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The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headline

In trials biofumigant crops reduced germination of *S. sclerotiorum* sclerotia by up to 70%, but were less effective against larger sclerotia. *S. subarctica*, a related species, has been identified on numerous crop hosts in Scotland.

Background

In the UK, carrot growers suffer estimated annual crop losses in excess of six million pounds due to Sclerotinia disease, with marketable yield predicted to be reduced by one tonne per hectare for each 1% increase in diseased roots (McQuilken, 2011; AHDB Horticulture Factsheet 19/11). Additionally, lettuce growers in the UK face potential crop losses of 15%, worth an estimated £12 million, due to Sclerotinia disease (DEFRA, 2013). *S. sclerotiorum* has a host range of over 400 plant species including important horticultural crops such as oilseed rape, lettuce, potato, peas, beans, sunflower, celery and some vegetable brassicas such as cabbage and swede. Many wild hosts and broad leaved weed species can also be infected including dandelion, fat hen, thistle and buttercup species.

The Pathogen - *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum (Lib.) de Bary is a plant pathogenic fungus that affects many economically important crops (Hegedus & Rimmer, 2005), with a world-wide distribution (Purdy, 1979) and a wide host range of over 400 plant species (Boland & Hall, 1994). Crops susceptible to sclerotinia disease include lettuce, oilseed rape, beans, peas, potatoes and carrots (Saharan & Mehta, 2008).

The long term survival structures for *S. sclerotiorum* are small black resting bodies called sclerotia (Willetts & Wong, 1980) which, when brought close to the soil surface, germinate carpogenically to produce mushroom-like apothecia. These then release air-borne ascospores which infect plants, upon which further sclerotia are formed and are returned to the soil (Bolton *et al.*, 2006). Sclerotia can also geminate myceliogenically to produce hyphae which can attack plant tissues directly (Bardin & Huang, 2001). The number of sclerotia produced by *S. sclerotiorum* on different plant tissues is variable and is an important factor in determining the inoculum levels in soil following an infected crop (Leiner & Winton, 2006).

A related species *S. subarctica* has been found in the UK (Clarkson *et al.*, 2010) on meadow buttercup and also more recently in a carrot crop in Scotland. Previously this pathogen has only been found in Norway (Holst-Jensen *et al.*, 1998) and Alaska (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. One aim of this work was therefore to establish the distribution and ecology of this species in the UK, on both crops and wild hosts.

Sclerotinia on Carrot

This project will focus on sclerotinia disease on carrots, as it 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). Previous research has shown differences in aggressiveness between isolates of *S. sclerotiorum* on carrots (Jensen *et al.*, 2008) and other crops. Possible pre-harvest resistance has been shown in glasshouse trials with carrots, (Foster *et al.*, 2008) although it is thought 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).

Control of Sclerotinia Disease

The most common approach to control of *S sclerotiorum* is to apply fungicides with the aim of killing ascospores before they infect plants, with the best protection obtained by spraying before canopy closure (McQuilken, 2011). Timing of spraying is critical to the effectiveness of protection provided by fungicides, and hence the extent of control by fungicides can be variable. Also, some of the effective active ingredients in fungicides currently 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). Clipping of carrot foliage to prevent lodging and hence plant to plant spread of infection between beds has also been found to protect against Sclerotinia disease in carrots (Kora *et al.*, 2005), as does applying optimum amounts of nitrogen to limit canopy growth and lodging (McQuilken, 2011). New control methods to reduce the viability of *S sclerotiorum* sclerotia in the soil would therefore be useful as a more long-term and sustainable control strategy.

Various non-organic soil amendments have been shown to inhibit sclerotial germination, such as potassium bicarbonate (Ordonez-Valencia *et al.*, 2009) and calcium cyanamide (Perlka®) (Huang *et al.*, 2006), but these are considered expensive by growers. There has

also been much research on biological control, with the fungus *Coniothyrium minitans* which parasitizes the sclerotia of *S. sclerotiorum,* now commercialised and marketed as Contans WG, although it does not always provide consistent results under field conditions (Fernando *et al.*, 2004).

It is thought that using *Brassica* green manure crops for biofumigation can potentially provide control against Sclerotinia disease (Porter *et al.*, 2002), but further work is needed to establish which crops work against which pathogens, as *Brassica juncea* (brown mustard) was found to be the only cruciferous plant to delay germination of *S. sclerotiorum* sclerotia in one study, (Smolinska & Horbowicz, 1999) yet *Brassica oleracea* var. *caulorapa* (kohlrabi) reduced mycelial growth in another (Fan *et al.*, 2008). Another study found that a blend of *Brassica napus* (oilseed rape) and *Brassica rapa* (field mustard) reduced the viability of sclerotia in the soil (Geier, 2009).

The main aim and objectives of the project were:

Aim: To identify potential new soil treatments for control of sclerotinia disease and to assess pathogen diversity.

Objectives:

i. To determine the effect of different biofumigation crops on the germination and survival of sclerotia of *Sclerotinia sclerotiorum*.
ii. To evaluate carrot varieties for susceptibility to *Sclerotinia sclerotiorum* and quantify production of sclerotia by different *S. sclerotiorum* genotypes.
iii. To investigate the incidence, diversity and epidemiology of *Sclerotinia subarctica.*

Summary

Objective 1: To determine the effect of biofumigation on the germination and survival of sclerotia of *Sclerotinia sclerotiorum*

Results from laboratory experiments showed that some biofumigant crops can significantly reduce carpogenic germination of *S. sclerotiorum* (Figure 1), with the most effective in soil box tests being *Raphanus sativus, which* reduced germination by 70%. The results from *in vitro* experiments testing direct effects of biofumigants on mycelial growth and carpogenic germination suggested that this reduction was caused by fungitoxic isothiocyanates being released from the plant material. Results from polytunnel experiments showed that three *Brassica juncea* biofumigation crops reduced germination of *S. sclerotiorum* sclerotia, but

this effect was not statistically significant, most likely due to insufficient germination in the control plots. Further work on methodology is required to enable assessment of biofumigant crops in a non-laboratory setting. An HPLC analysis showed a clear difference in the glucosinolate quantities in biofumigant crops grown at different times of year, with later sown crops (harvested June to November) having generally lower levels.

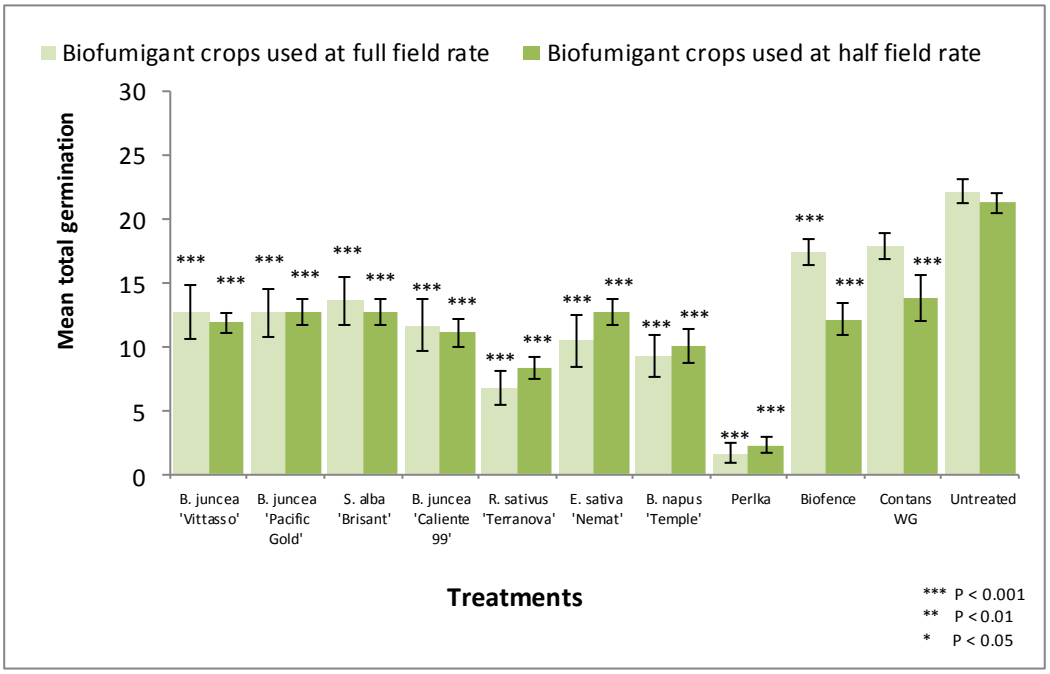


Figure 1 – The effect of biofumigant crops at full field and half field rates, *Brassica napus* 'Temple', Perlka®, Biofence and Contans WG on final germination of *S. sclerotiorum* sclerotia after 150 days in soil box trials.

Objective 2: To evaluate carrot varieties for susceptibility to *Sclerotinia sclerotiorum* and quantify production of sclerotia by different *S. sclerotiorum* genotypes

Carrot root inoculation experiments showed that the number of sclerotia produced on carrot roots is significantly affected by *S. sclerotiorum* isolate, possibly related to the survival strategy of the individual isolate. The weight of individual sclerotia produced by different isolates is 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. Some of the cultivars in the experiment produced very few sclerotia for either *S. sclerotiorum* isolate and may therefore be suitable for future breeding work. Whole carrot plant inoculation tests indicated that 'Little Finger' and 'Brasilia' may also be suitable cultivars for such a program, as they showed the slowest disease progression down the petiole compared to other carrot varieties such as Nairobi, Chantenay, Eskimo

and Narbonne. However, detached leaf inoculations produced results which were not correlated with the whole plant inoculations, with 'Brasilia' having the fastest rate of disease progression. This latter assay may not therefore be suitable for screening carrot varieties for resistance to *S. sclerotiorum.*

Objective 3: To investigate the diversity and epidemiology of *Sclerotinia subarctica*

S. subarctica was identified in East Scotland on numerous crop hosts in several locations whereas previously it had only been found in one location in England, on meadow buttercup. Population structure analysis showed that *S. subarctica* is genetically diverse in Scotland, and shared genotypes with isolates from Norway. The limited geographical distribution of *S. subarctica* may be due to its lower tolerance to high temperatures for mycelial growth compared with *S. sclerotiorum*, as well as its requirement for a longer period of cold conditioning before carpogenic germination can occur.

Although the full host range and epidemiology of *S. subarctica* are unknown, production of apothecia from germinating sclerotia has been observed in the lab and we assume therefore that ascospores are the main inoculum sources as for *S. sclerotiorum*. It is therefore very likely that fungicides applied for control of *S. sclerotiorum* will also control *S. subarctica.*

Conclusions

- In soil box experiments *Raphanus sativus* 'Terranova' reduced carpogenic germination of *S. sclerotiorum* sclerotia by 70% in comparison to the untreated control which compares well to the positive control Perlka® which reduced germination by 93%.
- Following inoculation with *S. sclerotiorum*, roots from some carrot lines produced very few sclerotia while others from whole plant tests showed slow disease progression and could therefore be used in future breeding programs.
- *S subarctica* is present on numerous crop and wild hosts in East Scotland, Norway and Sweden, often occurring in sympatry with *S. sclerotiorum*. *S. subarctica* genotypes were shared between Scotland and Norway, and between crop plants and meadow buttercup.
- Mycelial growth experiments indicated that *S. subarctica* has a lower tolerance to high temperatures for mycelial growth compared with *S. sclerotiorum*, as well as

requiring a longer period of cold conditioning at 5°C for rapid carpogenic germination, which may indicate adaptation colder climates.

Financial Benefits

Given the losses experienced in carrot and other susceptible crops, the potential financial benefits of reducing *S. sclerotiorum* inoculum through biofumigation or use of less susceptible cultivars could be substantial in the future. The next step is to confirm the efficacy of biofumigation in the field and to develop an integrated approach to Sclerotinia control which could include other techniques such as disease forecasting, canopy clipping (for carrot) and biological control using Contans.

Action Points

Biofumigation may be a useful addition to an integrated disease management system for *Sclerotinia sclerotiorum* - further polytunnel and field experiments are required to establish the best time of year to grow the biofumigation crop to maximize glucosinolate levels and biomass, as well as to determine the most effective method of incorporation.

SCIENCE SECTION

Introduction

The Pathogen - *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum (Lib.) de Bary is a plant pathogenic fungus that affects many economically important crops (Hegedus & Rimmer, 2005), with a world-wide distribution (Purdy, 1979) and a wide host range of over 400 plant species (Boland & Hall, 1994). Due to the large host range, the symptoms caused by *S. sclerotiorum* vary, but the white fluffy mycelial growth is an early symptom. Pale or dark brown lesions may be seen on the base of stems of herbaceous plants, often quickly covered by white mycelium, or infection may begin on a leaf and move into the stem (Saharan & Mehta, 2008). Multiple genotypes of *S. sclerotiorum* have been identified in the UK, with one genotype being found more frequently than the rest, at different locations and on different crops (Clarkson *et al.*, 2013).

The long term survival structures of *S. sclerotiorum* are small black resting bodies called sclerotia (Willetts & Wong, 1980) which when brought close to the soil surface germinate carpogenically to produce mushroom-like apothecia. These then release air-borne ascospores which infect plants, upon which further sclerotia are formed and are returned to the soil (Bolton *et al.*, 2006). Sclerotia can also geminate myceliogenically to produce hyphae which can attack plant tissues directly (Bardin & Huang, 2001). *S. sclerotiorum* therefore has both airborne and soilborne phases. The longevity of sclerotia is variable, being influenced by many factors including the time and depth of burial (Duncan *et al.*, 2006), and soil type (Merriman, 1976). The number of sclerotia produced by *S. sclerotiorum* on different plant tissues is also variable and is an important factor in determining the inoculum levels in soil following an infected crop. An infected cabbage head was found to produce 250 to 500 sclerotia, (Leiner & Winton, 2006) while an infected carrot root produced up to 30 (Jensen *et al.*, 2008).

A related species *Sclerotinia subarctica* has recently been identified in the UK (Clarkson *et al.*, 2010) after previously only being found in Norway on wild hosts (Holst-Jensen *et al.*, 1998) and on vegetable crops in Alaska (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. Further work is therefore required to establish the distribution and ecology of this species in the UK, on both crops and wild hosts.

Sclerotinia on Carrots

S. sclerotiorum has a host range of over 400 plant species including important horticultural crops such as oilseed rape, lettuce, potato, peas, beans, sunflower, celery and some vegetable brassicas such as cabbage and swede. Many wild hosts and broad leaved weed species can also be infected including dandelion, fat hen, thistle and buttercup species. This project focused on Sclerotinia disease on carrots, as it 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). In the UK, carrot growers suffer estimated annual crop losses in excess of six million pounds due to Sclerotinia disease, with marketable yield predicted to be reduced by one tonne per hectare for each 1% increase in diseased roots (McQuilken, 2011; AHDB Horticulture Factsheet 19/11). Additionally, lettuce growers in the UK face potential crop losses of 15%, worth an estimated £12 million, due to Sclerotinia disease (DEFRA, 2013).

Sclerotinia disease is a particular problem in temperate regions where carrots are stored for long periods (Kora *et al.*, 2005a). Previous research has shown differences in aggressiveness between isolates of *S. sclerotiorum* on carrots (Jensen *et al.*, 2008) which has also been demonstrated in other crops (e.g. Mei *et al.*, 2011). Infection 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 is suggested that under field conditions the pathogen enters the root via the crown of the plant (Jensen *et al.*, 2008), and experiments have shown 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.*, 1989).

Possible pre-harvest resistance has been shown in glasshouse experiments 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). However, it is thought 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).

Control of Sclerotinia Disease

In the absence of resistant crop cultivars control methods for Sclerotinia disease include fungicides, soil solarisation, biofumigation and cultural practices (Bardin & Huang, 2001). Fungicides are applied to kill ascospores before they infect plants, with the best protection obtained in carrots by spraying before canopy closure (McQuilken, 2011). Some of the effective active ingredients in fungicides currently 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). Even so, 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 (Wang *et al.*, 2009) (Liu *et al.*, 2009). Similarly, no resistance was found in Australian isolates from bean, where boscalid was the only fungicide registered for control (Jones *et al.*, 2011) and in Europe it was found that there has been no change in *S. sclerotiorum* sensitivity to boscalid since its introduction (Stammler *et al.*, 2007). However, *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), but none have yet been reported in the UK. No cross resistance was found between fludioxonil and carbendazim, suggesting that this active can be used in areas of carbendazim resistance (Kuang *et al.*, 2011).

Various non-organic soil amendments have been shown to inhibit sclerotial germination, such as potassium bicarbonate (Ordonez-Valencia *et al.*, 2009) and calcium cyanamide (Perlka®) (Huang *et al.*, 2006). Simply burying sclerotia to prevent carpogenic germination is also effective at reducing disease (Williams & Stelfox, 1980), but a subsequent cultivation could bring viable sclerotia back to the soil surface (Mitchell & Wheeler, 1990). Clipping of carrot foliage to prevent lodging and hence plant to plant spread of infection between beds was found to protect against Sclerotinia disease in carrots (Kora *et al.*, 2005), as does applying optimum amounts of nitrogen to limit canopy growth and lodging (McQuilken, 2011). Soil solarisation reduces the numbers of sclerotia in the soil, and also reduces the ability of surviving sclerotia to germinate carpogenically (Phillips, 1990).

Inhibition or suppression of carpogenic germination of *S. sclerotiorum* sclerotia has been achieved using various organic soil amendments, 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 mycoparasites such as *Trichoderma* spp. or *Coniothyrium minitans* (Huang *et al.*, 2005). There has been much research into these biological control agents, with *C. minitans* being commercialised and marketed as Contans WG, although it has not always provided consistent results under field

conditions (Fernando *et al.*, 2004). However, it was found to significantly reduce carpogenic germination when used in conjunction with a commercial NPK fertiliser (Yang *et al.*, 2011).

Biocidal activity of plant extracts such as glucosinolates have been reported in literature since the 19th century. Many *Brassica* spp. produce significant levels of glucosinolates, a secondary metabolite which themselves are not fungitoxic (Manici *et al.*, 1997), but are hydrolysed in the presence of water and endogenous myrosinase enzyme to release isothiocyanates (ITCs) which have a wide range of biocidal characteristics (Kurt *et al.*, 2011) and are acutely toxic to several pathogenic fungi (Chew, 1987). It has been found that even when ITCs are present in concentrations too low to suppress mycelial growth they can delay fungal sporulation (Drobnica *et al.*, 1967) and some of these natural ITCs are superior to the synthetic fumigant metham sodium (methyl isothiocyanate) in their ability to suppress fungi (Sarwar *et al.*, 1998).

The definitive mode of action of ITCs inhibiting fungal growth and other microorganisms is not known, but some hypotheses are:

i. Inactivation of intracellular enzymes by oxidative breakdown of -S-S bridges (Zsolnai, 1966)
ii. Uncoupler action of oxidative phosphorylation suggested from the inhibition of oxygen uptake of yeasts by ITCs (Kojima & Oawa, 1971)
iii. Inhibition of metabolic enzymes by thiocyanate radical, indicated as a degradation product of ITCs (Banks *et al.*, 1986)

Brassica green manures which are macerated and ploughed back into the soil, a process termed 'biofumigation', can potentially have at least two modes of action against plant pathogens; a direct effect of the ITCs, or an indirect effect of stimulating antagonistic (beneficial) organisms (Matthiessen & Kirkegaard, 2006). Using biofumigation crops 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). A study on a blend of *Brassica napus* and *Brassica campestris* showed a reduction in the level of viable sclerotia in the soil (Carr, 2003), but issues surrounding methods and rates of incorporation need to be resolved in order to gain consistent results (Geier, 2009), particularly as synthetic pure ITCs have been shown to reduce sclerotial viability *in vitro* (Kurt *et al.*, 2011). Also, further work is needed to establish which crops work against which pathogens, as *Brassica 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 (Smolinska & Horbowicz, 1999), yet *Brassica oleracea* var. *caulorapa* inhibited mycelial growth by 89.5% in another (Fan *et al.*, 2008).

The aim and objectives of this project were:

Aims: To identify potential new soil treatments for control of Sclerotinia disease and to assess pathogen diversity.

Objectives:

i. To determine the effect of different biofumigation crops on the germination and survival of sclerotia of *Sclerotinia sclerotiorum*.
ii. To evaluate carrot varieties for susceptibility to *Sclerotinia sclerotiorum* and quantify production of sclerotia by different *S. sclerotiorum* genotypes.
iii. To investigate the incidence, diversity and epidemiology of *Sclerotinia subarctica.*

Objective 1: Biofumigation as a Control Measure for *Sclerotinia sclerotiorum*

Biofumigation Soil Box Experiments

Materials and Methods

Soil box experiments (six in total) were set up to test the effect of 10 biofumigant and other soil treatments on the carpogenic germination of *S. sclerotiorum* sclerotia (Table 1). All biofumigant crops were used at either half or full field rate dry weight equivalents. Positive controls (Perlka® and Contans WG) and biofumigant treatment Biofence (mustard meal pellets) were used at full field rate to provide comparisons with biofumigation crops. Oilseed rape 'Temple' was used as a low glucosinolate *Brassica* control.

Table 1- Summary of treatments and rates used in soil box biofumigation experiments

Treatments	Full Field Rate (per soil box)	Half Field Rate (per soil box)
1. *Brassica juncea* 'Vittasso'	6g	3g
2. *Brassica juncea* 'Pacific Gold'	6g	3g
3. *Sinapis alba* 'Brisant'	6g	3g
4. *Brassica juncea* 'Caliente 99'	6g	3g
5. *Raphanus sativus* 'Terranova'	6g	3g
6. *Eruca sativa* 'Nemat'	6g	3g
7. *Brassica napus* 'Temple'	6g	3g
8. Perlka® (Calcium cyanamide)	0.43g	0.43g
9. Biofence (mustard meal pellets)	1.4g	1.4g
10. Contans WG (*Coniothyrium minitans*)	0.4g	0.4g
11. Untreated	-	-

All crops were grown in pots in a polytunnel and harvested within two weeks of first flowering. They were then dried at 80°C for 24 hours, milled to a fine powder and stored in sealed bags at -20°C. Compost (John Innes No 1) for use in experiments was passed through a 4 mm sieve and pasteurised by autoclaving at 110°C for 30 minutes. Sclerotia of *S. sclerotiorum* isolate L6 were produced by inoculating wheat grain in flasks with mycelial agar plugs and incubating them at 18°C for six weeks. The sclerotia were harvested by floating off the wheat grain, and dried overnight in a laminar flow cabinet. These sclerotia were then conditioned in pasteurised compost (30% moisture content) at 5°C for 40 days to enable rapid and reliable carpogenic germination.

Each biofumigant/soil treatment was mixed with pasteurised compost and 350g of the compost/treatment mixture placed into a 600ml clear plastic box. Preconditioned sclerotia (30) were laid out in a grid pattern (six by five) before adding another 50g of the mixture to

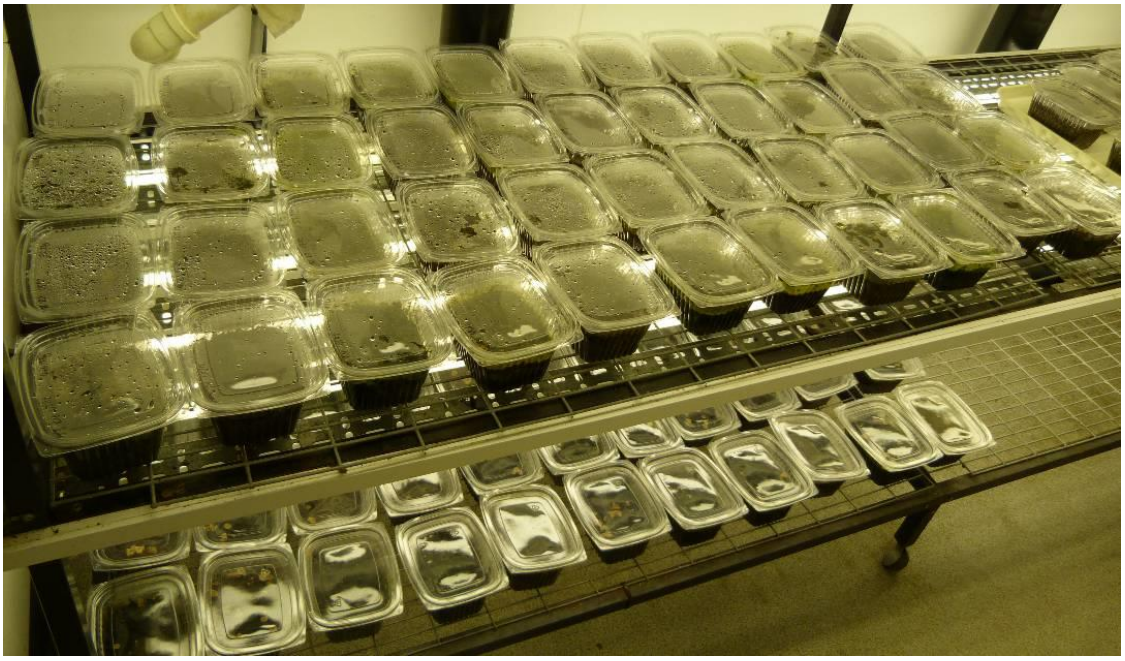


Figure 2: Soil box experiment to evaluate the effect of biofumigants on carpogenic germination of *S. sclerotiorum* laid out on shelving in a controlled environment room

cover the sclerotia. Water was added to give 30% moisture content, lids were then immediately placed onto the boxes and they were then weighed before being incubated in a controlled environment room at 15°C with lights (14h day). Four replicates of each treatment were set up in each experiment, arranged in a randomised block design with four rows and 11 columns (Figure 2). Every two weeks boxes were watered to bring them back to their original weight. The emergence of stipes or apothecia from sclerotia was recorded twice a week using a scale of one (stipe) to four (mature apothecium with wavy cap). Each soil box experiment was run for 150 days to fully assess the effects of the biofumigation treatments,

and the experiment was repeated six times (three at half field rate and three at full field rate equivalents). Results were (statistically) analysed using a Generalised Linear Model.

Results

The biofumigant crops and *Brassica napus* 'Temple' delayed carpogenic germination of the *S. sclerotiorum* sclerotia, and significantly reduced germination in comparison with the untreated control after 150 days in both the half rate and full rate experiments (Figure 3). *Raphanus sativus* 'Terranova' provided the greatest reduction in germination (70% in the full field rate experiments) compared to the untreated control. *Coniothyrium minitans* (Contans WG) and Biofence also reduced overall germination in comparison with the untreated control, and Perlka reduced germination by 93%.

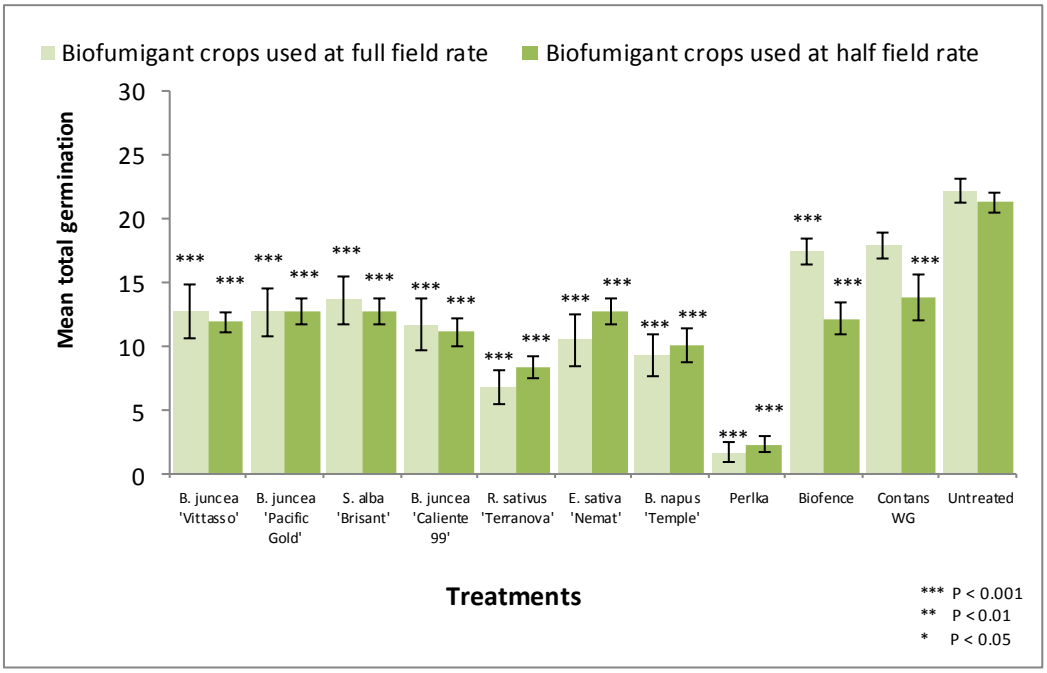


Figure 3: The effect of biofumigant crops, low glucosinolate *Brassica napus* 'Temple', Perlka®, Biofence and Contans WG on final germination of *S. sclerotiorum* sclerotia after 150 days in soil box trials. Error bars show SEM.

Effect of Size of *Sclerotinia sclerotiorum* Sclerotia on the Efficacy of Biofumigant Treatments

Materials and Methods

Further soil box experiments were carried out to test one biofumigant crop (*B. juncea* 'Caliente 99', full field rate equivalent) on germination of different sized sclerotia from the three *S. sclerotiorum* isolates L6, L17 and L44. The sclerotia (produced as described

previously) were 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 and three repeat experiments were carried out. Each soil box test was run for 150 days to fully assess the effects of the biofumigation treatments. Results were (statistically) analysed using an Analysis of Variance (ANOVA).

Results

The large sclerotia produced the most apothecia overall across all the *S. sclerotiorum* isolates (Table 2). For isolate L6, there was a significant ($p<0.05$) reduction in germination for sclerotia treated with *B. juncea* 'Caliente 99' for all three sizes, and 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 2). For *S. sclerotiorum* isolate L17, there were again significant differences between the treated and untreated sclerotia for all three sizes ($p<0.05$, Table 2) 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). 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. 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 2).

Table 2 - 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 soil box experiments.

***S. sclerotiorum* Isolate**	**Size of Sclerotia**	**% Reduction in Germination**	**Mean Number of Apothecia per Sclerotium**
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

In vitro Biofumigation Experiments: Effect of Biofumigants on Mycelial Growth of Sclerotinia sclerotiorum

Experiments reported in the Annual Report 2013 were carried out to test six biofumigant crops in vitro to determine whether they reduced or suppressed growth of *S. sclerotiorum* on agar, at a rate of 1 g and 2 g dried plant material. The rate of mycelial growth of *S. sclerotiorum* isolate L6 was reduced by all the biofumigant crops, with the greatest inhibition caused by Brassica juncea 'Pacific Gold' and 'Caliente 99' in both the 1g and 2g experiments. The low glucosinolate oilseed rape cultivar 'Temple' also slightly inhibited mycelial growth.

In vitro Biofumigation Experiments: Effect of Biofumigant Dose on Mycelial Growth of Sclerotinia sclerotiorum

Materials and Methods

Experiments were carried out to 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 biofumigant crops were grown, harvested and processed as described previously. One 5 mm mycelial plug of actively growing mycelium from *S. sclerotiorum* isolate L6 was placed in the centre of a PDA plate. The plate was inverted, and the dried plant material placed in the lid of the Petri dish and water added (Figure 5). An untreated control was also set up. All Petri dishes were immediately sealed with parafilm and placed into an incubator at 15°C in the dark. 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 1 g dried plant material were used in a total of six experiments, three experiments for each of the two biofumigant crops. Five replicates of each treatment were set up in each experiment, arranged in a randomised block design and mycelial growth was assessed twice a day for four days by measuring along an x and y axis of the radial growth.

Results

ED50 values for the dose response experiments were calculated by using Probit analysis (Finney, 1971), with a fitted term of LOG10 dose +0.01. 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).

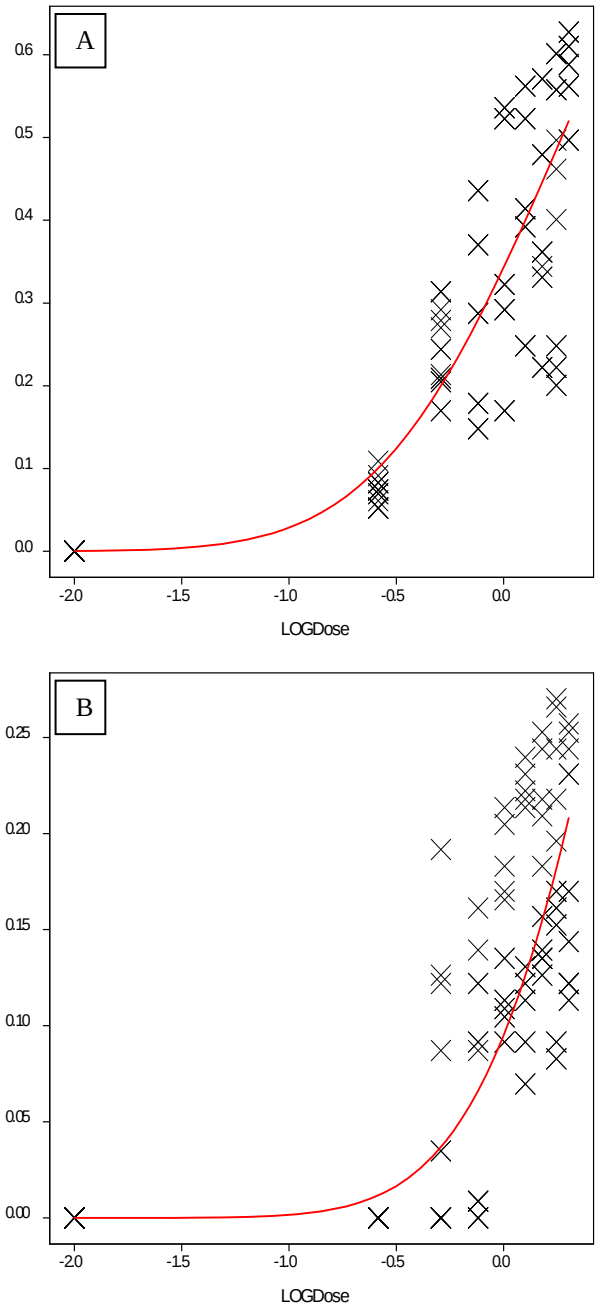


Figure 4 - Fitted (red line) and observed values (means across all replicates; black crosses) of the proportional response of mycelial growth of *S. sclerotiorum* isolate L6 to LOG dose of *B. juncea* ‘Caliente 99’ (A) and *S. alba* ‘Brisant’ (B) dried and milled plant material after 72 hours.

In vitro Biofumigation Experiments: Carpogenic Germination

Materials and Methods

Experiments were carried out to determine whether volatiles produced by the biofumigant crops have a direct effect on carpogenic germination of *S. sclerotiorum* sclerotia. A rate of either 1g or 2g of dried plant material was used in a total of six experiments (three experiments at each rate). All biofumigant crops and low glucosinolate *Brassica napus* 'Temple' were grown, harvested and processed as for the soil box experiments. Sclerotia of *S. sclerotiorum* isolate L6 were produced and conditioned as described previously. Sieved and pasteurised compost (John Innes No. 1, J. Arthur Bowers; 50g) was placed into a Petri dish and preconditioned sclerotia (20) were laid out in a grid pattern and pressed flat into the compost. Water was added to give 30% moisture content, and each Petri dish was placed into a 1200ml plastic box, together with a separate dish of the dried biofumigant crop (Figure 5). Water was added to the dried plant material and the lids immediately placed onto the boxes. They were then weighed before being incubated in a controlled environment room at 15°C in the dark.

Four replicates of each treatment were set up in each experiment, arranged in a randomised block design. Every two weeks, the Petri dishes were watered to bring them back to their original weight. The emergence of stipes or apothecia was recorded once a week and germinated sclerotia removed from the Petri dish. Each experiment was run for 80 days to fully assess the effects of the biofumigation treatments. For four experiments the biofumigant treatment was removed and germination was assessed for the following four weeks. Results were (statistically) analysed using a Generalised Linear Model.

Figure 5 - Petri dish with germinating *S. sclerotiorum* sclerotia and separate dish of biofumigation treatment in 1200ml plastic box

Results

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 6). 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 6).

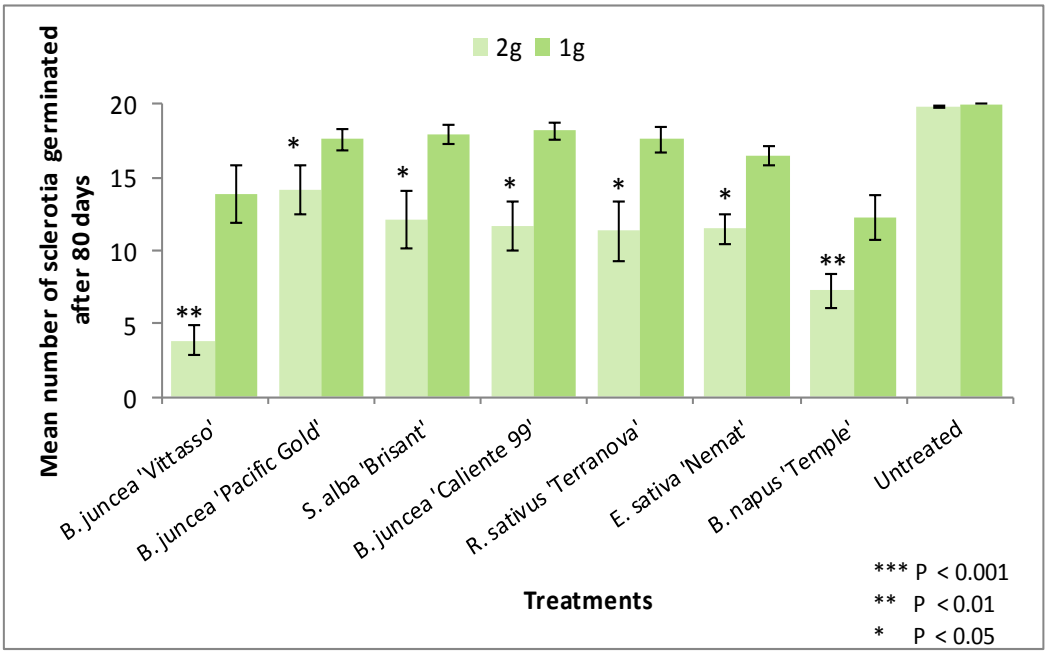


Figure 6 - The effect of 1 g and 2 g dried biofumigant crops and low glucosinolate *Brassica napus* 'Temple' on carpogenic germination of *S. sclerotiorum* sclerotia after 80 days in an *in vitro* trial

For the four experiments where the biofumigant treatment was removed and germination was assessed for the following four weeks, and there was further germination in all treatments except for *R. sativus* 'Terranova' at the 2 g rate (Figure 7). 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 7).

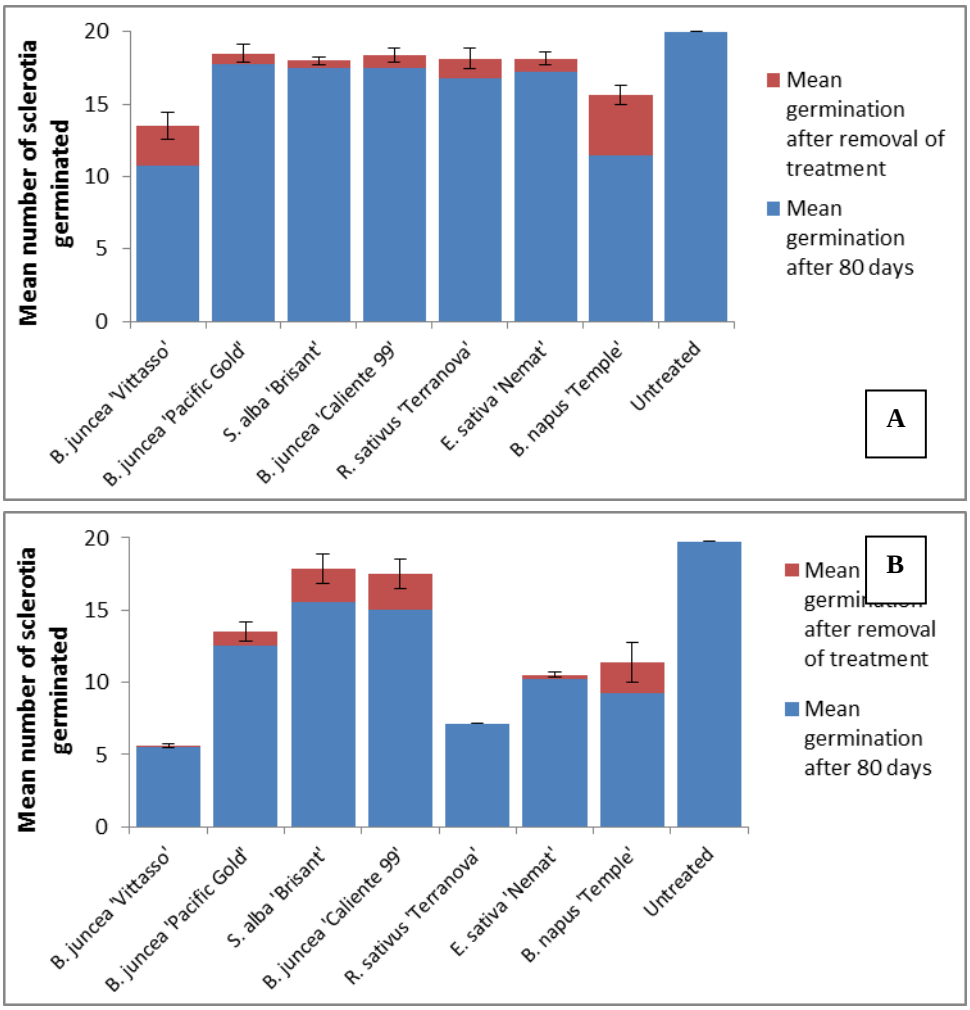


Figure 7 - The effect of biofumigant crops and *B. napus* 'Temple' on carpogenic germination of *Sclerotinia sclerotiorum* sclerotia *in vitro* after 80 days (blue bars) and then after removal of treatment for 4 weeks (red bars), using either 1 g (A) of 2 g (B) plant material. Error bars indicate SEM for mean germination after removal of treatment.

Polytunnel Biofumigation Experiment

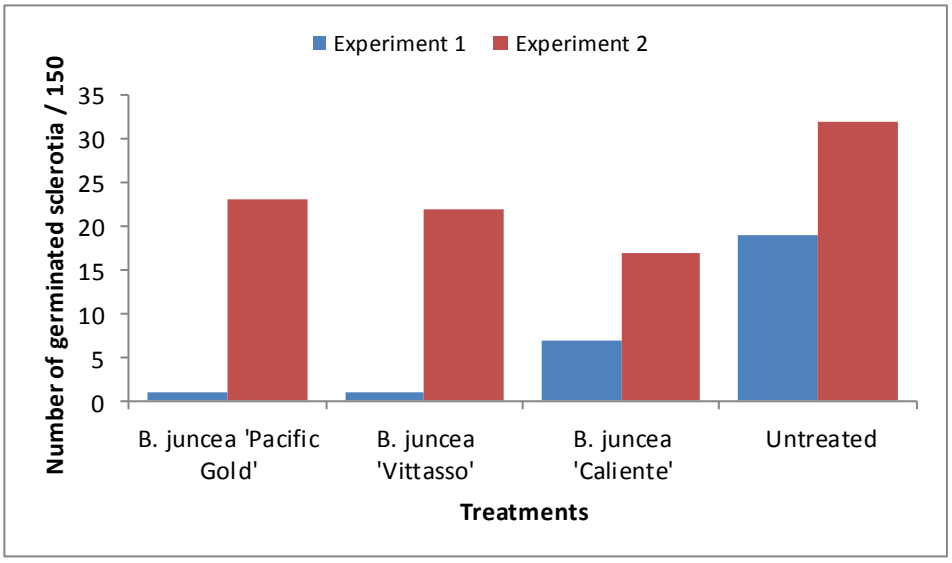
Materials and Methods

Two polytunnel experiments (2012, 2013) were set up to assess the effect of selected biofumigant crops (*B. juncea* 'Vittasso', 'Caliente 99' and 'Pacific Gold') on the viability of *S. sclerotiorum* sclerotia. The biofumigant crops were direct sown in separate plots in a polytunnel according to supplier's sowing rates, and grown until flowering. They were then harvested, shredded with an electric shredder (Bosch AXT 25 TC, HSS Hire Service Group Ltd, England) and dug into the plots. Preconditioned *S. sclerotiorum* sclerotia from isolate L6 (50) were placed into each bed, in two grids. Two weeks after incorporation the beds were planted with lettuce plugs. Once a week, the sclerotia were checked for germination.

Three replicates were arranged in a randomized block design and the experiment was repeated once. Results were (statistically) analysed using a Generalised Linear Model.

Results

There was limited germination in the untreated plots for both experiments, and whilst *B. juncea* 'Caliente 99' reduced germination by over 50% none of the treatments gave a significant reduction compared to the untreated plots (Figure 8). No Sclerotinia disease developed in the lettuce.



HPLC Analysis of Glucosinolates

As described in the Annual Report 2013 all biofumigant crops used in the experiments were analysed using High Performance Liquid Chromatography (HPLC) to assess any differences in the levels of glucosinolates in the crops harvested at different times of the year. For all the biofumigant crops except *B. napus* 'Temple' and *E. sativa* 'Nemat' the main glucosinolate was higher in the crops grown between December and June (in the glasshouse) than those grown between May and November (in the polytunnel).

Objective 2: Susceptibility of Carrots to *S. sclerotiorum*

Carrot Root Inoculations

As detailed in the Annual Report 2013, two experiments were carried out to assess the production of sclerotia by two *S. sclerotiorum* isolates (L6 and L44) on roots from a carrot diversity set consisting of 87 accessions grown at the Wellesbourne site by the Genetic Resources Unit.

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. 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.

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 ($p<0.05$). 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.

Whole Carrot Plant Inoculations

As detailed in the Annual Report 2013 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, 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.

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}) was significantly different from all accessions except accession 90 ($p<0.05$)

Detached Carrot Leaf Experiments

Methods and Materials

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) 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.

Table 3 - 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
7	Elite	Nairobi	shallow	semi-upright
n/a	Elite	Chantenay	shallow - medium	upright
n/a	Elite	Eskimo	deep	upright
n/a	Elite	Narbonne	unknown	upright
90	Mapping parent - wild	QAL	deep	upright
51	Cultivated	Little finger	shallow	prostrate
88	Mapping parent	MK2/00-1 MK300/00	deep	upright
86	Mapping parent - elite	Brasilia	shallow	upright
10	Advanced cultivar	Gold Pak	shallow	upright
80	Advanced cultivar	Altringham large red	shallow	upright

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. The length of the lesion along the petiole (mm) after four days was analysed using ANOVA. 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 (described in the 2013 Annual Report) and the detached leaf experiments.

Results

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 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 9).

Figure 9 - 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 4).

Table 4 - Rate of *S. sclerotiorum* (isolate L6) lesion development on detached leaves from different carrot accessions.

Carrot Diversity Set No.	Carrot Variety/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
Degrees of freedom		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 ($r = 0.1291$).

Objective 3: Epidemiology and control of *Sclerotinia subarctica*

Sampling of *Sclerotinia* Isolates and Molecular Identification of *S. subarctica*

Methods and Materials

Isolates (420) of *Sclerotinia* spp. were obtained from meadow buttercup and different crop plants in England, Scotland, Norway and Sweden (Table 5) while another 314 were also available from previous work from populations where S, subarctica was unedified. (Clarkson *et al*, unpublished). 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* 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 distilled 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 both 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, 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 5 -The population code, country, location, sampling year, plant host and sample size for isolates of *Sclerotinia* spp. collected 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
					420

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 reverse osmosis (RO) water and blotted dry on tissue (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 10).

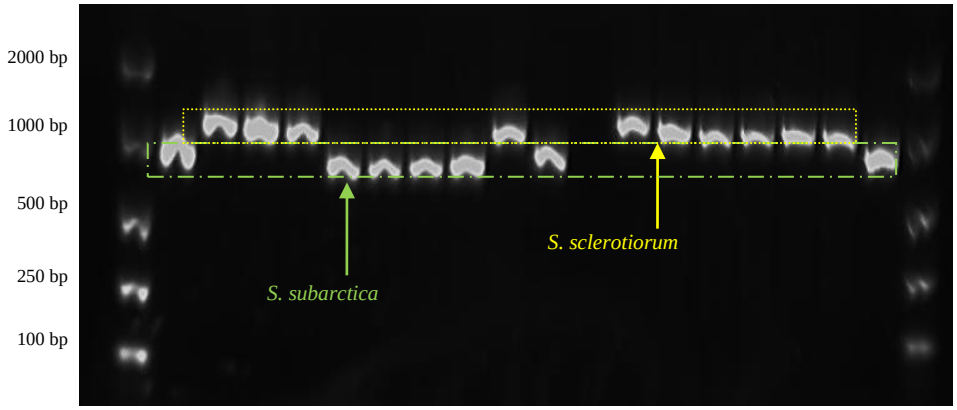


Figure 10 - Gel electrophoresis of the rDNA LSU amplicons for *S. sclerotiorum* (larger PCR product) and *S. subarctica* (smaller product).

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 was used to confirm species identity.

Results

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 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 samples. All sequences of the *S. subarctica* isolates were identical.

Table 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
			734	166	578

Molecular Characterisation of *Sclerotinia subarctica*

Methods and Materials

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 7) 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 7 - 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.

ARLEQUIN (Excoffier et al., 2005) was used to determine the haplotype frequency of all the *S. subarctica* 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). Population subdivision between *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).

Results

Microsatellite analysis of all the *S. subarctica* isolates resulted in 83 haplotypes from the 166 isolates from England, 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; Figure 11). Of these, 14 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 11) and another seven other haplotypes were common to both countries. 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 (Table 8). 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 8).

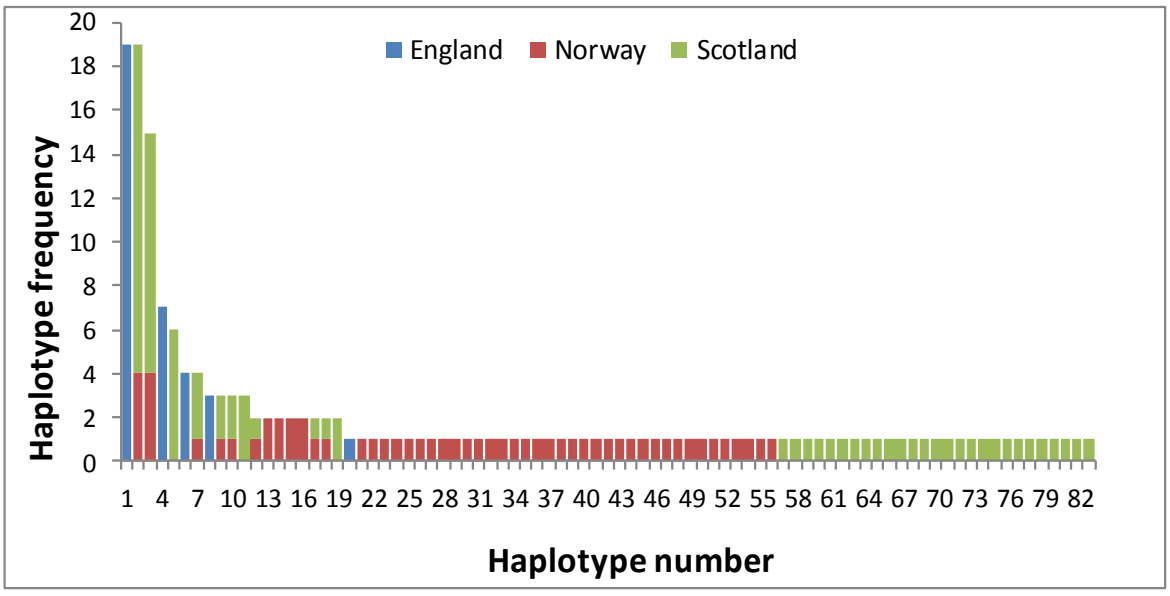


Figure 11 - Microsatellite haplotype frequency for *S. subarctica* isolates (n=166) from England, Scotland and Norway.

Table 8 - 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	0.336	0.419	0.499

[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 9). Gene diversity was lowest in England (0.34) and highest in Norway (0.499). 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 12).

Table 9 - 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 10).

Table 10 - Fixation index (R_{ST}) values for pairwise comparisons of *S. subarctica* populations from crop plants and meadow buttercups in England, Scotland, Norway and Sweden.

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

***=p<0.001

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.

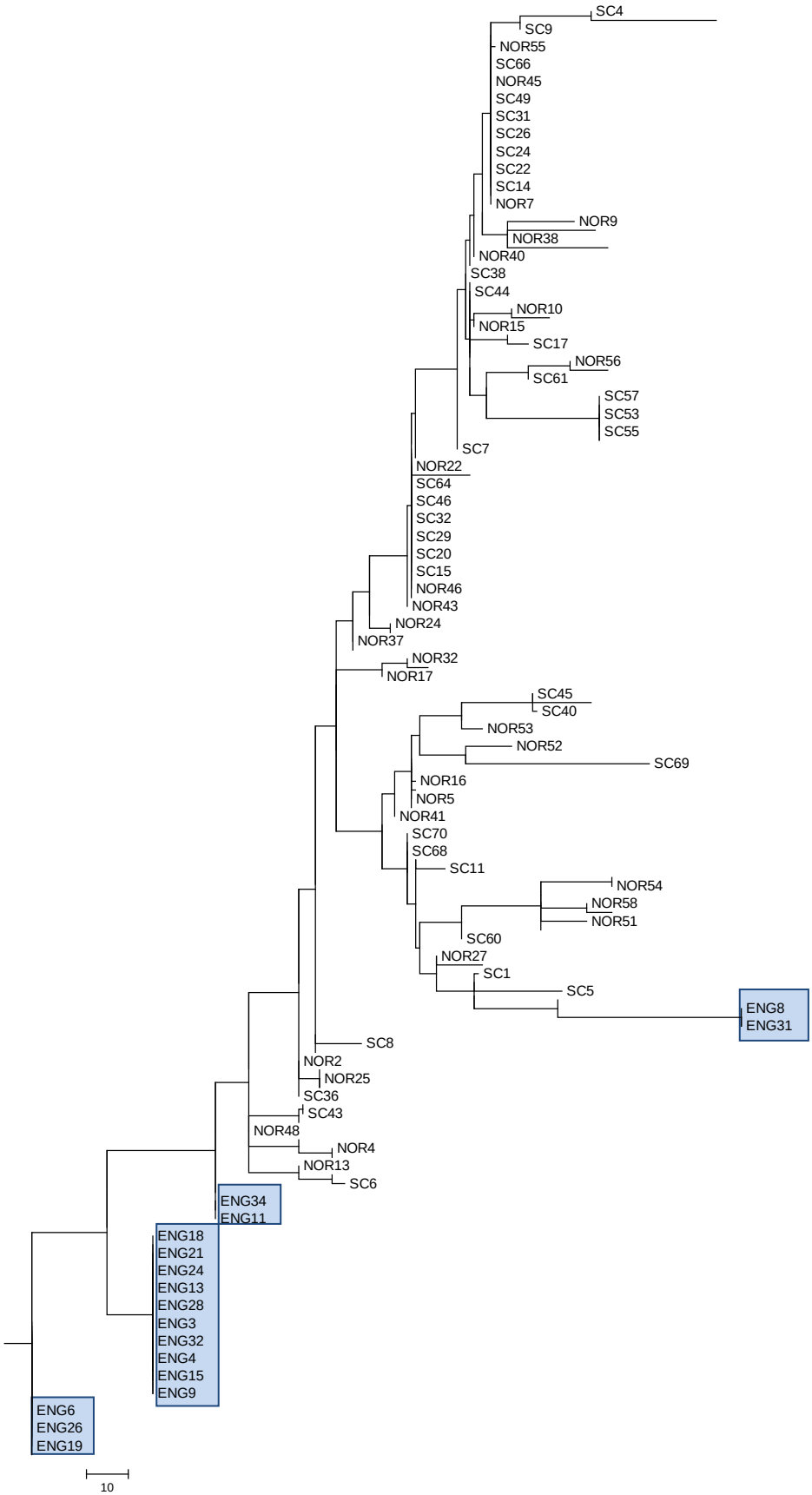


Figure 12 - Minimum evolution spanning tree of microsatellite haplotypes for *S. subarctica* isolates from Scotland (SC) England (ENG) and Norway (NOR). Grouping of haplotypes from England is highlighted in blue.

Mycelial Growth of *Sclerotinia* spp. at Different Temperatures

Methods and Materials

Experiments were carried out to test mycelial growth of *S. subarctica* in comparison to *S. sclerotiorum* at different temperatures. As detailed in the Annual Report 2013 the first experiment compared the growth of four *S. subarctica* isolates with a 'standard' *S. sclerotiorum* isolate L6 at four temperatures ranging from 5°C to 20°C. The second experiment compared the growth of five *S. subarctica* isolates with five *S. sclerotiorum* isolates at four different temperatures (25°C, 28°C, 30°C and 33°C).

S. sclerotiorum and *S. subarctica* isolates (Table 11) were selected based on their representing different microsatellite and IGS haplotypes. 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. The rate of mycelial growth and total colony size at 46 hours were analysed using Analysis of Variance (ANOVA).

Table 11 - *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
Liel17 a1	*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	

Results

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 12).

Table 12 - 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
L.S.D. @ 5%	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 12). 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 observed at 20°C (between 1.57 and 1.76 mm/hour). The slowest rate of growth for all isolates was at 5°C (between 0.26 and 0.36 mm/hour, Table 12).

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 13). 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 13). At 15°C the mean colony diameter of *S. subarctica* isolate PS13 was significantly greater ($p<0.05$) than all other isolates (Figure 13)

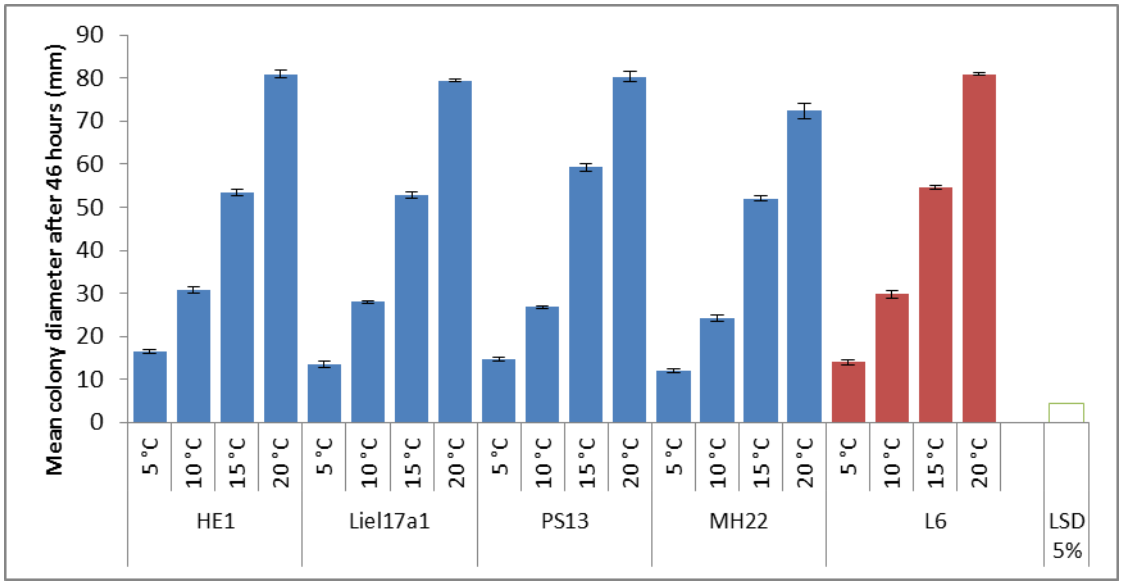


Figure 13 - Mean mycelial colony diameter (mm) of four *S. subarctica* isolates (blue bars) and one *S. sclerotiorum* isolate (red bars) after 46 hours at 5, 10, 15 and 20 °C. Error bars show SEM.

Temperature Range 25 to 33°C

There were significant differences ($p<0.05$) comparing growth rate between species (across all five isolates) at each temperature (Table 13). The fastest growth rate of 1.87 mm/hour was for *S. sclerotiorum* at 25°C, whilst *S. subarctica* had the slowest growth rate of 0.01 mm per hour at 33°C.

Table 13 - Mean mycelial growth rate (mm/hour) for across five *S. sclerotiorum* and five *S. subarctica* isolates at 25°C, 28°C, 30°C and 33°.. 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
L.S.D. @ 5%	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 14).

Table 14 - Mean mycelial growth rate (mm/hour) across 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).

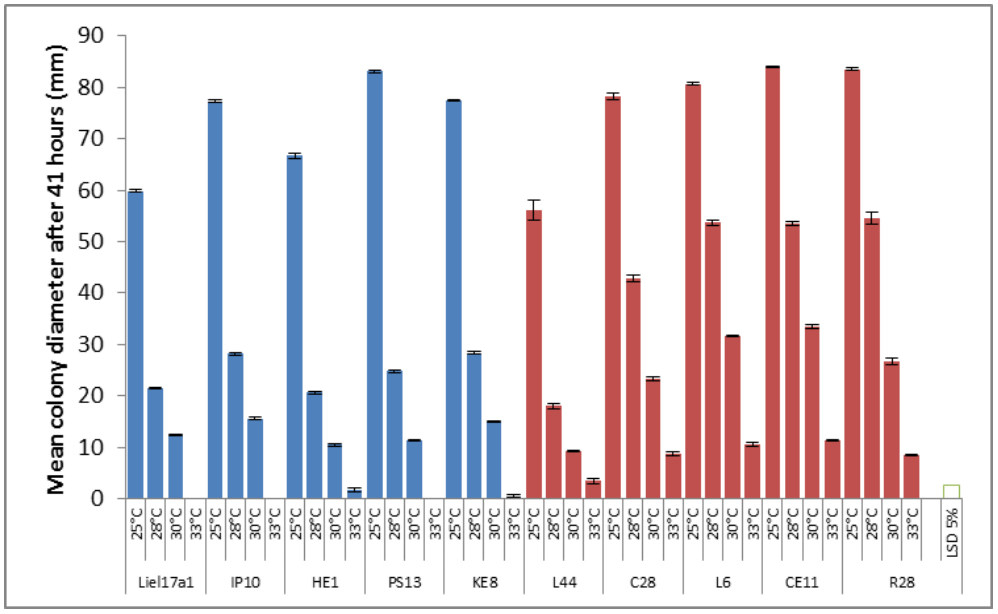
		Growth Rate (mm/hour)			
Isolate	**Species**	**25°C**	**28°C**	**30°C**	**33°C**
Liel17a1	*subarctica*	1.46 b	0.52 b	0.30 b	0 a
IP10	*subarctica*	1.88 d	0.69 d	0.38 c	0 a
HE1	*subarctica*	1.63 c	0.50 ab	0.26 ab	0.04 ab
PS13	*subarctica*	2.03 fg	0.61 c	0.28 ab	0 a
KE8	*subarctica*	1.89 d	0.69 d	0.37 c	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
L.S.D. @ 5%	0.06				
D.f.	429				

For the mean colony diameter after 46 hours there were significant differences (p<0.05) between species (across all five isolates) at each temperature (Table 15), 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 15 - Mean colony diameter (mm/hour) after 46 hours across five *S. sclerotiorum* and five *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
L.S.D. @ 5%	3.03	
D.f.	461	

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 14).

Conditioning Time for Carpogenic Germination of *S. subarctica*

Methods and Materials

Two 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 16). Isolates in each experiment were selected based on their representing different microsatellite and IGS haplotypes, 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 conditior 012). *S. sclerotio* Chapter 3) than L6 and has also been found previously to require longer periods of conditioning (personal communication, John Clarkson, 2012).

Figure 14 - 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.

Sclerotia were produced and positioned in pasteurised compost (30% moisture content) in Petri dishes (20 per dosh) as for the *in vitro* carpogenic germination experiments described previously. The sclerotia were incubated at 5°C in the dark for different durations after which they were moved to 15°C (in the dark). Every two weeks, water was added to the compost to maintain constant moisture content. The emergence of stipes was recorded

once a week for 180 days (apothecia are not produced in the dark). 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. The cumulative number of sclerotia germinated up to 21 days after being placed at 15 °C were (statistically) analysed using ANOVA.

Table 16 - *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

Results

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 after 21 days increased with increasing conditioning duration, up to 100 days where the maximum germination was 14.6 sclerotia (Figure 15). 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 15). 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 15). 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 15).

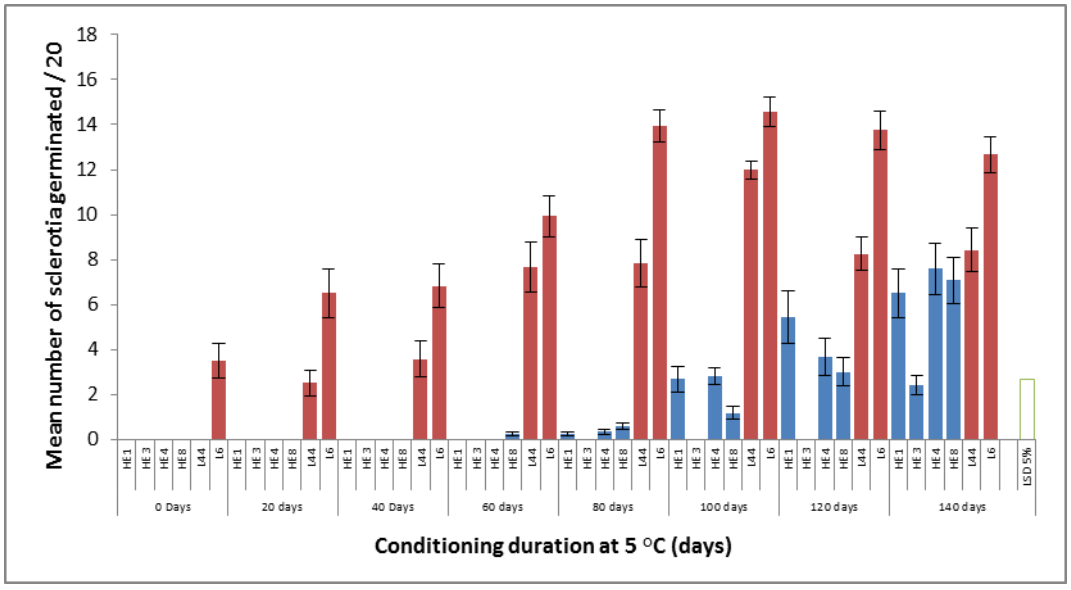


Figure 15 - Mean number of sclerotia of *S. subarctica* isolates HE1, HE3, HE4 and HE8 (blue bars) and *S. sclerotiorum* isolates L6 and L44 (red bars) germinated after different durations of conditioning at 5°C (0 to 140 days) followed by 21 days at 15°C. Error bars show SEM.

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 with no conditioning treatment (Figure 15). Mean germination for L6 reached a maximum of 20 sclerotia after 60 days conditioning at 5°C. *S. subarctica* isolate LSA1 first germinated after 20 days conditioning, while the remaining *S. subarctica* isolates germinated after 60 days conditioning (Figure 16). 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 16).

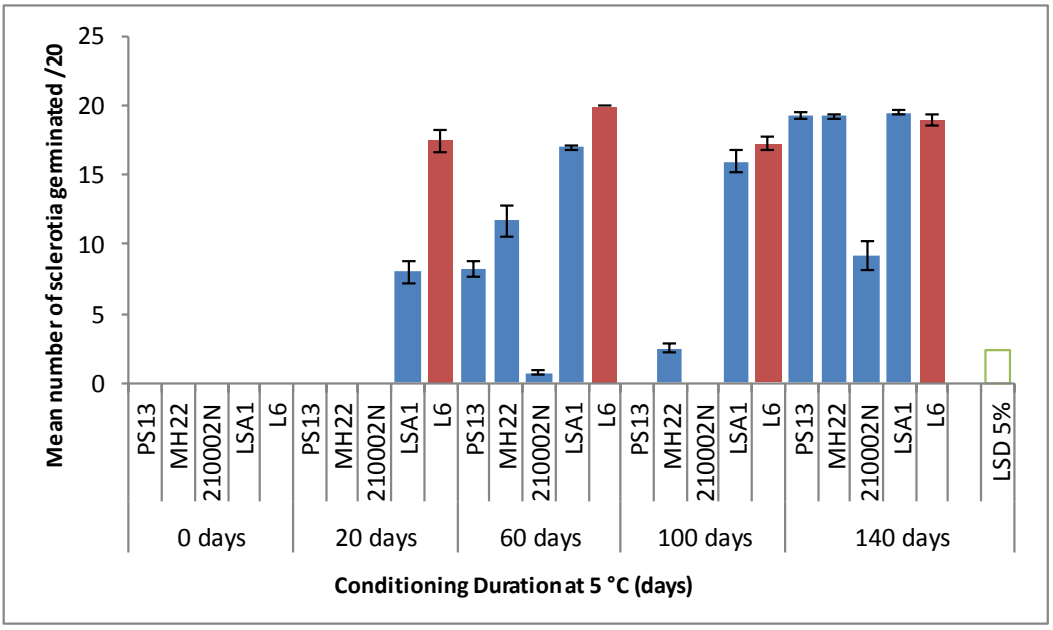


Figure 16 - Mean number of sclerotia of *S. subarctica* isolates PS13, MH22, 210002N and LSA1 (blue bars) and *S. sclerotiorum* isolate L6 (red bars) germinated after different periods of conditioning durations at 5°C (0 to 140 days) followed by 21 days at 15°C. Error bars show SEM.

Discussion

Objective 1: To determine the effect of biofumigation on the germination and survival of sclerotia of *Sclerotinia sclerotiorum*.

There have been very few studies examining the effects of biofumigant crops on the carpogenic germination of sclerotia of *S. sclerotiorum*. The results from this study showed that *Brassica juncea* 'Caliente 99', *Brassica juncea* 'Pacific Gold', *Brassica juncea* 'Vittasso', *Sinapis alba* 'Brisant', *Raphanus sativus* 'Terranova', and *Eruca sativa* 'Nemat' reduced 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.

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 observed for *S. trifoliorum* (Vleugels et al., 2013). In the soil box 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%. 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 sclerotia than the medium sclerotia and three times as many as the small sclerotia. Therefore, these results indicate

that biofumigation is potentially 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 the 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 used, including the low glucosinolate *B. napus* 'Temple'. Other 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 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).

In the current work, out of two biofumigant powders tested, the more effective biofumigant crop for suppressing mycelial growth of *S. sclerotiorum* was *B. juncea* 'Caliente 99', which has a greater dose response than *S. alba* 'Brisant'. The most effective crop for inhibition of carpogenic germination of *S. sclerotiorum* sclerotia varied between the soil box experiments and the *in vitro* experiments, being *R. sativus* 'Terranova' and *B. juncea* 'Vittasso' respectively. These discrepancies may be 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 soil box experiments 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* experiments 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 soil box experiments therefore provide a more reliable method for screening suitable biofumigant crops against this pathogen.

The HPLC analysis (detailed in the 2013 Annual Report) combined with the experiment results suggests there may be non-glucosinolate derived volatiles being released from *B. napus* 'Temple' which resulted in reduced germination of sclerotia. Small quantities of the glucosinolate sinigrin was detected in *B. napus* Temple but there were no other obvious peaks on the chromatograms to indicate the presence of a measurable quantity of any other glucosinolate. 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.* 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.

The polytunnel experiments did not produce significant reductions in germination of *S. sclerotiorum* sclerotia, and germination was low in the untreated plots. However, the reduction in germination in the treated plots does indicate that biofumigation has potential to be used as part of an integrated disease management system, and further work is required to improve experimental methods for testing biofumigation outside of the laboratory.

Objective 2: To evaluate carrot varieties for susceptibility to *Sclerotinia sclerotiorum* and quantify production of sclerotia by different *S. sclerotiorum* genotypes.

The detached carrot leaf inoculations produced clearly measurable *S. sclerotiorum* lesions, with results consistent across the replicates for each experiment and between experiments. Significant differences were found between the carrot accessions on the rate of 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 again substantial and 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.

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 qualitative resistance to *S. sclerotiorum*, if the

results are not relevant to those seen on whole plants. Detached leaf assays using mycelial plugs have been found to be very effective and reliable in screening soybean cultivars (Wegulo et al., 1998), and high positive correlation has been found between field inoculations on stems of *Brassica oleracea* and detached leaf assays (Mei et al., 2011), but not for *Brassica napus* cultivars where significant differences seen in field evaluation studies were not found in detached leaf assays (Bradley et al., 2006). However, results using homogenised mycelium sprayed or pipetted onto detached leaf soybean leaves did correlate with those from whole plants inoculated with agar plugs of mycelium placed onto a cut petiole (Chen & Wang, 2005). Additionally it is possible that using mycelial PDA plugs to inoculate 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). In the present study, significant differences were found using this method, although half of the ten carrot accessions tested had a mean rate of lesion development of between 11.2 and 12.5 mm per day suggesting that more accessions should be screened to identify a greater range of responses to *S. sclerotiorum*.

Despite the lack of a clear correlation between detached leaf and whole carrot plant inoculation tests, results from each indicate that 'Little Finger' and 'Brasilia' may be more resistant to Sclerotinia, and in the whole plant tests, these varieties showed the slowest disease progression down the petiole compared to other carrot varieties such as Nairobi, Chantenay, Eskimo and Narbonne.

Objective 3: To investigate the diversity and epidemiology of *Sclerotinia subarctica.*

The results from the current study show that *S. subarctica* is present in (East) 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.* 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 from different countries have been characterised using microsatellite markers.

The microsatellite analysis showed that each *S. subarctica* population consisted of multiple haplotypes with two haplotypes sampled at a higher frequency. Haplotype 1 consisted only of isolates sampled from buttercup in Hereford, 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 resulted in the apparent

high frequency of this haplotype. Haplotype 2 was present in 8 of the 21 populations, and was widely distributed across different host plants in both Scotland and Norway. The same *S. subarctica* haplotypes were sampled in both crop plants and meadow buttercup in Scotland (as found by Clarkson *et al.* (2013) for *S. sclerotiorum* populations in the UK), suggesting that wild hosts could 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).

Results from experiments examining the effect of temperature on *S. sclerotiorum* and *S. subarctica* suggests that *S. sclerotiorum* has a greater tolerance for high temperature for mycelial growth compared with *S. subarctica.* This might be one reason explaining different geographical distributions of the two species and their 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, further work with additional isolates of both species is required to verify these findings.

The conditioning experiments clearly showed that *S. subarctica* isolates vary from *S. sclerotiorum* isolates in their requirements for length of conditioning before they will germinate carpogenically, as no germination for any of the *S. subarctica* isolates was observed for a conditioning duration under 20 days, whereas *S. sclerotiorum* isolates germinated with no conditioning. The second experiment was not repeated and further experiments with these and additional isolates are required for 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).

Conclusions

Objective 1: To determine the effect of biofumigation on the germination and survival of sclerotia of *Sclerotinia sclerotiorum*.

- Volatiles released from biofumigation crops have a direct 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 are in direct contact with the sclerotia or in the vapour phase.
- In this study, the most effective crop for inhibition of carpogenic germination of *S. sclerotiorum* sclerotia was *R. sativus* 'Terranova' in the soil box experiments and *B. juncea* 'Vittasso' in the in vitro experiments. In the mycelial growth experiments *B. juncea* 'Caliente 99' was the most effective at inhibiting growth.
- Using soil box tests may be the best method for establishing the most effective biofumigant crops against resting propagules *S. sclerotiorum* and other soilborne fungal pathogens.
- There is potential for utilising biofumigation crops as part of an integrated disease management system against soil borne diseases such as *S. sclerotiorum*.

Objective 2: To evaluate carrot varieties for susceptibility to *Sclerotinia sclerotiorum* and quantify production of sclerotia by different *S. sclerotiorum* genotypes.

- The number of sclerotia produced on carrot roots was significantly affected by the *S. sclerotiorum* isolate, possibly related to the survival strategy of the individual isolate.
- The weight of individual sclerotia produced by different *S. sclerotiorum* isolates is influenced by carrot accession, but not by 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.
- Significant differences in the rate of *S. sclerotiorum* lesion progression were observed between different carrot accessions suggesting that some may be useful in future resistance breeding programmes.
- Detached carrot leaf inoculations could be used for *S. sclerotiorum* resistance screening, but further work is needed on a wider range of accessions and leaf age needs to be taken into account to ensure comparisons can be accurately made between whole plants and detached leaves.

Objective 3: To investigate the diversity and epidemiology of *Sclerotinia subarctica*

- *Sclerotinia subarctica* was present on numerous crop and wild hosts in East Scotland, Norway and Sweden.
- *S. subarctica* often occurs in sympatry with *S. sclerotiorum*.
- Some *S. subarctica* microsatellite haplotypes were shared between Scotland and Norway, and between crop plants and meadow buttercup.
- Isolates of *S. subarctica* have a lower tolerance to high temperatures for mycelial compared with *S. sclerotiorum*,
- Isolates of *S. subarctica* required 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 the temperature for cold conditioning of *S. subarctica* isolates to achieve rapid germination.

Knowledge and Technology Transfer

- Abstract for University of Warwick School of Life Sciences Postgraduate Symposium, March 2012.
- Poster presented at 2012 AHDB Horticulture studentship conference, July 2012.
- Presented at AAB IPM conference, October 2012.
- Invited presentation at RHS PhD Symposium, November 2012
- Poster at School of Life Sciences PhD Symposium, March 2013
- Invited presentation to 3rd Year BSc Horticulture Students at Pershore College, April 2013
- Presented at Warwick Crop Centre Seminar Series, July 2013
- Invited presentation and poster at AHDB Horticulture Studentship Conference, September 2013
- Poster at AHDB Crop Conference, September 2013
- Presented at BSPP Presidential Meeting 2013: Visions of plant disease management in 2050: metropolis, arcadia or dystopia?, 17th December 2013
- Invited presentation at AHDB Horticulture/BCGA Carrot Technical Seminar, 20th March 2014.
- Presented at Postgraduate Symposium, School of Life Sciences, 26th March 2014.
- Presented at Warwick Crop Centre seminar series, 5th June 2014.
- Presented at 5th International Biofumigation Symposium, 10th September 2014.

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