Study on interactions between *Sclerotium rolfsii* Sacc. and selected antagonists

by Acharaporn Na Lampang

Thesis submitted for the degree of
Doctor of Philosophy

in

The University of Adelaide
(Faculty of Agricultural and Natural Resource Sciences)

September 1994

Table of contents

Abstract

S. rolfsii is a soil-borne fungus causing stem rot diseases of monocotyledonous and dicotyledonous plants. Products developed for the biological control of this pathogen have not been widely accepted, suggesting a need for more information on interactions between potential antagonists, the pathogen and its hosts. This study was, therefore, carried out to evaluate, *in vitro* and *in vivo*, soil microorganisms potentially antagonistic to *S. rolfsii.*

S. rolfsii was isolated from a diseased capsicum plant and potential antagonists were obtained from disease-free areas of the same field. Two species of *Streptomyces*, one isolate each of *G. virens* and *T. harzianum*, selected on the basis of preliminary studies *in vitro*, were investigated as potential biocontrol agents.

In dual culture studies, both *G. virens* and *T. harzianum* reduced sclerotial viability, particularly in media low in nutrient resources, such as soil extract agar. *G. virens* and *T. harzianum* appeared to inhibit growth of *S. rolfsii in vitro* by production of non-volatile metabolites, as shown by cellophane overlay and culture filtrate studies. The effect varied depending upon culture age and medium. Likewise, *Streptomyces* spp. inhibited *S. rolfsii* by means of non-volatile metabolites. Inhibitory volatile compounds, also, appeared to be produced by *Streptomyces* spp. and *T. harzianum.*

An *in vitro* seedling bioassay was developed to evaluate the protective effect of potential antagonists on capsicum and wheat. *T. harzianum* and *G. virens* enhanced seedling survival to some extent, although the degree of protection depended upon species of antagonist and host. *Streptomyces* spp. had almost negligible effects on survival of the host in this assay.

Experiments conducted with seedlings in potting mix in humid chambers, indicated that application of the antagonists 14 days prior to inoculation with *S. rolfsii* gave the best protection. Generally, results were similar to those obtained in the *in vitro* bioassay. Application of *Streptomyces* s1 or s2 alone significantly increased

shoot dry weight of both host species, suggesting production of plant growth promoting factors.

It was concluded that all four potential antagonists enhanced survival of seedlings of capsicum and wheat. Shoot dry weight of wheat seedlings was enhanced when either *G. virens* or *Streptomyces* s1 was applied in the presence of *S. rolfsii*. Antibiosis and promotion of plant growth appeared to be involved.

This work contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by any other person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

SIGNED:..

DATE:.....1/9/94.....

Acknowledgements

I would like to express my gratitude to the Australian Government for the EMSS scholarship through AIDAB and the office of International Programs. I am also grateful to the Thai Government for the opportunity to pursue further study.

This thesis is a by-product of motivation and help in various aspects which I have received from people, with whom I have been associated during the course of this study. Without them, it would not be the way it is. For this reason, I would like to thank.....

My supervisors, Dr. E. Scott and Dr. K. Sivasithamparam, for their supervision, patience, understanding, support, encouragement, help and concern in various aspects. Special thanks go to Dr. Scott for assistance in preparation of the thesis.

Prof. Z. Punja, for his valuable advice on aspects of the research, Dr. G. Wildermuth and Dr. I. Porter, for valuable advice and information, Dr. J. Walker and Dr. M. Heap, for fungal isolates from different hosts at the initiation of this study.

Mr. K. Morton, for allowing me to take plant and soil samples from his property.

Dr. M. Priest and Dr. I. Kurtboke, for confirmation of identification of the antagonist species.

Dr. T. Rathjen and Dr. V. Vanstone, for supplying seeds

Prof. E. Ghisalberti, for allowing me to use his information on the organic chemistry aspect of work in this study, and also for friendship.

Dr. M. Tate, for his guidance and help in the initiation of work involving inorganic chemistry.

Ms. L. Giles, for help with statistical analysis and friendship.

Dr. S. Smith and Dr. J. Whipps, for their helpful discussion and advice.

Dr. K. Davies, for proof-reading the first draft of this thesis. Also, my deep appreciation for friendship, comfort, encouragement and help in typing part of the references.

Mrs. V. Hope, the Overseas Student Advisor, for her help professionally and personally in several aspects. Also, special appreciation for her warm and caring personality which has made me feel at home.

The Departmental laboratory manager Mr. T. Feckner, assistant lab. manager Mr. G. Taylor, and secretary Ms. A. Johnsen, for their co-operation and friendship. Special thanks go to Ms. J. March, for friendship and help with typing part of the references.

Mr. E. Nagy and Mr. P. Ingram, Plant Growth Services, for facilitating the growth room experiments and also for friendship.

Mr. C. Leaney and Mr. J. Nevermann, the Supply Unit, for their co-operation and friendship.

My late grandparents and my parents, to whom I dedicate this work, for their unconditional love, support, understanding and encouragement all my life.

All my friends, for their friendship, understanding and support. Special thanks go to "Friends in need are friends indeed", Varaporn Thonglairuam, Garry Rosewarne, Chernchoke Sornkwan, Reiny Schepper and Natalie Dillon.

Chapter 1

Introduction and literature review

1.1 Introduction

Sclerotium rolfsii Sacc. is a soil-borne, facultative plant parasitic fungus which causes stem rot, crown rot, sclerotium wilt and blight on various crops. The host range is well over 500 species, including both monocotyledonous and dicotyledonous plants (Punja, 1988). Diseases caused by this fungus are widespread in moist tropical and warm temperate areas where hot and humid conditions enhance growth and survival of the pathogen (Anon., 1971-1978).

Several methods for control of disease caused by *S. rolfsii* have been practised. Among them, chemical control is frequently used either as a sole method or as a component in a combined control strategy. Certain soil fumigants were found to be toxic to sclerotia leading to a reduction in disease incidence (Jenkins and Averre, 1986; McCarter, Jaworski, Johnson and Williamson, 1976). It has also been reported that fungicide application provided effective control on various crops in the field (Mukhopadhyay and Thakur, 1971; Punja, Grogan and Unruh, 1982). A major disadvantage of chemical control, however, is that large amounts of chemicals are usually required for effective control, resulting in a high cost of production. Furthermore, as environmental effects have been a major concern for the public in recent years, many researchers now have concentrated their work on alternatives to chemical control, especially biological methods. The use of microorganisms to control plant pathogenic fungi is being explored, and the most popular species of fungal biocontrol agents being studied belong to *Trichoderma* and related genera. In addition, antibiotic-producing bacteria, including actinomycetes, receive attention, but to a lesser extent. Fungal antagonists have been applied in different forms, aimed at protecting plants from infection by *S. rolfsii*, especially at the seedling stage. Subsequently, successful biological control of diseases caused by *S. rolfsii* in both laboratory and field conditions has been reported, with the majority being laboratory

studies (Elad, Chet and Katan, 1980; Ordentlich, Nachmias, Strashnov and Chet, 1990). In spite of the successes mentioned, few commercial products containing biocontrol agents active against *S. rolfsii* have achieved worldwide popularity. This suggests a need for more information and a better understanding of biological control of *S. rolfsii* in order to achieve successful control in nursery and field conditions. Therefore, this project was initiated in February 1990 with the main objectives as follows:

1. To isolate and identify microbes, fungi and actinomycetes in particular, potentially antagonistic against *S. rolfsii*

2. To study the interaction of *S. rolfsii* and selected microbial antagonists *in vitro* and *in vivo*

The research was directed towards biological control in warm temperate and tropical regions. A potting medium was used as a model system for the examination of biological control of S. rolfsii.

1.2 Literature review

Information on biology, ecology and control of *S. rolfsii* is reviewed in the following section, with the aim of providing an overview of the fungus itself and the diseases it causes. The review also presents previous attempts to control these diseases based on the knowledge of factors influencing growth and sclerotial production of the pathogen.

1.2.1 Taxonomy, host range, diseases and symptoms

S. rolfsii is the anamorph of the Basidiomycete *Athelia rolfsii* (Curzi) Tu and Kimbrough (1978). The teleomorph is rarely seen in nature (Aycock, 1966), and it is the anamorph which is of economic importance and is the subject of this study.

The pathogen is of economic importance on various cultivated plants. These include peanut (Branch and Csinos, 1987; Narain and Kar, 1990; Sahu, Narain and Swain, 1989; Shew, Wynne and Beute, 1987; Zeidan, Elad, Hadar and Chet, 1986), cowpea (Nwakpa and Ikotun, 1988), beet, brassica, citrus, cucumber, banana, potato, rice, sugar cane, wheat, barley (Domsch, Gams and Anderson, 1980), tomato (Agrios,

1988; Wokocha, 1990), carrot, celery, sweet corn, onion, eggplant (Agrios, 1988) and chilli (Wangikar, Somani and Bobade, 1988). It can also infect horticultural plants such as betelvine (Palakshappa, Srikant-Kulkarni, Hegde and Kulkarni, 1988) and ornamentals such as lily (Hsieh, Tu and Tsai, 1990).

Crown blight, root and crown rot, damping off of seedlings, stem canker, bulb and tuber rot, and fruit rot are diseases commonly caused by *S. rolfsii.* In the United States, they are often called "southern wilts or southern blights" because they are common and severe in the southern states (Agrios, 1988).

Infection usually occurs at soil level and extends a few cm above and below. The first visible symptoms on a host appear as yellowing or wilting of the lower leaves or dying back of leaves from tip to petiole. These symptoms then progress to the upper leaves (Agrios, 1988; Beniwal, Ahmed and Gorfu, 1992). Necrosis occurs prior to hyphal penetration of epidermis, cortex and stele. If infected plants have succulent stems, the stem may fall over, while plants with harder stems stand upright and begin to lose their leaves or to wilt. In the meantime, the fungus grows upward on/the plant, covering the stem lesion with a cottony white mass of mycelium, the upward advance of the fungus depending on the amount of moisture present. The fungus also moves downward into the roots and finally destroys the root system. Infected tissues are usually pale brown and soft but not watery. As the host is close to death or dead, resting bodies called sclerotia are formed on all infected tissues and on/in the nearby soil (Agrios, 1988; Wangikar *et al.*, 1988).

1.2.2 Pathogenicity

The initial contact of mycelium with the host and subsequent necrosis, as a result of the secretion of oxalic acid and polygalacturonases by *S. rolfsii*, are seen as key requirements for establishing infection by the pathogen (Punja, Huang and Jenkins, 1985). Oxalic acid sequesters calcium from the cell wall to form calcium oxalate (Punja and Jenkins, 1984b) and reduces the tissue pH to the optimum for endopolygalacturonase and cellulase activity (Bateman, 1972; Punja, Huang and Jenkins, 1985). It is also directly toxic to plant tissue (Franceschi and Horner,

1980). It has been noted that cellulases may play a secondary role in tissue destruction and disease development since they appear later in the sequence of enzyme production (Punja *et al.*, 1985).

Infective fungal hyphae contain numerous microbodies (Hänssler, Maxwell and Maxwell, 1978), the presumed site of oxalate synthesis (Armentrout, Graves and Maxwell, 1978) and vacuoles containing phosphatase (Hänssler, Maxwell and Maxwell, 1975; 1978). Pectic substances in host cell walls are depleted (Bateman, 1970) in advance of growing hyphae (Smith, Punja and Jenkins, 1984) and peroxidase is released from host cell walls by *S. rolfsii* through enzymatic action (Barnett, 1974). The end of the infection process is indicated by the necrotic and macerated appearance of tissues (Punja, 1985).

1.2.3 Transmission

S. rolfsii is capable of extensive saprophytic growth in surface layers of soil, on crop residues and weed hosts. The sclerotia formed are disseminated in infested soil, by contaminated tools (Agrios, 1988; Gurkin and Jenkins, 1985), humans, animals, water and wind (Anon., 1971-1978). They may be present on vegetative propagation material or as contaminants amongst seed (Agrios, 1988; Anon., 1971-1978). Although the teleomorph is infrequent in the field (Aycock, 1966), basidiospores can infect host tissues under greenhouse conditions (Punja and Grogan, 1983). At present, it is not known whether the teleomorph plays an important role in disease transmission in the field or not.

1.2.4 Favourable conditions for fungal growth and distribution of disease

The temperature range for hyphal extension and dry weight production is 8-40°C (Punja, 1985). However, the growth rate of the fungus and disease progression are highest at 25-33°C (Punja, 1988). *S. rolfsii* has been found in various soil types including open fallow soils, mangrove mud and poorly drained soils. The fungus is capable of growing in a wide pH range, from 1.4-8.6, optimum 3.0-6.5,

with initial retardation on near neutral or alkaline media (Domsch *et al.*, 1980; Sharma and Kaushal, 1979).

Although diseases caused by *S. rolfsii* occur primarily in warm climates, affecting plants in countries within 38° latitude on either side of the equator (Agrios, 1988), they are also found in warm temperate areas (Anon., 1971-1978).

In Australia, *S. rolfsii* has been recorded by the Queensland Department of Primary Industries as causing disease of peanut, soybean, sunflower, corn, wheat, barley, common oats, carrot, onion, cabbage, tomato, papaw, watermelon, rockmelon, strawberry, chrysanthemum, gerbera, snapdragon, iris, guinea grass, nutgrass, perennial ryegrass, red and white clover, subterranean clover and pig weed (Simmonds, 1966). *S. rolfsii* was first reported in wheat fields in Western Australia by Dewan and Sivasithamparam (1987), and was isolated from roots of wheat and ryegrass. Wheat var. Gamenya showed 12% pre-emergence and post-emergence death of seedlings. Other hosts showing disease in Western Australia were bromegrass (75.6% seedling death), barley (54.8%), subterranean clover (33.3%), lupin (82.4%) and oats (63.8%). These crops were grown in rotation with wheat and may serve to increase the inoculum levels of *S. rolfsii* in soil. This suggests the possibility that *S. rolfsii* diseases will become increasingly important in the future. The fungus has also been recorded as causing minor disease in wheat and barley in New South Wales (G. Wildermuth, pers. comm.). In 1990, *S. rolfsii* was isolated from several crops such as beans and peanuts in Queensland (K.J. Middleton and G. Wildermuth, pers. comm.), carrots in New South Wales and South Australia (J. Walker and M. Heap, pers. comm.), carnation in New South Wales (J. Walker, pers. comm.) and nerines in South Australia (M. Heap, pers. comm.). It was also found in capsicum grown at Penfield Gardens, South Australia in 1990.

In Thailand, the demand for wheat for human consumption has increased markedly in the past 10 years, leading to an increase in crop area. This increase in cultivation, combined with humid conditions, has led to increased incidence of

sclerotium wilt on wheat caused by *S. rolfsii*. The disease has become a significant threat, in terms of yield reduction, to growers (Chuaiprasit, 1990).

1.2.5 Colony morphology, and structure and composition of sclerotia

On potato dextrose agar, the colony is white and fluffy with branched mycelium that forms abundant sclerotia but is usually sterile (Agrios, 1988; Anon., 1971-1978). For the teleomorph, in general, basidial formation in culture occurs on media that are poor in nutrients, in older cultures grown at low-light intensities, and following the growth of mycelium transferred from a rich to a poor medium (Punja and Grogan, 1983; Punja, Grogan and Adams, 1982).

Sclerotia are formed laterally from main hyphal strands and their shape can be either globose or irregular. Young sclerotia are white, darkening as they mature (Willetts, 1972). Mature sclerotia are not connected to mycelial strands and have the size, shape, and colour of mustard seed (Agrios, 1988). They are composed of an outer melanized rind, two to four cell layers thick (Chet and Henis, 1968; Liu, Yu and Wu, 1977), cortex with faintly pigmented walls and an innermost region, the medulla, with colourless, unevenly thickened walls comprising loosely interwoven hyphae (Nair, White, Griffin and Blair, 1969; Chet, 1975). Walls of mature sclerotia contain chitin, laminarin and other ß-1,3 glucans (Chet and Henis, 1968) while sugars, lipids and glucans comprise the major storage reserves (Punja, 1985). Following sclerotium initiation and during the developmental stage, drops of exudate containing cations, proteins, carbohydrates, amino acids, enzymes and oxalic acid are formed on the sclerotial surface (Christias, 1980; Colotelo, 1978). Effects of exudation on activities of soil microorganisms and subsequent sclerotial germination will be reviewed in section **1.2.7.3.**

1.2.6 Factors influencing growth and sclerotial production of *S. rolfsii*

Growth and sclerotial production of *S. rolfsii* can be influenced by environmental factors, nutritional factors and chemicals. Environmental factors include temperature, aeration, moisture and light (Punja, 1985).

1.2.6.1 Environmental factors

High temperatures favour mycelial growth and sclerotial production of *S. rolfsii.* Maximum sclerotial formation occurs at 27-30°C (Mathur and Sarbhoy, 1976; Sharma and Kaushal, 1979). Fluctuation in temperature may affect the shape and size of the sclerotia (Aycock, 1966).

S. rolfsii is considered to be highly aerobic for growth. Nevertheless, this depends on levels of both oxygen and carbon dioxide (Aycock, 1966). It has been reported that oxygen levels as low as 1% (Mitchell and Mitchell, 1973) and 4% (Griffin and Nair, 1968) markedly retarded linear growth and dry weight of the fungus, respectively. Sclerotial initials in culture were inhibited by exposing the fungus either to oxygen below 15% or carbon dioxide above 1% (Kritzman, Chet and Henis, 1977) or 4% (Griffin and Nair, 1968). This may, in part, explain why sclerotia are most abundant within the top 8 to 10 cm of the soil profile (Punja, 1985). As the level of carbon dioxide was increased to above 15%, the rate of linear hyphal growth and mycelial dry weight in culture were reduced by 50% (Mitchell and Mitchell, 1973).

Sclerotia germinate only in relative humidity of approximately 100%, at rates influenced by age, drying or mechanical injury. Wet soil, shading and crowding of plants, leading to increased moisture levels at the soil surface, promote germination of the fungus and hence plant disease (Anon., 1971-1978). In addition, it has been found that disease incidence was greater at soil moisture-holding capacities (MHC) of 50-75% than at saturation (Punja and Jenkins, 1984a). As soil dries, infection occurs further below the surface (Anon., 1971-1978). Thus, thinning over-crowded plants may reduce disease incidence in areas with a history of *S. rolfsii* disease.

Sclerotial initials develop early under light. Both growth and sclerotial formation in cultures of *S. rolfsii* are greater under continuous light, especially blue light, compared to continuous darkness (Humpherson-Jones and Cooke, 1977; Miller and Liberta, 1977), but individual sclerotium dry weight is lower (Humpherson-Jones and Cooke, 1977). The abundant sclerotial production at the soil surface compared to that which occurs deeper in soil could, in part, be a response to light (Punja, 1985).

1.2.6.2 Nutritional factors and chemicals

The number and size of sclerotia are influenced by the source of carbon (Bahadur, Sinha and Upadhyaya, 1976; Sharma and Kaushal, 1979; Zoberi, 1980) or nitrogen in the medium (Mathur, 1977; Zoberi, 1980), C:N ratio of the medium (Punja, 1986) and by pH (Sharma and Kaushal, 1979). In general, media that support extensive growth, usually with high C:N ratios, also yield numerous sclerotia (Punja, 1985, 1986).

Sclerotial formation in culture can be inhibited at any stage, i.e. initiation, development or maturity (Henis and Chet, 1968; Willets, 1972). It can also be inhibited by low concentrations of several chemicals including 2-mercaptoethanol and related sulfhydryl compounds (Christias, 1975), carboximide fungicides (Fellman and Le Tourneau, 1983) and phenylthiourea (Le Tourneau, 1976). This is related to chemical control of *S. rolfsii*, reviewed in section **1.2.8.2.**

1.2.7 Forms of sclerotial germination

There are two forms of sclerotial germination, namely hyphal and eruptive. Hyphal germination is characterized by the growth of individual strands from the sclerotium surface; these hyphae originate from cells of the medulla, but their growth is not extensive unless an external source of nutrients is available (Chet, Timar and Henis, 1977). In contrast, eruptive germination is characterized by plugs or aggregates of mycelium bursting through the sclerotial rind (Punja and Grogan, 1981). The latter type of germination is independent of an external source of

nutrients. Nevertheless, prolonged exposure to microbial activities in non-sterile soil, together with the loss of endogenous reserves through nutrient leakage, can result in less germination and possibly shorten the period of survival, providing possibilities to manipulate disease control (Punja, 1985). For this reason, review in the next section will focus on factors affecting eruptive germination of sclerotia. These factors are classified as environmental, abiotic and biotic factors (Punja, 1985). Environmental factors include temperature, moisture and aeration, whereas abiotic factors comprise nutrients and chemicals including volatile compounds, and soil microorganisms are biotic factors.

1.2.7.1 Environmental factors

Eruptive germination on soil and agar is maximal at 21-30°C, and is rarely found below 15°C and above 36°C (Punja and Jenkins, 1984a). The optimum range of solute water potential (Y_S) and matric water potential (Y_m) is -2.5 to -10 bars and 0 to -1 bar respectively. Eruptive germination is completely inhibited at Ys lower than -60 bars and at Y_m less than -10 bars in a coarse sandy loam or at Y_m less than -15 bars in a fine sandy loam (Punja and Jenkins, 1984a). The ranges of carbon dioxide, oxygen and ethylene concentrations suitable for eruptive germination are 0.5-9%, 15-20.5% and 1-40 µg/ml, respectively. In addition, levels of carbon dioxide above 20% and oxygen below 3% are inhibitory to germination (Punja and Jenkins, 1984a). Furthermore, it has been reported that percentage germination is lower at soil depth below the top 2.5 cm than it is at the soil surface and is nil below 8 cm (Punja and Jenkins, 1984a). As results from subsequent studies have discounted the possibility that oxygen depletion or ethylene and carbon dioxide buildup are involved, physical pressure from the weight of soil over buried sclerotia has been speculated to be partly responsible for the inhibition of germination (Punja and Jenkins, 1984a). This provides the possibility of disease reduction by deep burying of sclerotia, which could be done by cultivation. However, the method may promote sclerotial germination through mechanical injuries, therefore it should be combined with other techniques.

1.2.7.2 Abiotic factors

On media that contain soluble nutrients, eruptive germination is inhibited and hyphal germination occurs instead. Furthermore, adding metabolizable carbon sources such as 20 mM glucose or sucrose to substrates low in nutrient status (e.g. water agar) also inhibited eruptive germination but stimulated hyphal germination (Punja, Jenkins and Grogan, 1984). Several volatile compounds, mainly alcohols and aldehydes at levels ranging from 1.6 to 6.1 µl per 60x15 mm Petri dish, stimulated sclerotium germination and increased the mycelial growth rate of *S. rolfsii* in water agar, without affecting dry weight (Punja *et al.* , 1984). This could be explained by the observation that many of the test alcohols are known constituents of plant tissue distillates, which may be released by autocatalytic reactions and trigger germination of sclerotia. These compounds may stimulate sclerotium germination directly by affecting enzyme activity or membrane permeability, or by acting as metabolic regulators (Fries, 1973). They also may promote germination indirectly by influencing the activities of resident soil microorganisms through nutrient exudation on the sclerotial surface (Gilbert and Linderman, 1971; Linderman and Gilbert, 1973). The increase in membrane permeability caused by exposing sclerotia to these volatile compounds may activate or mobilize compartmentalized glucanases that act on stored glucans, therefore, enhancing nutrient leakage leading to eruptive germination as a final result (Punja and Grogan, 1981). Salts containing the ammonium ion and/or carbonate and bicarbonate cations are toxic to sclerotia, especially at high pH as there is an additional effect of bicarbonate converted from carbon dioxide. The optimum range of pH for germination is 2-5, and a complete inhibition occurs at pH above 7 (Punja and Grogan, 1982). The practical use of carbonate and bicarbonate salts for controlling *S. rolfsii* diseases is demonstrated by application of ammonium fertilizers containing these cations (see section **1.2.8.1**).

1.2.7.3 Biotic factors

It has been shown that sclerotia formed in nonsterile soil germinate poorly and less consistently than those produced in laboratory cultures (Linderman and

Gilbert, 1973; Punja *et al.*, 1984), due to the presence of surface-contaminating microorganisms (Punja *et al.*, 1984). During the developmental stage, drops of nutrient exudate are formed on the sclerotial surface (see section **2.2.2**). This exudation enhances the activities of soil microorganisms that may stimulate or inhibit sclerotial germination in nonsterile soil (Gilbert and Linderman, 1971). Exudation is increased by drying sclerotia (Coley-Smith, Ghaffar and Javed, 1974; Punja and Grogan, 1981; Smith, 1972), exposing them to volatiles released from plant tissues (Punja *et al.*, 1984), imposing physical pressure (Punja and Jenkins, 1984a), treating with sodium hypochlorite (Linderman and Gilbert, 1973; Punja and Grogan, 1981) and heat (Lifshitz, Tabachnik, Katan and Chet, 1983). The increase in sclerotial exudation may increase microbial antagonism, resulting in accelerated death of sclerotia (Coley-Smith, Ghaffar and Javed, 1974; Smith, 1972). Furthermore, as the numbers and activities of antagonistic soil microorganisms can be enhanced by adding nitrogenous compounds (Henis and Chet, 1968) or plant residues and volatile extracts to soil (Gilbert and Linderman, 1971; Linderman and Gilbert, 1969; Linderman and Gilbert, 1973; Mixon and Curl, 1967), the application of these factors may increase the chance of survival for plants grown in areas prone to sclerotial infection. However, cost-benefit implications should not be neglected, particularly for volatile extracts which would be difficult to apply. Biotic factors are involved in the phenomenon of fungistasis, which is discussed in section **1.2.12.5**.

1.2.8 Disease control strategies

Knowledge of factors affecting the growth, sclerotial production and germination of *S. rolfsii* has led to disease control strategies which can be divided into two groups i.e. conventional and biological. Conventional disease control strategies comprise cultural practices and chemical application whereas the basis of biological control is dependent upon the effects of antagonistic soil microorganisms on the pathogen. These approaches are often combined as integrated control, aiming to improve the effectiveness of disease control for *S. rolfsii*, therefore, integrated control will be reviewed where appropriate.

1.2.8.1 Cultural practices

Carbonate and bicarbonate salts of potassium, sodium, lithium and ammonium have been reported to inhibit sclerotial germination of *S. rolfsii in vitro* (Punja and Grogan, 1982). Ammonium fertilizers such as urea and ammonium bicarbonate are fungicidal to sclerotia (Punja, Carter, Campbell and Rossell, 1986; Punja and Grogan, 1982), and may also limit disease indirectly by inducing host resistance (Henis and Chet, 1968). The application of calcium fertilizers such as calcium nitrate, calcium sulphate or calcium carbonate may provide some degree of disease control at low levels of disease severity (Punja *et al.*, 1985); it has been speculated that high calcium levels in plant tissues may partially offset the action of oxalic acid and cell wall-degrading enzymes produced by *S. rolfsii*, thereby, reducing disease severity (Punja *et al.*, 1985; Tarabcih and Al-Menoufi, 1987). This approach could be effective and economical where calcium supply in the soil is limiting, particularly in light, sandy soils.

Information from findings mentioned above has led to a number of successful field experiments, involving the application of calcium and ammonium fertilizers in an integrated control program with different fungicides and ploughing techniques (Gurkin and Jenkins, 1985; Jenkins and Averre, 1986; Punja *et al.*, 1986; Punja *et al.*, 1982).

Despite the fact that the germination of sclerotia is inhibited at pH above 7.0, an attempt to control the disease by the adjustment of soil pH through liming on golf greens failed (Punja *et al.*, 1982). This may be due to infection by sclerotia which had survived the period of increased pH, indicating the significance of sclerotia as a primary source of inoculum.

Although deep ploughing alone could remove inoculum from the upper 8-12 cm of the soil, it was observed that there was no effect on disease incidence where low inoculum levels of *S. rolfsii* resulted in high disease incidence (Gurkin and Jenkins, 1985; Punja *et al.*, 1985). This means that there is no simple extrapolation between inoculum levels and disease incidence. In addition, deep ploughing in conjunction with

other methods such as fungicide or fertilizer applications in an integrated control program has been found to be effective in reducing disease incidence (Jenkins and Averre, 1986; Punja *et al.*, 1986). This indicates that deep ploughing could be used effectively as a component in integrated control programs rather than as a sole method.

Solar heating (Elad, Katan and Chet, 1980b; Mihail and Alcorn, 1984; Porter and Merriman, 1983) or application of the antagonistic fungus *Trichoderma harzianum* Rifai, or a combination of both (Elad *et al.*, 1980b) were reported to reduce disease caused by *S. rolfsii*, with the combination being more effective than either method alone (Elad *et al.*, 1980b). Similar results were obtained in solarized soil amended with *Gliocladium virens* Miller, Giddens & Foster before solarization, when disease incidence in tomato was reduced by 49% compared to untreated controls (Ristaino, Perry and Lumsden, 1991). Results indicate the potential for successful disease control by incorporating solarization in the integrated control program.

Rotation of host crops and non-host, or less affected, crops may lower inoculum levels and, therefore, reduce disease incidence in subsequent years (Punja, 1985). This is supported by work on peanut rotated with onion, when it was found that sclerotia buried in an onion field were 43% less viable than those of the control, and disease incidence on peanut was markedly reduced (Zeidan *et al.*, 1986). However, this method alone may not always be effective since *S. rolfsii* has a wide host range, and sclerotia can persist on crop debris for up to 6 months (Tisdale, 1921). It may, again, be used in combination with other control strategies.

The addition of organic amendments such as compost (Gorodecki and Hadar, 1990; Mathur and Sinha, 1970; Mohapatra and Dash, 1990; Yuen and Raabe, 1984), or oat or corn straw (Gautum and Kolte, 1979; Mehrotra and Caludius, 1972), to soil has been found to reduce disease incidence, possibly due to the release of toxic ammonia or to increase in populations of resident antagonistic soil microorganisms (Gorodecki and Hadar, 1990; Mohapatra and Dash, 1990; Punja, 1985). This method may be used effectively on a small scale.

Screening for resistance to *S. rolfsii* is common in field crops, particularly legumes. Varietal differences in response to sclerotial infection were shown in cowpea and soybean, when a range of reaction from resistant to highly susceptible was observed (Nwakpa and Ikotun, 1988; Vallejos, 1988). Two of 12 Virginia peanut genotypes were selected on the basis of ratios of dead: living plants at infection sites (Shew *et al.*, 1987). Also, results from a field experiment on peanut artificially inoculated with *S. rolfsii* showed two of 16 test cultivars to have low disease incidence and high yield performance (Branch and Csinos, 1987). These two characteristics are perhaps the ultimate features that breeders are looking for in any crop. Since varietal differences in response to *S. rolfsii* infection do occur, the method may be used in conjunction with other methods in integrated control programs.

1.2.8.2 Chemical control

Chemicals have been applied successfully to control *S. rolfsii*. Methyl bromide, chloropicrin and metham-sodium are soil fumigants, toxic to sclerotia of *S. rolfsii*, which lead to a reduction in disease incidence (Jenkins and Averre, 1986; McCarter *et al.*, 1976). Also, captan, benomyl and mancozeb effectively inhibited mycelial growth of *S. rolfsii* on eggplant at 700 ppm *in vitro*, whereas the effective concentration used for soil treatment in the greenhouse was 600 ppm (Nwufo and Onyeagba, 1989). Fungicide treatments (benodanil, flutolanil, quintozene and Nor-Am SN 596), applied at planting, resulted in 98-100% protection to field-grown iris against infection by *S. rolfsii* after 9 months (Chastagner, Stalcy and Riley, 1990). Tebuconazole, an ergosterol biosynthesis inhibitor, and PCNB inhibited sclerotial formation both *in vitro* and in the field, with the former being more effective. (Brenneman, Murphy and Csinos, 1991). Soil drench with 0.2% tolclofos-methyl in an integrated control program gave the best control of *S. rolfsii*, followed by soil amendment with *Trichoderma harzianum* at 100 g/kg soil, and soil solarization with white polyethylene mulch for 30 days (Mohapatra and Dash, 1990). Because of environmental concerns, the use of certain of these chemicals, e.g. captan, benomyl, methyl bromide, is restricted or is to be discontinued (T.J. Wicks, pers. comm.).

As alternatives to synthetic compounds, chemicals from plants, which as natural products, may be preferable for the environment, are being investigated. Saponin, isolated from lucerne root, showed activity against some plant pathogenic fungi including *S. rolfsii* (Levy, Zehavi, Naim and Polacheck, 1989). Growth of *S. rolfsii in vitro* was reduced by 90% when saponin was applied at 40 µg/ml of agar medium. Using natural plant products could be a useful approach for disease control of *S. rolfsii* in the future, but needs further study.

Chemical control either alone or as a component in an integrated control program, provides satisfactory results. However, increasing environmental awareness around the world has stimulated interest in biological control. The concept and relevant aspects of biological control of *S. rolfsii* will be explored in the next section.

1.2.8.3 Biological control

A considerable number of research programs have focussed on biological control of plant diseases during the last three decades. In the broad sense, crop resistance to disease is the ideal means of controlling disease, as there is no requirement for additional input. However, many crops have little or no resistance to certain plant pathogens, therefore, biological control using microorganisms against plant pathogens is an attractive alternative method for disease control.

The term "biological control" is defined as a reduction in the pathogen inoculum or its disease-producing capacity by action of one or more organisms other than humans (Cook and Baker, 1983). Living organism(s) which affect disease either by their direct influence on the pathogen or indirectly through their effects on the host or environment, provide the basis of biological control regardless of its initiation (Sharma and Sankaran, 1988).

1.2.9 Antagonism

In natural conditions, microorganisms interact as a consequence of their growth and development (Singh and Faull, 1988). In biological control of plant disease, the relationship between two organisms can be categorized as an antagonistic

(parasitic) relationship where there is an advantage to one partner and a disadvantage to the other partner (Deacon, 1983). Microbial antagonism in biological control of plant disease is broadly based on three categories, i.e. competition, hyperparasitism and antibiosis.

Competition is defined as the injurious effect of one organism on another because of the utilization or removal of some resource of the environment including nutrients, oxygen and space (Clark, 1965). Space and oxygen are factors that can be changed in the rhizosphere or rhizoplane, leading to a detrimental effect on pathogen establishment. This is related to the occupation of a site by microorganisms which also diminishes the limited supply of nutrients in either environment. Carbon, nitrogen and vitamins determining the growth of, and infection by, soil-borne plant pathogens are important in this respect (Singh and Faull, 1988).

The term "hyperparasitism" covers a range of interactions including minor or major morphological disturbances, the overgrowth of hyphae of one fungus by another, penetration and direct parasitism by the production of haustoria, and the lysis of one hypha by another (Dennis and Webster, 1971c). Hyperparasitic interactions can be either biotrophic or necrotrophic, depending on the mode of parasitism and its effect on the host. Biotrophic parasites obtain nutrients from the living cells of the host via haustoria. Necrotrophic parasites acquire nutrients from dead host cells, usually killed by the parasite before it invades. This death is caused by toxins or extra-cellular enzymes. Coiling of one mycelium around another may indicate the occurrence of hyperparasitic interactions. The resting structures of fungi may also be attacked by hyperparasitic fungi. The degree of parasitism varies, depending upon several factors including changes in the C:N ratio, temperature, light, pH, and nutrients (Singh and Faull, 1988).

Antibiosis occurs when the production of antibiotics or toxic metabolites by microorganisms has a direct effect on another microorganism. *In vitro* studies have shown that certain fungi and bacteria are capable of producing volatile (Dennis and Webster, 1971b; Fries, 1973; Hutchinson, 1973) and non-volatile antibiotics

(Dennis and Webster, 1971a; Ghisalberti *et al.*, 1990; Ghisalberti and Sivasithamparam, 1991; Lumsden, Locke, Adkins, Walter and Ridout, 1992; Rothrock and Gottlieb, 1984; Utkhede, 1983), whereas information on antibiosis in potting mix or soil is limited. Although antibiotics are likely to be degraded by other microorganisms not affected by them and adsorbed onto clay colloids and humus particles in soil, their production by microorganisms in microhabitats may be ecologically significant in terms of biological control as these microbes occupy small substrate niches to protect themselves from invaders (Singh and Faull, 1988). Production and detection of these antibiotics in potting mix or soil, therefore, merit investigation.

1.2.10 Classification and biology of potential antagonists

Fungi, actinomycetes and other bacteria are commonly considered as potential biocontrol agents for soil-borne plant pathogens. This review will focus on the first two with special reference to *Trichoderma harzianum*, *Gliocladium virens* and *Streptomyces* spp., as these organisms were studied in this project.

Since there is a close resemblance between several *Trichoderma* spp., the concept of "species aggregate", an entity defined as an aggregation of morphologically very similar and often hardly separable species, was proposed (Rifai, 1969). This concept is followed in **1.2.12.4** where relevant papers are cited.

1.2.10.1 *T. harzianum*

T. harzianum is a hyphomycete with teleomorph that belongs to the genus *Hypocrea* (Rifai, 1969). Its morphology is very similar to *Trichoderma viride* Pers. ex. S. F. Gray and *Trichoderma aureoviride* Rifai and it is often mistakenly classified as one of these two (Rifai, 1969).

Colonies of the fungus grow rapidly on malt extract agar at 25 °C and form a smooth-surfaced, watery white and sparse mycelial mat which may be followed by development of aerial hyphae. Phialospores are produced singly, successively and accumulate at the tip of each phialide to form a globose conidial head. They are smooth-

walled, subglobose or short obovoid, often with a broad truncate base, pale green when viewed singly but appearing much darker in mass (Rifai, 1969).

T. harzianum is frequently found in relatively warm regions (Danielson and Davey, 1973) but also up to 3,450 m above sea level (Reeves and Horn, 1976). It has also been isolated from various soil types ranging from light sandy soil in Libya (Youssef, 1974) to forest soils in central Africa (Kobayasi, Matsushima, Takada and Hagiwara, 1977). Optimum temperature for growth is in the range 15-35 °C (Franz, 1975), and the optimum pH is between 3.7 and 4.7 (Danielson and Davey, 1973).

T. harzianum is an antagonist of several soil-borne plant pathogens including *S. rolfsii* (Henis and Papavizas, 1983). Some active isolates produce an anti-fungal antibiotic pyrone, with a distinctive coconut smell (Claydon, Allan, Hanson and Avent, 1987), and analogues (Ghisalberti, Narbey, Dewan and Sivasithamparam, 1990) which may be partly, if not totally, responsible for disease reduction.

1.2.10.2 *G. virens*

G. virens is also a hyphomycete with a teleomorph that belongs to the genus *Hypocrea* (Domsch *et al.*, 1980).

Colonies grow rapidly, reaching 5.8 cm in 5 days at 20 °C on oatmeal agar. Its distinctive characteristic is that phialides are appressed, bearing one large drop of green conidia on each whorl. Conidia are large with a range 4.5-6 x 3.5-4 µm, appearing short-ellipsoidal and smooth-walled (Domsch *et al.*, 1980).

G. virens is a common soil fungus (Domsch *et al.*, 1980). It has also been isolated from plant tissues including healthy and diseased strawberry roots in Illinois, USA (Nemec, 1970), and logs used in the cultivation of Shiitake mushroom, *Lentinus edodes*, in Japan (Morquer and Komatsu, 1968). Optimal growth occurs at 25-32 °C (Komatsu, 1976). Direct evidence on optimum pH for growth is lacking but it is expected to be similar to that of *Trichoderma*.

The fungus is known as a mycoparasite of soil-borne plant pathogens (Howell, 1982). It also produces the antifungal antibiotics gliotoxin (Jones and Hancock, 1988; Lumsden *et al.* , 1992), gliovirin (Howell and Stipanovic, 1983) and

viridin. The latter may be converted into the phytotoxin, viridiol (Jones and Hancock, 1987; Lumsden *et al.*, 1992). This possibly deleterious effect on plant growth should be taken into account in the critical amount of inoculum applied. Among the antibiotics produced, gliotoxin has been reported as the most effective against a range of soil-borne plant pathogens including *S. rolfsii* (Lumsden *et al.*,1992).

1.2.10.3 *Streptomyces* spp.

Streptomyces are actinomycetes of the class Streptomycetes. The genus comprises Gram-positive bacteria with G+C content in their DNA greater than 55 mol % (Goodfellow and Williams, 1983). The species are highly oxidative and form an extensive branching substratum and aerial "mycelium" (Goodfellow and Simpson, 1987). Fragmentation of the substrate mycelium is uncommon and spores are rarely produced on it. The aerial "hyphae" usually carry long, uniserial chains of spores, enclosed within a thin filamentous sheath (Goodfellow and Simpson, 1987).

Streptomycetes are classified according to pH and temperature requirements (Goodfellow and Simpson, 1987). Acidophilic streptomycetes grow between pH 3.5-6.5 (Hagedorn, 1976), neutrophilic at pH 5.0-9.0 with an optimum around neutrality (Kutzner, 1981; Williams, 1978), and alkalophilic species at pH above 7.0 (Mikami, Miyashita and Arai, 1982). Based on temperature preference, mesophilic streptomycetes develop within 14 days at 25-30 °C while thermophilic types grow within 5 days at 45-60 °C (Goodfellow and Simpson, 1987).

Streptomycetes are commonly found in soil, composts and fodders, and aquatic ecosystems. They can be pathogenic to plants, humans and animals (Goodfellow and Simpson, 1987). However, the scope of this review is limited to those in soil for their relevance in biocontrol of soil-borne plant pathogens.

Streptomycetes in soil exist for long periods as arthrospores or chlamydospores that germinate in the presence of exogenous nutrients (Lloyd, 1969; Mayfield, Williams, Ruddick and Hatfield, 1972). "Hyphae" of streptomycetes may rapidly colonize organic substrates such as root fragments and fungal hyphae, suggesting antagonistic potential. Colonies of streptomycetes are restricted to 20-

200 µm diam. Sporulation occurs as nutrients become exhausted (Mayfield *et al.*, 1972), and spores can be dispersed above soil by water movement and arthropods (Dzinogov, Marialigeti, Jagar, Contreras, Kondics and Szabo, 1982; Ruddick and Williams, 1972). Thus, soil and water management could be important factors contributing to successful biocontrol using streptomycetes.

Over 55% of the antibiotics detected between 1945 and 1978 (i.e. more than 5,000 compounds) originated from *Streptomyces* (Berdy, 1980). In addition, results from pot trials have shown that antibiotics are produced by *Streptomyces* spp. in both sterile (Rangaswami and Vidyasekaran,1963; Rothrock and Gottlieb,1984) and unsterile soil (Rothrock and Gottlieb,1984), leading to a complete inhibition of spore germination of *Helminthosporium sativum* Pam., King and Bakke in corn rhizospheres (Rangaswami and Vidyasekaran,1963), and reduction in both disease severity index and mean shoot fresh weight per pot for pea infected with *Rhizoctonia solani* (Rothrock and Gottlieb,1984). *Streptomyces* spp. isolated from soil on roots of Casuarinas in New South Wales inhibited growth of *Phytophthora cinnamomi in vitro* in dual culture (Gerrettson-Cornell, 1991). Also *Streptomyces* spp. may have contributed to disease suppression in bean plants artificially inoculated with sclerotia of *S. rolfsii* which had been exposed to sublethal temperatures (Lifshitz, Tabachnik, Katan and Chet, 1983). Reductions in saprophytic growth of *S. rolfsii* and mortality rates of barley plants inoculated with this pathogen in the presence of *Streptomyces*, were also observed (Singh and Dwivedi, 1987). Although *Streptomyces* spp. have considerable potential in biocontrol of *S. rolfsii*, little work has been done in this area and interactions between the organisms have not been studied in any detail.

1.2.11 Mechanisms of biological control of *S. rolfsii*

To control a soil-borne plant pathogen, an antagonist should rapidly colonise the soil in advance of the pathogen and/or compete successfully for substrates. The ability to produce antibiotics and/or interfere with growth and development are also desirable characteristics for the antagonist. Factors determining successful biological control of *S. rolfsii* comprise: inoculum of the pathogen and its colonisation

of host plants, competition, mycoparasitism, antibiosis and fungistasis (Campbell, 1989).

1.2.11.1 Inoculum of the pathogen and colonisation of the host

Although chemotaxis or chemotropism may occur with some fungi, generally it is the plant that grows towards the inoculum of the pathogen in soil. The inoculum potential of the pathogen may be lowered by competition with resident microorganisms, resulting in reduced colonisation of the plant by the pathogen. Thus inoculum potential and colonisation could be affected by the amount and activity of a biocontrol agent applied to potting mix or soil. Optimisation of the formulation, method of application and survival of these biocontrol agents would promote their competitiveness against the pathogen. This means that a critical amount of antagonist should be placed as close as possible to the proposed point of action in a form which will survive for the period of time required to protect the plant (Campbell, 1989).

The significance of inoculum potential of the pathogen in relation to biocontrol strategies was demonstrated in an attempt to control *Pythium ultimum* Trow, causal agent of post-emergence damping-off and root rot of cucumber, in a growth chamber (Wolffhechel and Funck-Jensen, 1992). Three antagonistic strains of both wild type and mutant *T. harzianum* and one wild type *G. virens* were applied either as conidial suspensions (direct inoculum) or with peat-bran as a food base. The test strains, applied as 1% peat-bran preparations (by volume of the sphagnum peat growth medium) simultaneously with the pathogen, 4 days before transplanting seedlings, were equally effective in reducing the disease index of cucumber 7 days after planting. Phytotoxicity and a reduction in efficacy of the application were observed when the rate of antagonist inoculum was increased to 5%. When applied as suspensions of 10^5, 10^6 and 10^7 conidia per ml of growth medium, however, the antagonists protected the plants without causing toxicity, suggesting that the production of phytotoxic substances could have been stimulated by the peat-bran mixture. It was also possible that *P. ultimum* had taken advantage of the excess

nutrient source to build up its inoculum potential, hence a carefully developed formulation of antagonist is desirable to avoid possible deleterious effects.

1.2.11.2 Competition

By its nature, competition is inhibitory but not lethal. Competition for infection sites was demonstrated in a study on soil suppressiveness to *Pythium ultimum* (Lifshitz, Sneh and Baker, 1984a). These authors found a decrease in the population density of the pathogen when bean leaves were periodically applied to a sandy loam soil. This suppression was accompanied by an increase in population density of an antagonist, *Pythium nunn* Lifshitz *et al.* (Lifshitz, Stangehellini and Baker, 1984b). When colonization of bean leaves by *P. nunn* and *P. ultimum* was followed in the soil (Paulitz and Baker, 1988), it was observed that reduction of sporangial germination of *P. ultimum* was induced by competition from *P. nunn*. Although, *P. nunn* occupied bean leaves after *P. ultimum*, it colonized almost all of the tissues and sporangial counts of the pathogen declined to low levels. A series of studies has shown lysis of germ tubes and reduction in size and vigour of secondary sporangia of *P. ultimum*, suggesting that mechanisms other competition, such as antibiosis and mycoparasitism, may have contributed to the ultimate decline in inoculum potential of *P. ultimum* (Lifshitz *et al.* 1984a).

As well as competition for infection sites, carbon competition and immobilisation have been investigated. Competition for carbon sources in a bark compost suppressive to *Pythium* spp. was shown as a rapid decline in levels of glucose, reducing sugars and total carbohydrates, compared to those in a conducive compost (Chen, Hoitink, Schmitthenner and Tüovinen, 1988). One microorganism may compete with others for carbon sources through immobilization. This was shown by a negative correlation between labile carbon content and formation of secondary sporangia of *P. ultimum* (Paulitz and Baker, 1988). In the same study, when *P. nunn* was added as an antagonist, a decrease in density of sporangia of *P. ultimum* was recorded, suggesting that available carbon was immobilized by this antagonist.

Although the C:N ratio is known to be important in growth of microorganisms, little has been reported on competition for nitrogen *per se*. It was found that the addition of chitin and lignin to soils induced suppression of bean root rot caused by *Fusarium solani* f. sp. *phaseoli* (Berk.) Snyder & Hansen (Maurer and Baker, 1965). Nevertheless, the addition of nitrogen to the system failed to demonstrate competition for nitrogen because suppression increased. The explanation could be that there was no strong competition for nitrogen in the soil tested and the nitrogen added was more beneficial to competitors than to the pathogen. Thus, successful biological control could be achieved by manipulating the type and amount of nitrogen added to the soil.

1.2.11.3 Mycoparasitism

An antagonist may simply use a pathogen as a food source. If the pathogen is a fungus, the antagonist is a mycoparasite which may cause exolysis of its host. Exolysis (heterolysis) is the destruction of a cell by enzymes of another organism and frequently results in the death of the attacked cell. Such enzymes include chitinases and cellulases (Campbell, 1989).

Trichoderma is recognised as a mycoparasite of several soil-borne plant pathogens. Its hyphae may penetrate resting structures such as sclerotia or may parasitize growing hyphae of the pathogen. Results from a recent study have shown that it is possible for *Trichoderma longibrachiatum* Rifai and *G. virens* to become established and antagonise *S. rolfsii* in low pH conditions, favourable for growth and development of the pathogen (Sreenivasaprasad and Manibhushanrao, 1990). Isolates of the antagonists grew well in acidic potato dextrose agar (PDA) and water agar (WA). The area of the colonies of mycoparasites invading the pathogen on dual culture plates was assessed at 48, 96 and 144 hours. Results revealed that, although *S. rolfsii* first over-grew all test mycoparasites, *G. virens* later invaded the pathogen. Using Scanning Electron Microscopy and Fluorescence Microscopy, it was demonstrated that the hyphae of antagonistic *Trichoderma* spp. and *G. virens* grew alongside those of *S. rolfsii*, and produced side branches which formed appressoria or hooks for attachment,

or coils around *S. rolfsii* hyphae (Elad, Chet, Boyle and Henis, 1983; Lim and Teh, 1990; Sreenivasaprasad and Manibhushanrao, 1990). Vacuolation, granulation and coagulation of the host cytoplasm and ultimately cell death were reported and loss of cytoplasm also occurred in cells of adjacent hyphae not in direct contact with the antagonistic hyphae (Lim and Teh, 1990). The mycoparasite sometimes penetrated the host mycelium by partially degrading its cell wall. As cell walls of *S. rolfsii* are composed of ß-1,3-glucan and chitin, the mode of antagonism was investigated through ß-1,3-glucanase and chitinase assays (Elad *et al.*, 1983). These authors demonstrated a doubling in the activities of glucanase and chitinase in dual cultures compared to those in pure cultures of either *S. rolfsii* or *T. harzianum*, implicating these enzymes in mycoparasitism.

1.2.11.4 Antibiotic production

Antibiotics produced by some microorganisms may have a deleterious effect on the growth and development of others, generally by a reduction or cessation of growth or sporulation, reduction in germination and/or deformation of hyphae (Campbell, 1989). Effects vary depending upon the susceptibility of the host to the antibiotic. If the antibiotic is lethal, then endolysis (the breakdown of the cytoplasm of a cell by the cell's own enzymes) may be involved. Since antibiotics are commonly produced by soil microorganisms, they are, therefore, assumed to be active in the soil (Campbell, 1989). However, reports on antibiotic production in soil are scarce. This could be due to a lack of techniques for assessment together with the possibility of these antibiotics being adsorbed by organic matter or clay particles (Singh and Faull, 1988).

In vitro studies have demonstrated production of volatile (Claydon *et al.*, 1987; Dennis and Webster, 1971b; Ghisalberti *et al.*, 1990) and non-volatile antibiotics (Dennis and Webster, 1971a; Lim and Teh, 1990: Ridout, Lumsden and Hruschka, 1992) by isolates of *Trichoderma* spp. and *Gliocladium virens.* Non-volatile antibiotics from *T. viride*, *T. hamatum* (Bon.) Bain, *T. polysporum* (Link ex Pers.) Rifai and *G. virens* were shown, by means of the cellophane overlay technique,

to affect fungi representing the various subdivisions (Dennis and Webster, 1971a). Observation of colony diameters and hyphae of the test fungi revealed both fungicidal and fungistatic effects of antibiotics produced by some isolates of *Trichoderma*; response depended on the test fungus used. There was considerable variation in the production of diffusible inhibitory substances within, as well as between, species-groups of *Trichoderma*. Antibiotic production by the most active isolates, i.e. *T. harzianum*, *T. hamatum*, *T. polysporum*, *T. koningii* Oud., *T. pseudokoningii* Rifai, *T. longibrachiatum*, *T. viride* and *G. virens*, was investigated further in liquid culture at pH 4.0 and 6.5 using Weindling's medium and a malt extract medium, of low and high nutrient status, respectively. Measurement of colony diameters of the test fungi showed that the *Trichoderma* isolates produced antibiotics at higher or similar pH in malt extract medium compared with the Weindling's medium. In contrast to the *Trichoderma* isolates, the production of antibiotics by *G. virens* in malt extract medium was much less than in Weindling's medium at both pH levels. These results indicated differences in conditions required for production of antibiotics by *Trichoderma* and *G. virens*. Therefore, production of antibiotics effective against certain plant pathogens in soil, by these antagonists, would depend upon the particular antagonist and the growing conditions. The antibiotics produced by *T. viride* and *T. hamatum* in this study were identified as trichodermin and U-21,963 ('Dermadine'), respectively (Dennis and Webster, 1971a). These antibiotics possessed both antifungal and antibacterial properties. In addition, gliotoxin and viridin were identified as antibiotics produced by *G. virens*. Also, using the cellophane overlay technique employed by Dennis and Webster (1971a), it was shown that all test *Trichoderma* spp. produced inhibitory metabolites which diffused through the medium (MEA) and inhibited the growth of test fungi including *S. rolfsii*. Colony growth of *S. rolfsii* was most affected by *T. koningii* followed by *T. harzianum* and *T. hamatum*, respectively (Lim and Teh, 1990).

Dennis and Webster (1971b) reported the effect of volatile antibiotics produced by 12 isolates of *Trichoderma* spp. and two of *G. virens* on *in vitro* growth of

the same set of fungi as in Dennis and Webster (1971a). When grown on 2% malt extract agar, *G. virens* did not produce volatile antibiotics, whereas isolates of *Trichoderma* produced a range of volatile antibiotics with different effects on different test fungi. The active metabolites had the same morphological effect on all the susceptible test fungi including *R. solani*; mycelial growth was stunted and hyphae were more highly branched than normal. Unlike the non-volatile compounds, no fungicidal effects were observed with any of the test fungi used. The active isolates of *T. viride*, *T. koningii* and *T. hamatum* were all characterized by a definite 'coconut' smell. Despite the failure of one such isolate of *T. koningii* to inhibit growth of *R. solani*, the authors reported that there was a strong correlation between production of the smell and production of volatile metabolites. The isolates were thought to produce either the same complex of metabolites in different proportions, or entirely different chemical compounds. Similar results were found in a study of the effects of volatile metabolites produced by *T. harzianum*, *T. hamatum* and *T. koningii* against *S. rolfsii in vitro* using the method of Dennis and Webster (1971b)(Lim and Teh, 1990). The authors reported that all the test *Trichoderma* spp. produced volatile metabolites inhibiting the colony growth of *S. rolfsii* in varying magnitudes. The inhibitory effect produced by volatile metabolites was almost 50% less than that produced by non-volatile metabolites. This experiment had a high coefficient of variation of the results (up to 48%), which perhaps reflected the difficulty of the assessment of these metabolites or variability in the production of metabolites.

Antibiotic efficacy of 300 isolates of actinomycetes was observed against six different soil-borne fungi *in vitro* using a modified agar-ring method (Turhan and Grossmann, 1986). These authors found variation between the test fungi in their sensitivity to the antibiotic effect of the actinomycete isolates. *Sclerotinia sclerotiorum* Lib. de By. was the most susceptible. Results suggested the potential of actinomycetes as a biocontrol agent for soil-borne plant pathogens. This was supported by a study on biological control of *S. rolfsii*, causing foot rot of barley (Singh and Dwivedi, 1987). Among the six isolates of antagonists tested, *Streptomyces diastaticus*

reduced growth of *S. rolfsii* by 39% in dual cultures, and reduced host plant mortality rates by almost 40% in pot experiments. These authors, however, found stronger inhibition effects, up to 88% reduction in growth of *S. rolfsii* and 70% reduction in mortality rates of host plants, with *Trichoderma* isolates in dual cultures and pot trials, respectively.

1.2.11.5 Fungistasis

Fungistasis has been studied since the 1960s. The term is defined as the imposition of dormancy, especially for fungal spores, by nutrient limitation, mainly a shortage of available carbon (Lockwood, 1986). Many pathogens produce resting structures of various kinds that remain dormant in soil until nutrients are available. Saprotrophic microorganisms may reduce the level of available carbon, causing fungistasis of the pathogen, preventing germination and subsequent infection. If available carbon is added above the needs of the saprotrophic competitors, then the germination of pathogens may be stimulated as fungistasis is broken (Campbell, 1989). Therefore, the practical use of fungistasis in biocontrol is in the manipulation of the carbon status to encourage the growth of saprotrophs at the expense of pathogens.

Fungistatic effects of organic amendments on survival of *S. rolfsii* causing betelvine disease, were investigated in pot experiments (Palakshappa *et al.*, 1989). Groundnut and safflower cakes applied to both sterile and non-sterile soils were the best soil amendments, reducing survival of sclerotia of *S. rolfsii* with maximum effect 15 days after application. Concurrently, there was an increase in microbial population, up to 45 days after the application. *T. viride* and *Streptomyces* spp. were among the microbes isolated from these induced-suppressive soils and showed antagonistic effects on *S. rolfsii*. These results demonstrated some role for fungistasis in biological control. Disease level was not mentioned, but it is assumed that the reduction in viability of sclerotia would have led to a reduction in disease incidence and/or severity.

Fungistasis can also be promoted by chemical application. The efficacy of 2-furfuraldehyde for control of *S. rolfsii* on lentil was studied in both laboratory and

greenhouse experiments (Canullo, Rodriguez-Kabana and Kloepper, 1992). Mycelial growth of the fungus was reduced proportionally with concentrations of 0.1-0.5 ml furfuraldehyde incorporated into agar medium, and viability of sclerotia diminished on exposure to 2-furfuraldehyde vapour. The chemical, when added to agar on which was plated soil dilutions, reduced the populations of fungi, including *Trichoderma* spp., and bacteria in untreated soil. On the contrary, repeated experiments in a greenhouse using natural soil treated with the fumigant showed increased populations of *Trichoderma* spp. and bacteria, but diminished numbers of actinomycetes. In addition, increasing dosages applied to soil artificially infested with *S. rolfsii* reduced the level of disease on the test plants. The discrepancy between results from laboratory and greenhouse experiments could be due to the fact that beneficial microorganisms were sensitive to furfuraldehyde concentations used for soil dilution plates, while adsorption by clay particles and organic matter may have reduced the effective concentration in soil. It could also due to errors in soil sampling methods.

1.2.12 Conclusion

Biological control may be one of the best alternatives to chemical control, considering the effect on environment. The successful application of a biocontrol agent, however, depends upon an understanding of interactions between the pathogen and antagonists with respect to the control mechanisms. This study, therefore, aimed at examining some aspects of the interactions between selected antagonists and *S. rolfsii*, and relating these to the efficacy of the potential biocontrol agents applied in simulated natural conditions.

Chapter 2

Isolation of microorganisms from a field site

2.1 Introduction

Soil is a complex and competitive environment for microorganisms (Harman and Lumsden, 1990). Disease suppressive soil is defined as soil in which disease development is suppressed when a pathogen is present or is introduced in the presence of a susceptible host (Cook, 1981; Schneider, 1982). Soil of the rhizosphere, i.e. around plant roots, can be unfavourable to pathogens due to the presence of antagonists as part of the rhizosphere community (Lynch, 1987). This soil in certain situations is regarded as 'suppressive' and provides a useful source of biocontrol agents. However, in the absence of identifiable disease suppressive soils, it is suggested that an alternative means of isolating biocontrol agents is to examine healthy plants in otherwise heavily infested agricultural sites (Lumsden and Lewis, 1989).

Bacteria and fungi are major components of the soil microbial community (Paul and Clark, 1989). Population counts of these microbes are commonly obtained from soil dilution plates and selective media (Crawford, Lynch, Whipps and Ousley, 1993; Martin, 1950; Papavizas and Lumsden, 1982; Rothrock and Gottlieb, 1984; Vickers and Williams, 1987). These populations are, at best, only rough estimates, since the microflora is very diverse and not all organisms can be cultured on laboratory media (Alexander, 1977). Potential antagonists may be isolated by means of selective media, and the sclerotia baiting technique, in particular, has been used to isolate microbes antagonistic against sclerotial-forming pathogens (Knudsen, Eschen, Dandurand and Bin, 1991; Trutmann and Keane, 1990; Zuberer, Kenerley and Jeger, 1988).

In this study, the sclerotia baiting technique was used to isolate fungi potentially antagonistic to *S. rolfsii*, while the soil dilution plate and selective medium techniques were used for the enumeration of soil microbial populations in order to

provide information on the microflora of the site. Representative actinomycete colonies growing on these plates were screened for potential antagonism to *S. rolfsii* on a "soil mimic medium" (1/5M32; Sivasithamparam, Parker and Edwards, 1979) and selected on the basis of inhibition zones around the actinomycete colonies. The aim was to obtain a small number of potential antagonists for detailed study.

2.2 Materials and methods

Medium formulations are given in **Appendix 1**.

2.2.1 Isolation of *S. rolfsii*

S. rolfsii was isolated from mature diseased capsicum grown at Penfield Gardens, South Australia in 1990. The soil was a sandy loam over permeable clay loam, pH 5.5. Sclerotia (Insert erratum 1 (page 162).) placed onto 18 ml potato dextrose agar (PDA) in 9 cm Petri plates and incubated at 25°C in the dark for 3 days. Cultures were identified with the aid of a microscope. They were used as sources of inoculum of the pathogen for further study. Sub-cultures were also made on PDA slants, incubated under the same conditions for 14 days, to confirm production of sclerotia, and kept at 4 °C as stock cultures.

2.2.2 Soil dilution plate method

Four 15x15 cm^3 soil samples were taken at random from root zones of plants in disease-free areas in the capsicum field described above. They were bulked and mixed thoroughly. Five Petri plates of each of four different agar media were prepared: a) 1/5M32, pH adjusted to 5.5, for total microbial populations (Sivasithamparam *et al.* , 1979); b) crystal violet agar (CVA) for isolation of Gram negative bacteria (Bakerspigel and Miller, 1953); c) rose bengal agar (RBA) for the total fungal population (Martin, 1950; Papavizas and Davey, 1959; Smith and Dawson, 1944) and d) casein glycerol medium (CGM) for isolation of Streptomycetes (Küster and Williams, 1964). Serial dilutions were prepared to 10^{-4} (Seeley and VanDemark, 1981) in Ringer's solution (McLean and Cook, 1941). One ml aliquots of diluted soil suspension were plated out. Five replicates were prepared for each dilution on each agar medium. The plates were incubated at 25°C in the dark and colonies

appearing on the media were recorded daily for 7 days. Anaerobic incubation was not attempted. Microorganisms from 1/5M32 plates were identified to major groups, and those from RBA and CGM to genus. Twenty common colony types of bacteria other than actinomycetes were characterised by Gram staining (Skerman, 1967), and tested for fluorescence on King's B medium (King, Ward and Raney, 1954).

2.2.3 Isolation and preliminary screening of antagonists

2.2.3.1 Actinomycetes

Twenty colony types of actinomycetes on CGM were identified to genus level using Shirling and Gottlieb's method (1966). Fourteen colonies of actinomycetes were purified and screened for antagonistic activity against *S. rolfsii* in dual cultures in Petri plates of 1/5M32 agar medium. Half of a Petri plate was evenly smeared with one isolate of actinomycetes using sterile cotton buds. These plates were incubated at 25°C in the dark for 7 days prior to inoculation of the pathogen. *S. rolfsii* was grown on 1/5M32 agar medium for 3 days. Plugs of mycelium were cut from the leading edge of these cultures using a 1.1 cm diam cork borer. A plug of *S. rolfsii* was then placed on the uninoculated half of each Petri plate. Controls consisted of *S. rolfsii* grown on 1/5M32. These plates were incubated at 25°C in the dark. There were ten replicates for each isolate. Growth of *S. rolfsii* was observed daily for 7 days.

2.2.3.2 Fungi

Twenty mature sclerotia were collected from 14 day-old cultures of *S. rolfsii*, and divided into four lots of five. Each lot was placed in a 4x5 cm muslin bag and incubated at 25°C for 7 days in soil associated with healthy plants from Penfield Gardens. Sclerotia were then retrieved and surface-sterilised with 10% White King domestic bleach (0.4% available chlorine) plus a drop of wetting agent (Tween 80) for 2 min. Sclerotia were then rinsed three times with sterile distilled water, 5 min each rinse. These sclerotia were placed on 18 ml of RBA, one per plate, and incubated at 25°C in the dark for up to 7 days. Colonies of fungi growing out from sclerotia were

sub-cultured on to malt extract agar (MEA), examined using a microscope and identified using Rifai's key (1969).

2.3 Results

2.3.1 Enumeration of microbial populations

Total counts of soil microbial populations are presented in **Table 2.3.1.1**. The majority of soil microbes isolated was bacteria. Colonies of bacteria other than actinomycetes developed most rapidly, followed by those of fungi and actinomycetes. Using selective media, microorganisms were identified to major genera, forms or types and enumerated (**Table 2.3.1.2**). There were approximately 9.4 x 10^3 colony forming units (cfu) of fungi isolated on RBA, per gram soil (**Table 2.3.1.2a**). About 50% of these fungal colonies sporulated (**Table 2.3.1.2b**). Among them, *Penicillium* was the predominant genus followed by *Aspergillus*, while *Trichoderma* and *Gliocladium* accounted for 8% of the sporulating colonies. Approximately 6.3 x 10^5 cfu of actinomycetes per gram soil, appeared on CGM (**Table 2.3.1.2c**). The majority of actinomycetes isolated belonged to the genus *Streptomyces*. Similar numbers of *Streptomyces* colonies with Rectus and Spira forms were observed. About 2.15 x 10^6 bacteria other than actinomycetes, per gram of soil, were isolated on to CVA (**Table 2.3.1.2d**). Examination of 20 representative colony types showed that most were Gram negative, with 85% rod-shaped, 75% spore-forming and 10% were fluorescent non-spore-formers.

2.3.2 Antagonist screening and identification

2.3.2.1 Actinomycetes

S. rolfsii completely over-grew all but three of the 14 isolates of actinomycetes tested. In dual cultures with two of these three actinomycete isolates, growth of the pathogen was sparse and stopped at the edge of the actinomycete colony, whereas when paired against the third isolate, colony growth of *S. rolfsii* was unusually fluffy and over-grew only two-thirds of the actinomycete colony. All three

Table 2.3.1.1 Total microbial populations (cfu x 10^4 per gram soil) on 1/5M32 agar medium over 7 days (mean of five replicates with standard error of the mean in parentheses).

Day	Microorganisms			Total
	Actinomycetes	Other bacteria	Fungi	
1	0.0	119.6 (2.72)	0.0	119.6
2	0.0	84.2 (1.84)	1.4 (0.22)	85.6
3	17.2 (0.52)	16.8 (0.72)	0.8 (0.18)	34.8
4	2.6 (0.36)	7.6 (0.46)	0.6 (0.22)	10.8
5	2.8 (0.34)	4.4 (0.22)	0.0	7.2
6	0.0	0.0	0.0	0.0
7	0.0	0.0	0.0	0.0
Total	22.6	232.6	2.8	258.0

Table 2.3.1.2 Enumeration of microorganisms

Table 2.3.1.2a Total fungi isolated from soil on to rose bengal agar over 7 days (cfu x 10^2 per gram soil), mean of five replicates. SE = standard error of the mean.

Day	1	2	3	4	5	6	7	Total
Mean	12.6	68.8	4.8	4.0	2.4	1.2	0.0	93.8
SE	0.51	0.97	0.38	0.32	0.25	0.20	0.00	

Table 2.3.1.2b Major genera of sporulating fungi, isolated from soil on to rose bengal agar over 7 days (cfu x 10^2 per gram soil), mean of five replicates. SE = standard error of the mean.

Genera	*Penicillium*	*Aspergillus*	*Trichoderma* & *Gliocladium*	Total
Mean	33.0	8.0	3.6	44.6
SE	1.48	0.71	0.60	

Table 2.3.1.2c Actinomycetes isolated from soil on to casein glycerol medium over 7 days (cfu x 10^4 per gram soil), mean of five replicates. SE = standard error of the mean.

Day	1	2	3	4	5	6	7	Total
Mean	0.0	0.0	30.6	20.0	6.2	3.6	2.2	62.6
SE	0.0	0.0	1.75	1.14	0.58	0.60	0.49	

Table 2.3.1.2d Bacteria other than actinomycetes isolated from soil on to crystal violet agar over 7 days (10^4 per gram soil), mean of five replicates. SE = standard error of the mean.

Day	1	2	3	4	5	6	7	Total
Mean	149.6	65.0	0.0	0.0	0.0	0.0	0.0	214.6
SE	2.99	3.36	0.00	0.00	0.00	0.00	0.00	

isolates were identified as *Streptomyces* and the two which caused sparse growth of *S. rolfsii* were considered to belong to the same species (I. Kurtböke, Soil Science and Plant Nutrition, University of Western Australia). Two isolates, one representing each species, were chosen for further study and coded s1, which caused sparse growth of *S. rolfsii*, and s2, which stimulated fluffy growth of the pathogen (**Plate 2.3.2.1**). They had Flexibilis and Rectus forms, respectively.

2.3.2.2 Fungi

All 20 baited sclerotia yielded fungal colonies other than *S. rolfsii*. Of the 20 colonies growing out from baited sclerotia, only 14 produced spores. Two most common sporulating isolates were identified as *T. harzianum* and *G. virens* (**Plate 2.3.2.2**), and identification was confirmed by M. Priest at the Biological and Chemical Research Institute, New South Wales. These isolates were selected for use in subsequent studies.

2.4 Discussion

The study reported in this chapter was undertaken to give estimates of microbial populations in soil, from which possible antagonists were isolated under the conditions applied. This approach only gives an overview of the soil microbial community. Insert erratum 2 (page 162). 1/5M32 was designed as a medium with nutrient status equivalent to that of natural agricultural soil (Sivasithamparam *et al.*, 1979). Therefore, it was assumed that this medium would allow isolation of a more representative soil microbial population than would rich media such as PDA and MEA, which have been used in such studies previously. Results obtained using 1/5M32 were in general agreement with previously published work (Ridge and Rovira, 1971; Sivasithamparam *et al.*, 1979). It is known that RBA inhibits growth of actinomycetes and most bacteria and reduces spreading of fungal colonies on plates prepared from some soils (Martin, 1950; Papavizas and Davey, 1959). In this study, RBA allowed estimation of the fungal population without interference from bacteria. Although several selective media for isolation of *Trichoderma* and *Gliocladium* have been improved (Davet, 1979; Elad and Chet, 1983; Papavizas and Lumsden, 1982), RBA

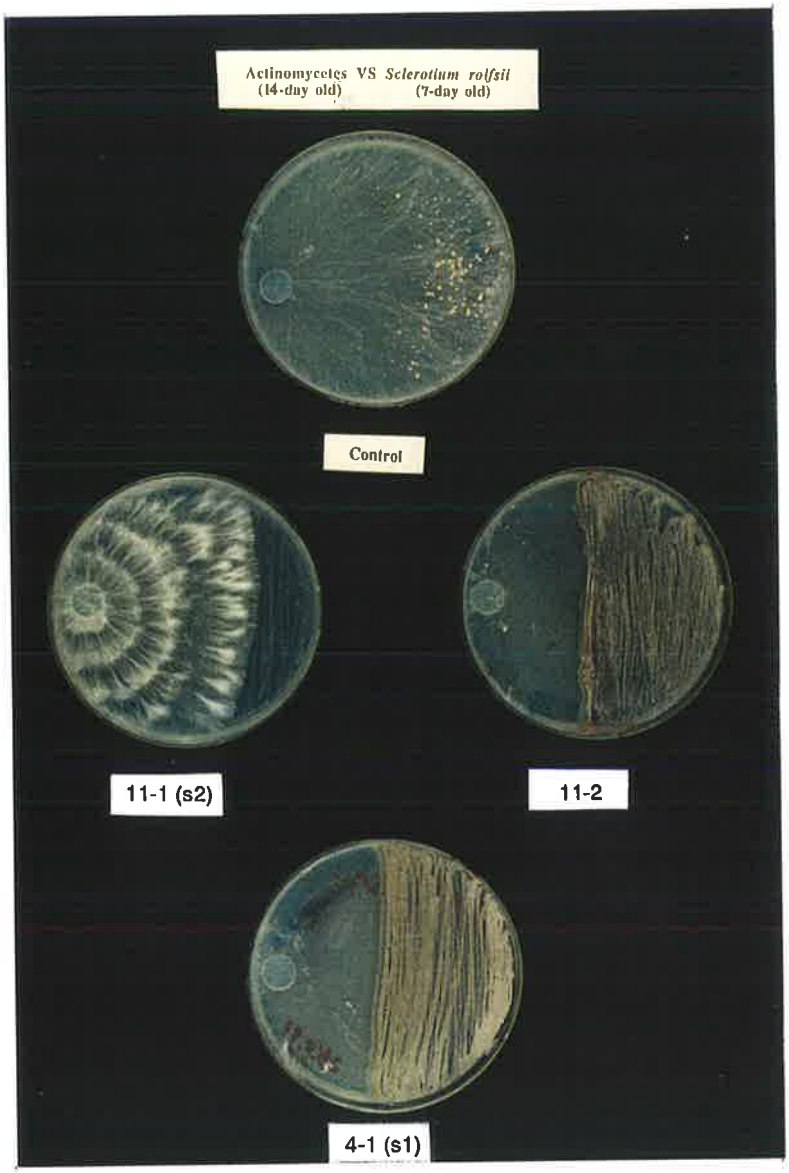

Actinomycetes VS *Sclerotium rolfsii*
(14-day old) (7-day old)
Control
11-1 (s2)
11-2
4-1 (s1)

Plate 2.3.2.1 Screening for actinomycetes potentially antagonistic to *S. rolfsii* by means of the dual culture technique, using 1/5M32 agar.

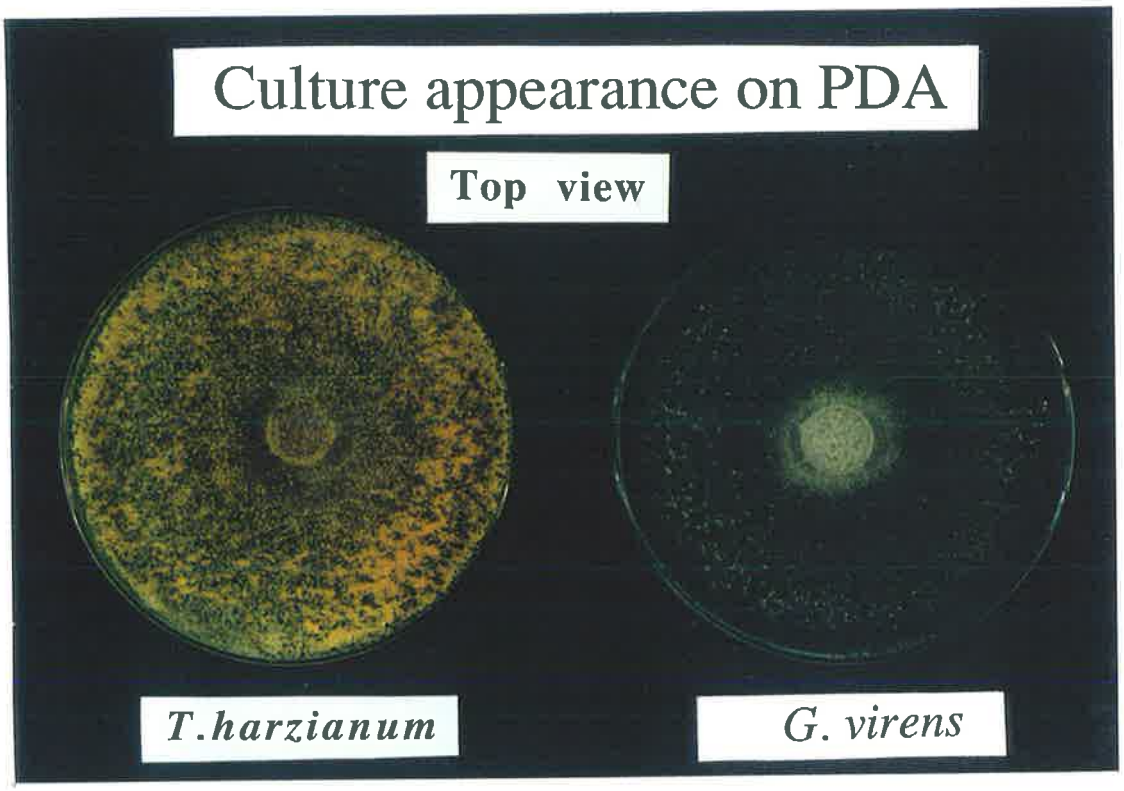

Culture appearance on PDA
Top view
T.harzianum
G. virens

Plate 2.3.2.2 Cultures of *T. harzianum* and *G. virens*, 7-days old on PDA.

as originally used by Martin (1950) was effective in this study. It has been reported that CVA inhibits growth of Gram positive bacteria (Bakerspigel and Miller, 1953); this was confirmed. Bacteria other than actinomycetes isolated were mainly Gram negative, rod shaped and spore-formers. Colonies with ability to fluoresce were likely to be *Pseudomonas* species. By subtracting numbers of Gram negative bacteria on CVA from the total counted on 1/5M32, the number of Gram positive bacteria was estimated to be 4.3×10^5 per gram soil. *Penicillium* and *Aspergillus* were the most common genera of soil fungi identified. In the rhizosphere community, possible interactions between these microorganisms in soil would have an impact on the success of biological control using soil microbial antagonists.

To initiate this study on interactions between *S. rolfsii* and selected antagonists, four isolates of soil microorganisms comprising two fungi and two actinomycetes were selected by means of the soil dilution plate, selective medium and sclerotial baiting technique, and investigated for potential as biocontrol agents in subsequent experiments.

Chapter 3

In vitro assessment of antagonism by selected antagonists against *S. rolfsii*

3.1 Introduction

Once potential antagonists have been isolated from soil, evaluation of their efficacy is necessary. *In vitro* and soil tests on efficacy of antagonists are based on possible modes of antagonism, comprising competition, parasitism and antibiosis (as discussed in section **1.2.9**). One of the techniques commonly used to detect antagonism *in vitro* is dual culture (Boosalis, 1964; Harman, Chet and Baker, 1980; Chet and Baker, 1981; Elad *et al.* , 1983), which allows observation of the effect of the antagonist on growth and survival of a pathogen. Nutrient levels can have an impact on this effect due to nutrient competition between the pathogen and the antagonist (Chen *et al.*, 1988; Paulitz and Baker, 1988), and this is discussed in section **1.2.11.2**. Antibiotics of a non-volatile nature, produced by some species of microorganisms known to be antagonistic to certain soil-borne pathogens, have been extensively studied by means of the cellophane overlay (Dennis and Webster, 1971a; Lim and Teh, 1990; Lockwood, 1959) and culture filtrate techniques (Dennis and Webster, 1971a; Ridout *et al.* , 1992)(as discussed in section **1.2.11.4**). The principle of these two methods is based on the production of active metabolites by the antagonist, which diffuse into agar medium and inhibit growth and survival of the pathogen.

The aim of the experiments reported in this chapter, was to evaluate the effect of selected antagonists on growth and survival of *S. rolfsii*, based on modes of antagonism. Experiments were, therefore, carried out *in vitro* to determine the effect of *T. harzianum* and *G. virens* on; a) sclerotial viability of *S. rolfsii* in dual cultures, in relation to nutrient status of the growth medium; b) interactions between the hyphae of these antagonists and the pathogen in dual cultures; and c) colony growth of *S. rolfsii*, by means of production of non-volatile metabolites on agar and in liquid media of different nutrient status. Selected experiments were repeated, with

appropriate modifications, with *Streptomyces* s1 and s2 as antagonists. Only one culture medium, 1/5M32, was used in studies of s1 and s2 because this formulation is suitable for both bacteria and fungi.

3.2 Materials and methods

Formulations of soil extract agar (SEA) and 1/6 neutral dox yeast (NDY) used in experiments reported in this chapter are given in **Appendix 1.**

The rationale for the experiment is stated briefly at the beginning of the section, where necessary.

3.2.1 Production of sclerotia of *S. rolfsii*

S. rolfsii was cultured on 1/5M32 agar plates, 9 cm diam, at 25°C in darkness for 3 days, after which disks, 1.1 cm diam, were cut from the colony margin and used to inoculate moistened carrot slices, each 1 mm thick, in Petri plates. Plates were sealed with Parafilm and incubated at 25°C in darkness for 10 days, by which time most sclerotia were mature. Mature sclerotia were used in all relevant experiments, unless otherwise stated.

3.2.2 Effects of *T. harzianum*, *G. virens* and nutrient status of the culture medium on sclerotial viability

Petri plates of water agar (WA; 15 g Oxoid No. 1 agar in 1 l distilled water), soil extract agar (SEA) and PDA (Oxoid), containing 18 ml medium per 9 cm diam plate, were prepared. Disks, 1.1 cm diam, were cut from the leading edge of 3 day-old cultures of *T. harzianum* and *G. virens* on PDA. A disk of the antagonist was placed on the agar surface, at one side of the prepared agar plate, 1 cm from the edge. A mature sclerotium was placed opposite the disk of the antagonist, at the same distance from the edge. There were five replicates for each combination of antagonist and culture medium. Control plates were prepared using a disk of uninoculated PDA instead of antagonist. Dual cultures were incubated at 25°C in the dark for 10 days, after which five sclerotia formed in each replicate of each of the three media were randomly collected. Each set of sclerotia was surface-sterilised (as in section **2.2.3.2**), and

plated out on one Petri plate of PDA to test viability. Sclerotium germination was recorded after incubation as above for 5 days. Statistical analysis was performed on these data using the GENSTAT 5 package (Lawes Agricultural Trust, Rothamsted Experiment Station, UK). Analysis of deviance (McCullagh and Nelder, 1983) was carried out instead of analysis of variance due to binomially distributed errors.

3.2.3 Studies on hyphal interactions between the fungal antagonists and *S. rolfsii*

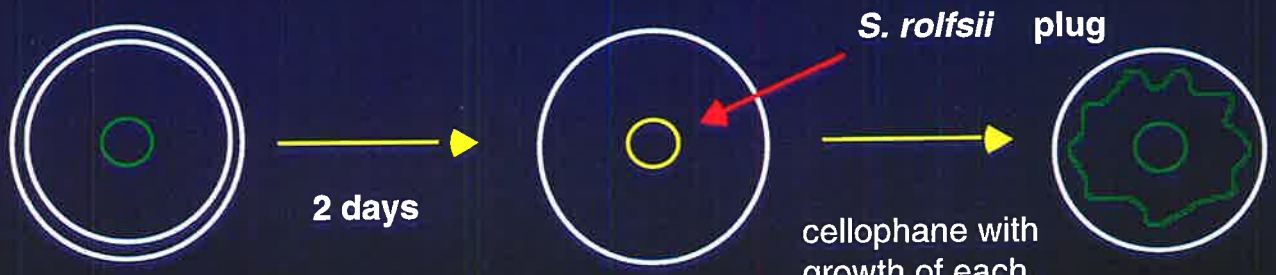
Petri plates of WA, 18 ml medium per 9 cm diam plate, were prepared. Porous uncoated cellophane (Australia Cellophane, Hawthorne, Victoria), 50 µm thick, was cut into 3.0x8.5 cm strips, and autoclaved in distilled water. One strip was placed at the centre of each plate. Disks of *T. harzianum* or *G. virens* and *S. rolfsii*, 1.1 cm diam, cut from the margin of 3 day-old cultures on 1/5M32 were placed 4.5 cm apart on the cellophane strip. There were ten replicates per antagonist X *S. rolfsii* treatment. Control plates were prepared using a disk of sterile 1/5M32 instead of antagonist. Petri plates were incubated at 25°C in darkness for 2 days, at which time mycelium of the pathogen slightly over-grew the colony of the paired antagonist. A piece of the cellophane, 2 cm wide, was cut from the interaction area and stained with lactophenol cotton blue. Hyphal interactions were observed using a Leitz Orthoplan microscope.

3.2.4 Effect of diffusible metabolites produced by *T. harzianum* and *G. virens* in different media, on growth of *S. rolfsii*, using the cellophane overlay technique

The procedure for the cellophane overlay technique is illustrated in **Plate 3.2.4.**

Petri plates of four agar media (1/6 NDY, 1/5M32, 1/2PDA and PDA) were prepared, 100 ml of medium per 15 cm diam plate. Cellophane circles, 14

The Cellophane Overlay Technique



T. harzianum

G. virens

in NDY/6,1/5 M32,

1/2 PDA, PDA.

Plate 3.2.4 The procedure for the cellophane overlay technique. *T. harzianum* or *G. virens* was grown on a circle of sterile cellophane placed on each of four agar media, for 2 days at 25°C in darkness. The cellophane with the adhering antagonist was then removed and a disk of *S. rolfsii* culture was placed on the agar medium at the point previously inoculated with the antagonist.

cm diam, were autoclaved in distilled water. A sterile circle of cellophane was placed over the agar surface in each Petri plate and dried in a laminar flow cabinet for 30 min. Disks, 1.1 cm in diam, were cut from the margin of 3 day-old cultures of *T. harzianum* and *G. virens* growing on 1/5M32 and one disk was placed on the centre of each cellophane circle. For controls, a disk of sterile 1/5M32 was used instead of the antagonist. The plates were incubated at 25°C in the dark for 2 days, after which the cellophane and adhering fungus were removed. A disk of *S. rolfsii*, 1.1 cm diam, was then placed on the agar medium, at the position previously occupied by the antagonist. The Petri plates were incubated in the dark at 25°C. Colony diameter of *S. rolfsii* was recorded daily for 3 days. There were ten replicates per treatment and the experiment was repeated once. Data from the last observations of both experiments were pooled and analysis of variance (ANOVA) was performed using the GENSTAT 5 package.

3.2.5 Effect of diffusible metabolites produced by *Streptomyces* s1 and s2 on growth of *S. rolfsii*, using the cellophane overlay technique

Procedures were similar to **Experiment 3.2.4**, except that 7 day-old cultures of s1 or s2 were evenly smeared on the whole surface of cellophane circles overlaid on 1/5M32 agar medium in 9 cm diam Petri plates. These plates were incubated at 25°C in the dark for 5 days before removal of cellophane and inoculation with *S. rolfsii*. Colony growth of *S. rolfsii* was recorded daily for 3 days, as mean of two measurements made at right angles as colonies were irregular. There were ten replicates per treatment and the experiment was repeated once. Data from the last observations of both experiments were pooled and analysed by ANOVA using the GENSTAT 5 package.

3.2.6 Studies on metabolites produced in liquid culture in two media by *T. harzianum* and *G. virens*

In the initial experiment (**3.2.6.1**), culture filtrates of *T. harzianum* and *G. virens* harvested from 1/5M32 broth and potato dextrose broth (PDB) at day 14 were more inhibitory to *S. rolfsii* than were those produced by day 28. Therefore, in

the next experiment (**3.2.6.2**), filtrates of antagonist cultures grown in these media were harvested on days 3, 7 and 14. Since results were inconclusive, a subsequent experiment (**3.2.6.3**) was designed in which filtrates of cultures in 1/5M32 were harvested on days 2, 3, 7 and 14, to determine the optimal time for assessment of metabolite production. In **Experiments 3.2.6.1-3**, cultures were initiated so that all harvests within an experiment were completed on the same day, to avoid possible deterioration of metabolites during storage.

3.2.6.1 Effect of filtrates from liquid cultures of *T. harzianum* and *G. virens* in two media, harvested at two times, on growth of *S. rolfsii*

The procedure for the culture filtrate technique is illustrated in **Plate 3.2.6.1**.

Liquid media of 1/5M32 and PDB were prepared in Schott bottles, 100 ml per bottle. Each bottle was inoculated with five 1.1 cm diam disks of one antagonist on 1/5M32 agar medium. These broth cultures were left on an orbital shaker at 150 rpm at 25°C in the dark for 14 and 28 days. They were then poured into sterile 250 ml centrifuge bottles through muslin cloth and mycelia were discarded. Filtrates were centrifuged at 10,000 rpm for 10 mins to precipitate spores and cell debris and the supernatants were passed through sterile millipore filters, 0.45 µm, using a vacuum pump. Culture filtrates were used immediately by incorporating at 20% into cooled molten 1/5M32 agar and dispensing into 5 cm diam Petri plates, approximately 8 ml per plate. There were six plates per antagonist for each harvest for each culture medium. Controls were prepared by incorporating PDB or 1/5M32 broth at 20% into cooled molten 1/5M32 agar. Disks of *S. rolfsii*, 4 mm diam, were cut from 3 day-old cultures growing on 1/5M32 agar and one disk was placed on the centre of each agar plate. Petri plates were incubated at 25°C in the dark. Colony diameter of *S. rolfsii* was recorded daily for 2 days. Data on day 2 were analysed statistically by ANOVA using the GENSTAT 5 package and Tukey's test was used for comparison between means

Culture filtrate incorporate into agar medium

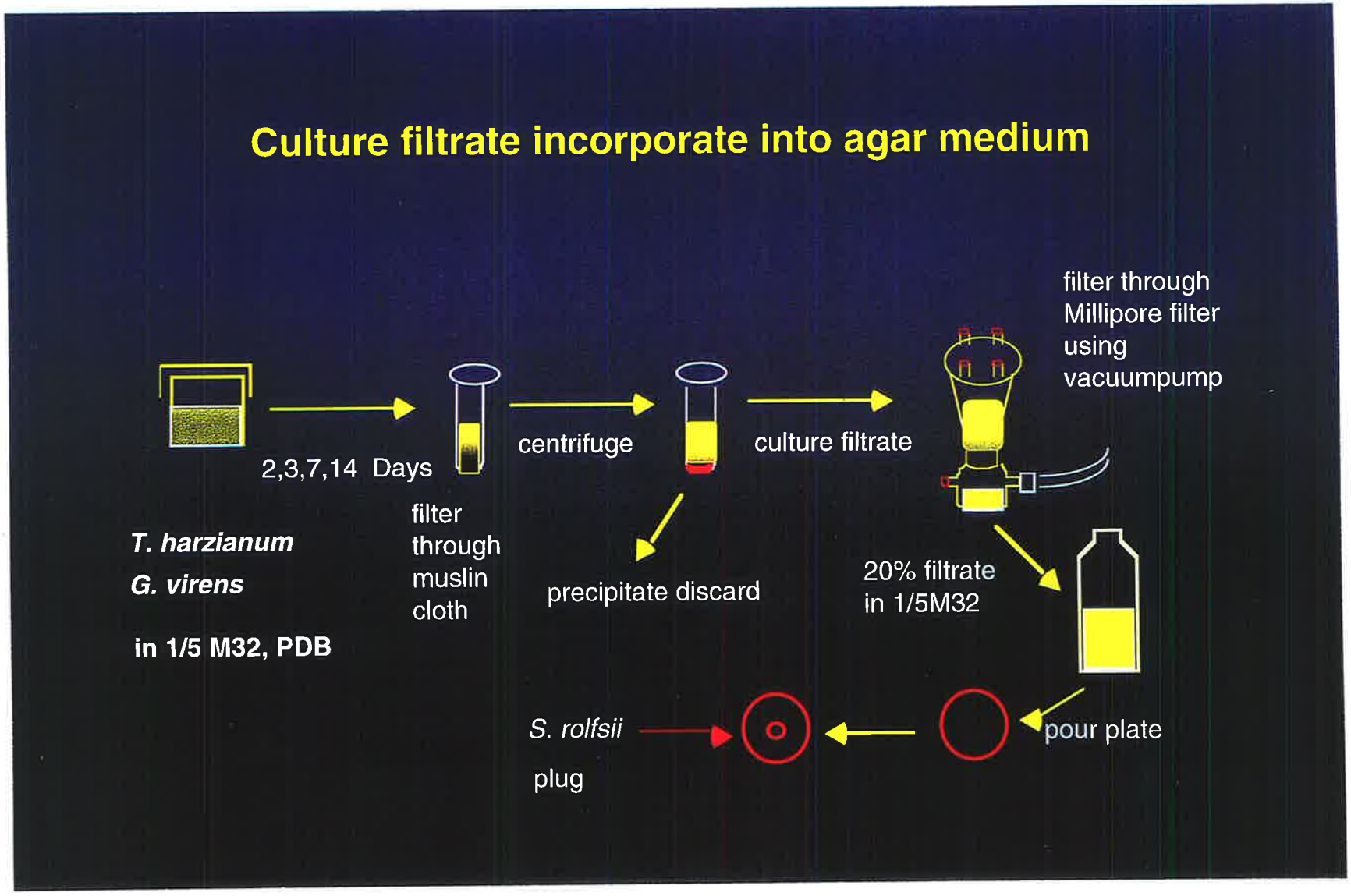


Plate 3.2.6.1 The procedure for the culture filtrate technique.

3.2.6.2 Effect of filtrates from liquid cultures of *T. harzianum* and *G. virens* in two media, harvested at three times, on growth of *S. rolfsii*

Procedures and statistical analysis were as in **Experiment 3.2.6.1**, except that the culture filtrates were harvested on days 3, 7 and 14.

3.2.6.3 Effect of filtrates from liquid cultures of *T. harzianum* and *G. virens* in 1/5M32, harvested at four times, on growth of *S. rolfsii*

Procedures and statistical analysis were as in **Experiment 3.2.6.1**, except that only 1/5M32 medium was used, and culture filtrates were harvested on days 2, 3, 7 and 14 .

3.2.7 Effect of filtrates from liquid cultures of *Streptomyces* s1 and s2 in 1/5M32, harvested at five times, on growth of *S. rolfsii*

Procedures were similar to those in **Experiment 3.2.6.3**, except that s1 and s2 were smeared evenly on the surface of 1/5M32 agar and incubated at 25°C in the dark for 7 days. Disks of 1.1 cm diam were cut from these cultures and used to inoculate 1/5M32 broth. Culture filtrates were harvested at 14 and 28 days after inoculation. Statistical analysis was performed on colony growth of *S. rolfsii* as in **Experiment 3.2.6.1.**

3.2.8 Identification of metabolites produced by *T. harzianum* and *G. virens* in liquid cultures, harvested after 31 days cultivation

G. virens was cultured in 1/5M32 broth (3 litres) for 31 days. The mycelium was removed by filtration and the filtrate was extracted with dichloromethane. The residue (70 mg) was separated into two fractions by chromatography on a Chromatotron instrument (silica gel; 1 mm plate) eluting with dichloromethane:ethyl acetate (1:1). The two fractions were further purified by preparative thin layer chromatography using the same solvent system. Spectroscopic

analysis of the two main fractions was carried out using nuclear magnetic resonance (NMR) and mass spectrometric (MS) techniques.

A culture of *T. harzianum*, grown in 1/5M32 and extracted as above, yielded only a small metabolite fraction. A culture of this fungus was also grown for 31 days in 1/5 PDB and the filtrate was extracted as above to give a fraction (20 mg) which was analysed by NMR techniques.

3.3 Results

3.3.1 Effects of *T. harzianum* and *G. virens* and nutrient status of the culture medium on sclerotial viability

There were significant interactions between treatments and media ($p<0.001$). In general, the proportion of viable sclerotia was reduced in the presence of *T. harzianum* and *G. virens*. This was particularly so on SEA which is intermediate in terms of nutrient status (**Figure 3.3.1**). The reduction caused by *G. virens* was greater than that caused by *T. harzianum* in all media except SEA, where their effects were similar.

3.3.2 Studies on hyphal interactions between the fungal antagonists and *S. rolfsii*

There was no evidence of extensive coiling, penetration or degeneration of *S. rolfsii* hyphae. Therefore, there is no Figure 3.3.2.

3.3.3 Effect of diffusible metabolites produced by *T. harzianum* and *G. virens* in different media, on growth of *S. rolfsii*, using the cellophane overlay technique

Growth of *S. rolfsii* was suppressed when *T. harzianum* or *G. virens* had been grown on the agar medium overlaid with cellophane prior to inoculation of the pathogen (**Figure 3.3.3**)(**Plate 3.3.3a-c**).

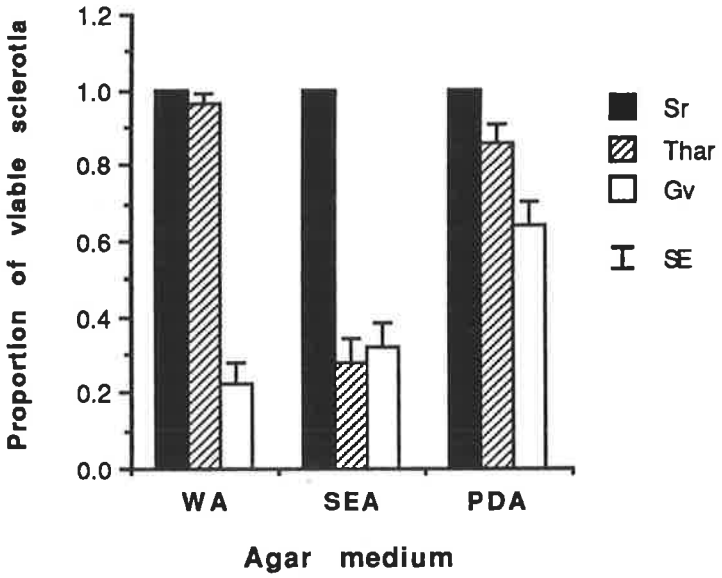


Figure 3.3.1 Effect of *T. harzianum* (Thar) or *G. virens* (Gv) on proportion of viable sclerotia of *S. rolfsii* in dual cultures, assayed on three different agar media. Sr = control, *S. rolfsii* alone. WA = water agar, SEA = soil extract agar, PDA = potato dextrose agar. Original data were means of five replicates. SE = standard error of the proportion.

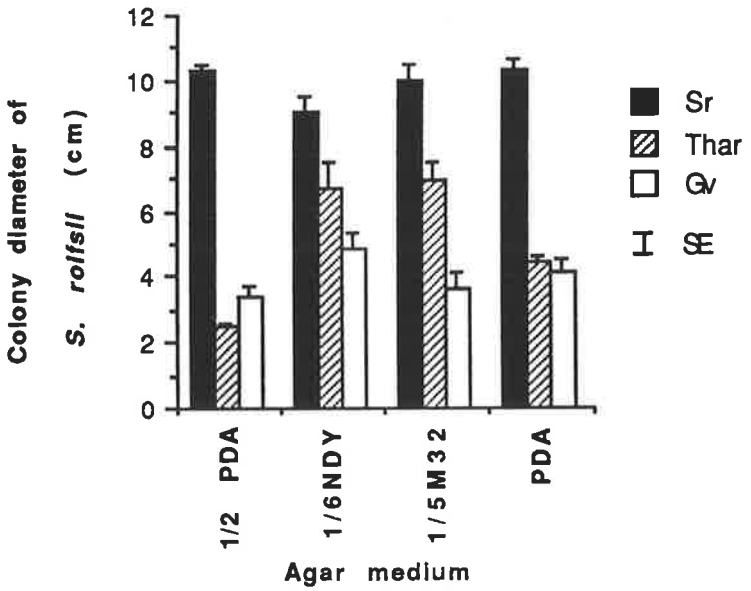
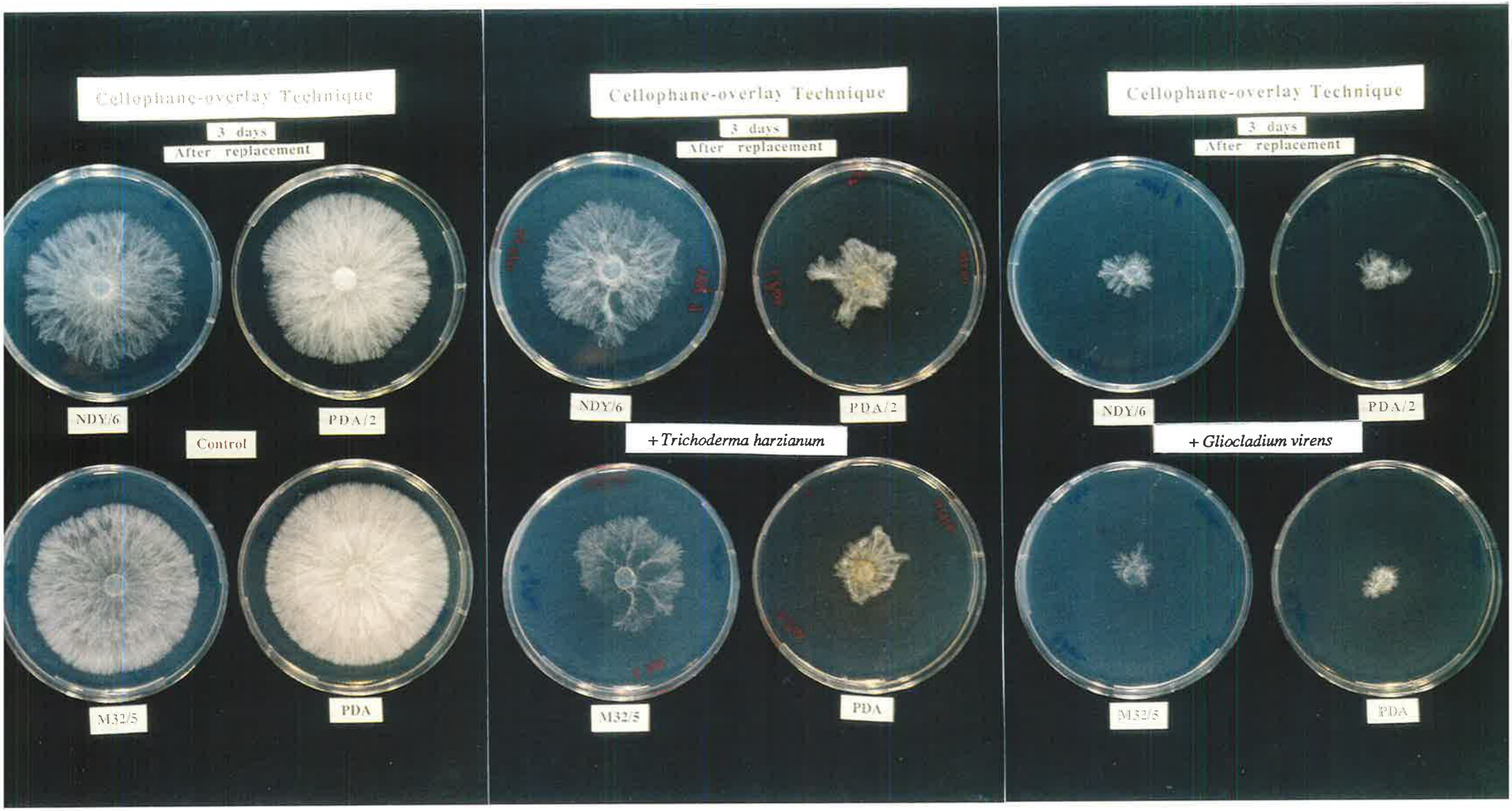


Figure 3.3.3 Effect of prior cultivation of *T. harzianum* (Thar) and *G. virens* (Gv) on growth of *S. rolfsii*, assayed on four agar media, using the cellophane overlay technique. Sr = control, *S. rolfsii* alone. Data are means of five replicates. SE = standard error of the mean.


Cellophane-overlay Technique
3 days
After replacement
NDY/6
PDA/2
Control
M32/5
PDA
Cellophane-overlay Technique
3 days
After replacement
NDY/6
PDA/2
+ Trichoderma harzianum
M32/5
PDA
Cellophane-overlay Technique
3 days
After replacement
NDY/6
PDA/2
+ Gliocladium virens
M32/5
PDA

a) b) c)

Plate 3.3.3a-c Inhibition of growth of *S. rolfsii* on different agar media by *T. harzianum* and *G. virens*, using the cellophane overlay technique. **a** = Control, *S. rolfsii* alone, **b** and **c** = *T harzianum* and *G. virens*, respectively, cultivated on the cellophane overlaid agar medium for 2 days prior to inoculation with *S. rolfsii*.

The inhibitory effect with prior cultivation of *G. virens* was greater than that of *T. harzianum* on 1/6NDY and 1/5M32 ($p<0.001$). In contrast, the effect caused by *G. virens* was less than that of *T. harzianum* on 1/2PDA. Their effects were similar on PDA.

It was noted that colony appearance of *S. rolfsii* in controls was sparse on 1/6 NDY and 1/5M32 agar, which were low and intermediate, respectively, in terms of nutrient status, whereas that in half and full strength PDA (rich medium) was dense. A feathery appearance or uneven growth, with mycelium growing towards the lid, away from the agar medium, was commonly observed in treatments with prior cultivation of *T. harzianum* or *G. virens*. Also, medium previously exposed to *T. harzianum* had yellow tints around the spot originally inoculated with this antagonist. This effect was not found with *G. virens*.

3.3.4 Effect of diffusible metabolites produced by *Streptomyces* s1 and s2 on growth of *S. rolfsii*, using the cellophane overlay technique

Colonies of *S. rolfsii* on control plates reached the plate margin within 3 days. Growth of *S. rolfsii* was reduced when s1 and s2 were inoculated on the cellophane overlaid agar medium prior to inoculation of the pathogen ($p<0.001$)(**Figure 3.3.4**). The degree of growth reduction caused by s1 was greater than that of s2.

S. rolfsii on medium previously overlaid with s1, commonly had limited fluffy mycelium growing from parts of the inoculum disk only, whereas sparse mycelium was associated with s2 (**Plate 3.3.4**). After 7 days, it was noted that sclerotia were not produced on agar medium previously exposed to s1, while only a few were observed on that exposed to s2.

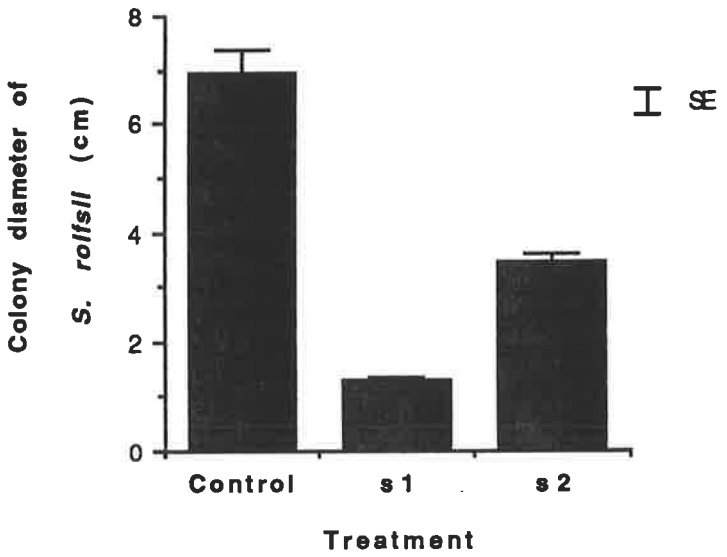


Figure 3.3.4 Effects of *Streptomyces* s1 and s2, on growth of *S. rolfsii* (Sr), assayed on 1/5M32 agar medium, using the cellophane overlay technique. Control = Sr alone. Data are means of five replicates. SE = standard error of the mean.

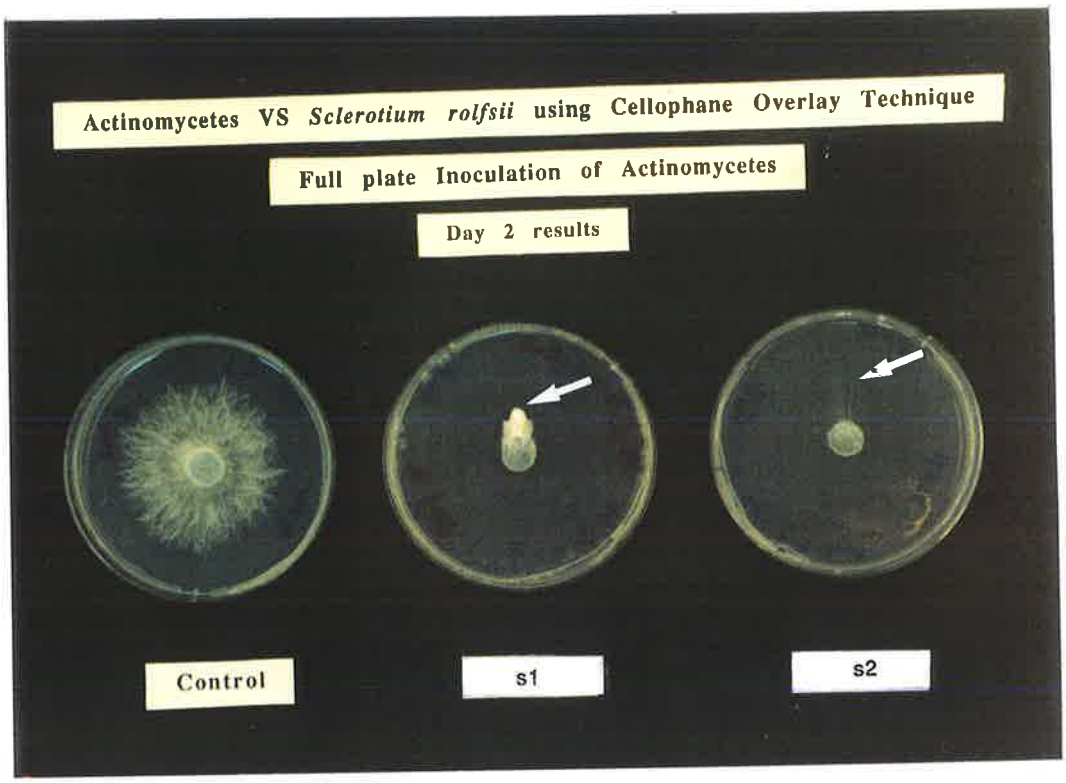

Actinomycetes VS *Sclerotium rolfsii* using Cellophane Overlay Technique
Full plate Inoculation of Actinomycetes
Day 2 results
Control
s1
s2

Plate 3.3.4 Inhibition of growth of *S. rolfsii* by *Streptomyces* s1 and s2 using the cellophane overlay technique. S*treptomyces* spp. were cultivated on cellophane overlaid 1/5M32 agar for 5 days prior to inoculation with *S. rolfsii*; the control was inoculated with the pathogen alone. Arrows show fluffy mycelium associated with s1, and sparse mycelium with s2.

3.3.5 Studies on inhibitory metabolites

3.3.5.1 Metabolites produced by *T. harzianum* and *G. virens* in liquid media, assayed on days 14 and 28

There were significant differences between treatments in colony diameter of *S. rolfsii* ($p<0.001$). Culture filtrates of both 14 and 28 day-old cultures of *T. harzianum* and *G. virens* grown in 1/5M32, inhibited growth of *S. rolfsii*, compared to that of the control (**Figure 3.3.5.1**). When the antagonists were cultured in PDB, culture filtrates of all except the 28 day-old culture of *G. virens* suppressed growth of *S. rolfsii*. The degree of inhibition was greater for culture filtrates of *T. harzianum*, compared to those of *G. virens*, particularly with 28 day-old filtrates.

In general, filtrates from 14 day-old cultures were slightly more inhibitory to *S. rolfsii* than were those from 28 day-old cultures, and filtrates from PDB cultures were more inhibitory than those from 1/5M32, with the exception of the 28 day-old *G. virens* mentioned above.

3.3.5.2 Effect of filtrates from liquid cultures of *T. harzianum* and *G. virens* in two media, harvested at three times, on growth of *S. rolfsii*

When cultures of the fungal antagonists were grown in 1/5M32 medium, only filtrate of 3 day-old culture of *G. virens* significantly reduced growth of *S. rolfsii* ($p<0.001$)(**Figure 3.3.5.2**). In contrast, when the antagonists were grown in PDB, growth of *S. rolfsii* was inhibited by filtrates from all ages of both cultures. The longer the cultures of antagonists were incubated, the greater the inhibitory effect of the culture filtrates on growth of *S. rolfsii*. The level of inhibition was greater with filtrates of *G. virens*, compared to those of *T. harzianum*.

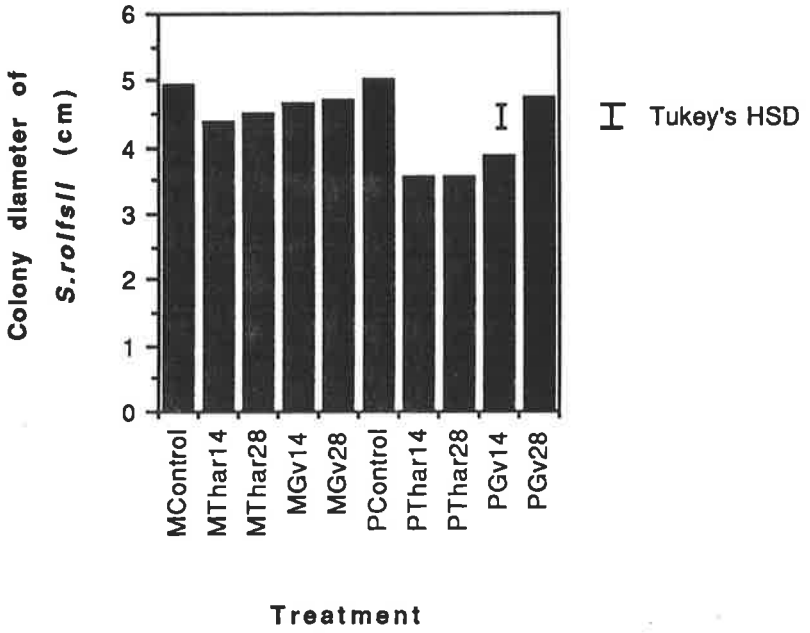


Figure 3.3.5.1 Effect of filtrates of *T. harzianum* (Thar) and *G. virens* (Gv) cultures grown in 1/5M32 broth (M) and potato dextrose broth (P), harvested on days 14 and 28, on growth of *S. rolfsii*. Data are means of six replicates.

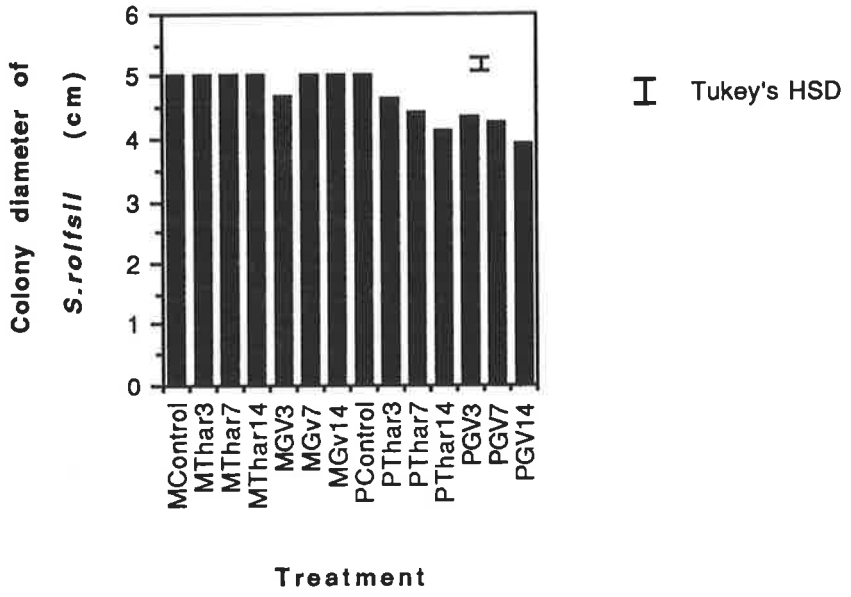


Figure 3.3.5.2 Effect of filtrates of *T. harzianum* (Thar) and *G. virens* (Gv) cultures grown in 1/5M32 (M) and potato dextrose broth (P), harvested on days 3, 7 and 14, on growth of *S. rolfsii*. Data are means of six replicates.

3.3.5.3 Effect of filtrates from liquid cultures of *T. harzianum* and *G. virens* in 1/5M32, harvested at four times, on growth of *S. rolfsii*

Culture filtrates of *T. harzianum* and *G. virens* significantly reduced growth of *S. rolfsii* in all treatments, compared to the control($p<0.001$)(**Figure 3.3.5.3**). The inhibitory effects caused by culture filtrates of *T. harzianum* were greater than those of *G. virens*, in all except for filtrate from 2 day-old cultures.

3.3.6 Effect of filtrates from liquid cultures of *Streptomyces* s1 and s2 in 1/5M32, harvested at five times, on growth of *S. rolfsii*

Culture filtrates of both s1 and s2 inhibited growth of *S. rolfsii* in all treatments, compared to the control ($p<0.001$)(**Figure 3.3.6**). In general, the longer the incubation time for cultures of s1, the greater was the inhibitory effect. On the contrary, the culture filtrate of s2 was most effective when the filtrate was harvested after 14 days cultivation, after which the effect was reduced.

3.3.7 Identification of metabolites produced by *T. harzianum* and *G. virens* in liquid culture, harvested after 31 days cultivation

Two compounds, 167/1 (4mg) and 167/3 (13mg), were recovered from cultures of *G. virens*. The NMR (^{1}H- and ^{13}C-NMR) and MS parameters for 167/1 correlated well with those reported in the literature for gliotoxin (**Figure 3.3.7a**)(E. L. Ghisalberti, pers. comm.). The second sample was deduced to be a mixture of two bismethylthiogliotoxin compounds (**Figure 3.3.7b**).

Only a small amount of metabolites was obtained from the broth cultures of *T. harzianum*. NMR analysis of the fraction obtained from the 1/5PDB culture broth indicated that it contained peptaibols (peptide metabolites). Pentyl pyrone was not detected.

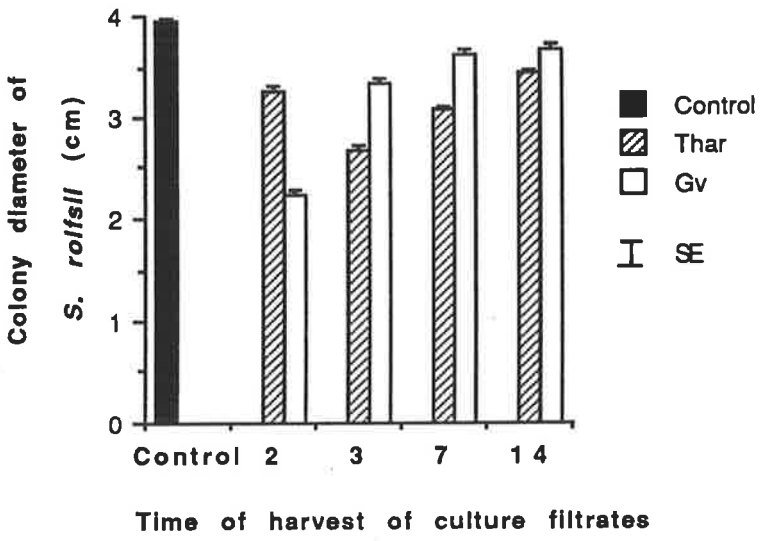


Figure 3.3.5.3 Effect of filtrates of *T. harzianum* (Thar) and *G. virens* (Gv) cultures grown in 1/5M32 medium, harvested on days 2, 3, 7 and 14, on growth of *S. rolfsii.* Data are means of six replicates. SE = standard error of the mean.

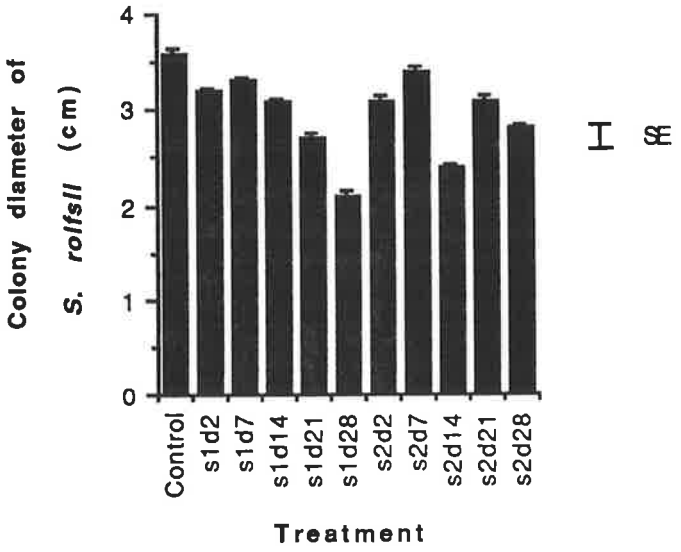
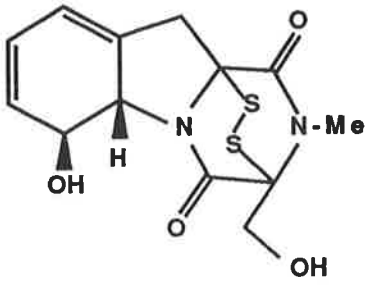
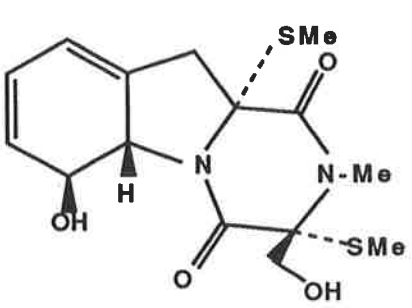
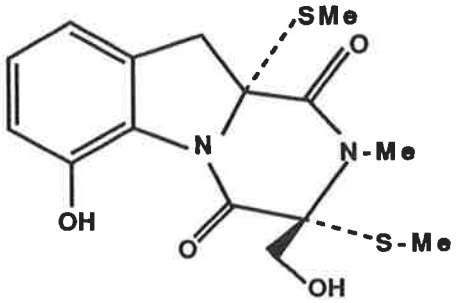


Figure 3.3.6 Effect of culture filtrates of *Streptomyces* s1 and s2 grown in 1/5M32 broth, harvested on days 2, 7, 14, 21 and 28, on growth of *S. rolfsii*. Data are means of six replicates. SE = standard error of the mean.



a) **167/1** Gliotoxin





bisdethiobis(methylthio)gliotoxin dehydro-bisdethiobis(methylthio)gliotoxin

b) **167/3** bismethylthiogliotoxin

Figure 3.3.7 Chemical structures of metabolite fractions recovered from liquid culture of *G. virens* (age 31 days).

3.4 Discussion

Since sclerotia are the primary source of inoculum for *S. rolfsii*, reduction of sclerotial viability would have a direct impact on soil inoculum, leading to a reduction of disease caused by the pathogen. Results from the dual culture experiment reported in this chapter demonstrated that the viability of sclerotia of *S. rolfsii* was reduced in the presence of both fungal antagonists tested, and that *G. virens* generally had a stronger effect than *T. harzianum* regardless of the nutrient levels in the medium. These findings indicate that *G. virens* could be a good candidate for biological control of *S. rolfsii* under conditions of competition for nutrients such as in potting mix or natural soil. Recent studies on the effects of *Trichoderma* spp. on growth of *S. rolfsii*, using the same dual culture technique, reported variable results with respect to inhibition of colony growth of the pathogen (Henis, Lewis and Papavizas, 1984; Lim and Teh, 1990; Sreenivasaprasad and Manibhushanrao, 1990). Although the dual culture technique has limited value in quantitative studies, e.g. for comparing efficacy of antagonists (Henis *et al.* , 1984; Sreenivasaprasad and Manibhushanrao, 1990), the method was used effectively in this study to examine survival of *S. rolfsii*, and may also be useful for such studies of other sclerotium-forming fungi. Although SEA was used in this experiment, it was replaced with 1/5M32, "soil mimic medium", in all subsequent experiments since the latter is a defined medium; thus variation inherent in natural soil was avoided.

Since studies of hyphal interactions between the two fungal antagonists (*T. harzianum* and *G. virens*) and *S. rolfsii* proved inconclusive, this aspect, including the possible production of lytic enzymes, was not pursued further. The work reported in this chapter was, then, focussed on the detection of inhibitory diffusible metabolites.

Results using the cellophane overlay technique indicated that metabolites were produced by all antagonists and diffused through the cellophane into the agar medium, inhibiting growth of *S. rolfsii*. The effects caused by the two fungal antagonists differed, depending upon the species and medium used. *G. virens* was more inhibitory to growth of *S. rolfsii* than was *T. harzianum* in low and intermediate

nutrient resource media (1/6NDY and 1/5M32, respectively). This inhibitory effect was in general agreement with that for sclerotial viability observed using the dual culture technique. Overall, results generally agree with those of previous studies of effects of metabolites produced by *Trichoderma* spp. on various test fungi, with the cellophane overlay technique, using either diluted malt extract agar and incubated in the light (Dennis and Webster, 1971a) or full strength of the same medium and incubated in the dark (Lim and Teh, 1990). When isolates of *Streptomyces* were tested using the same cellophane overlay technique, a strong inhibitory effect of diffusible metabolites was also shown, as reduction of sclerotium production as well as of colony growth. Sclerotium production was completely inhibited on agar medium previously exposed to s1, while only a few sclerotia were observed on that exposed to s2; although sclerotium viability could not, therefore, be assessed, s1 and s2 obviously had a strong detrimental effect on formation of these survival structures. The effects on sclerotium production together with colour change of the medium and variation in the appearance of the colony of the pathogen exposed to the four different antagonists, suggested that they may have produced different non-volatile metabolites.

Results from the cellophane overlay technique were confirmed and expanded in culture filtrate studies. When 1/5M32 was used, the inhibitory effects of metabolites produced by *G. virens* and *T. harzianum* early in the culturing period (2 and 3 days, respectively) were again apparent. Inhibitory effects of *T. harzianum* cultured in 1/5M32 broth (**Figure 3.3.5.1**) were not reproducible in the next experiment (**Figure 3.3.5.2**). Reasons for this were not clear, as procedures and conditions were standard, but variability in the antagonists or pathogen may have contributed to this. For *G. virens* grown in 1/5M32, it was shown that metabolites produced early in the culturing period (day 2) inhibited *S. rolfsii* more effectively than did those produced by *T. harzianum*, confirming results obtained with the cellophane overlay technique, in which the antagonist was cultured on the cellophane overlaid agar medium for 2 days prior to inoculation with *S. rolfsii*. Overall results from the culture filtrate study generally agree with those of similar

experiments carried out by Dennis and Webster (1971a). The authors found that the inhibitory effect of culture filtrates of *Trichoderma* spp. and *G. virens* on growth of the test fungi, reached a peak on day 5 of culturing. Observations made in the present study suggest that *T. harzianum* and *G. virens* may be effective as biocontrol agents against fast growing fungi such as *S. rolfsii* in soil.

Although PDB generally yielded fungal metabolites more inhibitory than those produced in 1/5M32, the latter was preferred as a test medium, since results were expected to be more applicable to potting mix or simulated natural soil.

Dennis and Webster (1971a) reported that Weindling's medium was excellent for the production of the metabolite gliotoxin by *G. virens* (details of the production of gliotoxin are discussed in section **1.2.11.4**). Chemical analysis of organic compounds produced by 31 day-old culture of *G. virens* in 1/5M32 broth, however, showed that the fungus produced gliotoxin and related compounds in 1/5M32. It is possible that these compounds can be produced in natural soil and, therefore, *G. virens* may be a good competitor under natural conditions.

Since the best inhibitory effects were found to be produced by young cultures of *T. harzianum* and *G. virens*, a preliminary study on water soluble compounds produced by young cultures of these two antagonists in 1/5M32, was initiated (data not presented). Results were, however, inconclusive, and the experiment had to be discontinued due to lack of time. It is possible that inhibitory metabolites produced in young cultures are water soluble (M. Tate, pers. comm.) and information obtained from such studies may enhance efficacy of antagonist application, therefore, this aspect merits further investigation.

Unlike those of the fungal antagonists, the culture filtrates of *Streptomyces* species most inhibitory to colony growth of *S. rolfsii* were found in ageing cultures. This tended to contradict results obtained by the cellophane overlay, in which 5-day-old cultures of s1 and s2 inhibited growth of *S. rolfsii*, and it is possible that cultures in liquid medium delayed the production of the inhibitory metabolites. This may be due to reduced aeration. It was also possible that metabolites produced by 5-day-old

cultures using the cellophane overlay technique differed from those produced by ageing liquid cultures. These aspects were not investigated further.

The slow growth of *Streptomyces* could be a disadvantage if they are to be applied as inoculum to control a fast-growing pathogen, unless a pre-incubation period is allowed.

Chapter 4

Study on volatile metabolites produced by antagonists *in vitro*

4.1 Introduction

The role of volatile compounds, like compounds of a non-volatile nature, in biological control of plant pathogens has been investigated. There have been extensive reports on the production of volatile metabolites by *Trichoderma* spp. *in vitro*, on the inhibitory effects of these metabolites on growth of several soil-borne fungal plant pathogens and, in some cases, also on diseases caused by these pathogens (Claydon *et al.*, 1987; Dennis and Webster, 1971b; Ghisalberti *et al.*, 1990; Simon, Dunlop, Ghisalberti and Sivasithamparam, 1988)(see section **1.2.11.4**). Evidence of the production of volatile compounds by *Trichoderma* in potting mix or soil, however, is limited. While the reason for this is not clear, it may be associated with the difficulty of detecting these compounds in such conditions. Evidence of production of volatile compounds by actinomycetes *in vitro* is limited, and few studies have been conducted with soil. Hora and Baker (1972) reported that although fungistasis was restored in sterile soil re-inoculated with fungi, bacteria and actinomycetes, volatile compounds were present only in treatments with actinomycetes. Since effects of volatile compounds produced by fungal and actinomycete antagonists may contribute to successful biological control of soil-borne plant pathogens, the subject merits investigation.

Media rich in nutrient resources, such as PDA and MEA, are commonly used for *in vitro* studies of the production of volatile compounds. It is, however, possible that organisms shown to produce volatile antibiotics in such artificial conditions may not produce detectable quantities, if at all, in soil, which is a heterogeneous substrate, sometimes low in nutrients. In addition, antagonistic species that produce non-volatile compounds may not necessarily produce volatiles. The aim of the study reported in this chapter was to determine whether volatile fungistatic compounds were produced *in*

vitro by selected antagonists in the "soil mimic medium", 1/5M32, and to evaluate their effects on growth of *S. rolfsii*, using the agar plate method originally used by Dennis and Webster (1971b).

4.2 Materials and methods

4.2.1 Ability of *T. harzianum* and *G. virens* to produce volatile compounds fungistatic to *S. rolfsii*

Petri plates of 1/5M32 agar medium, 18 ml per 9 cm diam plate, were inoculated with disks of *T. harzianum* and *G. virens* cut from the margin of 3 day-old cultures on 1/5M32 agar. A 1.1cm diam disk of each isolate was placed, separately, at the centre of each Petri plate. Cultures were incubated at 25°C in darkness for 2 days. Fresh plates of 1/5M32 were then inoculated with *S. rolfsii* as above and the bases of these plates inverted immediately over the bases of the plates containing the 2 day-old antagonists. The bases were taped together with Cellotape® adhesive tape. Control plates were prepared in the same way, except that an uninoculated plate of 1/5M32 was used instead of that with the antagonist. There were ten replicates per treatment. These plates were incubated at 25°C in the dark for 3 days. The experiment was repeated once. The colony diameter of *S. rolfsii* was measured and compared to that of the control. ANOVA was performed on the pooled data using the GENSTAT 5 package.

4.2.2 Ability of *Streptomyces* s1 and s2 to produce volatile compounds fungistatic to *S. rolfsii*

Petri plates of 1/5M32 agar medium, 18 ml per 9 cm diam plate, were inoculated with *Streptomyces* s1 or s2 by smearing inoculum all over the surface of the agar. These cultures were incubated in the dark for 14 and 28 days, respectively, to allow the *Streptomyces* to grow. Cultures were set up so that all treatments were ready for further procedures on the same day. Application of the pathogen, incubation of dual cultures, data collection and statistical analysis were as in section **4.2.1.**

4.3 Results

4.3.1 Ability of *T. harzianum* and *G. virens* to produce volatile compounds fungistatic to *S. rolfsii*

Growth of *S. rolfsii* was significantly reduced ($p<0.001$) in the presence of *T. harzianum*, although reduction was slight. *G. virens* did not affect growth of *S. rolfsii* (**Figure 4.3.1**). There were no other visible effects on colony morphology.

4.3.2 Ability of *Streptomyces* s1 and s2 to produce volatile compounds fungistatic to *S. rolfsii*

Growth of the pathogen was significantly inhibited ($p<0.001$), in all treatments except s2 inoculated 28 days before exposure (**Figure 4.3.2**). The longer the pre-inoculation period, the weaker the inhibitory effect ($p<0.001$). The decline in the effect was greater with s1, compared to that of s2 ($p<0.05$). Colony diameter was only slightly reduced, however, and there were no other visible effects on colony morphology.

4.4 Discussion

Results from the *in vitro* experiments reported in this chapter showed that growth of *S. rolfsii* was reduced by exposure to 2 day-old cultures of *T. harzianum*, 14 and 28 day-old cultures of s1 and 14 day-old cultures of s2, in closed conditions, using 1/5M32. This suggested the production of volatile compounds, inhibitory to the pathogen, by these antagonists. Although these differences were statistically significant, inhibition of the pathogen was, in all cases, slight.

Results for *T. harzianum* generally agree with findings from previous studies in which this species was tested against several fungi on media rich in nutrient resources, such as MEA and PDA. These fungi included *Sclerotinia sclerotiorum*, a sclerotium-forming plant pathogen (Claydon *et al.*, 1987), and *Gaeumannomyces graminis* var. *tritici* (Ghisalberti *et al.*, 1990). Since results reported here indicate that cultures of *T. harzianum* produced volatile compounds on the "soil mimic medium", it is possible that these compounds could also be produced in potting mix or

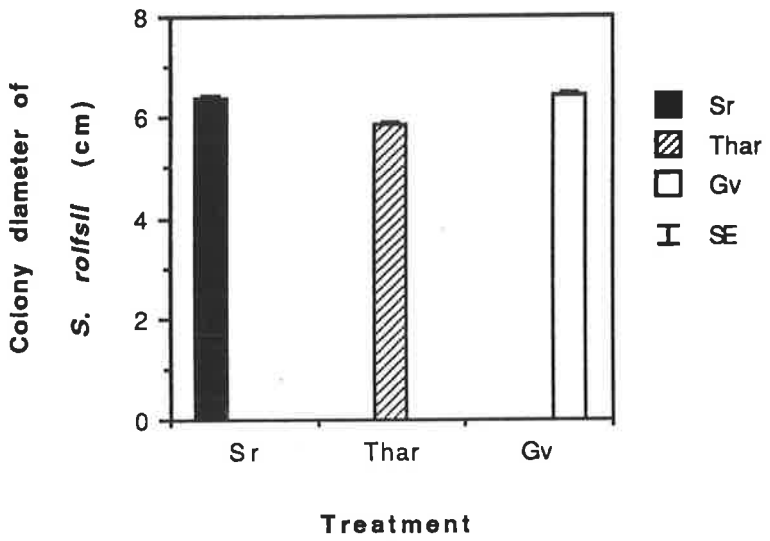


Figure 4.3.1 Effect of volatile compounds produced by *T. harzianum* (Thar) and *G. virens* (Gv), on *S. rolfsii* (Sr), shown by colony diameter at 3 days. Data are means of 20 replicates (data from repeated experiments were pooled for ANOVA). SE = standard error of the mean.

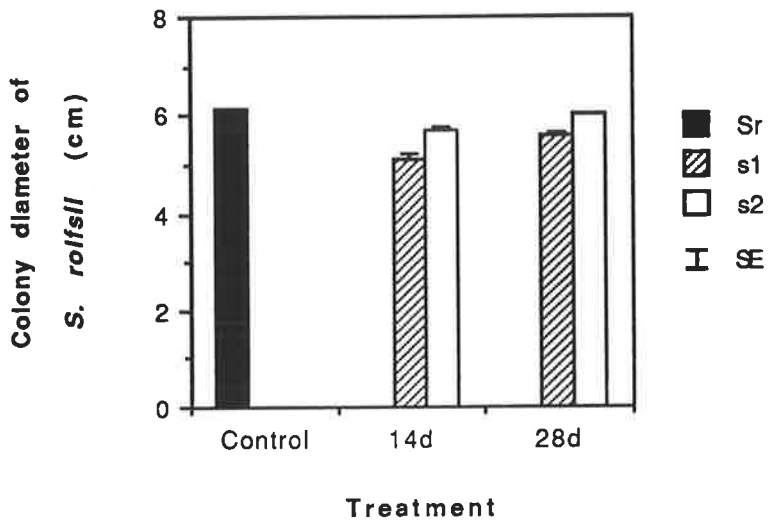


Figure 4.3.2 Effect of volatile compounds produced by *Streptomyces* s1 and s2, on *S. rolfsii* (Sr), shown by colony diameter at 3 days. Data are means of 20 replicates (data from repeated experiments were pooled for ANOVA). SE = standard error of the mean.

soil. The test isolate of *G. virens* failed to inhibit growth of *S. rolfsii* in the "soil mimic medium". These observations support the findings of Dennis and Webster (1971b) that *T. harzianum* produced volatile antibiotics on MEA medium whereas *G. virens* did not.

Some isolates of *T. harzianum* are known to produce volatile anti-fungal alkyl pyrones, with the most common being 6-*n*-pentyl-a-pyrone and analogues (Claydon *et al.*, 1987; Ghisalberti *et al.*, 1990). Chemical analysis of volatile compounds produced by young cultures of *T. harzianum* in 1/5M32 medium should be carried out in future in order to identify the active compounds involved.

Volatile compounds produced by *Streptomyces* spp. pre-incubated for 14 and 28 days reduced growth of *S. rolfsii*, and this effect was, again, statistically significant but slight. The inhibitory effect was reduced when the pre-incubation period was extended from 14 to 28 days, suggesting that the production and/or activity of these compounds declined with time. These results require confirmation *in vitro* and in soil conditions.

Since inhibition of *S. rolfsii* was slight compared to controls, these volatile compounds are unlikely to be a major factor in disease control in potting mix or soil, although in combination with other mechanisms such as production of non-volatile inhibitory metabolites and competition, they may contribute to biocontrol of this pathogen.

Results from **Chapter 3** and this chapter showed antibiosis to be involved in interactions between the antagonists and the pathogen. However, these interactions may be affected in one way or another by the presence of a host plant, and this will be addressed in subsequent chapters.

Chapter 5

Plant bioassay *in vitro*

5.1 Introduction

Although experiments with dual cultures and culture filtrates can provide useful information about possible mechanisms and antagonisms (see **Chapter 3**), it is necessary to demonstrate that antagonists can prevent or protect host plants from infection by the pathogen.

S. rolfsii is known to have a wide host range including monocotyledonous and dicotyledonous species (see sections **1.2.1** and **1.2.4**). There have been reports on varietal differences among field crops in response to *S. rolfsii* infection, with extensive information available on legumes (Branch and Csinos, 1987; Nwakpa and Ikotun, 1988; Shew *et al.*, 1987; Vallejos, 1988)(see section **1.2.8.1**) and to a lesser extent on cereals (Kilpatrick and Merkle, 1967). Development of a bioassay involving host plants requires selection of species or varieties that have an intermediate response to infection. Once the host is selected, the next step is to test the efficacy of the potential antagonists in providing protection against infection by the pathogen. *In vitro* study is known to give an indication of what may occur in nature. The use and potential of tissue culture material for screening for resistance in a number of host-pathogen systems have been well documented (Daub, 1986; Scott, Chambers and Son, 1994; Stephens and Elmer, 1988; Van den Bulk, 1991). Stephens and Elmer (1988) found a positive correlation between results from an *in vitro* bioassay using aseptically grown seedlings of *Asparagus* spp. and those of older plants in glasshouse conditions, in terms of disease response to *Fusarium oxysporum* f.sp. *asparagi* and *F. moniliforme*. Chambers (1994) adapted this approach and demonstrated protection of micropropagated shoots of chestnut from the crown rot pathogens *Phytophthora cinnamomi* and *P. citricola* by *Trichoderma hamatum*, *T. pseudokoningii* and *Gliocladium virens*. Shoots placed upright in agar medium at the interaction zone between pathogen and antagonist remained healthy 2 days after inoculation whereas

unprotected controls were dead. Such *in vitro* assays are potentially valuable as they allow rapid evaluation of large amounts of plant material with minimal time, space and effort, and have potential benefits in breeding and disease control programs. However, there have been few studies on *in vitro* bioassays for *S. rolfsii*, either for host-pathogen interactions or in the elucidation of biocontrol.

Protective effects have been assessed, in soil studies, using criteria such as the disease index (Rothrock and Gottlieb, 1984; Wolffhechel and Jensen, 1992), plant survival (Harman, Taylor and Stasz, 1989; Papavizas and Lewis, 1989) and mean shoot fresh weight (Rothrock and Gottlieb, 1984). Since plant survival is one of the major parameters determining the final yield, the experiments reported in this chapter were carried out to assess survival of hosts in the presence of the pathogen, with and without the antagonists, *in vitro*, with the aim of obtaining information about the ability of the antagonists to prevent or reduce disease.

5.2 Materials and methods

The rationale of the experiments is stated briefly at the beginning of each section, where necessary.

Wheat seeds used in experiments reported in this and subsequent chapters were provided by T. Rathjen and V. Vanstone, Department of Plant Science, Waite Agricultural Research Institute.

5.2.1 Pathogenicity of *S. rolfsii* on selected varieties of wheat

Since there is limited evidence of disease caused by *S. rolfsii* on wheat grown in South Australia, it was necessary to determine whether the strain of *S. rolfsii* originally isolated from diseased capsicum could infect wheat under certain conditions and, if so, whether there were differences among varieties in response to infection by the pathogen. Observations were expected to facilitate the choice of variety for subsequent experiments, and to provide information on varietal responses if the pathogen should become a major problem in wheat crops in Australia in the future. Therefore, this experiment was designed to test the pathogenicity of *S. rolfsii* on eight

varieties of wheat commonly grown in South Australia; "Halberd", "Machete", "Molineux", "Oxley", "Schomburgk", "Spear", "Tatiara" and "Warigal".

5.2.1.1 Preparation of seedlings

Seeds of each variety were separately surface-sterilised for 3 min with 10% White King domestic bleach (0.4% available chlorine) plus a drop of wetting agent (Tween 80). Seeds were then rinsed three times with sterile distilled water (SDW) and placed on filter paper (Whatman no. 1) moistened with SDW, in sterile Petri plates. These plates were incubated at 25°C in the dark for 3 days, by which time the plumule had emerged and crown roots developed.

5.2.1.2 Inoculation of seedlings

Disks of *S. rolfsii*, 1.1 cm diam, were cut from the leading edge of cultures grown on 1/5M32 agar medium for 3 days. Two disks were placed, 5 cm apart on filter paper moistened with SDW, in each Petri plate. Two seedlings of the same variety were placed, one on each disk, in one plate, ensuring that the stem base touched the disk. The control treatment was prepared using uninoculated disks of 1/5M32. Each disk represented one replicate, and there were ten replicates per treatment per wheat variety. Petri plates were incubated at 25°C in the dark. Development of infection was monitored and the number of seedlings surviving infection by *S. rolfsii* was recorded at termination of the experiment on day 7. The Chi-square (X^2) statistic was used to test for significant difference between treatments.

5.2.2 Effects of *S. rolfsii* on capsicum and wheat seedlings in the presence of *T. harzianum* and *G. virens*

5.2.2.1 Choice of variety and preparation of seedlings

Capsicum "Giant Bell" was chosen as one of the two hosts in this experiment since it was the original host of the test strain of *S. rolfsii*. Seeds were obtained from Arthur Yates & Co Pty Ltd, New South Wales, Australia. "Warigal" was chosen from the group of seven wheat varieties showing a similar response to infection

by *S. rolfsii* (see section **5.3.1**). For consistency, these two hosts were also used in subsequent experiments. Seedlings of wheat were prepared as in section **5.2.1.1**. Similar procedures were followed for capsicum seedlings, except that the time allowed for germination was 11 days, at which time the cotyledons were unfolded and the tap root developed.

5.2.2.2 Inoculation of seedlings

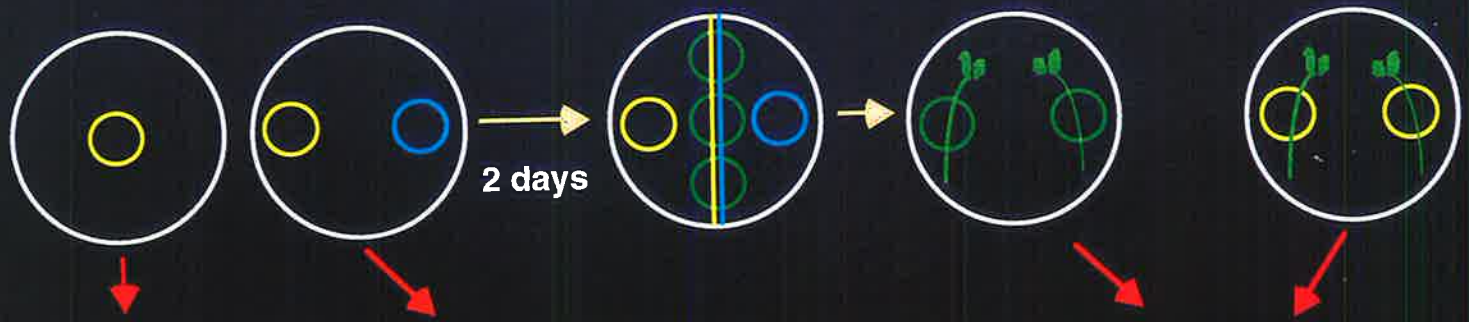
The experimental procedure is shown in **Plate 5.2.2.2**. Treatments consisted of monocultures of *S. rolfsii*, *T. harzianum* and *G. virens*, and combined cultures of *S. rolfsii* + *T. harzianum* and *S. rolfsii* + *G.virens*, respectively. Monocultures were prepared by growing each isolate on 1/5M32 agar medium for 3 days. Disks, 1.1 cm diam, were cut from the colony margin of these cultures. Combined cultures were prepared by growing each antagonist in dual culture with the pathogen on 1/5M32 at 25°C in the dark for 2 days, after which 1.1 cm diam disks were cut from interaction areas. The control treatment was 1.1 cm diam disks of uninoculated 1/5M32 agar.

Two identical disks were placed 5 cm apart on filter paper (Whatman no. 1) moistened with SDW, in a sterile Petri plate. Seedlings were placed on disks and incubated as in section **5.2.1.2**. There were ten replicates per treatment. The number of surviving seedlings was recorded daily for 7 days. Results from days 4 and 7 were tabulated separately according to species, pathogen and antagonist. Since all seedlings of the control and treatments with each species of antagonist alone were alive and healthy, a binomial model was fitted to the data for "*S. rolfsii*-inoculated" plants only. Log-linear models with Poisson error distribution were fitted to the data, and the resulting X^2 statistics used to test for differences between treatments.

5.2.3 Effects of *S. rolfsii* on capsicum and wheat seedlings in the presence of *Streptomyces* s1 and s2

Host varieties, preparation of seedlings, inoculation with *S. rolfsii* and controls were as in sections **5.2.2.1** and **5.2.2.2**. For preparation of monocultures

Plant Bioassay Technique



S. rolfsii

or *T. harzianum*

or *G. virens*

S. rolfsii + *T. harzianum*

or

S. rolfsii + *G. virens*

2 host spp. ; capsicum

wheat

Plate 5.2.2.2 Experimental procedure for the plant bioassay technique, using 2 day-old culture disks of *S. rolfsii*, *T. harzianum* or *G. virens* and disks from combined cultures of *S. rolfsii* and *T. harzianum* or *S. rolfsii* and *G. virens*. Two identical disks were placed on moistened filter paper in a Petri plate and one newly emerged seedling of wheat or capsicum was placed on each disk.

of *Streptomyces* s1 and s2, each species was smeared separately over the surface of 1/5M32 agar in 9 cm diam Petri plates. These cultures were incubated at 25°C in the dark for 7 days, after which 1.1 cm diam disks were cut. Combined cultures of *Streptomyces* and *S. rolfsii* were prepared by smearing half of a 9 cm diam Petri plate of 1/5M32 with each species of *Streptomyces*. These cultures were incubated at 25°C in the dark for 7 days prior to inoculation with *S. rolfsii*. One 1.1 cm diam disk of *S. rolfsii* cut from 3-day old cultures grown on 1/5M32 was then placed on the uninoculated half of the Petri plate, 4 cm from the edge of the *Streptomyces* growth. These dual cultures were incubated for a further 3 days under the same conditions, at which time mycelia of *S. rolfsii* had just reached colonies of *Streptomyces*. Disks, 1.1 cm diam, were cut from interaction areas. The experimental set up and statistical analysis on number of living seedlings were as in section **5.2.2.2**. Again, all seedlings of the control and treatments with each species of antagonist alone were alive and healthy, thus, only data for "*S. rolfsii*-inoculated" plants were analysed, as in section **5.2.2.2**.

5.3 Results

5.3.1 Pathogenicity of *S. rolfsii* on selected varieties of wheat

Significant differences in survival following inoculation with *S. rolfsii* were found among the eight varieties of wheat tested ($p<0.05$). "Spear" was the most susceptible, and the other varieties did not differ significantly (**Figure 5.3.1**).

5.3.2 Effects of S. rolfsii on capsicum and wheat seedlings in the presence of *T. harzianum* and *G. virens*

5.3.2a Day 4 results

Survival of capsicum overall following inoculation with *S. rolfsii* was significantly different from wheat ($p<0.05$).

S. rolfsii alone killed all capsicum seedlings (**Table 5.3.2a**). Three and six of ten seedlings survived inoculation with *S. rolfsii* in combination with *T.*

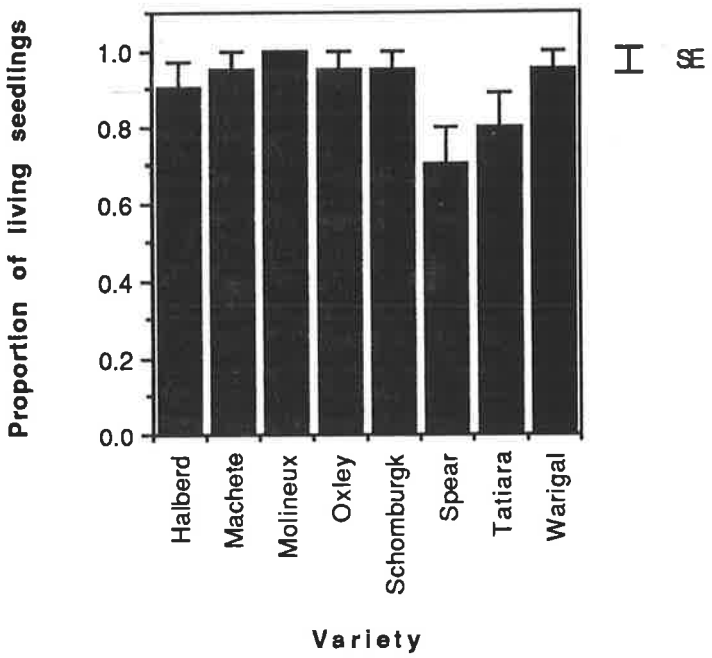


Figure 5.3.1 Proportion of seedlings of eight wheat varieties surviving 7 days after inoculation with *S. rolfsii*. Original data were the total number of seedlings surviving on ten replicate disks. SE = standard error of the proportions.

harzianum and *G. virens*, respectively; this difference between antagonists was not significant. In contrast, all wheat seedlings survived inoculation with *S. rolfsii* either alone or in combination with *T. harzianum* or *G. virens* ($p<0.01$).

It was noted that there was superficial mycelium of *S. rolfsii* on all seedlings surviving inoculation with the pathogen either alone or in combination with each species of the antagonist. Observations suggested that the pathogen colonised the host more quickly in the absence of *T. harzianum* and *G. virens* but growth rates were not measured.

5.3.2b Day 7 results

After 7 days, none of the capsicum seedlings exposed to *S. rolfsii* in combination with *T. harzianum* were alive, whereas five of ten survived inoculation with *S. rolfsii* and *G. virens* (**Table 5.3.2b**). In contrast, two of ten wheat seedlings survived inoculation with *S. rolfsii* alone, whereas nine of ten were alive following inoculation with *S. rolfsii* and either of the antagonists ($p<0.01$). Survival of host seedlings was greater when seedlings were inoculated with *S. rolfsii* and antagonist, compared to *S. rolfsii* alone ($p<0.05$).

There was less superficial mycelium on surviving wheat seedlings following inoculation with *S. rolfsii* alone compared to day 4, however, sclerotia had developed. These seedlings had discoloured stem bases, whereas those surviving following combined inoculation of the pathogen and antagonist were not discoloured. Conidia of the antagonists in the latter were produced on seedlings and filter paper.

5.3.3 Effects of *S. rolfsii* on capsicum and wheat in the presence of *Streptomyces* s1 and s2

5.3.3a Day 4 results

Survival of capsicum seedlings overall was greatly reduced by inoculation with *S. rolfsii* ($p<0.01$), whereas that of wheat was unaffected.

Table 5.3.2a Number of capsicum and wheat seedlings of ten replicates, surviving inoculation with *S. rolfsii* (Sr) alone or *S. rolfsii* in combination with *T. harzianum* (Thar) or *G. virens* (Gv), 4 and 7 days after inoculation. Only data for treatments inoculated with *S. rolfsii* are presented (see section **5.2.2.2**).

Table 5.3.2a Four days after inoculation.

Host	Sr	Sr+Thar	Sr+Gv	Total
Capsicum	0	3	6	9
Wheat	10	10	10	30
Total	10	13	16	
X^2 Host x pathogen, df_1	p<0.01			
X^2 Host, df_1	p<0.05			
X^2 antagonist, df_2	ns			
X^2 pathogen, df_1	p<0.05			

Table 5.3.2b Seven days after inoculation.

Host	Sr	Sr+Thar	Sr+Gv	Total
Capsicum	0	0	5	5
Wheat	2	9	9	20
Total	2	9	14	
X^2 Host x pathogen, df_1	p<0.01			
X^2 antagonist x pathogen, df_2	p<0.05			
X^2 pathogen, df_1	p<0.001			

All capsicum seedlings inoculated with *S. rolfsii* alone died (**Table 5.3.3a**). Six of ten survived inoculation with s1 and *S. rolfsii*, whereas none survived when s2 was used instead; this difference was, however, insignificant. In contrast, all wheat seedlings remained alive regardless of the treatment ($p<0.05$). However, it was observed that seedlings surviving inoculation with *S. rolfsii* either alone or in combination with *Streptomyces* spp. had superficial mycelium of *S. rolfsii* covering most of the plant.

5.3.3b Day 7 results

After 7 days, plant survival was severely reduced by inoculation with *S. rolfsii* ($p<0.001$). All, except two of ten capsicum seedlings inoculated with *S. rolfsii* and s1, had died (**Table 5.3.3b**). These two seedlings had superficial mycelium of *S. rolfsii* covering the whole plant and appeared unlikely to survive much longer. Sclerotia formed on plants and filter paper in all treatments inoculated with *S. rolfsii* either alone or in combination with antagonist.

5.4 Discussion

All eight varieties of wheat tested were infected by *S. rolfsii* (see section **5.3.1**). "Spear" was the most susceptible variety while others did not differ significantly in response to inoculation with *S. rolfsii*. Results were in general agreement with those of Kilpatrick and Merkle (1967) who reported differences among wheat varieties following inoculation with *S. rolfsii* in sand or soil under controlled conditions. They did not, however, test any of the varieties used in this study.

"Warigal" was arbitrarily chosen and used in the two subsequent experiments. When applied alone, *S. rolfsii* killed all seedlings of capsicum "Giant Bell" within 4 days (see section **5.3.2**), whereas *T. harzianum* or *G. virens* alone had no detrimental effects over 7 days. *T. harzianum* and *G. virens* appeared to provide some protection for capsicum initially, with 30% and 60% seedlings surviving after 4 days,

Table 5.3.3 Number of capsicum and wheat seedlings of ten replicates, surviving inoculation with *S. rolfsii* (Sr) alone or *S. rolfsii* in combination with *Streptomyces* s1 or s2, 4 and 7 days after inoculation. Only data for treatments inoculated with *S. rolfsii* are presented (see section **5.2.3**).

Table 5.3.3a Four days after inoculation.

Host	Sr	Sr+s1	Sr+s2	Total
Capsicum	0	6	0	6
Wheat	10	10	10	30
Total	10	16	10	
X^2 Host x antagonist x pathogen, df_2	$p<0.05$			
X^2 Host, df_1	$p<0.05$			
X^2 antagonist, df_2	ns			
X^2 pathogen, df_1	$p<0.01$			

Table 5.3.3b Seven days after inoculation.

Host	Sr	Sr+s1	Sr+s2	Total
Capsicum	0	2	0	2
Wheat	0	0	0	0
Total	0	2	0	
X^2 pathogen, df_1	$p<0.001$			

whereas by 7 days only *G. virens* had any protective effect, with 50% of seedlings remaining alive. Although no seedlings of "Warigal" inoculated with *S. rolfsii* alone had died by 4 days, the majority had died at 7 days, at which time the test was terminated. Both *T. harzianum* and *G. virens* were effective in enhancing survival of wheat inoculated with *S. rolfsii*, as most seedlings were still alive at the day 7 assessment. It had been decided that the experiments reported in sections **5.3.1** and **5.3.2** were to be terminated on day 7 since space and nutrient supply, as indicated by chlorosis, became limiting for the seedlings.

The experiment on pathogenicity of *S. rolfsii* on wheat varieties has shown that *S. rolfsii*, originally isolated from capsicum, can infect and kill wheat seedlings *in vitro* and that the degree of response depended upon variety. It was noted that the pathogenicity of *S. rolfsii* on "Warigal" reported in sections **5.3.2** and **5.3.3**, in which survival was similar, was greater than that in section **5.3.1**; conditions were identical in all three experiments and the reasons for this discrepancy are unknown. Information obtained from the *in vitro* bioassay is potentially valuable for allowing rapid evaluation of annuals such as cereals, providing that the *in vitro* results are positively correlated with those in potting mix or soil. However, this assay requires further assessment using these and other host plants.

Results from the plant bioassay for biocontrol reported in section **5.3.2** were in general agreement with those of Papavizas and Collins (1990). These authors incubated sclerotia of *S. rolfsii* for 5 days in soil amended with various concentrations of *G. virens* and found that up to 97% of these sclerotia were colonised by the antagonist. When surface-sterilised and used in a bioassay on excised hypocotyls of 9-day-old snap bean plants, the sclerotia were no longer infective. Despite the technical difference in that Papavizas and Collins (1990) used sclerotia, compared to mycelia in the experiment reported in this chapter, both tests indicated that a bioassay on host plant material *in vitro* was useful in providing a rapid indication of possible antagonism, prior to testing in soil or potting mix in glasshouse and field. It is suggested that this plant bioassay approach has the potential to facilitate studies of

biocontrol of *S. rolfsii* as well as other plant pathogenic fungi and bacteria. While the work of Chambers (1994) using *Trichoderma* and *Gliocladium* spp. to protect micropropagated chestnut from *Phytophthora* spp. supports this suggestion, further investigation is required.

Streptomyces s2 had no protective effect for capsicum and wheat, whereas s1 gave some protection up to 7 days after inoculation. Although the results are not strictly comparable, survival of capsicum was greater in the presence of s1 than of *T. harzianum* at both 4 and 7 days, and as good as *G. virens* at 4 days, whereas the *Streptomyces* isolates did not protect wheat at 7 days after inoculation. It may be that this bioassay is less appropriate for assessing efficacy of *Streptomyces* than for fungal antagonists, due to the slow growth rates of the former. Despite prior inoculation of *Streptomyces* on agar, *S. rolfsii* would still be able to grow faster on the stem base of the plant.

Experiments reported in this chapter provided evidence that some of the potential antagonists, particularly *G. virens*, may protect capsicum and wheat against *S. rolfsii*. These observations were made in a non-competitive environment, thus, it was necessary to evaluate the ability of these organisms to protect capsicum and wheat seedlings growing in potting mix.

Chapter 6

Study on pathogenicity of *S. rolfsii* in potting mix

6.1 Introduction

The test isolate of *S. rolfsii* was capable of infecting and killing capsicum and wheat seedlings *in vitro* (see **Chapter 5**), however, confirmation of pathogenicity on these hosts in "soil" was necessary. Field surveys have shown that this pathogen causes foot rot on capsicum under hot and humid conditions, resulting in a major yield loss in some cases (see section **1.2.4**). There is little information on pathogenicity of *S. rolfsii* on this host in controlled environments, however. Inoculation of wheat at seeding, using *S. rolfsii* at 0.5% weight/weight of ground-oat cultures in sand at 27°C in a glasshouse, caused up to 67% death of wheat seedlings (Kilpatrick and Merkle, 1967), whereas approximately 40% infection was found under natural conditions in the field (Simmonds, 1960). In experiments on snap bean in soil in the glasshouse, at temperatures between 23-30°C, inoculation with *S. rolfsii* before planting either as a known number of sclerotia per gram soil (Henis *et al.*, 1984; Papavizas and Collins, 1990) or as weight/weight of sclerotia/soil (Elad *et al.*, 1980a) caused up to 80% death of test plants.

The experiment reported in this chapter was carried out to investigate pathogenicity of the test isolate of *S. rolfsii* on capsicum and wheat seedlings in a potting mix under controlled conditions in a growth room. It was designed to allow observation of direct interactions between the pathogen and its hosts under conditions close to those in nature.

6.2 Materials and methods

6.2.1 Preparation of potting mix, seedlings and inoculum of *S. rolfsii*

Potting mix was prepared from recycled soil. Half a cubic metre of used and composted experimental soil (mixed University of California, John Innes and other

potting mixes composted for 2 years) was steam-sterilised at 100°C for 45 min. This mixture was cooled and the following ingredients were added; 1/5 m^3 Eurotorf peat moss, 500 g blood meal, 200 g agricultural lime, 200 g potassium sulphate and 100 g superphosphate. The mixture was thoroughly mixed and passed through a 1 cm-grid sieve. The pH of this final mixture was 5.5. Pots of 10 cm diam were filled with this mixture, and then placed in plastic trays, 45x70 cm, to soak with reverse osmosis (RO) water for 2 days. Excess water was then drained off.

Seeds of capsicum and wheat were germinated *in vitro* as in section **5.2.1.1.**

Inoculum of *S. rolfsii* was prepared on pieces of carrot. Carrot roots, approximately 2 cm diam, were washed, blotted dry and peeled using a sterilised knife blade. They were then cut into 2 mm thick slices. Four pieces of carrot, total weight approximately 3 g, were placed in each sterile Petri plate and inoculated with 50 sclerotia from 12 day-old cultures of *S. rolfsii* grown on 1/5M32 agar. Two ml SDW were added to each Petri plate to soften the carrot tissues and also to provide humid conditions in the plate. These Petri plates were incubated at 25°C in the dark for 10 days, at which time the carrot pieces were completely colonised by mycelium of *S. rolfsii*, with a combination of young, intermediate and mature sclerotia being present.

6.2.2 Preparation of treatments

Each host species was either inoculated with *S. rolfsii* (Sr) or left uninoculated (control). For the Sr treatment, the top 3 cm potting mix was temporarily removed from the pot. All colonised carrot pieces from one Petri plate were placed in each pot and spread evenly using a spatula. The potting mix was then replaced. One seedling was transplanted into each pot. One pot represented one replicate. There was a total of 20 replicates per treatment for each host. Controls were prepared without inoculum of *S. rolfsii*. The treatments were arranged in a completely randomized design (CRD). Preliminary experiments showed that a humid environment was necessary for disease development, thus a humid chamber was designed to give 80-95% relative humidity. This chamber comprised a plastic sheet, over a

supportive wire frame, which covered the tray containing the prepared pots. The experiment was conducted in a growth room at 25°C under banks of lights (Phillips TLD 58w/33, Cool white 2J, Australian AS 1021) with 12 h daylength. The number of living seedlings was recorded daily. The experiment was terminated 14 days after transplanting and inoculation with *S. rolfsii*. The number of surviving seedlings was tabulated according to host species and inoculation status. A model recognising the binomial error structure was fitted to the contingency table. X^2 tests were used in the assessment of species and inoculation effects.

6.3 Results

The difference between the overall number of non-inoculated and inoculated seedlings surviving was highly significant ($p<0.001$)(**Table 6.3**); all non-inoculated seedlings were alive. Capsicum and wheat did not differ significantly in terms of number of seedlings surviving inoculation with *S. rolfsii*. Most capsicum seedlings were dead within 4 days after transplanting and inoculation with *S. rolfsii* and all were dead within 8 days. At the end of the experiment, 14 days after inoculation, six of 20 wheat seedlings survived, however these were stunted, with leaf sheath discoloration and yellow leaf tips.

6.4 Discussion

The humid chamber provided an environment conducive to disease development and carrot pieces proved to be a satisfactory food base for inoculation of seedlings with *S. rolfsii*. *S. rolfsii* infected and caused death of seedlings of capsicum and wheat and was slightly more pathogenic on the former. Although death of capsicum seedlings was slightly delayed in the potting mix, results were consistent with those obtained *in vitro* (see section 5.3.2). In contrast, 70% of wheat seedlings inoculated in potting mix died within 14 days, although only 10% were dead within 7 days *in vitro*. Results obtained in potting mix were similar to those of Kilpatrick and Merkle (1967), who used colonised oats at 0.5% in soil (weight/weight) as the inoculum of

Table 6.3 Number living seedlings of capsicum and wheat out of 20, 14 days after transplanting and inoculation with *S. rolfsii.*

Host	Inoculation status		Total
	Non-inoculated	Inoculated	
capsicum	20	0	20
wheat	20	6	26
Total	40	6	
X^2 Inoculation x Host, df_1	ns		
X^2 Inoculation, df_1	$p<0.001$		
X^2 Host, df_1	ns		

S. rolfsii and found up to 67% post-emergence death of wheat seedlings in controlled conditions (see section **6.1**).

A delay of death of capsicum seedlings in potting mix compared to that *in vitro* may be due to initial antagonism of *S. rolfsii* by other microorganisms, which was temporary, and the pathogen was then able to infect as it was in the active mycelial stage. The discrepancy in results for wheat obtained in potting mix and *in vitro* suggests that the former provided a more conducive environment for pathogenicity of *S. rolfsii*.

It was concluded that the isolate of *S. rolfsii* tested was pathogenic on capsicum and wheat, and that the former seemed slightly more susceptible to disease. This experiment, therefore, confirmed pathogenicity of the test isolate of *S. rolfsii* and showed that satisfactory disease development occurred in the environmental conditions used. Thus effects of antagonistic microorganisms could be investigated with some confidence. Rapid development of disease due to *S. rolfsii* suggested that, to control the pathogen, a potential antagonist could require a pre-incubation period for establishment and this aspect needed investigation.

Chapter 7

Development of methods for application of potential antagonists to seedlings in potting mix

7.1 Introduction

It is known that *S. rolfsii* can infect and cause death of host plants, and that its interactions with other microorganisms, either naturally occurring or introduced into the community can change this effect (see section **1.2.8.3**).

Studies on biological control of soil-borne plant pathogens using fungal antagonists in controlled conditions have involved several methods of inoculating the antagonists. These have included direct application of spores to soil or potting mix, as a known number of spores to a unit weight of soil (Chet and Baker, 1981; Harman *et al.*, 1989; Henis *et al.*, 1984), or indirectly, mixed with a non-nutritional "carrier" such as white clay granules (Neypes, Lapis and Telan, 1988), lignite or coal substrates (Harman *et al.*, 1989), pyrax (Papavizas and Collins, 1990) or cereal bran-based substrates (Elad *et al.*, 1980; Ristaino *et al.*, 1991; Roiger and Jeffers, 1991; Wolffhechel and Funck-Jensen, 1992). Antagonists with "carriers" have been applied as seed dressings or soil treatment, whereas weight by weight or volume by volume of colonised substrates in soil or potting mix was used for cereal bran-based substrates.

Actinomycetes have been tested as potential antagonists, in the presence of a pathogen and a host, as a known number of colony forming units to a unit weight of soil (Crawford *et al.*, 1993), or as weight by weight of pellets of "mycelium" (Rothrock and Gottlieb, 1984) or both "mycelium" and spores, in soil (M. L., Lahdenpera, Kemira Oy, Finland, pers. comm.); information on the use of nutritional substrates is lacking. Although it seems that the direct application of actinomycete antagonists has given satisfactory results, the addition of nutrients may aid establishment. Thus, a food base with high nutrient content for prolific growth and spore production could improve the potential of the actinomycetes as antagonists.

Most of the studies on biological control of soil-borne plant pathogens under growth room and glasshouse conditions have reported some protective effects of antagonists tested. This was so whether the antagonist and the pathogen were inoculated at the same time, at planting (Crawford *et al.*, 1993), or at the same time but one week before planting (Rothrock and Gottlieb, 1984). However, it appeared that a pre-incubation time for actinomycete antagonists was necessary (Rothrock and Gottlieb, 1984).

Experiments reported in this chapter were carried out in potting mix in humid chambers in a growth room. The objectives were to determine a suitable food base for the potential antagonists and the effects of time and rate of application of these antagonists on survival and growth of selected hosts inoculated with *S. rolfsii*. In a preliminary experiment, different grains were screened for suitability as a nutritional supplement for *T. harzianum* and *G. virens*. The two fungal antagonists, growing on the chosen food base, were then tested for their protective effects against *S. rolfsii* on survival of seedlings of wheat and capsicum, at different rates and times of application. Since fungistasis occurred in some experiments, inocula of *S. rolfsii* of different ages were tested for pathogenicity on wheat in the absence and presence of antagonists. Also, experiments on the efficacy of *Streptomyces* spp. (growing on rolled oats) against *S. rolfsii* were carried out on capsicum comparing different rates of inoculum and pre-incubation of the antagonists, and on wheat investigating the effect of inoculum age of the pathogen in the presence of the antagonists.

7.2 Materials and methods

The rationale for the experiment is stated briefly at the beginning of each section, where necessary.

7.2.1 Test of food bases for suitability for *T. harzianum* and *G. virens*

Grains of wheat, oats and barley were tested as food bases for the fungal antagonists (**Figure 7.2.1**). Fifty grams of each type of grain were placed separately

in plastic containers, 6.5 cm diam and 8 cm height (Disposable Products Ltd., SA), and 62 ml double distilled water (DDW) added before closing. These containers of grain were then autoclaved at 121°C for 90 min.

T. harzianum and *G. virens* were grown separately on 1/5M32 agar at 25°C for 3 days. Disks were cut from cultures of each species and put in 1/5M32 broth, separately, at a rate of one disk for each 25 ml. These liquid cultures were incubated on an orbital shaker at 150 rpm at 25°C for 2 days. Suspensions of mycelial fragments of each of these cultures were made using a handheld food processor, operating at medium speed for 1 min. The blade of this processor was surface-sterilised by operating in 70% ethanol for 1 min, immersed in the ethanol for at least 1 h before and after use for each species, and rinsed by operating for 1 min in sterile DDW. Five ml of the mycelial suspension was added to sterilised grain in each container using a sterile pipette tip and shaken to ensure thorough mixing. There were ten replicates per fungus and food base combination. The containers of grain were incubated at 25°C in the dark for 7 days, at which time cultures were sporulating profusely.

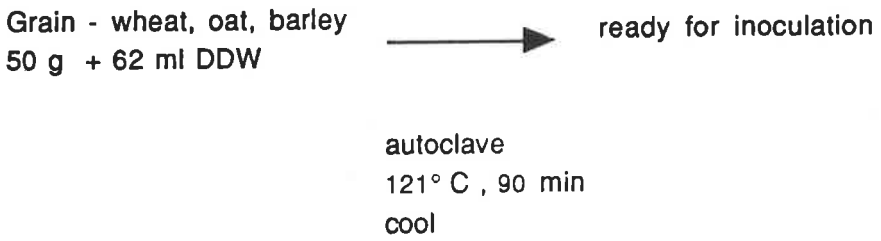
7.2.2 Effect of simultaneous inoculation with *S. rolfsii* and the fungal antagonists, on survival of wheat seedlings

Results from a preliminary experiment showed that the application of *T. harzianum* and *G. virens* in combination with *S. rolfsii* on day of transplanting did not protect seedlings of wheat (data not presented). The experiment described in this section was, therefore, carried out to investigate the effect of the combined application of the pathogen and the fungal antagonists 1 day before transplanting, on survival of wheat seedlings.

Inoculum of *S. rolfsii* was prepared in the form of colonised carrot pieces (see section **6.2.1**), whereas colonised oat grains were chosen as the food base for inocula of *T. harzianum* and *G. virens* (see section **7.2.1**).

Plastic pots, 10 cm diam, containing the potting mix (see section **6.2.1**) were used for eight treatments. These treatments comprised: uninoculated control; *S. rolfsii*; *T.*

Food base preparation



Inoculation of food base

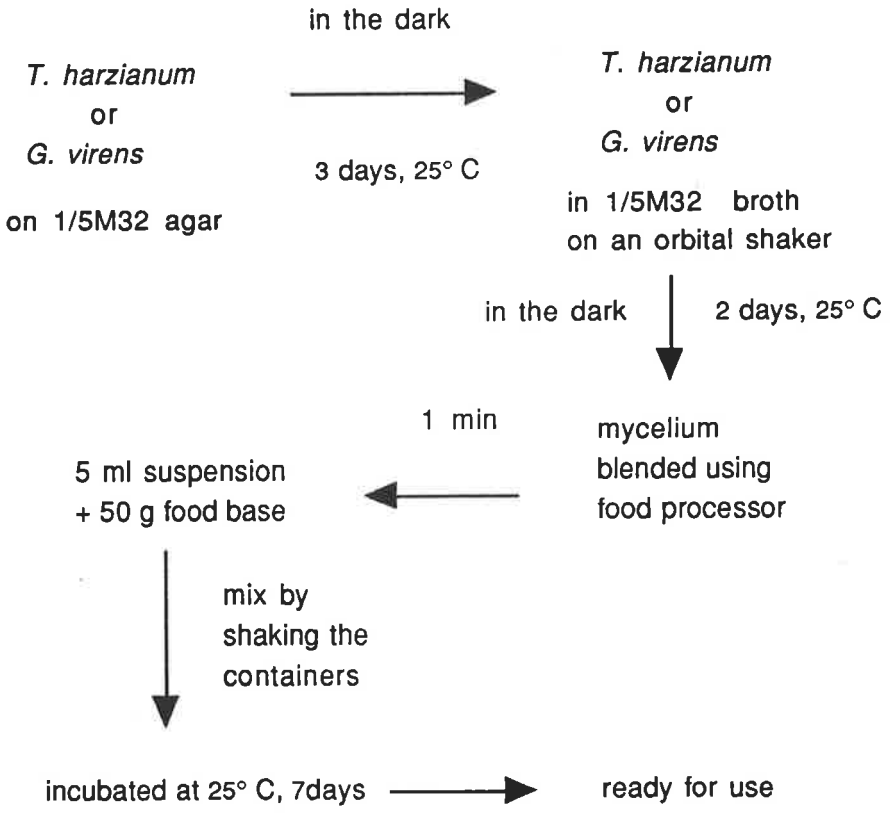


Figure 7.2.1 Preparation of *T. harzianum* and *G. virens* cultures on food bases

harzianum; *G. virens*; *S. rolfsii* + *T. harzianum*; *S. rolfsii* + *G. virens*; *T. harzianum* + *G. virens*; and *S. rolfsii* + *T. harzianum* + *G. virens*. They were arranged in a Randomized Complete Block Design (RCB) with 16 replicate pots per treatment.

Inoculation with the pathogen and/or antagonist for each treatment was carried out following temporary removal of the top 3 cm potting mix from the pot. For treatments involving both the pathogen and antagonist, 10 g of oat grains colonised with *T. harzianum* and/or *G. virens* were spread evenly over the surface, followed by 3 g of carrot pieces colonised with *S. rolfsii*. The potting mix was then replaced. Treatments inoculated with a single culture were prepared with the appropriate fungus as above, whereas the control was left uninoculated. Five seeds of wheat var. "Warigal" were sown in each pot 1 day after inoculation. Pots were incubated in humid chambers at 25°C in a growth room, under banks of fluorescent lights with 12 h daylength, as before (see section **6.2.2**), except that a clear plastic tube, 3.5x7.0 cm, sealed at the top with muslin cloth, was placed over each pot in order to prevent contamination between treatments. The appearance of small black flies in the growth room necessitated this precaution.

The number of living seedlings was recorded daily for 14 days after sowing. Binomial distributions were fitted to the data from day 14. Data were analysed statistically as proportion of living seedlings by accumulated analysis of deviance using the GENSTAT 5 package. A chi-square test (X^2) was used to test for significant differences of the deviances.

7.2.3 Effect of rate of inocula of *T. harzianum* and *G. virens*, applied 7 days prior to inoculation with *S. rolfsii*, on survival of wheat and capsicum seedlings

Results from the experiment described in the previous section showed that five seedlings per 10 cm diam pot in uninoculated controls as well as treatments were stunted 14 days after sowing (see section **7.3.2**), probably due to overcrowding. Since it was intended to observe plants over 28 days, only one plant per pot was used in subsequent experiments including the experiment described in this section. It was

also found that *T. harzianum* and *G. virens* applied at 10 g/pot on the same day as *S. rolfsii*, 1 day before transplanting, did not enhance survival of wheat seedlings (see section **7.3.2**); the experiment described in this section was then designed to investigate the effect of different rates of inocula of these two fungal antagonists applied 7 days before the application of *S. rolfsii*, on survival of wheat and capsicum seedlings. The investigation also determined whether the oat grains used as a food base for the antagonists had any effect on survival of these seedlings.

Preparation of oat grains colonised with *T. harzianum* or *G. virens*, carrot pieces colonised with *S. rolfsii* and preparation of pots and potting mix, were carried out as described previously (see sections **6.2.1** and **7.2.1**). Wheat and capsicum seeds were germinated 3 and 11 days, respectively, prior to planting (see section **5.2.1.1**).

There were two categories of uninoculated controls for each host species; one with sterilised oat grains added at 10, 20 and 30 g/pot, the other without additional oat grains. The remaining treatments included inoculation with *S. rolfsii*, *T. harzianum* or *G. virens* alone and *S. rolfsii* in combination with *T. harzianum* and/or *G. virens* (as in section **7.2.2**). The two antagonist preparations were applied in the relevant treatments at 10, 20 and 30 g/pot. The pathogen and antagonists were delivered to the potting mix (as in section **7.2.2**). One seedling was transplanted into each pot 7 days after the application of the pathogen and antagonists. The treatment combinations were arranged in a RCB with eight replicates. Pots were incubated in humid chambers at 25°C in a growth room, with 12 h daylength (as in section **7.2.2**).

The number of living plants was recorded daily for 14 days after seedling transfer and once a week thereafter. Data from day 28 were analysed statistically by accumulated analysis of deviance using the GENSTAT 5 package, and X^2 was used to test for significant differences of the deviances (as in section **7.2.2**).

Since the addition of sterilised oat grains to the uninoculated control treatment did not affect survival of capsicum seedlings and stunting was observed with the addition in excess of 10 g/pot (see section **7.3.3**), it was decided that this

treatment was unnecessary and, therefore, it was excluded from subsequent experiments.

7.2.4 Effect of rate of inocula and pre-incubation of *T. harzianum* and *G. virens* on survival of capsicum seedlings inoculated with *S. rolfsii*

Results from the experiment described in the previous section showed that neither rates of application nor 7 day pre-incubation period for *T. harzianum* and *G. virens* improved survival of host plants, and that oat grains added at 20 and 30 g/pot caused stunting (see section **7.3.3**). The experiment described in this section was aimed at confirming the effect of the two lower rates of inocula of these antagonists (10 and 20 g/pot), compared to that of the uninoculated control without sterilised oat grains added. It was also designed to investigate survival of capsicum when the antagonists were pre-incubated for 14 days prior to transplanting seedlings.

Formulation of inoculum of *T. harzianum*, *G. virens* and *S. rolfsii* and preparation of pots and potting mix were as before (see sections **6.2.1** and **7.2.1**). Seeds of capsicum were germinated 11 days before planting. The delivery method for the pathogen and antagonists was as in section **7.2.2**.

T. harzianum and/or *G. virens* were applied to the potting mix at 10 and 20 g/pot, either alone or in combination with *S. rolfsii*. The antagonists were added to the soil 14 days before transplanting seedlings, and *S. rolfsii* was applied at one of two times: a) at the same time as the antagonist, and (b) at time of transplanting seedlings. These treatments were compared with one negative and two positive controls. The negative control had neither the pathogen nor the antagonist application, whereas the positive controls had *S. rolfsii* inoculated at the time corresponding to (a) and (b). The treatments were arranged in a RCB with eight replicates. Pots were incubated in humid chambers at 25°C in a growth room, with 12 h daylength (as in section **7.2.2**).

The number of living plants was recorded daily for 28 days after transplanting. Data from day 28 were analysed statistically by accumulated analysis of

deviance using the GENSTAT 5 package, and X^2 was used to test for significant differences of the deviances (as in section **7.2.2**).

7.2.5 Effect of rate of inocula and pre-incubation of *Streptomyces* s1 and s2 on survival of capsicum seedlings inoculated with *S. rolfsii*

Method of preparation of rolled oats colonised with *Streptomyces* is shown in **Figure 7.2.5**. The food base for *Streptomyces* was prepared by adding 50 ml DDW to 50 g rolled oats in a plastic container, 6.5 cm diam and 8 cm height (Disposable Products Ltd., SA), and autoclaving at 121°C for 60 min.

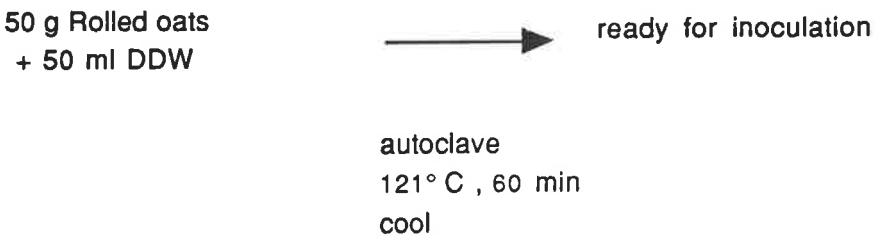
Streptomyces s1 and s2 were grown on 1/5M32 agar in 9 cm diam Petri plates for 7 days. Disks, 1.1 cm diam, were cut from cultures of each and put, separately, into 200 ml 1/5M32 broth at a rate of one disk for each 25 ml. Cultures were placed on an orbital shaker at 150 rpm at 25°C for 7 days, at which time spores were precipitated on the bottom of the container when left still. Supernatant from these liquid cultures was then drained off, leaving a spore suspension of approximately 50 ml for each of s1 and s2 in the container. These spore suspensions were then used to inoculate sterile rolled oats. One ml of spore suspension of each antagonist was thoroughly mixed with sterile rolled oats, using a sterile spatula. The inoculated rolled oats were spread in sterile Petri plates and incubated at 25°C in the dark for 7 days. Oats colonised with *Streptomyces* were then weighed into batches of 10 and 20 g.

Treatment preparation, growing conditions, data collection and statistical analysis on the number of living seedlings of capsicum were as described in section **7.2.4**.

7.2.6 Effect of age of *S. rolfsii* inoculum on survival of wheat seedlings in the presence of *T. harzianum* and *G. virens*

Preparation of inocula of *T. harzianum*, *G. virens* and *S. rolfsii*, pots and potting mix, were carried out as in sections **6.2.1** and **7.2.1**, except that carrot pieces were colonised with *S. rolfsii* for 5 and 10 days before use. Since results

Food base preparation



Inoculation of food base

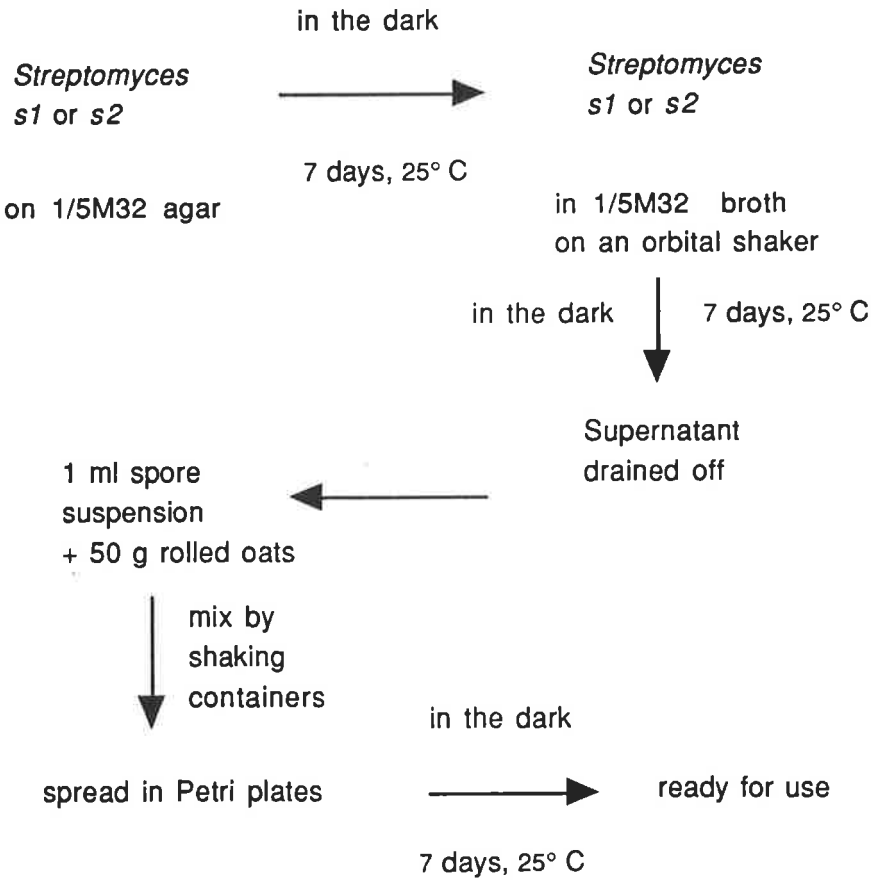


Figure 7.2.5 Preparation of *Streptomyces* spp. on rolled oats

reported in section **7.3.4** showed that the presence of *T. harzianum* or *G. virens* applied at 20 g/pot did not enhance seedling survival, compared to 10 g/pot, the antagonists were applied at 10 g each per pot only. Seeds of wheat var. "Warigal" were germinated 3 days before use. The delivery method for the pathogen and antagonists was as in section **7.2.2.**

T. harzianum and/or *G. virens* were applied to the potting mix 14 days before planting at 10 g/pot, either alone or as a mixed culture of two, and each or both in combination with 5 or 10 day-old inoculum of *S. rolfsii.* Treatments were compared with one negative and two positive controls. The negative control was not inoculated with any microorganisms. One of the positive controls was inoculated with 5 day-old *S. rolfsii* whereas a 10 day-old culture was used for the other.

The treatment layout was a RCB with eight replicates. The experiment was maintained in humid chambers at 25°C in a growth room with 12 h daylength as described previously (see section **7.2.2**). The number of living seedlings was recorded daily for 28 days. Data from day 28 were analysed statistically by accumulated analysis of deviance using the GENSTAT 5 package, and X^2 was used to test for significant differences of the deviances (as in section **7.2.2**).

7.2.7 Effect of age of *S. rolfsii* inoculum on survival of wheat seedlings in the presence of *Streptomyces* s1 and s2

Preparation of host, treatments and growing conditions were as in section **7.2.6**, except that s1 and s2 were used instead of *T. harzianum* and *G. virens*. Inocula of s1 and s2 on rolled oats were prepared as in section **7.2.5.** Data collection and statistical analysis were as in section **7.2.6**.

7.2.8 Test for fungistasis

Since fungistasis was suspected in experiments reported in sections **7.3.3** and **7.3.4**, a rapid test was designed to investigate the ability of sclerotia to germinate and infect plant tissue. Sclerotia were obtained from capsicum seedlings inoculated with *S. rolfsii* alone (see section **7.2.4** for treatment descriptions). Since capsicum

and wheat seedlings were unavailable at the time of this test, pieces of tomato leaf were used instead.

Sclerotia were retrieved from each of eight replicate pots of capsicum seedlings which had been inoculated with *S. rolfsii* alone a) at the time of transplanting the seedlings or (b) at the same time as the antagonist 14 days before transplanting (see section **7.2.4**). These sclerotia were surface-sterilised for 3 min using 10% White King domestic bleach (0.4% available chlorine), then rinsed three times in sterile DDW, 5 min for each rinse.

Two substrates were prepared: 1) 1/5M32 agar, pH adjusted to 5.5, in 9 cm diam Petri plates (see section **2.2.2**), and 2) Moistened potting mix, 0.3 cm deep, in 9 cm diam Petri plates (preparation of potting mix as in section **6.2.1**). Twenty surface-sterilised sclerotia from each of the eight replicate pots of the two *S. rolfsii* treatments were placed on each of these substrates. Sclerotia were placed separately, 5 cm apart, four on each plate. One piece of tomato leaf (0.3x1.0 cm) was placed next to each sclerotium. The plates were incubated in plastic trays at 25°C in a growth room, under banks of fluorescent lights with 12 h daylength (as in section **7.2.2**).

Germination of sclerotia on the two substrates and infection of tomato leaf pieces was observed daily for 7 days.

7.3 Results

7.3.1 Test of food bases for suitability for *T. harzianum* and *G. virens*

Visual inspection showed that *T. harzianum* and *G. virens* grew best and most rapidly on oat grain, compared to wheat and barley. A dense mat of mycelium with young conidia of these two antagonists was seen spreading through oat grains within 3 days of inoculation. Both antagonists sporulated most heavily on oat grains 7 days after inoculation (**Plate 7.3.1 a** and **b**). Oat grains were thus chosen as the food base for *T. harzianum* and *G. virens* for subsequent experiments.


oat
barley
wheat
Trichoderma harzianum growth
in different food bases
1-week old cultures
grain

a)


oat
barley
wheat
Gliocladium virens growth
in different food bases
1-week old cultures
grain

b)

Plate 7.3.1 Cultures of *T. harzianum* (a) and *G. virens* (b) in different food bases.

7.3.2 Effect of simultaneous inoculation with *S. rolfsii* and the fungal antagonists, on survival of wheat seedlings

Approximately 94% of the uninoculated wheat seeds germinated. The presence of *T. harzianum* and/or *G. virens* alone did not significantly affect survival of wheat seedlings ($P<0.05$) (**Figure 7.3.2**). Only a few seedlings survived inoculation with *S. rolfsii* alone. Only the application of *T. harzianum* together with *S. rolfsii* significantly improved survival of wheat seedlings, whereas that of *G. virens* and *S. rolfsii* or the combined application of *T. harzianum* and *G. virens* together with *S. rolfsii* did not.

It was noted that, with five seedlings per 10 cm diam pot, plants in uninoculated controls and in inoculated treatments were stunted 14 days after sowing.

7.3.3 Effect of rate of inocula of *T. harzianum* and *G. virens*, applied 7 days prior to inoculation with *S. rolfsii*, on survival of wheat and capsicum seedlings

Since all seedlings in the two sets of uninoculated control treatments, and *T. harzianum* and *G. virens* (alone) treatments remained alive after 28 days, only data from treatments inoculated with *S. rolfsii* are presented.

The rate of inoculum of the two fungal antagonists and the presence of *T. harzianum* had no significant effect on survival of seedlings. Given that there was no effect of rate of inoculum on survival, data were pooled over the three rates of inoculum of the two antagonists. Survival of wheat and capsicum seedlings differed in response to application of *T. harzianum*, *G. virens* and *S. rolfsii*. All wheat seedlings inoculated with *S. rolfsii* alone died (**Figure 7.3.3**). Survival of wheat seedlings was significantly improved with the application of *G. virens* in combination with *S. rolfsii* or *G. virens* and *T. harzianum* in combination with *S. rolfsii* ($P<0.01$) in comparison with those inoculated with *S. rolfsii* alone. In contrast, 50% of capsicum seedlings survived inoculation with *S. rolfsii* alone. Only *G. virens* and *T. harzianum* in combination with *S. rolfsii* gave significantly greater survival of capsicum than treatment with *S. rolfsii* alone ($p<0.01$).

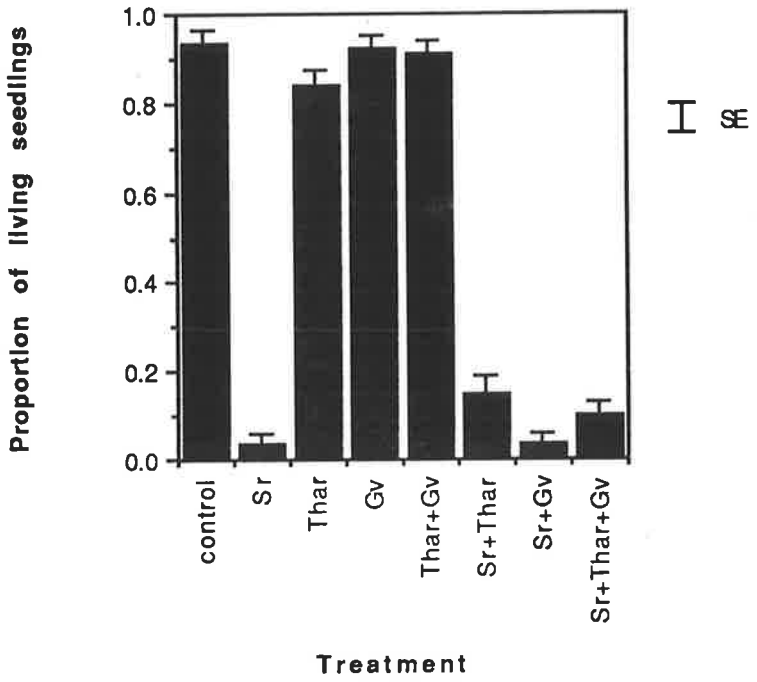


Figure 7.3.2 Proportion of wheat seedlings surviving 14 days after inoculation with *S. rolfsii* (Sr), *T. harzianum* (Thar) and/or *G. virens* (Gv) on the same day. Original data were the number of seedlings surviving out of five in each of 16 replicate pots. SE=standard error of the proportions.

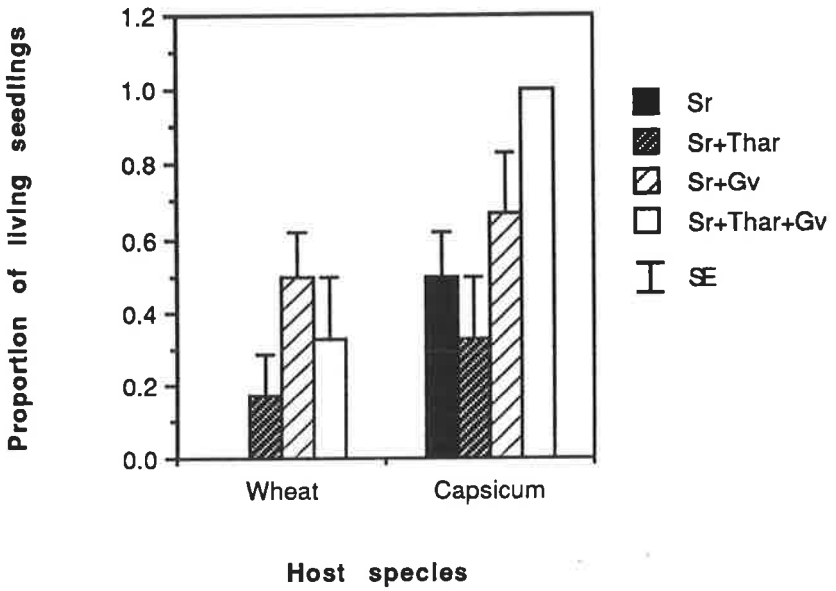


Figure 7.3.3 Proportion of wheat and capsicum seedlings inoculated with *S. rolfsii* surviving after 28 days in the presence of *T. harzianum* and *G. virens*. Data were pooled over the three rates of inoculum of antagonists (10, 20, 30 g/pot) each with eight replicates. SE = standard error of the proportions.

Stunting was observed in seedlings in the uninoculated control treatment with sterilised oat grains added at 20 and 30 g/pot and those in the treatments inoculated with the antagonists alone at 20 and 30 g/pot.

7.3.4 Effect of rate of inocula and pre-incubation of *T. harzianum* and *G. virens* on survival of capsicum seedlings inoculated with *S. rolfsii*

Since all seedlings in the uninoculated control, *T. harzianum* and *G. virens* (alone) treatments remained alive after 28 days, only data from treatments involving *S. rolfsii* are presented. Overall, there was significantly greater survival of capsicum seedlings when *S. rolfsii* and the two fungal antagonists were applied simultaneously 14 days before transplanting seedlings, in comparison to those inoculated with *S. rolfsii* on day of transplanting, 14 days after the application of the antagonists ($p<0.05$)(**Figure 7.3.4 a**). The only significantly different treatment ($p<0.01$), however, was the application of *S. rolfsii* alone; all capsicum seedlings survived when *S. rolfsii* was applied 14 days before transplanting seedlings, whereas approximately half survived when *S. rolfsii* was applied on day of transplanting.

Once the two *S. rolfsii* alone treatments were removed from the analysis to test for differences between the remaining treatments, survival of capsicum seedlings was unaffected by the time of inoculation with *S. rolfsii*, species or rate of inoculum of antagonists. There were, however, significant interactions between species of antagonists and rate of inoculum of antagonists, on survival of capsicum seedlings ($p<0.05$). All capsicum seedlings survived inoculation with *S. rolfsii* in the presence of *G. virens* applied at 10 g/pot, regardless of the time of inoculation with *S. rolfsii* (**Figure 7.3.4 b**). In contrast, fewer seedlings survived in the combination of *T. harzianum* and *S. rolfsii*. When the rate of inoculum of antagonists was increased to 20 g each/pot, survival of capsicum seedlings remained unchanged in all treatments, except *G. virens* in combination with *T. harzianum* and *S. rolfsii*, when it declined slightly but significantly ($p<0.05$).

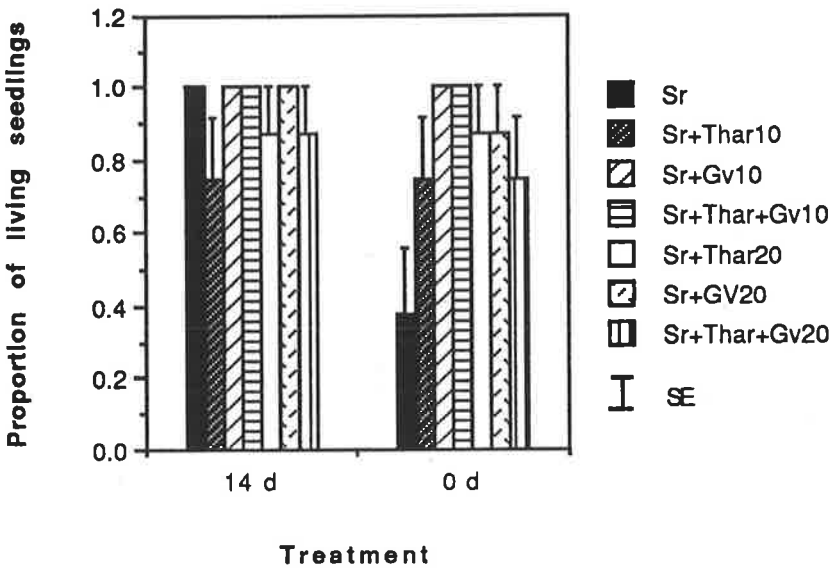


Figure 7.3.4 a Effect of time of inoculation with *S. rolfsii* (Sr), rate of inoculum of *T. harzianum* (Thar) and *G. virens* (Gv) and species of the antagonists, on the survival of capsicum seedlings at 28 days. Antagonists were applied in all relevant treatments 14 days before transplant, whereas *S. rolfsii* was applied at 14 days before transplant (14 d) or on day of transplant (0 d). Rate of inoculum: 10, 20 = 10 or 20 g each antagonist per pot. Original data were the total number of seedlings surviving in eight replicate pots. SE = standard error of the proportions.

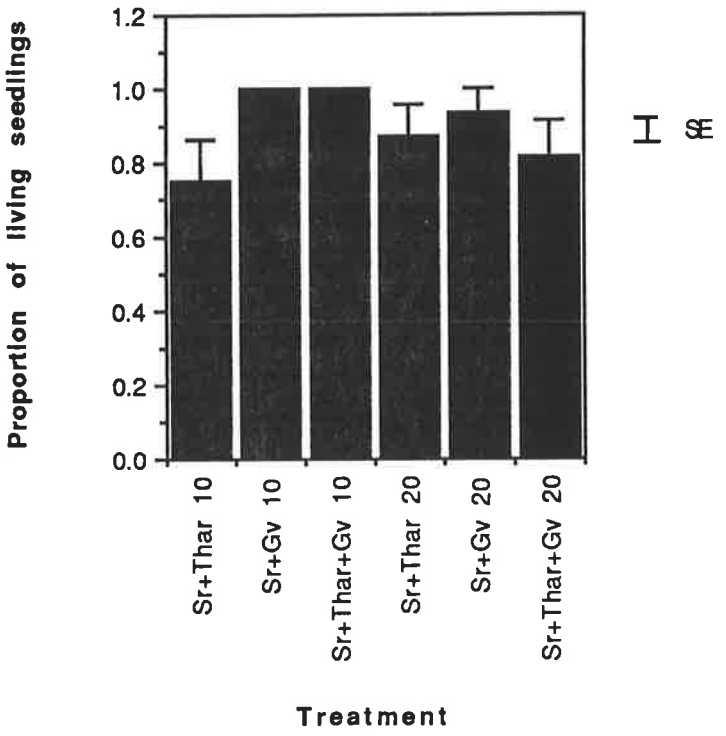


Figure 7.3.4 b Proportion of capsicum seedlings surviving (at 28 days) inoculation with *S. rolfsii* (Sr), as affected by rate of application of *T. harzianum* (Thar) and *G. virens* (Gv). Rate of antagonist: 10, 20 = 10 or 20 g each antagonist per pot. Original data were the total number of seedlings surviving in eight replicate pots. SE = standard error of the proportions.

7.3.5 Effect of rate of inocula and pre-incubation of *Streptomyces* s1 and s2 on survival of capsicum seedlings inoculated with *S. rolfsii*

Since the survival of capsicum seedlings was unaffected by the presence of *Streptomyces* s1 and/or s2 alone, only data from treatments inoculated with *S. rolfsii* are presented.

The rate of application of either *Streptomyces* s1 or s2 and the presence of s2 had no significant effects on seedlings of capsicum surviving inoculation with *S. rolfsii* (data not presented). Survival of wheat seedlings decreased with simultaneous application of *S. rolfsii* and s1 14 days before transplanting, compared to that with the application of *S. rolfsii* alone. In contrast, the survival was increased with the application of *S. rolfsii* on the day of transplanting, 14 days after the application of s1, in comparison to the application with *S. rolfsii* alone ($p<0.05$)(**Table 7.3.5**).

7.3.6 Effect of age of *S. rolfsii* inoculum on survival of wheat seedlings in the presence of *T. harzianum* and *G. virens*

Since all uninoculated seedlings and seedlings inoculated with *T. harzianum* and/or *G. virens* alone were alive after 28 days, only data from treatments inoculated with *S. rolfsii* are presented.

Fewer wheat seedlings survived following inoculation with 5 day-old cultures of *S. rolfsii* compared to 10 day-old cultures whether inoculated with *S. rolfsii* alone or *S. rolfsii* together with *T. harzianum* ($p<0.01$)(**Table 7.3.6**). The application of *G. virens* together with *S. rolfsii*, or *G. virens* in combination with *T. harzianum* and *S. rolfsii*, significantly improved survival of wheat seedlings overall, regardless of age of the pathogen ($p<0.001$). Such enhancement was not found when *T. harzianum* was applied together with *S. rolfsii*.

Table 7.3.5 Number of capsicum seedlings out of 16, surviving inoculation with *S. rolfsii* (Sr) alone or *S. rolfsii* together with s1. The antagonist was added 14 days before transplanting, whereas the pathogen was added 0 or 14 days before transplanting. Data were pooled over the two rates of inoculum of the antagonist (10 and 20 g/pot).

Time of inoculation with *S. rolfsii* (days before transplanting)	Sr	Sr+s1
14	15	13
0	10	12
Total	25	25
X^2 time x s1, df_1	($p<0.05$)	

Table 7.3.6 Number of wheat seedlings out of eight replicates, surviving inoculation with *S. rolfsii* (Sr) alone or *S. rolfsii* in combination with *T. harzianum* (Thar) and/or *G. virens* (Gv). Data were obtained 28 days after inoculation with 5 or 10 day-old cultures of *S. rolfsii*. Only data for treatments inoculated with *S. rolfsii* are presented.

Age of Sr (day-old)	Sr	Sr+Thar	Sr+Gv	Sr+Thar+Gv	Total
5	2	2	5	7	16
10	6	5	7	7	25
Total	8	7	12	14	
x^2 age, df_1	$p<0.01$				
x^2 Gv, df_1	$p<0.001$				

7.3.7 Effect of age of *S. rolfsii* inoculum on survival of wheat seedlings in the presence of *Streptomyces* s1 and s2

Since all uninoculated seedlings of wheat and those inoculated with s1 and/or s2 were alive after 28 days, only data from treatments involving *S. rolfsii* are presented.

Effects of age of *S. rolfsii* and the presence of s1 or s2 on survival of wheat seedlings were insignificant (data not shown). A few wheat seedlings survived inoculation with 5 or 10 day-old cultures of *S. rolfsii*, whereas none survived inoculation with 5 day-old *S. rolfsii* and s1. In contrast, survival of wheat seedlings inoculated with 10 day-old culture of *S. rolfsii* was improved when s1 was present ($p<0.001$)(**Figure 7.3.7 a**).

Interaction between inoculation with the pathogen and species of the antagonist was significant ($p<0.01$). There was a decrease in survival of wheat seedlings overall, regardless of the age of *S. rolfsii*, when s1 or s2 was applied together with *S. rolfsii* compared to the application of *S. rolfsii* alone. The survival was, however, improved slightly when s1 was applied in combination with s2 and *S. rolfsii* (**Figure 7.3.7 b**).

7.3.8 Test for fungistasis

All 160 sclerotia from each of the two treatments inoculated with *S. rolfsii* b) at 14 days before transplanting seedlings, and a) at time of transplanting seedlings, germinated on the agar plates and produced mycelium which infected tomato leaf pieces within 5 days. Percentage germination and infectivity of sclerotia on the potting mix after 7 days incubation was 32%, when the sclerotia had been obtained from potting mix inoculated with *S. rolfsii* 14 days before transplanting. Approximately 43% germination and infectivity occurred in sclerotia obtained from the treatment inoculated with *S. rolfsii* at the time of transplanting seedlings (**Table 7.3.8**).

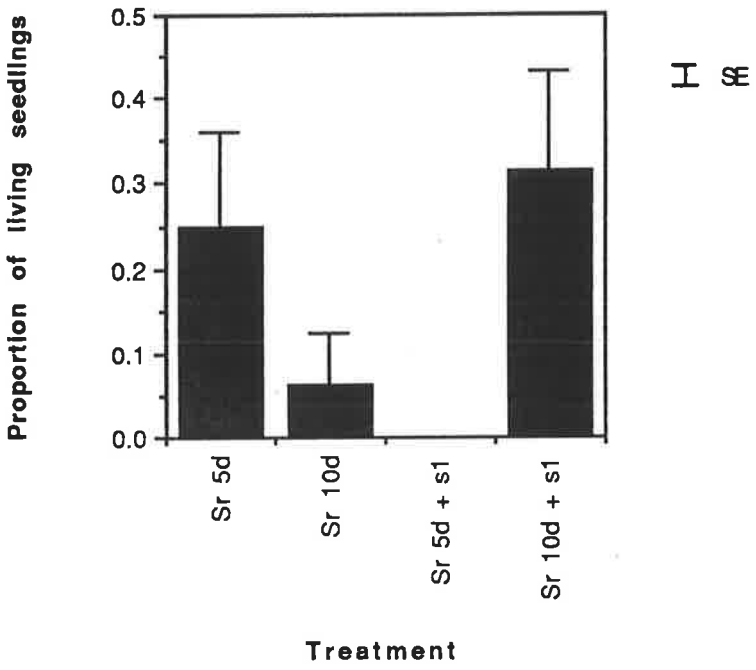


Figure 7.3.7 a Effects of inoculation with 5 and 10 day-old cultures of *S. rolfsii* (Sr 5d and Sr 10d, respectively), and the presence of *Streptomyces* s1 on the survival of wheat seedlings. Original data were the total number of seedlings surviving in eight replicates. SE= standard error of the proportions.

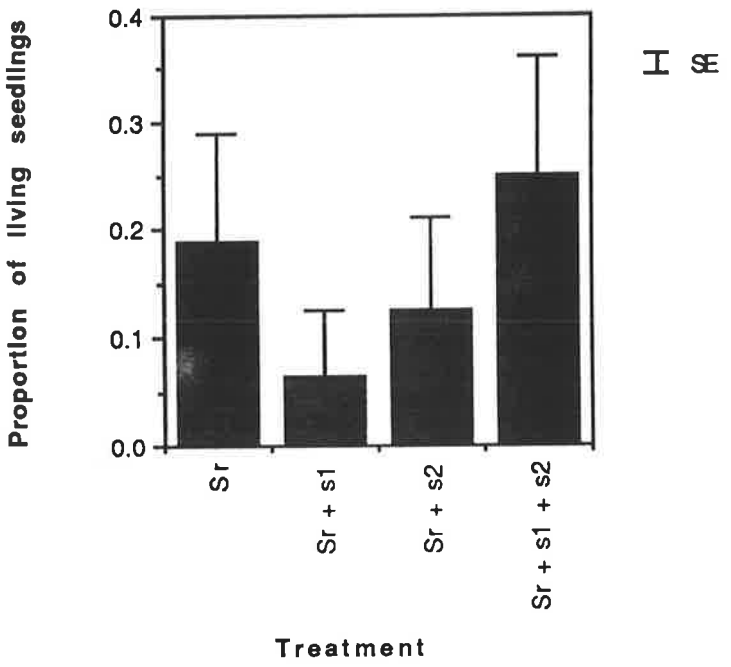


Figure 7.3.7 b Effects of *Streptomyces* s1 and s2 on the proportion of wheat seedlings surviving inoculation with *S. rolfsii* (Sr). Data for eight replicate pots of each of 5 and 10 day-old cultures of Sr were pooled. SE = standard error of the proportions.

Table 7.3.8 Percentage germination and infection by sclerotia from treatments inoculated with *S. rolfsii* alone at different times[a], on 1/5M32 agar plates or potting mix plates.

Time of inoculation with *S. rolfsii*	1/5M32 agar plates	potting mix
a) 14 days before transplanting	100	32.18
b) at time of transplanting	100	42.81

Note:

[a] There were 160 sclerotia from each time of inoculation in each germination medium. All sclerotia which germinated infected tomato leaf pieces.

7.4 Discussion

Sterilised oat grains were found to support good growth and sporulation of the test isolates of *T. harzianum* and *G. virens in vitro* and thus provided an efficient means of introducing inoculum to potting mix or soil. Controls showed that sterilised oat grain added at 10 g per 10 cm diam pot had no detrimental effects on seedling survival, whereas 20 or 30 g per pot caused stunting, possibly due to inhibition of root growth. Similar effects were apparent when the oat grains were colonised with *T. harzianum* or *G. virens*. However, 10 g of the antagonist inoculum was adequate to improve survival of capsicum seedlings (results reported in section **7.3.4**), and would be more economical in terms of resources than the higher rates. Although in some cases, combined application of either of the two fungal antagonists or *Streptomyces* species improved survival of the host plants, stunting occurred, again probably due to the large amounts of food base present, and the efficacy of smaller amounts of inoculum should be investigated.

The *Streptomyces* isolates grew well on rolled oats. The colonised substrate then provided a useful means of application of inoculum of the potential antagonists to potting mix or soil, as an alternative to direct application as previously reported (Crawford *et al.*,1993). This method could aid the establishment of *Streptomyces* in potting mix or soil.

Various problems occurred in the treatments inoculated with *S. rolfsii* alone. One was a lack of reproducibility of results. In the experiments reported in section **7.3.3**, all wheat seedlings were killed by inoculation with 10 day-old culture of *S. rolfsii* but only half of the capsicum seedlings died (**Figure 7.3.3 a** and **b**, respectively). This was rather surprising, as in preliminary experiments capsicum was more susceptible to inoculation with *S. rolfsii* than was wheat (data not shown). Also, 5 day-old cultures of *S. rolfsii* caused most deaths of wheat seedlings in the experiment **7.3.6**, whereas 10 day-old cultures of *S. rolfsii* caused most deaths in **7.3.7**. While the reasons for this were not obvious, variation in pathogenicity of *S. rolfsii* could be a contributing factor. In addition, in experiments reported in section

7.3.4, none of the wheat seedlings was killed when *S. rolfsii* was applied to potting mix 14 days before transplant, whereas less than half survived application of *S. rolfsii* on the day of transplant. This observation suggests that fungistasis had occurred. Fungistasis was also suspected to have occurred in the experiment reported in section **7.3.5**, when *S. rolfsii* killed only one of 16 capsicum seedlings when applied 14 days before transplant but six of 16 capsicum seedlings were killed when the pathogen was applied at time of transplant. The effect of fungistasis was demonstrated in the experiment reported in section **7.3.8**. Percentage germination and infection of tomato leaf tissue by sclerotia retrieved from the treatment in which *S. rolfsii* had been applied 14 days before transplant was 32%, compared to 43% when the pathogen had been applied on the day of transplant. Fungistasis in soil has long been known and is attributed to activity of soil microorganisms as well as chemical and physical properties of soil (see section **1.2.11.5**). Although inoculum of *S. rolfsii* was applied as mycelium on carrot pieces in an attempt to avoid fungistasis and ensure infectivity, this problem still occurred. Apparently, the use of older cultures of *S. rolfsii*, which had sclerotia at various stages of development, made the pathogen prone to fungistasis and prolonged exposure to the potting mix enhanced this phenomenon. This was probably due to the effects of naturally occurring microorganisms since the potting mix was pasteurised, not sterilised. Insert erratum 3 (page 162) Several methods have been used to detect fungistasis in soil and it has been measured as percentage germination of spores of various test fungi. Studies have included the use of cellulose films dusted with spores of *Penicillium nigricans* and buried in moist unsterile soil (Dobbs and Hinson, 1953), a serial dilution assay of soil water using *Botrytis allii* as a test fungus (Jefferys and Hemming, 1953), and the use of agar disks inoculated with spores of a range of fungi placed on filter-paper in contact with moist, unsterile soil (Jackson, 1958). The method described in section **7.2.6** was developed to give a simple and rapid test for both the detection of fungistasis affecting *S. rolfsii* and infectivity of the pathogen, whereas those described previously allowed the detection of fungistasis only.

T. harzianum and *G. virens* showed only slight or no protective effects on survival of wheat seedlings, when both antagonist and pathogen were added to the potting mix 14 days before transplanting seedlings. However, significant protection of seedlings of both wheat and capsicum occurred when these two antagonists were added to the potting mix 14 days before the pathogen. Similar results were obtained with *Streptomyces* s1 and s2. These results suggest that prior establishment of the potential antagonists could be useful for improving their efficacy. This suggestion is supported by results from experiments on beans artificially inoculated with *S. rolfsii* in pots, to which *T. hamatum* was added simultaneously at 10^6 conidia per gram soil (Chet and Baker, 1981). The authors found a 35% decrease in disease incidence at the first planting and a further 28% reduction with the second planting. Pre-incubation time for the potential antagonists, thus, merits further investigation. Apart from this, it is also possible that application of *S. rolfsii* 14 days prior to transplant allowed fungistasis to increase, hence the controls with application of the pathogen alone were less likely to die and thus effects of the antagonists were less likely to be statistically significant. It was, therefore, decided to exclude treatments involving the application of *S. rolfsii* 14 days before transplant from subsequent experiments.

Chapter 8

Effect of antagonists on survival and growth of host plants, in the absence and presence of *S. rolfsii* in potting mix

8.1 Introduction

In addition to reducing losses due to disease (see section **1.2.11.1-2** and **Chapter 7**), *Trichoderma* spp. have been reported to promote plant growth. The application of *Trichoderma harzianum* on wheat bran at 3 gram fresh weight per kilogram soil enhanced dry weight of bean seedlings 25 days after sowing (Elad, Chet and Katan, 1980a). Also, a conidial suspension of *T. harzianum* applied to natural soil at $5x10^6$ conidia per ml, under glasshouse conditions, resulted in 43% enhancement in dry weight of cucumber 15 days after sowing (Kleifeld and Chet, 1992). These authors also found a significant increase in shoot dry weight of tomato and tobacco following the application of two isolates of each of *T. harzianum* and *T. koningii* as peat-bran cultures to give 10^5 cfu per gram soil at planting.

Although there are fewer reports in the literature on biological control of soil-borne diseases by actinomycetes, effects of these antagonists on survival and growth of host plants have been documented. Results from glasshouse experiments showed a significant reduction in severity of damping-off caused by *Rhizoctonia solani* in pea seedlings when *Streptomyces hygroscopicus* var. *geldanus* was applied as mycelial pellets at 0.62 gram (wet weight) for each kilogram soil, 2-7 days prior to application of pathogen and planting. Shoot fresh weight was enhanced by 72% after 7 days, and phytotoxicity was not reported (Rothrock and Gottlieb, 1984). Likewise, simultaneous application of each of 12 isolates of actinomycetes and *P. ultimum* at seeding, at 10^5 cfu of antagonist per gram of potting mix, significantly improved plant survival and shoot dry weight of lettuce by up to 83% and 74%, respectively, 20 days after planting (Crawford *et al.*, 1993). Crawford *et al.* (1993) observed that one of these 12 isolates significantly enhanced the plant biomass in the absence of the

pathogen (data not shown), suggesting the production of plant growth promoting factors.

Although plant growth promotion by certain biological control agents has been reported, until mechanisms involved are understood, it is not possible to exploit this effect fully. Hence, production of plant growth promoting factors by *Trichoderma* spp. and actinomycetes merits investigation.

Experiments presented in **Chapter 7** suggested that pre-incubation of antagonists prior to transplanting of seedlings and inoculation with the pathogen enhanced plant survival, and that fungistasis occurred when the pathogen was applied to the potting mix 14 days prior to transplanting. The experiments reported in this chapter were, therefore, designed to investigate further the effects of application of the fungal and actinomycete antagonists 14 days prior to transplanting seedlings and inoculating with the pathogen. Antagonists were applied separately to avoid stunting due to large amounts of food base present. The antagonist inoculum was mixed with a portion of the potting mix instead of applying as a layer of inoculum in order to improve distribution. Based on observations of enhanced plant growth in certain treatments in previous experiments, shoot dry weights were recorded in addition to seedling survival. Fungal antagonists and *Streptomyces* spp. were evaluated simultaneously under the same conditions. Results, in terms of survival, from fungal and actinomycete experiments were analysed separately to provide continuity with previous experiments, whereas shoot dry weights were analysed together as one experiment to allow comparison between antagonists and hosts with respect to plant growth promotion.

8.2 Materials and methods

8.2.1 Effect of *T. harzianum* or *G. virens* applied 14 days prior to inoculation with *S. rolfsii* and transplanting, on survival of host seedlings

Preparation of potting mix and pots was as in section **6.2.1**. Seeds of wheat and capsicum were germinated for 3 and 11 days, respectively, as before. *T. harzianum* and *G. virens* were inoculated on oat grains and incubated for 7 days (see section **7.2.1**), and 10 g of colonised grain was used to inoculate each pot. Inoculum of *S. rolfsii* was 5 day-old mycelium on carrot pieces (see section **6.2.1**).

Treatments were: *T. harzianum*, *G. virens* or *S. rolfsii* alone and *T. harzianum* or *G. virens* and *S. rolfsii* applied to capsicum and wheat. Controls were uninoculated seedlings. There were eight replicates arranged in a RCB.

Application of antagonists was as follows: the top 3 cm of the potting mix was removed and 10 g of oat grain colonised with *T. harzianum* or *G. virens* was mixed with a small portion of the potting mix in the pot using a spatula. The potting mix which had been removed was then replaced. The pathogen was applied 14 days later, in the appropriate treatments, by making a 3 cm deep well in the centre of the potting mix, placing 3 g colonised carrot pieces in the well and mixing the inoculum into the potting mix with a spatula. At this time, one seedling was transplanted into each pot of each treatment including uninoculated controls. Throughout the experiment, pots with or without seedlings were incubated in humid chambers at 25°C under fluorescent lights with 12 h daylength in a growth room (as in section **7.2.2**). Moisture content of the potting mix was estimated using a "Plant saver" meter (manufacturer unknown, Taiwan) and pots watered to approximately soil moisture holding capacity.

The number of living seedlings was recorded at 7-day intervals for 28 days. The number of seedlings surviving 28 days after planting was analysed statistically by accumulated analysis of deviances using the GENSTAT 5 package, and X^2 was used to test for significant differences of the deviances. Shoot dry weights were obtained and analysed as described in section **8.2.3**.

8.2.2 Effect of *Streptomyces* s1 or s2 applied 14 days prior to inoculation with *S. rolfsii* and transplanting, on survival of host seedlings

Materials and methods were as in section **8.2.1**, except that *Streptomyces* s1 and s2, applied as colonised rolled oats (see section **7.2.6**) were used instead of *T. harzianum* and *G. virens*. Statistical analysis was performed on number of wheat and capsicum seedlings surviving 28 days after the application of *S. rolfsii*, using accumulated analysis of deviances as in section **8.2.1**. Shoot dry weights were obtained and analysed as described in section **8.2.3**.

8.2.3 Shoot dry weight

Seedlings were harvested 28 days after transplanting. Potting mix clinging to the roots was washed off gently in water. Shoots and roots were separated and blotted dry. They were then dried, individually, in an oven at 80°C for 2 days and weighed.

Root dry weights in some samples were negligible while in others roots had rotted completely. Dry weight of roots was, therefore, excluded from statistical analysis. Data for shoot dry weight of capsicum and wheat seedlings from both experiments were combined to compare responses between host species, and also antagonist species. Data were subjected to ANOVA, using the GENSTAT 5 package.

8.3 Results

8.3.1 Effect of *T. harzianum* or *G. virens* applied 14 days prior to inoculation with *S. rolfsii* and transplanting, on survival of host seedlings

Since all uninoculated wheat and capsicum seedlings and those inoculated with antagonist alone were alive at the termination of the experiment, only data for treatments involving *S. rolfsii* are presented.

Response, in terms of seedling survival, of capsicum and wheat was similar. Survival of seedlings treated with *T. harzianum* or *G. virens* in combination

with *S. rolfsii* was approximately three and four times greater, respectively, than that of seedlings inoculated with the pathogen alone ($p<0.01$)(**Table 8.3.1**).

It was noteworthy that seedlings surviving in combined applications of antagonist and pathogen were much smaller than the corresponding uninoculated controls. This effect was most pronounced in treatments involving *T. harzianum*. Some wheat seedlings inoculated with antagonist alone were larger than the uninoculated controls.

8.3.2 Effect of *Streptomyces* s1 or s2 applied 14 days prior to application of *S. rolfsii* and transplanting, on survival of host seedlings

Since all uninoculated wheat and capsicum seedlings were alive at the termination of the experiment, only data for treatments involving *S. rolfsii* are presented.

Response, in terms of seedling survival, of capsicum and wheat was similar. Survival of seedlings treated with *Streptomyces* s1 or s2 in combination with *S. rolfsii* was approximately twice that of seedlings inoculated with the pathogen alone ($p<0.01$)(**Table 8.3.2**).

It was noteworthy that seedlings surviving in combined applications of antagonist and pathogen were much smaller than the corresponding uninoculated controls. This effect was most pronounced in treatments involving s2. Some wheat seedlings inoculated with antagonist alone were larger than the uninoculated controls.

8.3.3 Shoot dry weight

The application of *S. rolfsii* alone significantly reduced shoot dry weight of capsicum and wheat seedlings compared to uninoculated controls ($p<0.001$)(**Figure 8.3.3**). Interaction between application of the pathogen and host species was significant at $p<0.001$. Application of *Streptomyces* s1 or s2 alone significantly increased shoot dry weight of both plant species ($p<0.001$). In treatments combining pathogen and

Table 8.3.1 Number of wheat and capsicum seedlings surviving inoculation with *S. rolfsii* (Sr), when *T. harzianum* (Thar) or *G. virens* (Gv) had been applied to the potting mix 14 days prior to application of the pathogen and transplanting of seedlings. Data are the number of seedlings surviving in eight replicate pots, one each pot.

Treatment	Host species		Total
	wheat	capsicum	
Sr	1	2	3
Sr + Thar	6	4	10
Sr + Gv	6	6	12
Total	13	12	
X^2 antagonist, df_2	($p<0.01$)		

Table 8.3.2 Number of wheat and capsicum seedlings surviving inoculation with *S. rolfsii* (Sr), when *Streptomyces* s1 or s2 had been applied to the potting mix 14 days prior to application of the pathogen and transplanting of seedlings. Data are the number of seedlings surviving in eight replicate pots, one each pot.

Treatment	Host species		Total
	wheat	capsicum	
Sr	4	2	6
Sr + s1	8	5	13
Sr + s2	7	7	14
Total	19	14	
X^2 antagonist, df_2	($p<0.01$)		

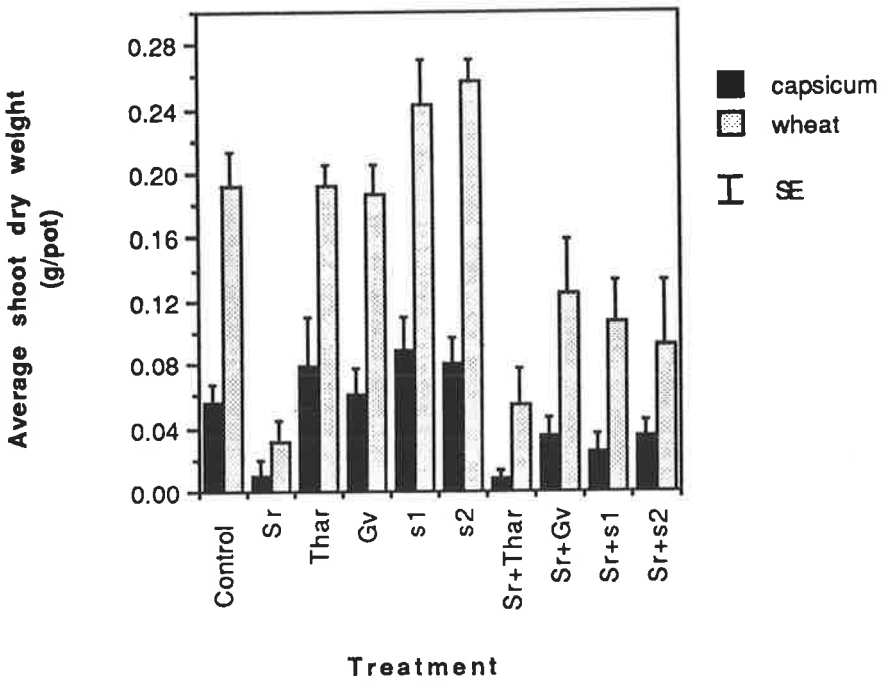


Figure 8.3.3 Average shoot dry weight of capsicum and wheat seedlings inoculated with *S. rolfsii* (Sr), *T. harzianum* (Thar), *G. virens* (Gv), *Streptomyces* s1 or s2, either alone or in combinations of the pathogen and each antagonist. The antagonists were applied to potting mix 14 days prior to application of the pathogen and transplanting of seedlings. Original data were obtained from eight replicate pots, one seedling each pot. SE = standard error of the mean.

antagonist, shoot dry weight of capsicum did not differ significantly from that in the treatment with *S. rolfsii* alone. In contrast, shoot dry weight of wheat seedlings was significantly improved with the application of either *G. virens* or s1 in combination with *S. rolfsii*, compared to *S. rolfsii* alone.

8.4 Discussion

T. harzianum and *G. virens* applied to potting mix at 10 g oat-grain inoculum per pot 14 days before application of *S. rolfsii* and transplanting of seedlings, enhanced survival of wheat and capsicum seedlings. Likewise, *Streptomyces* s1 and s2 prepared in a rolled-oat food base, enhanced seedling survival. These results demonstrate the efficacy of the delivery method developed in this study (see **Chapter 7**). When introduced into potting mix or soil, the antagonists may be exposed to fluctuations of temperature and competition with other soil microorganisms for nutrients. The extra nutrients supplied by the food base may be essential for the establishment of these antagonists and, hence, their performance as biocontrol agents. The significance of a food base has been well documented (Elad et al., 1980a; Ristaino et al., 1991; Wolffhechel and Funck-Jensen, 1992), but the possible benefits of a pre-incubation period for establishment of microorganisms introduced into soil or potting mix have not been considered.

The application of *Streptomyces* s1 or s2 alone gave a noticeable increase in the average shoot dry weight of wheat and capsicum seedlings, suggesting that plant growth promoting factors had been produced and/or induced. These results support the findings of Crawford *et al.* (1993) that one of 12 actinomycete strains, applied to potting mix at time of planting in a glasshouse, enhanced biomass of lettuce. *Streptomyces* isolates able to produce or induce plant growth promoting factors could enable plants to survive challenge with a pathogen. Enhancement of plant growth as well as antagonism of pathogens could contribute to the protective effect of biocontrol agents. This hypothesis is supported by results reported in experiment **8.3.3**. As well as enhancing survival of wheat seedlings, the application of s1 14 days prior to inoculation with *S. rolfsii* improved average shoot dry weight of wheat seedlings in comparison to the application of *S. rolfsii* alone. In contrast, application of s2 did not

enhance growth of either wheat or capsicum in the presence of the pathogen, and those plants surviving the combined application of the pathogen and s2 were stunted from 7 days after the application of the pathogen. This suggests that plant growth promoting effect did not have a major contribution to disease control in the case of s2. Application of *G. virens* together with *S. rolfsii* enhanced survival of wheat and improved shoot dry weight of wheat, with no indication of plant growth promoting effects by the antagonist. This suggests that mechanisms other than the production of plant growth promoting factors were operating. Previous studies have shown that *G. virens* may produce viridin which is converted to the phytotoxin viridiol (Jones and Hancock, 1987), but there was no evidence of phytotoxicity following the application of inoculum at 10 g per 10 cm diam pot. Application of *T. harzianum* in combination with *S. rolfsii*, although enhancing survival of wheat and capsicum, did not increase shoot dry weight of these plants significantly. Since there was no indication of plant growth promoting factors produced/induced, the enhancement of survival caused by *T. harzianum* in this instance could, again, be attributed to mechanisms other than the production of plant growth promoting factors.

Although survival of wheat and capsicum was enhanced with application of each of the four antagonists in combination with *S. rolfsii*, only wheat showed a significant improvement in shoot dry weight when *G. virens* or s1 was applied together with the pathogen. Since *S. rolfsii* alone had a strong impact on shoot dry weight of host plants, results suggest a difference between host species in response to species of the antagonists tested. Most studies on biological control of soil-borne plant pathogens have concentrated on species of antagonists and one or few host species, and information on response of different species of host to the selected antagonists is lacking. A study on mechanism of this response could enhance efficacy of antagonist application and, therefore, merits further investigation.

Chapter 9

General discussion

This study was designed to examine interactions between *Sclerotium rolfsii* and potential antagonists *in vitro* and to relate these to protection of host plants in potting mix. The warm, humid environment created in the growth room was designed to simulate conditions in tropical and subtropical regions, as well as those which might be expected in a close crop canopy in warm temperate regions. Such conditions favour development of Sclerotium diseases in many crops worldwide.

Results obtained in this study showed that the selected antagonist strains had a significant effect on growth and survival of *S. rolfsii*, and on development of disease on capsicum and wheat, *in vitro* and *in vivo*. Only one strain of *S. rolfsii* and two strains of each of the fungal and actinomycete antagonists were used in this study to allow detailed investigation of the interactions between the pathogen and antagonists *in vitro* and *in vivo*.

The strain of *S. rolfsii* used was isolated from field-grown capsicum. This fungus is known to be an aggressive soil-borne pathogen which kills host plants rapidly. However, development of disease on host plants inoculated with cultures of *S. rolfsii*, which had been grown on carrot pieces for 10 days before use, was found to be inconsistent. Such inoculum consisted of mycelium and sclerotia at different stages of development. This inconsistent pathogenicity appeared to be due largely to variation in inoculum and fungistasis, probably the result of interactions between the pathogen and other soil microorganisms. Fungistasis in natural soil has been studied as an indirect means of control of *S. rolfsii* disease (see section **1.2.11.5**) but has not been reported previously in experiments using potting mix. Fungistasis was reduced by using actively growing 5 day-old mycelium, again on carrot pieces, instead of older inoculum. Since only one strain of *S. rolfsii* was used, it is also possible that variation, perhaps due to mutation and sectoring, occurred and further studies of pathogenicity and confounding effects of fungistasis should involve a larger number of isolates.

With limited information, in terms of response to application of antagonists with or without *S. rolfsii*, on plant species other than legumes, capsicum and wheat were chosen as test plants in this study. Both species were susceptible to the strain of *S. rolfsii* used. Conditions in seedling bioassays on agar disks in Petri plates and in potting mix in humid chambers were equally conducive to disease development on capsicum. In contrast, growth room conditions were more conducive for initiation and development of disease in wheat. This difference may be due to the relative humidity needed for disease development on wheat; it is likely that relative humidity in Petri plates was less than optimal. The Petri plate bioassay for pathogenicity of *S. rolfsii* on eight wheat varieties commonly grown in South Australia showed that these varieties may be divided into two groups; moderately susceptible and moderately resistant, based on their susceptibility to the pathogen. These observations need to be confirmed by inoculation of seedlings in potting mix or soil in natural conditions. Although disease in this bioassay was less severe than expected, the method, perhaps with modification to optimise relative humidity, may be useful for rapid preliminary screening for resistance to *S. rolfsii* in breeding programs.

In order to facilitate comparisons between *in vitro* and *in vivo* experiments, culture media assumed to have nutrient status close to that of agricultural soil were included in experiments involving fungal antagonists *in vitro*. However, it appeared that inhibitory effects of antagonistic fungi were more pronounced when PDA or PDB were used. Dennis and Webster (1971a) have suggested previously that production of non-volatile antibiotics is enhanced in culture medium of high nutrient status without presenting supporting data, and the observations made in this study lend support to this suggestion. They also illustrate the need to follow up *in vitro* experiments with studies in potting mix or soil.

There was no convincing evidence of mycoparasitism of *S. rolfsii* by *T. harzianum* and *G. virens*, therefore, it was not considered as a major mechanism for control of the pathogen, whereas antibiosis was likely to be involved. Both *T. harzianum* and *G. virens* produced metabolites which reduced growth and viability of *S.*

rolfsii in dual cultures *in vitro*. The degree of reduction varied depending upon nutrient status of the growing medium and strain of antagonist. Viability of sclerotia of the pathogen was reduced to a similar level by metabolites from *T. harzianum* and *G. virens* in soil extract agar. Thus, both antagonists have the potential to reduce the primary source of inoculum of *S. rolfsii* in potting mix or soil in nursery or field conditions and, hence, reduce disease incidence.

By means of the cellophane overlay technique (Dennis and Webster, 1971a), both *T. harzianum* and *G. virens* were found to be capable of producing diffusible metabolites inhibiting growth of *S. rolfsii in vitro*, in a range of media from nutrient-poor (1/6NDY) to intermediate (1/5M32) and rich (1/2 PDA, PDA). As in dual culture experiments, the degree of inhibition was dependent upon nutrient status of the medium and strain of antagonist. Growth inhibition caused by *G. virens* was greater than that by *T. harzianum* in medium low and intermediate in terms of nutrient status. This indicated that *G. virens* could be a better antagonist than the latter under nutrient stress conditions, which are likely to occur in soil or potting mix due to competition among soil microorganisms.

Ability of *T. harzianum* and *G. virens* to produce inhibitory metabolites when grown in 1/5M32 or PDB was confirmed by means of the culture filtrate technique (Dennis and Webster, 1971a; Ridout *et al.*, 1992). In general, filtrates of cultures of *G. virens* and *T. harzianum* as young as 2 or 3 days-old were more inhibitory than those from older cultures. If such metabolites were produced rapidly in soil or potting mix, then these strains would have potential to control the fast-growing *S. rolfsii*. While there was some evidence for production of volatile metabolites by *T. harzianum*, inhibition was slight, and it was concluded that non-volatile metabolites were more likely to contribute to any antagonism of *S. rolfsii* by *T. harzianum* and *G. virens*.

In the plant bioassay in Petri plates, the host species differed in terms of survival following application of each fungal antagonist in combination with *S. rolfsii*. Survival of capsicum seedlings was enhanced by *G. virens* but not by *T. harzianum*. In

contrast, both antagonists enhanced survival of wheat seedlings to a similar level. The protective effects appeared to be related to the production of non-volatile and volatile compounds, discussed above. Beneficial effects of *G. virens* on survival of host plants were confirmed in pot experiments. *T. harzianum*, although it did not enhance survival of capsicum in the Petri plate bioassay, did improve survival of this host in potting mix, again suggesting that the *in vitro* bioassay requires some modification to improve correlation with *in vivo* experiments. As well as enhancing survival of both host species, application of *G. virens* improved shoot dry weight of wheat but not capsicum. There was no evidence of phytotoxicity due to production of viridiol (Jones and Hancock, 1987) at the level of inoculum used. Since there was no evidence of plant growth promotion by either *T. harzianum* or *G. virens* when applied alone in potting mix, protection of host plants by these two antagonists was likely to be due to the production of volatile and non-volatile metabolites by *T. harzianum* and non-volatile metabolites by *G. virens*. It was apparent that *G. virens* protected host plants more effectively than did *T. harzianum*. The experiments carried out in potting mix confirmed results of *in vitro* studies on effects of metabolites obtained using the cellophane overlay technique with 1/5M32, in particular.

By means of the cellophane overlay technique, it was observed that *Streptomyces* s1 and s2 produced diffusible metabolites reducing growth and sclerotium production of *S. rolfsii in vitro*, with s1 being the more effective. Inhibition of *S. rolfsii* by s1 and s2 in culture filtrate studies was greatest for filtrate harvested from 28 and 14 day-old cultures, respectively. While it was evident that volatile metabolites were produced *in vitro* by *Streptomyces* s1 and s2, inhibition of growth of *S. rolfsii* was slight, and it was concluded that non-volatile metabolites were, as with the fungal antagonists, more likely to be a major contributing factor to any antagonism of *S. rolfsii* by s1 and s2. Although these antagonists produced non-volatile and volatile inhibitors, they did not enhance survival of hosts in the Petri plate seedling bioassay. It may be that the small amounts of these compounds present or produced in the agar disks used in the bioassay were insufficient to provide any

protection to the seedlings, and that greater amounts of "mycelium" and nutrients are required. This suggestion is supported by the enhancement of survival of wheat and capsicum observed when either s1 or s2 was applied to potting mix in the rolled oat food base. In addition, these two *Streptomyces* strains appeared to promote growth, as indicated by increase in shoot dry weight of wheat and capsicum seedlings. The plant growth promotion effect of s1 was also observed in wheat inoculated with the pathogen and antagonist, whereas s2 did not enhance shoot dry weight of either host species in the presence of the pathogen. Results overall suggest that non-volatile, and perhaps volatile compounds also, together with plant growth promotion factors produced by the *Streptomyces* strains were responsible for protection of host plants against *S. rolfsii*, and that the level of protection depended upon the strain of the antagonist as well as response of host species to antagonist application.

Results obtained in experiments on seedlings in humid chambers (**Chapters 7 and 8**) indicated that allowing the antagonists to become established in the potting mix prior to introducing the pathogen and seedling reduced the pathogenic activity of *S. rolfsii*. This finding is supported by results from the study of *S. rolfsii* on beans in soil in the glasshouse by Chet and Baker (1981), using *Trichoderma hamatum*. These authors found that there was a further reduction in disease incidence on the host at a second planting, 14 days after the first planting and application of the antagonist (see section **7.4**). Likewise, supporting evidence on effect of pre-incubation period of *Streptomyces* in soil on pathogenic activity of a soil-borne plant pathogen was found in controlled conditions in the glasshouse by Rothrock and Gottlieb (1984). These authors reported a reduction in severity of damping-off caused by *Rhizoctonia solani* in pea seedlings by application of *Streptomyces hygroscopicus* var. *geldanus* 2-7 days prior to application of pathogen and planting (see section **8.1**). Although *in vitro* studies using cellophane overlay and culture filtrate techniques had shown that young (2-3 day-old) cultures of the fungal antagonists inhibited the pathogen effectively, it may be that in potting mix, a longer period is required for establishment and accumulation of effective levels of metabolites. Although the

pathogen may already be present in some field situations, prior application of antagonists may provide some protection in nursery operations, in soil not known to harbour the pathogen, or in soil replanted after sterilisation.

For an effective control of the fast-growing *S. rolfsii*, it is recommended that *G. virens* or *Streptomyces* s1 be applied to potting mix or soil as cultures on a cereal food base, prior to planting. The antagonist inoculum should be distributed throughout the growing substrate in order to improve control. In field conditions, this could be done simultaneously with ploughing. For a highly susceptible host, such as capsicum, seed dressing in addition to soil application may be necessary if there is a history of *S. rolfsii* disease. These organisms, therefore, have potential as commercial biological control agents for *S. rolfsii* in nursery and field crops, and should be studied and developed further. Insert erratum 4 (page 162).

Appendix 1

Media and solutions for isolation and culture of soil microorganisms

All media were autoclaved at 121°C for 20 min.

1/5 M32 agar

K_2HPO_4	0.5 g
Ferric tartrate (0.5% solution)	1.0 ml
$MnSO_4.7H_2O$	0.5 g
Glucose	5.0 g
Neutralised bacteriological peptone (Oxoid)	2.5 g
Yeast extract	0.5 g
Agar, bacteriological (Oxoid, Agar No. 1)	38.0 g
Trace elements	5.0 ml

(2 g $MnSO_4$ + 1 g Na_2MoO_4 +0.2 g $ZnSO_4$ + 0.1 g $CuSO_4$ in 1000 ml distilled water)

Distilled water	2494 ml

Crystal Violet Agar

Neutralised bacteriological peptone (Oxoid)	5.0 g
Beef extract ('LAB-LEMCO'powder)(Oxoid)	5.0 g
Agar, bacteriological (Oxoid, Agar No. 1)	15.0 g
Tap water to	1000 ml

Add 2 ppm crystal violet immediately before pouring agar.

Rose Bengal Agar

KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
Neutralised bacteriological peptone (Oxoid)	5.0 g
Dextrose	10.0 g
Rose bengal	0.03 g
Agar, bacteriological (Oxoid, Agar No. 1)	20 g
Distilled water to	1000 ml

Add 3 ml of a filter sterilised solution of 0.1% streptomycin sulphate immediately before pouring agar.

Casein Glycerol Medium (CGM)

Casein (Difco) "vitamin free"	0.3 g
Glycerol	10.0 g
KNO_3	2.0 g
NaCl	2.0 g
K_2HPO_4	2.0 g
$MgSO_4.7H_2O$	0.05 g
$CaCO_3$	0.02 g
$FeSO_4.7H_2O$	0.01 g
Agar, bacteriological (Oxoid, Agar No. 1)	15.0 g
Distilled water to	1000 ml

King's B Medium

Proteose peptone	20.0 g
Glycerol	10.0 g
K_2HPO_4	1.5 g
$MgSO_4$	1.5 g

Agar, bacteriological (Oxoid, Agar No. 1)	15.0 g
Distilled water to	1000 ml

Soil extract agar (SEA)

Soil	1 kg
Tap water	1 l

Soil was autoclaved in tap water at 121°C for 30 min. The supernatant was then filtered off and tap water added to make 1 l. Fifteen grams of agar, bacteriological (Oxoid, Agar No. 1), was added to this supernatant and the mixture was autoclaved as normal.

1/6 NDY

NaNO3	0.330 g
KH_2PO_4	0.166 g
$MgSO_4.7H_2O$	0.083 g
KCl	0.083 g
Yeast extract	0.083 g
Sucrose	5.0 g
Agar, bacteriological (Oxoid, Agar No. 1)	15.0 g
Distilled water to	1000 ml

Ringer's solution

NaCl	7.5 g
KCl	0.075 g
$CaCl_2$	0.1 g
$NaHCO_3$	0.1 g
Distilled water to	1000 ml

References

Agrios, G. N. 1988. Plant Pathology. Third Edition. Academic Press Inc., New York.

Alexander, M. 1977. Introduction to Soil Microbiology. 2nd Ed. John Wiley, New York.

Anon. 1971 - 1978. *Corticium rolfsii*. CMI Descriptions of Pathogenic Fungi and Bacteria No. 410. The Commonwealth Mycological Institute, Surrey, England.

Armentrout, V. N., Graves, L. B. .Jr. and Maxwell, D. P. 1978. Localization of enzymes of oxalate biosynthesis in microbodies of *Sclerotium rolfsii*. Phytopathology 68: 1597-1599.

Aycock, 1966. Stem rot and other diseases caused by *Sclerotium rolfsii*. North Carolina Agricultural Experimental Station Technical Bulletin no. 174.

Bahadur, P., Sinha, V. C. and Upadhyaya, Y. M. 1976. Effect of certain sugars and nitrogen sources on the growth of *Sclerotium rolfsii*. Indian Journal of Phytopathology 29: 426-427.

Baker, R. and Dickman, M. B. 1993. Biological control with fungi. In: Soil Microbial Ecology. F.B. Metting Jr (ed), pp. 275-305, Marcel Dekker Inc., New York.

Bakerspigel, A. and Miller, J. J. 1953. Comparison of oxgall, crystal violet, streptomycin and penicillin on bacterial growth inhibitors. Soil Science 76: 123-126.

Barnett, N. M. 1974. Release of peroxidase from soybean hypocotyl cell walls by *Sclerotium rolfsii* culture filtrates. Canadian Journal of Botany 52: 265 - 271.

Bateman, D. F. 1970. Depletion of the galacturonic acid content in bean hypocotyl cell walls during pathogenesis by *Rhizoctonia solani* and *Sclerotium rolfsii*. Phytopathology 60: 1846-1847.

Bateman, D. F. 1972. The polygalacturonase complex produced by *Sclerotium rolfsii*. Physiological Plant Pathology 2: 175-184.

Beniwal, S. P. S., Ahmed, S. and Gorfu, D. 1992. Wilt/root rot diseases of chickpea in Ethiopia. Tropical Pest Management 38 (1): 48-51.

Berdy, J. 1980. Recent advances in and prospects of antibiotics research. Process Biochemistry 15: 28-36.

Branch, W. D. and Csinos, A. S. 1987. Evaluation of peanut cultivars for resistance to field infection by *Sclerotium rolfsii*. Plant Disease 71 (3): 268-270.

Brenneman, T. B., Murphy, A. P. and Csinos, A. S. 1991. Activity of tebuconazole on *Sclerotium rolfsii* and *Rhizoctonia solani*, two soil-borne pathogens of peanut. Plant Disease 75 (7): 744-747.

Campbell, R. 1989. Biological control of microbial plant pathogens. Cambridge University Press, Cambridge.

Canullo, G. H., Rodriguez-Kabana, R., and Kloepper, J. W. 1992. Changes in soil microflora associated with control of *Sclerotium rolfsii* by furfuraldehyde. Biocontrol Science and Technology 2 (2): 159-169.

Chambers, S. M. 1994. Phytophthora rootrot of chestnut. PhD thesis, University of Adelaide, Australia.

Chastagner, G. A., Stalcy, J. M. and Riley, K. 1990. Control of *Sclerotium rolfsii* on bulbous iris and lilies with in-furrow fungicide application. Acta Horticulture 266: 457-467.

Chen, W., Hoitink, H. A. J., Schmitthenner, A. F. and Tüovinen, O. H. 1988. The role of microbial activity in suppression of damping-off caused by *Pythium ultimum.* Phytopathology 78 (3): 314-322.

Chet, I. 1975. Ultrastructural basis of sclerotial survival in soil. Microbial Ecology 2: 194-200.

Chet, I. and Henis, Y. 1968. The control mechanism of sclerotial formation in *Sclerotium rolfsii* Sacc. Journal of General Microbiology 54: 231-236.

Chet, I. and Henis, Y. 1968. The control mechanism of sclerotial formation in *Sclerotium rolfsii* Sacc. Journal of General Microbiology 54 : 231-236.

Chet, I. and Henis, Y. 1968. X-ray analysis of hyphal and sclerotial walls of *Sclerotium rolfsii* Sacc. Canadian Journal of Microbiology 14: 815-816.

Chet, I., and Baker, R. 1981. Isolation and biocontrol potential of Trichoderma hamatum from soil naturally suppressive to *Rhizoctonia solani.* Phytopathology 71: 286-290.

Chet, I., Timar, D. and Henis, Y. 1977. Physiological and ultrastructural changes occurring during germination of sclerotia of *Sclerotium rolfsii.* Canadian Journal of Botany 55: 1137-1142.

Christias, C. 1975. Specific inhibition of sclerotium formation by 2-mercaptoethanol and related sulfhydryl compounds in *Sclerotium rolfsii.* Canadian Journal of Microbiology 21: 1541-1547.

Christias, C. 1980. Nature of the sclerotial exudate of *Sclerotium rolfsii* Sacc. Soil Biology and Biochemistry 12: 199-201.

Chuaiprasit, C. 1990. 1990 National Barley and Wheat Conference. C. Chuaiprasit (ed) January 24 - 26, 1990, Chiang Rai, Thailand.

Clark, F.E. 1965. The Concept of Competition in Microbial Ecology. In: Ecology of Soil Borne Plant Pathogens. K. F. Baker and W. C. Snyder (eds). pp. 339 - 345, University of California Press, Berkeley.

Claydon, N., Allan, M., Hanson, J. R. and Avent, A. G. 1987. Antifungal alkyl pyrones of *Trichoderma harzianum.* Transactions of The British Mycological Society 88 (4): 503-514.

Coley-Smith, J. R., Ghaffar, A. and Javed, Z. V. R. 1974. The effect of dry conditions and subsequent leakage and rotting of fungal sclerotia. Soil Biology and Biochemistry 6: 307-312.

Colotelo, N. 1978. Fungal exudates. Canadian Journal of Microbiology 24: 1173-1181.

Cook, R. J. 1981. Biocontrol of Plant Pathogens: overview. In: Biological Control in Crop Production. G.C. Papavisas (ed). pp. 23 - 44, Beltsville Agricultural Research Center, 5th Symposium, Allanheld and Osmun, Totowa, New Jersey.

Cook, R. J. and Baker, K. F. 1983. The Nature and Practice of Biological Control of Plant Pathogens. St. Paul, Minnesota. The American Phytopathological Society.

Crawford, D. L., Lynch, J. M., Whipps, J. M., and Ousley, M. A. 1993. Isolation and characterization of actinomycete antagonists of a fungal root pathogen. Applied and Environmental Microbiology 59 (11): 3899-3905.

Danielson, R. M. and Davey, C. B. 1973. Non-nutritional factors affecting the growth of *Trichoderma* in culture. Soil Biology and Biochemistry 5: 495-504.

Daub, M. E. 1986. Tissue culture and the selection of resistance to pathogens. Annual Review of Phytopathology 24: 159-186.

Davet, P. 1979. Technique pour l'analyses des populations de Trichoderma et de *Gliocladium virens* dans le sol. Annual Review of Phytopathology. 11: 529-533.

Deacon, J. W. 1983. Microbial Control of Plant Pests and Diseases. Van Nostrand Reinhold (UK) Co. Ltd., England.

Dennis, C., and Webster, J. 1971a. Antagonistic properties of species-groups of *Trichoderma*. I. Production of non-volatile antibiotics. Transactions of the British Mycological Society 57: 25-39.

Dennis, C., and Webster, J. 1971b. Antagonistic properties of species-groups of *Trichoderma*. II. Production of volatile antibiotics. Transactions of the British Mycological Society 57:41-48.

Dennis, L. and Webster, J. 1971c. Antagonistic properties of species groups of *Trichoderma*. III Hyphal interaction. Transactions of the British Mycological Society 57: 363-369.

Dewan, M. M. and Sivasithamparam, K. 1987. First report of Sclerotium rolfsii on wheat in Western Australia. Plant Disease 71 (12): 1146.

Dobbs, C. G., and Hinson, W. H. 1953. A widespread fungistasis in soils. Nature. 172: 197-199.

Domsch, K. H., Gams, W. and Anderson, T. H. 1980. Compendium of Soil Fungi. Vols 1 and 2. Academic Press, New York.

Dzinogov, A. Marialigeti, K., Jager, K., Contreras, E. and Kondics, L. 1982. Studies on the microflora of millipedes (Diplopoda) I, A comparison of actinomycetes isolated from surface structures of the exoskeleton and the digestive tract. Pedobiologia 24: 1-7.

Elad, Y., and Chet, I. 1983. Improved selective media for isolation of *Trichoderma* spp. or *Fusarium* spp. Phytoparasitica 11: 55-58.

Elad, Y., Chet, I., and Katan, J. 1980a. *Trichoderma harzianum*: a biological agent effctive against *Sclerotium rolfsii* and *Rhizoctonia solani*. Phytopathology 70: 119-121.

Elad. Y., Chet, I., Boyle, P. and Henis, Y. 1983. Parasitism of *Trichoderma* spp. on *Rhizoctonia solani* and *Sclerotium rolfsii* - scanning electron microscopy and fluorescence microscopy. Phytopathology 73: 85-88.

Elad. Y., Katan, J. and Chet, I. 1980b. Physical, biological and chemical control integrated for soil-borne diseases in potatoes. Phytopathology 70: 418-422.

Fellman, J. and Le Tourneau, D. 1983. Effects of carboximide fungicides on growth and sclerotium formation of *Sclerotium rolfsii*. Transactions of the British Mycological Society 80: 170-172.

Franceschi, V. R. and Horner, H. T. Jr. 1980. Calcium oxalate crystals in plants. Botanical Review 46: 361-427.

Franz, G. 1975. Temperaturansprueche mikroskopischer bodenpilze ausklimatisch und geographphisch verschiedenen standorten. Zeitschrift für Pflanzenernakrung Dungung und Bodenkkude 1: 73-87.

Fries, N. 1973. Effects of volatile organic compounds on the growth and development of fungi. Transactions of the British Mycological Society 60: 1-21.

Gautum, M. and Kolte, S. J. 1979. Control of *Sclerotium* of sunflower through organic amendments of soil. Plant and Soil 53: 233-238.

Gerrettson-Cornell, L. 1991. Actinomycetes interaction with *Phytophthora cinnamomi* Rands. Phytophthora Newsletter 17: 31-32.

Ghisalberti, E. L. and Sivasithamparam, K. 1991. Review : Antifungal antibiotics produced by *Trichoderma* spp. Soil Biology and Biochemistry 23 (11): 1011-1020.

Ghisalberti, E. L., Narbey, M. J., Dewan, M. M., and Sivasithamparam, K. 1990. Variability among strains of *Trichoderma harzianum* in their ability to reduce take-all and to produce pyrones. Plant and Soil 121: 287-291.

Gilbert, R. G., and Linderman R. G. 1971. Increased activity of soil microorganisms near sclerotia of Sclerotium rolfsii. Canadian Journal of Microbiology 17: 557-562.

Goodfellow, M. and Simpson, K.E. 1987. Ecology of Streptomycetes. Frontiers in Applied Microbiology Vol. II: 97-125.

Goodfellow, M. and Williams, S. T. 1983. Ecology of actinomycetes. Annual Review of Microbiology 37: 189-216.

Gorodecki, B. and Hadar, Y. 1990. Suppression of *Rhizoctonia solani* and *Sclerotium rolfsii* diseases in container media containing composted separated cattle manure and composted grape marc. Crop Protection 9: 271-274.

Griffin, D. M. and Nair, N. G. 1968. Growth of *Sclerotium rolfsii* at different concentrations of oxygen and carbon dioxide. Journal of Experimental Botany 19: 812-816.

Gurkin, R. S. and Jenkins, S. F. 1985. Influence of cultural practices, fungicides, and inoculum placement on southern blight and Rhizoctonia crown rot of carrot. Plant Disease 69 (6): 477-481.

Hagedorn, C. 1976. Influences of soil acidity on *Streptomyces* populations inhabiting forest soils. Applied and Environmental Microbiology 32: 368-375.

Hänssler, G., Maxwell, D. P. and Maxwell, M. D. 1975. Demonstration of acid phosphatase-containing vacuoles in hyphal tip cells of *Sclerotium rolfsii*. Journal of Bacteriology 124: 997-1006.

Hänssler, G., Maxwell, D. P. and Maxwell, M. D. 1978. Ultrastructure and acid phosphatase chemistry of hyphae of *Sclerotium rolfsii* in hypocotyl tissue of *Phaseolus vulgaris*. Phytopathology 92: 157-167.

Harman, G. E., Chet, I., and Baker, R. 1980. *Trichoderma hamatum* effects on seed and seedling disease induced on radish and pea by *Pythium* spp. or *Rhizoctonia solani*. Phytopathology 70: 1167-1172.

Harman, G. E., Taylor, A. G., and Stasz, T. E. 1989. Combining effective strains of *Trichoderma harzianum* and solid matrix priming to improve biological seed treatments. Plant Disease 73: 631-637.

Harman, G.E. and Lumsden, R.D. 1990. Biological Disease Control. In: The Rhizosphere J.M. Lynch (ed). pp. 259-281 John Wiley & Sons Ltd., West Sussex, England.

Henis, Y. and Chet, I. 1968. The effect of nitrogenous amendments on the germinability of sclerotia of *Sclerotium rolfsii* and on their accompanying microflora. Phytopathology 58: 209-211.

Henis, Y. and Papavizas, G. C. 1983. Factors affecting germinability and susceptibility to attack of sclerotia of *Sclerotium rolfsii* by *Trichoderma harzianum* in field soil. Phytopathology 73 (10): 1469-1479.

Henis, Y., Lewis, J. A., and Papavizas, G. C. 1984. Interactions between *Sclerotium rolfsii* and *Trichoderma* spp.: Relationship between antagonism and disease control. Soil Biology and Biochemistry 16 (4): 391-395.

Hora, T. S. and Baker, R. 1972a. Extraction of a volatile factor from soil inducing fungistasis. Phytopathology 62 : 1475-1476.

Hora, T. S. and Baker, R. 1972b. Soil fungistasis : microflora producing a volatile inhibitor. Transactions of the British Mycological Society 59 : 491-500.

Hora, T. S. and Baker, R. 1974. Influence of a volatile in natural or limed soil on fungal spore and seed germination. Soil Biology and Biochemistry 6 : 257-261.

Howell, C. R. 1982. Effect of *Gliocladium virens* on *Pythium ultimum*, *Rhizoctonia solani*, and damping-off of cotton seedlings. Phytopathology 72: 496-498.

Howell, C. R. and Stipanovic, R. D. 1983. Gliovirin, a new antibiotic from *Gliocladium virens*, and its role in the biological control of *Pythium ultimum.* Canadian Journal of Microbiology 29: 321-324.

Hsieh, T. F., Tu, C. C. and Tsai, W. H. 1990. Effects of temperature and moisture on the occurrence of lily southern blight caused by *Sclerotium rolfsii* Sacc. Journal of Agricultural Research of China 39 (4); 315-324.

Humpherson-Jones, F. M. and Cooke, R. C. 1977. Morphogenesis in sclerotium-forming fungi. I. Effects of light on *Sclerotinia sclerotiorum*, *Sclerotium delphinii*, and *Sclerotium rolfsii.* New Phytologist 78: 171-180.

Hutchinson, S. A. 1973. Biological activity of volatile fungal metabolites. Annual Review of Phytopathology 11 : 223-246.

Jackson, R.M. 1958. An investigation of fungistasis in Nigerian soils. Journal of General Microbiology 18 : 248-258.

Jefferys, E. G. and Hemming, H. G. 1953. Fungistasis in soils. Nature 172 : 872-873.

Jenkins, S. F. and Averre, C. W. 1986. Problems and progress in integrated control of southern blight of vegetables. Plant Disease 70:614-619.

Jones, R. W. and Hancock, J. G. 1987. Conversion of viridin to viridiol by viridin-producing fungi. Canadian Journal of Microbiology 33 (11): 963-966.

Jones, R. W. and Hancock, J. G. 1988. Mechanism of gliotoxin action and factors mediating gliotoxin sensitivity. Journal of General Microbiology 134 (7): 2067-2076.

Kilpatrick, R. A. and Merkle, O. G. 1967. Seedling disease of wheat caused by *Sclerotium rolfsii*. Phytopathology 57: 538-540.

King, E. O., Ward, M. K. and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. Journal of Laboratory and Clinical Medicine 44: 301-307.

Kleifeld, O., and Chet, I. 1992. *Trichoderma harzianum*-interaction with plants and effect on growth response. Plant and Soil 144 (2): 267-272.

Knudsen, G. R. Eschen, D. J., Dandurand, L. M. and Bin, L. 1991. Potential for biocontrol of *Sclerotinia sclerotiorum* through colonization of sclerotia by *Trichoderma harzianum*. Plant Disease 7 : 466-470.

Kobayasi, Y., Matsushima, T., Takada, M. and Hagiwara, H. 1977. Reports of the Japanese Mycological Expedition to Mts. Ruwenzori, Central Africa. Transactions of Mycological Society (Japan) 18: 69-94.

Komatsu, M. 1976. Studies on *Hypocrea*, *Trichoderma* and allied fungi antagonistic to shiitake, *Lentinus edodes*. Report of the Tottori Mycological Institute (Japan) 13: 1-113.

Kritzman, G., Chet, I. and Henis, Y. 1977. Effect of carbon dioxide on growth and carbohydrate metabolism in *Sclerotium rolfsii*. Journal of General Microbiology 100: 167-175.

Küster, E. and Williams, S. T. 1964. Selection of media for isolation of Streptomyces. Nature 202: 928-929.

Kutzner, H. J. 1981. The family Streptomycetaceae. In: The Prokaryotes: A Handbook on Habitats, Isolation and Identification of Bacteria. Vol II. M. P. Starr, H. Stolp, H. G. Truper, A. Balows and H.G. Schlegel (eds). pp. 2028 - 2090. Springer-Verlag, Berlin.

Lahpendera, M. L. *Streptomyces*-A new tool for controlling plant diseases. Information published by Kemira Oy, Espoo Research Centre, Espoo, Finland.

Le Tourneau, D. 1976. Effects of phenylthiourea on growth and sclerotial formation of *Sclerotium rolfsii* and *Whetzelinia sclerotiorum*. Canadian Journal of Microbiology 22: 378-383.

Levy, M., Zehavi, U., Naim, M. and Polacheck, I. 1989. Isolation, structure determination, synthesis, and antifungal activity of a new native alfalfa-root saponin. Carbohydrate Research 193 (1): 115-123.

Lifshitz, R., Sneh, B. and Baker, R. 1984a. Soil suppressiveness to a plant pathogenic *Pythium* species. Phytopathology 74: 1054-1061.

Lifshitz, R., Stangehellini, M. E., and Baker, R. 1984b. A new species of *Pythium* isolated from soil in Colorado. Mycotaxon 20: 373-379.

Lifshitz, R., Tabachnik, M., Katan, J. and Chet, I. 1983. The effect of sublethal heating on sclerotia of *Sclerotium rolfsii*. Canadian Journal of Microbiology 29: 1607-1610.

Lim, T. K., and Teh, B. K. 1990. Antagonism *in vitro* of *Trichoderma* species against several basidiomycetous soil-borne pathogens and *Sclerotium rolfsii*. Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz 97 (1): 33-41.

Linderman, R. G. and Gilbert, R. G. 1973. Behaviour of sclerotia of *Sclerotium rolfsii* produced in soil or in culture regarding germination stimulation by volatiles, fungistasis, and sodium hypochlorite treatment. Phytopathology 63: 503-504.

Linderman, R.G. and Gilbert, R.G. 1969. Stimulation of Sclerotium rolfsii in soil by volatile components of alfalfa hay. Phytopathology 59: 1366-1372.

Liu, T. M., Yu, P. H. and Wu, L. C. 1977. The isolation, purification, and identification of dark pigment in sclerotia of *Sclerotium rolfsii* Sacc. Plant Protection Bulletin (Taiwan) 19: 223-237.

Lloyd, A. B. 1969. Behaviour of Streptomycetes in soil. Journal of General Microbiology 56: 165-170.

Lockwood, J. L. 1986. Soil-borne plant pathogens : concepts and connections. Phytopathology 76: 20-27.

Lockwood, J.L. 1959. *Streptomyces* spp. as a cause of natural fungitoxicity in soils. Phytopathology 49: 327-331.

Lumsden, R. D. and Lewis, J. A. 1989. VIII Selection, Production, Formulation and Commercial use of Plant Disease Biocontrol Fungi : problems and progress. In: Biotechnology of fungi for improving plant growth. J. M. Whipps and R. D. Lumsden (eds). pp. 171 - 190. Cambridge University Press, Cambridge.

Lumsden, R. D., Locke, J. C., Adkins, S. T., Walter, J. F. and Ridout, C. J. 1992. Isolation and localization of the antibiotic gliotoxin produced by *Gliocladium virens* from alginate prill in soil and soilless media. Phytopathology 82 (2): 230-235.

Lynch. J. M. 1987. Biological Control within Microbial Communities of the Rhizosphere, In: Ecology of Microbial Communities. M. Fletcher, T. R. G. Gray and J. G. Jones (eds). Cambridge University Press, Cambridge.

Martin, J.P. 1950. Use of acid, rose bengal, and streptomycin in the plate method for estimating soil fungi. Soil Science 69: 215-232.

Mathur, S. B. 1977. Physiological studies on *Sclerotium rolfsii*. II. Utilization of inorganic and organic nitrogen sources. Indian Journal of Phytopathology 30: 131-132.

Mathur, S. B. and Sarbhoy, A. K. 1976. Physiological studies on *Sclerotium rolfsii* causing root rot of sugarbeet. Indian Journal of Phytopathology 29: 454-455.

Mathur, S. B. and Sinha, S. 1970. Role of manuring in control of root-rot of guar (*Cyamopsis psoralioides* DC.) and wilt of gram (*Cicer arietinum* L.) caused by *Sclerotium rolfsii* Sacc. Mycopathologia et Mycologia Applicata 40: 155-159.

Maurer, C. L., and Baker, R. 1965. Ecology of plant pathogens in soil. II Influence of glucose, cellulose and inorganic nitrogen amendments on development of bean root rot. Phytopathology 55: 69-72.

Mayfield, C. I., Williams, S. T., Ruddick, S. M. and Hatfield, H.L. 1972. Studies on the ecology of actinomycetes in soil. IV. Observations on the form and growth of Streptomycetes in soil. Soil Biology and Biochemistry 4 : 79-91.

McCarter, S. M. Jaworski, C. A. Johnson, A. W. and Williamson, R. E. 1976. Efficacy of soil fumigants and methods of application for controlling southern blight of tomatoes grown for transplants. Phytopathology 66: 910-913.

McCullagh, P. and Nelder, J. A. 1983. Generalized Linear Models. Chapman and Hall, London.

McLean, R. C. and Cook, W. R. I. 1941. Plant Science Formulae. Chapter 8 Culture and nutrient solutions. MacMillan and Co., Ltd., London.

Mehrotra, R. S. and Caludius, G. R. 1972. Biological control of the root rot and wilt diseases of *Lens culinaris* Medic. Plant and Soil 36: 657-664.

Mihail, J. D. and Alcorn, S. M. 1984. Effects of soil solarization on *Macrophomina phaseolina* and *Sclerotium rolfsii*. Plant Disease 68: 156-159.

Mikami, Y., Miyashita, K. and Arai, T. 1982. Diaminopimelic acid profiles of alkalophilic and alkaline-resistant strains of actinomycetes. Journal of General Microbiology 128 (8): 1709-1712.

Miller, R. M. and Liberta, A. E. 1977. The effects of light and tyrosinase during sclerotium development in *Sclerotium rolfsii* Sacc. Canadian Journal of Microbiology 23: 278-287.

Mitchell, D.J. and Mitchell, J.E. 1973. Oxygen and carbon dioxide concentration effects on the growth and reproduction of *Aphanomyces eutiches* and certain other soil-borne plant pathogens. Phytopathology 63: 1053-1059.

Mixon, A. C. and Curl, E. A. 1967. Influence of plant residues on *Sclerotium rolfsii* and inhibitory soil miroorganisms. Crop Science 7: 641-644.

Mohapatra, P. D. and Dash, S. C. 1990. Efficacy of different methods of controlling *Sclerotium rolfsii* Sacc. in betelvine. Orissa Journal of Agricultural Research 3 (2): 126-129.

Morquer, R. and Komatsu, M. 1968. *Trichoderma* and *Gliocladium* found on bed logs of shiitake mushroom (*Lentinus edodes*) in Japan. Transactions of Mycological Society (Japan) 8: 136-140.

Mukhopadhyay, A. N. and Thakur, R. P. 1971. Control of Sclerotium root rot of sugarbeet with systemic fungicides. Plant Disease Reporter 55: 630-634.

Nair, N. G., White, N. H., Griffin, D. M. and Blair, S. 1969. Fine structure and electron cytochemical studies of *Sclerotium rolfsii* Sacc. Australian Journal of Biological Science 22: 639-652.

Narain, A. and Kar, A. K. 1990. Wilt of groundnut caused by *Sclerotium rolfsii*, *Fusarium* spp. and *Aspergillus niger*. Crop Research Hisar 3 (2): 257-262.

Narasimhan, R. 1969. Physiological studies on the genus *Sclerotium*. I. Effect of initial H-ion concentration on the growth of *Sclerotium rolfsii* and *Sclerotium oryzae* in different inorganic nitrogen media. Indian Journal of Phytopathology 22: 115-123.

Nemec, S. 1970. Fungi associated with strawberry root rot in Illinois. Mycopathologia et Mycologia Applicata 41: 331-346.

Neypes, M. V., Lapis, D. B. and Telan, I. F. 1988. *Trichoderma glaucum* Abbot for biological conrol of foot rot of wheat caused by *Sclerotium rolfsii* Sacc. Phillipine Agriculturist 71 (2): 157-163.

Nwakpa, F. I. and Ikotun, T. 1988. The reaction of cowpea varieties to infection by *Sclerotium rolfsii*. Tropical Grain Legume Bulletin 35: 32-34.

Nwufo, M. I. and Onyeagba, A. 1989. Studies on the foot and root rot diseases of eggplant (*Solanum melongena* L.) caused by *Sclerotium rolfsii* Sacc. in southern Nigeria. Beitrage zur Tropischen Landwirtschaft und Veterinarmedizin 27 (4): 449-453.

Ordentlich, A., Nachmias, A., Strashnov, Y. and Chet, I. 1990. Integrated control of soilborne plant pathogens. Phytoparasitica 18: 83.

Palakshappa, M. G., Srikant-Kulkarni, Hegde, R. K., and Kulkarni, S. 1988. Effect of organic amendments on the survival ability of *Sclerotium rolfsii* Sacc. a causal agent of foot rot of betelvine. Mysore Journal of Agricultural Sciences. 23 (3): 332-336.

Papavizas, G. C. and Collins, D. J. 1990. Influence of Gliocladium virens on germination and infectivity of Sclerotia of *Sclerotium rolfsii*. Phytopathology 80: 627-630.

Papavizas, G. C. and Davey, C. B. 1959. Evaluation of various media and antimicrobial agents for isolation of soil fungi. Soil Science 88: 112-117.

Papavizas, G. C., and Lumsden, R. D. 1982. Improved medium for isolation of Trichoderma spp. from soil. Plant Disease 66: 1019-1020.

Paul, E.A. and Clark, F.E. 1989. Components of the Soil Biota. In: Soil Microbiology and Biochemistry. pp. 49-72 Academic Press, Inc., San Diego, USA.

Paulitz, T. C., and Baker, R. 1988. Interactions between *Pythium nunn* and *Pythium ultimum* on bean leaves. Canadian Journal of Microbiology 34: 947-951.

Porter, I. J. and Merriman, P. R. 1983. Effects of solarisation of soil on nematode and fungal pathogens at two sites in Victoria. Soil Biology and Biochemistry 15 : 39-44.

Punja, Z. K. 1985. The biology, ecology and control of *Sclerotium rolfsii*. Annual Review of Phytopathology 23: 97-127.

Punja, Z. K. 1986. Effect of carbon and nitrogen step-down on sclerotium biomass and cord development in *Sclerotium rolfsii* and *S. delphinii*. Transactions of the British Mycological Society 86 (4): 537-544.

Punja, Z. K. 1988. *Sclerotium rolfsii* : Potential Impact on Wheat Production and Possible Means of Control. In: Wheat Production Constraints in Tropical Environments. A.R. Klatt (ed.). pp. 158 - 174, CIMMYT, Mexico.

Punja, Z. K. and Grogan, R. G. 1983. Germination and infection by basidiospores of *Athelia* (*Sclerotium*) *rolfsii*. Plant Disease 67 (8): 875-878.

Punja, Z. K. and Jenkins, S. F. 1984a. Influence of temperature, moisture, modified gaseous atmosphere, and depth in soil on eruptive sclerotial germination of *Sclerotium rolfsii*. Phytopathology 74: 749-754.

Punja, Z. K. and Jenkins, S. F. 1984b. Light and scanning electron microscopic observations of calcium oxalate crystals produced during growth of *Sclerotium rolfsii.* in culture and in infected tissue. Canadian Journal of Botany 62 (10): 2028-2032.

Punja, Z. K., Carter, J. D., Campbell, G. M. and Rossell, E. L. 1986. Effects of calcium and nitrogen fertilizers, fungicides, and tillage practices on incidence of *Sclerotium rolfsii* on processing carrots. Plant Disease 70 (9): 819-824.

Punja, Z. K., Carter, J. D., Campbell, G. M., & Rossell, E. L. (1986). Effects of calcium and nitrogen fertilizers, fungicides, and tillage practices on incidence of *Sclerotium rolfsii* on processing carrots (*Daucus carota*). Plant Disease 70 (9): 819-824.

Punja, Z. K., Grogan, R. G. and Adams, G. C. 1982. Influence of nutrition, environment, and the isolate, on basidiocarp formation, development, and structure in *Athelia (Sclerotium) rolfsii*. Mycologia 74 (6): 917-926.

Punja, Z. K., Grogan, R. G. and Unruh, T. 1982. Chemical control of *Sclerotium rolfsii* on golf greens in Northern California. Plant Disease 66 (1): 108-111.

Punja, Z. K., Huang, J. S. and Jenkins, S. F. 1985. Relationship of mycelial growth and production of oxalic acid and cell wall degrading enzyme to virulence in *Sclerotium rolfsii*. Canadian Journal of Plant Pathology 7 (2): 109-117.

Punja, Z. K., Jenkins, S. F. and Grogan, R. G. 1984. Effect of volatile compounds, nutrients and source of sclerotia on eruptive sclerotial germination of *Sclerotium rolfsii.* Phytopathology 74 (11): 1290-1295.

Punja, Z. K., Jenkins, S. F., & Grogan, R. G. 1984. Effect of volatile compounds, nutrients and source of sclerotia on eruptive sclerotial germination of *Sclerotium rolfsii.* Phytopathology 74 (11): 1290-1295.

Punja, Z.K. 1985. The biology, ecology, and control of *Sclerotium rolfsii.* Annual Review of Phytopathology 23: 97-127.

Punja, Z.K. and Grogan, R.G. 1981. Eruptive germination of sclerotia of *Sclerotium rolfsii.* Phytopathology 71: 1092-1099.

Punja, Z.K. and Grogan, R.G. 1982. Effects of inorganic salts, carbonate - bicarbonate anions, ammonia, and the modifying influence of pH on sclerotial germination of *Sclerotium rolfsii.* Phytopathology 72: 635-639.

Rangaswami, G., and Vidyasekaran, P. 1963. Antibiotic production by *Streptomyces* spp. in corn rhizosphere. Phytopathology 53: 995-997.

Reeves, F. B. and Horn, C. 1976. *Trichoderma* in Colorado. Mycotaxon 3: 337-344.

Ridge, E. H. and Rovira, A. D. 1971. Phosphatase activity of intact young wheat roots under sterile and non-sterile conditions. New Phytologist 70: 1017-1026.

Ridge, E. H. and Rovira, A. D. 1971. Phosphate activity of intact young wheat roots under sterile and non-sterile conditions. New Phytologist 70: 1017-1026.

Ridout, C. J., Lumsden, R. D., & Hruschka, W. R. 1992. Identification of mycelial polypeptides associated with gliotoxin-producing strains of the biocontrol fungus *Gliocladium virens*. Phytopathology 82 (4): 479-484.

Rifai, M. 1969. A revision of the genus *Trichoderma*. Mycological papers 116: 1-56.

Ristaino, J. B., Perry, K. B. and Lumsden, R. D. 1991. Effect of solarization and *Gliocladium virens* on sclerotia of *Sclerotium rolfsii*, soil microbiota, and the incidence of southern blight of tomato. Phytopathology 81 (10): 117-124.

Roiger, D.J. and Jeffers, S. N. 1991. Evaluation of *Trichoderma* spp. for biological control of Phytophora crown and rootrot of apple seedlings. Phytopathology 81: 910-917.

Rothrock, C. S., and Gottlieb, D. 1984. Role of antibiosis in antagonism of *Streptomyces hygroscopicus* var. *geldanus* to *Rhizoctonia solani* in soil. Canadian Journal of Microbiology 30: 1440-1447.

Ruddick, R. M. and williams, S. T. 1972. Studies on the ecology of actinomycetes in soil. V Some factors influencing the dispersal and adsorption of spores. Soil Biology and Biochemistry 4: 93-103.

Sahu, K. C., Narain, A. and Swain, N. C. 1989. Survival of *Sclerotium rolfsii* Sacc. pathogenic to groundnut plant in Orissa at different soil depth. Environmental Ecology 7 (4): 1031-1032.

Schneider, R. W. 1982. Suppressive Soils and Plant Diseases. The American Phytopathological Society, St. Paul, Minnesota.

Scott, E. S., Chambers, S. M. and Son, C. S. 1994. In vitro selection of disease resistant plants using tissue culture. Proceedings of the Symposium on biology and control of crop pathogens, BIOTROP Special Publication No. 54. SEAMEO BIOTROP, Bogor, Indonesia.

Seeley, H. W. and VanDemark, P. J. 1981. Microbes in action : a laboratory manual of microbiology. Third edition. W. H. Freeman and Company, U.S.A.

Seeley, H. W. Jr. and VanDemark, P. J. 1981. Microbes in action. A laboratory manual of microbiology. Third edition. W.H. Freeman and Company. USA.

Sharma, J. K. and Sankaran, K. V. 1988. Chapter 1 Biocontrol of Rust and Leaf Spot Diseases. In: Biocontrol of Plant Disease Vol II. K. G. Mukerji and K. L. Garg (eds). pp. 1 - 24, CRC Press, Inc., Florida.

Sharma, S. L. and Kaushal, B. R. 1979. Cultural and physiological studies with sunflower isolate of *Sclerotium rolfsii*. Indian Journal of Mycology and Plant Pathology 9: 105-107

Shew, B. B., Wynne, J. C. and Beute, M. K. 1987. Field, microplot, and greenhouse evaluations of resistance to *Sclerotium rolfsii* in peanut. Plant Disease 71: 188-191.

Shew, B. B., Wynne, J. C., & Beute, M. K. 1987. Field, microplot, and greenhouse evaluations of resistance to *Sclerotium rolfsii* in peanut. Plant Disease 71 (2): 188-191.

Shirling, E.B. and Gottlieb, D. 1966. Methods for characterization of *Streptomyces* species. International Journal of Systematic Bacteriology 16 (3): 313-340.

Simmonds, J. H. 1960. Science Branch. Plant Pathology Section. 1959-60 Report. Department of Agriculture, Queensland, pp. 62-64.

Simmonds, J. H. 1966. Host Index of Plant Diseases in Queensland. Queensland Department of Primary Industries, Brisbane.

Simon, A., Dunlop, R. W., Ghisalberti, E. L., and Sivasithamparam, K. 1988. *Trichoderma koningii* produces a pyrone compound with antibiotic properties. Soil Biology and Biochemistry 20 (2): 263-264.

Singh, J. and Faull, J. L. 1988. Chapter 12 Antagonism and Biological Control. In: Biocontrol of Plant Disease Vol II. K. G. Mukerji and K. L. Garg (eds). pp. 167-177. CRC Press Inc., Florida.

Singh, R. K., and Dwivedi, R. S. 1987. Studies on biological control of *Sclerotium rolfsii* Sacc. causing foot rot of barley. Acta-Botanica-Indica 15 (2): 160-164.

Sivasithamparam, K., Parker, C. A. and Edwards, C. S. 1979. Rhizosphere micro-organisms of seminal and nodal roots of wheat grown in pots. Soil Biology and Biochemistry 11: 155-160.

Skerman, V. B. D. 1967. A Guide to the Identification of the Genera of Bacteria. Williams and Wilkens Co., Maryland.

Smith, A. M. 1972. Biological control of fungal sclerotia in soil. Soil Biology and Biochemistry. 4: 131-134.

Smith, N. R. and Dawson, V. T. 1944. The bacleriostatic action of rose bengal in media used for the plate counts of soil fungi. Soil Science 58: 467-470.

Smith, V. L., Punja, Z. K. and Jenkins, S. F. 1984. A histopathological and SEM study of infection of host tissue by *Sclerotium rolfsii*. Phytopathology 74: 633.

Smith, V. L., Punja, Z. K., & Jenkins, S. F. 1986. A histological study of infection of host tissue by *Sclerotium rolfsii*. Phytopathology 76 (8): 755-759.

Sreenivasaprasad, S., and Manibhushanrao, K. 1990. Biocontrol potential of fungal antagonists *Gliocladium virens* and *Trichoderma longibrachiatum*. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz 97 (6): 570-579.

Stephens, C. T. and Elmer, W. H. 1988. An *in vitro* assay to evaluate sources of resistance in *Asparagus* spp. to Fusarium crown and rootrot. Plant Disease 72: 334-337.

Tarabcih, A. M. and Al-Menoufi, O. A. 1987. Effect of calcium carbonate on sunflower footrot disease caused by *Sclerotium rolfsii* Sacc. Alexandria Journal of Agricultural Research 32 (1): 325-332.

Tisdale, W. H. 1921. Two Sclerotium disease of rice. Journal of Agricultural Research 21: 649-658.

Trutmann, P. and Keane, P. J. 1990. *Trichoderma koningii* as a biological control agent for *Sclerotinia sclerotiorum* in Southern Australia. Soil Biology and Biochemistry 22 (1): 43-50.

Tu, C. C. and Kimbrough, J. W. 1978. Systematics and phylogeny of fungi in the Rhizoctonia complex. Botanical Gazette 139: 454-466.

Tukey, J. W. 1953. The Problem of Multiple Comparison, mimeographed publication. In: Applied Statistics for Engineers. W. Volk (ed). pp. 179, McGraw-Hill.

Turhan, G., and Grossmann, F. 1986. Investigation of a great number of actinomycete isolates on their antagonistic effects against soil-borne fungal plant pathogens by an improved method. Journal of Phytopathology 116 (3): 238-243.

Utkhede, R. S. 1983. Inhibition of *Phytophthora cactorum* by bacterial isolates and effects of chemical fungicides on their growth and antagonism. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz 90 (2): 140-145.

Vallejos, O. V. 1988. Effect of *Rhizoctonia solani* and *Sclerotium rolfsii* on 10 bean cultivars and lines in Lambayequc, Peru. Fitopatologia 23 (1): 22-25.

Van den Bulk, R. W. 1991. Application of cell and tissue culture and *in vitro* selection for disease resistance breeding - a review. Euphytica 56: 269-285.

Vickers, J. C., and Williams, S. T. 1987. An assessment of plate inoculation procedures for the enumeration and isolation of soil streptomycetes. Microbios Letters 35: 113-117.

Wangikar, P. D., Somani, R. B. and Bobade, K. P. 1988. Sclerotium collar rot - a new menace to chilli in Vidarbha. P K V Research Journal 12 (1): 88-89.

Willetts, H. J. 1972. The morphogenesis and possible evolutionary origins of fungal sclerotia. Biological Reviews 47: 515-536.

Willetts, H. J. 1971. The survival of fungal sclerotia under adverse environmental conditions. Biological Reviews 46 : 387-407.

Williams, S. T. 1978. Streptomycetes in the soil ecosystem. Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene. I. Abteilung, Supplement No. 6 . pp. 137-144.

Wokocha, R. C. 1990. Integrated control of *Sclerotium rolfsii* infection of tomato in the Nigerian Savanna : effect of *Trichoderma viride* and some fungicides. Crop Protection 9 (3): 231-234.

Wolffhechel, H. and Funck-Jensen, D. 1992. Use of *Trichoderma harzianum* and *Gliocladium virens* for the biological control of post-emergence damping-off and root rot of cucumbers caused by *Pythium ultimum.* Journal of Phytopathology 136 : 221-230.

Youssef, Y. A. 1974. On the fungal flora of Libyan soils. Archiv fuer Mikrobiologie 99: 167-171.

Yuen, G. Y. and Raabe, R. D. 1984. Effects of small-scale aerobic composting on survival of some fungal plant pathogens. Plant Disease 68: 134-136.

Zeidan, O., Elad, Y., Hadar, Y. and Chet, I. 1986. Integrating onion in crop rotation to control *Sclerotium rolfsii.* Plant Disease 70 (5): 426-428.

Zoberi, M. H. 1980. Some nutritional factors regulating formation of sclerotia of *Sclerotium rolfsii.* Canadian Journal of Botany 58: 2484-2490.

Zuberer, D. A., Kenerley, C. M., and Jeger, M. J. 1988. Populations of bacteria and actinomycetes associated with sclerotia of *Phymatotrichum omnivorum* buried in Houston black clay. Plant and Soil 112: 69-76.

Errata to be inserted as follows

1. page 30, 2.2.1, line 3. 'were surface sterilised with 10% White King domestic bleach (0.4% available chlorine) plus a drop of wetting agent (Tween 80) for 2 min, then rinsed three times with sterile distilled water, 5 min each rinse, and'

2. page 35, 2.4, line 4. '; the isolation system may not completely reflect the natural situation due to the limitation that only culturable organisms were studied, however, it facilitated laboratory studies and provided information about the soil microbial population and the occurrence of certain antagonists used in this study.'

3. page 115, line 19. 'Isolates of actinomycetes, bacteria and fungi all imposed some degree of fungistasis when used to infest sterilised soil (Lockwood, 1964), and similar inoculations of an artificail soil imposed a general microbiostasis (Ho and Ko, 1986).'

4. page 132, line12. 'The feasibility of adding antagonists to the potting mix or soil in the field is an important consideration. In particular, the antagonist should probably be applied as dried, rather than fresh, inoculum. Thus, future work should include study of formulation, quantity and method of application of antagonist inoculum required to give adequate control of disease. Shelf life of the antagonist formulation also should be considered. Such studies would provide information on the commercial viability of a biocontrol product developed using the antagonists studied in this project.'

5. page 144, second last reference. 'Ho, W. C. and Ko, W. H. 1986. Microbiostasis by nutrient deficiency shown in natural and synthetic soils. Journal of General Microbiology 132: 2807-2815.'

6. page 148, second last reference. 'Lockwood, J. L. 1964. Soil fungistasis. Annual Review of Phytopathology 2: 341-362.'