ASPECTS OF THE BIOLOGY OF THE SCLEROTIA OF *SCLEROTIUM CEPIVORUM*

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For my grandfather
who died shortly before this thesis was submitted
and whose footsteps I followed into science.

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The fungus *Sclerotium cepivorum* Berk. is the causal agent of onion white rot disease and reproduces solely by means of sclerotia that persist in the soil between susceptible crops. Sclerotia are, therefore, a vulnerable part of the lifecycle and disease control measures are being targeted towards them. These methods depend on a thorough knowledge of sclerotial biology. Three key aspects of sclerotial biology were investigated in the course of this research programme; the factors that affect survival of sclerotia in soil, the phenomenon of sclerotial dormancy and the relationship between inoculum levels in the soil and subsequent disease incidence.

The influence of soil type, location and sclerotial condition on survival of sclerotia was examined under field conditions. There was no significant difference in sclerotial survival in the two soil types tested (Patumahoe clay loam and Wakanui silt loam). Experiments were conducted at two locations (Auckland and Lincoln), which differed substantially in both average soil temperature and rainfall; Auckland having a warmer wetter climate. Location significantly affected sclerotial survival over time, with fewer sclerotia surviving at Auckland. The condition of the sclerotia, related to structural damage caused by desiccation and re-wetting in the field, was an important influence on survival. Most sclerotia (40 - 60%) decayed after just two months in soil, after which numbers remained relatively stable for up to 2 years. During the growing season, sclerotia became progressively more contaminated by other fungi and exhibited signs of desiccation. The sudden drop in viability over the first few

months in soil was attributed to damage caused by adverse environmental conditions and subsequent attack by microbes.

A study was made of the dormancy requirements of naturally-produced sclerotia in New Zealand soils. Dormancy was tested in sclerotia from 5 isolates buried in one of two different soils (Patumahoe clay loam and Wakanui silt loam). Two established methods were modified to test for dormancy in New Zealand conditions; one based in the laboratory and one in the field. In the laboratory-based experiment, < 40% of sclerotia germinated after 15 weeks in soil. Soil type did not significantly affect the length of dormancy in this study. The low rates of germination observed resulted in no significant differences among the isolates in the length of dormancy. This method was prone to problems caused by contamination by other fungi and is not recommended for use in New Zealand conditions. In the field trial, 47 – 72% of sclerotia had germinated or decayed after 4 months in soil, depending on the isolate. Sclerotia required 6 months in soil before high rates of germination occurred (89 - 98%). In the field trial, isolates differed in the amount of time required in soil before dormancy began to break (3 – 5 months). In both experiments described above, dormancy lasted several months longer than reported in the literature. Sclerotia collected from onions in a commercial field also exhibited sclerotial dormancy. This has implications for the use of germination stimulants (e.g. diallyl disuphide, DADS) in disease control in New Zealand. With dormancy lasting up to 6 months in some circumstances, a spring treatment of a germination stimulant would seem more appropriate than an autumn treatment following an outbreak of white rot. This is in line with overseas recommendations and should be practical as DADS can be applied while a cover crop is in place without any adverse effects. It may be necessary to delay planting until 8 weeks after the last DADS treatment, to ensure that seedlings are not exposed to a burst of germinating sclerotia.

Eleven field trials were conducted over three years. The aim of these was to describe the relationship between inoculum density (ID) in the soil at the time of planting and incidence of white rot at harvest. Soil samples were taken from each trial site and the numbers of sclerotia present in them determined. A novel method was developed to separate sclerotia from soil. This technique used the magnetic properties of haematite, an iron ore common in the soils of the Pukekohe region, to divide soil particles from sclerotia. Disease incidence, soil temperature and moisture were then recorded through the growing season. Curve-fitting was performed to determine the pattern of disease progress. In the first growing season (1998 / 99) data from 57% of the quadrats were sucessfully fitted to the gompertz model, one of the sigmoid curves. In the second year (1999 / 2000), low ID (and disease incidence) meant that only 26% of quadrats could be fitted to the gompertz model. In the third year, the pattern of disease was unlike that of the previous two seasons, with lower soil temperatures probably the cause of a later onset of white rot and continuous increase in disease incidence until harvest. In previous years, numbers of new infections had decreased in November - December. In 2000 / 01, the rate of new infections continued to increase into December. Data collected during the third year was fitted to exponential, log and linear models, with all data fitting one of these models.

There was a positive correlation in year one between inoculum density and time to maximum rate of disease increase (TMR), with high ID resulting in an earlier disease onset by approximately 4 weeks. A positive, but not statistically significant, trend was also observed between ID and both maximum rate of disease increase (MR) and the point of inflection in the curve (LI). No correlations between ID and these parameters were identified in 1999, due probably to the low levels of inoculum and disease observed. TMR, LI and MR were not calculated for the 2000 data, as these could not be fitted to the gompertz model.

It was not possible, in any of the years tested, to predict disease crop losses to white rot disease from inoculum levels at the beginning of the growing season. There could be a number of reasons for this; the study may not have been large enough to show any correlation between the two, even if one existed. A more likely explanation could be that pre-plant inoculum level influences only the time of disease onset, while the later progression of the disease may be driven by other, mainly environmental, factors.

This study investigated several features of sclerotial biology in *S. cepivorum*. Such information was required to underpin other research projects on the control of onion white rot. Data collected during the course of this study can be used in a range of applications, including the prediction of time of disease onset, the usefulness of rotation systems in disease control and in developing guidelines for the effective use of germination stimulants to reduce sclerotial numbers on soil.

Keywords: *Sclerotium cepivorum*, sclerotia, soil, New Zealand, survival, conditioning, dormancy, inoculum density, onion white rot.

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CHAPTER 1: INTRODUCTION

White rot is a disease of onions and related plants, caused by the fungus *Sclerotium cepivorum* Berk. The disease is important because not only does it affect one of New Zealand's major vegetable crops, but also it can be difficult to control and has caused significant economic losses over the past two decades. An overview of the existing information on *S. cepivorum* follows.

1.1 Background to *Sclerotium cepivorum* and *Allium* white rot disease

The 'form genus' *Sclerotium*, to which *S. cepivorum* belongs, was described (Saccardo 1911) on the basis of sterile mycelia and the possession of a sclerotium; a survival structure which was differentiated into three tissues; rind, cortex and medulla. The term 'form genus' indicates that the group is not a natural one but rather based on these shared common features. Not surprisingly therefore, since the original description was made, some of the species originally placed in *Sclerotium* are now assigned to different genera. The teleomorphs (sexual stages) of some species have been discovered and found to belong to a range of different taxonomic groups e.g. the basidiomycetes (*S. rolfsii* and *Athelia rolfsii*) and ascomycetes (*S. oryzae* and *Magnaporthe salvinii*) (Punja and Rahe 1992). This reflects the diversity of the fungi within this genus. *S. cepivorum* does not have a known teleomorph.

The most important defining feature of this group of fungi is the presence of vegetative, resting structures called sclerotia (Punja and Rahe 1992). The sclerotia of *S. cepivorum* are small, black and spherical. They are made up of a rind that is several cell layers thick but unlike some other members of the genus, they have no cortex, only an internal medulla made up of compact white hyphae (Backhouse and Stewart 1987). The medulla consists mainly of inter-hyphal spaces filled with polysaccharide

gel and medullary hyphae containing protein bodies (Backhouse and Stewart 1987). The black pigment melanin present in the rind and responsible for the dark colour is thought to act as a defence against drying out (Willetts 1971, Willetts and Bullock 1992). Sclerotia of *S. cepivorum* have been known to survive in field conditions in the U.K. for up to 20 years (Coley-Smith *et al.* 1990). Sclerotial survival is dealt with in detail in Chapter 2.

Sclerotia develop as follows (Littley and Rahe 1992) in response to the restriction of mycelial growth by culture vessels, damage to the mycelium, environmental cues and contact with a variety of chemicals / barriers (Willetts and Bullock 1992). Sclerotial initials appear as branched, looped hyphae. These hyphae then grow into bundles. An acellular matrix appears, progressively obscuring the sclerotia and becoming black as melanisation occurs. Finally, a layer of ovoid cells that will become the rind develops on the sclerotial surface (Littley and Rahe 1992).

Sclerotia form easily in artificial culture, for example on PDA (potato dextrose agar) when the colony has grown out to the edge of the Petri dish, after around 10 days at 18 - 20°C. They are roughly equal in size, 0.25 - 0.6 mm in diameter. In the field, larger sclerotia have been found, up to 2 cm in diameter. The large form has been reported in the U.K., Egypt, New Zealand and Australia (Backhouse and Stewart 1988, Georgy and Coley-Smith 1982, Metcalf *et al.* 1997). These larger sclerotia may represent a primitive apothecial stage. They can be aggregations of smaller sclerotia or, more commonly, one larger entity (Backhouse and Stewart 1988) and occur more frequently in some years than in others.

In addition to sclerotia, *S. cepivorum* produces micro-conidia that can be observed on mycelium emerging from the sclerotium (Punja and Rahe 1992). The function of these micro-conidia is largely unknown but recent work showed that, in very moist conditions, they germinated and

penetrated into host tissue (Gindro and Lhoste 1997). Enzyme activity within the micro-conidia was given as evidence for their infective potential.

S. cepivorum infects only *Allium* species (Coley-Smith 1959) and germinates only in the presence of members of the genus (e.g. onion, garlic, leek, chives, shallots) or extracts of them. These plants exude compounds from their roots that include S-alk(en)yl-L-cysteine sulphoxides. Microbes in the soil break down these compounds into substances including 1-propyl- and 2-propenyl (diallyl) disulphides and these trigger germination in sclerotia of *S. cepivorum* (Coley-Smith and King 1969, Coley-Smith *et al.* 1987, Dickinson and Coley-Smith 1970). A synthate of one of these exudates, diallyl disulphide (DADS), has been used to trigger germination at high levels (90 - 100%) in the absence of a suitable host crop (Coley-Smith and Parfitt 1986, Crowe *et al.* 1990). An aqueous solution of 0.1 - 0.2 g l^{-1} was sufficient to produce these results (Coley-Smith and Parfitt 1986).

Sclerotia, once produced, will not germinate in response to *Allium* extracts / exudates until they have been 'conditioned' for a variable period of time in soil. This phenomenon, known as constitutive dormancy, is dealt with in full in Chapter 3.

Germination of sclerotia occurs in two ways (Backhouse and Stewart 1987, Brix and Zinkernagel 1992, Crowe and Hall 1980, New *et al.* 1984). In eruptive germination, the first visible change is the appearance of a bulge on the sclerotial surface. A plug of hyphae then emerges from this swelling (Backhouse and Stewart 1987, New *et al.* 1984). This is the most common type of germination and occurs in unsterile soil as a reaction to the presence of a host (Brix and Zinkernagel 1992, Crowe and Hall 1980, New *et al.* 1984). Once germinated, the hyphae grow towards and penetrate host tissue, in response to an unknown chemical signal. They enter via weak areas, through the anticlinal wall junctions or

occasionally through the periclinal wall (Stewart *et al.* 1987). In sterile medium and (rarely) in field soil, germination involves the penetration of the rind by individual hyphae (Backhouse and Stewart 1987, New *et al.* 1984). These hyphae often form sclerotial initials, but these do not usually form a bundle, instead collapsing and lysing within a few days (New *et al.* 1984). While eruptive germination exhausts the reserve materials of the sclerotia, leaving empty fragments of rind, germination by individual hyphae does not require many reserves and the sclerotia are able to germinate again (New *et al.* 1984).

There is conflicting data on the effect of pH on germination, but cultured sclerotia can germinate at high levels over a wide range of pH values (4.5 - 7.8, Adams and Papavizas 1971). The higher end of the scale is generally favoured (90% germination at pH 6 and above, Adams and Papavizas 1971). Alternating wetting and drying treatments have been shown to reduce infection rates and germination (Adams and Papavizas 1971).

Temperature also has an effect on the fungus. *S. cepivorum* is more tolerant of low than of high temperatures; it can survive exposure to -14°C for 5 months (Willetts 1971). The optimum soil temperature for disease development is 13 - 18°C (Fullerton *et al.* 1994). Therefore, *S. cepivorum* is confined to temperate parts of the world or is active only in cooler winter months (Willetts 1971). The disease is at its height in months when the temperature is in the optimum range and onset of the disease can be delayed by cold weather until the soil temperature reaches this level (Fullerton *et al.* 1994).

1.2 The importance and world-wide distribution of the disease

Of all the diseases that affect onions, white rot is economically by far the most important. It has become more significant recently due to changing cultural practices, especially the use of short-term rotation systems. Rapid crop rotations were introduced to take full advantage of the limited

areas of land suitable for onion cultivation but have led to increasing incidence of the disease since the 1970s (Coley-Smith 1990).

While onion white rot was first reported in the U.K., it has since been found across Europe, in North and South America, Asia and Australasia (Mordue 1976). In New Zealand, white rot has been recorded at Pukekohe, near Auckland, since the 1960s (Dingley 1969) and in Marshlands, north of Christchurch, since the 1920s (Fullerton *et al.* 1994). Some years, in the 1980s and '90s, crops were abandoned due to very high levels of white rot (see Figure 1). Certain fields are so highly infested that it is now uneconomic to plant onions there (Fullerton and Stewart 1991).

Diseased plants marked with labels.

Figure 1. Crop showing disease symptoms.

White rot is notable not only because its incidence has increased but also because the plant that it affects is a large part of New Zealand's cropping industry. Onions are a major fresh vegetable crop in New Zealand, with

exports worth $79M in 2000 (source: the New Zealand Vegetable and Potato Growers Federation).

Seventy five percent of all New Zealand's onions are grown in the Pukekohe region (Fullerton and Stewart 1991, Fullerton *et al.* 1986). The remainder of commercial onion cultivation occurs in small areas of Canterbury such as Marshlands and Kaiapoi. New Zealand onions are exported to Europe and Asia, particularly Japan, Germany and the U.K. (source: the New Zealand Vegetable and Potato Growers Federation). The cultivar 'Pukekohe Longkeeper' has been used almost exclusively in the North Island but others such as 'Kiwi Gold' are becoming more common. Canterbury growers use a range of cultivars, for example 'Porters Early Globe' (A. Stewart, pers. comm). Onions in this country are planted in May - June (early season) or August - September (late season) and both plantings are harvested in the following December – March, with Canterbury growers harvesting one or two months later than their North Island counterparts. Incidence of white rot is more common in the early crop than in those planted later.

1.3 Disease symptoms and spread

The first sign of white rot is wilting and seedling death in the early months of the season (August or September), resembling 'damping off' (Fullerton *et al.* 1994). A change in leaf colour from the normal healthy bright green to a blue-green, characteristic of white rot, is also seen, as the fungus rots away the roots. Appearance of the disease in the very early months has little effect on eventual commercial yield (if <20% of plants are infected, A. Stewart, pers. comm.) as the smaller number of plants in the row benefit from reduced competition (Fullerton and Stewart 1991, Stewart and Fullerton 1991). When white rot occurs later in the year (from October to November and occasionally into December), the change in leaf colour to blue-green is followed by wilting, yellowing and plant collapse (Fullerton *et al.* 1994). The characteristic white mycelium, responsible for the name 'white rot', is visible on the base of the bulb (Fullerton *et al.* 1994) (see Figure 2). Later, black sclerotia are formed on

the surface of the bulb. Once infected, the bulb is no longer of commercial value; either it rots away or is so badly damaged as to be unsuitable for sale (Fullerton *et al.* 1994).

The disease is spread when sclerotia or infected material are moved by cultivation, on equipment or footwear (Fullerton *et al.* 1994). It is also transferred by animals, in windblown debris and irrigation water (Adams 1979, Fullerton *et al.* 1994, Mikhail *et al.* 1974). There is some spread of disease between plants in a row as a result of contact between the bulbs or roots (Scott 1956a and b). Spread over a distance of 20 cm has been shown (Entwistle and Munasinghe 1977) and when physical contact was prevented, disease incidence was significantly decreased or even halved (Entwistle and Munasinghe 1977, Scott 1956a and b). See diagram of life-cycle, Figure 3.

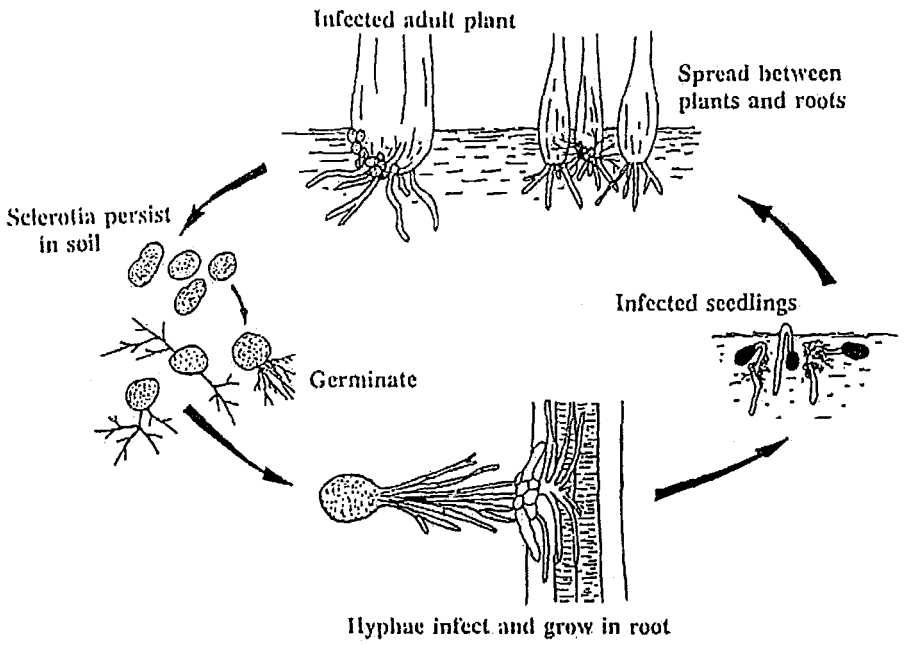
Reproduced with permission, Fullerton *et al.* 1994.

Figure 2. Typical symptoms of white rot.

1.4 Control measures

The earliest methods used to control onion white rot were cultural and included rotation (which is not very useful as the sclerotia are so long-lived), removal or avoidance of contaminated material / soil and the application of lime (Adams and Papavizas 1971). The purpose of these is to minimise the introduction and spread of sclerotia from infected fields.

Once sclerotia are present in a field, several methods can be used to reduce the level of inoculum in the soil. These include the induction of sclerotial germination in the absence of host plants. This technique works on the principle that *S. cepivorum* cannot survive as a saprophyte (Dhyani and Chauhan 1976, Scott 1956a). Therefore, if germination is triggered as a result of the application of *Allium* extracts or a synthate of them while there are no host plants available, the fungus starves.



Reproduced with permission, Fullerton *et al.* 1994.

Figure 3. Life cycle of *Sclerotium cepivorum*.

The main disadvantages of this technique are that the most widely used germination stimulant (DADS) is expensive and, as it is highly volatile, it can quickly evaporate in warm conditions (Coley-Smith and Parfitt 1986).

DADS is most effective when soil temperatures are 15 - 20°C for several weeks (Fullerton *et al.* 1994).

Another complication is that newly produced sclerotia will not respond to *Allium* extracts for some time after they are formed as a result of constitutive dormancy, so would not be susceptible to this form of control (see Chapter 3 for detail). On the positive side, DADS is not unpleasant to handle, can be manufactured in large quantities (Fullerton *et al.* 1994) and provides a specific method of control that targets sclerotia without the problems associated with chemical-based measures (e.g. residue build-up and reduced effectiveness of chemical controls over time). DADS has now been registered for use in New Zealand under the name Alli-Up (Stewart *et al.* 2001).

Other methods to reduce inoculum density are soil sterilisation and solarisation. Sterilisation uses chemicals that liberate methyl isothiocyanate into the soil (e.g. metham sodium) but this is expensive and has given only variable results (Coley-Smith 1990).

Soil solarisation works on the principle that high temperatures can decrease the levels of inoculum in the soil; sclerotia of *S. cepivorum* are unable to survive for long at temperatures of 35°C and above (Crowe and Hall 1980, Entwistle and Munasinghe 1978). This is reflected by the distribution of the disease, which is confined to cooler areas or winter months.

The usual method of solarisation is to spread clear plastic sheets over the ground to raise the soil temperature in the upper layer of the soil high enough to kill the sclerotia (Coley-Smith 1990). The technique was first employed in Israel and has been used successfully in New Zealand, Australia and Egypt (Coley-Smith 1990, McLean *et al.* 2001, Porter and Merriman 1983). In New Zealand, solarisation has been trialed in two regions (Canterbury and Marlborough) and resulted in a significant

reduction in numbers of viable sclerotia, by up to 100% (McLean *et al.* 2001).

Pesticides are the principle means of disease control in the growing crop. Many pesticides have been used against white rot including formalin, mercurous chloride, dichloran, pentachloronitrobenzene, benzimidazoles and more recently dicarboximides (Coley-Smith 1990, Fullerton *et al.* 1994). Benomyl (Benlate) was used quite successfully in some trials in the early 1970s but results varied and its use was not widespread (Fullerton *et al.* 1994).

The dicarboximides iprodione and vinclozolin (marketed as Rovral and Ronilan) were successfully used through the early 1980s as a foliar spray or as a combination of seed dressing (62.5 g a.i. kg^{-1} seed) and foliar sprays (0.75 kg a.i. ha^{-1}) (Fullerton *et al.* 1994, Stewart and Fullerton 1991) but were found to be inactive at Pukekohe by 1986 (Fullerton and Stewart 1991). In the U.K. it was also noticed that they became less effective after several years of use (Entwistle and Hawling 1984). This loss of activity was due to the phenomenon known as enhanced microbial degradation (Fullerton and Stewart 1991, Slade *et al.* 1992). It was discovered that both Rovral and Ronilan were being completely degraded within 7 days after three successive applications, due to the build-up in the soil of microbes able to degrade the compounds (Fullerton *et al.* 1994, Slade *et al.* 1992). Therefore, they became ineffective after several seasons of regular use.

Procymidone (marketed as Sumisclex) is a dicarboximide and is related to iprodione and vinclozolin. It was introduced to control white rot when the other two were found to be ineffective (Fullerton and Stewart 1991, Fullerton *et al.* 1994, Stewart and Fullerton 1991) and has been used to control white rot since then, reducing incidence by up to 95% in one trial (Stewart and Fullerton 1991), although its effectiveness has decreased in recent years (Stewart and Fullerton 1999). For some reason, not yet known, it is not subject to enhanced degradation (Slade *et al.* 1992).

Procymidone acts best as a seed dressing, followed by 3 - 4 foliar sprays, applied monthly (Stewart and Fullerton 1991, Stewart and Fullerton 1999). The seed dressing (5 g a.i. kg^{-1} seed) can protect the plants against white rot for the first 2 - 3 months, after which foliar sprays (0.75 kg a.i. ha^{-1}) are required to maintain control (Fullerton and Stewart 1991, Stewart and Fullerton 1991). A granular formulation of procymidone, applied at planting to the soil surface, has been added to these treatments in recent years (Stewart and Fullerton 1999). Procymidone (and in some cases iprodione and vinclozolin, Stewart and Fullerton 1991) can cause adverse effects on the onion seedling, known as phytotoxicity, when used as a seed dressing. The plants become yellow, their first leaves are deformed and in severe cases, the roots and base of the plant fail to develop properly (Fullerton *et al.* 1994). The condition is made worse by cold, wet weather and can be avoided if an application rate of less than 5 g procymidone kg^{-1} seed is used (Fullerton *et al.* 1994). Mild phytotoxicity disappears after some weeks (Stewart and Fullerton 1991).

Yet another group of chemicals, the triazoles (dimethylation inhibiting fungicides or DMIs), are the latest fungicides to be used widely in the control of white rot (Fullerton *et al.* 1994 and 1995) and have been found to be effective as foliar sprays, reducing disease incidence by up to 85% (Fullerton *et al.* 1995). They have also been used as a soil treatment (applied along the row) but one, tebuconazole, is not suitable as a seed dressing as it tends to be toxic to onion seed when used in this way (Fullerton *et al.* 1995). Marketed as Folicur, tebuconazole is now the most commonly used fungicide against white rot (250 g. a.i. l^{-1}) and has replaced Sumisclex as the main foliar spray (A. Stewart, pers. comm.). However, recent studies have shown that the effectiveness of Folicur is decreasing in the Pukekohe region, although not yet in Canterbury (Stewart and Fullerton 1999). There is no evidence of fungicide resistance or enhanced microbial degradation with this product.

Triadimenol, marketed as Cereous (and related to Folicur), is the most effective foliar spray at present and may replace Folicur but is not yet in common usage (Stewart and Fullerton 1999). Shirlan (active ingredient fluazinam), currently used against *Botrytis* and downy mildew, is successful against white rot but is not registered for this purpose (Stewart and Fullerton 1999).

Fungicides are only useful when applied at the correct time. If an unusual season develops, for example where the soil temperature does not reach the optimum range for *S. cepivorum* until relatively late in the season, fungicides cease to be effective unless their application times follow the progress of the disease. In 1992 and 1993, there were substantial losses to white rot at Pukekohe because soil temperatures remained at optimum levels after the last foliar spray had been used (Fullerton *et al.* 1994). Fungicides may also vary in their effectiveness, providing good disease control in some years and inadequate control in others. This has been attributed to changes in soil conditions, affecting the persistence and activity of the fungicide (Tyson *et al.* 1999).

Biocontrol agents have been trialed against *S. cepivorum*. Potential microbial antagonists were identified from unhealthy (non-germinating) sclerotia and field soil (e.g. Harrison and Stewart 1988, Kay and Stewart 1994, Moubasher *et al.* 1970, Stewart and Harrison 1988). Some fungi (*Chaetomium globosum*, *Coniothyrium minitans, Trichoderma hamatum, T. harzianum, T. koningii* and *T. virens*) have been shown to control *S. cepivorum* as efficiently or more so than Sumisclex by inhibiting its growth and causing sclerotial decay (Kay and Stewart 1994, McLean and Stewart 2000). They have been applied as a soil additive (sand:bran:fungal mix) and, in the form of a fungal homogenate, as a seed coating or soil application (Kay and Stewart 1994, McLean and Stewart 2000). Disease control rates of 50 – 70% have been achieved using *Trichoderma harzianum* as both pellets (10^4 colony forming units per pellet, applied to the planting row) and prills (10^5 cfu g^{-1} prill,

incorporated into the soil adjacent to the plants three times during the growing season, Stewart *et al.* 2001). This provided effective control for most of the year and work has been undertaken to develop a practical slow-release formulation (Stewart *et al.* 2001).

There are no onion cultivars that show a natural resistance to white rot and although 30 different *Allium* species have been tested, only one showed substantial resistance to the disease (Bansal and Broadhurst 1992). With no resistance existing in wild members of the *Allium* genus (Esler and Coley-Smith 1984), resistant varieties of commercial crops are unlikely to be found in the foreseeable future (Coley-Smith 1990, Fullerton *et al.* 1994). However, in Mexico a low level of resistance has been induced in garlic through irradiation and also in some onion cultivars in Canada (Stewart and Fullerton 1999). There is the option of introducing resistance to white rot with transgenic techniques. While this possibility is being explored (by Crop and Food Research Ltd., A. Stewart pers comm.), it may not be acceptable to consumers (Stewart and Fullerton 1999). Another possibility, to reduce susceptibility of crops to white rot, could be to reduce the volume of root exudates that trigger sclerotial germination or to change their nature so that they no longer trigger germination in *S. cepivorum*, but given that these compounds are primarily responsible for the flavour of the crop, such measures may not be acceptable (Coley-Smith 1990). Staff at Crop and Food Research Ltd. are currently evaluating plants with different sulphur metabolite profiles, to identify any with resistance to white rot disease (A. Stewart, pers. comm.).

None of the control measures detailed above are 100% successful. This has prompted the suggestion that integrated control packages could be the best method with which to limit the effect of *S. cepivorum* (Coley-Smith 1990, Entwistle 1990a, Stewart and Fullerton 1999, Stewart *et al.* 2001). Biological control agents and fungicide treatments could be used

to protect the growing crop while germination stimulants / solarisation can reduce inoculum density before planting (Stewart *et al.* 2001).

1.5 Project aims

The aim of the experiments outlined in this thesis was to investigate aspects of the biology of this pathogen, particularly some properties of the sclerotia, as these structures are the means by which the fungus reproduces itself. The key objectives were as follows.

1) Determine the rate of survival of sclerotia of *S. cepivorum* and the factors that influence their survival In New Zealand soils.

2) Test for the existence of sclerotial dormancy in a range of New Zealand isolates and determine how long this period of dormancy persists.

3) Describe the relationship between inoculum density and subsequent disease incidence in the Pukekohe region, South Auckland.

The survival studies (Objective 1) could provide information about the usefulness of rotational cropping systems in the control of white rot and the possible effectiveness of biological control agents with the potential to reduce sclerotial viability over time. Studies of dormancy (Objective 2) have value because constitutive dormancy is an almost universal property of sclerotia but remains widely misunderstood and is the subject of a highly contradictory literature. Also, for the effective use of germination stimulants such as DADS in disease control, it is necessary to know whether all isolates exhibit dormancy and for what length of time. Data on inoculum density and other determining factors (Objective 3) have been used to understand the mechanisms behind the development of disease epidemics. This information can then be applied to crop protection, as it allows one to predict disease progress and to take

appropriate remedial action. If a model for the effect of inoculum density and other factors on white rot were developed in New Zealand, then management practices such as rotation systems and spraying regimes could be adapted accordingly and informed decisions made about the suitability of land for onion cultivation.

CHAPTER 2: FACTORS THAT INFLUENCE SURVIVAL OF SCLEROTIA OF *S. CEPIVORUM* IN NEW ZEALAND SOILS.

2.1 Introduction

As sclerotia are the sole means by which the fungus survives between successive crops of onions, studies have been done to determine how long they can persist in the soil. Such information can be used to determine the usefulness of rotational cropping systems in the control of white rot and the likely effectiveness of biological control agents with the potential to parasitise sclerotia and thereby reduce their viability over time.

Variable results have been reported previously. In one study, 70% or more of the sclerotia remained viable after 20 years (Coley-Smith *et al.* 1990). Similarly, in California, viable sclerotia were recovered from fields that had not been planted in onions or other host crops for 10 - 15 years (Crowe *et al.* 1980). Other studies have described situations where sclerotia survived for much shorter periods of time. For example, it was found that sclerotia decayed steadily with time in British Columbia (Leggett and Rahe 1985, Leggett *et al.* 1983); only 24% of sclerotia survived burial for 16 months.

In a short-term study of sclerotial survival under New Zealand conditions, only 22% of sclerotia were alive after 3 months and 2 - 3% after 11 months (Alexander and Stewart 1994). However, even when there was substantial loss of sclerotia in the first few months after burial, the remaining sclerotia were able to survive long-term (Coley-Smith *et al.* 1990) and were sufficient to ensure continued maintenance of the fungal population in the soil.

Studies have been done, with inconsistent results, to determine if pure-culture sclerotia (raised on sterile wheat or maize) differ in their survival properties to those produced on onions. For example, 30% of sclerotia produced on onions survived 3 months compared to 90% of those from pure culture (Entwistle and Munasinghe 1975) while another study reported 'no obvious difference' between the survival of pure-culture and onion-produced sclerotia over 10 years (Coley-Smith *et al.* 1990).

There have been concerns that studies of survival could be confused by the production of secondary sclerotia. These are smaller sclerotia, which are produced from existing ones after the larger, parental sclerotia germinate (Somerville and Hall 1987). They are perhaps formed in response to harsh environmental conditions (Entwistle and Munasinghe 1978, Somerville and Hall 1987). However, these are produced so rarely (in 0.4% of sclerotia) that they are not likely to cause appreciable error in the results (Crowe and Hall 1980).

There is a variety of information on the effect of micro-organisms on long-term survival. A number of soilborne fungi have been shown to be associated with decay (Adams and Ayers 1981) and some have been investigated as potential biological controls (e.g. Kay and Stewart 1994, Moubasher *et al.* 1970). Some isolates were successful biocontrols while others had a variable effect or none (see Chapter 1).

A number of other factors may also adversely influence sclerotial survival. These include desiccation (Leggett and Rahe 1985, Leggett *et al.* 1983, Smith 1972), high soil temperatures (Coley-Smith 1990, Potter and Merriman 1983), flooding (Coley-Smith *et al.* 1990, Leggett and Rahe 1985) and the presence of high organic matter in the soil (Leggett *et al.* 1983).

The lifespan of sclerotia is ultimately limited by how long the internal medulla can supply nutrients. Over time, these sclerotial contents are

eroded (Backhouse and Stewart 1987, Coley-Smith *et al.* 1990). This could be due to the metabolic activity of the fungus (Backhouse and Stewart 1987) or leakage of nutrients due to damage caused by drying (Coley-Smith 1979).

To summarise, there is a great deal of conflicting data on the issue of sclerotial survival in *S. cepivorum.* These contradictions highlight the need for a detailed study of sclerotial survival in New Zealand soils.

Aim

The experiments reported in this chapter aim to determine the rate of survival of sclerotia of *S. cepivorum* and the factors that influence their survival In New Zealand soils.

2.2 Materials and Methods

2.2.1 Experiment 1

This experiment evaluated the survival of naturally-produced sclerotia in field soil and was designed to quantify the effect of soil type on survival.

A mixed population of sclerotia was collected from a field in Pukekohe. This was the sole source of sclerotia as the emphasis was on the influence of environmental factors rather than on any isolate effect. Sclerotia were collected in February 1998 (late-season for sclerotial production). Sclerotia were rubbed off infected onions and recovered by progressive wet-sieving (Crowe *et al.* 1980). The sieve sizes were 500, 250 and 150 µm. Only sclerotia from the 250 µm sieve were collected. After sieving, the still damp sclerotia were transferred onto filter paper and picked out individually under a microscope with fine forceps.

Sclerotia were counted into lots of 50, placed in nylon mesh bags (10 cm x 10 cm, Nybolt, mesh 85 µm) and buried at 10 cm depth. Each bag contained 20 g of white sand and 20 g of either of the two soil types used, sieved to < 150 µm.

The bagged sclerotia were buried at Mt. Albert Research Centre (Auckland) in either Patumahoe clay loam (pH 5.8 - 6.3, 4% organic matter) from Pukekohe, South Auckland or Wakanui silt loam (pH 6.9, 4% organic matter) from Kaiapoi, north of Christchurch. Pukekohe and northern Christchurch are the regions where most onion cultivation takes place in New Zealand. Each soil was contained in 6 plastic bins (47 cm diameter, 53 cm deep) which were sunk into the ground with only the top 1 - 2 cm of the bins exposed and arranged in a grid pattern with bins containing the two soil types placed alternately, approximately 1 m apart (see Figure 4). Gravel (5 cm layer) was spread in the base of the bins to aid drainage. Drainage holes (eight per bin, 1.5 cm in diameter) were drilled into the sides of the bins. The mesh bags (see Figure 5) containing

sclerotia were buried at 10 cm depth in one of the two soils (randomly, 8 per bin) with labels attached to allow for easy retrieval. A 'Tinitag' datalogger (Gemini Dataloggers, U.K.) was used to record soil temperature. Rainfall data was also collected. Bags were also buried in a commercial onion field at Pukekohe (not in bins) to determine whether placement of bags into bins influenced survival.

Figure 4. Bins of soil for survival experiments.

Sampling occurred every month for the first 4 months and every 4 months thereafter. At each sampling time, 4 replicate bags, each with 50 sclerotia, were removed at random from each treatment (soil type).

Sclerotia were recovered by wet-sieving from the bags and counted. To determine viability, sclerotia were surface-sterilised in 5% sodium hypochlorite for 5 min, washed in sterile distilled water and allowed to germinate on potato dextrose agar (PDA) (Coley-Smith and Javed 1970). The agar was amended with 50 mg l^{-1} each of penicillin and streptomycin (Sigma; penicillin G and streptomycin sulfate). Germination was scored after 5 - 7 days. Viability of sclerotia was 82% at the start of the experiment.

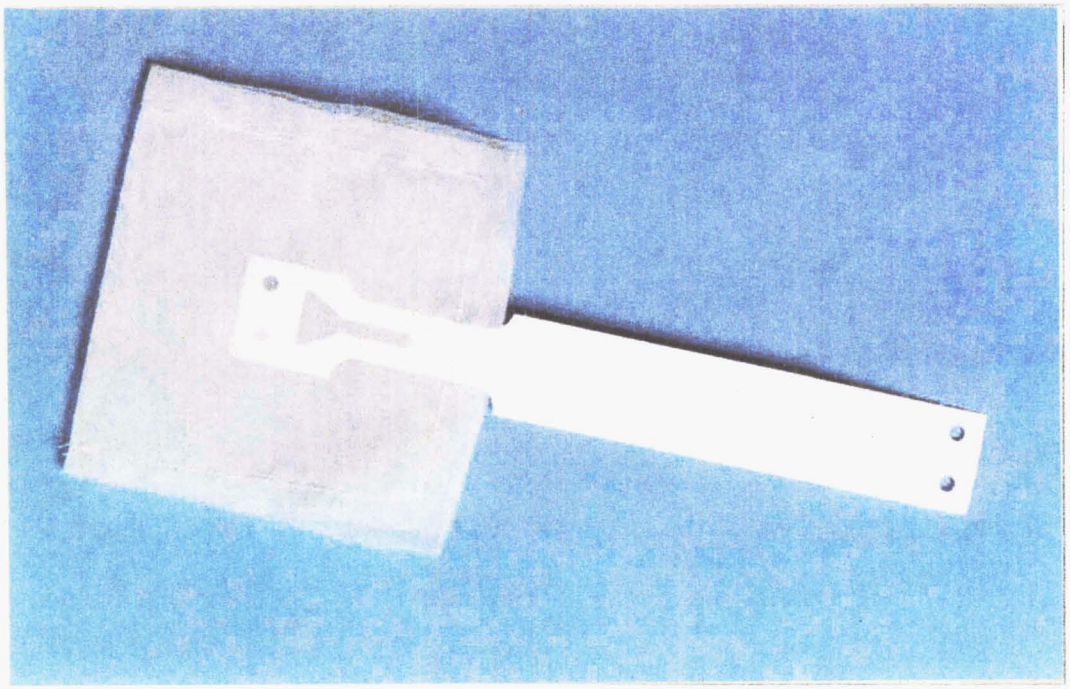


Figure 5. Nylon bag used to contain sclerotia.

Sclerotia were also stored with white sand in six sealed plastic containers (20 ml bottles with screw-top lids), incubated at 18 - 20°C in the dark and sclerotial viability regularly assessed as previously described. This was done for comparability with previous work, where the survival of sclerotia in storage was used as a control for assessments of viability in the field (Alexander and Stewart 1994).

The experiment was set up in February 1998. Results from the first assessment indicated an unusually high rate of sclerotial decay; therefore a repeat experiment was set up 4 weeks later. Preliminary analysis of the

data from each experiment revealed the same trends and so the data were combined as two replicates.

A sample of sclerotia collected was plated directly onto 5 mm discs of unamended PDA to assess the level of sclerotial contamination by other organisms at the start of the experiment. The proportion of sclerotia contaminated by other fungi or bacteria was estimated, using 100 sclerotia for each assessment. These contaminants were isolated, cultured on PDA (fungi) or nutrient agar (bacteria) and then classified into groups (Gram negative or positive for bacteria, genus for fungi).

2.2.2 Experiment 2

This experiment used early-season sclerotia, collected from recently infected bulbs and buried in two soil types at both Auckland and Lincoln. The methodology and trial design were the same as for 'Experiment 1' with the following modification; after wet-sieving, sclerotia were air-dried for 2 h before counting. Counting was then achieved by collecting the dry residue from the sieve onto a glass plate and selecting the sclerotia with a needle. Viability of sclerotia was 100% at the start of the experiment.

2.2.3 Data analysis

The data from the two experiments were studied using 'analysis of variance' or ANOVA, (programme supplied by Systat Inc., Evanston, USA), with soil type and location of trial site (climate) as factors. Data from each sampling interval were analysed separately, then an analysis done on overall effects over time.

2.2.4 Sclerotial ultra-structure

Sclerotia were examined for contaminants and /or damage that might contribute to a loss of viability. Approximately 6000 sclerotia were collected from 6 - 8 infected onions, taken from each of two locations in

the Pukekohe area in December 1999 and January 2000 (end of the growing season). After collection, the sclerotia were rubbed off the onions by wet-sieving and stored at 4°C until required (1 week).

The sclerotia were examined using the scanning electron microscope (SEM). The internal contents were examined using a modified 'cold stage' method, where sclerotia were suspended in water and the solution positioned in a well carved out of the carbon-based mountant 'Aquadug' (N. Andrews, pers. comm.) prior to freezing in liquid nitrogen. The mounted specimen was placed into liquid nitrogen at -150°C to cool the outer surface as rapidly as possible, then transferred under vacuum to the microscope. The specimen was cut in longitudinal section with a scalpel to expose the sclerotia and etched in the microscope (Leica, model 440) at -80°C to remove water crystals. The specimen was re-cooled to -150°C, placed in a sputter coater (Oxford cold stage system, model CT1500) and then coated with gold for 5 min at 1.2 KV and 50 milli-amps. Once the coating was completed, the specimen was put back into the microscope and observed. This process was repeated with subsamples of 50 - 100 sclerotia. The external rinds (n = 100, i.e two subsamples of 50 sclerotia) and internal structure (n = 50) of field-produced sclerotia were examined.

Loss of medullary contents was classified as follows; 'limited' = visible holes in the medulla mostly restricted to the area immediately beneath the rind, 'moderate' = many holes present, giving a sponge-like appearance to the medulla, 'severe' = holes throughout the medulla combined with hollowing of the central area.

Fifty sclerotia were also removed and plated onto discs of PDA and the proportion contaminated with fungi or bacteria determined (as previously).

2.2.5 Influence of drying treatment on sclerotial morphology

An experiment was set up to test whether dry conditions resulted in the symptoms that were observed in field-produced sclerotia. Four to 6 week old sclerotia were produced on onions in the laboratory (Coley-Smith 1985) and then placed in a sealed 50 ml glass Schott bottle, suspended in a small 2 cm^{-2} nylon bag above a 15 ml solution of concentrated sulphuric acid. The acid vapour acted as a desiccant. Sclerotia were alternately dried (for 2 - 3 days) and then rewetted by soaking in water (for 12 h). This treatment was continued for 1 or 3 weeks. If time had not been a limiting factor in this experiment, a slower and perhaps gentler desiccant (e.g. silica gel) could have been used in place of rapid desiccation by suphuric acid.

Controls consisted of sclerotia produced on onions but not subjected to wet and dry treatments. Samples were assessed using SEM techniques as described previously.

2.3 Results

2.3.1 Experiment 1

There was a marked decrease in sclerotial viability over the first 1 - 2 months after burial in soil, followed by a more gradual decline (Figure 6). After 1 month, numbers of viable late-season sclerotia dropped from 82% to 27% and 35% in Patumahoe clay loam and Wakanui silt loam respectively. For the remainder of the experiment, there was a relatively slow decline in numbers with 9% and 13% viable sclerotia remaining after 24 months in the two soil types. Soil type had no significant effect ($p = > 0.05$) on sclerotial viability. Placement of bags into bins of soil did not influence sclerotial survival since the pattern of survival was very similar to those buried in the field (data not shown).

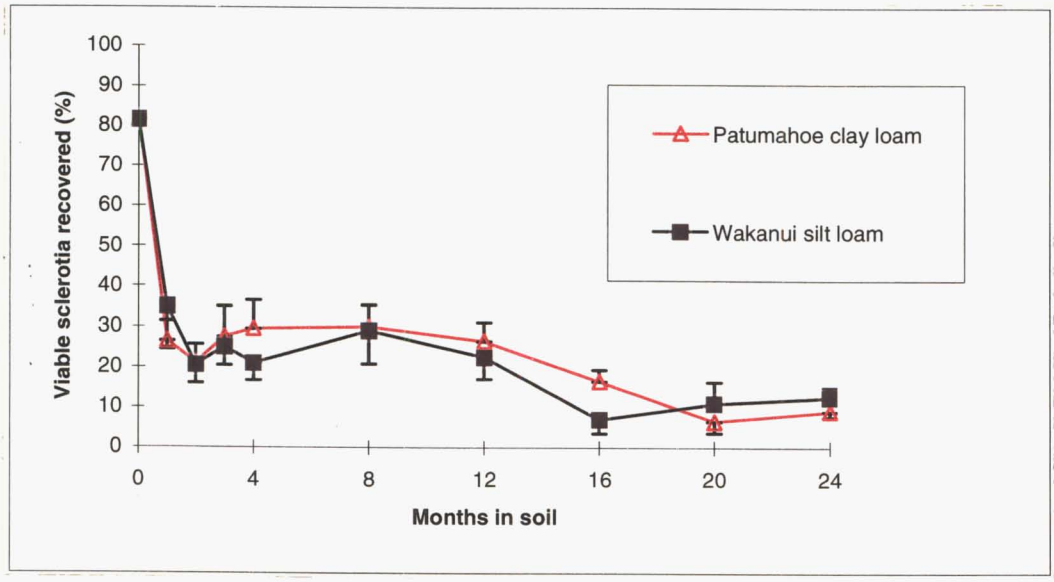
2.3.2 Experiment 2

A similar trend in sclerotial survival was observed in the second experiment. Early-season sclerotia also decayed rapidly; viability was reduced from 100% to 34% - 52% after 2 months in soil (Figure 7). After 12 months, viability of early-season sclerotia was 24% - 40% and after 20 months it was 20% - 29%. There was no significant difference in sclerotial survival in the two soil types. When data from each sampling interval were analysed separately, location had no significant effect on long-term survival (location was significant only at month 3, $p = < 0.05$). However, there was an overall effect; on average more sclerotia survived at Lincoln than at Auckland (see Table 7 in appendix 9.3.1 for ANOVA results). During the experiments, mean winter soil temperatures were 3 - 6°C higher in Auckland and annual rainfall 2.5 times that of Lincoln (Tables 3 and 4, in appendix 9.1, data from MetService New Zealand Ltd. and the National Institute of Water and Air).

2.3.3 Sclerotial contamination and damage

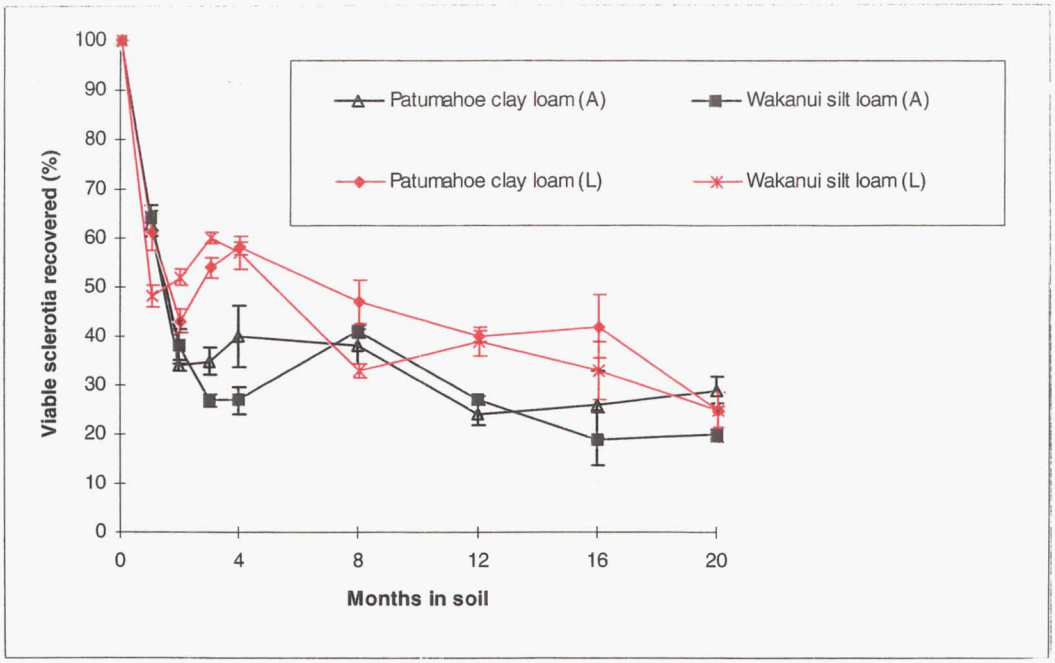
The fungi and bacteria from 100 sclerotia, from both early- and late-season populations, were isolated and classified into groups. Late-season sclerotia were heavily contaminated (100% by other fungi, mostly *Fusarium* and *Trichoderma* spp. and 46% by bacteria). Early-season sclerotia had fewer fungal contaminants (26%, predominantly *Penicillium* spp.) but 71% had bacteria on the sclerotial surface. Some of the late-season sclerotia appeared unhealthy at the start of the experiments. Many had a spongy texture. When cut in cross-section, some sclerotia had a hollow or dull-coloured central medulla. This damage was not seen in the early-season population, where sclerotia were relatively young, collected from recently infected bulbs.

The behaviour of sclerotia stored in sand differed substantially between early- and late-season populations. The early-season sample remained more than 98% viable for at least 20 months ('Experiment 2'), while the late-season controls decayed gradually and by 24 months only 35% were viable ('Experiment 1').



Standard error shown.

Figure 6. Survival of late-season sclerotia of *S. cepivorum* in two soil types.



(A) = set up at Auckland, (L) = set up at Lincoln. Standard error shown.

Figure 7. Survival of early-season sclerotia of *S. cepivorum* in two soil types, at two locations.

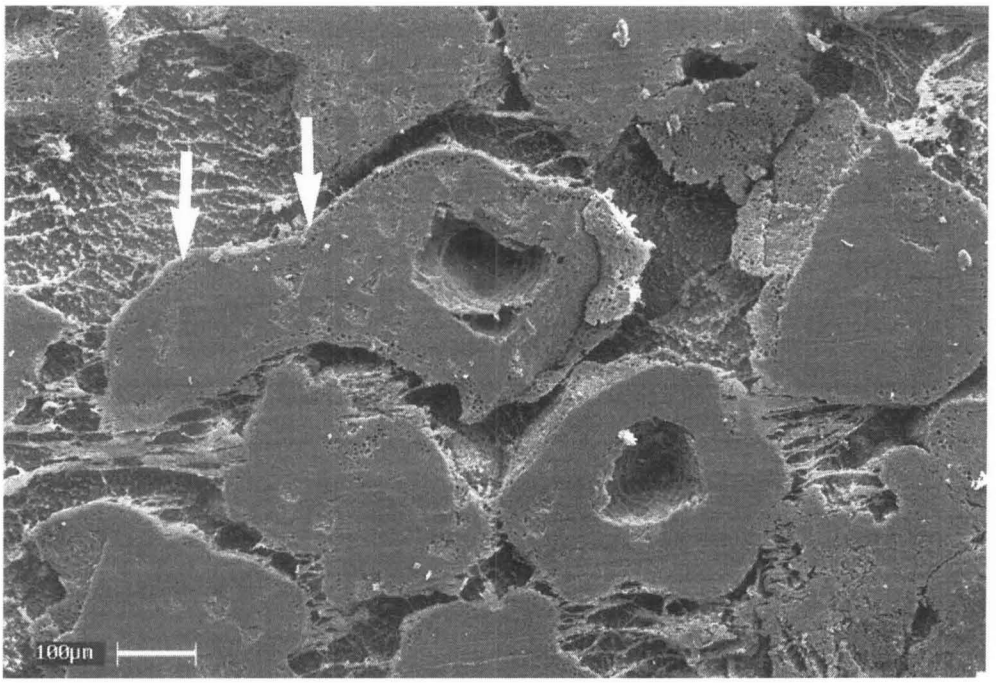
2.3.4 Sclerotial ultra-structure

Under SEM, the external rind of sclerotia appeared intact but most sclerotia were wrinkled or, in less severe cases, had depressions in their surface (3% no wrinkling, 75% light to moderate, 22% severe). Wrinkled sclerotia became turgid after soaking for 30 min in water.

On examination of the internal medulla, many sclerotia were found to have eroded hyphal contents (limited losses 32%, moderate 39%). This was most evident in the central part of the medulla (Figure 8). In extreme cases, the sclerotia were hollow (29%). Hollow sclerotia were also observed under the light microscope. No invading microbes were found inside the sclerotia that could account for this damage, although the external surfaces of most sclerotia were contaminated; 90% by bacteria and 84% by other fungi, mainly *Penicillium* spp. (n = 50). Therefore, it was assumed that the loss of hyphal contents was the result of metabolic activity or leakage. 'Healthy' sclerotia had a solid medulla and showed no erosion of hyphal contents (Figure 9).

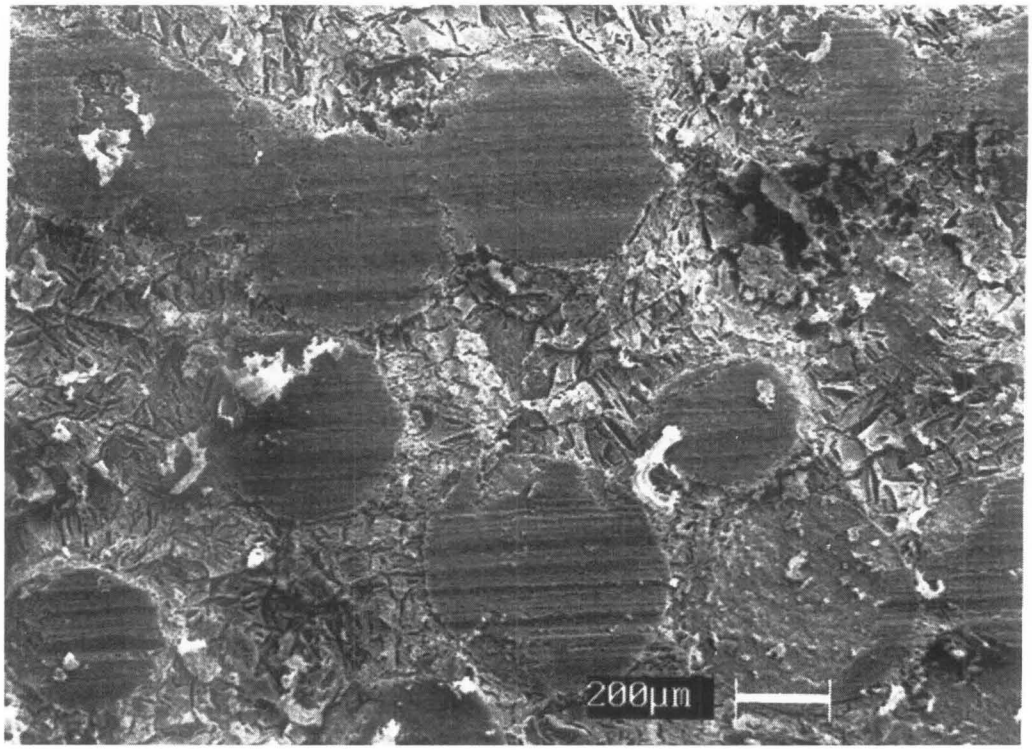
2.3.5 Influence of drying treatment on sclerotial morphology

Wrinkling of the sclerotial surface was visible after just 24 h of drying. After 1 week of alternate drying and wetting treatments, 98% were moderately wrinkled and 2% severely affected (n = 100). Extensive erosion of medullary contents was not visible at 1 week, but at 3 weeks 86% of sclerotia had moderate loss and 14% suffered more severe erosion with hollowing of the central medulla (n = 50). Controls showed none of these symptoms.



Sclerotia in cross-section showing loss of medullary contents (arrowed) and hollowing.

Figure 8. Sclerotia with loss of medullary contents.



'Healthy' sclerotia in cross-section with no loss of internal contents.

Figure 9. 'Healthy' sclerotia.

2.4 Discussion

Most sclerotia (40 - 60%) died in the first two months of incubation in soil, after which sclerotial viability declined more slowly. This rapid decline is unusual, in terms of most other work published overseas. There have been conflicting reports on the rate of sclerotial survival but most studies report a gradual decrease in viable sclerotia, as opposed to an early, steep decline (Coley-Smith and Sansford 1986, Coley-Smith *et al.* 1990, Leggett and Rahe 1985, Leggett *et al.* 1983).

Our results do, however, agree with the only previous study conducted in New Zealand (Alexander and Stewart 1994). These authors found that only 22% of sclerotia survived the first 3 months. They attributed this decline to high rainfall and associated degradation of sclerotia by micro-organisms. Another experiment, conducted in the U.K. (Entwistle and Munasinghe 1975) also showed that only 30% of sclerotia survived 3 months. Studies involving 'muck' soil (high in organic matter) in Canada reported only 24% viability after 16 months (Leggett and Rahe 1985, Leggett *et al.* 1983), although the decline was steady over time, not precipitous. Other researchers, while reporting that the remainder of the population persisted for 20 years, also noted that a proportion of naturally-produced sclerotia decayed rapidly, for an unknown reason (Coley-Smith *et al.* 1990). The results presented here are, therefore, not without precedent.

Many of the sclerotia collected in February (late-season) were in poor condition, presumably since they had been exposed to fluctuating environmental conditions and micro-organisms since they were produced (from September on). This may explain why late-season sclerotia were of lower viability than their early-season counterparts, at the start of the survival experiments (82% compared to 100%). Bearing in mind that

sclerotial age may influence survival, the second experiment was set up using early-season sclerotia. It was not possible to directly compare the survival rates of early- and late-season populations, as by necessity experiments were set up 8 - 9 months apart. However, some general trends can be seen. The most obvious difference between early- and late-season sclerotia was their condition. This included variation in the level and type of microbial contaminants present on their surface and also, to a lesser extent, the degree of structural damage present (spongy and occasionally hollow medullae were noticed only in the late-season population). Microbial action was not considered the main factor causing the early decline in viability since if it were, the early-season sclerotia, with far fewer fungal contaminants, would not have shown the same sudden decline in viability, albeit at a slightly lower level to the late-season sclerotia. Bacterial contamination was not considered to be as important an influence on survival as contamination by other fungi; even apparently healthy sclerotia generally have bacteria on their surface. Also, the fungi found on the sclerotia in these studies were those commonly employed as sclerotial parasites (e.g. *Trichoderma* spp.).

Soil type did not influence survival in this study, although only two were tested. In both experiments, sclerotia buried in either of the two soils exhibited the same pattern of sclerotial survival. This is at variance with the suggestion in some previous work that soil type (a combination of various physical characteristics and micro-flora) was an important governing factor (Leggett and Rahe 1985, Leggett *et al.* 1983 and others). It is possible that the factor(s) responsible for the early decline in viability had occurred while the sclerotia were maturing in fields at Pukekohe and therefore sclerotia collected from Pukekohe and subsequently buried at both Lincoln and Auckland soils showed the same survival trends.

When data from each sampling interval were treated separately, the location of the trial site had no significant effect on long-term survival of

sclerotia. However, significantly more sclerotia survived at Lincoln over time. This probably reflects the fact that Auckland experiences a warmer, damper climate than Lincoln, which may encourage decay by microbes.

It was postulated that the sclerotia had some inherent property that pre-disposed a certain number of them to die very rapidly on exposure to soil. This property seemed to develop over the growing season; late-season sclerotia showed structural damage not seen in the early-season population and the late-season controls decayed steadily with time while the early-season control group remained more than 98% viable for at least 20 months. Ultra-structural examination of sclerotia revealed signs of desiccation and loss of medullary contents.

Pitting of the sclerotial surface has been documented (Georgy and Coley-Smith 1982, Littley and Rahe 1992) and was attributed to the death of rind cells, as a result of dry conditions. These rind cells are then replaced by others from beneath (Backhouse and Stewart 1987). 'Abnormal sclerotia' of *Sclerotinia sclerotiorum* have been found with a wrinkled or grooved surface, a brownish non-viable central area in the medulla and a much reduced sclerotial viability (Huang 1982 and 1983). The lower survival rate was attributed to damage caused by environmental conditions as, in common with this study, no microbes were found inside the sclerotia. The wrinkled rind was described as the result of drying out, which in turn caused loss of nutrients (supported by Coley-Smith *et al.* 1990, Smith 1972), increased action by micro-organisms and a swift drop in viability. This supports both our observations of sclerotial structure and the trend for rapid decay during the first few months in soil.

Drying has long been associated with sclerotial decay and subsequent leakage of nutrients (Leggett and Rahe 1985, Leggett *et al.* 1983, Smith 1972). While there is some disagreement in the literature as to whether drying out adversely affects survival (Coley-Smith *et al.* 1974, Papavizas 1977), many researchers have reported a positive correlation between

desiccation and loss of fitness (e.g. Huang 1982 and 1983, Littley and Rahe 1992). Desiccation, in the literature, either occurred naturally or was induced by treatment with dry air or a chemical desiccant such as $CaCl_2$ (Coley-Smith *et al.* 1974, Huang 1982 and 1983, Smith 1972).

It is well documented that the leaching of nutrients pre-disposes sclerotia to attack by microbes (Coley-Smith *et al.* 1974, Huang 1982 and 1983, Smith 1972). One study stated that *Trichoderma hamatum* only invaded sclerotia after they had been dried and the authors proposed that this was because they were attracted to nutrients or to dead rind cells on the sclerotial surface (Coley-Smith *et al.* 1974). In our study, the rinds of the desiccated sclerotia examined by SEM were severely contaminated by other fungi as were those used in the survival experiments.

Loss of the contents of medullary hyphae and / or the presence of sclerotia with hollow centres has been demonstrated previously in sclerotia that have been some time in soil (Backhouse and Stewart 1987, Coley-Smith *et al.* 1990). The authors suggested that this was the result of physiological activity (i.e. the sclerotia were not entirely dormant) or leakage of cell contents as a result of wetting and drying. Some metabolic activity is required to sustain life and over time, the contents are steadily eroded. However, the loss of contents that was observed in a significant proportion of the population over a short 5-month growing season would appear too extensive to be due to normal metabolic activity alone.

As sclerotia form and mature, respiring hyphae become physiologically less active and go into a state of dormancy as sclerotial survival is ultimately limited by how long their food supply lasts. One possibility is that, in the relatively young sclerotia sampled, this process of 'shutting down' has not yet fully occurred which could explain the loss of medullary contents although it does not account for the wrinkled, desiccated

appearance of the sclerotia. An alternative explanation is, therefore, required.

The metabolic activity required to replace rind cells damaged by drying out could create a reduction in storage reserves. The loss of fluid, which caused the wrinkling of the sclerotial surface, may also have resulted in a loss of storage compounds. Even healthy sclerotia are known to leak amino acids and this is more pronounced in desiccated sclerotia (Huang 1982).

Artificial desiccation caused a wrinkled sclerotial surface and a reduction in hyphal contents similar to that observed in field-produced sclerotia. Also, sclerotia produced on inoculated onions in cool, moist conditions did not show the early mortality seen in the natural population (see Chapter 3; in dormancy Experiment 2, the controls were buried in soil in the same way as for the survival experiments and provide an indication of recovery rates of artificially-produced sclerotia over time). This provides additional support for the hypothesis that the survival properties of sclerotia in these trials were determined to a large extent by the environmental conditions under which they were formed.

2.5 Conclusions

Although most sclerotia decayed early, enough remained in the soil to continue the lifecycle of the fungus. One infected bulb can produce in excess of 1000 sclerotia; if even 10% of these survive, the inoculum level is still substantially increased. Environmental factors such as desiccation during the growing season may cause damage to the sclerotia, which could account for the early and substantial loss of viability observed.

CHAPTER 3: DORMANCY IN SCLEROTIA OF *SCLEROTIUM CEPIVORUM* IN NEW ZEALAND SOILS

3.1 Introduction

Allium spp. produce root exudates that contain S-alk(en)yl-L-cysteine sulphoxides (Coley-Smith and King 1969). Microbes in the soil break down these compounds into substances including 1-propyl- and 2-propenyl (diallyl) disulphides and these then trigger germination in sclerotia of *S. cepivorum* (Coley-Smith and King 1969, Coley-Smith *et al.* 1987, Dickinson and Coley-Smith 1970). This interaction is the basis of the host-specificity of *S. cepivorum*; *S. cepivorum* germinates only in response to these exudates.

Constitutive dormancy is the term used to describe why sclerotia will not germinate, although given the appropriate environmental cue(s), until they have been conditioned for some time in soil. Sclerotial dormancy occurs in a number of fungi (Coley-Smith and Cooke 1971, Willetts and Wong 1980) including *Sclerotinia sclerotiorum* (Dilliard *et al.* 1995, Phillips 1987), *Sclerotinia minor* (Adams and Tate 1976) and *Sclerotium rolfsii* (Chet 1969). Dormancy seems to prevent germination in adverse conditions (see section on 'exogenous' dormancy below) or in the absence of a host plant, where the fungus is host-specific. The majority of sclerotia of *S. cepivorum* exhibit constitutive dormancy, which prevents them from germinating when exposed to *Allium* exudates unless they have been conditioned by a period in unsterile soil (Brix and Zinkernagel 1992, Coley-Smith 1960, Coley-Smith *et al.* 1987).

Little is known of the physiological basis of sclerotial dormancy or how *Allium* extracts work to trigger germination (Coley-Smith *et al.* 1987). Not all isolates exhibit constitutive dormancy; sclerotia of some isolates

germinate at high levels directly after they mature whilst others require longer or shorter periods of conditioning in unsterile soil before dormancy breaks (Brix and Zinkernagel 1992). However, for most isolates, 1 - 3 months appears sufficient. Inconsistent reports on sclerotial dormancy are common in the literature. In most, but not all, previous work, naturally-produced sclerotia (arising from host plant infection) have shown higher rates of germination than those grown in pure culture (Coley-Smith *et al.* 1987). Variation in dormancy requirements has been attributed to genetic differences between isolates or to environmental influences on sclerotial germination, such as cultivation history, soil temperature / moisture and time of year (Brix and Zinkernagel 1992, Coley-Smith and Holt 1966, Coley-Smith *et al.* 1987, Gerbrandy 1992).

If artificially-produced sclerotia of *S. cepivorum* are left in axenic culture once they have matured, they remain dormant, in one experiment for as long as two years (Coley-Smith 1960, Coley-Smith *et al.* 1987). This provides evidence that some component of unsterile soil is responsible for the breakdown of constitutive dormancy, allowing sclerotia to respond to the presence of *Allium* species or their extracts. Perhaps time is needed for micro-organisms in the soil to affect the rind of newly-produced sclerotia, before the sclerotia can respond to *Allium* extracts. This could explain the function of unsterile soil in the breakdown of constitutive dormancy (not to be confused with 'exogenous' dormancy, imposed by adverse soil conditions, see following section for explanation). In a study involving *S. sclerotiorum* (Phillips 1987), it was noted that sclerotia broke dormancy more readily when conditioned in soil than when stored for a similar length of time in an artificial medium (moist vermiculite), implying that sclerotial age was less important than the microbial stimulation of the sclerotia by soil.

Various methods have been trialed to break dormancy. Exposure to low or high temperatures has given variable results (Brix and Zinkernagel 1992, Elnaghy *et al.* 1971, Gerbrandy 1989 and 1992) with different

isolates breaking dormancy after a variety of temperature treatments. Freezing and thawing treatments actually reduced the rates of germination in one trial (Brix and Zinkernagel 1992). Six out of ten isolates tested broke dormancy after drying for 48 h (Gerbrandy 1992). Abrasion of the rind seemed most successful in triggering dormant sclerotia to germinate (Coley-Smith 1960). If the rind is broken during freezing, this may also trigger germination (Brix and Zinkernagel 1992). There is some evidence to suggest that surface sterilisation with sodium hypochlorite breaks dormancy. Dormancy does not seem to be affected by pH as germination of conditioned sclerotia occurs over a wide range of pH values; 4.5 - 7.8 (Adams and Papavizas 1971, Coley-Smith 1960).

As a result of the host-specificity of *S. cepivorum*, artificial synthates of *Allium* exudates, especially diallyl disulphide (DADS), have been trialed as a disease control measure. They can be used to stimulate sclerotial germination in the absence of a suitable host plant. Once germinated, the fungus cannot survive as a saprophyte and dies rapidly. This method may, therefore, reduce the level of inoculum in the soil prior to planting the host crop. Treatment with DADS can reduce disease incidence from 45% to 5%, which is comparable to the effect of conventional fungicide applications (Tyson *et al.* 2000). For the effective use of DADS in disease control, it is necessary to know whether all isolates exhibit dormancy and for what length of time.

A further aim of such work is to determine whether sclerotia that are produced on infected plants early in the season may or may not overcome constitutive dormancy within a single growing season and thereby act as secondary inoculum for further infection. Studies of dormancy also have value in themselves as constitutive dormancy is an almost universal property of sclerotia, but remains widely misunderstood and is the subject of a highly contradictory literature.

Exogenous dormancy

In a related process known as 'exogenous dormancy', the soil environment, in particular soil microbes, has a constant inhibitory effect on sclerotial germination. This prevents sclerotia from germinating in the absence of suitable host plants. The presence of *Allium* species or their extracts overrides the negative influence of the soil and (when constitutive dormancy is not active) the result is germination.

Some work has been done on the basis of exogenous dormancy; water expressed from four soil types successfully suppressed sclerotial germination (King and Coley-Smith 1969). The inhibitory effect of the expressate was lost after autoclaving and reduced by membrane-filtration. It follows that the inhibitor is either destroyed by heat or is volatile (King and Coley-Smith 1969). Sclerotia germinate without DADS on sterile culture such as autoclaved soil, agar, filter paper and silica gel because the inhibitory action of soil microbes has been removed (Adams and Papavizas 1971, Coley-Smith and Holt 1966, Coley-Smith *et al.* 1967, King and Coley-Smith 1969).

Exogenous dormancy was not the subject of the present study, but to successfully examine constitutive dormancy it was necessary to be aware of the other process. Much of the ambiguity in the literature seems to be the result of authors confusing constitutive and exogenous dormancy, not making it clear which phenomenon they are discussing or perhaps being unsure of the distinction, if any, between the two; when faced with a population of sclerotia that will not germinate, it may be unclear which process is in action. In this thesis I follow the definition of Sussman and Halvorson (1966) who describe constitutive dormancy as an inherent inability of sclerotia to germinate, which is broken down by special stimuli not required for normal vegetative growth. Exogenous dormancy, on the other hand, is dependant solely on the environment, not on the sclerotia themselves and is broken by a return to conditions favouring growth (the presence of *Allium* exudates in this case).

These two types of dormancy may interact with each other and are closely associated with other factors affecting sclerotial germination. Therefore, while it is possible to treat exogenous and constitutive dormancy as separate processes, they should not be considered in isolation. Unless stated otherwise, the term 'dormancy' will refer to the constitutive, not the exogenous, type.

Aim

The aim of the experiments reported here was to determine the nature of dormancy in sclerotia of isolates of *S. cepivorum* and to develop a methodology with which to test for it. Two established methods were modified and tested to determine which was most useful to monitor sclerotial dormancy in New Zealand soil (Brix and Zinkernagel 1992, Coley-Smith *et al.* 1987).

3.2 Materials and methods

3.2.1 Experiment 1

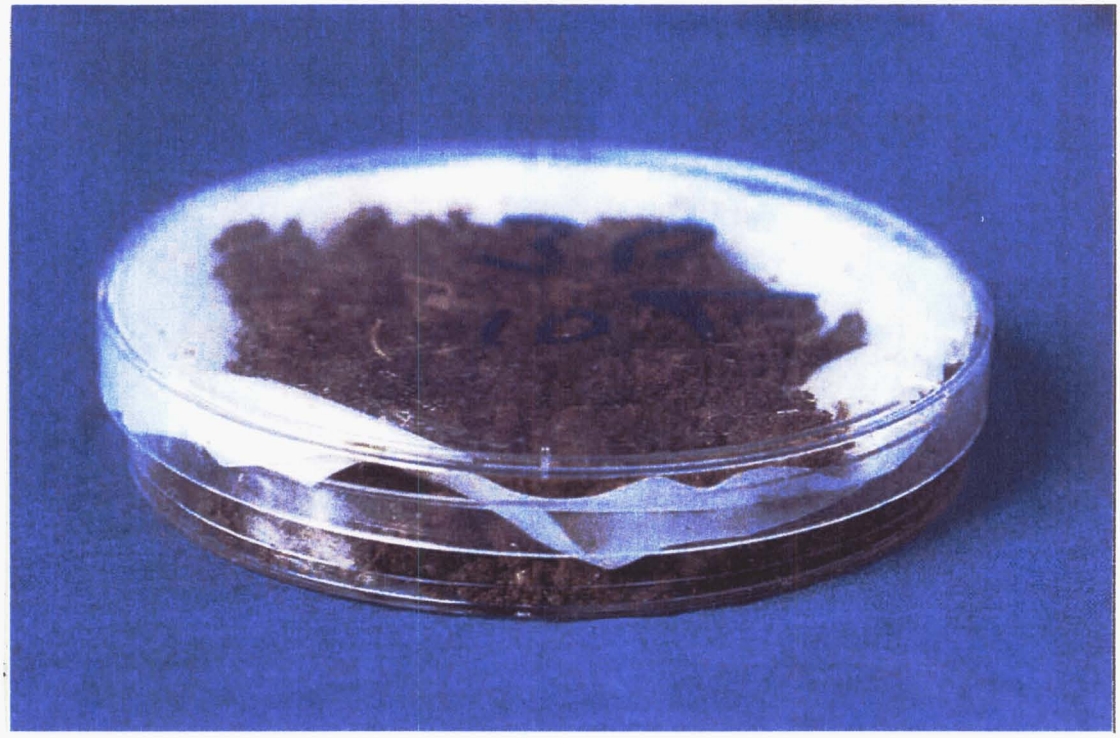
This experiment determined dormancy requirements of sclerotia of three isolates of *S. cepivorum*, two ('Sc3', 'master') from the Pukekohe region (South Auckland, North Island) and one ('Sc5') from Kaiapoi (north of Christchurch, South Island). As mentioned in Chapter 1, the majority of New Zealand's onion cultivation takes place in these two regions. Dormancy was tested in two soil types; Patumahoe clay loam (from Pukekohe) and Wakanui silt loam (from Kaiapoi).

Sclerotia were produced on onions in the laboratory (Coley-Smith 1985). Discs from the growing edge of a culture, grown on PDA, were inserted under flaps cut into the side of the onion bulbs (Coley-Smith 1985). The onions were incubated in open plastic bags for 7 days at 18°C, after which they were removed from the bags, placed in open shallow 2 l plastic containers and incubated a further 6 weeks at the same temperature, under an alternating 12 h light, 12 h dark regime (Coley-Smith 1985). The onions were kept moist but not waterlogged; water was sprinkled on them every second day. The cultivar 'Anna Marie' was used to produce inoculum as these onions produce a high number of sclerotia per bulb (K. McLean, pers. comm.). Once mature (6 weeks after inoculation), sclerotia were rubbed off the infected onions and recovered by progressive wet-sieving (washing sclerotia through a series of progressively finer sieves, Crowe *et al.* 1980). The sieve sizes were 500, 250 and 150 µm. Only sclerotia from the 250 µm sieve were collected by washing onto filter paper. They were then air-dried for up to 1 h, selected with a needle, counted and immediately placed in nylon mesh bags, 75 sclerotia per bag (bags 10 cm^{-2}, mesh 85µm, Scapa Filtration Ltd., Auckland). Each bag of sclerotia also contained a mixture of 20 g of white sand and 20 g of either of the two soil types used. The soil was sieved to less than 150 µm. (For illustration of nylon bag, see Figure 5, Chapter 2).

The bagged sclerotia were buried in Patumahoe clay loam (pH 5.8 - 6.3, 4% organic matter, Alexander and Stewart 1994) from Pukekohe or in Wakanui silt loam (pH 6.9, 4% organic matter) from Kaiapoi. Each soil type was contained in 6 plastic bins (47 cm diameter, 53 cm deep) as for the survival experiments; see Figure 4, Chapter 2. The bins were sunk into the ground, in a field at Lincoln University with only the top 1 - 2 cm of the bins exposed and arranged in a grid pattern with bins containing the two soil types placed alternately, approximately 1 m apart. Gravel (5 cm layer) was spread in the base of the bins to aid drainage. Drainage holes (8 per bin, 1.5 cm in diameter) were drilled into the sides of the bins. The mesh bags containing sclerotia were buried at 10 cm depth in one of the two soils, with labels attached to allow for easy retrieval. Twenty bags were buried in each bin of each soil type. A 'Tinitag' datalogger (Gemini Dataloggers, U.K.) was used to record soil temperature. Rainfall data was also collected. Regular hand-weeding kept grass cover sparse, to allow easy retrieval of bags. As there was no literature to the contrary, it was assumed that groundcover had no effect on sclerotial germination. Also, in the field, groundcover was limited for much of the growing season, until the onion crop became large and leafy, around October.

Five bags per isolate / soil type combination were chosen randomly and removed at weekly intervals for 15 weeks. Once removed from the field, the sclerotia were recovered onto a 250 µm sieve by wet-sieving, picked up with forceps and placed in 8.5 cm diameter Petri dishes between two layers of fresh soil (approximately 15 g in each layer). The sclerotia were kept separate from soil, to allow them to be recovered later, by two layers of nylon mesh fabric (Brix and Zinkernagel 1992, Coley-Smith *et al.* 1987). Sclerotia from individual bags were placed in separate dishes. There were 25 sclerotia in each dish, representing a randomly chosen subset of the 75 sclerotia originally placed in each bag. More sclerotia than needed were placed in each bag as a proportion was expected to

decay (for reasons other than DADS-dependant germination, as DADS had not yet been applied) during conditioning (see Chapter 1). Five Petri dishes in total were used for each isolate in each soil type. One ml of 0.08% DADS (aqueous solution, supplied by Elliott Chemicals Ltd., Auckland) was added to each of three Petri dishes (Brix and Zinkernagel 1992). The DADS was sterilised using a membrane filter (model Millex-gs, 0.22 µm, Milipore, Molsheim, France). The remaining two dishes were not exposed to DADS and acted as controls. The soil in the dishes was moistened with 1 ml 'Tween 20' solution (20 gl^{-1}, Brix and Zinkernagel 1992). Sterile water (1 ml) was added to controls in place of DADS. The dishes were then sealed with plastic film and kept in incubators at 18°C in the dark. (For Petri dish set-up, see Figure 10). Sclerotial germination was scored by lifting the nylon cloth and upper layer of soil off the sclerotia (Brix and Zinkernagel 1992, Coley-Smith and Parfitt 1986). Gentle pressure was then applied to the sclerotia, by folding them in a layer of mesh and squeezing them between the thumb and forefinger. Sclerotia were examined under the stereo microscope (x 12, Leica, model WILD MZ8). Those sclerotia that were hollow collapsed completely and were scored as germinated while 'dormant' sclerotia remained firm and intact. This gave a rapid assessment of germination. In pilot trials, germination was initially scored as the emergence of hyphae from the sclerotia. These hyphae were easily confused with or obscured by fungal contaminants on the sclerotial surface. Therefore, sclerotia were gently squeezed as described and hollow or spongy sclerotia (i.e. squashed flat) scored as 'germinated'. This eliminated the need to distinguish between any contaminants and emerging hyphae of *S. cepivorum*. To allow sufficient time for germinating sclerotia to be degraded completely, scoring was done 12 weeks after the application of DADS. The number of intact sclerotia was counted and these were assumed to be dormant.



Sclerotia placed between two layers of soil, kept separate by two layers of mesh fabric.

Figure 10. Petri dish set-up.

Dishes containing DADS were placed in a separate, identical incubator to the controls to prevent diffusion of DADS among the dishes (Brix and Zinkernagel 1992). A second quantity of DADS was applied 15 weeks after the initial dose (after scoring germination for the first time) and germination re-scored 12 weeks later. This second application was used to determine whether or not a single dose of DADS was sufficient to cause substantial sclerotial germination, or whether it acted as a primer, with high rates of germination occurring only after additional applications. The experiment was conducted during winter and spring 1999 (conditioning in soil took place between August and November).

Contamination of the Petri dishes (especially by *Talaromyces* spp.) had been a significant problem in pilot trials but did not occur in Experiment 1, due to several modifications already mentioned; germination rates were determined as described, the DADS was sterilised and the experiment was conducted in winter and spring, rather than summer, as high soil

temperatures have been shown to favour the growth of some *Talaromyces* spp. (Beauchat 1988).

3.2.2 Experiment 2

A second experiment was set up as described for Experiment 1, with the following changes. The nylon bags containing the sclerotia were smaller (5 cm^{-2}, with 5 g of sieved soil and 5 g of sand in each bag), dormancy was tested in one soil type only (Wakanui silt loam) and the bags were initially buried in a single bin of soil, not spread over 12 bins. DADS was applied once, not twice as in Experiment 1. The main difference between Experiments 1 and 2 was that, rather than removing the sclerotia from the bags after conditioning and placing into Petri dishes of soil, the treated bags were each removed from soil, injected with 1 ml of 0.08% DADS using a pipette (HTL, 200 – 1000 µl) and then reburied in a second adjacent bin of soil, to prevent any diffusion of DADS to controls. Separating treatment and control bags into two adjacent bins involved a risk of pseudo-replication. The necessary isolation of treatment bags, to prevent diffusion of DADS to controls, is a limitation of this type of experiment. The problem might have been solved by the use of very large bins (allowing for a sufficiently great distance between treatments and controls within a single bin) or the use of a high number of bins (although treatments and controls would still have to be replicated in isolation from each other and the numbers of bins involved were impractical in this case). Pseudo-replication was not, however, considered to be a major issue as other authors have successfully run experiments where controls and treatments were placed in separate incubators (Brix and Zinkernagel 1992) and there was no evidence from the survival studies that bins had any effect on the properties of the sclerotia (see Chapter 2).

For each isolate / soil type combination, at each sampling time, there were three treated bags and two untreated control bags, each bag containing 75 sclerotia. DADS was applied to treatment bags at monthly

intervals for 6 months. Scoring of germination was done 8 weeks after the DADS was applied; intact sclerotia recovered from the bags by wet-sieving were categorised as dormant and counts made.

The trial was lengthened from 4 months to 6 months when early results indicated that dormancy might last longer than previously thought. Five isolates were tested; the three from Experiment 1 and a further two isolates from Pukekohe ('smeeds', 'das'). The experiment was conducted between March and October 2000.

3.2.3 Experiment 3

Naturally-produced sclerotia, collected from onions in a commercial field, were exposed to DADS using the method described for Experiment 2. This was to determine whether sclerotia produced under field conditions behaved in the same way as those produced on artificially-infected onions in the laboratory. Sclerotia were collected in September 2000, from 30 newly-infected bulbs in a commercial field at Pukekohe. In this experiment, the sclerotia were believed to be 8 weeks old or less as there was no white rot in the collection area 8 weeks prior to sampling and a proportion of sclerotia had half-formed rinds (~25%), appearing pale-coloured under the microscope (x15), indicating that they were immature. Only black sclerotia with fully-formed rinds were used.

It was expected that a high proportion (up to 60%) of field-produced sclerotia would not survive the first few months in soil due to natural decay (see Chapter 2). This may be confused with decay following DADS-dependant germination. To prevent masking of the (presumably smaller) effect of the breakage of dormancy during the first half of the experiment, an increased number of replicates were used; 20 bags, each containing 100 sclerotia were buried. Half of these (10 replicate bags) were immediately exposed to DADS with the other half acting as controls. Those bags that had been treated with DADS were buried in a single bin with controls buried in a separate, adjacent bin to prevent any diffusion of

DADS from treated bags to controls. Bins contained Wakanui silt loam. All bags were recovered 8 weeks after DADS was applied and the sclerotia removed from the bags by wet-sieving and any sclerotia counted and assumed dormant. As there was a limited supply of sclerotia, a full-scale study, with multiple sampling intervals, was not possible. Therefore, the treated sclerotia were immediately exposed to DADS, not buried in soil for up to 4 or 6 months as in Experiments 1 and 2.

3.2.4 Data analysis

Data (numbers of sclerotia germinating) were analysed by factorial ANOVA (programme supplied by Systat Inc., Evanston, USA) with treatment, isolate, soil type and time spent in soil (if applicable) as factors. Data from each sampling interval were analysed separately and then, if more than one sampling interval, an analysis was done on the overall effect of treatments over time.

3.3 Results

3.3.1 Experiment 1

Results for 'Experiment 1' are shown in Table 1. As there was no significant effect of soil type ($p = >0.05$), the data from both soils were combined. A percentage of sclerotia (11 - 23%, range of means, all isolates) were able to germinate one month after treatment with DADS. Only 25 - 33% (range of means, all isolates) of sclerotia had germinated by week 15 with the remaining presumed dormant or otherwise unable to germinate. Fewer than 6% of sclerotia germinated or decayed in control dishes (those not exposed to DADS).

Despite the modifications made to the original methodology, there were problems with this trial. Dormancy did not break completely by the end of the experiment and there was considerable variation amongst the treated samples. Significantly more treated sclerotia germinated compared to the controls in weeks 3, 8 - 10 and 15 ('SC3'), from week 3 onwards with the exception of week 11 ('SC5') and all weeks except weeks 4 and 5 ('master'), (see appendix, Table 9). There was no significant difference between the dormancy requirements of the three isolates ($p=>0.05$) and there were no interaction effects (for ANOVA results, see Table 9 in appendix). When a second quantity of DADS was applied after the first, it had no further effect on germination. For this reason, only one application of DADS was used in Experiment 2.

3.3.2 Experiment 2

Results for Experiment 2 are shown in Figures 11a - 11e (standard deviation shown). As with Experiment 1, a proportion (23%, treatment mean of all isolates) of sclerotia were able to germinate after 1 month. After 4 months in soil, 47 - 72% of sclerotia had germinated with the remaining sclerotia presumed dormant (range of treatment means, all

isolates). By 6 months, dormancy had broken in 89 - 98% of sclerotia. Germination was simple to score with no contamination by other fungi as was experienced in pilot trials for Experiment 1. There was no significant difference in sclerotial germination between the isolates in the first 2 months (p=>0.05) but after month 3 significant differences occurred (for ANOVA results, see Table 10 in appendix) reflecting the difference in timing of dormancy breakage between isolates. By month 6, when all isolates had broken dormancy, there was again no difference between the isolates (p=>0.05).

In all isolates, at least 3 months in soil were required before treated sclerotia germinated at significantly higher rates to the controls. The breakage of dormancy (as measured by the difference between treatment and control bags) was statistically significant in most isolates by month 4 ('das', 'sc3', 'master'). The 'sc5' isolate began to break dormancy earlier, after conditioning for 3 months in soil. In the isolate 'smeeds', the difference between sclerotia exposed or not exposed to DADS was not statistically significant in the first four months but significant at month 5 and 6.

3.3.3 Experiment 3

This experiment tested for dormancy in sclerotia collected from a commercial field. After exposure to DADS, 51% (mean of 10 replicates, std = 12.8%) of sclerotia germinated or decayed. Significantly fewer sclerotia decayed (21%, mean of 10 replicates, std = 12.0%) when not exposed to DADS (p = 0.000). The sclerotia were assumed to have been only just mature at the start of the experiment (they were 8 weeks old or less) and had not been conditioned in soil, prior to treatment with DADS.

Table 1. Experiment 1: dormancy in three isolates of *S. cepivorum*.

Isolate	Weeks in soil	GERMINATED SCLEROTIA (%) + DADS[1]	- DADS[2]
'Master'	1	12±9*	2±4
	2	10±4*	2±2
	3	17±9*	0
	4	16±14	3±4
	5	15±16	1±2
	7	7±3*	2±4
	8	29±6*	1±2
	9	21±7*	1±2
	10	15±7*	2±4
	11	13±3*	1±2
	12	21±9*	3±4
	15	28±15*	4±3
'Sc3'	1	16±13	1±2
	2	21±13	4±6
	3	14±3	0
	4	11±8	4±5
	5	18±14	4±3
	7	13±7	5±5
	8	29±9*	3±2
	9	25±15*	2±2
	10	32±16*	2±2
	11	18±19	1±2
	12	16±13	2±2
	15	25±20	6±4
'Sc5'	1	11±9	1±2
	2	11±13	2±2
	3	13±3*	1±2
	4	23±11*	4±3
	5	14±6*	4±0
	7	16±6*	1±2
	8	44±14*	2±4
	9	41±17*	2±2
	10	35±16*	1±2
	11	27±22	3±2
	12	27±14*	0
	15	33±19*	2±2

1 = mean of six replicates. 2 = mean of four replicates.

Results from sclerotia buried in different soils have been combined here, as they were not significantly different. Standard deviation included.

* = ANOVA indicates treatment significantly different from control.

Figure 11. Experiment 2: breakage of dormancy in five isolates of *Sclerotium cepivorum*.

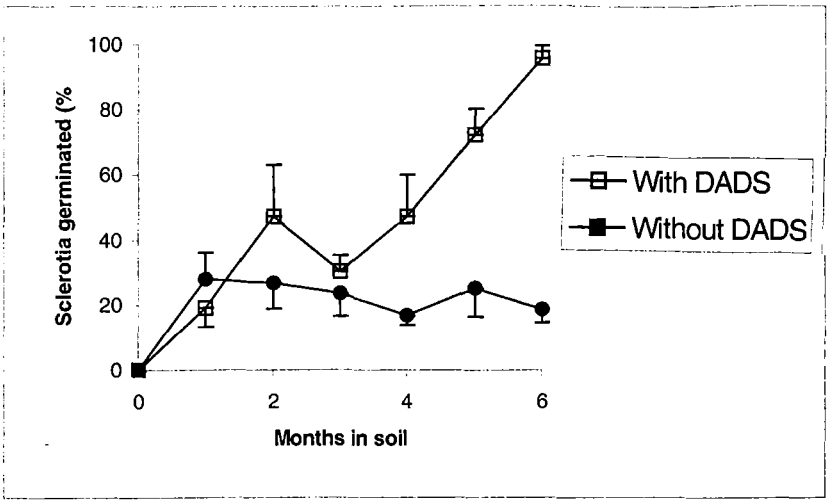


Figure 11a. 'Smeeds'

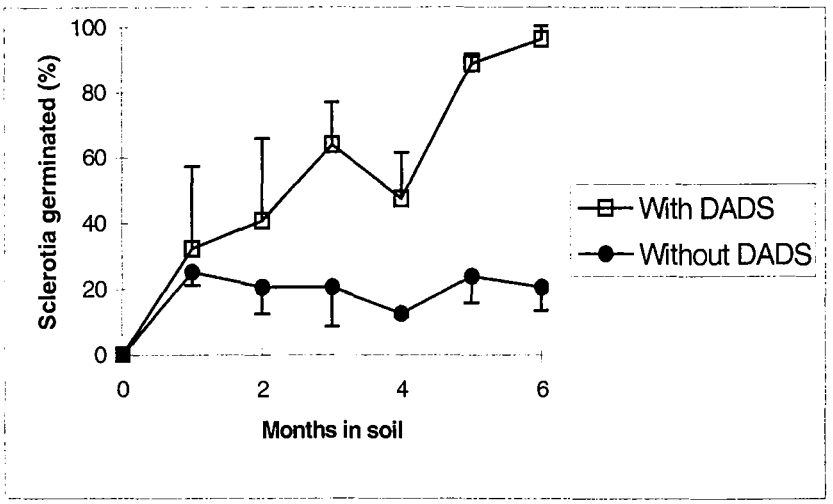


Figure 11b. 'Sc5'

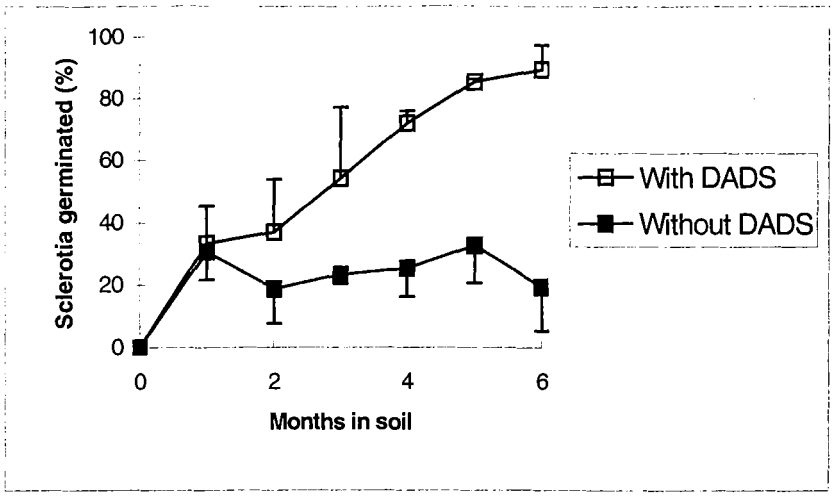


Figure 11c. 'Sc3'

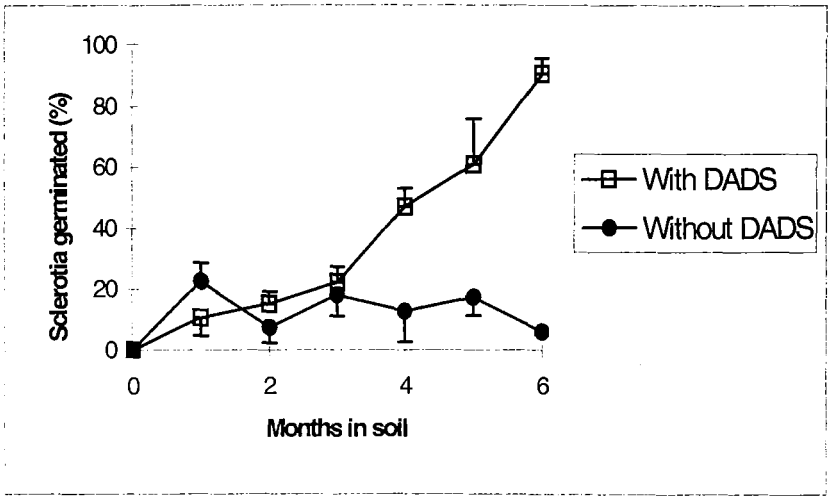


Figure 11d. 'Master'

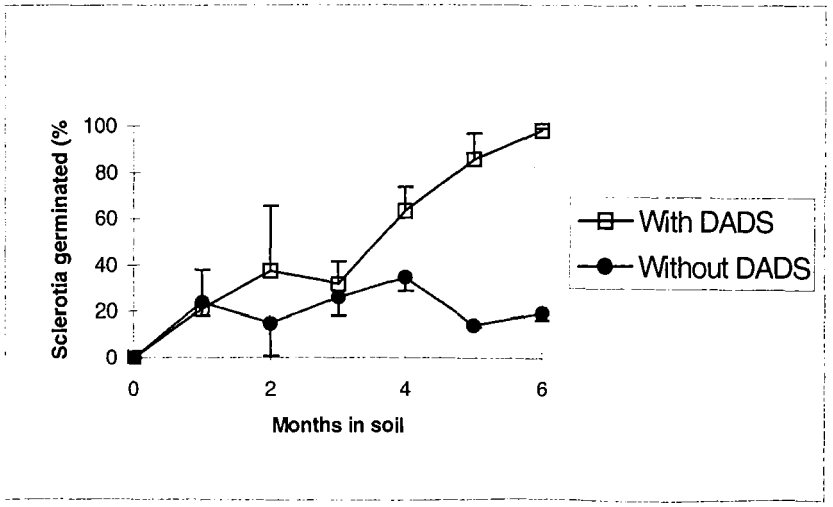


Figure 11e. 'Das'

3.4 Discussion

Dormancy was shown to exist in all isolates tested. In the field trial (Experiment 2), a minimum of 3 months in unsterile soil were required before statistically significant numbers of sclerotia were capable of germination (>20% of sclerotia, after 3 months). Germination rates of above 89% were only seen in sclerotia after incubation in soil for 6 months. This is longer than reported in most previous overseas work; high rates of germination (80% or above) were observed after 1 - 3 months in soil (Brix and Zinkernagel 1992, Coley-Smith *et al.* 1987). An exception to the general trend was one study (Gerbrandy 1992) where sclerotia from 3 out of 10 isolates remained dormant after a year, although no reason was postulated for this. The literature also describes variable results with some isolates germinating immediately (Brix and Zinkernagel 1992) and others, as in this study, requiring some time in soil, before substantial rates of germination occurred.

The laboratory-based method used in Experiment 1 gave far more variable results than the field-based technique and rates of germination were low (<35%) after almost 4 months in soil. This is somewhat lower than that seen in the field trial, over the same time period, and could be due to seasonality; germination rates may be 50% lower in summer than in winter (Gerbrandy 1990) but this experiment was conducted in winter / spring. Also, in pilot trials, which were conducted year-round (not all data shown, see Table 8 in appendix for results of one pilot trial), germination rates were uniformly low when this method was used. It is considered more likely that the presence of fungal contaminants resulted in an unfavourable environment, perhaps competing for nutrients or releasing compounds that inhibited sclerotial germination, thereby causing lower rates of germination, compared to the field-based study.

There was some potential for error in Experiment 1 as spongy sclerotia that were scored as 'germinated' could be the result of mycoparasitism

and decay but the fact that these were seen only in the plates that had been exposed to DADS provides some level of confidence.

In Experiment 1, sclerotia were buried in field-soil outdoors during conditioning and then a subset of these removed (25 out of the 75 originally buried in each bag) and DADS applied to those removed, to test for dormancy. It was expected that some of the sclerotia would decay during conditioning (see survival experiments, Chapter 2) and by removing and testing sclerotia after this had occurred, the expectation was that the loss of sclerotia, due to causes other than DADS-dependant germination, could be excluded from the analysis of dormancy. From the behaviour of the controls in Experiment 2, we know that on average 23 - 31% of sclerotia decayed naturally, in the first month after burial in soil, and that little further decay occurred after the first month (see Figure 11). This loss of sclerotia should not affect the analysis of dormancy in Experiment 1, as they decayed before DADS was applied.

The only way that loss of sclerotia during conditioning could affect the results is if the sclerotia that died, before DADS was applied, were significantly more (or less) likely to germinate in response to DADS than the rest of the sample. It is unlikely but possible that those sclerotia that were prone to early decay were also those most likely to germinate in the presence of DADS, as they may have had structural damage (as indicated by the survival studies involving field-produced sclerotia) and treatments that damage the rind have been shown to break dormancy. If this were true, the levels of dormancy found in Experiment 1 may be somewhat lower than they should have been. However, when examined under the dissecting microscope before the experiments were set up, the sclerotia used in Experiments 1 and 2 did not show the same structural damage observed in the field-produced sclerotia used in the survival work.

Soil type had no effect on rates of germination / dormancy in Experiment 1 but dormancy was tested in two soils only. It is possible that variation in microflora between the soil types could affect dormancy. However, this study supports those of other authors who found no significant differences between soils (e.g. Gerbrandy 1990).

In Experiment 2, there were significant differences in the timing of breakage of dormancy among the isolates; one isolate began to break dormancy after 3 months in soil, others after 4 or 5 months. While no differences in dormancy requirements were noticed among isolates in Experiment 1, this may be because the low rates of germination and high levels of statistical variation observed precluded the detection of any differences. A number of studies have found that sclerotial isolates require a variety of lengths of time in soil before dormancy breaks (Brix and Zinkernagel 1992, Gerbrandy 1992) and these have been attributed to a range of environmental variables (e.g. temperature, time of year) and / or genetic differences. With the high level of genetic diversity present in New Zealand cultures of *S. cepivorum* (Tyson *et al.* in press), it seems logical that isolates could differ in a number of their responses to environmental pressures and in their properties, including constitutive dormancy. However, as only five isolates were tested, a definitive statement of the effect of isolate on dormancy cannot be made. Although significant differences in the timing of dormancy were found, the overall trend was the same in all the isolates tested, with a gradual increase in germination over time and maximum germination occurring after 6 months conditioning in soil (Figure 11).

The method used in Experiment 2, where DADS was injected into bags, proved more effective than that used in Experiment 1. It was not prone to problems with contaminants on the sclerotial surface and the sclerotia were also not exposed to drying out, which sometimes occurred in the Petri dishes, despite the use of a wetting agent and the fact that the dishes were sealed with plastic wrap. The dry soil conditions in the dishes

may have contributed to the low rates of germination observed, although drying is usually associated with an increase, rather than a decrease, in sclerotial germination of *S. cepivorum* (Gerbrandy 1992). More of the control sclerotia germinated or decayed in the field-based Experiment 2, compared to the laboratory-based Experiment 1 but more of the treated sclerotia also germinated / decayed.

According to the literature, field-produced sclerotia may differ in their dormancy requirements from those produced on onions in the laboratory. Artificially-produced sclerotia may lose some of their infectivity after several generations in culture, resulting in a reduction of their ability to germinate (Gerbrandy 1990). This problem is usually minimised by cycling the fungus through a host plant, as done here to produce natural inoculum. Also, one of the three isolates tested in Experiment 1 ('master') was recently isolated from the field and in Experiment 2, three of the five isolates were recently isolated ('master', 'das', 'smeeds') and these new isolates behaved in the same way (showing a gradual breakage in dormancy) as those that had been in culture for some time.

Sclerotia produced in the field are subject to desiccation and subsequent loss of internal contents / microbial contamination (Harper *et al.* in prep.). This may cause field-produced sclerotia to have a far shorter dormancy period than those raised on onions under the controlled temperature and moisture of the laboratory; treatments reported to break dormancy are those that can affect the integrity of the rind e.g. abrasion, temperature changes (Coley-Smith 1960, Gerbrandy 1992). The damage caused by environmental conditions in the field may have the same effect on the rind, thereby increasing the speed with which sclerotia break dormancy.

To investigate this, sclerotia were collected from the field and tested for dormancy. While a percentage of sclerotia (30%, the difference between treatment and control means, Experiment 3) were able to germinate immediately, the remaining sclerotia appeared dormant. This indicates

that sclerotial dormancy is a real phenomenon and not an artifact of culturing methods commonly used to produce inoculum for this type of study. The percentage of sclerotia able to germinate immediately is similar to that seen in the other experiments (11 - 23%, range of mean germination rates observed after 1 month, in Exps. 1 and 2).

Dormancy trials involving field-produced sclerotia are problematical as it is extremely difficult to find a population of similar age to work with or indeed to establish the age of sclerotia in the field. Nonetheless, a group of just-mature sclerotia was sucessfully identified in a commercial field and sclerotial dormancy was observed in them.

The methods used to test for dormancy in Experiments 2 and 3 were the same and the results were very similar. Experiment 3, however, probably involved fewer genetic strains as sclerotia were collected from a small (360 m^{-2}) area within a single field whereas Experiment 2 involved five isolates from separate fields or regions.

3.4.1 Behaviour of controls

In these experiments, some decay and / or germination occurred in samples that had not been treated with DADS. Why was this? Firstly, a number of factors cause sclerotial decay (Chapter 2). Some damage may have occurred to the sclerotia when they were collected from the onions or while they were on the bulb. Some may have been unformed with immature or weak rinds. Mycoparasitism and other predators could account for some loss of sclerotia, or spontaneous germination in the absence of a host plant may have occurred; in one study, low temperatures (5°C) and a rapid temperature change (from 15°C to 5°C) gave high levels of germination in the absence of *Allium* spp. (Gerbrandy 1989). While no large temperature changes occurred during the set-up of the experiments, the literature provides evidence that germination without *Allium* spp. is possible under certain circumstances. All these factors may have contributed to the loss of up to 35% of sclerotial numbers in the

untreated controls (35% = highest mean germination rate recorded in controls in Exps. 1 – 3. Lowest = 0%).

3.4.2 The role of dormancy

The role of sclerotial dormancy is not entirely known but exogenous dormancy seems to be a fail-safe mechanism to prevent germination in adverse environmental conditions. So what benefits does constitutive dormancy have? Conditions can be favourable for germination and infection, yet no germination will occur if constitutive dormancy is active. First of all, dormancy may have no benefit. It may simply be a limitation of sclerotial structure; some time is required before sclerotia are conditioned to allow the perception of signals that trigger germination. However if this were true, selective pressure would surely have eliminated dormancy, yet it is a common occurrence in sclerotial fungi (Dillard *et al.* 1995). A more likely scenario, perhaps, is that staggering germination over a period of time is a survival mechanism (Gerbrandy 1990, Summerville and Hall 1987), allowing some control over what would otherwise be indiscriminate germination in the presence of a suitable host. If, for example, all sclerotia automatically germinated when a host was available and for some reason (e.g. dry weather in mid to late season) conditions did not favour the production of new sclerotia after infection, the result could be a reduction of inoculum numbers in the soil. With a percentage of sclerotia unlikely to germinate for six months or more after they are formed, this possibility is avoided. (For further discussion of the evolutionary significance of dormancy, see Chapter 5).

As a proportion of sclerotia were able to germinate almost immediately, some secondary infections may occur in nature, allowing newly-formed sclerotia to infect the crop in the same growing season. This supports those authors who found that sclerotia of some isolates did not show dormancy, while others had broken dormancy after only 1 month in soil (Brix and Zinkernagel 1992, Coley-Smith *et al.* 1987). Why a small percentage of sclerotia do not show dormancy is unknown. Again, it may

be due to genetic diversity among the isolates or perhaps some of the reasons that explain why untreated sclerotia decay could also explain why some sclerotia do not show dormancy (e.g. a number of sclerotia may suffer damage while maturing on the bulb, resulting in premature germination / breakage of dormancy).

3.4.3 Practical implications

As dormancy was found in all three experiments reported here, it should be considered when applying DADS to prevent disease. With dormancy lasting up to six months in some circumstances, a spring treatment would seem more appropriate than an autumn treatment following an outbreak of white rot. This is in line with overseas recommendations (Coley-Smith *et al.* 1987, Gerbrandy 1990) and should be practical as DADS can be applied while a cover crop is in place without any adverse effects (Tyson *et al.* 2000). It is necessary to delay planting until 8 weeks after the last DADS treatment, to ensure that seedlings are not exposed to a burst of germinating sclerotia (8 weeks after treatment with DADS no germinating sclerotia were seen). These recommendations are compatible with normal commercial practice, as DADS is usually injected into the soil in spring and / or autumn, 2 - 3 months prior to planting (Stewart and Fullerton 1999).

3.5 Conclusions

Dormancy was found to exist in all isolates tested and a conditioning period of 6 months in field soil was required before high rates of germination (>89%) could occur. This is longer than previously reported in the literature. However, dormancy may not last as long as this in the field situation as the structural damage commonly seen in field-produced sclerotia could cause them to germinate more readily than the sclerotia produced for these experiments. In all experiments, dormancy broke gradually with increasing proportions of sclerotia capable of germination

over time. A small proportion of sclerotia were able to germinate as soon as they were formed (30% in Experiment 3), suggesting that some secondary infections can occur in the field. Soil type had no influence on dormancy in this study.

In the field trial, different isolates varied in the duration of dormancy, with the breakage of dormancy becoming statistically significant after the sclerotia were exposed to soil for 3 - 5 months. There were no significant differences between the dormancy requirements of different isolates in laboratory-based trials but this probably reflected the low rates of germination observed when this method was used.

The field-based method was more effective than the laboratory-based technique; there were no problems with contaminating fungi or desiccation and the scoring of germination was more objective and accurate. This was the first study of sclerotial dormancy of *S. cepivorum* in New Zealand.

CHAPTER 4: THE RELATIONSHIP BETWEEN INOCULUM LEVEL AND INCIDENCE OF *ALLIUM* WHITE ROT.

4.1 Introduction

There have been a number of studies on the effect of inoculum density on *Allium* white rot disease (e.g. Abd El-Razik *et al.* 1985, Adams and Papavizas 1971, Crowe *et al.* 1980). In most but not all studies, the number of sclerotia in the soil at time of planting affected the progress of the disease and correlated with disease severity at the end of the growing season (Adams 1981, Adams and Papavizas 1971, Crowe *et al.* 1980). This has been shown for many diseases (e.g. *Sclerotium rolfsii*; Tomasino and Conway 1987, *Sclerotinia sclerotiorum*; Holley and Nelson 1986). Data on inoculum density (and other determining factors) have been used; to both understand the mechanisms behind the development of epidemics and to apply that information to crop protection, as it allows one to predict disease progress and to take appropriate remedial action.

If a model for the effect of inoculum density and other factors on white rot were developed in New Zealand, then management practices such as rotation systems and spraying regimes could be adapted accordingly and informed decisions made about the suitability of land for onion cultivation. Such models are created for a particular area or soil type, as climatic variation means that they are often only effective in the region for which they were formed.

4.1.1 Background to methods

There are several methods used to determine inoculum levels for soil-borne fungi and assess the impact on subsequent disease development. The first involves removing soil samples from the field and then assessing the inoculum density, in this case the number of sclerotia

present. After a crop is sown, disease incidence and yield in that crop are measured and correlated with pre-plant inoculum densities. Fields representing a range of inoculum densities and environmental conditions are used (Adams 1981).

The disadvantages of this method are that the inoculum densities may not be uniform and that plots of differing densities cannot often be chosen randomly (Johnson 1993). This can be overcome by repeating the experiment in the same area and / or over a number of years.

A second method involves the artificial creation of plots or soil boxes of known inoculum levels, in which plants are sown and disease levels monitored as above. Fumigation may be used to produce areas of low inoculum density (Johnson 1993). If required, inoculum can be introduced at various times during a growing season (Johnson 1993). If the inoculum is artificially produced, it must have similar activity and infective quality to naturally-produced inoculum.

The advantages of this method are that it is reproducible and the densities produced are more uniform than those natural densities used in field-based experiments. However, fumigation may affect other organisms and so indirectly affect the disease. There is no literature on the use of fumigation to alter numbers of sclerotia of *S. cepivorum* in this way.

Other techniques include the use of fungicides to inhibit or prevent disease and cultivars that are genetically identical except for their resistance to the disease (James and Teng 1979, Johnson 1993, Sumner *et al.* 1985). The first may be applicable but is not generally used (Johnson 1993) and has not been tried with onion white rot. The second is impossible as, so far, no onion cultivars have shown resistance to white rot (Adams and Papavizas 1971, Bansal and Broadhurst 1992,

Esler and Coley-Smith 1984), except a few lines in Canada, with only low levels of disease resistance (Stewart and Fullerton 1999).

4.1.2 Previous work on *S. cepivorum*

An early experiment on the relationship between inoculum density and incidence of white rot (Adams and Papavizas 1971) used soil artificially infested with known levels of inoculum. The number of plants infected depended in part on the isolate concerned but the authors reported a positive correlation between inoculum density and incidence (Adams and Papavizas 1971). Unusually high numbers of sclerotia were needed to give high rates of infection; most isolates caused 75% infection at 25 sclerotia g^{-1} of soil. The high numbers of sclerotia may have been needed because the sclerotia had been buried without conditioning and, therefore, some may have been dormant over the 4 week period during which incidence was recorded.

Natural inoculum levels have been found to be in the range 0.001 - 7 sclerotia g^{-1} of air-dried soil (Abd El-Razik *et al.* 1985, Adams 1981, Crowe *et al.* 1980, Utkhede *et al.* 1978). Such levels were accompanied by a wide range of disease incidence (0 – 100%) (Abd El-Razik *et al.* 1985, Adams 1981, Crowe *et al.* 1980). Work conducted under field conditions generally showed a positive relationship between levels of inoculum and disease incidence; increasing inoculum density resulted in earlier onset and greater incidence of disease (Adams 1981, Crowe *et al.* 1980). The term 'disease onset' is used here and elsewhere in this chapter to describe when the disease becomes detectable (usually when approximately 5% of the crop is infected). Other authors, however, reported no correlation (Utkhede *et al.* 1978) and observed white rot in fields where no viable sclerotia had been found.

Studies involving inoculum density are complicated by the fact that inoculum levels in the field are not uniform. This fact and spread of fungus from plant to plant along a row (Entwistle and Munasinghe 1977,

Scott 1956a and b) gives the disease a patchy distribution (Adams 1981, Crowe *et al.* 1980).

Aim

The aim of this work was to describe the relationship between inoculum density (and other factors) on progress and incidence of onion white rot disease in the Pukekohe area, South Auckland.

4.2 Methods

4.2.1 Method and analysis: field trials

The purpose of these experiments was to assess inoculum levels in field soil and attempt to correlate them with disease progression. Eleven fields trials were run in total, over three years. Inoculum densities were determined in commercial fields at Pukekohe. The level of inoculum was recorded in fields that were then planted with onions in the following season. The fields were chosen as far as possible to represent different inoculum densities using aerial photographs and disease histories provided by growers. The photographs show plants suffering from white rot as blue in colour or in extreme cases as bare patches in the field, see Figure 12. For obvious reasons, it was unlikely that commercial growers would plant areas with a history of high disease incidence into onions. Some land, however, was made available to Lincoln University in the 1998 / 99 growing season and zones of high inoculum density within the block were used.

4.2.1.1 Collection of soil samples

Using a trowel, soil samples were taken from the upper 10 cm of the soil (the approximate ploughing depth is 15 cm) as numbers of sclerotia have been found to be restricted to and uniformly distributed within this layer (Adams 1981). A soil corer was trialed as a means of soil sampling but proved impractical as the soil has a high clay content and was often water-logged, causing it to compact at the end of the corer. Soil sampling was done after bedding-up; before seedling emergence or when plants were at the 'hook' stage (Brewster 1994) in July and August.

Figure courtesy of A. Stewart. Bare areas in the field represent total crop loss to white rot.

Figure 12. Aerial view of field affected by white rot.

Figure 13. Soil sampling at a trial site.

Inoculum density was determined in each field, from a sampling area of 360 m^{-2}. Samples were taken from each of 24 quadrats within every 360 m^{-2} sampling area. From these 24 quadrats (each 1.5 m^{-2}), eight soil samples were removed and combined. The eight sub-samples were collected from along the length of each quadrat, around the central four rows of onions (there were eight or ten rows of plants running the length of each bed, depending on the type of seeding equipment used by individual growers). The 1.5 m^{-2} quadrats were set out along the beds of onions, with a buffer area of 1 m between them. The 360 m^{-2} plots were 11 beds wide (around 18 m, the width of a spray track) and 20 m long. Quadrats were not placed on the central three beds or the beds on the extreme edge of the plot; the tractor etc would drive over the middle bed during the course of routine crop maintenance and would have disturbed the soil and thereby perhaps the uniformity of the inoculum density in those central beds. The beds at the extreme edges acted as a buffer zone.

4.2.1.2 Processing soil samples

Following collection, soil samples from each 1.5 m^{-2} quadrat were processed separately as follows; the soil was air-dried for 12 h or until its weight had equilibrated. Fifty grams or 100 g of soil per quadrat were then removed from the combined sample (50 g per quadrat in the first year, and 100 g per quadrat in the following two years), soaked in water for 10 min (to soften lumps of dried soil) and washed through a stack of progressively finer sieves (sieves from Endecotts Ltd., London, England) for at least 5 min (Adams 1981, Crowe *et al.* 1980). The sieves ranged from 1.7 mm to 250 µm in pore size. Gentle pressure from the fingertips was required to work clay particles though the sieves. The residue from the sieves with pore size between 250 µm and 500 µm (the size of sclerotia of *S. cepivorum*) was washed onto filter paper and allowed to dry for 1 h at room temperature.

After wet-sieving, a large number of soil particles remained which appeared identical to sclerotia; they were dark-coloured, round and in the same size range. None of the existing methods were successful in removing these particles (wet-sieving, flotation in sucrose solution, dilution plate techniques, bleaching or a combination of the above, Adams 1989, McCain 1967, Papavizas 1972, Utkhede and Rahe 1979), so a novel technique was developed (Harper and Stewart 2000). The particles that resembled sclerotia consisted of concretions of clay containing haematite, a black iron oxide (Bakker *et al.* 1996). The magnetic properties of haematite were used to remove these particles as detailed below (Harper and Stewart 2000).

Once wet-sieved and dried, the residue was then run through a magnetic separator (at 1 amp current), of the type used for mineral separation (model L-1, S.G. Frantz Co. Inc., New Jersey, U.S.A.), to remove the soil concretions. The sclerotia were recovered with forceps from the non-magnetic, light-coloured fraction (Figures 15 and 16) that consisted of organic material and quartz.

The effectiveness of the 'magnetic separation' method was tested in a pilot trial. When soil samples were seeded with known numbers of sclerotia (5 - 25 sclerotia per sample), the method described resulted in 100% recovery of sclerotia in all cases. The recovered sclerotia were all viable, (as determined by the method described in Chapter 2).

Five fields were sampled in this way over the 1998 / 1999 season. The number of fields was reduced to three in subsequent seasons, to allow more soil per quadrat to be sampled (100 g instead of 50 g). More soil was required to provide greater detail of local variations in inoculum level. Inoculum density was expressed as the number of viable sclerotia g^{-1} air-dried soil.

4.2.1.3 Recording disease incidence

Once pre-plant inoculum density was recorded as above, onions were treated according to normal commercial practice. However, growers were asked to avoid the use of foliar sprays against white rot in the trial plots during the growing season as their application would have affected incidence and thereby confused the relationship between inoculum density and disease progression. Sumisclex seed dressings (5 g procymidone kg^{-1} seed) were tolerated as it was impossible to exclude them from the trial areas, due to the limitations of commercial seeding equipment and there was evidence that they no longer provided protection against white rot (Stewart and Fullerton 1999).

From early spring to harvest (August or September to December / January), the incidence of disease was recorded monthly by scoring numbers of diseased onions growing in the same 1.5 m^{-2} areas within which inoculum density had been recorded. Disease incidence was recorded in the central four rows of plants. The same plants were used in all subsequent sampling of incidence (approximately 80 -120 plants per quadrat). The growth stage of the plant was also noted (Brewster 1994). Numbers of diseased plants in each quadrat were converted to a percentage; these were then used in the analyses. The identity of *S. cepivorum* was from time to time confirmed by culturing the fungus from sclerotia collected from diseased plants. A 'Tinitag' datalogger (Gemini Loggers, U.K.) recorded temperature at 10 cm depth in the plots, every 3 h (for soil temperatures, see Figures 27 – 29 in appendix). Rainfall was also recorded, using an 'Enviroscan' (Sentek Pty Ltd, Kent Town, Australia, model RT6, supplied and monitored by Agriculture New Zealand Ltd.), which calculated soil moisture at 10 – 50 cm depth, using the ability of damp soil to conduct an electrical impulse (for Enviroscan printouts, see Figures 24 – 26 in appendix).

4.2.1.4 Analysis

The experiment was run over three seasons (starting June 1998, finishing December 2000). The relationship between inoculum density and disease progress was illustrated using curve-fitting techniques. After testing different models of disease progress, the gompertz equation was chosen for curve-fitting in 1998 and 1999. Exponential, log and linear models were also tested in 2000. The gompertz model is one of the sigmoid curves, commonly used to describe plant growth and disease progress and has been sucessfully used to model disease progress in a number of studies (Berger 1981, Viljanen-Rollinson *et al.* 1998).

The principle difference between the gompertz and the widely known logarithmic model is that the gompertz produces an asymmetric rather than a symmetric curve; a plot of the daily increase in disease would be skewed to the right (Berger 1981). It is described by the following; Y = a*exp(-exp(b-c*time)) where 'a' is the asymptotic disease incidence and 'Y' is disease incidence at any time (Berger 1981).

A number of parameters derived from the gompertz curve were correlated with inoculum density. There were TMR (time to maximum rate of disease increase), MR (maximum rate of disease increase), LI (level of disease at curve's point of inflection) and the asymptote (final level of disease incidence). These were calculated as follows; TMR = Ln(b)/c, MR = a*c/e, LI = a/e where a, b and c refer to the gompertz equation above and e is a constant (e = 2.71). 'B' and 'c' are shape parameters, used to derive LI and MR.

Data for analysis were split into categories based on the inoculum level in the soil at the beginning of the growing season. Decisions on where to set the cut-off points between the inoculum classed (e.g. between 'low' and 'moderate') were sometimes difficult and a number of different cut-offs were initially made. Those finally chosen achieved the best

disjuncture between the groups and allowed for roughly similar sample sizes within each inoculum class.

4.2.2 Method and analysis: glasshouse trial

The aim of this experiment was to determine the level of disease (as represented by mean percentage of infected plants) in soil of known inoculum densities, under conditions ideal for the disease. It was designed to provide a comparison with data from the field trials outlined above.

Sclerotia raised on wheat were used to inoculate soil at concentrations of 0.001, 0.01, 0.1, 1 and 10 sclerotia g^{-1} of air-dry soil; these concentrations reflect those found in the field. To produce sclerotia, 50 g of whole wheat was soaked in 45 ml water amended with 50 mg ml^{-1} chloramphenicol in 250 ml flasks overnight, then autoclaved at 121°C for 30 min on each of 2 successive days (Alexander and Stewart 1994). The flasks were each inoculated with four 5 mm disks from a 7 day old culture grown on PDA and incubated in the dark at 20°C for 6 weeks. At this stage, the sclerotia were collected by wet-sieving, contained in mesh bags and buried in soil (as described in Chapter 2). In this way, sclerotia were conditioned by a period of 12 months in soil before use in this experiment, to ensure that dormancy had broken and they were capable of infecting plants.

After conditioning, the sclerotia used to inoculate the soil were recovered from the mesh bags by progressive wet-sieving, collected from the 250 µm sieve, air-dried for 1 h and 10 x 1 g lots of the residue were weighed out and the numbers of sclerotia present in each of those 1 g lots were counted and averaged. This indicated the weight of inoculum needed to augment the soil to create the higher densities. The lower densities were made up by individually counting out the necessary number of sclerotia. Approximately equal numbers of sclerotia from each of three isolates of *S. cepivorum* were used to inoculate the soil. All isolates originated from the Pukekohe area ('sc3', 'sc5', 'sc10'). To ensure that the levels of

inoculum in the various treatments were correct, samples of the soil were wet-sieved and the sclerotia counted.

The infested soil was contained in pots (10 cm diameter and 15 cm deep), 2:1 with bedding mix (Southhort Ltd, Christchurch) and onion seed sown (approximately 12 seeds per pot). The seed was treated with 'Thiram 80W' (active ingredient thiram, 800 g kg^{-1} seed) to prevent 'damping off' disease. The pots were set out in the glasshouse using a randomised plot design (see Figure 15, set up of glasshouse trial). Once the seedlings emerged, they were thinned to four per pot. Incidence (numbers showing symptoms of white rot) was recorded weekly for a minimum of 10 (and up to 16) weeks, after which time the seedlings were forming bulbs.

Figure 14. Glasshouse trial.

There were 12 replications of 4 plants in each treatment (48 plants per treatment). Control pots with uninfested soil / bedding mix were included. The pots were incubated in a climate-controlled glasshouse where the average temperature was 20°C. Growth cabinets could have provided more stable temperature conditions but a glasshouse was used because it had the advantage of natural light conditions and the temperatures were close to those deemed optimum for disease development (15 - 20°C), except in mid-summer months (January – February). Unnatural light conditions in the growth cabinets would probably have prevented the onions from developing normally and this could then have affected the interaction between plant and disease to confuse the results.

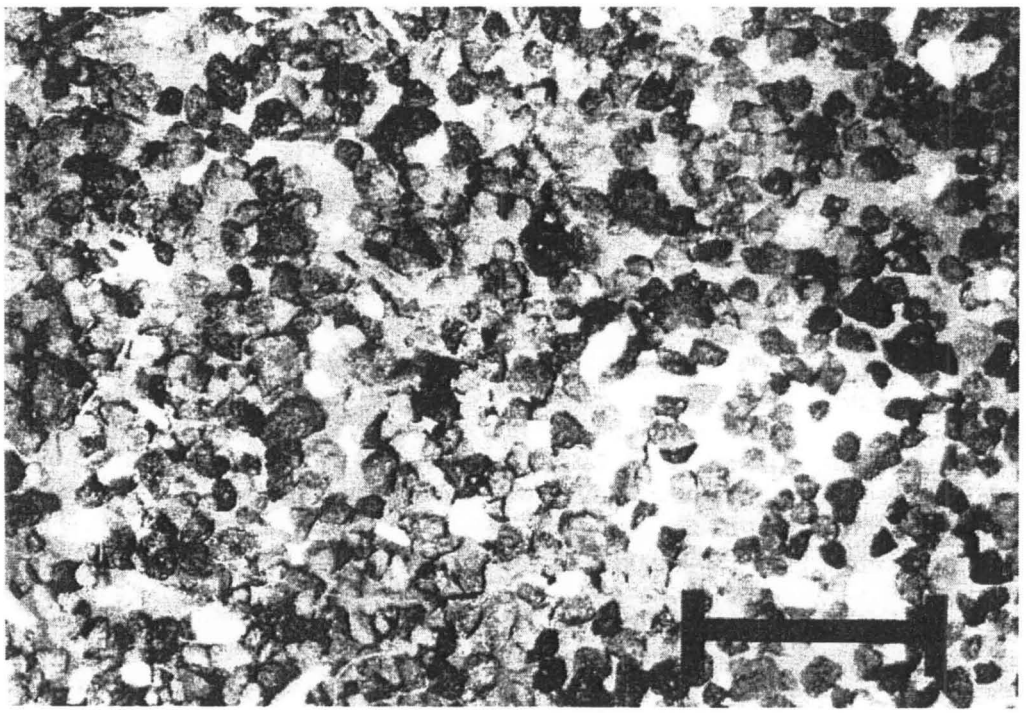
The soil, into which onions were planted, should ideally have been Patumahoe clay loam from Pukekohe, to allow comparison with the field trials. However, this was not available, so field soil from the Lincoln region (Templeton and Lismore silt loams, Cox 1978) was used. This soil contained no sclerotia and had no history of white rot. This experiment

was repeated three times: February – April 1999, August – October 1999, November 1999 – February 2000.

4.3 Results

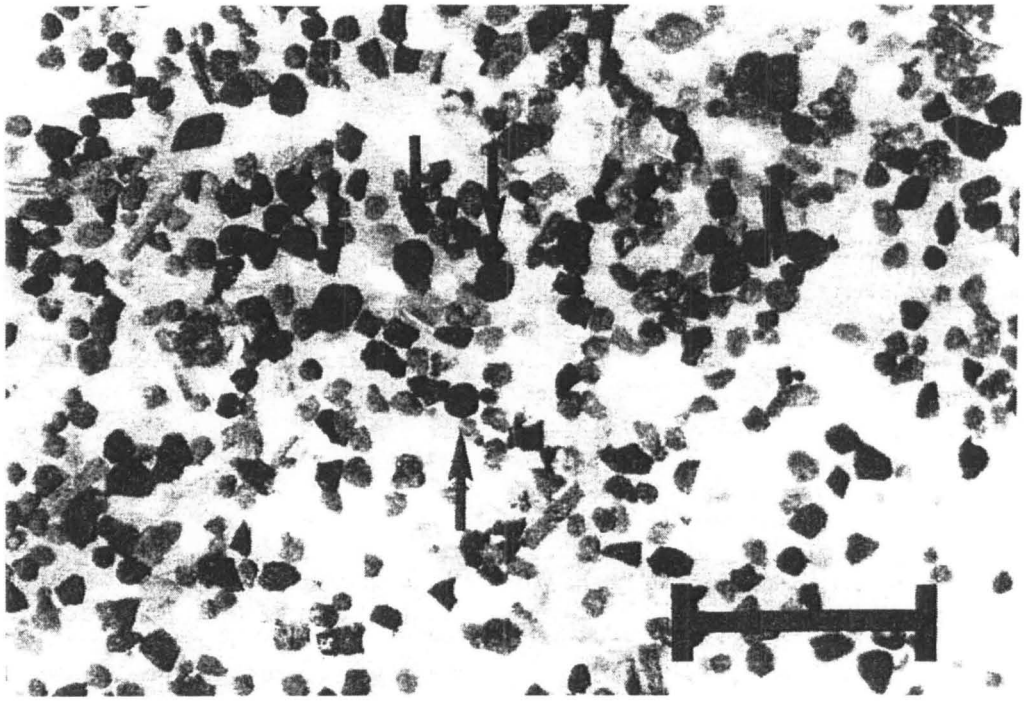
4.3.1 Methodology

A novel method was developed during the course of the field trials that enabled the number of sclerotia present in field soil to be determined (Harper and Stewart 2000). This technique used the magnetic properties of Patumahoe clay loam to remove concretions of clay from soil samples after wet-sieving. These clay particles strongly resembled sclerotia and had to be removed before one could pick out and count sclerotia. Figures 15 and 16 show a soil sample (100 g) before and after magnetic separation.



Soil sample immediately prior to magnetic separation. Sclerotia present in the sample cannot be distinguished from soil particles. Scale bar = 3 mm.

Figure 15. Sample before magnetic separation.



Soil sample after magnetic separation. Four sclerotia are arrowed, appearing as round, black objects, which may easily be picked out with forceps. Scale bar = 3 mm.

Figure 16. Sample after magnetic separation.

4.3.2 Field trials

Inoculum density was determined shortly after planting and then disease incidence recorded over the growing season, until as near harvest as possible. At the end of each trial, plants were at growth stage 8 or 9, with bulbs formed and upper parts bending under their own weight (8) or the neck soft and upper parts collapsing (9) (comparable with stages H and I, Brewster 1994). In any given year, fields could be planted several weeks apart. Therefore, incidence of white rot is shown relative to both calendar date (Figures 17, 22 and 23 below) and plant growth stage (Figures 31a–31c, see appendix). Both sets of curves are very similar.

4.3.2.1 Year one

Trials were run in five fields; 'bollard', 'makan', 'smeeds' 'jivan' and 'master' (see Figure 30 in appendix for location of trial sites). Inoculum levels (mean for each field) were as follows, in order of decreasing

inoculum density; 0.103 (bollard), 0.047 (makan), 0.038 (smeeds), 0.026 (jivan) and 0.020 sclerotia g^{-1} dry soil (master). Of these, bollard and makan were considered to have high mean inoculum density (≥ 0.04 sclerotia g^{-1} soil) while the rest fell into the 'moderate' category (0.02 – 0.04 sclerotia g^{-1} soil). Cumulative disease levels in these fields over the 1998 / 99 growing season are shown in Figure 17 (standard error shown).

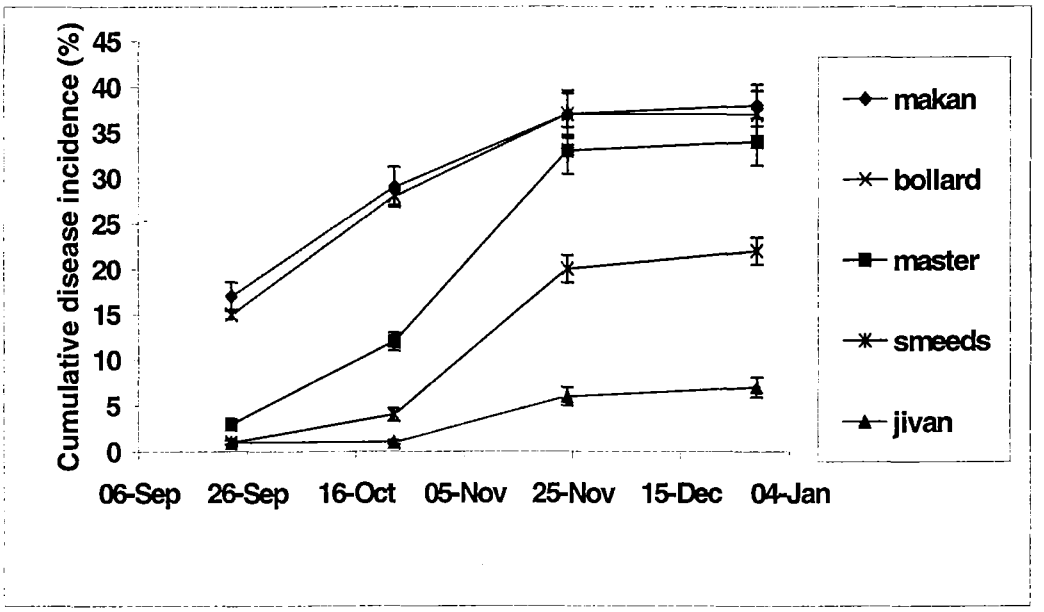


Figure 17. Disease progress in five fields, 1998 / 99.

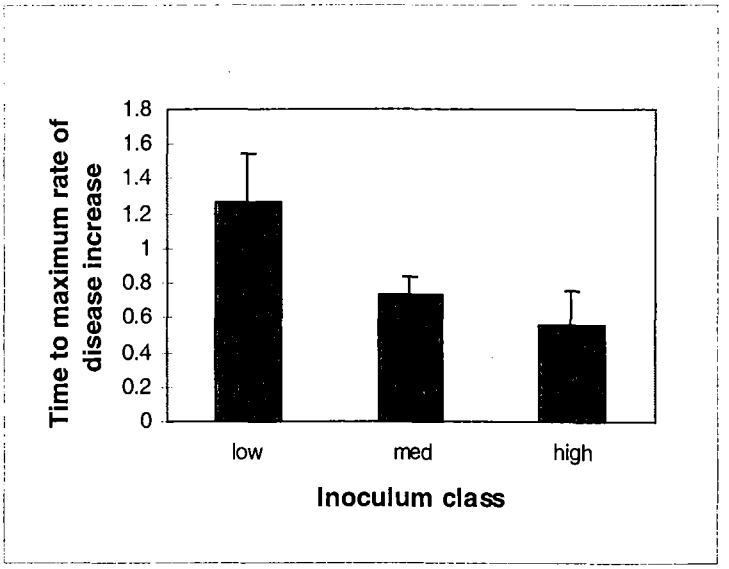
In the 1998 / 1999 season, the disease began relatively early in two of the five fields (those with the highest inoculum levels, makan and bollard). By September 1998, when the first measure of disease was taken, the disease had progressed past the lag phase of the disease progress curve. An entire gompertz curve could not therefore be fitted to data from these fields. Instead, they were fitted to a curve based on the gompertz model but without the lag phase. This curve is described here as the 'log model'. In subsequent seasons, the disease was recorded from one month earlier than in year one, to avoid missing the initial part of the curve.

A number of parameters, derived from the gompertz and log models, were correlated with inoculum density in the soil at planting. A positive

correlation was observed in year one, with increasing inoculum levels resulting in an earlier onset of disease (as measured by TMR, time to maximum rate of disease increase, see Figure 18). In those quadrats with a 'low' inoculum level, TMR was delayed by approximately 4 weeks, compared to those with 'moderate' or 'high' levels.

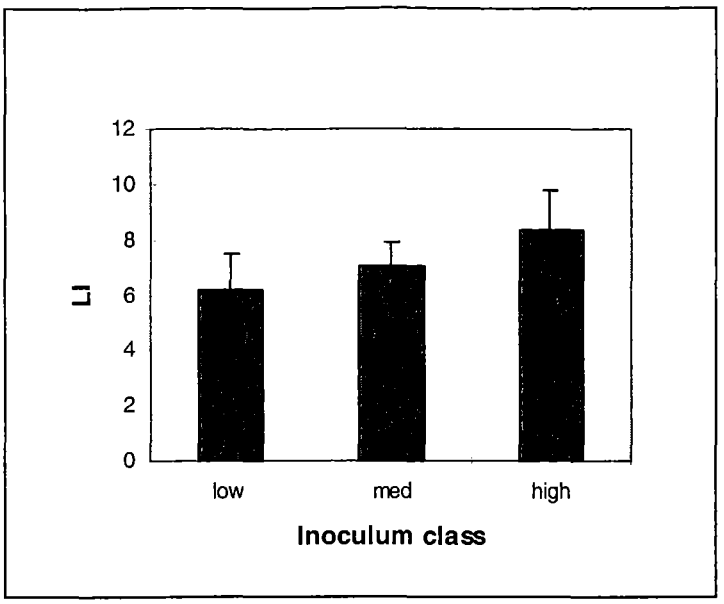
A positive trend was also observed between inoculum level and the point of inflection of the curve (LI, see Figure 19) and maximum rate of disease increase (MR, Figure 20), but these trends were not statistically significant. There was no obvious relationship between inoculum density and cumulative disease at the end of the growing season (Figure 21).

In the figures below (Figures 18 - 21) 'low' inoculum levels were classed as those soils with 0 or 1 sclerotium per 50 g of dry soil. 'Moderate' soils contained 2 - 4 sclerotia per 50 g soil and 'high' contained 5 - 10 sclerotia per sample.



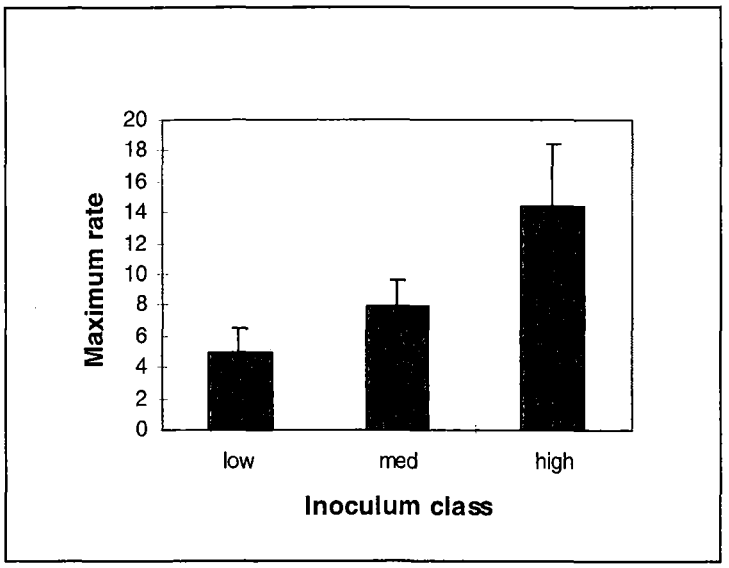
In quadrats with a low inoculum density, the disease required significantly more time to develop than in moderate or high inoculum areas. Data has been log transformed. Standard error shown. Y axis shows log of time (weeks into growing season).

Figure 18. Relationship between inoculum class and time to maximum rate of disease increase (TMR), 1998/99 season.



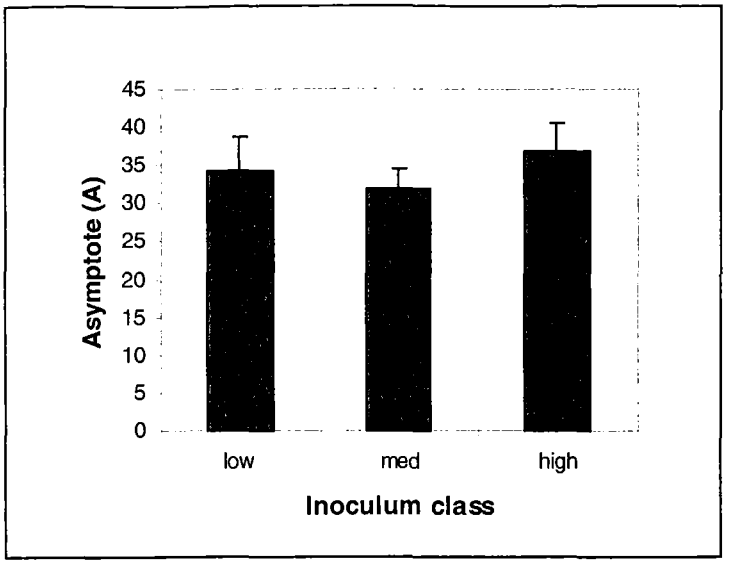
The following trend was observed; the higher the inoculum level, the higher the central point of the disease progress curve (LI). However, this was not statistically significant. Standard error shown. Y axis shows % disease incidence.

Figure 19. Relationship between inoculum class and point of inflection (LI), 1998/99 season.



The relationship between inoculum level and the speed with which the disease increased (maximum rate or MR). A positive, but not statistically significant, difference was observed. Standard error shown. Y axis shows % disease incidence / time.

Figure 20. Relationship between inoculum class and maximum rate of disease increase (MR), 1998/99 season.



There is no clear relationship between inoculum and final disease outcome; the bars do not increase steadily from left to right. Standard error shown. Y axis shows % disease incidence.

Figure 21. Relationship between inoculum class and disease at harvest, 1998/99 season.

Tests were performed to determine how closely the data followed the gompertz and log curves. In most quadrats (89%), the disease closely matched one of the predicted curves (gompertz or log). Of the 57% of quadrats fitted to the gompertz model, the mean corrected R^2 was 0.959 (±0.018, standard deviation). Of the quadrats fitted to the log model (32% of the total number), mean corrected R^2 was 0.999 (±0.045 standard deviation). Curves could not be fitted to the remaining 11% of quadrats.

4.3.2.2 Year two

Trial sites were set up in three fields. Inoculum densities (field means) were 0.016 (young), 0.007 (das) and 0.003 sclerotia g^{-1} soil (mcm). These inoculum levels were lower than those available in the previous year. The numbers of sclerotia in 'das' and 'mcm', in particular, were considered extremely low. Disease progression curves are shown in Figure 22 (standard error shown). When curve-fitting was performed, only 26% of quadrats fitted the gompertz model (corrected $R^2 > 0.5$). In the

remaining 74% of quadrats, no curve could be fitted, due to the consistently low levels of disease observed.

Three inoculum classes were created from data from the 1999 / 00 season; there were no sclerotia in the first class (n = 38), one sclerotium in the second (n = 20), and 2 or more in the third (n = 12). When inoculum density was correlated with a number of parameters, including cumulative disease incidence at the end of the season (expressed as the asymptote of the disease progress curve), the results were not statistically significant; p-values were 0.317 (asymptote), 0.175 (MR), 0.317 (LI) and 0.087 (TMR).

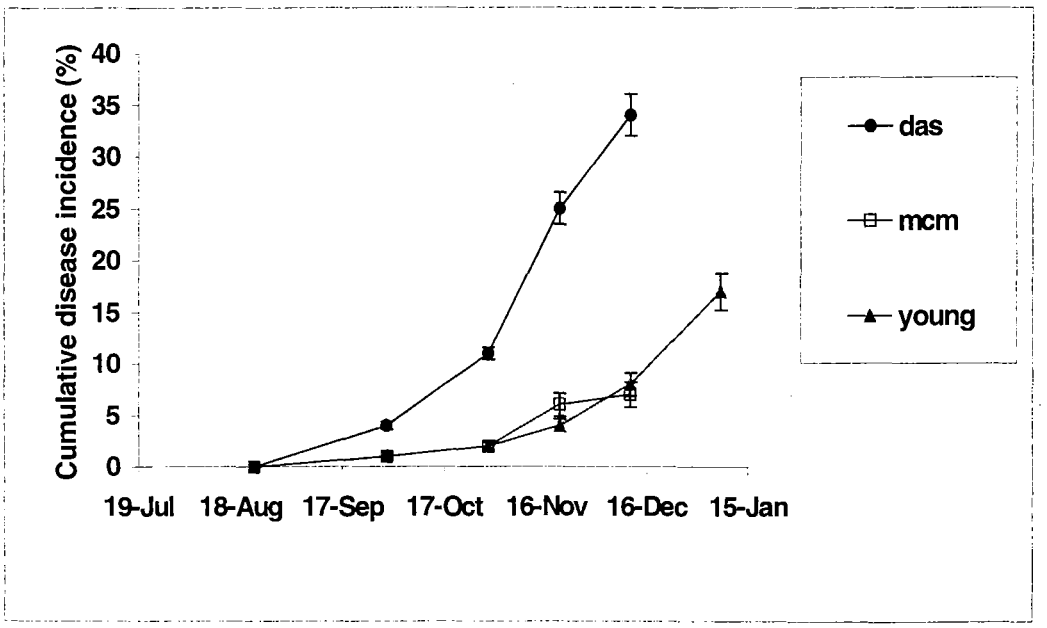


Figure 22. Disease progress curves in three fields, 1999 / 00.

4.3.2.3 Year three

Three field trials were run in 2000; 'makan2', 'master2' and 'union'. These first two fields were also used in the first year of trials. They contained 'moderate' levels of inoculum with 0.025 and 0.022 sclerotia g^{-1} soil respectively (field means, 'moderate' represents 0.02 – 0.04 sclerotia g^{-1} soil). In 'union', the soil contained extremely low numbers of sclerotia in the soil (0.001 sclerotia g^{-1}) and little or no white rot was observed in the

quadrats; for this reason data from this field were excluded from the statistical analysis. Disease progression curves are shown in Figure 23 (standard error shown).

In 2000, the soil temperatures were not consistently in the optimum range for disease development (13 - 18°C, Adams and Papavizas 1971, Stewart and Fullerton 1999) until early October (see Figure 29, appendix), 2 - 6 weeks later than in 1998 and 1999. Temperatures remained in the optimum range into December. The more usual pattern in Pukekohe is that temperatures reach the optimum range in September and then fall outside the range in October or early November (Fullerton *et al.* 1994, Stewart and Fullerton 1999), as occurred in 1998 and 1999.

In 2000, the disease did not begin to build up sharply until October and cumulative disease incidence kept increasing until the end of the season (Figure 23). This pattern was not the same as observed in the previous two years, where the number of new infections declined in December (Figures 17 and 22). As a result, the gompertz model did not fit the disease progression curves and consequently inflexion and asymptotic levels could not be estimated. Linear, log and exponential models were therefore also tested.

In 'makan2', 10 out of the 24 quadrats matched the exponential pattern (correlation coefficients were highest with this model), 10 out of 24 were fitted to the log and 4 out of 24 to the linear model. In 'master2', quadrats were fitted as follows; 5/24 exponential, 8/24 log, 11/24 linear. Mean raw R^2 of quadrats fitted to exponential model was 0.995 (0.012 standard deviation, n = 15). Mean raw R^2 of quadrats fitted to the log model was 0.992 (0.014 standard deviation, n = 18) and for those fitted to the linear model, mean R^2 was 0.998 (0.003 standard deviation, n = 15).

Three inoculum classes were formed with the data from two out of three fields (those with sufficient inoculum for analysis, makan2 and master2),

based on the number of sclerotia found in each quadrat; those with 0 or 1 sclerotia per soil sample (n = 16), those with 2 or 3 (n = 23) and those with 4, 5 or 6 sclerotia per sample (n = 9). Then the analysis was repeated with two inoculum classes (0,1,2 and 3,4,5,6 sclerotia, n = 26, n = 22 respectively). Disease incidence at harvest did not differ significantly between the inoculum density groups ($p > 0.05$).

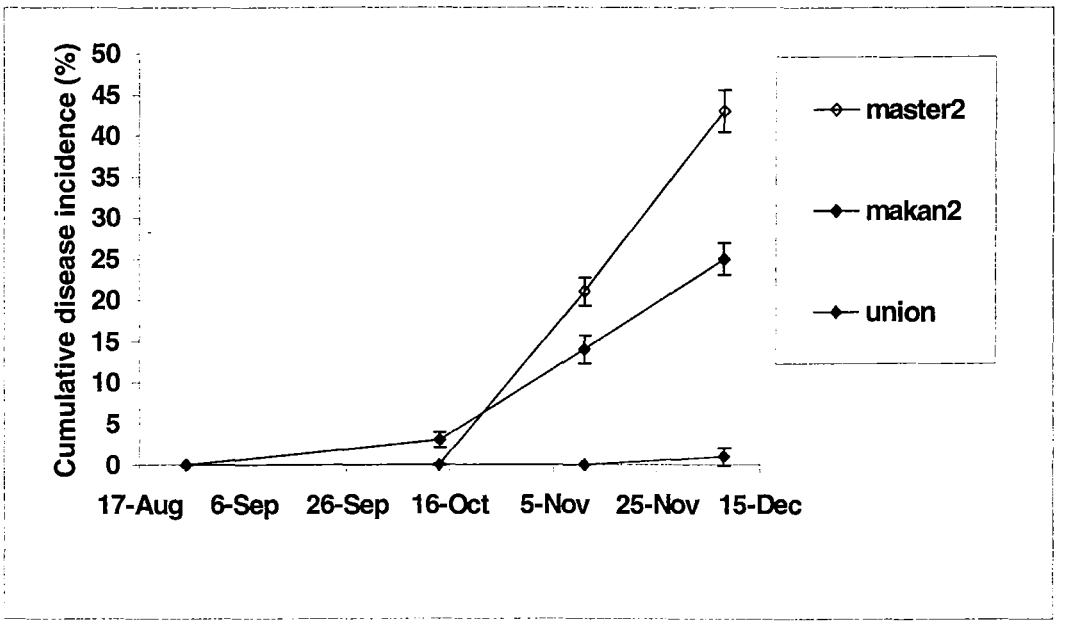


Figure 23. Disease progress curves in three fields, 2000.

4.3.2.4 Combined analysis

A combined ANOVA analysis of data from all three years was not performed as the levels of inoculum or weather / disease progression differed in each of these years, making it impractical to compare them. Gompertz curves could not be fitted to all data or to data collected in all three seasons; therefore it was not possible to compare inoculum density with TMR, MR etc in all years.

4.3.3 Glasshouse trial

This experiment was repeated three times. During all three trials, there was little or no white rot in all treatments. The maximum disease

incidence observed was 2%, in one treatment (that with the highest inoculum density) during the third experiment.

4.4 Discussion

4.4.1 Field trials

Inoculum levels in the quadrats ranged from 0 – 0.2 sclerotia g^{1-} soil, while disease incidence at harvest in the same areas was 0 – 74%. These inoculum levels are similar to those reported in the United States (Adams 1981, Crowe *et al.* 1980), and Canada (Utkhede *et al.* 1978) but considerably lower than those observed in Egypt (Abd El-Razik *et al.* 1985) where 0 - 7 sclerotia g^{-1} soil were found. The high level of inoculum required to produce peak disease frequency in Egypt (1.8 – 3.5 sclerotia g^{-1} soil required for 90 - 100% infection) was attributed to low virulence of the pathogen, the low susceptibility of onion cultivars and climate.

Inoculum density was extremely patchy and this was most pronounced in fields with a short history of the disease. It appeared that, where the disease had been present for some time, the inoculum level was more uniform (and the intensity of disease was greater) compared to fields with a relatively recent history of white rot. This supports other authors who suggest that uniformity of inoculum in the soil is an important determining factor in the severity of the disease (Crowe *et al.* 1980). This is probably because, as numbers of sclerotia in the soil are low, an even distribution of them makes it much more likely that successful infections will occur, compared to fields where the average inoculum level may be similar, but the sclerotia are concentrated in a few extremely small areas.

Patchy, low inoculum levels make an accurate assessment of sclerotial numbers difficult. Increasing the intensity of soil sampling can combat this; the level of sampling used in the present study was considerably in

excess of that reported in the literature (Abd El-Ravik *et al.* 1985, Adams 1981, Crowe *et al.* 1980, Utkhede *et al.* 1978).

In year one, there was a correlation between increasing inoculum density and an earlier onset of white rot (as determined by TMR, time to maximum rate of disease increase). This supports Crowe *et al.* 1980, who found that increasing inoculum density resulted in an earlier onset of disease symptoms, in addition to earlier appearance of disease foci and enhanced disease severity. There was, however, no similar relationship between inoculum level and other parameters (MR, LI) and there was no obvious association between TMR and inoculum density in subsequent trials. This was probably because in year two, inoculum levels were so low that no comparison with soils of higher inoculum content was possible that year, while in year three a different pattern of disease development was observed to that of previous seasons; the rate of new infections continued to rise until the end of the season, instead of flattening off from November onwards.

Although inoculum level may sometimes determine the time of disease onset (as seen in year one), the relationship between inoculum level and cumulative disease incidence at harvest was not statistically significant in any of the three years. There are a number of possible reasons to explain this. The first is that inoculum density does not affect disease severity. This seems highly unlikely in view of the fact that most of the literature on white rot reports a positive correlation between the two (e.g. Abd El-Ravik *et al.* 1985, Adams 1981, Adams and Papavizas 1971, Crowe *et al.* 1980), a rare exception being Utkhede *et al.* 1978. With the inoculum levels being generally very low in terms of numbers of propagules per unit soil, it would seem logical that increasing inoculum levels result in increasing rates of infection, at least in the early stages of an epidemic, before secondary infections (spread between plants) become more important.

It could also be that this study was too small to show a relationship between inoculum density and final disease outcome, even if one were present. This question can only be answered by more research. A further option is that the inoculum levels in Pukekohe soils are too low to show any correlation; previous studies have reported that sclerotial numbers similar to those at Pukekohe were accompanied by disease incidence of 10 - 80% (Crowe *et al.* 1980), too broad a range to be predictive. Also, the work that showed the strongest correlation between inoculum and disease levels (Abd El-Ravik *et al.* 1985) involved much higher inoculum densities than seen here (up to 3.5 compared to a maximum of 0.2 sclerotia g^{-1} soil). However, some other studies with comparable inoculum densities to those in Pukekohe have shown a positive correlation (Adams 1981, Crowe *et al.* 1980).

The most likely explanation is probably that inoculum density determines the time of disease onset at Pukekohe, but the later progression of the disease is determined by other, mainly environmental, factors. The observations made in 1998 seem to support this hypothesis (TMR was the only parameter that correlated with inoculum density).

Fields with similar inoculum densities sometimes had different disease outcomes (e.g. das and young, 1999 / 00, see Figure 22). Variability in the effect of inoculum density on incidence of white rot has been reported in previous studies (Crowe *et al.* 1980, Entwistle 1990b) and attributed to unfavourable conditions for sclerotial germination, either because of high soil temperatures (which can reduce rates of germination, Gerbrandy 1992) or because germination stimulants were not produced or retained in soil (Entwistle 1990b).

So if inoculum density has only a limited effect on disease progress, what other factors may drive an epidemic? Rainfall has no significant effect, according to long-term trials conducted in the Pukekohe region (Fullerton *et al.* 1994, Stewart and Fullerton 1999). Rainfall (supplemented by

irrigation) in 1998 – 2000 was consistent throughout the season with only short periods of dry weather (less than a week) occurring in November and December (1998 and 1999 seasons, see Figures 24 – 26 in appendix), when plants were under water-stress. Therefore, in these trials there was sufficient rainfall for soil moisture not to be a limiting factor in disease development. A minimum amount of moisture is almost certainly required for successful disease development, as dry conditions can adversely affect sclerotial survival (and presumably infection of host plants) (Leggett and Rahe 1985 and results in Chapter 2) but the rainfall experienced in Pukekohe must more than meet this requirement, as there has been no reported association between dry weather and a reduction in the rate of new infections (Fullerton *et al.* 1994, Stewart and Fullerton 1999).

Soil temperature has been correlated with an increase in disease incidence (Fullerton *et al.* 1994, Stewart and Fullerton 1999) and unusually late white rot epidemics in the mid 1990s were attributed to the fact that soil temperatures did not reach the optimum range for disease development (13 - 18°C, Adams and Papavizas 1971, Fullerton *et al.* 1994) until October, one month later than in most other years. During 1998 and 1999 seasons, soil temperature followed the most common pattern (Fullerton *et al.* 1994), reaching the optimum range in late August / September and falling outside the range in late October / early November, after which time the number of new infections began to decrease. In the 2000 season, the soil temperatures and pattern of disease increase were similar to those seen in the late-season outbreak of 1992 / 93. This would argue that soil temperature is a major factor controlling disease progression, once the limitations of an inoculum source and sufficient rainfall are met.

The peak in the rate of new infections occurred at various times during the season, seemingly dependant on both inoculum level and soil temperature; in the two fields with the highest average inoculum loads

('makan' and 'master', 1998 / 99) most infections occurred between the third week of September and the third week of October. Most fields, those with moderate levels of inoculum, showed highest number of new infections between the third week of October and the end of November (in 1998 / 99 and 1999 / 00, the most common pattern of disease development, Fullerton *et al.* 1994). Perhaps, as suggested earlier, inoculum density may affect disease progress early in the season (including when the maximum rate of new infections occurs) while the later progression of the disease (including percentage disease incidence at harvest) may be determined by other, mainly environmental factors. In 2000, when soil temperatures did not enter the optimum range until October but remained there through November, there were approximately equal numbers of new infections from the third week of October until mid December, when the trials were finished.

The peak in the rate of new infections (October / November) coincided with plant growth stages 6 – 8, (stage 7 = bulbing, see appendix, Figures 31a – 31c) in all three years. This could be because this is when soil temperatures are usually favourable to the fungus, but may also be because the level of root exudates, responsible for triggering sclerotial germination, peaks immediately prior to and during bulbing (Lancaster *et al.* 1984). As the plants produce more exudates, the exudates presumably extend further from the roots and more sclerotia are triggered to germinate in the root zone, leading to an increased likelihood of infection. Plant growth stage may therefore be an important factor in determining when the rate of new infections begins to increase.

The disease progress curves were similar when plotted against both time and plant growth stage. This is because growth stages converge in mid-season, with the result that plants mature at approximately the same time (Lancaster *et al.* 1986); early and late crops can be harvested together, despite the fact that they may have been planted several months apart (May - June and August – September). Plant growth is regulated by

temperature and day-length, rather than by the passing of time, so all onion crops form bulbs in mid and late November in Pukekohe (Lancaster *et al.* 1986).

Practical implications

The fact that high inoculum density may bring disease onset forward (as happened in 1998 / 99) means that some degree of prediction should be possible; growers whose fields have an inoculum load of above 0.04 g^{-1} soil (i.e. in the 'high' inoculum category) should be aware that the disease may, if weather conditions are conducive, become a problem in August - September, a month earlier than in fields with lower inoculum levels. Normal practice is to use Sumisclex seed treatments (in the past these have protected seedlings for up to 14 weeks after sowing but they no longer adequately control the disease, Stewart and Fullerton 1999). The first spray (either Folicur or Cereous) is applied from 6 - 10 weeks after sowing (usually early August) and then two more at monthly intervals (Fullerton *et al.* 1994). This should protect against an early onset of disease, assuming that the sprays remain effective. When soil temperatures remain in the optimum range for disease development into December, an additional spray to cover this time is required.

Deciding on a 'safe' level of inoculum (one resulting in commercially acceptable losses) is problematical; in quadrats where the soil sampling failed to find any sclerotia, there was a mean 14% incidence at harvest (±16% standard deviation, maximum 68%), reflecting the lower limit of soil sampling techniques. The 'safe threshold' may be as low as 0.005 sclerotia g^{-1} soil, as fields with inoculum levels of 0.005 sclerotia g^{-1} soil or less gave ≤ 7% disease incidence (commercially acceptable losses can be up to 10%). This limit is conservative; the sample areas were not treated with preventative sprays, so disease incidence in a field that is managed according to normal commercial practice will be much less.

4.4.2 Glasshouse trial

This experiment was repeated three times and during all three trials, little or no white rot was observed in any of the treatments. One possibility for this was that the onions did not develop normally under greenhouse conditions; they formed bulbs earlier than usual, probably as a result of abnormal light and temperature conditions (Lancaster *et al.* 1986). This irregular development could have affected the volume and / or nature of the root exudates that trigger sclerotial germination; the concentration of sulphoxides peaks at or just before bulbing (Lancaster *et al.* 1984). Other possibilities are that the unnatural soil conditions (soil was incorporated with bedding mix) had some effect or that the sclerotia had lost their pathogenicity, after time in artificial culture. Over the same timeframe, several other workers in the research group noticed that the isolates employed in their trials, particularly one known as Sc3 (one of the three isolates used in these glasshouse experiments), seemed to have lost their ability to infect onions. For this reason, a mix of isolates was used to create the treatments and although there was a little disease (2%) in the treatment with the highest inoculum density (during the third trial), it was not enough to support statistical analysis. The greenhouse trials were therefore abandoned after the third attempt. Fortunately, this was the minor component of the inoculum density study and the field trials stand alone.

4.5 Conclusions

Disease progress curves were sucessfully fitted to the gompertz model in two out of three sets of annual trials (to 57% and 26% of quadrats in 1998 and 1999), with an uncommon weather pattern influencing the progression of the disease in the third year (2000); in that year, data was fitted to log, linear and exponential models. Higher inoculum levels resulted in earlier onset of disease in the 1998 / 99 season, by approximately 4 weeks. Inoculum densities at planting did not correlate

with disease incidence in any of the three years tested. It appears that, while inoculum density may determine the time of disease onset at Pukekohe, the later progression of the disease is determined by other, mainly environmental, factors.

This research programme provided the kind of data required to form the basis of a predictive model for losses to white rot at Pukekohe. However, for this to be achieved, further research into factors affecting disease development, such as climatic variables, would be required. This would help growers to make informed decisions about the suitability of land for the cultivation of onions and enable better management of the disease. (For further discussion about the use of disease models, see Chapter 5).

CHAPTER 5: FINAL DISCUSSION

5.1 Introduction

Sclerotia are the only means by which this fungus can reproduce itself. They are, therefore, a vulnerable part of the lifecycle and control measures are being developed to target them (e.g. the use of diallyl disulfide as a germination stimulant). These methods depend on a thorough knowledge of sclerotial biology. Information gathered in the course of this research programme underpins other projects concerning the control of onion white rot.

The objectives of this thesis have been met. They were to

- determine the survival of sclerotia of *S. cepivorum* and to indicate which factors may be important influences on survival in New Zealand soils

- test for the existence of sclerotial dormancy in a range of New Zealand isolates and determine how long this period of dormancy persists

- describe the relationship between inoculum density and subsequent disease incidence in the Pukekohe region, South Auckland.

5.2 Sclerotial survival

The survival properties of naturally-produced sclerotia were tested over a timeframe of several years (see Chapter 2). Sclerotia were collected from commercial fields and buried in two different soil types and at two locations. The survival rates of sclerotia collected both early and late in the growing season were determined. Most sclerotia decayed rapidly in the first two months in soil, after which numbers remained relatively

stable. This sudden drop in viability was attributed to damage caused by desiccation and re-wetting in the field, probably resulting in leakage of nutrients and subsequent attack by microbes. Symptoms of desiccation, such as those seen in the field population, were induced by drying and re-wetting treatments in the laboratory. High annual rainfall with occasional periods of extremely dry weather in December / January occurred in Pukekohe during this study and are typical of the region (see climate data, Figures 24 – 29). Warm soil temperatures conducive to microbial activity also occurred; soil temperatures in the fields were between 10°C and 25°C for most of the growing season. This environment would provide the necessary conditions for structural damage to take place in the sclerotia.

Contrary to expectation, soil type had no effect on long-term survival. It may be that the properties of the two soils were similar (one was a silt loam and the other a clay loam, both with 4% organic matter). Location (related to climate) had only a minor effect in these trials. It is speculated that the structural damage, probably responsible for the early decline in viability, occurred while the sclerotia were maturing in fields at Pukekohe and, therefore, sclerotia collected from Pukekohe and subsequently buried at both Lincoln and Auckland showed the same survival trends, despite the fact that Lincoln has a different climate to Auckland.

The trend of an early decline in viability, perhaps caused by damage from adverse environmental conditions, is unusual in terms of previous work published on *Sclerotium cepivorum*. This highlights the value of New Zealand-based studies and the fact that it is often difficult to predict what will happen in this country by extrapolation from overseas work.

There are a number of reasons why the New Zealand situation may differ from that experienced in other countries. The horticultural soils here have been in cultivation for a relatively short period of time, compared to parts of Europe for example, and much of the farmland was under sub-tropical

rainforest in the recent past. The biodiversity of our soils is, therefore, both unique and diverse. This, in all probability, affects the interactions between micro-flora and the pathogen and, through selection pressure, the properties of the pathogen itself. *S. cepivorum* in New Zealand is genetically heterogeneous and some genetic groups may be unique to this country (Tyson *et al.* in press) despite the fact that it is presumably a recent import into this country from other regions where the disease is known, having arrived with or sometime after the introduction of *Allium* crops. The rapid appearance of unique characteristics in a pioneer population of organisms is a common biological phenomenon, known as 'rapid adaptive radiation'. These factors and climatic differences most likely explain why *S. cepivorum* may behave unusually in New Zealand, compared to that reported in overseas studies.

What are the implications for the commercial grower of the rapid, early loss of sclerotial viability observed? Due to the large numbers of new sclerotia produced on every infected bulb, even if 80% of sclerotia decay over the first 12 months in soil (maximum sclerotial decay seen in the survival trials after 12 months was 82%), this may still leave a significantly increased inoculum density. For example, if a low level of disease incidence is experienced in a field (10% of plants are infected), assuming an average planting density in the beds of 100 plants m^{-2}, and a conservative estimate of 1000 new sclerotia produced by every infected bulb, numbers of sclerotia will be increased by 2000 sclerotia m^{-2} area after 12 months, even if only 20% survive. If only 2% of sclerotia survive 3 years in soil (a figure taken from the survival studies), the inoculum density will still have increased by 200 sclerotia m^{-2}. Assuming a ploughing depth of 15 cm and calculating the volume of soil present in each 1 m^{-2} area within a field (150,000 cm^{-3}), 20% and then 2% sclerotial viability after 1 and 3 years results in an inoculum density of 0.013 and 0.001 sclerotia per cm^{-3} soil respectively. One cm^{-3} of fresh Pukekohe field soil weighs approximately 1 g when air-dry (mean weight 1.1 g). Inoculum densities per unit dry soil are therefore 0.013 and 0.001

sclerotia g^{-1} soil. This is a conservative estimate, based on observations of planting density and inoculum production in the field, and does not take into account any previously existing inoculum in the soil. Both of these inoculum densities would fall into the 'low' inoculum class described in Chapter 4.

If the same calculations were performed for fields with moderate (30%) and high (70%) levels of disease incidence, the following is found. One year after the epidemic with 30% disease incidence, inoculum density would be 0.040 sclerotia g^{-1} soil (a 'high' level, see Chapter 4), while after 3 years it drops to 0.004 sclerotia g^{-1} soil ('low'). For a serious epidemic with 70% of plants infected, the figures are 0.093 sclerotia g^{-1} soil (after 1 year, a very high ID) and 0.009 sclerotia g^{-1} soil (3 years, low).

From the above, it can be seen that a one-year rotation system would be unsafe, following all but the lowest level of disease. A 3-year rotation would result in most inoculum densities returning to the 'low' classification but significant white rot can still be expected at these levels (see Chapter 4). Therefore, a 4- or 5-year rotation is recommended for most circumstances. The use of solarisation and germination stimulants could enable shorter rotation systems to be used and provide an additional safeguard to further reduce sclerotial numbers in the soil.

5.3 Sclerotial dormancy

Dormancy was tested using modifications of two established methods, one field-based and one involving Petri dishes in the laboratory (see Chapter 3). The second technique was found to be prone to problems caused by contamination by other fungi, which grew over and obscured the sclerotia, making the scoring of germination difficult. The more effective, field-based method was then used to test dormancy requirements of five isolates. Although there were some differences between the isolates in terms of when dormancy began to break, all isolates required six months in soil before high rates of germination were achieved. This is several months longer than reported in most other

literature and has implications for the effective use of germination stimulants in disease control. It would be advisable to apply a germination stimulant, such as DADS, two or three months prior to planting a new crop of onions, rather than immediately following harvest of the previous onion crop. This would allow the maximum amount of time for sclerotia produced during the previous epidemic to break constitutive dormancy in soil before DADS is used. A gap of at least two months between application of DADS and planting would be necessary to prevent young seedlings from being exposed to germinating sclerotia.

As the early crop of onions is sown in midwinter, autumn (April) may therefore be the best time to apply DADS; this would allow sclerotia produced during the previous summer to condition for a minimum of three months in soil and DADS is most effective when soil temperatures are cool (Fullerton *et al.* 1994), as they generally are at this time. These recommendations are compatible with normal commercial practice, as DADS is usually injected into the soil in spring and / or autumn, 2 - 3 months prior to planting (Stewart and Fullerton 1999). Previous trials (Tyson *et al.* 2000) have shown that DADS may be applied in the presence of a cover crop, without any phytotoxic effects. They also indicated that both autumn and early-summer applications of DADS were highly effective at reducing disease incidence in a subsequent crop (Tyson *et al.* 2000).

The recommendations given above are made with regards to dormancy requirements of sclerotia and the limitations of DADS. Other considerations, such as crop rotation systems, local weather conditions and grower preferences should also be taken into account when deciding on an application strategy for germination stimulants.

Dormancy was found in both field-collected and artificially-produced sclerotia. This is useful as it suggests that artificially-produced sclerotia may be an accurate model for the dormancy requirements of sclerotia in

the commercial situation and this reduces some of the uncertainty previously involved in such studies.

As an interesting aside, DADS strongly stimulated the growth of some soil-borne fungi during the first, failed dormancy experiment (see appendix, Table 8) especially *Talaromyces* spp. If DADS enhances the growth of, for example, a biocontrol agent (some *Talaromyces* spp. have been trialed as sclerotial parasites, e.g. Madi *et al.* 1997) or simply promotes an active soil microflora, this may have additional benefits to the grower.

It is thought that exogenous dormancy acts as a 'fail-safe' mechanism, preventing sclerotia from germinating in adverse conditions or in the absence of a host plant when the fungus is host-specific. Exogenous and constitutive dormancy have both advantages and disadvantages. On the one hand, sclerotia must germinate to survive, as their internal nutrient supply cannot sustain them indefinitely and to complete their lifecycle they need a host. On the other hand, if they germinate in conditions that are not favourable to growth or disease development, they also die. The ability of sclerotia to both live for several years and to spread germination events over several months, is a powerful tool for survival. Constitutive dormancy may also have evolved to prevent self-competition (A. Stewart, pers. comm.); as sclerotia are relatively immobile in soil, the chances are that they will not disperse far from the parent bulb / mycelium. Therefore, if germination were possible immediately after sclerotia were formed, they would be in direct competition for a host with the parental mycelium.

Little is known about the mechanism controlling dormancy. There are a few theories about how constitutive dormancy is maintained or germination triggered, some of which may be applicable to *S. cepivorum*. Constitutive dormancy is common to a number of sclerotia-producing fungi (e.g. Coley-Smith and Cooke 1971, Dillard *et al.* 1995). The rind may play a major role; melanisation of the rind inhibits sclerotial

germination in *S. sclerotiorum* (Huang and Kokko 1989) and sclerotia of a number of *Sclerotinia* spp., when grown on media amended with melanin-inhibiting fungicides, do not show dormancy (Huang *et al.* 1990). Treatments that affect the integrity of the rind, such as abrasion, rapid temperature changes and desiccation, can break constitutive dormancy in *S. cepivorum* and other fungi (e.g. Brix and Zinkernagel 1992, Coley-Smith 1960, Gerbrandy 1992). Perhaps the rind serves to insulate receptors, responsible for triggering germination, from cues that might otherwise begin the process, though how they do this when the rind is permeable to water (Phillips 1987) is not known. How the S-alk(en)yl-L-cysteine sulphoxides from *Allium* exudates act to trigger germination in *S. cepivorum* is also unknown (Coley-Smith *et al.* 1987). If the rind has an important role, this could explain why some sclerotia require longer or shorter periods of conditioning before germination can occur; those sclerotia that break dormancy almost immediately after they are formed may have a weaker or thinner rind than those that require several months in soil before germinating. However, a damaged rind has also been linked to reduced sclerotial survival (see Chapter 2) and to have decreased rind integrity a requirement for germination, therefore, seems counterproductive.

The breakage of dormancy is probably more complex than simply weathering of the rind; perhaps a physiological change takes place during the period of dormancy. There has been the suggestion that at least part of the period of dormancy in *S. sclerotiorum* is taken up by the development of apothecial initials (Saito 1973). Until this has occurred, the sclerotial cannot germinate. Some similar physiological or structural change may be involved in constitutive dormancy of *S. cepivorum.*

In summary, sclerotial dormancy is an extremely complex survival strategy, influenced both by a range of environmental factors and the genetic make-up of the fungus. While we now know something about the

factors that trigger the breakage of dormancy, the physiological mechanism behind it remains largely unknown.

5.4 Inoculum density and disease incidence

A method was developed to isolate sclerotia from the soil types commonly found in the Pukekohe region. This made it possible to collect detailed data about the numbers of sclerotia present in soil. Inoculum level and disease incidence were measured over three years, at a total of 11 sites, and the information used to describe the relationship between these two parameters (see Chapter 4). Disease progress curves were sucessfully fitted to the gompertz model in two of the three years (57% and 26% of quadrats fitted this model in 1998, 1999 respectively), with an unusual weather pattern probably influencing the pattern of the disease in the third year (2000); in that year, data was fitted to log, linear and exponential models.

A prediction of disease progress from pre-plant inoculum density was not generally possible; in 1998 higher inoculum levels resulted in an earlier onset of disease, by up to 4 weeks, but it was not possible to anticipate the final asymptote of the disease progress curve, in any of the 11 trials, from inoculum density data. There are several possible reasons to explain why inoculum density did not appear to affect final disease outcome; the study may not have been large enough to detect any relationship between the two parameters, even if one were present. A more likely explanation, perhaps, is that pre-plant inoculum density affects the time of disease onset, while other, mainly environmental, factors drive the later progression of the disease. The most important of these other factors may well be the degree of root exudation from host plants (affected by plant growth stage and soil temperature) and the direct effect of soil temperature / moisture on the growth and infection processes of the fungus (A. Stewart, pers. comm.). There has been little work to date on the driving forces behind the production / persistence of onion root exudates in soil and the effect of these on sclerotial germination under field conditions.

To create a full-scale predictive model it would be necessary to collect similar data over several additional seasons. Combined with data on climate and the effect of spray regimes, this could be the basis of a computer-based management program such as that successfully used to manage cereal production / diseases in the U.K. (Decision Support System for Arable Crops or DESSAC, Audsley *et al.* 1997, Brooks 1998). For other examples of successful models used to manage plant diseases see Vincelli and Lorbeer 1989 (*Botrytis squamosa*), Kobayashi *et al.* 1995 (*Rhizoctonia solani*) and Twengstrom *et al.* 1998 (*Sclerotinia sclerotiorum*).

To create accurate models that can be used for the prediction of disease epidemics, a variety of information is required. For example, it may be necessary to quantify such things as the frequency of secondary infections (Gilligan 1990). Other factors that might be included in the model could be speed of root growth and the width of the pathozone (i.e. how far root exudates spread from onion roots, triggering germination). The pathozone, and hence the degree of exposure of sclerotia to *Allium* exudates, is affected by the growth-stage of the onion crop; the greatest concentration of sulphoxides occurs just prior to or during bulbing, Lancaster *et al.* 1984. All these parameters can affect disease progress and severity, in addition to local variation in inoculum levels.

It is not generally possible to infer biological processes from disease progress curves (Pfender 1982), but given biological information such as that detailed above, it should be possible to isolate the factors that control the development of an epidemic. A model of this complexity was far beyond the scope of this research programme. The information would however be extremely useful in identifying the factors that influence disease severity and showing which of these could be influenced to mitigate the effect of white rot on commercial yield.

5.5 Conclusions

The main findings of this thesis were as follows.

- The majority of sclerotia decay during the first few months in soil. This was attributed primarily to the effect of desiccation and re-wetting on sclerotial structure, which probably pre-disposed them to attack by microbes.

- Dormancy existed in all isolates tested and persisted for 6 months. One of the established methods used to study dormancy (using Petri dishes of soil in the laboratory) was not useful under New Zealand conditions but another (field-based) method proved effective.

- The relationship between inoculum level and disease incidence can, for most data, be described using the gompertz and other models. Higher inoculum levels resulted in earlier onset of disease in the 1998 / 99 season, but did not correlate with disease incidence at harvest.

5.6 Recommendations for future research

Future work could include a study of sclerotial survival over a longer timeframe (e.g. 5 years) to determine when all sclerotia have decayed or reached a 'safe' threshold (a safe level, resulting in commercially acceptable losses, may be as low as 0.005 sclerotia g^{-1} of dry soil, see Chapter 4).

One could also test dormancy requirements of a greater number of isolates in an increased variety of soil types, using the methods described in Chapter 3. Field trials could also be used to determine the level of inoculum both before and after DADS is applied; this would show how many sclerotia respond to DADS in the field situation and would be a measure of both sclerotial dormancy and the effectiveness of the

techniques used to apply DADS. The effect of DADS on subsequent disease incidence has been examined (Tyson *et al.* 2000) but not, as far as I am aware, the effect of DADS on numbers of sclerotia in field soil at Pukekohe. While changes in disease incidence probably reflect changes in inoculum levels, a small trial to look at the direct effect of DADS on sclerotia in the field may have benefit.

Finally, future research could involve the collection of more data on factors affecting disease development, with a view to creating a management tool for growers. Existing sources of information (of which there is a substantial amount, both published and unpublished) on past white rot epidemics, weather patterns etc in Pukekohe could be collated into a central database and used to supplement any new data.

In my opinion, of the potential research topics above, most benefit would be gained by further research into the factors controlling disease progression in Pukekohe. Data would need to be collected over several years, on inoculum density, weather parameters, root growth and cultivar susceptibility, amongst others. Modeling a biological system is always difficult, especially plant-pathogen interactions such as this, where environmental variables during the growing season can have considerable effect on the impact of the disease. The present study is only a starting point for this type of work. However, I see no reason why such studies should not be attempted as they have great potential as a tool for both growers and scientists. Overseas studies of this type have worked well (e.g. Audsley *et al.* 1997, Twengstrom *et al.* 1998) and are now routinely used to enhance production and facilitate precision management techniques.

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9. APPENDIX

9.1 Climate data

Soil temperature and rainfall at Lincoln and Mount Albert, Auckland, (used for trials concerning sclerotial survival or dormancy) are displayed in Tables 2 and 3. Soil moisture and temperature data (for 1998 – 2000, from sites used in the inoculum density / disease severity trials) are shown in Figures 24 – 29.

9.2 Location of trial sites in Pukekohe

Figure 30 shows the location of trial sites in Pukekohe, where inoculum density and disease severity were measured. The sites were as follows.

1998 season –

'bollard' (Bollard Rd., Tuakau),
'smeeds' (Smeeds Rd., Tuakau),
'jivan' (Parker Lane, north of Tuakau),
'makan' (McNally Rd, Pukekoke),
'master' (Mill Rd., Bombay).

1999 season –

'das' (Pukekohe East),
'young' (Union Rd., Mauku),
'mcmiken' (Titi Rd., Mauku).

2000 season –

'master' and 'makan' as in 1998
'union' (Union Rd., Mauku).

Table 2. Average soil temperature (°C) at 10 cm depth, 1998 - 2000*

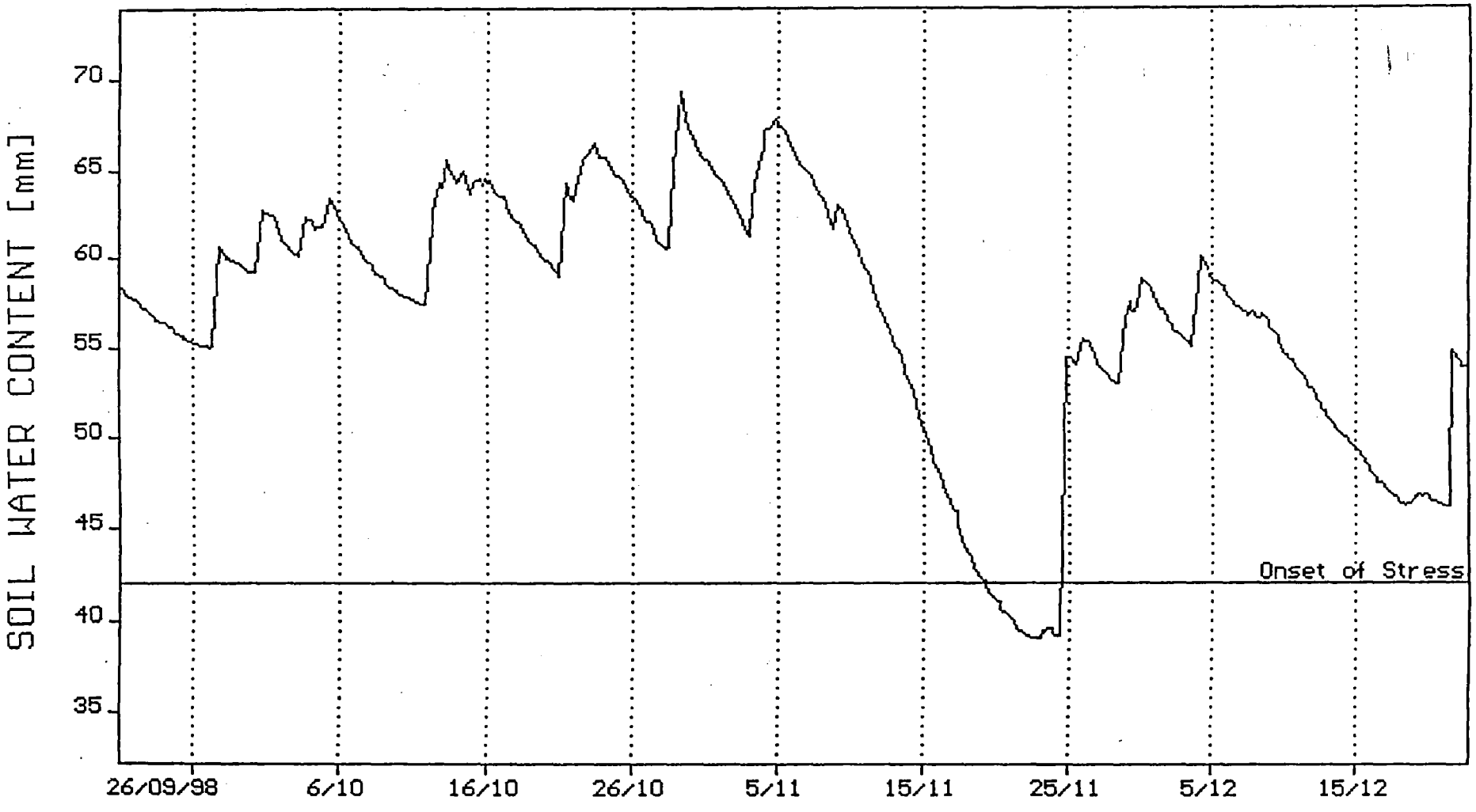
	jan	feb	mar	apr	may	jun	jul	aug	sep	oct	nov	dec
Auckland '98	20.3	22.0	19.9	17.4	14.7	13.0	12.0	11.4	13.0	15.4	17.6	19.3
Auckland '99	21.7	20.7	19.6	16.8	14.3	11.6	10.7	10.2	13.0	15.4	17.3	18.1
Auckland '00	19.2	20.0	18.7	16.7	15.0	11.9	12.0	11.0	12.1	14.4	15.1	19.1
Lincoln '98	20.2	22.3	17.5	13.4	10.1	6.3	6.6	6.1	9.6	12.9	15.5	18.9
Lincoln '99	20.9	20.1	17.2	12.7	9.8	6.6	6.2	6.5	9.6	12.9	14.4	16.4
Lincoln '00	17.8	17.7	16.0	12.4	7.7	5.6	6.2	5.1	7.9	9.9	12.9	16.2

*Courtesy Metservice N.Z. Ltd (Lincoln) and National Institute of Water and Air (Mt. Albert, Auckland).

Table 3. Total monthly rainfall (mm), 1998 - 2000*

Location & date	jan	feb	mar	apr	may	jun	jul	aug	sep	oct	nov	dec
Auckland '98	16.2	93.8	100.1	44.9	77.3	128.5	355.3	138.6	80.5	103.1	68.8	52.2
Auckland '99	103.8	57.7	72.9	165.7	55.2	110.2	130.7	113.2	69.6	96.6	172.5	42.8
Auckland '00	86.2	9.2	51.2	128.4	118.1	203.4	173.2	84.2	72.2	65.5	74.8	56.6
Lincoln '98	17.2	13.4	31.4	10.8	54.4	39.7	48.5	45.7	19.3	57.2	20.3	24.1
Lincoln '99	36.3	38.3	56.1	36.3	23.6	69.1	135.1	58.0	26.6	50.9	60.5	35.1
Lincoln '00	85.0	19.9	51.8	50.6	72.4	41.4	10.8	99.4	50.6	78.6	82.8	24.6

*Courtesy Metservice N.Z. Ltd (Lincoln) and National Institute of Water and Air (Mt. Albert, Auckland).



Note: plants in water-stress from 15 – 22 November.

Figure 24. Soil moisture at site used for inoculum density work, 1998 growing season.

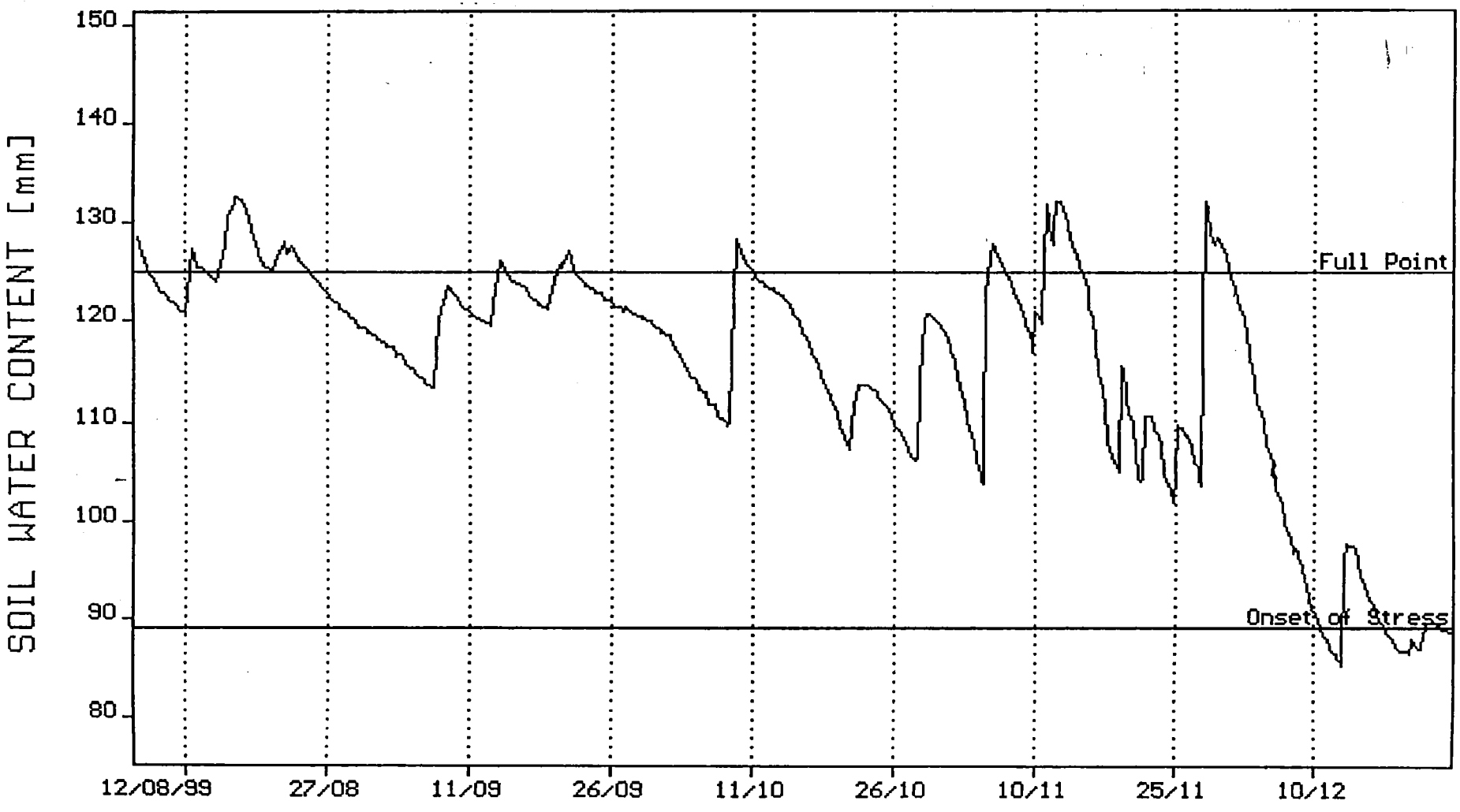


Figure 25. Soil moisture at site used for inoculum density work, 1999 growing season.

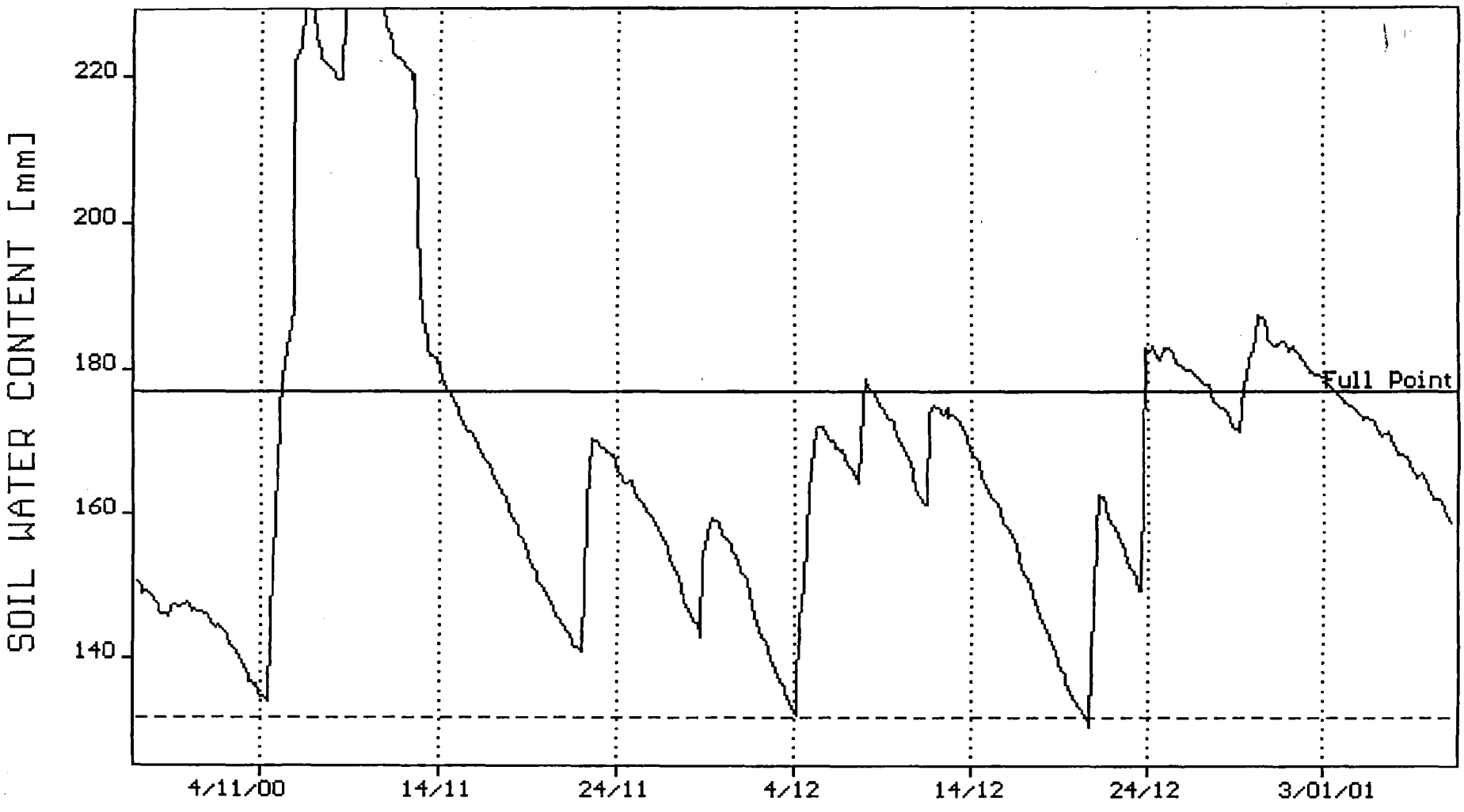
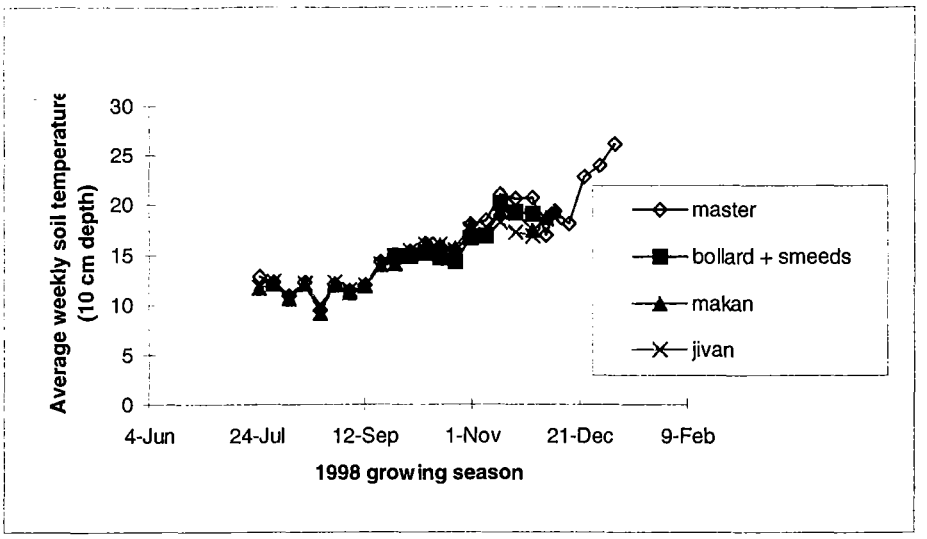
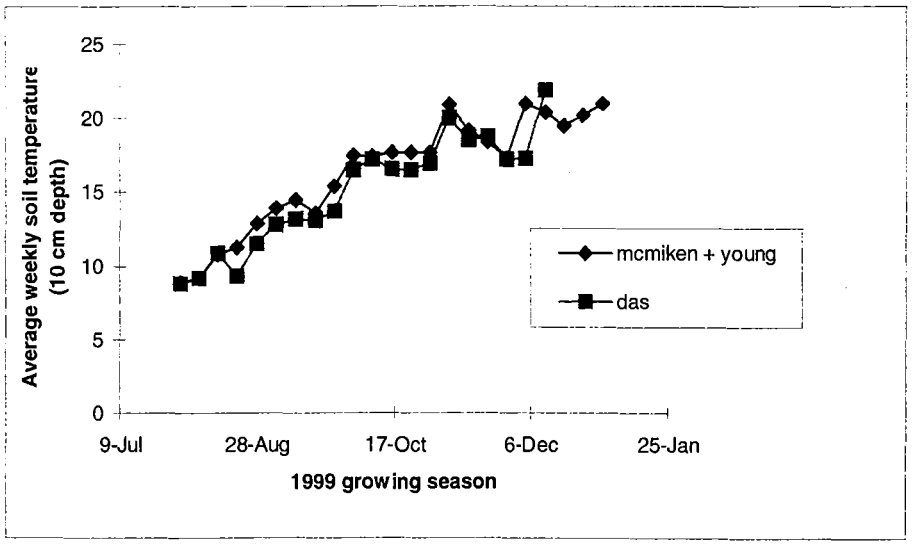


Figure 26. Soil moisture at site used for inoculum density work, 2000 growing season.



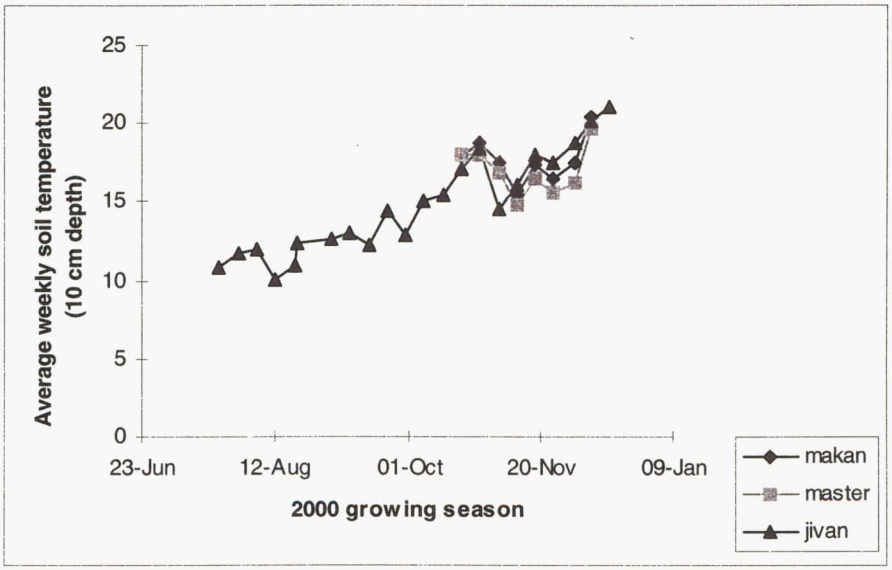
Note: temperatures very similar in all fields. Fields coded as 'bollard' and 'smeeds' were located near each other, hence the single data set for these two sites. Temperatures were recorded every 3 h, weekly means shown.

Figure 27. Soil temperatures at sites used for inoculum density work, 1998 growing season.



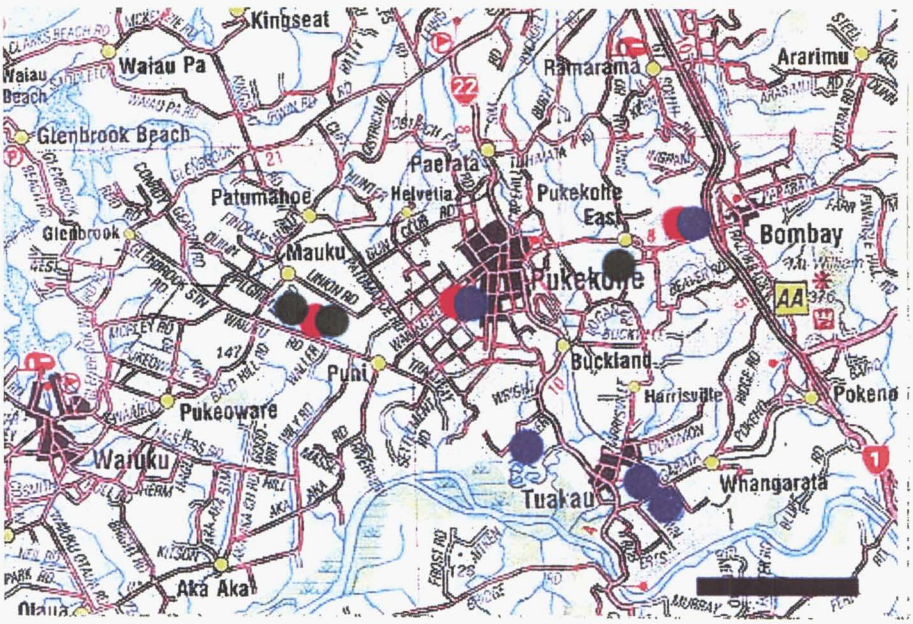
Fields coded as 'mcmiken' and 'young' were located near each other. Weekly means shown.

Figure 28. Soil temperatures at sites used for inoculum density work, 1999 growing season.



Data from 'jivan' courtesy of R. Wood, Vegcon Services. Although no trials were conducted in 'jivan' in 2000, these data are included to supplement those from 'makan' and 'master' sites. Figures 27 and 28 show that soil temperatures were similar in all fields, even though they were located across the Pukekohe region (Figure 30). Weekly mean temp. shown.

Figure 29. Soil temperatures at sites used for inoculum density work, 2000 growing season.



Blue = 1998/99 field sites, Red = 1999/00, Green = 2000

Scale bar at lower right = 5 km. Source: A.A. District Map, South Auckland and Coromandel

Figure 30. Location of trial sites in Pukekohe region.

9.3 Raw experimental data

9.3.1 Survival data

Data from the survival experiments are shown in Tables 4 - 6 below. Numbers represent mean viable sclerotia recovered from soil, as a percentage of the total number buried at the beginning of the trial. 'Controls' were sclerotia kept in sand in the laboratory. The majority of these data are presented (with standard errors) as Figures 6 and 7, Chapter 2. Experiments 1a and 1b below were combined as two replicates and renamed 'Experiment 1' in Chapter 2.

Table 4. Survival experiment 1a

Months in soil	0	1	2	3	4	8	12	16	20	24	28
Pukekohe soil	80	15	24	17	30	17	30	9	10	4	2
Kaiapoi soil	80	30	15	18	12	30	18	12	19	14	7
Field Pukekohe[A]	80	-	16	21	18	18	26	12	2	10	-
Control	80	-	93	82	74	74	52	50	14	34	-

A = Sclerotia buried in the field, not in bins of soil, to ensure that placing soil into bins does not affect sclerotial survival. This experiment involved 'late-season' sclerotia.

Table 5. Survival experiment 1b

Months in soil	0	1	2	3	4	8	12	16	20	24
Pukekohe soil	82	38	18	38	29	43	23	24	3	14
Kaiapoi soil	82	40	26	32	30	28	27	2	3	11
Control	82	68	65	66	62	60	54	37	29	37

Note: Pukekohe soil = Patumahoe clay loam. Kaiapoi soil = Wakanui silt loam
This experiment involved 'late-season' sclerotia.

Table 6. Survival experiment 2

Months in soil	0	1	2	3	4	8	12	16	20	24
Pukekohe soil (A)	100	63	34	35	40	38	24	26	29	B
Kaiapoi soil (A)	100	64	38	27	27	41	27	19	20	B
Pukekohe soil (L)	100	61	43	54	58	47	40	42	25	40
Kaiapoi soil (L)	100	48	52	60	57	33	39	33	25	23
Control	100	99	100	99	98	100	98	100	98	100

Note: (A) = set up at Auckland, (L) = set up at Lincoln. B = data not available (trial site in Auckland destroyed). This experiment involved 'early-season' sclerotia.

Table 7 shows results of an ANOVA examination of data collected during the survival experiments. This analysis is of overall effect of treatments over time. An analysis of data from each individual sampling point was also performed and differed from that shown in Table 7 only in one detail; location (a treatment tested in Experiment 2) was not significant at most time intervals (see Chapter 2), while over time there was a highly significant effect. Experiments 1 and 2 cannot be contrasted directly as they were set up at different times of year (see Chapter 2), but a comparison of the two (and so the survival properties of sclerotia collected in early- versus late-season) is included here for interest. Time was an important factor in both experiments; significantly more sclerotia died in the first two months than during the rest of the trial.

9.3.2 Dormancy data

Data from the full-scale dormancy pilot trial are shown in Table 8, with standard deviation. Sclerotial germination was recorded weekly for 13 weeks (data from selected weeks shown). Contamination by other fungi began at approximately week 9, obscuring the sclerotia, often inhibiting germination (note lower rates of germination after week 10) and probably aggravating the high rates of variation in the treated samples. Changes were subsequently made to the method to discourage contamination (see Chapter 3).

Table 7. ANOVA results for survival experiments.

Experiment	Treatment	P-value
Exp. 1	Soil type	0.195
	Time	0.005**
	Soil*time	0.520
Exp. 2	Location	0.000**
	Soil type	0.400
	Time	0.001**
	Location*soil	0.897
	Soil*time	0.652
	Location*time	0.036**
	Location*soil*time	0.792
Exp. 1&2	Season (early or late)	0.004**

Analysis done on overall effect of treatments over 16 months.

** = statistically significant

ANOVA results for dormancy Experiments 1 and 2 are shown in Tables 9 and 10 below. In Table 10, the upper section of the table refers to the effect of DADS on sclerotial germination; after conditioning for 3, 4 or 5 months in soil (depending on the isolate) significantly more sclerotia germinated when exposed to DADS than when not exposed to it. When DADS caused a significant increase in germination, this indicated that dormancy was beginning to break, so these results show how long dormancy persisted in sclerotia of the five isolates. The lower row in the table refers to the effect of isolate on the timing of dormancy; isolates differ significantly from each other after 3, 4 and 5 months in soil. For explanation of these results, see Chapter 3.

9.3.3 Inoculum density / disease incidence data

Cumulative disease incidence is plotted against plant growth stage in Figures 31a – 31c below. These data were collected during the experiments outlined in Chapter 4 (on the relationship between inoculum density and disease incidence).

Table 8. Results from dormancy pilot trial.

GERMINATED SCLEROTIA (%)			
Isolate	**Wks in soil**	**+DADS[1]**	**- DADS[2]**
'Master'	2	Nil	Nil
	4	3±2.3	Nil
	6	11±12.2	2±2.8
	8	57±15.1	Nil
	10	55±18.5	Nil
	12	20±6.9	Nil
'Sc3'	2	Nil	Nil
	4	11±6.1	Nil
	6	44±28.0	Nil
	8	43±12.9	2±2.8
	10	65±10.1	Nil
	12	40±14.4	2±2.8
'Sc5'	2	Nil	Nil
	4	12±10.6	Nil
	6	20±13.9	2±2.8
	8	76±24.3	2±2.8
	10	48±10.6	2±2.8
	12	32±25	2±2.8

1 = mean of 3 replicates, 2 = mean of 2 replicates

Table 9. ANOVA results for dormancy Experiment 1.

Isolate	Treatment	Wk1	Wk2	Wk3	Wk4	Wk5	Wk6	Wk8	Wk9	Wk10	Wk11	Wk12	Wk15
Sc3	+/- DADS	0.101	0.085	0.000	0.232	0.122	0.072	0.003	0.039	0.017	0.175	0.081	0.028
	Soil type	0.967	0.120	0.741	0.799	0.424	0.933	0.858	0.882	0.890	0.840	0.258	0.108
Sc5	+/- DADS	0.105	0.106	0.001	0.025	0.021	0.005	0.002	0.003	0.009	0.085	0.003	0.024
	Soil type	0.662	0.158	0.865	0.919	0.843	0.780	0.386	0.235	0.643	0.540	0.149	0.409
Master	+/- DADS	0.030	0.003	0.011	0.125	0.139	0.032	0.000	0.000	0.020	0.001	0.004	0.016
	Soil type	0.050	0.084	0.345	0.467	0.416	1.000	0.678	0.242	0.292	0.865	0.089	0.216
All	Isolate	≥0.05 [A]	≥0.05 [A]	0.894	0.360	0.815	0.229	0.453	0.280	0.128	0.421	0.616	0.408

There were no interaction effects ($p \geq 0.05$), data not shown. Interactions between both soil type / sclerotial germination and isolate / sclerotial germination were tested for. A = clear from graphs that no significant difference between isolates, so ANOVA test not required.

Table 10. ANOVA results for dormancy Experiment 2.

When significant	Isolate	Month1	Month2	Month3	Month4	Month5	Month6
≥ Month 4	Sc3			0.164	0.004	0.004	0.005
≥ Month 4	Master			0.525	0.018	0.032	0.000
≥ Month 4	Das	0.909 [A]	0.102 [A]	0.561	0.040	≤ 0.05 [B]	≤ 0.05 [B]
≥ Month 5	Smeeds			0.269	0.056	0.009	≤ 0.05 [B]
≥ Month 3	Sc5			0.033	0.045	≤ 0.05 [B]	≤ 0.05 [B]
Months 3,4 and 5	All	0.352	0.399	0.013	0.040	0.019	0.245

A = as the isolates did not differ significantly at months 1 and 2, a combined analysis was performed and these p-values refer to that analysis.

B = From graphs of data collected at these sampling intervals (see Figure 11), it was clear that significantly more sclerotia germinated when exposed to DADS, compared to control samples; an ANOVA test was not required.

Figures 31a - 31c. Disease progress curves, relative to plant growth stage.

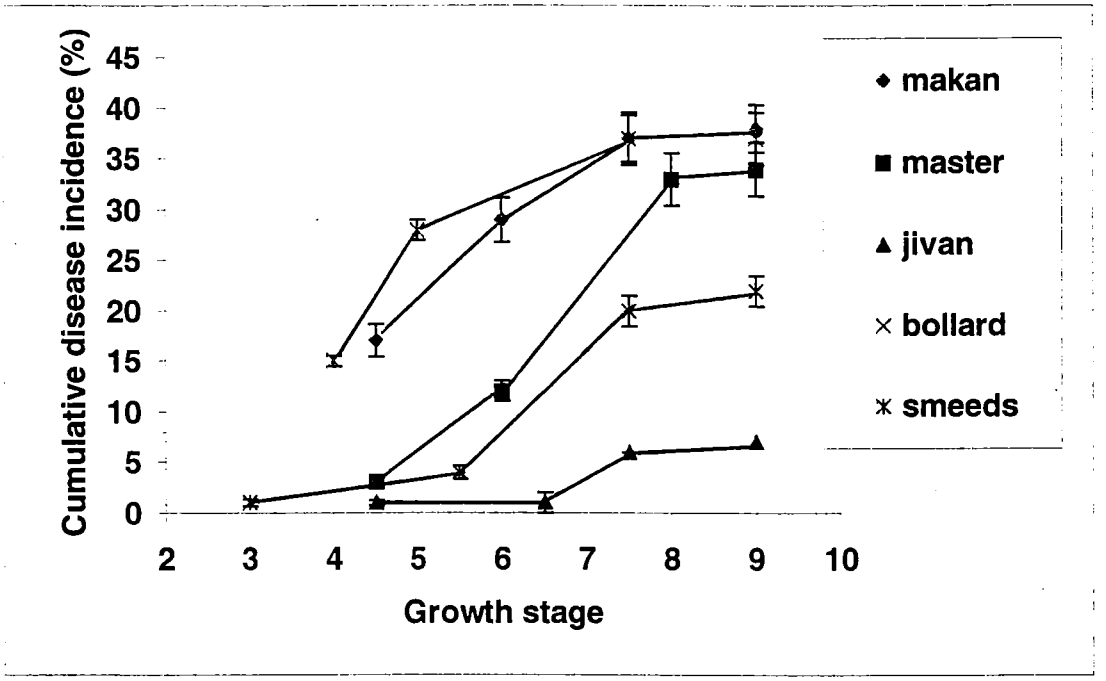


Figure 31a. Five fields, 1998.

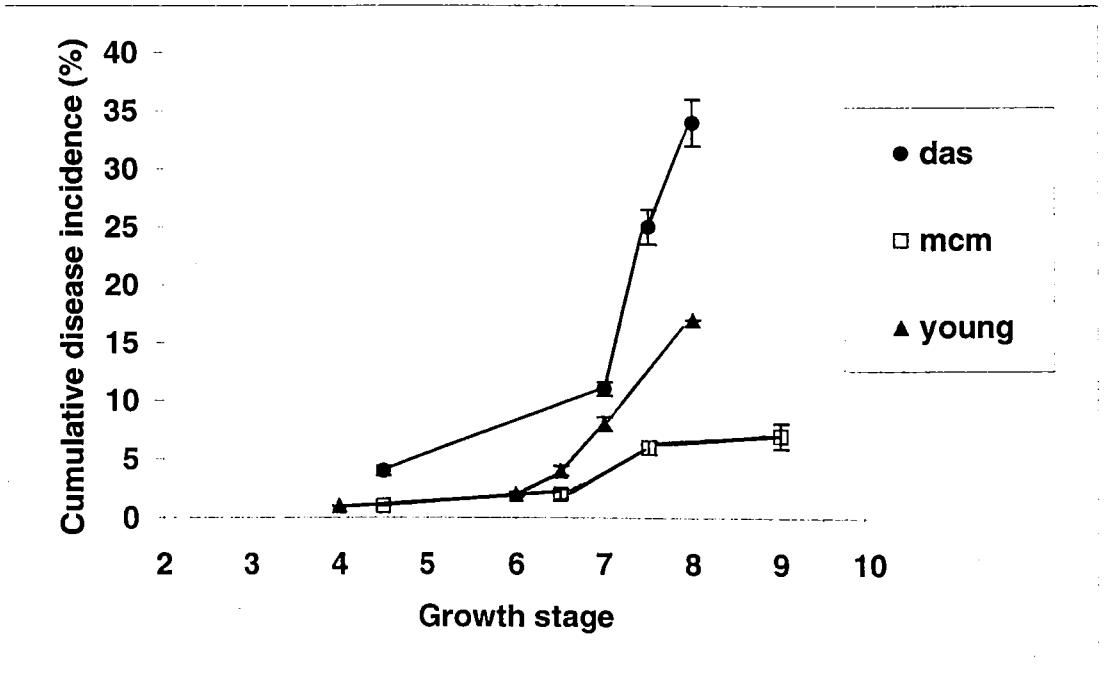


Figure 31b. Three fields, 1999.

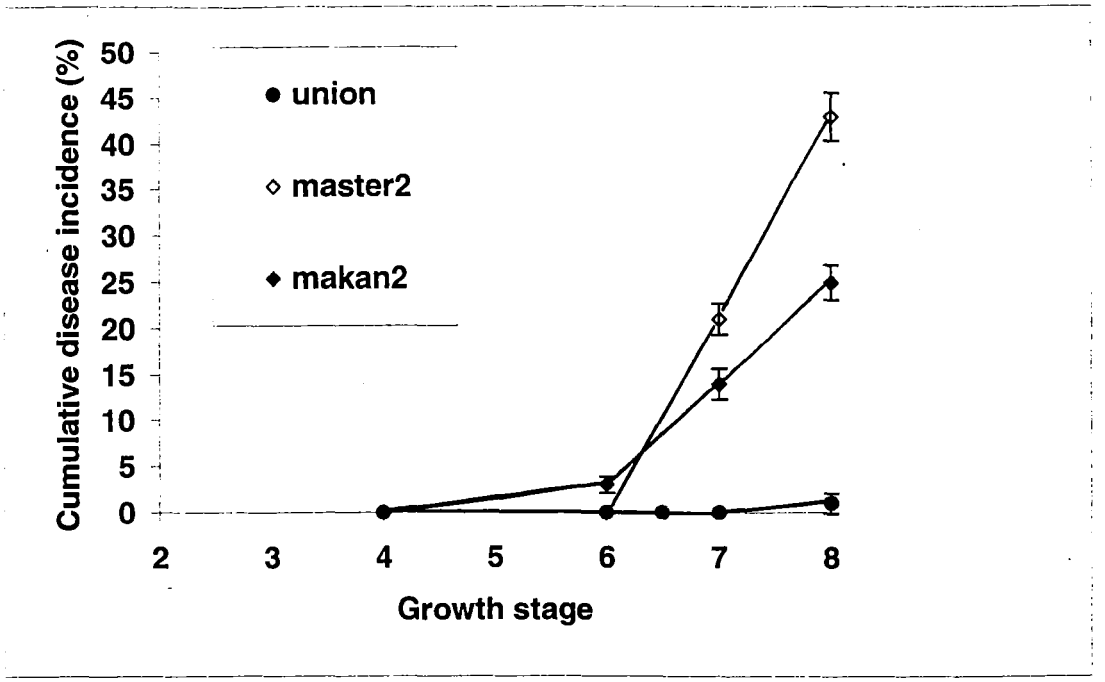


Figure 31c. Three fields, 2000.

Guide to Figure 31a – 31c, disease progress relative to growth stages.

Explanation of plant growth stages (Brewster 1994);

1 = Prior to germination.

2 = Hook appears above soil surface.

3 = First true leaf appears.

4 = Cotyledon senescence, second and third true leaves appear.

5 = Fourth leaf stage.

6 = Fall of first leaf. Leaves five, six and seven appear.

7 = Start of bulbing. The concentration of sulphoxides, responsible for triggering sclerotial germination, peaks now, Lancaster *et al.* 1984.

8 = Bulb swelling. Older leaves desiccate. Leaves fold under own weight.

9 = Neck softens, foliage collapses. Bulb reaches final size. Harvest can occur.

10 = Bulb ripens, outer skin dries, foliage senesces.

Raw data from the 11 field trials is shown below in Table 11. ID = inoculum density, or sclerotia per 100 g^{-1} soil, DI = disease incidence (cumulative).

Table 11. Raw data from inoculum density field trials.

Field	Quadrat	Plant Number	ID	DI at harvest (%)
Bollard	1	69	2	41
	2	26	10	23
	3	27	14	59
	4	27	14	74
	5	38	18	24
	6	29	19	38
	7	35	18	31
	8	32	10	25
	9	21	10	24
	10	41	4	48
	11	27	14	26
	12	35	12	37
	13	25	10	52
	14	32	8	25
	15	26	20	58
	16	22	6	64
	17	27	2	41
	18	32	6	19
	19	42	2	36
	20	40	4	50
	21	29	12	34
	22	26	14	27
	23	26	16	19
	24	24	4	14
Smeeds	1	114	2	10
	2	120	12	19
	3	108	6	20
	4	134	6	31
	5	122	0	18
	6	134	2	17
	7	130	2	19
	8	141	0	25
	9	134	2	19
	10	136	10	32
	11	120	4	28
	12	136	2	16
	13	120	2	23
	14	109	0	10
	15	127	2	17
	16	159	4	13
	17	136	14	23
	18	109	4	24
	19	101	8	31
	20	151	2	23
	21	110	0	25

	22	116	2	43
	23	114	0	23
	24	153	6	16
Jivan	1	115	6	15
	2	111	0	22
	3	118	0	5
	4	116	2	4
	5	130	0	5
	6	85	0	4
	7	111	8	4
	8	132	0	4
	9	111	0	4
	10	119	0	0
	11	108	2	0
	12	122	2	2
	13	94	0	12
	14	106	12	15
	15	111	2	13
	16	121	0	8
	17	94	4	12
	18	103	12	13
	19	115	0	10
	20	109	0	6
	21	114	4	3
	22	105	8	2
	23	125	0	11
	24	101	0	2
Master	1	108	4	17
	2	135	4	24
	3	117	2	25
	4	105	6	24
	5	100	2	N/A
	6	117	4	25
	7	120	0	21
	8	125	2	23
	9	110	0	26
	10	134	0	28
	11	119	2	19
	12	113	0	22
	13	96	2	48
	14	99	0	41
	15	144	0	30
	16	89	6	52
	17	96	0	47
	18	104	0	58
	19	124	4	37
	20	101	0	49
	21	103	0	47
	22	95	4	49

	23	98	0	44
	24	94	6	29
Makan	1	74	6	34
	2	77	2	35
	3	85	2	37
	4	68	4	48
	5	64	0	51
	6	79	2	47
	7	64	16	59
	8	100	4	43
	9	64	4	18
	10	79	10	44
	11	66	10	59
	12	76	16	50
	13	86	4	37
	14	94	0	21
	15	71	0	45
	16	69	6	46
	17	77	4	21
	18	79	8	41
	19	83	2	27
	20	75	4	48
	21	105	2	26
	22	94	0	34
	23	82	2	28
	24	90	4	31
	25	98	6	27
	26	96	2	22
	27	83	2	29
	28	82	2	38
Das	1	112	3	38
	2	118	0	22
	3	127	0	27
	4	120	1	33
	5	108	0	52
	6	107	1	54
	7	134	1	36
	8	137	2	31
	9	124	0	24
	10	107	0	26
	11	110	0	35
	12	113	2	26
	13	117	1	26
	14	74	0	37
	15	121	2	22
	16	115	0	30
	17	109	0	36
	18	67	0	43
	19	113	0	43

	20	97	1	30
	21	127	1	24
	22	89	1	54
	23	79	0	43
	24	98	1	31
Mcm	1	71	0	20
	2	82	0	16
	3	62	1	16
	4	59	0	12
	5	62	0	5
	6	70	0	6
	7	72	1	1
	8	56	0	4
	9	84	1	2
	10	80	0	4
	11	86	0	4
	12	74	0	0
	13	73	0	3
	14	86	0	9
	15	67	0	12
	16	74	0	5
	17	60	0	0
	18	59	0	10
	19	66	1	5
	20	54	2	4
	21	46	0	9
	22	86	0	1
	23	68	1	16
	24	73	0	8
Young	1	88	1	14
	2	86	0	11
	3	64	0	17
	4	102	0	7
	5	108	0	6
	6	106	13	19
	7	108	0	7
	8	121	1	12
	9	96	4	12
	10	113	1	10
	11	113	0	4
	12	98	0	6
	13	87	4	7
	14	72	1	18
	15	115	1	15
	16	116	1	25
	17	93	4	18
	18	115	0	18
	19	103	0	19
	20	116	3	14

	21	87	2	28
	22	91	1	19
	23	94	2	15
	24	89	0	9
Master2	1	91	0	21
	2	98	0	26
	3	101	0	32
	4	88	3	60
	5	95	0	41
	6	77	0	68
	7	89	3	47
	8	104	1	35
	9	84	3	71
	10	82	2	50
	11	106	2	44
	12	94	1	40
	13	100	2	34
	14	97	5	27
	15	110	3	36
	16	99	4	47
	17	103	0	38
	18	100	2	34
	19	89	6	53
	20	99	3	47
	21	95	3	53
	22	90	3	39
	23	91	1	52
	24	97	5	32
Makan2	1	60	3	35
	2	51	3	29
	3	73	2	26
	4	62	4	26
	5	81	4	46
	6	48	3	42
	7	54	1	28
	8	53	1	25
	9	64	4	23
	10	68	1	25
	11	61	5	15
	12	73	2	29
	13	50	0	18
	14	64	2	31
	15	65	3	25
	16	68	3	18
	17	72	2	6
	18	61	1	31
	19	62	2	19
	20	62	1	13
	21	52	4	21

	22	61	2	11
	23	58	0	28
	24	47	3	28
Union	1	113	0	0
	2	61	0	0
	3	122	1	0
	4	82	0	0
	5	85	0	2
	6	63	0	2
	7	92	0	4
	8	61	0	0
	9	69	0	0
	10	83	0	0
	11	55	0	0
	12	69	0	0
	13	77	0	0
	14	84	0	0
	15	75	0	0
	16	67	0	0
	17	95	0	1
	18	105	0	2
	19	116	0	1
	20	113	0	0
	21	101	0	1
	22	101	0	0
	23	109	0	0
	24	95	0	0