BIOLOGY AND CONTROL OF *SCLEROTIUM ROLFSII* ON GROUNDNUT IN SOUTH AFRICA

Thesis submitted in fulfilment of requirements for the degree Doctor of Philosophy in the Department of Plant Pathology, Faculty of Natural and Agricultural Sciences, University of the Free State

By

André John Cilliers

Promoter:

Prof. Z.A. Pretorius

Co-promoter:

Dr. P.S. van Wyk

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"Through the fish-eyed lens

Of tear-stained eyes,

I can barely define the shape

Of this moment in time"

From: "The Final Cut" - Pink Floyd

CONTENTS

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PREFACE

This thesis is a compilation of five manuscripts written over a period of 36 months. Since each chapter was prepared as an independent article, some repetition was unavoidable. At the time of submission the following chapters or parts thereof had been published or presented:

Chapter 2: CILLIERS, A.J., and PRETORIUS, Z.A. 1998. Mycelial compatibility groups of *Sclerotium rolfsii* in South Africa (Abstr.). South African Journal of Science Vol 94.

Chapter 3: CILLIERS, A.J., HERSELMAN, L., AND PRETORIUS, Z.A. 2000. Genetic variability within and among mycelial compatibility groups of *Sclerotium rolfsii* in South Africa. *Phytopathology* 90:1026-1031.

Chapter 3: HERSELMAN, L., CILLIERS, A.J., AND PRETORIUS, Z.A. 2000. Genetic variation among mycelial compatibility groups of *Sclerotium rolfsii* in South Africa. Durable Disease Resistance Symposium, Wageningen, Holland.

Chapter 4: CILLIERS, A.J., and PRETORIUS Z.A. 2000. Relative pathogenicity of mycelial compatibility groups of *Sclerotium rolfsii* to groundnut in South Africa. 12th Biennial congress of the Australasian Plant Pathology Society, Canberra Australia.

GENERAL INTRODUCTION

In South Africa *Sclerotium rolfsii* was first reported as a plant pathogen by Moore in 1926 but was not described on groundnut (peanut) until 1931 (Doidge and Bottomley, 1931). The fungus causes substantial losses to groundnut and other crop plants throughout the world. Groundnut plants are not only killed by the fungus, but kernels are discoloured by oxalic acid, leading to downgrading of consignments. Very little research has been done on *S. rolfsii* in South Africa. For example, no work has been done on the population structure of the pathogen and no formulations have yet been registered on groundnut for chemical control of *S. rolfsii*. This situation prompted the present study which investigates the population structure, genetic constitution, pathogenicity and control of the pathogen in South Africa.

The introductory chapter is a review of the fungus *Sclerotium rolfsii* over the past 16 years, with particular reference to its occurrence on groundnuts. A very comprehensive review was written by Punja in 1985. This chapter strives to cover the work done on this fungus on groundnut since that time. Specific questions posed in this chapter are addressed in later chapters.

In chapter 2 the population structure of *Sclerotium rolfsii* in South Africa is defined with regards to mycelial compatibility groups (MCGs). The issue is debated whether these MCGs can be linked to specific host plants or to geographical areas in South Africa, as has been suspected by other researchers. Suggestions are also made as to the possible mechanism whereby new MCGs arise.

The first study showing genetic variation between and within MCGs of *S. rolfsii*

by means of AFLP (Amplified Fragment Length Polymorphisims) analysis is reported in chapter 3. No study prior to this one has shown that isolates within a MCG can be distinguished from each other. MCGs were also clearly differentiated with this technique indicating that isolates within a MCG of *S. rolfsii* are not clonally derived.

The issue of relative pathogenicity of MCGs to specific crop plants, particularly groundnut, is investigated in chapter 4. The possibility that specific MCGs are pathogenic to specific crops has never been investigated before. This chapter also covers the issue of possible mating type switching in *S. rolfsii*, an issue never investigated before by other researchers.

The control of soil borne pathogens has always been a complex issue. No attempts have been made in South Africa to control *S. rolfsii* on groundnut by combining chemical, biological and cultural control measures. Chapter 5 investigates possible means of controlling the fungus by such an integrated approach. A fungicide was identified that inhibited *S. rolfsii* but not the antagonist *Trichoderma harzianum*. In combination with inversion ploughing and a decreased plant density, a co-ordinated strategy for managing stem rot of groundnut is proposed.

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CHAPTER 1

A REVIEW OF *SCLEROTIUM ROLFSII* ON GROUNDNUT WITH PARTICULAR REFERENCE TO BIOLOGY AND CONTROL

ABSTRACT

Sclerotium rolfsii is a wide host range pathogen that survives in the soil as dried sclerotia. The sexual stage, *Athelia rolfsii*, is known but does not occur commonly in nature. The pathogen occurs in most groundnut producing areas of the world where it causes severe damage both in plant mortality and reduced quality of groundnut kernels. For this reason a great deal of research has been done on the control of this fungus by means of biological, genetic, cultural and chemical control methods. The aim of this review is to summarise the biology and control of *S. rolfsii*, with particular reference to its occurrence on groundnut. Since a comprehensive review appeared in 1985, emphasis is placed on literature of the past 16 years.

INTRODUCTION

Sclerotium rolfsii Sacc., the causal organism of southern stem rot (SSR) or southern blight (Fig. 1 A) of groundnut (*Arachis hypogaea* L.), is widely distributed and was first recorded in South Africa on tobacco (*Nicotiana tabacum* L.) in 1926 (Moore, 1926) and on groundnut in 1931 (Doidge and Bottomley, 1931). World-wide, this pathogen has been reported to cause disease in more than 500 plant species (Aycock, 1966). The sexual state, *Athelia rolfsii* (Curzi) Tu & Kimbrough, has been induced in the laboratory (Punja and Grogan, 1983) but is not thought to occur commonly in nature. Since the

fungus was first observed in South Africa it has been reported from more than 30 hosts, including various crop plants (Doidge, 1950; Crous *et al.,* 2000). Since 1985 the pathogen has been reported on *Alstromeria* spp. in Canada (Chang and Mirza, 1994), perennial groundnut in Florida, USA (Stanley *et al.,* 1996), English walnut (*Juglans nigra* L.) in Europe (Belisario and Corazza, 1996), lisianthus (*Eustoma grandiflorum* (Raf.) Shinners) in the USA (McGovern, 2000) and sunflower (*Helianthus annuus* L.) in Italy (Infantino and Giambattista, 1997). Two comprehensive reviews of *Sclerotium rolfsii* have been written in the past: one by Aycock in 1966 and the other by Punja in 1985. Most of the research since the review by Punja (1985), however, has been on various aspects of the control of the fungus throughout the world, particularly chemical control. *Sclerotium rolfsii* does not produce asexual spores and overwinters as sclerotia (Fig. 1 B), the primary inocula, on plant debris and in soil (Punja, 1988). As a result, most control strategies target the fungus in the soil.

The population structure of *S. rolfsii* has been defined by the placing of isolates into different mycelial compatibility groups (MCGs) (Punja and Grogan, 1983). Studies of these groups have shown vague associations between MCGs and geographical area and/or host plant (Harlton *et al.,* 1995; Punja and Sun, 1997). Some genetic studies of MCGs have distinguished between groups but not among all isolates within MCGs (Harlton *et al.,* 1995; Punja and Sun, 1997).

Various aspects of the biology and life-cycle of the fungus have also been studied over the past fifteen years, including microscopic studies of the fungus (Robertson, 1992), induction of the teleomorph (Tu *et al.*, 1992) and sclerotial biogenesis (Zarini and Christias, 1997). Other studies have elucidated some of the factors influencing fungal host infection (Shew *et al.,* 1987; Bowen *et al.,* 1992; Starr *et al.,* 1996) and compared

inoculation techniques (Shokes *et al.,* 1996).

The fungus accounts annually for a loss of between 5-10 % in groundnut yields in the USA (Csinos *et al.,* 1983; Sturgeon, 1986) and is considered to be one of the major pests of groundnut (Wolf *et al.,* 1995). Groundnut yield was found to be inversely related to incidence of SSR and directly related to the number of years between crops (Bowen *et al.,* 1996). *Sclerotium rolfsii* has been associated with reduction of groundnut quality due to discolouration of the kernels (Fig. 1 C) by the fungus (Van Wyk and Cilliers, 1998). Chemicals for the control of SSR have also been shown to improve groundnut quality in combination with insecticides (Backman *et al.,* 1989).

By far the greatest research effort on this fungus has been aimed at various aspects of its control worldwide. In particular a significant amount of work has been conducted on the chemical control of SSR in the USA (Hagan *et al.,* 1988; Bowen *et al.,* 1992; Culbreath *et al.,* 1992; Minton *et al.,* 1993; Brenneman *et al.,* 1994; Damicone and Jackson, 1994; Culbreath *et al.,* 1995). Other approaches that have been investigated include biological control using various fungi and bacteria (Henis *et al.,* 1983; Elad *et al.,* 1984; Papavizas and Lewis, 1989; Papavizas and Collins, 1990; Latunde-Dada, 1993; Pleban *et al.,* 1995; Benhamou and Chet, 1996), genetic control (Grichar and Smith, 1992; Besler *et al.,* 1997) and control by tillage and other cultural practices (Colvin and Brecke, 1988; Colvin *et al.,* 1988; Minton *et al.,* 1990; Minton *et al.,* 199l). Progress has certainly been made worldwide for the protection of crop plants against this versatile pathogen.

The aim of this paper is to review the research done on the biology and control of the fungus, particularly on groundnut, since that time. It is appropriate, however, to briefly summarise the review by Punja (1985) in order to provide the necessary

background information.

SUMMARY OF A PREVIOUS REVIEW OF *SCLEROTIUM ROLFSII*

Punja (1985) thoroughly discussed the biology of the pathogen, including the factors affecting mycelial growth and sclerotial production. The factors that have been investigated include temperature, aeration, moisture and light.

Factors influencing fungal growth and sclerotial production

The occurrence of the fungus in warm regions of the world indicates that the pathogen has a high optimal growth temperature. The temperature range for hyphal extension and dry weight production is between 8 and 40 °C and the temperature of maximum growth and sclerotial production is between 27 and 30 °C. The fungus is considered to be highly aerobic, but mycelial growth and sclerotial production can be stimulated by low concentrations of carbon dioxide. Nevertheless, low (4 %) levels of oxygen retard linear fungal growth in culture.

Fungal growth is reported to decrease with increasing moisture and disease is greatest in well-drained, sandy soils or in soils with a high silt content that retained moisture. Growth of the fungus, as well as sclerotia production, are both greater under continuous light, especially blue light, than in darkness. The fungus grows well on a wide range or carbon sources, while optimum growth occurs at lower pH values and is markedly less above pH 8. Many scientific reports have shown that sclerotial formation in culture may be inhibited at various stages of development by low concentrations of unrelated chemicals, some of which also decrease mycelial growth.

Structure, composition and germination of sclerotia

The structure and composition of sclerotia are also discussed in detail in the review by Punja (1985). Sclerotia range in size from 0.5 - 2 mm in diameter and are formed laterally from main hyphal strands. Following sclerotial initiation and during development, drops of exudate containing various chemicals including oxalic acid, form on the sclerotial surface. Mature sclerotia are composed of three distinct layers or regions. The first is the outer melanized rind consisting of two to four cell layers. Beneath this is the cortical layer which in turn encloses the innermost medullary region. Mature sclerotia contain amino acids, sugars, fatty acids and lipids.

Two distinct forms of sclerotial germination occur namely hyphal and eruptive. In the case of hyphal germination, individual hyphae (originating from the cells of the medulla) grow from the surface of the sclerotium. In the case of eruptive germination, plugs or aggregates of mycelium burst from the sclerotium. Mature sclerotia appear to have little, if any, dormancy and germinate readily under favourable conditions. The type of sclerotial germination could affect disease incidence since mycelial growth and energy available for infection are affected by the type of sclerotial germination. A germination type requiring more energy would therefore have less energy available for infection.

Other factors affecting the germination of sclerotia are the assay substrate, nutrients, volatile compounds, chemicals, temperature, moisture, aeration and depth in soil. Media containing soluble nutrients apparently inhibit eruptive germination of sclerotia and only hyphal germination occurs. The addition or omission of chemicals to growth media can induce or inhibit eruptive sclerotial germination. Both types of germination are, however, inhibited above a pH of 7. Optimum pH for germination is

between 2 and 5. Optimum temperature for eruptive germination is between 21 and 30 °C with little germination occurring below 15 °C and above 36 °C. Germination of sclerotia decreases at soil depths below 2.5 cm and is zero at depths of 8 cm or more.

The presence of other microbes in the soil also appears to affect sclerotial germination. Sclerotia in non-sterile soil germinate poorly in comparison with those in laboratory cultures. This fact creates opportunities for biological control of the pathogen by inhibiting sclerotial germination or for cultural control by burying sclerotia deeply in the soil.

Factors influencing sclerotial survival

The biotic factors affecting survival of sclerotia in soil are linked to abiotic factors which predispose sclerotia to the action of antagonists or which increase nutrient leakage from sclerotia. Such factors include drying, heating, deep burial, exposure of sclerotia to chemicals and the induction of changes in the integrity of the sclerotial rind. Antagonistic fungi such as *Trichoderma* sp and *Aspergillus* sp are capable of penetrating the sclerotial rind and destroying the sclerotium. Possible control strategies could therefore include deep burying of sclerotia and the application of antagonistic organisms to the soil, both of which have been reported to reduce disease in the field. Other control strategies include chemical control. Numerous fungicides have been registered which inhibit sclerotial germination or mycelial growth of the fungus. The limiting factor, however, is that most of these chemicals have to be applied in large amounts in order to provide effective control, and even then efficacy can vary from crop to crop. Fumigation of soil with products such as methyl bromide is effective in destroying sclerotia. Disease can be decreased with the application of ammonium fertilizers such

as urea. The action of these fertilizers is unclear, but it appears that they may be toxic to sclerotia or they may limit disease by altering host susceptibility or populations of antagonistic microbes in the soil.

Cultural control practices, such as solar heating, have reduced disease and sclerotial numbers in the soil. Solar heating combined with the application of *Trichoderma harzianum* is more effective than any of these treatments alone. As mentioned before, both deep ploughing and crop rotation can reduce disease in the field. The addition of organic amendments to soil can also reduce disease. Such methods of control are possibly the most economical and can be combined with chemical and biological control. Several organisms have been reported to reduce mycelial growth and sclerotial production of *S. rolfsii*, including bacteria, actimomycetes, a mycorrhizal fungus and *Trichoderma* spp. Few studies have, however, proved the efficacy of these organisms in the field. The use of *Trichoderma* spp. shows a fair degree of control in the field at various rates of application.

Virulence and the fungal infection process

In the infection process of the host plant, it appears that cell death occurs prior to hyphal penetration due to the combined production of large quantities of oxalic acid and polygalacturonases. Oxalic acid is not only toxic to plant tissue, but also sequesters calcium from the cell wall of plant cells and lowers the tissue pH to the optimum for endopolygalacturonase and cellulase activity. Upon infection, hyphae grow through plant tissue both inter- and intracellularly with the end result being water-soaked, necrotic and macerated lesions in the plant tissue.

Pathogenicity and virulence of the fungus are influenced by a range of factors.

The production of oxalic acid appears to be of paramount importance in the infection process. The amount of oxalic acid produced depends on the specific isolate, the carbon and nitrogen source in the medium and the initial pH and presence of buffers in the medium. In view of this, possible control of the pathogen could be implemented by selecting crop plants with tolerance to oxalic acid. Even though cultivars with a fair degree of tolerance to the fungus have been registered, the mechanism(s) of tolerance are still largely unknown.

The fungal population and the teleomorph

The teleomorph state of *S. rolfsii* is described as *Athelia rolfsii*. The formation of basidia in culture depends on the isolate, the nutrient composition of the medium, the light intensity and the age of the culture. Basidia tend to form on nutrient-poor media and in older cultures at low light intensities. The addition of activated charcoal can also enhance fruiting in some isolates. A large degree of variation exists between single-basidiospore isolates of *A. rolfsii*, suggesting that field isolates may be heterokaryotic (Punja, 1985).

Punja (1985) noted that certain isolates from different geographical areas or host plants often produced a killing reaction (plasmolysis) when paired against each other. This observation manifests macroscopically as a barrage zone or aversion between the mycelia of two isolates. The reaction is indicative of genetic dissimilarities and isolates could be grouped according to these reactions. Isolates belonging to the same group often come from the same host plant or geographical area, although other groups contain isolates from different host plants and geographical areas.

Distribution of inoculum and diseased plants in a field are clustered. For this

reason soil sample size is imperative in any effort to determine the degree of infestation in a field by counting sclerotia. Larger sample sizes are more likely to give more accurate answers than smaller sample sizes which are subsequently bulked. The extraction procedure of the sclerotia from the soil may also influence the results of such a sampling. Advisory programs that attempt to identify infested fields should take such factors into account. Several methods are employed to determine the level of sclerotial infestation of the soil including treating soil with methanol to stimulate sclerotial germination and direct observation and enumeration of sclerotia; the so-called baiting method. Sclerotia may also be recovered from soil directly by wet-sieving, flotation-sieving or elutriation. Viability of these sclerotia can then be tested on agar media. Such media can be made selective by adding oxalate or gallic acid. Factors such as sampling time and location of the sample in relation to the host plant have to be considered in order to establish accurate correlations between inoculum density and disease incidence in a field.

Factors influencing disease incidence and severity

Disease incidence may be stimulated by fluctuations in temperature and moisture conditions. So too may the presence of organic matter such as defoliated leaves which can sustain mycelial growth. In groundnut, however, prevention of defoliation actually increased the disease severity by *S. rolfsii*. It was hypothesised that the leaf canopy created a microclimate on the soil surface that stimulated infection. Foliar fungicides may also have decreased the population of *Trichoderma* antagonists in the soil, thereby enhancing disease. High moisture and temperature conditions have been implicated with disease but free moisture is apparently not required for infection. The continuous

planting of susceptible crops on the same field has been suggested as a cause for increased disease severity whereas a one-year rotation with maize has been shown to reduce inoculum density.

The survival of sclerotia has been shown to be influenced by both biotic and abiotic factors. Temperatures of above 50 °C for extended periods of time are lethal and sublethal temperatures may also hasten death of sclerotia. Sclerotia show a lower survival rate in moist soils, with an even lower survival rate in moist soils with high temperatures. Little work has been done on the effect of low temperatures on sclerotial survival. Mycelium appears to survive better in sandy soils than in fine-textured soils while sclerotia survive longer in moist soil close to the surface than buried beneath the soil surface.

The above, in short, summarises the review of Punja (1985). This review should be read in combination with the one by Aycock (1966). What follows in the present study is a summary of most of the work done on this fungus since 1985. It does not, however, include all the references to *S. rolfsii*, but rather contains references pertinent to the topics that have been chosen for discussion.

BIOLOGY AND INDUCTION OF THE TELEOMORPH, *ATHELIA ROLFSII*

Various aspects of the biology of the fungus have been investigated, most of which are covered extensively in the review by Punja (1985). Subsequent work by Roberson (1992) on the distribution of actin in the hyphal tips of *S. rolfsii* using light microscopy and immunofluorescence techniques, however, revealed that the majority of the actin was localized in brightly florescent plaques that were principally localized in the first 10 to 12 µm of the hyphal tip. Most of these plaques were positioned in the peripheral

cytoplasmic regions. Coarse actin fibres were also observed in subapical regions parallel to the long hyphal axis and in association with septa (Roberson, 1992).

Punja and Damiani (1996) did a comparative study of the growth, morphology and physiology of three different *Sclerotium* species. All three species studied, *S. rolfsii, S. delphini* Welch and *S. coffeicola* Stahel, produced oxalic acid, pectinase and polyphenoloxidase with the highest production by *S. rolfsii.* The study showed that the three species could be distinguished from each other on the basis of colony characteristics such as morphology and sclerotium size, as well as differences in histological staining, composition of sclerotia, growth responses and temperature (Punja and Damiani, 1996).

The sexual state of *S. rolfsii, Athelia rolfsii*, has been induced in the laboratory (Punja and Grogan, 1983). Basidial formation in culture depends on several factors including isolate, the nutrient composition of the medium, the light intensity and the age of the culture (Punja *et al.,* 1982). Even laboratory induction has been found to be difficult, if not impossible (Tu *et al.,* 1992). A dependable method for basidia induction was described in 1992 by Tu *et al.* whereby a corn-leaf-culture method was used. All the teleomorph isolates induced in that study were positively identified as being *A. rolfsii.*

Early work in South Africa noted *S. rolfsii* as being toxic to different species of livestock (Terblanche and Rabie, 1967). The fungus was grown on maize and fed to ducklings, chickens, sheep, a horse and a heifer. Symptoms in the domestic livestock included anorexia, nervous disorders and reduction of growth rate. In another study ten years later a toxic amino acid was isolated from dried sclerotia of *S. rolfsii* (Potgieter *et al.*, 1977). A structure was assigned to the compound on the basis of available chemical and physical data, namely 2(S)3(R)-2-amino-3-hydroxypent-4-ynoic acid (Potgieter *et*

al., 1977).

GENETIC VARIABILITY AND MYCELIAL COMPATIBILITY GROUPS

The wide host range of *S. rolfsii* has prompted studies of the population as well as the genome of the fungus. Morphologically and pathogenically variable isolates from single-basidiospore strains of *S. rolfsii* have prompted researchers to suggest that field isolates may be hererokaryotic (Punja and Grogan, 1983; Punja *et al.*, 1985). Hyphal-tip cells of field isolates have also been shown to contain several nuclei, although discharged basidiospores are predominantly binucleate (Punja and Grogan, 1983). This condition may have arisen as a result of mitotic and not meiotic cell division (Punja and Grogan, 1983).

Punja and Grogan (1983) showed that *S. rolfsii* isolates can be placed in mycelial compatibility groups (MCG) based on mycelial interactions (Glass and Kuldau, 1992) between isolates similar to those that occur in vegetative compatibility groups (VCG) (Leslie, 1993). These interactions were initially associated with geographical area or host plant (Punja, 1985). In 1995 Nalim *et al.* did the first survey of MCGs in specific fields and in individual plants in Texas. The isolates in their study were placed into 25 MCGs and the DNA amplification patterns resulting from the use of the 18-base oligonucleotide primer NK2 and from restriction digests of the internal transcribed spacer (ITS) region of the rDNA were examined in a subset of 80 isolates representing 12 MCGs. Three NK2-amplified DNA patterns in genomic DNA and four *Mbo*I restriction digest patterns of the ITS region were found. All isolates from a single MCG gave identical patterns for each marker, and some of the MCGs shared the same ITS and NK2 patterns. It was further found that MCGs having the same DNA patterns were often

from the same field.

Harlton *et al.* (1995) screened a worldwide collection of *S. rolfsii* isolates and identified 49 MCGs from 119 isolates. Isolates from the same geographical area or host often grouped in the same MCG, but in some cases widely diverse isolates also grouped in the same MCG. Variation in ITS regions was examined following restriction enzyme digests. Restriction fragment length polymorphisms (RFLPs) were obtained with *Alu*I, *Hpa*II, *Rsa*I and *Mbo*I. The combined banding patterns for these four enzymes were used to characterize intra-specific variation within the *S. rolfsii* isolates. Twelve subspecific groupings were identified, some of which correlated with their MCG. However, some of the isolates within a MCG showed different ITS-RFLP patterns and certain patterns were also dispersed among MCGs. The total sum of digested fragment sizes was also found to exceed the undigested polymerase chain reaction product in several of the isolates. Harlton *et al.* (1995) maintained that this length discrepancy must have resulted from variation among rDNA copies in the presence of restriction sites. Segregation of two *Mbo*1 restriction patterns in the ITS region among 29 single-basidiospore strains derived from four parental field isolates of *S. rolfsii* with one pattern, suggested to the authors the presence of two distinct rDNA types in field isolates. They concluded that the rDNA types may reflect a heterokaryotic nuclear condition in field isolates of *S. rolfsii*.

In another study by Punja and Sun (1997) 128 isolates from 36 host species and 23 geographical regions were paired against each other and no less than 68 MCGs were identified. The isolates were then compared by means of RAPD-PCR (random amplified polymorphic DNA-polymerase chain reaction) amplifications with five primers. Polymorphic bands were scored and used in UPGMA (unweighted pair-group method,

arithmatic average) analysis. No relationship between host of origin and MCG was found, except that many isolates from the same host belonged to the same MCG. Conversely, isolates in a specific MCG could have originated from many different hosts. The UPGMA analysis revealed that isolates within the same MCG were genetically diverse, as were isolates from the same geographical area. Isolates from the same MCG did, however, tend to group closer together suggesting greater genetic similarity. Isolates from widely distant geographical locations were more distantly related and isolates with identical RAPD patterns were in the same MCG and were probably clonally derived (Punja and Sun, 1997).

In a more recent study Punja and Sun (2001) investigated the genetic relationship between 132 isolates of *S. rolfsii* collected from 1967 to 1997 from 36 different host species and 13 countries worldwide using RAPD analysis. A total of 71 MCGs were identified in this study, many of which were unique single-member groups. As was observed in other studies, these unique groups usually originated from widely separated geographical regions. Certain MCGs were also found to contain isolates from vastly different host plants and from widely different geographical areas. The RAPD analysis showed that isolates from different MCGs could be differentiated from each other using six primers. No discernable relationships between MCGs could be detected using UPGMA analysis. Isolates within an MCG were diverse but tended to cluster together. Certain members of some of the MCGs showed identical RAPD banding patterns and these isolates were considered to be clonally derived (Punja and Sun, 2000).

A study from Japan by Okabe and Matsumoto (2000) identified four MCGs from 132 isolates and four fields within a 120 m radius of each other. Isolates between MCGs and within MCGs were compared using RAPD analysis and isolates from the same MCG

collected from different geographical areas were genetically identical. Slight differences were, however, observed within MCG A where seven isolates differed slightly from the other in that they lacked one band in the OPB-13 pattern. These observations prompted the authors to suggest that minor somatic mutation could occur without affecting mycelial compatibility during clonal growth in field populations. Since two of the MCGs were collected in 1994 and 1997, Okabe and Matsumoto further suggested that MCGs are persistent over time.

No research has yet been done in South Africa MCGs of *S. rolfsii*. Isolates from various host plants and geographic localities should be collected and screened in order to determine whether there is any association between MCG, host plant and geographic area as was found by Punja and Sun (1997) and Harlton *et al.* (1995). Previous studies have not yet been able to genetically distinguish between all isolates of *S. rolfsii* within specified MCGs. AFLP (amplified fragment length polymorphisims) analysis is one genetic technique that has not yet been used to investigate the degree of similarity/dissimilarity between and within groups.

HOST INFECTION BY *SCLEROTIUM ROLFSII*

The infection process of *S. rolfsii* has been studied and described in previous papers (Punja *et al.*, 1985). One of the most important factors in the infection process is the production of oxalic acid (Punja *et al.*, 1985). It has even been speculated that cell death may occur prior to hyphal penetration due to the production of oxalic acid and polygalacturonases (Bateman, 1972). The wide host range of the fungus has been attributed to the production of non-specific cell-wall degrading enzymes which, in conjunction with the production of oxalic acid, overcome the defence mechanisms of

such a wide variety of plant species (Punja, 1985).

Subsequent work has shown that various factors affect the infection of groundnut plants by *Sclerotium rolfsii*. Infection is increased when plants are subjected to stress as a result of high temperatures in the field (Bowen *et al.*, 1992) or in the greenhouse (Shew *et al.*, 1987) and when irrigation is increased (Davis *et al.*, 1996) or as an additive effect with other groundnut pathogens such as the nematode *Meloidogyne arenaria* Neal (Starr *et al.*, 1996). Other reports have, however, shown that more disease was present in nonirrigated groundnut fields as opposed to those that received regular irrigation (Bowen, 1998).

Smith *et al.* (1986) found that mycelial aggregates functioning as infection cushions aided infection of *S. rolfsii* into host tissue (sugarbeet leaves, bean hypocotyls and carrot roots or petioles) by secretion of enzymes and oxalic acid, which resulted in cell death and collapse. Penetration occurred after death and collapse of the cells beneath the infection cushions. Infection was found to occur by means of penetration pegs which formed from appressoria. The cuticle was not dissolved in the process, but was rather pulled away from the epidermis, suggesting that cellular layers below the epidermis were dissolved by enzymatic activity. Hyphal growth within the host was both inter- and intracellular.

Shokes *et al.* (1996) compared six inoculation techniques in the greenhouse and field using groundnut as the host plant. The six techniques were (a) a germinating sclerotium on a 1-cm-diameter agar disk appressed to the base of each central stem; (b) mycelia of a composite of six isolates growing on sterilized oat seed placed on the soil near the base of each central stem in the greenhouse or in the centre of rows in the field; (c) 2-3 ml of a potato dextrose agar (PDA) slurry with actively growing mycelium

applied to the base of each central stem; (d) mycelia on toothpicks impregnated with potato dextrose broth inserted into the base of each central stem; (e) a toothpick with mycelia inserted into the soil near the base of each central stem; and (f) mycelia on PDB-impregnated clothespins clamped around the base of each central stem. The most effective techniques were the agar disk technique (a) and the clothespin technique (f).

The effect of the lesser cornstalk borer (LCB) on disease incidence has been investigated (Wolf *et al.,* 1995). A significant increase in disease incidence caused by *S. rolfsii* was observed when the treatment contained LCB larvae, as opposed to the treatment with *S. rolfsii* only. Other soil organisms have also been implicated in the increase of SSR in the field. Field observations have suggested that the nematode *Meloidogyne arenaria* may be associated with increased SSR incidence on groundnut (Starr *et al.,* 1996). However, these authors found no interaction between final nematode population densities and incidence of SSR. They concluded that the apparent disease complex between these two organisms on groundnut can be attributed to the additive effect of these two pathogens.

SCLEROTIAL BIOGENESIS, GERMINATION AND VIABILITY

Sclerotia are the resting structures whereby sclerotium-forming fungi survive for long periods of time in nature. Sclerotial biogenesis of *S. rolfsii* was studied microscopically by Zarini and Christias (1997) and three stages of sclerotial development were identified, namely sclerotial initials, development and mature stages. The initials developed from primordia, small tufts of mycelial hyphae loosely held together. The tufts became more dense and increased in size. The sclerotia then assumed their spherical shape during the development stage. Mature sclerotia are spherical and compact and completely

independent of their outer layer of hyphae. The skin surface of mature sclerotia are rough and scaly (Zarini and Christias, 1997). It has also been shown that sclerotial biogenesis in *S. rolfsii* is associated with lipid peroxidation (Georgiou, 1997). A direct relationship was found between the number of sclerotia formed and the lipid peroxidation levels in mycelial colonies and it was suggested that this phenomenon may be associated with the oxidative stress caused by growth conditions (Georgiou, 1997).

Sclerotial germination, as with spore germination of other fungi, is dependent on climatic and soil conditions. Shim and Starr (1997) determined that sclerotial germination and consequent infection of host tissue was most readily achieved in acidic soils. Disease did, however, develop at a soil pH of 8.7 and 9.8 even though it was significantly less than in acidic soils.

A correlation has been found between carbon loss from sclerotia, viability of these sclerotia and pathogenic aggressiveness (Hyakumachi and Lockwood, 1989). The level of endogenous carbon from ^{14}C-labelled sclerotia was monitored and found to decrease over a period of 50 days of incubation in soil. The ^{14}C loss was mainly attributed to ^{14}C and $^{14}CO_2$ loss into the soil. $^{14}CO_2$ accounted for 45.9-88.4 % of the total loss of ^{14}C, and loss through sclerotial respiration was estimated to be 42.2-77.2 % of the total ^{14}C loss during the same incubation time. Sclerotia incubated in the soil became dependent on nutrients for germination and lost viability when the ^{14}C loss reached 20 and 40% respectively. It was also found that radish (*Raphanus sativus* L.) seedlings that had been inoculated with sclerotia that had lost more than 20% of their ^{14}C had significantly longer shoots than seedlings inoculated with nonincubated sclerotia. Sclerotia of *S. rolfsii* were therefore able to germinate without exogenous nutrients until they had lost approximately 20% of their endogenous nutrients, and germination terminated after the

loss of approximately 40%. Pathogenic aggressiveness began to decline as nutrient independence began to decline (Hyakumachi and Lockwood, 1989).

DISEASE CONTROL

As stated earlier, most research during the recent 16 years has been directed at controlling the pathogen. Research done on biological, cultural and chemical control of the fungus prior to 1985 was extensively discussed by Punja (1985). Control efforts with regards to these three approaches as well as genetic control in groundnut since 1985, are discussed below.

Biological control

Control with *Trichoderma* spp: Many researchers have investigated the biological control of soilborne pathogens, including *S. rolfsii,* by using *Trichoderma* species (Henis *et al.,* 1983; Elad *et al.,* 1984; Latunde-Dada, 1993; Benhamou and Chet, 1996). *Trichoderma harzianum* Rifai and *T. koningii* Oudemans are the two species most commonly used. *Trichoderma koningii* was found to significantly reduce the symptoms of *S. rolfsii* damping off, blight and wilting of tomato plants (Latunde-Dada, 1993). Sclerotial counts in the soil were also reduced significantly by the antagonist and *Trichoderma* protected plants were more vigorous than unprotected plants (Latunde-Dada, 1993).

Certain factors have also been found to influence the susceptibility to attack of the sclerotia by *T. harzianum* (Henis and Papvizas, 1983). Exposure of sclerotia to relative humidity and heat triggered their germination and also increased their susceptibility to degradation by *T. harzianum* (Henis and Papvizas, 1983). Treatment

of sclerotia with metham-sodium before inoculation with *T. harzianum* resulted in increased sporulation of the antagonist on the sclerotia and consequently an increase in susceptibility of sclerotia to degradation by *T. harzianum* (Henis and Papvizas, 1983).

The parasitism of sclerotia of *S. rolfsii* by *T. harzianum* was investigated by Benhamou and Chet (1996). Their observations suggested that diffusible compounds were responsible for the observed disturbances in the cytoplasmic medullar region of the host sclerotia. Their study also indicated that production of cell wall-degrading enzymes by *T. harzianum* probably was not the first event involved in sclerotial decay as was suggested previously by Elad *et al.* (1984). The role of lectins of *S. rolfsii* in the parasitism of the pathogen by *Trichoderma* species has been studied (Barak *et al.*, 1985; Inbar and Chet, 1994). Inbar and Chet (1994) observed that hyphae of *T. harzianum* coiled tightly around nylon fibres coated with the newly isolated lectin. This was not observed in uncoated fibres. They suggested that the recognition and attachment of *T. harzianum* to the *S. rolfsii* cell surface was mediated by a complex agglutinating polymer which surrounds the host hyphae. This recognition initiated a sequence of events which eventually lead to the destruction of the host (Inbar and Chet, 1994).

Soil treated with thiourea, a slow-release N fertilizer compound, was found to have a higher population of *T. harzianum* relative to soils treated with other such compounds (Canullo *et al.*, 1992). Such chemical amendments, combined with a *Trichoderma* sp., may thus be an effective strategy to control *S. rolfsii*.

Control with other fungi: Various aspects of the influence of *Gliocladium virens* Miller, Giddens & Foster on *S. rolfsii* have been investigated (Papavizas and Lewis, 1989;

Papavizas and Collins, 1990; Lewis and Fravel, 1996) on tomato (*Lycopersicon esculentum* Mill.) (Ristaino *et al.,* 1991). Papavizas and Collins (1990) observed a positive correlation between the percentage of colonization of sclerotia by *G. virens* in soil and reduction of infectivity and germination for certain strains of *S. rolfsii.* A rapid (within 3 days in soil) loss of sclerotial viability was observed in the case of certain *S. rolfsii* strains, even at low concentrations of the antagonist. Lewis and Fravel (1996) found that *G. virens* was more effective than three *Trichoderma* species, including *T. harzianum*, in controlling *S. rolfsii* on snap bean (*Phaseolus vulgaris* L.).

Talaromyces flavus (Klöcker) A.C. Stolk & R.A. Samson, has also been suggested as a biocontrol agent against *S. rolfsii* (Madi *et al.,* 1997). A benomyl-resistant strain of *T. flavus*, which overproduced extracellular cell wall degrading enzymes, was found to exhibit enhanced antagonistic activity against *S. rolfsii* compared to the other strains (Madi *et al.,* 1997). Once again, the application of such an organism in combination with a fungicide such as benomyl, may effectively control the pathogen in the soil.

Control with bacterial species: Attempts have also been made to control *S. rolfsii* with bacterial species, particularly *Bacillus* species. *Bacillus cereus* Frankland and Frankland, *B. subtilis* (Ehrenberg) Cohn and *B. pumilis* Meyer and Gottheil all significantly decreased *S. rolfsii* incidence on bean (*Phaseolus vulgaris* L.) seedlings (Pleban *et al.,* 1995). A study of the pathogen in soil of bean plants has also shown that the bacterium *Serratia marcescens* Bizio was effective in significantly reducing disease by *S. rolfsii* (Ordentlich *et al.,* 1988). Since the sterile soil treatment and the raw soil treatment in this study produced equally good disease control, it was concluded that the

interaction between *S. marcescens* and *S. rolfsii* in soil is direct and does not involve other soil microbes (Ordentlich *et al.*, 1988).

Genetic control

Genetic control of SSR is an inexpensive and extremely effective means of disease control for the producer. Even though disease resistant cultivars are very expensive to develop, the control of disease by this means is environmentally friendly and inexpensive. Genetic control by means of resistant cultivars should, however, be incorporated into an integrated disease management program and should not be used as the only means of control.

Genetic resistance against SSR has been identified in certain groundnut cultivars and research in the USA has shown that the valentia market types are significantly more susceptible to SSR than the Spanish, runner or Virginia types (Branch and Csinos, 1987). As a group, the Spanish varieties were found to be the least susceptible of the four types (Branch and Csinos, 1987). Cultivars and breeding lines with spreading canopies such as NC Ac 17941A x Florigiant, have, however, been identified to exhibit partial resistance to SSR (Beute *et al.* 1986). In this study two cultivars with upright canopies (NC 2 and NC Ac 18016) were also identified with partial resistance to SSR. Resistant plants had fewer disease loci and lower plant mortality than susceptible genotypes (Beute *et al.*, 1986).

Besler *et al.* (1997) identified a Spanish-type (Tamspan 90) and a runner type (Tamrun 96) groundnut cultivar with resistance to the disease under naturally infested field conditions under irrigation. Grichar and Smith (1992) also identified a Spanish-type, Tx855138, with significantly more resistance than the other Spanish-types used in their

trial.

Smith *et al.* (1989) identified the cultivar TxAG-3, a runner type, as having resistance to SSR. This resistance was accompanied by stable yields under diseased or disease-free conditions, whereas the susceptible varieties showed dramatically reduced yields under disease conditions. This observation was also made by other researchers (Brenneman *et al.*, 1990a; Grichar and Smith, 1992). Resistance has also been identified in certain Virginia-type groundnuts in the USA (Brenneman *et al.*, 1990a). In this study NC 9, VA 81B and Early Bunch were the most resistant Virginia-types to SSR. The Virginia-types NC 2 and NC Ac 18016 showed resistance in another field study while NC Ac 18416 showed partial resistance to infection in the greenhouse (Shew *et al.*, 1987).

The USA commercial cultivar Southern Runner was found to have a degree of resistance to SSR when compared to three other commercial cultivars from the Georgia Peanut Breeding Program (Branch and Brenneman, 1993), to other runner types from the USA (Grichar and Smith, 1992) and to Florunner (Arnold *et al.*, 1988). The advanced breeding line GA T-2741, however, had a better yield and disease resistance than Southern Runner in the study in Georgia (Branch and Brenneman, 1993).

The groundnut cultivar FL MDR 98 has been identified as a cultivar with multiple-pest resistance (Gorbet *et al.*, 1998; Shokes and Gorbet, 1999). Among other diseases and pests, this cultivar was also noted to having resistance to SSR, and having higher yield than both Southern Runner and Florunner under diseased conditions (Gorbet *et al.*, 1998). Other studies have also identified the resistance in FL MDR 98 (Besler *et al.*, 1999). In the study by Besler *et al.* (1999) eight groundnut cultivars were compared for resistance against SSR and FL MDR 98 and TX 901338-2 were the most resistant while the cultivars Okrun and Florunner were the most susceptible (Besler *et al.*, 1999).

Okrun and Florunner were also the lowest yielding cultivars while TX 901338-2 and Tamrun 96 were the highest yielding of the eight tested cultivars. The groundnut cultivar Andru 93 has also been shown to have more resistance to SSR and higher yield under disease conditions than Georgia Green and Florunner in an integrated disease management program (Fajardo *et al.*, 1998).

Cultural control

Control of pests and diseases by tillage and cultural practices are an integral part of any integrated pest management system. Such control measures are often inexpensive and effective. Because *S. rolfsii* occurs primarily in the anamorph state in nature (Punja, 1985) and reproduces and survives as sclerotia in the soil (Punja, 1988), research has been done on the control of the pathogen in groundnut fields by tillage practices (Colvin and Brecke, 1988; Colvin *et al.,* 1988; Minton *et al.,* 1990; Grichar and Smith, 1991; Minton *et al.,* 1991).

Work done on different tillage systems (Grichar and Smith, 1991) and planter types (Wehtje *et al.,* 1994) showed that neither of these factors affect the disease incidence of *S. rolfsii* on groundnut. Grichar and Smith (1991) investigated the effect of full-, minimum- and no-tillage on disease yield and grade of groundnut. Under full tillage the cover crop was shredded, the soil was turned with a moldboard plough, disced and bedded and the beds were levelled for planting. The minimal-tillage system used was as above except that ploughing was omitted. For the no-tillage system, the cover-crop was shredded to a height of 25-30 cm and all the vegetation was killed by the application of a herbicide. The amount of pod disease caused by *S. rolfsii* did not differ between tillage systems. In a study by Minton *et al.* (1990) damage caused by *S. rolfsii*

was actually found to be greater with conventional tillage than with minimum tillage. Differences were, however, observed in grade, yield and disease between the different cultivars tested in the study by Grichar and Smith (1991). The yield was significantly higher in the full-tillage plots as compared to the low- and no-tillage plots. An increased yield as a result of improved tillage has also been found in other studies (Colvin *et al.,* 1988; Minton *et al.,* 1990; Minton *et al.,* 1991). In another study by Colvin and Brecke (1988), no yield differences were observed under different tillage systems. Grades of the groundnuts in the study by Grichar and Smith (1991) were significantly reduced by the no-tillage system. The study by Colvin and Brecke (1988), however, showed no differences in quality of graded groundnuts subjected to different tillage systems, even though the tillage systems used in their study differed slightly from those used by Grichar and Smith (1991).

Sclerotial germination has been found to be lower at depths below 2.5 cm than at the soil surface and germination is zero at depths of 8 cm or more (Punja, 1985). Since the fungus survives as sclerotia in the upper soil layers, deep ploughing has been suggested as a means of cultural control in the past (Punja, 1985). The studies done comparing tillage systems often included a no-tillage treatment where no ploughing whatsoever was applied to the field (Grichar and Boswell, 1987; Colvin *et al.,* 1988; Grichar and Smith, 1991). In most cases disease was not significantly reduced by ploughing, and in some cases was increased (Minton *et al.,* 1990). Conventional ploughing therefore does not seem to be a viable means of reducing disease.

Crop rotation between groundnut and cotton has been shown to reduce the incidence of SSR in groundnut (Rodríguez-Kábana *et al.,* 1991) as opposed to a system of groundnut monoculture. Irrigated groundnut fields have also been found to have a higher SSR disease incidence than nonirrigated fields (Brenneman, 1998). The irrigated

fields, however, also had higher yields. Both fields were cultivated under groundnut monoculture for 10 years and both the irrigated and nonirrigated fields showed a steady decline of 214 lb/A and 148 lb/A, respectively (Brenneman, 1998). In another contrasting study, however, SSR incidence in a groundnut field under monoculture declined over three years (Bowen, 1998). In this same study, earlier planted groundnut and rain-fed plots had more disease than later planted and irrigated plots (Bowen, 1998).

Chemical control

By far the most research that has been done on *S. rolfsii* over the past fifteen years has been on chemical control of SSR. Most of this work has been done in the USA, and has tested the effect of numerous fungicides and insecticides on disease incidence, yield and quality of groundnuts in American research plots and farmers fields. A distinct increase in yield of groundnut has been associated with effective chemical control of SSR in combination with (Jaks *et al.*, 1998) or without (Hagan *et al.*, 1988; Bowen *et al.*, 1992; Culbreath *et al.*, 1992; Minton *et al.*, 1993; Brenneman *et al.*, 1994; Damicone and Jackson, 1994; Culbreath *et al.*, 1995) the use of advisory and calendar spray programs. Recently, however, a study has shown that the continuous use of fungicides has caused reduced fungicide sensitivity among isolates of *S. rolfsii* (Franke *et al.* 1998). This study indicated that isolates from locations with the highest exposure history to fungicides had the lowest sensitivity to tebuconazole, flutolanil and PCNB (Franke *et al.* 1998).

Effect of fungicides: Various spray regimes and combinations of fungicides have been tested and found to be successful either in the control of *S. rolfsii* or else for the increase

of yield in diseased fields, or both (Brenneman and Culbreath, 1999; Lee *et al.*, 1999; Jackson and Damicone, 1999). Brenneman *et al.* (1991) compared the activity of tebuconazole with that of PCNB against *S. rolfsii.* The mean ED_{50} value for inhibition of mycelial growth in vitro was 0.08 µg/ml for tebuconazole and 3.9 µg/ml for PCNB. The ED_{50} values for inhibition of sclerotia formation were 0.13 and 4.99 µg/ml for tebuconazole and PCNB respectively. ED_{50} values for the inhibition of sclerotial initials were 0.14 and 2.75 µg/ml for tebuconazole and PCNB respectively. In the field tebuconazole provided good control of *S. rolfsii* on groundnut when applied seven times as a foliar spray at rates of 188-250 g/ha. Correlations were also found between the number of tebuconazole applications and the incidence of SSR (Brenneman and Culbreath, 1994). Bowen *et al.* (1997) tested the effect of flutolanil and tebuconazole for the control of SSR. The fungicides were combined with chlorothalonil and the most effective control of SSR was found when tebuconazole (14 to 28 % of the recommended rate) was combined with chlorothalonil at 67 % and flutolanil at 15 % of the recommended rate. Flutolanil has also been documented to improve groundnut quality when applied together with chlorothalonil (Backman *et al.,* 1989) and to reduce SSR when applied alone (Johnson *et al.* 2001). In the study by Backman *et al.* (1989) two other fungicides namely diniconazole and terbutrazole, also improved groundnut quality when tested in combination with chlorothalonil.

An organophosphorus compound called fosthiazate was tested for activity against SSR, nematodes and *Frankliniella* spp. However, this compound only had modest activity against SSR (Minton *et al.,* 1993). Ammonium bicarbonate was shown to be ineffective in controlling SSR in the field (Brenneman *et al.,* 1990b). The fungicide fluazinam was tested against *S. rolfsii in vitro* and found to be fungitoxic, even though

no field trials have yet been conducted to test the effect of this fungicide on *S. rolfsii* in the field (Smith *et al.,* 1992). In 1986 the fungicide flutolanil WP50 was evaluated for control of SSR and *Rhizoctonia solani* J.G. Kühn on groundnut and was shown to have excellent activity against both pathogens (Taylor, 1986). This fungicide was also able to be tank- mixed with *Cercospora* leaf spot treatments for combined applications.

Other application techniques have been investigated, including injection into irrigation water (chemigation) and foliar sprays (Brenneman *et al.,* 1994) of propiconazole at rates of 0.12-0.25 kg/ha. Chemigation resulted in 26 % less disease than the control plots whereas control by foliar spray was inconsistent. Certain chemical applications were thought to predispose groundnut plants to stem rot infection. Work done by Johnson *et al.* (1994) showed, however, that neither chloroacetamide herbicides nor chlorimuron predisposed groundnut to stem rot. Grichar (1995) compared seven fungicides at various rates for control of SSR and found that MON 2400 applied at a high rate at late groundnut flowering to early pegging was most effective (Grichar, 1995). SAN 619 and flutolanil were also effective in this study. Fungicide deposition, however, did not have a clear impact on disease control and yield (Grichar, 1995) even though flutolanil was found in a previous study (Minton *et al.,* 1991) to reduce disease by *S. rolfsii.*

Fungicides in combination with insecticides: In many instances the insecticide chlorpyrifos has been applied to control SSR, either alone or in combination with PCNB (pentachloronitrobenzene) (Hagan *et al.,* 1986; Minton *et al.,* 1990; Bowen *et al.,* 1992). Results have shown that chlorpyrifos alone, PCNB alone, and a combination of the two chemicals, significantly reduced SSR (Hagan *et al.,* 1986; Minton *et al.,* 1990). A study

in 1988 by Hagan and Weeks compared the effect of PCNB 10 G (11.2 kg a.i./ha) with chlorpyrifos 15 G (2.2 kg a.i./ha), ethoprop 15 G (3.3 kg ai/ha) and fonofos 10 g (2.2 kg a.i./ha) for the control of SSR in the field. Significant reductions in SSR were recorded for chlorpyrifos for all of the three years of the trial. This was attributed to the reduction of late season damage by soil pests. Significant reductions in disease were also recorded for ethoprop and fonofos for two of the three years. Disease suppression with fonofos, chlorpyrifos and ethoprop was similar to that by PCNB, but yields were not significantly increased. Application of PCNB did, however, increase the yield in all three years (Hagan and Weeks, 1988).

In another study by Hagan and Weeks (1986) the insecticide chlorpyrifos was compared to PCNB for control of SSR. Both pesticides alone and in combination with each other significantly reduced SSR in the field. The pesticides in combination, however, gave the best disease control and yield response.

The additional benefits of chlorpyrifos as a soil insecticide treatment in combination with standard (chlorothalonil) and experimental (tebuconazole) groundnut fungicide programs were investigated by Chapin and Thomas (1993). The combination of chlorpyrifos and chlorothalonil reduced the incidence of SSR and also brought about an increase in yield. In the tebuconazole program, however, chlorpyrifos did not influence disease incidence or yield significantly. Tebuconazole alone, however, reduced the incidence of SSR relative to the standard chlorothalonil program (Chapin and Thomas, 1993).

Bowen *et al.* (1992) monitored 44 individual sites over a period of 7 years and found that yields of chemically untreated fields averaged 785 kg/ha less than the plots receiving the most complete chemical treatments. Increase in yield potential was best with a combination of PCNB and chlorpyriphos. PCNB has also been tested in

combination with other insecticides for effective control of SSR (Hagan *et al.,* 1988). Various soil insecticides were tested (chlorpyriphos, ethoprop and fonofos) alone and in combination with PCNB and all were found to decrease disease incidence significantly and all treatments increased yield, except fonofos and ethoprop alone (Hagan *et al.,* 1988). With the recommended PCNB pattern of one or two applications at 0.56 kg/ha in a wide (30 cm) band over the row, variable control of *S. rolfsii* (23-90 %), and groundnut yield increases (102-915 kg/ha) were observed in five field trials (Damicone and Jackson, 1994).

Csinos (1985) compared chlorpyrifos and two hydrolysis products for activity against *S. rolfsii* with PCNB 10G in reducing radial growth, sclerotial initials formation and germination of mature sclerotia. The hydrolysis product 3,5,6-trichloro-2-pyridinol reduced radial growth at concentrations of >1 µg/ml, sclerotium formation at 1-25 µg/ml and sclerotial initials formation and sclerotial formation at 1-10 µg/ml. At a concentration of 25 µg/ml, 3,5,6-trichloro-2-pyridinol equalled PCNB in its antifungal activity.

Effect of application band width: A study by Brown *et al.* (1996) indicated that time of application and band width had little effect on the insecticidal or fungistatic activity of chlorpyriphos. The same has been observed for certain fungicides applied to groundnut (Brenneman, 1998). Different band width applications of PCNB were investigated by Hagan *et al.* (1991), and both the narrow band width (10 cm, 5.6 kg ai/ha PCNB) and the wide band width (25 cm, 11.2 kg ai/ha PCNB) reduced the incidence of SSR in the field. In another study undertaken in 1989 (Hagan and Weeks, 1989) the narrower band width application of PCNB produced significantly better yields than the standard 25 cm band width application. When Hagan *et al.* (1991) combined PCNB with ethoprop (5.6

+ 1.7 kg a.i./ha and 11.2 + 3.3 kg a.i./ha) yield was increased and disease was decreased. Another study on band width application found that PCNB, PCNB-chlorpyrifos and experimental compounds were equally effective in controlling SSR and increasing groundnut yield when either one quarter of the recommended rates of the fungicides were applied in a 10-15 cm band over the centre of the row or when the full recommended rates were applied in a 40 cm band (Csinos, 1986).

Csinos (1989) observed that narrow band width (10 or 15 cm) applications of the foliar-applied flutolanil 50WP and diniconazole 25WP tended to give increased yields and better disease control than with wider (30 or 41 cm) band width applications, although wider applications did give good control (Csinos, 1987). In the same study it was found that narrow band applications centred over the row at half the recommended rate per hectare of PCNB 10G, PCNB-chlorpyriphos 10-2G, ethoprop-PCNB 3-10G and PCNB 10G + ethoprop 10G did not control disease significantly less than the recommended rate of the material applied in wider bands. The results of the study indicated that reduced rates of fungicides targeted to the site of initial infection could give effective disease control. The effect of diniconazole 25W alone was compared with that of PCNB and PCNB + ethoprop and found to be more effective during one of two seasons tested in reducing disease caused by *S. rolfsii* (Hagan *et al.,* 1991).

Resistance to fungicides and effect of leafspot control: Resistance to PCNB could not be detected among 112 isolates of *S. rolfsii* from commercial groundnut fields in Oklahoma, USA (Damicone and Jackson, 1994).In a study by Shim *et al.* (1998) on isolates from Texas, however, 18 isolates out of the 377 tested isolates had greater tolerance ($P<0.05$) to PCNB than the standard sensitive isolate at a ED_{50} (dosage causing a 50 % reduction in growth) value of 10 µg PCNB/ml. ED_{50} values were

normally distributed about a mean of 2.86 ± 1.45 µg/ml in the study by Damicone and Jackson (1994). It has been shown, however, that PCNB tolerant isolates of *S. rolfsii* are significantly less aggressive than PCNB sensitive ones (Shim *et al.,* 1995).

Other early reports have suggested that leaf spot control and higher soil moisture, enhanced by a lush canopy of leaves, enhance infection by *S. rolfsii* (Backman *et al.,* 1975; Shew and Beute, 1984). The same findings have been made in the case of *Sclerotinia minor* Jagger, another soilborne pathogen of groundnut (Porter, 1980). The maintenance of a lush canopy of leaves appears to create a micro climate for development ofsoilborne diseases and it has been suggested that the fungicides applied against leaf spot diseases also reduce the population of *Trichoderma* in the soil, thereby enhancing the pathogenicity of *S. rolfsii* (Backman *et al.,* 1975). Certain of the leaf spot fungicides in the triazole family have, on the other hand, been shown to give excellent control of SSR. This effect was noted by Backman and Crawford (1985) after it was observed that certain leaf spot fungicides produced higher yields without improving leaf spot control over the standard chlorothalonil program.

CONCLUSION

Sclerotium rolfsii is a serious pathogen of crop plants throughout the world and can be a devastating pathogen of groundnut under favourable climatic conditions. Certain aspects of the biology of the fungus have been studied in detail over the past 16 years. Particular attention has been paid to the infection process of the fungus, the induction of the teleomorph and sclerotial biogenesis and germination.

The population biology of the pathogen is a topic which still has scope for research since it is unknown how mycelial compatibility groups originate. Vague associations have been observed between MCGs and host plant or geographic area,

but isolates from vastly different hosts and geographical areas in the same MCG suggest that other mechanisms or factors may cause new MCGs to arise within a population. It has been shown, however, that isolates within the same MCG are genetically more similar than isolates from different MCGs. The occurrence of the same MCG on the same specie of host plant, as was observed in many cases, raises the question as to whether an isolate would remain in its original MCG if it were to infect a different host plant. It could be possible that the pathogen, being a very versatile fungus, employs a different set of genes in the infection process of different host plants. The active genes in the pathogen (determined by the host plant) at the time of isolation of the fungus, could determine the MCG into which the isolate falls. Consequently the same isolate could sort into a different MCG if it is inoculated into and re-isolated from a different host plant.

Significant research effort has been employed to control the fungus by various means. Biological control with fungi and bacteria has been investigated. Two *Trichoderma* species have been used and the biochemistry of the pathogen/antagonist interaction has been investigated. Other fungal genera that have been used for control of the pathogen are *Gliocladium* and *Talaromyces*. The bacterial species used for biological control are from the genera *Bacillus* and *Serratia*. A fair degree of success has been obtained with this approach to disease control, but biological control is usually expensive and impractical due to a lack of consistent control under a range of environmental conditions.

Chemical control has been investigated in great depth in the USA and a host of fungicides, insecticides, nematicides and combinations of these have been suggested and used for the control of the fungus. These pesticides have also been used effectively in spray programs in the USA. Different application band widths of the pesticides have

been tested and different results obtained, some of which are conflicting. Pesticides or combinations thereof were, however, identified that controlled the disease, improved quality and increased groundnut yields.

Plant breeders have developed and identified groundnut cultivars and breeding lines with varying degrees of resistance to SSR. The use of such cultivars in combination with good tillage and cultural practices could effectively control the pathogen. Research has been done on the effect of different tillage strategies of groundnut on the incidence of SSR. Here too, however, results appear to be conflicting in some cases. Some research has shown that tillage does not affect disease severity while other studies have shown the opposite.

Previous research has answered many questions, but at the same time raised new ones. It is clear that more research is required before we will understand and control *Sclerotium rolfsii.* This situation is particulary apparent in South Africa, where few efforts have been directed towards controlling this destructive but fascinating fungus.

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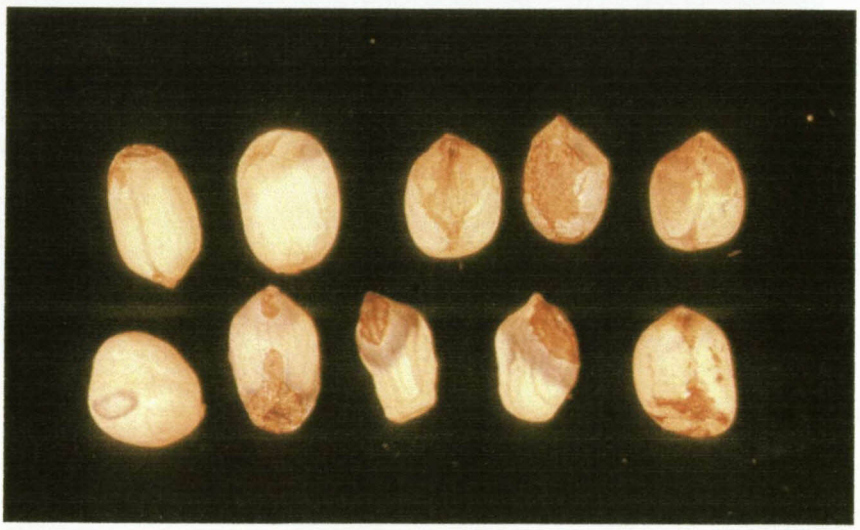
Figure 1. **A** Southern stem rot of groundnut caused by *Sclerotium rolfsii*. **B** Germinating sclerotia of *Sclerotium rolfsii* on potato dextrose agar. **C** Blue and discoloured groundnut kernels. Discolouration caused by *Sclerotium rolfsii* infection of groundnut plant.

A

B



C

CHAPTER 2

MYCELIAL COMPATIBILITY GROUPS OF *SCLEROTIUM ROLFSII* IN SOUTH AFRICA

ABSTRACT

Previous studies of the fungus *Sclerotium rolfsii* have shown that populations of the fungus can be defined by the placing of isolates into mycelial compatibility groups (MCG) based on mycelial interactions similar to those described for vegetative compatibility groups (VCG). No such study has been conducted in South Africa. A total of 121 isolates of *S. rolfsii* were collected from 15 localities on 7 different plant species, including *Arachis hypogaea* (groundnut), *Helianthus annuus* (sunflower), *Lupinus albus* (lupin), *Glycine max* (soybean), *Beta vulgaris* (beetroot), *Daucus carrota* (carrot) and *Valeriana officinalis*. Thirteen MCGs were identified, some containing isolates from the same host plant or geographic area, suggesting a possible relationship between MCG and host plant or locality. Other MCGs, however, contained isolates from a variety of hosts from various localities.

INTRODUCTION

Punja and Grogan (1983 a and b) showed that *Sclerotium rolfsii* Sacc. isolates can be placed into mycelial compatibility groups (MCGs), similar to those that occur in vegetative compatibility groups (VCGs) (Leslie, 1993), based on mycelial interactions (Glass and Kuldau, 1992) between isolates. Other studies have suggested that the role of MCGs and VCGs are important in defining field populations of fungi and facilitating

genetic exchange in fungal species where the teleomorph stage of the life cycle has a minimal impact on the disease cycle (Kohn *et al.,* 1991; Leslie, 1993). Some population studies of *S. rolfsii* have investigated genetic variation within and between MCGs. In 1995 Nalim *et al.* did the first survey of MCGs in specific fields and in individual plants in Texas. In their study isolates could be placed into 25 MCGs and the DNA amplification patterns resulting from the use of the 18-base oligonucleotide primer NK2, and from restriction digests of the internal transcribed spacer (ITS) region of the rDNA, were examined in a subset of 80 isolates representing 12 MCGs. All isolates from a single MCG gave identical patterns for each marker, and some of the MCGs shared the same ITS and NK2 patterns. It was further found that MCG having the same DNA patterns were often from the same field. Some studies have suggested that MCGs may be associated with either geographical area or host plant (Harlton *et al.,* 1995; Punja and Sun, 1997). Harlton *et al.* (1995) screened a worldwide collection of *S. rolfsii* isolates and identified 49 MCG from 119 isolates. Isolates from the same geographical area or host often grouped in the same MCG, but in some cases widely diverse isolates also grouped in the same MCG.

In a study by Punja and Sun (1997) 128 isolates from 36 host species and 23 geographical regions were paired against each other and no less than 68 MCGs were identified. No relationship between host of origin and MCG was found, except that many isolates from the same host belonged to the same MCG. Conversely, isolates in a specific MCG could have originated from many different hosts. An UPGMA analysis (a cluster analysis by the unweighted paired group method using arithmetic averages) revealed that isolates within the same MCG were genetically diverse, as were isolates from the same geographical area (Punja and Sun, 1997). Isolates from the same MCG

did, however, tend to group closer together suggesting greater genetic similarity. Isolates from widely distant geographical locations were more distantly related and isolates with identical RAPD patterns were in the same MCG and were probably clonally derived (Punja and Sun, 1997).

No such population study has yet been undertaken in South Africa. The aim of this study was to collect isolates of *S. rolfsii* from various crop plants in South Africa, to determine the MCGs of these isolates, and to establish the relationship between MCG, geographical area and host plant.

MATERIALS AND METHODS

Collection of isolates

During the 1997/1998, 1998/1999 and the 1999/2000 growth seasons isolates of *S. rolfsii* were collected from fourteen different localities in South Africa (Table 1, Fig. 1) and from seven different plants including various commercially produced crop plants. The geographical areas from which the isolates were collected were diverse, representing six of the nine provinces in South Africa. The localities varied with respect to altitude from locations at, or nearly at, sea level (George and Makathini) to localities at higher altitudes such as Potchefstroom, Bloemfontein or Bergville in the Drakensberg. Bergville being in the Drakensberg, however, has a different climate to that of Potchefstroom and Bloemfontein which are on the plateau. Isolates in the form of sclerotia or mycelium were taken from the following crop plants: *Arachis hypogaea* L. (groundnut), *Helianthus annuus* L. (sunflower), *Lupinus albus* L. (lupin), *Glycine max* (L.) Merr. (soybean), *Beta vulgaris* L. (beet) and *Daucus carrota* L. (carrot). The other plant species that yielded the fungus was *Valeriana officinalis* L., a medicinal herb found

chiefly in the temperate and cold regions of the northern hemisphere. One to four isolates were taken from each infected plant. Isolates that were collected from the same host during the same month of the season came from the same field. All isolates were identified by host plant, locality and date (month and year). In order to obtain permanent cultures, sclerotia or fungal mycelium from the infected plants were placed on 50 % malt extract agar (MEA) at room temperature (23 °C) and single hyphal tips were transferred from the germinating sclerotia or growing mycelium to potato dextrose agar (PDA) (Difco Laboratories, Detroit). Each culture was grown for 10 days until numerous sclerotia had formed. Cultures were transferred to PDA slants and stored at 5 °C until the MCGs were determined.

Mycelial compatibility groups

Single sclerotia from these stored *S. rolfsii* cultures were placed onto PDA plates for the determination of the MCG. Each isolate was paired at least once against itself as a control and six other isolates on 100 x 15 mm PDA plates. All pairings were conducted twice. The feasibility of the system was confirmed by pairing six of the isolates against each other using only two isolates per PDA dish. Isolates were placed in different MCGs based on the presence of an antagonistic zone, which indicates incompatibility, between two paired fungal isolates.

RESULTS

Collection of isolates

A total of 121 isolates from hyphal tips were collected from 15 localities throughout South Africa (Tables 1 and 2). Most of the isolates (34) originated from the Potchefstroom

area. Vaalharts yielded 13 isolates with Ottosdal and Viljoenskroon each yielding 11. Nine isolates came from Bergville and eight from Makathini. The other localities all produced between one and seven isolates. Isolates were obtained from the host plants as indicated, with most isolates (85) coming from groundnut. Sunflower yielded 11 isolates and nine were recovered from soybean. Eight isolates were from lupin and two from valerian and carrot, with a single isolate from beet.

Mycelial compatibility groups

Isolates paired with each other either showed clear barrier zones or the lack thereof (Fig. 2). Mycelia of isolates in the same MCG intermingled forming a white ridge indicating a compatible reaction. Mycelia of isolates from different MCGs formed a clear antagonistic zone in the area of mycelial contact, indicating an incompatible reaction (Fig. 1). Isolates from the same MCG appeared to form sclerotia in distinct patterns and grow at approximately the same speed. All isolates could be assigned to one of 13 MCGs which were then numbered from A through M. All duplicate pairings produced the same results. Reference isolates are kept at Pretoria (Plant Protection Research Institute) and are preserved under reference numbers PPRI 7018 to PPRI 7030.

DISCUSSION

There exists a substantial degree of genetic variation within the South African population of *S. rolfsii,* as reflected by the mycelial compatibility groups identified from a variety of plants at different localities. Punja and Grogan (1983 a and b) observed obvious morphological differences between isolates of *S. rolfsii* from different geographical areas and hosts, but noted that such differences could be influenced by media and

environment and could therefore not be used as a dependable criterion for grouping. Instead, isolates were grouped based on whether or not the hyphae were compatible, visually observed by the presence of an antagonistic or barrage zone between two incompatible isolates and the lack thereof in compatible isolates. In the case of incompatible reactions, hyphae in the zone of contact were lysed. Their results showed that isolates that appeared morphologically similar often grouped together (Punja and Grogan, 1983 a).

It has since been reported that several compatibility groups can occur in a specific geographic area or on the same host, or conversely, distribution may be limited to a specific group for a specific area (Punja and Grogan, 1983a; Stenlid, 1985; Brayford, 1990; Leslie, 1993; Punja and Sun, 1997). The South African MCGs do not appear to be strictly linked to a specific host or geographical area, but in some cases isolates grouped according to host or locality. The occurrence of a certain MCG (for example MCG I) on a single crop (soybean) and from a single locality (Bergville) tends to suggest that MCGs may be linked to host plant or locality. On the other hand, other MCGs such as MCG B and MCG C showed a wide host range and distribution. It is striking, however, how isolates from certain host plants fell into specific MCGs in some cases. Examples are the isolates from beet and valerian (MCG J), and the isolates from soybean (MCG I) which fell into an MCG of their own. No other isolates from the other crop plants could be placed into these groups. Harlton *et al.* (1995) identified 49 MCGs, 33 of which were represented by single isolates, from a worldwide collection of 119 isolates. Isolates from the same MCG in their study appeared to group together according to geographical area and host, although in some cases widely diverse isolates also grouped in the same MCG. Punja and Sun (1997), who identified 68 MCGs from

a collection of 128 isolates taken from 36 host species and 23 geographical regions, made the same observation. The manner in which new MCGs arise is not yet known (Harlton *et al.*, 1995). It has been suggested that a single VCG could predominate in an area if a fungal isolate infected a new host plant or colonised a new area and then spread vegetatively (Carlile, 1986). Consequently, it was suggested that, barring mutation, a field under monoculture year after year could eventually contain a single MCG. If genetic diversity occurs over time through mutation or other mechanisms of variation, new MCGs could emerge as adapted individuals which are distinguished from nonclonal individuals (Todd and Rayner, 1980).

The isolation of the same MCG from widely different geographical areas or hosts could be attributed to spread by agricultural practices (Harlton *et al.*, 1995), in particular via soil or seed. The results of this study could support this suggestion since there was generally a greater variety of MCGs collected from areas with the most agricultural activity, such as Potchefstroom and Viljoenskroon. MCGs represented by single isolates could have resulted from either geographic isolation (as is possible in the case of MCG I and MCG A) or recent colonization, as suggested by Harlton *et al.* (1995).

It could also be possible that the MCG of an isolate is influenced by the host plant or survival strategy (either parasitic or saprophytic), therefore a particular isolate may not always be compatible with itself. An isolate growing saprophytically may, by virtue of the genes expressed in its survival strategy, fall into a specific MCG while the same isolate growing parasitically may fall into another MCG. This could also explain why Harlton *et al.* (1995) found widely diverse isolates from their worldwide collection grouping in the same MCG and why there were no clear-cut groupings according to host plant or geographical area in their study or in this study. For example, the latter authors

collected two isolates, which differed with regards to MCG and ITS pattern, from groundnut and bean 10 m apart in Indonesia. It may depend on whether the isolate was a saprophyte or a pathogen at the time of isolation. It has been suggested that sexual reproduction may play a role in tropical climates. This could possibly be true since aerial infections of crops such as corn and cowpea have been reported and may have resulted from basidiospore inoculum (Punja, 1988).

Another mechanism whereby genetic diversity could have occurred is the phenomenon of parasexuality which has been reported in fungi such as *Fusarium* and *Aspergillus* (Kendrick, 1985). The mechanism occurs in non-sexually differentiated organs and follows the sequence of plasmogamy, karyogamy and meiosis (Ulloa and Hanlin, 2000). According to Kendrick (1985) this phenomenon probably is common among conidial fungi, but the frequency of occurrence is rare (occurring in fewer than one conidium in a million). At present the occurrence and role, if any, of parasexuality in *S. rolfsii* is unknown.

In the study by Nalim *et al.* (1995), 25 MCGs were identified from a total of 366 isolates collected over a period of nine years in Texas groundnut fields. This gives a ratio of one MCG for every 14.64 isolates tested. In the South African study presented here a total of 88 isolates were taken from groundnut fields and 10 MCGs were identified, giving a ratio of one new MCG for every 8.8 isolates tested. If the other crop plants are included in the mathematical ratio, a total of 121 isolates yielded 13 MCGs giving a ratio of one new MCG for every 9.3 isolates tested. Earlier Harlton *et al.* (1995) reported a ratio of 2.43 when a world collection of 119 *S. rolfsii* isolates were compared for MCGs, whereas Punja and Sun (1997) characterized an even more variable population, detecting a different MCG for every 1.88 isolates tested. The variability

observed in the present and previous studies most probably is related to the time the pathogen has been introduced to a certain geographical area and selection pressure exerted by different host plants, environment and activities such as agricultural practices. The variation detected in South Africa is not surprising considering the fact that the host plants comprised seven species, most of which are grown under different climatic conditions. Another factor could be the induction of the teleomorph stage of the fungus, which would give rise to genetic exchange and possible new MCGs. Even though the sexual stage is not common in nature, it may have been induced under suitable conditions.

Using MCGs as criterion, there clearly is a degree of genetic diversity within the South African population of this fungus. There appear to be vague associations between MCG and geographical area or host plant as has been found by other researchers (Harlton *et al.,* 1995; Punja and Sun, 1997). Work has been done in the past on the genetic structure of such populations. Such a study has, however, never been done in South Africa and could help to explain the structure of the population of *S. rolfsii* in this country.

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Table 1. Geographic locality from which isolates were collected, host plant of isolates, number of isolates per locality, date of collection and number of isolates from each mycelial compatibility group (MCG) of isolates from the population of *Sclerotium rolfsii* in South Africa

Locality	Host plant	Number of isolates	Date of collection	MCG
Barkly West	Groundnut	2	March 1998	C
Bergville	Soybean	9	June 1998	I
Bloemfontein	Beetroot	1	August 1999	J
Brits	Groundnut	4	March 1999	2 X H, 1 X E 1 X L
Brits	Groundnut	1	March 1998	C
Bushbuck Ridge	Groundnut	4	March 1998	D
George	Carrot	1	Unknown	H
Hoopstad	Groundnut	6	November 1998	4 X E, 1 x B 1 X D
Jacobsdal	Carrot	1	January 1999	E
Jacobsdal	Groundnut	3	February 2000	B
Krugersdorp	Valerian	2	December 1998	1 X C, 1 X J
Lichtenburg	Groundnut	7	February 1998	A
Makathini	Sunflower	5	May 1998	4 X F, 1 X G
Makathini	Groundnut	3	March 1998	F
Ottosdal	Groundnut	11	March 1999	7 X C, 2 X B 1 X H, 1 x F
Potchefstroom	Sunflower	5	February 1998	1 X D, 4 X B
Potchefstroom	Groundnut	13	February 1998	B
Potchefstroom	Lupin	8	February 1998	7 X C, 1 X B
Potchefstroom	Groundnut	6	April 1998	4 X B, 2 X E
Potchefstroom	Sunflower	1	March 2000	K
Potchefstroom	Groundnut	1	March 1998	E
Vaalharts	Groundnut	2	March 1998	B
Vaalharts	Groundnut	1	January 2000	D
Vaalharts	Groundnut	9	April 2000	8 X C, 1 X B
Vaalharts	Groundnut	1	February 2000	B
Viljoenskroon	Groundnut	5	May 2000	2 X C, 1 X D 1 X G, 1 X H
Viljoenskroon	Groundnut	2	January 2000	1 X F, 1 X E
Viljoenskroon	Groundnut	7	March 1998	4 X E, 1 X D 2 X M

Table 2. Thirteen mycelial compatibility groups (MCG) of *Sclerotium rolfsii* in South Africa showing the host plant from which isolates were collected, the geographic locality and the date of collection

MCG	Host Plant	Locality	Date
A	Groundnut	Lichtenburg	February 1998
B	Groundnut	Potchefstroom	February 1998
			April 1998
		Vaalharts	March 1998
			February 2000
			April 2000
		Jacobsdal	February 2000
		Ottosdal	March 1999
	Sunflower	Hoopstad	November 1998
	Lupin	Potchefstroom	February 1998
		Potchefstroom	February 1998
C	Groundnut	Barkly West	March 1998
		Brits	March 1998
		Ottosdal	March 1999
		Vaalharts	April 2000
		Viljoenskroon	May 2000
	Lupin	Potchefstroom	February 1998
	Valerian	Krugersdorp	December 1998
D	Groundnut	Bushbuck Ridge	March 1998
		Vaalharts	January 2000
		Hoopstad	November 1998
	Sunflower	Viljoenskroon	May 2000
		Potchefstroom	February 1998
E	Groundnut	Potchefstroom	March 1998
			April 1998
		Viljoenskroon	March 1998
			January 2000
		Brits	March 1999
		Hoopstad	November 1998
	Carrot	Jacobsdal	January 1999
F	Groundnut	Makathini	March 1998
		Viljoenskroon	January 2000
		Ottosdal	March 1999
	Sunflower	Makathini	May 1998
G	Groundnut	Viljoenskroon	May 2000
	Sunflower	Makathini	May 1998
H	Groundnut	Ottosdal	March 1999
		Brits	March 1999
		Viljoenskroon	May 2000
	Carrot	George	Unknown
I	Soybean	Bergville	June 1998
J	Beetroot	Bloemfontein	August 1999
	Valerian	Krugersdorp	December 1998
K	Sunflower	Potchefstroom	March 2000
L	Groundnut	Brits	March 1999
M	Groundnut	Viljoenskroon	May 2000

Figure 1. Map of South Africa showing the localities from which isolates were collected. 4 Barkly West, 3 Bergville, 2 Bloemfontein, 5 Brits, 15 Bushbuck Ridge, 1 George, 9 Hoopstad, 13 Jacobsdal, 6 Krugersdorp, 12 Lichtenburg, 14 Makathini, 11 Ottosdal, 7 Potchefstroom, 10 Vaalharts, 8 Viljoenskroon.

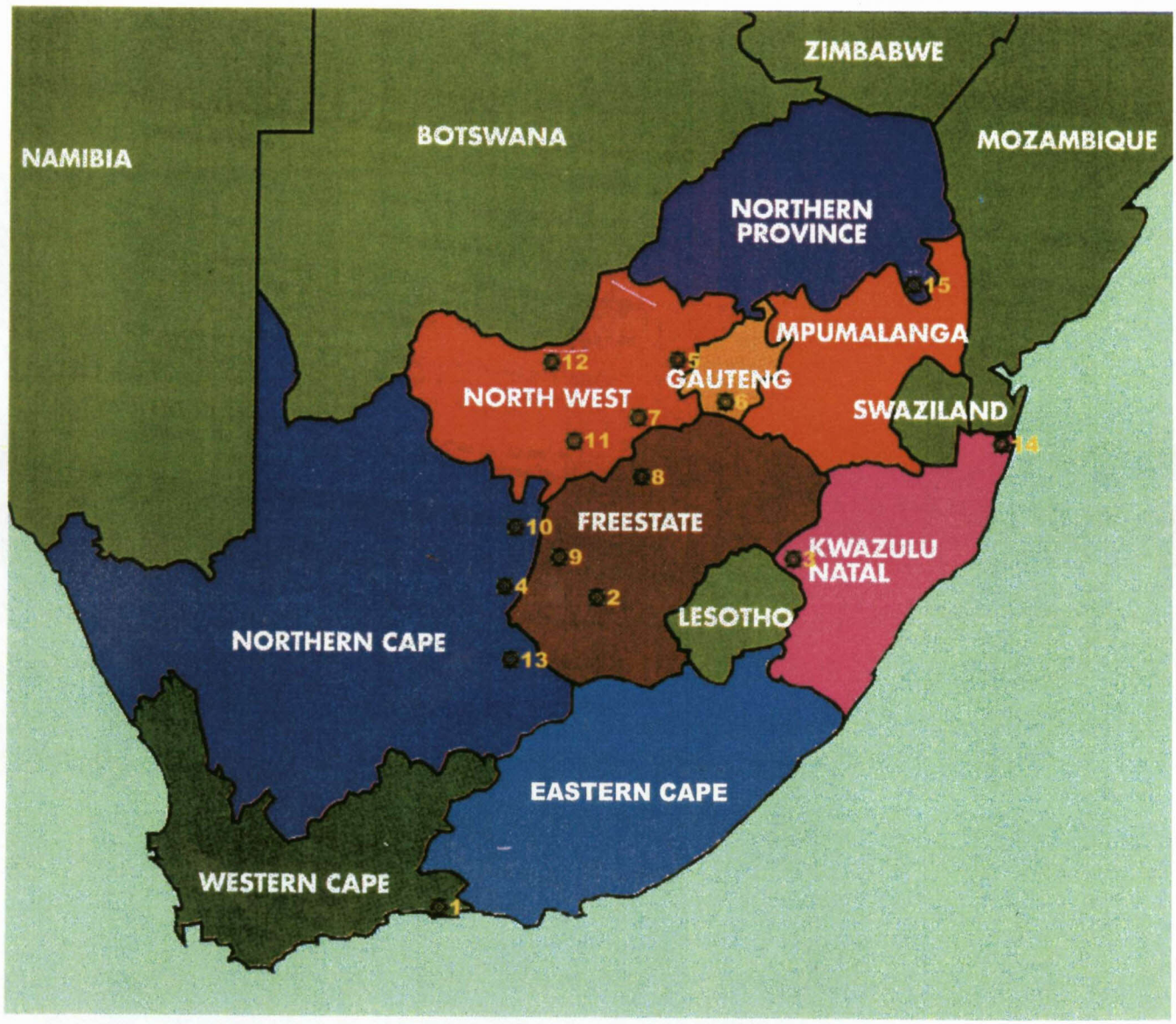
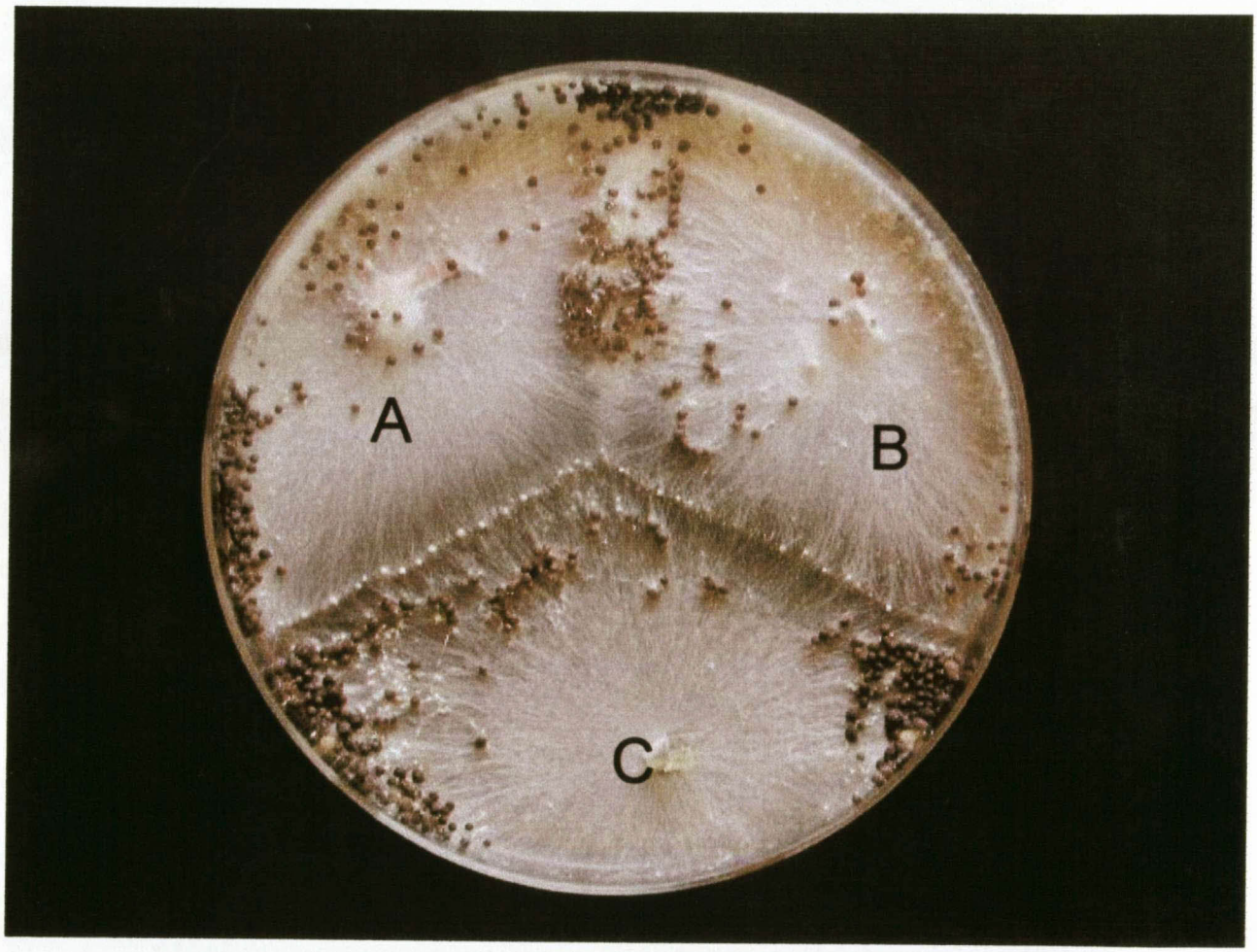

ZIMBABWE
NAMIBIA
BOTSWANA
MOZAMBIQUE
NORTHERN PROVINCE
15
MPUMALANGA
12
5
GAUTENG
NORTH WEST
6
7
SWAZILAND
11
14
8
10
FREESTATE
9
3
KWAZULU NATAL
4
2
LESOTHO
NORTHERN CAPE
13
EASTERN CAPE
WESTERN CAPE
1

Figure 2. Mycelial interactions between three isolates of *Sclerotium rolfsii.* A compatible reaction occurs between A and B (same mycelial compatibility group) and an incompatible reaction occurs between B and C and A and C (different mycelial compatibility groups).


A
B
C

CHAPTER 3

GENETIC VARIABILITY WITHIN AND AMONG MYCELIAL COMPATIBILITY GROUPS OF *SCLEROTIUM ROLFSII* IN SOUTH AFRICA

ABSTRACT

Isolates of *Sclerotium rolfsii*, the causal organism of stem rot or southern blight of groundnut, can be placed in mycelial compatibility groups (MCGs) based on hyphal interactions between isolates. The aim of this study was to determine whether AFLP analysis was a suitable technique to assess genetic variability between isolates and MCGs of *S. rolfsii*. For preliminary genetic analysis, 10 isolates were selected from each of two MCGs and compared with each other using the restriction enzymes *Eco*R1 and *Mse*1 and 4 primer pairs. Polymorphisims ranged from 10 to 36 per primer combination, with an average of 22.5. AFLP analysis clearly showed genotypic differences (22 %) among MCGs B and C with a maximum variation of 6.41 % between any two isolates per group using four primer pairs. Certain isolates could not be distinguished from each other. A more in depth study of 10 isolates from MCG B, using eight additional primer pairs, showed small genetic differences (a maximum of 4.2 % and a minimum of 0.2 %) between isolates. These results suggested that DNA could be pooled for comparison of MCGs. Pooled DNA from isolates within groups using 20 primer pairs confirmed differences between nine MCGs. This technique effectively differentiated MCGs of *S. rolfsii* from each other and also detected differences between isolates within a single MCG. Isolates within an MCG do not, however, appear to be clonally derived and it is suggested that sexual and asexual reproduction may be taking place simultaneously.

INTRODUCTION

Sclerotium rolfsii Sacc. causes disease in over 500 plant species throughout the world, including stem rot of groundnuts (Aycock, 1966; Punja, 1985). Stem rot is considered a serious disease of groundnut in South Africa, where it occurs commonly in the Vaalharts and Viljoenskroon production areas. This disease can be devastating in other areas where groundnut is grown. Yield losses amounting to $66 million have been estimated in Georgia, USA (University of Georgia Co-operative Extension Service estimates, 1988-1992) and losses of 5 to 10 % of the total crop in Alabama have been attributed to this disease (Sturgeon, 1986). The sexual state, *Athelia rolfsii* (Curzi) Tu & Kimbrough, has been induced in the laboratory (Punja and Grogan, 1983) but it is not thought to occur commonly in nature.

Punja and Grogan (1983) showed that *S. rolfsii* isolates can be placed in mycelial compatibility groups (MCG) based on mycelial interactions. They identified 25 groups from 72 isolates. When isolates from the same group were paired, hyphae intermingled with little or no cell death whereas isolates from different groups formed antagonistic zones that were accompanied by plasmolytic killing of hyphal cells. Nalim *et al.* (1995) identified 25 MCGs among *S. rolfsii* isolates collected from Texas. Using molecular markers, they found that all isolates within an MCG gave identical patterns and that some MCGs shared the same patterns for the internal transcribed spacer (ITS) regions and 18-base oligonucleotide primer, NK2. Harlton *et al.* (1995) screened a worldwide collection of *S. rolfsii* and found that isolates from the same geographical area or host often grouped in the same MCG. In some cases, however, widely diverse isolates also grouped in the same MCG. Variation in ITS regions revealed 12 subspecific groupings,

some of which correlated with their MCGs (Harlton *et al.* 1995). More recently, Punja and Sun (1997) paired 128 isolates of *S. rolfsii* from 36 host species and 23 geographical regions and detected 68 MCGs. A comparison of these isolates by means of RAPD-PCR amplifications showed no clear relationship between the host of origin and MCG, except that many isolates from the same host belonged to the same MCG. Conversely, isolates in a specific MCG could have originated from many different hosts.

In a more recent study Punja and Sun (2001), using RAPD analysis, investigated the genetic relationship between 132 isolates of *S. rolfsii* collected from 1967 to 1997 from 36 different host species and 13 countries. A total of 71 MCGs were identified in this study, many of which were unique single-member groups. As was observed in other studies, these unique groups usually originated from widely separated geographical regions. Certain MCGs were also found to contain isolates from vastly different host plants and from widely different geographical areas. The RAPD analysis showed that isolates from different MCGs could be differentiated from each other using six primers. No discernable relationships between MCGs could be detected using UPGMA analysis. Isolates within an MCG were diverse but tended to cluster together. Certain members of some of the MCGs showed identical RAPD banding patterns and these isolates were considered to be clonally derived (Punja and Sun, 2001).

A study from Japan by Okabe and Matsumoto (2000) identified four MCGs from 132 isolates and four fields within a 120 m radius of each other. Isolates between MCGs and within MCGs were compared using RAPD analysis and isolates from the same MCG collected from different geographical areas were genetically identical. Slight differences were, however, observed within MCG A where seven isolates differed slightly from the other in that they lacked one band in the OPB-13 pattern. These observations prompted

the authors to suggest that minor somatic mutation could occur without affecting mycelial compatibility during clonal growth in field populations. Since two of the MCGs were collected in 1994 and 1997, Okabe and Matsumoto further suggested that MCGs are persistent over time.

A similar study was conducted in South Africa where 13 MCGs were identified in a collection of 121 isolates taken from various host plants and diverse localities (see Chapter 2). Results of this study were consistent with those of Punja and Sun (1997) and Harlton *et al.* (1995) where isolates from the same locality or host plant often grouped in the same MCG, but in other cases isolates from widely different hosts or localities grouped in the same MCG.

Molecular markers are increasingly being used to characterize populations of plant pathogens (McDonald, 1987; Michelmore and Hubert, 1987). According to Majer *et al.* (1996) molecular markers may be used to evaluate levels of genetic diversity and phylogenetic relationships within and between species, and to identify particular races and pathotypes. Several different types of markers have been developed. Isozyme markers are relatively inexpensive and easy to use but tend to reveal low levels of polymorphisms in pathogenic fungi. RFLP (restriction fragment length polymorphism) markers may be highly informative if appropriate DNA probes are available, but in several studies little variation has been revealed. RAPD (randomly amplified polymorphic DNA) markers, although widely used, show different levels of success and suffer from lack of reproducibility (Majer *et al.* 1996). The latter authors suggested that AFLP (amplified fragment length polymorphism) fingerprinting is more suitable for detecting polymorphisms among fungal isolates, e.g. variability is assessed at a large number of independent loci, these markers are "neutral" (i.e. not subject to natural selection),

variation is revealed in any part of the genome, and data are reproducible and obtained quickly.

The objective of this study was to determine whether AFLP analysis could differentiate among South African isolates and MCGs of *S. rolfsii*.

MATERIALS AND METHODS

Selection of isolates

Isolates of nine MCGs (A to I, see Chapter 2) were randomly selected to study genetic differences between and within MCGs (Table 1).

DNA isolation

Isolates selected for AFLP analysis were cultured at 25 °C in a nutrient broth liquid medium which had been enriched with 16 g/l glucose. Each isolate was cultured in 20 ml medium in a 100-ml-capacity Erlenmeyer flask. After 2 weeks the mycelial mat was removed and freeze dried. DNA was isolated using a modified version of the method described by Graham *et al.* (1994). Approximately 750 µl CTAB buffer (100 mM Tris, pH 8.0; 20 mM EDTA, pH 8.0; 1.4 mM NaCl; 2 % CTAB [hexadecyltrimethylammonium bromide]; 0.2 % ß-mercaptho-ethanol) were added to 250 µl of the freeze dried mycelium ground to a fine powder. The suspension, contained in a 1.5 ml microfuge tube, was thoroughly mixed and incubated at 65°C for 1 h. Five hundred microlitre chloroform:isoamyl alcohol (24:1) were added and the suspension mixed by gentle inversion. After centrifugation at 12,000 g for 3 min, the upper aqueous layer was transferred to a fresh tube containing 500 µl isopropanol, mixed by gentle inversion and incubated at room temperature for 20 min. It was then centrifuged at 12,000 g for 5 min

before adding 500 µl 70 % (w/v) ethanol and incubated at room temperature for 20 min. DNA was precipitated at 12,000 g for 5 min, the pellet air-dried for 1 h, and resuspended in TE buffer (10 mM Tris-Cl, pH 8.0; 1 mM EDTA, pH 8.0). The resuspended DNA was extracted with 1/10 volume 7.5 M ammonium acetate and an equal volume chloroform:isoamyl alcohol (24:1). The aqueous layer was transferred to a fresh tube containing two volumes of cold absolute ethanol. The precipitated DNA was washed three times in cold 70 % ethanol, the pellet air-dried, and resuspended in TE buffer. The DNA was treated with RNase for 2 h at 37 °C. Concentration and purity of DNA were estimated by measuring absorbances at A_{260} and A_{280}. DNA samples were diluted to a working solution of 200 ng/µl.

AFLP analysis

AFLP analysis was performed following the protocol described by Vos *et al.* (1995) and the product manual supplied by Life Technologies Inc. (Gaithersburg, MD, USA), with minor modifications. Restriction enzymes *Eco*RI and *Mse*I were used to digest 500 ng/µl of *S. rolfsii* genomic DNA for 4 h and the reaction mix, without inactivation of the restriction endonucleases, was subjected to the overnight ligation of adapters at 37 °C, followed by preamplification. The ligation mix was not diluted prior to preamplification and the preamplification DNA was diluted only 1:5 prior to selective amplification. The selective amplification was conducted using two primers. The *Eco*RI primer contained two selective nucleotides and the *Mse*I primer three selective nucleotides, corresponding to the *Eco*RI and *Mse*I linkers obtained from Life Technologies Inc. (Gaithersburg, MD, USA).

Gel electrophoresis was performed as described by Vos *et al.* (1995) but

employing a 5 % denaturing polyacrylamide gel (19:1 acrylamide:bis-acrylamide; 7 M urea; 1x TBE buffer). Electrophoresis was performed at constant 80 W power for 2 h. For DNA visualization, AFLP gels were silver-stained following the protocol described by the Silver Sequence™ DNA Sequencing System manual supplied by Promega (Madison, WI, USA).

A total of 64 primer combinations (8 x 8) are available in the Life Technologies Inc. (Gaithersburg, MD, USA) AFLP kit. To establish whether this technique has the potential to differentiate between MCGs of *S. rolfsii*, four primer pair combinations were tested on 10 isolates from each of two randomly selected MCGs. The *Eco*RI primer E-AA (randomly selected) was used in combination with the following four possible *Mse*I primers: M-CAA, M-CAC, M-CAG and M-CAT (randomly selected). An additional eight primer pairs (Table 2) were then tested on 10 isolates from one MCG to determine the variation within a single group. DNA from various isolates (Table 3) within an MCG was pooled in order to compare groups with each other. This was done based on the relatively low between-isolate variation (maximum of 4.2 % and minimum of 0.2 %) observed when variation within MCG B was tested. This low variation indicated that a better representative sample for each group could be obtained by pooling the DNA. The pooled DNA would then include all possible variation within each group giving a better representation of the genotype of each MCG. Twenty different primer pair combinations were used to determine the genetic variability between the total number of MCGs detected. Reactions with each of the twenty primer pair combinations were repeated at least twice in independent experiments. Only reproducible results were used in statistical analyses.

Data analysis

The DNA bands obtained for each isolate or group of isolates were scored based on their presence (1) or absence (0). Only reliable and repeatable bands were considered. Pairwise genetic distances were expressed as the complement of Nei and Li's *F* statistic. Cluster analysis was done by the unweighted paired group method using arithmetic averages (UPGMA). All calculations were done with the aid of the program NTSYSpc version 2.02i (Exeter Software, New York, USA). The goodness-of-fit of phenograms was determined by computing a cophenetic value matrix using the COPH module of NTSYSpc and comparing this matrix with the SAHN tree matrix using the MXCOMP module. A cophenetic correlation of $r>0.9$ is considered a good fit.

RESULTS

AFLP analysis

Polymorphisms among isolates of *S. rolfsii* were frequent and easy to score (Fig. 1). Primer combinations varied in their ability to detect polymorphisms, ranging from 10 to 36 polymorphisms per primer combination. On average a primer combination detected 22.5 polymorphisms. The total number of polymorphisms detected with the 20 primer combinations among the nine groups was 449. A total of 668 fragments were amplified, with an average of 33.4 fragments per primer combination. Fragment sizes varied between 100 and 700 base pairs.

The AFLP analysis differentiated between MCGs B and C which were initially tested (Fig. 2). Clear differences were observed with each of the four primer pairs tested. The phenogram which was drawn using the pooled data of the four primer pairs showed a 22 % dissimilarity between MCGs B and C and a maximum variation of 6.41

% between isolates within an MCG. The cophenetic correlation value of r=0.985 indicated that the UPGMA cluster analysis was statistically significant. Not all isolates could be distinguished from each other, since only four primer pairs were tested and the main objective was not to individually distinguish isolates from one another, but to determine the genetic diversity within an MCG.

The twelve primer pairs revealed smaller differences within MCG B than the differences which were observed between B and C. The phenogram showed maximum differences of 4.2 % between the isolates (Fig. 3). A cophenetic correlation value of r=0.866 was obtained. The same general grouping was found between the isolates with these 12 primer pairs as was originally observed with the 4 primer pairs tested. The degree of relationship between the two phenograms was done by computing the cophenetic correlation, which gave a value of r=0.759, and the approximate Mantel t-test giving a value of t=3.417. Most of the lack of fit between the two phenograms can be attributed to isolates 61 and 138. All 10 isolates could be distinguished from each other using 12 primer pairs. The largest observed difference between two isolates was only 4.2 % providing justification for pooling DNA to investigate the variation between MCGs.

Using 20 primer pairs on the pooled DNA revealed clear differences between MCGs (Fig. 4). Differences between MCGs ranged from 9.3 % between groups A and F to 34.1 % between group D and the rest of the groups. A cophenetic correlation value of r=0.823 was obtained.

DISCUSSION

Sclerotium rolfsii has often been classified into MCGs (Punja and Grogan 1983, Nalim *et al.* 1995, Punja and Sun 1997) and attempts have been made to understand the

genetic structure of such populations (Nalim *et al.* 1995, Okabe and Matsumoto 2000, Punja and Sun 2000). In this study, we applied AFLP fingerprinting to show genetic variation within and between nine MCGs of *S. rolfsii* detected in South Africa.

From the present study, it was clear that isolates within an MCG were genetically diverse (Fig. 3). This is consistent with work done by Punja and Sun (2001) but in contrast with the suggestion by Nalim *et al.* (1995) that isolates belonging to a specific MCG are clonal, their hypothesis being supported by similarities in morphology and host specificity. They placed *S. rolfsii* isolates in 25 MCGs and the DNA amplification patterns resulting from the use of the 18-base oligonucleotide primer NK2 and from restriction digests of the ITS region of the rDNA were examined in a subset of 80 isolates representing 12 MCGs. Four of these MCGs were represented by one isolate only. Three NK2-amplified DNA patterns in genomic DNA and four *Mbo*I restriction digest patterns of the ITS region were found. All isolates from an MCG gave identical patterns for each marker, and some MCGs shared the same ITS and NK2 patterns. Furthermore, MCGs having the same DNA patterns were often from the same field.

The similar DNA amplification patterns observed within MCGs (Nalim *et al.* 1995) may be ascribed to the failure of the technique used to detect differences. In our study, AFLP fingerprints clearly differentiated among isolates within an MCG even though groups appeared morphologically identical and often came from the same host plant. Although isolates from the same MCG (10 isolates each from groups B and C) differed from one another, they tended to cluster closer together, suggesting a smaller degree of genetic variability within a group and a larger degree of variability between groups (Fig 2). The results from our study are consistent with those of Punja and Sun (1997, 2000) in many respects. They found that MCGs clustered together genetically and that there

was no correlation between geographical location and MCG. They did, however, find isolates with identical RAPD patterns which they hypothesised could be clonally derived. Among the isolates we tested, no two isolates had exactly the same banding pattern. Our data, thus did not suggest the domination of one particular genotype on a given crop.

Harlton *et al.* (1995) screened a worldwide collection of *S. rolfsii* isolates and identified 49 MCGs from 119 isolates. Isolates from the same geographical area or host often grouped in the same MCG, but in some cases widely diverse isolates also grouped in the same MCG. Variation in the ITS regions was examined following restriction enzyme digests. Some of the isolates within an MCG showed different ITS-RFLP pattens and certain patterns were also dispersed among MCGs. According to Harlton *et al.* (1995) inter- and intraspecific variation within the ITS-regions has been reported in several fungi, and is mostly due to deletion or insertion events in the ITSI and ITSII regions. It has been found that unique individuals are not necessarily correlated to host, nor are they restricted in geographical range (Harlton *et al.,* 1995). Clonally derived isolates within an MCG appeared to share ITS restriction site similarity (Harlton *et al.,* 1995). In contrast, members within one MCG that are subject to different selection, mutation, and drift could possess the same vegetative compatibility alleles but have different ITS sequences (Harlton *et al.,* 1995).

In our study, there was no apparent clustering according to host of origin and/or geographical area of the MCGs (Table 1). A cophenetic correlation value of r=0.003 indicated no correlation between cluster analysis based on genetic data and cluster analysis based on geographic data. The same was true (r=0.194) for cluster analysis of host versus genetic data. However, MCGs containing isolates from more than one

host and geographical area (Groups B, C and D), clustered separately from groups containing isolates from only one host and/or geographical area (Fig. 4). It would therefore appear that groups with a narrow host range are genetically dissimilar to those with a wider host range. Certain of these MCGs, e.g. groups A and F, are more closely related genetically and showed a smaller degree of genetic variation compared to others (Fig. 4). The same was true for individual isolates within an MCG, e.g. 41 and 42, 47 and 135, and 45 and 60 were closely related, respectively (Fig. 3), even though these isolates all came from different plants. Punja and Sun (1997) showed that isolates within the same MCG were genetically diverse, as were isolates from the same geographical area. Isolates from the same MCG in their study did, however, tend to group closer together suggesting greater genetic similarity. They concluded that isolates from widely distant geographical locations were more remotely related and isolates with identical RAPD patterns were in the same MCG and were probably clonally derived. We also observed, as did Harlton *et al.* (1995), that isolates from one geographical area e.g. Potchefstroom, frequently belonged to several MCGs (34 isolates were grouped into four groups, B, C, D and E). An MCG could also contain isolates from widely different geographical areas, e.g. group C contains isolates from Potchefstroom, Brits and Barkley-Wes. It was observed, however, that in certain cases isolates from the same geographical area and host plant grouped together, e.g. isolates from in Lichtenburg all fell in group A.

The occurrence of the same MCG on different host species (e.g. Group B) is reflective of the wide host range and spread of the pathogen. Also, the fact that isolates from within a given geographic area, e.g. Potchefstroom (Group B) were diverse, suggests that genetic changes had occurred within subpopulations. It may be possible,

with further field studies, to link specific MCGs or groups of MCGs to pathogenicity of a particular crop plant. It may also be possible, considering the variation between isolates within groups, to find genetic markers linked to pathogenicity. Such studies will, however, require controlled pathogenicity tests and growth studies in order to determine whether virulence in the field varies for a specific crop plant.

Based on AFLP fingerprints we conclude that genetic variation exists between *S. rolfsii* isolates of a single MCG. A larger degree of variation exists between MCGs, whether DNA from various isolates is pooled (Fig.4) or not (Fig. 2). According to Milgroom (1996) asexual reproduction results in offspring that are identical to each other and their parent (or nearly so, but for mutation), the result of which is a clonal population structure. These clonal populations have distinctive features, such as widespread occurrence of identical genotypes, absence of recombinant genotypes, and correlations between independent sets of genetic markers. On the other hand, the meiotic phase associated with sexual reproduction results in independent assortment of chromosomes and recombination within chromosomes, using greater genetic diversion (Milgroom 1996). The sexual state of the fungus, *Athelia rolfsii*, is not thought to occur commonly in nature, even though it has been induced in the laboratory (Punja and Grogan, 1983). In the light of the larger variation between MCGs and the smaller variation between isolates within MCGs (Fig. 3), it is suggested that sexual and asexual reproduction may occur simultaneously. A more in depth study of the genetic composition of MCGs of *S. rolfsii* is, however, required before the hypothesis can be rejected that the pathogen is reproducing largely in a clonal manner.

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Table 1. Locality, host, number of South African isolates of *Sclerotium rolfsii* studied and designated mycelial compatibility group

Locality[a]	Crop plant[b]	Number of isolates[c]	MCG[d]
Potchefstroom	*Arachis hypogaea*	22	B,E
Potchefstroom	*Helianthus annuus*	5	B,D
Potchefstroom	*Lupinus albus*	8	B,C
Lichtenburg	*A. hypogaea*	7	A
Viljoenskroon	*A. hypogaea*	4	E
Barkley West	*A. hypogaea*	2	C
Bushbuck Ridge	*A. hypogaea*	4	D
Brits	*A. hypogaea*	1	C
Vaalharts	*A. hypogaea*	2	B
Makathini	*A. hypogaea*	3	F
Makathini	*H. annuus*	5	F,G
George	*Daucus carota*	1	H
Bergville	*Glycine max*	9	I

[a] Locality in South Africa from which isolates were obtained.

[b] Crop plants from which isolates were obtained.

[c] Total number of isolates collected per specific host and locality.

[d] Mycelial compatibility grouping.

Table 2. Primer pairs tested on pooled DNA from nine different mycelial compatibility groups (MCGs) of *Sclerotium rolfsii*

	M[a]-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTC	M-CTT
E[b]-AA	x	x	x	x	x	x	x	x
E-AC	x	x	x	x				
E-TG					x	x	x	x
E-TT					x	x	x	x

[a] *Mse*I primers with three selective nucleotides used in AFLP analysis.

[b] *Eco*RI primers with two selective nucleotides used in AFLP analysis.

Table 3. Number of isolates of *Sclerotium rolfsii* used for pooled DNA analysis from nine different MCGs

MCG[a]	No. of isolates[b]	Isolate No.[c]
A	5	64,66,67,68,69
B	5	41,48,60,61,135
C	5	10,19,21,24,121
D	5	54,123,124,125,126
E	4	89,106,109,142
F	5	87,117,137,141,144
G	1	81
H	1	37
I	5	70,80,115,130,132

[a] Nine mycelial compatibility groups (MCGs) of *S. rolfsii* in South Africa.

[b] Number of isolates used per MCG in the AFLP analysis.

[c] Isolate numbers used in the AFLP analysis.

Figure 1. AFLP fingerprints of nine mycelial compatibility groups of *Sclerotium rolfsii* using the E-AA, M-CAG primer pair combination. Lane M represents the 100 bp molecular marker. MCG groups A-I are designated as in Table 1.

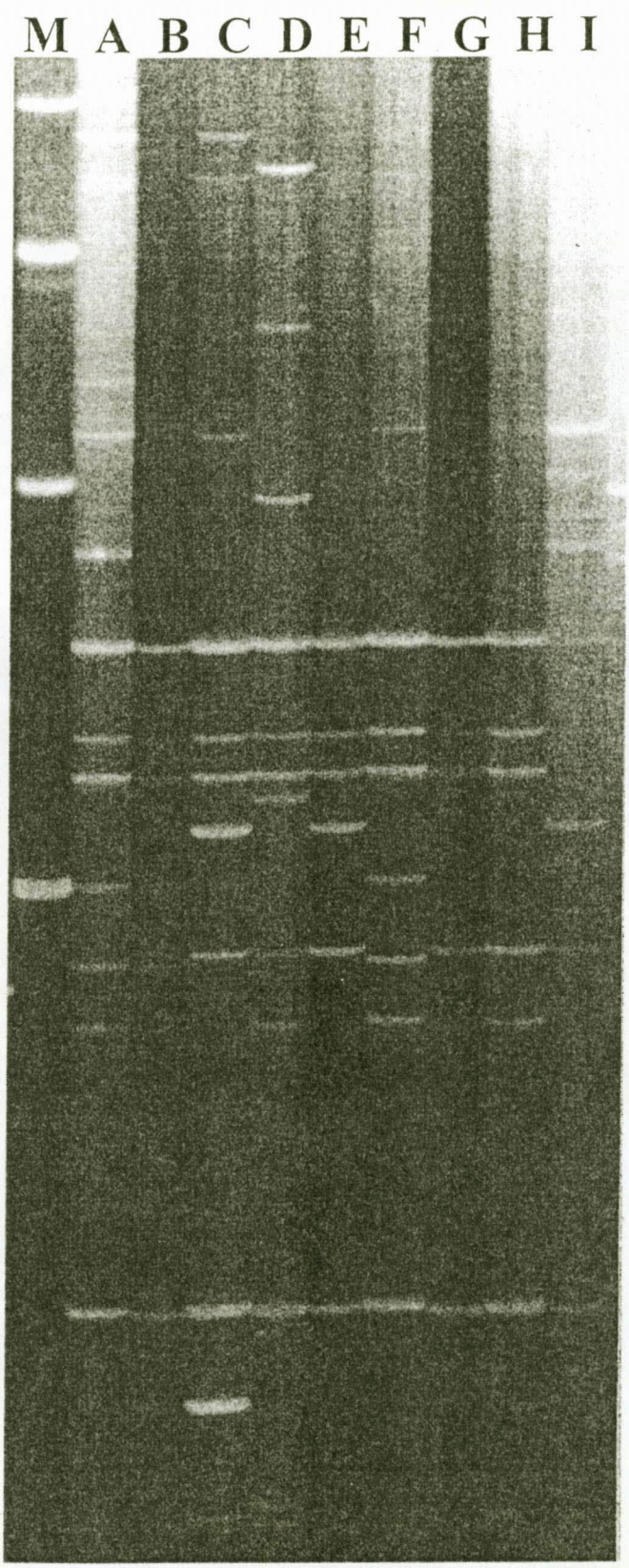

M A B C D E F G H I

Figure 2. Phenogram showing the relationship among 10 isolates each of *Sclerotium rolfsii* mycelial compatibility groups (MCGs) B and C. Data were generated by scoring AFLP fingerprints following application of four primer pair combinations. Bands were scored as present (1) or absent (0) and a similarity matrix was derived with the SIMGEND program (NTSYSpc version 2.02i) using Nei's distance. A phenogram was reproduced by the unweighted pair group method for arithmetic average (UPGMA) in the SAHN program.

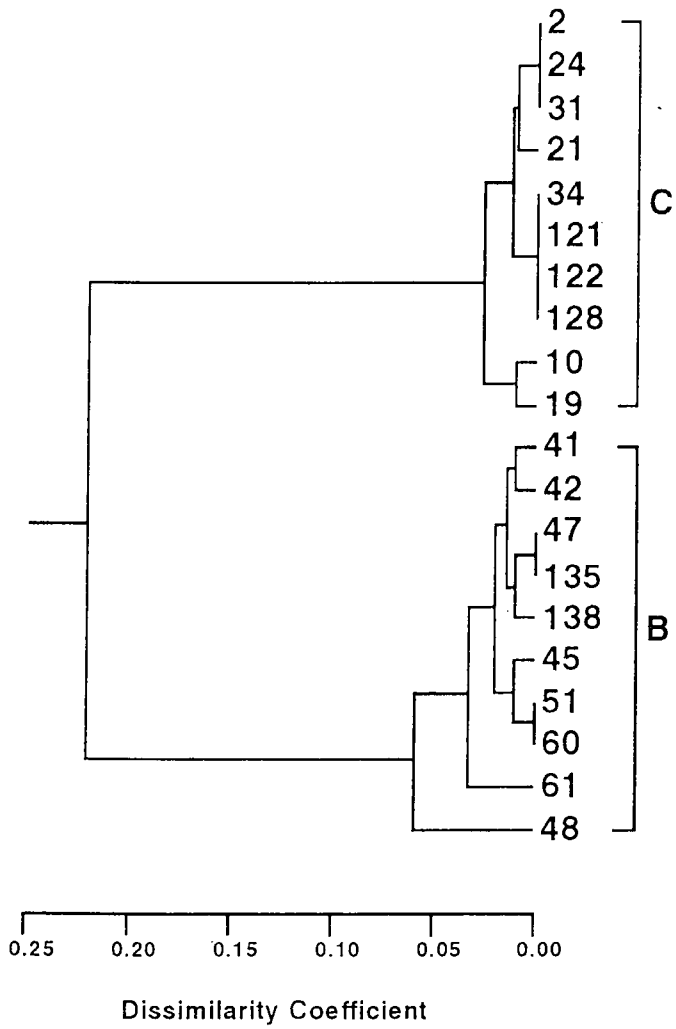

2
24
31
21
34
121
122
128
10
19
C
41
42
47
135
138
45
51
60
61
48
B
0.25
0.20
0.15
0.10
0.05
0.00
Dissimilarity Coefficient

Figure 3. Phenogram of 10 isololates of *Sclerotium rolfsii* mycelial compatibility group B. Data were generated by scoring AFLP fingerprints following application of 12 primer pair combinations and analyzed as described in Fig.2.

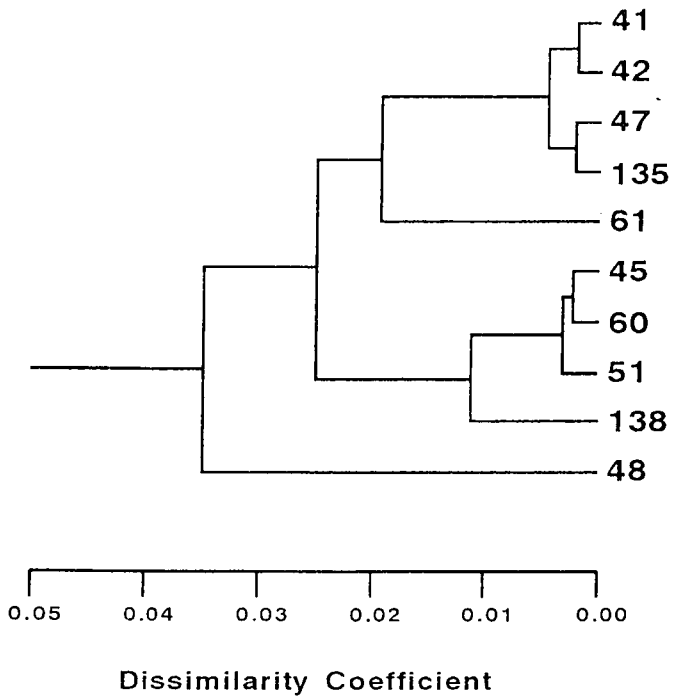
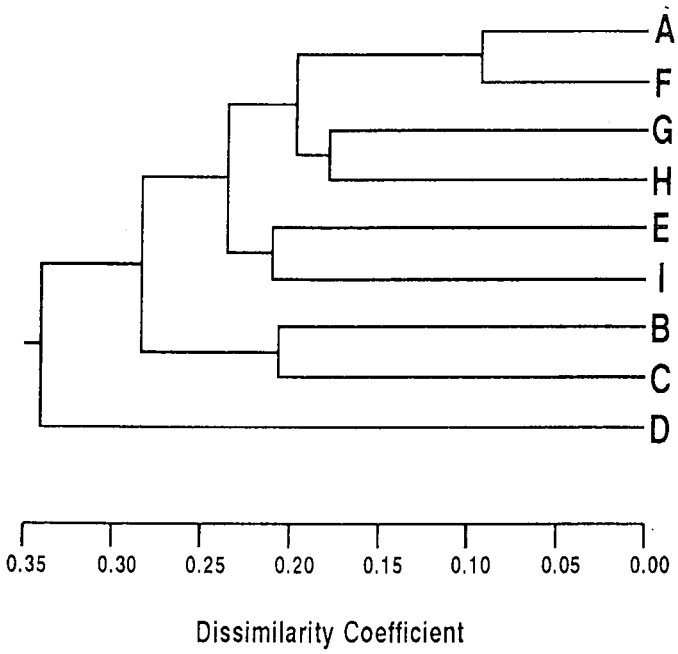

41
42
47
135
61
45
60
51
138
48
0.05
0.04
0.03
0.02
0.01
0.00
Dissimilarity Coefficient

Figure 4. Phenogram of nine mycelial compatibility groups of *Sclerotium rolfsii*. Data were generated by scoring AFLP fingerprints following application of 20 primer pair combinations and analyzed as described in Fig.2.


A
F
G
H
E
I
B
C
D
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Dissimilarity Coefficient

CHAPTER 4

MYCELIAL COMPATIBILITY GROUPS IN *SCLEROTIUM ROLFSII* IN RELATION TO PATHOGENICITY OF SELECTED CROP PLANTS

ABSTRACT

Sclerotium rolfsii causes disease of many crop plants throughout the world, including dry bean, groundnut, soy bean and sunflower. The population structure of this fungus has been defined in previous studies by placing isolates into mycelial compatibility groups (MCGs), similar to the vegetative compatibility groups described for other fungi. Various studies of populations of *S. rolfsii* have suggested a possible link between geographical locality and MCG. No studies have yet been conducted to determine a possible relationship between MCGs and pathogenicity to specific crop plants, or differences in pathogenicity of different isolates within a specific MCG. The aim of this study was to determine differences in pathogenicity between MCGs to four different crop plants in the field and also between isolates within MCGs, if indeed such differences exist. The four crop plants used were sunflower, soy bean, groundnut and dry bean. No differences in pathogenicity were found to exist between 10 isolates from two tested MCGs inoculated separately on groundnut. Inoculum comprising nine identified MCGs together, however, showed significant differences in pathogenicity to groundnut between the groups, with group E being the most pathogenic. Groundnut was the most diseased of the four crops inoculated, and MC group E was isolated more frequently than the other MCGs. Re-isolated cultures from trials involving the inoculation of specific isolates from specific MCGs were found to no longer be compatible with the originally inoculated

isolates. These results prompted the hypothesis that MCGs may not be fixed entities and may be subject to change, depending on environmental factors, growth strategy and host plant. In a trial conducted to test this hypothesis, however, no switching of MCGs was observed under controlled conditions.

INTRODUCTION

Sclerotium rolfsii Sacc. has been reported as a pathogen of numerous plants worldwide (Aycock, 1966). The population structure of this fungus has been researched by Punja and Grogan (1983) who showed that *S. rolfsii* isolates can be placed into mycelial compatibility groups (MCGs) based on mycelial interactions between isolates. The population of *S. rolfsii* in South Africa has since been studied and found to constitute several mycelial compatibility groups (see Chapter 2). Isolates within these groups were collected from numerous crop plants from diverse geographical localities throughout South Africa. Certain MCGs appeared to be specific to host plant or geographic location in South Africa, whereas others were isolated from several different crops and localities. These observations are consistent with those of other researchers (Harlton *et al.,* 1995; Punja and Sun, 1997).

Even though it has been suggested that certain MCGs may be specific to geographical localities, no work has yet been done to prove that certain MCGs may be specific to host plants. Differences in aggressiveness between isolates within a specific MCG on a specific crop plant have also never been documented in South Africa or elsewhere. The aim of this study was to determine whether any one of nine selected MCGs was more pathogenic to groundnut (*Arachis hypogaea* L.), *Helianthus annuus* L. (sunflower), *Glycine max* (L.) Merr. (soy bean) or *Phaseolus vulgaris* L. (dry bean),

four crop plants from which the pathogen has been reported in this country (Crous *et al.* 2000). Differences in pathogenicity between isolates within two MCGs were also investigated.

MATERIALS AND METHODS

Of 13 MCGs of *S. rolfsii* which were identified (see Chapter 2), nine were selected for the field trials. The selected MCGs were originally isolated in the field from carrot, groundnut, lupins, soy bean and sunflower from diverse localities in South Africa. The selected MCGs were represented by between 1 and 27 isolates in the culture collection.

Inoculum preparation

Inoculum of the pathogen was prepared by multiplying sclerotia of *S. rolfsii* on potato dextrose agar (PDA) (Difco Laboratories, Detroit) plates. Single sclerotia were taken from the agar slants and placed individually on single PDA plates. From these isolates subcultures were made which were incubated under white light at 23 °C. After four weeks the sclerotia were harvested by scraping them from the surface of the dry agar. Sclerotia were placed into airtight containers and stored at 5 °C. In the case of experiment A below, five randomly chosen isolates from each of the two MCGs were allowed to produce sclerotia and were harvested and stored as described above. For experiment B below, a representative isolate was chosen from each MCG and sclerotia were multiplied for inoculation in the field. In the case of experiment C, five isolates from each MCG were chosen randomly (except the two MCGs that were represented by one isolate only) and sclerotia were produced, harvested and stored as described above. Just before inoculation, equal amounts of sclerotia of each isolate (by mass) were mixed

so that the inoculum used for each MCG contained the pooled sclerotia of five different isolates. Isolates were chosen where the sclerotia appeared to be of similar size so that equal numbers of propagules were present in each inoculum sample.

Before inoculation, 20 sclerotia from each MCG or isolate for experiments A, B and C were tested for viability by plating on PDA. Sclerotia were noted as being viable if germination and consequent mycelial growth took place within 48 h.

Field inoculations

Field trials were conducted in the 1998/1999 and the 1999/2000 growing seasons at the Vaalharts Agricultural Research farm in the Northern Cape Province of South Africa. All trials used the cultivar Sellie planted in late October to early November, which coincides with the time of planting of all commercially produced groundnuts in the country. Seed was visually inspected for any signs of disease and dusted with thiram (120 g/100 kg seed) before planting. Because the soil type was homogenous and irrigation was applied to the entire block as a whole, the experiment was arranged according to a completely randomised design. No insect control was applied and weeding was done manually for all trials. Leaf diseases were controlled during the season using difenoconazole and later carbendazim plus flusilazole. The difenoconazole was applied six weeks after planting and the carbendazim plus flusilazole was applied every three weeks after that.

Experiment A: Pathogenicity of isolates within and between MCGs to groundnut

This trial was performed during the 1999/2000 growing season. Two MCGs were selected (C and E) and sclerotia were produced for each of five isolates from each

MCG. Three grams of dried sclerotia for each isolate were produced and divided into three equal portions of 1 g each to serve as inoculum for each plot with three repetitions for each isolate.

Plots were three rows X 1.3 m each with three repetitions. Each 1.3 m row contained 16 plants and each isolate was therefore tested against 48 plants. Each inoculated row was bordered on each side by an uninoculated row (taken as a control) to minimize cross infections. The spacing between rows was 0.45 m and inoculated rows were therefore 0.9 m apart. Each row was inoculated at 100 days after planting (DAP), with 1 g dried sclerotia. A shallow furrow (1 cm deep) was made 2 cm from the centre of the row of groundnut plants. The dry sclerotia were sprinkled evenly along the length of the furrow, covered with soil and compacted, leaving the sclerotia buried 1 cm below the soil surface. After one week, the field was flood irrigated. The field was subsequently irrigated weekly. Irrigation was applied from the top of the field so that the irrigation water moved across rows. No fungicides were applied to the field. The field was inspected for diseased plants every 14 days after inoculation until harvest at approximately 160 days. Three isolates were taken from each diseased plant. These isolates were paired with each other in order to establish whether they belonged to the same MCG. At harvest all plants were lifted and the roots and pods were examined for infection. All re-isolated cultures were paired in duplicate with the originally inoculated isolate in order to verify the MCG.

Diseased plants were expressed as a proportion (group proportions) of the total number of plants in the inoculated rows. A Scheffé test was used to compare the number of diseased plants per isolate and between the MCGs using group proportions where all proportions of all isolates in all combinations were compared.

Experiment B: Pathogenicity of MCGs to groundnut

This trial was conducted during the 1998/1999 and 1999/2000 growing seasons. For inoculation, plots three rows X 5 m each were selected in the middle of the field. Three repetitions, each containing 60 plants, were inoculated with 1 g of sclerotia of each of nine MCGs 100 DAP as described above. Three uninoculated 5 m rows were taken as controls. The MCGs were inoculated together to determine whether any of the groups would dominate in the presence of the other groups. The field was flood irrigated one week after inoculation and then weekly after inoculation. No fungicide was applied to the field which was inspected for dying plants at 14 day intervals after inoculation until harvest (160 days). Diseased plants were removed and three re-isolations were made from each diseased plant. These re-isolated cultures were paired with each other in order to establish whether they all belonged to the same MCG. If this was the case, they were then paired in duplicate against the MCGs used as inoculum and the MCG of the pathogenic isolate was determined. An analysis of variance (ANOVA) was done for the proportion of diseased plants per group (5% confidence interval) as in experiment A, and a Student-Newman-Keuls multiple range test was done.

Experiment C: Pathogenicity of MCGs to four crop plants

This trial was conducted during the 1999/2000 planting season. Another field was selected at Vaalharts Agricultural Research farm that had no previous history of *S. rolfsii* infection and planted with groundnut cv Sellie, sunflower cv RHA OR 251, dry bean cv Kranskop and soy bean cv Highveld Top, each in a 10 m X 10 m block. The soil type and the irrigation was homogenous for the four blocks. All seed was inspected for signs

of disease and dusted with thiram (120 g/100 kg seed) before planting. Plots were three rows X 5 m each with three repetitions in a completely randomised design. These three repetitions for each crop plant were inoculated with 1 gram of sclerotia of each of nine MCGs 100 DAP. In the case of the groundnuts, dry beans and soy beans, each 5 m row contained approximately 60 plants. The inter-row spacing of the sunflowers was such that there were approximately 30 plants per 5 m row. Three uninoculated 5 m rows of each crop were used as controls. No fungicide was applied to the field. The field was inspected for diseased plants at 14 day intervals after inoculation until harvest (160 days), and three re-isolations were made per plant as described above. These three re-isolated cultures were paired with each other in order to establish whether they all belonged to the same MCG. At harvest all plants were lifted and the roots were examined for infection by the fungus. The re-isolated cultures were then paired in duplicate against all nine MCGs used as inoculum and the MCG of the pathogen was determined.

MCG stability

To test whether isolates within an MCG are stable and remain in a specific MCG irrespective of growth strategy or host plant, six isolates from three different MCGs were selected and inoculated onto groundnut plants (cv Akwa) grown in sterile soil in 10 ℓ pots in a glasshouse. The plants were inoculated at 60 DAP by sprinkling 25 sclerotia around the stem of each plant to simulate natural infection. A mulch of sterile (autoclaved three times for 40 minutes) groundnut shells was used to cover the surface of each inoculated pot. Eight mulched control pots were left uninoculated.

The isolates chosen for the trial were originally recovered from groundnut (isolate

numbers 64 and 69 from MCG A and isolates 125 and 126 from MCG D) and soybean (isolate numbers 70 and 77 from MCG I). Twelve plants were inoculated with each of the chosen isolates. The pots were arranged in a completely randomised block in the glasshouse and the mean temperature was 20 °C at night and 26 °C during the day. All plants were lifted 135 DAP and re-isolations were made from diseased plant material. The isolates were then paired against the mother cultures which had been preserved at 5 °C to determine whether the isolates had switched MCG during the infection process.

RESULTS

Inoculum preparation

All selected isolates vigorously produced sclerotia on the PDA plates. Sclerotia were successfully harvested and stored. The sclerotia were 100 % viable within 48 h of plating on PDA.

Experiment A: Pathogenicity of isolates within and between MCGs to groundnut

Infection in the field was satisfactory and increased throughout the growing season for isolates from both MCGs (Fig. 1 A) with the first infected plants being observed 28 days after inoculation. A total of 49 diseased plants successfully yielded *S. rolfsii* upon re-isolation (Table 1) whereas no uninoculated control plants showed any symptoms of disease. This showed that irrigation did not shift sclerotia within the field. Of the diseased plants, 28 isolates were from MCG E inoculated rows and 21 from MCG C inoculated rows. Isolate 142 (group E) was the most pathogenic isolate and killed 10 plants, and group E isolates appeared to cause more disease than group C isolates did.

Compared group proportions of diseased plants, however, showed no differences between pathogenicity of MCGs. The computed t-value for differences between group proportions was 1.056, where a value of 1.96 was required for 5% significance. After comparing all proportions of all isolates in all combinations using a Scheffé test, it was found that none of the combinations exceeded the 5% Scheffé critical value 2.317, indicating that there was no significant difference between pathogenicity of isolates.

The re-isolations from the diseased plants yielded unexpected results. Many of the re-isolated cultures no longer matched the originally inoculated isolates (Table 1). All of the diseased plants yielded re-isolated cultures from one or more MCGs other than the one originally inoculated into the soil. Isolate 24 from group C, for example, yielded six diseased plants and re-isolated cultures belonged to four different MCGs, including the inoculated group C.

Experiment B: Pathogenicity of MCGs to groundnut

Infection of the crop plants in the field was satisfactory, with disease occurring in both growing seasons. Disease increased after inoculation (Fig. 1 B) with the first diseased plants being observed 28 days after inoculation. From this field trial, 57 diseased plants successfully yielded *S. rolfsii* over the two seasons. No control plants showed any signs of disease. Only one plant yielded more than one MCG and seven of the nine MCGs were recovered from the diseased plants. Amongst the recovered isolates certain MCGs dominated (Table 2). Almost three times more group E isolates were obtained from the diseased plants than from any other group during this season, indicating that group E was significantly more aggressive than any of the other groups. The second most readily recovered group, namely group A, yielded 12 isolates and was also

significantly more aggressive than the remaining seven MCGs (Table 2). All the isolates from MCGs A and E were originally isolated from groundnut in the field.

Experiment C: Pathogenicity of MCGs to four crop plants

Seventeen diseased plants were identified throughout the trial and *S. rolfsii* was isolated from all 17. Most disease occurred at the end of the growing season for all four crop plants (Fig. 1 C) with the first diseased plants of groundnut and dry bean being observed 42 days after inoculation. Diseased plants of sunflower and soy bean were only observed at harvest (Fig. 1 C). No control plants in any of the four crops were diseased. The disease was most severe in the groundnut block, yielding nine diseased plants (Table 3). The most commonly isolated MCG was E, comprising five of the nine isolates. Two new MCGs were also isolated from the diseased groundnut plants. Only one sunflower plant was diseased and MCG D was recovered from this plant. This MCG was originally isolated from sunflower. Two soy bean plants were diseased and both isolates belonged to MCG B, originally isolated from groundnut, sunflower and lupins in the field. Five dry bean plants were infected by *S. rolfsii* and four different MCGs were isolated from these plants (Table 3). These MCGs were originally isolated from groundnut, sunflower and lupin in the field.

MCG stability

Of the 72 plants that were inoculated, *S. rolfsii* was successfully recovered from 32 diseased plants. Of these, most isolates (14) came from MCG A with seven each from inoculated isolates 64 and 69. The two group I isolates, namely 70 and 77, respectively yielded five and six re-isolations. The least pathogenic MCG was group D where

isolates 125 and 126 caused disease of three and four plants respectively. No control plants showed any signs of disease. All isolates that were paired against the original mother cultures were found to be compatible with these cultures. No isolates underwent MCG switching in this trial.

DISCUSSION

Although it has never been suggested in previous studies that MCGs of *S. rolfsii* may not be fixed entities, (Harlton *et al.,* 1995; Punja and Sun, 1997) the results of this study indicate the possibility of exceptions to this conclusion. The possible role of insects was not investigated in the study however. The experiment conducted under controlled environment conditions showed that no MCG switching occurred. Experiment A suggested that there are no differences in pathogenicity to groundnut within the tested isolates in groups C and E respectively and that there were also no differences in pathogenicity between the two groups themselves. Taking these results into consideration and using representative isolates for each MCG in experiment B, however, clear differences were observed in pathogenicity between the nine groups. Group E clearly dominated in this study. This group also caused more disease in experiment A, even though this difference was not significant. The main difference between the two experiments was that in experiment A sclerotia of the tested isolates and groups were inoculated alone (not in combination with any other MCG) on the row whereas in experiment B all nine MCGs were inoculated together. Results from this trial suggest that group E may have successfully competed with the other MCGs and dominated over them, subsequently causing more disease. It could be hypothesised, therefore, that in combination with the other MCGs, group E is the most aggressive, causing the most

disease. In this case aggressiveness indicates the competitive ability of an isolate (Martens, 1985). Shaner *et al.* (1992) stated, however, that the term is not always used in this context by plant pathologists and that it could also mean the "disease-causing capacity" of an isolate in nature. In a situation where there is no competition between MCGs (as in experiment A), no difference in pathogenicity could be observed between the tested MCGs. Another possibility is that the other groups inoculated switched mating type under field conditions and were re-isolated as group E isolates. This is, however, unlikely considering that no mating type switching was observed in the glasshouse.

The unexpected results of this study came from experiment C, where it appeared that isolates did not remain true to their original MCG classification, and were re-isolated and found to belong to another MCG. Although the risk of cross contamination or presence of natural inoculum cannot be completely dismissed. The chances for contamination in this study were considered minimal since the experimental plot had no previous history of the disease and an uninoculated row was present between each inoculated row. There were also no diseased plants in any of the control rows. Preparation of the inoculum was performed under controlled laboratory conditions and contamination during this phase of the experiment is unlikely. The most logical explanation is that certain isolates of *S. rolfsii* underwent a change between the time of inoculation in the field and the time of re-isolation that brought about a switch in the MCG of the specific isolate. Such switches in mating type are not unknown in the fungal kingdom, and have been reported in filamentous ascomycete fungi (Perkins, 1987; Harrington and McNew, 1997). In ascomycetes this mating-type switching has been attributed to ascospore size, where large ascospores were found to give rise to

self-fertile colonies and the converse was true for small ascospores (Harrington and McNew, 1997). The results of the controlled trial, however, did not support the observations in the field. It might therefore be assumed that MCGs are stable entities that do not undergo change over a short period of time or without some external influences found in the field. If indeed switching did occur in the field, it could not be reflected in the controlled environment. The mechanism causing such possible switches is unknown and only hypotheses can be made from this study.

Certain possibilities may be considered should it be argued that switching did occur in the field. One possibility is that the teleomorph state was somehow induced and that the re-isolated cultures had a different mixture of nuclei, thus giving a different compatibility reaction. Another option is that mutation gave rise to different MCGs. The fact that this fungus appears to be highly adaptable, as reflected by its wide host range (Aycock, 1966), is another possible answer. It could also be possible that the specific host plant, or the growth strategy (pathogenic or saprophytic), may play a role in determining the MCG of an isolate, by either inducing the teleomorph (which could give rise to the exchange of nuclei) or by inducing the formation of heterokaryons in the asexual state which exchanged nuclei. It may be possible that some of the original isolates from the field were growing as saprophytes and not as pathogens when they were isolated. This may also have been true for the isolations in the field experiment. The fact that seven of the group C isolates were re-isolated as group E isolates in experiment A (Table 1) suggests a tendency of group C isolates to appear as group E isolates after passing as a pathogen through a groundnut plant. This could be an evolutionary trend, eventually culminating in a single MCG, or it may simply be that isolates pathogenic to groundnut emerge as group E isolates, assuming that certain

isolates are capable of falling into two or more MCGs. The results of experiment B, where group E appears to have dominated above the other groups, could serve to support this hypothesis. This implies that, given enough time and a monoculture system, one MCG will dominate in a given groundnut field. This, however, can only be true if it is accepted that there is a specific group at the end of the "evolutionary line" with regards to groundnut and that all isolates pathogenic to groundnut tend to evolve towards this end.

It is also notable that MCG H caused no disease in experiments B and C in spite of the fact that it was just as strongly represented in the inoculum as all the other MCGs. This particular MCG was represented by a single isolate from a host plant and geographical area far removed from the other MCGs (see Chapter 2). It was the only isolate taken from carrot and from a locality at sea level. This observation once again supports the hypothesis of the influence of the host plant and also suggests that geographical separation may play a role, as has been suggested in other studies (Harlton *et al.* 1995; Punja and Sun, 1997).

This study raises the possibility that MCGs of *S. rolfsii* may be subject to change in the field. Much more detailed research under controlled conditions with a range of isolates and hosts is needed. The conclusion that can be drawn from the pathogenicity studies above is simply that certain groups appear to dominate over others after pathogenesis of specific crop plants.

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Table 1. Isolation data from experiment A. Total number of diseased plants from plots infested with *Sclerotium rolfsii* isolates from two MCGs (E and C) and the number of isolates per MCG to which the reisolated cultures belonged

MCG E Isolate number	Total diseased plants. Number of isolates per MCG		MCG C Isolate number	Total diseased plants. Number of isolates per MCG	
142	10	9xE, 1xB	24	6	2xC, 2xB, 1xE, 1x new MCG
106	7	4xE, 2xC, 1xB	121	3	3xE
89	4	3xE, 1xG	19	4	2xC, 2xE
110	4	1xE, 1xD, 2xC	10	5	4xC, 1xE
109	3	2xE, 1x new MCG	21	3	2xB, 1xC
Total	28			21	

Table 2. Mycelial compatibility groups (MCGs) in *Sclerotium rolfsii*, number of re-isolations per MCG after inoculation of all nine MCGs to groundnut and the statistical variation between number of diseased plants between MCGs

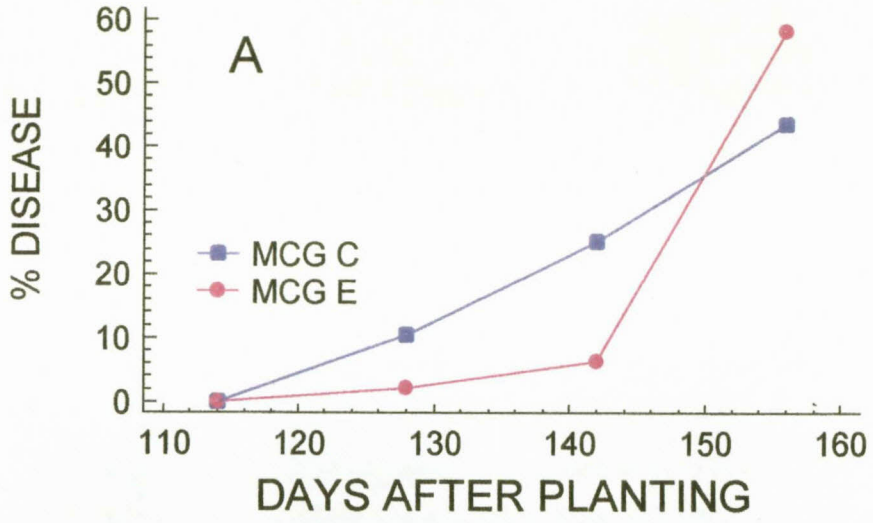
MCG	Re-isolations	Statistical variation[a]
A	12	b
B	6	c
C	0	d
D	1	cd
E	30	a
F	4	cd
G	3	cd
H	0	d
I	1	cd

[a] MCGs followed by the same letter do not differ significantly from each other using an ANOVA at 95% significance level.

Table 3. Number of diseased plants in each of four crop species, number of diseased plants, and number of isolates per MCG to which pathogenic isolates belonged

Crop plant	Number of diseased plants	Number of isolates per MCG
Dry bean	5	2xC, 1xD, 1xE, 1xF
Groundnut	9	1xA, 1xB, 5xE, 2x new MCG
Soy bean	2	2xB
Sunflower	1	1xD

Figure 1. A. Increase in number of groundnut plants killed by two different mycelial compatibility groups (C and E) of *Sclerotium rolfsii* from time of inoculation (100 days after planting) to harvest. **B.** Increase in number of diseased groundnut plants inoculated with nine different MCGs from time of inoculation (100 days after planting) until harvest. **C.** Increase in infection by *Sclerotium rolfsii* on dry bean, groundnut, soy bean and sunflower from time of inoculation (100 days after planting) until harvest.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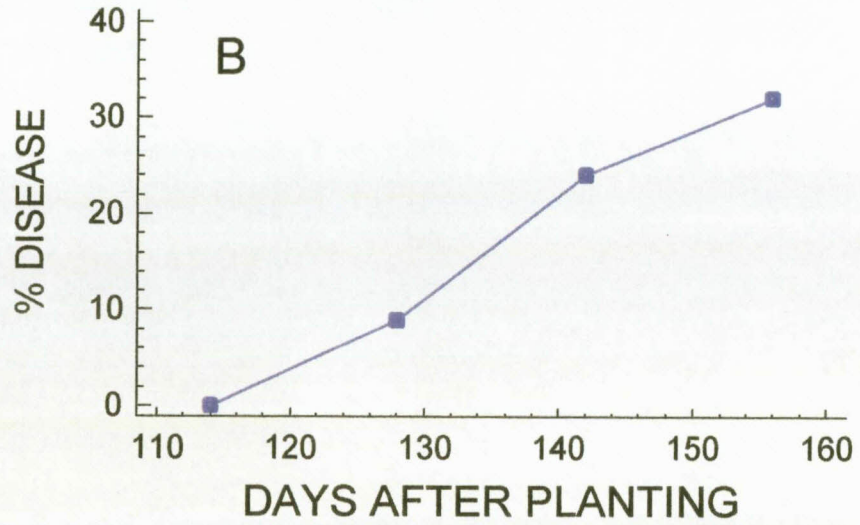
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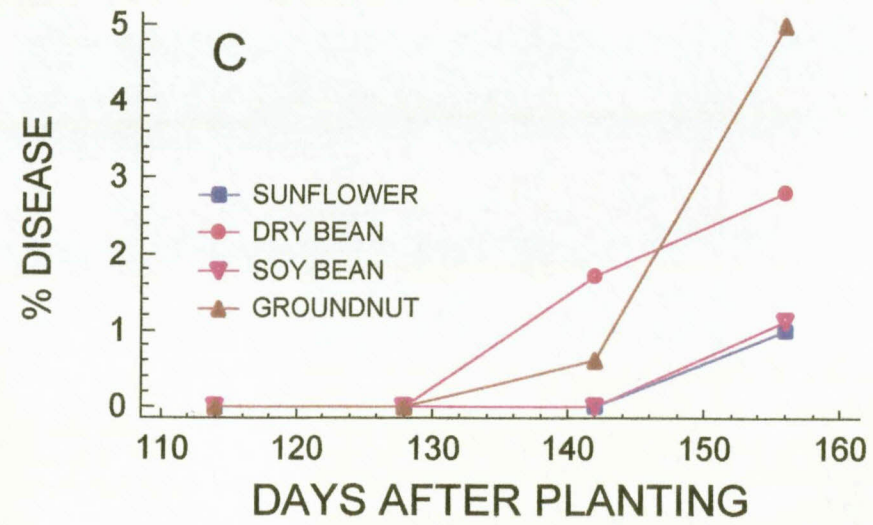
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DAYS AFTER PLANTING

CHAPTER 5

STRATEGIES FOR THE CONTROL OF *SCLEROTIUM ROLFSII* ON GROUNDNUT IN SOUTH AFRICA

ABSTRACT

Sclerotium rolfsii causes disease of numerous crop plants worldwide, including groundnuts. Control of this pathogen is difficult since it produces sclerotia which overwinter in the soil and serve as inoculum to cause disease the following season. Various chemical, biological and cultural control strategies have been suggested and implemented, some of which have reduced disease incidence in the field. No studies have yet been undertaken in South Africa to control this disease on groundnut, either chemically, biologically or by cultural practices. In this study, several strategies were investigated for the control of *S. rolfsii* on groundnuts. Difenoconazole (Score ®) was identified as a fungicide that could possibly be applied in combination with *Trichoderma harzianum*, a biological antagonist of *S. rolfsii*, rather than carbendazim plus flusilazole (Punch C ®) or chlorothalonil (Bravo ®). Difenoconazole significantly reduced the growth rate of *S. rolfsii* but not that of *T. harzianum*. The cultivation of infected fields with an inversion plough significantly reduced infection of groundnuts by *S. rolfsii* and improved the quality of the produce, although yield was not increased. Lower plant density increased the incidence of disease in an infected field, and is therefore not considered to be a viable form of cultural control.

INTRODUCTION

Sclerotium rolfsii Sacc., the causal organism of southern stem rot (SSR) or southern blight of groundnut, is widely distributed throughout the world (Aycock 1966), including South Africa (see Chapter 2). Control of the fungus is difficult since it does not produce asexual spores and overwinters as sclerotia, the primary inocula for the following season, on plant debris and in soil (Punja, 1988). Various methods of control have been investigated including genetic control (Branch and Csinos 1987; Smith *et al.*, 1989; Brenneman *et al.,* 1990; Besler *et al.,* 1997), chemical control (Hagan *et al.*, 1988; Bowen *et al.,* 1992; Culbreath *et al.,* 1992; Minton *et al.,* 1993; Brenneman *et al.,* 1994; Damicone and Jackson, 1994; Culbreath *et al.,* 1995), cultural practices (Gurkin and Jenkins, 1985; Punja *et al.,* 1986) and biological control, (Henis *et al.,* 1983; Elad *et al.,* 1984; Letunde-Dada, 1993; Benhamou and Chet, 1996) particularly with *Trichoderma* and *Gliocladium* species (Papavizas and Lewis, 1989; Papavizas and Collins, 1990; Ristaino *et al.,* 1991).

This study, to our knowledge, is the only attempt that has been made in this country to investigate possible control strategies for *S. rolfsii* by means of chemicals, biological agents and with cultural practices such as plant population density and inversion ploughing. The aim of this work was to identify a fungicide that could be applied in combination with biological control agents as well to evaluate the influence of plant density and inversion ploughing for the control of *S. rolfsii* on commercial groundnut fields in South Africa.

MATERIALS AND METHODS

Response of fungi to fungicides *in vitro*

From preliminary tests it appeared that difenoconazole was effective in suppressing the

growth of *S. rolfsii* with only a minimal effect on the growth of *T. harzianum* (P.S. van Wyk, personal communication). To verify this observation, three fungicides commonly used in South Africa for control of fungal leaf diseases on groundnut were chosen for testing against *S. rolfsii* and *T. harzianum*, each of which were represented by two randomly chosen isolates. The fungicides chosen were difenoconazole (supplied as Score®, 250 g/ℓ active ingredient), carbendazim and flusilazole (supplied as Punch C ®, 125 g/ℓ carbendazim and 250 g/ℓ flusilazole active ingredient) and chlorothalonil (supplied as Bravo®, 500 g/kg active ingredient). The dilutions registered on groundnuts in South Africa are 250 ml/100 ℓ water for difenoconazole, 350 ml/100 ℓ water for carbendazim/flusilazole, and 1.5 kg/100 ℓ water for chlorothalonil. Potato dextrose agar (PDA, Biolab by Merck, Midrand, South Africa) was autoclaved and the fungicides were added, according to the dilutions indicated in Table 1, to the warm agar just before pouring of the 90 mm petri dishes. These dilutions represent concentrations of 50%, 33%, 25% and 12% of the amount of registered active ingredient, respectively. Mycelium in the form of 5 mm agar plugs was transferred from actively growing mother cultures to the various chemically amended dishes. Unamended PDA dishes served as the controls for the four isolates. Plates were incubated at 30 °C. The daily growth rate of each isolate on the media containing the respective fungicides was determined for six days. Each treatment was replicated six times.

Results were statistically analysed by doing an ANOVA on the log (x +1) transformed data of all the growth rates from day 3 to day 6 and are presented as box plots.

Effect of commercial farming practices

The aim of these trials was to assess the effect of two control practices under

commercial farming conditions. All trials were conducted on farmers' fields and managed as any other commercial groundnut field. In 1999 two fields at different localities were selected which had severe epidemics of stem rot during the previous season on groundnut (cv Akwa).

Both fields received no irrigation and appeared to have homogenous soil type. Disease distribution during the previous season appeared to be homogenous. Both fields were prepared as follows: the stubble was disced, the field was ploughed and the seedbed was prepared by tilling the field.

Effect of inversion ploughing: The first field was used to evaluate the effect of inversion ploughing on SSR in the field. The 8 ha field was divided into twelve equal plots of 200 m x 66 m, six of which were ploughed with the inversion plough and six of which were ploughed with the conventional mould board at a depth of between 15 and 20 cm. The inversion plough consisted of double mouldboards for each row and effectively inverted the soil profile such that the top 20 cm of soil was buried and the 20 cm layer of soil below that was elevated to the soil surface (Fig. 1). On 1 December 1999 the field was planted with groundnut cv Akwa. The seeding rate was 50 kg seed (70/80 seed size) per hectare. This gave a stand of approximately 100 000 plants per hectare. Leaf diseases were controlled during the season using difenoconazole and later carbendazim plus flusilazole. The difenoconazole was applied six weeks after planting and the carbendazim plus flusilazole was applied every three weeks after that. All applications were done with a Jacto Condor spray boom with 6 hollow cone nozzles which delivered between 150 and 250 ℓ/ha. Weeds were controlled by spraying acetochlor (supplied as Harness ® and applied at 405 g/ha active ingredient) at planting at a dosage of 450 mℓ/ha. The herbicide dimethenamide (supplied as Frontier ® and

applied at 540 g/ha active ingredient) was added at 600 mℓ/ha to the difenoconazole application six weeks after planting. At planting 2:3:2 N:P:K (22) fertilizer was applied at 80 kg/ha onto the row. Disease was assessed at 120 DAP and again at harvest. Each plot was evaluated at 120 DAP by lifting two 5 m rows of groundnut plants. All plants infected with *S. rolfsii* were counted and disease was expressed as a percentage of the total number of plants lifted. At harvest (160 DAP) all plants were lifted and the above procedure was followed to determine the disease incidence. An ANOVA was done on the data of percentage infected plants to determine whether disease incidence differed significantly between treatments. The yield and grade of each plot was also determined at harvest.

Effect of plant density: Treatments consisted of three planting densities each replicated six times. Each plot was 10 m x 38 m in size. The field was planted on 7 December with cv Akwa at the various plant densities viz. 110 000 plants/ha, 78 000 plants/ha and 55 000 plants/ha. Fertilization, foliar disease control and weed control were similar to that described above. At harvest (160 DAP) each plot was sampled by lifting two random 5 m rows within each plot and the plants infected with *S. rolfsii* were counted and expressed as a percentage of the total number of plants in each row. An ANOVA was done on the percentage infected plants to determine whether disease incidence differed significantly between plant densities. The yield and grade of each plot were determined at harvest.

Effect of integrated disease management (IDM)

During the 2000/2001 season two field trials were conducted to test the combined effect of cultural, biological and chemical control of SSR of groundnut. The first trial was

conducted on a farmer's field near Viljoenskroon which had, for the previous two seasons, been under groundnut monoculture and had shown uniform infection by *S. rolfsii*. This trial was repeated, on a smaller scale, in artificially infested plots at Potchefstroom Agricultural Research farm.

IDM in a commercial field: The experiment at Viljoenskroon was arranged according to a randomised block design with two main treatments (inversion and conventional ploughing) and four sub-treatments (combined chemical and biological treatments, chemical treatment alone, biological treatment alone, and an untreated control). Subplots were 30 x 40 m in size and were replicated four times. Following the inversion and conventional ploughing, the entire field was tilled before planting Akwa, a popular commercial cultivar on 7 November 2000. Tillage was conducted in such a way that inoculum carry-over between the different ploughing treatments was minimized. Planting density was 110 000 plants/ha, as suggested by experiments conducted the previous year.

The biological treatment was applied at planting with a gravity fed dripper directly onto the planting furrow. The biological agent was a suspension of *T. harzianum* grown in the laboratory in a chemostat system using an isolate taken from a groundnut plant infected with SSR (obtained from P.S. van Wyk). The isolate was tested *in vitro* by pairing against three randomly chosen isolates on *S. rolfsii* taken from infected peanut plants. In each case parasitism of the *S. rolfsii* isolates by the *T. harzianum* isolate was observed. Parasitism occurred as described by Elad *et al.* (1984). The concentrated product (40×10^6 colony forming units/ml) was diluted with water at a ratio of 500 ml/50 ℓ and applied at 50 ℓ/ha. For the chemical treatment, difenoconazole (31 ml active ingredient per kg) was applied to the relevant plots when groundnut plants emerged 10

days after planting with a hand sprayer using a cone nozzle. The fungicide was diluted (0.31 ml/ℓ) and applied at a rate of 100 ℓ/ha. Practices similar to those used the previous season were followed for plant nutrition and the control of leaf diseases and weeds.

At harvest, disease incidence was assessed by lifting three 10 m rows in the middle of each plot, totalling 300 plants. Diseased plants were rated visually and a plant was taken as being diseased if there were signs of damage by *S. rolfsii* either to pods or stems of the plant. Disease data were converted to percentage disease per block. The same three rows of 10 m each were used to determine the pod yield and grade of the groundnuts after drying. The harvested groundnuts from the four repetitions per treatment were pooled for the grading in order to get a large enough sample.

The data were statistically analysed by doing an ANOVA (95% confidence level). LSD (least significant difference) values were determined using a Student T-test. All treatments were compared with each other and the control in order to determine whether any of the treatments had significantly decreased disease incidence or affected yield.

IDM in an artificially infested field: The trial described above was repeated in an artificially infested field but three tillage systems (mouldboard ploughing, inversion ploughing and no ploughing) were compared in randomised blocks with two main treatments (inversion and conventional ploughing) and four sub-treatments (combined chemical and biological treatments, chemical treatment alone, biological treatment alone, and an untreated control). Main-plots (tillage systems) were 10 m X 32 m and sub-plots (biological, chemical or control treatments) 5 m X 8 m (40 m^2) in size. There were two repetitions of each treatment combination. The inoculum was applied as dry sclerotia directly to the unploughed field four days prior to planting. Sclerotia of *S. rolfsii* were

produced in 2 ℓ erlenmeyer flasks on sterile groundnut shells. Each of 10 flasks were inoculated with a different isolate of *S. rolfsii* taken from diseased groundnut plants in the field. The isolates used were nos. 42, 106, 109, 137, 73, 144, 147, 187, 193 and 164. The contents of the flasks were mixed with coarse maize meal and scattered, at a rate of 31 g inoculum/maize meal mixture per m^2, evenly by hand over the field. This mixture contained approximately 200 sclerotia per 10 g. Even distribution was ensured by dividing the field and the inoculum into six equal portions and inoculating each portion separately. Following soil preparation according to the three ploughing systems mentioned, the entire field was tilled before planting with cv Akwa on 14 November 2000.

The application of biological and chemical control agents and field plot management were as described previously. Disease assessment and yield and grade determination were as above except that complete blocks of 40 m^2 were lifted and assessed. These also totalled 300 plants per block. The data were statistically analysed by doing an ANOVA (95% confidence level) and calculating LSD values. All treatments were compared with each other and the control in order to determine whether any of the treatments had significantly decreased disease incidence or affected yield.

RESULTS

Response of fungi to fungicides *in vitro*

The two *T. harzianum* isolates grew slightly faster than the *S. rolfsii* isolates on the chemically amended plates and control plates (Figs. 2 and 3). None of the fungal isolates tested showed any growth on the medium amended with carbendazim and flusilazole at any of the dilutions tested after 6 days. Both *T. harzianum* isolates grew on the plates containing difenoconazole and chlorothalonil at all the dilutions tested

(Figs. 2 and 3). The *S. rolfsii* isolates showed poor growth on the medium containing chlorothalonil, only growing at the weakest dilutions (12.5 % and 25 %). The growth of these isolates on the difenoconazole medium was virtually non-existent with only one repetition of isolate number two (S2) growing at the weakest (12.5 %) dilution. *Sclerotium rolfsii* isolate number one (S1) showed no growth on the medium containing difenoconazole at all the dilutions (Fig. 2 A-D).

On the media containing difenoconazole and chlorothalonil, the *T. harzianum* isolates showed signs of growth after three days of incubation. At most of the dilutions the *S. rolfsii* isolates only began to grow after four days of incubation. The control plates of all four isolates of both fungal species showed signs of rapid growth after only a day of incubation (Fig. 2 E). Colony size after the six day incubation period varied between the isolates and the fungal species on the different media. The isolates on the control plates all filled the petri dishes (90 mm) within six days. The fastest growing isolate was *T. harzianum* isolate number two (T2) which reached a diameter of 90 mm after five days incubation.

On chlorothalonil amended media the growth rate of only one of the *Trichoderma* isolates was significantly higher than that of the two *Sclerotium* isolates. However, the growth rate of both *Trichoderma* isolates was significantly higher on difenoconazole than that of the two *Sclerotium* isolates. The growth rate of all four fungal isolates generally appeared to be more rapid on chlorothalonil than on difenoconazole.

Effect of commercial farming practices

Effect of inversion ploughing: Diseased plants were easy to identify at harvest by the presence of sclerotia and mycelium. The percentage diseased plants was significantly higher in plots that had been ploughed with the conventional plough than those that had

been ploughed with the inversion plough, but disease incidence in the trial was low (Table 2). The grade of the plots that had been ploughed with the inversion plough showed a higher percentage of choice and diverse grade groundnuts and a lower percentage of crushing grade than the plot that had been ploughed with the conventional mould board plough. The yield from the plots that had been ploughed with the inversion plough was slightly lower than that of the plots ploughed with the conventional plough while the Rand/ton was slightly higher from the inversion ploughed plots as a result of the higher quality (2001 price was R 3600 for choice grade groundnuts).

Effect of plant density: The incidence of diseased plants differed significantly between all the plant densities, with the lowest plant density (55 000 plants/ha) having the highest percentage of diseased plants and the highest plant density (110 000 plants/ha) having the lowest percentage of diseased plants (Table 3). The best quality groundnuts were also from the plot with the highest plant density where the percentage of choice grade groundnuts was almost 20% higher than that of the other two plant densities (Table 3).

Effect of integrated disease management (IDM)

At harvest the mean disease incidence was 3.27 % at Potchefstroom and 3.75 % at Viljoenskroon. All of the treated and untreated plots had a minimum of 1.7 % diseased plants at Potchefstroom and 0.7 % diseased plants at Viljoenskroon.

IDM in a commercial field: None of the biological/chemical treatments significantly decreased disease incidence in the field or affected pod yield (Table 4) in 2001. The inversion plough did, however, significantly decrease disease incidence (Table 4), as was the case during the previous season. Similar to the previous season, the groundnut

pod yield was higher where the conventional plough was used, although the difference was not significant. Both the disease and yield data showed no significant interaction between tillage treatments (inversion plough and conventional plough) and the chemical/biological treatments.

As far as the quality of the kernels was concerned, there were no notable differences in the quality of the groundnuts between treatments (Table 4), except that for treatment A (the *Trichoderma* treatment) no choice grade kernels were identified in the grading process. As far as the crushing grade kernels were concerned, the highest percentage came from the control and the lowest percentage was from treatment C (the difenoconazole treatment). The percentage of choice grade kernels from the plots ploughed with the inversion plough was slightly lower than that of the plots where the conventional plough was used. The same was true for the Rand/ton where the conventionally ploughed plots gave higher values on average (Table 4).

IDM in an artificially infested field: Disease incidence was uniformly low, and none of the biological/chemical treatments significantly decreased disease incidence, or affected quality or yield (Table 5) when tested at a 95% confidence level. However, the highest yield was obtained from the inversion plough treatment and the lowest yield from the tilling only treatment. When tested at 94 % confidence, however, a significant difference was observed between the inversion plough treatment and the tillage only treatment. Both the disease and yield data showed no significant interaction between tillage treatments (inversion plough and conventional plough) and the chemical/biological treatments.

The results of the quality grading of the kernels from this trial showed that the highest percentage of choice grade kernels were from plots that had been treated with

the inversion plough (Table 5). The lowest percentage of choice grade kernels came from the tillage only treatment (Table 5). The highest percentage of crushing grade kernels was from the tillage only plots and the lowest percentage from the inversion ploughed plots. The same was true for the Rand/ton value which was highest for the inversion ploughed plots and lowest for the tillage only treatment (Table 5).

DISCUSSION

From previous studies and observations it was clear that certain strategies could be implemented to control *S. rolfsii* on groundnuts in South Africa and also to improve the quality of the harvested groundnut kernels. Currently various chemicals are registered for control of the disease in the USA, including tebuconazole, flutolanil and azoxystrobin. No genetic resistance exists within the currently available South African cultivars. One of the most important issues in the groundnut industry is quality. For this reason South Africa has a stringent groundnut grading system whereby groundnuts are classed as being either choice grade, diverse grade or crushing grade. Groundnut kernels are rejected on the basis of various criteria including the percentage of unhealthy, blemished and soiled kernels. The blue discolouration of the groundnut testa caused by oxalic acid as a result of kernel and pod infection by *S. rolfsii* (Bateman and Beer, 1965) causes an infected seed lot to have an inferior grading. Effective disease control, therefore, means that groundnut quality is improved.

Various factors have been found to be effective at controlling SSR. Sclerotial germination has been found to be lower at depths below 2.5 cm than at the soil surface and germination is nil at depths of 8 cm or more (Punja, 1985). Deep ploughing has thus been suggested as a means of cultural control (Punja, 1985), prompting the present investigation of inverting the upper soil layer, where the fungus survives as sclerotia,

with lower soil layers. Furthermore, the presence of a lush canopy of leaves, often caused by chemical control of leaf spot, may create a favourable microclimate and increase *S. rolfsii* disease severity as was found to be the case with *Sclerotinia minor* on groundnut (Porter, 1980). Previous reports have suggested that leaf spot control and higher soil moisture, enhanced by a lush canopy of leaves, enhance infection by *S. rolfsii* (Backman *et al.*, 1975; Shew and Beute, 1984). The maintenance of a lush canopy of leaves appears to create a micro climate for development ofsoilborne diseases and it has been suggested that the fungicides applied against leaf spot diseases also reduce the population of *Trichoderma* in the soil, thereby enhancing the pathogenicity of *S. rolfsii* (Backman *et al.,* 1975). A decreased plant density could reduce this effect and therefore reduce disease incidence. No previous studies, to our knowledge, have investigated the effect of plant density on disease incidence of SSR on groundnuts in this country or anywhere else.

The differences in disease severity in the plant density trial reported here (Table 3) can be attributed to wind erosion. The Viljoenskroon groundnut producing area in which this trial was conducted is notorious for wind erosion. The lower planting densities were more prone to become wind-blown causing windborne sand to damage the plants or to heap up around the stems of the plants and cover the runner stems, increasing the possibility of infection. It would therefore appear that, even though soilborne disease severity may also be stimulated by lush foliar growth of the canopy, decreasing the plant density is not a viable option to be considered for reducing *S. rolfsii* damage in areas that are prone to wind erosion. The quality of the groundnuts in this trial was also best at the highest plant density (Table 3). This observation is consistent with the disease severity observed at the three different plant densities.

A combination of biological and chemical control against SSR has also, to our

best knowledge, never been investigated in this country before. *Trichoderma harzianum* Rifai and *Trichoderma koningii* Oudemans are the two species that have most commonly been investigated for biological control of *S. rolfsii* (Latunde-Dada, 1993; Benhamou and Chet, 1996). Significant control of *S. rolfsii* has been reported on tomato with the application of *T. koningii* (Latunde-Dada, 1993). Tomato plants treated with the antagonist showed less disease, but were also more vigorous than untreated plants (Latunde-Dada, 1993). In a previous study where leaf spot fungicides were evaluated for their effect on soilborne pathogens of groundnut (Backman *et al.*, 1975), however, it was found that benomyl had a toxic effect to *Trichoderma* spp *in vitro*, while it did not affect *S. rolfsi*, and actually increased *S. rolfsii* infection in the field.

Canullo *et al.* (1992) used biological control along with a chemical amendment in an attempt to control *S. rolfsii*. In their study soil treated with a slow-release N fertilizer was found to have a higher population of *T. harzianum* relative to soils treated with other such compounds. In a study by Csinos *et al.* (1983) it was found that *T. harzianum* alone did not decrease disease incidence of *S. rolfsii*. When combined with PCNB at 11.2 kg/ha, however, disease was reduced. Other treatments in their study containing PCNB in combination with various insecticides and nematicides also reduced disease and increased yields. The application of a biological antagonist could, conceivably control *S. rolfsii* if combined with a fungicide which inhibits the pathogen but not the antagonist. It appeared from the *in vitro* growth study that difenoconazole could be applied in combination with *T. harzianum*, which has already been used as a biocontrol agent *of S. rolfsii*. The fungicide significantly reduced the growth rate of the pathogen while that of the biological antagonist was not significantly reduced. Commercial formulations of *T. harzianum* are available in this country (Dagutat Biolab. CC, PO Box 1551, Groenkloof, Pretoria and Trichoprotection®, Agrimm Technologies Ltd.,

Christchurch, New Zealand, distributed in South Africa by Hygrotech).

In the field experiments conducted at Viljoenskroon and Potchefstroom where the chemical and biological agents were tested in combination with different tillage practices, however, they had no effect on disease incidence or yield. The only observed effect was reduced disease in the commercial field with the use of the inversion plough, which was consistent over two seasons. The failure of the biological agent and the fungicide applied on the row to control disease may have been due to the fact that both applications were applied early in the season and the disease only appeared later. Since this was the first trial of its kind, different application times were not included in the trial planning. The *Trichoderma* was applied as an actively growing fungus while the *S. rolfsii* was probably still dormant in the field and by the time that the pathogen became active the antagonist was less able to have an effect on disease incidence. The same can be said for the fungicide application which should have been applied closer to the time of actual infection. In retrospect, therefore, these applications may have been effective if they were applied later in the season. A trial investigating different application times would provide the answer to this question.

The action of the inversion plough in our 1999 and 2000 studies effectively inverted the soil profile and buried sclerotia so deeply that disease severity was significantly decreased, even though yield was not improved in the naturally infected fields. The reason for the failure of the inversion plough to affect disease incidence in the smaller trial at Potchefstroom is not known. Disease incidence was low in all plots, making it more difficult to demonstrate differences. The smaller trial which was artificially inoculated did had the advantage above the commercial field trial of having uniform inoculum distribution which would eliminate the effect of inoculum aggregation in the field. The improved quality of the groundnuts from the fields that had been treated

with the inversion plough in 1999 can be attributed to less disease in this field. The disease data from 1999 confirms this since significantly less disease was present in the inversion ploughed portion of this field.

Previous tillage studies have shown variable effects on groundnut diseases. No differences in disease (*S. rolfsii*) severity was observed in a study by Grichar and Smith (1991) between the three different tillage systems tested. A possible explanation for this is that the plough used in their study did not bury sclerotia deeply enough, thereby not significantly reducing disease severity. In a study by Minton *et al.* (1990) damage caused by *S. rolfsii* was actually found to be greater with conventional tillage than with minimum tillage. In another study disease incidence was unaffected by tillage system (Grichar and Boswell, 1987). Work done on tillage systems (Grichar and Smith, 1991) and planter type (Wehtje *et al.*, 1994) showed that neither of these factors affect the disease incidence of *S. rolfsii* on groundnut. Increased yield and quality in groundnut has, however, been reported for full tillage systems as opposed to minimal tillage systems (Colvin *et al.*, 1988; Minton *et al.*, 1990; Grichar and Smith, 1991).

The quality data from the artificially infected field (2000) gave results that were consistent with those of the field trial of the previous year (1999). The highest percentage of choice grade groundnuts came from the plots treated with the inversion plough. The same was true for the Rand/ton values. It therefore appears that the use of an inversion plough reduces disease and improves the quality of the groundnut crop in an infected field. Grades of the groundnuts in the study by Grichar and Smith (1991) were significantly reduced by the no-tillage system, which is consistent with what was found in this study. The study by Colvin and Brecke (1988), however, showed no differences in quality of graded groundnuts subjected to different tillage systems.

The yield from the fields treated with the inversion plough were, however, not

increased. This is consistent with work done by Colvin and Brecke (1988) who also found no yield increase under different tillage systems. In their study, however, conventional ploughing was evaluated. The results were, however, in contrast with work done by Grichar and Smith (1991) who studied the effect of no-tillage, minimal-tillage and full-tillage on *S. rolfsii* disease severity, yield and grade. Under full tillage the cover crop was shredded, the soil was turned with a moldboard plough, disced and bedded and the beds were levelled for planting. The minimal-tillage system used was as above except that ploughing was omitted. For the no-tillage system, the cover-crop was shredded to a height of 25-30 cm and all the vegetation was killed by the application of a herbicide. The yield was significantly higher in the full-tillage plots as compared to the low- and no-tillage plots. An increased yield as a result of improved tillage has also been found in other studies (Colvin *et al.,* 1988; Minton *et al.,* 1990; Minton *et al.,* 1991). The artificially inoculated field trial at Potchefstroom (2000), however, did show that the highest yields were from the plots that had been ploughed with the inversion plough, even though disease and grade were unaffected.

In the commercial naturally infected field trials consistent results were not achieved with regards to crop value over the two seasons. The plots ploughed with the inversion plough in 2000 gave mean Rand/ton values that were lower than those from the plots treated with the conventional plough. The opposite was true in 1999. This could possibly be ascribed to the fact that there was a high incidence of black pod rot (*Chalara elegans*) present in the field at the time of harvest. This pod pathogen is known to reduce the quality of groundnut kernels in South Africa (Van Wyk and Cilliers, 1998).

Due to the action of the inversion plough a more powerful tractor is required to pull the plough through the soil, especially in soils with a high clay content. This will

increase fuel consumption during the preparation of a field for planting. The increased fuel cost would have to be justified by either increased quality or yield of the end product. Since neither of the commercial trials showed an increased yield due to the action of the plough, the feasibility of the inversion plough may be questioned. Additional studies with higher disease incidence would help to further define the value of this practice. Also, additional studies should be conducted to determine the effect of the inversion plough on the chemical, biological and physical properties of the soil. The 1999 trial did, however, show a higher Rand/ha value for the plots treated with the inversion plough. Also, if the plough is used only every three to five years with the use of a normal plough in between, the increased costs may be justified. It certainly appears to be possible to control *S. rolfsii* on groundnuts in South Africa and to improve kernel quality in the process. The use of an inversion plough and timely applications of fungicides or biological control agents may all be beneficial. Further studies are needed to better define their use patterns. Lower plant density is not a viable solution. An integrated disease management system combining these cultural, chemical and biological control strategies seems to be the answer to control SSR of groundnut in South Africa.

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Table 1. Dilutions of fungicide active ingredient tested against two isolates on *Sclerotium rolfsii* and two isolates of *Trichoderma harzianum*

	Concentration of active ingredient			
	50%	33%	25%	12%
Fungicide				
Chlorothalonil	3750 mg/ℓ	2500 mg/ℓ	1875 mg/ℓ	938 mg/ℓ
Difenoconazole	313 mg/ℓ	208 mg/ℓ	156 mg/ℓ	78 mg/ℓ
Carbendazim	219 mg/ℓ	146 mg/ℓ	110 mg/ℓ	55 mg/ℓ
flusilazole	438 mg/ℓ	292 mg/ℓ	219 mg/ℓ	109 mg/ℓ

Table 2. Incidence of stem rot before and at harvest, yield and grade of groundnuts from inversion and conventional plough treatments at Viljoenskroon during the 1999/2000 season

	Inversion plough	Conventional plough
Disease incidence*		
120 DAP	2.53a	4.19b
Harvest (150 DAP)	2.67a	4.61b
Grade(% of total)		
Choice	69	63
Diverse	22.4	21.1
Crushing	8.6	15.9
Value and yield		
Rand/ton[b]	4499.18	4464.64
Ton/ha	1.43	1.49

[a] Values in rows followed by the same letter do not differ significantly from each other.

[b] According 2000 groundnut price.

* Percentage of visually diseased plants.

Table 3. Incidence of stem rot before and at harvest, yield and grade of groundnuts from different plant densities at Viljoenskroon during the 1999/2000 season

	55 000 pph[a]	78 000 pph	110 000 pph
Disease incidence[b] *			
Harvest (150 DAP)	16.6 a	10.6 b	7.5 c
Grade (% of total)			
Choice	54.9	52.8	72.9
Diverse	35.7	13.2	20.1
Crushing	9.4	13.2	7.0
Value and yield			
Rand/ton[c]	2529	2072	2785
T/ha	1.23	1.82	2.01

[a] Plants per hectare.

[b] Values followed by the same letter do not differ significantly from each other.

[c] According 2000 groundnut price.

* Percentage of visually diseased plants.

Table 4. Incidence of *Sclerotium rolfsii* at harvest, kernel grade, pod yield and Rand value of groundnuts grown in different tillage and chemical and/or biological control treatments[a] in a naturally infected field trial at Viljoenskroon during 2000/2001

	Treatment				
	A	B	C	D	Mean[b]
Disease incidence [c]					
Inversion plough	2.5	3.1	2.8	3.1	2.9a
Conventional plough	4.5	4.2	5.8	4.3	4.7b
Mean[b]	3.5a	3.6a	4.3a	3.7a	
Grade (% of total)					
Choice grade					
Inversion plough	0	56.5	58.0	59.5	43.5
Conventional plough	0	59.3	60.8	62.0	45.5
Mean	0	57.9	59.4	67.1	
Diverse & standard grade					
Inversion plough	76.1	19.8	21.0	18.5	33.9
Conventional plough	75.5	20.0	21.5	17.3	33.6
Mean	75.8	19.9	21.3	17.9	
Crushing grade					
Inversion plough	24.0	23.8	21.0	22.0	22.7
Conventional plough	24.5	20.8	17.8	20.8	21
Mean	24.3	22.3	19.4	21.4	
Yield T/ha[d]					
Inversion plough	6843	7465	6538	6425	6818a
Conventional plough	7170	8323	6970	7653	7529a
Mean[b]	7007a	7894a	6754a	7039a	
Rand/ton					
Inversion plough	9097	11904	10531	10355	10472
Conventional plough	9307	14055	11702	13269	12083
Mean	9202	12979	11117	11812	

[a]**A**: *Trichoderma* treatment, **B**: *Trichoderma* and Difenoconazole (Score ®) treatment, **C**: Difenoconazole (Score ®) treatment, **D**: Control.

[b]Mean values in rows and columns followed by the same letter do not differ significantly from each other.

[c]LSD for chemical and/or biological control treatments = 1.7 and LSD for tillage treatments = 1.2.

[d]LSD for chemical and/or biological control treatments = 1403 and LSD for tillage treatments= 992.

Table 5. Incidence of *Sclerotium rolfsii* at harvest, kernel grade, pod yield and Rand value of groundnuts grown in different tillage and chemical and/or biological control treatments[a] in an artificially infected field trial at Potchefstroom during 2000/2001

	Treatment				
	A	B	C	D	Mean[b]
Disease incidence[c]					
Inversion plough	3	2.9	2.9	3.4	3.0a
Conventional plough	3.4	4.5	3.2	4.2	3.8a
Tillage only	3.2	2.5	3	3	3.0a
Mean[b]	3.2a	3.3a	3.0a	3.5a	
Grade (% of total)					
Choice grade					
Inversion plough	60.5	62.5	66.3	59.0	62.1
Conventional plough	0	63.5	67.3	64.5	48.8
Tillage only	0.0	0.0	59.3	60.5	30.0
Mean	20.2	42	64.3	61.3	47.0
Diverse & standard grade					
Inversion plough	18.3	21.0	16.8	17.8	18.5
Conventional plough	79.3	18.5	15.8	16.3	32.5
Tillage only	74.8	74.3	15.5	17.8	45.6
Mean	57.5	37.9	16.0	17.3	
Crushing grade					
Inversion plough	21.3	16.5	17.0	23.3	19.5
Conventional plough	20.5	18.0	17.0	19.3	18.7
Tillage only	25.3	25.8	25.3	21.8	24.6
Mean	22.4	20.1	19.8	21.5	
Yield T/ha[d]					
Inversion plough	6435	6515	6485	6275	6428a
Conventional plough	5545	5775	6645	6105	6018a
Tillage only	4615	5050	5615	5450	5183a
Mean[b]	5532a	5780a	6248a	5943a	
Rand/ton					
Inversion plough	912	1025	974	818	932.3
Conventional plough	684	882	1002	930	874.5
Tillage only	512	596	740	746	648.5
Mean	703	834	903	831	

[a] **A**: *Trichoderma* treatment, **B**: *Trichoderma* and Difenoconazole (Score ®) treatment, **C**: Difenoconazole (Score ®) treatment, **D**: Control.

[b] Mean values in rows and columns followed by the same letter do not differ significantly from each other.

[c] LSD for chemical and/or biological control treatments = 1.27 and LSD for tillage treatments = 1.10.

[d] LSD for chemical and/or biological control treatments = 1442 and LSD for tillage treatments= 1249.

Figure 1. Illustrated action of the double mouldboard inversion plough.

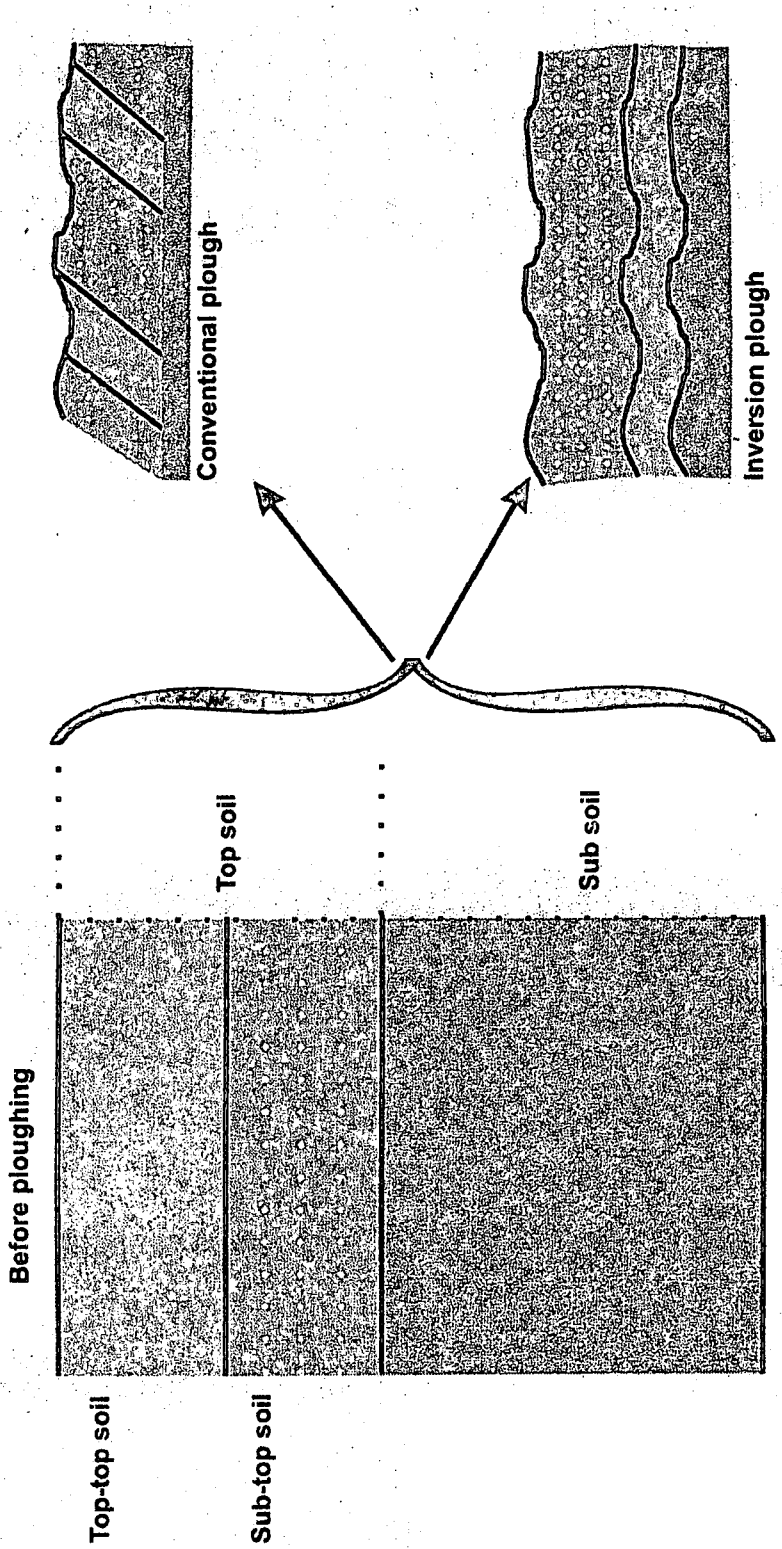
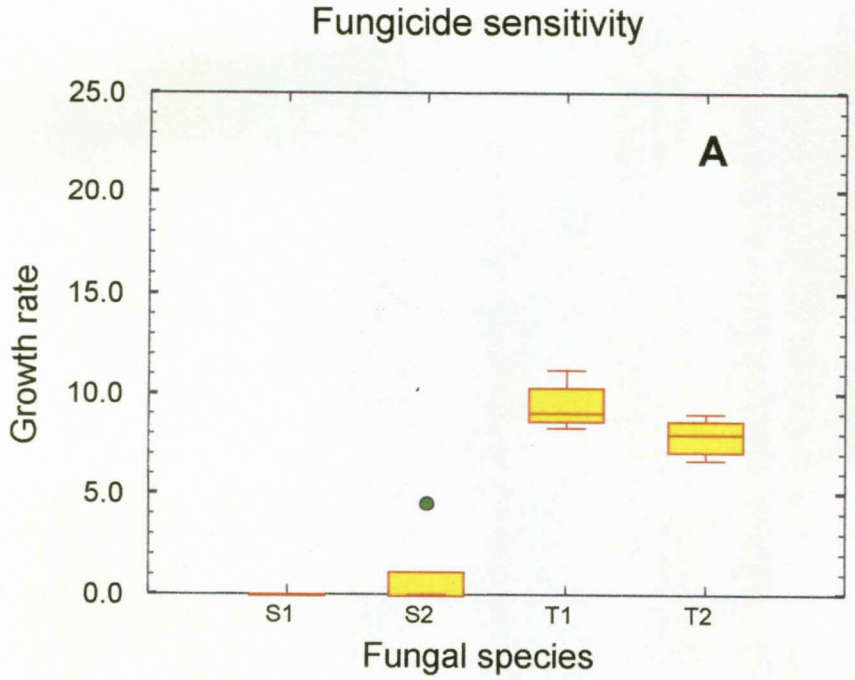
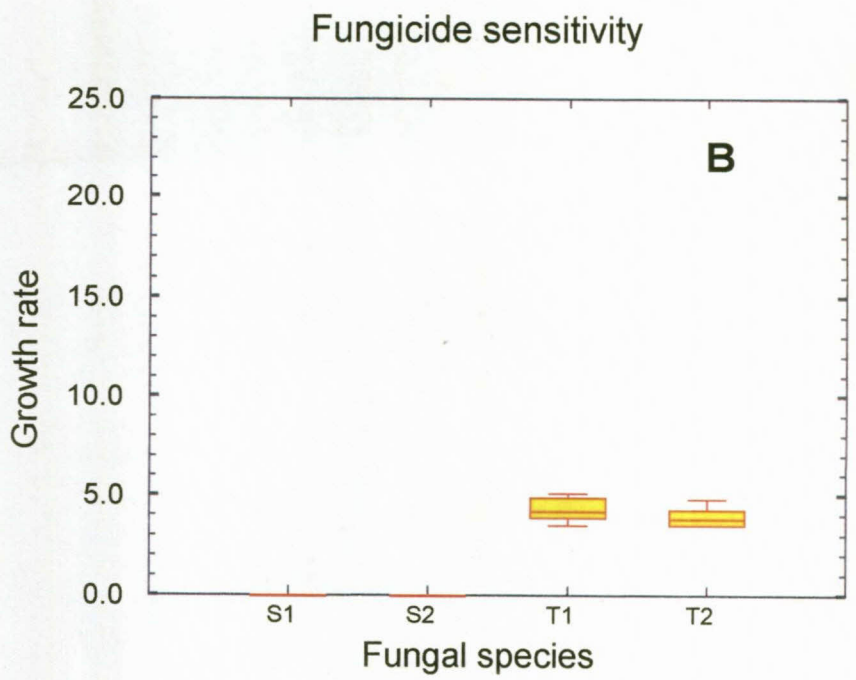
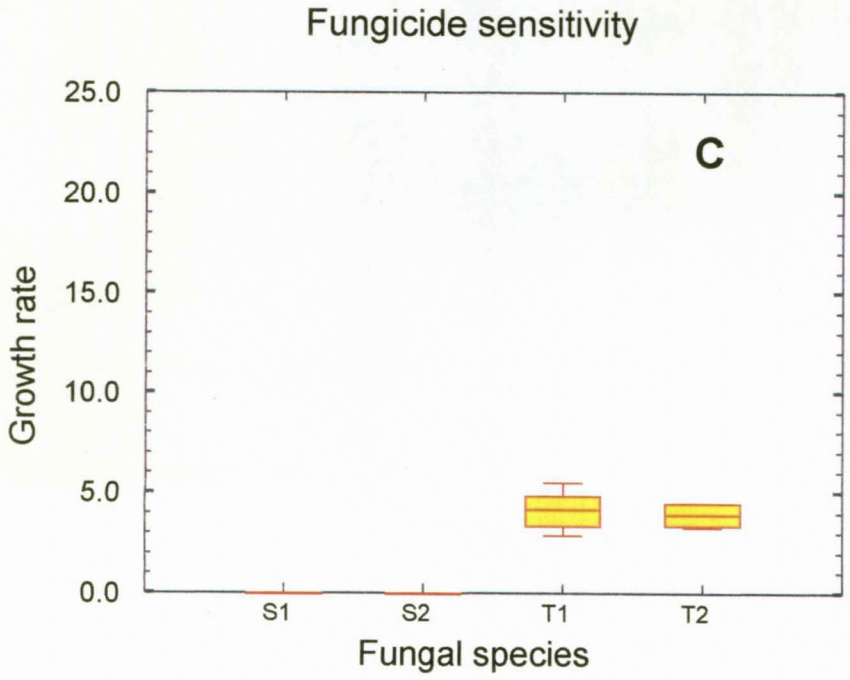
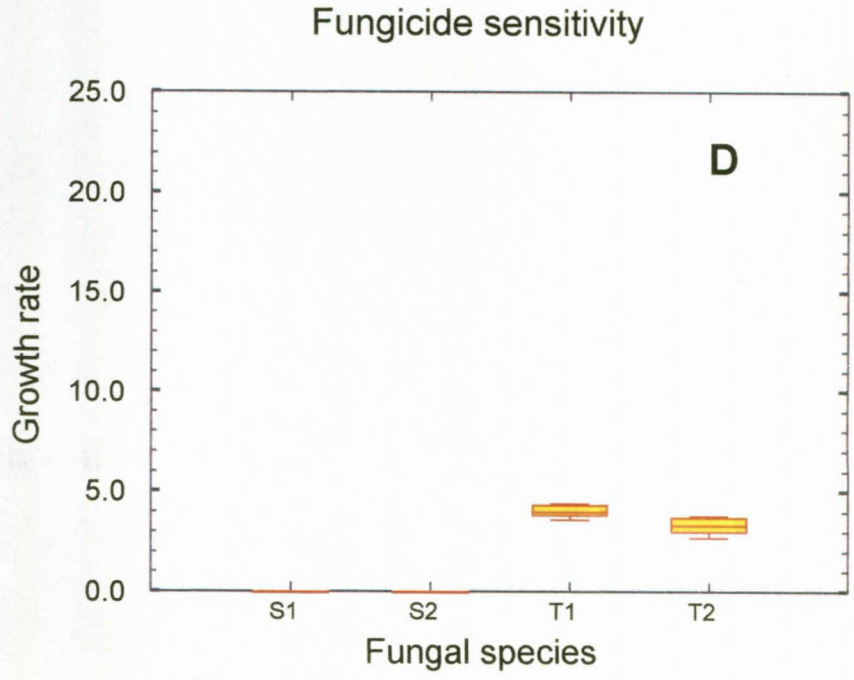

Before ploughing
Top-top soil
Sub-top soil
Top soil
Sub soil
Conventional plough
Inversion plough

Figure 2. Box plots of growth rates (in mm) of two *Trichoderma harzianum* isolates (T1 and T2) and two *Sclerotium rolfsii* isolates (S1 and S2) on PDA growth media containing four different concentrations of difenoconazole. The top and bottom of the box are the 25th and 75th percentiles. The length of the box is therefore the interquartile range (IQR) and represents the middle 50% of the data. A line is drawn through the middle of the box at the median (50 th percentile). The upper adjacent value (T-shaped line that extends from lower end of box) is the largest observation that is less than or equal to the 75th persentile plus 1.5 times IQR. The lower adjacent value (other T-shaped line) is the smallest observation that is greater than or equal to the 25 th percentile minus 1.5 times IQR. Growth rates of fungi on PDA amended with difenoconazole at **A**. 78 mg/ℓ **B**. 156 mg/ℓ **C**. 208 mg/ℓ **D**. 313 mg/ℓ and **E**. unamended.


Fungicide sensitivity
A
Growth rate
25.0
20.0
15.0
10.0
5.0
0.0
S1
S2
T1
T2
Fungal species


Fungicide sensitivity
B
Growth rate
25.0
20.0
15.0
10.0
5.0
0.0
S1
S2
T1
T2
Fungal species


Fungicide sensitivity
C
Growth rate
25.0
20.0
15.0
10.0
5.0
0.0
S1
S2
T1
T2
Fungal species


Fungicide sensitivity
D
Growth rate
25.0
20.0
15.0
10.0
5.0
0.0
S1
S2
T1
T2
Fungal species

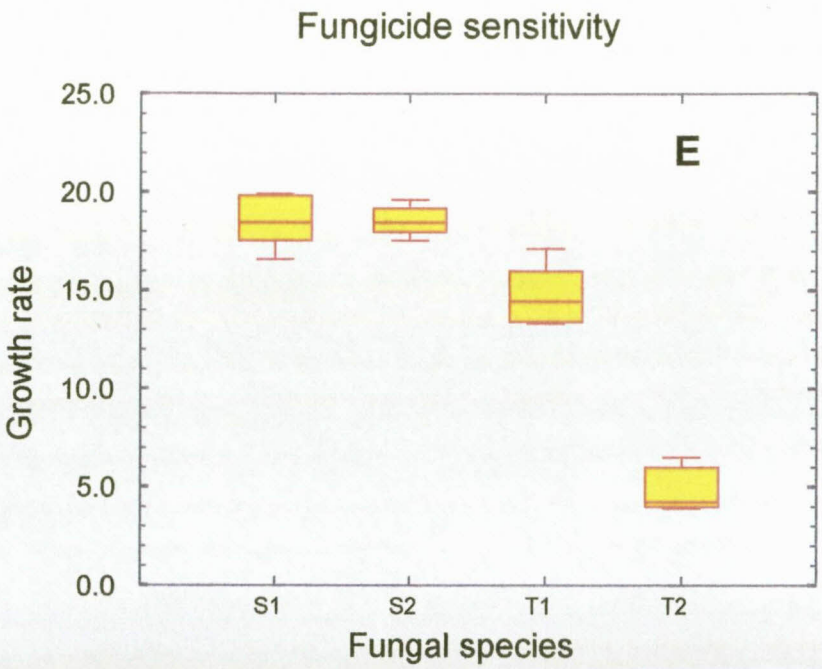
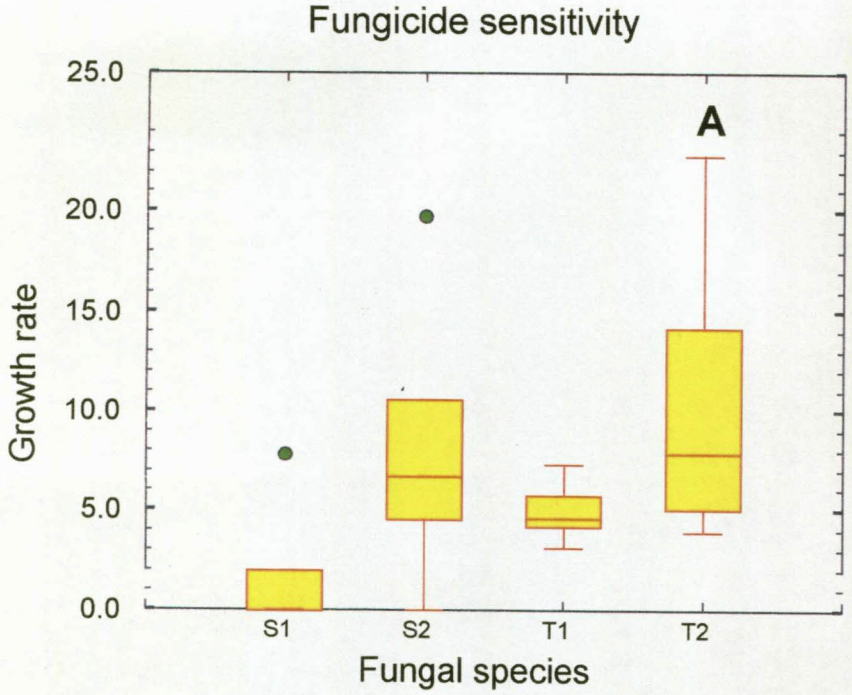
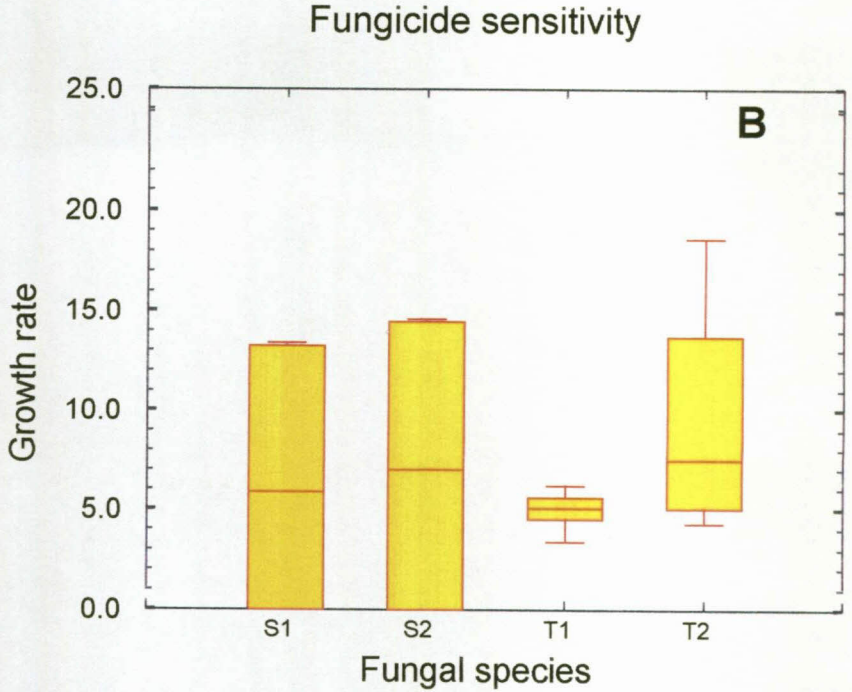
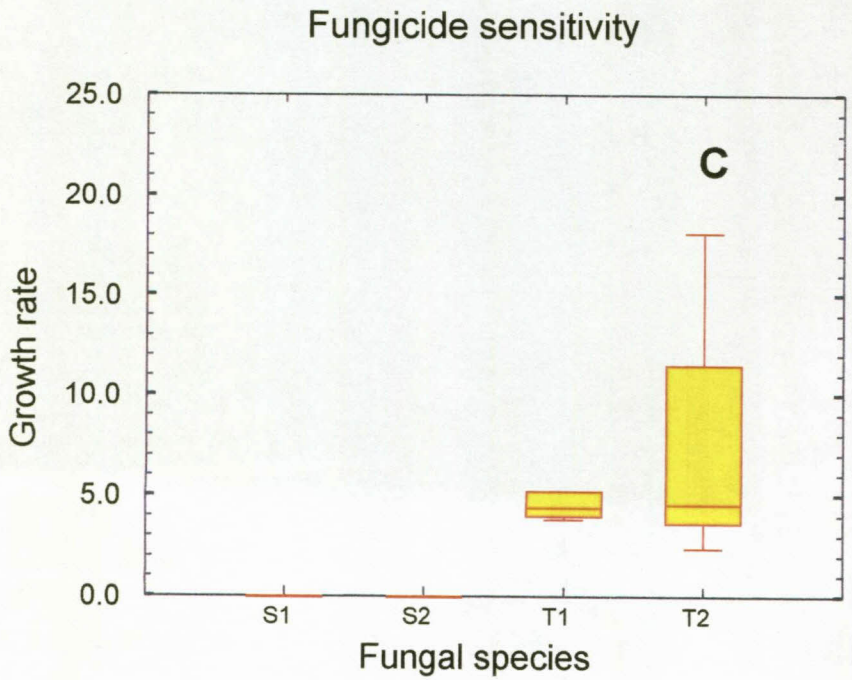
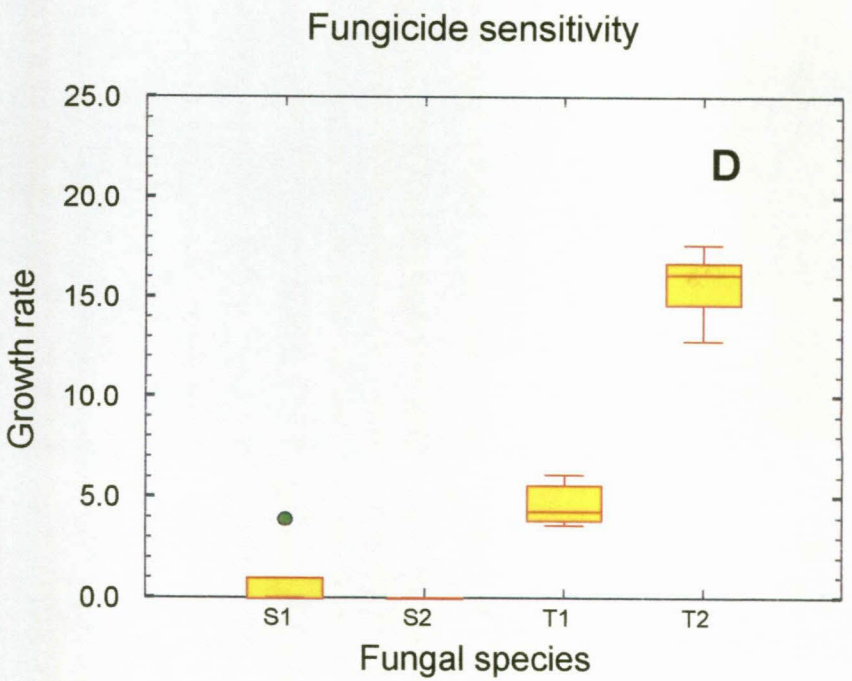

Fungicide sensitivity
E
25.0
20.0
15.0
10.0
5.0
0.0
Growth rate
S1
S2
T1
T2
Fungal species

Figure 3. Box plots of growth rates (in mm) of two *Trichoderma harzianum* isolates (T1 and T2) and two *Sclerotium rolfsii* isolates (S1 and S2) on PDA growth media amended with chlorothalonil at **A**. 938 mg/ℓ **B**. 1875 mg/ℓ **C**. 2500 mg/ℓ **D**.3750 mg/ℓ.


Fungicide sensitivity
A
Growth rate
25.0
20.0
15.0
10.0
5.0
0.0
S1
S2
T1
T2
Fungal species


Fungicide sensitivity
B
Growth rate
25.0
20.0
15.0
10.0
5.0
0.0
S1
S2
T1
T2
Fungal species


Fungicide sensitivity
C
Growth rate
25.0
20.0
15.0
10.0
5.0
0.0
S1
S2
T1
T2
Fungal species


Fungicide sensitivity
D
Growth rate
25.0
20.0
15.0
10.0
5.0
0.0
S1
S2
T1
T2
Fungal species

Summary

Information gleaned from this study has stimulated new questions regarding the genetic structure, population dynamics and control of *Sclerotium rolfsii*. This work can hopefully serve as a foundation for the further study of this fungus in South Africa and elsewhere in the world.

The population of the fungus in South Africa was studied and the 121 isolates collected were found to belong to 13 different mycelial compatibility groups (MCG), occurring on various crop and other plants in different geographic localities. Vague associations between MCG, host plant and geographical area could be observed.

The genetic fingerprinting technique of amplified fragment length polymorphisms was found to be an effective tool to distinguish between MCGs of *S. rolfsii*. This technique has never before been used for this purpose, even though other genetic techniques have been used in other studies. No previous study, however, was able to distinguish isolates within MCGs, as this study was able to do. The hypothesis was also made that isolates within a MCG are not clonally derived, based on the differences observed between isolates in the same MCG, the different host plants and the different geographic areas from which the isolates originated.

The pathogenicity of MCGs of *S. rolfsii* to groundnut appeared to be significantly different from group to group, but the results of field trials suggested that isolates were not fixed into specific MCGs, even though a controlled glasshouse experiment did not support this. The phenomenon of MCG switching has never been reported for this fungus, even though it has been recorded for other fungi. Certain isolates also appeared to be more pathogenic to groundnut than others. Mycelial compatibility group E consistently emerged as a virulent pathogen of groundnut. No groups were found to

be specific to any of three other crop plants tested.

The control of the fungus in groundnut fields was found to be possible with the use of an inversion plough in trials conducted over two consecutive seasons. A fungicide was identified that suppressed *S. rolfsii* but not *Trichoderma harzianum*, a naturally occurring biological antagonist of the pathogen in the soil. The application of the fungicide difenoconazole at 12.5% of the registered concentration for groundnuts did not reduce disease in the field. The application of *T. harzianum* and difenoconazole in the early stages of groundnut plant development had no effect on yield or disease incidence. Levels of disease in the field were found to increase with a decreased plant density. This observation was attributed to increased soil erosion in the field as a result of the sparser plant density.

Sclerotium rolfsii is a virulent pathogen of numerous crop plants in South Africa and this study has provided some answers to questions regarding the biology and control of this fungus. However, much still has to be discovered and may future scientists find some inspiration from what was done here.

Key words: *Sclerotium rolfsii*, southern stem rot, population, mycelial compatibility groups, AFLP analysis, pathogenicity, control, inversion plough, integrated pest management.

Opsomming

Die inligting wat in hierdie studie gegenereer is het nuwe vrae laat ontstaan oor die genetiese struktuur, populasie-dinamika en beheer van *Sclerotium rolfsii*. Hopelik sal hierdie werk dien as 'n basis vir verdere studies oor hierdie swam in Suid Afrika en elders in die wêreld.

Die populasie van die swam in Suid Afrika is bestudeer en 121 isolate, wat in 13 misiliêr-verenigbare groepe (MVGe) geklassifiseer kon word, is versamel. Die indeling van isolate binne hierdie groepe was tot 'n mindere mate gekoppel aan gasheerplant en/of geografiese gebied.

Die genetiese vingerafdruktegniek van geamplifiseerde fragmentlengte polimorfismes ("amplified fragment length polymorphisms" [AFLP]) kon effektief gebruik word om tussen die groepe te onderskei, sowel as tussen isolate in dieselfde groep. Hierdie tegniek is nog nooit voorheen gebruik vir hierdie doel nie, hoewel ander genetiese tegnieke wel in die verlede ondersoek is. Sodanige studies kon egter nog nooit tussen isolate binne dieselfde groep onderskei nie. Op grond van die variasie wat binne groepe met hierdie studie aangedui is kan die stelling ook gemaak word dat isolate binne 'n groep nie klonaal ontstaan nie en ook omdat isolate in dieselfde groep van verskillende gasheerplante en gebiede kan kom.

Die patogenisiteit van die verskillende groepe teenoor grondbone was verskillend tussen groepe, maar die data van veldproewe het daarop gedui dat isolate nie noodwendig tot 'n spesifieke MVG beperk is nie. 'n Onafhanklike proef, onder beheerde toestande in 'n glashuis, het egter gewys dat isolate nie binne groepe verander nie. Die verskynsel van verandering tussen MVGs is nog nooit vir *S. rolfsii* aangeteken nie, alhoewel dit by ander swamme waargeneem is. Sekere isolate was ook meer aggresief teenoor grondbone as ander binne dieselfde groep. Groep E was deurgaans die mees

virulente groep op grondbone. Geen groepe was spesifiek tot enige van die ander gewasse wat getoets is nie.

Beheer van grondboonstamvrot oor twee agtereenvolgende seisoene is verkry waar besmette lande met 'n inversieploeg bewerk is. 'n Swamdoder is ook geidentifiseer wat *S. rolfsii* onderdruk terwyl die patogeen se natuurlike antagonis, *Trichoderma harzianum*, nie benadeel word nie. Die toediening van die fungisied difenoconazole teen 12.5 % (31 g/kg aktiewe bestandeel) van die voorgestelde toedieningskonsentrasie vir blaarsiektes van grondbone, het nie *S. rolfsii* infeksie onder landtoestande beheer nie. Proefresultate het ook getoon dat die toediening van die biologiese middel in kombinasie met die swamdoder vroeg in die seisoen geen effek op siekte of opbrengs gehad het nie. Onder veldtoestande het siekte toegeneem soos plantdigtheid verlaag het. Die rede hiervoor is waarskynlik die skade veroorsaak deur wind-erosie, wat toeneem soos die plantpopulasie afneem.

Sclerotium rolfsii is 'n virulente patogeen van baie gewasse in Suid-Afrika en hierdie studie het slegs sekere antwoorde op vrae rondom die biologie en beheer verskaf. Die studieveld is dus nog oop vir verdere navorsing en die hoop word uitgespreek dat die huidige werk as inspirasie sal dien vir verdere navorsing oor hierdie onderwerp.