Biological control of *Sclerotium cepivorum* Berk. (Onion White Root Rot) using *Trichoderma koningii* Oudem.

by

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Submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy

University of Tasmania

Hobart

April 1997.

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Acknowledgements.

I am very grateful to my supervisor Dr Calum Wilson for his constructive criticism and useful advice over the past two years, and my former supervisor Dr Ralph Cruickshank (now retired) for mentorship in the projects first year. I would also like to thank Dr James Wong for his input in planning the work presented in Chapters III to V and Dr Jason Dennis for help with planning and maintenance of the trials presented in Chapters VI and VIII. I also thank Dr Caroline Mohammed, Dr A D Liyanage, and Dr Ali Salardini for useful discussions.

I would like to thank Mr Bill Peterson for technical advice, Mr Darren Bradford, Mrs Lyn Dow. Mrs Laura Maddock and Mrs Sally Jones for assistance in times of need, Mr Rob Tennant and Mr Weis Jablonski for assistance in electron microscopy, Mr Rod Teague and Mr Ted Garrard for advice in microtome technique as well as Dr David Ratkowsky and Mr Bruce McKorkell for statistical advice. Thanks to Dr Ian Pascoe and Mr Michael Fuhlbohm for providing cultures and Dr Stephen Parker for antimicrobial compound screening.

For funding I am indebted to the Australian Research Council, and the members of the Tasmanian Onion Industry Panel, in particular I thank Mr Tim Groom (Roberts Vegetables), Mr Patrick Johnstone (Perfecta Produce), Mr Dane Gilham (Vecon), Mr Don Faulkner (Clements Marshall), and Mr Russell Parbury (Forth Farm Produce) for their collaborations. Thanks to Mr Breven Howe (Vecon Oils) for collaboration in the germination stimulant trial (Ch. V:15). Thanks also to Mr John Bonney, Mr Kevin Goodwin and Mr Bill Scolyer for providing field trial sites.

For encouragement at all times I would like to thank my parents Robert and Gail, brothers Scott, Mark and Ben, and my two grandmothers Doreen and Stella. Most of all I thank my beloved wife Sharon, who has long endured life with a PhD student and has done everything possible to provide support.

Summary.

At the time this study commenced *Trichoderma koningii* strains had reduced incidence of *Sclerotium. cepivorum* infection of onions in field trials by 60%. The goal of the work was to try to improve *T. koningii* 's efficacy. To gain some understanding of the antagonism process histological and enzymatic studies of antagonism within infected onion roots were undertaken as well as ecological studies and field trial evaluation.

Histological studies confirmed.*S cepivorum* hyphae penetrated the epidermis of onion roots and grew into the hypodermis and cortex. In early stages of the infection only cells *S. cepivorum* grew through were lysed, as the infection developed cells were killed, and cell walls disintegrated in the zone ahead of the infection hyphae. The root epidermis and stele tissues were more resistant to hydrolysis than the cortex, resulting in formation of a cavity filled with *S. cepivorum* hyphae within the cortex. *S. cepivorum* was shown to produce three isozymes of polygalacturonase and three isozymes of pectinesterase in infected tissue. A series was noted in enzyme production with pectinesterases produced first on onion cell wall substrate, followed by polygalacturonase. A novel technique for localisation of pectolytic enzymes in infected tissue was developed by loading tissue segments into wells of an electrophoresis gel. Using this technique it was confirmed that distribution of pectolytic enzymes which diffused ahead of infection hyphae were correlated to cell wall dissolution. It was postulated that *S. cepivorum* may derive advantage from the resistance of the epidermis to hydrolysis, which may serve as a barrier to secondary invaders which may compete for nutrients or inhibit *S. cepivorum.*

When placed on the epidermis of healthy onion roots *T. koningii* (Tr5) was observed to grow in the epidermal mucilage without entering healthy epidermal tissue. When *T. koningii* was placed on the epidermis of *S. cepivorum* infected roots it was observed to actively colonise epidermal passage cells with little colonisation of other epidermal tissues, before branching and spreading throughout the infected or damaged tissues below. Passage cells appear to exhibit some differences in suberisation, and possibly lignification to other epidermal cells. Electrophoretic studies showed that *T. koningii* produced one polygalacturonase and two pectinesterase in liquid culture, and produced pectinases in damaged onion root tissues. Changes were observed in *S. cepivorum* hyphae when *T. koningii* colonised infected tissue, including detachment at the septa, dissolution of cell walls, and lysis of hyphal apices with release of the protoplasm. Contact between hyphae was not necessary for this lysis to occur.

An electrophoresis protocol developed in this study showed that *T. koningii* produced chitinolytic enzymes likely to be component of the antagonism process. The *T. koningii* chitinase complex consisted of at least four proteins, two endochitinases and two chitobiases. Electrophoresis of root segments in which lysis of *S. cepivorum* hyphae had occurred showed that *T. koningii* produced at least two chitinolytic enzymes (an endochitinases and a chitobiase) in these tissues. *T. koningii* was able to produce chitinolytic enzymes to use *S. cepivorum* sclerotia as a sole source of nutrition. Enzymes produced in degradation of crustacean chitin were the same ones produced to degrade *S. cepivorum* cell walls, which has useful implications for soil amendments.

Pot trials where *T. koningii* was added as a continuous band of inoculum just below germinating seeds, demonstrated that when *T. koningii* was well established in the rhizosphere it was able to prevent at least 82% of infections initiated 3cm below the onion base plate. A number of field amendment methods were investigated including fluid drill sowing of seed with living *T. koningii* mycelium and in furrow spore sprays, along with solid carriers including crabshell chitin, sawdust, and a peat/chitin/osmocote blend.

To monitor establishment in the rhizosphere a selective medium (RASP) was developed, which was used in combination with isozyme profiles to distinguish *Trichoderma* isolates growing on onion roots. Studies of rhizosphere establishment suggested that Tr5 had a poor ability to become established in soils of pH 7.5, however at pH 5.5 a high proportion of roots were colonised by Tr5. *S. cepivorum* infection is generally most severe in Tasmania when soil temperatures are in the 11 to 15°C range. Studies of the effects of soil temperature on biocontrol demonstrated that Tr5 was more able to suppress infections when soil temperature was 10 to 12°C than 15 to 18°C.

When Tr5 was well established in the rhizosphere a consistent ability to suppress between 63 to 79% of infections in soils with *S. cepivorum* sclerotial densities ranging from 10 to 100 sclerotia per kilogram was demonstrated. The ability of *S. cepivorum* sclerotia to infect the onion base plate decreased with increasing depth of burial, and Tr5 was further able to suppress a greater proportion of infections originating from a depth of 7cm than 4cm. This finding may have implications for integrated control, as sclerotia near the soil surface may be more readily stimulated to germinate by sclerotial germination stimulants, and integration of the two measures will be a subject of future work.

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1.0. Introduction:

Sclerotium cepivorum (Berk.), is the causal agent of onion white root rot, a disease which is the most serious threat to Tasmania's $40 million per annum onion export industry, and to onion growing throughout the world. While this disease can be controlled by fungicides the residues of these chemicals remain in the produce and reduce marketability of exported onions. The sustainability of effective fungicide treatments are additionally threatened by development of resistance by the pathogen, and build up of fungicide degrading micro organisms in the soil.

An isolate of *Trichoderma koningii* (Oudem.) introduced to the furrow as dormant propagules produced on grains of millet at the time of sowing, provided up to 60% reduction in incidence of *S. cepivorum* infections in field trials (Lacey and Wong, 1991). While this result was encouraging, the efficacy is not sufficient for commercial application, and it was not known how to improve this level of disease control.

To try to enhance control, a better understanding was needed of the mechanism by which *T. koningii* was able to prevent onions becoming infected. Preliminary investigations (Metcalf, 1993) included histological studies of the *S. cepivorum* infection process within onion roots, and the interactions between *T. koningii* and *S. cepivorum* hyphae. It was established that some root cells are lysed by *S. cepivorum* extracellular toxins ahead of the advancing infection hyphae, creating a damaged but uncolonised zone of root tissues in the path of the infection. *T. koningii* hypha were observed to colonise this zone of damaged cells, but not healthy root tissues. In roots which *T. koningii* had colonised damaged tissues, structural alterations were noticed in cell walls of *S. cepivorum* hyphae. Based on the ability of *T. koningii* to use chitin as a sole carbon source it was suggested that chitinolytic enzymes could have contributed to *S. cepivorum* lysis.

The *S. cepivorum* infection process needs to be more clearly understood before the way *T. koningii* modifies this process can be determined. The process of penetration of the root epidermis is well documented (Abd El-Razik *et al.*, 1973; Stewart *et al.*, 1989 a & b) however more information is needed concerning infection of the hypodermis, cortex and stele tissues following penetration. The relationship between infection hyphae and root cell death and tissue maceration needs to be studied in detail. Pectolytic enzymes which degrade plant cell walls are produced by *S. cepivorum in vitro* (Mankarios and Friend, 1980). The nature of the *S. cepivorum* pectolytic enzyme complex needs to be clarified. The relationships between spatial distribution of infection hyphae, cell death, tissue maceration and pectolytic enzyme distribution are studied in Chapter III.

The processes by which *T. koningii* is able to enter *S. cepivorum* infected onion roots, and inhibit the *S. cepivorum* infection need to be established. The physical interactions between hyphae of *T. koningii* and *S. cepivorum* are investigated with a view to understand what feature of *T. koningii* (eg. antibiotics, physical mycoparasitism, cell wall degrading enzymes) causes lysis of *S. cepivorum.* The processes by which *T. koningii* , which does not penetrate the epidermis of healthy onion roots, is able to enter the cortex and attack *S. cepivorum* infection hyphae needs to be established. Histological and enzymatic relationships of these processes are studied in Chapter IV.

Determination of the mechanism of biological control is the first step in understanding how to improve the efficacy of *T. koningii.* The next stage of development is to determine an appropriate method for production and soil augmentation, which is studied in Chapter V. Attempts at biological control of plant pathogens have often failed due to lack of understanding of the environmental conditions which favour establishment and antagonism by the biocontrol agent in the ecological niche in which it must provide control (Adams, 1990; Deacon, 1991). It is necessary to establish the ability of *T. koningii* to become established in the rhizosphere and suppress *S. cepivorum* infections under different soil pH and soil temperature conditions, and to determine how *T. koningii* might interact with *S. cepivorum* infections developing from different depths and varied densities of sclerotia. These properties are studies in Chapter VI. As it is important to be aware of any genetic variation in the pathogen in interpreting trial results from different locations, possible genetic variability in *S. cepivorum* isolates is studied in Chapter VII.

2.0. Literature Review- *Sclerotium cepivorum.* Berk.

2.1 Introduction.

Sclerotium cepivorum is commonly known as *Allium* white rot or Onion white rot. It is a member of the Agonomycetales (Mycelia Sterilia) first described on onions by Berkeley (1841) in the U.K. The fungus has no known teliamorph. Sclerotia are ultrastructurally most similar to *Sclerotinia* spp (Kohn and Grenville, 1989), and immunoblots have demonstrated that *S. cepivorum* sclerotial proteins cross react with antibodies to *Sclerotinia sclerotiorum* proteins while proteins of *Sclerotinia homeocarpa* and *Sclerotium rolfsii* do not (Novak and Kohn, 1991). Ribosomal DNA sequence indicates 98% similarity to fungi of the genus *Sclerotinia* (Carbone and Kohn, 1993).

A review of literature pertaining to control of *S. cepivorum* has been compiled by Entwistle (1990a). *S. cepivorum* is distributed worldwide, a map has been produced of areas of occurrence (Anon, 1990). There seem to be few countries with appropriate crops and climates where the disease is not found (Coley-Smith, 1990). *S. cepivorum* exclusively attacks the genus *Allium* (Coley-Smith and Holt, 1966), which includes onions, leeks, shallots, garlic, and chives. *S. cepivorum* should be classed as a root inhabiting fungus as opposed to a soil dweller (Scott, 1956a).

Initial stages of *S. cepivorum* infection are confined to the host root system (Scott, 1956b). The diseased plants tend to occur in clusters of a few, up to 40 or more adjacent plants and might become infected any time from emergence until harvest. Roots frequently extend horizontally providing a direct path for mycelial growth. Symptoms are not evident until the pathogen has colonised and partially rotted the stem plate or leaf sheaths. As the stem plate, roots and leaf sheaths are killed leaves become yellow and flaccid, ultimately the whole plant dies (Crowe and Hall 1980a).

Sclerotia are produced on the infected tissue and due to their persistence in soil symptoms of disease can occur on land that has been rested from onion production for many years (Coley-Smith 1959). Sclerotia have been recovered from fields where onions have not grown for 15 years (Crowe *et al.*,1980), and have survived burial for 20 years (Coley-Smith *et al.*, 1990). Symptoms are usually worse whenever a field previously infected with white rot is replanted.

2.2. Strains of *S. cepivorum*.

Isolates of *S. cepivorum* display noticeable variation in vigour and pathogenicity (Utkede and Rahe, 1983), it is difficult to distinguish which attributes might be used to distinguish strains from one another. Some observations of criteria which may distinguish isolates are listed in the following paragraphs.

Smith (1972) noted in methods for sclerotia production that it was necessary to place a piece of cellophane on the potato dextrose agar surface or the sclerotia formed within the agar. The isolate of *S. cepivorum* used herein predominantly formed sclerotia amid tufts of mycelium on the agar surface. Abd El-Razik *et al.* (1974) noted a loose correlation between pathogenicity and *in vitro* production of polygalacturonase. Considerable difference has been noted in the time of unsterile soil conditioning required before sclerotia of different *S. cepivorum* isolates (produced by either pure culture or on onion bulbs) could be stimulated to germinate by diallyl sulphide, and in the response of the isolate to freeze conditioning (Brix and Zinkernagel, 1992a). Differences between isolate's inclination to germinate in presence of *Allium* after storage in soil at a range of temperatures have also been noted (Gerbrandy, 1992), as have differences in mycelial growth at different temperatures (Walker, 1926).

2.3. Morphology.

Colonies on potato dextrose agar are white or faintly brownish grey, usually with a fairly even sheet of aerial mycelium but sometimes showing tufts of larger hyphae. Black spherical sclerotia of 200-500 µm diameter develop in diurnal zones.

2.3.1. Hyphal morphology.

Cells of primary hyphae are thin walled at the advancing edge of the colony with dense granular contents, usually 9-18 µm wide and 300-400 µm long, tips are sometimes dichotomously branched before the development of the first septum. Cells of secondary branched hyphae are narrower than those of the primary hyphae. Cell contents often show shrinkage of the cytoplasm when lactophenol blue stains are applied. A phialidic spermatial stage is present and occasionally small aggregates of cells superficially similar to those of the sclerotial rind occur (Mordue, 1976).

2.3.2. Sclerotial morphology.

Sclerotial initials arise from dichotomously branched hyphal tips , usually one initial is involved in the development of each sclerotium. Mature sclerotia show a sharply differentiated rind with evenly thickened strongly pigmented walls, a narrow cortex (2-3 cells thick) of thin walled isodiametric cells and a medulla of intertwined branched hyphae of smaller diameter than primary hyphae. Cortex and medulla cells show granular contents, rind cells do not. Inter-hyphal spaces of sclerotia contain a gelatinous material (Mordue , 1976).

The sclerotial walls are pigmented with melanin, sclerotia are 0.3 to 0.6mm in diameter and have a thin outer rind consisting of two layers of pigmented cells and a medullary tissue of thick hyphae (Coley-Smith and Cooke, 1971). No remnants of host tissue were seen inside sclerotia (Backhouse and Stewart, 1987).

The cells of the rind region are isodiametric and thick walled. The medullary hyphae are loosely arranged in a matrix of extracellular material and contain large vacuoles, the contents of these was not identified. The main material found in the cytoplasm which could function as a nutrient reserve, was strongly acidic protein identified by metachromatic staining with toluidine blue. Staining of the medulla with the periodic acid schiff reaction (to distinguish carbohydrates) indicated that intracellular polysaccharides were not a major component of the reserves of sclerotia of *S. cepivorum* (Backhouse and Stewart, 1987).

A larger sclerotial form (up to 1.5 cm diam) was first reported in Egypt (Georgy and Coley-Smith, 1982). It was postulated to be a vestige of the apethecium producing ancestors of *S. cepivorum* by Backhouse and Stewart (1988) who found it in New Zealand. The large sclerotial form has also been reported in North America (Crowe, 1995a), and was recently found in Tasmania (Chapter 7: Isolate variability).

2.4. Physiology.

2.4.1. Mycelium.

Mycelium appears as septate white hyphae. Hyphae tend to spread only a very short distance from the roots of plants and germinating sclerotia and cannot grow saprophytically so plants or their roots must be growing extremely close together for infection to spread from one plant to another (Scott 1956b). Hyphae may irradiate 1-2cm through the soil from roots (Crowe and Hall 1980b).

Mycelium can grow further in course textured soil in search of a host than in fine textured soil. Scott (1956a) measured growth from a cube of onion scale into unsterile soil particles of average diameter 4-7, 2-4, and 0-2 mm in diameter. At 6 days on King Norton's loam particles mycelium had extended 15 mm in the coarser division, 8.5 mm in the medium division, and 2.5 mm in the small division. In all cases hyphae died after 10 days in the absence of a host. Hyphae could not colonise unsterile soil from agar inocula.

The mycelium gives rise to micro-conidia which have never been known to germinate (Coley-Smith 1960). These are very small and are born on flask shaped phialides on short hyphal side branches. Sometimes these are produced on cushions or sporodochia. Their function is unknown.

2.4.2. Sclerotial physiology.

Sclerotia from field infected onion bulbs were incubated in waterlogged muck soil, and in dry laboratory conditions in muck soil for 8 months. The sclerotia from the wet soil displayed a hollow medulla region, whereas those from the dry soil had a centre filled with dense mycelium. Sclerotia from soil that had not grown an onion crop for three years showed considerable loss of the hyphae in the medullary region (Backhouse and Stewart, 1987). Leggett and Rahe (1985) suggested that this effect may be due to the increased rate of sclerotial decay in waterlogged soil. Backhouse and Stewart (1987) noted that the outer rind often contained dead or broken cells and New *et al.* (1984) reported collapsed surface cells by Scanning Electron Microscope observation. In healthy sclerotia, Backhouse and Stewart (1987) reported that an entire intact layer of healthy rind cells enclosed the medulla, implying that when rind cells die, medullary cells beneath differentiate to form new rind cells. This type of observation coupled with the loss of medullary tissue with ageing implies that the sclerotia do not become entirely dormant, but undergo a constant process of maintaining the integrity of the rind to protect the hyphae within. Comparison of S. E. M. micrographs of sclerotial surfaces revealed that field sclerotia had a more pitted, broken surface compared to culture sclerotia (Littley and Rahe, 1992).

2.4.3. Production of sclerotia.

Investigation of the stimulus for sclerotium production has looked into variables including culture age, nutrient availability, accumulation of staling products, mycelial injury, and barriers to colony expansion. Studies by Littley and Rahe (1991) concluded that the stimulus for sclerotium formation is provided by

restriction of growth, and this may be induced by physical, nutritional, competitive or antagonistic factors. The experiments of Littley and Rahe (1991) were conducted in potato dextrose media, it has not been determined whether growth restriction remains the stimulus when *S. cepivorum* utilises its normal polysaccharide carbon sources by production of polygalacturonases, pectinesterases, and cellulases (Abd-El-Razik *et al.*, 1974; Mankarios and Friend, 1980). Sclerotia produced in culture differ to those produced under field conditions, such as in structure of the rind, which is thicker and more fractured in laboratory sclerotia. Laboratory sclerotia may exhibit differences in germination responses due to differing environmental conditions at the time of formation (Littley and Rahe, 1992).

Sclerotium production could be greater at different stages in the development of the crop. Many plants do not become infected until late in the season, when plant size provides a large nutrient base for the production of sclerotia (Crowe *et al.*, 1980). The larger root system of older plants facilitates greater plant to plant spread. There is a possibility of sclerotia produced early in the season being stimulated to germinate later in the same season (Crowe *et al.*, 1980) having an exponential effect on inoculum production.

Methods for sclerotium production under unsterile conditions for experimental purposes have been developed (Coley-Smith, 1985).

2.4.4. Secondary sclerotia.

Sclerotia which germinate from appropriate stimuli which do not find onion roots to infect, may form secondary sclerotia. These may form near or even inside the rind of the decaying germinated parent sclerotium (Entwistle and Munasinghe, 1981a). Somerville and Hall (1987) reported that secondary sclerotia are smaller (0.09-0.35 mm) than primary sclerotia (0.2-0.6 mm), some rinds failed to melanize fully, and the rind was one or two cell layered and more pliable than in a primary sclerotium. Secondary sclerotia with partially melanized rinds failed to germinate. Secondary sclerotia were only formed after sclerotia germinated eruptively. Secondary sclerotia formed within 30 days of a six hour exposure to allyl sulphide. An average of 2.8 secondary sclerotia were formed per primary sclerotium, optimal temperature for this formation was 15-21°C, formation routinely occurred on media devoid of nutrients, and on water agar in the presence of allyl sulphide, and on plain glass cover slips. Eight out of sixty six secondary sclerotia that germinated eruptively after 48 hrs exposure to allyl sulphide on nutrient agar, were able to form tertiary sclerotia (Somerville and Hall, 1987).

2.4.5. Sclerotial dormancy & germination relationships.

Two types of sclerotial germination are reported. In the field sclerotia germinate by a plug of mycelium which erupts through the rind to infect onion roots (Coley-Smith, 1960). Under sterile conditions individual hypha are reported to emerge through the rind and grow out until the sclerotium is surrounded by them (Coley-Smith *et al.*,1967; Adams and Papavizas, 1971). The plug of mycelium which erupts through the rind originates from the cells directly beneath the rind (Backhouse and Stewart, 1987).

2.4.5.1. Stimulation to Germinate.

The stimulus for *S. cepivorum* sclerotia to germinate is chemical (Coley-Smith and Hickman, 1957). The stimulus is non specific in sterile soil (Coley-Smith *et al.*, 1967). The tip of the onion root encourages sclerotium germination more than the rest of the root (Coley-Smith, 1960; Coley-Smith and Cooke, 1971). The active compounds which stimulate germination were identified as volatile n-propyl and allyl sulphides (King and Coley-Smith, 1969). Merriman *et al.* (1981) reported that diallyl-disulphide and artificial onion oil, both diluted to 0.1% of original concentration and applied to soil, reduced recovery of sclerotia buried at 10cm by 75%. These compounds were as effective at inducing sclerotia to germinate at 0.1% as at 5% of the original materials concentration. Diallyl disulphide is of disease control significance and will be discussed later.

2.4.5.2. Dormancy in the Life Cycle.

The sclerotia are normally held in fungistatic dormancy in non-sterile soil but germinate in specific response to *Allium* spp. Dormancy of sclerotia produced in culture can be broken immediately by abrasion of the rind (Coley-Smith 1960). Control of dormancy is both exogenous because it appears to be expressed by soil mycostasis and is constitutive in that it is an innate property of the life cycle. The physical nature of the rind may play a role in maintaining dormancy. The exact mechanism by which exogenous dormancy is broken is not known (Coley-Smith and Cooke, 1971).

Under no set of environmental conditions will all the sclerotia in a soil sample germinate at once, and this has merit as a survival mechanism in itself (Coley-Smith and Cooke, 1971). Viability of sclerotia was unaffected by two years of laboratory dry storage; the pH and soil nutrient status had no effect on persistence

of sclerotia (Coley-Smith, 1959). Sclerotia produced on laboratory media do not germinate in response to *Allium* spp. for several weeks after they have been added to soil (Coley-Smith, 1960). Thirty days in unsterile soil can remove constitutive dormancy (Avila de Morena, 1991a). Sclerotia produced on onion bulbs in the field also undergo a period of constitutive dormancy. After four months burial percentage germination increased from 5% to 84.5% (Leggett *et al.*, 1983).

2.4.5.3. The release of sclerotial dormancy.

Coley-Smith *et al.* (1987) observed that like culture sclerotia, the sclerotia produced on onion bulbs underwent a period of constitutive dormancy during which low proportions of sclerotia could be induced to germinate using diallyl disulphide. This variable period was usually overcome by three months of incubation (conditioning) in unsterile soil. The dormancy tended to remain if the sclerotia were stored in sterile conditions (Coley-Smith *et al.*, 1987).

An alternative method for breaking constitutive dormancy was proposed by Elnaghy *et al.* (1971) who suggested that alternate freezing and thawing of sclerotia for 1 week might break constitutive dormancy. Brix and Zinkernagel (1992a) compared ten pure culture isolates of *S. cepivorum* under three treatments; (a) no conditioning, (b) freezing at -18°C for 20 hrs then thawing at 20 degrees for 4 hrs over three consecutive days, (c) soil conditioning treatments at 15°C in unsterile soil for 12 weeks. Sclerotia were incubated and percentage germination calculated in presence and absence of diallyl sulphide following these treatments. There was broad variability among isolates, though several general trends were evident. In absence of diallyl sulphide, up to 40% of sclerotia germinated after the freezing treatment, but a maximum of 6.3% germinated after the soil conditioning. In presence of diallyl sulphide, all isolates displayed higher percentage germination after soil conditioning than freezing, even though a few isolates displayed high germination with no conditioning but low germination after the freezing treatment. Overall the study indicated that each isolate must be considered independently, and that results obtained using one isolate can not be considered to pertain to any other isolate.

There have been some contradictions in literature concerning germination of sclerotia under pure culture and non-sterile conditions. A higher proportion of sclerotia from pure culture tend to maintain dormancy when exposed to *Allium* extracts in some (Coley-Smith, 1985; Coley-Smith *et al.*, 1987) , though not all experiments (Somerville and Hall, 1987; Brix and Zinkernagel, 1992a).

Isolates of *S. cepivorum* appear to display variations in the time of conditioning required for release of constitutive dormancy. For example, of four

isolates from which sclerotia were produced in pure culture, one (12/85) displayed 87.5 % germination after 0 weeks buried in unsterile soil and increased to 100 % germination by 6 weeks, another isolate (17/86) took until 10 weeks to display 88.9 % germination (Brix and Zinkernagel, 1992a). The variation was also evident for sclerotia produced on onion bulbs, though usually less than half the number of sclerotia of all four isolates produced on onion bulbs germinated when exposed to diallyl sulphide compared to those from pure culture after unsterile conditioning treatment from 0 - 12 weeks.

2.4.5.4. Sclerotium non-specific germination and decay.

Sclerotia decay shortly after germination and they do not get a chance to re-germinate if successful location and infection of a host is not achieved (Coley-Smith, 1959). Some level of germination does occur in the soil spontaneously, referred to as non-specific germination (Crowe and Hall, 1980b). Locke (1967) reported up to 17.5% of sclerotia germinating in this manner at 15°C in moist non-sterile soil (time period not specified), and this dropped to 2.5% at 10°C and 3% at 20°C, suggesting a temperature dependent effect. Tests comparing germination with and without garlic extracts showed that 2 to 6 out of 20 sclerotia germinated in absence of garlic as opposed to 15 to 20 with garlic (Crowe and Hall, 1980a). The influence of non susceptible plants was investigated (Coley-Smith and Holt, 1966), none were found to influence the germination of sclerotia. Non-specific germination in absence of a suitable host could account for reduction in inoculum over long time periods, but would do little to reduce inoculum potential in the short term.

The rate of sclerotial decay appears to be affected by temperature and moisture, and both factors are inter related. For example optimum temperature for sclerotium germination was 15 to 18°C, and non-specific germination occurred in a temperature range from 9 to 24°C (Crowe and Hall 1980b). Non-specific germination at all temperatures decreased as matric potential became drier than -300 millibars (field capacity) and also decreased as soil became wetter than this. Optimum temperature for germination also depends on the temperature history of the sclerotia. After storage at 5°C for 58 days fastest germination occurred in the 15-20°C range, whereas for sclerotia conditioned at 20°C, germination was highest in the at 10 and 5° C range (Gerbrandy, 1989).

While there is a range where non-specific germination and subsequent decay occurs, further decay seems to be brought about by extremes of wetting and drying. Smith (1972) reported that after 20 hrs of drying at 30°C and 30% relative humidity followed by 3 weeks incubation on moist filter paper, 85% of *S. cepivorum* sclerotia decayed. When sclerotia were air dried for 48-72 hours before being placed on the

soil surface in mesh bags for 4 months, only 80% of the sclerotia were recovered of which only 26.8% were viable, compared to 60 % where sclerotia were not dried before placement on the soil surface. A similar trend was observed for buried sclerotia, though percentage recovery was slightly higher for buried sclerotia (Leggett *et al.*, 1983). Further investigation of the effects of drying of sclerotia was made by drying sclerotia for 24 hrs over $CaSO_4$ in a desiccator, then weighing before drying in an oven at 160°C for 4 hrs and weighing again. It was determined that 24 hrs drying at air temperature in the desiccator had removed 97.7 % of the moisture content of the sclerotium (Leggett and Rahe, 1985), indicating a poor ability to retain moisture in dry air which can lead to loss of viability in appropriately dry conditions. Sclerotia which were air dried in the desiccator lost a large proportion of Gram negative bacteria from their surface mycoflora in comparison to sclerotia taken directly from onion bulbs. When air dried sclerotia were placed in unsterile soil for 96 hours, a large increase in surface mycoflora (predominantly *Trichoderma* spp.) was detected. This increase was not found when undried sclerotia direct from onion bulbs were incubated in the same soil for 96 hours. These results would tend to indicate that disruption of the sclerotial surface mycoflora could create a niche for opportunistic mycoparasites as an additional mechanism by which sclerotial drying could decrease survival. Colonisation of the sclerotial surface after drying would be aided by the nutrients which are leaked from the sclerotium in the hours after the period of drying. For example Coley-Smith *et al.* (1974) demonstrated increased leakage of ^{14}C labelled assimilates following periods drying longer than one day. In contrast, some workers who have performed similar experiments (Coley-Smith *et al.*, 1974; Papavizas, 1977) reported no sclerotial decay after 3 months burial. Coley-Smith and Sansford (1986) found that decay of sclerotia seemed to be associated with the prolonged waterlogging of the soil in the winter. Alexander and Stewart (1994) found that a large proportion of sclerotia (above 80%) decayed in the first three months following burial, whereas almost all of those which survived this period tended to remain viable after 11 months burial.

Some hints toward enhancing sclerotial decay exist. The stress resulting from sublethal heat injury during solarization or composting may weaken sclerotia of *S. cepivorum*, and decrease viability (Porter and Merriman 1983). Weakened sclerotia are more readily attacked by antagonists such as *Trichoderma harzianum* (Henis and Papavizas 1983).

2.5. Pathogenicity.

2.5.1. Inoculum density.

Inoculum density is not a finite determinant of disease as sclerotial germination is governed by conditioning, age, temperature, root exudates, microflora and many other factors. In one study, soil infested by 0.2 sclerotia per gram induced a 94.9% crop loss (Crowe *et al* .,1980), while five hundred times this level of inoculum was required to cause similar levels of infection in another study (Adams and Papavizas, 1971). Soil infested with between 0.001 and 1.0 sclerotia per gram of soil contained between 1.0 and 10.0 sclerotia /gram when the crop was harvested (Crowe *et al* ., 1980). Disease is also known to increase with increasing plant density due to increased root to root spread (Littley and Rahe, 1987).

2.5.2. Sclerotial Depth

Symptoms of infection tended to appear later in the season as the depth of inoculum burial increased, no infection was recorded when sclerotia were buried at 45cm (Crowe and Hall 1980a). Later infections have a larger host plant biomass to grow on and higher sclerotium production results. Crowe and Hall (1980a) found that infection in garlic was worse when sclerotia were 5cm deep in the soil and suggested that increased incidence was due to the high root density at this depth, which facilitated root to root spread as described by Scott (1956b).

When initial infection occurred within 1cm of the base plate, little spread in the crop resulted (Crowe and Hall, 1980a).

2.5.3. Temperature, Moisture and pH.

Walker (1926) reported most severe disease development in the 14 to 18°C range. However, temperature relationships for sclerotial germination vary according to the temperature history of the sclerotia, and the isolate under study (Gerbrandy, 1989; Gerbrandy, 1992). Once sclerotia germinate and infect plants, time for symptom development decreases with increasing temperature. Live mycelium (as pre germinated sclerotia) of *S. cepivorum* was introduced to healthy onion plants at temperatures ranging from 6 to 24°C. Infection symptoms took 5 times as long to be seen at 6°C than at 24°C (Crowe and Hall, 1980b).

Sclerotium germination can occur in soil of matric potential no drier than -300 millibars, although pre-germinated sclerotia can infect onion seedlings at

appropriate temperatures at matric potentials as dry as -3 bars (Crowe and Hall, 1980b).

Sclerotial germination was found to be greatest in culture media and soils of pH 5.0-5.5 (Adams and Papavizas, 1971). This result is taken in some caution as 5.4 was the original soil pH, and pH treatments were lowered or raised using $AlSO_4$ or lime, which may have altered germination relationships.

2.5.4. Pre-penetration growth on Host.

The pre-penetration growth of *S. cepivorum* on the epidermis of sterile onions which have been artificially inoculated is similar to pre-penetration growth on the surface of field grown infected onion roots (Stewart *et al.,* 1989b). *S. cepivorum* displays a distinctive pattern of growth on root tissue. Hyphae are closely appressed to the host surface and tend to grow along the lines of the longitudinal epidermal cell walls, often producing side branches which grow obliquely to these. When several branches occur in close proximity they tend to grow in all directions, these branches are often short, stubby, curved, and coiled, and closely septate (Abd-El-Razik *et al.*, 1973; Stewart *et al.*, 1989b).

Flentje (1957) suggested that a diffusible material might be present at a higher concentration between longitudinal cell wall junctions of plant roots and be responsible for attracting hyphae. Observations of *S. cepivorum* on onion roots would tend to support the hypothesis of Flentje (1957). Both stem and root tissues secrete a compound which induces the fungus to attempt penetration (Stewart *et al.*, 1989a), and is released in high concentration at the stem base. Coley-Smith (1960) has noted the root tip region to be the most stimulatory part of the onion to sclerotium germination.

2.5.5. Penetration of the roots:

Abd-El-Razik *et al.* (1973) reported that penetration occurred in several ways, the first being the formation of an infection cushion by one of four means: (1) a single hypha curling back and forth to form a knotted mat, (2) the formation of short stubby side branches that could aggregate to form a tuft-like infection cushion, (3) two or more hyphae with short stubby side branches aggregating to form an infection cushion, and (4) mass tangling of several hyphal tips curling back and forth.

The second means of penetration described was the formation of a simple appressorium from the swollen tip of a single hypha (Abd-El-Razik *et al* ., 1973). A third means of penetration was described by Stewart *et al* . (1989a) where the tips of

hyphae would grow between the junctions of cell walls , seemingly breaching the cuticle without formation of a penetration peg. Infection hyphae arising from this type of penetration are restricted to the upper walls of the epidermis, just below the cuticle. When this type of penetration occurs at the transition zone between root and stem, a club shaped swelling was reported immediately before penetration (Stewart *et al* . 1989a). Abd-El-Razik *et al.* (1973) reported that penetration never occurred via stomata even though hyphae were seen to grow across these openings.

No distinction was made between the different types of infection structures role in penetration in the study of Abd-El-Razik *et al.* (1973). Stewart *et al.* (1989b) reported that infection cushions were not formed on the roots, but only on tissue with a cuticle (ie the base of the stem and the leaves) and suggested that the cuticle offered a lot more resistance to penetration. The inability of a single hyphal tip to penetrate the cuticle possibly initiates a process of dichotomous branching resulting in a more complex infection structure which provides either greater mechanical force and/or a greater concentration of enzymes necessary for successful penetration. A mucilaginous material seems to be present in the infection cushion which could be involved in adherence to the host (Stewart *et al.*,1989a). Where an appressorium or infection cushion was adhered poorly to the host, penetration was not successful (Stewart *et al.*, 1989b). The reason for poor adherence was not determined.

2.5.6. Mechanism of Penetration:

The exact mechanism of penetration of the root epidermis by *S. cepivorum* has not been determined. Mechanisms of penetration which have been suggested for other pathogens include mechanical pressure (Politis and Wheeler, 1973), enzymatic dissolution (Rijkenberg *et al* ., 1980), and a combination of both (Sharman and Heale, 1977).

A discolouration of the infected tissues was reported by Abd-El-Razik *et al.* (1973) in tissues which appeared hydrolysed below infection cushions, which supports the enzyme activity hypothesis. Nevertheless the importance of adhesion shown by other workers supports the importance of mechanical penetration. Abd-El-Razik *et al.* (1973) reported no discolouration and death below single appressoria, and when Stewart *et al.* (1989b) removed infection cushions and appressoria from the host epidermis they observed ragged cuticular edges forced inward by the penetration peg. It seems likely that a combination of the two methods of penetration is involved.

2.5.7. Internal Infection.

Limited work has been done on exactly what happens after *S. cepivorum* enters the roots of the host. According to Abd-El-Razik *et al.* (1973) the penetration peg (which is narrower than the hyphae) enlarges to the normal diameter and grows both intra and intercellularly from one parenchyma cell to another completely disintegrating tissue. Ultimately, infection spreads to the vascular elements resulting in the death of phloem cells, this is followed by growth up into the stem to infect the stem and leaf base. As the infection advances, cell wall decomposition becomes evident in the cortical region, and parenchymatous cells in the stem and root cortex become completely disintegrated soon after invasion. Where disease spreads to leaves, the hollow regions of the leaf are free from mycelium (Abd-El-Razik *et al* ., 1973).

2.5.8. Oxalic acid production:

S. cepivorum produces oxalic acid in culture and in infected onion tissue (Stone and Armentrout, 1985). Culture filtrates of sodium polypectate medium on which *S. cepivorum* had been grown for 16 days contained 1.8 mg of oxalic acid / ml. Heat killed onion roots on which *S. cepivorum* had been grown for 16 days contained 13.6 mg of oxalic acid per gram dry weight of tissue. Non-inoculated onion tissue contained no detectable oxalic acid. Concentration of oxalic acid in inoculated live roots increases with time. Over 16 days, production of oxalic acid was highest in the first four days. Maceration of tissue increased in equivalent proportions to the concentration of oxalic acid in the root (Stone and Armentrout, 1985).

A species of the same genus, *Sclerotium rolfsii*, is known to produce oxalic acid in infected tissue (Punja and Jenkins, 1984). Oxalic acid and polygalacturonase are produced simultaneously by this pathogen, which cannot hydrolyse calcium pectate in absence of oxalate ions. Production of oxalic acid by *Sclerotium rolfsii* in infected tissue would create an acid environment favourable for polygalacturonase activity as well as sequestering calcium from the pectates of the cell walls (Bateman and Beer, 1965). Oxalic acid secreted by the pathogen must sequester calcium from other sources resulting in calcium oxalate formation (Rao and Towari, 1986). Calcium oxalate crystals are tetragonal to prismatic in shape and there is a consistency in size and shape of calcium oxalate crystals (Urbanis *et al* .,1978). Tetragonal crystals were present in tissue infected by *S. cepivorum* (Stewart *et al.*, 1989b); the identity of the crystals was not positively determined but were suspected to be oxalic acid.

Oxalic acid is also produced by the pathogen *Sclerotinia sclerotiorum* (Noyes and Hancock, 1981). Oxalic acid was detected in sap of *S. sclerotiorum* infected sunflower plants; the pH of sap taken from 2cm above an infection site was one pH unit lower than sap taken from a healthy plant. Presence of oxalic acid in leaf tissues was also correlated with wilting of leaves.

Oxalic acid may also have a role in pathogenesis by effecting the balance between cytoplasm soluble (cytosolic) calcium and extracellular calcium (Pluim *et al.*, 1994). Extracellular calcium, through calcium pectates, functions in cementing together adjacent cells. The divalent calcium cation is important for stabilising membrane structure by binding to negatively charged phospholipids on the exterior membrane surface. Cytosolic calcium is known to stimulate plant senescence by binding to calmodulin (a protein which activates many lipolytic enzymes) and is involved in many cellular processes (Poovaiah, 1985). Oxalic acid may also have a role in suppression of plant defence compounds by o-diphenol oxidase inhibition (Ferrar and Walker, 1993).

2.5.9. Pectolytic Enzyme Production.

The earliest report of pectolytic enzyme production by *S. cepivorum* was made by Abd-El-Razik *et al.* (1974). A range of isolates were tested for pathogenicity, and effects of media pH and temperature on polygalacturonase and pectinesterase production. Pectinesterase activity was measured by titration of carboxyl groups freed by hydrolysis of ester linkages by pectinesterase in culture filtrates. Reduction of viscosity in 1% pectin solution was used to assess polygalacturonase activity. Optimum temperature for polygalacturonase and pectinesterase production was in the 15 to 20°C range for all isolates. Optimum pH varied between isolates in the 3.0 to 7.0 range for polygalacturonase production, though all isolates displayed optimum activity of pectinesterase at a pH of 4.5. This study was undertaken prior to discovery of oxalic acid's involvement in the *S. cepivorum* infection process (Stone and Armentrout, 1985). It was not determined whether the different *S. cepivorum* isolates were successful in modifying the pH of the medium by secreting oxalic acid, which may be a reason for the polygalacturonase activity which varied with medium pH. By comparing enzyme activity to a pathogenicity disease index, Abd-El-Razik *et al.* (1974) made the preliminary conclusion that differences in polygalacturonase production, but not pectinesterase production were correlated with pathogenicity.

Two polygalacturonases are produced by *S. cepivorum* in culture. These enzymes cause ion leakage, cell wall collapse, and cell death in onion epidermal tissue (Mankarios and Friend, 1980). *S. cepivorum* has the ability to degrade

carboxy-methyl cellulose, xylan, galactan, araban, sodium polypectate, and citrus pectin in solution (Mankarios and Friend, 1980).

Metcalf (1993) showed, using electrophoresis by the method of Cruickshank and Pitt (1987), that an isolate of *S. cepivorum* from Tasmania (Sc-1), grown in citrus pectin containing culture media, produced two isozymes of polygalacturonase and a pectin esterase. By using the same electrophoresis method, but with infected onion root segments added to the electrophoresis gel wells, it was shown that these proteins are present within the onion root tissue ahead of the infection hyphae following penetration of the root epidermis, associated with disintegration of cell walls before direct contact with infection hyphae. The enzymes were present up to 12 mm ahead of hyphae however their activity was greatest in root segments colonised by hyphae.

S. cepivorum produces polygalacturonases which attack the terminal linkages of the pectin chains (exopolygalacturonases) and polygalacturonases which can break pectin chains in at any location (endopolygalacturonases). Favaron *et al.* (1993) found that *S. cepivorum* endopolygalacturonases tend to predominate over exopolygalacturonase. This was based on data obtained by measuring the reducing groups in aliquots taken from a viscosimeter at the time of 50% decrease in viscosity.

Favaron *et al.* (1993) found that onion and leek cell walls contain components (which they named Polygalacturonase Inhibitor Proteins) which inhibit the activity of polygalacturonases of a number of pathogens. This inhibition was assessed to be around 90% in the case of *Sclerotinia sclerotiorum* and *Macrophomina phaseolina*, though it was lower for *S. cepivorum* (10% for onions and 20% for leeks). This inhibition was destroyed after heating the cell wall extracts at 100 degrees for 20 minutes to denature proteins.

2.5.10. Other enzymatic weapons:

S. cepivorum produces cellulase, xylanase, galactanase and arabanase (Mankarios and Friend, 1980).

2.5.11. Spread of the Pathogen:

Harrison (1954) reported that *S. cepivorum* is spread by dissemination of sclerotia from infected bulbs and seedlings, adhering to farm implements and machinery, in used onion bags, in excreta of farm animals fed on diseased bulbs, and by wind and running water. No experimental results are given in support of these conclusions.

In the south east of England continuous cropping of infested land has encouraged the development of this disease problem (Coley-Smith 1990). Scott (1956a) demonstrated that *S. cepivorum* cannot spread saprophytically in soil, so spread is accomplished by transportation of the dormant sclerotia. Crowe and Hall (1980a) reported that plant to plant spread in the crop depends on the time and the depth of infection, root density, root distribution, and the number of individual root infections. The number of individual root infections depend on the root density and the inoculum density.

2.6. Control.

2.6.1. Detection of sclerotia in soil.

Samples of infested soil can be washed through two stacked sieves (0.595 mm openings and 0.210 mm openings) and the residues on the 0.210 mm sieve transferred to columns containing 2.5 M sucrose solution. After 2 hours the fractions collected in the upper portion of the sucrose solution were collected and washed in water on 0.210 mm sieves and examined by dissecting microscope to distinguish sclerotia from charcoal fragments (Utkede and Rahe, 1979).

2.6.2. Solarization.

Porter and Merriman (1985) have achieved some success in Australia with this method. Soil is covered in black plastic sheets to conserve solar radiation thus raising the temperature to levels lethal to sclerotia or more favourable for antagonistic microbes including *Trichoderma* spp. Obviously this method has limited usefulness for cooler climates.

Southern blight of peanuts caused by *Sclerotium rolfsii* was reduced from 65% to 8% infection by soil solarisation after soil was moistened by a single irrigation (Gristein *et al.*, 1979). A review of use of soil solarisation for the control of soil-borne pests has been compiled by Katan (1981).

Evidence exists that it may be possible to pre-condition sclerotia to germinate by temperature treatment, 75% of sclerotia which were pre-incubated at 30°C for 28 days, then incubated at 10°C for 50 to 60 days were induced to germinate in absence of *Allium* exudates. Only 15% of sclerotia which were not pre-incubated at 30°C before the same 10 degree incubation germinated. In general a period of incubation at high temperature followed by incubation at lower temperature stimulated germination of all of the isolates that were tested (Gerbrandy, 1992).

2.6.3. Flooding.

There may be some potential to de-infest fields by flooding, as prolonged periods of waterlogging have been associated with decline in sclerotial populations. In on study ten nylon covered PVC canisters each filled with 1000 sclerotia were buried at a depth of 10 cm in field soil which was subjected to flooding from April to October in 1992, 1993, both years, or not flooded (control), four replicates of each treatment were performed. After 1 month burial prior to flooding 920 (mean) sclerotia per canister were recovered. In the treatment flooded in both years, the number of viable sclerotia was 8 in October 1992 (survival in unflooded control: 455) , and 0 in October 1993 (survival in unflooded control: 422). However, in the treatment flooded in the 1993 season only, less viable sclerotia were recovered in the unflooded control (422) than in the treatment (670) (Crowe 1995b). These results could reflect a greater predisposition for sclerotial decline in the first twelve months after formation, other workers have reported extensive sclerotial decay in this period (Alexander and Stewart, 1994) or the results may be due to seasonal difference and random variation.

2.6.4. Breeding for resistance.

Breeding for resistance is likely to integrate well with the use of fungicides for effective control of *S. cepivorum* . (HRI) Wellesbourne is conducting a program involving challenge of cultivars with the disease (Bleasdale, 1992). Two types of resistance mechanisms to white rot have been identified. The first, non-stimulatory resistance, occurs where the stimulatory capacity of the root exudates is too weak to induce the sclerotia to germinate and attack the roots. Rahe (1981) in Canada showed a resistant cultivar to be less stimulatory to sclerotial germination than other cultivars. Greenhouse tests of 150 seedlots of *Allium cepa* and 30 different *Allium* spp. showed that low levels of disease incidence were correlated with production of root exudates that stimulated germination only weakly. Lower levels of flavour and odour compounds are associated with weak capacity to stimulate sclerotia to germinate, though sclerotial germination in root zones of plants originating as sets, is much faster than in those originating from seed (Coley-Smith *et al.*, 1987). The second mechanism is based on a tissue reaction which does not influence the number of infection sites but impedes the spread of the infection in the plant (Rahe, 1981; Brix and Zinkernagel, 1992b). One unidentified *Allium spp* . 'PI 259549' induced high levels of germination of sclerotia but exhibited low levels of infection. It was suspected that this species had some sort of tissue reaction resistance (Brix and Zinkernagel, 1992b).

suspected that this species had some sort of tissue reaction resistance (Brix and Zinkernagel, 1992b).

2.6.5. Sclerotium Germination Stimulants.

These compounds are imitations of the exudates of onion roots and when applied to the soil they trigger sclerotia to germinate. In absence of a suitable host, the hyphae from the germinated sclerotium die, thus reducing the *S. cepivorum* inoculum density. The most effective compound for this purpose developed so far has been diallyl-disulphide, which has been used to induce significant reductions in sclerotial numbers at certain concentrations. Summer treatments tend to provide smaller reductions than at other times of the year, which might be related to soil temperature (Coley-Smith *et al.*, 1986).

In another study which used garlic extract (which contains diallyl disulphide) to stimulate germination, it was found that soil needed to be wetter than -300 millibars, and warmer than 9°C. At a matric potential of 0 and temperature of 27°C, 100% of buried sclerotia germinated and a clear relationship between sclerotium germination and soil moisture content and temperature was shown (Crowe and Hall, 1980b). This use of sclerotial germination stimulants warrants further investigation as it can certainly act to reduce sclerotium numbers drastically. Coley-Smith (1990) describes much reduced disease incidence and marked increase in crop yield following diallyl disulphide treatment of soil.

2.6.6. Chemical Treatments.

2.6.6.1. Fungicides.

These are a fairly effective short term solution to the white rot problem, but cannot be relied upon in the long term due to problems such as development of resistance by the pathogen, build up in the soil of fungicide degrading micro-organisms, and withdrawal of chemicals from the market on safety grounds (Lacey and Wong, 1991). Chemically treated produce may contain residues and is less marketable in many parts of the world, including the United States and several European Economic Community nations. The Tasmanian and Victorian onion industries have successfully used Procymidione (Sumisclex 500WP ICI) to control white rot . Porter (1988) reported that seed dressings, surface sprays at sowing, and a surface spray at 10 weeks provided the best control. A research program based on chemical control has been underway in Tasmania since 1983 and led to registration of this chemical. (Wong and Maynard 1986). In the 1995/6 season the Tasmanian

onion industry ceased use of Sumisclex predominantly due to chemical residue considerations. The current recommendation is 1L Folicur 430 (Tebuconazole) applied in 125 kg of lime super per hectare at the time of sowing. It would appear that post emergence sprays of Folicur are not able to prevent infections, but are able to prevent a large portion of infected onions from rotting (Dennis, 1996). There have been several examples of this Folicur Lime Super treatment breaking down (ie not providing economic control) in commercial crops in the 1996/7 season (D. Fawkner, Clements Marshall Pty. Ltd.).

Avila De Moreno (1991b) tested Iprodione, Vinclozolin, and Carbendazim against *S. cepivorum* infection of garlic. Vinclozolin and Carbendazim applied 45 and 75 days after sowing gave the best control. Chemical treatments for control of *S. cepivorum* have been reviewed in detail by Entwistle (1990), who suggests that Iprodione may lose efficacy due to resistance in the pathogen and Dichloran has been rapidly degraded by microbes.

2.6.6.2. Soil Partial Sterilants.

Complete sterilisation is not practical for field soils, though some success has been obtained with soil partial sterilants, for example the methyl-isothiocyanate-liberating compounds such as metham sodium and Dazomet (Coley-Smith 1990). Invariably some sclerotia tend to survive the treatment (Coley-Smith, 1985).

Metham (Vapam) applied through sprinkler irrigation at a rate of 243 l/ha with 2.5 cm of water provided 94% control of white rot on bunching onions. The cost of this treatment was $383 (US, 1983), and it increased the value of yield per hectare from $4,449 to $37,686 (Adams and Johnstone ,1983).

2.6.7. Biological Control.

S. cepivorum offers three main opportunities for biocontrol; microbes which parasitise mycelium, microbes which parasitise sclerotia and microbes which convert onion root exudates to compounds other than those which stimulate sclerotial germination. Ghaffar (1969a) screened 123 fungi, 17 bacteria and 22 actinomycetes for *in-vitro* antagonism against *S. cepivorum*. The list of genera found to inhibit growth of *S. cepivorum* included *Trichoderma, Coniothyrium, Fusarium, Gliocladium, Aloeurisma, Cladosporium, Penicillium, Tilachlidium,* and *Helminthosporium. T. viride* coiled on *S. cepivorum* hyphae and *C. minitans* parasitised sclerotia. Jackson *et al* (1991a) reported *in vitro* screening methods for *S. cepivorum* antagonists. *Trichoderma pseudokoningii, T. viride, and Gliocladium virens* were identified as potential biocontrol agents. Subsequent studies of

nutritional means of enhancing antagonism by these agents against *S. cepivorum* indicated most suitable nitrogen sources were L-alanine, and bacteriological peptone, and most suitable carbon sources were glucose, galactose and sucrose (Jackson *et al.*, 1991b). *In vitro* screening for antagonists was also reported by Harrison and Stewart (1988) who identified *Trichoderma viride, Gliocladium virens, Coniothyrium minitans, Gliocladium roseum, Chaetomium globosum,* and *Penicillium expansum* isolates with potential for biocontrol based on inhibition and parasitism of mycelium and sclerotia. *In vitro* screens reported by Garcia (1992) obtained complete inhibition of radial growth of *S. cepivorum* by *Trichoderma* spp. after 10 days incubation in dual culture. Moubasher *et al.* (1970) raised the issue that *Allium* root exudates may inhibit spore germination of biocontrol fungi.

Penicillium nigricans reduced white rot incidence when agar cultures were incorporated into trial plot soil containing sclerotia. The amount of *P. nigricans* inoculum was very high in this study (1:4 w:w) (Ghaffar, 1969b). Seed treatments using a strain of this fungus did not provide significant season long protection from *S. cepivorum* (Utkede and Rahe, 1980).

Coniothyrium minitans was investigated for control of *S. cepivorum* by Ahmed and Tribe (1977). The biological control agent was applied to seeds as a pycnidial dust, the survival of onion seedlings was increased three and a half fold over the untreated control. *C. minitans* provided the same amount of control as the fungicide calomel also applied as a seed dressing. Protection over the length of a growing season was not tested. de Oliviera *et al.* (1984) reported antagonism against *S. cepivorum in-vitro* by this fungus, however no disease control in unsterile soil was recorded. *C. minitans* is well documented as a parasite of mycelium (Trutmann *et al.*, 1982) and sclerotia of many pathogenic fungi (Turner and Tribe, 1976; Huang and Hoes, 1976; Harrison and Stewart, 1988; Budge and Whipps, 1991; Gerlagh *et al.*, 1996). The ability of *C. minitans* ability to reduce formation of apothecia and subsequently airborne propagule dispersal (Trutmann *et al.*, 1982) provides a mechanism for combat of *Sclerotinia* infections which is lost against *S. cepivorum*. *S. cepivorum* sclerotia are possibly less vulnerable to this mycoparasite than those of *S. sclerotiorum* (Gerlagh *et al.*, 1996).

Trichoderma harzianum was reported to parasitise sclerotia of *S. cepivorum*. Five isolates of *S. cepivorum* varied in their tolerance to *T. harzianum* parasitism. Overall, germination of sclerotia was drastically reduced by treatment with *T. harzianum*, and sclerotia were more susceptible in darkness than in sunlight. Incorporation of *T. harzianum* into soil on barley grains at 2% of soil weight reduced incidence of *S. cepivorum* infection in glasshouse conditions by 64% (Abd-El-Moity and Shatla ,1981). A strain of *T. harzianum* tolerant to the fungicide iprodione provided a 70% disease reduction when applied in a cornmeal, wheatbran,

gliotoxin fermentation medium in the field when applied alone. It provided an 80% reduction in disease incidence when applied with iprodione. (Abd-El-Moity *et al.*, 1982). This *T. harzianum* strain was used in sowing of around 550 feddan (~200ha) of onion crops between 1980-83, and provided reductions from 85% infection to 10-15% infection in the 1981 season. In the 1982/3 season when the percentage of infection was considerably higher, it was noted that *T. harzianum* failed to control disease when (1) Temperature was unfavourable for antagonist establishment at the time of sowing, (2) Sandy soils with low organic content were sown, (3) Fields were irrigated more than 2 -3 times in the season, and (4) Weed control was poor. (Abd-El-Moity, 1983) These factors all effect the soil environment for pathogen and antagonist, and these observations demonstrate the specific favourable conditions a biocontrol agent requires to overcome *S. cepivorum.* This biological control agent was further developed as a calcium alginate granule formulation (Abd-El-Moity, 1986). *T. harzianum* is also known to parasitise sclerotia of *Sclerotium rolfsii* and *Rhizoctonia solani* (Wells *et al.*, 1972). *Trichoderma* isolates differ in their ability to penetrate *S. rolfsii* sclerotia (Henis *et al.*, 1983).

de Oliviera *et al.* (1984) reported some inhibition of *S. cepivorum* infections by *T. harzianum* applied as conidial suspension. Papavizas (1982) reported UV induced mutant *T. harzianum* strains tolerant of benomyl which provided 70% reductions in *S. cepivorum* infection while the parent strain provided no reduction. As Tasmanian seed is routinely coated with benomyl for control of *Botrytis allii* , benomyl tolerance would be a desirable attribute in a biocontrol agent.

The decay of *S. cepivorum* sclerotia was shown to proceed at a faster rate in the presence of *Sporidesmium sclerotivorum* than in soils where *S. sclerotivorum* was not present (Adams and Ayers 1981). This might implicate *S. sclerotivorum* as a sclerotial parasite, as it has been found in *S. cepivorum* infested soils in the U.K (Sansford *et al.*, 1987). *Verticillium tenerum* has been reported to parasitise sclerotia of *S. cepivorum*, but not mycelium, and reduced losses in pot trials by 54% (Gindrat and Corbaz, 1994). However there was some concern regarding the negative influence of *V. tenerum* on onion growth. *Teratosperma oligocladum* and *Laterispora brevirama* also parasitise sclerotia (Parfitt and Coley-Smith, 1983).

Isolates of *Chaetomium globosum* and *Trichoderma* species have shown ability to provide greater than 60% decrease in the percentage of onions which developed *S. cepivorum* infection (Kaye and Stewart, 1994a). Two treatments which provided the greatest percentage of decrease in infection, also had the lowest percentage of seed emergence while the treatment with the highest percentage of seed emergence demonstrated the lowest reduction in infection. This possibly indicates that the biological control agents task is easier when the seedlings are further apart minimising root to root spread of disease.

Utkhede and Rahe (1980) identified species of the bacteria *Bacillus subtilis* with *in vitro* antagonistic ability. Pot trials using an international selection of *S. cepivorum* isolates indicated that one isolate (B2) consistently reduced infections by in excess of 80% (Utkhede and Rahe,1983). B2 was reported to stimulate sclerotia to germinate while providing biological control. The two activities may be two totally independent mechanisms {ie. biocontrol via antimicrobial compounds, and increased germination of sclerotia by metabolism of onion root exudates} (Reddy *et al.*, 1992). In culture *B. subtilis* antagonism causes *S. cepivorum* hyphae to first display swollen mitochondria, then become more vacuolated than controls, which leads to bursting of hyphal walls and apices and leakage of cytoplasm. It was suggested that the effects were more likely due to disruption of metabolism than membrane or cytoskeleton alteration by antagonistic enzymes (Backhouse and Stewart, 1989). Establishment in the rhizosphere may be a problem in use of *B. subtilis* for biocontrol (Wong *et al.*, 1983).

The biological control agent used in this study is an isolate of *Trichoderma koningii*, and this is reviewed separately.

3.0 Literature Review-*Trichoderma koningii* Oudem.

3.1 Introduction.

Trichoderma spp. use as biological control agents have been the subject of an increasing number of research publications over the past three decades. There have been several reports of the antagonism of *Trichoderma* spp. against *S. cepivorum* (Moubasher *et al.*, 1970; Abd-El-Moity *et al.*, 1981; de Oliviera *et al.*, 1984; Leggett and Rahe, 1985; Kaye and Stewart, 1994a & b). *Trichoderma* spp. are efficient saprophytes and sclerotial parasitism is well documented (Dos Santos and Dhingra, 1982; Trutmann and Keane, 1990; Sandys-Winsch *et al.*, 1994). In the case of the Tasmanian onion industry, biological control using *Trichoderma* spp. would be a desirable replacement for fungicides which leave residues in produce making it less marketable.

A number of review articles have described use of *Trichoderma* spp. in biocontrol of plant pathogens (Henis and Chet, 1975; Papavizas and Lumsden, 1980 ; Papavizas, 1985; Adams, 1990; Baker, 1991; Whipps, 1992; Goldman *et al.*, 1994; Lumsden *et al.*, 1995; Samuels, 1996; Elad, 1996).

3.1.1. Taxonomy.

The genus *Trichoderma* was introduced by Persoon (1794). Rifai (1969) revised the existing taxonomy of the genus. Prior to this time there was some confusion between the genera *Gliocladium* and *Trichoderma*. The nine species defined by Rifai were: *Trichoderma piluliferum* Webster and Rifai., *Trichoderma polysporum* Rifai., *Trichoderma hamatum* (Bon) Bain., *Trichoderma koningii* Oudem., *Trichoderma aureoviride* Rifai., *Trichoderma harzianum* Rifai., *Trichoderma longibranchiatum* Rifai., *Trichoderma pseudokoningii* Rifai. and *Trichoderma viride* Pers ex S. F. Gray.

T. koningii was first described by Oudemans and Koning (1902). Rifai based the classification on similarity to drawings of the morphology of *T. koningii* by Oudemans, as the type cultures had been lost (Rifai, 1969). The species are more properly described as species aggregates as they are made up of more than one genetic entity that cannot be distinguished under the morphological characters used to define them (Meyer, 1991).

An additional two species; *Trichoderma citrinoviride* and *Trichoderma atroviride* were described by Bisset (1984). Doi *et al.* (1987) proposed a new section *Saturnisporum* for two new species *T. ghanense* and *T. saturnisporum*. Bisset (1991a) proposed that the genus *Trichoderma* be revised to consist of five

sections; *Pachybasium*, *Trichoderma*, *Saturnisporum* , *Longibranchiatum* and *Hypocreanum* . *T. koningii* was placed in section *Trichoderma*. Bissett (1991b) proposed that Rifai's *T. hamatum* species aggregate (which is within section *Pachybasium*) be segregated into ten new species. Bisset's taxonomic system like Rifai's was based on morphological criteria. Bisset has tended to split species based on discontinuous morphological criteria, whereas Rifai perceived a broader continuum of basic morphologies.

Evaluation of the Rifai taxonomic system by cladistic analysis of isoenzyme polymorphism has since confirmed that the morphologically defined species are widely divergent (Stasz *et al.*, 1989). Many isolates of *T. koningii* fitted into one cluster (Group G) of phylogenetically similar isolates, but other *T. koningii* isolates did not. Spore wall texture which has been used to distinguish *T. koningii* from *T. viride*, was not shown to be a consistent character for taxonomic purposes. Stasz *et al.* (1989) mentioned that the general pattern indicated *T. viride* to have arisen from an ancestral *T. koningii* type. Meyer (1991) noted two distinct types of conidial ornamentation within *T. viride,* restriction endonuclease digestion of mtDNA produced patterns in which presence or absence of certain bands correlated with the conidial types. This indicated that mitochondrial DNA characters may be a criterion for subdividing other *Trichoderma* species.

Trichoderma is the anomorph of the ascomycete genus *Hypocrea* (Samuels and Lodge, 1996). Rehner and Samuels (1994) compared *Hypocrea* anomorphs of *Trichoderma* and *Gliocladium* spp using sequences of rDNA. It was found that *T. viride* was arranged among the *Hypocrea* spp. The consequence of this finding is that the morphological stereotype of *Trichoderma* has to be modified to include the steriotypical *Gliocladium* morphology (Samuels, 1996), and where the name *Gliocladium* is used hereafter it this is recognised, however the name used on the original publication is retained. Countless issues remain to be resolved in systematics of *Trichoderma* and *Gliocladium* spp. A detailed review of these issues was recently published (Samuels, 1996).

3.2 Morphology.

3.2.1. Macromorphology.

Colonies grow rapidly at room temperature, and initially appear smooth, later appear hairy due to the appearance of aerial hyphae. Colony colour is initially white or clear but after production of phialospores (conidia) ranges from greenish white to dark green (Rifai, 1969).

3.2.2. Micromorphology.

The mycelium is made up of hyaline, highly ramified, septate, smooth walled, 2-10 μm diameter hyphae (Rifai., 1969). Intercalary or rarely terminal chlamydospores are typically formed, usually on the submerged hyphae, they are globose, ellipsoidal, and sometimes barrel shaped, smooth walled and may measure up to 12μm in diameter (Rifai, 1969).

The conidiophores are much branched, formed in compact or loose tufts in ring like zones which are less obvious as the colony becomes older due to aerial hyphae. The main branches of conidiophores, which on average measure about 4μm in diameter, put out several side branches in groups of two or three, which stand at more or less wide angles to their bearer. The length of the side branches increases markedly with distance from the apex of the main branch so that the outline of the branching system is conical or pyramidal. Smaller lateral branches are produced by these side branches and these may again branch, every side branch becoming more or less a miniature of its bearer. The phialides are ninepin shaped and narrower at the base than directly above the middle, then attenuated, sometimes rather abruptly, to their conical apices. They measure 7.5-12 x 2.5-3.5μm, but the phialides that terminate the branches of the conidiophores may be up to 30μm. Typically they stand at a wide angle to their bearer and arise more or less regularly in false verticils of up to five beneath the terminal phialide; occasionally they also arise singly or somewhat irregularly. The phialospores (conidia) are mostly elliptic-subcylindrical, occasionally oblong or almost angular, sometimes obovoid-ellipsoidal, usually with a distinct truncate base and rounded apex. They are 3-4.8 x 1.9-2.8μm smooth walled, pale green to green when viewed under the microscope but appear much darker in mass. These phialospores are produced singly and successively and accumulate in a globose conidial head at the tip of each phialide (Rifai, 1969).

3.3 Nutritional factors affecting growth.

Trichoderma spp. are saprophytic soil fungi and use a wide variety of carbon and nitrogen sources. Nutritional variations occur between strains of species, this is to be expected as taxonomy is based on morphology, not biochemical similarities such as Stasz *et al.* (1989) have described. C:N ratio's of 15:1 provided optimum biomass production in glucose alanine media (Jackson *et al.*, 1991c).

3.3.1. Nitrogen sources.

Aube and Gagnon (1969) tested growth of three strains of *Trichoderma viride* on a range of nitrogen sources. The response of each strain to N sources varied considerably. Overall, utilization of aspargine, and ammonium nitrate allowed greatest biomass production. In addition, ammonium nitrate supplements to the basal medium produced greater biomass than ammonia or nitrate alone. Tye and Willets (1973) recorded *T. lignorum*'s ability to use a wide range of organic compounds (not specified) or alternately urea, nitrate, nitrite, or ammonium ions as sources of cellular nitrogen (not quantified).

Danielson and Davey (1973c) tested a range of *Trichoderma* spp. for ability to use of a range of nitrogen sources. Considerable variation existed between individual *Trichoderma* spp. and between strains of a species. Relative to NH_4Cl (a known suitable nitrogen source), strains of *T. koningii* produced more biomass in media supplemented with urea (102%), casamino acids (120%), L-alanine (110%) and L-aspartic-acid (108 and 125%). NH_4Cl promoted 48% faster biomass production than KNO_3 (in a medium buffered with citrate). Conversely Ward and Henry (1961) using non-buffered media found NO_3 to be superior. "The apparent superiority of NO_3 in those studies was due almost certainly to the production of inhibitory H-ion concentrations in cultures with NH_4" (Danielson and Davey, 1973c).

Nitrogen sources are not necessarily germination promoters. Table 3(A). shows germination response of phialospores of six *Trichoderma* species to several nitrogen sources (Danielson and Davey, 1973d) In control treatments where no nitrogen source was added, approximately the same percentage (94%) of phialospores germinated as in treatments containing N sources which induced the most phialospores (95%) to germinate. Germination was lower than the untreated control in the presence of some N sources. Ammonium chloride, sodium nitrate, thymine, L-histidine, and D-aspartic acid were non inhibitory to phialospore germination.

Danielson and Davey (1973c & d) showed that nutritional requirements of different species and isolates of a single species vary. The best nitrogen sources were L-alanine, followed by casamino acids and ammonium chloride, with slower biomass production in media amended with potassium nitrate for the three *Trichoderma* species tested. Little biomass was produced when no nitrogen source was provided.

Jackson *et al.* (1991b) studied *T. pseudokoningii* and two strains of *T. viride* as well as *Gliocladium virens*. The nitrogen sources tested and results are shown in Table 3(B). *G. virens* produced the greatest amount of biomass in media amended

with KNO_3, overall the three *Trichoderma* spp. isolates produced the most biomass in media amended with L-alanine.

Table 3(A): Germination percentage of phialospores of *Trichoderma koningii* at pH 4.3 on a range of different nitrogen containing compounds (at 0.1%). (Source: Danielson and Davey, 1973d)

Amendment	13 hrs	25 hrs
None	94	ND *
Malt Extract	97	ND
Casamino Acid	87	ND
ß-Alanine	0	5
L-Cysteine.	0	29
D-Aspartic Acid	0	85
L-Alanine	8	ND
L-Histidine	48	91
L-Aspartic Acid	64	ND
Thymine	94	ND
NH_4Cl	91	ND
$NaNO_3$	95	ND

* ND = Not Determined

Table 3(B): Biomass produced (mg ml^{-1}) by *Gliocladium virens* and *Trichoderma* spp. after seven days at 25° C on different nitrogen sources with glucose as a carbon source (Source: Jackson *et al.*, 1991b).

N Source	*G. virens*	*T psuedokoningii*	*T. viride 1*	*T. viride 2*
None	0.0	0.06	0.04	0.0
L-alanine	6.15	5.73	10.10	6.82
NH_4Cl	2.67	3.18	2.21	2.20
CasaminoAcid	3.49	4.64	4.21	4.98
KNO_3	6.46	1.83	0.11	0.71

Diverse mixtures of nitrogen sources appear sustain better growth than single nitrogen sources alone. Watanabe *et al.* (1987) assessed biomass production by *T. viride*, *T. harzianum*, , *T. hamatum* *T. pseudokoningii* and *Gliocladium* strains in media amended with 2000 mg/L of nitrogen available as NH_4Cl, $NaNO_3$, or a commercial fertilizer "20-20-20" containing 10% urea, 6% NO_3, and 4% NH_4. *T. pseudokoningii* and *G. virens* produced more biomass in media amended with $NaNO_3$. *T. hamatum* (isolate IFO 31291) produced more biomass in media amended with NH_4Cl. All other isolates produced more biomass when provided with the diverse nitrogen source provided by 20-20-20 (eg *T. viride* 40% more, *T. hamatum* [TRI-4] 41% more) than on $NaNO_3$ or NH_4Cl.

Most studies have concentrated on the effect of nitrogen sources on growth and bioass production *in vitro*. However, Watanabe *et al.* (1987) also considered the effects of adding different amounts of fertiliser to soil on antagonistic activity of *Trichoderma* spp. Using 20-20-20 applied to the soil at 0 (control), 25, and 250 mg per kg of soil they determined the ability of antagonists to reduce survival of *Rhizoctonia solani* which had infected beet seeds in the soil. Three day old antagonist cultures produced on bran were added to the soil (5g per kg). Overall six of the seven isolates reduced survival of *R. solani* in the beet seed (irrespective of fertiliser concentration) by between 50 and 79 %. Addition of 20-20-20 did not improve the ability of germling preparations of six of the seven *Trichoderma* spp isolates to reduce survival of *R. solani.*

3.3.2. Carbon Sources.

The use of complex carbon sources by *Trichoderma* spp. is of interest to this study because these may selectively enhance the biological control agent over other soil organisms. Growth of *Trichoderma viride* on a range of monosaccharides, disaccharides, polysaccharides, and carbohydrate derivatives as carbon sources were assessed by Aube and Gagnon (1969). Growth rates of the three isolates varied in response to different carbon sources. The results of the more suitable C sources are shown in Table 3(C). Carbon sources which provided poor biomass production and were omitted from Table 3(C) included monosaccharide: arabinose, disaccharides: lactose and melibiose, polysaccharide: raffinose, and carbohydrate derivatives: L-rhamnose, salicin, dulcitol, and inisitol).

Many carbon sources were used by *T. viride* (Table 3.C), strains 1500 and 1503 grew well on more complex polysaccharides such as dextrin, glycogen and soluble starch. The disaccharide cellobiose proved as good a carbon source as the easily degradable monosaccharide sources for all three isolates. Sucrose was a good carbon source for strains 1500 and 1503, but mediocre for 1501. Maltose provided good biomass production for strain 1500 only. D-glucose was the best monosaccharide carbon source, though the three strains varied considerably in their use of the monosaccharides (Aube and Gagnon, 1969).

Table 3(C): Dry weight of mycelium (mg) produced by *T. viride* isolates 1500, 1501, and 1503 in 40mls of basal medium containing various carbon sources at 10g carbon per litre plus 18 mg/l nitrogen as aspargine (Source: Aube and Gagnon, 1969).

Group/C source	*T. viride* 1500	*T. viride* 1501	*T. viride* 1503
Monosaccharides			
D-Glucose	454.3	282.8	365.0
D-Ribose	447.9	133.9	331.0
D-Xylose	489.4	67.4	327.1
D-Fructose	384.6	231.9	328.8
D-Galactose	383.4	266.4	300.5
D-Mannose	427.3	296.6	347.5
None	59.2	40.6	52.4
Disaccharides			
Maltose	399.1	23.9	81.3
Sucrose	463.3	255.9	320.8
Cellobiose	460.1	303.0	372.7
Polysaccharides			
Starch (Soluble)	253.2	82.1	275.3
Dextrin	340.1	93.9	263.3
Glycogen	302.0	65.1	269.4
Carbohydrate derivatives			
D-sorbitol	347.4	91.7	286.6
D-mannitol	416.9	163.5	330.4
Glycerol	407.9	94.4	321.1

Danielson and Davey (1973c) assessed a range of carbon sources relative to dextrose. They tested growth of *T. viride, T. polysporum, T. harzianum* (2 strains), *T hamatum* (2 strains), *T. pseudokoningii* , and three strains of *T. koningii* (Table 3.D). *T. koningii* biomass production when grown on the polysaccharide dextrin averaged at 31% faster than on monosaccharide dextrose. This carbon source might be useful in selectively enhancing growth of *T. koningii.* Compounds which proved equivalent to, or better than dextrose for sustaining *T. koningii* included monosaccharides (D-fructose and D-ribose), disaccharide (cellobiose), alcohol: (glycerol) as well as dextrin. Tye and Willets (1973) reported *Trichoderma lignorum* capable of growth on methanol, methylamine, and formate, and have recorded growth on representative examples of polysaccharides, disaccharides, monosaccharides, purines, pyrimidines, amino acids, and TCA cycle acids. They do not elaborate further.

Table 3(D): Growth of *Trichoderma* strains on 31 potential carbon sources assessed as a percentage relative to dextrose (Source: Danielson and Davey, 1973c).

Carbon Source	*T. koningii* T12	*T.koningii* T5	*T. koningii* T1
Monosaccharides			
D-Fructose	ND *	106	114
D-Mannose	ND	82	95
D-Galactose	71	93	99
L-Sorbose	5	49	0
L-Rhamnose	10	8	11
L-Arabinose	49	39	57
D-Arabinose	28	48	9
D-Xylose	96	75	74
D-Ribose	75	117	124
Disaccharides			
Sucrose	58	0	87
D-Cellobiose	93	120	106
Polysaccharides			
Dextrin	137	140	116
Soluble starch	85	68	76
Inulin	0	0	101
Salicin	39	19	54
Dextrose yield (mg)	33	34	34

* Note- ND=not determined in this study

3.3.2.1. Utilization of chitin and laminarin.

Growth of *T. harzianum* on the polysaccharides laminarin and chitin was reported by Elad *et al.* (1982). This isolate isolate was capable of using *Sclerotium rolfsii* and *Rhizoctonia solani* cell walls as a sole carbon source and produced ß-1,3-glucanase and chitinase. ß-1,3-glucanase activity was 67% greater when laminarin was in a 3:1 (v/v) ratio with glucose than when the medium contained an equivalent amount of laminarin alone, indicating an inducing effect by glucose (however glucose induced lower ß-1,3-glucanase activity in absence of laminarin). This enzyme differs from cellulase which is inducible and is repressed by glucose (Bull, 1967). Chitinase and chitobiase are also repressed by presence of a more readily metabolised carbon source such as glucose or *N*-acetylglucoseamine. Carbohydrates structurally related or similar to chitin such as cellulose or chitosan did not promote incremental synthesis of chitobiase (Ulhoa and Peberdy, 1993). Chitobiase from *T. harzianum* has been purified and characterised (Ulhoa and Peberdy, 1991b).

Trichoderma spp. can also degrade cell walls of *Sclerotium rolfsii* (Artigues *et al.*, 1984), *Rhizoctonia solani* (Ridout *et al.* ,1988), and *Fusarium oxysporum* (Sivan and Chet, 1989a & b, Ordentlich *et al.*, 1991) This is discussed further in the section on lytic enzymes.

3.3.2.2. Utilization of cellulose.

Studies by Domsch and Gams (1969) of production of cellulases for degradation of carboxymethylcellulose by 40 species of fungi have shown species of *Trichoderma* to be among the most active degraders. The subject of cellulase activity and enzyme detection is discussed in a separate review (Ch. II:5).

Many fungi rapidly degrade cellulose but only a few produce cellulolytic enzymes of a high potency and stability. Of these *Trichoderma viride* was found a convenient source of enzymes capable of total hydrolysis of native insoluble cellulose to glucose (Mandels, Weber, and Parizek, 1971).

Danielson and Davey (1973c) studied degradation of powdered cellulose by six species (10 isolates) of *Trichoderma* including two isolates of *T. koningii* (see Table 3.E). *T. koningii* isolates were at 8 days the fastest (183), and third fastest (515), degraders of cellulose, but later were overtaken by four other *Trichoderma* isolates. *T. pseudokoningii* (444) degradation exemplifies the variance in time which different isolates take to adapt to using cellulose as a sole carbon source as it was among the slowest degraders at 8 and 15 days but by days 30 through to 60 had degraded the greatest percentage of cellulose.

Table 3(E): Degradation of powdered cellulose by *Trichoderma* species as determined by weight loss in liquid culture over a time period of 60 days.(expressed as percent degradation).(source: Danielson and Davey, 1973c)

Isolate	8 Days	15 Days	30 Days	60 Days
T. hamatum (652)	0	0.6	2.4	6.0
T.hamatum (690)	0	2.1	8.2	9.1
T.polysporum (262)	0.5	4.5	7.7	9.6
T.hamatum (454)	3.8	8.3	11.3	10.6
T.koningii(183)	4.8	6.7	9.2	10.8
T.koningii(515)	3.0	6.6	9.6	11.4
T.harzianum(33)	2.6	7.1	10.6	14.4
T.viride(67)	1.3	9.4	14.1	15.6
T.harzianum(790)	1.6	7.6	14.9	16.8
T.pseudokoningii	0.1	2.8	16.1	20.6
Leaf Litter	0	0	0	6.5

Degradation of pure cellulose does not represent behaviour in natural systems especially with rich sources of available nitrogen added. Danielson and Davey (1973c) compared degradation of purified cellulose to degradation of natural cellulose sources. *T. koningii* (183) was the most rapid degrader of green loblolly

pine needles at 1 month after incubation, and at 3 months the second most rapid, overtaken slightly by *T. hamatum* (454). *T. pseudokoningii* (444) which was the fastest degrader of pure cellulose was among the slower degraders of pine needles. Neither Hering (1967) using oak leaves nor Frankland (1969) using bracken petioles detected any change in cellulose composition after incubation with *Trichoderma* for 6 months.

3.3.2.3. Utilization of cellulose in rhizosphere colonisation.

Foster *et al* . (1983) demonstrated that mucilage overlying the root surface of the region of cell elongation constitutes the remains of the outer primary wall of the root epidermal cells. Microorganisms were embedded in this mucilage, and cellulose is a major component of primary cell walls. Ahmad and Baker (1987b) assessed the competitive saprophytic ability of several isolates of *Trichoderma* (four strains of *T.harzianum* [T-95, T12B, WT, and T-12] and one strain of *T. koningii* [T-8] and one of *T. viride* [T-S-1]). Previous studies (Ahmad and Baker, 1987a) had shown T-95 and T12b to be benomyl tolerant and rhizosphere competent (ie. capable of colonising the rhizosphere). Ahmad and Baker (1987b) found the same two strains had the greatest competitive saprophytic ability in non-sterile soil (determined by colonisation of buried fragments of wheat straw and cellophane disks). This was correlated with the ability to produce cellulase. Ahmad and Baker (1987b) concluded that rhizosphere competence is at least in part determined by competitive saprophytic ability to use substrates such as cellulose in the soil, implicating root mucilage as a substrate for rhizosphere colonisation. In support of this hypothesis *Gliocladium virens* isolates which were not able to use cellulose as a sole carbon source were not able to colonise the rhizosphere (Papavizas *et al.*, 1990).

3.3.2.4. Enhancement of Cellulase Activity.

It is desirable to take advantage of *Trichoderma* spp. cellulolytic enzymes in enhancing competitive saprophytic abilities and thus biocontrol. Studies have shown that it is possible to improve cellulase activity of strains by selecting for high cellulase producing mutants using a semi-quantitative plate assay method (Montencourt and Eveleigh, 1977). Mandels *et al.* (1971) induced mutations, which produced twice the cellulase activity of their parent strain by high energy irradiation. Troutman and Matejka (1978) induced tolerance to benomyl by gamma irradiation, but did not report the biocontrol abilities of these mutants. Papavizas, Lewis, and Abd-El-Moity (1982) induced benomyl tolerant biotypes which gave up to 38%

better biocontrol of *Sclerotium cepivorum* than the parent strain by a 100 minute ultra-violet irradiation under germicidal lamps in benomyl amended agar.

3.3.2.5. Utilisation of pectin.

T. koningii produces two inducible endopolygalacturonases [EC 3.2.1.15], These enzymes are catalytically similar to phytopathogenic polygalacturonases, however they are not able to bind to plant tissues exhibiting a difference in structure which could be responsible for the lack of pathogenicity toward plants (Fanelli *et al.*, 1978).

3.3.4. Other Nutritional Requirements.

The effects of salts, sulphur sources, and compounds such as vitamins on growth of *Trichoderma* has largely been ignored (Papavizas, 1985). Salts of magnesium may be important for growth of *T. viride* (Shukla and Mishra 1970). Addition of 3.28mg of iron /L to a glucose alanine medium increased biomass production, but 164 mg of iron /L was toxic to *Trichoderma* spp, the lack of Mg, P, K or S in the medium decreased growth of some isolates (Jackson *et al.*, 1991c).

3.4 Non-Nutritional Factors.

3.4.1. Temperature.

In southern Australia, low temperatures appear to restrict mycoparasitic activity of *T. koningii* in winter (Trutmannn and Keane, 1990). Less than 10% of *T. koningii* conidia could germinate below 10°C and no mycoparasitism occurred on *S. sclerotiorum* sclerotia. Highest mycoparasitism and conidium germination occurred from 15-30°C, and both dropped abruptly above 30°C (Trutmannn and Keane, 1990).

Danielson and Davey (1973b) have reported optimum growth of *Trichoderma* in culture at temperatures ranging from 22 to 34°C. However, *in vitro* tests of the effects of temperature on growth, represent growth in soil poorly. For example, the optimum *Trichoderma harzianum* growth range in artificial media was 27-30°C, but saprophytic colonisation ability in non sterile soil was greatest at 16-20°C. The overall temperature range for saprophytic growth was between 10 and 35°C (Eastburn and Butler, 1991).

Temperature has a differential effect on antagonistic activity of different isolates. Tronsmo and Dennis (1978) tested *T. koningii* as an antagonist against

Botrytis cinerea and *Mucor mucedo* at 5°, 10°, and 20° *in vitro*. Isolate *T. koningii* (123) was most strongly inhibitory against both test fungi at 20°C, while *T. koningii* (3) was most inhibitory to both test fungi at 5°C. This indicates the danger of selecting potential biological control agents in temperature conditions other than those that might be encountered in environment. Adaptation to the temperature of field soil in preparation of biocontrol amendments is important. *T. hamatum* was significantly more effective at reducing the survival of buried *Rhizoctonia solani* infested beet seeds when incubated in a vermiculite-wheat-bran mix at 5°C than at 25°C (Lewis *et al.*, 1991).

3.4.2. Soil Moisture.

The response to soil moisture content is a species dependant one (Widden and Abitbol, 1980). Growth on loblolly pine needles was determined at 46%, 103%, 144%, and 201% moisture content (based on oven dry weight). Three *T. koningii* isolates grew optimally at 201% moisture, and growth of all three decreased linearly with the moisture percentage. At 46% moisture, growth was one third of that at 201% (Danielson and Davey, 1973b).

Seasonal fluctuations in natural populations of *Trichoderma* spp. are known to be affected by soil moisture. Moisture content of soil sites naturally containing *T. harzianum* averaged at 18.3% by weight (range: 7.4% to 28.4%). Soil populations of *T. harzianum* (assessed on a Colony Forming Units per 50 mg soil sample basis) fluctuated seasonally, peaking from December to March in the northern hemisphere winter, and rising to a smaller peak again in early summer (Eastburn and Butler, 1988a). Soil moisture was related to the season and *Trichoderma* population size within individual samples were influenced by moisture. However, moisture content was not significantly related to the proportion of soil samples from which *Trichoderma* spp. were isolated (Eastburn and Butler, 1988a). Eastburn and Butler (1988b) found that *T. harzianum* population variation could not be related specifically to seasonal change. Population dynamics of *Trichoderma* spp. have proven difficult to study.

Fifty milligram soil samples were used in the studies of Eastburn and Butler (1988a & b), on the basis that this approached the growth range of the fungal thallus.In these studies population size was assessed by the Colony Forming Units method, which discounts mass conidium production due to harsh conditions, and could indicate larger numbers of propagules than actively growing unstressed mycelium. This type of conidium production in the unfavourable season could explain the twice yearly peaks Eastburn and Butler (1988a) reported. Further studies used colonisation of buried alfalfa stems as a measure of effect of soil

moisture on saprophytic colonisation (Eastburn and Butler, 1991). In these studies *T. harzianum* was increasingly active as matric potential decreased from 0 to -1.0 bar and was as active, or slightly less, down to -16 bars. Matric potentials drier than -16 bars were not tested.

3.4.3. Soil pH.

In solutions of pH 2.3, 2.6, 3.3, 3.7, 4.3, 4.7, 5.1, 5.6 and 6.2, buffered with 0.1M citrate, *T. koningii* (isolate T-12) grew optimally at pH 4.3, biomass production was at least 80% of this in all solutions above pH 2.6 and below pH 5.6. All other *Trichoderma* isolates tested produced maximum biomass between pH 3.7 to 4.7 (Danielson and Davey 1973b).

A number of reports suggest that soil suppressiveness is associated with lower pH which appears to favour proliferation of *Trichoderma* spp. (Chet and Baker, 1980; Chet and Baker, 1981). For example, in soils with a pH of 5.0 and 6.0 which were suppressive to *Rhizoctonia solani*, the inoculum density of *R. solani* was significantly lower than in soils of pH 7.0, 8.0, and 9.0. An antagonistic isolate of *T. harzianum* was present in this soil (Liu and Baker, 1980). *T. hamatum* was found to reduce *Rhizoctonia* damping-off of radish more effectively when the soil pH was reduced from 8.1 to 6.0 (Chet and Baker, 1981). It has also been noted that *Trichoderma* spp. were more frequently observed in soils amended with ammonium sulphate than in the same soils amended with lime. Single applications of lime caused a reduction in suppressiveness of *Gaeumannomyces graminis* (Simon *et al.*, 1988), and addition of lime to soil also inhibited the transfer of suppressiveness between soils (Simon and Sivasithamparam, 1988b). However Eastburn and Butler (1988b) found no correlation between soil pH and frequency of isolation of *T. harzianum* in 140 soil samples with pH ranging between 6.2 to 7.9. At pH 6.2, the isolate of *T. harzianum* used by Danielson and Davey (1973b) grew at only 33% of its maximum rate.

3.4.4. Carbon Dioxide.

T. koningii growth regulation by pH was shown to have a carbon dioxide dependant effect by Danielson and Davey (1973b). Growth at pH 7.7 would normally be 33% of growth at pH 4.4 in normal air. When 2% CO_2 modified atmosphere was provided, growth increased to 51% and when 10 % CO_2 was provided, growth increased to 65%.

3.4.5. Effect of Sunlight.

Some studies have reported mycoparasitic interactions which occurred only in complete darkness. Abd-El-Moity and Shatla (1981) suggested that light destroys toxins produced by *Trichoderma* spp. This is not an issue for soil-borne studies but should be considered in planning *in vitro* investigations.

3.5. Survival in the soil.

The abundance of *Trichoderma* species worldwide coupled with their ability to degrade various organic substrates, metabolic diversity, and resistance to microbial inhibitors, suggests that they may possess the ability to survive in many ecological niches depending on prevailing conditions and the strain involved (Papavizas, 1985).

Little is known about the fate of conidia and chlamydospores added to soil. Some conidia (about 3%) can survive at least 130 days in non rhizosphere soil (Papavizas, 1982). It is not possible to determine if these were the conidia originally added, as some may have germinated, forming new propagules within this period. Conidia added to soil without a food base cannot grow or proliferate (Papavizas *et al.*, 1984).

Chlamydospores are important structures which enable soil inhabiting fungi to survive and persist. They are asexually produced, thick walled, and arise from modifications of hyphal segments. They usually have the ability to withstand adverse environmental conditions (Griffiths, 1974). Park (1954) observed rapid chlamydospore formation in sterile soil extracts inoculated with *T. viride.* Chlamydospores were shown to survive better than conidia , and remained viable for at least 20 months (Caldwell, 1958). Chlamydospores tend to be formed at great density in freshly decomposing plant material (Lewis and Papavizas, 1984) and might have the ability to control plant disease by competitive infestation of organic debris, preventing other organisms from producing propagules on this substrate. Chlamydospores enter the soil as this material breaks down (Lewis and Papavizas, 1984). Chlamydospores seem to germinate and establish populations better in soil than conidia alone. Papavizas *et al.* (1984) compared adding air dried biomass produced in a fermentor (which includes chlamydospores), and conidia of *Trichoderma hamatum* to two soils. The number of colony forming units was determined by the dilution plate method. This data is shown in Table 3(F).

The data in Table 3(F) shows that the number of colony forming units produced in both soils over 130 days ranged from 260 to 2015 times as many propagules per gram of soil. Papavizas *et al.* (1984) concluded that the

chlamydospore should be considered the most important propagule for ensuring survival and proliferation in the soil due to its longer survival and more abundant production in liquid and solid media. Papavizas (1985) cites Papavizas *et al.* (1984) that hyphae also survive in soil. However hyphae without spores added to soil in this study were of *Talaromyces flavus*, while hyphae of *Trichoderma* were added as dried mycelial mats containing chlamydospores.

Table 3(F). Colony forming units of *T. hamatum* isolated from two soils as determined by the dilution plate method on a selective medium at 10, 20, 60, and 130 days after inoculation with conidia or fermented biomass (Source: Papavizas *et al.*, 1984).

	Colony forming units (x 10^3)/ gram of soil amended with:			
	conidia		fermenter biomass	
Days	Soil 1	Soil 2	Soil 1	Soil 2
10	4.2	4.6	2340	4080
20	4.0	2.6	1500	5600
60	3.4	1.8	880	1500
130	1.6	1.6	720	1020

Proliferation along the developing rhizosphere is one of the most important traits for antagonists applied to seed (Cook and Baker, 1983). In two separate studies *T. harzianum* strains were found to colonise both the top (soil surface) 1 cm of the rhizosphere and the root tip (in seedlings with 8 cm roots), in these two parts of the rhizosphere 10 times as many colony forming units were produced, as on the rest of the root (Ahmad and Baker,1987a; Sivan and Chet ,1989b).

3.5.1. Competition with other Soil Organisms.

Hubbard (1983) reported an isolate of *T. hamatum* which had good biocontrol abilities *in vitro* but when introduced to soils gave poor *Pythium* seed rot control. Production in the soil of extracellular iron chelating siderophores by a species of *Pseudomonas* inhibited growth of *T. hamatum* by 90% in media containing no iron (Fe^{++} and Fe^{+++}). In soils containing *Pythium* spp. where iron was increased by 7 µg/gram with *Pseudomonas* present, plant emergence was improved by 28% when *T. hamatum* was added. Hadar *et al.* (1984) provided a solution to this problem by selection of a Trichoderma isolate adapted to soils with low iron levels and was suppressive to disease. (Though the inoculum level required to achieve this control was 10^8 conidia per gram of soil, while a similar soil without Pseudomonads required 10^4 to 10^5 conidia for the same effect.).

Competition for iron is not the only mechanism of inhibition which Pseudomonads possess. Strain 2-79 of *P. fluorescens* produces the pigmented antibiotic phenazine-1-carboxylic acid (P.C.A.) and has been reported to produce this antibiotic on wheat roots grown in steamed and natural soil; this was shown to suppress saprophytic fungi (Thomashow *et al* .,1990). In Potato Dextrose Agar which is relatively high in iron (Bin *et al.*, 1991), radial growth of *T. harzianum* (ThzIDI) was inhibited by *P. flourescens*, which suggests that P.C.A. may have been a mechanism of inhibition in the soil (Bin *et al.*, 1991). The zone of inhibition on PDA was not as big as on lower Iron content King's B agar. The ability of ThzIDI to colonise sclerotia of *Sclerotinia sclerotiorum* was not inhibited in steamed natural soil where the native microbiota had been injured (Bin *et al.,* 1991). Further discussion of siderophore and antibiotic production by *Pseudomonas* spp. is beyond the aims of this review. For more detail the reader is referred to reviews by Defago and Keel (1995) and Voisard *et al.* (1994).

3.5.2. Assessment of survival and establishment in the soil.

Determination of growth and rhizosphere colonisation of substrate by *Trichoderma* spp. is a labour intensive and cumbersome process. Problems exist in qualitative measurement of rhizosphere colonisation, as presence of dormant conidia may skew the data. The two main methods used have been direct plating of root segments (Harman *et al.*, 1989) and serial dilution of washings from root segments (Ahmad and Baker, 1987a). Neither method is totally satisfactory (Sivan and Harman, 1991). Several useful *Trichoderma* selective media have been developed (eg Papavizas, 1982; Papavizas and Lumsden, 1982; Elad and Chet, 1983). Askew and Liang (1993) report that propamocarb suppresses Oomycetes and mildly inhibits *Aspergillus* and *Penicillium* while allowing *Trichoderma* growth. While useful for isolating *Trichoderma* spp., a problem may be encountered where more than one *Trichoderma* spp. is isolated and the morphology differs insufficiently to separate them.

In *Trichoderma*, characteristics such as resistance to benomyl or antibiotics have been induced, these can be a useful criteria in identification (Sivan and Harman, 1991). Several groups of researchers have attempted to develop enzyme linked immunosorbent assays (ELISA) to detect fungal propagules in the soil using polyclonal antisera, however these tend to cross react widely with related and unrelated species (Dewey *et al.*, 1992) making them unreliable. Reactivity of antisera raised against material associated with *Trichoderma* mycelium including extracellular protein is also known to vary with the substrate used for fungal growth (Carter and Lynch, 1991). With the development of monoclonal immunological

assays it has been possible to raise antibodies to *T. harzianum* mycelium (Thornton *et al.*, 1994) which cross react with mycelium of other *Trichoderma* spp. and their teleomorph *Hypocrea* but not with a range of other soil saprophytic fungi. An isolate specific ELISA test for *Trichoderma* propagules would be a powerful tool for ecological biocontrol work.

Another alternative in identification of biocontrol agents is comparison of isozyme profiles. Zamir and Chet (1985) successfully grouped 23 geographically diverse *T. harzianum* isolates into five isozyme types based on bands of alcohol dehydrogenase (EC 1.1.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glutamate oxaloacetate transaminase (EC 6.2.1.1), phosphoglucomutase (EC EC 2.7.5.1), superoxide dismutase (EC 1.15.1.1), malate dehydrogenase (EC 1.1.1.37) and phosphoglucoisomerase (EC 3.5.1.9). Methods of this type look only at a small part of the genome, though the ability to separate isolates within a species is useful, and the methods are not too time consuming.

Molecular methods supply more information for typing studies. Mills and Muthumeenakshi (1994) compared a group of *T. harzianum* strains, seen as pathogens by the mushroom industry, using restriction fragment length polymorphism (RFLP) of ribosomal and mitochondrial DNA, random amplified polymorphic DNA (RAPD) analysis, and nucleotide sequence of the internal transcribed spacer 1 region. All methods agreed that there were three groups of isolates, with limited variation within. Mitochondrial DNA RFLP comparisons indicated the greatest amount of variation among isolates. Nucleotide sequence had the advantage of allowing a quantitative comparison of evolutionary divergence to be made. DNA(RAPD) analysis has also been investigated by Zimand *et al.* (1994) who were able to distinguish between morphologically similar isolates using ten oligonucleotide primers and by Grebus *et al.* (1995) who reported distinction of 70 *Trichoderma* isolates at an isolate, species, and genus level, this allowed identification and monitoring of the *T. harzianum* Th382 strain population in biocontrol studies by using DNA(RAPD) banding patterns. Ten primers (Operon H kit) were used to generate the RAPD patterns.

While molecular tests are able to recognise differences and similarities in *Trichoderma* isolates, they are not able to confirm beyond all doubt that a *Trichoderma* isolate growing in soil or on plant tissue is descended from the original inoculum added in that experiment. The use of genetic markers expressed in normal mitotic development of the fungus is a powerful tool for studying biocontrol agent ecology. Expression of the *Eschlerishia coli uid* A ß-glucuronidase gene (GUS causes hyphae containing this gene to appear blue in presence of 5-bromo-4-chloro-3-indoyl-ß-D-glucuronide. This gene has been implanted into a strain of *T. harzianum* (Thrane *et al.* , 1995) and *T. koningii* (Dyer

et al., 1995) using the hygromycin-B resistance gene as a selective marker for isolation (Sivan *et al.*, 1992). It was necessary to study the transformants carefully to ensure that they were of similar efficacy to their wild type parent. A proportion of transformants may prove to be unstable (S. Dyer, pers. com.). Using the GUS marker, Green and Funck-Jensen (1995) were able to specifically observe colonisation behaviour in the cucumber rhizosphere by *T. harzianum* .

3.6. Plant Growth Increase.

Plant growth increases reported in presence of *Trichoderma* spp. (Windham *et al.*, 1986) were thought to be caused by reduction of minor pathogens, or a direct effect on plant growth. Tobacco and tomato seedlings in steamed soil (re-colonised by a number of fungi and bacteria, none of which reduced plant growth) had significantly higher emergence when *Trichoderma* spp. were introduced. Tobacco root and top weights increased by 266 and 291% respectively when *T. koningii* was introduced. The authors concluded that *Trichoderma* spp. may possess a growth stimulating factor. When *Trichoderma* spp. were separated from germinating seeds by a membrane, an increase in germination of tobacco, tomato, and corn seeds was reported. Cause of growth enhancement was not elucidated, though minor pathogen suppression and mycorrhizal activity were refuted. Other suggested mechanisms include production of plant hormones and vitamins, conversion of non-utilisable nutrients to a utilisable form, and stimulation of host defences including activities of hydrolytic enzymes and deposition of lignin (Inbar *et al.*, 1994). Kleifeld and Chet (1992) noted that an isolate of *T. harzianum* which enhanced growth of pepper plants was able to grow inside the roots in a way similar to mycorrhizae. Duffy *et al.* (1996) noted an increase growth rate of wheat in soils amended with an isolate of *T. koningii* which appeared to be more active in colonisation of crown rather than seminal roots. The roots were occasionally stunted and highly branched, but had no discolouration.

Reports also exist of growth inhibition by *Trichoderma* spp. Cutler *et al.*, (1991) reported that *T. koningii* produced cyclonerodiol, a compound also isolated from *Gibberella fujikuroi* with plant growth inhibitory activity. Menzies (1993) reported seedling inhibition of cucumber, pepper and tomato by a strain of *T. viride* isolated from tomato roots. It was suggested that *Trichoderma* spp. live in a delicate balance with host plants, and if an increase in host susceptibility occurs, *Trichoderma* may become a pathogen. Farr *et al.* (1989) report that *T. viride* may be pathogenic to 32 genera of host plants. Additionally, *T. koningii* has been reported to inhibit mycorrhizal associations (McAllister *et al.*, 1994; Rousseau *et al.*,

1996) which could be of concern to the present study as onions are well documented for increasing nutrient uptake by mycorrhizal associations (Brewster, 1994).

3.7 Mechanisms of Antagonism in Biocontrol.

The mechanisms by which *Trichoderma* spp. inhibit other organisms have not been precisely defined. Many antagonistic traits have been identified presumptively and suggested mechanisms include antibiosis, lysis, competition, and mycoparasitism (Papavizas, 1985). It has been suggested that a mass action effect accounts for inhibition, for instance the appressed growth habit of *T. harzianum* around hyphae of *R. solani* could involve antibiotic production, and direct parasitism, as well as lysis (Lewis and Papavizas, 1980). *T. harzianum* is able to parasitise both hyphae and sclerotia of *R. solani* , whereas some antagonists (eg *G. virens*) may only attack sclerotia (Mukherjee *et al.,* 1995). Both mechanisms should be considered in screening for sclerotial fungus antagonists (Sandys-Winsch *et al.*, 1994). In the case of *S. cepivorum* inhibition by *T. harzianum,* two factors were identified in sterile filtrates which caused radial growth inhibition; only one of which was denatured by autoclaving (Papavizas *et al.*, 1982).

While various mechanisms of antagonism have been documented, until they can be shown to occur in soil, plant tissues, and the natural environment the significance of any of the mechanisms of disease control observed *in vitro* should be viewed with reservation (Whipps, 1992).

3.7.1. Antibiosis.

In detection of antimicrobial compounds the growth stage of the cultures can be an important issue. Cultures may need to be in the growth decline phase before they produce high amounts of antibiotic metabolites. Nutrient stress in culture seems to most closely represent the conditions in which organisms find themselves in competition for limited nutrition in soil, and antibiotic production at this stage confers an advantage (Ghisalberti and Sivasithamparam, 1991). Nevertheless, there are some reports that inhibitors from young growing colonies inhibit pathogens more than those from old cultures (eg. Lewis and Papavizas, 1987).

In a recent review of antifungal metabolites produced by *Trichoderma* spp. (Ghisalberti and Sivasithamparam, 1991) three broad categories were described: leachable, volatile and peptaibol.

3.7.1.1. Leachable antifungal metabolites.

This group of compounds includes those with some mobility in water. Earliest reports of antibiotics produced by *Trichoderma* spp. include the discovery of gliotoxin production by *T. lignorum* (Wiendling, 1932; Jones and Hancock, 1988) and production of viridin by *T. viride* (Brian and McGowan, 1945). There is some confusion over the identity of these organisms however, as Dennis and Webster (1971a) believed that the isolates of Brian and McGowan (1945) were morphologically similar to *Gliocladium virens* and have listed other articles in which identity of the organism may be taxonomically incorrect. More recently *Gliocladium* has been included in genus *Trichoderma* (Sameuls, 1986).

Dennis and Webster (1971a) reported that of seven species of *Trichoderma* , none produced gliotoxin or viridin (while *G. virens* produced both of these) and that *T. viride* and *T. polysporum* produced another compound "trichodermin". Peptide antibiotics were produced by *T. polysporum, T. hamatum* , *T. koningii, T. harzianum, T. pseudokoningii* , *T. longibranchiatum,* and *T. viride.* Little characterisation of the compounds was undertaken. Polypeptides associated with gliotoxin production by *G. virens* have been identified with a view to enhance biocontrol performance by enhanced gliotoxin production (Ridout and Lumsden, 1993).

A group of antimicrobial compounds referred to as the koninginins have been more recently discovered. The first of these, koninginin A ($C_{16}H_{28}O_4$) was a white finely crystaline compound produced by a *T. koningii* strain isolated from a wilted ornamental plant. The compound was found to be inhibitory to etioliated wheat coleoptiles (Cutler *et al.*, 1989). A strain of *T. koningii* isolated from soil suppressive to *Gaeumannomyces graminis* var. *tricici* (*Ggt*) was found to produce 4,8-*di-hydroxy*-2-(1-*hydroxyheptyl*)-3,4,5,6,7,8-*hexahydro*-2H-1-*benzopyran*-5-*one* (later named koninginin D), this toxin was inhibitory to growth of *Ggt* in culture (Dunlop *et al.*, 1989). A further compound 8-*hydroxy*-2-(1-*hydroxyheptyl*)-3,4,5,6,7,8-*hexahydro*-2H-1-*benzopyran*-5-*one* (koninginin B; $C_{16}H_{26}O_4$) of similar structure to koninginin A was isolated from *T. koningii* by Cutler *et al.* (1991). Almassi *et al.* (1991) later reported koninginin B as a breakdown product of koninginin A. Two new compounds, 3-(*propenyl*)-4(*hexa*-2E,4E-*dien*-6-*yl*)*furan*-2(5H)-*one* and 3-(2-*hydroxypropyl*)-4-(*hexa*-2E,4E-*dien*-6-*yl*)*furan*-2(5H)-*one* inhibitory to *Ggt* were also noted (Almassi *et al.*, 1991). Koninginin (F) and a cyclic keto triol with antifungal activity were isolated by Ghisalberti and Rowland (1993). Koninginins C (Parker *et al.* ,1995a) and E (Parker *et al.*, 1995b) an isomer of koninginin B, have also been isolated from *T. koningii.*

Other reports of antifungal compounds include 1-hydroxy-3-methylanthraquinone , 1,8-*dihydroxy*-1-*methyl-anthraquinone* (Ghisalberti *et al.*, 1990), trichodermin (Godtfredsen and Vandegal, 1965), dermadin and trichoviridin (Tamura *et al.*, 1975). Diffusible antibiotics (not identified) were produced by *T. harzianum, T. viride, T. polysporum,* and *T. hamatum* which inhibited radial growth of *Phytophthora cactorum* when radial growth of the *Trichoderma* spp. was halted by benomyl added to agar (Lederer *et al.*, 1992).

3.7.1.2. Volatile antifungal compounds.

Reports of a strong coconut aroma produced by *Trichoderma spp.* have been made for some time (Bisby ,1939; Rifai ,1969; Dennis and Webster ,1971b). Isolates which were inhibitory to *R. solani* were the same ones which had the coconut aroma. Headspace gasses from a culture of *T. harzianum* inhibited growth of *Aspergillus niger* and several other saprophytes (Hutchinson and McGowan, 1972). They attributed this to the concentration of carbon dioxide and ethanol produced by *T. harzianum,* as these compounds were present in significant amounts and no coconut aroma was mentioned. Bruce *et al.* (1984) described efficacy of *Trichoderma spp.* in controlling wood rotting basidiomycetes. They believed water soluble antibiotics were involved in the observed inhibition and lysis of mycelium.

Claydon *et al* . (1987) tested two isolates of *T. harzianum* which were known to have the distinct coconut aroma and identified two compounds, 6-*n*-*pentyl*-2H-*pyran*-2-*one*, (6PAP, which appears to be the major compound of significance, $C_{10}H_{14}O_2$) and 6-n-*pentenyl*-2H-*pyran*-2-one ($C_{10}H_{12}O_2$), which they showed were inhibitory to *Rhizoctonia solani, Sclerotinia sclerotiorum, Phytophthora cinnamommi , Gaeumannomyces graminis, Pyrenochaeta lycopersici , Verticillium dahlii, V. fungicola, Phomopsis sclerotioides , Fusarium oxysporum* , and *Aspergillus flavus.* They also showed that the compounds reduced the rate of lettuce damping-off caused by *R. solani.*

At about the same time Claydon *et al.* (1987) discovered that 6PAP was produced by *T. harzianum* , Simon *et al.* (1988) reported the production of the same two compounds by *T. koningii* . Cultures were extracted using ethyl-acetate and the solvent evaporated under reduced pressure, thin layer chromatography using ethyl acetate as the solvent on silica gel detected compounds of R_f of 0.9. Exudates of the isolate of *T. koningii* grown on a dialysis membrane were inhibitory to the growth of *G. graminis* , *R. solani* , *P. cinnamommi* , *Pythium middletonii* , *F. oxysporum* , and *Bipolaris sorokiniana* . Ghisalberti *et al.*, (1990) found that *T. harzianum* isolates which produced 6PAP showed greater ability to suppress *Ggt* than isolates

which produced a range of other antimicrobial compounds. In another study production of 6PAP was found to be correlated with inhibition of *Rhizoctonia* root rot by a range of *T. koningii* isolates, while production of enzymes which degrade crabshell chitin was not (Worasatit *et al.*, 1994). While production of 6PAP appears to be a major inhibition mechanism it is worthy of mention that some *Trichoderma* spp. chitinolytic isozymes have been shown to be specifically induced by *R. solani* cell wall substrates (Haran *et al.*, 1996). These may not be produced in degradation of crabshell chitin, and this type of induced enzyme activity would possibly not have been detected in the experiments of Worasit *et al.* (1994).

3.7.1.3. Peptaibol antifungal compounds.

Peptaibol membrane-active metabolites include suzukacillin (Ooka *et al.* 1966; Irmscher and Jung, 1977), alamethacin from *T. viride* (Irmsher and Jung, 1977), paracelsin from *T. reesii* (Bruckner *et al.*, 1984), and trichlorzianins from *T. harzianum* (Schirmböck *et al.*, 1994). Peptaibols form reversible voltage dependant ion channels in membranes (Fox and Richards, 1982) and cause irreversible membrane damage at minimal concentrations of 10^{-5} or 3 x 10^{-5} M, respectively (Irmscher and Jung, 1977; Bruckner *et. al.*, 1984). Production of peptaibols together with cell wall degrading enzymes by *T. harzianum* were reported to be induced by *B. cinerea* cell walls (Schirmböck *et al.*, 1994).

3.7.2. Mycoparasitism .

3.7.2.1 Mycoparasitic Coiling.

Weindling (1932) made the first report of coiling of *Trichoderma lignorum* around hyphae. Webster and Lomas (1964) showed that strains discribed by Weindling (1932) were actually *Gliocladium* . Other reports exist of coiling activity by *Trichoderma* spp. around hyphae of pathogens (see Dennis and Webster, 1971c). These authors reported coiling by *T. koningii* on hyphae of *R. solani* , *Fommes annosus* , *F. oxysporum* , *Pyronema domesticum* , *Mucor hiemalis* , and *Pythium ultimum.* Trutmannn and Keane (1990) reported coiling on *Sclerotinia sclerotiorum* .

Three types of hyphal interactions were reported by Dennis and Webster (1971c):

(1) Numerous short branches were produced by the main hyphae and these coiled around the susceptible host hypha.

(2) The main hypha coiled around the susceptible host hypha at a narrow angle. (ie

fewer coils per length of hypha)

(3) The *Trichoderma* hyphae followed the host hyphae and at intervals produced short branches that coiled around the host hyphae.

When this occurred *Trichoderma* hyphae grew in a zigzag manner along the host hyphae. All three types of coiling were seen on all test fungi by each species of *Trichoderma* tested. Prior to coiling, vacuolation, coagulation of the cytoplasm, and sometimes bursting of the hyphae (*F. annosus* and *R. solani*) were induced by the antibiotic producing strains, but not by the other strains. Experiments using plastic threads of the same width as hyphae of *Pythium* spp. determined that coiling was not simply a touch response as no coiling on the plastic threads was observed.

Penetration of hyphae was observed by Dennis and Webster (1971c) only for *Pythium* spp. Durrell (1966) has reported penetration on a range of oomycetes and higher fungi. Appressoria like structures have been reported in penetration of *S. sclerotiorum* hyphae by *T. koningii* (Trutmann and Keane, 1990) and *C. minitans* (Trutmann *et al.* 1982). *T. harzianum* produced similar structures in penetration of *S. sclerotiorum* (Inbar *et al.*, 1996), *R. solani* and *S. rolfsii* (Elad, *et al.*, 1983).

3.7.2.2. Host Specific Recognition in Mycoparasitism.

Host specific recognition is involved in mycoparisitism. Lectins are sugar binding proteins which make cells adhere. After *R. solani* hyphae were exposed for 30 minutes to a 20mM saccharide solution of D or L-fucose, L-methyl-fucoside and D or L-galactose attachment of red blood cells was inhibited. This led to an assumption that lectins were involved in binding, and when *Trichoderma* cell walls components were separated, galactose was identified as a component of the cell wall (Elad *et al.,* 1983). Free floating conidia of *Trichoderma* were attracted (adhered) to hypha of *R. solani* in masses (Elad *et al.*, 1983). A similar lectin relationship appears to exist between *Trichoderma* spp. and *Scletotium rolfsii* (Barak *et al.,* 1985). More direct evidence for the role of lectins in mycoparasitism was obtained when *T. harzianum* was grown on nylon fibres treated with a purified *S. rolfsii* lectin. The mycoparasite coiled around the fibres in a similar way to when it encountered *S. rolfsii* hyphae. When *T. harzianum* was grown on purified agglutinin treated fibres appressorium like bodies were produced, but not when it was grown on untreated fibres or blocked activated fibres (Inbar and Chet, 1992; Inbar and Chet, 1994). One *T. viride* strain was reported to secrete extracellular lectins with affinity for D-galactose, though not for *N*-acetylglucosamine (Neethling and Nevalainen, 1996).

T harzianum has been demonstrated by Transmission Electron Microscopy and Scanning Electron Microscopy to penetrate cell walls of *Rhizoctonia solani* to

cause extensive damage such as cell wall alteration, aggregation of cytoplasm, retraction of the plasma membrane from the cell wall, and ultimately death (Benhamou and Chet ,1993). In this process *T. harzianum* first coils around the host. T.E.M. observations indicated that the interaction was mediated by a fine external matrix which originated from *R. solani* hyphae. The polysaccharide nature of this matrix was demonstrated by affinity for a gold complexed lectin . The occurrence of a significant amount of galactose residues in this external matrix suggests that receptors (lectins) with galactose binding affinity are present at the cell surface of *Trichoderma* hyphae. This could account for the behaviour of *Trichoderma* following adhesion to the host, as it coils close to the host surface.

Some mycoparasites are specifically induced to germinate in response to the pathogen. *Sporidesmium sclerotivorum* macroconidia may be induced to germinate in response to compounds (tentatively called Sporigermins) which are present in the rind of *Sclerotinia minor* sclerotia. Consistent with this discovery *S. sclerotivorum* did not parasitise the mycelium of *S. minor* , and it was postulated that this may be an adaptation to sclerotia residing in the soil for long periods, whereas hyphae are present only for a brief portion of the life cycle (Mischke *et al.,* 1995).

3.7.3. Lytic enzymes.

Research on mycoparasitism as a mechanism of biocontrol raises two additional questions: is physical contact necessary for host destruction, and how important are enzymes and extracellular compounds for host destruction in mycoparasitism? The host may be softened by toxic metabolic products and enzymes, before disorganisation and death occur (Papavizas, 1985).

Lytic activity of fungal antagonists including *Trichoderma* is mainly due to the lytic enzymes ß-1,3-glucanase, a semi- constitutive enzyme induced by laminarin, starch, xylose, mannitol, and glycerol (Reese and Mandels, 1959) and chitinase, an inducible enzyme excreted in media where chitin or its oligomers are the sole carbon source (Monreal and Reese, 1969; Sivan and Chet, 1989a).

Different strains of *T. harzianum* effective in controlling *R. solani* were found to produce ß-1,3-glucanase and chitinase in cultures containing cell walls of this pathogen as a sole carbon source (Ridout *et al.*, 1986). Electrophoretic separation of proteins showed that more extracellular proteins were induced by cell walls than by glucose. The composition of the extracellular enzyme complex of a single *T. harzianum* isolate altered when different isolates of *R. solani* were used as the chitin source.

The cell walls of higher fungi (eg *Sclerotium rolfsii* , *Rhizoctonia solani*) are composed of ß-1,3-glucan and chitin (Chet, Henis, and Mitchell 1967). Oomycete

(eg *Pythium aphinidermatum*) cell walls are predominantly composed of cellulose and ß-1,3-glucan (Bartnicki-Garcia, 1973). Lysis of the pathogen *Sclerotium rolfsii* has been shown to occur in presence of chitinases produced by *Serratia marcescens.* Crude chitinases caused swift swelling of the hyphal tips often followed by bursting (Ordentlich et *al.*, 1988). Hyphal tips are composed of oligomers of *N*-acetyl-glucosamine and *β*-1,3-glucan. Ordentlich *et al.*(1988) suggested that chitinases produced by *S. marcescens* attacked the hyphal tip region probably causing a release of *S. rolfsii* *β*-1,3- glucanase which together with the *S. marcescens* chitinase completely degrades the hyphae.

Control of some pathogens has been correlated with enzyme activity. Three isolates of *T. harzianum* (TH203,TH250,TH294) were tested for activity of ß-1,3-glucanase, chitinase, and cellulase, on cell walls of *Pythium aphanidermatum* , *Rhizoctonia solani* , and *Sclerotium rolfsii.* Isolate TH294 displayed the greatest cellulase, chitinase, and ß-1,3-glucanase activity of all isolates with *P. aphanidermatum* cell walls as the carbon source, but its activity on *R. solani* and *S. rolfsii* cell walls was poor. When inhibitory properties against infection of bean seedlings were determined, TH294 gave superior control of *P. aphanidermatum* and poor control of *R. solani* and *S. rolfsii* infection. Similarly TH250 which had poor chitinase activity on *S. rolfsii* and good chitinase activity on *R. solani* gave poor *S. rolfsii* and good *R. solani* control (Elad *et al.*, 1982).

The degree of inhibition which *Trichoderma harzianum* (P1) is able to exert on the target fungus is proportional to the level of chitin in the cell walls of the host (Lorito *et al.*, 1993b). Production and elongation of germ tubes by conidia of *Botrytis cinerea, Fusarium solani, Ustilago avenae, Uncinula necator, Fusarium graminearum, Saccharomyces cerevisiae* and *Trichoderma harzianum* (22) was strongly inhibited by *T. harzianum* (P1) chitobiosidase and endochitinase at a range of concentrations up to 1000 µg /ml (Table 3G). The combination of the two enzymes did not inhibit germination and growth of *Pythium ultimum* sporangia (which do not contain chitin) and *T. harzianum* (P1)'s own conidia. Lorito *et al.* (1993b) suggested that resistance of *T. harzianum* P1 to the presence of its own enzymes might be in the form of an inhibitor produced in culture or a modification of cell wall composition. The combination of endochitinase and chitobiosidase was a more powerful inhibitor of spore germination and growth than either enzyme alone (Table 3.G). A similar synergism occurs between chitinolytic enzymes and peptaibol antibiotics to inhibit germ tube elongation of *B. cinerea* (Schirmböck *et al.* , 1994). Similarly, endochitinases and gliotoxin of *G. virens* applied alone had little or no inhibitory effect on germination of *B. cinerea* spores. However in combination caused 95% inhibition. Concentrations as low as 25 µg /ml of

endochitinase increased the effect of 1 µg /ml of gliotoxin from 20% inhibition to above 80% (Di Pietro *et al.*, 1993).

Table 3(G): The *in vitro* inhibitory effect (ED_{50}: derived by regression analysis of growth response curves) for enzymes from *Trichoderma harzianum* strain P1 on spore germination and cell replication (ge/re) and germ tube elongation (Source:Lorito *et al.*, 1993b).

Fungus	ED_{50} for enzymes (µg / ml $^{-1}$)					
	Chitobiosidase		Endochitinase		Chitob + Endochit	
	ge/re	elong	ge/re	elong	ge/re	elong
B. cinerea	152	125	41	58	10	24
F. solani	165	168	110	67	30	28
U. avenae	179	--	135	--	34	--
U. necator	180	173	35	30	13	10
T.harzianum (22)	62	162	90	35	ND	ND
S. cerevisiae	490	--	535	--	>400	--
F. graminearum	125	132	100	70	ND	ND
T. harzianum (P1)	>1000	>1000	>1000	>1000	>1000	>1000
P. ultimum	>1000	>1000	>1000	>1000	>1000	>1000

Production of chitinolytic enzymes is by no means a determinant of control of all fungal pathogens with cell walls containing chitin. One strain of *T. harzianum* (T-203) strongly mycoparasitic to *R. solani* and *P. aphinidermatum* failed to parasitise *F. oxysporum* (Sivan and Chet, 1989a). Comparison of induction and activity of lytic enzymes produced by T-203 and *T. harzianum* (T-35) which effectively controls *Fusarium* wilt, determined that in liquid media with chitin and laminarin as a sole carbon sources, both strains produced similar quantities of 1,3-ß-glucanase and chitinase. When the sole carbon source was *F. oxysporum* cell walls, T-203 produced 90% more chitinolytic enzymes than T-35, but not more 1,3-ß-glucanase (Sivan and Chet, 1989a). Neither T-35 nor T-203 parasitised *F. oxysporum* hyphae, which were concluded to be more resistant to mycoparasitism than *R. solani* and *S. rolfsii*. *F. oxysporum* cell walls contain a higher proportion of protein than many other fungal pathogens (Sivan and Chet, 1989a). T-35 control of *F. oxysporum* (Sivan and Chet, 1986; Sivan *et al.* 1987) was suggested to be due to competition or antibiosis (Sivan and Chet, 1989a). A similar finding was made by Ordentlich *et al.* (1991) who assessed the ß-1,3-glucanase and chitinase activity of three *Trichoderma* strains against *F. oxysporum f. sp. vasinfectum* and *F. oxysporum f. sp. melonis* cell walls, and drew comparison to biocontrol efficacy for biocontrol of *Fusarium* wilt of cotton and muskmelon. Enzyme activity was not related to biocontrol capability. Biocontrol of *F. oxysporum* at the rhizosphere level possibly involves more complex or subtle interactions which are not detected *in vitro*, such as antibiosis or competition for nutrients (Ordentlich *et al.*, 1991).

R. solani hyphae are susceptible to membrane disruption caused by secretions of *T. hamatum* , *T. harzianum* , and *G. virens* causing leakage of soluble protein, carbohydrates, salts and amino acids from the cytoplasm (Lewis and Papavizas, 1987). Acetylglucosamine, a product of chitin hydrolysis, was not detected in cultures indicating that antibiotics or other enzymes were involved. Autoclaving of filtrates, which could denature enzymes and toxins, reduced leakage by 75-80%, and it was suggested that the leakage inducer may be a membrane disrupting enzyme. {Note- Published prior to the discovery of *Trichoderma* low molecular weight pyrone antibiotics (Claydon *et al* .,1987) which might be evaporated by heat treatment. Peptaibol antibiotics were also not discounted by Lederer *et al.* (1992).} Filtrates of germling cultures with actively growing hyphae appear to induce more cytoplasmic leakage than older culture filtrates (Lewis and Papavizas, 1987).

Activity of chitinases in lysis of *R. solani* by *T. harzianum* has been implied by scanning electron microscopy and transmission electron microscopy studies where *N*-acetylglucosamine residues were specifically labelled with a wheat germ agglutinin gold complex lectin (Benhamou and Chet, 1993). The series of events in lysis commenced with mycoparasitic coiling and binding close to the cell wall. Evidence of chitinase involvement occurred after binding when as alteration of *N*-acetylglucosamine residues occurred at a higher level first in the outer *R. solani* cell wall then in the inner cell wall. It was proposed that a gradual production of chitinases by *Trichoderma* is necessary for penetration through the host cell wall and that chitin oligomers released from the outer host cell wall act as elicitors for further enzyme production. Other enzymes such as lipases, proteases, and ß-1,4-glucanases are likely to be involved in a co-ordinated attack on the host (Benhamou and Chet, 1993; Benhamou and Chet, 1996). Retraction of the plasma membrane from the cell wall and cytoplasm aggregation were observed as the attack progressed.

Similar studies of the effects of *T. harzianum* cell wall degrading enzymes on *Botrytis cinerea*, showed that degradation of chitin was a relatively late event in the antagonism process. Retraction of cytoplasm from the cell wall, and disorganisation of organelles occurring before loss of viability. Little or no chitinolytic degradation was observed even when the cytoplasm was completely disorganised leaving an empty hyphal shell (sometimes with *T. harzianum* growing inside it). However, by the tenth day after contact, extensive degradation of the cell wall chitin had occurred. This implied that some *Trichoderma* isolates display an antagonistic phase where antibiotics are produced, followed by a saprophytic phase involving chitinolytic enzymes (Belanger *et al.*, 1995), although no investigation was made of the timing of antibiotic production. *T. harzianum* is also known to produce chitinolytic isozymes in a specific and regulated series during degradation of the

host. Sodium dodecyl sulphate polyacrylamide gel electrophoresis and western blot analysis using rabbit polyclonal antibodies were ued to study the series of enzyme production, and indicated both the chitinolytic isozymes which were produced, and the series they were produced in, varied between *R. solani* and *S. rolfsii* (Haran *et al.*, 1996).

Trichoderma spp. are not necessarily immune to the attack of chitinases despite their ability to use them to inhibit other fungi. *T. hamatum* spore germination was 50% inhibited by 8 μg /ml^{-1} of thorn apple, tobacco or wheat chitinase and 2μg/ ml^{-1} inhibited 50% of hyphal elongation (Broekaert *et al.*, 1988). It was not determined whether this *T. hamatum* isolate was itself a chitinase producer. Lysis of *T. viride* hyphal tips due to plant chitinases was suggested to be due to disruption of the balance between chitin formation and tip expansion (Schlumbaum *et al.*, 1986).

Genetic engineering using genes encoding chitinase offers potential for improving efficacy of biocontrol agents by increasing production of enzymes. Genes for production of chitinase may be introduced to organisms of greater rhizosphere competence, increasing the size of th environment where the enzymes can act. For example, *Pseudomonas* spp. with increased ability to suppress *F. oxysporum* were produced by transfer of chitinase encoded genes from *Serratia marcescens* (Sundheim *et al.*, 1988). Protoplasts of *T. harzianum* contain multiple nuclei (up to 10, though usually 4 to 6; Sivan *et al.*, 1990), and procedures have been described to successfully transfer nuclei (Stasz, *et al.*, 1988; Sivan, *et al.*, 1990) and plasmids (Herrera-Estrella *et al.*, 1990) between protoplasts. Protoplast fusion has successfully produced prototrophic *T. harzianum* strains with better overgrowth ability *in vitro* against *R. solani, S. rolfsii,* and *P. aphanidermatum.* than the parent strains (Pe'er and Chet, 1989), and induced prototrophs with improved rhizosphere competence over both parental strains (Sivan and Harman, 1991). Protoplast fusion between different species of *Trichoderma* has also been acheived (Toyama *et al.*, 1984).

3.7.4. Competition.

Biological control resulting from competition has no effect on the viability of the pathogen (Baker, 1981). Competition for nutrients, primarily carbon, nitrogen, and iron may result in biological control of soil-borne plant pathogens (Scher and Baker, 1982). Carbon and nitrogen compounds have been identified which are required for chlamydospore germination in *Fusarium solani f. sp. phaseoli* (Cook and Schroth, 1965). Sneh *et al.* (1984) demonstrated that asparagine and glucose are important for germination of chlamydospores of *F. oxysporum f. sp. lini.* Elad and

Baker (1985) found that the inhibition of *F. oxysporum* , *F. solani, and F. graminearum* chlamydospore germination was reversed by the addition of iron to the system. Germination of chlamydospores is one of the most sensitive stages in the life cycle of *Fusarium* spp. (Baker, 1981).

Germination of *F. oxysporum* chlamydospores was inhibited by the addition of *T. harzianum* (T-35) pre-germinated conidia when 0.05 and 0.01 mg/g of soil of glucose and asparagine were added as food base. However when the same amendments were increased to 0.3 and 0.06 mg/g of soil the inhibitory effect was nullified (Sivan and Chet, 1989b).

When *T. harzianum* (T-35) was added to non-rhizosphere soil on wheat bran preparation it had only a slight effect on survival of *F. oxysporum* . However application of T-35 as a wheat bran peat preparation, conidial suspension, or seed treatment significantly reduced pathogen propagule counts in the rhizosphere soil of melon and cotton which further suggests a competitive mechanism of biocontrol (Sivan and Chet, 1989b).

3.7.5. Antifungal Synergistic Interactions.

Inhibitory mechanisms in biological control may be produced and or enhanced by the interactions of two different organisms to produce an effect which is greater than the summation of the inhibitory properties of either organism.. Biocontrol strains of two bacteria (*Pseudomonas putida* and *Enterobacter cloaceae*) and chitinolytic enzymes from *T. harzianum* (P1) were combined and tested for inhibition of spore germination and germ tube elongation against *Botrytis cinerea* , *Fusarium oxysporum*, and *Uncinulata necator.* The cells of *E. cloaceae*, but not *P. putida* synergistically increased the inhibitory effect of chitinolytic enzymes on both spore germination and germ tube elongation. Culture filtrate of *E. cloacea* combined with *T. harzianum* chitinolytic enzymes generated only an additive response indicating that the presence of the bacterial cells was required for a synergistic effect. Chitinolytic enzyme activity in the presence of chitinous substrates enhanced growth of *E. cloaceae* and enhanced it's ability to bind to the hyphae of the pathogens, even at concentrations as low as 8-16 $\mu g/ml^{-1}$ irrespective of high concentrations of D-glucose and sucrose in the medium (Lorito *et al.*, 1993a). It was suggested that the synergistic effect might result from cell wall degrading enzymes somehow removing sugar inhibition on receptor sites on the bacterial cells and enhance the number of sugar residues available for attachment of bacteria on the fungal cell walls.

While combinations of biological control agents may provide a dual mechanism for disease suppression, care must be taken that they do not compete or

inhibit each other. In one study where *T. koningii* was found to be compatible with fluorescent *Pseudomonas* spp. in biocontrol of *Gaeumannomyces graminis*, the two biocontrol agents appeared to have efficacy in separate regions of the root system (Duffy *et al.*, 1996). Bacteria from *G. graminis* conducive soil inhibit *T. koningii* significantly more than bacteria from suppressive soil (Simon and Sivasithamparam, 1988c).

3.8 Application of biological control.

3.8.1. Production and Delivery Systems.

One of the most critical obstacles to biocontrol by direct massive augmentation or with seed treatment, has been the lack of knowledge of methods of mass culturing and delivering the fungi (Papavizas, 1985). Recently it has become apparent that a major obstacle in the commercial development of a biocontrol product has been problems associated with "scale-up" operations (Lumsden *et al.*, 1995).

3.8.1.1. Natural Introduction Media.

Solid materials which have been used to mass culture and introduce biocontrol fungi include peanut shells (Backman and Rodriguez-Kabana, 1975), bark pellets (Sundheim, 1977), and a sand-cornmeal mix (Lewis and Papavizas, 1980). Composted hardwood bark colonised by *T. harzianum* was suppressive to *R. solani* damping-off of radish when the compost was incubated for six weeks (Nelson *et al.*, 1983). *T. koningii* spore suspensions have been sprayed onto foliage of cowpea to prevent *R. solani* web blight with some success (Latunde-Dada, 1991).

Abd-El-Moity and Shatla (1981) tried amending *S. cepivorum* infested soil with *T. harzianum* on various raw plant materials. Reductions in disease incidence were: bean straw 7.5%, wheat straw 35%, and barley grain 71%. They do not specify the concentration of propagules each treatment contained. Sivan *et al.* (1984) compared a range of different materials for producing propagules of *T. harzianum* . Results (Table 3H) showed that the wheat-bran-peat mix was the best medium tested for production of propagules in a short time period and was the best for propagule survival in storage.

Table 3(H): Growth and survival of propagules of *Trichoderma harzianum* on a range of natural substrates (Source:Sivan *et al.*, 1984).

	CFU/gram of substrate after incubation for:	
Substrate	7 Days	1 Year
Wheat bran	4000	12.0
Peat	450	7.3
Wheat-bran-peat	4900	450.0
Compost	590	0.04
Wheat straw	490	0.22
Cotton Straw	210	0.21

Easy preparation of media for augmentation of *Trichoderma* spp. into soil is desirable. Maintenance of sterile conditions can be a problem in large scale production. Lewis *et al.* (1991) developed a system which did not require sterile conditions, called V.B.A.-F.B. (Vermiculite Bran Acid-Fermented Biomass). This consists of vermiculite mixed with powdered wheat bran which is heated uncovered at 70° C for three days to decrease microbial contaminants, then 0.05M HCl (to discourage growth of bacteria and encourage *Trichoderma* propagules to germinate) is mixed in with *Trichoderma* biomass produced by the liquid fermentation method of Papavizas *et al.* (1984). This dormant biomass is incubated for 2 days prior to introduction to soil so germling hyphae are the main propagule. *T. hamatum* introduced to soil in F.B.A.-F.B. dramatically reduced survival of *R. solani* on infested beet seeds.

Use of processed manure as a carrier for biocontrol agents has advantages, as it can be added in large quantities to support proliferation and growth and biocontrol agents can be easily incorporated. A processed manure powder, kaolinite, and conidia mixture was used to establish *T. harzianum* in soil. It was determined that it was necessary for manure pellets to contain 10^6 conidia /g before *T. harzianum* was detected in 100% of 0.15g samples taken from the soil (Kok *et al.*, 1996).

3.8.1.2. Liquid Fermentation Media.

A technique for large scale production of biocontrol fungi on cheap available materials was developed by Papavizas *et al.* (1984). Biomass was produced in 20L polypropylene carboys with screw caps into which stoppers with aeration tubes leading to the bottom of the container were fitted. Air passed through a 3µm filter aerated and agitated the broth. The food source was 30g of molasses, and 5g of brewers yeast per Litre. Vessel and media were autoclaved for 2 x 1hr before inoculation. When 2 litres of *T. hamatum* inoculum was added the media was ready for use in 10 days.

3.8.1.3. Synthetic Carriers.

Several synthetic materials for application of biocontrol fungi have been developed. Kuek *et al.* (1992) cultured mycelium of mycorrhizal inoculants on and within hydrogel beads 2.5mm in diameter which support and protect intact mycelium through storage and introduction to soil, and promote rapid outgrowth of the hyphae. This material could be spread in soil like fertilizer or could be precisely placed using a fluid drill. Lewis and Fravel (1996) describe a formulation of Pyrax ABB (a pyrophyllite silicate) and fungal biomass (9:1, w/w) produced by liquid fermentation (Papavizas *et al.*, 1984) which provided a base for *G. virens* to colonise soil for control of *S. rolfsii* . Nutrients from the fermented biomass trapped during filtration provide adequate nutrition for proliferation, as the carrier is inert. Alginate prills formed by blending fungal biomass with sodium alginate, which is then added drop by drop into a solution of calcium chloride, to form a spherical bead can be readily produced in large quantities (Abd-El-Moity, 1986). Various organic nutrition sources may be included in the biomass blend to enhance growth of the biocontrol agent (Lewis *et al.*, 1996). Prill amendments with most potential included wheat bran, soy fibre, and chitin. While most carriers involve solid materials, amendment of *T. harzianum* propagules by fluid drill gel was used for control *S. rolfsii* southern blight of apple seedlings. *T. harzianum* did not become adequately established for disease suppression and it was suggested that incorporation of organic or chemical nutrient sources into the gel should be investigated (Conway, 1986).

Speed of spore germination by one biological control agent from hydrogel beads has been enhanced by addition of osmoregulants such as P. E. G. (Polyethylene glycol). Knudsen *et al* . (1991) enhanced *T. harzianum* hyphal growth from alginate pellets over a 72 hour period by about 30% by adding P. E. G. solution instead of water (20 or 40g P.E.G. per 100g pellet). This increase is the rate of germination, not the total amount of inoculum which will germinate.

3.8.2. Addition to the soil.

Applications of *Trichoderma* spp. as seed dressings have been suggested as an alternative to introducing them into soil. This method requires smaller amounts of material than in furrow or broadcast applications (Papavizas ,1985). Antagonists applied to seeds may have a chance to be the first organisms to establish in the developing rhizosphere (Chao *et al.*, 1986). Proliferation of the antagonist along the developing root system is one of the most important traits for antagonists applied to seeds (Cook and Baker, 1983). Percolated water enhances the downward

movement of both bacterial and fungal propagules in the rhizosphere (Chao *et al.*, 1986).

Seed treatments have been reported to control *Pythium* spp. (Wright, 1956; Liu and Vaughan, 1965). Harman *et al.* (1980) reported that seed treatments with the biological control agent *Trichoderma hamatum* protected seedlings from attack by *Pythium* spp. or *Rhizoctonia solani* . As a consequence of the seed treatment, numbers of *Trichoderma* propagules increased approximately 100 fold and the soil became mildly suppressive to these pathogens. Harman *et al.* (1981) sought to improve reliability of biocontrol by addition of amendments which would support the continued protection of the host. Cultures of *T. hamatum* were grown on PDA and were allowed to dry out before being ground using a mortar and pestle and sieved through a 589 μm^2 screen. Conidia from the dried PDA culture were applied at 3% (W:V) to water containing a spreading agent (Pelgel®). *R. solani* attack on peas was reduced by 10% when *T. hamatum* was added as a seed dressing in this way. However, when *T. hamatum* with chitin was amended disease incidence decreased by 51%. *T. hamatum* grown on ground *R. solani* cell walls reduced disease incidence by 37%, however cellulose amendments gave poorer control than *T. hamatum* alone. As simple sugars were present from the PDA, this type of effect might be due to catabolite repression (Montencourt and Eveleigh, 1977). An interesting comparison would have been made if the *Trichoderma* cultures had been grown on the polysaccharide amendment, rather than PDA.

T. koningii and *T. harzianum* introduced to soil as seed treatments did not move into soil due to the deleterious effects of soil microflora. such as *P. fluorescens* (Chao *et al.*, 1986).

It seems that the choice of introduction medium and method can influence the potential of individual biological control agents considerably in the control of onion white root rot. Sodium alginate pellets, soil additives, and seed coatings were used to introduce five potential biocontrol agents (*Chaetomium globosum, G. virens, T. viride , T. harzianum* and *Trichoderma* spp.) to soil in a study to find a biological control for *S. cepivorum* (Kay and Stewart, 1994a). Table 3(I) shows some results from this study which was conducted in 400 x 800 x 200 mm soil boxes in open air conditions. In soil containing 0.8g of pre-incubated sclerotia added as a layer 50 mm below the surface, alginate pellets or sand/bran medium were added at a concentration of 0.1% per gram of dry soil. Results showed that some of the potential biocontrol agents varied in efficacy when applied by different methods, eg. *Chaetomium globosum* displayed good efficacy when applied by sand bran soil additive (18% infection) but provided poor control (99% wilt) when applied as a seed dressing. In general, sand/bran medium was a better amendment strategy than

seed dressings. Alginate was not as good an amendment strategy as sand/bran, but was better than seed dressings.

Table 3(I): Percentage *S. cepivorum* infection at 10 weeks after sowing onion seed with biocontrol agents added by either sand bran medium, alginate pellets, or as a seed dressing (Source:Kay and Stewart, 1994a).

	% wilt ten weeks after sowing.		
	Sand/Bran	Alginate	Seed Dressing
Chaetomium globosum	18 d	53 b	99 a
Gliocladium virens	41 bc	45 b	43 b
Trichoderma viride	29 cd	44 b	38 c
T. harzianum	31 cd	39 c	78 a
Trichoderma spp (C62)	21 d	75 a	73 a
Disc H_2O control	61 b	76 a	65
No Treatment at all			81 a

3.9. Compatibility With Existing Commercial Practice.

Vegetable industries use a range of fungicides to control various fungal pathogens. Biocontrol preparations have sometimes formed a component of fungicide spray programs (Elad and Kirshner, 1992; Elad *et al.*, 1993). A biocontrol agent for *S. cepivorum* in Tasmania would need to be compatible with the benlate:thiram seed dressing for control of *Botrytis allii* and lower fungi. Field isolated strains of *Chaetomium globosum*, *T. harzianum* , *T. viride* and *Trichoderma* spp. with biological control potential for *S. cepivorum* were screened on fungicide amended agar for sensitivity to fungicides used by the New Zealand onion industry (Kaye and Stewart, 1994b). Some potential biocontrol agents were highly sensitive to benomyl (Table 3.J).

Table 3(J): Concentrations of six fungicides which inhibited 50% of antagonist growth compared to untreated controls (source: Kay and Stewart, 1994b).

Isolate	Fungicide sensitivity ED_{50} (µg/ml)					
	benomyl	captan	iprodione	mancozeb	procymidone	thiram
C. globosum	0.1	81.6	3.3	>100	3.0	58.4
T. harzanum	0.2	>100	1.4	>100	0.9	65.6
T. viride	0.3	>100	2.1	>100	1.6	65.8
Trichoderma spp	0.3	>100	1.3	>100	1.1	>100

While growth on P.D.A. does not exclusively represent activity in soil, this gives some indication of the potential effect that such chemicals use in crops may have on specific fungal isolates. Kay and Stewart (1994b) induced mutations of the

four antagonists used in their study by UV irradiation. In presence of fungicides, soil box trials indicated that these antagonists had similar efficacy (occasionally better) than the wild strains. Tolerance to benomyl was not induced in this study, though other workers using similar techniques (Papavizas *et al.*, 1982; Papavizas and Lewis, 1983) have reported successful benomyl resistance induction. There have been some reports of benomyl tolerant mutants losing their efficacy for biocontrol of *Sclerotium rolfsii* (Papavizas *et al.*, 1990), though Papavizas *et al.* (1982) also reported UV induced benomyl resistant strains of *T. harzianum* which provided an 80% reduction in infection of onions by *S. cepivorum* (in pot trials), whereas the wild (benomyl sensitive) parent strain provided no disease suppression. The universal use of Benomyl as a seed dressing for control of *Botrytis allii* might cause a problem in commercialisation of benomyl sensitive biological control agents.

4.0. Literature Review- Fungal Cell Walls and their Degradation.

4.1. Constituents.

Cell walls of higher fungi are predominantly composed of polysaccharides, and these can be divided into two groups on the basis of function and physical form. The first, skeletal polysaccharides, are insoluble and highly crystalline homopolymers and include chitin and ß-linked glucans. The second, matrix polysaccharides, are amorphous or slightly crystalline, and are mostly water soluble; they include homopolymers, heteropolymers and polysaccharide complexes (Peberdy, 1990).

4.2. Cell wall synthesis.

The microfibrils of the skeletal components (1-3)-ß-D/(1-6)-ß-D-glucan and chitin (glucosaminoglycan) are embedded in a matrix (Bartnicki-Garcia, 1973; Wessels *et al.*, 1990) and are normally located toward the inner surface of the wall. The matrix is composed mainly of α-glucans (*s*-glucan, a *a*-1,3-homopolymer of glucose, and nigeran, α-1,3- and α-1,4- linked glucan) and glycoproteins (Peberdy, 1990).

Miscellaneous components associated with fungal cell walls also include *D*-galactosamine polymers, chitosan, polyuronides, melanins, and lipids (Peberdy, 1990).

4.2.1. Chitin.

Chitin chains are synthesised at the hyphal apex and are initially present in a non-crystalline state. Some hydrogen bonding between chain molecules occurs soon after polymerisation leading to formation of crystalline microfibrils (Vermeulen and Wessels, 1986)

4.2.2. ß- glucan.

In some fungi, eg *Agaricus bisporus* and *Schizphyllum commune* (1-3)-ß-D/(1-6)-ß-D-glucans are covalently linked to chitin in the cell walls, though (1-3)-ß-D/(1-6)-ß-D-glucans from cell walls of different fungi vary in affinity for different solvents (Sietsma and Wessels, 1981). Evidence for the existence of covalent bonds between glucans and chitin is supported by the results of kinetic experiments where labelled acetylglucosamine and glucose precursors were incorporated into growing

apices (Wessels *et al.*, 1983). Labelled water soluble glucans passed through the [1 M KOH] alkali soluble glucan fraction of the apices to the alkali insoluble glucan fraction found 0.6μm from the tip (Sonnenberg *et al.*, 1982). The water and/or alkali soluble fractions are more concentrated in the first 4μm of the apex. As the apex grows, the amount of alkali insoluble material increases approximately three fold by the time it is 0.6 μm from the tip.

4.3. Cell Wall Degradation.

4.3.1. Enzymes.

Literature regarding chitin degrading enzyme nomenclature has been confused, clarification was provided by Harman *et al.* (1993).

Enzymes which have activity against chitin are referred to as chitinolytic enzymes collectively (Harman *et al.*, 1993). Enzymes that cleave at random points in the chitin chain are referred to as endochitinases (EC 3.2.1.14.) and they require at least the tetramer of chitin for activity. The enzyme that releases chitin dimers (chitobiose) from the end of chains is called 1,4-ß-chitobiosidase (chitobiosidase) and it requires at least the trimer of chitin to act. Enzymes that release monomeric units from the chitin chain end are exochitinases (eg *N*- acetyl-ß-glucosaminidase [EC 3.2.1.30.]) and they require at least the dimer of chitin to act (Harman *et al* , 1993; Tronsmo and Harman, 1993). The preceding definition of chitobiosidase is the same as that given for exochitinase by some authors (eg Robbins *et al.* , 1988; McCreath and Gooday, 1992; Sahai and Manocha, 1993). Chitobiosidase is a preferable term because exochitinase has also been used to describe enzymes that release monomeric units from chitin (Tronsmo and Harman, 1993).

Addition of more easily metabolised carbon sources induced repression of exochitinase and *N*- acetylglucosaminidase production by *T. harzianum* (Ulhoa and Peberdy, 1993). Note, these authors refer to *N*-acetylglucosaminidase (EC 3.2.1.30) as chitobiase.

Enzymes of the *T. harzianum* (P1) chitinase complex were purified by Harman *et al.* (1993). Fractionation of concentrated dialysed culture filtrate using gel filtration on sephacryl S-300 separated several enzymes by molecular weight. The first large peak of activity (Set 1) occurred between fractions 80 - 110 and consisted of glucosaminidase and a smaller quantity of chitobiosidase activity. Another peak (Set 2) of chitobiosidase was detected together with some endochitinase in fractions 110 - 120. Fractions 150-190 (Set 3) contained most of the endochitinase. Electrophoresis in native, sodium dodecyl sulphate (SDS) and iso-electric focussing gels showed that the (Set 3) endochitinase consisted of a single

41 kilodalton protein. After purification the chitobiosidase consisted of three closely spaced protein bands on S.D.S. gels, the largest (40 kilodaltons) stained most intensely, the 38 kilodalton one stained weakly, and a 35 kilodalton one stained intermediately. Optimum pH for activity of the endochitinase was at pH 4.0 and gradually declined to pH 7.0, optimum for chitobiosidases was determined to be in the general range from 4.0 to 7.0. Haran *et al.* (1995) additionally report production of *N*-acetylglucosaminidase (73 kDa) and endochitinase (52 kDa) by *T. harzianum* (strain TM). The strains 41 kDa protein reacted with polyclonal antibodies (SDS - polyacrylamide gel electrophoresis western blot analysis) raised against the 41 kDa protein produced by PI strain used by Harman *et al.* (1993). This suggests that the other isozymes were not closely serologically related to this protein. De la Cruz *et al.* (1992) reported procedures for purification and characterisation of *T. harzianum* chitinases also. It was noted that two of the chitinases purified (Chit 33kDa and Chit 37kDa) were not able to act on insoluble chitin in the absence of a third protein (Chit 42kDa). Genes encoding the 42 kDa chitinolytic enzyme have recently been characterised (Carsolio *et al.*, 1994).

4.3.2. Substrate.

Molano *et al.* (1979) added wheatgerm chitinase to a reaction mixture in which chitin was synthesised by *S. cerevisiae* (a yeast) chitin synthetase. Nascent chitin chains were rapidly degraded. However, if chitin was first allowed to accumulate and chitinase was then added, the rate of chitin hydrolysis decreased by approximately two orders of magnitude. This result implies that if chitin chains have already associated into fibrils by hydrogen bonding, the activity of the enzyme becomes more difficult. This shows that aggregation of the chitin chains is not simultaneous with synthesis, and suggests that crystallisation might be a spontaneous process.

Vermeulen and Wessels (1984) showed evidence that the chitin microfibrils in apical tips of hyphae are highly susceptible to chitinase degradation, but that they become more resistant as they move further down the hyphae to sub apical hyphal walls . Formation of the hydrogen bonds between adjacent molecules leading to crystallisation seems to lessen the susceptibility towards chitinase (Vermeulen and Wessels, 1986). Non growing hyphal tips were also more resistant to degradation after incorporation of *N*- acetylglucosamine (Vermeulen and Wessels, 1984).

4.4. Methods of chitinase assay.

4.4.1. Viscometric.

Determination of chitinase activity is an area fraught with serious problems and pitfalls. Insolubility of the substrate in aqueous solutions has made kinetic (eg viscometry) measurements difficult, and their interpretation uncertain (Cabib, 1987).

4.4.2. Colourimetric/Turbidometric.

Determination of the reducing power of chitinase has routinely been performed using the specific colorimetric reaction of *N*-acetyl-hexosamines with p-dimethylaminobenzaldehyde (Reissig *et al.* 1955). However, *N*- acetylhexosamines substituted in C-4 do not yield colour in this reaction (Cabib, 1987). Since chitinases do not give rise to free GlcNAc this procedure is not useful unless an excess of endochitinase-free ß-*N*-acetyl-hexosaminidase is present, this can be added after incubation (Cabib, 1987). Alternatively chitin could be separated by filtration (Cabib and Bowers, 1971) or by centrifugation (Boller *et al.* 1983) before the ß-*N*-acetyl-hexosaminidase is added in a second incubation.

4.4.3. Electrophoresis.

Zone electrophoresis by the method of Paigan (1956), with modifications of Cota-Robles *et al.* (1957) was used by Berger and Reynolds (1958) to investigate the chitinase system of *Streptomyces griseus* using swan potato starch beds made up in a 0.2 M phosphate buffer. They detected chitobiase and chitinase isozymes which migrated cathodically and a chitinase which migrated anodically. The zone electrophoresis can be followed up by cutting the isozyme bands from the bed and purifying them by dialysis and re-running on fresh starch columns before testing their ability to release *N*- acetylglucosamine by colourimetric means.

Trudel and Asselin (1989) described two systems of polyacrylamide gel electrophoresis for detection of chitinolytic enzymes under native and S.D.S. denatured conditions. The fluorescent stain used in this technique, "Calcoflour White" is a disodium salt of 4,4'-bis-[4-analino-bis-diethyl-amino-S-triazin-2-ylamino]-2,2'-stilbene-disulphonic acid, when combined with chitin (or another polysaccharide with ß configuration) and irradiated with UV light shows positive fluorescence. It is thought the sites of stain binding are hydroxyl groups in the ß-configured polymers (Maeda and Ishida, 1967).

4.4.3.1. Polyacrylamide Gel Electrophoresis (PAGE) under native Conditions.

Trudel and Asselin (1989) describe electrophoresis systems for detection of chitinases. Polyacrylamide gel electrophoresis under native conditions as for the Reisfeld *et al.* (1962) method (pH 4.3) has been performed using 15% polyacrylamide gels with 5% stacking gels. Samples containing 15% sucrose and 0.01% methylene blue, were loaded and electrophoresis performed at room temperature for 3 hrs at 30 mA. An alternative system of polyacrylamide gel electrophoresis under native conditions is the system of Davis (1964) conducted at pH 8.9 and using 0.01% bromophenol blue as a tracker dye. Electrophoresis for the Davis system was performed at room temperature for 65 minutes at 20 mA.

Electrophoresis gels were incubated in 150mM sodium acetate buffer at pH 5.0 for 5 minutes, then covered with an overlay, containing 0.01 % glycol chitin in 100 mM sodium acetate buffer pH 5.0. After incubation for 1 hr under moist conditions at 37°C in a plastic container gels were removed and incubated with 0.01 % Calcoflour white in 500 mM Tris-HCl pH 8.9. This was incubated for 5 minutes before the calcoflour white was removed and the gels are incubated for 1 hr in distilled water. Lytic zones on the overlay gel can be identified then photographed under Ultra Violet light. Corresponding proteins in the separating gel can be stained with coomassie blue or silver nitrate, this technique detected well defined bands, not smears (Trudel and Asselin, 1989).

4.4.3.2. Polyacrylamide gel electrophoresis (PAGE) under denaturing conditions.

Techniques of sodium dodecyl sulphate polyacrylamide gel electrophoresis such as developed by Laemmli (1970) can be used to seperate proteins into individual polypeptide chains based on molecular weight can be used to isolate enzymes. Sodium dodecyl sulphate (S.D.S.) minimises the native charge differences so all proteins migrate as anions as a result of the complex formed with S.D.S. This method of electrophoresis was employed by Molano *et al.* (1979) to determine the molecular weight of endochitinase from wheat germ by comparison to standards of known molecular weight.

Trudel and Asselin (1989) describe S.D.S. polyacrylamide gel electrophoresis using 10-15% (W:V) polyacrylamide gradient gels containing 0.01% glycol chitin and 0.1% S.D.S. with 5% polyacrylamide stacking gels containing 0.1% S.D.S. Samples were boiled for 5 minutes with 15% sucrose and 2.5% S.D.S. in 125 mM Tris-HCl (pH 6.7) with or without 2% ß-mercaptoethanol. 0.01% bromophenol blue was the tracker dye. Electrophoresis was performed at room temperature for 65 minutes at 20 mA.

After S.D.S. electrophoresis gels were incubated for 2hrs at 37°C with reciprocal shaking in a 100 mM sodium acetate buffer (pH 5.0) containing 1% (V:V) Triton X-100 purified through a mixed bed resin de-ionising column. Gels were then stained, destained, viewed and photographed as for native gels.

Cherif and Benhamou (1990) used S.D.S. polyacrylamide gel electrophoresis with calcoflour white staining to detect chitinases produced by *Trichoderma* spp. in degradation of *Fusarium oxysporum f. sp. radicis-lycopersici* cell walls. Their results indicated that at least three major, and five minor chitinases were produced. Some chitinases were produced early after contact between the antagonist and the pathogen cell walls, others were gradually excreted into the medium. When *Trichoderma* was grown in glucose media for seven days no chitinases were detected.

Tronsmo and Harman (1993) describe a system of polyacrylamide gel electrophoresis using a Phastsystem (Pharmacia LKB Biotechnology , Uppsala, Sweden). Enzyme activity was detected on 20% native gels or sodium dodecyl sulphate (SDS) gels using agarose overlay gels containing 100 mM potassium phosphate at pH 6.7 (agarose gels) or 100 mM sodium acetate at pH 4.8 (phast gels) and enzyme substrates for the three chitinolytic enzymes in question. The agarose substrate suspensions were dissolved in a microwave oven and cooled at 42° C prior to application to gels. For visualisation of activity, gels were soaked in 25% V:V aqueous isopropanol for 15 minutes and rinsed in distilled water before application of the overlay. Overlays were poured onto a glass plate and the separating gel was placed face down on top of the molten agar suspension. 300 micrograms per ml of substrate were used to detect enzyme activity, these were: 4-methylumbelliferyl-*N*-ß-D-glucosaminide {abbrev- 4MU-(GlcNAc)} , 4-methylumelliferyl-ß-D-*N,N'*-diacetylchitobioside {abbrev- 4MU-(GlcNAc)2} , or 4-methylumbelliferyl-ß-D-*N,N',N''*-triacetylchitotriose (abbrev- 4MU-(GlcNAc)3} to detect *N*- acetyl-ß-glucosaminidase, chitobiosidase, and endochitinase, respectively. Bands were evident when viewed under ultraviolet light after about 5 minutes incubation at 25°C (Tronsmo and Harman, 1993). The interpretation of Tronsmo and Harman (1993) on the mode of activity of each enzyme, was based on which 4-methylumbelliferone bearing substrate activity was detected upon. The interpretation of Tronsmo and Harman (1993) of each enzymes mode of activity appears to differ from the interpretation of Haran *et al.* (1995), who reported proteins which acted on two of the three or all three substrates. Haran *et al.*, (1995) considered proteins with activity on 4MU-(GlcNAc)2 and 4MU-(GlcNAc)3 but not 4MU-(GlcNAc) to be endochitinases, and proteins with activity on all three substrates to be *N*-acetyl-ß-glucosaminidases. The interpretation of Haran *et al.* (1995) is followed in the present study, as it is logical that glucosaminidases should act on all three substrates.

Greater sensitivity in detection of chitinase isozymes was recently achieved by western blot analysis of electrophoretically separated proteins using rabbit polyclonal antisera (Haran *et al.*, 1996). The method allowed presence of known chitinase isozymes which were not detected due to low concentration in antagonism assays using 4-methyumbelliferone substrates to be detected. However the antibodies cross reacted with several other proteins which makes the exclusive use of the method unreliable. Haran *et al.* (1996) also used 4-methylumbelliferyl substrates for detection of chitinolytic enzymes in general by adding both 4MU-$(GlcNAc)_2$ and 4MU-$(GlcNAc)_3$ to the overlay gel.

4.4.4. Cytochemical Labelling.

Cherif and Benhamou (1990) have used Transmission Electron Microscopy combined with specific cytochemical labelling of *N*-acetylglucosamine residues using wheat germ agglutinin, a lectin, to study cell wall degradation. Ovomucoid, a water soluble glycoprotein isolated from egg white was chosen for binding to wheat germ agglutinin, and conjugated to colloidal gold particles which can be detected by the T.E.M . *N*-acetylglucosamine was labelled in cell walls of *Trichoderma* spp. and *Fusarium oxysporum* f. sp. *radicis- lycopersici* growing in culture together. In *Trichoderma* spp the distribution of *N*- acetylglucosamine was found to be even over the cell walls, perhaps preferentially associated with the inner cell wall layers, this arrangement did not alter after 7 days of incubation in the presence of *F. oxysporum* f. sp. *radicis-lycopersici* during which *Trichoderma* was shown to be producing chitin degrading enzymes. When incubated alone, *F. oxysporum* f. sp. *radicis-lycopersici* cell wall *N*- acetylglucosamine was distributed evenly over the cell walls but gradually after 3 days incubation with *Trichoderma* the *N*- acetylglucosamine was released into the surrounding medium, and by 7 days most cells had died and their cell walls fell apart.

Some questions need to be further addressed from the work of Cherif and Benhamou (1990). Results show *N*-acetylglucosamine present in cell walls before chitinolytic enzymes are introduced, after these enzymes are present it is released. Clarification is needed as to whether the *N*-acetylglucosamine was originally present as monomeric units in the cell wall matrix and was then released by the action of endochitinases and chitobiosidases on other components of the cell wall (chitin polymers and structural fibrils), or the monomers were the actual degradation products of the chain polymers and fibrils released by the activity of an exochitinase (Data using S.D.S. P.A.G.E. showed that degradation of chitin with release of *N*-acetylglucosamine was occurring, presumably by activity of *N*-acetyl-β-

glucosaminidase). Authors do not mention whether *N*-acetylglucosamine can be degraded further after being bound by the lectin.

Chitinase mediated fungal growth inhibition, whether that of plants (Nielsen *et al* , 1994; Benhamou *et al.*, 1993) or other fungi (Cherif and Benhamou, 1990; Benhamou and Chet, 1993) has been found to coincide with apical swelling and lysis of hyphae. Exposure of hyphae to bean endochitinases for 30 minutes induced release of chitin oligomers from the cell wall. These were detected by cytochemical labelling of *Rhizoctonia solani* *N*-acetylglucosamine residues (Benhamou *et al* .,1993). The process ultimately leads to retraction of the plasma membrane from the cell wall and distortion and breakdown of the cell wall. It was noted that hyphal apices seemed to be highly accessible to chitinases, often swelling after exposure (Benhamou *et al* . 1993), leading to bursting of the hyphal tip as has been reported for tobacco plant chitinase on *Phycomyces blakesleeanus* (Broekaert *et al.*, 1988). In support of these results, a clear picture of action of sugar beet chitinases on tips of *Cercospora beticola* was gained by incubating young mycelia in 40 µl of P.D.B. containing 30-90 u/Ci/ml of tritium labelled *N*-acetylglucosamine ([^{3}H]GlcNac). After incubation for 20-60 min the pulse labelling was either terminated by soaking in an ice cold 6% (W/V) trichloroacetic acid bath, or mycelium was further allowed to grow by incubation in P.D.B. for four hours followed by dehydration in a 70 - 99% ethanol gradient. Specimens were incubated with 1-10 µg of chitinase in 40 µl Tris-HCl at pH 8 at 30°C for 1-20 hrs. The reaction was then terminated by dipping in a trichloroacetic acid bath. To visualise the radioactive depositions in the fungal specimen , the slide was coated in an autoradiographic emulsion (Ilford K5, Ilford Ltd, London, UK.) and exposed for 7 days at 4°C before developing. Autoradiographs showed that labelled [^{3}H]GlcNac in apices was more readily released by chitinases than labelled [^{3}H]GlcNac in the mature cell walls in the zone behind the hyphal apex (Nielsen *et al.* , 1994). This information is compatible with the model for cell wall synthesis and structure of *Schizophyllum commune* (Wessels, 1986) that exposed nascent chitin fibres in the apex of growing hyphae are more amenable to hydrolysis by chitinase, whereas in the mature cell wall chitin fibres are cross linked to form a chitin-glucan complex which may be covered by other polysaccharide and protein layers which make the chitin less accessible to hydrolysis.

5.0 Literature Review: Cellulose degradation.

5.1. Cellulose degradation.

Literature on the subject of cellulose degradation pathways may be confusing as the original model of Reese *et al.* (1950) which involved a series of enzymes (C_1 followed by C_X followed by ß-glucosidase) was a widely accepted hypothesis for many years. Later work by Erikkson (1969) suggested that the mechanism of degradation was reversed, ie the C_X enzymes (or endo-1,4-ß glucanases (EC 3.2.1.4), of which *T. koningii* produces five [Wood ,1972; Wood, 1975]) first attack native cellulose chains and open up the 1,4-ß-glucosidic bonds giving rise to both reducing and non reducing end groups. The C_1 enzymes (or exo-1,3-ß-glucanase (EC 3.2.1.91), a single enzyme in the case of *T. koningii* [Wood, 1972]) liberates cellobiose units from the non-reducing end of the polymer. 1,4-ß-glucosidase (EC 3.2.1.21) hydrolyses cellobiose and other water soluble cellodextrins to glucose. This mechanism was shown to apply to *T. koningii* (Halliwell *et al.* ,1972; Wood and McCrae, 1972) as well as other fungi reviewed by Erikkson *et al.* (1990). Erikkson *et al.* (1990) proposed that the names C_1 and C_X should be avoided in future as they cause confusion, particularly as their reaction sequence is the reverse of that initially suggested. The final step in breakdown is the activity of 1,4-ß-glucosidases causing hydrolysis of cellobiose and other water soluble cellodextrins to glucose (Erikkson, 1990).

Trichoderma viride has been well studied as a producer of cellulase and produces a stable extracellular cellulase complex capable of attacking crystalline cellulose (Montencourt and Eveleigh, 1977). This type of action appears to be absent in many other microbial cellulases (Mandels, 1975). The action of the cellulase complex seems to be controlled by a represser-inducer system in which sophorose serves as an inducer. Inductive formation of cellulase was markedly affected by addition of glucose (Nisizawa, Suzuki and Nisizawa, 1972) where cellulase activity in 2 hours was approximately 4 times greater when glucose concentration was 1mM/L than when it was 100 mM/L. Cellulase activity also tripled at glucose concentrations of 1, 10, and 100 mM/L when sophorose concentration of the media was increased from 0.1mM/L to 0.5 mM/L. This phenomenon has been called catabolite repression, and is also induced by other cellulose degradation products such as cellobiose (Montencourt and Eveleigh, 1977). The phenomenon may also be partly repressed by presence of starch (Taj-Aldeen, 1993).

5.2. Cellulase detection methods.

5.2.1. Electrophoresis.

Polyacrylamide gel electrophoresis appears to be a suitable analytical procedure for detection of cellulase activity. The technique combines the advantage of enzyme identification with capacity to separate protein bands so that even slight differences in molecular weight are detected in the presence of sodium dodecyl sulphate (Schwarz *et al.*, 1987). Polysaccharides containing contiguous B-(1-4) linked D-glucopyranosyl units can be stained by congo red which has provided the basis for a sensitive test for detecting colonies of cellulase producing micro-organisms (Teather and Wood, 1982).

One difficulty in electrophoresis technique is in bringing the enzyme into contact with the substrate, as even soluble cellulose derivatives such as carboxymethyl cellulose cannot be expected to diffuse inside polyacrylamide gels because of their high molecular weight. Diffusion of renatured enzymes out of the gel is slow and inefficient.

5.2.2. Cutting of gels

Rather than attempting to stain zones of enzyme activity and facing the difficulties of diffusion associated with proteins and cellulose, Saddler and Khan (1981) performed electrophoresis in 7.5% and 15% polyacrylamide gels (pH 9.5) which were run for 50 mm. The gel was then cut into 25 slices, each 2 mm long. Slices were then extracted with 1 ml of 0.2 M citrate-phosphate buffer pH 5.0 containing 10 mg of substrate (either carboxymethyl cellulose, avicel, or cellobiose). Gel slices were eluted for 24 hrs at 4°C, and assayed for activity of various cellulases by colorimetric procedures.

5.2.3. Agar gel replicas.

Beguin (1983) visualised the protein bands with endo-1,4-B-glucanase activity after electrophoresis in the presence of sodium dodecyl sulphate by blotting the slab gels on CMC agar. Upon incubation and staining of the replicas with congo red, the locations of the endoglucanases were revealed by their lack of colour. Band contrast was found to semi-quantitatively brighten with increased enzyme concentration. Staining of slab gels with coomassie blue for protein and

comparison to bands of known molecular weight can be used to determine molecular weight of the cellulases.

5.2.4. Incorporation of substrates into gels.

Schwarz *et al.* (1987) developed a technique whereby endoglucanases, 1,4-*B*- D- glucan cellobiohydrolases (another term referring to exo-1'3-*B*- glucanase, the enzyme which splits cellobiose units from non reducing ends of cellulose chains) and protein could be stained in the same gel. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% acrylamide gels in presence of SDS as described by Laemmli (1970). *B*-glucan or carboxymethylcellulose were incorporated into the gel before polymerisation. Before protein samples were loaded they were heated for 10 minutes at 80°C in 2% SDS. Electrophoresis was conducted at 40°C at a constant current at 30 mA. After electrophoresis gels were washed five times for 30 minutes in cold 0.1 M succinate buffer pH 5.8, containing 10 mM dithiothreitol. Gels were then submersed in 0.1 M succinate buffer (pH 5.8) and incubated for 30 minutes at 60 degrees. For activity staining of cellobiohydrolase activity incubations were carried out in the presence of 1 mM 4-methylumbelliferyl-*B*-D- cellobioside and positive bands were detected by fluorescence under UV 340nm. Endo-1,4-*B*-glucanases were visualised by staining the gel with congo red (1 mg/ml) for 10 minutes at room temperature and destaining in 1 M NaCl for another 10 minutes.

The method of embedding substrates in polyacrylamide gels is favourable to use of overlay gels. Renatured enzymes may be anchored to gels by the folding of polypeptides around the gel matrix as the enzymes are renatured in SDS PAGE.

5.2.5. Substrate adherence.

Mathew and Koteswara (1992) described a method whereby Endo-1,4-ß-D-glucanase can be detected in a single gel electrophoresis procedure without need to incorporate the substrate into the gel. PAGE was performed under native conditions by the method of Davis (1964) at 20 mA in 7.5% acrylamide gels 1.5 mm thick at 15°C. Gels were pre-run for 4 hrs at 10 mA.

After electrophoresis gels were transferred to 1% carboxymethylcellulose in 0.1M citrate buffer (pH 4.8) and incubated for 15 minutes at 50° C, before being transferred to 0.1% solution of congo red and incubated at 25° C with gentle shaking for 10 minutes. Gels were destained by washing in 1M NaCl. Activity bands were visible as yellowish clearances on a dark red background.

This previously unreported property of carboxymethylcellulose to bind to acrylamide provides a useful, fast and simple alternative to stacking gels. The method depends on degradation of the adhered carboxymethylcellulose making cleared zones appear. It was not determined whether exo-1,3-ß-glucanases and 1,4-ß-glucosidases had any effect on the substrate, and it may be that some of the clear zones detected result from these enzymes.

Chapter III: The *Sclerotium cepivorum* infection process:

6.0. Histological Studies.

6.1. Introduction.

It is necessary to more clearly understand the development of *S. cepivorum* infections in order to understand how the infection process might be inhibited by potential biocontrol agents such as *T. koningii.* The mechanisms by which *S. cepivorum* attacks and penetrates the onion root epidermis have been well documented (Abd-El-Razik *et al.*, 1973; Stewart *et al.*, 1989a; Stewart *et al.*, 1989b). Results of a preliminary study (Metcalf, 1993) supported these reports that host cell penetration occurred by production of appressoria, infection cushions of four types, and hyphal tips growing in between junctions of cell walls. Stewart *et al.* (1989a) suggested that the infection cushions were formed in response to difficulties in penetrating the root surface as it was noted that they were formed only on bulb surface regions where a cuticle was present, and not on roots. Little information has been published regarding the behaviour of *S. cepivorum* following penetration of the epidermis, though Stewart *et al.* (1989b) noted extensive degradation of the epidermis 2-3 cells ahead of advancing hyphae, and formation of a cavity beneath infection cushions. Abd-El-Razik *et al.* (1973) noted that *S. cepivorum* advanced intra and intercellularly causing extensive disintegration of root cortical parenchyma cells. This study aims to investigate the relationships between spatial distribution of hyphae, host cell death and cell wall degradation in development of *S. cepivorum* infections.

6.2. Materials and Methods.

Onion seedlings produced under sterile conditions were infected by pre-germinated sclerotia by the methods described for the infection bioassay in Appendix C. Distinction of living from dead tissues was made by the Feulgen stain described in Appendix D and wax embedded before sectioning as described in Appendix D. The experiment was repeated over 100 times. Slides were examined by light microscope. Some nuclei were seen to be only faintly stainable, this implies that denaturation of nuclei is a gradual process following cell death. In this study it is considered that presence of a stainable nucleus is not absolute evidence of a living cell, though absence of a clearly stainable nucleus is evidence of a dead cell.

6.3. Results.

S. cepivorum hyphae expanded over the petri dish base and along the onion root, appressoria and infection cushions often formed in an attempt to penetrate the petri dish base (Figure 6.A.). Examination of the underside of the infection cushions revealed hundreds of closely packed hyphal tips closely pressed to the plastic, the base of the infection cushion was surrounded in a thick clear fluid, there was no direct evidence that this material was secreted by the hyphae.

The early stages of the infection process (1-24 hrs) involved penetration of the epidermis and initial colonisation of the cortex. This involved degradation of only the cell wall material in the direct path of the hyphae, and lysis of those root cells through which hyphae actually grew. In the following 12 hours hyphae gradually increased in number and thickly colonised the parts of the root below the infection site (Fig 6.B.). At this stage some root cell nuclei, 1-2 cells from the infection started to show signs of deterioration. As the numbers of hyphae increased the tissues appeared weakened and cell walls were seen to disintegrate in the path of the infection front (Figure 6.C.). Sometimes a few individual hyphae were observed to grow into the solid tissues in advance of the infection front (Figure 6.D.). Infection hyphae sometimes penetrated the vascular tissues, and proceeded to grow in the direction of the onion bulb (Figure 6.H.). Whether these hyphae preceed or follow the cortical infection hypha varied between replicates. As the infection front advanced toward the bulb a cavity formed in its path which became filled by swollen vacuolate hyphae (Figure 6.E.). Although the cortical tissues were liquefied, the epidermis almost always remained intact (Figure 6.F.), often as a single cellular layer. Most (but not all) epidermal cells in this tissue lacked Feulgen stainable nuclei. The tissues within the endodermis were resistant to hydrolysis along the length of the root despite all tissues surrounding them being liquefied (Figure 6.G.). The internal infection tended to proceed ahead of the external infection by a distance which increased as the infection became more developed (As a typical example: in a 5 cm long seedling root inoculated at the tip, when external hyphae had grown 2 cm, the internal hyphae would have grown 2.5-3.5 cm).

x 100

Figure 6(A): Underside of an *S. cepivorum* infection cushion: The infection cushion formed in an attempt to penetrate the base of a plastic petri dish.

Stain: Feulgen x1000

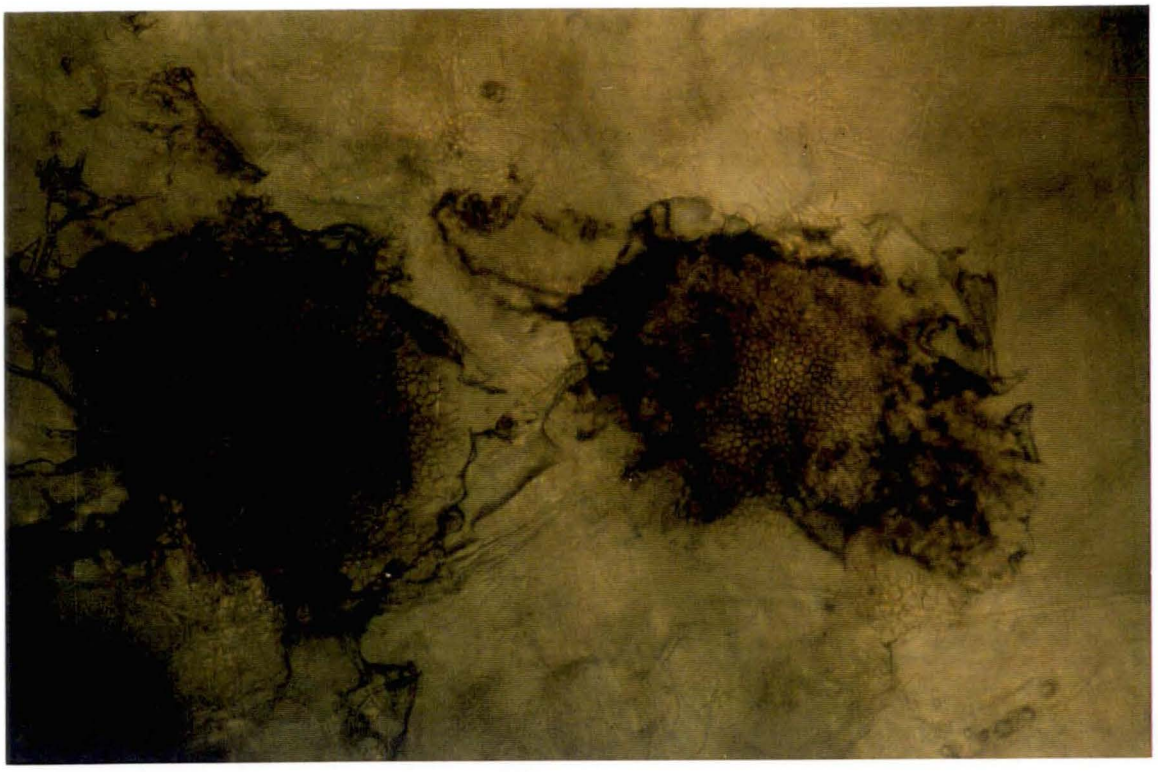
Figure 6(B): *S. cepivorum* hypha in the cortex: After penetrating into the cortex *S. cepivorum* hyphae (arrow) branch and increase in number within the onion tissues.

Stain: Ruthenium Red x1000

Figure 6(C): Cortical cell wall disintegration: Host cortical cell walls disintegrate in the path of the *S. cepivorum* infection hyphae.

Stain:Feulgen x 400

Figure 6(D): *S. cepivorum* infection: A few hyphae (arrows) occasionally grew ahead of the infection front into intact tissues. Many nuclei were still stainable in this zone but looked to be at least partially degraded.

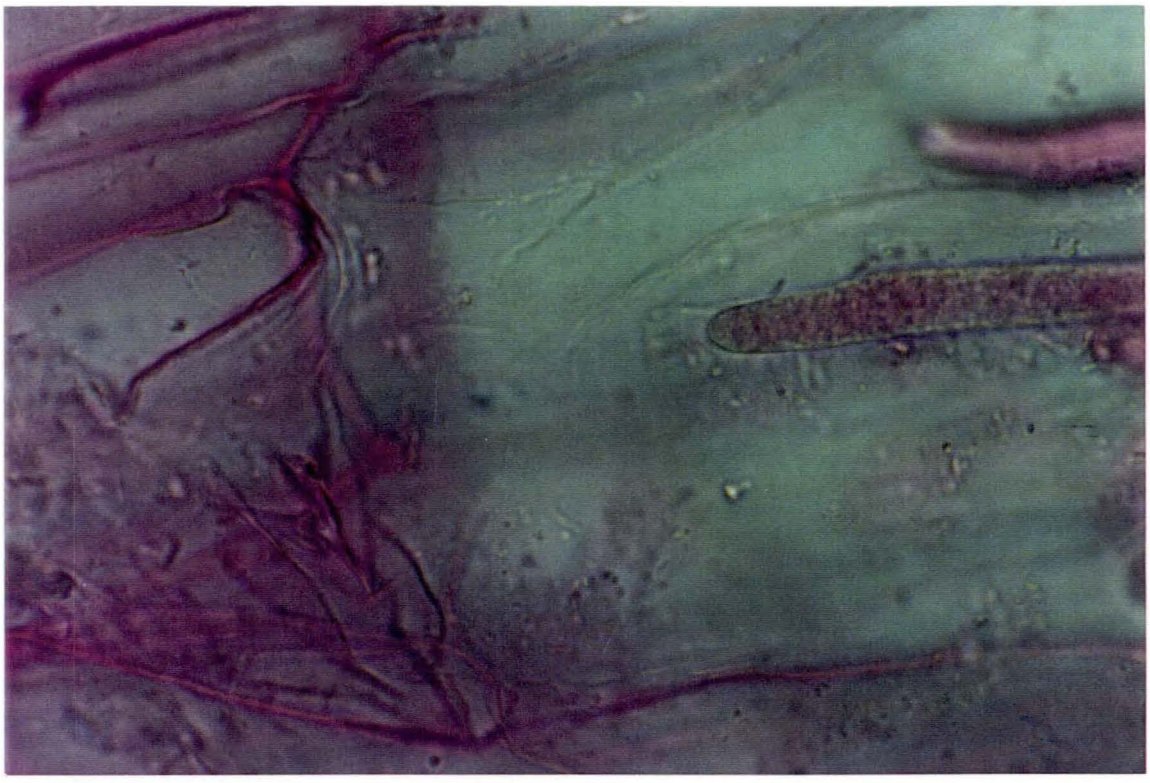


x 100

Figure 6(A): Underside of an *S. cepivorum* infection cushion: The infection cushion formed in an attempt to penetrate the base of a plastic petri dish.

Stain: Feulgen x1000

Figure 6(B): *S. cepivorum* hypha in the cortex: After penetrating into the cortex *S. cepivorum* hyphae (arrow) branch and increase in number within the onion tissues.



Stain: Ruthenium Red x1000

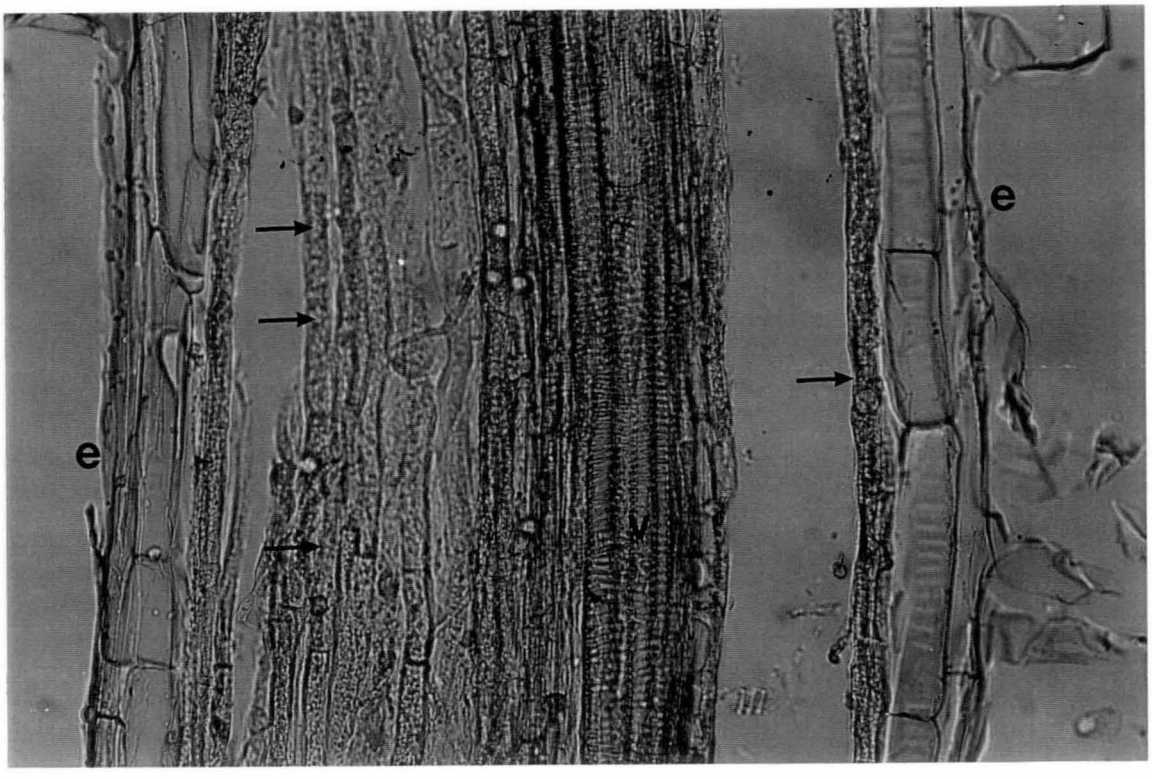
Figure 6(C): Cortical cell wall disintegration: Host cortical cell walls disintegrate in the path of the *S. cepivorum* infection hyphae.

Stain:Feulgen x 400

Figure 6(D): *S. cepivorum* infection: A few hyphae (arrows) occasionally grew ahead of the infection front into intact tissues. Many nuclei were still stainable in this zone but looked to be at least partially degraded.

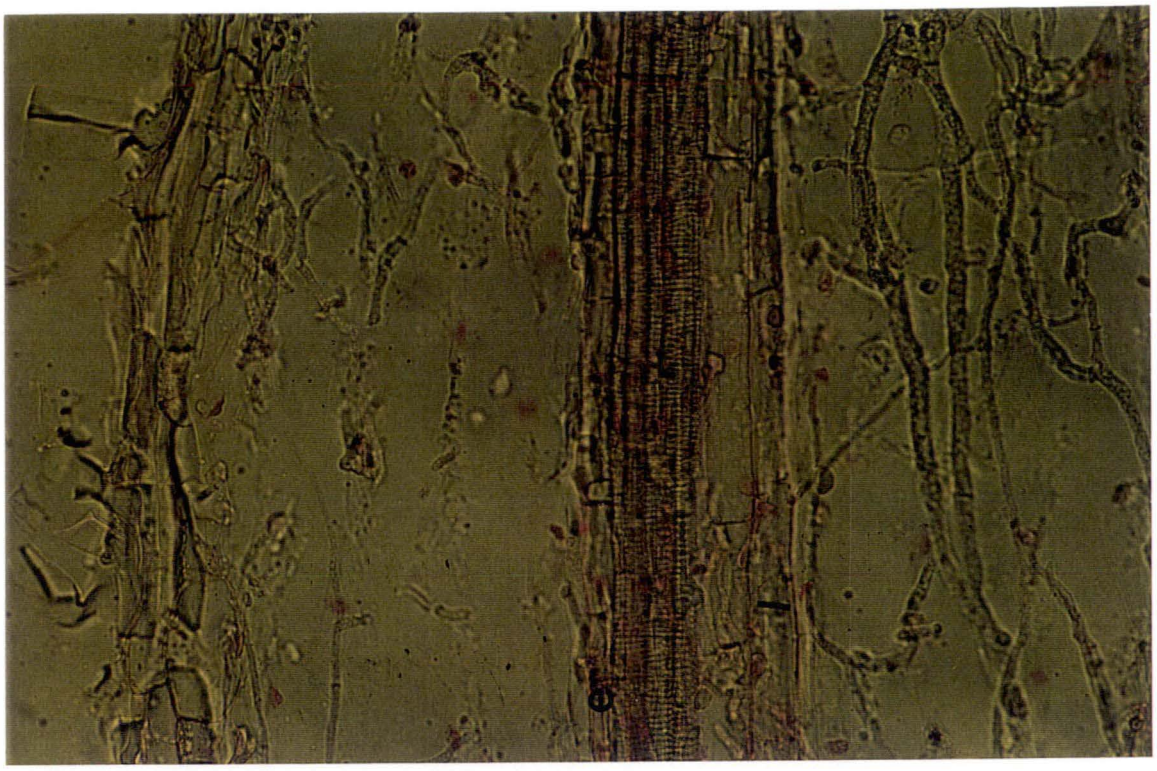
Stain: Feulgen x 200

Figure 6(E) Degraded cortical tissues: A cavity formed in the path of the infection front. Nuclei within the stele have retained the Feulgen stain (arrows).



Stain:Feulgen x 400

Figure 6(F): Hyphae in cortical cavity: Though tissues in the cortex became liquefied along a large portion of the root and filled by enlarged *S. cepivorum* hyphae (arrows), the epidermis (e) and vascular tissues (v) remain in tact.



Stain: Feulgen x 200
Figure 6(G): The Stele: Tissue within the endodermis (e) remained intact despite the tissues surrounding them being hydrolysed and filled by *S. cepivorum* hyphae.

Stain: Feulgen x 400
Figure 6(H): *S. cepivorum* hyphae colonise the stele: Hyphae were observed growing through xylem vessels toward the onion bulb base plate.

The spatial relationships between advancing masses of hyphal tips and the zone of cell death varied. In some replicates, the foremost hyphal tips grew among cells which had clearly stainable nuclei, though in the majority of replicates the cell walls dissolved to form a cavity (as in Figure 6.F.), well in advance (200-1000μm) of the mass of hyphae. Where this occurred, there was normally a zone, for a distance of several cortical cells (500-2000μm) ahead of the dissolving cell walls, where the majority (>75%) of cortical cells lacked stainable nuclei. The frequency of stainable nuclei in cortical cells increased with distance from the hyphal tips until the root section appeared healthy (around 3000μm in front of the most advanced hyphal tips). Where the infection front was not far below the bulb base, parts of the lower bulb lacked stainable nuclei. Occasionally diffuse stained nuclei could be seen among partially decomposed cell walls floating in the infection cavity (Figure 6.I.; 6.G.), which suggests that the Feulgen stain shows intact chromosomal material, as opposed to nuclei of cells living at the time of fixation.

Stain: Feulgen x 400

Figure 6(I): Nuclei in a partial state of degradation are occasionally seen in the infection cavity, indicating that presence of a nucleus does not imply a healthy cell. Epidermal and hypodermal cell layers are intact. Root hairs (h), Intact nuclei (i), decomposing nuclei (d)

6.4. Discussion.

Initial studies of the *S. cepivorum* infection process in onion roots (Metcalf, 1993) utilised actively growing mycelium from agar colonies. Potential problems existed with this method as culture media may contain accumulated phytotoxins, and would probably have a greater number of infective hyphal tips than a germinating sclerotium, perhaps resulting in greater disease expression. A germinating sclerotium was the most appropriate way to infect the root. In initial tests a sclerotium was placed on tap water agar with an onion root tip beside it. By this method sclerotial germination was slow and unreliable, and in the week or more it might take for hypha to appear the seedling would begin to show signs of lack of nutrition. This time lag was overcome by pre-germinating sclerotia so hyphal tips appeared before introducing the sclerotium to an onion root tip on a cube of soil agar. Pre-germination of sclerotia was expected to present a difficulty due to constitutive dormancy of culture sclerotia (Coley-Smith, 1960) which is maintained under sterile conditions (Coley-Smith *et al.*, 1987) and may take months to break under non sterile conditions (Leggett *et al.*, 1983). However a procedure was developed whereby dormant sterile culture sclerotia germination could be reliably induced for infection experiments. Dormancy characteristics of different *S. cepivorum* isolates are known to vary (Brix and Zinkernagel, 1992a) so the technique may not be repeatable for other isolates. Also it is possible that handling sclerotia with forceps abraded the rind, which can release dormancy (Coley-Smith, 1960). Sclerotium germination of isolate Sc4 under these conditions was almost always of type 2, where single hypha emerged through the rind (Coley-Smith *et al.*, 1967). This mode of germination is common on agar or sterilised soil (Adams and Papavizas, 1971). Type two germination initially lacked the vigour of eruptive germination, however by the time the sclerotium was introduced to the onion seedling the growth of hypha was of equal vigour to eruptive germination. The simulated process of root infection is as close as is possible (under the sterile conditions necessary to this study) to processes in non sterile soil.

The results of this study support the findings of Stewart *et al.* (1989a) that *S. cepivorum* penetrates the root epidermis by production of appressoria and infection cushions and hyphae disappearing between cell wall junctions. Infection cushions were seen to form on leaves, the bulb base and the roots. Penetration looked to be by a combination of mechanical and enzymatic means. In previous work (Metcalf, 1993) it was noted that the underside of the appressorium developed from a round into a cone shape which pressed with increasing depth into the cell wall. Ultimately the cone would develop a point and puncture the cell wall allowing the hyphal tip to enter. This type of penetration would be consistent with effects reported previously

such as recurling of the epidermal cell wall beside the enlarging infection peg (Stewart *et al.*, 1989a). The underside of *S. cepivorum* infection cushions was constructed of closely packed swollen hyphal tips (Figure 6.A.) as have been reported by other workers (Stewart *et al.*,1989a). The reason for infection cushion formation has not been positively determined. Stewart *et al.* (1989b) suggested that the complexity of infection structures was in response to the resistance of the tissues to penetration. Formation of infection cushions on root tissue in the present study does not oppose this hypothesis as varieties of onion and possibly strains of *S. cepivorum* differ. It is reasonable to expect that a high number of appressoria, and the influence of cell wall degrading enzymes, would serve to concentrate the pressure that *S. cepivorum* is able to place on the part of the epidermis beneath the infection cushion. The mucilaginous material in the viscinity of the infection cushion base was also reported by Stewart *et al.* (1989a).

It would appear that in the early stages of infection following penetration of the epidermis, cell death is limited to the cells through which hyphae actually grow, hypha were seen to grow through the epidermal and hypodermal layers and into the cortex intracellularly. Abd El-Razik *et al.*(1973) reported both intercellular and intracellular growth, this aspect of the *S. cepivorum* infection process differs from post penetration pathogenesis in *Sclerotinia sclerotiorum* and *S. minor* infection of beans where infection hyphae invade host tissue exclusively intercellularly (Lumsden and Dow, 1973).

On further colonisation nuclei were either lacking or only faintly stainable in tissue surrounding hyphae (Figure 6.D.). Similar phenomena have been reported in *S. sclerotiorum* infections where host cells are known to die in advance of hyphae (Hancock, 1972). In many examples, the cell walls in the path of the foremost infection hypha tips dissolved shortly before the hyphal tips reached them (Figure 6.C), which is in contrast to advancing *S. sclerotiorum* cortical infections which develop exclusively between cells (Lumsden and Dow, 1973). The advanced cell wall degradation provides evidence of extracellular cell wall degrading enzyme activity. The zone over which these enzymes were active was difficult to determine by microscopic examination. Figure 6(E) clearly shows an example where hypha are not seen within 2-3 cells from the front of dissolving cell walls. However, in an adjacent microtome section, foremost hyphal tips were only about the length of one root cell from the foremost dissolved cell walls. In Figure 6(E) nuclei within the stele have retained the Feulgen stain, whereas almost all cells in the epidermis and hypodermis on the other side of the infection cavity lack nuclei, suggesting that the tissues of the stele have a greater resistance to hydrolysis.

In sections of the root on the tip end of the infection front the cortical cavity is well formed, and is filled with large and vacuolate *S. cepivorum* hypha (Figure

6.F). Even when total hydrolysis of the cortex has taken place, the epidermis, hypodermis and tissues within the endodermis tend to remain intact (Figure 6.E.; 6.F.; 6.G.; 6.I.). Composition of these tissues is likely to contribute to their resistance to hydrolysis. The epidermis and hypodermis contain cellulose and lipids, the hypodermis is surrounded by a multi layered suberin lamella (Petersen *et al.*, 1978). Staining for lignin indicated that epidermal, hypodermal and cortical cells are not lignified. Histochemical Transmission Electron Microscope staining for phenolic compounds has shown that these are abundant in the epidermis and to a lesser extent in the hypodermis. Tests for phenolics were consistently negative in the cortex (Peterson *et al.*, 1978). Studies on colour reactions with p-nitranaline and sulphanilic reagent and binding of ferric ions, particularly in the outer tangential wall of epidermal cells have suggested that the phenolic compounds present may be di-hydroxy phenols according to the criteria of Smith *et al.*(1969), in their Table 16.2. Phenolic compounds are well documented as inhibitors of fungal pathogens in general (Agrios, 1988), and are known to be inhibitors of extracellular polygalacturonase activity (Farkas and Kiraly, 1962). Phenolic inhibition of exopolygalacturonase of *Sclerotinia* spp., which have extracellular diffusible enzymes and produce oxalic acid (Noyes and Hancock, 1981) in a similar manner to *S. cepivorum* (Figures 7.A, C. D and E (Ch. III:7); Stone and Armentrout, 1985), has been studied in detail (Byrde, Fielding and Williams, 1960).

Ultrastructural studies have reported resistance of the epidermis and hypodermis of onion roots to dissolution. Exposure to drizelase (a commercial mixture of cellulases and pectinases) and to concentrated sulphuric acid, resulted in hydrolysis of the cortical tissue and formation of a sheath consisting of the intact epidermal and hypodermal layers cell walls (Peterson *et al.*, 1978; Clarkson *et al.*, 1978). Also, studies of the permeability of epidermal and hypodermal layers have indicated that resistance of the apoplastic route across the epidermis and hypodermis clearly increases with molecular dimensions of the permeant solutes (Clarkson *et al.*, 1978). Therefore, high molecular weight extracellular enzymes would have difficulty moving into the tissues of the hypodermal and epidermal layers to act on the cell walls. Plasmodesmatal pits (Scott, *et al.* , 1956) seem to be involved in movement of molecules across these layers, and it is known that the resistance of the apoplastic route increases with molecular dimensions of the permeant solutes (Clarkson *et al* ., 1978). In *Phytopthora parasitica* infection of tobacco roots, pit membranes in have been shown to become clogged with released cell wall materials (Benhamou and Cote, 1992), which could inhibit movement of enzymes and toxins of pathogen origin into the endodermal tissues.

S. cepivorum could derive advantage from the resistance of the epidermis to degradation in that the epidermis can then serve as a barrier to prevent colonisation

of the infected root by saprophytes which could compete for nutrients, produce toxins or enzymes which may damage *S. cepivorum*, denature its extracellular proteins, and/or inhibit sclerotium formation. It is interesting to note that in stem tissue where Stewart *et al.* (1989b) reported that epidermal cells can be degraded 2 to 3 cells in advance of hyphae, the cuticle is not degraded, similarly providing a barrier between hyphae and the external environment. This strategy is also demonstrated by *S. sclerotiorum* infections (Lumsden and Dow, 1973).

Infection often spreads into the vascular tissues and sometimes the foremost infection hyphae were seen advancing in the xylem (Figure 6.H.). The stele tended to remain intact despite this infection, even if it became filled with *S. cepivorum* hyphae. Stainable nuclei often remained in the cells surrounding the xylem where almost all cortical cells were seen to be lacking nuclei (Figure 6.E.). Resistance of the onion root stele to pectinases and cellulases which dissolved the cortex has been previously reported (Clarkson *et al.*, 1978) but the mechanism by which it resists degradation has not been determined. Endodermal bands surrounding the stele in other plant species are known to contain relatively more lignin and suberin than other primary walls (Bonnet, 1968) and sometimes phenolic compounds (Van Fleet, 1961). Whether plasmodesmata in this zone inhibit transport of high molecular weight proteins warrants investigation.

The extensive cell wall degradation in advance of *S. cepivorum* hyphae plays an important role in the infection process. Production of oxalic acid and pectinases which operate synergistically (Stone and Armentrout, 1985) is likely to be involved. Relationships of *S. cepivorum* pectolytic enzymes to the advance of infection hyphae are investigated in Chapter III(7).

Chapter III: The *S. cepivorum* infection process:

7.0: Enzymatic relationships:

7.1. Introduction:

Results of histological studies in Chapter III(6) indicate that extensive cell wall degradation (predominantly in the onion root cortex and often in advance of hyphae) are a feature of the infection process of *S. cepivorum.* Onion cell wall composition has been documented (Mankarios et *al.,* 1980), and pectin (a polymer of d-galacturonic acid) was shown to be the major component (42.4% of the cell wall). Production of two polygalacturonase enzymes (Mankarios and Friend, 1980; Favaron *et al.*, 1993) and a pectinesterase (Abd-El -Razik *et al.*, 1974) by *S. cepivorum,* with the ability to degrade pectin have been reported. Additionally cellulases, arabinases, and xylanases are known to be produced (Mankarios and Friend, 1980). However the relative importance and role each enzyme plays in the onion root infection process has not been determined. In *Sclerotinia* spp. which share high genetic similarity with *S. cepivorum* (Carbone and Kohn, 1993), extracellular polygalacturonases and pectinesterases form a co-ordinated component in development of lesions, and are known to alter cell wall materials 2 to 3 cells in advance of hyphae (Lumsden, 1979). It is likely that pectolytic enzymes of *S. cepivorum* play a similar role in infection of onion tissue, however while *Sclerotinia* spp. infect at least 225 host genera (Purdy, 1979), *S. cepivorum* has evolved to exclusively recognise and infect the genus *Allium* with great efficiency. Additionally, activity of *S. sclerotiorum* pectolytic enzymes is inhibited by onion cell wall extracts (Echandi and Walker, 1957). It might then be expected for *S. cepivorum* to display considerable specific adaptation to infection of its host. It is the aim of this chapter to determine what pectolytic enzymes are produced by the *S. cepivorum* isolate (Sc4) during the infection process, and to determine the spatial relationships which exist between hyphae and pectolytic enzymes in infected onion roots.

7.2. Materials and Methods:

Infected onion roots (see Appendix C) were cut into 2 mm segments and placed consecutively into the wells of electrophoresis gels for detection of pectolytic enzymes. Wells were topped up with distilled water and electrophoresis was performed by the methods described in Appendix E. Results of three replicates are presented. Following electrophoresis, root sections were retrieved from wells, cut

longitudinally in half and examined microscopically to determine whether *S. cepivorum* hyphae were present in the section.

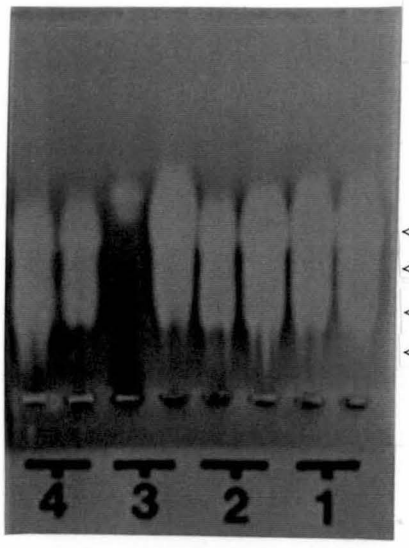
S. cepivorum was also added as a $5mm^2$ cube of growing mycelium on onion agar (Appendix B) to 2mls of liquid culture fluid containing 20 % macerated onion in a 5ml Bijou bottle and incubated for 1, 2 ,3 , and 4 weeks at 10°C in darkness. To detect pectinases, samples (20 µl) of this culture fluid were then added to wells of an electrophoresis gel, before electrophoresis by the method described in Appendix E was performed for comparison of polygalacturonase and pectinesterase isozymes.

7.2. Results:

In liquid culture (20% onion tissue in distilled water) *S. cepivorum* produced two polygalacturonase (PG) isozymes (R_f 0.08 and 0.16) and two pectinesterase (PE) isozymes (R_f 0.28 and 0.40) (Figure 7.A). Isozyme patterns changed with fungal development. Young cultures produced large amounts of the two PE proteins, while little or no PG was detected. As physiological age increased PE (R_f 0.28 and 0.40) activity diminished, PG was only readily detected in cultures showing some sclerotial formation (Figure 7.A.; 3 weeks; replicate 1). However, in other work with citrus pectin as the sole carbon source, PG was detected without sclerotial formation (Chapter VII: Figure 21.G). In an attempt to quantify the relative sensitivity of the method for enzyme detection, electrophoresis of 1:10 dilutions of culture fluids which had similar activity to those used to produce Figure 7(A) was performed, activity bands were not as clearly pronounced, but remained clearly detectable (results not presented).

Non infected onion root tissue (Figure 7.B) produced a native pectinesterase (Rf 0.13), which was also detected in infected roots. The PG and PE isozymes produced in infected roots (Figures 7.C., 7.D. and 7.E) are of similar R_f to bands produced in liquid culture. Two additional isozymes cathodically migrating PG (Figure 7.E.) and PE (Figures 7.D. and 7.E.) were detected in infected root tissue. The onion roots loaded into the electrophoresis gel in Figures 7.C, D and E. show the native onion pectinesterases as well as the polygalacturonase and pectinesterase isozymes which are produced by *S. cepivorum* as it infected the roots.

Following electrophoresis, sections from each root were examined and foremost internal and external infection hyphae were identified (Table 7.F.). Progress of external infection always lagged behind internal infection. PG's and PE's were found migrating ahead of the foremost infection hyphae. The R_f 0.40 PE was the most noticeably mobile in the roots. Similar results were observed in preliminary studies (Metcalf, 1993).



Weeks Incubation

Figure 7.(A): Pectinase electrophoresis gel showing isozymes of polygalacturonase (PG) and pectinesterase (PE) produced by *S. cepivorum* (Sc4) in liquid culture:. Dark zones indicate PG, light zones indicate PE. Culture grown *S. cepivorum* produced PG R_f 0.08 and 0.16 bands and PE Rf 0.28 and 0.40. Each band may consist of many smaller isozymes. Two replicate cultures were incubated for 1, 2, 3 or 4 weeks at 25°C.

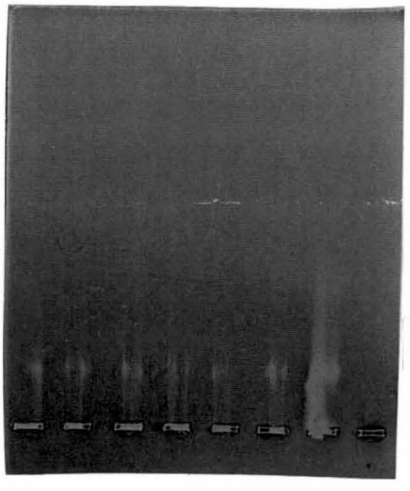


Figure 7(B): Pectinase electrophoresis gel showing isozymes produced by a non infected onion root: Zones of pectinesterase (R_f 0.13) native to the plant have been detected.

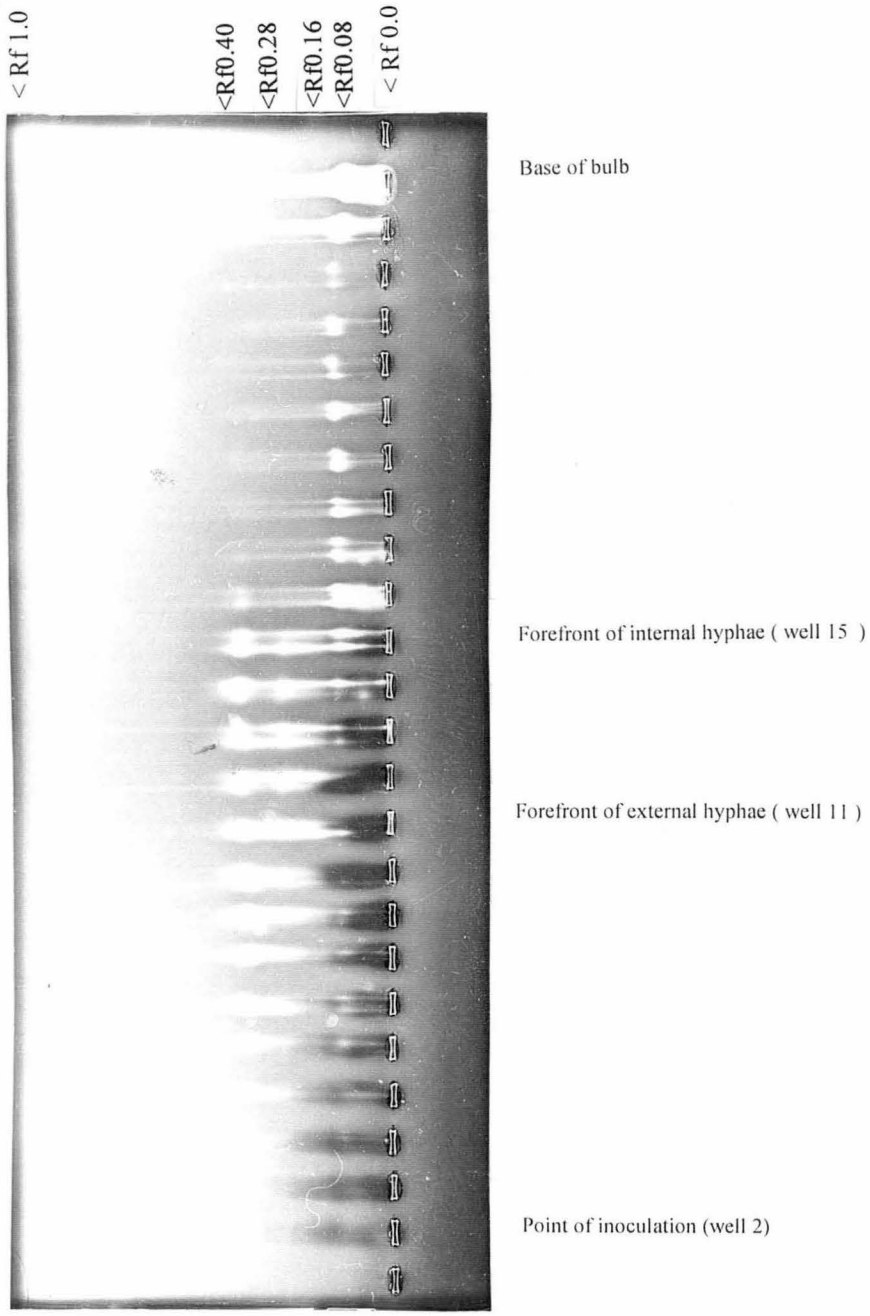


Figure 7(C) Pectinase electrophoresis gel showing isozymes produced in an onion root infected by *S. cepivorum* : Microscopic examination of root segments showed that the infection hyphae had advanced as far as well 15. Pectinases have diffused into adjacent root sections. Light zones indicate pectinesterase (PE), Dark zones indicate polygalacturonase (PG).

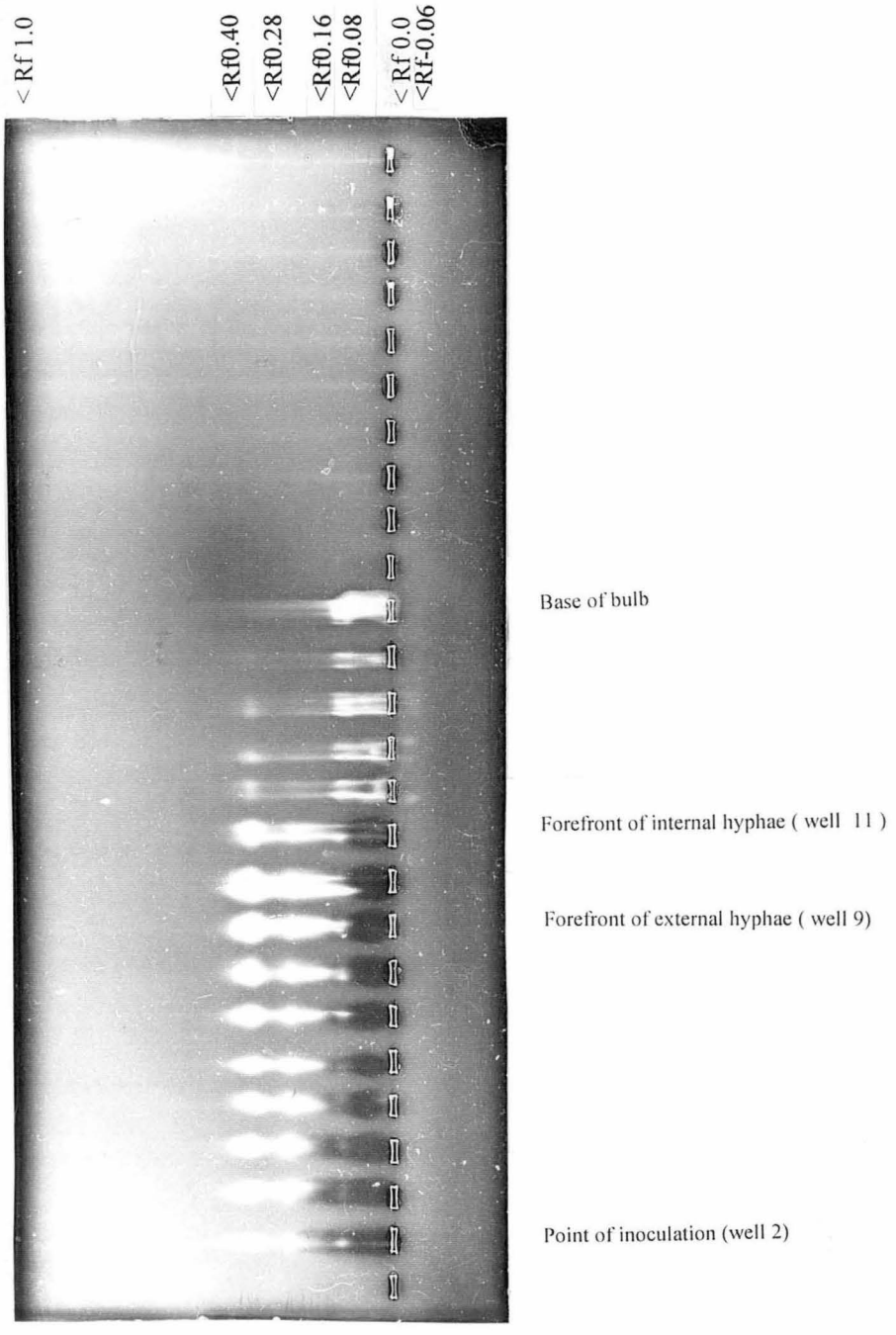


Figure 7(D): Pectinase electrophoresis gel showing isozymes produced in an onion root infected by *S. cepivorum*:: Microscopic examination of the root segments following electrophoresis showed that the foremost infection hyphae had grown into the section in well 11, pectinases have diffused ahead of the infection into several adjacent sections (Light zones indicate PE, dark zones indicate PG).

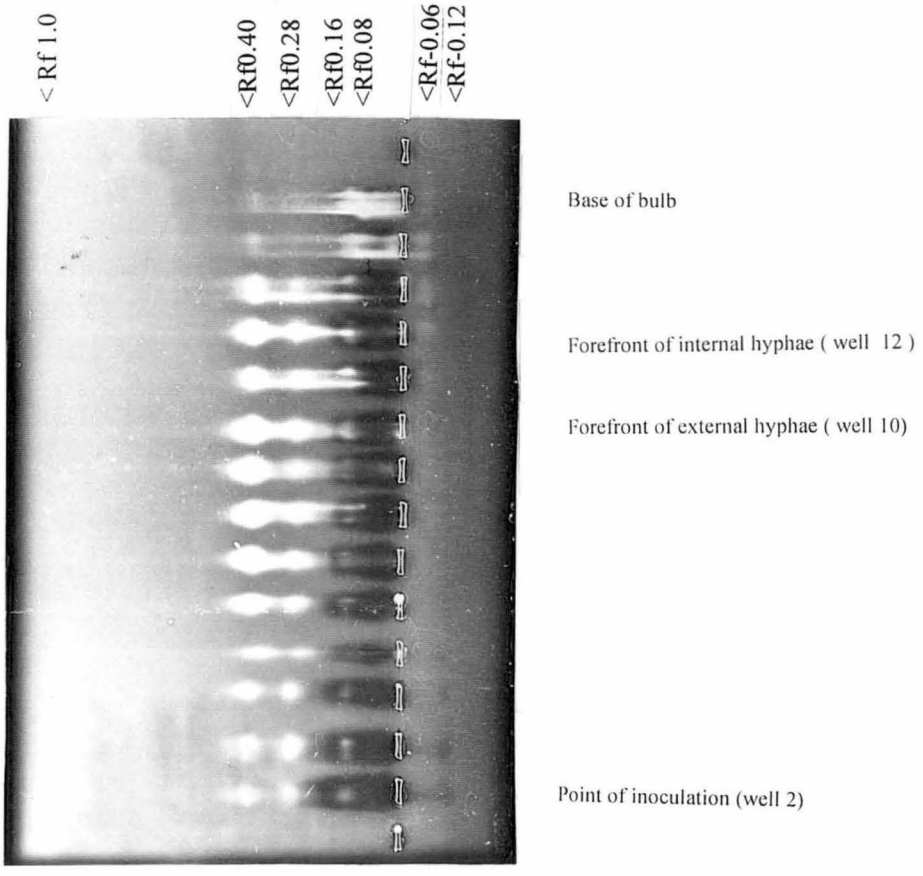


Figure 7.(E): Pectinase electrophoresis gel showing isozymes produced in an onion root infected by *S. cepivorum* : Microscopic examination showed that foremost infection hyphae had grown as far as well 12, the pectinases had diffused into several adjacent sections (light zones indicate PE; dark zones indicate PG).

Table 7(F): Distribution of hyphae, polygalacturonases (PG) and pectinesterases (PE) of *S. cepivorum* in onion roots 96 hours after infection at the tip by single germinating sclerotium.

Replicate	7(C)*	7(D)*	7(E)*	Range#
Root tip in well number	2	2	2	
Internal Infection front	30mm	22mm	24mm	
External Infection front	22mm	18mm	20mm	-4 to -8 mm
PG R_f 0.08	+ 2mm	+ 2mm	+ 6mm	+2 to +6mm
PG R_f 0.16	0	- 4mm	+ 2mm	-4 to +2mm
PG R_f -0.12	nd	nd	- 16mm	
PE Rf 0.28	0	0	+2	0 to +2mm
PE Rf 0.40	+ 4mm	+ 6mm	+ 6mm	+4 to +6mm
PE Rf -0.06	nd	+ 6mm	+6mm	+6mm
Total root length	48mm	30mm	28mm	

Notes to table 7.(F):
*-Figures indicate the number of millimetres in front of or behind the foremost internal infection hyphae where the foremost activity of the isozyme was detected in onion roots in electrophoresis gels 7.C., 7.D. and 7.E.
#- range = the range in mm of the foremost activity of the isozyme in relation to the infection front.

7.4 Discussion:

In studies by Lumsden (1976), pectolytic enzymes of *S. sclerotiorum* in infected tissue were localised by plating them on a pectate medium in which enzyme activity was detected by appearance of opaque halo's surrounding the tissue. In the present study a novel technique has been developed where pathogens enzymes have been localised by loading tissue segments directly into electrophoresis gels. The method has advantages over Lumsden's method in that different proteins with identical activity can be clearly distinguished. The method is no more time consuming, and a permanent record is readily produced. It has not been established whether the method is able to detect the total of pectolytic enzyme activity present in each root segment, as it is possible that a proportion of each isozyme may be too tightly bound to the cell walls to migrate. Close examination of zones of PE R_f -0.06 activity in Figures 7(D) and 7(E) indicates that the band has been detected as two streaks, each originating from either end of the well. This suggests that this isozyme was not able to migrate directly out of each root section by passing through the epidermis, and proteins which have been detected have migrated from the two cut faces of the root segment. Therefore the method probably provides a slightly conservative indication of protein diffusion ahead of hyphae. More precise localisation of the sites of pectolytic enzyme activity at cellular level might be

accomplished by electron microscopy using colloidal gold labelling of the *Aplysia* gonad lectin which specifically recognises galacturonic acid molecules (Benhamou *et al.*, 1988; Benhamou and Côte, 1992; Benhamou, 1996). Polyclonal antibodies have also been used to localise pectolytic proteins of *Sclerotinia fructigena* in infected tissue, however results may be difficult to interpret due to cross reactions (Hislop *et al.*, 1974).

The changes in the series of enzymes produced in culture including reduction in activity of the Rf 0.28 PE and corresponding increase in activity of PG Rf 0.08 and 0.16 appear to correspond to the time of sclerotial formation, this phenomenon has been observed in other replications, and using other *S. cepivorum* isolates (not presented). While sclerotial fungi are known to produce pectinases in a consistent sequence which subsequently depolymerise complex pectic polysaccharides (Cruickshank, 1983; Errampelli and Kohn, 1995), the ready detection of PG in young cultures of the same isolate incubated in pectolytic enzyme medium which contains citrus pectin (Ch. VII., Figure 21.G) suggests that the effect is related to substrate. It is not clear whether rhamnogalacturonan chains in onion cell walls are as readily accessible to the PG of *S. cepivorum* in macerated onion bulb tissue as in pectinase medium. Some degradation of cell wall lipids in the macerated bulb tissue may be required before PG can act. More information is needed on cell wall structure.

In onion root tissue the Rf 0.40 pectinesterase, which appears to be the most diffusible isozyme, is likely to reach tissue and act upon it before polygalacturonase. PE demethylates pectin in the middle lamellae to form pectate. Demethylated pectin (as sodium polypectate) is known to be a more favourable substrate than citrus pectin for *S. cepivorum* polygalacturonase (Mankarios and Friend, 1980). Pectate is also a more preferable substrate than methylated pectin for *S. sclerotiorum* pectolytic enzymes (Lumsden, 1976). The PG Rf 0.08 also diffused ahead of the foremost infection hyphae in all three replicates and in combination with the diffusible PE bands seems likely to be a major contributor to the advanced cell wall degradation. Although PG Rf 0.16 did diffuse 2mm ahead of hyphal tips in one replicate, this enzyme appeared to be most active in heavily degraded tissue closer to the initial point of inoculation, possibly acting on solubilised galacturonan chains. This, combined with the lower amounts of PE which tended to be detected closer to the point of inoculation, tends to agree with the shift in activity from PE to PG in increasingly physiologically aged onion macerate cultures. The trend is also similar to *S. sclerotiorum* infections, where PE was more active at advancing margins than older parts of lesions while PG was detected in advancing margins of lesions, but more active in older parts of lesions (Lumsden, 1976).

The evidence that *S. cepivorum* pectinases are present in the root tissues ahead of the advancing hyphae correlates well with histological evidence collected in Chapter III(6). Extracellular PE and PG are present in the zone where cell wall degradation in advance of infection hypha occurred. Many nuclei were not stainable by the Feulgen method in this tissue, however, there is no evidence that PE and PG are primary agents of cell death. *S. cepivorum* produces oxalic acid (Stone and Armentrout, 1985), and in the related species *Sclerotium rolfsii* oxalic acid has been shown to move systemically ahead of the infection (Bateman and Beer, 1965) sequestering calcium from cell walls and affecting the balance of intra and extracellular calcium (Pluim *et al* ., 1994). This is important in cementing cell walls together, stabilising membrane structure, and other cellular processes (Poovaiah, 1985). In preliminary studies, Metcalf (1993) noted that large numbers of tetragonal crystals of similar form to calcium oxalate were precipitated in the cortex and the xylem of onion roots in advance of hyphae. Preliminary histochemical identification by the paper chromatography method of Stone and Armentrout (1985) was made. Oxalic acid is known to act synergistically with PG by lowering the pH to the optimum for enzyme activity (Stone and Armentrout, 1985). Whether spatial relationships of oxalic acid in the *S. cepivorum* infection process are the same as for *S. rolfsii* (Bateman and Beer, 1965) also needs further histochemical investigation. The role of *S. cepivorum* cellulases and xylanases (Mankarios and Friend, 1980) in cell death and cell wall hydrolysis warrants further investigation.

Chapter IV: Interactions between *T. koningii* and *S. cepivorum.*

8.0: *In vitro* interactions.

8.1 Introduction:

Observations of antagonism made *in vitro* do not necessarily represent antagonism in the soil ecosystem, however they may provide some insight into the mechanism of antagonism. Lysis of host hyphae without physical contact is suggestive of production of diffusible non-volatile antibiotics (Dennis and Webster, 1971a; Lederer *et al.*, 1992), Inhibition of growth in non fluid conditions might imply volatile antibiotic production (Dennis and Webster, 1971b; Claydon *et al.*, 1987; Simon *et al.*, 1988). Coiling of antagonist hyphae on the host might imply mycoparasitic activity (Dennis and Webster, 1971c; Trutmann and Keane, 1990) and degradation of the host cell wall suggests production of chitinolytic enzymes by the antagonist (Ridout *et al.*, 1986; Ordentlich *et al.* 1988; Benhamou and Chet, 1993).

The aim of this study is to gain some preliminary insight into the mechanism of biocontrol from observing the hyphal interactions *in vitro*.

8.2 Materials and Methods:

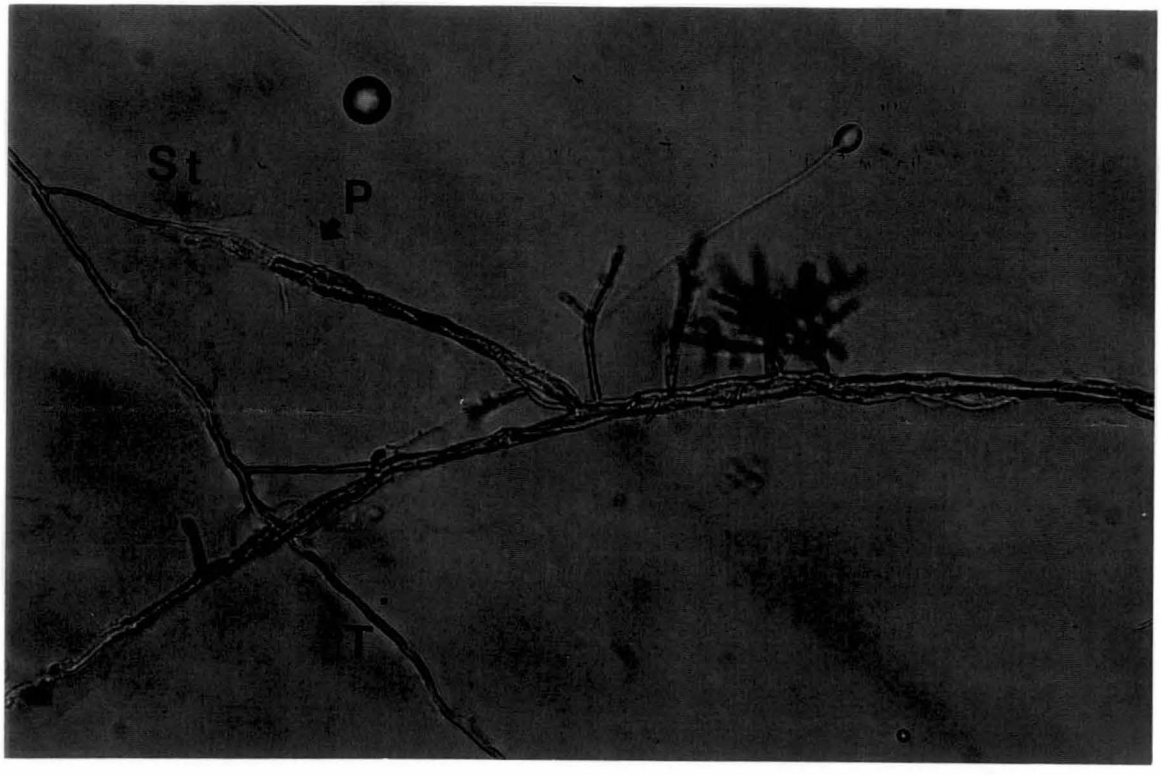
The antagonism bioassay (Ch. IV:9-11; methods described in Appendix C) involved placing cubes of agar containing growing *T. koningii* (Tr5) and *S. cepivorum* (Sc4) mycelium onto the base of an empty petri dish to colonise or infect an onion root. As hyphae radially spread from the soil agar the interactions between *T. koningii* and *S. cepivorum* hyphae could be casually observed through the bottom of the petri dish inverted on the microscope stage. The petri dish base was too thick for observation above x 200 magnification. These plates were incubated at 20° C in darkness for four days. The experiment was replicated over 100 times.

8.3. Results:

Mycelium of *T. koningii* was inclined to spread thinly over the petri dish base. When the hyphae of *S. cepivorum* and *T. koningii* met, mycoparasitic behaviour by *T. koningii* was observed. In many examples direct physical contact between the two fungi appeared necessary for lysis of *S. cepivorum* hyphae. Figure 8(A) displays an example where hyphal tips of *S. cepivorum* and *T. koningii* made contact while growing from opposite directions. *T. koningii* hyphae have grown onto and along those of *S. cepivorum*, changing direction of growth to follow the

larger *S. cepivorum* hyphae where they have branched. The initials of *T. koningii* conidiophores can be seen originating from *T. koningii* hyphae in contact with *S. cepivorum* hyphae. The only evidence of *S. cepivorum* cell lysis in Figure 8(A) is at the two *S. cepivorum* hyphal tips where the cell walls are seen to be decaying as *T. koningii* grows across them. At both sites a pool of fluid can be seen. The only sites in this replicate where *S. cepivorum* cells were clearly lysed are surrounded by this fluid. (there is no clear evidence that the fluid originated from either fungus). In identical replicates where mycoparasitism was at a more advanced stage than Figure 8(A) pools of fluid appeared to originate from condensation droplets. Hyphae of *T. koningii* were observed growing along cell walls of branching *S. cepivorum* hyphae (Figure 8.B.). *S. cepivorum* hyphae were observed to be partially dissolved in this process and *T. koningii* had thickly colonised the remnants. Where *S. cepivorum* cell walls were partially dissolved *T. koningii* was most often closely associated with the *S. cepivorum* cell wall, although at one point where *S. cepivorum* hypha branched, *T. koningii* hypha followed only the lower branch but the higher branch was also observed to be dissolving. In further replicates, *T. koningii* hyphae were observed growing through the path of almost completely dissolved cell walls. *S. cepivorum* infection cushions were noted in a partially degraded state on several occasions (Figure 8.C.). As the degradation process proceeded *S. cepivorum* hyphae were observed in partially decayed condition in close proximity to *T. koningii* hyphae without physical contact. *S. cepivorum* hyphae in close proximity to *T. koningii* became increasingly difficult to distinguish from the fluid in which they were observed. Figures 8(D), 8(E) and 8(F) are a connected series of microphotographs where in Figure 8(D) some *S. cepivorum* hyphae are only distinguishable as faintly visible cell wall remnants with *T. koningii* growing in close proximity. *T. koningii* hyphae are difficult to distinguish in the area in Figure 8(E) in which some *S cepivorum* septa appear to be beginning to become detached, however a single unbranched *T. koningii* hyphal tip can be distinguished growing over the cell walls in Figure 8(F). It is possible that *T. koningii* may have grown intracellularly through the *S. cepivorum* hypha in Figure 8(E). However, as another *T. koningii* hypha crossed the path of the *S. cepivorum* hypha, and the photograph aspect is through the petri dish base, there is no solid evidence that the *T. koningii* hypha pictured in Figure 8(F) has reached this location by intracellular growth. Cell walls of the sub apical region of *S. cepivorum* hyphae pictured in Figure 8(F) have been partially decomposed in the zone ahead of the growing *T. koningii* hyphae. A curious splattered formation is noted at the partially decomposed *S. cepivorum* apex. Similar phenomena were observed on approximately 1% of *S. cepivorum* apices growing in pools of fluid which also contained *T. koningii* (Figures 8.G. and 8.H.). The phenomenon of swelling and lysis of tips was occasionally observed of

S. cepivorum hyphae which were not growing near *T. koningii* hyphae (Figure 8.I.), swelling and lysis of *T. koningii* hyphal tips was observed on a few occasions (around 0.1%) also (Figure 8.J.)



x 100

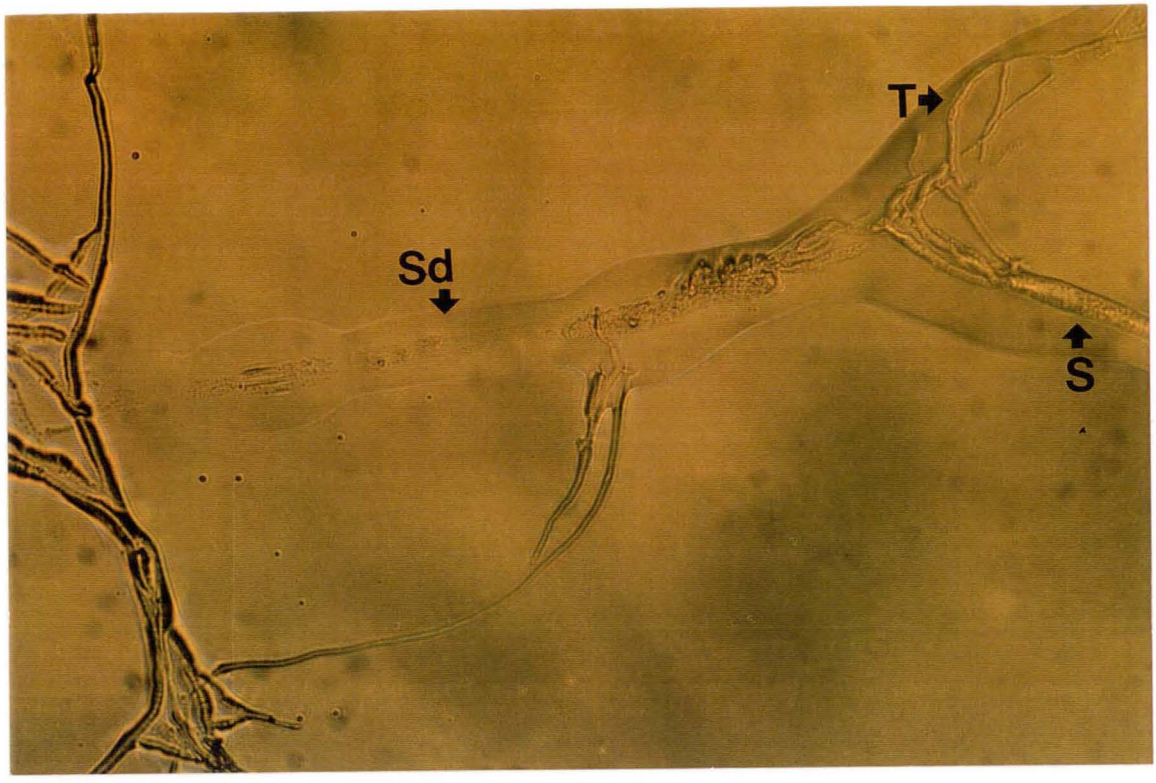
Figure 8(A): Hyphae of *T. koningii* (T) growing over the larger hyphae of *S. cepivorum*: Antagonism is viewed through the base of a sealed petri dish. Lysis of *S. cepivorum* mycelium is evident only at its hyphal tips (St) where a pool of fluid (P) of uncertain origin was found.

x 200

Figure 8(B): *T. koningii* hyphae growing in close association with cell walls of *S. cepivorum* : *S. cepivorum* hyphae are in partially dissolved condition. At one point (A) an *S. cepivorum* hyphal branch with no physical contact with *T. koningii* is partially dissolved.

x 200

Figure 8(C): *T.koningii* hyphae growing through the liquefied remains of *S. cepivorum* hyphae: An *S. cepivorum* infection cushion produced on the petri dish base can be seen in partially degraded condition (arrow).

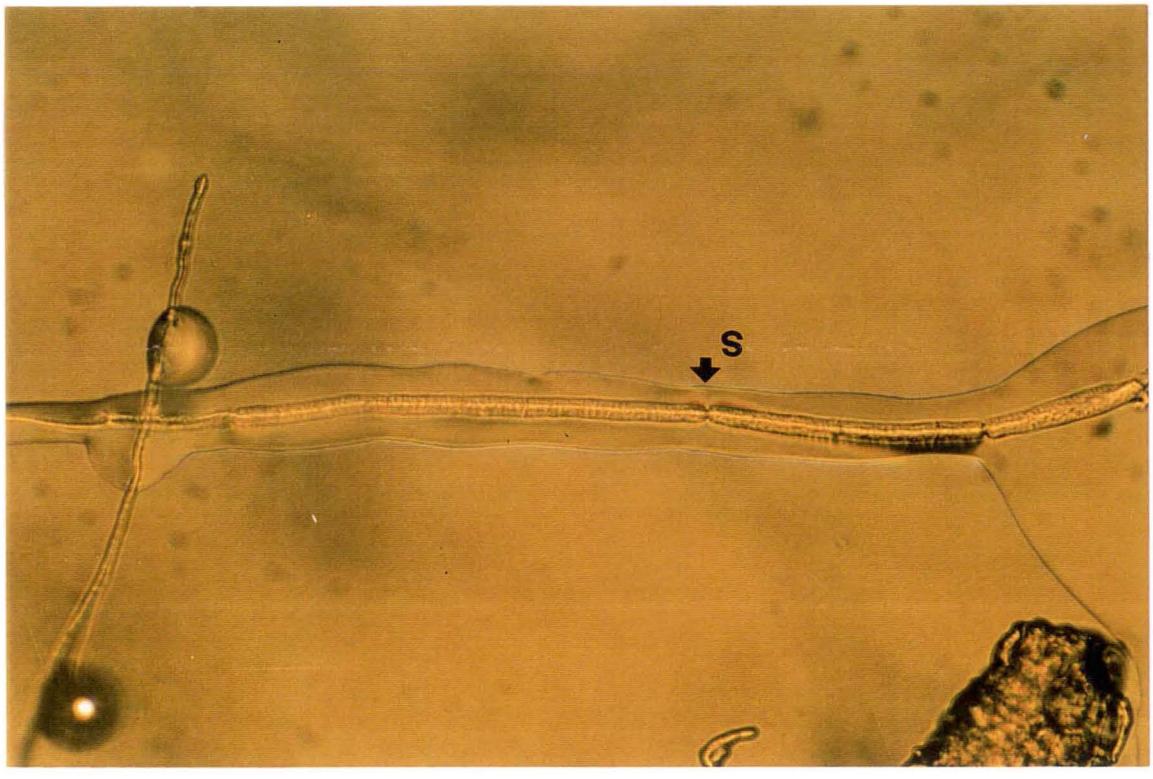


Joins 8(E)

x 200

Figure 8(D): Degradation of hyphae: *S. cepivorum* (S) and *T. koningii* (T) growing on the base of a sealed petri dish, viewed through the bottom of the petri dish. *S. cepivorum* hyphae have been almost completely degraded at some points (Sd). (1 in series of 3)

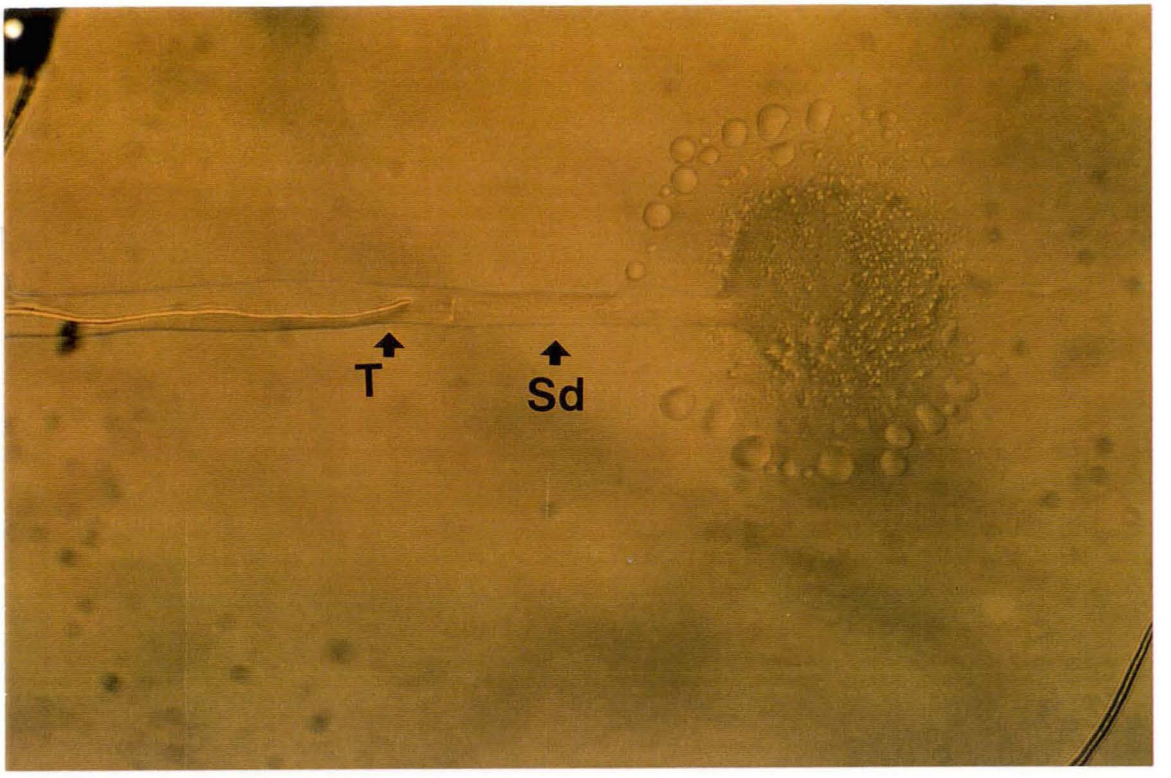
Joins 8(D)



Joins 8(E)

Figure 8(E) *S. cepivorum* hypha in pool of fluid: *S. cepivorum* hypha showing early signs of separation at the septa (s).

Joins 8(E)



x 200

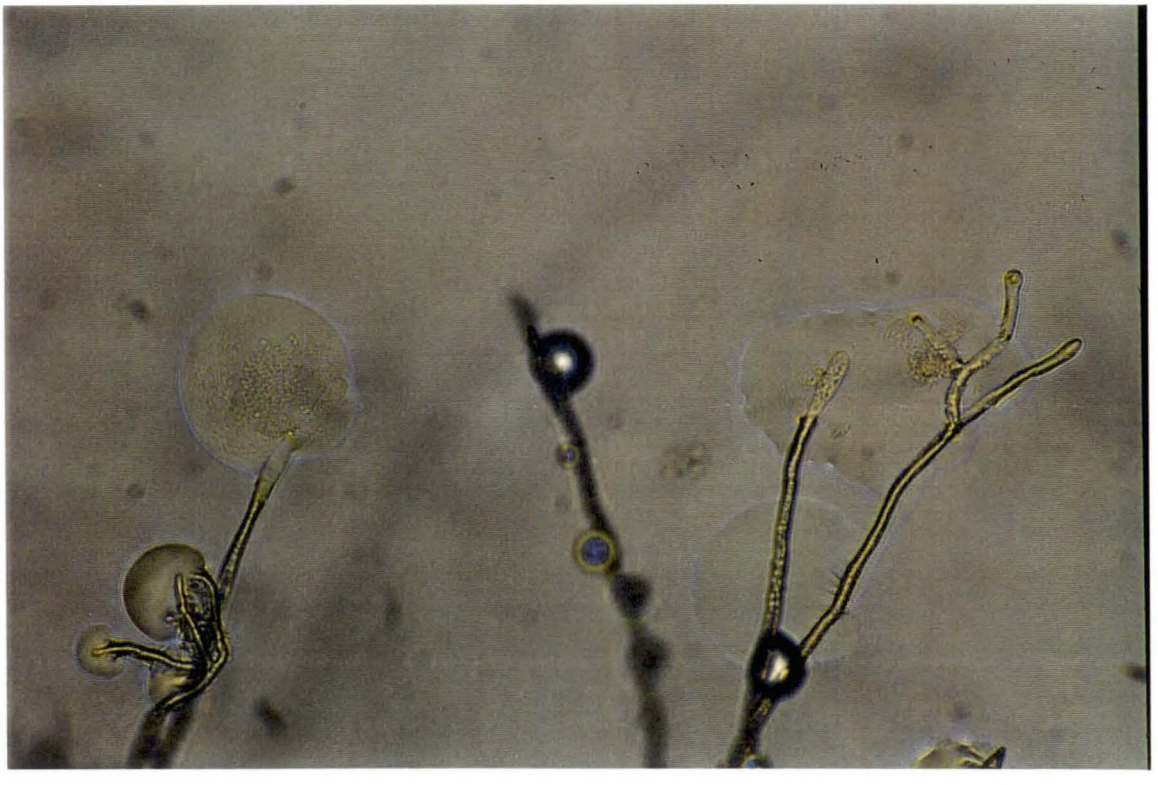
Figure 8(F): Lysed *S. cepivorum* hyphal tip: Cell walls of *S. cepivorum* in partially degraded condition (Sd), a *T. koningii* hypha (T) is growing in close contact with the *S. cepivorum* cell walls. The *S. cepivorum* hyphal apex has been lysed and released protoplasm.

x 200

Figure 8(G): Swollen and lysed *S. cepivorum* hyphal apex in close proximity to *T. koningii*: Protoplasm has been released (arrow).

x 100

Figure 8(H): Swollen and lysed *S. cepivorum* hyphal apex (Sl) The hypha originated from a pool of fluid on the petri dish base containing growing *T. koningii* hyphae and decaying *S. cepivorum* hyphae. The smaller circle is the swollen apex, a larger pool of fluid surrounds the apex.



x 200

Figure 8(I): *S. cepivorum* hyphal tips: Apices were occasionally seen swollen and lysed without *T. koningii* hypha in close proximity.

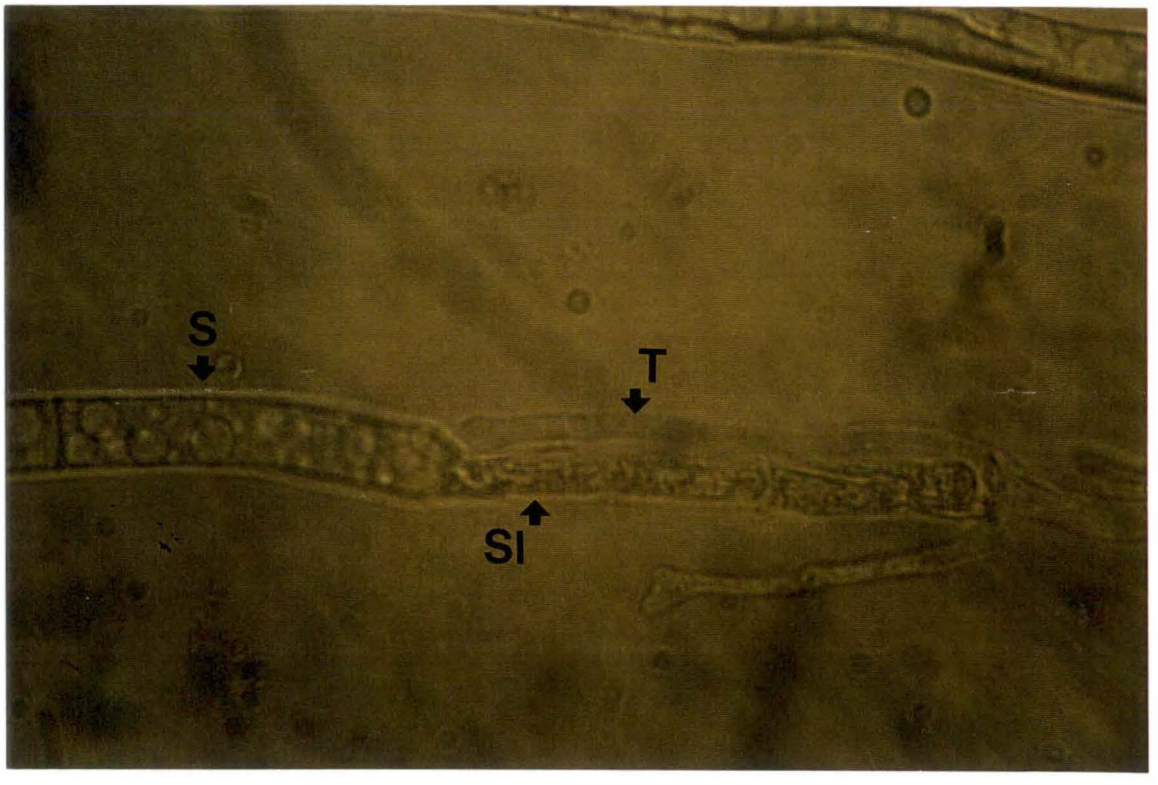
x 200

Figure 8(J): *T. koningii* hyphal apices: *T. koningii* hyphal tips were seen swollen and lysed on a few occasions.

In Figure 8(K). two side branches are seen emerging from a *T. koningii* hypha growing at 90° to a larger *S. cepivorum* hypha, mycoparasitic activity has taken place as *T. koningii* growing apices have followed the *S. cepivorum* hypha, producing short, stubby side branches which have colonised the decaying *S. cepivorum* cell walls and in general have not colonised the surrounding petri dish surface. Although examples of lysis without physical contact have been seen in this study, the example in Figure 8(K) shows *S. cepivorum* hyphal cells have not been lysed until the apices of *T. koningii* hypha made physical contact with them as shown in enlargement in Figure 8(L).

x 200

Figure 8(K) Mycoparasitism: *T. koningii* (T) hyphae have grown along *S. cepivorum.* hyphae (S), *S. cepivorum* cells have been killed as the *T. koningii* apex reached them. Point D is the segment pictured at x1000 in Figure 8(L).



x 1000

Figure 8(L) Mycoparasitism: Mycoparasitic action of *T. koningii* (T) against *S. cepivorum* (S). *S. cepivorum* cells in contact with *T. koningii* have been lysed (Sl).

8.4. Discussion:

The biochemical environment for interactions observed in the petri dish base is likely to be of lower complexity than that in infected tissue (Ch. IV:9-11), containing both fungi as well as their metabolites and extracellular proteins, but lacking plant polysaccharides and secondary metabolites. Chitin present in *S. cepivorum* cell walls would be a major available carbon source (possibly enhancing activity of *T. koningii* chitinolytic enzymes). Physical contact was not essential for *T. koningii* lysis of *S. cepivorum* when a fluid medium was present between the two. When a fluid medium was not present, only *S. cepivorum* cells physically contacted by *T. koningii* were lysed (Figure 8.K.). This suggests that extracellular proteins or antimicrobial compounds produced by *T. koningii* diffuse through the fluid and would not otherwise be able to reach and attack *S. cepivorum* . Similar single cellular lysis of *Sclerotinia minor* cells has been reported in presence of *Gliocladium virens* (Burgess and Hepworth, 1996). A fine extracellular matrix between hyphae of host and antagonist has been reported in mycoparasitism of *R. solani* by *T. harzianum* (Benhamou and Chet, 1993). This type of matrix could mediate transport of the lytic enzymes in mycoparasitism where no extracellular fluid is present.

Intracellular growth by *Trichoderma harzianum* in antagonism of *S. cepivorum* has been reported (de Oliviera *et al.*, 1984), however no conclusive evidence of this phenomenon was recorded in this study. Given the close association between *T. koningii* and *S. cepivorum* which often occurs in antagonism (Figures 8.B; 8.K.) and the manner in which *T. koningii* was observed following the path of *S. cepivorum* hypha through pools of fluid containing dissolving *S. cepivorum* hyphae (Figure 8.C.), it is reasonable to expect that *T. koningii* could exhibit this behaviour as a random, rather than as an active colonisation process. In mycoparasitism of *Sclerotium rolfsii*, *Trichoderma harzianum* has been reported to produce appressorium like structures involved in penetration of the *S. rolfsii* cell wall (Elad *et al.*, 1983), but no such structures were observed in this study. The mycoparasitic interaction observed in this study appears to be a less specifically co-ordinated attack.

A curious formation around a lysed *S. cepivorum* hyphal apex was noted in Figure 8.F. This is similar to the phenomenon of chitinase induced hyphal tip swelling and lysis which has been observed in *S. rolfsii* (Ordentich *et al.*, 1988), *Sclerotinia minor* (Burgess and Hepworth, 1996), *Botrytis cinerea* (Lorito *et al.*, 1993); Di Pietro *et al.*, 1993), *Phycomyces blakesleeanus* (Broekaert *et al.*, 1988), *Rhizoctonia solani* (Benhamou *et al.*, 1993), *Fusarium solani f. sp. lycopersici* , *Fusarium solani f. sp. phaseoli* , *Penicillium digitatum* , and *Trichoderma viride*

(Mauch *et al.*, 1988). The phenomenon only accounted for about 1% of all lysed *S. cepivorum* hyphae, the remainder of lysed apices decayed in the pools of fluid. It is likely that *T. koningii* chitinolytic enzymes could be produced under these conditions (Ch IV:11). Bursting of apices in the presence of cell wall degrading enzymes is in agreement with the accepted model of fungal cell wall synthesis and structure proposed by Wessels (1986), whereby the hyphal apex grows in a delicate balance between degradation and synthesis of wall polymers. The high turgor pressure of hyphal cells forces broken chitin microfibrils away from one another before synthesising enzymes can insert new microfibrils or extend the broken ones. The newly synthesised (and nascent) microfibrils in the apex are more readily attacked by chitinolytic enzymes than other regions of the cell wall (Vermuelen and Wessels, 1984). Interference with the cell wall metabolism upsets the balance between cell wall lysis and synthesis during hyphal tip extension. The cytoskeleton is less able to contend with cell turgor pressure, resulting in bursting (Wessels, 1986). In some examples, of hyphal tip bursting cytoplasm leaking from the lysed apex could clearly be seen (Figure 8.G.). Sometimes a less noticeable dome shaped swelling of the apex was seen (Figure 8.H.). Hyphal tip bursting was observed on a few occasions when *T. koningii* was not in close proximity to the burst tip (Figure 8.I.) which is compatible with the growth model of Wessels (1986) as the petri dish base contains little nutrition and growth requires synthesis of chitin fibrils to maintain the balance in the growing apex. However, bursting was more common in the presence of *T. koningii* . *T. koningii* hyphae growing on the nutrient devoid petri dish base were also seen with burst apices on a few occasions (Figure 8.J.) and similar phenomena may be responsible. *T. koningii* hypha were never seen with burst apices in the pools of fluid.

Chapter IV: Interactions between *S. cepivorum* and *T. koningii*.

9.0. Interactions within onion roots:

9.1. Introduction:

In order to enhance the disease control efficacy of *T. koningii* it is important to understand the mechanism by which it is able to restrict *S. cepivorum* infection hyphae from infecting the onion bulb. The growth behaviour of *T. koningii* on infected roots is an important aspect of this interaction. Some aspects of the behaviour of *T. koningii* on the surface of onion roots have been previously described (Metcalf, 1993), and were generally reported to grow through the onion root surface mucilage in an irregular branched pattern, occasionally producing chlamydospores. The aim of these experiments was to determine how *T. koningii* modifies the normal infection process as described in Chapter III, and how it interacts with the advancing *S. cepivorum* infection and the onion root tissue.

9.2. Materials and Methods:

Onion seedlings grown by the methods described in Appendix C.1 were placed in the petri dish base with the root tip in close proximity to a pre-germinated *S. cepivorum* sclerotium by the method described in Appendix C.2. A cube of soil agar (Appendix A) containing growing *T. koningii* mycelium was placed below the root, at a point between the *S. cepivorum* infection and the bulb base. After incubation (Appendix C.4) the root sections were stained by the Feulgen method, dehydrated and embedded in paraffin, microtome sectioned and mounted by the procedure described in Appendix D. The experiment was replicated over 100 times. In some preliminary replicates specimens were stained with 0.1% Ruthenium Red, destained in distilled water and hand sectioned using a razor blade. To observe *T. koningii* growth behaviour on healthy onion roots, roots were inoculated with *T. koningii* only and incubated for 96 hours before staining by the Feulgen method. These roots were not microtome sectioned before microscopic examination. For closer examination of passage cells, root segments from onion seedlings grown under sterile conditions by the methods described in Appendix C.1. were viewed with an environmental scanning electron microscope (Phillips ElectroScan, model 2020) mounted in a fully hydrated state on a cold stage (4-7°C).

9.3. Results:

In absence of *S. cepivorum* infection, *T. koningii* hyphae tended to be associated with the onion root mucilage layer whereas *S. cepivorum* hyphae were closely appressed to the root surface. *T. koningii* hypha were seen growing on the root by dissecting microscope, sometimes producing conidiophores which grew out from the root surface (Figure 9.A.). *T. koningii* hyphae were never observed entering root cells or growing into root tissues except where onion seedlings had become in the petri dish for several days and become nutrient depleted, or the seedlings were severely stressed from moisture depletion, where upon *T. koningii* hypha would rapidly colonise the cortical region of dead or perhaps weakened root tissues.

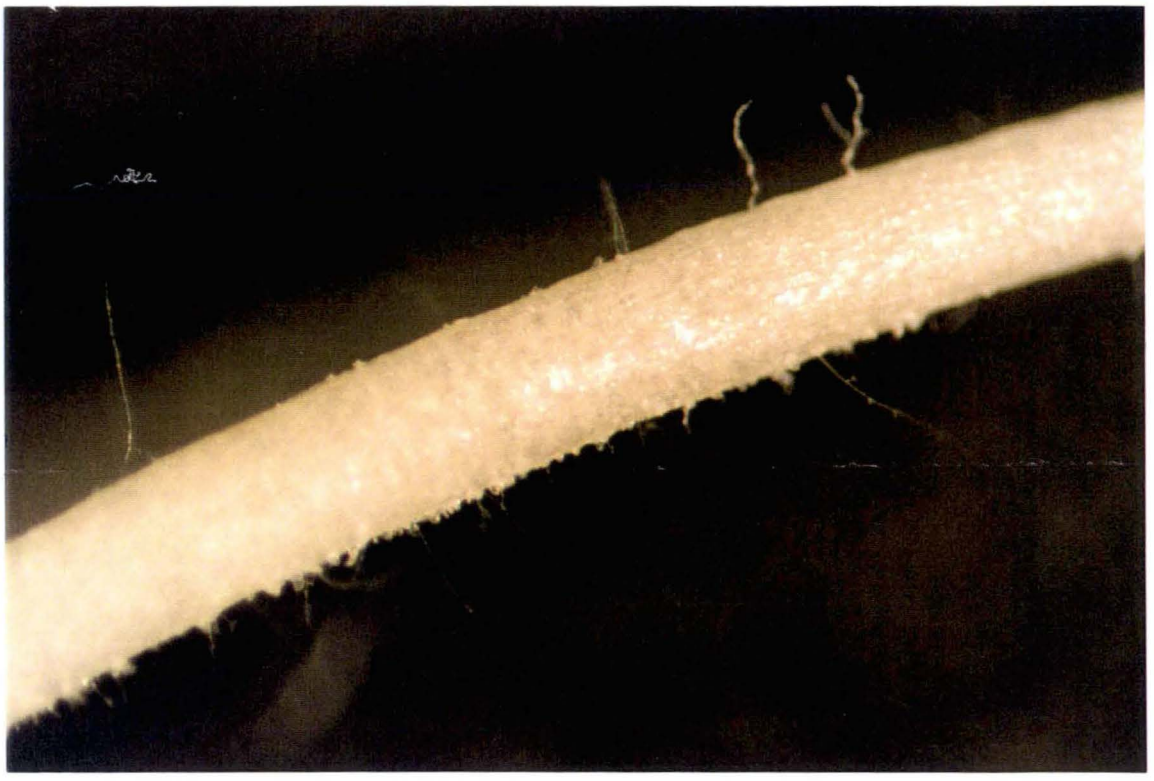
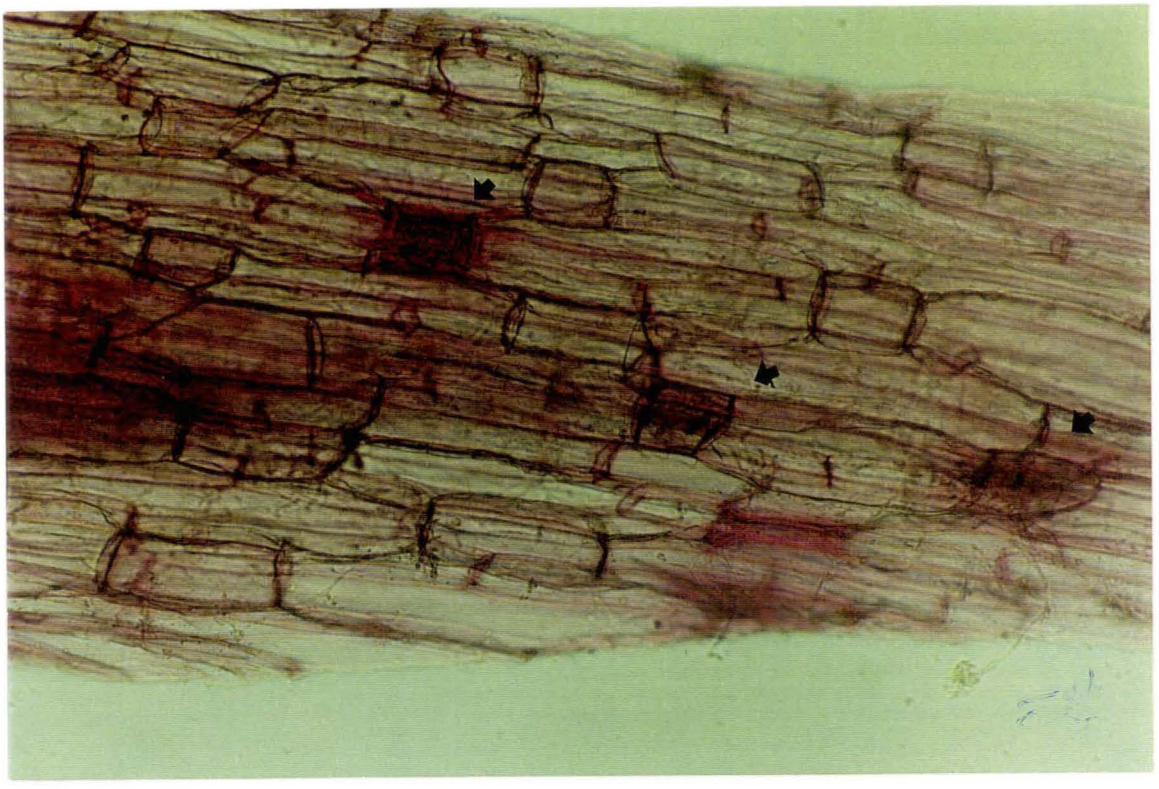


Figure 9(A): Conidiophores : *T. koningii* produced spore structures on the onion root surface as hyphae grow through the root epidermal mucilage.

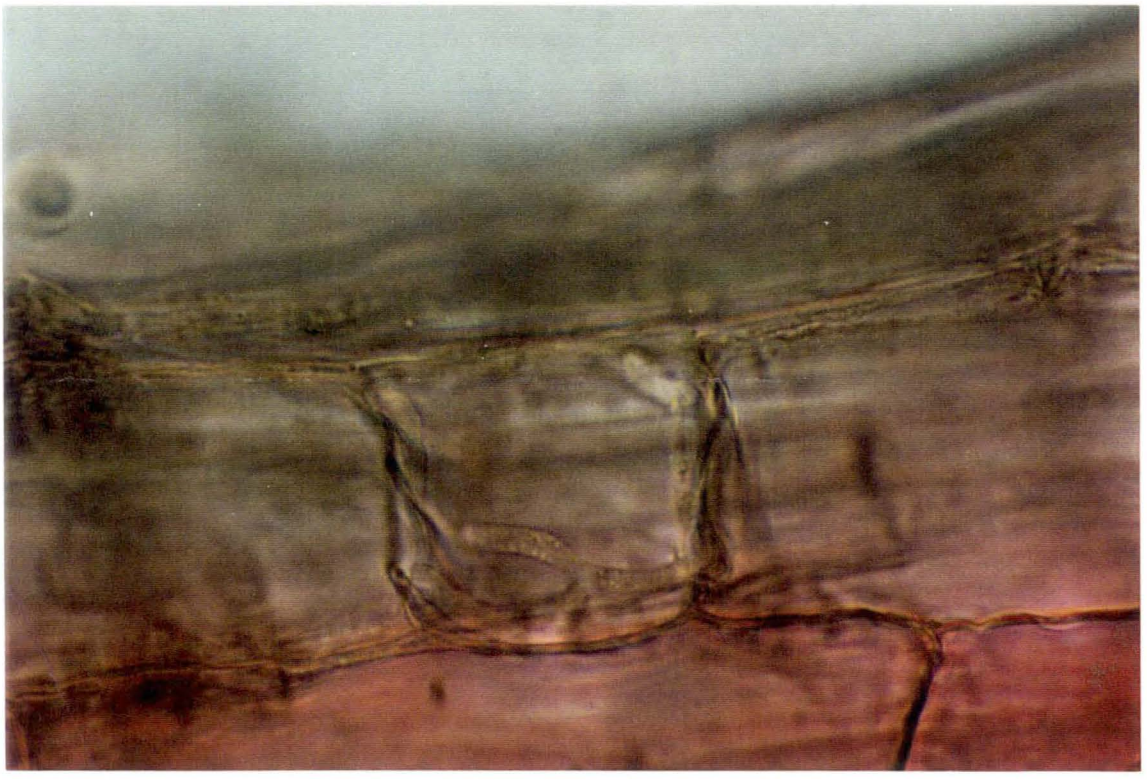
Light microscope studies of the epidermis of onion roots infected with *S. cepivorum* onto which *T. koningii* was inoculated showed that the most common interaction when the two fungi made physical contact was for *T. koningii* to grow across the *S. cepivorum* hypha without any antagonistic response. In root epidermal mucilage, a range of nutrition sources including cellulose and pectin would be available, while on the petri dish base (Ch. IV:8) nutrition is limited. As time proceeded *S. cepivorum* hyphae were often seen in a decaying condition on the epidermis. However mycoparasitic coiling was never observed on the root epidermis. In regions of onion roots where cells lacked Feulgen stainable nuclei, *T. koningii* hyphae were frequently observed specifically colonising cube shaped epidermal passage cells (Figure 9.B). This behaviour was not observed in the absence of *S. cepivorum.* These cells retained Ruthenium Red (specific for pectin) when they were colonised by *T. koningii* . *T. koningii* hyphae were often observed coiling within the cell walls of passage cells (Figure 9.C.) and fully colonising the space before exhibiting any colonisation of surrounding tissues (Figure 9.D.). It was common for a clump of *T. koningii* hyphae to form covering the colonised passage cells (Figures 9.D.; 9.E.; 9.G.; and 9.I.). These clumps bear some resemblance to one of the types of *S. cepivorum* infection cushions (Abd-El-Razik *et al.*, 1973). These clumps were not formed on longer epidermal cells. In some examples *T. koningii* hypha could be seen growing through root cortical tissues with no stainable nuclei near the forefront infection hypha (Figure 9.F.) of *S. cepivorum* . Where the colonised passage cell is on a section of epidermis with degraded hypodermal and cortical tissue below, *T. koningii* hyphae branched rapidly and colonised the cortical cavity (Figure 9.G; Figure 9.H.). In examples where the hyphae of *S. cepivorum* were present in the cavity directly below the passage cells, *T. koningii* hyphae were seen growing among the *S. cepivorum* hyphae (Figure 9.I.) which appear highly vacuolate and swollen, and often become detached at the septa.

Evidence of passage cell colonisation was also provided by examination of root segments removed from gel wells after electrophoresis (Ch. IV:10). After segments were bisected, the inner wall of the epidermis, which was often the only cellular layer remaining after *S. cepivorum* degraded the cortex and hypodermis, could be examined by microscope. In some examples a dark precipitate was deposited in passage cells (Figure 9.J.), and on closer inspection this material was appeared to be of similar form to calcium oxalate crystals. *T. koningii* hypha were frequently seen to emerge into the cavity via epidermal passage cells and spread radially in every direction to colonise the root cavity (Figure 9.K.).



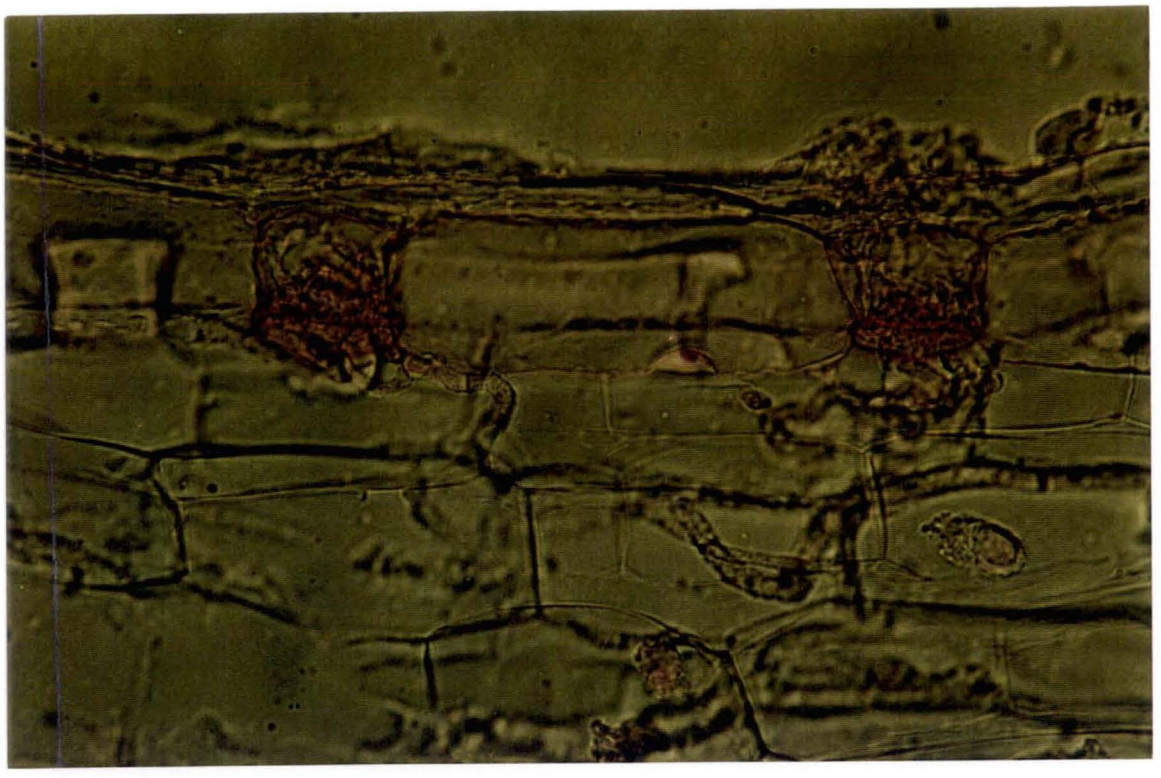
Stain: Ruthenium Red x 200

Figure 9(B): *T. koningii* colonising the infected onion root epidermis: Arrows indicate passage cells which *T. koningii* has colonised. Examples which have been colonised by *T. koningii* here have retained Ruthenium Red which stains pectin.



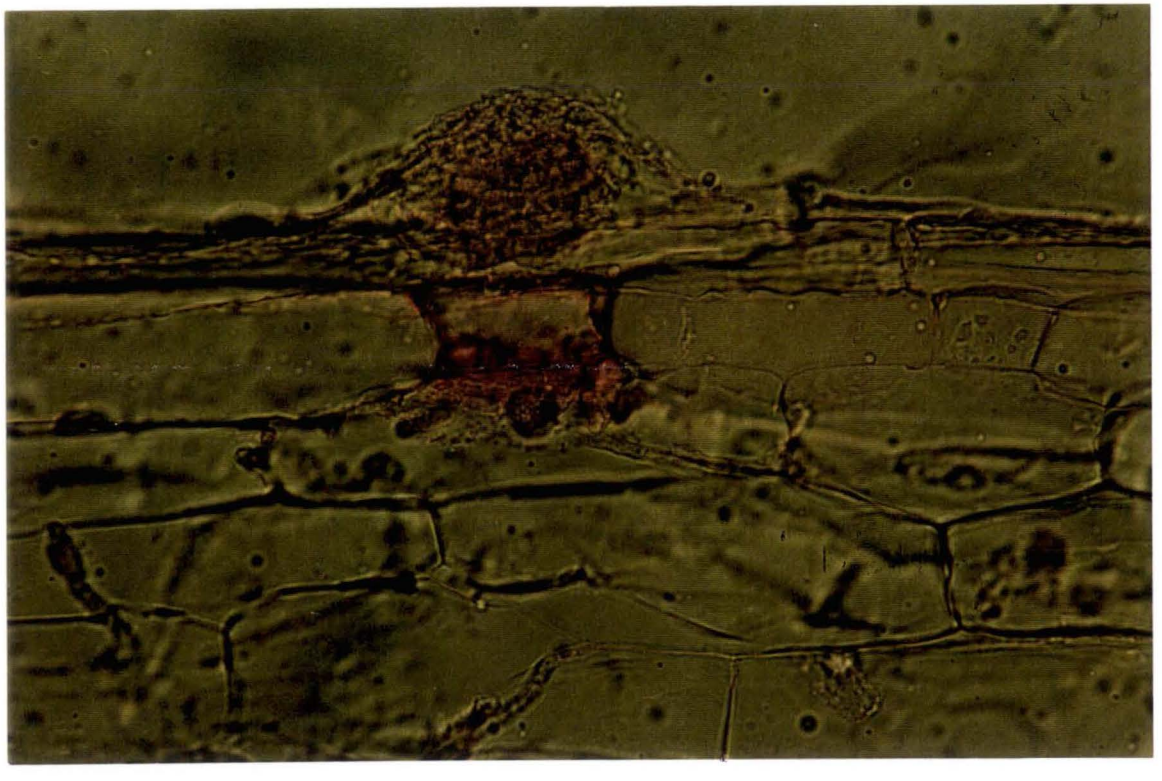
Stain: Ruthenium Red x 1000.

Figure 9(C): Hypha of *T. koningii* growing into and colonising an epidermal passage cell: Slight adjustment of the focus revealed more similar hyphal coils within this cell.



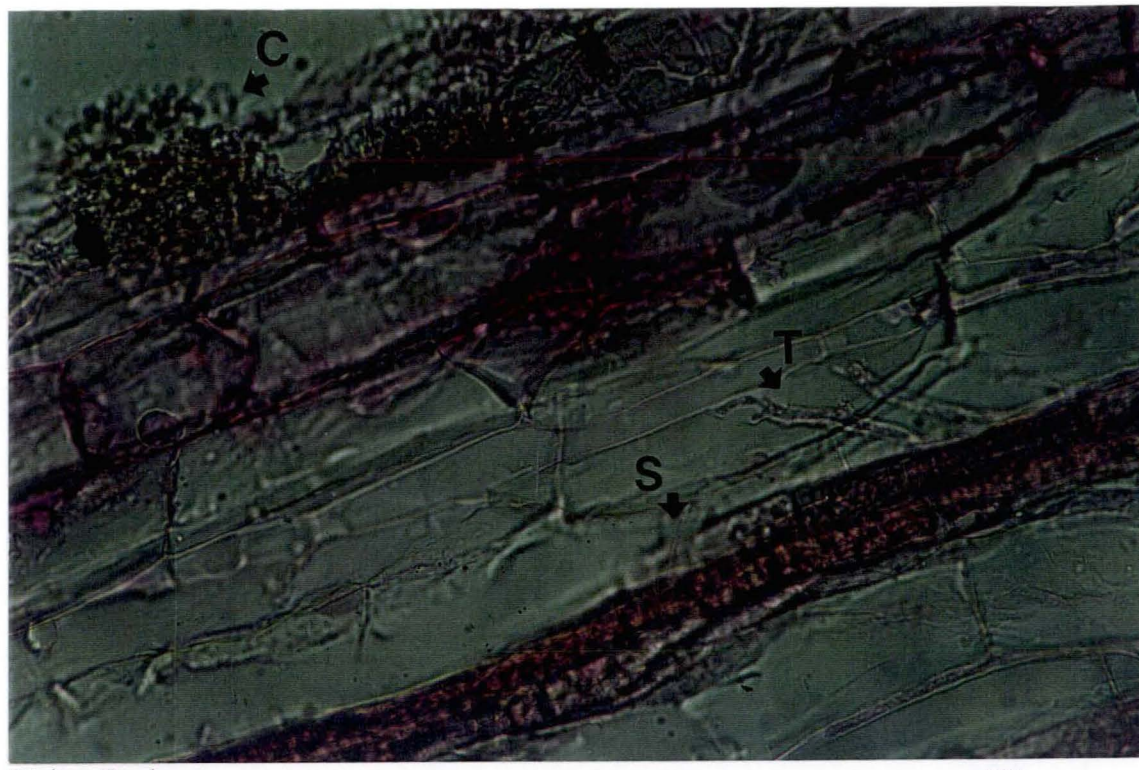
Stain: Feulgen x 400

Figure 9(D): *T. koningii* hyphae starting to appear in the epidermal and hypodermal cells surrounding the passage cells: Staining revealed that most cells lack clearly stainable nuclei.



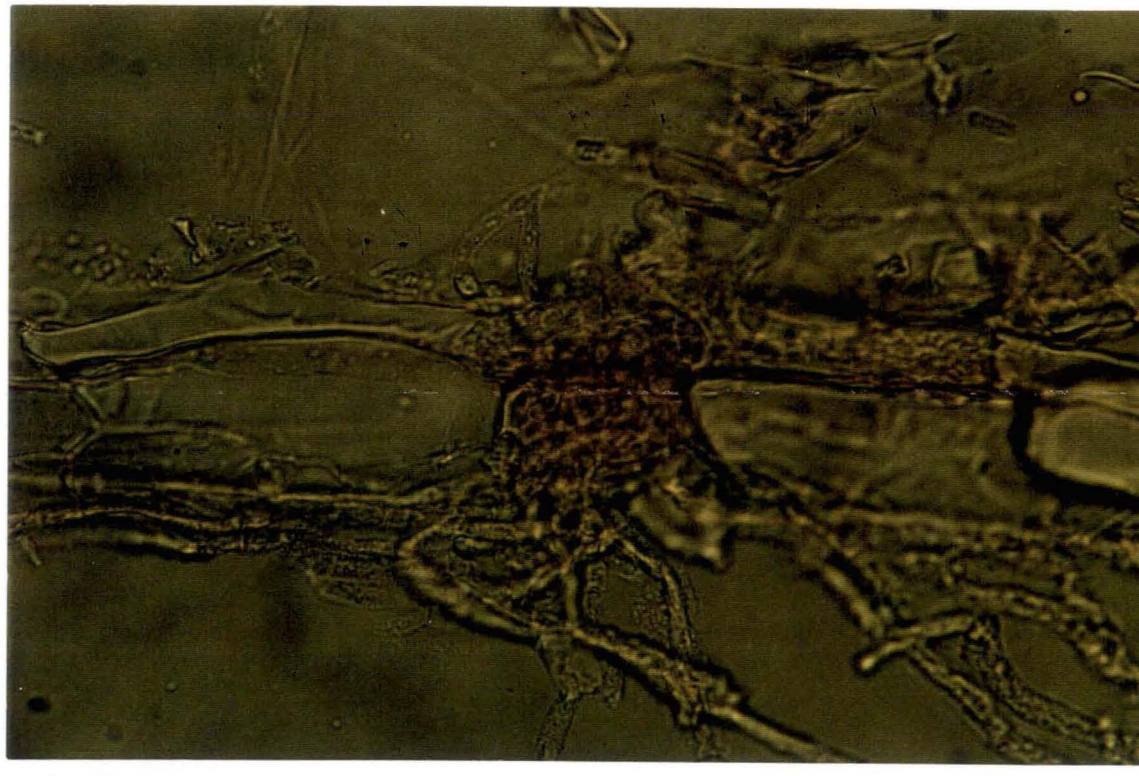
Stain: Feulgen x 400

Figure 9(E): Clump of *T. koningii* hypha: Clumps formed on the root covering the surface of an epidermal passage cell which *T. koningii* has colonised.



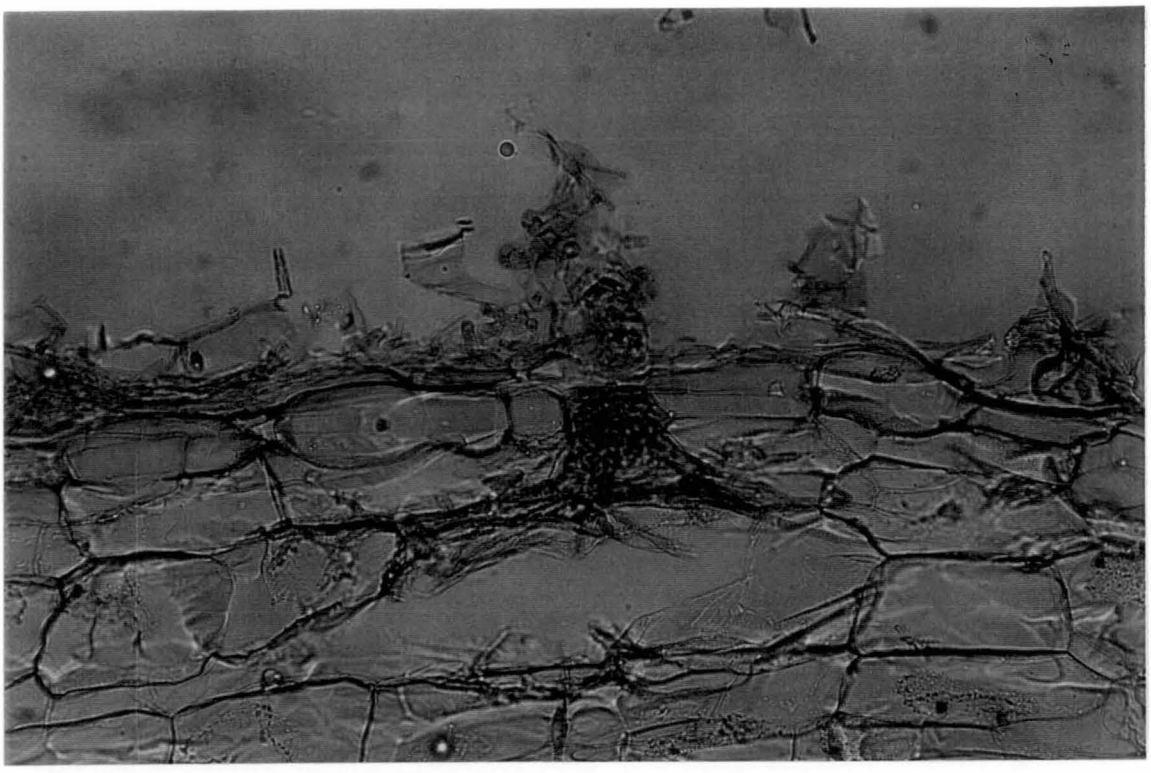
Stain : Feulgen x 400

Figure 9(F): Clumps of *T. koningii* hypha : Clumps (C) formed on the root epidermis, *T. koningii* hypha (T) can be seen growing through the cortex toward the forefront *S. cepivorum* hyphal tips (S). Note- not all hypha of either fungus or passage cells in close proximity are evident as the microtome slice has excluded them.



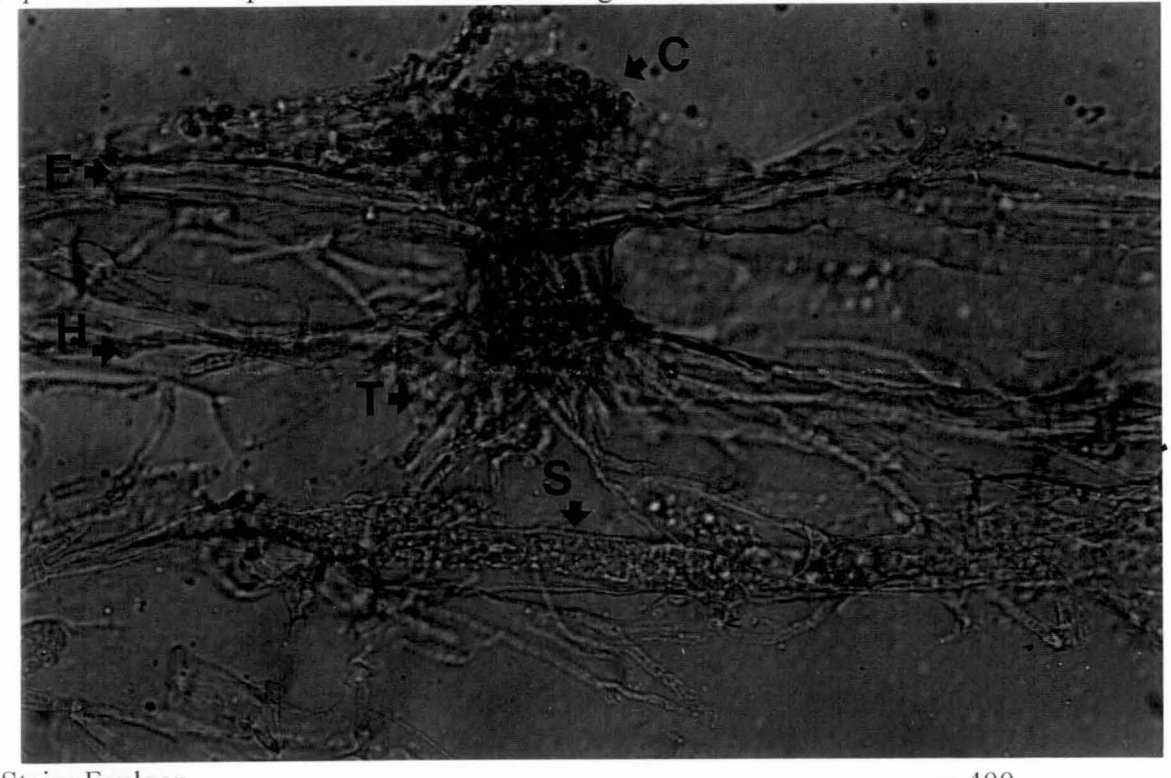
Stain: Feulgen x400

Figure 9(G): *T. koningii* hypha colonising short cells: *T. koningii* hyphae growing from outside the root epidermis to colonise an epidermal passage cell without colonising surrounding longer epidermal cells, before branching rapidly to grow into the root cortical cavity.



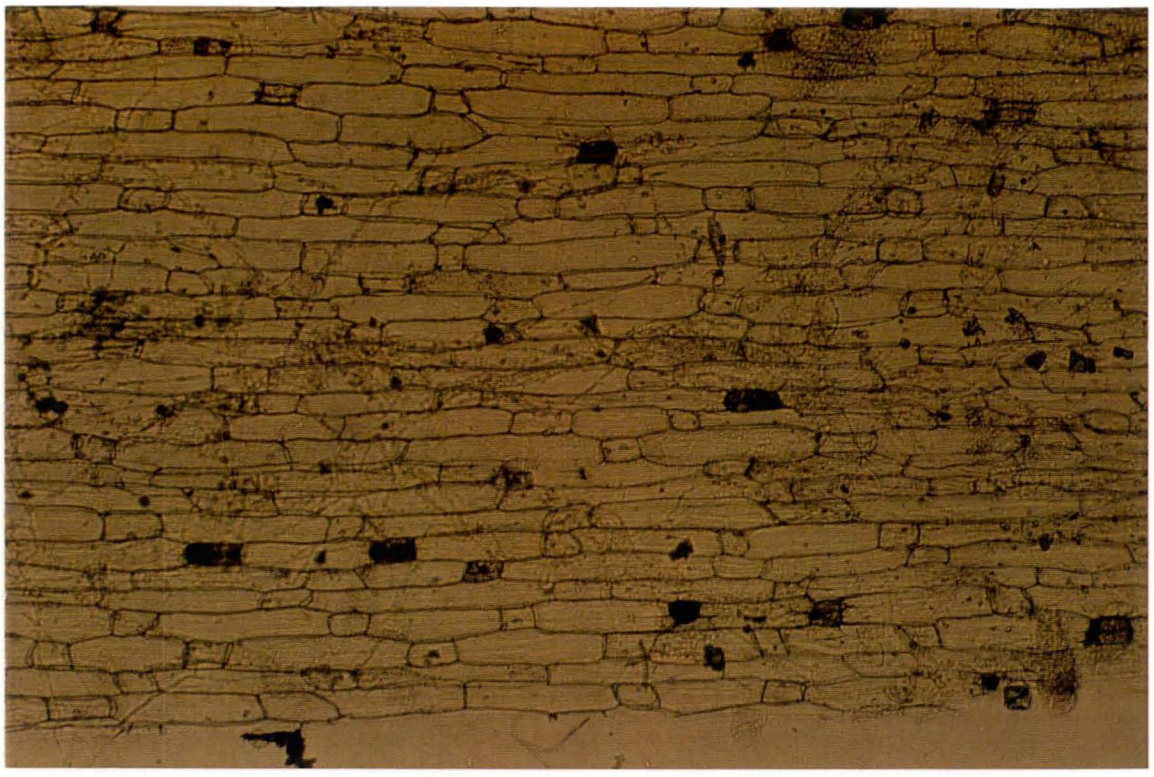
Stain : Feulgen x 200

Figure 9(H): *T. koningii* colonising epidermal passage cell: *T. koningii* has colonised the passage cell without colonising surrounding long epidermal cells, and grown into the cortical cavity below. Root cells lacked stainable nuclei. Hypha of *S. cepivorum* are not present in this immediate region of this section.



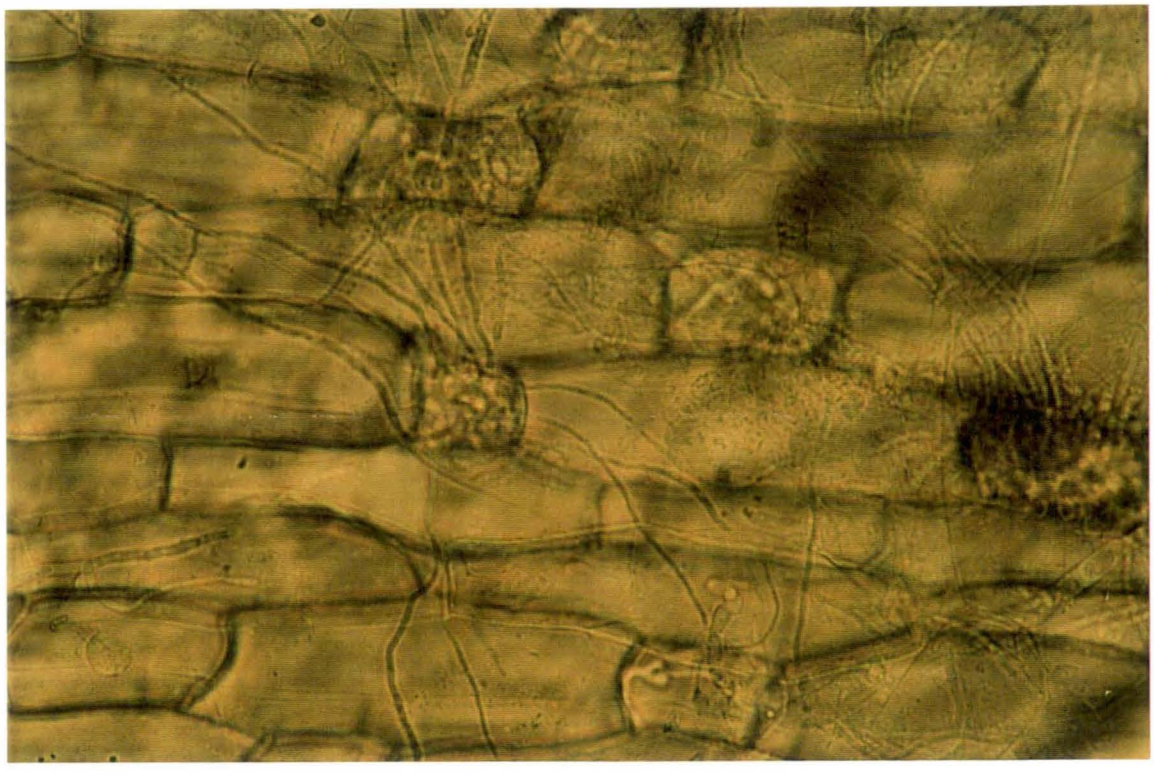
Stain: Feulgen x 400

Figure 9(I): *T. koningii* colonising epidermal passage cell: Via the passage cell *T. koningii* (T) has grown into the cortical cavity containing *S. cepivorum* (S) hyphae which are starting to detach at the septa. A clump of *T. koningii* hypha (C) cover the passage cell on the outer epidermis (E), the hypodermis (H) is partially degraded



No Stain x400

Figure 9(J): Dark precipitate in passage cells: The inner side of the epidermis is viewed following total hydrolysis of the cortical and hypodermal layers by *S. cepivorum* cell wall degrading enzymes. A dark crystaline precipitate commonly deposited in passage cells was of similar form to calcium oxalate.

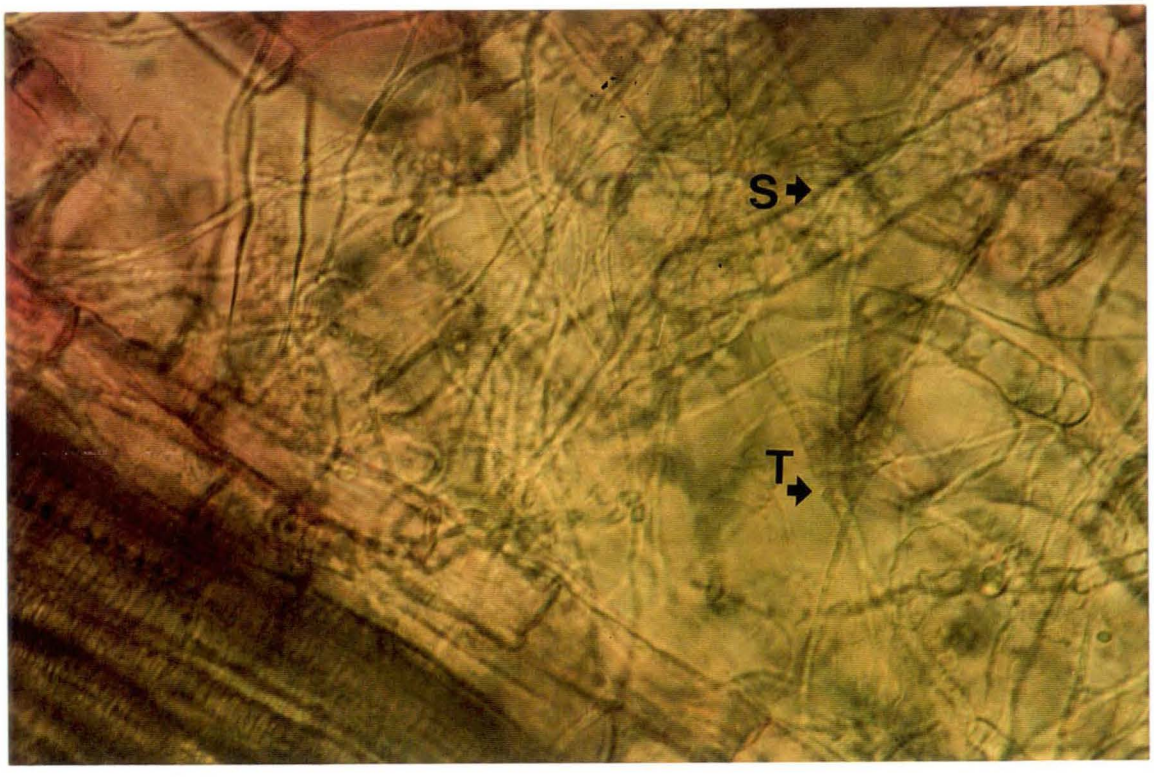


No stain x 400

Figure 9(K): Passage cells: Cells have been colonised by *T. koningii* , hyphae have grown in all directions to colonise the cavity. The photograph is taken from an inside the epidermis perspective, the hypodermis has been degraded leaving only epidermal tissues.

After the cavity was colonised by *T. koningii* , changes became evident in the *S. cepivorum* hypha which were similar to those described for antagonistic activity on the petri dish base (Figure 9.L ; 9.I), with *S. cepivorum* hypha becoming swollen and vacuolate, detaching from other cells at the septa (9.M), and ultimately become crumpled and degrading. Inside the root cavity lysis involving close physical contact of hypha was not observed.

A more natural view of the root epidermis was obtained by environmental scanning electron microscopy of fully hydrated root segments. Outer tangential walls of epidermal cells were found to be convex, cell wall junctions were sunken between cells. The surface of passage cells did not protrude any further than cell wall junctions giving them a sunken appearance (Figure 9.N).

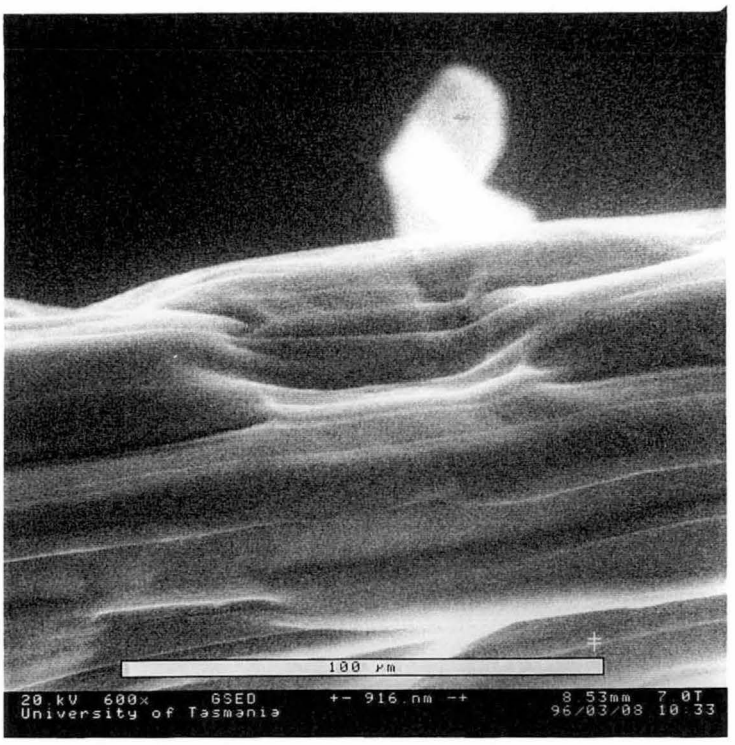


Stain: Feulgen x 400

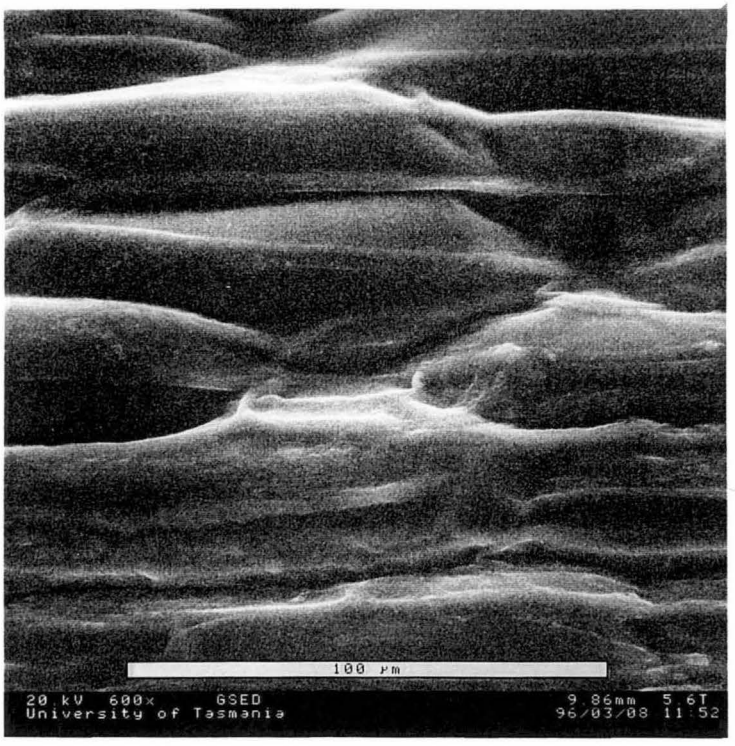
Figure 9.(L): The cortical cavity : Inside the degraded cortex of an infected onion root, *S. cepivorum* hypha (S) are vacuolate and many cells have become detached at the septa, *T. koningii* (T) hypha have colonised the cortical region.

Stain: Feulgen x 200

9.(M): *S. cepivorum* hyphae: In the root cortex, *T. koningii* hyphae were in the tissues surrounding *S. cepivorum* hyphae, some tips were seen to be lysed (L) and becoming detached at the septa.



(A)



(B)

9(N) A & B. Epidermal Passage Cells: Environmental Scanning Electron Micrographs of the onion root epidermis showing passage cells which appear to be sunken by comparison to the convex outer cell walls of larger epidermal cells.

7.4. Discussion:

T. koningii hypha inoculated onto onion roots were able to colonise the outer epidermis by growing through the surface mucilage. *T. koningii* hyphae were never seen to enter root cells of healthy specimens. If the root was under stress from lack of moisture or lack of nutrition *T. koningii* hyphae were seen growing into and degrading the damaged root tissues, producing chlamydospores within the tissues and conidia at the root surface.

In roots colonised by *S. cepivorum* , microscopic examination of Ruthenium Red and Feulgen stained segments indicated that while *T. koningii* grew over the epidermis, it actively colonised certain cells, shorter in length than other epidermal cells, known as passage cells (Figure 9.B.). It is possible that the initial reason for preferential colonisation of these cells is nutritional, as staining with Ruthenium Red (specific for pectin) showed that some colonised passage cells stained more strongly for pectin than surrounding cells. However this could be attributable to disruption of the cell wall, as passage cells in healthy roots did not show any greater affinity for this stain. Passage cell colonisation was not observed in healthy roots inoculated with *T. koningii* .

According to Von Guttenberg (1968), in plants which have a long and short celled hypodermis , the shorter passage cells are unsuberised. Peterson *et al.* (1978) found that the outer tangential wall of the epidermal layer of onion roots becomes suberised about 1.5 cm from the root apex, and it was suggested that passage cells become suberised more slowly than longer cells. Therefore, in some parts of the root a differential suberisation occurs. In the root epidermis of *Flaxinus americana* (the white ash) passage cells are the only cells which are not suberised (Brundrett *et al.*, 1991), and in the orchid *Cattleya aurantiaca* passage cells are the only epidermal cells which are not lignified (Brundrett *et al.*, 1988). Additionally, short cortical cells are known to contain a greater number of plasmodesmatal pits per unit surface area than longer cells (Scott *et al.*, 1956). Whether this extends to the hypodermis and epidermis which would allow greater access to high molecular weight extracellular proteins of *S. cepivorum* or *T. koningii,* is not known. It is also unknown whether passage cells are slowly elongating. If so, the deposition of cellulose microfibrils at these sites, as described by Scott *et al.* (1956), would provide an excellent source of nutrition for *T. koningii* .

The normal sequence of events associated with passage cell colonisation was for *T. koningii* hypha to coil within the cell until it became filled with mycelium, often forming a mycelial clump above the cell. The reason for these formations is unknown, the structures bear some similarity to *S. cepivorum* infection cushions (Ch. III:6). However as the passage cells are already dead, appear to offer little

resistance to colonisation by *T. koningii* , and clumps are not seen until after the cell is well colonised, this seems an unlikely function. The structure is possibly the response to abundant nutrition. Whatever the reason for formation of the structure, it is feasible that the clump could inhibit other saprophytes from colonising the root cortex via passage cells, allowing *T. koningii* to colonise the cortex with less competition from other organisms.

In this and other histological studies, *S. cepivorum* has not shown preference for passage cells as penetration sites. This is the first incidence of which the author is aware of entry of root tissue by *Trichoderma* spp. by colonisation of passage cells. However there are several reports of mycorrhizal fungi entering roots via these cells. For example, in another member of the Lilliaceae, *Smilacina racemosa,* versicular arbuscular mycorrhizae gain access to the root cortex via passage cells (Brundrett and Kendrick, 1988), and Hussey (1982) found that entry of VAM fungi into asparagus roots was restricted to certain exodermal short cells, which were not suberised. The phenomenon of coiling within passage cells is also common in colonisation of roots by VAM (Brundrett *et al.*, 1996). While it has been reported for *Trichoderma* spp. to grow into healthy root tissue in a similar way to mycorrhizae (Kleifield and Chet, 1992) and this has been associated with increases in plant growth, there is no indication of mycorrhizal activity as the fungus appears to colonise only the damaged tissues.

The growth of *T. koningii* into infected tissues suggests that *T. koningii* competes for nutrition with *S. cepivorum*. The relationships of *T. koningii* and *S. cepivorum* cell wall degrading enzymes are investigated in Ch. IV:10. The types of changes which were observed in *S. cepivorum* hyphae after *T. koningii* grew into the infected tissue suggested that contact between the two fungi was not necessary for lysis of *S. cepivorum* hyphae and a diffusible principle is likely to be involved. The mechanism of lysis is further investigated in Ch. IV:11. The dissolution of cell walls, lysis of hyphal apices, and detachment of hyphae at the septa would suggest that extracellular enzymes are likely to be involved.

Chapter IV: Interactions between *T. koningii* and *S. cepivorum.*

10.0: Pectolytic enzyme relationships.

10.1. Introduction.

Pectolytic enzymes have been well established as a major component of the attack on plant cell walls by pathogens (Collmer and Keene, 1986). The ability to produce pectinases does not, however indicate that an organism possesses pathogenic abilities (Bateman and Millar, 1966), as many micro organisms encounter pectin in their normal metabolism and saprophytic growth. Relationships between spatial distribution of onion root cell death and *S. cepivorum* infection hyphae seem to involve some cell lysis ahead of advancing infection hyphae particularly in the cortex (Ch. III:6). Relationships between distribution of cell wall degradation and *S. cepivorum* pectolytic enzymes have been established (Ch. III:7). Some tissues, particularly the epidermis, hypodermis and stele were more resistant to degradation than the cortex, possibly due to suberisation (Bonnet, 1968), inhibition of pectolytic enzymes by presence of phenolic compounds (Petersen *et al.*,1978) and/or resistance to movement of large molecules such as proteins across the apoplastic route (Clarkson *et al.*, 1978). In colonisation of the root, *T. koningii* grows through the onion root surface mucilage. The predominant pathway to colonising internal damaged or hydrolysed tissues appeared to be via passage cells in the epidermis, which possibly exhibit some differences in cell wall composition (Petersen *et al.* 1978; see also Ch. IV:9) and plasmodesmatal pit density (Scott *et al.*, 1956) to other cells. The aim of these experiments was to investigate pectolytic enzyme production by *T. koningii* and the involvement of *T. koningii* pectolytic enzymes during colonisation of *S. cepivorum* infected roots by *T. koningii.*

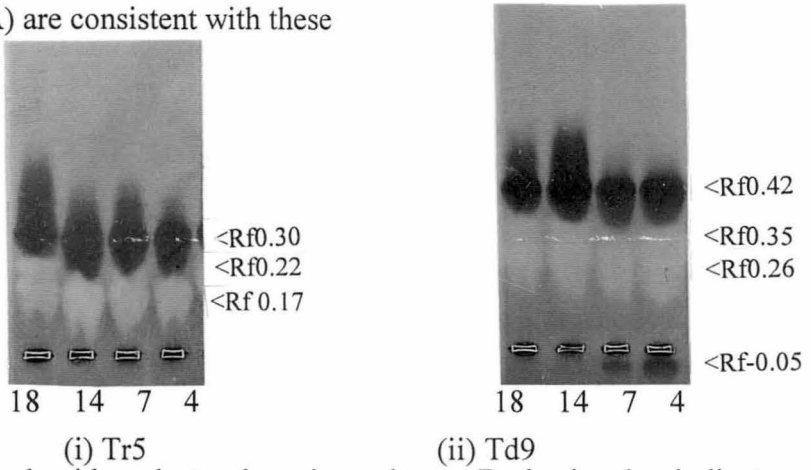
10.2. Materials and Methods:

For characterisation of pectolytic enzyme production, *T. koningii* (Tr5) and another *Trichoderma* spp. (Td9) isolated from parasitised *S. cepivorum* sclerotia at Scolyer's trial site (Ch. V:14) were grown in 5ml Bijou bottles containing 2 mls of pectinase medium (Appendix B.3) under static conditions at 25°C for 4, 7, 14, and 18 days. 20µl samples were withdrawn from cultures and loaded into electrophoresis gels. Similar experiments were repeated four times. Isozyme analysis for detection of polygalacturonase (PG) and pectinesterase (PE) was performed as described in Appendix E.

Onion roots were infected by *S. cepivorum* (Sc4) and colonised by *T. koningii* (Tr5) under sterile conditions by the procedure described in Appendix C. Following incubation for 72-96 hours in presence of both fungi roots were cut into 2 mm sections and loaded in sequence into pectinase electrophoresis gels. Electrophoresis was performed as described in Appendix E. Following electrophoresis root segments were retrieved from wells and examined by light microscope to determine the level of *S. cepivorum* infection and *T. koningii* colonisation. Six replicates of the experiment were performed for detection of pectinases in onion roots, three are presented herein.

10.3 Results.

Figure 10(A) is a polyacrylamide electrophoresis gel displaying the pectolytic enzyme system of *T. koningii* (Tr5) and another *Trichoderma* spp. (Td9). Tr5 produced a PG isozyme (R_f 0.30; extremities ranging from R_f 0.20-46) and two PE isozymes (R_f 0.17; extremities ranging from R_f 0.08-18, and R_f 0.22; extremities ranging from R_f 0.20-25). *T. koningii* therefore has the capacity to compete for the carbon sources *S. cepivorum* uses in its pathogenic attack on the roots. Td9 produced isozymes of differing R_f to Tr5 including a cathodic migrating PG. In both isolates there was some change in isozyme production at progressing stages of physiological development. In Tr5 this was most notably production of the PE R_f 0.22 in 18 day old cultures which was not detected in younger cultures. The Tr5 PG and PE isozyme profile was examined in two other replicates (not presented), results presented in Figure 10(A) are consistent with these



(i) Tr5 (ii) Td9

Figure 10(A): Polyacrylamide electrophoresis gels: Dark bands indicate polygalacturonase (PG), light bands indicate pectinesterase (PE), numbers indicate days incubation in pectinase medium at 25°C.

(i)*T. koningii* (Tr5) produced a PG isozyme R_f 0.30 (extremities ranging from R_f 0.20-46) and two PE isozymes R_f 0.17 (extremities ranging from R_f 0.08-18) and R_f 0.22 (extremities ranging from R_f 0.20-25).

(ii)*Trichoderma* spp. (Td9) produced two PG bands R_f 0.42 (extremities R_f 0.34-55) and R_f -0.05, and two PE bands R_f 0.35 (extremities R_f 0.30-8) and R_f 0.26 (extremities R_f 0.28-15).

Figures 10(B) and 10(C) display examples of replicates of where the hypha of *T. koningii* and *S. cepivorum* did not make physical contact in or on the root according to microscopic examination. Electrophoresis was performed just before the foremost *S. cepivorum* hyphal tips reached the part of the root colonised by *T koningii*, though *S. cepivorum* PG had diffused into this zone.

Figure 10(B) is a contact print of an electrophoresis gel into which sections of an onion root in which the antagonism experiment was performed were loaded in sequential order from the root tip (well 2) to the bulb base (well 25). A PE (R_f 0.13) native to the plant was detected in all wells. *S. cepivorum* hypha were present externally on root segments loaded into wells 2 to 16 and internally in wells 2 to 10. All three PG isozymes (R_f 0.08, 0.16, -0.12) known to be produced by *S. cepivorum* (see Ch. III:7) are detected in wells 2 to 15. *S. cepivorum* PE isozymes (R_f 0.28 and 0.40) were detected in wells 2 to 6. *T. koningii* did not enter this onion root, remaining in the onion root surface mucilage on the outside of the epidermis of root segments loaded into wells 18 to 21. *T. koningii* PE was not detected. However *T. koningii* PG R_f 0.30 was detected in wells 16 to 19. *S. cepivorum* PG and PE were not detected in root segments where *T. koningii* enzymes were detected.

Figure 10(C) displays record of a replicate where *T. koningii* was physically and enzymatically more active than in Figure 10(B). In this example, *S. cepivorum* PG has diffused at least 2mm ahead of the infection and well into the zone where *T. koningii* pectinases are detected. The *S. cepivorum* R_f 0.40 PE, which appears to be the most diffusible isozyme (Ch. III:7), has not diffused ahead of the infection hyphae. *T. koningii* hypha have not entered the internal tissues. *T. koningii* PG and PE activity was detected in tissues on the root tip side of the area that *T. koningii* had colonised indicating some diffusion of *T. koningii* proteins.

Where the two fungi did come into contact, and could both be seen colonising the root cortical cavity the isozymes of both were detected in the infection cavity (Figure 10.D.). *T. koningii* PG activity has increased in quantity in the area around well 15, where the root would possibly be injured by the diffusing *S. cepivorum* PE proteins.

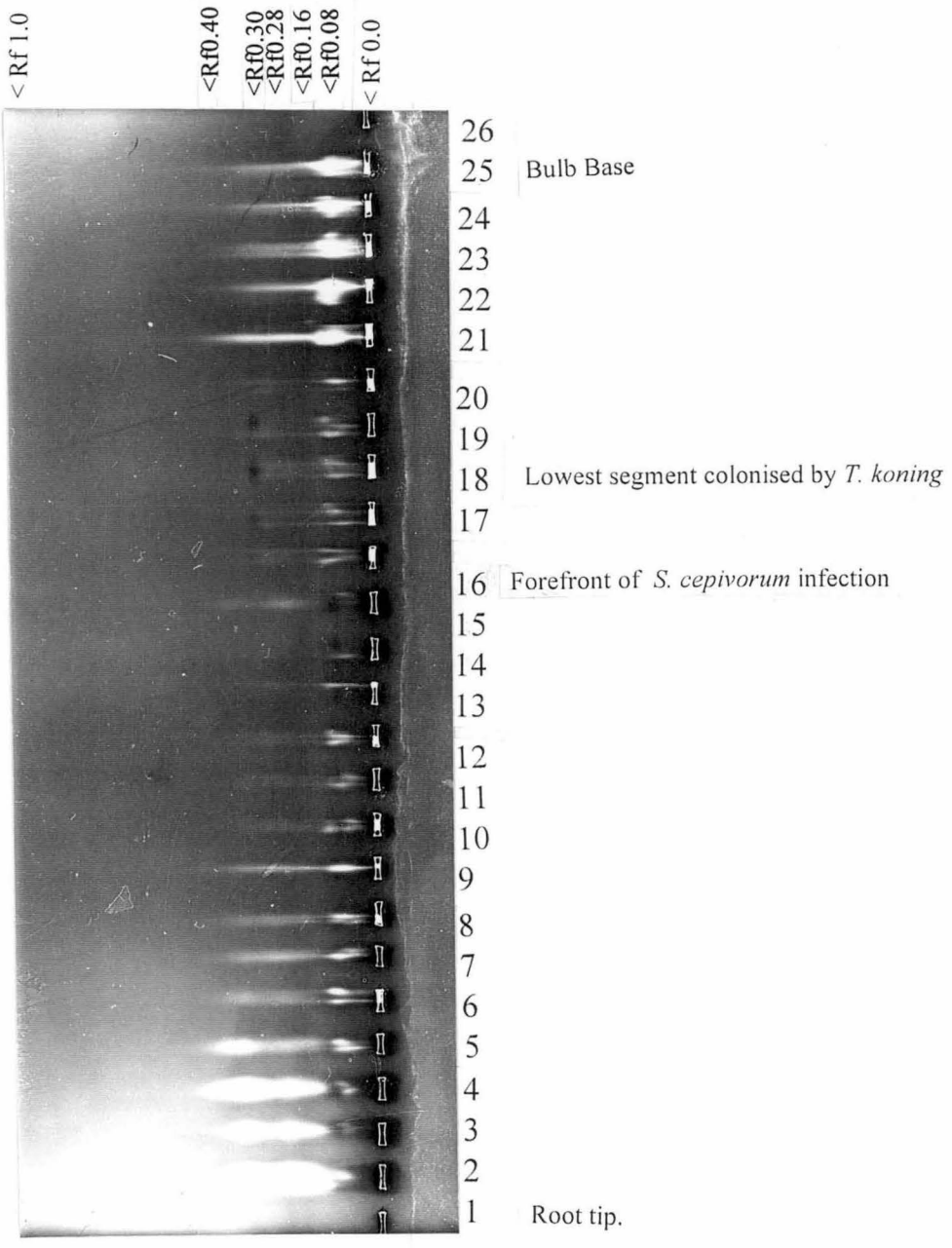


Figure 10(B): Pectinase electrophoresis gel showing spatial distribution of PG and PE in an onion root: *S. cepivorum* PE (light bands, R_f 0.28 and 0.40) are detected in wells 2 to 6 and PG (dark bands, R_f 0.08, 0.16, -0.12) activity was detected in wells 2 to 15, *T. koningii* PG (R_f 0.30) was detected in wells 16 to 19. Root components and distribution of *S. cepivorum* and *T. koningii* hyphae are marked. A band of PE R_f 0.13 native to the plant has been detected in all wells, a streak associated with this band is strongly detected in wells 21-25, the light streaks between R_f 0.2 to 0.5 in wells 7 to 9 are most probably of plant origin.

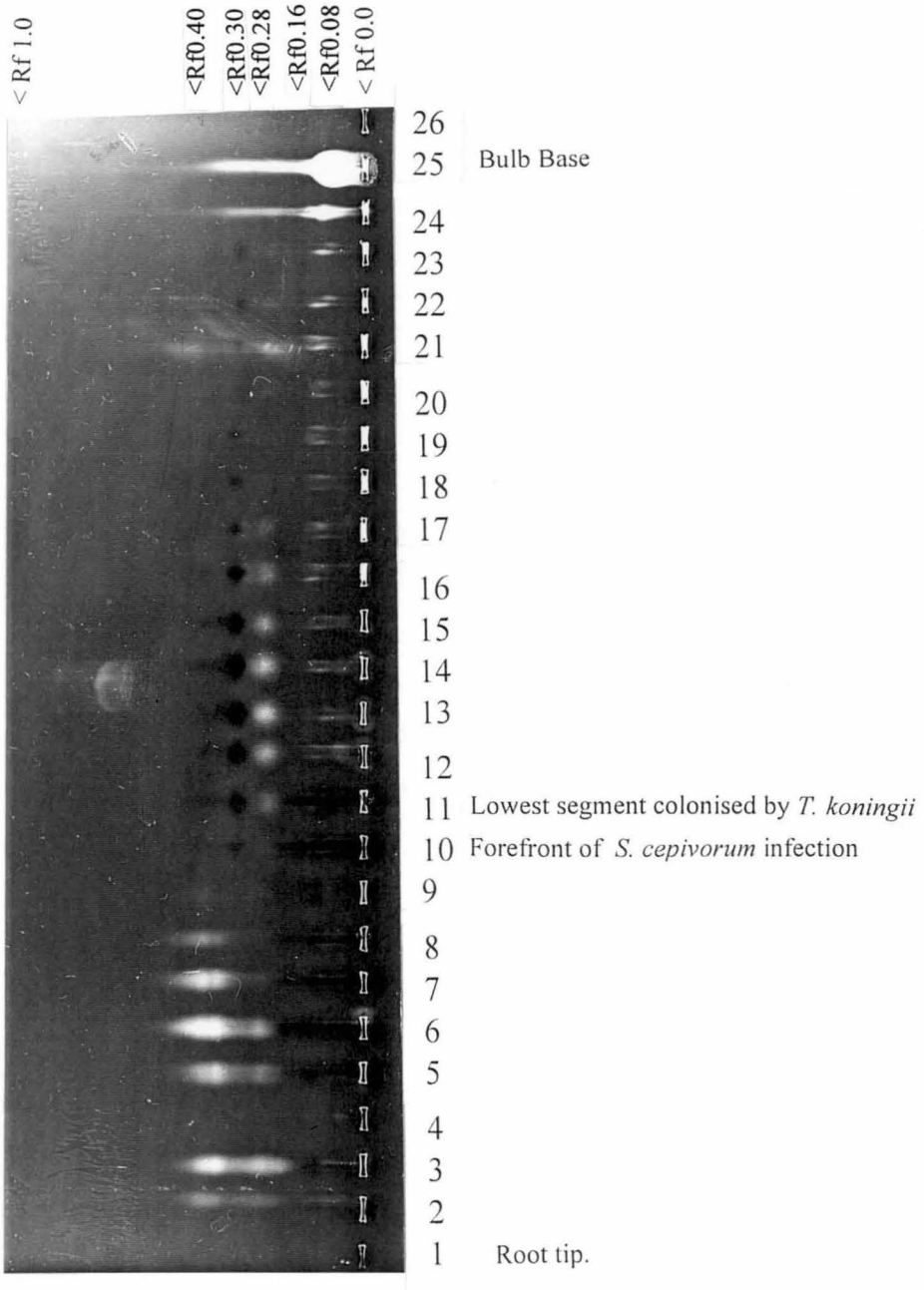


Figure 10.(C): Pectinase electrophoresis gel showing spatial distribution of pectinases in an onion root: *S. cepivorum* pectinesterase R_f 0.40 was detected in wells 2 to 9 and R_f 0.28 to 0.5 in wells 2 to 7. *S. cepivorum* PG bands (R_f 0.08, 0.16, -0.12) were detected in wells 2 to 11. *T. koningii* PG R_f 0.30 is detected in wells 10 to 23 and PE R_f 0.22 is detected in wells 10 to 17. Root components are marked. PE of plant origin R_f 0.13 is detected in wells 12 to 25.

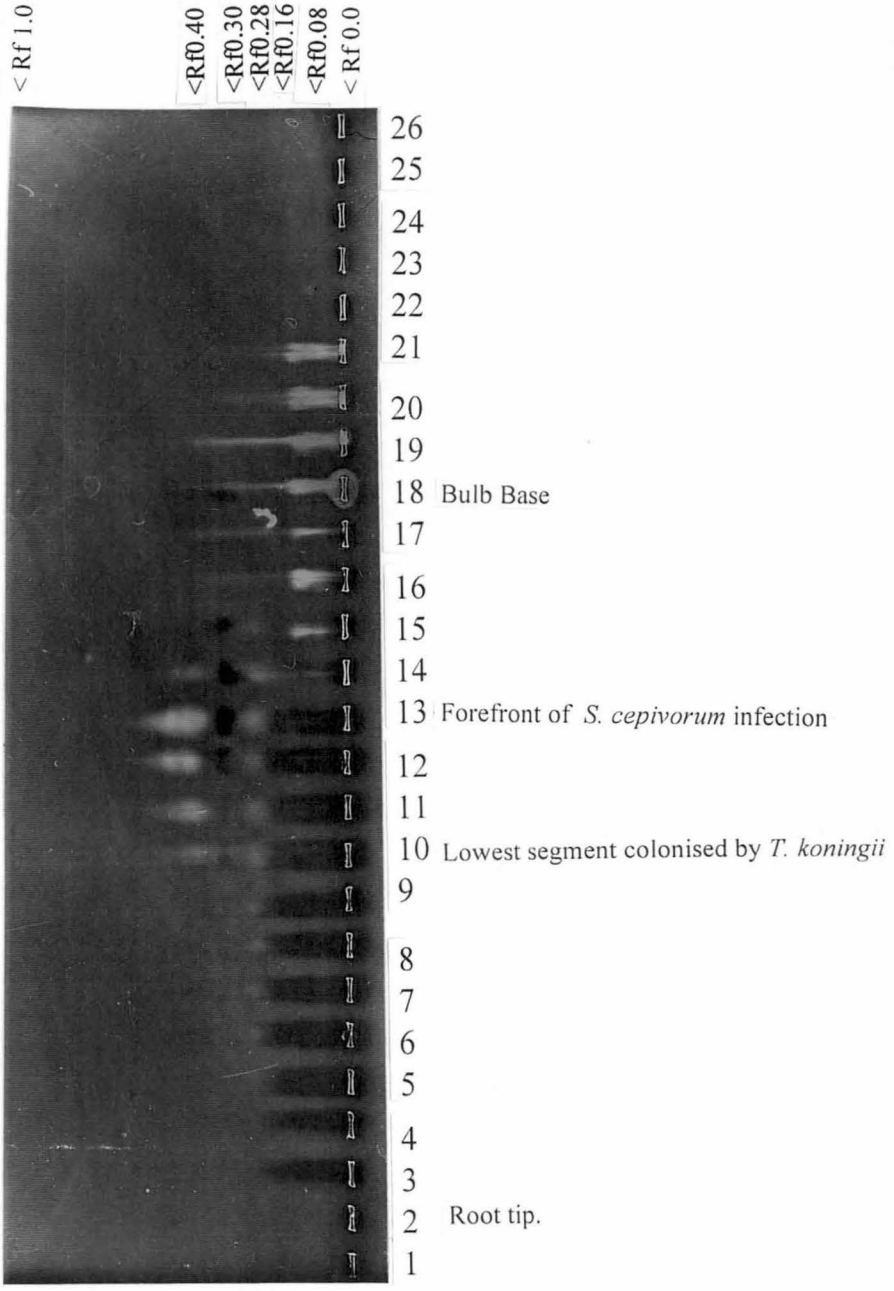


Figure 10.(D): Pectinase electrophoresis gel showing spatial distribution of pectinases in an onion root: *S. cepivorum* PG isozymes (R_f 0.08, 0.16, -0.12) were detected from wells 3 to 13 and PE isozymes (R_f 0.40) were detected in wells 10 to 14. *T. koningii* PG R_f 0.30 is detected in wells 10 to 15. The PE band detected of R_f 0.24 detected in wells 4 to 15 may originate from either fungus. Root components are marked. PE R_f 0.13 has been detected in wells 11 to 21. In lane 18 a small zone of PG R_f 0.30 is attributable to *T. koningii* which was noted to have sporulated on the cap of a root emerging from the base plate.

10.4. Discussion.

When *T. koningii* was grown in culture with pectin as the sole carbon source, proteins with pectinesterase (R_f 0.17 and 0.22) and polygalacturonase (R_f 0.30) activity were detected (Figure 10.A). Some change in relative activity of different proteins was observed at different culture ages. Isozymes of another *Trichoderma* spp. (Td9), isolated from parasitised *S. cepivorum* sclerotia, were of greater mobility than those of Tr5. However like Tr5 the PG band was of greater R_f than the PE, a pattern which appears to be common among Tasmanian *Trichoderma* isolates(Ch VI:16-19). The cathodically migrating PG of Td9 was not detected in older cultures suggesting a change in enzyme induction as substrate was utilised.

Production of PG prior to colonising any internal tissues, suggests utilisation of the root surface mucilage for nutrition. This layer contains polyphenolic compounds (Petersen *et al.*, 1978) which may hinder pectolytic enzyme activity. In example 10(B) where *S. cepivorum* infection was on the lower regions of the root, the *S. cepivorum* infection had not developed to the point where extracellular enzymes had diffused very far ahead of the internal infection. This piece of evidence suggests that it would be unlikely that many root cells would have been injured by *S. cepivorum* toxins in the zone ahead of the infection and below the epidermal area colonised by *T. koningii*, which perhaps suggests why *T. koningii* had remained on the epidermis. However presence of other toxins such as oxalic acid and cellulase were not examined and are likely to be important in pathogenesis.

In replicate 10(D) where *T. koningii* had grown into the root cortical cavity, pectolytic enzymes of both fungi were detected in wells 9 to 14 probably competing for similar substrates. In root antagonism experiments overall, *S. cepivorum* PE in particular did not diffuse in detectable quantities as far ahead of *S. cepivorum* hyphae as was found in the *S. cepivorum* infection bioassays (Ch. III:7). It is possible that some diffusible extracellular protein such as the polygalacturonase inhibitor proteins described by Favaron *et al.* (1993) produced by *T. koningii* could be denaturing *S. cepivorum* isozymes.

The available nutrition of pectin in dead tissues of the infected root provides one explanation for *T. koningii* colonisation of cortical tissue. *T. koningii* produced PG in all replicates whether it grew in epidermal mucilage or the cortical tissue. In healthy tissue however, the integrity of the root epidermis appeared to be to regulated by *T. koningii* 's ability to colonise the cortex. In studies of PG produced by another *T. koningii* isolate, Fanelli *et al.* (1978) found that *T. koningii* PG was not absorbed by plant tissue in the way phytopathogenic fungi polygalacturonases were (Cervone *et al.*, 1978). They proposed that the glycosidic moiety of the enzyme mediates recognition of host tissues, and that the *T. koningii* proteins were

not able to bind to the plant tissues, and this difference in structure could account for the lack of pathogenicity. This result considered, it is reasonable to suggest that *T. koningii* PG and PE isozymes detected in root segments where *T. koningii* was present only in the epidermal mucilage have been degrading the free oligosacharides in the epidermal mucilage, and are not able to act on the cell walls. Microscopic studies have shown that epidermal passage cells are important in colonisation of infected root tissue. These cells possibly exhibit some differences in cell wall structure to normal root epidermal cells, including lignification (Brundrette *et al.*, 1988), suberisation (Peterson *et al.*, 1978) and plasmodesmatal pit density (Scott *et al.*, 1956). A hypothesis which warrants testing is that it may be possible for *T. koningii* pectolytic enzymes to more readily utilise pectin at these sites. Certainly Ruthenium Red (stain specific for pectin) had greater affinity for some passage cells which *T. koningii* had colonised than other epidermal tissue (Figure 9.B.). It is possible that *T. koningii* isozymes produced within infected root tissue utilise oligosaccharides liberated by *S. cepivorum* PG. *T. koningii* pectolytic enzymes may have difficulty penetrating the healthy plant tissues, which could explain why the fungus appears to be restricted to colonisation of dead tissue.

In total, six replications of this experiment for pectinase detection were performed, and one limitation on interpretation of the results is that each is only a sample of the distribution of isozymes at a precise moment in a continuing process which sometimes had a variable outcome (ie occasionally Tr5 did not successfully inhibit the infection).

In summary, *S. cepivorum* hyphae produce PG and PE which diffuse into tissues, hydrolysing cortical tissue and many cells die in advance of the hyphae. The epidermis and hypodermis are more resistant to degradation, and tend to remain intact while cortical tissues dissolve. The *T. koningii* hyphae remain on the outside of the epidermis while these tissues are healthy, often producing polygalacturonase and pectinesterase as it degrades pectin in the epidermal mucilage. When the epidermal cells have been injured by the diffusible pectinases produced by *S. cepivorum* , *T. koningii* is observed to colonise epidermal passage cells and grow into the dead tissues or cavity of the cortex, presumably by competing for the same carbon sources as *S. cepivorum*. *T. koningii* has not acted to damage healthy tissues in these experiments, this is consistent with the findings of Fanelli *et al.*, (1978) that *T. koningii* pectinases are not absorbed by plant tissues in the same way as those of phytopathogenic fungi. The epidermis is dead, yet neither fungus seems to be able to colonise it except for the passage cells. As described in Ch. III(6) there is evidence that the epidermis and hypodermis are more resistant to degradation than the cortex perhaps due to composition of phenolic compounds and suberisation. Plasmodesmata may also hinder movement of enzymes into these tissues.

Chapter IV: Interactions between *T. koningii* and *S. cepivorum* :

11.0: Chitinolytic enzyme relationships.

11.1 Introduction:

Microscopic observations of structural changes in *S. cepivorum* hyphae in the presence of *T. koningii* in the petri dish base (Ch. IV:8) and in onion roots (Ch. IV:9) coupled with the lack of detectable antimicrobial compound production (Metcalf, 1993) lead to studies to determine whether *T. koningii* could produce fungal cell wall degrading chitinolytic enzymes.

The nomenclature for chitinolytic enzymes used herein follows that of Tronsmo and Harman (1993). Enzymes that cleave at random points in the chitin chain are referred to as endochitinases (EC 3.2.1.14.) and they require at least the tetramer of chitin for activity. The enzyme that releases chitin dimers (chitobiose) from the end of chains is called 1,4-ß-chitobiosidase (chitobiosidase), and it requires at least the trimer of chitin to act. Exo-enzymes that release monomeric units from the chitin chain end are *N*-acetyl-ß-glucosaminidases (EC 3.2.1.30.) hereafter referred to as glucosaminidases, these require at least the dimer of chitin to act. The preceding definition of chitobiosidase is the same as that given for exochitinase by some authors (eg Robbins *et al.* , 1988; McCreath and Gooday, 1992; Haran *et al.*, 1995).

Although electrophoresis systems for the detection of chitinolytic enzymes in general (Trudel and Asselin, 1989; Ulhoa and Peberdy, 1991a, Cherif and Benhamou, 1990) and specific systems for detection of endochitinases, glucosaminidases, and chitobiosidases (Tronsmo and Harman, 1993; Lorito *et al.*, 1993) have been developed, their use is cumbersome due to the need to denature and renature enzymes after SDS-PAGE (Cherif and Benhamou, 1990) or use overlay gels containing the substrate (Trudel and Asselin, 1989; Haran *et al.*, 1996). The system described herein uses electrophoresis at temperatures too cold for enzymes to act allowing incorporation of the substrate directly into the gel. Additionally, use of a citric acid buffer system (Cruickshank and Pitt, 1987) and incubation in malic acid before staining, produced crisper bands than conventional systems. Literature pertaining to the development of this system and the structure of fungal cell walls has been reviewed (Ch. II:4).

Chitin is insoluble in water and water soluble glycol chitin was adapted by Trudel and Asselin (1989) as a useful substrate. Various protein stains including silver nitrate, coomassie blue, and calcoflour white have been used to detect chitinases. The method reported herein adapts substrates and reagents used in the

technique of Trudel and Asselin (1989) to the system of polyacrylamide gel electrophoresis under native conditions for detection of pectin degrading enzymes without the need to use overlay gels developed by Cruickshank and Wade (1980). It is the aim of these experiments to detect and identify any chitinolytic enzymes produced by *T. koningii* and to determine if these are produced following colonisation of *S. cepivorum* infected onion roots by *T. koningii* where *S. cepivorum* hyphae have been seen to be in a state of degradation.

11.2. Materials and Methods:

Chitinolytic enzymes were produced in chitinolytic enzyme medium (Appendix B) containing either 1g/100 mls of purified crabshell chitin, 1g/100 mls of ground *S. cepivorum* sclerotia, or in distilled water containing 1g/100mls of ground *S. cepivorum* sclerotia. Two millilitres of these solutions was inoculated with a 0.5mm cube of purified crabshell chitin agar containing *T. koningii* incubated at 25 °C in 5ml McCartney bottles under static conditions for the periods specified.

To detect whether chitinases were produced by *T. koningii* in presence of *S. cepivorum* hyphae the antagonism bioassay experiment was performed (Appendix C), and resultant root segments were loaded into the wells of electrophoresis gels. Electrophoresis was performed to detect chitinolytic enzymes using glycol chitin as a substrate (Appendix E). The lack of specificity of this substrate made positive identification of some isozymes impossible due to chitinolytic enzymes of plant origin. In contrast, methylumbelliferone liberating substrates are more specific, but less replication is possible due to expense of these substrates. Electrophoresis gels contained 4-methylumbelliferyl-*N*-acetyl-B-D-glucosaminide [4-MU-(GlcNAc)], 4-methylumbelliferyl-B-D-*N,N'*-diacetyl-chitobioside [4-MU-$(GlcNAc)_2$] and 4-methylumbelliferyl-B-D-*N,N',N''*-triacetylchitotriose [4-MU-$(GlcNAc)_3$] as dimeric, trimeric and tetrameric substrates, respectively. Only hydrolytic reaction at the aryl glycosidic bond which links the 4-methylumbelliferyl residue (which is terminal) is detected by appearance of a UV fluorescent band.

Three onion root segments were loaded into each 4-methylumbelliferyl substrate containing gel type, the first was a healthy root segment, the second a root segment infected by *S. cepivorum* and the third a root segment infected by *S. cepivorum* then colonised by *T. koningii.*

For detection of chitinases produced by both fungi in the absence of non host chitin (constitutive chitinases) PAGE using glycol chitin as substrate was performed using crude culture fluid from *S. cepivorum* and *T. koningii* grown in 2.0 mls of 20% onion macerate culture at 25°C for 2 weeks.

11.3. Results:

Electrophoresis for detection of enzymes which attacked glycol chitin was performed. Two buffer systems were compared to find one which gave clearer results for chitinases from the same culture. Figure 11(A) is a gel incorporating a citric acid buffer while Figure 11(B) incorporated a sodium acetate buffer.

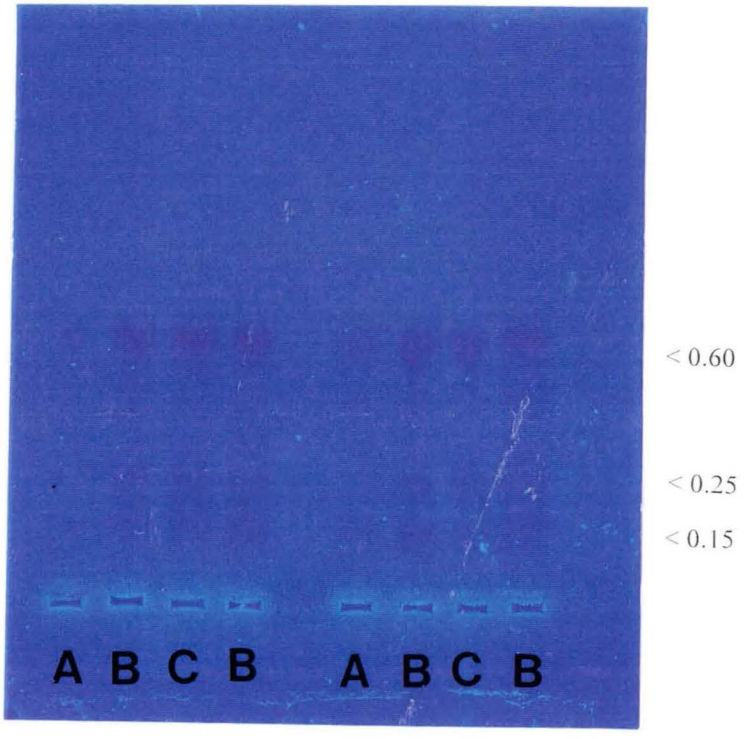


Figure 11(A): Polyacrylamide gel electrophoresis for detection of chitinolytic enzymes: The gel incorporated a citric acid buffer system and glycol chitin as substrate. Cultures of *T. koningii* were A) 2 months, B) 1 month and C) 1 week old. Three isozymes were evident R_f 0.15, 0.25, and 0.60.

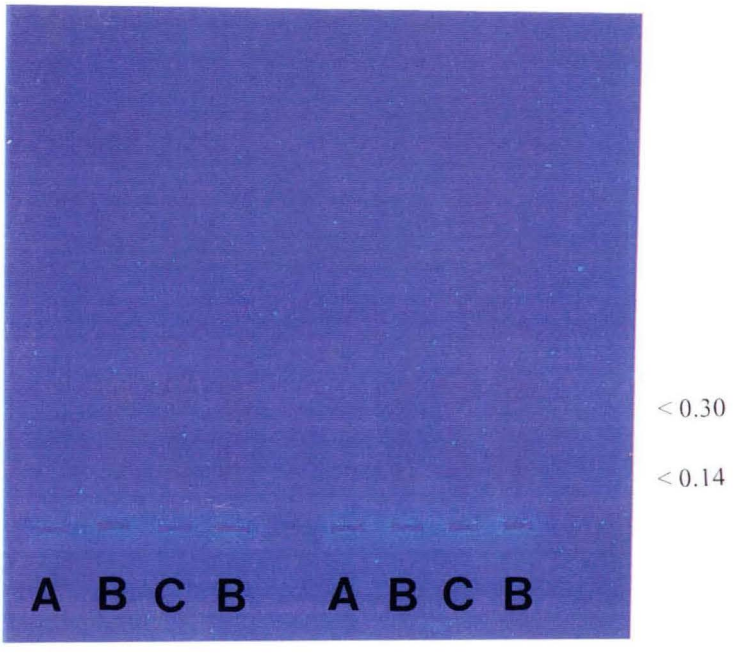


Figure 11(B): Polyacrylamide gel electrophoresis for detection of chitinolytic enzymes : The gel incorporated a sodium acetate buffer system and glycol chitin as substrate. Cultures are A) 2 months, B) 1 month and C) 1 week old. Two isozymes have been detected R_f 0.14, 0.30.

After development the gel shown in Figure 11(A) displayed at least three chitinolytic bands, possibly consisting of a number of smaller isozymes. These were of Rf 0.60, 0.25, and 0.15. Isozymes did not appear to vary with the age of the cultures. The citric acid buffer system used for Figure 11(A) provided better separation, and clearer detection of isozymes than the sodium acetate system used for Figure 11(B) and was adopted for future experiments.

Specific identification of the mode of activity of each enzyme was performed using 4-MU-(GlcNAc) as substrate. Two isozymes were detected at Rf 0.15 and Rf 0.24 (Figure 11.C). When 4-MU-$(GlcNAc)_2$ was used as substrate four isozymes were detected of Rf 0.15, Rf 0.24, Rf 0.46, and Rf 0.62 (Figure 11.D). When 4-MU-$(GlcNAc)_3$ was used as substrate two isozymes were detected of Rf 0.46 and Rf 0.62 (Figure 11.E).

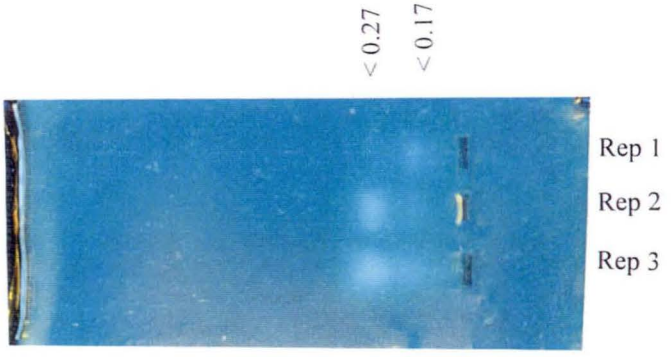


Figure 11(C) Polyacrylamide gel electrophoresis for detection of chitinolytic enzymes using4-methylumbelliferyl-*N*- acetyl- B-D- Glucosaminide as substrate: Photograph is under 344 nm UV. Two isozymes produced by *T. koningii* were detected of Rf 0.15 and Rf 0.24 are detected as fluorescent bands.

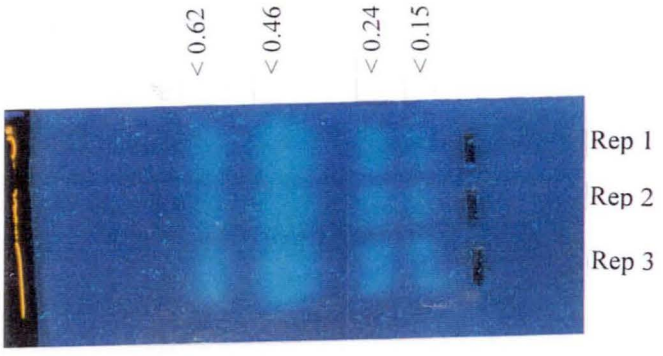


Figure 11(D).: Polyacrylamide gel electrophoresis for detection of chitinolytic enzymes using 4-methylumbelliferyl-B-D-*N,N'*-diacetylchitobioside as substrate: Four Isozymes produced by *T. koningii* were detected of R_f 0.15, 0.24, 0.46, R_f 0.62 as fluorescent bands.

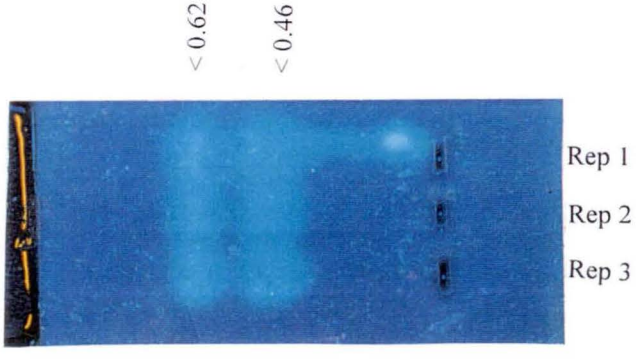


Figure 11(E): Polyacrylamide gel electrophoresis for detection of chitinolytic enzymes using 4-methylumbelliferyl-B-D-*N,N',N''*-triacetylchitotriose as substrate: Two isozymes produced by *T. koningii* R_f 0.46 and R_f 0.62 have been detected as fluorescent bands.

The systems established for detection of proteins which degrade crabshell chitin in liquid cultures were used to determine whether *T. koningii* produced the same proteins in degradation of *S. cepivorum* cell walls. When grown in culture containing ground *S. cepivorum* sclerotia as a nutrition source, *T. koningii* produced proteins of the same R_f as were detected when it was grown on purified crabshell chitin (Figure 11.F). An isozyme not previously detected using glycol chitin as substrate was detected of R_f 0.46 in all treatments. *T. koningii* has been able to use *S. cepivorum* sclerotia as a sole source of all nutrients. The most active isozymes from cultures containing ground *S. cepivorum* sclerotia are fainter than those from cultures where additional nutrients were added.

Antagonism bioassays (Appendix 2) whereby *T koningii* was allowed to colonise *S. cepivorum* infected root material before roots were sectioned and loaded into electrophoresis gels for detection of chitinolytic enzymes are shown in Figure 11(G). Comparison of bands produced in lane 10 (uninfected onion root) to those in lanes 2 to 9 indicate which zones may be attributed to indigenous plant chitinases. Additional bands were detected in lanes 6 to 9 (*T. koningii* colonised segments) of R_f 0.26, 0.40, 0.70, and 0.85. A faint band was also detected at 0.55 in lane 9 only. The isozymes of *T. koningii* have migrated slightly further in Figure 11(G) than in some previous replicates so that isozyme R_f values are 0.19, 0.29, 0.55 and 0.70. Bands with equivalent R_f to *T. koningii* chitinases detected in the root were of R_f 0.70, and 0.55. All other chitinolytic proteins from the root segments were not of similar R_f to *T. koningii* chitinolytic enzymes. In another replicate, where similar bands were active (Gel 35, not presented) the R_f 0.85 protein was detected in a healthy onion root segment. There is not sufficient evidence that the streaks detected in wells 2 to 4 from R_f 0.2 to 0.4 are *T. koningii* chitinolytic enzymes. It is not possible to tell whether the *T. koningii* R_f 0.26 chitinase is produced in the root, due to the mass of plant chitinase of similar R_f.

Electrophoresis for detection of chitinolytic enzymes produced constitutively (in absence of chitinous substrate) by both *T. koningii* and *S. cepivorum* in liquid onion macerate culture was performed using glycol chitin as substrate. No production of constitutively produced chitinolytic enzymes was detected by either fungus.

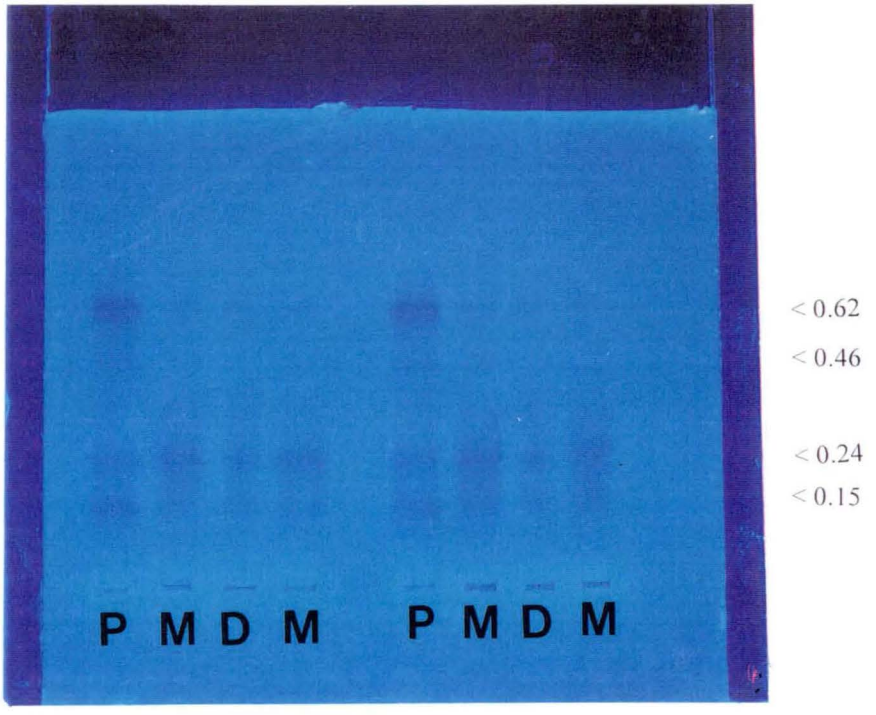


Figure 11(F): Polyacrylamide gel electrophoresis for detection of chitinolytic enzymes: Bands were detected in *T. koningii* culture containing purified crabshell chitin (P), ground *S. cepivorum* sclerotia with added mineral salts (M), and ground *S. cepivorum* sclerotia in distilled water (D). Bands of activity are detected in all lanes of R_f 0.15, 0.24, 0.46 and 0.62.

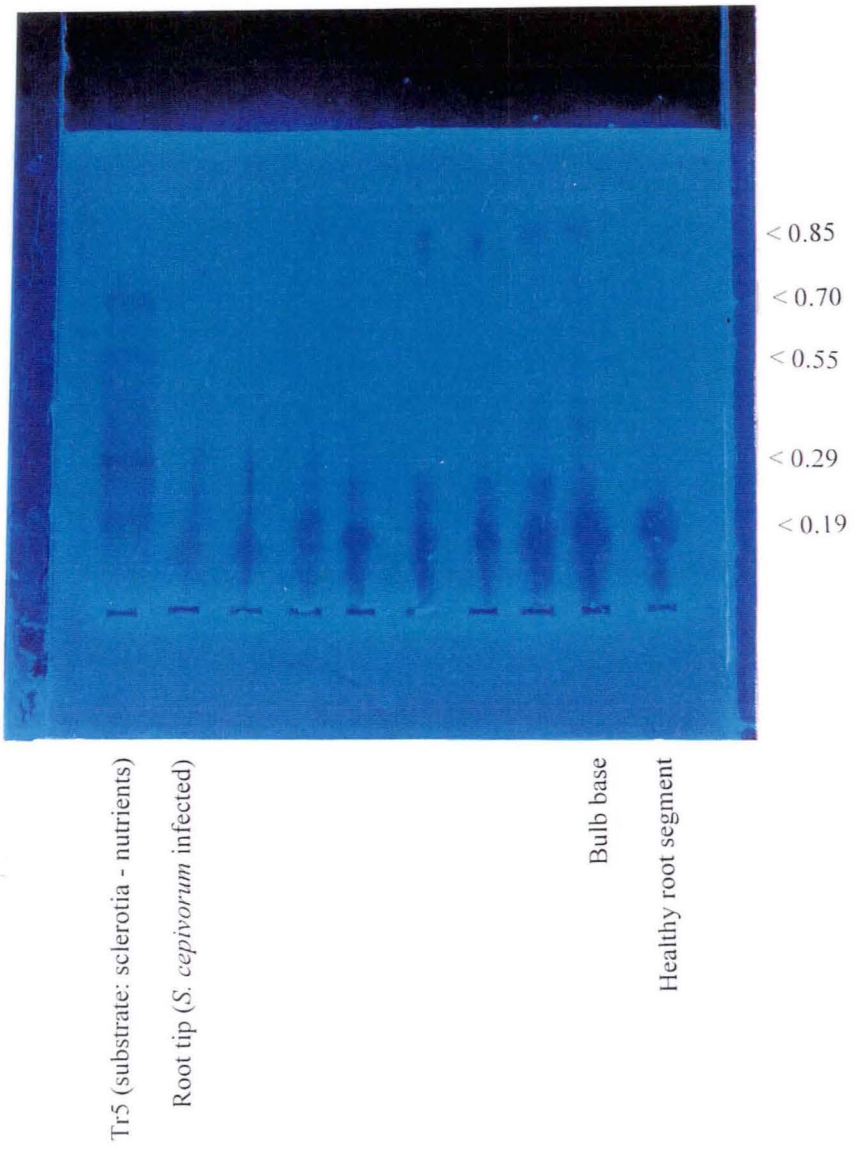


Figure 11(G): Polyacrylamide gel electrophoresis for detection of chitinolytic enzyme activity in the antagonism bioassay: Lane 1 shows the *T. koningii* chitinolytic enzyme complex (lane 10) shows activity produced by a healthy onion root segment (not colonised by *T. koningii* or *S. cepivorum*). The root tip (point of *S. cepivorum* inoculation) was placed in well 2, higher segments were colonised by *T. koningii.*

When *T. koningii* colonised onion roots which were infected by *S. cepivorum* , proteins of R_f 0.2-0.35 with activity on 4-MU-(GlcNAc) were detected (Figure 11.H), these were not detected in roots infected by *S. cepivorum* in isolation. *T. koningii* cultures with purified crabshell chitin as the carbon source produced chitinolytic enzymes of R_f 0.15 and 0.24, (Figure 11.C) the latter corresponds to the R_f of proteins in Figure 11(H).

Proteins with activity on 4-MU-$(GlcNAc)_2$ of R_f 0.0-0.2 were detected in onion roots infected by *S. cepivorum* but not in healthy onion roots (Figure 11.I.). In *S. cepivorum* infected roots which had been colonised by *T. koningii* , the R_f 0.0 to 0.2 proteins native to the plant were detected as well as isozymes of R_f 0.23-33 and 0.46. These two bands are of similar R_f to proteins detected using this substrate when *T. koningii* cultures were grown on purified chitin (Figure 11.D.). On further replications of the experiment, in which all three lanes were loaded with *S. cepivorum* infected roots colonised by *T. koningii*, isozymes of R_f 0.24 and 0.46 were detected as well as a band of activity R_f 0.0-0.1 probably of *S. cepivorum* or plant origin (Figure 11.K).

In the first antagonism experiments using 4-MU-$(GlcNAc)_3$ as substrate, no isozymes of similar R_f to those produced when *T. koningii* was grown in chitinolytic enzyme medium were detected in infected roots colonised by *T. koningii.* However, after 12 hours further incubation, pale yellow bands of R_f 0.0 to 0.3 appeared only in the *T koningii* colonised root segment (Figure 11.J). In three further replications of the antagonism bioassay presented in Figure 11(J) isozymes of Rf 0.46 were detected immediately after incubation, each lane contained a root segment colonised by both fungi (Figure 11.L).

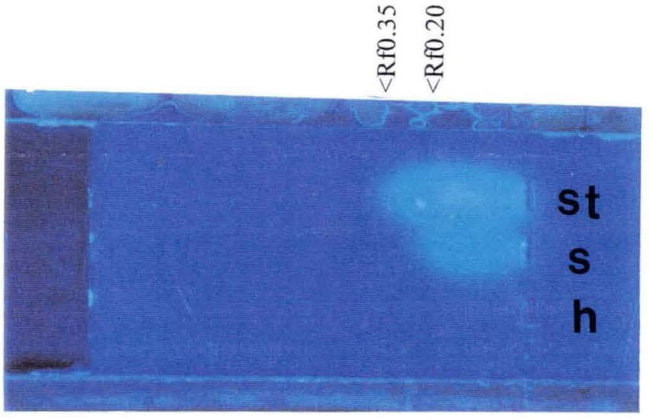


Figure 11(H): Electrophoresis gel containing the substrate 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide for detection of chitinolytic enzymes: No chitinolytic enzymes were detected in healthy roots (h), a band of R_f 0.00-0.2 was detected when the root was infected by *S. cepivorum* (S), when *T. koningii* was also present (ST) a continuous zone of activity was detected from R_f 0.0 to 0.35.

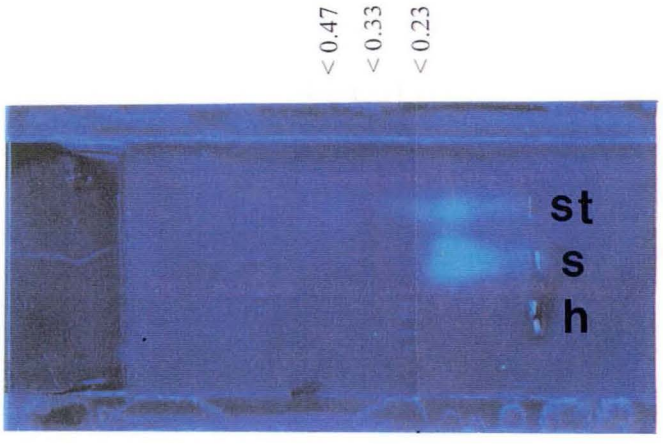


Figure 11(I): Electrophoresis gel containing the substrate 4-methylumbelliferyl-*N*-acetyl-B-D-*N,N'*-diacetylchitobioside: No activity of chitinolytic enzymes was detected in healthy roots (h), bands of activity (R_f 0.0-0.2) were detected in *S. cepivorum* infected roots (S), in *S. cepivorum* infected roots colonised by *T. koningii* (ST) additional bands of R_f 0.23-33, and 0.47 were detected.

Figure 11(J): Electrophoresis gel containing the substrate 4-methylumbelliferyl-B-D-*N,N',N''*- triacetylchitotriose: No activity was detected until 12 after initial incubation

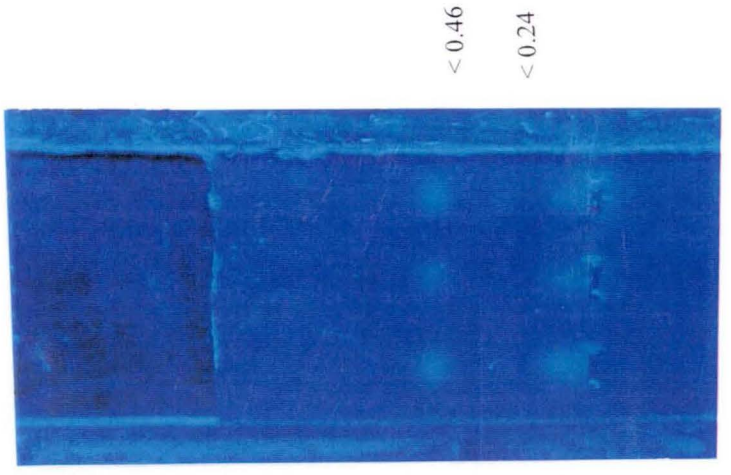


Figure 11(K) Electrophoresis gel containing the substrate 4-methylumbelliferyl-*N*-acetyl-B-D-*N,N'*-diacetylchitobioside: All three lanes were loaded with root segments infected by *S. cepivorum* which were colonised by *T. koningii* . In each replicate isozymes of R_f 0.24 and 0.46 have been detected.

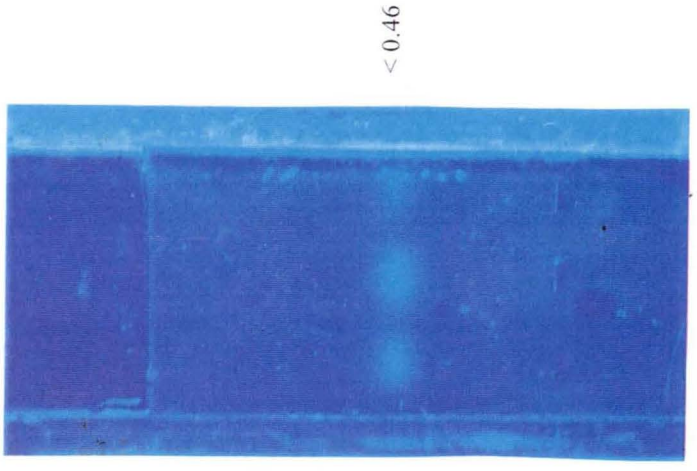


Figure 11(L): Electrophoresis gel containing the substrate 4-methylumbelliferyl-B-D-*N,N',N''*- triacetylchitotriose: All three lanes were loaded with root segments infected by *S. cepivorum* which were colonised by *T. koningii* isozymes of R_f 0.46 were detected in each lane.

11.4. Discussion.

Structural changes of *S. cepivorum* hypha in presence of *T. koningii* hyphae implied that cell walls were dissolving in antagonism. Lack of detection of antimicrobial compound production by Tr5 (Metcalf, 1993) led to studies to detect enzymes which degraded the major fungal cell wall polysaccharide chitin. Studies which have investigated mycoparasitic interactions have examined degradation of cell wall polysaccharides in liquid culture (de la Cruz *et al.*, 1993; Garcia *et al.*, 1994; Haran *et al.*, 1995; Shirmböck *et al.*, 1994). The series of chitinases produced in direct mycoparasitic confrontation have been recently characterised (Haran *et al.*, 1996). Other workers have conducted histological studies of effects of chitinolytic enzymes on host cell wall dissolution (Benhamou and Chet, 1993; Benhamou and Chet, 1996). Production of chitinolytic enzymes by *Trichoderma* spp. during mycoparasitic interactions within host tissue has been the subject of few if any studies.

Through modification of existing techniques, a chitinolytic enzyme polyacrylamide gel electrophoresis system was developed by combining a citric acid buffer system (Cruickshank and Pitt, 1987) with glycol chitin substrate (Trudel and Asselin, 1989). A sodium acetate buffer system (Di Pietro *et al.* , 1993) was shown to be inferior (Figure 11.B) to one based on citric acid (Figure 11.A). Gels were run at temperatures too cold for the enzymes to act on the substrate as they migrated (2-4°C), allowing incorporation of the substrate into the original gel, and making overlay gels unnecessary (overlay gels were found to be cumbersome and of poor resolution, results not presented). Following electrophoresis, the gel was incubated in 0.1M malic acid at 25°C, causing a gradual pH change in the gel, allowing each enzyme to act as the pH passed through its optimum range for activity. Calcoflour white (Maeda and Isheda, 1967; Trudel and Asselin, 1989) successfully stained undegraded glycol chitin in the gels. An alternative approach published during preparation of this thesis detected total chitinolytic enzyme activity by addition of both 4-MU-(GlcNAc) and 4-MU-$(GlcNAc)_2$ to overlay gels. And recognised specific proteins using polyclonal antibodies (Haran *et al.*, 1996).

The identical isozymes produced by *T. koningii* in degradation of purified crabshell chitin and ground *S. cepivorum* sclerotia may have useful implications for soil amendments. If the chitinolytic enzymes used to attack the pathogen are those which degrade crabshells, addition of crabshell chitin to the soil may stimulate antagonistic activity of *T. koningii* (Further examined in Chapter V:13 & 14).

Further investigation of chitinolytic enzyme activity was made using specific substrates. Tronsmo and Harman (1993) detected chitinolytic enzymes of *T.*

harzianum using these substrates following native or SDS PAGE by use of overlay gels. Detection of chitinase activity is based on the liberation of fluorescent 4-methylumbelliferone (4-MU) by the hydrolytic reaction at the aryl glycosidic bond between acetylglucosamine residues. Other bonds in the substrate may be cleaved, but only cleavage of the 4-MU group is detected.

The chitinolytic isozymes of R_f 0.15 and 0.24 detected using 4-MU-(GlcNAc) (Figure 11.C), four chitinolytic isozymes R_f 0.15, 0.24, 0.46 and 0.62 detected using 4-MU-$(GlcNAc)_2$ (Figure 11.D) and two chitinolytic isozymes R_f 0.46 and 0.62 detected using 4-MU-$(GlcNAc)_3$ (Figure 11.E) closely agree in R_f with the bands detected using the non specific substrate glycol chitin (Figure 11.F).

Tronsmo and Harman (1993) considered that 4-MU-(GlcNAc), 4-MU-$(GlcNAc)_2$ and 4-MU-$(GlcNAc)_3$ specifically detect glucosaminidases, chitobiosidases and endochitinases respectively. Studies using lysozyme (EC. 3.2.1.17) have shown that lysozymes binding subsites B, C and D with 4-MU-$(GlcNAc)_3$ and 4-MU-$(GlcNAc)_2$ were in common (Yang and Hamaguchi, 1980; Yang *et al.*, 1981). The protein being active against the aryl glycosidic bond linking the methylumbelliferyl group to the *N*--acetyl-glucosamine chain of both substrates. The activity of proteins of identical R_f on more than one substrate as shown in this study would be consistent with a similar model.

The lack of activity of the *T. koningii* chitinolytic proteins R_f 0.46 and 0.62 on 4-MU-(GlcNAc) precludes the possibility that they are glucosaminidases, whilst activity on both 4-MU-$(GlcNAc)_2$ and 4-MU-$(GlcNAc)_3$ suggests no preference for liberation of chitobiose, therefore these proteins are endochitinases. They cannot act on less than trimeric substrate.

The activity of chitinolytic isozymes of R_f 0.15 and 0.24 on 4-MU-$(GlcNAc)_2$ suggests that the proteins are chitobiosidases. However chitobiosidase activity on 4-MU-$(GlcNAc)_3$ would liberate two products; chitobiose and 4-MU-(GlcNAc). As it is known that the two proteins can hydrolyse 4-MU-(GlcNAc), they do not appear to have chitobiosidase activity. The lack of activity on 4-MU-$(GlcNAc)_3$ suggests that the proteins are not endochitinases as these can cleave randomly at internal sites. The isozymes also appear unlikely to be glucosaminidases, these would be expected to be capable of activity on the terminal 4-MU group of 4-MU-$(GlcNAc)_3$. The definitions of these three types of chitinolytic enzymes are not adequate to explain the demonstrated activity of these two proteins. One definition which fits more closely is chitobiase (EC. 3.2.1.30). Zikakis and Castle (1988) describe chitobiases as glucosaminidases which hydrolyse chitobiose to GlcNAc, and can also hydrolyse chitotriose and chitotetraose, but at decreasing rates as the number of GlcNAc residues increases. This definition would fit the demonstrated activity of these proteins on 4-MU-(GlcNAc) and 4-MU-

(GlcNAc)$_2$. The weight of evidence suggests that the two proteins had no activity on 4-MU-(GlcNAc)$_3$ with the exception of faint activity detected after 12 hours incubation in Figure 11(J). The term chitobiase is tentatively used to refer to these proteins hereafter.

T. koningii produces pectinesterase and polygalacturonase which degrade pectin as a carbon source as it colonises the diseased onion roots (Ch. IV.10). Chitin in the hypha of *S. cepivorum* is another available carbon source and production of chitinolytic enzymes by *T. koningii* during colonisation of infected roots provides some explanation for the observed septal detachment and hyphal degradation seen in *S. cepivorum* infected onion roots when *T. koningii* is present (Ch. IV.9). As no chitinolytic enzymes were detected in 20% onion medium in which *T. koningii* was grown it seems reasonable to assume that chitinases produced constitutively by *T. koningii* are of insufficient quantity to be detected without concentration. Chitinolytic enzymes are known to be produced constitutively by *Trichoderma harzianum* prior to antagonism of *Sclerotium rolfsii* (Haran *et al.*, 1996) however Haran *et al.* concentrated these proteins prior to electrophoresis and detection. Chitinolytic enzymes of *T. koningii* detected in the antagonism bioassay may be produced constitutively in processes such as hyphal tip extension, however the observed increase in enzyme production to a concentration which was detectable is likely to be induced.

Electrophoresis of proteins from onion roots from the antagonism bioassay has shown that several chitinolytic isozymes not attributable to plant or *S. cepivorum* origin were present (Figure 11.G). These were of similar R_f to the*T. koningii* endochitinases produced in culture which in this gel were detected at R_f 0.70 and 0.55. Specific substrate systems made isozyme identification easier. Bands of R_f 0.0-0.2 with activity on 4-MU-(GlcNAc) and 4-MU-(GlcNAc)$_2$ were produced in *S. cepivorum* infected roots. These were not present in non-infected roots and they may have a role in hyphal tip extension by *S. cepivorum*, or *S. cepivorum* may produce additional chitinolytic enzymes when placed under physiological stress, such as in the early stages of mycoparasitic activity. The stress of infection could also induce chitinolytic enzyme production by the plant as a defence. Mycorrhizal Zygomycetes are known to trigger chitinase synthesis in *Allium porrum* roots (Spanu *et al.*, 1989), though in pea roots production of some chitinase isozymes may cease when the plant is under nutrient stress, in order to encourage mycorrhizal colonisation (Dassi *et al.*, 1996).

In infected roots where *T. koningii* was present, additional activity on 4-MU-(GlcNAc) and 4-MU-(GlcNAc)$_2$ was detected of R_f 0.23 - 0.33. This activity may originate from the R_f 0.24 chitobiase. Activity of R_f 0.46 detected using 4-MU-(GlcNAc)$_2$ (Figures 11.I. and 11.K) and 4-MU-(GlcNAc)$_3$ (Figure 11.L) is likely to

be the *T. koningii* R_f 0.46 endochitinase. The R_f 0.62 endochitinase was not detected in infected root tissue using 4-MU-$(GlcNAc)_2$ or 4-MU-$(GlcNAc)_3$. However, a faint band with general chitinolytic activity of similar R_f to this isozyme was detected in Figure 11(G). Figure 11(F) showed that this protein was less active in degradation of *S. cepivorum* sclerotia than purified crabshell chitin, and it is possible that concentration of this protein may be too low for detection. Alternately the isozymes may be produced in a series, and the protein may not have been induced when the root was sampled. Certainly the related species *Trichoderma harzianum* is known to produce a changing series of glucosaminidases in lysis and degradation of living *Sclerotium rolfsii* mycelium in which some proteins increase and then diminish activity (Inbar and Chet, 1995). *T. harzianum* is also known to produce a differing series of chitinolytic enzymes during antagonism of *S. rolfsii* to those produced in antagonism of *Rhizoctonia solani.* (Haran *et al.*, 1996).

While the evidence shows that chitobiase (R_f 0.24) and endochitinase (R_f 0.46) were produced when *T. koningii* colonised infected tissue, this is not evidence that chitinolytic enzymes are the agents causing death of *S. cepivorum* hyphae. It has been suggested that chitinolytic enzymes serve to weaken the cell wall of the pathogen which in turn facilitates diffusion of antibiotics into the cytoplasmic membrane, and that chitinases later play a role in the saprophytic phase of growth (Worasit *et al* ., 1994). However Metcalf (1993) failed to identify antibiotic production by this isolate of *T. koningii* and further examination of Tr5 by a specialist in *Trichoderma* antimicrobial compounds concluded that no koninginins, 6-pentyl-a-pyrone or harzianopyridone were detectable (Dr Stephen Parker, HortResearch, Ruakura, NZ; analysis performed by methods of Parker *et al.*, 1995a & b). There have been other reports of antagonistic *Trichoderma* spp. isolates which produced chitinolytic enzymes but no detectable antibiotics (Chet and Baker, 1980; Chet and Baker, 1981) and some antagonists of *S. cepivorum* produced no known antifungal metabolites (Jackson *et al.*, 1991b). If Tr5 does produce antibiotics it is possible that they are produced at other stages of physiological development to those tested (eg. *T. harzianum* isolate 73 produced increased quantities of antibiotics on ageing [Ghisalberti and Sivasithamparam, 1991]).

Chapter IV: Interactions between *T. koningii* and *S. cepivorum.*

12.0: Development of a *Trichoderma* Selective Medium:

12.1. Introduction:

Much of this study has examined the importance of establishment of *Trichoderma* spp. on onion root surfaces. It was therefore necessary to develop a selective medium which could be used to determine the proportion of onion roots that are colonised. Several selective media have been developed for the isolation of *Trichoderma* propagules from soil. Some groups (Liu and Baker, 1980; Papavizas, 1982) have modified a medium developed by Martin (1950) which incorporated rose bengal and streptomycin sulphate acidified to pH 4.0 to suit the soil ecosystem and *Trichoderma* strain under study. Other workers have developed PDA (Kok *et al.*, 1996) and V8 juice (Papavizas and Lumsden, 1982) media with addition of fungicides and antibiotics. Elad *et al.* (1981) found p-diethylaminobenzenediazo sodium sulphonate (Dexon 60% WP Farbenfabrik Bayer A. G., Germany) enhanced growth and sporulation of *Trichoderma* spp.. Harman *et al.* (1989) used a medium composed of mineral salts and sucrose amended with chlorotetracycline, and the fungicides captan and ronilan. Monosaccharide carbon sources have predominantly been used. The ability of Tr5 to degrade cellulose as a sole carbon source (Wood and Kellogg, 1988; Erikssen *et al.*,1990) provided an opportunity to exclude other soil microbes which are less able to use cellulose, in particular *Rhizopus* spp. which can overgrow *Trichoderma* spp. (Papavizas and Lumsden, 1982).

Several developmental stages of the medium have been used in different chapters of this study. The goal was to preferentially isolate *Trichoderma* spp. and in particular isolate Tr5 from onion roots taken from non sterile soil.

12.2. Materials and Methods:

The basal medium was mineral salts solution (Appendix B.7). All quantities are given per Litre. Table 12(A) lists amendments to this solution for each developmental stage of the selective medium. Antibiotics, fungicides and rose bengal were added after the medium had cooled to around 60°C in 10mls of a sterile distilled water solution without filter sterilisation.

Table 12(A):Amendments to 1L of mineral salts solution to make various developmental stages in RASP selective medium.

Period of use	1993	1993	1993	1994/5	1995/6
Name	DCS	RBSC	RANS	RAS	RASP
dextrose	10g				
cellulose		10g	10g	5g	5g
streptomycin sulphate	0.1g	0.1g	0.1g	0.05g	0.05g
cycloheximide	0.07g				
chlorotetracycline	0.05g				
nystatin			40µg		
rose bengal		0.03 g	0.03g	0.016g	0.016
allisan (dichloran)			4mg	4mg	4mg
previcur (propamocarb)					3.2ml
pH	7	7	7	4	4
Agar	20	20	20	30	30

note: -Cellulose was purified cellulose powder (Sigma)
-Allisan syn- Dichloran/Botran (Schering Pty Ltd)
-Previcur = 600g/L propamocarb (Schering PtyLtd)
-pH adjusted using HCl.

To assess the pH for optimum growth, RAS medium was buffered to pH 2, 3, 4, 5, 6, and 7 using HCl. Three replicates of each pH treatment were performed. A 5mm cube of mineral salts agar with no added carbon source was placed in the centre of each plate. Three colony diameter measurements were made of each replicate at 48 and 96 hours after inoculation from which an average was taken.

12.3. Results:

The first attempt at developing a selective medium (DCS) was a failure. Growth of *Trichoderma* spp. was limited to sporulation on the plated material if any growth occurred at all, no contaminants grew. Variation of antibiotic components showed cyclohexamide and chlortetracycline inhibited *T. koningii* therefore these were excluded.

In RBSC medium, the addition of rose bengal (30mg/L) successfully limited radial growth of fungi including *Aspergillus* and *Penicillium* and enhanced sporulation for identification of *Trichoderma* isolates. The polysaccharide cellulose replaced dextrose as the carbon source. This step reduced isolation of undesired bacteria and fungi and decreased vigour of *Rhizopus* spp.

In RANS medium the fungicide Allisan was added at a rate of 4mg per litre. This step reduced contamination with *Penicillium* , *Botrytis* and *Rhizopus* spp. The concentration of rose bengal was found to be high enough to cause lysis of Tr5 hyphal tips before sporulation, and after experimentation was lowered to 16mg/L. Nystatin did not enhance selectivity and was not included in RAS. Tr5 radial growth

was greatest at pH 4.0. (Figure 12.B) Hyphae did not spread beyond the original inoculum at pH 2.0. pH 4.0 was selected for future work.

Penicillium spp. and some lower fungi still occasionally formed colonies on the medium. The final amendment of 3.2ml per Litre of Previcur eliminated lower fungi and further reduced colonisation by *Penicillium* spp. No restriction of *Trichoderma* isolation efficiency was observed.

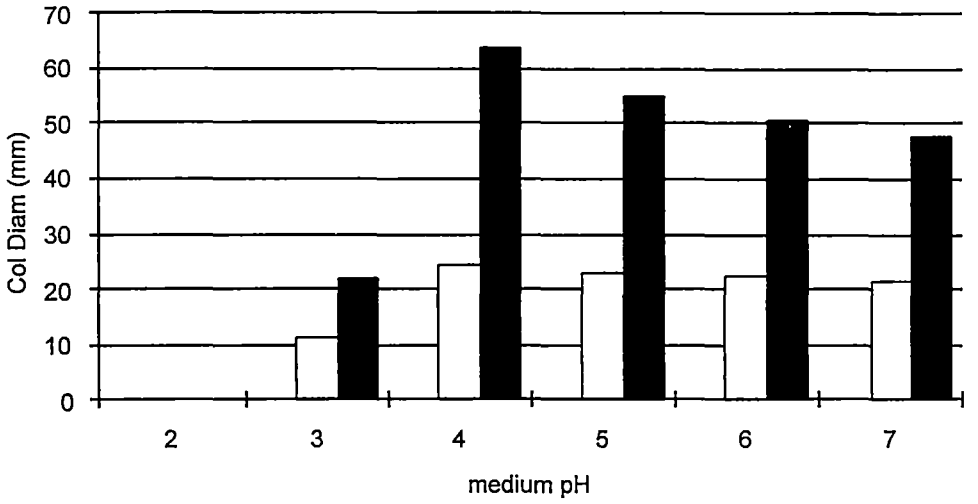


Figure 12(B): Radial growth of *T. koningii* added to plates of RAS agar buffered at pH 2, 3, 4 ,5 , 6, and 7 measured at 48 (light) and 96 (dark) hours.

12.4. Discussion.

The need to specifically adapt selective media to the microbial ecosystem and *Trichoderma* spp. under study is exemplified by inhibition of Tr5 by chlortetracycline which was a component of the selective media of Papavizas and Lumsden (1982), Chao *et al.* (1986) and Harman *et al* (1989). Optimisation of media pH was important both to enhance Tr5 growth and to suppress some microbial competitors (Martin, 1950). pH may also influence ability to utilise cellulose as a carbon source. *T. koningii* (Tr5) produces separate isozymes of exocellobiohydrolase and ß-glucosidase (data not presented) which degrade cellulose. Cellulase isozymes of some microbes have specific and sometimes separate pH optima (McCarthy, 1987). pH 4.0 was a desirable H+ concentration both for enhancement of Tr5 growth and suppression of some competitors (Martin, 1950). Three important selective ingredients in RASP (Rose Bengal, Streptomycin and acidification) were present in the medium of Martin (1950). Allisan has been previously used in selective media for inhibition of *Aspergillus* spp. (Bell and Crawford, 1967). Previcur was recommended for suppression of Oomycetes and *Penicillium* (Askew and Liang, 1993). Selective enhancement by cellulose

amendment has been previously used in one *Trichoderma* selective medium (Montencourt and Eveleigh, 1977).

Incubation on RASP medium at 25°C reliably enumerated *Trichoderma* spp . from soil or onion roots. It provides a useful tool for ecological studies. Tr5 possibly has a competitive advantage over some other *Trichoderma* spp. when grown on this medium (Chapter VI:16).

Chapter V: Field amendment methodologies.

13.0: The effect of nutrient amendments and application techniques on establishment of *T. koningii* (Tr5) in field soils: 1993/4 trials.

13.1. Introduction.

Lacey and Wong (1991) previously demonstrated 60% reductions in field incidence of *S. cepivorum* infections using *Trichoderma* spp. when inoculum was amended as dried spores and mycelium raised on white millet. Strategies for soil amendment of biological control agents have been discussed in Chapter 2. In addition to seed dressings (Harman *et al.*, 1981) carriers include vermiculite/wheat/bran (Lewis *et al.*, 1991), peat/bran (Wolffhechel and Funck-Jensen, 1992), corn cobs, peanut hulls, soy fibre, crabshells (Lewis *et al.*, 1996), processed manure (Kok *et al.*, 1996), hydrogel beads (Kuek *et al.*, 1992), inert clay carriers (Lewis and Fravel, 1996), alginate pellets (Knudsen *et al.*, 1991) dried fermented biomass (Papivizas *et al.*, 1984). Fluid drill sowing which injects seed into the furrow in a gel, has been previously used for amendment of *Trichoderma* spp. by Conway (1986) who recommended investigation of organic amendments in gels. The aims of these trials were to :

(1) Compare amendment using actively growing mycelium as opposed to dormant spores.

(2) Evaluate amendment using in-furrow-sprays and fluid drill sowing (Currah *et al.*, 1974).

(3) To investigate amendment of Tr5 with complex carbon sources to determine whether they would provide a selective advantage for establishment in the soil.

(4) Comparison of biocontrol strategies to the commercial fungicide practice.

13.2. Materials and Methods.

Three potential field sites were examined. Nine soil samples were taken from each site and numbers of sclerotia were assessed by wet sieving of 200g samples (see Appendix F). The Bonney and Goodwin sites were assessed to contain relatively evenly distributed inoculum densities, and had been sites of considerable loss in previous seasons. Soil pH was assessed in 5:1 distilled water to soil suspension and was determined to be 6.30 at Bonney's and 6.37 at Goodwin's.

13.2.1. Inoculum production.

Millet inoculum and liquid fermented inoculum were prepared as described in Appendix A. Carbon sources added to the fluid drilling gel (10g/L) were purified microgranular cellulose (Treatment H), crabshell chitin (treatment I), and wholemeal flour (Treatment K), and cellulose and chitin (Treatment J, 5g/L each). Spore suspensions for in furrow sprays were prepared by adding 2 litres of distilled water to 5L flasks of millet inoculum at sporulative stage which were then vigorously shaken. The suspension of millet and spores was passed through a 0.1mM sieve to yield a green solution of spores. 0.5g/L of Phostrogen (a commercial plant food; Phostrogen Australia PtyLtd) was added to the suspension before spraying.

13.2.2. Inoculum Density:

The number of colony forming units in each of the amendment materials was assessed by serial dilution plating where 1 gram (or millilitre) of material was added to 9mls of sterile distilled water and the resulting suspension was diluted to 10^{-6}. 0.1ml of each serial dilution was added to a plate of potato dextrose agar (see Appendix B; media) using sterile technique and spread over the agar. After 24hrs incubation at 25_C the plates were examined for colonies of *Trichoderma* .

13.2.3. Treatments:

The treatment list and rates of application are summarised in Table 13(A). Tr5 was applied using dried or wet (undried) millet (B,C) fluid drill culture (H,I,J,K) or in furrow sprays (L,M,N,O,P). Control treatments included; untreated (A), seed dressing of cellulose and chitin without Tr5 spray (F), Flocal seed dressing (D), Flocal + cellulose and chitin seed dressing (G), and Flocal seed dressing with Sumisclex in Lime Super (E), the commercial fungicide recommendation for 1993/4 season. Control treatments where autoclaved millet or autoclaved Tr5 grown on millet were amended to determine whether any reductions in infection were due to the amendment of organic matter or due to Tr5 were not included as the size of the infested area was a consideration and it has been suggested that controls of this type do not verify biocontrol efficacy (Baker *et al.*, 1984). Fluid drill sowing was simulated by mixing 120 seeds in 60 mls of liquid culture in a syringe which was injected as a continuous bead along the furrow. In-furrow spraying was simulated using a hand pump action sprayer.

Treatments were arranged in a randomised complete block design. Each treatment was replicated six times, each plot was 1.6m wide (commercial bed spacing) and 60cm long, containing 8 rows, 20 cm apart. One hundred and twenty seeds were added to each plot with the intention of thinning all plots to 100 (one seed every 4cm). The percentage of emerged seedlings which became infected by *S. cepivorum* was analysed by one way analysis of variance and LSD (0.05) test.

Table 13(A). Treatment list for field trials in the 1993/4 season.

Treatment	Tr5 Millet	Fluid drill gel	In Furrow spray	Fungicide
A Untreated				
B Dry Millet	110kg/ha			
C Wet Millet	220kg/ha			
D Flocol sd				+
E Flo/Sumi				++
F sd Cl + Ch				
G Flo sd Cl+Ch				+
H fd Cellulose		600L/ha		
I fd Chitin		600L/ha		
J fd Cl + Ch		600L/ha		
K fd Flour		600L/ha		
L ifs Cellulose			600L/ha	
M ifs Chitin			600L/ha	
N ifs Cl + Ch			600L/ha	
O ifs flour			600L/ha	
P ifs -			600L/ha	

notes to Table 13(A):

-No amendments to untreated control
-Seed source was regular creamgold supplied by Vecon PtyLtd, no chemical treatment
-millet inoculum prepared as per methods in appendix XX
-Flocol SD= seed dressing of Flocol applied at 50ml/kg of seed.
-Flo/Sumi= Flocol seed dressing with Sumisclex in lime/superphosphate carrier consisting of 4kg Sumisclex and 200kg of lime/superphosphate (applied at a rate of 200kg/ha).
-Cl = cellulose , Ch = chitin from crabshells
-fd = seed and Tr5 sown by the fluid drill method with the designated carbon source
-ifs= seed sown as normal with seed dressing carbon source 1.0g, phostrogen 0.5g per kilogram of seed and phostrogen, Tr5 added as a spore spray.

The trial was sown at Bonney's site on 22/7/1993 and at Goodwin's site on 3/8/1993. Emergence and disease incidence were recorded at fortnightly intervals. Diseased seedlings were taken to the laboratory and examined to confirm the cause of death.

At Bonney's site, death due to *S. cepivorum* infection was very rarely encountered until 26/10/1993 while at Goodwin's *S. cepivorum* infection was prevalent from 30/9/1993. The percentages of disease presented are taken from these dates. Infected seedlings were marked using white nursery tags to prevent double

counting. The trials were destructively sampled on 24/2/94 (Bonney's) and 7/3/95 (Goodwin's), at the start of autumn.

13.3. Results.

Sclerotial density in each of nine 200g samples from the two trial sites was:
Bonney's: 23, 10, 12, 9, 11, 14, 20, 11, 13 ; Average 13.7 (68/kg)
Goodwins: 21, 220, 21, 8, 7, 12, 22, 10, 98; Average 46.6 (233/kg)

The density of *Trichoderma* propagules in most amendment materials was in the range of 10^5 to 10^6 colony forming units per gram (Table 13.B). Millet inoculum added to soil and used to make a spore spray was from the same supply. The cultures used at Goodwin's had been inoculated on the same day but were sown two weeks later. In general the fermented liquid cultures used at Goodwin's site contained more colony forming units than those used at Bonney's. The inoculum density of the liquid chitin and cellulose + chitin cultures used at Bonney's site were far below the range of the other cultures.

Table 13(B): The number of Tr5 colony forming units (spores and mycelium) in 1 gram of the amendment materials tested in the 1993/4 trials.

Material	Bonneys (CFU)	Goodwins (CFU)
Millet	5.6×10^5	5.6×10^5
In-furrow spore spray	2.5×10^6	2.5×10^6
FD Cellulose	3.2×10^6	6.3×10^6
FD Chitin	6.0×10^2	5.6×10^5
FD Cellulose and Chitin	3.9×10^3	1.1×10^6
FD Flour	2.1×10^6	1.3×10^6

Note: In FD Cellulose cultures, conidia appeared to be the main propagule whereas mats of mycelium including chlamydospores were the main propagule in chitin cultures. FD Flour and Cl + Ch cultures were a blend of the two.

Emergence (see Table 13.C) in the presence of Tr5 millet inoculum was not significantly different to the untreated control. The in-furrow spray without nutrients (P) provided good emergence at both sites (significantly better at Bonney's only), other in-furrow spray treatments (L,M,N,O) displayed emergence similar to the untreated control (A) and non inoculated dressed seed (F). At Goodwin's site fluid drill sowing provided poor emergence (treatments H, I, J and K), significantly lower than in the untreated control. However at Bonney's, emergence in these treatments

was similar to the untreated control. Flocal seed dressings used alone (D) and together with Sumisclex/Lime/Super (E) provided good emergence at both sites.

The overall level of disease at Bonney's site (Table 13.C) was relatively low (12.94% infection in the untreated control) compared to Goodwin's site (70.09% infection in the untreated control). At Bonney's site only Tr5 treatments I (fluid drill sown Tr5 with chitin) and N (in furrow spray with cellulose and chitin seed dressing), and fungicide treatments E (Flocal seed dressing with Sumisclex/lime/super) and G (Seed dressing of Flocal with cellulose and chitin) provided significant reductions in disease in comparison to the untreated control. At Goodwins site all treatments except F (seed dressing of cellulose and chitin), K (fluid drill sown Tr5 with flour), and M (in furrow spray with seed dressing of chitin) successfully reduced disease. Reductions varied from 21.82% (I: fluid drill sown Tr5 with cellulose and chitin) to 80.04% (E: Flocal seed dressing with Sumisclex/lime/super). The best disease reduction in a biological control treatment in Goodwin's trial was provided by application of wet (40.09%) and dried (39.65%) millet. There was no significant difference in disease between treatments of dried or wet millet in either trial. Fluid drill and in furrow spray applications on the whole provided similar (Bonney's) or less (Goodwin's) disease control than the millet treatments. Fluid drill sowing of Tr5 using chitin, which provided the best biocontrol reduction in disease in Bonney's trial (57.8%), did not perform as well in Goodwin's trial with a reduction of 22.45%.

The commercially successful treatment of Flocal seed dressing with Sumisclex/Lime/Super (E) provided a 64% reduction in disease at Bonney's site which was not significantly better than several of the biocontrol treatments (B,H,N and O). However, at Goodwin's site, this treatment was singularly effective with a disease incidence decrease of 80%. The level of disease suppression provided by Tr5 millet treatments was in a similar range to that provided by Flocal seed dressings (D) alone.

Table 13(C) The number of seedlings emerged, percentage of infection, and reduction in disease incidence by comparison to the untreated control in Bonney's and Goodwin's trials.

	Bonney's Trial			Goodwin's Trial		
	Emergence	% Infect	% Inf red	Emergence	% Infect	% Inf red
A)Untreated	80.6	12.94 a		93.8	70.09 a	
B)Dry Millet	96.6	7.62 abcde	41.1%	93.6	42.29 de	39.65%
C)Wet Millet	95.6	8.48 abcd	34.4%	98.1	41.99 e	40.09%
D)Flocal SD	103.3 *	8.82 abcd	31.8%	101.5	40.94 e	41.58%
E)Flo/Sum	96.8	4.14 de	64.4%	106.3	13.98 f	80.04%
F)SD Cl+Ch	75.5	9.69 abcd	25.1%	102.1	58.36 abc	16.72%
G)FlsdCl+Ch	80.3	2.50 e	80.6%	94.3	40.09 e	42.78%
H)FD Cl	82.8	7.50 abcde	42.0%	62.3 #	54.72 bc	21.91%
I)FD Ch	80.0	5.46 cde	57.8%	75.1 #	54.35 bc	22.45%
J)FD Cl+Ch	86.1	9.47 abcd	26.8%	55.8 #	54.97 bc	21.82%
K)FD Flour	71.6	10.64 abc	17.7%	58.8 #	60.96 ab	13.02%
L)IFS Cell	80.3	10.66 abc	17.6%	88.6	50.08 bcde	28.54%
M)IFS Chit	83.6	8.63 abcd	33.3%	101.6	59.15 abc	15.60%
N)IFSCl+Ch	79.6	6.35 bcde	50.9%	94.5	52.64 bcd	24.88%
O)IFS Flour	74.1	7.96 abcde	38.4%	100.6	49.31 cde	29.63%
P)IFS NoNut	107.1 *	11.91 ab	7.9%	100.3	53.82 bc	23.20%

* Significantly higher emergence than the untreated control according to LSD (P=0.05).
# Significantly lower emergence than the untreated control according to LSD (P=0.05).
% infection values from each trial site followed by the same letter are not significantly different according to LSD (P=0.05). df=95, Error mean square=26.64 (Bonney) and 97.08(Goodwin).

13.4. Discussion.

In some trials (eg Ch 18: Inoculum Density) onion emergence was reduced following amendment of Tr5 colonised millet. In these trials where 120 seeds were added to each plot emergence was not affected by amendment of Tr5 as millet inoculum. Amendment of Tr5 using in-furrow sprays on uncoated seed (P) provided significantly higher emergence than in the untreated controls (A), though not when the seed was nutrient coated (L, M, N and O). As emergence in these treatments was not significantly different in Goodwin's trial, or in Scolyer's trial (Chapter 14) this may be a random occurrence.

Sowing by the fluid drill method provided similar emergence to the untreated control at Bonney's site. However, emergence under this sowing method was quite poor in Goodwin's trial. Examination of the CFU counts (Table 13.B) on this material for treatment J (FD cellulose and chitin) would suggest that the lower emergence could be consistent with higher Tr5 inoculum densities, however this interpretation is not supported by the CFU counts for fluid drilled cellulose (H) and flour (K) cultures, where emergence at Goodwin's site is a lot lower than Bonney's in both cases, and both treatments had CFU counts in the 10^6 range. The cultures used at Goodwin's were two weeks older than those used at Bonney's. As the CFU counts for cellulose and flour cultures did not rise in this time, it implies that they had reached the growth peak. Cultures in this stage of the growth cycle are likely to contain accumulated secondary metabolites and staling products, and it may be that these compounds could have reduced emergence at Goodwin's site.

The inoculum level at Bonney's site (average 69 sclerotia/kg) suggests that severe disease should be expected under conducive conditions, particularly as the grower had lost virtually the entire crop in that part of the paddock in the previous season. As the majority of sclerotia at the site were only one year old there is a possibility that there had been insufficient conditioning to release constitutive dormancy (Coley-Smith *et al.*, 1987; Brix and Zinkernagel, 1992a). However, no crop had been grown at Goodwin's site (Ave: 239 sclerotia/ kg) in the previous season. For several season's prior to this there had been onion white root rot trials on the plot and the 70% disease incidence could be expected. In reality the total loss was greater as another 20% of onions were lost to miscellaneous causes including *Ulocladium, Alternaria, Botrytis* and cut worm (data not presented).

The amendment of Tr5 on millet in either dried (B) or actively growing (C) form made no difference to disease incidence. The reductions in disease using this treatment was not as good as anticipated based on the work of Lacey and Wong (1991) who showed 60% disease reduction using *T. koningii* strain TrA in field trials. In concurrently run pot trials, Tr5 provided superior disease control to TrA and was designated for use in future studies. It is possible that the rate of Tr5 millet amendment was too low for adequate Tr5 root colonisation. Certainly in the soils and conditions used for the root colonisation studies (Ch. VI:20) this rate of application would have resulted in no more than 60% root colonisation, but this relationship may vary with environmental conditions and between soils.

It is difficult to quantify whether some proportion of suppression may be due to organic matter amendment rather than the biocontrol agent. Lacey and Wong (1991) showed that disease in presence of autoclaved Tr5 millet inoculum was not significantly different to untreated controls. However, amendment of autoclaved

biocontrol agent or autoclaved millet as a control would not necessarily resolve this issue (Baker *et al.*, 1984).

While fluid drill systems have been used to apply fungicides for control of white rot (Entwistle and Munasinghe, 1981b) this is the first attempt to use fluid drilling to introduce biological control agents for control of *S. cepivorum.* Conway (1986) used the fluid drill system for amendment of biocontrol agents for *S. rolfsii* . The only fluid drill sown treatment which provided significant reductions in disease incidence at both sites was treatment I amended with chitin. This treatment gel had the lowest Tr5 CFU count at the time of amendment at both sites and was especially low (600 CFU / ml) when applied in Bonney's trial where the best disease reduction was achieved. The gel used by Conway (1986) contained 10^6 propagules per ml, predominantly chlamydospores rehydrated 30 minutes before application. A possible explanation for the high efficacy of the chitin culture at Bonney's site may be that the material was predominantly comprised of chlamydospores and mycelium whereas other treatments had high numbers of conidia. There have been reports of superior *Trichoderma* spp. colonisation using low propagule numbers of chlamydospores compared to high numbers of conidia (Papivizas *et al.* ,1984). Unfortunately propagule types were not precisely quantified. The concentration of conidia in inoculum may not be a final determinant in any case. For example Lewis *et al* . (1996) reported two commercial biocontrol preparations of the same agent with 1000 fold difference in inoculum density but equivalent efficacy, and Kok *et al.* (1996) amended *T. harzianum* in equal volumes of manure carrier with inoculum densities ranging from 10^3 to 10^7 and found that all soil populations stabilised to CFU counts within one exponential order of each other after 125 days. While CFU of soil populations of sporulative fungi is a less than optimal method for assessing saprophytic activity, this suggests that if a low rate of amendment is sufficient to colonise the niche there is no value in additional propagules in equivalent volumes of carrier (Kok *et al.*, 1996), suggesting that the soil environment ultimately regulates the level of proliferation of an introduced fungus.

The amendment of Tr5 as spore sprays has resulted in significantly less (50.9%) disease in Bonney's trial in treatment N (IFS+ cellulose and chitin seed dressing), and smaller reductions in treatments L (cellulose), N, O (flour), and P (no nutrients) in Goodwin's trial. Treatment P was not expected to provide disease suppression as it is documented that conidia with no added food base remain in fungistatic dormancy in soil (Papavizas *et al.*, 1984). There was however a small reduction in disease in this treatment at Goodwin's site suggesting that some colonisation occurred. The use of uninoculated cellulose and chitin seed dressings (F) have not significantly suppressed disease. Therefore we are able to conclude that disease suppression observed in other seed dressing treatments is due to activity of

Tr5. The rationale behind use of chitin in seed dressing was that there may be some enhancement of Tr5's chitinolytic enzyme activity which appears to be an important aspect of biocontrol (Chapter IV. 11). Cellulose was included to investigate it's potential as a selective food base. Additionally polysaccharide soil amendments may be more effective when used in combinations. There is no evidence that either rationale has been effective though it is interesting to note that control using inoculated seed dressings of cellulose (L) and chitin (M) together add up to exactly the level of disease control achieved in treatment O (cellulose+chitin) at Bonney's site. However this additive trend was not confirmed in Goodwin's trial. In studies by Maurer and Baker (1964) chitin amendments were found to be more effective in disease suppression when amended together with lignin. In another study where chitinolytic *Trichoderma hamatum* was added to cellulose or chitin seed dressings for control of *Rhizoctonia* and *Pythium* chitin was found to be of high efficacy, however the conidia were applied in the seed dressing rather than as a spray (Harman, *et al.*, 1981). The use of in-furrow sprays with soil amendments warrants further investigation as it would be more easily integrated into current industry practices. However there is an unresolved issue of whether coating nutrients onto seed has deleterious effects on emergence. Such effects on emergence seem to be less than that of fluid drill sowing, and in-furrow sprays would be more easily adapted to industry practice than fluid drilling which can be cumbersome in practice (D. O'Connor, vegetable consultant , UK, pers comm). Chitin amendments alone also deserve investigation as these have been shown to be effective for control of some pathogens (Mitchell, 1963; Henis and Chet, 1975). Mitchell and Alexander (1963) suggested that the influence of chitin amendments may result in an increase in the population of mycolytic micro-organisms.

Chapter V: Field amendment methodologies.

14.0: The effect of nutrient amendments and application techniques on establishment of *T. koningii* (Tr5) in field soils: the 1994/5 trial.

14.1. Introduction.

The 1993/4 trials investigated amendment of Tr5 using predominantly liquid materials. These materials sometimes provided better control than the solid material millet but overall were inconsistent between sites. Millet amendment provided only around 40% disease control, but the data had indicated that the method was repeatable and it was therefore logical to build on this method using information gained from experimenting with other techniques. Chitin and other carbon source amendments provided sufficient disease suppression that they deserve further investigation though coating these nutrients directly onto seed appeared to be a hazard in terms of emergence. In-furrow sprays in absence of seed dressings had provided good emergence, but without a colonisation base these sprays provided little disease suppression. In inoculum depth trial A (Ch. VI:16) Tr5 demonstrated high efficacy when applied as a layer below the seed. As fertilizer is commonly applied as a band below seed in Tasmania, placement of Tr5 in this way was a practical option. Therefore, comparison of amendment below as opposed to beside seed is warranted.

Amendment carriers suitable for commercial application would need to be available at low cost and in abundant supply and be easily produced. Laboratory grade cellulose for example would be too expensive. Carriers investigated in Scolyer's trial included millet inoculum, crabshell chitin, a peat/ chitin/osmocote mixture, chitin and sawdust. These treatments were investigated with and without Tr5 in-furrow spore suspension sprays, and sown beside and 1cm below the seed. In-furrow sprays alone were tested to determine whether some proportion of disease control was due to the spray rather than the carrier. Also amendment of Tr5 raised in liquid culture incorporating crabshell chitin and sown by the fluid drill method, and amendment of Tr5 colonised sawdust were investigated. Chitin amendments are known to have some disease suppressing properties (Mitchell, 1963). Therefore autoclaved chitin with no amendment of Tr5 was included to determine if a proportion of any disease suppression could be due to the amendment rather than Tr5. The best commercial fungicide practice (Flocal seed dressing with Sumisclex/lime super) was included for comparison to commercial practice. For comparison to standard industry practice a replication of the Tr5 millet treatment using seed dressed with Benlate/Thiram was included.

14.2. Materials and Methods.

The site for the trial was selected for uniformly spread *S. cepivorum* sclerotia of appropriate density (assessed by wet sieving) and area at Mr Bill Scolyer's property at Kindred in north west Tasmania.
Nine 200g samples were wet sieved to determine the inoculum density.

Solid amendment materials which were pre colonised by Tr5 as described in Appendix A (Mass Culture Methods) were amended all at a rate of 110 kg/ha. Inoculum density of each amendment material was determined by the dilution plate assay (Ch. V:13.2.2.).

The trial site was worked to a commercial standard seed bed, and each plot was one metre long and consisted of 6 furrows 15cm apart. There was a 30cm buffer between each plot along the bed and a 60 cm space for the tractor wheel between plots. The trial was arranged in a randomised complete block design. Six replicates of each treatment were performed.

The treatment details are listed in Table 14(B). Carrier material sown below seed was laid in a 1cm deeper furrow, buried with 1cm of soil, then seed was added. Appropriate in-furrow sprays were applied to seed before burial. The emergence data was analysed by one way analysis of variance and LSD (0.05) test.

14.3. Results and Discussion.

Nine 200g samples were sieved to determine the inoculum density of the site, results were: 7, 14, 7, 11, 17, 12, 11, 59, 5. Ave: 15.8 (79 sclerotia per kilogram). *Trichoderma* spp. were isolated from these sclerotia. One of these isolates (Td9) is under evaluation in 1996/7 field trials.

Inoculum density of each amendment material as determined by the dilution plate method is presented in Table 14(A). None of the other carriers had the profuse green sporulation which was observed for millet.

Table 14(A). Inoculum density of the different carriers.

Carrier	Colony Forming Units per gram of inoculum.
Peat/ Chitin/ Osmocote.	6.3×10^3
Sawdust	1.0×10^3
Chitin/ Sawdust	3.6×10^3
Millet	6.0×10^5
Chitin	9.4×10^4
FD Chitin	9.0×10^2
In Furrow Spore Spray	1.0×10^6

Figures are averages of two plate replicates withdrawn from the same dilution.

Inadequate disease was recorded in the trial for interpretation. Infection was entirely absent from most treatments. A crop had been grown at the site in the previous season so it is possible that many sclerotia had not been released from constitutive dormancy. The site was well watered and grown as for a commercial crop and the result is difficult to explain.

Some useful information is provided by the emergence data (Table 14.B) which shows that treatment M (Peat/chitin/osmocote inoculum with no in furrow spray) provided significantly better emergence than the untreated control (A) and two treatments, B and D (millet inoculum applied 1cm below the seed with and without in furrow sprays respectively) displayed significantly worse emergence than the untreated control. Emergence in all other treatments was not significantly different to the untreated control.

Comparison of the different amendment strategies by analysis of variance showed that there were no differences in emergence between the different amendment materials. Excellent emergence was experienced using in-furrow sprays in trials at Bonney's and Goodwin's sites, however there was no significant difference between emergence in the presence or absence of these sprays in Scolyer's trial. Emergence has been significantly greater when amendment materials were placed beside the seed than when they were placed in a layer 1cm below the seed, all eight means for seed sown beside inoculum exceed the corresponding mean for seed sown 1cm above inoculum (Below Ave: 70.3 , Beside Ave 81.55 ; Lsd(0.05)=5.81). It is speculated that the greater disruption to the seed bed at sowing allowed seed to slip deeper, which is known to reduce emergence.

Table 14(B): Treatment list for the 1994/5 field trial.

Code	carrier	Beside seed	Below seed	IF spray	Emergence
A	Untreated				78.3 bcdef
B	millet		11g	60ml	60.6 h
C	millet	11g		60ml	77.5 cdef
D	millet		11g		65.5 gh
E	millet	11g			79.5 abcd
F	chitin		11g	60ml	68.0 efgh
G	chitin	11g		60ml	81.8 abcd
H	chitin		11g		74.0 cdefg
I	chitin	11g			75.3 cdefg
J	P.C.O.		11g	60ml	71.1 defgh
K	P.C.O.	11g		60ml	89.1 ab
L	P.C.O.		11g		71.8 defgh
M	P.C.O.	11g			90.6 a
N	Chit/sawdust		11g	60ml	74.5 cdefg
O	Chit/sawdust	11g		60ml	79.3 abcde
P	Chit/sawdust		11g		77.5 cdef
Q	Chit/sawdust	11g			79.3 abcde
R	IFS only			60ml	85.5 abc
S	Sawdust	11g			80.5 abcd
T	FD Chitin	60ml			71.5 defgh
U	Flo/sd+ Sum/LS				67.8 fgh
V	millet + BT SD				77.8 bcdef
W	Autoclaved Chitin	11g			80.3 abcd

Emergence values followed by the same letter are not significantly different according to LSD(0.05). df=137, error mean square = 98.32, P value 2.303×10^{-5}.

A self criticism of the experimental design is that chitin is the only carrier where uninoculated controls were performed. At the time the trial was planned the goal was to screen for a carrier which could provide better Tr5 establishment than the millet and it was desirable to screen as many methods as possible. There is debate over whether such treatments are a valid control in any case (Baker *et al*, 1984).

Chapter V: Field amendment methodologies.

15.0: Integration of biological control and sclerotial germination stimulants.

15.1. Introduction.

Sclerotial germination stimulants have been discussed in Chapter 2 (Literature Review; *Sclerotium cepivorum*) and involve stimulation of *S. cepivorum* sclerotial germination by onion root exudate compounds in the absence of onions, under which circumstances *S. cepivorum* mycelium cannot find a suitable host plant to re form sclerotia and eventually dies. The use of this method in combination with biological control might provide a better opportunity for onion production at infested sites without use of fungicides.

Allyl-sulphides have been extensively investigated for use as sclerotial germination stimulants (Somerville and Hall, 1987; Coley-Smith *et al* , 1987; Brix and Zinkernagel, 1992a). The exact mechanism by which sclerotia specifically recognise these compounds has not been precisely determined. The Tasmanian company Vecon Oils Pty Ltd is a commercial distiller of onion oil for use in the food industry. Onion oil distillation has a waste product (onion mash water) which still contains many of the original allyl and alkyl sulphide compounds likely to stimulate sclerotia to germinate (B Howe, pers comm from GLC analysis). Tens of thousands of litres of this material is presently produced and disposed of each season, and could potentially be used as a germination stimulant.

This study investigated the use of this material as a germination stimulant and the potential for integration of this with biological control using *Trichoderma koningii* (Tr5).

15.2. Materials and Methods.

15.2.1. *In-vitro* studies on sclerotial germination.

To investigate the potential for use of the different germination stimulants from *Allium*, *S. cepivorum* (Sc4) sclerotia which had been taken from a six month old and long dormant sterile culture, were placed using forceps on the surface of soil agar (see Appendix B). A 1cm diameter well was cut in the centre of the agar using a glass cylinder, to which 0.5mls of the stimulant was added. Approximately twenty sclerotia were placed in each plate. Each stimulant was used at concentrations of 1%, 10% and 100% diluted in distilled water as appropriate. Controls were performed using distilled water. Petri plates were sealed with parafilm and stored in

darkness at room temperature for five days before they were examined by dissecting microscope for germinating sclerotia and the percentage of germination was calculated. The products tested included mash water which originated from a commercial distillation boiling vessel, condensate water which is water that condensed with the onion oil following distillation, artificial oil which is a food additive, and burned oil which is heat damaged distilled onion oil. Three replicates of each treatment were performed. Results were analysed by one way analysis of variance with mean separation by LSD test.

15.2.2. Field Trials:

To examine the influence of onion mash waste water and Tr5 on disease expression, trials were conducted in the 1994/5 season at a property near Forth in northwest Tasmania owned by Vecon Pty Ltd. The infested soil area was a long thin headland. Prior to the trial, the sclerotial density was determined by wet sieving 200g samples (see Appendix F). The sclerotial counts were 13, 7, 4, 14, 6, and 31 (average 62.5 sclerotia per soil kg). Another potential site was rejected as inoculum density was lower and more variable.

The onion mash water waste was applied on 29/6/1994 (winter) at a rate of 24L per M^2 at concentrations of 100%, 10%, 1% and 0%. Dilutions were made using tap water. The list of treatments is shown in Table 15(A). The trial site was two rows wide and divided into four blocks containing all eight treatments. The layout is shown in Table 15(B). 0.5m buffer zones were left between plots. The mash water was applied using a watering can, and a $1m^2$ frame was placed around the treatment area to prevent horizontal run-off. Soil temperature at a depth of 5cm at the time of treatment was 8°C. Immediately before the mash water was applied, soil samples were taken from the top ten centimetres of soil in each plot (four trowel loads of soil were taken from separate parts of each plot and mixed together in a plastic sample bag which was tied to prevent moisture loss). Samples were again taken three weeks after the mash water application. Samples were stored at air temperature for a few days before the density of inoculum was determined by wet sieving by the methods described in Appendix F.

Tr5 was sprinkled in the furrow as millet inoculum (see Appendix A) at a rate of 110 kg/ha at the time of sowing (25/8/96). One metre square plots consisted of six furrows to which 120 seeds were sown (one per 5cm). The emergence was considered to be complete in early November 1994. Disease was assessed at fortnightly intervals thereafter, infected plants were marked using plastic nursery tags to prevent double counting. The trial was destructively sampled on 2/3/1995.

Table 15(A): Treatment list for onion mash water : biocontrol trial. Twenty four litres of onion mash waste water applied by infiltration at 0, 1, 10, and 100% of original concentration. Fifty seven days after mash water application, onion seed together with (+) or without (-) Tr5 at a rate of 110 Kg/ha was sown in the plots.

Treatment	MW conc	Tr5
A	0%	+
B	0%	-
C	1%	+
D	1%	-
E	10%	+
F	10%	-
G	100%	+
H	100%	-

Table 15(B). Layout of the trial site for the onion mash waste water : biocontrol trial. Twenty four litres of onion mash waste water applied by infiltration at 0, 1, 10, and 100% of original concentration. Fifty seven days after mash water application onion seed together with (+) or without (-) Tr5 at a rate of 110 Kg/ha was sown in the plots.

100+	100-
0-	0+
10+	10-
1-	1+
10-	10+
100+	100-
1+	1-
0-	0+
0+	0-
1-	1+
100-	100+
10+	10-
1-	1+
10+	10-
0-	0+
100+	100-

15.2.3. Root isolation:

To establish how effectively Tr5 had colonised roots in the trial, roots samples were taken at the time of harvest. After bulbs were uprooted, roots were inserted into snap seal plastic bags and broken from the bulb without contacting any surfaces other than the plastic bag. In the laboratory, five centimetre long segments were cut aseptically and placed in the centre of plates of RAS selective medium (Ch. IV:12). After two weeks incubation, plates were inspected for colonisation by *Trichoderma* spp. Any *Trichoderma* spp. present were sub cultured to eliminate

contaminants and incubated in pectinase medium for three weeks before isozyme profiling for polygalacturonase, pectinesterase, and ribonuclease to determine whether the bands were similar to those known to be produced by Tr5 (Ch. IV:10).

15.3. Results.

15.3.1. *In vitro* studies on sclerotial germination.

The germination of sclerotia in presence of various *Allium* root exudate compounds was recorded after five days storage in darkness at room temperature (Table 15.C). In the three untreated controls, only one sclerotium out of the total 180 germinated. Overall the most effective stimulant was condensate water which induced 62% of sclerotia to germinate at full concentration, 41.2% when diluted to 10% and 18.2% when diluted to 1%. Mash water was the second best stimulant which induced 48.9%, 19.6, and 12.2% germination at 100, 10 and 1% concentration respectively. Onion oil failed to induce sclerotial germination at the two higher concentrations, though at 1% dilution 27% of sclerotia were induced to germinate. Artificial onion oil displayed a similar pattern to the onion oil with low germination at the two higher concentrations and 40.5% germination the two lower concentrations. In all cases sclerotial germination was of the non-eruptive type (Coley-Smith *et al.* 1967). In several examples secondary sclerotia were noted to form in the mash water and condensate plates on the soil agar surface atop a white clump of mucilage which appeared to be of fungal origin. Only one example was noted where a secondary sclerotium formed with a fully melanised rind (see Figure 15.D). Three weeks after formation this sclerotium was transferred to the surface of a plate of onion agar, however no colonies developed. Statistical analysis was performed using LSD (0.05).

Table 15(C): The percentage of sclerotia which germinated in sealed petri dishes of soil agar to which 0.5 mls of *Allium* root exudate compounds were added.

Treatment	Dilution	Germ %	lsd=12.0
Distilled water		0	f
Distilled water		0.33	e
Distilled water		0	f
mash water	100%	48.93	b
mash water	10%	19.6	cd
mash water	1%	12.2	de
condensate	100%	62.1	a
condensate	10%	41.2	b
condensate	1%	18.2	cd
onion oil	100%	0	f
onion oil	10%	0	f
onion oil	1%	27.63	c
artificial oil	100%	2.06	e
artificial oil	10%	1.23	e
artificial oil	1%	40.5	b

-germination percentages followed by the same letter are not significantly different according to LSD(0.05). df=63, error mean square=10.62, P value= 0.000593

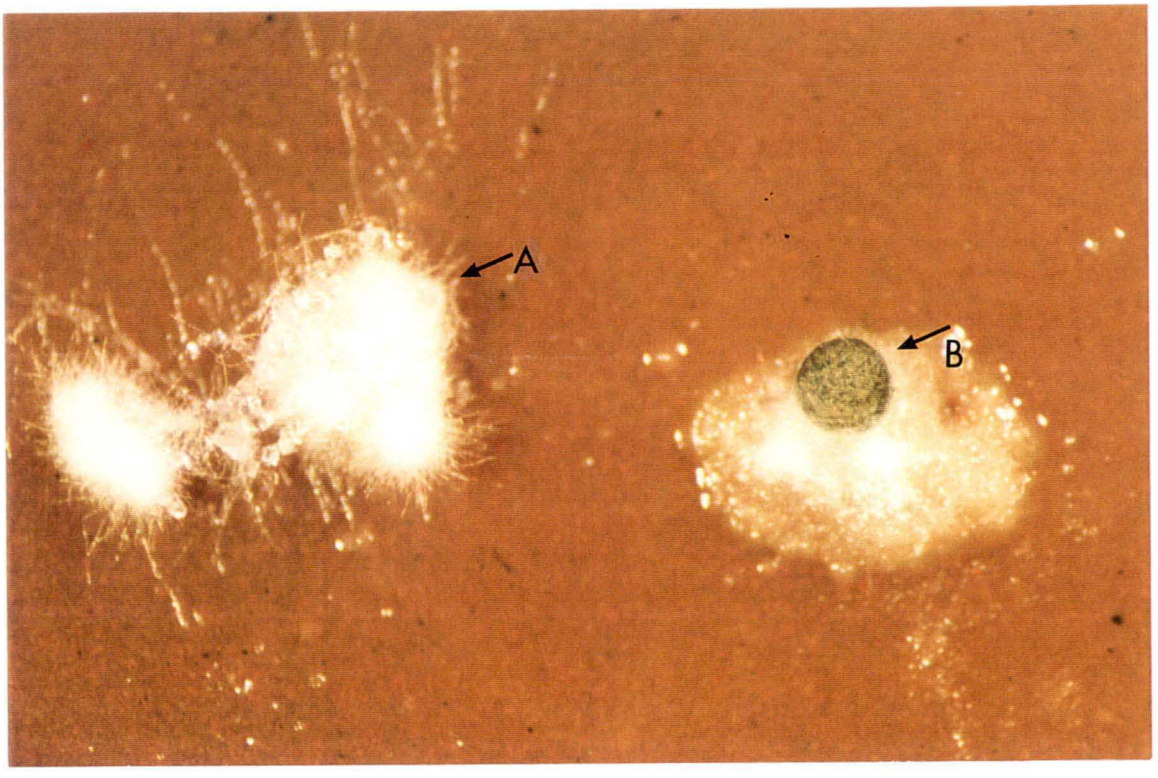


Figure 15(D). Secondary sclerotia formed on the surface of soil agar. Most (A) but not all (B) failed to develop fully melanised rind.

15.3.2. Field Trials:

The average numbers of *S. cepivorum* sclerotia which were found in 200g soil samples taken from each plot before and six weeks after mash water application are presented in Table 15(E). There was a reduction in the average numbers of sclerotia in all treatments including the untreated control. The greatest reduction occurred following the 1% and 10% mash water dilutions. The only statistically significant reduction was in the 1% treatment, though the values for the 10% rate are exactly the value of LSD (3.25) apart. It is surprising to note that the reduction in sclerotial numbers was less in the 100% treatment (17.9%) than in the untreated control (24.6%).

Table 15(E). The mean number of intact *S. cepivorum* sclerotia found in 200g soil samples taken from 8 replicate plots in the germination stimulant trial either before treatment with onion mash water, or 8 weeks after onion mash water was applied.

Rate	Before treatment	8 weeks after treatment	% decrease
water only	12.75	9.62	24.6%
1%	10.62	7.10*	33.2%
10%	9.75	6.5	33.4%
100%	7.00	5.75	17.9%

-note: treatment means with * in the after column had significantly lower numbers of sclerotia after the application of onion mash water than before according to students T test (0.05) assuming unequal population variances.

Emergence in the stimulant/biocontrol trial was poor in all treatments (see Table 15.F.). The only treatment where more than half of the seed emerged on average was D (1% mash water - Tr5), and this figure was mostly due to a single plot where 113 seedlings emerged. There were no significant differences in emergence in the presence or absence of Tr5, or related to the mash water treatment. Disease incidence was high in all treatments irrespective of the amendment of Tr5 or mash water. Two way analysis of variance indicated that there was no evidence of differences among the means.

Table 15(F): The number of seedlings which emerged from 120 seeds sown and percentage of seedlings which became infected in plots which had been treated with 0%, 1%, 10%, or 100% onion mash waste water which were either amended (+) or not amended (-) with Tr5.

MW rate	-Tr5		+Tr5		MW% infect
	emergence	% infection	emergence	% infection	
0%	52.5 ab	58.48	44.0 b	67.16	62.8
1%	69 a	68.76	39.0 b	59.82	64.2
10%	48.75 b	59.18	49.5ab	46.68	52.9
100%	50.25 ab	69.00	55.5 ab	48.63	58.8

Note: -numbers followed by the same letter are not significantly different according to LSD(0.05).
-emergence= seedling emergence per plot of 120 seeds.
-MW rate= the dilution of onion mash waste water applied to the treatment at a rate of $24L/m^2$
-MW% infect= percentage of plants which became infected in all plots treated with that concentration of mash water irrespecive of whether Tr5 was added.
-two way analysis of variance indicated that there was no evidence of differences among means.
-Interaction P value 0.48. Error mean square 359.01.

15.3.3. Root Sample Isolations.

Of the thirty two samples taken from both Tr5 amended and unamended plots, nine and ten respectively were colonised by *Trichoderma* spp (Table 15.G.). There was no evidence of any influence of the mash water amendment on *Trichoderma spp.* colonisation. Only 2 and 1 respectively of these were morphologically similar to Tr5. Isozyme profiles for pectinases distinguished three isolates from amended and six from unamended plots which had isozyme profiles indistinguishable from Tr5 (Figure 15.H). Ribonuclease isozymes were determined to be unreliable in further investigations and are not presented here.

Table 15(G): Isolation of *Trichoderma* spp. from thirty two onion root samples from plots amended and unamended with Tr5 in the stimulant/biocontrol trial.

Tr5	Total *Trichoderma*	Morph similar	profile similar
-	10	2	6
+	9	1	3

Total Trich= total isolations of Trichoderma spp from 32 samples.
Morph similar= morphologically similar to Tr5.
Profile similar= pectinase profile not distinguishable from that of Tr5.

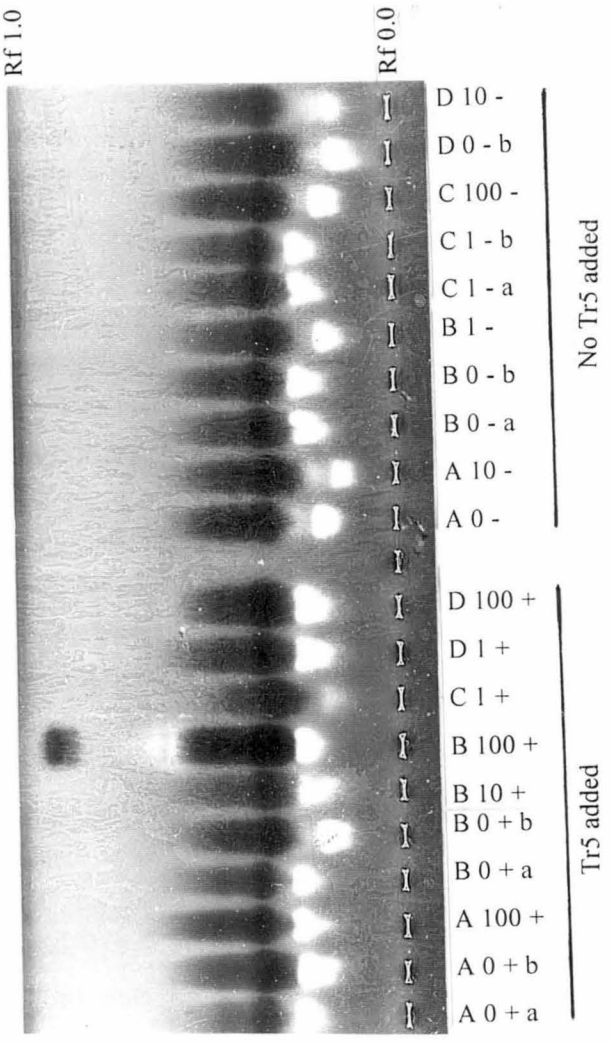


Figure 15(H): Contact print of a polyacrylamide electrophoresis gel for the detection of polygalacturonase (dark zones) and pectinesterase (light zones). *Trichoderma spp.* isolated from roots in the stimulant/biocontrol trial grown on pectinase medium at 25°C for 21 days.

15.4. Discussion.

In the *in-vitro* assay, sclerotia which were fully dormant were induced to germinate to some extent by all of the materials tested. This is in contrast to prior studies which show that sclerotia are expected to display constitutive dormancy (Coley-Smith *et al.*, 1987) before conditioning in unsterile soil. The onion seedling infections performed *in vitro* (Chapters III & IV) where sclerotia were pre-germinated also demonstrated that a large proportion of Sc4 sclerotia were not subject to constitutive dormancy. Other workers have established that a minority of isolates are able to germinate without conditioning (Brix and Zinkernagel ,1992a). It should be noted that in this study, sclerotia were individually picked up using fine forceps, which may have abraded the sclerotial rind, and can release dormancy (Coley-Smith, 1959).

Under laboratory conditions in this assay, the most effective stimulant for sclerotial germination was condensate water. However this material would not be available in sufficient quantities for commercial application. The next most effective stimulant was the mash waste water which induced 48.9% of sclerotia to germinate. Thousands of litres of this material are available and are currently pumped into the sewage system by Vecon Oils. Therefore it was decided to evaluate this material in field studies. The burned onion oil was tested in its most concentrated form, and induced 27% germination when diluted to 1%. The viability of the sclerotia in the 100% and 10% plates was not investigated further, but it may be that the high concentration of oil and associated volatile compounds were toxic to the sclerotia, and lower concentrations may have induced greater germination. This material has a market value around A$1000 per litre so only second rate (burned) oil would be available. Artificial onion oil might be more economical than distilled onion oil. It induced 40% of sclerotia to germinate at the lower concentration. This material could possibly be toxic at the higher concentration.

The formation, and partial formation of secondary sclerotia was unexpected as there was no solid tissue, though the condensate and mash water might be expected to contain some soluble nutrients. The only secondary sclerotium which fully formed did not germinate when placed on onion agar, however it may be showing constitutively dormancy.

In the field, decreases in raw sclerotial numbers were detected in all treatments in the three weeks between application of mash water and the second sampling including decreases in the untreated control. The decrease was statistically significant only in the 1% treatment. The decrease could be interpreted to be due to either non-specific germination and decay including mycoparasitism which would have occurred regardless of the treatment, or to stimulated germination. As a

decrease in raw numbers of sclerotia was present in the untreated control plots natural decay would seem the most likely reason for the decline, though it is possible that the volatile disulphides could induce sclerotia to germinate in untreated control plots, from treated plots half a metre away. In a recent commercial scale investigations of diallyl disulphide application, Dennis and Stagg (1996) employed 10m buffers between all plots to avoid this possibility. It is noteworthy that the smallest percentage decrease in sclerotial numbers occurred under the highest mash water concentration which had been expected to be the most stimulatory. One possible explanation is that the higher soluble nutrition allowed more secondary sclerotia to form.

It is an unfortunate co-incidence that pre-application soil samples revealed progressively lower numbers of sclerotia as the mash water concentration designated to them increased. The plots where mash water was applied at the higher rate would therefore be expected to be less severely challenged by *S. cepivorum* irrespective of the treatment. In spite of this, Table 15(F) shows that the percentage of infection was little different at any level of the mash water treatment strata.

The addition of Tr5 made no difference to the percentage of infection. Root sampling would suggest that Tr5 establishment on the root surface was very poor, so a higher rate of application is required or we need to better understand how to enhance Tr5 saprophytic proliferation. Only about one third of roots were colonised by any *Trichoderma* spp.. Amendment of Tr5 did not affect colonisation rate. Isozyme profiles for pectinase activity excluded many of these isolates from the Tr5 type group as did colony morphology. In the process of sieving sclerotia from soil samples many sclerotia were incubated on onion agar in an attempt to isolate *S. cepivorum* mycelium. One *Trichoderma* spp. native to this soil (Isolate Td7) was isolated from a parasitised sclerotium, and could not be separated from Tr5 based on its pectinase or ribonuclease profile, but had differing morphology in similar fashion to some of the *Trichoderma* spp. isolated from root samples which similar profiles.

The concept of using a two fisted approach for control of *S. cepivorum* remains a desirable goal. Since this work was completed trials on soil pH, inoculum density, temperature, and depth of inoculum and rate of application (Chapter VI) have enhanced our knowledge of how to best use Tr5 considerably. Sclerotial germination stimulants (diallyl disulphide) are presently under evaluation on a much larger scale by other members of our group (Dennis and Stagg, 1996). Once both efforts have achieved the maximum disease reduction possible independently further attention will be given to integration of the two methods.

Chapter VI: Ecological Studies.

16.0: The influence of *S. cepivorum* inoculum depth on disease incidence and biocontrol efficacy.

16.1. Introduction:

In laboratory work (Chapters III & IV) it has been established that *Trichoderma koningii* (Tr5) was able to attack *S. cepivorum* mycelium in onion roots as infections moved toward the bulb. The following trial was the next step in the development process in establishing whether Tr5's efficacy could also be demonstrated under non-sterile conditions where onion roots were exposed to *S. cepivorum* inoculum, and infections had to pass through a band where *T. koningii* inoculum was added, to infect the bulb base. As it would appear that there must be some damage to root tissues before *T. koningii* will colonise the root infection cavity and challenge *S. cepivorum*, the distance between the infection point and the base plate may influence the respective level of disease control. Therefore, *S. cepivorum* inoculum was buried at different depths.

One initial goal of this PhD program was to evaluate whether the efficacy of the selected biocontrol agent, Tr5, for antagonism against *S. cepivorum* might be enhanced by addition to the soil with a food base which would selectively enhance establishment in the rhizosphere. In Trial 1, Tr5 was therefore applied on a range of carbohydrate sources of varying complexity including purified cellulose. As production of chitinolytic enzymes seemed likely to be an important component of Tr5's antagonistic ability, the polysaccharide chitin (from crabshells) was included.

The influence of the depth of sclerotia in the soil on disease incidence has been previously investigated in garlic (Crowe and Hall, 1980a). Sclerotia buried at a depth of 30cm were able to germinate and infect garlic plants. The greatest disease incidence resulted from sclerotia placed at intermediate depths (10-15 cm) where the number of individual infection loci (groups of adjacent diseased plants) was intermediate, but the number of infected plants per disease locus was high due to infections spreading between roots of adjacent plants in the 5 to 7.5 cm zone of high root density.

Inoculum depth trial 2 replicates and follows on from results of Trial 1. To assess whether lower disease incidence from deeper inoculum in trial 1 was due to lower sclerotial germination, or the greater distance infections had to travel to reach the bulb base, *S. cepivorum* sclerotia were buried in nylon mesh bags at the level where the inoculum was placed in each pot.

When plants become infected in Tr5 treated pots a question is raised as to whether Tr5 was unsuccessful in challenging the infection, or whether Tr5 had not colonised the roots on which infection passed from the buried sclerotia to the bulb base. To investigate this question, root samples were taken from bulbs and incubated on a *Trichoderma* selective medium to assess whether Tr5 was present on the roots. As some isolations were morphologically different to Tr5, pectinase isozyme profiles were used to test the accuracy of the morphological identification.

16.2. Materials and Methods:

16.2.1. Trial 1.

The trial was conducted in a shade house at the University of Tasmania Horticultural Research Centre in the 1993/4 season. Steam treated potting mix was the root medium (20kg sand, 20kg peat, 140g lime, 80g dolomite, 120g osmocote, steamed for 45 minutes). Plastic pots 12 cm in diameter and 13cm deep were used.

S. cepivorum (Isolate Sc4) sclerotia were produced on plates of potato dextrose agar (appendix B), the cultures were 4 weeks old and had appeared to have no surviving vegetative mycelium whn examined by microscope. The contents of the plate (which contained 500-1000 sclerotia) was removed and placed as a single piece on the potting mix at the appropriate depth and the pot was filled to a depth of 1 cm below the rim.

T. koningii (Tr5) inoculum was produced on mineral salts agar (Appendix B) amended with 10g of either; crabshell chitin, cellulose, flour, 5g cellulose + 5g chitin , no added carbon source, or on Potato Dextrose Agar (see Appendix A). Treatments are listed in Table 16(A).

Table 16(A): Treatment list showing letter codes for inoculum depth trial 1 run in the 1993/4 season.

Amendment	*S.cepivorum* Absent	*S. cepivorum* 4cm	*S.cepivorum* 7cm	*S.cepivorum* 10cm
No Tr5	A	B	C	D
Tr5+Flour	E	F	G	H
Tr5+Cell+Chit	I	J	K	L
Tr5+Chitin	M	N	O	P
Tr5+Cellulose	Q	R	S	T
Tr5+P.D.A.	U	V	W	X
Tr5+M.S.A.	Y	Z	Za	Zb

Notes to Table 16(A):
-Carrier refers to the type of agar used to grow Tr5 and add it to the pot.
-Cell + Chit refers to half cellulose and half chitin
-PDA refers to potato dextrose agar
-M.S.A. refers to mineral salts agar with no added Carbon source.

At the time of sowing the appropriate agar for the Tr5 treatment was removed from the petri dish as a single piece and placed on the soil surface, thirty seeds were placed on top of the agar, and covered with a 1cm layer of potting mix.

To inhibit *T. koningii* from spreading to untreated controls by aerial sporulation and water splash each pot was encircled in a stiff plastic sheath to a height of 30cm above the rim (open at the top).

The trial was sown in mid December (Summer) 1993. Six replicates of each treatment were performed. Pots were arranged randomly and were watered daily by hand. Seedling emergence was assessed one month after sowing and observations of disease were made at fortnightly intervals with the final destructive sampling at 17 weeks after sowing. *S. cepivorum* infection was judged to have occurred when sclerotia had formed on the onion base.

Statistical analysis was performed using the two way anova to assess interaction between inoculum depth and biocontrol efficacy which indicated pairwise comparison using individual one way anova and LSD tests across each stratum to be appropriate (McPherson, 1992).

16.2.2. Trial 2.

Preparation.

The soil used for the trial was a kraznozem from north west Tasmania (pH 6.2). Five 200g samples of the soil were sieved to by the methods of McCain (1967) to ensure no sclerotia were present. Black PVC soil bags 20cm diameter, 34 cm deep which held 8 kg of air dried soil were used. Sclerotia were provided by Dr J J C Dennis (DPIF), collected from field infected bulbs from north west Tasmania six months before the trial, air dried and stored in paper bags in darkness. The list of treatments is presented in Table 16(B).

Table 16(B): Treatments in Inoculum Depth Trial 2. Sclerotia were buried at 4, 7, 10 and 20 cm, or not added. Ten grams per pot of Tr5 millet was added to treated pots to provide a band 1cm below the seed.

Treatment: Pot No	Depth of buried sclerotia.	Presence/absence of *T. koningii* band
A: 1-20	-	+
B: 21-40	4	+
C: 41-60	7	+
D: 61-80	10	+
E: 81-100	20	+
F: 101-120	-	-
G: 121-140	4	-
H: 141-160	7	-
I: 161-180	10	-
J: 181- 200	20	-

Pots were filled with soil to the designated depth below the rim, 38 mg of sclerotia (about 400; the amount added in the inoculum density trial to supply 50 sclerotia per kilogram of soil) were evenly sprinkled over the soil, the pot was then topped up to a point 5 cm below the rim where 10g (3180 kg/ha) of *T. koningii* (Tr5) millet inoculum (Appendix A) was added as a layer. The inoculum was covered with 1cm of soil, 20 onion seeds (var: regular creamgold) were added and the seed was covered in a further 1cm of soil. To minimise concerns that *T. koningii* could spread from treated to untreated pots in water splash hessian bags filled with pine bark were placed in between twenty blocks of alternating groups of ten treated or untreated pots. Each block containing two pots of every Tr5+ or Tr5- treatment in random arrangement. The perimeter of the block of pots was encircled with the same bags to prevent solarisation effects.

The trial was sown in spring 1995 and monitored at fortnightly intervals, diseased seedlings were marked with white nursery tags to prevent double counting. All treatments were destructively sampled 29 weeks later.

Sclerotial depth interactions.

To assess the interaction between depth of burial, presence or absence of Tr5, and the *Allium* root exudate germination stimulus, nylon mesh bags each containing 30 sclerotia were buried in each pot at the same level as other *S. cepivorum* inoculum. These were retrieved at the time of harvest, and stored individually in snap seal plastic bags for two days until they were examined by dissecting microscope to assess the number of viable sclerotia remaining. To assess what proportions of the decrease in numbers of sclerotia in the bags was due to

germination and what proportion was due to mycoparasitism and natural decay, additional bags were buried in pots with no onions , and with and without Tr5 at each depth.

Root Colonisation.

To assess how well Tr5 had colonised roots in the treated plots, and whether Tr5 had spread to the untreated controls, root samples were taken, incubated and identified by the same procedures described in Appendix G.

16.3. Results:

16.3.1. Inoculum depth pot trial 1.

The results of seedling emergence and percentage *S. cepivorum* infection are presented in Table 16(C). Of the 30 seeds added to each pot directly on top of the Tr5 inoculum the average emergence ranged from 20.33 to 26.0 plants per pot. Neither the presence of *T. koningii* or of *S. cepivorum* at any depth affected the average number of seeds which emerged. However there are a few comparisons where the percentage infection difference should be interpreted cautiously due to significantly differing emergence (eg disease incidence in treatments F and G should be compared to each other with caution, though both can both be compared to Treatments B, C and E; Table 16(C).

The percentage infection data was analysed using the two way analysis of variance (Figure 16.D). An interaction was shown (P=0.05). Pairwise comparisons using LSD for individual strata were made (see Table 16(C).

In absence of Tr5 the disease was most severe (43.93%) when *S. cepivorum* inoculum was buried at 4cm. At 7cm infection was only 7.56% (82% less) and significantly different at the 0.001% level. In treatment D where sclerotia were buried at 10 cm disease incidence was only 0.92% (98% less than at 4cm). The relationship is described graphically in Figure 16(E).

Table 16(C): Seedling emergence and percentage disease incidence when *S. cepivorum* sclerotia were buried at 4, 7 and 10 cm below the soil surface and Tr5 was added at 1cm directly below the seed.

	S.cepivorum Depth	Tr5 +C source	Average Ω seedling emergence (out of 30/pot)	% infection 4cm ¥	% infection 7cm ¥	% infection 10cm ¥	LSD † * .05 ** .01 ***.001
A	-	-	24.00 abcd				
B	4	-	23.50 abcd	43.93 a			(18.4)
C	7	-	25.33 abc		7.56 a		***
D	10	-	20.66 cd			0.92 a	***
E	-	flour	24.50 abcd				
F	4	flour	25.83 ab	8.00 b			(11.13)
G	7	flour	20.66 cd		0.00 b		
H	10	flour	23.50 abcd			0.00 a	
I	-	cell+chit	24.33 abcd				
J	4	cell+chit	24.83 abcd	1.90 b			(3.62)
K	7	cell+chit	21.16 abcd		0.83 b		
L	10	cell+chit	24.00 abcd			0.00 a	
M	-	chitin	24.16 abcd				
N	4	chitin	20.83 bcd	7.09 b			(7.41)
O	7	chitin	26.00 a		2.77 b		
P	10	chitin	22.00 abcd			0.00 a	
Q	-	cellulose	21.16 abcd				
R	4	cellulose	20.50 cd	8.01 b			(4.32)
S	7	cellulose	24.16 abcd		0.00 b		**
T	10	cellulose	20.00 cd			0.00 a	**
U	-	P.D.A.	22.50 abcd				
V	4	P.D.A.	23.66 abcd	4.55 b			(4.49)
W	7	P.D.A.	22.66 abcd		1.90 b		
X	10	P.D.A.	23.16 abcd			0.75 a	
Y	-	M.S.A.	20.33 cd				
Z	4	M.S.A.	23.50 abcd	2.20 b			(1.27)
Za	7	M.S.A.	22.33abcd		0.66 b		*
Zb	10	M.S.A.	23.16abcd			1.58 a	
		LSD 0.05 individual depth stratum		13.29	4.65	2.814	

Ω- Numbers in the emergence column followed by the same letter are not significantly different.

¥- LSD are calculated from individual Anova for each depth strata and exclude the no sclerotia control. Numbers followed by the same letter are not significantly different.

†- LSD are calculated from individual Anova for each Tr5/Carbon source stratum and exclude the no sclerotia control. 7 and 10 cm figures followed by * indicate significantly lower infection than the 4cm treatment for that Tr5/Carbon source stratum. Figures in brackets in the 4cm rows of this column indicate the LSD (0.05) for that individual stratum.

Table 16(D): Two way analysis of variance for Inoculum depth trial percentage infection data.

Source of Variation:	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Tr5/C strata	3600.3617	6	600.0602	10.8882	2.124E-09	2.1861
Inoc dep	2627.9292	2	1313.9646	23.8421	2.905E-09	3.0828
Interaction	4346.6852	12	362.2237	6.5726	1.216E-08	1.8455
Error	5786.6442	105	55.1108			
Total	16361.62	125				

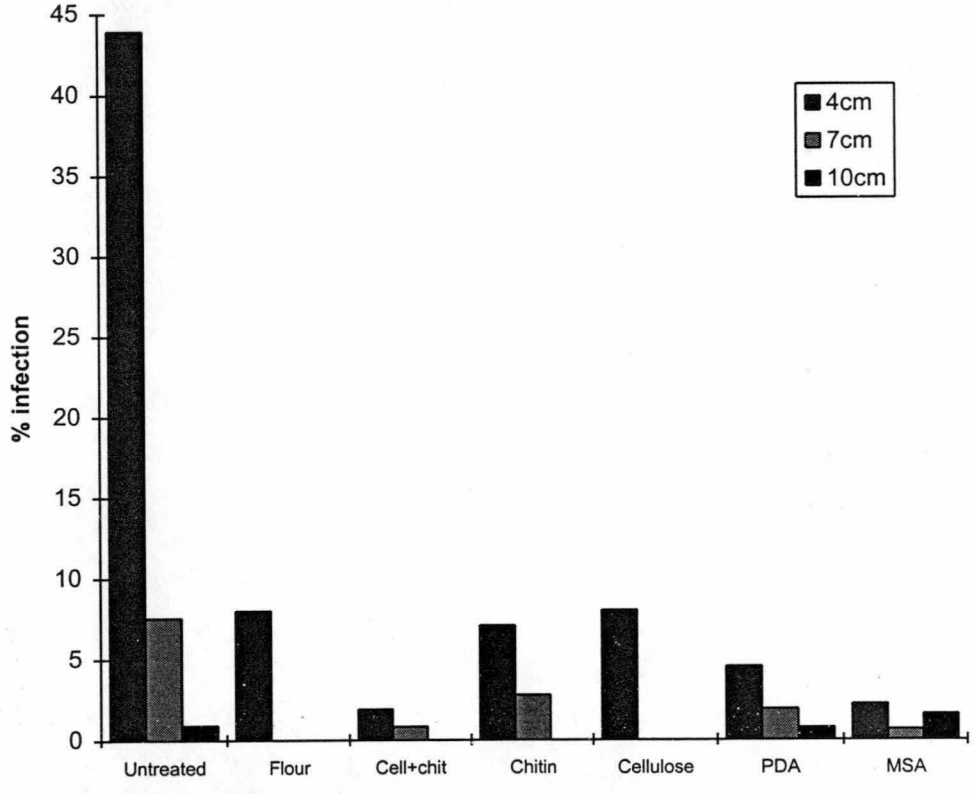


Figure 16(E): Percentage of *S. cepivorum* infection when sclerotia were buried at depths of 4, 7 and 10 cm. Tr5 was added as agar plate inocula at 1cm depth grown on flour, cellulose , chitin, cellulose and chitin (50:50), PDA and with no added carbon source (MSA), Tr5 was absent in the untreated control.

In all treatments where Tr5 was present in the 4 and 7 cm depth strata there was a significant decrease in the level of disease. In the 4cm strata this decrease from 43.95% infection in the untreated control ranged between 8.01% (82% less) and 1.90 % (95% less). There were no significant differences between any of the Tr5 introduction media. At the 7cm depth, disease was reduced from 7.56% in Treatment C to less than 2.77% in all cases. In the Tr5+cellulose(S) and Tr5+flour(G) treatments there was no disease. At the 10cm sclerotial depth the disease was very low in the untreated control (D) where one seedling died in a single replicate. There was no disease in any other treatment.

16.3.2. Inoculum Depth Trial 2.

16.3.2.1 Infection Interactions:

The results for seedling emergence and infection are presented in Table 16(F). Of 20 seeds sown in each pot an average of 11.15 emerged in Tr5 treated pots and 12.22 emerged in untreated pots. Examination of the Tr5 unamended pots emergence column shows that emergence was significantly higher where sclerotia where buried at 10 and 20cm, than at 4cm. This was not evident in Tr5 treated pots where there were there were no significant differences in emergence between different sclerotial depth treatments, however where no sclerotia were added emergence was significantly lower than when sclerotia were added at 4, 10 or 20cm.

The interaction of Tr5 and inoculum depth was examined by two way analysis of variance (Table 16.H.). There was no interaction between the depth of sclerotia and the Tr5 strata. The means were therefore compared across each strata level independently using LSD.

The percentages of infection in presence and absence of Tr5 are expressed graphically in Figure 16(G). Infection was perhaps lower than expected (17.05%) in the 4cm treatment lacking Tr5. The level of disease was 42% less in the 7cm non amended treatment, though this was not significantly different (P=0.05). Percentage infection was significantly lower (56%) than the 4cm in the 10cm non amended treatment. However the comparison should be drawn in caution as emergence was significantly greater than in the 4cm treatment. Similarly caution should be used in examination of the 86.1% lower infection recorded from 20cm deep sclerotia than 4cm deep sclerotia.

In treatments to which Tr5 was added, the average percentage of infection was always less than the corresponding unamended sclerotial depth control (Table 16.F.). In the 4cm treatment, 7.47% of plants became infected. Sclerotia buried at 7cm and 10cm infected 2.19% and 3.07% of plants respectively (not a significant

decrease). Sclerotia buried at 20cm in Tr5 treated pots caused 1.3% disease, significantly less disease than the Tr5 amended 4cm treatment (82.1%).

In both the 4 and 7cm strata the disease was significantly lower (56.2% & 77.9% respectively) in Tr5 treated pots by comparison to treatments without Tr5. However, at 10 and 20 cm, the reductions in disease by comparison to untreated controls were not significant, though this is to be expected when the percentage of infection in untreated controls is low.

Table 16(F): The effects on disease incidence of the interaction between depth of *S. cepivorum* inoculum and presence or absence of Tr5.

S. c	No *T. koningii* (Tr5)				+ *T. koningii* (Tr5)				
	Emerg	**% Inf**	**St Dev**	**dep%**	**Emerg**	**% Inf**	**St Dev**	**dep%**	**% Dec**
0	11.55 bc	0.00			8.9 d	0.00			
4	10.3 cd	17.05a	16.13		11.45 bc	7.47a	13.00		56.2*
7	12.2 bc	9.88ab	12.12	42.0%	10.75 cd	2.19ab	4.53	70.6	77.9*
10	12.9 ab	7.50b	16.38	56.0%	12.75 ab	3.07ab	9.09	58.9	59.1
20	14.15a	2.36b	5.25	86.1%	11.9 bc	1.33b	3.37	82.1	44.6
		lsd 0.05 =8.4				lsd 0.05 =5.3			

Notes to Table 16(F):.
S. c= Depth at which a layer of 38mg of *S. cepivorum* sclerotia was buried in the pots.
Emerg= Average number of emerged seedlings from 20 seeds sown
% inf= percentage of seedlings which became infected by *S. cepivorum*.
St Dev= standard deviation
dep%= percentage decrease in the of the number infections compared to that at 4cm.
% Dec= percentage decrease in infection for that depth stratum following amendment of Tr5.
* in % Dec column indicates significant decrease in infection in Tr5 amended treatment compared to unamended treatment of equivalent depth.
Numbers in the % inf columns followed by the same letter do not differ significantly according to LSD(0.05).

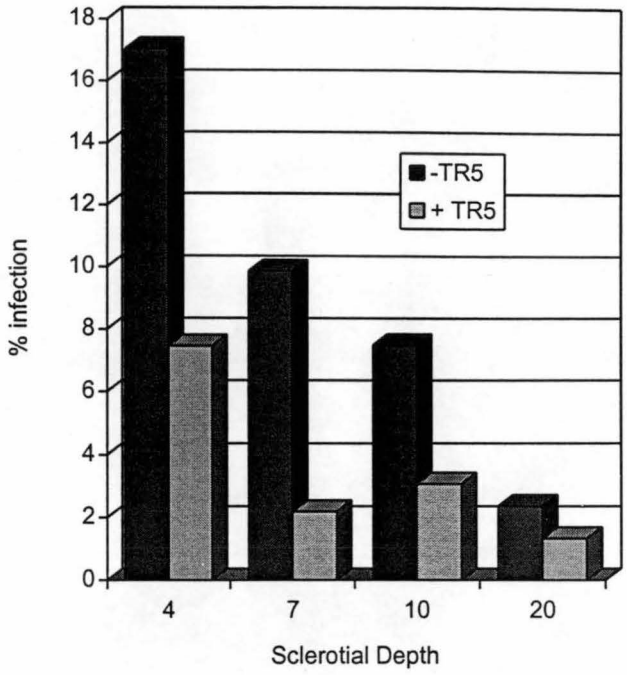


Figure 16(G): Relationship between the disease incidence caused by *S. cepivorum* inoculum buried at four depths in the soil and corresponding level of disease when Tr5 was added.

Table 16(H): Two way analysis of variance on *S. cepivorum* disease incidence in inoculum depth trial 2.

Strata	SS	df	MS	F	P-value	F.crit
Depth	2092.24	3	697.41	5.676	0.0010342	2.664
Tr5	1266.18	1	1266.18	10.306	0.00161746	3.903
Interaction	539.90	3	179.96	1.464	0.22638382	2.664
Error	18673.20	152	122.85			
Total	22571.53	159				

16.3.2.1. Survival of Buried Sclerotia.

The average number of sclerotia which were assessed to be healthy from the 20 replicate bags from each treatment is presented in Table 16(I).

Table 16(I): Average number of sclerotia assessed to be healthy from 30 originally buried in nylon mesh bags in the inoculum depth trial pots at 4, 7, 10 or 20 cm in pots amended or unamended with Tr5 and with and without onions.

	Depth	+ Onions	- Onions	Onion mean
Tr5 -	4	7.45def	8.5cde	7.96
	7	10.3abc	10.2bc	10.25
	10	6.05f	10.5abc	8.2
	20	12.4ab	7.3def	9.85
	mean	9.05	9.12	9.07
Tr5 +	4	7.15ef	8.3cde	7.72
	7	6.95ef	6.0f	6.47
	10	7.6def	7.6def	7.6
	20	9.5cd	12.6a	11.05
	mean	8.42	8.86	8.21
Tr5+ & - mean				
	4	7.3	8.4	7.84B
	7	8.6	8.1	8.36B
	10	6.3	9.05	7.93B
	20	10.95	9.95	10.4A
	mean	8.28Notes to Table 20(E):	8.87	**8.6**

Numbers followed by the same letter do not differ according to LSD(P=0.05)
-lower case letters represent LSD test on all data
-upper case letters represent LSD test between depth strata
-Tr5 + and Tr5 - strata were not significantly different.

Table 16(J): Analysis of variance of the buried sclerotia survival data.

Source	df	SS	MS	Fcrit	F tab (0.05)
Onion	1	16.2	16.2	1.17	3.84
Tr5	1	61.25	61.25	4.43*	3.84
Depth	3	375.62	125.20	9.07***	2.63
Onion:Tr5	1	11.2	11	0.81	3.84
Onion:Depth	3	131.81	43.93	3.18**	2.63
Tr5:Depth	3	262.68	87.56	6.34***	2.63
Onion:Tr5:Depth	3	427.84	142.61	10.33***	2.63
Error	304	4224.2	13.8		
Total	319	5510.8			

*= evidence of significant differences at P=0.05 level
**=evidence of significant differences at P=0.025 level
***= evidence of significant differences at P=0.005 level

The range of individual observations which Table 16(I) represents were highly variable, and preliminary examination of the means did not implicate any likely trends. Around two thirds of sclerotia decayed in all treatments irrespective of

presence of onions or Tr5. Green sporulation was seen on the surface of some decayed sclerotia. Some of these were placed on RASP medium and *Trichoderma* spp. morphologically different to Tr5 were isolated.

Further examination was made using factorial analysis of variance (Figure 16.J.) to examine interactions between the three strata. The ANOVA implied that there was evidence of differences according to the presence of Tr5, however analysis using the LSD showed that there was no significant difference between numbers of surviving sclerotia in the presence or absence of Tr5 across the overall depth and onion strata.

The ANOVA also implicated depth as a strata where there was highly significant evidence of differences among data sets. Examination of this stratum independently reveals that the sclerotial survival was significantly greater at 20cm than the three higher depths. It warrants mention that the figures represent only an overall 8.8% (26% as opposed to 34.8%) higher sclerotial survival at the 20 cm depth.

There was no evidence of interaction between the presence or absence of onions, or of Tr5. However the anova implicated that sclerotial depth interacted with both of these strata. Examination of the interaction is presented in Table 16(K) which shows evidence of significantly higher sclerotial viability at the 7cm level in absence of Tr5 but not at any other level. Examination of the Onion:Depth interaction is presented in Table 16(L) which shows that there were no significant differences between sclerotial survival in the presence or absence of onions at each depth. The differences among means implicated by the anova seem to reflect the higher sclerotial survival at 20 cm. -Onion totals exceed +Onion totals at 4 and 10, but not 7 and 20cm, there is no evidence of real differences among means.

Table 16(K): Examination of the Tr5:depth interaction.

Scler Depth	- Tr5	+ Tr5	(-T)-(+T)	SS	Fcalc	F0.05
4	319	309	10	1.25	0.03	2.68
7	410	259	151	285.5	6.89***	2.68
10	331	304	27	9.11	0.22	2.68
20	394	442	-48	28.8	0.69	2.68

Note: SS derived by $[(-T)-(+T)]^2$/treatments x reps.
df=3 and 319
Error Mean Square =13.8
***= evidence of real differences among means at 0.005 level

Table 16(L): Examination of the onions:depth interaction.

Scler Depth	- O	+ O	(-O) -(+O)	SS	Fcalc	F0.05
4	336	292	44	50.62	0.584	2.68
7	324	345	-21	5.51	0.133	2.68
10	362	273	89	99	2.39	2.68
20	398	438	-40	20	0.483	2.68

Note: SS derived by $[(-O)-(+O)]^2$/treatments x reps.
df=3 and 319
Error Mean Square =13.8

16.3.2.1. Rhizosphere colonisation.

Table 16(M) displays the average frequency of isolation of *Trichoderma* spp. and of isolates morphologically indistinguishable from Tr5 which were isolated from root samples taken in the Tr5 present (+) and Tr5 absent (-) non infected controls. Colonisation by Tr5 type isolates was detected on 89 of the total 100 samples. In the untreated control two Tr5 type isolates were detected. The total *Trichoderma* spp. isolation was significantly greater in Tr5 inoculated pots. On four occasions more than one isolate was present on the one plate (colonial boundaries were invariably distinct) and in this case two isolations were recorded. In three cases no *Trichoderma* spp. were detected.

Table 16(M): Average number of *Trichoderma* spp. and of isolates morphologically similar to Tr5 which were isolated from 5 root samples taken from each pot in treatments A and F.

Treatment	Tr5	Total Isol^{n} *	Tr5 type*	% Tr5 +
A	+	5.1a	4.45a	89%
F	-	3.7b	0.1b	2%

*numbers followed by same letter not significantly different according to LSD (0.05)

To test the accuracy of the morphological assessment 40 isolates from each treatment were isozyme profiled for polygalacturonase and pectinesterase activity. Tr5's profiles have been characterised (Chapter IV:10) and production of any bands not known to be produced by Tr5 resulted in exclusion of the isolate. Of 33 isolates thought to be of Tr5 type, all were found to produce only isozymes known in Tr5. Of 48 isolates morphologically assessed to be other than Tr5, twelve produced no bands which would not have excluded them from the Tr5 type group had they been incorrectly assessed. Figure 16(N) is an example of a pectinase gel in which 20

bands which would not have excluded them from the Tr5 type group had they been incorrectly assessed. Figure 16(N) is an example of a pectinase gel in which 20 *Trichoderma* isolates collected from treatment F, and all morphologically assessed to be other than Tr5 type were isozyme profiled. Isolates F101d, F102b, F105e, F106a, F108a and F110c display profiles which would not have excluded them from the Tr5 type group had they been assessed to be of that group.

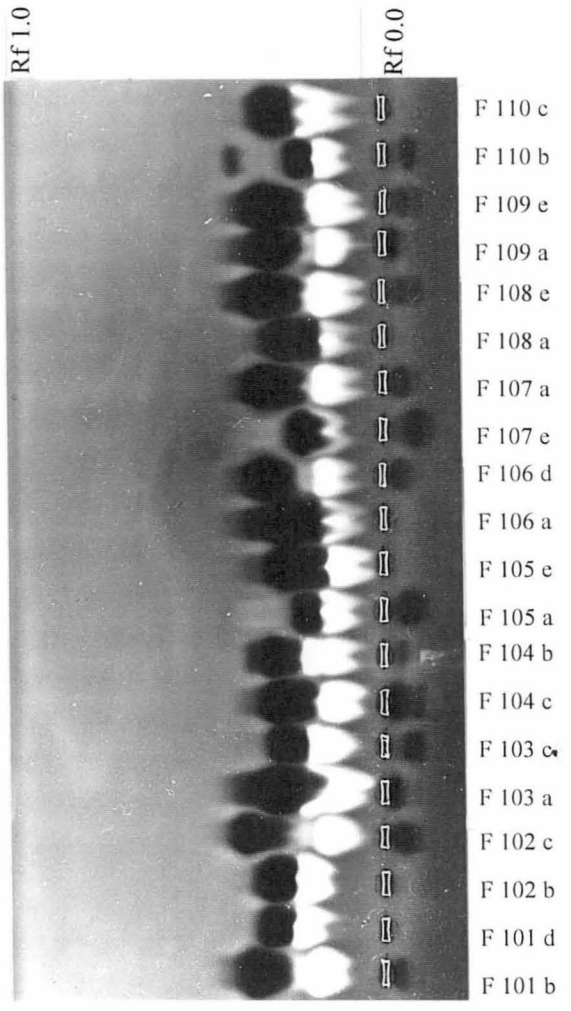


Figure 16(N): Polyacrylamide gel electrophoresis for the detection of pectinase. Light zones=polygalacturonase, dark zones indicate pectinesterase. A range of *Trichoderma* spp. assessed morphologically to be different to Tr5 were isozymprofiled. All produced some bands not known to be produced by Tr5 with the exceptions of isolates F101d, F102b, F105e, F106a, F108a and F110c.

16.4. Discussion:

The findings concerning the influence of sclerotial depth on infection have demonstrated that there was a significant decrease in infection as sclerotia were buried deeper in the soil. This is in general agreement with the findings of Crowe and Hall (1980a) who recorded the greatest number of garlic infection loci (groups of infected adjacent plants) when sclerotia were buried at a depth of 5cm. It was suggested that infection decreased from sclerotia in the top 2.5 cm, because root density was low, and root to root spread was limited. One area of discrepancy between this and the data of Crowe and Hall (1980a), is in the infections from sclerotia buried at 10cm. In the trials of Crowe and Hall (1980a) infection from deeper inoculum occurred late in the season when plants had large root systems. Individual loci included four times as many plants at 10cm as at 5cm, but were less common. In contrast, in trial 1 there was negligible infection from inoculum depths of 10cm in a trial of similar timescale. In general, Crowe and Hall considered differences in infection from sclerotia at different depths to be related to extensive root to root spread when sclerotia germinated in the layer where root density was highest, and they did not regard the greater distance an infection had to travel from greater depths (and consequent greater opportunity for suppressive antagonists) to be an important factor. In potting mix used in trial 1 (which did contain indigenous *Trichoderma* spp.), natural antagonism may have played a greater role than in soil used by Crowe and Hall (1980a), in limiting root to root spread.

In trial 1, developing infections within roots had to pass a band pre-colonised by Tr5 inoculum to reach the bulb, in addition to saprophytically dispersed Tr5 inoculum. In field trials infections in Tr5 treated strata could be interpreted as; a) poor efficacy, or b) poor Tr5 establishment. However, in this trial, all roots had to make contact with Tr5 inoculum. The range of results obtained using this experimental design would indicate that while Tr5 suppresses 82 to 95% of infections, a proportion of infections originating at both 4 and 7 cm manage to pass Tr5. Infection then multiplied by the process of root to root spread (Scott, 1956b) results in around 10% of the untreated level of infections. The proportion of infections which passed through the Tr5 layer was reasonably proportional between sclerotial depths (eg the percentage reductions in infection for treatments Z and ZA compared to 4 and 7 cm untreated controls were 95 and 87% respectively).

An early goal of the Tr5 biocontrol project was to investigate whether addition of complex carbon sources to soil with Tr5 would give it a selective advantage over other soil organisms (Harman *et al.* 1981). It was hypothesised that addition of chitin might stimulate production of Tr5 chitinases likely to be involved in antagonism. The disaccharide and polysaccharide carbon sources on which Tr5

has been grown in this work have not provided better control than when Tr5 was added with no added carbon source grown on Mineral Salts Agar. However the rationale of carbon source addition was to serve as a food base for radial colonisation (excepting chitin) and inoculum addition as a complete agar layer was not a test of this.

Investigation of the effect of adding the complex carbon sources in absence of Tr5 was not made due to time and labour limitations. However, the evidence that Tr5 in treatments Z and Za (with no added carbon source) provided decreases in disease not significantly different (and in most cases better) than the complex carbon source treatments, suggests that the infection decreases are not due to amendment of the carbon sources in isolation.

In trial 2 the use of Krasnozem soil rather than potting mix more closely reflects commercial onion growing. Tr5 was added to soil as millet inoculum, rather than on a range of complex carbohydrate carriers. In the strata not amended with Tr5, emergence has tended to increase as the depth of inoculum increased. While it is possible that some plants may have been infected before emergence assessment, emergence in the non-infected control was not significantly different to any but the 20cm treatment, so the trend is most likely due to random occurrence. The trend is not clearly evident in the Tr5 treated strata, though it might be expected to be less evident subject to the efficacy of Tr5.

The overall trend detected in trial 1, for disease incidence caused by deeper sclerotia to be lower is supported by the results of trial 2. However it is less pronounced. In Trial 1, disease incidence in the 4cm untreated control was more severe (43.9%) than trial two (17.05%), though disease incidence at 7cm was comparable (7.46% vs 9.88%). In Trial 1 sclerotia buried at 10cm caused 0.92% infection, but infection from 10cm was greater (7.5%) in trial 2. The 20cm treatment was absent in trial 1, though trial 2 demonstrated that a small proportion of disease was induced by infections originating at this depth. Plant density may explain part of the disease incidence differences. The average spacing was one seedling per $4.8cm^2$ in trial 1 compared to one seedling per $30.5cm^2$ in trial 2. Root to root spread would then be expected to have had greater opportunity at the higher plant density in trial 1, although trial 2 was of longer duration (29 as opposed to 17 weeks) and onion root systems are known to increase in density per cm^3 with the age of the plants (Greenwood *et al.*, 1982). Root constriction in pots confounds this issue.

The root medium is likely also, to play a role in the differences in disease incidence. The soil used for Trial 2 hosted numerous isolates of *Trichoderma* spp, One isolate from this soil (Td22) was evaluated as a biological control agent in the following seasons in field trials, and provided 92% disease suppression (Dennis *et al.*, 1997). It is difficult to interpret the extent to which natural suppression may be

involved in low disease incidence. The buried sclerotia assay has shown that extensive sclerotial decay occurred in this soil under all treatments.

Sclerotia buried at 7cm resulted in 42% less disease than at 4cm, however placing sclerotia at 10cm resulted in only 24% less disease than at 7cm, which was not significantly lower as might be expected from the results of Trial 1. Disease incidence in Trial 1 may have been influenced by 18% higher emergence in the 10 than the 7 cm treatment, which would provide greater opportunity for root to root spread. In Trial 2 a significantly lower number of sclerotia remained viable at 10 than 7 cm, so sclerotial germination may have been greater and plants more severely challenged. Another explanation may be provided by the root density at differing depths. In garlic, root density is highest in the 5 to 7.5 cm band, and infections originating below this must pass this band. Spread of infection between roots in this band results in a higher numbers of plants per disease locus (Crowe and Hall, 1980a). Onions and garlic have similarly structured root systems (Brewster ,1994), and so root to root spread as infections reached the zone of higher root density could have resulted in the similar disease incidence from these two treatments.

The rhizosphere colonisation study conducted in trial 2 used samples from the non-infected controls taken at the time of harvest. This method had the advantage of sampling from a full population, rather than only from those onions which survived (possibly due to Tr5's presence). The disadvantage is that there is not an opportunity to compare Tr5 root colonisation to disease incidence in individual pots. *Trichoderma* spp. were isolated from 74% of roots in untreated controls. Tr5 had not extensively spread to untreated controls, as only 2% of these roots were colonised by Tr5 type isolates. The presence of so many native *Trichoderma* spp. provides one plausible explanation for the low disease incidence in untreated controls. The large proportion of sclerotia in mesh bags which were in decayed condition may also have been influenced by native *Trichoderma* spp. The isolation of *Trichoderma* spp. from decayed sclerotia adds to this hypothesis.

Trichoderma isolates which could not be distinguished from Tr5 were isolated from 89% of root samples taken from treatment A. It has not been proven that these isolates were Tr5. However, if we allow this assumption, this result implies that around one in ten developing infections would not be challenged by Tr5 (although root samples were only 1cm long and Tr5 might have colonised lower regions of the root). The influence of addition of Tr5 to the rhizosphere on native *Trichoderma* spp. is not known. The isolation of Tr5 type and native type isolates from the same root segment implies that Tr5 did not entirely dominate the soil ecological niche which *Trichoderma* spp. occupy, yet on the RASP medium plate colonies invariably defined individual territories implying competition and incompatibility. The isolation of native isolates from 74% of root segments in

unamended controls and native isolates from 13% of root samples from Tr5 treated pots could be interpreted to mean that Tr5 suppressed native species, or Tr5 may out compete other *Trichoderma* isolates on the RASP medium, which was specifically designed for optimum Tr5 growth.

In Trial 2 disease incidence in the 4cm sclerotial depth treatment was significantly reduced (56.2%) by addition of Tr5, however this was lower than the corresponding reduction (82-95%) in trial 1. The rhizosphere colonisation study has suggested that Tr5 type isolates were present on around 89% of roots, and consequently, it is suggested that infections of the remaining roots by *S. cepivorum* are less likely to be challenged and more easily reach the bulb. In trial 1 inoculum was added as a complete layer so it is feasible that all roots were colonised by Tr5. In trial 2 infections which were able to reach the baseplate may have been able to spread directly from bulb to bulb, as bulbs often touched, particularly at the base. Infections were recorded in only seven treatment B pots (compared to fifteen treatment G pots), five of which had multiple infection. In the temperature trial (Ch. VI.20) photographs were taken of above ground mycelium spreading between seedlings. The phenomenon has also been described by Entwistle (1990).

Tr5 amendment had greater efficacy in preventing infections originating at 7cm than 4cm. The deeper infection point would mean that infections must travel further through the root cortex to reach the base plate, allowing greater opportunity for Tr5 to colonise the damaged and infected tissues and cut off the infection. This further decreases the opportunity for infection by root to root spread in this zone of high root density. In the top few centimetres of the pot the soil may dry out faster than in the rest of the pot, activity of *Trichoderma* spp. may decrease as matric potential of soil dries from -2 to -14 bars (Eastburn and Butler, 1991). In the 0 to 4 cm layer, growth may be more often disrupted than in the 4 to 7cm layer where many infections originating from 7cm may be challenged. The reduction in disease is again lower than the 89% root colonisation would lead us to anticipate, so a proportion of infections must be out competing Tr5 from this depth as well. It is noteworthy that examination of the Tr5:Depth interaction (see Table 16.K.) showed that 7cm was the only band where there was a significant decrease in the number of sclerotia which survived burial in the presence of Tr5. This was the first evidence encountered that Tr5 may have activity as a sclerotial parasite. This result was not expected, as the reduction in survival from 34.1% to 21.6% seems unlikely to be a significant suppressive force in a soil where 66% of sclerotia decayed in absence of both Tr5 and *Allium* roots. However the result does suggest that Tr5 was most active in the 7cm layer, which is quite consistent with disease incidence. It is well established that *Trichoderma* spp. populations vary exponentially at different depths in the soil profile. Soil pH, temperature and plant spp. were shown to alter the

population depth relationship (Ahmad and Baker, 1987a). *Trichoderma* spp. populations were usually 10-100 fold lower (according to colony forming units) in the top 3cm than in the 4-8 cm layer (Ahmad and Baker, 1987a; Sivan and Chet, 1989b), though another study found few *T. harzianum* propagules more than 3cm below the seed (Chao *et al.*,1986).

The level of suppression of infections originating from 10cm by addition of Tr5 was lower than for infections originating at 7cm (59.1%), this was not significantly different from disease incidence in Tr5's presence from 7cm. Disease incidence was so low in the 20cm untreated control that comparison is difficult to draw, and extra caution is necessary due to the significantly differing emergence figures.

The initial goal of burying sclerotia in nylon mesh bags in each pot was to determine whether observed differences in disease incidence could be attributed to differences in sclerotial germination. This was not successful as differences in sclerotial survival were not detected even in presence or absence of onions. The interactions detected at 7cm is supported by the observed disease control at this depth. However it should be taken in caution, as in a data set where so many potential interactions are present, there is always a chance that one interaction will randomly occur which supports an expected outcome. It is interesting to note that across all other strata, the sclerotial viability was significantly greater for sclerotia buried at 20cm which provides some evidence that lower sclerotial germination occurred at this depth which might be correlated with the lower disease incidence. Certainly these sclerotia are the most removed from both influencing factors (ie Tr5 and onions), it is therefore frustrating that the lowest sclerotial survival within the 20cm stratum was recorded in pots subject to neither influence.

The extensive sclerotial decay detected across all treatment strata was higher than expected. Decline in sclerotial numbers can be accounted for by the combined processes of sclerotial germination due to *Allium* exudates and sclerotial decay due to mycoparasitism. Comparable studies have reported 80% germination of sclerotia over 20 weeks of burial 7.5cm below onion plants (Coley-Smith, 1985), which is consistent with the results of this study, except for treatments where sclerotia were buried in the absence of onions, where no real differences between the numbers of sclerotia which remained viable in the presence or absence of onions were found. It is possible that some sclerotial germination occurred in the absence of onions due to volatile *Allium* exudates from neighbouring pots, though it seems unlikely that the stimulus would induce similar levels of germination to when sclerotia were actually among onion roots. The mesh bags normally contained the remnants of many partially decomposed sclerotia, and this combined with occasional observation of green sporulation from which *Trichoderma* spp. were isolated would suggest an

alternate explanation that the great majority of sclerotia were parasitised, and did not germinate. While it is known that sclerotia can survive for many years (Coley-Smith *et al.*, 1990), it is also known that as many as 85% of sclerotia can decay in the first three months following burial in soil, and those which survive this period seem to be of greater longevity (Alexander and Stewart, 1994). An extensive population of native *Trichoderma* spp. was known to be present in this soil, some of which may be sclerotial mycoparasites. If the latter explanation were correct, the reason for no difference between sclerotial recovery from pots with and without onions is probably that many sclerotia were parasitised before they sensed the germination stimulus (as most disease was not evident until a few weeks before harvest), and mycoparasitic decay has overshadowed the decline in numbers due to germination as only 30% of the sclerotia remained in a viable state when they began to germinate.

Chapter VI: Ecological studies.

17.0: The relationships of *S. cepivorum* inoculum density to *T. koningii* biocontrol efficacy.

17.1. Introduction:

When white rot is first encountered in a paddock the number of infections is tolerably low and confined to a small patch. In subsequent crops inoculum density increases more than ten fold. The percentage of infections is proportional to inoculum density, for example increasing from 7.5% at a sclerotial density of 0.4 /Kg of soil, to 53.1% at 17 /kg and 94.9% at 200/Kg (Crowe *et al.*, 1980). This trend loosely agrees with findings of field trials run as part of this study (See Ch V.), though other influences are important (eg soil moisture and temperature).

It would be desirable to be able to assess the sclerotial density at a site, and use this information to assist in making decisions on whether biological control is likely to be an effective control alternative, as a sclerotial density threshold may exist beyond which biocontrol is not an effective option. The aim of the trial was to relate the amount of inoculum in the soil to disease incidence, and determine the range of *T. koningii* 's efficacy in relation to inoculum density.

17.2. Materials and Methods:

17.2.1. Preparation.

Pots and soil were as for Inoculum Depth Trial 2 (Ch.VI:16). To determine volumes of inoculum to add to pots 5 lots of 10 mg of sclerotia were counted and determined to contain a mean of 105 sclerotia. Rates of inoculum density used were 0, 10, 25, 50 and 100 sclerotia per kg of soil and accordingly lots of 0, 7.6, 19, 38, and 76 mg of sclerotia were added (with 5g NPK [8:4:10] fertiliser) to 8kg lots of soil and thoroughly mixed by tipping between two trays then into the soil bags.

T. koningii (Tr5) inoculum was produced by the methods described in Appendix A.1. (Millet Inoculum). Tr5 millet inoculum was added at 0g, 5g (1590 kg/ha), 10g (3180 kg/ha) and 15g (4770 kg/ha). A summary of treatments appears in Table 17(A).

Table 17(A): Treatment list for the inoculum density trial showing pot numbers, sclerotia per kilogram and volume of *T. koningii* inoculum.

Treatment(Pot Nos)	Sclerotia / Kg	Tr5 (grams)
A (521-530)	0	0
B (531-540)	0	5
C (541-550)	0	10
D (551-560)	0	15
E (561-570)	10	0
F (571-580)	10	5
G (581-590)	25	0
H (591-600)	25	5
I (601-610)	50	0
J (611-620)	50	5
K (621-630)	50	10
L (631-640)	50	15
M (641-650)	100	0
N (651-660)	100	5
O (661-670)	100	10
P (671-680)	100	15

Trials were sown on 24/9/95 (spring). Treatments were replicated ten times. Twenty seeds were sown per pot with the intention of thinning to 10 after emergence. Tr5 inoculum was sprinkled evenly among the seed which was then covered with 1cm of soil.

The pots were arranged randomly. To avoid concerns that pots at the outside of the block would be exposed to higher soil temperature by sunlight on the black plastic, the outer pots were surrounded by hessian bags filled with pine bark.

The ability of *T. koningii* to spread from treated to untreated control pots was of concern, so all untreated pots were grouped together in five separate groups at regular spacing through the block of treated pots, separated by hessian bags filled with pine bark.

17.2.2. Assessment of disease.

Emergence was recorded six weeks after sowing. Pots were examined for disease at fortnightly intervals and infected seedlings marked using white nursery tags. Seedlings which were killed by agents other than *S. cepivorum* (eg *Botrytis* or insects) were recorded separately. Destructive sampling was made on 16/4/96 (29 weeks after sowing).

17.2.3. Assessment of Tr5 root colonisation.

Assessment of the proportion of roots on which *T. koningii* had become established was made directly after each onion was uprooted for treatments A, B, C, D, M, N, O and P (400 samples in all) by methods described in Appendix G. To test the accuracy of the morphological assessment the polygalacturonase, pectinesterase and ribonuclease isozyme profiles of a subsample of 13 randomly selected isolates from each treatment were examined. A $2mm^2$ piece of inoculum from each test isolate was added to 2ml of pectinase medium (Appendix B.3.) in a 5ml Bijou bottle and incubated for 7 days at 25°C. Electrophoresis was performed by the methods of Cruickshank and Pitt (1987) (Appendix E.1.). Isozymes of pectinase and ribonuclease were compared to the isozymes known to be produced by Tr5 through its life cycle (Chapter IV; 10). Production of any bands not known to be produced by Tr5 resulted in exclusion of the isolate.

17.2.4. Analysis.

Statistical analysis of emergence was performed using the One Way Analysis of Variance and Least Significant Difference of means calculated as $LSD=t^{0.05(df)}Ã(2\{S_Y\}/n)$ where S_Y= the error mean square, $t^{0.05(df)}$= degrees of freedom and n = replicate number. For analysis of percentage infection data any values from pots where five or less seedlings had emerged were discarded (if no seedlings emerged as happened in two replicates the % wilt of zero was not a fair value). Pairwise comparisons were made where uneven replicate numbers were involved, LSD calculated as $t^{0.05(df)}Ã(1/n_1 + 1/n_2)Ã(S_Y)$.

17.3. Results:

17.3.1. Seedling Infection.

The results of seedling emergence and incidence of *S. cepivorum* infection under conditions of 0,10,25,50, and 100 sclerotia /Kg soil, and with *T. koningii* applied at three rates are presented in Table 17(B). The percentage emergence was disappointing (39-63%) so thinning to even numbers was omitted. Two treatments (O & P) where *S. cepivorum* was applied at the highest inoculum density and Tr5 was applied at 10 and 15 g/pot displayed significantly lower emergence than when Tr5 was applied at 5 g/pot (N) and not applied (O). Although the trend is not

significantly different from untreated controls in any other treatments the average emergence did tend to be slightly lower at the higher Tr5 rates.

The incidence of disease in all untreated controls was lower than expected. The disease incidence at 10 sclerotia /kg was significantly lower (47%) in comparison to that at 25 sclerotia /kg. The percentage disease might have been expected to increase further for the 50 and 100 sclerotia /Kg treatments, however no significant increase in infection was recorded and the actual figures were lower.

At the lower two sclerotial densities Tr5 was applied only at the lower rate. At 10 sclerotia /Kg the percentage of infections in the unamended control was reduced by 78.5% when Tr5 was present. This is not significantly different within the overall body of data. At 25 sclerotia / kg disease in the Tr5 amended treatment was reduced from 27.5% to 5.7% (significantly different), a percentage infection reduction almost identical to that at 10 sclerotia / Kg soil.

In Treatments I, J, K, and L where sclerotial density was 50 sclerotia / Kg, disease was reduced from 19.4% to 6.6% at the 5g rate and further to 5.1% at the 10g rate. At the 15g rate infection was higher (7.1%) though this increase was not significant.

At the 100 sclerotia / Kg density there is a question of whether comparison of treatments O and P to the untreated control would be valid, as emergence is significantly lower. At this sclerotial concentration the percentage of infection was reduced by 74% at the 5g Tr5 rate than in the unamended control.

Table 17(B): Effects of sclerotial inoculum density and addition of *T. koningii* millet inoculum on *S. cepivorum* infections.

Treatment	sclerotia /kg	Tr5 Millet	Emergence	% Infection	%wilt reduction
A	0	0	12.5a	0	
B	0	5	11.1a	0	
C	0	10	10.4ab	0	
D	0	15	10.6ab	0	
E	10	0	12.5a	12.75bc	
F	10	5	10.8ab	2.73c	78.5
G	25	0	12.3a	27.53a	
H	25	5	10.3ab	5.78c	79.0
I	50	0	12.4a	19.40ab	
J	50	5	10.9a	6.62c	65.8
K	50	10	10ab	5.10c	73.7
L	50	15	10.6ab	7.12c	63.2
M	100	0	12a	21.29ab	
N	100	5	11.3a	5.50c	74.1
O	100	10	7.8b	5.15c	75.8 *
P	100	15	7.8b	4.88c	77.0 *

-Values within the same column followed by the same letter are not significantly different.
- Not a valid comparison due to significantly differing emergence.

17.3.2. Root Colonisation and isolate identification.

Table 17(C) shows the average number of *Trichoderma* isolates, and those of Tr5 type morphology isolated from roots. From eight of the one hundred samples taken from untreated controls, isolates which were morphologically indistinguishable from Tr5 were isolated. At all three rates of Tr5 addition, the Tr5 like isolates were present on at least 92% of root samples. The frequency of isolation of native *Trichoderma* spp. from untreated controls was higher than expected. These were not studied in detail though at least ten morphological types could be distinguished. Occasionally plates were encountered where two *Trichoderma* isolates had colonised separate parts of a plate with distinct boundaries. Both were counted in this case which has resulted in mean total isolation figures in excess of 5 in treatments C and D.

Table 17(C): Average number of *Trichoderma* spp. and of isolates morphologically similar to Tr5 which were isolated from 5 root samples taken from each pot in eight treatments in the inoculum density trial.

Treatment	Tr5 rate	Total Isol^{n}#	Tr5 type*	% Tr5 type
A	0	3.3 b	0.3 b	6 %
B	5g	4.8 a	4.8 a	96 %
C	10g	5.3 a	4.9 a	98 %
D	15g	5.3 a	4.8 a	96 %
M	0	2.6 b	0.5 b	10 %
N	5g	5 a	4.9 a	98 %
O	10g	5 a	4.6 a	92 %
P	15g	5 a	4.7 a	94 %

* Values followed by the same letter are not significantly different. LSD(t=0.05)=0.596
# Values followed by the same letter are not significantly different. LSD(t=0.05)=0.818
-Values greater than 5 have sometimes been obtained where more than one isolate grew from a single sample
Isol^{n}=Total number of Trichoderma spp. isolations.

To test the accuracy of the morphological assessments, isozyme profiles for polygalacturonase and pectinesterase were performed. The isozymes produced by Tr5 through its life cycle have been characterised (Chapter IV:10) and production of any bands not known to be produced by Tr5 resulted in exclusion of the isolate. Figure 17(D) is an example of a gel where these profiles have been examined. Isolate P671^{e} is an example of an isolate eliminated from the list, its profile being similar to Td9 (Chapter IV:10). In total 100 isolates were profiled. Of 77 isolates assessed to be morphologically similar to Tr5, two produced banding patterns which were different to that produced by Tr5. Of 23 isolates assessed to be morphologically different to Tr5, 17 produced isozymes not known to be produced by Tr5, and six produced isozymes indistinguishable from Tr5.

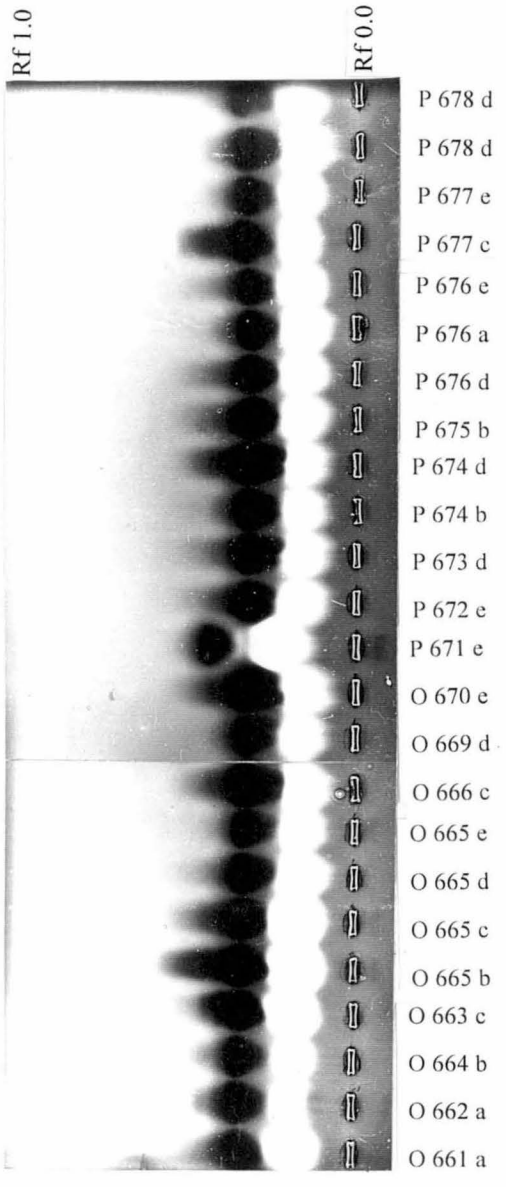


Figure 17(D). Polyacrylamide electrophoresis gel for the detection of polygalacturonase (PG, dark zones) and pectinesterase (PE, light zones). Profiles are from randomly selected isolates from treatments O and P. All isolates profiled here were assessed to be morphologically of the Tr5 type. One isolate ($P671^{e}$) displayed a PE (R_f 0.30) and PG (R_f -0.05) which are not known to be produced by Tr5.

17.4. Discussion:

In trials run at field sites in 1994/5 season, sample isolations indicated that the proportion of roots on which Tr5 became established was usually less than 20%. For this reason the rate of inoculum addition was increased on that previously used. The 5g rate would introduce 0.06% by weight of inoculum carrier to the soil. Comparable rates have been used in similar studies, (eg 0.1%, Kay and Stewart, 1994a), though the number of colony forming units per gram of carrier probably differs.

Although the trend is only significant at the highest sclerotial concentration, emergence has tended to be slightly lower when Tr5 was present than when it was absent. This is a trend which should be monitored closely in future work. Although, even if Tr5 was causing slightly lower emergence, this would not be a deleterious concern for commercial production in any case (D. Faulkner, pers comm). It has been established that Tr5 is inclined to actively colonise and degrade health compromised onion root tissue, and how Tr5 might react to presence of sublethal seed borne pathogens (eg *Botrytis alli*) will be investigated in future work.

The percentage of disease in untreated controls at 50 and 100 sclerotia per kilogram of soil is lower than expected given that percentage disease was 27.5% at 25 sclerotia / Kg. At similar densities higher infection rates have been reported (87% at 68 sclerotia /kg (Crowe *et al.,* 1980); 68% at 10 sclerotia/kg (Hall and Sommerville, 1983). A high level of variability seems characteristic of the disease and differences in age, moisture and temperature history affect the proportion of sclerotia which germinate (Gerbrandy, 1992 ; Coley-Smith *et al.,* 1987; Papavizas, 1977; Brix and Zinkernagel, 1992a). Once one seedling within a replicate became infected there is an increased probability of other infections due to root to root spread, exemplified in treatment H where three replications had at least two infections, while the rest had none.

From results of previous field trials it was expected that there might be an overall decrease in the efficacy of the biocontrol agent as *S. cepivorum* inoculum density increased. However the percentage of wilt reduction in Tr5 treated pots in comparison to untreated controls has been relatively constant at between 63.2 to 79.0%. There would seem to be no improvement in efficacy beyond that achieved at the lowest rate of Tr5 addition. This result would seem to be explained by the root isolation data in Table 17(C) which shows that root colonisation by isolates of Tr5 type averaged at 97% at the lowest rate of Tr5 addition, and was not significantly different at the higher rates. With 97% of roots colonised by Tr5 type organisms at the lower rate of inoculum, addition an efficacy improvement would be unlikely. The amount of inoculum required for full root colonisation is obviously less than the

five gram rate, and an efficacy gradient might more likely be demonstrated if 5g had been the highest of the three rates. However, this relationship may vary in different seasons and under different environmental conditions and in different soils.

The morphological assessment used to differentiate Tr5 from other *Trichoderma* isolates is by no means an absolute measure of colony identification. In two of 77 isolates (2.5%) an incorrect assessment was made, the actual error is possibly a little higher, as six of 23 isolates (26%) morphologically different from *T. koningii* produced isozymes which would not have excluded them from the Tr5 type group had an incorrect morphological assessment been made.

The root colonisation data in Table 17(C) indicates that there was 8% colonisation of the two untreated controls by Tr5 type isolates. Unfortunately it is not known whether Tr5 colonised these pots by airborne spore dispersal, or the Tr5 type was native to the soil at a low density (which is a reasonable explanation as Tr5 was first isolated from soils from this district). Molecular analysis (eg PCR) might further determine if these isolates were genetically homologous to Tr5, however this would not resolve the question of their origin.

A significantly lower proportion of total *Trichoderma* spp. were isolated from roots in untreated controls (average 2.95 of 5) than in Tr5 treated pots. This data does show however that almost 60% of root samples were colonised by various *Trichoderma* spp. where no Tr5 amendment was used. The level of natural suppression which these species may provide is unknown. However, in screening for biocontrol agents for future studies (data not presented), seven out of ten of *Trichoderma* spp. isolates from these untreated controls caused lysis of *S. cepivorum in vitro* of type B (Ghaffar, 1969; Harrison and Stewart, 1988), with lysis of hyphal tips and degradation of hyphae. It is possible that the soil used has some level of natural suppression and this hypothesis would be generally supported by the low level of disease in untreated controls. This implies that addition of Tr5 merely tops up existing suppression, and disease incidence might have been higher in a soil with a lower indigenous *Trichoderma* population. The soil had not been used for commercial cropping for several years, and might have hosted a more diverse and less disrupted soil ecosystem than frequently cropped soil.

It is unfortunate that the higher range of sclerotial densities examined in the study did not induce 100% disease, as the goals of the trial had been to establish whether some sclerotial density threshold existed beyond which Tr5 was of limited efficacy. Similar trials in soil with a history of chemical application and cultivation would make an interesting comparison.

Chapter VI: Ecological studies:

18.0: The effects of soil pH on *Sclerotium cepivorum* infection and biological control by *T. koningii.*

18.1. Introduction:

Trichoderma spp. are well documented as being enhanced by acidic soil conditions (Chet and Baker, 1980; Chet and Baker, 1981; Liu and Baker, 1980). Microbial activity appears to be reduced in acidified soils, and the change in microbial balance allows an increase in activity of *Trichoderma* spp. which in turn further suppresses activity of other microbes (Simon and Sivasithamparam, 1988c). It appears that increasing soil pH by amendment of lime can remove pathogen suppressiveness (Simon *et al.*, 1988).

It is desirable to be able to amend *T. koningii* (Tr5) to soil to fully suppress *S. cepivorum* infections. Complete disease suppression has not been achieved by use of different amendment methodologies (Chapter V) or increased rates of Tr5 application (Ch. VI; 18). An obvious strategy to improve efficacy is to create soil conditions more favourable for Tr5 activity. Tr5 has been shown to grow optimally at pH 4.0 in culture media. The aims of the trial were to evaluate any differences in *S. cepivorum* infection at different pH, and whether the efficacy of Tr5 was altered at different pH.

In other studies, soil pH has been altered by amendment of elemental sulphur (Hausenbuiller, 1984) and ammonium sulphate (Simon and Sivasithamparam, 1988a & b). In the krasnozem soil it was necessary to add excessive amounts of ammonium sulphate to acidify soil, and this raised concerns that the unusually high nitrogen levels might induce greater than normal saprophytic proliferation of Tr5 at all soil pH's. Ferrous sulphate was found to more easily acidify the krasnozem soil, the over abundance of iron is not a problem for onions even in acid soils (Salardini, 1995) though there may be some negative effects on fungi which need to be determined.

18.2. Materials and Methods:

18.2.1: Trial preparation.

Krasnozem soil was collected from a site without a history of *Allium* cropping. No sclerotia were recovered from this soil by wet sieving by the methods described in Appendix F. The soil pH (originally 6.2) was adjusted by buffering all soil to pH 4.5 by adding 14g /Kg of soil of ferrous sulphate, before re adjusting using

calcium carbonate ($CaCO_3$). Calcium carbonate was added at concentrations of 6g, 10g, and 14g /Kg to adjust lots of soil to pH 5.5, 6.5 and 7.5 soils respectively. Twenty replicates were performed of each treatment, each in 20cm diameter 34cm deep pots. Within the four pH levels there were four treatments;

1) *S. cepivorum* alone,
2) *S. cepivorum* with autoclaved grains of millet,
3) *S. cepivorum* with Tr5 amended as millet inoculum,
4) Tr5 amended as millet inoculum in absence of *S. cepivorum.*

S. cepivorum sclerotia (38 mg) were mixed evenly through the soil of each pot providing a concentration of 50 sclerotia per kilogram of soil. Tr5 millet inoculum (Appendix A) or autoclaved millet was amended at a rate of 5g/pot (1590kg/ha). pH tests were performed on all pots after emergence to ensure that the pH had not diverged from what it had been before sowing. Pots were re-examined for pH at the conclusion of the trial.

The disease incidence data was analysed by two way analysis of variance. There was no evidence of interaction between the strate so individual levels of each strata were analysed by independent one way analysis of variance and LSD test.

18.2.2. Soil pH influence on *S. cepivorum* sclerotia.

Thirty sclerotia collected from infected bulbs in the preceding season by Dr J J C Dennis (DPIF) were buried in nylon mesh bags at a depth of 7cm in ten of the twenty replicates of each treatment where *S. cepivorum* inoculum was present. Bags of 30 sclerotia were also buried in pots where no seed was sown, with and without Tr5 at each pH in order to determine how many sclerotia remained viable in absence of host root exudates. This was done to determine what portion of any decline in sclerotial numbers was due to non-specific germination and mycoparasitic decay, as opposed to stimulated germination. After destructive sampling of the trial, bags were recovered from soil, washed gently and the numbers of intact healthy sclerotia were counted. This assessment was based on retention of the normal healthy springiness when gently squeezed using tweezers.

The numbers of sclerotia which remained viable were first analysed by LSD test which showed that there was no difference between means in presence or absence of Tr5 or autoclaved millet. In both the -Tr5 and +Tr5 strata there were significant differences between the number of sclerotia which remained in viable condition where onions were present and absent. The differences in sclerotial survival across pH, Onion, and biocontrol amendment strata were further examined by 2 x 2 x 2 factorial analysis of variance.

18.2.3. Assessment of Tr5 root colonisation.

The proportion of roots colonised by Tr5 under the different treatment regimes were assessed by taking 100 root samples (five per pot) at the time of harvest from treatments S, T, U, W, X and Y. These were incubated on RASP medium and grouped based on morphological features by the procedure described in Appendix G. A sub sample of one in four isolates were analysed for pectinase isozyme profiles by the methods described in Appendix E.1.

18.2.4. *In vitro* effect of ferrous sulphate and calcium carbonate.

Air dried soil was briefly ground using a mortar and pestle to reduce size of aggregates to a powdery consistency and 100g aliquots were mixed with 100mls of distilled water and 4g of agar. Soil was amended with ferrous sulphate at the rate it was used in this trial and in the pH root colonisation assay (Chapter IV; 20) and calcium carbonate at the rate it was used in this trial. Soil suspensions were amended with;

1) 14g/kg $FeSO_4$ + 6g/kg $CaCO_3$ (final pH 5.5).
2) 14g/kg $FeSO_4$ + 0.1ml NaOH (final pH 5.5).
3) 0.1ml HCl (final pH 5.5).
4) 2g/kg $FeSO_4$ (final pH 5.5).
5) Non amended (pH 6.2).

The soil media were autoclaved and poured into petri plates. A $2mm^2$ cube of tap water agar Tr5 inoculum was added to the centre of each plate, plates were sealed using parafilm and incubated at 25°C for 58 hours in darkness. Diameter of colonies was measured at three points 120°apart. Seven replicates of each treatment were conducted. The averages of three measurements from each plate were collated and analysed by one way analysis of variance and LSD test (0.05).

19.3. Results:

19.3.1 Disease Incidence.

With soil pH buffered to 4.5 growth of onions was very poor. The pH 6.5 treatments were found to have diverged from this pH, and therefore, the pH 4.5 and 6.5 infection strata were excluded from the analysis. Following analysis of the remaining treatments, soil pH was shown to have an influence on seedling emergence (Table 18.A). At pH 5.5 emergence was significantly lower than at pH

7.5 in all treatments except where Tr5 was amended in absence of *S. cepivorum* . There was no difference between emergence in the presence or absence of Tr5 or autoclaved millet.

Table 18(A): Emergence of onion seedlings and incidence of *S. cepivorum* infections in soils buffered to pH 5.5 and 7.5.

Amendment	pH 5.5		pH 7.5	
	Emergence	% infection	Emergence	% infection
S.cepivorum alone	9.7 de	18.5 A	13.5 a	7.1 B
S.cepivorum+millet	11.4 cd	12.3	13.2 ab	10.2
S.cepivorum+Tr5	8.7 e	10.0	14.2 a	6.2
Tr5	10.9 cd		12.2 bc	

note: Emergence represents the number of twenty seeds added which emerged.
Emergence was analysed by one way analysis of variance and LSD (0.05) values followed by the same letter are not significantly different.
% infection represents the percentage of seedlings which were infected by *S. cepivorum.*
% infection was analysed by 2 way analysis of variance (P value=0.406, error mean square=267.62, Total df=119), followed by separate one way analysis and LSD (0.05) test of individual strata.
Capital letters in the % infection columns indicate a significant difference in *S. cepivorum* infections in untreated controls of pH 5.5 and 7.5, no other significant differences were found.

Disease incidence was low throughout the trial at both soil pH. There were no significant differences between disease incidence in the presence or absence of Tr5 or autoclaved millet. However incidence of infection in untreated controls was significantly higher at pH 5.5 than at pH 7.5 (Figure 18.B).

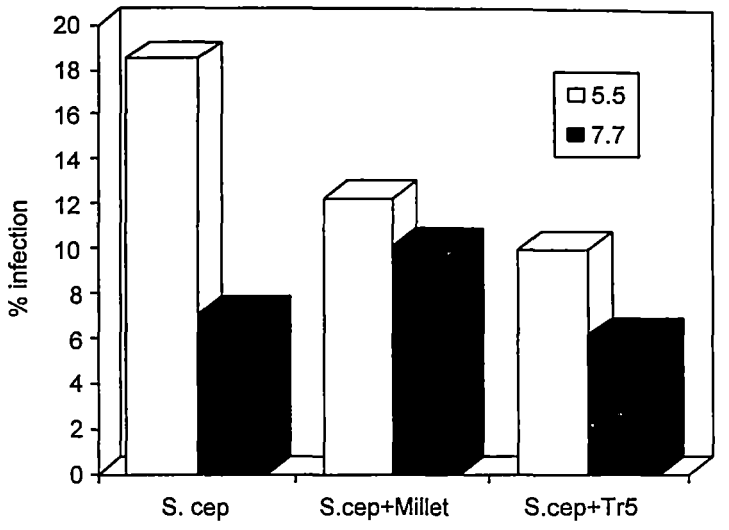


Figure 18(B): The incidence of *S. cepivorum* infections in soils buffered to pH 5.5 and 7.5. Treatments were amended with *S. cepivorum* alone (S. cep) or treated with 5g/pot of autoclaved millet (S. cep+Millet) or Tr5 millet inoculum (S. cep + Tr5).

19.3.2. Assessment of the influence of soil pH on sclerotia.

Following burial there was no significant difference between the numbers of sclerotia which remained viable in nylon mesh bags irrespective of whether they were unamended or amended with Tr5 or autoclaved millet. However, within both the Tr5 amended and unamended treatments, significantly more sclerotia remained viable in treatments where no onions had been planted than when onions were planted (Table 18.C).

Factorial analysis (2 x 2 x 2) across the three strata (Table 18.D) confirmed that there was evidence of differences among mean numbers of surviving sclerotia in onions present or absent strata. In all cases significantly more sclerotia remained intact in the absence of onions roots. Evidence of differences among mean numbers of surviving sclerotia was also detected across the pH levels. This seems to reflect the significantly lower sclerotial survival at pH 7.5 in the absence of Tr5. An interaction was also detected between onion and Tr5 presence or absence. Examination of this interaction (Table 19.E) shows that there is greater sclerotial survival in absence of both onions and Tr5.

Table 18(C): Average number of sclerotia remaining in viable condition from 30 originally buried in nylon mesh bags at a depth of 7cm in pots of soil either of pH 5.5 or 7.5, either amended with 5g of Tr5 millet inoculum (+ Tr5) or autoclaved millet (+ Millet) or unamended, which either had onions growing in the pots (+) or had no onions (-).

Soil pH	Onions	Unamended	+ Tr5	+ Millet
pH 5.5	+	5.0 d	6.7 cd	5.1 d
	-	12.2 a	10.8 a	nd
pH 7.5	+	5.0 d	5.8 cd	4.5 d
	-	8.1 bc	10.6 ab	nd

Notes to Table 18(C).
-values followed by the same letter do not differ significantly according to LSD (0.05) = 2.50
-nd=not determined

Table 18(D): 2 x 2 x 2 factorial analysis of variance on numbers of surviving sclerotia.

Source of var	df	SS	MS	F	Fcrit
Onion	1	460.8	460.8	58.55 *	4.0
Tr5	1	2.45	2.45	0.31	4.0
pH	1	33.8	33.8	4.29 *	4.0
Onion:Tr5	1	51.2	51.2	6.50 *	4.0
Onion:pH	1	14.45	14.45	1.83	4.0
Tr5:pH	1	5.0	5.0	0.63	4.0
Onion:Tr5:pH	1	27.05	27.05	3.43	4.0
Error	72	567.2	7.87		
Total	79				

Table 18(E): Examination of the Onion:Tr5 interaction.

	+Tr5	-Tr5	(+T) -(-T)	SS	Fcalc	Fcrit
-Onion	228	189	39	76.05	9.66*	4.00
+Onion	100	125	-25	31.25	3.97	4.00

Notes to Table 18(E)
SS derived by $[(-T)-(+T)]^2$/ treatments(2) x reps(10).
df(0.05)=1 and 79 (value of 4.0 is for 60df, 120 gives 3.92 so borderline case)
error mean square=7.8.
*=implies significantly greater sclerotial survival across strata (in absence of onions and Tr5).

18.3.3. Root colonisation by Tr5.

Overall, there was no difference between the size of the Tr5 type, or total *Trichoderma* spp. populations between the treatments of pH 5.5 and 7.5. The frequency of isolation of fungi morphologically similar to Tr5, and of total isolation of *Trichoderma* spp is displayed in Table 18(F). Two way analysis of variance indicated that there was no interaction between the variables of the pH and amendment strata for isolations of Tr5 type fungi or of total *Trichoderma* spp. isolations. Significantly more fungi morphologically indistinguishable from Tr5 were isolated from onion roots in Tr5 amended pots than were isolated from unamended, or autoclaved millet amended pots. The amendment of autoclaved millet made no difference to the number of Tr5 type isolations compared to the unamended control at either pH.

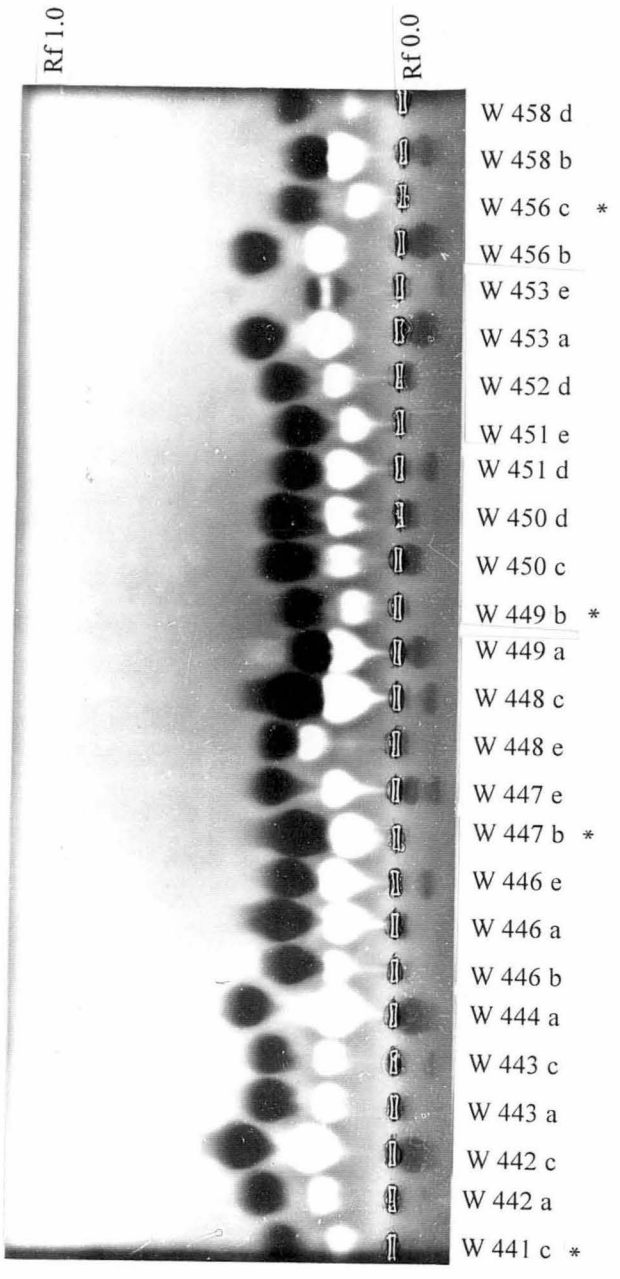
The total number of *Trichoderma* spp. isolations was significantly greater in Tr5 amended pots than in unamended, or millet amended pots. *Trichoderma* spp were isolated from 81% and 71% of roots in Tr5 amended treatments of pH 5.5 and 7.5 respectively. Identical numbers of *Trichoderma* spp. were isolated from roots in unamended and millet amended treatments at both pH.

Table 18(F): Isolation frequency of all *Trichoderma* spp., and of isolates morphologically similar to Tr5 from onion roots in soils of pH 5.5 and 7.5 amended with 5g/pot of Tr5 millet inoculum, autoclaved millet, or unamended.

pH	Amendment	Tr5 type		Total *Trichoderma*	
		Mean	% Col^{zn}	Mean	% Col^{zn}
5.5	*S.cepivorum*	0.1 b	2%	1.85 b	37%
	S.cepivorum+Millet	0.25 b	5%	1.85 b	37%
	S.cepivorum+Tr5	3.25 a	65%	4.05 a	81%
7.5	*S.cepivorum*	0.25 b	5%	1.80 b	36%
	S.cepivorum+Millet	0 b	0%	1.80 b	36%
	S.cepivorum+Tr5	2.85 a	57%	3.55 a	71%

Notes to Table 18(F):
-numbers in mean column indicate the average number of isolations from five root samples taken from each of 20 replicates of each treatment.
-"Tr5 type" indicates isolates morphologically indistinguishable from Tr5.
-"Total *Trichoderma* " indicates the total number of isolations of fungi of genus *Trichoderma*..
- No interaction was present between pH and amendment strata for isolation of Tr5 type (Interaction P-value 0.465) or Total *Trichoderma* spp. (interaction P-value 0.67) according to 2 way analysis of variance.
-Tr5 type isolations and Total *Trichoderma* isolations were each analysed by one way analysis of variance. Figures in the same column followed by the same letter are not significantly different.
-% Col^{zn} = percentage of root samples colonised by Tr5 type isolates or *Trichoderma* spp.

Isozyme profiles for polygalacturonase and pectinesterase (eg Figure 18.G) showed that of fifty two isolates assessed morphologically to be of Tr5 type eight (15%) were found to produce isozymes not known to be produced by Tr5. Of one hundred and three isolates assessed not to be morphologically of Tr5 type, twenty one (20%) would not have been excluded from the Tr5 type group based on their pectinase profiles. Figure 18(G) displays the profiles of 26 *Trichoderma* spp isolated from roots in the unamended control of pH 7.5. Isolate $W449^{b}$ (Figure 18.G) displays a profile typical of Tr5, there are five profiles of isolates which were assessed morphologically to be other than Tr5 type shown on this gel which would not be excluded from the Tr5 type group based solely on their pectinase profiles.



* = morphologically indistinguishable from Tr5

Figure 18(G): Contact print of polyacrylamide gel electrophoresis for the detection of pectinases. Light zones indicate isozymes of pectinesterase, dark zones indicate polygalacturonase. Isolates W 441^{c}, W447^{b}, W456^{c} and W449^{b} were assessed to be morphologically similar to Tr5 and display profiles typical of Tr5. Isolates W443^{a}, W446^{a} ,W446^{b}, W451^{e}, and W458^{d} display profiles which would not technically exclude them from the Tr5 type group had they been assessed morphologically to be of this type.

Table 18(H): Colony diameter of Tr5 in soil agar with pH adjusted by different methods.

Strategy	$FeSO_4$	HCl	$CaCO_3$	NaOH	pH	Ave Diam
1	14g/kg		6g/kg		5.5	40.25 B
2	14g/kg			0.1ml (5M)	5.5	40.77 B
3		0.1ml (8M)			5.5	51.64 A
4	2g/kg				5.5	52.77 A
5					6.2	50.59 A

Notes to Table 18(H):
-Ave Diam= Average diameter derived from seven replicates, three measurements taken on each replicate.
-LSD(0.05) =3.69.
-M= Molar
-numbers followed by the same letter are not significantly different.

There was no significant difference in the radial growth of Tr5 in presence of 14g/kg of ferrous sulphate when the pH was raised to 5.5 using $CaCO_3$ or NaOH (Table 18.H). However, radial growth was significantly greater when pH was buffered to 5.5 using 2g/kg of ferrous sulphate. There was no significant difference in radial growth when this lower rate of ferrous sulphate addition was replaced with hydrochloric acid, or when the pH was allowed to remain at 6.2.

18.4. Discussion:

In other trials (eg VI:17 the inoculum density trial) it was noted that onion seedling emergence in Tr5 colonised millet amended treatments was slightly lower than in unamended controls. In this trial there were no such differences, but there was notable reduction in emergence at lower pH. Those seedlings which became established at pH 5.5 grew and matured adequately. Some seed may have been better adapted to pH 5.5 and many of the larger bulbs from pH 5.5 soil have been retained with the intention of selecting for greater tolerance of lower pH. The regular creamgold seed is commonly used in soils in the pH 6.0 to 6.5 range.

The incidence of disease in unamended controls being significantly lower at pH 7.5 than at pH 5.5 is difficult to explain. Previous studies of the influence of soil pH on *S. cepivorum* germination and infection have found infection to be one quarter as severe at pH 5.0 as at pH 7.0. (Adams and Papavizas, 1971). Additionally Coley-Smith (1960) reported increased sclerotial germination in lime amended soil. Examination of the root colonisation data indicates that similar percentages of roots (36 and 37%) were colonised by native *Trichoderma* spp, so there is no obvious evidence of differences in natural suppressiveness according to isolation of this fungus from the rhizosphere. Also the average numbers of buried sclerotia which remained in viable condition in these two treatments was identical implying that both

treatments have been subjected to a similar infection challenge (There is a possibility though that a larger proportion of the decline in sclerotial numbers may be attributable to decay rather than germination at pH 7.5). The total number of plants in the pH 7.5 pots was 28% higher than at pH 5.5. Higher plant density can result in greater root to root spread (Crowe and Hall 1980a) and therefore greater disease incidence. Alternately the death of each seedling at higher plant density contributes less to the percentage of disease incidence. Infections were recorded in 11 of 20 (average=1.72 infections) pots at pH 5.5 and 10 of 20 (ave=1.05 infections) at pH 7.5. The only other obvious variable between the two treatments outside of the actual pH difference is the abundance of calcium carbonate, which was amended at 6g / Kg at pH 5.5 and 14g /Kg at pH 7.5. Calcium is less available to plants at pH 5.5 than 7.5 (Buckman and Brady, 1965). The amendment of soil using calcium carbonate has caused reductions in infection of carrots by the related fungus *Sclerotium rolfsii* (Punja *et al.*, 1986). Increased calcium levels in plant tissues results in precipitation of oxalic acid (which acts synergistically with the pathogens pectin degrading enzymes) as calcium oxalate which decreases pathogen vigour (Punja, 1989). Hence it is possible that higher levels of calcium have affected the ability of *S. cepivorum* to penetrate and attack onion tissue. Harrison (1954) reported that white rot was more severe in acid (pH 5-6.5) soils than alkaline soils (pH 7.5) and white rot had not been reported from natural limestone areas of Victoria (Australia). The conflicting results of Adams and Papavizas (1971) who used lime to raise soil pH but recorded higher infection in lime amended soil may be explained by soil type differences and their use of "hydrated lime" [$Ca(OH)_2$] whereas the present study used crushed limestone ($CaCO_3$). It is necessary to add 35% more $CaCO_3$ than $Ca(OH)_2$ for an equivalent pH change due to differing molecular weight, therefore more Ca^{2+} are released (Adams and Papavizas do not state the volume of $Ca(OH)_2$ used). Additionally Ca_2^+ ions from $Ca(OH)_2$ are directly exchanged onto clay particles whereas Ca_2^+ from $CaCO_3$ appear to be first released into solution (Hausenbuiller, 1984). Soil amendment using $Ca(OH)_2$ may not provide as much soluble calcium to neutralise the pathogens oxalic acid as amendment of $CaCO_3$. Certainly Punja (1989) has reported greater suppression of *S. rolfsii* infection of carrots following amendment of $CaCO_3$ than $Ca(OH)_2$. The influence of calcium is to be investigated as a component of future studies.

Disease suppression in the pH trial was poorer than expected based on concurrent work. In the inoculum density trial, sampling of roots in Tr5 amended treatments indicated that 97% of roots were colonised by Tr5 type isolates, and 74% reductions in disease incidence were recorded. In the pH trial, root colonisation at pH 5.5 and 7.5 was 65 and 57% respectively. The insignificant reductions in disease

incidence seem likely to be due to poor root colonisation, as if Tr5 was not on the root surface when they became infected no disease suppression can be expected.

Amendment of Tr5 colonised millet had a greater disease ameliorating effect at pH 5.5 than at pH 7.5, although in general the disease reduction was poor in comparison to other trials (eg Ch VI; 16 & 17.). Comparison of root colonisation data between trials correlates well with disease suppression results. In the inoculum density trial (Ch. IV; 17) 97% of roots were colonised by Tr5 type fungi following amendment with a similar rate of inoculum, and this provided a 74% disease reduction. In this trial root colonisation was 65 and 57% at pH 5.5 and 6.5, respectively. Insignificant reductions in infection in Tr5 amended treatments were recorded.

The poor root colonisation at pH 7.5 correlates with data collected in the soil pH assay. Amendment of Tr5 at 1000 Kg/ha resulted in 58% root colonisation by Tr5 type isolates. At pH 5.5 however there is an inconsistency between the data as Tr5 type colonies were isolated from 92% of roots after amendment of Tr5 at a rate of 100kg/ha (one sixteenth of the rate used in this experiment). One difference between the two experiments is that the root samples for the soil pH assay were taken at 8 and 10 weeks after sowing, whereas the soil pH trial samples were taken at the time of harvest (29 weeks after sowing). The lower colonisation may then reflect a decrease in Tr5 colonisation toward the end of the season either due to succession or cooler temperatures. Another difference is in the method used to buffer the soil. The pH 5.5 soil in the pH trial was buffered by amendment of 14g/kg of ferrous sulphate and 6g/kg of calcium carbonate, whereas in the soil pH assay the only amendment at this pH was 4g/kg of ferrous sulphate. As the pH trial, inoculum density and inoculum depth trials were concurrently run using the same soil, site and Tr5 rate, the remaining variables are the higher rate of ferrous sulphate amendment and the presence of 6g /Kg of calcium carbonate. The results of the *in vitro* growth bioassay (Table 18.H) show Tr5's vigour under sterile conditions on soil media containing pH amendments used in the pH trial and colonisation assay. In soil acidified using ferrous sulphate at 14g/kg, growth of Tr5 did not differ when pH was re-adjusted using $CaCO_3$ (1) or NaOH (2). There is no evidence that the poor colonisation in this treatment is due to the presence of $CaCO_3$, though the possibility should not be discarded. Addition of calcium carbonate to soil suppressive to *Gaeumannomyces graminis* var *tritici* has been shown to alter the microbial balance of soil bacteria and decrease activity of *Trichoderma* spp (Simon and Sivasithamparam, 1988c). As greater radial growth was recorded in soils of the same pH acidified using 2g/kg of $FeSO_4$ (3) and HCl (4), it is possible that $FeSO_4$ amendment at higher rates has decreased the vigour of Tr5. If this also occurs in saprophytic growth it could contribute to the poor root establishment. As radial

growth of Tr5 was no different when soil was acidified using $FeSO_4$ at the lower rate, HCl, or without amendment, $FeSO_4$ added at the lower rate has probably not inhibited Tr5 establishment. Further discussion of the influence of soil pH on saprophytic colonisation by *Trichoderma* spp. is undertaken in Chapter VI; 19.

Chapter VI: Ecological Studies.

19.0: The effects of soil pH on root colonisation by *Trichoderma koningii* (Tr5).

19.1. Introduction:

The proliferation and activity of *Trichoderma* spp are known to be favoured by low soil pH conditions (Liu and Baker, 1980; Cook and Baker, 1983; Papavizas, 1985; Simon and Sivasithamparam, 1988c). Under laboratory conditions *T. koningii* (Tr5) has been shown to grow most rapidly at pH 4.0 (Ch. IV:12.), and poorly below pH 3.0 and above pH 8.0.

It is suggested that Tr5 must be on the surface of onion roots to prevent *S. cepivorum* infections from developing (Chapter IV). In contrast, in trials where root establishment has been successful, good disease suppression has been achieved (eg Chapter VI:17), in trials where root establishment was poor, disease suppression has been low (Ch VI:18). It is economically and practically desirable to add Tr5 to the soil by production and augmentation of the lowest possible volume of inoculum. The aim of this work was to determine the pH range at which Tr5 can be added to the soil in the lowest possible quantity to achieve maximal colonisation of onion roots.

19.2. Materials and Methods:

Soils of pH below 5.5 were found to be too acidic for normal development of regular creamgold onions (Ch. VI:19), therefore pH treatments in the range of 5.5, 6.5, and 7.5 were investigated. As the original pH of the soil was close to 6.5 the easiest way to raise and lower the pH would have been by addition of either ferrous sulphate or calcium carbonate. To avoid concerns that this method would lead to nutritional inconsistency between the strata all treatments were first acidified to pH 5.5 using ferrous sulphate then adjusted using calcium carbonate at a variable rate.

Soil trays 25 x 30 cm long and 15 cm deep were filled with kraznozem soil similar to that used for commercial onion growing in Tasmania. The natural pH of the soil was around 6.2. By addition of ferrous sulphate the soil pH of all soil was acidified to 5.5, then pH 6.5 and 7.5 treatments were adjusted using different amounts of calcium carbonate. Soil was then moistened and left for two weeks. The pH was re-checked before sowing to ensure there had been no change in pH due to soil buffering.

Around two hundred seeds (var: Regular Creamgold) were sown during summer in each tray. Tr5 was added as millet inocula (Appendix A) at rates of 0,

100, 500 and 1000 kilograms per hectare sprinkled evenly among the seed. Seed and Tr5 were then covered in 1cm of soil.

Five replicates were performed of each treatment. Ten root samples were taken from each pot at eight and at ten weeks after sowing. Samples were taken by uprooting seedlings and inserting the roots into snap seal plastic bags. The uppermost 1cm of root was cut off using sterile technique and incubated on RASP medium for 2 weeks at 25°C.

Colonies were assessed to be morphologically similar or dissimilar to Tr5, and to test the accuracy of the morphological assessment, pectinase isozyme profiles of a 25% sub sample of the 972 *Trichoderma* isolates were performed (Appendix E). At the conclusion of the experiment, the pH of soil in all trays was rechecked to determine whether any change had taken place during the experiment.

Results were analysed by two way analysis of variance and LSD test. Two sets of data are presented, one for isolates of Tr5 type and another for total *Trichoderma* spp isolations.

19.3. Results:

Incubation of root segments on RASP medium indicated that large and diverse *Trichoderma* spp. populations were indigenous to the soil. Modification of the soil pH did not significantly alter the frequency with which indigenous *Trichoderma* spp. were isolated from onion roots. Where Tr5 had been amended at any rate, the isolation of other *Trichoderma* spp. was lower in general. Two way analysis of variance indicated that no interaction was present between the rate of Tr5 addition and the pH, in either the Tr5 type only ($P=0.185$, $df=6,59$), or the total *Trichoderma* spp. ($P=0.465$, $df= 6,59$) data. The levels of either strata were therefore examined independently.

Within the untreated control (0 Tr5) there was no significant effect of pH on the frequency with which Tr5 type isolates were encountered (Table 19.A.). When Tr5 millet was added at a rate of 100kg/ha significantly more (Average 18.4) root samples were colonised by Tr5 type isolates at pH 5.5 than at 6.5 and 7.5. There were no differences in colonisation by Tr5 type isolates at the 500kg/ha rate. At a rate of 1000kg/ha significantly more Tr5 type isolates were present on roots at pH 5.5 and 6.5 than at pH 7.5.

At pH 5.5, all three rates of Tr5 millet amendment resulted in greater numbers of Tr5 type isolations than where no Tr5 was added (Table 19.A; Figure 19.C.). At pH 6.5, significantly more Tr5 type fungi were isolated when soil was amended with 100 and 500 kg/ha of Tr5 millet inoculum than in the unamended control. Further increasing the amendment rate resulted in greater root colonisation

by Tr5 type isolates at pH 6.5. At pH 7.5 only addition of Tr5 millet at the 500 kg/ha rate resulted in greater colonisation of roots by Tr5 type isolates than in the unamended control.

The total number of *Trichoderma* spp. including Tr5 type fungi isolated from roots did not differ significantly at each pH level except where Tr5 was added at the maximum rate (1000 kg/ha), where significantly more *Trichoderma* spp. were isolated at pH 6.5 than 7.5 (Table 19.B; Figure 19.D.). Significantly higher proportions of roots were colonised by *Trichoderma* spp. in Tr5 amended treatments than in unamended controls at each pH with the exception of the pH 6.5 treatment amended with 500kg/ha. The only treatment where 100% of root samples were colonised was where soil of pH 6.5 was amended with 1000kg/ha of Tr5 millet inoculum.

After the final sampling, the soil pH of all trays was re-measured to ensure that no unknown changes had occurred to the pH. Three divergences from expected pH were identified, replicate Jb which had been pH 7.5 retested at pH 6.5, replicate Gd which had been pH 6.5 retested at pH 6.0, and replicate De which had been pH 5.5 retested at pH 6.0.

To test the accuracy of the morphological classification, pectinase isozyme profiles for polygalacturonase and pectinesterase were performed for a total of 194 isolates. Figure 19(E) is a contact print of an electrophoresis gel which exemplifies the results. One hundred and five of these isolates had been morphologically assessed to be of Tr5 type, two were found to produce bands not known to be produced by Tr5. Of eighty six isolates morphologically assessed not to be of Tr5 type, 46 could not be excluded from the Tr5 type group based on their profiles.

Table 19(A): Mean isolations of Tr5 type isolates from 20 roots of onions where soil of pH 5.5, 6.5, and 7.5 was amended with 0, 100, 500, and 1000 kg/ha of Tr5 millet inoculum.

pH	0	100	500	1000
5.5	4.0 (b)	18.4 (a) A	15.0 (a)	16.4 (a) A
6.5	1.6 [c]	11.6 [b] B	10.4 [b]	19.6 [a] A
7.5	3.2 {b}	10.6 {ab} B	13.4 {a}	11.6 {ab} B

Notes to table 19(A):
-means followed by the same letter in brackets represent LSD 0.05 tests across individual pH levels and do not differ significantly according to LSD (0.05). Different brackets are used for LSD tests at the three pH levels.
-means followed by the same letter in capitals represent tests across individual Tr5 rate levels and do not differ significantly according to LSD (0.05). Where capitals are absent no significant differences are evident among means at that inoculum rate.
-interaction between pH and amendment rate was non significant (P=0.185; df=6,59)

Table 19(B): Mean isolations of all *Trichoderma* spp from 20 root samples of onions where soils of pH 5.5, 6.5, and 7.5 was amended with 0, 100, 500, and 1000 kg/ha of Tr5 millet inoculum.

pH	0	100	500	1000	
5.5	11.8 (c)	19.2 (ab)	15.6 (b)	19.4 (a)	AB
6.5	11.2 [c]	16.0 [ab]	15.0 [bc]	20.0 [a]	A
7.5	12.4 {b}	18.0 {a}	18.2 {a}	17.6 {a}	B

Notes to table 19(B):

-means followed by the same letter in brackets represent LSD 0.05 tests across individual pH levels and do not differ significantly according to LSD (0.05). Different brackets are used for LSD tests at the three pH levels.

-means followed by the same letter in capitals represent tests across individual Tr5 rate levels and do not differ significantly according to LSD (0.05). Where capitals are absent no significant differences are evident among means at that inoculum rate.

- Interaction between pH and amendment rate was non significant (P=0.465, df= 6, 59).

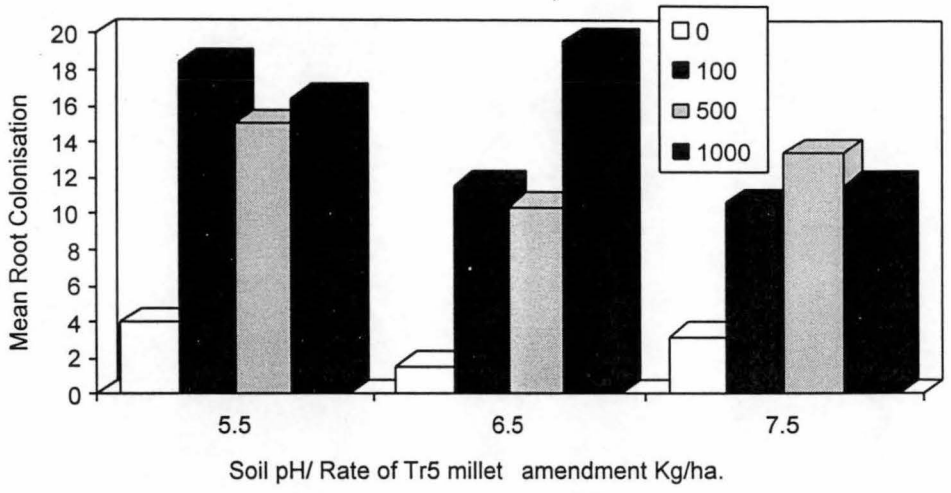


Figure 19(C): Average number of 20 roots samples taken from each replicate from which isolates not morphologically distinguishable from Tr5 were isolated under different soil pH and Tr5 rate of addition treatments.

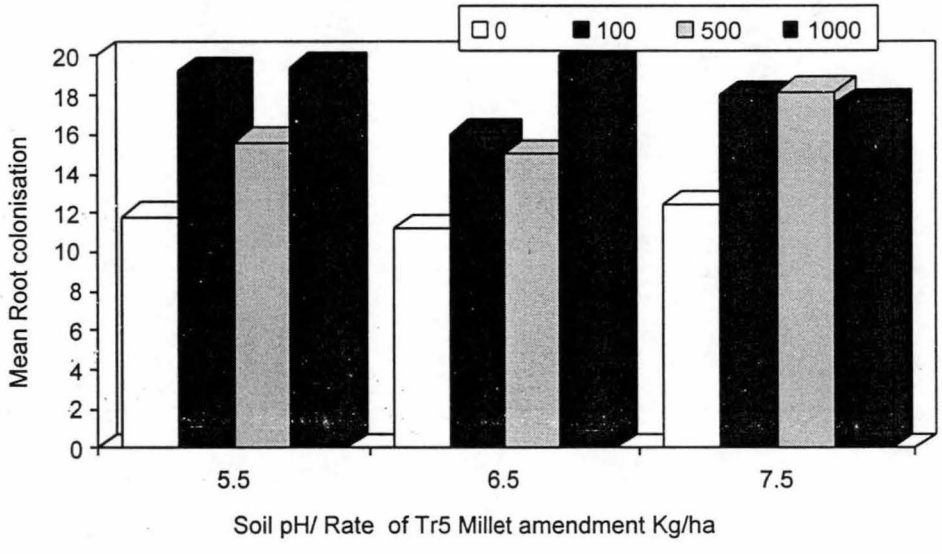


Figure 19(D): Average number of roots from 20 samples taken per replicate from which *Trichoderma* spp were isolated under different soil pH and Tr5 addition treatments.

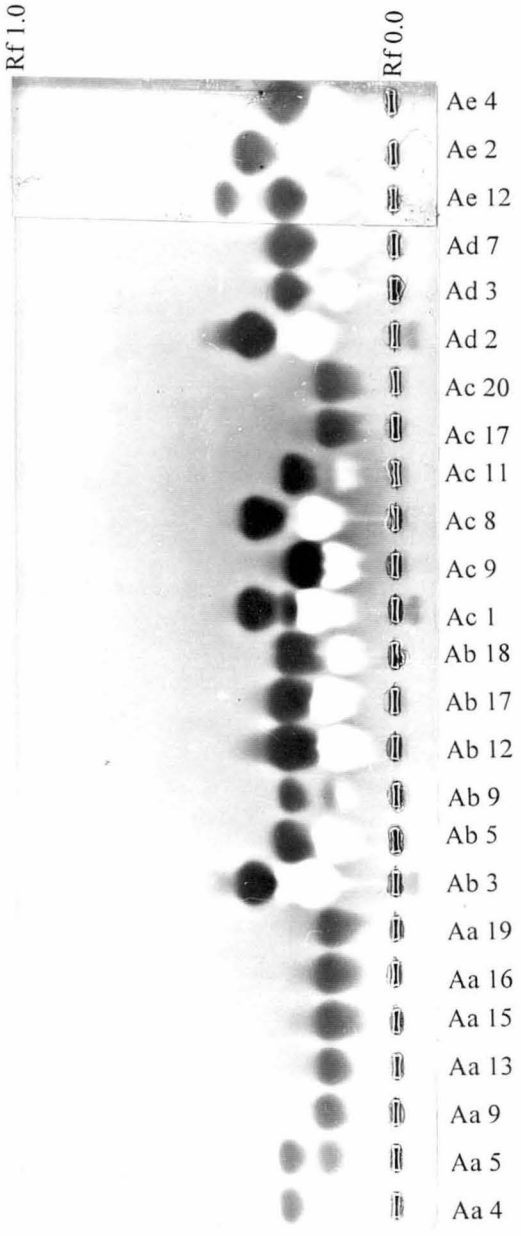


Figure 19(E) Contact print of a polyacrylamide electrophoresis gel for the detection of Pectinase, light zones indicate polygalacturonase, dark zones indicate pectinesterase. Isolate Ab12 was assessed to be of Tr5 type morphologically and displays bands typical of those known to be produced by Tr5. Isolates Ad7, Ad3, Ac11, Ab18 and Ab17 were of differing morphology to Tr5, however they could not be distinguished from Tr5 based on pectinase profiles.

19.4. Discussion:

The root isolation data shows that the soil used for this assay hosted an extensive and variable *Trichoderma* spp. population. At least five different types can be distinguished from samples taken from the pH 5.5 untreated control (refer to Figure 19(E); Aa5, Aa9, Ab3, Ab5 & Ac1) and many more morphological types were distinguished. In treatments where Tr5 was not added to the soil, around 59% of onion root samples were colonised by *Trichoderma* spp. across the pH range. The contribution these isolates make to disease suppression is unknown. Within the timescale of this trial there was no difference in the magnitude of the indigenous *Trichoderma* population. There may be some preliminary evidence of change in the composition of the *Trichoderma* population however, as some of the pectinase profile types encountered commonly in the pH 5.5 and 6.5 untreated controls were not encountered at pH 7.5 (eg. in untreated controls profile type of isolate Aa9 was encountered in seven of 30 profiles from pH 5.5, nine of 29 profiles at pH 6.5 and none of 27 profiles at pH 7.5.). Species composition of *Trichoderma* populations are known to fluctuate seasonally (Widden and Abitbol, 1980). If the microbial balance has altered in a similar manner due to pH, colonisation of the pH 5.5 rhizosphere for Tr5 may involve competing within a quite different rhizosphere environment to that at pH 7.5.

Addition of Tr5 to soil caused a significant increase in the total number of *Trichoderma* spp. isolated in all cases except for the 100 and 1000kg treatments of Tr5/ha at pH 7.5. In only one treatment (pH 6.5, 1000kg/ha) *Trichoderma* spp. were isolated from every single root. Given that around 59% of roots were colonised by indigenous *Trichoderma* spp. in untreated controls (leaving 41% uncolonised), and the average isolation of Tr5 type isolates across all amended treatments was 70%, it follows that Tr5 is occupying part of the niche normally occupied by other *Trichoderma* spp. In the Inoculum Depth trial where similar phenomena were demonstrated, the issue of whether results reflected Tr5 being better adapted than indigenous isolates in colonisation of RASP medium was raised. If Tr5 was co-existing with indigenous isolates (as would be the case under this hypothesis), it is most unusual that the only treatment where 100% root colonisation was achieved was where 98% of roots were colonised by Tr5 type isolates. It follows therefore that Tr5 amendment must have some form of suppressive effect on indigenous populations beyond simple competition for space and nutrition. The consequences of this are not known, but it is reasonable to speculate that some part of the soils natural disease suppression ability may be lost, resulting in a more severe infection challenge which Tr5 must additionally overcome.

The initial aim of the work was to find a pH range where the best possible Tr5 establishment could be accomplished at the lowest possible rate of inoculum addition. The best establishment of the assay was achieved at pH 6.5 after addition of millet inoculum at a rate of 1000kg/ha. While the establishment under these conditions was excellent, the rate of addition is high, and the 92% establishment achieved at pH 5.5 after addition of one tenth the amount of inoculum would appear *prima facie* to be a superior alternative. More work would be needed to determine whether onion production would be adversely affected at this pH. According to Brewster (1994) onion production is optimal toward the lower end of the pH 6 - 7 range, but growth is satisfactory at pH 4.0 in high organic fraction soils.

At pH 5.5, the 100 kg/ha treatment has provided the same root colonisation to that of the two higher amendment rates. High proportions of root colonisation at pH 6.5 were not achieved until the rate reached 1000 kg/ha. At pH 7.5 all three rate treatments appear to be of similar proportion to the 100 and 500 kg treatments at pH 6.5. It is not known what rate of Tr5 addition would be required to lift root colonisation at pH 7.5. Examination of root colonisation at pH 7.5 in Chapter VI:19 where 59% root colonisation by Tr5 type isolates was present at the time of harvest, would indicate that 1500 kg/ha was not enough inoculum for full rhizosphere colonisation at the end of the season. It may be that a niche of fixed size exists in pH 7.5 soil which Tr5 is able to fully occupy even at a low rate of application, but that Tr5 is not able to occupy a larger portion of the rhizosphere, irrespective of the amendment rate.

It is difficult to interpret the effect that the three divergences from specified pH may have had on root colonisation, the changes are likely to have occurred gradually. In replicate Jb where increased acidity from pH 7.5 to 6.5 was detected, Tr5 type isolates were isolated from 19 of the 20 samples. The average of the other four replicates of this treatment was 8.5 (having been 10.6 including Jb) raising the issue of whether the change caused greater colonisation. In treatment F (pH 6.5, 100kg/ha) the isolation frequencies were; 8, 11, 13, 6 and 20 (average 11.6). Although isolation in treatment Jb was within the range of the treatment F replicates, it was still unusually high, and is outside the range of the other J replicates. Whether the result has been biased or not, colonisation was too low for good control in any case.

In self criticism it is worth considering that the sampling method, which is a test of competitive saprophytic ability (Ahmad and Baker, 1987b) assessed only the presence or absence of *Trichoderma* spp. propagules of any type on onion roots approximately 0.5 to 1.5 cm below the base plate. In studies which have attempted to quantify the number of *Trichoderma* propagules (colony forming units-CFU) it has been noted that more than ten times the number of CFU are often present in the

top 2cm of soil than in the zone between 2-8cm, and some variation in this relationship according to pH in the 5 to 7 range has been recorded (Ahmad and Baker, 1987a). This may have implications for biocontrol agent efficacy, as there may be some differences in Tr5's ability to prevent infections originating at different depth (Ch VI: 16). The test of competitive saprophytic ability was chosen over CFU in this study as it is possible to sample from a larger number of sites, and CFU counts do not necessarily determine whether a fungus is actively growing, however some workers have obtained a clearer understanding of rhizophere activity of *Trichoderma* spp. by using a combination of the two methods (Kok *et al.*, 1996).

The morphological classification of isolates was tested by isozyme profiling for pectinase, and 98.1% of isolates morphologically assessed to be similar to Tr5 displayed profiles consistent with the Tr5 pectinase profile. Of the isolates morphologically assessed to be other than Tr5 type, 53% did not display any bands which would have excluded them from the Tr5 type group had they been accidentally placed in this group. As two isolates with profiles other than Tr5 type were found in the Tr5 type group there are probably at least two more isolates in the Tr5 type group which are not Tr5. A more critical interpretation of this isozyme result is that 53% of the isolates morphologically assessed to be Tr5 type are not Tr5 type, and the method cannot prove otherwise. As isolates in the group assessed to be other than Tr5 have different morphology to Tr5, it is clear that the non Tr5 grouping is reliable.

Chapter VI: Ecological studies:

20.0: Temperature Trial.

20.1. Introduction.

White rot is a cool weather disease (Adams and Springer, 1977). While relationships have been established between soil temperature and *S. cepivorum* sclerotial germination (Crowe and Hall, 1980b) it is known that periods of conditioning, and the isolate under study cause this relationship to vary (Gerbrandy, 1992). The temperature relationships of the Tasmanian strain of *S. cepivorum* have not been established, and it is not known how Tr5 might interact with soil temperature and the pathogen. It is desirable to be able to predict from environmental conditions any period when the biocontrol agent might have poor efficacy, so the crop might be saved by early lifting or application of a fungicide.

The goal of the trial was to investigate the influence of temperature on incidence of *S. cepivorum* infection and biocontrol using *T. koningii* (Tr5) and the influence of a sudden change in temperature on this relationship.

20.2. Materials and Methods.

To facilitate this trial, two glasshouses at the University of Tasmania (Hobart) were fitted with industrial air conditioning systems to modify air temperature. The two thermostats were set to 10 and 20°C. The temperature of the air in each was monitored by thermometer. A temperature data logger probe was buried at a depth of 10cm in pots in each glasshouse to monitor soil temperature. To ensure equal soil moisture relationships, tensiometers buried at 15cm in pots measured moisture tension in each glasshouse, moisture tension was not allowed to remain above 10 centibars.

Two tonnes of krasnozem soil similar to that used for onion production was transported from Devonport for the trial (same source as other pot trials). Sclerotia were provided by Dr J Dennis (DPIF), collected from field infected bulbs from north west Tasmania six months before the trial, air dried and stored in paper bags in darkness. Thirty eight milligrams of sclerotia were added (with 5g NPK [8:4:10] fertilizer) to 8kg lots of soil and thoroughly mixed by tipping between two trays then into black PVC soil bags 20cm diameter, 34 cm deep. This provided an inoculum density of 50 sclerotia per kilogram of soil. Tr5 was amended as millet inoculum (Appendix A) at a rate of 1590 kg/ ha. The trial was sown in spring of 1995 and ran for 6 months. Ten replicates of each treatment were performed. Twenty seeds were added to each pot at a depth of 1cm with the millet inoculum. To assess the influence of the temperature regimes on

S. cepivorum, nylon mesh bags containing 30 sclerotia were buried in all pots amended with *S. cepivorum* at a depth of 7cm. These were recovered at destructive sampling and the number of viable sclerotia remaining were assessed.

Four temperature strategies were investigated:

(1) Six months of the cooler season (10/10).

(2) Three months of cooler season followed by three months of warmer season (10/20)

(3) Three months of warmer season followed by three months of the cooler season (20/10).

(4) Six months of the warmer season(20/20).

These strategies were replicated across five treatments:

(1) *S. cepivorum.*

(2) *S. cepivorum* + *T. koningii* millet inoculum.

(3) *S. cepivorum* + autoclaved millet.

(4) no amendment.

(5) *T. koningii* millet inoculum only.

The treatments are listed in Table 20(A). Disease was assessed at fortnightly intervals, and infected plants were marked using nursery tags to prevent double counting. Pots were arranged in alternating blocks of Tr5 + and Tr5 - to minimise spread of the biocontrol agent to untreated controls by water splash.

Root samples were taken from non-infected controls at the end of the second month. One centimetre segments were incubated on RASP medium (Ch. IV:12 B) at 20°C for 2 weeks before they were assessed as either + or - *Trichoderma* spp. No further characterisation of these isolates was made.

Table 20(A): Treatment list for the 1996/7 glasshouse temperature trial. Glasshouse air conditioner thermostats were set to 10 and 20°C. + and - indicate presence or absence of *S. cepivorum* and *T. koningii*.. Treatments F to J were shifted from the 10 to 20°C glasshouse after three months. Treatments K to O were shifted from the 20 to 10° glasshouse at the same time.

Treatment	Month1-3	Month4-6	*S cepivorum*	*T.koningii*
A: 681-690	10	10	+	-
B: 691-700	10	10	+	+
C: 701-710	10	10	+	AM
D: 711-720	10	10	-	-
E: 721-730	10	10	-	+
F: 731-740	10	20	+	-
G: 741-750	10	20	+	+
H: 751-760	10	20	+	AM
I: 761-770	10	20	-	-
J: 771-780	10	20	-	+
K: 781-790	20	10	+	-
L: 791-800	20	10	+	+
M: 801-810	20	10	+	AM
N: 811-820	20	10	-	-
O: 821-830	20	10	-	+
P: 831-840	20	20	+	-
Q: 841-850	20	20	+	+
R: 851-860	20	20	+	AM
S: 861-870	20	20	-	-
T: 871-880	20	20	-	+

AM= autoclaved millet.
note: 10 and 20 refer to the air temperatures in °C the thermostats were set to, not the soil temperature.

20.3. Results.

Air temperature thermostats were set to 10 and 20°C in the two glasshouses. Air temperature fluctuated by 2-3°C above and below these temperatures. Soil temperature in each glasshouse fluctuated diurnally by around 5°C. Figure 20(B) shows the mean monthly temperatures 10cm deep in soil in each glasshouse during the trial. Throughout the season, soil temperature was 5-6°C higher in the warmer glasshouse. Figure 20(B) omits an instance at the end of the second month where the cool glasshouse air conditioner broke down while the researcher was inspecting trials in Devonport and the temperature rose above 20°C for four days.

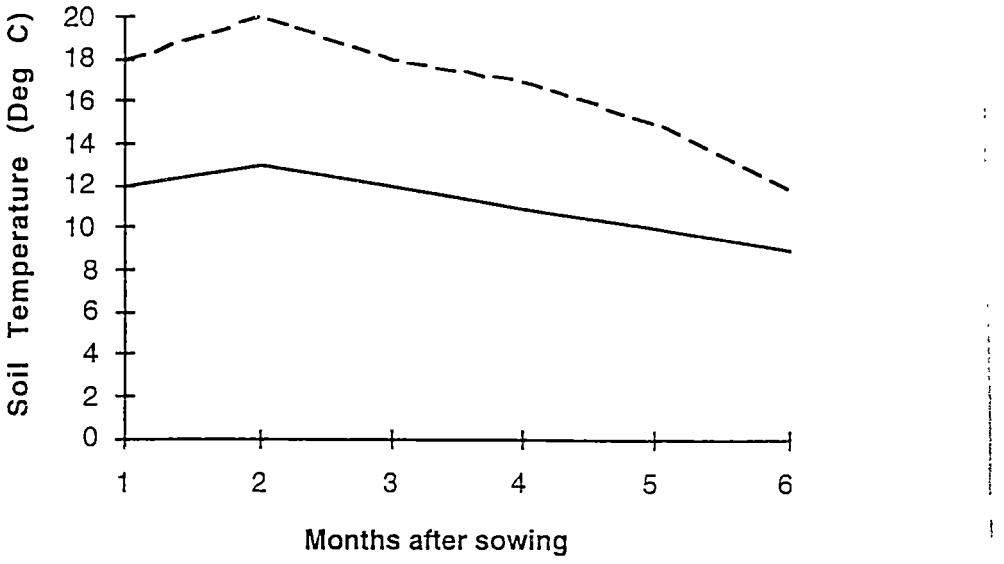


Figure 20(B) The mean monthly temperature taken 10 cm deep in pots of soil in the 10° (____) and 20°C (_ _ _) glasshouses.

Comparison of treatments D and I to N and O (Table 20.C) shows that emergence did not differ between the two temperature strategies in non-infected controls. Identical treatments under the same temperature tend to be in agreement, with the exception of treatments B and G. In the cool season simulation emergence was significantly poorer in both Tr5 and autoclaved millet amended treatments than non Tr5 amended treatments. This was not found in plants grown in the warmer glasshouse, with the exception of lower emergence in Tr5 amended non-inoculated controls as indicated by comparison of treatments O and T to N and S.

Three dates were considered to be important in the development of disease, these were: (1) the end of month three when pots were transferred between glasshouses (2) the end of month four when the influence of this change could be seen (3) destructive sampling at the end of month six. Disease incidence at these three dates in shown in Table 20(C).

In the first three months, *S. cepivorum* infections in untreated controls developed more rapidly in the cooler glasshouse (A and F) than the warmer one (K and P). In the fourth month, disease incidence in pots transferred from cool to warm (F) and warm to cool (K) glasshouses proceeded at a rate which did not differ from pots remaining at the original temperature. In the last two months disease in pots which had spent all six months in the cool glasshouse more than tripled from 21.4% to 69.2%, but the rate of infection was slower in pots transferred to the warm glasshouse, increasing from 28.0% to 42.7%. In treatment P, which spent the entire trial in the warmer glasshouse, disease increased at a steady rate from 7.36% at 3 months to 27.79% at 6 months. In treatment K which was moved from the warm to the cool glasshouse, infection rate increased from 1.38% to 38.15% in the last three months.

The efficacy of Tr5 was greatest in the full cool season simulation, ultimately providing 73.5% suppression of disease. In treatment G, which was transferred to the warmer glasshouse at 3 months, no disease was recorded in Tr5 treated pots in the first four months. However in the last two months in the warmer glasshouse the efficacy of Tr5 to limit disease was poor, with a disease level not significantly different from the untreated control (F). In both treatments which began the season in the warmer glasshouse Tr5 was also ineffective at limiting disease.

Disease incidence in pots amended with autoclaved millet was not significantly different to *S. cepivorum* only treatments in all temperature strata.

The sclerotia which were buried in nylon mesh bags in each pot were recovered following destructive sampling and counted. The number of intact viable sclerotia (from the original thirty) are presented in Table 20(D). Sclerotia buried in pots of soil without onion seedlings were included to determine what proportion of the decline in sclerotial numbers was due to mycoparasitism or non-specific germination or decay. There are no overriding differences in sclerotial survival associated with the different temperature treatments, around two thirds of all sclerotia decayed in the absence of onions and Tr5. An average of 5.08 more sclerotia per bag remained viable where onions were absent, suggesting that approximately 17% of sclerotia may have germinated in response to onion roots. No differences were evident between sclerotial survival in the presence or absence of Tr5 or autoclaved millet.

No root samples were taken specifically for determination of Tr5 root colonisation. However, in development of the RASP medium root samples were taken from the non-inoculated controls of this trial to determine the selectivity of the medium. The only characterisation made was whether a fungus of the Genus *Trichoderma* grew on the plate, although the raw numbers do provide some measure of the relative size of *Trichoderma* spp. populations. The results shown in Figure 20 (E) indicate that around 20% of roots in untreated/non infected controls were colonised by *Trichoderma* spp., and 96% of roots in Tr5 amended/non infected controls were colonised by various *Trichoderma* spp. No differences were evident between cool and warm glasshouses. It was not likely that differences would be detected as the rate of amendment was high.

Table 20(C) Emergence and disease incidence in the temperature trial as observed at 3, 4 and 6 months after sowing.

	Treatment	Emergence	% infection at 3 month	% infection at 4 month	% infection at 6month
cool:cool	A (S)	13.2 a	17.44 ab	21.48 ab	69.27 a
	B (ST)	10.7 bcd	2.39 c	2.15 cd	18.39 cd
	C (SA)	8.6 de	2.22 c	2.00 cd	53.22 ab
	D (-)	13.4 a			
	E (T)	9.5 cde			
cool:warm	F (S)	12.7 ab	20.33 a	28.03 a	42.79 abc
	G (ST)	8.4 e	0 c	0.00 d	21.11 c
	H(SA)	8.7 de	7.40 bc	6.66 bcd	17.45 c
	I (-)	13 a			
	J (T)	8.8 de			
warm:cool	K (S)	12.9 ab	1.38 c	5.41 cd	38.15 bc
	L (ST)	12 ab	0 c	5.00 cd	43.86 abc
	M(SA)	12.1 ab	0 c	0.00 d	29.26 bc
	N (-)	12.1 ab			
	O (T)	9.6 cde			
warm:warm	P (S)	11.5 abc	7.36 bc	13.33abcd	27.79 bc
	Q (ST)	12.4 ab	7.93 bc	16.74 abc	39.00 bc
	R(AM)	10.7 bcd	2.77 c	2.5 bcd	19.61 c
	S (-)	11.7 abc			
	T (T)	9.2 de			
	LSD (0.05)	2.22	11.96	15.18	27.19

Note: - Numbers followed by the same letter are not significantly different according to LSD.
- S=*S. cepivorum*, T= Tr5, A= Autoclaved millet.
- % inf = % of onions infected by *S. cepivorum* .

Table 20(D): Intact sclerotia remaining from bags of 30 which were buried in pots for the duration of the temperature trial in presence or absence of onions and Tr5 and under different temperature strategies.

Temperature, Onion strategy	*S.cepivorum* only	*S.cepivorum* + Tr5	*S.cepivorum* + AM
10/10 + onions	3.5 f	5.1 def	5.3 def
10/10 - onions	12.3 a	7.8 bcd	nd
10/20 + onions	4.8 ef	5.2 def	8.4 bc
10/20 - onions	7.1 cde	12.2 a	nd
20/10 + onions	5 def	5.2 def	6.4 cdef
20/10 - onions	7.5 bcde	12.9 a	nd
20/20 + onions	6 cdef	7.2 cde	4.7 ef
20/20 - onions	12.5 a	10.4 ab	nd

Note: -10 and 20 refer to the air temperature thermostat setting, not soil temperature.
-Numbers followed by the same letter are not significantly different according to LSD.
-nd= not determined

Table 20(E): Number of root samples taken, and *Trichoderma* spp. isolated from root samples taken from the non-infected controls two months after the trial was set up and incubated on RASP medium.

Temp: Treatment.	Total samples	Total *Trichoderma* spp	Percentage *Trichoderma* Colonisation
10°C - Tr5 (D, I)	35	7	20%
10°C + Tr5 (E, J)	34	34	97.1%
20°C - Tr5 (N, S)	38	9	23.6%
20°C + Tr5 (O, T)	25	24	96%

Note: 10 and 20°C refer to air temperature thermostat settings, not soil temperature.

20.4. Discussion.

The annual soil temperature monthly averages for the seasons from 1990-1995 in north west Tasmania are shown in figure 20(F). Higher and lower fluctuations for short periods are common. The warmer glasshouse typically simulates soil temperatures in the months from December to February. The cooler glasshouse simulates typical soil temperatures in the October - November period, and last crops lifted in March. The cool:warm strategy would be the most similar to onion crops sown around August and lifted in February.

According to Brewster (1994) the minimum temperature for optimum onion seed germination is 13° C. The cooler glasshouse, with soil temperature of 12-13°C was close to this limit. There has been no difference between germination in untreated and non infected controls at the two temperatures. In the cooler glasshouse, the 20-30% lower emergence in Tr5 amended treatments appears to be related to the addition of organic material rather than the Tr5 as similarly low emergence is evident in autoclaved millet amended treatments. Possibly the addition of this food base could induce greater microbial activity which could influence nutrient uptake in the soil environment, or activity of minor seedling pathogens. The warmer glasshouse did not demonstrate the same phenomenon, seedling vigour was probably stronger at this temperature and germinating seeds were able to overcome the difficulty. Soil temperatures at the time (May to September) of commercial sowing (and sowing of other trials in this study) would be around 4°C lower than those in the cool glasshouse at the time of emergence.

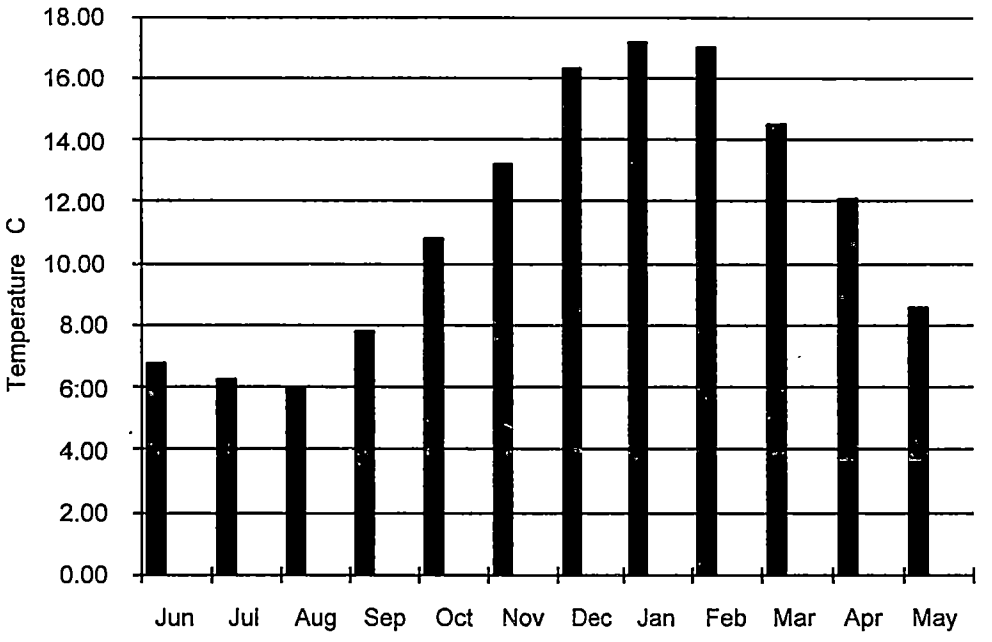


Figure 20(F): Average monthly soil temperature for the years 1990 - 95 at a depth of 10cm taken at the Forthside Vegetable Research Station (north west Tasmania).

In the first three months after emergence, the number of *S. cepivorum* infections steadily increased in both untreated controls in the cool glasshouse but was significantly lower in the warmer glasshouse. The increased severity of infection at this soil temperature is consistent with the general incidence of *S. cepivorum* infections in Tasmania, where disease is normally most severe from October to mid-December where soil temperatures are normally in the 11 to 15°C range, and less severe in mid summer soil temperatures above 15°C. Similarly in New Zealand

severe disease late in the season has been associated with lower than normal soil temperature in the September -October period (Fullerton *et al.*, 1994) with the rate of infection levelling off as soil temperature rose above 18°C. Relationships of temperature to sclerotial germination vary according to the isolate under study (Gerbrandy, 1989; Gerbrandy, 1992). The isolate used by Crowe and Hall (1980b) displayed optimum (78%) germination at 18°C but around one quarter of this level of germination at 12°C at a range of matric potentials. Adams and Papivizas (1971) demonstrated a similar relationship. One tendency common to all isolates tested by Gerbrandy (1992) was that a greater percentage of sclerotial germination occurred when sclerotia were stored at 20°C for 8 weeks followed by storage at 5°C for 8 weeks, than for sclerotia stored at 20°C for 16 weeks. The sclerotia used in the present study had been stored at air temperature in the laboratory (fluctuating between 12 and 20°C) for six months prior to the trial and would have experienced a drop in temperature which could have encouraged germination in this way.

While plants which remained in the cooler glasshouse after three months continued to develop infections at a steady rate to finally reach 69.27% infection, plants moved to the warmer glasshouse continued to become infected at a similar rate for the next month, before the rate of infection slowed. Although the mean daily temperature in the warmer glasshouse was in the 14-16°C range in the last month of the trial infection reached only 42.79% . The continued infection in the fourth month may have been from infections already initiated before the pots were moved to the warm glasshouse. Established infections are known to develop faster in warmer soils (Crowe and Hall, 1980b).

Pots not amended with Tr5 held in the warmer glasshouse for the duration of the trial had significantly lower infection than those held in the cool glasshouse. Most of these infections occurred in the last month of the trial when soil temperature was in the 14-16°C range. Virtually no infections were present in pots transferred from the warm to cool glasshouse before they experienced a drop in soil temperature from 18-20°C to 10-12°C. Consistent with the trend identified by Gerbrandy (1992), it appears that a large proportion of the sclerotia then germinated to induce 38.15% infection. During the same period disease incidence in pots which spent the entire season in the cool glasshouse increased by 51%.

As in the inoculum depth trial and soil pH trials sclerotia buried in mesh bags have decreased in number by around 70% in the absence of onions due to decay and non specific germination. There is too much variability in the numbers of those which remain to detect any real differences due to the effect of temperature or presence of Tr5 inoculum or autoclaved millet. Greater numbers of viable sclerotia remained in treatments where no onions were present. It is worthy of mention that the treatment with most severe disease (cool:cool) had the greatest number of viable sclerotia remaining.

While Tr5 efficacy in the full cool season simulation has been similar to that in other pot trials (eg Inoculum density, Inoculum depth), Tr5 has failed to provide control in treatments which spent the first three months in the warmer glasshouse, and performed poorly in those transferred from warm to cool conditions. Under this temperature regime comparison of treatments P and Q would suggest that Tr5 was of low efficacy in the first three months in this glasshouse. During this period good disease suppression was recorded in the cooler glasshouse, suggesting that Tr5 was more able to establish itself in the cooler soil, than the warm soil. No data was collected specifically to determine whether there were any differences in Tr5 establishment under the two temperature strategies. The data shown in Table 20(E) does not prove that Tr5 became established, but the total *Trichoderma* populations were of similar magnitude at the two temperatures (the possibility cannot be excluded that amendment of Tr5 millet inoculum encouraged another native species to proliferate). Poor performance of Tr5 in the warmer soil may also be linked to greater vigour of *S. cepivorum*. This hypothesis is supported by Crowe and Hall (1980b) who showed that once roots have become infected, the infection develops 20-30% faster (varying at different matric potential) at 18°C than at 12°C. However under laboratory conditions in the antagonism bioassay (Chapter IV) which was performed at 20°C, pre-germinated sclerotia were used to infect roots and Tr5 was able to suppress *S. cepivorum* infection. There may be some physiological differences between the Tasmanian *S. cepivorum* isolate and that used by Crowe and Hall (1980b).

Autoclaved millet has been amended as a control to investigate whether the efficacy of the Tr5 millet treatment is due to the presence of the fungus, or partially attributable to organic amendment. There is an issue of whether addition of undigested organic matter constitutes a valid control for an experiment of this type, as this may induce greater microbial activity leading to biological control in itself (Baker *et al.* ,1984) which is especially a concern where the rate of amendment is high as in this trial. Under the cool:warm, warm:cool, and warm:warm strategies there are no significant differences between disease incidence in untreated controls, millet amended and Tr5 amended treatments. There may be some form of disease suppression under warmer conditions resulting from this amendment, as in all three of these temperature strategies the actual disease percentages are lower than in untreated controls and Tr5 amended treatments.

These results indicate that Tr5 is likely to retain reasonable efficacy in cool conditions in the September to November period when soil temperatures are in the 8 to 14°C range, and many sclerotia germinate. However there is reason for concern that if there should be a dip in soil temperature in January when sclerotia again germinate followed by warm weather when infections develop fast, Tr5 may not be able to successfully challenge the infection.

Chapter VII:

21.0: Isolate variability in *S. cepivorum* .

21.1. Introduction:

S. cepivorum has been generally considered to be a genetically homogenous pathogen, though there have been reports on isolate variability based on several criteria including polygalacturonase production (Abd-El-Razik *et al.*, 1974), germination following conditioning treatments (Brix and Zinkernagel, 1992a; Gerbrandy, 1992) size of sclerotia, colony colour, morphology and rate of radial growth (Stewart, 1990).

Genetic variability between *S. cepivorum* at trial sites could contribute to some of the unexpected disease incidences which have been demonstrated (Ch. V), though localised climatic factors are probably more important.

In sieving sclerotia to assess the influence of onion mash water waste on *S. cepivorum* sclerotial germination (Chapter V:15), *S. cepivorum* sclerotia were trapped in a 0.297mm sieve for counting. While the typical *S. cepivorum* sclerotium was recognisable, they were often detected as two joined sclerotia or as a rugby ball shaped body. Additionally, sclerotia were encountered in the 0.297mm sieve which were two thirds of the diameter of those known to be *S. cepivorum*. But for being lodged in fragments of decomposing plant tissue, these sclerotia would have passed through the sieve un-noticed. As there was a concern that these sclerotia may be a smaller isolate of *S. cepivorum*, a 0.210mm sieve was added to each sample and the sclerotia were counted. The sclerotia in the 0.210mm sieve were approximately twice as numerous as those in the 0.297mm sieve. Consequently, it was necessary to determine whether these sclerotia were *S. cepivorum* .

21.2. Materials and Methods.

21.2.1. Isolates

Fungal isolates were collected from a range of sources in north west Tasmania (Table 21.A).

Table 21(A). The source of isolates in this study.

Isolate	Site	Isolation date	Source
Sc1	unknown	1990	provided by Dr James Wong
Sc4	Bonney	1993	infected bulb
Sc5	Bonney	1993	sieved from soil
Sc7	Goodwin	1993	infected bulb
Sc8	Vecon (oil)	1994	sieved from soil
Sc9	Harding	1994	sieved from soil
Sc10	Harding	1994	sieved from soil
Sc12	Harding	1994	sieved from soil
Sc13	Harding	1994	sieved from soil
Sc14	Scolyer	1994	seived from soil
Sc16	Addison	1996	infected bulb

21.2.2. Koch's postulates.

To confirm pathogenicity, axenic culture sclerotia of isolates Sc1, Sc4, Sc5, Sc7, Sc8, Sc10, Sc12, and Sc14 were buried 4cm deep in potting mix (20 kg peat, 20kg sand, 140g lime, 80g dolomite, 120g osmocote, steam treated) in pots and a dormant onion bulb which had been inspected by dissecting microscope for adhering propagules of *S. cepivorum* was planted on the surface. Pots (15cm diameter) were left for three months (during spring) in a shade house and regularly watered. When bulbs were seen to have become infected, sections of root and base plate tissue were taken to the laboratory and plated on onion agar containing 0.1% streptomycin sulphate, and incubated at room temperature for 4-7 days before they were inspected for mycelium and then sclerotia.

21.2.3. Isozyme Profiles.

Isozyme profiles were made for polygalacturonase, pectinesterase and ribonuclease by the methods of Cruickshank and Pitt (1987) (Appendix E; Electrophoresis Methods). Cultures were incubated on a medium composed of 20% macerated onion tissue in distilled water at 25°C for 1, 2, 3 and 4 weeks under static conditions in darkness

Isolates of *Macrophomina phaseolina* were supplied by Michael Fulbohm (University of Queensland). Isolates of *Colletotrichum coccodes* were supplied by Ian Pascoe (Knoxfield Laboratories, Agriculture Victoria). These fungi , Sc8 and Sc4 were incubated in 2.0mls of pectinase medium (Appendix B; media) at 25°C for the specified periods.

21.3. Results.

21.3.1. Culture Characteristics.

Isolates were obtained in pure culture on onion agar (Appendix B; media). In comparison to a typical *S. cepivorum* isolate Sc4 which has with sclerotia ranging from 300 to 500µm diameter, isolates Sc8, Sc9, Sc10, Sc12 and Sc13 had sclerotia of 200 to 300µm diameter. Microscopic examination showed that the smaller sclerotial fungi developed sclerotia within the agar and almost always within a tiny fragment of onion tissue, whereas Sc4 sclerotia formed among tufts of mycelium on the agar surface. Calcium oxalate crystals (Stone and Armentrout, 1985) which are common in Sc4 culture were infrequently formed in cultures of the smaller sclerotial fungi.

Isolate Sc9 was isolated from Harding's soil from a sclerotium of typical *S. cepivorum* size. Mycelium was of similar dimensions and calcium oxalate crystals were noted precipitated in onion agar culture. When sclerotia formed they were 4 - 8mm in diameter. After a further 2 weeks sparse conidiophores were seen similar to *Botrytis* spp..

Isolate Sc16 was found on the base of bulbs showing infection typical of *S. cepivorum* late in the 1995/6 season. However, the sclerotium was many times larger than those normal to *S. cepivorum* (Figure 21b), and was surrounded by smaller sclerotia. After surface sterilisation in 2% sodium hypochlorite for 2 minutes the sclerotium was cut into three segments which were incubated separately. The sclerotia which formed following germination of each segment were of similar size to sclerotia of isolates Sc 4,5,7, and 14. In the same season

(1995/6) large *S. cepivorum* sclerotia were collected from three sites near Devonport and a glasshouse trial 300km south in Hobart.

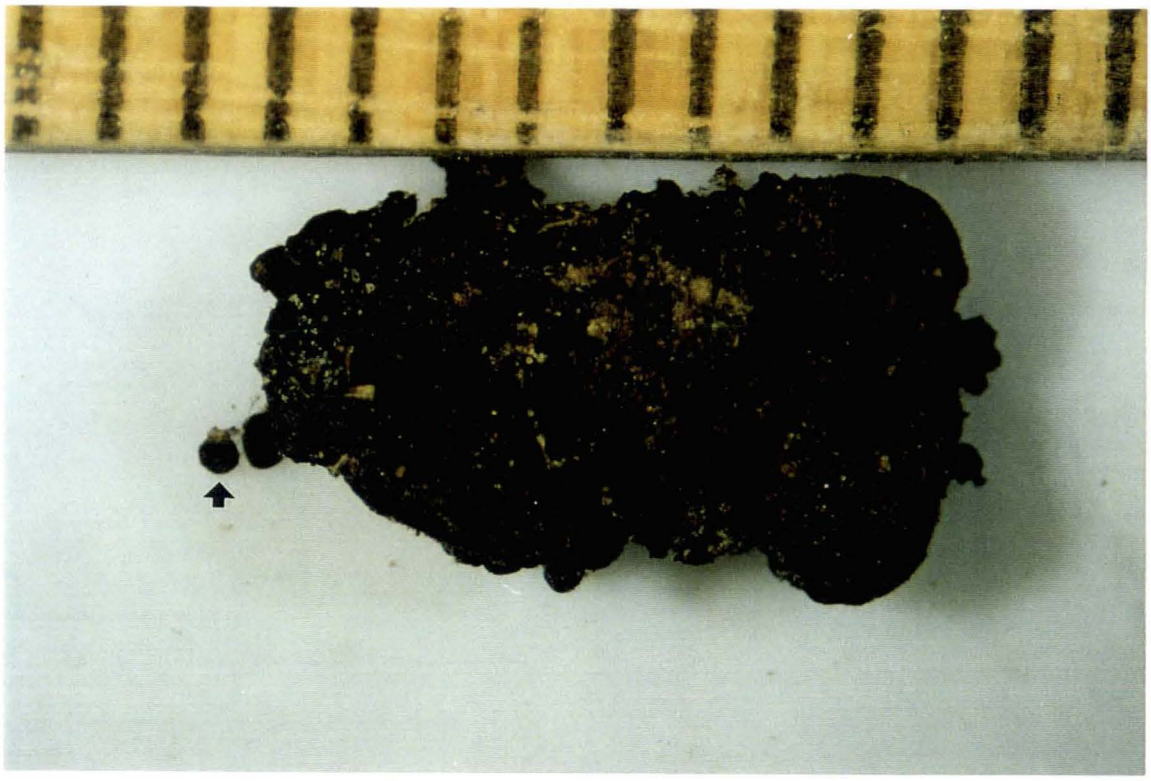


Figure 21(B): The large sclerotial form of *S. cepivorum*. This example was collected near Devonport, Tasmania in January 1996 (scale in mm). A normal sized sclerotium (arrowed) is shown to the left of the large sclerotium.

21.3.2. Koch's Postulates:

S. cepivorum was successfully re-isolated from bulbs showing distinct white rot symptoms grown in steam sterilised potting mix amended with sclerotia of isolates Sc1, Sc4, Sc7, and Sc14. The Sc5 amended bulb did not become infected. Bulbs from pots to which isolates Sc8, Sc10 and Sc12 were added showed different symptoms to the former group with drying off of green leaf tissue, loss of virtually all roots, and loss of tissues surrounding the base plate which in the most extreme case resulted in the protrusion of base plate from the bottom of the bulb (Figure 21.C). Dark sclerotia formed in the dry scale tissue surrounding the base plate. Fungi identical to those inoculated were re-isolated for isolates Sc8 and Sc12. Bulbs were left in the pots and Sc8, Sc10 and Sc12

inoculated bulbs developed a rot of the core tissue which appeared to spread from the base plate after a further two months. No isolations were made from this rotted tissue though black sclerotia were numerous in the scale tissue surrounding the rot. The fungus was first encountered in sieved soil samples from the Vecon site in August 1994. By January 1995 a morphologically identical fungus had been isolated from sclerotia on the base plates of onions which had dried off and stopped growing but not rotted at Vecon's, Harding's, Scolyer's and Goodwin's sites. Field collected specimens (Figure 21.D) were not as severely decayed surrounding the base plate as those artificially inoculated. Thirty specimens with similar symptoms to Figure 21(D) were stored in the laboratory. Over six months almost all developed an internal rot which appeared to spead from the base. The cause was not confirmed, but symptoms were dissimilar to infection caused by *Botrytis allii.*

Figure 21(C): Onion base tissue from a bulb inoculated with isolate Sc12. Scales from the tissue surrounding the base plate have progressively decayed to the stage where the base plate protrudes, this specimen displays more severe loss of base scale tissue than field collected specimens.

Figure 21(D): Onion bulb collected at Scolyer's trial site with black sclerotia embedded in scale tissue surrounding the base plate. Fungi morphologically similar to isolate Sc8 were isolated from bulbs showing similar symptoms.

21.3.3. Isozyme Profiles.

Isozyme profiles by the method of Cruickshank and Pitt (1987) for polygalacturonase (PG) and pectinesterase (PE), and ribonuclease (RN) were made for all isolates. Three distinct profile groups were detected, these are displayed in Figure 21(E). Details of the profile groups were as follows:

-group 1
- -PE R_f 0.10-0.30
  R_f 0.40-0.55
- -PG R_f 0.15 (occasionally numerous small bands from 0.05-0.35)
- -RN R_f 0.14, 0.70, 0.90

(isolates: Sc1, Sc4, Sc5, Sc7, Sc14, Sc16)

-group 2
- -PE R_f 0.10-0.40 (made of numerous small bands)
- -PG R_f 0.70
- -RN R_f 0.12, 0.45. 0.60

(isolates: Sc8, Sc10, Sc12, Sc13

-group 3
- -PE R_f 0.15-0.40, 0.50-0.65
- -PG
- -RN R_f 0.20, 0.48, 0.56.

(isolate: Sc9)

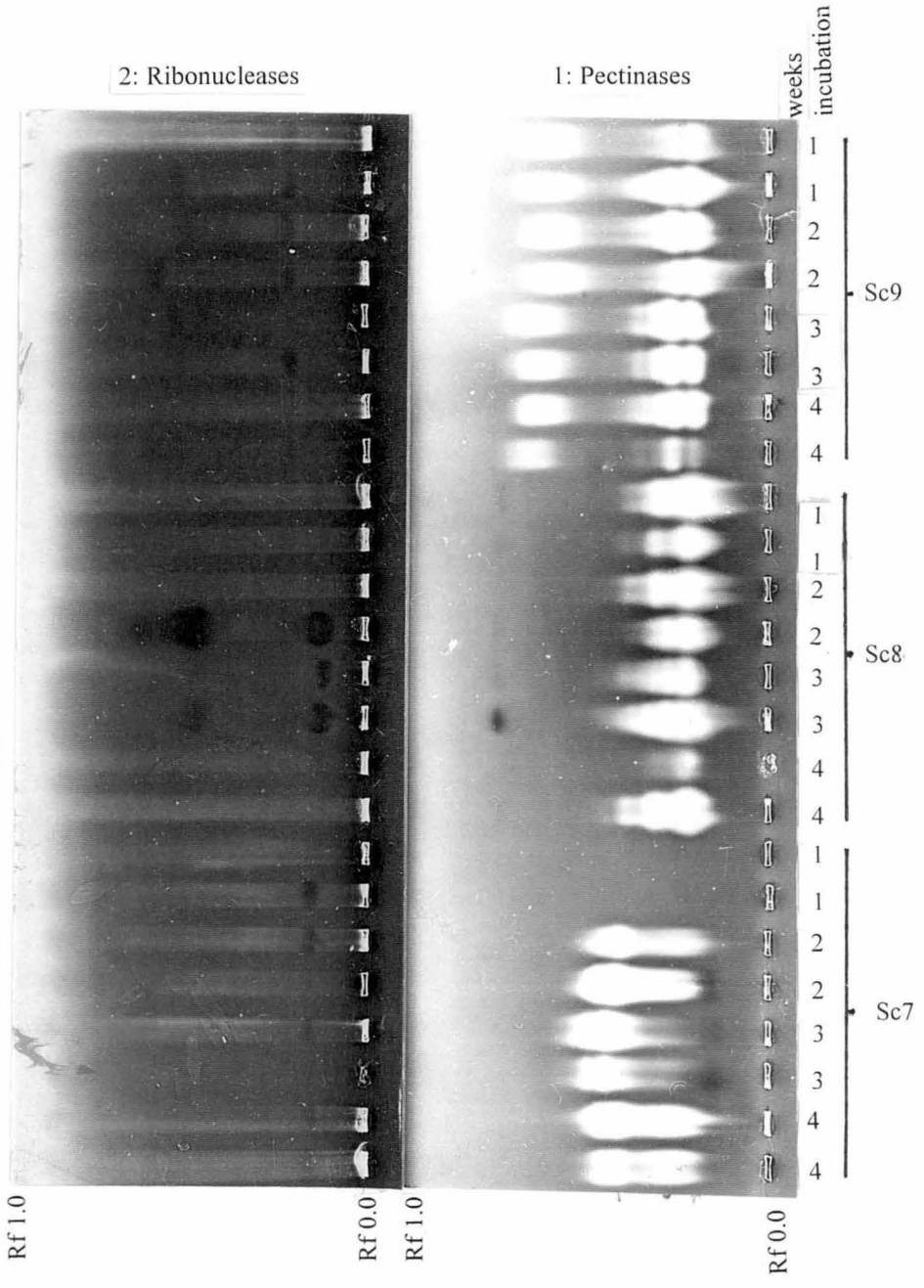


Figure 21(E): Isozyme profiles for isolates Sc7, Sc8, and Sc9 for production of (1) polygalacturonase (dark zones) and pectinesterase (light zones) and (2) ribonuclease. Cultures grown in 20% onion medium at 25°C for 1, 2, 3 and 4 weeks. Two replicates of each age are presented.

To test the identity of the larger sclerotial form (Sc16) pectinase isozyme profiles were compared to those of isolate Sc4. The mycelium was taken as hyphal tips 2cm from the germinating sclerotium in the agar. Figure 21(F) shows that the pectinase profile of Sc16 at 7 and 14 days was identical to the pectinase profile of Sc4.

To investigate the identity of isolate Sc8 pectinase profiles were compared to those of *Macrophomina phaseolina* and *Colletotrichum coccodes*. The profile of Sc8 was dissimilar to both of these pathogens (Figure 21.G).

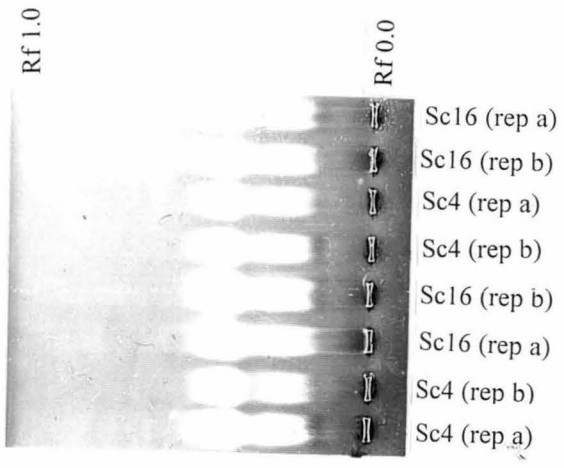


Figure 21(F): Isozyme profiles for polygalacturonase (dark zones) and pectinesterase (light zones) of isolates Sc4 (confirmed by Koch's postulates to exhibit symptoms typical of *S. cepivorum*) and Sc16 (the larger sclerotial from) grown in 20% onion medium at 25°C for 7 and 14 days (two replicates of each presented).

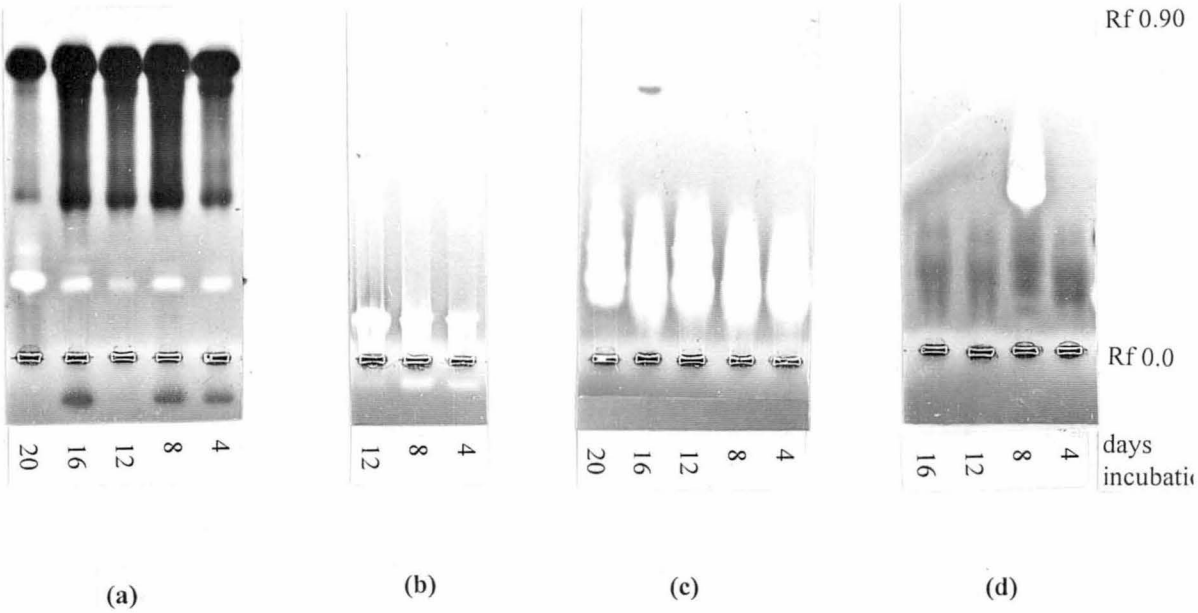


Figure 21(G): Isozyme profiles for pectinase of *Macrophomina phaseolina* (a) and *Colletotrichum coccodes* (b), Sc8 (c) and Sc4 (d) grown on pectinase medium at 25°C for the periods specified.

21.4. Discussion.

In selection of trial sites and assessment of sclerotial densities as in Chapter V:15 (the sclerotial germination stimulant trial) populations of sclerotia have been assessed by wet sieving. If this data is to be relied upon it is important to know whether all of the sclerotia counted are those of *S. cepivorum.* In this work two fungi have been detected which can potentially mislead sclerotial density counts. The upper size limit of Sc8 sclerotia is large enough for some sclerotia to be trapped in the 0.297mm sieve. Based on sclerotial morphology these are indistinguishable from *S. cepivorum* . Additionally, isolate Sc9, which has a pectinase isozyme profile (Figure 21.E.) similar to *Botrytis cinerea* (Cruickshank, 1988) produces some proportion of sclerotia which are small and round enough to be mistaken for *S. cepivorum* in sieve samples.

According to pectinase and ribonuclease isozyme profiles there is no difference between the isolates morphologically similar to Sc4 from the other sites. However, there is a genetic difference between *S. cepivorum* (Sc4) and the smaller sclerotial fungi. According to pathogenicity confirmation by Koch's postulates the smaller sclerotial fungus displays different infection symptoms to *S. cepivorum* . Based on this it seems unlikely that Sc8 is a different strain of *S. cepivorum* . The morphological similarity and lack of all sporulative structures

excepting sclerotia would suggest that the fungus is a member of the Agonomycetales (syn- *Mycelia Sterilia*). The fungus bore some similarity to descriptions of one fungus in this group, *Sclerotium bataticola* (syn- *Macrophomina phaseolina*) (Holliday and Punithalingam, 1970) however no pycnidia were formed. *M. phaseolina* has been documented as a pathogen of onions causing drying and disintegration of infected scale with hard shiny black sclerotia of 50-150µm in diameter formed in the scale tissue commonly known as "Charcoal Rot" (Sumner, 1995). Comparison of pectinase profiles of *M. phaseolina* isolated from mungbeans in Queensland to Sc8 indicated that *M. phaseolina* had numerous bands absent in Sc8 (Figure 21.G). Culture morphology of the two fungi was also distinctly different. Dr Ian Pascoe (Diagnostician, Institute for Horticultural Development, Knoxfield, Victoria) suggested the fungus might be a strain of *Colletotrichum coccodes* which had lost the ability to produce setae, and that the fungus demonstrated growth of aerial mycelium on Oatmeal Agar which is often characteristic of *Botrytis* spp. Pectinase isozyme profiles of *C. coccodes* were dissimilar to Sc8. Comparison of the Sc8 isozyme profile to a group of 26 *Botrytis* spp. pectinase isozyme profiles compiled by Cruickshank (1988) indicated high similarity to the profile of *Botrytis ficariarum* (CBS 176.63). As yet cultures of this fungus have not been obtained. However, Hennebert and Groves (1962) describe this fungus as having sclerotia 3-12 x 0.5-1.5mm in size but sometimes larger so this seems an unlikely identity. One other member of the genus *Sclerotium* which the fungus bears similarity to is *Sclerotium gladioli* (Whetzel, 1945) (Syn- *Stromatinia gladioli*) which is the cause of Dry Rot of *Gladiolus* corms (Drayton, 1934). Like Sc8, *S. gladioli* has white silky mycelium and sclerotia 115-229 x 105-208µm in diameter (Massey, 1928). This fungus has been isolated in southern Tasmania. Isozyme profiles for pectinase performed by the methods used herein but with the pectinase medium amended with glucose, by Dr R H Cruickshank detected PE bands of R_f 0.15-0.50 which does loosely fit the PE profile of Sc8, the PG of R_f 0.70 sometimes detected in Sc8 was not detected in *S. gladeoli.* (R. H. Cruickshank, 1982, *Unpubl*). Dr Cruickshank's *S. gladioli* cultures have since been discarded. If the fungus is not *S. gladioli* it may be an undescribed species, reference cultures have not yet been obtained.

Identification of the Sc8 type isolates is important, as this fungal type seems to be widespread in north west Tasmania and is possibly causing some losses attributed to *S. cepivorum* by growers. The propensity of infected bulbs to rot in storage remains unconfirmed, but would pose a serious economic problem. The isolation of the fungus from Scolyer's trial site, coupled with extremely low incidence of *S. cepivorum* infection, leads to speculation of what proportion of

the 79 sclerotia per kilogram counted in soil samples from this site might have actually been the smaller sclerotial fungus.

The discovery of the larger sclerotial form of *S. cepivorum* is the first report of this phenomenon in Australia. A critical explanation would be that the bulb was co-infected by *Botrytis* spp.. However, pectinase isozyme profiles confirm that the sclerotium was *S. cepivorum* (Figure 21.F). This structure has previously been reported from Egypt (Georgy and Coley-Smith, 1982), New Zealand (Backhouse and Stewart, 1988) and North America (Crowe, 1995a). The structure had a number of normal size sclerotia embedded in it and seemed to be an extension of the phenomenon where *S. cepivorum* sclerotia are formed as double or multiple lobes. Backhouse and Stewart (1988) suggested the phenomenon may be a remnant of an ancestral sexual phase.

Chapter VIII: General Discussion.

This study commenced due to the serious threat to Tasmania'a onion exports ($40 million per annum) caused by onion white root rot (*Sclerotium cepivorum*). At this time, control was solely dependant on the fungicide Sumisclex 500 WP (Wong and Maynard, 1986) which is no longer used due to concerns over chemical residue which can remain in the produce. The chemical recommended in 1996/7 season (Folicur 430) has failed to provide effective disease control in following correct application in several commercial crops. Development of a non-chemical control measure for *S. cepivorum* is highly desirable for sustainable onion production in Tasmania.

Lacey and Wong (1991) demonstrated 60% reductions in disease incidence using *T. koningii* (strain TrA) amended to soil at the time of sowing as millet inoculum applied at a rate of 110 kg/ ha. While this was an encouraging result, it is far below what would be considered a commercially acceptable level of disease control. The overall goal of this project was to develop an understanding of the biological control system from a histological, histochemical and ecological perspective, to determine how to use *T. koningii* (Tr5) to provide efficient disease control, and to understand the reason for any loss of efficacy. Detailed study of a single biocontrol isolate was considered more likely to lead to sustainable disease control than mass screening of potential biocontrol isolates with less understanding of their mode of action.

To understand how Tr5 prevented developing infections from reaching the base plate a more detailed understanding of the *S. cepivorum* infection process was necessary. The process of penetration of the onion root epidermis has been documented (Abd El-Razik *et al* ., 1973; Stewart *et al.,* 1989a & b). The results presented in Ch. III(6) further our knowledge of the development of internal root infections following penetration, showing that initially, cell death is confined to those cells through which *S. cepivorum* actually grows, but as the infection develops, considerable cell death and cell wall degradation occurs in advance of hyphae. Cortical tissues are more readily degraded than those of the epidermis and stele, resulting in formation of a cavity filled with hyphae. It was suggested that in soil the undegraded epidermis may serve as a barrier which excludes competitive or antagonistic saprophytes from *S. cepivorum* hyphae (Ch. III:6). This finding may have implications for the broader study of control of *S. cepivorum*. For example onion cultivars with an epidermis more readily degraded may allow greater microbial competition within infected tissue.

Previous studies on *S. cepivorum* cell wall degrading enzymes have identified two pectinesterases (Mankarios and Friend, 1980; Favaron *et al.*, 1993) and a polygalacturonase (Abd El-Razik *et al.*, 1974). Studies presented in Ch. III(7)

identified three proteins with pectinesterase and three proteins with polygalacturonase activity which were produced by *S. cepivorum* during infection of onion roots. It has been established that pectinesterases of *S. sclerotiorum* diffuse ahead of hyphae (Lumsden, 1976), and it was confirmed that *S. cepivorum* pectolytic enzymes also exhibit this behaviour. Pectinesterases appeared to be the first pectolytic enzymes to act on the cell walls by demethylating pectin both in infected roots and in culture before polygalacturonase (Ch. III:7). Polygalacturonase acts more readily on demethylated pectin (Mankarios and Friend, 1980). A novel technique for localisation of pectin degrading enzyme activity in infected tissue by loading tissue segments into the wells of an electrophoresis gel has been developed. The method is a powerful tool as it allows identification of individual proteins, and can be performed under native conditions. To determine whether *S. cepivorum* pectinases are primary agents of the root cell death which has been observed (Ch. III:6) would require more work on distribution of cellulases, proteases and oxalic acid.

When *T. koningii* was introduced to the surface of onion roots it grew through epidermal mucilage but did not penetrate the epidermis of healthy roots. On the surface of infected roots it was discovered that *T. koningii* actively colonised epidermal passage cells (Ch. IV:9). These cells appear to become suberised more slowly than other epidermal cells (Peterson *et al.*, 1978). In some other Lilliaceae members they are not lignified (Brundrett *et al.*, 1988), and they may have a greater number of plasmodesmatal pits per unit surface area (Scott *et al.*, 1956). Colonisation of tissues by *Trichoderma* spp. via passage cells has not previously been reported. However in another member of the Lilliaceae, *Smilicina racemosa* (Brundrett and Kendrick, 1988) and Asparagus (Hussey, 1982), vesicular arbuscular mycorrhizas gain access to the root cortex via these cells. Growth of *Trichoderma* spp. inside roots in similar form to mycorrhiza has been reported (Kleifield and Chet, 1992), but it is unlikely that Tr5 is exhibiting this type of association as the phenomenon was observed solely on infected roots. Following initial colonisation of the passage cells, Tr5 coiled to fill the cell with mycelium, a mechanism which could prevent other soil micro-organisms from entering the cortical tissue by this path, before spreading into infected cortical tissue.

Little information is available concerning pectin degrading enzymes of *T. koningii.* Production of two polygalacturonases was reported (Fanelli *et al.*, 1978), both were glycoproteins which were not absorbed by plant tissue. Cervone *et al.* (1977) suggested that the glycosidic moiety of enzymes mediates recognition of the host by phytopathogenic fungi, and Fanelli *et al.*, (1978) suggested that differences in structure of *T. koningii* polygalacturonases may account for the lack of pathogenicity. Results presented in Chapter IV(10) established that Tr5 produced a polygalacturonase and two pectinesterases in liquid culture. Polygalacturonase was

produced as *T. koningii* grew in root epidermal mucilage before colonising infected tissue. It was suggested that *T. koningii* pectolytic enzymes are not able to act on epidermal tissue (Ch. IV:10), but that passage cells may be more favourable sites for activity and consequently are actively colonised. As *T. koningii* was not noted to be associated with these cells in healthy roots it is possible that the cells must be injured by *S. cepivorum* toxins before *T. koningii* colonises them. On cucumber roots *T. harzianum* has been noted to actively colonise wounded epidermal cells (Green and Funck-Jensen, 1985). After entering the root via passage cells, *T. koningii* appeared to be limited to colonisation of damaged tissue. *T. koningii* produced both polygalacturonase and pectinesterase to degrade infected tissue. When *T. koningii* was present within the root *S. cepivorum*, pectinesterase in particular was notably less detectable in advance of the infection. Zimand *et al.* (1996) recently reported reductions in *Botrytis cinerea* polygalacturonase in bean leaf lesions in presence of *T. harzianum*. As in the present study these authors were undecided whether the effect was due to reduction in *B. cinerea* biomass, direct effect on enzyme production or secretion, or activity of *Trichoderma* proteases. Possible denaturation of *S. cepivorum* pectinases by *T. koningii* would be a worthy avenue for future investigations.

Observation of hyphal interactions between *S. cepivorum* and *T. koningii* indicated that physical contact between the two fungi was only necessary for lysis of *S. cepivorum* hyphae when no aqueous medium was present between the two (Ch. IV:8) This suggested that a non volatile principle such as antibiotics or cell wall degrading enzymes, was responsible for lysis. Within onion roots, *S. cepivorum* hyphae were seen to begin to decay as *T. koningii* colonised the infected tissue. *S. cepivorum* hyphae were seen to become detached at septa, sometimes with bursting of hyphal tips. These types of changes are characteristic of the effects of chitinolytic enzymes on fungal hyphae (Ordentlich *et al.*, 1988; di Pietro *et al.*, 1993; Lorito *et al.*, 1993). These observations combined with lack of detection of antimicrobial compounds lead to study of chitinolytic enzyme production by *T. koningii* .

Previous studies of chitinolytic enzymes in degradation of fungal cell walls have investigated degradation of cell wall polysaccharides in liquid culture (de la Cruz *et al.*, 1993; Garcia *et al.*, 1994; Haran *et al.*, 1995; Schirmböck *et al.*, 1994). Histological studies have established the role of chitinolytic enzymes on host cell wall dissolution (Benhamou and Chet, 1993; Benhamou and Chet, 1996). In direct mycoparasitic confrontation between *T. harzianum* and *S. rolfsii* it was recently established that a series of chitinolytic enzymes are produced (Haran *et al.*, 1996). Within host tissue a diverse array of nutrition sources are present and little information is available concerning whether production of chitinolytic enzymes in liquid culture or direct mycoparasitism is of a similar nature to that observed within host tissue. In culture, *T. koningii* produced four proteins with chitinolytic activity

(Ch. IV:11). Definitions of chitinolytic enzymes clarified by Harman *et al.*, (1992) describe two of these proteins (R_f 0.46 and 0.62) as endochitinases. However, these definitions are not adequate for explanation of the remaining two proteins (R_f 0.15 and 0.24) to which the term chitobiase (Zikakis and Castle, 1988) has been applied (Ch. IV:11). In liquid culture, chitinolytic isozymes produced by Tr5 for degradation of purified crabshell chitin were the same as those produced for degradation of *S. cepivorum* sclerotia. In *S. cepivorum* infected roots, *T. koningii* (Tr5) produced the Rf 0.46 endochitinase and Rf 0.24 chitobiase; these isozymes are likely to contribute to the observed *S. cepivorum* cell wall degradation. No series of isozyme production was noted as has been reported for *T. harzianum* (Haran *et al.*, 1996). It is not certain whether presence of these enzymes is induced or constitutive, but the increase in production to detectable concentration is likely to be induced. While chitinolytic enzymes have been found to be produced by *T. koningii* in infected tissue, these are not claimed to be the sole mechanism of lysis. However this is not the only example of a successful antagonist from which no antimicrobial compounds could be detected (Chet and Baker, 1980; Chet and Baker, 1981, Jackson *et al.*, 1991b).

For studies of chitinolytic enzyme production within infected tissue it was desirable to be able to conduct electrophoresis under native conditions (eg boiling in SDS buffers was not possible) with minimal tissue disruption so root segments could later be inspected by microscope. Through modification of pre-existing techniques a chitinolytic enzyme detection system combining a citric acid buffer (Cruickshank and Pitt, 1987) with glycol chitin substrate (Trudel and Asselin, 1989) was developed (Ch. IV:11). Electrophoresis at 2-4°C prevented enzymes from acting on the substrate as they migrated, eliminating need for substrate containing overlay gels. Calcoflour white (Maeda and Isheda, 1967; Trudel and Asselin, 1989) successfully stained undegraded glycol chitin in the gels. For more specific characterisation of mode of activity either 4-MU-(GlcNAc), 4-MU-$(GlcNAc)_2$ or 4-MU-$(GlcNAc)_3$ were added to gels (Appendix E). An alternative approach published during preparation of this manuscript detected total chitinolytic enzyme activity by addition of both 4-MU-(GlcNAc) and 4-MU-$(GlcNAc)_2$ to overlay gels (Haran *et al.*, 1996). Specific proteins were recognised in gels using polyclonal antibodies. This technique would be useful for study of bands obscured in the gel by plant chitinases of similar R_f.

Studies presented in Ch. III & IV enabled a hypothesis for the mechanism of disease suppression to be established. This work was performed under sterile conditions, and would be difficult if not impossible to conduct in unsterile soil. However, until the mode of action can be shown to occur in natural environments, mechanisms of disease control obtained *in vitro* should be viewed with reservation (Whipps, 1992). The core issue of the mechanism of disease suppression hypothesis advanced in Chapters III and IV is that when an *S. cepivorum* infection is advancing

within an onion root toward the bulb base, *T. koningii* is able to prevent the advance of the hyphae if present on the root epidermis. The mode of action hypothesis was first tested under unsterile conditions in inoculum depth pot trial 1 (Ch. VI:16) where infections originating from layers of *S. cepivorum* sclerotia buried at different depths in soil had to pass a Tr5 pre-colonised band to infect the bulb. This trial demonstrated that Tr5 was able to successfully challenge at least 82% of infections initiated 3cm below the base plate. The greater the distance an infection had to travel to reach the base plate, the greater the chance that it could be successfully challenged by Tr5. This result does not confirm that the mechanism of suppression hypothesis is expressed in natural environments, but does confirm that by some means Tr5 is able to prevent infection hyphae within the root cortex from advancing to the base plate, which is a firm basis for continuation of Tr5's evaluation.

In inoculum depth pot trial 1, Tr5 was added to the pot as a continuous layer. Full rhizosphere establishment was provided with minimal requirement for saprophytic growth. In commercial field conditions Tr5 would need to be added in some form of carrier and from this base compete saprophytically for establishment on onion roots. Having the biocontrol agent in an active state in the right place at the right time is often the key to successful biocontrol (Lumsden *et al.*, 1995). The overriding requirement was to determine the means and conditions for delivery which would allow successful rhizosphere establishment. Three important components of this were; firstly to investigate appropriate carrier and delivery systems which will encourage Tr5 proliferation in soil (Chapter V), secondly to investigate ecological conditions which would most favour Tr5 establishment (Chapter VI:18-20), and thirdly to investigate how Tr5 might interact with conditions related to the pathogen (Chapter VI:16-17).

Lacey and Wong (1991) amended *Trichoderma* spp. to soil as dormant conidia grown on millet grains. Application of this material in an actively growing state did not improve efficacy over dormant preparations (Ch. V:13). Amendment of *Trichoderma* spp. for control of *S. cepivorum* using fluid drill techniques (Currah *et al.*, 1974) has not been previously attempted. Overall this technique did not improve efficacy beyond that of millet amendment, and would probably prove to be cumbersome in practice. Amendment using in-furrow sprays of Tr5 conidia also did not improve disease suppression including when additional nutrients were applied to the seed (Ch. V:13). Investigation of carriers continued in the following season; materials included sawdust, crabshell chitin, peat/chitin/osmocote mixtures, and millet applied below and beside seed and in combination with in-furrow spore sprays. Due to low disease incidence little information was gathered on the effects of these materials on efficacy, though considerable experience was gathered on Tr5 mass production. Amendment of Tr5 as millet inoculum remains the most reliable amendment strategy. The issue of carriers may require several more seasons of

evaluation. Techniques for monitoring the proliferation of Tr5 in soil by root sampling (Ch. VI) provide a useful tool for determination of a minimum rate of amendment for future field trials.

Ecology of biocontrol agents has been too often ignored in biocontrol studies (Deacon, 1990). In order for Tr5 to become established in the rhizosphere it is necessary not only to add it to the soil in an appropriate form, but to know that it is able to germinate, grow and compete within the soil ecosystem. Some attributes of the soil (eg pH and nutritional status) can be modified to create an environment in which some organisms compete better than others, possibly shifting the biological balance to the extent that *T. koningii* may become more readily established. Other soil conditions are less readily controlled (eg temperature) and it is necessary to understand how well *T. koningii* might become established and compete under these conditions.

To monitor proliferation of *T. koningii* a system for quantifying establishment and differentiating Tr5 was established. Other workers have used incubation of root segments (Harman *et al.*, 1989), serial dilution (Ahmad and Baker, 1987a), and ELISA testing (Thornton *et al.*, 1994), together with isolate identification by RFLP and DNA(RAPD) analysis (Mills and Muthumeenakshi, 1994), labelling with fluorescent marker genes (Green and Funck-Jensen, 1995), resistance to fungicides or antibiotics (Sivan and Harman, 1991), isozyme profiling (Zamir and Chet, 1985) and cultural characteristics (Seaby, 1996). Incubation of root segments on RASP selective medium (Ch. IV:12) provided insight into composition of the native *Trichoderma* population of the soil as well as the relative success of Tr5 amendment methodologies. Polygalacturonase and pectinesterase isozyme profiles (Cruickshank and Pitt, 1987) tested the morphological characteristics used to differentiate native isolates from Tr5 (Ch. VI:16-19). From this work a useful tool has been developed for quantifying what portion of the root system was colonised by Tr5. Pectinase isozyme profiles have not previously been used for characterisation of *Trichoderma* spp., although they are recognised criteria in species identification of *Penicillium* (Cruickshank and Pitt, 1987), *Sclerotinia* (Cruickshank, 1983), *Rhizoctonia* (Cruickshank, 1990) and *Botrytis* (Cruickshank, 1987).

It is well established that *Trichoderma* spp. are enhanced by acidified soil conditions (Chet and Baker, 1980; Chet and Baker, 1981). The change in pH alters the microbial balance and allows an increase in activity of *Trichoderma* spp. (Simon and Sivasithamparam, 1988c). Acidification of soil within the range which will not effect nutrient uptake (Hausenbuiller, 1984) is seen as a reasonable proposition. Trial results were complicated by what appears to be a suppressive effect of ferrous sulphate on *T. koningii* and calcium on *S. cepivorum.* (Ch. VI:18). However, investigation of saprophytic establishment of Tr5 in a further trial (Ch. VI:19) demonstrated that Tr5 had a poor ability to become established in soils of pH 7.5

even at high amendment rates, which has implications concerning use of Tr5 at infested sites with high pH. The high rates of root colonisation noted at low rates of Tr5 amendment at pH 5.5 are an encouraging result, as it is desirable to acheive maximum root colonisation from the lowest possible rate of Tr5 amendment. The possibility of soil pH modification for improvement of Tr5 establishment is an avenue which could be further pursued.

Sclerotium cepivorum infection is favoured by cool soil temperatures (Adams, 1977; Fullerton *et al.*, 1994). Temperature relationships for germination of sclerotia are known to vary between isolates (Gerbrandy, 1992), but disease is generally most severe during the months when soil temperatures are in the 11 to 15°C range in Tasmania (Ch. VI:20). The ability of Tr5 to establish on roots and combat infections at different temperatures is important information for commercial application of biocontrol, as it is desirable to be able to predict the effects of weather conditions on efficacy. The results presented in Chapter VI(20) confirm that the Tasmanian strain of *S. cepivorum* (Ch. VII) was most active under cooler conditions. Sudden drops in temperature resulted in a rapid increase in infection, which is consistent with the findings of Gerbrandy (1992) concerning effects of temperature on sclerotial germination. The ability of Tr5 to prevent infections appeared to be greater under cooler temperatures, similar to the time of the Tasmanian season when losses to *S. cepivorum* are most severe. The poorer ability of Tr5 to prevent infections under warmer conditions may be related to faster growth of *S. cepivorum* (Crowe and Hall, 1980b). While it is propitious that Tr5 is most able to suppress infections under the temperature conditions where losses to *S. cepivorum* are most severe, apparent reduction in ability to suppress infection under warmer conditions (Ch. VI:20) is a problem, as late season infections need to be graded out before packaging. To try to overcome this shortcoming there is a need for soil temperature monitoring of all trials. It would be worth screening for a compatible biocontrol agent which was more active in warm conditions, and could be applied in combination with Tr5.

S. cepivorum infection tends to be mild when it is first encountered in a paddock. Inoculum density increases exponentially in the next crop and this increase is proportional to the level of infection (Crowe *et al.*, 1980), though soil temperature is another important influence. The possibility that a sclerotial density threshold may exist beyond which *T. koningii* is unlikely to provide effective control is a concern for commercial application of biocontrol. If such a threshold were established biocontrol might be considered a suitable treatment for the first onion crop following initial detection, possibly preventing a mild problem from becoming severe. In the inoculum density trial (Ch. VI:17) Tr5 displayed a relatively constant ability to suppress 63 to 79% of infections at inoculum densities ranging from 10 to 100 sclerotia per kilogram of soil. Although as high as 98% of roots were assessed to be

colonised by isolates which were morphologically and enzymatically indistinguishable from Tr5, a proportion of *S. cepivorum* infections were able to reach the bulb. This proportion may represent the extent of suppression which can be achieved using Tr5. The results of Inoculum Depth Trial 2 (Ch. VI:16) where it was confirmed that the ability of sclerotia to successfully infect the bulb decreases with greater depth of burial, also showed that Tr5 was able to suppress a greater proportion of infections originating from 7cm, than 4cm. Once mycelium emanating from these sclerotia penetrates roots it is possible that infection is able to reach the bulb base before Tr5 can effectively colonise the infected tissue.

The information that sclerotia buried at some depths are more able to successfully infect the base plate has implications for integrated control of the disease. Sclerotia near the soil surface may be more readily exposed to sclerotial germination stimulants than those buried deeper in the soil profile, and it may be possible to target these sclerotia. While the use of onion mash waste water as a sclerotial germination stimulant (Ch. V:15) did not provide significant disease reductions (Ch. V:15) a number of research groups have demonstrated reductions in inoculum using these compounds (Davies and Coley-Smith, 1990; Crowe *et al.*, 1994; Moreno and Pale, 1994; Dennis, 1997) and a future direction of research in Tasmania will be to develop an integrated control strategy, involving a combination of these two environmentally acceptable control measures. It would appear that neither measure is currently able to stand alone for commercial control of *S. cepivorum* , as some sclerotia may not be induced to germinate due to inadequate conditioning for release of constitutive dormancy (Coley-Smith *et al.*, 1987), and results of Chapter VI:16 and 17 demonstrate that even with full root colonisation Tr5 cannot provide complete disease suppression.

By developing an understanding of the mode of action of *T. koningii* and its ability to become established as a competitive saprophyte under a range of ecological conditions, and also by understanding the ecological features of the pathogen which enable it to sometimes out compete the biological control agent, it appears that a predictable level of disease control can be achieved. However biocontrol is an ecological interaction which varies according to environmental conditions, and there is every reason to expect that repeated application of a single agent for control of the disease would ultimately select for a strain of *S. cepivorum* which could overcome *T. koningii. T. koningii* (Tr5) has demonstrated sufficient efficacy that its evaluation should continue. However, it would be inadvisable to rely on Tr5 in isolation as a control measure, and future research should be directed toward screening which includes study of the ecology of biological control agents with alternative modes of action which can ultimately be applied in a mixture which attacks multiple components of the life cycle of *S. cepivorum* , including resting sclerotia.

Ultimately, the foundation of the Tasmanian onion industry's problem with *S. cepivorum* may rest with the unending desire to apply current season control measures, rather than trying to improve the overall health (eg structure, organic matter, microbial diversity) of its soils, where populations of microbes suppressive to pathogen's such as *S. cepivorum* could become permanently established.

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Appendix A: Methods for Tr5 inoculum production:

A.1) Tr5 Millet Inoculum.

Materials:

White Millet	1kg,
Phostrogen *	2.5g
tap water	500ml.

(* commercial plant food; Phostrogen Australia PtyLtd)

Millet and Phostrogen were added to a 5L conical flask and shaken before the tap water was added, the mouth was covered in aluminium foil. The flasks were autoclaved and allowed to cool before inoculation. Most profuse growth was achieved by pouring the autoclaved millet into ethanol sterilised plastic trays about 3cm deep. Cultures were incubated in darkness at 20°C until covered in green sporulation.

CFU count: 5.6×10^5 spores / gram.

A1.2. Liquid Fermentation.

T. koningii (Tr5) cultures for fluid drilling treatments were produced by liquid fermentation by methods adapted from Papavizas *et al.* (1984). Ten litre pyrex flasks were sealed with rubber stoppers with holes bored for aeration tubes. 10L of mineral salts solution (Appendix B.7) containing 10g/L of the appropriate carbon source. Air was delivered by a fish tank pump via 0.22µm air filters. The air tube end was suspended 1 inch from the bottom of the flask Following autoclaving, and cooling, the cultures were inoculated with Tr5 from tap water agar agar culture.

The culture fluid produced by this method was for fluid drill sowing and contained 0.2% agar. Nutrients were added as for mineral salts solution (see Appendix 2). The appropriate carbon source was added at 10g/L. The solution was buffered to pH 4.0. After autoclaving the solution was cooled before inoculation with Tr5.

A.1.3. Chitin culture:

Cultures were grown in 2L conical flasks containing:

50g x crabshell chitin (sigma).

10g x Phostrogen.

90ml x water.

Which were autoclaved for 45 minutes and allowed to cool before inoculation with Tr5 and were incubated at 25°C for 4 weeks with weekly aeration in a laminar flow and occasional shaking.

A.1.4. Sawdust culture:

Cultures were grown in 2L conical flasks containing:

100g x sawdust (from sanding eucalypt floorboards)
20g x Phostrogen
90ml x water

Prepared and incubated as for chitin culture.

A.1.5. Chitin/Sawdust culture:

Cultures were grown in 5L conical flasks containing:

150g x sawdust
150g x crabshell chitin
60g x Phostrogen
740ml x water

autoclaved and incubated as for chitin culture.

A.1.6. Peat/Chitin/Osmocote culture:

cultures were grown in 5L conical flasks containing:

200g x sand
300g x peat
100g x chitin
50g x 4 month release osmocote
250 ml of water
0.2g of phostrogen

autoclaved and incubated as for chitin culture.

Appendix B: media procedures:

B.1. Chitinolytic enzyme medium:

$(NH_4)_2SO_4$ . . .	0.2 g
KH_2PO_4	0.4 g
Na_2HPO_4	0.6 g
$FeSO_47H_20$. . . .	0.02 g
$CaCl_2$	100 ug
H_3BO_3	1 ug
$MnSO_4$	1 ug
$ZnSO_4$	7 ug
Purified Crabshell Chitin .	1.0 g

Per 100 mls of distilled water.
Adjusted to pH 4.0 using HCl.

To determine the pH optimum for *T. koningii* chitinolytic enzyme medium, plates of pure chitin agar were prepared and buffered at pH 3, 4, 5, 6, 7 and 8 using HCl and NaOH. Composed of the same constituents as the medium. Five replicates of each pH were inoculated with *T. koningii* in a $2mm^3$ cube of tapwater agar. Measurements of radial growth were made at 48 and 96 hours. The fastest colony growth was at pH4.0.

The same medium was used to invstigate the chitinolytic enzymes produced by *T. koningii* in response to growth on sclerotia as a sole carbon source by substituting 1.0 g of ground *S. cepivorum* pure culture sclerotia for the chitin.

B.2. Soil Agar:

Soil agar was made by adding 50 g of Kraznozem soil from Mr John Bonney's property at Forth (Tasmania) and 10 g of Agar to 450 ml of tapwater in a 500ml Schott bottle before Autoclaving, and pouring into petri dishes.

B.3. Pectolytic enzyme medium:

The medium for production of *T. koningii* pectolytic enzymes consisted of:

$NH_4H_2PO_4$	0.9 g
$(NH_4)_2HPO_4$	2.0 g
$MgSO_47H_2O$	0.1 g
KCl	0.5 g
Citrus pectin	10 g

per litre of distilled water, buffered to pH 4.0

B.4. Onion Pectinase Media:

S. cepivorum pectolytic enzymes were produced on a media composed of 200g of onion cell wall tissue, and 800ml of distilled water. 2ml aliquots were added to 5ml bijou bottles. *S. cepivorum* was added as a 2mm cube of onion agar and incubated at 10°C.

B.5. Onion Agar:

S. cepivorum isolates were maintained on a medium composed of 200 g of onion cell wall tissue from onion bulbs, 800 ml of distilled water and 20 g of agar. The cell walls in the water were macerated in a blender for 5 minutes before being poured into schott bottles, autoclaved , and poured into petri dishes.

B.6. Potato Dextrose Agar.

Medium composed of 50g of peeled potato blended in 450ml of water sieved through cheesecloth then poured into a schott bottle with 10g dextrose and 10g agar. Autoclaved before pouring into petri dished.

B.7. Mineral Salts Solution:

$(NH_4)_2SO_4$ . . .	2 g
KH_2PO_4 . . .	4 g
Na_2HPO_4 . . .	6 g
$FeSO_47H_20$. . . .	0.2 g
$CaCl_2$	1 mg
H_3BO_3 . . .	10 ug
$MnSO_4$ . . .	10 ug
$ZnSO_4$ . . .	70 ug
Distilled Water	1 litre.

The mineral salts solution was used as a standard additive in making laboratory media with purified sole carbon sources (eg pectin, cellulose, purified crabshell chitin) , in these cases the carbon sources were added at 10g per litre. Agar was added at 10g per litre.

Appendix C: Methods for Infection and Antagonism Bioassays.

C.1. Production of sterile seedlings:

Onion seeds (regular creamgold variety) were soaked in 2% sodium hypochlorite in an ultrasound bath for 1 minute to assist penetration of sodium hypochlorite into seed coat cracks. Seed was then washed in sterile distilled water and placed on plates of agar containing 5 g /L of a commercial plant food "Phostrogen" to germinate. Seedlings were grown until stem and root tissue differentiated and roots were at least 3 cms long (3 to 4 weeks old). These plates were incubated at room temperature resting on their ends to encourage roots to grow downward and straight

C.2. Pre Germination of Sclerotia:

S. cepivorum sclerotia (Strain Sc4) were placed on soil agar (Appendix B). A drop of sterile onion mash water (provided by Vecon Oils), was placed in the centre of the plate which was sealed with parafilm. After 2 days hyphae were seen erupting from about 60% of the sclerotia and growing into the soil agar. Germination was conducted at 10°C.

C.3. Seedling infection procedure(The Infection Bioassay):

A 0.5 cm cube of soil agar containing a pre germinated sclerotium was cut from the plate and placed on the base of a sterile petri dish. An onion seedling was placed in the petri dish with the root tip in close proximity to the sclerotium. An autoclaved piece of paper towelling soaked in sterile distilled water was placed in the petri dish to maintain moist humidity before the petri dish was sealed with parafilm. The petri dish was then incubated until the infection was developed (about 48-72 hrs at 20°C in darkness) before embedment, sectioning and examination.

C.4. *T. koningii* inoculation (The Antagonism Bioassay):

Procedure for this experiment was the same as the infection bioassay, but a cube of soil agar containing growing *T. koningii* mycelium was placed between the developing *S. cepivorum* infection and the bulb so that the interaction of the two fungi as *S. cepivorum* hyphae attempted to grow past *T. koningii* to infect the bulb could be observed. Incubation was at 20°C for 72-96 hrs, and was conducted in darkness. The experiment was not sampled until dissecting microscope observation indicated that hyphae of both fungi had been growing on the same section of the root for some time. The experiment was replicated well over 100 times. Observations made in Chapter IV:8 were made during the course of these experiments by viewing hyphal interactions as the two fungi spread across the petri dish base, and in Chapter IV:9 by microscopic examination of microtome sections of the roots, Chapter IV:10 by loading segments into pectinase electrophoresis gels to detect distribution of polygalacturonases and pectinesterases, and in Chapter IV:11 by loading tissue segments into electrophoresis gels for detection of chitinolytic enzymes.

Appendix E: Electrophoresis Procedures.

E.1. Electrophoresis for the detection of pectolytic enzymes:

Pectolytic enzyme production was detected by the method of Cruickshank and Pitt (1987) using polyacrylamide gel electrophoresis to produce zymograms.

Gel moulds were made of glass and perspex plates 82mm by 165mm separated by glass strips 2.0 mm thick by 4mm wide cemented to the glass plate on each side. Well moulds each of 4.5 µl volume were provided by cementing 26 perspex blocks 1.0 mm wide, 3.0 mm long, and 1.5 mm deep along a line 15 mm from the bottom edge of the perspex plate. The plates were sealed together on three sides with lightweight vinyl-backed adhesive tape, with the lower corners being sealed by molten dental wax.

A discontinuous buffer system was used. Gels were buffered at pH 8.7, by adding tris(hydroxymethyl)aminomethane, 4.598 g, and citric acid monohydrate, 0.525 g per litre. Electrode tank buffer at pH 8.7 was composed of boric acid 7.22g and sodium tetraborate decahydrate, 15.75g per litre.

Acrylamide gel mixture was prepared by dissolving 0.1g of citrus pectin in 100mls of gel buffer with magnetic stirring, followed by addition of 10g of acrylamide, 0.25g *N,N'*methylenebisacrylamide and 0.1 ml *N,N,N',N'* tetramethylethylenediamide. Just prior to pouring, 0.1g ammonium persulphate crystals were dissolved in the mixture. Gel moulds were held obliquely in initial filling to prevent air bubbles from being trapped in the gel moulds. The mixture was sufficient for three gels which polymerised in about 10 minutes. Tape and perspex plate were removed leaving the gel supported on the glass plate.

Electrophoresis was carried out at 2-4°C. A few drops of kerosene were placed on copper plates to improve contact, the glass plate containing the gel was placed on this. The copper plate was suspended 7 cm above the tank floor and each tank contained 500 ml of borate buffer. A platinum wire electrode was immersed across each tank adjacent to the central partition. Electrical connection was made using hospital lint wicks. Power was provided by a 12mA constant current per gel.

A small spot of 0.05% bromophenol blue in gel buffer was applied to the cathodic (sample) end of the gel. Hospital lint wicks were applied to the gel ends. Power was supplied until the dye had migrated to 50 mm beyond the wells, which took about 90 minutes..

After electrophoresis a cut was made at the buffer front and the excess gel discarded. Each gel was incubated at 25° C in 100ml of 0.1 M malic acid which caused a gradual pH change in the gel to pH 3.0 in 90 minutes, allowing each enzyme to act on the pectin, while passing through a suitable pH range. Several changes of

distilled water followed and the gels were soaked overnight in 0.01% ruthenium red. Zones produced by the action of pectin esterase were dark, zones produced by action of polygalacturonase were colourless. Pectin lyase produces cleared zones, in presence of calcium ions.

A permanent record of results was made by direct photographic printing onto high contrast photographic paper showing negative contrast, ie polygalacturonase appeared as black areas and pectin esterase as white.

E.1.1. Detection of Ribonucleases.

Electrophoresis for detection of ribonucleases was performed as for pectinases, however 0.03% Na salt of high molecular weight ribosomal RNA from wheat germ was substituted in place of citrus pectin. Following elctrophoresis gels were incubated in 0.1M $CH_3COONa3H_2O$ for 1 hr at room temperature, before being soaked overnight at 4°C in 0.05% acridine orange solution.

E.2. Electrophoresis for the Detection of Chitinolytic enzymes:

E.2.1. Purification of Chitin:

Chitin was purified by the method of Shimahara and Takiguchi (1988) in three stages.

Step 1: Demineralisation: Dried shell chips were immersed in 1L of 2 N HCl at room temperature for 48 hrs, and the HCl was changed after the first 24 hrs. The chips were then washed in de-ionised water until the pH became neutral

Step 2: Deproteination: the demineralised shell chips were added to 1L of 1N aqueous NaOH in which they were boiled for 36 hrs with occasional stirring, distilled water was added as the vaporisation proceeded. The exhausted alkaline solution was exchanged for a fresh one after the first six hours.

Step 3: Lipids and pigments were eliminated by adding the chips from step 2 to 500mls of 95% ethanol for 6 hours, the chips were then collected ad rewashed in ethanol before air drying.

The yield from this procedure has been reported to be 17.5% deacetylated.

E.2.2. Preparation of Glycol Chitin:

Glycol chitin was a desirable substrate for chitinase assay as it is soluble in water whereas pure chitin is insoluble. Preparation of glycol chitin was by the method of Hirano (1988).

4g chitin purified by the method of Shimahara and Takiguchi (1988) was ground by hammermill to 1mm^2 chips then by mortar and pestle to a powder, then slurried in 16 mls of 60% NaOH solution containing 0.2% sodium dodecyl sulphate at 4 degrees C for 1 hour, then frozen at -20 degrees overnight. This frozen alkali chitin was then gradually added to 70 mls of 2-propanol containing 9.5 g of chloroacetic acid in an ice bath for 30 minutes then stirred mechanically at room temperature for one hour. The product was collected by filtration through a glass filter, and washed well with ethanol to give a powdered material. This material was suspended in 300 ml of water to give a viscous solution.

The viscous solution was re cooled in an ice bath and 8 mls of acetic anhydride was added (drop by drop). The product was dialysed against running water for 2 days then dialysed against distilled water for 1 day. The dialysed solution was centrifuged at 5000 rpm for 20 minutes to remove insoluble material, and 3 volumes of acetone were added and the solution was allowed to stand overnight. The solution was again centrifuged and the gel product was washed in acetone, then suspended in 30 mls of ethanol, then collected by filtration and air dried. The solid dry glycol chitin yield was 2.04 grams.

E 2.3. Electrophoresis.

Gel Moulds: Gel moulds were made of glass plates 82 mm^2 separated by glass strips 3.5 mm thick by 4 mm wide cemented to the glass plate at each side. Well moulds each of 15 ul volume were provided by cementing 10 perspex blocks 1.5 mm wide, 4.0 mm long, and 2.5 mm deep along a line 15 mm from the bottom edge of the perspex plate. The plates were sealed together on three sides with a lightweight vinyl backed adhesive tape, with the lower corners being further sealed with molten dental wax.

Buffer Systems: Two gel buffer systems were examined: 1) discontinuous buffer system after Poulik (1957) where the gels were buffered at pH 8.7 by adding Tris(hydroxymethyl)aminomethane, 4.598 g, and citric acid monohydrate, 0.525 g per litre. 2) a buffer comprising the same concentration of Tris and 4.590 g of sodium acetate per litre.

The electrode tank buffer was composed of boric acid, 7 22g and sodium tetraborate decahydrate, 15.75 g per litre.

Gel Slab Formula: The gel slab was composed of:

Buffer (citric acid or sodium acetate)	20	ml
N,N'-Methyene Bis Acrylamide	0.05	g

Acrylamide	2.0	g
N,N,N',N'-tetramethylethylenediamide	20	ul
Ammonium persulphate	0.03	g
Glycol Chitin	0.03	g

In early experiments 0.03 grams of finely ground purified chitin was included in the place of glycol chitin, unfortunately the enzymes were not able to sufficiently degrade this substrate to show clear isozyme bands.

The gel slab mix began to polymerise after ammonium persulphate was added. The mix was poured into the mould taking care to exclude air bubbles and allowed to stand for approximately 20 minutes to set.

Electrophoresis was carried out at 2-4°C under the same electrical system as is described for pectin degrading enzymes (Appendix E.1). Gels were allowed to run until a spot of bromophenol blue tracker dye placed just below the wells had migrated 50 mm past the wells.

E.2 4. Development and Staining:

After removal from the glass plates the gels were incubated in 0.1 M Malic Acid at 25°C. This caused a gradual pH change in the gel from 8.7 (citric) or 9.9 (sodium acetate) to 3.0 allowing each enzyme to act as the pH passed through its optimum range. Gels were then taken from the malic acid, rinsed in distilled water, then placed in 0.01% Calcoflour White (W:V) in 500mM Tris(hydroxymethyl)aminomethane adjusted to pH 8.9 with HCl. Gels were soaked in 50 mls of this solution for 20 minutes then placed in distilled water overnight before photographing. Calcoflour White is a fluorescent stain which shows up well under 3660 nm UV light. The camera lens was inserted through a hole into a box which excluded sunlight and was illuminated with 3660 nm light, exposure of the film for 7 seconds gave clear image of the dark zones where chitinase isozymes had degraded the substrate.

E.2 5. Specific enzyme substrates:

The use of glycol chitin as a substrate detects chitinolytic enzymes generally. However, to identify enzyme mode of activity it is necessary to use more specific substrates.

Gels moulds of 27 x 72 x 2.5 mm were prepared, constituents of these gels were:

Buffer (citric acid)	5	ml
N,N'-Methylene Bis Acrylamide	0.0125 g	
Acrylamide	0.5	g
N,N,N',N'-tetramethylethylenediamide	5	ul
Ammonium persulphate	0.0075 g	
Substrate *	0.0012 g*	

*Substrate refers to either·

- 4-methylumbelliferyl-*N*- acetyl- B-D- Glucosaminide.
- 4-methylumbelliferyl-B-D-*N,N'*-diacetylchitobioside.
- 4-methylumbelliferyl-B-D-*N,N',N"*-triacetylchitotriose.

(all substrates from Sigma).

15 ul drops of pure chitin culture media in which *T. koningii* had been incubated for various time periods was added to the wells and electrophoresis was performed as for the larger gels but at 10 mA. After electrophoresis gels were incubated for 1 hour in 0.1 M malic acid. Zones of activity were detected by production of fluorescent bands by the release of 4-methylumbelliferone.

Appendix F: Methods for assessing sclerotial populations in soil samples.

200g soil samples were placed in a 0.595mm Endecott sieve, on top of a 0.297 mm Endecott sieve. Soil was gently washed using water until all aggregates smaller than 0.595 passed through into the 0.297 mm sieve. Contents of the 0.297mm sieve was gently washed until particles smaller than 0.297mm passed though the sieve. Contents of the 0.297mm sieve was washed into a petri dish and examined by dissecting microscope for *S. cepivorum* sclerotia. Sclerotia were gently squeezed using fine forceps do determine whether they were healthy, before being removed to a storage bottle.

Appendix G: Sampling onion roots for colonisation by *Trichoderma* spp.

Assessment of the proportion of roots on which *T. koningii* had become established was made directly after each onion was uprooted at the time of destructive sampling. Without contact with hands or soil 5cm long root samples were inserted into snap seal airtight plastic bags and cut off using the seal. The top 1cm of each root sample was removed using aseptic technique within a laminar flow cabinet and incubated on RASP medium (Chapter IV; 12.0) for 14 days at 25_C. All plates were assessed as either of three categories; 1) No *Trichoderma* spp isolated, 2) *Trichoderma* spp. morphologically different to *T. koningii* (Tr5) , 3) isolates morphologically indistinguishable from *T. koningii* (Tr5). This assessment was based on morphological appearance (eg; colour of sporulation, structure of the conidiophore, pattern of sporulation, fluffiness). As specified in individual chapters, the accuracy of the morphological assessment was tested by comparison of the polygalacturonase, pectinesterase and ribonuclease isozyme profiles of a subsample of isolates from each treatment to that of Tr5 (Chapter IV; 10).