Project title:	Pathogen diversity, epidemiology and control of Sclerotinia disease in vegetable crops
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Project leader:	Dr John Clarkson, Warwick Crop Centre, University of Warwick, CV35 9EF
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Location of project:	Warwick Crop Centre, University of Warwick, School of Life Sciences, Wellesbourne, Warwick, CV35 9EF
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Date project commenced:	3 October 2011
Expected completion date:	31 December 2014

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 *Raphanus sativus* 'Terranova' reduced carpogenic germination of *S. sclerotiorum* sclerotia to produce apothecium by 73% in comparison to the untreated control, which compares well to the positive control Perlka® which reduced germination by 82%.

Background

The Pathogen – Sclerotinia sclerotiorum

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

The long term survival structures for *S. sclerotiorum* are small black resting bodies called sclerotia (Willetts and 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 and 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 and 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 is 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). The timing of spraying is critical to the effectiveness of protection provided by fungicides, so new control methods to reduce the viability of sclerotia in the soil would help to eliminate this issue. Also, some of the effective active ingredients in fungicides currently used routinely against sclerotinia disease such as boscalid, carbendazim, cyprodinil, fludioxonil (Matheron and Porchas, 2008), azoxystrobin and difenoconazole are classed as medium to high risk for resistance (McQuilken, 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), but these are considered expensive by growers. 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). There has also been much research on biological control, with the fungus *Coniothyrium minitans* (which parasitises 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 and 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 aims and objectives of this project are:

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

Objectives:

i. To determine the effect of organic soil amendments 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 diversity, epidemiology and control of *Sclerotinia subarctica.*

Summary

Objective 1 - To determine the effect of organic soil amendments on the survival of sclerotia of Sclerotinia sclerotiorum.

Results from trials showed that the majority of biofumigant crops tested can significantly reduce carpogenic germination of *S. sclerotiorum* (Figure 1), and the results from *in vitro* trials testing direct effects of biofumigants on mycelial growth and carpogenic germination suggests that this is caused by fungitoxic isothiocyanates being released from the plant material. However, although the low glucosinolate oilseed rape cultivar, *Brassica napus* 'Temple' reduced carpogenic germination, HPLC analysis confirmed that it does not contain significant levels of glucosinolates. This suggests that there are other volatile compounds being released from 'Temple' which affects the germination of sclerotia.

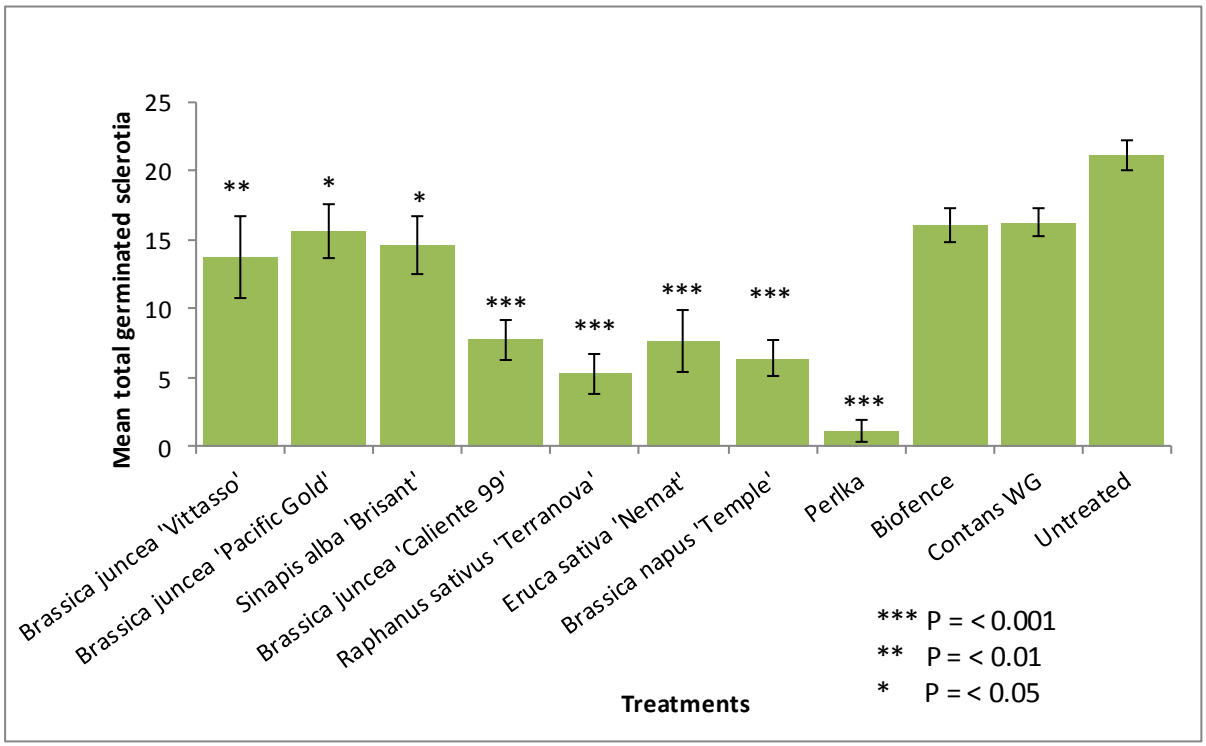

Figure 1 – The effect of biofumigant crops at full field rates, *Brassica napus* 'Temple', Perlka®, Biofence and Contans WG on final germination of *S. sclerotiorum* sclerotia after 150 days in a soil box experiment at 15°C.

The HPLC analysis showed a clear difference in the glucosinolate quantities in biofumigant crops grown at different times of year in the polytunnel. Further examination of this data will indicate whether this is due to changing temperatures across the cropping dates, or pest damage. A current polytunnel trial will help to assess the effectiveness of the biofumigant crops on carpogenic germination in a more realistic setting, as well as determining effects on subsequent disease incidence.

Objective 2 - To determine the aggressiveness of different Sclerotinia genotypes and species on commercial carrot varieties and quantify production of sclerotia.

Carrot root inoculations repeated as in year one showed that there is consistent variation in the number and size of sclerotia produced by two different isolates of *S. sclerotiorum* (L6 and L44). The size of sclerotia may affect their survival in soil, and the number of apothecia produced, therefore having a direct impact on the relative frequencies of each isolate. Some of the cultivars in the trial produced very few sclerotia for either *S. sclerotiorum* isolate and may therefore be suitable for future breeding work. Whole carrot plant inoculation trials 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.

Objective 4 - Epidemiology and control of Sclerotinia subarctica

The results from the microsatellite genotyping of *S. subarctica* isolates collected in 2012 shows that there is considerable diversity amongst isolates from Scotland, in comparison to isolates from England (Herefordshire). This could indicate that sexual reproduction is occurring in Scotland where the conditions may be more favourable for this species. Research investigating the chilling time required for rapid carpogenic germination will help determine the conditions required by this species, although mycelial growth trials showed no significance differences between *S. subarctica* isolates and a *S. sclerotiorum* isolate in their response to temperature.

Conclusions

- In soil box trials *Raphanus sativus* 'Terranova' reduced carpogenic germination of *S. sclerotiorum* sclerotia by 73% in comparison to the untreated control which compares well to the positive control Perlka® which reduced germination by 82%
- The best overall control in soil box trials and direct *in vitro* tests was achieved by *Brassica juncea* 'Caliente', which also had the highest levels of the glucosinolate sinigrin out of all the brown mustards.
- Roots from some carrot lines produce very few sclerotia while others from whole plant tests show slow disease progression after inoculation and could therefore be used in future breeding programs.
- Results from genotyping suggest that *S. subarctica* isolates are more diverse in Scotland compared to those found in Herefordshire.

Financial Benefits

Financial benefits have yet to be established – further details on this expected at the end of the project.

Action Points

Experiments are still underway to establish proof of concept, so no action points at present.

SCIENCE SECTION

Introduction

The Pathogen – Sclerotinia sclerotiorum

Sclerotinia sclerotiorum (Lib.) de Bary is a plant pathogenic fungus that affects many economically important crops (Hegedus and Rimmer, 2005), with a world-wide distribution (Purdy, 1979) and a wide host range of over 400 plant species (Boland and 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 and 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 and it is thought that the genotypes vary in their aggressiveness (Clarkson *et al.*, 2013).

The long term survival structures for *S. sclerotiorum* are small black resting bodies called sclerotia (Willetts and 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 and Huang, 2001). *S. sclerotiorum* therefore functions as both an airborne and soil borne pathogen. 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 and 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 required to establish the distribution and ecology of this species in the UK, on both crops and wild hosts (Clarkson *et al.*, 2013).

Sclerotinia on Carrots

This project focuses 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). It 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) as also demonstrated in other crops (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 trials show 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 trials with carrots, one defence mechanism being leaf abscission after infection of the petiole (Foster *et al.*, 2008) and a second being a structural barrier of lignin, diphenols, suberin flavanols, peroxidases and phenolases (Craft and 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 and 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 and 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, but 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). 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 effective at reducing disease (Williams and Stelfox, 1980), but a subsequent cultivation could bring viable sclerotia back to the soil surface (Mitchell and 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 has been 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 abilities 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 and Oawa, 1971)
iii. Inhibition of metabolic enzymes by thiocyanate radical, indicated as a degradation product of ITCs (Banks *et al.*, 1986)

Brassica green manures can have several modes of action – a direct effect of the ITCs against a pathogen or an indirect effect by stimulating beneficial organisms (Matthiessen and Kirkegaard, 2006). Using these green manure crops for biofumigation can provide control against Sclerotinia disease (Porter *et al.*, 2002), but has not yet been shown to have a consistent significant effect on viability of sclerotia (Matthiessen and Kirkegaard, 2002). A study on a blend of *Brassica napus & Brassica campestris* showed a reduction in the level of viable sclerotia in the soil (Carr, 2003), so it seems issues surrounding methods and rates of incorporation need to be resolved in order to gain consistent results (Geier, 2009), particularly as synthetic pure ITCs significantly 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 and Horbowicz, 1999) yet *Brassica oleracea* var. *caulorapa* inhibited mycelial growth by 89.5% in another (Fan *et al.*, 2008).

The aims and objectives of this project are:

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

Objectives:

i. To determine the effect of organic soil amendments 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 diversity, epidemiology and control of *Sclerotinia subarctica.*

Objective 1 – Organic soil amendments

Biofumigation Soil Box Trials

Materials and Methods

Soil box trials (six in total) were set up to test the effect of 10 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 trials

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 4mm 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 with 30% moisture 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 cover the sclerotia. Water was added to give 30% moisture content, lids were then

immediately placed onto the boxes and they were 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 trial, arranged in a randomised block design with four rows and 11 columns on a single shelf in the controlled environment room (Figure 2). Every two weeks the boxes were watered to bring them back to their original weight. The emergence of stipes or apothecia was recorded twice a week using a scale of one (stipe) to four (mature apothecium with wavy cap).

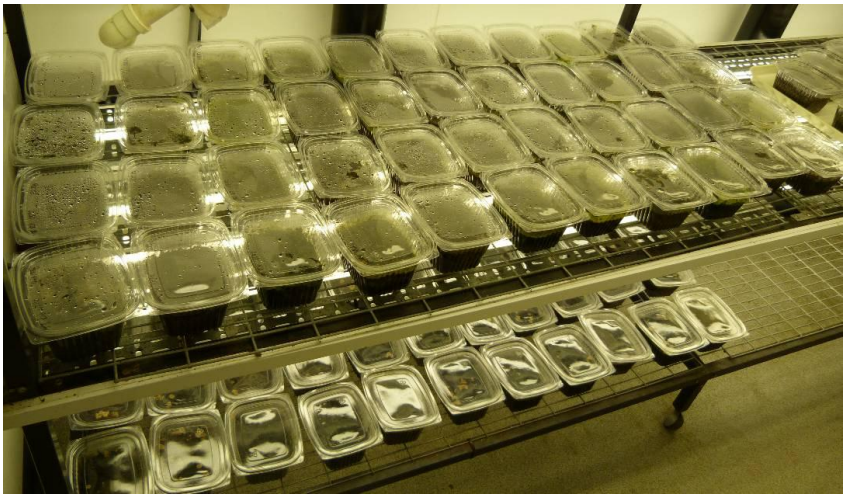

Figure 2 - Soil box trials to evaluate the effect of biofumigants on carpogenic germination of *S. sclerotiorum* laid out on shelving in a controlled environment room

Results

Each soil box trial was run for at least 150 days to fully assess the effects of the biofumigation treatments. Therefore, at the time of writing only four out of six trials have finished (two at half field rate and two at full field rate equivalent treatments), and these results have been (statistically) analysed using a Generalised Linear Model. The

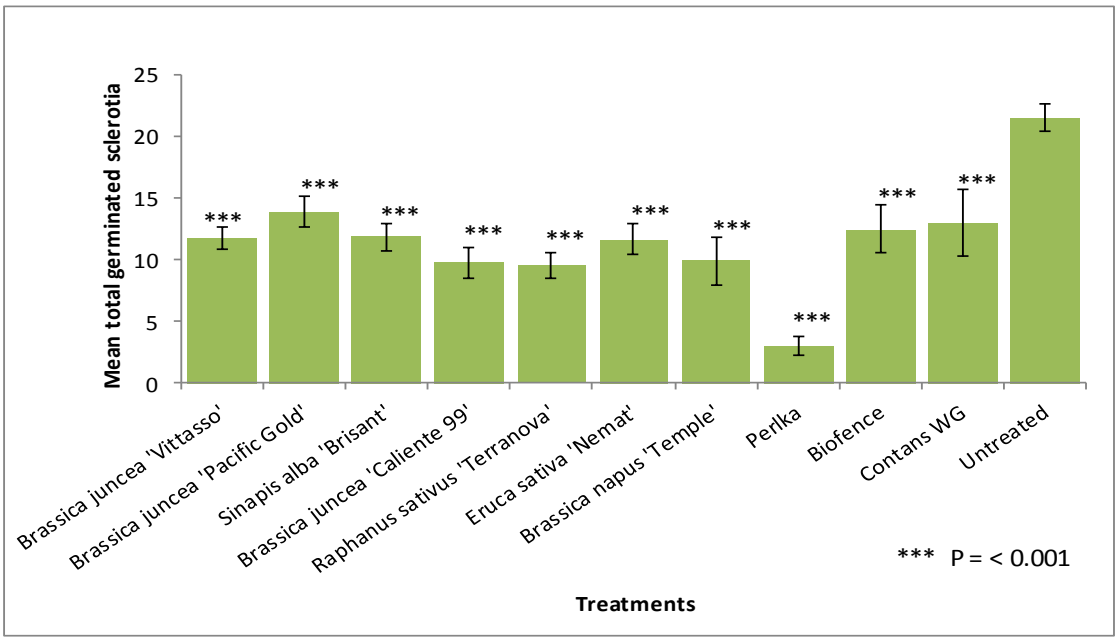

Figure 3 – The effect of biofumigant crops (half field rates), low glucosinolate *Brassica napus* 'Temple', Perlka®, Biofence and Contans WG on final germination of *S. sclerotiorum* sclerotia after 150 days in a soil box trial at 15°C

biofumigant crops and *Brassica napus* 'Temple' delayed carpogenic germination of the sclerotia, and significantly reduced germination in comparison with the untreated control after 150 days in both the half rate (Figure 3) and full rate trials (Figure 4). *Raphanus sativus* 'Terranova' provided the greatest reduction in germination (73% in the full field rate trials) 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 82%.

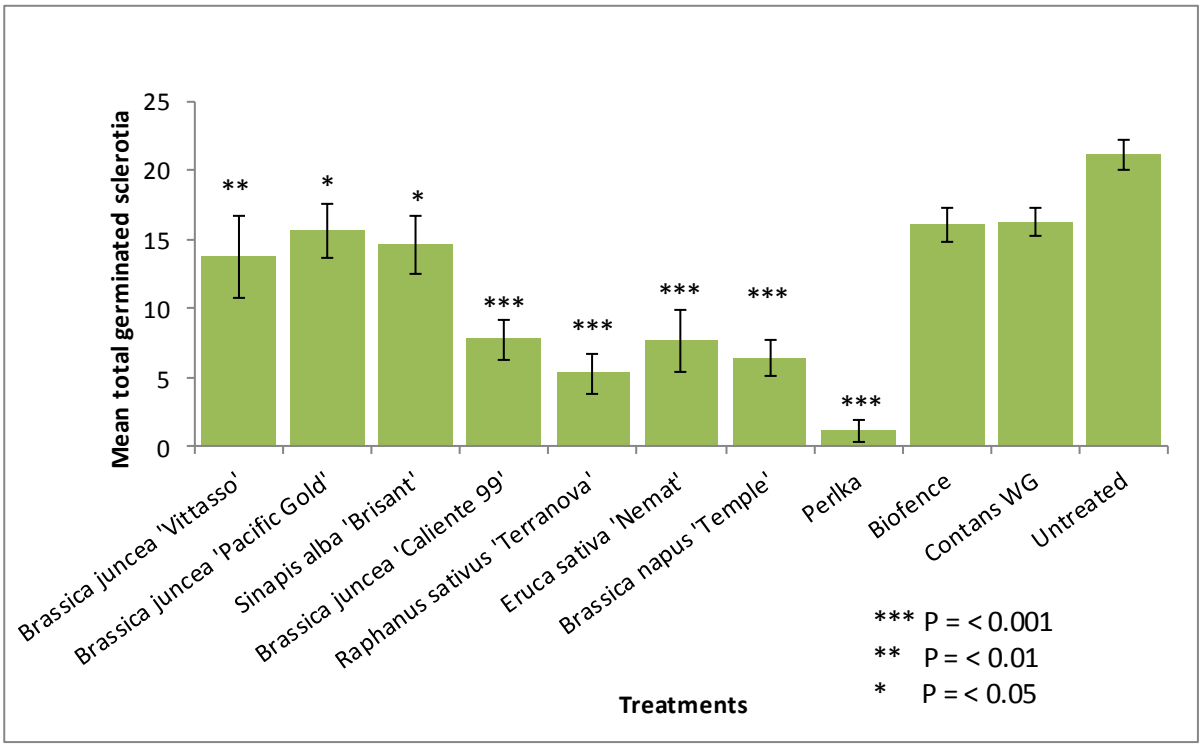

Figure 4 – The effect of biofumigant crops (full field rates), low glucosinolate *Brassica napus* 'Temple', Perlka®, Biofence and Contans WG on final germination of *S. sclerotiorum* sclerotia after 150 days in a soil box trial at 15°C

In vitro Biofumigation Trials – Mycelial Growth

Materials and Methods

Trials were carried out to test six biofumigant crops *in vitro* to determine whether they reduced or suppressed growth of *S. sclerotiorum* on agar. All biofumigant crops and low glucosinolate *Brassica napus* 'Temple' were grown, harvested and processed as for the soil

box trials. One 5mm 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 rate of either 1g or 2g of dried plant material was used in a total of six trials (three trials at each rate) with either 10ml or 20ml of water respectively. Five replicates of each treatment were set up in each trial, 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.

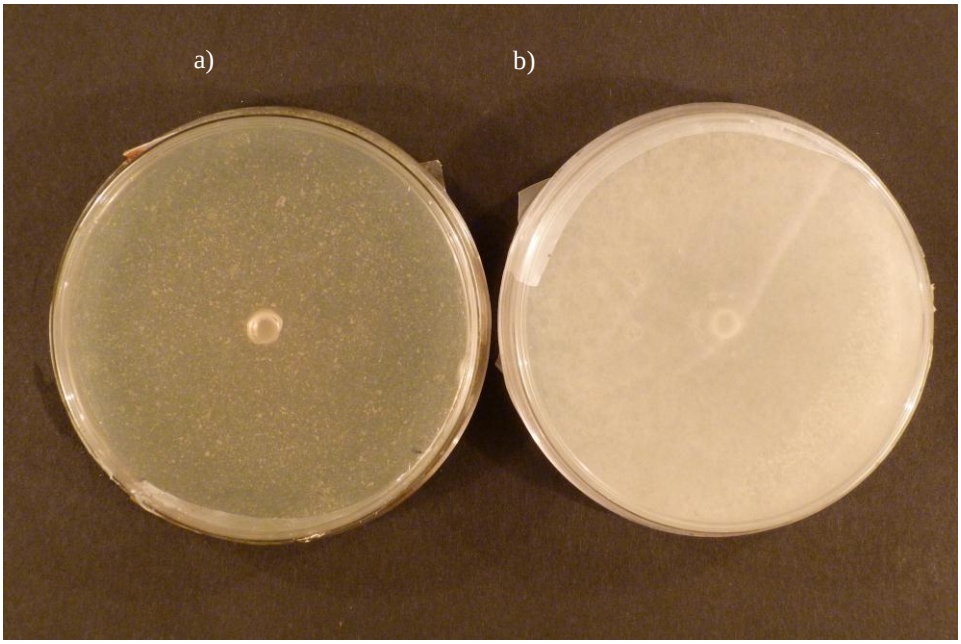

Figure 5 – Growth of *S. sclerotiorum* on PDA after 4 days for a) 2g *Brassica juncea* 'Pacific Gold' dried plant material and RO water and b) untreated .

Results

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 trials (Figures 6 and 7). The low glucosinolate oilseed rape cultivar 'Temple' also slightly inhibited mycelial growth.

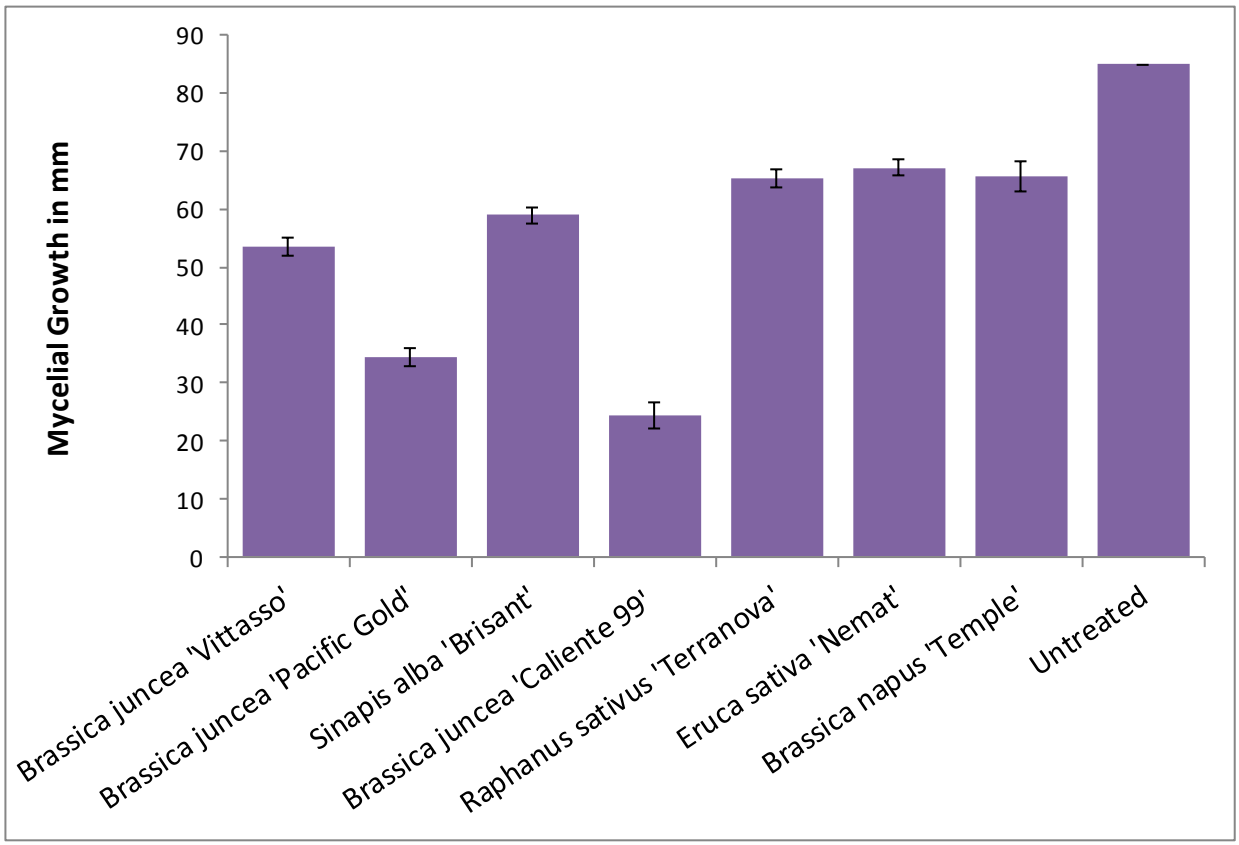

Figure 7 – The effect of 1g of dried biofumigant crops on mycelial growth of *S. sclerotiorum* after four days

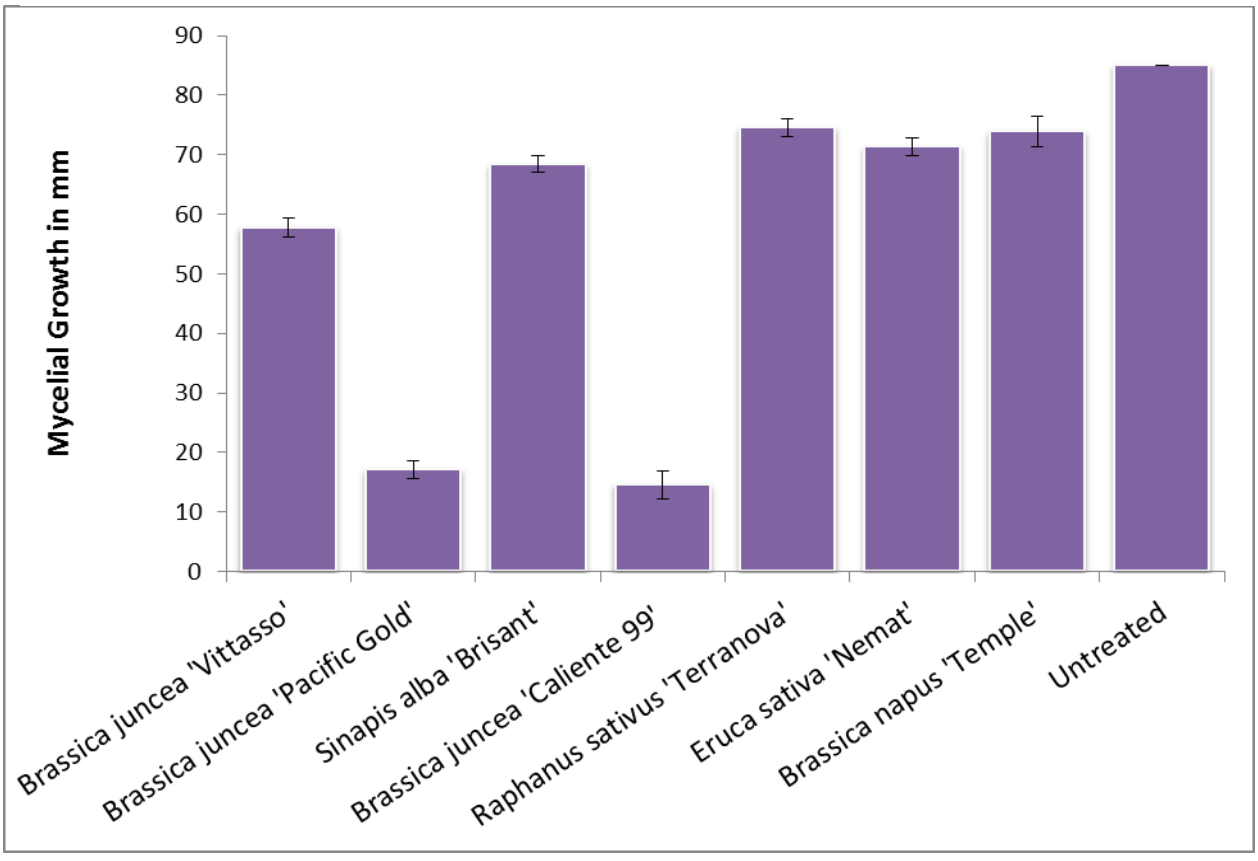

Figure 6 - The effect of 2g of dried biofumigant crops on mycelial growth of *S. sclerotiorum* after four days

In vitro Biofumigation Trials – Carpogenic Germination

Materials and Methods

Trials were carried out to determine whether the 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 trials (three trials at each rate). All biofumigant crops and low glucosinolate *Brassica napus* 'Temple' were grown, harvested and processed as for the soil box trials. Sclerotia of *S. sclerotiorum* isolate L6 were produced and conditioned as for the soil box trials.

Sieved and pasteurised compost (John Innes No. 1, J. Arthur Bowers; 50g) was placed into a Petri dish. 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 8). Water was added to the treatment and the lids immediately placed onto the boxes and they were 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 trial, 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.

Figure 8 - Petri dish with germinating sclerotia and separate dish of biofumigation treatment in 1200ml plastic box

Results

Each trial was run for at least 80 days to assess the effects of the biofumigation treatments and at the time of writing only four out of six trials have finished (two at each rate). There was limited reduction in germination at the rate of 1g of biofumigant crop (Figure 9), but at the rate of 2g *Brassica juncea* 'Vittasso' reduced germination by 84% in comparison with the untreated control. The low glucosinolate *Brassica napus* 'Temple' reduced germination by 67% and *Brassica juncea* 'Caliente 99' reduced germination by 56% (Figure 10).

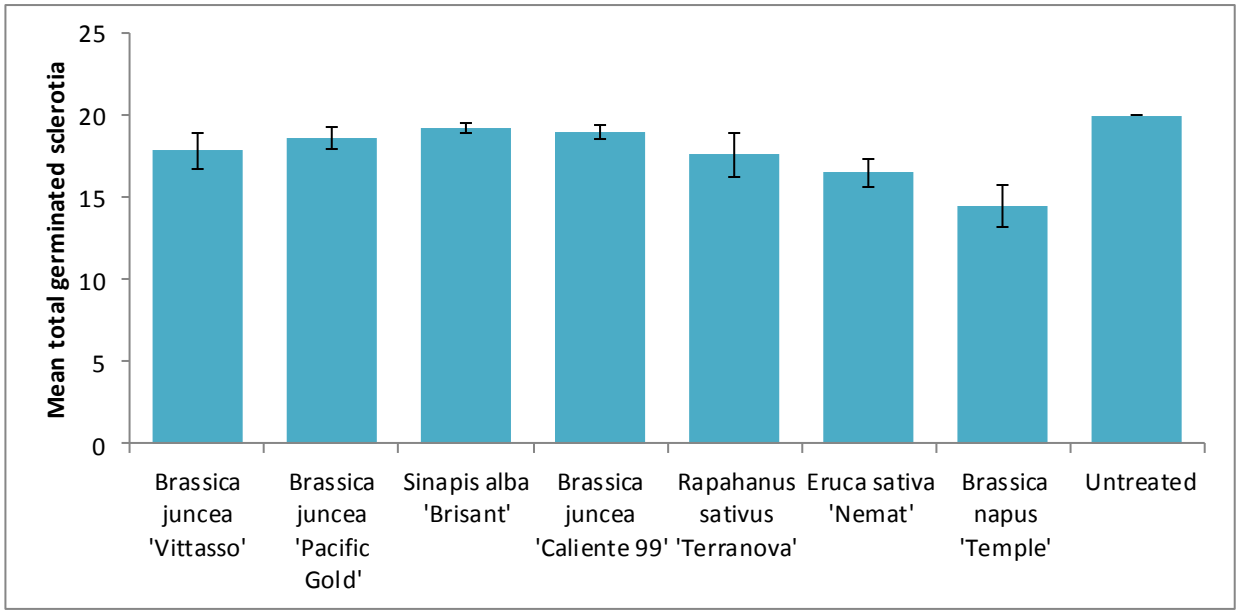

Figure 9 - The effect of 1g dried biofumigant crops and low glucosinolate *Brassica napus* 'Temple' on carpogenic germination of *S. sclerotiorum* sclerotia after 80 days in an *in vitro* trial

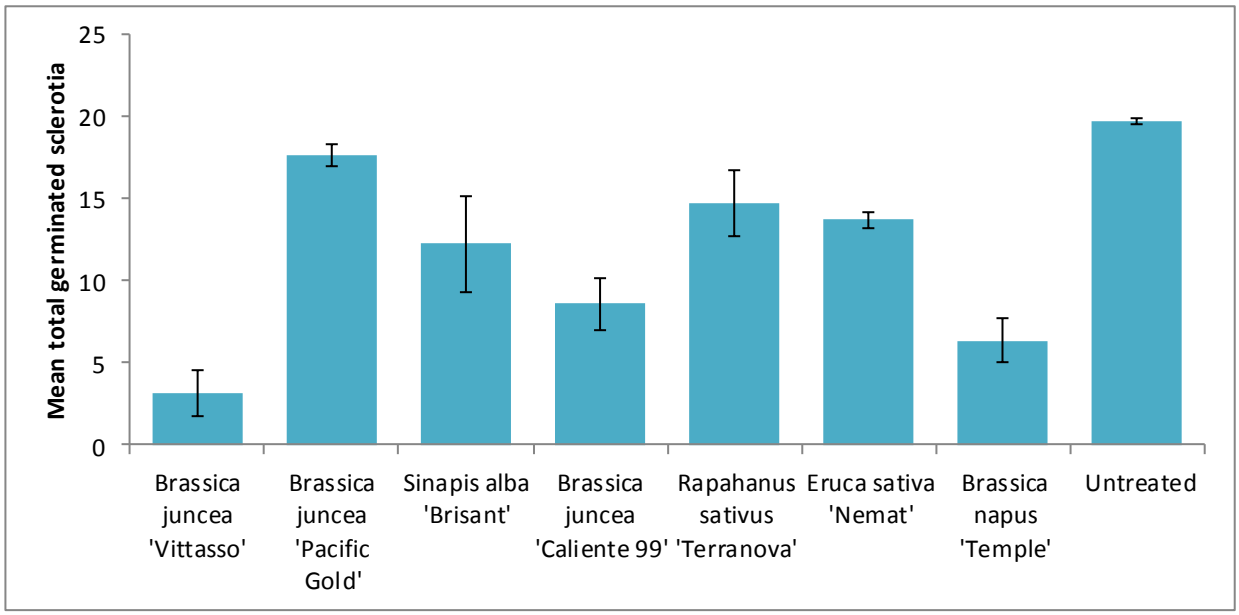

Figure 10 - The effect of 2g dried biofumigant crops and low glucosinolate *Brassica napus* 'Temple' on carpogenic germination of *S. sclerotiorum* sclerotia after 80 days in an *in vitro* trial

Polytunnel Biofumigation Trial

Materials and Methods

A polytunnel trial is currently being set up to assess the effect of selected biofumigant crops on viability of sclerotia and subsequent disease incidence in a lettuce crop. Three replicates of four treatments were arranged in a randomized block design (Figure 11). The biofumigant crops were sown according to supplier's sowing rates, and will be grown for eight weeks, or until flowering. They will then be shredded and rotavated into the beds and 50 sclerotia will be placed into each bed, in two grids of 25. Two weeks after incorporation the beds will be planted with lettuce. Once a week the sclerotia will be checked for germination and the lettuce will be checked for signs of disease.

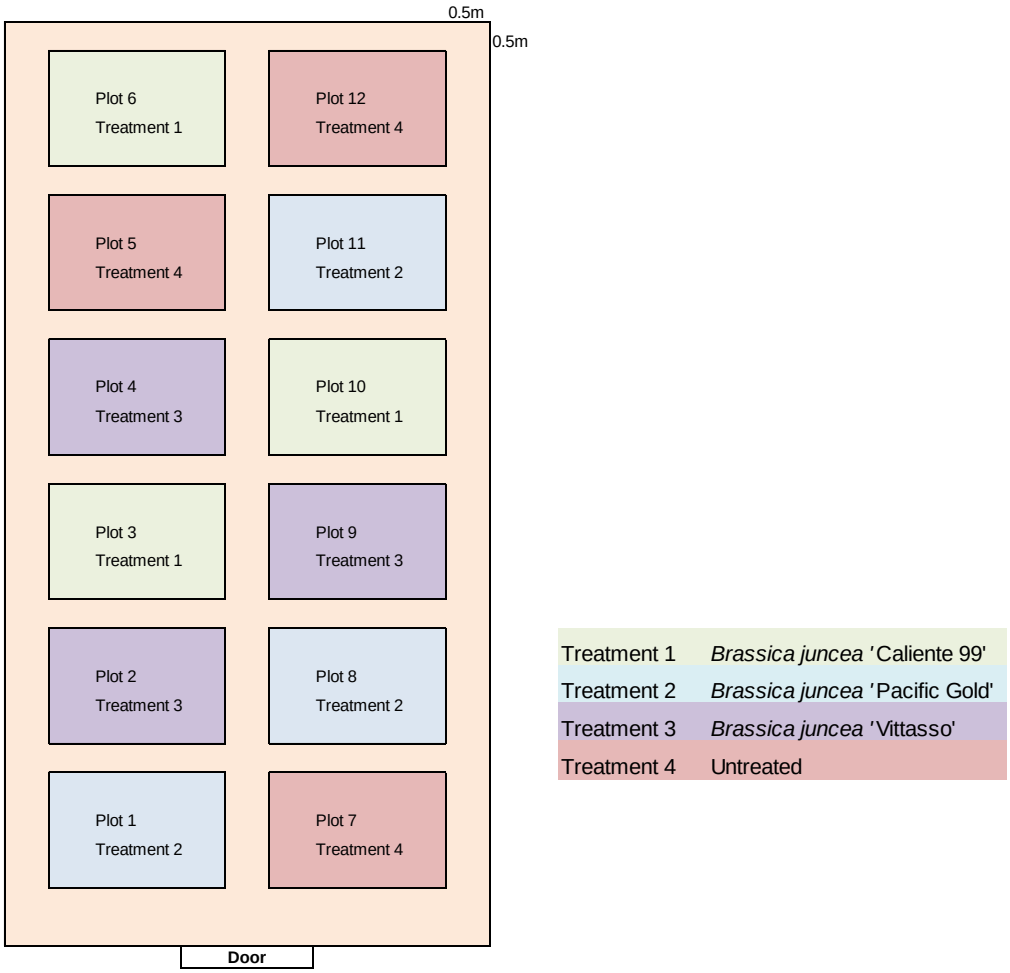

Figure 11 - Experimental plot layout for polytunnel biofumigation trial

Results

The trial has been running for three weeks so there are no results at the time of writing.

HPLC Analysis of Glucosinolates

Materials and Methods

All biofumigant crops used in the trials 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. All extractions were carried out on crops grown, harvested, dried and stored as for the soil box trials.

RO water (100ml) was brought to boiling point in a round bottomed flask with some anti-bumping granules. Plant material (1g) was then added and the mixture kept at boiling point for 30 minutes and condensed using a reflux condenser. The mixture was allowed to cool and then filtered through a 25µm syringe filter. HPLC analysis was undertaken using a HP Agilent 110 series system with a UV diode array detector. Separations were at approximately 23°C on a reverse-phased Zorbax SB-Aq 4.6 x 250mm 5 µm column (Agilent), with a running pressure of approximately 43 bar. An eluent of 0.025M $CH_3CO_2NH_4$ (Ammonium Acetate) was used with a pump rate of 1ml/min and an injection volume of 20µl (Table 2). The retention times of the glucosinolates varied from three minutes to eight minutes (Table 3) with detection at 228 nm and 242 nm for detecting any breakdown of glucosinolates to isothiocyanates. 1000ppm and 100ppm standards (Phytolab GmbH & Co) were run in between every 3 samples.

Table 2 - Flow gradient used in HPLC analysis of glucosinolates of biofumigant crops.

Time (mins)(Cumulative)	Ammonium Acetate	Acetonitrile
0	99%	1%
3	99%	1%
4	50%	50%
20	50%	50%
21	99%	1%
26	99%	1%

Table 3 - Retention times of glucosinolates in HPLC analysis

Crops	Glucosinolate	Time (mins)
Brassica juncea / Brassica napus	Sinigrin	3.4
Sinapis alba	Sinalbin	5.6
Raphanus sativus	Glucoraphenin	4.2
Eruca sativa	Glucoerucin	7.8

Results

Only one extraction for each crop at each harvest date has been completed at the time of writing. The highest levels of the glucosinolate sinigrin were found in *Brassica juncea* 'Caliente 99' at the first three harvest times (Figure 11), peaking at 26.5 µmol/g^{-1} dw. Glucosinolate levels generally decreased between the first harvest date of 27th June 2012 and the last harvest date of 5th November 2012. Very small amounts of sinigrin were detected in *Brassica napus* 'Temple' and glucoerucin in *Eruca sativa* 'Nemat' was undetectable due to a contamination peak obscuring the peak on the chromatogram.

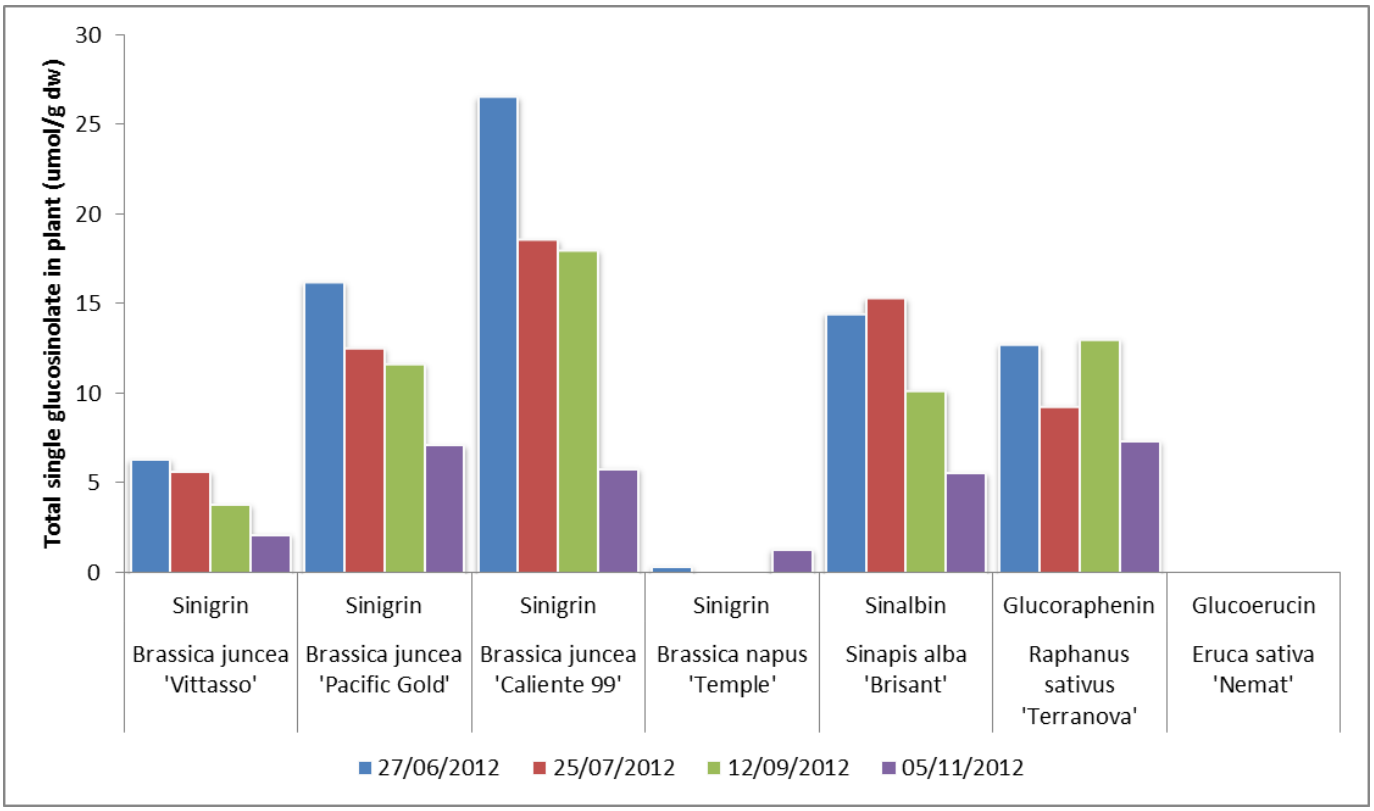

Figure 11 – Single glucosinolate levels (sinigrin, sinalbin, glucoraphenin and glucoerucin) as determined by HPLC analysis of four different crops of biofumigant plants. Glucoerucin was undetectable due to a contamination peak on the chromatogram.

Objective 2 – Aggressiveness of *Sclerotinia sclerotiorum* isolates and production of sclerotia

Carrot Root Inoculation

Materials and Methods

Two trials were carried out to assess the production of sclerotia by two *S. sclerotiorum* isolates (L6 and L44) on roots from a carrot diversity set grown at the Wellesbourne site by the Genetic Resources Unit. Previously, isolate L6 has been found to produce large numbers of small sclerotia while isolate L44 produces small numbers of larger sclerotia. A 5mm plug of mycelium was placed into the centre of each carrot root incubated on damp tissue in bagged trays at 13°C (Figure 12). In both trials four replicate roots for each of 88 accessions (where available at harvest) for each *S. sclerotiorum* isolate were inoculated. Sclerotia were retrieved, counted and weighed once they were mature.

Figure 12 - Carrot roots incubated on damp tissue, three weeks after being inoculated with *S. sclerotiorum* isolate L44.

Results

Statistical analysis was carried out using a restricted (or residual) maximum likelihood. Isolate L6 produced an average of 0.612 sclerotia g^{-1} of carrot root tissue, while L44 produced just over half that amount, at 0.347 sclerotia g^{-1}. Some of the accessions produced very few sclerotia for either *S. sclerotiorum* isolate, but generally more sclerotia were produced by isolate L6 than isolate L44 (Figure 13).

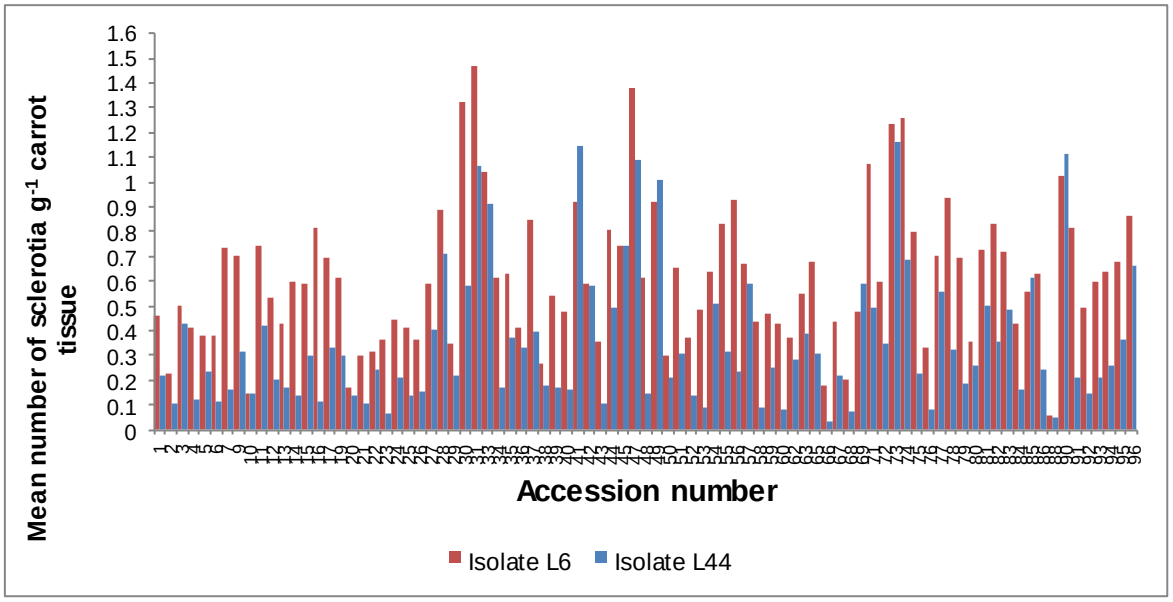

Figure 13 - Mean numbers of sclerotia produced per 1g of carrot tissue, for *S. sclerotiorum* isolates L6 and L44, organised in order of carrot accession number.

Whole Carrot Plant Inoculation

Materials and Methods

Whole carrot plant inoculations to assess the susceptibility of different cultivars and accessions to *S. sclerotiorum* have been carried out in three trials. The results from the carrot root inoculation trial, together with root position and leaf growth habit was taken into account to obtain a diverse range of varieties to test (Table 4).

Table 4 - Varieties used in whole carrot plant inoculation trial, and their growth habits and sclerotia production on roots.

Carrot Diversity Set No.	Group	Name	Root position in soil	Leaf growth habit	L6 sclerotia production	L44 sclerotia production
7	Elite	Nairobi	shallow	semi-upright	high	low
	Elite	Chantenay	shallow - medium	upright	low - med	low
	Elite	Eskimo	deep	upright		
	Elite	Narbonne				
90	Mapping parent - wild	QAL	deep	upright	high	med
86	Mapping parent - elite	Brasilia	shallow	upright	med	low
93	Mapping parent	USDA 9304B	shallow	upright	low	low
92	Mapping parent	USDA 7262B	deep	upright	low	low
30	Wild	7159	deep	prostrate	high	med
51	Cultivated	Little finger	shallow	prostrate	high	med

Carrot plants were grown in 3L deep pots in a polytunnel. At 18 weeks old, six plants of each cultivar were moved to a glasshouse and three leaves on each plant were inoculated by cutting off the leaf and placing a pipette tip with a mycelial plug inside onto the cut end (Figure 14). The plants were then covered with a plastic bag to maintain humidity for three days, and sprayed with water three times a day.

The distance from where the petiole meets the crown of the plant to the edge of any lesion on the petiole was measured, and progression of infection into the crown of the plant was scored from zero (no infection in crown) to four (crown diseased and rotten). The plants were assessed twice a week, for a total of four weeks.

Figure 14 - Carrot plants in a glasshouse with pipette tip inoculations of *S. sclerotiorum* on three petioles per plant.

Results

All carrot plants in the trials became infected, with many showing infection in the crown of the plant by the end of four weeks. The rate of lesion spread down the petiole was slowest in Brasilia and Little Finger (Figure 15), both of them being lines from the carrot diversity set.

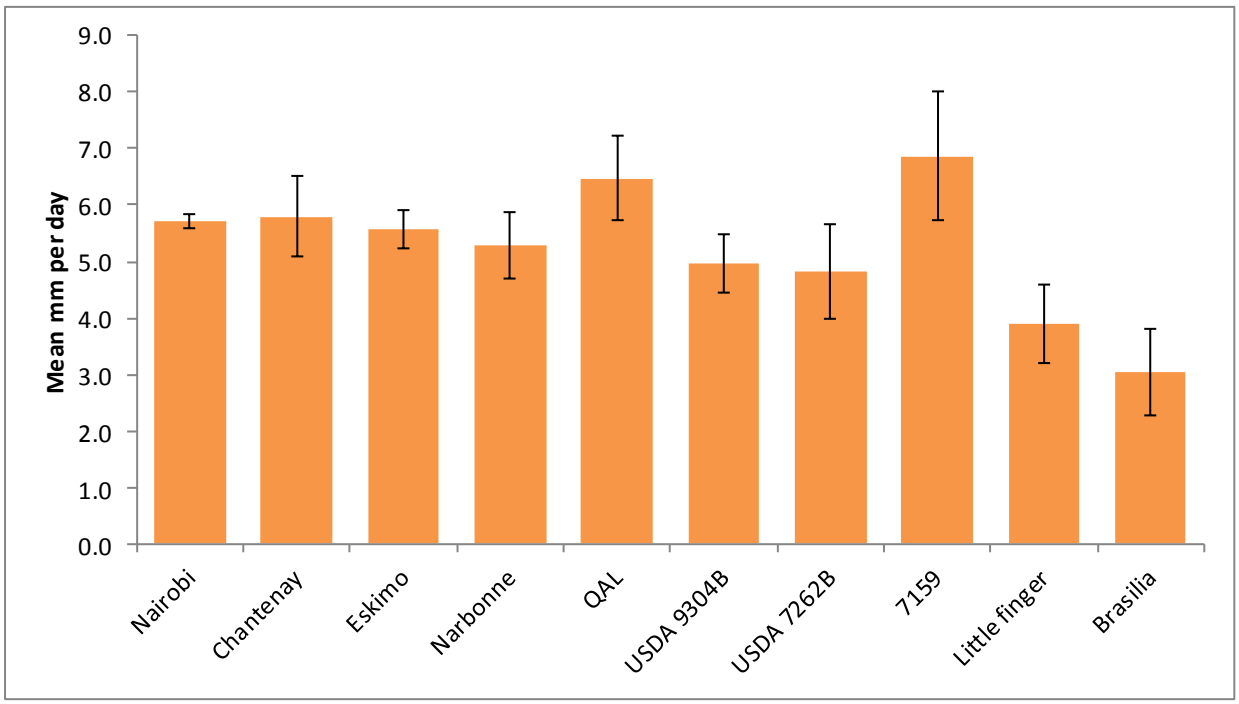

Figure 15 - Mean growth rate of *S. sclerotiorum* (mm day^{-1}) along petioles over four weeks for different carrot varieties/lines.

Objective 3 – Epidemiology and control of *Sclerotinia subarctica*

Microsatellite Markers

Methods and Materials

Samples of *Sclerotinia* spp. sclerotia were collected from buttercups and different crops in Scotland (pea, potato and swede), from crops in Norway (lettuce, swede and artichoke) and from lettuce in Sweden. Genomic DNA was extracted from freeze-dried mycelium for 193 isolates using a DNeasy plant mini kit. Identification of *S. subarctica* isolates was made by PCR amplification of the large subunit ribosomal DNA (Holst-Jensen *et al.*, 1998). The 32 *S. subarctica* isolates identified were then characterised using eight microsatellite markers in two separate multiplexed PCR reactions, four loci per reaction (Winton *et al.*, 2007). Primer mix one contained MS01, MS03, MS06 and MS08 and primer mix two contained MS02, MS04, MS05, and MS07. PCR amplification was carried out with thermocycling parameters of 95°C for 15 min; 35 cycles of 94°C for 30s, 55°C for 90s, 69°C for 75s; 60°C for 30min and then a hold at less than 12°C. All products were sized using an ABI Prism 3100 Genetic Analyser.

Results

The microsatellite data from the 2012 *S. subarctica* isolates has been compared with data from 2011 and previous work carried out on isolates obtained in 2009 and 2010. All Scottish, Norwegian and Swedish *S. subarctica* isolates are different genotypes from any of the isolates collected in Herefordshire (Figure 16). Only four genotypes were found within 33 isolates from Herefordshire, while 23 genotypes were present in 42 isolates from Scotland. Some of the isolates from Scottish buttercup and crops in Scotland shared genotypes. However, 32 of the isolates did not share a genotype with any other isolates currently found.

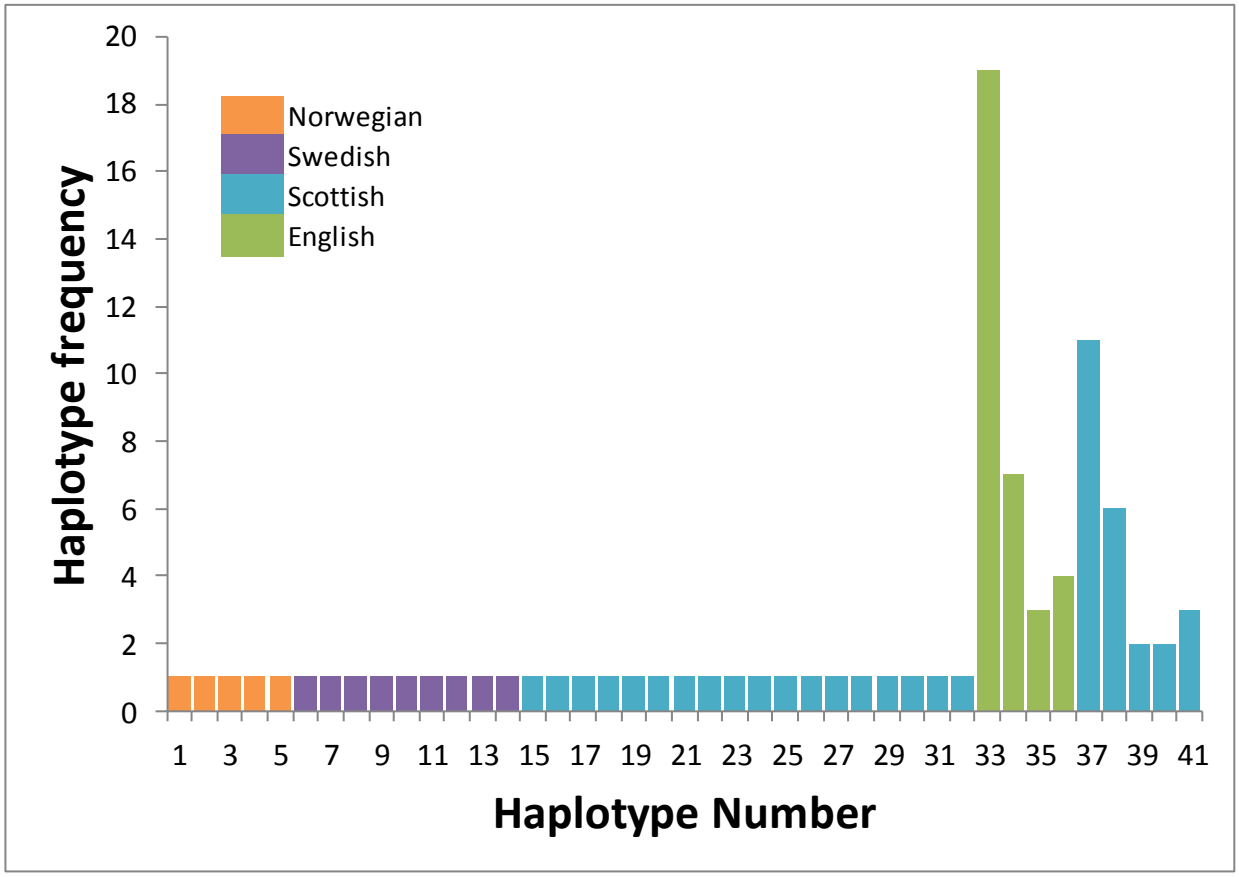

Figure 16 – Haplotype frequency found amongst *S. subarctica* isolates from Norway, Sweden, England and Scotland as determined by microsatellite marker analysis

Mycelial Growth of Sclerotinia spp. at Different Temperatures

Methods and Materials

Trials were carried out to test mycelial growth of *S. subarctica* in comparison to *S. sclerotiorum* at four different temperatures (5°C, 10°C, 15°C and 20°C). One 5mm mycelial plug of actively growing mycelium from each of four *S. subarctica* isolates and one *S. sclerotiorum* isolate was placed in the centre of a PDA plate and then placed into incubators in the dark. Three replicates of each treatment were set up in each trial, 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. At the time of writing only one of three trials has been completed.

Results

There was no significant difference in the rate of growth of the *S. subarctica* isolates (HE1, Liel17a1, PS13 and MH22) in comparison with the *S. sclerotiorum* isolate L6 (Figure 17) at any of the four temperatures.

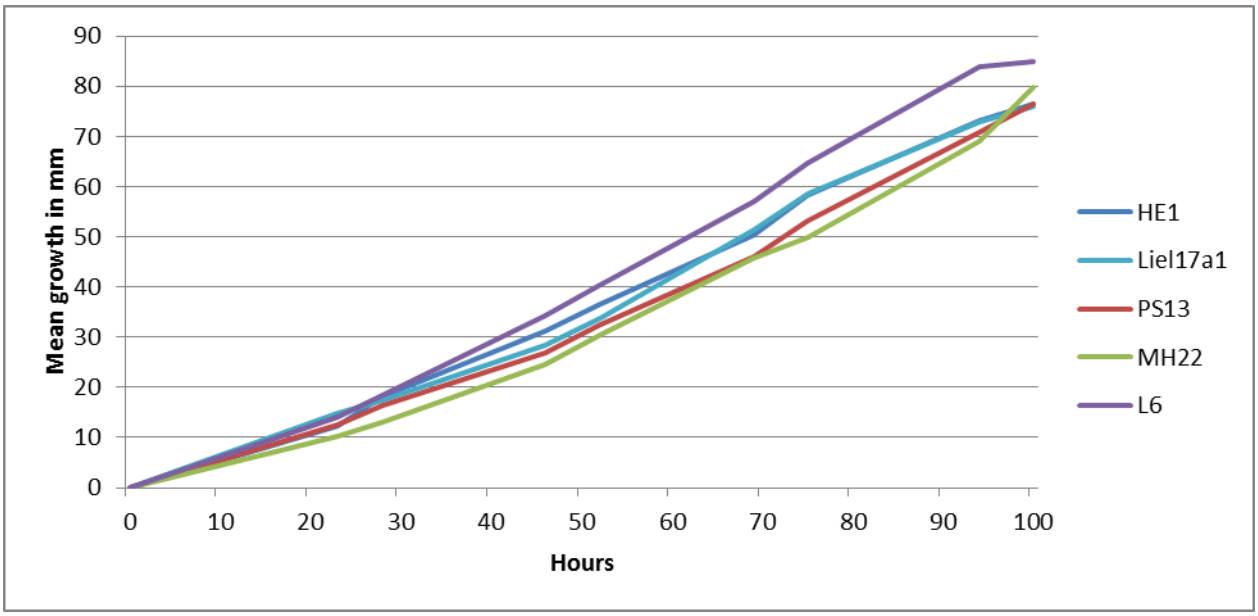

Figure 17 - Mean rate of mycelial growth at 10° for four *S. subarctica* isolates (HE1, Liel17a1, PS13 and MH22) and one *S. sclerotiorum* isolate (L6)

Conditioning Time for Carpogenic Germination of *S. subarctica*

Methods and Materials

Trials have been set up to ascertain the optimum length of chilling time at 5°C required for rapid carpogenic germination of *S. subarctica* sclerotia, and for comparison with *S. sclerotiorum*. Sclerotia of *S. sclerotiorum* isolates L6 and L44, and sclerotia of *S. subarctica* HE1, HE3, HE4 and HE8 were produced as for the soil box trials.

Sieved and pasteurised compost (John Innes No. 1, J. Arthur Bowers, 50g) was placed into a Petri dish. 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 weighed before being incubated for different durations in the dark (Table 6). After the incubation period at 5°C the Petri dishes were moved to a controlled environment room at 15°C in the dark.

Table 6 - Chilling times used for treatments in carpogenic germination of *S. subarctica* trials

Treatment Number	***Duration at 5°C (days)***
1	0
2	20
3	40
4	60
5	80
6	100
7	120
8	140

Four replicates of each treatment were set up in each trial, 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 was recorded once a week and germinated sclerotia removed from the Petri dish.

Results

Trials are still ongoing so no results available at the time of writing.

Discussion

Objective 1 - To determine the effect of organic soil amendments on the survival of sclerotia of Sclerotinia sclerotiorum.

Results from the soil box trials show that biofumigant crops can significantly reduce carpogenic germination of *S. sclerotiorum*, and the results from the *in vitro* trials indicates that this is caused directly by ITCs being released from the plant material, at least in the case of the brown mustards (*Brassica juncea)*. However, the low glucosinolate oilseed rape cultivar, *Brassica napus* 'Temple' also reduced carpogenic germination and the HPLC analysis confirmed that it does not contain significant levels of glucosinolates. This suggests that there may be other volatile compounds being released from 'Temple' which affects the germination of sclerotia. Future work will now look at the effect of sclerotial size on biofumigation efficacy.

The HPLC analysis showed a clear difference in the glucosinolate quantities in crops grown at different times. Further examination of this data will indicate whether this is due to changing temperatures across the cropping dates, or pest damage. The polytunnel trial will help to assess the effectiveness of the biofumigant crops on carpogenic germination in a realistic setting, as well as determining effects on disease incidence which may be reduced if sclerotia are germinating under stress due to the effects of the biofumigation.

Objective 2 - To determine the aggressiveness of different Sclerotinia genotypes and species on commercial carrot varieties and quantify production of sclerotia.

The repeated carrot root inoculations showed that there is consistent variation in the number and sizes of sclerotia produced by the two different isolates of *S. sclerotiorum*. The size of sclerotia may affect their survival in soil, and the number of apothecia produced, therefore having a direct impact on the relative frequencies of each isolate. Some of the cultivars in the trial produced very few sclerotia for either *S. sclerotiorum* isolate and would therefore be suitable for future breeding work. The whole carrot plant inoculation trials indicated that 'Little Finger' and 'Brasilia' may be suitable cultivars for such a program, by having the slowest disease progression down the petiole.

Objective 4 - Epidemiology and control of Sclerotinia subarctica.

The results from the genotyping analysis showed that there is considerable diversity in isolates of *S. subarctica* in Scotland, in comparison to isolates from England (Herefordshire). This may indicate that sexual reproduction is occurring in Scotland where the conditions may be more favourable for this species. The research investigating requirements for carpogenic germination will help to determine the conditions required by this species, although the mycelial growth trials showed no significance differences between *S. subarctica* isolates and a *S. sclerotiorum* isolate.

Conclusions

- In soil box trials *Raphanus sativus* 'Terranova' reduced carpogenic germination of *S. sclerotiorum* sclerotia by 73% in comparison to the untreated control which compares well to the positive control Perlka® which reduced germination by 82%
- The best overall control in soil box trials and direct *in vitro* tests was achieved by *Brassica juncea* 'Caliente', which also had the highest levels of the glucosinolate sinigrin out of all the brown mustards.
- Roots from some carrot lines produce very few sclerotia while others from whole plant tests show slow disease progression after inoculation and could therefore be used in future breeding programs.
- Results from genotyping suggest that *S. subarctica* isolates are more diverse in Scotland compared to those found in Herefordshire.

Knowledge and Technology Transfer

- 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 HDC Studentship Conference, September 2013
- Poster at AHDB Crop Conference, September 2013

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