Pathogen Diversity, Epidemiology and Control of Sclerotinia Disease in Vegetable Crops

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List of Abbreviations

µl	Microlitre
µm	Micrometre
µmol	Micromole
AFG	Afghanistan
AMOVA	Analysis of Molecular Variance
ANOVA	Analysis of Variance
ARG	Argentina
ATP	Adenosine triphosphate
AUS	Australia
bp	Base pair
BRA	Brazil
C	Celsius
CDS	Carrot Diversity Set
CHE	Switzerland
CHN	China
cm	Centimetre
CZE	Czech Republic
d	Day
d.f.	Degrees of freedom
DNA	Deoxyribonucleic acid
dw	Dry weight
DZA	Algeria
ED	Effective Dose
EGY	Egypt
ESP	Spain
ETH	Ethiopia
F	Forward
FRA	France
g	Gram
GBR	Great Britain
GLM	Generalised Linear Model
GSL	Glucosinolate
h	Hour
ha	Hectare
HPLC	High performance liquid chromatography
IGS	Intergenic spacer region
IND	India
IRN	Iran
ISR	Israel
ITC	Isothiocyanate
ITS	Internal transcribed spacer region
JPN	Japan
kg	Kilogram
km	Kilometre
L	Litre
LBY	Libya
LOG	Logarithm
LSD	Least Significant Difference
LSU	Large subunit
M	Molar mass
m	Metre
MAR	Morocco
mAU	Milli-absorbance unit
MCG	Mycelial Compatibility Group
mg	Milligram
min	minute
ml	Millilitre
n	Number
NB	Nota bene
ng	Nanogram
NLD	Netherlands
nm	Nanometre
No.	Number
NZL	New Zealand
p	Probability
PAK	Pakistan
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
pH	Power of hydrogen
POL	Poland
ppm	Parts per million
PRT	Portugal
R	Reverse
rDNA	Ribosomal deoxyribonucleic acid
REML	Restricted or residual maximum likelihood
RNA	Ribonucleic acid
RO	Reverse osmosis
RP	Reversed-phase
rRNA	Ribosomal ribonucleic acid
S	Sulphur
s	Second
SEM	Standard Error of the Mean
sp.	Species
spp.	Species
SUN	Suriname
SWE	Sweden
SYR	Syria
TNF	Tumour Necrosis Factor protein
TUR	Turkey
UK	United Kingdom
USA	United States of America
UV	Ultra violet
v/v	Volume by volume
w/w	Weight by weight

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Declaration

This thesis is presented in accordance with the regulations for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. The work in this thesis has been undertaken by the author except where otherwise stated.

Summary

Sclerotinia sclerotiorum is a necrotrophic fungal pathogen with a worldwide distribution and a wide host range, including many economically important crops. The control strategies for this pathogen and related species include using fungicides, biological control agents and cultural practices such as crop rotations. However, the genetic diversity and the long term survival structures (sclerotia) of this pathogen, combined with the recent discovery of the related species *S. subarctica* in England and the need for growers to implement integrated disease management strategies means that new control measures need to be sought. Biofumigation, using green manures which are macerated and ploughed into the soil, may be a useful new control approach in an integrated programme.

Microcosm and *in vitro* experiments clearly showed that volatiles released from biofumigation crops have a direct inhibitory effect on the mycelial growth and carpogenic germination of *S. sclerotiorum* sclerotia. The most effective biofumigation crop for inhibiting carpogenic germination varied depending on whether the volatiles released from the biofumigant crops were in direct contact with the sclerotia when the most effective crop was *Raphanus sativus* 'Terranova', or in the vapour phase when the most effective crop was *B. juncea* 'Vittasso'.

Carrot root inoculations showed that the number of sclerotia produced on carrot roots was significantly affected by *S. sclerotiorum* isolate. However, the results also showed that the weight of individual sclerotia produced by different isolates was influenced by carrot accession, but not by *S. sclerotiorum* isolate. Additionally, the carrot plant and detached leaf inoculations showed significant differences in the rate of lesion progression of *S. sclerotiorum* on different carrot accessions, indicating differences in susceptibility to the pathogen.

S. subarctica microsatellite haplotypes identified in this research were shown to be shared between Scotland and Norway, and between crop plants and meadow buttercup. However, the English population did not share any microsatellite haplotypes with any other population, and analysis indicated that this *S. subarctica* population in England may be isolated and inbred.

1. Introduction

1.1 Taxonomy of *Sclerotinia* species

Sclerotinia is a genus of fungi in the family Sclerotiniaceae which currently comprises 13 named species (Table 1.1) (NCBI, 2014) including three economically important necrotrophic plant pathogens: *S. sclerotiorum* (Lib.) de Bary, *S. trifoliorum* Erikss. and *S. minor* Jagger. (Kohn, 1979). It also includes *S. subarctica* nom. prov. which has not been formally described and its host range has yet to be published, but it causes symptoms similar to *S. sclerotiorum* (Clarkson *et al.*, 2010).

Table 1.1 - The taxonomic hierarchy of the family Sclerotiniaceae (ITIS, 2014) and 13 named species (NCBI, 2014).

Kingdom	Fungi
Subkingdom	Dikarya
Division	Ascomycota
Subdivision	Pezizomycotina
Class	Leotiomycetes
Subclass	Leotiomycetidae
Order	Helotiales Nannf., 1932
Family	Sclerotiniaceae Whetzel ex Whetzel, 1945
Genus	*Sclerotinia*
Species	*Sclerotinia borealis* Bubák & Vleugel 1917
	Sclerotinia bulborum (Wakker) Sacc. 1889
	Sclerotinia glacialis Graf & Schumacher
	Sclerotinia homoeocarpa F. T. Benn. 1937
	Sclerotinia minor Jagger 1920
	Sclerotinia nivalis Saito 1997
	Sclerotinia pirolae Grosse
	Sclerotinia sclerotiorum (Lib.) de Bary 1884
	Sclerotinia spermophila Noble 1948
	Sclerotinia subarctica nom. prov.
	Sclerotinia tetraspora Holst-Jensen & T. Schumach. 1994
	Sclerotinia trifoliorum Erikss. 1880
	Sclerotinia veratri E. K. Cash & R. W. Davidson 1933

1.2 Host Range and Economic Importance of *Sclerotinia* spp.

The majority of this introduction focuses on *Sclerotinia sclerotiorum* due to the research carried out on this species, with information included on related species where this is available. *S. sclerotiorum* affects many economically important crops (Hegedus & Rimmer, 2005) and has a world-wide distribution (Purdy, 1979) (Figure 1.1).

S. sclerotiorum has a host range of over 400 plant species (Boland & Hall, 1994), whereas just over 90 species are hosts for *S. minor* (Melzer *et al.*, 1997). Crops susceptible to *S. sclerotiorum* and *S. minor* include lettuce, beans, peas, potatoes and carrots, while *S. sclerotiorum* also infects the extensively grown and economically important crop oilseed rape (*Brassica napus*). The host range of *S. trifoliorum* is more limited, being found mainly on forage and vegetable legumes (Willetts & Wong, 1980). *S. subarctica* has recently been identified in the UK on meadow buttercup (Clarkson *et al.*, 2010) after previously only being found in Norway on the wild hosts meadow buttercup, dandelion, and yellow marsh marigold (Holst-Jensen *et al.*, 1998) and on a range of vegetable crops in Alaska, including potato, lettuce, cabbage, bean, and squash (Winton *et al.*, 2006). The symptoms caused by *S. subarctica* are very similar to *S. sclerotiorum* and therefore the former may be undetected in crops in the UK (Clarkson *et al.*, 2013).

Yield losses caused by *Sclerotinia* spp. can vary, but are sometimes as high as 100 % (Purdy, 1979). Losses in fruit and vegetable crops can be due to produce rotting in the field prior to harvest, as well as in storage (Willetts & Wong, 1980) or a reduction in quality of harvested produce. Where crops have been grown for seed yields can be affected by a reduction in seed size from premature ripening of infected

plants, as well as harvested seed becoming contaminated with sclerotia (Morrall & Dueck, 1982). The biggest yield losses are in crops such as oilseed rape, which is extensively grown in many areas worldwide.

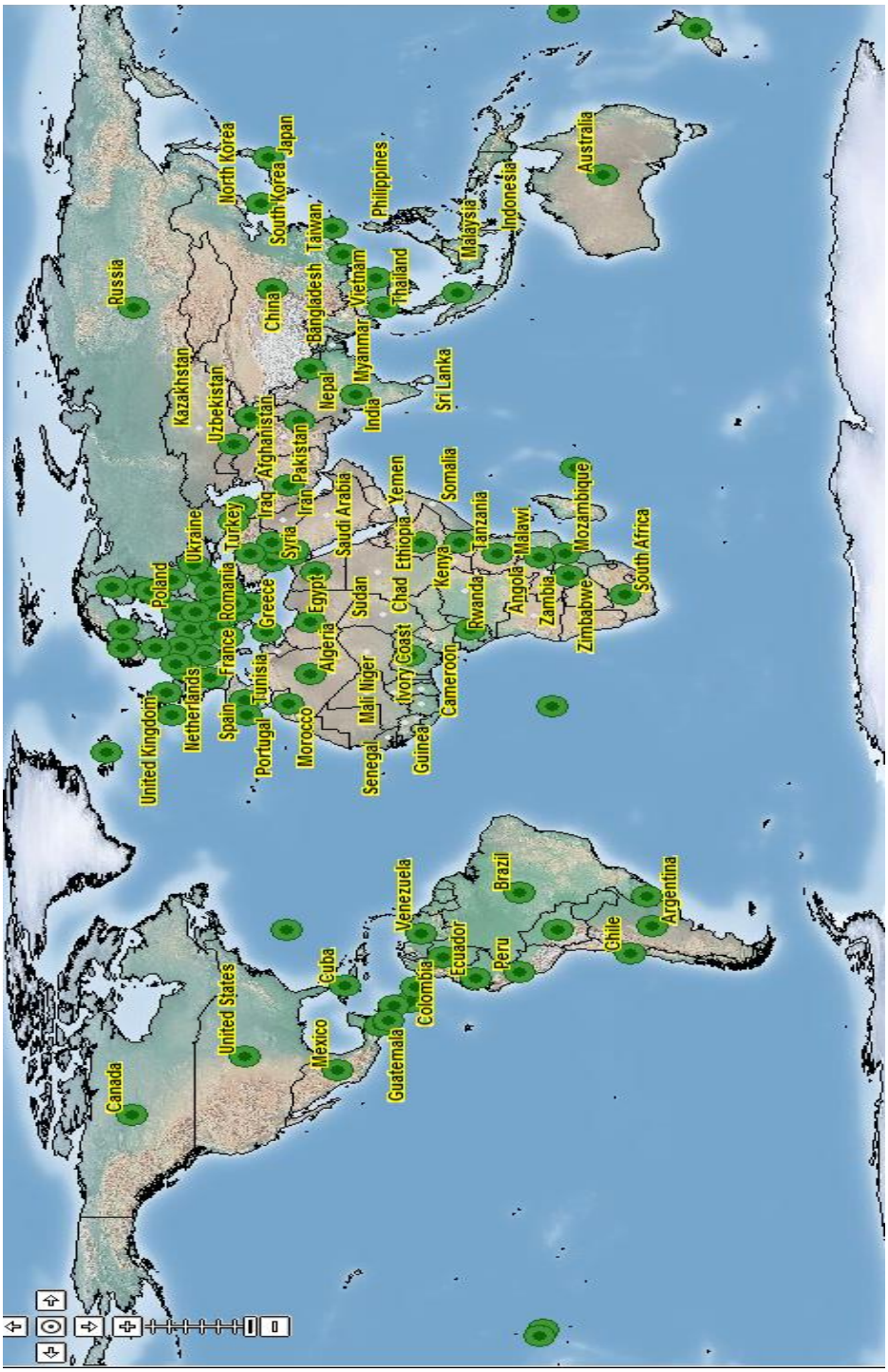

Figure 1.1 - Disease map showing the global distribution of *Sclerotinia sclerotiorum*. Green markers indicate countries where Sclerotinia disease has been reported. (Plantwise, 2005)

In 2011, a total of 33.7 million hectares (ha) of oilseed rape were planted worldwide, yielding 62.6 million tonnes worth over $35 billion. In the UK 705,000 ha of oilseed rape were grown in 2011, worth $1.6 billion (FAO, 2014). Yields can be halved when an oilseed rape crop is severely affected by Sclerotinia disease (Pope *et al.*, 1989; Berry *et al.*, 2014) and between 1991 and 2002 losses in the USA were estimated to have a direct economic impact of $94 million (Lamey, 2003).

Losses in other crops can also be significant; based on yield losses and market values of the crop it is estimated USA soybean growers lost $560 million due to Sclerotinia disease in 2009 (Peltier *et al.*, 2012), and the disease caused an average yield reduction in bean fields in Nebraska of 13% over four years (Steadman, 1983). As a result, in 2002 the National Sclerotinia Initiative was established in the USA to coordinate research on *Sclerotinia* disease across a range of crops (USDA, 2014). In the UK, carrot growers suffer estimated annual crop losses in excess of six million pounds due to the disease, with marketable yield predicted to be reduced by one tonne per hectare for each 1% increase in diseased roots (McQuilken, 2011). Additionally, lettuce growers in the UK face potential crop losses of 15%, worth an estimated £12 million, due to Sclerotinia disease (DEFRA, 2013).

1.3 Life Cycle and Epidemiology of *Sclerotinia* spp.

1.3.1 Sclerotia and Survival

The long term survival structures for *Sclerotinia* spp. are small black resting bodies called sclerotia (Willetts & Wong, 1980). Sclerotia of *S. sclerotiorum, S. trifoliorum* and *S. subarctica* can germinate carpogenically when brought close to the soil

Figure 1.2 – Sclerotia of *S. sclerotiorum* formed on lettuce plants (top left), an apothecium in the field (top right), sclerotia of *S. sclerotiorum* forming on inoculated carrot roots (bottom left) and apothecia produced from *S. sclerotiorum* sclerotia (bottom right).

surface to produce mushroom-like apothecia (Figure 1.2). The apothecia release air-borne ascospores which infect plants, upon which further sclerotia are formed and are returned to the soil (Figure 1.3). However, *S. minor* is thought to mainly infect plants by myceliogenic germination to produce hyphae which attack plant tissues directly, and apothecia are rarely seen in the field (Abawi & Grogan, 1979) A sclerotium is a hyphal aggregate, with a black outer rind containing melanin and a light coloured interior known as the medulla which contains proteins and carbohydrates (Le Tourneau, 1979).

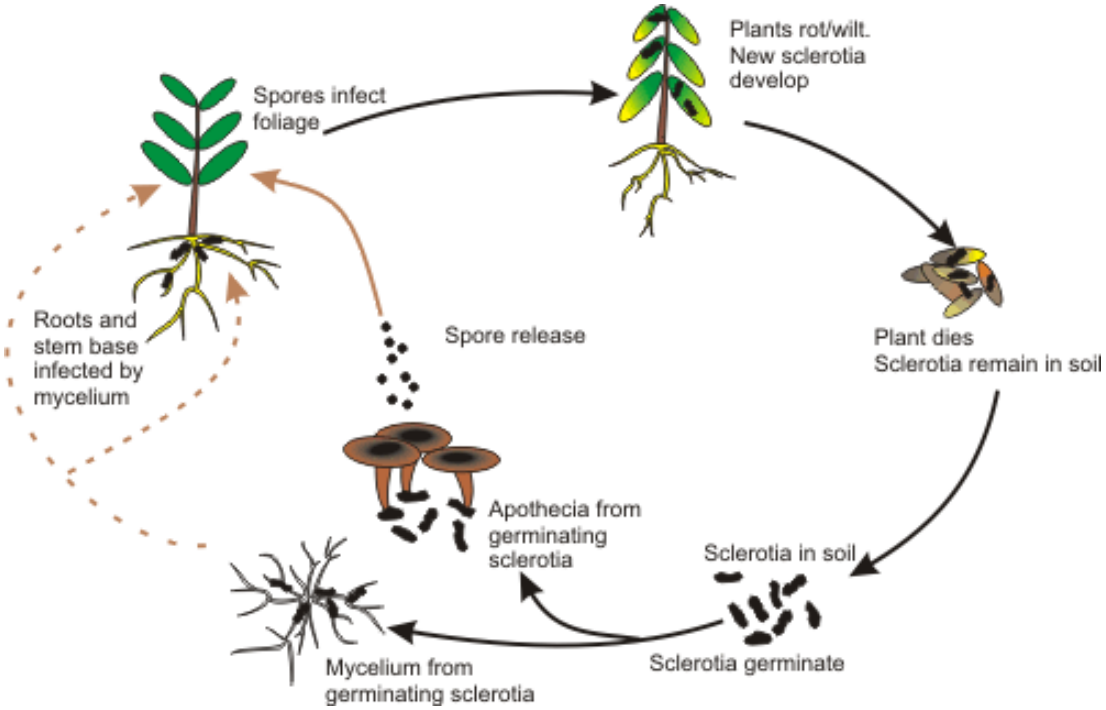

Figure 1.3 - *Sclerotinia sclerotiorum* life cycle (HDC, 2012)

Sclerotia are generally produced when mycelial growth of the pathogen encounters limited nutrients (Christias & Lockwood, 1973), and their longevity is variable, being influenced by many factors including the time and depth of burial (Duncan *et al.*, 2006), and soil type (Merriman, 1976). Under suitable conditions of temperature and moisture a large percentage of *S. sclerotiorum* sclerotia can survive at least three years (Ćosić *et al.*, 2012) although other studies estimate sclerotia remaining viable for up to eight years (Adams & Ayers, 1979).

The number of sclerotia produced by *S. sclerotiorum* on different plant tissues is variable and an important factor in determining the inoculum levels in soil following an infected crop. Infected cabbage heads were found to produce between 250 and 500 sclerotia, (Leiner & Winton, 2006), while infected carrot roots produced up to 30 (Jensen *et al.*, 2008). In combinable peas it has been estimated that approximately two sclerotia are returned to each m^2 of soil for each 1% disease incidence (Archer *et al.*, 1992). Additionally, different isolates of *S. sclerotiorum* have been found to vary in their ability to form sclerotia. For instance, in a project at Warwick HRI, *S. sclerotiorum* isolate L6 was found to produce large numbers of small sclerotia while isolate L44 produced small numbers of larger sclerotia across several crop plants (lettuce, oilseed rape, bean) (Young *et al.*, 2013).

1.3.2 Germination of Sclerotia, Ascospore Dispersal, Infection and Disease Development

S. sclerotiorum sclerotia often require a period of conditioning before they can germinate carpogenically to produce apothecia, and this is affected by many environmental factors including temperature, light, and water availability (Phillips,

1987; Hao *et al.*, 2003), as well as the temperature at which the sclerotia were formed (Huang & Kozub, 1991). In temperate regions a combination of low temperatures over a period of time, followed by an increase in temperature and adequate soil moisture is required for production of apothecia (Bardin & Huang, 2001). They are usually produced after canopy closure in crops due to shading helping to maintain the high soil moisture required (Bolton *et al.*, 2006). An apothecium can produce up to sixteen hundred ascospores per hour, equivalent to 7.7 x 10^5 ascospores over a twenty day period (Clarkson *et al.*, 2003), and depending on environmental conditions these ascospores can survive on plant tissue for approximately two weeks (Caesar & Pearson, 1983). It has been shown that ascospores can travel for several kilometres in air currents (Li *et al.*, 1994), although most remain in the field in which they were released (Wegulo *et al.*, 1998).

S. sclerotiorum ascospores can germinate on healthy plant tissue, but need a film of water and an exogenous nutrient source such as senescent tissues (flower petals, leaves and pollen grains) to enable subsequent infection of a host (Bardin & Huang, 2001). Flowering time is therefore considered a critical forecasting factor for Sclerotinia disease in crops such as oilseed rape (Bolton *et al.*, 2006), whereas in crops such as carrots infection is generally first seen on petioles of lodged senescing leaves (McQuilken, 2011). Environmental conditions are important for infection, with approximately 48-72 h of continuous leaf wetness needed for ascospores to infect host plants (Abawi & Grogan, 1979). Mycelium grows on the senescing plant material and can enter through the stomata of the host plant, or penetrate the cuticle using enzymes or mechanical force (Bolton *et al.*, 2006). When interacting with a host plant, *S. sclerotiorum* secretes a range of cell wall degrading enzymes which enables penetration, maceration of plant tissues and degradation of cell wall

components (Lumsden, 1979; Riou *et al.*, 1991). It has also been shown that *S. sclerotiorum* produces oxalic acid which is involved in deregulating guard cell function, causing open stomata in advance of invading hyphae (Guimarães & Stotz, 2004). Other studies investigating oxalic acid as a pathogenicity factor of *S. sclerotiorum* showed that initially the pathogen generates a reducing environment in host cells which suppresses host defense responses, including the oxidative burst and callose deposition (Williams *et al.*, 2011). Once infection is established it then induces the generation of plant reactive oxygen species leading to programmed cell death of host tissue (Williams *et al.*, 2011). The association of oxalic acid production with pathogenicity of *Sclerotinia* spp. has been ratified by the development of mutants which do not produce oxalic acid but still have the full complement of cell wall degrading enzymes. These mutant strains were found to be non-pathogenic on dry bean (Godoy *et al.*, 1990). Similarly, much work has been carried out on transgenic plants transformed to express an oxalate oxidase gene, resulting in plants which have enhanced levels of resistance to *Sclerotinia* spp. (Donaldson *et al.*, 2001; Livingstone *et al.*, 2005; Dias *et al.*, 2006; Dong *et al.*, 2008; Cunha *et al.*, 2010).

Due to the wide host range of *S. sclerotiorum* disease symptoms vary, but white fluffy mycelial growth is characteristic on infected plants. Pale or dark brown lesions may be seen on the base of stems of herbaceous plants, often quickly covered by white mycelium (Figure 1.4), or infection may begin on a leaf and move into the stem. As the disease progresses into the main stems wilting typically occurs, and sclerotia are formed either inside infected tissue or on the tissue surface (Bolton *et al.*, 2006).

Figure 1.4 - Sclerotinia disease on lettuce (left) and carrot (right) in the field, showing the characteristic symptoms of white fluffy mycelium and brown lesions.

1.4 *Sclerotinia sclerotiorum* on Carrot

The research in this thesis focuses particularly on Sclerotinia disease on carrots, but much of the work is generally applicable to other susceptible crops. *S. sclerotiorum* is one of the most economically important diseases affecting carrot production worldwide (Kora *et al.*, 2005) and has been reported in over twenty carrot producing countries (Kora *et al.*, 2003). It is a particular problem in temperate regions where carrots are stored for long periods (Kora *et al.*, 2005a) and can cause post-harvest epidemics in storage (Foster *et al.*, 2008).

Infection of carrots by *S. sclerotiorum* is normally via ascospores landing on damaged or senescing leaves, which then germinate and infect tissue. Spore release from apothecia can occur throughout the growing season from June to September, with optimal conditions for foliage infection being four days continuous leaf wetness

with an air temperature of 10 to 18°C (McQuilken, 2011). It has been shown that under field conditions the pathogen enters the carrot root via the crown of the plant (Jensen *et al.*, 2008), and other work shows that it is unlikely that carrot roots are directly infected by mycelium germinating from sclerotia in the soil surrounding the carrot roots (Finlayson *et al.*, 1989b). It has been suggested that control of Sclerotinia disease in carrots is best obtained by preventing leaf infection and reducing the quantity of sclerotia in the soil (McQuilken, 2011).

1.5 Diversity of *Sclerotinia sclerotiorum*

S. sclerotiorum is mainly homothallic and population studies in the UK, North America and Canada on the pathogen have shown a predominately clonal mode of reproduction (Kohli *et al.*, 1995; Cubeta *et al.*, 1997; Clarkson *et al.*, 2013). Individual isolates can be classified into clonal lineages using independent markers such as mycelial compatibility groups (MCG) (defined by the ability of different isolates to fuse and form one cohesive colony), microsatellites or DNA fingerprinting (Kohn *et al.*, 1991; Sirjusingh & Kohn, 2001).

The population structure of *S. sclerotiorum* has been studied in the UK on both crop plants and meadow buttercup. Based on analysis of microsatellite markers multiple haplotypes of *S. sclerotiorum* were identified, with one haplotype being found more frequently than the rest, at different locations and on different crops (Clarkson *et al.*, 2013). This multi-clonal structure based on microsatellites has been observed in several previous studies, including populations in Alaska (Winton *et al.*, 2006), Australia (Sexton & Howlett, 2004), and Iran (Hemmati *et al.*, 2009). The reasons for the success of certain clones is not understood, but could be due to traits such as

sclerotial germination and survival, effectiveness of dispersal of ascospores or aggressiveness (Clarkson *et al*., 2013).

Research has shown that generally microsatellite haplotypes and DNA fingerprints are closely associated with MCGs, (Kohn *et al.*, 1991; Clarkson *et al.*, 2013), but a few studies have noted a de-coupling of MCGs and microsatellite markers or DNA fingerprints, e.g. isolates from potato in North America (Atallah *et al.*, 2004) and from buttercup in Norway (Kohn, 1995). In these studies, the hypothesis of random mating based on linkage disequilibrium measures of molecular data could not be rejected, suggesting that outcrossing is occurring, although there is no known mechanism for this. Direct evidence for outcrossing has been demonstrated when ascospores collected from a single apothecium were found to belong to more than one MCG (Atallah *et al*., 2004).

1.6 Methods for Control of Sclerotinia Disease

1.6.1 Plant Resistance

There is a general lack of any high level resistance in most of the major crops affected by Sclerotinia disease (Bolton *et al.*, 2006), with many breeders and growers relying on partial resistance in crops such as soybean (Boland & Hall, 1987), pea (Porter *et al.*, 2009), sunflower (Godoy *et al.*, 2005) and various *Brassica* species (Mei *et al.*, 2011; Mei *et al.*, 2012) to provide some disease control. Possible pre-harvest resistance has been demonstrated in glasshouse trials with carrots, one defence mechanism being leaf abscission after infection of the petiole (Foster *et al.*, 2008) and a second being a structural barrier of lignin, diphenols, suberin flavanols,

peroxidases and phenolases (Craft & Audia, 1962), which slow or stop progression of the pathogen from an infected petiole into the crown (Foster *et al.,* 2008).

Wild relatives of cultivated crops potentially provide sources of greater resistance, and Garg *et al.* (2010) reported increased levels of resistance against *S. sclerotiorum* through introgression of three wild crucifers into cultivated *Brassica*. Further work assessing a range of *Brassica* species has identified other sources of resistance that could be used in future breeding programmes (Uloth *et al.*, 2013), together with associated quantitative trait loci (QTL) to enable screening of genotypes as well as phenotypes (Bolton *et al.*, 2006). Various quantitative genetic traits have been identified in connection with resistance to *S. sclerotiorum,* including on soybean (Arahana *et al.*, 2001), sunflower (Mestries *et al.*, 1998) and oilseed rape (Wu *et al.*, 2014).

1.6.2 Fungicides

Fungicides are the major control method for Sclerotinia disease and are used on a range of crops, such as oilseed rape, soybean, carrots and lettuce (Budge & Whipps, 2001; Kora *et al.,* 2005a; Bolton *et al.,* 2006). The fungicides are applied in order to kill ascospores before they infect plants, with the best protection in carrot crops obtained by spraying before canopy closure (McQuilken, 2011). However, limits on the permitted number of sprays make it essential to time applications in order to control disease effectively (Clarkson *et al.*, 2007) and forecasting systems have been developed for some crops to ensure fungicides are used efficiently (McDonald & Boland, 2004).

In addition to the problems associated with timing of fungicide applications, some of the effective active ingredients in fungicides used routinely against Sclerotinia disease such as boscalid, carbendazim, cyprodinil, fludioxonil (Matheron & Porchas, 2008), azoxystrobin and difenoconazole are classed as medium to high risk for resistance (McQuilken, 2011). *S. sclerotiorum* isolates with resistance to carbendazim have been found in both China (Yin *et al.*, 2010) and in several regions of France (Kaczmar *et al.*, 2000). No resistance was found to fludioxonil, suggesting that this active can be used in areas of carbendazim resistance (Kuang *et al.*, 2011). Additionally, no resistance has been found to boscalid when tested against isolates of *S. sclerotiorum* from China, although boscalid was not being used in China at the time of the studies (Liu *et al.*, 2009; Wang *et al.*, 2009), and no resistance was found in Australian isolates from bean fields, where boscalid was the only fungicide registered for control (Jones *et al.*, 2011). Similarly, it was found that there has been no change in *S. sclerotiorum* sensitivity to boscalid since its introduction in Europe, although very few resistance studies have been carried out (Stammler *et al.*, 2007).

1.6.3 Soil Fumigation

Chemical soil fumigants such as methyl bromide and metam sodium have been used as soil treatments against a range of pests and pathogens, and are very effective at reducing the viability of sclerotia of *Sclerotinia* spp. (Ben-Yephet, 1988; O'Neill & Rickwood, 2002; Ceustermans *et al.*, 2010). The world's largest user of methyl bromide was the USA, where it was used for crops such as strawberry, pepper and tomato (Rosskopf *et al.*, 2005). It was also used extensively as a pre planting treatment for lettuce in Belgium and Italy (Saharan & Mehta, 2008; Ceustermans *et*

al., 2010), and in cut flower and bulb production in the UK (O'Neill & Rickwood, 2002). However, due to its contribution to the depletion of the ozone layer its use was prohibited in the developed world in 2005 (DEFRA, 2010).

The less potent metam sodium (sodium *N*-methyldithiocarbamate) has been used world-wide since the 1950s to control various soil borne pests and pathogens in moderately intensive production systems for crops such as potato (Matthiessen & Kirkegaard, 2006; Cox, 2006). However, it is also being withdrawn from the market, with approval for essential uses in the UK up to 31st December 2014 only (HSE, 2014).

1.6.4 Cultural Control and Soil Solarisation

Crop rotations with non-host crops such as wheat or corn can be used to achieve a reduction in either the numbers of *S. sclerotiorum* sclerotia in the soil (Gracia-Garza *et al.*, 2002), or the number of apothecia (Mueller *et al.*, 2002), but given the large host range of the pathogen the number of crops suitable for rotation is limited (Peltier *et al.*, 2012). Simply burying sclerotia to prevent carpogenic germination is effective at reducing disease (Williams & Stelfox, 1980), and Mueller *et al.* (2002) found burying sclerotia deeper than 10 cm in the soil by moldboard ploughing delayed the emergence of apothecia when compared to a no tillage plot. However, a subsequent cultivation could bring viable sclerotia back to the soil surface (Mitchell & Wheeler, 1990). Other studies have found fewer apothecia and a reduced level of disease when using no-till systems (Kurle *et al.*, 2001; Gracia-Garza *et al.*, 2002).

Close crop canopies and lodging of foliage favours Sclerotinia disease development, and early planting of crops with a narrow row width, a high planting density and high soil fertility all help to accelerate canopy closure (Peltier *et al.*, 2012). Changing these practices may also reduce crop yields, but clipping of carrot foliage to prevent lodging and hence reduce plant to plant spread of infection between beds was found to protect against Sclerotinia disease in carrots (Kora *et al.*, 2005). Additionally applying optimum amounts of nitrogen helps to limit canopy growth and lodging (McQuilken, 2011).

Soil solarisation uses clear plastic laid on the soil surface for several weeks during hot seasons, so the sun can penetrate and heat up the ground. Temperatures of 40 °C and upwards can be achieved, which facilitate microbial colonisation and degradation of weakened sclerotia, therefore reducing the viability of sclerotia in the top 10 cm of soil by over 90%, and reducing the ability of surviving sclerotia to germinate carpogenically (Ben-Yephet, 1988; Phillips, 1990; Swaminathan *et al.*, 1999). However, solarisation is most effective during the middle of the cropping season making it an expensive control option due to the loss of land for several weeks combined with the cost of the polythene (Phillips, 1990).

1.6.5 Soil Amendments and Biological Control

Various non-organic soil amendments have been shown to inhibit sclerotial germination of *S. sclerotiorum*, such as potassium bicarbonate (Ordonez-Valencia *et al.*, 2009) and the fertiliser calcium cyanamide (Perlka®) (Huang *et al.*, 2006). However, growers have found Perlka® too expensive for it to be economically viable

for use in oilseed rape (Blake, 2008). Organic matter can also affect the survival of sclerotia (Coley-Smith & Cooke, 1971), and the addition of compost to soil was shown to significantly reduce carpogenic germination, possibly due to the microbes present (Couper *et al.*, 2001). It can also inhibit sclerotia production *in vitro* (Mello *et al.*, 2005), but another study found high levels of organic matter increased carpogenic germination (Ferraz *et al.*, 1999).

Many different organic amendments have been shown to inhibit carpogenic germination of *S. sclerotiorum* sclerotia, including fish meal, bone meal, raw cattle manure (Huang *et al.*, 2002), fowl manure and lucerne hay (Asirifi *et al.*, 1994), and some amendments can be even more effective when combined with biological control agents such as *Trichoderma* spp. or *Coniothyrium minitans* (Huang *et al.*, 2005). These agents have been the subject of extensive research (Budge & Whipps, 1991; McQuilken *et al.*, 1995; McQuilken & Chalton, 2009; Jones *et al.*, 2014), with *C. minitans* being commercialised and marketed as Contans WG. However, this product has not always provided consistent results under field conditions (Fernando *et al.*, 2004).

1.6.6 Biofumigation

Many *Brassica* spp. produce significant levels of glucosinolate (GSL) compounds, the concentration of which can vary greatly between tissues within a single plant (Gupta *et al.*, 2012). When a plant cell is ruptured as a result of pathogen attack or mechanical wounding the GSLs come into contact with the enzyme myrosinase (held separately in the plant cells from the GSLs) and are hydrolysed in the presence of water to release various products, including isothiocyanates (ITCs) (Vig *et al.*,

2009). ITCs have a wide range of biocidal characteristics (Kurt *et al.*, 2011) and are acutely toxic to several pathogenic fungi (Chew, 1987). Due to the volatility of ITCs the term 'biofumigation' was coined to describe the process of using *Brassica* crops as green manures which are macerated and incorporated into the ground to suppress soil borne pests and diseases (Kirkegaard *et al.*, 1993; Kirkegaard, 2009).

Using selected *Brassica* crops for biofumigation can provide control against Sclerotinia disease (Porter *et al.*, 2002), but has not yet been shown to have a consistent significant effect on viability of sclerotia (Matthiessen & Kirkegaard, 2002). Synthetic pure ITCs significantly reduce sclerotial viability *in vitro* (Kurt *et al.*, 2011) and it has been shown that volatiles from *Brassica* crops can also inhibit the formation of sclerotia *in vitro* (Ojaghian *et al.*, 2012), potentially helping to reduce levels of inoculum in the soil.

There is a possibility of utilising *Brassica* cultivars bred for high ITC levels in an integrated disease management system against *S. sclerotiorum* (Pung *et al.*, 2004), although a greater understanding is needed concerning the major mechanisms involved in pathogen suppression by biofumigants generally (Motisi *et al.*, 2010). For instance, it has been found that incorporating broccoli residues both with and without added myrosinase into soil stimulates microbial activity (Omirou *et al.*, 2011), which raises questions regarding whether ITCs are directly responsible for suppressing plant pathogenic fungi or whether there is an indirect effect of increasing microbial activity and biomass. Hollister *et al.* (2013) found large increases in bacterial taxa associated with fungal disease suppression following the soil incorporation of *Brassica* seed meals. Other studies have shown non-detrimental transient changes to microbial communities in the soil following incorporation of

Brassicas (Potgieter *et al*., 2013), and an increase in soil organic matter and mineral nutrients with a minimal effect on the root colonisation of arbuscular mycorrhizal fungi (Koron *et al.*, 2014).

With regard to using biofumigant crops in an integrated disease management system, work has been carried out to combine them with the biocontrol agent *Bacillus amyloliquefaciens* to control Phytophthora blight in pepper, and this combination of treatments gave the greatest reduction in disease incidence (Wang *et al.*, 2014). Biofumigation has also been investigated for use as postharvest control against various fungal pathogens, significantly reducing decay in strawberries caused by *Botrytis cinerea* in one study (Ugolini *et al.*, 2014).

In Italy biofumigation products have been developed, resulting in pellets, meals and liquid products being marketed (in general terms) as fertilisers and feeds with soil conditioning and plant health promoting effects (Lazzeri *et al.*, 2013). These seed meals have been shown to be effective for control of apple replant disease (Mazzola *et al.*, 2007) and for control of *Phytophthora nicotianae* in greenhouse trials with pepper plants (Morales-Rodríguez *et al.*, 2013). These products may be advantageous when consideration is given to the potential problems of the biofumigation plants themselves being host to the pathogen of interest and increasing pathogen inoculum potential (Gilardi *et al.*, 2013), as well as issues surrounding the limitation of incorporating biofumigant crops into rotations.

1.7 Project Aims and Objectives

The overall aim of this research was to evaluate biofumigation treatments for control of Sclerotinia disease caused by *S. sclerotiorum,* and to assess pathogen diversity, with a focus on *S. subarctica*. The specific objectives were to:

1. Determine the effect of biofumigants on the germination and survival of sclerotia of *Sclerotinia sclerotiorum*.

2. Evaluate carrot varieties for susceptibility to *Sclerotinia sclerotiorum* and quantify production of sclerotia by different *S. sclerotiorum* isolates.

3. Investigate the population diversity and epidemiology of *Sclerotinia subarctica.*

1.8 Thesis Structure

Chapter 2 – Frequency and diversity of *Sclerotinia* spp. in the UK and Norway

Describes the sampling of *Sclerotinia* spp. in the UK and Norway, and the molecular analysis of *S. sclerotiorum* and *S. subarctica* on crop and wild hosts using microsatellite markers and IGS sequencing.

Chapter 3 – Pathogenicity of *Sclerotinia sclerotiorum* on carrots

Describes the root, whole plant and detached leaf inoculation experiments carried out to ascertain the production of sclerotia by two different *S. sclerotiorum* genotypes, and to evaluate the susceptibility of different carrot cultivars to Sclerotinia disease.

Chapter 4 – Biofumigation as a control measure against Sclerotinia disease

Describes the *in vitro* and microcosm experiments conducted to determine the effect of volatiles released from *Brassica* tissues on both the mycelial growth and carpogenic germination of *S. sclerotiorum* sclerotia.

Chapter 5 – Temperature effects on the growth of *Sclerotinia subarctica*

Describes the experiments assessing the length of conditioning (chilling) time required for carpogenic germination of sclerotia of *S. subarctica*, and the effect of temperature on mycelial growth of *S. subarctica* and *S. sclerotiorum.*

Chapter 6 – General Discussion

This chapter concludes the thesis with a discussion of all the results from the previous chapters, and outlines potential future work in light of the conclusions drawn.

2. Frequency and diversity of *Sclerotinia* spp. in the UK and Norway

2.1 Introduction

Sclerotinia sclerotiorum is a homothallic fungus and hence sexual reproduction through self-fertilisation to produce apothecia would be expected to result in a clonal population structure. *S. sclerotiorum* population studies in the UK, North America and Canada have observed a multi-clonal structure on a range of crops (Kohli *et al.*, 1995; Cubeta *et al.*, 1997; Clarkson *et al.*, 2013), with some evidence of outcrossing in studies undertaken in Australia and North America (Atallah *et al.*, 2004; Sexton & Howlett, 2004). Classification of individual isolates into clonal lineages has been carried out using independent markers such as mycelial compatibility groups (MCGs), microsatellites, DNA fingerprinting (Kohn *et al.*, 1991; Sirjusingh & Kohn, 2001) and sequence data (Malvárez *et al.*, 2007).

DNA fingerprinting, based on restriction fragment length polymorphisms (RFLP), has been widely used in the past for characterisation of *S. sclerotiorum* isolates, as Kohn *et al.* (1991) developed a putative transposable element pLK44.20 which was used as a probe in Southern Hybridisations to produce a DNA fingerprint. The widespread use of this probe allowed comparative studies of different populations, and led to a database containing fingerprints of more than three thousand *S. sclerotiorum* isolates from a range of hosts and geographical locations (Hambleton *et al.*, 2002). Microsatellite markers have since superseded the use of DNA fingerprinting in many population studies of fungal pathogens such as *S. sclerotiorum*, as they are generally more informative when investigating intra-specific genetic variation, and also more easily quantified.

Microsatellite loci have high mutation rates and are therefore often multiallelic, the different alleles being scored for phylogenetic inference. Sirjusingh and Kohn (2001) identified 25 microsatellite loci for *S. sclerotiorum*, and most subsequent studies have used a subset of these markers (Sexton & Howlett, 2004; Clarkson *et al.*, 2013) although some have used the full set (Atallah *et al.*, 2004). Sequence data has also been used to genotype *S. sclerotiorum* isolates with intergenic spacer region (IGS) sequences being more informative than the commonly sequenced internal transcribed spacer region (ITS) sequences (Carbone & Kohn, 2001b).

The population structure of *S. sclerotiorum* even within single fields has generally been found to consist of one or a few clones found at high frequency with a large number of other clones sampled only once (Kohn, 1995). Additionally, the predominant *S. sclerotiorum* clones at a local scale have, in many cases, been found over a wider geographic area as well as being found repeatedly over a number of years in the same location (Kohn *et al.*, 1991; Kohli *et al.*, 1995). In the UK, Clarkson *et al.* (2013) found 228 microsatellite haplotypes within 384 isolates of *S. sclerotiorum* from 12 populations from both crop plants and meadow buttercup (*Ranunculus acris*), with one haplotype found at high frequency in all but one isolated meadow buttercup population. This multi-clonal structure based on microsatellites has also been observed in *S. sclerotiorum* populations in Alaska (Winton et al., 2006), Australia (Sexton & Howlett, 2004), and Iran (Hemmati et al., 2009).

There is, however, a limit to the geographic distribution of *S. sclerotiorum* clones, with DNA fingerprinting showing that none of the clones from oilseed rape in western Canada (Kohn *et al.*, 1991; Kohli *et al.*, 1992; Kohli *et al.*, 1995) and

soybean in Ontario and Quebec (Hambleton *et al.*, 2002) were shared with those found in populations from locations and crops in the USA (Cubeta *et al.,* 1997; Malvárez *et al.,* 2007). Similarly, in Eastern Australia *S. sclerotiorum* populations on oilseed rape in areas more than 400 km apart were found to be genetically distinct (Sexton & Howlett, 2004). The use of sequence data allows different population studies to be directly compared which is difficult for microsatellite data. For instance, Clarkson *et al.* (2013) found 14 IGS haplotypes within the 12 *S. sclerotiorum* UK populations, with six IGS haplotypes exclusive to buttercup. Three of these *S. sclerotiorum* IGS haplotypes were also identified in populations from Canada, the USA and New Zealand (IGS1, IGS2 and IGS3) and another IGS haplotype was also shared with a population in Norway (IGS6). In the same study, it was also observed that *S. sclerotiorum* isolates with the same microsatellite haplotype generally also had the same IGS haplotype (Clarkson *et al.*, 2013).

The related species *Sclerotinia subarctica* (also known as *Sclerotinia* sp. 1) appears very similar in culture to *S. sclerotiorum,* and was first found in Norway on wild hosts, then on potatoes (Holst-Jensen *et al.,* 1998). Holst-Jensen *et al.* (1998) highlighted the phylogenetic closeness of *S. subarctica* to other *Sclerotinia* spp., differentiating between them by examining the variation in the ITS regions ITS1 and ITS2, as well as the 5.8S rRNA gene. Additionally, they found that *S. subarctica* lacks an intron in the large subunit (LSU) rDNA compared with *S. sclerotiorum,* which can be visualised by electrophoresis of polymerase chain reaction (PCR) products and allows *S. sclerotiorum* and *S. subarctica* to be distinguished (Holst-Jensen *et al.*, 1998; Holst-Jensen *et al.*, 1999). *S. subarctica* has been reported on vegetable crops in Alaska, where it comprised 46% of *Sclerotinia* spp. isolates obtained (Winton *et al.*, 2006)

When *S. subarctica* was discovered in the UK on meadow buttercup 15 of 32 *Sclerotinia* spp. isolates selected at random were found to be *S. subarctica*, identified by amplification and sequencing of the ITS region. All of the ITS sequences for the 15 *S. subarctica* isolates were identical, and their identity was further confirmed by amplification of the LSU (Clarkson *et al.*, 2010). In order to investigate the structure and diversity of *S. subarctica* populations Winton *et al.* (2007) identified eight polymorphic microsatellite loci and developed primer pairs to characterise them. From these loci, eighteen alleles were observed across the 41 isolates tested, with only four haploid multilocus genotypes across all the alleles.

Other than the Winton *et al.* (2007) study, little is currently known about *S. subarctica* incidence and diversity, with no studies in the UK or other Northern European countries. The aims of the work described in this chapter were:

- To establish the frequency of *S. subarctica* in the UK and Norway.
- To genotype *S. subarctica* isolates from the UK and Norway using microsatellite markers.
- To genotype *S. sclerotiorum* isolates from Norway using microsatellite markers and IGS sequences.
- To compare *S. sclerotiorum* IGS sequences from Norway with those from other countries (Canada, USA, Australia, Norway, UK, New Zealand) which are available from Genbank or from the study of Clarkson *et al.* (2013) and further unpublished UK populations.

2.2 Materials and Methods

2.2.1 Sampling of *Sclerotinia* isolates

Isolates of *Sclerotinia* spp. were obtained from meadow buttercup and different crop plants in England, Scotland, Norway and Sweden (Table 2.1). Isolates were also obtained from previous unpublished work by Clarkson *et al.*(Table 2.4) The majority of samples from crops were obtained as sclerotia by third parties who were requested to collect samples at spatially separate points across a field/plot, usually down transects a minimum 3 m apart, ideally 10 m apart. The numbers of sclerotia received from a sampling location varied due to differences in Sclerotinia disease incidence.

Cultures of *Sclerotinia* spp. were obtained from individual sclerotia by surface sterilising them in a solution of 50% sodium hypochlorite (11 – 14% available chlorine, VWR International Ltd, UK) and 50% ethanol (v/v) for 4 minutes with agitation. This was followed by two washes in sterile reverse osmosis (RO) water for 2 minutes. The sclerotia were then bisected and placed cut side down onto potato dextrose agar (PDA; Oxoid Ltd, UK) and incubated at 18°C. After 2 to 3 days the actively growing mycelium for each isolate was subcultured onto five PDA plates using agar plugs from the leading edge. These plates were incubated at 18°C for 6 weeks or until sclerotia had formed and matured, after which they were stored at 5°C, and at -20°C in potato dextrose broth (PDB; Formedium, UK) with 10% glycerol (Sigma-Aldrich Company Ltd, UK). These stock sclerotia were used to initiate new cultures as required.

Isolation of *Sclerotinia* spp. from meadow buttercup followed the method described by Clarkson *et al.* (2013). Briefly, this was done by sampling flowers from five

plants showing symptoms of infection (Figure 2.1), which were collected at 40 points at 10 m intervals along transects, with flowers from each plant stored separately. The flowers were then incubated on damp tissue paper in sealed plastic boxes at room temperature (approximately 22°C) for 4 weeks. Sclerotia formed on the damp tissue paper were then picked off and cultured as described above.

Table 2.1 –The population code, country, location, sampling year, plant host and sample size for isolates of *Sclerotinia* spp. examined in this study.

Code	Country	Location	Sampling Year(s)	Plant host	Number of Isolates
N	England	Edwinstowe, Nottinghamshire	2012	Carrot	40
Y	England	Coxwold, North Yorkshire	2012	Carrot	32
C	Scotland	Muirhead, Lanarkshire	2012	Carrot	20
EYE	Scotland	Eyemouth, Berwickshire	2013	Potato	34
FOR	Scotland	Forfar, Angus	2013	Carrot	10
GLA	Scotland	Glamis, Angus	2013	Carrot	12
IBE	Scotland	Isla Bend	2012	Potato	18
LAN	Scotland	Meigle, Perthshire	2013	Potato	26
MEI	Scotland	Meigle, Perthshire	2012	Pea	39
RED	Scotland	Redford, Angus	2013	Potato	17
SOR	Scotland	Forfar, Angus	2013	Oilseed rape	15
TYN	Scotland	Tyninghame, East Lothian	2012	Swede	28
BUP	Norway	Buskerud	2013	Pumpkin	1
BUS	Norway	Buskerud	2012, 2013	Lettuce	17
HED	Norway	Hedmark	2013	Carrot	2
NOR	Norway	Norway	2013	Oilseed rape	20
NTC	Norway	Nord-Trøndelag	2013	Chinese cabbage	1
NTP	Norway	Nord-Trøndelag	2013	Potato	1
NTR	Norway	Nord-Trøndelag	2013	Lettuce	3
OPP	Norway	Oppland	2013	Cabbage	1
OST	Norway	Østfold	2012, 2013	Clover, celery & Jerusalem artichoke	9
ROG	Norway	Rogaland	2012, 2013	Lettuce	39
VAG	Norway	Vest-Adger	2013	Lettuce	6
VES	Norway	Vestfold	2012, 2013	Lettuce & swede	5
ALM	Sweden	Almhaga	2012	Lettuce	7
KAR	Sweden	Karsholm	2012	Lettuce	10
TRA	Sweden	Tranägen	2012	Lettuce	7

Figure 2.1 – flowers of meadow buttercup (*Ranunculus acris*) showing symptoms of *Sclerotinia* disease.

2.2.2 Molecular Identification of *Sclerotinia subarctica*

Sclerotinia spp. cultures were initiated from stock sclerotia and incubated on PDA at 18°C for 3 to 4 days to produce actively growing colonies. Three agar plugs were taken from the leading edge, placed into Petri dishes containing half strength PDB, and incubated at 18°C for 3 days. The agar plugs were then removed and the mycelial mat washed twice in sterilised RO water and blotted dry on tissue paper (KimTech; Kimberly-Clark Ltd, UK) before being freeze-dried overnight. Genomic DNA was extracted from the freeze-dried mycelium using a DNeasy Plant Mini Kit (Qiagen Ltd, UK) following the manufacturer's protocol.

S. subarctica isolates were initially identified by PCR amplification of the large subunit (LSU) ribosomal DNA, where a large (304bp) intron is absent in *S. subarctica* compared to *S. sclerotiorum* (Holst-Jensen *et al.*, 1998). The PCR reaction mixture of 25 µl consisted of 1 x REDTaq ReadyMix PCR reaction mix (Sigma-Aldrich, UK), LR5 primer and LROR primers (Vilgalys & Hester, 1990)

(0.4 µmol L^{-1}) and approximately 10 ng DNA template. PCR amplification was carried out with thermal cycling parameters of 94°C for 2 min; 35 cycles of 94°C for 60 s, 52°C for 60 s, 72°C for 60 s; 72°C for 10 min and then a hold at 12°C. PCR products were visualised on a 1.5% agarose gel with a DNA ladder (EasyLadder I, Bioline Reagents Ltd, UK; Figure 2.2).

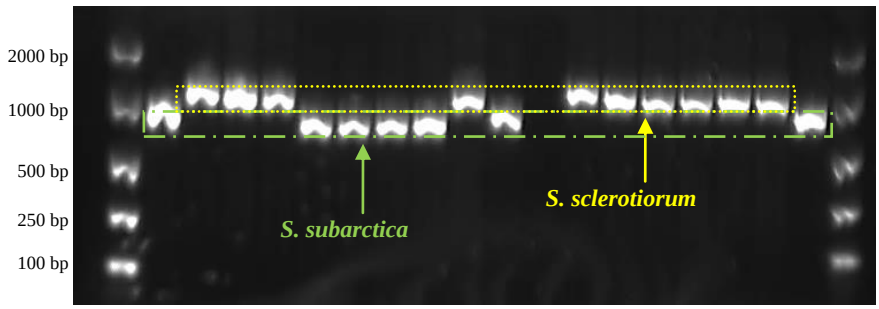

Figure 2.2 - Gel electrophoresis of the rDNA LSU amplicons for *S. sclerotiorum* (larger PCR product) and *S. subarctica* (smaller product).

The identification of *S. subarctica* isolates was subsequently confirmed by PCR amplification and sequencing of the rRNA ITS region. The PCR reaction mixture of 25 µl consisted of 1 x REDTaq ReadyMix PCR reaction mix (Sigma-Aldrich, UK), modified standard ITS primers (White *et al.*, 1990) developed by Clarkson (unpublished) for *S. sclerotiorum* – ITS2AF (TCGTAACAAGGTTTCCGTAGG) and ITS2AR (CGCCGTTACTGAGGTAATCC; 0.4 µmol L^{-1} each) and approximately 10 ng DNA template. PCR amplification was carried out with thermal cycling parameters of 94°C for 2 min ; 40 cycles of 94°C for 15 s, 59°C for 15 s, 72°C for 30 s; 72°C for 10 min and then a hold at 12°C. BLAST analysis (Altschul *et al.*, 1990) was used to confirm species identity.

2.2.3 Molecular Characterisation of *Sclerotinia subarctica*

Isolates identified as *S. subarctica* were characterised using eight microsatellite markers in two separate multiplexed PCR reactions with fluorescent-labelled primer pairs (Applied Biosystems, UK), four loci per reaction (Table 2.2) as developed by Winton *et al.* (2007) . Primer mix 1 contained MS01, MS03, MS06 and MS08 and primer mix 2 contained MS02, MS04, MS05, and MS07.

Table 2.2 - The repeat motif, primer sequence and amplicon size range for the microsatellite loci used to characterise *S. subarctica* isolates (Winton *et al.*, 2007).

Locus	Repeat Motif	Primer sequence (5'-3')	Range (bp)
MS01	$(GAAA)_n$	F: GATGTTTGAGTCTCCGTGAT R: TGACAGTGGCTGAGTTAATG	130-150
MS02	$(TCAC)_n(TGTC)_n$	F: ATAGCGATGAGTACAGTCCC R: AATACTCCGGGACAGACAG	176-180
MS03	$(GTAT)_n$	F: TCCCGATAGGTTATCGTTGTT R: AATGTTGTGGAGAAGGTCAC	185-191
MS04	$(ATAC)_n(ATACC)_n$	F: CATAACGGGAAGACATTCAT R: CGGAGATTGATCTGTCATTT	188-213
MS05	$(GTT)_n$	F: GTTACCGATTTATTTGTGCC R: TTCGATTCTTTCGTATGGT	318-331
MS06	$(TCTT)_n(CCCTA)_n$	F: AAATACCCAAAGCCATCC R: GTGATTGGGATAAACAGGAA	373-408
MS07	$(GTTT)_n(GGTT)_n$	F: AGGAAACCCTCCATGTTTAT R: CAAGAAGCAGAGACACAACA	361-369
MS08	$(ACCA)_n$	F: GCGGTGGTTTAGTATTATGC R: TCGTTAACAGGATATTGGCT	372-380

The PCR reaction mixture of 20 µl consisted of 1 x QIAGEN Multiplex PCR Master Mix, 0.5 x Q solution, primer mix (0.4 µmol L^{-1}) and approximately 10 ng DNA template (Winton *et al.*, 2006). PCR amplification was carried out with thermal cycling parameters of 95°C for 15 min; 35 cycles of 94°C for 30 s, 55°C for 90 s, 69°C for 75 s; 60°C for 30 min and then a hold at 12°C. PCR products were visualised on a 1.5% agarose gel to confirm amplification and two separate PCR amplifications per locus were carried out for each isolate to ensure reproducibility of results. All PCR products were sized by Eurofins (Germany) using an ABI 3130xl genetic analyser and allele sizes were determined using GeneMarker (Version 1.6;

SoftGenetics, USA). FLEXIBIN (Amos *et al*., 2007) was used to bin allele sizes and estimate the relative number of repeats for each locus.

2.2.4 Molecular Characterisation of Norwegian *S. sclerotiorum* isolates

Isolates identified as *S. sclerotiorum* from Norway were characterised using eight microsatellite markers (Sirjusingh & Kohn, 2001) and sequencing part of the intergenic spacer (IGS) region of the rRNA gene. Two separate multiplexed PCR reactions with fluorescent-labelled primer pairs (Applied Biosystems, UK) were carried out, with three (7-2, 8-3, 92-4) or five (13-2, 17-3, 55-4, 110-4, 114-4) loci per reaction (Sirjusingh & Kohn, 2001) (Table 2.3). The PCR reaction mixture of 10 µl consisted of 1 x QIAGEN Multiplex PCR Master Mix, 0.5 x Q solution, forward and reverse primer pairs (0.2 µmol L^{-1}) and approximately 10 ng DNA template (Winton *et al.*, 2006). PCR amplification was carried out with thermal cycling parameters of 95 °C for 15 min; 35 cycles of 94 °C for 30 s, 55 °C for 90 s, 69 °C for 75 s; 69 °C for 75 s and then a hold at 12 °C. PCR products were visualised on a 1.5 % agarose gel to confirm amplification and two separate PCR amplifications per locus were carried out for each isolate to ensure reproducibility of results. All products were sized by Eurofins (Germany) using an ABI 3130xl genetic analyser and allele sizes were determined using GeneMarker (Version 1.6; SoftGenetics, USA). FLEXIBIN (Amos *et al*., 2007) was used to bin allele sizes and estimate the number of repeats for each locus.

Table 2.3 - The repeat motif, primer sequence and amplicon size range for the microsatellite loci used to characterise Norwegian *S. sclerotiorum* isolates (Sirjusingh & Kohn, 2001).

Locus	Repeat Motif	Primer sequence (5'-3')	Range (bp)
7-2	$(GA)_n$	F: TTTGCGTATTATGGTGGGC R: ATGGCGCAACTCTCAATAGG	160-172
13-2	$(GTGGT)_n$	F: TCTACCCAAGCTTCAGTATTCC R: GAACTGGTTAATTGTCTCGG	284-304
8-3	CA_n	F: CACTCGCTTCTCCATCTCC R: GCTTGATTAGTTGGTTGGCA	251-271
17-3	$(TTA)_n$	F: TCATAGTGAGTGCATGATGCC R: CAGGGATGACTTTGGAATGG	345-390
55-4	$TACA_n$	F: GTTTTCGGTTGTGTGCTGG R: GCTCGTTCAAGCTCAGCAAG	173-221
92-4	$(CT)_n$	F:TCGCCTCAGAAGAATGTGC R: AGCGGGTTACAAGGAGATGG	374-378
110-4	$(TATG)_n$	F: ATCCCTAACATCCCTAACGC R: GGAGAATTGAAGAATTGAATGC	362-378
114-4	$(AGAT)_n(AAGC)_n$	F: GCTCCTGTATATACCATGTCTTG R: GGACTTTCGGACATGATGAT	351-391

PCR primers IGS2F (TTACAAAGATCCTCTTTCCATTCT**)** and IGS2R (GCCTTTACAGGCTGACTCTTC) (Clarkson *et al.,* 2013) were used to amplify an 834 bp (approx.) fragment of the IGS region of the ribosomal RNA gene. The PCR reaction mixture of 25 µl consisted of 0.5 x REDTaq ReadyMix PCR reaction mix (Sigma-Aldrich, UK), IGS2F and IGS2R primers (4 µmol L^{-1}) and approximately 10-30 ng DNA template. PCR amplification was carried out with thermal cycling parameters of 94°C for 2 min, 40 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 2 min followed by 72°C for 10 min and a hold of 12°C. PCR products were visualised on a 1.5% agarose gel to confirm amplification and then purified using the QIAquick PCR purification kit (Qiagen, UK), according to the manufacturer's protocol. Sequencing of the purified products was carried out by GATC Biotech (Germany).

2.2.5 Analysis of *S. sclerotiorum* and *S. subarctica* Microsatellite Data

ARLEQUIN (Excoffier *et al*., 2005) was used to determine the haplotype frequency of all the *S. subarctica* isolates and the Norwegian *S. sclerotiorum* isolates based on the relative number of repeats at each microsatellite locus, as well as identify shared haplotypes, calculate Nei's unbiased gene diversity (expected heterozygosity) and generate data for a minimum spanning tree (computed from a matrix of pairwise distances calculated between all pairs of haplotypes), which was then visualised using TREEVIEW (Page, 1996). The *S. sclerotiorum* data set was expanded using microsatellite data from the work of Clarkson *et al.* (2013), together with unpublished data from UK and Australian populations (Table 2.4).

Population subdivision between *S. sclerotiorum* and *S. subarctica* isolates from different countries was estimated through pairwise comparisons of R_{ST} (Slatkin, 1995), a statistic which uses a stepwise mutation model appropriate for microsatellites, and significance was tested by permuting (1023) haplotypes between populations. ARLEQUIN was also used to perform a hierarchical analysis of molecular variance (AMOVA) with isolates grouped by country of origin to test the hypothesis that these groups were different. Significance of the resulting F-statistics was tested using the inbuilt non-parametric permutation approach (1023 permutations).

Microsatellite data for the *S. subarctica* isolates and the Norwegian *S. sclerotiorum* isolates were analysed by a Bayesian population structure approach using STRUCTURE v2.3.3 (Falush *et al.*, 2003) to determine the most probable number of genetic clusters (K) with no pre-consideration of geographic origin. Six independent runs were carried out using a burn-in period of 500,000 Markov Chain Monte Carlo

iterations and a 500,000 run-length implemented using the admixture model and correlated allele frequencies for K values between 1 and 6. The python script structureHarvester.py v0.6.92 (Earl & von Holdt, 2012) was used to summarise the STRUCTURE output, producing ΔK values using the Evanno method (Evanno *et al.*, 2005) to estimate the most probable value of K.

Table 2.4 – Hosts and locations for additional *S. sclerotiorum* and *S. subarctica*[1] isolates genotyped by microsatellite and IGS sequencing from the work of Clarkson *et al.* (2013) (highlighted in grey) and previously unpublished data used for comparison with isolates from Norway genotyped in this study.

Code	Country	Location	Sampling Year	Plant Host	Number of Isolates
C	England	Blyth, Nottinghamshire	2005	Carrot cv 'Nairobi'	32
CE	England	Methwold, Norfolk	2009	Celery cv 'Victoria'	32
DG	England	Deans Green, Warwickshire	2009	Meadow buttercup	32
EV	Wales	Elan Valley, Powys	2009	Meadow buttercup	32
HE[1,2]	England	Michaelchurch Escley, Herefordshire	2009	Meadow buttercup	32
L	England	Petworth, Sussex	2005	Lettuce cv 'Silverado'	32
O	England	Preston Wynn, Herefordshire	2005	Oilseed rape cv 'Winner'	32
P	England	Sutton St Nicholas, Herefordshire	2009	Pea cv 'Setchey'	32
R	England	Holywell, Warwickshire	2007	Meadow buttercup	32
S	England	Holywell, Warwickshire	2008	Meadow buttercup	32
T	England	Deans Green, Warwickshire	2008	Meadow buttercup	32
X	England	Preston Wynn, Herefordshire	2007	Oilseed rape cv 'Lioness'	32
V	England	Vowchurch, Herefordshire	2009	Oilseed Rape cv unknown	32
CC[1,2]	England	Michaelchurch Escley, Herefordshire	2010	Meadow buttercup	32
QM	England	Michaelchurch Escley, Herefordshire	2010	Meadow buttercup	32
UM	England	Upwood, Cambridgeshire	2010	Meadow buttercup	32
VT	England	Vowchurch, Herefordshire	2010	Oilseed Rape	32
SB	England	Sutton Bridge, Lincolnshire	2010	Oilseed Rape cv 'Catana'	32
CY[2]	England	Michaelchurch Escley, Herefordshire	2011	Meadow buttercup	24
SC[1]	Scotland	Balbeggie, Perthshire	2010	Carrot cv 'Nairobi'	32
LI	Scotland	Dunfermiline, Fife	2011	Meadow buttercup	23
BO	Scotland	Borrowstoun, Perthshire	2011	Meadow buttercup	32
BOM[1]	Scotland	Bo'ness, West Lothian	2011,2012	Meadow buttercup	89
LIE[1]	Scotland	Dunfermiline, Fife	2012	Meadow buttercup	56
AUS	Australia	Narra Tarra	2009/2010	Oilseed Rape	5
AUS	Australia	Walkaway site 1	2009/2010	Oilseed Rape	3
AUS	Australia	Walkaway site 2	2009/2010	Oilseed Rape	5
AUS	Australia	East Chapman site 1	2009/2010	Oilseed Rape	1
AUS	Australia	Moonyoonooka	2009/2010	*Lupinus angustifolius*	3
AUS	Australia	Walkaway site 3	2009/2010	*Lupinus angustifolius*	4
AUS	Australia	East Chapman site	2009/2010	Oilseed Rape	5
AUS	Australia	East Chapman site	2009/2010	Oilseed Rape	4
AUS	Australia	Walkaway site 4	2009/2010	Oilseed Rape	5
AUS	Australia	Kendenup	2009/2010	Oilseed Rape	3
AUS	Australia	Mt. Baker site 1	2009/2010	Oilseed Rape	5
AUS	Australia	Moonyoonooka	2009/2010	*Lupinus angustifolius*	2
AUS	Australia	Naragulu	2009/2010	Oilseed Rape	4
AUS	Australia	Mt. Barker site 2	2004	Oilseed Rape	4
AUS	Australia	Walkaway site 5	2004	Oilseed Rape	5
AUS	Australia	Perth Metro area	2009/2010	Cabbage	1
AUS	Australia	Binningup	2009/2010	Carrot	1

[1]Samples which included *S. subarctica* isolates
[2]The same site sampled in different years

2.2.6 Analysis of ITS and IGS Sequence Data for *S. sclerotiorum* and *S. subarctica*

ITS sequences obtained for all the *S. subarctica* isolates were aligned with those from representatives of different *Sclerotinia* spp. and ITS haplotypes using the ClustalW algorithm implemented in MEGA v6 (Tamura *et al.*, 2013) and a phylogenetic tree was drawn using the 'minimum evolution' option. Sequence identity was confirmed by BLAST analysis. Representative isolates included were: *S. subarctica* EF091810 - Winton *et al*. (2006); *S. trifoliorum* AY187068 – Powers *et al*. (unpublished); *S. minor* AB516661 - Uzuhashi *et al.* (2010); *S. sclerotiorum* isolate KJ744364 – Liebe & Varrelmann (unpublished); *S. sclerotiorum* isolate KJ817041 – Lee *et al.* (unpublished); *S. sclerotiorum* isolates L44, C32, PE7, T31 and L37 – Clarkson *et al*. (unpublished).

IGS sequences for the Norwegian *S. sclerotiorum* isolates were aligned using the ClustalW algorithm implemented in MEGA v6 (Tamura *et al.*, 2013) and a phylogenetic tree was drawn using the 'minimum evolution' option. Sequences were then compared with representative haplotypes from the previous population study of Clarkson *et al.* (2013) and other populations (unpublished; Table 2.4) in a second phylogenetic tree. DNASP v. 5 (Librado & Rozas, 2009) was used for calculation of haplotype diversity and also used to examine the subdivision between country populations using pairwise comparisons of the nearest neighbour statistic, with significance calculated with 1000 permutations. A median joining network of IGS haplotypes was constructed in NETWORK v. 4.6 (Bandelt *et al.*, 1999) (Fluxus Technology, USA) and further expanded using IGS sequence data from Clarkson *et al.* (2013), unpublished UK and Australian sequences (Table 2.4), and sequence data

from GenBank for isolates from Canada, New Zealand, Norway and the USA (Carbone & Kohn, 2001a) (Table 2.5).

Table 2.5 - Host and locations for *Sclerotinia sclerotiorum* isolates with published IGS sequences used in the median joining network for comparison with IGS sequences from Norwegian isolates obtained in the present study (Carbone & Kohn, 2001a).

Country	Location	Sampling Year	Plant Host	Number of Isolates
Canada	Alberta	1992	Oilseed rape	40
USA	Georgia	1990-1992, 1997	Oilseed rape	48
USA	Alabama	1997	Oilseed rape	15
USA	Alabama	1997	Radish	1
USA	Louisiana	1996	Cabbage	16
USA	Louisiana	1996	Groundnut	5
USA	Louisiana	1996	*Geranium* sp.	1
USA	North Carolina	1996	Cabbage	40
USA	North Carolina	1991, 1994-1995	Tobacco	10
New Zealand	unknown	1993-1997	Kiwi fruit	38
New Zealand	unknown	1994	Hemp	1
USA	New York	1996	Cabbage	31
Norway	Sandvika	1993-1994	Lesser celandine	51
Norway	Vestfold	1993	Lesser celandine	28

2.3 Results

2.3.1 Molecular Identification of *Sclerotinia subarctica*

Amplification of the LSU rDNA for a total of 734 isolates from England, Scotland, Norway and Sweden (including those from previous work by Clarkson *et al*., unpublished) identified 166 isolates as *S. subarctica* (Table 2.6). *S. subarctica* was present in a wide range of crop hosts, such as lettuce, potato, Jerusalem artichoke, swede, pea, carrot and celery, as well as on meadow buttercup in Scotland. *S. subarctica* was found in the majority of locations sampled in Scotland, Norway and Sweden, but not identified in the two sites sampled in England in 2011 (Edwinstowe, Notts; Coxwold, N. York). *S. subarctica* has only been found in one site in England (HE, CC), which has been sampled three times.

Amplification and sequencing of the rRNA ITS region followed by BLAST analysis and visualisation of representative sequences from each population in a minimum evolution spanning tree (constructed to include other known *Sclerotinia* spp.) confirmed the identification of the 166 *S. subarctica* isolates, from a total of 21 sampling sites (Figure 2.3). All sequences of the *S. subarctica* isolates were identical.

2.3.2 Molecular Characterisation of *Sclerotinia subarctica* Isolates

Microsatellite analysis of all the *S. subarctica* isolates resulted in 83 haplotypes from the 166 isolates from England, Scotland, Norway and Sweden (Figure 2.4). Of these, 8 haplotypes were shared between two or more locations, with two haplotypes found at a much higher frequency than the rest (haplotypes 1 and 2, 19 isolates). Haplotype

1 was only found in one location, from meadow buttercup sampled in different years in Herefordshire, England (CC, HE). Haplotype 2 was found in Norway and Scotland, on crop hosts and meadow buttercup (Figure 2.4).

Table 2.6 – Isolates identified as either *S. sclerotiorum* or *S. subarctica* by amplification of the LSU rDNA from samples taken from a range of crop plants and wild hosts from 2009 to 2013 in England, Scotland, Sweden and Norway. Isolates highlighted in grey are from previous work by Clarkson *et al*. (unpublished).

Code	Country	Plant host	Number of Isolates	Number identified as *S. subarctica*	Number identified as *S. sclerotiorum*
CC	England	Meadow buttercup	89	19	70
HE	England	Meadow buttercup	40	15	35
N	England	Carrot	40	0	40
Y	England	Carrot	32	0	32
BOM	Scotland	Meadow buttercup	89	4	85
LIE	Scotland	Meadow buttercup	56	13	43
SC	Scotland	Carrot	40	7	33
C	Scotland	Carrot	20	0	20
EYE	Scotland	Potato	34	18	16
FOR	Scotland	Carrot	10	0	10
GLA	Scotland	Carrot	12	0	12
IBE	Scotland	Potato	18	6	12
LAN	Scotland	Potato , Saxon	26	12	14
MEI	Scotland	Pea	39	12	27
RED	Scotland	Potato, Rooster	17	2	15
SOR	Scotland	Oilseed rape	15	0	15
TYN	Scotland	Swede	28	0	28
BUP1	Norway	Pumpkin	1	0	1
BUS1	Norway	Lettuce	17	5	12
HED1	Norway	Carrot	2	2	0
NOR1	Norway	Unknown	20	5	15
NTC1	Norway	Chinese cabbage	1	0	1
NTP1	Norway	Potato	1	0	1
NTR1	Norway	lettuce	3	3	0
OPP1	Norway	Cabbage	1	0	1
OST1	Norway	Clover & Jerusalem artichoke	9	6	3
ROG1	Norway	Lettuce	39	21	18
VAG1	Norway	Lettuce	6	5	1
VES1	Norway	Lettuce & swede	5	2	3
ALM1	Sweden	Lettuce	7	3	4
KAR1	Sweden	Lettuce	10	2	8
TRA1	Sweden	Lettuce	7	4	3

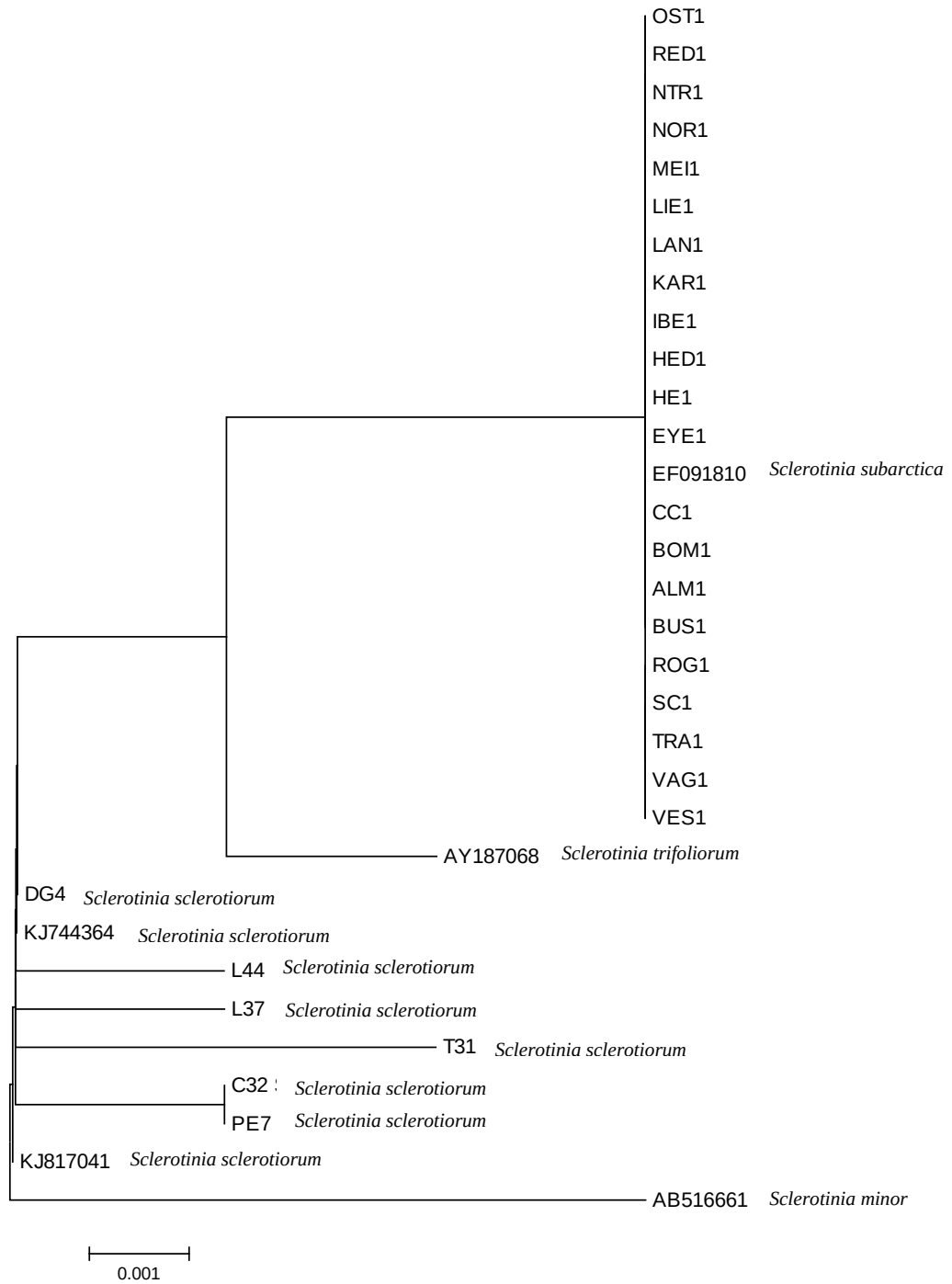

Figure 2.3 – Phylogenetic tree for ITS sequence data showing representative *S. subarctica* isolates from 21 sampling sites together with previously sequenced *Sclerotinia* spp. isolates - *S. subarctica* isolate EF091810 - Winton *et al*. (2006); *S. trifoliorum* isolates AY187068 – Powers *et al*. (unpublished); *S. minor* isolate AB516661 – Uzuhashi *et al.* (2010); *S. sclerotiorum* isolate KJ744364 - Liebe & Varrelmann (unpublished); *S. sclerotiorum* isolate KJ817041 – Lee *et al.* (unpublished) *S. sclerotiorum* isolates L44, C32, DG4, PE7, T31 and L37 – Clarkson *et al*. (unpublished).

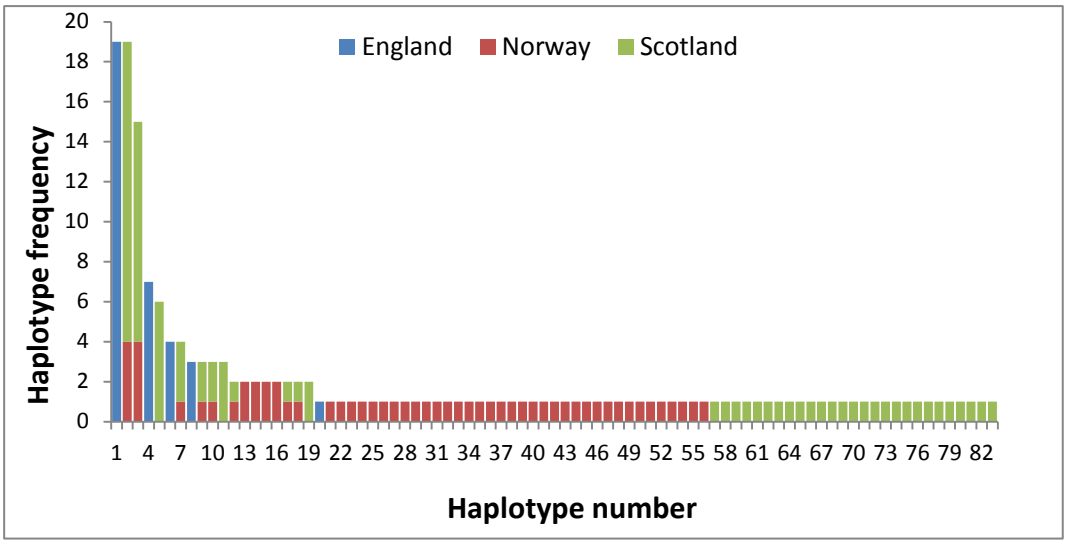

Figure 2.4 - Microsatellite haplotype frequency for *S. subarctica* isolates (n=166) from Scotland, England and Norway.

Analysis of all the *S. subarctica* isolates resulted in 1 to 4 alleles per locus for England, and 2 to 8 alleles for both Scotland and Norway (Norway and Sweden were grouped due to the small number of isolates from Sweden, and will now be referred to as Norway; Table 2.7). There were shared alleles across all loci, with 1 to 2 private (exclusive) alleles per locus for English isolates, 1 to 4 for Scottish isolates and 1 to 3 for Norwegian isolates. The gene diversity at each locus for England was 0 to 0.63, for Scotland 0.15 to 0.73, and for Norway 0.34 to 0.77 (Table 2.7).

Table 2.7 – Summary of microsatellite locus data for *S. subarctica* isolates from England, Scotland and Norway.

Microsatellite Locus[1]	Allele Size range England (bp)	Allele Size range Scotland (bp)	Allele Size range Norway (bp)	Number of Alleles England	Number of Alleles Scotland	Number of Alleles Norway	Number of Private Alleles England[2]	Number of Private Alleles Scotland[2]	Number of Private Alleles Norway[2]	Gene Diversity England	Gene Diversity Scotland	Gene Diversity Norway
MS01	129-147	128-185	127-162	3	6	8	1	1	3	0.358	0.515	0.683
MS02	173-175	161-193	162-181	1	6	4	0	2	0	0.000	0.352	0.368
MS03	192-203	170-194	170-193	2	3	4	1	0	1	0.166	0.442	0.399
MS04	188-190	175-200	178-212	1	8	6	0	4	2	0.000	0.733	0.766
MS05	319-346	317-333	317-331	3	4	2	1	0	0	0.358	0.592	0.479
MS06	378-425	348-416	369-408	4	6	4	1	2	0	0.629	0.417	0.622
MS07	372-389	361-375	361-383	3	2	5	2	0	3	0.580	0.151	0.336
MS08	377-395	370-384	370-392	3	2	5	1	0	3	0.597	0.151	0.336
Mean	-	-	-	2.5	4.625	4.75	0.875	1.125	1.5			

[1]Locus as defined by Winton *et al.* (2007)
[2]Number of alleles not found in any other population

Within each country the number of *S. subarctica* microsatellite haplotypes ranged from 5 to 48, while the number of unique haplotypes ranged from 5 to 40 (Table 2.8). Gene diversity within each country was in the range 0.34 to 0.50 (Table 2.8). There was an obvious grouping of English haplotypes from meadow buttercup when the data was viewed in a minimum spanning tree, but not for haplotypes from Norway and Scotland (Figure 2.5).

Table 2.8 - Microsatellite haplotype frequency and gene diversity for *S. subarctica* populations from England, Scotland, and Norway.

Country	Sample size	Number of haplotypes	Number of unique haplotypes[1]	Gene Diversity
England	34	5	5	0.336
Scotland	74	38	30	0.419
Norway	58	48	40	0.499

[1]Number of haplotypes not found in any other population

The fixation index (R_{ST}) values indicated significant differentiation of the *S. subarctica* populations from Norway, England and Scotland ($p < 0.001$; Table 2.9).

Table 2.9 – Fixation index (R_{ST}) values for pairwise comparisons of *S. subarctica* populations from crop plants and meadow buttercups in England, Scotland, Norway and Sweden. *** $p<0.001$

	Norway	England	Scotland
Norway			
England	0.589***		
Scotland	0.368***	0.637***	

R_{ST}-based AMOVA analysis with an imposed structure of grouping isolates according to country showed that 55.54% ($p<0.001$) of the molecular variance was within the *S. subarctica* populations, while 44.46% ($p<0.001$) was between populations, indicating that geographic origin was significant.

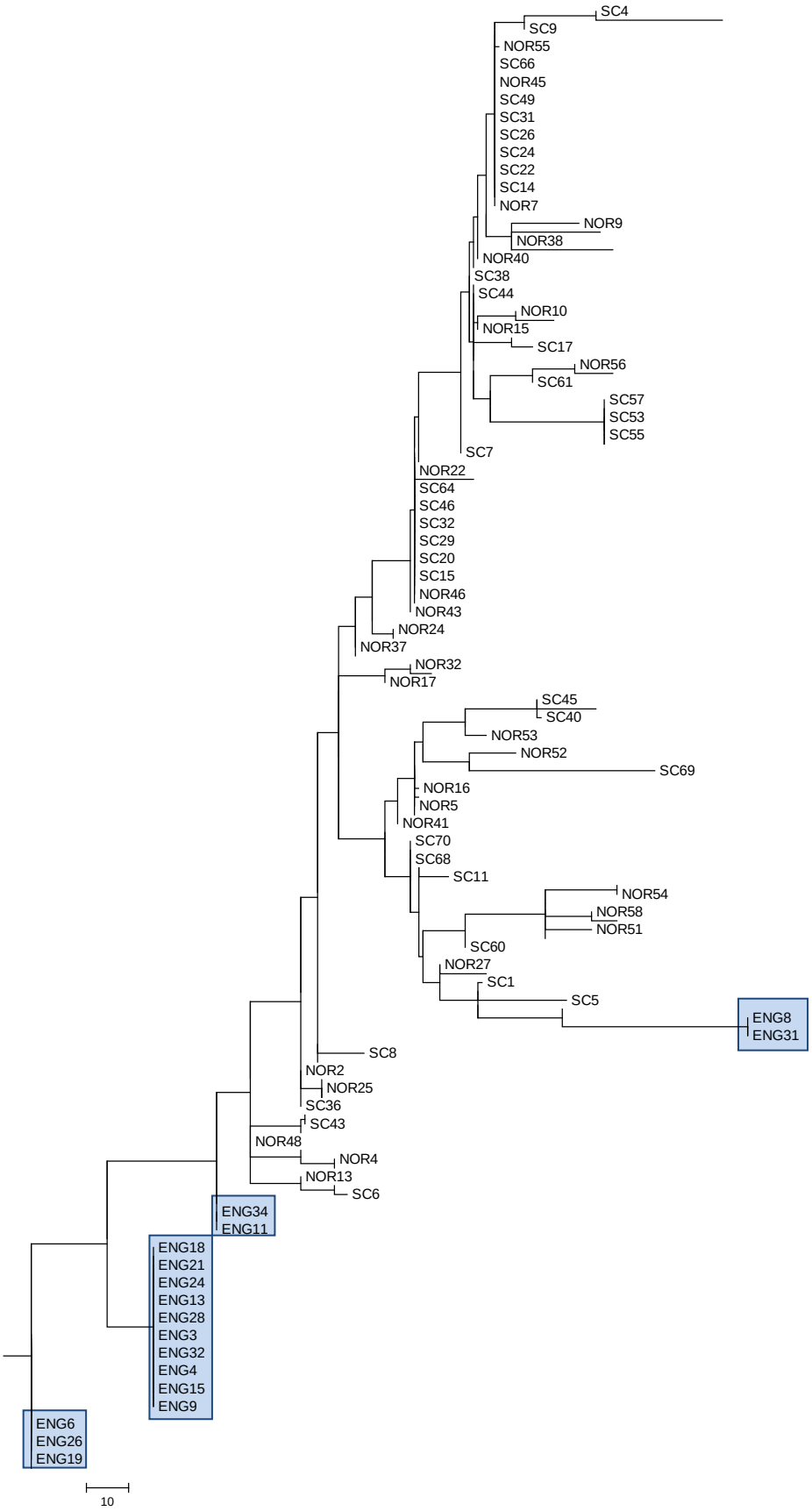

Figure 2.5 - Minimum evolution spanning tree of microsatellite haplotypes for *S. subarctica* isolates from Scotland (SC) England (ENG) and Norway (NOR). Grouping of haplotypes from England is highlighted in blue.

2.3.3 Population Structure of *Sclerotinia subarctica* Isolates

The Bayesian cluster analysis of the microsatellite data from *S. subarctica* using STRUCTURE suggested the number of genetically distinct ancestral populations was K = 3, the value associated with the highest value of ΔK. Examining the probability of each isolate belonging to each of the three populations showed that the majority of isolates from buttercup in England belonged to the blue population (Figure 2.6), whereas the majority of isolates from Scotland and Norway belonged to the red population (Figure 2.6). There were some isolates from each of the three populations which belonged to the green population.

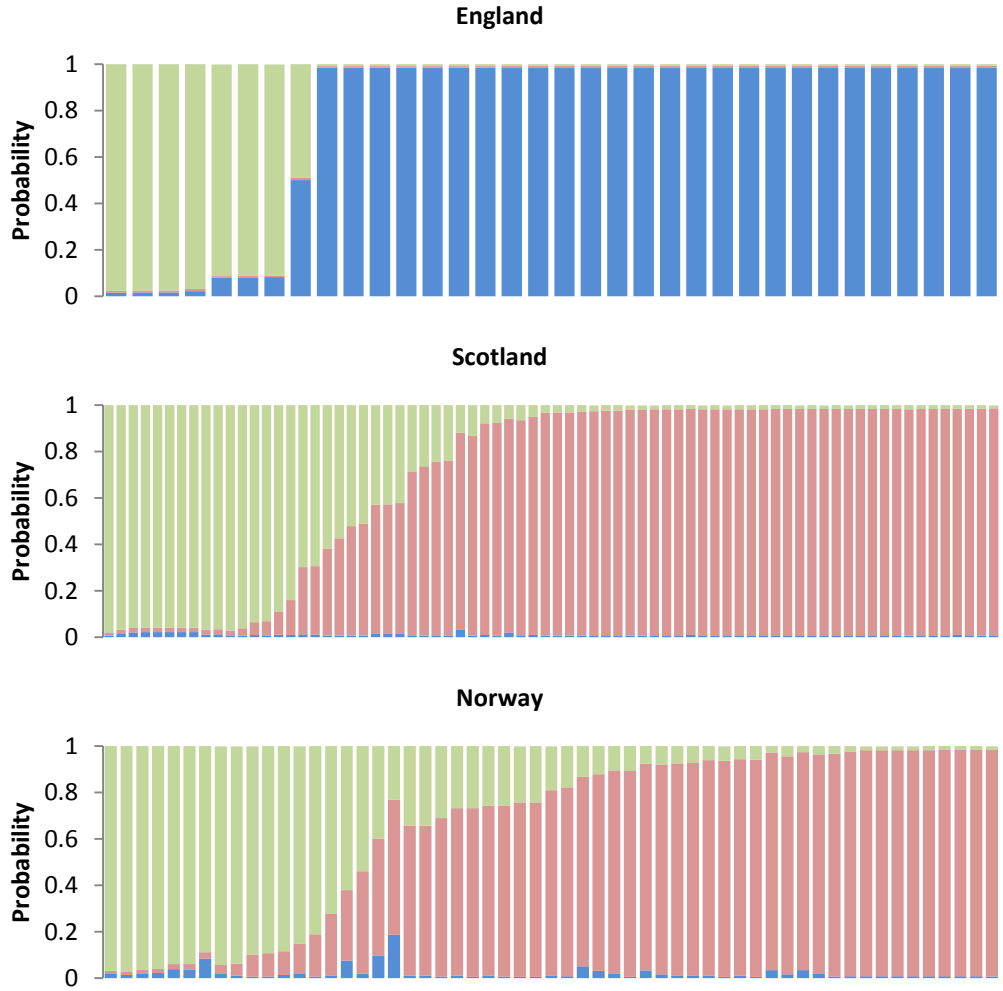

Figure 2.6 – Bar plot showing the assignment of *S. subarctica* isolates from England, Scotland and Norway to three populations using STRUCTURE. Each bar represents a single isolate and the three colours indicate the probability of each isolate belonging to a population.

2.3.4 Molecular Characterisation of Norwegian *Sclerotinia sclerotiorum* Isolates

Microsatellite analysis of the Norwegian *S. sclerotiorum* isolates resulted in 50 haplotypes from 54 isolates. Of these haplotypes, four were shared between two or more locations in Norway, but there were no shared haplotypes with UK (Clarkson *et al.*, 2013)(Clarkson *et al.*, unpublished) or Australian isolates (unpublished) (Figure 2.7).

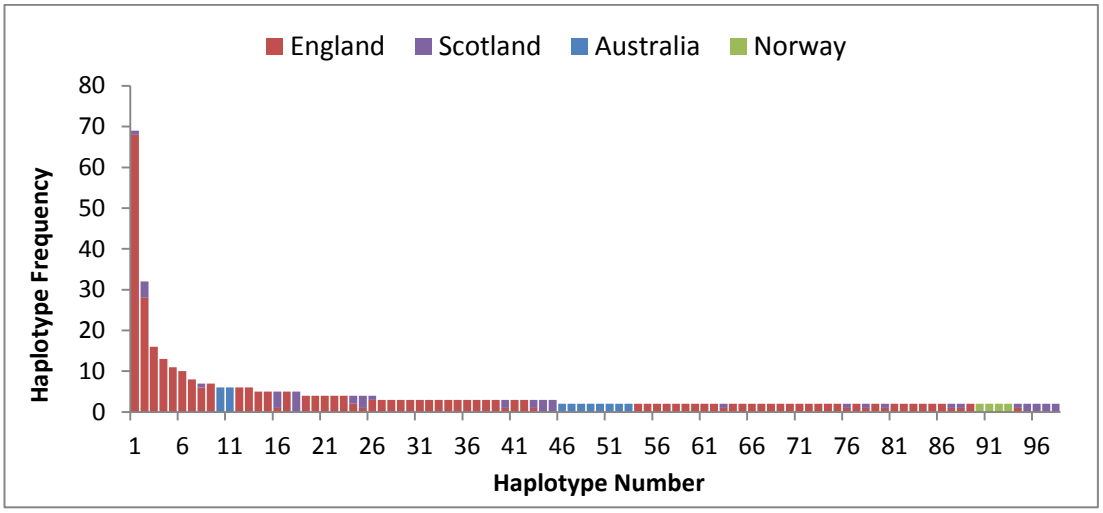

Figure 2.7 - Microsatellite haplotype frequency for *S. sclerotiorum* populations (n=801) from England, Scotland, Norway and Australia for 98 haplotypes comprising more than one isolate. The remaining 385 haplotypes were each represented by a single isolate.

Gene diversity for the Norwegian population was 0.652, which was similar to the gene diversity for the populations from England, Scotland and Australia of 0.696, 0.581 and 0.686 respectively (Table 2.10). There was no obvious grouping of the microsatellite haplotypes by location when the data was viewing in a minimum spanning tree (data not shown).

Table 2.10 - Microsatellite haplotype frequency and gene diversity for *S. sclerotiorum* populations from England, Scotland, Australia and Norway.

Country	Sample size	No. of haplotypes	No. of unique haplotypes[1]	Gene Diversity
England	600	343	266	0.696
Scotland	87	64	41	0.581
Australia	60	42	32	0.686
Norway	54	50	46	0.652

[1]Number of haplotypes not found in any other population

The fixation index (R_{ST}) values indicated significant differentiation of *S. sclerotiorum* populations between different countries ($p<0.001$, $p<0.01$; Table 2.11). R_{ST}-based AMOVA analysis with an imposed structure of grouping populations according to country showed that 89.74% of the molecular variance was within the *S. sclerotiorum* populations ($p<0.001$), while 10.26% was between populations ($p<0.001$), indicating that geographic origin was significant.

Table 2.11 – Fixation index (R_{ST}) values for pairwise comparisons of *S. sclerotiorum* populations from England, Scotland, Australia and Norway. **p<0.01, ***p<0.001

	Australia	England	Norway	Scotland
Australia				
England	0.186***			
Norway	0.200***	0.096***		
Scotland	0.152***	0.015**	0.068***	

2.3.5 Population Structure of Norwegian *Sclerotinia sclerotiorum* Isolates

The Bayesian cluster analysis with the microsatellite data suggested the number of genetically distinct ancestral populations for the Norwegian *S. sclerotiorum* isolates was K = 3, the value associated with the highest value of ΔK. Examining the probability of each isolate belonging to each population showed a roughly even distribution of the isolates between the three populations (Figure 2.8).

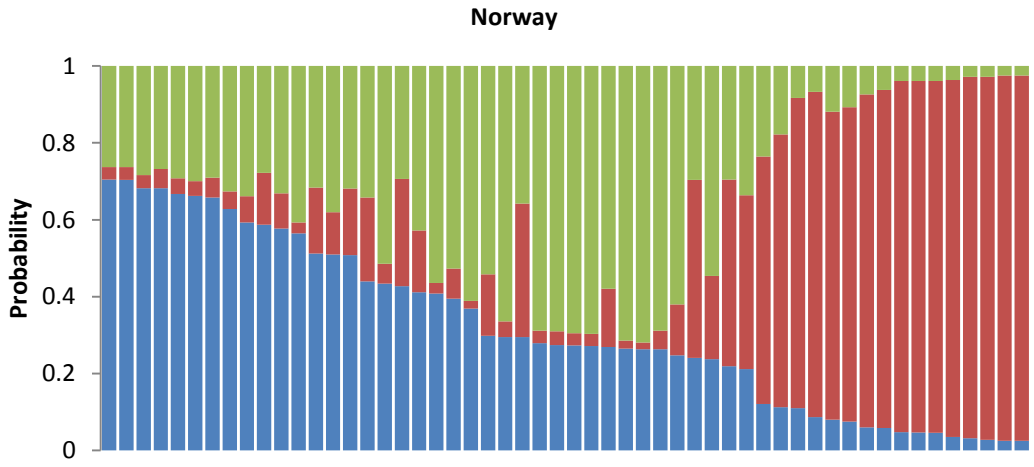

Figure 2.8 - Bar plot showing the assignment of *S. sclerotiorum* isolates from Norway to three populations using STRUCTURE. Each bar represents a single isolate and the three colours indicate the probability of each isolate belonging to a population.

The Bayesian cluster analysis with the microsatellite data for populations of *S. sclerotiorum* from England, Scotland, Australia and Norway suggested the number of genetically distinct ancestral populations for *S. sclerotiorum* isolates was K = 4, the value associated with the highest value of ΔK. Examining the probability of each isolate belonging to each population showed that the majority of isolates from Norway and Australia belonged to the blue population, while the isolates from England and Scotland were mainly split between the other three populations (purple, green and red; Figure 2.9).

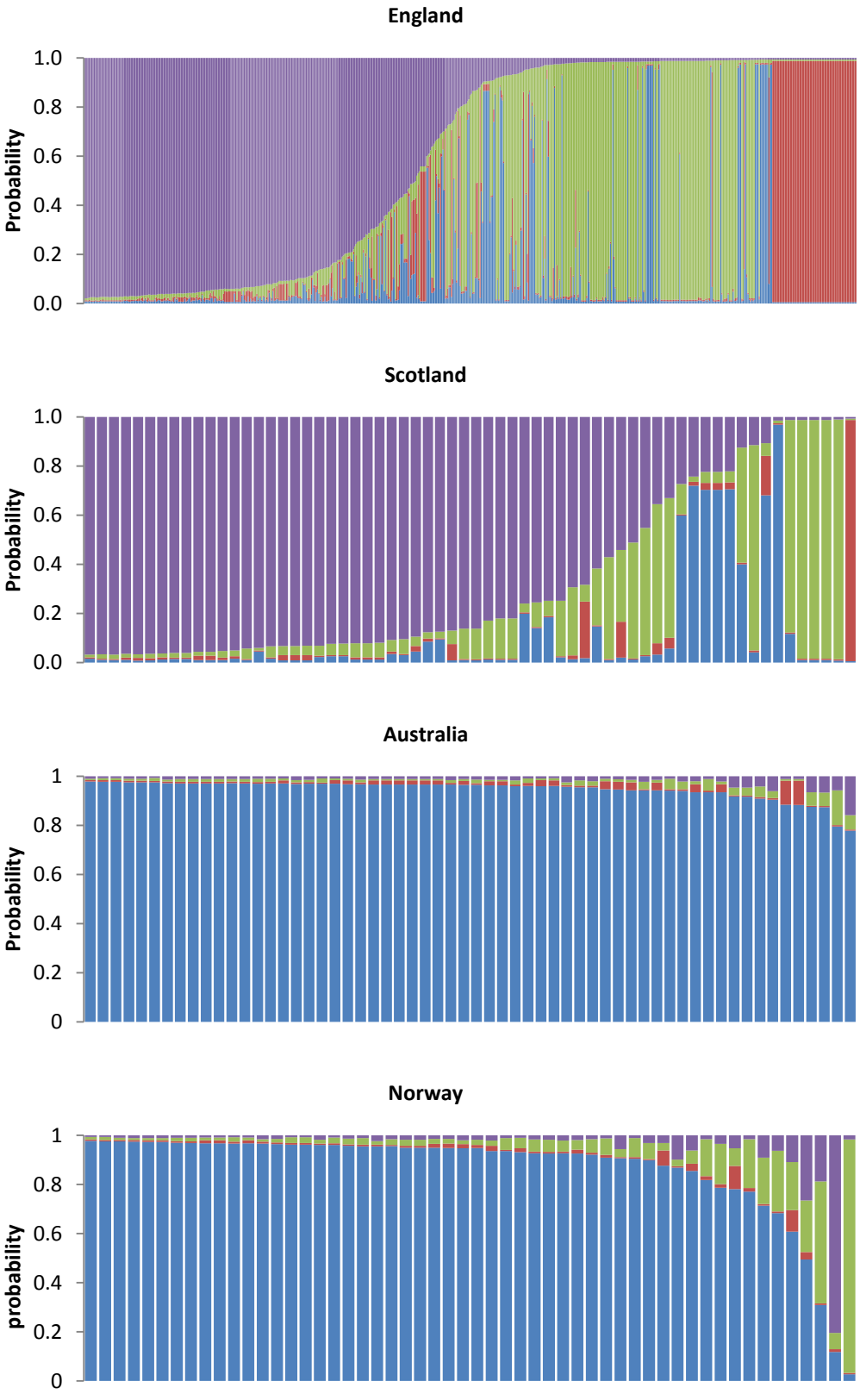

Figure 2.9 - Bar plot showing the assignment of *S. sclerotiorum* isolates from England, Scotland, Australia and Norway to four populations using STRUCTURE. Each bar represents a single isolate and the four colours indicate the probability of each isolate belonging to a population.

2.3.6 Analysis of Sequence Data for *S. sclerotiorum* Isolates

Within the 54 Norwegian *S. sclerotiorum* isolates eight IGS haplotypes were identified (Table 2.12) with IGS haplotype 2 being the most common. When the IGS sequence data for the Norwegian isolates were viewed in a phylogenetic tree and compared with previous published sequences in a median joining network it indicated three haplotypes not found in other countries, IGS24, IGS25 and IGS26 (Figures 2.10 and 2.11).

Within the total of 1,123 *S. sclerotiorum* isolates from all countries, 26 haplotypes were identified from the eight country populations (Table 2.12). The number of haplotypes in each population ranged from 3 to 17 and haplotype diversity ranged from 0.38 (Norway) to 0.78 (USA). The Norwegian population shared haplotypes with all the other populations (Table 2.12; Figure 2.11). The nearest neighbour statistic (Snn) values showed significant subdivision between all of the pairwise comparisons of the IGS sequences for the *S. sclerotiorum* isolates from different countries (Table 2.13).

Table 2.12 - IGS haplotype frequency and diversity for *S. sclerotiorum* populations from USA, Canada, New Zealand, Norway (Lesser Celandine), England, Scotland, Australia and Norway (samples from current study).

Haplotype	USA[1]	Canada[1]	New Zealand[1]	Norway - Lesser Celandine[1]	England[2,3]	Scotland[2,3]	Australia[3]	Norway
1	0	21	10	1	44	16	0	3
2	20	3	12	14	179	49	0	41
3	60	16	17	0	297	12	9	3
4	0	0	0	0	15	0	0	0
5	15	0	0	0	5	1	29	1
6	0	0	0	57	19	6	0	0
7	41	0	0	0	17	1	15	0
8	0	0	0	0	10	0	0	0
9	0	0	0	0	4	0	0	0
10	0	0	0	0	1	0	0	0
11	0	0	0	0	1	0	0	0
12	0	0	0	0	2	0	0	0
13	0	0	0	0	2	0	0	0
14	0	0	0	0	2	0	0	0
15	13	0	0	0	0	0	0	0
16	18	0	0	0	0	0	0	1
17	0	0	0	7	0	0	0	0
18	0	0	0	0	1	0	0	0
19	0	0	0	0	1	0	0	0
20	0	0	0	0	1	0	0	0
21	0	0	0	0	0	0	6	0
22	0	0	0	0	0	0	1	0
23	0	0	0	0	0	1	0	0
24	0	0	0	0	0	0	0	1
25	0	0	0	0	0	0	0	1
26	0	0	0	0	0	0	0	1
Haplotype Diversity	0.775	0.573	0.667	0.446	0.659	0.632	0.682	0.377

[1] *S. sclerotiorum* IGS sequences published by Carbone and Kohn (2001a)
[2] Includes *S. sclerotiorum* IGS sequences published by Clarkson *et al.* (2013)
[3] Includes *S. sclerotiorum* IGS sequences unpublished by Clarkson *et al.*

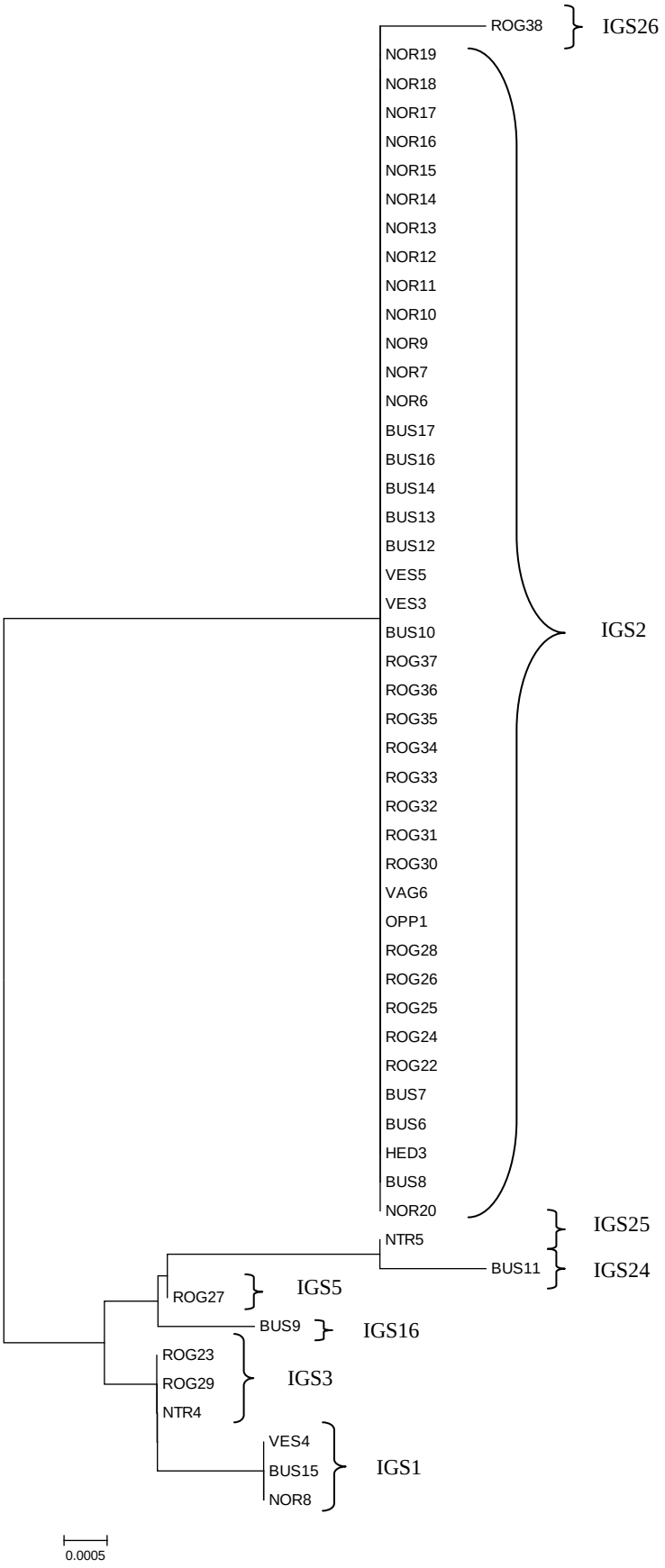

Figure 2.10 - Phylogenetic tree from IGS sequence data for Norwegian *S. sclerotiorum* isolates, with IGS haplotype number indicated by the groupings of isolates.

Table 2.13 – Nearest neighbour statistic (Snn values) for *S. sclerotiorum* populations from USA, Canada, New Zealand, Norway (Lesser Celandine), England, Scotland, Australia and Norway. *p<0.05,**p<0.01, ***p<0.001

	USA[1]	Canada[1]	New Zealand[1]	Norway - Lesser Celandine[1]	England[2,3]	Scotland[2,3]	Australia[3]	Norway
USA								
Canada	0.849***							
New Zealand	0.795***	0.543*						
Norway - Lesser Celandine	0.927***	0.939***	0.869***					
England	0.779***	0.898***	0.888*	0.916***				
Scotland	0.783***	0.695***	0.626**	0.787***	0.796***			
Australia	0.742***	0.880***	0.876***	0.993***	0.936***	0.887***		
Norway	0.824***	0.811***	0.667***	0.821***	0.871***	0.565**	0.922***	

[1] *S. sclerotiorum* IGS sequences published by Carbone and Kohn (2001a)
[2] Includes *S. sclerotiorum* IGS sequences published by Clarkson *et al.* (2013)
[3] Includes *S. sclerotiorum* IGS sequences unpublished by Clarkson *et al.*

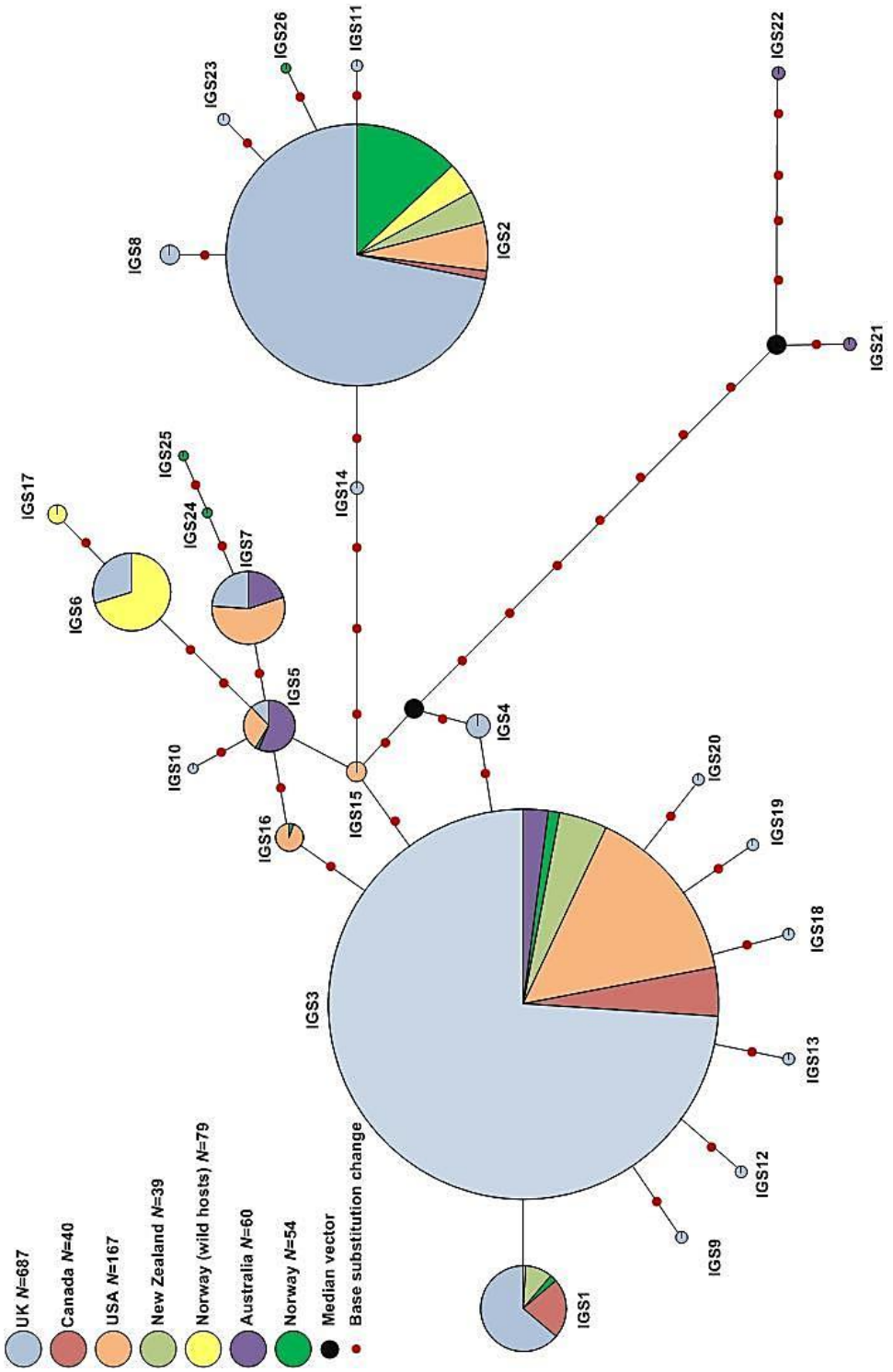

Figure 2.11 – Median joining network showing phylogenetic relationships between IGS haplotypes for *S. sclerotiorum* populations from UK, Canada, USA, New Zealand, Norway (Lesser Celandine), Australia and Norway. The size of each circle is proportional to the haplotype frequency and branch lengths are proportional the number of base substitution changes. The median vectors represent an hypothesised haplotype required to connect existing haplotypes within the network (after Clarkson *et al.* (2013).

2.4 Discussion

There has only been one other population study for *Sclerotinia subarctica* with isolates sampled in Alaska (Winton *et al.*, 2006). Overall, the results from the current study show that *S. subarctica* is present in Scotland on a range of crop plants and also the wild host meadow buttercup, as well as in various locations and crop plants in Norway, and on lettuce in Sweden. The pathogen was often found occurring in sympatry with *S. sclerotiorum.* In England, however, *S. subarctica* was only found in one location on meadow buttercup. This is the first time that *S. subarctica* has been found on crop plants in the UK and Norway, and also the first time that a large number of *S. subarctica* isolates have been characterised using microsatellite markers, enabling comparisons of populations from England, Scotland and Norway. *S. sclerotiorum* isolates from Norway were also genotyped for the first time using microsatellites, and IGS sequencing allowed comparison with populations from other countries.

The microsatellite analysis showed that *S. subarctica* populations from England, Scotland and Norway consisted of multiple haplotypes with two haplotypes sampled at a higher frequency. Haplotype 1 consisted only of isolates sampled from buttercup in Hereford, England, a population which was intensively sampled by Clarkson *et al.* (2013) and is the first and only location where *S. subarctica* has been found in England (Clarkson *et al.*, 2010). This intensive sampling was the reason for the apparent high frequency of haplotype 1. In contrast, haplotype 2 was present in both Scotland and Norway and was distributed widely across different host plants. The same *S. subarctica* haplotypes were identified in both crop plants and meadow buttercup in Scotland, as also found by Clarkson *et al.* (2013) for *S. sclerotiorum*

populations in the UK, suggesting that wild hosts can potentially act as a source of inoculum of *S. subarctica*, as well as enabling the pathogen to survive in the absence of a susceptible crop host (Phillips, 1992). The *S. subarctica* populations in the UK, Norway and Sweden also showed the same multiclonal structure as has been observed in the related species *S. sclerotiorum* in numerous countries (Kohn *et al.*, 1991; Kohli *et al.*, 1995; Sexton & Howlett, 2004; Winton *et al.*, 2006; Hemmati *et al.*, 2009; Clarkson *et al.*, 2013).

Winton *et al.* (2007) found only four microsatellite haplotypes from 41 isolates of *S. subarctica*, with significant linkage disequilibrium between the eight microsatellite loci, suggesting clonal reproduction and a lack of evidence of outcrossing. In the present study 83 microsatellite haplotypes were found from the 166 isolates, with 14 shared between two or more populations and 83% (69 isolates) had unique haplotypes. This is much greater than the 10% found in *S. subarctica* in Alaska by Winton *et al.* (2007).

The number of alleles found across all eight microsatellite loci ranged from one to four for England, and two to eight for both Scotland and Norway, with these two populations having a greater number of alleles than found in Alaska (two to three). Two of the loci (MS02 and MS04) were not polymorphic for the isolates from England. Gene diversity per locus for Scotland and Norway ranged from 0.15 to 0.77, whereas the gene diversity seen by Winton *et al.* (2007) ranged from 0.31 to 0.62. The sample size of 41 isolates used by Winton *et al.* (2007) is comparable to the sample sizes of 34 (England), 74 (Scotland) and 58 (Norway) used in the present study and, therefore, equivalent levels of gene diversity would be expected, particularly for England and Norway. The *S. subarctica* isolates from Norway and

Scotland are more genetically diverse than those found in Alaska, whereas the isolates from England are less diverse than all the other countries.

The Baysian cluster analysis using STRUCTURE showed that the majority of *S. subarctica* isolates from the English population (all from meadow buttercup in Herefordshire) belonged to a single population, while the Scottish and Norwegian isolates were assigned to two other populations. Additionally, the analysis of the microsatellite data showed that the English population did not share any haplotypes with any other population, and the minimum evolution spanning tree showed the English isolates clustered together by themselves in four separate clades. Moreover, the R_{ST}-based AMOVA showed the English population was significantly differentiated from the Scottish and Norwegian populations, and they had the lowest mean gene diversity across all the microsatellite loci, as well as the lowest gene diversity for all but two of the eight loci. All this evidence suggests that the population of *S. subarctica* in England is an isolated inbred population, such as has been observed for *S. sclerotiorum* populations on Lesser Celandine in Norway by Kohn (1995), and on meadow buttercup in Wales by Clarkson *et al.* (2013).

The presence of the *S. subarctica* population in Herefordshire is difficult to explain, and it is unlikely that it was introduced by plant material or soil contaminated with sclerotia, as the meadow is estimated to be over 150 years old (Clarkson *et al.*, 2013). However, it is not isolated from agricultural production as *S. sclerotiorum* (but not *S. subarctica*) was detected in oilseed rape only 6 km away (Vowchurch V, VT, Table 2.4). Moreover, *S. subarctica* was also absent from another buttercup meadow in very close proximity (Michaelchurch Escley, QM, Table 2.14). This *S. subarctica* population in England, therefore, warrants further investigation to

determine whether this species can be found in other neighbouring meadows, and to determine whether it has any other attributes of an isolated population.

The microsatellite analysis of the Norwegian *S. sclerotiorum* isolates showed that this population consisted of multiple haplotypes, of which four were shared between two or more sampling locations in Norway but none were shared with English, Scottish or Australian *S. sclerotiorum* populations. This is in direct contrast to the Norwegian *S. subarctica* populations, where haplotypes were shared with isolates found in Scotland. The Scottish *S. sclerotiorum* samples were from only three different sites, two of which were buttercup meadows, whereas the Scottish *S. subarctica* samples were from eight different sites, including the same two buttercup meadows. However, three of the *S. subarctica* microsatellite haplotypes were shared between Scottish meadow buttercup and crop plant hosts in Norway.

The large number of *S. sclerotiorum* isolates sampled from the UK, but with no microsatellite haplotypes in common with Norway, suggests that the transmission of *S. sclerotiorum* and *S. subarctica* may be different and should be researched further. Additionally, further work is required to examine whether the sharing of *S. subarctica* haplotypes between the two countries is related to the biology of this species, which is discussed in Chapter 5. The two species may have arrived in each country by different routes, i.e. *S. subarctica* appears to be commonly found in Northern regions (Alaska and Norway) so it is possible that it came to Scotland via trade routes from Norway, whereas *S. sclerotiorum* is distributed globally, and therefore may have arrived in both England and Norway at the same time but via separate routes. Moreover, it is possible that the microsatellites evolve at a slower rate in *S. subarctica* than in *S. sclerotiorum*, as has been observed in related species

of rice, where the African cultivated rice *Oryza glaberrima* was found to have a population mutation rate four times slower than the Asian cultivated rice *O. sativa* (Gao & Xu, 2008). A greater number of alleles for each microsatellite locus was found for *S. sclerotiorum* (5 to 17) than for *S. subarctica* (1 to 8), which indicates a slower mutation rate in *S. subarctica*, so the same microsatellite haplotypes may be more likely to be found in different countries for this species than for *S. sclerotiorum.*

In total, 50 microsatellite haplotypes were found within 54 Norwegian *S. sclerotiorum* isolates, and 85% (46 isolates) had unique haplotypes. Unique haplotype proportions in *S. sclerotiorum* have been found to vary from 11 % in vegetable crops in Alaska (Winton *et al.*, 2006) to 89 % in potatoes in Washington, USA (Atallah *et al.*, 2004), so the present study falls within this large range. Gene diversity of the Norwegian *S. sclerotiorum* population in the present study was similar to the levels seen in Australian populations (Sexton & Howlett, 2004), and the levels found in various English populations by Clarkson *et al.* (2013).

The Baysian cluster analysis of the Norwegian isolates only, indicated three populations, the same as found for UK *S. sclerotiorum* populations (Clarkson *et al.*, 2013). This was not the case in Iran where only one population was inferred by STRUCTURE for 276 isolates representing 37 *S. sclerotiorum* populations from oilseed rape in four northern provinces (Hemmati *et al.*, 2009). The R_{ST}-based AMOVA showed that the Norwegian *S. sclerotiorum* isolates were significantly differentiated from each other and from the Australian, English and Scottish populations. However, the Baysian cluster analysis using STRUCTURE showed that the majority of Norwegian and Australian isolates belonged to the same population,

whereas the UK isolates belonged to a different population. The Norwegian and Australian *S. sclerotiorum* isolates are therefore distinct from the English and Scottish populations as evidenced by the lack of any shared microsatellite haplotypes, and an unambiguous separation of isolates based on cluster designation in the STRUCTURE analysis.

Although no microsatellite haplotypes were shared between Australian and Norwegian *S. sclerotiorum* populations, the results from the STRUTURE analysis suggesting that the majority of these isolates belonged to the same population may be in part due to STRUCTURE being influenced by variation in the sample sizes. Other limitations of the software which have been highlighted when examining human population structure, including forcing STRUCTURE to place individuals into too few clusters (Kalinowski, 2011). Additionally, recent forensic guidelines for population surveys using microsatellite data recommend a minimum of 500 samples (Carracedo *et al.*, 2013) in order to reliably ascertain the variability in a population (Porras-Hurtado *et al.*, 2013).

The IGS sequence data provided a lower level of phylogenetic resolution than the microsatellite data for the Norwegian *S. sclerotiorum* isolates, with eight haplotypes identified within the eight sampling sites. Clarkson *et al.* (2013) found 14 IGS haplotypes from 12 UK *S. sclerotiorum* populations in a much larger sample size of 384 isolates. IGS sequencing allowed comparisons with previous studies, and the frequency and relationship of the IGS haplotypes seen in the phylogenetic network suggests the global distribution of a small number of common haplotypes, with lower frequency haplotypes often emerging from these at the local scale (Clarkson *et al.*, 2013). It is possible that these common haplotypes have been transported around

the world by the movement of soil, seeds or plant material contaminated with sclerotia (Malvárez *et al.*, 2007). Using microsatellite markers to analyse population structure gives a higher resolution and is useful at the local scale (Sirjusingh & Kohn, 2001), but comparisons between studies is difficult due to differences in design, methodology and execution (Rollins *et al.*, 2014). Using a standard isolate as well as increasing sample sizes would help to enable comparison between studies carried out by different research groups (Rollins *et al.*, 2014). Additionally, future work needs to utilise the latest tools available for investigating population structure, and now that the genome of *S. sclerotiorum* has been sequenced (Amselem *et al.*, 2011) a panel of single nucleotide polymorphisms (SNPs) can be developed to screen for markers to clarify the population structure at a deeper level, identifying divergence genes in haplotypes from different populations (Rollins *et al.*, 2014).

The diversity of *S. sclerotiorum* may be related to its wide host range (Boland & Hall, 1994) and the worldwide distribution of this pathogen (Purdy, 1979). The same may be true for *S. subarctica*, although a host range for this species has not been published and its distribution appears more limited. However, (Clarkson *et al.*, 2010) found *S. subarctica* readily infected plants and detached leaves of oilseed rape, and the results from the present study shows it is present on a range of crop plants and meadow buttercup in Scotland, Norway and Sweden. The success of certain clones of both species is not understood, but for *S. sclerotiorum* it has been suggested that it could be due to traits which help reproduction and spread, although Kull *et al.* (2004) found that aggressiveness was not associated with clone frequency. It is possible that sclerotial survival and germination are important factors and further work is required to examine this in more detail.

3. Susceptibility and Sclerotial Production of *Sclerotinia sclerotiorum* on Carrots

3.1 Introduction

Carrots (*Daucus carota* L.) are grown across the world in a wide range of climates (Foster *et al.*, 2008) and are valued for their health benefits and flavour (Kora *et al.*, 2003). Around 1.2 million hectares of carrots were harvested worldwide in 2012, giving a yield of 36.9 million tonnes and a value of $12.6 billion (FAO, 2014). In the UK 664,000 tonnes of carrots were produced in 2012 (Eurostat, 2014), with a production value of $389 million (FAO, 2014). They are commonly grown on a five to six year rotation, which can include other crops susceptible to *S. sclerotiorum* such as oilseed rape or potatoes (personal communication, Martin Evans, 2012).

Sclerotinia disease on carrots was first reported on field carrots in Belgium in 1860, and first described on stored carrots in 1871 (Mukula, 1957; Kora *et al.*, 2003). In the field in Canada Kora *et al.* (2005a) found Sclerotinia disease epidemics in carrots starting in mid-August, occurring after a combination of events including ascospores being detected in the crop, canopy closure, prolonged periods of high soil moisture and leaf wetness. The presence of apothecia or ascospores have been consistently associated with the appearance of symptoms of Sclerotinia disease in the field (Foster *et al.*, 2011), suggesting that ascospores are the primary inoculum source for initiation of epidemics in carrot, as is also the case with oilseed rape (Morrall & Dueck, 1982), lettuce (Clarkson *et al.*, 2004), and bean (Boland & Hall, 1987).

The symptoms of Sclerotinia disease on carrots, such as white mycelium, are first seen on senescing leaves in contact with the soil (Kora *et al.*, 2005a), and other

studies also report a link between lodging of canopies and senescing plant material with susceptibility to *S. sclerotiorum* (Couper *et al.*, 2001). Glasshouse experiments have also shown that Sclerotinia disease develops more quickly from artificial ascospore inoculations on damaged carrot foliage than on undamaged foliage (McQuilken & Chalton, 2009). It has been suggested that in the field the pathogen enters the root via the crown of the plant as it is unlikely that carrot roots are directly infected by mycelium germinating from sclerotia in the surrounding soil (Finlayson *et al.*, 1989b).

In the USA serious losses of marketable carrot roots have been reported to occur due to Sclerotinia disease during storage (Hansen *et al.*, 2001). Post-harvest epidemics of Sclerotinia disease on stored carrot roots mainly originates from infected foliage (Finlayson *et al.*, 1989b) and any mycelium growing from infected roots can spread into adjacent healthy roots (Kora *et al.*, 2005a). Several methods are used for storing carrots, varying with the countries in which they are produced. Methods include ventilated ambient air storage, temperature controlled refrigerated storage or storing them in the ground covered with straw and polythene sheeting (Kora *et al.*, 2008). This last method is used in the UK, with carrots stored in the ground over the winter months, covered with a layer of straw and plastic to protect them from frost and to keep them dark to prevent growth. Under the straw any Sclerotinia disease which was present in the crop before strawing down can spread via mycelium in a similar way observed in carrot roots in cold stores (personal communication, Martin Evans, 2012). Post-harvest Sclerotinia disease epidemics can substantially affect the number of marketable roots and their shelf life during storage and transportation (Kora *et al.*, 2003).

There is little published research on pre-harvest resistance in carrots to *S. sclerotiorum,* and no reports of any genotypes with high level resistance. In most cultivated crop hosts there is no qualitative resistance to *S. sclerotiorum* (Kora *et al.*, 2003) but various quantitative resistance traits have been identified in connection with resistance to *S. sclerotiorum*, on hosts such as soybean (Arahana *et al.*, 2001), sunflower (Mestries *et al.*, 1998) and oilseed rape (Wu *et al.*, 2014). Although there are no published studies on potential genetic interactions between carrot cultivars and *S. sclerotiorum*, Foster *et al.* (2008) found that inoculating petioles with the pathogen elicits the formation of structural barriers at the petiole base, demonstrating the existence of genetically regulated defence mechanisms in carrot against *S. sclerotiorum*. Differences have been seen in the level of Sclerotinia disease between carrot cultivars in long term storage, and Finlayson *et al.* (1989a) found the response of carrot cultivars to field infection and storage decay were correlated with variations in the permeability and integrity of root cell membranes as induced by *S. sclerotiorum in vitro*. It was suggested that these results indicate the presence of quantitative resistance of root tissues to attack by *S. sclerotiorum*, which is why there is varying susceptibility to the pathogen between cultivars (Kora *et al.*, 2003).

There have been several studies investigating foliar trimming of carrot crops to improve airflow through the canopy, improve fungicide penetration and remove older senescing leaves which act as an entry point for *S. sclerotiorum* (Kora *et al.*, 2005; McDonald *et al.*, 2007). Similarly, the canopy architecture of a susceptible crop may provide unfavourable conditions for disease development, leading to disease escape (Agrios, 2005). Different crop plant cultivars may be characterised by features such as upright foliage reducing lodging; light airy foliage allowing air movement which provides a microclimate less favourable to disease development;

and large internodes reducing disease severity (Coyne *et al.*, 1974; Blad *et al.*, 1978; Calonnec *et al.*, 2013; Tivoli *et al.*, 2013). Variation in leaf morphology between different carrot cultivars and wild types provides a further potential source of breeding material to achieve a canopy architecture which is unfavourable for disease development (Jensen *et al*., 2008).

To enable rapid screening of crop cultivars to find sources of resistance against *S. sclerotiorum* methods have been developed using detached cotyledon or detached stems, and compared to results on whole plants (Garg *et al.*, 2008; Mei *et al.*, 2012). This enables high throughput of cultivars in an economically viable way, and has often been found to be a reliable screening method (Garg *et al.*, 2008). However, there has been no published research applying this method to screening of carrot cultivars for resistance to *S. sclerotiorum*, but work has been carried out to develop detached leaf protocols for screening resistance in carrot to *Botrytis cinerea* using agar plugs and conidial suspensions (Baranski *et al*., 2006). Similarly, for *Alternaria dauci* comparisons have been made between detached carrot leaf and whole carrot plant inoculations, but there was a low correlation in resistance levels observed between the two inoculation methods (Pawelec *et al*., 2006).

The aims of the work described in this chapter were:

- To quantify the production of sclerotia by two *S. sclerotiorum* isolates on harvested carrot roots from a large range of carrot accessions.
- To determine if there are differences in *S. sclerotiorum* lesion development in whole carrot plants and detached leaves from selected accessions.

3.2 Materials and Methods

3.2.1 Carrot Root Inoculation

Carrot root inoculations to assess the production of sclerotia by two *S. sclerotiorum* isolates (L6 and L44) were carried out on roots from a carrot diversity set, consisting of 87 accessions, grown in the field at Wellesbourne by the Genetic Resources Unit. These were sown in April 2011, in drills 2 cm deep and 15 cm apart. There was no application of fertiliser, insecticides or fungicides. The diversity set consisted of a mixture of wild species, elite cultivars, mapping parents and cultivated varieties (Figure 3.1, Table 3.1). Roots were harvested on 24th November 2011 and 6th December 2011, cleaned with tap water, air dried and individually weighed. The roots harvested in November 2011 were immediately used in the experiment and those harvested in December 2011 were stored in open bags at 5°C until they were used in a repeat experiment on 23rd March 2012.

Each root was checked for any disease symptoms before the centre of each root was inoculated with a 5 mm agar plug of *S. sclerotiorum* mycelium from a 3 day old actively growing colony of isolate L6 or L44. The roots were then incubated at 13°C on damp tissue in 20 L plastic (bakery) trays enclosed in plastic bags. After one week any roots not showing signs of infection were pierced with a needle under the mycelial plug. The carrots were kept damp by opening the bags and spraying the trays with water once a week to maintain humidity and encourage disease development. Four replicate carrot roots for each accession (where available at harvest) for each *S. sclerotiorum* isolate were inoculated, and two repeat experiments were carried out. Sclerotia were retrieved, counted and weighed once they were mature, after approximately six weeks.

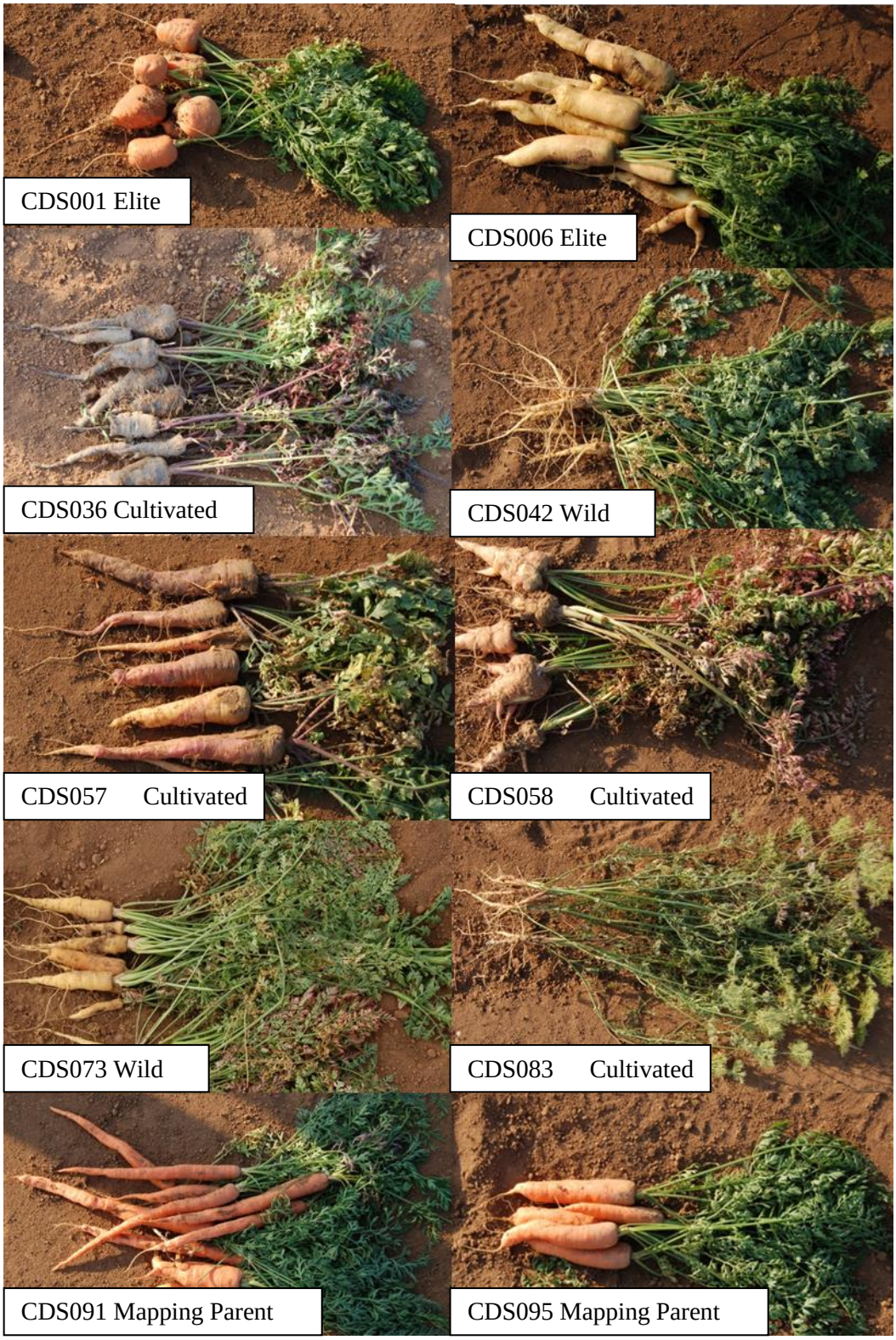

Figure 3.1 – a selection of the carrot roots harvested from the carrot diversity set used for inoculation with *Sclerotinia sclerotiorum*. CDS numbers refer to the carrot diversity set number given to each accession.

Table 3.1 - Details of the 87 carrot accessions used for root inoculations with *Sclerotinia sclerotiorum*.

Carrot Diversity Set Number	Group	Genus	Species	Subtaxa	Acession Name	Type	Country of Origin	Root shape
CDS001	elite	*Daucus*	*carota*		PARMEX			Round
CDS002	elite	*Daucus*	*carota*		AUTUMN KING 2 VITA LONGA			Tapering
CDS003	elite	*Daucus*	*carota*		AMSTERDAM 2 SWEETHEART			Oblong
CDS004	elite	*Daucus*	*carota*		DEEP PURPLE F1			Tapering
CDS005	elite	*Daucus*	*carota*		YELLOWSTONE F1			Tapering
CDS006	elite	*Daucus*	*carota*		WHITE SATIN			Tapering
CDS007	elite	*Daucus*	*carota*		NAIROBI			Tapering
CDS009	elite	*Daucus*	*carota*		PURPLE HAZE			Tapering
CDS010	cultivated	*Daucus*	*carota*		GOLD PAK	CARROT-IMPERATOR	USA	Tapering
CDS011	cultivated	*Daucus*	*carota*		PERSIA NO 242	CARROT	IRN	Tapering
CDS012	cultivated	*Daucus*	*carota*		RED ELEPHANT	CARROT-ST VALERY	GBR	Tapering
CDS013	cultivated	*Daucus*	*carota*		KURODA GOSUN	CARROT-CHANTENAY	JPN	Tapering
CDS014	cultivated	*Daucus*	*carota*		ANNUAL RED RAWALPINDI	CARROT	PAK	Tapering
CDS015	cultivated	*Daucus*	*carota*		AMSTERDAM FORCING	CARROT-AMSTERDAM	GBR	Tapering
CDS016	cultivated	*Daucus*	*carota*		TOPWEIGHT	CARROT	AUS	Tapering
CDS017	cultivated	*Daucus*	*carota*		WESTERN RED	CARROT	AUS	Tapering
CDS019	cultivated	*Daucus*	*carota*		JAMES SCARLET INTERMEDIATE	CARROT	GBR	Tapering
CDS020	cultivated	*Daucus*	*carota*		LONG RED SURREY	CARROT-LONG ORANGE	GBR	Tapering
CDS021	cultivated	*Daucus*	*carota*		EARLY FRENCH FRAME	CARROT	GBR	Round
CDS022	cultivated	*Daucus*	*carota*		EGMONT GOLD	FODDER CARROT	NZL	Obtriangular
CDS023	cultivated	*Daucus*	*carota*		TARANAKI STRONG TOP	FODDER CARROT	NZL	Tapering
CDS024	wild	*Daucus*	*carota*	*azoricus*		WILD SPECIES		Tapering
CDS025	cultivated	*Daucus*	*carota*		AZERBAIJAN LOCAL	RED/WHITE CARROT	SUN	Tapering
CDS026	cultivated	*Daucus*	*carota*		WHITE DAGHESTAN LOCAL	WHITE CARROT	SUN	Tapering
CDS027	cultivated	*Daucus*	*carota*		PUSA KESAR	CARROT	IND	Tapering
CDS028	cultivated	*Daucus*	*carota*		MUSCADE D'ALGER	CARROT-SUB TROP	MAR	Tapering
CDS029	cultivated	*Daucus*	*carota*		TROPICAL	CARROT-CHANTENAY	BRA	Tapering
CDS030	wild	*Daucus*	*carota*	*gingidium*		WILD SPECIES	PRT	Tapering
CDS031	wild	*Daucus*	*carota*	*gummifer*		WILD SPECIES	GBR	Tapering
CDS033	wild	*Daucus*	*capillifolius*			WILD SPECIES	LBY	Tapering
CDS034	cultivated	*Daucus*	*carota*		CRIOLLA	CARROT-CHANTENAY	ARG	Obtriangular
CDS035	cultivated	*Daucus*	*carota*		BRASILIA	CARROT-NANTES	BRA	Tapering
CDS036	cultivated	*Daucus*	*carota*			CARROT-BLACK	IND	Tapering
CDS037	wild	*Daucus*	*carota*	*maritimus*		WILD SPECIES	FRA	Tapering
CDS038	cultivated	*Daucus*	*carota*		BLANCHE A COLLET VERT HORS TERRE	FODDER CARROT		Tapering
CDS039	cultivated	*Daucus*	*carota*		LANGE ROTE STUMPFE OHNE HERZ	CARROT-BERLICUM		Tapering
CDS040	cultivated	*Daucus*	*carota*		ROYAL STAR	CARROT	AUS	Tapering
CDS041	cultivated	*Daucus*	*carota*		SRUDHI	CARROT	DZA	Tapering
CDS042	wild	*Daucus*	*carota*			WILD SPECIES	GBR	Tapering
CDS043	cultivated	*Daucus*	*carota*		WHITE BELGIAN	FODDER CARROT	GBR	Tapering
CDS044	wild	*Daucus*	*carota*	*commutatus*		WILD SPECIES	FRA	Tapering
CDS045	wild	*Daucus*	*carota*	*gadecaei*		WILD SPECIES	FRA	Tapering
CDS047	wild	*Daucus*	*carota*			WILD SPECIES	CHE	Tapering
CDS048	wild	*Daucus*	*carota*			WILD SPECIES	POL	Tapering
CDS049	wild	*Daucus*	*carota*			WILD SPECIES	CSK	Tapering
CDS050	cultivated	*Daucus*	*carota*		ST VALERY	CARROT-ST VALERY		Tapering
CDS051	cultivated	*Daucus*	*carota*		LITTLE FINGER	CARROT-AMSTERDAM		Tapering
CDS052	cultivated	*Daucus*	*carota*	*sativus*	CENOURA	CARROT	PRT	Tapering
CDS053	cultivated	*Daucus*	*carota*			CARROT	ESP	Tapering
CDS054	cultivated	*Daucus*	*carota*			BLACK CARROT	IND	Tapering
CDS055	cultivated	*Daucus*	*carota*			CARROT	TUR	Tapering
CDS056	cultivated	*Daucus*	*carota*			BLACK CARROT	TUR	Tapering
CDS057	cultivated	*Daucus*	*carota*		BELEDI	CARROT	SYR	Tapering
CDS058	cultivated	*Daucus*	*carota*		GAJAR	BROWN-RED CARROT	IND	Tapering
CDS059	cultivated	*Daucus*	*carota*			CARROT	TUR	Tapering
CDS060	cultivated	*Daucus*	*carota*		LONG RED	LONG RED CARROT	ETH	Obtriangular
CDS062	cultivated	*Daucus*	*carota*			PURPLE CARROT	AFG	Tapering
CDS063	cultivated	*Daucus*	*carota*		GARGA SERK	RED CARROT	PAK	unknown
CDS065	cultivated	*Daucus*	*carota*		HAVIJ	CARROT	IRN	Tapering
CDS066	cultivated	*Daucus*	*carota*			CARROT	SWE	Obtriangular
CDS067	cultivated	*Daucus*	*carota*		SWEETCROP	CARROT	NZL	Obtriangular
CDS068	cultivated	*Daucus*	*carota*		YIELD HEAVY	CARROT	NLD	Tapering
CDS069	cultivated	*Daucus*	*carota*		ZARDAK TBUR	CARROT	AFG	Tapering
CDS071	cultivated	*Daucus*	*carota*		KINTOKI	CARROT-RED	JPN	Tapering
CDS072	cultivated	*Daucus*	*carota*			CARROT	TUR	Tapering
CDS073	wild	*Daucus*	*carota*		BINYOMINA NO 1	WILD CARROT	ISR	Tapering
CDS074	cultivated	*Daucus*	*carota*		RED CARROT	CARROT	CHN	Tapering
CDS075	cultivated	*Daucus*	*carota*		YELLOW CARROT 12	CARROT	CHN	Tapering
CDS076	cultivated	*Daucus*	*carota*		GELBE WORTEL	CARROT	NLD	Tapering
CDS077	cultivated	*Daucus*	*carota*		MORADA DEL MAESTRAZGO	CARROT-MIXED COLOURS	ESP	Tapering
CDS078	cultivated	*Daucus*	*carota*		YAMANOUCHI ISHYAKU SENKO	CARROT	JPN	Tapering
CDS079	cultivated	*Daucus*	*carota*		ALTRINGHAM	CARROT	GBR	Obtriangular
CDS080	cultivated	*Daucus*	*carota*		ALTRINGHAM LARGE RED	CARROT	CZE	Tapering
CDS081	cultivated	*Daucus*	*carota*		T-29	CARROT	PAK	Tapering
CDS082	cultivated	*Daucus*	*carota*		HARUMAKI KINKOU 5 SUN	CARROT	JPN	Tapering
CDS083	cultivated	*Daucus*	*carota*		RED CARROT II	RED CARROT	EGY	Tapering
CDS084	cultivated	*Daucus*	*carota*		MIRZAMUSHUK	CARROT	SUN	Obtriangular
CDS085	wild	*Daucus*	*carota*			WILD CARROT	SUN	Tapering
CDS086	mapping parent	*Daucus*	*carota*		BRASILIA			Tapering
CDS088	mapping parent	*Daucus*	*carota*		MK2/00-1 MK300/00			Tapering
CDS090	mapping parent	*Daucus*	*carota*	*carota*	QAL			Tapering
CDS091	mapping parent	*Daucus*	*carota*		USDA 0493B			Tapering
CDS092	mapping parent	*Daucus*	*carota*		USDA 7262B			Tapering
CDS093	mapping parent	*Daucus*	*carota*		USDA 9304B			Tapering
CDS094	mapping parent	*Daucus*	*carota*		YL1/01-1			Tapering
CDS095	mapping parent	*Daucus*	*carota*		USDA B6274B			unknown
CDS096	wild	*Daucus*	*carota*	*hispanicus*				Tapering

3.2.2 Whole Carrot Plant Inoculation

Whole carrot plant inoculations to assess the susceptibility of different cultivars and accessions to *S. sclerotiorum* were carried out in glasshouse experiments. The results from the carrot root inoculation experiments (3.2.1), together with root position and leaf growth habit as determined by the Genetic Resources Unit was taken into account to select a diverse range of accessions and varieties to test (Table 3.2).

Table 3.2 – Varieties/accessions used in whole carrot plant and detached leaf inoculation experiments, and their growth habits.

Carrot Diversity Set No.	Group	Name	Root position in soil	Leaf growth habit	Used in Whole Plant Inoculation	Used in Detached Leaf Inoculation
7	Elite	Nairobi	shallow	semi-upright	x	x
n/a	Elite	Chantenay	shallow - medium	upright	x	x
n/a	Elite	Eskimo	deep	upright	x	x
n/a	Elite	Narbonne	unknown	upright	x	x
90	Mapping parent - wild	QAL	deep	upright	x	x
93	Mapping parent	USDA 9304B	shallow	upright	x	
92	Mapping parent	USDA 7262B	deep	upright	x	
30	Wild	7159	deep	prostrate	x	
51	Cultivated	Little finger	shallow	prostrate	x	x
88	Mapping parent	MK2/00-1 MK300/00	deep	upright		x
86	Mapping parent - elite	Brasilia	shallow	upright		x
10	Advanced cultivar	Gold Pak	shallow	upright		x
80	Advanced cultivar	Altringham large red	shallow	upright		x

Ten carrot plants of each variety/accession were grown from seed in Levington M2 compost (Everris, UK) in 3 L deep pots in a polytunnel with three repeated sowings on 30th March 2012, 4th May 2012 and 22nd June 2012. After 18 weeks, six plants of each cultivar were moved to a glasshouse with a mean temperature of approximately 20°C. Three leaf petioles on each plant were inoculated by cutting off the leaflets and placing a pipette tip with a mycelial plug, taken from the actively growing edge of a three day old culture of *S. sclerotiorum* isolate L6, onto the cut end of the petiole (Figure 3.2). Plants were then covered with a plastic bag to maintain humidity for

three days, after which the bags were removed and the plants misted with water during each night to promote disease development.

Figure 3.2 - Carrot plants inoculated with *S. sclerotiorum* using mycelial plugs in pipette tips placed onto the end of cut petioles.

To assess disease development the healthy petiole tissue between the crown of the plant and the lesion edge was measured (Figure 3.3). Progression of infection into the crown of the plant was scored using 0 (no infection in crown), 1 (brown lesion visible), 2 (brown lesion and some mycelium visible), 3 (brown lesion and large amount of mycelium) and 4 (crown rotten and sclerotia being formed). Plants were arranged in a randomised block design, with six replicates for each carrot accession and the experiment was repeated three times. Disease development was assessed twice a week, for a total of four weeks.

Figure 3.3 – Detail of carrot petiole inoculated with *Sclerotinia sclerotiorum* using a mycelial plug in a pipette tip, and the zone of measurement to assess disease progress (healthy petiole tissue between the crown and the lesion edge, indicated by the arrow).

3.2.3 Detached Carrot Leaf Inoculation

Detached carrot leaf inoculations were carried out to assess the suitability of this method to test the susceptibility of different accessions to *S. sclerotiorum*. Preliminary experiments were carried out to evaluate the method and to assess the reproducibility of the results. These experiments used three replicates of six leaves from four elite carrot accessions (Nairobi, Narbonne, Chantenay and Eskimo), in three repeated experiments. The experiments were conducted as described below for a wider range of accessions.

In subsequent larger scale experiments a range of carrot accessions were selected for testing (Table 3.2) based on the results from the root and whole carrot plant inoculations. All plants were grown from seed in compost (Levington F2 + sand, Everris) in module trays in a controlled environment room at 20°C under white fluorescent lights (12 h day) for eight weeks. Five leaves of each accession were cut and trimmed to 15 cm before being placed into clear plastic boxes on damp tissue paper (Figure 3.4).

Figure 3.4 – Detached leaves of carrot accession 'Narbonne' inoculated with *Sclerotinia sclerotiorum*

A 5 mm mycelial plug of *S. sclerotiorum* isolate L6 was placed onto the cut end of each leaf and the boxes sealed in clear plastic gripper bags before being incubated in a controlled environment room under white fluorescent lights (14 h light, 10 h dark) at 15°C. The boxes were arranged in a randomised block design with three replicates for each carrot variety and the experiment was repeated three times. Lesion size on the carrot petioles was measured from the cut end after four days.

3.2.4 Statistical Analyses

All statistical analysis was carried out in Genstat® (13th edition, VSN international Ltd.). For the carrot root inoculation experiments the weight per sclerotium and the number of sclerotia g^{-1} of carrot root tissue were log transformed to satisfy the requirements of homogeneity of variance and reduce the influence of residuals, with the addition of 0.05 to allow for any nil counts. Transformed data were then analysed using restricted or residual maximum likelihood (REML) variance components analyses with a fixed effects structure of isolate by accession and a random effects structure of tray number.

For the whole carrot plant experiments the progression of the pathogen down the petiole was calculated as a mean rate of mm d^{-1} which was analysed using analysis of variance (ANOVA), with a blocking structure of experiment by replicate by leaf.

For both the preliminary and full detached leaf experiments the length of the lesion along the petiole (mm) after four days was analysed using ANOVA, with a blocking structure as described for the whole carrot plant experiments. The rate of lesion development was also calculated based on the lesion length after 4 days and

Pearson's simple correlation coefficient calculated to determine if there was a relationship between the rates of lesion development over four days in the whole carrot plant experiments and the detached leaf experiments.

Interpretations of the analyses and comparisons of treatment means were carried out by comparing REML or ANOVA treatment means using the approximate least significant difference values (LSD) at the 5% level.

3.3 Results

3.3.1 Carrot Root Inoculation

Across all the carrot accessions *S. sclerotiorum* isolate L44 produced sclerotia with a mean weight of 0.03 g, compared to 0.02 g for isolate L6. The maximum weight of an individual sclerotium produced by both isolates was very similar, being 0.14 g for isolate L44 and 0.15 g for isolate L6 across all carrot accessions (Figure 3.5).

Greater differences were observed in the number of sclerotia produced by each isolate, with L6 producing a mean of 0.6 sclerotia g^{-1} of carrot root tissue across all accessions, and L44 producing a mean of 0.3 sclerotia g^{-1}. Again, the maximum number of sclerotia g^{-1} carrot root tissue produced by each isolate was very similar with 3.3 sclerotia for L44 and 3.2 for L6 (Figure 3.5).

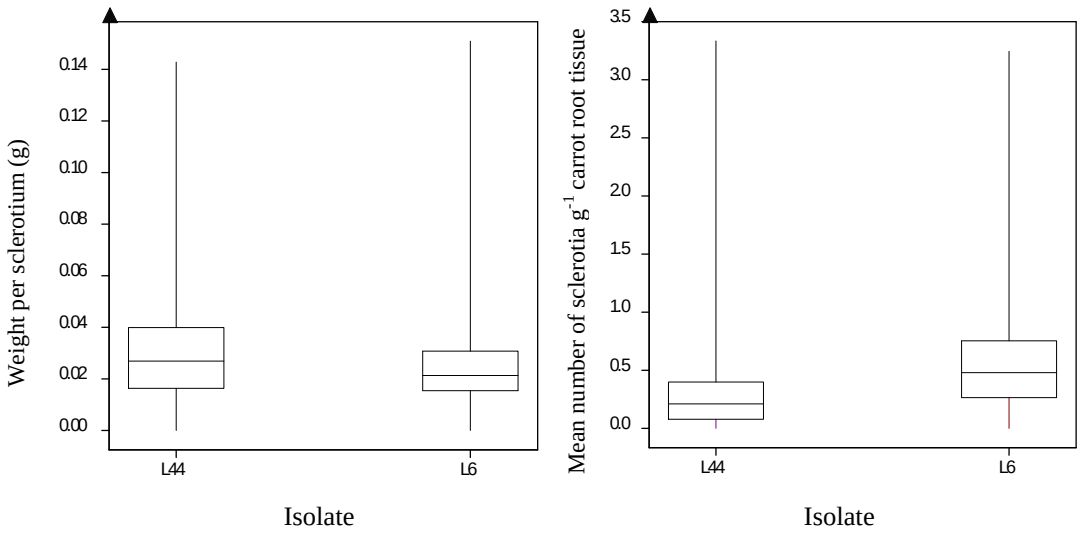

Figure 3.5 - Box and whisker plots of the numbers of sclerotia produced g^{-1} carrot root tissue (right), and the weight per sclerotium produced (left), for *S. sclerotiorum* isolates L6 and L44 over all carrot accessions. Whiskers show the minimum and maximum values of all the data.

When data for each *S. sclerotiorum* isolate and the individual carrot accessions was examined, the minimum mean weight of a sclerotium was 0.01 g for isolate L6 (accession 76, cultivated type; significantly different from 5 other accessions ($p<0.05$)) and 0.008 g for L44 (accession 24, wild type; significantly different from 14 other accessions ($p<0.05$)). The maximum mean weight of a sclerotium was 0.06 g for isolate L6 (accession 85, wild type; significantly different from 29 other accessions ($p<0.05$)) and 0.07 g for L44 (accession 59, cultivated type; significantly different from 73 other accessions ($p<0.05$)) (Figure 3.6).

The minimum number of sclerotia produced g^{-1} carrot root tissue was 0.06 for L6 (accession 88, mapping parent; significantly different from 48 other accessions ($p<0.05$)) and 0.03 for L44 (accession 66, cultivated type; significantly different from 38 other accessions ($p<0.05$)). The maximum number of sclerotia produced g^{-1} carrot root tissue was 1.5 for L6 (accession 31, wild type; significantly different from 54 other accessions ($p<0.05$)) and 1.2 for L44 (accession 73, wild type; significantly different from 43 other accessions ($p<0.05$)) (Figure 3.6).

The REML analysis of the weight of sclerotia produced showed no significant effect of *S. sclerotiorum* isolate, but did show a significant effect of carrot accession ($p<0.001$) and a significant interaction between isolate and accession ($p<0.05$). The REML analysis of numbers of sclerotia produced showed a significant effect of *S. sclerotiorum* isolate ($p<0.001$) and of carrot accession ($p<0.001$), but no significant interaction between isolate and accession.

Comparing log transformed means with a 5% LSD of 0.4 (d.f. 790) there were significant differences in the weight of individual sclerotia produced between the two *S. sclerotiorum* isolates for accession numbers 34 (cultivated type) and 88

(mapping parent). Comparing LOG transformed means with a 5% LSD of 1.3 (d.f. 790) there were fifteen carrot accessions for which there were significant differences in the number of sclerotia produced g^{-1} carrot root tissue between the two *S. sclerotiorum* isolates – accession numbers 14, 20, 23, 25, 39, 43, 58, 66, 67, 76, 79 (all cultivated types), 24, 49 (wild types), 9 (elite cultivar) and 88 (mapping parent).

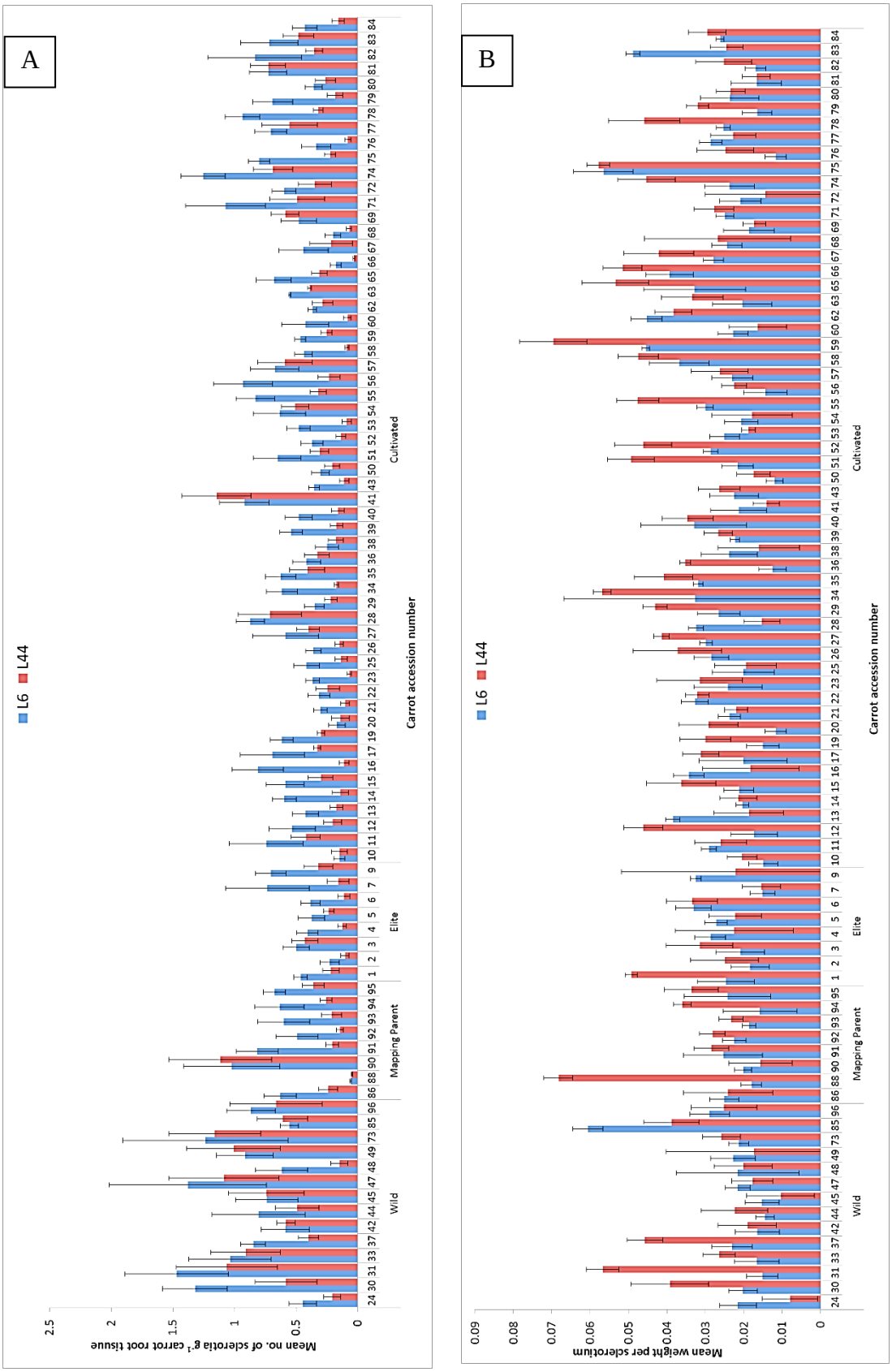

Figure 3.6 – Mean numbers of sclerotia produced g^{-1} carrot root tissue (A) and mean weight per sclerotium (B) produced on roots of 87 carrot accessions for *Sclerotinia sclerotiorum* isolates L6 (blue) and L44 (red), sorted by accession type. Error bars show SEM.

3.3.2 Whole Carrot Plant Inoculation

Following inoculation with *S. sclerotiorum* isolate L6 all the carrot plant petioles in experiments became infected, and for most of the accessions disease progressed into the crown. In some cases, sclerotia were also formed in the crown of the plant by the end of four weeks (Figure 3.7).

Figure 3.7 – Following inoculation of petioles with *Sclerotinia sclerotiorum* lesions developed and progressed down the petiole (left), into the crown where white mycelium and sclerotia developed (right).

The ANOVA of the rate of *S. sclerotiorum* lesion development showed a significant effect of carrot accession ($p<0.001$) and was slowest in accession 51 (Little finger, cultivated type, 3.8 mm day^{-1}) which was significantly different from all other accessions ($p<0.05$). The fastest rate was observed in accession 30 (7159, wild type, 6.87 mm day^{-1}), which was significantly different ($p<0.05$) from all accessions except accession 90 (Table 3.3). However, accessions 51 and 30 had the lowest mean crown infection scores. The two accessions 93 and 92 with the highest mean crown infection scores (USDA 9304B and USDA 7262B, mapping parents) had relatively slow rates of lesion development (4.9 mm and 4.8 mm day^{-1} respectively; Table 3.3).

Table 3.3 – The mean rates of *S. sclerotiorum* (isolate L6) lesion development and mean crown infection scores for nine carrot accessions.

Carrot Diversity Set No.	Carrot Cultivar/Accession Name	Mean Rate of Lesion Development per Day (mm) [1]	Mean Crown Infection Scores 0 (no infection) to 4 (crown diseased and rotten)
7	Nairobi	5.71 c	2
n/a	Chantenay	5.80 cd	2
n/a	Eskimo	5.58 bc	2
n/a	Narbonne	5.27 abc	2
90	QAL	6.48 de	2
93	USDA 9304B	4.98 ab	4
92	USDA 7262B	4.83 a	4
30	7159	6.87 e	1
51	Little finger	3.89 f	1
d.f		424	
5% LSD		0.73	

[1] Numbers followed by a different letter are significantly different at the 5% level.

3.3.3 Detached Leaf Inoculation

Preliminary Experiments

All the petioles of the four elite carrot accessions used in these experiments became infected, and at the end of the experiments (four days) dark brown lesions had developed from the inoculated cut end. The ANOVA of the rate of lesion development (mm day^{-1}) showed a significant effect of the carrot accessions ($p<0.001$). The mean rate of lesion development ranged from 11.6 mm day^{-1} for Narbonne and 15.3 mm day^{-1} for Chantenay, the latter being significantly greater than the other accessions ($p<0.05$; Table 3.4).

Final Experiments

As observed in the preliminary experiments, the greatest rate of lesion development for the four elite cultivars was observed for Chantenay (11.5 mm day^{-1}), with the lowest on Narbonne (9.8 mm day^{-1}; Figure 3.8).

Figure 3.8 - Lesions caused by *S. sclerotiorum* isolate L6 after 4 days on detached carrot leaves, varieties Chantenay (top) and Narbonne (bottom)

The ANOVA of the rate of lesion development showed a significant effect of carrot accession ($p<0.001$). Across the carrot accessions the lowest rate of lesion development was 7.2 mm day^{-1} for accession 90 (QAL, mapping parent), which was significantly slower than all the other accessions ($p<0.05$). The greatest rate of lesion development was observed for accession 86 (Brasilia, mapping parent, 12.7 mm day^{-1}), which was significantly faster than five other accessions ($p<0.05$; Table 3.4).

Table 3.4 - Rate of *S. sclerotiorum* (isolate L6) lesion development on detached leaves for different carrot accessions.

Carrot Diversity Set No.	Carrot Cultivar/Accession	Mean rate of lesion spread per Day (mm)[1] Preliminary Experiments		Mean rate of lesion spread per Day (mm)[1] Final Experiments	
7	Nairobi	12.4	a	10	a
	Chantenay	15.3	b	11.5	bc
	Eskimo	13	a	11.3	b
	Narbonne	11.6	a	9.8	a
88	MK2/00-1 MK300/00	-		11.7	bc
86	Brasilia	-		12.7	c
10	Gold Pak	-		11.8	bc
90	QAL	-		7.2	d
80	Altringham large red	-		12.5	bc
51	Little finger	-		9.7	a
d.f.		105		396	
5% LSD		1.6		1.3	

[1] Numbers followed by a different letter are significantly different using 5% LSD.

The results of the rate of lesion development in mm day^{-1} for the six varieties Nairobi, Little finger, QAL, Chantenay, Narbonne and Eskimo used in both the whole plant and detached leaf experiments were not highly correlated (r = 0.1291).

3.4 Discussion

The results from the present study show that different *Sclerotinia sclerotiorum* isolates produce different numbers of sclerotia, and that *S. sclerotiorum* lesion development varies between carrot accessions, indicating differences in susceptibility between cultivars. This is the first time that variation in numbers and weight of sclerotia produced by *S. sclerotiorum* has been assessed on a carrot diversity set, and also the first time detached leaf assays have been assessed for their suitability for screening for resistance to *S. sclerotiorum* in carrot. There have been limited studies examining the numbers of sclerotia produced by different *S. sclerotiorum* isolates on a range of crops, and few have examined the susceptibility of different carrot accessions to *S. sclerotiorum*.

Results from the carrot root inoculations showed a difference in the production of sclerotia between the two *S. sclerotiorum* isolates L6 and L44, with L44 producing approximately half the number produced by L6. This confirms the research of Young *et al.* (2013), where it was reported that L6 produced higher numbers of sclerotia than L44 across artificially inoculated oilseed rape, potato, bean, pea and carrot plants. Similarly, Akram *et al.* (2008) found significant differences in the numbers of sclerotia produced by 16 isolates of *S. sclerotiorum* on chickpea. However, whilst Leiner and Winton (2006) reported consistent and significant differences in the numbers of sclerotia produced by two *S. sclerotiorum* isolates on cabbage and carrot tissue, they did not find significant differences between the same isolates on lettuce and celery.

Significant differences in the number of sclerotia produced by two different *S. sclerotiorum* isolates on cultivated and wild carrot roots have been seen previously,

with the two different wild roots producing between 0.7 and 1.6 sclerotia g^{-1} carrot root tissue, and the cultivar 'Bolero' producing 0.3 sclerotia g^{-1} carrot root tissue (Jensen *et al.*, 2008). Greater numbers of sclerotia were also produced on wild carrot types in the present study, with numbers ranging from 0.2 to 1.4 sclerotia g^{-1} carrot root tissue, and the elite types, cultivated types and mapping parents producing 0.03 to 1.1 sclerotia g^{-1} carrot root tissue. However, whilst *S. sclerotiorum* isolate and carrot accession had a significant influence on the number of sclerotia produced, there was no significant interaction between the two, indicating that the number of sclerotia produced is dependent on an individual *S. sclerotiorum* isolate. It has been suggested that the number of sclerotia produced by different *S. sclerotiorum* isolates can be used as a measure of aggressiveness, as it can be correlated with an isolate's ability to form lesions on host plants (Durman *et al.*, 2003; Kull *et al.*, 2004; Leiner & Winton, 2006; Jensen *et al.*, 2008). However, a study on 205 isolates from sunflower found no correlation between aggressiveness of isolates and sclerotial production (Li *et al.*, 2008).

Leiner and Winton (2006) showed that carrot tissue contained more non-structural carbohydrates than celery, and the sclerotia produced on carrot tissue were four to five times bigger. Carbohydrate content could, therefore, be a useful indicator of production of *S. sclerotiorum* sclerotia on different cultivars of crop plants. In the present study, the weight of individual sclerotia produced by different *S. sclerotiorum* isolates was found to be influenced by carrot accession, but not by *S. sclerotiorum* isolate. Isolate L44 produced approximately half the (mean) number of sclerotia than was produced by isolate L6 (0.3 sclerotia g^{-1} carrot root tissue compared to 0.6 sclerotia g^{-1} carrot root tissue). However, the mean weight of the sclerotia was comparable between isolates, (0.03 g for L44 and 0.02 g for L6).

Similarly, Jensen *et al.* (2008) found one *S. sclerotiorum* isolate produced half the number of sclerotia (0.3 sclerotia g^{-1} carrot root tissue) of that produced by another isolate (0.6 sclerotia g^{-1} carrot root tissue), but the mean weight of sclerotia was 0.19 g compared to 0.24 g respectively. This therefore suggests that a *S. sclerotiorum* isolate which produces fewer sclerotia is not necessarily producing bigger sclerotia, and may be at a competitive disadvantage with isolates that produce sclerotia in greater numbers.

The weight of sclerotia can influence the carpogenic germination and survival of sclerotia, with smaller sclerotia (1 to 3 mg) being recovered in the field more frequently than larger sclerotia (14 to 40 mg) six years after an initial outbreak of Sclerotinia disease on lettuce. In this study by Ben-Yephet *et al.* (1993), recovery of the bigger sclerotia decreased from 17.6 % in year one to 4.8 % in year six, whereas recovery of the smaller sclerotia increased from 71.4 % in the first year to 77.7 % in year six. They also observed that the bigger sclerotia were more likely to germinate carpogenically, with 31 % germination compared to 11 % in the smaller sclerotia. The larger sclerotia were also found to produce over four times more apothecia per sclerotium.

There was significant variation in the number and weight of sclerotia produced on the 87 carrot accessions used in the root inoculation experiments. This may be due to variation in carbohydrate content as suggested by Leiner and Winton (2006), or it may be due to variations in the permeability and integrity of root cell membranes as found by Finlayson *et al.* (1989a), indicating the presence of quantitative resistance of root tissues to *S. sclerotiorum* (Kora *et al.*, 2003). Further work could utilise the results from this large scale screening to select carrots which sustain low numbers of

sclerotia, whilst considering other traits such as yield, storage and taste, a trait which may also depend on the carbohydrate content of the carrot.

The whole carrot plant inoculation experiments showed significant variation in the rate of lesion development down the petioles of different carrot accessions, with those having the slowest and fastest rates being significantly different from all other accessions. The difference between the fastest rate on accession 7159 of 6.9 mm per day and the slowest rate on Little finger of 3.9 mm per day was substantial, indicating that breeding for quantitative resistance would be possible. Foster *et al.* (2008) demonstrated the formation of structural barriers at the petiole base by inoculating petioles which had been cut to 3 cm above the crown of the carrot 'Enterprise', known to be a susceptible cultivar. They found that the mycelium within the inoculated petioles progressed to the crown at a rate of 7.4 mm ± 1.2 mm per day, which is comparable with the fastest mean rate in the present study.

Both the accession with the fastest lesion development rate and the accession with the slowest rate also had the lowest mean crown infection scores, suggesting that these two measures of disease are not related. These differences in tissue type susceptibility have also been seen in *Brassica napus* between stem and cotyledon tissues (Garg *et al.*, 2008), and would need to be considered in breeding programmes. The differences in the susceptibility of the carrot petioles and the crown did not seem to be due to leaf abscission as was observed by Foster *et al.* (2008), where inoculated petioles detached from the crown during watering. It may be that the defence response was, therefore, a structural barrier, also observed by Foster *et al.* (2008). This would be comprised of lignin, diphenols, suberin flavanols, phenolases and peroxidases, known to be produced by carrots in response to

wounding or pathogen attack (Craft & Audia, 1962). Heale and Sharman (1977) found an increase in peroxidase activity and lignin synthesis in carrot tissue when inoculated with *Botrytis cinerea*, and lignin was also observed by Rittinger *et al.* (1987) to be present within carrot root cell walls 11 days after wounding. Additionally, over-expressing peroxidise from rice in transgenic carrots has been found to result in higher levels of accumulated lignin, which were further increased following inoculation with *S. sclerotiorum* (Wally & Punja, 2010).

The detached leaf inoculations produced clearly measurable lesions, with results consistent across the replicates for each experiment and between experiments. Significant differences were found between the carrot accessions for the rate of *S. sclerotiorum* lesion development, with the slowest rate in accession QAL at 7.2 mm per day being significantly different from all the other accessions. The difference between the slowest and the fastest rate of 12.7 mm per day on accession Brasilia is substantial, and again indicates a possible defence response. Some of the leaves in the experiments became pinched at the leading edge of the lesion, which may indicate a structural barrier defence response as previously discussed.

The low correlation between the results in the whole carrot plant experiments and the detached leaf experiments raises the question of whether a detached leaf protocol is suitable for screening carrot accessions for quantitative resistance, if the results are not comparable to those observed on whole plants. Detached leaf assays using mycelial plugs have been found to be very effective and reliable in screening soybean cultivars for *S. sclerotiorum* resistance (Wegulo *et al.*, 1998), and a high positive correlation was found between field inoculations on stems of *Brassica oleracea* and detached leaf assays (Mei *et al.*, 2011). However, this was not the case

for *Brassica napus* where significant differences seen in field evaluation studies between cultivars were not found in the detached leaf assays (Bradley *et al.*, 2006). Moreover, it is possible that using agar plugs to inoculate plants with *S. sclerotiorum* provides an energy source which allows the pathogen to ramify too rapidly for a host plant to engage defence response, therefore making it difficult to distinguish resistant genotypes (Garg *et al.*, 2008). However, in the present study significant differences were found using this method, and previous work has shown that using homogenised mycelium sprayed or pipetted onto soybean in detached leaf assays, the results were significantly correlated with those obtained by inoculating whole plants using mycelial plugs placed onto a cut petiole (Chen & Wang, 2005).

Since *S. sclerotiorum* ascospores are suggested as the primary inoculum source for the initiation of pre-harvest epidemics in carrot (Foster *et al.*, 2011) a detached leaf assay using ascospores as the inoculum would potentially provide a more accurate assessment of resistance responses in carrot accessions. However, Baranski *et al.* (2006) found higher discrimination between carrot cultivars on whole carrot plant inoculations than in detached leaf inoculations using conidial suspensions of *Alternaria dauci*. Similarly, Pawelec *et al.* (2006) found *Botrytis cinerea* disease symptoms were unreliably produced on detached carrot leaves when inoculation was carried out using a suspension of conidia, whereas inoculation with agar plugs allowed discrimination between carrot genotypes differing in their susceptibility to the pathogen.

The leaves used in the present study in the detached leaf experiments were from eight week old plants grown in module trays, whereas in the whole plant experiments they were 18 weeks old and grown in deep three litre pots. Age-related or ontogenic

resistance is thought to pay an important role in the difference in the susceptibility of leaves of carrots to *S. sclerotiorum* (Geary, 1978). The leaves which were inoculated on the whole plants were chosen to be fresh, non-senescing and as upright as possible, but the age difference and the conditions under which they were grown may have had an impact on the results of the inoculations.

Ontogenic resistance is expressed in young leaves, where they react with hypersensitivity to *S. sclerotiorum* (Geary, 1978). It is thought the reaction is due to the rapid induction of antifungal compounds, and the ontogenic resistance decreases with age causing older leaves to be more susceptible to infection (Kora *et al.*, 2003). This is also observed in storage of carrot roots, where resistance to pathogens decreases as the roots age, largely due to the corresponding reduction in their ability to gather antifungal compounds to the site of infection (Dennis, 1983). Any future work on detached carrot leaf inoculations with *S. sclerotiorum* therefore needs to ensure the age of the leaves is appropriate for comparison to whole plant inoculations.

Overall, breeding for a carrot which is less susceptible to *S. sclerotiorum* could be carried out by choosing an accession which produces fewer sclerotia, has a low rate of lesion development on petioles, and a low crown infection rate. The carrot diversity set maintained by the Genetic Resources Unit provides a resource which could be screened for *S. sclerotiorum* lesion development in its entirety, given that only a small number of accessions were assessed in the current project. Using the whole diversity set would also provide an opportunity to examine in greater detail the suitability of using detached leaf assays to screen carrots for resistance to *S. sclerotiorum*. Additionally, further work on architecture based resistance (i.e. disease

escape) using ascospores would provide data based on a realistic model of *S. sclerotiorum* infection in the field, but it may be difficult to ensure reproducible results, as well as time consuming to produce a large quantity of ascospores for any work to be carried out in a controlled environment. Any future breeding programme would also need to take in account the numerous traits required for both producers (e.g. yield, ease of processing) and consumers (e.g. flavour, colour and storage).

4. Biofumigation as a Control Measure against *Sclerotinia* Disease

4.1 Introduction

As discussed in Chapter 1 the term 'biofumigation' was coined to describe the process of using *Brassica* crops as green manures which are macerated and incorporated into the ground to suppress soil borne pests and diseases (Kirkegaard *et al.*, 1993). Many *Brassica* spp. produce significant levels of glucosinolates (GSL), which are held in plant cells separately from the enzyme myrosinase and are not fungitoxic (Manici *et al.*, 1997). When a plant cell is ruptured as a result of pest or pathogen attack, or mechanical wounding, the GSLs and myrosinase come into contact and are hydrolysed in the presence of water to release various products, including isothiocyanates (ITCs) (Vig *et al.*, 2009) (Figure 4.1) which have a wide range of biocidal characteristics (Kurt *et al.*, 2011) and are acutely toxic to several pathogenic fungi (Chew, 1987). The incorporation of biofumigant crops can also have indirect effects on the pest or pathogen due to changes in populations of antagonistic organisms, as well as suppressive effects due to compounds released from the tissues that are not related to GSLs or ITCs (Matthiessen & Kirkegaard, 2006).

4.1.1 Glucosinolates and Isothiocyanates

There are 16 families of dicotyledonous angiosperms which have been identified as containing GSLs (Fahey *et al.*, 2001), which are sulphur-containing secondary plant metabolites (Mithen, 2001). These are classed as β-thioglucoside N-hydroxysulfates, with a side chain (R) and a sulphur-linked β-d-glucopyranose moiety (Figure 4.1)

(Fahey *et al*., 2001) and are divided into aliphatic, aromatic and indole GSLs according to the type of side chain (Fenwick *et al*., 1983).

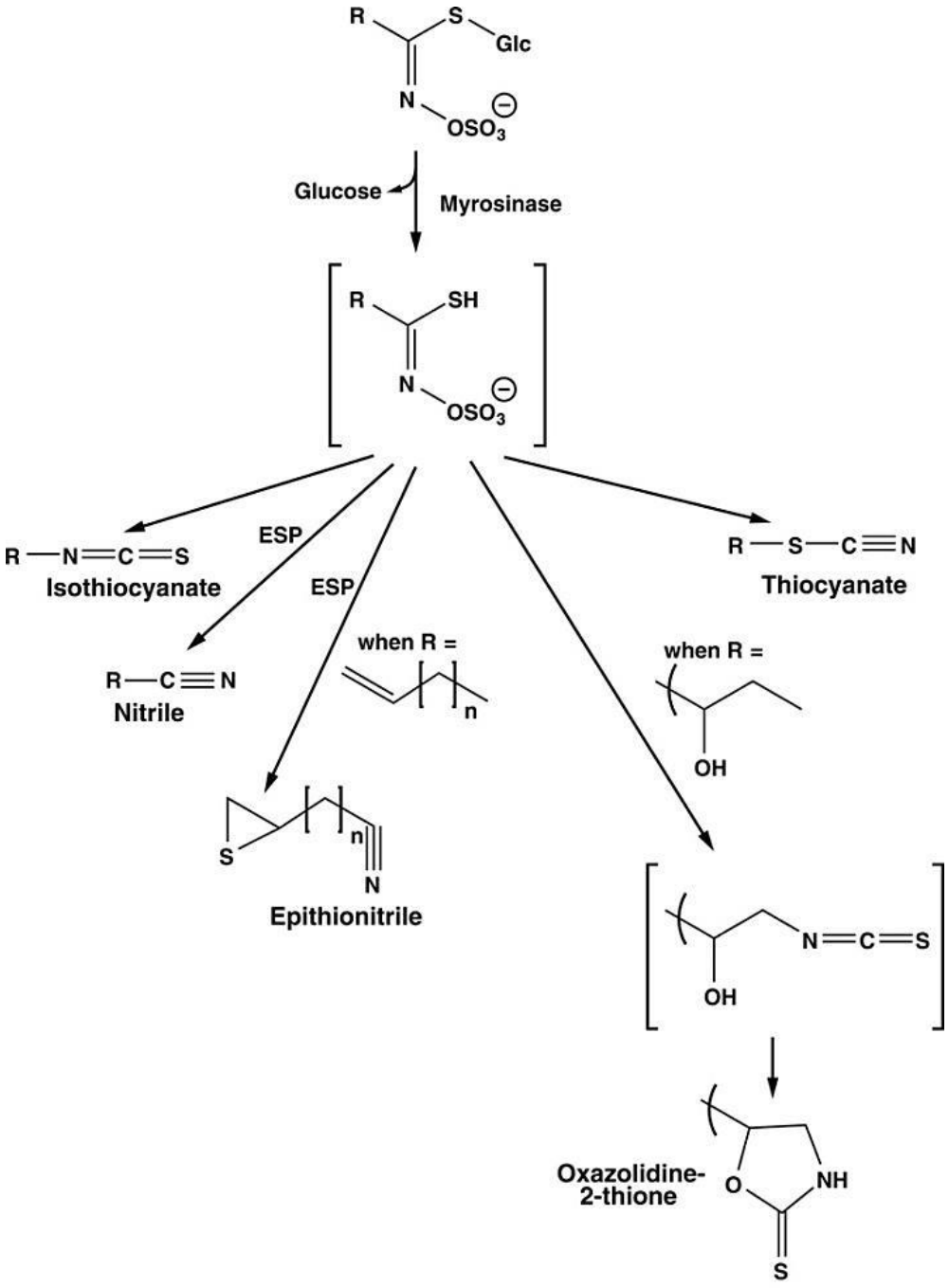

Figure 4.1 - The chemical structure of glucosinolates with an outline of glucosinolate hydrolysis and resulting products. The brackets indicate unstable intermediates. ESP: epithiospecifier protein; R: variable side chain (Halkier & Gershenzon, 2006).

GSL content can vary in the same crop grown in different years (Bangarwa *et al.*, 2011) and between tissues within a single plant, such as between leaves and seeds (Gupta *et al.*, 2012), or between leaves and flower buds. In kale (*B. oleracea acephala* group) the total GSL content in the leaves was found to increase with age up to early flowering, when the aliphatic GSLs decreased in the leaves as it increased in the flower buds (Velasco *et al.*, 2007). Environmental factors such as temperature and day length has been shown to affect GSL content in broccoli (*B. oleracea* L. var. italic), with levels of total GSL concentrations increasing with higher temperatures (Steindal *et al.*, 2013). In brown mustards (*B. juncea* cultivars) the concentrations of allyl ITC produced has been found to vary between cultivars and development stage, with some cultivars producing the greatest concentrations at the appearance of the first flower, and others producing the greatest quantity once the seeds have matured (Taylor *et al.*, 2014).

The definitive mode of action of ITCs inhibiting fungal growth and other microorganisms is not known, but some hypotheses are: inactivation of intracellular enzymes by oxidative breakdown of -S-S bridges (Zsolnai, 1966); inhibition of the coupling between the phosphorylation reactions and electron transport, therefore hindering the ATP synthesis, suggested from the inhibition of oxygen uptake of yeasts by ITCs (Kojima & Oawa, 1971); inhibition of metabolic enzymes by the thiocyanate radical, indicated as a degradation product of ITCs (Banks *et al.*, 1986); inhibition of Tumour Necrosis Factor proteins, involved in extrinsic apoptosis, or induction of intrinsic apoptosis, depending on the type of ITC (Molina-Vargas, 2013).

4.1.2 Using Biofumigation against Sclerotinia Disease

Due to the volatility of ITCs *Brassica* crops can be used to suppress soil borne pests and diseases (Kirkegaard *et al.*, 1993) and have been found to be effective against some nematodes, *Verticillium dahlia*, *Rhizoctonia solani* and *Pyrenochaeta lycopersici* (Matthiessen & Kirkegaard, 2006). Some natural ITCs were shown to be superior to the synthetic ITC fumigant metam sodium (methyl isothiocyanate) in their ability to suppress fungi (Sarwar *et al.*, 1998).

There are few studies investigating the use of biofumigant *Brassica* green manure crops for control of Sclerotinia disease, with inconsistent results in field and polytunnel experiments. Porter *et al.* (2002) found the biofumigant crops BQ Mulch (a blend of *Brassica napus* cultivars) and *Brassica juncea* 'Fumus' significantly reduced the levels of disease on lettuce crops caused by *S. minor*, whereas Bensen *et al.* (2009) observed significant short-term reductions in *S. minor* on lettuce when using *Sinapis alba* 'Ida Gold' and *B. juncea* 'ISCI 61' mustards, but no effect in longer field experiments. Similarly, Geier (2009) found biofumigation using *B. juncea* 'Pacific Gold' in a polytunnel had no significant effect on the carpogenic germination of *S. sclerotiorum* sclerotia.

However, laboratory approaches have shown the potential of using biofumigation crops for control of *S. sclerotiorum*. For instance, Kurt *et al.* (2011) found synthetic (pure) ITCs significantly reduced mycelial growth of *S. sclerotiorum* and sclerotial viability *in vitro*, with six of the seven ITCs tested providing complete suppression of sclerotial germination. *B. juncea* was found to be the only cruciferous plant to affect sclerotial viability of *S. sclerotiorum* in one study, delaying myceliogenic germination by seven days, although after ten days approximately 80 % of the

sclerotia germinated (Smolinska & Horbowicz, 1999). However, whilst Rahimi *et al.* (2013) and Ojaghian *et al.* (2012) also found *B. juncea* varieties to be the most effective in inhibiting mycelial growth, other studies have found *B. oleracea* var. *caulorapa* and *B. nigra* to inhibit mycelial growth of *S. sclerotiorum* by 89.5 % (Fan *et al.*, 2008) and 100 % respectively (Rahmanpour *et al.*, 2013).

There is also evidence that the size of *S. sclerotiorum* sclerotia may affect the efficacy of biofumigation. Smolinska and Horbowicz (1999) found a greater reduction in mycelial germination of smaller sclerotia produced by *S. cepivorum* than the larger sclerotia produced by *S. sclerotiorum* when they were exposed to volatiles from *B. juncea* plant tissue. This was also the case for plant seeds, where germination inhibition of smaller seeds was greater than larger seeds when exposed to volatiles from *B. juncea* and *B. nigra* plant tissue (Oleszek, 1987).

It is clear that there is variation in the efficacy of different *Brassica* species on the growth and viability of *S. sclerotiorum*, which can in part be related to their GSL content and the potential for conversion to ITCs. However, many studies do not quantify the GSL content of the *Brassica* species used which means any effects seen cannot be definitively attributed to biofumigation, and also limits the ability to make comparisons between crops used in different studies (Matthiessen & Kirkegaard, 2006). Reversed-phase (RP) high performance liquid chromatography (HPLC) has generally been used for analysis of GSLs because it can detect them in both intact and desulfated forms (Tsao *et al.*, 2002). These methods have been optimised to enable identification and quantification of GSLs, which can be used as an indirect measure of the biofumigation potential of plants (Wathelet *et al.*, 2004).

The aims of the work described in this chapter were:

- To determine whether biofumigation using different *Brassica* spp. can reduce carpogenic germination of *S. sclerotiorum* sclerotia, and if there is variation in efficacy.

- To determine the effects of biofumigation crops on mycelial growth of *S. sclerotiorum*.

4.2. Methods and Materials

4.2.1 Production of Plant Material

All biofumigant crops (*B. juncea, B. napus, Eruca sativa, Raphanus sativus* and *Sinapis alba*) were grown in 7.5 L pots, (five seeds per pot, in Levington M2 compost (Scotts)) in a polytunnel or glasshouse with supplemental lighting, and whole plants harvested within two weeks of first flowering (approximately eight weeks after sowing). Temperatures were recorded by the on site weather station and by the glasshouse climate control computer. Harvested plants were immediately placed in an oven at 80°C for 24 hours, then milled to a fine powder (Brook Compton Series 2000 mill, England) and stored in sealed plastic bags at -20°C. Repeat sowings were carried out throughout the year (Table 4.1).

Table 4.1 - Details of sowing and harvest dates for all biofumigant crops used in experiments.

	Sown	Harvested
Glasshouse	20/12/2011	16/02/2012
	08/02/2012	04/04/2012
	06/03/2012	01/05/2012
	26/03/2012	29/05/2012
	12/04/2012	27/06/2012
Polytunnel	28/05/2012	25/07/2012
	26/07/2012	12/09/2012
	20/08/2012	05/11/2012

4.2.2 HPLC Analysis of Glucosinolates

Quantification of the main GSL in each biofumigant crop species was carried out using a simplified extraction and HPLC method adapted from Tsao *et al.* (2002). All extractions were carried out on a random bulked sample of the total dried plant material produced at each harvest date.

To extract the GSLs, RO water (100 ml) was brought to boiling point on a heating mantle in a round bottomed flask with some anti-bumping granules (VWR International Ltd, UK). Dried and milled plant material (1 g) was then added and the mixture kept at boiling point for 30 minutes with a reflux condenser. The mixture was allowed to cool before filtration using a 25 µm syringe filter. HPLC analysis was then undertaken using a HP Agilent 110 series system with a UV diode array detector. Separations were at approximately 24°C on a reverse-phased Zorbax SB-Aq 4.6 x 250 mm 5 µm column (Agilent Technologies, U.S.A), with a running pressure of approximately 43 bar. An eluent of 0.025 M $CH_3CO_2NH_4$ (ammonium acetate) pH 6.75 was used with acetonitrile and a pump rate of 1 ml/min and an injection volume of 20 µl. The gradient was increased from 99% ammonium acetate to 50% at six minutes, and then back to 99% at 21 minutes, for a total run time of 26 minutes.

The retention times of the GSLs detected varied from 3 to 8 minutes, with detection at 228 nm. To identify any breakdown of GSLs to ITCs detection was also run at 242 nm, as at this wavelength ITCs show as a larger peak than the same peak detected at 228 nm (Tsao *et al.*, 2002). 1000 ppm and 100 ppm standards of each pure glucosinolate (Phytolab GmbH & Co, Germany) were run in between every 3 samples, and a 1000 ppm standard of pure allyl ITC (Sigma Aldrich, UK) was run once to verify the peak size difference between wavelengths.

4.2.3 Production of Sclerotia

Sterile stock *S. sclerotiorum* sclerotia (produced as described in Chapter 2) were used as necessary to initiate new cultures when required. For use in the biofumigation experiments sclerotia of *S. sclerotiorum* isolates L6, L17 and L44 (isolated from lettuce, Petworth, Sussex, in 2005) were produced as described by Clarkson *et al.* (2003). Isolate L6 was used in all experiments where only one isolate was required. This isolate was selected as being highly pathogenic, quick growing and consistently able to produce apothecia. Large numbers of sclerotia were produced in 500 ml flasks on sterile wheat grain (50 g wheat, 70 ml water, autoclaved at 121°C for 15 minutes) by inoculating with three mycelial agar plugs from the leading edge of a 3 day old actively growing *S. sclerotiorum* culture on PDA, and incubating in the dark at 18°C for six weeks. The flasks were shaken once a week to ensure complete colonisation of the wheat grain and to prevent sclerotia clumping together. The sclerotia were harvested by floating off the wheat grain with a 2 mm sieve, and dried overnight in a laminar flow cabinet. They were stored at room temperature in paper bags until required for use in experiments.

4.2.4 Microcosm Experiments – Effect of Biofumigant Treatments on Carpogenic Germination of *S. sclerotiorum* Sclerotia

Microcosm experiments were set up to test the effect of six biofumigant plants and four other treatments (Table 4.2) on the carpogenic germination of *S. sclerotiorum* sclerotia (2-4 mm in size, isolate L6). These experiments were initially conducted using fresh plant tissue, but the breakdown of the plant tissue over the course of the experiments resulted in inconsistencies in the water content of the compost used.

These initial experiments with fresh plant tissue (macerated in a food processor) were carried out as described below, using the fresh weight equivalents of the half or full field rate. These quantities were calculated by using the highest predicted biomass given by the seed companies, and the suggested incorporation depth of the biomass, and extrapolating that down to equivalent quantities required for a 600 ml box with an incorporation depth of 6.5 cm. This information for fresh weight of plants to be used in the boxes was changed to a dry weight equivalent by weighing out three fresh samples of each crop, oven drying at 80°C for 24 hours and then reweighing the samples.

Positive control treatments of two products previously reported to have activity against *S. sclerotiorum* sclerotia (the fertiliser Perlka® and the biological control Contans WG, a formulation of the mycoparasite *Coniothyrium minitans*), and the biofumigant treatment Biofence (mustard meal pellets) were used to provide comparisons with biofumigation crops, and were used at the equivalent field rates suggested by the manufacturers. *Brassica napus* 'Temple', a commercially grown oilseed rape cultivar, was used as a low glucosinolate *Brassica* control (Table 4.2).

The method for the microcosm experiments was adapted from Young *et al*. (2004). *S. sclerotiorum* sclerotia were cold conditioned at 5°C for 40 days in 2 mm mesh bags in pasteurised compost (John Innes No 1, J. Arthur Bowers, passed through a 4 mm sieve, double bagged and pasteurised by autoclaving at 110°C for 30 minutes) with 30% w/w moisture content to ensure subsequent carpogenic germination. Moisture content was calculated by weighing three samples of the compost before and after oven drying for 24 hours at 80°C.

Table 4.2 - Summary of treatments used in microcosm experiments. Positive control treatments are highlighted.

Treatments	Main Glucosinolate of Crops	Full Field Rate (g per box)	Half Field Rate (g per box)
1. *Brassica juncea* 'Vittasso'	Sinigrin	6	3
2. *Brassica juncea* 'Pacific Gold'	Sinigrin	6	3
3. *Sinapis alba* 'Brisant'	Sinalbin	6	3
4. *Brassica juncea* 'Caliente 99'	Sinigrin	6	3
5. *Raphanus sativus* 'Terranova'	Glucoraphenin	6	3
6. *Eruca sativa* 'Nemat'	Glucoerucin	6	3
7. *Brassica napus* 'Temple'	Glucobrassicanapin / Sinigrin	6	3
8. Perlka® (Calcium cyanamide)	n/a	0.43 *	n/a
9. Biofence (mustard meal pellets)	n/a	1.4 *	n/a
10. Contans WG (*Coniothyrium minitans*)	n/a	0.4 *	n/a
11. Untreated	n/a	0	0

* Based on field rates of Perlka® (400 kg/ha), Biofence (3000 kg/ha) and Contans WG (8 kg/ha)

Each biofumigant/soil treatment was mixed with pasteurised compost (John Innes No 1, J. Arthur Bowers) and 350 g of the compost/treatment mixture was placed into a 600 ml clear plastic box (Malsar Kest Ltd, UK). Preconditioned *S. sclerotiorum* sclerotia (30) were laid out in a grid pattern (six by five) before adding another 50 g of the compost mixture to cover the sclerotia and an appropriate amount of water to give 30% (w/w) moisture content. Lids were then immediately placed onto the boxes which were weighed before being incubated in a controlled environment room at 15°C with white fluorescent lighting (14 h day). To reduce the loss of volatiles from the biofumigant treatments the four replicate boxes for one treatment were set up together in their entirety, before moving on to the next. To maintain constant moisture levels water was added to the compost every two weeks to bring the boxes back to their original weight. Germination of sclerotia was recorded twice a week as emergence of stipes or apothecia (Figure 4.2).

Figure 4.2 - Stipes and apothecia germinating from *S. sclerotiorum* sclerotia.

Four replicate boxes were set up for each treatment, arranged in a randomised block design with four rows and 11 columns. In the initial experiments with fresh plant material two repeat experiments were carried out, and for the full experiments with dried and milled plant material three repeat experiments were carried out for plant treatments at full rate and half rate respectively. The other treatments (Biofence, Perlka®, and Contans WG) included at full field rate equivalents each time.

4.2.5 Microcosm Experiments – Effect of Size of *Sclerotinia sclerotiorum* Sclerotia on the Efficacy of Biofumigant Treatments

Experiments were set up to test the effect of the biofumigant *B. juncea* 'Caliente 99' (used at full field rate equivalent) on germination of different sized sclerotia from three *S. sclerotiorum* isolates: L6, L17 and L44. The sclerotia were produced as before but passed through sieves to separate them into three different size grades: large = >5.6 mm, medium = 4-5.6 mm and small = 2-4 mm. Boxes were set up as before with three replicates of each size grade (Table 4.3) and three repeat experiments were carried out.

Table 4.3 - Treatments used in microcosm experiments to determine the effect of the biofumigant *B. juncea* 'Caliente 99' on carpogenic germination of three size grades of *S. sclerotiorum* sclerotia.

Treatment number	*S. sclerotiorum* isolate	Size of sclerotia (large = >5.6 mm, medium = 4-5.6 mm, small = 2-4 mm)	Treatment
1	L6	Small	*B. juncea* 'Caliente 99' 6 g
2	L6	Medium	*B. juncea* 'Caliente 99' 6 g
3	L6	Large	*B. juncea* 'Caliente 99' 6 g
4	L6	Small	Untreated
5	L6	Medium	Untreated
6	L6	Large	Untreated
7	L17	Small	*B. juncea* 'Caliente 99' 6 g
8	L17	Medium	*B. juncea* 'Caliente 99' 6 g
9	L17	Large	*B. juncea* 'Caliente 99' 6 g
10	L17	Small	Untreated
11	L17	Medium	Untreated
12	L17	Large	Untreated
13	L44	Small	*B. juncea* 'Caliente 99' 6 g
14	L44	Medium	*B. juncea* 'Caliente 99' 6 g
15	L44	Large	*B. juncea* 'Caliente 99' 6 g
16	L44	Small	Untreated
17	L44	Medium	Untreated
18	L44	Large	Untreated

4.2.6 *In vitro* Experiments – Effect of Biofumigants on Mycelial Growth of *Sclerotinia sclerotiorum*

In vitro experiments were carried out to test the direct effect of volatiles (potentially ITCs) released from the six biofumigant plants (Table 4.2) and oilseed rape on mycelial growth of *S. sclerotiorum* using a method adapted from Sexton *et al.* (1999). A 5 mm mycelial plug of actively growing mycelium from *S. sclerotiorum* isolate L6 was placed in the centre of a 9 cm Petri dish containing potato dextrose agar (PDA; Merck), the dish inverted, and dried plant material (1 or 2 g) placed in the lid. Water was then added at a rate of 10 ml per 1 g dried plant material and the Petri dish immediately sealed with Parafilm® M (Bemis Co. Inc., U.S.A.). An untreated control consisted of only water in the lid.

All Petri dishes were placed at 15°C in the dark and mycelial growth of *S. sclerotiorum* was assessed twice a day for four days by measuring the radial growth along the x and y axis (Figure 4.3). There were five replicate plates for each treatment arranged in a randomised block design and the experiment was repeated three times for each rate of plant material.

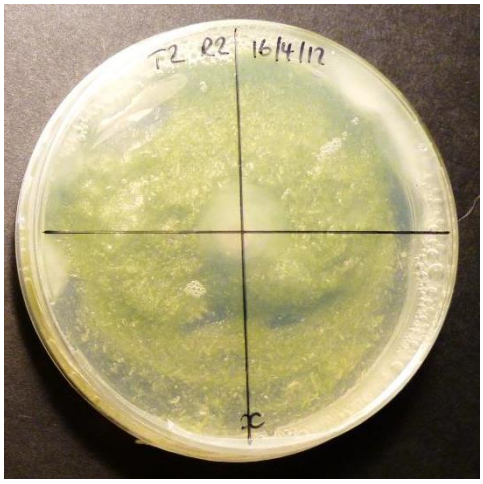

Figure 4.3 – Inverted Petri dish of PDA inoculated with *S. sclerotiorum*, with biofumigant plant material placed in the lid.

4.2.7 *In vitro* Experiments –Effect of Biofumigant Dose on Mycelial Growth of *Sclerotinia sclerotiorum*

Experiments were carried out to determine the dose response curve and effective doses to provide 50 % inhibition (ED50) for two biofumigant crops – *B. juncea* 'Caliente 99' and *S. alba* 'Brisant'. The method for these experiments was as described in 4.2.6 above, but using a range of dried plant material quantities (0.25 g, 0.5 g, 0.75 g, 1 g, 1.25 g, 1.5 g, 1.75 g and 2 g) and water at the rate of 10 ml per 1g dried plant material.

4.2.8 *In vitro* Experiments –Effect of Biofumigants on Carpogenic Germination of *Sclerotinia sclerotiorum* Sclerotia

Experiments were carried out to test the direct effect of volatiles (potentially ITCs) released from the six biofumigant plants and oilseed rape on carpogenic germination of *S. sclerotiorum* sclerotia. Pasteurised compost (50 g, 30% w/w moisture content) was placed in a 9 cm Petri dish and preconditioned *S. sclerotiorum* sclerotia from isolate L6 (20) laid out in a grid pattern and pressed flat into the compost. Each Petri dish was placed into a 1200 ml plastic box (Malsar Kest Ltd, UK), together with a separate round plastic dish (6 cm diameter, 3 cm depth) containing either 1 g or 2 g of the dried plant material (Figure 4.4). Water (10 ml per 1 g dried plant material) was added and the lids immediately placed on the plastic boxes. Boxes were weighed before being incubated in a controlled environment room at 15°C in the dark and the emergence of stipes or apothecia recorded once a week for 80 days.

Figure 4.4 – Petri dish with germinating *S. sclerotiorum* sclerotia with a separate dish containing biofumigant plant material

Every two weeks the compost in the Petri dishes was watered to bring them back to their original weight. Control boxes were set up with just water added in the separate dish. Four replicates for each treatment were arranged in a randomised block design and the experiment was repeated three times for each of the two rates of plant material. For two of the three experiments (for both the 1 g and 2 g plant material quantities) the dish containing the treatment was removed after 80 days and germination continued to be monitored once a week for a total of four weeks, to assess whether the sclerotia were killed by the biofumigant treatments, or whether germination was being suppressed.

4.2.9 Plant Material Batches Used in Biofumigation Experiments

Due to the variation in quantities of plant material harvested at each date for drying and milling it was not always possible to use the same batch of plant material for all repeat experiments. A record of which harvest batches were used in each experiment was maintained so results could be analysed with GSL levels if required (Table 4.4).

Table 4.4 – The harvest batches of biofumigant crops used in each repeat biofumigation experiment.

	Plant Material State as Used (fresh or dry)	Experiment Repeat Number	Rate (field rate equivalent or quantity in g)	Crop Harvest Batch Date
Microcosm experiments	Fresh	1	Full	16/02/2012
	Fresh	2	Half	04/04/2012
	Fresh	3	Full	01/05/2012
	Fresh	4	Half	29/05/2012
	Dry	5	Full	25/07/2012
	Dry	6	Half	25/07/2012
	Dry	7	Full	25/07/2012
	Dry	8	Half	25/07/2012
	Dry	9	Full	12/09/2012
	Dry	10	Half	12/09/2012
Microcosm experiments - sized sclerotia	Dry	1	Full	12/09/2012
	Dry	2	Full	05/11/2012
	Dry	3	Full	05/11/2012
Mycelial growth and rate	Dry	1	1g	25/07/2012
	Dry	2	2g	25/07/2012
	Dry	3	1g	25/07/2012
	Dry	4	2g	25/07/2012
	Dry	5	1g	25/07/2012
	Dry	6	2g	25/07/2012
Mycelial growth - Dose response 'Brisant'	Dry	1	n/a	25/07/2012
	Dry	2	n/a	25/07/2012
	Dry	3	n/a	25/07/2012
Mycelial growth - dose response 'Caliente 99'	Dry	1	n/a	12/09/2012
	Dry	2	n/a	12/09/2012
	Dry	3	n/a	12/09/2012
***In vitro* carpogenic germination**	Dry	1	1g	27/06/2012
	Dry	2	2g	25/07/2012
	Dry	3	1g	25/07/2012
	Dry	4	2g	25/07/2012
	Dry	5	1g	25/07/2012
	Dry	6	2g	12/09/2012

4.2.10 Statistical Analyses

All statistical analyses were carried out using Genstat® (13th edition, VSN International Ltd). The number of sclerotia germinating in the initial, full rate and half rate microcosm experiments (after 150 days) and in both carpogenic germination *in vitro* experiments (1g and 2g experiments, after 80 days) were analysed using a Generalised Linear Model (GLM) and logistic regression, with fitted terms of replicate + treatment and an estimated dispersion parameter. Interpretations from the GLM and logistic regression were made by comparing t probabilities calculated with reference to the untreated control for each set of experiments.

The number of sclerotia germinating in the microcosm experiments testing the effect of sclerotial size were analysed using Analysis of Variance (ANOVA) with a blocking structure of trial x replicates, and interpretations were carried out by comparing ANOVA treatment means using the approximate least significant difference values (LSD) at the 5% level.

For the mycelial growth experiments the growth measurements over time were fitted to logistic curves and a rate of growth calculated. The percentage mycelial growth inhibition was calculated at the time the untreated controls had grown to the edge of the Petri dish (between 66 and 73 hours after the start of the experiment), for the experiments for all crops and the dose response experiments by $I = ((C-T)/C) \times 100$, where I = % inhibition of mycelial growth, C = Untreated control mycelial growth, T = Treated plate mycelial growth.

ED50 values for the dose response experiments were calculated by using Probit analysis (Finney, 1971), with a fitted term of LOG10 dose +0.01. The logistic curve parameters and the percentage of mycelial growth inhibition were analysed using ANOVA with a blocking structure of replicates, and interpretations were carried out by comparing ANOVA treatment means using the approximate least significant difference values (LSD) at the 5% level.

4.3 Results

4.3.1 HPLC Analysis of Glucosinolates

Quantification of the main GSL in each biofumigant crop species was carried out as described in Section 4.2.2. The HPLC analysis clearly showed peaks on the chromatograms at retention times comparable with the GSL standards used (Figures 4.5 and 4.6).

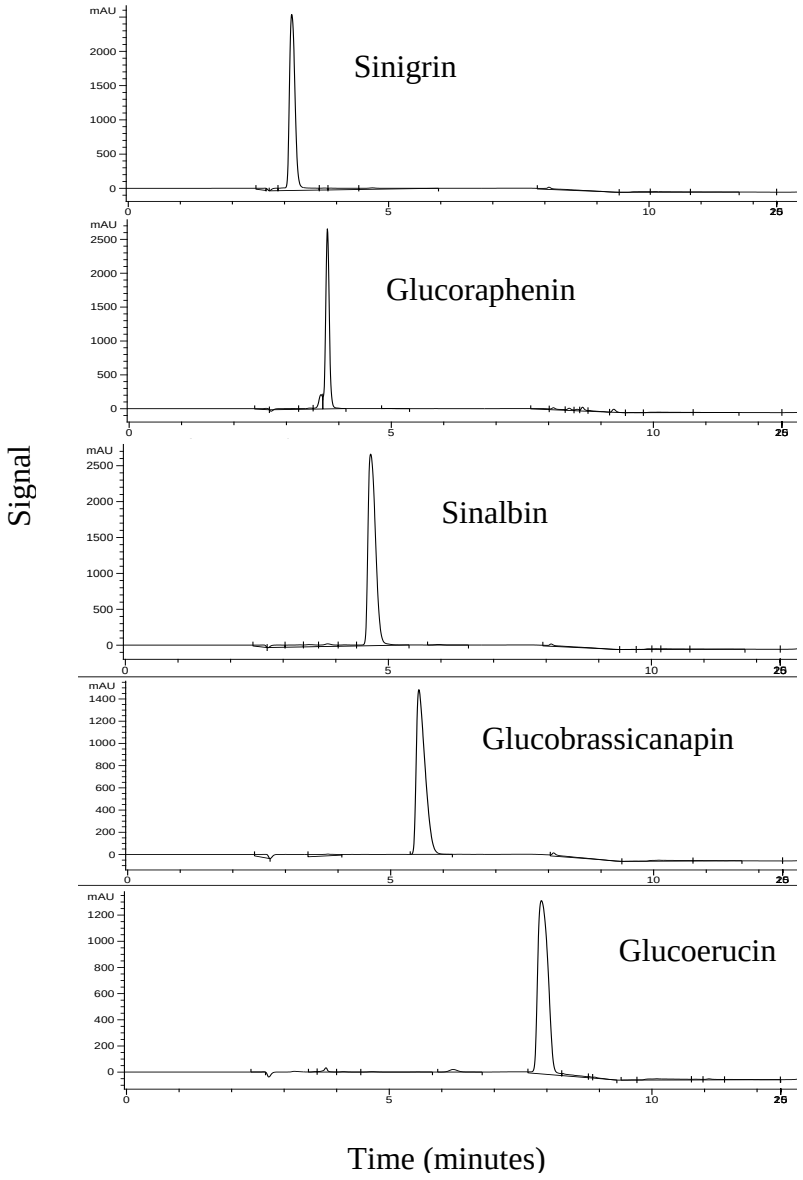

Figure 4.5 - Chromatograms showing the 1000 ppm glucosinolate standards (sinigrin, glucoraphenin, sinalbin, glucobrassicanapin and glucoerucin) detected at 228 nm.

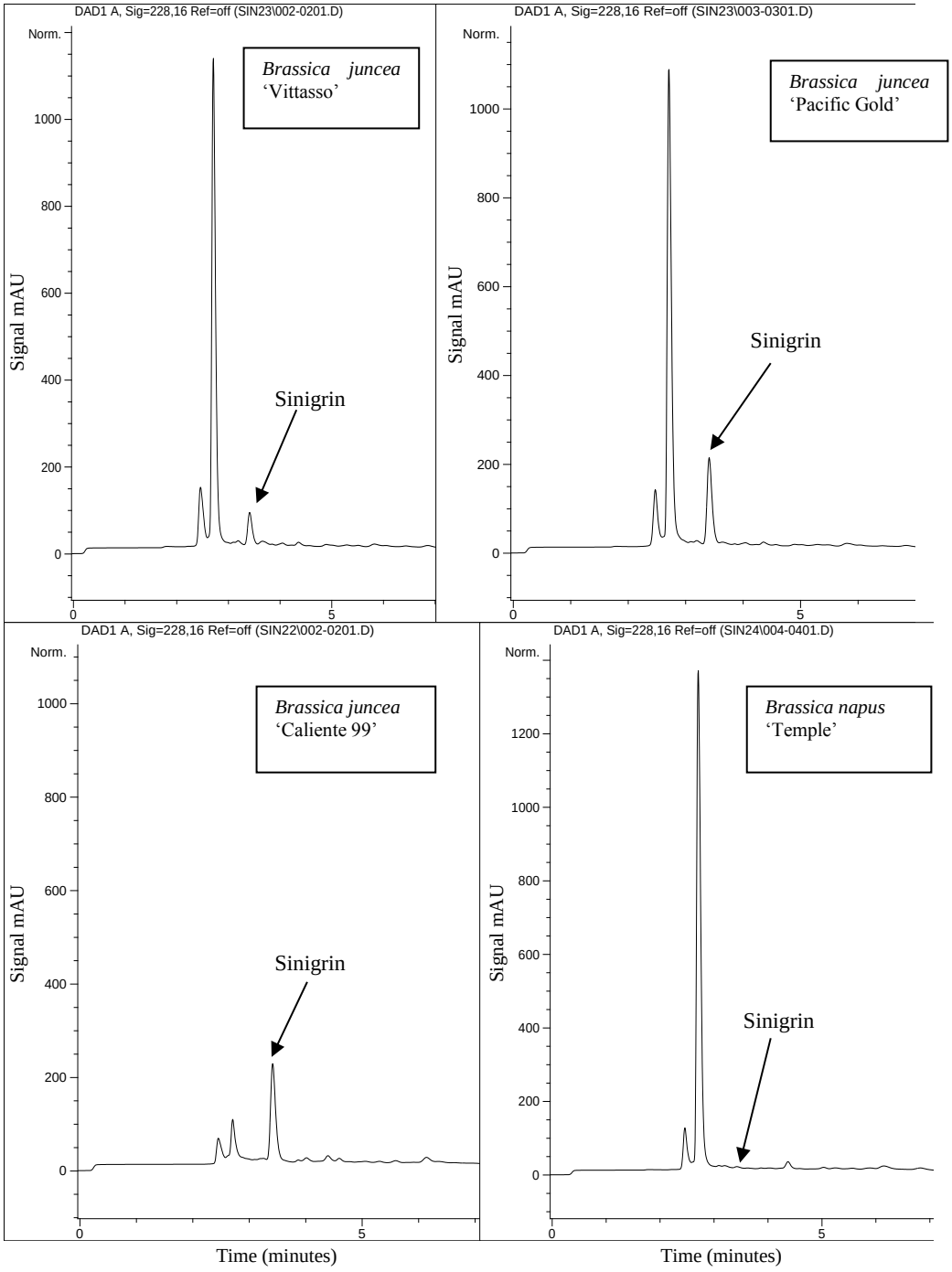

Figure 4.6 – Example chromatograms for four of the biofumigant crops harvested on 27th June 2012, showing peaks detected at 228 nm indicating the quantity of the glucosinolate sinigrin present in the sample.

As expected, all *B. juncea* samples contained detectable amounts of sinigrin, *S. alba* samples contained detectable levels of sinalbin, *R. sativus* contained detectable levels of glucoraphanin and *E. sativa* contained detectable levels of glucoerucin. There were no unexpected peaks detected in any samples which would indicate the presence of a large quantity of another GSL.

The highest level of the GSL sinigrin was found in *B. juncea* 'Pacific Gold' harvested on 4th April 2012 (33.29 µmol/g^{-1} dw, Figure 4.7). Of the *B. juncea* cultivars the lowest levels of sinigrin at each harvest date were found in *B. juncea* 'Vittasso', ranging from 2.16 µmol/g^{-1} dw for the crop harvested 5th November 2011 to 19.65 µmol/g^{-1} dw for the crop harvested 4th April 2012. The highest level of sinalbin of 31.08 µmol/g^{-1} dw in *S. alba* 'Brisant' was found in the crop harvested 1st May 2012, and the highest level of glucoerucin of 25.66 µmol/g^{-1} dw in *E. sativa* 'Nemat' was found in the crop harvested 25th July 2012. The highest level of glucoraphenin in *R. sativus* 'Terranova' of 17.73 µmol/g^{-1} dw was found in the crop harvested 29th May 2012.

Very low levels of sinigrin were found in *B. napus* 'Temple' for only two of the eight harvest dates (27th June 2012 and 5th November 2012) and no other GSL was detected by HPLC analysis. For all crops except *B. napus* 'Temple' and *E. sativa* 'Nemat' the lowest levels of GSLs were seen in the crop harvested 5th November 2012. No conversion to ITCs resulting from either the extraction method, or occurring from elution during the HPLC analysis were seen for any of the samples using detection at 242 nm.

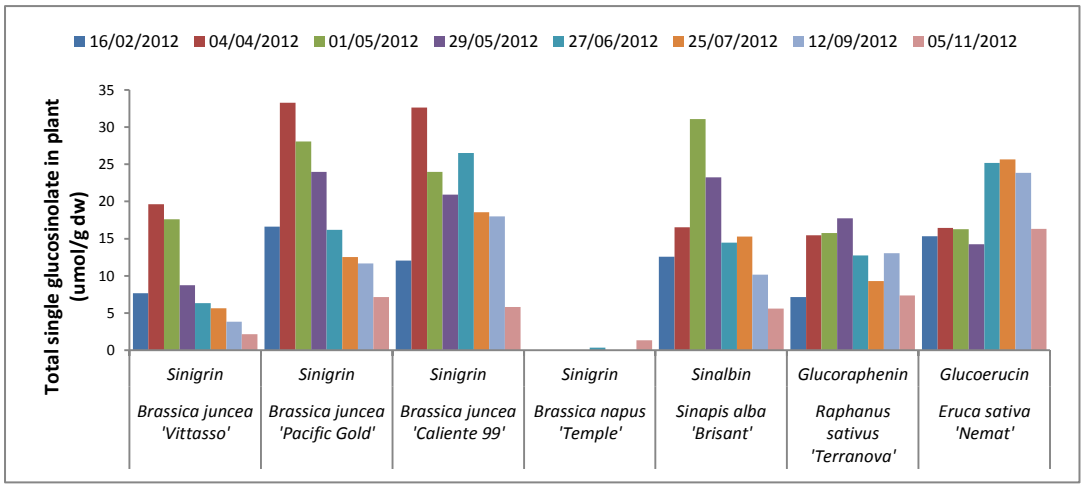

Figure 4.7- Single glucosinolate levels (sinigrin, sinalbin, glucoraphenin and glucoerucin) as determined by HPLC analysis of six different biofumigant plants and *B. napus* 'Temple' grown in a glasshouse (harvest dates 16/2/12, 4/4/12, 1/5/12, 29/5/12 and 27/6/12) and in a polytunnel (harvest dates 25/7/12, 12/9/12 and 5/11/12).

For all the biofumigant crops except *B. napus* 'Temple' and *E. sativa* 'Nemat' the mean, maximum and minimum quantities of single glucosinolate was higher in the crops grown between December and June (in the glasshouse) than those grown between May and November (in the polytunnel; Figure 4.8).

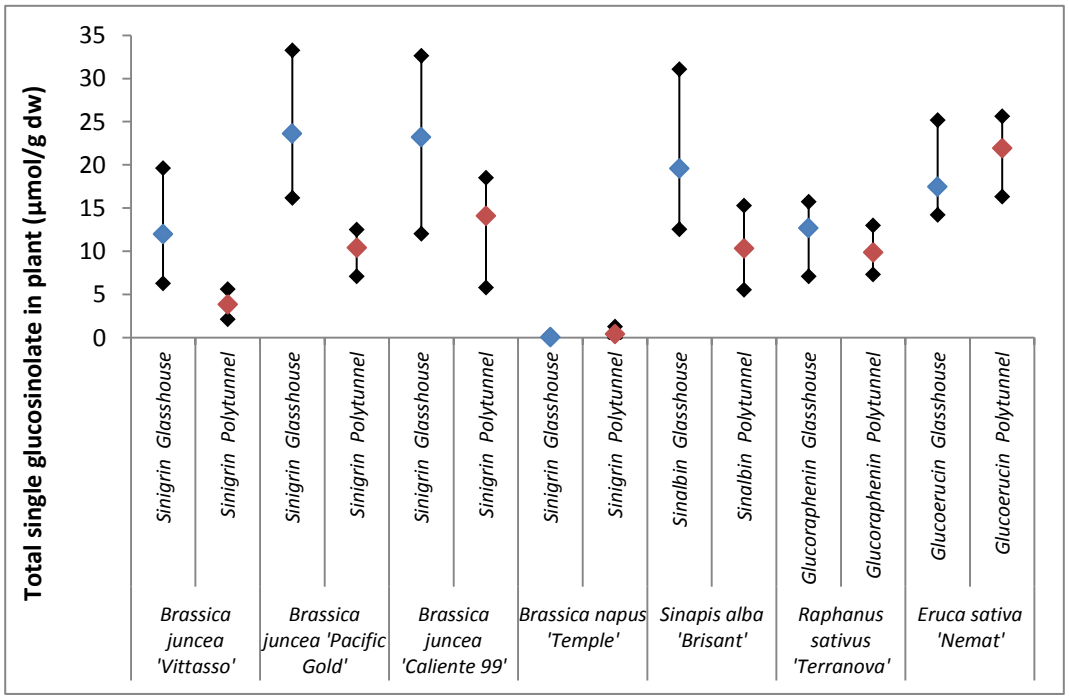

Figure 4.8 – Mean, maximum and minimum total single glucosinolate quantities as determined by HPLC analysis of six different biofumigant crops and *B. napus* 'Temple', grown in a glasshouse (blue) and in a polytunnel (red).

The highest temperature during the growth of the biofumigant crops was observed in the glasshouse, reaching a maximum of 33°C at the end of March 2012, with a minimum temperature of 15.6°C over the same period (Figure 4.9). For the polytunnel, the maximum temperature was 28.1°C at the end of July 2012, with the minimum being -0.1°C in the middle of October (Figure 4.9), based on weather station data.

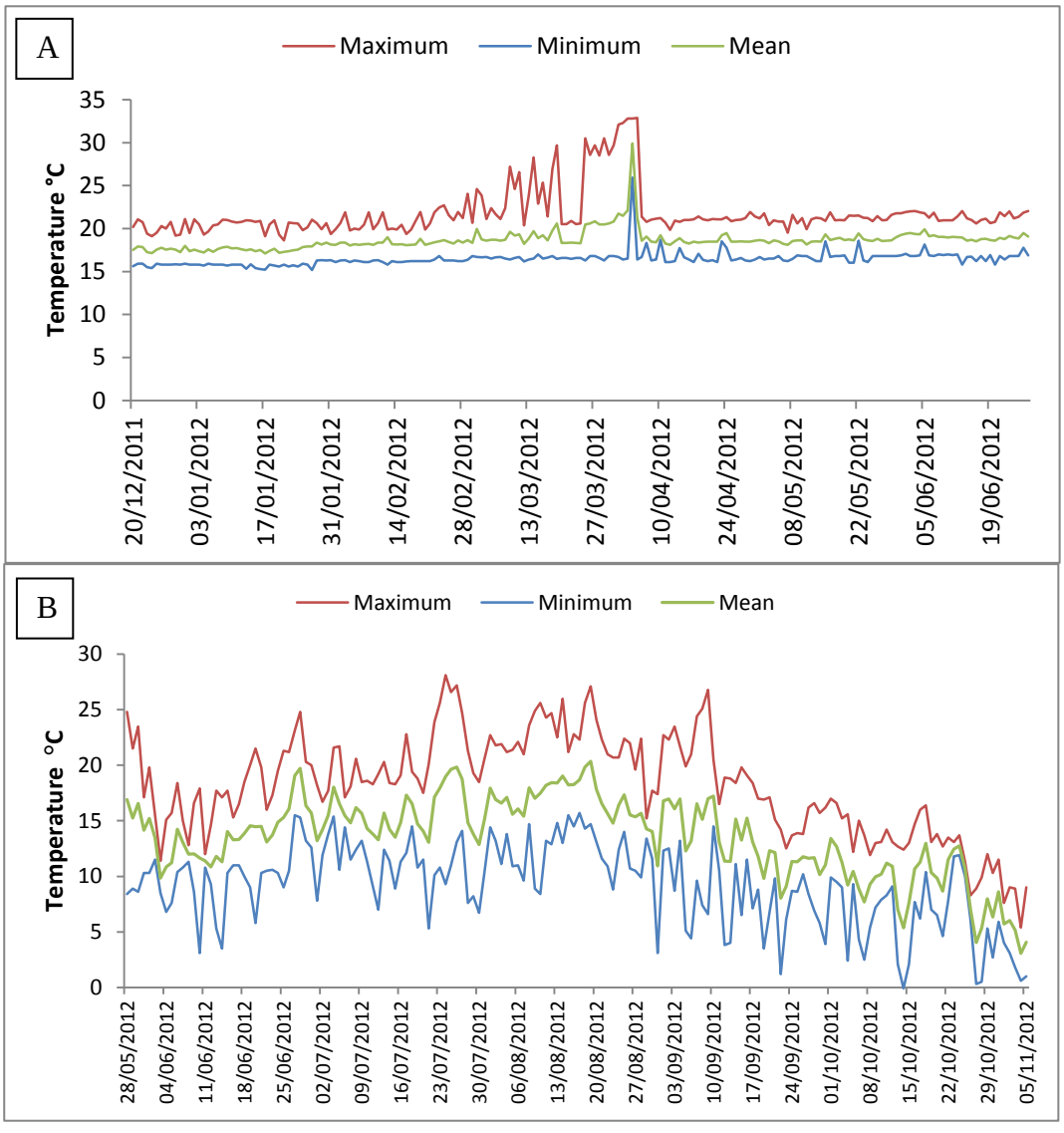

Figure 4.9 – Daily maximum (red line), minimum (blue line) and mean (green line) temperatures in glasshouse compartment (A, period 20/12/2011 to 27/6/2012) and onsite weather station (B, period 28/5/2012 to 5/11/2012) whilst biofumigant crops were grown.

4.3.2 Microcosm Experiments – Effect of Biofumigant Treatments on Carpogenic Germination of *S. sclerotiorum* Sclerotia

4.3.2.1 Initial Experiments Using Fresh Plant Material

Microcosm experiments were set up to test the effect of six biofumigant plants and four other treatments on the carpogenic germination of *S. sclerotiorum* sclerotia. All the biofumigant crops and *Brassica napus* 'Temple' significantly reduced germination of *S. sclerotiorum* sclerotia in comparison with the untreated control after 150 days when used at full field rate. In the half field rate experiments *B. juncea* 'Vittasso', *R. sativus* 'Terranova' and *E. sativa* 'Nemat' significantly reduced germination (Figure 4.10).

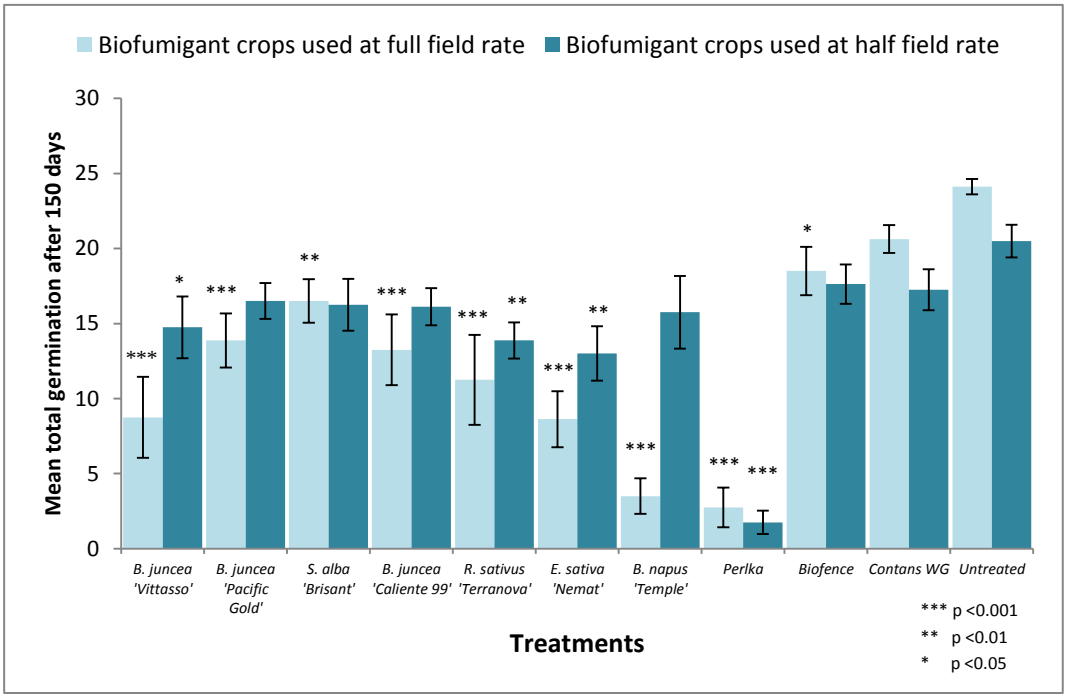

Figure 4.10 - The effect of biofumigant crops, low glucosinolate *B. napus* 'Temple', Perlka®, Biofence and Contans WG in initial experiments using fresh plant material, on the carpogenic germination of *S. sclerotiorum* sclerotia after 150 days in microcosm experiments. Error bars indicate SEM. NB – Positive control treatments Perlka®, Biofence and Contans WG were used at full field rate equivalents in all experiments.

Of the biofumigant treatments, *E. sativa* 'Nemat' provided the greatest reduction in germination with a mean total germination of 8.6 sclerotia compared to 24.1 in the untreated control (full rate experiments). The low glucosinolate *B. napus* 'Temple' reduced mean germination to 3.5 sclerotia in the full rate experiments, and to 15.8 in the half rate experiments. Of the positive control treatments, Perlka® reduced mean germination to 1.8 sclerotia in the half rate experiments; Biofence significantly reduced mean germination in comparison with the untreated control in the full rate experiments and Contans WG did not significantly reduce germination. Five of the biofumigation treatments (*B. juncea* 'Vittasso', *B. juncea* 'Pacific Gold', *B. juncea* 'Caliente 99', *R. sativus* 'Terranova' and *E. sativa* 'Nemat') resulted in greater reductions in germination when used at the full field rate in comparison to being used at half field rate.

Variation in the water content of the boxes was observed during the course of these microcosm experiments with fresh plant material, apparently caused by the degradation of the plant material over the course of the experiments.

4.3.2.2 Experiments Using Dried Plant Material

All the biofumigant crops and *B. napus* 'Temple' significantly reduced germination of *S. sclerotiorum* sclerotia in comparison with the untreated control after 150 days for both the half rate and full rate experiments (Figure 4.11).

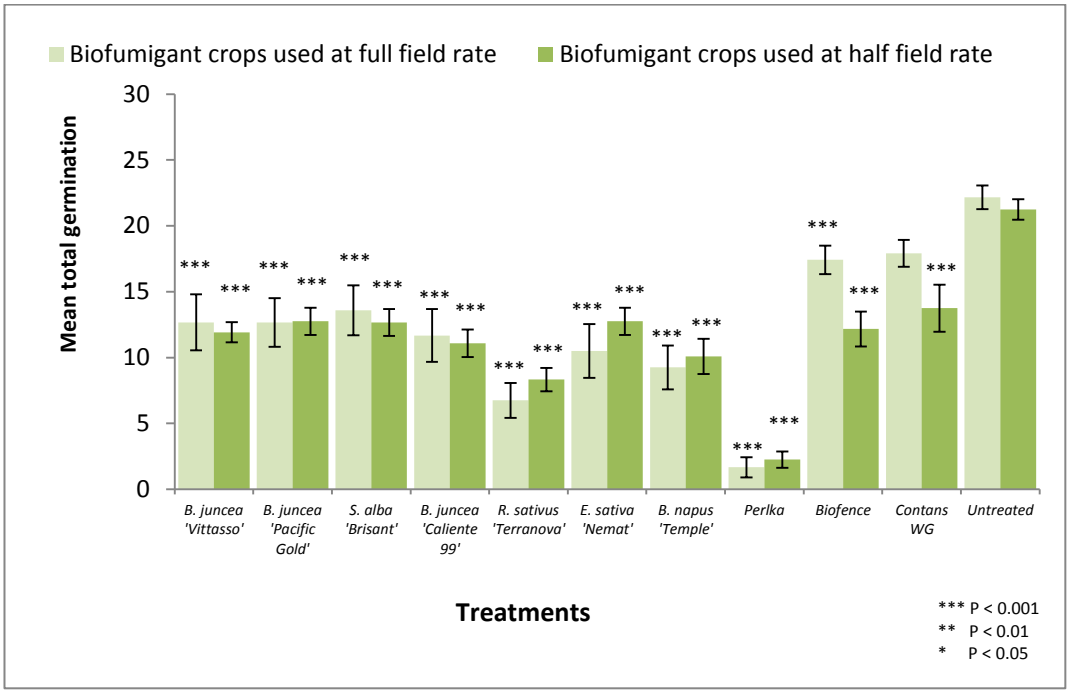

Figure 4.11 - The effect of biofumigant crops, low glucosinolate *B. napus* 'Temple', Perlka®, Biofence and Contans WG on the carpogenic germination of *S. sclerotiorum* sclerotia after 150 days in microcosm experiments. Error bars indicate SEM. NB – Positive control treatments Perlka®, Biofence and Contans WG were used at full field rates in all experiments.

Of the biofumigant crops, *R. sativus* 'Terranova' provided the greatest reduction in germination with a mean total germination of 6.8 sclerotia compared to 22.1 in the untreated control (full rate experiments). The low glucosinolate *B. napus* 'Temple' reduced mean germination to 9.3 sclerotia. Of the positive controls (used at full field rates in both sets of experiments) the greatest reduction in germination was observed with Perlka®. There were only small differences between the germination in the half rate experiments and the full rate experiments.

4.3.3 Microcosm Experiments – Effect of Size of *Sclerotinia sclerotiorum* Sclerotia on the Efficacy of Biofumigant Treatments

Experiments were set up to test the effect of the biofumigant *B. juncea* 'Caliente 99' on germination of different sized sclerotia from three *S. sclerotiorum* isolates: L6, L17 and L44. The mean number of apothecia produced per sclerotium by the different sizes of sclerotia ranged from 0.9 (L17 small sclerotia) to 3.1 (L6 large sclerotia) with the large sclerotia producing the most apothecia overall across all isolates (Table 4.5). For *S. sclerotiorum* isolate L6 there was a significant reduction in germination for sclerotia treated with *B. juncea* 'Caliente 99' compared to the untreated sclerotia for all three sizes ($p<0.05$) (Figure 4.12). The efficacy of biofumigation was greatest in the medium sized sclerotia, reducing germination by 60.8%, and lowest in the large sclerotia where germination was reduced by 37.1% (Table 4.5). For *S. sclerotiorum* isolate L17, there were again significant differences between the treated and untreated sclerotia for all three sizes ($p<0.05$) (Figure 4.12) and the efficacy of biofumigation was greatest for the small sclerotia (92.4% reduction in germination) and lowest for the large sclerotia (75.3% reduction in germination, Table 4.5). For isolate L44 there was no significant difference between the treated and untreated sclerotia for all three sizes, but there was very low germination in the untreated controls (Figure 4.12). Across all *S. sclerotiorum* isolates the efficacy of biofumigation was 72-75% for small and medium sclerotia, compared to 57.5% for the large sclerotia (Table 4.5).

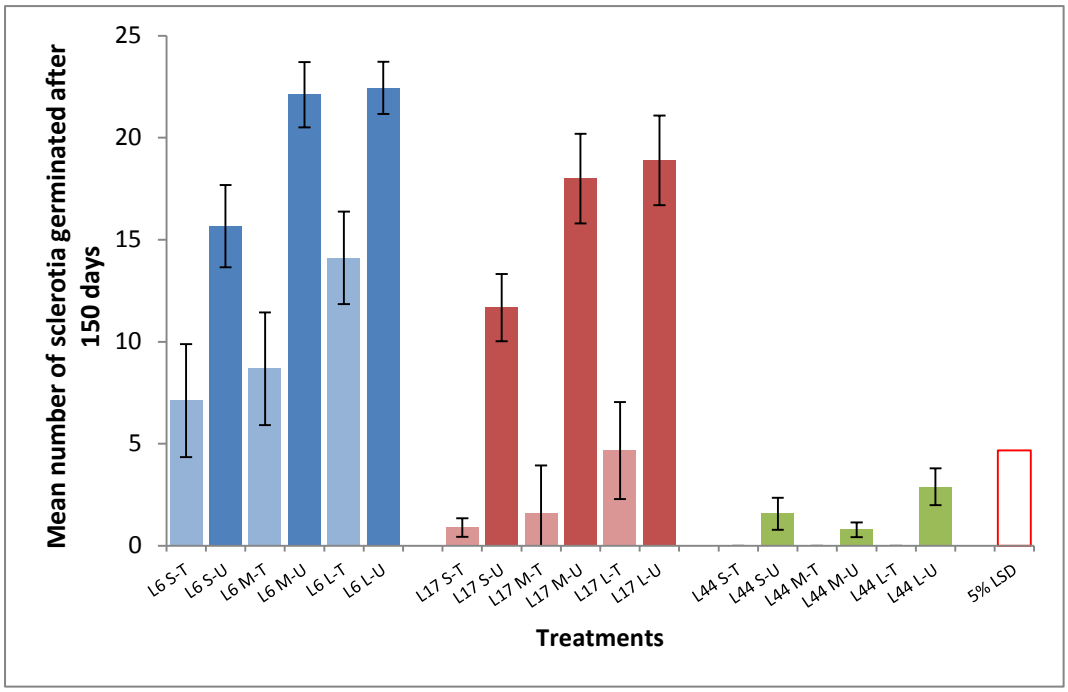

Figure 4.12 - The effect of biofumigant crop *B. juncea* 'Caliente 99'on the carpogenic germination of three different sizes of *S. sclerotiorum* sclerotia of isolates L6, L17 and L44 after 150 days in microcosm experiments. S-T = small sclerotia treated, S-U = small sclerotia untreated, M-T = medium sclerotia treated, M-U = medium sclerotia untreated, L-T = large sclerotia treated, L-U = large sclerotia untreated. Error bars indicate SEM.

Table 4.5 – The percentage reduction in germination and mean number of apothecia per sclerotium of three different sizes of *S. sclerotiorum* sclerotia for isolates L6, L44 and L17 when treated with biofumigant crop *B. juncea* 'Caliente 99' after 150 days in microcosm experiments.

***S. sclerotiorum* Isolate**	**Size of Sclerotia**	**% Reduction in Germination for Biofumigation Treatment**	**Mean Number of Apothecia per Sclerotium (for all treatments)**
L6	Small	54.6	1.1
L6	Medium	60.8	1.7
L6	Large	37.1	3.1
L17	Small	92.4	1
L17	Medium	91.3	1.3
L17	Large	75.3	2.9
L44	Small	100.0	0.9
L44	Medium	100.0	1.4
L44	Large	100.0	2.6
All isolates	Small	72.3	1
All isolates	Medium	74.9	1.5
All isolates	Large	57.5	2.9

4.3.4 *In vitro* Experiments – Effect of Biofumigants on Mycelial Growth of *Sclerotinia sclerotiorum*

4.3.4.1 Growth Rate

In vitro experiments were carried out to test the direct effect of volatiles (potentially ITCs) released from the six biofumigant plants and oilseed rape on mycelial growth of *S. sclerotiorum.* The rate of mycelial growth of *S. sclerotiorum* isolate L6 was reduced for all six biofumigant treatments using 1 g plant material, with growth rates ranging from 0.9 to 1.5 mm per hour compared to 1.6 mm per hour for the untreated control. This was significant for *B. juncea* 'Pacific Gold', 'Caliente 99' and 'Vittasso', *S. alba* 'Brisant' and *B. napus* 'Temple' ($p<0.05$) (Table 4.6). In experiments using 2 g plant material the rate of mycelial growth was again reduced for all six treatments, but this was only significant for *B. juncea* 'Caliente 99', 'Vittasso' and 'Pacific Gold' ($p<0.05$), with growth rates ranging from 0.5 to 1.3 mm per hour (Table 4.6).

B. juncea 'Caliente 99' was the only biofumigation treatment to show a substantial mycelial growth rate reduction when the plant material quantity used was increased from 1 g to 2 g, and in contrast there was an increase in mycelial growth rate for *B. juncea* 'Pacific Gold' and *S. alba* 'Brisant' treatments (Table 4.6).

Table 4.6- The effect of dried biofumigant crops and low glucosinolate *Brassica napus* 'Temple' on mycelial growth rate (based on logistic curves) of *S. sclerotiorum* isolate L6 over four days. * indicates significant difference to the untreated control at p<0.05.

	Growth rate (mm/hour)			
Treatments	**1g**		**2g**	
Brassica juncea 'Pacific Gold'	0.92	*	1.28	*
Brassica juncea 'Vittasso'	1.36	*	1.20	*
Brassica juncea 'Caliente 99'	1.01	*	0.54	*
Raphanus sativus 'Terranova'	1.59		1.54	
Sinapis alba 'Brisant'	1.44	*	1.59	
Eruca sativa 'Nemat'	1.51		1.47	
Brassica napus 'Temple'	1.46	*	1.52	
Untreated	1.63		1.54	
5% LSD	0.16		0.20	
d.f.	95		91	

4.3.4.2 Mycelial Growth Inhibition

After 73 hours (the time at which the mycelial growth of the untreated *S. sclerotiorum* control reached the edge of the Petri dish) there were significant differences in the percentage reduction of mycelial growth compared to the untreated control in both the experiments using 1 g plant material and the experiments using 2 g plant material. The greatest inhibition was for *B. juncea* 'Caliente 99', at 72.8% for 1 g plant material and 85.3% for 2 g plant material. Each of the brown mustard (*B. juncea)* cultivars inhibited mycelial growth more than any of the other treatments. The lowest inhibition of 14.6% was observed for *R. sativus* 'Terranova' at a rate of 2 g, and only *B. juncea* 'Pacific Gold' and 'Caliente 99' gave greater inhibition of mycelial growth at the higher rate of 2 g s compared to 1 g plant material (Figure 4.13).

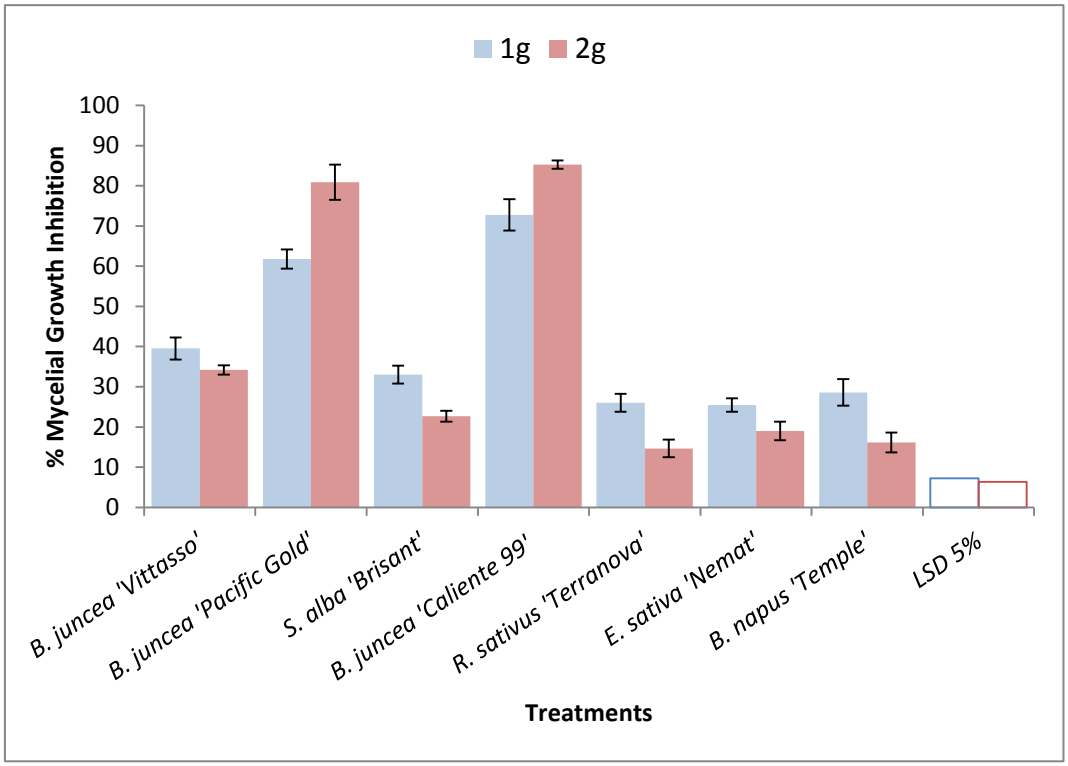

Figure 4.13 – The effect of biofumigant crops and *B. napus* 'Temple' on the inhibition of mycelial growth of *S. sclerotiorum* isolate L6 compared to the untreated control after 73 hours, using either 1 g or 2 g plant material. Error bars indicate SEM.

4.3.5 *In vitro* Experiments – Effect of Biofumigant Dose on Mycelial Growth of *Sclerotinia sclerotiorum*

Experiments were carried out to determine the dose response curve and ED50 for two biofumigant crops – *B. juncea* 'Caliente 99' and *S. alba* 'Brisant'. There was a clear dose response in the inhibition of mycelial growth of *S. sclerotiorum* isolate L6 after 72 hours when treated with different quantities of biofumigant crop *B. juncea* 'Caliente 99' or *S. alba* 'Brisant'. The ED50 for *B. juncea* 'Caliente 99' was calculated as 1.86 g, whereas for *S. alba* 'Brisant' the ED50 was 6.31 g (Figure 4.14).

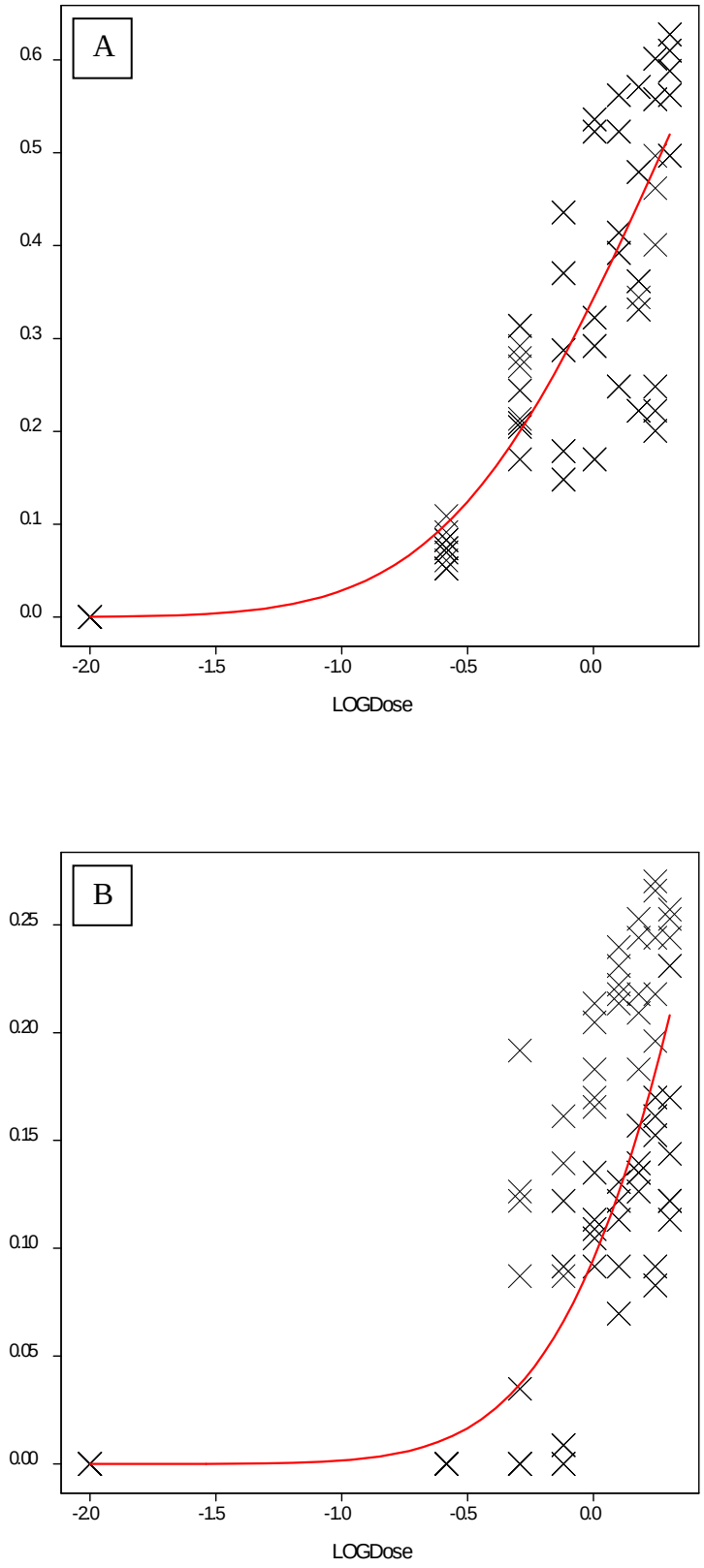

Figure 4.14 - Fitted (red line) and observed values (means across all replicates; black crosses) of the proportional response of mycelial growth of *S. sclerotiorum* isolate L6 to LOG dose of *B. juncea* 'Caliente 99' (A) and *S. alba* 'Brisant' (B) dried and milled plant material after 72 hours.

4.3.6 *In vitro* Experiments – Effect of Biofumigants on Carpogenic Germination of *Sclerotinia sclerotiorum* Sclerotia

Experiments were carried out to test the direct effect of volatiles (potentially ITCs) released from the six biofumigant plants and oilseed rape on carpogenic germination of *S. sclerotiorum* sclerotia. There was limited or no reduction in the germination of *S. sclerotiorum* sclerotia using 1 g biofumigant crop, but all treatments significantly reduced germination using 2 g plant material (Figure 4.15). The most effective treatment was *B. juncea* 'Vittasso' with a mean germination of 3.8 sclerotia after 80 days in the 2 g experiments, compared to 19.8 sclerotia germinating in the untreated control. The least effective treatment using 2 g plant material was *B. juncea* 'Pacific Gold' with a mean germination of 14.2 sclerotia. *B. napus* 'Temple' significantly reduced germination in the 2 g experiments ($p>0.01$) with a mean of 7.3 sclerotia germinating, and was the most effective treatment in the experiments with 1 g plant material with a mean of 12.3 sclerotia germinating (Figure 4.15).

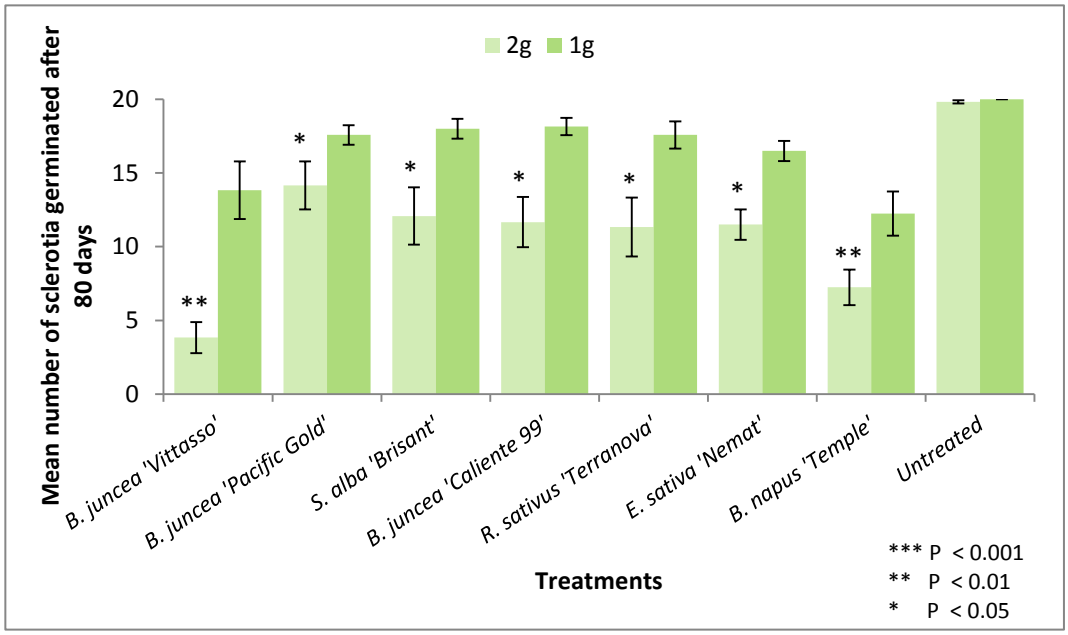

Figure 4.15 - The effect of biofumigant crops and *B. napus* 'Temple' on carpogenic germination of *Sclerotinia sclerotiorum* sclerotia after 80 days in an *in vitro* experiment. Error bars indicate SEM.

During the course of the experiments stunted black stipes were observed emerging from some of the germinating sclerotia in a few of the treatments. This may indicate that the sclerotia were under stress due to their exposure to volatiles from the biofumigant crops (Figure 4.16).

Figure 4.16 – *S. sclerotiorum* sclerotia from the *in vitro* biofumigation experiments for carpogenic germination. Sclerotia with blackened stipes on the left and those with normal stipes on the right.

For the four experiments where the biofumigant treatment was removed and germination assessed for the following four weeks, there was further germination in all treatments except for *R. sativus* 'Terranova' at the 2 g rate (Figure 4.17). The greatest additional germination was seen in *B. napus* 'Temple' at the 1 g rate, with a mean of 4.1 sclerotia germinating after removal of the treatment. The lowest additional germination was seen in *B. juncea* 'Vittasso' at the 2 g rate, with a mean of 0.1 sclerotia. For *B. juncea* 'Vittasso', *R. sativus* 'Terranova', *E. sativa* 'Nemat' and *B. napus* 'Temple' there was more germination after removal of the 1 g treatments than the 2 g treatments (Figure 4.17).

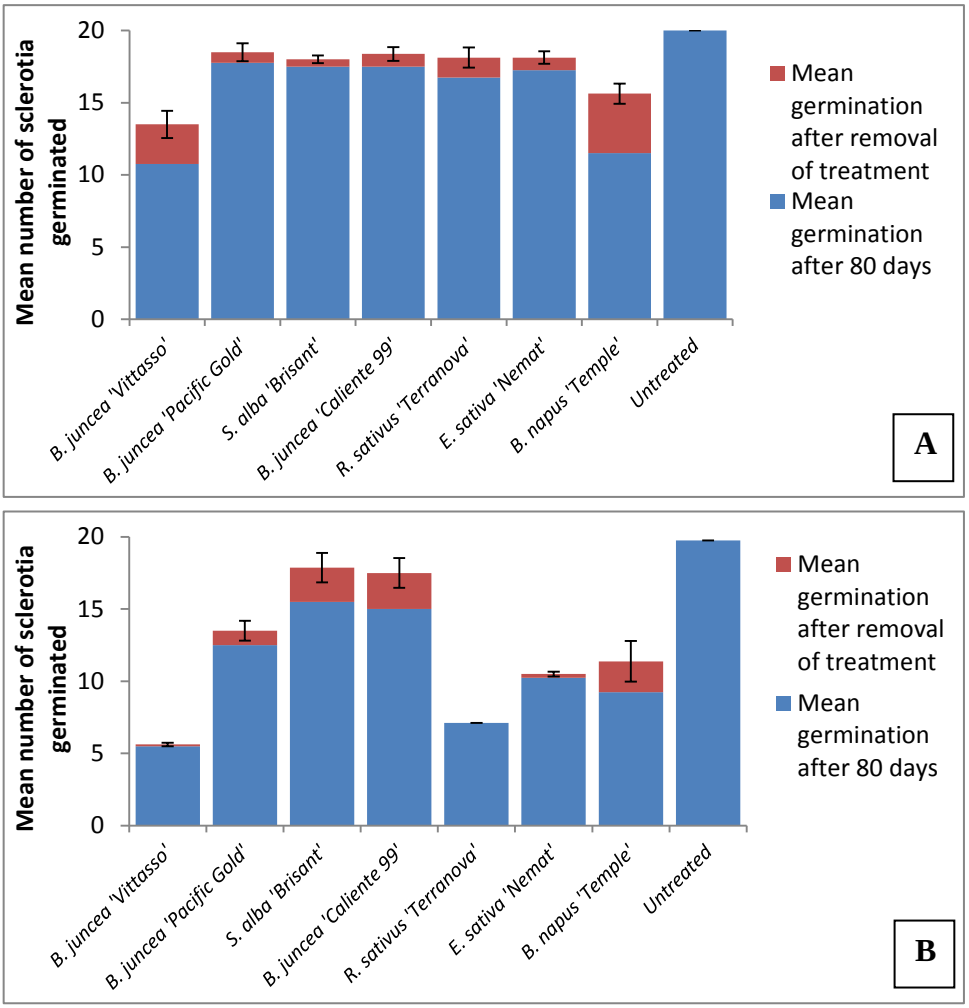

Figure 4.17 - The effect of biofumigant crops and *B. napus* 'Temple' on carpogenic germination of *Sclerotinia sclerotiorum* sclerotia *in vitro* after 80 days (blue bars) and then after removal of treatment for 4 weeks red bars), using either 1 g (A) of 2 g (B) plant material. Error bars indicate SEM for mean germination after removal of treatment.

4.4 Discussion

There have been very few studies examining the effects of biofumigant crops on the carpogenic germination of sclerotia of *S. sclerotiorum*. The results from the present study showed that *Brassica juncea* 'Caliente 99', *Brassica juncea* 'Pacific Gold', *Brassica juncea* 'Vittasso', *Sinapis alba* 'Brisant', *Raphanus sativus* 'Terranova', and *Eruca sativa* 'Nemat' reduce carpogenic germination of *S. sclerotiorum* sclerotia, and also indicated that this effect is caused directly by volatiles, most likely ITCs, released from the plant material. This is the first time volatiles from crops bred specifically for biofumigation have been shown to have a direct effect on the carpogenic germination of *S. sclerotiorum* sclerotia.

The HPLC analysis of the main GSLs in the crops used in the experiments showed a wide variation in GSL content, both between the cultivars of the same species, and within cultivars at different cropping dates. There were differences in the levels of GSLs between crops harvested at different times for use in different experiments. For example, for the harvest dates 25th July 2012 and 12th September 2012 (plant material used in the microcosm experiments) the differences were in the range 0.6 to 5.1 µmol/g dw. However, the statistical analysis of the results for each assay showed no significant differences between repeat experiments.

For all the biofumigation crops the levels of GSL generally declined with later sowing times. This trend is, however, potentially confounded by the first sowings being in a glasshouse, and the later sowings being in a polytunnel. The decline in GSLs for later sown crops confirms the results of Smolinska and Horbowicz (1999), who found that levels of ITCs decreased from their spring crop to the winter crop for both *B. juncea* and *S. alba*, with allyl ITC (derived from sinigrin) decreasing in *B.*

juncea from 648 to 336 µg/g^{-1} dw. Sarwar *et al.* (1998) also found greater quantities of GSLs in spring sown *B. juncea* and *B. napus* than autumn sown, with total GSLs in the shoots increasing from an average across all crops of 20.5 to 35.2 µmol/g^{-1} dw. These studies indicate that light levels and temperature influence the quantity of GSLs produced, and they have been found to be under circadian light regulation in *Arabidopsis*, with more GSLs produced during the day (Huseby *et al.*, 2013). In the present study the greatest quantity of sinigrin (19.6 to 33.3 µmol/g^{-1} dw) was found in all the *B. juncea* cultivars for the earlier crops grown in the glasshouse, at higher temperatures than seen in the later sown crops in the polytunnel. This is similar to the findings of a study where levels of total GSLs in *Brassica oleracea* increased from 23.5 to 33.2 mg/g^{-1} dw when grown at 21°C instead of 15°C (Steindal *et al.*, 2013). However, the time of year of sowing and resulting variance in environmental conditions had different effects on the GSL levels for the different species grown in the present study, and this was particularly clear in the glucoerucin levels in *E. sativa* 'Nemat', which produced higher or similar levels of GSL when grown in the polytunnel than in the glasshouse. It is clear that this is an area which warrants further work to ensure optimum growing conditions to balance biomass and GSL concentrations for particular biofumigation crops.

In the microcosm experiments using dried plant material the biofumigant with the greatest effect on germination of *S. sclerotiorum* sclerotia was *R. sativus* 'Terranova', whereas in the initial experiments using fresh plant material the most effective treatment was *E. sativa* 'Nemat'. However, there were differences in the efficacy of all the plant treatments between experiments using fresh material and those using dried material, and this is most likely due to the differences observed in water content in the boxes, with some becoming waterlogged as the fresh plant

material broke down. This may have impacted on the viability of the *S. sclerotiorum* sclerotia as sclerotia recovered from flooded soils have been shown previously to have reduced viability (Matheron & Porchas, 2005). Using dried plant material enabled the water content in the boxes to be maintained at a consistent level throughout the experiments, ensuring the repeatability of conditions and results. The GSLs preserved during the drying and milling process, as evidenced by the HPLC analysis, were comparable with previously reported levels of GSL content, and were within the range observed by Kirkegaard and Sarwar (1998) (0.2 to 44.9 μmol/g^{-1} dw) across the 80 *Brassica* spp. they sampled. However, it has been found that the preservation of GSLs through drying varies with the temperature and length of drying time, as well as between species. Lazzeri *et al.* (2004) found that in five high glucosinolate producing plants between 16.1% and 62.9% GSLs were retained after drying at 40°C for three days, and drying at 100°C to 250°C for 10 to 20 minutes retained as much as 80% of GSLs. Using gas-chromatography mass-spectrometry and *in vitro* mycelial growth assays to assess fungitoxic activity towards *Pythium* and *Rhizoctonia* they also found that the enzyme myrosinase was sufficiently preserved after drying to catalyse GSL hydrolysis, and inhibition of mycelial growth was not increased by the addition of exogenous myrosinase (Lazzeri *et al.*, 2004). Eylen *et al.* (2008) found broccoli myrosinase was stable until 45°C, with activity reduced by more than 95% after 10 min at 70°C. However, from the inhibition of *S. sclerotiorum* mycelial growth by the dried biofumigant plant material in the present study it is most likely that both GSLs and myrosinase were sufficiently preserved during processing of the plant material to enable ITC production, although further analysis would be needed to unequivocally confirm this.

If the myrosinase was inactivated by the drying process the reduction in germination of sclerotia in the microcosm experiments could potentially be attributed to changes in the soil microbial population, due to the addition of the dried plant material. One study found both intact and denatured *Brassica* seed meals significantly reduced *Rhizoctonia solani* infection of winter wheat seedlings (Handiseni *et al.*, 2013). This experimental approach could be used to expand on the microcosm experiments in the current study, by autoclaving biofumigant plants and comparing their efficacy with non-autoclaved plants, to ascertain whether the mechanism involved in the reduction in germination of *S. sclerotiorum* sclerotia is due to the conversion of GSLs to ITCs, or due to changes in the soil microbial population. This would be a valuable addition to the *in vitro* experiments which deliberately sought to establish an effect of volatiles released from the biofumigants.

Some studies have found that different isolates of *S. sclerotiorum* consistently produce different sizes of sclerotia (Akram *et al.*, 2008; Li *et al.*, 2008) and this has also been seen in *S. trifoliorum* (Vleugels *et al.*, 2013). In the microcosm experiments assessing the effect of sclerotial size on the efficacy of biofumigation, the large *S. sclerotiorum* sclerotia were less affected with germination reduced by only 57%, compared to the small and medium sclerotia where germination was reduced by over 70%. Additionally, the larger sclerotia germinated more consistently in the present study, something also observed by Dillard *et al.* (1995), and produced twice the mean number of apothecia per sclerotium than the medium sclerotia, and three times as many as the small sclerotia. Therefore, results from the present study indicate that biofumigation is less effective against large *S. sclerotiorum* sclerotia which have the greater inoculum potential. Similarly, Smolinska and Horbowicz (1999) found that volatiles from *B. juncea* plant tissue gave a greater reduction in

mycelial germination of the smaller sclerotia produced by *Sclerotium cepivorum* than the larger sclerotia produced by *S. sclerotiorum*, but this may also in part be due to different sensitivities of the species investigated. It is possible that biofumigation will be more effective against the smaller sclerotia produced by *S. trifoliorum* and *S. minor*, and less effective against the larger sclerotia produced by *S. subarctica.* Further work with a wider range of *S. sclerotiorum* isolates and with different species is needed to confirm these results.

In the *in vitro* carpogenic germination experiments *B. juncea* 'Vittasso' was the most effective biofumigant in inhibiting germination. All of the crops significantly reduced germination at the higher rate of 2 g, including the low glucosinolate *B. napus* 'Temple'. Many studies using a similar method of exposing *S. sclerotiorum* sclerotia to ITCs produced via the addition of water to milled dried plant material have assessed the viability of the sclerotia by myceliogenic germination, rather than carpogenic germination (Smolinska & Horbowicz, 1999). In one study total GSL extracted from *B. juncea* cultivars and a myrosinase solution were added to vials containing *S. sclerotiorum* sclerotia and sterile soil, sealed for 24 hours, and then the sclerotia were removed and plated onto fresh sterile soil where carpogenic germination was assessed over six weeks. They found that all 11 *B. juncea* accessions tested reduced germination, with *B. juncea* 'Pacific Gold' being the most effective with complete inhibition of germination with GSL extracted from 0.5 g plant material (Bomford, 2009). However, the assumption was made that if sclerotia did not germinate after six weeks then they were dead, which may not have been the case. In the present study it was found that for some treatments up to four *S. sclerotiorum* sclerotia (of 20) germinated after the treatments were removed from the boxes, which indicates that at least some of the sclerotia were not killed by the

biofumigant treatments, but that carpogenic germination was suppressed. However, suppression of carpogenic germination may still be a valuable control measure in the field. In order to assess viability the sclerotia could have been retrieved, bisected and plated onto PDA or water agar to see if they would germinate myceliogenically (Hao *et al.*, 2003).

Kurt *et al.* (2011) examined the volatile effects of a range of pure ITCs on carpogenic germination of *S. sclerotiorum* sclerotia *in vitro* and found that all the pure ITCs tested, except phenyl and 2- phenylethyl at their lowest concentrations significantly inhibited the production of apothecia compared with the untreated controls. Butyl and benzyl ITCs gave the greatest reduction of 92.5% at their highest concentrations, which are aliphatic and aromatic GSLs respectively. In the present study, the most effective treatment for reducing carpogenic germination *in vitro* was *B. juncea* 'Vittasso'. Assuming the volatiles responsible are ITCs, the main GSL sinigrin in this species would produce the aliphatic allyl ITC. Kurt *et al.* (2011) also found that allyl ITC reduced *S. sclerotiorum* germination by between 60% and 75%, dose dependent, which is comparable with the reduction observed for *B. juncea* 'Vittasso' of 81% (2 g experiments) in this study. However, based on the HPLC analysis this cultivar has the lowest GSL content for each harvest date out of all the *B. juncea* cultivars used in the experiments. This could be due to the efficacy of the conversion of GSLs to ITCs which has been shown to vary between *Brassica* spp. when fresh material is incorporated into the soil. For instance, Morra and Kirkegaard (2002) found a lower conversion efficiency for a high GSL *B. juncea* cultivar (0.6%) than for a low GSL cultivar (1.6%). They also found that freezing and thawing plant tissue increased ITC release efficiencies to 26%, although they did not test dried milled plant material (Morra & Kirkegaard, 2002). It is therefore possible that the

conversion efficiency of GSLs to ITCs was greater for *B. juncea* 'Vittasso' than in the other cultivars tested here, even though the GSL content was lower.

In the current work the most effective biofumigant crop for suppressing mycelial growth of *S. sclerotiorum* was *B. juncea* 'Caliente 99'. Ojaghian *et al.* (2012) reported that fresh macerated tissues of a *B. juncea* cultivar were the most effective in reducing mycelial growth of *S. sclerotiorum*, when compared to *B. napus* and *B. campestris*. They also found that fresh macerated tissues provided greater inhibition than dried irradiated tissues. Larkin and Griffin (2007) used 1g fresh macerated plant material and found that the greatest inhibition of 90.2% of mycelial growth of *S. sclerotiorum* was obtained with a *B. juncea* cultivar, slightly higher than the 85.3% found in the present study using a rate of 2 g dried plant material. They also found that a *B. napus* cultivar with low glucosinolate content resulted in a mycelial growth reduction of 20.4%, and that a *Sinapis alba* cultivar reduced mycelial growth by 23.7%, both lower than the reductions found in the present study of 28.6 % (*B. napus* 'Temple') and 33% (*S. alba* 'Brisant') respectively. The 1 g fresh plant material used by Larkin and Griffin (2007) is likely to have a much lower GSL content than the dried plant material used here, but they did not quantify the GSL content of the crops used in their experiments so comparisons are difficult.

In another study using freeze dried plant material the inhibitory effects of different parts of plants (seed stem, leaf, petiole and root) against mycelial growth of *S. sclerotiorum* was tested over 48 hours, and it was found that the leaf, petiole, and roots of a *B. juncea* cultivar were the most effective, with agar plates treated with leaf and petioles still showing 100% inhibition for 24 hours after the treatment had been removed (Rahimi *et al.*, 2013). It is possible that had these experiments been

continued for a few more days the pathogen may have recovered, as was found in similar *in vitro* tests run over nine days using cultivars of *B. juncea, B, napus, B. rapa, S. alba* and *R. sativus.* Here, *B. juncea* was the only treatment which delayed mycelial growth, but only for ten days (Smolinska & Horbowicz, 1999). In a similar approach, Rahmanpour *et al.* (2013) did not test *B. juncea* cultivars, but found that 5 mg of freeze dried leaf and petiole tissue of a condiment variety of *B. nigra* completely inhibited mycelial growth of *S. sclerotiorum* after 24 hours and this inhibition continued for two days after the treatments were removed. The main GSL in *B. nigra* is sinigrin, which is the same as found in *B. juncea*. Rahmanpour *et al.* (2013) did not analyse the GSLs in the crops used in their experiments, but these studies using plant material in either fresh or dried forms and the present work indicate that sinigrin and its conversion to allyl ITC is the most effective GSL for inhibiting mycelial growth of *S. sclerotiorum*. This confirms the results of Kurt *et al.* (2011) using pure ITCs, where they found methyl and allyl ITCs gave the greatest inhibition over 5 days.

The variation in the ability of each individual biofumigant crop to inhibit mycelial growth or carpogenic germination in the microcosm and *in vitro* experiments is not unexpected. Kurt *et al.* (2011) found that different pure aliphatic and aromatic ITCs were effective against mycelial growth or carpogenic germination, but this depended on whether they were used as vapour or contact phases. They observed aliphatic ITCs (derived from GSLs such as sinigrin, glucoerucin and glucoraphenin) to be more effective than aromatic ITCs (derived from GSLs such as sinalbin) at inhibiting mycelial growth in the vapour phase, with the opposite being true when the ITCs were incorporated into the PDA. This is in agreement with the present study where the only biofumigant with a main GSL which would hydrolyse to produce an

aromatic ITC was *S. alba* 'Brisant'. This biofumigant was more effective at inhibiting carpogenic germination in the microcosm experiments where the ITCs released from the plant material would have been in contact with the sclerotia, than in the *in vitro* carpogenic experiments using the vapour phase. However, in the mycelial growth experiments it resulted in greater inhibition than some of the aliphatic GSL containing crops.

The most effective crop for inhibition of carpogenic germination of *S. sclerotiorum* sclerotia varied between the microcosm experiments and the *in vitro* experiments, and was *R. sativus* 'Terranova' and *B. juncea* 'Vittasso' respectively. In the mycelial growth experiments *B. juncea* 'Caliente 99' was the most effective at inhibiting growth. It is suggested that these differences are possibly due to a combination of differences between contact phase and vapour phase effects, the different quantities of plant material used, (i.e. 6 g in a full field rate microcosm experiment and a maximum of 2 g in the *in vitro* experiments), and a variation in susceptibility of the different tissues, (i.e. sclerotia vs. mycelium). However, it also indicates that whilst the *in vitro* trials are a useful tool to ascertain the direct effects of volatiles, they may not be as valuable for determining the most effective biofumigant crop to be used in the field against resting propagules of a pathogen such as *S. sclerotiorum*. The microcosm experiments therefore provide a more reliable method for screening suitable biofumigant crops against this pathogen.

The HPLC analysis combined with the experimental results suggests there may be non-glucosinolate derived volatiles being released from *B. napus* 'Temple' which resulted in reduced germination of sclerotia. Previously it has been reported that synthetic pure butyl ITC, which is derived from the parent GSL gluconapin and is

commonly found in *B. napus* and *B. juncea*, is the most effective ITC in inhibiting carpogenic germination of *S. sclerotiorum* sclerotia (Kurt *et al.*, 2011). However, the HPLC analysis conducted for the present study detected only small quantities of the GSL sinigrin in *B. napus* Temple and there were no other obvious peaks on the chromatograms to indicate the presence of a measurable quantity of any other GSL. Similarly, Smolinska and Horbowicz (1999) detected no measurable amount of ITCs in two out of the four *B. napus* cultivars in their experiments. The inhibition of carpogenic germination of *S. sclerotiorum* sclerotia by *B. napus* 'Temple' cannot be explained by interaction with soil organisms, due to the deliberate methods employed to ascertain a direct mode of action of the biofumigant crops against *S. sclerotiorum.* Additionally, one study found that a low glucosinolate *B. napus* cultivar reduced carpogenic germination of sclerotia by 44%, but did not affect myceliogenic germination (Dandurand *et al.*, 2000). This is similar to the results from the present study, where *B. napus* 'Temple' was very effective in reducing carpogenic germination, but amongst the least effective treatments in inhibiting mycelial growth. It was suggested by Bending and Lincoln (1999) that the biofumigant properties of *B. juncea* were due to the combined effect of small quantities of ITCs, and large quantities of less toxic non-glucosinolate derived volatile S-containing compounds, such as carbon-disulphide, dimethyl-disulphide, dimethyl-sulphide and methanethiol. These compounds, amongst others such as fatty-acid derivatives, were found in *B. napus* by Tollsten and Bergström (1988) so may be responsible for the results in the present study, and warrant further research.

5. Temperature effects on the germination and growth of *Sclerotinia subarctica*

5.1 Introduction

Sclerotinia subarctica is a species related to *S. sclerotiorum* and was first identified in the UK after being isolated from *Ranuculus acris* (meadow buttercup) in 2009 (Clarkson *et al.*, 2010). It has previously been found on the wild plants *Taraxacum* sp. (dandelion), *Caltha palustris* (yellow marsh marigold) and *Aconitum septentrionale* (northern wolfsbane), as well as potato in Norway (Holst-Jensen *et al.*, 1998). In Alaska, Winton *et al.* (2006) identified *S. subarctica* on lettuce, cabbage, bean, squash and potato in sympatry with *S. sclerotiorum*. As discussed in Chapter 2, *S. subarctica* has been found in only one location in England (on meadow buttercup) and on numerous crop plants in Eastern Scotland. It was suggested by Winton *et al.* (2006) that *S. subarctica* is endemic to higher latitudes, and one hypothesis is that the climate in Scotland is more favourable to this species.

Morphologically *S. sclerotiorum* and *S. subarctica* are indistinguishable (Holst-Jensen *et al.*, 1998) although *S. subarctica* has generally been found to produce fewer but larger sclerotia *in vitro* than *S. sclerotiorum* (Clarkson *et al.*, 2010) (Figure 5.1). As discussed in Chapter 3, the size of a sclerotium may influence subsequent carpogenic germination and survival, and Ben-Yephet *et al.* (1993) found bigger *S. sclerotiorum* sclerotia were more likely to germinate and produce apothecia, although fewer were recovered from the field than smaller sclerotia several years later. They suggested that this could be due to greater survival rates of the smaller sclerotia, or a higher relative rate of apothecial production by the larger sclerotia. It was hypothesised by Winton *et al.* (2006) that larger sclerotia confers a competitive

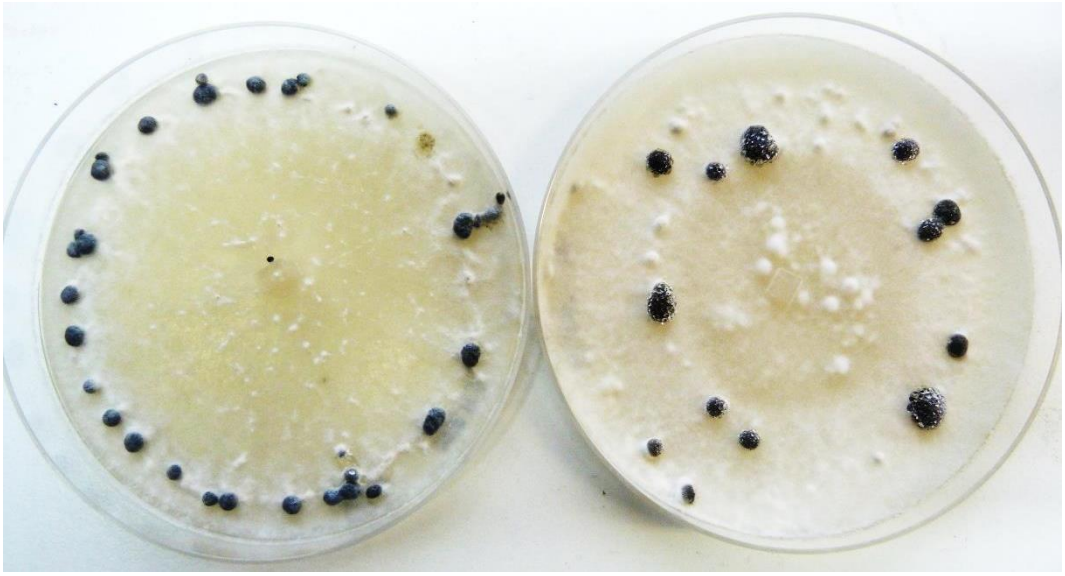

Figure 5.1 - PDA plates of a *S. sclerotiorum* isolate (left) and *S. subarctica* isolate (right) showing the differences in numbers and sizes of sclerotia typically produced in culture by the two species.

advantage, by enabling survival over winter in the extremely low temperatures experienced in Alaska.

For carpogenic germination to occur a sclerotium must be fully mature, with a complete outer rind (Purdy, 1979; Abawi & Grogan, 1979). 'Conditioning' sclerotia at low temperatures in moist conditions has been found to reduce the length of time for carpogenic germination to occur, as well as reducing the time to maximum germination (Phillips, 1986). The optimum temperatures for conditioning to ensure rapid carpogenic germination of *S. sclerotiorum* sclerotia has been found to vary between isolates, with a temperature range of 4 to 16°C suggested by Dillard *et al.* (1995). The standard treatments used by researchers are reported to be 4, 5 or 10°C for 4 weeks (Mylchreest & Wheeler, 1987; Huang & Kozub, 1989; Smith & Boland, 1989; Sansford & Coley-Smith, 1992; Clarkson *et al.*, 2007). It has also been demonstrated that the temperature at which sclerotia are formed affects the requirement for cold conditioning (Huang & Kozub, 1993). Isolates from different geographic regions can therefore vary in their need for chilling, with those

originating in tropical regions not requiring any cold treatment to germinate carpogenically (Huang & Kozub, 1991; Bolton *et al.*, 2006).

For the majority of crops Sclerotinia disease is caused by ascospores released from apothecia formed following carpogenic germination of sclerotia (Schwartz & Steadman, 1978; Abawi & Grogan, 1979). Sclerotia are also capable of myceliogenic germination, which is influenced by a range of environmental factors. At 100% relative humidity desiccated *S. sclerotiorum* sclerotia germinate myceliogenically more readily than fresh sclerotia (Huang *et al.*, 1998). Disease in the field initiated by myceliogenic germination is rarely seen, but has been reported on sunflower (Bardin & Huang, 2001) and can cause post-harvest disease outbreaks in crops such as carrots (Lumsden, 1979; Finlayson *et al.*, 1989b; Foster *et al.*, 2008). Sclerotia can also be induced to germinate myceliogenically, rather than carpogenically, by freezing (Huang, 1991). This response is thought to be due to injury to the rind of the sclerotia, as only immature sclerotia without a completely melanised rind, or mature sclerotia with an injured rind, will readily germinate myceliogenically (Huang, 1985).

The mycelial growth of *S. sclerotiorum in vitro* is affected primarily by temperature and, depending on the isolate and other conditions such as nutrients and pH of the media, growth is commonly seen in the temperature range between 0 and 30°C (Le Tourneau, 1979). Abawi and Grogan (1975) found incubating *S. sclerotiorum* at 20 to 25°C resulted in the greatest rate of mycelial growth, with only a small amount of growth observed at 5°C and no measurable growth at 30°C. They also observed that whilst more sclerotia were produced on PDA plates at 25°C, the sclerotia produced at 10 and 15°C were larger. Conversely, *S. minor* was shown to produce larger

sclerotia at higher temperatures, but had a similar optimum temperature for mycelial growth of 18°C, with growth occurring in the range 6 to 30°C (Imolehin *et al.*, 1980), which is comparable with *S. sclerotiorum.*

Little is currently known about *S. subarctica* in terms of its biology and epidemiology. Identifying the conditions required for this pathogen to grow and reproduce may help to explain why it is restricted to specific geographic areas and is not as widespread as *S. sclerotiorum*. The aims of the work described in this chapter were:

- To investigate the effect of conditioning time on the carpogenic germination of *S. subarctica* sclerotia, for comparison with *S. sclerotiorum.*
- To investigate the effect of temperature on mycelial growth of *S. subarctica*, for comparison with *S. sclerotiorum.*

5.2 Materials and Methods

5.2.1 Production of *Sclerotinia sclerotiorum* and *S. subarctica* Sclerotia

Sterile stock sclerotia of *S. sclerotiorum* and *S. subarctica* (produced as described in Chapter 2) were used as necessary to initiate actively growing cultures as required. Sclerotia of different *S. sclerotiorum* and *S. subarctica* isolates were then produced on wheat grain as described by Clarkson *et al.* (2003) and outlined in Chapter 4**.**

5.2.2 Effect of Temperature on Mycelial Growth of *S. sclerotiorum* and *S. subarctica* Isolates

In vitro experiments were carried out to test the effect of temperature on the mycelial growth of *S. subarctica* and *S. sclerotiorum*. The first experiment compared the growth of four *S. subarctica* isolates with a 'standard' *S. sclerotiorum* isolate L6 at four temperatures ranging from 5 to 20°C (Table 5.1), while the second compared the growth of five *S. subarctica* isolates with five *S. sclerotiorum* isolates at four temperatures ranging from 25 to 33°C (Table 5.1). Isolates were selected based on their representing different microsatellite and IGS haplotypes according to the analysis outlined in Chapter 2. Additionally, only a limited number of *S. subarctica* isolates were initially available, hence their use in experiment one.

A 5 mm mycelial plug of actively growing mycelium from each *Sclerotinia* isolate was placed in the centre of a 9 cm Petri dish containing potato dextrose agar (PDA; Merck), and incubated at the different temperature treatments in the dark. Mycelial growth was assessed twice a day for up to 168 hours by measuring the radial growth along the x and y axis. There were four replicate plates for each temperature

treatment arranged in a randomised block design and each experiment was repeated three times.

Table 5.1 - *S. sclerotiorum* and *S. subarctica* isolates used in the mycelial growth experiments at 5°C, 10°C, 15°C and 20°C (Experiment 1) and 25°C, 28°C, 30°C and 33°C (Experiment 2).

Isolate	Species	Location	Crop Type	Year Isolated	Microsatellite Haplotype	IGS Haplotype	Experiment	
L6	*sclerotiorum*	Petworth, Sussex, England	Lettuce	2005	3	2	1	2
L44	*sclerotiorum*	Petworth, Sussex, England	Lettuce	2005	57	6		2
C28	*sclerotiorum*	Blyth, Northamptonshire	Carrot	2005	17	1		2
CE11	*sclerotiorum*	Methwold, Norfolk	Celery	2009	1	3		2
R28	*sclerotiorum*	Holywell, Warwickshire	Buttercup	2007	6	4		2
Liel17a1	*subarctica*	Dunfermline, Fife, Scotland	Buttercup	2012	5	n/a	1	2
IP10	*subarctica*	Isla Bend, Perthshire, Scotland	Potato	2012	2	n/a		2
HE1	*subarctica*	Michaelchurch Escley, Herefordshire, England	Buttercup	2009	1	n/a	1	2
PS13	*subarctica*	Meigle, Perthshire, Scotland	Pea	2012	3	n/a	1	2
KE8	*subarctica*	Eyemouth, Berwickshire, Scotland	Potato	2013	6	n/a		2
MH22	*subarctica*	Millerhill, Midlothian, Scotland	Swede	2012	48	n/a	1	

5.2.3 Effect of Conditioning Time Duration on Carpogenic Germination of *S. sclerotiorum* and *S. subarctica* Isolates

Experiments were carried out to determine the effect of conditioning duration (chilling) at 5°C on subsequent carpogenic germination at 15°C for different *S. subarctica* and *S. sclerotiorum* isolates (Table 5.2). Isolates were selected based on them representing different microsatellite and IGS haplotypes according to the analysis as outlined in Chapter 2, as well as which *S. subarctica* isolates were available before the start of the experiments.

S. sclerotiorum L6 was used as a 'standard' isolate as it has previously been found to germinate carpogenically reliably with a conditioning time of 30 days (personal communication, John Clarkson, 2012). *S. sclerotiorum* isolate L44 was used as it produces larger sclerotia (as discussed in Chapter 3) than L6, and L44 has also been

found previously to require longer periods of conditioning (personal communication, John Clarkson, 2012).

Table 5.2 - *S. sclerotiorum* and *S. subarctica* isolates used in carpogenic germination experiments at 0, 20, 40, 60, 80, 100, 120 and 140 days at 5°C (Experiment 1) and 0, 20, 60, 120 and 140 days at 5°C (Experiment 2).

Isolate	Species	Location	Crop Type	Year Isolated	Microsatellite Haplotype	IGS Haplotype	Experiment	
L6	*sclerotiorum*	Petworth, Sussex, England	Lettuce	2005	3	2	1	2
L44	*sclerotiorum*	Petworth, Sussex, England	Lettuce	2005	57	6	1	
HE1	*subarctica*	Michaelchurch Escley, Herefordshire, England	Buttercup	2009	1	n/a	1	
HE3	*subarctica*	Michaelchurch Escley, Herefordshire, England	Buttercup	2009	4	n/a	1	
HE4	*subarctica*	Michaelchurch Escley, Herefordshire, England	Buttercup	2009	9	n/a	1	
HE8	*subarctica*	Michaelchurch Escley, Herefordshire, England	Buttercup	2009	7	n/a	1	
210002N	*subarctica*	Rogaland, Norway	Lettuce	2012	74	n/a		2
LSA1	*subarctica*	Almhaga, Sweden	Lettuce	2012	20	n/a		2
PS13	*subarctica*	Meigle, Perthshire, Scotland	Pea	2012	3	n/a		2
MH22	*subarctica*	Millerhill, Midlothian, Scotland	Swede	2012	48	n/a		2

Compost (John Innes No 1, J. Arthur Bowers) for use in experiments was passed through a 4 mm sieve and pasteurised by autoclaving at 110°C for 30 minutes. Moisture content was calculated by weighing three samples of the compost before oven drying them for 24 hours at 80°C. After drying the samples were reweighed and the moisture content calculated. The pasteurised compost (50 g, 30% w/w moisture content) was placed in 9 cm Petri dishes, and sclerotia (20) of each isolate were laid out in a grid pattern and pressed flat into the compost. The lids were placed onto the Petri dishes, which were then weighed and placed into sealed plastic boxes (Sealfresh Giant Storer, Stewart, UK). The sclerotia were incubated at 5°C in the dark for different durations after which they were moved to 15°C (in the dark). The emergence of stipes was recorded once a week for 180 days. By incubating the Petri dishes in the dark stipes are readily produced but apothecia are not.

Every two weeks water was added to the compost in the Petri dishes to bring them back to their original weight. Four replicates for each temperature/isolate treatment

were arranged in a randomised block design and experiment 1 was repeated three times. Experiment 2 was carried out only once.

5.2.4 Statistical Analyses

All statistical analysis was carried out in Genstat® (13th edition, VSN international Ltd.). For the mycelial growth experiments the rate of growth was calculated at 46 hours. Rate of growth and total colony size at 46 hours were analysed using Analysis of Variance (ANOVA) with a blocking structure of trial x replicates, and significant differences were identified by comparing ANOVA treatment means using the approximate least significant difference values (LSD) at the 5% level. ANOVA was carried out for both individual isolates and also for isolates grouped by species.

For the conditioning duration experiments, the cumulative number of sclerotia germinated up to 21 days after being placed at 15°C were analysed using ANOVA with a blocking structure of trial x replicates, and significant differences were identified by comparing ANOVA treatment means using the approximate least significant difference values (LSD) at the 5% level.

5.3 Results

5.3.1 Effect of Temperature on Mycelial Growth of *S. sclerotiorum* and *S. subarctica* Isolates

In vitro experiments were carried out to test the effect of a range of temperatures on mycelial growth of different *S. subarctica* and *S. sclerotiorum* isolates.

Temperature Range 5 to 20°C

In the first experiments comparing the growth of four *S. subarctica* isolates with *S. sclerotiorum* isolate L6 at four temperatures ranging between 5 and 20°C there were significant differences ($p<0.05$) between some of the isolates at the different temperatures (Table 5.3).

Table 5.3 - Mean mycelial growth rate (mm/hour) for *S. sclerotiorum* (isolate L6) and *S. subarctica* (isolates HE1, Liel17a1, PS13 and MH22) at 5°C, 10°C, 15°C and 20°C. Numbers followed by different letters within a column are significantly different from each other ($p<0.05$).

		Growth Rate (mm/hour)			
Isolate	**Species**	**5°C**	**10°C**	**15°C**	**20°C**
HE1	*subarctica*	0.36 a	0.67 a	1.16 a	1.76 a
Liel17a1	*subarctica*	0.29 ab	0.61 ab	1.15 a	1.73 a
PS13	*subarctica*	0.32 ab	0.58 ab	1.29 b	1.75 a
MH22	*subarctica*	0.26 b	0.53 b	1.13 a	1.57 b
L6	*sclerotiorum*	0.30 ab	0.65 a	1.18 a	1.76 a
5% LSD	0.09				
d.f.	152				

At 10 and 20°C the rate of mycelial growth of *S. sclerotiorum* isolate L6 was significantly greater ($p<0.05$) than *S. subarctica* isolate MH22 (Table 5.3). At 15°C the rate of mycelial growth of *S. subarctica* isolate PS13 was significantly greater ($p<0.05$) than all other isolates (Table 5.3). The fastest rate of growth for all isolates,

regardless of species, was seen at 20°C (between 1.57 and 1.76 mm/hour). The slowest rate of growth for all isolates was seen at 5°C (between 0.26 and 0.36 mm/hour, Table 5.3).

The mean colony diameter after 46 hours for all the individual isolates was lowest at 5°C (12.11 to 15.56 mm) and greatest at 20°C (72.33 to 80.94 mm, Figure 5.2). At 10 and 20°C the mean colony diameter of *S. sclerotiorum* isolate L6 was significantly greater ($p<0.05$) than *S. subarctica* isolate MH22 (Figure 5.2). At 15°C the mean colony diameter of *S. subarctica* isolate PS13 was significantly greater ($p<0.05$) than all other isolates (Figure 5.2)

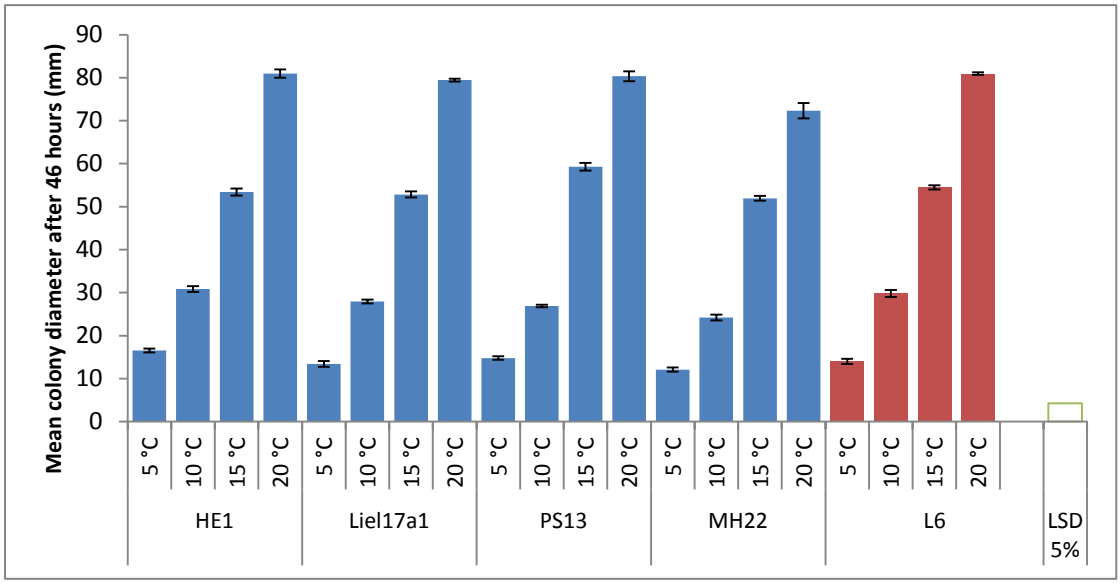

Figure 5.2 - Mean mycelial colony diameter (mm) of four *S. subarctica* isolates (shown in blue) and one *S. sclerotiorum* isolate (shown in red) after 46 hours at 5, 10, 15 and 20 °C. Error bars show SEM.

Temperature Range 25 to 33°C

In the set of experiments comparing the growth of five *S. subarctica* isolates with five *S. sclerotiorum* isolates at four temperatures ranging between 25 and 33°C there were significant differences ($p<0.05$) in the mean growth rate between species at each temperature (Table 5.4). The fastest mean growth rate of 1.87 mm/hour was for *S. sclerotiorum* at 25°C, whilst *S. subarctica* had the slowest mean growth rate of 0.01 mm per hour at 33°C.

Table 5.4 - Mean mycelial growth rate (mm/hour) for *S. sclerotiorum* and *S. subarctica* at 25°C, 28°C, 30°C and 33°C. Numbers followed by different letters within a row are significantly different from each other ($p<0.05$).

Temperature (°C)	*S. sclerotiorum* Growth Rate (mm/hour)	*S. subarctica* Growth Rate (mm/hour)
25	1.87 a	1.78 b
28	1.09 a	0.60 b
30	0.61 a	0.32 b
33	0.21 a	0.01 b
5% LSD	0.07	
d.f.	461	

For the growth rate of individual isolates there were significant differences ($p<0.05$) in the rate of growth at the different temperatures, with all the *S. sclerotiorum* isolates except L44 having a significantly faster growth rate ($p<0.05$) than the *S. subarctica* isolates at 28, 30 and 33°C (Table 5.5).

Table 5.5 - Mean mycelial growth rate (mm/hour) for five *S. sclerotiorum* isolates and five *S. subarctica* isolates at 25°C, 28°C, 30°C and 33°C. Numbers followed by different letters within a column are significantly different from each other ($p<0.05$).

		Growth Rate (mm/hour)			
Isolate	**Species**	**25°C**	**28°C**	**30°C**	**33°C**
Liel17a1	*subarctica*	1.46 b	0.52 b	0.30 b	0 a
IP10	*subarctica*	1.88 d	0.69 d	0.38 c	0 a
HE1	*subarctica*	1.63 c	0.50 ab	0.26 ab	0.04 ab
PS13	*subarctica*	2.03 fg	0.61 c	0.28 ab	0 a
KE8	*subarctica*	1.89 d	0.69 d	0.37 c	0.01 a
L44	*sclerotiorum*	1.37 a	0.44 a	0.23 a	0.08 b
C28	*sclerotiorum*	1.91 de	1.04 e	0.57 d	0.21 cd
L6	*sclerotiorum*	1.97 ef	1.31 f	0.77 f	0.26 cd
CE11	*sclerotiorum*	2.05 g	1.30 f	0.82 f	0.28 d
R28	*sclerotiorum*	2.04 g	1.33 f	0.65 e	0.20 c
5% LSD	0.06				
d.f.	429				

For the mean colony diameter after 46 hours there were significant differences ($p<0.05$) between species at each temperature (Table 5.6), with the largest colony size for both *S. sclerotiorum* and *S. subarctica* at 25°C (76.52 and 72.88 mm) and the smallest at 33°C (8.48 and 0.43 mm).

Table 5.6 - Mean colony diameter (mm/hour) after 46 hours for *S. sclerotiorum* and *S. subarctica* at 25°C, 28°C, 30°C and 33°C. Numbers followed by different letters within a row are significantly different from each other ($p<0.05$).

Temperature (°C)	***S. sclerotiorum* Colony Diameter (mm) After 41 hours**	***S. subarctica* Colony Diameter (mm) After 41 hours**
25	76.52 a	72.88 b
28	44.53 a	24.69 b
30	24.92 a	12.97 b
33	8.48 a	0.43 b
5% LSD	3.03	
d.f.	461	

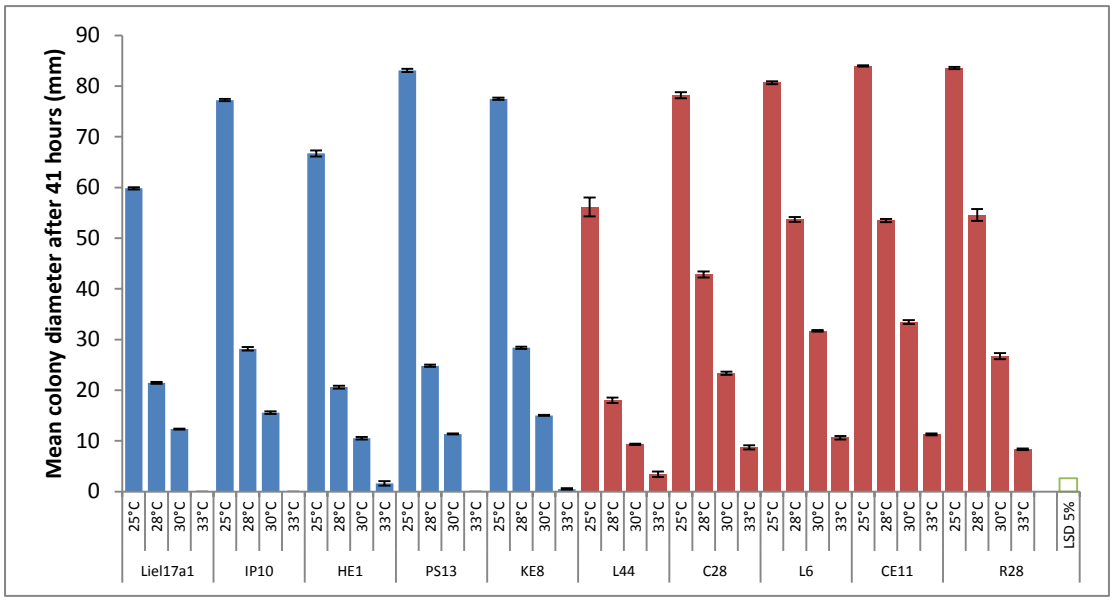

Figure 5.3 - Mean mycelial colony diameter (mm) of five *S. subarctica* isolates (shown in blue) and five *S. sclerotiorum* isolates (shown in red) after 46 hours at 25, 28, 30 and 33°C. Error bars show SEM.

At 28, 30 and 33°C, the mean colony diameter for all the *S. subarctica* isolates was significantly different ($p<0.05$) from all the *S. sclerotiorum* isolates, except for isolate L44. At 25°C, the mean colony diameter was significantly smaller ($p<0.05$) for L44 (56.17 mm) than for all other isolates (59.83 to 83.96 mm, Figure 5.3).

5.3.2 Effect of Conditioning Time Duration on Carpogenic Germination of *S. sclerotiorum* and *S. subarctica* Isolates

In the first of the experiments carried out to test the effect of conditioning (chilling) duration at 5°C on subsequent germination at 15°C for different *S. subarctica* and *S. sclerotiorum* isolates, a mean of 3.5 sclerotia (out of 20) for *S. sclerotiorum* isolate L6 had germinated in the treatment where sclerotia received no conditioning. In contrast, none of the *S. subarctica* isolates nor the other *S. sclerotiorum* isolate (L44) germinated without cold treatment. Mean germination for *S. sclerotiorum* isolate L6

increased with increasing conditioning duration, up to 100 days where the maximum germination was 14.6 sclerotia (Figure 5.4). For *S. sclerotiorum* isolate L44, germination was first observed after 20 days conditioning, with a mean of 2.5 sclerotia, and again germination increased with increasing conditioning time, up to 100 days with a maximum mean germination of 12.0 sclerotia (Figure 5.4). The mean number of sclerotia germinated for L6 was significantly greater than L44 at all conditioning durations, except for 100 days ($p<0.05$).

For the *S. subarctica* isolates, no germination was observed for conditioning durations of less than 60 days, and for the majority of *S. subarctica* isolates (HE1, HE4 and HE8) germination started after 80 days conditioning. Germination increased with conditioning duration and maximum germination was observed after 140 days (HE1 6.5; HE4 7.6; and HE8 7.1 sclerotia germinated). No germination was observed for HE3 below a duration of 140 days conditioning, and germination for this isolate was significantly less than all other isolates at all conditioning times ($p<0.05$, Figure 5.4). The germination of *S. sclerotiorum* isolate L6 was significantly greater than all the *S. subarctica* isolates at all conditioning times ($p<0.05$). Isolate L44 had significantly greater germination than all the *S. subarctica* isolates except after 140 days conditioning where it was only significantly greater than HE3 ($p<0.05$, Figure 5.4).

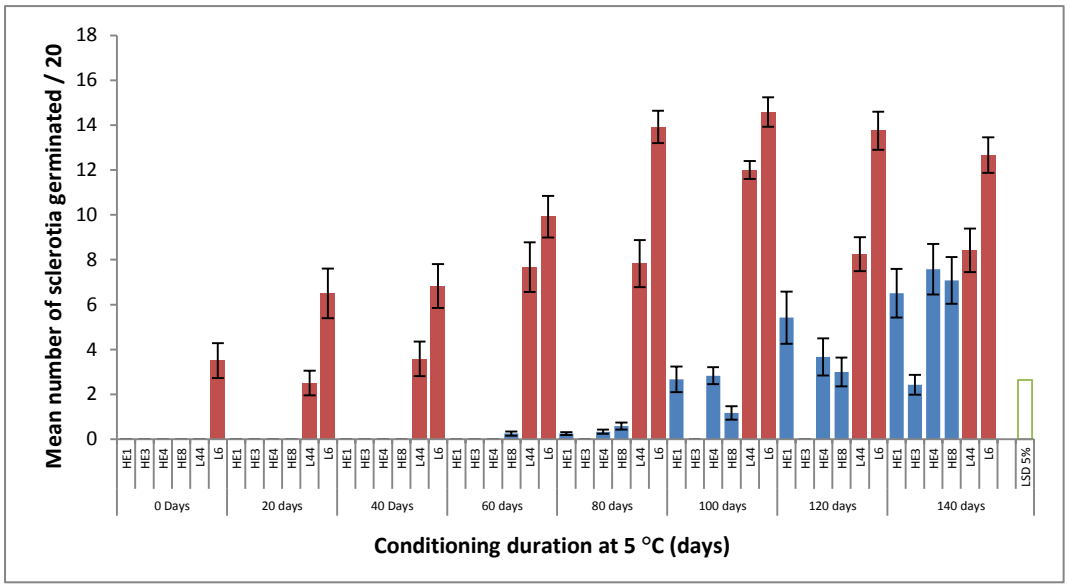

Figure 5.4 - Mean number of sclerotia of *S. subarctica* isolates HE1, HE3, HE4 and HE8 (shown in blue) and *S. sclerotiorum* isolates L6 and L44 (shown in red) germinated after different durations of conditioning at 5°C (0 to 140 days) followed by 21 days at 15°C. Error bars show SEM.

In experiment 2 which used a different range of *S. subarctica* isolates to compare with *S. sclerotiorum* isolate L6, there was no germination for any isolate in the treatment where sclerotia received no conditioning (Figure 5.5). Mean germination for L6 reached maximum (20 sclerotia) after 60 days conditioning. *S. subarctica* isolate LSA1 first germinated after 20 days conditioning, while the remaining *S. subarctica* isolates germinated after 60 days conditioning (Figure 5.5). The maximum mean germination for all the *S. subarctica* isolates was after 140 days conditioning (PS13 19.25; MH22 19.25; 210002N 9.25; and LSA1 19.5 sclerotia germinated). This was significantly greater than for any other conditioning duration ($p<0.05$). Isolate L6 showed significantly greater germination at 20 and 60 days conditioning duration compared to all the *S. subarctica* isolates, whereas after 100 days conditioning germination was significantly greater than only three of the four *S. subarctica* isolates (PS13, MH22 and 210002N). After 140 days conditioning germination of L6 was only significantly greater than 210002N ($p<0.05$) (Figure 5.5).

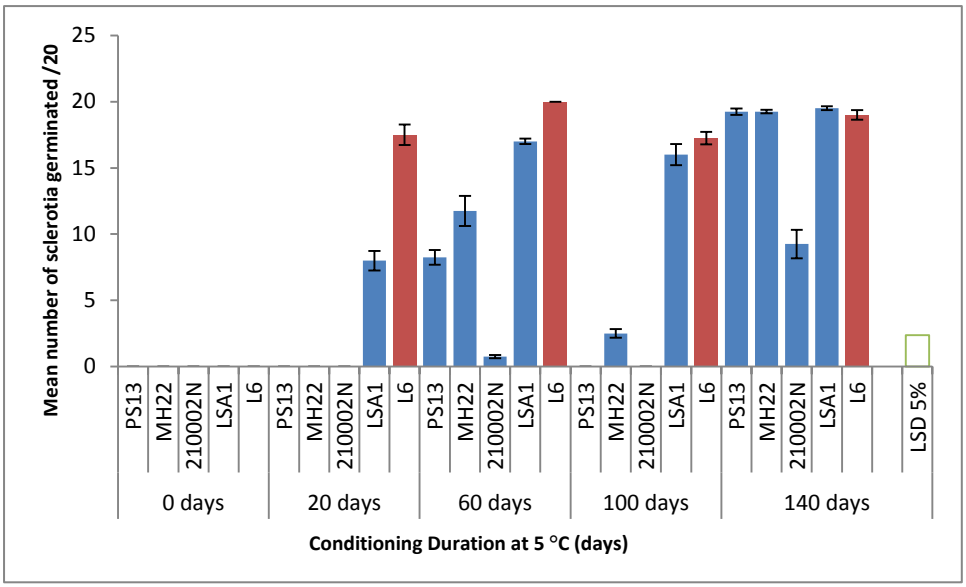

Figure 5.5 - Mean number of sclerotia of *S. subarctica* isolates PS13, MH22, 210002N and LSA1 (shown in blue) and *S. sclerotiorum* isolate L6 (shown in red) germinated after different periods of conditioning durations at 5°C (0 to 140 days) followed by 21 days at 15°C. Error bars show SEM.

5.4 Discussion

There has been no previous research concerning the effect of environmental factors on *Sclerotinia subarctica*, and hence this is the first time that the effects of temperature on mycelial growth and carpogenic germination of this pathogen have been investigated. As discussed in Chapter 2, the distribution of *S. subarctica* is more limited than the closely related *S. sclerotiorum*, with *S. subarctica* isolates frequently identified in Scotland and Norway, while in England this species has only been found in one location out of 22 sampled. A possible hypothesis for this distribution is that the climatic conditions at higher latitudes are more favourable for the survival, growth and sclerotial germination of *S. subarctica*. For example, climate data from 1981 to 2010 shows that Eastern Scotland has an annual average minimum temperature 2°C colder than Southern England, with between 10 and 45 more air frost days, and between 20 and 50 more ground frost days (Anonymous, 2014).

The mycelial growth at 5 to 25°C of the *S. subarctica* isolates used in the present study was similar to that observed for *S. sclerotiorum*, and is within the temperature range suggested by Le Tourneau (1979) of 0 to 30°C. When the cultures were incubated at 20 and 25°C the total mean colony diameter of all the *S. subarctica* isolates was the greatest, at 78.26 mm and 72.88 mm. Abawi and Grogan (1975) also recorded the greatest mycelial growth for *S. sclerotiorum* isolates from bean fields in New York at 20 and 25°C, of approximately 60 mm and 70 mm after 48 hours. However, they found no growth at 30°C, in contrast to the present study where all *S. sclerotiorum* and *S. subarctica* isolates grew at this temperature, and at 33°C two of the *S. subarctica* isolates grew. However, at both 30 and 33°C the mean colony

diameter for the *S. subarctica* isolates was significantly less than four out of the five *S. sclerotiorum* isolates. Wu *et al.* (2008) found that mycelial growth was greatest for both *S. sclerotiorum* (from lettuce and cauliflower in California) and *S. minor* (from lettuce in California and peanut in Oklahoma) isolates at 25°C, and also observed some growth at 30°C. They noted that growth of *S. sclerotiorum* was more rapid than *S. minor* at 20°C (11.1 mm/ day^{-1} compared to 7.1 mm/day^{-1}) and also at 25°C, but was almost equal at the lower temperature range 5 to 15°C. No significant difference in rate of growth at lower temperatures between *S. sclerotiorum* and *S. subarctica* were seen in this study, but was observed at the higher temperatures where both the rate of growth and the mean colony diameter were significantly greater for *S. sclerotiorum*.

The results therefore indicate that *S. sclerotiorum* may have a higher temperature tolerance for mycelial growth than *S. subarctica.* This might be one reason explaining different geographical distributions of the two species and indicates potential adaptation to local environments. *S. sclerotiorum* occurs worldwide and is adapted to environments over a broad temperature range (Purdy, 1979), while *S. subarctica* appears to be adapted to a smaller range of regions with colder weather. However, given the diversity of both species (as discussed in Chapter 2) further work with a greater number of isolates of both species is required to verify these findings.

The conditioning experiments suggested that the *S. subarctica* isolates tested required longer durations of conditioning at 5°C than the *S. sclerotiorum* isolates, as in contrast to *S. sclerotiorum* no germination occurred for the *S. subarctica* isolates for conditioning durations of less than 20 days. The second experiment was not repeated due to time constraints caused by sampling, identification and

characterisation of *S. subarctica* isolates. Further experiments with these and additional isolates are required for more definitive conclusions to be drawn. However, all the isolates for this experiment were from different geographical regions (Eastern Scotland, Norway and Sweden), while in the first experiments all the *S. subarctica* isolates originated from one location in Herefordshire, England. As isolates from different geographic regions have been found to vary in their need for a period of cold conditioning (Huang & Kozub, 1991; Bolton *et al.*, 2006) it is likely that differences between *Sclerotinia* spp. and between isolates of the same species in the length of conditioning time required for carpogenic germination to occur is due to genetic variability, and related to local adaptations to environmental and ecological conditions (Clarkson *et al.*, 2007).

For all the *S. subarctica* isolates used in the conditioning experiments the longest chilling time of 140 days at 5°C resulted in the greatest germination at 15°C. This was significantly different from the two *S. sclerotiorum* isolates used in the same experiments, both of which showed the greatest germination after 100 days at 5°C. A general increase in the numbers of sclerotia germinating was seen for all isolates as the conditioning duration increased from 0 to 100 days, and this increase in germination continued for the *S. subarctica* isolates up to 140 days. An increase in germination with increased conditioning duration has been observed previously for *S. sclerotiorum*, where sclerotia for an isolate from snap beans in New York were incubated at 8°C for up to eight weeks (Dillard *et al.*, 1995). Clarkson *et al.* (2007) found that increasing the duration of conditioning at 4°C for two *S. sclerotiorum* isolates (from lettuce in Norfolk and Cheshire) decreased the length of time to germination, but there was little decrease in time to germination for conditioning durations of more than 100 days, which is consistent with the results from the

present study. Mylchreest and Wheeler (1987) found a wide variation (6 to 20 weeks) for 35 *S. sclerotiorum* isolates (from 17 different hosts in England and Scotland) to germinate carpogenically at 10°C, after conditioning at 4°C. They suggested that modifications of their method would be required to determine the optimum temperature regime for each individual isolate. The ideal temperature for conditioning to ensure rapid carpogenic germination of *S. sclerotiorum* sclerotia varies between isolates, with Dillard *et al.* (1995) suggesting a temperature range of 4 to 16°C, and Clarkson *et al.* (2007) finding conditioning at 4 to 10°C reduced mean germination times in comparison with conditioning at 13 to 20°C. It is possible therefore, that a lower conditioning temperature would reduce the conditioning duration required by *S. subarctica* sclerotia to germinate carpogenically.

The size of sclerotia produced by *S. subarctica* may have an impact both on the length of conditioning time required for carpogenic germination and sclerotial survival (Ben-Yephet *et al.*, 1993; Winton *et al.*, 2006). *S. sclerotiorum* isolate L44 was deliberately used as a comparison with the *S. subarctica* isolates as it generally produces larger sclerotia, close in size to those produced by *S. subarctica.* However, L44 had significantly more sclerotia germinating than the *S. subarctica* isolates for different conditioning durations, except at 140 days. L44 also required 40 days less conditioning to reach maximum germination, but conditioning for more than 100 days unexpectedly caused a significant reduction in germination. Interestingly, in the mycelial growth experiments at higher temperatures L44 also had a similar growth rate and colony size to the *S. subarctica* isolates. Further work should be carried out on *S. sclerotiorum* and *S. subarctica* isolates deliberately selected to have different sized sclerotia, to determine both the optimum duration and temperature of cold conditioning for rapid germination for both species. Additionally, sclerotia from

individual isolates could be also graded by size to see whether this affects the conditioning time required, particularly given that the processes involved in carpogenic germination of sclerotia are not well understood (Le Tourneau, 1979).

6. General Discussion

Sclerotinia disease caused by *Sclerotinia sclerotiorum* continues to be a major problem in many vegetable crops, causing yield losses in the field, and post-harvest epidemics in storage, particularly in carrots. There have been limited studies examining the numbers of sclerotia produced by different *S. sclerotiorum* isolates on a range of crops, an important factor in determining the levels of inoculum in the field following disease outbreak, and few have examined the susceptibility of different carrot accessions to *S. sclerotiorum*. The pathogen is very diverse in the UK but little is known of how this impacts on disease development and control, and there has only been only one population study for the related species *S. subarctica,* with isolates from Alaska (Winton *et al.*, 2006). This species was identified in England in 2010, and its host range and geographical distribution in the UK is unknown.

The effectiveness of current control measures for Sclerotinia disease is threatened by the reduction in active ingredients available, and the increased risk of pathogen resistance to the fungicides used. Furthermore, growers need to use integrated disease management systems in order for them to abide by the regulations implemented under the Sustainable Use Directive (Pendergrast, 2013). In an integrated disease management system, using *Brassica* crops for biofumigation could potentially provide control of *S. sclerotiorum* by directly affecting the viability of sclerotia, but there have been few studies examining this.

The aims of this research, as outlined in Chapter 1, were to evaluate biofumigation treatments for control of Sclerotinia disease caused by *S. sclerotiorum,* and to assess pathogen diversity, with a focus on *S. subarctica*. The specific objectives were to:

1. Determine the effect of biofumigants on the germination and survival of sclerotia of *Sclerotinia sclerotiorum*.
2. Evaluate carrot varieties for susceptibility to *Sclerotinia sclerotiorum* and quantify production of sclerotia by different *S. sclerotiorum* isolates.
3. Investigate the population diversity and epidemiology of *Sclerotinia subarctica.*

The results from the microcosm and *in vitro* experiments clearly showed that volatiles released from biofumigation crops have a direct inhibitory effect on the mycelial growth and carpogenic germination of *S. sclerotiorum* sclerotia. The most effective biofumigation crop for inhibiting carpogenic germination varied depending on whether the volatiles released from the biofumigant crops were in direct contact with the sclerotia (as in the microcosm experiments) when the most effective crop was *Raphanus sativus* 'Terranova', or in the vapour phase (as in the *in vitro* experiments) when the most effective crop was *B. juncea* 'Vittasso'. Additionally, a different brown mustard (*B. juncea* 'Caliente 99') was most the effective in inhibiting mycelial growth, indicating differences in the susceptibility of the different fungal tissues, i.e. mycelium vs. sclerotia. Therefore, using microcosm experiments is a more suitable method for establishing the most effective biofumigant crops against resting propagules of soil borne fungal pathogens, as it tests the biofumigants in an assay which simulates how they would be applied in a field situation, against sclerotia rather than mycelium. The *in vitro* assays were, however, invaluable for establishing the direct effect of volatiles released from the biofumigant crops.

The reduction in the effectiveness of biofumigation for larger *S. sclerotiorum* sclerotia highlights how this control method must be viewed as part of an integrated disease management system, and not a stand-alone treatment. The level of control achieved by biofumigation alone means it cannot be brought in as a direct replacement for chemical soil fumigants or foliar fungicide sprays, and should instead be used in combination with crop rotations, biological controls (such as Contans WG), and disease forecasting. Further research needs to look at combining biofumigation with Contans WG to establish how they will work together and to ensure that the volatiles released from biofumigant crops do not have an adverse effect on the biological control used. Additionally, a delayed application of Contans WG, some time after biofumigants have been incorporated, may help to reduce any negative impact of ITCs on the biological control agent.

The unexplained effectiveness of the low GSL *B. napus* 'Temple' needs to be further investigated to understand which compounds are responsible for this cultivar inhibiting germination of *S. sclerotiorum* sclerotia. Once the mechanism behind this inhibition is understood, future research could look beyond only using high GSL *Brassica* spp. for biofumigation. Instead, a range of crops with different compounds which are effective at inhibiting sclerotial germination could be used in the field. This would not only widen this area of research considerably, but also help to prevent pathogens such as *S. sclerotiorum* becoming resistant to the volatiles released following the hydrolysis of GSLs.

Whilst finding control measures which target *S. sclerotiorum* sclerotia, rather than ascospores, will help to reduce the levels of inoculum in the soil, quantifying the number of sclerotia produced by different crop accessions and breeding a variety that

produces small sclerotia in low numbers is also important. The results from the carrot root inoculations showed that the number of sclerotia produced on carrot roots was significantly affected by the *S. sclerotiorum* isolate, which is possibly related to the survival strategy of the individual isolate. However, the results also showed that the weight of individual sclerotia produced by different isolates was influenced by carrot accession, but not by *S. sclerotiorum* isolate. This may be due to the nutritional content of plant tissue, or due to variations in the permeability and integrity of root cell membranes. Further work could utilise the results from this large scale screening to select carrots which sustain low numbers of sclerotia. Additionally, the carrot plant and detached leaf inoculations showed significant differences in the rate of lesion progression of *S. sclerotiorum* on different carrot accessions, indicating differences in susceptibility to the pathogen. Detached leaf inoculations could be used for resistance screening, but further work is needed to assess a wider range of accessions, as well as to determine whether inoculations with ascospores could provide reliable and reproducible results, and also to determine whether the results on detached leaves are comparable with inoculations on whole carrot plants.

Additionally, future work should look at architecture based resistance, using ascospores as inoculum to determine the extent to which canopy architecture can facilitate disease escape. To carry out this work in a controlled environment it would be ideal to use a wind tunnel where ascospores could be distributed at specific wind speeds through a crop canopy. However, the quantity of ascospores required for these types of experiment would be time consuming, and therefore expensive, to produce. Alternatively, field based experiments could be conducted with pre conditioned sclerotia placed amongst the carrot crop to ascertain differences in

disease incidence amongst the varying foliage types. Any future breeding programme would also need to take in account the numerous traits required by both carrot producers and consumers. When balancing desirable traits with disease resistance it should be taken into consideration that while the carbohydrate content of the carrot may be an important factor in the numbers of sclerotia produced, it may also affect taste, an important trait for consumers.

The diversity of *S. sclerotiorum* also needs to be taken into account when screening crop plants for susceptibility. The research carried out in this study only used two *S. sclerotiorum* genotypes for the root inoculations, and one genotype for the whole plant and detached leaf inoculations. Additionally, the results from this study showed that the related species *S. subarctica* is present on numerous crop and wild hosts in Eastern Scotland, following its identification in England in 2010 (Clarkson *et al.*, 2010). This species needs to be included when screening for susceptibility, as its host range has not been published and it was often found occurring in sympatry with *S. sclerotiorum.*

S. subarctica microsatellite haplotypes identified in this research were shown to be shared between Scotland and Norway, and between crop plants and meadow buttercup. However, the English population did not share any microsatellite haplotypes with any other population, and they clustered together by themselves in the minimum evolution spanning tree. Additionally, they were significantly differentiated from the Scottish and Norwegian populations, they had the lowest mean gene diversity across all the microsatellite loci, and the lowest gene diversity for all but two of the eight loci. These findings indicate that this *S. subarctica* population in England may be isolated and inbred, as seen for *S. sclerotiorum*

populations on Lesser Celandine in Norway by Kohn (1995), and on meadow buttercup in Wales by Clarkson *et al.* (2013). However, it is not isolated from agricultural production, or other buttercup meadows fields, yet has not been found in sampling conducted in the surrounding area. Further sampling in surrounding fields should be carried to determine whether this species can be found in any other wild hosts or crop plants in the area. This population in England could also be compared with further *S. subarctica* isolates to assess whether it has any other attributes of an isolated population.

In contrast to the *S. subarctica* microsatellite haplotypes, analysis of the Norwegian *S. sclerotiorum* isolates showed that no haplotypes were shared with English, Scottish or Australian *S. sclerotiorum* populations. It may be that the two species arrived in Norway and England via different routes, with *S. subarctica* spreading to Scotland from Northern regions where it has been previously identified (Alaska and Norway), and *S. sclerotiorum* arriving in each country at around the same time but by separate paths due to its global distribution. Additionally, the rate of evolution of microsatellites in both species may be different and could influence the interpretation of the population structure of *S. subarctica.* The latest tools for investigating population structure can now utilise the genome of *S. sclerotiorum* (Amselem *et al*., 2011), using SNPs to screen for markers to identify divergence genes in haplotypes from different populations (Rollins *et al.*, 2014).

As well as the possibility of different routes into different countries, and the question of the differences in the rate of evolution of microsatellite markers for *Sclerotinia* spp., *S. sclerotiorum* occurs worldwide and is adapted to environments over a broad temperature range (Purdy, 1979), while *S. subarctica* appears to be adapted to a

limited range of regions with colder climates. In this study it was found that the mycelial growth of *S. subarctica* isolates is similar to *S. sclerotiorum* isolates between 5 and 25°C, but significantly slower at 28 to 33°C. This indicates that they may have a lower temperature tolerance for mycelial growth than isolates of the related species *S. sclerotiorum*, possibly due to adaptations by each species to their local environments. The isolates of *S. subarctica* were also found to require a longer period of cold conditioning at 5°C for rapid carpogenic germination than *S. sclerotiorum*, although the amount of time required varied between isolates. Further work is need to establish the optimum length of time and temperature for cold conditioning of *S. subarctica* isolates to achieve rapid germination. This should be conducted using a wider range of both *S .subarctica* and *S. sclerotiorum* isolates, given the variation not only between but also within species. Also, given the lack of knowledge surrounding the mechanisms and processes involved in carpogenic germination of sclerotia, screening a range of sclerotia sizes, both between and within isolates, would help to establish the importance of sclerotial size on the conditioning time required for carpogenic germination.

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