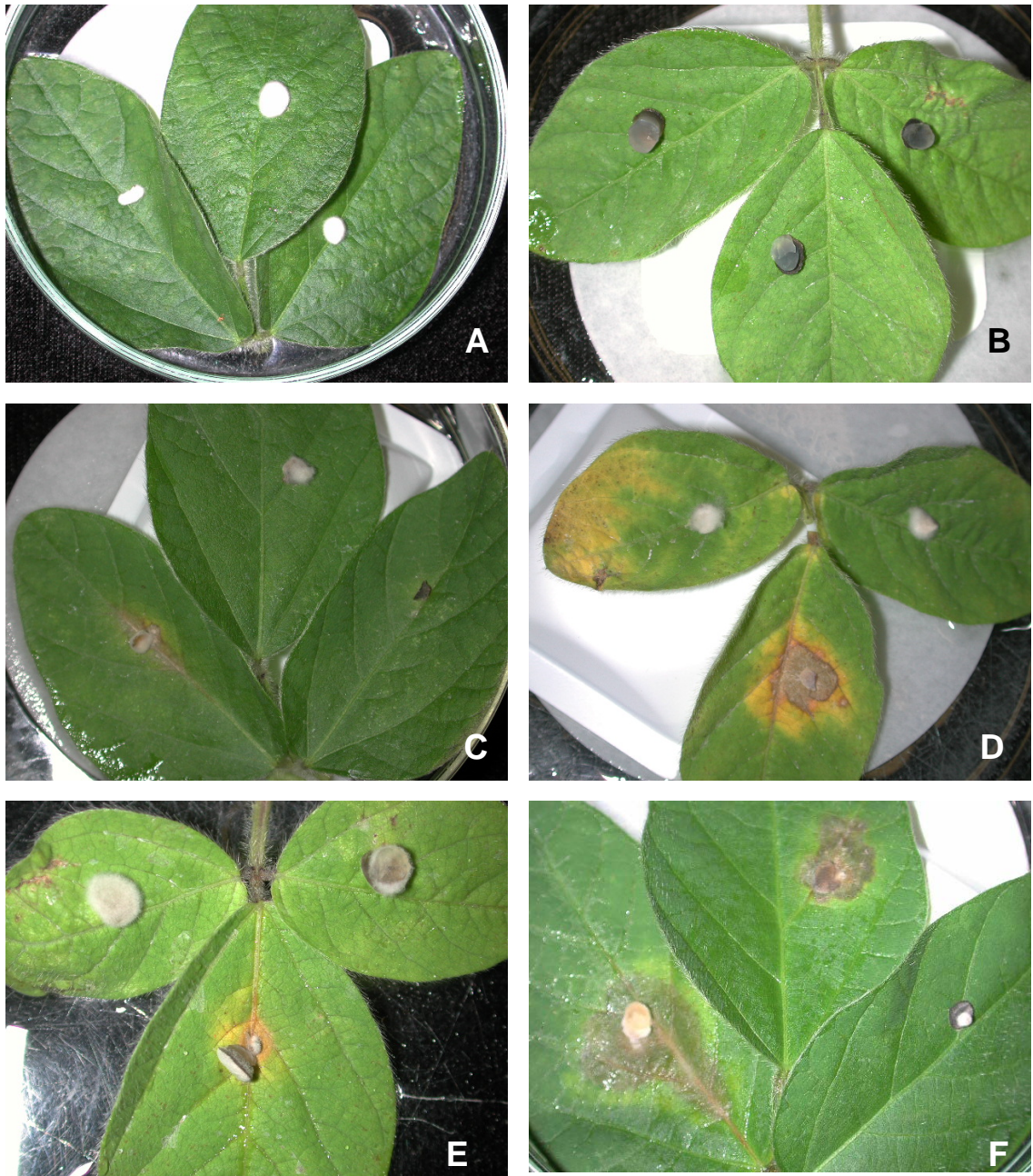*Sclerotinia sclerotiorum* is a devastating and cosmopolitan pathogen especially on agronomically important crops such as soybean, sunflower, dry bean, chickpea and peanut. Breeding for disease resistance remains an important disease control strategy and variation within the pathogen population could significantly affect the stability of the host response to infection. Eighteen isolates collected from infected commercial fields were grown at 15°C, 20°C, 25°C and 30°C on PDA to determine differences in growth requirements. All isolates grew optimally at 20-25 °C and growth was significantly reduced at 30 °C. However, significant isolate x temperature interactions were recorded and indications are that some isolates could still grow well at the higher temperature. Oxalic acid production by isolates has been correlated with pathogenicity and determinations of oxalic acid production were done *in vitro* using bromophenol blue as indicator. Variation within populations was also determined using Amplified fragment length polymorphism assays. Greenhouse experiments to ascertain whether the significant variation within the isolates tested could be related to pathogenicity were conducted using cultivars, SNK 500 and PAN 520 RR and inoculating plants *in situ* using a detached leaf assay. No significant correlation between oxalic acid production and pathogenicity was recorded. Low infection levels were however recorded. AFLP analysis revealed small differences among isolates with similarity correlation ranging from 0.72 to 0.97. A larger sample from the local *S. sclerotiorum* population is required to determine hetero- or homogeneity among isolates.

5.2. Introduction

Sclerotinia sclerotiorum is a devastating and cosmopolitan pathogen (Bolton, Thomma & Nelson, 2006), with a broad host range of over 400 species. This enables populations to persist and spread easily (Boland & Hall, 1994) especially on agronomically important crops such as soybean, sunflower, dry bean, chickpea and peanut. This is mainly due to a lack of genetic resistance, efficacious chemical fungicides and biological control agents and, hence difficulty in disease control. Identifying the modes of pathogenicity of *Sclerotinia* is important in the identification of effective alternate control strategies among agronomically important crops since previous control measures have failed (Cessna *et al.*, 2000).

The secretion of oxalic acid (ethanedioic acid) by *S. sclerotiorum* appears to be an essential determinant of pathogenicity (Godoy *et al.* 1990). Isolates of the pathogen differ in the production of oxalic acid and the host appears to be able to exert some kind of selective pressure on *S. sclerotiorum* populations in the release of these acids (Durman, Menendez & Godeas, 2005). Tu (1989) found that oxalic acid has an effect on the chloroplasts, other cellular organelles and the plasma membrane by altering membranes and disrupting the function of these organelles. According to Bateman and De Beer (1965) this results from the removal of calcium ions bound to pectins resulting in the exposure of cells to fungal enzymes. Acid secretion alters the pH of infected tissue to those favourable for cell wall degrading enzymes (Tu, 1985).

Guimaraes and Stotz (2004) found evidence of guard cell disfunction in plants infected with *S. sclerotiorum* that resulted in the inability of stomatal pores to close in the dark. Stomata appeared to remain open in advance of invading hyphae, indicating that oxalic acid may be produced ahead of the advancing hyphae thus exposing stomata to hyphal penetration and secondary colonization. Another mode of action appears to be via the suppression of the oxidative burst in the host plant which is normally associated with resistance to pathogen attacks. The oxidative burst is the controlled release of O_2- and H_2O_2

at the site of infection. In soybeans and tobacco, oxalate secreted by *S. sclerotiorum* effectively blocks the burst, thus blocking the defense signal to activate oxidase (Cessna *et al.* 2000). Lettuce plants with a oxalate decarboxylase gene were resistant to *S. sclerotiorum* even when the gene was incorporated into previously highly susceptible cultivars (Dias *et al.* 2006). The resistant white bean cultivar, ExRico 23, showed tolerance of the plasma membrane to oxalic acid and greater membrane stability (Tu, 1989).

An understanding of the genetic structure of the pathogen population is especially important when devising disease management and resistance-screening strategies (Sexton & Howlett, 2004). Information on the genetic structure of *S. sclerotiorum* isolates could assist in the breeding of resistant and improved cultivars with durable resistance (Zhao & Meng, 2003). It is especially important to know if new pathogen genotypes have evolved that are better adapted to, and more pathogenic on soybean (Hambleton, Walker & Kohn, 2002). Sun *et al.* (2005) found that 61.2% of 170 isolates compared were polymorphic with the bands for each primer pair differing by 0.0% to 86.7% using random amplified polymorphic DNA markers. Viji *et al.* (2004) found that the population of *S. sclerotiorum* used in their study consisted of multiple genotypes which corresponded with a high level of differences between Sclerotinia populations from New South Wales and Victoria in Australia (Sexton & Howlett, 2004).

The current study was undertaken to firstly determine variation in *S. sclerotiorum* isolates collected from soybean and sunflower in South African commercial production fields and secondly to investigate pathogenicity of isolates.

5.3. Materials and Methods

5.3.1. Laboratory evaluation

5.3.1.1. Stock cultures

Sclerotia were collected from infected plants from 18 commercial soybean and sunflower fields at various localities (Table 5.1). These were washed in running tap water, surface sterilized in 3% sodium hypochlorite and rinsed twice in sterile distilled water. Sclerotia were aseptically cut into smaller pieces (± 3 mm lengths) plated onto potato dextrose agar (PDA, Biolab) and incubated at 22°C for three days. Resultant cultures served as stock cultures for further study.

5.3.1.2. Temperature Growth Studies

Five mm agar plugs were cut from actively growing stock cultures and placed in the centre of a 90mm PDA dish and grown at 4 temperatures *viz.* 15°C, 20°C, 25°C and 30°C. Each treatment was replicated 5 times. Colony diameter (mm) was determined as average of two perpindicular measurements, measured daily for three days.

5.3.1.3. Oxalic acid production

Oxalic acid production by each isolate was determined using the method of Durman *et al.,* (2005). PDA was prepared and 50 mg ℓ^{-1} of bromophenol blue ($C_{19}H_{10}Br_4O_5S$) (Saarchem) was added. Agar was autoclaved for 20 min at 121°C and upon cooling to 50°C, was decanted into 90 mm Petri dishes. Agar plugs were cut from the stock cultures with a five mm cork borer and placed in the centre of the PDA-bromophenol blue agar plates. Cultures were incubated at room temperature on a laboratory bench and the resulting yellow halo was recorded as evidence of oxalic acid production by the respective isolates. The radius of the yellow halo was measured daily for three days.

5.3.1.4. Amplified fragment length polymorphism

5.3.1.4.1. Sample material

Stock cultures were sub-cultured on PDA for 7 days and the resultant sclerotia harvested and freeze-dried for 48 h in a Virtis Freezemobile II. Samples were stored in a -80°C freezer until further use.

5.3.1.4.2. DNA extraction

5clerotia were ground to a fine powder using a Qiagen TissueLyser for 3 min at 30 rs^{-1}. Total genomic DNA was isolated using the CTAB (hexadecyktrimethylammonium bromide) DNA isolation method (Saghai-Maroof *et al.*, 1984). Approximately 250 µl of finely grounded sclerotia was mixed with 750 µl CTAB buffer consisting of 100 mM Tris-HCl , 20 mM EDTA (ethylenedriamineteracetate), 1.4 M NaCl, 2% (w/v) CTAB and 0.2% (v/v) β-mercapto-ethanol was added. The mixture was shaken and incubated at 65°C for 1h.

Chloroform extractions were done by adding 500 µl chloroform:isoamylalcohol [24:1 (v/v)] and centrifuging the suspension for 5 min at 12 000 g. After centrifugation DNA was precipitated by adding 500 µl isopropanol and incubating at room temperature for 20 min. Tubes were centrifuged for 5 min at 12 000 g, the supernatant was discarded and tubes drained upside down. The pellet DNA was washed at room temperature by adding 500 µl ice-cold 70% (v/v) ethanol and incubation for 20 min. The suspension was centrifuged at 12 000 g for 5 min and supernatant was discarded. The resultant pellet was air-dried for 1 h at room temperature. DNA was resuspended in 200 µl TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 4°C overnight. DNase-free RNasesA (0.1µg µl^{-1}) was added and tubes were inbcubated at 37°C for 2 h. DNA was precipitated with 0.75 M ammonium acetate and 200 µl choloroform:isoamyl

alcohol [24:1 (v/v)] and centrifuged for 5 min at 12 000 g. DNA was precipitated from the aqueous phase overnight in 500 µl ice-cold ethanol (100%).

Tubes were centrifuged for 15 min at 12 000 g and the supernatant was discarded. DNA was washed twice with 500 µl 70% ice-cold ethanol by centrifugation for 10 min each time and supernatant was discarded. Tubes were drained and placed upside down to air-dry. The pellet was resuspended in 50 µl TE-buffer (pH 8.0) and incubated at 37°C for 2 h before the DNA concentration was determined.

5.3.1.4.3. DNA concentration

DNA concentrations were determined using a spectrophotometer and measuring the absorbances at 260 nm and 280 nm. The DNA concentration was calculated using the formula [DNA = optical density (OD260) x dilution factor x constant (50 µg ml^{-1}). DNA samples were diluted to a working concentration of 200 ng µl^{-1} in sterile distilled water and stored at 4°C. The integrity and concentration of the DNA was confirmed by 0.8% (w/v) agarose gel electrophoresis for 45 min at 80 V in 1 x UNTAN buffer (40 mM Tris-HCl, 2 mM EDTA, pH ajusted to 7.4 with acetic acid) with visualisation under the UV light after staining with ethidium bromide.

5.3.1.4.1. AFLP procedure

Genomic DNA (± 1.0 mg) was digested with 4 u 1 x *MSE* I (New England Biolabs) and 1 x *MSE* I buffer [50mM NaCL, 10 mM Tris-HCl, 10 mM MgCl$_2$, 1 mM dithiothroitol (DTT), pH 7.9] at 37°C for 5 hours, followed by digestion with 5 u *Eco* RI (Roche Diagnosticts) EcoR1 at 37°C overnight. Ligation of adapters was done by adding 0.4 mM adebosine triphosphate (ATP), 50 pmol MSE I-adapter, 5 pmol /ECO RI-adapter, 1 x T4 DNA ligase buffer (66 mM Tris-HCL, pH 7.6, 6.6 mM MgCl$_2$, 10 mM DTT, 66 mM ATP, and 1 u T4 DNA ligase (USB Corporation), followed by overnight incubation at 16°C

A 50 µl pre-selective PCR reaction was performed using a DNA Engine DYAD™ Peltier thermal cycler, using 5 µl undiluted restriction/ligation product, 30 ng of each selective pre-selective primer (*MSE*-C and *Eco*-A primers (Table 5.2), 1 x Promega plymerase buffer, 2 mM MgCl$_2$, dNTPs and 0.02 u go Taq ® Flexi DNA Polymerase Promega). The cycling programme consisted of an initial denaturation stem at 94°C for 5 min, followed by 30 cycles of 94°C for 30s, 56°C for 1 min and 72°C for 1 min and a final elongation step at 72°C for 5 min. The quantity and quality of pre-selective reactions were determined by 1.5% (w/v) agarose gel electrophoresis for 45 min at 80 V. Based on electrophoresis results reactions were diluted accordingly (1:5 or 1:10).

Selective amplification was done in a 20 µl reaction volume containing 5 µl of the diluted pre-selective DNA 1 x Promega polymerase buffer, 2 mM MgCl$_2$ 200 µM dNTPs, 100 µg ml^{-1} biovine serum albumin, 30 ng MSE I – selective primer, 30 ng Eco RI – selective primer and 0.75 u GoTaq® Flexi DNA Polymerase (Promega). The cycling programme for selective amplification consisted of an initial denaturation step at 94°C for 5 min, followed by one cycle fo 94°C for 30 s, 65°C for 30 s and 72°C for 1 min. The annealing temperature was lowered by 1°C per cycle for 8 cycles, followed by 25 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min and a final elongation step at 72°C for 2 min. Selective reactions were mixed with 20 µl formamide loading dye [98% (v/v) de-ionised formamide, 10 mM EDAT pH 8.0, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol] and denatured at 95°C for 5 min. Mixture were immediately placed on ice prior to loading/ PCA products (5 µl) were separated through a 5% denaturing polyacylamide gel (19: acrylamide: bis-acrylamide, 7M uica and 1 x TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 20 mM EDTA)].

The reaction was run at a constant power of 80 V for approximately 2 h on a Dedicated Height Sequencer (model #DH-400-93 C.B.S Scentific Co.) gel system. Amplified fragments were visualized using the protocal of the Silver Sequence ™ DNA sequencing system manual applied by Promega. Stained gels were left upright to air-dry and photographed by exposing photographic paper (ILFORD MULTIGRADE N RC DE LUXE) placed under the gel to dim

light for approximately 20s. This produced a negative image of the same size as the gel. AFLP fragments were determined by comparison with a 100 bp DNA ladder (Promega).

5.3.1.4.5. Statistical analysis

AFLP data were scored as discrete variables, "1" for present and "0" for absente. NTSYS pc. Version 2.2 (Rohlf, 1998) was used to compute similarity coefficients for qualitative data. Data were used to calculate pair-wise genetic distances which were expressed as complements of Jaccard (1908) coefficients. Cluster analysis was done to determine associations between the 18 genotypes using the UPGMA (Unweighted pair group method using arithmetic averages) clustering method.

5.3.2. Greenhouse evaluation

5.3.3.1. Pathogenicity evaluation

5.3.3.1.1. Plant production

Pathogenicity tests of the isolates was conducted on two soybean cultivars,from the National Cultivar Trails planted in the greenhouse, i.e. SNK 500, a susceptible cultivar and PAN 520 RR, a moderately resistant cultivar. One liter pots were filled with steam sterilized soil (composition of soil:peatmoss 1:1), overseeded with each cultivar and watered daily. After emergence plants were thinned to four plants per pot. Plants were maintained at a 22/18°C day/night regime with a 14/10 hours light/dark cycle. A randomized block design was used with two cultivars, three replications and 18 isolates as treatment variables.

5.3.3.1.2. Inoculum production

Each isolate was grown on PDA for three days. Potato dextrose broth (PDB) was prepared by cutting 200g of unpeeled, washed potatoes into cubes and boiling these in 1 ℓ of water. The potatoes were drained and the remaining water was topped up with water to 1 ℓ. Dextrose (20g) was added. The procedure was repeated to obtain 1½ ℓ of PDB. The suspension is autoclaved for 20 min. at 121°C and left to cool. An agar plug was cut from the three day old stock cultures of *S. sclerotiorum* and placed in an Erlenmeyer flask containing 80 ml of the broth. The flasks were placed on a shaker and incubated at room temperature for three days. Resultant colonies were homogenized for 15–20 seconds to ensure the mycelium is evenly suspended.

Mycelia suspensions in PDB for each isolate were applied as a foliar spray to each cultivar (60ml per isolate for both cultivars). After inoculation, pots were covered in transparent plastic bags, with four holes at the top to allow gas exchange while maintaining high humidity for optimum disease development.

Nine days after inoculation bags were removed. The amount of leaf damage, degree of wilting and the number of dead plants were recorded every fourth day using the following scale:

Leaf and stem symptoms were assessed as follows:
0 = no visible symptoms on leaves;
1 = leaves showing small symptoms (spots, browning of leaves and curling up);
2 = Some leaves yellow, large brown lesion visible or lesion visible underneath the straw, moving downward on the stem;
3 = infected leaves and petioles are brown, mycelium growth visible in some cases, lesions moved further down stems, leaves start to die;
4 = 90% of leaves are dead, curled up or have been shed. Leaves and apical meristem wilted and lodging occurs. Mycelial growth visible;

5 = all leaves infected, dead or shed, mycelial growth and sclerotia visible whole plant dead in some cases.

Wilting were assessed according to the following scale:

0% = no wilting symptoms visible;

5% = infected leaves wilted and dead, new small leaves on apical meristem dead;

30% = Infected leaves and stems wilted and dead;

50% = top part of plant wilted and dead;

80% = 90% of plant wilted and dead, only unifoliate leaves visible;

100% = Entire plant wilted and dead;

After 20 days, the trail was terminated

5.3.3. Detached leaf assay

A detached leaf assay was conducted to evaluate isolate pathogenicity and aggressiveness according to leaf lesions in the laboratory. Leaves from soybean cultivar SNK 500 grown to V4 growth stage in the greenhouse were cut and three leaves were placed in each of the 162 sterile glass Petri dishes lined with moist filter paper i.e. 18 isolates and three petridishes per isolates (replicates). A 5 mm agar plug from the respective stock cultures of *S. sclerotiorum* was placed in the centre of the leaf. Lesions were measure daily for seven days. Each isolate assay was repeated 3 times.

5.3.4. Data analysis

Data were analyzed using the NCSS program (Hintze, 2001). An ANOVA was conducted for each isolate, cultivar and isolate x cultivar interaction. Means were compared using Fishers Least Significance Difference (LSD) at P = 0.05.

5.4. Results

5.4.1. Laboratory evaluation

5.4.1.1. Temperature Growth Study

Mean colony diameter of isolates (Table 5.3) differed significantly (P<0.05) with temperature. Maximum growth occurred at 20-25°C (62.31 mm and 58.49 mm respectively) but was significantly inhibited at 30ºC (17.37 mm). Mean isolate diameters over all temperatures did not differ greatly except for isolate 3 (36.23 mm)(P<0.05).

Significant (P<0.05) interaction between isolates and temperature were recorded. Isolate 4 yielded good growth at 30ºC compared with remaining isolates while isolates 2 and 3 were particularly sensitive to the higher temperature. Isolate 3 and 16 were more sensitive to lower temperatures (Table 5.3) as indicated by significantly reduced colony diameters compared with remaining isolates. Data thus indicate that isolates of the *S. sclerotiorum* population differ in their adaptation to different temperature conditions.

5.4.1.2. Oxalic acid production

Significant differences (P<0.05) were recorded in the secretion of oxalic acid by isolates of *S. sclerotiorum* (Figure 5.5). Mean halo diameters of the isolates ranged from 11.31 mm to 54.95 mm, indicating large differences in the ability of isolates to produce oxalic acid. Isolates 12, 15 and 16 had the lowest means of 11.31 mm,13.11 mm and 15.67 respectively compared with remaining isolates (Table 5.4).

5.4.1.3. AFLP fingerprinting

Primer combination E-ACT/M-CTG amplified the highest number of polymorphic fragments (12) of the four primer combinations used (Figure 5.6). A total of 101

fragments were amplified using four AFLP primer combinations of which 63 (64%) were polymorphic among the 18 genotypes. The number of polymorphic fragments per primer combination range form 7 to 12 with an average of 9.5. All 18 genotypes could be distinguished uniquely from each other using only four primer combinations. Jaccard's similarity coefficients ranges from 0.72 to 0.97. The most dissimilar genotypes was isolates 7 and 14 while the most similar isolates were isolates 9 and 11. A dendogram was constructed using Jaccard's similarity coefficients and the UPGMA clustering method. The 18 genotypes were clearly divided into two main groups, I and II (Figure 5.1). In group I a total variation of 83.08 % were observed which is near similar to group II (83.90%) Within these two groups however, no clear differences could be discerned based on host plant or locality. Isolates 1 and 6 and isolates 3 and 4 from sunflower showed a 94.75% and 93.42% similarity respectively. Isolates 14, 17 and 18 from soybeans showed a 93.98 % but isolate 10 and 15, also collected from soybeans only yielded in a 86.25% similarity. These isolates were collected from different localities (Table 5.1). Isolate 9 and 11 had the highest genetic similarity of 97.26%.

5.4.2. Greenhouse evaluation

5.4.2.1. Pathogenicity evaluation

Wilting incidence was low (<10 %) (data not shown). Significant differences ($P<0.05$) in leaf lesion development were, however recorded with isolate 15 yielding a mean rating of 1.9 compared with isolate 1, with a rating of 0.1. No cultivar x isolate interaction was recorded. Although there was a tendency for increased lesion development with oxalic production by isolates, correlations between these variables were non-significant. Wilting and leaf lesion development are illustrated in Figure 5.3.

5.4.2.2. Detached leaf assay

The detached leaf assay indicated that isolates 4 and 16 had mean lesion diameters of 13.55 mm and 17.79 mm (Table 5.4). The remaining isolates resulted in low mean lesion sizes. Lesions however were slow to develop and failed in some cases to develop at all despite leaves being inoculated with actively growing cultures of the same age. Lesion development is illustrated in Figure 5.2.

5.5. Discussion

Temperature had the greatest effect on growth of *S. sclerotiorum* isolates and, with the exception of isolate 3, the highest mean colony diameters were obtained at 20–25°C. This is the optimal temperature range generally reported in a number of other studies (Abawi & Grogan, 1979; Philips & Botha, 1990). All isolates showed reduced growth at 30°C which indicates that pathogenicity is significantly suppressed by high temperatures (Van den Berg & Lentz. 1968; Vuong et al., 2004). However, the ability of some isolates to endure higher temperatures, eg. isolate 4, suggests that, depending on the population structure present, significant pathogen activity could still be expected under higher temperature conditions. The pathogen population used in the current study was relatively small and a larger sample would be required to determine the size of the higher temperature-tolerant component of the local *S. sclerotiorum* population and the potential risk it may have for soybean production in warmer areas.

Oxalic acid production similarly differed between isolates but no relationship with host plant or locality was found. According to Godoy *et al.,* (1990) higher amounts of oxalic acid secretion relate to higher pathogenicity. Durman *et al,* (2005) reported differences in the ability of isolates to release oxalic acid, especially on different hosts. This suggests that the host plant could exert some kind of selective pressure on *S. sclerotiorum* isolates to produce oxalic acid. The current study however failed to yield any relationship between oxalic

acid production and lesion development. Some isolates, eg. isolate 5, which has a halo diameter similar to isolate 4, failed to induce a lesion on detached leaves while the latter produced a significant lesion (13.55 mm). Noyes & Hancock (1981) also reported that wilting of plants in the greenhouse was correlated with oxalic acid production. This could not however be confirmed in the current study due to the low infection levels.

AFLP results obtained indicated relatively small genetic differences between isolates used in the current study, although this could be attributed to the small sample size used. Kohn *et al.* (1991) found that field populations of *S. sclerotiorum* were genetically heterogenous while studies in New South Wales and Victoria in Australia (Sexton & Howlett, 2004) indicated that moderate to high levels of differentiation occur within populations of the pathogen. Noonan *et al.* (1996), on the other hand found geographic and phytotypic differences between samples while still displaying a high level of genetic homogeneity. A larger sample size and samples from various continents may be useful in determining the genetic differences found in *S. sclerotiorum*.

Although the current study would suggest that isolates of *S. sclerotiorum* in South Africa may differ, the extent and significance of these differences in terms of host, locality and, in particular, pathogenicity is not clear. Further studies using a larger population sample are warranted.

5.6. References

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Table 5.1: Sources of isolates of *S. sclerotiorum* used to determine isolate variation, genetic analysis and pathogenicity

Isolate	Genus and species	Source	Location (South Africa)	Host	Date
1	*S. sclerotiorum*	Sclerotia – Field	Greytown	Sunflower (*Helianthus annuus*)	April 2006
2	*S. sclerotiorum*	Sclerotia – Field	Bethlehem	Soybeans (*Glycine max*)	April 2006
3	*S. sclerotiorum*	(ARC Grain Crops Institute, Potchefstroom) Grown on PDA	Potchefstroom	Sunflower (*Helianthus annuus*)	April 2006
4	*S. sclerotiorum*	(ARC Grain Crops Institute, Potchefstroom) Grown on PDA	Potchefstroom	Sunflower (*Helianthus annuus*)	April 2006
5	*S. sclerotiorum*	(ARC Grain Crops Institute, Potchefstroom)	Potchefstroom	Sunflower (*Helianthus annuus*)	April 2006
6	*S. sclerotiorum*	(ARC Grain Crops Institute, Potchefstroom) Grown on PDA	Potchefstroom	Sunflower (*Helianthus annuus*)	April 2006
7	*S. sclerotiorum*	Sclerotia – Field	Kinross	Soybean (*Glycine max*)	March 2006
8	*S. sclerotiorum*	Sclerotia – Field	Kinross	Soybean (*Glycine max*)	March 2006
9	*S. sclerotiorum*	Sclerotia – Field	Kinross	Soybean (*Glycine max*)	March 2006
10	*S. sclerotiorum*	Sclerotia – Field	Naboomspruit	Soybean (*Glycine max*)	March 2006
11	*S. sclerotiorum*	Sclerotia – Field	Kinross	Sugar bean (*Phaseolus vulgaris*)	March 2006
12	*S. sclerotiorum*	Sclerotia – Field	Kinross	Soybean (*Glycine max*)	March 2006
13	*S. sclerotiorum*	Sclerotia – Field	Kinross	Sunflower (*Helianthus annuus*)	March 2006
14	*S. sclerotiorum*	Sclerotia – Field	Ermelo	Soybean (*Glycine max*)	March 2006
15	*S. sclerotiorum*	Sclerotia – Field	Greytown	Soybean (*Glycine max*)	March 2006
16	*S. sclerotiorum*	Sclerotia – Field	Standerton	Sunflower (*Helianthus annuus*)	March 2006
17	*S. sclerotiorum*	Sclerotia – Field	Leslie	Soybean (*Glycine max*)	November 2006
18	*S. sclerotiorum*	Sclerotia – Field	Bergville	Soybean (*Glycine max*)	February 2007

Table 5.2: Adapters and primers used in AFLP analyses of *S. sclerotiorum* populations

Adapters/primers	Sequences (5'-3')
Eco RI - adapters	CTCGTAGACTGCGTACC AATTGGTACGCAGTCTAC
Mse I - adapters	GACGATGAGTCCTGAG TACTCAGGACTCAT
Pre-selective primers	
Eco-A	GACTGCGTACCAATTCA
Mse-C	GATGAGTCCTGAGTAAC
Selective primers	
E-AGC : M-CTA	GAGTGCGTACCAATTCAGC GATGAGTCCTGAGTAACTA
E-ACT : M-CTG	GAGTGCGTACCAATTCACT GATGAGTCCTGAGTAACTG
E-AGC : M-CTC	GAGTGCGTACCAATTAGC GATGAGTCCTGAGTAACTC
E-ACC : M-CAG	GAGTGCGTACCAATTCACC GATGAGTCCTGAGTAACAG

Table 5.3: Mean colony diameter of *S. sclerotiorum* isolates on PDA at different temperatures

Isolate	Temperature				
	15°C	20°C	25°C	30°C	Mean
1	48.63	58.16	59.8	17.78	**46.09**
2	44.37	59.54	59.78	9.9	**43.40**
3	36.55	51.99	46.74	9.64	**36.23**
4	48.49	60.71	61.17	28.75	**49.78**
5	46.58	58.97	59.95	19.53	**46.26**
6	49.66	63.37	59.46	15.66	**47.04**
7	50.68	64.25	57.85	19.82	**49.78**
8	47.01	63.06	57.52	14.87	**45.62**
9	47.54	66.18	60.52	16.76	**47.75**
10	48.74	62.59	60.13	18.47	**47.48**
11	46.32	63.52	54.3	16.37	**45.13**
12	49.85	65.05	61.66	19.46	**49.01**
13	46.76	62.99	58.33	18.16	**46.56**
14	49.29	65.63	61.7	18.03	**48.66**
15	46.94	62.6	58.74	19.94	**47.06**
16	31.14	63.34	60.54	13.75	**42.19**
17	48.7	64.48	59.33	18.75	**47.82**
18	49.57	65.03	55.3	17	**46.73**
Mean	**49.27**	**62.30**	**58.49**	**17.37**	**46.86**
LSD (P>0.05)	**Isolate Means** **Temperature Means** **Interaction Means**		13.25 5.57 3.21		

Table 5.4: Mean halo size due to oxalic acid production, lesion diameter after inoculation with *S. sclerotiorum* on detached soybean leaves and leaf lesion rating of inoculated soybean plants in the greenhouse

Isolate	(a) Oxalic acid	(b) Leaf assay	(c)Leaf lesions (1-5)		
			SNK500	PAN 520 RR	Mean
1	50.45	0.00	0.2	0.0	0.1
2	54.95	0.00	2.2	1.0	1.6
3	49.24	0.00	1.4	1.0	1.2
4	51.70	13.55	1.0	1.0	1.0
5	53.17	0.00	1.4	2.0	1.7
6	42.38	5.86	1.0	1.0	1.0
7	49.75	0.00	1.2	1.2	1.2
8	52.40	0.00	2.0	1.2	1.6
9	53.61	0.33	1.0	1.0	1.0
10	42.27	0.00	1.2	1.0	1.1
11	30.15	0.00	0.8	1.0	0.9
12	11.31	0.30	1.8	1.0	1.4
13	45.65	0.85	0.8	2.0	1.4
14	15.55	7.59	0.4	1.8	1.1
15	13.11	5.31	1.8	2.0	1.9
16	15.67	17.79	1.2	1.0	1.1
17	42.30	0.00	1.0	0.0	0.5
18	43.87	2.15	1.0	0.0	0.5
LSD (P<0.05):	2.55	4.95	Interaction =0.07		0.10

a) Mean diameter of oxalic acid secretion (yellow halo (mm)) on PDA augmented with Bromophenol blue.

b) Lesion diameter (mm) of soybean leaves inoculated with *S. sclerotiorum* on a detached leaf assay.

c) Leaf lesion evaluation of cultivars inoculated with *S. sclerotiorum* in the Greenhouse

Table 5.5: Similarity correlation among the 18 *S. sclerotiorum* isolates from AFLP analysis

|	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	1.00																	
2	0.89	1.000																
3	0.87	0.95	1.0000															
4	0.82	0.88	0.93	1.000														
5	0.85	0.95	0.90	0.91	1.000													
6	0.95	0.87	0.85	0.80	0.83	1.000												
7	0.78	0.86	0.84	0.81	0.85	0.74	1.000											
8	0.86	0.82	0.80	0.75	0.78	0.87	0.86	1.000										
9	0.91	0.86	0.83	0.78	0.81	0.88	0.79	0.92	1.000									
10	0.83	0.80	0.80	0.78	0.77	0.85	0.83	0.82	0.80	1.000								
11	0.93	0.88	0.86	0.80	0.84	0.91	0.81	0.92	0.97	0.80	1.000							
12	0.91	0.84	0.82	0.77	0.80	0.94	0.80	0.88	0.90	0.85	0.92	1.000						
13	0.83	0.85	0.85	0.82	0.80	0.78	0.84	0.80	0.79	0.85	0.79	0.78	1.000					
14	0.91	0.82	0.80	0.79	0.78	0.91	0.72	0.80	0.83	0.83	0.85	0.88	0.78	1.000				
15	0.86	0.86	0.86	0.80	0.81	0.86	0.79	0.79	0.80	0.86	0.82	0.85	0.81	0.85	1.000			
16	0.86	0.91	0.91	0.87	0.88	0.86	0.83	0.78	0.82	0.81	0.84	0.83	0.88	0.81	0.84	1.000		
17	0.91	0.81	0.79	0.80	0.81	0.93	0.75	0.81	0.82	0.84	0.84	0.89	0.75	0.94	0.86	0.81	1.000	
18	0.91	0.81	0.79	0.81	0.82	0.89	0.75	0.83	0.85	0.80	0.87	0.86	0.75	0.88	0.85	0.80	0.94	1.00
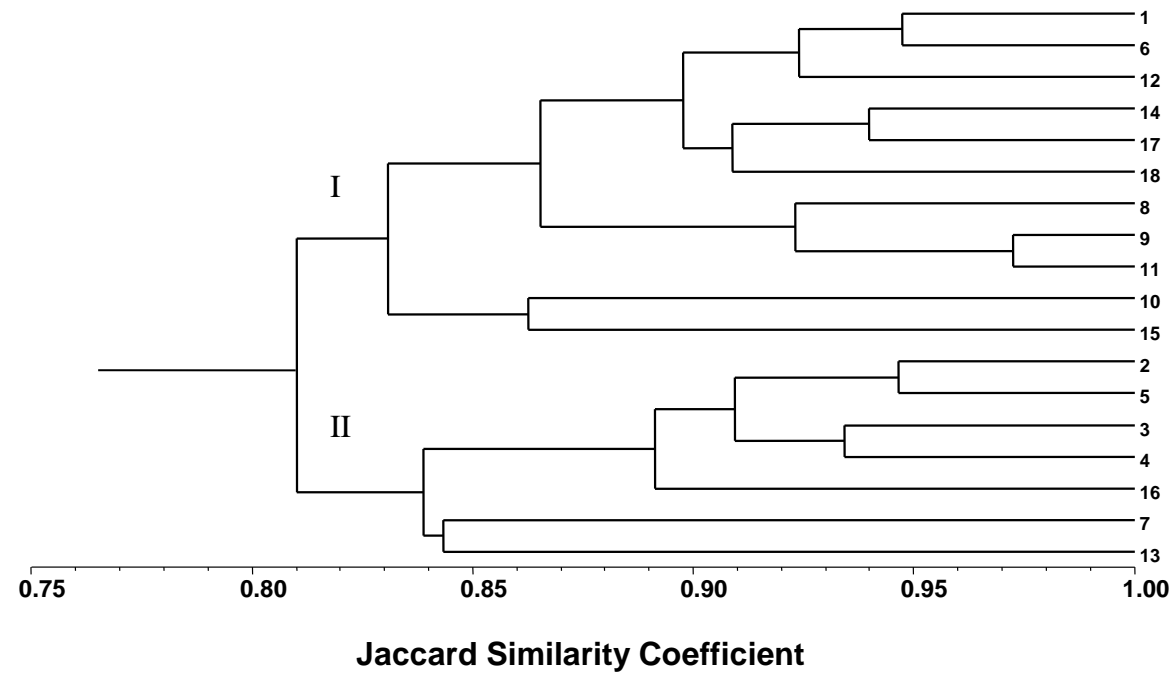
Figure 5.1: Dendogram based on AFLP data contructed for 18 *S. sclerotiorum* isolates using Jaccard's coefficient of similarity and UPGMA cultering.
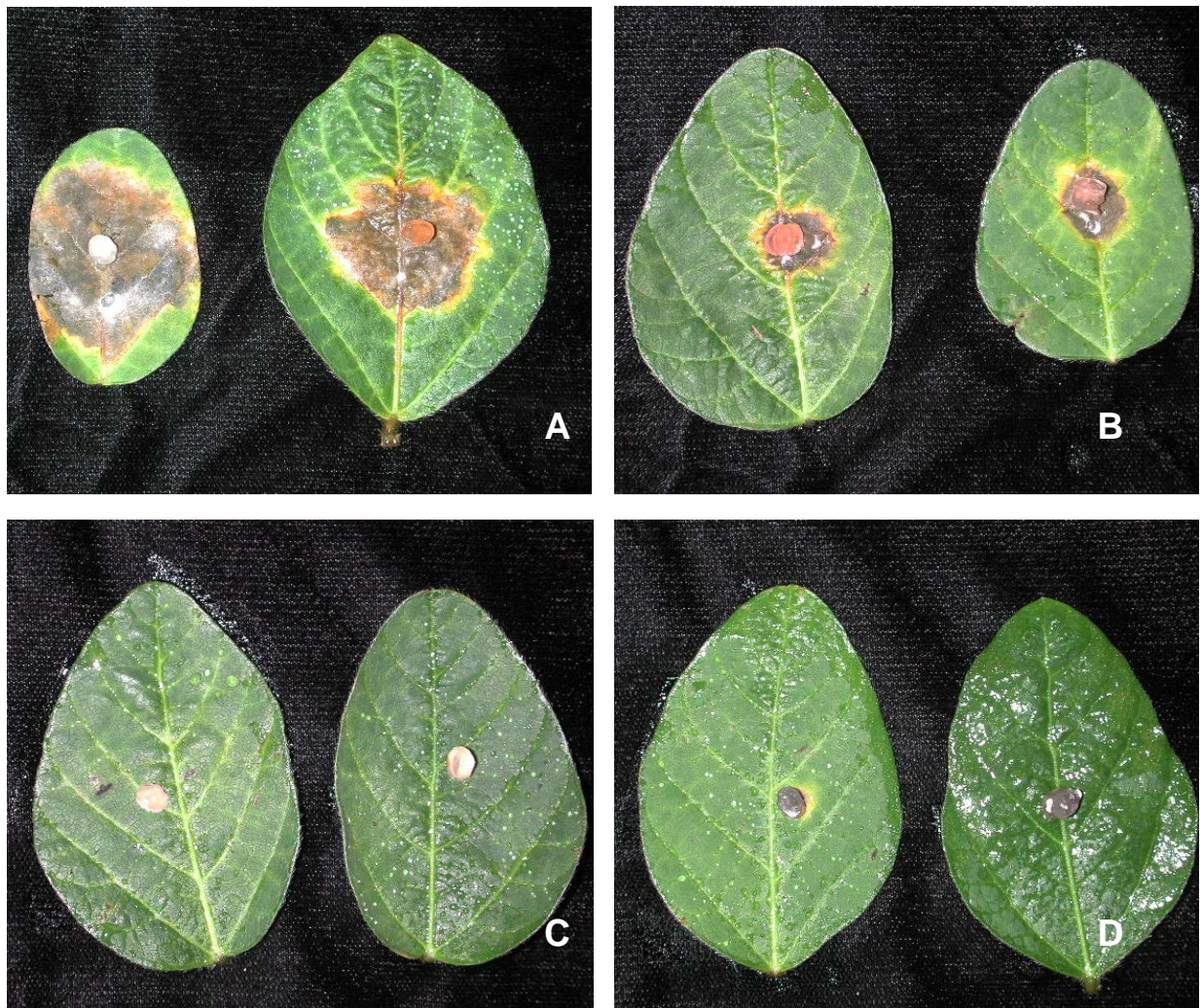
Figure 5.2: Detached leaf assay indicating differences in lesion size due to *Sclerotinia sclerotiorum* isolate variation: (a) isolate 16 (b) isolate 6 (c) isolate 5 and (d) isolate 8.

Figure 5.3: Greenhouse evaluation of pathogenicity showing leaf damage and wilting incidence due to colonization by isolate 2 of *S. sclerotiorum* on soybean cultivars- (a) SNK 500 (b) PAN 520 RR.
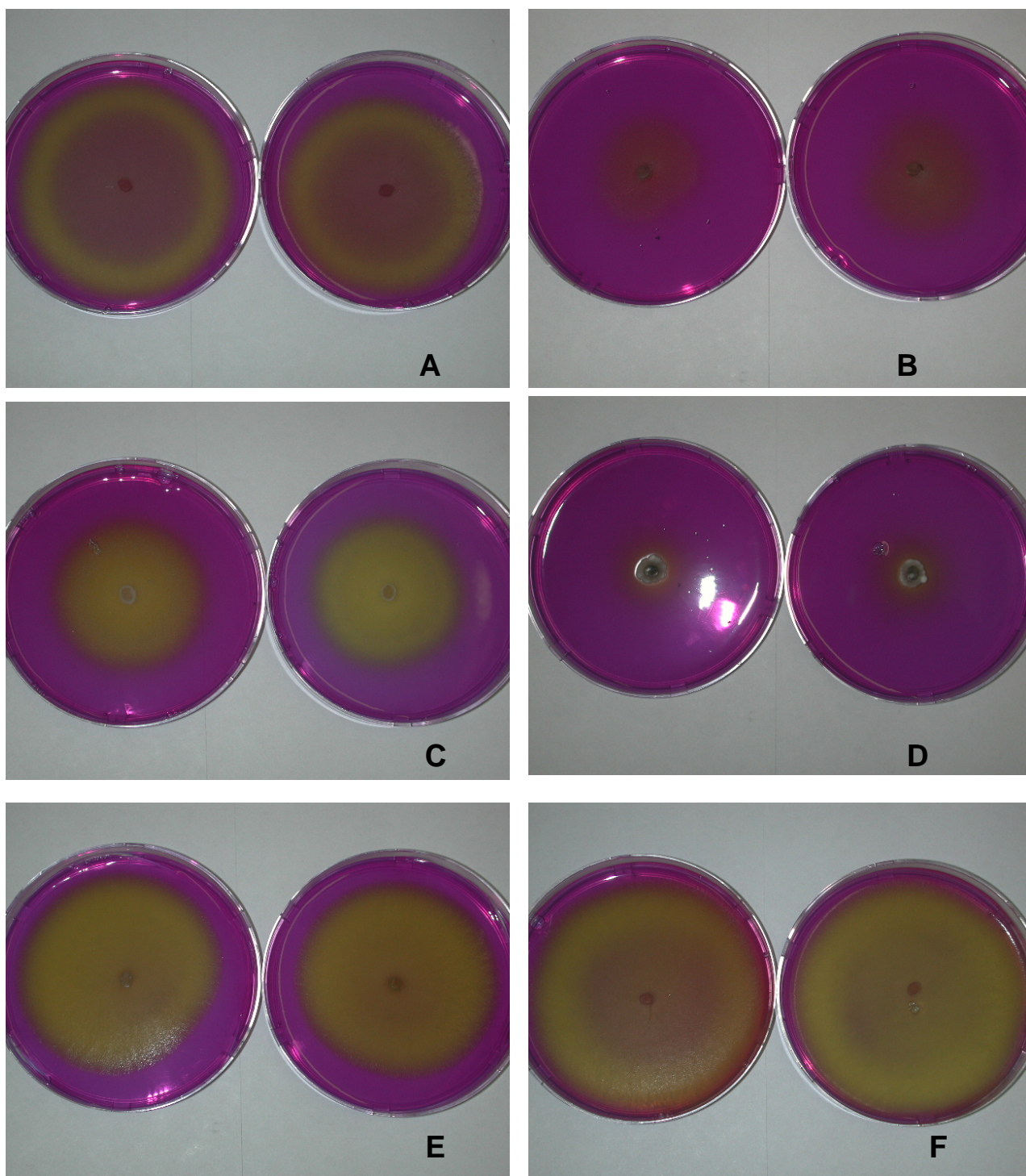
Figure 5.5. Oxalic acid production (yellow halo) by *S. sclerotiorum* isolates *in vitro* on PDA ammended with bromophenol blue (a) isolate 7 (b) isolate 11 (c) isolate 10 (d) isolate 12 (e) isolate 18 and (f) isolate 2.

Primer Combination 1:
*EcoR*1: AGC; *MSe*I: CTA

Primer Combination 2:
*EcoR*1: ACT; *MSe*I: CTG

Primer Combination 3:
*EcoR*1: AGC; *MSe*I: CTC
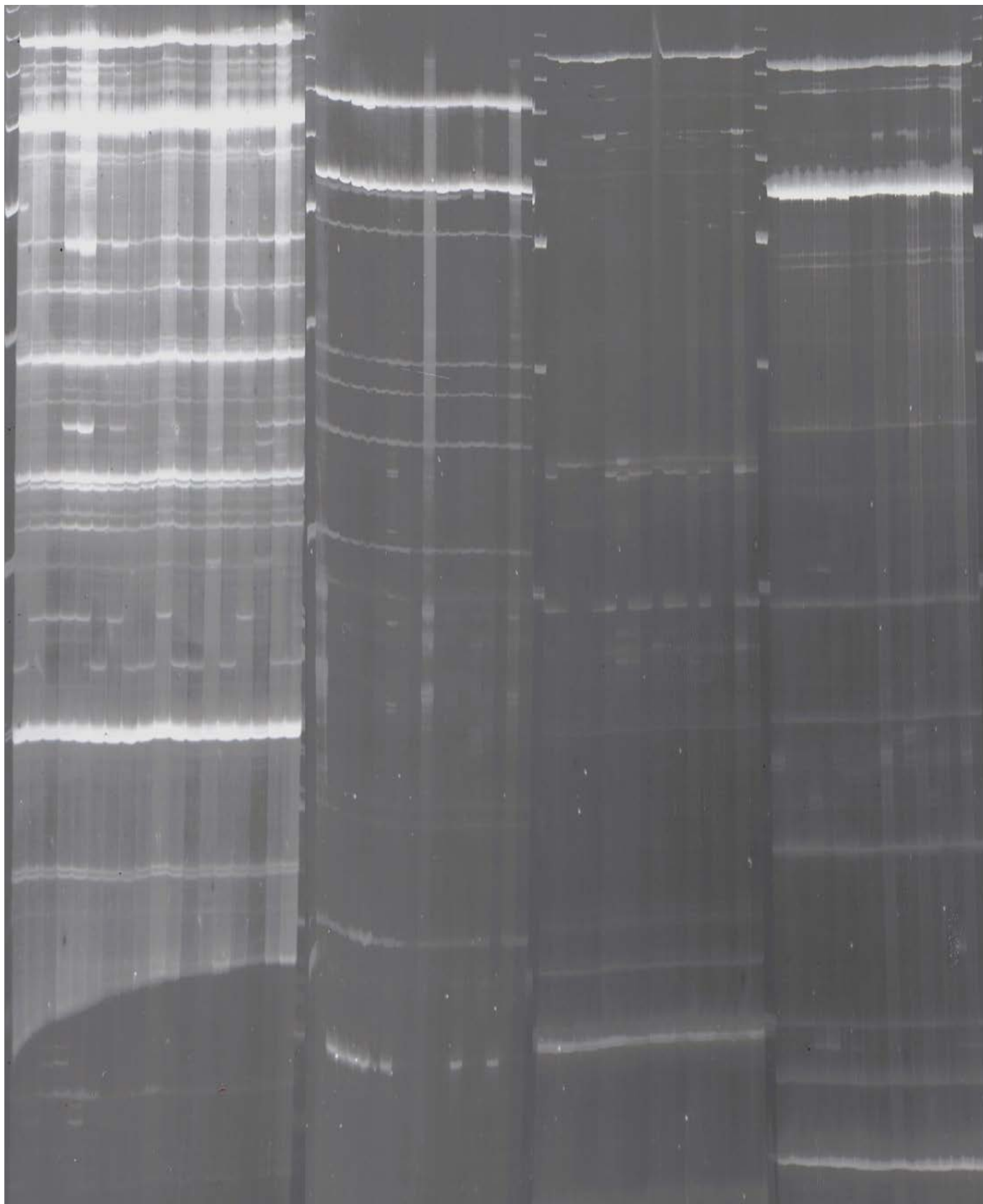
Primer Combination 4:
*EcoR*1: ACC; *MSe*I: CAG

Figure 5.6: AFLP fragments constructed for 18 *S. sclerotiorum* isolates using the four primer combinations determined with a 100 bp DNA ladder.

Summary

- *Sclerotinia sclerotiorum* (Lib.) de Bary causes destructive diseases on numerous plant species worldwide. The pathogen has a host range of more than 400 plant species including agricultural crops such as sunflower, dry beans, soybeans, potatoes, carrots and groundnuts. It was first reported on soybean as causing Sclerotinia stem rot in South Africa in 1979 and is currently common throughout the local production areas. To date no reliable and economically feasible method of control has been developed and local cultivars lack significant levels of resistance. As a result, the disease causes extensive quantitative as well as qualitative losses.

- The objectives of this study were to compare various inoculation techniques for *S. sclerotiorum* on soybeans in the greenhouse; determine the optimum conditions for infection, evaluate commercial cultivars for resistance to Sclerotinia stem rot in greenhouse; to determine an economically feasible and effective control method (chemical and biological) for Sclerotinia stem rot and to determine the genetic relationship and isolate variation of various *S. sclerotiorum* isolates collected from local production fields.

- Greenhouse evaluations of six different inoculation techniques on four soybean cultivars were conducted. Leaf damage and wilting incidence were evaluated using a 0–5 disease rating scale. Infection levels induced by various inoculation techniques differed and a spray mycelium method proved to be the most consistent and effective. An inoculation technique x cultivar interaction was recorded, especially when wounding of plants was included.

- Temperature and leaf wetness duration studies were conducted in the greenhouse. Optimum temperatures for disease development were determined at 20.90-22.75°C. Results indicated that high humidity or free moisture are critical for disease development and disease incidence and severity increased with increasing RH. Field evaluations indicated that the optimum temperature and humidity were 22.75°C and 95.37% respectively. Multiple regression analyses indicated that these were the only variables significantly related to disease potential.

- Various chemical and biological control agents are commercially available but these fail to control the disease effectively. Laboratory results revealed that chemicals applied at the regulatory rate failed to control mycelium growth effectively as opposed to the double rates which were effective throughout the evaluations. Biological control has the potential to act as a mycoparasite and suppress *S. sclerotiorum.* However further research is needed to optimize effectiveness. Benomyl and procymidone proved to be the most effective treatments in the laboratory and greenhouse. Application time relative to inoculation had a small but significant effect, indicating the need for correct application time.

- Growth studies, oxalic acid analysis, AFLP analysis and pathogenicity studies indicated genetic variation among the 18 isolates used in the current study. Isolates reacted differently to the various temperatures during growth studies and optimum growth was observed at 20°C. Some isolates were able to grow more readily at higher temperature than others. Isolates varied significantly in oxalic acid production but no correlation was recorded with the AFLP results which indicated small variation within isolates. Oxalic acid production by isolates could also not be correlated with pathogenicity although isolates varied in their ability to cause wilting and leaf damage.

Opsomming

- *Sclerotinia sclerotiorum* (Lib) de Bary is 'n vernietigende patogeen op verskeie plante wêreldwyd. Die patogeen het 'n gasheer reeks van meer as 400 plant spesies en kom veral voor op agronomiese belangrike gewasse soos sonneblom, droë bone, sojabone, aartappels, wortels and grondbone. Dit is die eerste keer op sojabone in Suid-Afrika geraporteer in 1979 en kom tans algemeen voor in die plaaslike produksiegebiede. 'n Betroubare en ekonomies bekostigbare metode van beheer is tans nie beskikbaar nie en betekenisvolle weerstand in die plaaslike sojaboon kultivars teen Sclerotinia stamvrot ontbreek wat ernstige oesverliese tot gevolg het.

- Die doel van die studie was om verskeie inokulasie metodes te evalueer in die glashuis; om optimale omstandighede vir infeksie vas te stel; om huidige kommersiële kultivars vir weerstand teen sclerotinia stamvrot te evalueer in die glashuis; om 'n ekonomies bekostigbare en effektiewe beheer metode te ontwikkel (biologies en chemies) en om die isolaat variasie en patogenisiteit van verskeie *S. sclerotiorum* isolate vanaf plaaslike produksiegebiede vas te stel.

- Glashuisevaluasies van ses verskillende inokulasie metodes op vier sojaboon kultivars is gedoen. Die blaarskade en voorkoms van verwelking is vasgestel op 'n 0-5 siekte skaal. Siektevoorkoms het getoon dat die verkeie metodes van mekaar verskil en dat die 'n spuit muselium metode die mees effektiefste was. Duidelike verkille tussen kultivars is gevind. Daar is gevind dat kultivars verskillend reageer teenoor die verskillende inokulasiemetodes wat gebruik is, veral waar besering van die plant weefsel teenwoordig is.

- Temperatuur en blaaroppervlak-vog studies is in die glashuis gedoen. Die optimale temperatuur vir die ontwikkeling van Sclerotinia stamvrot is vasgestel as 20.90°C en 22.75°C. Hoë humiditeit of vog is noodsaaklik vir siekte ontwikkeling en die siekte het toegeneem in beide die glashuis en die veld soos die RH verhoog het. Veld-evaluasie het getoon dat die optimum temperatuur en RH bereken was by 22.75°C en 95.37% onderskeidelik. Veelvoudige regressie analises het getoon dat hierdie die enigste veranderlikes was wat met siekte ontwikkeling gepaard gegaan het.

- Verskeie chemiese en biologiese beheer-metodes is kommersiëel beskikbaar, maar beheer van Sclerotinia stamvrot deur die agente ontbreek steeds. Laboratorium resultate het getoon dat die chemiese middels toegedien teen die gespesifiseerde dosis nie die fungus effektief kon beheer nie teenoor die dubbel-dosis wat regdeur die evaluasie effektief was. Biologiese beheer-agente het die vermoë en potensiaal om *S. sclerotiorum* te beheer maar verdere navorsing is nodig om die effektiwiteit te verhoog. Benomyl en Sumisclex was die mees effektiefste agente gedurende die studie in die laboratorium en die glashuis. Siekte onderdrukking het verskil tussen behandelings. Toedienings-tyd het slegs 'n klein invloed gehad wat die tekort vir die korrekte toedienings tyd uitwys.

- Isolaatvariasie en patogenisiteitverskille is gevind tussen die verskillende isolate. Isolate het verskillend gereageer op die temperature gedurende die groeistudie evaluasie en die optimale swamgroei was gevind by 20°C. Isolate het merkbaar verskil in die vermoë om oksaalsuur te produseer. Geen korrelasie is egter gevind tussen laasgenoemde resultate en die AFLP resultate wat getoon het dat daar slegs 'n klein variasie tussen die isolate is nie. Patogenisiteit evaluasie in die glashuis het getoon dat

isolate verskil in die vermoë on blaarskade en verwelking by kultivars te veroorsaak.