Project title:	Carrots: Control of carrot cavity spot through the use of pre-crop green manures/biofumigation.
Project number:	FV405
Project leader:	John Clarkson, Warwick Crop Centre, School of Life Sciences, University of Warwick, Wellesbourne Campus. Wellesbourne, Warwick, CV35 9EF.
Report:	Annual Report Year 1
Previous report:	None
Key staff:	Andrew Taylor Claire Handy Steve Ellis (ADAS)
Location of project:	Warwick Crop Centre
Industry Representative:	John Kenyon, Marsh Farm, Marsh Road Hesketh Bank, Preston PR4 6XT
Date project commenced:	01/09/12
Date project completed (or expected completion date):	31/01/16

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

Pythium violae was detected in soil from around mature carrots in crops drilled after the incorporation of different biofumigant and green manure crops. However, cavity spot disease failed to develop significantly in any of these treatments or the untreated control. The potential of these crops against cavity spot could not therefore be determined due to the low disease levels.

Background

Cavity spot is the major disease of carrots in the UK and is caused mainly by *Pythium violae*. Control of the disease largely relies on early application of fungicides but enhanced degradation of these products may also be a problem in some soils. Long rotations between carrot crops are also recommended. Although cavity spot has been studied in a number of different projects over the years, the dynamics of the pathogen have only begun to be addressed recently, following the development of a specific DNA-based PCR test for *P. violae* and its use in UK-focused research (Anon, 2009). However, this test cannot be used to predict disease before carrot planting (Barbara, 2007), or in the autumn to assess disease risk in strawed down crops, as there is evidence that final levels of disease are to a large extent driven by environmental conditions such as soil moisture (Barbara, 2010; Martin, 2011).

Recently, there has been increased interest in the potential use of biofumigation and green manure crops to control soilborne diseases such as *P. violae*. Green manure crops aim to enhance soil health and fertility with the potential added benefit of encouraging soil microbial activity which may suppress certain plant pathogens. In previous work, a clover/ryegrass mix or a potato crop grown before carrots reduced cavity spot disease whereas other preceding crops such as forage rape and wheat maintained or enhanced *P. violae* levels (Kretzschmar, 2009). It was proposed that this effect was due to the relative ability of different plants or their associated microbiota to sustain or suppress the pathogen.

Biofumigation involves crushing and incorporating specific biofumigant crops into the soil. This process, when carried out under high soil moisture conditions, allows the conversion of glucosinolate compounds to isothiocyanates (ITCs) which are toxic to a range of soil microorganisms. Biofumigation using a brown mustard resulted in good control of cavity spot in one experiment previously (Anon, 2009), but there was seemingly little effect on *P. violae* levels as detected by PCR. This suggested that disease suppression may actually be

due to the build-up of competing soil microbiota following incorporation of the biofumigant, a hypothesis also supported by the observation that soil pasteurisation using the chemical fertiliser calcium cyanamide resulted in an increase in cavity spot (Anon, 2009).

In addition to cavity spot caused by *P. violae*, carrot crops are also increasingly affected by Sclerotinia disease caused by *Sclerotinia sclerotiorum*. *S. sclerotiorum* survives in the soil as sclerotia which when brought close to the soil surface germinate carpogenically to produce mushroom-like apothecia. These then release air-borne ascospores which infect plants. Additional problems in carrot crops such as fanging and other root disorders may also occur due to free-living nematodes (particularly stubby-root and needle nematodes), although there is still scant evidence for the extent and type of symptoms they cause (Ellis, 2000). Biofumigation / green manures may therefore also have some benefit against these other carrot problems.

The main aim of this project was therefore to test the effect of biofumigant mustards and green manure crops on the dynamics of *P. violae* and cavity spot disease. Secondary aims of the project were to 1) determine the dynamics of free-living nematodes during the carrot growing season and assess fanging and 2) test the effect of biofumigant mustards and green manure crops on germination of *S. sclerotiorum* sclerotia

Summary

Field experiments and assessments

Two field experiments were carried out in Cottage Field at Wellesbourne (where cavity spot was previously known to develop), primarily to test the effect of different biofumigant / green manure crops on cavity spot disease and the dynamics of *P. violae*. At the same time, the effects of these treatments on free-living nematodes, carrot fanging and germination of *S. sclerotiorum* sclerotia were also assessed. The first experiment established biofumigant / green manure crop treatments (two mustards, wheat, clover-rye mix, forage rape) in September 2012 which were incorporated in late May 2013. The second established biofumigant treatments (two mustards) in March 2013 which were incorporated in early June 2013. For both experiments, biofumigants / green manures were sown in beds and carrots drilled approximately two weeks after incorporation. Mustard cultivars used were the brown mustard Caliente 119 and the white mustard Brisant, both of which have been bred for high glucosinolate content. The forage rape cv. Hobson was included as a low glucosinolate Brassica ‘control’, while the wheat cv. Consort and the clover rye mix (cvs. Hobson / Merwi) were included as they had previously been shown to maintain / suppress cavity spot respectively. Biofumigant / green manure crops were netted or fleeced to prevent pigeon/rabbit damage, subsequently crushed / chopped using a flail topper and

incorporated using a bed former when the mustard crops were at full flower (when maximum glucosinolate levels occur). Irrigation was applied immediately to all treatments, ensuring adequate moisture to allow the conversion of glucosinolates to ITCs in the two mustard crops. For the autumn experiment only, sclerotia of *S. sclerotiorum* were buried in each treatment plot immediately after incorporation (but before irrigation) to assess germination and production of apothecia. Following incorporation of the autumn / spring biofumigant / green manure crops, carrots (cv. Nairobi) were drilled and all plots irrigated regularly to encourage development of cavity spot. Leaf samples from brown / white mustards and forage rape were taken to quantify the levels of glucosinolates using HPLC. Soil and / or small carrot root samples (40 roots per treatment) were taken during the season for PCR detection of *P. violae*, counts of free-living nematodes and to assess cavity spot disease levels and fanging. The main assessments of cavity spot and fanging were carried out for two large harvests of carrot roots (160 roots per treatment) before and after strawing down of the carrot crops.

Direct effect of biofumigant plants on *P. violae*

Additional work was carried out as part of a University of Warwick Summer Studentship to determine if ITC volatiles released from selected biofumigants including brown and white mustards had a direct effect on *P. violae in vitro*. For each biofumigant, the growth of *P. violae* mycelium was assessed on agar in an inverted Petri dish where water was added to dried, milled plant material in the lid to liberate ITCs.

Results and conclusions

Biofumigant / green manure crop growth and glucosinolate levels

The autumn-sown biofumigants / green manures over-wintered well but a cold spring resulted in a relatively late flowering time and initially a slow development of the spring-sown mustard crops, even though these were fleeced. Autumn and spring-sown mustard crops were in full flower in late May / early June respectively. This is the optimum time for incorporation (highest glucosinolate levels) and hence for carrot growers who drill mainly in April and May, or even earlier, the period for growing mustard biofumigants to this stage is short. Glucosinolate levels in the brown and white mustard crops were also lower compared with those obtained using the same mustard types when gown in a polytunnel in June (Clarkson, 2013). The most likely reason for this was the combination of low temperature and short day-length in winter / early spring, both of which have been demonstrated to reduce glucosinolate production.

Dynamics of *P. violae*

P. violae was detected by PCR in all treatment plots (from soil or soil from around carrots) for at least one sampling time over the duration of the autumn and spring-sown experiments (Fig. 1). However, based on the number of plots per treatment testing positive for the pathogen, there was no clear effect of biofumigation / green manure treatments. Generally, *P. violae* dynamics followed a similar pattern for all the treatments plots and there were three sampling times for the autumn-sown experiment where the pathogen was detected in most plots; 19/02/13 (biofumigants/green manures semi-mature), 20/09/13 (subsequent carrot crops mature) and 06/03/14 (post carrot crop strawing down). The general increase in *P. violae* levels in late autumn as the carrot crops matured has been observed previously (Anon, 2009). However, an apparent decrease in the pathogen in October / November 2013 in the presence of overwintered carrots is unexpected but examination of recorded weather data suggests that there was a drier period during this time (data not shown). Detection of *P. violae* in February 2013 in the absence of carrots was also unexpected and requires further investigation.

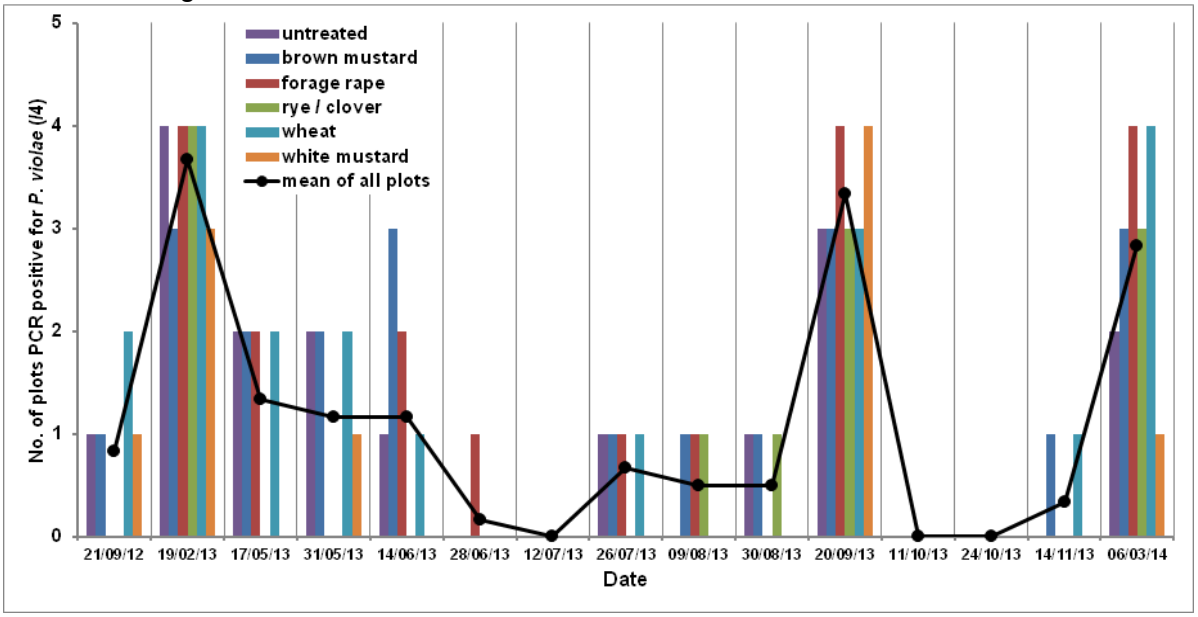


Figure 1. Number of plots (out of a maximum of four) positive for *P. violae* using PCR for autumn-sown biofumigant / green manure crops. Samples for PCR were from bare soil or soil from around plants or from around carrots roots.

Development of cavity spot and effect of biofumigants / green manures

Cavity spot levels as recorded for the same ten roots used for PCR detection remained low throughout the season for both autumn and spring-sown biofumigant / green manure experiments with an average of less than two lesions per root. Cavity spot incidence (presence of one lesion or more) was variable and ranged between 40 and 70%. Similarly, cavity spot levels in the larger root samples (160 roots per treatment) harvested pre- and post- strawing down of the carrot crops on 14/11/13 and 06/03/14 were also low for both

experiments. Here, the average number of lesions per root was less than one and cavity spot incidence (presence of one lesion or more) varied between 23 and 42%. Due to these low disease levels, it was not possible to determine any effect of the treatments.

Effect of biofumigants / green manures on free-living nematodes and carrot fanging

Initial counts showed that *Trichodorus* spp. (stubby root), *Tylenchorynchus* / *Helicotylenchus* spp. (stunt / spiral), *Pratylenchus* spp. (root lesion) and *Longidorus* spp. (needle) nematodes were all present in Cottage Field while other free-living nematode types were absent or below detectable levels. However, only *Longidorus* spp. were present in sufficient numbers (>200/L soil) to cause damage throughout the field experiments. For both autumn and spring experiments, numbers of these and all the other nematode spp. declined considerably following incorporation of the biofumigants / green manures but this effect was observed for all the treatments including the untreated (fallow) control. This led to the conclusion that the treatments themselves had no effect on free-living nematodes and hence the decline may have been due to other factors, most likely the tilling operations involved in incorporating the treatments and drilling subsequent carrot crops.

Effect of biofumigants / green manures on germination of *S. sclerotiorum* sclerotia

In the autumn-sown experiment, final mean percentage germination for buried *S. sclerotiorum* sclerotia were 65, 61, 73, 54, 57 and 71% for brown mustard, white mustard, forage rape, rye / clover mix, wheat and untreated control treatments respectively. Statistical analysis showed that there was a small significant effect on germination for the rye/grass clover treatment only. This was in contrast to findings by Clarkson (2013) under controlled conditions (in enclosed boxes) where a brown mustard significantly reduced carpogenic germination by 63% compared to the untreated control.

Direct effect of biofumigant plants on *P. violae in vitro*

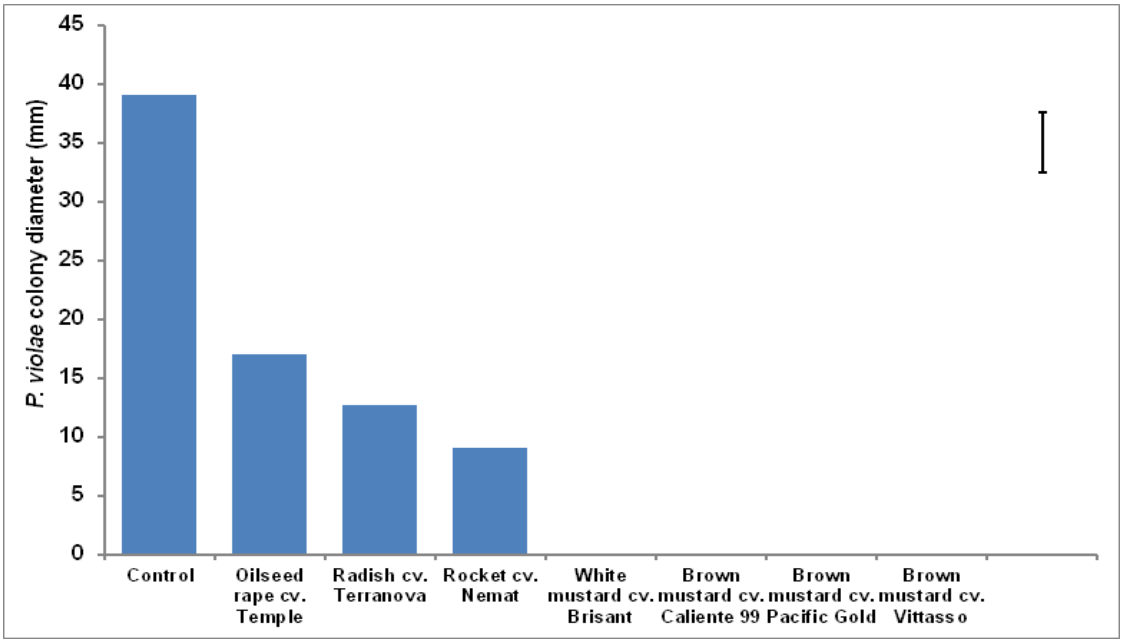
All biofumigant treatments significantly inhibited the growth of *P. violae* by more than 50% on agar. The brown and white mustards tested completely inhibited growth of *P. violae* (Fig. 2). This is the first evidence that such biofumigants may have a direct effect on the pathogen rather than potentially reducing disease through increased microbial activity as previously suggested.

Overall conclusions

Although ITCs from mustard biofumigants can inhibit both *P. violae* and *S. sclerotiorum*, it is clear that demonstrating this effect in the field is challenging. The primary problem was the low levels of cavity spot developing in experiments despite the extensive use of irrigation in an attempt to promote disease. However, it is important to note that the efficacy of

biofumigation can be affected by any agronomic and environmental factors which result in reduced growth and low glucosinolate levels in the biofumigants, poor conversion of glucosinolates to ITCs due to inefficient crushing / incorporation of plant material and inadequate soil moisture levels as well as potential loss of ITCs through escape of volatiles in the air or dissolved in percolating water. The latter could be prevented by the use of polythene covers post-incorporation as used in the original work which demonstrated that brown mustards could reduce cavity spot (Anon, 2009) but this was deemed impractical and potentially costly by the industry.

Figure 2. Effect of biofumigants on growth of *P. violae* on agar after 7 days at 20°C. Bar indicates least Significant Difference (LSD, $P \leq 0.05$) between treatments.



Recommendations for work in Year 2 for consideration by BCGA

1) Include one or two grower sites to test selected biofumigants / green manures to reduce the risk of low cavity spot levels.
2) Reduce the number of biofumigant / green manure treatments to allow increased monitoring of *P. violae* dynamics by PCR detection and more accurate assessment of cavity spot disease levels through increased sampling of larger plots.
3) Reconsider using polythene covers to maximise biofumigation potential of mustards.
4) Cease monitoring of free-living nematodes and fanging to increase project focus on cavity spot and free up resources.

Financial Benefits

None at this time.

Action Points

None at this time.

SCIENCE SECTION

Introduction

Cavity spot is the major disease of carrots in the UK and is caused mainly by *Pythium violae* and less frequently by *P. sulcatum*. In other countries, *Pythium* species such as *P. vipa* and *P. intermedium* have also been associated with the disease. In addition, other fungi may cause similar root lesions or invade cavities e.g. *Cylindrocarpon destructans*, *Mycocentrospora acerina*.

Control of cavity spot is difficult and although application of appropriate fungicides can reduce disease by more than 50%, these must be applied early before disease is apparent. Enhanced degradation of these products may also be a problem in some soils. Long rotations between carrot crops are also recommended although some reports have suggested that there was no build-up of cavity spot disease over four years of continuous carrot cropping (Anon, 2009). Many other plant species including other crop plants can also potentially be asymptomatic hosts of *P. violae* (Kretzschmar, 2009).

Although cavity spot has been studied in a number of different Defra and HDC projects over the years, the dynamics of the pathogen have only begun to be addressed recently, following the development of a specific PCR test for *P. violae* in Norway (Klemsdal et al., 2008) and its validation and use in UK-focused research (e.g. Anon, 2009). However, the PCR test cannot be used to predict disease before carrot planting (Barbara, 2007), or in the autumn to assess disease risk in strawed down crops, as there is evidence that final levels of disease are to a large extent driven by environmental conditions such as soil moisture (Barbara, 2010; Martin, 2011). Hence, initial *P. violae* inoculum levels may be less important. The typical dynamics of the pathogen mirrors the development of the carrot crop, being undetectable in early spring, rising to a peak as the roots begin to mature in July / August, and rapidly declining in October / November (Anon, 2009). However, this may vary from year to year and year-round dynamics of *P. violae* have yet to be explored.

The interest in the potential use of biofumigation and green manure crops to control soilborne diseases such as *P. violae* has increased in recent years. Green manure crops aim to enhance soil health and fertility with the potential added benefit of encouraging soil microbial activity which may suppress certain plant pathogens. In previous work (Kretzschmar, 2009), a clover/ryegrass mix or a potato crop grown before carrots was found to reduce cavity spot disease whereas other preceding crops such as forage rape and wheat maintained or enhanced *P. violae* levels. It was proposed that this effect was due to

the relative ability of different plants or their associated microbiota to sustain or suppress the pathogen.

Biofumigation involves crushing and incorporating specific biofumigant crops into the soil. This process, when carried out under high soil moisture conditions, allows the conversion of glucosinolate compounds to isothiocyanates (ITCs) which are toxic to a range of soil microorganisms. Glucosinolates occur at high levels in certain *Brassica* and related crucifer crops such as mustards, For instance brown mustard (*B. juncea*) produces the glucosinolate sinigrin (allyl glucosinolate) which is converted to allyl isothiocyanate while white mustard (*Sinapis alba*) produces the glucosinolate sinalbin (hydroxybenzyl glucosinolate) which is converted to hydroxybenzyl isothiocyanate. Biofumigation using a brown mustard (*Brassica juncea*) previously resulted in good control of cavity spot (Anon, 2009), but in this case there was seemingly little effect on pathogen levels as detected by PCR. This suggested a different mode of action such as the build-up of suppressive soil microbiota following incorporation of the biofumigant. The potential importance of microbiota generally for the suppression of cavity spot was also supported by the observation that soil pasteurisation using the chemical fertiliser calcium cyanamide resulted in an increase in cavity spot disease.

In addition to cavity spot caused by *P. violae*, carrot crops are also increasingly affected by Sclerotinia disease caused by *Sclerotinia sclerotiorum*. *S. sclerotiorum* survives in the soil as sclerotia 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). Additional problems in carrot crops such as fanging and other root disorders may also occur due to free-living nematodes (particularly stubby-root and needle nematodes), although there is still scant evidence for the extent and type of symptoms they cause (Ellis, 2000). Biofumigation / green manures may therefore also have some benefit against these other carrot problems.

The main aim of this project was therefore to:

- Test the effect of biofumigant mustards and green manure crops on the dynamics of *P. violae* and cavity spot disease

Secondary aims of the project were to:

- Determine the dynamics of free-living nematodes during the carrot growing season and assess fanging.

- Test the effect of biofumigant mustards and green manure crops on germination of *S. sclerotiorum* sclerotia

Materials and methods

Field experiments

Two field experiments were carried out in Cottage Field at Wellesbourne (where cavity spot was previously known to develop) to test the effect of different biofumigant / green manure crops primarily on cavity spot disease and the dynamics of *P. violae*. At the same time, the effects of these treatments on free-living nematodes, carrot fanging and germination of *S. sclerotiorum* sclerotia were also assessed.

The first experiment established biofumigant / green manure crop treatments (two mustards, wheat, clover-rye mix, forage rape) in 1.83 m beds in September 2012 which were incorporated in late May 2013 while the second established biofumigant treatments (two mustards) in March 2013 which were incorporated in early June 2013 (Table 1). All biofumigants/green manures were drilled in 1.83 m beds in 8 rows at seed rates shown in Table 1. For both experiments, carrots were drilled approximately two weeks after incorporation. The mustard cultivars used were the brown mustard Caliente 119 and the white mustard Brisant, both of which have been bred for high glucosinolate content. The forage rape cv. Hobson was included as a low glucosinolate Brassica 'control', while the wheat cv. Consort and the clover rye mix (cvs. Hobson / Merwi) were included as they had previously been shown to maintain / suppress cavity spot respectively (Kretzschmar, 2009).

The autumn-sown biofumigant / green manure crops were netted until incorporation to prevent pigeon/rabbit damage while spring biofumigants and both carrot crops were fleeced until they were well established, to prevent the same problems and avoid any carrot fly. All biofumigants / green manures were crushed / chopped using a Khun flail topper and incorporated using a bed former when the mustard crops were at full flower (corresponding to when maximum glucosinolate levels occur). Irrigation was then applied immediately to all treatments, ensuring that adequate moisture was present to allow the conversion of glucosinolates to ITCs in the two mustard crops. For the autumn experiment only, sclerotia of *S. sclerotiorum* were buried in each treatment plot immediately after incorporation (but before irrigation) to assess germination and production of apothecia as described below.

For both autumn and spring-sown experiments, there were four replicate plots per treatment (4 m x 1.83 m) each separated by 2 m arranged in an augmented Trojan square design. The autumn experiment was arranged in 6 rows (across beds) x 4 columns (down beds)

with each treatment represented once in each row across the 6 beds. In both experiments, each treatment bed was separated by a complete untreated (fallow) bed and following incorporation of biofumigant / green manure crops, carrots (cv. Nairobi) were drilled in all the beds over each experimental area in four rows at a rate of 100 seeds per linear m. All plots were irrigated regularly to encourage development of cavity spot.

Leaf samples from each plot of the spring and autumn-sown brown and white mustards were taken on the day of incorporation to quantify the levels of sinigrin and sinalbin glucosinolates respectively at flowering using HPLC as outlined in by Clarkson (2013). For comparison, the presence of any glucosinolates was also quantified for the (low glucosinolate) forage rape.

Soil and carrot root samples, as described in detail below, were taken throughout the season for PCR detection of *P. violae*, counts of free-living nematodes and to assess cavity spot disease levels and fanging (Table 2). Assessment of cavity spot and fanging were also carried out for two large harvests of carrot roots on two occasions before and after strawing down of the carrot crops on 18/11/13.

Table 1. Summary of biofumigant / green manure crop treatments, sowing and incorporation dates and subsequent carrot crop sowing dates

Treatment	Sowing date	Seed rate	Incorporation date	Carrot sowing date
autumn crops				
brown mustard (cv. Caliente 119)	21/09/12	1.5 g m^{-2}	20/05/13	03/06/13
white mustard (cv. Brisant)	21/09/12	2.5 g m^{-2}	20/05/13	03/06/13
forage rape (cv. Hobson)	21/09/12	1.0 g m^{-2}	20/05/13	03/06/13
rye / clover mix (cv. Hobson / Merwi)	21/09/12	18.0 / 0.7 g m^{-2}	20/05/13	03/06/13
Wheat (cv. TBC)	21/09/12	15.7 g m^{-2}	20/05/13	03/06/13
untreated	-	-	-	03/06/13
spring crops				
brown mustard (cv. Caliente 119)	05/03/13	1.5 g m^{-2}	03/06/13	17/06/13
white mustard (cv. Brisant)	05/03/13	2.5 g m^{-2}	03/06/13	17/06/13
untreated	-	-	-	17/06/13

PCR detection of *P. violae,* cavity spot monitoring and disease assessments

Soil samples for PCR detection of *P. violae* were taken from bare soil, from between plants of developing crops, or from around carrots roots lifted at regular intervals during the growing season depending on the time of year (Table 2). Samples were taken between September 2012 (autumn drilling of biofumigants / green manures) and March 2014 (final disease assessment of carrot crops), and therefore included samples pre- and post-biofumigant / green manure incorporation and during development of the carrots crops. For soil samples taken in the absence of a crop or early on in crop development, a total of approximately 10 g of soil (from a depth of 8-12 cm) was taken from a five point 'W' sampling of each plot (2 g per sampling point), air dried for two days on the lab bench at room temperature and thoroughly mixed in a plastic bag before DNA extraction. For soil samples taken from around carrot roots, 10 roots were lifted from a five point 'W' sampling for each plot (40 roots per treatment) and any large lumps of soil attached removed and discarded before air drying for two days at room temperature. The soil from around each of the 10 carrots was then brushed off, combined and thoroughly mixed before DNA extraction. DNA extractions from all soil samples were carried out using 0.25 g soil and the PowerSoil DNA Isolation Kit (Camb Bio, UK). DNA samples were then stored at -20°C and subsequently used for PCR detection of *P. violae* in 20 µl reactions using the specific primers published by Klemsdal et al. (2008). Amplified PCR products were visualised by gel electrophoresis with an expected product size for *P. violae* of 352 bp. Thermo cycling parameters for the PCR reactions were 93°C for 2 min, followed by 40 cycles of 93°C 60s', 60°C 60s, 72°C 60s and finally 72°C for 10 min.

Cavity spot lesions were also counted on each of the 10 carrot roots that were sampled from treatment plots for *P. violae* PCR detection as a way of monitoring disease levels over time. In addition, because of the spatial variation and sporadic nature of the disease, two large harvests of 40 roots per plot (160 roots per treatment) were carried out on 21/10/13 (pre-strawing down) and 07/03/14 (post-strawing down) to properly assess the potential effect of the biofumigant / green manure treatments. For these large carrot samples, ANOVA was carried out in Genstat to assess any significant effects of the treatments.

Burial of *S. sclerotiorum* sclerotia and germination assessments

Sclerotia of *S. sclerotiorum* isolate L6 were produced as described by Clarkson (2013) by inoculating wheat grain in flasks with agar plugs from an actively growing culture on potato dextrose agar (PDA) and incubating at 18°C for six weeks. The sclerotia that were formed were harvested by floating off the wheat grain, and dried overnight in a laminar flow cabinet. Sclerotia were then conditioned in pasteurised compost at 30% moisture at 5°C for 40 days

to enable rapid and reliable carpogenic germination. Conditioned sclerotia (40 per plot, 160 per treatment) were then buried in a grid pattern in each plot (2 cm depth) for the autumn experiment only on 20/05/13 immediately after incorporation but before the irrigation was applied. Germination to produce apothecia was then recorded weekly from 28/08/13 to 18/11/13. ANOVA was carried out in Genstat to assess any significant effects of biofumigant / green manure treatments on final percentage germination of sclerotia.

Free-living nematode and carrot fanging assessments

Soil samples for free-living nematode counts from each plot were taken just before incorporation of biofumigants / green manures for autumn / spring-sown crops and approximately 14 days later, a few days before sowing of subsequent carrot crops (Table 2). All plots were sampled again .on 06/11/13 just before strawing down, approximately two weeks after the first of the larger harvest of carrot roots. Samples consisted of approximately 2 kg soil from a 5 point 'W' sampling from each plot (400 g / point) at 8-12 cm depth, and were sent to Dr Steve Ellis (ADAS, High Mowthorpe) for free-living nematode counts of *Trichodorus* spp. (Stubby Root), *Tylenchorynchus* / *Helicotylenchus* spp. (Stunt / Spiral), *Heterodera* spp. (Cyst), *Pratylenchus* spp. (Root Lesion), *Longidorus* spp. (Needle), *Xiphinema* spp. (Dagger), *Ditylenchus* spp. (Stem) and *Meloidogyne* spp. (Root knot). In addition, an initial sampling was also done in autumn 2012, just after biofumigants/ green manures were sown, where six 2 kg samples were taken from a 5 point 'W' sampling across the entire experimental area. The number of fanged carrots was assessed for the samples of 10 carrots per plot used for PCR detection of *P. violae* throughout the season and also for the two larger harvests of 40 carrots per plot carried out on 21/10/13 and 07/03/14, pre- and post- strawing down of carrots crops. For these large carrot samples, ANOVA was carried out in Genstat to assess any significant effects of the biofumigation / green manure treatments.

Table 2. Summary of *P. violae* PCR detection, cavity spot disease, fanging and free-living nematode count assessments

	autumn-sown experiment				spring-sown experiment			
Date	**PCR**	**Cavity spot**	**Fanging**	**Nematode count**	**PCR**	**Cavity spot**	**Fanging**	**Nematode count**
21/09/12	root zone soil	-	-	-	-	-	-	-
21/09/12	**Biofumigants / green manures sown**							
25/09/12	-	-	-	root zone soil*	-	-	-	root zone soil*
19/02/13	root zone soil	-	-	-	root zone soil	-	-	-
05/03/13					**Biofumigants / green manures sown**			
17/05/13	root zone soil	-	-	root zone soil	-	-	-	-
20/05/13	**Biofumigants / green manures incorporated**							
31/05/13	root zone soil	-	-	root zone soil	root zone soil	-	-	root zone soil
03/06/13	**Carrots sown**				**Biofumigants / green manures incorporated**			
14/06/13	root zone soil	-	-	-	root zone soil	-	-	root zone soil
17/06/13					**Carrots sown**			
28/06/13	root zone soil	-	-	-	root zone soil	-	-	-
12/07/13	root zone soil	-	-	-	root zone soil	-	-	-
26/07/13	root zone soil	-	-	-	root zone soil	-	-	-
09/08/13	root zone soil	-	-	-	root zone soil	-	10 carrot roots	-
30/08/13	10 carrot roots	10 carrot roots	10 carrot roots	-	10 carrot roots	10 carrot roots	10 carrot roots	-
20/09/13	10 carrot roots	10 carrot roots	10 carrot roots	-	10 carrot roots	10 carrot roots	10 carrot roots	-
11/10/13	10 carrot roots	10 carrot roots	10 carrot roots	-	10 carrot roots	10 carrot roots	40 carrot roots	-
21/10/13	-	40 carrot roots	40 carrot roots	-	-	40 carrot roots	10 carrot roots	-
24/10/13	10 carrot roots	10 carrot roots	10 carrot roots	-	10 carrot roots	10 carrot roots	10 carrot roots	-
06/11/13	-	-	-	root zone soil	-	-	-	root zone soil
14/11/13	10 carrot roots	10 carrot roots	10 carrot roots	-	10 carrot roots	10 carrot roots	10 carrot roots	-
18/11/13	**Carrots strawed down**				**Carrots strawed down**			
06/03/14	10 carrot roots	10 carrot roots	10 carrot roots	-	10 carrot roots	10 carrot roots	40 carrot roots	-
07/03/14	-	40 carrot roots	40 carrot roots	-	-	40 carrot roots	40 carrot roots	-

Direct effect of biofumigant plants on *P. violae in vitro*

Additional work, not included in the original project proposal, was carried out as part of a University of Warwick Summer Studentship to determine if ITC volatiles released from biofumigants had a direct effect on *P. violae in vitro*. The biofumigants tested were three varieties of brown mustard (*Brassica juncea*, cvs. Pacific Gold, Vittasso and Caliente 99), fodder radish (*Raphanus sativus*, cv. Terranova), white mustard (*Sinapis alba*, cv. Brisant), rocket (*Eruca sativa* cv. Nemat. Oilseed rape (*Brassica napus* cv. Temple) was also included as a non-biofumigant control. A *P. violae* isolate obtained previously from an infected carrot in Cottage Field, Wellesbourne was grown on PDA for 6 days at 20°C. For each biofumigant, a 5 mm agar plug of actively growing *P. violae* mycelium was placed at the centre of a PDA plate which was then inverted. Dried, milled plant material (2 g, prepared from poly-tunnel grown biofumigant plants as described by Clarkson [2013]) was then placed in the lid and 15ml of water added. Petri dishes were immediately sealed and incubated at 20°C and the growth of *P. violae* assessed by measuring the colony diameter in the X and Y axis after 7 days. Data were analysed by ANOVA in Genstat to determine any significant effects of the treatments.

Results

Field experiments

Biofumigant / green manure crop growth and glucosinolate levels

The autumn-sown biofumigants / green manures over-wintered well but a cold spring resulted in a relatively late flowering time and initially a slow development of the spring-sown crops, even though these were fleeced. Both autumn and spring-sown crops were in full flower in late May / early June and hence incorporation times were 20/5/13 and 03/06/13 respectively. Mean glucosinolate levels for autumn and spring-sown brown mustard crops (sinigrin) were 0.68 and 0.20 µmol/g respectively and for white mustard (sinalbin) were 3.29 and 1.18 µmol/g respectively. The fodder rape samples did not contain detectable amounts of glucosinolates.

PCR detection and dynamics of *P. violae*

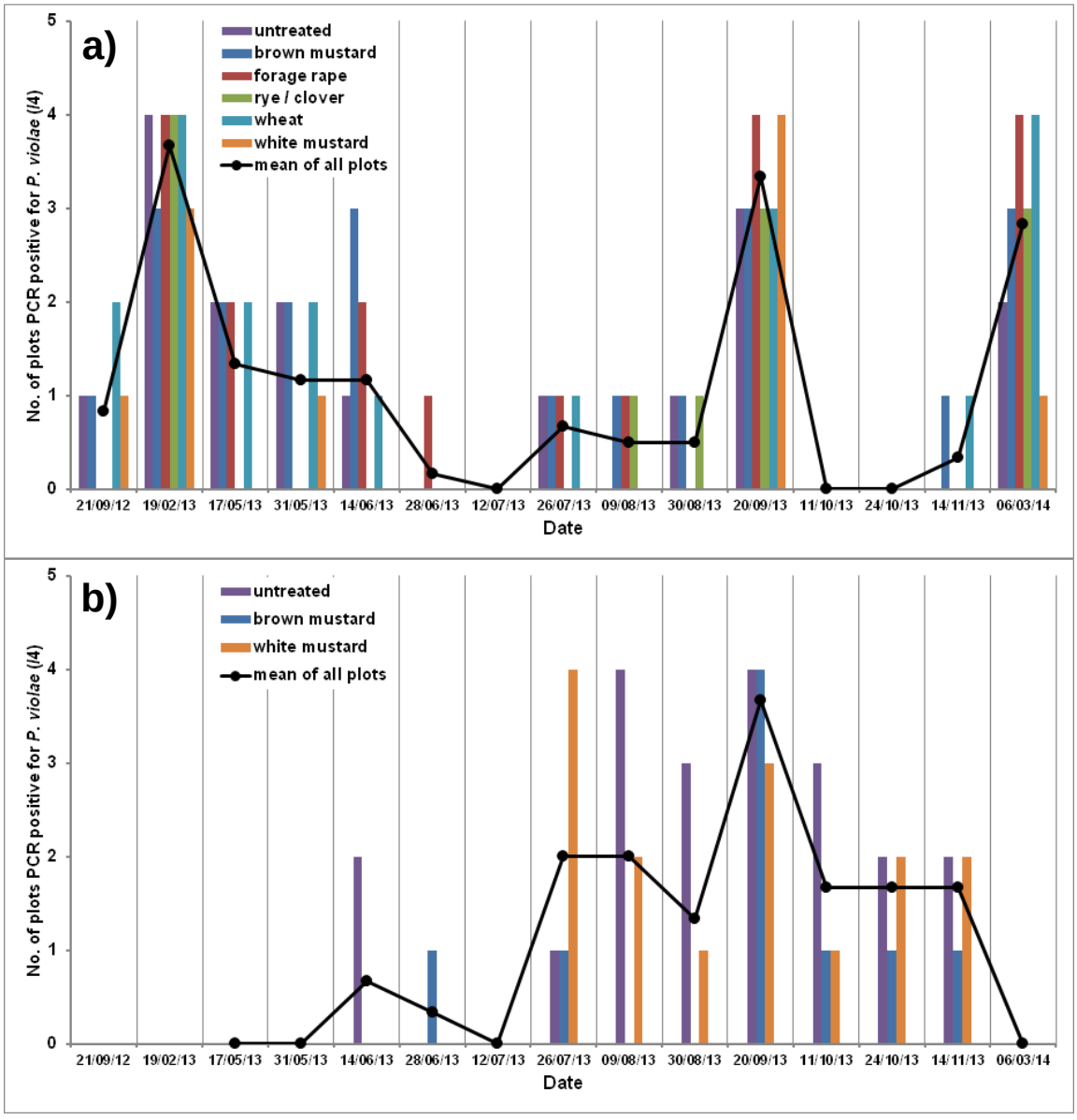
P. violae was detected by PCR in all treatment plots for at least one sampling time over the duration of the autumn and spring-sown experiments. When the number of plots per treatment testing positive for *P. violae* was examined, there was no clear effect of biofumigation / green manure treatment on the pathogen (Fig. 3). Generally, *P. violae* dynamics followed a similar pattern over all the treatments plots and there were three sampling times for the autumn-sown experiment where the pathogen was detected in most plots; 19/02/13 (biofumigants/green manures semi-mature), 20/09/13 (subsequent carrot crop mature) and 06/03/14 (post carrot crop strawing down). Between these times, *P. violae* was not detected or only in a relatively small number of plots. The pattern was less clear for the spring-sown experiment (although there was no sampling for these plots on 19/02/13) but there was again a peak of detection in most plots on 20/09/13. However in contrast, there was no *P. violae* detected post strawing down on 06/03/14.

Development of cavity spot

Cavity spot levels in the same ten roots used for PCR detection remained low throughout the season for both autumn and spring-sown biofumigant / green manure experiments (Fig. 4). There was therefore no clear effect of any of the treatments on disease levels for these small root samples. The proportion of roots affected (incidence) was greatest for both experiments for the assessment on 24/10/13 and ranged between 0.43-0.65 (43-65%) for the autumn-sown treatments and 0.50-0.70 (50-70%) for the spring-sown treatments (Fig. 4cd). However, the corresponding severity of cavity spot disease for this assessment was very low with a maximum ranging between 0.68-1.00 lesions/root for the autumn and 1.13-1.63 lesions per root for the spring-sown experiments respectively (Fig. 4ab). Over all the

six disease assessments carried out for both autumn and spring-sown experiments on these small numbers of roots, cavity spot incidence and severity increased from the first assessment on 30/08/14 (0-0.08 lesions/root) to the maximum levels on 24/10/14. However, the last two assessments pre- (14/11/13) and post- (06/03/14) strawing down resulted in lower disease levels. The small number of roots sampled and low disease levels precluded any statistical analysis to assess the effect of biofumigant / green manure treatments on cavity spot.

Figure 3. Number of plots (out of a maximum of four) positive for *P. violae* using PCR for a) autumn and b) spring-sown biofumigant / green manure crops. Samples for PCR were from bare soil or soil from around plants (21/09/12-26/07/130 or from around carrots roots (30/08/13-06/03/14).



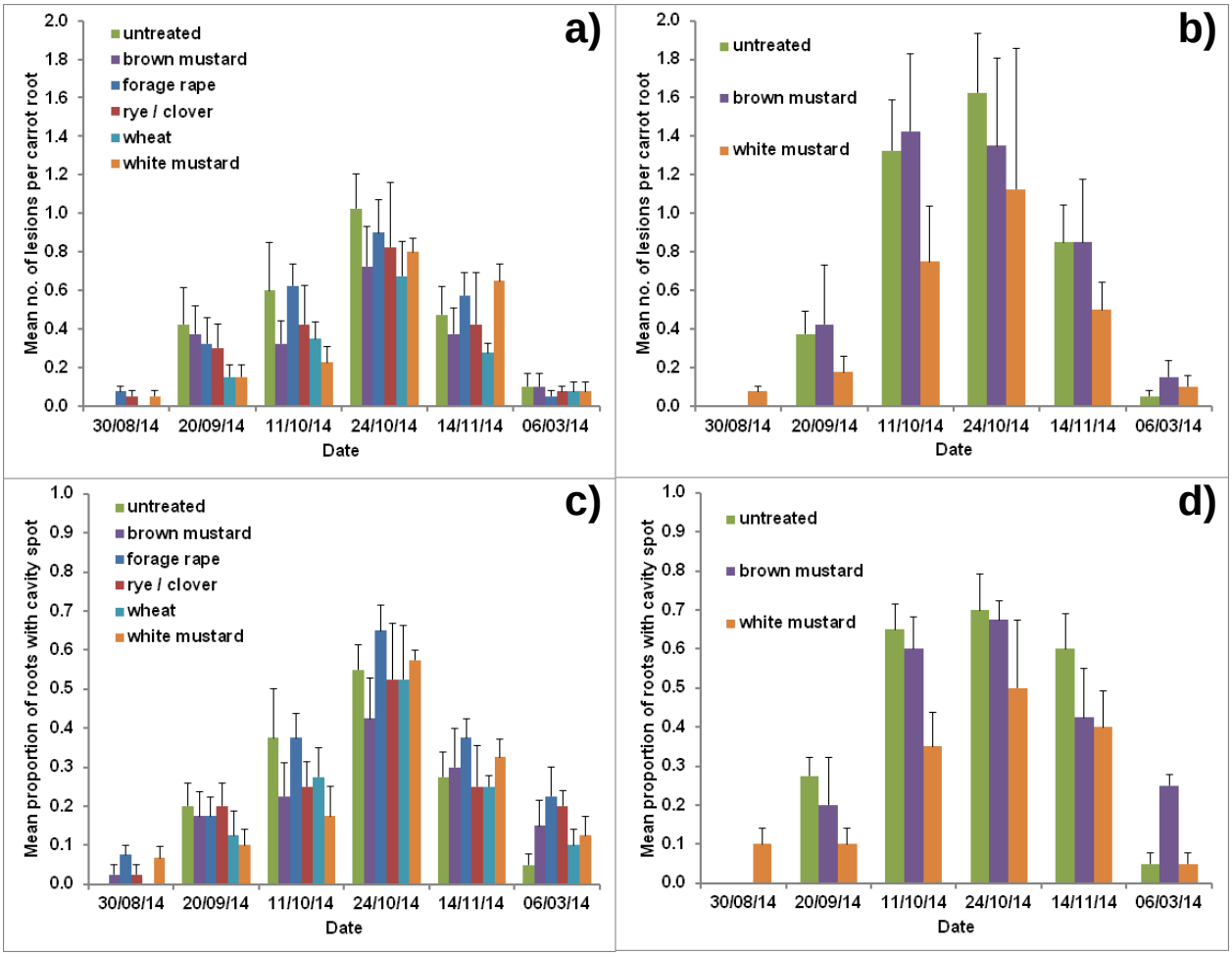


Figure 4. Cavity spot disease severity (mean number of lesions per carrot root) and incidence (mean proportion of roots affected) for a,b) autumn and b,c) spring-sown biofumigant / green manure crops over time for 10 carrot root samples per plot (40 roots per treatment). Error bars are standard error of the mean.

Effect of biofumigants / green manures on cavity spot

Cavity spot levels in the larger root samples for both autumn and spring-sown biofumigant / green manure experiments (40 roots per plot, 160 roots per treatment), harvested pre- and post- strawing down of the carrot crops on 14/11/13 and 06/03/14, were low (Fig. 5). Statistical analysis using ANOVA showed that there was no significant effect of any of the treatments on cavity disease levels. Across all treatments and both assessment times, the proportion of roots affected with cavity spot varied between 0.23-0.42 (23-34%) for the autumn-sown experiment and 0.34-0.42 (34-42%) for the spring-sown experiment (Fig 5cd). The corresponding mean number of lesions per root varied between 0.28-0.49 for the autumn-sown experiment and 0.41-0.71 for the spring-sown experiment (Fig 5ab).

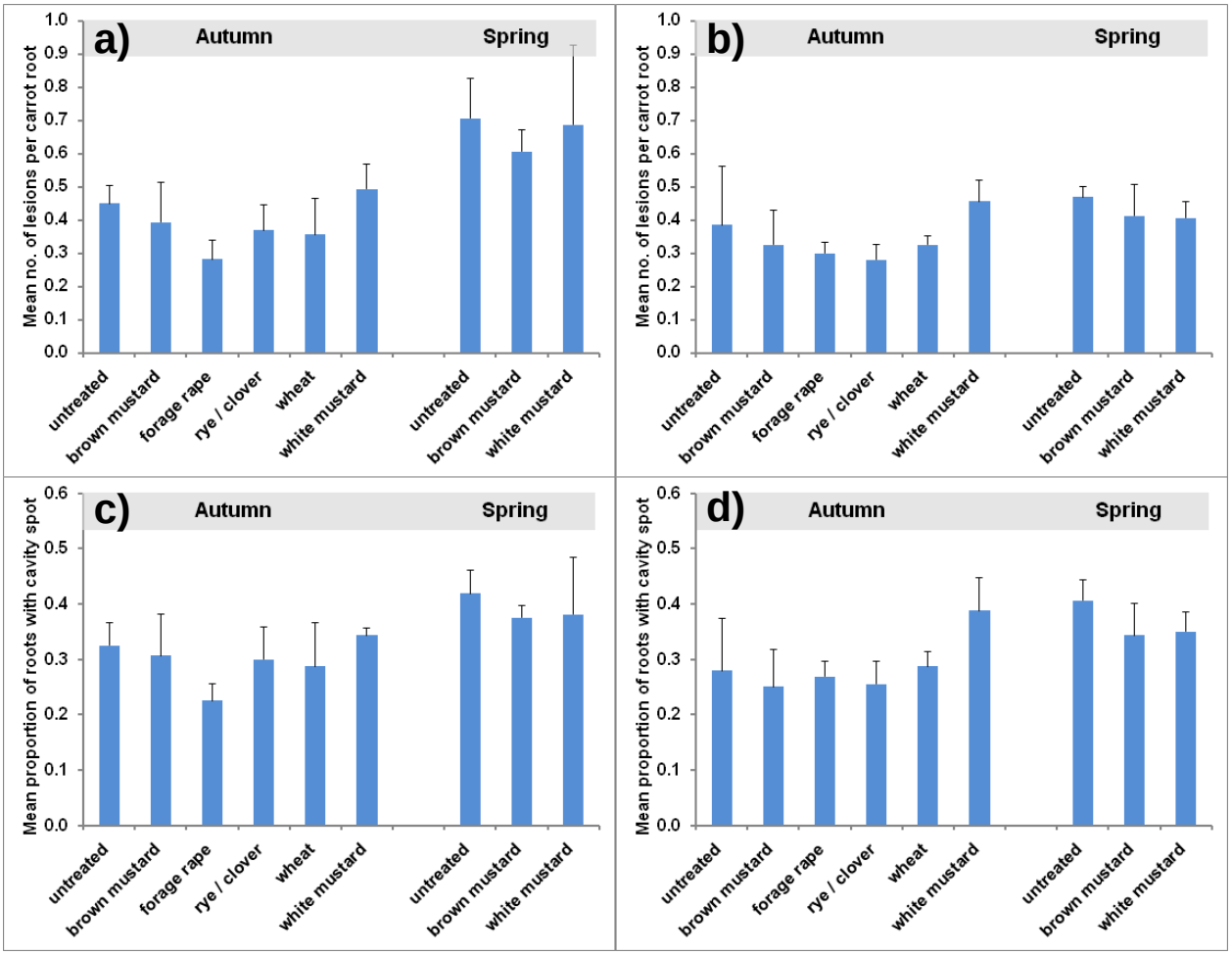


Figure 5. Cavity spot disease severity (mean number of lesions per carrot root) and incidence (mean proportion of roots affected) for autumn and spring-sown biofumigant / green manure crops for 40 carrot root samples per plot (160 roots per treatment) on a,c) 21/10/13 (pre strawing down) and on b,d) 07/03/14 (post strawing down). Error bars are standard error of the mean.

Effect of biofumigants / green manure treatments on free-living nematodes and carrot fanging

Initial nematode counts on 25/09/12 from the six points in the experimental area showed that *Trichodorus* (mean 271 /L soil), *Tylenchorynchus* / *Helicotylenchus* (mean 3604 /L soil), *Pratylenchus* (mean 25 / L soil) and *Longidorus* (584 / L soil) were present in Cottage Field while the other nematode types were absent or below detectable levels, a situation which continued for subsequent assessments.

For the autumn-sown experiment, levels of *Trichodorus, Tylenchorynchus* / *Helicotylenchus* and *Longidorus* recorded in the assessment pre-incorporation of the biofumigants / green manures on 17/05/13 were reduced in all treatments including the untreated control in the post-incorporation assessment 14 days later on 31/05/13 (Fig. 6). Over all the treatments, *Trichodorus*, *Tylenchorynchus* / *Helicotylenchus* and *Longidorus* were reduced from mean levels of 116, 3737 and 397 nematodes /L soil to 27, 1892, and 119 nematodes /L soil respectively. There was therefore no apparent effect of particular treatments. Levels of *Pratylenchus* were generally low (<38 nematodes /L soil) or absent for all treatments in both assessments.

For the spring-sown experiment, levels of *Trichodorus, Tylenchorynchus* / *Helicotylenchus* and *Longidorus* were much lower initially in the assessment pre-incorporation of biofumigants on 31/05/13 compared to the autumn-sown experiment but were reduced in all treatments including the untreated control in the post-incorporation assessment 14 days later on 16/06/13 (Fig. 7). Over all the treatments, *Trichodorus*, *Tylenchorynchus* / *Helicotylenchus* and *Longidorus* were reduced from mean levels of 38, 1952 and 680 nematodes /L soil to 17, 1404 and 321nematodes /L soil respectively. There was therefore no apparent effect of particular treatments. In contrast, levels of *Pratylenchus* increased in all treatments but were still generally low (<38 nematodes /L soil).

The final assessment of free-living nematodes on 06/11/13 before strawing down revealed a general decline in all populations since the previous samplings in late May / early June and again there was no consistent effect of treatments (Fig. 8). Over all the autumn-sown treatments, mean levels of *Trichodorus*, *Tylenchorynchus* / *Helicotylenchus* and *Longidorus* levels were 3, 1070, 18 and 183 nematodes /L soil respectively. Over all the spring-sown treatments, mean levels of *Trichodorus*, *Tylenchorynchus* / *Helicotylenchus* and *Longidorus* levels were 0, 567, 27 and 291 nematodes /L soil respectively.

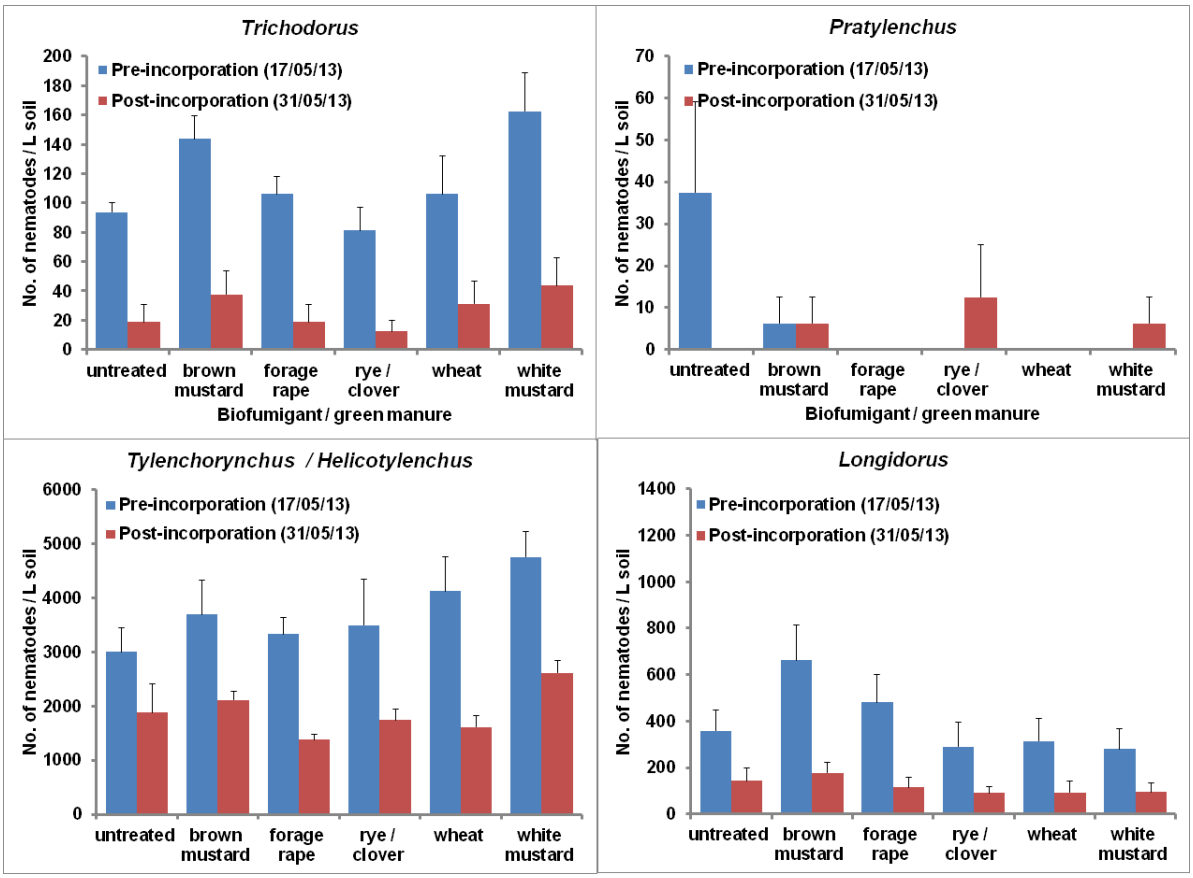


Figure 6. Free-living nematode counts pre- and post-incorporation of biofumigants / green manures for autumn-sown experiment. Error bars are standard error of the mean.

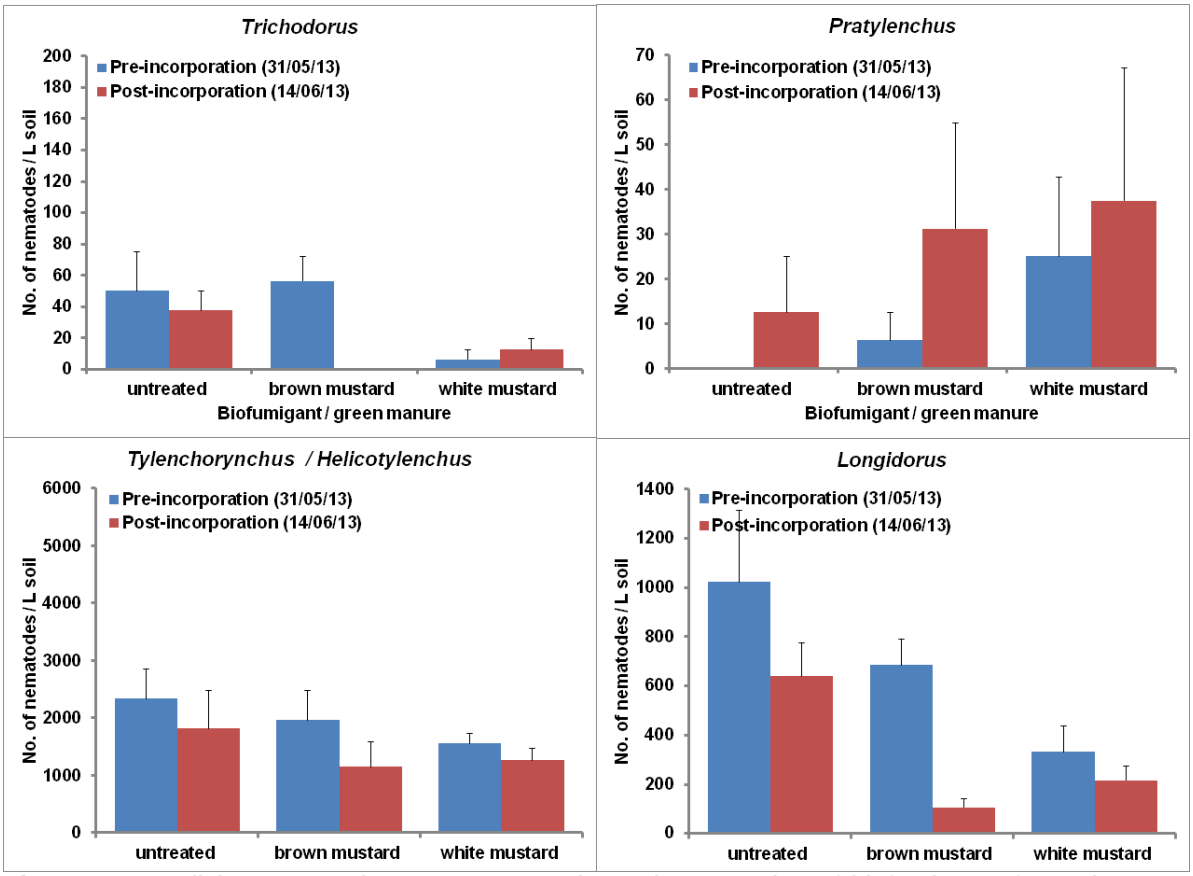


Figure 7. Free-living nematode counts pre- and post-incorporation of biofumigants for spring-sown experiment. Error bars are standard error of the mean.

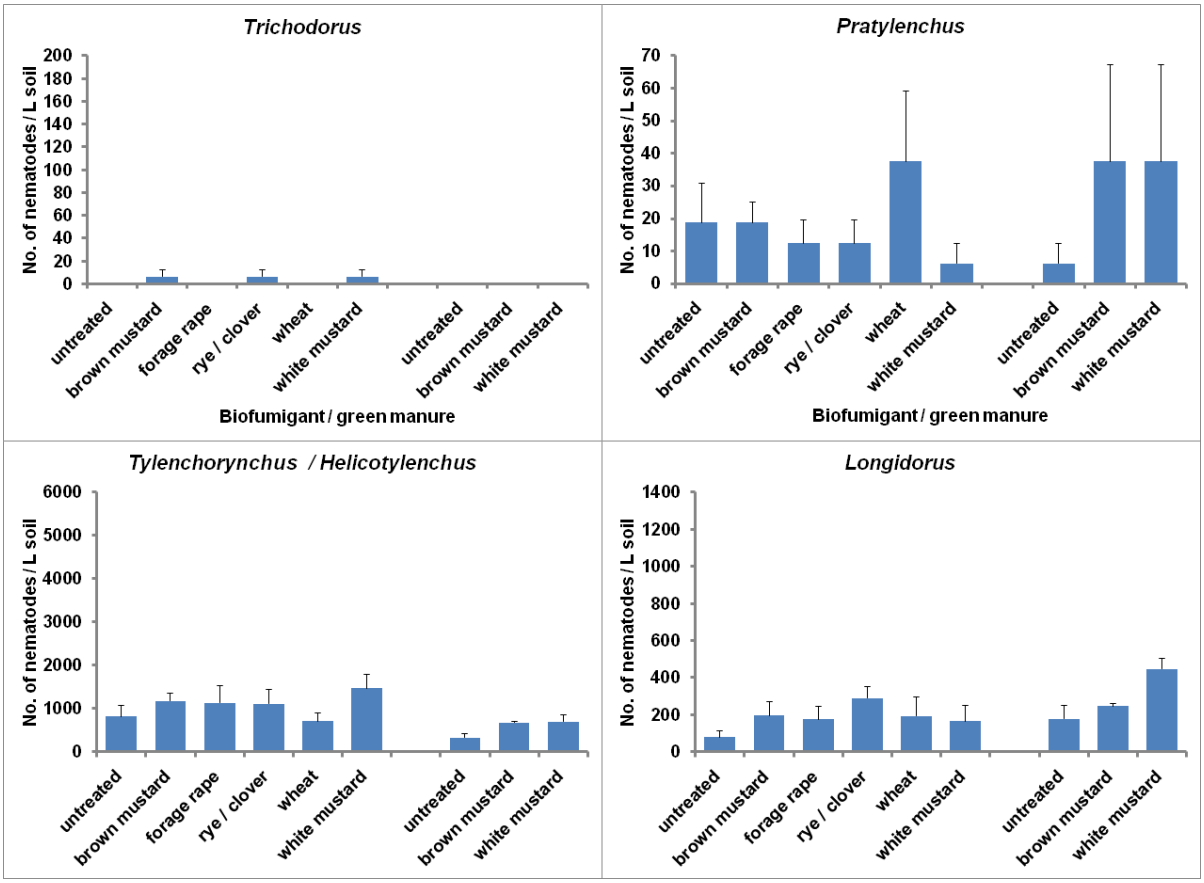


Figure 8. Free-living nematode counts for biofumigant/green manure treatments on 06/11/13. Error bars are standard error of the mean.

The proportion of fanged carrot roots for both autumn and spring-sown experiments in the 10 carrot root samples was inconsistent and varied over the season (0.05-0.35, 5-35%) and there was no clear effect of any biofumigant / green manure treatment (Fig. 9ab). For the 40 carrot root samples assessed on 21/10/13 and 07/03/14, there was some indication that the brown mustard treatment reduced fanging in the autumn-sown crop but this was not evident in the spring-sown crop (Fig. 9 cd). However, statistical analysis using ANOVA showed that there was no significant effect of any of the treatments on the proportion of fanged carrots.

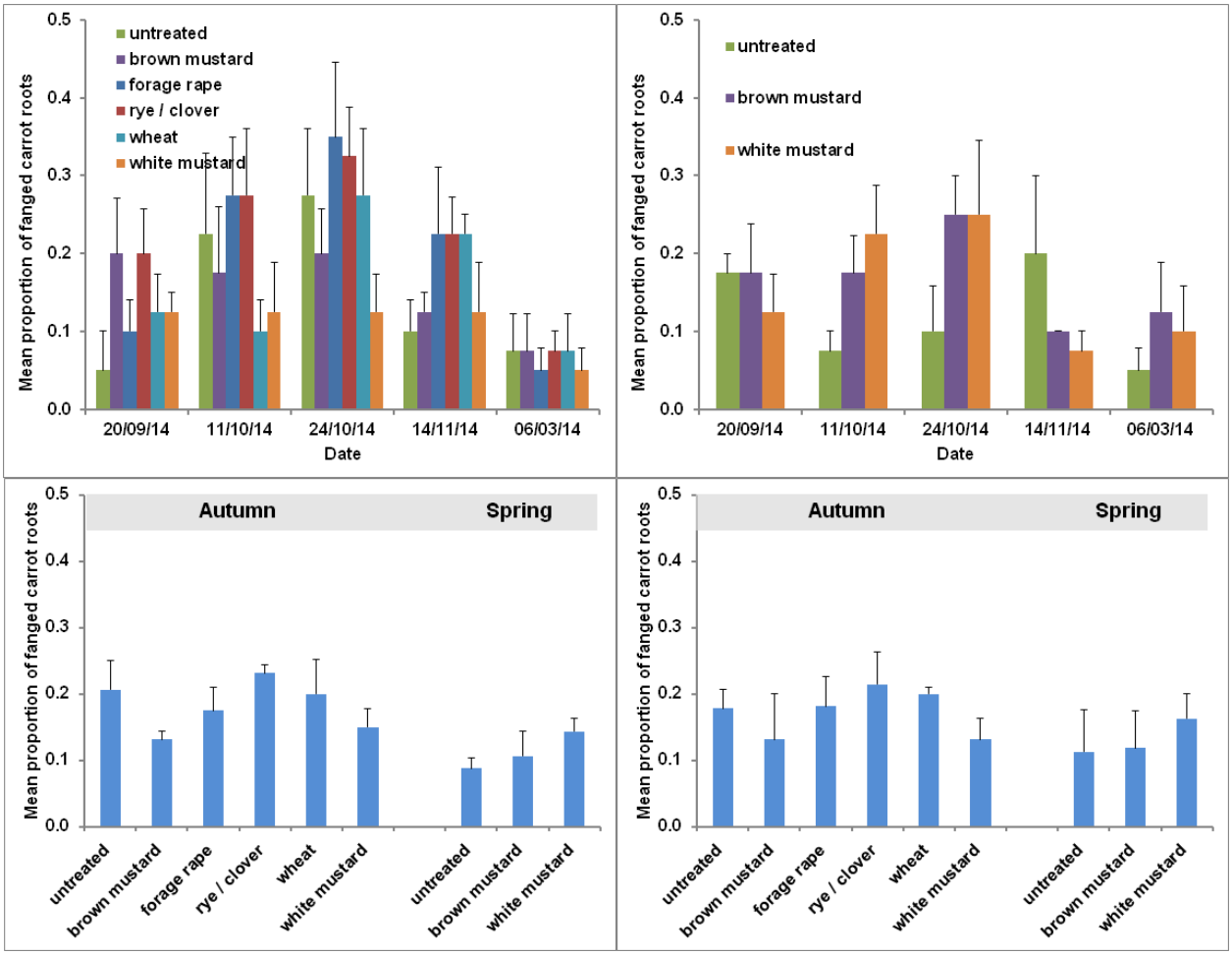


Figure 9. Mean proportion of fanged carrot roots for a) autumn-sown biofumigants / green manures for 10 carrot root samples / plot (40 roots per treatment) over time, b) spring-sown biofumigants for 10 carrot root samples / plot (40 roots per treatment) over time, c) autumn and spring-sown biofumigants / green manures for 40 carrot root samples per plot (160 roots per treatment) on 21/10/13 (pre strawing down) and d) autumn and spring-sown biofumigants / green manures for 40 carrot root samples per plot (160 roots per treatment) on 07/03/14 (post strawing down). Error bars are standard error of the mean.

Effect of biofumigants / green manure treatments on germination of *S. sclerotiorum* sclerotia

In the autumn-sown experiment, final mean percentage germination for buried *S. sclerotiorum* sclerotia were 65, 61, 73, 54, 57 and 71% for brown mustard, white mustard, forage rape, rye / clover mix, wheat and untreated control treatments respectively. Statistical analysis showed that there was a small significant effect on germination for the rye/grass clover treatment only (P = 0.045).

Direct effect of biofumigant plants on *P. violae in vitro*

All biofumigant treatments and *B. napus* cv. Temple significantly inhibited the growth of *P. violae* ($P \leq 0.05$, Fig. 10) by more than 50%. All the brown mustard cultivars and also the white mustard cultivar completely inhibited growth of *P. violae*.

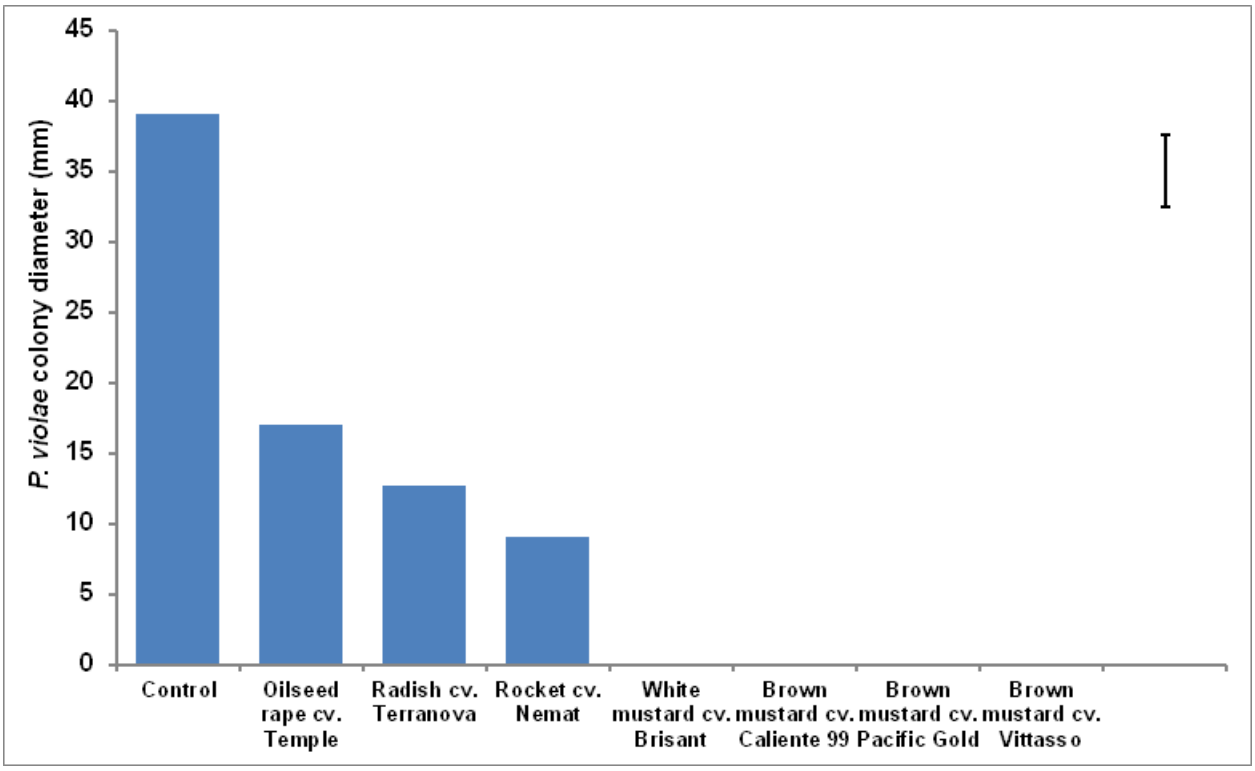


Figure 10. Effect of biofumigants on growth of *P. violae* on agar after 7 days and 20°C. Bar indicates least Significant Difference (LSD, $P \leq 0.05$) between treatments.

Discussion

The autumn-sown green manures clover/ryegrass, wheat and forage rape overwintered well in this first year of experiments as might be expected. The brown and white mustards also established and survived the winter despite their potentially being unadapted to cold conditions. Full flowering for both autumn and spring-sown mustards (fleeced) occurred in late May / early June, having been delayed by a cold spring, and there was only a few weeks difference in flowering time. It is well established that the highest glucosinolate levels in mustards and other brassica crops generally occur at full flowering (Clossais-Besnard & Larher, 1991; Bellostas et al., 2007) and hence ideally, the biofumigant crops should be grown to this stage before incorporation. Hence, for carrot growers who drill mainly in April and May or even earlier, the period for growing either autumn or spring-sown biofumigants until full flowering is short. Hence, if biofumigants were to be utilised by growers, then it is likely they would have to be incorporated before full flowering when glucosinolate levels are lower. Levels of the glucosinolate sinigrin in the flowering brown mustard crops in the autumn and spring-sown biofumigants in this project ranged between 0.20 and 0.68 µmol/g while those for sinalbin in the white mustard crops ranged between 1.18 and 3.29 µmol/g. These are much lower levels compared with those obtained using the same mustard types raised in a polytunnel in June 2012, where levels of sinigrin and sinalbin were 26.5 and 14.5 µmol/g respectively (Clarkson, 2013). In comparison, published levels for sinigrin and sinalbin in field crops have been reported in the range 0.1-18.7 µmol/g and 9.0-14.4 µmol/g respectively (Kirkegaard & Sarwar, 1998). The reason for the low glucosinolate levels in the mustard crops here was most likely due to a combination of low temperature and short day-length, both of which have been demonstrated to reduce glucosinolate production in brassicas (Smolinska and Horbowicz, 1999; Steindal et al., 2013).

The PCR test was used to establish the presence / absence of *P. violae* in the treatment plots using one soil sample per plot (approx. 10g). This comprised smaller combined soil samples from five areas in each plot or from around the outside of 10 carrot roots from different areas in each plot, giving a total of four soil samples per treatment. This may not give an accurate indication of *P. violae* incidence especially as only 0.25 g of soil is taken from each sample for DNA extraction. However, if the total number of PCR samples over all the treatment plots is taken into account (therefore assuming no effect of treatment), then this gives 24 samples in total at each time point for the autumn-sown experiment which probably does give a reasonable indication of pathogen incidence and dynamics over time as indicated in the results section. The spatial variation in *P. violae* incidence in the field and the small soil sample sizes required for DNA extraction and subsequent PCR detection means that assessing pathogen dynamics is challenging and therefore requires multiple

samples and PCR tests. This technical aspect of the work will be investigated further in a recently funded HDC PhD studentship starting in October 2014. Despite these potential problems and using the PCR results from all plots in each experiment, there was a general increase in the number of plots testing positive for *P. violae* in late autumn as the carrot crops matured as has been observed previously (Anon, 2009). However, an apparent decrease in the pathogen in October / November 2013 in the presence of overwintered carrots is unexpected but examination of recorded weather data suggests that there was a drier period during this time (data not shown). Detection of *P. violae* in February 2013 in the absence of carrots was also unexpected and requires further investigation.

Although *P. violae* was detected by PCR, cavity spot disease failed to develop to significant levels in the carrot crops for either the autumn or spring-sown experiments, despite the use of regular irrigation which has previously been shown to promote disease (Barbara, 2010). Although the proportion of carrots affected with cavity spot reached a maximum of between 43 and 70% in the 10 root assessment made on 24/10/13 for autumn and spring experiments, this is, however, based on the presence of only one or two lesions and a small sample size. On average, fewer than two lesions / root developed in any of the treatments over all the disease assessments made using 10 root samples per plot. The apparent decrease in cavity spot levels in subsequent assessments after 24/10/13 is difficult to explain although it possible that the few cavities that were formed by *P. violae* were subsequently invaded by other soilborne fungi resulting in a change in symptoms. The 10 root samples were used primarily to obtain soil from around the carrots for PCR detection of *P. violae* but disease levels were also assessed as a way of monitoring disease progress and potentially timing the larger 40 root assessments. Overall however, it is clear that sampling only 10 carrots per plot (40 carrots per treatment) resulted in a high degree of variability in levels of cavity spot recorded between replicate plots and between different assessments at the low disease levels encountered in 2013. Therefore, although the soil from around these samples was useful for monitoring *P. violae* dynamics by PCR, more carrots would be required per sample to give more certainty in quantifying disease levels over time due to the sporadic and patchy nature of cavity spot.

Cavity spot levels for assessments on 21/10/13 (pre-strawing down) and 07/03/14 (post-strawing down) using the larger samples of 40 roots per plot (160 roots per treatment) were more consistent but disease levels were still very low for autumn and spring experiments with an average of less than one lesion per root over all treatments and between 23 and 42% roots affected. Again this incidence is based on the presence of only one or two lesions. Overall the low levels of cavity spot in the autumn and spring experiments meant

that it was not possible to detect any significant effects of the biofumigant / green manure treatments.

Free-living nematode counts showed detectable levels of *Trichodorus, Tylenchorynchus* / *Helicotylenchus* and *Longidorus* in Cottage Field at Wellesbourne. Of these species, *Trichodorus* and *Longidorus* spp. are potentially the most damaging to carrots. Anecdotal thresholds suggest that numbers of *Trichodorus* spp. in excess of 200/L soil and of *Longidorus* spp. in excess of 50/L soil would justify nematicide treatment (Dr Steve Ellis, personal communication). Numbers of both species were therefore above these thresholds in the initial sampling at sowing of the Autumn biofumigants (25/09/12) with numbers of Longidorus spp. particularly high (> 11 times the threshold). Subsequent counts pre- / post-incorporation of biofumigants / green manures for these two nematodes showed that *Trichodorus* spp. numbers fell below the damaging threshold in all treatments but that numbers of *Longidorus* spp. although declining, remained above the threshold. Therefore root fanging might have been expected as a result of feeding by *Longidorus* spp and these numbers should in principal have provided a robust test of the biofumigant/green manure treatments. Anecdotal thresholds for *Tylenchorynchus* spp. and *Pratylenchus* spp. are estimated to be about 10,000/L soil and 2,500/L soil respectively so both these species were below levels thought to be damaging for all the assessments carried out.

The levels of all nematode species clearly declined in all treatments including the untreated control following incorporation of biofumigants / green manures for both autumn and spring experiments. This suggests that the treatments themselves had no effect on free-living nematodes and hence the decline may have been due to other factors, most likely the tilling operations involved in incorporating the treatments and drilling subsequent carrot crops. Assessment of fanging in the 10 root carrot samples was again variable, but more consistent for the 40 root samples although there were no significant differences between treatments. However, the brown mustard treatment slightly reduced fanging compared to the untreated control but only in the autumn experiment and this was not associated with low numbers of any of the different free-living nematode species including *Longidorus* spp. which were the only spp. above the damaging threshold. There was also no relationship between nematode numbers and carrot fanging.

Although the rye/clover green manure slightly reduced the germination of *S. sclerotiorum* sclerotia, the biofumigant mustard crops treatments had no effect. This is in contrast to findings by Clarkson (2013) under controlled conditions (in enclosed boxes) where the brown mustard Caliente 99 significantly reduced carpogenic germination by 63% compared to the untreated control. However in the same experiment, the white mustard Brisant was

less effective, reducing germination by only 31%. The inhibitory effect of both these treatments was shown to be due to the direct action of volatile ITCs.

Using a similar approach (Clarkson, 2013), the in-vitro experiments reported here showed that volatile ITCs from a range of biofumigants, including white and brown mustards as used in the field experiments, also inhibited growth of *P. violae* on agar. This is the first evidence that such biofumigants may have a direct effect on the pathogen rather than potentially reducing disease through increasing microbial activity as previously suggested (see introduction).

Although it is clear from experiments under controlled conditions that ITCs from mustard biofumigants can inhibit both *P. violae* and *S. sclerotiorum*, translating this effect into the field will be challenging, as there are a number of factors that can affect the efficacy of the biofumigation process. These include agronomic and environmental factors which result in poor growth and low glucosinolate levels in the biofumigants (as outlined above), poor conversion of glucosinolates to ITCs due to inefficient crushing / incorporation of plant material or inadequate soil moisture levels and potential loss of ITCs through escape of volatiles in the air or dissolved in percolating water (Matthiesson & Kirkegaard, 2006). The latter could be prevented by the use of polythene covers post-incorporation as used in the original work which demonstrated that brown mustards could reduce cavity spot (Anon, 2009) but this was deemed impractical and potentially costly by the industry.

Future work in Year 2 will continue to focus on the potential of biofumigants / green manures to reduce cavity spot in consultation with HDC and BCGA. However the following recommendations are made based on Year 1 results:

- Include one or two grower sites to test selected biofumigants / green manures to reduce the risk of low cavity spot levels.
- Reduce the number of biofumigant / green manure treatments to allow increased monitoring of *P. violae* dynamics by PCR detection and more accurate assessment of cavity spot disease levels through increased sampling of larger plots.
- Reconsider using polythene covers to maximise biofumigation potential of mustards.
- Cease monitoring of free-living nematodes and fanging to increase project focus on cavity spot.

Conclusions

- autumn and spring-sown biofumigant mustard crops can be grown under UK conditions, but glucosinolate levels at flowering were low.
- The time period for growing mustard crops until full flower in advance of carrot sowing is short.
- Although *P. violae* was detected in all treatments plots by PCR, cavity spot failed to develop and hence the effect of biofumigant / green manure treatments on disease could not be assessed.
- There was no little or no effect of biofumigant / green manure treatments on germination of *S. sclerotiorum* sclerotia, free-living nematodes or carrot fanging.
- Additional in vitro experiments showed that mustard ITCs inhibited growth of *P. violae* on agar.

Knowledge and Technology Transfer

- 21/11/13: Presentation at the UK Carrot and Onion Conference: Challenges for control of cavity spot and Sclerotinia in carrot.
- 16/01/14: project update presentation to the BCGA

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