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SCLEROTIA OF *SCLEROTIUM CEPIVORUM*:

FACTORS AFFECTING THEIR FORMATION, SURVIVAL AND

INFECTION OF ONIONS

by

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B.Sc., University of Victoria, 1979

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APPROVAL

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Factors affecting their formation

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ABSTRACT

Initiation of sclerotia of *Sclerotium cepivorum in vitro* was triggered by physical, chemical, nutritional or biological conditions that restricted further growth and expansion of mycelium into new substrate. Sclerotial initials, formed by hyphae branching and looping back upon themselves, were the first stage of sclerotial development. Over time, the number and size of initials increased, and bundles of mycelium aggregated into spherical forms that are characteristic of sclerotia. These aggregates produced an acellular matrix which progressively obscured the surface hyphae and became black. A layer of ovoid rind cells developed beneath the acellular matrix at the surface of the sclerotium. In general, the rind of sclerotia aged in dry conditions had a broken, irregular appearance, compared with that of fresh sclerotia or sclerotia aged under moist, axenic conditions. Sclerotia aged under dry conditions developed 1 to 4 layers of rind cells, while sclerotia kept moist developed only 1 or 2 layers. Structural differences observed between laboratory-produced and natural sclerotia are attributed to differences in moisture conditions under which they matured and aged. Environmental factors influenced the ability of sclerotia to survive, germinate and infect onions. Survival of sclerotia in flooded soil was reduced. Neither immersion in sterile water nor anaerobic conditions alone equalled the effect of flooding, which suggests the involvement of a microflora adapted to flooded, anaerobic soils. Mineral soils from Aldergrove and Summerland, British Columbia, and muck soil from Cloverdale, British Columbia did not show any evidence of suppressiveness to

onion white rot, while muck soil from Burnaby, British Columbia previously reported to be suppressive to white rot was suppressive. The Aldergrove, Summerland and Cloverdale soils became suppressive following autoclaving. This suppressiveness was associated with increased populations of microorganisms compared with those in raw soil. The suppressiveness of Burnaby muck soil was eliminated by autoclaving. Exposure of sclerotia to high sucrose concentrations while recovering them from soil did not affect white rot levels in subsequent experiments.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

A. INTRODUCTION

White rot of *Allium* species, caused by the fungus *Sclerotium cepivorum* Berk. was first described by Berkeley in England in 1841 (Jones and Mann 1963). It is a common disease world-wide and can cause severe crop losses (Adams 1981, Crowe *et al.* 1980, Croxall *et al.* 1953, Merriman and Isaacs 1980, Utkhede 1982). The pathogen is an imperfect fungus with strong taxonomic links to the *Sclerotiniaceae* (Jones *et al.* 1972, Kohn and Grenville 1989 a, b, Novak and Kohn 1988, 1991), although molecular evidence from restriction fragment length polymorphisms suggest that *S. cepivorum* is a strong, distinct taxonomic entity (Kohn *et al.* 1988). The fungus produces abundant black, spherical sclerotia about 200 to 500 µm in diameter (Mordue 1976), although it occasionally produces larger, irregularly shaped sclerotia similar in appearance to those of *Botrytis* spp. (Backhouse and Stewart 1988, Georgy and Coley-Smith 1982). It has been speculated that the large sclerotia may be a vestigial apothecial stage of *S. cepivorum* (Backhouse and Stewart 1988). *S. cepivorum* has no known sexual stage, but produces microconidia which have no known function (Coley-Smith 1960, New *et al.* 1984, Kohn and Grenville 1989 a).

The only known means of survival in *S. cepivorum* is by sclerotia. In nature, the sclerotia are held in dormancy by mycostasis induced by non-sterile field soil (Allen and Young 1968), and germinate and infect only in the presence of a host plant (Coley-Smith and Hickman 1957, Coley-Smith and Holt 1966, Coley-Smith *et al.*

1967, Elnaghy *et al.* 1971, King and Coley-Smith 1969 a, b). The sclerotia can remain viable in soil for long periods, with estimates of longevity ranging from 10 months to more than 20 years (Coley-Smith 1959, Coley-Smith and Sansford 1987, Coley-Smith *et al.* 1990, Crowe *et al.* 1980, Leggett 1983). Dispersal in the field is limited, as the mycelium has only limited ability to grow through soil (Scott 1956 a), but the sclerotia can be carried by wind, water and domestic animals either alone or on infected plant material (Mikhail *et al.* 1974, Utkhede 1982).

Sclerotia are composed of a central medulla and an outer rind. The medulla is made up of interwoven hyphae of moderate wall thickness with the interhyphal spaces filled with a polysaccharide gel, while the rind is composed of thick-walled ovoid cells surrounded by an acellular matrix (Backhouse and Stewart 1987, Entwistle and Munasinghe 1981 b, Kohn and Grenville 1989 a, b, Leggett and Rahe 1985). The medullary cells are full of protein bodies (Backhouse and Stewart 1987) and carbohydrates (Kohn and Grenville 1989 a), while the rind cells are rich in metachromatic granules, probably polyphosphate (Kohn and Grenville 1989 a). The rind cell walls are also rich in phenolic compounds (Kohn and Grenville 1989 a). The composition of the acellular rind matrix is unknown, but is probably rich in melanin, as has been reported for *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Bell and Wheeler 1986), which would be consistent with the role of the rind in survival and persistence (Backhouse and Stewart 1987).

The nutritional requirements of *S. cepivorum* are not specialized and it grows well on a wide variety of general purpose media, including potato dextrose agar (Utkhede and Rahe 1979), malt extract agar (Scott 1956), Czapek Dox agar (Papavizas 1970), sand-cornmeal medium (Coley-Smith and Holt 1966), perlite-cornmeal medium (Esler and Coley-Smith 1984), oatmeal-sugar-sand medium (Allen and Young 1968), oat seed/nutrient broth medium (Sommerville and Hall 1987), and sterile sugar beet seed (Entwistle and Munasinghe 1981 b). Semi-selective media for recovery of *S. cepivorum* have also been developed (Coley-Smith and Javed 1970, Papavizas 1972). Experiments to produce a defined growth medium showed that a wide variety of mono-, di-, tri- and polysaccharides are suitable as carbon sources and most amino acids, casein, casamino acids, inorganic nitrate and ammonium are suitable nitrogen sources (Townsend 1957, Papavizas 1970).

The host range of *S. cepivorum* is limited to the genus *Allium* and a few closely related members of the Liliaceae (Coley-Smith 1960, Coley-Smith and Holt 1966, Coley-Smith *et al.* 1967, Esler and Coley-Smith 1984). *S. cepivorum* has been induced to infect a variety of non-*Allium* plants under sterile conditions with high levels of inoculum (Esler and Coley-Smith 1984, Young and Allen 1969), but infections of those species by *S. cepivorum* in nature are unknown. The basis of this host specificity involves the response of the sclerotia to volatile sulphur compounds associated with host plant root exudates. The sclerotia will germinate spontaneously in a sterile environment (Coley-Smith *et al.* 1967), but can overcome the soil-

3

based mycostasis only in response to the host root exudates (Coley-Smith et al. 1987, King and Coley-Smith 1968, Somerville and Hall 1987). While many of these stimulatory compounds exhibit some antibiotic properties, the breaking of mycostasis does not appear to function in that manner (Coley-Smith et al. 1968). Host volatiles are produced by microbial metabolism of the root exudates, mostly S-alk(en)yl-L-cysteine sulphoxides (Coley-Smith and King 1969, Coley-Smith et al. 1967, Esler and Coley-Smith 1983, King and Coley-Smith 1969 a) into 1-propyl disulphide and 2-propenyl (= diallyl) disulphide (Coley-Smith and King 1969, Coley-Smith and Cooke 1971, King and Coley-Smith 1969 a). The sclerotia can support populations of bacteria that grow on carbohydrates exuded by the sclerotia (Coley-Smith and Dickenson 1971). Large differences in susceptibility exist among *Allium* spp. (Adams 1981, Coley-Smith 1986 a, b, Coley-Smith and Esler 1983), which seem to be based on differences in the concentration and composition of precursors to stimulatory volatiles contained in the host tissue (Coley-Smith 1986 a, b, Esler and Coley-Smith 1983). Those *Allium* species containing principally S-methyl-L-cysteine sulphoxide had low flavour and odour levels and were not very stimulatory of sclerotial germination, while those containing mostly S-1 or S-2-propenyl-L-cysteine sulphoxide had high flavour and odour levels and were very stimulatory to sclerotial germination (Esler and Coley-Smith 1983).

When stimulated to germinate, the sclerotia undergo eruptive germination, where a plug of mycelium breaks through the sclerotial rind at a single point (Coley-Smith 1960, New et al. 1984). Mycelial

germination, in which mycelial strands emerge from many points on the sclerotial rind also occurs, but in nature the normal mode of germination resulting in infection is thought to be the eruptive or plug germination (New *et al.* 1984, Sommerville and Hall 1987). The sclerotia usually germinate only once, although there are reports of formation of secondary sclerotia (Entwistle and Munasinghe 1981 b, Sommerville and Hall 1987). During primary infection, sclerotia germinate within 10 mm of the root (Coley-Smith 1960) and hyphae of *S. cepivorum* penetrate and colonize the tissue inter and intracellularly, destroying the parenchymatous tissue (Abd-El-Razik *et al.* 1973) with the involvement of polygalacturonase and cellulase, among other factors (Mankarios and Friend 1980). Infection cushions are formed by branching of a single hypha on stem tissue, while simple appresorium-like structures are formed by hyphal tips for penetration on roots (Stewart *et al.* 1989 a, b). While the fungus follows the lines of the cell walls during growth outside the host, the stimulus for forming infection structures is probably chemical (Stewart *et al.* 1989 a, b). There is some disagreement about the site of primary infection. Primary infection has been reported to occur principally on the roots, with the infection spreading up the roots to the stem base and bulb (Crowe and Hall 1980 a) or primarily at the stem base (Stewart *et al.* 1989 a). Secondary infection can occur when the fungus spreads from plant to plant, usually where roots or bulbs come in contact with each other (Crowe and Hall 1980 a, Ryan and Kavanaugh 1976, Scott 1956 b), as the ability of the fungus to grow in non-sterile soil is severely limited (Scott 1956 a). Host plant density plays an important role in disease severity (Burdon and Chilvers

1982, Littley and Rahe 1987), probably both by affecting the frequency with which a root encounters a sclerotium to initiate a primary infection and the ease with which plant to plant secondary infections can occur (Crowe *et al.* 1980, Crowe and Hall 1980 a, Ryan and Kavanaugh 1976, Scott 1956 b).

Optimum temperatures for mycelial growth *in vitro* are between 20° and 25° C, while sclerotial germination is optimum between pH 4.8 and 5.3 (Adams and Papavizas 1971, Locke 1967). There is evidence that if sclerotia are exposed for a period of time at 5 to 10° C prior to stimulation by *Allium* extracts, they will germinate quickly in the 10 to 20° C range (Gerbrandy 1989). Sclerotial germination in soil is reported to be optimal at soil moisture levels around field capacity (y_m = -300 mb) and disease development in soil in containers was limited to y_m values between -45 mb and -3 bar (Crowe and Hall 1980 b). Disease in the field is favoured by cool conditions (about 15° C) in fairly dry soil of pH 6 or greater (Adams and Papavizas 1971, Walker 1924), but freezing and thawing reduces germinability (Brix and Zinkernagel 1992 a). Sclerotial survival is reduced by high soil temperatures (greater than 40° C) and extremely low soil moisture (-1200 bars) (Adams 1987). As these conditions are not likely to occur except near the soil surface, they would have little impact on disease levels. Infection can take place throughout the year, but in England tends to be greatest in late spring and summer (Entwistle and Munasinghe 1976). In fall planted onions in the United States, infection takes place mostly in the fall (Adams and Springer 1977). Both of these reports place infection in the cooler part of the growing

season, when soil temperatures would be near 15° C, favouring rapid germination (Gerbrandy 1989). Symptom development on onions is more rapid with increasing temperature between 6° and 24° C (Crowe and Hall 1980 b).

As the sclerotia may remain dormant in the soil for many years, control of the disease is difficult. Early attempts at chemical control using calomel (mercurous chloride) (Croxall et al. 1953, Leach and Seyman 1957) and other mercury compounds (Ali et al. 1976, Leach and Seyman 1957, Utkhede 1982) were quite successful, but environmental and regulatory considerations now prevent their use. A wide variety of fungicides have been evaluated as control measures, but only a few have proven to be commercially useful (Table 1). There are no fungicides currently recommended for white rot control in British Columbia (Anon. 1991). Fungicides have not proven useful in reducing sclerotial populations in soil (De Resende and Zambolim 1987 a). In addition to experiments with fungicides, work has been done with various methods of fungicide placement and application (Entwistle and Munasinghe 1980, Fullerton and Stewart 1991, Leach and Seyman 1957, Littley and Rahe 1983 a, Krauthausen and Schietinger 1989, Porter et al. 1991, Rahe and Littley 1981 b, Stewart and Fullerton 1991), with varying success.

Development of resistance to fungicides in S. cepivorum has not been a problem in practice. Resistance to dicloran has been reported under laboratory conditions (Littley and Rahe 1984) and in the field (Locke 1969), but dicloran has largely fallen out of use for white rot control.

7

Table 1. Summary of results of fungicide trials for control of white rot caused by *Sclerotium cepivorum*.

Chemical	Efficacy[1]	Farm use?	References
benomyl	+/++	No	Ali *et al.* 1976
captafol	-	No	Utkhede and Rahe 1979
captan	-	No	Utkhede and Rahe 1979
carboxin	-	No	Utkhede and Rahe 1979
dicloran	-/+++	Yes	Abd-El-Razik *et al.* 1988, Ali *et al.* 1976, Fletcher *et al.* 1971, Oudemans and Edgington 1984, Utkhede and Rahe 1979, Valdes and Edgington 1986
furalaxyl	-	No	Utkhede and Rahe 1979
iprodione	+/+++	Yes	Abd-El-Razik *et al.* 1988, Entwistle and Munasinghe 1981 a, Fullerton and Stewart 1991, Littley and Rahe 1984 a, Oudemans *et al.* 1983, 1984, Rahe and Littley 1981 a, b, de Resende and Zambolim 1987 b, Utkhede and Rahe 1979
mancozeb	-	No	Fletcher *et al.* 1971
myclozolin	+/+++	No	Krauthausen and Schietinger 1989, Oudemans and Edgington 1984, Rahe and Littley 1981 a, b, 1982
nuarimol	-/+	No	Utkhede and Rahe 1979
procymidone	-/++/+++	No	Fullerton and Stewart 1991, Krauthausen and Schietinger 1989, Oudemans and Edgington 1984, Porter *et al.* 1991, Stewart and Fullerton 1991.
quintozene	+++	Yes	Ali *et al.* 1976, Leach and Seyman 1957, Utkhede 1982
thiophanate methyl	-/+	No	Entwistle and Munasinghe 1981 a, Utkhede and Rahe 1979
thiram	-	No	Utkhede and Rahe 1979
triademefon	-	No	Oudemans and Edgington 1984, Utkhede and Rahe 1979
triadimenol	-/+	No	Utkhede and Rahe 1979
vinclozolin	+/++/+++	No	Fullerton and Stewart 1991, Littley and Rahe 1983 a, b, 1984 a, Rahe and Littley 1981 a, b, 1982, 1983, Utkhede and Rahe 1979
zineb	-	No	Fletcher *et al.* 1971

[1] Compared to untreated control taken at 100% disease, +++ is ≤25% disease, ++ is ≤50%, + is ≤75%, - is ineffective. Variations in efficacy ratings reflect reported differences between trials.

Resistance to the dicarboximide fungicides, of which iprodione is the most widely used for white rot control, has been reported from *in vitro* studies (Littley and Rahe 1984), but no resistant isolates have been recovered from the field (Entwistle 1984). Failure to control the disease with iprodione in the field has been attributed to the development of iprodione-degrading microflora in treated fields (Walker *et al.* 1986). Decline in efficacy of the dicarboximide fungicide myclozolin after only three seasons of testing have been reported (Krauthausen and Schietinger 1989). In general, fungicide treatment of soil can impair onion growth and vigour, and should not be overused (de Bertoldi *et al.* 1978, Entwistle *et al.* 1981, Powell 1975).

There have been several reports of successful biological control of white rot, but none have emerged as a commercially useful control (Entwistle 1988). One of the earliest claims for biological control of white rot was using *Penicillium nigricans* (Ghaffar 1969). However, as control was only achieved in greenhouse trials over the course of two weeks at a rate equivalent to 200,000 kg of amendment per ha, this report has to be regarded as of mainly academic interest. Ahmed and Tribe (1977) were able to achieve significant control of white rot with the hyperparasite *Coniothyrium minitans*. Control was equal to that provided by mercurous chloride, without the phytotoxicity caused by that chemical. A promising avenue of investigation has been focussed on *Trichoderma harzianum*. Abd-El-Moity and Shatla (1981) were able to achieve season-long white rot control in the field, comparable to the use of dicloran, by inoculating plots with *T. harzianum*. By inducing fungicide tolerance in *T. harzianum*, Abd-El-Moity *et al.*

9

(1982) were able to combine the biocontrol agent with iprodione in the field to obtain significant season-long white rot control, significantly better than either the fungicide or *T. harzianum* alone. While work with *T. harzianum* for control of other diseases has continued (Bin *et al.* 1991), progress on controlling *S. cepivorum* with *T. harzianum* seems to have run into technical difficulties (Jackson *et al.* 1991 a). In the Fraser Valley mucklands of British Columbia, *Bacillus subtilis* has been reported to provide significant season long control of white rot (Utkhede and Rahe 1980 b) but the control was not consistent from year to year (Rahe and Littley 1981 b). Work with the mycoparasite *Sporidesmium sclerotivorum* (Ayers and Adams 1979, Adams 1987 a) holds some promise for control of white rot, although research has been concentrated mainly on sclerotial pathogens other than *S. cepivorum* (Adams 1987 a, b, Bullock *et al.* 1986). Many other organisms have been reported to show some activity against *S. cepivorum* under favourable conditions, but field exploitation of this potential has not occurred (Abd-El-Razik *et al.* 1985, Entwistle 1988, Stewart and Harrison 1988).

Progress on biological control of sclerotial plant diseases has been slow. Many of the earlier efforts suffered from a poor understanding of the ecological relationships of the organisms involved and from poor technique (Leggett 1983, Papavizas 1973, Smith 1972). In the last decade, however, there has been more systematic research on screening methods for detection of potential biocontrol agents (Jackson *et al.* 1991 b, Kenerley and Stack 1987, Stack *et al.* 1985), and on application and delivery methods (Jackson

et al. 1991 a) that promise to improve success rates of biocontrol programs in the future. Intriguing discoveries with biocontrol potential for other sclerotial fungi, such as the use of suppressive composts (Hadar and Gorodecki 1991) and mycophagous insects (Anas and Reeleder 1987, 1988 a, b, Anas *et al.* 1989) may eventually be of use for control of *S. cepivorum*.

Cultural control, focussed mostly on inoculum reduction, has also received research attention. White rot can remain a threat to *Allium* crops for years after a susceptible crop is grown in infested soil, because the sclerotia remain dormant and viable in soil for considerable lengths of time (Coley-Smith 1959, Coley-Smith and Sansford 1987, Crowe *et al.* 1980, Leggett 1983). However, the fungus has extremely limited saprophytic ability in soil (Scott 1956 a) and most sclerotia die if they germinate and do not infect a host, despite a limited ability to produce secondary sclerotia (Crowe and Hall 1980 b, Entwistle and Munasinghe 1981 b, Sommerville and Hall 1987). Therefore, inducing sclerotia to germinate in the absence of a susceptible host offers a potential means of inoculum reduction. Normally, the sclerotia are maintained in a dormant state by non-sterile soil, germinating only in the presence of the host plant (Allen and Young 1968, Coley-Smith and Hickman 1957, Coley-Smith and Holt 1966, Coley-Smith *et al.* 1967, Elnaghy *et al.* 1971, King and Coley-Smith 1969 a, b). The sclerotia are stimulated to break dormancy and germinate by several volatile allyl and propyl sulphides produced by microbial metabolism of allyl cysteine sulphoxides contained in many *Allium* root exudates (Coley-Smith and King 1969,

11

Esler and Coley-Smith 1983, King and Coley-Smith 1969 a). Merriman and others have pioneered the use of onion oil, both natural and artificial, as a means of stimulating germination of sclerotia in the absence of the host, thereby reducing inoculum levels (Abd-El-Razik *et al.* 1988, Entwistle *et al.* 1982, Merriman and Sutherland 1978, Merriman *et al.* 1980, 1981, Utkhede and Rahe 1982). While some of these efforts are very promising, results have been erratic (Abd-El-Razik *et al.* 1988, Coley-Smith and Parfitt 1986). More recent work has found distinct seasonal variation in the response of sclerotia to artificial germination stimulants, based on temperature differences (Coley-Smith and Parfitt 1986, Davies 1990, Entwistle *et al.* 1982, Sommerville and Hall 1987) and isolate differences in dose responses (Brix and Zinkernagel 1992 a). This discovery has lead to refinements in the technique that are beginning to show real promise (Crowe *et al.* 1991).

Another approach to inoculum reduction is the use of a trap crop. When a crop of susceptible plants is grown in infested soil, sclerotia germinate and infections occur. If the crop is then destroyed before the pathogen reproduces, inoculum is reduced. This approach was tested by Merriman and Isaacs (1978), but the trap crop of onions had little effect on the population of sclerotia in the soil, so the method may not hold much promise.

Crop rotation is a method that has been successfully used as a means of inoculum reduction in many crops, but has been widely viewed as impractical for onion white rot control due to the persistence and longevity of the sclerotia. Banks and Edgington

(1989) found that a one year rotation to carrots significantly reduced the population of sclerotia in soil, compared to fallow soil or soil planted to onions. The remaining sclerotial population was still high enough that no difference in subsequent disease levels could be observed. Various rotations of cotton, legumes and grains have been reported to reduce sclerotial populations of *S. cepivorum*, an effect enhanced by the presence of *T. harzianum* (Abdel-Rahman *et al.* 1990)

The use of soil amendments has also attracted the attention of some researchers. Incorporation of dried, powdered leaves of *Eucalyptus rostrata* provided significant control of white rot in inoculated soil in pots (Ismail *et al.* 1990, Salama *et al.* 1988). While the amendment had some effects on the levels of phenols and pectolytic enzymes in the plants (Ismail *et al.* 1990), the control seemed to operate in the soil, where there were elevated populations of microorganisms in the amended treatments (Salama *et al.* 1989). Unfortunately, the effect required the addition of one part leaf powder to two parts soil (Salama *et al.* 1988), making it impractical for field use. Joshi (1988) found that incorporation of a winter cover crop of *Brassica juncea* into field soil significantly reduced sclerotia populations, but did not result in a decrease in disease in a subsequent onion crop. This reduction in population probably resulted from stimulation of sclerotia germination by allylisothiocyanate produced by the rotting *B. juncea* plants. While not present in *Allium* spp., this chemical is stimulatory to germination of sclerotia of *S. cepivorum* (Coley-Smith and Parfitt 1983).

Some workers in Egypt have reported success in reducing white rot incidence by the use of pre-planting solarization of the soil (Matrod *et al.* 1991, Satour *et al.* 1991).

Work on the effects of soil fertility (Sirry *et al.* 1974 a) and irrigation regimes (Sirry *et al.* 1974 b) on white rot incidence probably merits further examination given recent progress in this area with other sclerotial pathogens (Canullo and Rodriguez-Kabana 1990, Fang and Liu 1988).

Flooding of infested soil to reduce inoculum levels was first suggested by Leggett and Rahe (1985), to explain the differences they observed between the survival of sclerotia in Fraser Valley muck soils and that reported elsewhere. Alternate wetting and drying of sclerotia of *S. cepivorum* has been shown to result in increased levels of nutrient leakage from sclerotia, ultimately leading to death of the sclerotia (Adams 1987, Leggett *et al.* 1983, Leggett and Rahe 1985, Papavizas 1977) and other sclerotial fungi (Adams 1987, Coley-Smith *et al.* 1974, Papavizas 1977, Smith 1972, Smith *et al.* 1989, Willetts 1971). Joshi (1988) found that flooding significantly increased the rate of decline of sclerotial populations in soil and that incorporation of *B. juncea* residues significantly enhanced the effect. Banks and Edgington (1989) reported that flooding for 3 months over the winter significantly decreased sclerotial populations and subsequent disease levels. They suggested that an integrated approach using crop rotation, flooding and fungicides would provide more satisfactory disease control. Coley-Smith *et al.* (1990) have shown that flooding

significantly enhanced sclerotial decay, but judged the rate of decline of sclerotial populations to be too slow to be of use in the field.

Breeding for disease resistance has shown limited promise. Population level resistance has been reported in some onion and leek cultivars (Coley-Smith 1986 a, Coley-Smith and Esler 1983, Utkhede and Rahe 1978 b, 1980 a, VanderMeer *et al.* 1983) and plant introduction lines (Utkhede and Rahe 1978 a), but these differences were often small and varied among seed lots (Coley-Smith and Esler 1983, Rahe 1986). These differences were not due to differences in the ability of *S. cepivorum* to colonize root tissues (Coley-Smith 1986 a). Differences in susceptibility have not been found among garlic cultivars (Coley-Smith and Entwistle 1988). There is good evidence that differences in susceptibility to white rot are based on differences in the ability of various *Allium* cultivars and species to stimulate sclerotial germination (Coley-Smith *et al.* 1987, Brix and Zinkernagel 1992 b). If this is so, breeding for field resistance may not be useful, as it may select for low levels of the flavour and odour compounds that are the precursors of the volatile germination stimulants. As *Allium* species are grown primarily for flavour and odour characteristics, this is undesirable. In addition, as various *Allium* species are typically grown at different densities, the effect of host density on disease incidence (Littley and Rahe 1987) may confound comparisons of cultivars and species (Rahe 1981), although Coley-Smith (1986 a) found that leeks supported slower plant to plant spread than did several other *Allium* species.

15

What emerges from the foregoing discussion is a picture of a disease organism with a simple asexual life cycle, a single long-lived perennating structure, and a limited host range, yet one that has proven to be extremely difficult to control. The reasons for this inability to develop effective control measures revolve around characteristics of the sclerotia. Crop rotation is of limited value due to the persistence of the sclerotia. Disease resistance and host specificity are based on the response of the sclerotia to host produced germination stimulants. Biological and cultural control methods act by maintaining mycostasis on the sclerotia or by causing death of the sclerotia. Inoculum reduction is seen as the best long term control. Chemical control is difficult due to the resistant and quiescent nature of the sclerotia and the limited time and space when the mycelium is exposed and vulnerable. There appears little hope of a single, effective control strategy for *Allium* white rot. Only by an understanding of the biology and ecology of this organism can an effective integrated approach be developed that will allow the farming of *Allium* crops in the presence of *S. cepivorum.*

The objective of this thesis was to examine some of the characteristics of sclerotia of *S. cepivorum* that could influence its remarkable reproductive and survival abilities. One major emphasis is on factors that induce the fungus to initiate sclerotia formation and on the morphogenic process that leads to this long-lived resistant structure. The second emphasis is on the examination of selected factors that influence the ability of sclerotia to survive in soil, and to infect a host. These include the effects of flooding on sclerotial

survival and the influence of soil microflora on infection ability of this pathogen.

B. FORMATION OF SCLEROTIA

Reproduction and dispersal of propagules are important aspects in the life cycle of any organism. A great deal of research has been conducted on factors responsible for the dormancy of sclerotia of *S. cepivorum* and breaking that mycostatic dormancy (with its link to host specificity). However, factors affecting sclerotial formation have not received much attention. This part of the thesis addresses that aspect. The first section examines the factors that trigger sclerotium formation to understand what causes the fungus to switch from myceliogenic growth to sclerotium formation.

The second section describes the morphological aspects of sclerotium formation. The structure of the sclerotium is probably an important factor for its persistence in soil and for resistance to attack by parasites and predators. Understanding the mechanisms leading from relatively unorganized myceliogenic growth to sclerotial morphogenesis may aid in understanding the factors which contribute to the success of the sclerotium as a perennating structure.

I. Initiation of Sclerotia

The extent of production of sclerotia in the field in the Fraser Valley of British Columbia is extremely variable, with infected plants producing abundant sclerotia in some years and few or none in other years. The factors responsible for initiation of sclerotia in the field are unknown. In culture, initiation and development of sclerotia of *S. cepivorum* appear to occur simultaneously over an entire culture dish. This suggests that an environmental cue or signal, rather than the maturation or aging of the mycelium, initiates formation of the sclerotia.

While the factors responsible for initiating sclerotium formation have been extensively studied in other fungi, only Christias and Lockwood (1973) have examined this phenomenon in *S. cepivorum*. They found that sclerotia were initiated when mycelium of *S. cepivorum* was transferred from a nutrient-rich medium to a nutrient-poor medium. As nutrient deprivation would probably not occur simultaneously over the entire culture, it seemed probable that the mechanism may be more subtle or complex than this result would suggest. Townsend (1957) found that high nutrient levels lead to large numbers of sclerotial initials, but retarded maturation of sclerotia.

Factors affecting sclerotium initiation in *Sclerotium rolfsii* Sacc. have been studied extensively and include restriction of growth by the culture dish (Wheeler and Waller 1965, Zoberi 1980). *S. rolfsii* is slower to initiate sclerotia when cultured on poor media (Wheeler and

Sharan 1965) but when transferred from a rich medium to a poor one, sclerotia are induced (Christias and Lockwood 1973). Punja (1986) found that when growth of *S. rolfsii* was inhibited as the fungus grew from a nitrogen-rich medium to a nitrogen-poor medium, sclerotium formation was induced without wall contact. When the growth rate was slowed by restricting the level of carbon in the medium, sclerotium initiation was reduced. Hadar *et al.* (1983) found that depletion of glucose in the medium would restrict mycelial growth of *S. rolfsii* and induce sclerotium formation.

The role of staling products in the initiation of sclerotia in *S. rolfsii* is less clear. Wheeler and Waller (1965) reported that staling products did not play a role in inducing sclerotial initiation in *S. rolfsii*, while Humpherson-Jones and Cooke (1977 a) reported that staling products in the medium induced sclerotium formation. Light has also been reported to influence sclerotium formation in *S. rolfsii* (Humpherson-Jones and Cooke 1977 a, Trevethick and Cooke 1973). Henis *et al.* (1965) reported that cutting the mycelium with a cork borer induced initiation of sclerotium formation in *S. rolfsii*. Chet and Henis (1975) suggested that a variety of antibiotics that induce sclerotium formation in *S. rolfsii* act by inhibiting cell wall synthesis.

The factors affecting initiation of sclerotia in other sclerotium-forming species are diverse. Henis and Inbar (1968) reported that initiation of sclerotium formation in *Rhizoctonia solani* occurred when growth was restricted by a heat-stable antibiotic produced by *B. subtilis* in culture. Light (Marukawa *et al.* 1975 a, b, Trevethick and Cooke 1973), staling products (Bedi 1958, Humpherson-Jones and

20

Cooke 1977 c, Vega *et al.* 1970), nutritional factors (Chet and Henis 1975, Rai and Agnihotri 1971, Trevethick and Cooke 1971, Wang and Le Tourneau 1972, Willetts 1972, Wong and Willetts 1974), temperature (Bedi 1962, Rawn 1990), specific morphogenic factors (Willetts 1972, Chet and Henis 1975), and endogenous rhythms (Humpherson-Jones and Cooke 1977 b) have all been suggested to influence initiation of sclerotium formation in other species.

A series of experiments was conducted to evaluate some of these factors for inducing sclerotium formation in *S. cepivorum*. The first study tested whether sclerotium initiation was a function of elapsed time. Nutrient availability, accumulation of staling products, mycelial injury, and various barriers to colony expansion were also tested as potential environmental cues for initiation of sclerotium formation.

Materials and Methods

General

All stock and experimental cultures of *S. cepivorum* were obtained from a single sclerotium isolate recovered from a commercial vegetable farm near Cloverdale, B.C. and were maintained on Difco potato dextrose agar (PDA) at $17°\pm1°$ C in the dark, unless otherwise stated. Inoculum for initiation of new cultures consisted of a 5 mm disk of mycelium cut from the actively growing margin of a 6 to 7-day old culture. Time at which melanization of the sclerotia was sufficient to be seen in the mycelium by the unaided eye was considered to be time of sclerotium formation.

Elapsed Time

To test whether sclerotium formation was a function of elapsed time, Petri dishes of four different diameters were filled to approximately the same depth with PDA. These were 135 mm diameter (150 mm nominal) containing 45 ml, 85 mm diameter (100 mm nominal) containing 18 ml, 57 mm diameter (60 mm nominal) containing 9 ml, and 33 mm diameter (35 mm nominal) containing 4.5 ml. The PDA was poured at the same time from the same batch using an automatic pipetor. Ten replications of each dish size were inoculated with *S. cepivorum*. Colony diameter was measured daily and the time of sclerotium formation was noted.

Nutrient Availability

To test whether limited nutrient availability could initiate sclerotium formation, *S. cepivorum* was inoculated onto 100 x 15 mm Petri dishes containing either 15 ml of water agar (WA), 1/10, 1/2 or full-strength PDA. All media contained 1.5% agar. Each treatment was replicated 10 times. The plates were examined daily, and colony diameter and time of sclerotia formation were recorded.

Nutrient Gradient

A third experiment tested the hypothesis that a threshold nutrient concentration could induce sclerotium formation. Ten replicate 100 x 15 mm Petri plates, each with 18 ml of WA, were allowed to solidify on a slant such that one side of the plate had a thickness of less than 1 mm, while the opposite side was about 8 mm

thick. Then, 18 ml of PDA was added to the dish (laid flat) to form a gradient of the same dimensions as the WA, but in the opposite direction. Thus, the medium was the same thickness over the entire plate, but the thickness (and hence the amount) of the nutrient portion of the medium varied continuously across the plate. Control plates contained 36 ml of PDA. The centre of each plate was inoculated with *S. cepivorum.*

Staling Products

Experiment A

The effect of the accumulation of staling products in the medium on the induction of sclerotium formation was tested. The first treatment consisted of *S. cepivorum* growing on about 35 ml of potato dextrose broth (PDB) in 25 x 100 mm Petri dishes. The mycelium was contained within the Petri dish by a circular screen which measured 80 mm in diameter by 20 mm deep with a mesh size of 0.5 mm. These cultures were incubated without agitation at $17^\circ \pm 1^\circ$ C in the dark. The second treatment was identical, except that every second day the screen containing the mycelium was removed from the PDB, rinsed twice in sterile distilled water and placed in fresh PDB. Both treatments were replicated six times.

Experiment B

Six replications of four treatments, each based on 18 ml of PDB in 100 x 25 mm Petri dishes were used, with the addition of 6 ml of one of PDB (control), sterile distilled water (dilution control), 'new stale'

PDB, or 'old stale' PDB. The 'new stale' PDB was from a PDB culture of *S. cepivorum*, inoculated about 2 months previously. The 'old stale' PDB was from a PDB culture inoculated about 14 months earlier. Both stale media were sterilized by filtration through a 0.45 µm membrane filter and were plated onto PDA and incubated to check for sterility.

Mycelial Injury

The effect of mycelial injury on inducing sclerotium formation was tested. Five days after inoculation on PDA, the mycelium of *S. cepivorum* in the treatment plates was sliced with a sterile scalpel at about 1 cm intervals over the entire plate, while the control plates were left untouched. Each treatment was replicated six times. All plates were then incubated and the time of sclerotium formation in each treatment was noted.

Barriers to Colony Expansion

Physical Barrier

A series of experiments was performed to examine the effects of various barriers to colony expansion as inducers of sclerotium formation. The effect of a physical barrier to colony expansion was tested using barriers made by cutting the lid of a 60 x 15 mm polystyrene Petri dish in half and placing one half of the lid in an inverted position onto the bottom of a 100 x 15 mm dish. The 100 x 15 mm Petri dish was then filled with about 30 ml PDA. The rim of the cut lid then formed a vertical semi-circular barrier in the medium about 2/3 of the distance from the centre to the edge of the dish. Control

24

plates contained 30 ml of PDA with no barrier. Ten replicate plates for each treatment were inoculated in the centre with *S. cepivorum.*

Antibiotic Barrier

Utkhede and Rahe (1980) reported that *B. subtilis* strain EBW2 produces a diffusible antibiotic that inhibits the growth of *S. cepivorum in vitro.* The inhibition zone produced by this strain of *B. subtilis* was used as a barrier to colony growth and expansion. EBW2 was streaked onto 100 x 15 mm PDA plates in a circle with a radius of about 30 mm and a 5 mm plug of *S. cepivorum* was placed in the centre. Control plates had no bacteria.

Competition Barrier

To test whether the effect on the initiation of sclerotium formation by *B. subtilis* was due to a restriction of growth and not due to a specific antibiotic effect, *S. cepivorum* cultures were plated against themselves to provide a restriction of growth without antibiotic effects. The control plates were 100 x 15 mm Petri dishes of PDA inoculated with one plug of *S. cepivorum* about 1 cm from one edge of the dish. The treatment (dual) plates were inoculated with two plugs of *S. cepivorum* on opposite sides of the dish, each about 1 cm from the edge of the dish.

Nutrient Barrier

The effectiveness of a nutrient step-down as a barrier to colony expansion and inducer of sclerotium formation was tested. Metal cookie tins (190 mm in diameter by 70 mm deep) were used as Petri

dishes to provide a large surface area for growth. Each tin was divided into two sections by a 10 mm high glass strip held in place with silicone rubber caulking. For the nutrient barrier dishes, one half of the dish was filled with PDA to a point where the liquid medium would just cover the top of the glass strip, but would be prevented from spilling into the other compartment by surface tension. When the PDA solidified, the other half of the dish was filled with WA to the same level. This provided a continuous growth surface with a diffusion barrier between the two media. Control dishes were filled with PDA on both sides of the barrier. All dishes were inoculated on the PDA, 10 mm from the centre of the barrier.

Results

Elapsed Time

Rate of radial growth of the colonies was the same in all treatments, until contact was made with the edge of the dish (Figure 1).

Sclerotia formed 7, 8, 9 and 10 days after inoculation in the 33, 57, 85 and 135 mm dish sizes, respectively. In all cases, sclerotia were visible 4 days after the plate was fully colonized. No significant variation occurred among replications within a treatment in the time to sclerotium formation.
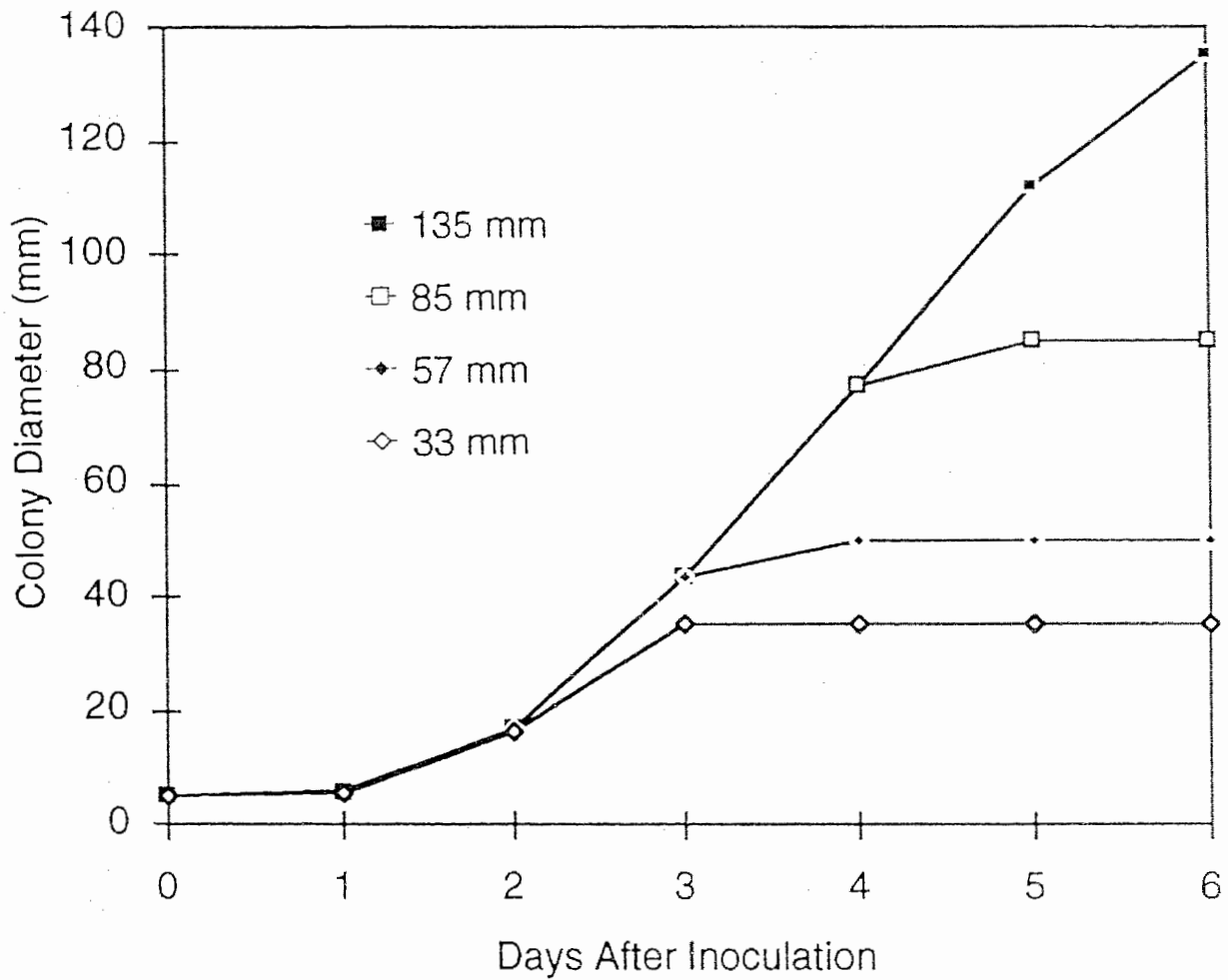
Figure 1. Growth of colonies of *Sclerotium cepivorum* on potato dextrose agar in various dish sizes.

Nutrient Availability

The rate of colony growth depended on nutrient concentration, with full-strength PDA producing the highest growth rate and WA the lowest (Figure 2).

Sclerotia formed 7, 8, 11 and 25 days after inoculation on full, 1/2 and 1/10 strength PDA and WA, respectively, 3 days after reaching the edge of the dish in the case of the PDA treatments. Mycelial growth was extremely sparse on WA, with only a few mycelial strands reaching the edge of the dishes. No variation in time to sclerotium formation occurred among replications within a treatment.

Nutrient Gradient

Sclerotia developed in the control plates 9 days after inoculation, 4 days after the mycelium reached the walls of the dish. Sclerotia developed 11 days after inoculation in the nutrient gradient plates and 4 days after the mycelium reached the walls of the dish. The growth rate on the gradient plates was slower than on the control plates containing full depth PDA. Sclerotia formed simultaneously over the entire surface of the medium, irrespective of the gradient. Sclerotia were much more numerous where the PDA was deep than where it was shallow.
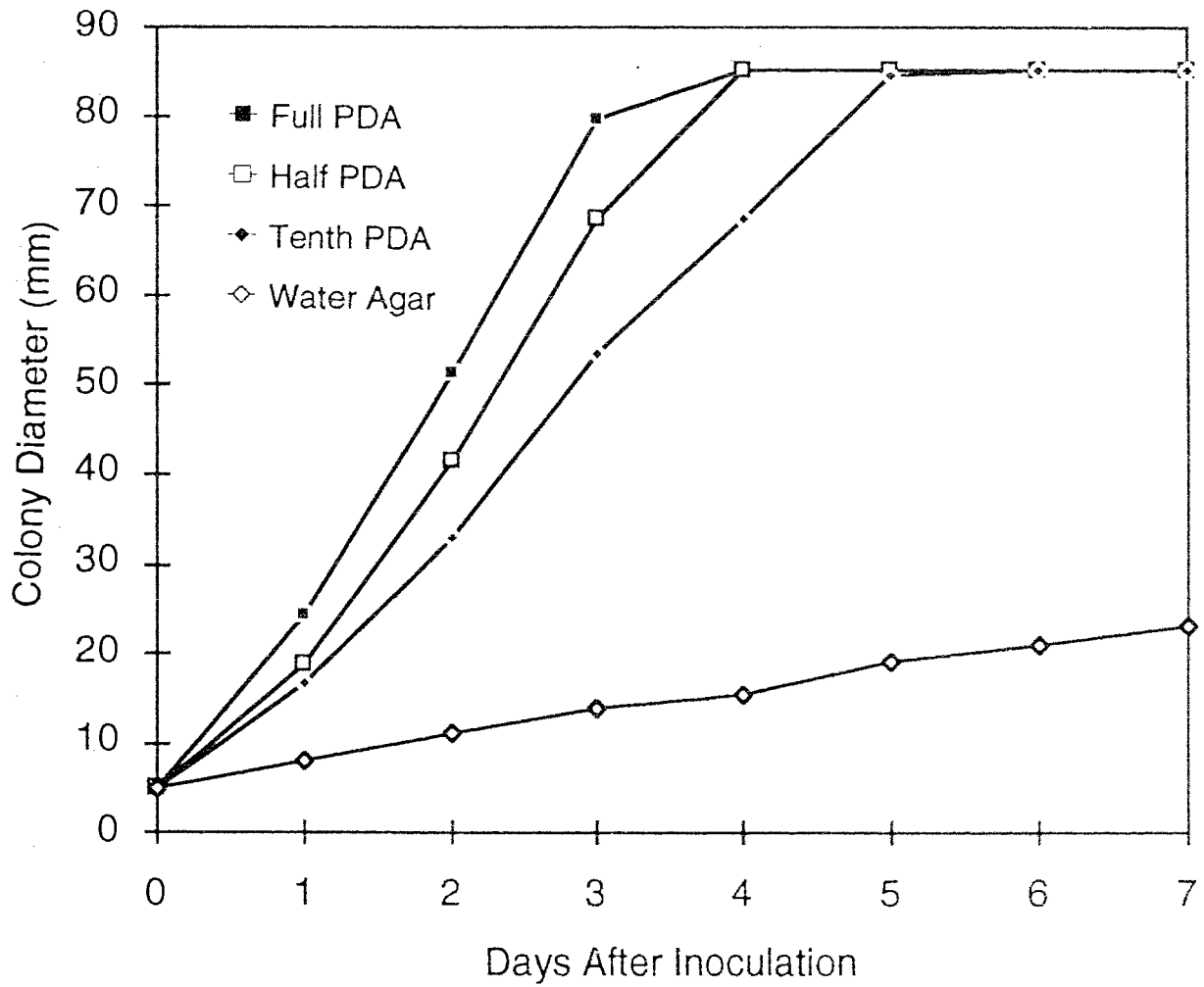
Figure 2. Growth of colonies of *Sclerotium cepivorum* on full, 1/2, and 1/10 strength potato dextrose agar (PDA) and on water agar.

Staling Products

In the fresh medium experiment, sclerotia developed in both treatments 13 days after inoculation in all replications. Sclerotia initially formed in both treatments in a central circular area about 3 cm in diameter, but by 20 days after inoculation sclerotia had formed over the entire colony. Sclerotia were more numerous in the treatment with frequent medium changes.

In the old media experiment, none of the filtered old media produced any colonies when streaked onto PDA. Twelve days after inoculation, all cultures of *S. cepivorum* formed sclerotia in all replications of both treatments. No differences were observed among treatments in number, pattern or density of sclerotia.

Mycelial Injury

Nine days after inoculation, sclerotia formed in all cultures, in both the treatment and control, in all replications.

Barriers to Colony Expansion

Physical Barrier

Sclerotia appeared inside the barrier 6 days after inoculation, and while the medium behind the barrier was beginning to be colonized at that time, sclerotia did not form beyond the barrier for a further 4 days.

Antibiotic Barrier

When the mycelium of *S. cepivorum* grew toward the colony of *B. subtilis,* a clear inhibition zone 10 to 15 mm wide with blackened margins was formed where neither organism grew. This interaction has been described by Utkhede and Rahe (1980). Sclerotia formed in colonies of *S. cepivorum* growing adjacent to *B. subtilis* 7 days after reaching the inhibition zone, 3 days before they formed in colonies in the control plates. Colonies growing on the control plates formed sclerotia 5 days after reaching the edge of the plate.

Competition Barrier

In the dual *S. cepivorum* plates, sclerotia had formed over all of the dual plates 11 days after inoculation, but had formed only between the inoculation plug and the nearest dish wall in the singly inoculated plates. There was no evidence of any inhibition between the two colonies in the doubly inoculated plates.

Nutrient Barrier

Sclerotia formed 7 days after inoculation in the area between the point of inoculation and the WA portion of the plate. The colony reached the sides of the dish in the PDA portion 6 days after inoculation and sclerotium formation occurred in that area 10 days after inoculation. Very little growth took place on the WA and the growth decreased with increasing distance from the PDA. No sclerotia were formed on WA after 20 days.

<u>Discussion</u>

The results of the elapsed time experiment support the hypothesis that initiation of sclerotia in cultures of *S. cepivorum* is associated with full colonization of the surface of the growth medium. This result implies that formation of sclerotia is unrelated to mycelial maturation (and, therefore, the total elapsed time from inoculation), but is associated with the elapsed time from full colonization of the substrate. The initiation of sclerotium formation in this system cannot be associated with the fungus achieving a critical biomass, as this would have been achieved by all treatments at the same time, as the growth rates were similar.

Therefore, what quality of the fully colonized substrate is the trigger for sclerotium initiation? If limited nutrient availability *per se* was the trigger for sclerotium formation in *S. cepivorum*, one would have expected to see sclerotia form earlier in the dilute media. In fact, the opposite occurred; both growth of the fungus and initiation of sclerotia was positively correlated with increasing nutrient concentration, as can be seen in Figure 2. Therefore, this model is rejected. The dilute media restricted the growth rate of the colony and delayed the full colonization of the substrate. This is similar to the results reported by Wheeler and Sharan (1965) for *S. rolfsii*.

Both of the staling products experiments directly tested the hypothesis that an accumulation of staling or signal compounds in the medium is responsible for triggering formation of sclerotia. The fact that regular changes of medium did not delay formation of sclerotia in

the fresh medium experiment argues against the staling products hypothesis. If the accumulation of a staling product or signal compound is a trigger for sclerotium induction, the addition of stale medium should hasten sclerotium formation. However, the addition of stale medium to the growth medium of S. cepivorum had no effect on the induction of sclerotium formation. From the results of these two experiments it is concluded that the accumulation of staling or signal products in the medium does not trigger sclerotium formation in S. cepivorum.

From the results of the mycelial injury trial, it is concluded that mycelial injury does not play a role in induction of sclerotium formation under these conditions. The physical barrier experiment, however, showed that restriction of colony expansion by the side wall of a Petri dish induced sclerotium formation. A similar effect was reported by Wheeler and Waller (1965) and Zoberi (1980) in S. rolfsii. These results also show that the restriction of growth by antibiosis was a triggering factor for sclerotium formation. This result is similar to the report by Henis and Inbar (1968) of sclerotium formation by R. solani when growth was restricted by a heat-stable antibiotic produced by B. subtilis in culture.

It is possible that the triggering of sclerotium formation by antibiosis observed in the bacterial barrier trial was the result of a specific biochemical or physiological effect of the bacterial antibiotic, rather than a non-specific restriction of growth. The dual cultures of the same isolate of S. cepivorum showed that non-specific restriction of growth and expansion by competition in the absence of antibiosis

33

was an effective trigger of sclerotium formation. This result strengthens the conclusion that the triggering of sclerotium formation by bacterial inhibition was due to a non-specific restriction of growth and not a specific response to the antibiotic. Similarly, the triggering of sclerotium formation by a nutritional barrier, i.e. growing from a rich medium to a poor medium, was likely due to non-specific restriction of growth. This is consistent with the results reported for *S. cepivorum* and *S. rolfsii* by Christias and Lockwood (1973) and for *S. rolfsii* by Hadar *et al.* (1983) and Punja (1986).

The evidence presented here shows that restriction of mycelial growth in *S. cepivorum* after a period of rapid growth and expansion initiates sclerotium formation. Whether the restriction of growth is physical, nutritional, through competition or antagonism seems to be irrelevant; it is the restriction of growth that is the trigger. Physical injury of the mycelium and staling products do not play a role in inducing sclerotium formation in *S. cepivorum*. Growth in a nutrient deprived substrate is slower, so it takes longer to 'run out of room' in a substrate of a given size. Townsend (1957) showed that in general, conditons favouring mycelial growth favoured initiation of large numbers of sclerotia, but did not favour sclerotial development and maturation. The original observation that sclerotia form simultaneously over the entire dish is an artifact of inoculating plates in the centre, so that the mycelium reaches all sides of the dish at the same time.

Presumably, sclerotium formation on *Allium* hosts is triggered when further colonization of the host is prevented. The nature of this

delimitation of colonization would make an interesting subject for further investigation.

II. Sclerotial Morphogenesis

Once the signal for initiation of sclerotial formation has occurred, the elaboration of unorganized mycelium into an organized sclerotium must begin. Sclerotial ontogeny of *S. cepivorum* is only partially known. A description of the formation of sclerotia of *S. cepivorum* using light microscopy has been published (Townsend and Willetts 1954), but this work covered only the early stages of sclerotium development. The anatomy and histochemistry of mature and germinating sclerotia of *S. cepivorum* have been described (Backhouse and Stewart 1987, Jones *et al.* 1972, Kohn and Grenville 1989 a, b, New *et al.* 1984). As there is no published description of the entire process of formation, maturation and aging of sclerotia of *S. cepivorum*, the ontogeny of sclerotia of *S. cepivorum in vitro* and the effects of maturation and aging on the structure of the sclerotia were examined.

Sclerotia of *S. cepivorum* produced axenically on sand-cornmeal medium have been reported to decay more rapidly under field conditions than field-collected sclerotia (Leggett 1983). Coley-Smith (1985) has expressed concerns about differences in the behaviour of sclerotia produced on laboratory media compared with that of natural sclerotia. In general, the relationship between the survival of fungal sclerotia in the field and their origin is variable. Survival of laboratory-produced sclerotia has been found to be shorter (*Macrophomina phaseolina*, Short *et al.* 1980), the same (*Sclerotinia minor*, Imolehin and Grogan 1980) or longer (*S. sclerotiorum*, Merriman 1976) than that of field-collected sclerotia.

36

Leggett (1983) attributed the reduced survival of laboratory-produced sclerotia of *S. cepivorum* to differences in rind structure, since laboratory-produced sclerotia had a thicker, more fractured rind than field collected sclerotia. Backhouse and Stewart (1987) surmised that maintenance of an intact rind was a factor in survival of sclerotia of *S. cepivorum.* Stewart and Harrison (1988) reported that several mycoparasites capable of killing sclerotia of *S. cepivorum* gained entry to the sclerotia through gaps in the rind. Other workers have postulated an important role for rind integrity in sclerotial survival in other fungi (Chet 1969, Coley-Smith and Cooke 1971, Linderman and Gilbert 1973).

The nature of the differences between field and laboratory-produced sclerotia are complex. Laboratory-produced sclerotia are generally produced axenically, without the influences of microbial competition, predation, parasitism or antibiosis. When axenically-produced sclerotia are introduced to soil, they acquire a microflora which may or may not be similar to that of natural sclerotia. Naturally produced sclerotia are subject to fluctuations in moisture, temperature and aeration which laboratory-produced sclerotia do not generally experience. There is often an age difference between field collected and laboratory-produced sclerotia in experimental systems. Natural sclerotia must either be collected from infected onions in the field and stored until used, or recovered from soil. Consequently, the sclerotia will be several to many months old when examined. Laboratory-produced sclerotia, on the other hand, can be produced year-round and are of known age.

With these variables in mind, sclerotia that had been produced and stored under various conditions were examined to assess whether the differences reported by Leggett (1983) between laboratory-produced and natural sclerotia were due to the method of production *per se*, or to some other factor correlated with the method of production.

Materials and Methods

Sclerotia of *S. cepivorum* were collected from infected onions in a commercial onion farm near Cloverdale, British Columbia. They were stored in field soil in open 2L clay pots with a drain hole in the bottom, outdoors, exposed to fall weather conditions, until used. An isolate of *S. cepivorum* obtained from a single sclerotium from this collection was the source of all laboratory-produced sclerotia used in this study. The fungus was cultured on PDA in 100 x 15 mm petri dishes at $17° \pm 1°$ C in the dark. All plates were inoculated with a 5 mm diameter agar plug taken from the edge of an actively growing culture of *S. cepivorum* inoculated 7 days earlier. Field collected sclerotia were recovered from the soil in the clay pots using the method of Vimard *et al.* (1986).

To examine the morphogenesis of sclerotia of *S. cepivorum*, PDA plates inoculated as described above were sampled daily from a circle with a radius of about 1.5 cm from the centre of the dish. Samples consisted of 5 mm diameter plugs cut out of the PDA with a cork borer, which were then processed for scanning electron microscopy (SEM) or light microscopy (LM).

Samples for SEM were treated with a version of the ligand-mediated osmium coating technique (Kelley *et al.* 1973). They were fixed in 4% glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.2) and were post-fixed in 1% OsO_4 in the same buffer. After thorough rinsing in buffer, they were immersed in a saturated aqueous solution of thiocarbohydrazide (Eastman Organic Chemicals, Rochester, New York) for 10 minutes, rinsed thoroughly in buffer and re-treated with 1% OsO_4 in buffer for 1 hour. The fixed agar plugs were then completely dehydrated in a graded ethanol series in 10% increments over 24 h, followed by a graded amyl acetate/ethanol series in 25% increments over 24 h. The plugs were then critical-point dried in CO_2. Dried plugs were affixed to aluminum SEM stubs with graphite glue, gold coated in a vacuum evaporator and examined with an ETEC Autoscan SEM (ETEC Corporation, Hayward, California) at 20 kV.

Samples for LM were trimmed to cubes with sides of about 2 mm, fixed in 4% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 24 h and dehydrated in a graded ethanol series in 10% increments over 24 h. They were then infiltrated and embedded in JB4 plastic (J.B. EM Services, Pointe Claire, Quebec) in closed BEEM capsules. Blocks were sectioned (1 - 1.5 µm) using a glass knife on a Reichert® ultramicrotome, and stained with toluidine blue containing 1% sodium borate, with heat, for 3 minutes.

To examine changes in sclerotial morphology over time under different conditions, sclerotia from different treatments were examined using SEM and LM by the methods described above. 'Old

dry axenic sclerotia' were obtained from a sand-cornmeal (20:1) culture of *S. cepivorum* inoculated 12 months earlier and allowed to dry axenically after sclerotium formation. 'Old moist axenic sclerotia' were obtained from PDA plates inoculated 12 months earlier and sealed with Parafilm® (American National Can Company, Greenwich, Ct.) to prevent desiccation. 'Axenic onion grown sclerotia' were produced aseptically on onions following the method of Coley-Smith (1985). Field collected sclerotia were recovered from storage in soil in pots as described above.

Results

At 6 to 7 days after inoculation, bundles of hyphae began to appear in the mycelium (Fig. 3). These were formed by hyphal filaments branching and folding back upon themselves to produce bundles of more or less parallel hyphae (Fig. 4). Over the next 2 days, these strands developed to produce curving, hyphal bundles in the mycelial mat (Fig. 5). In cross section at this stage, there was very little specialized structure, and the orientation of the hyphae was not highly organized (Fig. 6).

Figs. 3 - 6. Sclerotium development in *Sclerotium cepivorum* 6 to 8 days after inoculation onto potato dextrose agar. Fig. 3. Mycelium 6 days after inoculation. Note the developing sclerotial initial (arrow), formed by hyphal filaments branching and folding back upon themselves to produce bundles of more or less parallel hyphae. x200. Fig. 4. Closer view of sclerotial initial, 6 days after inoculation. x1750. Fig. 5. Sclerotial initials develop into theses bundles of hyphae, 8 days after inoculation. x1115. Fig. 6. Cross section of hyphal bundle, 8 days after inoculation. x230.

42
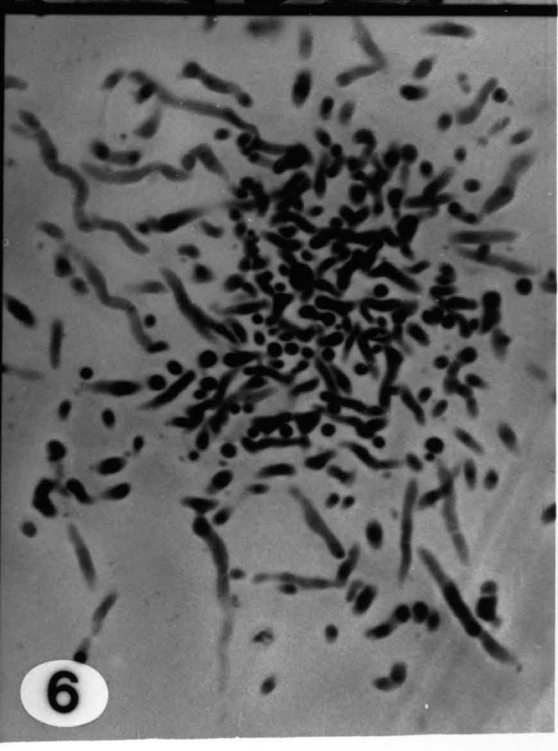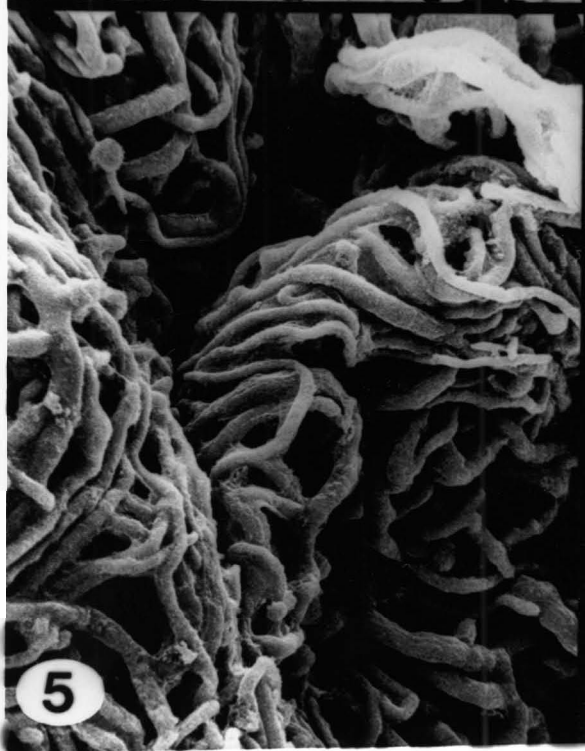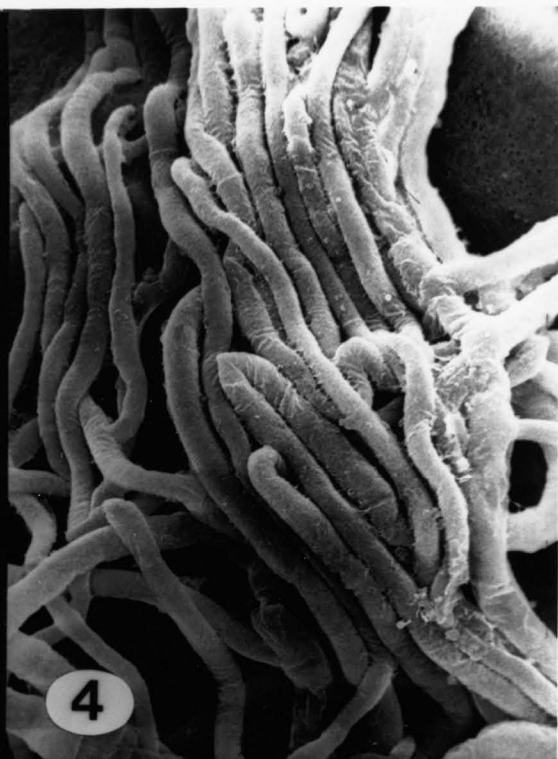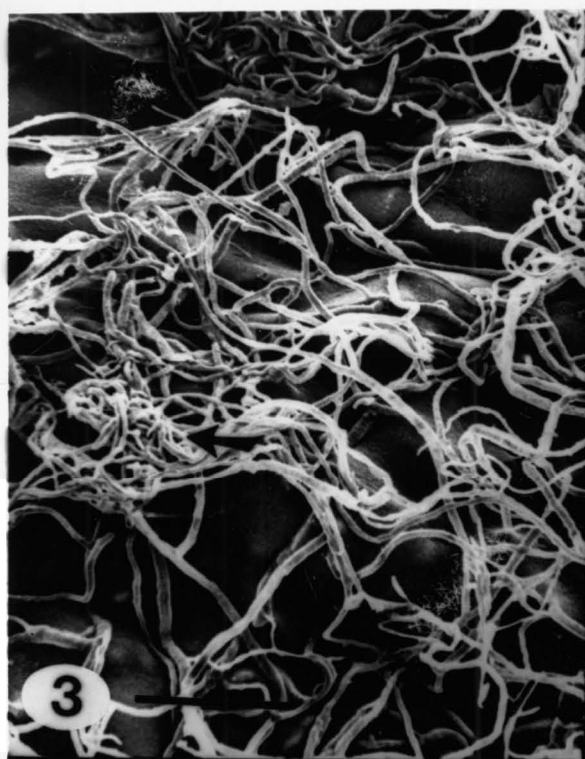
Growth of these hyphal bundles occurred, so that by 12 days they had grown to be a ball of hyphal strands, with most of the hyphae on the surface lying parallel to the surface of the new sclerotium (Fig 7). Phialides bearing microconidia were visible at this time (Fig 7). In cross section, the structure of the forming sclerotium was well established by this time (Fig. 11). The orientation of the mycelium in the medulla of the forming sclerotium was random, while the hyphae that eventually form the rind were lying parallel to the surface. The intensity of staining of the rind cells was noticeably greater than that of the medullary cells at this stage. An extracellular matrix began to appear on the rind, gradually covering and obscuring the hyphae (Fig. 8). The ends of a few hyphae projected from the surface at this time (Fig. 9). This period of differentiation was associated with the contents of the rind cells staining more darkly, when seen in cross section (Fig. 12). Much of the darkly staining rind cell contents was abundant granules showing strong metachromatic staining. By 13 to 14 days, the extracellular matrix had completely obscured the rind hyphae, and melanization had occurred (Fig. 10). The outer surface was smooth, with only a few hyphae adhering to the matrix surface.

In sclerotia aged 12 months in moist conditions on PDA, the rind layer was thicker and more broken in appearance than the freshly produced sclerotia on PDA (Fig. 13), and the rind layer was one to two cells thick. Many of the outermost rind cells appeared to be empty and some had broken and collapsed. The medullary cells were now filled with granules showing strong metachromatic staining.

Figs. 7 - 10. Sclerotium development in *Sclerotium cepivorum* 12 to 14 days after inoculation onto potato dextrose agar. Fig. 7. Newly formed sclerotium, 12 days after inoculation. Note that the majority of hyphal strands are lying parallel to the surface. Phialides bearing microconidia are visible in the upper right corner of the micrograph (arrow). x415. Fig. 8. Matrix beginning to obscure the rind hyphae on newly formed sclerotium, 12 days after inoculation. x1475. Fig. 9. Maturing sclerotia, showing rind matrix forming. Note projecting hyphal tips in the area between the sclerotia. x735. Fig. 10. Completely formed sclerotium. Note how the extracellular matrix has obscured the rind hyphae. x385.
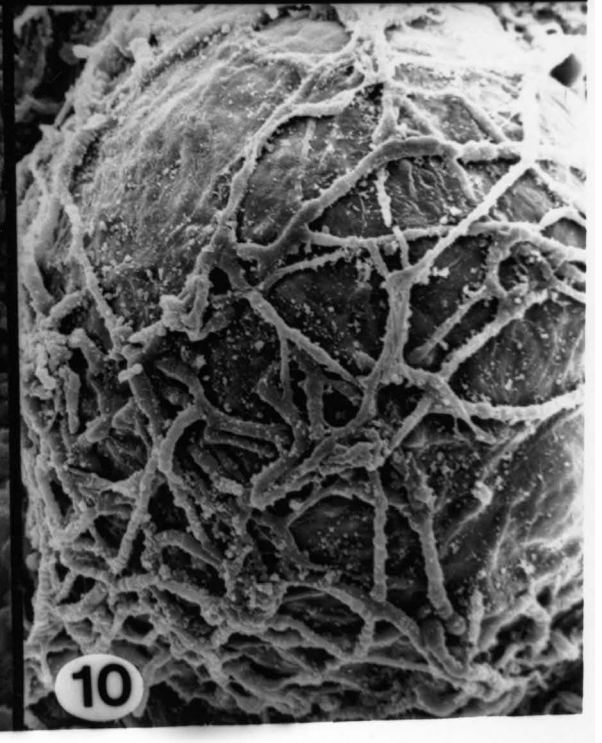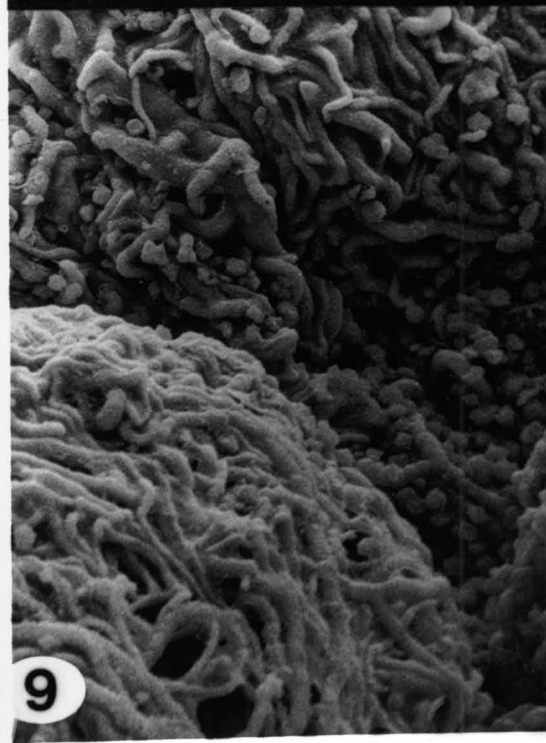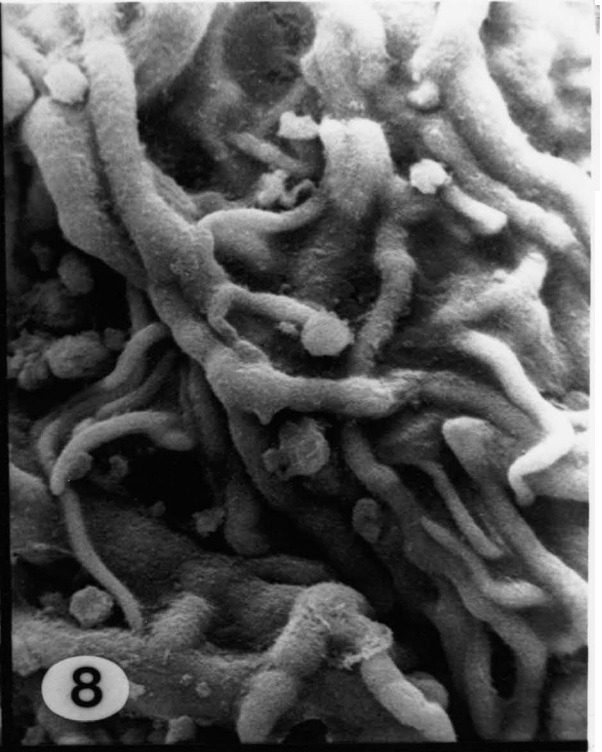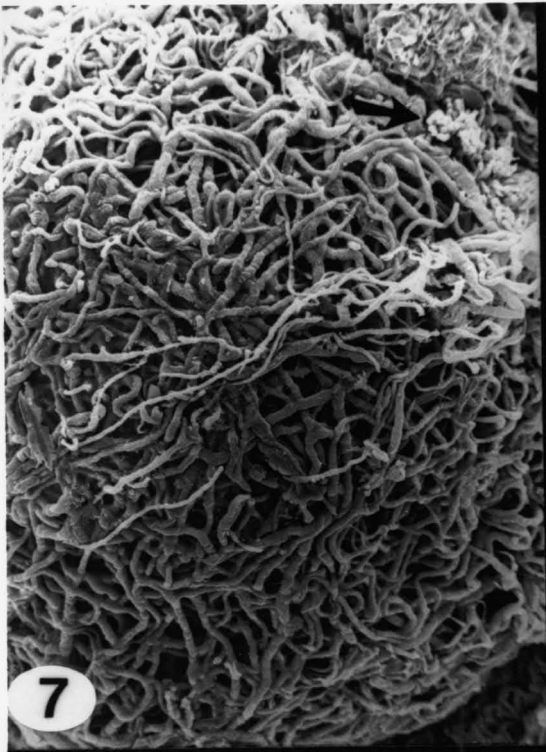
Sclerotia which had been aged 12 months in dry axenic sand cultures typically developed a much thicker rind than freshly produced sclerotia from PDA, up to four cells thick in places (Fig. 14). This was similar to the appearance of field collected sclerotia (Fig 15) which had been exposed to natural wetting and drying. In these sclerotia, most of the rind cells appeared to contain cytoplasm. The broken appearance of the rind appeared to be due to the collapse of outer rind cells.

PDA-grown sclerotia kept in moist axenic conditions for 12 months did not develop the thickened rind of the axenic dried sclerotia of the same age (Fig. 16). The rind layer on these sclerotia appeared comparable to that of the freshly produced sclerotia (Fig. 17). There was little of the broken appearance of the rind associated with sclerotia from dry conditions (Figs. 14 and 15).

Most sclerotia produced axenically on onions appeared similar to those produced on PDA (Fig. 18), although a few onion-produced sclerotia did show intermediate rind thicknesses. The outermost rind cells contained cytoplasm, although it appeared that many of the pits on the surface of the rind were the remains of collapsed cells.

A comparison of SEM micrographs of sclerotial surfaces revealed that the field sclerotia had a much more pitted, broken appearance (Fig. 19) than freshly produced sclerotia from PDA (Fig. 10).

Figs. 11 - 16. Cross sections of sclerotia of *Sclerotium cepivorum* at various stages of development. Fig. 11. Newly formed sclerotium, 12 days after inoculation on potato dextrose agar. The orientation of the mycelium in the medulla is random, while the rind hyphae run parallel to the surface of the sclerotium. x310. Fig.12. Newly formed sclerotium, 14 days after inoculation. Note the intense staining of the rind cells. x310. Fig.13. Sclerotium, 30 days after inoculation. Rind is more broken in appearance and some of the outermost rind cells are empty. x455. Fig.14. Sclerotium from dry axenic sand culture, approximately 12 months old. The rind is thick and most rind cells contain cytoplasm. x765. Fig.15. Field collected sclerotium approximately 14 months old. Note thickness and broken appearance of the rind. x455. Fig 16. PDA-grown sclerotia kept moist 12 months. The rind is thin and intact. x455.
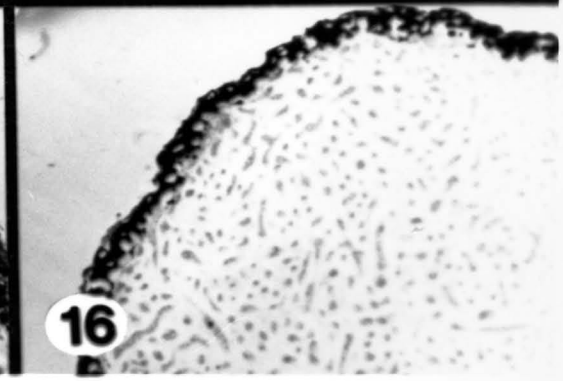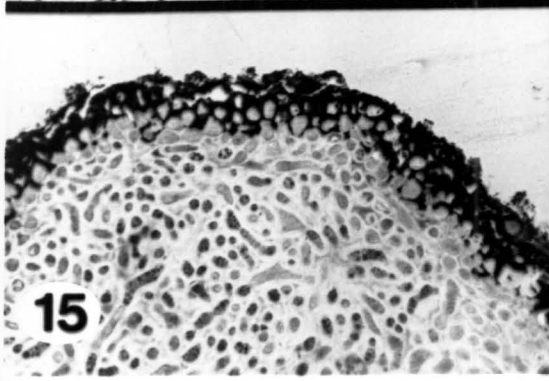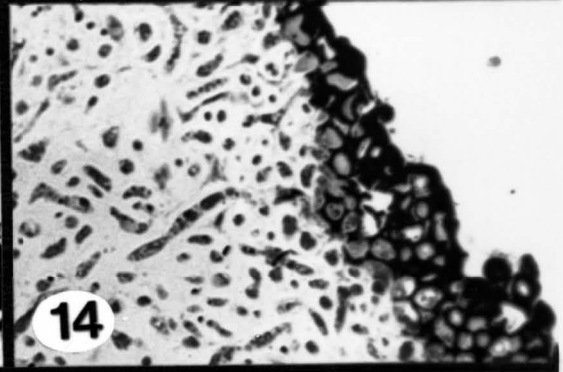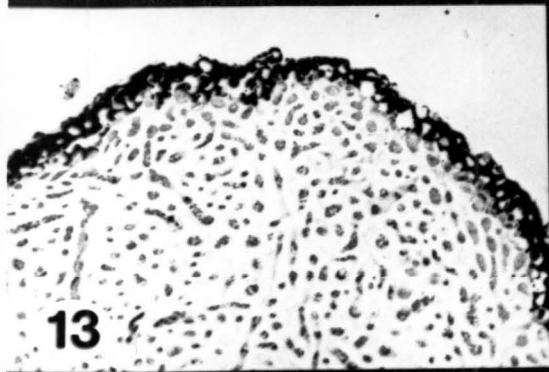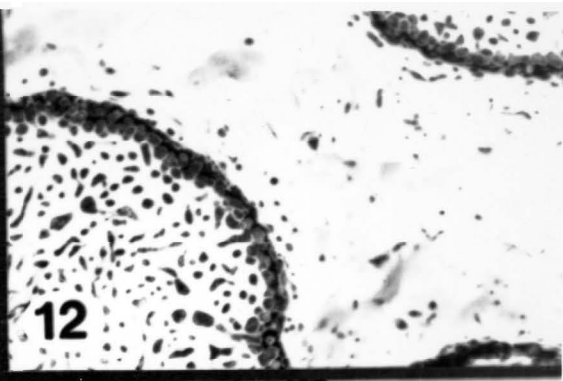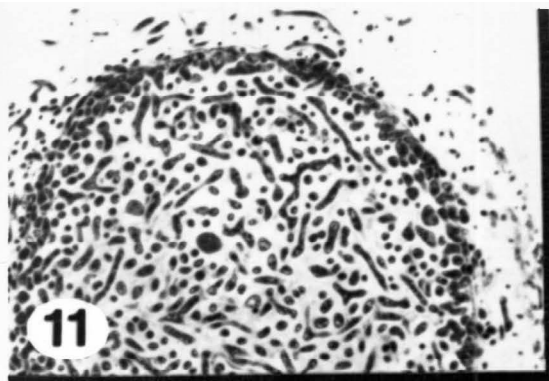
Figs. 17 - 19. Sclerotia of *Sclerotium cepivorum* from various sources. Fig. 17. Freshly produced sclerotia from potato dextrose agar. The rind is thin and intact. x455. Fig. 18. Sclerotia produced axenically on onions. Note the similarity to freshly produced sclerotia from PDA. x455. Fig. 19. Field collected sclerotium. Note the broken, pitted appearance. x505.
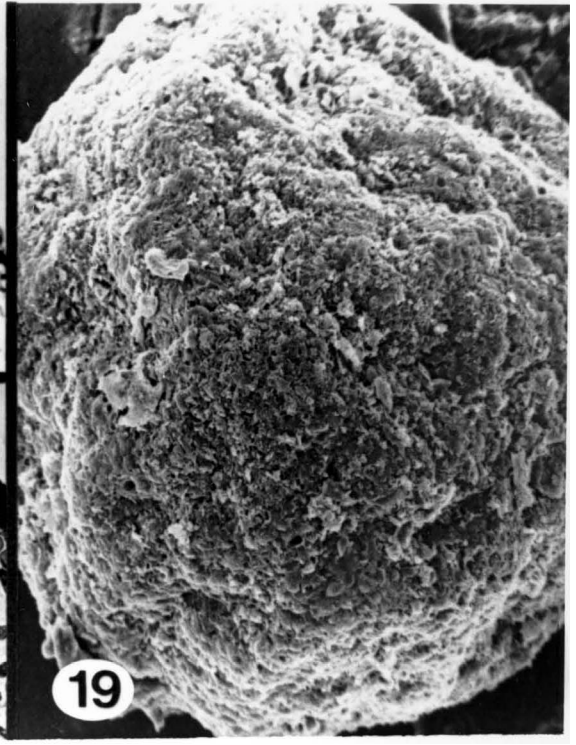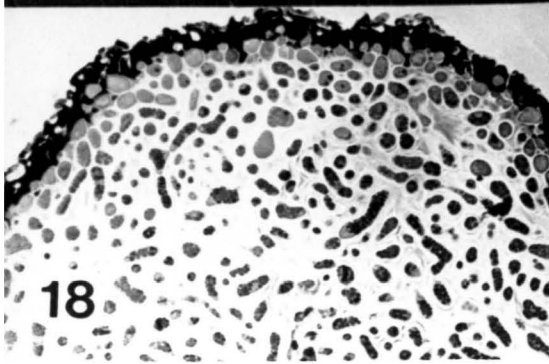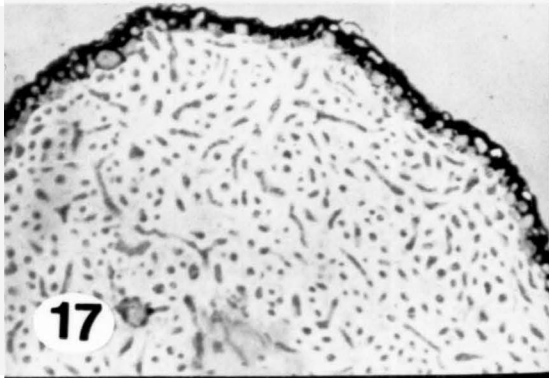
Discussion

Sclerotia of *S. cepivorum* were formed when the hyphae began branching and folding back upon themselves to form tight parallel bundles. This early phase of sclerotium formation was unlike that described by Townsend and Willetts (1954), who described a branching pattern that was initially dichotomous and became progressively more irregular. In more than 25 sclerotia from the Cloverdale isolate examined by SEM, this branching pattern of development was never observed. As the observations presented here and those of Townsend and Willetts (1954) were based on examination of undisturbed mycelium, the observed difference was not likely an artifact of the techniques used. Townsend and Willetts (1954) do not state the origin of the isolate of *S. cepivorum* they used, so it is possible that this is a difference between isolates. The pattern of sclerotial development observed here does not closely correspond with the categories proposed by Willetts (1972).

The observed morphology of the mature sclerotia was consistent with that described in previously published reports (Backhouse and Stewart 1987, Kohn and Grenville 1989 a, b, Leggett and Rahe 1985).

The relatively unorganized elaboration of the forming sclerotium continued for about 10 days, at which time the hyphae that make up the rind of the sclerotium began growing parallel to the surface of the forming sclerotium. The 'palisade' arrangement of hyphae reported by Willetts and Wong (1971) in *Sclerotinia*

51

sclerotiorum and by Bullock *et al.* (1980) in *Sclerotinia minor* could be seen to a very limited extent in *S. cepivorum* (Fig. 9), but the majority of the rind area was made up of hyphal strands lying parallel to the surface of the sclerotium. The appearance of phialides bearing microconidia at this stage, seen by Coley-Smith (1960) and New *et al.* (1984) in germinating sclerotia and by Kohn and Grenville (1989 a) on the mycelial weft in culture, is noteworthy because this fungus is not known to produce any functional spores or to undergo sexual reproduction (Kohn and Grenville 1989 a). The extracellular rind matrix began to appear shortly after this, covering and obscuring the rind hyphae. This matrix was likely produced by the differentiating rind cells, which remain alive in mature sclerotia (Kohn and Grenville 1989 b). The differentiation of the cortical cells proceeded, with the contents staining more strongly, similar to that which has been reported in *S. rolfsii* (Willetts 1969). The metachromatic staining cytoplasmic granules in the medulla have been shown to be membrane-bound protein bodies (Backhouse and Stewart 1987, Kohn and Grenville 1989 a, b) and a few polyphosphate granules (Backhouse and Stewart 1987). The darkening of the acellular rind matrix and the rind cell walls, which occurred by 13 to 14 days, was probably due to melanin. Melanin has been reported to occur in the rind of *Botrytis cinerea* and *S. sclerotiorum* (Bell and Wheeler 1986). The rind cell walls of *S. cepivorum* are known to contain significant amounts of phenolic compounds (Kohn and Grenville 1989 b). This is consistent with the role of the rind in survival and persistence of sclerotia in soil (Backhouse and Stewart 1987).

It appears that as the sclerotia age, especially under dry conditions, the outer layer of cells may die, giving the surface an irregular, pitted appearance. This is consistent with the observations of Backhouse and Stewart (1987) who hypothesized that as rind cells died or become damaged, they are replaced by differentiation of new rind cells beneath. These observations indicate that the loss and replacement of rind cells was more pronounced under dry conditions. This is consistent with the observations of several authors that drying accelerates the rate of decay of sclerotia (Coley-Smith *et al.* 1974, Leggett and Rahe 1985, Smith 1972).

When sclerotia from a variety of sources were compared, those produced axenically on onions and those from PDA appeared to be similar. In addition, sclerotia produced on onions in the field (and subjected to natural drying) and on artificial media and subsequently dried, were similar. From this it was concluded that the source of the sclerotia is less important than the moisture stresses to which the sclerotia were subsequently subjected, in determining the appearance of the rind. The differences in behaviour of field and laboratory-produced sclerotia that have been reported in germination and survival studies (Coley-Smith 1985, Leggett *et al.* 1983) are probably due to factors other than differences in rind structure.

C. SURVIVAL OF SCLEROTIA

When an onion root comes sufficiently close to a sclerotium, the microbially metabolized root exudates induce sclerotial germination, leading to infection. In a natural ecosystem, where *Allium* hosts are scarce and unevenly distributed, a sclerotium may have to persist a very long time before it is encountered by a host root. Thus, to be successful, sclerotia have to be very long-lived, which is the case.

Disease management aimed at this part of the life cycle is accomplished by reduction of inoculum potential, and can be achieved in one of three ways. The sclerotium may remain dormant until its energy reserves are exhausted and it dies. Interfering with stimulation by host volatiles, increasing mycostasis by increasing or altering microbial activity in soil, or long term crop rotation to 'outwait' the pathogen are examples of this strategy. The sclerotium can be induced to germinate in the absence of the host and, failing to infect, it dies. Artificial germination stimulants and incorporation of crop residues of *Brassica juncea* are examples of this strategy. Finally, the sclerotium can be killed directly by other organisms, chemicals, solarization or flooding.

This portion of the thesis investigates one of these strategies - flooding as a means of white rot management. While only a little work has been done on control of *S. cepivorum* with flooding (Banks and Edgington 1989, Joshi 1988), it is a strategy that may be of use in the Fraser Valley and which may already be serving to naturally reduce inoculum potential without human intervention (Leggett and Rahe

1985). To be used effectively, an understanding of the aspects of flooding that are crucial to inoculum reduction is needed so that these may be utilized to maximize disease control.

Effects of Flooded and Anaerobic Conditions on Survival of Sclerotia

One of the factors making *Allium* white rot such a disease control problem is the extreme longevity of its sclerotia, estimated at up to 20 years (Coley-Smith *et al.* 1990). In the Cloverdale mucklands of the Fraser Valley of British Columbia, however, estimates of the half-life of sclerotia of *S. cepivorum* range from 0.8 to 2.2 years (Leggett 1983). It is of interest to establish why this difference exists and to examine the possibility of exploiting the factors behind this difference in a disease control strategy.

The most striking difference between the Cloverdale mucklands and most other onion growing areas in the world is that the Cloverdale fields are subject to flooding for portions of the winter. As flooded soil becomes anaerobic, it is plausible that this condition causes the sclerotia to die prematurely. Winter flooding has been postulated as being responsible for the rapid decay of sclerotia of *S. cepivorum* in the Fraser Valley (Leggett *et al.* 1983, Leggett and Rahe 1985). Flooding has been shown to be effective in reducing both sclerotial numbers and subsequent disease levels in field plots in Ontario muck soils (Banks and Edgington 1989) and in nearly eliminating survival of both new and existing sclerotia in an infected garlic crop (Crowe and Debons 1992). Sclerotial survival has also been shown to be reduced by high soil moisture, especially in conjunction with warm temperatures (Crowe and Hall 1980 b).

Deliberate flooding of crop land for control of pests and diseases is a very old idea. The Chinese have long recognized the benefits of rotations of paddy rice with cotton for control of *Fusarium oxysporum* f. sp. *vasinfectum* (Cook 1981 a). Early in this century, European grape growers used flooding in a partially successful attempt to control the Phylloxera root aphid and the technique was used commercially in California for control of wireworms in the 1930's (Newhall 1955).

The most obvious and dramatic effects of flooding on soil are the reduction in aeration, the increase in the activity of anaerobic organisms and the biological production of ethylene and other hydrocarbon gases, nitrous oxide, carbon dioxide, organic sulphur compounds and other gasses (Brown 1986, Cook 1981 b, Smith and Restall 1971).

Lack of oxygen is not always the most important factor in anaerobic soils. The suppression of microsclerotia production in *Verticillium dahliae* under anaerobic conditions was correlated with increased carbon dioxide and decreased oxygen levels. Either factor alone was less effective (Ioannou *et al.* 1977 a, b). The suppression of microsclerotia production in *V. dahliae* can be induced either by flooding or by incubating moist soil under nitrogen (Menzies 1962). Water has the disadvantage that it is a relatively good solvent for oxygen. Thus it can be difficult to achieve anaerobic conditions in soil where percolation or evaporation make frequent replenishing of the floodwaters necessary (Ioannou *et al.* 1977 a, Menzies 1970).

Flooding is not widely used for disease control, primarily due to the limited number of areas where it is feasible. Flooding has been successfully used to control *Fusarium* in two areas. Paddy rice in rotation with cotton is used to control *F. oxysporum* f. sp. *vasinfectum* in China (Cook 1981 a) and flooding has been extensively used in Central America to control *F. oxysporum* f. sp. *cubense* (Newcombe 1960, Newhall 1955, Zentmeyer and Bald 1977). *F. moniliforme, F. graminearum, F. solani,* and *F. oxysporum* f. sp. *nivale* have all been reportedly eliminated from non-sterile soil by saturation with water (Stover 1953). The mechanism of reduction of *Fusarium* in flooded soil appears to be due to the prevention of formation of chlamydospores due to high levels of carbon dioxide (Bourret *et al.* 1968, Louvet 1970, Newcombe 1960, Stover and Freiberg 1958).

In the vegetable growing areas of the Florida Everglades, annual flooding has been routinely used to control *Sclerotinia sclerotiorum* in commercial lettuce and celery crops (Genung 1976). In this region, it is possible to get 90 to 100% decay of sclerotia in 23 to 30 days at 20° C with flooding (Moore 1949). It has been shown that more sclerotia rot as soil moisture increases and carpogenic germination and production of apothecia were strongly suppressed in saturated soil (Teo and Morrall 1985, Teo *et al.* 1985). Rotation with paddy rice has been shown to successfully control *S. sclerotiorum* (Stoner and Moore 1953). Flooding has also been shown to reduce survival of sclerotia of *Rhizoctonia solani* (Lee 1985).

Flooding can also be an effective means of reducing populations of microsclerotia of V. dahliae and V. albo-atrum. While some authors reported that microsclerotia can be killed by flooding (Butterfield et al. 1978, Green 1980, Menzies 1962), other studies indicate that production of microsclerotia can be suppressed by flooding but there is no effect on pre-existing microsclerotia (Ioannou et al. 1977 a, b, c). The degree to which microsclerotial populations are reduced depends on the extent of the anaerobiosis (Butterfield et al. 1978, Ioannou et al. 1977 b) and the soil temperature (Green 1980). There is evidence of a diffusible bacterial toxin in this system and the suppression of microsclerotia is greater when nutrient is added to the system (Menzies 1962). Suppression of microsclerotia production in flooded soil has been shown to be related to both oxygen and carbon dioxide concentrations (Ioannou et al. 1977 a).

Field trials using rotation with paddy rice that resulted in a 95% reduction in inoculum levels also gave reduction in Verticillium wilt of cotton the following year (Butterfield et al. 1978). In contrast, flooded tomato fields where only a 45% reduction in inoculum was achieved showed no reduction in disease (Ioannou et al. 1977 b).

An interesting case is the proposed biological control of a complex of root canker basidiomycete pathogens in New Zealand by flooding. As this disease does not occur on poorly drained land, flooding seemed a reasonable avenue for investigation. Taylor and Guy (1981) found that Peniophora sacrata, one of the main pathogens in the disease complex, survived for five weeks at 20° C in

sterile or non-sterile moist soil or sterile flooded soil, but did not survive in non-sterile flooded soil. Further, they found that in 24 of 32 soils tested, the addition of 0.5% natural soil to the sterile flooded treatment eliminated *P. sacrata* within five weeks.

Taylor and Guy (1981) isolated a variety of facultative and obligate anaerobic bacteria and tested these against *P. sacrata* in dual culture plates. The most promising of these (*Bacillus cereus, B. megaterium, B. cereus* var. *mycoides, B. polymyxa* and three isolates each of three *Clostridium* species) were tested in soil individually, but none controlled the fungus very well. *P. sacrata* survived for 5 weeks with a mixture of four *Bacillus* isolates, all isolates of each of the *Clostridium* species and a mixture of all the *Clostridium* isolates, but did not survive a mixture of all 13 isolates. However, this combination was still not as effective as natural soil. This evidence suggests suppression of this disease in wet soils is due to a biological factor, is mediated through a complex of anaerobic bacteria acting in concert, and probably involves organisms or factors as yet undetected (Taylor and Guy 1981).

The cause of mortality of sclerotia of *S. cepivorum* in flooded soil could be due to excess water levels *per se* or to the resulting lack of oxygen, or both. These effects could be mediated biologically (different parasites and competitors will be present under anaerobic conditions than in aerobic conditions), chemically (muck soils can form organic sulphur compounds that could induce sclerotial germination (Brown 1986)), or purely physically.

The objective of this investigation was to elucidate the roles of excess soil water and anaerobic conditions in the reduced survival of sclerotia in flooded soil. Survival was compared in flooded soil, flooded aerated soil, normal soil and normal soil under an anaerobic atmosphere.

Materials and Methods

Soil used in the first experiment was collected from a commercial vegetable farm near Cloverdale, British Columbia, partially dried to facilitate handling and sieved (5 mm mesh) to remove roots and rocks. Sclerotia of *S. cepivorum* were collected from infected onions in a commercial onion farm near Cloverdale, British Columbia. They were stored in field soil in open 2L clay pots with a drain hole in the bottom, outdoors, exposed to fall weather conditions, until used. Four-litre glass jars were each filled with about 2 L of soil, and five nylon mesh bags each containing 25 sclerotia and about 20 ml of field soil were buried in each container. Initial viability of a sample of 30 sclerotia was assessed using the method of Vimard *et al.* (1986). Each treatment was replicated five times, and each container constituted a replication. Treatments were normal soil, where moderate moisture levels were maintained throughout (soil was kept visibly moist, but below field capacity); flooded soil, which was kept with at least 2 cm of water covering the soil surface at all times; aerated soil, which was kept flooded but aerated by bubbling air through an aquarium airstone at the bottom of the container at about 1L/min.; and nitrogen, where soil was kept at moderate

moisture levels (about 60% of field capacity) in an atmosphere of pure nitrogen. All containers were incubated at 17°±1° C in the dark. One bag was removed from each container at 1, 2, 3, 4.5 and 6.5 months and the number of viable sclerotia remaining therein was assessed by the method of Vimard *et al.* (1986).

In a second experiment, the role of water alone in the survival of sclerotia, in the absence of the activity of soil microorganisms was examined. Axenic sclerotia were taken from PDA plates that had been allowed to dry. Treatments consisted of 10 axenic sclerotia placed in sterile 18 mm culture tubes with or without 10 ml of sterile distilled water (SDW). Sclerotia were tested for initial viability by plating 20 onto PDA, incubating at 17° ± 1° C in the dark and rating germination. Each treatment was replicated three times for each sampling. The tubes were incubated at 17° ± 1° C in the dark. At 28, 48, 103, 125 and 248 days, sclerotia were recovered and plated onto PDA, incubated at 17° ± 1° C in the dark and checked for germination. Three tubes of each treatment were recovered at each sampling time.

Results

Initial viability of sclerotia was 96.7 %. Viability in all treatments had declined rapidly by the first sampling time. After the initial decline of viable sclerotia, viability remained at about 40% in the normal soil treatment, while viability in the flooded soil treatment continued to decline until no viable sclerotia could be recovered after 98 days. Viability in the aerated/flooded soil and nitrogen treatments was intermediate, and reached zero after 150 days (Fig. 20). Analysis of

covariance on log transformed data for recovery of viable sclerotia, with time as the covariate showed a highly significant difference in the rate of decline in viability among treatments (P=0.0002). Regression of log transformed percent viable sclerotia recovered on time was highly significant (P<0.01) for all treatments and there was a significant difference among slopes (P=0.015).

In the second experiment, initial viability of sclerotia was 90.0%. Viability in sterile water was undiminished after 125 days, while viability of sclerotia in dry tubes declined after 48 days. This difference was consistent to the end of the experiment (250 days), with the sclerotia in sterile water consistently exhibiting higher viability than the sclerotia in dry tubes (Fig. 21). All sclerotia from the dry tube treatment plated at 103 days were contaminated with bacteria and did not germinate. These results were deleted from the analysis.
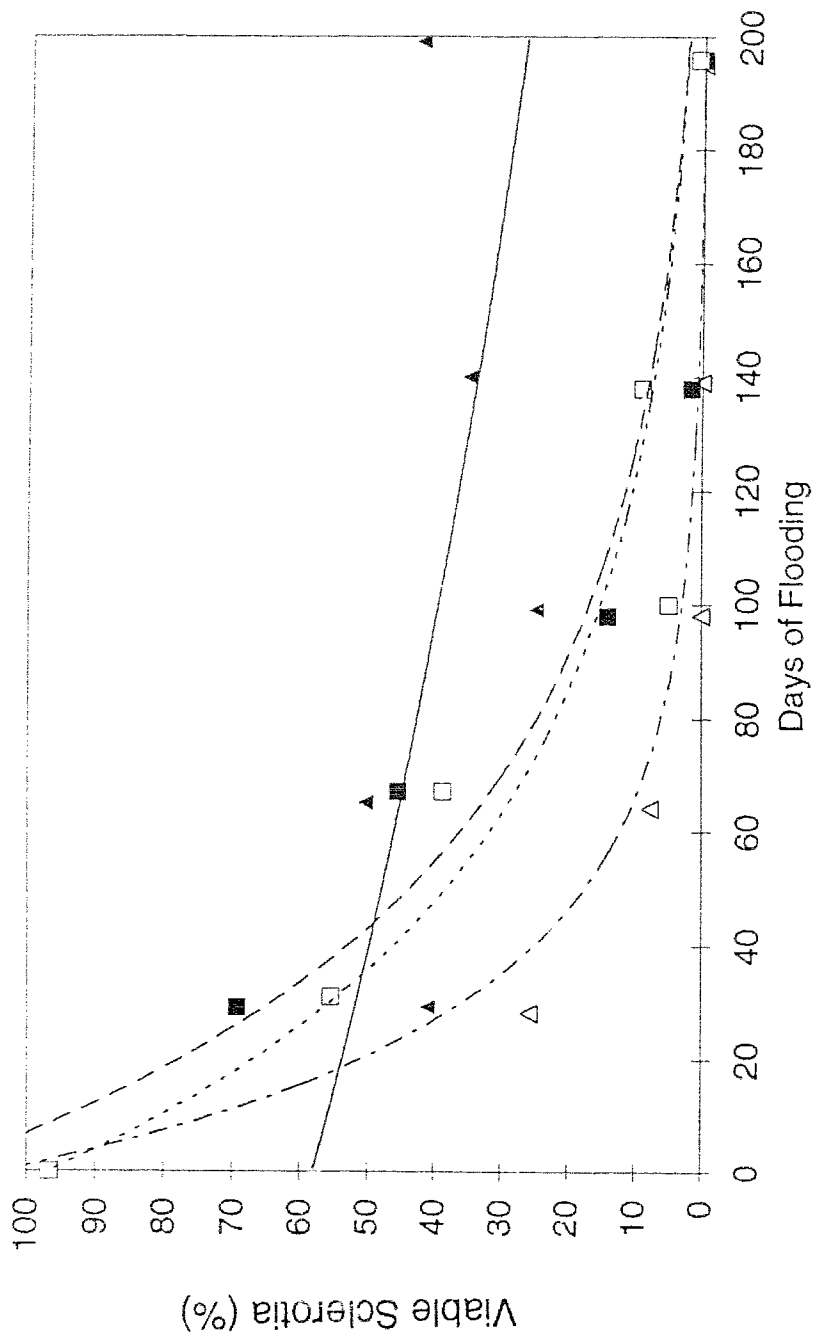
Figure 20. Recovery of viable sclerotia *Sclerotium cepivorum* over time from normal soil
——▲——, flooded soil ----△----, normal soil under a nitrogen atmosphere --□-- and soil
flooded with aerated water ----■----.
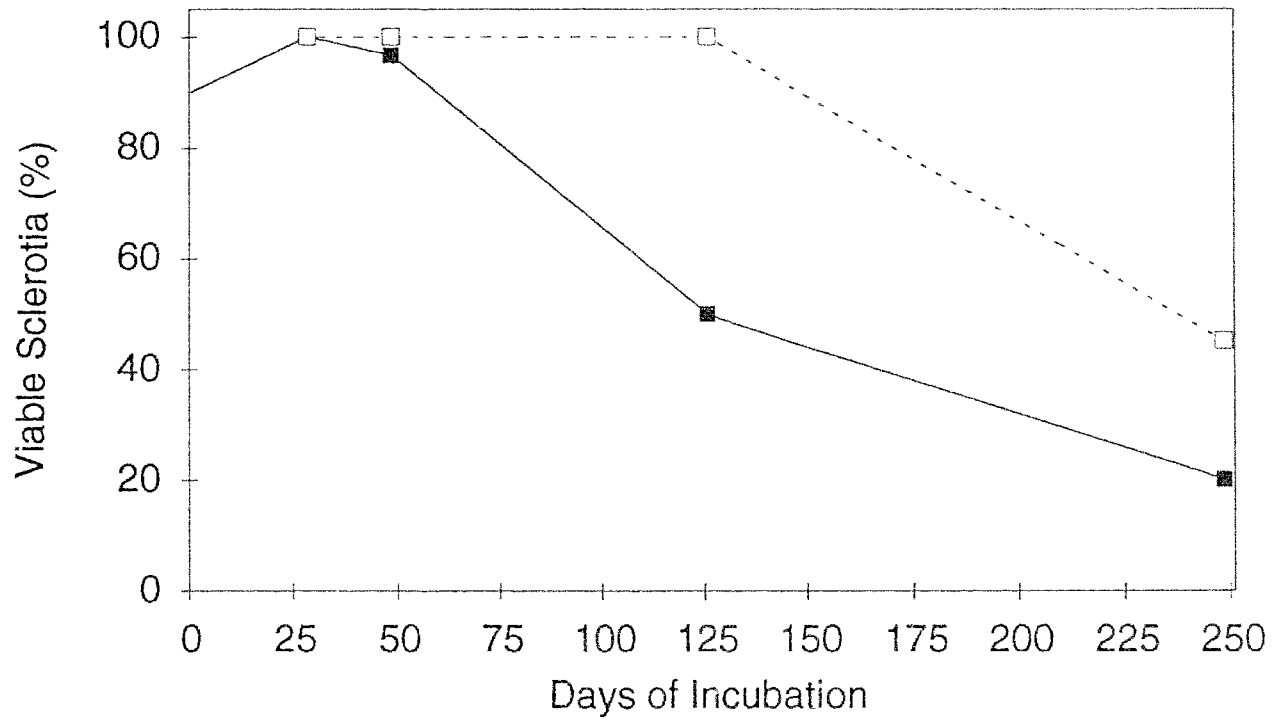
Figure 21. Survival of sclerotia of *Sclerotium cepivorum* in sterile water – –□– – or dry tubes ——■——. Viability expressed as % sclerotia germinating on potato dextrose agar (PDA) at 17° ± 1° C in the dark.

Discussion

Flooding dramatically reduced the survival of sclerotia of *S. cepivorum* in Cloverdale muck soil under laboratory conditions at 17° C. This result is consistent with the hypothesis that the winter flooding of fields in Cloverdale is the cause of the short half-lives of the sclerotia (Leggett 1983) and is consistent with the reduction in sclerotial survival reported for flooded field trials by Banks and Edgington (1989) and Crowe and Debons (1992). The incubation of the experiments at 17° C means that extrapolation of these results to the field must be done with caution. Crowe and Debons (1992) reported a substantial reduction in sclerotial survival from in-season flooding, when soil temperatures ranged from 10° to 25° C. The reductions in sclerotial numbers and viability reported by Banks and Edgington (1989) occurred both with winter and spring flooding, both of which would have involved soil temperatures well below 17°. The over-wintering decay of sclerotia reported by Leggett and Rahe (1985) in the Fraser Valley occurred in soil temperatures that ranged from about 10° C to just below 0°. Crowe and Hall (1980 b) reported that decay of sclerotia in saturated soil increased with increasing temperature, for temperatures of 6° C or greater. They found no decay at 0° or 4° C. However, for all temperatures above 6°, decay was maximal in saturated soil and decreased with decreasing soil moisture. Thus, while decay is greater at higher temperatures, winter flooding does reduce sclerotial populations and temperatures that permit decay of sclerotia in saturated soil do occur for substantial periods while the soil in Cloverdale is flooded.

Excess water alone (the aerated treatment) or anaerobic conditions alone (the nitrogen treatment) did not completely mimic either the flooded or normal conditions. It should be noted that the level of aeration in the aerated-flooded treatment was not equivalent to that of the normal treatment, as air from the airstone did not reach all parts of the soil equally. Likewise, the nitrogen treatment would have taken some time to become anaerobic as the nitrogen gas would not displace the air in the soil as effectively as water. Thus, within the limits of this experimental system, it appears that both water and anaerobic conditions are required for the full effect of flooding on sclerotial survival to occur. This would suggest that there is a biological component involved in the decline in sclerotial viability under flooded conditions. An experiment with better aeration of the flooded, aerated treatment and better purging of the air in the soil in the nitrogen treatment would be needed to confirm these conclusions.

The presence of water alone, under sterile conditions, did not reduce survival of sclerotia as much as occurred in flooded soil. In fact, keeping the sclerotia under dry conditions led to a more rapid decline in viability than occurred in aseptic, flooded conditions. As this system was aseptic, sclerotia in the dry conditions would have died from desiccation. With the small volume of water in the culture tube in the sterile water treatments, anaerobic conditions would be unlikely to develop, especially in the absence of any microflora to deplete the oxygen in the system. It should be noted that the sclerotia used in this experiment had been dried before use. It has been reported that sclerotia of *S. cepivorum* that have been dried and rewetted decay

more quickly under field conditions than those that have not been dried (Coley-Smith *et al.* 1974, Leggett and Rahe 1985, Smith 1972). Despite this, the sclerotia that had been dried and rewetted had a higher survival in this sterile system than those dried and not rewetted.

D. FACTORS AFFECTING INFECTION AND INOCULUM INCREASE

The capacity of germination stimulants to negate the effect of soil mycostasis in *S. cepivorum* is variable. Utkhede *et al.* (1978) reported there were fields in the Fraser Valley in which viable sclerotia of *S. cepivorum* and conditions suitable for infection were present, but in which no disease occurred. Entwistle (1986) found no consistent relationship between sclerotial density in soil and resulting disease levels. Crowe *et al* (1980) found that disease levels increased as preplanting inoculum levels increased. Adams (1981) was able to fit a quadratic curve to the relationship between sclerotial density in soil and white rot in bunching onions in New Jersey. If the numbers of sclerotia found by Utkhede *et al.* (1978) in the Fraser Valley occurred in New Jersey, substantial amounts of white rot could be predicted to occur (Adams 1981), but did not. Utkhede *et al.* (1978) suggested that the muck soil in their survey area was suppressive to white rot. Leggett (1983) found that when this soil was autoclaved and reinoculated with sclerotia of *S. cepivorum*, significantly higher levels of disease occurred than in the raw soil with the same level of inoculum. This suggests that this soil is suppressive (Baker and Cook 1982, Cook and Baker 1983). While Leggett (1983) could not correlate a factor or factors with this suppressiveness, any level of natural control of a disease is worth further investigation.

Soil microflora play an important role in the expression of onion white rot, both by imposition of mycostasis on the sclerotia (Coley-Smith *et al.* 1967, Coley-Smith *et al.* 1987, King and Coley-Smith

1968) and by conversion of host root exudates to stimulatory volatiles (Coley-Smith and King 1969, Coley-Smith *et al.* 1967, Esler and Coley-Smith 1983, King and Coley-Smith 1969 a). It is likely that one factor responsible for the variability in the relationship between inoculum density and disease reported by several authors (Adams 1982, Entwistle 1986, Utkhede *et al.* 1978) is variation in the microflora present in different soils. Any perturbation of the populations of soil microflora could reasonably be expected to affect the expression of white rot in that soil. Despite this, work on biological control of onion white rot is still done in axenic (Abd-El-Moity and Shatla 1981, Jackson *et al.* 1991) and microbiologically simplified (Abd-El-Moity and Shatla 1981) environments. Many environmental variables can affect soil microflora, as well as the soil physical environment and the host plant condition. This section of the thesis examines the effects of autoclaving as a severe perturbation of the normal microflora in several soils, and studies the effects of changes in host physiology on the expression of onion white rot at a constant inoculum density.

Effect of Soil Autoclaving on White Rot

Leggett (1983) reported that significantly more white rot occurred on onions grown in autoclaved soil with added inoculum of *S. cepivorum* than in raw soil. Regardless of soil treatment, there was no significant difference in white rot levels when laboratory-produced or field-collected sclerotia were used as inoculum. The soil used in Leggett's experiments was from a commercial vegetable farm in Burnaby, British Columbia reported to be suppressive to white rot (Utkhede *et al.* 1978). The experiments reported here were designed to re-examine the effect of soil autoclaving on white rot infection of onions under several conditions and in several soils.

Materials and Methods

General

Four soils were used. The Cloverdale muck soil was from an uninfested field on a commercial vegetable farm near Cloverdale, British Columbia, as in previous experiments. The Burnaby muck soil was from a white rot infested field on a commercial vegetable farm in Burnaby, British Columbia, the same soil as used by Leggett (1983). Aldergrove sandy loam soil was from an apple orchard near Aldergrove, British Columbia and Summerland silt was collected from an uncultivated area near Summerland, British Columbia. The Cloverdale muck soil was used in all experiments, and the Burnaby, Aldergrove and Summerland soils were used in only the soil types experiment. Each soil was partially dried as needed, to facilitate

handling and passed through a 5 mm sieve to remove rocks and roots. Soil was then moistened to approximately field capacity and autoclaved twice (20 min, 121° C) on successive days in double thickness autoclavable plastic bags (50 cm x 80 cm), 3 L per bag. Autoclaved soils were stored in the sealed bags until used.

Sclerotia used were collected from infected onions in a commercial onion farm near Cloverdale, British Columbia. They were stored in field soil in open 2L clay pots with a drain hole in the bottom, outdoors, exposed to fall weather conditions, until used. Laboratory-grown sclerotia of *S. cepivorum* were produced from a single-sclerotium isolate from the field-collected sclerotia in sand-cornmeal medium (100:1) in 2 L flasks and were kept sterile and moist until used. Onion *cv.* Improved Autumn Spice seeds, all from the same seed lot, were used for all experiments. For surface sterilization, seeds were immersed in 1% NaOCl for 3 minutes and rinsed three times in sterile distilled water (SDW).

The experiments were conducted in 11 cm diameter plastic pots filled with soil. Four 5 cm deep holes were made in the soil in each pot, ten sclerotia were placed into each hole, soil was added to a depth of 1.5 cm, then two seeds were placed into each and covered with soil.

The pots in the soil autoclaving experiment were kept under a 16 h photoperiod and 18/13 °C diurnal temperature regime in a controlled environment chamber. Pots in the other three experiments were incubated under a diurnal cycle of temperature ranging from 9 to 16 °C and a 16 h photoperiod. All pots were saturated with SDW at

the beginning of each experiment and resaturated with SDW whenever the plants were judged to be close to wilting.

Pots were examined every 3 or 4 days and dead or obviously dying plants were removed for confirmation of infection by *S. cepivorum*. Plant tissue was surface sterilized in 1% NaOCl for 1 minute, then plated onto PDA and held at $17°$ C $\pm$ $1°$ C. Emerging fungi were subcultured as necessary, and colonies producing sclerotia typical of *S. cepivorum* were recorded.

Soil Autoclaving

The goal of this experiment was to compare the amounts of white rot developing on onions grown in autoclaved and untreated Cloverdale muck soil amended with equal numbers of either laboratory-produced or field-collected sclerotia. The experiment was conducted as a completely randomized design with five replications and was terminated 91 days after seeding. Samples of the sclerotia used for inoculum were assessed for viability at the beginning of the experiment. Thirty sclerotia of each type were surface sterilized in 1% NaOCl for 1 minute, rinsed twice in SDW, crushed and plated onto PDA. Plates were incubated at $17°$ C $\pm$ $1°$ C in the dark and examined 5 days later.

The relative abundance of fungi, bacteria and actinomycetes in the various treatments at the end of the experiment was estimated by dilution plating. Two 1 g soil samples were taken from each of two pots for each treatment and dilution plated onto rose bengal agar (RBA) for fungi, starch casein agar (SCA) for actinomycetes and

Thornton's agar (TA) for bacteria (Johnson and Curl 1972). Plates were incubated at room temperature in the light for 6 to 8 days. Selected colonies from RBA and SCA plates were subcultured onto PDA for purification and identification. Sclerotia were recovered from four pots of each soil treatment at the end of the experiment and assayed for viability by the method of Vimard et al. (1986).

A simplified version of this experiment was repeated using Cloverdale muck soil and field-collected sclerotia of S. cepivorum. Soil treatments were raw soil and autoclaved soil, both amended with sclerotia as before, and one check treatment of raw soil not amended with sclerotia of S. cepivorum. The experimental design was completely randomized with five replications. The trial was harvested 104 days after seeding, at which time the plants were well bulbed and the tops were going down.

Soil Recolonization

The goal of this experiment was to compare development of white rot on onions growing in autoclaved soils given different amounts of exposure to non-sterile air prior to amendment with sclerotia and seeding. Cloverdale muck soil and field-collected sclerotia were used. Treatments were autoclaved soil with 0, 4 or 16 days of exposure to air before adding sclerotia and seeding, and raw soil with and without added sclerotia (controls). The air exposure treatments consisted of placing the autoclaved soil in uncovered autoclaved trays in a plant growth room for 4 or 16 days before seeding. Seeds were either surface sterilized or untreated. Each pot

was put into a 25 mm deep petri dish to prevent cross contamination through watering. Experimental design was completely randomized, fully crossed (five soil conditions, two seed conditions) with five replications. The experiment was terminated 134 days after seeding, by which time the plants had bulbed and the tops were going down, making it impossible to distinguish between disease and maturity on the basis of top symptoms. Data were analysed by logistic regression using a generalized linear model procedure in GLIM (Release 3, Numerical Algorithms Group, Royal Statistical Society, Oxford, U.K.).

Soil Types

This experiment was designed to compare the response of white rot infection to autoclaving treatment in Cloverdale, Burnaby, Aldergrove and Summerland soils. Treatments were the four soils, autoclaved and raw, using field-collected sclerotia as inoculum. The trial was set up as a completely randomized design with five replications. Five replications of each soil type (not autoclaved) were seeded as an uninoculated check. The experiment was terminated 210 days after seeding, by which time all the plants had bulbed and the tops had gone down. Data were analysed using the GLM procedure of Minitab (Release 8, Minitab Inc., State College, Pa.).

Results

Soil Autoclaving

Initial viability of laboratory sclerotia and field-collected sclerotia was >93% and >83% respectively. Differences in seedling emergence between soil treatments and sources of sclerotia were not significant (P=0.73, ANOVA). No plants died in the autoclaved soil treatments, whereas considerable mortality occurred in the raw soil, of which approximately 46% was due to white rot (Table 2). The type of sclerotia used as inoculum did not affect emergence (P=0.73), plant mortality (P=0.82) or confirmed white rot (P=0.93). There were no significant interactions between soil treatment and sclerotia type.

Significantly higher populations of actinomycetes (P<0.0001), bacteria (P<0.0001), and fungi (P<0.001), as indicated by colony forming units (cfu), occurred in the autoclaved soil than in the raw soil at the termination of the experiment (Table 3).

Source of sclerotia had no effect on populations of actinomycetes (P=0.13) or bacteria (P=0.62), but did affect the fungal population (P=0.0009) (Table 4). The interaction of sclerotia source and soil treatment on fungal populations was significant (P<0.0001) Significantly higher fungal populations occurred in the autoclaved soil/field-collected sclerotia combination than in the autoclaved soil/lab sclerotia combination (P≤0.05), while both autoclaved treatments resulted in significantly higher fungal populations than in the raw soil treatments (P≤0.05) (Table 4).

Table 2. Effect of soil treatment and origin of sclerotia of *Sclerotium cepivorum* on emergence, mortality and white rot in *Allium cepa* growing[1] in Cloverdale muck soil.

Soil Treatment	Sclerotia Type	Emergence (%)	Mortality (%)	White Rot Confirmed (%)
Raw	Field	75.0 a[2]	67.0 a[2]	45.8 a[2]
	Lab	77.5 a	63.3 a	46.2 a
Autoclaved	Field	82.5 a	0.0 b	0.0 b
	Lab	75.0 a	0.0 b	0.0 b

[1] The experiment was terminated 91 days after seeding.

[2] Means within a column followed by the same letter do not differ ($P \leq 0.05$) according to Duncan's Multiple Range Test.

Table 3. Effect of soil treatment on populations of soil microorganisms (cfu) occurring after 91 days of exposure to non-sterile conditions and growth of onions.

Soil Treatment	Actinomycetes	Bacteria	Fungi
Autoclaved	12.5×10^6 a[1]	56.5×10^5 a[1]	7.7×10^5 a[1]
Raw	1.2×10^6 b	9.5×10^5 b	1.4×10^5 b

[1] Means within a column followed by the same letter do not differ ($P \leq 0.05$) according to Duncan's Multiple Range Test.

Table 4. Effect of soil treatment and source of *Sclerotium cepivorum* sclerotia on fungal population (cfu) in soil 91 days after amendment with sclerotia and seeding with *Allium cepa*.

	Source of Sclerotia	
Soil Treatment	Lab	Field
Autoclaved	$4.4 \times 10^5 b^1$	$10.9 \times 10^5 a$
Raw	$1.2 \times 10^5 c$	$1.5 \times 10^5 c$

[1] Means followed by the same letter do not differ ($P \leq 0.05$) according to Duncan's Multiple Range Test.

Table 5. Recovery and viability of sclerotia of *Sclerotium cepivorum* at
the end of the soil autoclaving experiment.

Soil Treatment	Recovered (%)[1]	Viability (%)[2]
Autoclaved	54.4 a[3]	88.4 a[3]
Raw	146.9 a	76.7 a

[1] Sclerotia recovered as a percentage of the number of sclerotia used
as inoculum.

[2] Viable sclerotia as a percentage of the number of sclerotia
recovered.

[3] Means within a column followed by the same letter do not differ
($P \leq 0.05$) according to Duncan's Multiple Range Test.

Differences between treatments in the numbers and viability of sclerotia recovered at the end of the experiment were not significant (Table 5). The greater than 100% recovery of sclerotia in the raw soil was due to formation of new sclerotia on onions in two of the five replicate pots. This caused extreme variance in the data.

When the experiment was repeated, autoclaving Cloverdale muck soil completely suppressed the occurrence of white rot. White rot occurred in 80.8% (SD 5.6%) of plants growing in raw inoculated soil. The inoculated autoclaved soil and the uninoculated raw soil did not produce any white rot infections.

Soil Recolonization

Emergence of seedlings from the surface sterilized seeds in the raw soil was anomalously low (Table 6). There were no significant differences in emergence among any of the remaining soil treatments, or between sterilized and non-sterilized seed treatments.

White rot developed in all treatments except the 4 day with non-autoclaved seeds, and the uninoculated controls. The uninoculated controls were not included in any further analysis. The occurrence of white rot in all of the autoclaved treatments was reduced or delayed compared with its occurrence in the raw soil treatments (Fig. 22).

Table 6. Emergence (%) of *Allium cepa* seedlings 10 days after seeding into raw, autoclaved and autoclaved-recolonized muck soil.

Soil Treatment/ Condition at time of seeding	Sterilized Seeds	Non-sterilized Seeds
Raw/Natural	15.0 a [1]	67.5 bc
Autoclaved/Recolonized 0 Days	67.5 bc	77.5 c
Autoclaved/Recolonized 4 Days	77.5 c	72.5 bc
Autoclaved/Recolonized 16 Days	65.0 bc	72.5 bc

[1] Means followed by the same letter do not differ (*P*<0.05) according to Duncan's Multiple Range Test.

Diseased plants were first detected in the inoculated raw soil (26 days after seeding), followed by the 16 day (88 days after seeding), the 0 day (96 days) and the 4 day treatment (102 days). Curves produced by logistic regression of proportion white rot on days after seeding were highly significant ($P<0.0001$). Soil treatment had a highly significant effect ($P<0.0001$), and seed treatment had a significant effect ($P=0.019$). While the differences between seed treatments were statistically significant, their magnitude was small and the direction of their effect in different soil treatments was sometimes opposite (Fig. 22).

White rot levels at harvest were highest in the raw and 0 day treatments and lowest in the 4 and 16 day treatments (Table 7). Mean time of disease onset was significantly delayed in all of the autoclaved treatments compared with the raw soil (Table 7). While the autoclaved/0 day treatment significantly delayed the appearance of white rot, the level of white rot at harvest was not significantly different from that in the raw soil. Seed treatment had no effect on final white rot levels ($P=0.84$).

Soil Types

Over the course of this experiment, 1% of the plants in the uninoculated Cloverdale soil and 5% of the plants in the uninoculated Burnaby soil became infected with white rot, while the uninoculated Aldergrove and Summerland soils did not produce any white rot infections.

As can be seen in Figure 23, autoclaving the Aldergrove and Summerland soils reduced white rot, while in the autoclaved Cloverdale soil the disease was entirely suppressed. Autoclaving the Burnaby soil delayed the occurrence of white rot, but once disease occurred its progress was rapid and white rot levels surpassed those in the raw soil. White rot was strongly influenced by soil origin ($P=0.043$) and autoclaving treatment ($P<0.001$) and soil and autoclaving showed a significant interaction ($P<0.001$). The rate of disease increase was significantly higher in the raw soil than in the autoclaved treatments for the Cloverdale and Summerland soils ($P<0.0005$). The opposite was true for Aldergrove soil ($P<0.05$) and the Burnaby soil ($P<0.0005$) where the rate of disease increase was significantly higher in the autoclaved than in the raw soil, although the difference was much larger between the two treatments in the Burnaby soil than in the Aldergrove soil. The Burnaby and Aldergrove soils showed significant delays in the time of disease onset in the autoclaved treatments compared to the raw soils ($P<0.0025$), while the Summerland soil did not ($P>0.50$).

Table 7. Effect of soil autoclaving and duration of exposure to non-
sterile air before seeding on mean time of first detection of
white rot symptoms and level of white rot at harvest in
onions growing in muck soil.

Soil Treatment/ Condition at time of seeding	Mean time of first symptom detection (days after seeding)	% White rot at harvest
Raw/Natural	58.7 a[1]	69.3 a[1]
Autoclaved/Recolonized 0 Days	108.6 b	62.4 a
Autoclaved/Recolonized 4 Days	168.8 c	27.3 b
Autoclaved/Recolonized 16 Days	100.1 b	22.6 b

[1] Means followed by the same letter do not differ ($P \leq 0.05$) according
to Duncan's Multiple Range Test.
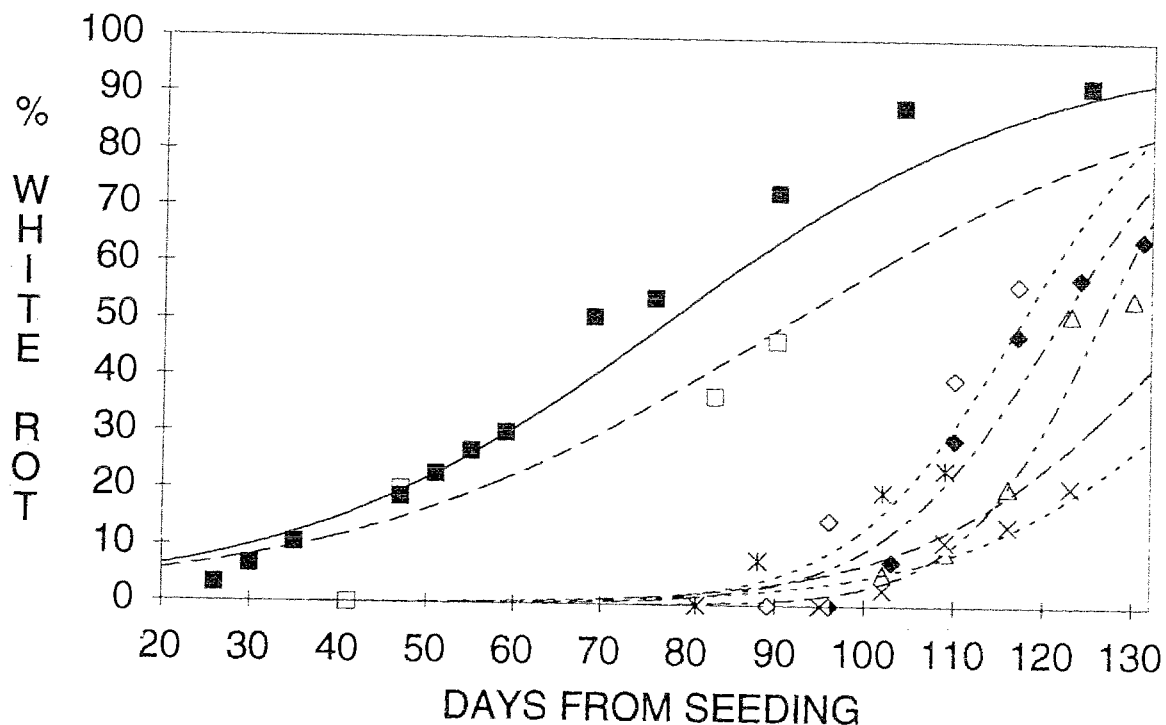
Figure 22. Progress of onion white rot in untreated and autoclaved Cloverdale soil exposed to airborne micro-organisms 0, 4 or 16 days before inoculation with *Sclerotium cepivorum* and seeding with *Allium cepa*.: Raw soil, untreated seeds —■—; raw soil, surface sterilized seeds --□--; autoclaved soil exposed 0 days, surface sterilized seeds ----◇----; autoclaved soil exposed 0 days, untreated seeds ---◆---; autoclaved soil exposed 4 days, surface sterilized seeds ----△----; autoclaved soil exposed 16 days, untreated seeds ----✕----; autoclaved soil exposed 16 days, surface sterilized seeds --✕--.

CLOVERDALE SOIL

BURNABY SOIL

ALDERGROVE SOIL

SUMMERLAND SOIL
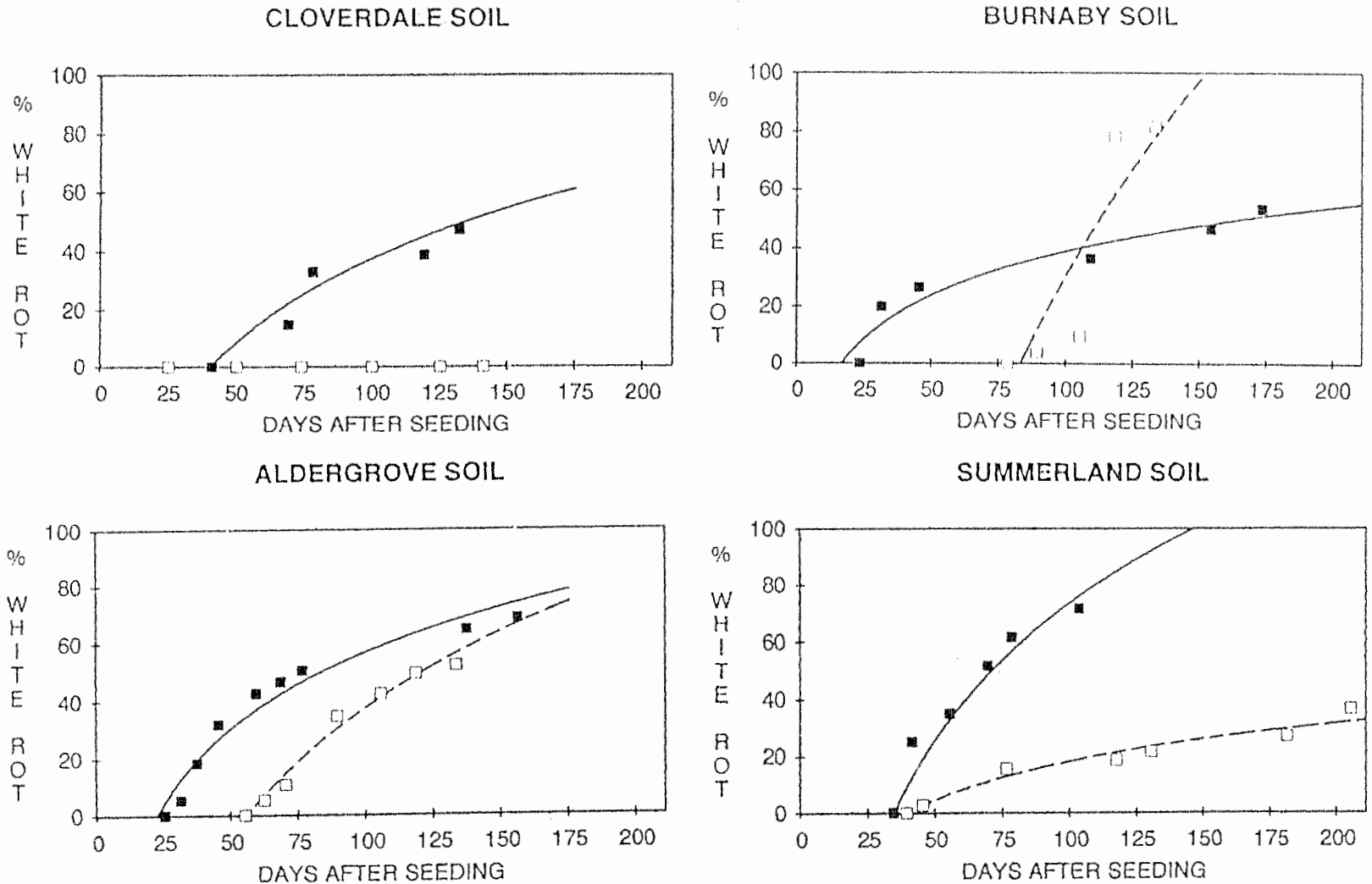
Figure 23. Soil associated variation in the effect of prior soil autoclaving on the development of white rot on onions growing in four different soils. White rot in normal soil was significantly different (P<0.001, logistic regression, GLM) than in autoclaved soil.

—■— Normal soil, --□-- Autoclaved soil

Discussion

Microorganisms other than *S. cepivorum* contribute to the expression of white rot of *Allium* spp. In natural environments bacteria, actinomycetes and fungi function in the phenomenon of mycostasis and sclerotial dormancy (Allen and Young 1968, Coley-Smith *et al.* 1967, Coley-Smith and Dickenson 1971, Coley-Smith *et al.* 1968), metabolism of *Allium* root exudates to volatile sulphides and disulphides (Coley-Smith *et al.* 1967, King and Coley-Smith 1969a) that stimulate germination of sclerotia (Coley-Smith and King 1969, Esler and Coley-Smith 1983, King and Coley-Smith 1968), and as hyperparasites of the pathogen (Ahmed and Tribe 1977, Adams 1987 a, b). Thus it is expected that conditions that affect microbial activity and the balance of microorganisms in soil would affect the relationship between inoculum and white rot.

Autoclaving of soil significantly altered the progress of white rot on onions. The effect generally was to suppress development of the disease in autoclaved soil subsequently recolonized naturally from unsterile air, from onion seeds and from sclerotia of *S. cepivorum* added after autoclaving, but different effects were observed in different soils. Autoclaving suppressed white rot levels in the Cloverdale soil in the two soil autoclaving experiments and in the Cloverdale, Aldergrove and Summerland soils in the soil types experiment. In the case of the soil recolonization trial, autoclaving suppressed white rot in two of the three autoclaved treatments. The final white rot level in the autoclaved/0 day treatment was not different

from that in the raw soil, but the onset of disease was delayed an average of 50 days in the autoclaved soil relative to that in the raw soil. Although white rot was delayed or suppressed by autoclaving in three of the four soils used in these experiments, it seems that whatever factor was responsible for the delay in white rot infection did not affect the progress of the disease once infection occurred.

In contrast to the suppression of disease in the Cloverdale, Aldergrove and Summerland soils, an increase in white rot was associated with autoclaving the Burnaby muck soil in the soil types experiment, although the time of disease onset was delayed in the autoclaved Burnaby soil. The final level of white rot was higher in the autoclaved Burnaby soil than in the raw soil, the only instance in which this occurred. This result in the Burnaby soil is consistent with the results reported by Leggett (1983) in the same Burnaby soil.

Why are the results in the Cloverdale, Aldergrove and Summerland soils different from those in the Burnaby soil? The Cloverdale soil responded similarly in four experiments and the response of the Burnaby soil was consistent with the results reported by Leggett (1983). It has been suggested on the basis of indirect evidence that the Burnaby soil is suppressive to white rot (Utkhede *et al.* 1978). It is expected that whatever suppressiveness, competition or antagonism is present in a soil would be destroyed by autoclaving and the soil would become conducive to disease (Baker and Cook 1982, Cook and Baker 1983). This is consistent with the behaviour of the Burnaby soil, but is contrary to the behaviour of the other three.

Two explanations can be offered regarding the suppression of white rot associated with soil autoclaving. The first is that a flush of microbial growth associated with recolonization following autoclaving resulted in conditions that were inhibitory to sclerotial germination, which may have been due to non-specific mycostasis (Allen and Young 1968, Coley-Smith *et al.* 1967, Coley-Smith and Dickenson 1971, Coley-Smith *et al.* 1968) The increased populations of fungi, bacteria and actinomycetes observed in the autoclaved soil treatments in the soil autoclaving experiment are consistent with this view. The fact that viable sclerotia were recovered at the end of the experiment, despite the suppression of the disease, further supports the mycostasis hypothesis.

The unusual nature of host specificity and germination responses of *S. cepivorum* suggests a second explanation. Sclerotia of *S. cepivorum* will germinate in non-sterile soil only in the presence of an *Allium* host (Coley-Smith 1960), or various volatile sulphide compounds (Coley-Smith and Cooke 1971, Coley-Smith and King 1969, King and Coley-Smith 1969) which result from the microbial conversion of *Allium* root exudates (Coley-Smith and King 1969, King and Coley-Smith 1968, 1969). Root exudation from the onions growing in the autoclaved soils may have been different from that occurring in the untreated soils, or the conversion of those exudates to stimulatory volatiles may not have occurred as they did in the untreated soils. If the reestablishment of a microflora in the autoclaved soil did not include or was antagonistic to the reestablishment of microorganisms responsible for the conversion of

Allium root exudates to stimulatory volatiles, then the sclerotia would be unable to overcome soil mycostasis and would be unable to germinate and infect the host.

Germination of sclerotia of *S. cepivorum* occurs spontaneously under aseptic conditions (Coley-Smith *et al.* 1967). The recovery of viable sclerotia from the autoclaved treatment in the soil autoclaving experiment shows that the suppression of white rot was not due to sclerotia spontaneously germinating and dying in the autoclaved Cloverdale soil before the opportunity for infection occurred. It can therefore be inferred that lack of infection was due to lack of germination. Once conversion of root exudates became possible, the sclerotia would respond, the apparent suppressiveness would disappear and infections could occur. The disease progress curves in the Cloverdale, Summerland and Aldergrove soils suggest that suppressiveness following autoclaving was a temporary phenomenon.

The effect of autoclaving the Burnaby soil is not explained by either of these interpretations. The autoclaved Burnaby soil, after being initially suppressive, became more conducive than the raw soil. Perhaps the Burnaby soil is naturally suppressive, as Utkhede *et al.* (1978) speculated, and this condition was destroyed by autoclaving. The fact that higher white rot levels did not develop in autoclaved Cloverdale, Aldergrove and Summerland soils than in the corresponding raw soils indicates a lack of suppressiveness in those raw soils. That suppressiveness of the Burnaby soil was destroyed by autoclaving and was not re-established by random recolonization of

the soil suggests that the natural suppressiveness of this soil may have been contributed by one or more specific organisms, rather than by a high level of general mycostasis. However, Leggett (1983) conducted a comprehensive comparative analysis of the microflora of Burnaby and Cloverdale muck soils and was not able to identify any specific factor as responsible for the reputed suppressiveness of the Burnaby soil.

These results confirm the importance of soil microbial activity in the expression of white rot and suggest two lines of further inquiry in potential controls for this disease. First, given the role of increased levels of microbial activity in suppressing white rot infection, it would be useful to pursue experiments with organic amendments or other treatments that can enhance microbial activity. Second, the confirmatory evidence of the suppressive nature of the Burnaby soil should be followed up with additional attempts to elucidate what characteristic of that soil or its microflora imparts suppressiveness. Obviously, if the factor responsible for the suppressiveness can be transferred to or enhanced in other soils, it would be of use in white rot control. Additional understanding of the nature of the suppressiveness could have significant implications for white rot control.

E. DISCUSSION

Allium white rot has proven to be an extremely difficult disease to control, yet the causal organism has a simple asexual life cycle, a single perennating structure, and a limited host range. The remarkable properties of the sclerotium are probably the key to the success of *S. cepivorum* as a plant pathogen. The extraordinary persistence of the sclerotia limits the value of crop rotation. The response of the sclerotia to volatile germination stimulants is the basis of host specificity in this disease. Biological and cultural controls are usually aimed at reducing sclerotial populations. Success of chemical control has been limited, as the sclerotia are extremely resistant when dormant and the mycelium is only briefly exposed and vulnerable during infection. Despite much research work on many strategies, there appears to be little hope of a single, effective control for *Allium* white rot. In this thesis I have added to the understanding of the biology and ecology of this organism with the hope that effective integrated approaches to control might eventually be developed that will allow the large scale farming of *Allium* crops in the presence of *S. cepivorum*.

This thesis examined a few of the characteristics of sclerotia of *S. cepivorum* that affect the difficulty in controlling this disease. The year to year variability in the production of sclerotia observed in the field has important implications in disease control, especially for strategies relying on inoculum reduction. The data presented here show that the formation of sclerotia of *S. cepivorum in vitro* can be

93

triggered by a physical, chemical, nutritional or biological restriction of growth and expansion of the mycelium into new substrate. If a similar mechanism operates in the host plant, one could speculate on the form that restriction of mycelial expansion takes *in vivo*. Since apparently uncolonized tissue often remains when sclerotium formation occurs, the restriction of expansion occurs before the fungus has run out of tissue to colonize. This raises the possibility of tissue resistance in the host plant, a phenomenon that has not yet been described for this host/pathogen interaction. If this is true, then there may be more room for progress in breeding than previous efforts would indicate. It also raises the possibility that adverse conditions leading to a reduction in plant growth may trigger sclerotium formation. Based on the data presented here on the initiation of sclerotium formation, work needs to be done to identify the factors responsible for initiating sclerotium formation in the host plant. Such knowledge could lead to the development of management techniques that would reduce the formation of new sclerotia when a susceptible crop is grown in infested land, making inoculum reduction techniques more feasible in the long term.

This thesis describes the complete process of sclerotium formation in *S. cepivorum*, from initiation through maturation and aging. The early steps in morphogenesis observed here differ from those reported by Townsend and Willetts (1954). While differences in some morphological characteristics between isolates of a species are not uncommon, a process so fundamental as sclerotial morphogenesis would be expected to be highly uniform. There is,

however, close agreement between the observations presented here and published descriptions of the structure of mature sclerotia (Backhouse and Stewart 1987, Entwistle and Munasinghe 1981 a, Kohn and Grenville 1989 a, b, Leggett and Rahe 1985). Townsend and Willetts (1954) do not state the origin of their isolate(s) of *S. cepivorum*, and do not state whether they examined more than one isolate of the fungus. In the work presented in this thesis, morphogenesis was observed for only one isolate, so further discussion of the possible reasons for the differences in the morphogenic process should await examination of more isolates from diverse locations. On the other hand, many isolates were represented in the examinations of mature and aged sclerotia and, aside from the environmentally-induced differences in rind thickness, their structure was remarkably similar.

Leggett (1983) reported that laboratory-produced sclerotia decayed in soil more rapidly than field-collected sclerotia. Differences in rind structure were linked to the source of the sclerotia, with the laboratory-produced sclerotia possessing a thicker, more broken rind. It is probable that the differences attributed to sclerotial origin were due to differences in the moisture conditions under which the two groups of sclerotia matured and aged. Previous experimental work has shown that sclerotia that have been dried decay more rapidly in field soil than those not dried (Leggett *et al.* 1983, Leggett and Rahe 1985, Smith 1972). If rind structure is dependent on moisture relations and not sclerotial origin, the accelerated decay observed in laboratory-produced sclerotia may be consistent with the accelerated

decay seen in dried sclerotia. Periods of drying in the field may eventually prove to be as useful as flooding in inoculum reduction schemes.

Reduction in survival of sclerotia under flooded conditions in soil confirms the results reported by Joshi (1988), Coley-Smith *et al.* (1990), and Valdes and Edgington (1986). Since neither excess water nor anaerobiosis alone mimicked flooding in these experiments, this effect probably involved an anaerobic microflora adapted to survival in flooded, anaerobic soils. If this is borne out, then steps could be taken to enhance the populations and activity of the microflora to increase the effectiveness of inoculum reduction. The addition of organic matter to flooded soil has been reported to enhance sclerotial decay (Joshi 1988), which may act through providing an energy base for the increase in anaerobic microbial populations, as well as by accelerating the development of anaerobic conditions.

The confirmation of the suppressiveness of the Burnaby muck soil, postulated by Utkhede *et al.* (1978), is potentially valuable. Further work is needed to identify the factor or factors responsible for suppressiveness. Since suppressiveness was destroyed by autoclaving, the suppressive factor is likely biological. If this is the case, it may be possible to inoculate non-suppressive soils, such as those from Cloverdale, Aldergrove and Summerland, to convert them to suppressiveness. In a suppressive environment, all other white rot control measures could be expected to be more effective. The induction of suppressiveness associated with the flush of microbial growth following autoclaving in the non-suppressive soils, reinforces

the importance of the sclerotia-soil microflora interaction in the expression of white rot. It would not be surprising if much of the variability in results from a wide range of white rot control experiments around the world is due to differences in the microbiological status of the growth media in which the experiments were done.

F. APPENDIX I

Suitability of Sucrose Centrifugation as a Means of Recovery of Sclerotia of *Sclerotium cepivorum* from soil

Most of the field-collected sclerotia used in the research reported in this thesis were isolated from soil by sucrose centrifugation using the technique of Vimard *et al.* (1986). Fungistasis can be imposed on fungal sclerotia by exogenous microorganisms, particularly bacteria (Bristow and Lockwood 1975, Hsu and Lockwood 1973). This is particularly true for *S. cepivorum* where dormancy has been shown to be a biological phenomenon of external origin (Coley-Smith 1960, Coley-Smith *et al.* 1967). Microorganisms are stimulated by sclerotial exudates, imposing stasis on the sclerotium until the stasis is overcome by specific host-derived volatiles (Allen and Young 1968, Coley-Smith and Cooke 1971, Dickenson and Coley-Smith 1970, Hsu and Lockwood 1973). It seemed desirable to determine if addition of sucrose to the sclerotia during sucrose centrifugation treatment had any effect on the level or speed of disease development in this system, as the addition of sucrose could lead to increased microbial activity on the sclerotial surface and possibly alter the length or intensity of mycostasis. In addition, several other authors have developed or used techniques for isolation of sclerotia from soil that involve the addition of sucrose (Crowe *et al.* 1980, Utkhede and Rahe 1979, Banks and Edgington 1989), so this question may have wider relevance.

Materials and Methods

Sclerotia of *S. cepivorum* were collected from infected onions in a commercial onion farm near Cloverdale, British Columbia. They were stored in field soil in open 2L clay pots with a drain hole in the bottom, outdoors, exposed to the weather, until used. All sclerotia were assessed for viability at the beginning of the experiment as follows. They were surface sterilized in 1% NaOCl for 1 minute, rinsed twice in sterile distilled water (SDW), split open by squeezing with forceps and plated onto PDA. Plates were incubated at 18° C in the dark and examined 5 days later.

Cloverdale muck soil from an uninfested field on a commercial vegetable farm near Cloverdale, British Columbia, was used. The soil was partially dried to facilitate handling, and passed through a 5 mm sieve to remove rocks and roots. The experiments were conducted in 11 cm diameter plastic pots filled with soil. Four 5 cm deep holes were made in the soil in each pot, ten sclerotia were placed into each hole, soil was added to a depth of 1.5 cm, then two seeds were placed into each and covered with soil. Onion seeds *cv.* Improved Autumn Spice (Buckerfields Ltd., Langley, British Columbia) were used. All pots were saturated with water at the beginning of the experiment and resaturated whenever the plants were judged to be close to wilting. Each treatment was replicated five times.

Treatments of sclerotia were as follows: (i) sucrose centrifugation (Vimard *et al.* 1986) or by (ii) wet sieving (Utkhede and Rahe 1979) without sucrose floatation. Pots were incubated in trays in

a waterbath to provide a root zone temperature of about 17° C under a 12 h photoperiod. Pots were examined every 3 or 4 days and plants which were dead or obviously dying were removed for confirmation of white rot infection. Roots and the stem base of these plants were surface sterilized in 1% NaOCl for 1 minute, then plated onto PDA and incubated at 18° C until sclerotia typical of *S. cepivorum* developed. The experiment was terminated 120 days after seeding when all plants were removed and roots and the stem bases of these plants were plated as above.

Results

Initial viability of the sclerotia obtained by sucrose centrifugation and by wet sieving was 96.3% and 94.8 % respectively. This difference was not significant ($P=0.78$). Mean white rot at harvest in the wet sieving sclerotia treatment and the sucrose centrifugation treatment was 46.9% and 32.4% respectively. This difference was not significant ($P=0.48$) (Table 8).

Table 8. Effect of sucrose centrifugation on viability of sclerotia of *Sclerotium cepivorum* and their ability to infect *Allium cepa*.

Sclerotial Recovery Method	Initial Viability of Sclerotia (%)	Mean White Rot at Harvest (%)
Wet Sieving	94.8 a[1]	46.9 b[1]
Sucrose Centrifugation	96.3 a	32.4 b

[1] Means within a column followed by the same letter do not differ ($P<0.05$) according to Duncan's Multiple Range Test.

Discussion

Exposure of sclerotia of *S. cepivorum* to sucrose during recovery from soil had no significant effect on the level of disease observed in this study. Whether this treatment had any effect on sclerotial dormancy or survival was not tested, but the interpretation of disease incidence in this system does not appear to be compromised by the use of the sucrose centrifugation technique. The use of sucrose centrifugation to recover sclerotia from soil for use as inoculum in the other experiments reported here is therefore reasonable, and desirable because of its efficiency.

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