CONTROL OF ONION WHITE ROT USING BENEFICIAL MICROORGANISMS AND SOIL SOLARISATION

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The fungal isolates *Chaetomium globosum* (A53), *Coniothyrium minitans* (A69), *Trichoderma harzianum* (C52), *Trichoderma koningii* (C60, C62) and *Trichoderma viride* (D73) were confirmed as antagonists of *Sclerotium cepivorum*, the causal agent of onion white rot. In dual culture with *S. cepivorum,* these isolates produced inhibition zones and colonised pathogen hyphae. When agar was amended with culture filtrates of *C. globosum* and *T. harzianum*, the growth of *S. cepivorum* was distorted or unusual, indicating the production of antibiotics.

The ability of these isolates to reduce the incidence of onion white rot was tested in a glasshouse trial in artificially infested (1 sclerotium per gram soil) Wakanui silt loam soil. A fungal homogenate of each of the fungal antagonists was cultured in a sand:bran mix and added to the soil in polystyrene boxes (59 x 33 x 19cm), at two different application times. Application 1 was applied to half of the polystyrene boxes, two weeks before onion seed planting and application 2 was applied to the remaining soil boxes at the time of onion seed planting. *T. harzianum* (C52) applied to the soil two weeks before onion seed planting resulted in disease control equivalent to the fungicide (procymidone) seed treatment (82.9%). When the fungal treatments were applied to the soil at the time of planting, all treatments afforded disease control equivalent to the procymidone control. A significantly lower seedling emergence resulted with the application of the fungal treatments compared with the procymidone control treatment; irrespective of fungal application time, however, a significantly greater number of seedlings emerged when the fungal treatments were applied at the time of planting rather than two weeks before planting. *T. harzianum* and *T. koningii* (C62) were selected for further investigation as these species established and grew well on a range of media, unlike *Chaetomium globosum* and *Coniothyrium minitans*. In a second

glasshouse trial, *T. harzianum* and *T. koningii* were applied to the soil at the time of planting as onion seed coatings or alginate pellets. Mixtures of the two fungi were also prepared and a combination of *Trichoderma* spp. (Trichopel) was also included as a treatment. Unfortunately, only low numbers of white rot diseased seedlings were evident and no significant treatment effects were observed for the duration of the trial. No disease developed in a field trial established to compare commercially prepared pellets of *T. harzianum*, *T. koningii* and combinations of the two *Trichoderma* spp. The lack of treatment effects was a result of low amounts of pathogen inoculum rather than the failure of the fungal antagonist delivery system.

S. cepivorum sclerotial viability was greatly reduced when sclerotia were incubated in soil and exposed to continuous temperatures ranging from 20 to 50+°C in incubators. Temperatures above 40°C reduced sclerotial viability to 0% after a 1 day exposure period, whereas at 20°C, sclerotial viability was reduced to 10.7% after 28 days. Temperatures above 20-25°C adversely affected sclerotial viability, although a lower temperature for a longer period did not cause the same extent of sclerotial degradation as a higher temperature for a shorter period of time. A four week soil solarisation trial did not reduce sclerotial viability when artificially produced *S. cepivorum* sclerotia were buried in the soil at 10 and 20cm depths under polythene. The solarisation treatment raised the average soil temperature by 7°C. While there was a significant difference in the maximum and minimum temperatures recorded at 10 and 20cm in both solarised and non solarised soil, the increase in temperature at the shallower depths did not affect sclerotial viability.

Increases in temperature adversely affected sclerotial viability, although in Canterbury the use of soil solarisation alone did not reduce sclerotial viability. Beneficial microorganisms could be combined with soil solarisation, either following soil solarisation at the time of onion seed planting or heat tolerant microorganisms could be incorporated into the soil before soil solarisation treatment. An integrated control approach could provide a commercially viable programme for control of onion white rot.

Key words: *Sclerotium cepivorum*, sclerotia, control, onion white rot, antagonistic fungi, *Chaetomium globosum, Coniothyrium minitans, Trichoderma harzianum, T. koningii, T. viride*, heat, soil solarisation.

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~CHAPTER ONE~
INTRODUCTION

1.1 The Disease.

White rot of onions (*Allium cepa* L.) is one of the most important and destructive diseases of *Allium* species. Onion white rot is caused by the soil borne fungus *Sclerotium cepivorum* Berk. which was first described in England by Berkeley in 1841 (Walker, 1924). Fifty years later, Voglino reported white rot to be a serious disease of garlic in Italy (Voglino, 1903 in Walker, 1924) and today, onion white rot is present in most countries where the climate is favourable for disease development (Entwistle, 1990).

In New Zealand, white rot was first recorded in the Marshlands area, Christchurch in 1922 (Cunningham, 1922). The disease spread to Blenheim and Invercargill by 1955 and by 1960 white rot appeared in Pukekohe, South Auckland (Beeson, 1960 in Dingley, 1969).

Onions are and have been a major export crop of New Zealand. Over the past five years, onion exports have increased, making onions the biggest vegetable export earner in New Zealand (Anon, 1995). In the 1994-1995 season, the onion export industry earned over $92 million, an 11% increase from the 1993-1994 season (Anon, 1995). Garlic exports earned just over $3 million in the 1994-1995 season and shallots and leeks earned nearly $200,000 (Anon, 1995).

Onions are a valuable crop to New Zealand and the control of pests and diseases which threaten the market position of onions is important. Onion white rot being one of the most serious diseases affecting onions, has been widely studied. *S. cepivorum* is known to affect several *Allium* species including: onion, *Allium cepa* L.; welsh onion, *Allium fistulosum* L.; leek, *Allium porrum* L.; garlic, *Allium sativum* L. and shallot, *Allium ascalonicum* L. (Walker, 1924).

1.2 Symptoms, Causal Organism and Disease Cycle.

The first indication of white rot infection is a change in leaf colour from bright green to blue green. The outer leaves of the onion then change to a yellowish colour and begin to die back from the tips (Plate 1.1). Subsequently, the whole plant wilts and dies (Fullerton *et al.*, 1994). Below ground, the roots are gradually destroyed, enabling the plant to be easily

Plate 1.1 Early disease symptoms of onion white rot.

pulled from the soil. *S. cepivorum* also causes a semi-watery decay of the scales which is associated with the growth of superficial white mycelium on the affected area (Tims, 1948; Fullerton *et al.*, 1994). Within several days, the mycelium darkens and is transformed into large numbers of sclerotia (Plate 1.2) (Fullerton *et al.*, 1994).

Sclerotia are the only reproductive structures of *S. cepivorum* as no perfect stage has yet been described (Crowe, 1995) and no asexual spores are produced. The sclerotia are black, uniformly round and are 200-500µm in diameter (Mordue, 1976). A narrow, smooth or pitted rind surrounds a medulla of compact interwoven hyphae and a gelatinous material is often present in the interhyphal spaces (Mordue, 1976). Occasionally, larger more irregular sclerotia have been produced, measuring 3-15mm in length. Research has shown a number of these large sclerotia to be aggregations of the smaller more common sized sclerotia (Georgy & Coley-Smith, 1982), although, some aggregations have been reported as unified structures (Backhouse & Stewart, 1988).

The protective nature of the sclerotial rind enables the sclerotia to remain viable in the soil for a substantial length of time. Plant decay and harvesting procedures result in the release of sclerotia into the soil. In California, Crowe *et al.* (1980) reported the occurrence of viable sclerotia from fields where *Allium* species had not grown for 10-15 years. Trials in the United Kingdom have shown 70, 75 and 80% viability of sclerotia when buried for 4 years at depths of 3, 6 and 9 inches respectively (Coley-Smith, 1959) and after 20 years burial at depths greater than 7cm, over 70% of the recovered sclerotia were viable (Coley Smith *et al*, 1990). In contrast, Legget *et al.* (1983) reported sclerotial decay 4 months after burial in muck soil in British Columbia and a study undertaken in Auckland, New Zealand also reported rapid loss of sclerotial viability with only 10-15% of the original sclerotial population remaining after 3 months (Fullerton *et al.*, 1994). The variation in sclerotial viability may be the result of interactions between environmental and biological factors (Fullerton *et al.*, 1994); for example, the pretreatment of sclerotia, drying periods before soil inoculation, or perhaps the variation between naturally produced sclerotia and laboratory-grown sclerotia could cause differences in sclerotia viability (Legget *et al.*, 1983).

Sclerotia will germinate only in the presence of *Allium* roots. The alkyl-cysteine sulphoxides exuded by *Allium* roots are broken down by soil microorganisms to form thiols

Plate 1.2 *Sclerotium cepivorum* sclerotia on the bulb of a diseased onion.

and sulphide compounds which stimulate *S. cepivorum* sclerotia to germinate (Coley-Smith & King, 1969). Undiluted garlic extracts, when applied to *S. cepivorum* sclerotia in soil, caused 100% sclerotial germination after 5 weeks and 72% of the sclerotia germinated when the garlic extract was diluted 1000 times (King & Coley-Smith, 1968). Germination of sclerotia is indicated initially by the formation of bulges on the rind surface (Coley-Smith, 1960). Although fine hyphal threads can project from the sclerotium at this time, it would appear more common for the rind to split and a plug of medullary hyphae to erupt from the sclerotium (Coley-Smith, 1960) (Figure 1.1). The optimum temperature range for germination is 13-18°C (Asthana, 1947).

Infection of the basal stem tissue occurs via the production of infection cushions, whilst penetration of the roots occurs via direct penetration of hyphal tips between or within epidermal cell walls (Stewart *et al.*, 1989a) (Figure 1.1). Fungal mycelium grows internally, beneath the cuticle, killing epidermal cells ahead of the advancing hyphae (Stewart *et al.*, 1989b). *S. cepivorum* has been shown to produce a range of cell wall degrading enzymes in onion tissue (Mankarios & Friend, 1980). Fungal mycelium can be spread from plant to plant, however, as *S. cepivorum* has no saprophytic ability (Scott, 1956a) the mycelium cannot survive in the soil for any length of time. Whole planting rows become infected due to root contact between neighbouring plants (Scott, 1956b) (Figure 1.1). Hence, spacing of onions is a major consideration for disease control. *S. cepivorum* can also be spread further via contamination of drainage water, equipment including tractors and harvesting containers, footwear, infected seeds and/or transplants and also by animals, in that they may feed upon infected plants and defecate viable sclerotia at a distance away (Fullerton *et al.*, 1994).

1.3 Disease Control.

Initially, the control of onion white rot relied upon cultural practices such as collection and destruction of infected plants, crop rotations (Brien *et al.*, 1959) and avoidance of infested land (Letham & Cother, 1982). However, as the disease became increasingly widespread and persistent, the use of fungicides became almost exclusive. More recently, concerns associated with environmental impacts of such fungicidal chemicals, the increasing development as well as registration costs and the reduction of fungicide effectiveness due to resistance and enhanced microbial degradation, has called for alternative control measures (Entwistle, 1992). Alternative strategies include sclerotial germination stimulants, soil

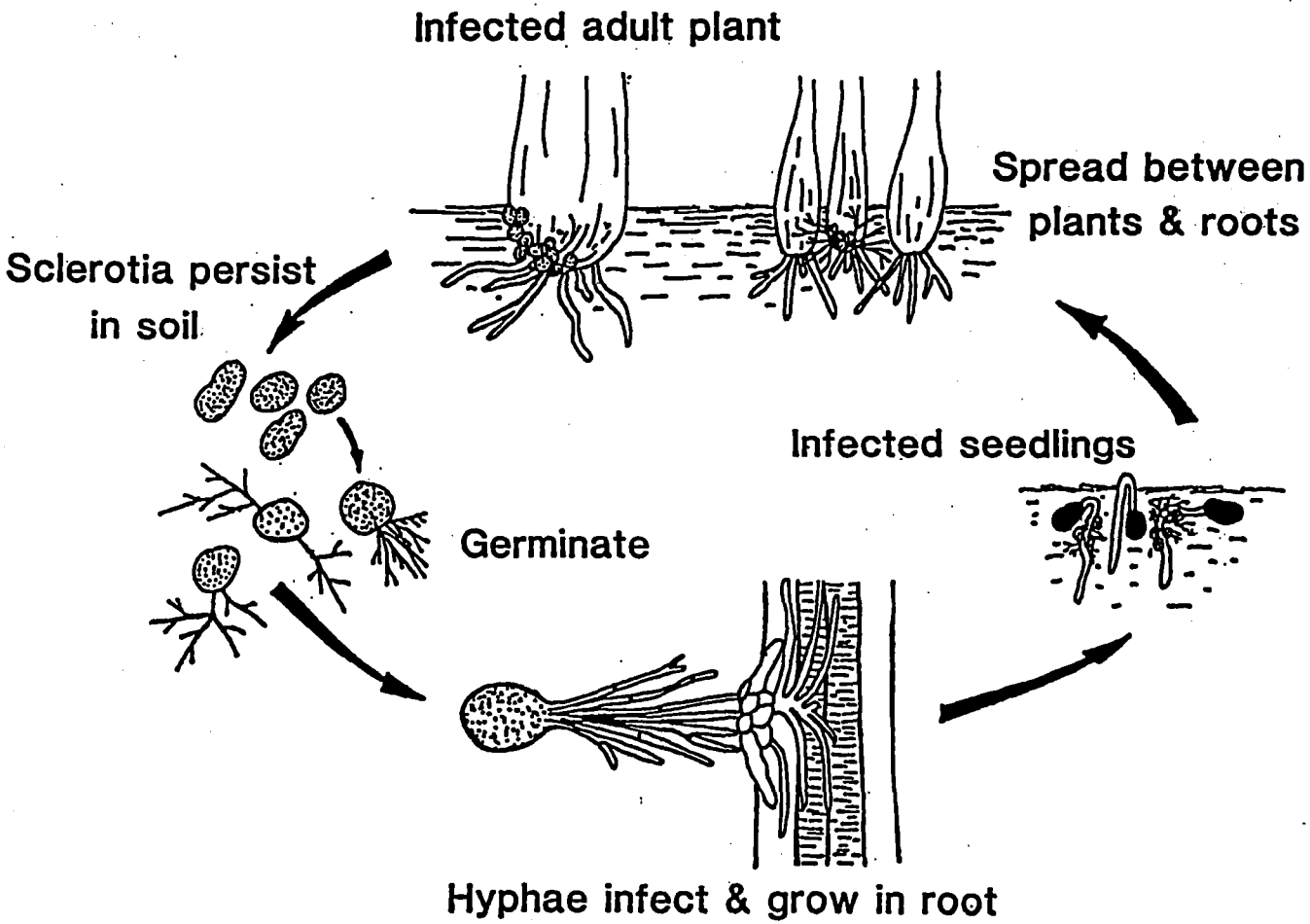


Figure 1.1 Disease cycle of onion white rot.

partial sterilisation and biological control (use of beneficial microorganisms).

1.3.1 **Fungicidal Control.**

Over the past forty years, various chemical compounds have been tested for control of onion white rot; including: calomel, benomyl, dicloran, thiophanate-methyl and botran (Booer, 1945, 1946; Croxall *et al.*, 1953; Entwistle & Munasinghe, 1973; Maloy & Machtmes, 1974; Ryan & Kavanagh, 1976). A number of factors associated with these compounds has led to unsatisfactory control of onion white rot, for example, insufficient concentrations and inappropriate application methods. In addition, levels of control have been inconsistent from one season to the next (Entwistle & Munasinghe, 1973; Maloy & Machtmes, 1974) and some chemicals have exhibited phytotoxicity.

Successful control of onion white rot was obtained after the introduction of systemic fungicides. The dicarboximides, iprodione and vinclozolin, were first trialed in New Zealand during 1979 and were highly effective in controlling onion white rot (Wood, 1980). Success with iprodione was also reported in the United Kingdom where a combined seed and stem base treatment were used (Entwistle & Munasinghe, 1980a,b). Unfortunately, in both the United Kingdom and New Zealand, the continued use of iprodione and vinclozolin has led to a decrease in effectiveness due to enhanced degradation of the chemicals by soil microorganisms (Entwistle, 1983; Walker *et al.*, 1986; Slade *et al.*, 1992). In New Zealand, experiments undertaken to examine the increased degradation of iprodione and vinclozolin, have shown that repeated applications of the chemicals to Patumahoe clay loam soil resulted in increased rates of fungicidal degradation. Three applications of each fungicide were made at five week intervals to separate soil samples. A 50% reduction in the concentration of iprodione was evident 35 days after the first application of iprodione, whereas after the second application a 50% reduction in the fungicide was obtained after two days. Vinclozolin showed a similar trend with a reduction of 50% after 22 days following application one and after approximately one day with application two (Slade *et al.*, 1992).

The related dicarboximide, procymidone has been shown to be less prone to enhanced degradation and is now the predominant dicarboximide in use in New Zealand. Effective control has been achieved using a seed treatment (5.0g a.i./kg seed) followed by four foliar sprays (0.75kg a.i./ha) applied at monthly intervals (Fullerton & Stewart, 1991; Stewart & Fullerton, 1991). A dispersible granule, applied at 0.05 or 0.1g a.i./m, was also shown to be

very effective in New Zealand (Stewart & Fullerton, 1991), however, success was limited in Australia (Porter *et al.*, 1991). It is risky to rely on one group of fungicides and within New Zealand, alternative fungicides such as the triazole group have been tested and subsequently registered for onion white rot control (Fullerton *et al.*, 1994). Preliminary trials with tebuconazole and triadimenol have shown tebuconazole and triadimenol foliar sprays, following procymidone seed treatment to give almost complete control of onion white rot. Soil sprays of tebuconazole and combinations of soil and foliar sprays were also effective in controlling onion white rot, with only 0.5% of the seedlings diseased after 7 months (Fullerton *et al.*, 1995). While providing excellent control, some applications (tebuconazole seed treatment) were phytotoxic and there were problems with residue levels (A. Stewart pers. comm.).

1.3.2 **Sclerotial Germination Stimulants.**

Sclerotial germination stimulants are applied to the soil to induce sclerotial germination and hence reduce inoculum density before the *Allium* crops are planted. Germinated sclerotia are susceptible to antagonism, competition, starvation, lysis and soil animal actions (Merriman *et al.*, 1980; Utkhede & Rahe, 1982) and without the presence of a host crop, germinated sclerotia will die. This is the basis for using sclerotial germination stimulants as a promising control strategy.

Successful reductions in inoculum levels of *S. cepivorum* have been reported using onion oil and juice, garlic juice, diallyl disulphides (DADS) and eucalyptus leaves (Merriman *et al.*, 1980; Utkhede & Rahe, 1982; Coley-Smith & Parfitt, 1986; Somerville & Hall, 1987; Ismail *et al.*, 1990). Utkhede and Rahe (1982) reported a 73% reduction in white rot incidence when onion oil was applied one month before planting. Although excellent in its control, onion oil is prohibitively expensive. A cheaper alternative is artificially produced onion oil, however, Merriman *et al.* (1980) reported a large variation in the disease control achieved between regions. Research in the United Kingdom has determined several important factors when using DADS to control onion white rot. DADS is temperature and season sensitive. The optimum temperature is 10-15°C and temperatures hotter than 15°C decrease the control effect of DADS (Coley-Smith & Parfitt, 1986). The volume of water added to apply DADS in the field also influences the control effect and generally the greater the volume of water the greater the control of onion white rot (Coley-Smith *et al.*, 1986). Volumes of 0.05% DADS applied at rates of 25 l/m^2, 12.5 l/m^2 and 6.25 l/m^2 to *S.*

cepivorum infested soil have significantly controlled onion white rot by 94, 90 and 80% respectively (Davies & Coley-Smith, 1990). Ismail *et al.* (1990) also reported a reduction in white rot disease with the incorporation of *Eucalyptus rostrata* leaves into infested soil. Soil amended with Eucalyptus leaves at the time of planting or one month before, decreased the percentage of onion white rot infection from 45% under control conditions to 16% and 13%, respectively. The onset of disease was also delayed and diseased seedlings did not appear until 4 months after planting (Ismail *et al.*, 1990).

1.3.3 **Soil Partial Sterilisation.**

Soil partial sterilisation is aimed at reducing pathogen inoculum levels in the soil. This can be achieved by using either the sun's energy (solarisation) or by chemical application. The most commonly used chemicals are methyl isothiocyanate liberating compounds such as metham, metham sodium, dazomet and dichloropropene (Adams & Johnston, 1983; Davies & Coley-Smith, 1986; Keer, 1986; Wong & Maynard, 1986; Davies, 1990). However, variable results have been obtained and the chemical effect on sclerotia has not always correlated well with disease control (Keer, 1986). Chemical sterilisation is also expensive, indicating that widespread use may be limited to smaller areas. Adams and Johnston (1983) reported a cheaper alternative method where the sterilant metham sodium was applied to the soil using an overhead sprinkler at half the concentration of other methods, and still provided effective control. Although inconsistent results have been obtained using chemical soil sterilants, the use of these compounds could be applicable to integrated control programmes.

Another form of soil partial sterilisation is solarisation, the use of the sun to heat soil. This is not a new phenomenon (Grooshevoy, 1939; Avidov, 1956) however, modern solarisation was pioneered in Israel (Katan *et al.*, 1976; Katan, 1981). The technique involves mulching the soil with polyethylene (PE) sheeting during the hottest months of the year. The purpose is to increase the temperatures such that soil borne inoculum is eliminated or at least reduced. Polyethylene can be mulched or laid in flat sheets close to the soil and tucked down into the soil, either method is effective, however, a minimum air space between the soil and the PE is superior in that the insulating effect of the air layer can be minimised (Stapleton & DeVay, 1986). The use of more than one layer of sheeting, often with an air space between the two has also been reported as successful (Ben-Yephet *et al.*, 1987; Duff & Connelly, 1993). For example, Ben-Yephet *et al.* (1987) reported a 97% reduction in

Fusarium oxysporum f. sp. *vasinfectum* (Atkinson) Snyder & Hansen chlamydospores buried at 30cm after 31 days of double layer solarisation compared to only 58% reduction of chlamydospores under a single layer of PE. Transparent PE of 50μm thickness is most commonly used due to its low cost, high transmittance of irradiation and strength value (Porter & Merriman, 1983,1985; Satour *et al.*, 1989). Although the use of coloured PE has been reported (Alkayssi & Alkaraghouli, 1991), there is a tendency for it to absorb heat rather than transmitting it (DeVay, 1991). Alternatives to PE include: polyvinyl chloride and ethylene vinyl acetate (DeVay, 1991).

Soil wetness is a key factor to consider in solarisation. The wetter the soil before the PE is laid the greater the success of inoculum reduction. Plots may also be irrigated while solarisation takes place (Katan *et al.*, 1976). The wetness of the soil increases the thermal sensitivity of the soil microflora and fauna as well as increasing heat transfer or conduction in the soil (Mahrer *et al.*, 1984). Matrod *et al.* (1991) reported that although temperatures were higher in the top fraction of tarped dry soils than wet soils, *S. cepivorum* sclerotial degradation was higher in moist soils.

The use of soil solarisation to control soil borne diseases has, in the past, been confined to the hotter regions of the world such as Israel (Grinstein *et al.*, 1979; Katan *et al.*, 1976); North Carolina (Ristaino *et al.*, 1991); Arizona (Mihail & Alcorn, 1984) and Jordan (Abu-Irmailch, 1991). Success in marginal regions of the world have also been reported, for example, in Greece (Tjamos, 1991) and Japan (Horiuchi, 1991). Solarisation to control onion white rot has been undertaken in the hot regions of Egypt (Satour *et al.*, 1989,1991) and the marginal regions of Spain (Basallote-Ureba & Melero-Vara, 1993) and Australia (Porter & Merriman, 1983,1985). In Egypt, there was no onion white rot infection following a six week solarisation period. The onion plants were taller, heavier and had a larger bulb size than onions planted in non solarised soil (Satour *et al.*, 1989). In Spain, a similar trend was shown, with no viable sclerotia detected after soil solarisation. When the soil was planted with onions, a low disease incidence was evident (Basallote-Ureba & Melero-Vara, 1993). While research conducted in Spain and Egypt reported successful reductions in *S. cepivorum* inoculum, no detail was given on the temperatures lethal to *S. cepivorum* sclerotia. However, *in vitro* experiments undertaken by Adams (1987) reported the effect of temperature upon sclerotial germination. The LD_{50} of *S. cepivorum* sclerotia was achieved when sclerotia were incubated at continuous temperatures of 35, 40, 45 and

50°C for 129.6, 9.5, 1.7 and 0.8 hours, respectively. Porter and Merriman (1983) reported a similar decline in sclerotial viability with increasing temperature. In their study, sclerotia were incubated at a range of temperatures between 25 and 50°C for 6 hours and then maintained at 25°C for 18 hours to simulate daily temperature fluctuations for a two week period. Their results showed 81% viability of sclerotia from soil at 40°C in contrast to 2% sclerotial viability from soil at 45°C. To date, control of onion white rot with soil solarisation has not been attempted in New Zealand, although soil solarisation has been trialed in Canterbury to control weeds (Alexander, 1990). The soil temperature data from Alexander (1990) indicates that soil solarisation in the Canterbury region may be a viable option for onion white rot control and is worthy of investigation.

1.3.4 **Biological Control.**

Biological control is most completely defined as "the reduction of inoculum density or disease-producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host or antagonist, or by mass introduction of one or more antagonists" (Baker & Cook, 1974). Biological control is a viable option when considering control strategies for onion white rot, given that other systems have often proved ineffective and/or inappropriate. Crop rotations are not a suitable method of control for onion white rot due to the persistence of *S. cepivorum* sclerotia in the soil (Coley-Smith, 1959). Fungicides, whilst effective in many instances, are expensive to produce and register and often have associated political, environmental and economic constraints (Lewis & Papavizas, 1991) and breeding programmes worldwide have not yet found a suitable resistant variety.

A number of microorganisms have been identified as potential biological control agents for onion white rot control (Table 1.1). Although several actinomycete and bacterial species have been identified, these have not been included in Table 1.1 as this study will concentrate only on fungal biological control agents. Bacterial species (*Bacillus* spp.), while appearing successful in overseas studies have not been successful at controlling onion white rot in New Zealand (A. Stewart pers. comm.). Ghaffar (1969a) pioneered *in vitro* selection of *S. cepivorum* antagonists, and more recently, Harrison and Stewart (1988) identified *Chaetomium globosum* Kunze. ex Steudel., *Coniothyrium minitans* Campbell, *Gliocladium roseum* Bainer, *Gliocladium virens* Miller, Giddens & Foster (recently renamed *Trichoderma virens*) and *Trichoderma viride* Pers. ex Gray as promising control agents.

Table1.1 Fungal species antagonistic towards *Sclerotium cepivorum*

Fungal Species	Reference
Aleurisma carnis	Ghaffar, 1969a.
Chaetomium globosum	Harrison & Stewart, 1988; Stewart & Harrison, 1988; Kay & Stewart, 1994.
Coniothyrium minitans	Ghaffar, 1969a; Turner & Tribe, 1976; Ahmed & Tribe, 1977; de Oliveira *et al.*, 1984; Legget 1986; Harrison & Stewart, 1988; Stewart & Harrison, 1988; Kay & Stewart, 1994.
Fusarium spp.	Ghaffar, 1969a; Legget & Rahe, 1985; Harrison & Stewart, 1988.
Gliocladium roseum	Harrison & Stewart, 1988; Stewart & Harrison, 1988; Kay & Stewart, 1994.
Gliocladium virens	Harrison & Stewart, 1988; Stewart & Harrison, 1988; Kay & Stewart, 1994.
Penicillium expansum	Ghaffar, 1969a; Utkhede & Rahe, 1980; Legget, 1986; Harrison & Stewart, 1988; Stewart & Harrison, 1988.
Penicillium nigricans	Ghaffar, 1969a,b; Utkhede & Rahe, 1980*.
Penicillium spp.	de Oliveira *et al.*, 1984; Legget & Rahe, 1985; Abd-El-Razik *et al.*, 1986; Harrison & Stewart, 1988.
Sporidesmium sclerotivorum	Ayers & Adams, 1979; Adams, 1987; Adams & Ayers, 1981*.
Teratosperma oligocladium	Ayers & Adams, 1981.
Trichoderma harzianum	Abd-El-Moity & Shatla, 1981*; Papavizas *et al.*, 1982; de Oliveira *et al.*, 1984; Abd-El-Razik *et al.*, 1985; Kay & Stewart, 1994.
Trichoderma viride	Ghaffar, 1969a; Harrison & Stewart, 1988; Kay & Stewart, 1994.
Trichoderma spp.	Legget & Rahe, 1985; Harrison & Stewart, 1988; Kay & Stewart, 1994.

* Field trials conducted with the plant pathogen and antagonist.

Modified from Kay, 1991.

Glasshouse and field trials have shown onion seeds coated with *C. minitans* pycnidial dust (Ahmed & Tribe, 1977) and *Trichoderma harzianum* Rifai conidial suspensions (de Oliveira *et al.*, 1984) to result in the control of onion white rot. Glasshouse trials involving a number of antagonists and application methods showed *C. globosum* and a species of *Trichoderma* to reduce disease levels by 67% and 73% respectively, when applied as soil additives, compared to 50% disease in the control (Kay & Stewart, 1994).

Biological control agents can exhibit three modes of action against a pathogen namely: antibiosis, parasitism and competition (Baker, 1968). Antibiosis can be defined as antagonism of the fungal plant pathogen mediated by specific or non-specific metabolites produced by the microorganism (Fravel, 1988; Jackson, 1965). Antibiosis can be most readily detected in culture. Dual plate assays, where agar plugs of the antagonist and the pathogen are placed on opposite sides of an agar plate, show antibiotic production by the development of a zone of inhibition between the two cultures. *Penicillium* spp. (Ghaffar, 1969a) and *C. globosum* (Harrison & Stewart, 1988) have shown this interaction type when grown with *S. cepivorum.*

Mycoparasitism is the parasitic interaction between two fungal species (Sundheim & Tronsmo, 1988). The parasitic fungus invades the living body of the plant pathogen and feeds and reproduces itself at the host's expense while not contributing to its welfare (Burge, 1988). *In vitro* tests have confirmed *Trichoderma* spp. as mycoparasites (Harrison & Stewart, 1988). *Trichoderma koningii* Oudemans, although not extensively studied in conjunction with *S. cepivorum,* has shown control potential with *Sclerotinia sclerotiorum* (Lib) de Bary. In culture, *T. koningii* was shown to parasitize 100% of *S. sclerotiorum* sclerotia (dos Santos & Dhingra, 1982).

Competition can be defined as "the endeavor of two or more organisms to gain the measure each wants from the supply....of a substrate, in the specific form and under specific conditions in which that substrate is presented....when that substrate is not sufficient for both." (Clark, 1965). *In vitro* agar screenings have shown, *Gliocladium virens, G. roseum, C. minitans* (Harrison & Stewart, 1988) and *T. viride* (Ghaffar, 1969a; Harrison & Stewart, 1988) to outcompete *S. cepivorum* for nutrients and also to overgrow the *S. cepivorum* colonies.

The fungal modes of action, while defined separately, are not mutually exclusive. Biological control of onion white rot may involve more than one mechanism, for example, in culture, *T. harzianum* hyphae coiled around *S. cepivorum* hyphae and produce cell wall degrading enzymes to aid penetration, although occasionally, *T. harzianum* will penetrate *S. cepivorum* hyphae directly (de Oliveira *et al.*, 1984). *T. harzianum* has also been shown to produce antibiotics in culture to inhibit *S. cepivorum* growth before the colonies meet (de Oliveira *et al.*, 1984) and degradation of *S. cepivorum* sclerotia has also been reported (Abd-El-Moity & Shatla, 1981). The antagonism exhibited by some fungi is not necessarily correlated with disease control and developmental features such as slow growth can often result in the fungus being unsuitable for control applications, for example *Sporidesmium sclerotivorum* Uecker, Ayers et Adams (Harrison, 1987). Therefore, a knowledge of fungal modes of action is important to the development of successful control strategies. Early attempts at biological control were often unsuccessful due to the use of inappropriate application methods (Garrett, 1956). It is now recognised that delivery systems for biological control agents must enable the microorganism to survive, establish, proliferate and become active in a foreign environment. For the microorganism to be delivered to the soil in such a way that the above requirements are met, an understanding of how the microorganism antagonises its host must be known. Fungi that antagonise by antibiotics would be most effective when applied as seed coatings or root dips. The antibiotics produced would be able to inhibit the growth of the pathogen near the seed or seedling, hence enabling the *Allium* crop to grow past infection stages. In contrast, mycoparasitic antagonists are more likely to be efficient when delivered as bulk carriers or alginate pellets to the soil before planting. The earlier addition of these fungi would allow them to become established, antagonise the pathogen and reduce inoculum levels before the *Allium* crop is planted. In addition to understanding the mode of action, it is important to be familiar with disease epidemiology, such as the pathogen life cycle, survival and infection mechanisms and disease spread, for only when these factors are taken into consideration, can a successful control programme be developed.

1.4 Aims and Objectives.

The research into biological control of onion white rot undertaken in this study was a continuation and extension of an existing biological control programme, involving microoganisms antagonistic towards *S. cepivorum*. Both *in vitro* and *in situ* experiments were conducted using fungi previously identified as antagonistic towards *S. cepivorum*

(Ghaffar, 1969a; Harrison & Stewart, 1988; Kay & Stewart, 1994). Soil solarisation is a new aspect of cultural control to be trialed in the Canterbury region and previous research (Alexander, 1990) indicated that soil solarisation may be a viable option for disease control.

The combination of these biological and cultural systems could provide effective, low cost control of onion white rot. The use of soil solarisation may reduce the amount of disease in subsequent *Allium* crops (Satour *et al.*, 1989). Fungal antagonists could be incorporated into the soil before solarisation or perhaps applied in conjunction with planting, to reduce disease incidence to insignificant levels.

1.4.1 **Specific Objectives.**

1. To evaluate the ability of promising fungal antagonists to control *S. cepivorum* in a glasshouse and field situation.
2. To determine the most appropriate time to apply fungal antagonists to the soil to maximise disease control.
3. To examine the effect of a range of temperatures on sclerotial viability.
4. To determine whether soil solarisation is a suitable disease control option in the Canterbury region.

~CHAPTER TWO~

GLASSHOUSE TRIAL AND ASSOCIATED *IN VITRO* STUDIES.

GLASSHOUSE TRIAL.

2.1 Introduction.

All the experiments and trials described in this study were conducted using the same isolate of *S. cepivorum* (E68), originally isolated from a diseased onion. This isolate of *S. cepivorum* grew well on both agar and organic substrates and produced sclerotia abundantly. Isolates of *C. globosum, T. harzianum, T. koningii* and *T. viride* have been previously trialed against *S. cepivorum* and reported as possible antagonists (Kay & Stewart, 1994). Successful control of *S. cepivorum* with *T. koningii* has been reported in the United Kingdom and Australia (A. Stewart pers. comm.). *C. minitans* also appears successful against onion white rot (Ahmed & Tribe, 1977; Harrison & Stewart, 1988; Stewart & Harrison, 1988). Based on previous research, the isolates: *Chaetomium globosum* (A53), *Coniothyrium minitans* (A69), *Trichoderma harzianum* (C52), *Trichoderma koningii* (C60, C62) and *Trichoderma viride* (D73) were chosen for further study (Table 2.1). Isolate C62 was previously unidentified and a preliminary identification of *Trichoderma koningii* has been made based on Rifai's key (Rifai, 1969). All cultures were maintained on slope tubes of Potato dextrose agar (PDA) (Appendix 1.1) at 4°C in the dark. When required, the cultures were transferred to PDA plates and grown at 20°C in a 12 hour light, 12 hour dark cycle incubator.

Screening all six fungal isolates against *S. cepivorum* in a field situation was impractical. Smaller scale glasshouse trials can be used to give an indication of how potential fungal antagonists may work under a wider field situation. The advantage to these trials is that a number of antagonists can be tested in a relatively small area and with relative ease. The disadvantage is that there can be some variability in disease control afforded by the same isolates between glasshouse and field trials (Papvizas, 1985; Lewis *et al.*, 1990). Nevertheless, the best potential biological control agents can be selected for larger scale field or commercial situation trials. Glasshouse and pot trials have previously shown successful reductions in onion white rot using fungi (Ahmed & Tribe, 1977; Abd-El-Moity & Shatla, 1981; Kay and Stewart 1994).

Table 2.1 Origin of *Sclerotium cepivorum* and fungal antagonists

Isolate	Original isolate number	Isolate number	Supplier	Isolation date	Source
Chaetomium globosum	422	A53	ICMP[1]	1944	rotting fabric, unknown
Coniothyrium minitans		A69	LUMCC[2]	1990	sclerotium of *Sclerotinia minor*, Pukekohe, NZ
Sclerotium cepivorum		E68	LUMCC	1990	diseased onion tissue, unknown
Trichoderma harzianum	3099	C52	ICMP	1971	*Eucalyptus* bark, Palmerston North, NZ
Trichoderma koningii	255809	C60	CMI[3]	1978	carrot, Norfolk, UK
Trichoderma koningii	T1	C62	LUMCC	1988	soil, Pukekohe, NZ
Trichoderma viride		D73	LUMCC	1986	soil, Pukekohe, NZ

[1] International Collection of Microorganisms from Plants, Landcare Research New Zealand Ltd., Mount Albert Research Centre, 120 Mt Albert Rd, Private Bag 92169, Auckland, New Zealand.
[2] Lincoln University Microbial Culture Collection.
[3] Commonwealth Mycological Institute, Kew, Surrey, England.

The objectives of this trial were to determine the ability of a range of fungi to control onion white rot disease in a glasshouse situation and to determine the best time to apply a biological control agent to the soil, relative to onion seed sowing, to maximise disease control.

2.2 Methods and Materials.

2.2.1 Production, harvesting and conditioning of *S. cepivorum* sclerotia.

Production of Sclerotia.

Sclerotia of *S. cepivorum* were produced on whole wheat grains (Plate 2.1) (Backhouse & Stewart, 1989). Fifty grams of whole wheat grains were added to each of 20 250ml conical flasks. Forty-five millilitres of 0.0025% (w/v) chloramphenicol was added to each flask and the flasks were left overnight. The wheat flasks were autoclaved at 121°C and 15 psi for 30 minutes on two consecutive days. When cool, each flask was inoculated with four 5mm disks of *S. cepivorum* taken from the actively growing edge of a seven day old culture grown on PDA. The flasks were incubated in the dark at 20°C for between six and eight weeks, and shaken at weekly intervals to ensure an even distribution of the mycelium. During the first three weeks of incubation, 0.5ml of sterile distilled water (SDW) was added if the flasks appeared dry, to encourage mycelial growth (A. Stewart pers. comm.).

Harvesting of Sclerotia.

The sclerotia were harvested from the wheat grains using progressive wet sieving through 850µm, 500µm and 250µm sieves (Kay & Stewart, 1994). Only sclerotia measuring 500µm in diameter were used in the trials and the sclerotia were air dried on sterile Whatman No. 1 filter paper for 24 hours before immediate use. The sclerotia used after this stage were termed "fresh".

Conditioning of Sclerotia.

For the glasshouse and soil temperature studies, the sclerotia were conditioned before they were incorporated into the soil. The sclerotia were loosely contained in polyester mesh (URE Pacific) bags (85µm pore size, 20cm x 20cm) that were buried in liver pails (18cm x 18cm x 19cm) filled with Wakanui silt loam soil (pH 5.1). The pails were incubated between 15-18°C and the moisture content of the soil was maintained between 40-50%. The sclerotia were incubated in this fashion for a minimum of two months, in order to

Plate 2.1 *Sclerotium cepivorum* sclerotia grown on whole wheat.

overcome constitutive dormancy (Coley-Smith, 1960). These sclerotia were termed "conditioned".

2.2.2 Sclerotial viability assay.

Before both the fresh and conditioned sclerotia were used, a sample of 100 sclerotia were surface sterilised. Previous investigations in this study determined that a concentration of 0.25% sodium hypochlorite (NaOCl) for one minute reduced the level of surface contaminants but allowed a high level of germination (>80%). Following treatment with NaOCl, the sclerotia were washed in five changes of SDW, touched to Whatman No.1 filter paper using sterile forceps to absorb excess liquid and placed onto PDA droplets. The sclerotia were examined daily for ten days and the number of germinated sclerotia were recorded.

2.2.3 Glasshouse trial.

Large polystyrene boxes, 59 x 33 x 19cm were filled to within 5cm of the top with shredded Wakanui silt loam soil. Conditioned sclerotia of *S. cepivorum* were incorporated into the sieved soil to give an inoculum density of one sclerotium per gram of soil (dry soil weight).

2.2.3.1 Preparation of Antagonist Inoculum.

The fungal antagonists *Chaetomium globosum* (A53), *Coniothyrium minitans* (A69), *Trichoderma harzianum* (C52), *T. koningii* (C60, C62) and *T. viride* (D73) were cultured for 14 days in 250ml conical flasks each containing 100ml of Molassess Yeast Extract (MYE) broth (Papavizas *et al.*, 1984; Kay & Stewart, 1994) (Appendix 1.2). The flasks were inoculated with two 5mm disks of the fungal antagonist taken from the actively growing edge of a five day old colony, grown on PDA. Each treatment was replicated twice and the flasks were incubated on a rotary shaker in the dark at 25°C. After 14 days, 100ml of SDW was added to each flask and mixed thoroughly. The two replicate flasks for each treatment were combined and blended in a 2 speed Waring Commercial Blendor®, on low speed, for 30 seconds in three bursts. This procedure gave a fine spore/mycelium homogenate (<150μm).

Thirty-five grams of sand (<800μm) and 35g of ground wheat bran (<800μm) were placed in a 1l flask and autoclaved for 30 minutes, at 121°C and 15 psi on two successive days. Once cool, 70ml of an antagonist homogenate was added to the flask and the contents

shaken. Seventy millilitres of uninoculated MYE broth replaced the antagonist homogenate as a control. The flasks containing *Chaetomium globosum* and *Coniothyrium minitans* were incubated in the dark for nine days at 25°C before immediate application to the soil and flasks containing *T. harzianum, T. koningii* and *T. viride* were incubated in the same fashion for five days before immediate application to the soil. This was to ensure that the slow growing isolates, *Chaetomium globosum* and *Coniothyrium minitans,* reached the same approximate concentration as the faster growing *Trichoderma* isolates at the time of soil inoculation.

Before each sand:bran antagonist mix was applied to the soil, a colony forming unit (CFU) assay was performed to ensure a minimum of 2 x 10^6 CFUs per $10cm^3$ of sand:bran mix (Lewis & Papavizas, 1984) was added to each box (Table 2.5).

2.2.3.2 Colony Forming Unit assay.

A modification of the method of Whipps *et al.*, (1989) was used to determine the number of CFUs for each fungal antagonist. Amounts of each sand:bran antagonist mix ($10cm^3$) were added to 90ml of sterile 0.01% Water agar (WA) (Appendix 1.3). The fungal antagonist/WA combinations were shaken on a wrist action shaker at a medium to fast speed for 10 minutes and, after standing for a further 20 minutes, a dilution series was made by transferring 10ml to 90ml of WA to a dilution of 10^{10}. Aliquot's of the fungal antagonist/WA combinations (0.5ml) at each dilution were pipetted onto the surface of PDA plates (three plates for each dilution) amended with 50ppm streptomycin and 50ppm penicillin G. Fungal colonies were counted within five days and the number of CFUs/$10cm^3$ soil was calculated.

The antagonists were applied to the soil at two different times.

Application 1.

Two weeks before onion seed planting, $10cm^3$ of sand:bran antagonist mix was added to the soil and mixed thoroughly. Three parallel trenches, 30mm deep, were made longitudinally in each box. The untreated onion seed (Pukekohe Long Keeper (Regular) May and Ryan) was planted at approximately 20mm intervals and covered with soil already infested with the fungal antagonists.

Application 2.

The sand:bran antagonist mix was added at the time of planting. Three parallel trenches, 30mm deep, were made longitudinally in each box. Amounts of the sand:bran antagonist mix (10cm^3) were mixed thoroughly into 500g of Wakanui silt loam soil and placed in the trenches. The untreated onion seed (Pukekohe Long Keeper (Regular) May and Ryan) was then planted in the trenches at 20mm intervals, and covered with antagonist infested soil.

The trial was set up in a glasshouse and the boxes were completely randomised within the glasshouse. The boxes were placed in individual watering trays to avoid cross contamination and were watered once a week from the watering trays, for the duration of the trial. Weeding took place by hand. Three weeks after planting, the seedlings were drenched with Captan (3.25g a.i./l/80cm^3) to control damping-off.

Each box contained one treatment, with 3 rows of approximately 25 seedlings (80 seedlings in each box) representing a single observation. There were eight treatments; 6 fungal treatments and 2 controls: pathogen only (unamended soil) and procymidone coated seed (Sumisclex 25 1g a.i./100g seed). An uninoculated sand:bran control was not included, since it would be impossible to determine whether any observed effect on disease was a direct effect of the addition of bran or the effect of increased soil microorganism populations, stimulated by the bran. There were two different application times; application 1 was two weeks before onion seed planting and application 2 was at the time of onion seed planting. Each treatment was replicated five times.

Assessment.

Three and five weeks after seed sowing, seedling emergence was counted. Following this, the seedlings were examined weekly for 12 weeks (Kay, 1991), for visible symptoms of white rot. Instead of removing diseased seedlings, coloured toothpicks, a different colour each week, were placed next to the diseased seedlings (Plate 2.2). This was considered preferable as valuable sources of inoculum would be lost upon seedling removal. Periodically, several diseased seedlings were removed, and surface sterilised in 0.25% NaOCl for two minutes and washed in five changes of SDW. The diseased tissue was blotted dry on sterile Whatman No. 1 filter paper and placed on WA (Appendix 1.4) plates to minimise contamination from soil microorganisms. Once the fungi grew from the diseased tissue, a small section of mycelium was removed and placed onto PDA plates. The PDA

Plate 2.2 Onion seedlings showing white rot disease symptoms. Assessments made at weekly intervals and diseased seedlings marked with different coloured toothpicks.

plates were examined daily for the appearance of *S. cepivorum* to ensure correct diagnosis of the disease. The results for seedling emergence and post emergence cumulative disease for each fungal treatment were analysed using a General Linear Model (GLM) Analysis of Variance (ANOVA) with treatment and application as the variables. The results for seedling emergence and post emergence cumulative disease for each treatment at each application time were analysed using a GLM ANOVA with treatment, emergence and disease as the variables.

2.3 Results.

There was a significant difference ($p \leq 0.05$) in the number of emerged and post emergence diseased seedlings between each application time (Table 2.2). There was no significant interaction between the fungal treatments and the time of application (Appendix 2.1). The fungal treatments and the control treatments were separated into application times for further analysis.

There was a significant difference ($p \leq 0.05$) in the number of emerged and post emergence diseased seedlings between treatments in application 1 and also in application 2 when the control treatments were included in the analysis (Appendix 2.1). The results are presented in Tables 2.3 and 2.4. The procymidone control had the highest number of germinated seedlings (97.5%) and this was significantly different ($p \leq 0.05$) from all other treatments except the application of *T. koningii* (C60) (87.5%), (C62) (78.0%) and *C. minitans* (83.5%) at the time of planting. There was no significant difference ($p \leq 0.05$) in the number of emerged seedlings between any of the fungal treatments and the pathogen control, irrespective of when the treatments were applied to the soil.

Disease was initiated early in the trial. Three weeks after planting, diseased seedlings were observed. Some seedlings were discoloured and lying on the soil surface. Isolations from the diseased tissue diagnosed the disease as white rot. The first disease assessment was brought forward to five weeks after planting rather than the proposed six week disease assessment. Over 50% of the total white rot infections were recorded in the first seven weeks. *C. globosum, T. harzianum, T. koningii* (C62) and both the pathogen and procymidone control treatments have been selected to show this trend (Figure 2.1). The addition of fungi to soil two weeks before seed planting failed to control initial onion white rot infection as compared to the fungi applied at the time of planting. At the first disease

Table 2.2 Mean number of emerged and white rot diseased seedlings after treatment with fungal antagonists, both two weeks before seed planting and at the time of seed planting

Application time	Emergence		Disease	
Two weeks before planting	57.2 ±0.35	b	12.5 ±0.48	a
At the time of planting	65.9 ±0.48	a	7.6 ±0.53	b

Table 2.3 The mean number of emerged and white rot diseased seedlings after treatment with fungal antagonists two weeks before onion seed planting

Treatment	Emergence[1]	% Emerge.	Disease[2]	% Disease[3]	% Control[4]
Control-pathogen only	58.8 ±3.40 b[5]	73.5	23.4 ±2.58 a	40.0	-
Control-procymidone	78.0 ±1.26 a	97.5	4.0 ±1.61 d	5.1	82.9
Chaetomium globosum	55.6 ±2.18 b	69.5	16.8 ±1.85 b	30.2	28.3
Coniothyrium minitans	60.6 ±1.03 b	75.8	11.8 ±1.32 bc	19.5	49.6
Trichoderma harzianum	56.2 ±2.13 b	70.3	9.6 ±1.69 cd	17.1	54.0
Trichoderma koningii (C60)	55.4 ±1.66 b	69.3	10.8 ±2.35 bc	19.5	53.8
Trichoderma koningii (C62)	57.6 ±2.24 b	72.0	14.4 ±2.46 bc	20.0	38.5
Trichoderma viride	57.6 ±2.16 b	72.0	11.4 ±2.82 bc	19.8	51.3

Table 2.4 The mean number of emerged and white rot diseased seedlings after treatment with fungal antagonists at the time of onion seed planting

Treatment	Emergence[1]	% Emerge.	Disease[2]	% Disease[3]	% Control[4]
Control-pathogen only	58.8 ±3.40 b[5]	73.5	23.4 ±2.58 a	40.0	-
Control-procymidone	78.0 ±1.26 a	97.5	4.0 ±1.61 b	5.1	82.9
Chaetomium globosum	64.0 ±4.51 b	80.0	11.4 ±4.52 b	17.8	51.3
Coniothyrium minitans	66.8 ±3.06 ab	83.5	8.4 ±2.54 b	12.5	64.1
Trichoderma harzianum	65.2 ±3.01 b	81.5	5.0 ±0.95 b	7.6	78.6
Trichoderma koningii (C60)	70.0 ±4.12 ab	87.5	9.8 ±3.67 b	14.0	61.5
Trichoderma koningii (C62)	62.4 ±4.25 ab	78.0	3.4 ±1.17 b	5.4	63.2
Trichoderma viride	66.8 ±1.96 b	83.5	8.6 ±3.12 b	12.8	85.5

[1] The mean number of seedlings emerged for each treatment five weeks after planting. n = 80.
[2] The mean number of post emergence diseased seedlings for each treatment after 16 weeks.
[3] Percentage of diseased seedlings relative to the number of emerged seedlings for each treatment.
[4] Percentage disease control for each treatment relative to the number of diseased seedlings associated with the pathogen control.
[5] Mean values followed by the same letter do not differ significantly ($p \leq 0.05$), within each table according to a LSD test.

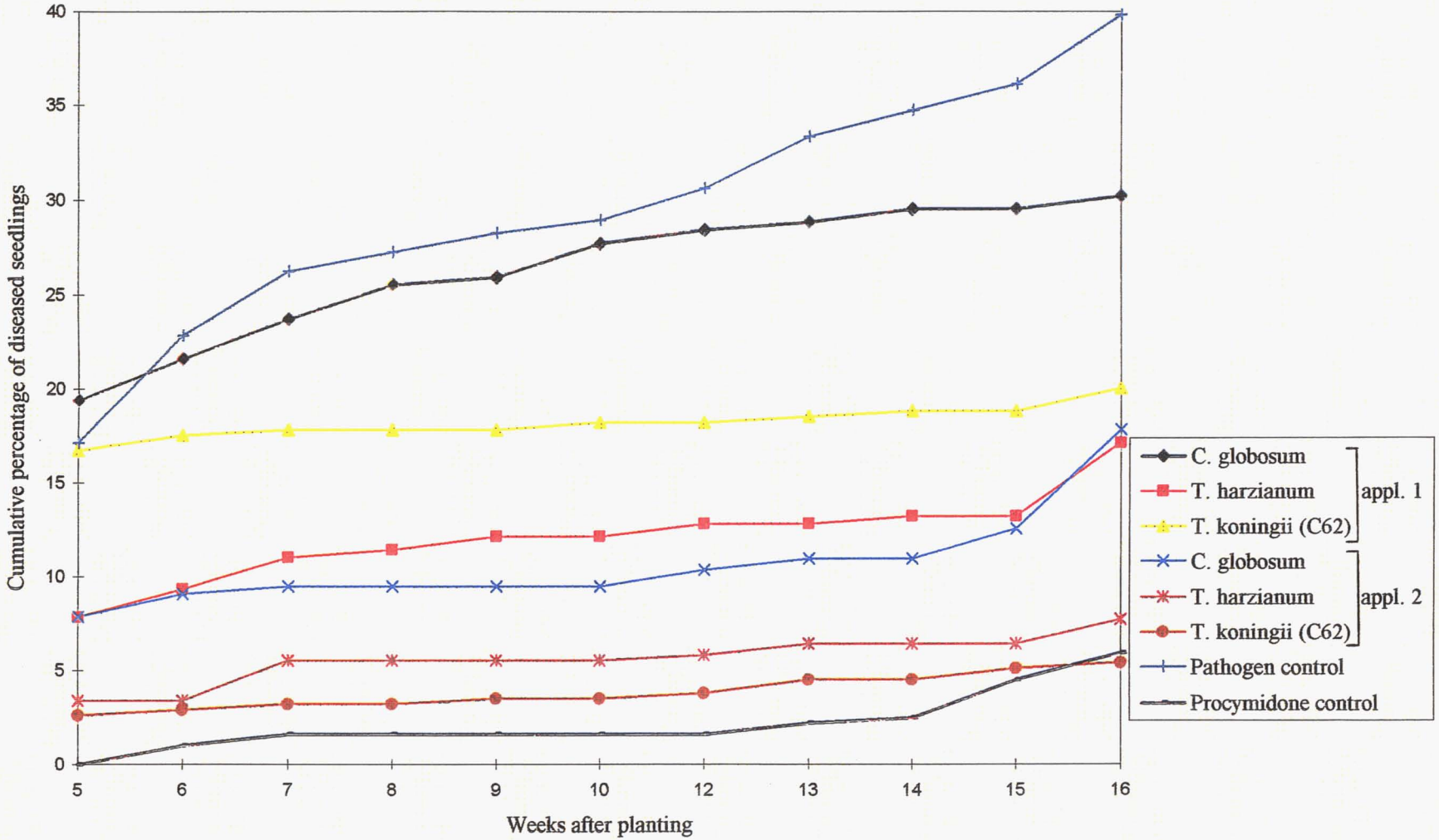


Figure 2.1 Cumulative percentage of white rot diseased onion seedlings for selected fungal treatments, glasshouse trial.

assessment, the application of *C. globosum* and *T. koningii* two weeks before planting resulted in disease levels equivalent to the pathogen control (19.8%). In contrast, *T. harzianum* and *T. koningii* applied at the time of planting resulted in low levels of disease (4.0 and 3.4% respectively) which were not significantly different ($p \leq 0.05$) to the procymidone control (0%) (Appendix 2.1). The number of diseased seedlings in all treatments increased slowly for the duration of the trial and after 16 weeks, 117 seedlings were infected with onion white rot in the pathogen control treatment. The seedlings showed characteristic onion white rot symptoms including the blueing of foliage, leaf tip dieback and a patchy distribution of diseased seedlings within each box (Plate 2.3).

After 16 weeks, 40% of the emerged seedlings were diseased in the pathogen control; a significantly greater ($p \leq 0.05$) amount of disease than any other treatment in both applications (Tables 2.3 and 2.4). When the fungal treatments were applied two weeks before planting, inoculum concentrations ranged from 1.93×10^3 to 1.04×10^8 CFUs in $10cm^3$ of soil (Table 2.5) and disease was significantly less ($p \leq 0.05$) in the procymidone control (5.1%) than all other fungal treatments except *T. harzianum* (17.1%). The application of *T. harzianum* two weeks before planting, resulted in a significantly lower ($p \leq 0.05$) number of diseased seedlings than *C. globosum*, but the number of diseased seedlings associated with the application of *T. harzianum*, was not significantly different ($p \leq 0.05$) from the other fungal treatments. When the fungal treatments were applied to the soil at the time of planting, inoculum concentrations ranged from 6.55×10^4 to 1.70×10^5 CFUs in $10cm^3$ of soil (Table 2.5) and there was no significant difference ($p \leq 0.05$) in the number of diseased seedlings between the procymidone control and the fungal treatments.

2.4 Discussion.

Much of the early disease was not recorded (Figure 2.1). Onion white rot does not usually infect seedlings until approximately six weeks after sowing (A. Stewart pers. comm.). This early infection of white rot could have caused a pre-emergence damping off as 73.5% of the seedlings emerged in the pathogen control compared to 97.5% seedling emergence with the procymidone control. The procymidone coated seeds were protected from infection before germination. The early infection of *S. cepivorum* could have also caused a post-emergence damping-off. Initially, the dying seedlings were thought to be infected with *Pythium* or *Phytophthora*. Isolations confirmed the disease to be white rot and in hindsight, disease assessments should have commenced three weeks after planting.

Plate 2.3 Patchy distribution of onion white rot diseased seedlings in soil boxes, glasshouse trial.

Table 2.5 Number of colony forming units in $10cm^3$ of soil for each fungal treatment, when applied to the soil two weeks before and at the time of seed planting

Treatment	Applied to the soil two weeks before planting[1]	Applied to the soil at the time of planting[2]
Chaetomium globosum	7.70×10^{3} (3)	7.84×10^{4}
Coniothyrium minitans	3.47×10^{4}	1.04×10^{5}
Trichoderma harzianum	1.04×10^{8}	6.55×10^{4}
Trichoderma koningii (C60)	2.32×10^{3}	1.70×10^{5}
Trichoderma koningii (C62)	1.93×10^{3}	1.04×10^{5}
Trichoderma viride	1.17×10^{7}	7.84×10^{4}

[1] The fungal treatments were mixed with 17.25 kg of soil.
[2] The fungal treatments were mixed with 500g of soil.
[3] CFU/$10cm^3$ soil.

The addition of antagonistic fungi to the soil at the time of planting gave greater control of onion white rot than when applied two weeks before planting. With this method, the fungi were mixed with 500g of soil and added only to the planting furrow. Thus, a greater concentration of the fungal antagonist was closer to the seed, providing greater protection than when the fungi were mixed into the whole box, two weeks before onion seed planting (Table 2.5). A much higher concentration of *T. harzianum* and *T. viride* was added to the soil two weeks before planting, than the other treatments. The higher concentration of *T. harzianum* and *T. viride* did not provide much greater control of onion white rot than the other fungal treatments applied two weeks before planting (Table 2.3) and hence, probably did not influence disease control levels.

The addition of fungi to the soil two weeks before planting was hypothesised to give the fungi time to establish and proliferate, conditions that are imperative for the survival of soil microorganisms. It would have been expected that the fungi applied well in advance of seed planting would inhibit the infection of onion white rot to a greater degree than when applied at the time of onion seed planting. This was not the case. The introduction of extra nutrients in the form of bran may have stimulated resident populations of soil fungi (Mulligan *et al.*, 1995; Paulitz & Baker, 1987). Competition between resident soil microorganisms and the introduced antagonist fungi could have occurred and resulted in reduced antagonistic capabilities or perhaps total suppression of the introduced fungi. This could explain the reduced emergence and greater amount of disease observed than when the fungi were applied at the time of planting.

The procymidone coated seed provided 82.9% disease control and was most effective in the first 12 weeks, after which, a greater number of onion seedlings became infected with onion white rot. This is to be expected as in a commercial situation a seed coating would be followed by foliar sprays to maintain chemical efficacy (Fullerton & Stewart, 1991; Stewart & Fullerton, 1991).

Although all fungal treatments applied at the time of planting provided disease control equivalent to that afforded by the procymidone coated seeds, *T. harzianum* (C52) and *T. koningii* (C62) were chosen for further study for several reasons. Firstly, these two species established and proliferated well on a range of media, compared to *Chaetomium globosum* and *Coniothyrium minitans*. There was not enough time between trials to maximise the

growth of *Chaetomium globosum* and *Coniothyrium minitans*, hence these species were not investigated further. Secondly, *T. harzianum* and *T. koningii* have been reported as successful biological control agents by other researchers. *T. harzianum* has previously been reported to reduce *S. cepivorum* infection from 84% to 29% in greenhouse trials (Abd-El-Moity & Shatla, 1981) and *T. harzianum* has also been reported successful against *Sclerotinia sclerotiorum* (Singh, 1991) and *Rhizoctonia solani* Kuhn (Lewis & Papavizas, 1985b). Similarly, *T. koningii* reduced onion white rot disease by 60% when incorporated in a millet formulation and added to the soil at seed planting (Wong *et al.*, 1995) and *T. koningii* (C62), the same isolate as used in this study, provided 79% disease control of onion white rot, when incorporated into the soil in a sand:bran mix (Kay & Stewart, 1994).

Although an indication of antagonism can be obtained from the glasshouse trial and previous literature based on similar isolates (Kay & Stewart, 1994), the level of control did vary between the same isolates when similar methodology was used. For example *C. globosum* gave poor control of *S. cepivorum* in this research but provided significantly better control of onion white rot than *T. harzianum, T. viride* and *G. virens*, when studied previously (Kay and Stewart, 1994). These discrepancies and the lack of biological control data on isolates such as *T. koningii*, prompted some *in vitro* assays to determine the activity of these isolates. While these studies were conducted, a second glasshouse trial and field trial were established to examine the potential of *T. harzianum* and *T. koningii* as biological control agents of onion white rot using a range of delivery systems.

IN VITRO STUDIES - DUAL PLATE AND AMENDED AGAR ASSAYS.

2.5 Introduction.

In vitro assays are commonly used when initially identifying antagonistic microbial species (Ghaffar, 1969a) and also when investigating antagonistic interactions (Stewart & Harrison, 1988). Dual plate and amended agar were the two assays chosen to examine the antagonistic activities of *C. globosum, C. minitans, T. harzianum, T. koningii* and *T. viride.*

The dual plate technique involves inoculating an agar plate with both the pathogen and fungal antagonist at opposite sides of the plate. Various interactions can be observed when the two cultures grow towards one another. Dual plate assays are particularly good at detecting the production of inhibitory compounds as indicated by the production of zones of

inhibition. The relative competitive ability of both organisms in culture can also be examined.

The amended agar assay is a more sensitive assay used to detect the production of inhibitory compounds by the antagonist when grown in liquid culture. PDA is amended with the culture filtrate and inoculated with the pathogen and the pathogen colony is monitored to detect abnormal growth.

The objectives of these experiments were to examine the antagonistic interactions in closer detail and to gain an insight into the possible modes of action of the antagonist fungi in relation to *S. cepivorum*.

2.6 Dual Plate Assay.

2.6.1 Methods and Materials.

PDA plates were inoculated at opposite sides (50-60mm apart) with 5mm plugs of fungal mycelium taken from the actively growing edge of a five day old *S. cepivorum* culture and fungal antagonist cultures grown on PDA. This was the case for all fungal antagonist isolates except *C. minitans*, where a nine day old culture was used due to the slow growth of this fungus. Each *S. cepivorum*/fungal antagonist combination was replicated three times. Single and dual inoculations of *S. cepivorum* were included as controls. The plates were sealed with polythene wrap, inverted and incubated at 20°C in the dark. The radii (mm) of both the *S. cepivorum* and the fungal antagonist were measured daily for 14 days or until the colonies reached the plate edge. The measurements were made from the edge of the mycelial plug towards the other agar plug along pre-drawn lines. The distance between the leading edges of the two colonies was also measured each day. The morphology of each colony was examined daily and any changes such as the colouration of either colony including colouration at colony edges, the production of spores or sclerotia and cessation of colony growth, were recorded.

Upon completion of the experiment, the plates were examined in detail and the interaction between *S. cepivorum* and the fungal antagonist was described according to the following criteria (Ghaffar, 1969a; Harrison & Stewart, 1988; Kay, 1991).

A. The hyphae of the two colonies intermingle but remain clearly distinguishable.

B. The growing margins of the two colonies meet, *S. cepivorum* is inhibited and overgrown by the fungal antagonist.

C. The hyphae of the two organisms approach one another and stop growing.

D. The growth of *S. cepivorum* is inhibited at a distance leaving a clear zone of inhibition between the two organisms.

Interaction types B and D were considered to be antagonistic.

Analysis.

The radii (mm) of the *S. cepivorum* colonies at day eight were analysed with a one way ANOVA of fungal growth. Day eight was the time taken for the single inoculation of *S. cepivorum* to grow across the plate.

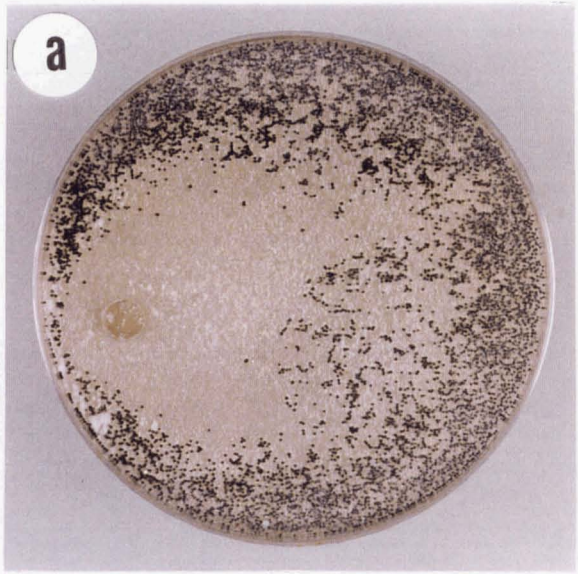
2.6.2 **Results.**

All fungal antagonists significantly reduced ($p \leq 0.05$) the growth of *S. cepivorum* in dual culture after eight days compared to the *S. cepivorum* control (Table 2.6) (Appendix 2.2). The single inoculation *S. cepivorum* control, grew across the PDA plate in eight days (Plate 2.4.a). A dual culture control was also set up to ensure that there was no antagonism between *S. cepivorum* colonies in dual culture (type **A** interaction). In this case, the two colonies met and sclerotia developed at the periphery of each colony (the middle of the plate). The *C. globosum* isolate inhibited the growth of *S. cepivorum* at a distance (type **D** interaction, Plate 2.4.b). *C. globosum* turned the agar a yellowish orange colour and the leading edge of the *S. cepivorum* colony was discoloured and brown by day 14, as compared to the control which had a healthy leading edge with mature sclerotia formed by day 14. The *C. minitans* isolate produced a **C** type interaction where the two fungal colonies met and stopped growing (Plate 2.4.c). A dark, dense line developed between the two colonies and *S. cepivorum* began producing sclerotia by day ten, the same time as the control plates. The *Trichoderma* isolates, significantly reduced ($p \leq 0.05$) the growth of *S. cepivorum* compared to the other fungal antagonists (Table 2.6) and the mean colony growth of *S. cepivorum* was similar when grown with *T. harzianum* (19.0mm), *T. koningii* (C60) (18.7mm), (C62) (19.7mm) and *T. viride* (19.0mm). All the *Trichoderma* isolates produced a type **B** interaction (Plate 2.4.d) and the *S. cepivorum* colonies were completely covered. The health of the *S. cepivorum* colonies was difficult to determine since only the lower surface of the agar plate could be examined, however, the colonies appeared brown

Table 2.6 Dual plate interactions between *Sclerotium cepivorum* and the fungal antagonists

Fungal antagonist	*S. cepivorum* Colony growth[1]	Inhibition zone[2]	Interaction type[3]
Sclerotium cepivorum	69.7 ±0.33 a[4]	-	-
Chaetomium globosum	32.7 ±0.88 c	5.7 ±0.94	D
Coniothyrium minitans	53.0 ±1.00 b	-	C
Trichoderma harzianum	19.0 ±1.00 d	-	B
Trichoderma koningii (C60)	18.7 ±1.33 d	-	B
Trichoderma koningii (C62)	19.7 ±0.33 d	-	B
Trichoderma viride	19.0 ±0.58 d	-	B

[1] Mean *S. cepivorum* colony radius (mm) ± standard error.
[2] Inhibition zones were assessed on the day that *S. cepivorum* ceased growing.
[3] Refer to text (Section 2.6.1) for definition of interaction types.
[4] Mean values followed by the same letter do not differ significantly ($p \leq 0.05$) with a LSD test.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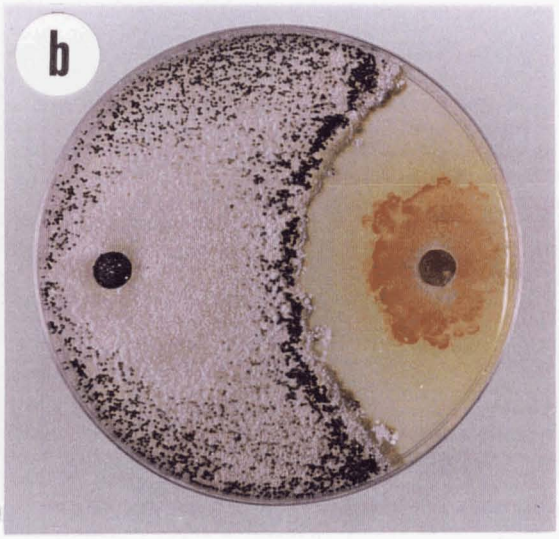
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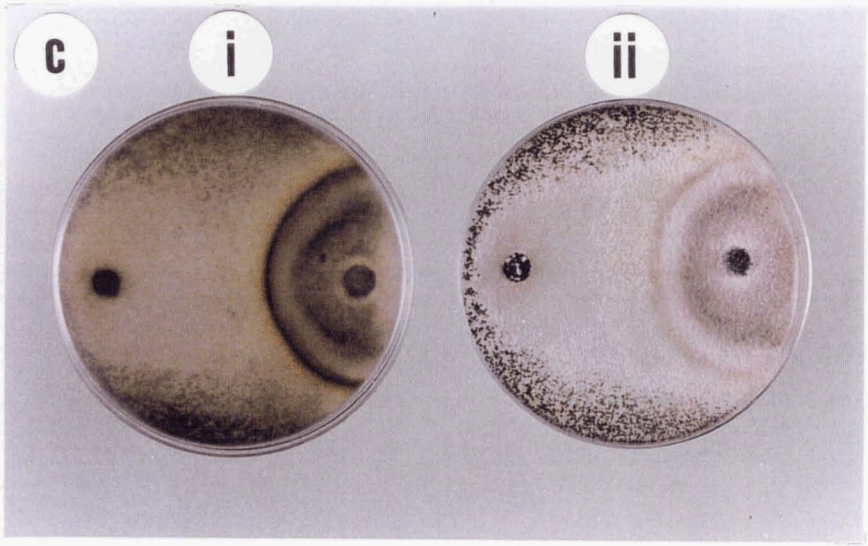
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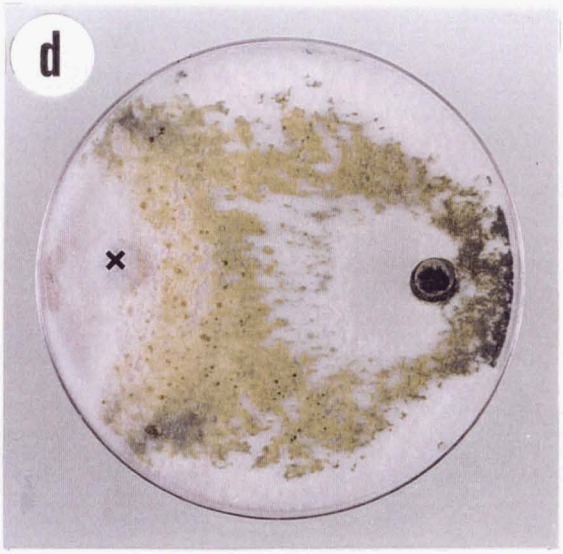
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and discoloured. Production of *S. cepivorum* sclerotia was inhibited and *Trichoderma* spore production was evident after day ten.

2.7 Amended agar assay.

2.7.1 Methods and Materials.

Two hundred and fifty millilitre conical flasks containing 100ml of MYE broth were autoclaved for 30 minutes at 15 psi on two successive days. Once the broth had cooled, each flask was inoculated with two 5mm disks of each fungal antagonist taken from the actively growing edge of a five day old colony, grown on PDA (Kay, 1991). Two replicate flasks were made for each fungal antagonist and the control treatments were *S. cepivorum* or uninoculated agar disks. The inoculated flasks were incubated at room temperature (18-20°C) in the dark, on a rotary shaker (Abd-El-Moity & Shatla, 1981).

After 14 days, the cultures were removed from the shaker and the two flasks for each fungal antagonist were combined. The liquid medium was separated by suction filtration. The resultant culture filtrate was centrifuged at 500 rpm for ten minutes. Ten millilitre aliquot's of the centrifuged filtrate were filter sterilised (to remove any bacterial contamination) with a 0.2μm millipore filter, directly into cooled PDA (≤55°C) at a concentration of 10% (v:v) (Abd-El-Moity & Shatla, 1981; Kay, 1991). The amended agar was poured into Petri dishes, and upon solidification, the plates were centrally inoculated with a 5mm disk taken from the actively growing edge of a five day old *S. cepivorum* culture grown on PDA. PDA amended with uninoculated MYE broth acted as the control treatment. Three replicate plates of each fungal antagonist, *S. cepivorum* or uninoculated agar were prepared and the plates were sealed with polythene wrap, inverted and incubated in the dark at 20°C.

Assessment.

The radius (mm) of each *S. cepivorum* colony was measured in four directions along pre-drawn lines, each day for seven days or until the colony reached the plate edge. The mean daily radial growth was calculated (excluding growth at day one as such growth is often not representative of normal fungal growth). A one-way ANOVA on the growth of *S. cepivorum* was performed.

2.7.2 Results.

The effect of fungal culture filtrates on *S. cepivorum* growth is shown in Table 2.7. *C. minitans, T. koningii* and *T. viride* did not significantly affect the growth of the *S. cepivorum* colonies ($p \leq 0.05$) compared to the control plates (Table 2.7) (Appendix 2.3). *C. globosum* and *T. harzianum* did significantly reduce the growth rate of *S. cepivorum* with 79.7 and 23.4% inhibition, respectively. The *S. cepivorum* colonies associated with the control, *C. minitans, T. koningii* and *T. viride* amended agar grew across the agar plate and began to produce sclerotia at the periphery of the plate after seven days. The *S. cepivorum* colony grown on the *T. harzianum* culture filtrate amended agar grew across the agar almost to the plate edge and a few sclerotia had developed by the completion of the trial, on day seven. Culture filtrate from the *C. globosum* isolate substantially altered the growth of *S. cepivorum* and sclerotia began to develop around the *S. cepivorum* plug in the centre of the plate, rather than around the plate edge.

2.8 Discussion.

In the dual plate assay, the interactions between the fungal antagonists and *S. cepivorum* were the same as the interactions recorded by Kay (1991). *C. globosum* inhibited the growth of *S. cepivorum* at a distance which was consistent with the production of inhibitory compounds as previously reported (Domsch *et al.*, 1980; Kay, 1991), however, in this study the width of the inhibition zone was much less than when examined five years ago (Kay, 1991). This difference could be due to the age of the culture. This isolate of *C. globosum* was first isolated in 1944 and has been in culture ever since. The *C. globosum* culture had lost the ability to sporulate and the fungal colony edges were very uneven, indicating poor and inconsistent growth (A. Stewart, pers. comm.).

Although the dual plate results parallelled those of Kay (1991), the amended agar results were very different. Kay (1991) reported a 12 and 20% reduction in *S. cepivorum* growth on agar amended with *T. viride* and *T. koningii* (C62) respectively, while this study showed less than 3% inhibition for both *T. viride, T. koningii* (C60, C62) and also for *C. minitans*. The variation between these two studies could be due to the type of molasses used. This study used a very thick molasses requiring only 10g, whereas Kay (1991) used 30g of a thinner molasses. Alternatively, the cultures could have lost the ability to produce antibiotics after long term storage. While the effect of light has been shown to influence the production of inhibitory compounds (Abd-El-Moity & Shatla, 1981), this was not an issue

Table 2.7 Mean daily growth of *Sclerotium cepivorum* on agar amended with culture filtrate of fungal antagonists

Antagonist filtrate	*S. cepivorum* colony growth[1]	Percentage inhibition[2]
Control - Uninoculated MYE broth	12.8 ±0.03 a[3]	-
Sclerotium cepivorum	12.6 ±0.00 a	1.6
Chaetomium globosum	2.6 ±0.05 c	79.7
Coniothyrium minitans	12.5 ±0.06 a	2.3
Trichoderma harzianum	9.8 ±0.03 b	23.4
Trichoderma koningii (C60)	12.8 ±0.03 a	0.0
Trichoderma koningii (C62)	12.7 ±0.07 a	0.8
Trichoderma viride	12.4 ±0.59 a	3.1

[1] Mean daily growth rate of *S. cepivorum* (mm) ± standard error.
[2] Percentage inhibition of the *S. cepivorum* colony relative to the uninoculated MYE broth control.
[3] Mean values followed by the same letter do not differ significantly ($p \leq 0.05$) with a LSD test.

in this study and that of Kay (1991), since the culture filtrates were grown in the dark in both studies.

The dual plate assay does not always detect the production of inhibitory compounds. *T. harzianum* produced a C type interaction and covered the *S. cepivorum* colony with hyphae and spores. When the culture filtrate amended agar was inoculated with *S. cepivorum*, the growth of the *S. cepivorum* colony was inhibited by 23.4%, indicating the production of inhibitory compounds. *Trichoderma* spp. are known to produce antibiotics and metabolites (Dennis & Webster, 1971a,b). *T. harzianum* has been shown to produce 6-pentyl-alpha-pyrone (6PAP) (Dodd-Wilson, 1996), however, this culture did not impart the characteristic coconut aroma. The production of 6PAP or any other antibiotic could have occurred when *T. harzianum* was introduced into the soil in the glasshouse trial and may have been responsible for the reduction in onion white rot disease. If antibiotics or metabolites were produced then production probably occurred in both applications. The earlier introduction of the fungal treatments could have enabled the antibiotics to breakdown before onion seed planting occurred. If the antagonist fungi were suppressed by resident microorganisms, then antibiotic production would have ceased for the remainder of the trial.

The *in vitro* assays have provided explanations and valuable information regarding the antagonistic ability of the six fungal antagonists. *C. globosum* may have lost some of its antibiotic activity and this may explain some of the reduced biological control activity in this study compared to Kay (1991). *T. harzianum* and *T. koningii* (C62) are still potentially effective biological control agents of onion white rot. Antibiotic production may be a possible mode of action for *T. harzianum* but the production of antibiotics does not appear plausible for *T. koningii*.

~CHAPTER THREE~

INVESTIGATIONS OF DELIVERY SYSTEMS FOR *TRICHODERMA HARZIANUM* AND *TRICHODERMA KONINGII*

3.1 Introduction.

A glasshouse and field trial were conducted to investigate further the potential of *Trichoderma harzianum* (C52) and *T. koningii* (C62) as biological control agents. In a commercial situation, pellet formulations and seed coatings are two of the most feasible and practical options for applying biological control agents to soil. The use of bulk carriers, as previously trialed, is limited as large volumes of material must be worked with, added to the soil and stored. Seed coatings and pellets can be used with standard planting equipment, the products are easily handled and do not require large areas for storage. Another advantage is that the biological control agent is delivered directly to the planting furrow, providing maximum protection for the seed. Pellet formulations have been used successfully in conjunction with biological control agents. *Gliocladium virens* controlled *Pythium ultimum* Trow in ornamentals when incorporated into the soil as pellets (Lumsden & Locke, 1989). *G. virens* has also prevented damping-off of snap bean (Papavizas & Lewis, 1989), cotton and sugar beet (Lewis & Papavizas, 1987). Seed coatings have not been as successful as pellet applications, although *Trichoderma* strains as seed coats were able to control pre-emergence damping-off caused by *P. ultimum* in cucumber seeds (Harman & Taylor, 1988).

Combinations of fungal species are thought to be able to provide greater control of plant pathogens than applications of individual isolates (Deacon, 1994). Individual fungal isolates can occupy only a limited niche range when in competition with other microorganisms, such as in a soil environment, where the resident microorganisms have already adapted to the prevalent conditions. Although there are isolated successful examples of control by individual fungal isolates such as the protection of pine stumps against *Heterobasidion annosum* (Fries). Bref. with *Peniophora gigantea* (Fries.) (Rishbeth, 1963); it is unlikely that one fungal isolate would be able to provide successful control of a plant pathogen for a continuous period of time. There are no successful examples of control of fungal plant pathogens, where single isolates of biological control agents have been introduced into a soil environment. Isolates of *Idriella bolleyi* (Sprague) von Arx have shown markedly different levels of water stress tolerance *in vitro* (Douglas & Deacon, 1994). The

combination of these *Idriella* isolates could result in greater control of the cereal pathogens *Gaeumannomyces graminis* (Saccardo) von Arx & Olivier and *Fusarium* spp., as the introduction of more than one species could occupy a greater number of niches. Hence, the combination of *T. harzianum* and *T. koningii* was considered worthy of investigation. The glasshouse trial incorporated pellet formulations and onion seed coatings of *T. harzianum* and *T. koningii* both individually and combined together. The glasshouse trial also incorporated Trichopel. Trichopel is a combination of *Trichoderma* strains, coated onto barley grains to form pellets. Agrimm Technologies (Appendix 3.1) market Trichopel as a biological soil conditioner claimed to have disease suppressive abilities and has been shown to promote plant growth (Hunt, 1994). The rationale for the incorporation of Trichopel in the soil box trial was two-fold, firstly, to determine the ability of the combination of *Trichoderma* strains in Trichopel to control onion white rot compared to standard fungicide control and, secondly, to determine the effect, if any, on onion plant growth. The field trial incorporated pellet formulations of both *T. harzianum* and *T. koningii* prepared individually and in combination. These pellets were also prepared by Agrimm Technologies and the fungi were pelleted onto barley grains.

The objectives of the glasshouse and field trial were to determine the effect of different delivery systems on the success of *T. harzianum* (C52) and *T. koningii* (C62) to control onion white rot and also to determine the effect of Trichopel *Trichoderma* strains on onion white rot disease control.

3.2 Methods and Materials.

3.2.1 Glasshouse Trial.

3.2.1.1 Pellet Formulation.

T. harzianum and *T. koningii* were grown on V-8 juice agar (Appendix 1.5) plates for 12 days under continuous fluorescent light between 22-25°C. Ten millilitres of Tween 20 solution (Appendix 1.6) was added to each plate and the surface of the colony was rubbed with a sterile hockey stick to produce a conidial suspension; haemocytometer spore counts were performed before pellet formation. Ten millilitres of the conidial suspension was added to a 1l conical flask containing 500ml of fermentation medium (MYE broth) (Papavizas *et al.*, 1984). The cultures were incubated on a rotary shaker in the dark for eight days at room temperature (18-22°C). After incubation, the fungal biomass was separated from the

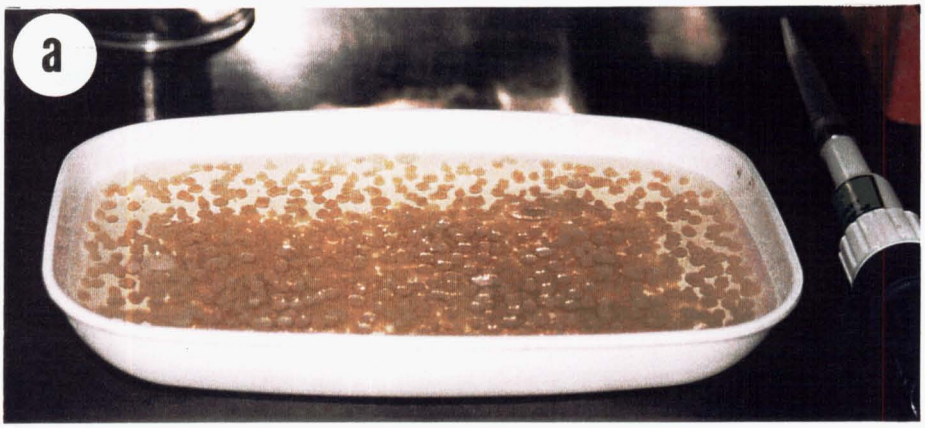
liquid using filtration and the mycelial mats were dried in flowing air for two days and ground (<425μm) in a mortar and pestle.

Alginate pellets containing fungal material were made by the methods of Walker & Connick (1983) and Lewis & Papavizas (1985a) modified as follows. Sodium alginate (Hopkin & Williams Ltd) was dissolved in distilled water (20g/750ml) at 40°C on a stirring hot plate in a 1l Duran bottle. Ground wheat bran (<850μm particle size) was suspended in water (50g/250ml) in a 500ml Duran bottle. Both Duran bottles and contents were autoclaved for 30 minutes at 121°C and 15 psi. When cool, the preparations were transferred to a sterile milkshake blender attachment with 3.5g/l ground fungal biomass for both the *T. harzianum* and *T. koningii* individual pellets. For the *T. harzianum/T. koningii* combination, 1.75g of *T. harzianum* and 1.75g of *T. koningii* was added and comminuted for 30 seconds at high speed. Uninoculated fermentation medium acted as the control treatment and pellets were formed using the same method as the inoculated medium.

The mixture containing the fungus, bran and alginate was added dropwise, using a sterile pipette (Kay & Stewart, 1994), into 500ml of gellant solution (0.25 M calcium chloride ($CaCl_2$), pH 5.4). Each droplet gelled as the fungal preparation came in contact with the $CaCl_2$ solution and distinct beads formed (Plate 3.1a). After 20 minutes, the bead like pellets were separated from the solution by gentle filtration (Plate 3.1b), washed and air dried for 24 hours in flowing air at 25°C. The dried pellets measured 1-2mm in diameter (Plate 3.1c).

3.2.1.2 Seed Coating.

Five grams of ground wheat bran (<850μm) was placed in a 100ml beaker and autoclaved at 121°C and 15 psi for 25 minutes on two successive days. Once cooled, 0.25g of methyl cellulose and 25ml of inoculated fermentation medium (as prepared for the pellets) for both *T. harzianum* and *T. koningii* individual seed coatings and 12.5ml of both *T. harzianum* and *T. koningii* for the combination coating was added and mixed. Ten grams of onion seed was then added and the mixture was stirred to coat the onion seeds. The fungal coated seed mixture was spread out on sterile filter paper and placed in the laminar flow unit for 12 hours to dry (Plate 3.1d). Once dry, the seeds were separated by gentle rubbing.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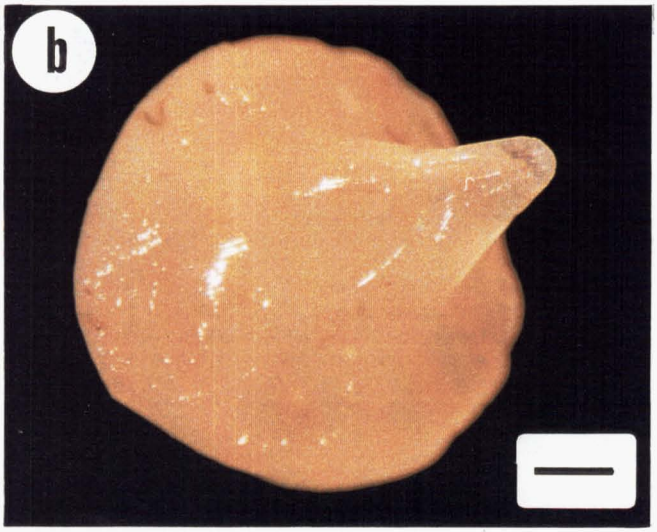
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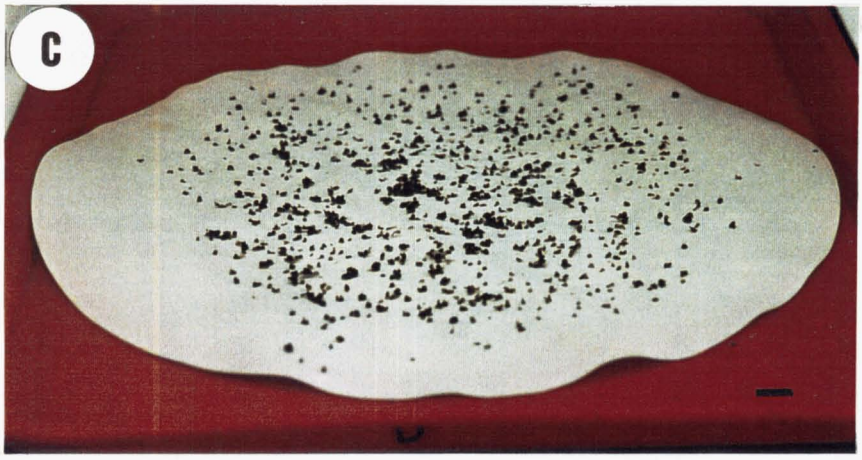
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A colony forming unit assay (Whipps *et al.*, 1989) was performed and $10cm^3$ of the pellets and coated onion seeds were added to 90ml of sterile 0.01% WA (Section 2.2.3.2). The number of colony forming units per $10cm^3$ was determined for both the pellet and seed coat formulations (Table 3.1).

3.2.1.3 Glasshouse Trial design.

Large polystyrene boxes, 59 x 33 x 19cm were half filled with shredded Wakanui silt loam soil. Sclerotia of *Sclerotium cepivorum* (Section 2.2.1) that had been preincubated in soil for one month, were incorporated into the shredded soil to give an inoculum concentration of one sclerotium/g soil (dry soil weight).

The trial was set up in a glasshouse and after 12 days, the trial was moved outside to a shaded area, in an effort to lower the soil temperature. The boxes were arranged in five replicate blocks of nine boxes to account for the variability associated with shading both within the glasshouse and outside. The boxes were placed in individual watering trays to avoid cross contamination and were watered three or four times a week, from the watering trays, for the duration of the trial, to maintain soil moisture. Weeding took place by hand.

Each box contained one treatment, with three rows of 25 seedlings (75 seedlings in each box) representing a single observation. One pellet/seed was placed adjacent to the seed in the planting furrow for the Trichopel treatment and two pellets/seed were placed adjacent to the seed in the planting furrow for the *T. harzianum, T. koningii* and *T. harzianum/T. koningii* pellet formulations. There were nine treatments; *T. harzianum* seed coating, *T. harzianum* pellet, *T. koningii* seed coating, *T. koningii* pellet, *T. harzianum/T. koningii* seed coating, *T. harzianum/T. koningii* pellet, Trichopel, pathogen control and a procymidone chemical (Sumisclex 25 1g a.i./100g seed) control. An uninoculated pellet control was not included as it would confound the results (Section 2.2.3).

Assessment.

Two and four weeks after seed sowing, seedling emergence was counted. Following this, the seedlings were examined at weekly intervals for 12 weeks for visible symptoms of white rot. Diseased seedlings were marked with coloured toothpicks, as previously described (Section 2.2.3). At the end of the trial, all remaining healthy seedlings were removed and below ground disease symptoms were recorded. The results for seedling emergence and

below ground disease symptoms were recorded. The results for seedling emergence and post emergence cumulative disease for each treatment were analysed using a Balanced ANOVA.

The fresh and dry weights of the remaining healthy seedlings after 12 weeks were also recorded for each treatment in each replicate. If there was no significant difference in emergence and disease control between the treatments, then the fresh and dry weights of the seedlings were analysed using a Balanced ANOVA to determine any plant growth effects from the *Trichoderma* spp.

3.2.2 **Field trial.**

A field trial was conducted at Lincoln University, Canterbury in a plot of Wakanui silt loam soil (7 x 15m) that had lain uncultivated for over one year. *S. cepivorum* sclerotia and the wheat grains (Section 2.2.1) were incorporated into the soil to a depth of 20cm at a concentration of 1 sclerotium/g soil, seven months before the plot was planted with onion seed. Before the start of the trial, ten 50g soil samples were randomly taken from the plot and combined. One hundred gram sub samples of the bulked soil sample were wet sieved through 850µm, 500µm and 250µm sieves to recover the sclerotia. The number of recovered sclerotia was calculated per 100g of soil to determine the actual population of sclerotia. Before planting, the plot was prepared in a manner similar to that of commercial onion production, that is typical weed control and soil preparation by rotary hoeing.

Onion seed (Pukekohe Long Keeper (Regular) May and Ryan) was pretreated with thiram (32g a.i./100g seed), to control damping-off. The seed was coated by adding 4.5ml of water for every 100g of seed and 1.5ml of water for every 10g of fungicide powder, to the dry onion seed. The onion seed was stirred until all the seed was wet. The fungicide was then added and stirred until the seed was evenly coated and dry (A. Stewart pers. comm.).

T. harzianum and *T. koningii* were applied to the planting furrow, with the onion seed as pellets, formulated by Agrimm Technologies. Formulations of *T. harzianum* and *T. koningii* were coated onto barley grains either individually or as a mixture. A CFU count was performed, where $10cm^3$ of each pellet formulation was added to 90ml of 0.01% WA (Section 2.2.3.2). The number of CFU/$10cm^3$ was calculated (Table 3.4).

The onion seed was sown in 1.5 m longitudinal rows (Figure 3.1). There were three longitudinal planting beds (1m x 15m) across the plot (7m x 15m). Within each planting bed were six onion seed beds separated by 0.8m guard row. Within each onion seed bed there were four planting rows which contained 75 seedlings per row at a spacing of 20mm and a depth of 20-40mm (Kay, 1991) (300 seedlings in each onion planting bed). Each onion planting bed contained one treatment of which there were six: *T. harzianum* pellet, *T. koningii* pellet, *T. harzianum/T. koningii* pellet, sterile pellet, procymidone chemical control (Sumisclex 25 1g a.i./100g seed), and onion seed treated only with thiram (32g a.i./100g seed) and each treatment was represented in each of the three longitudinal planting beds. Planting was made in early November and one pellet/onion seed was added to the planting furrow.

Assessment.

Seedling emergence was counted three and five weeks after sowing. White rot infected plants were counted at fortnightly intervals after the first emergence count. The trial continued through until January, when final counts were made on the total number of plants surviving per treatment. The results for seedling emergence and post emergence cumulative disease for each treatment were analysed using a Balanced ANOVA.

3.3 Results.

3.3.1 Glasshouse Trial.

The concentration of fungal inoculum for the seed coatings ranged from 1 x 10^5 CFUs/10cm^3 (*T. koningii*) to 4 x 10^5 CFUs/10cm^3 (*T. harzianum* and the *T. harzianum/T. koningii* combination) (Table 3.1). The pellet fungal counts for all but the Trichopel (1 x 10^5 CFUs/10cm^3) were zero (Table 3.1). Both *T. harzianum* and *T. koningii* had viable spores on the V-8 juice agar plates (Table 3.2). The variation in seed coat and pellet fungal concentrations had no significant treatment effect ($p \leq 0.05$) on seedling emergence and there was no significant replicate effect ($p \leq 0.05$) between any of the treatments (Appendix 3.2).

Above ground disease symptoms were scarce and many of the diseased seedlings were not diagnosed until harvest (Appendix 3.3). The pathogen control treatment had only 16% of the seedlings diseased with onion white rot (Table 3.3) and the *T. harzianum/T. koningii* seed coat application resulted in 5.4% of the seedlings diseased. There was no significant

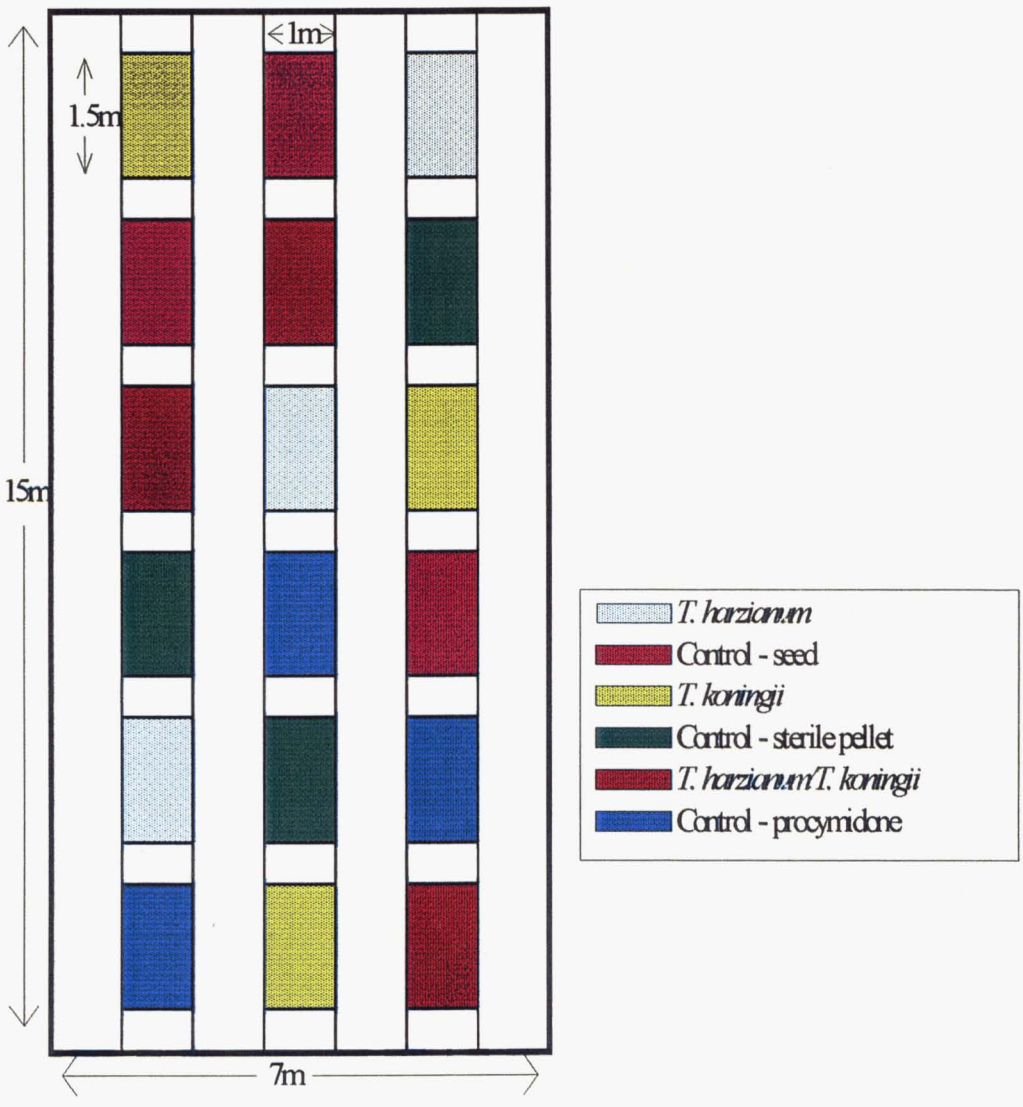


Figure 3.1 Field trial design layout.

Table 3.1 Colony forming unit counts from coated seeds and pellets for the glasshouse trial

Treatment	Seed coating	Pellet
T. harzianum	4×10^5	0
T. koningii	1×10^5	0
T. harzianum/T. koningii	4×10^5	0
Trichopel	-	1×10^5

Note: values are expressed as colony forming units/$10cm^3$.

Table 3.2 Colony forming unit counts before seed coat and pellet formulation (from V-8 juice agar plates) for the glasshouse trial

Treatment	Plates used for seed coat formulation	Plates used for pellet formulation
T. harzianum	6.3×10^5	1.0×10^6
T. koningii	3.0×10^5	3.5×10^5

Note: values are expressed as colony forming units/ml.

Table 3.3 The mean number of emerged and onion white rot diseased seedlings in the glasshouse trial for each treatment

Treatment	Emergence[1]	% Emergence	Disease[2]	% Disease[3]
Control-pathogen	56.8 ±1.77 a[4]	75.7	9.2 ±3.70 a	16.0
Control-procymidone	56.8 ±2.60 a	75.7	6.4 ±4.27 a	11.2
T. harzianum seed coat	49.8 ±4.16 a	66.4	12.0 ±4.39 a	24.0
T. harzianum pellet	55.8 ±1.08 a	74.4	14.6 ±4.61 a	26.0
T. koningii seed coat	54.4 ±3.70 a	72.5	14.4 ±6.54 a	26.0
T. koningii pellet	57.0 ±2.35 a	76.0	9.8 ±5.30 a	17.0
T. harzianum/T. koningii seed coat	48.2 ±3.25 a	64.3	2.6 ±1.44 a	5.4
T. harzianum/T. koningii pellet	54.6 ±3.25 a	72.8	11.2 ±2.03 a	20.5
Trichopel	58.0 ±0.95 a	77.3	5.4 ±1.81 a	9.3

[1] The mean number of seedlings emerged for each treatment four weeks after planting. n = 75.
[2] The mean number of post emergence diseased seedlings for each treatment after 12 weeks.
[3] Percentage of diseased seedlings relative to the number of emerged seedlings for each treatment.
[4] Mean values followed by the same letter do not differ significantly ($p \leq 0.05$) according to a LSD test.

difference between treatments ($p \leq 0.05$) and no significant replicate effect in the number of diseased seedlings (Appendix 3.2).

There was a large variation in the fresh and dry weights of onion plants recorded for each box (Appendix 3.4), however, there was no significant effect on fresh and dry weights by treatment and no significant replicate effect ($p \leq 0.05$) (Appendix 3.4).

3.3.2 **Field Trial.**

An average of nine sclerotia were recovered in 100g of soil, indicating that the actual concentration of sclerotia was closer to 0.1 sclerotium/g of soil, rather than 1 sclerotium/g of soil (Appendix 3.5). The concentration of fungal inoculum for the commercially prepared pellets ranged from 2×10^5 CFUs/10cm^3 (*T. koningii*) to 6×10^5 CFUs/10cm^3 (*T. harzianum*) (Table 3.4). Onion seed emergence ranged from 52.4 to 86.5% after five weeks (Table 3.5). The addition of *T. harzianum*/*T. koningii* pellets (5.3×10^5 CFUs/10cm^3) resulted in a significantly greater ($p \leq 0.05$) seedling emergence than all other treatments (Table 3.5) (Appendix 3.6).

Only one onion white rot diseased seedling was recorded (in one of the *T. harzianum* treated plots) over the period of the trial and therefore no statistical analysis was performed.

3.4 Discussion.

The glasshouse trial was established while the CFU assay (Section 2.2.3.2) was being performed and the lack of viable inocula in the pellets was not discovered until after planting. There were a number of possible explanations for the loss of viability in the pellets. Firstly, during pellet formulation, it was noted that on occasions the pipette tip became blocked by bran flakes. This could have prevented the fungal biomass containing viable inoculum from passing into the pellets. Secondly, the pH of the gellant solution was recorded as pH 11 and hydrochloric acid (HCl) had to be added to obtain the recommended pH of 5.4 (Lewis & Papavizas, 1985a). This excess acid could have adversely affected the viability of the fungal biomass. The pellet applications should have resulted in a high number of diseased seedlings, since no viable inoculum was actually added. Instead, disease levels were low for all treatments. The seed coats of *T. harzianum* and *T. koningii* had a higher CFU count per 10cm^3 than in the first glasshouse trial, hence some reduction in the number of diseased seedlings would have been expected, since viable inocula was present.

Table 3.4 Colony forming unit counts from commercially prepared pellets for the field trial

Pellet Formulation	CFU/10cm^3
Sterile pellet	-
T. harzianum	6 x 10^5
T. koningii	2 x 10^5
T. harzianum/T. koningii	5.3 x 10^5

Table 3.5 Mean number of emerged seedlings in the field trial for each treatment

Treatment	Emergence[1]		% Emergence
Control-untreated seed	173.7 ±2.33	b[2]	57.9
Control-procymidone	191.3 ±12.78	b	63.7
T. harzianum pellet	157.3 ±8.95	b	52.4
T. koningii pellet	184.3 ±16.76	b	61.4
T. harzianum/T. koningii pellet	259.7 ±20.42	a	86.5
Sterile pellet	190.3 ±37.25	b	53.4

[1] Mean number of seedlings emerged for each treatment five weeks after planting. n = 300.
[2] Mean values followed by the same letter do not differ significantly ($p \leq 0.05$) according to a LSD test.

The control provided by the seed coating treatments was not significantly greater than the pellet treatments and this lack of significance was influenced by the large variation in the number of diseased seedlings between the replicates.

The low number of diseased seedlings recorded in both trials was unusual and disappointing, however, factors such as temperature, soil moisture and sclerotial health could have adversely affected disease development. Temperature could have influenced the amount of disease. In the glasshouse trial, the seedlings spent the first 12 days after planting in the glasshouse, where the average air temperature was 22°C and the soil temperature ranged from 17.3 to 23°C. The optimum sclerotial germination temperature is 13-18°C (Asthana, 1947). These higher temperatures could have impeded disease development.

A high level of moisture was maintained in the soil boxes, in an effort to lower the soil temperature and encourage disease. This excess moisture could have caused pre-emergence damping-off, which could explain the low seedling emergence (54.6%). Alternatively, the excess moisture may have adversely affected the health of the sclerotia. The detrimental effect of moisture on sclerotial viability is well documented (Coley-Smith & Cooke, 1971; Smith, 1972c; Crowe & Hall, 1980b; Legget & Rahe, 1985).

Five hundred micrometre and 250μm diameter sclerotia with an average of 62% viability were used in the glasshouse trial. The combination of two sizes of sclerotia with a lower than normal viability could not be helped, because the sclerotia initially conditioned for use became contaminated and two sizes had to be combined to provide enough inoculum to give one sclerotium/g of soil. The smaller sclerotia may not have been able to withstand environmental conditions such as excess moisture as well as the larger sclerotia, and the number of viable sclerotia in the soil may, therefore, have been reduced.

The sclerotia used in the first glasshouse trial were incubated for two and a half months and had 82% viability and the sclerotia used in this trial were incubated for one month and had 62% viability. Thus, the length of the conditioning period could also have influenced the ability of the sclerotia to respond to onion exudates and germinate. The length of the conditioning period is of major importance in breaking constitutive dormancy. From this

work, it appears that a conditioning period of one month is insufficient to enable maximal germination.

In the field, sufficient sclerotia were added to ensure one sclerotium for every gram of soil, however, sampling before planting indicated a much lower concentration. The sclerotia were incubated in the soil for seven months prior to planting. The long incubation period may have affected the viability of the sclerotia and although sclerotia can remain viable for considerable lengths of time (Coley-Smith, 1959; Coley-Smith *et al.*, 1990), there have been reports of substantial losses of sclerotial viability after periods of two years (Legget *et al.*, 1983) or even after periods as short as three months (Fullerton *et al.*, 1994). The lower number of viable sclerotia is likely to have influenced disease development in the field.

The main factors influencing the low disease in the glasshouse trial would have been: the health of the sclerotia, the excess moisture and the initial high temperatures. In the field, the number of viable sclerotia were reduced by reasons unknown. This reduction most probably contributed to the lack of disease. The time of planting and the environmental conditions were marginal for disease development and also may have influenced the onset of disease.

The glasshouse and field trials would be worthwhile repeating as no significant treatment effects were determined. The development of an improved seed coating procedure would also be beneficial to ensure a greater concentration of viable inocula, was introduced into the soil.

~CHAPTER FOUR~

THE EFFECT OF SOIL TEMPERATURE ON SCLEROTIAL VIABILITY

4.1 Introduction.

Applying heat by solarisation to *S. cepivorum* infested soil is a cheap, non hazardous method to reduce or eliminate the number of viable sclerotia. Increases in soil temperature also reduced the number of viable *Sclerotinia minor* (Jagger), *S. sclerotiorum* and *Sclerotium rolfsii* (Saccardo) sclerotia (Mihail & Alcorn, 1984; Vannacci *et al.*, 1988; Phillips, 1990). While the use of polythene to heat the soil (solarisation) is not a new application, within New Zealand its use has been limited. New Zealand has a relatively cold climate compared to Egypt and Israel, where soil solarisation is a common practice to partially sterilise the soil. However, in Canterbury, a soil solarisation study was conducted to control weeds in brassica crops (Alexander, 1990). The temperatures recorded under polythene, when correlated with data from overseas research on loss of sclerotial viability with increased temperature (Porter & Merriman, 1983; Adams, 1987), prompted the idea that summer temperatures in Canterbury may be high enough to reduce the viability of *S. cepivorum* sclerotia.

The objectives of these studies were to determine the diurnal range of soil temperatures recorded under polythene in Canterbury and to determine the effect of temperature on sclerotial viability using *in vitro* studies. The results of these studies determined the value of a soil solarisation trial aimed to reduce *S. cepivorum* sclerotial viability.

SECTION ONE

4.2 PRELIMINARY SOIL SOLARISATION TRIAL.

4.2.1 Methods and Materials.

The trial was conducted at the Field Service Centre, Lincoln University, Canterbury in Wakanui silt loam soil for four weeks (20th Dec 1994-16th Jan 1995). The plots (3m x 3m) were arranged in a block of four separated on all sides by a 0.5m guard row. The plots were irrigated to saturation and on the following day (Mihail & Alcorn, 1984) transparent polythene (PE) (Permathane Plastics) 50μm thick (Satour *et al.*, 1989; Duff & Connelly, 1993) was laid over two of the four plots (Plate 4.1). The edges of the polythene were

Plate 4.1 Preliminary soil solarisation field trial design layout.

buried in the soil (Alexander, 1990) to a depth of 10cm. The uncovered plots were hand irrigated once a week and weeds were removed by hand.

Temperature sensors (Phillips KTY83-110) encased in stainless steel tubes were placed in the soil at two depths (10cm and 20cm) in each of the four plots. The soil temperature was recorded every three hours using a data logger (Datataker DT600) for the duration of the trial.

Analysis.

The maximum and minimum soil temperatures for each plot were analysed using a Balanced ANOVA with depth and solarised or non solarised as the variables.

4.2.2 **Results.**

The maximum soil temperatures were significantly different ($p \leq 0.05$) between solarised and non solarised soil but the minimum temperatures were not (Appendix 4.1). The maximum temperatures recorded at both 10 and 20cm were significantly different ($p \leq 0.05$), whereas the minimum temperatures were only marginally significant between the two depths (Appendix 4.1). There was no significant interaction between the solarisation treatment and the depths at which the maximum and minimum temperatures were recorded. The mean daily maximum and minimum temperatures for both solarised and non solarised soil at 10 and 20cm are presented in Figures 4.1 and 4.2. The highest temperatures were recorded at a depth of 10cm under polythene and there was a much greater fluctuation in the daily maximum temperature than at any other depth. The temperature at 10cm under solarised conditions ranged from 12 to 44°C compared to 9 to 28°C under non solarised conditions at the same depth. The average soil temperature in the solarised soil was 27.2°C compared to 20.3°C in the non solarised soil. The maximum and minimum temperatures at 20cm under polythene, were higher than the minimum temperatures recorded at 10cm under polythene. The total number of hours in solarised soil, when temperatures above 20 and 30°C were recorded at 20cm was 1296 and 333 respectively, compared to 1260 and 519 at 10cm. In non solarised soil, the total number of hours when temperatures above 20 and 30°C were recorded at 20cm was 414 and 0 respectively, compared to 561 and 72 at 10cm. (Table 4.1). This data clearly shows that higher temperatures were reached and maintained for longer periods of time at 10cm than at 20cm in both solarised and non solarised soil, the exception being the number of hours above 20°C at 20cm in solarised soil. This increase

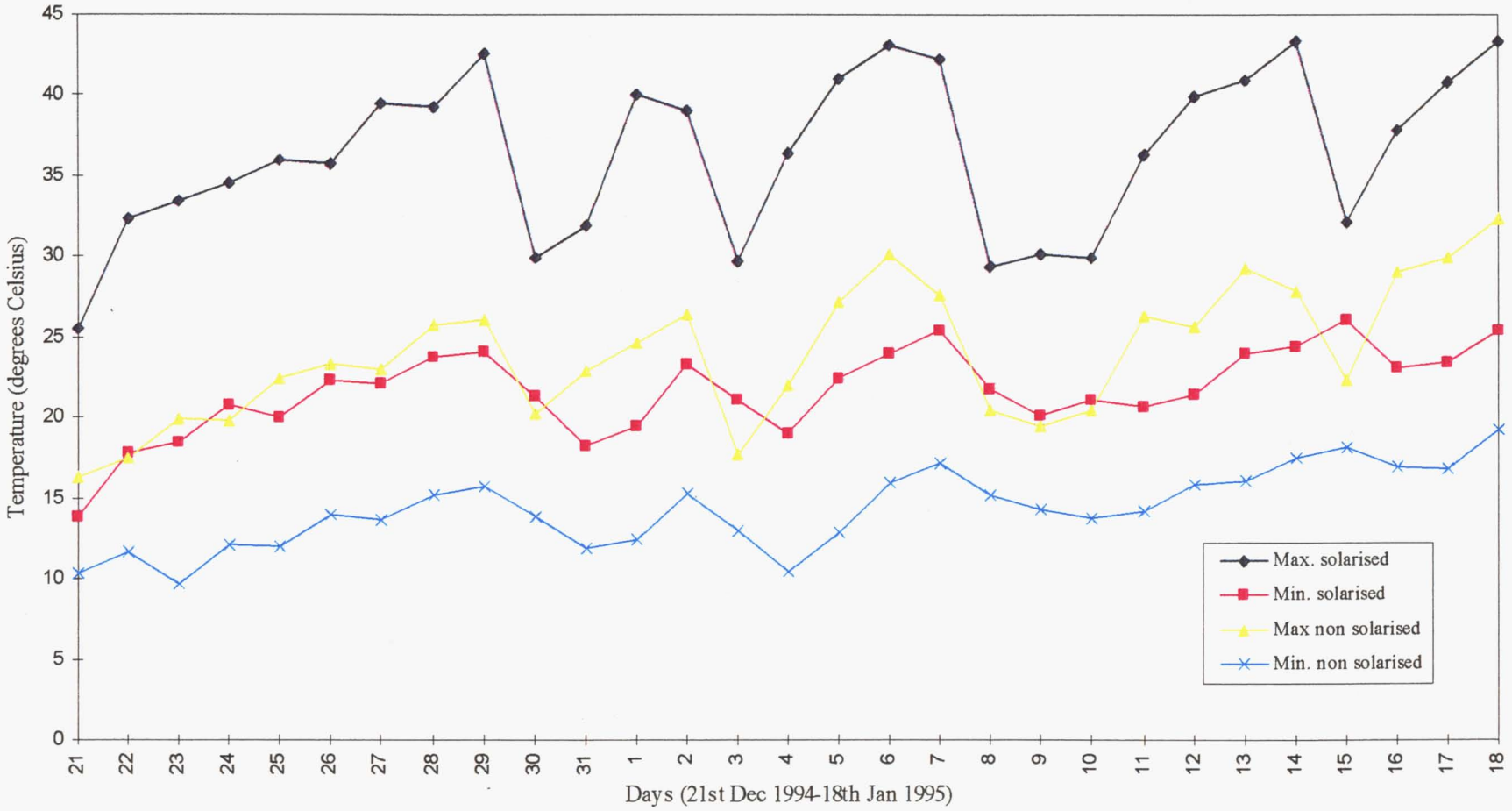


Figure 4.1 The mean maximum and minimum temperature recorded each day from solarised and non solarised soil at 10cm, preliminary soil solarisation trial.

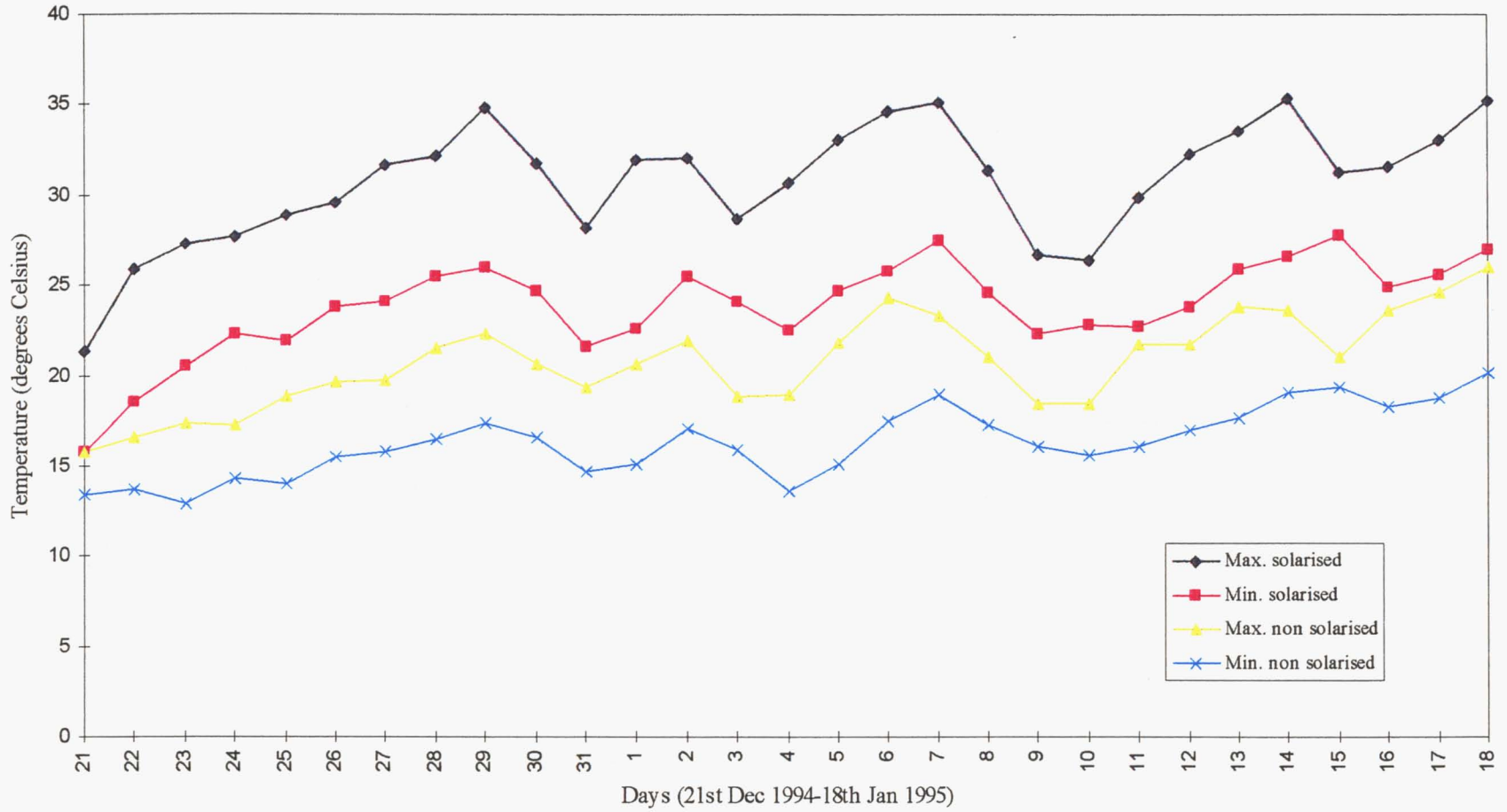


Figure 4.2 The mean maximum and minimum temperature recorded each day from solarised and non solarised soil at 20cm, preliminary soil solarisation trial.

Table 4.1 Approximate total number of hours spent above selected temperatures at 10 and 20cm in solarised and non solarised soil

Temp.	Solarised		Non solarised	
	10cm	20cm	10cm	20cm
>40	96	-	-	-
>35	246	18	-	-
>30	519	333	72	-
>20	1260	1296	561	414

Note: The hours are only approximate values, as the nature of the data does not provide enough detail to determine the exact number of hours spent at each temperature.

was mainly influenced by the temperatures recorded between 20 and 30°C (Table 4.1). The number of hours recorded above 20°C is most probably an over estimate in that the temperature was not recorded often enough to gain an accurate number of hours.

4.2.3 **Discussion.**

The large fluctuations in maximum temperatures at 10cm in the solarised plots would be expected as the top fraction of the soil is exposed to extremes of heat and cold and would be more sensitive to small changes in air temperature. The temperature fluctuations could be important for reducing sclerotial viability in the upper portions of the soil (Kye & Kim, 1985). The minimum temperature is higher at 20cm soil depths, than at 10cm depths, as the soil retains more heat and would not be influenced by small air temperature fluctuations.

The solarisation treatment raised the average soil temperature by 7°C, compared to the non solarised soil. The temperature values obtained for the solarised plots are probably under estimates of the temperatures that could be obtained with improved methods. The saturation of the soil made hand laying of the polythene very difficult. In a commercial situation, the polythene would be laid using specialised equipment that would ensure a level soil surface and a minimum of air between the polythene and the soil. Thus, an increase in maximum temperatures under solarisation would be anticipated. In Australia, the average maximum and minimum soil temperature was 33.7 and 24.0°C respectively, under polythene at a depth of 15cm, which represented a reduction of 65% in *S. cepivorum* sclerotial viability (Porter & Merriman, 1983). These temperatures compared favourably with the temperatures recorded at the 10cm depth under polythene. Sclerotial viability was also reduced at temperatures greater than 20°C in abnormally dry or moist soils (Crowe & Hall, 1980b).

Temperature has a major effect on sclerotial viability. The interactions of soil temperature with soil moisture, composition and microflora are also important in reducing sclerotial viability. These interactions are complex and difficult to examine together, therefore a laboratory based study examining the effect of a range of temperatures on sclerotial viability was devised. The sclerotia were incubated at temperatures ranging from 20 to 50+°C, these temperatures represented those recorded in the field, with the exception of 50°C which, although not recorded, was considered to be obtainable under optimised solarisation conditions.

SECTION TWO

4.3 THE INFLUENCE OF TEMPERATURE ON SCLEROTIAL VIABILITY.

4.3.1 Methods and Materials.

Fresh sclerotia (Section 2.2.1) were counted into lots of 100 and each lot was placed in a polyester mesh (URE Pacific) bag (85µm pore size, 8cm x 8cm) with a marker attached for location purposes (Plate 4.2) (Coley-Smith, 1985; Alexander, 1992).

Liver pails (18cm x 18cm x 19cm) were filled to within 10mm of the top with shredded, unsterile Wakanui silt loam soil. Three bags of sclerotia were selected randomly and buried randomly in each liver pail at a depth of 8cm. Seven liver pails were maintained at each of the following temperatures: 20, 30, 35, 40, 45 and 50+°C. The range of temperatures chosen, was similar to those used by other researchers (Porter & Merriman, 1983; Adams, 1987). A control treatment was also included where sclerotia were incubated at 20°C in a glass vial. The actual soil temperature in the liver pails at a depth of 8cm was periodically monitored (Appendix 4.2), using a soil temperature probe, to ensure stable temperatures within each pail. The average moisture content was maintained at 25% to simulate field conditions. The pails were weighed with a moisture content of 25% and water was added throughout the trial to maintain an approximate 25% soil moisture content by pail weight. There were seven assessment times: 0.25, 0.5, 1, 2, 8, 16 and 28 days after the start of the experiment for each temperature, at which three bags of sclerotia from each temperature, at each time, were selected randomly and assessed.

Assessment.

The sclerotia were surface sterilised in 0.25% NaOCl for one minute, washed in five changes of SDW and blotted dry on sterile Whatman No. 1 filter paper. The sclerotia were then placed individually using sterile forceps onto isolated PDA droplets in Petri dishes. The plates were sealed with polythene wrap and incubated in the dark at room temperature (18-20°C). Viability was initially determined on the ability of the sclerotia to germinate, and germination was recorded every second day for ten days by observation under the stereo microscope. The percentage of sclerotia which germinated was recorded and the sclerotia which had not germinated after ten days were surface sterilised again, cut into halves and examined. If the medulla was white and healthy, the sclerotia were classed as viable.

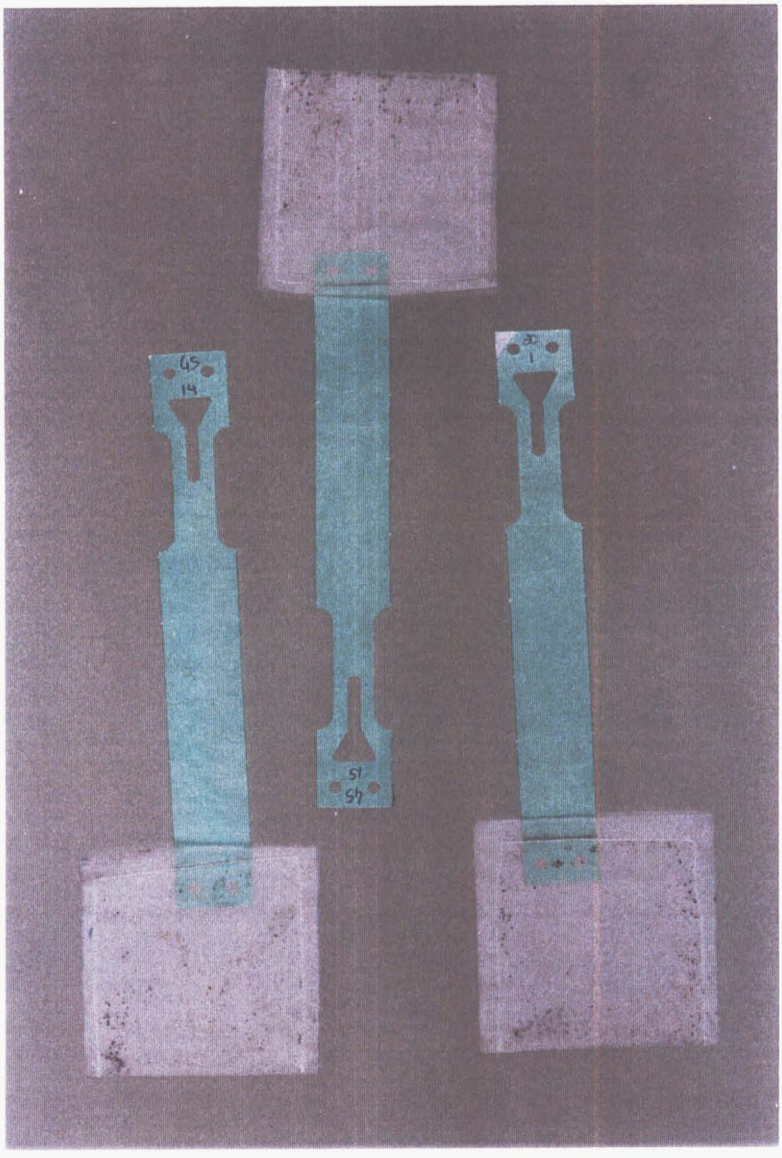


Plate 4.2 Polyester mesh bags containing *Sclerotium cepivorum* sclerotia.

The results were analysed using a Balanced ANOVA with the variables of temperature and length of exposure period. A probit analysis was performed to determine the temperature required at each exposure period to reduce sclerotial viability by 95% (LD_{95}). The data was converted to thermal days to compare the effects of short exposure periods at higher temperatures with longer exposure periods at lower temperatures on sclerotial viability. The effect of base temperatures on sclerotial viability was also determined, by subtracting temperatures of 10, 20, 25 and 30°C from the temperature before calculating the thermal day period.

4.3.2 **Results.**

There was a significant difference ($p \leq 0.05$) in sclerotial viability, when the sclerotia were incubated at different temperatures and also after varying lengths of exposure periods (Appendix 4.3). There was also a significant interaction between the temperature and the length of exposure period. There was no significant reduction ($p \leq 0.05$) in the number of viable control sclerotia, recovered over the 28 day period with 88-100% viability recorded (Table 4.2). At 20°C, there was no significant reduction ($p \leq 0.05$) in sclerotial viability after soil incubation for 0.25, 0.5 and 8 days. Unusually, a significantly greater number of viable sclerotia were recovered after 8 days soil incubation compared to after 1 day. Viability was significantly reduced after 28 days incubation with only 10.7% of the sclerotia remaining viable. At 30°C, sclerotial viability was significantly reduced to 46% after incubation for 1 day in the soil. None of the sclerotia were viable after 16 days. The loss in sclerotial viability at 30°C was likely to be an under estimate as the incubator could not consistently maintain 30°C for the duration of the experiment. At 35°C, there was no significant difference in the number of viable sclerotia recovered after 0.25 and 0.5 days incubation. Viability was significantly reduced to 32% after 1 day and after 8 days the percentage viability was zero. Six hours incubation at 40°C was sufficient to significantly reduce sclerotial viability to 31% with a further reduction to 0% after 1 day. This trend continued with increasing temperature. At 45°C, 13.7% of the sclerotia were viable after 0.25 days in the soil and after 0.5 days sclerotial viability was reduced to zero. At 50°C sclerotial viability was lost within the first 0.25 day incubation period.

Higher temperatures were required at shorter exposure periods to reduce sclerotial viability by 95% (LD_{95}) (Table 4.3). Temperatures greater than 40°C were required for periods of

Table 4.2 The mean number of viable *Sclerotium cepivorum* sclerotia recovered from a range of temperatures after selected exposure periods

Temp.	Days													
	0.25		0.5		1		2		8		16		28	
cont.[1]	100.0	a[2]	95.0	a	88.3	a	93.0	a	91.0	a	98.7	a	98.0	a
20°C	100.0	a	96.7	a	58.3	cd	70.3	bc	84.0	ab	47.0	de	10.7	gh
30°C	90.3	a	99.0	a	46.0	e	27.0	fg	36.0	ef	0	h	0	h
35°C	97.0	a	100.0	a	32.0	ef	33.7	ef	0	h	0	h	0	h
40°C	31.0	ef	3	h	0	h	2.3	h	0	h	0	h	0	h
45°C	13.7	gh	0	h	0	h	0	h	0	h	0	h	0	h
50°C	0	h	0	h	0	h	0	h	0	h	0	h	0	h

[1] cont. = control, sclerotia stored in a glass vial at 20°C in the dark.

[2] Mean values within each column and across each row, followed by the same letter do not differ significantly ($p \leq 0.05$) according to a LSD test. n=100.

Table 4.3 Temperature required at selected exposure periods to reduce *Sclerotium cepivorum* sclerotial viability by 95%

Exposure period (Days)	Temperature (°C)
0.25	47.4
0.5	45.2
1	41.9
2	40.7
8	35.0
16	24.8
28	24.3

up to 2 days to reduce sclerotial viability to 5%. In contrast, LD_{95} temperatures of 24.8 and 24.3 °C were required at 16 and 28 day exposure periods, respectively.

Thermal day conversions for the temperature data are presented in Table 4.4. Equivalent thermal day periods do not result in equivalent losses in sclerotial viability. Sclerotial viability was affected more by exposure to high temperatures for short periods of time than lower temperatures for longer periods of time. For example, after exposure to 35°C for 8 days, sclerotial viability was reduced to 21% but only to 52% after exposure to 20°C for 14 days, for the same thermal day treatment (280). Similarly, after 2 days at 40°C (i.e. 80 thermal days), only 3% of the sclerotia remained viable, whereas 79% of the sclerotia remained viable after 4 days at 20°C.

Equivalent losses in sclerotial viability were evident at different thermal day periods (Table 4.5). For example 20% of the sclerotia remained viable after 345 and also after 157.5 thermal days. The subtraction of selected base temperatures resulted in a similar number of thermal days at base temperatures of 20-25°C. A base temperature of 20-25°C indicated that temperatures of 20-25°C and above adversely affected sclerotial viability. Temperatures less than 20°C had little effect on sclerotial viability.

A selection of germinated sclerotia are shown in Plate 4.3. A number of fungal species were observed to colonise the ungerminated sclerotia (Plate 4.4). These colonised sclerotia had been incubated in the soil for 8 days or longer. After 16 and 28 days at 20°C, some of the ungerminated sclerotia were colonised with isolates of *Mucor*, *Trichoderma* and three unidentified bacterial species. At 30°C, a variety of fungal species colonised the sclerotia (Plate 4.4a,b), these were mainly isolates of *Trichoderma*, however, species of *Fusarium*, *Gliocladium*, *Verticillium* and an isolate of *Paecilomyces lilacinus* (Thom) Samson were also evident. At 35°C, all the ungerminated sclerotia were contaminated with one Mucoracious fungal species (Plate 4.4c) and at 40°C and 45°C, an isolate of *Aspergillus* contaminated all of the ungerminated sclerotia (Plate 4.4d). At 50°C, none of the ungerminated sclerotia were contaminated.

4.3.3 Discussion.

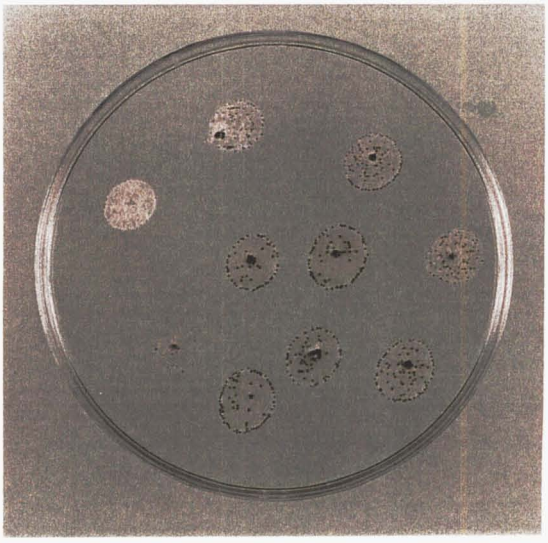
An increase in soil temperature caused a decrease in sclerotial viability. The sclerotia readily decayed when incubated for short periods at temperatures above 40°C. Adams

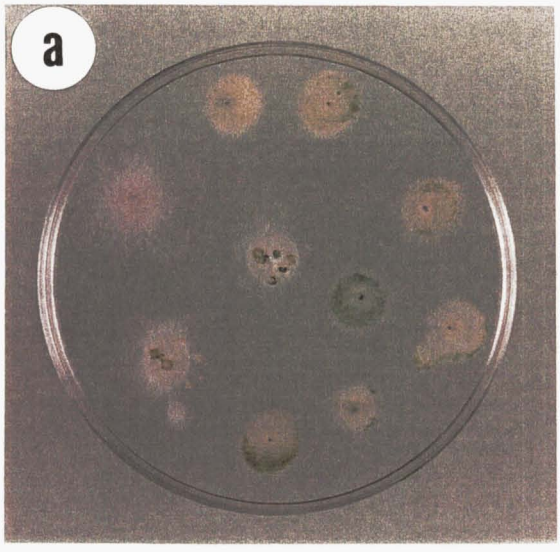
Table 4.4 The number and percentage of viable *Sclerotium cepivorum* sclerotia at selected thermal days

°C	Days	Thermal Days	Number of viable sclerotia	Percentage of viable sclerotia
20	14	280	156	52
35	8	280	62	21
20	4	80	238	79
40	2	80	8	3
20	10.5	210	186	62
30	7	210	110	37
35	6	210	69	23
20	16.5	330	135	45
30	11	330	90	30

Table 4.5 The number of thermal days at selected base temperatures for thermal day periods with equivalent losses in *Sclerotium cepivorum* sclerotial viability

% viable Sclerotia	°C	Days	Thermal Days	Thermal days Base temperatures (°C)			
				10	20	25	30
20	30	11.5	345	230	115	57.5	0
20	35	4.5	157.5	112.5	67.5	45	22.5
1	30	16	480	320	160	80	0
1	40	5	200	150	100	75	50
3	20	30.5	610	305	0	-	-
3	40	0.5	20	15	10	-	-
46	20	16	320	160	0	-	-
46	30	2	60	40	20	-	-

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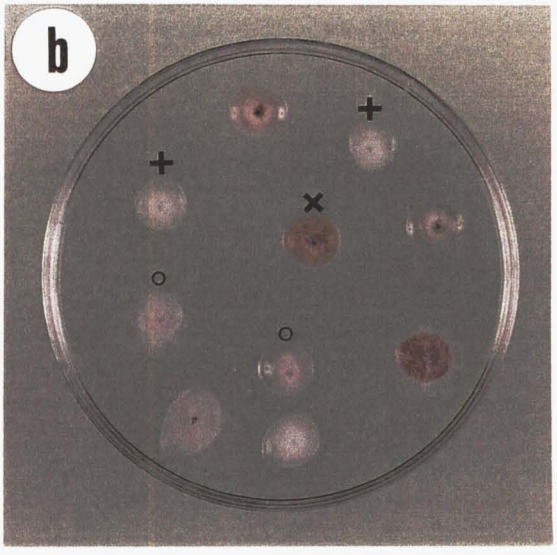
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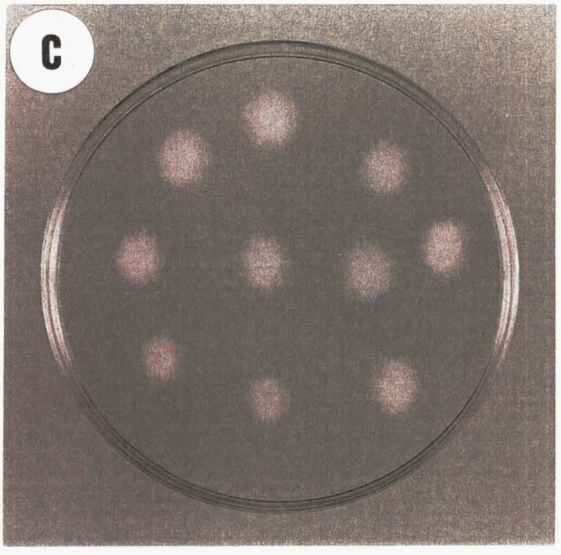
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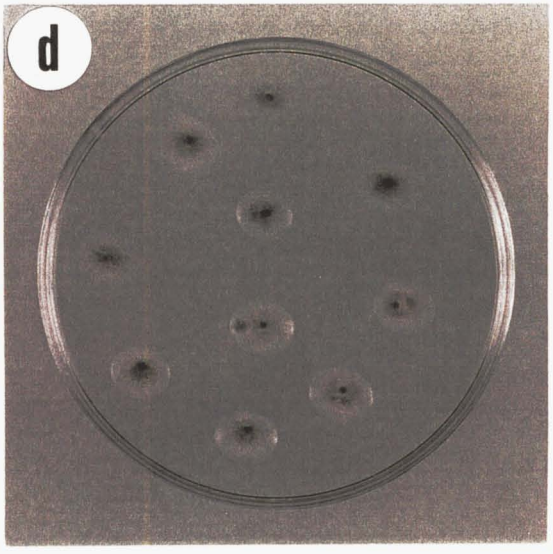
d

(1987) reported that *S. cepivorum* sclerotia were most sensitive to temperatures of 40-50°C. The equivalent thermal day data indicated that although higher temperatures maintained for short periods of time may have the same number of thermal days as lower temperatures maintained for longer periods, the resulting number of viable sclerotia is not equivalent, with the former treatment being more effective in reducing sclerotial viability. This has important implications in a field situation, in that higher temperatures must be reached if a rapid decrease in the number of viable sclerotia is to occur. In Canterbury, temperatures greater than 40°C can be reached at depths of 10cm (Figure 4.1), however, achieving higher temperatures at 20cm is likely to be more important as far as disease control is concerned because the onion roots grow deep in the soil and can stimulate sclerotial germination at lower depths. Crowe and Hall (1980a) reported onion roots to grow 16cm in 4.5 weeks. The preparation of the soil for onion sowing also brings viable sclerotia from greater depths to the surface.

Temperatures above 40°C are lethal to sclerotia. Losses in viability at higher temperatures were most probably a direct effect of temperature. Sublethal temperatures and fluctuating temperatures are reported to weaken the sclerotia and increase leakage of organic substances, leading to stimulated reproduction of soil microorganisms and increasing sclerotial vulnerability to antagonistic microflora (Smith 1972b; Katan *et al.*, 1976; Lifshitz *et al.*, 1983). A field trial conducted in New Zealand, reported less than 15% sclerotial recovery when buried in soil for 6 months at temperatures ranging between 9.1 and 22.1°C (Alexander, 1992). In this study, at 20 and 30°C, sclerotial viability was reduced to 10.7 and 0% after 28 and 16 days respectively, and the LD_{95} temperatures for 28 and 16 day exposure periods are 24.3 and 24.8°C respectively. In the field, the minimum soil temperature at a depth of 20cm under polythene, remained above 20°C for 26 continuous days (Figure 4.2). Temperatures above 24°C were maintained only for three continuous days. The minimum temperature at 20cm in untarped soil never reached 20°C. When correlated with the incubator trial results, this suggests that under polythene, temperatures of 24°C are needed to reduce sclerotial viability by 95% in four weeks at the 20cm depth. Alternatively, temperatures of 20 to 24°C could reduce sclerotial viability by 95% if a longer solarisation period was used. Contrary to these results, Adams (1987) reported 88-100% sclerotial viability at 25 and 30°C after 30 days of continuous heating in soil. Although temperatures above 30°C were reached at lower depths in the soil solarisation study, the longest period of constant heat was approximately 12 hours, which would bring

about only a very small reduction in sclerotial viability. Constant temperatures are rarely found in the field and it is likely that the temperature fluctuations will influence sclerotial viability (Kye & Kim, 1985). In an Australian study (Porter and Merriman, 1983), sclerotia were incubated in soil at 40 and 45°C for 6 hours, the temperature was then decreased to 25°C and maintained for 18 hours. The sclerotia were incubated in this fashion for two weeks to simulate field conditions. After two weeks, 81 and 2% of the sclerotia remained viable at 40 and 45°C, respectively. These results indicated that fluctuations in temperature are only effective at high temperatures. Temperature fluctuations in the field may vary between regions and would be related to environmental factors.

The influence of moisture, soil composition and soil microflora would be important in reducing sclerotial viability at sublethal temperatures as temperatures would not be high enough to directly affect sclerotial viability. The control treatment sclerotia, stored in a glass vial, maintained a high level of viability at 20°C for 28 days. In contrast, when the sclerotia were incubated at the same temperature for 28 days in soil, the viability decreased by 90%. Soil moisture may be a contributing factor. Whilst the liver pails were maintained at approximately 25% soil moisture capacity, the soil at higher temperatures was prone to drying out. Soils with a low water availability do not affect the germination and survival of *S. cepivorum* sclerotia (Coley-Smith *et al.*, 1974), nor do dry soils promote sclerotial decay (Papavizas, 1977). Hence, the effect of overdry soils probably did not influence sclerotial viability. The 24 hour air drying period before experimental use, did not affect sclerotial viability in this study as the viability remained above 80%. The detrimental effect of drying and rewetting on sclerotial viability has been documented (Smith, 1972a; Papavizas, 1977; Sunder *et al.*, 1990), however, wetting and drying cycles would be more influential in the field. This loss of viability at a constant moisture may be an under estimate of the losses obtained in the field as wet/dry cycles would contribute to sclerotial degradation.

Another factor influencing the health of the sclerotia would be the composition of the soil, the addition of clay or organic matter could alter the transmittance of heat through the soil due to the greater retention of water. Clay soils usually contain higher moisture levels than other soils, Alexander (1992) used a Patumahoe clay loam soil, this could explain the experienced 85% loss of sclerotial viability after 6 months incubation. The pH level of the soil could also affect the viability of the sclerotia when combined with heat. Although no reduction in sclerotial viability was recorded when the sclerotia were incubated for 21

months in pH 4.2-7.2 soil (Coley-Smith, 1959), the application of heat could alter the effect of pH. For example, 96% of buried sclerotia incubated at 35°C in pH 6.4 soil remained viable after 32 days (Adams, 1987), however, in pH 5.1 soil, 0% of the sclerotia maintained at 35°C were viable after 28 days, in this study. The loss of sclerotial viability could be the result of a direct effect of pH or perhaps the effect could be indirect, through the altered composition of the soil microflora in response to pH.

The natural soil microflora could also affect sclerotial viability. Fungal species are frequently isolated from soil, hence the appearance of fungi from heated soils is not unusual. At 40 and 45°C, the dominant fungal species was *Aspergillus*, while nonpathogenic towards onion, propagule suspensions of *Aspergillus candidus* Link. have reduced onion white rot in glasshouse trials (Abd-El-Razik *et al.*, 1985). Research examining the effect of soil heating on microflora populations, found that *Aspergillus* spp. survived heat treatment and populations often increased (Tjamos & Paplomatas, 1988; Dwivedi, 1991). *Aspergillus* spp. are not able to decay intact sclerotia, however, *Aspergillus* spp. are commonly associated with decayed sclerotia (Phillips, 1990). Phillips (1990) also suggested that *Aspergillus* spp. may be able to colonise heat exposed sclerotia as they are in a weakened state.

Isolates of *Trichoderma*, *Fusarium*, *Penicillium* and *P. lilacinus* were also among those fungal species isolated from the incubated sclerotia. *Trichoderma* spp. are known antagonists of *S. cepivorum* (Abd-El-Moity & Shatla, 1981; Harrison & Stewart, 1988; Kay & Stewart, 1994), and *Penicillium* spp. have been reported to control *S. cepivorum in vitro* (Ghaffar, 1969a; Utkhede & Rahe, 1980). Moubasher *et al.* (1970) isolated species of *Trichoderma, Fusarium* and *Penicillium* from *S. cepivorum* sclerotia and isolates of *Penicillium* and *Paecilomyces* from the soil surrounding the roots of infected onions. It is questionable what role the fungal colonists have in the decline in sclerotial viability. This assay could not determine whether the sclerotial colonists had penetrated the sclerotia or were present only on the surface of the sclerotial rind and it is possible that certain fungal species could have survived the sterilisation procedure. The loss of sclerotial viability due to fungal contaminants in this experiment could be an over estimate since any fungi present on the surface of the sclerotia could have overgrown *S. cepivorum* on the PDA droplet.

The results from both the preliminary soil solarisation trial and the effect of temperature on sclerotial viability experiment indicated that soil solarisation could be a viable control option for onion white rot and a further trial is worthy of investigation. Although lethal temperatures would rarely be reached in Canterbury, the detriment of sublethal temperatures to sclerotia has been established. The stimulation of natural populations of soil microorganisms by heat is also an aspect to be further studied. *Aspergillus* spp. and *Trichoderma viride* have been shown to tolerate adverse temperature conditions (Pullman *et al.*, 1984 in Dwivedi, 1991). These species would be worthy of further investigation to determine the potential for integration of heat tolerant microorganisms into a soil solarisation programme to control onion white rot.

SECTION THREE

4.4 SOIL SOLARISATION.

4.4.1 Methods and Materials.

The trial was conducted at the Biological Husbandry Unit, Lincoln University, Canterbury on Wakanui silt loam soil, that was artificially infested with *S. cepivorum* sclerotia.

S. cepivorum sclerotia both fresh and conditioned (Section 2.2.1) were counted into lots of 50 and each lot was placed in a polyester mesh (URE Pacific) bag (85µm pore size, 10cm x 10cm) with a marker attached for location purposes (Coley-Smith, 1985; Alexander, 1992). A control treatment was also included where the sclerotia were maintained in a glass vial, at 20°C for the duration of the trial.

The trial ran for a period of four weeks (13th December, 1995-10th January, 1996). The plots (3m x 6m) were arranged randomly across four planting beds, with two plots in each bed. Each plot contained 12 bags of sclerotia which were placed 0.8m apart in a grid arrangement. Three bags containing fresh sclerotia were placed randomly at a depth of 10cm and the remaining three at 20cm. Three bags containing conditioned sclerotia were placed randomly at a depth of 10cm and the remaining three at a depth of 20cm. The plots were irrigated to saturation and on the following day (Mihail & Alcorn, 1984) transparent polythene (PE) (Permathane Plastics) 50µm thick (Satour *et al.*, 1989; Duff & Connelly, 1993) was laid over four (one in each planting bed) of the plots (plots 1, 4, 6 and 8) and the edges were buried in the soil (Alexander, 1990) to a depth of 10cm (Plate 4.5). The four

Plate 4.5 Soil solarisation field trial design layout. Plots 1 and 2.

(one in each planting bed) uncovered plots (plots 2, 3, 5 and 7) were sprinkler irrigated once a week and the soil surface was lightly hoed at weekly intervals to remove weeds.

Temperature sensors (Phillips KTY83-110) encased in stainless steel tubes were placed in the soil at two depths, 10cm and 20cm in each of the eight plots. The soil temperature was recorded every half an hour using a field data logger (Datataker DT600) for the duration of the trial.

Assessment.

Upon completion of the solarisation treatment, the sclerotia were retrieved from the bags. The percentage recovery of the sclerotia was calculated following which the sclerotia were surface sterilised in a 0.25% NaOCl solution for one minute, washed in five changes of SDW and blotted dry on Whatman No.1 filter paper. The sclerotia were then placed individually using sterile forceps, onto isolated PDA droplets in Petri dishes. The plates were sealed with polythene wrap and incubated in the dark at room temperature (18-20°C) for ten days.

Viability was initially classified on the ability of the sclerotia to germinate, and germination was recorded every second day for ten days by observation under the stereo microscope. The percentage of sclerotia which germinated was recorded and after ten days the sclerotia which had not germinated were cut into halves and examined. Sclerotia with a healthy white medulla were recorded as viable. The results were analysed using a Balanced ANOVA with soil depth and solarised or non solarised as the variables.

4.4.2 **Results.**

The depth at which the sclerotia were buried did not significantly ($p \leq 0.05$) affect viability (Appendix 4.4), therefore, the number of viable sclerotia at 10 and 20cm were combined for analysis.

Over 85% of the fresh sclerotia were recovered intact from the bags in both the solarised and non solarised soil compared to 100% for the control sclerotia stored in a glass vial. The remainder of the sclerotia were disintegrated. The solarisation treatment did not significantly affect ($p \leq 0.05$) sclerotial viability, even though there was a wide range in the percentage of viable sclerotia recovered from the plots (Table 4.6). There was a significant

Table 4.6 Mean number of viable fresh *Sclerotium cepivorum* sclerotia from each plot

Treatment	Plot number	% recovered sclerotia	% viable sclerotia
Control	-	100	99.3
Solarised	1	83.7	42.6
Non solarised	2	95.0	72.3
Non solarised	3	95.7	70.0
Solarised	4	95.3	72.0
Non solarised	5	98.7	75.7
Solarised	6	89.7	64.3
Non solarised	7	95.3	68.9
Solarised	8	80.3	25.7

n=50

replicate effect and a significant interaction between the solarisation treatment and replicate (Appendix 4.4).

The conditioned sclerotia had a very low recovery rate (16.5%) compared to 91.7% for the fresh sclerotia or 100% for both the fresh and conditioned control sclerotia in glass vials. The number of recovered viable conditioned sclerotia was exceptionally low (1%) as was the viability of the conditioned control sclerotia (15.3%). The majority of the conditioned soil incubated sclerotia were disintegrated and only fragments of sclerotial rind remained in the bags. Any sclerotia observed to be intact, either disintegrated when touched with forceps or were flattened against the sides of the bags. The data for the conditioned sclerotia was not analysed as the recovery rate was so low. There were a number of fungal species that colonised the sclerotia.

Both the fresh and conditioned sclerotia were colonised by a number of fungal species. These included: species of *Verticillium*, *Penicillium*, *Fusarium*, *Trichoderma* and an isolate of *P. lilacinus*. A number of unidentified species also colonised the sclerotia. *Aspergillus* spp. were also present, however, only in conjunction with the fresh sclerotia.

The soil temperatures were very similar within the solarised plots and within the non solarised plots. The mean maximum and minimum temperatures for the solarised and non solarised soil, at 10 and 20cm are presented in Figures 4.3 and 4.4. The maximum and minimum temperatures were significantly different ($p \leq 0.05$) in the solarised plots than in the non solarised plots and there was a significant replicate effect for the minimum temperatures but not the maximum temperatures. There was no significant interaction between replicate and the solarisation treatment (Appendix 4.4). The maximum and minimum temperatures at 10cm and 20cm were significantly different ($p \leq 0.05$) and there was a significant interaction between depth and temperature (Appendix 4.4). At 10cm, the maximum temperature was higher in the solarised and non solarised soil, compared to the maximum temperatures at 20cm in both treatments. The minimum temperatures in the solarised and non solarised soil were higher at 20cm than at 10cm. The hottest temperatures were recorded in early January and the coolest were in mid December. The temperatures in the solarised plots ranged from 16.0 to 43°C, and in the non solarised soil, the temperatures ranged between 13.0 and 29°C. The average soil temperature in the solarised soil was 27.6°C compared to 20.3°C in the non solarised soil. Higher temperatures were reached and maintained for a

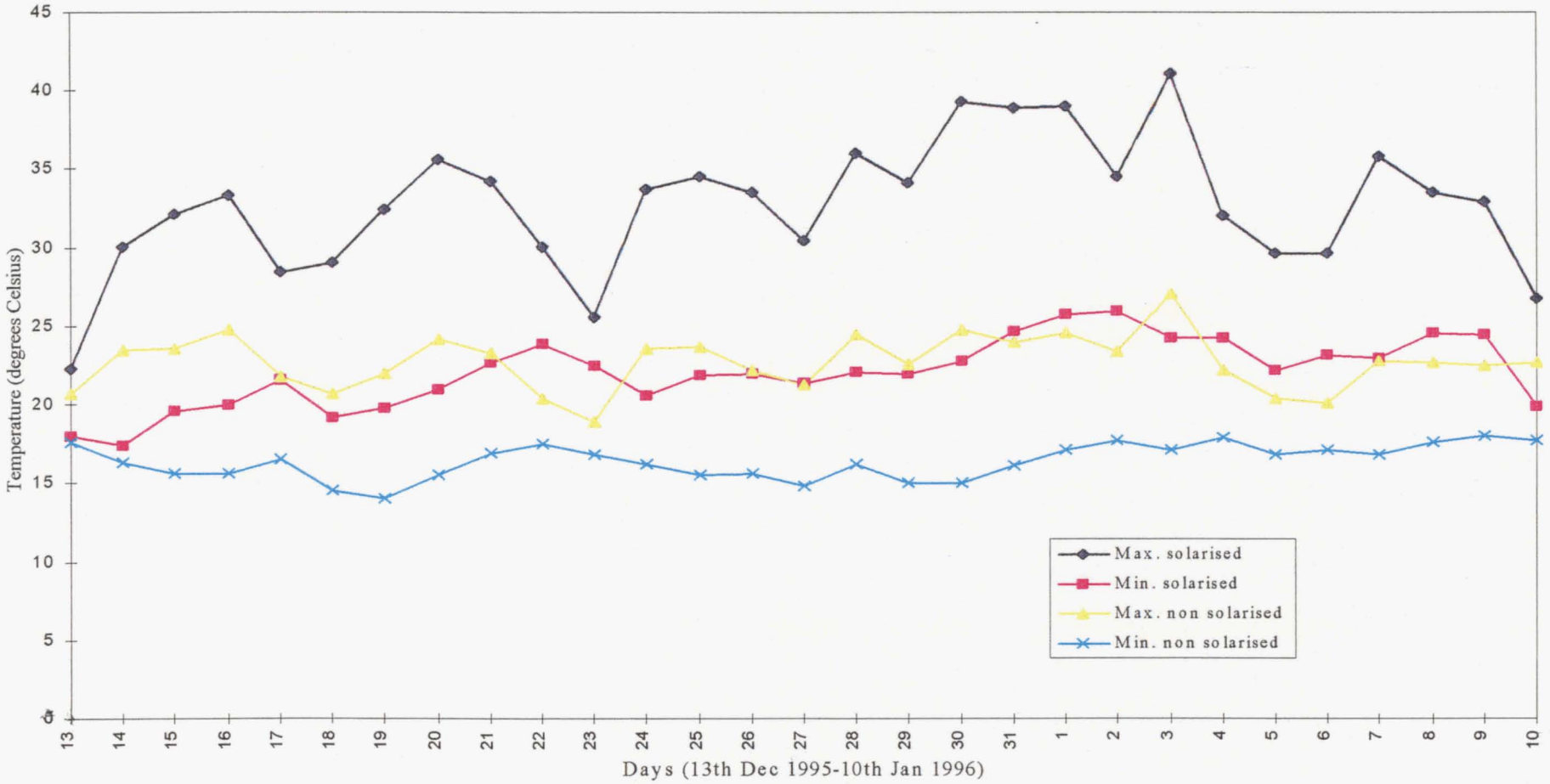

Figure 4.3 The mean daily maximum and minimum soil temperature for solarised and non solarised soil at 10cm, soil solarisation trial.

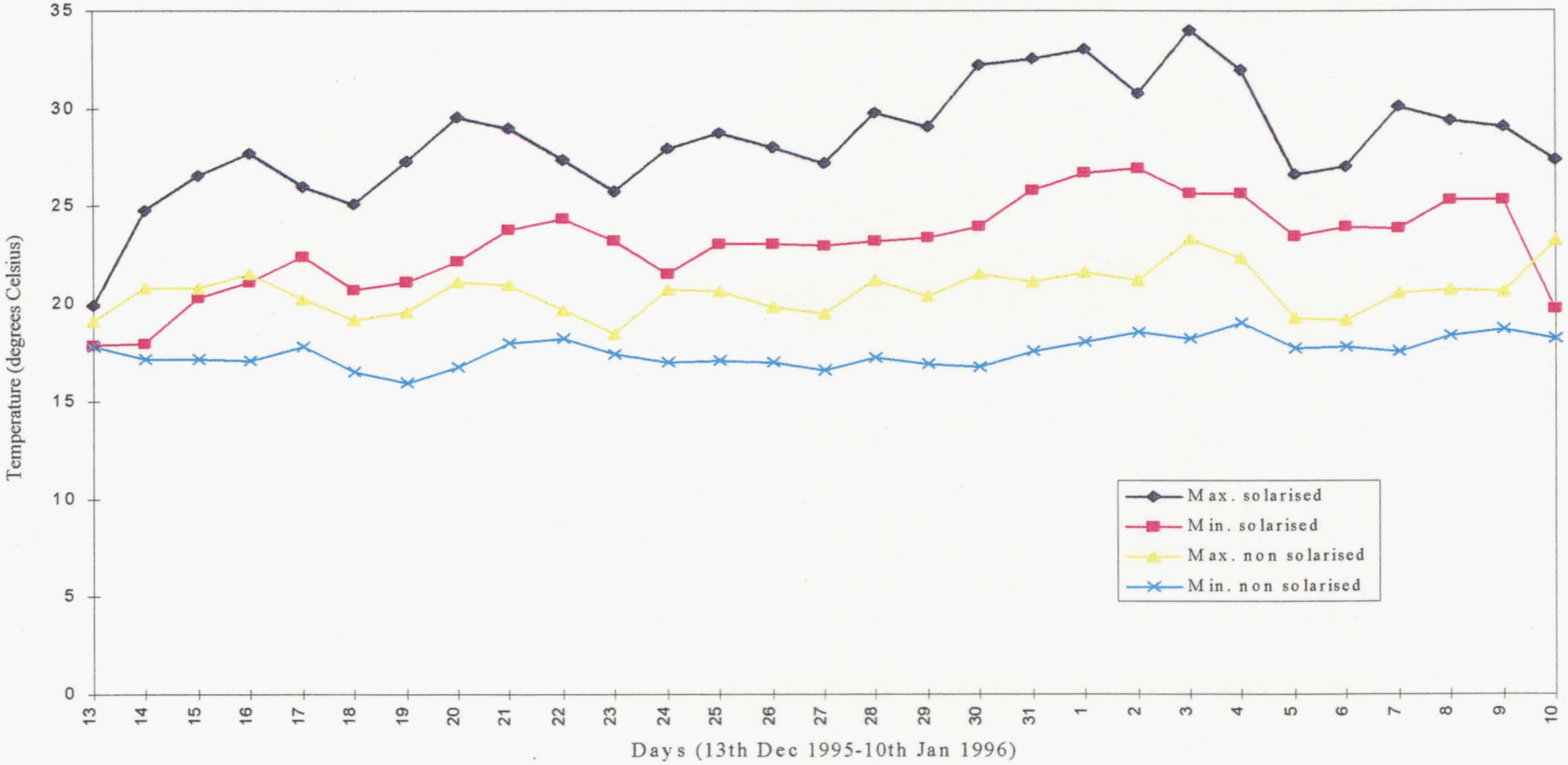

Figure 4.4 The mean daily maximum and minimum soil temperatures for solarised and non solarised soil at 20cm, soil solarisation trial.

longer period of time in the solarised soil compared to non solarised soil (Table 4.7). Temperatures above 20°C were maintained for 1897 and 2545.5 hours at 10 and 20cm respectively, in solarised soil. In contrast, in non solarised soil, temperatures above 20°C were maintained for longer periods (930.5 hours) at 10cm than 20cm (641 hours).

4.4.3 Discussion.

The low recovery rate and viability of the conditioned sclerotia was not surprising, given that problems with the sclerotial conditioning period had already been recognised (Section 3.4). In previous trials, the conditioned sclerotia had either been contaminated or had a low viability (62%), which was probably due to the short, one month conditioning period. In this trial, the sclerotia were conditioned for three months and had a viability of 76% at the start of the trial. When stored in a glass vial at 20°C for one month, following the conditioning period, sclerotial viability decreased substantially even though a high number of intact sclerotia were recovered. In soil, both the recovery rate and sclerotial viability were significantly decreased after a one month incubation period. The sclerotia in the glass vial did not disintegrate, however, the soil incubated sclerotia did. The sclerotia were unhealthy and the conditioning period was the most likely cause. A three month conditioning period should have been sufficient to break constitutive dormancy (Coley-Smith, 1960), hence the soil composition, moisture and incubation temperature could be influencing factors.

Temperatures recorded under the polythene were similar to those recorded in the previous year. An increase in the maximum temperatures was predicted with improved polythene laying techniques. Even though, with this soil solarisation trial, much greater attention was paid to levelling the soil and ensuring minimal air spaces between the polythene and soil, an increase in temperature was not evident. While the higher temperatures were not obtained, there was an increase in the number of hours above 20°C at both depths in solarised and non solarised soil for this trial compared to the preliminary investigation. The mean air temperature was slightly higher for this trial (16.8°C) compared to the mean air temperature experienced in the preliminary field trial (15.6°C). In contrast, the amount of solar radiation was higher for the preliminary field trial (786.60 MJ/m^2) compared to this trial (710.35 MJ/m^2) (Cherry, 1994, 1995a,b,c, 1996). This higher amount of solar radiation could account for the greater number of days above 40°C recorded in the preliminary trial.

Table 4.7 Total number of hours spent above selected temperatures at 10 and 20cm in solarised and non solarised soil

Temp.	Solarised		Non solarised	
	10cm	20cm	10cm	20cm
>40	13	-	-	-
>35	140	2.5	-	-
>30	551	243	-	-
>20	1897	2545.5	930.5	641

The preliminary temperature and heat sensitivity trials indicated that a 90% reduction in sclerotial viability was possible at 20°C after 28 days in soil. The sclerotia in the solarised soil in this trial were incubated above 20°C for 26 consecutive days and sclerotial viability was not significantly decreased. The large variation in the number of recovered sclerotia from the polyester mesh bags influenced the lack of significance. Twenty treatment blocks would be required to provide the chance of detecting a significant solarisation effect, rather than four blocks. Alternatively, a larger number of sclerotia per treatment block would be needed to detect a significant difference. The effect of soil moisture, composition and microflora could be maximised to advantage the soil solarisation process.

The detrimental effect of drying and rewetting cycles on sclerotial viability has already been established (Section 4.3.3). Excess moisture is hypothesised to be detrimental to *S. cepivorum* sclerotial viability (Legget & Rahe, 1985) and sclerotial decay is rapid in saturated soils at high temperatures (Crowe & Hall, 1980b). Irrigation beneath the polythene could result in greater losses in sclerotial viability. Drip systems (Katan *et al.*, 1976; Dwivedi, 1991) and furrow irrigation (Pullman *et al.*, 1979; Satour *et al.*, 1989) have been used for this purpose.

Soil solarisation may increase pathogen vulnerability to soil microorganisms or increase populations of antagonistic microflora and hence increase the parasitic and lytic effects on the sclerotia (Katan *et al.*, 1976). Increased colonisation of *S. cepivorum* sclerotia by soil microorganisms has also been reported, following treatment with sublethal temperatures (Entwistle & Munasinghe, 1990). The increased microbial activity could explain why 100% of the sclerotia could not be recovered from the bags. A number of fungal species were isolated from the sclerotia when plated onto PDA droplets. Species of *Verticillium, Penicillium, Fusarium, Trichoderma* and an isolate of *P. lilacinus* appeared in conjunction with both the fresh and conditioned sclerotia. These are the same species as those identified from the incubator trial. An isolate of *Aspergillus* was also present, but only on the fresh sclerotia. Although research suggested that *Aspergillus* spp. could be secondary colonists of sclerotia (Phillips, 1990), The isolate of *Aspergillus* in this study was not associated with the conditioned sclerotia which were in a substantially weakened state. The *Aspergillus* isolate most probably survived the surface sterilisation procedure and overgrew the sclerotia on agar droplet

Although the weather was not particular warm and the amount of solar radiation was not as high as a usual summer, soil solarisation would probably not significantly reduce the number of viable sclerotia. The integration of biological control agents or perhaps sclerotial germination stimulants with a soil solarisation programme may provide significant reductions in sclerotial viability.

~CHAPTER FIVE~

CONCLUDING DISCUSSION

Onion white rot is a destructive disease of *Allium* species. Currently in New Zealand, control of onion white rot relies on the dicarboximide procymidone, and the triazole group of systemic fungicides. The reduction of chemical applications to vegetable crops is desirable. This study investigated the potential of several previously identified fungal antagonists to control *Sclerotium cepivorum* and to determine a suitable delivery system for successful control agents. Cultural control measures such as soil solarisation were also investigated to determine the suitability of the Canterbury climate for such a control option.

Chaetomium globosum, Coniothyrium minitans, Trichoderma harzianum, T. koningii and *T. viride*, were all shown to be successful biological control agents for reducing the amount of onion white rot disease while not compromising seedling emergence, when applied in bulk carrier formulations, in a glasshouse trial. These results are in accordance with Kay and Stewart (1994). Stewart and Harrison (1988) first reported *C. globosum* as a mycoparasite of *S. cepivorum.* In this study, *C. globosum* reduced *S. cepivorum* disease significantly in relation to the pathogen control treatment as was previously reported (Kay & Stewart, 1994). This control was probably the result of antibiotic production as *C. globosum* was shown to produce inhibition zones *in vitro* as also previously recorded (Harrison & Stewart, 1988; Kay, 1991). *C. minitans* was reported as a sclerotial parasite (Harrison & Stewart, 1988) and successful control of onion white rot has been reported (Ahmed & Tribe, 1977), as with this study. Ahmed and Tribe (1977) conducted their trial in seedling trays containing small amounts of soil with 240mg of sclerotia. This small amount of soil in comparison to the large amount used in this study could have resulted in the sclerotia being much closer together than with this trial and the effect of *C. minitans* could have been greater. Entwistle (1988) commented that mycelial growth of *C. minitans* is limited in soil and hyphae would be unable to spread from one sclerotium to the next unless the sclerotia were clumped together.

Trichoderma species grow quickly, sporulate abundantly and are not medium selective compared to genera such as *Chaetomium, Coniothyrium, Sporidesmium* and *Teratosperma* (Adams, 1987; Harrison, 1987; Cook & Baker, 1989; Alexander, 1992). *Trichoderma* species have been reported to antagonise sclerotial fungi in three ways, production of

antibiotics (Ghaffar, 1969a; Dennis & Webster, 1971a,b; de Oliveira *et al.*, 1984), parasitism (dos Santos & Dhingra, 1982; de Oliveira *et al.*, 1984) and competition for space and/or nutrients (Harrison & Stewart, 1988). This study supported the production of antibiotics with the inhibition of *S. cepivorum* growth on agar amended with culture filtrates of *T. harzianum*. In dual culture, all *Trichoderma* species overgrew *S. cepivorum* indicating effective competition for space and/or nutrients. Competition could have also occurred when the *Trichoderma* species were introduced into the soil in the glasshouse trial, as Lewis and Papavizas (1984) reported abundant proliferation when *Trichoderma* mycelial bran combinations were incorporated into the soil. Successful control of onion white rot was reported with applications of *T. harzianum* spore suspensions to heat sterilised soil infested with *S. cepivorum* (Abd-El-Moity & Shatla, 1981). The results of this study supports the success of *T. harzianum*. In contrast, other researchers have reported isolates of *T. harzianum* as poor biological control agents (Kay & Stewart, 1994; Basallote *et al.*, 1995). Although similar bulk carrier formulations were used to apply *T. harzianum* to the soil, the inoculum levels were not specified. It is highly possible that the differences in the efficiency of *T. harzianum* between the studies could be related to inoculum levels. Basallote *et al.* (1995) also applied *T. harzianum* to garlic and *T. harzianum* may have reacted differently to garlic compared to onion varieties. *T. koningii* has not been widely investigated as a biological control agent of onion white rot although successful control of onion white rot was achieved in this study, with concentrations of 1.04×10^5 wheat bran formulations. Similar success has also been reported (Kay & Stewart, 1994; Wong *et al.*, 1995). In contrast, *T. koningii* was reported unsuccessful (Basallote *et al.*, 1995) and again inoculum levels could vary between the studies.

The application of potential biological control agents to the soil before planting of susceptible crops has not been widely investigated. In this study, the addition of fungi two weeks before planting resulted in significantly less plant emergence and significantly greater disease than when the fungi were applied at the time of planting. In contrast, significant reductions in onion white rot were reported with applications of *Penicillium godlewskii* (Zaleski) and *Aspergillus candidus* 15 days before planting (Abd-El-Razik *et al.*, 1985). Similarly, applications of *C. minitans* significantly controlled *Sclerotinia* disease on glasshouse lettuce crops when applied to soil after harvest, approximately one week before the sowing of a second crop (Whipps & Budge, 1992). While neither *Penicillium* nor *Aspergillus* species were trialed in this study and although *Aspergillus* is reported as a

secondary colonist rather than an initial parasite (Phillips, 1990), the success of these fungi could be the result of the application method or perhaps the environmental conditions in the glasshouse influenced the action of the *Penicillium* and *Aspergillus* species. The addition of *C. minitans* in a maize perlite preparation may have advantaged the survival and subsequent action of *C. minitans* rather than the sand:bran formulation used in this study. Different sclerotial fungal species may also respond differently to the applications of the same fungal antagonist.

An unsuccessful attempt was made to determine the effect of delivery systems of *T. harzianum* and *T. koningii* on onion white rot disease control, in both the glasshouse and the field. Seed coats and pellets using *T. harzianum* (Abd-El-Moity, 1986) and *T. viride* (Kay, 1991) have previously been reported as successful against onion white rot. A brief report (Hughes & Wong, 1986) also suggested *Trichoderma* spp. coated seeds were successful at reducing onion white rot. The lack of success with the seed coatings and pellets in this study was most probably related to the pathogen inoculum levels rather than the application of the biological control agent. Continued applications of *S. cepivorum* sclerotia to this field site in forth coming seasons will result in higher levels of inoculum and hopefully antagonist application effects will become apparent. The development of an improved seed coating procedure would be advantageous and a trial involving the effect of *T. harzianum* and *T. koningii* seed coats and commercial pellet formulations on onion white rot disease incidence would be worthwhile repeating.

A limitation associated with this research was the conditioning treatment of the sclerotia. The sclerotial conditioning treatment used in this study was not ideal for the *S. cepivorum* isolate used. Coley-Smith (1960) recommended a three month incubation period to break constitutive dormancy. While Coley-Smith and co-workers experienced no loss in viability with this length of conditioning period (Esler & Coley-Smith, 1983; Coley-Smith, 1986), it was probably too harsh for the *S. cepivorum* isolate used in this study. In a later paper, Coley-Smith (1985) reported a shorter period of one to two months was needed to break constitutive dormancy, for sclerotia produced on onion bulbs, rather than pure culture produced sclerotia as used in this study. Brix and Zinkernagel (1992) reported that conditioning periods between four and ten weeks were required to break dormancy in a range of *S. cepivorum* isolates. In this study, a four week conditioning period resulted in 62% of the sclerotia germinated, with 82 and 76% of the sclerotia germinated after two and

a half and three months, respectively. This research and others (Sommerville & Hall, 1987) suggested that constitutive dormancy periods may be isolate specific. The *S. cepivorum* isolate used in this study (E68) probably requires a period of two to two and a half months for maximum germination. Although the production methods of *S. cepivorum* sclerotia varied considerably (Coley-Smith & Javed, 1970; Adams & Papavizas, 1971; Ahmed & Tribe, 1977; Abd-El-Moity & Shatla, 1981; Esler & Coley-Smith, 1983) this is unlikely to have affected sclerotial viability as this study used an identical method to Kay and Stewart (1994) and viability was considerably lower after the conditioning period in this study than in previous studies (Kay, 1991; Kay & Stewart, 1994). The methods of storage of *S. cepivorum* isolates also varied and although agar is commonly employed (Harrison & Stewart, 1988; Kay & Stewart, 1994), Coley-Smith (1985) used a soil burial method. When cultures were required, the sclerotia were retrieved from bags in the soil, grown on agar and the agar was used to inoculate onion bulbs. The continual soil storage of sclerotia may enable the sclerotia to endure soil environments to a greater extent than agar stored cultures. The main problem with the loss in sclerotial viability in this study lies with the conditioning period and the conditions of that period. Further investigations would be worthwhile to determine specific conditioning treatments for isolates of *S. cepivorum.*

Soil solarisation was not successful at reducing the number of viable sclerotia in Canterbury, even though successful reductions in soil borne pathogens in climates marginal for soil solarisation have been reported (Garibaldi & Gullino, 1991). Temperatures in Canterbury are not as hot as those recorded in other marginal regions such as Australia, where polythene covered soil reached maximum temperatures of 42.3 and 38.3°C at 15 and 25cm depths respectively, whereas untarped soil reached temperatures of 35.9 and 33.3°C at the same depths (Porter & Merriman, 1983). At constant sublethal temperatures sclerotial viability was reduced to 10.7% at 20°C and 0% at 30°C after 28 and 16 days exposure, respectively. These results conflict with Australian studies, where temperatures less than 40°C did not reduce sclerotial viability (Porter & Merriman, 1983). Similarly, Adams (1987) reported 88 to 100% sclerotial survival at 25 and 30°C after 30 days. These differences could be the result of isolate variation in response to heat, although Stewart (1990) reported little variation in sclerotial pathogenicity of different *S. cepivorum* isolates to temperatures ranging from 5 to 30°C. A possible explanation for the response of *S. cepivorum* isolates to heat could be the variation in methodology. Small amounts of moist *S. cepivorum* sclerotia infested soil were placed in glass vials and exposed to temperatures

ranging from 25 to 50°C (Porter and Merriman, 1983; Adams, 1987), whereas this study used sclerotia contained in polyester mesh bags that were placed into liver pails containing soil. While the sclerotia were incubated at varying temperatures, no mention was made of how the temperature was monitored and it is possible that the soil temperature was not as high as predicted.

The thermal time data indicated two mechanisms for reducing sclerotial viability. At temperatures greater than 40°C the reduction in sclerotial viability was most likely a direct effect of temperature, whereas at sublethal temperatures, the interactions of temperature, moisture and soil microorganisms most probably contributed to the reduction in sclerotial viability. These results support the theories of other researchers (Katan *et al.*, 1976; Katan, 1981; Stapleton & DeVay, 1986; Vannacci *et al.*, 1988). In countries with cold climates by soil solarisation standards, the influence of moisture and soil microflora are more important as temperatures high enough to directly affect sclerotial viability are not obtainable. Moisture is detrimental to sclerotial viability and flooding has been reported as a successful control measure (Legget & Rahe, 1985; Crowe & Carlson, 1995). Increasing soil moisture under the polythene, could cause greater losses in sclerotial viability. The soil moisture content was also hypothesised to have influenced sclerotial viability during the conditioning period, either directly or as a result of the increased microbial activity within the soil.

While no attempt was made to examine the effect of soil solarisation on disease incidence in subsequent onion crops in this study, the 49% reduction in sclerotial viability, while not significant, may have reduced sclerotial viability enough to cause significant reductions in disease incidence in onion crops. Porter and Merriman (1985) reported a 32% reduction in *S. cepivorum* sclerotial viability after a four week soil solarisation period. This reduction in sclerotial viability resulted in 35% of the seedlings diseased compared to 53% in the non solarised soil. The correlation of decreases in sclerotial viability with subsequent disease incidence would be worthwhile investigating to determine the decrease in viability required to significantly reduce the number of diseased plants.

Cost comparisons between current chemical control measures and the use of soil solarisation would be valuable, as the cost associated with the use of soil solarisation may limit its usage in some regions. While cost calculations for the use of soil solarisation have not been made, the costs associated with polythene removal and disposal and also the

amount of time the land is out of production are of primary concern. Discing, burning, physical removal and storage are suggested options for removal and disposal of polythene (Brown *et al.*, 1991). Environmental pollution problems associated with some of these methods would need to be addressed if soil solarisation was to be implemented in Canterbury. The amount of time the land was out of production would also need to be taken into consideration. In New Zealand, onions are sown in autumn and spring. A four week solarisation programme would not interfere with early maturing varieties of autumn sown onions as harvesting occurs in December. Soil solarisation would be harder to implement with spring sown onions as the onion crop is sown in December and harvested in January/February. If the grower wanted to solarise the soil then an onion crop would have to be missed. Phillips (1990) reported this as one of the major limiting factors with the control of *S. sclerotiorum* diseases.

The use of soil solarisation alone is not likely to be sufficient in controlling onion white rot. The addition of heat tolerant microorganisms to the soil before soil solarisation may be a way to maximise the potential of soil solarisation. Preliminary investigations with this study have shown an isolate of *Trichoderma viride* (D77) to proliferate and sporulate on agar at temperatures as high as 30°C, whereas, the growth rate of *T. harzianum* and *T. koningii* was significantly reduced at these higher temperatures. *S. cepivorum* was not able to grow at temperatures as high as 30°C. While research has not been undertaken to examine the effect of fungal additions before soil solarisation, the addition of *T. harzianum* to solarised soil before crop planting, successfully controlled *R. solani* infections in Iris bulbs (Chet *et al.*, 1982), and *S. rolfsii* disease in potatoes (Elad *et al.*, 1980).

The addition of soil amendments such as crop residues could also be utilised to reduce sclerotial viability. Brassica plants have volatile sulphur containing compounds including: isothiocyanates, sulphides, disulphides, trisulphides and mercaptans (Lewis & Papavizas, 1970) within their tissues. *S. cepivorum* is sensitive to sulphur containing compounds. Sclerotia germinate in response to sulphides and mercaptans have been shown to inhibit sclerotial germination of *S. cepivorum* (Coley-Smith & King, 1969). When broccoli debris was incorporated into soil infested with *S. cepivorum* sclerotia the percentage of diseased onion plants was reduced from 93.7% to 56.2%. The treatment was even more effective if heat was incorporated and solarising the soil resulted in only 6.2% of the seedlings diseased (Zavaleta-Mejia *et al.*, 1990). The use of crop residues may be more convenient for smaller

scale farmers that rotate *Allium* species with other vegetables or have access to large amounts of vegetable debris, rather than large scale *Allium* farmers. Germination stimulants such as diallyl disulphide (DADS) may be more accessible and could reduce sclerotial viability if incorporated into solarised soil before planting. The application of DADS before solarisation would probably be ineffective in reducing sclerotial viability as the control effect of DADS is decreased with temperatures greater than 15°C (Coley-Smith & Parfitt, 1986).

The future of onion white rot control lies with integrated control systems including biological and cultural controls. Soil solarisation is a possible control method for onion white rot and the incorporation of excess moisture, beneficial microorganisms, crop residues and/or germination stimulants may maximise the potential of soil solarisation as a control option. The long term effects of soil solarisation have been documented (Tjamos & Paplomatas, 1988; Satour *et al.*, 1989) and disease suppression is evident from one season to the next, indicating that costs of a solarisation treatment would not need to be incurred every year. A control strategy involving periodic solarisation treatment and the application of beneficial microorganisms in an easily dispersible form could prove an effective control programme, that would be readily accepted by commercial growers.

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PERSONNAL COMMUNICATIONS.

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~APPENDICES~

Appendix 1: Growing media and solutions.

1.1 PDA (potato dextrose agar)

39.0g Potato dextrose agar (Gibco)

2g Davis (NZ) agar

1l water

Preparation: all ingredients were combined in a 1l Duran bottle and autoclaved at 121°C and 15 psi for 15 minutes.

1.2 MYE (molasses yeast extract broth)

10g molasses (Redlabel)

1.6g yeast extract

1l water

Preparation: all ingredients were combined in a 1l Duran bottle and autoclaved at 121°C and 15 psi for 25 minutes.

1.3 0.01% WA (water agar)

0.1g Davis (NZ) agar

1l water

Preparation: all ingredients were combined in a 1l Duran bottle and autoclaved at 121°C and 15 psi for 15 minutes.

1.4 WA (water agar)

20g Davis (NZ) agar

1l water

Preparation: all ingredients were combined in a 1l Duran bottle and autoclaved at 121°C and 15 psi for 15 minutes.

1.5 V-8 juice agar

200ml V-8 juice

15g Davis (NZ) agar

6.0ml 1.0_N NaOH

800ml water

Preparation: all ingredients were combined in a 1l Duran bottle and autoclaved at 121°C and 15 psi for 15 minutes.

1.6 Tween 20 solution

1ml Tween 20

1l water

Preparation: all ingredients were combined in a 1l Duran bottle and autoclaved at 121°C and 15 psi for 15 minutes.

Appendix Two: Results data and analyses for Chapter Two.

Glasshouse Trial.

2.1 ANOVA calculations.

GLM ANOVA for emergence, application 1 and application 2 without the controls

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Application	1	1382.40	1382.40	1382.40	26.11	0.000
Treat	5	250.13	250.13	50.03	0.94	0.461
Application* Treat	5	361.80	361.80	72.36	1.37	0.254
Error	48	2541.60	2541.60	52.95		
Total	59	4535.93				

GLM ANOVA for disease, application 1 and application 2 without the controls

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Application	1	350.42	350.42	4350.42	10.51	0.002
Treat	5	253.15	253.15	50.63	1.52	0.202
Application*Treat	5	134.48	134.48	26.90	0.81	0.551
Error	48	1600.80	1600.80	33.35		
Total	59	2338.85				

GLM ANOVA for emergence, application 1 and the controls

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatments	7	1938.30	1938.30	276.90	12.00	0.000
Error	31	715.60	715.60	23.08		
Total	38	2653.90				

GLM ANOVA for emergence, application 2 and the controls

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatments	7	1531.99	1531.99	218.86	2.77	0.023
Error	31	2447.60	2447.60	78.95		
Total	38	3979.59				

GLM ANOVA for disease, application 1 and the controls, at trial completion

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatments	7	933.79	933.79	133.40	5.81	0.000
Error	31	711.95	711.95	22.97		
Total	38	1645.74				

GLM ANOVA for disease, application 2 and the controls, at trial completion

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatments	7	1338.94	1338.94	191.28	4.91	0.001
Error	31	1208.75	1208.75	38.99		
Total	38	2547.69				

GLM ANOVA for disease, application 1 and the controls, five weeks after planting

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatments	7	495.99	495.99	70.86	4.66	0.001
Error	31	471.60	471.60	15.21		
Total	38	967.59				

GLM ANOVA for disease, application 2 and the controls, five weeks after planting

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatments	7	502.09	502.09	71.73	4.95	0.001
Error	31	449.60	449.60	14.50		
Total	38	951.69				

***In vitro* assays.**

2.2 Dual Plate Assay.

One-way ANOVA for the mean growth of *Sclerotium cepivorum*

Source	DF	SS	MS	F	P
Fungi	6	7559.1	1259.9	575.15	0.000
Error	14	30.7	2.2		
Total	20	7589.8			

2.3 Amended agar assay.

One-way ANOVA for the mean rate of growth of *Sclerotium cepivorum*

Source	DF	SS	MS	F	P
Fungi	7	265.983	37.998	277.19	0.000
Error	16	2.193	0.137		
Total	23	268.176			

Appendix Three: Results data and analyses for Chapter Three.

Glasshouse Trial.

3.1 Agrimm Technologies contact address.

Agrimm Technologies

P.O. Box 13-245

Fitzgerald Ave

Christchurch.

3.2 ANOVA calculations.

Balanced ANOVA for the number of emerged seedlings for each treatment

Source	DF	SS	MS	F	P
Treatments	8	462.40	57.80	1.68	0.141
Replicate	4	124.58	31.14	0.91	0.471
Error	32	1097.82	34.31		
Total	44	1684.80			

Balanced ANOVA for the number of diseased seedlings for each treatment

Source	DF	SS	MS	F	P
Treatments	8	666.84	83.36	1.01	0.445
Replicate	4	424.80	106.20	1.29	0.294
Error	32	2629.60	82.18		
Total	44	3721.24			

3.3 Cumulative number of diseased seedlings

		Number of diseased seedlings, recorded at weekly intervals									
Treatment	Rep #.	4	5	6	7	8	9	10	11	12	Harvest
T. harzianum	1	4	4	6	6	8	10	14	15	17	10
seed coat	2	0	0	0	4	4	4	4	4	4	9
	3	0	0	0	0	0	0	0	0	0	0
	4	1	1	1	1	3	3	3	4	5	6
	5	0	0	1	1	1	4	6	6	6	2
T. harzianum	1	3	3	3	5	5	7	7	9	11	12
pellet	2	0	0	0	0	0	0	0	0	0	0
	3	5	5	5	5	5	5	6	7	7	9
	4	4	6	6	6	7	8	9	9	9	16
	5	4	4	4	4	4	4	4	4	4	5
T. koningii	1	5	6	6	6	10	11	11	16	17	10
seed coat	2	1	2	2	4	4	4	4	4	5	28
	3	0	0	0	0	0	0	0	0	0	0
	4	1	1	1	1	1	1	1	1	1	4
	5	0	0	0	0	0	0	0	0	0	7
T. koningii	1	0	0	0	0	0	0	0	0	0	0
pellet	2	0	2	2	2	2	2	2	2	2	7
	3	2	2	2	3	3	5	5	5	7	4
	4	1	1	1	1	1	3	3	3	3	26
	5	0	0	0	0	0	0	0	0	0	0
T. harzianum/T. koningii	1	0	1	1	1	1	4	4	4	4	3
seed coat	2	0	0	0	0	0	0	0	0	0	0
	3	1	1	1	1	1	1	1	1	1	0
	4	0	0	0	0	0	0	0	0	0	0
	5	1	1	2	2	2	3	3	3	3	2
T. harzianum/T. koningii	1	0	0	0	0	2	2	2	2	2	7
pellet	2	0	0	0	3	3	3	3	4	4	14
	3	0	3	3	3	3	3	3	3	3	7
	4	0	0	1	1	2	2	2	3	3	3
	5	1	1	1	3	3	3	6	6	6	7
Trichopel	1	4	4	4	4	4	8	8	8	8	3
	2	0	0	0	0	1	1	1	1	1	7
	3	0	0	0	0	0	0	0	0	0	3
	4	0	0	0	0	0	0	0	0	0	1
	5	1	1	2	2	3	3	3	4	4	0
Control	1	0	0	0	0	0	0	0	0	0	0
pathogen	2	0	0	4	4	4	7	7	7	10	12
	3	0	2	2	4	4	6	6	6	6	2
	4	2	2	2	2	2	2	3	3	3	8
	5	2	2	2	2	2	2	2	2	2	3
Control	1	0	0	0	1	1	1	1	1	2	3
procymidone	2	0	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0	0	0
	4	1	1	1	1	2	2	2	2	2	21
	5	0	0	0	1	1	1	2	2	3	1

3.4 Fresh and dry weight data and Balanced ANOVA calculations for the glasshouse trial.

The mean fresh weight and dry weight (grams) for each treatment after 12 weeks

Treatment	Fresh Weight	Dry Weight
Control-pathogen	235.6 ±34.29	25.5 ±4.11
Control-procymidone	195.8 ±39.31	20.4 ±3.79
T. harzianum seed coat	180.1 ±39.63	18.4 ±4.38
T. harzianum pellet	243.9 ±13.33	23.9 ±2.91
T. koningii seed coat	175.0 ±34.76	19.0 ±3.93
T. koningii pellet	241.7 ±32.96	26.3 ±3.55
T. harzianum/*T. koningii* mix seed coat	202.1 ±10.20	20.6 ±0.92
T. harzianum/*T. koningii* mix pellet	237.4 ±26.97	25.8 ±3.24
Trichopel	207.9 ±12.04	23.0 ±1.16

Balanced ANOVA for the fresh weight for each treatment

Source	DF	SS	MS	F	P
Treatments	8	29235	3654	0.83	0.585
Replicate	4	13475	3369	0.76	0.557
Error	32	141309	4416		
Total	44	184019			

BalancedANOVA for the dry weight for each treatment

Source	DF	SS	MS	F	P
Treatments	8	370.49	46.31	0.79	0.618
Replicate	4	108.16	27.04	0.46	0.765
Error	32	1885.42	58.92		
Total	44	2364.07			

Field Trial.

3.5 Raw data for the number of *Sclerotium cepivorum* sclerotia recovered from the plot by soil sieving.

Number of *Sclerotium cepivorum* sclerotia recovered from soil samples taken ramdomly from the plot before onion seed planting

Replicate	Number of sclerotia
1	5
2	14
3	8
4	11
5	7
Mean	9

Note: sclerotia were recovered from 100g of soil.

3.6 ANOVA calculations.

Balanced ANOVA for the number of emerged seedlings for each treatment

Source	DF	SS	MS	F	P
Treatments	5	18525	3705	3.63	0.039
Replicate	2	3810	1905	1.87	0.204
Error	10	10194	1019		
Total	17	32529			

Appendix Four: Results data and analyses for Chapter Four.

SECTION ONE: PRELIMINARY SOIL SOLARISATION TRIAL

4.1 ANOVA Calculations.

Balanced ANOVA for maximum temperature in solarised and non solarised soil at 10 and 20cm

Source	DF	SS	MS	F	P
Solar	1	211.151	211.151	39.45	0.024
Error 1	2	5.352	2.676		
Depth	1	95.911	95.911	54.46	0.018
Depth*Solar	1	2.101	2.101	1.19	0.389
Error 2	2	3.522	1.761		
Total	7	318.039			

Balanced ANOVA for minimum temperature solarised and non solarised soil at 10 and 20cm

Source	DF	SS	MS	F	P
Solar	1	25.2050	25.2050	3.32	0.210
Error 1	2	7.585	3.79		
Depth	1	13.0050	13.0050	19.93	0.047
Depth*Solar	1	0.9800	0.9800	1.50	0.345
Error 2	2	1.3050	0.6525		
Total	7	48.0800			

SECTION TWO: THE INFLUENCE OF SOIL TEMPERATURE ON SCLEROTIAL VIABILITY

4.2 Temperature soil readings.

The temperature of the soil was monitored using a soil temperature probe that was planted into the soil to a depth of 8cm. The pails in which the temperature was monitored were chosen randomly. The experiment ran for a period of 28 days and began on the 4th of July 1995 and concluded on the 1st of August 1995.

Soil Temperature at each incubator temperature for selected times

Date	Pail	Incubator temperature (°C)	Soil temperature
30/6/95	7	50	49.8
	5	45	43.1
	3	40	39.6
	6	35	35.1
	5	30	33.0
	4	20	18.7
3/7/95	5	50	48.6
	7	45	44.3
	4	40	39.6
	2	35	34.4
	3	30	32.0
	2	20	19.3
7/7/95	5	50	46.1
	3	45	44.1
	4	40	40.1
	2	35	34.5
	6	30	31.2
	4	20	18.9
11/7/95	4	50	48.8
	1	45	44.3
	7	40	39.9
	1	35	34.5
	4	30	28.0
	1	20	18.4
17/7/95	7	50	49.9
	6	45	44.7
	5	40	41.3
	6	35	34.8
	6	30	27.0
	5	20	18.1
21/7/95	3	50	48.1
	2	45	44.6
	3	40	40.2
	8	35	34.1
	1	30	31.0
	6	20	19.6
28/7/95	4	50	49.7
	5	45	44.7
	7	40	39.9
	1	35	35.0
	3	30	26.0
	1	20	20.0

4.3 ANOVA Calculations.

Balanced ANOVA for the number of viable *S. cepivorum* sclerotia at selected temperatures after selected exposure periods

Source	DF	SS	MS	F	P
Temp	6	164991.1	274980.5	254.31	0.000
Time	6	37430.7	6238.5	57.69	0.000
Temp*Time	36	46789.8	1299.7	12.02	0.000
Error	98	10596.7	108.1		
Total	146	259808.4			

SECTION THREE: SOIL SOLARISATION TRIAL

4.4 ANOVA Calculations.

Balanced ANOVA for sclerotial viability in solarised and non solarised soil at 10 and 20cm

Source	DF	SS	MS	F	P
Solar	1	1621.69	1621.69	5.21	0.107
Replicate	3	1236.56	412.19	6.90	0.001
Solar*Replicate	3	933.56	311.19	5.21	0.004
Depth	1	196.02	196.02	3.28	0.078
Solar*Depth	1	25.52	25.52	0.43	0.517
Error	38	2271.63	59.78		
Total	47	6284.98			

Balanced ANOVA for maximum temperature in solarised and non solarised soil at 10 and 20cm

Source	DF	SS	MS	F	P
Solar	1	576.000	576.000	681.66	0.000
Replicate	3	8.265	2.755	1.39	0.334
Solar*Replicate	3	2.535	0.845	0.43	0.742
Depth	1	103.022	103.022	51.92	0.000
Solar*Depth	1	16.403	16.403	8.27	0.028
Error	6	11.905	1.984		
Total	15	718.130			

Balanced ANOVA for minimum temperature in solarised and non solarised soil at 10cm and 20cm

Source	DF	SS	MS	F	P
Solar	1	29.4306	29.4306	209.28	0.000
Replicate	3	2.5369	0.8456	6.01	0.031
Solar*Replicate	3	1.2219	0.4073	2.90	0.124
Depth	1	5.4056	5.4056	38.44	0.001
Solar*Depth	1	2.3256	2.3256	16.54	0.007
Error	6	0.8438	0.1406		
Total	15	41.7644			